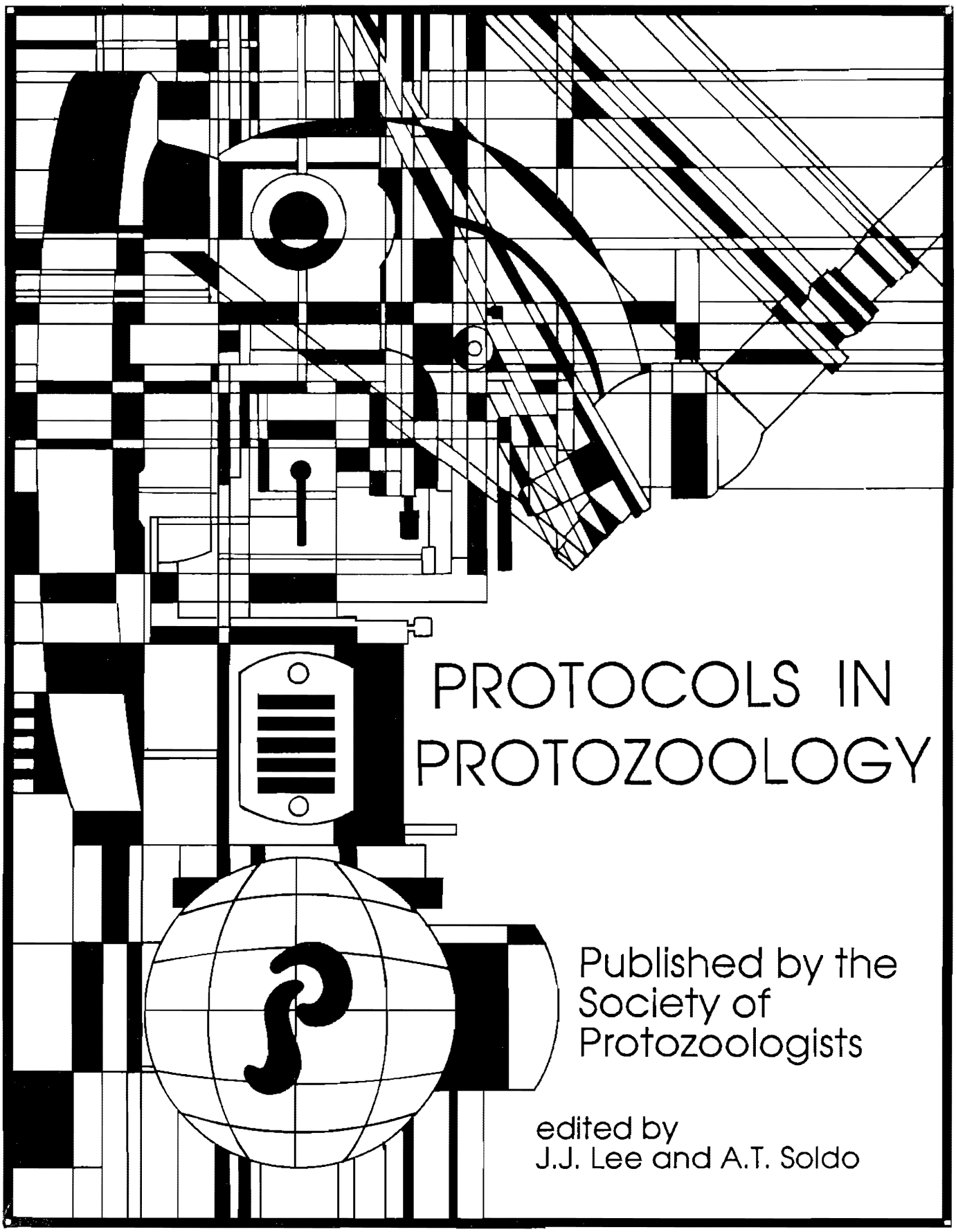

PROTOCOLS IN PROTOZOOLOGY

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edited by
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Forward

The Society of Protozoologists is very pleased to bring this manual to fruition. We intend to issue supplements which update a particular protocol or expand the series to cover additional topics. This first volume has greater coverage of some aspects of the field than others. In time we hope this will all even out. We invite all Protozoologists to share their experiences with the rest of us. No special invitation is needed; just contact one of the editors with your ideas. We, in turn, will respond with instructions for preparation of a protocol. The individual authors and the editors would appreciate knowing about any particular difficulties following a particular protocol encountered by readers. We will attempt to respond with an amended protocol in a future supplement. In consonance with its aims, the society has attempted to produce this book at the lowest possible price so that it is more affordable for most students. This is the Society's first attempt at desk-top publishing. The individual authors and/or the editors produced laser printed copy for reproduction by Allen Press. Since all the labor was donated to keep the costs as low as possible, we hope that users will purchase copies of this book rather than make photocopies of it. It is the only way the Society can recoup the funds that we have invested in its production.

It is with great respect and fondness that we dedicate this first volume to our late colleague, Dr William Balamuth. A manual of this type had long been one of his dreams. Encouraged by Dr Seymour H. Hutner, former chair of the Special Publications Committee, Dr Balamuth was working on this project at the time of his passing. With the aid of some of his former students and colleagues, some of the protocols he was working on will appear in future supplements. The editors wish to thank the officers of the Society for their suggestions and encouragement. We are particularly grateful to our treasurer Dr Brower Burchill for his advice on many aspects of this project. We are grateful to many members of the Special Publications Committee who, over the years, gave generously of their time and advice.

John J. Lee Anthony T. Soldo March 1992

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AXENIC ISOLATION OF DIATOMS AND CHLOROPHYTES AS FOOD FOR PROTOZOA

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Introduction

Although it is not widely known, it is very simple to isolate axenic cultures of diatoms and chlorophytes by cloning them on agar. The medium for isolation can be as simple as an agar-solidified sterile water from the collection site or as complicated as the series of differential media employed by Lee and coworkers (2) in their classical paper on salt marsh epiphytic diatom communities. They supplemented their media with nitrate, phosphate, vitamins (e.g., biotin, thiamine, or B_{12}),or simple substrates (e.g., acetate, lactate, glycine, or glycerol).

Protocol

1. Collect a sample of water from the pond, lake, or marine habitat from which you intend to isolate your algae.
2. Filter the water through # 1 Filter paper.
3. Divide the water into two fractions: Medium A (unenriched) and Medium B (enriched).
4. Add soil extract (5 ml/100ml), $NaNO_3$, or NH_4NO_3 (10 mg/100 ml), and $NaH_2PO_4.H_2O$ (5mg/100 ml) to 95 ml of the water to make Medium B.
   Adjust the pH of the water if it has changed (e.g. coastal sea water should be pH 8.1).
5. Add a good quality agar (e.g. Noble; 15g/l) to both media. If the agar is not of high quality, agar flakes will make it difficult to see the algal clones on the medium.
6. Boil to dissolve the agar.
7. Autoclave for 15 minutes.
8. Transfer the medium from the autoclave to a water bath at 60° C.
9. Just before pouring the petri plates, Aseptically add an antibiotic-antimycotic mixture (1 ml/100 ml; GIBCO Laboratories catalog #600-5240; contains: Penicillin 10,000 units,streptomycin 10,000µg, and Fungizone 25 µg/ ml). Mix thoroughly and pour the plates.
10. Take a small sample(<1 cm^2 surface) of a seaweed or similar aquatic substrate and transfer it to a 100 ml milk dilution bottle containing sterile water of the the same osmolarity (e.g., pond or sea water) as the sample. Shake vigorously to dislodge the epiphytes.
    -or-
    Take a plankton sample and examine it by eye. If it is optically transparent it can be used directly as an inoculum. If it is turbid it should be diluted so that the concentration of the protists is approximately 1 X 10^{-2} to 1 X 10^{-3} per ml.
11. Pipet 0.1 ml of the sample onto the surface of one of the agar plates prepared above.
12. Using an alcohol-sterilized and flamed bent glass rod, spread the inoculum over the surface of the plate.

11.Pipet 0.1 ml of the sample onto the surface of one of the agar plates prepared above.
12.Using an alcohol-sterilized and flamed bent glass rod, spread the inoculum over the surface of the plate.
13.Place the inoculated plates into plastic sandwich bags and seal them or tape them closed.
14.Incubate the plates, agar side up, near an artificial or indirect natural light source (30-50μE).
15.After the plates have been incubated for two weeks, remove them from the plastic bags and examine them under a disscecting microscope. Chlorophytes, rhodophytes, dinoflagellates, and some diatoms tend to form circular, entire, and raised colonies. Many other diatoms for colonies with irregular boarders, rectilinear colonies, colonies in the form of long chains, colonies with compact centers and motile diatoms at the edges, colonies with various types of punctuated growth patterns. Keys to a variety of colonies and illustrations have been published previously(2).
16.Choose well separated colonies and ring them with a wax pencil on the bottom of the dish.
17.Pick a colony with the aid of an alcohol-sterilized and flamed spatula(e.g.,Fisher Scientific Co., catalog #21-401-10, tip hand ground to a width of 1 mm) and transfer it to a liquid medium of the same type you used for the initial isolation.
18.Incubate in the light for 7-10 days.
19.Restreak on media without antibiotics to test for contamination.
20.The organisms can be grown in test tubes or flasks as food for protozoa.

COMMENTS

Our group has used this technique to isolate food to feed foraminifera(1,4), ciliates(6), nematodes (7,8), and copepods(5). In addition we have used axenic cultures of algae isolated from a particular habitat in our "baited-bloom" approach to the isolation of ciliates for mariculture(elsewhere in these **Protocols**).

LITERATURE CITED

1. Lee, J J & Bock, W D. 1976 The importance of feeding in two species of soritid foraminifera with algal symbionts. *Bull. Mar. Sci.* **26**:530-537.
2.---------, McEnery, M E, Kennedy, E M, & Rubin, H. 1975. A nutritional analysis of a sublittoral diatom assembage epiphytic on *Enteromorpha* from a Long Island salt marsh. *J Phycol* **11**:14-49.
3.---------, ---------- & Rubin, H A. 1969. Quantitative studies on the growth of *Allogromia laticollaris. J. Protozool.* **15**:377-395.
4.--------- Sang, K, ter Kuile, B, Strauss, E, Lee, P J & Faber, W W. 1991 Nutritional and related experiments on laboratory maintenance of three species of symbiont-bearing foraminifera. *Mar. Biol.* **109**:417-425.
5.-------- . Tietjen, J H & Garrison, J R. 1976 Seasonal switching in the nutritional requirements of *Nitocra typica,* an harpaticoid copepod from salt marsh *aufwuchs* commumities. *Trans Amer. Micros. Soc.***95:** 628-637.
6. Rubin, H A and Lee J J 1976.Informational energy flow as an aspect of the ecological efficiency of marine ciliates. *J. Theor. Biol.* **62**: 69-91.
7.Tietjen, J H & Lee, J J. 1977. Feeding behavior of marine nematodes. *In:* **Biology of Marine Benthos**(Coull, B C ed.) Univ. South Carolina Press, Columbia, SC pp.21-35.
8.----------& -------- 1973. Life history and feeding habits of the marine nematode, *Chromodorina macrolaimoides* Steiner. *Oecologia (BERLIN)***12**: 303-314.

A BAITED- BLOOM APPROACH TOWARD THE ISOLATION OF MARINE CILIATES AS POTENTIAL FOOD FOR MARICULTURE

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Introduction

Many coastal and estuarine marine fish have first-feeding larvae with very small and feeble jaws. The parents breed in shallow embayments and salt marshes which teem with protists and micrometazoa. Ciliates are about the right size to serve as food for the first-feeding fish larvae and they swim slowly in the microhabitats where the larvae forage. When we were approached to assist in a mariculture venture, the practical aspects and the magnitude of the problem of supplying of culturing and harvesting >1X 10^9 ciliates/day seemed formatable. There seemed to be two reasonable, but different, approaches toward growing this many ciliates relatively inexpensively: 1)isolate from nature a ciliate of approximately the right size(we were looking for one about 100µm long) and which swam in the water column close to the surface and then screen food organisms which would support its rapid growth ; or 2) select a food organism which could be grown very densely and easily in large batch culture and use it as bait to select a ciliate from a natural collection. Based on our previous nutritional experiments with ciliates(1) we thought the latter might be the simplest and most direct approach. It worked the first time we tried it and we have used when needed for maricultural experiments. Since we inoculated small natural samples into very dense cultures we named the technique the "baited-bloom" approach.

Protocol

1. Select or isolate an alga (see Lee 1992, elsewhere in these **Protocols**) which will grow very densely (>1 X 10^6 cells/ml) in culture. We have used successfully a culture of *Dunaliella salina* which the senior author isolated from the Towd Point salt marsh, Southampton, L.I., N.Y. It grows very well in a simple enriched seawater medium (Erdschrieber: filtered natural sea water 95 ml%,NH_4 NO_3 10mg%, NaH_2PO_4 5mg%, soil extract 5ml%).
2. Inoculate and grow one or two racks (~80 test tubes,10 ml/tube) of dense cultures of the alga. At the same time inoculate 250 and 500 ml screw- capped Erlenmeyer flasks of the same alga. The cultures should be incubated in moderate light (~30-50 µE m^{-2})
3. Place the test tube cultures into an insulated cooler and transport them to the collection vessel or field site.
4. With the aid of sterile pipettes, take aseptic samples of water from the upper cm of the water column and transfer 0.5 ml to each test tube with a dense culture of algae.
5. Incubate in the laboratory the tubes for 3 days at environmental temperature(20-25°).
6. Some of the tubes will be cleared in 3 days. Actively dividing ciliates can be viewed through the walls of the tubes under a good quality dissecting microscope.
7. Choose tubes with the appropriate sized ciliates and inoculate the ciliates into the 250 ml flasks of algae inoculated above(step 2).
8. Maintenance of the cultures is a matter of choice. They can be maintained in log phase by

transfer to dense algal cultures every three days. If neglected, the cultures will decline for the next 3 weeks, but can be transfered reliably after a month.

Scaling up is really a function of the facilities available at the mariculture site. The following was the proceedure we followed at the ILOR National Center For Mariculture, Elat Israel.

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9. Inoculate several 10 liter carboys with the algal(*Dunaliella salina*) culture. Incubate them in the light.
10. When the algal cultures have grown, take one and inoculate it into two or more 30 liter plastic bags. Incubate them before a bank of fluorescent lamps. Timing is everything, do not do anything with the second carboy until the algae in the plastic bags are near maximum density.
11. Inoculate the second carboy with ciliates. Inoculate fresh back-up carboys with algae.
12. When the algae in the plastic bags reach maximum density (>1 X 10^6/ml)take the culture in one (or two) of them and inoculate the next sized vessel. In the National Center For Mariculture in Elat this was an outdoor raceway. Inoculate back-up 30 liter plastic bags with algae
13. When the algae in the raceways are about to reach maximum density, inoculate the ciliates from the second carboy into several plastic bags with dense cultures of algae.
14. Several (2 or 3) days later transfer the algae from the raceways and the ciliates to the next largest vessel. In the IOLR NCM in Elat this was a 22,000 liter plastic tank.

COMMENTS

It really is practical to grow large numbers of ciliates for use in mariculture. When it comes to mass cultures the trick is in the timing. The food always has to be in excess.Since such a large amount labor, energy and other resources are commited to mass culture, back up cultures have to be available in case of failure. Unknown factors enter into calculations of yields once cultures are no longer axenic or synxenic. Ciliate yields and generation times which were completely predictable in gnotobiotic culture fell to less than 25% of potential by the time we reached the 22,000 liter culture vessel. This meant that we needed to devote more space and effort into growing food for the ciliates and more time and space was needed than was predicted at the onset.

LITERATURE CITED

1. Rubin, H A and Lee J J 1976.Informational energy flow as an aspect of the ecological efficiency of marine ciliates. *J. Theor. Biol.* **62**: 69-91.

CLONING CILIATES AND OTHER PROTOZOA USING THE SILICONE OIL PLATING PROCEDURE

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Introduction

The standard procedure for cloning microorganisms by plating on nutrient agar or gelatin and observing colony formation after a period of growth has had only limited success with protozoa (1,2). Except for some flagellates (3-5), a few small amoebae (6,7) and a slime mold (8), most protozoa, ciliates in particular, do not readily form discrete colonies on solid surfaces. The method described here is based on an entirely different principle than that used for these microorganisms. In the Silicone Oil Plating Procedure (SOPP)(9), suspensions of ciliates (or other protozoa) in culture medium are added to a volume of silicone oil and mixed vigorously to form an unstable emulsion. The mixture is poured into a small plastic petri dish where microdroplets form between the hydrophobic surface of the oil and the petri dish, some containing individual organisms. After a suitable period of incubation, these individuals grow and divide to form a clone.

Protocol

Materials

1. Silicone oil (viscosity standard) - 9.6 centipoises, d = 0.94 g/cc, Brookfield Laboratories, Inc., Stoughton, MA, USA.
2. Sterile plastic petri dishes (35 x 10 mm), Falcon, Inc.

Procedure

1. Prepare a suspension of ciliates (or other protozoa) in axenic or bacterized medium at a concentration of about 500 per ml.
2. Add 200 μl (100 ciliates) to 3 ml sterile silicone oil in a screw-cap tube. Vortex vigorously for 10 to 15 seconds and immediately pour the mixture into the petri dish.
3. Stand 15 to 30 minutes to allow the microdroplets to form and incubate at 20 -27°C for 5 to seven days.
4. Examine the plate in a dissecting microscope at 10 - 30 X magnification. Microdroplets containing ciliate (protozoan) populations may be transferred, by means of sterile micropipettes ("pulled" in a gas flame to a diameter of 100 to 200 μl), to larger volumes of culture medium and maintained by serial transfer.

Comments

1. An inverse relationship exists between the number and size (volume) of the microdroplets and the volume plated. Thus, plating 100 μl results in the formation of about 3000 microdroplets per plate; 200μl, 1000 microdroplets per plate; 300μl, 700 per plate; 400 μl, 300 per plate. From these data it can be calculated that the average volume of a microdroplet is $\approx$ 0.03 μl when 100 μl is plated; plating 400 μl of the culture medium results in the formation of microdroplets with a volume of $\approx$ 1.22 μl. This information is important in determining the probability that a given microdroplet containing a population of protozoa is derived from a single individual. This probability may be calculated from the so-called **Poisson** distribution formula:

$$p_r = s^r e^{-s}/r!$$

where r = the **actual** number of protozoa per microdroplet (0, 1, $\geq$2).
s = the **average** number of protozoa per microdroplet.

Hence, after plating a sample containing an average of (s) protozoa, the probability (p), of microdroplets devoid of protozoa (r = 0) is $p_o = e^{-s}$; the frequency of microdroplets containing only one protozoan per microdroplet (r = 1) is $p^1 = se^{-s}$ and so forth. Therefore, if in our example, we dilute a culture to 500 organisms per ml and plate 200 μl, s = 0.1 (since 200 μl produces 1000 microdroplets) we should expect 905 droplets (.905 x 1000) to lack protozoa, 91 droplets (.091 x 1000) to contain one organism each and the remainder to contain $\geq$ 2 protozoa each. Under these conditions, the probability that a population of protozoa in a microdroplet was derived from a single organism is about 0.91. Higher probabilities may be achieved simply by plating fewer organisms per plate.

2. The most significant advantage of SOPP is that the oil prevents loss of microdroplets because of evaporation. Despite the extremely small volumes contained in the microdroplets, plates may be routinely incubated for periods of up to 10 days without loss of microdroplets due to evaporation.

3. Gas exchange across the interface between the hydrophilic aqueous microdroplet and the hydrophobic silicone oil is significantly greater than that observed for water/air interfaces making SOPP eminently suitable for cultivation of highly aerobic organisms.

4. A major drawback of the procedure is that an excess of lipids in the culture medium interferes with the formation of microdroplets due to their surfactant properties. This difficultly may be overcome by reducing the concentration of lipids in the culture medium or by plating the organisms in smaller volumes of culture medium.

LITERATURE CITED

1. Provasoli, L. 1958. A catalogue of laboratory strains of free-living and parasitic protozoa (with sources from which they may be obtained and directions for their maintenance). J. Protozool.**5**,1-38.
2. West. RA, Barbera, PW, Kolar, Jr, Murrell, CB. 1962. The agar layer method for determining the activity of diverse materials against selected protozoa. J. Protozool.**9**:65-73.
3. Asami, K, Nodake,Y, & Ueono, T. 1955. Cultivation of Trichomonas vaginalis on solid medium. Exp. Parasitol. **4**:34-9.
4. McCalla, DR. 1966. Action of some analogs of nitrosoguanidine on the chloroplast of Euglena gracilis. J. Protozool. **13**:472-4.
5. Padan, E, Ginzburg, D, & Shilo, M. 1967. Growth and colony formation of the phytoflagellate Pyremenisium parvum Carter on solid medium. J. Protozool. **14**:477-80.
6. Neff, RJ. 1958. Mechanisms of purifying amoebae by migration on agar surfaces. J. Protozool. **5**:226-31.
7. Samuels, R. 1962. Agar techniques for colonizing and cloning trichomonads. J. Protozool. **9**:103-7.
8. Lazlo, WR. 1961. Obtaining the slime mold Fuligo septica in pure culture. J. Protozool. **8**:97.
9. Soldo, AT & Brickson, SA. 1980. A simple method for plating and cloning ciliates and other protozoa. J. Protozool. **27**:328-31.

ISOLATION AND PURIFICATION OF BACTERIAL SYMBIONTS (XENOSOMES) FROM AXENICALLY CULTIVATED MARINE CILIATES

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Introduction

The method described here was designed specifically to isolate bacterial symbionts (xenosomes) from Parauronema acutum, a marine hymenostome ciliate. It involves passing a homogenate of axenically cultivated xenosome-bearing cells through a paper pulp column (1) followed by final purification on a Percoll gradient (2). With modifications, it may be used for the isolation of symbionts from fresh water ciliates as well. We have successfully applied it to the isolation of lambda, mu and pi symbionts of Paramecium, a fresh water ciliate.

Protocol

Materials and Supplies:

1. 3 cm dia. x 30 cm glass column equipped with a tightly-sealed rubber stopper at one end. Push through the stopper a 19 gauge hypodermic needle so that the upper portion is flush with its upper surface. This is best done by countersinking a hole in the stopper to accommodate the flared portion of the needle. Attach a length of plastic tubing 0.034"in dia. to the needle.
2. Instant Sea Water (d = 1.015 g/cc) (ISW) prepared as follows: One pound Aquamarine salts (Aquamarine, Inc) dissolved in about 8 liters of distilled water. Adjust to 1.030 g/cc with the aid of a hydrometer, filter and store at 4°C. Before use dilute with an equal volume of distilled water.
3. Percoll (Pharmacia, Inc.)

Preparation of paper pulp column:

1. Take four circles of Whatman #1 filter paper (15 cm dia.- about 6.5 gms) and tear into pieces. Place these in a Waring blendor. Add 200 ml ISW and blend for 5 seconds. Pour paper pulp into a 600 ml beaker and rinse in 200 ml ISW. Combine for a total of 400 ml of paper pulp.
2. Place a 3 cm dia. nylon filter (80 mesh) on top of the rubber stopper, followed by a 3 cm dia. plastic screen. Tear one coffee filter in small pieces, crumple and stuff lightly into the column to form a support for the paper pulp. Add about 150 ml of the paper pulp to the column. Drain by gravity to pack the pulp but do not allow the column to become dry. Wash with about 100 ml ISW.

A-4.2

Preparation of Percoll Gradients:

1. For each batch of 500 ml culture prepare 2 x 15 ml Corex tubes, each containing 4 ml Percoll, 1 ml distilled water and 5 ml ISW (d = 1.030 g/cc). Mix by inversion.
2. Chill in an ice bath and store at 4°C until ready for use.

Isolation of Xenosomes

1. Harvest 500 ml of symbiont-bearing P. acutum cells. Wash twice with 100 ml volumes of ISW and resuspend the pelleted cells in 50 ml ISW. Pour into a chilled Waring blendor and homogenize (6 x 15-second bursts). (To reduce foaming, spray the insides of the Waring Blendor with silicone-based anti-foam agent before blending). Check microscopically to insure that breakage is complete.
2. Pour the blendate into the paper pulp column. Allow the first 30 ml (or so) of the eluate to pass through the column. Use this to wash into the column the remainder of the blendate. Then collect 4 x 45 ml volumes of the eluate in each of 4 x 50 capacity round-bottomed Nalgene tubes in an ice bucket.
3. Centrifuge the eluates in the SS-34 head of the Sorvall refrigerated centrifuge at 8000 rpm (7719 x g) for 15 minutes at 4°C. Decant the supernate and combine the pellets in a final volume of 5.0 ml ISW. Layer 2.5 ml portions over each of the Percoll gradients and centrifuge in the SS-34 head at 13,000 rpm (20,000 x g) for 30 minutes.
4. Each gradient will contain two bands. Aspirate to remove the upper band, and continue to a point just above the lower, xenosome-containing band. Re-suspend xenosomes in both lower bands and combine in a final volume of 25 ml ISW. Transfer to a 30 ml capacity Corex tube and centrifuge at 16,000 rpm (30,000 x g) for 15 min at 4°C. The xenosomes deposit as a loosely packed layer just above the densely packed, transparent Percoll pellet. Remove the xenosome-containing band with washings to a 1.5 ml Eppendorf, dilute to 1.5 ml with ISW and microfuge (14,000 x g) 5 minutes. Carefully remove the clear supernate and store the pellet at -80°C.

Comments

1. For each 500 ml of culture processed (3 -4 $x10^8$) cells the yield of xenosomes is about 5 x 10^{10}.
2. Xenosomes obtained in step 4 may still contain a small Percoll pellet. For most uses, this is not a problem. If desired, the Percoll may be removed by re-suspending the xenosomes in 25 ml ISW and centrifuging once again in the SS-34 head of the Servall at 16,000 rpm (30,000 x g) for 15 minutes at 4°C.
3. When applying the method to the isolation of symbionts from fresh water ciliates, use isotonic salt solution in place of ISW.

REFERENCES CITED

1. Preer, JR Jr. 1970. Isolation and purification (of symbionts in Paramecium). Methods Cell. Physiol.,**4**:315-318.
2. Soldo, AT, Brickson, SA, & Castiglione, GA. 1987. Inclusion bodies in xenosomes purified on Percoll gradients as revealed by electron microscopy. J. Protozool.**34**:6-9.

ISOLATION OF ENDOSYMBIOTIC ALGAE FROM LARGER FORAMINIFERA

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Introduction

Larger foraminifera are the hosts for a wide variety of different types of endosymbiotic algae (reviewed in 2). Using the techniques outlined below, we have succeeded in isolating in axenic culture the endosymbiotic chlorophytes, rhodophytes, and dinoflagellates from archaids, soritids, peneroplids, and the endosymbiotic diatoms from calcarinids, numulitids, amphisteginids, and alveolinids(1,3-8). The differences in surface texture and epibiotic colonization among the various types of larger foraminifera present different challanges to the investigator, but the basic technique is the same. The most difficult isolations were those of the endosymbiotic dinoflagellates from soritids. The surfaces of these particular hosts are heavily colonized by bacteria, diatoms, and chlorophytes. Surface crevices and the endolithic nature of some of the chlorophytes compound the dificulty and make it very hard to dislodge the potential contaminating epibionts from these these hosts.

Protocol

1. Collect the larger faraminifera from the marine habitat. SCUBA is most commonly used for harvesting the foraminifera from the sea, but some species (e.g., *Archaias, Sorites* and *Cyclorbiculina*) occur on sea grasses in very shallow water (<2m).
2. After returning to the collection to the laboratory, sort the foraminifera with the aid of a dissecting microscope. Sometimes it is convient to let the foraminifera sort themselves from non-living matter by standing glass microscope slides in the containers with them. The foraminifera will crawl up the slides over night. The slides can be removed and placed in petri dishes. When the dishes with the slides are placed under a dissecting microscope, the foraminifera are easily identified and picked with a sable brush. If the foraminifera have been collected by harvesting substrates (e.g., *Thalassia, Halophila,* sediment, or stones) on(or in) which they occur, the foraminifera first must be washed, or brushed, off the substrate.
3. Transfer the foraminifera to the first well of a sterile 9-hole spot plate containing aseptically added sterile seawater.
4. Place the spot plate under a dissecting microscope.
5. With the aid of a pair of sable artist brushes (00-0000), Carefully and vigorously brush the external surfaces of the foraminifera. One brush is used to hold the foraminifer the other is used to brush it. After the they have been used to perform the brushing in one well, the brushes are placed in 70% ethyl alcohol for 5 minutes and placed aside to dry before being used again. With a fresh brush transfer the foraminifera to the next well with sterile seawater.
6. Repeat step 5 another 17 times.
7. With the aid of a fresh sable brush, transfer each foraminifer to its own well with sterile seawater. Consistant with the experimental setup, examine each one at the highest

6. Repeat step 5 another 17 times.
7. With the aid of a fresh sable brush, transfer each foraminifer to its own well with sterile seawater. Consistant with the experimental setup, examine each one at the highest magnification available. One can use a 40X water immersion lens in a compound microscope if it is available. If the foraminifera appear free of external contamination, proceed to step 8; if not proceed to step 7a.

7a. It was not possible to remove all external algae from the surfaces of *Amphisorous hemprichii, Sorites marginalis, or Marginopora kudakajimensis* by only brushing and washing(4). The following additional steps were necessary for them. The seawater in each well is withdrawn by pipette and replaced with seawater which contains either 0.01N acetic acid, 10μM EDTA, or 0.01N HCl. Each of these decalcifying agents worked equally well. Observe under a dissecting microscope. The foraminifer should have a normally extended feeding web and the shell should be dissolving slowly(turning translucent, with no bubbles). If the dissolution process slows down withdraw the decalcifying medium and replace it with a fresh one. When the process is complete, wash each foraminifer in a well by removing the medium by pipet and gently replacing it with fresh sterile media. this can be repeated until no external algae are observed. After disrupting the foraminifer with a sterile pasteur pipete, proceed to step 9.

8. Sterilize, with the aid of flaming alcohol, a pair of fine forcepts (e.g., Dumont #5). Use one to tightly hold the foraminifer and the other to destroy and break open the test.
9. Aseptically pipete 0.1ml of the contents of each well into a test tube containing one of the following agar solidified (15g/l) media: Seawater, Erdschreiber[Add soil extract (5 ml/100ml), $NaNO_3$, or NH_4NO_3 (10 mg/100 ml), and $NaH_2PO_4.H_2O$ (5mg/100 ml) to 95 ml of the local seawater;Adjust the pH of the water if it has changed (e.g. coastal sea water should be pH 8.1], or medium "S"(Appendix). Just before pouring the petri plates, aseptically add an antibiotic-antimycotic mixture (1 ml/100 ml; GIBCO Laboratories catalog #600-5240; contains: Penicillin 10,000 units,streptomycin 10,000μg, and Fungizone 25 μg/ ml). Mix thoroughly and pour the plates. Spread the inoculum over the surface of the plates with the aid of a alcohol sterilized bent glass rod. Liquid media can also be used. With the difficult dinoflagellate -bearing hosts we found it advantageous to add GeO_2(1-10mg/l) to the media to inhibit the growth of any remaining diatoms.
10. Place the inoculated plates into plastic sandwich bags and seal them or tape them closed.
11. Incubate the plates, agar side up, near an artificial or indirect natural light source (30-50μE).
12. After the plates have been incubated for two weeks, remove them from the plastic bags and examine them under a disscecting microscope. Chlorophytes, rhodophytes, dinoflagellates, and some diatoms tend to form circular, entire, and raised colonies. Many other diatoms for colonies with irregular boarders, rectilinear colonies, colonies in the form of long chains, colonies with compact centers and motile diatoms at the edges, colonies with various types of punctuated growth patterns. Keys to a variety of colonies and illustrations have been published previously(1).
13. Choose well separated colonies and ring them with a wax pencil on the bottom of the dish.
14. Pick a colony with the aid of an alcohol-sterilized and flamed spatula(e.g.,Fisher Scientific Co., catalog #21-401-10, tip hand ground to a width of 1 mm) and transfer it to a liquid medium of the same type you used for the initial isolation.
15. Incubate in the light for 7-10 days.
16. Restreak on media without antibiotics to test for contamination.

COMMENTS

As mentioned above this protocol has been sucessfully employed to isolate the algal endosymbionts from a variety of larger foraminifera. The success rate for isolating the algae in culture has been close to 100% in recent years, but it was as low as 20% in the beginning(3). If one uses solid media one always gets axenic clones of the endosymbionts. One disadvantage is that it is possible to miss a second, or minor, endosymbiont if the colony types are similar and the number of colonies sampled is small. Transfer of the entire contents of the well to liquid media makes it easier to recognize several symbionts in the same host because they will both grow in the medium. It adds an extra step because if more than one species is present, the isolates may have to be separated by cloning them on agar.

LITERATURE CITED

1.Lee, J J. 1990. Fine structure of the rhodophycean *Porphyridium pupureum* in situ in *Peneroplis pertusus* (Forskol) and *P. acicularis* (Batsch) and in axenic culture. *J. Foram. Res.* **20**:162-169.
2.------- & Anderson, O R. 1991 Symbiosis in foraminifera.In: **Biology of Foraminifera** (Lee, J J & Anderson, O R, eds). Academic Press, London.pp157-220.
3.--------, Crockett, L J , Hagen, J & Stone, R. 1974. The taxonomic identity and physiological ecology of *Chlamydomonas hedleyi* sp. nov., algal flagellate symbiont from the foraminifer *Archaias angulatus. British J. Phycol.* **9**:407-422.
4.-------- & Lawrence, C. 1990. Endosymbiotic dinoflagellates from the larger foraminifera *Amphisorus hemprichi* and *Sorites marginalis*. In: **Endocytobiology IV** (Nardon, P, Gianinazzi-Pearson, V, Grenier, A M, Margulis, L & Smith, D C. eds) Institut National de la Recherche Agronomique, Paris
5.---------, McEnery, M E, Kennedy, E M, & Rubin, H. 1975. A nutritional analysis of a sublittoral diatom assembage epiphytic on *Enteromorpha* from a Long Island salt marsh. *J Phycol* **11**:14-49.
6.--------, ----------, ter Kuile, b, Erez, J, Röttger, R, Rockwell, R F, Faber, W W & Lagziel, A. 1989. Identification and distribution of endosymbiotic diatoms in larger foraminifera. *Micropaleontology* **35**:353-366.
7.-------, Faber, W W,Jr, Nathanson, B, Röttger, R, Nishihira, M. 1992. Endosymbiotic diatoms from larger foraminifera collected in Pacific Habitats. *Symbiosis* **13**: (in Press)
8.------- & Reimer, C W. 1984. Isolation and identification of endosymbiotic diatoms from larger foraminifera of the Great barrier Reef, Australia, Makapuu tide pool, Oahu, Hawaii, and the Gulf of Elat, Israel, with description of new species,*Amphora roettgerii, Navicula hanseniana,* and *Nitzschia frustulum* variety *symbiotica* . **Proc. VII Inter. Symp. Living and Fossil Diatoms**(Mann, D G, ed.)Koeltz Scientific Books, Koenigstein,Germany

APPENDIX

Synthetic medium "S" for the isolation and maintance of endosymbiotic algae from Foraminifera.

	Concentration
NaCl	2.5%
$MgSO_4.7H_2O$	0.9%
KCl	0.07%
$CaCl_2$	0.03%
NH_4NO_3	0.025%
Na glycerol PO4	0.005%
$NaSiO_3.9H_20$	0.007%
P II Metals *	3ml%
Tris	0.1%
Vitamin B_{12}	0.1μm
NTA	0.0007%
NaH_2CO_3	0.01%
Vitamins 8A*	1ml%
Na Acetate	1mM

*For source see (6)

CULTIVATION OF SELECTED PHOTOSYNTHETIC CRYPTOMONADS

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INTRODUCTION

Most photosynthetic Cryptomonads are marine. *Campylomonas* and *Komma* appear to be restricted to freshwater. Protocols for the axenic and xenic cultivation of selected genera are presented below.

PROTOCOL

Freshwater, Xenic, *Campylomonas*, *Komma*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

DY IV [1]

$MgSO4{\cdot}7H_2O$	50.0 mg
KCl	3.0 mg
NH_4Cl	2.7 mg
Na_2NO_3	20.0 mg
ß-Na_2 glycerophosphate	2.2 mg
H_3BO_3	0.8 mg
Na_2EDTA	8.0 mg
$NaSiO_3{\cdot}9H_2O$	14.0 mg
$FeCl_3$	1.0 mg
$CaCl_2$	75.0 mg
MES* (Sigma #M8250)	200.0 mg
Vitamin Solution (see below)	1.0 ml
Trace Metals Solution (see below)	1.0 ml
Glass distilled H_2O	998.0 ml

*MES = (2-[N-Morpholino]ethanesulfonic acid)

Vitamin Solution

Vitamin B_{12}	1.0 mg
Biotin	1.0 mg

Thiamine HCl	200.0 mg
Glass distilled H_2O	1.0 L

Dissolve components in H_2O, dispense in 10.0 ml aliquots and store at -20° C until needed.

Trace Metals Solution

$MnCl_2$	200.0 mg
$MnSO_4$	40.0 mg
$CoCl_2$	8.0 mg
Ma_2MoO_4	20.0 mg
Na_2VO_4	2.0 mg
H_2SeO_3	2.0 mg
Glass distilled H_2O	1.0 L

Add in order indicated, dissolving each component before the addition of the next.

3. Combine components and adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. The light intensity, photoperiod, and the incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Marine, Xenic

Falcomonas, Plagioselmis, Proteromonas, Protochrysis, Pyrenomonas, Rhinomonas, Rhodomonas, Teleaulax

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

f/2-si Medium [2]

$NaNO_3$ stock solution (see below)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ stock solution (see below)	1.0 ml
Trace metals solution	1.0 ml
Vitamin Solution	0.5 ml
Filtered seawater*	1.0 L

$NaNO_3$ Stock Solution

$NaNO_3$	75.0 g
Glass distilled H_2O	1.0 L

$NaH_2PO_4 \cdot H_2O$ Stock Solution

$NaH_2PO_4 \cdot H_2O$	5.0 g
Glass distilled H_2O	1.0 L

Trace Metals Solution

Na_2EDTA	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$CuSO_4 \cdot 5H_2O$ (0.98g/100ml)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (2.2g/100ml)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (1.0g/100ml)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (1.8g/100ml)	1.0 ml
$NaMoO_4 \cdot 2H_2O$(0.63g/100ml)	1.0 ml
Glass distilled H_2O	1.0 L

Vitamin Solution

Vitamin B_{12}	1.0 mg
Biotin	1.0 mg
Thiamine HCl	200.0 mg
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O, dispense in 10 ml aliquots and store at -20° C until needed.

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Combine components and adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. The light intensity, photoperiod, and the incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Marine, Axenic, *Chroomonas*, *Hemiselmis*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

DCM Medium [1]

Tris	5.0 g
Na_2 acetate$\cdot 3H_2O$	0.5 g
Na glutamate	0.5 g
DL-Glycine	0.5 g
Sucrose	0.5 g
ß-Na_2 glycerophosphate	0.4 g
$NaNO_3$ stock solution (7.5 g/100 ml)	6.7 ml
Lactic acid	1.0 ml

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Glycerine (10.0 g/100ml)	5.0 ml
Trace Metals solution (see below)	1.0 ml
NH_4Cl (0.27 g/100 ml)	1.0 ml
H_2SeO_3(0.13 mg/100 ml)	1.0 ml
Vitamin solution	1.0 ml
Natural seawater*	800.0 ml
Double glass distilled H_2O	200.0 ml

Trace Metals Solution

Na_2EDTA	0.686 g
$FeCl_3 \cdot 6H_2O$	0.315 g
$CuSO_4 \cdot 5H_2O$ (0.98 g/100 ml)	0.05 ml
$ZnSO_4 \cdot 7H_2O$ (2.2 g/100 ml)	3.0 ml
$CoCl_2 \cdot 6H_2O$ (1.0 g/100 ml)	0.1 ml
$MnCl_2 \cdot 4H_2O$ (1.8 g/100 ml)	2.0 ml
$NaMoO_4 \cdot 2H_2O$ (0.63 g/100 ml)	0.1 ml
Glass distilled H_2O to	100.0 ml

Vitamin Solution

Vitamin B_{12}	1.0 mg
Thiamine HCl	2000.0 mg
Biotin	1.0 mg
Folic acid	2.0 mg
PABA	1.0 mg
Niacin	100.0 mg
Inositol	1000.0 mg
Ca pantothenate	200.0 mg
Pyridoxine	100.0 mg
Glass distilled H_2O	1.0 L

Store frozen at -20° C until needed.

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Combine components and adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 $\mu Ein/m^2/s$ irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

Some strains of *Rhodomonas* can be cultivated axenically on f/2-si medium [1]. Artificial seawater may be substituted for natural seawater. There are many sources for artificial seawater

mixes. These mixes may vary in quality from lot to lot. Medium perpared with a new lot of artificial seawater should be tested in parallel with a medium prepared with a currently used lot to determine if it supports equivalent growth.

LITERATURE CITED

1. Anderson, RA, Jacobson, DM & Sexton, JP 1991. Provasoli-Guillard Center for Culture of Marine Phytoplankton. Catalogue of Strains. Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, MA 98 pp.

2. Guillard, RRL 1975. Culture of phytoplankton for feeding marine invertebrate animals using a new lot of artificial seawater. Smith, WL & Chanley, MH, ed., Plenum Press, New York pp. 26-60.

A-7.1

CULTIVATION OF SELECTED COLORLESS CRYPTOMONADS

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INTRODUCTION

The most common genera of colorless Cryptomonads are readily cultivated as monoprotist cultures with bacteria as a food source. There exist both freshwater and seawater strains of *Chilomonas* and *Goniomonas*. Only strains of *Chilomonas* have been reported to be cultivable under axenic conditions. Methods for cultivation of freshwater and seawater strains of both genera and the axenic cultivation of freshwater strains of *Chilomonas* are provided [1,2].

PROTOCOL

Freshwater, Xenic, *Chilomonas*, *Goniomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.

5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0	g
Glucose	0.16	g
Agar	20.0	g
Glass distilled H_2O	800.0	ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Cereal Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C overnight.
14. Aseptically add 0.1 ml of a growing protist culture which is at or near peak density to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Marine, Xenic, *Chilomonas, Goniomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Cereal Modified Grass Infusion Medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.
**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold

under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solutions prepared in steps 3 and 4.
6. Aseptically distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

PROTOCOL

Freshwater, Axenic, *Chilomonas*

1. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

CHM medium [2]

Sodium acetate·$3H_2O$	1.0 g
Lab Lemco Powder* (cat. #CM15)	1.0 g
Glass distilled H_2O	1000.0 g

*Distributed in the United States by Unipath Incorporated, Columbia, MD 21045

3. Combine the ingredients and distribute in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes. Autoclave at 121° C for 15 min with caps loosened one half turn.
4. When cooled inoculate fresh tube of medium with 0.1 ml of a peak culture.
5. Transfer at 7-14 d intervals.

COMMENTS

Members of the genera *Chilomonas* and *Goniomonas* have never been reported to form cysts. They must, therefore, be passaged at regular intervals or the culture will be lost.

LITERATURE CITED

1. Nerad, TA 1991. Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.
2. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of Strains 1988. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.

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CULTIVATION OF PHOTOSYNTHETIC DINOFLAGELLATES

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INTRODUCTION

Methods for the xenic cultivation of selected freshwater and marine photosynthetic dinoflagellates are presented below. In addition a method for the axenic cultivation of selected marine photosynthetic dinoflagellates is also included.

PROTOCOL

Marine, Xenic
Amphidinium, Glenodinium, Prorocentrum, Scrippsiella

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

ASW Medium [1]

Artificial seawater*	33.6 g
Tricine	0.5 g
Extra salts Solution (see below)	3.75 ml
Soil Extract Stock (see below)	25.0 ml
Vitamin Solution (see below)	4.0 ml
Glass distilled H_2O	1.0 L

Extra Salts Solution

$NaNO_3$	30.0 g
Na_2HPO_4	1.2 g
K_2HPO_4	1.0 g
Glass distilled H_2O	1.0 L

Soil Extract Stock

Air-dried rich organic soil	333.0 g
Glass distilled H_2O	667.0 ml

Adjust to pH 8.0 with 1N NaOH or 1N HCl and autoclave for 1 h at 121° C. Decant and filter through Whatman #1 filter.

Vitamin Solution

Biotin	0.2 mg

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Calcium pantothenate	20.0 mg
Vitamin B_{12}	4.0 g
Folic acid	0.4 g
Inositol	1000.0 mg
Nicotinic acid	20.0 g
Thiamine HCl	100.0 g
Thymine	600.0 mg
Glass distilled H_2O	1.0 L

After mixing, filter sterilize using a 0.2μm filter. Store in 10.0 ml aliquots at -30° C until needed.

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Add 25.0 ml soil extract stock and 3.75 ml extra salts solution to artificial seawater and Tricine and make up to 1.0 L with glass distilled H_2O. After dissolving, adjust pH to 7.6 - 7.8 with 1N NaOH or 1N HCl.
4. Autoclave for 15 min at 121° C.
5. When cooled, aseptically add filter sterilized Vitamin Solution.
6. Aseptically dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
7. Aseptically inoculate a tube with a 0.1 ml aliquot of a growing culture at or near peak density.
8. Loosen caps one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
9. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Xenic, *Woloszynskia*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

DM Medium [1]

$Ca(NO_3)_2 \cdot 4H_2O$ (2.0 g/100.0 ml)	1.0 ml
H_2PO_4 (1.24 g/100.0 ml)	1.0 ml
$MgSO_4 \cdot 7H_2O$ (2.5 g/100.0 ml)	1.0 ml
$NaHCO_3$ (1.59 g/100.0 ml)	1.0 ml
EDTA Solution (see below)	1.0 ml
Trace metals solution (see below)	1.0 ml
Vitamin solution (see below)	1.0 ml
Na_2SiO_3 (1.22 g/100.0 ml)	1.0 ml
Glass distilled H_2O	1.0 L

EDTA Solution

FeNa EDTA	225.0 mg
Na_2 EDTA	225.0 mg
Glass distilled H_2O	1.0 L

Trace Metals Solution

H_3BO_3	248.0 mg
$MnCl_2 \cdot 4H_2O$	139.0 mg
$(NH_4)6Mo_2O_{24} \cdot 4H_2O$	100.0 mg

Vitamin Solution

Vitamin B_{12}	4.0 mg
Vitamin B_1	4.0 mg
Biotin	4.0 mg
Glass distilled H_2O	100.0 ml

3. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes, loosen cap one half turn and autoclave for 15 min at 121° C.
4. Allow to cool and aseptically inoculate a tube with a 0.1 ml aliquot of a growing culture at or near peak density.
5. Loosen caps one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
6. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Marine, Axenic, *Amphidinium*, *Prorocentrum*, *Scrippsiella*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

E26: ANT Medium [1]

E26 Medium (see below)	500.0 mg
Antia's Medium (see below)	500.0 ml

E26 Medium

Nutrient solution (see below)	500.0 ml
Artificial seawater*	500.0 ml

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

Nutrient Solution

KNO_3	100.0 mg
$MgSO_4 \cdot 7H_2O$	100.0 mg
K_2HPO_4	10.0 mg
Vitamin B_{12}	0.1 mg

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Vitamin B_1	0.05 mg
Soil extract stock (see below)	50.0 ml
Glass distilled H_2O	450.0 L

Soil Extract Stock

Air-dried soil	330.0 g
Glass distilled H_2O	670.0 ml

Adust pH to 8.0 with 1N NaOH or 1N HCl and autoclave for 1 h at 121° C. Allow soil to settle and filter sterilize.

Antia's Medium

Tris	200.0 mg
KNO_3	50.0 mg
$NaH_2PO_4 \cdot 2H_2O$	7.8 mg
Vitamin B_1	500.0 mg
Na_2 EDTA	8.1 mg
Glycine	300.0 mg
Trace Metals Solution (see below)	2.5 mg
Glass distilled H_2O	1.0 L

Trace Metals Solution

$FeCl_3$*	650.0 mg
$Na_2MoO_4 \cdot 2H_2O$	97.0 mg
$MnSO_4 \cdot 4H_2O$	450.0 mg
$ZnSO_4 \cdot 7H_2O$	230.0 mg
$CuSO_4 \cdot 5H_2O$	10.0 mg
$CaSO_4 \cdot 7H_2O$	5.0 mg
Glass distilled H_2O	1.0 L

*Dissolve $FeCl_2$ in 1.0 ml concentrated HCl.

3. Autoclave E26 Medium and Antia's Medium separately for 20 min at 121° C.
4. When cooled, combine equal volumes of the two solutions.
5. Aseptically dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
6. Aseptically inoculate a tube with a 0.1 ml aliquot of a growing culture at or near peak density.
7. Loosen caps one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

Dinoflagellates are often very difficult to cultivate in high densities.

LITERATURE CITED

1. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of Strains 1988. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.

A-9.1

CULTIVATION OF COLORLESS DINOFLAGELLATES

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INTRODUCTION

Colorless dinoflagellates of the genera *Crypthecodinium*, *Gyrodinium*, *Noctiluca*, and *Oxyrrhis* have been cultivated axenically in a number of empirical formulations [1,2,3,4]. The first colorless dinoflagellate to be maintained axenically, *Crypthecodinium cohnii*, was cultivated on an empirical formulation consisting of peptone, yeast autolysate and acetate [4]. A completely defined medium for the growth of this species was reported in 1962 [5]. The cultivation protocol below can be used for the maintenance of most of the sibling species of this marine dinoflagellate.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

A_2E_6 Medium

NaCl	23.48 g
$MgCl_2 \cdot 6H_2O$	10.63 g
Na_2SO_4	3.92 g
$CaCl_2$ (anhydrous)	1.11 g
KCl	0.66 g
$NaHCO_3$	0.19 g
KBr	0.1 g
H_3BO_3	0.03 g
$SrCl_2 \cdot 6H_2O$	0.04 g
Metal Mixture (see below)	3.0 ml
$FeCl_3 \cdot 6H_2O$	0.01 g
Na_2 Glycerophosphate	0.15 g
$(NH_4)_2SO_4$	0.05 g
Tris Buffer	3.0 g
Vitamin Solution (see below)	1.0 ml
K_2HPO_4	0.01 g
Glucose	3.0 g
Glutamic acid	1.5 g

A-9.2

Glass distilled H_2O . . . . . 1.0 L
Adjust pH to 6.4 - 6.6. Dispense 5 ml per 16 x 125 mm screw capped test tube and autoclave.

3. Adjust pH to 6.4-6.6 with 1N NaOH or 1N HCl.
4. Distribute medium in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes, loosen caps one half turn and autoclave for 15 min at 121° C. Allow medium to cool before inoculation.
5. Inoculate a tube of medium prepared in step 4 with 0.1 ml from a culture at or near peak density.
6. Loosen the cap one half turn and incubate at 25° C.
7. Repeat steps 5-6 at 10-14 d interval.

COMMENTS

Growth of most strains of *Crypthecodinium cohnii* is excellent using the A_2E_6 medium. However, a few strains can only be maintained by adding 0.2% (w/v) Casein Hydrolysate.

LITERATURE CITED

1. Droop, MR 1959b. Water-soluble factors in the nutrition of *Oxyrrhis marina*. *J. Mar. Biol. Assoc.* **38**:605-620.
2. Lee, RE 1977. Saprophytic and phagocytic isolates of the colorless heterotrophic dinoflagellates *Gyrodinium lebouriae* Herdman. *J. Mar Biol. Ass.* **57**:303-315.
3. McGinn, MP & Gold, K 1969. Axenic cultivation of *Noctiluca scintillans*. *J. Protozool.* **16**(suppl.):33.
4. Pringsheim, EG 1956. Micro-organisms from decaying seaweed. *Nature* **178**:480-481.
5. Provasoli, L & Gold, K 1962. Nutrition of the American strain of *Gyrodinium cohnii*. *Arch. Mikrobiol.* **42**:196-203.

A-10.1

CULTIVATION OF PHOTOSYNTHETIC EUGLENIDS

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INTRODUCTION

Many species of photosynthetic euglenids can easily be grown on an axenic liquid medium developed by Dr. Seymour H. Hunter or on agar slants. For most strains cultivation at temperatures of 22-25° C will result in excellent growth if an appropriate light intensity and light/dark cycle are provided [1].

PROTOCOL

Liquid Medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

KH_2PO_4	0.02 g
K-citrate·H_2O	0.04 g
$MgSO_4 \cdot 3H_2O$	0.02 g
Trypticase (BBL)	0.6 g
Liver concentrate NF #XI powder* (ICN Biomedicals, Inc. #100377)	0.2 g
Glass distilled H_2O	1.0 L

*Equivalent to Liver-extract concentrate (1:20). Yeast extract (0.4 g), thiamine HCl (0.4 mg) and vitamin B_{12} (0.5 μg) may be substituted for the liver component.

4. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
5. Autoclave 15 min at 121° C. Leave caps loosened about one half turn while autoclaving. Tighten the caps immediately upon removing the tubes from the autoclave. Cool medium before use.
6. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
7. Keep cap loosened a half turn and incubate at 20-25° C under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.

8. For routine maintenance subculture every 10-14 d. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Agar Medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the component should be obtained from the company to assure growth.
2. Medium formulation:

Yeast extract	1.0 g
Beef extract	1.0 g
Tryptose	2.0 g
$FeSO_4$	Trace
Glucose	10.0 g
Agar	5.0 g
Glass distilled H_2O	1.0 L

3. Adjust pH to 7.2 with 20% (w/v) NaOH.
4. Autoclave 30 min at 121° C in a flask with the screw-cap loosened about one half turn.
5. While still liquid dispense 5 ml of medium per 16 x 125 mm screw-capped test tube. Place tubes on a horizontal slant and allow to cool until the agar has hardened.
6. Inoculate medium with a growing culture at or near peak density using an inoculating loop. The inoculum should be spread from the base of the slant to within no more than one centimeter of the top of the slant by a back and forth motion.
7. Keep cap loosened one half turn and incubate at 20-25° C under 50-100 $\mu Ein/m^2/s$ irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. For routine maintenance subculture every 1-4 wk. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

The second medium can also be used as a liquid medium. Eliminate agar and reduce concentration of the dry ingredients to $^1/_3$ of those listed above.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.

A-11.1

CULTIVATION OF OSMOTROPHIC EUGLENIDS

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INTRODUCTION

There are a number of genera of euglenids which lack chloroplasts and are osmotrophic. A single protocol which supports the growth of members of six genera is presented below, as well as, protocols for the axenic cultivation of *Astasia* and *Khawkinea* [1,2,3].

PROTOCOL

Xenic
Gyropaigne, Hyalophacus, Menoidium
Parmidium, Rhabdomonas, Rhabdospira

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Soil/H_2O Biphasic Medium [3]

Rich Organic Soil (Air-dried)	125.0 g
Glass distilled H_2O	875.0 ml

3. Place soil in the bottom of a flask and gently overlay with the glass distilled H_2O, etc.
4. Autoclave at 121° C for 15 min. Allow to cool overnight.
5. Repeat step 4.
6. Gently remove the supernatant and filter sterilize.
7. Dispense in 10.0 ml aliquots to T-25 tissue culture flasks.
8. Inoculate flasks with 0.1 ml from a culture at or near peak density.
9. Incubate at 20-25° C with the cap screwed on tightly.
10. Transfer at 7-14 d intervals. Some strains can be maintained for days or weeks after they have reached peak density without transfers. To avoid losing the cultures, transfer at regular intervals.

A-11.2

PROTOCOL

Axenic, *Astasia*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Hutner's Medium for *Euglena* [1]

KH_2PO_4	0.02 g
K-citrate·H_2O	0.04 g
$MgSO_4 \cdot 3H_2O$	0.02 g
Trypticase (BBL 1921)	0.6 g
Na acetate	0.1 g
Liver concentrate NF #XI powder* (ICN Biomedicals, Inc. #100377)	0.2 g
Glass distilled H_2O	1.0 L

*Can be substituted with yeast extract (0.4g), thiamine HCl (0.4 mg) and Vitamin B12 (0.5 μg) may be substituted for the Liver L.

3. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-caped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Incubate at 20-25° C with the cap loosened one half turn.
7. Transfer at 7-14 d intervals. Some strains can be maintained for days or weeks after they have reached peak density without transfers. To avoid losing the cultures, transfer at regular intervals.

PROTOCOL

Axenic, *Khawkinea*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

EG Medium [2]

Na acetate	1.0 g
Beef extract	1.0 g
Tryptone (Difco #0123)	2.0 g
Yeast extract	2.0 g
$CaCl_2$	0.01 g
Glass distilled H_2O	1.0 L

3. Dispense in 5.0 ml aliquot to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Incubate at 20-25° C with the cap loosened one half turn.

7. Transfer at 7-14 d intervals. Some strains can be maintained for days or weeks after they have reached peak density without transfers. To avoid losing the cultures, transfer at regular intervals.

COMMENTS

Cysts have not been reported in any of these genera. Therefore, if the cultures are not passaged regularly they will be lost from culture.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.
2. George, EA 1976. Culture Centre of Algae and Protozoa List of Strains 1976. (3rd ed.) Institute of Terrestrial Ecology, Natural Environment Research Council, Cambridge, England. 120 pp.
3. Pringsheim, EG 1946. The biphasic or soil-water culture method for growing algae and flagellata. *J. Ecol.* **33**:193-204.

CULTIVATION OF *ISONEMA* AND RELATED FLAGELLATES

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INTRODUCTION

Isonema and related flagellates (*Diplonema* and *Rhynchopus*) can be cultivated as monoprotist cultures with mixed bacteria as the food source or axenically. Most strains can be cultivated in the axenic state.

PROTOCOL

Xenic

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Cereal Modified Grass Infusion Medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.
**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St.

ouis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seatwer.
4. Add 2.5 g rye cerophyl to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solutions prepared in steps 3 and 4. Autoclave for 30 min at 121° C. Allow to cool.
6. Aseptically distribute medium in 10 ml amounts to T-25 plastic tissue culture flasks and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with the flagellate.
7. Aseptically inoculate the flask with a 0.1 ml aliquot of a growing culture at or near peak density.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

PROTOCOL

Axenic

1. Medium formulation:

Artificial seawater*	1.0 L
Enrichment solution (see below)	10.0 ml
Vitamin solution (see below)	1.0 ml

Enrichment Solution

$Na_2EDTA{\cdot}2H_2O$	0.553 g
$NaNO_3$	4.667 g
$Na_2SiO_3{\cdot}9H_2O$	3.000 g
Na_2glycerol$\cdot PO_4$	0.667 g
H_3BO_3	0.380 g
$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$	0.234 g
$FeCl_3{\cdot}6H_2O$	0.016 g
$MnSO_4{\cdot}4H_2O$	0.054 g
$ZnSO_4{\cdot}7H_2O$	7.3 mg
$CoSO_4{\cdot}7H_2O$	1.6 mg
Glass distilled H_2O	1.0 L

Neutralize Na_2SiO_3 with 1N HCl.

Vitamin Solution

Thiamine	0.1 g
Vitamin B_{12}	2.0 mg
Biotin	1.0 mg

Prepare artificial seawater according to the package directions. Filter sterilize in a 0.2 μm filter.

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated

carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

2. Autoclave artificial seawater for 15 min at 121° C.
3. Combine ingredients of the enrichment solution in the order indicated and filter sterilize.
4. Combine ingredients of the vitamin solution in the order indicated and filter sterilize.
5. After the artificial seawater has cooled, aseptically combine the three solutions in the proportions indicated.
6. Distribute 9.0 ml to a T-25 plastic tissue culture flask and aseptically add 1.0 ml (10% v/v) of heat-inactivated (56° C for 30 min) horse serum.
7. Aseptically inoculate the flask with 0.1 ml aliquot of a growing culture.
8. Incubate at 25° C for 7-14 d and repeat step 7.

COMMENTS

Most species of *Diplonema*, *Isonema*, and *Rhynchopus* are marine. The only species reported from freshwater, *D. ambulator*, was isolated from the tissues of *Crytocoryne*, a freshwater aquarium plant. The axenic medium for the marine species was developed by Nerad [1]. The medium for the axenic cultivation of the only freshwater species [2] is essentially the same as that for the marine species except that the basal medium is Sonneborn's *Paramecium* medium (see Cultivation of Selected Cryptomonads). This medium was suggested by Nerad for the axenic cultivation of the freshwater species. Bacterized cultivation is essentially identical to that for other bactivorous marine protists. For larger volumes of culture increase inoculum, medium and vessel size proportionally.

LITERATURE CITED

1. Nerad, TA 1990. The Life History, Cytology and Taxonomy of *Isonema* and *Isonema*-like Flagellates. Ph.D. Thesis, University of Maryland, College Park, MD, 229 pp.
2. Triemer, RE & Ott, DW 1990. Ultrastructure of *Diplonema ambulator* Larsen and Patterson (Euglenozoa) and its relationship to *Isonema*. *Europ. J. Protistol.* **25**:316-320.

A-13.1

CULTIVATION OF SELECTED PHOTOSYNTHETIC CHRYSOMONADS

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INTRODUCTION

Many genera of photosynthetic chrysomonads can be cultivated under xenic conditions. Protocols for the xenic cultivation of marine and freshwaters forms are presented below. Protocols for the axenic cultivation of freshwater representatives of *Ochromonas* and *Poteriochromonas* are also presented.

PROTOCOL

Xenic, Freshwater

Chromophyton, Chromulina, Chrysochaete, Chrysocaccus, Chrysosphaera, Epichrysis, Epipyxis, Hibberdia, Lagynion, Lepochromulina, Mallomonas, Ochromonas, Phaeoplaea, Phaeoschizochlamys, Phaethamnion, Poteriochromonas, Stichogloea, Synura

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

DY III Medium [3]

ß-NA_2-glycerophosphate	100.0 mg
NH_4NO_3	200.0 mg
$CaCl_2 \cdot 2H_2O$	750.0 mg
$NaSO_3 \cdot 9H_2O$	150.0 mg
$MgSO_4 \cdot 7H_2O$	500.0 mg
KCl	30.0 mg
Na_2EDTA	80.0 mg
Fe(electrolytic)*	7.0 mg
MnCl	2.0 mg
$ZnSO_4 \cdot 7H_2O$	0.4 mg
$CoCl_2 \cdot 6H_2O$	0.08 mg
$Na_2MoO_4 \cdot 2H_2O$	0.2 mg
H_3BO_3	50.0 mg

A-13.2

MES**(Sigma M8250)	200.0 mg
Vitamin Solution (see below)	1.0 ml
Glass distilled H_2O	1.0 L

Vitamin Solution

Thiamine HCl	0.02 mg
Vitamin B_{12}	0.50 mg
Biotin	0.50 mg
Glass distilled H_2O	1.0 L

*Dissolve 70 mg of iron wire in 10 ml of concentrated HCl.
**MES = (2-[N-Morhpoline]ethanesulfonic acid)

3. Adjust pH to 6.8 with 2N NaOH or 2N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Xenic, *Ochromonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Fluid Thioglycollate Medium

Fluid Thioglycollate Powder (BBL #11260)	29.5 g
Glass distilled H_2O	1.0 L

3. Combine components and boil to dissolve. Allow to cool and adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Axenic, *Poteriochromonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Poteriochromonas Medium [1]

Bacto-Vitamin free casamino acids	10.0	g
Bacto-Dextrose	20.0	g
Diamnonium Hydrogen Citrate	1.6	g
KH_2PO_4	0.6	g
$MgSO_4 \cdot 7H_2O$	0.4	g
$CaCl_3$	0.3	g
EDTA	0.1	g
$MnSO_4 \cdot H_2O$	0.123	g
$ZnSO_4 \cdot 7H_2O$	0.22	g
Thiamine·HCl	4.0	mg
Tween 80	2.0	ml
$FeSO_4 \cdot 7H_2O$	20.0	mg
$CoSO_4 \cdot 7H_2O$	6.0	mg
$CuSO_4 \cdot 5H_2O$	0.8	mg
H_3BO_3	1.2	mg
KI	0.02	mg
$Na_2MoO_4 \cdot 2H_2O$	0.1	g
DL-Tryptophan	0.2	g
DL-Methionine	0.4	g
L-Cystine	0.2	g
Choline Chloride	4.0	mg
Inositol	20.0	mg
PABA	2.0	mg
Vitamin B_{12}	0.4	mg
Biotin	0.02	mg
Glass distilled H_2O	1.0	L

3. Adjust final pH to 5.8. Heat medium to boiling for 2-3 min.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 $\mu Ein/m^2/s$ irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

A-13.4

PROTOCOL

Xenic, Marine

Chrysoderma, Chrysophaera, Girandyopsis, Pelagococcus, Sarcinochrysis, Stichochrysis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

f/2-si Medium [2]

$NaNO_3$ stock solution (see below)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ stock solution (see below)	1.0 ml
Trace metals solution	1.0 ml
Vitamin Solution	0.5 ml
Filtered seawater*	1.0 L

$NaNO_3$ Stock Solution

$NaNO_3$	75.0 g
Glass distilled H_2O	1.0 L

$NaH_2PO_4 \cdot H_2O$ Stock Solution

$NaH_2PO_4 \cdot H_2O$	5.0 g
Glass distilled H_2O	1.0 L

Trace Metals Solution

Na_2EDTA	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$CuSO_4 \cdot 5H_2O$ (0.98g/100ml)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (2.2g/100ml)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (1.0g/100ml)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (1.8g/100ml)	1.0 ml
$NaMoO_4 \cdot 2H_2O$(0.63g/100ml)	1.0 ml
Glass distilled H_2O	1.0 L

Vitamin Solution

Vitamin B_{12}	1.0 mg
Biotin	1.0 mg
Thiamine HCl	200.0 mg
Glass distilled H_2O	1.0 L

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Combine components, adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.

7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

Artificial seawater may be substituted for natural seawater. There are many sources for artificial seawater mixes. These mixes may vary in quality from lot to lot. Medium perpared with a new lot of artificial seawater should be tested in parallel with medium prepared with a currently used lot to determine if it supports equivalent growth.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.
2. Guillard, RRL 1975. Culture of phytoplankton for feeding marine invertebrate Animals. Smith, WL & Chanley, MH, ed., Plenum Press, New York pp. 26-60.
3. Lehman, TL 1976. Ecological and nutritional studies on *Dinobryon* Ehrenb.: seasonal periodicity and the phosphate toxicity problem. *Limnol. Oceanogr.* **21**:646-658.

CULTIVATION OF *BICOSOECIDS*

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INTRODUCTION

Both marine and freshwater bicosoecids can be readily cultivated as monoprotist cultures with bacteria as a food source [1]. The genus *Bicosoeca* has both marine and freshwater members. The genera *Pseudobodo* and *Cafeteria* appear to be restricted to the marine environment. There have been no reports of axenic cultivation of bicosoecids.

PROTOCOL

Freshwater, *Bicosoeca*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.

7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is at or near peak density to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Marine, *Bicosoeca, Cafeteria, Pseudobodo*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Modified Cereal Grass Infusion Medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St.

Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solutions prepared in steps 3 and 4. Autolclave for 30 min at 121° C. Allow to cool.
6. Aseptically distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with protists. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [2] for the cultivation of paramecia. Recent changes in bacterial systematic have determined the strain to be an non-pathogenic strain of *K. pneumoniae*. It is recommended that similar strains be used. For best results it is important to use only *K. pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity.

LITERATURE CITED

1. Nerad, TA 1991. Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.
2. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-148.

A-15.1

CULTIVATION OF SELECTED PRYMNESIIDS

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INTRODUCTION

All prymnesiids are marine. Several protocols for both the non-axenic and axenic cultivation are presented.

PROTOCOL

Xenic

Chrysochromulina, Coccolithus, Cricosphaera, Diacronema, Dicrateria, Emiliania, Hymenomonas, Imautonia, Isochrysis, Monochrysis, Ochrosphaera, Pavlova, Prymnesium, Pseudoisochrysis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

ASW Medium [2]

Artificial seawater*	33.6 g
Tricine	0.5 g
Extra Salts Solution (see below)	3.75 ml
Soil Extract Stock (see below)	25.0 ml
Vitamin Solution (see below)	4.0 ml
Glass distilled H_2O	1.0 L

Extra Salts Solution

$NaNO_3$	30.0 g
Na_2HPO_4	1.2 g
K_2HPO_4	1.0 g
Glass distilled H_2O	1.0 L

Soil Extract Stock

Air-dried rich organic soil	333.0 g
Glass distilled H_2O	667.0 ml

Adjust to pH 8.0 with 1N NaOH or 1N HCl and autoclave for 1 h at 121° C. Decant and filter through Whatman #1 filter.

A-15.2

Vitamin Solution

Biotin	0.2 mg
Calcium pantothenate	20.0 mg
Vitamin B_{12}	4.0 g
Folic acid	0.4 g
Inositol	1000.0 mg
Nicotinic acid	20.0 g
Thiamine HCl	100.0 g
Thymine	600.0 mg
Glass distilled H_2O	1.0 L

After mixing, filter sterilize using a 0.2μm filter. Store in 10.0 ml aliquots at -30° C until needed.

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Add 25.0 ml soil extract stock and 3.75 ml extra salts solution to artificial seawater and Tricine and make up to 1.0 L with glass distilled H_2O. After dissolving, adjust pH to 7.6 - 7.8 with 1N NaOH or 1N HCl.
4. Autoclave for 15 min at 121° C.
5. When cooled, aseptically add filter sterilized Vitamin Solution.
6. Aseptically dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
7. Aseptically inoculate a tube with a 0.1 ml aliquot of a growing culture at or near peak density.
8. Loosen caps one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
9. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Axenic
Apistonema, Emiliania, Hymenomonas, Isochrysis, Pavlova, Prymnesium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

S88 Medium [3]

NaCl	16.0 g
KCl	0.4 g
$MgSO_4 \cdot 7H_2O$	2.5 g
$CaSO_4 \cdot 2H_2O$	0.5 g
$SrCl_2 \cdot 6H_2O$	6.5 mg

$AlCl_3 \cdot 6H_2O$	0.25 mg
RbCl	0.10 mg
$LiCl \cdot H_2O$	0.05 mg
KI	0.025 mg
KBr	32.5 mg
Glycylglycine	500.0 mg
Glycine	250.0 mg
KNO_3	100.0 mg
K_2HPO_4	10.0 mg
Na_2HPO_4	50.0 mg
$FeSO_4 \cdot 7H_2O$	2.5 mg
$ZnSO_4 \cdot 7H_2O$	22.0 mg
$CuSO_4 \cdot 5H_2O$	19.6 mg
$CuSO_4 \cdot 7H_2O$	2.38 mg
$NaMoO_4 \cdot 2H_2O$	1.26 mg
Vitamin Solution	1.0 ml
Glass distilled H_2O	1.0 L

Vitamin Solution

Vitamin B_{12}	0.1 mg
Thiamine HCl	50.0 mg
Glass distilled H_2O	1.0 L

After mixing filter sterilize using a 0.2μm filter. Dissolve vitamins and store solution in 10.0 ml aliquots at -20° C until needed.

3. Adjust pH to 8.0 with 1N NaOH or 1N HCl and dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C.
5. Inoculate cooled medium with 0.1 ml of a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Axenic

Coccolithus, Dicrateria, Emiliania, Imantomia, Isochrysis, Ochrosphaera, Pavlova, Pleurochrysis, Pseudoisochrysis, Syracosphaera

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

f/2-si Medium [1]

$NaNO_3$ stock solution (see below)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ stock solution (see below)	1.0 ml
Trace metals solution	1.0 ml
Vitamin Solution	0.5 ml

A-15.4

Filtered seawater*	1.0 L

$NaNO_3$ Stock Solution

$NaNO_3$	75.0 g
Glass distilled H_2O	1.0 L

$NaH_2PO_4 \cdot H_2O$ Stock Solution

$NaH_2PO_4 \cdot H_2O$	5.0 g
Glass distilled H_2O	1.0 L

Trace Metals Solution

Na_2EDTA	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$CuSO_4 \cdot 5H_2O$ (0.98g/100ml)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (2.2g/100ml)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (1.0g/100ml)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (1.8g/100ml)	1.0 ml
$NaMoO_4 \cdot 2H_2O$(0.63g/100ml)	1.0 ml
Glass distilled H_2O	1.0 L

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

COMMENTS

Artificial seawater may be substituted for natural seawater. There are many sources for artificial seawater mixes. These mixes may vary in quality from lot to lot. Medium perpared with a new lot of artificial seawater should be tested in parallel with a medium prepared with a currently used lot to determine if it supports equivalent growth.

LITERATURE CITED

1. Guillard, RRL 1975. Culture of phytoplankton for feeding marine invertebrates. In: Culture of marine invertebrate animals. Smith, WL & Chanley, MH, ed., Plenum Press, New York pp. 26-60.
2. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of strains 1988. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.
3. Turner, MF 1979. Nutrition of some marine microalgae with special references to vitamin requirements and utilization of nitrogen and carbon sources. *J. Mar. Biol. Ass.* **59**:535-552.

CULTIVATION OF SELECTED VOLVOCIDS OTHER THAN *CHLAMYDOMONAS*

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INTRODUCTION

Many genera of volvocids can be cultivated under xenic conditions. One protocol is presented below for the xenic cultivation of eight freshwater genera. A number of protocols for the axenic cultivation of selected genera are also provided.

PROTOCOL

Freshwater, Xenic
Chlorogonium, Eudorina, Gonium, Haematococcus, Lobomonas, Pandorina, Pteromonas, Volvox

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

JM Medium [4]

$Ca(NO_3)_2 \cdot 4H_2O$ Solution (2.0 g/100 ml)	1.0 ml
KH_2PO_4 Solution (1.24 g/ 100 ml)	1.0 ml
$MgSO_4 \cdot 7H_2O$ Solution (5.0 g/100 ml	1.0 ml
$NaHCO_3$ Solution (1.59 g/100 ml)	1.0 ml
EDTA Solution (see below)	1.0 ml
Trace Metals Solution (see below)	1.0 ml
Vitamin Solution (see below)	1.0 ml
$NaNO_3$ solution (8.0 g/100 ml)	1.0 ml
$Na_2HPO_4 \cdot 12H_2O$ (3.6 g/100 ml)	1.0 ml
Glass distilled H_2O	991.0 ml

EDTA Solution

EDTA-Ferric sodium salt (Sigma E6760)	225.0 mg
Na_2EDTA	225.0 mg
Glass distilled H_2O	100.0 ml

After mixing filter sterilize using a 0.2μm filter.

Trace Metals Solution

H_3BO_3	248.0 mg
$MnCl_2 \cdot 4H_2O$	139.0 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	100.0 mg
Glass distilled H_2O	100.0 ml

After mixing filter sterilize using a 0.2μm filter.

Vitamin Solution

Biotin	40.0 mg
Thiamine HCl	40.0 mg
Vitamin B12	40.0 mg
Glass distilled H_2O	1.0 L

After mixing filter sterilize using a 0.2μm filter. Dissolve vitamins in glass distilled H_2O and store at -20° C until needed.

3. Combine components, adjust pH to 6.8 either with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow to cool before inoculating
6. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Axenic, *Eudorina*, *Gonium*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

VT Medium [2]

$Ca(NO_3)_2 \cdot 4H_2O$	117.8 mg
ß-Na_2 glycerophosphate	50.0 mg
$MgSO_4 \cdot 7H_2O$	40.0 mg
KCl	50.0 mg
Glycylglycine	500.0 mg
Vitamin Solution (see below)	1.0 ml
PIV Metals Solution (see below)	3.0 ml
Glass distilled H_2O	996.0 ml

Vitamin Solution

Biotin	0.01 mg
Thiamine HCl	1.0 mg
Vitamin B_{12}	0.01 mg
Glass distilled H_2O	1.0 L

After mixing filter sterilize using a 0.2μm filter. Dissolve vitamins in glass distilled H_2O and store at -20° C until needed.

PIV Metals Solution

$FeCl_3 \cdot 6H_2O$	196.0 mg
$MnCl_2 \cdot 4H_2O$	36.0 mg
$ZnSO_4 \cdot 7H_2O$	22.0 mg
$CoCl_2 \cdot 6H_2O$	4.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Na_2EDTA \cdot 2H_2O$	1.0 g
Glass distilled H_2O	1.0 L

After mixing filter sterilize using a 0.2μm filter.

3. Adjust pH to 7.5 with 1N NaOH or 1N HCl.
4. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
5. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow to cool before inoculating.
6. Inoculate medium with 0.1 ml of a growing culture at or near peak density.
7. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
8. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Axenic, *Haematococcus*, *Lobomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Proteose Agar [3]

Proteose Peptone (Difco #0120)	1.0 g
Agar	15.0 g
Bristol's Solution (see below)	1.0 L

Bristol's Solution

$NaNO_3$ Solution (2.5 g/100 ml)	10.0 ml
$CaCl_2$ Solution (0.25 g/100 ml)	10.0 ml
$MgSO_4 \cdot 7H_2O$ Solution (0.75 g/100 ml)	10.0 ml
K_2HPO_4 Solution (0.75 g/100 ml)	10.0 ml
KH_2PO_4 Solution (1.75 g/100 ml)	10.0 ml
NaCl Solution (0.25 g/100 ml)	10.0 ml
$FeCl_3$ Solution (1.0 g/ 100 ml)	0.05 ml
Glass distilled H_2O	940.0 ml

After mixing filter sterilize using a 0.2μm filter.

3. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow to cool before inoculting.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.

7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Carteria, Chlorogonium
Freshwater, Axenic

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

AF-6 Medium [5]

$NaNO_3$	140.0 mg
NH_4NO_3	22.0 mg
$MgSO_4 \cdot 7H_2O$	30.0 mg
KH_2PO_4	10.0 mg
K_2HPO_4	5.0 mg
$CaCl_2 \cdot 2H_2O$	10.0 mg
Fe-citrate	2.0 mg
Citric acid	2.0 mg
MES* (Sigma M8250)	400.0 mg
Vitamin Solution	1.0 ml
PIV Metals Solution	3.0 ml
Glass distilled H_2O	996.0 ml

*MES = (2-[N-Morhpoline]ethanesulfonic acid)

Vitamin Solution

Biotin	0.2 mg
Thiamine HCl	1.0 mg
Vitamin B_6	0.1 mg
Vitamin B_{12}	0.1 mg
Glass distilled H_2O	1.0 L

PIV Metals Solution

$FeCl_3 \cdot 6H_2O$	196.0 mg
$MnCl_2 \cdot 4H_2O$	36.0 mg
$ZnSO_4 \cdot 7H_2O$	22.0 mg
$CoCl_2 \cdot 6H_2O$	4.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Na_2EDTA \cdot 2H_2O$	1.0 g
Glass distilled H_2O	1.0 L

3. Adjust pH to 6.6 with 1N HCl or 1N NaOH and dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.

6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Freshwater, Axenic, *Brachiomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Trebouxia Agar [3]

Proteose Peptone (Difco #0120)	10.0 g
Glucose	20.0 g
Agar	15.0 g
Soil extract (see below)	140.0 ml
Bristol's Solution	850.0 ml

Soil extract

African violet soil	77.0 g
Na_2CO_3	0.2 g
Glass distilled H_2O	200.0 ml

Autoclave for 1 h. Allow to cool and filter through Whatman #1 filter paper.

Bristol's Solution

$NaNO_3$ Solution (2.5 g/100 ml)	10.0 ml
$CaCl_2$ Solution (0.25 g/100 ml)	10.0 ml
$MgSO_4 \cdot 7H_2O$ Solution (0.75 g/100 ml)	10.0 ml
K_2HPO_4 Solution (0.75 g/100 ml)	10.0 ml
KH_2PO_4 Solution (1.75 g/100 ml)	10.0 ml
NaCl Solution (0.25 g/100 ml)	10.0 ml
$FeCl_3$ Solution (1.0 g/ 100 ml)	0.05 ml
Glass distilled H_2O	940.0 ml

3. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
4. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
5. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
6. The transfer interval will vary with the strain cultivated. Intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

A-16.6

PROTOCOL

Marine, Axenic, *Dunaliella*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

DV Medium [1]

NaCl	18.0 g
KCl	600.0 mg
$MgSO_4 \cdot 7H_2O$	5.0 g
$NaNO_3$	500.0 mg
$CaCl_2$	100.0 mg
Tris	1.0 g
$Na_2SiO_3 \cdot 9H_2O$	200.0 mg
K_2HPO_4	30.0 mg
$FeCl_2$	0.1 mg
NTA	100.0 mg
Trace metals solution (see below)	30.0 ml
Vitamin solution (see below)	10.0 ml
Natural seawater*	1.0 L

Trace Metals Solution

Na_2EDTA	100.0 mg
$FeCl_2$	1.0 mg
H_3BO_3	2.0 mg
$MnCl_2$	4.0 mg
$ZnCl_2$	0.5 mg
$CoCl_2$	0.1 mg
Glass distilled H_2O	100.0 ml

Vitamin Solution

Thiamine HCl	20.0 mg
Biotin	0.05 mg
Vitamin B_{12}	30.0 mg
Folic Acid	0.025 mg
p-Aminobenzoic Acid	1.0 mg
Nicotinic Acid	10.0 mg
Thymine	50.0 mg
Inositol	100.0 mg
Putrescine·2HCl	4.0 mg
Riboflavin	0.5 mg
Pyridoxamine·2HCl	2.0 mg
Orotic Acid	26.0 mg
Folinic Acid	0.02 mg
Ca pantothenate	10. mg
Pyridoxine·2HCl	4.0 mg
Glass distilled H_2O	100.0 ml

Filter sterilize and store in 10.0 ml aliquots at -20° C until needed.

3. Combine all but the Vitamin Solution.

4. Allow medium to cool and aseptically add the appropriate amount of the Vitamin Solution.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

Some strains of *Chloromonas* can be cultivated axenically on EGM or PP Medium [4] or C Medium [5]. *Stephanosphaera* and *Pandorina* can be cultivated axenically using S66:E5 [4] and CA Medium [5] respectively.

LITERATURE CITED

1. Provasoli, L 1963. Growing Marine seaweeds. In: Proc. 4th Inter. Seaweed Symp., p. 9-17.
2. Provasoli, L & Pinter, I 1959. Artificial media for freshwater algae: Problems and suggestions. In: The Ecology of Algae Tyron, CA & Hartman, RT, ed. pp.84-96.
3. Starr, RC 1978. The Culture Collection of Algae at the University of Texas at Austin. *J. Phycol.* **14**(suppl.):47-100.
4. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of strains 1988. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.
5. Watanabe, MM & Satake, KN 1991. NIES-Collection List of Strains. 3rd edition, Microalgae and Protozoa. The National Institution Environmental Studies, Tsukuba, Japan 163 pp.

CULTIVATION OF SELECTED OSMOTROPHIC VOLVOCIDS

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INTRODUCTION

Polytoma and *Polytomella* species can be easily grown on an axenic liquid medium originally developed by Dr. Seymour H. Hutner for *Euglena*. For most strains cultivation at temperatures of 22-25° C in a room without the use of an incubator to maintain a constant temperature will result in excellent growth. For the best results, however, conditions should be controlled as much as possible.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If a the name of a company is listed with a specific component, the component should be obtained from the company to assure growth.
2. Medium formulation:

Hutner's medium for Euglena [1]

KH_2PO_4	0.02 g
K-citrate·H_2O	0.04 g
$MgSO_4 \cdot 3H_2O$	0.02 g
Trypticase (BBL)	0.6 g
Liver concentrate NF #XI powder* (ICN Biomedicals, Inc. #100377)	0.2 g
Glass distilled H_2O	1.0 L

*Yeast extract (0.4 g), thiamine HCl (0.4 mg) and vitamin B_{12} (0.5 μg) may be substituted for the liver component.

4. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
5. Autoclave 15 min at 121° C. Leave caps loosened about one half turn while autoclaving. Tighten the caps immediately upon removing the tubes from the autoclave. Cool medium before use.
6. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
7. Keep cap loosened one half turn and incubate at 20-25° C under 50-100 μEin/m^2/s irradiance. Incubate under 14/10 h light-dark photoperiod.

8. For routine maintenance subculture every 10-14 d. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.

CULTIVATION OF *CHLAMYDOMONAS*

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INTRODUCTION

Species of *Chlamydomonas* have proven to be widely distributed and extensively used as model organisms. The genus is most readily cultured and maintained as a living culture when grown on solidified medium. Protocols using several different approaches with solidified medium are provided here.

PROTOCOL

Agar Medium [1]

1. Medium ingredients should be added in the sequence listed. If a the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Yeast extract	1.0 g
Beef extract	1.0 g
Tryptose	2.0 g
$FeSO_4$	Trace
Glucose	10.0 g
Agar	15.0 g
Glass distilled H_2O	1.0 L

3. Adjust pH to 7.2 with 20% (w/v) NaOH.
4. Autoclave 30 min at 121° C in a flask with the screw-cap loosened about one half turn.
5. While still liquid dispense 5 ml of medium per 16 x 125 mm screw-capped test tube. Cool each tube until hardened on a 15° to the horizon.
6. Inoculate medium with a growing culture at or near peak density using an inoculating loop. The inoculum should be spread from the base of the slant to within no more than a cm of the top of the slant by a back and forth motion.
7. Keep cap loosened one half turn and incubate at 20-25° C under 50-100 μEin/m^2/s. Incubate under a 14/10 h light-dark cycle.
8. For routine maintenance subculture every 1-4 wk. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

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PROTOCOL

Enriched Agar Medium [1]

1. Medium ingredients should be added in the sequence listed. If a the name of a company is listed with a specific component, the medium component should be obtain from the company to assure growth.
2. Medium formulation:

Na Acetate (anhydrous)	2.0 g
Tryptone (Difco #0123)	2.0 g
Yeast extract (Difco #0127)	2.0 g
Bacto-agar (Difco #0140)	15.0 g
Glass distilled H_2O to	1.0 L

3. Heat under low temperature to dissolve ingredients.
4. Autoclave 30 min at 121° C in a flask with the screw-cap loosened about one half turn.
5. While still liquid dispense 5 ml of medium per 16 x 125 mm screw-capped test tube. Place on a 15° horizontal slant and cool until hardened.
6. Inoculate medium from a growing culture at or near peak density using an inoculating loop. The inoculum should be spread from the base of the slant to within no more than 1 cm of the top of the slant by a back and forth motion.
7. Keep cap loosened one half turn and incubate at 20-25° C under 50-100 μEin/m^2/s. Incubate under a 14/10 h light-dark cycle.
8. For routine maintenance subculture every 1-4 wk. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Proteose Agar Medium [1]

1. Medium ingredients should be added in the sequence listed. If a the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Proteose peptone (Difco #0120)	1.0 g
Agar	15.0 g
Bristol's Solution (see below)	1.0 L

Bristol's Solution

$NaNO_3$ stock solution (2.5g/100ml)	10.0 ml
$CaCl_2$ stock solution (0.25g/100ml	10.0 ml
$MgSO_4 \cdot 7H_2O$ stock solution (0.75g/100ml)	10.0 ml
K_2HPO_4 stock solution (0.75g/100ml)	10.0 ml
KH_2PO_4 stock solution (1.75g/100ml)	10.0 ml
NaCl stock solution (0.25g/100ml)	10.0 ml
Glass distilled H_2O	940.0 ml

Filter sterilize using a 0.2μm filter.

*All stock solutions are prepared in glass distilled H_2O. Add a drop of 1.0% $FeCl_3$ solution or 2.0 ml of minor element solution (see [2]).

3. Use low heat to dissolve ingredients.
4. Autoclave 30 min at 121° C in a flask with the screw-cap loosened about one half turn.
5. While still liquid dispense 5 ml of medium per 16 x 125 mm screw-capped test tube. Place on a 15° horizontal slant and cool until hardened.
6. Inoculate medium from a growing culture at or near peak density using an inoculating loop. The inoculum should be spread from the base of the slant to within no more than a cm of the top of the slant by a back and forth motion.
7. Keep cap loosened one half turn and incubate at 20-25° C under under 50-100 μEin/m^2/s. Incubate under a 14/10 h light-dark cycle.
8. For routine maintenance subculture every 1-4 wk. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

The agar slant should not reach the neck of the test tube. The length of the slant should be between 6-8 cm for a 16 x 125 mm screw-capped test tube.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.
2. Trelease, SF & Trelease, H 1935. Changes in hydrogen-ion concentration of culture solutions containing nitrate and ammonium nitrogen. *Am. J. Bot.* **22**:520-542.

CULTIVATION OF SELECTED PRASINOMONADS

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INTRODUCTION

Many genera of prasinomonads can be easily cultivated. The protocols for the axenic and non-axenic cultivation of several marine and freshwater prasinomonads are provided below.

PROTOCOL

Freshwater, Xenic, *Nephroselmis*, *Pedinomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Bold's Basal Medium [1]

Solution 1 (see below)	10.0 ml
Solution 2 (see below)	10.0 ml
Solution 3 (see below)	10.0 ml
Solution 4 (see below)	10.0 ml
Solution 5 (see below)	1.0 ml
Solution 6 (see below)	1.0 ml
Solution 7 (see below)	1.0 ml
Solution 8 (see below)	1.0 ml
Glass distilled H_2O	956.0 ml

Solution 1

$CaCl_2 \cdot 2H_2O$	2.5 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

Solution 2

$NaNO_3$	25.0 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

A-19.2

Solution 3

$MgSO_4 \cdot 7H_2O$	7.5 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

Solution 4

NaCl	2.5 g
K_2HPO_4	7.5 g
KH_2PO_4	17.5 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

Solution 5

EDTA	50.0 g
KOH	31.0 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

Solution 6

$FeSO_4.7H_2O$	4.98 g
Acidified H_2O*	1.0 L

*1.0 ml of concentrated H_2SO_4 dissolved in 1.0 L distilled H_2O

Solution 7

H_3BO_3	11.42 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

Solution 8

$ZnSO_4 \cdot 7H_2O$	8.82 g
$MnCl_2 \cdot 4H_2O$	1.44 g
MoO_3	0.71 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$CoNO_3 \cdot 6H_2O$	0.49 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

3. Autoclave solution 8 for 15 min at 121° C to dissolve the components. Allow to cool.
4. Add solutions in the order indicated to glass distilled H_2O while stirring.
5. Filter sterilize using a 0.2μm filter.
6. Aseptically dispense 5.0 ml of medium per 16 x 125 mm screw-capped test tubes.
7. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
8. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light/dark photoperiod.
9. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

A-19.3

PROTOCOL

Freshwater, Axenic, *Monomastix*, *Pedinomonas*, *Tetraselmis*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

C Medium [3]

$Ca(NO_3)_2 \cdot 4H_2O$	150.0 mg
KNO_3	100.0 mg
ß-Na_2 glycerophosphate	50.0 mg
$MgSO_4.7H_2O$	40.0 mg
Vitamin Solution (see below)	1.0 ml
PIV Metals Solution (see below)	3.0 ml
Tris	500.0 mg
Glass distilled H_2O	996.0 ml

Vitamin Solution

Vitamin B_{12}	0.1 mg
Biotin	0.1 mg
Thiamine HCl	10.0 mg
Glass distilled H_2O	1.0 L

PIV Metals Solution [4]

$FeCl_3 \cdot 6H_2O$	196.0 mg
$MnCl_2 \cdot 4H_2O$	36.0 mg
$ZnSO_4 \cdot 7H_2O$	22.0 mg
$CoCl_2 \cdot 6H_2O$	4.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Na_2EDTA \cdot 2H_2O$	1.0 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

3. Adjust pH to 7.5 and dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
7. The transfer interval will vary with the strain cultivated. The light intensity, photoperiod and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

A-19.4

PROTOCOL

Marine, Axenic
Mantoniella, Micromonas,
Nephroselmis, Pyramimonas, Tetraselmis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

AF-6 Medium [6]

$NaNO_3$	140.0 mg
NH_4NO_3	22.0 mg
$MgSO_4 \cdot 7H_2O$	30.0 mg
KH_2PO_4	10.0 mg
K_2HPO_4	5.0 mg
$CaCl_2 \cdot 2H_2O$	10.0 mg
Fe-citrate	2.0 mg
Citric acid	2.0 mg
MES* (Sigma M8250)	400.0 mg
Vitamin Solution	1.0 ml
PIV Metals Solution	3.0 ml
Glass distilled H_2O	996.0 ml

*MES = (2-[N-Morpholino]ethanesulfonic acid)

Vitamin Solution

Biotin	2.0 mg
Thiamine HCl	10.0 mg
Vitamin B_6	1.0 mg
Vitamin B_{12}	1.0 mg
Glass distilled H_2O	1.0 L

Dissolve vitamins in glass distilled H_2O and store in 10.0 ml aliquots at -20° C until needed.

PIV Metals Solution [4]

$FeCl_3 \cdot 6H_2O$	196.0 mg
$MnCl_2 \cdot 4H_2O$	36.0 mg
$ZnSO_4 \cdot 7H_2O$	22.0 mg
$CoCl_2 \cdot 6H_2O$	4.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Na_2EDTA \cdot 2H_2O$	1.0 g
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter.

3. Adjust pH to 6.6 with 1N HCl or 1N NaOH and dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.

7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Marine, Xenic
Mantoniella, Micromonas
Nephroselmis, Pyramimonas, Tetraselmis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

HESNW Medium [2]

Vitamin Solution (see below)	1.0 ml
Enrichment Solution (see below)	10.0 ml
Natural seawater*	989.0 ml

Vitamin Solution

Thiamine HCl	100.0 mg
Vitamin B_{12}	2.0 mg
Biotin	1.0 mg
Glass distilled H_2O	1.0 L

Filter sterilize using a 0.2μm filter. Dispense in 10.0 ml aliquots and store frozen at -20° C until needed.

Enrichment Solution

$Na_2EDTA \cdot 2H_2O$	0.553 g
$NaNO_3$	4.667 g
$Na_2SiO_3 \cdot 9H_2O$	3.000 g
ß-Na_2 glycerophosphate	0.667 g
H_3BO_3	0.380 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.234 g
$FeCl_3 \cdot 6H_2O$	0.016 g
$MnSO_4 \cdot 4H_2O$	0.054 g
$ZnSO_4 \cdot 7H_2O$	7.3 mg
$CoSO_4 \cdot 7H_2O$	1.6 mg
Glass distilled H_2O	1.0 L

After addition of the $Na_2SiO_3 \cdot 9H2O$ neutralize the solution with 1N HCl. Repeat filtration phase.

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.

6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.
7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

PROTOCOL

Marine, Axenic, *Tetraselmis*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Tetraselmis Medium
(R Lewin, personal communication)

Na glutamate	1.7 g
ß-Na_2 glycerophosphate	0.1 g
Vitamin Solution (see below)	10.0 ml
Trace Element Solution (see below)	1.0 ml
Natural seawater*	750.0 ml
Glass distilled H_2O	239.0 ml

Vitamin Solution

Vitamin B_{12}	1.0 mg
Thiamine HCl	1.0 g
Glass distilled H_2O	100.0 ml

Filter sterilize. Dispense in 10.0 ml aliquots and store at -20° C until needed.

Trace Element Solution

Na_2-EDTA	400.0 mg
$FeCl_3 \cdot 6H_2O$	19.6 mg
$MnSO_4 \cdot 4H_2O$	65.6 mg
H_3BO_3	456.0 mg
$ZnSO_4 \cdot 7H_2O$	8.8 mg
$CoSO_4 \cdot 7H_2O$	1.92 mg
Glass distilled H_2O	100.0 ml

*Natural seawater can be obtained from Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes.
4. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
5. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
6. Loosen cap one half turn and incubate at the appropriate temperature under 50-100 μEin/m^2/s irradiance. Maintain under a 14/10 h light-dark photoperiod.

7. The transfer interval will vary with the strain cultivated. Light intensity, photoperiod, and incubation temperature will affect the transfer interval. Some strains can be maintained for several weeks past peak density and can be transferred at less frequent intervals.

COMMENTS

Representatives of the genera *Mantoniella* [5] and *Pterosperma* [6] can be maintained axenically in media not detailed here. Artificial seawater may be substituted for natural seawater. There are many sources for artificial seawater mixes. These mixes may vary in quality from lot to lot. Medium perpared with a new lot of artificial seawater should be tested in parallel with a medium prepared with a currently used lot to determine if it supports equivalent growth.

LITERATURE CITED

1. Bischoff, HW & Bold, HC 1963. Phycological Studies. IV. Some algae from Enchanted Rock and related algal species. The Univ. of Texas Pub. No. 6318. 95 pp.
2. Harrison, PJ, Waters, RE & Taylor, FJR 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. *J. Phycol.* **16**:28-35.
3. Ichimura, T 1971. Sexual cell division and conjugation-papilla formulation in sexual reproduction of *Closterium strigosum*. In: Proc. 7th Inter. Seaweed Symp., pp. 208-214.
4. Provasoli, L & Pinter, IJ 1959. Artificial media for fresh-water algae: Problems and suggestions. In: The Ecology of Algae, Tryon, CA & Hartmann, RT, ed., pp. 84-96.
5. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of strains 1988. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.
6. Watanabe, MM & Satake, KN 1991. NIES-Collection List of Strains. 3rd edition. Microalgae and Protozoa. The National Institution of Environmental Studies, Tsukuba, Japan 163 pp.

CULTIVATION OF CHOANOFLAGELLATES

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INTRODUCTION

There are both freshwater and marine representatives of choanoflagellates. Some species are planktonic and others are found in shallow waters. The planktonic forms do best when cultivated in medium that supports low densities of bacteria while other forms do best when cultivated with high densities of bacteria. There have been reports of axenic cultivation of choanoflagellates [1] but only protocols for bacterized cultivation are given below.

PROTOCOL

Marine, Planktonic, *Acanthoecopsis*, *Diaphanoeca*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Marine Flagellate Medium [2]

Filter sterilized artificial seawater*	15.0 ml
Autoclaved rice grains (Uncle Ben's Natural Whole Grain Rice**)	2.0 g

*Can be obtained from a grocery store.
**There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Fill a 20 x 150 mm screw-capped test tube approximately $^1/_3$ full with rice grains. Autoclave at 121° C for 20 min. Loosen cap one half turn prior to autoclaving.
4. Prepare artificial seawater according to package directions. Filter sterilize.
5. Add 15.0 ml aliquots of seawater to T-25 tissue culture flasks and then aseptically add two rice grains.

6. Vigorously agitate the culture to be transferred and aseptically transfer 0.1 ml to a fresh flask of medium. Screw caps on tightly. The incubation temperature will vary with the strain. Incubate at 4-10° C for *Acanthoecopsis*, or 4-18° C for *Diaphanoeca*.
7. Transfer at 14-28 d intervals.

PROTOCOL

Marine, *Monosiga*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium	
2x Artificial seawater*	500.0 ml
2x Cereal modified grass infusion medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium	
Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.
**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solution prepared in steps 3 and 4. Autoclave for 15 min at 121° C. Allow to cool.
6. Aseptically distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with protists. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

A-20.3

PROTOCOL

Freshwater, *Monosiga, Salpingoeca*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is at or near peak density to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

A-20.4

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [3] for the cultivation of paramecia. Recent changes in bacterial systematics have determined it to be a non-pathogenic strain of *Klebsiella pneumoniae*. For best results it is important to use only an equivalent *K. pneumoniae* strain as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity.

LITERATURE CITED

1. Gold, K, Pfister, RM & Liguori, R 1970. Axenic cultivation and electron microscopy of two species of Choanoflagellida. *J. Protozool.* **17**:210-212
2. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.
3. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-148.

CULTIVATION OF *PROTEROMONAS*

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INTRODUCTION

Only a single species of proteromonad is reported to have been isolated and cultured, *Proteromonas lacertae* [1]. The species living in the cloaca of lizards was adapted to grow on a medium developed by Louis S. Diamond for the axenic cultivation of trichomonads. Whether the same medium will support the growth of other species is unknown, but Jaroslav Kulda details the approach used for both isolation and axenization of the lizard species [1]. The approach could be used in attempts to culture other species.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company for the best or even to obtain any growth.
2. Medium formulation:

Trypticase (BBL #11921)	20.0 g
Yeast extract	10.0 g
Maltose	5.0 g
L-Cysteine hydrochloride	1.0 g
L-Ascorbic acid	0.2 g
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
Noble Agar (Difco #0142)	0.5 g
Glass distilled H_2O	900.0 ml

3. Add all the dry materials except the agar to the glass distilled H_2O.
4. Adjust to pH 7.0 with 20% NaOH.
5. Add the agar and heat to dissolve.
6. Dispense 9.0 ml of medium per 16 x 125 mm screw-capped test tube.
7. Autoclave 15 min at 121° C. Leave caps loosened about one half turn while autoclaving. Tighten the caps immediately upon removing the tubes from the autoclave. Cool medium before use.

8. Just prior to use add 0.5 ml of heat-inactivated horse serum. To heat inactivate the horse serum aseptically dispense 50.0 ml into a 125 ml screw-capped bottle. Immerse the bottle to a level just above the serum in a water bath set at 56° C for 30 min.
9. Inoculate medium with 0.1 ml from a growing culture at the end of logarithmic growth or just beginning stationary phase.
10. Tighten cap and incubate at 20-22° C.
11. For routine maintenance subculture every 10-14 d.

COMMENTS

Kulda reports an equal amount of Tryptone L 42 (Oxoid) can be substituted for Trypticase. The substitution has not been tested by the authors of the protocol. The medium can be stored for 10 d at 4° C. Cells grow poorly or not at all in medium older than 10 d. Keep tubes tightly capped at all times.

LITERATURE CITED

1. Kulda, J 1973. Axenic cultivation of *Proteromonas lacertae-viridis*. *J. Protozool.* **20**:536.

A-22.1

CULTIVATION OF TRICHOMONADS

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INTRODUCTION

There are several media for the axenic cultivation of trichomonads. Two of the more commonly used are detailed here. Among the best have been those developed by Louis S. Diamond [1]. The cultivation methods for *Dientamoeba* and *Trichomonas tenax* are similar to those for *Entamoeba* and are detailed with these amebae. In fact, these amebal media will support the growth of many trichomonads, but will not be repeated here.

PROTOCOL

TYM Medium

1. Medium ingredients should be added in the sequence listed. If a the name of a company is listed with a specific component, the medium component should be obtain from the company for the best or even to obtain any growth.
2. Medium formulation:

Basal Medium	
Trypticase (BBL #11921)	20.0 g
Yeast extract	10.0 g
Maltose	5.0 g
L-Cysteine hydrochloride	1.0 g
L-Ascorbic acid	0.2 g
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
Noble Agar (Difco #0142)	0.5 g
Glass distilled H_2O	900.0 ml

3. Add all the dry materials except the agar to the glass distilled H_2O.
4. Adjust pH with 20% NaOH. See Table 1 for pH used.
5. Add the agar and heat to dissolve.
6. Dispense 9.0 ml of medium per 16 x 125 mm screw-capped test tube.
7. Autoclave 15 min at 121° C. Leave caps loosened about one half turn while autoclaving. Tighten the caps immediately upon removing the tubes from the autoclave. Cool medium before use.
8. Just prior to use add 0.5 ml of heat-inactivated serum required for the species/strain. See Table 1 for the serum to be used. To heat-inactivate serum, aseptically dispense 50.0 ml into

a 125 ml screw-capped bottle. Immerse covering the serum volume in a water bath set at 56° C for 30 min.

9. Inoculate medium with 0.1 ml from growing stock culture at end of logarithmic growth or just entering stationary phase.
10. Tighten cap and incubate at 35° C.
11. For routine maintenance subculture every 48-96 h depending upon the species and strain.

TABLE 1

The pH of the medium and the serum used influence optimum growth of strains. Usually pH is more important. Possible combinations which are known to support certain strains are listed in this table.

pH	Serum	Genus
6.0	Horse	*Trichomonas*
6.0-6.5	Lamb + 0.1 ml Dubos Serum (Difco #0292)	*Pentatrichomonas, Trichomonas, Tritrichomonas*
7.0	Horse	*Pentatrichomonas, Trichomonas, Tritrichomonas*
	Lamb	*Hypotrichomonas*
7.2	Horse	*Trichomonas, Tritrichomonas*
	Lamb	*Hypotrichomonas, Tetratrichomonas, Trichomitus, Trichomonas, Tritrichomonas*

PROTOCOL

CTLM Medium [2]
(*Monocercomonas, Tetratrichomonas, Trichomonas, Tritrichomonas*)

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company for the best or even to obtain any growth.
2. Medium formulation:

Basal Medium

Liver Infusion Broth (Difco #0269)	250.000 ml
10x Ringer Salt Solution (see below)	75.000 ml
Tryptose (Difco #0124)	25.000 g
L-Cysteine HCl	1.750 g
Maltose	1.250 g
L-Ascorbic Acid	0.250 g
$NaHCO_3$	0.075 g
Agar	1.150 g
Glass distilled H_2O	675.000 ml

10x Ringers Salt Solution

NaCl	9.00 g
KCl	0.42 g
$CaCl_2$	0.24 g
Glass distilled H_2O	100.00 ml

3. Add all the dry materials except the agar to the glass distilled H_2O.
4. Adjust pH with 20% NaOH to 6.0 for *Tetratrichomonas gallinarum* and *Trichomonas vaginalis*, 7.0 for *Monocermonas*, 7.3 for *Trichomonas gallinae*, 7.5 for *Tritrichomonas foetus*.
5. Add the agar and heat to dissolve.
6. Dispense 9.0 ml of medium per 16 x 125 mm screw-capped test tube.
7. Autoclave 15 min at 121° C. Leave caps loosened about one half turn while autoclaving. Tighten the caps immediately upon removing the tubes from the autoclave. Cool medium before use.
8. Just prior to use add 0.5 ml of heat-inactivated horse serum. To prepare heat-inactivated serum, aseptically dispense 50.0 ml into a 125 ml screw-capped bottle. Immerse to a level sufficient to cover the serum in a water bath set at 56° C for 30 min.
9. Inoculate medium with 0.1 ml from growing culture at or near peak density.
10. Tighten cap and incubate vertically at 35° C.
11. For routine maintenance subculture every 48-96 h depending upon the species and strain.

COMMENTS

The medium can be stored for about 7-10 d under refrigeration at 5-9° C without the serum component. Cells grow poorly or not at all in older medium. Keep tubes tightly capped at all times.

LITERATURE CITED

1. Diamond, LS 1957. The axenic cultivation of various trichomonads of animals and man. *J. Parasitol.* **43**:488-490.
2. McEntegart, MG 1954. The maintenance of stock strains of trichomonads by freezing. *J. Hyg.* **52**:545-550.

A-23.1

CULTIVATION OF FREE-LIVING DIPLOMONADS

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INTRODUCTION

Two free-living diplomonads, *Trepomonas agilis* and *Hexamita inflata*, can be cultivated in monoprotist culture in media with starch and mixed bacteria as food sources. Only one diplomonad other than *Giardia* has been axenically cultivated [1,2].

PROTOCOL

Axenic, *Hexamita inflata*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Peptone	5.0 g
Yeast extract	1.0 g
Glucose	2.0 g
Cysteine-HCl	0.2 g
Artificial seawater	900.0 ml

Artificial seawater

NaCl	22.0 g
KCl	0.6 g
$CaCl_2 \cdot 2H_2O$	1.0 g
$MgCl_2 \cdot 2H_2O$	2.9 g
Na_2SO_4	2.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Na_2HPO_4	0.1 g
KH_2PO_4	0.005 g
$NaHCO_3$	0.03 g
Glass distilled H_2O	1.0 L

3. Adjust pH to 7.5.
4. Dispense 9.0 ml of medium per 16 x 125 mm screw-capped test tube.
5. Autoclave 30 min at 121° C.

6. Allow medium to cool and add 1.0 ml heat-inactivated bovine serum (56° C for 30 min) to each tube.
7. Aseptically inoculate a 0.1 ml aliquot of a growing culture into a fresh tube of medium.
8. Incubate at 15-20° C for 2-3 d and repeat step 7.

PROTOCOL

Xenic, *Trepomonas agilis*

1. Media ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

(TYGM-9 Medium)

K_2HPO_4	2.8 g
KH_2PO_4	0.4 g
Casein Digest (BBL #97023)	2.0 g
Yeast Extract (BBL #11928)	1.0 g
NaCl	7.5 g
Gastric Mucin (USB #16030)	2.0 g
Glass distilled H_2O	970.0 ml

3. Autoclave for 15 min and allow to cool.
4. Add 30 ml heat-inactivated (56° C for 30 min) bovine serum and 0.5 ml sterile Tween 80 solution (10% v/v in absolute ethanol, filter-sterilize with a nucleopore filter).
5. Aseptically dispense 8.0 ml per 16 x 125 mm screw-capped test tube.
6. Aseptically add 0.5 ml per tube of rice starch suspension prepared as follows: Heat sterilize 0.5 g rice starch (BDH chemicals Ltd.(#30263)-available from Gaillard Schlesinger Inc., Carle Place, Long Island, NY) at 150° C for 2 h. After sterilization aseptically add 9.5 ml of sterile phosphate buffered saline (PBS) solution (see below) at pH 7.4. PBS should be added just prior to use and extra rice starch suspension should not be kept.

PBS

NaCl	0.85 g
KH_2PO_4	0.53 g
K_2HPO_4	1.06 g
Glass distilled H_2O	100.0 ml

Autoclave at 121° C for 15 min.

7. Inoculate tube medium aseptically with 0.1 ml from growing stock culture.
8. Tighten cap and incubate on a 15° horizontal slant at 25° C.
9. For routine maintenance subculture weekly.

A-23.3

PROTOCOL

Xenic, *Hexamita inflata*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

TYGM-9 Medium (see previous protocol)	3.0 ml
Cereal grass infusion medium	9.0 ml

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Prepare TYGM-9 as in the protocol above for *Trepomonas agilis*.
4. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
5. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
6. Filter through Whatman #1 filter paper.
7. Add 0.5 g Na_2HPO_4.
8. Autoclave for 30 min at 121° C. Allow to cool.
9. Thoroughly suspend the starch in TYGM-9 medium and combine 3.0 ml with 9.0 ml bacterized cereal grass infusion medium in a 16 x 125 mm screw-cap test tube.
10. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast Extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

11. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
12. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
13. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
14. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
15. Inoculate tube of medium aseptically with 0.1 ml from a growing stock culture.

16.Tighten the cap and incubate on a 15° horizontal slant at 25° C.
17.For routine maintenance subculture at 3-4 d intervals.

COMMENTS

The medium for the axenic cultivation of *Hexamita* can be used for initial establishment of an axenic culture. Once established a simpler medium reported by Khouw and McCurdy, Jr. [1] can be used. Details of the temperature range, pH tolerance and salinity tolerance of *Hexamita inflata* are reported in Khouw and McCurdy, Jr. [1].

LITERATURE CITED

1. Khouw, BT & McCurdy, HD, Jr 1968. Nutritional studies of a flagellated protozoan *Hexamita inflata* from the Canadian oyster, *Crassostrea virginica*. *Can. Microbiol.* **14**:817-82.
2. Khouw, BT, McCurdy, HD, & Drinnan, RE 1968. The axenic cultivation of *Hexamita inflata* from *Crassostrea virginica*. *Can. J. Microbiol.* **14**:184-185.

A-24.1

CULTIVATION OF FREE-LIVING BODONIDS

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INTRODUCTION

Many bodonids can be readily cultivated as monoprotist cultures with bacteria as the food source. Members of the genera *Bodo, Cryptobia* and *Rhynchomonas* are encountered in both freshwater and seawater environments. *Cruzella* and *Dimastigella* have only been reported from seawater and freshwater respectively. Only members of the genus *Bodo* have been established in axenic culture [1].

PROTOCOL

Freshwater, Xenic
Bodo, Cryptobia, Dimastigella, Rhynchomonas

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, *Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.

4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast Extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Marine, Xenic, *Cryptobia*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Marine Flagellate Medium [2]

Filter sterilized artificial seawater*	15.0 ml
Autoclaved rice grains (Uncle Ben's Natural Whole Grain Rice**)	2.0 g

*Can be obtained from a grocery store.

**There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Fill a 20 x 150 mm screw-capped test tube approximately $^1/_3$ full with rice grains. Autoclave at 121° C for 20 min. Loosen cap one half turn prior to autoclaving.
4. Prepare artificial seawater according to package directions. Filter sterilize.

5. Add 15.0 ml aliquots of seawater to T-25 tissue culture flasks and then aseptically add two rice grains.
6. Vigorously agitate the culture to be transferred and aseptically transfer 0.1 ml to a fresh flask of medium. Screw caps on tightly. The incubation temperature will vary with the strain.
7. Transfer at 14-28 d intervals.

PROTOCOL

Marine, Xenic, *Bodo, Cruzella, Rhynchomonas*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Cereal Modified Grass Infusion Medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.
**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solutions prepared in steps 3 and 4.
6. Aseptically distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

A-24.4

PROTOCOL

Freshwater, Axenic, *Bodo*

1. Prepare heat-killed *Klebsiella pneumoniae*.
2. Inoculate a loopful of *Klebsiella pneumoniae* into 5 ml of Nutrient Broth (Difco #0001). Incubate at 35° C overnight.
3. Add 0.5 ml of bacterized broth prepared in step 2 to each of ten 1 L Erlenmeyer flasks each containing 250 ml of Nutrient Broth. Incubate cultures at 35° C for 24 h.
4. Aseptically transfer bacterial suspensions to 500 ml screw-capped centrifuge bottles. Fill bottles with a maximum of 400 ml. Centrifuge in a refrigerated Sorvall Centrifuge at 5000 RPM for 10 min.
5. Decant supernatant and resuspend pellets in Page's Balanced Salt Solution (see below). Pool all suspensions in a single bottle and centrifuge as in step 4.

Page's Balanced Salt Solution

Solution 1 (see below)	500.0 ml
Solution 2 (see below)	500.0 ml

Solution 1

Na_2HPO_4	2.84 g
KH_2PO_4	2.72 g
Glass distilled H_2O	500.0 ml

Autoclave for 20 min at 121° C. Loosen cap one half turn prior to autoclaving.

Solution 2

$MgSO_4 \cdot 7H_2O$	8.0 mg
$CaCl_2 \cdot 2H_2O$	8.0 mg
NaCl	0.24 g
Glass distilled H_2O	500.0 mg

Autoclave for 20 min at 121° C. Loosen cap one half turn prior to autoclaving. When both solutions have cooled mix 1:1.

6. Discard supernatant and resuspend pellet in Page's Balanced Salt Solution (final volume of cell suspension should be equal to approximately 400 ml)
7. Repeat step 6 twice more.
8. Resuspend the final pellet in less than 100 ml of Page's Balanced Salt Solution. Make sure cells are thoroughly suspended.
9. Transfer to a 125 ml screw-capped serum bottle and dilute to a final volume of 100 ml with Page's Balanced Salt Solution.
10. Do a serial dilution of the suspension prepared in step 9. Carry dilution out to 10^{-9} dilution. Plate 0.1 ml aliquots in triplicate from the 10^{-7} -10^{-9} dilution tubes. Place the aliquots in the center of 100 mm petri plates and spread evenly over the surfaces with a spread bar. Incubate plates at 35° C overnight.
11. Place bottle prepared in step 9 in a 60° C water bath to a level such that the liquid level of the water bath is above that of the suspension in the bottle. At 10 min intervals swirl the bottle. Incubate for a total of 30 min Allow the bottle to cool to room temperature. This treatment should kill all bacterial cells.
12. As a check for viable cells, add 3 drops of the cell suspension prepared in step 11 to the edge of a 100 mm petri plate. Place the plate vertically to allow the drops to move to the opposite edge. Incubate plate at 35° C for 48 h.
13. Determine bacterial cell concentration from plates prepared in step 10.

14. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
15. Medium formulation:

Cereal Grass Infusion Medium and Vitamins

Cereal grass infusion medium (see below)	990.0 ml
Vitamin solution (see below)	10.0 ml

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

Vitamin Solution

Calcium pantothenate	0.05 g
Nicotinamide	0.05 g
Pyridoxal·HCl	0.05 g
Pyridoxamine·HCl	0.025 g
Riboflavin	0.05 g
Folic acid	0.025 g
Thiamine·HCl	0.15 g
Biotin	0.0125 mg
DL-Thioctic acid	0.5 ml
Glass distilled H_2O	100.0 ml

16. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
17. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
18. Filter through Whatman #1 filter paper.
19. Add 0.5 g Na_2HPO_4.
20. Add 10.0 ml of Vitamin Solution to 990.0 ml of grass infusion medium.
21. Dispense 5.0 ml of medium per 16 x 125 mm screw-capped test tube.
22. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
23. Add volume of the heat-killed bacteria to cereal grass infusion medium to bring the final concentration to 10^6 bacteria/ml.
24. Inoculate 0.1 ml of a culture of *Bodo* at peak density.
25. Incubate tube with cap loosened one half turn on a 15° horizontal slant.
26. Transfer every 7-10 d by repeating steps 23-25.

A-24.6

COMMENTS

The strain of *Aerobacter aerogenes* used by T.M. Sonneborn [3] for the cultivation of paramecia. Recent changes in bacterial systematics have determined it to be a non-pathogenic strain of *Klebsiella pneumoniae*. It is recommended a similar strain be used. For best results it is important to use only *K. pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity. Despite numerous attempts the heat-killed bacteria used for the axenic cultivation of *Bodo* could not be replaced with liquid components [1].

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1982. Axenic cultivation of *Bodo edax* and Bodo ucinatus and some observations on feeding rate in monoxenic cultures. *J. Protozool.* **29**:290.
2. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.
3. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-148.

A-25.1

AXENIC CULTIVATION OF *CRYPTOBIA*

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INTRODUCTION

Of the parasitic bodonids only *Cryptobia* species have been cultured. Those isolated from fish are cultured similar to trypanosomatid species on blood based media. Although blood from a variety of mammals, including humans, can be used, for practical purposes rabbit blood is used.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtain from the company to the best or even to obtain any growth.
2. Medium formulation:

Diphasic Blood Agar Medium [1]

Beef broth powder (Difco #0131)	25.0 g
Neopeptone (Difco #0119)	10.0 g
NaCl	2.5 g
Agar	10.0 g
Glass distilled H_2O	250.0 ml

3. Add 25.0 g of beef broth powder to the glass distilled H_2O and infuse by bringing to a rapid boil for 3-5 min, stirring constantly.
4. Filter through Whatman #2V filter paper.
5. Add the remaining components and heat to boiling.
6. Once the preparation reaches boiling remove from heat and filter through Whatman #2V filter paper.
7. Adjust to pH 7.2-7.4 with 1 N NaOH.
8. Autoclave 20 min at 121° C.
9. Cool in a 50° C water bath for 30 min and aseptically add 30% (v/v) sterile, defibrinated rabbit blood.
10. Dispense 5 ml per 16 x 125 mm screw-capped test tube, slant and cool. This step must be done rapidly or the preparation will begin to gel before being dispensed.
11. Aseptically add 3.0 ml per tube of sterile Locke's Solution (see below) as an overlay.

Locke's Solution

NaCl	8.0 g
KCl	0.2 g

A-25.2

$CaCl_2$	0.2 g
KH_2PO_4	0.3 g
Glucose	2.5 g
Glass distilled H_2O	1.0 L

13. Inoculate with 0.1 ml from growing stock culture.
14. Tighten cap and incubate vertically at 25° C.
15. For routine maintenance subculture weekly.

COMMENTS

If poor or no growth is obtained reduce the Locke's Solution overlay to 1.0 ml. For larger volumes of culture increase inoculum, medium and vessel size proportionally. The medium is stable for approximately one month at refrigerated temperatures (5-9° C). As the medium ages the color will change from a bright red to purple. Growth will decrease as the color changes. The rabbit blood is defibrinated by aseptically adding sterile glass beads to a flask containing the whole blood. The flask is then swirled gently for 10 min. The fibrin will adhere to the beads and thereby be removed from the serum. Defibrinated rabbit blood can be obtained from Granite Diagnostics Inc., Box 908, 1308 Rainey Street, Burlington, NC.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.

AXENIC CULTIVATION OF INSECT AND PLANT TRYPANOSOMATIDS

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INTRODUCTION

Trypanosomatid species isolated from insects or plants belonging to the genera, *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Leptomonas*, and *Phytomonas* can often easily be grown on an axenic medium developed by Hunter and coworkers [1]. For most strains cultivation at temperatures at 22-25° C will result in excellent growth. Some strains my require the supplementing of the basic medium with heat-inactivated serum in concentrations of 5-15% (v/v). Inactivated fetal calf serum is usually the best serum to use as a supplement. The serum should not be added to the medium unless there is poor growth. Most species can also be grown on a blood based medium. Blood from a variety of mammals, including human, can be used but for practical purposes rabbit blood is generally employed. There are many media which have been developed for the cultivation of this group of flagellates. The media detailed in the protocols below provide good yields for many of the genera.

PROTOCOL

Hutner's Medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Trypticase (BBL #11921)	6.0 g
Yeast extract	1.0 g
Liver concentrate NF #XI powder (ICN Biomedicals, Inc. #100377)	0.1 g
Sucrose	15.0 g
Hemin solution (see below)	5.0 ml
Glass distilled H_2O	1.0 L

*Equivalent to Liver-extract concentrate (1:20).

Hemin Solution

Triethanolamine (TEA)	2.5 ml
Hemin	25.0 mg

Glass distilled H_2O	2.5 ml

3. Adjust to pH 7.8 with 20% (w/v) NaOH.
4. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
5. Autoclave 30 min at 121° C. Cool medium before use.
6. Inoculate a 0.1 ml aliquot from a growing stock culture at or near peak density into 5 ml of medium.
7. Tighten cap and incubate vertically at 25° C.
8. For routine maintenance subculture weekly.

PROTOCOL

Mansour's Medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Beef broth powder (Difco #0131)	5.0	g
Neopeptone (Difco #0119)	4.0	g
NaCl	1.60	g
KCl	0.04	g
$CaCl_2$	0.06	g
Glucose	0.50	g
KH_2PO_4	0.06	g
Glass distilled H_2O	200.0	ml

3. Add the beef broth powder to the glass distilled H_2O and infuse by bringing to a rapid boil for 3-5 min, stirring constantly.
4. Filter through Whatman #2V filter paper.
5. Add the remaining ingredients while stirring.
6. Heat to boiling and restore volume to 200.0 ml with glass distilled H_2O.
7. Adjust to pH 7.2 with 1 N NaOH.
8. Filter through Whatman #2V filter paper.
9. Autoclave 30 min at 121° C.
10. Cool and aseptically add lysed rabbit blood solution that is prepared as follows: Mix 10 ml of whole rabbit blood with 10 ml of glass distilled H_2O and freeze and thaw twice. By alternately plunging into liquid nitrogen and thawing in a 35° C water bath.
11. Mix thoroughly by stirring and dispense in 5 ml aliquots to 16 x 125 mm screw-capped test tubes.
12. Inoculate 0.1 ml from a growing stock culture into 5 ml of medium.
13. Tighten cap and incubate vertically at 25° C.
14. For routine maintenance subculture weekly.

PROTOCOL

Diphasic Blood Agar Medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, it should be obtained from the company to assure growth.

2. Medium formulation:

Beef Broth Powder (Difco #0131)	25.0 g
Neopeptone (Difco #0119)	10.0 g
NaCl	2.5 g
Agar	10.0 g
Glass distilled H_2O	250.0 ml

3. Add 25.0 g of beef broth powder to the glass distilled H_2O and infuse by bringing to a rapid boil for 3-5 min, stirring constantly.
4. Filter through Whatman #2V filter paper 4.
5. Add the remaining components and heat to boiling.
6. Once the preparation reaches boiling remove from heat and filter through Whatman #2V filter paper.
7. Adjust to pH 7.2-7.4 with 1N NaOH.
8. Autoclave 20 min at 121° C.
9. Allow to cool until the preparation may be held comfortably in the hand.
10. Aseptically add 30% sterile, defibrinated rabbit blood.
11. Dispense 5 ml per 16 x 125 mm screw-capped test tube, slant and cool. This step must be done rapidly or the preparation will begin to gel before dispensed.
12. Add aseptically 3.0 ml sterile Locke's Solution (see below) after cooling as an overlay.

Locke's Solution

NaCl	8.0 g
KCl	0.2 g
$CaCl_2$	0.2 g
KH_2PO_4	0.3 g
Glucose	2.5 g
Glass distilled H_2O	1.0 L

13. Inoculate 0.1 ml growing stock culture into 5 ml of medium.
14. Tighten cap and incubate at 25° C.
15. For routine maintenance subculture weekly.

COMMENTS

A slight hemin precipitate is normal following autoclaving of Hutner's medium. In vigorous cultures the masses of cells will be observed as a white ring at the miniscus in the culture vessel. A film of cells will form up the sides of the vessel. If good growth is not obtained in Biphasic Blood Agar Medium reduce the Locke's Solution overlay to 1.0 ml. For larger volumes of culture increase inoculum, medium and vessel size proportionally. The blood based media are stable for approximately one month at refrigerated temperatures (5-9° C). As the medium ages the color will change from a bright red to purple. Growth will decrease as the color changes. The rabbit blood is defibrinated by aseptically adding sterile glass beads to a flask containing the whole blood. The flask is then swirled gently for 10 min. The fibrin will adhere to the beads and thereby be removed from the serum. Defibrinated rabbit blood can be obtained from Granite Diagnostics Inc., Box 908, 1308 Rainey Street, Burlington, NC.

A-26.4

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.

CULTIVATION OF BACTERIVOROUS FLAGELLATES

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INTRODUCTION

Many heterotrophic flagellates can be cultivated as monoprotist cultures with bacteria as a food source [1]. Members of the genera *Metopion* and *Massisteria* have to date only been reported from marine environments, *Amphimonas* and *Apusomonas* only from freshwaters and *Amastigomonas*, *Cercomonas*, *Heteromita*, *Jakoba*, *Paraphysomonas*, *Percolomonas* and *Spumella* from both environments.

PROTOCOL

Freshwater

Amastigomonas, *Apusomonas*, *Cercomonas*, *Heteromita*, *Jakoba*, *Paraphysomonas*, *Reclinomonas*, *Percolomonas*, *Spumella*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract	4.0	g
Glucose	0.16	g
Agar	20.0	g
Glass distilled H_2O	800.0	ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is at or near peak density to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance subculture weekly.

PROTOCOL

Marine

Amastigamoeba, Cercomonas, Heteromita, Jakoba
Metopion, Paraphysomonas, Percolomonas, Massisteria, Spumella

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Cereal grass infusion medium (see below)	500.0 ml

2x Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated

carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants. **This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper. Autoclave for 15 min at 121° C.
5. Allow solution prepared in step 4 to cool and combine equal amounts of this solution and the solution prepared in step 3.
6. Distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

COMMENTS

Only certain members of *Cercomonas, Heteromita, Jakoba, Percolomonas* and *Spumella* have been reported to form resistant stages in their life cycles. These strains may not need to be passaged as frequently as those strains not forming cysts. However, not all cysts are stable for long periods of time. Each strain should be studied on a case by case basis to determine the appropriate transfer interval.

LITERATURE CITED

1. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.

A-28.1

CULTIVATION OF *THERATROMYXA*

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INTRODUCTION

Theratromyxa weberi is a nematophagous ameba. It can be maintained on a number of different genera of nematodes [1]. The cultivation protocol of Sayre and Nerad [2] is detailed here.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 10.0 ml in T-25 flask.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

A-28.2

Agar Medium for *Klebsiella pneumoniae*

Yeast extract	4.0	g
Glucose	0.16	g
Agar	20.0	g
Glass distilled H_2O	800.0	ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Cereal Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing nematode culture which is at or near peak density into two T-25 flask containing 10 ml of fresh medium. Incubate at 25° C.
15. Detach cysts of *Theratromyxa* by rubbing sterile cotton swab over the bottom of a flask culture. Agitate vigorously and transfer 0.1 ml to one of the flasks set up in step 14.
16. When the amebae have almost completely eliminated the nematode, either transfer to a freshly inoculated culture of the nematode as in step 15 or store flask refrigerated (5-9° C) for up to 2 months. Nematode cultures may also be maintained refrigerated for up to 2 mo.
17. Before re-activating a refrigerated culture of the ameba first re-activate a refrigerated nematode culture (see step 14).
18. Repeat steps 15-16.

COMMENTS

The resting cysts of *Theratromyxa* are stable at refrigerated temperatures for many years (R Sayre, personal communication). The cysts, however, do not tolerate drying.

LITERATURE CITED

1. Sayre, RM 1973. *Theratromya weberi*, an amoeba predatory on plant parasitic nematodes. *J. Nematol.* 5:258-264.
2. Sayre, RM & Nerad, TA 1990. A nematode-destroying amoeba. In: Plant Nematology Laboratory Handbook, Zuckerman, BM, Ma, WF & Krusberg, LR ed. University of Massachusetts Press pp. 238-241.

CULTIVATION OF BACTERIVOROUS FRESHWATER AMEBAE

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INTRODUCTION

Many genera of amebae can be easily maintained as monoprotist cultures with bacteria as the food source [1]. Strains of the following genera can be maintained in this manner: *Echinamoeba, Filamoeba, Glaeseria, Mayorella, Nuclearia, Paraflabellula, Platyamoeba, Rosculus, Saccamoeba, Stachyamoeba, Vannella, Vexillifera.* Many strains can be cultivated on agar but some do best in liquid culture. Only strains of one the above genera, *Nuclearia*, have been reported to be cultivable in the axenic state.

PROTOCOL

Agar Plate Cultivation
Non-nutrient Agar

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Non-nutrient Agar	
Agar	15.0 g
Glass distilled H_2O	1.0 L

3. Autoclave at 121° C for 15 min.
4. Allow to cool in a 50° C water bath.
5. Aseptically dispense in 40.0 ml aliquots to 15 x 100 mm petri plates. Allow to harden.
6. Streak plate prepared in step 5 with *Klebsiella pneumoniae* or other suitable bacterium. Incubate at 35° C overnight.
7. Remove an agar block (~5 mm^2) with trophozoites or cysts from the edge of an agar plate and invert the block onto the plate prepared in step 6.
8. Wrap the entire edge of the plate with parafilm and incubate at the appropriate temperature.
9. Repeat steps 6-8 on a 14-21 d interval.

A-29.2

PROTOCOL

Freshwater Ameba Agar, Agar Plate Cultivation

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Freshwater Amoeba Medium

Malt extract	0.1 g
Yeast extract	0.1 g
Bacto-agar (Difco #0140)	10.0 g
Glass distilled H_2O	1.0 L

3. Dissolve first two components in glass distilled H_2O, add agar and autoclave at 121° C for 20 min.
4. Allow to cool in a 50° C water bath.
5. Aseptically dispense in 40.0 ml aliquots to 15 x 100 mm petri plates. Allow to harden.
6. Streak plate prepared in step 5 with *Klebsiella pneumoniae* or other suitable bacterium. Incubate at 35° C overnight.
7. Remove an agar block (~5 mm^2) with trophozoites or cysts from the edge of an agar plate and invert the block onto the plate prepared in step 6.
8. Wrap the entire edge of the plate with parafilm and incubate at the appropriate temperature.
9. Repeat steps 6-8 on a 14-21 d interval.

PROTOCOL

Agar Plate Cultivation
PYb medium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

PYb Agar

Proteose peptone	1.0 g
Yeast extract	1.0 g
Agar	20.0 g
Glass distilled H_2O	900.0 ml

3. Dissolve first two components in glass distilled H_2O while stirring and then add agar.
4. Autoclave at 121° C for 20 min.
5. Allow to cool in a 50° C water bath.
6. Aseptically add the following autoclaved solutions (autoclave solutions separately) to the medium prepared in step 5.

0.05 M $CaCl_2$ (0.56g/100ml)	4.0 ml
0.4 M $MgSo_4.7H_2O$ (9.86g/100ml)	2.5 ml
0.25 M Na_2HPO_4 (3.55g/100ml)	8.0 ml
0.25 M KH_2PO_4 (3.65g/100ml)	32.0 ml

7. Mix thoroughly by swirling the flask after each solution is introduced.
8. Increase the final volume to 1.0 L with sterile glass distilled H_2O. Mix by swirling.
9. Aseptically dispense in 40.0 ml aliquots to 15 x 100 mm petri plates. Allow to harden.
10. Streak plate prepared in step 5 with *Klebsiella pneumoniae* or other suitable bacterium. Incubate at 35° C overnight.
11. Remove an agar block (~5 mm^2) with trophozoites or cysts from the edge of an agar plate and invert the block onto the plate prepared in step 6.
12. Wrap the entire edge of the plate with parafilm and incubate at the appropriate temperature.
13. Repeat steps 10-12 on a 14-21 d interval.

PROTOCOL

Liquid cultivation

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast Extract (Difco #0127)	4.0	g
Glucose	0.16	g
Agar	20.0	g
Glass distilled H_2O	800.0	ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.

11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate cereal grass infusion medium with a loopful of bacteria from a stock slant and incubate at 30° C overnight.
14. Aseptically add 0.1 ml of a growing protist culture which is at or near peak density to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Axenic, *Nuclearia*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

M7 Medium [1]

L-Methionine solution (1.5g/L)	30.0 ml
Glucose solution (270.0g/L)	20.0 ml
Buffer solution (see below)	20.0 ml
Yeast extract solution (25.0g/L)	200.0 ml
Heat-inactivated lamb serum	200.0 ml
Glass distilled H_2O	530.0 ml

Buffer Solution

KH_2PO_4	18.1 g
Na_2HPO_4	25.0 g
Glass distilled H_2O	1.0 L

3. Sterilize the first four solutions separately and combine aseptically.
4. Aseptically distribute in 4.0 ml aliquots to 16 x 125 mm plastic screw-capped test tubes.
5. Add 1.0 ml of heat-inactivated lamb serum to a tube of medium prepared in step 4 just before use.
6. Vigorously agitate an actively growing culture of the protist and transfer a 0.1 ml aliquot to a fresh tube of medium prepared in step 5.
7. Screw the cap on tightly and incubate on a 15° slant at 25° C.
8. The amebae will form an almost continuous sheet of cells on the bottom surface of the test tube.
9. Repeat steps 6 & 7 at 10-14 d intervals.

COMMENTS

The bacterial density will be a function of the agar medium used. The bacterial density will increase as one goes from non-nutrient agar to Freshwater Ameba Agar to PYb Agar. Not all strains of a given genus can tolerate the high bacterial densities encountered on PYb agar. If amebae are to be harvested from agar plates use 2.0 g/L of agar. This will make the surface very firm and will diminish the likelihood of gouging of the plates during harvesting when using a

spread bar. If growth is poor on agar, transfer to liquid medium. Although only one of the amebae listed above has been cultivated axenically, many more are probably cultivable in this state. As of yet, axenization of most genera have not been attempted.

LITERATURE CITED

1. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.

CULTIVATION OF BACTERIVOROUS MARINE AMEBAE

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INTRODUCTION

Strains of marine amebae of the following genera: *Flabellula, Heteramoeba, Lingulamoeba, Mayorella, Nolandella, Paraflabellula, Paramoeba, Platyamoeba, Pseudoparamoeba, Rhizamoeba, Vannella, Vexillifera,* can be maintained as monoprotist cultures with bacteria as a food source [1]. Most can be maintained on agar surfaces while others do best in liquid media. No strains have been reported to be cultivable in the axenic state.

PROTOCOL

Agar Medium
Marine Ameba Agar

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Malt extract	0.1 g
Yeast extract	0.1 g
Agar	10.0 g
Artificial seawater*	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

3. Add the first two components to artificial seawater and dissolve.
4. Add agar and autoclave for 20 min at 121° C.
5. Allow to cool in a 50° C water bath.
6. Aseptically dispense in 40.0 ml aliquots to 15 x 100 mm petri plates. Allow to cool.
7. Remove an agar block (~5mm^2) with trophozoites or cysts from the edge of an agar plate culture and invert onto a plate prepared in step 6.
8. Wrap the entire circumference of the plate with parafilm and incubate at the appropriate temperature.
9. Repeat steps 6-8 on a 7-14 d interval.

A-30.2

PROTOCOL

Liquid Cultivation

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium	
2x Artificial seawater*	500.0 ml
2x Modified cereal grass infusion medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium	
Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.
**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper.
5. Combine equal amounts of the solutions prepared in steps 3 and 4.
6. Aseptically distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

COMMENTS

The bacterial density will be a function of the concentration of the malt extract and the yeast extract in the agar medium. The concentration of each component may be increased to 1.0 g/L. Not all strains of amebae can tolerate bacteria at high densities. High bacterial densities will result in higher yields of amebae. If amebae are to be harvested from agar plates use 2.0 g/L of agar. This will make the surface very firm and will diminish the likelihood of gouging of the plates during harvesting when using a spread bar.

LITERATURE CITED

1. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.

A-31.2

Prepare artificial seawater according to the package directions. Filter sterilize in a 0.2 μm filter.

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Autoclave artificial seawater for 15 min at 121° C.
4. Combine ingredients of the enrichment solution in the order indicated and filter sterilize.
5. Combine ingredients of the vitamin solution in the order indicated and filter sterilize.
6. After the artificial seawater has cooled, aseptically combine the three solutions in the proportions indicated.
7. Distribute 9.0 ml to a T-25 plastic tissue culture flask and aseptically add 1.0 ml (10% v/v) of heat-inactivated (56° C for 30 min) horse serum.
8. Aseptically inoculate the flask with 0.1 ml aliquot of a growing culture.
9. Incubate at 25° C for 7-14 d and repeat step 7.

COMMENTS

The taxonomic position of this organism is uncertain. It forms a highly reticulate plasmodium while actively growing and as the cultures ages parts of the plasmodium separate and detach from the surface of the vessel, round up and become free-floating. These are not true cyst stages but appear to aid as a dispersed stage for this organism.

LITERATURE CITED

1. Kumar, SR 1980. Morphology and taxonomy of the protist *Gymnophrydium marinum* Dangeard from the North Sea. *Bot. Mar.* **23**:353-360.

CULTURE AND MAINTENANCE OF BENTHIC FORAMINIFERA

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Introduction

If a few relatively simple procedures are followed, benthic foraminifera are relatively easy to maintain or culture in the laboratory (reviewed 1-3, 5). A distinction has been made between the two. Maintenance being the ability to keep the foraminifera alive in the laboratory for sustained periods(days-years); they usually feed and grow, but rarely reproduce. Cultured foraminifera grow and reproduce to produce successive generations in the laboratory. Although fewer than 1% of modern foraminiferal species have been maintained in the laboratory to date (1), no researcher should be discouraged from the attempt.

Protocol

1. Collect the larger faraminifera from the marine habitat. SCUBA is most commonly used for harvesting larger foraminifera from the sea, but some larger species (e.g., *Archaias, Sorites* and *Cyclorbiculina*) and many ordinary sized foraminifera occur on sea grasses and sea weeds in very shallow water (<2m). Some foraminifera are abundant in the upper cm of sediment. These can be harvested by using an ordinary kitchen spatula.
2. If the foraminifera have been collected by harvesting substrates (e.g.,*Ulva, Enteromorpha, Thalassia, Halophila,* sediment or stones) on(or in) which they occur, the foraminifera first must be washed, or brushed, off the substrate. This can be acomplished in the field by placing the sea grasses or sea weeds into plastic buckets filled with seawater and rubbing them together to dislodge the epiphytic communities and the foraminifera within them.
3. Remove the seaweeds and sea grasses from the bucket after they have been thoroughly rubbed.
4. Decant the contents of the bucket through a coarse sieve(3mm). If standard laboratory sieves are not available an ordinary plastic kitchen colander can be used. If ordinary sized foraminifera are being collected, a smaller sized sieve may also be used.
5. Let the bucket stand still for several minutes so that the foraminifera will settle to the bottom.
6. Avoiding agitation, decant the bucket carefully so that only the foraminifera and sediment remain. If crude fortuitous agnotobiotic cultures will be set up (step 12) save some of the water with its epiphytes for later use.
7. Add sea water to the bucket, mix well, and repeat steps 5 and 6.
8. With the aid of a wide-necked funnel, pour some of the sediment into the bottom of a wide-necked bottle. Overlay with sea water. Care should be taken not to place too much sediment (≤30% total volume)into any sample bottle.
9. Place bottles into an insulated container for protection from heat when being transported to the laboratory.
10. After returning to the collection to the laboratory, the investigator has a number of choices: 1) inoculate the sample into a circulating marine aquarium or chemostat(8); 2) set up fortuitous agnotobiotic cultures; 3) set up partially gnotobiotic cultures. Each of these methods has advantages and disadvantages with respect to effort and reliability. A number of

species are easily maintained in circulating marine aquaria. The aquarium must get 8 h moderate indirect light. The aquarium can be inoculated with the raw mixed collection, or with species selected under a dissecting microscope from it(see below).The epiphytic algae saved from step 6 may be added to the aquarium at this time. Aquaria fed with flowing seawater at many marine stations can also be used to maintain and culture foraminifera.

11. While it is practical to sort the foraminifera with the aid of a dissecting microscope upon arrival at the laboratory, it is more convient to let the foraminifera sort themselves from non-living matter. The collection is placed into stacking culture dishes (Carolina Supply Co. [Wheaton]cat, no. 41684) or deep petri dishes (Corning cat nos. 3160-152, 324 or 3140). Then glass microscope slides are stood in the sediment or leaned against the sides of the containers. The foraminifera will crawl up the slides over night. The slides can be removed and placed in petri dishes containing a thin layer of sea water. When the dishes with the slides are placed under a dissecting microscope, the foraminifera are easily identified and picked with a sable brush.
12. If agnotobiotic fortuitous cultures are being set up, the epiphytes saved in step 6 can be inoculated into stacking culture dishes or deep dishes. Cover the dishes with another dish, a glass cover or plastic wrap.
13. If gnotobiotic cultures are to be set up,transfer the foraminifera to the first well of a sterile 9-hole spot plate containing aseptically added sterile seawater.
14. Place the spot plate under a dissecting microscope. Add sterile sea water or erdschrieber medium [soil extract (5 ml/100ml), $NaNO_3$, or NH_4NO_3 (10 mg/100 ml), and $NaH_2PO_4.H_2O$ (5mg/100 ml) to 95 ml of the sea water; Adjust the pH of the water if it has changed (e.g. coastal sea water should be pH 8.1)] to all the other wells.
15. With the aid of a pair of sable artist brushes (00-0000), Carefully and vigorously brush the external surfaces of the foraminifera. One brush is used to hold the foraminifer the other is used to brush it. After the they have been used to perform the brushing in one well, the brushes are placed in 70% ethyl alcohol for 5 minutes and placed aside to dry before being used again. With a fresh brush transfer the foraminifera to the next well with sterile seawater.
16. Repeat step 15 another 17 times.
17. The foraminifera can then be aseptically inoculated into test tubes, tissue culture flasks, or glass petri dishes containing sterile media. If the later are used, the petri dishes should be incubated inside a larger petri dish with a piece of moist filter paper on the bottom to retard evaporation. Sea water or erdschrieber can be used as the medium. The latter encourages the growth of the algae in the culture vessel.
18. Two approaches to food have been used successfully. The food can be either alive or freshly killed(4). Heat killing is easily accomplished by placing test tubes with the algae into a hot (50°) water bath for 10-30 minutes before inoculating them. Inoculate food organisms. These can be isolated previously from the same community(See elsewhere in these **Protocols**). A mixture of some of the following algae may also be good food: *Cylindrotheca closterium; Nitzschia acicularis; Nitzschia spp.; Fragilaria construens; Achnanthes hauckiana, Navicula spp.; Chlorella sp.; Dunaliella salina.* These are available from the American Type Culture Collection, 12301 Parklawn Drive, Rocklawn, MD 20852 or Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575. An antibiotic-antimycotic mixture (1 ml/100 ml; GIBCO Laboratories catalog #600-5240; contains: Penicillin 10,000 units,streptomycin 10,000µg, and Fungizone 25 µg/ ml) can be added aseptically to the cultures to retard bacterial growth.
19. The culture dishes should be incubated in moderate indirect light near a window, or in front of a light bank. Incubation temperature should be close to the temperature of the sea where the foraminifera were collected.
20. The cultures must be examined weekly. At that time the medium should be changed and the culture fed.

COMMENTS

It is fairly well known that resource partitioning is an important aspect of the niches of foraminifera(9). Food quality seems very important in setting up cultures of these protozoa (5-8).Foraminifera can be grown in agnotobiotic cultures for many years. After a while the food organisms in the culture seem balanced with the foraminifera or the culture fails. Although foraminifera can grow for many years in chemostats and in aquaria with recirculating sea water, aquaria with running sea water are less reliable. Organisms which are recruited to the aquaria from the sea may compete, and eventually replace, the foraminifera. On the other hand, gnotobiotic cultures of some species of foraminifera have been maintained in the laboratory for decades.

LITERATURE CITED

1.Anderson, O R , Lee, J J & Faber, W W, Jr. 1991. Collection, maintenance and culture methods for the study of living foraminifera. *In:* Lee, J J & Anderson, O R (eds). Biology of the Foraminifera. Academic Press, London. pp.335-358.

2.Arnold, Z M. 1954. Culture methods in the study of living foraminifera. *Paleontol.* **28**: 404-416.

3. Arnold, Z M. 1974. Field and laboratory techniques for the study of living foraminifera. *In*: Hedley,R H & Adams, C G (eds). Foraminifera. Vol. 1. Academic Press, London. pp153-206.

4.Bradshaw, J S 1955. Preliminary laboratory experiments on the ecology of foraminiferal populations. *Micropaleontol.* **1**:351-358.

5. Lee,J J. 1974. Towards understanding the niche of foraminifera. *In*: Hedley, R H & Adams, C G (eds). Foraminifera. Vol. 1. Academic Press, London. pp. 208-257.

6. -------- & Bock, W D. 1976 The importance of feeding in two species of soritid foraminifera with algal symbionts. *Bull. Mar. Sci.* **26**:530-537.

7.---------, McEnery, M E & Rubin, H A. 1969. Quantitative studies on the growth of *Allogromia laticollaris*. *J. Protozool.* **15**:377-395.

8.--------- Sang, K, ter Kuile, B, Strauss, E, Lee, P J & Faber, W W,Jr. 1991 Nutritional and related experiments on laboratory maintenance of three species of symbiont-bearing foraminifera. *Mar. Biol.* **109**:417-425.

9.Muller, W A. 1975. Competition for food and other niche related studies of three species of salt marsh foraminifera. *Mar. Biol.* **31**:339-351.

A-33.1

CULTIVATION OF MYXOMYCOTANS

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INTRODUCTION

Many genera of acellular slime molds have been established in culture with bacteria or yeast as food organisms using the protocols described herein. Protocols for the axenic cultivation cultivation of *Physarum* and *Dictyostelium* are also provided.

PROTOCOL

Acytostelium, Badhamia, Dictyostelium, Didymium, Physarum, Polysphondylium, Stemonitis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Cornmeal Agar	
Cornmeal Agar (Difco #0386)	17.0 g
Glass distilled H_2O	1.0 L

3. Add powder to glass distilled H_2O and autoclave for 15 min at 121° C. (For some strains use only 8.5 g Cornmeal Agar per L of distilled H_2O). Allow medium to cool in a 50° C water bath for 30 min and distribute aseptically in 20.0 ml aliquots to 15 x 100 mm petri plates.
4. Allow plates to cool and add 0.1 ml of a suspension of the food organism.
5. Evenly distribute the suspension over the plate using a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).
6. Remove an agar block (~5 mm^2) containing sporocarps and invert it at the edge of the plate prepared in step 5.
7. Seal the plate shut with parafilm or adhesive tape by wrapping the complete circumference of the plate edge.
8. Invert the plate and incubate at 18-20° C.
9. Repeat steps 6-8 monthly.

A-33.2

PROTOCOL

Echinostelium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

GPY/10 Medium [4]

KH_2PO_4	1.5 g
Na_2HPO_4	0.915 g
Glucose	1.0 g
Peptone	1.0 g
Yeast extract	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Agar	15.0 g
Glass distilled H_2O	1.0 L

3. Adjust pH to 6.6-6.8 with 1N HCl or 1N NaOH and autoclave for 15 min at 121° C. Allow medium to cool in a 50° C water bath for 30 min and distribute aseptically in 20.0 ml aliquots to 15 x 100 mm petri plates.
4. Allow plates to cool and add 0.1 ml of a suspension of the food organism.
5. Evenly distribute the suspension over the plate using a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).
6. Remove an agar block (~5 mm^2) containing sporocarps and invert it at the edge of the plate prepared in step 5.
7. Seal the plate shut with parafilm or adhesive tape by wrapping the complete circumference of the plate edge.
8. Invert the plate and incubate at 18-20° C.
9. Repeat steps 6-8 monthly.

PROTOCOL

Axenic, *Dictyostelium*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Dictyostelium Medium [4]

Peptone (Oxoid L-37)	14.3 g
Yeast Extract (Oxoid L-21)	7.15 g
Glucose	15.4 g
$Na_2HPO_4 \cdot 12H_2O$	1.28 g
KH_2PO_4	0.49 g
Glass distilled H_2O	1.0 L

3. Autoclave for 15 min at 121° C.

4. Allow to cool and dispense in 1.0 ml aliquots to T-25 tissue culture flasks.
5. Inoculate with 0.1 ml from a culture at or near peak density.
6. Incubate at 25° C with the cap screwed on tightly.
7. Transfer every 14 d.

PROTOCOL

Axenic, *Physarum*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (part 1):

N plus C Medium [4]

Citric acid·H_2O	4.0	g
$FeCl_2$·$4H_2O$	0.06	g
$MgSO_4$·$7H_2O$	0.6	g
$CaCl_2$·$2H_2O$	0.6	g
$ZnSO_4$·$7H_2O$	0.034	g
Bacto Tryptone (Difco #0123)	10.0	g
Dextrose	10.0	g
Yeast Extract (Difco #0127)	1.5	g
KH_2PO_4	2.0	g
Glass distilled H_2O	1.0	L

3. Adjust pH to 4.6 with 1N KOH and autoclave for 15 min at 121° C.
4. Allow to cool and distribute 10.0 ml/T-25 tissue culture flask.
5. Medium formulation (part 2):

Hemin Solution

Hemin	25.0	mg
NaOH	0.1	g
Glass distilled H_2O	100.0	ml

6. Autoclave for 15 min at 121° C.
7. Allow to cool and aseptically add 0.1 ml of hemin solution to flask of medium prepared in step 4.
8. Inoculate flask prepared in step 7 with 0.1 ml of a culture at or near peak density.
9. Incubate at 25° C with the cap screwed on tightly for 14 d.
10. Agitate the culture vigorously and transfer 0.1 ml to a fresh flask of medium at each subculturing.

COMMENTS

In addition to species of *Physarum* and *Dictyostelium*, species of *Polysphondylium* [3] have been cultivated on empirical axenic media. Some species of both genera have been cultivated in completely defined axenic media: *Dictyostelium discoideum* [1], *Physarum flaviconum* and *P. rigidum* [2], and *P. polycephalum* [5].

LITERATURE CITED

1. Frenkel, J & Kessin, R 1977. A defined minimal medium for axenic stains of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci.* **74**:2157-2161.
2. Henney, HR, Jr & Lynch, T 1969. Growth of *Physarum flavicomum* and *Physarum nigidum* in chemically defined minimal media. *J. Bacteriol.* **99**:531-534.
3. Hohl, H-S & Raper, KB 1963. Nutrition of cellular slime molds II. Growth of *Polysphondylium pallidum* in axenic culture. *J. Bacteriol.* **85**:199-206.
4. Jong, SC & Edwards, MJ ed. 1991. American Type Culture Collection Catalogue of Filamentous Fungi Eighteenth edt. American Type Culture Collection, Rockville, MD 667 pp.
5. McCullough, CHR & Dee, J 1976. Defined and semi-defined media for the growth of amoebae of *Physarum polycephalum*. *J. Gen. Microbiol.* **95**:151-158.

CULTIVATION OF PROTOSTELIDS

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INTRODUCTION

Most protostelids can be cultivated with bacteria or yeast as food organisms. The only known exception are species of *Ceratiomyxa*, which have not yet been established in culture. The choice of a suitable food organism, pH, and agar concentration may be critical to establish growth. It may also be necessary to make an infusion from the material from which the protostelid was isolated to obtain growth and sporocarp formation [1]. No protostelid has yet been grown axenically.

PROTOCOL

Cavostelium, Protostelium, Schizoplasmodiopsis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Cornmeal Agar

Cornmeal Agar (Difco #0386)	17.0 g
Glass distilled H_2O	1.0 L

3. Add powder to glass distilled H_2O and autoclave for 15 min at 121° C. (For some strains use only 8.5 g Cornmeal Agar per L of distilled H_2O). Allow medium to cool in a 50° C water bath for 30 min and distribute aseptically in 24.0 ml aliquots to 15 x 100 mm petri plates.
4. Allow plates to cool and add 0.1 ml of a suspension of the food organism.
5. Evenly distribute the suspension over the plate using a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).
6. Remove an agar block (~5 mm^2) containing sporocarps and invert it at the edge of the plate prepared in step 5.
7. Seal the plate shut with parafilm or adhesive tape by wrapping the complete circumference of the plate edge.
8. Invert the plate and incubate at 18-20° C.
9. Repeat steps 6-8 monthly.

A-34.2

PROTOCOL

Cavostelium, Ceratiomyxella, Nematostelium, Protostelium, Schizoplasmodium, Schizoplasmodiopsis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

wMY Medium [1]

Malt extract	0.02	g
Yeast extract	0.02	g
K_2HPO_4	0.75	g
Agar	15.0	g
Glass distilled H_2O	1.0	L

3. Adjust pH as desired with 5% (v/v) lactic acid or 1N NaOH and autoclave for 15 min at 121° C.
4. Allow medium to cool in a 50° C water bath for 30 min and distribute aseptically in 24.0 ml aliquots to 15 x 100 mm petri plates.
5. Allow plates to cool and add 0.1 ml of a suspension of the food organism.
6. Evenly distribute the suspension over the plate using a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).
7. Remove an agar block (~5 mm^2) containing sporocarps and invert it at the edge of the plate prepared in step 5.
8. Seal the plate shut with parafilm or adhesive tape by wrapping the complete circumference of the plate edge.
9. Invert the plate and incubate at 18-20° C.
10. Repeat steps 6-8 monthly.

PROTOCOL

Cavostelium, Ceratiomyxella, Nematostelium, Protostelium, Schizoplasmodium, Schizoplasmodiopsis

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

HI Agar [1]

Hay*	2.5 g
Glass distilled H_2O	1.0 L
Agar	15.0 g

*In place of hay it may be necessary to use ground bark or leaves. Grind in a mortar and pestle.

3. Boil for 5 min and filter through a Whatman #1 filter.

4. Adjust pH as desired with 5% (v/v) lactic acid or 1N NaOH; add agar and autoclave for 15 min at 121° C.
5. Allow medium to cool in a 50° C water bath for 30 min and distribute aseptically in 20.0 ml aliquots to 15 x 100 mm petri plates.
6. Allow plates to cool and add 0.1 ml of a suspension of the food organism.
7. Remove an agar block (~5 mm^2) containing sporocarps and invert it at the edge of the plate prepared in step 5.
8. Seal the plate shut with parafilm or adhesive tape by wrapping the complete circumference of the plate edge.
9. Invert the plate and incubate at 18-20° C.
10. Repeat steps 6-8 monthly.

COMMENTS

At the Culture Collection of Algae and Protozoa (CCAP) in England *Planoprotostelium* and *Protostelium* are cultivated on a variant of Cornmeal Agar which contains 2.0 g of glucose per liter [2]. If growth is not achieved with either Cornmeal Agar or supplemented Cornmeal Agar, then it nay be necessary to try the second or third protocol.

LITERATURE CITED

1. Spiegel, FW 1990. Phylum Plasmodial Slime Molds: Class Protostelida In: Handbook of Protoctista: The Structure, Cultivation, Habitats and Life Histories of the Eurkaryotic Microorganisms and their descendants Exclusive of Animal, Plants and Fungi, Margulis, L, Corliss, JO, Melkonian, M, & Chapman, DJ, ed. 1990. Jones & Bartlett Publishers, Boston, MA, USA. pp. 484-497.
2. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of Strains. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.

LABORATORY MAINTENANCE CULTURES OF PLANKTONIC FORAMINIFERA AND RADIOLARIA

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INTRODUCTION

Planktonic foraminifera (Fig. 1) and radiolaria (Fig. 2) are exclusively open-ocean, floating sarcodines occurring in the major oceans of the world. They undoubtedly have adapted to the very uniform and pure conditions of open-ocean environments over long evolutionary time periods. Hence. they require special care in culture to ensure that the water is of the purist quality and freshly collected from open-ocean sources. At present it is not possible to culture planktonic foraminifera or radiolaria for more than one generation in the laboratory, but small individuals collected from the ocean can be grown to maturity in maintenance cultures.

The planktonic foraminifera (Class: Granuloreticulosea) secrete shells of calcium carbonate forming whorled chambers that are broadly classified into two groups (1) non-spinose species (lacking surface spines) and typically forming somewhat flattened, spiral shells, and (2) spinose species with more globose chambers ornamented usually with many radiating spines anchored within the outer layers of the shell wall. There are about 45 commonly occurring extant species. Some species of planktonic foraminifera (especially spinose ones) harbor algal symbionts and dwell for most of their life span in near-surface water where there is sufficient illumination to support symbiont photosynthesis. The symbionts are typically dinoflagellates, but yellow-brown pigmented symbionts categorized as chrysophycophytes are also found in some species. During the day, symbionts of spinose species are distributed within strands of cytoplasm along the spines, but at night are withdrawn near to or into the shell. Most non-spinose species lack symbionts. These are often deeper dwelling at low latitudes, or occur in surface water during the cooler seasons of the year in subtropical locations.

Feeding occurs by snaring protists or small crustacea within the feeding rhizopodia that radiate outward from the shell surface and tips of the spines. At regular intervals (approximately monthly for some species) reproduction occurs by release of hundreds of thousands of flagellated gametes. Preceding gamete release, the planktonic foraminifera retracts its rhizopodia and sheds its spines,if present, and begins sinking in the water column. These events occur within a period of 1 to 2 days. Subsequently,

small juvenile individuals can usually be found in the surface water within a period of about one week. Information on the biology, distribution, and paleoecology of planktonic foraminifera has been summarized in major reviews [e.g., 2, 3, 5-9].

Radiolaria (Classes: Polycystinea and Phaeodarea) secrete siliceous skeletons, or some larger species lack skeletons. There are at least 200 commonly occurring extant species. The organization of the cell cytoplasm and surrounding skeleton varies from spherical to flattened spheroidal, discoidal, quadrangular, or conical. Skeletal structures occur as simple needle-like deposits in the cytoplasm, or form elaborate latticed or porous skeletons of the most exquisite and delicate geometry enclosing the central cell mass. The major cell mass is concentrated into a central capsule surrounded by a capsular wall. Cytoplasmic strands emerge through pores in the capsular wall and form a radiating mass of axopodia. The localization of the pores, their pattern in the capsular wall, and arrangement of emergent axopodia varies among major taxonomic groups. Siliceous skeletons are secreted within the peripheral cytoplasm and are frequently ensheathed by a layer of cytoplasm that is connected to the axopodia. Many species contain algal symbionts (dinoflagellates, prasinomonads, and prymnesiads) usually located within the axopodial array. The symbionts are distributed toward the periphery of the axopodial array during the day and withdrawn inward at night.

Protists and small crustacean prey are captured on the surface of the axopodia. Smaller protists are engulfed into food vacuoles, whereas larger prey are invaded by specialized rhizopodia that extract soft tissue and enclose it within food vacuoles. Reproduction occurs by release of numerous flagellated swarmers after retraction of the external cytoplasm and sinking in the water column as occurs with planktonic foraminifera. The swarmers contain a crystal of strontium sulfate within a cytoplasmic vacuole. Information on biology and distribution can be found in Anderson [1].

Small and intermediate-size planktonic foraminifera and radiolaria, collected from the environment with plankton nets or captured in glass jars held in the hand of a SCUBA diver, can be grown to maturity in laboratory culture in sufficient numbers to allow observational and experimental studies. The techniques for collection, sorting, and placing of the individuals in culture are similar for planktonic foraminifera and radiolaria. They will be described together. Where appropriate, special treatment required by one of the groups will be noted.

PROTOCOLS

1. Collection of planktonic foraminifera and radiolaria.

The choice of a sampling site is critical. Since these are open-ocean protists, they must be collected in deep water of sufficient high quality but sufficiently close to the culturing laboratory to permit timely processing of the sample. It is possible to maintain cultures aboard an ocean vessel if it is sufficiently stable, or preferably, the work is done at an island laboratory near open ocean sites. Marine biological laboratories at Barbados, Bermuda, Curaçao, Jamaica, and other coastal locations near deep ocean water are usually good sites. Some species may be obtained within a reasonable distance from south Florida and southern California. The oceanic deep water must be within approximately 1 to 2 miles from the coast to allow sufficiently rapid transportation. It is not possible to collect samples from most continental coastal locations due to the wide continental shelf. The planktonic foraminifera and radiolaria do not survive in shelf water. It is usually

wise to make a trial collection at a prospective site, or ask someone there to obtain a fixed plankton sample to be sent to you for examination. Species available at a given site may vary substantially from one season to the next or over a period of days or weeks depending on current patterns and surface hydrological conditions. If you cannot visit a site, it is useful to request that several preserved plankton samples taken at intervals of several days be sent to you for examination. In general, equatorial and low latitude open ocean locations are highly productive and fairly predictable sources of some species of planktonic foraminifera and/or radiolaria. Higher latitude locations tend to be richer in smaller radiolaria and less rich in planktonic foraminifera. A zoogeographic review of planktonic foraminiferal abundances by Bé [6] is useful in predicting good collection sites at a given time of year. Further information on radiolarian abundances and collection sites can be found in Anderson [1]. Nigrini and Moore [11] and Lombari and Boden [10] provide additional data on taxonomic descriptions and distribution of modern radiolaria.

Many species of planktonic foraminifera and radiolaria are sufficiently robust that they can be collected by plankton nets (75 µm mesh for larger individuals, or 35 µm mesh for smaller individuals). To prevent injury as much as possible, the nets should be deployed from a drifting boat, not from a boat under power. The current is usually sufficient to accumulate a good catch within 3 to 5 minutes. By examining the suspension in the cod-end of the net, it is possible to judge how dense the catch is. The suspension should be lightly turbid, but not so dense that it looks optically dark. The cod-end sample is immediately decanted into a one gallon plastic jar and filled with seawater from the collection site. The plastic jar must be non-toxic. A Nalgene™ jar is satisfactory. The added seawater dilutes the sample and prevents excessive clumping during transportation to the laboratory. Place the samples in an insulated case to protect them against excessive heat. Use as many plankton tows as necessary to obtain sufficient individuals for your proposed culturing work.

Due to the fragileness of some species, it is preferable to collect individuals in small pint-sized glass jars while held in the hand of SCUBA divers. Only experienced and fully certified SCUBA divers should be permitted to collect samples. Planktonic foraminifera and radiolaria that are sufficiently large to be visualized while floating in the water column can be collected by SCUBA methods. This limits the method somewhat. The method is presented in summary. More detailed accounts are presented elsewhere [5, 8]. Divers work in pairs for safety and to improve collection efficiency. A nylon mesh bag containing glass pint jars for collecting individuals is attached to a nylon rope tethered to the boat. The collecting jars should have plastic tops without liners. The liners may have toxic substances. The jars should be filled with water to reduce buoyancy. Each jar is opened underwater prior to use and cleansed by swirling in some seawater using the fingers. A second empty nylon mesh bag is attached lower on the rope and is used to hold jars containing collected individuals. The SCUBA divers take a position in the water where they can look up against the dark surface of the hull of the boat. The light rays penetrating the water cause the floating planktonic foraminifera or radiolaria to scintillate, and they can be identified more easily. When an organism floats past the diver, the lid of the jar is gently tilted open and the turbulence causes the plankter to be swept into the jar. The lid is immediately secured and the jar placed in the receiving bag. It is absolutely essential to tighten the lid securely to prevent leakage during the return journey to the laboratory. Upon completion of the collecting trip, the samples are placed in an insulated container to protect them from excessive heat. If the boat tends to drift away too rapidly from the divers, one or more plankton nets can be deployed to act as sea-anchors and slow the rate of drift. All collecting jars must be kept

scrupulously clean and not used for other purposes. They can be washed with tap water between uses, but cleaning with detergent is not recommended.

2. Preparing Collected Samples for Culture

Samples collected by plankton nets must be immediately examined on return to the laboratory to separate the planktonic foraminifera and/or radiolaria from other matter in the sample jar. The jars are allowed to stand sufficiently long for most of the particles to settle. The major part of the water is poured off and the sample resuspended. Approximately 100 ml is poured into a 200 ml container (a pyrex crystallization dish is appropriate) and examined using a dissecting microscope with oblique illumination. Planktonic foraminifera and/ or radiolaria that are sufficiently free from other particles can be identified to species level if necessary. They are drawn up into a large bore pasteur pipette fitted with a rubber bulb and transferred to another dish containing approximately 100 ml of seawater collected at the sampling site. During the next several hours, the healthy planktonic foraminifera or radiolaria will gradually purge themselves of adhering debris by cytoplasmic streaming. The debris particles are expelled at the tip of the rhizopodia. For exact taxonomic identification, it may be necessary to examine the separated individuals using a compound microscope. An inverted microscope is particularly useful since the pyrex dish can be placed on the stage, and sufficient detail observed through the bottom of the glass to identify taxonomic features. Clean, robust individuals are removed by drawing them into a large bore pasteur pipette and transferred individually into culture containers as described in section 3. If the individuals are very large, the tip of a pasteur pipette is scored with a file and broken off, a rubber bulb is attached to the truncated tip, and the individual organism is drawn up into the broad opposite end of the pipette.

SCUBA-collected individuals can be examined within the glass jars using a dissecting microscope and identified to species level. If necessary, they can be transferred to a smaller container using a large-bore pasteur pipette and observed with an inverted compound microscope as described above. Healthy individuals are transferred to culture containers.

3. Culture Containers

Optically clear glass vials (30 mm diameter x 90 mm height), containing c. 30 ml of seawater from the open ocean sampling site, are preferred for culturing individual organisms. Do not place more than one individual per vial since they become entangled and eventually cannibalize one another. Each vial is closed with a plastic cap or with a piece of cling-type plastic film to prevent evaporation. The seawater should be freshly collected or if necessary filtered through 0.45 μm pore-size Millipore filter and stored for short periods of time in the refrigerator. **The filter should be rinsed several times with freshwater before use to remove any residual chemicals that may be present from the manufacturing process. Also, any filtered seawater must be allowed to equilibrate with the atmosphere before use to replace oxygen that is excluded during the filtering process.** Some species will die rapidly if placed in Millipore-filtered seawater that has not been re-oxygenated. Success of the culture program depends to a great extent on the quality of the seawater. Contaminated seawater will cause premature death or physiological abnormalities. Carefully monitor the treatment of the seawater sample to prevent contamination. As a general rule, the less done to the water after collection, the better.

4. Culture Apparatus

The temperature of the culture vials must be carefully controlled. Algal symbiont-bearing planktonic foraminifera and radiolaria require adequate illumination to sustain symbiont photosynthesis. Most symbiont-bearing planktonic foraminifera will not survive without adequate illumination and will reproduce prematurely if kept for a day or more without light. The culture apparatus used in our work [4] is shown in Fig. 3. The culture vials are partially immersed in temperature controlled baths to a depth equivalent to the level of the seawater in the vial. Each bath contains a thermostated circulator heater that can be set to a desired constant temperature. A cold water supply serves as a heat sink for each bath. For maintenance culture, the temperature is set equivalent to the seawater temperature at the collecting site or a few degrees Celsius cooler. Slightly cooler water reduces metabolic stress and may extend the vigor and longevity of the cultures. Fluorescent illumination can be supplied from below as shown in the figure, or suspended above the baths. In the latter case, it is preferable to use a clear plastic cap or a clear cling-type plastic film to close the vial to allow sufficient light penetration into the vial.

It is also possible to simply stack the vials in wire racks in front of a vertical bank of fluorescent lights. But, this does not allow exact control of the temperature and the room must be well air-conditioned, and the air circulated with a fan to prevent overheating. This is inadequate, however, for experimental work.

The fluorescent lights in the culture apparatus are set for a 12 h light/ 12 h dark cycle or to a different regime if variations in diel light cycle are part of an experiment. Light intensity can be varied by enclosing the container with the vials in a neutral density nylon mesh with sufficient layers to produce the desired reduction in light intensity. For normal growth conditions, light intensity of approximately 150 to 350 $\mu E/m^2/s$ is adequate. A blue filter of appropriate color quality can be interposed between the lights and the culture containers to produce shorter wavelength illumination characteristic of greater depths in the ocean. In general, it is important to carefully monitor the constant temperature apparatus to be certain that the system is functioning properly. The longevity of some planktonic foraminifera and radiolaria can be several weeks in culture, and it is very disconcerting to lose an experimental treatment after several weeks because of a failure of the constant temperature apparatus.

In general, it is wise to monitor the health and growth of the cultured organism each day by observing it with an inverted compound microscope. The organism can be examined through the bottom of the vial. If it is floating too high in the culture vial, a gentle squirt of culture water from the tip of a pasteur pipette will drive the individual toward the base of the vial. An ocular micrometer permits measurements within 2 to 3 μm accuracy for recording growth. Planktonic foraminifera grow by adding new chambers at regular intervals when well fed and healthy. The chambers are large and clearly visible. Many radiolaria grow by increments, but the silica is added more gradually through accretion. So, the geometry of the growing skeleton does not change so dramatically at each step as occurs with the planktonic foraminifera. A record is kept of the length of rhizopodia, axopodia, and condition and density of symbionts (when present), color, evidence of cytoplasmic motility, skeletal size, and general appearance of the central cytoplasmic mass.

It is essential to regularly open the vials to allow gas exchange with the atmosphere, This is typically done each day when the individuals are observed with the inverted microscope to determine their vitality and size as described above.

5. Sources of Food

Planktonic foraminifera vary widely in their natural food sources. In general, juvenile stages consume protists and will grow well if offered a mixture of laboratory cultured diatoms, coccolithophorida, or other small flagellates. Mature spinose species are largely omnivorous to carnivorous. *Hastigerina pelagica* is largely or exclusively carnivorous consuming small crustacea (e.g., copepods or even crab zoea). Other spinose planktonic foraminifera (e.g., *Globigerinoides sacculifer* and *Orbulina universa*) are omnivorous, but will survive well in culture on a carnivorous diet.

Artemia (brine shrimp) nauplii grown in the laboratory from dried cysts that can be purchased in most pet supply stores is a very good food source for many spinose species. One-day-old nauplii can be collected by shining light in one side of the culture dish containing seawater. The nauplii are phototactic and congregate near the light source where they can be drawn into a pasteur pipette. They are rinsed in seawater obtained at the open-ocean collecting site, individually drawn up into a pasteur pipette and gently expelled near the periphery of the rhizopodia of the planktonic foraminifera. The nauplius will be snared in the rhizopodia and gradually consumed over a period of an hour or more depending on the nutritional state of the foraminifera. In general, feeding one nauplius per day is sufficient to maintain good growth of most planktonic foraminifera. If the planktonic foraminifera is not large enough to snare a living nauplius, the nauplii are cut into smaller pieces. Several nauplii are transferred in a drop of water on the surface of a glass slide and rapidly cut into smaller pieces using a fine razor blade, with a chopping action. The small pieces of the nauplius are pipetted into the vicinity of the rhizopodial halo of the planktonic foraminifera. Non-spinose planktonic foraminifera will accept small nauplii or pieces of nauplii placed near the rhizopodial periphery. In general, non-spinose species do not float in laboratory culture, but become attached to the bottom of the culture vessel. They still protrude feeding rhizopodia and will consume prey and grow by new chamber addition. Typically, spinose and non-spinose species will add new chambers nearly daily under good growth conditions with daily feeding. Overfeeding should be avoided as this leads to excessive buildup of detrital matter in the cytoplasm.

Radiolarian species vary markedly in size, and thus require different food sources depending on their natural prey type and capacity to snare prey. The larger spumellaridan species such as *Thalassicolla nucleata*, *Physematium muelleri*, and spongiose skeletal spongodrymid-type species will accept *Artemia* nauplii or pieces of nauplii. For smaller species, it is preferable to maintain a mixture of algal species in the laboratory for food as recommended above for juvenile planktonic foraminifera. Or, if the culture system is illuminated, simply using unfiltered seawater from the sampling site will provide sufficient natural prey to maintain good growth of many smaller species of radiolaria (e.g., *Spongaster tetras*, *Dictyocoryne* spp., *Euchitonia elegans* and *Didymocyrtis tetrathalamus*). In some cases bacterial prey has been found in digestive vacuoles of smaller radiolaria, and the unfiltered seawater is a good source of these monera, including possibly cyanobacteria. It is wise to examine the cultures daily and transfer the radiolarian to new unfiltered seawater if an excessive growth of diatoms or other protists occurs. A moderate quantity of protists in the culture vial is desirable to maintain good nutrition of the radiolarian. As needed, the radiolarian is transferred by drawing it up into the large end of a pasteur pipette. In general, the smaller species are more delicate and do better with the least handling. Transfer them only as required, preferably about once every two weeks if necessary.

COMMENTS

It is difficult to predict in advance which species found at a particular collecting site will grow best in laboratory maintenance cultures. If you have not identified a particular species for culture work, it is advisable to try culturing several individuals of each of the major species obtained at a collecting site to assess their vitality in culture. Sometimes it is necessary to repeat the trial cultures for several days or weeks since natural variations in some populations of planktonic foraminifera and radiolaria affect vitality of a sample collected at a particular site and at a given time. In general, however, work at our laboratory suggests the following guidelines. Among the planktonic foraminifera, the following species are particularly suitable for laboratory maintenance culture and experimental work: spinose species, *Globigerinoides sacculifer*, *Orbulina universa*, *Globigerinella aequilateralis*, and *Hastigerina pelagica*; and the non-spinose species, *Globorotalia truncatulinoides*, and *Globorotalia hirsuta*. *Globigerinoides ruber*, though widely distributed in the natural environment, is not easily managed in laboratory culture. The individuals tend to shed their spines repeatedly, require more illumination than other species to maintain a robust appearance, and often die earlier.

Among the radiolaria, larger spumellaridan species are often most robust in culture including the skeletonless species *Thalassicolla nucleata*, *Physematium muelleri* (containing only skeletal spicules), *Thalassolampe margarodes,* and larger spongiose spheroidal skeletal spongodrymid-type species. Smaller spumellaridans that survive well in culture include *Spongaster tetras*, *Euchitonia elegans*, *Didymocyrtis tetrathalamus*, and *Spongodiscus* spp. Most Nassellarida are small and often more difficult to observe in culture vials. The large gelatinous colonial radiolaria, abundant in subtropical latitudes in summer, especially in the Sargasso Sea, are usually readily maintained in culture in pyrex culture dishes (containing 100 to 200 ml seawater). They should be illuminated and kept moderately cool (20^{o} to 25^{o} C). Occasional *Artemia* nauplii (about twice weekly or more often) can be supplied as food.

Larger individuals of planktonic foraminifera and many radiolarian species can be expected to begin reproduction relatively soon after collection, especially if well fed. Be alert to the signs of reproduction, including withdrawal of peripheral cytoplasm, a gradual loss of color of the remaining, usually abundant, central cytoplasm, formation of a whitish mass of developing reproductive cells in the central cytoplasm, and eventual release of reproductive cells that rapidly swim outward and away from the gradually emptied test. If the organisms seem to suddenly sink to the bottom of the culture vessel, this may be early stages of reproduction. Dying individuals can often be distinguished from contracted reproductive ones by the appearance of the remaining cytoplasm which often is heavily granular, sometimes remains pigmented even in later moribund stages, and usually is attacked by numerous scavenger flagellates that swarm near or around the surface.

When working with a new species in culture, one of the major requirements is finding an appropriate source of food. If natural prey in the seawater obtained at the collection site does not provide good growth, try establishing a mixed culture of microzoa liberated from the cytoplasm of very freshly collected individuals of the planktonic foraminifera or radiolaria you are trying to culture. Crush several of the freshly collected individuals and add them to the culture media in test tubes that are illuminated.

Sometimes, the mixture of microbiota that bloom in such a tube may serve as food if offered sparingly to the planktonic foraminifera and radiolaria in your culture vessels. If you find that a particular mixture of food organisms works well, transfer the cultures to new media at regular and frequent intervals to ensure a vigorous and healthy source of food organisms. Otherwise, one or more of the more rapidly growing individuals will completely overtake the food supply. It is often difficult to maintain such mixed cultures for very long, and it may be necessary to begin again if your food supply becomes less effective with time.

Radiolaria require a source of silicate for growth. Usually enough silicate is available in the original seawater, or dissolved from the glass vial, to sustain growth. It is important, therefore, to use glass culture containers. It may be advisable to experiment with adding a clean sand grain for additional silicate, if growth is not adequate.

LITERATURE CITED

1. Anderson, O R 1983 Radiolaria. Springer-Verlag, New York.

2. Anderson, O R 1984 Cellular specialization and reproduction in planktonic foraminifera and radiolaria. *In*: Steidinger, K A & Walker, L M (eds), Marine Plankton Life Cycle Strategies. Chemical Rubber Company Press, Boca Raton.

3. Anderson, O R 1988 Comparative Protozoology: Ecology, Physiology, Life History. Springer-Verlag, New York.

4. Anderson, O R, Bennett, P & Bryan, M 1989 Experimental and observational studies of radiolarian physiological ecology: 3. Effects of temperature, salinity and light intensity on the growth and survival of *Spongaster tetras tetras* maintained in laboratory culture. *Mar. Micropaleontol.* **14**: 275-282.

5. Anderson, O R, Lee J J, & Faber, W W Jr 1991 Collection, maintenance and culture methods for the study of living foraminifera. *In*: Lee, J J & Anderson, O R (eds), Biology of Foraminifera. Academic Press, London, pp. 335-357.

6. Bé, A W H 1977 An ecological, zoogeographic and taxonomic review of recent planktonic foraminifera. *In*: Ramsay, A T S (ed), Oceanic Micropaleontology, Vol. 1. Academic Press, London, pp. 1-100.

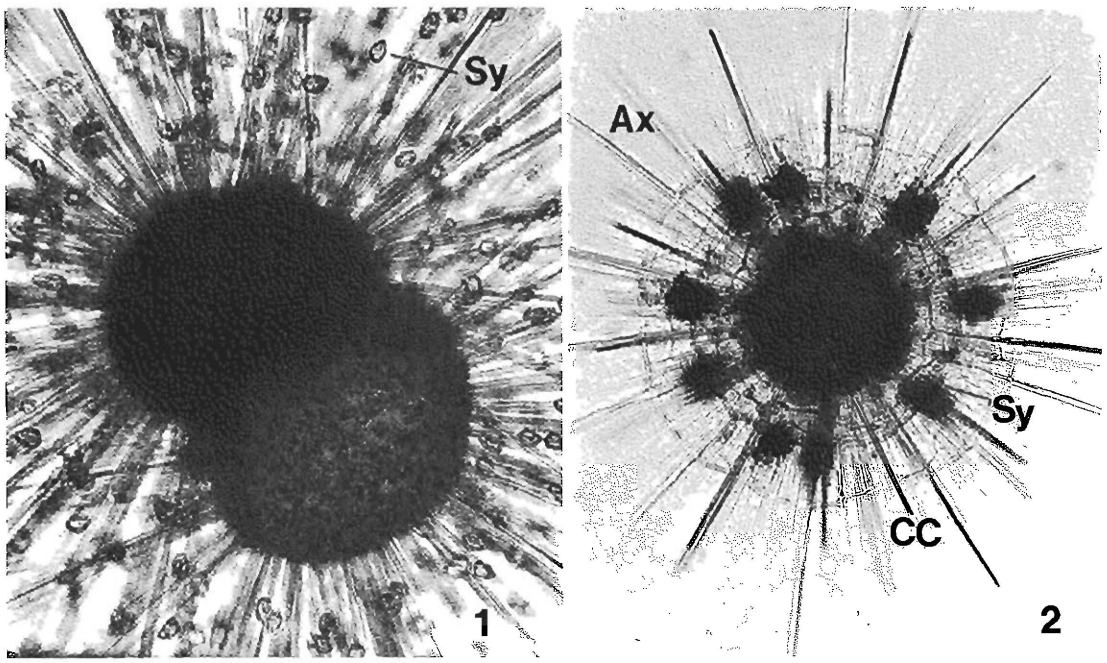
7. Bé, A W H, Hemleben, Ch, Anderson, O R, Spindler, M, Hacunda, J, & Tuntivate-Choy, S 1977 Laboratory and field observations of living planktonic foraminifera. *Micropaleontology* (NY), **25**: 155-179.

8. Hemleben, Ch, Spindler, M, & Anderson O R 1989 Modern Planktonic Foraminifera. Springer-Verlag, New York.

9. Lee, J J & Anderson O R 1991 Biology of Foraminifera. Academic Press, London.

10. Lombari, G & Boden, G 1985 Modern Radiolarian Global Distributions, Special Publication No. 16A. Cushman Foundation for Foraminiferal Research, Washington, DC.

11. Nigrini, C & Moore, T C 1979 A Guide to Modern Radiolaria. Special Publication No. 16. Cushman Foundation for Foraminiferal Research, Washington, DC.



Figs. 1 & 2. Living planktonic foraminifera and radiolaria. 1. Symbiont-bearing (Sy) planktonic foraminifera showing the spiral calcitic shell with radiating spines and halo of rhizopodia. 2. Radiolarian with symbionts (Sy) in the peripheral halo of axopodia (Ax) emerging from the denser cytoplasm in the central capsule (CC).

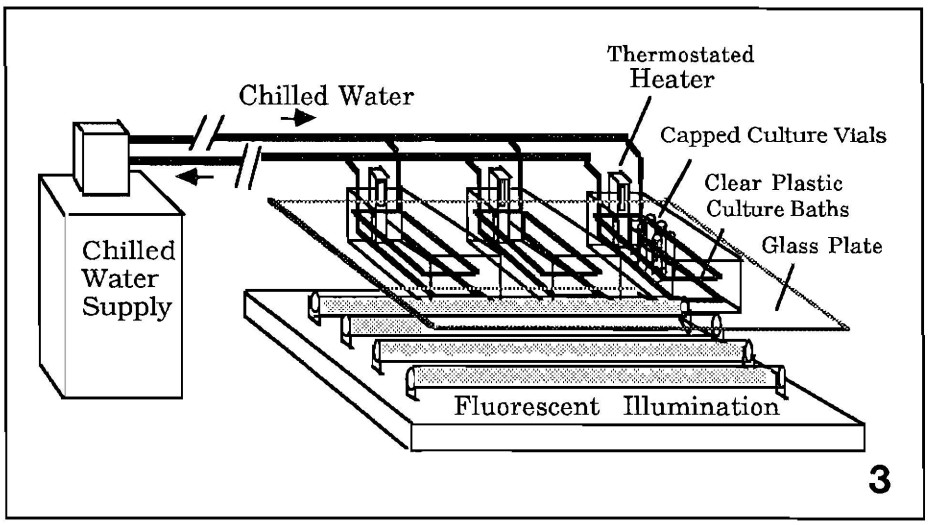


Fig. 3 Illuminated, constant-temperature culture apparatus. A fluorescent fixture provides illumination. Capped culture vials are partially immersed in culture baths. Each bath contains a thermostated, circulating heater to maintain a constant temperature. The chilled water circulating through copper tubing in the water bath is a heat sink. Adapted from Anderson, et al. [4].

A-36.1

CULTIVATION OF LABYRINTHULA

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INTRODUCTION

Labyrinthula is best cultivated monoxenically with bacteria or yeast as a food source [1]. One protocol is presented below.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Agar with 1% Serum [1]

Heat-inactivated horse serum	10.0 ml
Agar	12.0 g
Artificial seawater*	990.0 ml

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

3. Autoclave medium without horse serum at 121° C for 20 min.
4. Allow to cool to 50° C in a water bath.
5. Aseptically add the heat-inactivated horse serum and swirl to distribute evenly.
6. Aseptically dispense in 40.0 ml aliquots to 15 x 100 mm petri dishes. Allow to solidify.
7. Distribute food organisms over the surface of the plate. Incubate at 25° C overnight.
8. Aseptically remove an agar block (~5 mm^2) from the edge of a plate culture with active cells.
9. Invert the agar block at the edge of a plate prepared in step 7.
10. Repeat steps 7-9 at 14-28 d intervals.

COMMENTS

Some strains will be lost after prolonged cultivation. It is best to cryopreserve strains soon after isolation.

LITERATURE CITED

1. Jong, SC & MJ Gantt ed. 1987. American Type Culture Collection Catalogue of Fungi/Yeasts, 17th edition, American Type Culture Collection, Rockville, MD 532 pp.

CULTIVATION OF *PLASMODIUM*

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INTRODUCTION

Human malarial species can be maintained in primates or by *in vitro* methods [2]. Since *in vitro* methods are probably the most practicable for most laboratories the simplest *in vitro* method is detailed here. The *in vitro* cultivation has been semi-automated and there are methods to produce "large" quantities. The reader is referred to literature for the latter [1,3]. The maintenance of rodent *Plasmodium* is also provided *in vivo*.

PROTOCOL

In vitro Cultivation
Candle Jar Method
Plasmodium falciparum

1. Obtain one pint of human A+ blood as freshly drawn as possible from a local blood bank. If the blood can be obtained already separated into a cell pack and plasma, (it usually is no more expensive to obtain in this configuration than as whole blood) it will save several preparative steps.
2. If the blood is obtained as whole blood aseptically distribute in 100 ml aliquots to 125 ml screw-capped bottles and allow bottles to remain undistributed refrigerated (5-9° C) for 1-2 d. The red blood cells will settle and the serum can be removed with a pipette.
3. To 100 ml of serum add 1.0 ml of 10% (w/v) $CaCl_2$ prepared in glass distilled H_2O. Aseptically add an autoclaved stirring bar and stir at room temperature for 30 min. A clot will form over the stirring bar. Do not stir too rapidly or this will inhibit clot formation. Stir at medium setting. Serum allowed to warm to room temperature prior to addition of $CaCl_2$ will clot faster than cold serum.
4. With a pipette distribute serum (less the clot) in 10.0 ml aliquots to plastic screw-capped 16 x 125 mm and store at -20° C until needed.
5. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
6. Medium formulation:

RP medium

Solution 1 (see below)	960.0 ml
Solution 2 (see below)	40.0 ml
Solution 3 (see below)	1.0 ml

A-37.2

Solution 1

RPMI - 1640 powder (GIBCO 430-1800EB)	10.4 g
HEPES (Research Organics Inc. 6003H)	5.94 g
Glass redistilled H_2O	960.0 ml

Filter sterilize using a 0.2μm filter.

Solution 2

$NaHCO_3$	5.0 g
Glass redistilled H_2O	100.0 ml

Filter sterilize using a 0.2μm filter.

Solution 3

Hypoxanthine	1.5 mg
Glass redistilled H_2O	100.0 ml

Filter sterilize using a 0.2μm filter.

7. To prepare Solution 1 first dissolve the RPMI - 1640 powder in 900.0 ml of glass redistilled H_2O. Then dissolve the HEPES and dilute to 960 ml with glass redistilled H_2O. Filter sterilize. Can be stored refrigerated (5-9° C) for up to one month.
8. To prepare Solution 2 dissolve 5.0 g of $NaHCO_3$ in 100.0 ml of glass redistilled H_2O and filter sterilize. Can be stored refrigerated (5-9° C) for up to one month.
9. To prepare Solution 3 dissolve 15.0 mg of hypoxanthine in 100 ml of glass redistilled H_2O at 80° C. Mix thoroughly and transfer 10 ml of this solution to 90 ml of glass redistilled H_2O at 80° C.
10. Before use add 4.2 ml of solution 2 and 0.1 ml of solution 3 to 100 m of solution 1 and mix thoroughly. This solution can be stored refrigerated (5-9° C) for up to a week.
11. To 8.5 ml of the solution prepared in step 8 add 1.5 ml of declotted human serum prepared in step 4. This constitutes the complete liquid medium used for the cultivation of the parasite.
12. Wash red blood cells as follows: Transfer 5.0 ml of red cell pack to a 15.0 ml centrifuge tube and add ~10 ml of medium (less serum) prepared in step 8. Centrifuge at 850 g for 5 min.
13. Decant supernatant and repeat step 10.
14. Decant supernatant and repeat step 10.
15. Decant supernatant and add a volume of complete medium (see Step 9) equal to the volume of the cell pack. This constitutes a 50% uninfected blood suspension.
16. *In vitro* strains of *Plasmodium falciparum* can be cultivated in a 250 mm (ID) desiccator equipped with a 230 mm diameter desiccator plate with a $^7/_8$" center hole. Four to five candles of various heights are spaced about the surface of the plate and one is positioned beneath the plate under the center hole.
17. On the fourth day after inoculation swirl each petri dish to suspend the red blood cells and using an inoculating loop steak a loopful on a microscope slide. Allow slide to air dry and then Giemsa stain. Count the number of infected red blood cells versus uninfected red blood cells. A minimum of 500 red blood cells should be counted. A red blood cell may be multiply infected, but it is counted as a single infected cell.
18. If the average parasitemia is 5-10% the culture can be passaged.
19. Resuspend the red blood cells from all petri dishes and pool. Centrifuge at 850 g for 7 min.
20. Remove as much of the supernatant as possible using a pipette and resuspend the cells with a volume of complete medium equal to the volume of the cell pack. This represents a 50% infected blood suspension.
21. The desired parasitemia (0.1-0.2%) is obtained by diluting the 50% infected blood suspension with the 50% uninfected suspension (prepared in step 13 of previous protocol). For example if the parasitemia of the 50% infected blood suspension is 10% then if 0.1 ml of this suspension is diluted with 9.9 of 50% uninfected blood, a final parasitemia of 0.1% would be achieved.
22. To prepare 4 cultures of an 8.0% blood suspension, dilute 1.0 ml of the 50% blood suspension with the adjusted parasitemia with 5.0 ml of the complete medium. Dispense 1.5 ml of this preparation into 3.5 cm petri dishes. Place petri dishes on the desiccator plate.

23. Place a layer of high vacuum silicone grease (Dow Corning) along the rim of the desiccator.
24. Light all candles.
25. Turn sleeve of desiccator lid such that the vent is open and securely place the lid on the desiccator. Make sure the layer of high vacuum silicone grease (Dow Corning) is even and there are no air spaces to the outside.
26. Allow the candles to burn until the last one is almost extinguished. Then turn the sleeve of the desiccator lid to close the vent.
27. Tape the lid of the desiccator with masking tape. This will prevent the lid from sliding while incubating.
28. Place the desiccator at 37° C.
29. Feed the cultures daily as follows: Gently remove the desiccator from the incubator taking care not to agitate the cultures. Remove the masking tape and gently slide off the desiccator lid and with as little agitation as possible place the petri plates in a laminar flow hood.
30. Gently remove the lid, tilt the plates, and aspirate the medium covering the red blood cells using a Pasteur pipette. Avoid sucking the blood cells into the pipette.
31. After the medium has been removed add 1.5 ml of fresh complete medium to each 35 mm plate or 12.0 ml of medium if 100 mm plates are used.
32. When parasitemia is between 10-15%, subculture (usually every 4 d).

PROTOCOL

In vivo cultivation in mice
Plasmodium berghei berghei, Plasmodium vinckei vinckei

1. Inoculate 0.1 ml of a infected blood suspension obtained either from cryopreserved stock or an infected animal (see below) intraperitoneally into a 6-9 wk old mouse using a 1.0 ml syringe equipped with a 27 gauge ½ inch needle.
2. Monitor the mouse daily. Normal healthy mice have well groomed fur. The fur of infected mice appears progressively ruffled. Eventually the mouse will become sluggish and will adopt a hunched posture.
3. Do tail clips to determine the level of the parasitemia when mice begin to appear ill. Grip a mouse by the tail and allow it to hang downward with its head inside a cage. Place the wire lid on top of the cage to secure the tail against the top of the cage. Hold the tail with index finger and thumb of one hand near the base of the tail. With the fore finger and thumb of the other hand securely grip the tail and run these fingers back and forth along the tail several times. This will cause the tail to become engorged with blood.
4. With a sterile pair of scissors clip the very tip of the tail and force a drop of blood to the tip of the tail by running a thumb and fore finger from the base of the tail toward the tip.
5. Place the drop of blood at the edge of a microscope previously cleaned with 70% ethanol slide and make a blood smear. Allow to air dry.
6. Stain with Giemsa and count the number of infected red blood cells versus the uninfected cells. A minimum of 500 red blood cells should be counted. A red blood cell may be multiply infected, but it is counted as a single infected cell.
7. The level of parasitemia before the mouse will succumb will vary with the strain used. Monitoring on a daily basis will alert the experimenter as to when the strain should be passage.
8. To passage the strain remove blood from the infected mouse using cardiac puncture. In a laminar flow hood ventilated to the outside add one capful of the Metofane (Pitman-Moore, Inc., Washington Cross, NJ, cat. #55685) to a wad of cotton at the bottom of a 1 gallon jar. Place a wire mesh screen over the top of the cotton and tightly secure the lid. Allow the jar to remain undisturbed for 10 min. Remove lid of jar and add infected mouse. When mouse is thoroughly anaesthetized, tie it down firmly with its stomach upward. Thoroughly alcohol the chest with 70% denatured alcohol.

9. Puncture the heart using a 1.0 ml syringe equipped with a 27 gauge ½ inch needle. Prior to using add 0.4 ml of Yaeger's anticoagulant solution (see below) to the syringe. Move the plunger of the syringe back and forth several times to distribute the anticoagulant. Adjust the final volume of the anticoagulant to 0.1 ml.

Yaeger's Anticoagulant

Sodium citrate	1.33 g
Citric acid	0.47 g
Dextrose	3.00 g
Sodium heparin	0.2 g
Glass distilled H_2O	100.0 ml

10. Draw blood into the syringe by gently pulling the plunger outward. When blood is no longer obtainable or the mouse has died remove needle and invert the syringe several times to mix the anticoagulant evenly with the blood.
11. Remove air bubbles from the syringe. Place the syringe in a vertical position with the needle pointing upward. Place the tip of the needle on the surface of a cotton ball previously thoroughly alcoholed (squeeze the cotton ball so that it is moist but not dripping wet). With the index finger flick the top of the syringe several times to allow the air bubbles to coalesce and move to the top of the syringe body. Gently push in the plunger to remove the air pocket. It may be necessary to repeat this procedure several times to remove most air bubbles. When a steady stream of blood exits the needle, the blood is ready for injection.
12. Inject 0.1 ml of the infected blood suspension into each uninfected mouse.
13. Subculture as needed.

COMMENTS

Plasmodium falciparum can be cultivated without the addition of hypoxanthine but yields will be much lower (5-8% vs. 15-20%). Morakuta and Charuchinda report a salt solution which allows storage of red blood cells for up to 2 months [4]. *Plasmodium berghei* can be cultured *in vitro* for extended periods [5]. Red blood cells from the hamster, quinea pig and mouse supported optimal growth of *P. berghei* [6].

LITERATURE CITED

1. Butcher, GA 1981. A simple automated device for maintenance of *Plasmodium falciparum*: Continuous cultivation in a suspension culture. *Ann. Trop. Med. & Parasitol.* **75**:111-113.
2. Jensen, JB & Trager, W 1977. *Plasmodium falciparum* in culture: Use of out-dated erythrocytes and description of the candle jar method. *J. Parasitol.* **63**:883-886.
3. Jensen, JB, Trager, W & Doherty, J 1979. *Plasmodium falciparum*: Continuous cultivation in a semi-automated apparatus. *Exp. Parasitol.* **48**:36-41.
4. Morakote, N & Charuchinda, K. 1988. Storage of red cells for *in vitro* cultivation of *Plasmodium falciparum*. *Ann. Trop. Med. & Parasitol.* **82**:625-626.
5. Ramaiya, ML & Renapurkar, DM 1988. Use of various animal erythrocytes for *in vitro* cultivation of *Plasmodium berghei*. *Int. J. Parasitol.* **18**:639-641.
6. Ramiaya, ML, Kamath, V, & Renapurkar, DM 1987. Long term *in vitro* cultivation of *Plasmodium berghei*. *Int. J. Parasitol.* **17**:1329-1332.

CULTIVATION OF *TOXOPLASMA*

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INTRODUCTION

Strains of *Toxoplasma gondii* can be cultivated either *in vitro* in a cell line [1,4] or *in vivo* in mice. Protocols for both cultivation methods are provided.

PROTOCOL

In vitro

Cultivation in a cell line

1. A foreskin fibroblast cell live is one of several cell lines that can serve as a host for *Toxoplasma gondii*. The cell line can be maintained in the medium listed in step 2.
2. Medium formulation:

Toxoplasma Medium

L-Amino Acids

Arginine	0.105 g
Cystine	0.024 g
Glutamine	0.292 g
Histidine	0.031 g
Isoleucine	0.052 g
Leucine	0.052 g
Lysine	0.058 g
Methionine	0.015 g
Phenylalanine	0.032 g
Threonine	0.048 g
Tryptophase	0.010 g
Tyrosine	0.036 g
Valine	0.046 g

Vitamins

Choline	1.0 mg
Folic acid	1.0 mg
Inositol	2.0 mg
Nicotinamide	1.0 mg
Pantothenic acid	1.0 mg
Pyridoxal•HCl	1.0 mg
Riboflavin	0.1 mg
Thiamine•HCl	1.0 mg

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Earle's Salts

NaCl	6.8 g
KCl	0.4 g
$NaH_2PO_4 \cdot H_2O$	0.125 g
$CaCl_2$	0.2 g
$MgSO_4$	0.1 g
$NaHCO_3$	2.2 g
Glucose	1.0 g
Phenol red	0.05 g

3. The medium ingredients should be added to 1.0 L of glass distilled H_2O in the order indicated.
4. Adjust pH to 7.2-7.4.
5. Filter sterilize using a 0.2μm filter.
6. To 900 ml of medium add 100 ml of heat-inactivated fetal calf serum (HIFCS) before use. To inactivate serum, place 100 ml in a 125 ml screw-capped bottle and transfer to a 56° C water bath for 30 min.
7. Add 10 ml of complete medium to T-25 flasks.
8. When the cell line has formed a contiguous monolayer, remove the overlying medium with a pipette. Add 2.0 ml of 0.25% (w/v) Trypsin Solution dissolved in Hanks' Balanced Salt Solution (see below). Gently distribute the Trypsin Solution over the monolayer, remove the Trypsin Solution and place the flask at 35° C for 10 min.

Hanks' Balanced Salt Solution

NaCl	8000.0 mg
KCl	400.0 mg
$Na_2HPO_4 \cdot 2H_2O$	60.0 mg
KH_2PO_4	140.0 mg
$CaCl_2$	200.0 mg
$MgSO_4 \cdot 7H_2O$	350.0 mg
$NaHCO_3$	20.0 mg
Phenol red	20.0 mg
Glucose	1000.0 mg
Glass distilled H_2O	1000.0 ml

9. Add 2.0 ml of fresh complete medium and detach any cells still adherent by alternately aspirating the medium into a pipette and discharging the contents rapidly over the monolayer.
10. Distribute the cell suspension in 0.5 ml aliquots to T-25 flasks prepared in Step 7.
11. Outgas flasks with 95% air, 5% CO_2 gas mixture for 10 seconds and immediately screw caps on tightly.
12. Incubate flasks at 35° C and change medium twice a week.
13. When the monolayer of the cell line is complete remove the medium and replace with 10 ml of *Toxoplasma* Medium prepared in the above protocol but use 3% (v/v) HIFCS rather than 10% (v/v).
14. A culture of *Toxoplasma* that has almost completely destroyed the cell line is ready for subculture.
15. Vigorously agitate the culture and aseptically transfer 0.1 ml and 0.25 ml aliquots to fresh confluent cell monolayers prepared in step 13.
16. Outgas with 95% air, 5% CO_2 gas mixture and immediately tightly close cap. Incubate at 35° C.
17. Subculture at 7-14 d intervals from time of inoculation of the monolayer.

PROTOCOL

In vivo cultivation in mice

1. Inoculate 0.1 ml of a tachyzoite suspension obtained either from a cryopreserved stock or an infected animal (see below) at a concentration of 10^6 cells/ml intraperitoneally into a 6-9 wk old mouse using a 1.0 ml syringe equipped with a 27 gauge ½ inch needle.
2. Monitor the mouse daily. Normal healthy mice have well groomed fur. The fur of infected mice appears progressively ruffled. Eventually the mouse will become more sluggish and will adopt a hunched appearance. The abdomen will also become progressively distended. The infection will ultimately result in death of the mouse.
3. Kill the mouse using cervical dislocation. Hold the tail of the mouse with one hand and place the mouse on the wire lid of a cage. The mouse will normally grip the lid with its fore paws. Pull the tail gently and with the thumb and fore finger of the opposite hand firmly press the neck of the mouse against the cage. Pull the tail until the skull has been completely separated from the spinal column.
4. Inject 2.0 ml of Tyrodes Salt Solution aseptically into the peritoneum using a 27 gauge, ½ inch needle attached to a 5 ml syringe. Massage the abdomen and aseptically remove as much fluid as possible with a 20 gauge 1½ inch needle attached to a 5 ml syringe.

Tyrodes Salt Solution

NaCl	8.0 g
KCl	0.2 g
$CaCl_2$	0.2 g
$MgCl_2 \cdot H_2O$	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.05 g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

5. Inoculate 0.2 ml of the recovered peritoneal fluid per mouse to subculture.

COMMENTS

Toxoplasma gondii can be cultivated in a non-adherent cell line [3] and this would reduce the number of steps in the protocol and avoid the use of Trypsin. The serum component can be replaced with various proteins [2].

LITERATURE CITED

1. Frenkel, JK & Pfefferkorn, LC 1984. Method for the immunization of animals and men against *Toxoplasma gondii*. *U.S. Patent* **4**:473549.
2. Hermentin, K, Aver, H & Aspack, H 1987. In vitro cultivation of *Toxoplasma gondii* under defined, serum-free conditions. *J. Parasitol.* **73**:1276-1277.
3. Noreiga, FR & Hauser, WE, Jr 1988. *Toxoplasma gondii* maintenance in tissue culture: A new efficient method for culturing RH tachyzoites. *J. Parasitol.* **74**:495-499.
4. Pfefferkorn, ER & Pfefferkorn, LC 1976. *Toxoplasma gondii*: Isolation and preliminary characterization of temperature-sensitive mutants. *Exp. Parasitol.* **39**:365-376.

CULTIVATION OF *BABESIA*

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INTRODUCTION

Various species of *Babesia* can be cultivated *in vivo* in the appropriate host species. One species *B. bigemina*, has been adapted to *in vitro* cultivation [1]. The method presented below will focus on the *in vivo* cultivation of *B. microtia* in mice. **Since some species are infective to humans, safety should be a primary concern.** Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

In vivo cultivation
Babesia

1. Inoculate 0.1 ml of an infected blood suspension obtained either from cryopreserved stock or an infected animal (see below) intraperitoneally into a 6-9 wk old mouse using a 1.0 ml syringe equipped with a 27 gauge ½ inch needle.
2. Monitor the mouse daily. Normal healthy mice have well groomed fur. The fur of infected mice appears progressively ruffled. Eventually the mouse will become sluggish and will adopt a hunched posture.
3. Do tail clips to determine the level of the parasitemia when mice begin to appear ill. Grip a mouse by the tail and allow it to hang downward with its head inside a cage. Place the wire lid on top of the cage to secure the tail against the top of the cage. Hold the tail with index finger and thumb of one hand near the base of the tail. With the fore finger and thumb of the other hand securely grip the tail and run these fingers back and forth along the tail several times. This will cause the tail to become engorged with blood.
4. With a sterile pair of scissors clip the very tip of the tail and force a drop of blood to the tip of the tail by running a thumb and fore finger from the base of the tail toward the tip.
5. Place the drop of blood at the edge of a microscope slide previously cleaned with 70% ethanol and make a blood smear. Allow to air dry.
6. Stain with Giemsa and count the number of infected red blood cells versus the uninfected cells. A minimum of 500 red blood cells should be counted. A red blood cell may be multiply infected, but it is counted as a single infected cell.
7. The level of parasitemia before the mouse will succumb will vary with the strain used. Monitoring on a daily basis will alert the experimenter as to when the strain should be passage.
8. To passage the strain remove blood from the infected mouse using cardiac puncture. In a laminar flood hood ventilated to the outside add one capful of the Metofane (Pitman-Moore, Inc., Washington Cross, NJ, cat. #55685) to a wad of cotton at the bottom of a 1 gallon jar. Place a wire mesh screen over the top of the cotton and tightly secure the lid. Allow the jar to remain

undisturbed for 10 min. Remove lid of jar and add infected mouse. When mouse is thoroughly anaesthetized, tie it down firmly with its stomach upward. Thoroughly alcohol the chest with 70% denatured alcohol.

9. Puncture the heart using a 1.0 ml syringe equipped with a 27 gauge ½ inch needle. Prior to using add 0.4 ml of Yaeger's anticoagulant solution (see below) to the syringe. Move the plunger of the syringe back and forth several times to distribute the anticoagulant. Adjust the final volume of the anticoagulant to 0.1 ml.

Yaeger's Anticoagulant

Sodium citrate	1.33 g
Citric acid	0.47 g
Dextrose	3.00 g
Sodium heparin (NBCo)*	0.2 g
Glass distilled H_2O	100.0 ml

*Nutritional Biochemical Corporation

10. Draw blood into the syringe by gently pulling the plunger outward. When blood is no longer obtainable or the mouse has died remove needle and invert the syringe several times to mix the anticoagulant evenly with the blood.
11. Remove air bubbles from the syringe. Place the syringe in a vertical position with the needle pointing upward. Place the tip of the needle on the surface of a cotton ball previously thoroughly alcoholed (squeeze the cotton ball so that it is moist but not dripping wet). With the index finger flick the top of the syringe several times to allow the air bubbles to coalesce and move to the top of the syringe body. Gently push in the plunger to remove the air pocket. It may be necessary to repeat this procedure several times to remove most air bubbles. When a steady stream of blood exits the needle, the blood is ready for injection.
12. **With safety precautions** in mind, inject 0.1 ml of the infected blood suspension into each uninfected mouse.
13. Subculture as needed.

COMMENTS

Parasitemias can reach levels of greater than 60%.

LITERATURE CITED

1. Vega, CA, Buening, GM, Green, TJ & Carson, CA 1985. *In vitro* cultivation of *Babesia bigemina*. *Am. J. Vet. Res.* **46**:446-420.

CULTIVATION OF *STEPHANOPOGON*

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INTRODUCTION

Lipscomb and Corliss reported successful cultivation of *Stephanopogon apogon* for months using filtered seawater, split peas and unidentified bacteria [1]. The species can be cultivated to high densities using the method detailed below.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater	500.0 ml
2x Cereal grass infusion medium (see below)	500.0 ml

2x Cereal Grass Infusion Medium

Powdered cereal grass leaves*	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper. Autoclave for 15 min at 121° C.
5. Allow solution prepared in step 4 to cool and combine equal amounts of this solution and the solution prepared in step 3.
6. Distribute medium in 10.0 ml amounts in a T-25 flask and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.
9. Vigorously agitate a flask of a culture of *Rhynchomonas nasuta* at peak density and transfer to a 15.0 ml centrifuge tube. Centrifuge at 850 g for 5 min. Discard supernatant and resuspend pellet with artificial seawater to a final volume of approximately 14.0 ml. Centrifuge as above.
10. Discard supernatant and resuspend pellet with artificial seawater. Centrifuge as in step 9.
11. Discard supernatant. Resuspend pellet with supernatant to a final volume of approximately 1.0 ml.
12. Medium formulation:

Marine Flagellate Medium [2]

Filter sterilized artificial seawater	15.0 ml
Autoclaved rice grains (Uncle Ben's Natural Whole Grain Rice*)	2.0

*Can be obtained from grocery store.

13. Fill a 20 x 150 mm screw-capped test tube approximately $^1/_3$ full with rice grains. Autoclave at 121° C for 20 min. Loosen cap one half turn prior to autoclaving.
14. Prepare artificial seawater according to package directions. Filter sterilize.
15. Add 15.0 ml aliquots of seawater to T-25 tissue culture flasks and then aseptically add two rice grains.
16. Add 1.0 ml of concentrated suspension of *Rhynchomonas nasuta* prepared in step 11.
17. Vigorously agitate the culture of *Stephanopogon* to be transferred and aseptically transfer 0.1 ml to a flask prepared in step 16.
18. Incubate flask at 25° C with cap on tight.
19. Transfer when most prey have been eliminated.

COMMENTS

For routine maintenance of small populations cultivate *Rhynchomonas* in Marine Flagellate Medium rather than in Seawater Cereal Leaf Medium. Yields of *Stephanopogon* are proportional to densities of *Rhynchomonas* offered as a food source.

LITERATURE CITED

1. Lipscomb, DL & Corliss, JO 1982. *Stephanopogon*, a phylogenetically important "ciliate", shown by ultrastructural studies to be a flagellate. *Science* **215**:303-304.
2. Nerad, TA ed. 1991. American Type Culture Collection Catalogue of Protists, 17th edition, American Type Culture Collection, Rockville, MD 88 pp.

CULTIVATION OF SELECTED KARYORELICTEANS

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INTRODUCTION

Most Karyorelicteans are sand-dwelling. *Trachelorаphis* spp. from both estuarine and marine sediments have been cultured [1]. Of non-sand dwelling Karyorelicteans strains belonging to the genus *Protocruzia* have been successfully cultivated (A Soldo, personal communication).

PROTOCOL
Trachelorаphis

1. Filter sterilize water from the collection site.
2. Medium formulation:

Filter sterilized site H_2O	100.0 ml
Streptomycin-SO_4	10.0 mg

3. Aseptically distribute in 3.0 ml aliquots into 3.5 cm sterile plastic petri dishes.
4. Add crumbled freshly boiled egg yolk. The pieces of egg should be no larger than 1 mm and there should be no more than 6 pieces per dish.
5. Inoculate dish with 5-6 ciliates and incubate at a temperature equivalent to the temperature of the collection site water from which the isolation was made.
6. Every 5-7 d add a few pieces of freshly boiled egg yolk.
7. Every 2-4 wk subculture into fresh medium. The organisms are transferred by picking individuals with the aid of a mouth pipette and a dissecting microscope.
8. Repeat steps 5-7.

PROTOCOL
Protocruzia

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.

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2. Medium formulation:

Seawater Cereal Grass Infusion Medium	
2x Artificial seawater*	500.0 ml
2x Cereal grass infusion medium (see below)	500.0 ml

2X Cereal Grass Infusion Medium	
Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml distilled H_2O and boil for 5 min. Add distilled water to compensate for evaporation. Filter through Whatman #1 filter paper. Autoclave for 15 min at 121° C.
5. Allow solution prepared in step 4 to cool and combine equal amounts of this solution and the solution prepared in step 3.
6. Distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

COMMENTS

Protocruzia does not form a resistant stage in its life cycle and must therefore be passaged regularly. One strain of *Protocruzia* originally isolated from the bottom of a 10 gallon aquarium containing very old, mostly evaporated, mesosaline (~15 $^{o}/_{oo}$) Chesapeake Bay water (E Small, unpublished observation), has been maintained for several years (A Soldo, personal communications) using the protocol listed above. Other strains isolated from the Atlantic Ocean off the coast of Connecticut and Mexico have been maintained as monoprotist cultures using the same protocol. The *K. pneumoniae* strain used is the same strain originally used by TM Sonneborn for the cultivation of paramecia and was known as *Aerobacter aeorgenes* [2]. With changes in bacterial systematics it has been identified as a nonpathogenic strain of *K. pneumoniae*. It is recommended that an equivalent strain be used.

LITERATURE CITED

1. Lenk, SE, Small, EB, & Gunderson J 1984. Preliminary observations on feeding in the psammobiotic ciliate *Trachelioraphis*. *Origins of Life* **13**:229-234.
2. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-148.

CULTIVATION OF SELECTED HETEROTRICHS

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INTRODUCTION

Many of the heterotrichs are very large and are therefore ideal for study in the classroom. Several of these genera are pigmented. *Blepharisma* is pink and some species of *Stentor* have a bluish tint. Some members of these genera are colorless. Other colorless genera are *Spirostomum* and *Fabrea*. These organisms can be maintained as monoprotist cultures with bacteria as a food source. *Fabrea* is found in seawater and the other genera are common in freshwater.

PROTOCOL

Blepharisma

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.

7. Autoclave for 20 min at 121° C.
8. In advance place wheat kernels in a 20 x 125 mm screw-capped test tube to a depth of approximately 5 cm. Add a few drops of glass distilled H_2O and with the cap loosened one half turn, autoclave for 30 min at 121° C. Place the tube in a 70° C incubator overnight to remove any moisture. Allow the tube to cool to room temperature, screw the cap on tight and store refrigerated (5-9° C) until needed. Wheat kernels may be obtained from a grocery store.
9. Place 200 ml of medium prepared in step 7 into an 11 cm biological finger bowl containing approximately 200 ml of medium. Add one wheat kernel.
10. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

11. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
12. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
13. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
14. Inoculate finger bowl prepared in step 9 with a loopful of bacteria from a stock slant and incubate at 30° C overnight.
15. Inoculate 1 ml of a growing or encysted culture into medium prepared in step 14.
16. Place another empty finger bowl on top of the culture to reduce moisture loss and incubate in the dark at 25° C.
17. Repeat steps 14-16 at monthly intervals.

PROTOCOL

Spirostomum, Stentor

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Soil/H_2O Biphasic Medium [1]

Rich Organic Soil (Air-dried)	125.0 g
Glass distilled H_2O	875.0 ml

3. Place soil in the bottom of a flask and gently overlay with the glass distilled H_2O, etc.
4. Autoclave at 121° C for 15 min. Allow to cool overnight.
5. Repeat step 4.
6. In advance place wheat kernels in a 20 x 125 mm screw-capped test tube to a depth of approximately 5 cm. Add a few drops of glass distilled H2O and with the cap loosened one half turn, autoclave for 30 min at 121° C. Place the tube in a 70 C incubator overnight to remove any moisture. Allow the tube to cool to room temperature, screw the cap on tight and store refrigerated (5-9° C) until needed. Wheat kernels may be obtained from a grocery store.
7. Allow the flask prepared in step 5 to cool, swirl vigorously and transfer 200 ml to an 11 cm diameter biological finger bowl. Add one autoclaved wheat kernel.
8. Inoculate 1 ml of a growing culture into medium prepared in step 8.

9. Place another empty finger bowl on top of the culture to reduce moisture loss and incubate at 25° C.
10. Repeat steps 8-10 at monthly intervals.

PROTOCOL

Fabrea

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Fabrea Medium [A Repak, personal communication]

Artificial seawater*	750.0 g
Glass distilled H_2O	250.0 ml

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 2.0 g of activated carbon powder, shake and leave overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

3. Adjust pH to between 7.5-8.0 with 0.1N NaOH or 0.1N HCL.
4. Autoclave for 20 min at 121° C.
5. In advance place barley kernels in a 20 x 125 mm screw-capped test tube to a depth of approximately 5 cm. Add a few drops of glass distilled H_2O and with the cap loosened one half turn, autoclave for 30 min at 121° C. Place the tube in a 70° C incubator overnight to remove any moisture. Allow the tube to cool to room temperature, screw the cap on tight and store refrigerated (5-9° C) until needed. Wheat kernels may be obtained from a grocery store.
6. Place 200 ml of medium prepared in step 3 in an 11 cm biological finger bowl. Add ten sterilized barley kernels.
7. Medium formulation (Part 2):

Agar Medium for *Escherichia coli** or *Bacillus subtilis*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Artificial seawater	800.0 ml

*Use a non-mucoid forming strain.

8. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
9. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
10. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
11. Inoculate finger bowl prepared in step 9 with a loopful of bacteria from a stock slant and incubate at 30° C overnight.
12. Inoculate 1 ml of a growing or encysted culture into medium prepared in step 4.

13. Place another empty finger bowl on top of the culture to reduce moisture loss and incubate at 25° C.
14. Repeat steps 11-13 at monthly intervals.

COMMENTS

Species of *Spirostomum* and *Stentor* do not encyst and therefore must be transferred at regular intervals or they will be lost from culture. Not all species of *Blepharisma* and *Fabrea* encyst and even those strains that do form cysts when initially isolated from nature may lose that ability after repeated transfers. Not all species of *Stentor* can be maintained with bacteria as the sole food source. The addition of other small ciliates (e.g. *Cyclidium* and *Tetrahymena*), non-photosynthetic flagellates (e.g. *Chilomonas*), and photosynthetic flagellates (e.g. *Chlamydomonas*) may stimulate growth. While *Fabrea salina* grows very well with *Dunaliella tertiolecta* and *Rhodomonas lens* as food sources, it loses its ability to encyst (A Repak, personal communication).

LITERATURE CITED

1. Thompson, AS, Rhodes, JC & Pettman, I 1988. Culture Collection of Algae and Protozoa Catalogue of Strains. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Cumbria, United Kingdom. 164 pp.

CULTIVATION OF *SOROGENA*

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INTRODUCTION

Sorogena is colpodid ciliate which preys upon *Colpoda*. It is the only colpodid known to form sorocarps. The methods used for cultivation were developed by Olive and Blanton [1].

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

HI Agar	
Agar	15.0 g
Hay Infusion Broth (see below)	1.0 L

Hay Infusion Broth	
Dried hay	2.5 g
Glass distilled H_2O	1.0 L

3. Add dried hay to a flask and add 1.0 L glass distilled H_2O. Place the flask in a boiling water bath for 30 min. Filter through #1 Whatman filter paper. Return volume to 1.0 L with glass distilled H_2O.
4. Add 15.0 g of agar to the broth prepared in step 3. Heat to dissolve, adjust pH to 7.0 with either 5% (v/v) lactic acid or 1N NaOH prepared in glass distilled H_2O, and autoclave for 20 min at 121° C.
5. Cool agar to 45° C and distribute 15.0 ml aliquots to 20 x 100 mm petri plates. Allow to solidify.
6. Medium formulation (Part 2):

HI/LY Broth	
Lactose	0.2 g
Yeast extract	0.1 g
Hay Infusion Broth (see step 3)	1.0 L

7. Add lactose and yeast extract to Hay Infusion Broth.
8. Adjust pH to 6.0 with 5% (v/v) lactic acid and autoclave 20 min at 121° C.

9. When solution prepared in step 8 is cooled aseptically add 15.0 ml aliquots as an overlay to plates prepared in step 5.
10. Rub the bottom and sides of a culture with encysted *Colpoda* and sorocarps of *Sorogena* with a spread bar. Sorocarps of *Sorogena* will be evident at the air-liquid interface and attached to the surface film after 7-14 d. Transfer 0.1 ml aliquots to the plates prepared in step 9. Repeat step 10.

COMMENTS

The cysts of both *Colpoda* and *Sorogena* can be dried. For long term storage, the cultures can be allowed to air dry. To revive a dried culture add HI broth and resume normal transfer procedures after the culture has been established.

LITERATURE CITED

1. Olive, LS & Blanton, RL 1980. Aerial sorocarp development by the aggregative ciliate, *Sorogena stoianovitchae*. *J. Protozool.* **27**:293-299.

CULTIVATION OF CILIATES PREDATORY ON OTHER PROTOZOA

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INTRODUCTION

A number of genera of ciliates have members that are predatory on other protozoans. These genera are dispersed amongst various classes within the Phylum Ciliophora. Cultivation protocols for some of the most common genera encountered in nature, namely, *Coleps*, *Didinium*, *Dileptus*, *Euplotes*, *Platyophora*, *Spathidium* and *Protospathidium* are presented below.

PROTOCOL

Coleps

1. Filter sterilize spring or pond water.
2. Place 10.0 ml of filtered medium in a T-25 tissue culture flask.
3. Prepare the following medium for the cultivation of *Tetrahymena*:

Proteose Peptone (Difco #0120)	1.0 g
Glucose	1.0 g
Glass distilled H_2O	100.0 ml

4. Apply low heat and dissolve the ingredients.
5. Dispense 5.0 ml of medium per 16 x 125 mm screw-capped test tube.
6. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
7. Inoculate medium with 0.1 ml from a growing culture at or near peak density.
8. Incubate at 20-25° C for 7-10 d.
9. Aseptically transfer 1.0 ml of a culture of *Tetrahymena* prepared in step 8 to a 15 ml plastic screw-capped centrifuge tube.
10. Add 13.0 ml of filter sterilized pond water prepared in step 1, mix by inverting several times and centrifuge at 300 g for 3 min.
11. Remove all but 1.0 ml of the supernatant.
12. Repeat steps 9-11 twice.
13. Resuspend the cell pellet in the remaining supernatant and add to the flask prepared in step 2.
14. Add 0.1 ml of a growing culture of *Coleps*.
15. Incubate the flask at 25° C.
16. Repeat steps 9-15 at 10-14 day intervals.

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PROTOCOL

Didinium, Dileptus, Euplotes, Platyophrya, Spathidium, Protospathidium

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Autoclave for 20 min at 121° C.
8. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

9. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
10. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
11. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
12. Inoculate T-25 flask with 10.0 ml of fresh medium prepared in step 7 with a loopful of the bacteria from a slant prepared in step 11 and incubate at 30° C overnight.
13. Inoculate 0.1 ml of a growing culture of the appropriate protozoan into medium prepared in step 13. *Bodo* serves as a food source for smaller strains of *Platyophrya, Protospathidium* and, *Spathidium, Paramecium* serves as a food source for *Didinium*, and *Tetrahymena* serves as a food source for *Dileptus* and larger species of *Euplotes, Platyophrya* and *Spathidium*.
14. Allow the food source to clear the bacterial suspension. The time will vary with the strain of protozoan used.
15. Inoculate the cleared flask with 0.1 ml of a growing or encysted culture of the predator.

16.Repeat steps 12-15 every 14-21 d.

COMMENTS

The cysts of *Didinium*, *Dileptus*, and *Platyophrya* can be dried. For long term storage, the cultures can be allowed to air dry. To revive a dried culture add a cleared culture of the appropriate food source and resume normal transfer procedures after the culture has been established. Some species of *Euplotes* form a temporary cyst but this does not withstand drying.

A-45.1

CULTIVATION OF SUCTORIANS

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INTRODUCTION

Many suctorians can be maintained in xenic culture with the appropriate food source added at intervals. One species of *Tokophrya, T. infusionum*, has been successfully established in monoxenic bacteria-free culture with *Tetrahymena* as the only added food source [1]. Protocols for monoxenic cultivation of *Tokophrya* and the xenic cultivation of *Heliophrya* and *Tokophrya* are provided.

PROTOCOL

Paramecium as a food source,
Xenic, *Heliophrya, Tokophrya*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Non-nutrient Agar

Agar	15.0 g
Glass distilled H_2O	1.0 L

3. Autoclave at 121° C for 15 min.
4. Allow to cool in a 50° C water bath.
5. Aseptically dispense in 40.0 ml aliquots to 20 x 100 mm petri plates. Allow to harden.
6. Aseptically add 20.0 ml of filter sterilized spring water (spring water may be obtained from most biological supply houses) to the surface of an agar plate prepared in step 5.
7. Medium formulation (Part 2):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed

a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

8. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
9. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
10. Filter through Whatman #1 filter paper.
11. Add 0.5 g Na_2HPO_4.
12. Autoclave for 20 min at 121° C. Allow to cool.
13. Medium formulation (Part 3):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

14. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
15. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
16. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
17. Inoculate T-25 flask with 10.0 ml of fresh medium prepared in step 12 and add a loopful of the bacteria from a stock slant prepared in step 11 and incubate at 30° C overnight.
18. Inoculate 0.1 ml of a growing culture of *Paramecium*.
19. Allow *Paramecium* to clear the bacterial suspension. The time will vary with the strain of used.
20. Aseptically transfer the contents of a cleared *Paramecium* culture to a 15 ml plastic screw-capped centrifuge tube.
21. Add filter sterilized spring water prepared in step 6 to fill the tube, mix by inverting several times and centrifuge at 300 g for 3 min.
22. Remove all but 1.0 ml of the supernatant.
23. Repeat steps 20-22 twice.
24. Resuspend the cell pellet in the remaining supernatant and add to the plate prepared in step 7.
25. Add 0.1 ml of a growing culture of *Tokophrya* or *Heliophrya*. The suctorians will be attached to the agar surface and will be suspended in the surface film. Alternately aspirate the culture fluid into a pipette and expell it onto the surface of the agar to obtain a uniform suspension of cells.
26. Incubate the plates at 25° C.
27. Repeat steps 17-26 at 10-14 day intervals.

PROTOCOL

Tetrahymena as a food source,
Monoxenic, *Tokophrya*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.

2. Medium formulation:

Proteose Peptone (Difco #0120)	1.0 g
Glucose	1.0 g
Glass distilled H_2O	100.0 ml

3. Apply low heat and dissolve the ingredients.
4. Dispense 10.0 ml of medium per 20 x 125 mm screw-capped test tube.
5. Loosen caps one half turn and autoclave for 15 min at 121° C. Cool medium before use.
6. Inoculate medium with 0.1 ml from a growing culture of *Tetrahymena* at or near peak density.
7. Incubate at 20-25° C for 7-10 d.
8. Aseptically transfer 1.0 ml of the suspension of *Tetrahymena* to a culture of *Tokophrya* that has eliminated prey organisms. Do not add excessive numbers of *Tetrahymena*. Do not exceed a ratio of 2-3 *Tetrahymena*: *Tokophrya*.
9. Feed every 2-3 days or when prey organisms have been largely eliminated.
10. Every 10-14 days transfer the *Tokophrya* as follows: Scrape the curface of glass with a cotton swab near the air/medium interface to detach the *Tokophrya* cells. Aseptically transfer 0.1 ml to a fresh tube of medium.
11. Add 1.0 ml of a culture of *Tetrahymena* at or near peak density.
12. Repeat steps 9-11.

COMMENTS

Overfeeding of *Heliophrya* or *Tokophrya* may result in monster formation. Attempt to maintain a ratio of 2-3 prey organisms per each suctorian. If the number of abnormal suctorian is high reduce the feeding interval.

LITERATURE CITED

1. Rudzinska, MA 1972. Ultrastructural localization of acid phosphatase in feeding *Tokophra infusionum*. *J. Protozool.* **19**:618-629.

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CULTIVATION OF *PARAMECIUM*

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INTRODUCTION

Most strains of *Paramecium* can be cultivated as monoprotist cultures with bacteria as a food source. It is also possible to cultivate most strains axenically in an empirical formulation [7] and some in a completely defined axenic medium [4]. Thiele, *et al.* [6] report the cultivation of axenic strains in mass quantities [6]. Only methods for bacterized cultivation and axenic cultivation in an empirical medium are described.

PROTOCOL

Xenic

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.

7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast Extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Axenic

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Proteose Peptone (Difco #012-01)	10.0 g
Trypticase (BBL #11921)	5.0 g
Yeast RNA (Sigma R6625)	1.0 g
MgSO4·7H2O	0.5 g
Stigmasterol Solution (see below)	2.5 ml
Emcol Solution (see below)	5.0 ml
Vitamin Solution (see below)	10.0 ml
Glass redistilled H_2O	1.0 L

Stigmasterol Solution

Stigmasterol (Sigma S6126)	200.0 mg
Absolute ethanol	100.0 ml

TEM-4T Solution

TEM-4T*	2.0 g
Absolute ethanol	100.0 ml

Vitamin Solution

Calcium pantothenate	0.05 g
Nicotinamide	0.05 g
Pyridoxal·HCl	0.05 g

Pyridoxamine•HCl	0.025 g
Riboflavin	0.05 g
Folic acid	0.025 g
Thiamine•HCl	0.15 g
Biotin	0.0125mg
DL-Thioctic acid	0.5 ml
Glass distilled H_2O	100.0 ml

Filter sterilize using a 0.2μm filter.

*TEM-4T was produced by Hachmeister Inc., Pittsburgh, PA. Hachmeister discontinued production of TEM-4T and replaced it with Emcol. The company no longer exists. Please see comments for possible substitution if neither TEM-4T or Emcol is on hand.

3. Adjust pH to 7.0 with 1N NaOH dispense in 5.0 ml aliquots per 16 x 125 mm screw-capped test tube and autoclave at 121° C for 15 min.
4. When cool add 0.1 ml of a peak culture to a fresh tube of medium. Incubate at 25° C with caps loosened one half turn.
5. Transfer at intervals of 7-14 d. After reaching peak density the culture will abruptly die off. The transfer interval may vary with the strain and the medium component used [6].

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [5] for the cultivation of paramecia, has been found to be an non-pathogenic strain of *Klebsiella pneumoniae*. It is recommended an equivalent strain be used. For best results it is important to use only *K. pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity. TEM-4T was replaced with phosphatidylethanolamines (PE) (Sigma) by Fok and Allen [1] for the axenic cultivation of *Paramecium caudatum*. The ratio of phosphatidylethanolamine: stigmasterol was found to be important for optimization of growth. The importance of the ratio of TEM-4T: stigmasterol for the growth of *P. aurelia* was reported by Soldo and van Wagtendonk [3]. If phosphatidylethanolamine is substituted for TEM-4T it may be necessary to adjust the ratio of PE: stigmasterol to optimize growth of *P. aurelia*. Skoczylas and van Wagtendonk [2] were able to replace the TEM-4T component with an ethyl ether extract from linseeds. Although the yield was reduced in medium with linseed extract, the cells could be maintained for longer periods between subcultures than in media with TEM-4T.

LITERATURE CITED

1. Fox, AF & Allen, RD 1979. Axenic *Paramecium caudatum*. I. Mass culture and structure. *J. Protozool.* **26**: 463-470.
2. Skoczylas, B & Wagtendonk, WJ Van 1975. A maintenance medium for the axenic culture of *Paramecium aurelia*. *Acta Protozoologica* **14**:379-383.
3. Soldo, AT & Wagtendonk, WJ Van 1968. Lipid interrelationships in the growth of *Paramecium aurelia*, stock 297. *J. Gen. Microbiol.* **53**:341-348.
4. Soldo, AT & Wagtendonk, WJ Van 1969. The nutrition of *Paramecium aurelia*, stock 299. *J. Protozool.* **16**:500-506.
5. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-148.
6. Thiele, J, Honer-Schmid, O, Wahl, J, Klerfeld, G & Schultz, J 1980. A new method for axenic mass cultivation of *Paramecium tetraurelia*. *J. Protozool.* **27**:118-121.

7. Wagtendonk, WJ Van & Soldo, AT 1970. Methods used in the axenic cultivation of *Paramecium aurelia*. In: Methods in Cell Physiology, Prescott, DM ed., Vol. 4, Academic Press, New York, pp. 117-130.

CULTIVATION OF PARAMECIUM IN CHEMICALLY DEFINED MEDIUM

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Introduction

The medium described here was specifically designed for the cultivation of symbiont lambda-bearing and lambda-free strains of Paramecium octaurelia (1). It will also support the growth of several other symbiont-bearing and symbiont-free strains of Paramecium , and a large number of fresh water ciliates including Tetrahymena, Colpoda and related strains. It should prove useful as a base medium for studies of the comparative nutrition of these and related organisms.

Protocol

Stock mixes and solutions:

1. Amino acid mix (gms): L-alanine, 6.0; L-arginine,6.0; L-asparagine, 1.8; L-aspartic acid, 2.0; L-glutamic acid, 8.0; glycine,1.5; L-histidine,2.0; L-isoleucine, 2.5; L-leucine, 2.5; L-lysine HCl, 5.0; L-methionine, 3.0; L-phenylalanine, 2.5; L-proline, 1.5; DL-serine, 6.0; DL-threonine, 7.0; L-tryptophan, 1.5; L-tyrosine, 1.5 and DL-valine.

2. Nucleic acid mix (gms): Adenosine, 1.5; guanosine-2',3' phosphate, 2.5; cytidine, 1.5; uridine, and thymidine, 1.375.

Weigh appropriate amounts of each compound, place in a dry wide-mouth plastic bottles equipped with a tight-fitting cap. Place each mix on a mechanical shaker and agitate for 2 to 3 hours at room temperature. Store (cap-removed) in a desiccator at room temperature in the dark.

3. Vitamin mix (mg): Biotin, 0.01*; folic acid, 50; nicotinamide, 50; d-pantothenate, Ca, 100; pyridoxal-HCl, 50; riboflavin, 50; thiamine HCl, 150 and DL-6-thioctic acid, 1.0.
(* from a more concentrated stock).

Suspend the vitamins in 100 ml distilled water while under constant stirring (some are not soluble at these concentrations) and dispense in small volumes in screw-cap tubes. Flush with nitrogen, store at -20°C, thaw only once.

4. Fatty acids mix (mg): Oleic acid, 200; stearic acid, 100; palmitic acid, 150; linoleic acid, 10, and linolenic acid, 2.5.

Disperse the fatty acids in distilled water under constant stirring and adjust to Ph 10 with diethylamine to dissolve. Flush with nitrogen, dispense in small volumes, store at -20°c and thaw only once.

5. Metal mix (mg): $Fe(NH_4)_2(SO_4)_2.6H_2O$, 140; $ZnSO_4.H_2O$, 44.3; $MnCl_2.H_2O$, 14.4 and

$CuCl_2.H_2O$, 2.1.
Dissolve in 100 ml distilled water and store at 4°C.

6. Salt solutions (gms):
$CaCl_2.2H_2O$, 1.84
$MgCl_2.6H_2O$, 1.69
$(NH_4)_2SO_4$, 0.20
K_3PO_4 1.125
Weigh each salt **individually** and dilute to 100 ml. Store at 4°C. Do **not** prepare as a single mixture.

7. Stigmasterol: Dissolve 40 mg in 100 ml **absolute** ethanol. Store in a **tightly-fitting** cap and store at 4°C.

8. Cephalin: Disperse 0.5 gms (with the aid of a mortar and pestle) in 100 ml distilled and store in small portions, dispense in small portions. Store at -20°C and use only once.

9. Na Acetate: Dissolve 0.5 gms in 100 ml distilled water. Store at 4°C.

To prepare 100 ml of medium:

1. Dissolve 643 mg of the amino acid mix and 125 mg of the nucleic acid mix in 50 ml distilled water.
2. Add, under constant stirring, in the order given the following stock solutions:
10 ml Na acetate
5 ml Cephalin
1 ml Vitamins
1 ml Fatty Acids
1 ml $CaCl_2.H_2O$
1 ml $MgCl_2.6H_2O$
1 ml $(NH_4)_2SO_4$
1 ml Metals
1 ml K_3PO_4
0.5 ml Stigmasterol
3. Adjust the pH to 7.2 and dilute to volume. Dispense 5 ml per tube 16 x 150, cap and sterilize and $121_{o}C$ for 15 minutes.

4. When cool, inoculate with Paramecium (or other ciliates) and maintain stocks by weekly transfer at 27°C in the dark.

Comments

1. The medium will support the growth of P. octaurelia at populations ranging from 15,000 to 20,000 per ml. In the absence of cephalin, transplantable growth is possible, but only at populations not exceeding about 3,000 per ml.
2. A number of phopholipids including phosphatidyl serine, phosphatidyl inositol and phosphatidyl ethanolamine can replace cephalin for growth.
3. Serine will replace glycine for growth provided thymidine is present in the medium.
4. In the absence of thymidine, comparatively high levels of folic acid are required for optimal growth.
5. In the absence of the non-essential amino acids (alanine, asparagine, aspartic and glutamic acids) growth may be be restored to optimal levels by the addition of higher amounts of acetate. Pyruvate is about as effective as acetate in this respect. Glucose and certain other carbohydrates

are not.

LITERATURE CITED

1. Soldo, AT, & Van Wagtendonk, WJ. 1969. The nutrition of Paramecium aurelia stock 299. J. Protozool.**16**:500-506.

A-48.1

CULTIVATION OF *TETRAHYMENA*

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INTRODUCTION

There are a number of media which have been developed for the cultivation of *Tetrahymena*. Many species are relatively easy to cultivate as monoxenic cultures on the same cereal grass infusion medium used to culture *Paramecium* or as axenic cultures. Among the axenic media several have been selected either for simplicity or a specialized use which has broad application.

PROTOCOL

Xenic

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4 (see step 6)	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered cereal grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered cereal grass leaves rather than rye leaves.

3. Add 2.5 g of powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.

8. Loosen cap half a turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation:

Agar Medium for *Klebsiella pneumoniae*

Yeast extract	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Cereal Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened a half a turn.
15. For routine maintenance of a population subculture weekly.

PROTOCOL

Axenic, Proteose Peptone & Glucose

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Proteose Peptone (Difco #0120)	1.0 g
Glucose	1.0 g
Glass distilled H_2O	100.0 ml

3. Apply low heat and dissolve the ingredients.
4. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
5. Autoclave for 15 min at 121° C. Loosen cap half a turn prior to autoclaving. Cool medium before use.
6. Inoculate medium with 0.1 ml from a growing culture at the end of logarithmic growth or just beginning stationary phase.
7. Incubate at 20-25° C with cap loosened half a turn.
8. For routine maintenance subculture every 10-14 d.

PROTOCOL

Axenic, Proteose Peptone & Tryptone

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.

2. Medium formulation:

Proteose Peptone (Difco #0120)	5.0 g
Tryptone (Difco #0123)	5.0 g
K_2HPO_4	0.2 g
Glass distilled H_2O	1.0 L

3. Apply low heat and dissolve.
4. The pH is adjusted to 7.2 with 20% (w/v) NaOH.
5. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
6. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
7. Inoculate medium with 0.1 ml from a culture at or near peak density.
8. Incubate at 20-25° C with cap loosened half a turn.
9. For routine maintenance subculture every 10-14 d.

PROTOCOL

Axenic, Haskins Agar Medium [1]

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth
2. Medium formulation:

Dextrin	8.0 g
Na-acetate	0.6 g
Yeast extract	5.0 g
Liver extract	0.6 g
Tryptone (Difco #0123)	5.0 g
Agar	16.0 g
Glass distilled H_2O	1.0 L

3. Apply low heat and dissolve ingredients.
4. The pH is adjusted to 7.2-7.4 with 20% NaOH.
5. Dispense aseptically while still warm 2 ml of medium per 16 x 125 mm screw-capped test tube.
6. Autoclave for 15 min at 121° C. Loosen cap half a turn prior to autoclaving. Place on a 15° horizontal slant and cool.
7. After setting, aseptically dispense 2 ml sterile glass distilled H_2O as overlay to cover slants almost completely.
8. Inoculate medium with 0.1 ml from a growing culture.
9. Incubate at 25° C with cap loosened half a turn for 1 d. Then place at 18° C.
10. For routine maintenance subculture every 3-6 wk.

COMMENTS

The bacterial species originally used by TM Sonneborn was known as *Aerobacter aerogenes* [2]. With changes in bacterial systematics it has been identified as a nonpathogenic strain of *Klebsiella pneumoniae*. It is recommended an equivalent strain be used. For best results it is also important to maintain monoxenic conditions for bacterized cultivation, thus all cultures should be handled with complete aseptic techniques. Periodically both bacterial stock and protist stock cultures should be

tested to assure the presence of only the single strain of bacteria. For medium with agar, the slant should not extend all the way to the top of the test tube. It should be at least 2-3 cm from the top.

LITERATURE CITED

1. Cote, R, Daggett, P-M, Gantt, MJ, Hay, R, Jong, S-C & Pienta, P 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD, 98 pp.
2. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-147.

CULTIVATION OF SELECTED HYMENOSTOMES

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INTRODUCTION

Members of the genera *Colpidium* and *Glaucoma* can be grown as monoprotist cultures with bacteria as the food source. Both can be cultivated axenically in simple empirical formulations [1,2].

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

A-49.2

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance subculture weekly.

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [3] for the cultivation of paramecia. Recent bacterial systematics have determined it to be non-pathogenic strain of *Klebsiella pneumoniae*. It is recommended an euquivalent strains be used. For best results it is important to use only *K. pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity.

LITERATURE CITED

1. Kidder, GW, Dewey, VC & Fuller, RC 1954. Nitrogen requirements of *Glaucoma scintillans* and *Colpidium campylum*. *Proc. Soc. Exp. Biol. Med.* **86**:685-689.
2. Peterson, RE 1942. Essential factors for the growth of the ciliate *Colpidium campylum*. *J. Biol. Chem.* **146**:537-545.
3. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-147.

CULTIVATION OF SCUTICOCILIATES

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INTRODUCTION

Strains of freshwater scuticociliates belonging to the genera *Cinetochilum*, *Cyclidium*, *Homalogastra*, *Sphenostomella*, the brackish water scuticocilate *Potomacus*, and marine scuticociliates belonging to the genera *Anophryoides*, *Metanophrys*, *Miamiensis*, *Parauronema*, *Paranophrys*, *Pseudocohnilembus*, and *Uronema* can be readily maintained as monoprotist cultures with bacteria as the food source. In addition, strains of *Potomacus* and all of the marine genera mentioned above except *Glauconema* can be maintained in the axenic state in media developed by Soldo and Merlin [2] or modifications of these media (A Soldo, personal communication). An axenic medium defined except for the lipid components was developed by Soldo and Merlin [3] for the cultivation of *Parauronema acutum* and a completely defined medium has been developed for the cultivation of *Uronema marinum* [1]. Protocol for bacterized and axenic cultivation using empirical formulations are detailed below.

PROTOCOL

Freshwater, Xenic
Cinetochilum, Cyclidium, Homalogastra, Sphenostomella

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal

Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Cereal Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance subculture weekly.

PROTOCOL

Brackish water, Xenic, *Potomacus*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Half-Strength Seawater Cereal Grass Infusion Medium

Artificial seawater*	500.0 ml
2x Modified Cereal Grass Infusion Medium	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth. As an alternative to artificial seawater, natural seawater can be obtained from the Marine Biological Laboratory in Woods Hole, MA or from Real Ocean Inc., 1605 Water Street, Long Beach, CA 90802. To each liter of natural seawater add 5.0 g of activated carbon powder, shake and leave

overnight. Filter through Whatman 934-HA glass fiber filter. Treatment with the carbon will remove potentially toxic organic contaminants.

**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add recommended amount of salt/unit volume to prepare artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper. Autoclave for 15 min at 121° C.
5. Allow solution prepared in step 4 to cool and combine equal amounts of this solution and the solution prepared in step 3.
6. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.
7. Distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
8. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
9. Incubate at 25° C for 7-14 d and repeat steps 6-8.

PROTOCOL

Marine, Xenic

Anophryoides, Glauconema, Metanophrys, Miamiensis
Paranophrys, Parauronema, Pseudocohnilembus

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Seawater Cereal Grass Infusion Medium

2x Artificial seawater*	500.0 ml
2x Modified cereal grass infusion medium (see below)	500.0 ml

2x Modified Cereal Grass Infusion Medium

Powdered cereal grass leaves**	5.0 g
Glass distilled H_2O	1.0 L

*There are many sources of artificial seawater. Formulations from the same manufacturer vary from lot to lot. A new lot of artificial seawater should be tested in parallel with a currently used lot to determine if it supports equivalent growth.

A-50.4

**This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add twice the recommended amount of salt/unit volume to prepare 2x artificial seawater.
4. Add 2.5 g powdered cereal grass leaves to 500 ml glass distilled H_2O and boil for 5 min. Add glass distilled H_2O to compensate for evaporation. Filter through Whatman #1 filter paper. Autoclave for 15 min at 121° C.
5. Allow solution prepared in step 4 to cool and combine equal amounts of this solution and the solution prepared in step 3.
6. Distribute medium in 5.0 ml amounts into 16 x 125 mm screw-capped test tubes and inoculate with *Klebsiella pneumoniae* approximately 24 h prior to inoculation with ciliates. Loosen caps one half turn and incubate at 25° C.
7. Aseptically inoculate bacterized medium with a 0.1 ml aliquot of a growing culture. Keep the cap loosened one half turn after inoculation.
8. Incubate at 25° C for 7-14 d and repeat steps 6-8.

PROTOCOL

Brackish water, Axenic, *Potomacus*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

PP Medium

10x Concentrated Stock Solution (see previous protocol)	100.0 ml
Glass distilled H_2O	733.0 ml
Artificial Seawater (Aqua-Marin*) (see previous protocol)	167.0 ml

3. Follow steps 3-8 of previous protocol
4. Combine 100.0 ml of the 10x Concentrated Stock Solution with 733.0 ml of glass distilled H_2O. Mix thoroughly.
5. Prepare Aqua-Marin artificial seawater using glass distilled H_2O according to package directions. The specific gravity should be 1.030. Use a hydrometer to determine the specific gravity. If the specific gravity is greater than 1.030 add glass distilled H_2O to adjust. If the specific gravity is less the 1.030 add salts.
6. Add 167 ml of the artificial seawater to solution prepared in step 4. Mix thoroughly.
7. Follow steps 12-14 of the previous protocol but note that the final specific gravity of the growth medium should be 1.005 rather than 1.015. Use a hydrometer to determine the specific gravity. If the specific gravity is greater than 1.005 add glass distilled H_2O to adjust. If the specific gravity is less the 1.005 add salts.

A-50.5

PROTOCOL

Marine, Axenic

Anophryoides, Metanophrys, Miamiensis, Paranophrys, Parauronema, Uronema

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

MA Medium

10x Concentrated Stock Solution*	100.0 ml
Glass distilled H_2O	400.0 ml
Artificial Seawater (Aqua-Marin*) (specific gravity 1.030)	500.0 ml

10x Concentrated Stock Solution

Asolectin Emulsion (see below)	100.0 ml
Vitamin Solution (see below)	20.0 ml
Proteose Peptone (Difco #0120)	100.0 g
Trypticase (BBL #11921)	100.0 g
Yeast RNA (Sigma R6625)	10.0 g
Glass distilled H_2O	1.0 L

Asolectin Emulsion

Asolectin**	2.0 g
Glass distilled H_2O	100.0 ml

Vitamin Solution

Biotin (dissolve in ethanol, 1.0 µg/ml)	10.0 ml
Folic Acid (Sigma F7876)	50.0 mg
Nicotinamide	50.0 mg
Calcium D-Pantothenate (Sigma T2250)	100.0 mg
Pyridoxal•HCl	50.0 mg
Riboflavin (Sigma R4500)	50.0 mg
Thiamine•HCl (Sigma T4625)	150.0 mg
DL-6 Thioctic acid	1.0 mg
Glass distilled H_2O	100.0 ml

*Aqua-Marin can be obtained from: Aquatrol, Inc., 237-H North Euclid Street, Anaheim, CA.

**The company which manufactured Asolectin, Associated Concentrates of Woodside, NY, is no longer in business. A product which may support equivalent growth, soybean L-alpha-Lecithin (cat. #429415) is available from Calbiochem, Inc.

3. Prepare the Vitamin Solution by mixing components under constant stirring. For long-term storage of this component dispense in 2.0 ml aliquots in 16 x 125 mm screw-capped test tubes, flush with nitrogen, and store at -20° C.
4. Prepare the Asolectin Emulsion by dissolving the asolectin in glass distilled H_2O at 80° C.
5. Next prepare the 10x Concentrated Stock Solution by dissolving the Proteose Peptone, Trypticase, and Yeast RNA in 100.0 ml of glass distilled at 80° C.
6. Add 100.0 ml of the Asolectin solution prepared in step 4 with constant stirring to the solution prepared in step 5.

7. Add 20.0 ml of the Vitamin Solution with constant stirring to solution prepared in step 6.
8. Adjust pH of solution prepared in step 7 to 7.2 and bring final volume to 1.0 liter with glass distilled H_2O to complete the 10x Concentrated Stock Solution. This component may be stored long term in 100.0 ml aliquots at -20° C.
9. Combine 100.0 ml of the 10x Concentrated Stock Solution with 400.0 ml of glass distilled H_2O. Mix thoroughly.
10. Prepare Aqua-Marin artificial seawater using glass distilled H_2O according to package directions. The specific gravity should be 1.030. Use a hydrometer to determine the specific gravity. If the specific gravity is greater than 1.030 add glass distilled H_2O to adjust. If the specific gravity is less the 1.030 add salts.
11. Add 500.0 ml of the artificial seawater to solution prepared in step 9. Mix thoroughly.
12. Dispense in 5.0 ml aliquots to 16 x 125 mm screw-capped test tubes and autoclave at 121° C for 15 min with the caps loosened one half turn. The final specific gravity should be 1.015. Use a hydrometer to determine the specific gravity. If the specific gravity is greater than 1.015 add glass distilled H_2O to adjust. If the specific gravity is less the 1.015 add salts.
13. Aseptically transfer 0.1 ml of a growing culture to a fresh tube of medium and incubate at 25° C with caps loosened one half turn. Incubate in the vertical position for 14 d.
14. Transfer as above at biweekly intervals.

PROTOCOL

Marine, Axenic, *Pseudocohnilembus*

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Stock extract (see below)	500.0 ml
2x concentrated MA Medium	500.0 ml

Stock Extract

Powdered cereal grass leaves*	5.0 g
Brown rice (Uncle Ben's Natural Whole Grain Rice)**	5.0 g
Yeast extract (Difco #0127)	5.0 g
Dried seaweed (Wel-Pac Musubi Nori)**	5.0 g
Glass distilled H_2O	500.0 ml

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

**Can be obtained from grocery or health food stores.

3. Grind the brown rice and dried seaweed components in a mortar and pestle before weighing.
4. Add all components of the stock extract to 500.0 ml of glass distilled H_2O. Boil for 5 min, filter three times through Whatman #1 filter paper while hot. Cool, adjust pH to 7.2 and bring volume back to 500.0 ml with glass distilled H_2O.
5. Prepare 500.0 ml of 2x concentrated MA medium. See first protocol for axenic cultivation for the formulation and preparation of MA medium. Increase the concentration of all components two times.
6. Follow steps 12-14 of the first protocol for axenic cultivation.

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [4] for the cultivation of paramecia. Recent bacterial systematics have determined it to be non-pathogenic strain of *Klebsiella pneumoniae*. It is recommended a similar strain be used. For best results it is important to use only *Klebsiella pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity.

LITERATURE CITED

1. Hanna, BA & Lilly, DM 1974. Growth of *Uronema marinum* in chemically defined medium . *Marine Biology* **26**:153-160.
2. Soldo, AT & Merlin, EJ 1972. The cultivation of symbiote-free marine ciliates in axenic medium. *J. Protozool.* **19**:519-525.
3. Soldo, AT & Merlin, EJ 1977. The nutrition of *Parauronema acutum*. *J. Protozool.* **24**:556-562.
4. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-147.

A-51.1

CULTIVATION OF SELECTED COLPODID CILIATES

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INTRODUCTION

It is relatively easy to cultivate strains of *Colpoda*, *Cyrtolophosis*, and *Tillina* as monoprotist cultures on the same cereal grass infusion medium used to culture *Paramecium*. *Sorogena* and *Platyophrya* can be easily maintained as diprotist cultures with *Colpoda* and *Bodo* as food sources, respectively.

PROTOCOL

Colpoda, Cyrtolophosis, Tillina

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4 (see step 6.)	0.5 g
Glass distilled H_2O	1.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sells Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal rye grass to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.

6. Add 0.5 g Na_2HPO_4.
7. Dispense medium in 500 ml screw-capped bottles.
8. Loosen cap half a turn and autoclave for 15 min at 121° C. Cool medium before use.
9. Medium formulation (Part 2):

Agar for *Klebsiella pneumoniae*

Yeast extract	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen cap one half turn and autoclave for 15 min at 121° C. Slant at 15° angle to the horizon. Cool medium before use.
12. Solution 2 is used to grow the bacterial food source. Inoculate slant aseptically with the aid of a loop. Incubate 48 h at 25° C. Then place at 4° C. Subculture every 3-4 wk.
13. With the aid of a loop inoculate solution 1 from a stock bacterial culture 24 h prior to inoculation with protists and incubate at 30° C.
14. To medium previously inoculated with bacteria inoculate aseptically with 0.1 ml of a growing protist culture which is in late log or early stationary phase. Incubate at 25° C. Keep cap loosened one half turn.
15. For routine maintenance of a trophozoite population subculture weekly.

COMMENTS

The bacterial species originally used by TM Sonneborn was known as *Aerobacter aerogenes* [1]. With changes in bacterial systematics it has been identified as a nonpathogenic strain of *Klebsiella pneumoniae*. It is recommended that an equivalent strain be used. Although there are other species of bacteria present in the colpodid cultures it is important that only *K. pneumoniae* be added routinely as a food source. For best results it is important that cultures be handled using aseptic techniques. Periodically the bacterial stock culture should be tested for purity. The methods described here are for maintenance as actively growing trophozoite cultures. Cultures will encyst if not routinely subcultured and will remain viable for variable periods dependent upon the strain. The cysts may be stored at refrigerated temperature (5-9° C) to prolong viability. Establishment of an actively growing culture from cysts can be accomplished by simply decanting the culture fluid and replacing with fresh medium bacterized with *Klebsiella*.

LITERATURE CITED

1. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-147.

A-52.1

CULTIVATION OF SELECTED PERITRICHS

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INTRODUCTION

Opisthonecta (= *Telotrochidium*), *Rhabdostyla*, and *Vorticella* strains can be cultivated as monoprotist cultures with bacteria as the food source. Members of the genera *Opisthonecta* and *Opercularia* have been successfully cultivated in soluble axenic media [1,2]. Only the protocol for bacterized cultivation is presented below.

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation (Part 1):

Cereal Grass Infusion Medium

Powdered cereal grass leaves*	2.5 g
Na_2HPO_4	0.5 g
Glass distilled H_2O	10.0 L

*This component was originally Cerophyl, a product manufactured by Cerophyl Laboratories, Inc. Kansas City, MO and consisted of a mixture of powdered wheat, rye, oat and barley leaves. When Cerophyl Laboratories, Inc. closed, Agri-tech of Kansas City, MO marketed a product with the same name that consisted of powdered rye grass leaves only. Agri-tech also no longer exists. However, a lot of Agri-tech Cerophyl is still available under the name Cerophyll from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, NY 14692. A product equivalent to the Cerophyl is still produced by Dairy Goat Nutrition P.O. Box 22363, Kansas City, MO 64113 and is sold under the name Grass Media Culture. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 sell Cereal Leaves Product (cat #C-7141) which is similar but is composed of powdered wheat leaves rather than rye leaves.

3. Add powdered cereal grass leaves to glass distilled H_2O and boil for 5 min.
4. Add glass distilled H_2O to compensate for H_2O lost by evaporation.
5. Filter through Whatman #1 filter paper.
6. Add 0.5 g Na_2HPO_4.
7. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
8. Loosen cap one half turn and autoclave for 15 min at 121° C. Cool medium before use.

9. Medium formulation (Part 2):

Agar Medium for *Klebsiella pneumoniae*

Yeast extract (Difco #0127)	4.0 g
Glucose	0.16 g
Agar	20.0 g
Glass distilled H_2O	800.0 ml

10. Dispense 5 ml of medium per 16 x 125 mm screw-capped test tube.
11. Loosen caps one half turn and autoclave for 15 min at 121° C. Allow medium to cool and solidify at a 15° horizontal slant.
12. Streak slant with an inoculating loop. Incubate 48 h at 25° C. Then place under refrigeration (5-9° C). Subculture every 3-4 wk.
13. Inoculate Cereal Grass Infusion Medium with a loopful of bacteria from a stock slant and incubate at 30° C.
14. Aseptically add 0.1 ml of a growing protist culture which is in late log or early stationary phase to a tube of fresh medium. Incubate at 25° C. Keep caps loosened one half turn.
15. For routine maintenance of a population subculture weekly.

COMMENTS

The strain of *Aerobacter aerogenes* used by TM Sonneborn [3] for the cultivation of paramecia, has been found to be a nonpathogenic strain of *Klebsiella pneumoniae*. It is recommended that a similar strain be used. For best results it is important to use only *K. pneumoniae* as the added food source. Although the protists can be grown with other bacterial species present, one should passage the cultures using aseptic techniques to avoid the introduction of unfavorable bacterial species. Periodically the bacterial stock culture of *Klebsiella* should be tested to assure its purity.

LITERATURE CITED

1. Finley, HE & McLaughlin, D 1965. Cultivation of the peritrich *Telotrochidium henneguyi* in axenic and non-axenic media. *J. Protozool.* **12**:41-47.
2. McLaughlin, D, Johnson, GR & Bradley, CJ 1974. Growth of the peritrich *Opercularia coarctata* in axenic culture. *J. Protozool.* **21**: 561-564.
3. Sonneborn, TM 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.* **113**:87-147.

A-53.1

COLLECTION AND CULTIVATION OF HISTOPHAGOUS CILIATES

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INTRODUCTION

Histophagous ciliates, primarily hymenostome and scuticociliate taxa, can be collected from marine and freshwater environments by using baited traps (e.g., a perforated 35mm plastic film canister containing small pieces of animal tissues). Best results are usually obtained if the trap is suspended near the bottom of relatively calm water and retrieved in 1-6 hrs. Many histophagous ciliates can be maintained in culture if furnished an appropriate diet of metazoan tissue. Most species appear to prefer tissues of aquatic or edaphic invertebrates, but some histophages have been successfully reared on vertebrate tissue. Culture procedures are relatively simple; however, histophages should be fed regularly and care must be taken to avoid fouling of the culture medium.

PROTOCOL

1. Histophages removed from tissue traps may be free swimming or embedded in the bait. In either case, they will likely be well fed trophonts and appear as robust, slowly swimming cells that are darkly pigmented due to a large amount of ingested tissue.
2. To initiate cultures, place one or more well fed trophonts in a covered 80 x 40 mm crystallizing dish containing 50-75 ml autoclaved culture fluid (freshwater or seawater as appropriate).
3. Feed cultures when trophonts have completely digested their food vacuoles (1-3 d depending on species and amount of food ingested) and divide to produce theronts. Theronts are slender, rapidly swimming cells that lack digestive vacuoles and are transparent.
4. Feeding is accomplished by adding one or two small pieces (greater or equal to 1 mm^3) of fresh or frozen (preferably stored under liquid nitrogen) metazoan tissue to the culture dish. Behavior of the theronts should be monitored to assure that they accumulate around the food and begin feeding.
5. When the ciliates have finished eating (one to several hours) they will migrate away from their food. Any remaining tissue should then be carefully removed from the culture dish to avoid bacterial contamination.
6. Feed cultures whenever theronts become common (1-3 d intervals). Subculture monthly, or sooner if the culture fluid becomes cloudy from bacterial contamination, by following steps 2-5.

A-53.2

COMMENTS

Histophagous ciliates tend to be non-selective feeders as indicated by the large variety of animal tissue that has been used for their collection and cultivation [1,2,4,]. Animal tissues that have been used include annelids (*Glycera* sp.; *Tubifex* sp.), amphibians (*Rana catesbeiana*), chironomid larvae (*Chironomus* sp.), echinoderms (*Asterias glacialis*), fish (*Lebistes reticulatus*), mammal liver (*Bos bos*), mollusc (*Crassostrea virginica*), and sponges (*Ficulina ficus*). Obligate histophages are usually grown in bacterized cultures; however, Mugard and Rouyer successfully maintained *Ophryoglena mucifera* in axenic culture by feeding the ciliates sterilized rat brain [3]. Facultatively histophagous species of *Tetrahymena* spp. have also been cultured axenically [5].

LITERATURE CITED

1. Lynn, DH 1975. The life cycle of the histophagous ciliate *Tetrahymena corlissi* Thompson, 1955. *J. Protozool.* **22**:188-195.
2. Mugard, H 1949. Contribution á l'étude des infusoires hyménostomes histophagous. Ann. Sci. Nat. Zool. (sér. 11) **10**:171-268.
3. Mugard, H & Rouyer, M 1966. Cultures axeniques D'*Ophryoglena mucifera*, infusoire, hymenstome histophage. *Protistolgica* **2**:53-55.
4. Savioe, A 1968. Les ciliés histophages en biologie cellulaire. Ann. Univ. Ferrara (N.S., Sect III) **3**:65-71.
5. Thompson, JC Jr. 1958. Experimental infections of various animals with strains of the genus *Tetrahymena*. *J. Protozool.* **5**:203-205.

A-54.1

CULTIVATION OF *BLASTOCYSTIS*

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INTRODUCTION

Blastocystis hominis can be readily cultivated *in vitro* [1].

PROTOCOL

1. Medium ingredients should be added in the sequence listed. If the name of a company is listed with a specific component, the medium component should be obtained from the company to assure growth.
2. Medium formulation:

Blastocystis Egg Medium

Whole emulsified egg*	783.0 ml
Stone's Modification of Locke's Solution (see below)	217.0 ml

Stone Modification of Locke's Solution

NaCl	8.0 g
$CaCl_2$	0.2 g
KCl	0.2 g
$MgCl_2 \cdot 6H_2O$	0.01 g
$Na_2 \cdot HPO_4$	2.0 g
$NaHCO_3$	0.4 g
KH_2PO_4	0.3 g
Glass distilled H_2O	1.0 L

*Use only fresh **fertile** eggs (One source for such eggs is Truslow Farms, Inc., Chestertown, Maryland, USA.)

3. Filter lightly beaten eggs (emulsified) through gauze into a filtration flask.
4. Place under vacuum to draw out air bubbles.
5. Dispense in 4.0 ml amounts to 16 x 125 mm screw-capped test tubes. Inspissate (place in an 80° C incubator for 10 min) at a 30° slant. Allow to cool.
6. Add a 4.5 ml overlay of Stone's Modification of Locke's Solution. Rubber stopper (size 000) the tubes and autoclave in a press at 121° C for 5 min.
7. Remove the rubber stoppers after the medium is cool and replace aseptically with screw caps.

8. Add approximately 1.5 ml of heat inactivated (56° C for 30 min) horse serum (the final concentration will be 25% (v/v)) to each tube.
9. Loosen caps one full turn and place tubes in an anaerobic jar. Add a BBL GasPak (one anaerobic system GasPak per BBL GasPak 100 anaerobic culture jar). Close the vessel securely and incubate at 35° C for at least 48 h. The palladium catalyst in the GasPak jar should be replaced biweekly with fresh catalyst.
10. Remove growing cultures and uninoculated tubes of reduced medium immediately from the anaerobic jar and screw caps down tightly.
11. The cells are concentrated at the bottom of the liquid overlay. Carefully introduce a sterile Pasteur pipette aseptically through the air-liquid interface. Expel air from the pipette bulb prior to passage into the liquid and move the tip of the pipette to the cell mass and aspirate approximately one third of the mass into the pipette. Tighten the cap immediately unless the culture is to be promptly transferred to an anaerobic jar.
12. Loosen cap one full turn and place freshly inoculated culture, as well as, uninoculated tubes of medium (these tubes will be needed for subsequent transfers) into the anaerobic jar. Prepare a GasPak and quickly seal and incubate at 35° C.

COMMENTS

Avoid expulsion of air bubbles into the medium during transfers. These organisms are very sensitive to oxygen. Work quickly when transferring culture to avoid prolonged exposure to air.

LITERATURE CITED

1. Molet, B, Werler, C & Kremer, M 1981. *Blastocystis hominis* improved axenic cultivation. *Trans. Roy. Soc. Trop. Med. Hyg.* **75**:752-753.

CULTIVATION OF ANAEROBIC RUMEN FUNGI

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INTRODUCTION

Anaerobic phycomycetes have been isolated from the rumen of sheep, cattle, goats, musk oxen, reindeer, camel and the kangaroo [1]. They have also been found in the cecum of the horse, rhinoceros and elephant. It therefore appears that they are an indiginous part of the microbial flora of herbivores on a highly fibrous diet [1]. Biochemically the rumen fungi have many features in common with the anaerobic hydrogenosome containing protozoa [2]. No ribosomal analysis is available for the rumen phycomycetes to indicate if a common lineage exists between these two genera. However, it has recently been reported that based on ribosomal RNA homologies the oomycetes are closely related to the chrysomonad line [3].

PROTOCOL

A medium based upon that of Diamond [4] is used to isolate the fungi from rumen fluid [5].

1. Medium formulation:

Basal Medium	
Tryptone	20.0 g
Yeast extract	10.0 g
Maltose	2.0 g
L-cysteine HCl	1.0 g
L-ascorbic acid	0.2 g
Agar	1.0 g
Centrifuged rumen fluid	100.0 ml
Glass distilled H_2O	790.0 ml

Antibiotic Solution	
Na benzylpenicillin	10.0×10^6 U
Streptomycin sulfate	1.0 g
Glass distilled H_2O	10.0 ml

2. Autoclave 15 lb/in^2, 15 min.
3. Cool to 50° C add 10 ml filter-sterilized antibiotic solution, and 100 ml heat-inactivated sheep serum (56° C for 30 min).
4. Gas with CO_2 until agar gel.

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Inoculum is prepared from filtered rumen fluid. Centrifuge the filtrate at 1000xg for 15 min at room temperature, wash the sediment twice with sterile centrifuged rumen fluid, resuspend in sterile centrifuged rumen fluid and 0.2 ml is layered onto the surface of the medium. The tube is gased with CO_2 for 15 min and incubated at 39°C. After 48 h the top 5 cm of the medium is discarded and 1 cm of the underlaying sloppy agar containing *Neocallimastix* zoospores is overlaid onto fresh medium. After transfer the culture is shaken to distribute the inoculum in the top 6 mm of medium and incubated at 39°C for 48 h. Repeating these steps results in a monoculture of *Neocallimastix* sp.

PROTOCOL

Axenic cultivation in non-defined medium of *Neocallimastix* sp., *Sphaeromonas* and *Piromonas* [6].

1. Medium formulation:

Salts Solution I

K_2HPO_4	3.0 g/L

Salts Solution II

KH_2PO_4	3.0 g/L
$(NH_4)_2SO_4$	6.0 g/L
NaCl	16.0 g/L
$MgSO_4$ (anhydrous)	0.6 g/L
$CaCl_2$	0.6 g/L

Basal Medium

Salts solution I	150.0 ml
Salts solution II	150.0 ml
Bacto casitone	10.0 g
Yeast extract	2.5 g
Centrifuged rumen fluid*	200.0 ml
Cysteine-HCl	1.0 g
$NaHCO_3$	6.0 g
Maltose (or cellobiose)	5.0 g
Resazurin (0.1%)	3.0 ml
Glass distilled H_2O	500.0 ml

*Rumen fluid filtered through one layer of muslin and centrifuged at 20,000 g for 1 h at 4° C.

2. Gas with CO_2 for 30 min until resazurin is reduced.
3. Dispense 10 ml into Hungate tubes under an atmosphere of CO2 and tightly cap.
4. Autoclave 15 lb/in^2, 15 min.
5. When cool add 0.2 ml of filter-sterilized (0.2 um filter) vitamin solution per tube and 0.2 ml of antibiotic solution.

Vitamin Solution

Thiamin HCl	1.0 mg
Riboflavin	20.0 mg
Ca pantothenate	60.0 mg

Nicotinic acid	100.0 mg
Nicotinamide	100.0 mg
Folic acid	5.0 mg
Cyanocobalamin	20.0 mg
Biotin	20.0 mg
Pyridoxine HCl	10.0 mg
4-aminobenzoic acid	5.0 mg
Glass distilled H_2O	100.0 ml

Antibiotic Solution

Ampicillin	100.0 mg
Chloramphenicol	50.0 mg
Methanol	20.0 ml

6. Incubate at 39°C.

PROTOCOL

A semi-defined medium has been described which avoids the inclusion ofcentrifuged rumen fluid [7]. A modification of this is presented which reduces the potential formation of struvite in the original formulation caused by the high concentration of calcium, ammonia and phosphate. The anhydrous salts of magnesium and calcium internationalize the medium with respect to humidity changes. Other minor changes have also been made.

1. Medium formulation:

Basal Mix I

KCl	0.6 g
NaC0.6 g	
$MgSO_4$(anhydrous)	0.5 g
$CaCl_2$ (anhydrous)	0.1 g
$Fe(NH_4)_2SO_4$	0.2 g

Basal Mix II

KH_2PO_4	0.5 g
Trypticase	1.0 g
Yeast extract	0.5 g
PIPES buffer	1.5 g

Fatty Acid Solution

Acetic acid	6.85 ml
Propionic acid	3.00 ml
Butyric acid	1.84 ml
2-methylbutyric acid	0.55 ml
Isobutyric acid	0.47 ml
Valeric acid	0.55 ml
Isovaleric acid	0.55 ml
Adjust to pH 7.5 with 1M NaOH water to	1.0 L

Trace Elements

$MnCl_2.H_2O$	0.25 g
$NiCl_2.6H_2O$	0.25 g

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$NaMoO_4.2H_2O$	0.25 g
H_3BO_3	0.25 g
$CoCl_2.6H_2O$	0.05 g
SeO_2	0.05 g
$NaVO_3.4H_2O$	0.05 g
$ZnSO_4.7H_2O$	0.025g
$CuCl_2.2H_2O$	0.025g
0.2 M HCl	1.0 L

Vitamin Solution

1,4-naphthoquinone	0.25 g
Ca pantothenate	0.20 g
Nicotinamide	0.20 g
Na riboflavin phosphate.$2H_2O$	0.20 g
Thiamin HCl	0.20 g
Pyridoxine HCl	0.20 g
Biotin	0.025 g
Folic acid	0.025 g
Cyanocobalamin	0.025 g
P-aminobenzoic acid	0.025 g
HEPES 5mM	1.0 L
Coenzyme M (Na 2-mercaptoethane sulfonic acid; Pierce Chem. Co., Rockford, Il.)	40.0 mg
Hemin solution (10 mg in 100 ml of 50% Quadrol)	10.0 ml
Resazurin solution (1g/L)	1.0 ml

Reducing Agents

$NaS.9H_2O$	12.0 g
L-cysteine HCl	25.0 g
Na Hydroxymethane sulfinic acid.$2H_2O$, Aldrich Chem. Co., Milwaukee, Wi.	5.0 g
$NaHCO_3$	4.0 g

Carbohydrates

Glucose	3.75 g
Cellulose	10.0 g

2. Basal mix I is composed in a volume of 780 ml; once in solution thecomponents of basal mix II are gradually added.
3. 10 ml each of the trace elements, fatty acid solution, and hemin solutionare added.
4. Add Coenzyme M and 1 ml of resazurin.
5. To this the reducing agents, $NaHCO_3$ and carbohydrates are added.
6. Add 10 ml of the vitamin solution.
7. The medium is brought to 1 L and gased for 30 min with CO_2 prior to autoclaving at 15 lb/in^2 for 15 min.

COMMENTS

The usual anaerobic techniques should be employed when handling rumen fungi. The media described are suitable for the isolation and cultivation of fungi from different ruminants. The

media can be made selective for the different species by varying the carbon source eg. inulin selects for the growth of *Neocallimastix* spp. [8]. Sporogenesis by rumen phycomycetes can be induced by an extract prepared from oats [9].

LITERATURE CITED

1. Orpin, CG 1988. Nutrition and biochemistry of anaerobic chytridiomycetes. *Biosystems* **21**:365-370.
2. Yarlett, N, Orpin, CG, Munn, EA, Yarlett, NC & Greenwood, C 1986. Hydrogenosomes in the rumen *Neocallimastix patriciarum*. *Biochem. J.* **236**:729-739.
3. Cedergren, R, Gray, MW, Abel, Y & Sankoff, D 1988. The evolutionary relationships among known life forms. *J. Mol. Evol.* **28**:98-112.
4. Diamond, LS 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. *Parasitol.* **43**:488-490.
5. Orpin, CG 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* **91**:249-262.
6. Kemp, P, Lander, DJ & Orpin, CG 1984. The lipids of the rumen fungus *Piromonas communis*. *J. Gen. Microbiol.* **130**:27-37.
7. Lowe, SE, Theodorou, MK, Trinci, APJ & Hespell, RB 1985. Growth of anaerobic rumen fungi on defined and semi defined media lacking rumen fluid. *J. Gen. Microbiol.* **131**:2225-2229.
8. Phillips, MW & Gordon, GLR 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. *Biosystems* **21**:377-383.
9. Orpin, CG 1974. The rumen flagellate *Callimastix frontalis*: Does sequestration occur? J. Gen. Microbiol. **84**:395-398.

PRESERVATION OF PROTOZOA CRYOPRESERVATION, DRYING AND FREEZE-DRYING GENERAL METHODS AND DEFINITION OF TERMS

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INTRODUCTION

A variety of techniques have been used to maintain protozoan strains in the laboratory for long periods of time without frequent subculturing. The method that assures the longest viability without a change in characteristics known today is to stabilize at **cryogenic temperatures** (below -100° C). If stored below -130° C, viability can be maintained indefinitely as far as is currently known. This temperature is near the **glass phase transition temperature** (-139° C for pure H_2O and higher for solutions; the temperature at which there is no translational motion of molecules and therefore no chemical reactions) of most solutions. At this temperature viability is only being effected by the penetration of cosmic rays through the storage vessel. It has been estimated that the half-life of frozen preparations stored in this manner is 10,000 to 30,000 years. Numerous strains can also be kept for long periods of time at relatively higher **ultra-low temperatures** (-70° to -100° C) in a constant temperature mechanical freezer, but as the storage temperature increases above -130° C the length of viability decreases. Strains which form a resistant stage (i.e., cysts) in their life history often can also be maintained as **dried preparations** (unfrozen preparations from which the H_2O has been removed through evaporation) [7,9]. These strains as well as certain strains without a known resistant stage in the life history but which can tolerate harsh conditions can be preserved as **freeze-dried preparations** (frozen preparations from which the H_2O has been removed through sublimation) [3,6]. Although less stable than cryopreserved material, dried and freeze-dried preparations have the practical advantage of less expensive storage and can be transported more economically. The authors of this protocol have detailed separate protocols for the preservation methods of a wide array of protozoa. It is simply not practical or within the scope of this manual to address all the variations known to be required for all species and strains for each genus for which a methodology is outlined. If the investigator is attempting to cryopreserve a member of a particular genus it is recommended to first follow the separate protocol detailing the method for a particular genus. If the protocol yields less than satisfactory or negative results, then it may be necessary to modify the procedure. The purpose of this protocol is to suggest what might be considered to modify a particular approach and to suggest how to approach the possible preservation of protozoa for which protocols have not been detailed in this manual. The critical steps in cryopreservation, freeze-drying, and drying are reviewed in protocol format here. Terms used throughout the detailed protocols are explained here.

A-56.2

PROTOCOL

Cryopreservation, strains cultured on medium

1. Harvest when cultures are at or near peak density. Harvesting cells that are actively dividing or are beginning to decline after reaching peak density usually results in no or low numbers of viable cells after cryopreservation. Concentrate the cells as ***gently*** as possible. There are five basic harvesting methods. Select the least harsh method possible for a particular strain. The cells are usually concentrated to a level twice that required. (further detailed in steps 3 & 5).

    a) Allow the cells to settle to the bottom of a culture vessel and remove the overlying liquid medium with a pipette. Cells normally maintained in medium that contains a small amount of agar, such as trichomonads, should be cultivated in the medium without agar prior to freezing. Cultivate on a horizontal slant rather than vertically. They can be harvested by placing the culture tubes vertically and allowing the cells to settle.
    b) Use gravity filtration through a Nitex screen (a finely woven nylon cloth) which does not allow passage of the cells.
    c) For strains which attach to the wall of the culture vessel e. g., amebae, gently remove most of the overlying medium with a pipette. To detach the cells, ice the culture for 10 min, then tilt the vessel back and forth using the small amount of medium remaining to wash the cells off the vessel walls.
    d) For strains grown on agar, to harvest, add the cryoprotective solution in the final concentration desired (further details in steps 2-5) and suspend cells by rubbing the agar surface with a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).
    e) If centrifugation is required use the lowest speed that will allow pelleting of the cells. After resuspension transfer to a T-25 tissue culture flask at double the final concentration of cells desired and allow to remain undisturbed for 1 h prior to addition of the cryoprotective solution which is also prepared at double the final desired concentration (detailed in step 5).

2. Although others have been tried and some of them are useful, there are three **cryoprotectants** (substances which ameliorate the damage done as a result of freezing) which are generally used: dimethyl sulfoxide (**DMSO**), glycerol, and methanol. DMSO is the most widely used and is generally the best. Since it is hygroscopic it should be stored in small quantities. After opening a new bottle, sterilize by filtration using a 0.22 μm Teflon filter (Millipore), dispense in 10 ml aliquots and keep frozen. This will reduce the likelihood of dilution of the DMSO with H_2O. The concentration of DMSO used varies from 2.5-12.5% (v/v). For many photosynthetic strains methanol is the preferred cryoprotectant. Methanol is generally used at a concentration ranging from 3-5% (v/v). Absolute methanol does not require sterilization. Glycerol is the least effective of the cryoprotectants but certain strains, e.g. *Crypthecodinium*, can only be cryopreserved using this compound. Glycerol is generally used at concentrations ranging from 7.5-12% (v/v). Glycerol is sterilized by dispensing 10 ml quantities into 16 x 125 mm screw-capped tubes and autoclaving for 15 min at 121° C. The caps, which have been loosened half a turn prior to autoclaving are tightened only after the glycerol has fully returned to room temperature otherwise a partial pressure differential can be created which will result in air being sucked into the test tube when first used. For some organisms, e. g. *Toxoplasma* and *Blastocystis*, DMSO and glycerol are used in combination. In some cases various sugars, serum, and bovine serum albumin fraction V

(**BSA** - Sigma A-4503) may be combined with the primary cryoprotective agent. For a strain belonging to one of the genera listed in a detailed protocol use the cryoprotectant at the concentration listed. If recovery is not obtained or if not certain of the best cryoprotectant and/or concentration to use for a strain, especially if a never before cryopreserved strain, some experimentation is required. If it is the first attempt for an organism not belonging to a genus for which a detailed protocol is given, it is best to begin by using methanol for photosynthetic strains and DMSO for non-photosynthetic strains. Begin by doing a "test freeze" using a range of concentrations with a few **cryules** (vials made of plastic that can tolerate cryogenic temperatures even down to -196° C [12]) at each concentration. For methanol initially use 3%, 5%, 7%, and 9% (v/v) and for DMSO use 5%, 7.5%, 10%, and 12.5% (v/v). If recovery is not obtained with methanol, use DMSO (see step 6 for important comments on DMSO). If recovery is not obtained with DMSO use glycerol at concentrations of 5%, 7.5%, 10%, and 12.5% (v/v). Determine the concentration of the cryoprotectant that yields the highest recovery. Recoveries may be further enhanced by decreasing or increasing the concentration of the cryoprotective agent in 0.5% steps from the best value determined after a "test-free".

3. In general, the final concentration of cells desired after the cryoprotective solution is added is a function of the size of the organism. The final concentration of cells desired after the cryoprotective solution is added ranges from 1.0×10^5 to 5.0×10^7 cells/ml. Cells greater than 100 μm are preserved at $1\text{-}5 \times 10^5$ cells/ml; cells between 50-100 μm are preserved at $1\text{-}5 \times 10^6$ cells/ml; and cells less than 50 μm are preserved at $1\text{-}5 \times 10^7$ cells/ml. This is a general "rule" and if it is followed the number of viable cells recovered should be adequate, but it may be necessary to vary the cell concentration above or below the suggested level to optimize the number of viable cells recovered.
4. The next step is to prepare the cryoprotective solution. Glycerol and methanol solutions can be prepared at room temperature (20-23° C), but DMSO solutions should be prepared as follows particularly if the medium has large amounts of protein, e. g., contains serum:

    a) Place the required amount of DMSO in a test tube and incubate the tube in an ice bath for 10 min. The DMSO will solidify under these conditions.
    b) Add ½ of the appropriate amount of ice cold medium to the solidified DMSO and invert several times until the DMSO is in solution. Place the tube in ice for 5 min and then add the remainder of the cold medium.

    When DMSO is mixed with H_2O an exothermic reaction occurs and this can elevate the temperature of the solution sufficiently to precipitate proteins and certain salts. If the DMSO solution is prepared as described the problem will not be encountered. Never use a DMSO solution if it is warmer than room temperature.
5. Except for those strains cultivated on agar, the cryoprotective solution is added as a double concentrated stock and mixed 1:1 with the cell suspension. For sensitive organisms, the concentrated cryoprotective solution should be added in three equal aliquots at 2 min intervals. Dispense 0.5 ml of the final suspension into sterile plastic screw-capped cryules. For organisms particularly sensitive to osmotic shock during the thawing procedure, cryopreservation of 1.0 ml aliquots may be beneficial. As a frozen preparation thaws the channels between crystals of frozen H_2O that contain the cells thaw first. Therefore, the cells are initially bathed in a hyperosmotic milieu. As the frozen crystals of H_2O thaw this lowers the osmolarity of the extracellular environment. As the volume of the frozen suspension increases the rate at which this dilution occurs will be reduced. **SAFETY PRECAUTION:** Although glass cryules are made, it is recommended that they not be used. If used, do not seal, but cotton plug. **Store in the vapor phase of a liquid nitrogen freezer** or a mechanical refrigerator. Although glass cryules can be heat sealed, the authors recommend that the vials not be sealed for several reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage.

When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials they must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION WITH INFECTIOUS AGENTS IS POSSIBLE WITH SEALED GLASS VIALS.**

6. The time from the mixing of the cryoprotective solution with the cells to the initiation of the cooling cycle, **equilibration time**, should be at least 10 min but not longer than 60 min. Exposure of the cells to the cryoprotectant for too long may be detrimental to the cells. Tolerance to DMSO will vary with the organism and should be determined prior to cryopreservation in the following manner:

    a) Prepare a range of concentrations in the growth medium of the cryoprotectant to be used, usually in increasing increments of 2.5% (v/v) concentrations.
    b) Mix the cells and the cryoprotective solutions and incubate at room temperature (20-23° C). Observe at 15 min intervals up to 1 h. Determine the maximum time that the cells can tolerate the maximum concentration of the cryoprotectant.

7. Best results are generally, but not always, obtained by using a controlled cooling rate. This is somewhat of a misnomer because the rate is regulated only down to the point at which the ampules are transferred to liquid nitrogen. Also in actuality it is the rate of cooling of the chamber that is regulated. The ampule temperature passively follows changes in the chamber temperature. There are three cooling cycles routinely used.

    a) The simplest controlled cooling cycle, is -1° C/min from 25° C to -40° C with compensation for the **heat of fusion** (the heat liberated when liquid H_2O is converted to ice. Heat will continue to be produced until most of the H_2O has entered the solid state. There is often a plateau in the cooling cycle at this point. Some controlled rate freezing units can compensate for this heat by appropriately programming the cooling of the chamber. The plateau can be eliminated in some cases). The cryules are plunged into liquid nitrogen from -40° C. This cooling cycle is often accompanied by **supercooling**, i.e., the cooling of a solution below its freezing point (the freezing point normally lies between -2° to -6° C). When the heat of fusion occurs in a supercooled solution, an abrupt rise in temperature of the sample will occur and the temperature will rise from the supercooled temperature to the freezing point of the solution. This will be witnessed as a sharp blip on the recording chart. Supercooling can be a significant problem for certain sensitive strains.
    b) Some protozoa sensitive to supercooling can be cryopreserved using a triphasic cooling cycle developed to solve the problem [4]. The triphasic cooling cycle is -10° C/min. from 25° C to the heat of fusion; -1° C/min from the heat of fusion to -40° C. The cryules are then plunged into liquid nitrogen. The one disadvantage of this cooling cycle is that certain strains may be sensitive to **cold shock**, i.e., sensitivity to rapid cooling in the liquid phase.
    c) In order to minimize cold shock, but to avoid supercooling, a compromise cooling cycle has proven to be effective [8]. Begin at -1° C/min from 25° C to 4° C; -10° C/min from 4° C to the heat of fusion; -1° C/min from the heat of fusion to -40° C. The cryules are then plunged into liquid nitrogen.

The order of the cooling cycles listed is in the order of the sophistication of the equipment required to perform the procedure. Although often better results will be obtained with the second and third cooling cycles listed, adequate results may be obtained with the first cooling cycle which requires the least sophisticated equipment. In order to perform any of the controlled cooling cycles listed above a programmable freezing unit which can compensate for the heat of fusion is required. Two machines which have been successfully used are the Cryo-Med Model 900 and 1010 units. The initial cooling cycle of choice, the

last listed, may not be satisfactory for all strains. Therefore, if poor results are encountered then use the first cooling cycle, and, if results continue to be less than satisfactory the second. In some cases, however, uncontrolled cooling can yield sufficiently high recovery and in other cases very rapid uncontrolled cooling is essential for recovery, e. g. directly plunging into liquid nitrogen. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min can be achieved using several different relatively inexpensive commercially available devices listed next:
Bicell: manufactured by Nihon Freezer Co, LTD, 19-4,3-Chome, Yushima, Bunkyo-Ku, Tokyo, Japan.

Advantages: Least expensive; isopropyl alcohol not required.
Disadvantages: Only 7 ampules can be frozen in one vessel.

Cryopreservation Apparatus: Cat. #9001 manufactured by Cambridge Biotech Corp., Worcester, MA 01605 USA.

Advantages: Holds 24 ampoules
Disadvantages: Most expensive; ampoules are partially immersed in isopropyl alcohol and therefore ampule labels must be alcohol insoluble; screw-driver or similar device needed to pry lid off to transfer ampules to nitrogen freezer.

Nalgene 1°C Freezing Container: Cat. #5100-0001 manufactured by Nalge Co., Rochester, NY 14602 USA.

Advantages: Although isopropyl alcohol is required the ampules are not immersed in it; holds 18 ampules.
Disadvantages: Screw-capped lid can stick after freezing; a person with a large hands is required to unscrew the lid to transfer the ampules to nitrogen freezer.

Another approach for strains which can be cryopreserved using an uncontrolled cooling cycle is to place cryules on the bottom of a constant temperature mechanical refrigerator set between -55 to -70° C for 1 h and then transfer to a liquid nitrogen refrigerator or, if none is available, store the vials in an ultra-low constant temperature mechanical refrigerator [1,5,10,11].

8. Store frozen specimens at or below -130° C for optimum stability. If preparations are stored at higher temperatures monitor at least at 6 month intervals to determine stability.
9. To establish a culture from the frozen preparation one of the following methods is usually employed:

a) Transfer a frozen cryule directly from storage to a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate. For most strains the thawed contents can be added directly to fresh medium. For some strains which are sensitive to osmotic shock increasing the volume of the frozen suspension from 0.5 ml to 1.0 ml can be beneficial. As the volume of the frozen suspension is increased, the time for contents of the ampoule to completely thaw will be increased. (See step 5 for details).

b) Recovery of some sensitive strains can be enhanced by increasing the osmolarity of the medium used for initial establishment of the culture [8]. For some, but not all, strains e. g. *Tetrahymena*, *Euplotes* and *Glaucoma*, it may be critical to survival. Remove the frozen cryule from storage and aseptically add 0.5 ml of cultivation medium containing 8% (w/v) sucrose. Screw the cap on tightly and place in a 35° C water bath. Transfer the thawed contents to medium with 4% (w/v) sucrose. For axenic strains incubate in the normal fashion, but transfer to medium without sucrose when the culture has reached peak density. For strains grown with bacteria on the day following thawing and incubation at normal cultivation temperature, pick viable cells with a drawn out Pasteur pipette and transfer to fresh medium without sucrose.

c) For sensitive marine organisms and some freshwater organisms place the frozen cryule directly from storage to a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate. Gently remove contents from thawed ampoule with a Pasteur pipette and expel slowly into a T-25 tissue culture flask. Place the flask in a horizontal position and incubate at room temperature (20-23° C). At 15 min intervals add dropwise 0.25 ml of the appropriate medium. Continue until the final volume is 2.0 ml. After the final aliquot is added allow the culture to remain undisturbed for 15 min, then add dropwise 0.5 ml of the appropriate medium at 15 min intervals until the final volume is 4.0 ml. Allow the culture to incubate undisturbed overnight. The next morning slowly add 4.0 ml of the appropriate fresh medium and again allow the culture to incubate undisturbed until the following morning. During the morning of third day after thawing, if the culture has either free swimming cells or unattached nonmotile cells, gently agitate the flask and transfer 1 ml to a fresh T-25 flask containing 10 ml of the appropriate medium. Incubate thereafter using normal protocol. Maintain both cultures in parallel. If the culture has attached cells, gently remove all but approximately 1 ml of culture fluid with a pipette and transfer this material to a fresh T-25 flask. Add 10 ml of the appropriate medium to the decanted flask. Maintain both cultures in parallel.

PROTOCOL

Cryopreservation, *in vivo* cultivated strains

1. Harvest from an infected animal using one of the following methods:

   a) For blood parasites, harvest when the parasitemia is at or near its peak. The blood is withdrawn from the host animal using a syringe containing a small amount of anticoagulant, usually a solution containing heparin.
   b) For tissue other than blood, remove the infected organ(s) from the host animal(s) immediately after the host(s) has been killed and place in a sterile tissue homogenizer. Add aseptically an equivalent volume of the appropriate balance salt solution and homogenize. If found within lungs, e.g., *Pneumocystis*, lungs are not ground but extensively lavaged with an appropriate saline solution.
   c) For parasites of intraperitoneal macrophages, kill the host animal(s) and using a needle attached to a syringe, aseptically transfer the appropriate salt solution to the peritoneal cavity. Massage the abdomen for several min and aseptically remove all peritoneal fluid. To avoid contaminating the preparation, the side wall of the body cavity is pulled outward forming a pocket with the needle opening orientated away from the organs in the cavity.
   d) For intestinal parasites, the feces are collected and the parasites are washed free of debris using an appropriate saline solution.

2. Glycerol is used at concentrations range from 6.0-28% (v/v) and DMSO usually at 5-7.5% (v/v) [1]. If not using a detailed protocol, to determine the best concentration of the cryoprotective agent a "test freeze" is done with varying concentrations. Prepare a range of concentrations of the cryoprotective agent in the salt solution used to harvest the parasite. Usually a series of concentrations of the cryoprotective agent in 2.5% (v/v) incremental steps in the beginning at 5.0% are tested. All vials are frozen at the same time using the same cooling cycle.
3. The final concentration of extracellular parasites usually ranges between 10^7-10^8 cells/ml, but optimum results may require an increase or a decrease outside of this range.

4. Prepare the cryoprotective solution. Glycerol solutions can be prepared at room temperature, but DMSO solutions should be prepared as follows particularly if the medium has large amounts of protein, i.e., contains serum:

    a) Place the required amount of DMSO in a test tube and incubate the tube in an ice bath for 10 min. The DMSO will solidify under these conditions.
    b) Add ½ of the appropriate amount of ice cold medium to the solidified DMSO and invert several times until the DMSO is in solution. Place the tube in ice for 5 min and then add the remainder of the cold medium.

    When DMSO is mixed with H_2O an exothermic reaction occurs and this can elevate the temperature of the solution sufficiently to precipitate proteins and certain salts. If the DMSO solution is prepared as described the problem will not be encountered. Never use a DMSO solution if it is warmer than room temperature.
5. The cryoprotective solution is usually added as a double concentrated stock and mixed 1:1 with the parasite suspension. For sensitive organisms, the concentrated cryoprotective solution should be added in three equal aliquots at 2 min intervals. Dispense 0.5 ml of the final suspension into sterile plastic screw-capped cryules (**FOR SAFETY CONSIDERATIONS IN SELECTING AND HANDLING AMPULES** see Step 5 of the previous protocol). For organisms, particularly sensitive to osmotic shock during the thawing procedure, cryopreservation of 1.0 ml aliquots may be beneficial.
6. The time from the mixing of the cryoprotective solution with the cells to the initiation of the cooling cycle should be at least 10 min but not longer than 60 min. Exposure of the cells to the cryoprotectant for too long may be detrimental.
7. Strains are generally cryopreserved using a controlled cooling rate for a portion of the cooling cycle. Some parasites can or must be cryopreserved using an uncontrolled rapid cooling cycle. For controlled cooling, the following cooling cycle is effective for many strains: -1° C/min from 25° C to -40° C with compensation for the heat of fusion. The ampules are plunged into liquid nitrogen from -40° C and stored. The cycle requires specialized equipment (See preceding protocol for type of equipment and alternatives.). An effective uncontrolled cooling cycle for intracellular blood parasites is to place prepared cryules into a dry-ice ethanol bath (approximately -80° C) for 2 min. The ampules are then directly transferred to liquid nitrogen, if available, and subsequently stored in a liquid nitrogen freezer, or if none is available, in the coldest ultra-low constant temperature mechanical freezer available.
8. Store frozen specimens at or below -130° C for optimum stability. If preparations are stored at higher temperatures monitor at least at 6 month intervals to determine stability.
9. To establish an infection from the frozen preparation transfer a frozen cryule directly from storage to a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate. Immediately after thawing, do not leave in the water bath, aseptically **AND WITH SAFETY PRECAUTIONS IN MIND**, aspirate the entire contents of the cryule into a 1 ml syringe fitted with the appropriate size needle and inoculate intraperitoneally into a single uninfected host or for intestinal or lung parasites, inoculate orally after anaesthetizing the host. The newly infected host should be carefully monitored. The course of the infection may be longer or shorter than usual dependent upon the number of viable cells recovered.

A-56.8

PROTOCOL

Freeze-drying
Single Vial Manifold Method [1]
(Fig. 1)

1. Prepare in advance a sterile 12% (w/v) sucrose solution in glass distilled H_2O.
2. Harvest cells from a culture which is at or near peak density. For broth cultures concentrate cells by centrifuging at 200 g for 5 min. Resuspend cell pellet in 5.0 ml of broth and determine cell concentration. Adjust concentration to 10^8 cells/ml with fresh broth. Note the volume and centrifuge as above. Discard supernatant and replace with an equal volume of 12% sucrose solution. For agar slant or plate cultures suspend cells directly in the 12% sucrose solution. Adjust concentration to 10^8/ml with the 12% sucrose solution.
3. Dispense cell suspension in 0.1 ml aliquots into sterile glass cotton-plugged 1.0 ml bulb type (teardrop) freeze-drying ampules.
4. Just prior to freeze-drying a moisture trap (condenser) attached to a vacuum pump is prepared by placing a trimmed block of dry-ice which just fits into a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added just to the top of the condenser containing the dry-ice. An extender is placed on top of the condenser and the interior filled with dry-ice pellets.
5. Prepare a dry-ice Cellosolve bath in a stainless steel tray. A manifold suspended above the bath is attached to the trap prepared in step 4 using Tygon tubing.
6. The ampules are quick frozen by swirling when the vials are rapidly immersed in the bath. While still in the bath each vial is attached by Tygon tubing to the manifold. The bulb portion should be fully immersed in the bath. After all of the ampules have been attached the vacuum is turned on. The vacuum should be pumped down to at least 30 μm of Hg.
7. Keep under vacuum for at least 12 h. The dry-ice Cellosolve bath will return to room temperature during the 12 h period.
8. While still under vacuum raise the ampules from the bath. Dry the bulb portion of the vials.
9. Seal under 30 μm of Hg. At the point at which the neck of the ampule is attached to the tubing heat the glass with the hottest part of an air/gas flame until red hot. While slowly pulling the vial the narrowing neck is separated and sealed.
10. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered.
11. To establish a culture from the dried state aseptically add 0.1 ml of sterile glass distilled H_2O or sterile fresh broth. After the pellet dissolves dispense into fresh medium. Incubate using the protocol for routine cultivation. For sensitive strains use the following protocol [8], a modification of a protocol developed for bacteria sensitive to freeze-drying [2]:

    a) Place the freeze-dried vial and a fresh tube of modified medium (containing 12% (w/v) sucrose and with the pH adjusted to 7.0) in an ice bath.
    b) Aseptically add 0.5 ml of the cold modified medium to the freeze-dried vial and when the pellet is rehydrated transfer the contents from the vial to the tube of medium.
    c) Place the test tube at the appropriate temperature.

    It is critical to follow these instructions to obtain consistent recovery of sensitive strains from the freeze-dried state.

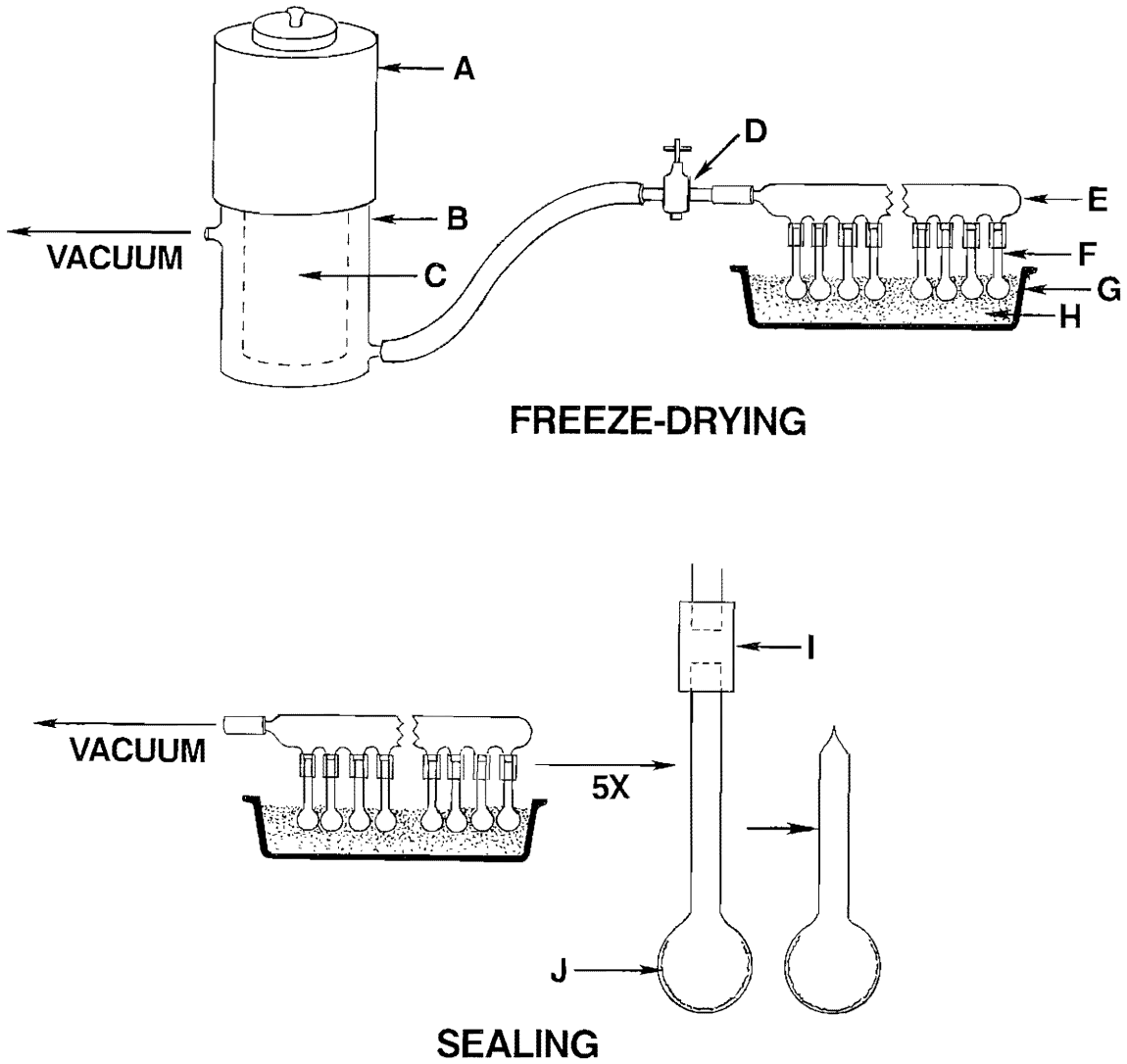


Fig. 1. Manifold method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) manifold; (F) teardrop ampule*; (G) stainless steel pan (H) dry-ice Cellosolve bath; (I) rubber sleeve connecting vial to manifold; (J) thin film of freeze-dried cell suspension.

*The teardrop ampule can be custom made by Kontes Glass Co. The outer diameter of the neck is 8 mm and the inner diameter is 6 mm. The neck length is 125 mm. The diameter of the bulb is 15 mm.

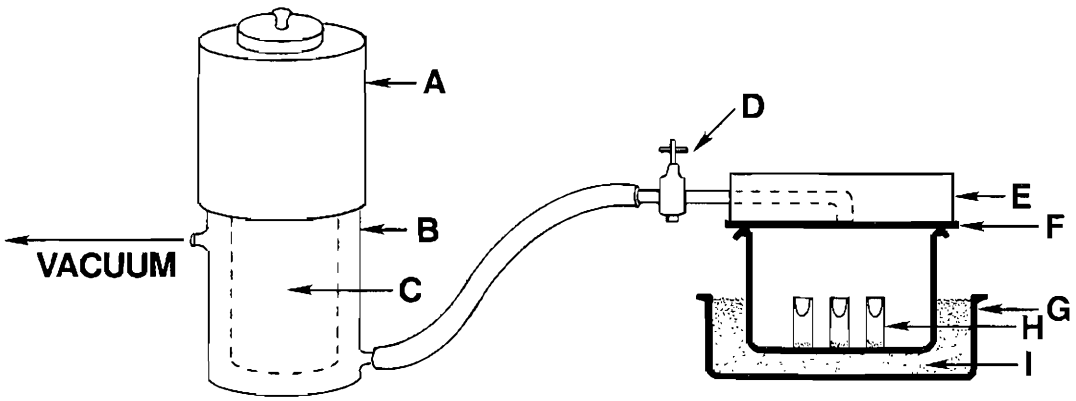
A-56.10

PROTOCOL

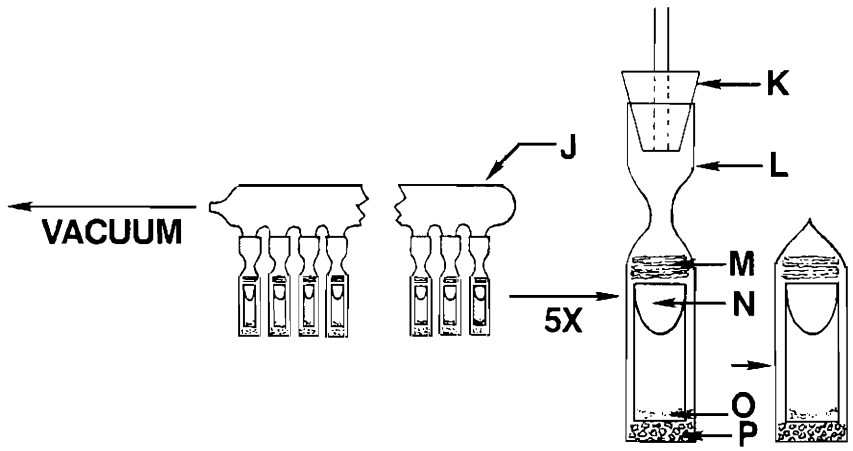
Freeze-drying
Double Vial Method [1]
(Fig. 2)

1. Prepare in advance a sterile 20% (w/v) suspension of skim milk (Difco #0032) in glass distilled H_2O or a 12% (w/v) sucrose plus 4% (w/v) BSA (Bovine Serum Albumin Fraction V - Sigma A-4503) solution in the growth medium. Prepare the latter as follows:

    a) Add 12.0 g sucrose to 100.0 ml of medium and dissolve thoroughly.
    b) Divide the above solution into four equal aliquots and transfer each to a separate 50 ml plastic screw-capped centrifuge tube. Add 1.0 g of BSA to each tube and agitate vigorously until dissolved. A considerable amount of foam will overly the liquid.
    c) Centrifuge at 850 g for 10 min to eliminate the layer of foam.
    d) Pool the solutions in a single vessel and filter sterilize. Store refrigerated (5-9° C) for up to 1 year.
2. Harvest cells from a culture which is at or near peak density. For broth cultures concentrate using the most gentle method available. Resuspend and pool the cell pellets to the desired volume with fresh broth medium. For agar slant or plate cultures add 3.0 ml of broth and wash cells into suspension.
3. For both broth and agar slant cultures adjust concentration of cells to 10^8/ml concentrate. Adjust concentration of cells from agar cultures with the protective solution but for broth cultures centrifuge as above and resuspend pellet with a necessary volume of protective solution to bring the concentration of cells to the desired level.
4. Dispense the cell suspension in 0.2 ml aliquots into sterile glass cotton-plugged 11.5 x 35.0 mm inner shell freeze-drying glass ampules (Glass Vials, Inc.).
5. Place ampules in a stainless steel container and place the container onto the bottom of a mechanical freezer set between -55 to -70° C for 1 h. It is important to maximize recovery in the initial cryopreservation step. Recovery may be improved if a controlled cooling rate is employed (see step 7 of the first protocol). When the ampules reach -40° C they are transferred to a stainless steel container that has been on the bottom of a mechanical freezer set between -55 to -70° C for 1 h.
6. Just prior to freeze-drying a moisture trap condenser attached to a vacuum pump is prepared by fitting a trimmed block of dry-ice which just fits inside a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added just to the top of the condenser containing the dry-ice.
7. Place container with frozen ampules on a 2.5 cm thick layer of freshly prepared dry-ice snow. Pack snow around sides of container for several hours. Place the Atmo-vac plate cover (Refrigeration for Sciences Inc.) over the pan with the ampules and place under vacuum of at least 30 μm of Hg. The cover is attached to the condenser by Tygon tubing that will not collapse. Keep under vacuum for at least 12 h. All the dry-ice snow will have evaporated. Turn off the vacuum pump and slowly introduce air which has been previously passed over a desiccant to minimize introduction of moisture.
8. In advance the 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
9. Allow outer shell vials to cool in a dry cabinet (10% or least relative humidity) and then insert inner shell vial with the freeze-dried preparation. On top of the inner vial a wad of glass



FREEZE-DRYING



SEALING

Fig. 2. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

fiber paper is inserted to prevent exposure of the freeze-dried preparation to excessive heat during flame sealing.

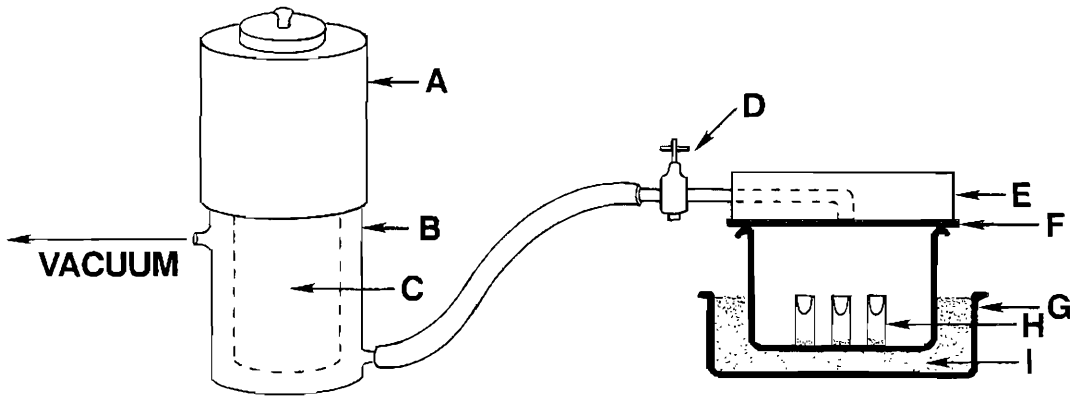
10. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.
11. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered. However, unsealed vials can be stored in the vapor phase of a liquid nitrogen freezer. Store directly after step 5 is completed. **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
12. To establish a culture from the freeze-dried state add 0.5 ml of sterile glass distilled H_2O or sterile fresh broth medium. After pellet dissolves dispense into fresh medium.
    For sensitive strains use the following protocol, a modification of a protocol used for bacteria sensitive to freeze-drying [2]:

    a) Place the freeze-dried vial and a fresh tube of modified medium (containing 12% (w/v) sucrose and with the pH adjusted to 7.0) in an ice bath.
    b) Aseptically add 0.5 ml of the cold modified medium to the freeze-dried vial and when the pellet is rehydrated transfer the contents from the vial to the tube of medium.
    c) Place the test tube at the appropriate temperature. It is critical to follow these instructions to obtain consistent recovery of sensitive strains from the freeze-dried state.

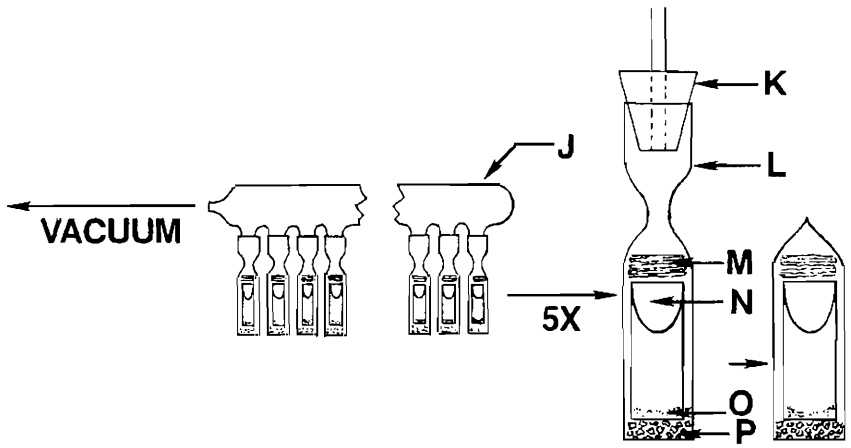
PROTOCOL

Drying [1]
(Fig. 3, sealing only)

1. Sterilize cotton-plugged 11.5 x 35.0 mm inner shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper. The mulched paper is prepared by soaking Whatman #1 filter in glass distilled H_2O and shredding in a Waring blender. Sufficient wet mulched paper is added to cover the bottom of the vials. The paper should not be packed. The vials are plugged with cotton, autoclaved for 20 min at 121° C, and then placed in a 70° C incubator until the mulched paper has dried (overnight is usually sufficient).
2. Harvest cells.
3. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
4. Dispense cells in 0.3 ml aliquots into the cotton-plugged sterile inner shell vials containing dried, mulched filter paper. Cut the tops of the cotton plugs off at the lip of the vial with a pair of scissors.
5. Place the ampules in a 25° C incubator for 14 d. After drying, place under vacuum in a desiccator for 7 d at room temperature (20-23° C). If preparations are to be stored as frozen preparations proceed to step 9.
6. In advance 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the inner shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.



FREEZE-DRYING



SEALING

Fig. 3. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

7. Allow outer shell vials to cool in a dry cabinet (10% or less relative humidity) and insert dried preparation. On top of the inner shell vial a wad of glass fiber paper is inserted to prevent exposure of the dried cells to excess heat during flame sealing.
8. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.
9. Sealed vials can be stored at room temperature but shelf-life of the preparation will be extended as the storage temperature is lowered. Unsealed vials can be stored in the vapor phase of a liquid nitrogen freezer. Store directly after step 5 is completed. **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
10. To establish a culture from the dried state aseptically add 0.5 ml of growth medium to each vial. For strains maintained on agar plates aseptically transfer the filter pellet to an agar plate and tease apart the pellet. Add the remainder of the liquid from the vial to the plate. For strains maintained in liquid culture incubate the vial in an upright position at 25°C. Monitor daily and when motile cells are observed, transfer the fluid from the vial to fresh medium and incubate upright under standard conditions. Handle routinely thereafter.

COMMENTS

Many protozoans that form cysts can be preserved by drying. However, not all cysts can be dried using the protocol given above. Cysts that cannot be dried may be amenable to freeze-drying.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.
2. Choate, RV & Alexander, MT 1967. The effect of rehydration temperature and rehydration medium on the viability of freeze-dried *Spirillum atlanticum*. *Cryobiol.* **3**:419-422.
3. Daggett, P-M & Nerad, TA 1982. Freeze-drying methods for amoeba and flagellates. *J. Protozool.* **29**:477.
4. ___ 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
5. Hwang, S-W & Horneland, W 1965. Survival of algal cultures after freezing by controlled and uncontrolled cooling. *Cryobiol.* 1: 305-311.
6. McGrath, MS, Daggett, P-M & Dilworth, S 1978. Freeze-Drying of algae: Chlorophyta and Chrysophyta. *J. Phycol.* **14**:521-525.
7. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525
8. Nerad, TA 1991. Chapter 10: Protozoa and Algae pp. 25-30. In: Simione FP & Brown, EM, ed. ATCC Preservation Methods: Freezing and Freeze-drying. 2nd edt. American Type Culture Collection. Rockville, MD 42 pp.
9. Reed, RB, Simione, FP, Jr & McGrath, MS 1976. Preservation of encysted *Polytomella*. *J. Gen. Microbiol.* **97**:29-34.
10. Simione, FP, Jr & Daggett, P-M 1976. Freeze-preservation of pathogenic and non-pathogenic *Naegleria* species. *J. Parasitol.* **62**:49.

11.___ 1977. Recovery of a marine dinoflagellate following controlled and uncontrolled freezing. *Cryobiol.* **14**:362-366.
12.___, McGrath, MS & Alexander, MT 1977. The use of plastic ampules for freeze preservation of microorganisms. *Cryobiol.* **14**:500-502.

A-57.1

CRYOPRESERVATION OF CRYPTOMONADS

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INTRODUCTION

Strains belonging to two genera of cryptomonads, *Chroomonas* and *Goniomonas*, are known to be amenable to cryopreservation. It is likely that other cryptomonads may be cryopreserved by the same or similar protocols.

PROTOCOL

Chroomonas

1. Harvest cells from a culture(s) that is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 24% (v/v) solution of sterile glycerol in fresh medium.
4. Mix the cells and the glycerol preparations in equal portions. The final concentration thus will be 10^7 cells/ml in 12% (v/v) glycerol after mixing. The time from the mixing of the cell preparation and glycerol solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. If the culture is not axenic add 0.1 ml of a Penicillin/Streptomycin Solution (see following) to inhibit growth of the bacteria.

Penicillin/Streptomycin Solution

Penicillin G (sodium)	10^6 Units
Streptomycin sulfate	10^6 μg
Glass distilled H_2O	100.0 ml

Dissolve components in distilled H_2O and then filter sterilize. Can be obtained from GIBCO (cat #600-5140).

10. Incubate the culture under an appropriate light/dark cycle and temperature. Thereafter, follow the protocol for routine maintenance.

PROTOCOL

Goniomonas

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium. Thereafter, follow the protocol for routine maintenance.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.

A-58.1

CRYOPRESERVATION OF DINOFLAGELLATES

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INTRODUCTION

Only a limited number of species of dinoflagellates have been successfully cryopreserved. Better success is achieved by using a controlled rate of cooling. Different degrees of survival occur, even within a genus, depending upon the cryoprotective agent [1] and the type of cooling rate [2] used. Methods applicable to *Amphidinium* and *Crypthecodinium* are provided. The methods may have application to other dinoflagellates. The first protocol is an uncontrolled cooling method which can be used for *Crypthecodinium* strains. The controlled cooling methods differ in rates of cooling. Both methods are provided here.

PROTOCOL

Uncontrolled Cooling, *Crypthecodinium*

1. Harvest cells from a culture which is at or near peak density by gentle centrifugation at 100 g for 5 min. For most strains the cell suspension can be used directly without centrifugation. Harvesting cells in as gentle a manner as possible will enhance recovery.
2. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile glycerol in fresh medium.
4. Mix the cell preparation and the glycerol solution in equal portions. Thus, the final concentration of the preparation will be 7.5% (v/v) glycerol and 10^6 cells/ml. The cooling cycle should be initiated no less 15 min and no longer than 60 min after addition of the glycerol to the cell suspension.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. After dispensing place ampules at the bottom of a mechanical freezer set at between -55 to -70° C for 1 h.
7. The frozen preparations may be stored in the mechanical freezer until needed or after I h the ampules are rapidly plunged into liquid nitrogen and stored in either the vapor or liquid phase of a nitrogen refrigerator. Store glass vials in vapor phase only.
8. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed to a level sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube. Incubate vertically at 25° C with the caps loosened one half turn. Thereafter follow routine protocol for cultivation.

A-58.2

PROTOCOL

Controlled Cooling, *Crypthecodinium*

1. Follow steps 1-5 in previous protocol.
2. For step 6 in the previous protocol substitute the following: Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C ampules are plunged into liquid nitrogen.
3. Store ampules in either the vapor or liquid phase of a nitrogen refrigerator. Store glass vials in vapor phase only.
4. Follow steps 8-9 of previous protocol.

PROTOCOL

Controlled Cooling, *Amphidinium*

1. Harvest cells from a culture which is at or near peak density. If the cell concentration in the culture vessel is sufficiently high (2 x 10^6 cells/ml), do not centrifuge, otherwise adjust concentration by centrifuging at 200 g for 2 min. Adjust concentration by addition of fresh medium.
2. While cells are centrifuging prepare a 18% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
3. Mix the cell preparation and the DMSO solution in equal portions. Thus, the final concentration of the preparation will be 9% (v/v) DMSO and 10^6cells/ml. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
4. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -50° C, then plunge vials into liquid nitrogen.
6. Store ampules in either the vapor or liquid phase of a nitrogen refrigerator. Store glass vials in vapor phase only.
7. Follow steps 8-9 of first protocol.

COMMENTS

Recovery of *Crypthecodinium* is generally best with glycerol [1].

LITERATURE CITED

1. Simione, FP, Jr & Daggett, P-M 1977. Recovery of a marine dinoflagellate following controlled and uncontrolled freezing. *Cryobiol.* **14**:362-366.
2. Daggett, P-M & Nerad, T 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

A-59.1

CRYOPRESERVATION OF EUGLENIDS

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INTRODUCTION

Only by using controlled rates of cooling have euglenids been successfully cryopreserved. Methods applicable to *Astasia*, *Entosiphon*, *Euglena* and *Khawkinea* are provided. The methods may be applicable to other euglenids. There are several methods which can be used for *Euglena* [1]. The best current method, using methanol as the cryoprotective agent, is detailed.

PROTOCOL

Astasia & *Khawkinea*

1. Harvest cells from a culture which is at or near peak density by centrifugation at 200 g at 2 min.
2. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. After mixing the final concentration will be 10^6 cells/ml and 5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. One of two cooling cycles can be used. 1) From room temperature cool at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, begin and maintain a rate at -1° C/min through heat of fusion. At -40° C plunge vials into liquid nitrogen. 2) From room temperature cool at -1° C/min. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. At -40° C plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase, if screw-capped cryules, of a nitrogen refrigerator. Store glass vials in vapor phase only.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the vial to a level just sufficient to cover the frozen material. Do not agitate.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of fresh medium.

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PROTOCOL
Entosiphon

1. Harvest cells from a culture which is at or near peak density by centrifugation at 200 g for 2 min.
2. Adjust concentration of cells for 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 25% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. After mixing the final concentration will be 10_6 cells/ml and 12.5% (v/v) DMSO. The time from mixing of the cell preparation and the DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. Follow steps 7-9 of protocol from the previous protocol.

PROTOCOL
Euglena

1. Harvest cells from a culture which is at or near peak density by centrifugation at 200 g for 2 min.
2. Adjust concentration of cells for 2 x 10^6/ml in fresh medium.
3. Will cells are centrifuging prepare a 6% (v/v) solution of methanol in fresh medium.
4. Mix the cell preparation and the methanol solution in equal portions. After mixing the final concentration will be 10^6 cells/ml and 3% (v/v) methanol. The time from mixing the cell preparation and the methanol solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. Follow steps 7-9 from *Astasia* protocol.

COMMENTS

Glycerol has been used at a concentration of 7.5% (v/v) for the cryopreservation of *Euglena* [1]. The cells are initially nonmotile for 3-6 d after thawing and recoveries are much lower than those obtained by using methanol.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I.P. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae, Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.

A-60.1

CRYOPRESERVATION OF *ISONEMA* AND RELATED FLAGELLATES

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INTRODUCTION

Axenic and bacterized strains of *Diplonema*, *Isonema*, and *Rhynchopus* are readily amenable to cryopreservation [1].

PROTOCOL

Uncontrolled Cooling

1. Cultures are grown in T-25 tissue culture flasks.
2. Strains that attach to the vessel can be harvested as follows: Remove all but approximately 1.0 ml of the culture fluid without agitating and then vigorously agitate to detach the cells. For strains which do not have an attached gliding stage in their life cycle distribute the cell suspensions to 15 ml centrifuge tubes and centrifuge at 300 g for 5 min.
3. Adjust concentration of cells to 2.0 x 10^6 - 10^7 cells/ml. For strains which have attached cells this can usually be achieved without centrifugation.
4. Prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The sterile DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
5. Mix the concentrated cell suspension and the DMSO in equal portions. The final concentration will be 10^6 - 10^7 cell/ml and 10.0% (v/v) DMSO. The time from mixing of the cell preparation and the DMSO solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
8. Store ampules in the vapor or liquid phase of a nitrogen refrigerator.
9. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed to a level sufficient to cover the frozen material. Do not agitate the vial.
10. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 10.0 ml of fresh medium in a T-25 flask. Incubate at 25° C. Thereafter, follow routine cultivation protocol.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Nerad, TA 1990. The Life History, Cytology and Taxonomy of *Isonema* and *Isonema*-like Flagellates. Ph.D. Thesis, University of MD, College Park, MD, 229 pp.

CRYOPRESERVATION OF BICOSOECIDS

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INTRODUCTION

Strains belonging to the two bicosoecid genera *Bicosoeca* and *Pseudobodo* are known to be amenable to a method of cryopreservation. A third genus recently described, *Cafeteria*, requires another, but similar method. Other similar flagellates perhaps could be cryopreserved by the same or similar protocols.

PROTOCOL

Bicosoeca & *Pseudobodo*

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 7.5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

A-61.2

PROTOCOL

Cafeteria

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells for 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

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CRYOPRESERVATION OF CHRYSOMONADS

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INTRODUCTION

Chrysomonads have been successfully cryopreserved only by using controlled rates of cooling. Methods applicable to *Ochromonas danica* [2] and *Poteriochromonas malhamensis* (*O. malhamensis*) [1] are provided. The methods may be applicable to other photosynthetic chrysomonads, particularly the method for the latter species using methanol as the cryoprotective agent.

PROTOCOL

Ochromonas danica

1. Harvest cells from a culture which is at or near peak density by centrifugation at 200 g for 5 min. If the density is sufficiently high (see Step 2), it may not be necessary to concentrate cells by centrifugation. Handling of cells as gently as possible prior to freezing will enhance recovery.
2. Adjust concentration of cells to 2 x 10^7/ml with fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. Thus, the final concentration will be 10^7 cells/ml and 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials.
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min from 25° C to -40° C. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. At -40° C plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Do not completely immerse the vial. It should be in the H_2O sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium. Incubate using routine protocol for cultivation.

A-62.2

PROTOCOL

Poteriochromonas malhamensis (*O. malhamensis*)

1. A near peak density test tube culture is used. Cells are not concentrated further.
2. Prepare a 20% (v/v) methanol solution in fresh medium.
3. Mix the culture and the methanol solution previously prepared in equal portions. The final concentration thus will be a cell preparation in 10% (v/v) methanol. The time from mixing of the cell preparation and the methanol solution before the freezing press is begun should be no less than 15 min and no longer than 60 min.
4. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
6. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
7. To establish a culture aseptically add 0.5 ml of fresh medium to the ampule before placing it in a 35° C water bath. Transfer thawed contents to a 16 x 125 mm screw-capped test tube containing 4.0 ml of fresh medium. Allow to stand for 30 min at room temperature (20-23° C). At the end of 30 min agitate gently by inversion several times and transfer 0.5 ml to a fresh test tube with 4.5 ml of medium. Incubate both cultures using normal protocol. In most cases only the diluted material will produce a culture. Thereafter follow routine cultivation protocol.

COMMENTS

Motile cells are not immediately evident in cultures initiated from thawed material. *Ochromonas danica* cells are not motile for 7-14 d and those of *Poteriochromonas malhamensis* for 2-7 d. *Ochromonas danica* and *P. malhamensis* cultures established from cryopreserved material retain ability to act as bioassay organisms for the determination of thiamin [2] and vitamin B_{12} respectively (unpublished observations). Although methanol has not been used to date as a cryoprotectant for *O. danica* recovery may be higher using this cryoprotective agent rather than DMSO. A concentration of 10% (v/v) DMSO was ineffective as a cryoprotectant for *P. malhamensis* (unpublished observations). A 10-fold dilution of the initial test tube culture established is very beneficial for *P. malhamensis*. In fact, in most cases a culture will be established only in the diluted tube. This type of treatment after thawing may be beneficial for other sensitive chrysomonads.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S, Simione, FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae, Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.
2. McGrath, MS & Daggett, P-M 1977. Cryopreservation of *Ochromonas* in liquid nitrogen with reproductivity of thiamin assay. *J. Protozool.* **26**:280-281.

CRYOPRESERVATION OF PRYMNESIIDS

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INTRODUCTION

Pavlova is amenable to one of the simplest methods for cryopreservation of protists. Whether the method can be applied [1] to other prymnesiids is unknown, but success with the one genus would suggest others would also be readily cryopreserved by the same or similar protocols [1].

PROTOCOL

1. Harvest cells from a culture which is at or near peak density by centrifugation at 1,300 g for 5 min.
2. Adjust concentration of cells for 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 24% (v/v) solution of sterile glycerol in fresh medium.
4. Mix the cells and the glycerol preparations in equal portions. The final concentration thus will be 10^7 cells/ml in 12% (v/v) glycerol after mixing. The time from the mixing of the cell preparation and glycerol solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. If the culture is not axenic add 0.1 ml of a Penicillin/Streptomycin Solution (see following) to inhibit growth of the bacteria.

Penicillin/Streptomycin Solution

Penicillin G (sodium)	10^6	Units
Streptomycin sulfate	10^6	μg
Glass distilled H_2O	100.0	ml

Dissolve components in glass distilled H_2O and then filter sterilize. The solution can be obtained from GIBCO (cat. #600-5140).

10. Incubate the culture under an appropriate light/dark cycle and temperature. Thereafter, follow the protocol for routine maintenance.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae, Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.

A-64.1

LONG TERM MAINTENANCE OF VOLVOCIDS BY DRYING AND CRYOPRESERVATION

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INTRODUCTION

Several genera are known to be easily maintained for long periods of time by cryopreservation. In the case of two genera, *Haematococcus* and *Polytomella*, the approach is to use the cyst stage in the life history which permits storage as either dried or frozen preparations [2,3]. Essential differences in the methods employed for other genera are cooling rates, type of cryoprotective agent, concentration of cryoprotective agent, and method of recovery. More than one method may be applicable. Several methods are provided here in detail. Although there are minor variations it is essential for successful results they be followed.

PROTOCOL

Uncontrolled Cooling *Lobomonas*

1. Harvest cells from a culture at or near peak density. For broth cultures centrifuge at 300 g for 5 min. For agar slant cultures 3.0 ml of broth medium containing the final concentration of cryoprotective agent is added to each slant and the cells are washed into suspension. (For agar cultures review next two steps before adding solution to cultures.)
2. For broth cultures adjust final concentration of cells to 2 x 10^6/ml. For agar cultures, adjust to 10^6/ml by adding fresh cryoprotective solution.
3. For use with broth cultures prepare a 14% (v/v) solution of sterile DMSO in fresh medium. For use with agar slant cultures a 7% (v/v) sterile DMSO solution is prepared in fresh broth medium. In both cases the DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. For broth cultures mix the cell preparation and the DMSO solution in equal portions. When the two solutions are mixed the final concentrations will be 10^6 cells/ml in a 7% DMSO solution. *For slant cultures this step is not done.*
5. Dispense cell suspensions in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation). The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should be no less than 15 min and no longer than 60 min.
6. After dispensing place ampules at the bottom of a mechanical freezer set between -55 to -70° C for 1 h.

7. The frozen preparations may be stored in the mechanical freezer until needed or after I h the ampules are rapidly plunged into liquid nitrogen and then stored in either the vapor or liquid phase of a nitrogen refrigerator. Store glass ampoules in vapor phase only.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate the vial while thawing.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of the appropriate broth or dispense the contents evenly over the surface of the appropriate agar medium in a 15 x 100 mm petri dish. Incubate using the protocol for routine cultivation.

PROTOCOL

Controlled Cooling *Brachiomonas, Haematococcus, Lobomonas* & *Polytomella*

1. Follow steps 1-5 of the first protocol except for *Polytomella* substitute 20% or 10% (v/v) glycerol respectively.
2. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -55° C. If the freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. At -55° C plunge vials into liquid nitrogen.
3. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
4. Follow steps 8 & 9 of first protocol.

PROTOCOL

Controlled Cooling, *Chlorogonium, Dunaliella Polytoma* & *Polytomella*

1. Follow steps 1-5 of first protocol substituting 22% or 11% (v/v) DMSO respectively for some strains *Dunaliella* or 20% or 10% (v/v) methanol respectively for *Polytoma* and some strains of *Dunaliella* or 10% or 5% DMSO respectively for *Chlorogonium*, and *Polytomella* in step 3.
2. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a cooling rate of -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge cryules into liquid nitrogen.
3. A frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
4. Follow steps 8 & 9 of first protocol.

PROTOCOL

Controlled Cooling, *Dunaliella* & *Polytoma*

1. Follow completely the previous protocol substituting 20% (v/v) methanol for *Dunaliella* and 10% (v/v) methanol for *Polytoma.*

A-64.3

PROTOCOL

Drying, *Haematococcus* & *Polytomella*
(Fig. 1, sealing only)

1. Sterilize cotton-plugged 11.5 x 35.0 mm inner shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper. The mulched paper is prepared by soaking Whatman #1 filter in glass distilled H_2O and shredding in a Waring blender. Sufficient wet mulched paper is added to cover the bottom of the vials. The paper should not be packed. The vials are plugged with cotton, autoclaved for 20 min at 121° C, and then placed in a 70° C incubator until the mulched paper has dried (overnight is usually sufficient).
2. Harvest cells by centrifugation at 300 g for 5 min.
3. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
4. Dispense cells in 0.3 ml aliquots into the cotton-plugged sterile inner shell vials containing dried, mulched filter paper. Cut the tops of the cotton plugs off at the lip of the vial with a pair of scissors.
5. Place the ampules in a 25° C incubator for 14 d. After drying, place under vacuum in a desiccator for 7 d at room temperature (20-23° C). If preparations are to be stored as frozen preparations proceed to step 9.
6. In advance 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the inner shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
7. Allow outer shell vials to cool in a dry cabinet (10% or less relative humidity) and insert dried preparation. On top of the inner shell vial a wad of glass fiber paper is inserted to prevent exposure of the dried cells to excess heat during flame sealing.
8. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.
9. Sealed vials can be stored at room temperature but shelf-life of the preparation will be extended as the storage temperature is lowered. However, unsealed vials can be stored in the vapor phase of a liquid nitrogen freezer. Store directly after step 5 is completed. **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
10. To establish a culture from the dried state aseptically add 0.5 ml and incubate upright under standard conditions. Observe daily for motile cells and when observed, subculture into 5.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube. Handle routinely thereafter.

COMMENTS

Recovery will be greater using a controlled cooling rate [1]. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

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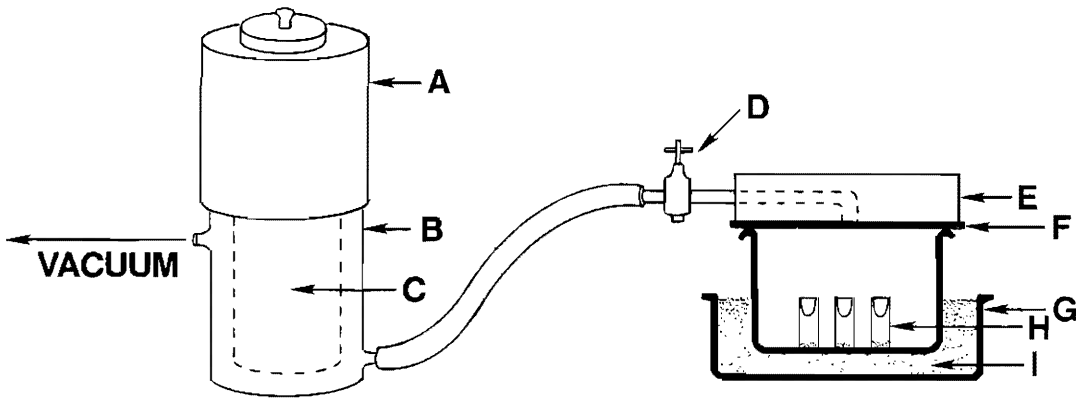
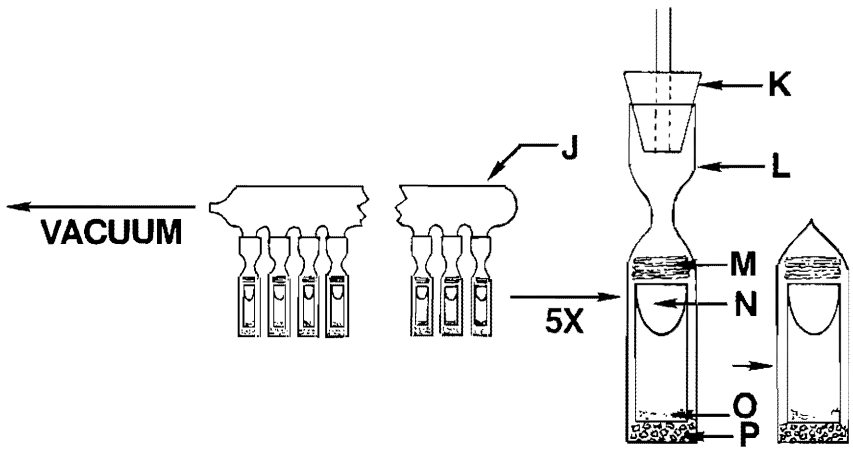


FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rat for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
2. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525.
3. Reed, RF, Simione FP, Jr & McGrath, MS 1976. Preservation of encysted *Polytomella*. *J. Gen. Microbiol.* **97**:29 -34.

LONG TERM MAINTENANCE OF *CHLAMYDOMONAS* BY CRYOPRESERVATION AND FREEZE-DRYING

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INTRODUCTION

Many species can easily be maintained for long periods of time by cryopreservation. Mutant strains retain marker traits. Methods employing either uncontrolled or controlled cooling rates can be used [1,2]. Both methods are provided here in detail with minor variations which may be more suitable for some strains. Many strains may also be preserved using freeze-drying methods [3]. Recovery is much higher when strains are cryopreserved but freeze-dried preparations can be stored at much higher temperature and can be conveniently distributed in this configuration.

PROTOCOL

Cryopreservation, Uncontrolled Cooling

1. Harvest cells from a culture in late logarithmic to early stationary phase of growth. For broth cultures use centrifugation at 300 g for 5 min. For agar slant cultures the cells are washed into suspension by adding broth medium containing the final concentration of cryoprotective agent to each slant.
2. For broth cultures adjust concentration of cells to 2 x 10^6/ml. For agar slant cultures adjust cell concentration to 10^6/ml.
3. For use with broth cultures prepare a 10% (v/v) solution of sterile DMSO in fresh medium. For use with agar slant cultures a 5% (v/v) DMSO solution is prepared in a fresh broth medium. In both cases the DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. For broth cultures mix the cell preparation and the DMSO solution in equal portions. When the two solutions are mixed the final concentrations will be 10^6 cells/ml in a 5% DMSO solution. *For slant cultures this step is not done.*
5. Dispense cell suspension in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation). The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should be no less than 15 min and no longer than 60 min.
6. After dispensing place ampules at the bottom of a mechanical freezer set between -55 to -70° C for 1 h.
7. The frozen preparations may be stored in the mechanical freezer (-55 to -70° C) until needed or after 1 h the ampules are then rapidly plunged into liquid nitrogen and stored in either the vapor or liquid phase, if screw-capped cryules of a nitrogen refrigerator.

8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate while thawing.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of the appropriate broth in a 16 x 125 mm screw-capped test tube or dispense the contents evenly over the surface of the appropriate agar medium in a 15 x 100 mm petri dish. Incubate and thereafter use the protocol for routine cultivation.

PROTOCOL

Cryopreservation, Controlled Cooling

1. Follow steps 1-5 of the previous protocol.
2. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -55° C. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. At -55° C plunge vials into liquid nitrogen. Or cool at -10° C/min from room temperature to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
3. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
4. Follow steps 8 & 9 of the previous protocol.

PROTOCOL

Freeze-drying, Uncontrolled Cooling,
Single Vial Manifold Method
(Fig. 1)

1. Prepare in advance a sterile 12% (w/v) sucrose solution in glass distilled H_2O.
2. Harvest cells from a culture which is in late logarithmic to early stationary phase of growth. For broth cultures concentrate cells by centrifuging at 200 g for 5 min. Resuspend cell pellet in 5.0 ml of broth and determine cell concentration. Adjust concentration to 10^8 cells/ml with fresh broth. Note the volume and centrifuge as above. Discard supernatant and replace with an equal volume of 12% sucrose solution. For agar slant or plate cultures suspend cells directly in the 12% sucrose solution. Adjust concentration to 10^8/ml with the 12% sucrose solution.
3. Dispense cell suspension in 0.1 ml aliquots into sterile glass cotton-plugged 1.0 ml bulb type (teardrop) freeze-drying ampules.
4. Just prior to freeze-drying a moisture trap (condenser) attached to a vacuum pump is prepared by placing a trimmed block of dry-ice which just fits into a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added just to the top of the condenser containing the dry-ice. A manifold suspended above a dry-ice Cellosolve bath is attached to the trap by Tygon tubing.
5. The ampules are quick frozen by swirling when the vials are rapidly immersed in the bath. While still in the bath each vial is attached by Tygon tubing to the manifold. The bulb portion should be fully immersed in the bath. After all of the ampules have been attached the vacuum is turned on. The vacuum should be pumped down to at least 30 μm of Hg.
6. Keep under vacuum for at least 12 h. All the dry-ice cellosolve bath will return to room temperature during the 12 h period.
7. While still under vacuum raise the ampules from the bath. Dry the bulb portion of the vials.

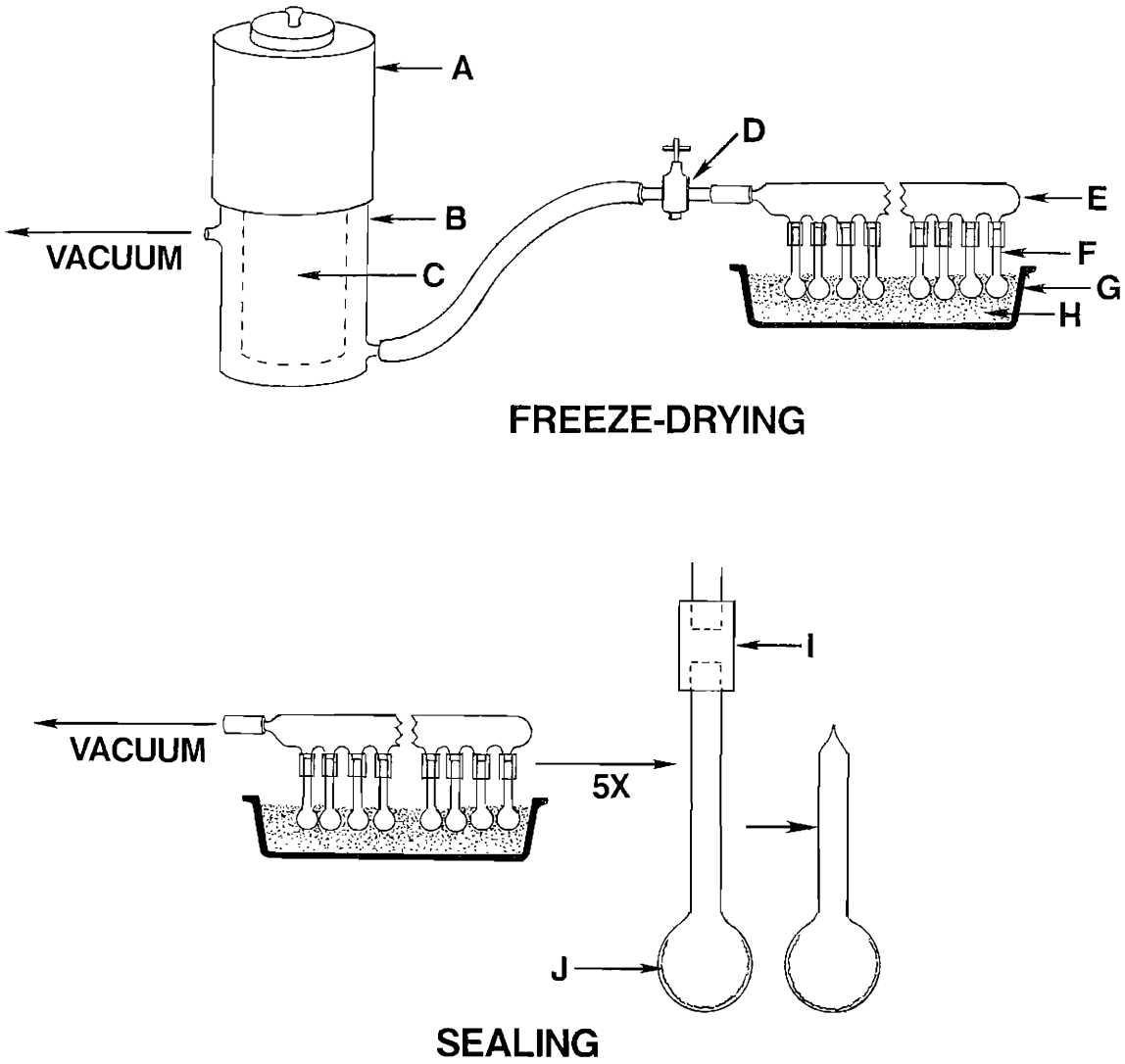


Fig. 1. Manifold method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) manifold; (F) teardrop ampule*; (G) stainless steel pan (H) dry-ice Cellosolve bath; (I) rubber sleeve connecting vial to manifold; (J) thin film of freeze-dried cell suspension.

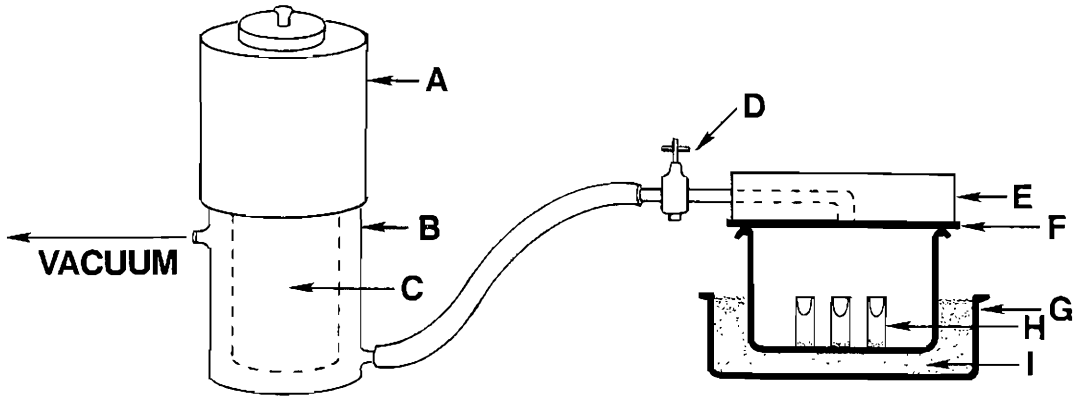
*The teardrop ampule can be custom made by Kontes Glass Co. The outer diameter of the neck is 8 mm and the inner diameter is 6 mm. The neck length is 125 mm. The diameter of the bulb is 15 mm.

8. Seal under 30 μm of Hg. At the point at which the neck of the ampule is attached to the tubing heat the glass with the hottest part of an air/gas flame until red hot. While slowly pulling the vial the narrowing neck is separated and sealed.
9. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered.
10. To establish a culture from the dried state aseptically add 0.1 ml of sterile glass distilled H_2O or sterile fresh broth. After the pellet dissolves dispense into 5.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube. Incubate using the protocol for routine cultivation.

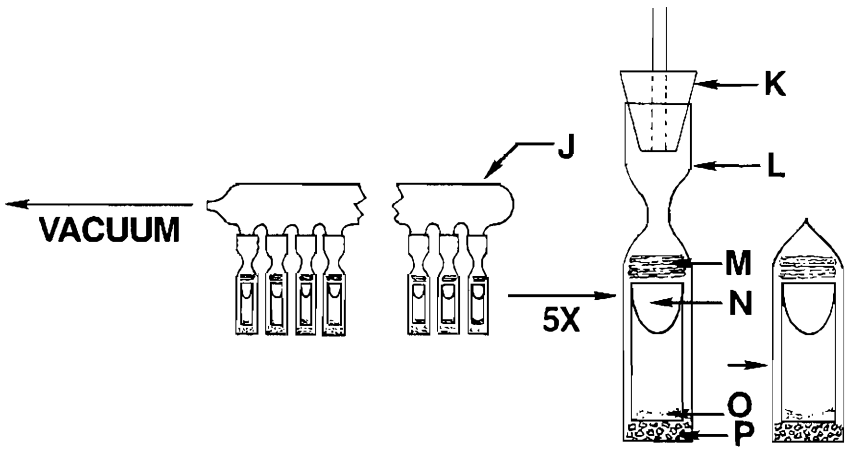
PROTOCOL

Freeze-drying, Uncontrolled Cooling,
Double Vial Method
(Fig. 2)

1. Prepare in advance a sterile 20% (w/v) suspension of skim milk (Difco #0032) in glass distilled H_2O.
2. Harvest cells from a culture which is in late logarithmic to early stationary phase of growth. For broth cultures concentrate the cells by centrifuging at 200 g for 5 min. Resuspend and pool the cell pellets to the desired volume with fresh broth medium. For agar slant or plate cultures add 3.0 ml of broth and wash cells into suspension.
3. For both broth and agar slant cultures adjust concentration of cells to 10^8/ml concentrate. Adjust concentration of cells from agar cultures with the protective solution but for broth cultures centrifuge as above and resuspend pellet with a necessary volume of protective solution to bring the concentration of cells to the desired level.
4. Dispense the cell suspension in 0.5 ml aliquots into sterile glass cotton-plugged 11.5 x 35.0 mm inner shell freeze-drying glass ampules (Glass Vials, Inc.).
5. Place ampules in a stainless steel container and place the container onto the bottom of a mechanical freezer set between -55 to -70° C for 1 h.
6. Just prior to freeze-drying a moisture trap condenser attached to a vacuum pump is prepared by fitting a trimmed block of dry-ice which just fits inside a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added just to the top of the condenser containing the dry-ice.
7. Place container with frozen ampules on a 2.54 cm thick layer of freshly prepared dry-ice snow. Pack snow around sides of container for several h. Place the Atmo-vac plate cover (Refrigeration for Sciences Inc.), over the pan with the ampules and place under vacuum of at least 30 μm of Hg. The cover is attached to the condenser by Tygon tubing which will not collapse. Keep under vacuum for at least 12 h. All the dry-ice snow will have evaporated. Turn off the vacuum pump and slowly introduce air which has been previously passed over a desiccant to minimize introduction of moisture.
8. In advance the 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
9. Allow outer shell vials to cool in a dry cabinet (10% or least relative humidity) and then insert inner shell vial with the freeze-dried preparation. On top of the inner vial a wad of glass fiber paper is inserted to prevent exposure of the freeze-dried preparation to excessive heat during flame sealing.
10. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair



FREEZE-DRYING



SEALING

Fig. 2. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

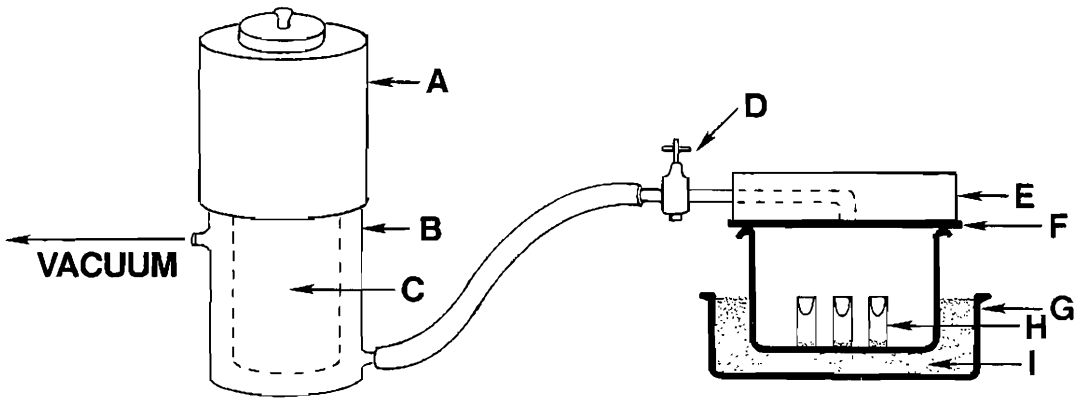
of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.

11. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered. However, unsealed vials can be stored in the vapor phase of a liquid nitrogen freezer. Store directly after step 5 is completed. **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
12. To establish a culture from the freeze-dried state add 0.5 ml of sterile glass distilled H_2O or sterile fresh broth medium. After pellet dissolves dispense into 5.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube.

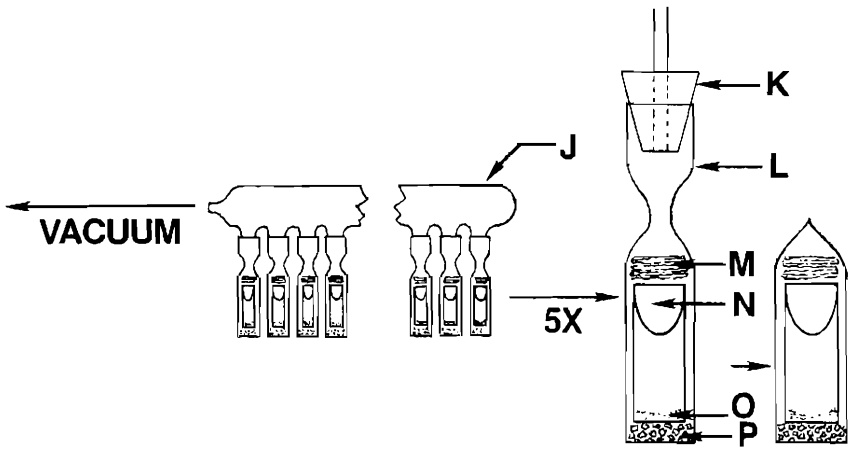
PROTOCOL

Freeze-Drying, Controlled Cooling, Double Vial Method
(Fig. 3, sealing only)

1. Prepare in advance a sterile 12% (w/v) sucrose plus 4% (w/v) BSA (Bovine Serum Album Fraction V - Sigma A-4503) solution in the appropriate medium.
2. Prepare outer vials (Glass Vials, Inc., 14.25 mm x 85.0 mm) by placing a small amount of silica gel granules (Fisher Scientific, grade 42, 6-16 mesh, "Tel-Tale" brand) in the vial to cover about half of the bottom. Add a small cotton wad to cushion the inner vial, and heat at 100° C overnight. The silica gel should be dark blue after heating and serves as a moisture indicator during storage. Place vials in a dry box (<10% relative humidity) to cool.
3. Harvest cells from a culture in logarithmic to early stationary phase of growth. For broth cultures concentrate cells by centrifugation at 200 g for 5 min. Resuspend cell pellet to a final volume of 5.0 ml with fresh broth and determine cell concentration. Adjust concentration to 10^8/ml with fresh medium.
4. Dispense cell suspension in 0.25 ml aliquots cells in cotton-plugged inner shell vials. Cut off portion of cotton plugs above lip of vial with a pair of scissors.
5. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -55° C. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. At -55° C plunge vials into liquid nitrogen. Or cool at -10° C/min from room temperature to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
6. Transfer ampules from liquid nitrogen to a slotted tray of a commercial freeze-dryer that has been equilibrated to a shelf temperature of -40° C. Proceed quickly to avoid unnecessary warming of the frozen preparations. If ampules must be stored, place in the vapor phase of a nitrogen refrigerator until ready for transfer to the commercial freeze-dryer.
7. Dispense 0.25 ml of suspending medium only (no cells) into two inner shell vials and place them in slots at the end of the tray on the shelf nearest the door of the freeze-dryer. Insert thermocouple probes.
8. Plug the thermocouple probes into the jacks on the inside of the chamber. Allow the vials containing the thermocouple probes to cool to -40° C.
9. Raise the shelf temperature to -30° C and freeze dry at that temperature for at least 18 h. Then increase the shelf temperature in 10° C increments, holding at each temperature at least one h. This can be done manually or with a programmer.
10. Record the condenser, shelf and specimen temperatures and the vacuum gauge readings throughout the cycle.



FREEZE-DRYING



SEALING

Fig. 3. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

11. When the shelf temperature reaches 0° C, hold at that temperature overnight. Adjust shelf temperature to 37° C the next morning. Product temperature must be at 25° C before the cycle is complete. If necessary, air injection can be used to elevate the pressure to improve heat input to the product and accelerate the drying. Turn off the programmer, turn off the shelf heat and bleed short bursts of air through a Pall filter (Pall Corp.) until the product temperature is approximately 25° C.
12. Turn off the vacuum pump. Using a nitrogen gas cylinder that has been stored at 2-8° C, back fill the chamber with nitrogen gas through a 0.22 μm Pall filter. Do not allow the condenser temperature to go above -50° C. It may be necessary to run the nitrogen gas through a copper coil immersed in liquid nitrogen to maintain condenser temperature.
13. Remove the inner vials from the freeze-dryer to a dry box, where the outer vials are cooling. Insert the inner vials into the outer vials in the dry cabinet. Tamp a ¼-inch plug of glass fiber paper (Whatman #1821-915) above the cotton-plugged inner vial.
14. Remove the vials from the dry cabinet. Heat the outer vial in an air-gas rotating the vial and keeping the flame just above the glass fiber paper until the glass begins to constrict. Pull the top of the vial slowly with forceps until the constriction is a narrow capillary tube. Cool the vials in a dry cabinet.
15. Attach each vial to a port of a manifold. Each port has a single-holed rubber stopper which fits the open end of the vial. Evacuate the system to less than 50 μm.
16. Seal the vials at the capillary using a double flame air-gas torch.
17. Vials are tested with a high-induction spark to indicate the absence of air. The tip of the spark tester (Electro-Technic Products) is held close to the top of the outer vial. If the vacuum is intact, the inside of the vial will glow purple.
18. Store vials at 2-8° C. To open vials, heat the top of the outer vial in a flame, then squirt a few drops of H_2O on the hot top to crack the glass. Strike with a file or pencil to remove the top. Remove the fiber paper insulation and the inner vial. Use forceps to gently remove the cotton plug and rehydrate with 0.3-0.4 ml of appropriate broth medium. When resuspended, transfer the contents to 5 ml of broth.

COMMENTS

Recovery will be greater using a controlled cooling rate [1]. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). Glycerol can be used in place of DMSO, but usually recoveries are better with DMSO. For further details on the freeze-drying procedures for double vials consult [4] and for single vials consult [5]. Avoid frost-free freezers for long term storage of freeze-dried preparations. The temperature cycling of the units will shorten their shelf life. Better growth is often obtained when the recovered cell suspension is placed on the surface of an agar plate rather than in broth.

LITERATURE CITED

1. McGrath, MS & Daggett, P-M 1977. First successful preservation of several algal genera. *USFCC Newsletter* **7**:12-14.
2. ___ 1977. Cryopreservation of flagellar mutants of *Chlamydomonas reinhardtii*. *Can. J. Bot.* **55**:1794-1796.
3. ___ & Dilworth, S 1978. Freeze-drying of algae: Chlorophyta and Chrysophyta. *J. Phycol.* **14**:521-525.
4. Weiss, FA. 1957 Maintenance and preservation of cultures. In: Manual of Microbiological Methods. Conn, HJ, ed., McGraw-Hill, Inc., New York, pp. 99-119.
5. Choate, RV & Alexander, MT 1967. The effect of rehydration temperature and rehydration medium on the viability of freeze-dried *Spirillum atlanticum*. *Cryobiol.* **3**:419-422.

CRYOPRESERVATION OF CHOANOFLAGELLATES

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INTRODUCTION

Several genera of choanoflagellates have proven to be amenable to long term maintenance by cryopreservation. There is no universal method, even though there are shared aspects to the protocols detailed here. The differences are critical to success for a particular genus. Which methodology is most likely applicable to other choanoflagellate genera is difficult to predict. For practical reasons it is recommended to first try the simplest method. Methods for the genera *Acanthoecopsis*, *Diaphanoeca*, *Monosiga*, and *Salpingoeca* are detailed here.

PROTOCOL

Acanthoecopsis

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile glycerol in fresh medium.
4. Mix the cell and glycerol preparations in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 7.5% (v/v) glycerol. The time from the mixing of the cell preparation and glycerol stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into fresh medium. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

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PROTOCOL

Diaphanoeca

1. Harvest cells from a culture which is at or near peak density. Use a rubber policemen or a spatula to detach cells. Gently centrifuge the cell suspension at 200 g for 2 min.
2. Adjust concentration of cells for 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. Chill the DMSO preparation to 4° C prior to use.
4. Mix the cell preparation and the DMSO solution in three equal portions at two min intervals. All manipulations should be at 4° C. Either a cold block or crushed ice in a container can be used to keep the preparations cool. The final concentration of the preparation will thus be 10^7 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From 4° C cool at -1° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state aseptically add 0.5 ml of fresh medium into ampule over the frozen material and place into a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. In this case *gently* agitate the vial while thawing to minimize the possibility of thermal shock.
9. Immediately after thawing, do not leave in the water bath, quickly, aseptically remove the contents of the ampule and place into 9.5 ml fresh medium in a T-25 tissue culture flask. Incubate the culture between 5-9° C. Once the culture has been established follow the protocol for routine maintenance.

PROTOCOL

Monosiga & *Salpingoeca*

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells for 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 7.5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.

8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into fresh medium. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

A-67.1

CRYOPRESERVATION OF *PROTEROMONAS*

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INTRODUCTION

Proteromonas is readily amenable to cryopreservation. There are two simple methods which can be used. Either an uncontrolled or a controlled cooling rate can be used [1,2]. Both methods are provided here.

PROTOCOL

Uncontrolled Cooling

1. Harvest cells from a culture which is at or near peak density by centrifugation at 1,300 g for 5 min.
2. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. Thus, the final concentration of the preparation will equal 5% (v/v) DMSO and 10^6 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after the addition of DMSO to the cell suspension.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or plastic screw-capped cryules (special plastic vials for cryopreservation).
6. After dispensing place ampules at the bottom of a mechanical freezer set between -55 to -70° C freezer for 1 h.
7. The ampules are then rapidly plunged into liquid nitrogen. Storage is in either the vapor or liquid phase of a nitrogen refrigerator. Ampules may be stored in a mechanical freezer set at -55° C or lower. Viability is dependent upon storage temperature. The colder the temperature the longer the stability.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the content of the ampule and inoculate into 9.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube.

A-67.2

PROTOCOL

Controlled Cooling

1. Follow steps 1-5 in previous protocol.
2. Place ampules in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
3. Follow steps 8 and 9 of first protocol.

COMMENTS

Recovery will be greater using a controlled cooling rate. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
2. Simione, FP, Jr & Daggett, P-M 1976. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. *J. Parasitol.* **62**:49.

A-68.1

CRYOPRESERVATION OF TRICHOMONADS

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INTRODUCTION

Trichomonad species belonging to the genera, *Hypotrichomonas*, *Monocercomonas*, *Pentatrichomonas*, *Tetratrichomonas*, *Trichomitus*, *Trichomonas* and *Tritrichomonas* can easily be maintained for long periods of time by cryopreservation. Species of *Dientamoeba* are preserved by another method (See protocol on cryopreservation of *Entamoeba* and *Dientamoeba*). One method requires the use of an ultra-low mechanical refrigerator and the other requires specialized equipment for cryopreservation of cells [1]. Both methods are detailed.

PROTOCOL

Uncontrolled Cooling

1. Harvest cells from a culture which is at or near peak density by centrifugation at 1300 g for 5 min. The cells grown in a medium containing agar are concentrated by centrifugation a solid pellet does not form. The soft pellet is resuspended to desired cell concentration with agar-free supernatant.
2. Adjust concentration of cells to 2 x 10^6 - 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. Thus, the final concentration will be 10^6 - 10^7 and 5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. After dispensing place ampules at the bottom of a mechanical freezer cooled to between -55 to -70° C for 1 h.
7. The frozen preparations may be stored in the mechanical freezer set at -55 to -70° C or rapidly plunged into liquid nitrogen and stored in either the vapor or liquid phase of a nitrogen refrigerator. Frozen preparations stored below -130° C are stabile indefinitely. Those stored at temperatures above -130° C are progressively less stabile as the storage temperature is elevated. **Vials should not be stored above -55° C.**
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the vial just sufficient to cover the frozen material. Do not agitate the vial.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 9.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube.

PROTOCOL

Controlled Cooling

1. Follow steps 1-5 in previous protocol.
2. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge ampules into liquid nitrogen.
3. Follow steps 7-9 of the previous protocol.

COMMENTS

Recovery from the frozen state will be higher using controlled versus uncontrolled cooling [2]. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). The protocols listed have not been optimized, but give consistent high recoveries which allow establishment of cultures from the frozen state.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae, Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.
2. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

CRYOPRESERVATION OF *GIARDIA*

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INTRODUCTION

Giardia is readily amenable to cryopreservation. There are two simple methods which can be used. Either an uncontrolled or a controlled cooling rate can be used [1,2]. Both methods are provided here.

PROTOCOL

Uncontrolled Cooling

1. Harvest cells from a culture which is at or near peak density. To detach cells from the wall of the culture tubes place in crushed ice or a chill block for 10 min. Invert tubes several times until the majority of the cells are in suspension. Centrifuge tubes at 1,300 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. Before centrifuging prepare a 24% (v/v) solution of sterile DMSO in fresh medium modified to contain 8% (w/v) sucrose. The solution is prepared as follows:

    a) Add sucrose to fresh medium and filter sterilize through a 0.2 μm filter.
    b) Add 2.4 ml of DMSO to a 20 x 150 mm screw-capped test tube which has been in an ice bath for 10 min.
    c) Allow the DMSO to solidify (~5 min) and then add 7.6 ml of ice cold medium containing sucrose
    d) Invert several times to dissolve DMSO
    e) Allow to warm to room temperature

4. Mix the cell preparation and the DMSO in equal portions. Thus, the final concentration of the preparation will equal 12% (v/v) DMSO + 4% sucrose (w/v) and 10^7 cells/ml.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules. (Special plastic vials for cryopreservation).
6. Place ampules at the bottom of a mechanical freezer set between -55 to -70° C for 1 h. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after the addition of DMSO + sucrose to the cell suspension.
7. Rapidly plunge ampules into liquid nitrogen. Store in either the vapor or liquid phase of a nitrogen refrigerator. Ampules may be stored in a mechanical freezer set at -70° C. Do not store above -70° C. Stability is dependent upon storage temperature. Store in a liquid nitrogen refrigerator (below -130° C) insures indefinite stability.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the content of the ampule and inoculate into 13.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube. Incubate in the normal fashion.

PROTOCOL

Controlled Cooling

1. Follow steps 1-5 in previous protocol.
2. Place ampules in controlled rate freezing unit. From room temperature cool at -10° C/min to the heat of fusion. If the freezing unit can compensate for the heat of fusion cool at -1° C/min through the heat of fusion to -40° C, then plunge into liquid nitrogen.
3. Follow steps 8 and 9 of first protocol.

COMMENTS

Recovery will be greater using a controlled cooling rate. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Simione FP, Jr & Daggett, P-M 1976. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. *J. Parasitol.* **62**:49.
2. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

A-70.1

CRYOPRESERVATION OF DIPLOMONADS OTHER THAN *GIARDIA*

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INTRODUCTION

There are two genera of diplomonads other than *Giardia*, *Hexamita* and *Trepomonas*, that are known to be amenable to cryopreservation. The method differs from that which is best for *Giardia*. Whether the method can be applied to other diplomonads is unknown, but it would warrant to first attempt the protocol detailed here before trying another method.

PROTOCOL

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

A-70.2

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

CRYOPRESERVATION OF TERMITE FLAGELLATES

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INTRODUCTION

Only one species of termite flagellate, *Tricercomitus divergens*, has been reported to be cryopreservable [1]. The protocol detailed below may be applicable to other termite flagellates.

PROTOCOL

1. In advance prepare a 40% (v/v) glycerol solution in fresh growth medium.
2. When the culture is at or near peak density (~3 x 10^5 cells/ml) mix the culture and the 40% glycerol solution in a 1:1 ratio.
3. The final concentration of the preparation will be 20% (v/v) glycerol and 1.5 x 10^5 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
4. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to -70° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. When the ampules reach -70° C they may be stored at that temperature in a mechanical refrigerator or they may be plunged into liquid nitrogen and then stored in the vapor phase of a liquid nitrogen refrigerator.
6. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
7. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium.

COMMENTS

Recovery of *Tricercomitus* was reported after storage at -70° C for 20 weeks [1]. Even though frozen preparations may be stored for convenience at -70° C it is recommended by the authors here that frozen material be stored in liquid nitrogen. This assures almost indefinite stability. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

A-71.2

LITERATURE CITED

1. Yamin, MA 1978. Axenic cultivation of the flagellate *Tricercomitus divergens* Kirby from the termite *Cryptotermes cavifrons* Banks. *J. Parasitol.* **64**:1122-1123.

A-72.1

CRYOPRESERVATION OF TRYPANOSOMATID PARASITES OF VERTEBRATES

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INTRODUCTION

Trypanosomatid parasites from vertebrates belonging to the genera, *Endotrypanum*, *Leishmania* and *Trypanosoma*, whether maintained *in vitro* or *in vivo*, are amenable to cryopreservation. Both uncontrolled [1,2,4] and controlled [3] cooling rates can be used. Any laboratory with an ultra-low freezer can maintain strains for long periods of time. Strains are known to have been viable after decades of storage only at ultra-low temperatures (R. Yaeger personal communication) **Since some species are infective to humans, safety should be a primary concern.** Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

Uncontrolled Cooling for *in vitro* Cultures

1. Harvest cells from a culture which is at or near peak density by centrifugation at 1,300 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. The final concentration will be 10^7 cells/ml and 5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation). **SAFETY PRECAUTION:** Although glass cryules can be heat sealed the authors recommend that the vials not be sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS.**
6. After dispensing place ampules at the bottom of a mechanical freezer set at between -55

to -70° C for 1 h.

7. The frozen preparations may be stored in the mechanical freezer until needed or rapidly plunged into liquid nitrogen and stored in either the vapor or liquid phase of a nitrogen refrigerator. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically transfer the contents of the ampule into the appropriate medium.

PROTOCOL

Uncontrolled Cooling for *Leishmania* maintained *in vivo*

1. Prior to beginning the procedure a glass tissue homogenizer is sterilized. The homogenizer should not be assembled prior to autoclaving.
2. In advance the following salt solution should be prepared:

Sodium citrate	1.2 g
NaCl	1.0 g
Glass distilled H_2O	90.0 ml

Dissolve components in glass distilled H_2O and filter sterilize.

3. Prepare a 10% (v/v) solution of sterile DMSO in the salt solution. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use. A 20% sterile (v/v) glycerol solution can also be used instead of DMSO.
4. Aseptically remove the infected spleen(s) from the host animal(s) immediately after the host has been killed and place in the tissue homogenizer. Add a volume of the salt solution approximately equal to the volume of the spleen(s).
5. Homogenize the tissue.
6. Mix the homogenate and the DMSO (or glycerol solution) in equal portions. After mixing the final concentration of the cryoprotective agent will be 5% (v/v) DMSO or 10% (v/v) glycerol. The time from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
7. Follow Steps 5-8 of protocol 1.
8. Immediately after thawing, do not leave in the water bath, aseptically **AND WITH SAFETY PRECAUTIONS IN MIND**, aspirate the entire contents into a 1 ml syringe and inoculate intraperitoneally into a single uninfected host. The newly infected host should be carefully monitored. The course of the infection may be longer or shorter than usual dependent on percent recovery of the parasite from the frozen state.

PROTOCOL

Uncontrolled Cooling for *Trypanosoma* maintained *in vivo*

1. In advance the following solution should be prepared:

Tyrodes Salt Solution:

NaCl	8.0 g
KCl	0.2 g
$CaCl_2$	0.2 g

$MgCl_2 \cdot H_2O$	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.05g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize.

2. Prepare a 10% (v/v) solution of sterile DMSO in Tyrodes Salt Solution. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use. A 20% (v/v) sterile glycerol solution can also be used.
3. Prepare a syringe equipped with the appropriate gauge needle as follows: Draw Yaeger's anticoagulant solution (see below) into the syringe. Move the plunger of the syringe back and forth several times to distribute the anticoagulant. Adjust the final volume of the anticoagulant to 1/10 th of the amount of blood normally obtained from the host animal.

Sodium citrate	1.33g
Citric acid	0.47g
Dextrose	3.00g
Sodium heparin	0.2 g
Glass distilled H_2O	100.0 ml

4. Draw blood into the syringe by gently pulling the plunger outward. When blood is no longer obtainable or the mouse has died remove needle and invert the syringe several times to mix the anticoagulant evenly with the blood. If clotting occurs during extraction of blood insufficient anticoagulant has been used.
5. Transfer the collected blood to a 16 x 125 mm screw-capped test tube and determine the volume by drawing the blood into a pipette. Expel the blood back into the test tube after the volume has been determined.
6. Mix the heparinized blood and the DMSO (or glycerol solution) in equal portions. If any clotting has occurred **do not use**. After mixing the final concentration of the cryoprotective agent will be 5% (v/v) DMSO or 10% (v/v) glycerol. The time from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
7. Follow steps 5-8 of the first protocol.
8. Immediately after thawing, do not leave in the water bath, aseptically **AND WITH SAFETY PRECAUTIONS IN MIND**, aspirate the entire contents into a 1 ml syringe and inoculate parasite intraperitoneally into a single uninfected host. The newly infected host should be carefully monitored. The course of the infection may be longer or shorter than usual dependent on percent recovery of the parasite from the frozen state.

PROTOCOL

Controlled Cooling

1. For any of the previous protocols substitute the cooling rate which follows: Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.

COMMENTS

Recovery will be greater using a controlled cooling rate. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). Strains belonging to the genera are available from the ATCC.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.
2. Allain, D 1964. Evaluation of the viability and pathogenicity of hemoflagellates after freezing and storage. *J. Parasitol.* **50**:604-607.
3. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
4. Simione, FP, Jr & Daggett, P-M 1976. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. *J. Parasitol.* **62**:49.

A-73.1

CRYOPRESERVATION AND FREEZE-DRYING OF INSECT AND PLANT TRYPANOSOMATIDS

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INTRODUCTION

Trypanosomatids isolated from insects belonging to the genera, *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Leptomonas*, and *Phytomonas* can easily be cryopreserved. There are two simple methods which can be used. One method requires the use of an ultra-low refrigerator [4] and other requires specialized equipment [2]. Both methods are provided here. Some *Crithidia* species can be freeze-dried [1,3].

PROTOCOL

Uncontrolled Cooling

1. Harvest cells from a culture which is at or near peak density by centrifugation at 1,300 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration will be 10^7 cells/ml and 5% (v/v) DMSO The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. After dispensing place ampules at the bottom of a mechanical freezer set between -55 to -70° C for 1 h.
7. The frozen preparations may be stored in the mechanical freezer or the ampules may be rapidly plunged into liquid nitrogen and stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the vial to a level just sufficient to cover the frozen material. Do not agitate.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium.

A-73.2

PROTOCOL
Controlled Cooling

1. Follow steps 1-5 in previous protocol.
2. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
3. Follow steps 7-9 of previous protocol.

PROTOCOL
Crithidia, Freeze-drying

1. In advance prepare growth medium supplemented with 12% (w/v) sucrose plus 4% (w/v) BSA (Bovine Serum Albumin Fraction V Sigma A-4503). Prepare 100 ml of the protective solution as follows:

    a) Add 12.0 g sucrose to 100.0 ml of medium and dissolve thoroughly.
    b) Divide the above solution into four equal aliquots and transfer each to a separate 50 ml plastic screw-capped centrifuge tube. Add 1.0 g of BSA to each tube and agitate vigorously until dissolved. A considerable amount of foam will overly the liquid.
    c) Centrifuge at 850 g for 10 min to eliminate the layer of foam.
    d) Pool the solutions in a single vessel and filter sterilize. Store refrigerated (5-9° C) for up to 1 year.

2. Harvest cultures that are at or near peak density. Centrifuge at 850 g for 5 min. Resuspend pellets with a small amount of supernatant and pool in a single centrifuge tube.
3. Determine the cell concentration using a hemocytometer and also measure the total volume of the cell preparation. Make a note of the total volume. Determine the volume of protective solution needed to yield the final desired concentration of 2×10^8 cells/ml using the following formula:

    a) Cell concentration x total volume (ml) = total number of cells.
    b) Total number of cells divided by 2×10^8 cells/ml desired = ml of protective solution required.

    Centrifuge at 850 g, discard the supernatant and resuspend the pellet with a volume of protective solution determined in b) above.
4. Distribute the cell suspension in 0.25 ml aliquots into 2 ml serum vials (Wheaton Scientific #066534). Aseptically place sterile butyl rubber stoppers (West #1012-3511) securely on vials. Also dispense 0.4 ml of the protective solution without the cells into vials. Immerse the tip of a freeze-dryer probe into each of these vials and secure it to the vial using tape (these probes will be hooked to the freeze-dryer and will monitor the drying during the freeze-drying cycle).
5. Place vials in a controlled rate freezing unit. Cool at -1° C/min from room temperature to -40° C. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion.
6. Transfer ampules to a pan containing dry-ice snow and place within a laminar flow hood. Aseptically remove and discard stoppers. Transfer ampules to a slotted tray of a commercial freeze-dryer that also has been placed on dry ice. Place the ampules with the probes at the

front of the tray near and feed the probe wires through the small port on the side. Gently place a sterile butyl rubber stopper on each vial in a manner that will allow exposure of the slot of the stopper so that H_2O vapor can escape during freeze-drying.

7. Gently place a 3 mm thick piece of acrylic (platen) on top of the rubber stoppers to prevent movement during transport. At the end of the freeze-drying run the platen will serve to push the rubber stoppers into the vials.
8. Slide the metal cover over the ampules and transfer to a freeze-dryer with the shelf temperature set at -40° C. Place a sterile cotton wad over the Pall filter holder.
9. Plug the thermocouple probes into the jacks on the inside of the chamber. Close the chamber door.
10. Raise the shelf temperature to -30° C and freeze-dry at that temperature for at least 18 h. Then increase the shelf temperature in 10° C increments, holding at each temperature at least one h. This can be done manually or with a programmer.
11. When the product temperature reaches 25° C and the vacuum is 50 μm of Hg or less the cycle is complete. If necessary, air injection can be used to elevate the pressure to improve heat input to the product and accelerate the drying. Turn off the programmer, turn off the shelf heat and bleed short bursts of air through a Pall filter (Pall Corp.) until the product temperature is approximately 25° C. Allow the vacuum to once again reach less than 50 μm of Hg.
12. Turn off the vacuum pump. Using a nitrogen gas cylinder that has been stored at 2-8° C, back fill the chamber with nitrogen gas through a 0.22 μm Pall filter. Do not allow the condenser temperature to go above -50° C. It may be necessary to run the nitrogen gas through a copper coil immersed in liquid nitrogen to maintain condenser temperature.
13. Remove tray from freeze-dryer. Slide the lid back and push down the acrylic platen to seal the vials.
14. Seal the vials with aluminum caps (Wheaton Scientific #224192-01) and store at -60° C.
15. To establish a culture from a freeze-dried vial proceed as follows:

    a) In advance prepare growth medium supplemented with 12% (w/v) sucrose and with pH adjusted to 7.0. Filter sterilize. Place a 5.0 ml aliquot in a 16 x 125 mm screw-capped test tube and place this in an ice bath.
    b) Remove aluminum crimped cap and aseptically remove butyl rubber stopper. Add 0.5 ml of the ice cold medium prepared above and allow the pellet to completely rehydrate. Add the rehydrated pellet to the remainder of the ice cold medium (4.5 ml). Place the tube upright with the cap screwed on tightly and incubate at 25° C.

16. If the established culture is observed immediately using an inverted microscope will observe numerous active cells. The number of active cells steadily declines as the medium warms and very few motile cells are evident after ½ h. Active cells are again observed 3-4 d after initial establishment of the culture.

COMMENTS

Recovery of *Crithidia* from the freeze-dried state is very low using the protocol above. If rehydration is attempted with supplemented medium at room temperature (20-23° C) recovery will not be achieved. If rehydration is attempted with ice cold supplemented medium only 1 in 10^6 cells survives freeze-drying. If supplemented ice cold medium is first adjusted to pH 7.0 the recovery is increased 10 fold. Recovery will be greater using a controlled cooling rate. If a controlled rate freezing unit is not available, a cooling cycle which closely approximately a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device

A-73.4

(Cryopreservation Apparatus Cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1982. Freeze-drying methods for amoeba and flagellates. *J. Protozool.* **29**:477.
2. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
3. Daggett, PM & Simione, FP, Jr 1976. The lyophilization of two species of *Crithidia*. *J. Protozool.* **23**:9A.
4. Simione, FP, Jr & Daggett, P-M 1976. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. *J. Parasitol.* **62**:49.

A-74.1

LONG TERM MAINTENANCE OF FREE-LIVING BODONIDS BY CRYOPRESERVATION AND DRYING

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INTRODUCTION

The following five genera of free-living bodonids are amenable to cryopreservation: *Bodo*, *Cruzella*, *Cryptobia*, *Dimastigella*, and *Rhynchomonas*. Both seawater and freshwater species have been cryopreserved. *Dimastigella* appears to be restricted to freshwater and *Cruzella* to seawater. There are freshwater and seawater representatives of *Bodo*, *Cryptobia*, and *Rhynchomonas*. Some members of *Bodo*, *Cryptobia*, and *Dimastigella* produce a resistant cyst stage in their life cycle. Those strains with resistant stages can often be dried.

PROTOCOL

Cryopreservation

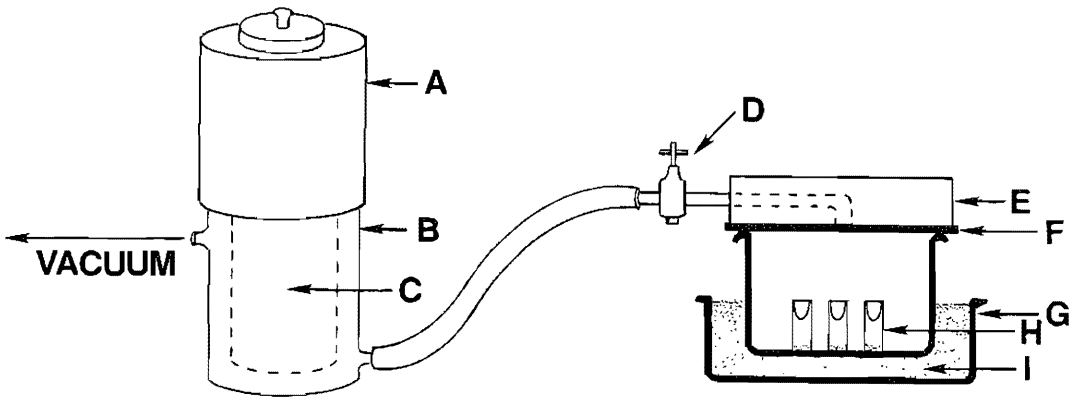
1. Harvest cells from a culture which is at or near peak density. If the strain encysts allow cysts to form before harvesting. Centrifuge at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 2x concentrated cryoprotective solution of DMSO or glycerol. For *Cruzella*, *Dimastigella* and *Rhynchomonas* prepare a 20% (v/v) solution of sterile DMSO in fresh medium. For *Bodo* prepare a 10% or 20% (v/v) DMSO solution in the growth medium. For *Cryptobia* prepare a 15% (v/v) glycerol in growth medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 5% or 10% (v/v) DMSO or 7.5% (v/v) glycerol. The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask for most strains. For some strains of *Bodo* recovery will be enhanced using the following thawing protocol:

a) Immediately after thawing, do not leave in the water bath, gently remove the contents of the ampule with a Pasteur pipette and expel slowly into a T-25 tissue culture flask. Incubate at room temperature (20-23° C) for 15 min.
b) At 15 min intervals add 0.25 ml of the appropriate medium dropwise. Continue until the final volume is 2.0 ml.
c) Allow the flask to remain undisturbed for 15 min.
d) Add 0.5 ml of the appropriate medium dropwise at 15 min intervals until the volume is 4.0 ml.
e) Allow the flask to remain undisturbed overnight.
f) In the morning of d 2 slowly add 4.0 ml of the appropriate medium. Allow to remain undisturbed overnight.
g) Add 8.0 ml of fresh bacterized medium. Incubate at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

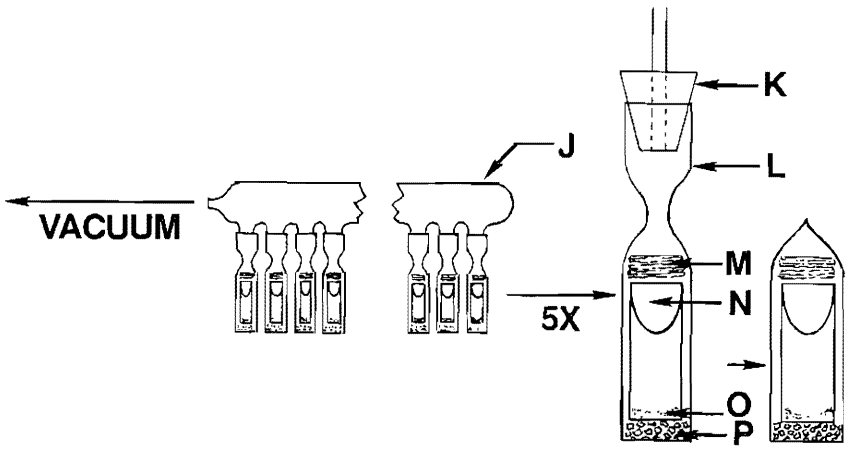
PROTOCOL

Drying, *Bodo* and *Dimastigella*
(Fig. 1, sealing only)

1. In advance sterilize glass cotton-plugged 11.5 x 35.0 mm shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper are prepared. Whatman #1 filter paper is soaked in glass distilled H_2O and then blended in a blender. About a third of volume of the vial is filled with the wet mulched paper inserted into the vials. The paper should not be packed. The vial is cotton plugged and the vials heat-sterilized (121° C for 24 h).
2. Grow flagellates in 500 ml bottles. Allow cysts to form.
3. Harvest the cysts by scraping the sides to suspend cysts. Centrifuge at 850 g for 5 min. After each spin collect and pool pellets.
4. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
5. Dispense final cysts in 0.3 ml aliquots into sterile shell vials containing dried, mulched filter paper.
6. Place the ampules in a 25° C for 14 d. After drying, place for 7 d in a vacuum desiccator under vacuum at room temperature (20-23° C).
7. In advance 14.25 x 85.0 mm outer shell freeze-drying glass vials are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
8. To cooled outer shell vials in a dry cabinet (10% or least relative humidity) the small vial with the dried preparation is inserted. On top of the smaller vial is added an ~64 mm wide wade of glass fiber paper is inserted.
9. The vials are removed from the dry cabinet to be sealed. The sealing of the outer vial is accomplished by rotating the vial in the hottest part of an air/gas flame and slowly pulling with forceps until a narrow neck is formed. The appearance is like a wine glass with a stem. After cooling the vial is attached to a port manifold. Once all the vials have been attached, the manifold is evacuated to 50 μm of Hg. To assure an evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch where the "stem" leaves the base of the outer shell vial.
10. Although vials can be stored at room temperature viability is extended if storage is at -4° C to -70° C. Preparations can be stored in the vapor phase of a nitrogen freezer. If storage is in



FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

nitrogen vapor, **FOR SAFETY REASONS DO NOT PUT VIALS INTO OUTER SHELL VIALS AND SEAL.** The cotton-plugged shell vial preparation is stored after step 6 is completed.

11. To establish a culture from the dried state aseptically add 1.0 ml of fresh medium and incubate at appropriate temperature. Observe daily for motile cells and when observed, transfer to a 16 x 125 mm containing 5.0 ml of fresh medium. Thereafter, follow routine maintenance protocol.

COMMENTS

Cryptobia strains that form cysts are probably also amenable to drying using the protocol described above. If a controlled rate freezer is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

A-75.1

LONG TERM MAINTENANCE MICROFLAGELLATES OF UNCERTAIN TAXONOMIC AFFINITY BY DRYING AND CRYOPRESERVATION

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INTRODUCTION

A cryopreservation method allows strains belonging to a number of microflagellate genera long term maintenance by cryogenic storage. The broad application of the method suggests it could be applied to other microflagellates. The protocol detailed here is known to be applicable to the following genera: *Amastigomonas*, *Apusomonas*, *Bordnamonas*, *Cercomonas*, *Heteromita*, *Jakoba*, *Metopion*, *Massisteria*, *Oikomonas*, and *Percolomonas*. Strains belonging to two genera, *Cercomonas* and *Heteromita*, can also be maintained in the dried state by a protocol used for maintaining other encysting protists [1]. The drying protocol, detailed here, may be applicable to other encysting microflagellates.

PROTOCOL

Cryopreservation

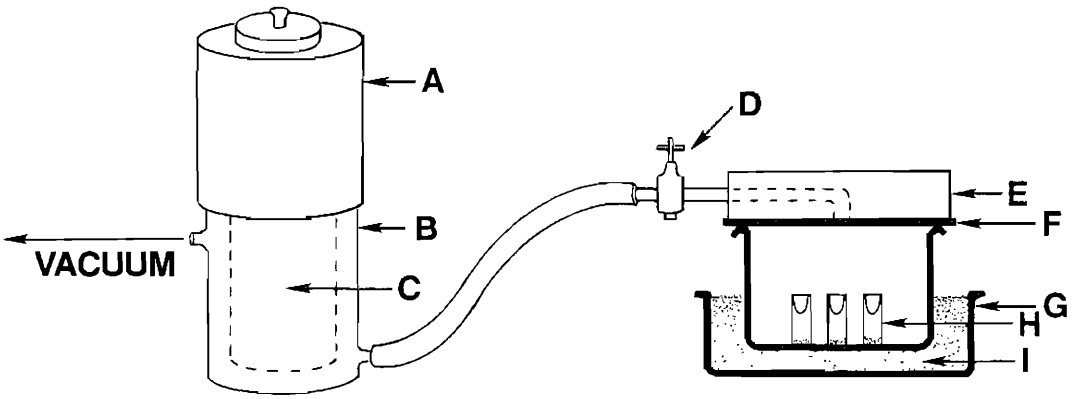
1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. Incubate at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

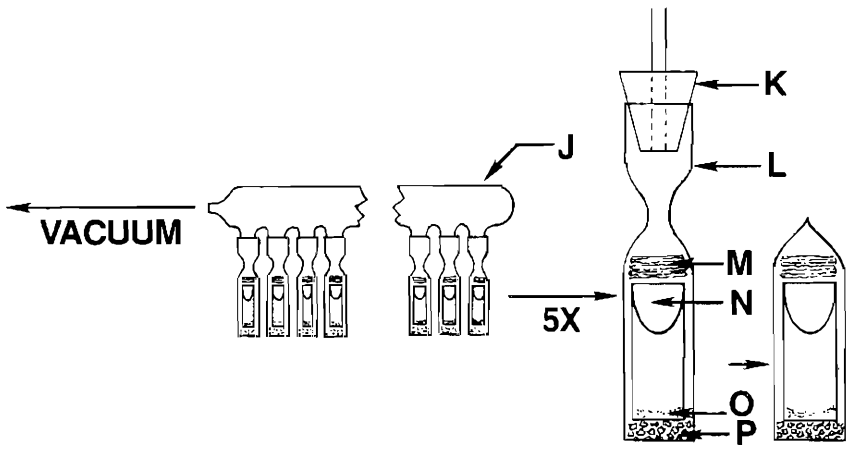
PROTOCOL

Drying *Cercomonas* & *Heteromita*
(Fig. 1, sealing only)

1. In advance sterilize glass cotton-plugged 11.5 x 35.0 mm shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper are prepared. Whatman #1 filter paper is soaked in glass distilled H_2O and then blended in a blender. About a third of volume of the vial is filled with the wet mulched paper inserted into the vials. The paper should not be packed. The vial is cotton plugged and the vials heat-sterilized (121° C for 24 h).
2. Grow flagellates in 500 ml bottles. Allow cysts to form.
3. Harvest the cysts by scraping the sides to suspend cysts. Centrifuge at 850 g for 5 min. After each spin collect and pool pellets.
4. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
5. Dispense final cysts in 0.3 ml aliquots into sterile shell vials containing dried, mulched filter paper.
6. Place the ampules in a 25° C for 14 d. After drying, place for 7 d in a vacuum desiccator under vacuum at room temperature (20-23° C).
7. In advance 14.25 x 85.0 mm outer shell freeze-drying glass vials are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
8. To cooled outer shell vials in a dry cabinet (10% or least relative humidity) the small vial with the freeze-dried preparation is inserted. On top of the smaller vial is added an ~64 mm wide wade of glass fiber paper is inserted.
9. The vials are removed from the dry cabinet to be sealed. The sealing of the outer vial is accomplished by rotating the vial in the hottest part of an air/gas flame and slowly pulling with forceps until a narrow neck is formed. The appearance is like a wine glass with a stem. After cooling the vial is attached to a port manifold. Once all the vials have been attached, the manifold is evacuated to 50 μm of Hg. To assure an evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch where the "stem" leaves the base of the outer shell vial.
10. Although vials can be stored at room temperature viability is extended if storage is at -4° C to -70° C. Preparations can be stored in the vapor phase of a nitrogen freezer. If storage is in nitrogen vapor, **FOR SAFETY REASONS DO NOT PUT VIALS INTO OUTER SHELL VIALS AND SEAL**. The cotton-plugged shell vial preparation is stored after step 6 is completed.
11. To establish a culture from the dried state aseptically add 1.0 ml of fresh medium and incubate at appropriate temperature. Observe daily for motile cells and when observed, transfer to a 16 x 125 mm 5.0 ml of fresh medium. Thereafter, follow routine maintenance protocol.



FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

A-75.4

COMMENTS

Dried material should not be stored in direct sun light. The colder the storage temperature for dried material the longer the viability. Dried preparations should not be kept in a **frost-free freezer**. The periodic frost-free heat cycle will reduce the longevity of stored dried material. Sealed preparations **should not be stored in a liquid nitrogen refrigerator** since the liquid may penetrate the vial through micro-fractures. The vial may **explode** as the trapped nitrogen is converted to the gaseous phase when it is returned to room temperature.

LITERATURE CITED

1. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525.

A-76.1

LONG TERM MAINTENANCE OF LARGE FRESHWATER/TERRESTRIAL AMEBAE BY DRYING AND CRYOPRESERVATION

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INTRODUCTION

To extend intervals required for routine maintenance some large amebas which can form cysts can be either dried or cryopreserved and stored at cryogenic temperatures. Cysts are often viable for extended periods of time in nature. By applying preservation technology the viability of the cyst form can be extended reducing the need to excyst and subculture stocks. A cryopreservation method for *Leptomyxa* and *Theratromyxa* and a drying method [1] applicable to the latter are detailed here.

PROTOCOL

Cryopreservation

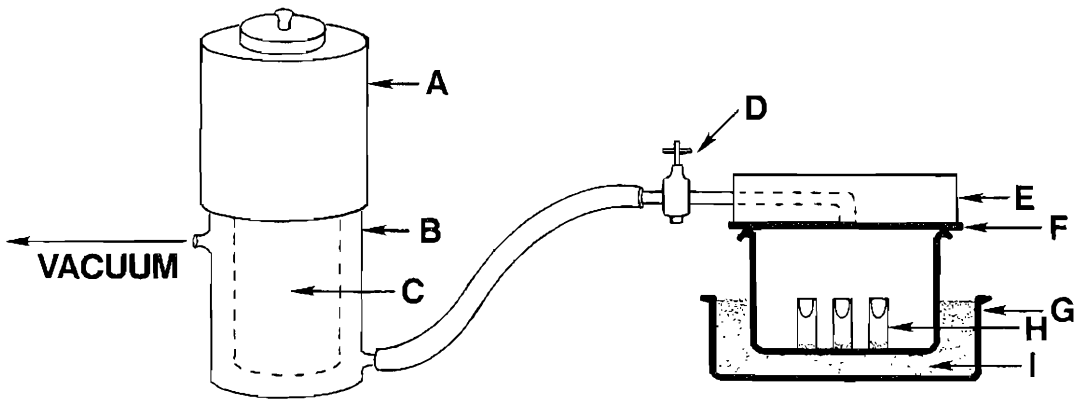
1. Allow cultures in 500 ml bottles to encyst. Harvest the cysts by scraping the sides to suspend cysts. Centrifuge at 850 g for 5 min. After each spin collect and pool pellets.
2. Adjust concentration of cysts to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO if cryopreserving *Leptomyxa*; 18% (v/v) solution of sterile DMSO if cryopreserving *Theratromyxa* in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 5% or 9% (v/v) DMSO respectively. The time from the mixing of the cell preparation and DMSO stock solution before the cooling cycle is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into 9.5 ml of fresh medium in a T-25 tissue culture flask. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

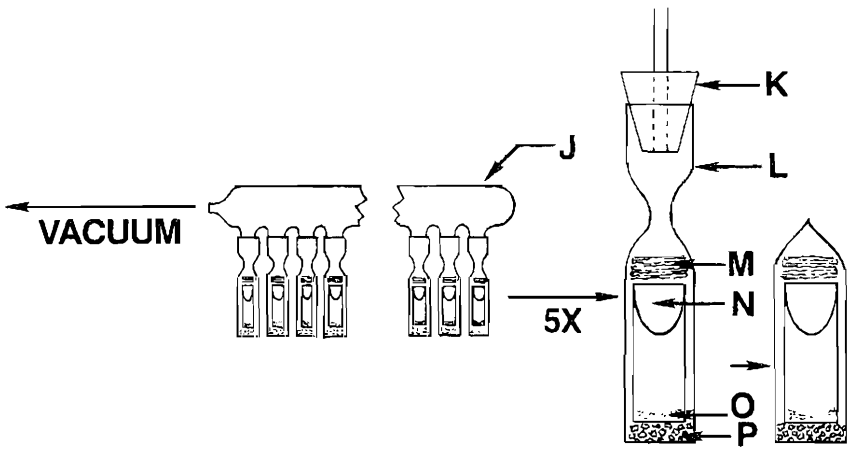
PROTOCOL

Drying *Leptomyxa*
(Fig. 1, sealing only)

1. In advance sterilize glass cotton-plugged 11.5 x 35.0 mm shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper are prepared. Whatman #1 filter paper is soaked in glass distilled H_2O and then blended in a blender. About a third of volume of the vial is filled with the wet mulched paper inserted into the vials. The paper should not be packed. The vial is cotton plugged and the vials heat-sterilized (121° C for 24 h).
2. Grow amebas in 500 ml bottles. Allow cysts to form.
3. Harvest the cysts by scraping the sides to suspend cysts. Centrifuge at 850 g for 5 min. After each spin collect and pool pellets.
4. Adjust concentration of cells for 2 x 10^6/ml in fresh medium.
5. Dispense final cysts in 0.3 ml aliquots into sterile shell vials containing dried, mulched filter paper.
6. Place the ampules in a 25° C for 14 d. After drying, place for 7 d in a vacuum desiccator under vacuum at room temperature (20-23° C).
7. In advance 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
8. To cooled outer shell vials in a dry cabinet (10% or least relative humidity) the small vial with the dried preparation is inserted. On top of the smaller vial is added an ~64 mm wide wad of glass fiber paper is inserted.
9. The vials are removed from the dry cabinet to be sealed. The sealing of the outer vial is accomplished by rotating the vial in the hottest part of an air/gas flame and slowly pulling with forceps until a narrow neck is formed. The appearance is like a wine glass with a stem. After cooling the vial is attached to a port manifold. Once all the vials have been attached, the manifold is evacuated to 50 μm of Hg. To assure an evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch where the "stem" leaves the base of the outer shell vial.
10. Although vials can be stored at room temperature viability is extended if storage is at -4° C to -70° C. Preparations can be stored in the vapor phase of a nitrogen freezer. If storage is in nitrogen vapor, **FOR SAFETY REASONS DO NOT PUT VIALS INTO OUTER SHELL VIALS AND SEAL**. The cotton-plugged shell vial preparation is stored after step 6 is completed.
11. To establish a culture from the dried state aseptically add 1.0 ml of fresh medium and incubate at appropriate temperature. Observe daily for motile cells and when observed, transfer to a 16 x 125 mm 5.0 ml of fresh medium. Thereafter, follow routine maintenance protocol.



FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

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COMMENTS

Dried material should not be stored in direct sun light. The colder the storage temperature for dried material the longer the viability. Dried preparations should not be kept in a **frost-free freezer**. The periodic frost-free heat cycle will reduce the longevity of stored dried material. Sealed preparations **should not be stored in a liquid nitrogen refrigerator** since the liquid may penetrate the vial through micro-fractures. The vial may **explode** as the trapped nitrogen is converted to the gaseous phase when it is returned to room temperature.

LITERATURE CITED

1. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525.

LONG TERM MAINTENANCE OF FREE-LIVING AMEBAE INCLUDING AMEBOFLAGELLATES

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INTRODUCTION

Many free-living amebae, including amphizoic ameboflagellates, are amenable to cryopreservation and stored indefinitely at cryogenic temperatures [1]. The genera which are known to amenable to the cryogenic storage include the following: *Acanthamoeba, Adelphamoeba, Comandonia, Echinamoeba, Flabellula, Hartmannella, Heteramoeba, Lingulamoeba, Naegleria, Nuclearia, Paraflabellula, Paramoeba, Paratetramitus, Protacanthamoeba, Rosculus, Saccamoeba, Singhamoeba, Tetramitus, Trichamoeba, Vahlkampfia, Vannella*, and *Willaertia*. The cryopreservation protocols detailed here may be applicable to other genera of amebae. Amebae of the following genera that form a resistant cyst stage can also be preserved in the dried state: *Acanthamoeba, Adelphamoeba, Comandonia, Hartmannella, Heteramoeba, Naegleria, Paratetramitus, Protacanthamoeba, Singhamoeba, Tetramitus, Vahlkampfia*, and *Willaertia* using our method applicable to other protists [3]. There have been few attempts to freeze-dry protozoa. A method developed for the axenic cyst stage of *Acanthamoeba* [2] is presented below.

PROTOCOL

Cryopreservation, Uncontrolled Cooling
Acanthamoeba, Adelphamoeba, Naegleria, Tetramitus, Vahlkampfia

1. Allow the cells to encyst. To detach cysts from plate cultures flush with fresh broth. The surface of the plate is rubbed gently with a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame) to detach adhering amebae. When grown in broth medium rub the bottom of the flask with a sterile cotton swab to detach cysts.
2. If the cyst concentration exceeds the required level do not centrifuge, but adjust concentration to 2 x 10^6 cysts/ml with fresh broth medium. If the concentration is too low, centrifuge at 600 g for 5 min and resuspend the pellet with the volume of fresh required to yield the desired concentration.
3. Prepare a 10% (v/v) solution of sterile DMSO in fresh broth medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.

4. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will 10^6 cysts/ml in 5% (v/v) DMSO.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in the bottom of a mechanical freezer set between -55° to -70° C for 2 h, then plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. Immerse to a level sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate onto fresh agar medium.

PROTOCOL

Cryopreservation, Controlled Cooling
Acanthamoeba, Adelphamoeba, Paratetramitus, Protacanthamoeba, Saccamoeba, Tetramitus

1. Follow steps 1-5 of the preceding protocol, except at step 3 substitute 6% (v/v) DMSO for strains of *Saccamoeba* (i. e. final concentration of DMSO will be 3% (v/v) for *Saccamoeba* strains).
2. At step 6 substitute the following: Place vials in a controlled rate freezing unit. Cool at -1° C/min from 25° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen. Then follow steps 7-9.

PROTOCOL

Cryopreservation, Controlled Cooling
Hartmannella, Naegleria, Nuclearia, Rosculus, Saccamoeba, Vahlkampfia, Vannella, Willaertia

1. Follow steps 1-5 of the first protocol, except at step 3 substitute 15% (v/v) DMSO for strains of *Hartmannella, Naegleria, Nuclearia, Rosculus, Vahlkampfia, Vannella,* and *Willaertia* and 6% (v/v) DMSO for strains of *Saccamoeba* (i.e. final concentration of DMSO will be 3.0% (v/v) for *Saccamoeba* strains and 7.5% (v/v) for the other genera).
2. At step 6 substitute the following: Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen. Then follow steps 7-9.

PROTOCOL

Cryopreservation, Controlled Cooling
Comandonia, Echinamoeba, Flabellula, Paraflabellula, Singhamoeba, Trichamoeba

1. Follow preceding protocol steps 1-5, except at step 3 substitute 15% (v/v) DMSO for strains of *Comandonia* and *Paraflabellula* (i.e. final concentration of DMSO will be 7.5% (v/v) for strains for these two genera).
2. At step 6 substitute the following: Place vials in a controlled rate freezing unit. Cool at -1° C/min from 25° C to 4° C, then -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion to -40° C. Then plunge ampules into liquid nitrogen. Then follow steps 7-9.

PROTOCOL

Drying
Acanthamoeba, Adelphamoeba, Comandonia, Hartmannella, Heteramoeba, Naegleria, Paratetramitus, Protacanthamoeba, Singhamoeba, Tetramitus, Vahlkampfia, and *Willaertia*
(Fig. 1, sealing only)

1. In advance sterile glass cotton-plugged 11.5 x 35.0 mm shell freeze-drying glass ampules (Glass Vials, Inc.) containing mulched paper are prepared. Whatman #1 filter paper is soaked in glass distilled H_2O and then blended in a blender. Add sufficient wet mulched paper to just cover the bottom of the vial. The paper should not be packed. The vials are cotton plugged autoclaved at 121° C for 30 min. The vials are then placed in a 70° C oven overnight to allow them to dry thoroughly.
2. Harvest cultures when they have encysted.
3. The cysts are washed from the agar plates fresh broth medium. Centrifuge at 200 g for 5 min. After each centrifugation collect and pool pellets.
4. Adjust concentration of cysts to 10^7 - 10^8/ml in fresh broth medium.
5. Dispense final cyst suspensions in 0.1 ml aliquots into sterile shell vials containing dried, mulched filter paper.
6. Place the ampules in a 25° C incubator for 7 d. After drying, place for 7 d in a vacuum desiccator under vacuum at room temperature (20-23° C).
7. In advance the 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is heated for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
8. Allow outer shell vials to cool in a dry cabinet (10% or least relative humidity) and then insert inner shell vial with the dried preparation. On top of the inner vial a wad of glass fiber paper is inserted to prevent exposure of the freeze-dried preparation to excessive heat during flame sealing.
9. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved

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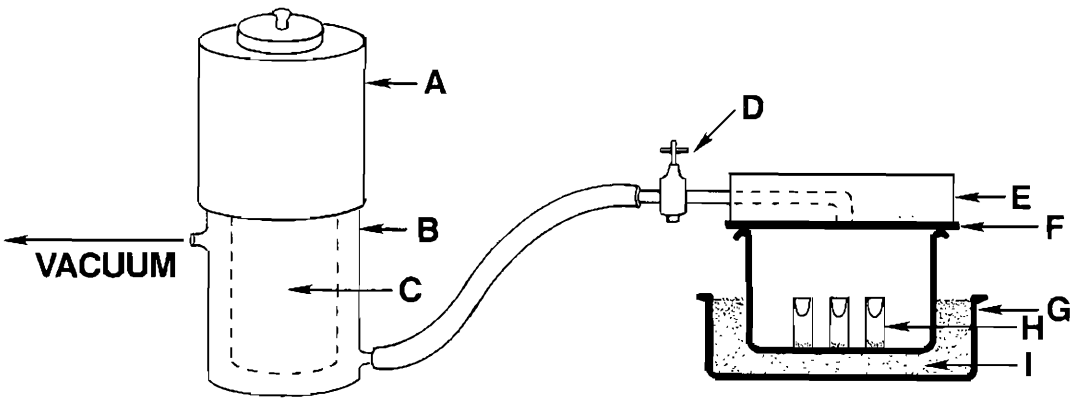
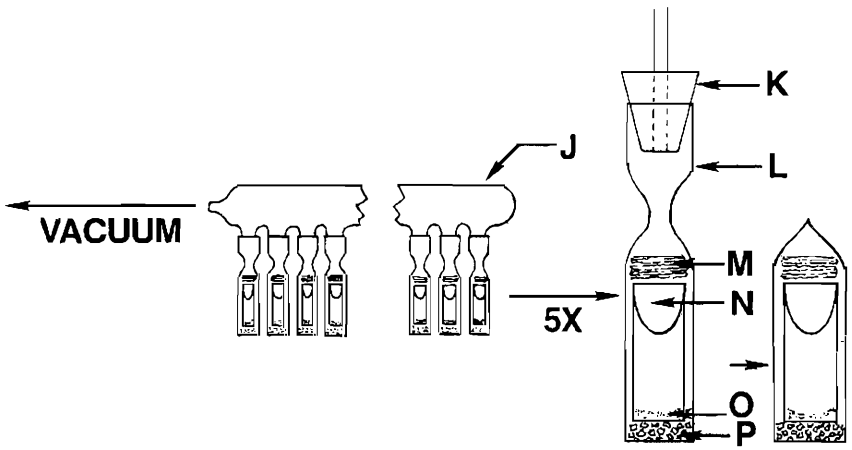


FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.

10. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered. However, **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
11. To establish a culture from the freeze-dried state add 1.0 ml of sterile glass distilled H_2O to the vial. The pellet of filter paper is then removed aseptically with a pair of sterile forceps and teased apart with the forceps and a sterile dissecting needle on the surface of an agar medium plate. The fluid remaining in the vial is then distributed evenly over the surface of the agar medium plate. Incubate at the appropriate temperature.
12. Thereafter follow protocol for culturing.

PROTOCOL

Freeze-drying, *Acanthamoeba*
(Fig. 2, sealing only)

1. Prepare outer vials (Glass Vials, Inc., 14.25 mm x 85.0 mm) by placing a small amount of silica gel granules (Fisher Scientific, grade 42, 6-16 mesh, "Tel-Tale" brand) in the vial to cover about half of the bottom. Add a small cotton wad to cushion the inner vial, and heat at 100° C overnight. The silica gel should be dark blue after heating and serves as a moisture indicator during storage. Place vials in a dry box (<10% relative humidity) to cool.
2. Harvest broth cultures at or near peak density as follows: Gently aspirate most of the culture fluid overlying the cells. Then place the flasks on ice for 10 min. Swirl the flasks to suspend the cells and transfer the cell suspensions to 15 ml plastic centrifuge tubes. Centrifuge at 500 g for 5 min and pool the cell pellets in a single tube.
3. Centrifuge as in step 3, discard the supernatant and resuspend the pellet with Page's Balanced Salt Solution (see below). Fill the tube to the 14 ml mark.

Page's Balanced Salt Solution

Solution 1 (see below)	500.0 ml
Solution 2 (see below)	500.0 ml

Solution 1

Na_2HPO_4	2.84 g
KH_2PO_4	2.72 g
Glass distilled H_2O	500.0 ml

Solution 2

$MgSO_4 \cdot 7H_2O$	8.0 mg
$CaCl_2 \cdot 2H_2O$	8.0 mg
NaCl	0.24 g
Glass distilled H_2O	500.0 mg

Autoclave for 20 min at 121° C. Loosen cap one half turn prior to autoclaving. When both solutions have cooled mix 1:1.

4. Repeat step 4 twice.
5. Repeat step 4 but resuspend the pellet with only 5 ml of Page's Balanced Salt Solution. Thoroughly suspend the cells by inverting several times and distribute 0.5 ml aliquots to the surfaces of non-nutrient agar plates. Distribute the cells evenly over the surface using a spread bar (a spread bar can be made from a Pasteur pipette using the following technique:

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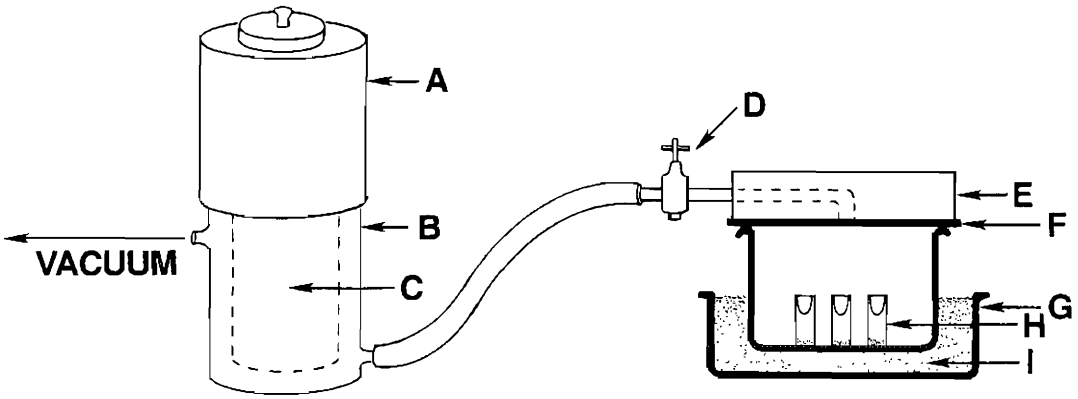
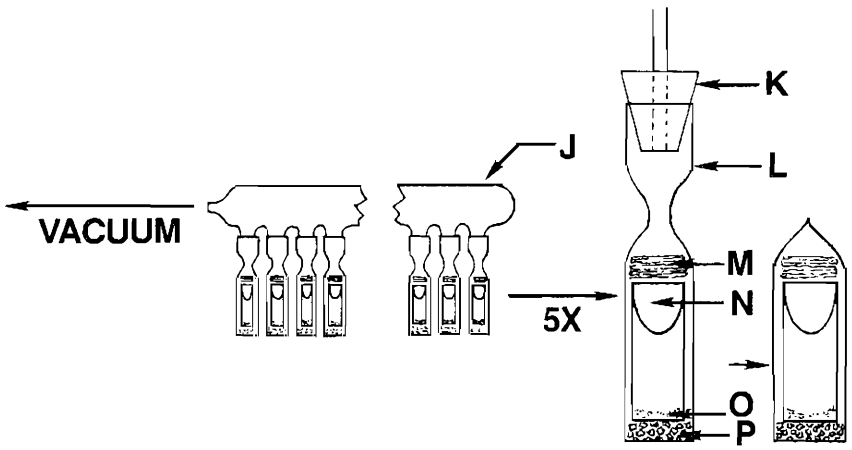


FREEZE-DRYING



SEALING

Fig. 2. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame).

6. Seal the plates shut with parafilm or adhesive tape by wrapping the complete circumference. Places the plates upright at 25° C until the cells encyst. The time will vary with the strain but usually the majority of the cells will have encysted within 7 d.
7. Flood the plates with Page's Balanced Salt Solution and rub the surfaces with a spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame). Transfer the cyst suspensions to 15 ml plastic centrifuge tubes and centrifuge at 500 g for 5 min. Resuspend the pellets and pool in a single centrifuge tube.
8. Determine the cyst concentration using a hemocytometer. The cyst concentration should be 10^7/ml. If the concentration is above this level adjust the concentration with Page's Balanced Salt Solution and note the final volume. If the concentration is below the desired level centrifuge at 500 g, discard the supernatant and resuspend the pellet with a volume of Page's Balanced Salt Solution to achieve the desired level. Again make a note of the final volume.
9. Centrifuge as above, discard the supernatant and replace with an equal volume of the growth medium supplemented with 12% (w/v) sucrose plus 4% (w/v) BSA (Bovine Serum Albumin Fraction V Sigma A-4503). Prepare 100 ml of the protective solution as follows:

    a) Add 12.0 g sucrose to 100.0 ml of medium and dissolve thoroughly.
    b) Divide the above solution into four equal aliquots and transfer each to a separate 50 ml plastic screw-capped centrifuge tube. Add 1.0 g of BSA to each tube and agitate vigorously until dissolved. A considerable amount of foam will overly the liquid.
    c) Centrifuge at 850 g for 10 min to eliminate the layer of foam.
    d) Pool the solutions in a single vessel and filter sterilize. Store refrigerated (5-9° C) for up to 1 y.

10. Dispense cell suspension in 0.25 ml aliquots into cotton-plugged 11.5 x 35.0 mm inner shell freeze-drying glass ampules (Glass Vials, Inc.). Cut off portion of cotton plugs above lip of vial with a pair of scissors.
11. Place vials in a controlled rate freezing unit. Cool at -1° C/min from room temperature to -40° C. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion. Plunge the ampules into liquid nitrogen.
12. Transfer ampules directly to a slotted tray of a commercial freeze-dryer that has been equilibrated to a shelf temperature of -40° C. Proceed quickly to avoid unnecessary warming of the frozen preparations. If ampules must be stored, place in the vapor phase of a nitrogen refrigerator until ready for transfer to the commercial freeze-dryer.
13. Dispense 0.25 ml of suspending medium only (no cells) into two inner shell vials and place them in slots at the end of the tray on the shelf nearest the door of the freeze-dryer. Insert thermocouple probes.
14. Plug the thermocouple probes into the jacks on the inside of the chamber. Allow the vials containing the thermocouple probes to cool to -40° C.
15. Raise the shelf temperature to -30° C and freeze dry at that temperature for at least 18 h. Then increase the shelf temperature in 10° C increments, holding at each temperature at least one h. This can be done manually or with a programmer.
16. When the shelf temperature reaches 0° C, hold at that temperature overnight. Adjust shelf temperature to 37° C the next morning. Product temperature must be at 25° C before the cycle is complete. If necessary, air injection can be used to elevate the pressure to improve heat input to the product and accelerate the drying. Turn off the programmer, turn off the shelf heat and bleed short bursts of air through a Pall filter (Pall Corp.) until the product temperature is approximately 25° C.

17. Turn off the vacuum pump. Using a nitrogen gas cylinder that has been stored at 2-8° C, back fill the chamber with nitrogen gas through a 0.22 micron Pall filter. Do not allow the condenser temperature to go above -50° C. It may be necessary to run the nitrogen gas through a copper coil immersed in liquid nitrogen to maintain condenser temperature.
18. Remove the inner vials from the freeze-dryer to a dry box, where the outer vials are cooling. Insert the inner vials into the outer vials in the dry cabinet. Tamp a ¼-inch plug of glass fiber paper (Whatman #1821-915) above the cotton-plugged inner vial.
19. Remove the vials from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. The vials are then placed back in the dry box to cool.
20. After cooling each vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg.
21. To assure that evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The tip of the spark tester (Electro-Technic Products) is held close to the top of the outer vial. If the vacuum is intact, the inside of the vial will glow purple.
22. The vials are then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.
23. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered. However, **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN FREEZER.**
24. To open vials, heat the top of the outer vial in a flame, then squirt a few drops of H_2O on the hot top to crack the glass. Strike with a file or pencil to remove the top. Remove the fiber paper insulation and the inner vial. Use forceps to gently remove the cotton plug and rehydrate with 0.25 ml of appropriate broth medium. When resuspended, transfer the contents to 5 ml of broth.

COMMENTS

Glass cryules are not **recommended** for cryopreservation. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells, 2) Minor fractures which are not easily detectable can be present which permits the entry of liquid nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas, and 3) To open sealed vials they must be broken open and even with the greatest care injury may occur. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.** If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.
2. Daggett, P-M & Nerad, TA 1982. Freeze-drying methods for some amebae and flagellates. *J. Protozool.* **29**:477.
3. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525.

A-78.1

CRYOPRESERVATION OF SCALED AND TESTATE AMEBAE

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INTRODUCTION

Scaled and testate amebae can be maintained for long periods of time by cryopreservation. The best results are obtained when a specific controlled rate of cooling is used [1]. Methods using two different cooling rates are provided. The first protocol usually provides the best results. Specialized equipment is needed for the methods detailed here. The methods provided are known to be applicable to members of the genera: *Cochliopodium, Capsellina, Chlamydophrys, Gocevia* and *Trichosphaerium*. For strains which are sensitive to cold shock, rapid cooling in the liquid phase may be detrimental. The second protocol may be better. It is possible that the methods can be used for other genera of scaled and testate amebae.

PROTOCOL 1

Capsellina, Chlamydophrys, Cochliopodium, Gocevia

1. For *Capsellina, Chlamydophrys*, and *Cochliopodium* harvest cells from a culture which is at or near peak density. For *Gocevia* allow the culture to encyst before harvesting. To detach cells from plate cultures flush with fresh broth. The surface of the plate is rubbed gently with a spread bar or a rubber policeman to detach adhering amebae. When grown in broth medium agitate the vessel to detach cells. For *Gocevia* rub the bottom of the flask with a sterile cotton swamp to detach cysts.
2. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low concentrate by centrifugation at 600 g for 5 min.
3. While cells are centrifuging prepare a 10% (v/v) solution of sterile DMSO in fresh broth medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will 10^6 cells/ml in a 5% (v/v) DMSO.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.

8. To establish a culture from the frozen state place a cryule in a water bath at 35° C. Immerse the vial just sufficient to cover the frozen material.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh broth medium or onto solidified medium in a petri plate.

PROTOCOL 2

Capsellina, Chlamydophrys, Cochliopodium, Gocevia

1. Follow steps 1-5 of the previous protocol.
2. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min from 25° C to -40° C. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. At -40° C plunge vials into liquid nitrogen.
3. Follow steps 7-9 of the previous protocol.

PROTOCOL

Trichosphaerium

1. Harvest cells from a culture which is at or near peak density. Cells are detached from the surface of a T-25 tissue culture flask by rubbing with a sterile cotton swab.
2. After detaching cells concentrate by centrifugation at 225 g for 5 min. Adjust concentration of cells to 2 x 10^5 ml in fresh broth medium.
3. While cells are centrifuging prepare at 15% (v/v) solution of sterile DMSO in sterile seawater. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Add a volume of the DMSO solution equal to the cell suspension volume but add in 3 equal aliquots at 2 min intervals. Thus, the final concentration will be 10^5 cells/ml in 7.5% (v/v) DMSO.
5. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min from 25° C to -40° C. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. At -40° C plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place a cryule in a water bath at 35° C. Immerse the vial just sufficient to cover the frozen material.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate the entire contents into a T-25 flask containing 10 ml of fresh medium **without** the food source added. The cells are left undisturbed in the dark at 25° C for 3-7 d before the food source is added. The delaying of the addition of the food source decreases the interval to the establishment of a vigorous culture.

COMMENTS

Recovery will be greater using a controlled cooling rate. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

A-79.1

CRYOPRESERVATION OF *GYMNOPHRYDIUM*

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INTRODUCTION

Gymnophrydium is an organism with uncertain taxonomic affinity. There are only two species recognized within the genus [1,2]. Only one has been successfully cultivated, *G. marinum* [3]. This organism forms a highly reticulate plasmodium and as the culture ages various sections of the plasmodium become increasing refractile, round up and become free-floating.

PROTOCOL

1. Harvest a culture that is just beginning to form free-floating spherical bodies as follows: Remove most of the culture fluid with a pipette.
2. Transfer the flask to an ice bath for 10 min.
3. Prepare a solution 20% (v/v) glycerol in the growth medium.
4. Gently rock the flask back and forth to suspend cells.
5. Measure volume of suspension and add an equal volume of cryoprotectant solution prepared in step 3 in three aliquots at 2 min intervals. The final concentration will be 10% (v/v) glycerol.
6. Dispense in 0.5 ml aliquots to 1.0 - 2.0 ml plastic screw-capped cryules (special cryules for cryopreservation).
7. After dispensing place the ampules in a controlled rate freezing apparatus and cool using the following cycle: -1° C/min from room temperature to 4° C; -10° C/min from 4° C to the heat of fusion; -1° C/min from the heat of fusion to -40° C; then plunge into liquid nitrogen.
8. Storage is either in the vapor or liquid phase of a nitrogen refrigerator.
9. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule.
10. Immediately after thawing, do not leave in the water bath, aseptically remove the content of the ampule and gently inoculate into an empty T-25 flask, i.e. without fresh medium, tissue culture flask. Place the flask in a horizontal position and incubate at room temperature.
11. At 15 min intervals add 0.25 ml of the appropriate medium dropwise. Continue until the final volume is 2.0 ml. After the final aliquot is added allow the flask to remain undisturbed for 15 min.
12. Add 0.5 ml of the appropriate medium dropwise at 15 min intervals until the final volume is 4.0 ml. Allow the flask to remain undisturbed over night.
13. In the morning of day 2 slowly add 4.0 ml of the appropriate fresh medium. Allow to remain undisturbed overnight.
14. In the morning of day 3 proceed as follows: Gently remove all but approximately 1 ml of the culture fluid with a pipette and transfer this material to a fresh T-25 flask. Add 10 ml of the appropriate medium to the decanted flask. Maintain both cultures under parallel conditions.

A-79.2

COMMENTS

The thawing protocol using a slow dilution of the thawed material with fresh medium [4] is essential to assure recovery. It may be possible to cryopreserve these organisms using a less complicated cooling cycle. If a controlled rate freeze unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Dangeard, P 1968. Sur une Proteomyxee (Protiste plasmodial) développée dans des cultures d'algues marines et sur son rattachment au genre *Gymnophrydium* de P.A. Dangeard. *C.r. hebd. Seances Acad. Sci, Ser. D.* **266**:1279-1282.
2. Dangeard, PA 1910. Études sur le development et la structure des organismes inferieurs. *Botaniste* **11**:1-332.
3. Kumar, SR 1980. Morphology and taxonomy of the protist *Gymnophrydium marinum* Dangeard from the North Sea. *Bot. Mar.* **23**:353-360.
4. Nerad, TA 1991. Chapter 10: Protozoa and Algae pp. 25-30. In: Simione FP & Brown, EM ed. ATCC Preservation Methods: Freezing and Freeze-drying. 2nd edition. American Type Culture Collection. Rockville, MD 42 pp.

A-80.1

CRYOPRESERVATION OF FORAMINIFERANS

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INTRODUCTION

To date only a single species of *Allogromia* has been successfully cryopreserved [1]. The method employed follows:

PROTOCOL

1. Harvest cells from a near to peak density culture. Agitate the cultures to detach the cells, transfer cell suspensions to 15 ml plastic centrifuge tubes and allow cells to settle.
2. Reduce volume in each tube to approximately 1.0 ml, resuspend cells and pool to a final volume of ~10 ml.
3. Determine cell concentration using a hemocytometer. Adjust concentration to 2.0 x 10^5 cells/ml with fresh medium. It may not be necessary to concentrate the cells further. If the cells must be concentrated, allow cells to settle as in step 1. Adjust final volume to yield desired cell concentration. The cells may be concentrated using centrifugation but centrifuge at the lowest speed that will allow pelleting of the cells.
4. Prepare a 20% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
5. Add a total volume of DMSO solution equal to the volume of the cell preparation but in 3 equal aliquots at 2 min intervals. Mix thoroughly by inverting the tube several times after the introduction of each aliquots of the DMSO solution. The final concentration will be 10^5 cells/ml and 10% (v/v) DMSO.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. After dispensing place the ampules in a controlled rate freezing apparatus and cool using the following cycle: -1° C/min from room temperature to 4° C; -10° C/min from 4° C to the heat of fusion; -1° C/min from the heat of fusion to -40° C; then plunge into liquid nitrogen.
8. Storage is either in the vapor or liquid phase of a nitrogen refrigerator.
9. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule.
10. Immediately after thawing, do not leave in the water bath, aseptically remove the content of the ampule and gently inoculate into an empty T-25, i.e. without fresh medium, tissue culture flask. Place the flask in a horizontal position and incubate at room temperature.
11. At 15 min intervals add 0.25 ml of the appropriate medium dropwise. Continue until the final volume is 2.0 ml. After the final aliquot is added allow the flask to remain undisturbed for 15 min.

12. Add 0.5 ml of the appropriate medium dropwise at 15 min intervals until the final volume is 4.0 ml. Allow the flask to remain undisturbed over night.
13. In the morning of day 2 slowly add 4.0 ml of the appropriate fresh medium. Allow to remain undisturbed overnight.
14. In the morning of day 3 proceed as follows: Gently remove all but approximately 1 ml of the culture fluid with a pipette and transfer this material to a fresh T-25 flask. Add 10 ml of the appropriate medium to the decanted flask. Maintain both cultures under parallel conditions.

COMMENTS

The slow dilution of the thawed material with growth medium [1] is essential to insure recovery. This may not be necessary for all foraminiferans.

LITERATURE CITED

1. Nerad, TA 1991. Chapter 10: Protozoa and Algae pp. 25-30. In: Simione FP & Brown, EM ed. ATCC Preservation Methods: Freezing and Freeze-drying. 2nd edition. American Type Culture Collection. Rockville, MD 42 pp.

A-81.1

CRYOPRESERVATION OF *BLASTOCYSTIS*

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INTRODUCTION

The only species within the genus *Blastocystis*, *B. hominis*, is cryopreservable. The protocol is detailed below.

PROTOCOL

1. In advance prepare a 14% (v/v) sterile glycerol plus 14% (v/v) sterile DMSO solution in Stone's Modification of Locke's Solution (see below) in the following manner: Combine 0.84 ml of sterile glycerol and 0.84 ml sterile DMSO in a 16 x 125 mm screw-capped test tube. When the glycerol and DMSO are combined chemical heat will be liberated. Allow the solution to return to room temperature. To the above solution add 4.32 ml of Stone's Modification of Locke's Solution. Mix by inverting several times, loosen cap one full turn and place in an anaerobic jar with an anaerobic Gaspak for 2-3 d (one BBL anaerobic system GasPak per BBL GasPak 100 anaerobic culture jar).

Stone's Modification of Locke's Solution

NaCl	8.0 g
$CaCl_2$	0.2 g
KCl	0.2 g
$MgCl_2 \cdot 6H_2O$	0.01 g
$Na_2 \cdot HPO_4$	2.0 g
$NaHCO_3$	0.4 g
KH_2PO_4	0.3 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize.

2. When the test tube cultures are at or near peak density (2-3 d old) remove the tubes from the anaerobic jar and immediately screw the caps on tightly. One by one gently remove the cells from the bottom of the egg medium slants and pool in a single 16 x 125 mm screw-capped test tube (work quickly to avoid prolonged exposure to air). Determine the cell concentration using a hemocytometer. Adjust the concentration to 1.0 - 2.0 x 10^7 cells/ml. If the concentration is too high, adjust using the overlay from a reduced (low redox potential) tube of medium. Reduced medium is prepared by loosening the caps of test tubes of growth medium one full turn and placing in an anaerobic jar which contains a BBL Gaspak (one anaerobic system GasPak per BBL GasPak 100 anaerobic culture jar) for 2-3 d. If the concentration is too low, centrifuge at 500 g for 5 min and resuspend the pellet with

a volume of supernatant to yield the desired concentration. Measure the pooled cell volume of the cell suspension by drawing it up gently into a pipette. To the cell suspension add an equal volume of the cryoprotective solution prepared in step 1 and gently mix by aspirating it into a pipette and expelling gently at the bottom of the test tube.

3. The final concentration of the preparation will be 7% (v/v) glycerol and 7% (v/v) DMSO and 5.0×10^6 to 1.0×10^7 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the cryoprotective solution to the cells.
4. Gently dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Plunge ampules into liquid nitrogen and then store in the vapor phase of a liquid nitrogen refrigerator.
6. Two d before attempting to establish a culture from the frozen state prepare a tube of reduced medium (see step 2).
7. When the medium is ready for inoculation place the frozen ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
8. Immediately after thawing, do not leave in the water bath, aseptically, gently lower a sterile Pasteur pipette from which the air has been expunged to the bottom of the liquid in the ampoule and slowly aspirate the entire contents into the pipette. **Be careful to minimize agitation of the fluid and do not introduce air bubbles at any time. These organisms are sensitive to oxygen.**
9. Add the cell suspension to the bottom of the liquid overlay of the reduced tube of medium prepared in step 6. **Avoid the expulsion of air bubbles from the tip of the pipette.**
10. With the cap of the test tube loosened one full turn place it in an anaerobic jar which contains a BBL Gaspak (one anaerobic system GasPak per BBL GasPak 100 anaerobic culture jar). Close the vessel securely and incubate at 35° C.
11. Thereafter maintain using standard protocol.

COMMENTS

Work as quickly as possible to avoid exposure of the cells to excessive amounts of air. These organisms are sensitive to air. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

A-82.1

CRYOPRESERVATION AND FREEZE-DRYING OF SLIME MOLDS

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INTRODUCTION

Many genera of slime molds can cryopreserved. Species of the following 11 genera have been cryopreserved using the protocols described herein: *Acytostelium, Badhamia, Cavostelium, Dictyostelium, Didymium, Echinostelium, Physarum, Polysphondilium, Protostelium, Schizoplasmodiopsis*, and *Stemonitis* [1]. These methods may also be applicable to other genera. The resting stages, cysts and/or spores, of some species can also be freeze-dried [1,2].

PROTOCOL

Cryopreservation
Strains cultivated with bacteria or yeast

1. Allow agar cultures to encyst or form sporocarps. Flood the plates with a 10% (v/v) sterile glycerol solution prepared in fresh broth medium. Rub the surface of the agar plates with a sterile spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame) to help detach adhering cells.
2. Transfer the resulting cell suspension to a 15.0 ml plastic centrifuge tube. Determine the cell concentration using a hemocytometer. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low, concentrate by centrifugation at 600 g for 5 min.
3. The final concentration of the preparation will be 10% (v/v) glycerol and 10^6 cells/ml. The cooling cycle should be initiated no less 15 min and no longer than 60 min after addition of the glycerol to the cells.
4. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge ampules into liquid nitrogen. Store ampules in the vapor phase of a liquid nitrogen refrigerator.
6. To establish a culture from the frozen state transfer to a water bath set at 35° C. Do not completely immerse the vial. Immerse to a level sufficient to cover the frozen material. Do not agitate the vial.

7. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate onto the surface of the appropriate agar medium.

PROTOCOL

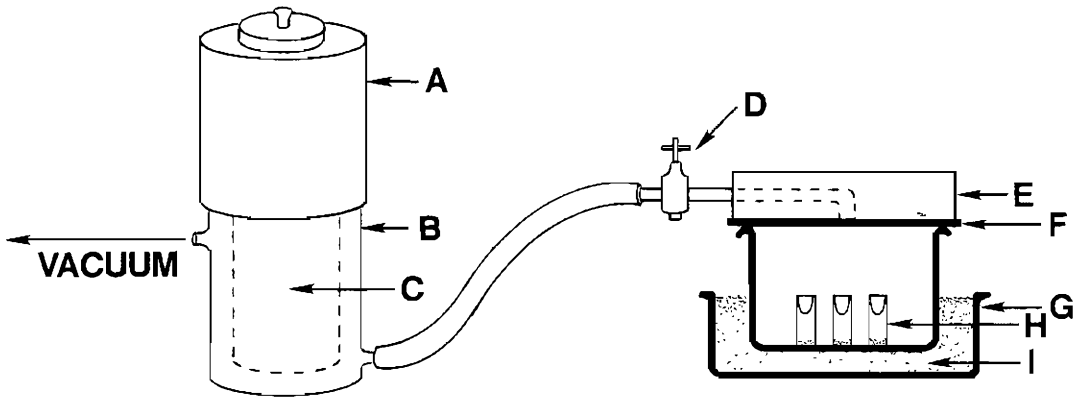
Cryopreservation
Axenic Liquid Cultures

1. In advance prepare a 20% (v/v) sterile glycerol solution in fresh broth medium.
2. Harvest a culture at or near peak density in the following manner: Gently remove as much culture fluid overlying the adhering cells as possible. Place the flasks on ice for 10 min and then gently swirl to suspend the cells.
3. Transfer the resulting cell suspension to a 15.0 ml plastic centrifuge tube. Determine the cell concentration using a hemocytometer. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low, concentrate by centrifugation at 400 g for 5 min.
4. Mix the cell suspension and the 20% (v/v) glycerol solution in a 1:1 ratio.
5. The final concentration of the preparation will be 10% (v/v) glycerol and 10^6 cells/ml. The cooling cycle should be initiated no less 15 min and no longer than 60 min after addition of the glycerol to the cells.
6. Follow steps 4-6 in the first protocol.
7. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 10.0 ml of fresh medium in a T-25 flask. Thereafter follow standard protocol for maintenance.

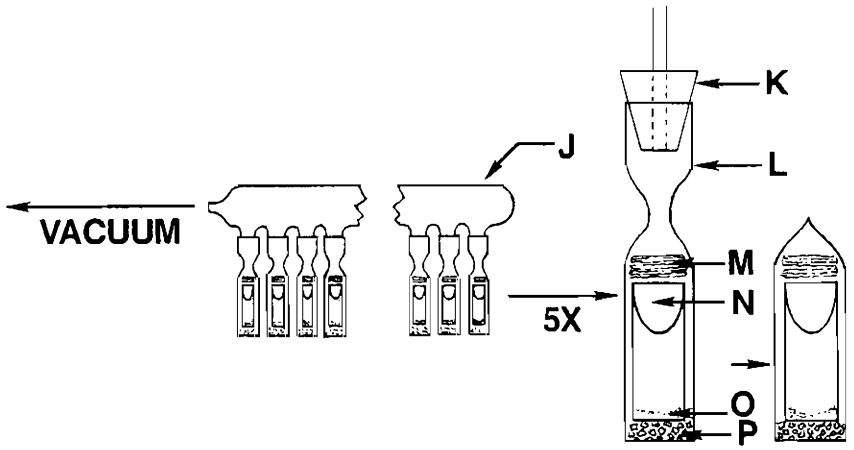
PROTOCOL

Freeze-drying
(Fig. 1)

1. Prepare in advance a sterile 20% (w/v) skim milk (Difco 0032) suspension.
2. Prepare outer vials (Glass Vials, Inc., 14.25 mm x 85.0 mm) by placing a small amount of silica gel granules (Fisher Scientific, grade 42, 6-16 mesh, "Tel-Tale" brand) in the vial to cover about half of the bottom. Add a small cotton wad to cushion the inner vial and heat in a 100° C oven overnight. The silica gel should be dark blue after heating and serves as a moisture indicator during storage. Place vials in a dry box (<10% relative humidity) to cool.
3. Agar cultures that have encysted or formed sporocarps are flooded with fresh broth medium. The surface of agar can be gently rubbed with a sterile spread bar (a spread bar can be made from a Pasteur pipette using the following technique: Heat the pipette at an area about 4-5 cm from the tip. Allow the pipette tip to drop at a 90° angle to the horizontal axis and then seal the end of the pipette in the flame) to help detach adhering cells.
4. Transfer the resulting cell suspension to a 15.0 ml plastic centrifuge tube. Determine the cell concentration using a hemocytometer. The final cell concentration desired is 10^8/ml. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells with fresh broth medium. Note the final volume of the suspension. Centrifuge the suspension at 500 g for 5 min, remove as much supernatant as possible, and suspend the cell pellet with a volume of the 20% skim milk suspension, equivalent to the volume of the supernatant removed. If the cell concentration is too low, centrifuge at 500 g for 5 min. Resuspend the cell pellet with a volume of the 20% skim milk suspension that will yield the final desired cell concentration.



FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

5. Dispense cell/skim milk suspension in 0.25 ml aliquots in cotton-plugged inner shell vials. Cut off portion of cotton plugs above lip of vial with a pair of scissors.
6. Place vials in a stainless steel pan and then transfer to a mechanical freezer set at - 55° C for 2 h. Alternatively place the vials in a controlled rate freezing unit and cool from room temperature at -1° C/min. If freezing unit can compensate for the heat of fusion, maintain a rate of -1° C/min through heat of fusion to -55° C. Transfer the vials to a stainless steel pan in a the mechanical refrigerator set at -55° C.
7. Just prior to freeze-drying a moisture trap condenser attached to a vacuum pump is prepared by fitting a trimmed block of dry-ice which just fits snugly into a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added over the dry-ice to a level up to the lip of the condenser.
8. Place container with frozen ampules on a 3 cm thick layer of freshly prepared dry-ice snow. Pack snow around sides of container for several h. Place the Atmo-vac plate cover (Refrigeration for Sciences Inc.) over the pan with the ampules and place under vacuum of at least 30 μm of Hg. The cover is attached to the condenser by Tygon tubing which will not collapse. Keep under vacuum for at least 12 h, after which all of the dry-ice snow will have evaporated. Turn off the vacuum pump and slowly introduce air which has been previously passed over a desiccant to minimize introduction of moisture.
9. In advance the 14.25 x 85.0 mm outer shell freeze-drying glass vials (Glass Vials, Inc.) are prepared. The bottom of the vial is covered with 6-16 mesh silica gel granules on top of which a cotton wad is added to provide cushioning for the smaller shell vial. At this stage the preparation is placed in a 100° C oven for 12 h. The silica gel should have turned dark blue and will serve as a moisture indicator during storage.
10. Allow outer shell vials to cool in a dry cabinet (10% or least relative humidity) and then insert inner shell vial with the freeze-dried preparation. On top of the inner vial a wad of glass fiber paper is inserted to prevent exposure of the freeze-dried preparation to excessive heat during flame sealing.
11. The vials are removed from the dry cabinet to be flame sealed. The outer vial is rotated in the hottest part of an air/gas flame approximately 0.5 cm above the wad of glass fiber paper. The bottom of the vial is held in one hand and the lip of the vial is pulled slowly using a pair of forceps until a narrow neck is formed. After cooling the vial is securely affixed to a number 00 stopper attached to a port manifold. Once all the vials have been affixed, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch at the narrowing of the vial above the glass fiber paper.
12. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered. However, **FOR SAFETY REASONS DO NOT STORE SEALED VIALS IN A LIQUID NITROGEN REFRIGERATOR.**
13. To establish a culture from the freeze-dried state add 0.25 ml of sterile glass distilled H_2O or sterile fresh broth medium. After pellet dissolves dispense onto the surface of fresh agar medium. Incubate at the appropriate temperature.
14. Thereafter follow standard protocol for culturing.

COMMENTS

Glass cryules are **not recommended** for cryopreservation. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells, 2) Minor fractures which are not easily detectable can be present which permits the entry of liquid nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas, and 3) To open sealed vials they must be broken open and

even with the greatest care injury may occur. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.** If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51pp.
2. Davis, EE 1965. Preservation of Myxomycetes. *Mycologia* **57**:986-988.

A-83.1

CRYOPRESERVATION OF LABYRINTHULIDS

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INTRODUCTION

The genus *Labyrinthula* can be maintained for long periods by cryopreservation and storage at cryogenic temperatures [1,2]. The protocol detailed here may also be applicable to other genera.

PROTOCOL

1. Harvest an agar cultures at or near peak density as follows: Flood with a 10% (v/v) glycerol solution prepared in fresh broth medium. Harvesting cells in as gentle a manner as possible will enhance recovery. The surface of the plates can be gently rubbed with a sterile spread bar to help detach adhering cells. When grown in broth medium agitate the vessel to detach cells and add an equal volume of 20% (v/v) sterile glycerol solution prepared in fresh broth medium.
2. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low, concentrate by centrifugation at 600 g for 5 min.
3. The final concentration of the preparation will be 10% (v/v) glycerol and 10^6 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
4. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At - 40° C the ampules are plunged into liquid nitrogen. The ampules are stored in the vapor phase of a liquid refrigerator.
6. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
7. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium.

COMMENTS

Although glass cryules can be used it is **not recommended**. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with

the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.
2. Davis, EE 1965. Preservation of Myxomycetes. *Mycologia* **57**:986-988.

A-84.1

CRYOPRESERVATION OF *PLASMODIUM*

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INTRODUCTION

Both *in vivo* and *in vitro* cultivated strains of *Plasmodium* can be easily kept for long periods by cryopreservation and storage at cryogenic temperatures [1]. No special equipment is required, but liquid nitrogen must be available. **Since some species of the parasite are infective to humans, safety should be a primary concern.** Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

In vivo, all *Plasmodium* species except
P. berghei and *P. vinckei*

1. In advance prepare a 30% (v/v) sterile glycerol solution in Alsever's solution (see below).

NaCl	4.2 g
Na_3 citrate·$2H_2O$	8.0 g
Glucose	20.5 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O, adjust pH to 6.1 with 10% (w/v) citric acid and filter sterilize. The solution can be obtained from GIBCO (cat #670-5190).

2. Prepare a syringe equipped with the appropriate gauge needle as follows: Draw Yaeger's anticoagulant solution (see below) into the syringe. Move the plunger of the syringe back and forth several times to distribute the anticoagulant. Adjust the final volume of the anticoagulant to 1/10 th of the amount of blood normally obtained from the host animal.

Sodium citrate	1.33 g
Citric acid	0.47 g
Dextrose	3.00 g
Sodium heparin	0.2 g
Glass distilled H_2O	100.0 ml

3. Draw blood into the syringe by gently pulling the plunger outward. When blood is no longer obtainable or the mouse has died remove needle and invert the syringe several times to mix the anticoagulant evenly with the blood.
4. Transfer the collected blood to a 16 x 125 mm screw-capped test tube and determine the volume by drawing the blood into a pipette. Expel the blood into the test tube after the volume has been determined.

5. Mix the heparinized blood with the 30% glycerol solution in a 2:1 ratio. If any clotting has occurred **do not use**. After mixing the final concentration of the cryoprotective agent will be 10% (v/v). The mixture should be placed in a 4° C ice bath. The time from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation). Filled ampules should be placed in a 4° C ice bath. Be careful not to immerse the ampules to the level of the vial cap. **SAFETY PRECAUTION:** Although glass cryules can be heat sealed the authors recommend that the vials not be sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open a sealed vial it must be broken. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS.**
7. Directly from 4° C plunge into liquid nitrogen.
8. The frozen preparations may be stored in a mechanical freezer until needed, but storage in either the vapor or liquid phase of a nitrogen refrigerator is recommend for the longest viability. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**
9. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
10. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule into a syringe and inoculate into an uninfected mouse. Follow the protocol for maintenance *in vivo*. The course of the infection may be longer or shorter than usual dependent on percent recovery of the parasite from the frozen state.

PROTOCOL

In vivo, P. berghei, P. vinckei

1. Follow the first protocol but substitute 19.6% (v/v) glycerol for 30% (v/v) glycerol in step 1 and mix 15 parts blood to 85 parts of cryoprotective solution in step 3. The final concentration of the cryoprotective agent will be 16.7% (v/v).

PROTOCOL

In vitro

1. In advance prepare a 28% (v/v) glycerol + 0.65% (w/v) NaCl + 3.5% (w/v) sorbitol solution in glass distilled H_2O. Autoclave for 15 min at 121° C.
2. Also in advance prepare a dry-ice alcohol bath such that the temperature of the bath is -80° C.
3. Infected red blood cells from culture are centrifuged for 7 min at 850 g.
4. The supernatant is removed and the pellet formed is resuspended in an equal volume of the cryoprotective solution.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation). **SAFETY PRECAUTION:** Although glass cryules can be heat sealed the authors recommend that the vials not be sealed for three reasons: (1) Even with the greatest care, any heating of the vial can kill the cells in solution. (2) Minor fractures which are not immediately detectable

can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. (3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS.**

6. Directly plunge the ampules in the -80° C dry-ice alcohol bath for 2 min. The time from the mixing of the infected cells with the cryoprotective solution to plunging should be not less that 15 min and no more than 60 min.
7. The frozen preparations may be stored in the mechanical freezer until needed, but storage in either the vapor or liquid phase of a nitrogen refrigerator is recommend for the longest viability. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C with the cap slightly loosened. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. After thawing transfer to a centrifuge tube and centrifuge for 7 min at 850 g.
10. Remove the supernatant and resuspend cells (usually about 0.2 ml) in an equal volume of sterile 3.5% (w/v) NaCl and centrifuge again for 7 min at 850 g.
11. Remove supernatant and resuspend in a equal volume of fresh cultivation medium and centrifuge again.
12. Repeat previous step.
13. Remove supernatant and resuspend in a equal volume of fresh cultivation medium. Add 50% solution of washed uninfected red blood cells in fresh medium to give a final volume of 1.0 ml.
14. Add 5.0 ml of fresh medium to the preceding preparation and dispense in 1.0 - 2.0 ml aliquots into each of four 3.5 cm petri dishes. Incubate according to normal cultivation protocol.
15. Follow the protocol for maintenance *in vitro* thereafter.

COMMENTS

Although glass cryules can be used it is **not recommended**. If it is absolutely necessary to use glass vials they should be cotton-plugged and not heat sealed for three reasons: (1) Even with the greatest care, excessive heating of the vial can kill the cells in solution. (2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. (3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS. DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.

A-85.1

CRYOPRESERVATION OF *TOXOPLASMA GONDII*

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INTRODUCTION

Toxoplasma gondii, whether maintained *in vitro* or *in vivo*, is amenable to cryopreservation for long term maintenance. **Since the parasite is infective to humans, safety should be a primary concern**. Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

In vivo

1. In advance the following sterile solution should be prepared:

Tyrodes Salt Solution

NaCl	8.0 g
KCl	0.2 g
$CaCl_2$	0.2 g
$MgCl_2 \cdot H_2O$	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.05 g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize.

2. Prepare a combined solution of 10% (v/v) sterile DMSO and 10% (v/v) sterile glycerol in Tyrodes Salt Solution. The solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
3. Harvest tachyzoites from one or more infected mice using the following procedure: Kill mice using cervical dislocation and stake onto a dissecting board with the abdomen facing upward. Wash abdominal area with 70% denatured alcohol. **CAUTION: Do not use an excessive amount. Be aware of any nearby open flames.** Inject 2.0 ml of Tyrodes solution aseptically into the peritoneum using a 27 gauge, ½ inch needle attached to a 5 ml syringe. Massage the belly and aseptically remove as much fluid as possible with a 20 gauge 1½ inch needle attached to a 5 ml syringe. The fluid obtained should be translucent. If more than one infected mouse is used, pool the material obtained.
4. Adjust the concentration of tachyzoites to between 2.0×10^6 - 10^7 cells/ml with Tyrodes Salt Solution.
5. Mix the peritoneal fluid and the cryoprotective solution in equal portions. The final concentration will be 5% (v/v) DMSO + 5% (v/v) glycerol, 10^6 - 10^7 cells/ml. The time

from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.

6. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
8. Store in either the vapor or liquid phase of a nitrogen refrigerator.
9. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
10. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule into a 1.0 ml syringe fitted with a 27 gauge ½ inch needle and inoculate intraperitoneally into an uninfected mouse. Follow the protocol for maintenance *in vivo*.

PROTOCOL

In vitro

1. Prepare a combined solution of 10% (v/v) sterile DMSO, 10% (v/v) glycerol and 17% (v/v) heat-inactivated (30 min at 56° C) fetal calf serum (HIFCS) in the medium used to grow the cells. Prepare 10 ml of cryoprotective solution in the following manner:

    a) Add 1.0 ml of DMSO in a 16 x 125 mm screw-capped test tube
    b) Add 1.0 ml of glycerol and mix thoroughly. This solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature.
    c) Add 6.3 ml of growth medium without serum and mix thoroughly.
    d) Add 1.7 ml of HIFCS and mix thoroughly.

2. Harvest the cultures by gently agitating the contents of each flask and transfer into 15 ml plastic centrifuge tubes. The attached remaining tissue culture cells (both infected and uninfected) in each flask are detached as follows. Add 1.0 ml of 0.25% (w/v) trypsin dissolved in Hanks' Balanced Salt Solution (see below), distributing the trypsin solution evenly over the cell layer by gentle movement of the flask. Remove the trypsin solution and then incubate the flasks at 35° C for 10 min. Forcefully expel 10 ml of fresh medium onto the monolayer of one of the flasks using a pipette. Remove the cell suspension and expel it onto the monolayer of the next flask and so forth, until all the flasks have been flushed. Remove the cell suspension from the place in a 15 ml plastic centrifuge tube. Centrifuge the cell suspensions at 850 g for 10 min. The cell pellets are pooled and resuspended with supernatant to a final volume of approximately 5 ml.

Hanks' Balanced Salt Solution (HBSS)

NaCl	8.0 g
KCl	0.4 g
$Na_2HPO_4 \cdot 2H_2O$	0.06 g
KH_2PO_4	0.06 g
$CaCl_2$	0.14 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$NaHCO_3$	0.35 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize. The solution can be obtained from GIBCO (cat #310-4020)

3. Adjust concentration to tachyzoites to between 2.0 x 10^7 - 10^8 cells/ml with fresh medium formula for HBSS.
4. Mix the cell preparation and the cryoprotective solution in equal portions. The final concentration will be 10^7 - 10^3 cells/ml in 5% (v/v) DMSO + 5% (v/v) glycerol + 10% (v/v) fetal calf serum. The time from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into an uninfected cell monolayer which has been previously established. Follow the protocol for maintenance *in vitro*.

COMMENTS

Recovery will be greater using a controlled cooling rate [1,2]. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). An alternative approach is to use an uncontrolled rate of cooling. Place ampules on the bottom of a mechanical freezer set at between -55 to -70° C for 1 h. Remove and plunge into liquid nitrogen, then store in the vapor phase of a nitrogen freezer [1]. Although glass cryules can be used it is **not recommended**. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution, 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials they must be broken open. Even with the greatest care injury **AUTOINOCULATION IS POSSIBLE. DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.
2. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

A-86.1

CRYOPRESERVATION OF SELECTED COCCIDIA

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INTRODUCTION

Eimeria and *Cryptosporidium* have been reported to be amenable to cryopreservation for long term maintenance [1,3]. **Since these parasites may be infective to humans, safety should be a primary concern.** Careful planning before a protocol is essential to exercising the required caution. The protocol for *Eimeria* is presented below.

PROTOCOL

1. For *in vivo* strains harvest oocysts from feces of host and clean with Clorox. For *in vitro* strains harvest from cell culture monolayer.
2. Centrifuge at 3,000 g for 10 min.
3. Discard supernatant and resuspend in Hanks' Balanced Salt Solution (see below) and centrifuge as in step 2.

Hanks' Balanced Salt Solution (HBSS)

NaCl	8.0 g
KCl	0.4 g
$Na_2HPO_4 \cdot 2H_2O$	0.06 g
KH_2PO_4	0.06 g
$CaCl_2$	0.14 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$NaHCO_3$	0.35 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize. The solution can be obtained from GIBCO (cat #310-4020).

4. Repeat step 3.
5. Discard supernatant and resuspend in HBSS.
6. Break the oocyst walls to release sporocysts using the following technique: Aseptically add sterilized glass beads and vortex for 10 min.
7. Separate the suspension from the glass beads and centrifuge as in step 2. Discard supernatant and suspend pellet in 4.5 ml of HBSS supplemented with 0.5 ml of heat-inactivated fetal calf serum. Sporozoites can be excysted from sporocysts by suspending in 0.25% (w/v) trypsin plus 0.75% (w/v) bile salt solution at 37° C. Optimum excystment temperature will

depend on the body temperature of the host. Centrifuge to concentrate and resuspend sporozoite pellet in HBSS. Adjust concentration to 2.0 x 10^6 sporozoites/ml.

8. Add dropwise an equal volume of a 15% (v/v) sterile DMSO solution prepared in HBSS. When the DMSO is mixed with the medium chemical heat is produced. Allow the mixture to return to room temperature before adding to the cells.
9. The final concentration will be 7.5% (v/v) DMSO and 10^8 cells/ml.
10. Equilibrate at room temperature for 16-19 h if sporocysts are used or 15-60 min if sporozoites are used.
11. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
12. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
13. Store in either the vapor or liquid phase of a nitrogen refrigerator.
14. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
15. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule into a 1 dram vial and dropwise add 2.0 ml of HBSS.
16. If sporocysts were frozen, orally administer the entire volume to a chicken that is 1-5 weeks old into a T-25 flask containing a monolayer of the appropriate cell line.

COMMENTS

Not all coccidians are as readily amenable to cryopreservation as are species of Eimeria. Although the successful preservation of oocysts of *Cryptosporidium* has been reported [3], attempts by others to cryopreserve the sporozoites or the oocysts have been unsuccessful [2]. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). An alternative approach is to use an uncontrolled rate of cooling. Place ampules on the bottom of a mechanical freezer set at between -55 to -70° C for 1 h. Remove and plunge into liquid nitrogen, then store in the vapor phase of a nitrogen freezer [1]. Although glass cryules can be used it is **not recommended**. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution, 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials they must be broken open. Even with the greatest care injury **AUTOINOCULATION IS POSSIBLE. DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Doran, DJ 1969. Freezing excysted coccidial sporozoites. *J. Parasitol.* **55**:1229-1233.
2. Fayer, R, Nerad, T, Rall, W, Lindsay, DS & Blagburn, BL 1991. Studies on cryopreservation of *Cryptosporidium parvum*. *J. Parasitol.* **77**:357-361.
3. Rossi, P, Pozio, E & Besse, MG 1990. Cryopreservation of *Cryptosporidium* sp. oocysts. *Trans. R. Soc. Trop. Med. Hyg.* **84**:68.

A-87.1

CRYOPRESERVATION OF *PNEUMOCYSTIS*

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INTRODUCTION

Pneumocystis carinii appears to be a very hardy organism and can survive exposure to cryogenic temperatures following controlled or uncontrolled cooling (M. Cushion, personal communication). The protocol developed by Armstrong and Richards [1] with variations is presented below. **Since these parasites may be infective to humans, safety should be a primary concern.** Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

1. Kill infected rats by cervical dislocation.
2. Aseptically remove lungs.
3. Place lungs in 30 ml of chilled phosphate buffered saline (PBS) in a sterile 125 ml cotton-plugged Erlenmeyer flask. Aseptically add a sterilized stirring bar and stir vigorously in a walk-in refrigerator for 60 min.
4. Aseptically remove the cell suspension excluding the lungs and transfer to a 50 ml screw-capped centrifuge tube that can withstand high speed centrifugation.
5. Centrifuge at 1,300 g for 10 min under refrigeration.
6. Discard the supernatant and resuspend the cell pellet in 30 ml of PBS. Centrifuge as above.
7. Repeat step 6.
8. Discard the supernatant and resuspend the pellet in 3 ml of PBS supplemented with 20% (v/v) heat-inactivated fetal calf serum (HIFCS).
9. Prepare a 20% (v/v) glycerol solution in PBS.
10. Mix the cell suspension with the cryoprotective solution in a 1:1 ratio. The final concentration will be 10% (v/v) glycerol in PBS with a final concentration of 10% (v/v) HIFCS.
11. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
12. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C transfer directly to a mechanical refrigerator set at -70° C or plunge into liquid nitrogen.
13. Store in the mechanical refrigerator or either the vapor or liquid phase of a nitrogen refrigerator.
14. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
15. Anaesthetize two immunosuppressed rats and allow them each to aspirate 0.25 ml aliquots of the thawed suspension.

A-87.2

COMMENTS

As prepared above *P. carinii* cannot be enumerated because it is mixed with lung cells and both are entrained in mucoid material. The preparation can be purified to a great extent by passage through filters of various porosities [1]. Armstrong and Richards [2] reported storage of frozen preparations at -70° C for 7 months with no apparent loss of viability. Storage at -130° C or lower will ensure indefinite stability. *P. carinii* has been successfully cryopreserved using 7.5% (v/v) DMSO as a cryoprotectant (M. Cushion, personal communication). If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA). An alternative approach is to use an uncontrolled rate of cooling. Place ampules on the bottom of a mechanical freezer set at between -55 to -70° C for 1 h. Remove and plunge into liquid nitrogen, then store in the vapor phase of a nitrogen freezer. Although glass cryules can be used it is **not recommended**. If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution, 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials they must be broken open. Even with the greatest care injury **AUTOINOCULATION IS POSSIBLE. DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Armstrong, MYK & Richards, FF 1989. Propagation and purification of rat *Pneumocystis carinii* in short-term cell culture. *J. Protozool.* **36**:245-275.
2. Wakefield, AE, Hopkin, JM, Burns, J, Hipkiss, JB, Steward, TJ, & Moxon, ER 1989. Cloning of DNA from *Pneumocystis carinii*. *J. Protozool.* **36**:5S-7S

A-88.1

CRYOPRESERVATION OF *BABESIA*

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INTRODUCTION

Babesia is easily kept for long term maintenance by cryopreservation and storage at cryogenic temperatures. No special equipment is required, but liquid nitrogen must be available [1]. **Since the parasite is infective to humans, safety should be a primary concern**. Careful planning before a protocol is essential to exercising the required caution.

PROTOCOL

1. In advance the following sterile solution should be prepared:

Tyrodes Salt Solution

NaCl	8.0 g
KCl	0.2 g
$CaCl_2$	0.2 g
$MgCl_2 \cdot H_2O$	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.05g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize.

2. Prepare a 10% (v/v) solution of sterile DMSO or 20% (v/v) sterile glycerol in the Tyrodes Salt Solution. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
3. Prepare a syringe equipped with the appropriate gauge needle as follows: Draw Yaeger's anticoagulant solution (see below) into the syringe. Move the plunger of the syringe back and forth several times to distribute the anticoagulant. Adjust the final volume of the anticoagulant to 1/10 th of the amount of blood normally obtained from the host animal.

Sodium citrate	1.33 g
Citric acid	0.47 g
Dextrose	3.00 g
Sodium heparin	0.2 g
Glass distilled H_2O	100.0 ml

4. Draw blood into the syringe by gently pulling the plunger outward. When blood is no longer obtainable or the mouse has died remove needle and invert the syringe several times to mix the anticoagulant evenly with the blood. If clotting occurs during extraction of blood insufficient anticoagulant has been used.
5. Transfer the collected blood to a 16 x 125 mm screw-capped test tube and determine the volume by drawing the blood into a pipette. Expel the blood into the test tube after the volume has been determined.
6. Mix the heparinized blood and the DMSO (or glycerol solution) in equal portions. If any clotting has occurred **do not use**. After mixing the final concentration of the cryoprotective agent will be 5% (v/v) DMSO or 10% (v/v) glycerol. The time from the mixing of the cell preparation and cryoprotective solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
7. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or sterile plastic screw-capped cryules (special plastic vials for cryopreservation). **SAFETY PRECAUTION:** Although glass cryules can be heat sealed the authors recommend that the vials not be sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS.**
8. Plunge vials directly into liquid nitrogen.
9. The frozen preparations may be stored in either the vapor or liquid phase of a nitrogen refrigerator. **DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**
10. To establish an infection from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
11. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule into a syringe and inoculate into an uninfected mouse. Follow the protocol for maintenance *in vivo*. The course of the infection may be longer or shorter than usual dependent on percent recovery of the parasite from the frozen state.

COMMENTS

Although glass cryules can be used it is **not recommended.** If it is absolutely necessary to use glass vials they should be cotton-plugged and not be heat sealed for three reasons: 1) Even with the greatest care, any heating of the vial can kill the cells in solution. 2) Minor fractures which are not immediately detectable can be present which permits the entry of nitrogen during storage. When a sealed ampule is removed from storage the rapid change in temperature can result in explosion of the vial due to expanding nitrogen gas. 3) To open sealed vials it must be broken open. Even with the greatest care injury can occur. **AUTOINOCULATION IS POSSIBLE WITH SEALED GLASS VIALS. DO NOT STORE COTTON-PLUGGED GLASS VIALS DIRECTLY IN LIQUID NITROGEN.**

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I Laboratory Manual on Preservation Freezing and Freeze-drying. American Type Culture Collection, Rockville, MD, 51 pp.

A-89.1

LONG TERM MAINTENANCE OF MICROSPORIDA BY FREEZE-DRYING AND CRYOPRESERVATION

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INTRODUCTION

Only a limited number of species of microsporida, belonging mostly to *Nosema*, have been reported to be successfully cryopreserved or freeze-dried [1]. The known methods may have application to genera other than those reported. The first protocol detail an uncontrolled cooling method. A protocol for the freeze-drying of *Pleistophora* (Sherlock, Personal communication). In both protocols, the spore stage which is naturally resistant to environmental stress, is utilized.

PROTOCOL

Uncontrolled Cooling

1. Harvest spores and concentrate to at least 4.0 x 10^7/ml.
2. To the spore preparation add a volume 100% (v/v) sterile glycerol in volume equal to the spore suspension volume.
3. Thoroughly mix the spore preparation and the glycerol. The final concentration of this preparation is 2.0 x 10^7/ml spore in 50% (v/v) glycerol. The time from the mixing of the cell preparation an the glycerol solution should be no less than 15 min and no more than 60 min.
4. Dispense in 1.0 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
5. After dispensing directly plunge the cryules into liquid nitrogen.
6. Store the frozen preparations either the vapor or liquid phase of a nitrogen refrigerator.
7. To establish a culture from the frozen state place a cryule in a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material.
8. Immediately after thawing, do not leave in the water bath, dispense thawed material over insect food.

A-89.2

PROTOCOL

Freeze-drying, Uncontrolled cooling, *Pleistophora*
(Fig. 1)

1. Remove the gut of an infected insect.
2. Place the gut aliquots into sterile glass cotton-plugged 1.0 ml freeze-drying tubular ampules (Bellco Glass Co., 8 mm outside diameter)
3. Repeat the process for each ampule until the total number of ampules have been prepared.
4. Place in a mechanical refrigerator set at -70° C for at least 2 h.
5. Just prior to freeze-drying a moisture trap (condenser) attached to a vacuum pump is prepared by placing a trimmed block of dry-ice which just fits into a stainless steel drum manifold (Virtis Model #6211 0245). Cellosolve (Fisher Scientific E-180) is added just to the top of the condenser containing the dry-ice.
6. While still frozen and in the freezer each vial is attached by Tygon tubing to a manifold. The manifold is connected to the moisture trap which should be located outside the freezer.
7. After all the ampules have been attached the vacuum is turned on. The pressure should be approximately 100 μm of Hg.
8. Keep under vacuum for at least 24 h.
9. After 24 h vacuum is discontinued and the ampules are removed from the freezer.
10. The neck of the ampule is partially constricted by heating the glass with the hottest part of an air/gas flame until red hot near the upper part of the neck. Slowly pull the vial using forceps to make a constriction.
11. Resume the vacuum at room temperature for 3 h at a pressure of 40 μm of Hg.
12. Complete the sealing of the ampule while under vacuum as follows: heat the glass the hottest part of an air/gas flame in the area of the constriction. Slowly pull the vial using forceps to close until sealed.
13. Sealed vials can be stored at room temperature but viability will be extended for longer periods as the storage temperature is lowered.
14. To establish a culture from the freeze-dried state aseptically add 0.5 ml of glass distilled H_2O. Remove the contents and homogenize or grind carefully for 2-5 min.
15. Dispense rehydrated, homogenized preparation over food to be fed to insects. Thereafter follow routine protocol for *in vivo* cultivation.

LITERATURE CITED

1. Alexander, M, Daggett, P-M, Gherna, R, Jong, S & Simione, FP, Jr 1980. American Type Culture Collection Methods. I. Laboratory Manual on Preservation Freezing and Freeze-drying As Applied to Algae Bacteria, Fungi and Protozoa. American Type Culture Collection, Rockville, MD, 51 pp.

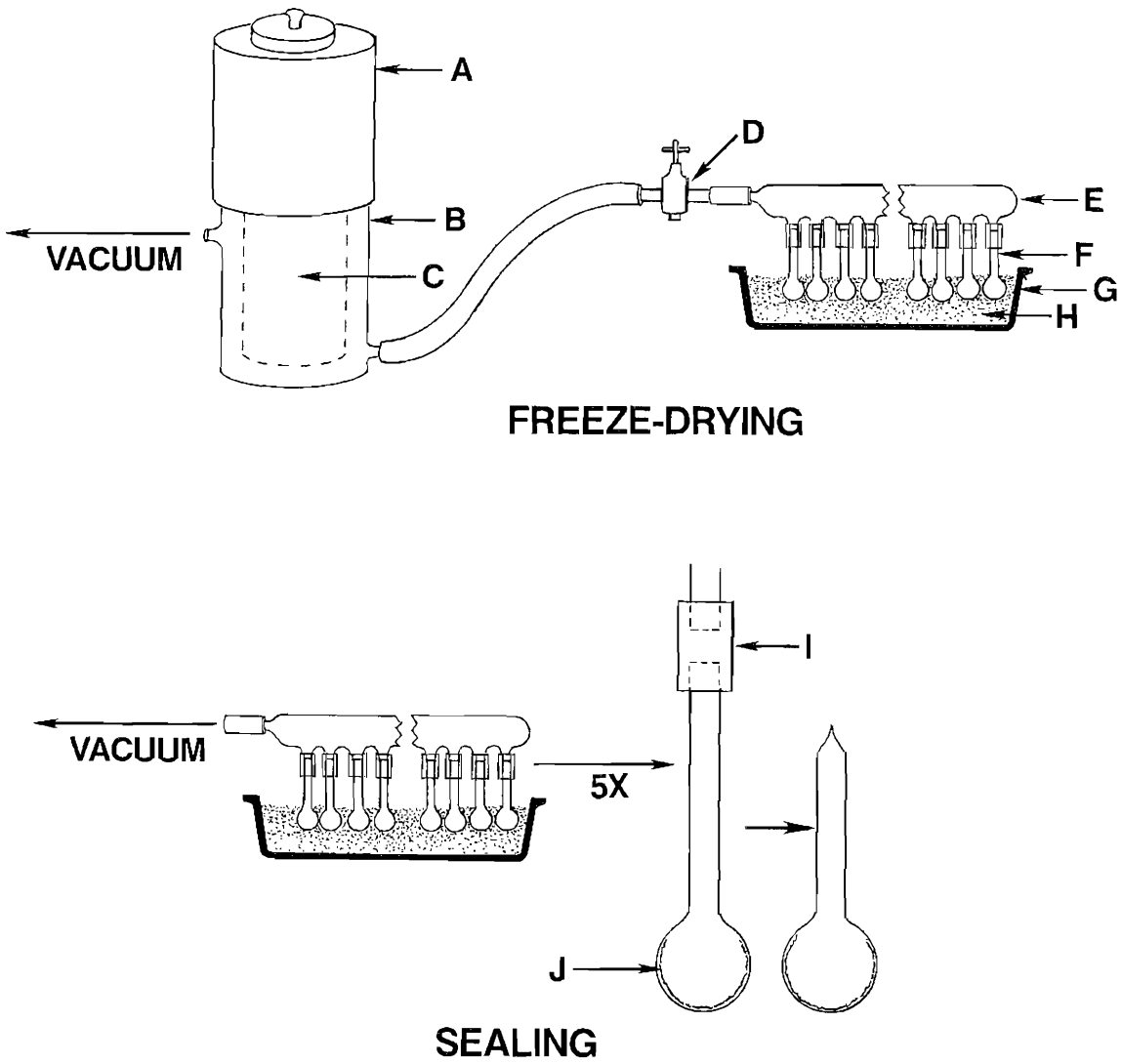

Fig. 1. Manifold method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) manifold; (F) teardrop ampule*; (G) stainless steel pan (H) dry-ice Cellosolve bath; (I) rubber sleeve connecting vial to manifold; (J) thin film of freeze-dried cell suspension.

*The teardrop ampule can be custom made by Kontes Glass Co. The outer diameter of the neck is 8 mm and the inner diameter is 6 mm. The neck length is 125 mm. The diameter of the bulb is 15 mm.

A-90.1

CRYOPRESERVATION OF *PARAMECIUM*

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INTRODUCTION

Paramecia can only be cryopreserved using a controlled three step cooling rate [1]. One method is generally applicable, but certain species or strains require modified methods. Differences between methods may seem in some cases to be minor, but substantial differences in results are obtained by making these modifications.

PROTOCOL

Paramecium aurelia complex, *P. jenningsi*,
P. polycarum, *P. sonneborni*

1. Prepare in advance the following cryoprotective solution:

DMSO	1.5 ml
Fresh growth medium without bacteria	7.5 ml
$MgCl_2$ (0.5 mM)	0.5 ml
$CaCl_2$ (0.5 mM)	0.5 ml

Mix the components in the order listed. Before adding the $MgCl_2$ and $CaCl_2$ allow the solution to return to room temperature. When the medium is added to the DMSO the solution will warm up due to chemical heat.

2. Harvest cells from a culture which is at or near peak density by filtration and centrifugation at 200 g for 1 min.
3. Adjust concentration of cells for 2 x 10^5 cells/ml in fresh medium.
4. Mix the cell preparation and the cryoprotective solution in equal portions.
5. Dispense in 1.0 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place ampules in a controlled rate freezing unit. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after the addition of the DMSO to the cell preparation. From 25° C cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a cooling rate of -1° C/min through heat of fusion to -45° C. Plunge ampules into liquid nitrogen.
7. Ampules are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state add 1.0 ml of Modified Exhausted Cerophyl (see below) to a frozen ampule in a 35° C water bath. Do not completely immerse the ampule. It should be immersed into the water sufficient to cover the frozen material. Do not agitate.

A-90.2

Modified Exhausted Cereal Grass Infusion Medium

Stock Vitamin Solution	10.0 ml
Stigmasterol Solution	2.5 ml
Exhausted Cereal Grass Infusion Medium*	987.5 ml

Vitamin Stock Solution [2]

Calcium pantothenate	0.05 g
Nicotinamide	0.05 g
Pyridoxal·HCl	0.05 g
Riboflavin	0.05 g
Pyridoxamine·HCl	0.025 g
Folic acid	0.025 g
Thiamine·HCl	0.15 g
Biotin	0.0125 mg
DL-thioctic acid	0.5 mg

Stigmasterol Solution

Stigmasterol	200.0 mg
Absolute ethanol	100.0 ml

*Bacterized Cereal Grass Infusion Medium cleared by growth of paramecia, and filter sterilized.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 15.0 ml of modified exhausted cerophyl overlayed onto the surface of a 20 x 100 mm petri plate containing non-nutrient agar.
10. Once established follow routine protocol for routine strain maintenance.

PROTOCOL

Paramecium caudatum & *Paramecium multimicronucleatum*

1. For step 1 of the first protocol substitute 15% (v/v) sterile DMSO and 2.5% (w/v) Bovine Serum Album Fraction V (Sigma) in fresh medium without bacteria.
2. For step 8 of the first protocol substitute the following: Approximately 1.0 ml of bacterized medium 802 is added to the ampule before placing it a water bath at 35° C. When completely thawed, the contents are added to a petri plate with non-nutrient agar having an overlay of 15 ml of bacterized medium. The plate is then incubated at 25° C. Once a vigorous culture is established follow procedure for routine maintenance.

COMMENTS

Despite numerous attempts axenically cultivated strains have not been successfully cryopreserved. In addition neither a *Chlorella*-bearing or symbiont-free strain of *P. bursaria* could be cryopreserved using the protocols above (unpublished observation)

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
2. Van Wagtendonk, W & Soldo, AT 1970. Methods used in the axenic cultivation of *Paramecium aurelia*. *Meth. Cell Physiol.* **4**:117-130.

A-91.1

CRYOPRESERVATION OF *TETRAHYMENA*

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INTRODUCTION

There are several methods which have been successfully used for cryopreservation of *Tetrahymena*. In some cases more than one method may be applied to a given species. On the other hand there are instances in which a species is known to be amenable only to a single method. Differences between methods may seem in some cases to be minor, but substantial differences in results are obtained by making these modifications. Better success is achieved by using controlled rates of cooling [1]. Several applicable methods are provided here.

PROTOCOL

Tetrahymena pyriformis complex and related species

1. *Tetrahymena* to be cryopreserved are grown in a medium with the following formulation:

Proteose peptone	5.0 g
Tryptone	5.0 g
K_2HPO_4	0.2 g
Glucose	1.0 g
Liver Extract	0.1 g
Yeast Extract	0.1 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and autoclave for 15 min at 121° C.

2. Harvest cells from a culture which is at or near peak density by centrifugation at 300 g for 2 min.
3. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
4. While cells are centrifuging prepare a 22% (v/v) sterile solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
5. Add a volume of the DMSO solution equal to the cell suspension volume but add in 3 equal aliquots at 2 min intervals. Thus, the final concentration of the preparation will equal 11% (v/v) DMSO and 10^6 cells /ml.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place the ampules in a controlled rate freezing unit. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after the addition of the DMSO to the cell

preparation. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -50° C ampules are plunged into liquid nitrogen.

8. Storage is in either the vapor or liquid phase of a nitrogen refrigerator.
9. To establish a culture from the frozen state place an ampule in a water bath at 35° C. Just prior to placing in the water bath aseptically add 0.5 ml of sterile Dryl's Salt Solution to the ampule. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule.

Dryl's Salt Solution (components are 0.1M)

$N_aH_2PO_4 \cdot 1H_2O$	10.0 ml
$Na_2HPO_4 \cdot 7H_2O$	10.0 ml
Sodium citrate$\cdot 2H_2O$	20.0 ml
$CaCl_2 \cdot 2H_2O$	15.0 ml
Glass distilled H_2O	945.0 ml

Mix the first 3 components with H_2O before adding $CaC1_2$ to avoid precipitation of Ca salts.

10. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of fresh medium in a 16 x 125 mm screw-capped test tube with a slightly loosened cap. Incubate at 25° C.

PROTOCOL

Tetrahymena patula

1. Harvest cells from a culture which is at or near peak density by centrifugation at 200 g for 1 min.
2. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 22% (v/v) solution of sterile DMSO in sterile glass distilled H_2O. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in 3 equal portions totaling a volume equal to that of the cell suspension in 2 min intervals. After mixing the final concentration will be 10^6 cells/ml and 11% (v/v) DMSO. The time from mixing of the cell preparation and the DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place the ampules in a controlled rate freezing unit.
7. Ampules are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath at 35° C. Just prior to placing in the water bath aseptically add 0.5 ml of sterile modified PYNFH medium (see below) containing 4% (w/v) sucrose to the ampule. Immerse the ampule just sufficient to cover the frozen material. Do not agitate the ampule. Incubate at 25° C.

Modified PYNFH Medium

Peptone (Difco #0118)	10.0 g
Yeast extract (Difco #0127)	10.0 g
Yeast nucleic acid	1.0 g
Folic acid	15.0 mg

Hemin	15.0 mg
Glass distilled H_2O	880.0 ml

Autoclave for 15 min at 121° C. Aseptically add 20 ml of buffer solution (see below). Aseptically add 100 ml of heat-inactivated calf or fetal calf serum. Adjust final pH to 6.5.

Buffer solution

KH_2PO_4	18.1 g
Na_2HPO_4	25.0 g
Glass distilled H_2O	1.0 L

Dissolve components in glass distilled H_2O and filter sterilize.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 9.0 ml of fresh modified PYNFH medium with 4% sucrose (w/v) in a T-25 tissue culture flask. Incubate at 25° C.
10. After culture has been established subculture into fresh normal medium without sucrose.

PROTOCOL

Generally applicable method

1. Harvest cells from a culture which is at or near peak density by centrifugation 200 g for 1 min.
2. Adjust concentration of cells for 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 22% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. After mixing the final concentration will be 10_6 cells/ml and 11% (v/v) DMSO. The time from mixing of the cell preparation and the DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place ampules in a controlled rate freezing unit. From 25° C cool at -2° C/min to heat of fusion (-2 to - 4° C). Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain a rate at -1° C/min through heat of fusion. Continue at a rate of -1° C/min from the heat of fusion to -50° C then plunge ampules into liquid nitrogen.
7. Ampules are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath at 35° C. Do not completely immerse the ampule. It should be immersed into the H_2O sufficient to cover the frozen material.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml fresh medium in a 16 x 125 mm screw-capped test with slightly loosened cap. Incubate at 25° C.

COMMENTS

The first protocol provided recommends a particular medium for the growth of cells prior to cryopreservation. The third protocol may be used for most *Tetrahymena* species, but is particularly recommended for *T. asiatica*, *T. australis*, *T. corlissi*, *T. leucophrys*, and *T. nanneyi*.

Addition of sucrose to the thawing medium first used to cultivate can enhance recovery of most strains. A controlled rate freezing unit which can compensate for the heat of fusion will give the best results. If a controlled rate freezing unit is not available an uncontrolled cooling rate can be use for some strains. One of the above protocols is followed, except the ampules are placed in the bottom of a mechanical refrigerator set at least at -55° C for at least 1 h. The ampules are then plunged in liquid nitrogen. Some strains can also be stored in a mechanical freezer set at for at least -55° C, but the length of viability will usually be less than if stored in a nitrogen refrigerator.

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.

A-92.1

CRYOPRESERVATION OF NON-ENCYSTING CILIATES

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INTRODUCTION

While some non-encysting ciliates are quite easy to cryopreserve, others are quite difficult and modifications of the normal thawing procedure are required [1]. The cryopreservation protocols for a diversity of non-encysting ciliates are provided below including methods applicable to cyrtophorids, euplotids, haptorids, peritrichs, scuticociliates and suctorians.

PROTOCOL

Haptorid: *Protospathidium*
Scuticocilates: *Anophryoides, Cinetochilum, Miamiensis, Metanophrys, Paranophrys, Parauronema, Potomacus*

1. Harvest a culture at or near peak density (one d after most of the prey organisms have been eliminated) as follows: Agitate the flask gently and transfer the cells to 15 ml plastic centrifuge tubes. Centrifuge at 300 g for 5 min.
2. Resuspend the cell pellets with supernatant and adjust the concentration to 2 x 10^5 - 10^6 cells/ml. Place the concentrated cell suspension in a T-25 tissue culture flask. Incubate the flask horizontally with the cap screwed on tightly at 25° C for 1 h.
3. Prepare a 18 - 22% (v/v) sterile DMSO solution in the growth medium (the concentration will vary with the strain being cryopreserved). The solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Add a volume of the cryoprotective solution prepared in step 3 equal to the volume of the cell suspension but add it in 3 equal aliquots at 2 min intervals.
5. The final concentration of the preparation will be 9-11% (v/v) DMSO and 10^5 - 10^6 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to 4° C. At 4° C cool at -10° C/min until the heat of fusion is reached. If freezing unit can compensate for the heat of fusion, cool at a rate of -1° C/min through heat of fusion to -40° C. Plunge ampules into liquid nitrogen and store in the vapor phase in a liquid nitrogen refrigerator.

8. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, gently remove the contents of the ampule with a Pasteur pipette and expel slowly into a T-25 tissue culture flask. Incubate at room temperature (20-23° C) for 15 min.
10. At 15 min intervals add 0.25 ml of the appropriate medium dropwise. Continue until the final volume is 2.0 ml.
11. Allow the flask to remain undisturbed for 15 min.
12. Add 0.5 ml of the appropriate medium dropwise at 15 min intervals until the volume is 4.0 ml.
13. Allow the flask to remain undisturbed overnight.
14. In the morning of day 2 slowly add 4.0 ml of the appropriate medium. Allow to remain undisturbed overnight.
15. Add 8.0 ml of fresh bacterized medium.

PROTOCOL

Cyrtophorid: *Chilodonella*
Euplotids: *Aspidisca, Euplotes*
Peritrichs: *Rhabdostyla, Vorticella*

1. Harvest a culture at or near peak density as follows: Agitate the flask vigorously and transfer the cells to 15 ml plastic centrifuge tubes. Centrifuge at 300 g for 5 min.
2. Resuspend the cell pellets with supernatant and adjust the concentration to 2×10^5.
3. Prepare a 18-22% (v/v) sterile DMSO solution in the growth medium (the concentration of DMSO required will vary with the strain being cryopreserved). The solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell suspension and the cryoprotective solution in a 1:1 ratio.
5. The final concentration of the preparation will be 9-11% (v/v) DMSO and 10^5 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. From 25° C cool at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, cool at a rate of -1° C/min through heat of fusion to -40° C. Plunge ampules into liquid nitrogen and store in the vapor phase in a liquid nitrogen refrigerator.
8. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, transfer the entire contents to a 15.0 ml overlay of the appropriate medium on a 20 x 100 mm petri plate containing non-nutrient agar.
10. Incubate the plate at the normal growth temperature for 24 h.
11. Add the appropriate prey organism and thereafter maintain using the standard cultivation protocol.

A-92.3

PROTOCOL

Suctorians: *Heliophrya, Tokophrya*

1. Harvest a culture at or near peak density (two days after most prey organisms have been eliminated) as follows: Scrap the surface of the agar plates with a sterile cotton swab and transfer the cell suspensions to 15 ml plastic centrifuge tubes. Centrifuge at 300 g for 5 min.
2. Resuspend the cell pellets with supernatant and adjust the concentration to 2 x 10^5.
3. Prepare a 20% (v/v) sterile DMSO solution in the growth medium. The solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell suspension and the cryoprotective solution in a 1:1 ratio.
5. The final concentration of the preparation will be 10% (v/v) DMSO and 10^5 cells/ml. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, continue to cool at a rate of -1° C/min through heat of fusion to -40° C. Plunge ampules into liquid nitrogen and store in the vapor phase in a liquid nitrogen refrigerator.
8. To establish a culture from the frozen state transfer an ampule directly from the storage temperature to a water bath set at 35° C. Do not completely immerse the vial. It should be immersed into the H_2O sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, transfer the entire contents to a non-nutrient agar plate with the appropriate liquid overlay.
10. Incubate the plates at the normal growth temperature for 1 d before adding food organisms.

PROTOCOL

Euplotids: *Euplotes*
Hymenostomatids: *Glaucoma*

1. Harvest a culture at or near peak density (two days after most prey organisms have been eliminated for *Euplotes* strains fed with *Tetrahymena*) as follows: Transfer the cells to 15 ml plastic centrifuge tubes. Centrifuge at 200 g for 5 min.
2. Resuspend the cell pellets with supernatant and adjust the concentration to 2 x 10^5 cells/ml for *Euplotes* and 2 x 10^6 cells/ml for *Glaucoma*.
3. For *Euplotes* prepare an 11% (v/v) and for *Glaucoma* a 20% (v/v) sterile DMSO solution in the growth medium. The solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Add a volume of the cryoprotective solution prepared in step 3 equal to the volume of the cell suspension but add it in 3 equal aliquots at 2 min intervals.
5. The final concentration of the preparation will be 5.5% (v/v) DMSO and 10^5 cells/ml for *Euplotes* and 10% (v/v) DMSO and 10^6 cells/ml for *Glaucoma*. The cooling cycle should be initiated no less than 15 min and no longer than 60 min after addition of the glycerol to the cells.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place vials in a controlled rate freezing unit. From 25° C cool at -1° C/min to 4° C. Cool at a rate of -10° C/min from 4° C to the heat of fusion. If freezing unit can compensate for the

heat of fusion, cool at a rate of -1° C/min through heat of fusion to -40° C. Plunge ampules into liquid nitrogen and store in the vapor phase in a liquid nitrogen refrigerator.

8. To establish a culture from the frozen state proceed as follows: Aseptically open the vial and add 0.5 ml of filter sterilized exhausted medium (previously used to grow the protozoan and cleared of the food source) containing 8% (w/v) sucrose. Transfer the vial to a 35° C water bath. Do not completely immerse the vial. It should be immersed into the water sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, gently remove the contents of the ampule with a Pasteur pipette and expel slowly into a 15 x 60 mm petri plate. Incubate at room temperature (20-23° C) for 15 min.
10. At 15 min intervals add 0.25 ml of the filter sterilized exhausted medium containing 4% (w/v) sucrose dropwise. Continue until the final volume is 2.0 ml.
11. Allow the petri plate to remain undisturbed for 15 min.
12. Add 0.5 ml of the appropriate medium dropwise at 15 min intervals until the volume is 4.0 ml.
13. Allow the petri plate to remain undisturbed overnight.
14. In the morning of day 2 remove the motile cells with a drawn out Pasteur pipette and transfer to 2.0 ml of filter sterilized exhausted medium containing 2% (w/v) sucrose in a 10 x 35 mm petri plate. Allow the cells to remain undisturbed for 2 h.
15. With a drawn out Pasteur pipette transfer the motile cells to 2.0 ml of filter sterilized exhausted medium without sucrose in a 10 x 35 mm petri plate. Allow the cells to remain undisturbed for 2 h.
16. For *Glaucoma* transfer the entire volume from the petri plate to 8.0 ml of fresh bacterized medium in a T-25 flask. For *Euplotes* transfer the entire volume to a T-25 flask containing 8.0 ml of the appropriate medium containing the prey organism *Tetrahymena*.
17. Maintain using normal cultivation protocol.

COMMENTS

It is impossible to specify the concentration of DMSO required for the successful recovery of a particular strain from the frozen state. If a ciliate can be assigned to a particular genus but has never been cryopreserved it is best to initially experiment with the DMSO concentrations specified above for that genus. The thawing procedure outlined in the first protocol may not be necessary for all strains but it is best to use this procedure initially. The thawing procedure outlined in the last protocol may not be necessary for all strains. If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

LITERATURE CITED

1. Nerad, TA 1991. Chapter 10: Protozoa and Algae pp. 25-30. In: Simione FP & Brown, EM, ed. ATCC Preservation Methods: Freezing and Freeze-drying. 2nd edt. American Type Culture Collection. Rockville, MD 42 pp.

A-93.1

LONG TERM MAINTENANCE OF SELECTED ENCYSTING CILIATES BY DRYING AND CRYOPRESERVATION

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INTRODUCTION

Many encysting ciliates are readily amenable to cryopreservation. Approaches useful for some heterotrichs, haptorids, pharyngophorids, scuticociliates, peritrichs and colpodids are provided. The known methods may have application to genera other than those reported. In the case of *Blepharisma* the method described has proven to be useful only to a single species, *B. stoltei* [1]. The same method is not successful with non-encysting species. Since a naturally occurring stage in the life history resistant to adverse conditions occurs in culture, the cyst stage is often selected to be the form used in a preservation method [2].

PROTOCOL

Blepharisma
Cryopreservation

1. Cultures are grown in 11 cm biological bowls containing approximately 200 ml of medium with one wheat kernel. Harvest when all or most cells have encysted. Allow cysts to settle by gravity in a 500 ml bottle. Wash 5 times in supernatant from finger bowls.
2. Adjust concentration of cells to 2 x 10^6/ml with medium.
3. Prepare a 15% (v/v) solution of sterile DMSO in fresh medium. The DMSO sterile solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will be 10^6 cells/ml and 7.5% (v/v) DMSO. The time from mixing of the cell preparation and the DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. Cool at -1° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place a cryule in a water bath at 35° C. Immerse the vial just sufficient to cover the frozen material.

9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into an 11 cm biological bowl containing approximately 150 ml of half strength medium plus one wheat kernel. Excystment usually occurs within 2-3 d. When the culture is established, trophozoites can be transferred to full strength medium. Thereafter, the protocol for routine maintenance is followed.

PROTOCOL

Cryopreservation, *Didinium*

1. Grow cells in large volumes. Allow cysts to form.
2. Suspend the cysts by scraping the sides of the vessel with a rubber policeman. Transfer cyst suspension to centrifuge tubes and centrifuge at 200 g for 5 min.
3. Adjust concentration of cysts to 2 x 10^6/ml in fresh medium.
4. While cells are centrifuging prepare a 20% (v/v) sterile solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
5. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will be 10_6 cells/ml preparation and the DMSO stock solution and 10% (v/v) DMSO. The time from mixing of the cell than 15 min and no longer than 60 min.
6. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml sterile glass cotton-plugged vials or 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
7. Place the ampules in a controlled rate freezing unit. From 25° C cool at -1° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -50° C plunge into liquid nitrogen.
8. The frozen preparations may be stored in a mechanical freezer until needed or in either the vapor or liquid phase of screw-capped cryule of a nitrogen refrigerator.
9. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. To improve recovery, aseptically add 0.5 ml of sterile Dryl's Salt Solution just prior to placing in the water bath. Do not completely immerse the ampule. It should be immersed into the H_2O sufficient to cover the frozen material.

Dryl's Salt Solution (components are 0.1M)

$NaH_2PO_4 \cdot H_2O$	10.0 ml
$Na_2HPO_4 \cdot 7H_2O$	10.0 ml
Sodium citrate$\cdot 2H_2O$	20.0 ml
$CaCl_2 \cdot 2H_2O$	15.0 ml
Glass distilled H_2O	945.0 ml

Mix the first 3 components with H_2O before adding $CaCl_2$ to avoid precipitation of Ca salts. Filter sterilize.

10. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into 5.0 ml of Rye Grass (Cerophyl) Infusion Medium which has been inoculated 24 h earlier with bacteria for the growth of Paramecium. Then inoculate culture with 0.1 ml from an actively growing Paramecium culture and incubate at 25° C.

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PROTOCOL

Cryopreservation
Colpoda, Dileptus, Homalogastra, Platyophrya, Pseudocohnilembus, Sorogena, Tillina, Sphenostomella, and *Vorticella*

1. Allow the culture to encyst before harvesting. To detach cysts, agitate vigorously or rub gently with a sterile cotton swab, spread bar or a rubber policeman.
2. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low, concentrate by centrifugation at 600 g for 5 min.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile DMSO in fresh medium. For *Platyophrya* and *Pseudocohnilembus* use 20% (v/v) DMSO. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will be 10^6 cells/ml. The final concentration of DMSO will be 10.0% (v/v) for *Platyophrya* and *Pseudocohnilembus* and 7.5% (v/v) for the others. The time from the mixing of the cell preparation and the DMSO stock solution should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. Cool at -10° C/min from 25° C to the heat of fusion. If freezing unit can compensate for the heat of fusion, cool at -1° C/min through heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place a cryule in a water bath at 35° C. Immerse the vial just sufficient to cover the frozen material.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium.
10. For *Colpoda, Homalogastra, Opisthonecta, Tillina, Sorogena, Sphenostomella* and *Vorticella,* observe daily for motile cells and when observed, transfer to a T-25 tissue culture flask containing 10.0 ml of Rye Grass (Cerophyl) Infusion Medium inoculated 24 h previously with *Klebsiella pneumoniae*. For *Didinium* inoculate into a T-25 flask containing an actively growing culture of *Paramecium*. For *Dileptus* inoculate into a flask containing an actively growing culture of *Tetrahymena* and for *Platyophrya* inoculate into actively growing *Bodo* culture.

PROTOCOL

Cryopreservation
Glauconema

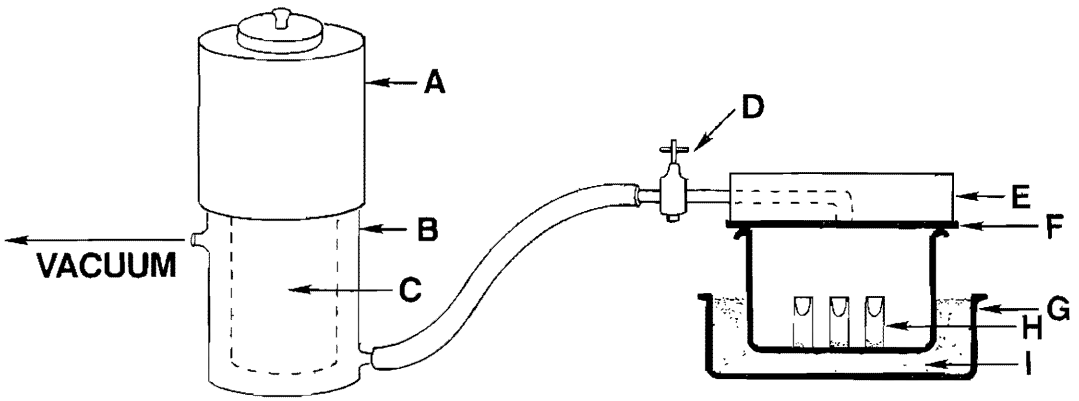
1. Harvest cells from a culture which is at or near peak density. Do not use an encysted culture.
2. If the cell concentration exceeds the required level do not centrifuge, but adjust concentration of cells to 2 x 10^6/ml with fresh broth medium. If the concentration is too low, concentrate by centrifugation at 600 g for 5 min.
3. While cells are centrifuging prepare a 25% (v/v) solution of sterile DMSO in fresh broth medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.

4. Mix the cell preparation and the DMSO solution in equal portions. Thus the final concentration will 10^6 cells/ml in 12.5% DMSO (v/v). The time from the mixing of the cell preparation and the DMSO stock solution should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots into 1.0 - 2.0 ml plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. Cool at -1° C/min from 25° C to 4° C, then -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain cooling rate at -1° C/min through heat of fusion to -40° C, then plunge vials into liquid nitrogen.
7. The frozen preparations are stored in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place a cryule in a water bath at 35° C. Immerse the vial just sufficient to cover the frozen material.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and inoculate into fresh medium.

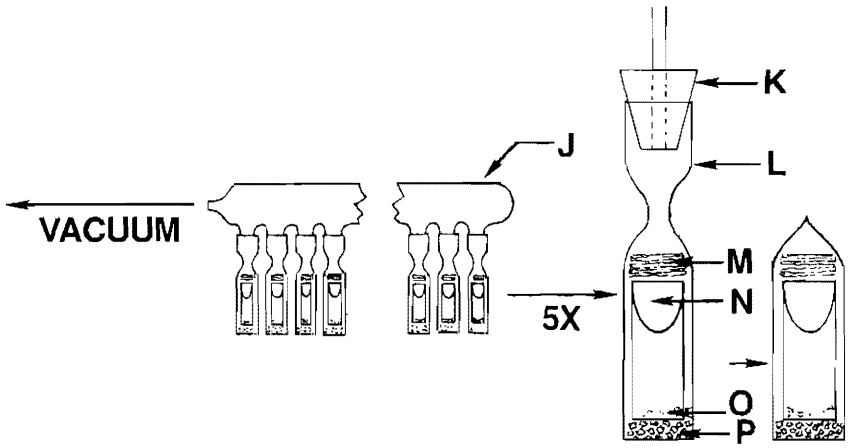
PROTOCOL

Drying
Colpoda, Homalogastra, Opisthonecta, Platyophrya
Tillina, Sorogena, Sphenostomella, and *Vorticella*
(Fig. 1, sealing only)

1. In advance prepare sterile glass cotton-plugged 11.5 x 35.0 mm shell vials (Glass Vials, Inc.) containing mulched filter paper. Whatman #1 filter paper is soaked in distilled H_2O and then shredded in a blender. Add enough of the wet mulched paper to cover the bottom of the vials. The paper should not be packed. Cotton plug the vials and autoclave. After autoclaving place vials in a 70° C incubator overnight.
2. Grow ciliates in volumes of 500 ml or more. Allow cysts to form.
3. Suspend the cysts by scraping the sides of the vessel. Centrifuge the cyst suspension at 200 g for 5 min. Pool pellets and resuspend in a single vessel.
4. Adjust concentration of cells for 2 x 10^6/ml in fresh medium.
5. Dispense final cyst suspension in 0.3 ml aliquots into sterile shell vials containing dried, mulched filter paper.
6. Place the ampules at 25° C for 14 d. After drying, place for 7 d in a desiccator under vacuum at room temperature (20-23° C).
7. In advance prepare 14.25 x 85.0 mm outer shell glass vials (Glass Vials, Inc.). Cover the bottom of the vial with 6-16 mesh silica gel granules and add a thin layer of cotton to provide cushioning for the inner shell vial. Heat the preparation for 12 h. The silica gel will turn dark blue and will serve as a moisture indicator during storage.
8. Cool outer shell vials in a dry cabinet (10% or less relative humidity) and insert the dried cyst preparations. On top of the inner vial insert a 64 mm wide wad of glass fiber paper.
9. Remove vials from the dry cabinet and seal the outer vial as follows: rotate the vial in the hottest part of an air/gas flame and slowly pull with forceps until a narrow neck is formed. The appearance is like a wine glass with a stem. After cooling the vial is attached to a port manifold. Once all the vials have been attached, the manifold is evacuated to 50 μm of Hg. To assure evacuation of the vials has been achieved each vial is tested using a high induction electrical spark. The vial is then sealed with a double flame air/gas torch where the "stem" leaves the base of the outer shell vial.
10. Although vials can be stored at room temperature viability is extended if storage is at -4° C to -70° C. Preparations can be stored in the vapor phase of a nitrogen freezer but for safety reasons do not seal. Store only cotton-plugged inner shell vial preparations.
11. To establish a culture from the dried state aseptically add 1.0 ml of Rye Grass (Cerophyl) Infusion Medium which has been inoculated with *Klebsiella pneumoniae* 24 h previously and



FREEZE-DRYING



SEALING

Fig. 1. Batch method of freeze-drying. (A) Condenser extender filled with dry-ice pellets; (B) Vir-Tis condenser; (C) condenser interior filled with dry-ice and Cellosolve; (D) valve controlling access to vacuum; (E) plexiglass portion of Atmo-vac plate*; (F) rubber portion of Atmo-vac plate; (G) stainless steel pan; (H) inner shell vial with cotton top cut off; (I) crushed dry-ice; (J) manifold; (K) number 00 stopper; (L) outer shell vial; (M) glass fiber wad; (N) cotton plug of inner shell vial; (O) freeze-dried cell suspension; (P) silica gel.

*The manufacturer no longer exists. If not already owned a similar device can be custom constructed. The dimensions of the plexiglass are 2.8 cm x 17.5 cm x 17.5 cm. A rubber pad is glued to the bottom of the plate and has a thickness of 0.6 cm. A 1.4 cm diameter channel through the plexiglass plate leads from the valve to a 1.4 cm diameter hole running up from the center of the plate. The holes intersect at a right angle.

incubate at 25° C in the dark. For *Colpoda, Homalogastra, Opisthonecta, Tillina, Sorogena* and *Vorticella*, observe daily for motile cells and when observed, transfer to a T-25 tissue culture flask containing 10.0 ml of Rye Grass (Cerophyl) Infusion Medium inoculated 24 h previously with *Klebsiella pneumoniae*. For *Didinium* inoculate into a T-25 flask containing an actively growing culture of *Paramecium*. For *Dileptus* inoculate into a flask containing an actively growing culture of *Tetrahymena* and for *Platyophrya* inoculate into actively growing culture of *Bodo*.

COMMENTS

Dried material should not be stored in direct sun light. The colder the storage temperature the longer the viability. Dried material should **not be kept in a frost-free freezer**. The periodic frost-free heat cycle to prevent the formation of ice will reduce longevity. Dried material can be kept in a nitrogen freezer, but **extreme caution should be used**. Only unsealed cotton-plugged inner vial preparations should be stored under these conditions.

LITERATURE CITED

1. Daggett, P-M & Nerad, TA 1984. A triphasic freezing rate for the preservation of sensitive protozoan strains. Proc. 4th Interl. Congr. Cult. Coll., Kocur, M & Da Silva, E, ed., World Federation of Culture Collections, pp. 163-167.
2. McGrath, MS, Daggett, P-M & Nerad, TA 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Microsc. Soc.* **96**:519-525.

A-94.1

CRYOPRESERVATION OF *STEPHANOPOGON*

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INTRODUCTION

Two species of *Stephanopogon*, *S. apogon* and *S. colpoda*, are known to be amenable to long term maintenance by cryopreservation. It is likely that other species could be preserved by the same or a similar protocol.

PROTOCOL

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells to 2 x 10^6/ml in fresh medium.
3. While cells are centrifuging prepare a 20% (v/v) sterile solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^6 cells/ml in 10% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots in 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to -40° C. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. At -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Once fully thawed immediately remove ampoule from water bath. For *S. apogon* aseptically inoculate the contents into a T-25 tissue culture flask containing a vigorous culture of *Rhynchomonas nasuta*. For *S. colpoda* inoculate into 10 ml of medium in a T-25 tissue culture flask.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device

A-94.2

(Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street, Worcester, MA 01605 USA).

A-95.1

CRYOPRESERVATION OF PRASINOMONADS

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INTRODUCTION

Tetraselmis is amenable to cryopreservation. The method described below can probably be applied to other prasinomonads.

PROTOCOL

1. Harvest cells from a culture which is at or near peak density by centrifugation at 850 g for 5 min.
2. Adjust concentration of cells for 2 x 10^7/ml in fresh medium.
3. While cells are centrifuging prepare a 15% (v/v) solution of sterile DMSO in fresh medium. The DMSO solution when first prepared will warm up due to chemical heat when mixed with the medium. The solution should be allowed to return to room temperature prior to use.
4. Mix the cell preparation and the DMSO in equal portions. The final concentration of the preparation will thus be 10^7 cells/ml in 7.5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process is begun should be no less than 15 min and no longer than 60 min.
5. Dispense in 0.5 ml aliquots 1.0 - 2.0 ml sterile plastic screw-capped cryules (special plastic vials for cryopreservation).
6. Place vials in a controlled rate freezing unit. From room temperature cool at -1° C/min to 4° C; continue at -10° C/min to the heat of fusion. If freezing unit can compensate for the heat of fusion, maintain rate at -1° C/min through heat of fusion. Continue at -1° C/min; at -40° C plunge into liquid nitrogen.
7. Store in either the vapor or liquid phase of a nitrogen refrigerator.
8. To establish a culture from the frozen state place an ampule in a water bath set at 35° C. Immerse the cryule just sufficient to cover the frozen material. Do not agitate the vial.
9. Immediately after thawing, do not leave in the water bath, aseptically remove the contents of the ampule and place into fresh medium. Incubate the culture at the appropriate temperature. Thereafter, follow the protocol for routine maintenance.

COMMENTS

If a controlled rate freezing unit is not available a cooling cycle which closely approximates a -1° C/min cooling rate can be achieved using a relatively inexpensive commercially available device (Cryopreservation Apparatus cat. #9001, Cambridge Biotech Corporation 365 Plantation Street,

Worcester, MA 01605 USA).

B. ECOLOGICAL METHODS

1. Plankton-sampling-freshwater. Bland J. Finlay & Barbara E. Guhl
2. Benthic sampling-freshwater. Bland J. Finlay & Barbara E. Guhl
3. A simple method for obtaining concentrated populations of protists from sediments. Eugene B. Small
4. A simple method for obtaining concentrated populations of protists from sediment water. Eugene B. Small
5. Sampling and enumerating soil protozoa. Stuart S. Bamforth
6. Enumerating soil testate amoebae by direct counting. Erna Aescht & Wilhelm Foissner
7. Enumerating active soil ciliates by direct counting. Erna Aescht & Wilhelm Foissner
8. Enumeration of protists and small metazoans in activated sludge. Hannes Augustin & Wilhelm Foissner
9. Collecting aufwuchs on artificial substrata. J. R. Pratt & R. L. Kepner, Jr.
10. Estimating the species richness of soil protozoa using the "non-flooded petri dish method". Wilhelm Foissner
11. Evaluating water quality using protozoa and saprobity indexes. Wilhelm Foissner.
12. Biomonitoring using protozoans. J. R. Pratt & J. M. Balczom
13. Determination of phagotrophic protist feeding rates *in situ*. Evelyn B. Sherr & Barry Sherr
14. Qualitative study and quantitative analysis of behavior of ciliated protozoa: principles, techniques, tricks. Nicola Ricci
15. Cover slip traps: simple samplers for sessile protists. Eugene B. Small

PLANKTON SAMPLING - FRESHWATER

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INTRODUCTION

No single sampling strategy can be used for all investigations of planktonic protozoa in all types of fresh water body. In deciding upon a strategy, particular regard should be paid to the size categories of protozoa being studied; the methods adopted to study the heterotrophic nanoplankton for example are quite different to those used in tackling the large microaerobic ciliates. It may also be useful to gather relevant information concerning the biological productivity and the physical and chemical characteristics of the water body; especially, in the case of stratified water bodies, the steepness of chemical gradients. This will help greatly in choosing a sampling device: there is, for example, little point in pump sampling small volumes of water down the vertical profile of a high mountain lake if the principal requirement is to obtain a large biomass of planktonic ciliates. Protozoan abundance is roughly proportional to overall productivity and pump sampling, although ideal for sampling across steep chemical gradients (and obtaining simultaneous measurements of the chemical profile [3, 4, 8]) is likely to be a waste of time in a high mountain lake.

The sampling strategy decided upon will necessarily become more rigorous and complicated as the diversity of investigated protozoa increases, as the study becomes quantitative, and as the choice of initial treatment of live samples becomes constrained by the requirements of subsequent treatment processes, eg those associated with silver staining or transmission electron microscopy. The following protocol is based on our experience of sampling a variety of fresh water bodies for most types of free-living aquatic protozoa which were subsequently subjected to a range of investigative procedures in the laboratory.

PROTOCOL

The recommended protocol is summarised in the following flow chart. The notes referred

(1) All of these point samplers will ensure that water is collected at a single depth with a vertical error of only a few centimeters [2, 3]. One of the simplest devices is the bottle illustrated. It is enclosed by a weighted frame and the bung is removed by tugging sharply on the string. Bottles such as this are relatively easy to use at depths up to 12-15m [5], but at greater depths, it becomes difficult to verify that the bottle is opening at the correct depth (when used to a depth of 12m in a particularly calm water body, the actual sampling depth was verified by monitoring the time taken for the evacuated air bubbles to reach the surface after pulling the bung).

Multiple syringe samplers [9] permit small volumes of water to be simultaneously collected over a complete vertical profile of up to about 2m [2, 3, 4]. They are very useful in ponds and small lakes, but of little use (because of their restricted length) in large lakes with weak chemical gradients.

Integrating samplers (eg Friedinger, Ruttner) are tubes of perspex or steel, of various lengths up to 1m. A messenger is sent down a wire to close the tube at the required depth. These samplers collect an `integrated' sample corresponding to the length of the tube. Relatively large volumes of water can be quickly collected using these samplers and they are ideal for large lakes.

(2) Some protozoa may become unrecognisable after fixation. It is usually easier to identify fixed protozoa if they have previously been seen alive. Low power (eg 100x) observation of the natural community in a Sedgewick-Rafter chamber with a thin cover slip (or another similar chamber) will usually suffice. Unproductive waters may need to be gently centrifuged (1,000 x g; 1min, then remove and examine the loose pellet as quickly as possible) or, in the case of larger protozoa, concentrated by sieving (nylon mesh is commercially available with pore sizes from 10µm upwards).

(3) Naked amoebae are difficult to count accurately in fresh water samples: most are very small (<10µm), they may be detectable only when they move, and many will be unrecognisable when associated (as they often are) with flocs and other particles. There is no reliable and tested method of directly enumerating these amoebae in samples of fresh water. It is unlikely that a direct method will be developed in the near future and the best approach is to try a method of enrichment cultivation. A reasonable starting point would be the method described by [10] and used to estimate the abundance of estuarine amoebae. Water samples were concentrated 10-fold or 15-fold by centrifugation (2,000 x g; 10min) prior to plating out 50, 5µl drops of the concentrated suspension onto nutrient agar. The agar surfaces were examined after 3 to 4 weeks incubation. The frequency of positive growth was then used as an estimate of abundance in the original sample (e.g. 4 positive results in 50 drops being equivalent to 16 amoebae per ml of the concentrated sample, with the assumption that a single amoeba or one viable cyst gave rise to each positive result). It is unrealistic to assume that enrichment methods provide suitable conditions for the growth of all amoebae present and it is likely that estimates of population abundance obtained using such methods are underestimates of true densities. Suggested adaptations for enumerating freshwater amoebae would include plating out onto non-nutrient agar previously streaked with *Escherichia coli*, and, in addition, inoculating liquid culture media in sterile plastic multi-well containers (eg 5µl into 100µl Prescott & Carrier's solution) containing *E. coli* or small centric diatoms, or both, as food. Recipes for culture solutions and further details of methods for culturing naked amoebae are described in [6].

(4) DAPI (4'6-diamidino-2-phenylindole) is a DNA-specific fluorochrome. It also binds non-specifically to cytoplasm. It fluoresces brilliant blue-white following excitation with ultraviolet. Prepare a stock solution by adding 10ml distilled water to a 1mg vial of DAPI. When stored at 4°C in the dark, this solution remains usable for at least 6 months.

(5) Blue excitation will reveal red autofluorescence of chlorophyll a. Heterotrophic cells will usually appear green or blue, in response to blue or UV excitation respectively. Heterotrophic flagellates (chrysomonads, kinetoplastids, choanoflagellates) and small ciliates (especially oligotrichs, tintinnids and scuticociliates) can often be identified, at least within these broad taxonomic categories.
Enumeration is best achieved using a squared graticule (eg 10 x 10) inserted in one of the eyepieces. If possible, use a relatively low power oil immersion objective (eg 54x or 63x) for detecting and enumerating protozoa, then switch to a 100x objective for further identification. In all cases use objectives with the highest numerical aperture available.

(6) Lugol's iodine is prepared by dissolving 10g iodine and 20g potassium iodide in 200ml distilled water with optional acidification by adding 20ml glacial acetic acid. The solution is stored in the dark.

(7) Having obtained a fixed sample, there are two simple ways of examining it quantitatively. (a) It can be put into a circular, shallow (eg 2cm tall) sedimentation chamber and allowed to settle. The chamber is then placed on the stage of an inverted microscope and the protozoa are enumerated. The principal advantage of this method is that high power objectives can be used, although in practice, they are of little use with organisms that are not sufficiently flattened. Another drawback is that the smaller protozoa make take a long time to settle. (b) The alternative is to use a Sedgewick-Rafter or other similar chamber with a shallow depth. The SR holds 1 ml of sample, the chamber has a depth of only 1mm and the base is calibrated with 1,000 squares with sides of 1mm. Thus the volume lying above each square is 1µl. The principal drawback with the SR is the requirement to focus through at least 1mm of water, so microscope magnifications are limited to about 160x (and even this is only possible by capping the chamber with a thin coverslip).
If the ciliates are extremely numerous in the chamber, a sub-sample of several transects can be counted without disturbing the chamber contents. The precision of the count will obviously improve as the number in the count increases. If the protozoa are arranged randomly in the chamber (this is usually true, or approximately so), the Poisson series is a suitable model, the variance will be equal to the mean, and we can expect 95% confidence limits of ± 20% on a count of 100 protozoa (i.e. having counted 100 organisms, there is a one in twenty chance of being wrong that the true value lies between 80 and 120). The confidence limits reduce to ± 10% for a count of 400.

(8) Provides good preservation of many fragile ciliates(eg *Loxodes*.) Fix sample in a glass centrifuge tube for at least 10min. Centrifuge fixed cells, then replace supernatant with buffer or distilled water.

(9) The fixative may precipitate if the water contains much hydrogen sulphide [8].

(10) Champy is 1% chromic acid (7 parts), 3% potassium dichromate (7 parts), 2% osmium tetroxide (4 parts). Add water sample to freshly prepared fixative (the first two constituents can be mixed and kept indefinitely. Fix for at least 5min, then centrifuge, decant supernatant and replace with da Fano's fluid (cobalt nitrate 1g, sodium chloride 1g, formalin 10ml, distilled water 90ml). This fixed material is suitable for Chatton-Lwoff silver staining [6, 7].

(11) This fixed material may then be post-fixed with 2% osmium tetroxide in preparation for electron microscopy. Na-cacodylate is regarded by some as being superior to phosphate buffers, mainly because the latter can precipitate within fixed cells. This is unlikely to be a problem at the light microscope level of investigation.

(12) It is of course also possible to take the lower densities of protozoa in unproductive waters and prepare them for silver staining and electron microscopy (fixatives as listed for high ciliate abundance). There are however significant practical problems in handling large volumes of OsO_4-fixed water. It is preferable to fix the living material collected on fine mesh sieves (see note 2). Concentration and fixation should be carried out in the boat or elsewhere on site.

LITERATURE CITED

1. Berninger, U-G, Finlay, B .J., & Kuuppo-Leinikki P. 1991. Protozoan control of bacterial abundances in freshwater. *Limnol Oceanogr* **36**: 139-147.
2. Finlay, B. J.. 1981. Oxygen availability and seasonal migrations of ciliated protozoa in a freshwater lake. *J Gen Microbiol* **123**: 173-178.
3. Finlay, B. J., Span, A. S. W., & Harman, J. M. P. 1983a. Nitrate respiration in primitive eukaryotes. *Nature* **303**: 333-336.
4. Finlay, B. J., Hetherington, N. B. & Davison, W. 1983b. Active biological participation in lacustrine barium chemistry. *Geochim Cosmochim Acta* **47**: 1325-1329.
5. Finlay, B. J., Curds, C. R., Bamforth, S. S. & Bafort, J. M. 1987. Ciliated protozoa and other microorganisms from two African soda lakes (Lake Nakuru and Lake Simbi, Kenya). *Arch Protistenk* **133**: 81-91.
6. Finlay, B. J., Rogerson, A., & Cowling, A. J. 1988a. A Beginner's Guide to the Collection, Isolation, Cultivation and Identification of Freshwater Protozoa. CCAP, Ambleside, U.K.
7. Finlay, B. J., Berninger, U-G., Clarke, K. J., Cowling, A. J., Hindle, R. M. & Rogerson, A. 1988b. On the abundance and distribution of protozoa and their food in a productive freshwater pond. *Europ J Protistol* **23**: 205-217.
8. Finlay, B. J., Clarke, K. J., Vicente, E. & Miracle, M. R. 1991. Anaerobic ciliates from a sulphide-rich solution lake in Spain. *Europ J Protistol* **27**: 14-49.
9. Heaney, S. I. 1974. A pneumatically-operated sampler for close intervals of depth. *Freshwater Biol* **4**: 103-106.
10. Rogerson, A. & Laybourn-Parry, J. 1992. The abundance of marine naked amoebae in the water column of the Clyde Estuary. *Estuar Coast Shelf Sci* (in press).

PROTOCOL FOR SAMPLING PLANKTONIC PROTOZOA

Sampling Devices (1)

Point Samplers

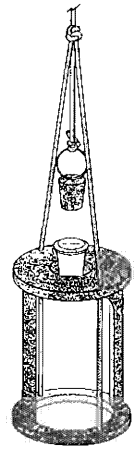
Peristaltic Pump
Simple Bottle Sampler
Multiple Syringe Sampler

Integrating Samplers

Friedinger Sampler
Ruttner Sampler

Microscopic observation of live material **(2)**

Examination and enumeration of FIXED protozoa



Simple Point Sampling Bottle

Protozoa <10µm in size (mainly heterotrophic flagellates and 'nano'-ciliates) (3)

Add 7.5 ml water sample to 2.5 ml of 4% glutaraldehyde in 0.1M Na-cacodylate buffer pH 7. Store at 0-4°C until required [eg 1].

Add aqueous DAPI (final concentration 10µg/ml). Leave for 5 min. **(4)**

Concentrate by vacuum filtering 2-10ml onto a black, 25mm, 0.2µm polycarbonate membrane (eg Nuclepore). Placing the filter on top of a cellulose filter with a larger pore size leads to a better distribution of the vacuum.

Place filter on glass slide, add a drop of immersion oil to top surface of filter, and lower microscope into oil. Observe and enumerate protozoa using epifluorescence microscopy **(5)**

Individual protozoa >10µm in size (mainly ciliates)

Low ciliate abundance
(<5 per ml)

Fixation with either saturated $HgCl_2$: (1 part fixative: 19 parts water sample) or acidified Lugol's iodine **(6)** (1 part fixative: 99 parts water sample), and concentration by gravity sedimentation or filtration. **(12)**

High Ciliate abundance
(>5 per ml)

Fix volume required to obtain at least 100 ciliates. Fix with one of the following:

Osmium tetroxide
add 5ml of sample to 0.6ml 2% OsO_4 in a glass centrifuge tube. **(8)(9)**

Champy
add 5.5ml of sample to 4.5ml Champy in a glass centrifuge tube. **(9)(10)**

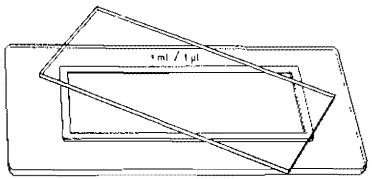
Mercuric chloride
add 9.5ml of sample to 0.5ml saturated $HgCl_2$. **(9)**

Lugol's iodine
add 9.8ml of sample to 0.2ml Lugol's iodine. **(6)(9)**

Formaldehyde
add 9ml of sample to 1ml 40% formaldehyde solution (formalin).

Glutaraldehyde
add 5ml of sample to 5ml 4% glutaraldehyde in 0.1M Na-cacodylate buffer (pH 7). **(11)**

Transfer fixed material to a Sedgwick-Rafter chamber or other suitable counting chamber, before or after centrifugation (2000 x g; 1min) depending on cell density. **(7)**



Sedgwick-Rafter Chamber

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BENTHIC SAMPLING - FRESHWATER

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INTRODUCTION

This contribution describes procedures for (a) obtaining quantitative samples of the freshwater benthos, and (b) determining the abundance of protozoa and their spatial distribution in the benthos. The procedures described are restricted to the analysis of soft sediments. For enumeration of benthic protozoa living in streams, and on macrophytes, see [1]. This protocol concentrates on methods suitable mainly for ciliates.

Anyone studying benthic freshwater protozoa will have to cope with several significant problems. The most serious is that there is no simple, direct method of extracting protozoa. The seawater-ice technique, which works well with marine sediments (see Benthic marine sampling) composed of relatively large sand particles, is of little use with freshwater sediments because the silt and clay fraction is washed through with the protozoa. The simplest alternative, which works well with aerobic and microaerobic protozoa, especially ciliates, is to put the sediment and some overlying water in a stoppered test tube. As the sediment consumes oxygen and becomes anoxic, it forces the protozoa to migrate into the overlying water in search of the upwards retreating oxygen. This is a laboratory model of what happens in stratified lakes [6]. In the laboratory, the process usually takes several days at room temperature. Although this method leads to an effective extraction of many organisms (and is very useful as a first step for isolating organisms into cultures), it does not give reliable quantitative results as the benthic community is likely to change due to laboratory conditions (eg temperature, development of different microbial communities).

The second extraction method we would recommend is a little more elaborate; it does require some specialised equipment, and it is probably useful only for isolating ciliates. `Electromigration' techniques (e.g. [9]) exploit the tendency of many ciliates to orient themselves in an electric field and to swim from the anode to the cathode. Our experience is that the technique does not work equally well for all ciliate species and for waters differing in chemical composition, so it is of dubious value for truly quantitative studies, but it does work with remarkable efficiency for some species, including some anaerobic organisms.

Another serious problem concerns microscopic examination of the sediment. If you put a cover glass on the preparation it may be possible to use high power objectives, but many of the smaller protozoa will remain hidden by particle aggregates. If the cover glass is removed, the smaller protozoa can be revealed or stimulated into motion by probing with a

needle, but observation will be confined to what can be achieved with low power objectives.

Beware of the obvious temptation to chemically fix the sediment in the expectation that it can be examined at a later date. Most protozoa become unrecognisable in fixed sediment. All samples which are taken for quantitative work have to be counted live, preferably on the day of collection. Benthic protozoa are very sensitive to changes in environmental conditions (eg light and especially temperature) and the number of organisms counted tends to decline after collection of a sample. Enumeration of ciliates after storage overnight, even in a refrigerated room, and in the dark, may lead to counts three to four-fold smaller than those obtained immediately after collection!

PROTOCOL

The protocol described below covers the quantitative analysis of the freshwater benthic protozoan (especially ciliate) fauna. It is assumed that the investigator has access to a device for obtaining core samples. This may simply be a rigid plastic tube pushed into the sediment, or it may be any of a variety of sophisticated samplers designed to be lowered from a boat into soft freshwater sediments (a bibliography of samplers is published in [3] and [4]). Core samplers generally permit protozoan quantification on an areal basis together with simultaneous measurement of chemical profiles (eg [5]).

Notes referring to the flow chart are as follows:

(1) Since most benthic protozoa are aerobic or microaerobic, they are confined to a relatively shallow oxygenated layer at the surface of the sediment (often 1cm or less), while the deeper layers become anaerobic due to the small pore volume of freshwater sediments and the limited vertical transport of oxygen by diffusion. Ciliates which are capable of living anaerobically are also usually found within the top few centimeters; partly because they have a very limited capacity to burrow through fine sediments and partly because the food concentration is higher at the sediment-water interface. It is therefore often sufficient to sample the surface layer of the sediment in order to determine protozoan abundance. Occasionally, anaerobic ciliates may be forced into deeper sediment layers, eg if the top sediment layer becomes suddenly oxygenated following a storm. Some anaerobic amoebae and flagellates may penetrate to greater depth, so it is useful to sample a depth profile in the sediment. In this case samples are usually taken with syringes inserted through the wall of the sample tube (see flow chart) or as 1cm slices from the top 6 - 8cm. The latter is most easily achieved by first removing the water overlying the sediment, then extruding the core by pushing it upwards in the tube.

(2) Ciliates die off quickly once they are removed from the core. Zoochlorellae-bearing organisms are especially liable to burst when they are exposed to bright light on the microscope. To keep the counting procedure as short as possible it is advisable to become familiar with the major species present before enumeration. Single organisms can be picked out from the chamber using a micropipette (eg a Pasteur pipette drawn out to a very fine tip over a flame) and then identified at higher magnifications.

(3) Both counting methods involve the direct observation of organisms on a microscopic slide. The larger the area covered by the sub-sample on the slide the more difficult it is to count high numbers of organisms without repeat counts of individual cells. When a 5µl sub-sample of sediment is diluted on a slide, the upper limit of numbers of organisms that can be

counted is 7 to 10. This depends on the texture of the sediment and the species composition.

(4) The flask containing diluted sediment is analogous to a single `sampling unit' and the drops taken from it are then sub-samples of that `sampling unit' [7]. If it is assumed that the 5µl drops are removed randomly from the flask of diluted sediment, the numbers of organisms in a series of drops will fit a Poisson distribution. The sum of all drops taken can be regarded as being equivalent to one single large drop, i.e. a large sub-sample. For a count of 100 organisms the 95% confidence limits are ± 20%, i.e. the odds are 19 to 1 that the true population mean lies within the limits calculated. Note however that this tells us something only about the precision of the sample mean. It tells us nothing about the true population mean, so we cannot, by using this dilution method, put confidence limits on our estimate of the number of organisms per ml sediment.

At low concentrations of cells it may not be possible to count the required number of drops for 100 cells because the counting procedure becomes too time consuming and the organisms start to die off in the dilution. The number of drops then has to be reduced but one should be aware about the reduced precision of the calculated values.

(5) With this method the `sample' is the sediment core and the `sampling units' are the 5µl drops. If at least 30 drops are removed (counts will almost certainly need to be transformed if n<30 [see 2] and it will usually be a more worthwhile effort to increase the number of sampling units), we can use normal methods in the calculation of the precision of the sample mean. The sample mean (x) is an estimate of the true population mean (µ). There is only one value for x at any one time, but , the sample mean, will vary from core to core. If we calculate 95% confidence limits for the sample mean, the odds are 19 to 1 that the true population mean lies within the limits calculated.

As an example, the numbers of protozoa counted in each of 48, 5µl drops were as follows:

8, 11, 7, 6, 11, 3, 2, 6, 5, 8,
7, 7, 3, 6, 1, 2, 6, 1, 2, 10,
2, 1, 5, 6, 5, 3, 2, 2, 15, 6,
5, 0, 3, 1, 2, 7, 4, 2, 2, 6,
16, 4, 6, 1, 3, 4, 5, 8,

n =48, x = 4.96, s =3.51

where s is the standard deviation.

The Standard error of the mean (S.E.M.) is s/ n and the 95% confidence limits are 2 x S.E.M. Thus, the population mean with calculated 95% limits is 992 ± 203 protozoa per ml sediment.

LITERATURE CITED

1. Baldock, BM, Baker, JH & Sleigh, MA. 1983. Abundance and productivity of protozoa in chalk streams. *Holarctic Ecology* **6**:238-246.
2. Elliott, JM. 1983. Some Methods for the Statistical Analysis of Samples of Benthic

Invertebrates. *Scient. Publs. Freshwat. Biol. Assoc.* **25**, Ambleside, UK.

3. Elliott, JM & Tullett PA. 1978. A Bibliography of Samplers for Benthic Invertebrates. *Occ. Publs. Freshwat. Biol. Assoc.* **4**, Ambleside, UK (Out of print).

4. Elliott, JM & Tullett PA. 1983. A Supplement to a Bibliography of Samplers for Benthic Invertebrates. *Occ. Publs. Freshwat. Biol. Assoc.* **20**, Ambleside, UK.

5. Finlay, BJ. 1980. Temporal and vertical distribution of ciliophoran communities in the benthos of a small eutrophic loch with particular reference to the redox profile. *Freshwat. Biol.* **10**:15-34.

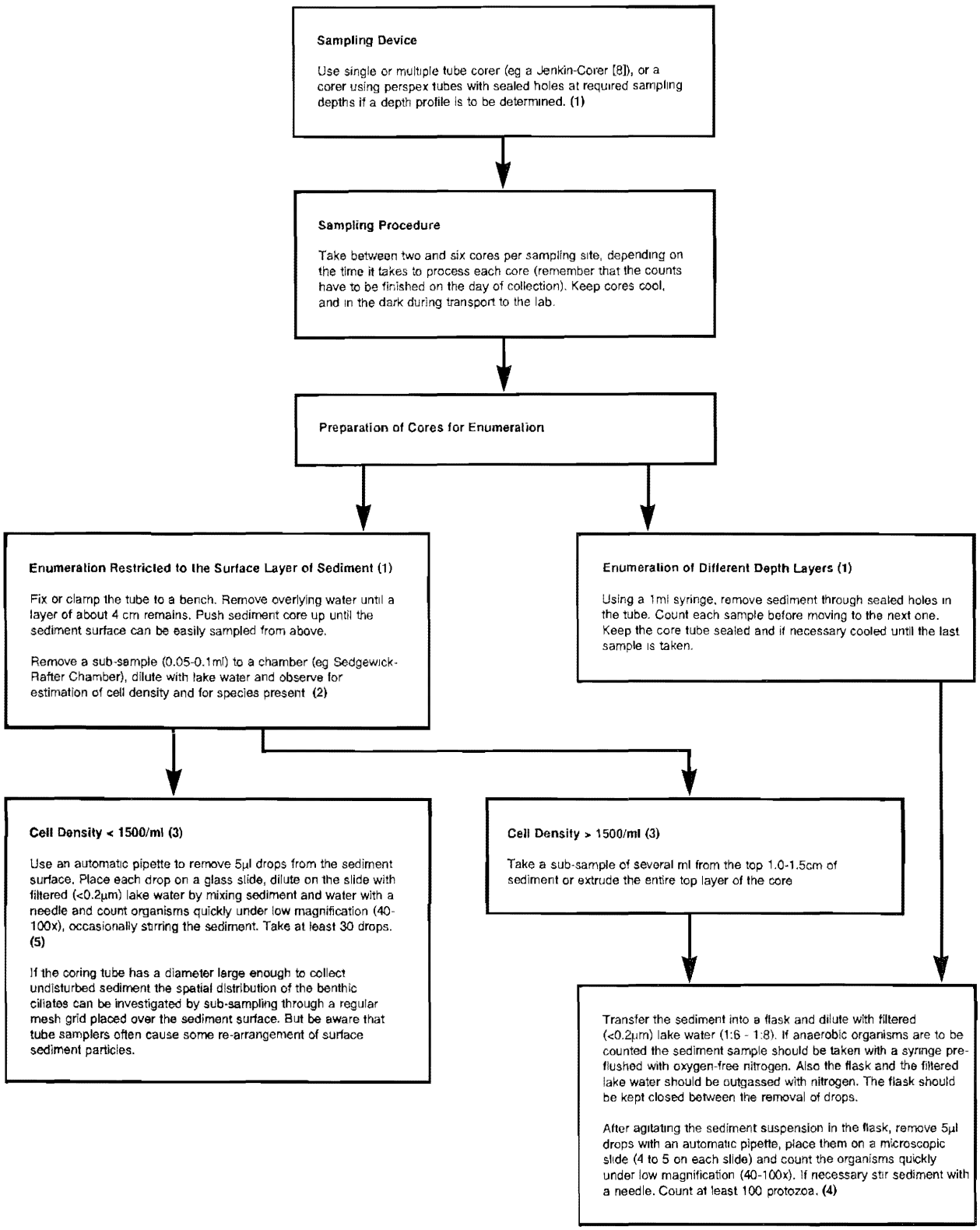
6. Finlay, BJ. 1981. Oxygen availability and seasonal migrations of ciliated protozoa in a freshwater lake. *J. Gen. Microbiol.* **123**:173-178.

7. Finlay, BJ, Laybourn, J & Strachan, I. 1979. A technique for the enumeration of benthic ciliated protozoa. *Oecologia* **39**: 375-377.

8. Ohnstad, FR, and Jones JG. 1982. The Jenkin Surface-Mud Sampler - a Manual. *Occ. Publs. Freshwat. Biol. Assoc.* **15**, Ambleside, UK.

9. Wagener, S, Stumm, CK, & Vogels GD. 1986. Electromigration, a tool for studies on anaerobic ciliates. *FEMS Microb. Ecol.* **38**:197-203.

PROTOCOL FOR SAMPLING BENTHIC PROTOZOA (ESPECIALLY CILIATES)



A SIMPLE METHOD FOR OBTAINING CONCENTRATED POPULATIONS OF PROTISTS FROM SEDIMENTS

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Introduction

Marine and estuarine sediments of varying microporal size provide microhabitats for many protistan organisms (1-4). Protists of different phylum-level taxa can be easily extracted from sandy sediments by means of a nested series of plastic cups, dishes, and nytex nylon (or a piece of panty- hose nylon). Protistan fauna at different depths in the sediment can readily be collected and then be examined microscopically. This technique is a simplification of a method originally described by Uhlig (5).

Protocol

1. Collect moist sediment from a coastal marsh or a fine (125-250 μm) sandy sediment from a back bay. The intraporal spaces between the sand grains hold the water containing the protists. The sand samples may be collected in various sized containers (e.g. 5 quart pails, plastic syringe tubes, etc. A carefully collected tube sample will often display a colored stratification: an uppermost layer which is pale red-yellow-gold in color, a lower layer, black in color and a thin mid-layer which is gray. These zones reflect the relative amounts of dissolved oxygen contained within the sediment water: top layer, aerobic; bottom layer, anaerobic, and in between a transition zone referred to as the RDL, redox discontinuity layer (See Ref. 1). If carefully taken back to the lab and subsequently "sliced" into separate samples, major differences will be noted in the richness of species and types of organisms encountered. If possible, transport these sediment samples to the laboratory in insulated containers (e.g. styrofoam chests) so that temperatures of the sediments remain relatively constant. Also, collect a quart or more of overlying water from as close as possible to the sample site. Begin the extraction procedure detailed below as soon as possible to avoid killing protists in the uppermost aerobic layer.

2. In the laboratory, assemble the simple modified Uhlig device as shown in Fig. 1. Note that the nytex nylon layer is held in place between the 2 plastic cups; this holds the sandy sediment sample in place. The nytex nylon has a pore spacing of 50-85 μm and is sufficient to hold the sandy sediments. One may substitute nylon netting from commercially produced panty-hose provided that the panty-hose material is suitably stretched to allow for similar porous spacing as in the nytex.

3. Filter the water through Whatman #1 filter paper. Use this filtered water to fill the lower petri dish in which the upper surface of the water will be in contact with the nytex nylon.

4. Add a quantity of the sand sample to the nested cups so that they are ~ 3/4 filled with sediment. Sediments in tube samples may be separated into 3 layers, an anaerobic black layer, a discolored RDL layer and an aerobic layer and placed in separate Uhlig devices.

5. To fill the space above the sediment, add filtered water. This will cause the bottom small petri dish to overflow gradually into the standard petri dish below.

6. Over time protists will slowly migrate out of the sediments into the small petri dish. The time required for this to take place varies with the species.

7. Cover the nested cups with the other half of the standard petri dish to prevent the sediment from drying out.

8. To sample, simply remove the small bottom petri dish which contains the migrated protists. To re-sample this same sediment, replace with another small petri dish, add filtered water, and replace sediment-containing cups, You may use the overflow water to pour back into the top of sediment samples or add additional water. Be mindful to remove air bubbles from the interface of the small petri dish and the nytex nylon of the cups.

9. It is best to sample at 12 hour intervals over a period 72 hours.

10. Samplers may be washed in tap water, dried, and used again.

11. To observe the protists, place a clean microscope slide on the stage of a compound microscope. Carefully set the small petri dish on the slide and then use the 10x microscopic objective to observe the extracted faunule. One may rack down the condenser to obtain greater depth of field. Otherwise, use standard glass pipettes to make temporary slide mounts for observation under the microscope at higher magnifications.

Comments

I have used this procedure for more than twenty years and have examined hundreds of sand dwelling protists, especially ciliates, from a wide variety of benthic microhabitats. Sampling may be done quantitatively (6) as well as qualitatively. The faunal composition will vary with the season, the salinity (especially in estuaries), the relative profusion of food organisms (e.g. different sulfur bacteria), and the relative abundance or lack of oxygen. Note: you may want to sample the colorless sediment layer below the black sulfide zone for methanogenic anaerobic protists. Ciliates from sediments may be found year round (certainly in Maryland's Chesapeake Bay country) even when the temperature of the sediment is a few degrees above freezing. It is possible to cultivate many of the protists encountered by using a combination of antibiotics and cooked egg yolk added to the initial petri dishes (7).

REFERENCES CITED

1. Fenchel, T. 1969. The ecology of marine microbenthos IV. Structure and function of the benthic ecosystem, its chemical and physical factors and the microfauna communities with special reference to the ciliated protozoa. Ophelia 6: 1-182.

2. Dragesco, J. 1960. Cilies mesop sammiques litoraux. Systematique, morphologia, ecologie. Trav. Stat. Biol. Roscoff (N.S.) 12: 1-356.

3. Patterson, D. J., Larsen, J., & Corliss, J. O. 1989. The ecology of heterotrophic flagellates and ciliates in marine sediments. Progress in Protistology 3: 185-277.

4. Corliss, J. O., Hartwig, E. & Lenk, S. E. 1988. Protozoa: Ciliophora. In: Introduction to the Study of Meiofauna. Eds. Higgins, R. P. & Thiel, H. Smithsonian Press, Washington, D.C.

5. Uhlig, G. 1968. Quantitative methods in the study of interstidial fauna. Trans. Amer. microsc. Soc. 87: 226-232.

6. Boynton, J. E. & Small, E. B. 1984. Ciliates by the slice. Sci. Teacher 51: 34-38.

7. Lenk, S. E., Small, E. B., & Gunderson, J. 1984. Preliminary observations of feeding in the psammobiotic ciliate Trachelorophis. Origins of Life 13: 229-234.

B-3.4

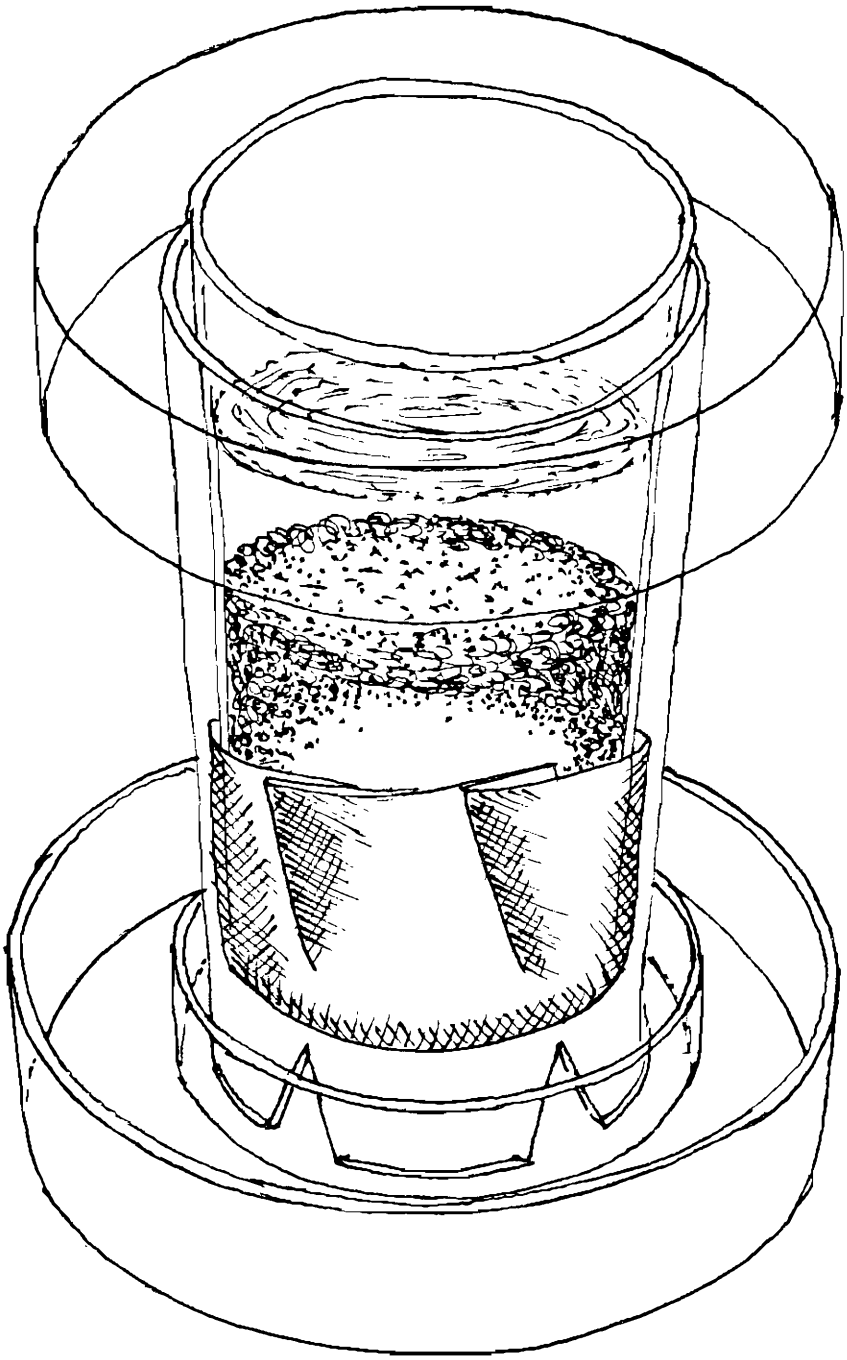

Fig. 1. Modified Uhlig protistan extractor. Intact at top and bottom are standard petri dishes for control of over flow and loss of water due to desiccation, respectively. Interior to the bottom standard petri dish sits a smaller plastic petri dish to which filtered water is added to the level of the nytex nylon wedged between the two soft plastic cups. The bottoms of the cups have been so cut that the inner one sits firmly inside the outer one, and the outer one is slightly longer to allow the protists to migrate out of the sediments (held in place by the nytex nylon) and into the water of the lower small petri dish. On top of the sediment sample a layer of filtered water has been added.

A SIMPLE METHOD FOR OBTAINING CONCENTRATED POPULATIONS OF PROTISTS FROM SEDIMENT WATER

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Introduction

A simple method of attracting ciliate protists to prey bacteria utilizes a baited trap containing an initially sterile food source. These traps may be conveniently placed at the bottom of marine, estuarine, or freshwater sites where bactivorous protists are known or suspected to be present. The trap selectively excludes micrometazoan predators so that over the course of a few days a large population of ciliates may be encountered feeding on the prey bacteria. This trapping technique has been successfully used in coastal estuaries of the eastern United States and Somalia on the Indian Ocean as well as in the marine caves of Bermuda (1).

Protocol

1. As shown in Figs. 1a & b, fashion a trap from a 35mm soft plastic film canister with a snap-top lid, Cut out spaces for three windows. Cover each space with nylon mesh screening (200-350 μm pore size) and cement in place with Dow Corning Aquarium silicon glue. Such traps then may be bobbered, weighted or otherwise positioned at a specific sampling site.

2. Several traps may be taken to a field site to be sampled.

3. The trap may then be baited with your choice of a sterile food-stuff that will permit the growth of resident bacteria that will serve as prey for the protists. We have used sterile canned tuna fish, canned crab meat and boiled wheat grains. The amount used per trap should be about the size of a pea.

4. The traps are then left in the habitat for varying periods depending upon the local water temperature. Two or three days is sufficient for traps left at sites where the water temperature is ~20°C or warmer.

5. When the traps are to be recovered from the collecting site, a clean plastic sandwich bag is carefully placed around the trap container to prevent accidental loss of the sample.

6. Traps containing water in bags are then carefully placed in cooler chests and returned to the laboratory as quickly as possible.

7. Once in the laboratory, the contents of a trap sample may be carefully placed in a sterile covered petri dish and be examined microscopically. Clones may then be isolated or the protists fixed for cytological purposes.

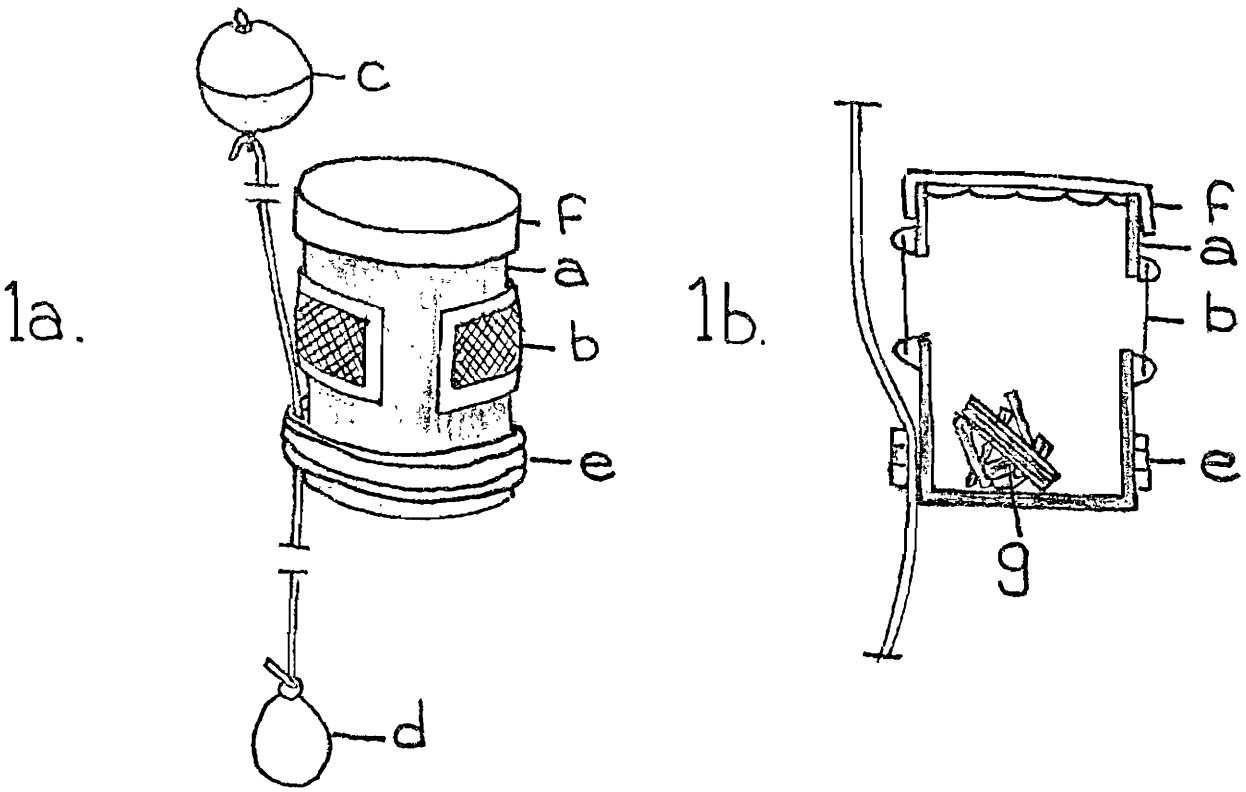
B-4.2

Comments

This technique, originally designed to sample marine caves, has been successfully used to sample many other types of habitats, especially bottom-water interfaces in which a rich detritus layer may be found. The technique also works well for sampling aquaria.

REFERENCES CITED

1. Small, E. B., Heisler, J. Sniezek, J., & Iliffe, T. 1986. Glauconema bermudense n. sp. (Scuticociliatida Oligohymenophorea), a troglobitic ciliophoran from bermudian marine caves. Stygologia 2: 167-179.



Whole and cut away views of 35mm film canister baited traps for protists, **a** = 35 mm film canister trap, **b** = window of trap covered with 200-350 μm nylon screening secured to canister with silicon aquarium cement, **c** = fishing bobber attached to line with lead weight **d** at opposite end, **e** = rubber band, **f** = snap top lid,and **g** = small portion of tuna fish as bait.

SAMPLING AND ENUMERATING SOIL PROTOZOA

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Introduction

Litters and soils are mosaics of microhabitats, therefore composite samples are collected from an area, mixed well, and subsamples used for identifications of species and estimations of numbers. Portions of the samples should also be measured for moisture content and pH, because the presence and abundance of many species reflect these two parameters.

Many protozoa in soils are encysted. Direct counts of testacea (4, 9, 10) distinguish between active, encysted forms and empty tests, direct ciliate count (9, 13) estimate the number of active ciliates, but most probable number (MPN) culture methods (5, 15, 16) must be used for the more abundant small flagellates and amoebae. Culture methods give only total numbers in the soil and do not distinguish between active and encysted forms.

Protocols

A. Sampling

1. Collect 6-12 samples by spatula or cork borer (about 15 mm diameter) from 0-3 cm soil depth, in a 4-16 square meter area; and preferably several such areas in a forest, grassland, agricultural field, etc.
2. Mix the samples thoroughly and withdraw subsamples for the studies described below.

B. Species Richness

1. Place 10-50 g of litter or soil sample, at least 1 cm deep, in a 10-15 cm diameter petri dish.
2. Saturate, but do not flood with distilled water. Water should be added until 5-20 ml will drain off when soil is gently pressed with a finger. This run-off contains the protozoa.
3. Examination schedule and usual succession of protozoa:
   Days 2-3 Small flagellates, colpodids, some other ciliates
   5-6 Small ciliates, hypotrichs
   8-10 Hypotrichs, testacea
   15-20 Mainly testacea
4. Most amoebae will be found by streaking two "sine waves"of a bacterial suspension on a plate of water agar, and by adding two straight streaks of the sample suspension bissecting the bacterial streaks. Examine for amoebae migrating into the bacterial streaks and beyond, on days 4-6.
5. Additional (especially small) testacea will be found on permanent direct count slides described below.
6. Taxonomic references:
   General: Lee et al (11); Lousier & Bamforth (12)
   Gymnamoebae: Page (14); Bovee (1)
   Testacea: Cash et al (2,3); Bovee (1); Foissner (9)

Ciliates: Foissner (7, 8, 9,)

C. MPN Dilution Method for Quantitative Estimations

1. Singh method:
    a. Embed 8 polypropylene or glass rings in agar per petri dish for 15 dishes (=levels).
    b. Prepare an initial 1:5 soil suspension, using soil extract as the diluent, and then make two-fold dilutions (1:10, 1:20, 1:40, etc.).
    c. Pipette 0.5 ml of each dilution into each of 8 rings to obtain 15 levels.
    d. Examine at 4-day intervals. After a week, add suspension of a soil bacterium, to induce excysted protozoa (mainly amoebae) to appear.
    e. Count number of negative rings and consult appropriate tables (e.g., to estimate numbers).(6)
2. Stout modification:
    a. Using larger rings, embed 10 rings per level for 3 levels.
    b. Prepare three ten-fold dilutions. Dilution range depends on soils: 10^1 to 10^3 for nutrient poor, 10^3 to 10^5 for very rich soils.
    c. Pipette 1.0 ml of each dilution into 10 rings to obtain 3 levels.
    d. Examine and consult tables, according to 1-d, 1-e above.
3. Darbyshire et al modification:
    a. Prepare a two-fold dilution series (1-b above).
    b. Pipette 0.05 ml of each dilution into 8 wells of a 96-well microtiter plate.
    c. Examine and consult tables according to 1-d, 1-e above.

D. Fresh Direct Counts for Ciliates and Testacea

1. To a small amount, 0.2 to 0.4 g, of fresh soil, add 3-6 ml of soil extract, and examine dropwise, on the day of sampling for ciliates.
2. Add 5 ml of phenolic aniline blue to another 0.1 ml of the fresh soil and allow to stand overnight. (Wash out the aniline blue by centrifugation and macerate the pellet. Add 0.5 ml of albumin-glkycerin or 0.5% agar to 5 ml of the pellet suspension, and immediately examine dropwise.
3. The amount of dilution depends upon soil type and abundance of protozoa.
4. Phenolic aniline blue: 5% phenol, 30 ml, 1% aniline blue, 2 ml; glacial acetic acid, 8 ml. Stand 1 hour before using.

E. Permanent Direct Count for Testacea: Couteaux Method

1. Fix 0.25 g fresh sample in 1 ml Bouin-Hollande solution for 24 hours.
2. Stain with 3 ml of 1% xylidine ponceau 2R (Aldrich Chem. Co. Milwaukee, WI) for 30 minutes.
3. Dilute with distilled water to 250 ml.
4. Agitate several hours to dislodge testacea from soil particles.
5. Filter 5 ml through Millipore filter (diameter 25 mm, pore size 0.45 m) under vacuum pressure.
6. Air dry the filter.
7. Clear in xylene.
8. Mount in Canada balsam or other mounting medium.
9. Examine under phase contrast, 400X magnification, a known area of the whole filter.
10. Bouin-Hollande solution: 6.25 copper acetate; 250 ml distilled water; 10 ml picric acid. Filter. Add 25 ml of 40% formalin and 2.5 ml glacial acetic acid.

F. Permanent Direct Count for Testacea: Korgonova & Geltser

1. Place 5 g soil in 50 ml water in a 250 ml flask. Soak several hours.
2. Shake 10 minutes; or on shaker several hours.
3. Pipette one drop (=0.05 ml) from center of flask to clean slide and add one drop of 0.5% agar.
4. Spread with thin glass rod over 8 square cm area.
5. Air dry the slide.
6. Stain with 1% phenolic erythrosin for 1 hour.
7. Put slide through 3 water rinses and air dry.
8. Examine as E-9 above.
9. Phenolic erythrosin: 1 g erythrosin in 100 ml of 5% phenol.

Comments

Testacea and ciliates can serve as bioindicators of soils (9). Testacean species may be grouped into four ecological types according to the shape of the shell: disc (Arcella), vaulted (acrostome), globose (axial), and wedge (plagiostome). These form a series from moist (Arcella) to dry (wedge). There are some exceptions to this scheme, but graphing these proportions give information about moisture stability.

For ciliates, if the number of colpodid species is divided by the number of polyhymenophoran species, a "C/P ratio" is obtained: >1.0 indicates a stressed or low organic soil, and <1.0 a more moisture stable soil, often rich in ciliate species.

Jaccard and Sorensen indices may be used for testacean and ciliate species to compare different soil habitats.

LITERATURE CITED

1. Bovee, E.C. 1985. Rhizopoda. In Lee, JJ, Hutner, SH, & Bovee, EC, eds: An Illustrated Guide to the Protozoa. Lawrence Kansas, Allen Press.
2. Cash, J. 1905, 1909. The British Freshwater Rhizopoda and Heliozoa. Vols 1,2. London, The Ray Society.
3. _____ & Wailes, GH. 1915, 1918. The British Freshwater Rhizopoda and Heliozoa, Vols 3,4. London, The Ray Society.
4. Couteaux, M-M. 1967. Une technique d'observation des thecamoebiens du sol pour l'estimation de leur densite absolue. Revue Ecol. Biol. Sol. 4: 593-596.
5. Darbyshire, JF, Wheatley, RE, Greaves, MP, & Inkson, RHE. 1974. A rapid micromethod for estimating bacterial and protozoan populations in soil. Revue Ecol. Biol. Sol. 11: 465-475.
6. Fisher, RA & Yates, F. 1963. Statistical Tables in Biological, Agricultural, and Medical Research. Edinburgh, Oliver & Boyd.
7. Foissner, W. 1982. Okologie und Taxonomie der Hypotrichida (Protozoa: Ciliophora) einger osterreischer Boden. Arch. Protistenk. 126: 19-143.
8. _____ 1985. Klassification und Phylogenie der Colpodea (Protozoa: Ciliophora). Arch. Protistenk. 129: 239-290.
9. _____ 1987. Soil Protozoa: Fundamental problems, ecological significance, adaptations in ciliates and testaceans, and guide to the literature. Progr. Protistol. 2: 69-212.
10. Korgonova, GA & Geltser, JG. 1977. Stained smears for the study of soil Testacida (Protozoa, Rhizopoda). Pedobiologia. 17: 222-225.
11. Lee, JJ, Hutner, SH, & Bovee, EC. 1985. An Illustrated Guide to the Protozoa. Lawrence, Kansas, Allen Press.
12. Lousier, JD, & Bamforth, SS. Soil Protozoa. In: Dindal, DL: Soil Biology Guide. New York, John Wiley. pp 97-136.
13. Luftenegger, G, Petz, W, Foissner, W, & Adam, H. 1988. The efficiency of a direct counting method in estimating the numbers of microscopic soil organisms. Pedobiologia. 31: 95-101.
14. Page, FC. 1988. A new key to freshwater and soil Gymnamoebae. Ambleside, England: Freshwater Biological Association.
15. Singh, BN. 1955. Culturing soil protozoa and estimating their numbers in soil. In: Kevan, DK McE: Soil Zoology. London, Butterworths. pp 403-411.
16. Stout, JD. 1962. An estimation of microfaunal populations in soils and forest litter. J. Soil Sci. 13: 314-320.

ENUMERATING SOIL TESTATE AMOEBAE BY DIRECT COUNTING

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INTRODUCTION

Testate amoebae are usually enumerated by direct microscopy of aqueous soil suspensions (4-9). Various modifications are used worldwide (e. g., 1, 2). We recommend the method of Lüftenegger et al. (6) because recovery experiments revealed a mean efficiency of 86% and the inspected sample mass is sufficient to record even the more rare, euedaphic species (Fig. 2, 3).

PROTOCOL

1. Put a certain amount of fresh (wet) soil, i. e. 1-2 g arable land or grassland or 0.5 g forest litter, in a centrifuge tube by taking 10-20 portions with tweezers from different sites of the sample.
   Remarks: No systematic studies about distribution of testate amoebae in soil are known. Usually, 10-20 soil cores are collected from the area studied and thoroughly mixed to a bulk sample.
2. Fix and stain sample with about 7 ml phenolic aniline blue at least overnight. Mix thoroughly by shaking at least ten times.
   Remarks: Samples can be stored in this condition for years. Centrifuge tubes with screw-tops are ideal for mixing and storing such samples. If suspension becomes colourless after a few hours (sometimes with calcareous soils), centrifugate sample and replace colourless solution by fresh phenolic aniline blue.
3. Wash content of storage vessel into a calibrated cylinder and fill up to 100 ml with distilled water. Close cylinder with parafilm and mix thoroughly by shaking at least ten times.
4. Take a 1 ml subsample from suspension using a 5 ml calibrated pipette cut off at the 1 ml marking to prevent selective sampling of small soil particles.
   Remarks: This step must be done quickly to minimize sedimentation. Collected sample mass corresponds to 0.01-0.02 g and 0.005 g fresh (wet) soil and litter, respectively (see step 1). Dilution depends mainly on soil type. Soils with a high clay content or with high numbers of testate amoebae need a higher dilution than humic or weakly populated soils. The 1 ml sample should be diluted with some water if suspension is too dense.
5. Examine whole subsample by placing suspension dropwise (about 0.1 ml) on grease-free slide. Use a compound microscope and a magnification of at least X100 (objective 10:1, ocular X10). Full (dark blue stained cytoplasm) and empty tests (unstained or light blue) are easily distinguished from unstained, inorganic soil particles.
   Remarks: Add 0.1 ml albumen-glycerol to 1 ml soil suspension if soil particles tend to aggregate on the slide. Preparations should be investigated without coverslip because species identification often requires that tests are turned with a mounted eye lash or isolated with a micropipette. Isolated species can be stored in a moist chamber (e. g., a covered petri dish with damp filter paper covering its bottom) for later identification. However, it is recommended to get acquainted with the respective species inventory

beforehand to restrict time-consuming identification during enumeration.

Stable voucher specimens can be obtained with the following method: collect tests with a micropipette and place them onto a slide covered with a thin, dry layer of albumen-glycerol; dry preparation at room temperature; transfer slide to xylene overnight and mount in synthetic medium (e. g., Eukitt, Euparal). To avoid destruction of voluminous tests support coverslip corners by small pieces of coverslip glass.

6. Repeat steps 4 and 5 to record at least 15-30 (arable land) or 50-70 (grassland, forest) full tests.
   Remarks: An experienced worker needs about 8 hours for the microscopical examination (counting) of 0.1 g soil from arable land or grassland and about 4 hours for 0.005 g forest litter. In bulked samples, the individual minimal area is usually approached with the masses mentioned above. A complete species inventory needs more detailed investigations, e. g., the flotation of empty tests by gas bubbles (3, 8). Repeated investigations of some soils with the method described showed that 2-5 samples distributed over one year yield approximately 50-80% of the species found in 10 samples investigated over two years (Fig. 1).

REAGENTS

a) Phenolic aniline blue (mix components and filter; stable for years)
   15 parts phenol solution (C_6H_5OH; preparation: dissolve 5 g phenol in 100 ml distilled water)
   1 part aniline blue solution ($C_{32}H_{25}N_3Na_2O_9S_3$; preparation: dissolve 1 g aniline blue in 100 ml distilled water)
   4 parts glacial acetic acid (= concentrated acetic acid; $C_2H_4O_2$)
b) Albumen-glycerol
   Use self-made (see Foissner's protargol protocol) or commercial product (e. g., Merck)

CALCULATION

Numbers are calculated per g dry mass of soil and/or as individuals per square meter. Accordingly, the water content and/or the bulk density of the respective soil layer must be determined by standard methods (see textbooks on soil investigation).

$$I\ g^{-1}\ dm = \frac{lwm}{wm \cdot dm} \qquad I\ m^{-2} = \frac{lwm}{wm \cdot dm} \cdot b \cdot d \cdot 10^4$$

b bulk density in g cm^{-3}
d depth (cm) of soil layer sampled (e. g., 5 cm)
dm dry mass of soil expressed from 0.0 to 1.0 (e. g., 0.4 if soil contained 60 % water)
I individual number (abundance)
lwm total individual number counted in wet mass (wm) of soil
wm wet (fresh) mass (in gram) of soil examined (e. g., 0.005 g forest litter)
10^4 factor to relate bulk density to 1 m^2 (= 10000 cm^2)

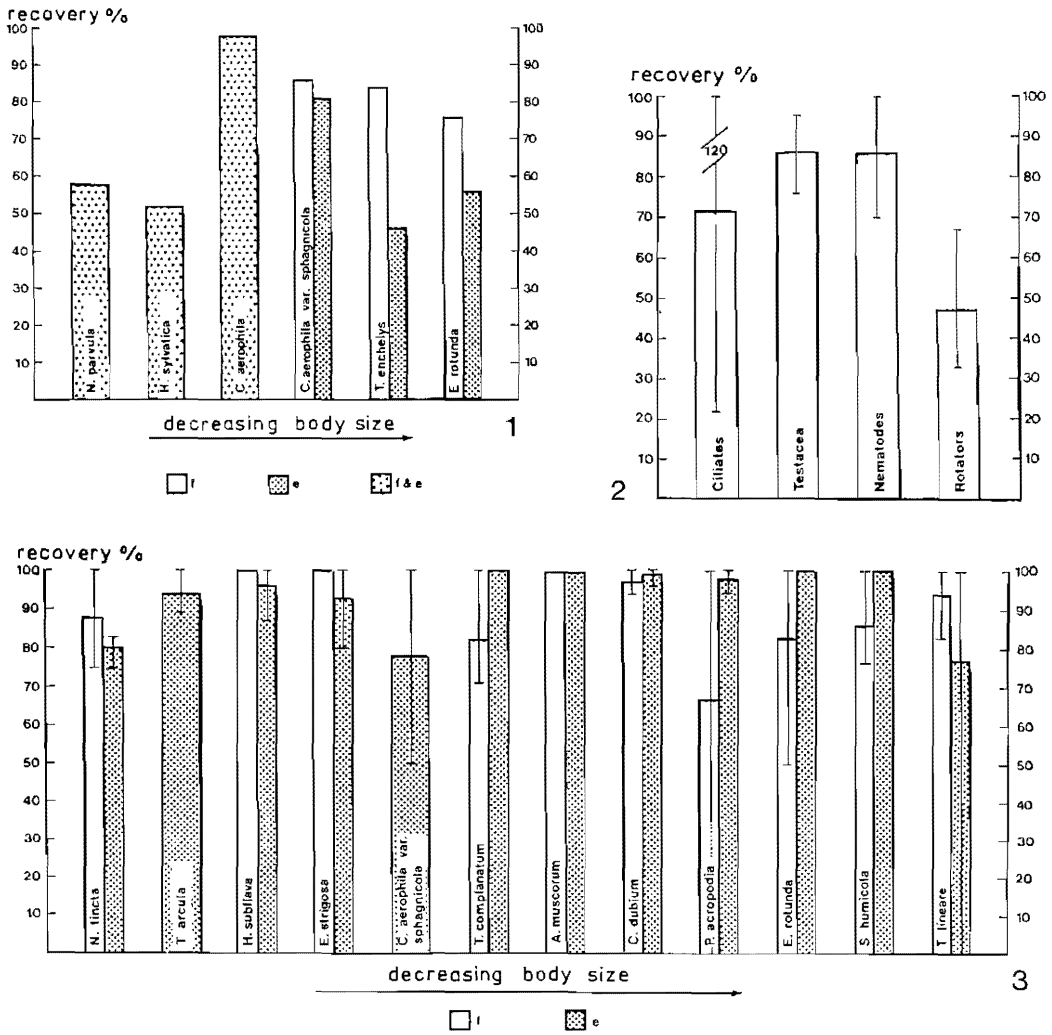

Fig. 1. Cumulative totals of testacean species from 10 sampling occasions over 27 months at three sites in Austria (from [4]). ---------- beech forest, - - - - - xerothermic uncultivated grassland, ... wheat field (see step 6 of protocol for explanation).

Fig. 2, 3. Recovery rates of testacean species (from [6]). **2.** Single species experiments (n = 1) with mineral soil from a levelled ski slope (0-3 cm) and from a mixed deciduous forest (5-10 cm), respectively. **3.** Multiple species experiments (n = 3) with spruce forest litter. e, empty tests; f, full tests.

LITERATURE CITED

1. Couteaux, M-M. 1967. Une technique d'observation des thécamoebiens du sol pour l'estimation de leur densité absolue. *Revue Écol. Biol. Sol* **4**:593-596.
2. Couteaux, M-M. 1975. Estimation quantitative des thécamoebiens édaphiques par rapport à la surface du sol. *C. r. hebd. Séanc. Acad. Sci., Paris* **281**:739-741.
3. Décloitre, L. 1960. Perfectionnements apportés à un appareil pour une technique d'isolement des microorganisms du sol, des mousses, des eaux. *Int. Revue ges. Hydrobiol.* **45**:169-171.
4. Foissner, W. 1987. Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to the literature. *Progr. Protistol.* **2**:69-212.
5. Lousier, J D & Parkinson, D. 1981. Evaluation of a membrane filter technique to count soil and litter testacea. *Soil Biol. Biochem.* **13**:209-213.
6. Lüftenegger, G, Petz, W, Foissner, W & Adam, H. 1988. The efficiency of a direct counting method in estimating the numbers of microscopic soil organisms. *Pedobiologia* **31**:95-101.
7. Meisterfeld, R. 1989. Die Bedeutung der Protozoen im Kohlenstoffhaushalt eines Kalkbuchenwaldes (Zur Funktion der Fauna in einem Mullbuchenwald 3). *Verh. Ges. Ökol.* **17**:221-227.
8. Schönborn, W. 1989. Urtiere (Protozoa). *In:* Dunger, W & Fiedler, H J (ed.), Methoden der Bodenbiologie. Fischer, Jena, pp. 282-290.
9. Wanner, M. 1991. Zur Ökologie von Thekamöben (Protozoa: Rhizopoda) in süddeutschen Wäldern. *Arch. Protistenk.* **140**:237-288.

ENUMERATING ACTIVE SOIL CILIATES BY DIRECT COUNTING

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INTRODUCTION

Ciliates cannot be directly extracted from soil. Therefore, enumeration involving various culture techniques have been suggested (2, 7, 8). However, these methods can only estimate the abundance of active + cystic cells. Thus, direct methods, i. e. inspection of soil suspensions, must be used for counting active ciliates (1, 4). Various direct methods are known: Couteaux & Palka (3) use millipore filters and Griffiths & Ritz (5) density gradient centrifugation with subsequent fluorescent staining. However, these techniques require fixation and staining of cells which is complicated and limits identification of species. We thus recommend to count the living cells. The mean efficiency of the procedure described below is 72% (6; Fig. 3). Bamforth (1) combines direct counting with a culture technique to estimate the numbers of active and cystic ciliates.

PROTOCOL

1. Take 10 portions of fresh (wet) soil, about 0.01 g each (in total 0.1 g), with tweezers from different sites of the sample and collect them in a small vessel.
   Remarks: No systematic studies about distribution of ciliates in soil are known. Usually, 10-20 soil cores are collected from the area studied and thoroughly mixed to a bulk sample. Ciliates must be counted on the day of sampling due to their ability to encyst rapidly.
2. Add 1-3 ml diluted soil extract and mix thoroughly with a glass-stick to obtain a fine-grained suspension.
   Remarks: The diluted soil extract used prevents delicate ciliates from bursting. Dilution depends mainly on soil type. Soils with a high clay content or with high numbers of ciliates need a higher dilution than humic or weakly populated soils.
3. Place suspension dropwise (about 0.1 ml) on grease-free slide and examine without coverslip under a compound microscope at X40 magnification (objective 4:1, ocular X10). Ciliates are rather easily recognized due to their mobility.
   Remarks: Preparations should be investigated without coverslip because species identification sometimes requires that cells are isolated with a micropipette. Isolated specimens can be stored in a moist chamber (e. g., a covered petri dish with damp filter paper covering its bottom) for later identification. However, it is recommended to get acquainted with the respective species inventory beforehand to restrict time-consuming identification during enumeration. Stable voucher specimens of ciliates are obtained by the methods described in chapter C of this book.

   There is a strong positive correlation between body size and percentage of recovery (Fig. 3). By using a higher magnification estimates could probably be improved, but the working time would increase too much for practical purposes, e. g. bioindication studies.
4. Repeat steps 1-3 until at least 0.4 g fresh (wet) soil is examined.
   Remarks: Dividing the total sample into 0.1 g portions reduces the risk of excystment. An experienced worker needs 2-4 hours for the microscopical examination (counting)

of 0.4 g soil. Experiments showed that 0.4 g soil wet mass is usually sufficient to obtain a representative estimation of the individual abundance and species composition (Fig. 1, 2). In soils with moderately high numbers of active ciliates (> 50 individuals per g wet mass) about 85% of the species, which are found in 1 g, occur in the 0.4 g subsample. This seems to be an acceptable compromise between working time and winning of additional information. For soils with few ciliates, a sample mass of 0.4 g is obviously too small. A rather complete species inventory can be obtained with the "non-flooded petri dish method" (see this protocol).

Note: Evolved soils usually contain few (< 50 individuals per g wet mass of soil) active ciliates due to the inhibitory effects of ciliatostasis (see [4] for detailed information).

REAGENTS

a) Soil extract (easily colonized by bacteria or fungi; check before use and eventually filter and autoclave again)
   300 g soil from sample site
   ad 1000 ml distilled water
   Boil for 10 minutes, decant, filter and autoclave
b) Diluted soil extract (prepare before use)
   1 part soil extract (see above)
   5 parts distilled water
   Adjust to pH of soil investigated with HCl or NaOH

CALCULATION

Numbers are calculated per g dry mass of soil and/or as individuals per square meter. Accordingly, the water content and/or the bulk density of the respective soil layer must be determined by standard methods (see textbooks on soil investigation).

$$I\ g^{-1}\ dm = \frac{Iwm}{wm \cdot dm} \qquad I\ m^{-2} = \frac{Iwm}{wm \cdot dm} \cdot b \cdot d \cdot 10^4$$

b bulk density in g cm^{-3}
d depth (cm) of soil layer sampled (e. g., 5 cm)
dm dry mass of soil expressed from 0.0 to 1.0 (e. g., 0.4 if soil contained 60 % water)
I individual number (abundance)
Iwm total individual number counted in wet mass (wm) of soil
wm wet (fresh) mass (in gram) of soil examined (e. g., 0.005 g forest litter)
10^4 factor to relate bulk density to 1 m^2 (= 10000 cm^2)

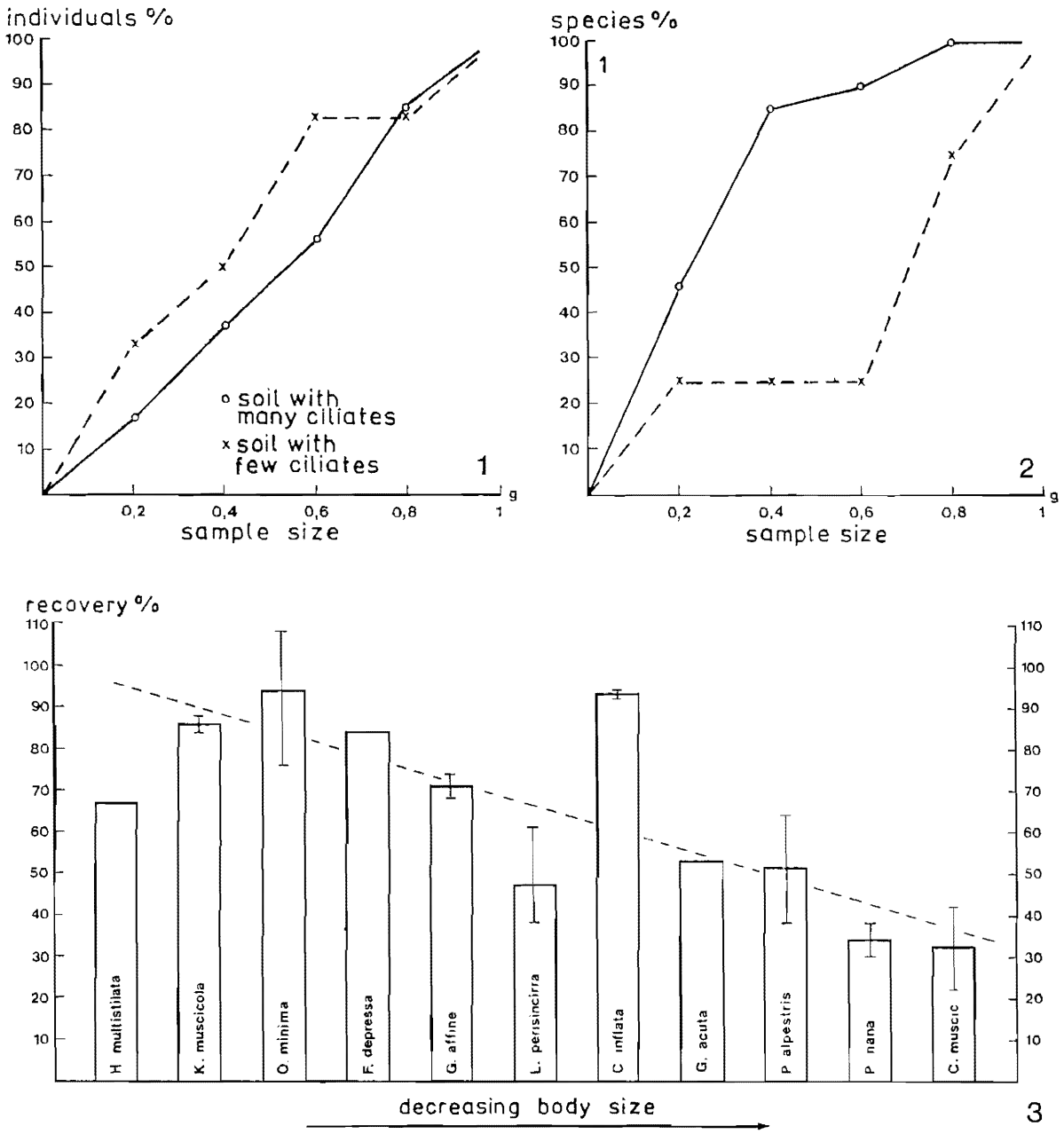

Fig. 1, 2. Test for optimal sample size for ciliate counting (from [6]). **1.** The individuals found in 1 g fresh soil of a levelled ski slope represent 100%. The 1 g soil sample was counted by subsampling of 0.2 g (5 x 0.2 g). The curves are almost straight, indicating a homogeneous distribution of the organisms. **2.** The same as in Fig. 1 was done for the species number (see remarks at step 4 of the protocol for further explanation).

Fig. 3. Recovery rates of ciliate species. Single species experiments with sterilized soils (from [6]). Correlation (r_s = 0.7) between body size and percentage of recovery is indicated by broken line.

LITERATURE CITED

1. Bamforth, S S. 1991. Enumeration of soil ciliate active forms and cysts by a direct count method. *Agric. Ecosyst. Environm.* **34**:209-212.
2. Cutler, D W. 1920. A method for estimating the number of active protozoa in the soil. *J. agric. Sci., Camb.* **10**:135-143.
3. Couteaux, M-M & Palka, L. 1988. A direct counting method for soil ciliates. *Soil Biol. Biochem.* **20**:7-10.
4. Foissner, W. 1987. Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to the literature. *Progr. Protistol.* **2**:69-212.
5. Griffiths, B S & Ritz, K. 1988. A technique to extract, enumerate and measure protozoa from mineral soils. *Soil Biol. Biochem.* **20**:163-173.
6. Lüftenegger, G, Petz, W, Foissner, W & Adam, H. 1988. The efficiency of a direct counting method in estimating the numbers of microscopic soil organisms. *Pedobiologia* **31**:95-101.
7. Singh, B N. 1946. A method of estimating the numbers of soil protozoa, especially amoebae, based on their differential feeding on bacteria. *Ann. appl. Biol.* **33**:112-120.
8. Stout, J D. Biological studies of some tussock-grassland soils VII. Protozoa. *N. Z. Jl agric. Res.* **1**:947-984.

ENUMERATION OF PROTISTS AND SMALL METAZOANS IN ACTIVATED SLUDGE

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INTRODUCTION

Augustin et al. (2) standardized a method for enumerating active protists and small metazoans in activated sludge. This direct counting method recovers an average of 85 % ciliates (n = 165 countings), 95 % nematodes (n = 10), 84 % rotators (n = 10), and 78 % testate amoebae (n = 10). The recovery rate is correlated to the size of the organisms (Fig. 2) and is not influenced by sludge structure. This simple method seems to be an acceptable compromise between time spent and degree of accuracy. Hiller (4) described another simple method for estimating individual numbers of organisms and particles; however, this technique requires that objects are non-motile.

PROTOCOL

1. Place 10 µl fresh, well-mixed activated sludge on one of both central areas of a Thoma counting chamber (Fig. 1).
   Remarks: Use a rather large-bored pipette (c. 0,5-1 mm), e. g. "Assipette-digital". Activated sludge containing very many organisms should be diluted (for example 1:1) with liquid obtained by centrifugation of the sludge investigated. It is recommended to get acquainted with the respective species inventory beforehand to restrict time-consuming identification during enumeration.
2. Cover droplet with coverslip (c. 18 x 18 mm).
   Remarks: The 10 µl sludge used fill exactly the hatched area of the chamber (Fig. 1).
3. Count organisms using a compound microscope and a magnification of 100:1.
   Remarks: Count the complete area of 14,5 x 7,0 mm (see explanation to Fig. 1). Counting 5 x 10 µl activated sludge needs about 2 hours. Very small protists, e. g. heterotrophic flagellates and most naked amoebae, must be counted with the oil immersion objective at a magnification of 1000:1. For such organisms it is sufficient to count 5 nl of sludge which is obtained by appropriate dilution (1).
4. Repeat steps 1 to 3 of protocol 4 times.
   Remarks: A statistical evaluation is possible because 5 separate subsamples are counted. The dispersion of the organisms usually follows a Poisson distribution which, however, should be tested with the dispersion index (4). A computer program is available (5) for the calculation of the dispersion index, the arithmetic mean with confidence interval, the coefficient of variation, the standard deviation, the number of individuals per milliliter extrapolated from arithmetic mean, and the percentage confidence limits.

B-8.2

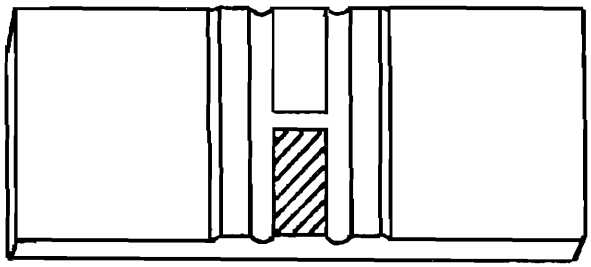

Fig. 1. Thoma counting chamber (from [2]). Unlike its normal use, the whole hatched area (not only the grid) carrying a capacity of 10 µl is counted.

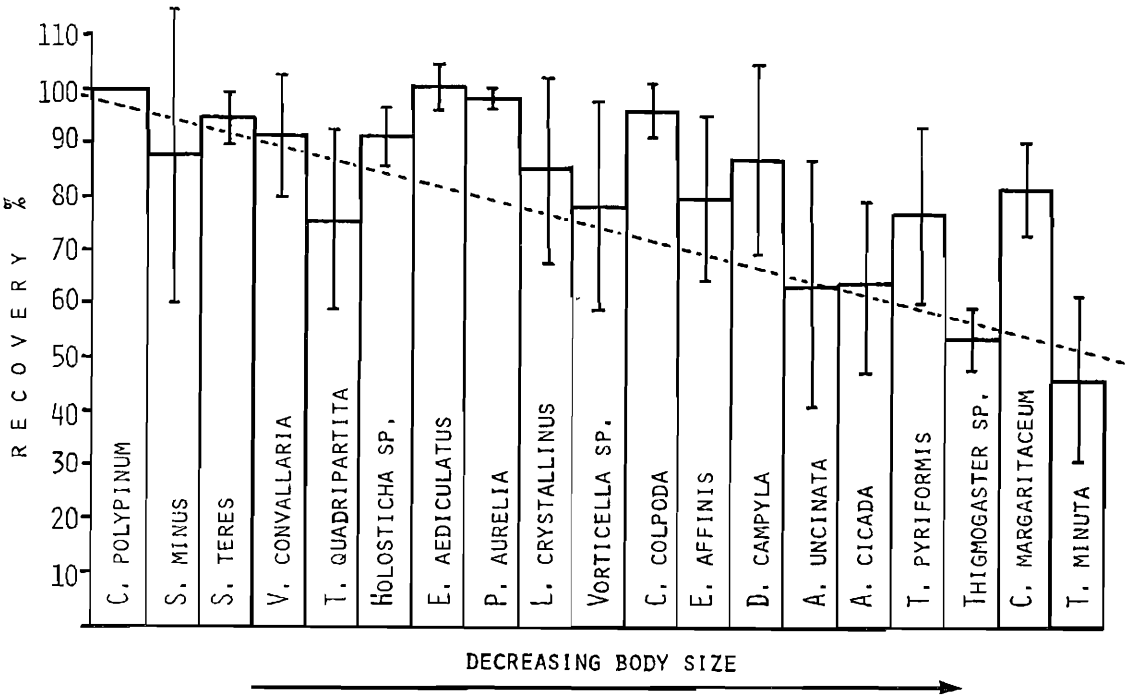

Fig. 2. Percentage recovery with standard deviation (vertical bars) for some ciliate species and correlation between body size and recovery rate (dotted line) in activated sludge (from [2]). *Colpidium colpoda*: number (n) of experiments = 20; *Cinetochilum margaritaceum*, *Euplotes aediculatus*, *Vorticella convallaria*: n = 15; *Dexiostoma campyla*, *Euplotes affinis*, *Litonotus crystallinus*, *Tetrahymena pyriformis*, *Vorticella* sp.: n = 10; *Acineria uncinata*, *Aspidisca cicada*, *Carchesium polypinum*, *Holosticha* sp., *Paramecium aurelia*, *Spirostomum minus*, *S. teres*, *Thigmogaster* sp., *Tokophrya quadripartita*, *Trochilia minuta*: n = 5.

LITERATURE CITED

1. Aescht, E & Foissner, W. 1992. Biology of an activated sludge plant heavily loaded with organic wastes from a pharmaceutical company. *Arch. Hydrobiol.* (in press).
2. Augustin, H, Foissner, W & Bauer, R. 1989. Die Zählung von Protozoen und kleinen Metazoen im Belebtschlamm. *Acta hydrochim. hydrobiol.* **17**:375-386.
3. Hiller, S A. 1991. Eine zeitsparende qualitative Auszählmethode hoher Effizienz zur Schätzung der Partikeldichte in Zählkammern. *Lab. med.* **15**:147-154.
4. Kalbe, L & Recknagel, R-D. 1987. Zur statistischen Auswertung von mikroskopischen Plankton- und Partikelzählungen. *Acta hydrochim. hydrobiol.* **15**:471-480.
5. Unterweger, A & Augustin, H. 1990. A computer program for quantitative and qualitative estimation of activated sludge biocenoses in purification plants. *J. Protozool.* **37**:63A (Abstract 301).

COLLECTING *AUFWUCHS* ON ARTIFICIAL SUBSTRATA

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INTRODUCTION

Many protista are adapted to life on surfaces. Surfaces are used as anchors for filtering (e.g., peritrichs) or intercepting (e.g., suctorians) feeding forms. Many protozoa graze upon bacteria, fungi, or algae comprising surface biofilms, or prey on micrometazoa such as rotifers. Surface-dwelling forms have often been described in the plankton, but are usually tychoplanktonic or associated with surfaces such as algal and fungal filament mats [e.g. 6, 10], filament balls [e.g. 11], macrophyte fragments [e.g. 15], or suspended micro-aggregates [7]. Submerged surfaces are often scoured, particularly in streams and shallow littoral zones, hence it is difficult to determine the age or developmental state of any natural *Aufwuchs* assemblage.

Artificial substrata provide a means of controlling community development and comparing sites with differing (perhaps incomparable) natural surfaces. Artificial substrates are commonly used to reduce the spatial heterogeneity that is usually associated with natural substrata [12]. Aufwuchs communities are increasingly used to evaluate and monitor responses to contamination in ecotoxicological studies. These communities are used to examine the bioaccumulation of toxins, influences on productivity and other functional responses, in taxonomic surveys, and in other ecological investigations. Artificial substrata may be used in biomonitoring work which examines natural microbial colonists, (see Pratt and Balczon, this volume).

Many artificial materials are used [1, 3], including polyurethane foam units (PFUs), styrofoam spheres and blocks, plastic petri dishes, glass and acrylic slides, glass coverslips, rods and tubes, Plexiglas discs, bricks, glazed and unglazed clay tiles, cut rock surfaces, clay flowerpots, wooden dowels and sticks, plastic (Mylar® and Teflon®) tape and artificial plastic plants. Extensive reviews of earlier [13] and more recent [2, 5] use of numerous artificial substrata are available. The following protocol is recommended for the deployment and collection of PF substrates, but basic principles apply to the usage of all artificial substrates.

B-9.2

PROTOCOL

1. Polyurethane foam units (6 x 5 x 4 cm blocks) should be tied firmly in the center (we normally use 16 ply cotton string), and then tied to an anchoring rope (Fig. 1). The number of PFUs used will depend on the purpose of investigation.
2. Substrata are placed in the aquatic system of interest by tying the anchoring rope to a suitable anchor. For all artificial substrata, anchoring is necessary to ensure recovery and to keep substrata on-site. Standard cement (cinder) blocks or bricks are usually adequate in all but the swiftest flowing waters. It is best to overestimate the anchor size necessary to secure PFUs rather than risk their loss during high flow or rough water conditions. Alternate anchoring configurations are illustrated in Fig. 2.

3. PFUs colonize more uniformly if they are wet when first deployed, and many other substrata need some "conditioning" to assure that the surface is chemical-free and prepared for colonizing organisms. This can be accomplished by an overnight soak in distilled water or by repeatedly squeezing the PF block underwater at the collecting site. This removes air bubbles, reduces the buoyancy of the substrata, and washes out possible contaminants.

4. An appropriate exposure period for any type of substrate may be determined by preliminary study. Substrata are placed at a collection site of interest and allowed to accumulate species by colonization from natural sources [4]. Colonization is more rapid in flowing waters than in lakes or ponds. Typical exposure periods for PFU substrata are 1-14 d in streams or estuaries, and up to 35 d in lakes or ponds. Longer periods are normally needed for flat substrata such as slides or clay tiles. The optimum exposure period will vary with the trophic and physico-chemical status of the system under consideration. Colonization is often diagnostic, and time-dependent sampling over an expanding time scale (e.g. 1, 2, 4, 8, 16 d) can identify an acceptably long, but convenient, interval over which to conduct studies. Alternatively, the colonization process itself may be followed, as microbial community succession becomes of increasing interest to investigators.

5. Collect substrata by removing the mass of connected blocks from the water after cutting the anchoring rope. Allow the substrata to float directly into a bucket of water collected on-site. Substrata may be carefully transferred into a water-filled insulated container to maintain in situ temperature.

6. Individual substrata can then be cut from the anchor rope and placed in collecting (Whirl-pak®) bags. It is best to "harvest" the substrata upon return to the lab. Substrata placed in labeled, 500 ml (18 oz) Whirl-pak® bags with a small amount of site-water (150 - 250 ml) transport quite well.

7. At the lab, the excess water is carefully poured from the collection bag. The PF substrate, still soaked to capacity, is then carefully squeezed into a beaker, and the sample is allowed to settle. Approximately 65 ml of sample is contained in an individual substratum. All substrata should be squeezed in a consistently forceful manner to remove as much sample as possible.

8. When examining the live organisms it is advisable to complete microscopic examination of samples within 12 h of sample collection. Once communities are removed from the "source" ecosystem, species composition changes occur and continue at an accelerated rate following squeezing of the PFUs.

COMMENTS (use of other substrata)

Floating substrata

Glass and acrylic slides - Glass slides may be deployed in several ways, but a conventional periphytometer (diatometer) is usually the most convenient. Certain periphytometers are simply floating slide boxes with open tops and bottoms to allow water to flow through. Other diatometers may be purchased or constructed which suspend slides in either surface (Fig. 3) or bottom waters, but all include some provision for floating and anchoring. Variations on the two-dimensional slide substratum are the benthic wedge, glass capillary tubes and other means of exposing glass or acrylic surfaces at depth. In lentic systems, slides should be oriented vertically in pairs, and, in lotic systems, placement should be vertical, such that the long axis of each slide is oriented parallel to the current.

Slides are usually collected from the periphytometer individually, although the entire substratum array may be collected by removing it from its anchor and placing it in a water-filled tray, pan, or bucket. Aufwuchs biomass on two-dimensional substrata is easily removed so care must be taken to avoid losing the attached organisms. Though associated with surfaces, many motile protista are especially vulnerable to dislodgement.

Individual slides can be examined *in toto.* If slides are exposed as back-to-back pairs, they can be split and observed directly without cleaning. Commonly the colonists are removed by scraping with a single-edged razor blade and the collected sample suspended in site water (filter sterilized if necessary).

Benthic substrata

Clay (ceramic) tiles - An increasingly popular artificial substrate, clay tiles are inexpensive and easy to use, yet provide a more realistic colonizable surface than glass or plastics. Unglazed, "quarry" or ceramic floor tiles may be cut into convenient sizes (usually from 5 x 5 to 20 x 20 cm) and placed in frames in flowing waters, or directly on top of existing substrates in smaller lentic systems. Other studies have used clay tiles to line the bottoms of artificial stream channels, providing discrete, benthic units which are easily sampled.

Entire tiles may be collected and placed directly into sample bags while still submerged. Alternatively, defined areas of tile surface may be "capped" with petri dish lids or bottle caps by firmly holding the "cap" against the colonized surface while pulling the substrata from the water. Surface area external to that enclosed by the "cap" may be removed by scraping and/or brushing and discarded. Quantitative samples may thus be obtained while avoiding the loss of organisms to shear forces associated with removal from the water.

Petri dishes - Petri dishes may be exposed using several methods, but the general approach is to colonize the inside of the bottom portion of the dish [14]. Retain the top for collection. Snap-fit dish lids are especially useful because the dish can be collected with the contents

safely sealed for transport. Bottoms are exposed horizontally in the water column (although vertical exposure should be acceptable. Several dishes may be exposed simultaneously using hand-built racks. Dishes may be held in place using rubber bands. Alternatively, flotation collars of styrofoam (foamed polystyrene) may be used. These are easily constructed from disposable drink cups.

Collection of each petri dish requires placing the lid of the dish firmly on the bottom under the water to trap specimens and water. The dish is then inverted and retrieved. When retrieving any substratum, the goal is to minimize the effects of surface tension forces which strip away unattached organisms as the substratum is pulled from the water. Viewing petri dish specimens at low power on an inverted microscope is recommended.

LITERATURE CITED

1. Aloi, J. E. 1990. A critical review of recent freshwater periphyton field methods. *Can. J. Fish. Aquat. Sci.* **47**:656-670.

2. Austin, A., Lang, S., & Pomeroy, M. 1981. Simple methods for sampling periphyton with observations on sampler design criteria. *Hydrobiol.* **85**:33-47.

3. Bamforth, S. S. 1982. The variety of artificial substrates used for microfauna., p.115-129. *In:* J Cairns, Jr. (ed.) Artificial Substrates. Ann Arbor Science Publishers, Inc., Ann Arbor, MI.

4. Cairns, J., Jr, Kuhn, D.L., & Plafkin, J. L. 1979. p.34-57 *In:* RL Weitzel (ed.) Methods and measurement of periphyton communities: a review., ASTM STP 690, American Society for Testing and Materials, Philadelphia, PA.

5. Cairns, J., Jr. 1982. Artificial substrates. Ann Arbor Science Publishers, Inc. Ann Arbor, MI.

6. Fauré-Fremiet, E. 1951. Associations infusoriennes à *Beggiatoa*. Hydrobiol. 3:65-71.

7. Goldman, J. 1984. Ocean nutrient cycles. p.137-170 *In* Flows of energy and materials in marine ecosystems: Theory and practice. *NATO Conf. Ser. 4, Mar. Sci.* **13**. Plenum Press.

8. Patrick, R, Hohn, MH, & Wallace, JH. 1954. A new method for determining the pattern of the diatom flora. *Notulae Naturae, Acad. Nat. Sci. Phil.* **259**:1-12.

9. Patrick, R., & Reimer, C. W. 1966. The diatoms of the United States I. Acad. Nat. Sci. Phila., Monogr. 13. 688 pp.

10. Picken, L. E. R. 1937. The structure of some protozoan communities. *J. Ecol.* **25**:368-384.

11. Pratt, JR, & Rosen, BH. 1983. Associations of species of *Vorticella* (Peritrichida) and planktonic algae. *Trans. Am. Microsc. Soc.* **102**:48-54.

12. Schindler, DW, Frost, VE, & Schmidt, RV. 1973. Production of epilithiphyton in two lakes of the Experimental Lakes Area, northwestern Ontario. *J. Fish. Res. Board Can.* **30**:1511-1524.

13. Sladeckova, A. 1962. Limnological investigation methods for the periphyton (*aufwuchs*) community. *Bot. Rev.* **28**:286-350.

14. Spoon, DM, & Burbanck, WD. 1967. A new method for collecting sessile ciliates in plastic petri dishes with tight-fitting lids. *J. Protozool.* **14**:735-739.

15. Walker, I. 1985. On the structure and ecology of the micro-fauna in the Central Amazonian forest stream 'Igarape de Cachoeira.' *Hydrobiol.* **122**:137-152.

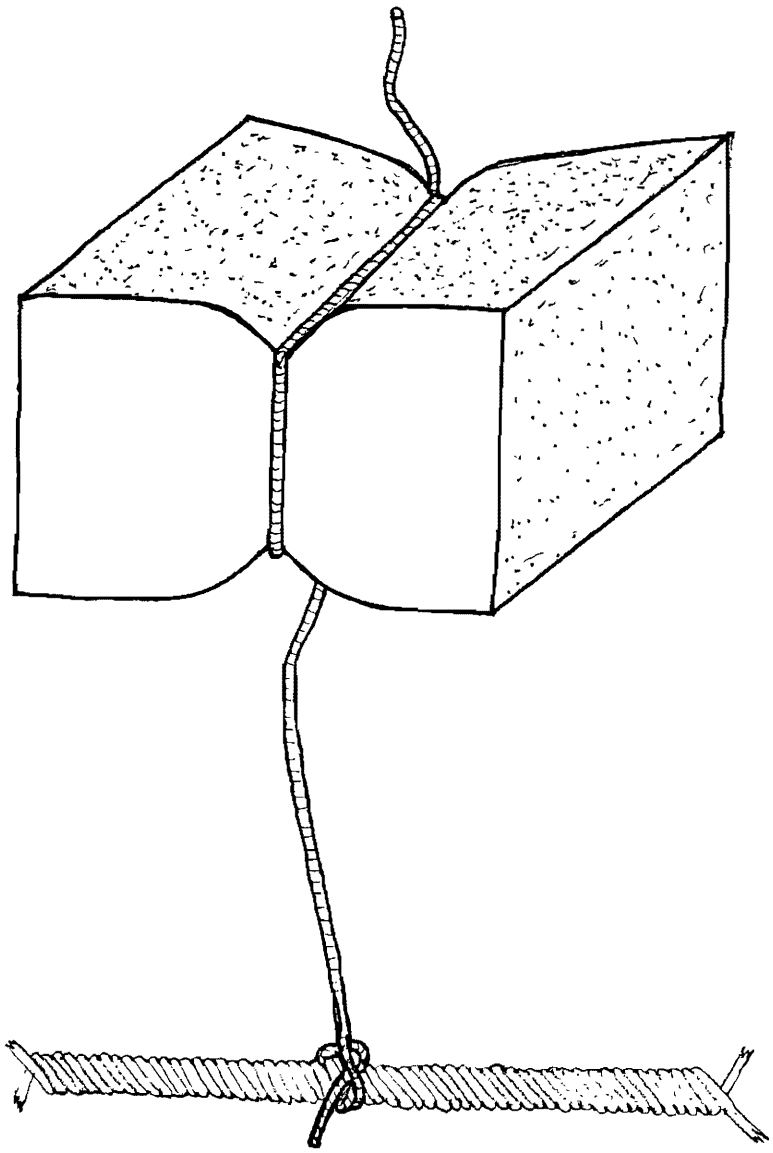

Figure 1. Polyurethane foam substrata for collection of *Aufwuchs.*

B-9.6

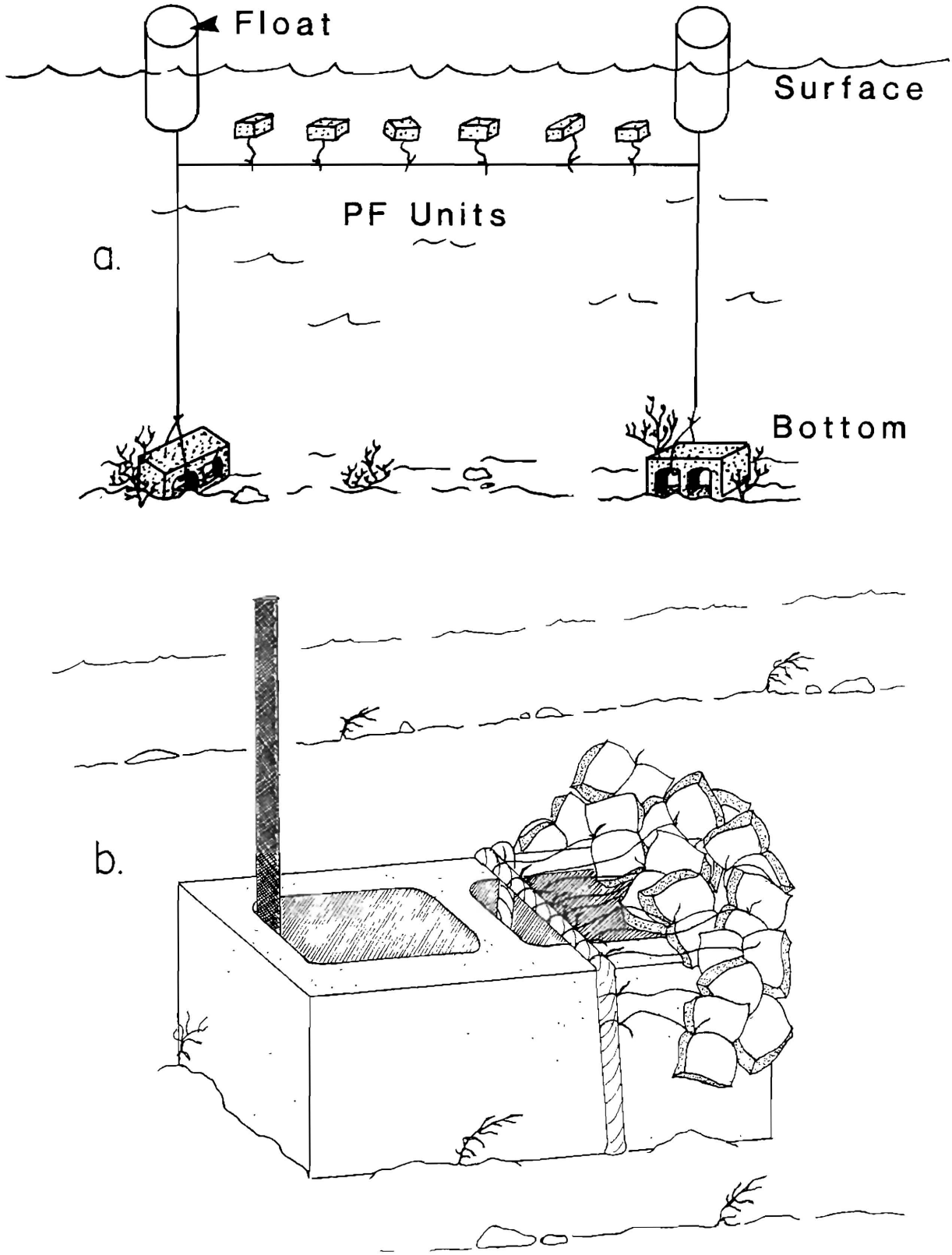

Figure 2. Example PF block anchoring configurations in (a) lentic and (b) lotic systems.

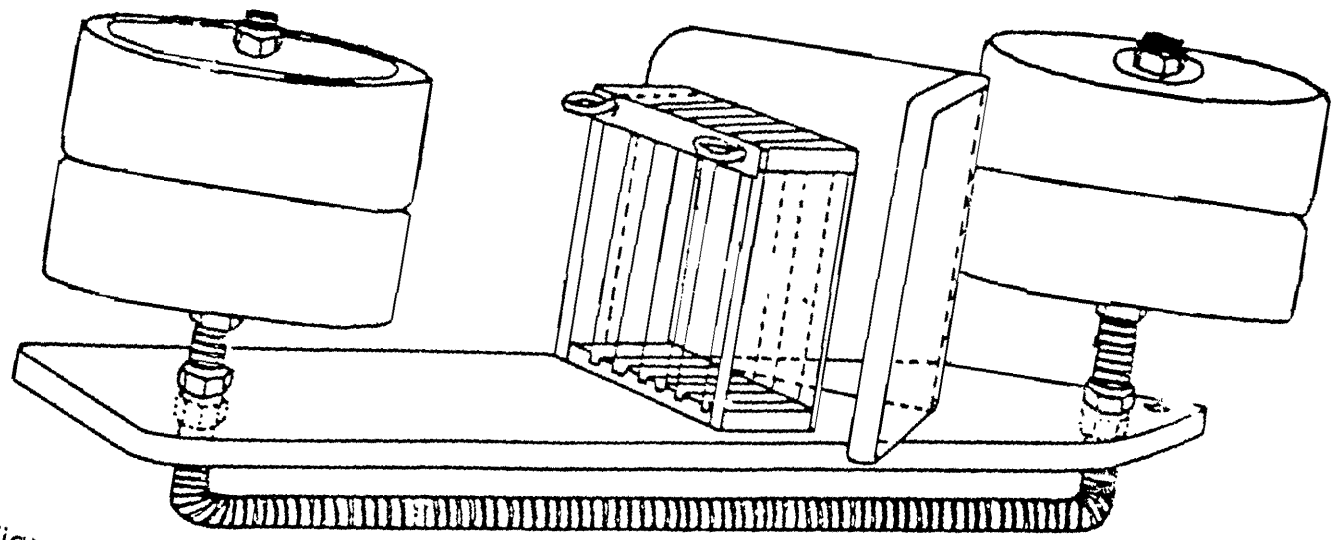

Figure 3. Example floating diatometer (the Catherwood diatometer [8]) with slides oriented parallel to the current in surface waters. (Figure from [9].)

ESTIMATING THE SPECIES RICHNESS OF SOIL PROTOZOA USING THE "NON-FLOODED PETRI DISH METHOD"

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INTRODUCTION

Many methods have been recommended for the estimation of the species richness of soil protozoa. The best method for testate amoebae is the careful inspection of watered soil suspensions and the flotation of empty tests by gas bubbles (1, 3).

Estimation of the richness in the other groups of soil protozoa (flagellates, naked amoebae, ciliates) is much more difficult, because these cannot be directly extracted so successfully from the soil. Therefore, enumeration involving various more or less complicated culture techniques have been suggested (e. g., 5). A very simple and highly effective "non-flooded petri dish method" was independently described by Varga (6), Starr (4) and Foissner (2).

PROTOCOL

1. Put 10-50 g of a fresh or air-dried soil or litter sample in a petri dish with 10-15 cm diameter.
2. Saturate but do not flood the sample with distilled water. Water should be added to the sample until 5-20 ml will drain off when the petri dish is tilted (45°) and the soil is gently pressed with a finger. Complete saturation will need up to 12 hours. Check, thus, culture after this time.
3. Cover petri dish and pinch a clip between bottom and lid to enable gas exchange.
4. Inspect cultures on days 2, 6, 12, 20 and 30 by taking a few milliliters from the run-off which contains a fauna of ciliates, flagellates, and naked amoebae, often unexpectedly rich. Later inspections add but few species.

COMMENTS

1. Air-dried soils yield often more individuals and species, probably due to reduced microbiostasis.
2. The sample should contain much litter and plant debris and must be spread over the bottom of the petri dish in at least a 1 cm thick layer.
3. Sample (soil) must not be flooded!
4. The run-off is often very rich in individuals and thus ideal for preparations, such as silver staining.
5. No systematic comparisons with other techniques are known. So many new species of soil ciliates have been discovered by myself with this method that it may be argued that it is more effective than other more frequently used and more complicated techniques. Repeated investigations of some soils showed that 2-5 samples distributed over one year produce 50-80 % of the species found in 10 samples investigated over two years. Thus, the method is not perfect and workers should be encouraged to look for a better alternative.

LITERATURE CITED

1. Décloitre, L. 1960. Perfectionnements apportés à un appareil pour une technique d'isolement des microorganisms du sol, des mousses, des eaux. *Int. Revue ges. Hydrobiol.* **45**:169-171.
2. Foissner, W. 1987. Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to the literature. *Progr. Protistol.* **2**:69-212.
3. Schönborn, W. 1989. Urtiere (Protozoa). *In:* Dunger, W & Fiedler, H J (ed.), Methoden der Bodenbiologie. G. Fischer, Jena, pp. 282-290.
4. Starr, R C. 1973. Special methods - dry soil samples. *In:* Stein, J R (ed.), Handbook of Phycological Methods. Culture Methods & Growth Measurements. Cambridge University Press, Cambridge, pp. 159-167.
5. Stout, J D. 1958. Biological studies of some tussock-grassland soils VII. Protozoa. *N. Z. Jl agric. Res.* **1**:947-984.
6. Varga, L. 1959. Untersuchungen über die Mikrofauna der Waldstreu einiger Waldtypen im Bükkgebirge (Ungarn). *Acta zool. hung.* **4**:443-478.

EVALUATING WATER QUALITY USING PROTOZOA AND SAPROBITY INDEXES

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INTRODUCTION

The main objective of this protocol is to make familiar protozoologists and instructors with the saprobic classification of heterotrophic protists (Table 1). Many are useful bioindicators in aquatic ecosystems, especially for the load of running waters with easily degradable organic wastes and for habitats devoid of, or significantly depleted in, molecular oxygen. Protozoa, thus, play a major role in the so-called "saprobic system" which is widely used for evaluating and assessing water quality in Central Europe and in the former Soviet Union. Detailed discussions about the pros and cons of the saprobic system and of water quality assessment in general were provided by, e. g., Amavis & Smeets (2), Moog (8) and Sladecek (11). The most widely used classification scheme distinguishes four zones of pollution using specific chemical (e. g., oxygen content) and biological (saprobic organisms) parameters (2, 8, 10, 11, 16):
(1) Polysaprobity. A zone of gross pollution with organic matter, very little or no dissolved oxygen. Few species, especially bacteria and heterotrophic protists, occur with high individual numbers.
(2) Alpha-mesosaprobity. A zone where some oxygen is present and mineralization commences. More species are present than in the polysaprobic zone. Bacteria and protists still dominate.
(3) Beta-mesosaprobity. A zone where decomposition products approach mineralization and the oxygen deficit is small. A great variety of protists, plants and animals occur in considerable numbers.
(4) Oligosaprobity. Mineralization of organic matter is completed and the water is saturated with oxygen. A great variety of plants and animals occur with low individual numbers. Protists are scarce in this zone.

Usually, many indicator organisms (often > 40 species) from various groups of animals (heterotrophic protists, macro-invertebrates ...) and plants (bacteria, autotrophic protists ...) are used for water quality surveys. Such large assemblages can be hardly assessed by simply looking at the saprobic classification of the individual species. Thus, many "biotic indexes" have been suggested to reduce the complexity of organism communities to simple, quantitative measures (see [14] for an excellent review). In this protocol two indexes are shown; both are widely used in Europe and are, in our experience, excellent tools for assessing the quality of running waters.

THE SAPROBITY INDEX OF PANTLE & BUCK

$$SIPB = \frac{\Sigma (N \cdot SI_i)}{\Sigma N}$$

N	estimated individual number for each species; 1 = few, 3 = many, 5 = very many (mass occurrence)
SI_i	saprobity index of species i (take it from table 3)
SIPB	saprobity index of Pantle & Buck (10)

Calculation example (for a community with 7 species)

Species	N	SI	$N \cdot SI_i$
A	1	2.8	2.8
B	3	1.9	5.7
C	3	3.5	10.5
D	5	3.8	19.0
E	1	2.1	2.1
F	3	3.0	9.0
G	3	4.8	14.4
Σ	19		63.5

$$SIPB = \frac{63.5}{19} ;$$

SIPB = 3.3

Classification

SIPB 1.0 - 1.5 clean = oligosaprobity = water quality class I; signal colour: blue
1.5 - 2.5 slightly to moderately polluted = beta-mesosaprobity = water quality class II; signal colour: green
2.5 - 3.5 heavily polluted = alpha-mesosaprobity = water quality class III; signal colour: yellow
3.5 - ≥ 4.0 very heavily polluted = polysaprobity = water quality class IV; signal colour: red

THE SAPROBITY INDEX OF ZELINKA & MARVAN

$$SIZM = \frac{\Sigma (N \cdot I \cdot r_i)}{\Sigma (N \cdot I)} ;$$

to be calculated separately for each saprobic class (oligosaprobity, beta-mesosaprobity ...)

N counted or estimated individual number for each species; if estimated, a similar (e. g., 1, 2, 3, 5, 7, 9) or the same ranking scale can be used as for Pantle & Buck's index
I indicative weight of species (take it from table 3)
r_i relative number (proportion) of a species in a saprobic class (saprobic valencies in table 3)
SIZM saprobity index of Zelinka & Marvan (16)

Calculation example (for a community with 7 species and counted individual numbers)

Species	N	I	r_i (saprobic valency)[1]					Calculation (N . I . r_i)					N . I
			x	o	b	a	p	x	o	b	a	p	
A	69	1	0	1	4	4	1	0	69	276	276	69	69
B	31	3	0	6	4	0	0	0	558	372	0	0	93
C	30	5	0	0	0	1	9	0	0	0	150	1350	150
D	42	2	0	0	2	5	3	0	0	168	420	252	84
E	8	1	0	2	4	3	1	0	16	32	24	8	8
F	120	4	0	0	1	8	1	0	0	480	3840	480	480
G	5	3	0	0	5	5	0	0	0	75	75	0	15
Σ								0	643	1403	4785	2159	899
$SIZM = \frac{\Sigma (N \cdot I \cdot r_i)}{\Sigma (N \cdot I)}$								0	0.7	1.6	5.3	2.4	

[1] x, xenosaprobity; o, oligosaprobity; b, beta-mesosaprobity; a, alpha-mesosaprobity; p, polysaprobity.

Classification

The classification is the same as for Pantle & Buck's index. However, Zelinka & Marvan's method shows the pollution situation more detailed because the saprobity index is calculated separately for each saprobic class. The highest value, 5.3 in our example, determines the water quality class (III in our example = heavily polluted); the neighbouring values show how sharply the water quality class is circumscribed. The sum of all saprobic classes used gives always 10, like the sum of the proportions of the saprobic valency of each species. It is very impressive to illustrate the proportions of the saprobic classes (oligosaprobity ...) with a block diagram using the above mentioned signal colours (blue for oligosaprobity ...; Fig. 1).

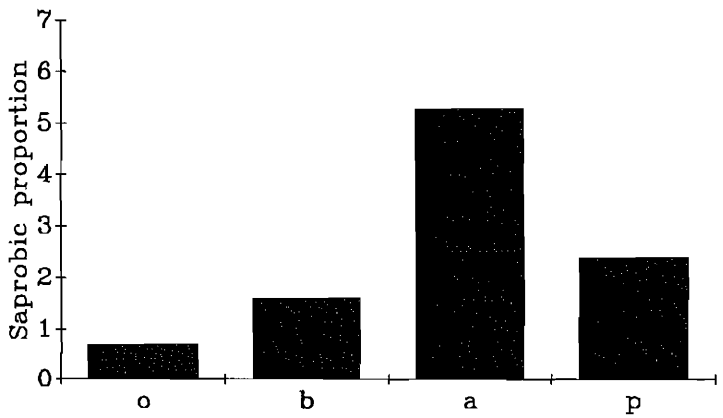

Fig. 1. Block diagram of the saprobic proportions shown in the calculation example.

COMMENTS

1. Direct stream bed sampling is more efficient and practical than artificial substrate sampling (7).
2. A meaningful calculation of the saprobity index needs at least 15 well-identified indicator species.
3. Saprobity indexes are rather simple mathematical constructs and must thus be checked against other parameters, e. g. chemical indicators.
4. Tümpling (13) describes a statistics for comparing saprobity indices in space and time.
5. Saprobity (= pollution with degradable organic wastes) should be assessed using several groups of indicator organisms (protists, macro-invertebrates ...) rather than single types which often prefer a certain saprobic level. Heterotrophic protists, for instance, are very useful indicators for alpha- and polysaprobity, whereas stone-flies are confined to oligo- and beta-mesosaprobic biotopes.
6. The saprobic system cannot be applied to waters which receive poisonous or non-biodegradable wastes.

Table 1. Saprobic classification of heterotrophic protists[1].

Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Flagellates								
Actinomonas mirabilis KENT	a	0	2	3	5	0	2	2.3
Actinomonas vernalis STOKES	b-a	0	2	4	4	0	2	2.2
Actinomonas sp.	b-a	0	2	4	4	0	2	2.2
Amphidinium larvale LINDQUIST	a	0	0	0	10	0	5	3.0
Amphimonas globosa KENT	b-a	0	0	5	5	0	3	2.5
Ancyromonas sigmoides KENT	a	0	0	2	8	0	4	2.8
Anisonema acinus DUJARDIN	b-a	0	0	5	5	0	3	2.5
Anisonema ovale KLEBS	b-a	0	0	5	5	0	3	2.5
Anisonema striatum KLEBS	b	0	0	8	2	0	4	2.2
Anisonema truncatum STEIN	b	0	0	7	3	0	4	2.3
Anisonema variabile KLEBS	b	0	0	8	2	0	4	2.2
Anisonema sp.	b-a	0	0	6	4	0	3	2.4
Anthophysa vegetans (MUELLER)	a	0	0	0	8	2	4	3.2
Anthophysa steinii SENN	a-b	0	0	4	6	0	3	2.6
Astasia curvata KLEBS	a-p	0	0	0	6	4	3	3.4
Astasia dangeardii LEMMERMANN	p-i	0	0	0	1	9	5	4.4 E
Astasia granulata PRINGSHEIM	a-b	0	0	4	6	0	3	2.6
Astasia inflata KLEBS	a	0	0	0	10	0	5	3.0
Astasia inflata f.fusiformis (SKUJA)	a	0	0	0	10	0	5	3.0
Astasia klebsii LEMMERMANN	p	0	0	0	3	7	4	3.7
Astasia linearis PRINGSHEIM	a	0	0	0	10	0	5	3.0
Astasia longa PRINGSHEIM	a	0	0	0	10	0	5	3.0
Astasia quartana (MOROFF)	a-p	0	0	0	5	5	3	3.5
Astasia sagittifera SKUJA	a	0	0	0	10	0	5	3.0
Astasia torta PRINGSHEIM	a-p	0	0	0	6	4	3	3.4
Astasia sp.	a	0	0	0	7	3	4	3.3
Astrosiga radiata ZACHARIAS	o-b	0	6	4	0	0	3	1.4
Aulacomonas hyalina SKUJA	b	0	3	7	0	0	4	1.7
Bernardinium bernardiense CHODAT	o-b	0	5	5	0	0	3	1.5
Bicosoeca campanulata (LACKEY)	a	0	0	3	7	0	4	2.7
Bicosoeca conica LEMMERMANN	b	0	0	10	0	0	5	2.0

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Bicosoeca kepneri REYNOLDS	b-o	0	4	6	0	0	3	1.6	
Bicosoeca lacustris CLARK	b-a	0	0	5	5	0	3	2.5	
Bicosoeca mitra FOTT	o-a	0	3	4	3	0	2	2.0	
Bicosoeca pascheri CONRAD	b	0	2	8	0	0	4	1.8	
Bicosoeca petiolata (STEIN)	b-a	0	0	5	5	0	3	2.5	
Bicosoeca planktonica KISSELEV	b	0	2	8	0	0	4	1.8	
Bicosoeca oculata ZACHARIAS	b	0	0	10	0	0	5	2.0	
Bicosoeca ovata LEMMERMANN	b	0	0	10	0	0	5	2.0	
Bicosoeca urceolata FOTT	b	0	0	10	0	0	5	2.0	
Bicosoeca sp.	b-a	0	2	5	3	0	2	2.1	
Bodo angustus (DUJARDIN)	a-p	0	0	0	6	4	3	3.5	E
Bodo caudatus (DUJARDIN)	p	0	0	0	3	7	4	3.7	E
Bodo celer KLEBS	m	0	0	0	0	10	5	5.6	E
Bodo edax KLEBS	m	0	0	0	1	9	5	5.0	E
Bodo erectus (RUEHLE)	a-p	0	0	0	5	5	3	3.5	
Bodo fusiformis (STOKES)	p-i	0	0	0	0	10	5	4.7	E
Bodo globosus STEIN	m	0	0	0	1	9	5	5.0	E
Bodo lens (MUELLER)	a	0	0	0	10	0	5	3.0	
Bodo ludibundus (KENT)	a	0	0	0	7	3	4	3.3	
Bodo minimus (KLEBS)	m	0	0	0	1	9	5	5.0	E
Bodo mutabilis KLEBS	p	0	0	0	0	10	5	4.0	
Bodo obovatus LEMMERMANN	p-i	0	0	0	0	10	5	4.5	E
Bodo ovatus (DUJARDIN)	a	0	0	0	9	1	5	3.1	
Bodo putrinus (STOKES)	m	0	0	0	0	10	5	5.9	
Bodo repens KLEBS	a	0	0	0	7	3	4	3.3	
Bodo saltans EHRENBERG	a	0	0	1	7	2	3	3.1	
Bodo uncinatus (KENT)	a-p	0	0	0	6	4	3	3.4	
Bodo sp.	p-a	0	0	0	4	6	3	3.4	
Cephalothamnion cyclopum STEIN	b	0	0	10	0	0	5	2.0	
Cercobodo agilis (MOROFF)	m	0	0	0	1	9	5	5.0	E
Cercobodo bodo (MEYER)	a	0	0	0	8	2	4	3.2	
Cercobodo crassicauda (DUJARDIN)	m	0	0	0	0	10	5	5.9	E
Cercobodo digitalis (MEYER)	b-a	0	0	5	5	0	3	2.5	
Cercobodo grandis (MASKELL)	m	0	0	0	1	9	5	5.0	E
Cercobodo longicauda (DUJARDIN)	m	0	0	0	10	0	5	5.9	E
Cercobodo ovatus (KLEBS)	b-a	0	0	5	5	0	3	2.5	
Cercobodo radiatus (KLEBS)	a-p	0	0	0	5	5	3	3.5	
Cercobodo simplex (MOROFF)	m	0	0	0	1	9	5	5.0	E
Cercobodo varians SKUJA	m	0	0	0	0	10	5	5.6	E
Cercobodo sp.	p-a	0	0	1	3	6	2	3.5	
Chilomonas bacillaris JAVORNICKY	a	0	0	0	10	0	5	3.0	
Chilomonas insignis (SKUJA)	a	0	0	0	10	0	5	3.0	
Chilomonas oblonga PASCHER	a	0	0	0	10	0	5	3.0	
Chilomonas oblonga f. minor (CZOSNOWSKI)	a	0	0	0	10	0	5	3.0	
Chilomonas paramecium EHRENBERG	a	0	0	2	6	2	3	3.0	
Chilomonas sp.	a	0	0	0	8	2	4	3.2	
Cladomonas fruticulosa STEIN	a	0	0	2	8	0	4	2.8	
Cladonema laxum KENT	b	0	0	10	0	0	5	2.0	
Codonobotrys physalis PASCHER	b	0	0	10	0	0	5	2.0	
Codonocladium umbellatum (TATEM)	b	0	0	9	1	0	5	2.1	
Codonodendron dinobryoideum (LEMMERMANN)	b	0	0	10	0	0	5	2.0	
Codonodendron ocellatum PASCHER	b	0	0	10	0	0	5	2.0	
Codonomonas mitra (FOTT)	o-a	0	3	4	3	0	2	2.0	

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Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Codonomonas mitra var. succica SKUJA	b	0	0	10	0	0	5	2.0
Codonomonas pascheri VAN GOOR	b	0	2	8	0	0	4	1.8
Codonomonas urceolata (FOTT)	b	0	0	10	0	0	5	2.0
Codonomonas sp.	b	0	1	7	2	0	3	2.1
Codonosiga botrytis (EHRENBERG)	a	0	1	3	5	1	1	2.6
Codonosigopsis robini SENN	b	0	0	10	0	0	5	2.0
Collodictyon triciliatum CARTER	a	0	0	1	8	1	4	3.0
Cryptoaulax akopos SKUJA	a	0	0	2	8	0	4	2.8
Cyathomonas truncata (EHRENBERG)	b-a	0	0	4	5	1	2	2.7
Cyclidiopsis acus KORSCHIKOFF	b-a	0	0	5	5	0	3	2.5
Dallingeria drysdali KENT	p-i	0	0	0	0	10	5	4.0 E
Desmarella moniliformis KENT	b-a	0	0	4	6	0	3	2.6
Dinema griseolum PERTY	b-a	0	0	5	5	0	3	2.5
Diploeca flava (KORSCHIKOFF)	b	0	0	10	0	0	5	2.0
Diplosiga francei LEMMERMANN	b	0	0	10	0	0	5	2.0
Diplosiga socialis FRENZEL	o	0	8	2	0	0	4	1.2
Diplosigopsis affinis LEMMERMANN	b	0	0	10	0	0	5	2.0
Diplosigopsis entzii FRANCE	o-b	0	5	5	0	0	3	1.5
Distigma curvatum PRINGSHEIM	a-p	0	0	0	6	4	3	3.4
Distigma proteus EHRENBERG	a	0	0	3	7	0	4	2.7
Entosiphon obliquus KLEBS	a	0	0	0	10	0	5	3.0
Entosiphon ovatum STOKES	a	0	0	0	10	0	5	3.0
Entosiphon sulcatum (DUJARDIN)	a	0	0	0	10	0	5	3.0
Euglenopsis vorax KLEBS	p	0	0	0	3	7	4	3.7
Furcilla lobosa STOKES	p-a	0	0	0	4	6	3	3.6
Furcilla trifurca HUBER-PESTALOZZI	a	0	0	0	10	0	5	3.0
Gyrodinium hyalinum (SCHILLING)	a	0	0	0	10	0	5	3.0
Gymnodinium lantzschii var. *lantzschii* JAVORNICKY	o	0	10	0	0	0	5	1.0
Gymnodinium lantzschii var. *rhinophoron* JAVORNICKY	b-a	0	0	6	4	0	3	2.4
Gymnodinium sp.	o-a	0	3	4	3	0	2	2.0
Gyromitus cordiformis SKUJA	o-b	0	5	5	0	0	3	1.5
Gyropaigne kosmos SKUJA	b-a	0	0	5	5	0	3	2.5
Gyropaigne spirale (MATVIENKO)	o	0	8	2	0	0	4	1.2
Helikotropis okteus POCHMANN	b	0	0	10	0	0	5	2.0
Helkesimastix faecicola WOODCOCK	p	0	0	0	3	7	4	3.7
Heteronema acus (EHRENBERG)	b-a	0	0	5	5	0	3	2.5
Heteronema acutissimum LEMMERMANN	b	0	0	8	2	0	4	2.2
Heteronema nebulosum (DUJARDIN)	b-a	0	0	5	5	0	3	2.5
Heteronema scabrum CYRUS	a	0	0	0	8	2	4	3.2
Heteronema sp.	b-a	0	0	4	5	1	2	2.7
Hexamita crassus KLEBS	m	0	0	0	0	10	5	5.9 E
Hexamita fissus KLEBS	m	0	0	0	0	10	5	5.9 E
Hexamita fusiformis KLEBS	m	0	0	0	0	10	5	5.9 E
Hexamita inflatus DUJARDIN	m	0	0	0	0	10	5	5.9 E
Hexamita pusillus KLEBS	m	0	0	0	0	10	5	5.9 E
Hexamita sp.	m	0	0	0	0	10	5	5.9 E
Hyaliella polytomoides PASCHER	a	0	0	0	10	0	5	3.0
Hyalogonium klebsii PASCHER	p	0	0	0	0	10	5	4.0
Katablepharis hyalurus SKUJA	a	0	0	0	10	0	5	3.0
Katablepharis notonectoides SKUJA	a	0	0	0	10	0	5	3.0
Katablepharis ovalis SKUJA	a	0	0	0	10	0	5	3.0
Katodinium fungiforme (ANISIMOVA)	b	0	0	7	3	0	4	2.3
Katodinium piscinale FOTT	a	0	0	0	10	0	5	3.0

Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Katodinium tetragonops (HARRIS)	b	0	0	8	2	0	4	2.2
Katodinium vorticella (STEIN)	a	0	0	1	9	0	5	2.9
Katodinium sp.	a-b	0	0	0	4	6	3	2.6
Khawkinea ocellata (KHAWKINE)	a	0	0	0	7	3	4	3.3
Khawkinea quartana (MOROFF)	a-p	0	0	0	5	5	3	3.5
Lagenoeca globulosa FRANCE	b	0	0	10	0	0	5	2.0
Lagenoeca obovata LEMMERMANN	b	0	0	10	0	0	5	2.0
Mastigamoeba gigantea (PROWAZEK)	p	0	0	0	0	10	5	4.0 E
Mastigamoeba invertens KLEBS	p-a	0	0	0	5	5	3	3.5
Mastigamoeba limax MOROFF	m-i	0	0	0	0	10	5	5.5 E
Mastigamoeba reptans STOKES	p	0	0	0	1	9	5	3.9
Mastigamoeba trichophora LAUTERBORN	i	0	0	0	0	10	5	5.0 E
Mastigamoeba sp.	a-i	0	0	0	3	7	4	4.3 E
Mastigella penardi LEMMERMANN	a-p	0	0	0	5	5	3	3.5
Mastigella radicula (MOROFF)	a-p	0	0	0	6	4	3	3.4
Menoidium cultellus PRINGSHEIM	a	0	0	0	10	0	5	3.0
Menoidium falcatum ZACHARIAS	a-b	0	0	4	6	0	3	2.6
Menoidium incurvum (FRESENIUS)	a	0	0	0	10	0	5	3.0
Menoidium minimum MATVIENKO	a	0	0	2	8	0	4	2.8
Menoidium pellucidum PERTY	a	0	0	2	8	0	4	2.8
Menoidium tortuosum SENN	a	0	0	0	10	0	5	3.0
Menoidium sp.	a-b	0	0	3	7	0	3	2.7
Monadodendron bennettii (KENT)	b-a	0	0	4	6	0	3	2.6
Monadodendron distans PASCHER	b	0	0	10	0	0	5	2.0
Monas arhabdomonas (FISCH)	m	0	0	0	1	9	5	5.0 E
Monas cylindrica SKUJA	b-a	0	0	5	5	0	3	2.5
Monas elongata (STOKES)	b-a	0	0	4	4	2	2	2.8
Monas guttula EHRENBERG	a	0	0	3	6	1	3	2.8
Monas minima MEYER	a-p	0	0	1	4	5	2	3.5 E
Monas obliqua SCHEWIAKOFF	b	0	1	7	2	0	3	2.1
Monas ocellata (SAUERFELD)	p-i	0	0	0	0	10	5	4.5 E
Monas sociabilis MEYER	m	0	0	0	0	10	5	5.9 E
Monas uniguttata SKUJA	a	0	0	3	7	0	4	2.7
Monas vivipara EHRENBERG	p	0	0	0	2	8	4	4.0 E
Monas vulgaris (CIENKOWSKI)	m	0	0	0	0	10	5	5.9 E
Monosiga ovata KENT	o	0	7	3	0	0	4	1.3
Multicilia lacustris LAUTERBORN	m	0	0	0	0	10	5	5.9 E
Notosolenus apocamptus STOKES	a	0	0	3	7	0	4	2.7
Notosolenus orbicularis STOKES	a	0	0	0	10	0	5	3.0
Oikomonas mutabilis KENT	p-i	0	0	0	0	10	5	4.7 E
Oikomonas socialis MOROFF	m	0	0	0	0	10	5	5.6 E
Oikomonas termo (EHRENBERG)	a	0	0	0	7	3	4	3.3
Pachysoeca massartii ELLIS	b-a	0	0	5	5	0	3	2.5
Pachysoeca obliqua FOTT	o-a	0	3	4	3	0	2	2.0
Pachysoeca ruttneri (BOURRELLY)	o-a	0	3	4	3	0	2	2.0
Parabodo sacculiferus SKUJA	m	0	0	0	0	10	5	5.5 E
Paramastix coronifera SKUJA	b-a	0	0	5	5	0	3	2.5
Paraphysomonas vestita STOKES	a	0	0	2	7	1	3	2.9
Peranema cuneatum PLAYFAIR	b-a	0	0	5	5	0	3	2.5
Peranema granuliferum PENARD	b	0	0	10	0	0	5	2.0
Peranema trichophorum (EHRENBERG)	a	0	0	3	7	0	4	2.7
Petalomonas abscissa (DUJARDIN)	a	0	0	0	10	0	5	3.0
Petalomonas alata STOKES	b	0	1	8	1	0	4	2.0

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Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Petalomonas angusta (KLEBS)	b-a	0	0	5	5	0	3	2.5	
Petalomonas applanata SKUJA	b	0	2	6	2	0	3	2.0	
Petalomonas carinata FRANCE	o	0	8	2	0	0	4	1.2	
Petalomonas curvata SKUJA	b	0	2	5	3	0	2	2.1	
Petalomonas inflexa KLEBS	b-a	0	0	5	5	0	3	2.5	
Petalomonas involuta SKUJA	b	0	1	6	3	0	3	2.2	
Petalomonas irregularis SKUJA	b	0	2	6	2	0	3	2.0	
Petalomonas klinostoma SKUJA	b	0	2	6	2	0	3	2.0	
Petalomonas mediocanellata STEIN	a	0	0	0	10	0	5	3.0	
Petalomonas mira AWERINZEW	b-a	0	0	5	5	0	3	2.5	
Petalomonas platyrhyncha SKUJA	b	0	0	10	0	0	5	2.0	
Petalomonas polytaphrena SKUJA	o-b	0	6	4	0	0	3	1.4	
Petalomonas praegnans SKUJA	o-b	0	6	4	0	0	3	1.4	
Petalomonas prototheca SKUJA	o	0	7	3	0	0	4	1.3	
Petalomonas punctato-striata SKUJA	o	0	8	2	0	0	4	1.2	
Petalomonas pusilla SKUJA	a	0	0	0	10	0	5	3.0	
Petalomonas quadrilineata PENARD	o-a	0	3	4	3	0	2	2.0	
Petalomonas scutulum SKUJA	a-b	0	0	4	6	0	3	2.6	
Petalomonas sexlobata KLEBS	b-o	0	5	5	0	0	3	1.5	
Petalomonas sinuata STEIN	a-b	0	0	4	6	0	3	2.6	
Petalomonas steinii KLEBS	a-b	0	0	4	6	0	3	2.6	
Petalomonas sulcata STOKES	o	0	8	2	0	0	4	1.2	
Petalomonas unguiformis SKUJA	b	0	1	7	2	0	3	2.1	
Petalomonas vulgaris SKUJA	a	0	0	2	8	0	4	2.8	
Phyllomitus amylophagus KLEBS	a-p	0	0	0	5	5	3	3.5	
Physomonas vestita STOKES	a	0	0	2	7	1	3	2.9	
Pleuromonas jaculans PERTY	a	0	0	0	8	2	4	3.2	
Polytoma caudatum KORSCHIKOFF	m	0	0	0	0	10	5	5.9	E
Polytoma fusiforme KORSCHIKOFF	m	0	0	0	0	10	5	5.9	E
Polytoma obtusum PASCHER	m	0	0	0	0	10	5	5.9	E
Polytoma ocellatum FRANCE	m	0	0	0	0	10	5	5.9	E
Polytoma papillatum PASCHER	m	0	0	0	0	10	5	5.9	E
Polytoma tetraolare PASCHER	m	0	0	0	0	10	5	5.9	E
Polytoma uvella EHRENBERG	m	0	0	0	0	10	5	6.0	E
Polytoma sp.	m	0	0	0	0	10	5	5.9	E
Polytomella agilis ARAGAO	p	0	0	0	0	10	5	4.0	
Polytomella caeca PRINGSHEIM	p	0	0	0	2	8	4	3.8	
Poteriodendron petiolatum STEIN	b-a	0	0	5	5	0	3	2.5	
Protaspis obovata SKUJA	b-a	0	0	4	6	0	3	2.6	
Protospongia haeckeli KENT	o	0	10	0	0	0	5	1.0	
Pseudobodo minimus HOLLANDE	a	0	0	0	10	0	5	3.0	
Rhabdomonas costata (KORSCHIKOFF)	o-a	0	3	4	3	0	2	2.0	
Rhabdomonas incurva FRESENIUS	a-p	0	0	0	6	4	3	3.4	
Rhabdomonas minima (MATVIENKO)	b	0	2	7	1	0	3	1.9	
Rhabdomonas spiralis PRINGSHEIM	o-b	0	4	6	0	0	3	1.6	
Rhipidodendron splendidum STEIN	b	0	3	5	2	0	2	1.9	
Rhynchomonas nasuta (STOKES)	b	0	0	8	2	0	4	2.2	
Salpingoeca amphoridium CLARK	b	0	1	6	3	0	3	2.2	
Salpingoeca buetschlii LEMMERMANN	b	0	0	10	0	0	5	2.0	
Salpingoeca flava KORSCHIKOFF	b	0	0	10	0	0	5	2.0	
Salpingoeca frequentissima ZACHARIAS	o-b	0	4	6	0	0	3	1.6	
Salpingoeca fusiformis KENT	b	0	0	10	0	0	5	2.0	
Salpingoeca gracilis CLARK	b	0	0	10	0	0	5	2.0	

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Salpingoeca macrostoma KORSCHIKOFF	b	0	0	10	0	0	5	2.0	
Salpingoeca oblonga STEIN	b-a	0	0	6	4	0	3	2.4	
Salpingoeca riethi FOTT	b	0	0	10	0	0	5	2.0	
Salpingoeca vaginicola STEIN	b	0	0	10	0	0	5	2.0	
Salpingoeca sp.	b	0	1	8	1	0	4	2.0	
Scytomonas pusilla STEIN	a	0	0	0	10	0	5	3.0	
Sphaeroeca volvox LAUTERBORN	a	0	0	0	8	2	4	3.2	
Sphenomonas quadrangularis STEIN	a	0	0	0	10	0	5	3.0	
Sphenomonas teres (STEIN)	a-p	0	0	0	5	5	3	3.5	
Spironema multiciliatum KLEBS	a	0	0	0	10	0	5	3.0	
Spongomonas uvella STEIN	o-b	0	5	5	0	0	3	1.5	
Stelexmonas dichotoma LACKEY	o-b	0	5	5	0	0	3	1.5	
Sterromonas formicina KENT	b-a	0	0	6	4	0	3	2.4	
Stephanocodon irregularis PASCHER	b-a	0	0	5	5	0	3	2.5	
Stephanocodon socialis (LAUTERBORN)	b	0	0	10	0	0	5	2.0	
Streptomonas cordata (PERTY)	b-a	0	0	5	5	0	3	2.5	
Tetrablepharis multifilis (KLEBS)	p	0	0	0	0	10	5	4.0	E
Tetramitus descissus PERTY	m	0	0	0	0	10	5	5.9	E
Tetramitus pyriformis KLEBS	m	0	0	0	0	10	5	5.9	E
Tetramitus rostratus PERTY	m	0	0	0	0	10	5	5.5	E
Tetramitus sulcatus KLEBS	m	0	0	0	0	10	5	5.5	E
Tetramitus sp.	m	0	0	0	0	10	5	5.5	E
Thylacomonas compressa SCHEWIAKOFF	b	0	0	7	3	0	4	2.3	
Toussetia polytomoides PASCHER	p	0	0	0	0	10	5	4.1	E
Trepomonas agilis DUJARDIN	m	0	0	0	0	10	5	5.9	E
Trepomonas rotans KLEBS	m	0	0	0	0	10	5	5.9	E
Trepomonas steinii KLEBS	m	0	0	0	0	10	5	5.5	E
Trepomonas sp.	m	0	0	0	0	10	5	5.5	E
Trigonomonas compressa KLEBS	m	0	0	0	0	10	5	6.0	E
Trigonomonas cyrusii CYRUS & SLADECEK	m	0	0	0	0	10	5	5.9	E
Trigonomonas inflata SKUJA	m	0	0	0	0	10	5	5.9	E
Trigonomonas tortuosa SKUJA	m	0	0	0	0	10	5	5.9	E
Trigonomonas sp.	m	0	0	0	0	10	5	5.9	E
Tropidoscyphus octocostatus STEIN	a	0	0	0	10	0	5	3.0	
Urceolus costatus LEMMERMANN	b	0	0	7	3	0	4	2.3	
Urceolus cyclostomus (STEIN)	b-a	0	0	4	6	0	3	2.6	
Urceolus cyrusorum CYRUS & SLADECEK	a	0	0	0	10	0	5	3.0	
Urophagus caudatus SKUJA	m	0	0	0	0	10	5	5.9	E
Urophagus rostratus (KLEBS)	m-p	0	0	0	0	10	5	5.1	E
Naked amoebae									
Amoeba chlorochlamys LAUTERBORN	m	0	0	0	0	10	5	5.9	E
Amoeba proteus (PALLAS)	b	0	0	8	2	0	4	2.2	
Astramoeba radiosa EHRENBERG	a-b	0	0	5	4	1	2	2.6	
Hartmanella sp.	p	0	0	1	3	6	2	3.5	
Hyalodiscus rubicundus HERTWIG & LESSER	b	0	2	8	0	0	4	1.8	
Mayorella vespertilio (PENARD)	o-b	0	5	5	0	0	3	1.5	
Nuclearia radians (GREEFF)	o	0	8	2	0	0	4	1.2	
Pelomyxa palustris GREEFF	p	0	0	0	0	10	5	4.0	
Thecamoeba verrucosa EHRENBERG	b	0	2	8	0	0	4	1.8	
Vahlkampfia guttula (DUJARDIN)	p-a	0	0	0	4	6	3	3.6	
Vahlkampfia limax (DUJARDIN)	p	0	0	0	0	10	5	4.2	E
Vampyrella pendula CIENKOWSKI	o-b	0	5	5	0	0	3	1.5	

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Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Testate amoebae								
Arcella dentata EHRENBERG	o	0	7	3	0	0	4	1.3
Arcella discoides EHRENBERG	b	0	2	5	3	0	2	2.1
Arcella hemisphaerica PERTY	b-a	0	0	4	5	1	2	2.7
Arcella rotundata PLAYFAIR	b	0	0	9	1	0	5	2.1
Arcella vulgaris EHRENBERG	b	0	1	7	2	0	3	2.1
Arcella sp.	b-a	0	2	4	4	0	2	2.2
Centropyxis aculeata (EHRENBERG)	b-o	1	3	4	2	0	1	1.7
Centropyxis aerophila DEFLANDRE	o-b	0	5	4	1	0	2	1.6
Centropyxis discoides PENARD	o-b	0	6	4	0	0	3	1.4
Centropyxis ecornis (EHRENBERG)	b	0	1	8	1	0	4	2.0
Centropyxis orbicularis DEFLANDRE	b-o	0	4	5	1	0	2	1.7
Centropyxis platystoma (PENARD)	o-b	0	4	5	1	0	2	1.7
Centropyxis sylvatica DEFLANDRE	o	0	7	3	0	0	4	1.3
Centropyxis sp.	o-b	0	3	5	2	0	2	1.9
Chlamydophrys minor BELAR	b-a	0	0	4	6	0	3	2.6
Chlamydophrys stercorea CIENKOWSKI	o-a	0	3	4	3	0	2	2.0
Cochliopodium bilimbosum (AUERBACH)	b	0	2	5	3	0	2	2.1
Cryptodifflugia compressa PENARD	o	0	7	3	0	0	4	1.3
Cryptodifflugia oviformis PENARD	b	0	2	5	3	0	2	2.1
Cyphoderia ampulla (EHRENBERG)	o-b	0	6	4	0	0	3	1.4
Cyphoderia trochus PENARD	b	0	1	8	1	0	4	2.0
Difflugia acuminata EHRENBERG	o	0	8	2	0	0	4	1.2
Difflugia bacillifera PENARD	o	0	9	1	0	0	5	1.1
Difflugia capreolata PENARD	b	0	1	8	1	0	4	2.0
Difflugia corona TARANEK	b	0	2	7	1	0	3	1.9
Difflugia curvicaulis PENARD	b	0	2	6	2	0	3	2.0
Difflugia difficilis THOMAS	o-a	0	3	4	3	0	2	2.0
Difflugia elegans PENARD	o	0	8	2	0	0	4	1.2
Difflugia fallax PENARD	o-b	0	5	4	1	0	2	1.6
Difflugia globulosa DUJARDIN	o-b	0	6	4	0	0	3	1.4
Difflugia gramen PENARD	b-a	0	1	5	4	0	2	2.3
Difflugia hydrostatica ZACHARIAS	o	0	10	0	0	0	5	1.0
Difflugia limnetica LEVANDER	o	0	7	3	0	0	4	1.3
Difflugia lobostoma LEIDY	b	0	1	7	2	0	3	2.1
Difflugia minuta RAMPI	o-b	0	4	4	2	0	2	1.8
Difflugia oblonga EHRENBERG	o-b	0	5	5	0	0	3	1.5
Difflugia oblonga var.nodosa LEIDY	o-b	0	6	4	0	0	3	1.4
Difflugia pristis PENARD	o-b	0	6	4	0	0	3	1.4
Difflugia pyriformis PERTY	o-b	0	5	5	0	0	3	1.5
Difflugia sp.	o-a	0	4	3	3	0	2	1.9
Diplophrys archeri BARKER	b-a	0	0	6	4	0	3	2.4
Euglypha acanthophora (EHRENBERG)	b	0	2	6	2	0	3	2.0
Euglypha alveolata DUJARDIN	b	0	2	6	2	0	3	2.0
Euglypha aspera PENARD	b	0	3	7	0	0	4	1.7
Euglypha ciliata (EHRENBERG)	b	0	3	7	0	0	4	1.7
Euglypha laevis PERTY	b	0	1	7	2	0	3	2.1
Euglypha sp.	b-o	0	3	6	1	0	3	1.8
Gromia brunneri BLANC	b	0	3	7	0	0	4	1.7
Gromia fluviatilis DUJARDIN	o	0	8	2	0	0	4	1.2
Lesquereusia spiralis (EHRENBERG)	o	2	8	0	0	0	4	0.8
Microchlamys patella (CLAPAREDE & LACHMANN)	o-b	0	4	6	0	0	3	1.6
Nebela collaris (EHRENBERG)	o	0	8	2	0	0	4	1.2

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Nebela militaris PENARD	o	1	8	1	0	0	4	1.0	
Nebela tubulosa PENARD	o	0	8	2	0	0	4	1.2	
Nebela sp.	o-b	0	6	4	0	0	3	1.4	
Pamphagus hyalinus LEIDY	o	0	7	3	0	0	4	1.3	
Pamphagus mutabilis BAILEY	b	0	3	7	0	0	4	1.7	
Paulinella chromatophora LAUTERBORN	b	0	3	7	0	0	4	1.7	
Pontigulasia bigibbosa PENARD	b	0	2	6	2	0	3	2.0	
Pseudodifflugia fulva (ARCHER)	o-a	0	3	4	3	0	2	2.0	
Pseudodifflugia globulosa STEPANEK	o-a	0	3	4	3	0	2	2.0	
Pseudodifflugia gracilis SCHLUMBERGER	b	0	2	6	2	0	3	2.0	
Pseudodifflugia orchas STEPANEK	b-a	0	0	5	5	0	3	2.5	
Pseudodifflugia senartensis COUTEAUX	b	0	3	6	1	0	3	1.8	
Pseudodifflugia sp.	b	0	2	6	2	0	3	2.0	
Quadrulella symmetrica (WALLICH)	b	0	3	5	2	0	2	1.9	
Trinema enchelys (EHRENBERG)	b	0	2	6	2	0	3	2.0	
Trinema lineare PENARD	b	0	3	6	1	0	3	1.8	
Heliozoa									
Acanthocystis pectinata PENARD	b	0	2	8	0	0	4	1.8	
Acanthocystis penardi WAILES	o-b	0	6	4	0	0	3	1.4	
Acanthocystis turfacea CARTER	o	0	8	2	0	0	4	1.2	
Actinophrys sol EHRENBERG	b-a	0	0	4	6	0	3	2.6	
Actinosphaerium eichhorni (EHRENBERG)	o-b	0	4	6	0	0	3	1.6	
Belenocystis tubistella RAINER	o	0	10	0	0	0	5	1.0	
Chlamydaster sterni RAINER	o	0	8	2	0	0	4	1.2	
Choanocystis aculeata (HERTWIG & LESSER)	b	0	2	8	0	0	4	1.8	
Clathrella foreli PENARD	o	0	9	1	0	0	5	1.1	
Clathrulina elegans CIENKOWSKI	b	0	2	6	2	0	3	2.0	
Elaeorhanis cincta GREEFF	o	0	8	2	0	0	4	1.2	
Heterophrys fockii ARCHER	o	0	9	1	0	0	5	1.1	
Heterophrys myriopoda ARCHER	o	0	8	2	0	0	4	1.2	
Lithocolla globosa SCHULZE	o	0	9	1	0	0	5	1.1	
Pterocystis echinata (RAINER)	o	0	10	0	0	0	5	1.0	
Raphidiophrys elegans HERTWIG & LESSER	b	0	1	9	0	0	5	1.9	
Raphidiophrys intermedia PENARD	o	0	10	0	0	0	5	1.0	
Raphidiophrys pallida SCHULZE	b	0	3	7	0	0	4	1.7	
Raphidiophrys symmetrica PENARD	o-b	0	5	5	0	0	3	1.5	
Raphidocystis glutinosa PENARD	o	0	7	3	0	0	4	1.3	
Raphidocystis tubifera PENARD	o	0	8	2	0	0	4	1.2	
Ciliophora									
Acineria incurvata DUJARDIN, 1841	p-i	0	0	0	0	10	5	4.5	E
Acineria uncinata TUCOLESCO, 1962	a-p	0	0	2	4	4	2	3.2	
Acineta flava KELLICOTT, 1885	b	0	1	7	2	0	3	2.1	
Acineta grandis KENT, 1882	b-a	0	0	4	6	0	3	2.6	
Acineta tuberosa (PALLAS, 1766)	a	0	0	1	6	3	3	3.2	
Acineta sp.	a-b	0	0	4	5	1	2	2.7	
Acinetides lacustris (STOKES, 1886)	p-a	0	0	0	4	6	3	3.6	
Actinobolina radians (STEIN, 1867)	b	0	1	7	2	0	3	2.1	
Actinobolina vorax (WENRICH,1929)	o	0	7	3	0	0	4	1.3	
Amphileptus carchesii STEIN, 1867	a	0	0	1	8	1	4	3.0	
Amphileptus claparedii STEIN, 1867	a	0	0	2	8	0	4	2.8	
Amphileptus meleagris (EHRENBERG, 1835)	a	0	0	0	10	0	5	3.0	
Amphileptus pleurosigma (STOKES, 1884)	b-a	0	0	5	5	0	3	2.5	
Amphileptus punctatus (KAHL, 1926)	a	0	0	1	9	0	5	2.9	

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Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Amphileptus rotundus (KAHL, 1926)	a	0	0	1	8	1	4	3.0	
Amphileptus trachelioides (ZACHARIAS, 1894)	o	0	7	3	0	0	4	1.3	
Amphileptus sp.	a	0	1	2	6	1	1	2.7	
Askenasia volvox (EICHWALD, 1852)	b	0	1	6	3	0	3	2.2	
Aspidisca cicada (MUELLER, 1786)	a-b	0	0	4	5	1	2	2.7	
Aspidisca lynceus (MUELLER, 1773)	b-a	0	1	4	4	1	1	2.5	
Aspidisca turrita (EHRENBERG, 1831)	a-b	0	0	4	6	0	3	2.6	
Aspidisca sp.	b-a	0	0	5	5	0	3	2.5	
Astylozoon fallax ENGELMANN, 1862	b-a	0	0	5	5	0	3	2.5	
Astylozoon faurei KAHL, 1935	b-a	0	0	5	5	0	3	2.5	
Blepharisma coeruleum GAJEVSKAJA, 1927	b	0	2	8	0	0	4	1.8	
Blepharisma lateritium (EHRENBERG, 1831)	b	0	2	8	0	0	4	1.8	
Bothrostoma sp.	p-i	0	0	0	0	10	5	4.0	E
Brachonella sp.	p-i	0	0	0	0	10	5	4.0	E
Bursaria truncatella MUELLER, 1773	b-a	0	2	4	3	1	1	2.3	
Bursaridium pseudobursaria (FAURÉ-FREMIET, 1924)	o-b	0	6	4	0	0	3	1.4	
Bursellopsis spumosa (SCHMIDT, 1921)	o	0	7	3	0	0	4	1.3	
Caenomorpha lauterborni KAHL, 1927	p-i	0	0	0	0	10	5	4.0	E
Caenomorpha medusula PERTY, 1852	p-i	0	0	0	0	10	5	4.0	E
Caenomorpha sapropelica KAHL, 1927	p-i	0	0	0	0	10	5	4.0	E
Caenomorpha uniserialis LEVANDER, 1894	p-i	0	0	0	0	10	5	4.0	E
Caenomorpha sp.	p-i	0	0	0	0	10	5	4.0	E
Calyptotricha lanuginosa (PENARD, 1922)	a	0	0	3	7	0	4	2.7	
Campanella umbellaria (LINNAEUS, 1758)	a-b	0	0	3	6	1	3	2.8	
Carchesium pectinatum (ZACHARIAS, 1897)	o-b	0	6	4	0	0	3	1.4	
Carchesium polypinum (LINNAEUS, 1758)	a	0	0	2	7	1	3	2.9	
Chaenea limicola LAUTERBORN, 1901	p	0	0	0	0	10	5	4.0	
Chaenea teres (DUJARDIN, 1841)	b	0	0	7	3	0	4	2.3	
Chaenea vorax QUENNERSTEDT, 1867	b	0	0	10	0	0	5	2.0	
Chaetospira muelleri LACHMANN, 1856	b	0	1	8	1	0	4	2.0	
Chaetospira remex (HUDSON, 1875)	b-a	0	1	5	4	0	2	2.3	
Chilodonella uncinata (EHRENBERG, 1838)	a	0	0	2	6	2	3	3.0	
Chilodontopsis depressa (PERTY, 1852)	b	0	1	7	2	0	3	2.1	
Chilodontopsis muscorum KAHL, 1931	a	0	0	0	10	0	5	3.0	
Chilodontopsis vorax (STOKES, 1887)	b-a	0	0	5	5	0	3	2.5	
Chlamydonella alpestris FOISSNER, 1979	b-a	0	2	4	4	0	2	2.2	
Chlamydonellopsis plurivacuolata BLATTERER & FOISSNER, 1990	b-a	0	0	5	5	0	3	2.5	
Cinetochilum margaritaceum (EHRENBERG, 1831)	b-p	0	1	3	3	3	1	2.8	
Climacostomum virens (EHRENBERG, 1838)	b	0	0	8	2	0	4	2.2	
Codonella cratera (LEIDY, 1877)	b-o	0	4	6	0	0	3	1.6	
Cohnilembus verminus (MUELLER, 1786)	a-b	0	0	4	6	0	3	2.6	
Cohnilembus vexillarius (KAHL, 1926)	b	0	0	10	0	0	5	2.0	
Cohnilembus sp.	b-a	0	0	5	5	0	3	2.5	
Coleps bicuspis NOLAND, 1925	b-a	0	0	7	3	0	4	2.3	
Coleps hirtus (MUELLER, 1786)	b-a	0	0	5	5	0	3	2.5	
Coleps sp.	a-b	0	0	4	6	0	3	2.6	
Colpidium colpoda (LOSANA, 1829)	p-i	0	0	0	2	8	4	4.0	E
Colpidium kleini FOISSNER, 1969	p	0	0	0	3	7	4	3.7	
Colpidium sp. (sensu lato)	p-i	0	0	0	3	7	4	3.7	E
Colpoda cucullus (MUELLER, 1773)	p-a	0	0	0	4	6	3	3.6	
Colpoda ecaudata (LIEBMANN, 1936)	p-i	0	0	0	1	9	5	3.9	
Colpoda inflata (STOKES, 1884)	a-p	0	0	0	5	5	3	3.5	

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Colpoda magna GRUBER, 1880	a-p	0	0	2	5	3	2	3.1	
Colpoda steinii MAUPAS, 1883[3]	a-p	0	0	0	5	5	3	3.5	
Colpoda steinii MAUPAS, 1883[4]	b-a	0	2	4	3	1	1	2.3	
Condylostoma vorticella (EHRENBERG, 1833)	b-a	0	1	6	3	0	3	2.2	
Cothurnia annulata STOKES, 1885	o-b	0	6	4	0	0	3	1.4	
Cristigera media KAHL, 1928	p-i	0	0	0	0	10	5	4.4	E
Ctedoctema acanthocrypta STOKES, 1884	b	0	1	8	1	0	4	2.0	
Cyclidium citrullus (COHN, 1866)	a	0	0	1	8	1	4	3.0	
Cyclidium elongatum (SCHEWIAKOFF, 1889)	b-a	0	0	5	5	0	3	2.5	
Cyclidium glaucoma MUELLER, 1773	a	0	0	0	9	1	5	3.1	
Cyclidium heptatrichum SCHEWIAKOFF, 1893	b	0	0	8	2	0	4	2.2	
Cyclidium oblongum KAHL, 1931	a-b	0	0	4	6	0	3	2.6	
Cyclidium singulare (KAHL, 1926)	a	0	0	0	10	0	5	3.0	
Cyclidium versatile PENARD, 1922	a-b	0	2	3	5	0	2	2.3	
Cyclidium sp.	a-b	0	0	4	6	0	3	2.6	
Cyrtolophosis mucicola STOKES, 1885	b-p	0	1	2	4	3	1	2.9	
Dendrosoma radians EHRENBERG, 1838	b-a	0	0	5	5	0	3	2.5	
Dexiostoma campyla (STOKES, 1886)	p-i	0	0	0	1	9	5	4.2	E
Dexiotricha plagia STOKES, 1885	a	0	0	2	6	2	3	3.0	
Dexiotricha sp.	p	0	0	0	1	9	5	4.5	E
Dexiotrichides centralis (STOKES, 1885)	p-i	0	0	0	0	10	5	4.5	E
Didinium cinctum VOIGT, 1902	o	0	8	2	0	0	4	1.2	
Didinium nasutum (MUELLER, 1773)	b-a	0	2	4	4	0	2	2.2	
Dileptus anser (MUELLER, 1773)	b-o	0	4	6	0	0	3	1.6	
Dileptus conspicuus KAHL, 1931	a	0	0	0	10	0	5	3.0	
Dileptus gigas (CLAPAREDE & LACHMANN, 1859)	b	0	0	7	3	0	4	2.3	
Dileptus margaritifer (EHRENBERG, 1833)	b-o	0	4	6	0	0	3	1.6	
Dileptus monilatus (STOKES, 1886)	b	0	0	7	3	0	4	2.3	
Dileptus sp.	a	0	1	3	6	0	2	2.5	
Discomorphella lauterborni (WETZEL, 1928)	p-i	0	0	0	0	10	5	4.4	E
Discomorphella pectinata (LEVANDER, 1894)	p-i	0	0	0	0	10	5	4.5	E
Disematostoma buetschlii LAUTERBORN, 1894	b	0	1	7	2	0	3	2.1	
Disematostoma tetraedricum (FAURÉ-FREMIET, 1924)	b	0	0	10	0	0	5	2.0	
Drepanomonas dentata FRESENIUS, 1858	o	0	8	2	0	0	4	1.2	
Drepanomonas revoluta PENARD, 1922	a-p	0	0	0	5	5	3	3.5	
Dysteria fluviatilis (STEIN, 1859)	b	0	0	8	2	0	4	2.2	
Enchelyodon elegans (KAHL, 1926)	a	0	0	0	10	0	5	3.0	
Enchelyodon fusidens KAHL, 1930	a	0	0	0	10	0	5	3.0	
Enchelyomorpha vermicularis (SMITH, 1899)	p-m	0	0	0	0	10	5	5.5	E
Enchelys gasterosteus KAHL, 1926	b-a	0	0	5	5	0	3	2.5	
Enchelys pupa (MUELLER, 1786)	b-a	0	0	5	5	0	3	2.5	
Epalxella sp.	p-i	0	0	0	0	10	5	4.5	E
Epenardia myriophylli (PENARD, 1922)	a-b	0	0	4	6	0	3	2.6	
Epistylis chrysemydis BISHOP & JAHN, 1941	a	0	0	2	6	2	3	3.0	
Epistylis coronata NUSCH, 1970	a	0	0	0	10	0	5	3.0	
Epistylis digitalis (LINNAEUS, 1758)	o-b	0	5	5	0	0	3	1.5	
Epistylis entzii STILLER, 1935	a	0	0	2	7	1	3	2.9	
Epistylis galea EHRENBERG, 1831	a-b	0	0	3	7	0	4	2.7	
Epistylis hentscheli KAHL, 1935	a-b	0	0	3	6	1	3	2.8	
Epistylis nympharum ENGELMANN, 1862	o-a	0	3	4	3	0	2	2.0	
Epistylis plicatilis EHRENBERG, 1831	a-b	0	0	3	6	1	3	2.8	
Epistylis procumbens ZACHARIAS, 1897	o-b	0	5	5	0	0	3	1.5	
Epistylis sp.	b-a	0	0	5	5	0	3	2.5	

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Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Euplotes aediculatus PIERSON, 1943	a	0	0	1	9	0	5	2.9	
Euplotes affinis (DUJARDIN, 1841)	b-a	0	0	5	4	1	2	2.6	
Euplotes eurystomus WRZESNIOWSKI, 1870	a	0	0	2	6	2	3	3.0	
Euplotes moebiusi KAHL, 1932	a	0	0	2	7	1	3	2.9	
Euplotes patella (MUELLER, 1773)	b	0	0	7	3	0	4	2.3	
Euplotes sp.	a	0	0	3	6	1	3	2.8	
Frontonia acuminata (EHRENBERG, 1833)	o	0	6	4	0	0	3	1.4	
Frontonia atra (EHRENBERG, 1833)	b	0	0	10	0	0	5	2.0	
Frontonia leucas (EHRENBERG, 1833)	o-p	0	2	3	3	2	1	2.5	
Frontonia vesiculosa DaCUNHA, 1913	b	0	1	6	3	0	3	2.2	
Frontonia sp.	b	0	1	6	3	0	3	2.2	
Gastronauta clatratus DEROUX, 1976	b-a	0	2	4	4	0	2	2.2	
Gastronauta membranaceus BUETSCHLI, 1889	b	0	2	6	2	0	3	2.0	
Gastrostyla mystacea (STEIN, 1859)	p	0	0	0	3	7	4	3.7	
Gastrostyla steinii ENGELMANN, 1862	a	0	0	2	7	1	3	2.9	
Glaucoma reniforme SCHEWIAKOFF, 1892	p	0	0	0	2	8	4	3.8	
Glaucoma scintillans EHRENBERG, 1830	p-i	0	0	0	1	9	5	4.2	E
Glaucoma sp.	p-a	0	0	1	4	5	2	3.4	
Halteria chlorelligera KAHL, 1932	o	0	8	2	0	0	4	1.2	
Halteria grandinella (MUELLER, 1773)	b-a	0	1	6	3	0	3	2.2	
Halteria sp.	b-o	0	4	5	1	0	2	1.7	
Hastatella radians ERLANGER, 1890	b-a	0	1	6	3	0	3	2.2	
Heliophrya minima (RIEDER, 1936)	b-a	0	0	5	5	0	3	2.5	
Heliophrya rotunda (HENTSCHEL, 1916)	b-a	0	0	5	5	0	3	2.5	
Hexotricha caudata LACKEY, 1925	p-m	0	0	0	0	10	5	5.0	E
Histriculus vorax (STOKES, 1891)	a	0	0	0	10	0	5	3.0	
Holophrya nigricans LAUTERBORN, 1894	b	0	0	10	0	0	5	2.0	
Holosticha kessleri (WRZESNIOWSKI, 1877)	a-b	0	0	4	5	1	2	2.7	
Holosticha monilata KAHL, 1928	a-b	0	0	3	6	1	3	2.8	
Holosticha multistilata KAHL, 1928	a-b	0	0	4	5	1	2	2.7	
Holosticha pullaster (MUELLER, 1773)	b-a	0	1	4	4	1	1	2.5	
Homalozoon vermiculare (STOKES, 1887)	b-a	0	2	4	4	0	2	2.2	
Hypotrichidium conicum ILOWAISKY, 1921	b-p	0	0	3	4	3	2	3.0	
Kellicotta cuspidata (KELLICOTT, 1885)	b-a	0	0	5	5	0	3	2.5	
Kerona pediculus (MUELLER, 1773)	b-o	0	4	5	1	0	2	1.7	
Lacrymaria olor (MUELLER, 1786)	b	0	2	6	2	0	3	2.0	
Lagenophrys vaginicola STEIN, 1852	o	0	9	1	0	0	5	1.1	
Lagynophrya acuminata KAHL, 1935	o	0	8	2	0	0	4	1.2	
Lagynus cucumis (PENARD, 1922)	p	0	0	0	0	10	5	4.0	
Lagynus elegans (ENGELMANN, 1862)	p-i	0	0	0	0	10	5	4.0	E
Lembadion bullinum (MUELLER, 1786)	b	0	0	9	1	0	5	2.1	
Lembadion lucens (MASKELL, 1887)	b	0	0	9	1	0	5	2.1	
Lembadion magnum (STOKES, 1887)	b	0	2	8	0	0	4	1.8	
Leptopharynx costatus MERMOD, 1914	o-b	0	5	5	0	0	3	1.5	
Litonotus anguilla (KAHL, 1931)	b-a	0	0	5	5	0	3	2.5	
Litonotus carinatus STOKES, 1885	b-a	0	0	5	5	0	3	2.5	
Litonotus crystallinus (VUXANOVICI, 1960)	b-a	0	0	5	5	0	3	2.5	
Litonotus cygnus (MUELLER, 1773)	b	0	0	10	0	0	5	2.0	
Litonotus fasciola (MUELLER, 1773)	a	0	0	1	8	1	4	3.0	
Litonotus fusidens (KAHL, 1926)	b-p	0	0	3	4	3	2	3.0	
Litonotus hirundo (PENARD, 1922)	a	0	0	1	8	1	4	3.0	
Litonotus lamella (MUELLER, 1773)	a	0	0	2	8	0	4	2.8	
Litonotus procerus (PENARD, 1922)	o-b	0	5	5	0	0	3	1.5	

Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Litonotus varsaviensis WRZESNIOWSKI, 1870	b-a	0	0	5	5	0	3	2.5
Litonotus varsaviensis f. polysaprobica SRAMEK-HUSEK, 1954	p-i	0	0	0	1	9	5	3.9 E
Litonotus sp.	a	0	0	1	7	2	3	3.1
Loxocephalus granulosus KENT, 1881	a	0	0	2	8	0	4	2.8
Loxocephalus luridus EBERHARD, 1862	a	0	0	0	10	0	5	3.0
Loxodes magnus STOKES, 1887	p	0	0	0	3	7	4	3.7
Loxodes rostrum (MUELLER, 1773)	a-b	0	0	4	6	0	3	2.6
Loxodes striatus (ENGELMANN, 1862)	a	0	0	1	6	3	3	3.2
Loxodes sp.	b-p	0	0	2	5	3	2	3.1
Loxophyllum helus (STOKES, 1884)	b	0	0	10	0	0	5	2.0
Loxophyllum meleagris (MUELLER, 1773)	b	0	0	8	2	0	4	2.2
Loxophyllum utriculariae (PENARD, 1922)	b	0	1	8	1	0	4	2.0
Loxophyllum sp.	b	0	1	6	3	0	3	2.2
Marituja pelagica GAJEVSKAJA, 1928	o	0	10	0	0	0	5	1.0
Mesodinium acarus STEIN, 1863	b	0	0	7	3	0	4	2.3
Mesodinium cinctum CALKINS, 1902	b	0	0	6	3	1	3	2.5
Mesodinium pulex (CLAPAREDE & LACHMANN, 1859)	b	0	2	6	2	0	3	2.0
Mesodinium sp.	b	0	1	6	3	0	3	2.2
Metacineta mystacina (EHRENBERG, 1831)	b-a	0	0	5	5	0	3	2.5
Metopus sp.	p-i	0	0	0	1	9	5	4.4 E
Microthorax pusillus ENGELMANN, 1862	a	0	0	2	8	0	4	2.8
Microthorax sulcatus ENGELMANN, 1862	b	0	0	10	0	0	5	2.0
Monodinium balbianii FABRE-DOMERGUE, 1888	b-o	0	4	5	1	0	2	1.7
Mucophrya pelagica GAJEVSKAJA, 1928	o	0	10	0	0	0	5	1.0
Multifasciculatum elongatum (CLAPAREDE & LACHMANN, 1859)	a	0	0	1	9	0	5	2.9
Myrionecta rubra (LOHMANN, 1908)	o	0	10	0	0	0	5	1.0
Nassula flava CLAPAREDE & LACHMANN, 1859	a-b	0	0	4	6	0	3	2.6
Nassula gracilis KAHL, 1931	a	0	0	2	8	0	4	2.8
Nassula ornata EHRENBERG, 1833	b-a	0	0	5	5	0	3	2.5
Nassula sp.	a	0	0	3	6	1	3	2.8
Nassulopsis elegans (EHRENBERG, 1833)	b	0	1	8	1	0	4	2.0
Obertrumia aurea (EHRENBERG, 1833)	b-a	0	0	6	4	0	3	2.4
Odontochlamys alpestris FOISSNER, 1981	b-a	0	0	5	5	0	3	2.5
Opercularia articulata GOLDFUSS, 1820	a-b	0	1	3	5	1	1	2.6
Opercularia coarctata (CLAPAREDE & LACHMANN, 1858)	a	0	0	2	7	1	3	2.9
Opercularia nutans (EHRENBERG, 1831)	b-a	0	0	5	5	0	3	2.5
Opercularia sp.	b-a	0	0	5	5	0	3	2.5
Ophrydium crassicaule PENARD, 1922	b-a	0	0	5	5	0	3	2.5
Ophrydium eutrophicum FOISSNER, 1979	b-a	0	1	6	3	0	0	2.2
Ophrydium sessile KENT, 1882	a-b	0	2	3	5	0	2	2.3
Ophrydium versatile (MUELLER, 1786)	o	0	8	2	0	0	4	1.2
Ophrydium sp.	b-a	0	2	4	4	0	2	2.2
Ophryoglena atra LIEBERKÜHN, 1856	b	0	0	10	0	0	5	2.0
Ophryoglena flava (EHRENBERG, 1833)	b	0	0	10	0	0	5	2.0
Ophryoglena oblonga GAJEVSKAJA, 1927	b	0	2	8	0	0	4	1.8
Opisthonecta henneguyi FAURÉ-FREMIET, 1906	b-p	0	0	3	4	3	2	3.0
Oxytricha chlorelligera KAHL, 1932	a	0	0	0	10	0	5	3.0
Oxytricha fallax STEIN, 1859	a	0	0	1	8	1	4	3.0
Oxytricha ferruginea STEIN, 1859	o	0	7	3	0	0	4	1.3
Oxytricha haematoplasma BLATTERER & FOISSNER, 1990	b-a	0	0	6	4	0	3	2.4

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Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Oxytricha hymenostoma STOKES, 1887	p	0	0	0	2	8	4	3.8	
Oxytricha saprobia KAHL, 1932	a-p	0	0	0	6	4	3	3.4	
Oxytricha setigera STOKES, 1891	a-b	0	0	4	6	0	3	2.6	
Oxytricha similis ENGELMANN, 1862	b-a	0	0	5	5	0	3	2.5	
Papillorhabdos carchesii FOISSNER, 1984	a	0	0	2	7	1	3	2.9	
Paracolpidium truncatum (STOKES, 1885)	a	0	0	2	6	2	3	3.0	
Paradileptus elephantinus (SVEC, 1897)	b	0	3	6	1	0	3	1.8	
Paramecium aurelia-Complex	b-a	0	0	5	5	0	3	2.5	
Paramecium bursaria (EHRENBERG, 1831)	b	0	0	7	3	0	4	2.3	
Paramecium calkinsi WOODRUFF, 1921	a	0	0	0	10	0	5	3.0	
Paramecium caudatum EHRENBERG, 1833	a	0	0	0	7	3	4	3.3	E
Paramecium putrinum CLAPAREDE & LACHMANN, 1859	p-i	0	0	0	1	9	5	3.9	E
Paramecium woodruffi WENRICH, 1928	p-i	0	0	0	0	10	5	4.2	E
Paraurostyla viridis (STEIN, 1859)	b-a	0	0	5	5	0	3	2.5	
Paraurostyla weissei (STEIN, 1859)	a	0	0	2	7	1	3	2.9	
Pelagohalteria cirrifera (KAHL, 1932)	o-b	0	6	4	0	0	3	1.4	
Pelodinium reniforme LAUTERBORN, 1908	p-i	0	0	0	0	10	5	4.5	E
Phascolodon vorticella STEIN, 1859	b-a	0	0	6	4	0	3	2.4	
Phialina coronata (CLAPAREDE & LACHMANN, 1859)	b	0	1	8	1	0	4	2.0	
Phialina pupula (MUELLER, 1773)	b	0	0	10	0	0	5	2.0	
Philasterides armata (KAHL, 1926)	b-a	0	0	5	5	0	3	2.5	
Placus luciae (KAHL, 1926)	o-b	0	5	5	0	0	3	1.5	
Placus ovum (KAHL, 1926)	b	0	0	10	0	0	5	2.0	
Plagiocampa longis KAHL, 1927	a	0	0	0	10	0	5	3.0	
Plagiopyla nasuta STEIN, 1860	p-i	0	0	0	0	10	5	4.5	E
Plagiopyla simplex WETZEL, 1928	p-i	0	0	0	0	10	5	4.5	E
Platycola decumbens (EHRENBERG, 1830)	b-a	0	2	4	4	0	2	2.2	
Platynematum sociale (PENARD, 1922)	a	0	0	2	7	1	3	2.9	
Platyophrya vorax KAHL, 1926	p-i	0	0	0	0	10	5	4.5	E
Pleuronema coronatum KENT, 1881	b	0	0	7	3	0	4	2.3	
Pleuronema crassum DUJARDIN, 1841	o	0	10	0	0	0	5	1.0	
Pleuronema setigerum CALKINS, 1902	b	0	0	10	0	0	5	2.0	
Pleurotricha grandis STEIN, 1859	b	0	0	10	0	0	5	2.0	
Podophrya fixa (MUELLER, 1786)	a	0	0	1	7	2	3	3.1	
Podophrya maupasii BUETSCHLI, 1889	a	0	0	1	9	0	5	2.9	
Prodiscophrya collini (ROOT, 1914)	a-i	0	0	1	5	4	2	3.4	
Prorodon ovum (EHRENBERG, 1831)	o-b	0	5	5	0	0	3	1.5	
Prorodon platyodon BLOCHMANN, 1895	b	0	0	10	0	0	5	2.0	
Prorodon teres EHRENBERG, 1833	a	0	0	1	9	0	5	2.9	
Prorodon viridis KAHL, 1927	a	0	0	1	6	3	3	3.2	
Prorodon sp.	b-a	0	1	4	4	1	1	2.5	
Pseudoblepharisma tenue (KAHL, 1926)	p	0	0	0	3	7	4	3.7	
Pseudochilodonopsis algivora (KAHL, 1931)[3]	a	0	0	0	10	0	5	3.0	
Pseudochilodonopsis algivora (KAHL, 1931)[4]	a-b	0	0	5	5	0	3	2.5	
Pseudochilodonopsis fluviatilis FOISSNER, 1988	b-a	0	0	5	3	2	2	2.7	
Pseudochilodonopsis piscatoris (BLOCHMANN, 1895)	b	0	0	7	3	0	4	2.3	
Pseudocohnilembus pusillus (QUENNERSTEDT, 1869)	a-p	0	0	0	5	5	3	3.5	
Pseudomicrothorax agilis MERMOD, 1914	b	0	0	10	0	0	5	2.0	
Pseudoprorodon ellipticus KAHL, 1930	b-a	0	0	5	5	0	3	2.5	
Pseudoprorodon niveus (EHRENBERG, 1833)	o	0	10	0	0	0	5	1.0	
Pseudovorticella chlamydophora (PENARD, 1922)	b-a	0	0	5	5	0	3	2.5	
Pseudovorticella monilata (TATEM, 1870)	b-a	0	1	5	4	0	2	2.3	
Pyxicola carteri KENT, 1882	o-b	0	5	5	0	0	3	1.5	

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Rhabdostyla inclinans (MUELLER, 1773)	a	0	0	0	10	0	5	3.0	
Saprodinium dentatum (LAUTERBORN, 1901)	p-i	0	0	0	0	10	5	4.5	E
Saprodinium putrinium LACKEY, 1925	p-i	0	0	0	0	10	5	4.5	E
Saprodinium sp.	p-i	0	0	0	0	10	5	4.5	E
Sathrophilus mobilis (KAHL, 1926)	b-a	0	0	6	4	0	3	2.4	
Sathrophilus muscorum (KAHL, 1931)	b-a	0	0	5	5	0	3	2.5	
Scyphidia rugosa DUJARDIN, 1841	a	0	0	0	8	2	4	3.2	
Spathidium depressum KAHL, 1930	o	0	7	3	0	0	4	1.3	
Spathidium faurei KAHL, 1930	o	0	7	3	0	0	4	1.3	
Spathidium gibbum KAHL, 1930	a	0	0	0	10	0	5	3.0	
Spathidium spathula (MUELLER, 1773)	o-b	0	5	3	2	0	2	1.7	
Spathidium sp.	b-a	0	2	4	3	1	1	2.3	
Sphaerophrya magna MAUPAS, 1881	p	0	0	0	2	8	4	3.8	
Sphaerophrya pusilla CLAPAREDE & LACHMANN, 1859	a	0	0	0	10	0	5	3.0	
Sphaerophrya soliformis LAUTERBORN, 1908	p	0	0	0	1	9	5	3.9	
Sphaerophrya stentoris MAUPAS, 1881	a-b	0	1	4	5	0	2	2.4	
Sphaerophrya sp.	p-a	0	0	1	4	5	2	3.4	
Spirostomum ambiguum (MUELLER, 1786)	a	0	0	2	6	2	3	3.0	
Spirostomum caudatum (MUELLER, 1786)	o-b	0	6	4	0	0	3	1.4	
Spirostomum minus (ROUX, 1901)	a-b	0	0	3	6	1	3	2.8	
Spirostomum teres CLAPAREDE & LACHMANN, 1858	p	0	0	1	2	7	3	3.6	
Staurophrya elegans ZACHARIAS, 1893	o-a	0	3	4	3	0	2	2.0	
Steinia platystoma (EHRENBERG, 1831)	b-a	0	0	6	4	0	3	2.4	
Stentor amethystinus LEIDY, 1880	b	0	2	6	2	0	3	2.0	
Stentor coeruleus (PALLAS, 1766)	a-b	0	0	4	6	0	3	2.6	
Stentor igneus EHRENBERG, 1838	b	0	0	7	3	0	4	2.3	
Stentor muelleri EHRENBERG, 1831	b-a	0	0	5	5	0	3	2.5	
Stentor multiformis (MUELLER, 1786)	b-a	0	0	5	5	0	3	2.5	
Stentor niger (MUELLER, 1773)	o-b	0	6	4	0	0	3	1.4	
Stentor polymorphus (MUELLER, 1773)	b-a	0	0	5	5	0	3	2.5	
Stentor roeselii EHRENBERG, 1835	b-a	0	1	4	5	0	2	2.4	
Stentor sp.	b-a	0	1	5	4	0	2	2.3	
Sterkiella histriomuscorum (FOISSNER, BLATTERER, BERGER & KOHMANN, 1991)	a	0	0	2	6	2	3	3.0	
Stichotricha aculeata WRZESNIOWSKI, 1866	b-a	0	1	5	4	0	2	2.3	
Stichotricha secunda PERTY, 1849	o	0	7	3	0	0	4	1.3	
Stokesia vernalis WENRICH, 1929	o-b	0	5	5	0	0	3	1.5	
Strobilidium caudatum (FROMENTEL, 1876)	o-b	0	5	5	0	0	3	1.5	
Strobilidium humile PENARD, 1922	b	0	2	8	0	0	4	1.8	
Strombidium viride STEIN, 1867	b	0	1	8	1	0	4	2.0	
Stylonychia mytilus-complex	a	0	0	1	9	0	5	2.9	
Stylonychia pustulata (MUELLER, 1786)	b	0	1	7	2	0	3	2.1	
Stylonychia putrina STOKES, 1885	a	0	0	2	7	1	3	2.9	
Stylonychia stylomuscorum (FOISSNER, BLATTERER, BERGER & KOHMANN, 1991)	b	0	0	10	0	0	5	2.0	
Stylonychia vorax STOKES, 1885	b	0	0	10	0	0	5	2.0	
Stylonychia sp.	b-a	0	0	5	5	0	3	2.5	
Supraspathidium vermiforme (PENARD, 1922)	a	0	0	0	8	2	4	3.2	
Tachysoma bicirratum FOISSNER, BLATTERER, BERGER & KOHMANN, 1991	a-p	0	0	2	4	4	2	3.2	
Tachysoma pellionellum (MUELLER, 1773)	b-a	0	1	4	4	1	1	2.5	
Tetrahymena pyriformis-complex	a-i	0	0	0	3	7	4	4.1	E

B-11.18

Taxa	S	Saprobic valency[2]					I	SI	
		x	o	b	a	p			
Thigmogaster oppositevacuolatus AUGUSTIN & FOISSNER, 1989	a-b	0	0	3	5	2	2	2.9	
Thigmogaster potamophilus FOISSNER, 1988	b-a	0	0	5	5	0	3	2.5	
Thuricola folliculata KENT, 1881	b	0	2	6	2	0	3	2.0	
Thuricola kellicottiana (STOKES, 1887)	b	0	2	7	1	0	3	1.9	
Thuricola vasiformis HAMMANN, 1952	a	0	0	0	10	0	5	3.0	
Thuricola sp.	b-a	0	0	5	5	0	3	2.5	
Tintinnidium fluviatile (STEIN, 1863)	o-b	0	5	5	0	0	3	1.5	
Tintinnidium pusillum ENTZ, 1909	b	0	0	8	2	0	4	2.2	
Tintinnidium semiciliatum (STERKI, 1879)	b	0	2	6	2	0	3	2.0	
Tintinnopsis cylindrata KOFOID & CAMPBELL, 1929	b	0	0	7	3	0	4	2.3	
Tokophrya carchesii (CLAPAREDE & LACHMANN, 1859)	a	0	0	2	7	1	3	2.9	
Tokophrya infusionum (STEIN, 1859)	b-a	0	2	5	3	0	2	2.1	
Tokophrya lemnarum (STEIN, 1859)	a	0	0	1	7	2	3	3.1	
Tokophrya quadripartita (CLAPAREDE & LACHMANN, 1859)	a-b	0	0	3	5	2	2	2.9	
Tokophrya sp.	a-b	0	0	3	5	2	2	2.9	
Trachelius ovum (EHRENBERG, 1831)	b	0	1	7	2	0	3	2.1	
Trachelophyllum apiculatum (PERTY, 1852)	b-a	0	0	5	5	0	3	2.5	
Trachelophyllum brachypharynx LEVANDER, 1894	a	0	0	0	10	0	5	3.0	
Trachelophyllum pusillum (PERTY, 1852)	b-a	0	0	5	3	2	2	2.7	
Trachelophyllum sp.	a-b	0	0	4	5	1	2	2.7	
Trichodina pediculus EHRENBERG, 1831	b	0	2	6	2	0	3	2.0	
Trichospira inversa (CLAPAREDE & LACHMANN, 1859)	a-p	0	0	0	5	5	3	3.5	
Trimyema compressum LACKEY, 1925	p-m	0	0	0	0	10	5	5.0	E
Trithigmostoma cucullulus (MUELLER, 1786)	a-p	0	0	2	5	3	2	3.1	
Trithigmostoma srameki FOISSNER, 1988	b-a	0	1	6	3	0	3	2.2	
Trithigmostoma steini (BLOCHMANN, 1895)	b-a	0	1	6	3	0	3	2.2	
Trochilia minuta (ROUX, 1899)	b-a	0	0	5	5	0	3	2.5	
Trochilioides recta (KAHL, 1928)	a	0	0	0	10	0	5	3.0	
Tropidoatractus acuminatus LEVANDER, 1894	p-i	0	0	0	0	10	5	4.5	E
Urocentrum turbo (MUELLER, 1786)	b	0	0	7	3	0	4	2.3	
Uroleptus gallina (MUELLER, 1786)	b	0	0	10	0	0	5	2.0	
Uroleptus musculus (KAHL, 1932)	a	0	1	8	1	0	4	3.0	
Uroleptus piscis (MUELLER, 1773)	a	0	0	3	7	0	4	2.7	
Uroleptus rattulus STEIN, 1859	b	0	0	10	0	0	5	2.0	
Uronema marinum DUJARDIN, 1841	a	0	0	1	8	1	4	3.0	
Uronema parduczi FOISSNER, 1971	a	0	0	1	8	1	4	3.0	
Urostyla grandis EHRENBERG, 1830	a	0	0	3	7	0	4	2.7	
Urotricha agilis (STOKES, 1886)	a	0	0	0	10	0	5	3.0	
Urotricha armata KAHL, 1927	a	0	0	2	8	0	4	2.8	
Urotricha farcta CLAPAREDE & LACHMANN, 1859	a-b	0	0	4	6	0	3	2.6	
Urotricha globosa SCHEWIAKOFF, 1892	b	0	0	7	3	0	4	2.3	
Urotricha ovata KAHL, 1926	a-p	0	0	0	6	4	3	3.4	
Urotricha sp.	a	0	0	2	6	2	3	3.0	
Urozona buetschlii SCHEWIAKOFF, 1889	p-i	0	0	0	0	10	5	4.5	E
Vaginicola ingenita (MUELLER, 1786)	b	0	2	6	2	0	3	2.0	
Vaginicola tincta EHRENBERG, 1830	o-b	0	5	5	0	0	3	1.5	
Vaginicola sp.	b	0	2	6	2	0	3	2.0	
Vorticella aquadulcis-complex[5]	b-a	0	2	5	3	0	2	2.1	
Vorticella campanula EHRENBERG, 1831	b-a	0	1	4	5	0	2	2.4	
Vorticella convallaria-complex[6]	a	0	1	2	6	1	2	2.7	
Vorticella fromenteli KAHL, 1935	a	0	0	2	8	0	4	2.8	

Taxa	S	Saprobic valency[2]					I	SI
		x	o	b	a	p		
Vorticella infusionum-complex[7]	p-a	0	0	1	4	5	2	3.4
Vorticella marginata STILLER, 1931	b	0	2	8	0	0	4	1.8
Vorticella mayeri FAURÉ-FREMIET, 1920	b	0	0	10	0	0	5	2.0
Vorticella microstoma-complex[8]	p-a	0	0	0	5	5	3	3.5
Vorticella natans (FAURÉ-FREMIET, 1924)	b	0	0	10	0	0	5	2.0
Vorticella octava-complex[9]	b-a	0	2	4	4	0	2	2.2
Vorticella picta (EHRENBERG, 1831)	b	0	2	6	2	0	3	2.0
Zoothamnium arbuscula (EHRENBERG, 1831)	b-a	0	1	6	3	0	3	2.2
Zoothamnium kentii GRENFELL, 1884	b-a	0	0	5	5	0	3	2.5
Zoothamnium procerius KAHL, 1935	b-a	0	0	5	5	0	3	2.5
Zoothamnium sp.	b-a	0	0	5	5	0	3	2.5

[1] Flagellates and amoebae were copied from Sladecek et al. (12) and Wegl (15). Their nomenclature, taxonomy and saprobic assessment need major revision (cp. [3]); some nomenclatural improvements were accomplished with several revisions (1, 4, 9). The list for the ciliates is also based on Sladecek et al. (12) and Wegl (15), but nomenclature and taxonomy are according to the improved list of Foissner (3). The revision of the saprobic classification of the ciliates is in progress and complete for cyrtophorids, oligotrichs, hypotrichs, colpodids, peritrichs, heterotrichs and odontostomatids (5, 6).

[2] Abbreviations: a, alpha-mesosaprobity; b, beta-mesosaprobity; E, eusaprobity (worse than polysaprobity, comprises raw, concentrated or very little diluted industrial wastes or waters with a very high load of organic matter undergoing anaerobic decomposition by microorganisms); I, indicative weight of species ranging from 5 to 1; o, oligosaprobity; p, polysaprobity; S, indication of saprobic assessment by simple letter; SI, saprobic index ranging from 0-8 (0 = xenosaprobity, 1 = oligosaprobity, 2 = beta-mesosaprobity, 3 = alpha-mesosaprobity, 4 = polysaprobity, 5 = isosaprobity, 6 = metasaprobity, 7 = hypersaprobity, 8 = ultrasaprobity); x, xenosaprobity.
Notes: The saprobic valencies and the indicative weights are for the limnosaprobic area; however, the saprobic assessment (S) and the saprobic index (SI) includes both limnosaprobity (xenosaprobity, oligosaprobity, beta-mesosaprobity, alpha-mesosaprobity, polysaprobity) and eusaprobity (isosaprobity, metasaprobity, hypersaprobity, ultrasaprobity). The letter "E" marks cases where both values do not correspond, i. e. the saprobic index (SI) is > 4.0 (11). Pollution increases from xenosaprobity → ultrasaprobity.

[3] If abundant or very abundant.

[4] If sparse.

[5] Includes *V. octava* of Sladecek's list.

[6] Includes *V. convallaria, V. citrina, V. nebulifera* and *V. similis* of Sladecek's list.

[7] Includes *V. cupifera, V, hians, V. microstoma* (pro parte), *V. microstoma* f. *elongata, V. microstoma* f. *monilata* and *V. microstoma* f. *turgescens* of Sladecek's list.

[8] Includes *V. aequilata* and *V. microstoma* (pro parte) of Sladecek's list.

[9] Includes *V. hamata*, but not *V. octava* sensu Noland & Finley and Sladecek.

LITERATURE CITED

1. Aescht, E & Foissner, W. 1989. Stamm: Rhizopoda (U.-Kl. Testacealobosia, Testaceafilosia). *Catalogus Faunae Austriae* **Ia**:1-79.

2. Amavis, R & Smeets, J. 1975 (ed.). Principles and methods for determining ecological criteria on hydrobiocenoses. Pergamon Press, Frankfurt am Main, Oxford, New York, Ontario, Paris.

3. Foissner, W. 1988. Taxonomic and nomenclatural revision of Sladecek's list of ciliates (Protozoa: Ciliophora) as indicators of water quality. *Hydrobiologia* **166**:1-64.
4. Foissner, W. 1991. Diversity and ecology of soil flagellates. *In:* Patterson, D J & Larsen, J. (ed.), The Biology of Free-living Heterotrophic Flagellates. Clarendon Press, Oxford, pp. 93-112.
5. Foissner, W, Berger, H, Blatterer, H & Kohmann, F. 1992. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems - Band II: Peritricha, Heterotrichida, Odontostomatida. *Informationsberichte des Bayerischen Landesamtes für Wasserwirtschaft* (in press).
6. Foissner, W, Blatterer, H, Berger, H & Kohmann, F. 1991. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems - Band I: Cyrtophorida, Oligotrichida, Hypotrichia, Colpodea. *Informationsberichte des Bayerischen Landesamtes für Wasserwirtschaft* **1/91**:1-478.
7. Foissner, W, Unterweger, A & Henschel, T. 1992. Comparison of direct stream bed and artificial substrate sampling of ciliates (Protozoa, Ciliophora) in a mesosaprobic river. *Limnologica (Berlin)* (in press).
8. Moog, O. (1991). Biologische Parameter zum Bewerten der Gewässergüte von Fliessgewässern. *Landschaftswasserbau* **11**:235-266.
9. Page, F C & Siemensma, F J. 1991. Nackte Rhizopoda und Heliozoea. Fischer, Stuttgart & New York.
10. Pantle, R & Buck, H. 1955. Die biologische Überwachung der Gewässer und die Darstellung der Ergebnisse. *Gas- u. WassFach (Wasser/Abwasser)* **96**:604-620.
11. Sladecek, V. 1973. System of water quality from the biological point of view. *Arch. Hydrobiol. Beih. Ergebn. Limnol. Planktonk.* **7**:IV + 1-218.
12. Sladecek, V, Zelinka, M, Rothschein, J & Moravcova, V. 1981. Biologicky rozbor povrchove vody. Komentar k CSN 830532 - casti 6: Stanoveni saprobniho indexu. Vydalo Vydavatelstvi Uradu pro normalizaci a mereni, Praha, 186 pp. (in Czech).
13. Tümpling, W v. 1966. Über die statistische Sicherheit soziologischer Methoden in der biologischen Gewässeranalyse. *Limnologica (Berlin)* **4**:235-244.
14. Washington, H G. 1984. Diversity, biotic and similarity indices. A review with special relevance to aquatic ecosystems. *Wat. Res.* **18**:653-694.
15. Wegl, R. 1983. Index für die Limnosaprobität. *Wass. Abwass. Wien* **26**:1-175.
16. Zelinka, M & Marvan, P. 1961. Zur Präzisierung der biologischen Klassifikation der Reinheit fließender Gewässer. *Arch. Hydrobiol.* **57**:389-407.

BIOMONITORING USING PROTOZOANS

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INTRODUCTION

Biological monitoring, defined as surveillance using living organisms to determine whether the environment is favorable for organisms [2], has been an integral part of environmental protection for decades. The method described here utilizes protozoan communities collected on polyurethane foam units (PFUs) for the routine monitoring of lotic ecosystems, but other Aufwuchs collectors may be appropriate. These communities have been shown to be sensitive to anthropogenic stress [6], and the importance of microbial communities in ecosystem processes is well established [3].

Advantages of using protozoan communities collected on PFUs include sampling simplicity, rapid responses to stress, taxonomic and functional diversity, and use of naturally derived, ubiquitous organisms. Limitations include the labor intensive nature of taxonomic work, and the need for taxonomic expertise. A further difficulty lies in translating changes in microbial community structure and function into meaningful effects on higher organisms. But these microbes play essential roles in
nutrient cycling, and they are likely links in food chains to higher organisms.

PROTOCOL

The method that follows is best termed an in situ bioassay. The PFUs (or alternative artificial substrata) are colonized at a reference site and subsequently redistributed along a reference-impact-recovery gradient. After an exposure period, the PFUs are harvested and analyzed. This is contrasted with a stream survey, in which PFUs are simply placed at various sites along a reference-impact-recovery gradient and allowed to colonize. The PFUs are then

harvested and analyzed. Both the traditional stream survey and the community transplantation in situ bioassay can be used to address questions of environmental change.

1. In flowing water, PFUs should be placed at an unpolluted (reference) site upstream of the potential impact site (see Pratt and Kepner, this volume, for the PFU sampling protocol), and allowed to colonize for 4-10 days (7 d typical). The PFUs should be tied three or four to a strand of rope. The number of strands placed into the stream for colonization depends on the number of stations to sampled (see no. 3).

2. Collect the rope strands with PFUs by cutting the strand and allowing the substrata to float, en masse, into a bucket filled with water, without actually removing the PFUs from the water (this will minimize the loss of biomass from the artificial substrates).

3. The strands of colonized PFUs should then be placed at several sites within the stream. At least three PFUs should be placed at each site to account for variability among the PFUs. Sites should be selected to characterize habitats both upstream and downstream of the potential impact area (e.g. a point-source discharge) (Figure 1). Also, transplanting to both "clean" and "impacted" sites accounts for community changes due to natural ecosystem differences. Strands of PFUs should be placed directly upstream and downstream of the potential impact site. The downstream impact site should be just far enough downstream of the point source to assure adequate mixing of the effluent with the stream water. The other downstream PFU strands should be spaced such that an observable downstream recovery can be detected. It is usually necessary to have the farthest downstream PFUs at least 5-10 km from the point source. Alternatively, if there is a tributary confluence prior to recovery, then PFUs can be placed in the receiving stream to determine whether recovery has occurred after the confluence. From our experience, a total of six to eight stations is adequate to observe impact and downstream recovery of the protozoan community, unless multiple impacts are present.

4. After one week at the various sites the PFUs can be collected and returned to the laboratory for subsequent analysis. The method of collection is described in Pratt and Kepner, this volume. Briefly, each PFU is placed individually into a labelled Whirlpak® bag (without removing the PFU from the water) and the PFUs are returned to the laboratory in an insulated container.

5. In the laboratory, the PFU contents are squeezed into labelled containers. The material is allowed to settle for at least five minutes. Slides of living material can be prepared by pipetting 1-2 drops of the settled material onto a glass slide and covering with a 22 mm square, No. 1 coverslip. Slides can then be scanned for protozoan taxa. At least two coverslips of material should be scanned [1]. Alternatively, the sample can be mixed to homogeneity and a known aliquot of the material can be transferred to a counting chamber for a quantitative estimate of protozoan numbers. Subsamples can also be fixed for subsequent identification and verification, or the determination of abundance for particular taxa amenable to fixation; however, fixatives are often selective and may not preserve all taxa.

6. Subsamples from the substrate can also be used for biomass determinations such as total protein, chlorophyll a, ash- free dry-weight, adenosine triphosphate, carbohydrate content, etc. [7].

7. Analysis. The data from the observation of living material can be analyzed by comparing the species richness at the various sites, beginning with the most upstream site and ending with the most downstream site. Changes in species richness are likely to be obvious if there is a significant impact due to the point source. The use of standard inferential statistics (e.g. analysis of variance) to compare the sites is inappropriate because the sites are spatially autocorrelated [5].

One can also pool data from the upstream sites and build a 95% confidence interval for the mean of the unimpacted sites. The other sites can be compared to this confidence interval and any values falling outside this range are likely to be impacted. Data can also be analyzed in terms of the functional groups of protozoa; that is, by enumerating the number of taxa in each of the functional groups and comparing the sites to determine if shifts in community composition have occurred [8,9].

Fixed material can be used to enumerate species richness and abundance. From these data, diversity indices (e.g. Shannon-Weaver) can be calculated which help elucidate changes in community composition. Indices should be used with caution because they can mask changes in community composition [4].

An alternative method for analyzing community structure is the use of community similarity or distance measures [10,11]. This method uses presence/absence or abundance data from the species richness study to compare inferentially the communities at the various sampling stations. These analyses can also provide information on "resistant" or "sensitive" species.

The non-taxonomic data (protein, chlorophyll a, etc.) can be analyzed graphically, as was the species richness data. These data are useful because while the number of species at a site may be unaffected, the total biomass (whether autotrophic or heterotrophic) may be affected.

COMMENTS

1. This protocol can be modified to monitor lentic systems as well, simply by deploying the strands of PFUs at sites which would be analogous to the reference-impact-recovery gradient described above.

2. It is important not to colonize the PFUs for too long. Substrates that are in a source ecosystem will have an initial colonization period that contains a maximum in the number of species, which is followed by a period in which the species richness declines [8]. Therefore, PFUs should be placed in streams for only 7-10 days for colonization, and should be placed in lakes, ponds, and reservoirs for only 2-4 weeks.

LITERATURE CITED

1. Cairns, J Jr, Plafkin, JL, & Kuhn, DL. 1979 Protozoan colonization of artificial substrates. *In:* Weitzel, RL (Ed.), Methods and Measurement of Periphyton Communities: A Review. American Society for Testing and Materials, Philadelphia, pp. 39-57.

2. Cairns, J Jr, & Pratt, JR. In Press A history of biological monitoring using invertebrates. *In:* Resh, VH, & Rosenberg, DM (Eds.), Biomonitoring and Freshwater Invertebrates. Chapman & Hall Publ., New York, NY.

3. Fenchel, T. 1987 Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Springer-Verlag Publ., New York, NY, 197 pp.

4. Green, RH. 1979 Sampling Design and Statistical Methods for Environmental Biologists. John Wiley & Sons Publ., New York, NY, 257 pp.

5. Hurlbert, SH. 1984 Pseudoreplication and the design of ecological field experiments. *Ecol. Monogr.*, 54:187-211.

6. Pratt, JR. 1990 Aquatic community response to stress: prediction and detection of adverse effects. *In:* Landis, WG, & van der Schalie, WH, Eds., Aquatic Toxicology and Risk Assessment: Thirteenth Volume, ASTM STP 1096, American Society for Testing and Materials, Philadelphia, pp. 16-26.

7. Pratt, JR, & Bowers, NJ. 1990 A microcosm procedure for estimating ecological effects of chemicals and mixtures. *Tox. Assess.*, **5**:189-205.

8. Pratt, JR, & Cairns, J Jr. 1985 Functional groups in the protozoa: roles in differing ecosystems. *J. Protozool.*, **32**: 415-423.

9. Pratt, JR, Horwitz, R, & Cairns, J Jr. 1987 Protozoan communities of the Flint River-Lake Blackshear ecosystem (Georgia, USA). *Hydrobiologia*, **148**:159-174.

10. Pratt, JR, & Smith, EP. 1991 Significance of change in community structure: a new method for testing differences. *In:* Biological Criteria: Research and Regulation, EPA-440/5-91-005, 172 pp.

11. Smith, EP, Pontasch, KW, & Cairns, J Jr. 1990 Community similarity and the analysis of multispecies environmental data: a unified statistical approach. *Water Res.*, **24**:507-514.

ADDITIONAL REFERENCE

Cairns, J Jr. 1982 Artificial Substrates. Ann Arbor Science Publishers Inc., Ann Arbor, MI, 279 pp.

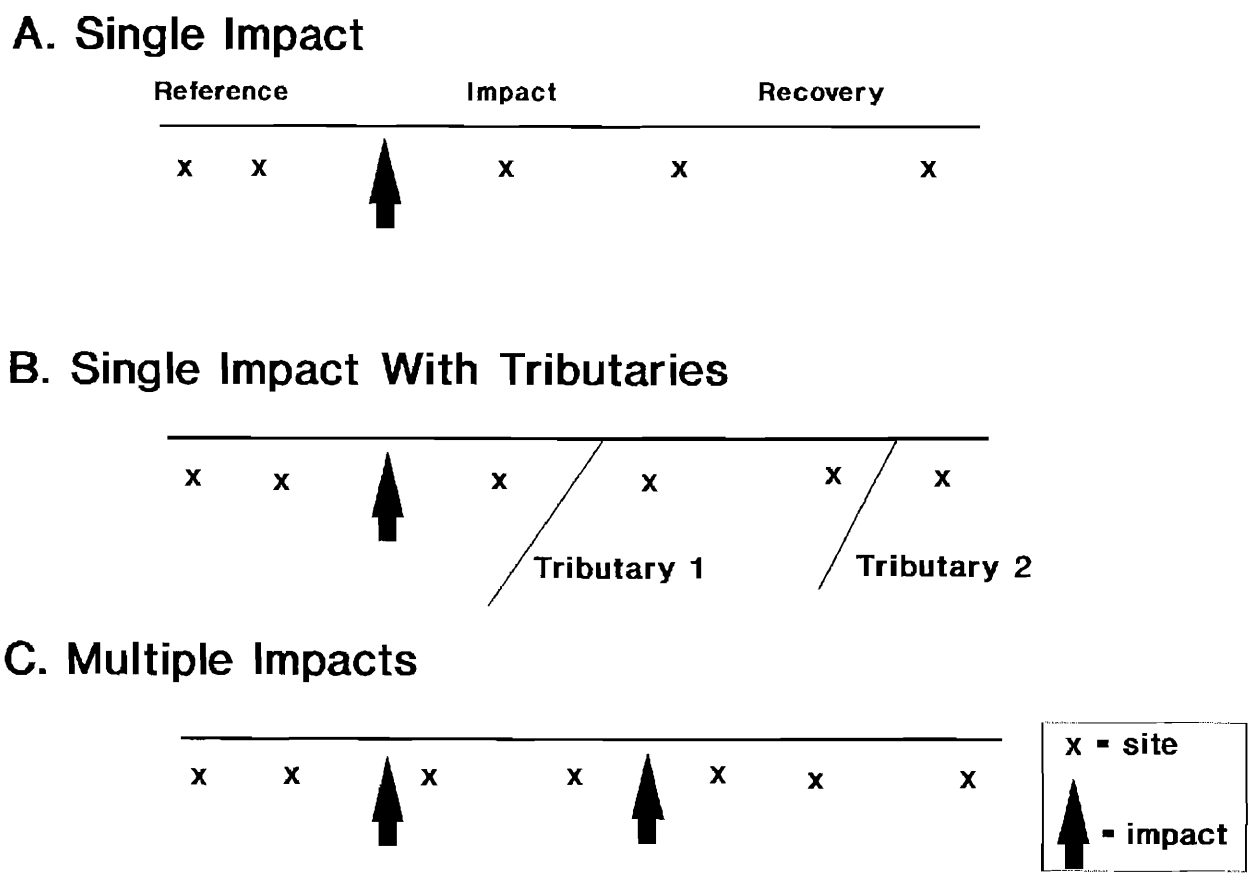

Fig. 1. Artificial substrata sampling strategy for various biomonitoring situations.

DETERMINATION OF PHAGOTROPHIC PROTIST FEEDING RATES IN SITU

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INTRODUCTION

Phagotrophic protists are major consumers of bacteria and phytoplankton in almost all aquatic habitats (1,2). Free-living flagellates have also recently been demonstrated to be capable of ingesting viral-sized particles (3) and high molecular weight dextran molecules (4). A number of methods for quantifying in situ grazing rates of protists on specific prey have been employed; each has both advantages and disadvantages (Table 1). Here we provide a short protocol for two methods: 1) a short-time course assay: use of fluorescently labeled prey to assay grazing on bacteria and small phytoplankton cells and 2) a long-time course assay: use of prokaryotic inhibitors to assay bacterivory. Other methods include the dilution approach of Landry and Hassett (5, 6, 7, 8, 9), size-selective filtration (10, 11), and uptake/disappearance of radiolabeled prey (12, 13, 14, 15). We are currently in the process of developing a new technique, the ammonium release method (ARM), in which grazing rate is determined by following the rate of release of excreted ^{16}N-ammonium from ^{15}N-labeled prey. The dilution method currently appears to be the grazing technique most widely used and most completely tested, however we do not ourselves have direct experience with this approach. Of course, in laboratory experiments with cultures of single species of predator and prey, the simpler approach of time course enumeration of predator and prey cell abundance, employing the equations of Frost (16) to determine clearance and ingestion rates, can be used effectively (e. g. 17, 18). However, prey abundances in laboratory culture experiments are typically orders of magnitude greater than in situ prey abundance, thus extrapolation of these results to the field should be made with caution.

I. UPTAKE OF FLUORESCENTLY LABELED PREY (FLP)

PROTOCOL

1. Obtain or prepare FLP as appropriate:

a. Fluorescently labeled microspheres (FLM). Plastic microspheres which fluoresce in yellow-green, blue, or red wavelengths, in sizes ranging from 0.05 μm to 90 μm in diameter, may be ordered from the company **Polysciences, Inc.**, Warrington, PA, phone: 215-343-6484. The FLM are sent as a concentrated suspension in deionized water, and must be diluted in into a few ml of deionized water to make a working stock. A few μl of the working stock are then pipetted into two ml of water and collected onto a 0.2 μm membrane filter for direct count enumeration via epifluorescence microscopy to determine the FLM concentration in the stock vial. For FLM < 0.2 μm in diameter, which cannot be quantitatively collected onto the membrane filter, a two-step procedure may be used to determine the concentration of < 0.2 μm FLM in the stock: 1) into 2 ml

of deionized water, pipette a few μl of the < 0.2 μm sized FLM stock and then add a few μl of 0.5 μm sized FLM; mix, pipette a small volume of the FLM mixture directly onto a slide, and determine the proportional abundance of < 0.2 μm : 0.5 μm FLM via epifluorescence microscopy; 2) filter 1 ml of the FLM mixture onto a membrane filter and determine the abundance of 0.5 μm FLM; the abundance of < 0.2 μm FLM is then the product of the ratio of small:large FLM and the concentration of the larger FLM.

The tendency of the FLM to clump may be overcome by aging the FLM in 5 mg ml^{-1} of bovine serum albumin for 24 hours (19).

Selection by protists against FLM compared to other prey has been shown in several studies (20,21,22).

b. Fluorescently labeled bacteria (FLB) or phytoplankton (FLA). Our procedure for staining bacteria or small algal cells with the fluorochrome DTAF is not very difficult (20). It does, however heat-kill the cells, destroy motility, and shrink algal cells, so the resulting FL prey are not truly comparable to living prey. However, FLB and FLA are both prefered over similarly-sized FLM (20, 21, 22). The basic procedure for preparation of FLB is: Suspend a dense concentration of bacteria (10^8 to 10^{10} ml^{-1}) or phytoplankton (10^6 to 10^8 ml^{-1}) in about 10 ml of phosphate buffered saline solution, pH 9; add 2 mg of DTAF (Sigma, Inc., St. Louis, MO, USA) and incubate at 60°C for two hours. Then centrifuge down the cells to a pellet, decant the DTAF solution, and wash 2-3 times with the phosphate buffered saline. After the final wash, resuspend the cells in 10-20 ml of pyrophosphate (PP_i-saline buffer (0.89 g of Na_4 pyrophosphate in 100 ml deionized water, add NaCl to equal the salinity of the prey cell medium, or 0.85% salt for freshwater). 1-2 ml aliquots of the FLB or FLA suspension can be stored frozen until required. See Sherr et al. 1987 (20), 1989 (23) and 1991 (22) for additional details.

J. Gonzalez was able to prepared fluorescently labeled viruses (FLV) by incubating a bacteriophage suspension with DTAF overnight at 5°C (3).

2. Protocol for short-term FLP uptake assays:

a. Decide on approximate concentration of FLP to be used in the experiment. We recommend FLP concentrations on the order of 10^7 FLV ml^{-1}, 10^6 FLB ml^{-1} for flagellate uptake, 10^5 FLB ml^{-1} for ciliate uptake, and 10^3 FLA ml^{-1}. For FLB, we use concentrations of about 5% of total bacterioplankton standing stock for ciliate grazing experiments, and of 30-50% of bacterioplankton standing stock for flagellate grazing experiments. From the concentrated FLP stock solution, make a diluted stock of appropriate concentration of FLP, if necessary, so that a volume of between 20 - 200 μl of diluted FLP stock can be added to the experimental containers to yield the desired concentration. Make up the diluted stock in PP_i-saline buffer, and sonicate for several seconds using a microtip at 30-W of power to disperse FLP clumps. It may be necessary in some cases to filter the FLP diluted stock through a 3 μm membrane filter in order to remove large clumps of cells.

b. Prepare experimental containers: We use Whirl-pak bags, pre-treated for hours or overnight with a small volume of 10% HCl, then copiously rinsed with first tap water, then deionized water, and a final rinse with sample water.

c. Add known volumes of sample (50 - 400 ml) to individual labeled Whirl-pak bags; place the bags in 1 liter beakers half-full of sample water, or of water of appropriate salinity at the same temperature as the sample. Place the beakers containing the bagged samples in the dark at the appropriate temperature. Let the protists recover from handling shock for 0.5 - 1 hr.

d. Sonicate/vortex the FLP diluted stock, add the pre-determined amount, and gently but quickly mix the FLB added to each sample by swirling or upending the sealed bag several times.

e. At selected time intervals, withdraw 10 to 20 ml subsamples from each bag and immediately preserve in separate labeled vials. Depending on the water temperature, sampling intervals may range between 5 - 15 min. over a total period of 20 - 90 min. Recommended preservation methods are Lugol + formalin + sodium thiosulfate (22) or 4% vol/vol ice cold glutaraldehyde of appropriate salinity (22). These preservation techniques have been shown to prevent loss of food vacuole contents in flagellates and ciliates.

f. For selected subsamples filter small aliquots of sample onto 0.2 μm plain membrane filtes and enumerate FLP to quantify the actual concentration of FLP in the experimenal bags.

g. Stain subsamples with DAPI (50 μl of 1 mg DAPI/ml per 5 ml of sample, incubate at least 7 minutes), filter onto 0.8 μm-black membrane filters, mount onto glass slides, and either inspect immediately or freeze at -20°C. When examining the prepared filters via epifluorescence microscopy, use a lower magnification (200 - 500 X) to locate larger-sized protists, and 1000 X for nanoflagellates, with the uv light filter set to visualize DAPI - stained cells. For each cell inspected, switch to the blue-light filter set to enumerate the FLP contained in the cell. For larger protists, focus up and down to ensure that all ingested FLP are counted. FLB and FLA often appear brighter inside the protists' food vacuoles than they do on the filter. For each time subsample, calculate FLP/cell. Total number of protists/ml should be determined also by counting flagellates or ciliates over a known area of the filter, for selected subsamples.

h. Plot FLP/protist versus time. A regression of the linear portion of the uptake curve yields a rate of FLP uptake, which is converted to clearance rate by dividing the value for FLP/cell/hr by the concentration of FLP per nl or μl. Multiply the per-cell clearance rates by the total number of protists/ml to obtain a clearance rate for the entire community of flagellates or ciliates present in the sample, for similarly-sized prey as the FLP used.

COMMENTS

1. This short-term uptake method does not quantify all of the potential grazing on the prey cells of interest, only that grazing due to the protistan cells observed in the samples. Larger, rarer grazers, benthic grazers, or viral lysis are sources of cell mortality which are not accounted for by this method. Also, the question of extent of selectivity for or against added FLP remains open. Significant selection against added FLP will result in underestimation of the true grazing rate.

2. To obtain adequate uptake rates on the short term, FLA must be added at relatively high concentrations compared to standing stocks of small phytoplankton cells found in most natural waters (22). This will also change the grazing rate compared to that of in situ conditions.

3. Both flagellates and ciliates may show higher grazing rates on larger-sized bacterial cells, thus care should be taken to use FLB of about the same average cell size as the natural bacterioplankton.

4. FLP uptake experiments may be designed to investigate aspects of the physiological and behavioral ecology of protists in culture or in situ, e.g. effect of temperature or prey type on prey digestion time (24), or variation in clearance rate depending on prey size (25) or initial abundance (22).

II. USE OF PROKARYOTIC INHIBITORS TO ASSAY BACTERIVORY

Despite concerns about long-term (> 18-24 hr) grazing experiments involving manipulation of the microbial community (see COMMENTS), we did get results consistent with other grazing rate methods using a prokaryotic inhibitor approach (26). The inhibitor mixture, vancomycin + penicillin, was chosen after preliminary evaluation of the effects of a number of prokaryotic inhibitors on both bacteria and protists (26). The use of eukaryotic inhibitors to assay grazing rates is not recommended, since the inhibitors may affect eukaryotic algae as well as phagotrophic protists, and uncoupling of the protist-bacteria trophic link can lead to decreases in bacterial growth rates (26, 27, 28).

B-13.4

PROTOCOL

1. Prepare at minimum duplicate experimental containers (we use Whirl-pak bags treated as described above) for the experimental treatments:

a. Whole water, no added inhibitors (bacterial growth + grazing)

b. Whole water, prokaryotic inhibitors added (grazing only)

c. 0.8 μm screened water, prokaryotic inhibitors added (non-grazing check for bacterial lysis) (it's a good idea to screen the water twice through 0.8 μm to ensure removal of the smallest bacterivores).

2. Add subsamples of whole water (200 - 400 ml) to each of the experimental bags, and place in 1 liter beakers half full of water of the same temperature and salinity as the sample.

3. Add prokaryotic inhibitors at concentrations of 200 mg l^{-1} of vancomycin (added as a preweighed powder) and 1 mg l^{-1} of penicillin (from a stock solution of 1 mg ml^{-1}). Gently mix into the inhibitor treatments. Incubate in the dark at in situ temperature.

4. Sample each container for bacterial abundance at time 0 and every 4-6 hours thereafter for periods of 18-24 hours. Data should be obtained for at least 4-6 time points. We recommend the acridine orange direct count (AODC) method for bacterial enumeration (29, 30). Also sample at the beginning and end of the experiment to determine protist abundance and general composition of the protistan community.

5. Perform linear regression analysis for the data sets obtained for each of the treatments. Rate of bacterivory is calculated as the difference between the rate of change of bacterial abundance in the control and inhibited whole water treatments, after correcting for any non-grazing bacterial cell loss observed in the 0.8 μm-screened inhibitor treatment. We found non-grazing cell loss to be on average about 1/5 of the cell loss attributed to grazing (26); such cell loss may be due to lysis as a result of viral infection (31) or to effects of the prokaryotic inhibitor. A check for growth of bacterivorous flagellates in the 0.8 μm screened water should also be made at the end of the experiment.

COMMENTS

1) In our experience, manipulations which decouple the predator-prey link, e.g. size selective filtration and use of eukaryotic inhibitors, can lead to experimental artifacts (26).

2) A concern in all long-time course experiments is the possibility of shifts in species composition and/or metabolic rates of predator and prey assemblages during time course experiments longer than the average doubling times of microbial populations, which could bias the experimental results away from actual in situ rates (32).

3) Prokaryotic inhibitors may become less effective at higher temperatures (> 20° C).

4) For a more in-depth assessment of measurement of protistan bacterivory, see the review of McManus & Fuhrman 1988 (33).

LITERATURE CITED

1.Porter, K G,Sherr, E B, Sherr, B F, Pace, M, & Sanders, R W. 1985 Protozoa in planktonic food webs. J Protozool 32:409-415.
2.Sherr, B, & Sherr, E. 1989 Trophic impacts of phagotrophic protozoa in pelagic foodwebs. In: Hattori, T, et al. (ed), Recent Advances in Microbial Ecology. Japan Scientific Societies Press, Tokyo, Japan, pp.388-393.
3.Gonzalez, J M, Suttle, C A, Sherr, E B, & Sherr, B F. Submitted Virus ingestion by marine protists. Nature.
4.Sherr, E B. 1988 Direct use of high molecular weight polysaccharide by heterotrophic flagellates. Nature 335:348-351.
5.Landry, M R, & Hassett, R P. 1982 Estimating the grazing impact of marine micro-zooplankton. Mar Biol 67:283-288.
6.Landry, M R, Haas, L W, & Fagerness, V L. 1984. Dynamics of microbial plankton communities: experiments in Kaneohe Bay, Hawaii. Mar Ecol Prog Ser 16:127-133.
7.Tremaine, S C, & Mills, A I. 1987 Tests of the critical assumptions of the dilution method for estimating bacterivory by microeukaryotes. Appl Environ Microbiol 53:2914-2921.
8.Gifford, D J. 1988 Impact of grazing by microzooplankton in the Northwest Arm of Halifax Harbour, Nova Scotia. Mar Ecol Prog Ser 47:249-258.
9.Gallegos, C L. 1989 Microzooplankton grazing on phytoplankton in the Rhode River, Maryland: nonlinear feeding kinetics. Mar Ecol Prog Ser 57:23-33.
10.Wright,R T, & Coffin, R B. 1984 Measuring microzooplankton grazing on planktonic marine bacteria by its impact on bacterial production. Microb Ecol 10:137-149.
11.Verity,P G. 1986 Grazing of phototrophic nanoplankton by microzooplankton in Narrangansett Bay. Mar Ecol Prog Ser 29:105-115.
12.Lessard, E & Swift, E. 1985 Species-specific grazing rates of heterotrophic dinoflagellates in oceanic waters, measured with a dual-label radioisotope technique. Mar Biol 87:289-296.
13.Servais, P, Billen, G, & Vives-Rego, J. 1985 Rate of bacterial mortality in aquatic environments. Appl Environ Microbiol 49:1448-1454.
14.Wikner,J, Andersson, A, Normark, S, & Hagstrom, A. 1986 Use of genetically marked minicells as a probe in measurement of predation on bacteria in aquatic environments.
15.Wikner, J, Rassoulzadegan, F, & Hagstrom, A. 1990 Periodic bacterivore activity balances bacterial growth in the marine environment. Limnol Oceanogr 35:313-324.
16.Frost, B W. 1972 Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod Calanus pacificus. Limnol Oceanogr 17:805-815.
17.Heinbokel, J F. 1978 Studies on the functional role of tintinnids in the Southern California Bight. I Grazing and growth rates in laboratory culture. Mar Biol 47:177-189.
18.Verity, P G. 1991 Measurement and simulation of prey uptake by marine planktonic ciliates fed plastidic and aplastidic nanoplankton. Limnol Oceanogr 36:729-750.
19.Pace, M L, & Bailiff, M D. 1987 An evaluation of a fluorescent microsphere technique for measuring grazing rates of phagotrophic microorganisms. Mar Ecol Prog Ser 40:185-193.
20.Sherr,B F, Sherr, E B, Fallon, R D. 1987 Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. Appl Environ Microbiol 53:958-965.
21.Nygaard, K, Borsheim, K Y, & Thingstad, T F. 1988 Grazing rates on bacteria by marine heterotrophic microflagellates compared to uptake rates of bacterial-sized monodispersed fluorescent latex beads. Mar Ecol Prog Ser 44:159-165.
22.Sherr, E B, Sherr, B F, & McDaniel, J. 1991 Clearance rates of < 6 μm fluorescently labeled algae (FLA) by estuarine protozoa: potential grazing impact of flagellates and ciliates. Mar Ecol Prog Ser 69:81-92.
23.Sherr, B F, Sherr, E B, & Pedros-Alio, C. 1989 Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine water. Mar Ecol Prog Ser 54:209-219.

24.Sherr, B F, Sherr, E B, & Rassoulzadegan, F. 1988 Rates of digestion of bacteria by marine phagotrophic protozoa: temperature dependence. Appl Environ Microbiol 54:1091-1095.
25.Gonzalez, J M, Sherr, E B, & Sherr B F. 1990 Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. Appl Environ Microbiol 56:583-589.
26.Sherr, B F, Sherr, E B, Andrew, T L, Fallon, R D, & Newell, S Y. 1986 Trophic interactions between heterotrophic protozoa and bacterioplankton in estuarine water analyzed with selective metabolic inhibitors. Mar Ecol Prog Ser 32:169-179.
27.Taylor, G T, & Pace, M L. 1987 Validity of eukaryotic inhibitors for assessing production and grazing mortality of marine bacterioplankton. Appl Environ Microbiol 53:119-128.
28.Tremaine, S C & Mills A L. 1987 Inadequacy of the eukaryotic inhibitor cycloheximide in studies of protozoan grazing on bacteria at a freshwater-sediment interface. Appl Environ Microbiol 53:1969-1972.
29.Hobbie, J E, Daley, R J, & Jasper, S. 1977 Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228.
30.Kirchman, D L, Sogda, J, Kapuscinski, R, & Mitchell, R. 1982 Statistical analysis of the direct count method for enumerating bacteria. Appl Environ Microbiol 43:769-776.
31.Heldal, M & Bratbak, G. 1991 Production and decay of viruses in aquatic environments. Mar Ecol Prog Ser 72:205-212.
32.Ferguson, R L, Buckley E N, & Palumbo A V. 1984 Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 47:49-55.
33.McManus, G B & Fuhrman, J A. 1988 Control of marine bacterioplankton populations: measurement and significance of grazing. Hydrobiologia 159:51-62
34.Borsheim, K Y. 1984 Clearance rates of bacteria-sized particles by freshwater ciliates, measured with monodisperse fluorescent latex beads. Oecologia 63:286-288.
35.Hollibaugh, J T, Fuhrman, J A, & Azam, F. 1980 Radioactive labeling of natural assemblages of bacterioplankton for use in trophic studies. Limnol Oceanogr 25:172-181.
36.Fuhrman, J A & McManus, G B. 1984 Do bacteria-sized marine eukaryotes consume significant bacterial production? Science 224:1257-1260.
37.Campbell, L & Carpenter, E J. 1986 Estimating the grazing pressure of heterotrophic nanoplankton on Synecococcus spp. using the seawater dilution and selective inhibitor techniques. Mar Ecol Prog Ser 33:121-129.

Table 1. Summary of techniques used to directly measure in situ protistan grazing rates on bacteria and phytoplankton (updated from Table 1 of Gifford, 1988, Ref. 8).

METHOD	ADVANTAGES	DISADVANTAGES	REFERENCES
Particle uptake: fluorescent microspheres, fluorescently labled bacterial or algal prey (FLP)	Direct measure of ingestion, cell-specific rates, identifies which groups of protists ingest which types of prey, short incubation times	Discrimination for or against added FLP can bias results, all sources of prey mortality are not assessed	Borsheim 1984 (34), Nygaard et al. 1988 (21), Sherr et al. 1987 (20), 1989 (23), 1991 (22)
Particle uptake: radiolabeled prey, dual-label approach, ^{3}H for bacteria, ^{14}C for phytoplankton	Direct measure of ingestion, cell-specific rates, uses live, natural prey, short incubation times	Works best with larger protists which can be easily separated from labeled prey, other sources of prey mortality not assessed	Hollibaugh et al. 1980 (35) Lessard & Swift 1985 (12)
Disappearance of labeled particles: radiolabeled natural bacterioplankton or E. coli minicells	Measure of total prey mortality, minimal manipulation of in situ microbial community	^{3}H labeled bacteria method requires long incubation times and may underestimate mortality, E. coli minicell method is complex	Servais et al. 1985 (13), Wikner et al. 1986 (14), 1990 (15)
Size fractionation to separate predator & prey	Quantitative, determines what size classes of protists have the greatest impact on the prey studied	Uncouples predator-prey feedback processes (e.g. nutrient recycling), may change prey growth rates, incomplete separation of predator & prey may occur, long incubation time	Wright & Coffin 1984 (10), Verity 1986 (11)
Selective metabolic inhibitors to minimize prey growth or predator grazing	Quantitative, uses intact microbial community	Inhibitors may affect non-target organisms or may not completely inhibit target group, eukaryotic inhibitors uncouples feedback processes, long incubation time	Furhman & McManus 1984 (36), Sherr et al. 1986 (26), Taylor & Pace 1987 (27), Tremaine & Mills 1987 (28), Campbell & Carpenter 1986 (37)

Range of dilutions of whole water to proportionally decrease grazing rates	Quantitative, straightforward, gives estimates of rates of prey growth as well as mortality, widely used, assumptions have been tested	May uncouple predator-prey feedback, rates of protist grazing may change with decrease in prey density, long incubation time	Landry & Hassett 1982 (5), Landry et al. 1984 (6), Tremaine & Mills 1987 (7), Gifford 1988 (8), Gallegos 1989 (9)
Ammonium release method (ARM): release of ^{15}N-labeled ammonium from ^{15}N-labeled prey	Measures turnover of specific prey organisms within intact assemblages, can give an idea of extent of nitrogen recycling (number of trophic steps) for a particular type of prey	Still in the process of development, reuptake of released ammonium could be a problem, may be difficult to apply in oligotrophic systems, long incubation time	Suzuki et al. unpublished data

QUALITATIVE STUDY AND QUANTATIVE ANALYSIS OF BEHAVIOR OF CILIATED PROTOZOA: PRINCIPLES, TECHNIQUES, TRICKS

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INTRODUCTION

The analysis of Protozoan behavior is a relatively neglected aspect of their biology. The theoretical rationals and the practical outcomes of this kind of scientific investigation are not immediately obvious to most protozoologists. Why study the behavior of Protozoa? The study of behavior is intrinsically interesting, intellectually challenging, practically important(7). Organisms use locomotory behavior to adapt to their environment; it is an interface of reconciling internal requirements and external constraints, so that behavior itself in any protozoon is a relevant adaptive character(4). Ciliates are "small organisms" (2) and this implies that they experience a Reynolds' number ($Re<<1$) which describes their locomotion as very similar to that of a man swimming in honey! A ciliate moves about in its environment without physical inertia, under severe viscoelastic conditions, which are capable of stopping a *Paramecium* in about 0.5 μm when its cilia quit beating. In other words the locomotory state of a protozoon mirrors faithfully, in time and space, the beating state of its ciliary engines. This significance of conclusion may be fully understood if we consider that the ciliary beat is completely controlled, in both direction and frequency, by the electrophysiological activity of the cell. This means that by monitoring the behavior of a certain experimental population of ciliates, we monitor the general condition of its electrophysiology, point by point, instant by instant (5, 6).

Eibl-Eibesfeldt (1) introduced the concept of an ethogram as a precise catalogue of all the behavioral patterns of a large animal species. when applied to ciliates, the ethogram is a useful tool for describing their behavior. This is very likely due to their fairly "simple" behavior (10, 11).Ethological analyses have been applied to monitor environmental conditions (12, 13). The components of a standard ethogram of a ciliate are as follows:

A. Swimming (Fig. 1 A). Ciliates swim through water with a helicoidal movement, described by the pitch and radius of the helicoid, the correction angle (formed by two successive traits of the

helicoid, during the Stop-and-Reorientation Reaction SRR), the translation velocity (namely the velocity of the ciliate along the straight, central axis of the helicoid) and by the real velocity (namely the true velocity of the ciliate along the helicoidal path itself). All of these measurements can be expressed in µm (absolute values) or in Relative Units (RU), defined as to the species-specific length of the species studied.

B. Creeping (Fig. 1 B, Fig. 2, Fig. 3). Each track consists of a regular sequence of well-defined elements alternatively belonging either to the Long Lasting Elements (LLE) or to the Short Lasting Elements (SLE); the first group (Fig. 2 A, Fig. 3 C) is formed by rightward arcs (A+) and leftward arcs (A-) (both characterized by frequency of occurrence: %, length: l, radius: r, central angle: ß°, velocity: v) and segments (S: frequency, length, velocity). Among the SLE (Fig. 2 B, Fig. 3 D) Continuous Trajectory Change (CTC), Smooth Trajectory Change (STC), Rough Trajectory Change (RTC) and the Side-Stepping Reaction (SSR) are described by the correction angle (a°) and their relative frequencies of occurrence; the SSR is also characterized by a third element, the length of the backward motion. Creeping velocity and temporal frequency of track interruption are the final two of the 43 parameters, forming the ethogram (Fig. 1).

PROTOCOL

The basic scheme of the present protocol is summarized in Table I, where the successive steps of the whole process are indicated on the left; each step is characterized by the instruments, the materials and the techniques required for the step itself and by its outcome. The single steps are briefly explained in the following sections, according to the terminology used in the table.

A. Cultivation

Cultivation is treated in other protocols in this book. It is important to use standardized cultivition conditions to have standard populations because behavior itself is seriously affected by changes of both external (temperature, pH, ionic composition of the medium, light etc.) and internal factors (growth rate, cell cycle phase, life cycle phase, clonal age, degree of starvation etc.).

COMMENT

It is essential that every item of glassware be carefully washed before coming into contact with experimental populations. During processing in the glass factory,many substances unknown to the operator are used (some of which are tensioactive) so that persistent traces of them remain on new beakers, flasks, test tubes, microscope slides etc. While these substances may be quite irrelevant for some experiments, they are uncontrolled variables for studying behavior (9).

B. Collecting ciliates.

Once the cultures are grown, the cells must be collected. Depending on species, different

techniques are used,: (a) mild centrifugation (2-4 x g); (b) slow filtration through a nylon net, with openings of known dimensions; (c) Pasteur pipettes.

Comment

Our experience suggests the greatest attention must be used in this phase of handling, to avoid any unnecessary mechanical stress. This sort of extra stimulation, affects the general state of the experimental populations in such a way that their locomotory behavior is severely altered. The recovery period, namely the time lag during which the cells are kept still in order to let them rest, is to some extent proportional to the intensity of mechanical stress, which is quite difficult to judge even for a well trained operator. This phase must also be considered the time to remove organic debris, which would affect the general patterns of behavior of certain species: for this reason, again, extreme caution must be used.

C. The standard populations.

If we want to have repeatible results and to draw reliable conclusions, experimental populations must be maintained under controlled conditions. The medium in which the cells are kept during the experiment must have a known composition and no effect on the physiology of the species studied. A synthetic medium(SMB)can be used for *O. bifaria* but not for *Blepharisma* (8). SMB affects the locomotory patterns of *Blepharisma* to such an extent that it can not be used for this kind of research. The presence of any organic debris has to be avoided with the greatest care, because it is perceived by the creeping cells, which react to it by altering their behavior either negatively (avoidances) or positively (tropisms).

D. The observation apparatus.

a) The Standard Microchamber (SM) (Fig. 4 A).

A SM consists of a microscope slide (scrupulously washed) with a coverslip, held still above it by means of 4 pieces of plasticine: the droplet with the cells is added by a pipette in the middle of the coverslip. There must be absolutely no contact between the medium and the plasticine itself to avoid any unwanted, uncontrolled chemical contamination.

Several comments must be made:

(1) The distance between slide and coverslip must be at least 2-3 mm: this enables us to distinguish cells in dorsal view from those in ventral view (which will be clearly out of focus), thus avoiding misleading interpretations of the geometry of the tracks (Fig. 4 A, lower part).

(2) Surfaces of slide and coverslip must be parallel to avoid optical distortions and unwanted extra lights, due to the mirror effect of oblique glass surfaces.

(3) The size of the drop must be neither too small (the border effect may become prevalent thus inducing unwanted consequences on behavior of ciliates) nor too large (otherwise the oxygen dissolved in the water will be too little, even dropping down to zero, in the center of the SM). An average roundish drop, about 2-3 cm in diameter, is perfectly suitable.

(4) Cell density of the experimental populations must be neither too low (in this case the

tracks observed would be too rare) nor too high (too many cell-to-cell contacts cannot but affect an ethogram):for *O. bifaria* 6-13cells/mm^2 is optimal. (5) The temperature must be as close as possible to that of the cultures, in order to avoid unwanted shocks.
(6) Once the SM has been set up, fiber glass cool light apparatus must be used, to avoid any progressive increase of the temperature.

b) The open microchamber.

Its use becomes necessary when extensive observations (> 30') must be made. The droplet of culture is placed on the slide by pipette, without any cover system: to avoid water evaporation (which would be a dramatic environmental change) the slide must be placed in a Petri dish (Fig. 4 B), with the bottom covered by wet paper, so working as a sort of small damp chamber. To allow dark-field observations, the slide is kept above a central "window" in the paper by means of two glass rods used as mechanical supports.

E. Recording sessions

The microchamber (either standard or open model) is inserted onto the base of a stereomicroscope coupled to a TV camera: our model is a Wild M 420 (a perfectly stable instrument) lying on a thick marble shelf, deeply embedded in a thick wall to exclude both vibrations and other unwanted stimuli. Observations and recordings must be made under dark-field conditions (white cells in a black background).Before we solved the problem, the temperature in the experimental set ups rose during long recording sessions, but the use of two external fiber glass lights (cool light) solved this problem. The two light-guides must be placed almost parallel to the upper surface of the microchamber, facing each other in order to produce a uniformly illuminated field, with a perfectly black background. A stroboscopic light may be added to illuminate the moving ciliates at a chosen frequency: the best frequency is one which enables us to have the images of the ciliates enhanced by extra light, neither overlapping each other along the tracks, nor too far away from each other. It is essential that this optimisation problem be solved, since the white shining images of ciliates along (and within) a track clearly mark the space/time relationships. When the microchamber is properly placed and illuminated, the cells are added by glass pipette: 1-2 min must elapse before recording the cellular locomotion, to let them settle down and get acquainted with their new environment.
At this point, the videotape must be clearly and unmistakably labelled with a number which documents the experiment(recording conditions, date, temperature, light intensity, species and strain, culture conditions, etc.).
Magnification is an important factor: if low, it allows a good spatial covering which, in turn, enables long recordings before any particular cell creeps beyond the TV camera field. The disadvantage is that no exact resolution of the behavioral patterns into their qualitative elements and quantitative parameters will be possible during the successive analytical phases . The reverse is true for high enlargements: while good qualitative and quantitative analysis becomes possible, no long track will be recorded and the overall view of the general creeping pattern will be lost! For *O. bifaria* we found that 20-30 X was the optimal magnification. This system is not totally defined, because the video tape can be analyzed in different TV systems. To prevent errors, we record a stage micrometer slide for each different enlargement on every tape used to record the steps of the experiment. Thus a simple, direct measurement of the length units used on the slide on the TV screen will indicate the true enlargement of that tape. For

experiments requiring very long recording sessions (many hours) the following procedure works well. The experimental populations are recorded from 0 to 20 min and, then left to settle; the next recording will be made from 1 h to 1 h 20 min from onset and so on. This procedure is very convenient because (a) it covers long periods of time; (b) it saves conspicuous quantities of videotape; (c) information loss is not significant: it must be clear that, on average, a 20 min videotape recording session contains an almost endless amount of data, certainly sufficient to analyze and draw an ethogram!

COMMENTS

1. The magnification must be recorded so that measurements are accurate.
2. Zoom lenses are quite suitable and useful for continuous filming of experimental populations, when passing from one enlargement to another: there is no "black-hole" in the TV recording.
3. Great care must be used to have standard populations that are representative of the species studied.
4. Always be sure to have a sufficiently large number of tracks recorded: better to have one tape more than necessary, than one less.
5. In our experience, it is desirable to use the same cultures for different recording sessions and as controls.
6. Although the ethogram of *O. bifaria* drawn by a beginner in 1991 does not differ significantly from the original data (1979), when one puts the results together into a single group, the standard deviations become very large, thus making the interpretation of experimental data far more complex than with possible with more homogeneous populations!
7. For each experimental treatment, a control ethogram must be drawn evey time: although the same average data are found in different recording sessions, the possibility of having the same population of ciliates for both controls and experimentally treated cells enables us to have far smaller standard deviations to evaluate even the slightest effects, even on one single parameter of the ethogram: this procedure actually enables us to fully exploit the potential of ethographic analysis.
8. During every recording session, one has to avoid moving the observation apparatus in the horizontal plane. If this is not observed, it becomes almost impossible to find the true coordinates (x, y) of the ciliate positions, foiling geometric analysis.
9.The direction of movement of a ciliate along a particular track (whether we are dealing with a leftward or a rightward arc) can be shown in photographic records by interposition of a grey filter (which reduces light quantity by one half) between the TV screen and the camera (kept still by the tripod) during the last half second of the exposure.

F. Visualization of the Tracks.

An unsophisticated 35 mm camera mounted on a tripod may be used to record images from the TV screen. It has to be positioned so that it will record the entire screen. Once the camera is stabilized, the lens focused and the diaphragm is opened to its proper setting, exposure time has to be determined. The following factors must be taken into account: (a) exposures too short (0-1 sec) do not allow the recording of tracks long enough to be measured; (b) exposures too long (> 15 sec) produce a messy overlapping of tracks, which hinders any fruitful analysis. As

an example, we use 5 sec exposures for the ethograms of *O. bifaria*.

COMMENTS

1. Take as many pictures as necessary to describe the tracks of a certain population: we generally use up at least two photographic peels (36 frames x 2) for each population at each recording session.
2. Take a picture of the TV recorded stage micrometer slide at the different enlargements used for the tracks: it will make measuring the true linear enlargement of the recordings easy.
3. The TV records must be photographed randomly, to avoid biased observation and/or analysis.
4. When processing the photographic films, every effort must be made to achieve the greatest contrast, to obtain negatives allowing the clearest study.
5. A good quality negative has a transparent background, grey tracks, along which dark-black spots indicate the positions of the ciliate evidenced by the stroboscopic light.

G. Hand-recorded tracks.
An overhead projection sheet taped on the TV screen can be used to copy the successive positions of a ciliate in the videotape recordings. If the species studied moves too slowly, the successive positions of a specimen along its track should be recorded every 5, 10, or even 20 frames (write the chosen frequency along the track itself.) The overall result of such a recording procedure is that each track is represented by a series of dots (if the operator is only interested in the centre of gravity of the ciliate: this choice is more convenient when the mere geometry of the track is to be analyzed) or of irregular geometric figures (circles, ellipses and so on) which indicate the body shape, if the operator preferes to record the position of the ciliate's entire body; this choice is usually made to analyze the way (parallel, obliquous, orthogonal) the ciliate moves along the track.

H. Track analysis.
The program we use is a very simple one, thoroughly described by Russo et al. (17); it ensures several basic advantages, with respect to the previous one, made by hand-made analysis: (a) far lesser subjectivity of analysis; (b) the automatic calculations of the geometric parameters enables the operator to analyze up to 10 times as many tracks as previously; (c) the average standard deviation of any parameter is dramatically reduced.
The basic, introductory part of the program divides the digitizer as in Fig. 5 B: the upper, right stripe (called "Operations Stripe"), divided in many rectangles enables the operator to choose the different operations required for drawing an ethogram, just by pointing the pen of the digitizer at the chosen rectangle.
I - The first operation is the "normalization" of the digitizer: the operator chooses the area to be analyzed, pointing the pen at its lower left corner (Fig. 5 B: LL) and then at its upper right corner (ibid.; UR): the first one represents the origin of both abscissas and ordinates.
II - The correct enlargement is chosen by pointing the pen at the extremes of the unit length, as recorded on the scale of the stage micrometer slide. From this point the program will calculate the apparent distances on the digitizer, converting them directly to µm.
III - Next the frequency of recording is selected. When photographic negatives are studied the

frequency corresponds to the number of flashes per second of the stroboscope; in the case of hand-drawn records it will be that indicated by the operator as number of frames per spot or per body profile. From this point the dimension "time" will be automatically calculated and stored in the files.
After choosing the normalized area, enlargement and recording frequency, the geometric acquisition of the tracks can begin: (a) photographic negatives are projected directly on to the digitizer by a slide projector.
(b) hand-drawn sketches: the transparent sheet is placed carefully on to the digitizer and taped in place. In both cases, the digitizer pen is pointed to the centers of the successive positions (from 1 to n) of the organism along its track; the points are memorized in RAM as $x_1\ y_1$, $x_2\ y_2$,...., $x_{n-1}\ y_{n-1}$, $x_n\ y_n$. If many tracks are recorded in that particular frame (either photographic or hand drawn), the acquisition can be done in series, the beginning of a new track being automatically recognized by the program, on the basis of the distance (d > 10 RU) between the last point of one track and the first of the successive. At the end of the acquisition the CRT shows all the acquired points.

Analysis of the elements of a track.
Any one of the already acquired tracks can be analyzed without restriction: the analysis in itself must be always made from the first (p) to the last (p) point of that particular track.
The first LLE (cf. Introduction) one can consider is the one which follows the first SLE (cf. Introduction, Fig. 1), to avoid any systematic underestimation. The length of the first LLE of the recorded track is likely to be far shorter than its true value, because the beginning of the recording never coincides with the beginning of the LLE itself.

Analysis of the LLE.
The operator points the pen to the rectangle of the "Operations Stripe" indicating the LLE (namely A-, S, A+) to be analyzed after choosing it, (s) the pen is pointed at the first and at the last point of that LLE and the monitor shows immediately the theoretical geometric interpolation: if the fitting between the theoretical and real curve is statistically satisfactory, the radius (r), the angle at the center (ß°), the length (l) and the cell velocity along that LLE (v) are shown on the monitor and acquired in a new file. If the fitting is not satisfactory the operator points the pen at the rectangle "correction" of the "Operations Stripe" and repeats the steps changing the end of the LLE analyzed, in such a way that the fitting may be found statistically significant.
Analysis of the SLE
These are recognized by the program itself, whenever the operator chooses the next LLE.
If a CTC is present (Fig. 6 A) the operator chooses the next LLE (A-, S, A+ according to what he observes along the track; in the example of Fig. 6 A: A- after a S) and acquires as its first point (Ai) the last of the preceding LLE (Sf): the program will automatically acquire a CTC between the S and the A- storing it together with its correction angle a°, in the succession representing that track.
If a STC is present (Fig. 6 B) the operator chooses (in the Operation Stripe) the next LLE which is (by definition of STC) opposite to the preceding and with a very short radius (1-2 times the species specific length):

in our example after A_i- - A_f-, the series A_i+ - A_f+ will follow. The third LLE (according to the definition of STC) will again be in the same sense as the first: in our example a leftward arc A_i- - A_f- ... series follows so that the program stores a succession like A- - A+ - A- (or more rarely like A+ - A- - A+), the radius of the second arc being very short. When all these conditions are fulfilled, the program recognizes a STC, measures its correction angle, stores it and the data describing it properly, in the succession of elements constituting the track.

If a RTC is present, after acquiring the first LLE (A- in Fig. 6 C) and after choosing the proper second LLE (S in the same example), the operator acquires as its first point (Si) the point successive to the last point (A-f) of the first LLE: the program automatically recognizes a RTC, calculates its correction angle and stores the data properly in the succession of elements forming the track.

If a SSR is recognized by the operator, the track is acquired by pointing the pen to the successive positions of the cells (Fig. 6 D). Doing this, the single point 4 will be acquired also as the point n. 6, after the extreme position n. 5 and before the n. 7, first point of the next LLE (Fig. 6 D, acquisition). When the SSR has to be analyzed the operator follows this procedure: the preceding LLE (in our example A-) ends with extreme position Af; the next LLE to be chosen is the Segment (S) and the positions to be pointed to are # 5 and # 6, which will become Si and Sf, respectively: a "negative" space, corresponding to the backward motion of the ciliate, is introduced and a SSR recognized. As soon as the operator chooses the third LLE (in our example an A-), and points the pen at position 6, now become A_i-, the correction angle is measured, the program stores properly (in the general analytical representation of the track) an SSR, the length of its backward motion, the width of its correction angle.

The succession of operative steps B_1 - B_2 - B_3-B_4 etc. creates a succession of LLE and SLE, each one with its quantitative parameters. When a track finishes, the program recognizes the long distance between the final point of the "n" track and the initial point of the (n + 1) track, so that it stores a "Gap" signal which automatically prepares the storage of the data of the next track. The points and the data describing the tracks of the same negative (or sketch) are then separatedly stored into a single file, the correspondence between files and negatives being one-to-one.

The statistical processing is quite simple. The data of different files are fed into Stat Pal, Marcel Dekker Inc, or Statgraphic, GSS Inc, for instance, and a series of means, standard deviations, percentages, correlations may be obtained. The complex of the statistical data describing the different parameters of the different behavioral elements represents the ethogram of the species, or the ethogram of a certain population experimentally treated in a certain way.

COMMENTS

1.The analysis of swimming in 3 dimensions is not yet practical. Only the tracks perpendicular to the optical axis of the microscope-TV system can be analyzed fruitfully: their percentage is always very small, so that their relative rarity hinders the usefulness of this movement for

experimental analysis of treated cells. It has been demonstrated that a ciliate swims forward along a helicoid, whose radius and pitch can be easily measured provided that properly recorded tracks are available: the analysis still has to be fully done by hand, making it more inconvenient and less frequently used.
2. In our experience it is better to keep the old tapes, instead of using them over and over again; this enables us not only to study old tracks again with a new perspective.

Acknowledgements

This chapter is the result of the intelligent and friendly cooperation of the members of the PROTO-ETHOLOGY group of Pisa University: Dr. R. Banchetti, Dr. A. Russo, Dr. F. Erra. The author is deeply indebted to Dr. R. Banchetti for her sympathetic and capable technical and scientific assistance.

LITERATURE CITED

1. Eibl-Eibesfeldt, I. 1967 Grundriss der Vergleichenden Verhaltensforschung. Ethologie. R Piper Verlag, Muenchen, Germany.
2. Fenchel, T. 1987 Ecology of Protozoa. The Biology of Free-living Phagotrophic Protists. Science Tech Publishers, Madison, Wisconsin.
3. Harrison, FW, & Corliss, JO. 1991 Microscopic Anatomy of Invertebrates. Vol. 1, Wiley-Liss Publ. New York U.S.A.
4. Hinde, RA. 1966 Animal Behaviour. A synthesis of Ethology and Comparative Psychology. McGraw-Hill Book Company, New York, U.S.A.
5. Machemer, H, & Deitmer, JW. 1987 From structure to behavior: *Stylonychia* as a model system for cellular physiology. *Progr.Protistol.* **2**:213-330.
6. ____ 1988 Electrophysiology. In: Goertz, HD (ed), *Paramecium*, pp.216-35, Springer Verlag, Berlin, Germany.
7. Martin, P, & Bateson, P. 1986 Measuring Behaviour. An introductory guide. Cambridge University Press, Cambridge, Great Britain.
8. Miyake, A. 1968 Induction of conjugation by cell free fluid in the ciliate *Blepharisma. Proc Jap Acad* **44**:837-41.
9. Ricci, N. 1981 The ethogram of *Oxytricha bifaria* Stokes. I. The motile behaviour. *Acta Protozool.* **20**:393-410.
10. ____ 1989 Microhabitats of Ciliates: specific adaptations to different substrates. *Limnol.Oceanogr.* **34**:1089-97.
11.____ 1990 The behaviour of ciliated Protozoa. *Anim.Behav.* **40**:1048-69.
12.____ 1991 Protozoa as tools in the pollution assessment. *Mar. Pollut. Bull.* **22**:265-68.
13.____ Etho-ecology of Ciliates: a reappraisal of their adaptive biology, an insight in their environmental constraints. *Acta Protozool.* in press.
14.____ Banchetti, R, & Pelamatti, R. 1989 The reversible carnivorism of *Oxytricha bifaria*. *Hydrobiologia* **182**:115-20.
15.____ Erra, F, Russo, A, & Banchetti, R. 1989 Substrates determine spatial distribution of Oxytricha bifaria. *J. Protozool.* **3**:567-71.
16.____ Erra, F, Russo, A, & Banchetti, R. 1991 The air-water interface: a microhabitat for hypotrichous settlers (Protista, Ciliata). *Limnol. Oceanogr.* **36**:
17.Russo, A, Gualtieri, P, & Ricci, N. 1988 A semi-automatic computerized analysis of tracks of Ciliates. *Experientia* **44**:277-80.

TABLE I - A schematic synthesis of the successive steps (left column) leading to the ethogram. Each step is described in terms of both instruments, materials, techniques required and output produced. A more articulated description and discussion of the process are given in the text.

A FLOW CHART FOR AN ETHOLOGICAL ANALYSIS OF CILIATES

	STEPS	Instruments & materials	Techniques	Output
I	Cultures	-thermostatic chamber -food -glassware -slides and coverslips	-culturing protocols -collecting protocols	-standard populations
II	Recording Sessions	-microscopes -TV camera, recorder, screen -cold light -stroboscopic light -micrometric slide -TV tapes	-peculiar illumination -TV recording standard techniques and tricks	-tracks recorded on videotapes
III	Track Visualization	-acetate sheets & pencils -photocamera tripod -films	-hand drawing -dark field, time exposure photograph	-drawn tracks -35 mm negatives
IV	Track Analysis	-projector -digitizer -PC 486/33 IBM compatible -software I	-semi-automatic analysis	-files (succession of LLE and SLE described by their parameters)
V	Statistical Processing of data	-PC 486/33 IBM compatible -software II	-statistical tests	-means, standard deviations hystograms, etc... correlations forming up the ETHOGRAM

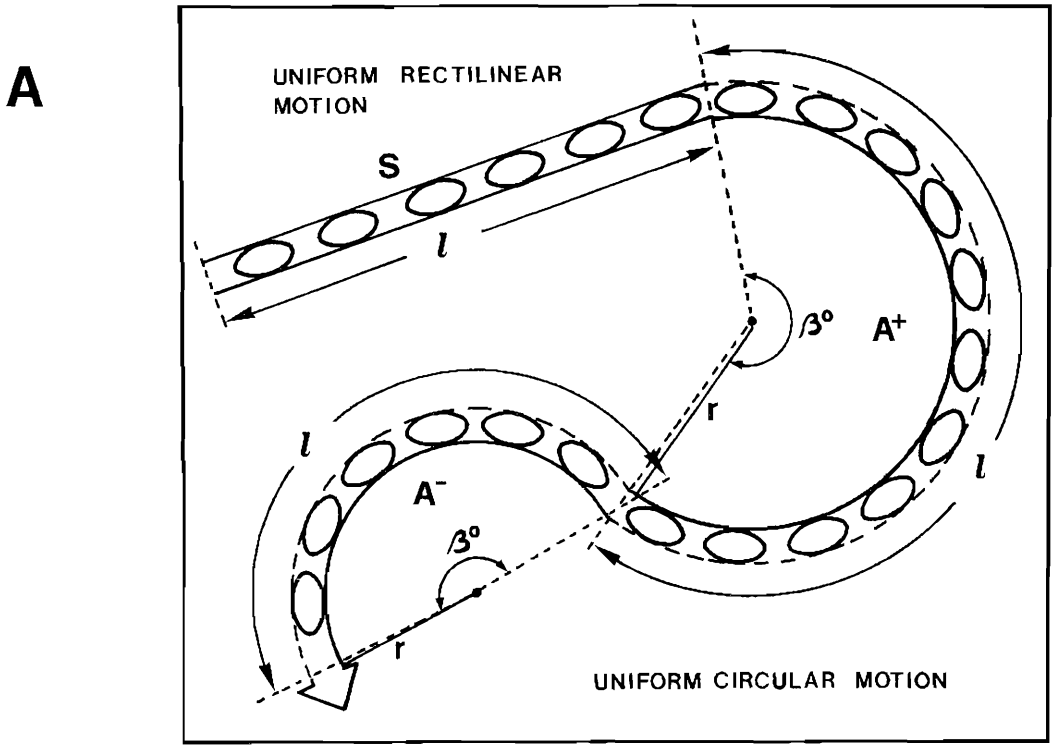
A
SWIMMING

pitch (p)	µm	1
	RU	2
radius (r)	µm	3
	RU	4
Stop Reor. Reaction(SRR)	α°	5
translation velocity(tv)	µm/sec	6
	RU/sec	7
real velocity(vr)	µm/sec	8
	RU/sec	9

B
CREEPING

Long Lasting Elements	A^+	frequency	%	10
		length	µm	11
			RU	12
		radius	µm	13
			RU	14
		central angle	β°	15
		velocity	µm/sec	16
			RU/sec	17
	S	frequency	%	18
		length	µm	19
			RU	20
		velocity	µm/sec	21
			RU/sec	22
	A^-	frequency	%	23
		length	µm	24
			RU	25
		radius	µm	26
			RU	27
		central angle	β°	28
		velocity	µm/sec	29
			RU/sec	30
Short Lasting Elements	CTC	frequency	%	31
		correction angle	α°	32
	STC	frequency	%	33
		correction angle	α°	34
	RTC	frequency	%	35
		correction angle	α°	36
	SSR	frequency	%	37
		correction angle	α°	38
		backward motion	µm	39
			RU	40

Fig. 1 - The qualitative elements and quantitative parameters forming up an ethogram are given, together with their units and progessive number: they have been grouped on the left to describe the swimming and on the right for creeping.



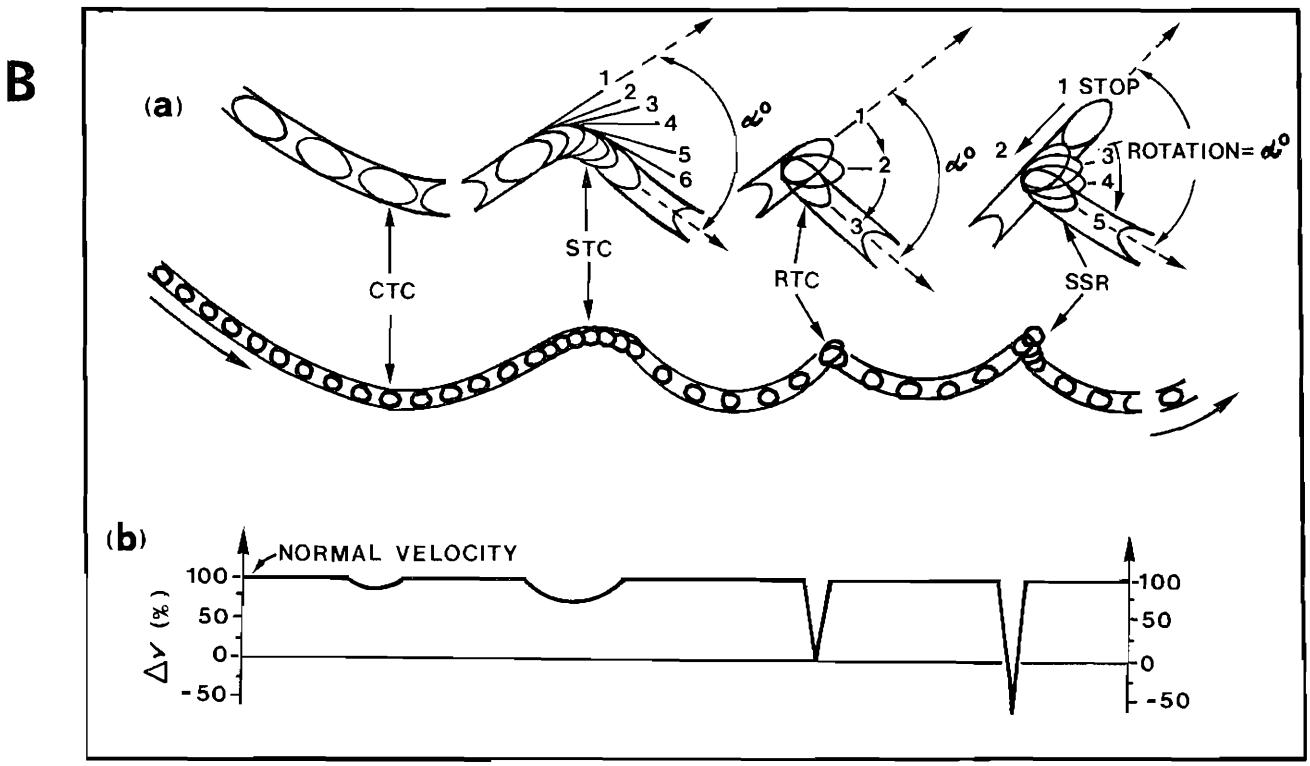
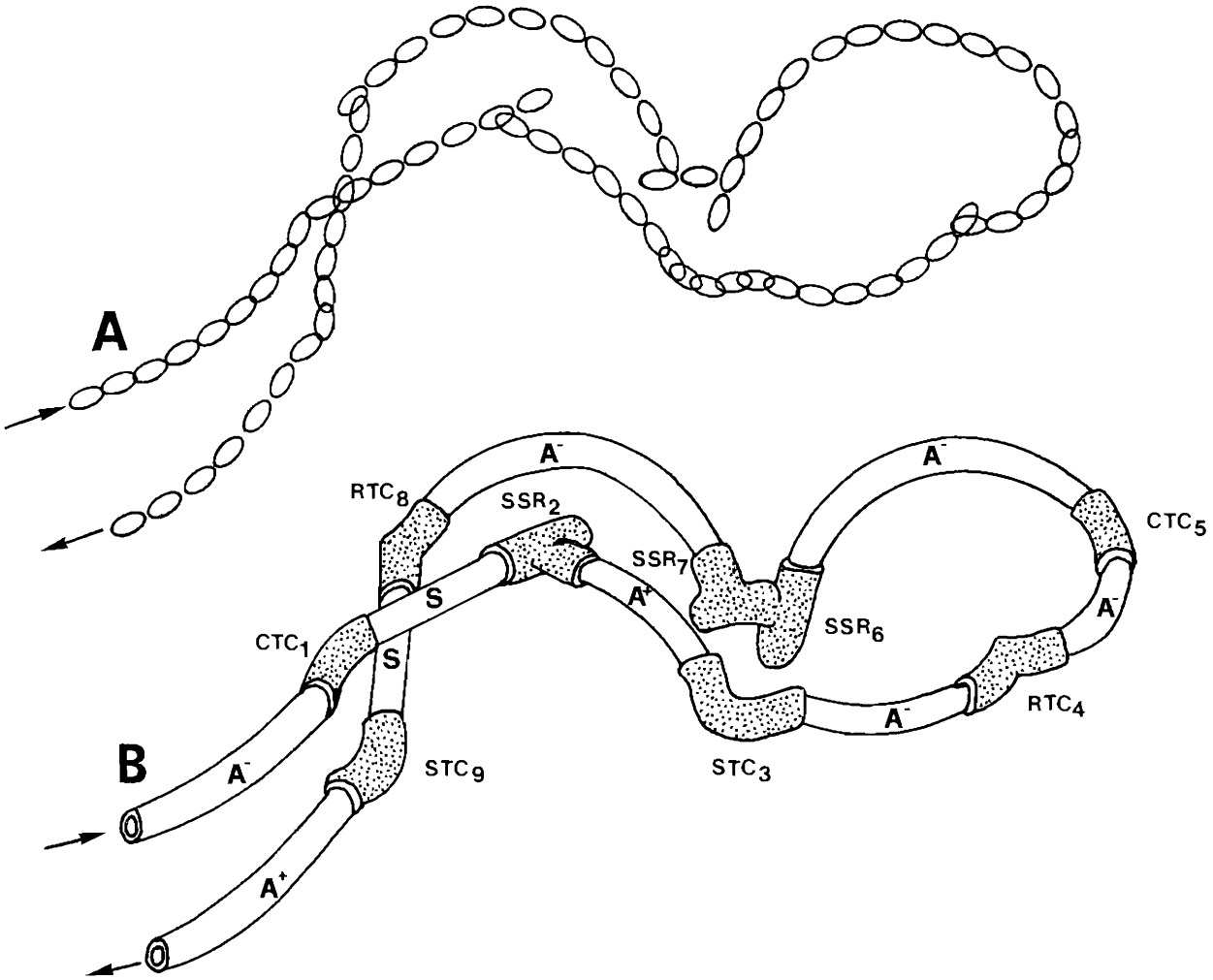


Fig. 2 - The elements of the standard ethogram of ciliates; A: the 3 Long Lasting Elements, LLE, are the rectilinear segments (S), the rightward arcs (A+), the leftward arcs (A-); in the scheme their parameters are also indicated; B: the 4 Short Lasting Elements, SLE, are the Continuous Trajectory Change, CTC, the Smooth Trajectory Change, STC, the Rough Trajectory Change, RTC, and the Side Stepping Reaction, SSR; their correction angles, a°, and the SSR's backward motion are also indicated.



C LLE

SYMBOL			
ELEMENT	A^-	S	A^+
RADIUS	r	–	r
LENGTH	ℓ	ℓ	ℓ
ANGLE	$\beta°$	–	$\beta°$
FREQUENCY	%	%	%

D SLE

SYMBOL				
ELEMENT	*CTC*	*STC*	*RTC*	*SSR*
CORRECTION ANGLE	$\alpha°$	$\alpha°$	$\alpha°$	$\alpha°$
BACKWARD MOTION	—	—	—	μm
FREQUENCY	%	%	%	%

Fig. 3 - A: an example of a track as recorded by hand directly from the TV screen; B: its analysis in terms of LLE and SLE; C: the parameters describing the different LLE; D: the parameters describing the different SLE.

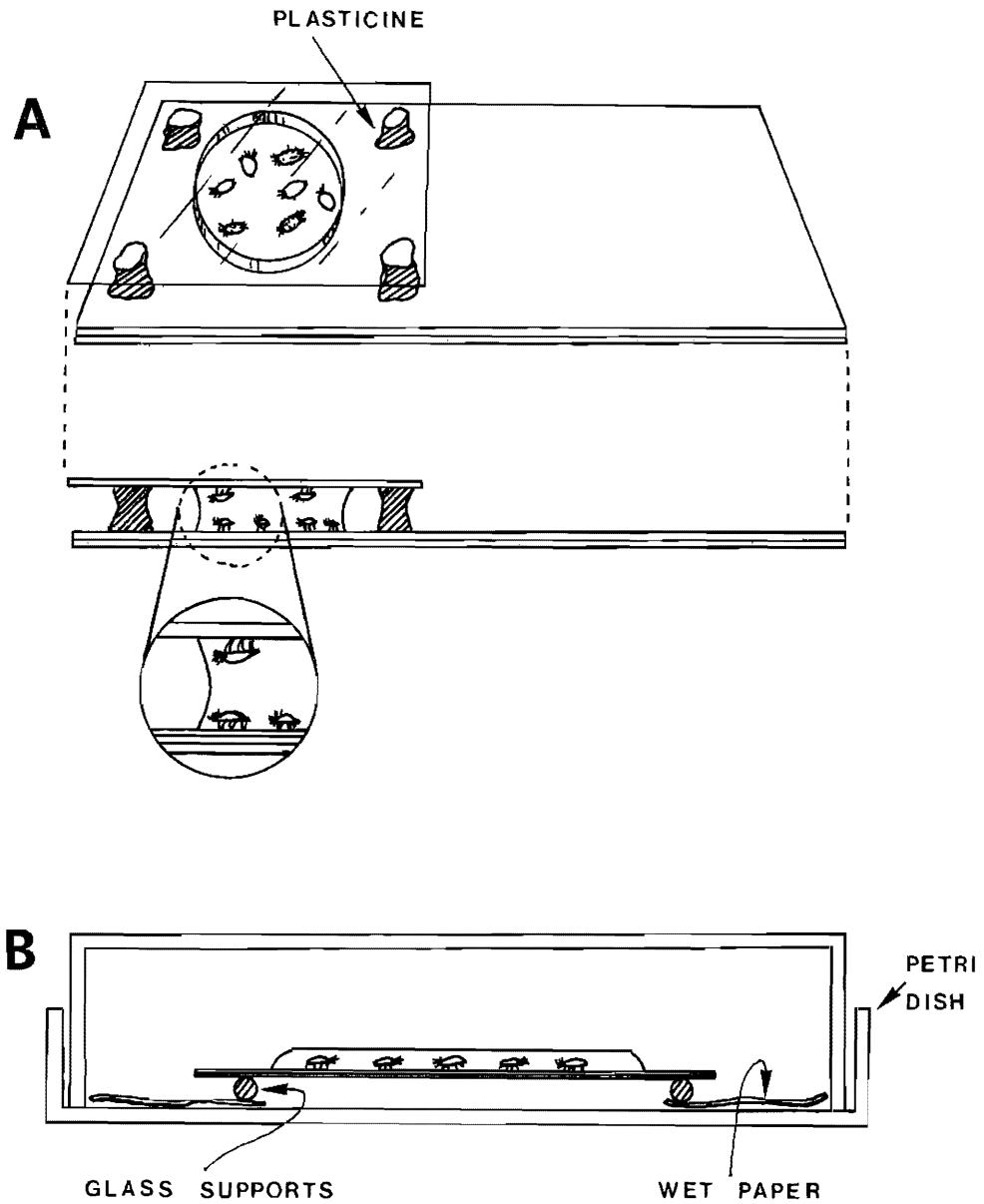


Fig. 4 - A: the Standard Apparatus to study and to record the locomotion of ciliates. B: the open microchamber.

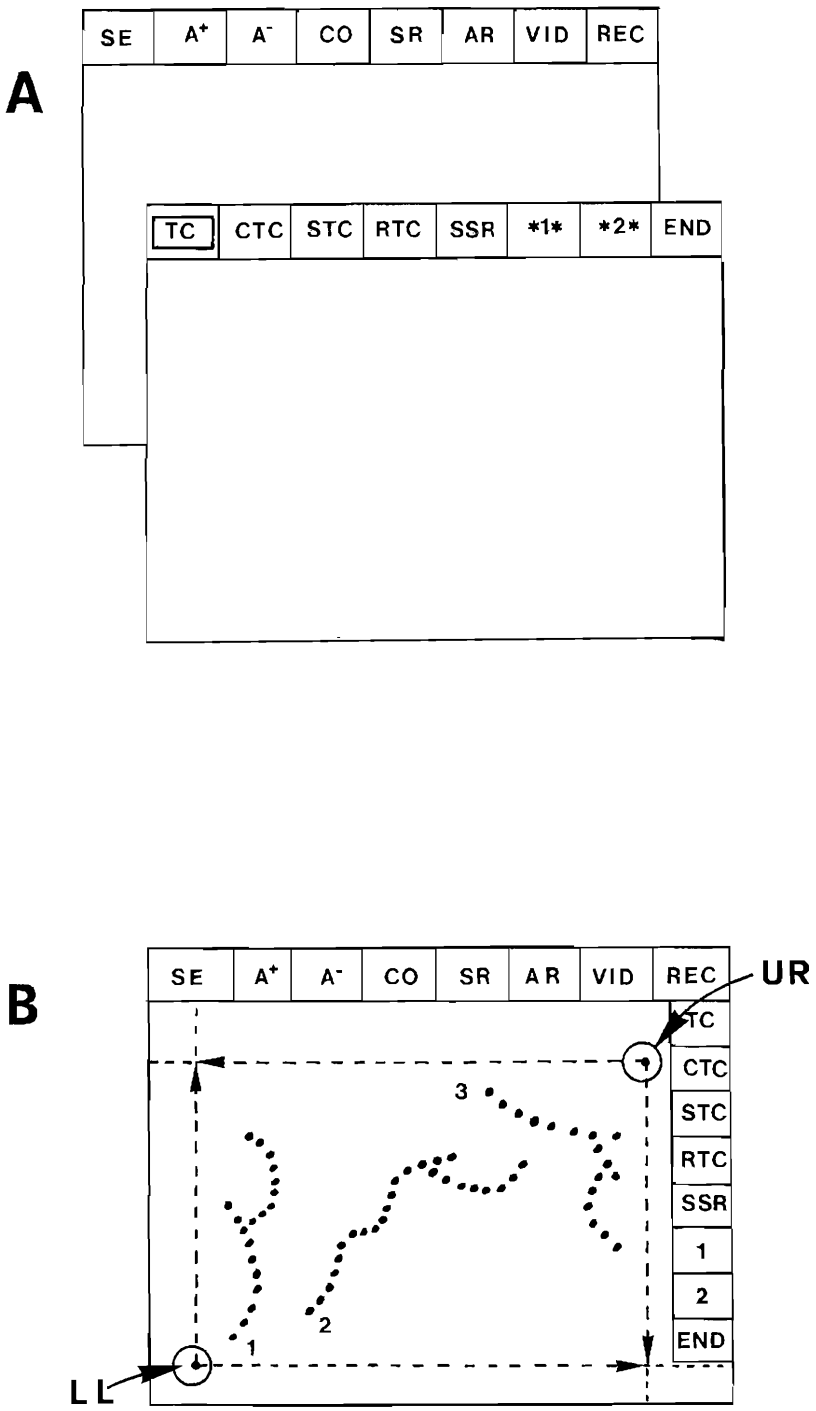


Fig. 5 - A: the schematic representation of what is shown on the TV screen, according to the rectangule pointed at by the pen. B: the operations STRIPE occupies the upper and the right sides of the digitizer; its remaining area may be "normalized" as exemplified in the scheme: LL = the Left Lower point which is the origin of the abscissas and of the ordinates; UR = Upper Right point.

B-14.16

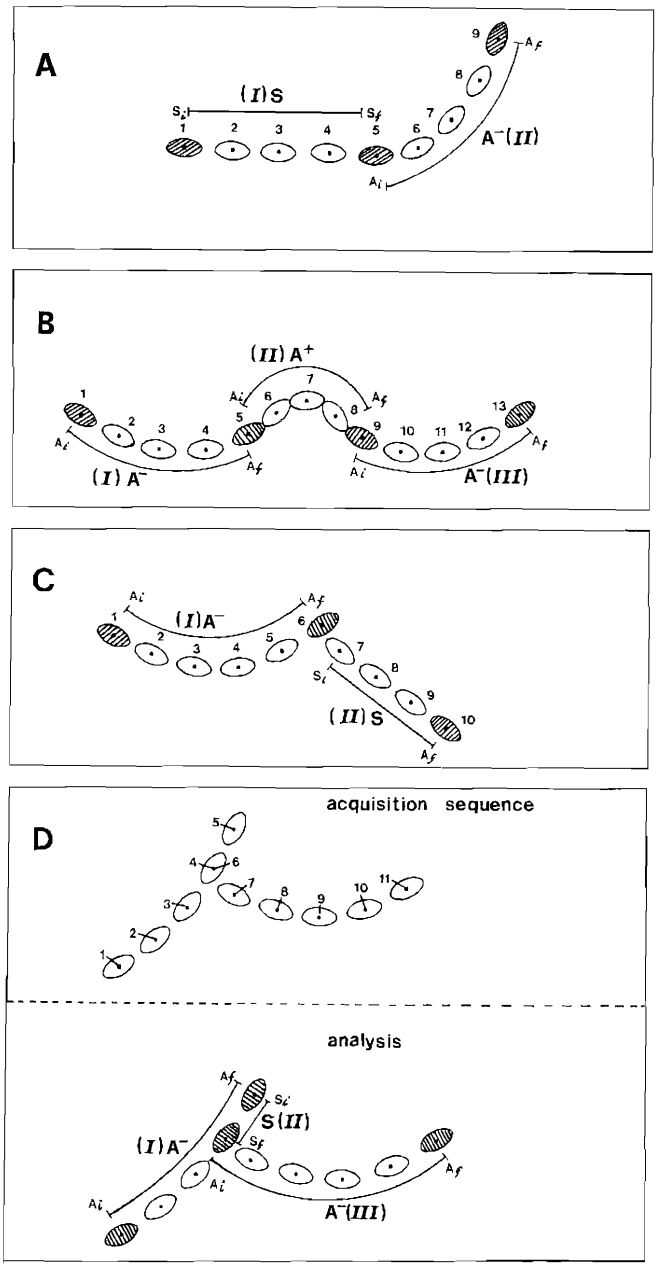


Fig. 6 - The analysis of the SLE. A: the first LLE is a segment: (I) S; the pen is pointed at the initial (1=Si) point and, then, to the final (5=Sf) point of the segment; the second LLE is a leftward arc: A- (II): the pen is pointed at the initial (5=Ai) point and then to the final one (9=Af). B: the first LLE is a leftward arc: (I) A-: the penis pointed at its initial (1=Ai) and final (5=Af) points; the second LLE is a rightward arc: (II) A+: the pen is pointe at its initial (5=Ai) and final (9=Af) points; the third LLE is a leftward arc: (III) A-: the pen is pointed at its initial (9=Ai) and final points (13=Af). A STC is recognized, measured and stored automatically. C: the first LLE is a leftward arc: (I) A-: the pen is pointed at its initial (1=Ai) and final (6=Af) points; the second LLE is a segment: (II) S; the pen is pointed at its initial (7=Si) and final (10=Sf) points. An RTC is recognized, measured and stored automatically. D: the first LLE is a leftward arc: (I) A-: the pen is pointed at its initial (1=Ai) and final (5=Af) points; the second LLE is a segment: (II) S: the pen is pointed at its initial (5=Si) and final (6=Sf) points; the third element is a leftward arc: (III) A-: the pen is pointed at its initial (6=Ai) and final (11=Af) points. A SSR is recognized, measured and stored automatically.

COVER SLIP TRAPS: SIMPLE SAMPLERS FOR SESSILE PROTISTS

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Introduction

For directly sampling aquatic habitats for sessile protists, "traps" which hold standard 1 x 3" microscope slides have long been used for fresh water (1) and more recently marine benthic habitats (2). Plastic petri dishes (3) have also been for this purpose. With the increased costs of cytological staining procedures like protargol staining that utilize expensive reagents like silver albuminose and gold chloride smaller sampling surfaces of glass coverslips (25 mm^2) have come into greater use. Herein is reported a simple coverslip-holding device which may be used in fresh water, estuarine, and marine microhabitats.

Protocol

1. As is illustrated in Fig. 1, a 7.5 cm piece of standard 3/4 "(OD) tygon plastic tubing is notched on the ends and is incised at ~ 1 cm intervals with a single edged razor blade. There is sufficient length for 6 such incisions. Standard coverslips of No. 1 thickness, 25 mm^2 may then be inserted. For additional support, a rubber band may be fitted over the coverslip pairs and into the notched ends of the piece of tubing. Nylon twine may be used to tie one end to the tubing and the other to a suitable stake on land.

2. Samplers may be left at sampling sites for varying periods depending upon the water temperature and the relative suspected abundance of sessile protists. In the mid-atlantic states area where mid-summer water temperatures range from ~ 25 to 30°C, 2 days is sufficient time for the colonization of sessile protists.

3. To eliminate invertebrate predation enclose the tubing trap in a small sleeve covered by plankton netting.

4. The ensembles may then be placed in suitable small glass jars and taken back to the laboratory for direct microscopic observation. For detailed cytological studies, the coverslips may be transferred into Coplin coverslip jars (A. H. Thomas & Co.) containing a fixative such as Bouin's (4) for protargol silver staining.

Comments

In addition to attached protists, particularly peritrich and suctorian ciliates, other non-attached ciliates, flagellates, and amoeboid protists may be present. These may commonly include predators and other members of the protistan community attached to the glass surface.

LITERATURE CITED

1. Sladeckova, A. 1962. Limnological methods for periphyton ("aufwuchs") community. Bot. Rev. 28: 286-350.

2. Deroux, G. 1970 le serie "chlamydonelliene" chez les Chlamydodontidae (holotriches, cyptophorine Faure - Fremiet). Protistologica 6: 155-182.

3. Spoon, D. M. & Burbanck, W. D. 1967. A new method for collecting sessile ciliates in plastic petri dishes with tight-fitting lids. J. Protozool. 14: 735-739.

4. Lee, J. J. Jr, Small, E. B., Lynn, D. H., & Bovee, E. C. 1985. Introduction to protozoa and methods used to study them. In: Lee, J. J., Hutner, S. H. & Bovee, E. C. Eds. Illustrated Guide to the Protozoa. Soc. Protozool. Lawrence, Kansas pp. 1-7.

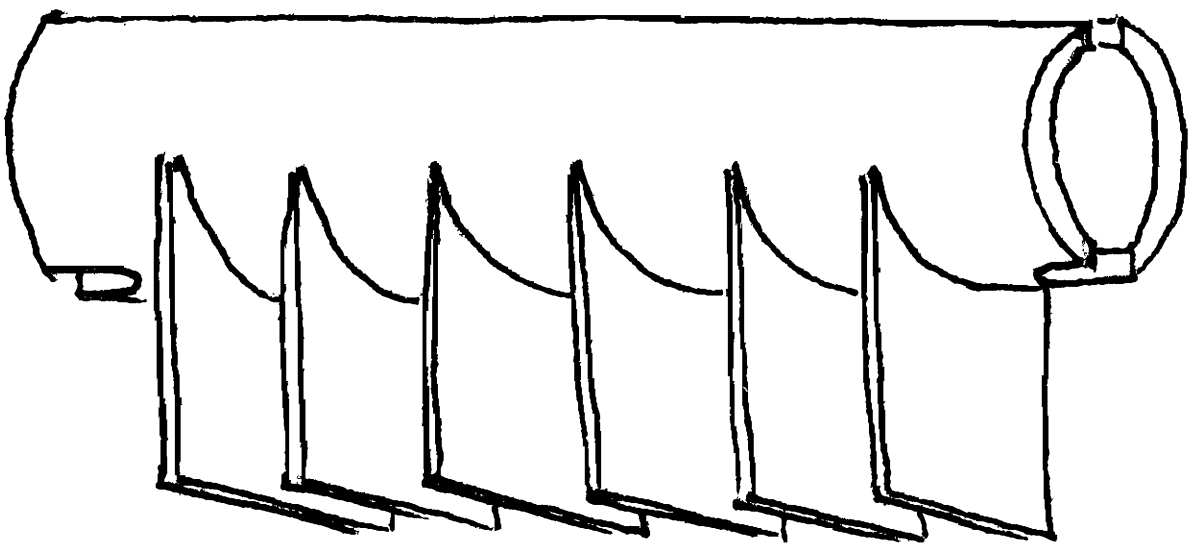

Fig. 1. Apparatus

C. FIXATION, STAINING, LIGHT AND ELECTRON MICROSCOPICAL TECHNIQUES

1. Immobilization methods for protozoa. Arthur J. Repak
2. Photomicrography. Arthur J. Repak
3. Attachment of sessile and thigmotatic protozoa to coverglasses. Bruce F. Hill
4. Protargol staining. Denis H. Lynn
5. A short procedure for portargol staining. Barry J. Wicklow & Bruce F. Hill
6. Protargol methods. Wilhelm Foissner
7. The silver carbonate methods. Wilhelm Foissner
8. The "wet" silver nitrate method. Wilhelm Foissner
9. Supravital staining with methyl green-pyronin. Wilhelm Foissner
10. Observing living ciliates. Wilhelm Foissner
11. The "dry" silver nitrate method. Wilhelm Foissner
12. Feulgen staining the nuclei of foraminifera. John J. Lee & Jan Pawlowski
13. Fixation for immunolabelling of cells and tissue for TEM. Jerome J. Paulin
14. Freeze drying of cells for scanning electron microscopy. Jerome J. Paulin
15. Rapid freezing and freeze substitution of protozoa for transmission and scanning electron microscopy. Mark A. Farmer
16. General comments on fixation of protozoa and transmission electron microcopy. Jerome J. Paulin
17. Fixation of protozoa with combinations of glutaraldehyde, formaldehyde and osmium tetroxide. Jerome J. Paulin
18. Poly-L-Lysine adhesive for cell suspension and reference to other adhesives used in scanning electronmicroscopy. Jerome J. Paulin
19. Preparation of cells for scanning electron microscopy. Jerome J. Paulin
20. Preparation of samples for scanning electron microscopy. Wilhelm Foissner
21. Ruthenium red as a stain. Jerome J. Paulin
22. Cytochemical localization of acid phosphatase (lysosomal and digestive activity marker enzyme). O. Roger Anderson

IMMOBILIZATION METHODS FOR PROTOZOA C-1.1

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INTRODUCTION

The details of living, rapidly moving protists such as protozoa are difficult to visualize without the aid of various immobilization methods. The techniques are varied, ranging from the use of physical methods of impeding movement e.g. methylcellulose (16), chemical techniques, e.g. narcotization (3,27), biological i.e. immunological methods (1) to behavioral (28). The choice is up to the investigator and may depend upon the purpose of the particular investigation at a particular moment.

PROTOCOL

I. PHYSICAL METHODS

A. AGITATION

Centrifugation at moderate speeds can slow or stop protozoa (e.g. *Paramecium caudatum* and *Amoeba proteus*) for several minutes. The same result can be achieved by vigorously hand shaking a culture in a test tube (14,15).

1. Place 3 ml of concentrated cells in 15 ml vials.
2. Shake with a mechanical agitator (e.g. modified blood pipet shaker; Fisher Scientific, Pittsburgh, PA 15219; Catalog # 13-718, or platform shaker; Fisher catalog # 14-251-200) set at 370-436 oscillations per minute with an amplitude of 4 in.

COMMENTS

Recovery varies with times of agitation. Agitations of 5 min required 1 h or longer. Periods longer than 10-20 min at 370-436 oscillations/min leads to thixotropic collapse of many organisms. Interestingly, periods of 15 sec stimulate an increase rather than a decrease in ingestion rate (15).

B. COMPRESSION

1. Cover glass method:
    a. Place organism into a small drop of culture fluid on to a glass slide. Cover with a coverslip.

b. Withdraw fluid from edge of the coverslip using filter paper until sufficient pressure is applied to stop organism.

c. Alternately allow time for evaporation of fluid from beneath the coverslip as a result of exposure to substage illumination. In time organisms will be stopped by pressure of coverslip.

COMMENTS

The length of time available for studying the living organism with this method depends upon time and the rate of evaporation of the fluid under the cover slip (11).

2. Roto-Compressor (invented by A.A. Schaeffer) [originally made by Biological Institute of Philadelphia, 250 Broad St., Philadelphia, PA] exerts a controlled vertical compression without shear force between a coverslip and a glass plate (35). See Fig 1.

3. Handiwrap™ - According to the Spoon (30) this technique can be used either for compression or counting aerobic organisms e.g *Cochliopodium, Epistylis, Heliophyra erhardi, Paramecium* sp. and *Tetrahymena.*

a. Preparation:

(1) Place thin plastic film between a thicker plastic sheet and a sheet of paper. Cut into 1 cm squares with tiny grid markings (~700µm) in the Handiwrap™ aligned properly. Film sticks to plastic sheet as a result of static electricity.

(2) Place 0.1 ml containing the organisms to be studied onto a plastic slide (RINZL™).

(3) Using one or two pair of watchmaker's forceps, remove plastic film from plastic sheet.

(4) Place film square over drop on plastic slide. Be sure to that side with square markings is on the upper surface. Caution - avoid fingers, tears or folds

(5) Rock slide from slide to side to spread material evenly under film.

(6) If the preparation is sealed with a coat of high vacuum silicone grease, water evaporation will be prevented and the preparation will be long lived (10+ days).

A bright field microscope with achromatic objectives, e.g. A.O. Microstar are recommended. Apochromatic lens show up differences in water thickness requiring constant focusing. Phase contrast and Nomarski interference phase microscopy have been employed using a drop of immersion oil on the plastic film for use with 44X and 100X objectives. To avoid oil from seeping under film use high vacuum silicone grease to seal film.

C. VISCOUS MATERIAL

1. Detain™ - [Ward's Natural Science Establishment, Inc., 5100 West Henrietta Rd., P.O.Box 92912,Rochester, NY 14692-9012; Catalog # 37 W7950]

According to the supplier, Detain™ is a 1% (w/v)aqueous solution of a polyether with a molecular weight of > 5 X 10^6 displaying high drag and elastic recoil properties along with low toxicity. The exact chemical nature of the material is a trade secret. The resin decreases protist swimming velocity without harming the cells for longer than 24 h. The material also does not inhibit cell division. It has been reported to have been tested on a variety of protists e.g.*Amoeba, Blepharisma, Bursaria, Colpidium, Chlamydomonas, Eudorina, Euglena, Euplotes, Pandorina, Paramecium, Pelomyxa, Phacus, Peridinium, Spirostomum, Stentor, Synura, Vorticella, Volvox.* This high molecular weight polymer easily degrades (monomer MW 50,000) in the presence of ultraviolet light. Detain™ also forms a thick seal if left out allowing for study of live organisms for longer than one day.

a. Preparation:
The 1% (w/v) stock solution may be used directly. The solution is also effective at concentrations of 0.25 -0.5% (w/v). To prepare further dilutions of stock mix slowly and thoroughly with an appropriate volume of distilled water or sea water using a glass or plastic stirring rod at 20°C.

b. Application: For wet mount place 2 drops on a slide and add 1 drop containing organisms in respective culture medium. Add coverslip. No mixing necessary.

2. Dialyzed Gum Arabic - (Acacia) [Aldrich Chemical Company, Inc., P.O.Box 2060, Milwaukee, WI 53201; Catalog # 26,077-0]

a. Preparation:
Mix powder to desire viscosity (range 1.084-15.2; pH 7)in distilled water. An Ostwald viscosimeter at 17°C was used to determine relative viscosities (23).
b. Application: For wet mounts place about 1µl of protists directly into 1 ml of gum arabic solution After 5 min transfer organism to 1 ml of fresh portion of viscous reagent.

Gum arabic is non-toxic to *P. caudatum* (23).

3. Methylcellulose (Methocel™) 10% (w/v). [Fisher Scientific, Pittsburgh, PA. 15219; Catalog #M352-500].

a. Preparation:
(1) Mix a 10gm methylcellulose with 50 ml of hot , not boiling, distilled water with stirring.
(2) Remove from heat and allow to stand for 20 min.
(3) Add 50 ml cool distilled water and mix thoroughly.
(4) Place at 5-10°C until solution becomes transparent. (5) Dispense into dropping bottles.

Solution is stable at room temperature. Does not pose a safety hazard. May be kept indefinitely.

b. Application:
(1) Make a ring on the surface of a glass slide.
(2) Place the medium containing the organisms into the middle of the ring.
(3) Mount a coverslip.

(4) Gradually the methylcellulose moves inward and immobilization occurs (10, 28).

The use of methylcellulose introduces some optical problems. Light is diffused by the methyl cellulose masking some of the details of the organisms.

4. 3% (w/v) Polyacrylamide hydrazide (PAAH) - Sigma Chemical Co., P.O.Box 14508, St.Louis, MO.63178; Catalog # P9905. Used as a slowing agent for ciliary beat (6, 9).

a. Preparation:
(1) The gel.
(a) Add 3 g of dry polymer to 100 ml of distilled water and stir magnetically overnight at room temperature.
(b) Buffer solution by the addition of solid MOPS salt and adjust pH as desired with 1N NaOH or 1N HCl.
(c) Autoclave final solution before use.

(2) Crosslinking Reagent
(a)Using glyoxal monohydrate (90% pure, BDH), prepare a 20% (w/v) solution.

B. Application:
(1) Add cells directly to the polymer solution over ice.
(2) Add crosslinking agent as follows:
(a) Add 0.47 ml to 9.43 ml of PAAH.
(b) Let stand over ice for 1 h.

Not tested on any protozoa to our knowledge, original work on *Streptomyces clavuligerus* (6) and marine mussels *Modiolus demissus* and *Mytilus edulis* (9). **Caution**: Highly toxic and an irritant. Can be absorbed through unbroken skin and causes central nervous system paralysis.

D. TEMPERATURE

Cold temperatures generally exert an slowing effect on the forward motion of free living protists (e.g. *P. caudatum, S. ambiguum, S. coeruleus*, (14,32,33).

1. Place contractile protozoa with fairly rigid pellicles into a drop of warmed saline gelatin [Sigma Chemical Co., P.O. Box 14508, St.Louis, MO 63178; Catalog # C1013 or C1138] (.05% NaCl in 10% (w/v) gelatin) on a warmed glass slide.
2. Stir with a warmed glass rod.
3. Place into a moist chamber, e.g. petri dish with a piece of moisten towel, in the refrigerator at 5-10°C for 2 min.
4. Remove and place for a few minutes in 10% (v/v) Ethanol (26).

Also warm temperatures (30°-40°C) have been reported to be useful to immobilize *Carchesium, Paramecium,* and *S. niger* (10,14,19).

E. PHYSICAL RESTRICTION

1. Break a coverslip into fragments.
2. With a pair of fine forceps, carefully place pieces of the coverslip onto a glass slide.
3. Place a small drop of medium containing the organisms to be studied between the fragments.
4. Mount a coverslip over the drop. The fragments will allow the study of the organisms within the small droplet.
5. The edges of the coverslip may be coated with vaseline to prevent dehydration (27).

OR

1. Place a small amount of cotton or lens paper that has been teased apart on a glass slide.
2. Add a drop of culture media containing organisms to be studied in the center of the material.
3. Mount a coverslip.

Objective is to create small "cells" surrounded by fibers in which the protists are contained (17).

F. AGAR

A technique recommended for slowing and studying ciliates by immobilizing the organisms on a layer of 1% (w/v) agar [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog # A 7049 or Agar Noble - Difco Laboratories, P.O.Box 332058, Detroit, MI 48232; Catalog # DFO142-02-7] on a glass slide.

1. Heat water to dissolve agar.
2. Pour a drop or two of warm agar over slide and allow it to cool and solidify.
3. Place small drop with ciliates on agar and mount a coverslip.

Ciliates stick to agar (4).

G. RADIATION

1. Ultraviolet

Many studies have shown that ultraviolet radiation can be utilized to immobilize a variety of protozoa ranging from flagellates such as *Astasia sp.* and *Euglena gracilis* to sarcodinids, e.g. *A. proteus* and ciliates e.g. *Blepharisma undulans, Bursaria truncatella, Colpidium colpoda, Dysteria sp., Euplotes sp., Fabrea salina, Nassula sp., P. multimicronucleatum, P. caudatum, P.bursaria, P. aurelia, Stylonychia curvata, and Tetrahymena geleii.* The intensity and time of exposure vary with the kind of protozoan and species. Flagellates required the least dosage (intensity x time), i.e. 25% as compared to that required to stop 50% of the individual *P. multimicronucleatum* in a given culture. *Amoeba* required approximately 40% dosage for immobilization. *Fabrea* was the most resistent to ultraviolet light (896% dosage) while *Euplotes* could be stopped with 7%. The wavelength

effective in immobilization ranged from approximately 250 to 300 nm (7,8).

2. alpha Radiation

In one study *Polytomella uvella* was subjected to alpha radiation at a dosage of 1/_^{2}/sec which resulted in some organisms slowing and moving erratically while others became immobilized (12,14).

3. X-Radiation

Dunaliella salina was immobilized by an X-ray dose of 1828 r with a 2 % survival rate. *Pandorina morum* was stopped by 400,000 r. Most sarcodinids were stopped by radiation ranging from 75,000-300,000 but either died or became abnormal in form. *Paramecium caudatum* was stilled by exposure to 400,000-500,000 r but died. Other ciliates were either behaviorly affected or died (14).

II. CHEMICAL METHODS

A. INORGANIC CHEMICALS

A few drops of the following solutions will generally either temporarily or permanently stop the movement of most fresh water protozoa e.g. *Blepharisma, P. caudatum, S. coerleus*:

1. Aluminum trichloride (.011 mM) - affects the forward motion of ciliates, e.g. *P. caudatum* (3).

2. Carbon dioxide

   a. Slowly add charged water (club soda) to a concentrated drop of protozoa on a microscope slide; or bubble gas from a CO_2 generator through a tube (e.g. micropipette) into the culture (4).

3. 3% (w/v) aq. cupric acetate for freshwater protists (16) e.g. *Chlamydomonas.*

4. 1% (w/v) aq. cupric sulfate for freshwater protozoa (10) e.g *Dinobryon, Synura, Uroglena, Volvox.*

5. 2.5% (w/v) $MgCl_2.6H_2O$ for freshwater organisms (22).

6. 7.5 % (w/v) magnesium chloride ($MgCl_2.6H_2O$) for marine* forms (4,22).

7. 20%(w/v) magnesium sulfate ($MgSO_4.7H_2O$) for marine* organisms (4,22).

8. 0.001 - .01% (w/v) nickel sulfate anesthetizes cilia (35) but not myonemes(13,31) e.g *Paramecium, Stentor.*

9. 0.5% KCl anesthetizes myonemes e.g. *S. coeruleus* (14).

10. 0.34-1.36 % (w/v) KH_2PO_4 is recommended as a non-toxic immobilizer for *P.caudatum, Ophryoglena mucifera, E.patella, Kahlia acrobates, S. coeruleus, V. microstoma.*Time for immobilization is roughly 10 to 20 min (21).

11. 1% (w/v) NaBr for freshwater forms. Effect not reversible, e.g. *S. coeruleus* (14).

12. 1% (w/v) NaI or KI for fresh water organisms. Effects reversible e.g. *S. coeruleus* (14).

* For marine forms, dilute salt solutions with equal volumes of sea water.

Either add drops directly to the medium containing the protozoa to be studied or to the side of the coverslip and draw the medium through with a piece of paper toweling or filter paper placed at opposite as an absorber (4,22).

B. ORGANIC CHEMICALS

The following are regarded as being narcotics. It is recommended that they be applied gently and slowly to contractile organisms while in their expanded state.

1. 10% (v/v) absolute ethanol - add a little at a time waiting for excitation to subside before adding more (22).

2. Butyn (butacaine sulfate) [Sigma Chemical Co.,P.O.Box 14508, St.Louis, MO 63178; Catalog # B0647]

a. Add 0.1% aq. solution, drop by drop to concentrated culture of organisms (4).

3. Chloral hydrate (2% trichlorohydrate acetaldehyde) [Sigma Chemical Co.,P.O.Box 14508, St.Louis, MO 63178; Catalog # C8383] (22).

a. Add crystals to water of culture or slowly add 2% aq. solution to cultures.

Caution: avoid contact with skin, mouth and eyes (4). Special permit required.

4. Chloretone (0.005-0.1% 1,1,1-trichloro-2-methyl-2-propanol) [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog # T5138] (22).

a. Add drop by drop unit the organisms are quiet.

Caution: Toxic. May kill organisms.

5. Chloroform - **not** recommended for use alone. Highly toxic.

a. To apply add slowly with dropper placed under the surface of the drop (4).

6. Clove oil [Sigma Chemical Co.,P.O.Box 14508, St.Louis, MO 63178; Catalog # C

a. Concentrate organisms in a small amount of water.

b. Place tip of pipette below surface and add 1 or 2 drops at a time (4).

7. Corri's solution (15).

96% methanol -	10 ml
chloroform -	0.15 ml
distilled water -	90 ml

8. A mixture of curare and strychnine sulfate anesthetizes myonemes but not cilia e.g. *S. coeruleus* (32).

9. N-Ethylurethane (0.3-1%) [Aldrich Chemical Co., Inc., P.O.Box 2060, Milwaukee, WI 53201; Catalog # E5,122-8]

a. Add directly to medium (22).

10. 0.25% Hydrochlorate of hydroxylamine [Aldrich Chemical Co.,Inc.,p.o.bOX 2060, Milwaukee, WI 53201; Catalog # 25,558-0] neutralized with sodium carbonate slows cilia and relaxes myonemes (14).

11. Iodoacetate [Aldrich Chemical Co., Inc., P.O.Box 2060, Milwaukee, WI 53201; Catalog # 16,054-7] inhibits flagellar movements of *Euglena gracilis* (14).

12. Menthol

a. add a few crystals to surface of water in a test tube or on a glass slide. Recommended for sessile organisms e.g. *Vorticella* (22).

13. Mercaptoethanol [Aldrich Chemical Co., Inc., P.O.Box 2060, Milwaukee, WI 53201; Catalog # M370-1] reduces amoeboid movement in *Amoeba proteus* (37).

14. Metabolic inhibitors of actomyosin-like ATP-ases stop flagellar, ciliary and pseudopodia motion. Examples: acrodinium-Cl (acriflavin), para-chloromercuri-benzoic acid (PCMB), salicyl-hydroxymercuric-methanoxypropyl-amido-orthoacetate (Salygran) (14).

15. Poly(Ethylene Oxide) (1% w/v polyox resin WSR 301) - Union Carbide Co., P.O.Box 8720, Charleston, WV 25303-0720) (31).

a. Polyox resin WSR 301- prepare fresh by slowly dissolving 1 gm of powder in 100 ml distilled water at 22°C.

b. Add 3 drops of Polyox to 1 drop of culture with protozoa.

c. Mount coverslip and set slide aside for 15 min at 22°C before observing.

Used with *B. undulans, Didinium nasutum, E. gracilis, P.aurelia, Prorodon platyodon.*

16. Polystryene(Petri dishes)[Falcon Standard dishes available from Fisher Scientific, Pittsburgh, PA 15219; Catalog # 08-757-100B].

(1) Add a few drop of concentrated cells ($5x10^5$/ml) from growth medium to about 4 ml of 10 mM Tris in a 60 X 15 mm plastic Petri dish.
(2) The addition of growth medium (e.g. proteose peptone) to the Petri dish restores cells to active motility.

It is important that cells be washed free of growth media using Tris before immobilization. Plastic polystyrene coverslips may be substituted for Petri dishes. Immobilization does not interfere with conjugation or cell division but the time for division completion is lengthened from 20 min to 1-2 h in *Tetrahymena thermophilia*. This technique allows for prolonged microscopic observation; electrophysiology, U.V. or laser microbeam irradiation, microinjection and organelle transplantation (36).

17. Polyvinyl alcohol (15% w/v) [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog # P81763] (13).

(1) Slowly add 15 g PVA to 100 ml distilled water.
(2) Place in water bath at 80°C and heat with continuous stirring until the solution becomes the consistency of thick molasses.
(3) Filter through 2 layers of cheese cloth to remove lumps.
(4) Allow solution to stand for several hours or longer while it clears to transparency.
(5) Store in capped bottles or vials.
(6) Add drop PVA to a drop of solution with ciliates for use.

18. Protamine sulfate (0.015-.05% w/v) - [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog #P4020].

Use this positively charged polyelectrolyte to coat glass slides in advance of use. Employed successfully against *Euglena, Halteria, Nyctotherus, Opalina, P. aurelia, P. caudatum, Stylocnychia, Tetrahymena.* and for intestinal ciliates of the sea urchin *Stongylocentrotus droebachiensis* (in sea water)(20).

a. Stock solution - 0.1% (w/v) protamine sulfate kept in cold. N.B. 1 ml aliquots of stock solution can be kept frozen and diluted before use.

b.Slide Preparation

(1) Clean glass slides with 95% ethanol.
(2) Place a 1.5 cm circle on one side with melted paraffin to form a shallow container in which protozoa can be restrained without compression.

(3) Fill circle with a few drops of freshly prepared protamine sulfate solution in distilled water.
(4) Allow 15 min for protamine absorption to occur.
(5) Drain off solution and rinse with medium in which protozoa are to be examined.
(6) Add a drop of concentrated protozoa and mount a cover glass.

Air dried slides can be stored in the refrigerator for up to 15 days.

19. Pyroligneous acid (Wood Distillate) [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog # W 2000]
a. vapors from which acts similarly to that of tobacco smoke but more slowly (22).

20. Rousselet's solution - 0.6% procaine HCl 10 ml
10% (v/v) aq. soln. methanol 1 ml
Must be prepared fresh. Used with *Vorticella* (26).

21. Sodium amytal (sodium 5-ethyl,5-isopentalbarbiturate) saturated solution;used with hypotrichs e.g. *Euplotes & Oxytricha* (19).

(1) Place a thin cover slide on a dissecting microscope stage.
(2) Mark with a vaseline ring 3mm in diameter.
(3) Add a few specimens to center of ring and remove excess fluid.
(4) Add one or two drops to fluid.
(5) Lower slide to vaseline.
(6) After about 20 to 30 min transfer ciliates to medium and reseal.

Organisms will remain active for 60 min or so at a reduced rate of activity.

22. Tobacco smoke - recommended as a narcotic for ciliates and flagellates. Fill a short tube with smoke. Invert over the mouth of the tube a slide upon which a drop containing the ciliates has been placed. Check under low power and remove slide as soon as desire action is achieved ($^1/_4$-1 min) (22).

III. BIOLOGICAL METHODS

Immobilization antigens have been reported in *P. aurelia, P.caudatum, P. multimicronucleatum, P. bursaria, T. pyriformis* and *Pandorina morum* (1). The antigens are water soluble proteins located in the pellicle and cilia. When cells are exposed to homologous antisera, the cilia clump, movement ceases and the cells are immobilized (18,24-26).

A. Preparation of Antisera

1. Prepare whole homogenates from 1-2 concentrates of cells.

2. Intravenous injections (each consisting of approximately 10,000 cells) given every four days for a total of injections.
3. Bleedings to be made 1 week after last injection.

B. Extraction and Purification of Antigen

1. Mixture B: 0.9% NaCl in 0.01 M Sodium phosphate @ pH 7 - 1 part
   30 % Ethanol - 1 part
2. Add volumes of mixture B to 1 volume of packed cells.
3. Extract at 2°C for 1 h.
4. Centrifuge for 3 min at 25,000 g and discard precipitate.
5. Lower pH to 2.0 by adding 0.2N HCl slowly with stirring.
6. Centrifuge and discard precipitate.
7. Raise pH to 7.0 by adding 0.2N NaOH.
8. Centrifuge and discard precipitate.
9. Add saturated ammonium sulfate to 55% saturation.
10. Centrifuge and retain precipitate.
11. Redissolve the precipitate in the desired solvent.
12. Centrifuge and retain supernatant.

Approximate yield = 1mg/ml of packed cells (18).
Apply to a concentration of organisms in a small drop on glass slide.

IV.BEHAVIORAL METHODS

I. Nigrosin [Sigma Chemical Co, P.O.Box 14508, St.Louis, MO 63178; Catalog # N 4754] - Congo Red [Sigma Chemical Co., P.O.Box 14508, St.Louis, MO 63178; Catalog # C6767] Stained Yeast (5,28).

(1) Mix 3 gm compressed yeast with 30 mg Congo Red in 10 ml distilled water plus a pinch of NaCl.
(2) Boil gently for 10 min. Allow to cool to room temperature before use.
(3)Dip glass needle in the stained yeast and transfer to drop containing protozoa, e.g. ciliate on glass slide. Drop should turn pink not red.
(4) Similarly add a small amount of Nigrosin to same drop and stir.
(5) Cover and seal.

Protozoa (e.g. *Blepharisma, Chilomonas paramecicium, Colpidium, Euglena, Paramecium, Spirostomum*) will slow as they begin to feed on the stained yeast within 10 min. Upon feeding on the Congo red stained yeast food vacuoles will form. The Bright orange (pH 5+) yeast inside the vacuoles will turn blue (pH 3 or less) to intermediate shades of purple. The nigrosin will only cover the surface of the ciliate revealing details of the pellicle, cilia and buccal structures. Flagella of flagellates will be revealed along with pellicular detail.

LITERATURE CITED

1. Allen S.L. 1967. Chemical Genetics of Protozoa, Chapter 13 *In*: Florkin, M., Scheer, B.T. and Kidder, G.W.(eds.) Chemical Zoology, Academic Press, New York. **1**: 617-69.
2. Andrejewa, E.W. 1931. Zur Frage über die physikalischchemische Bestimmung der Korrelation einger physiologischer Prozesse be Paramecium caudatum. *Arch. Protistenk.* **73**: 36-360.
3. Balamuth, W. 1966. Syllabus of Laboratory Exercises and Lecture Summaries. NSF Summer Institute in Protozoology. University of California, Berkley.
4. Behringer, M.J. 1989. Techniques and materials in Biology. 2nd ed. Krieger publishing Co., Melbourne, FL. pp. 50.
5. Buck, J.B.193. Quieting *Paramecium* for class study.*Science* **97**: 9.
6.Freeman, A & Aharonowitz, Y. 1981. Immobilization of microbial cells in crosslinked, prepolymerized, linear polyacrylamide gels: Antibiotic production by immobilized Streptomyces clavuligerus cells. *Biotecnol.Bioeng.*, **23**: 2747-2759.
7. Giese, A.C. 1967. Effects of radiation upon protozoa in Chen, T.T.(ed.) Research in Protozoology. Pergamon Press, Oxford. **2**: 292-295.
8._____. 1953. Protozoa in photobiological research. *Physiol.Zool.*,**26**: 1-22.
9. Gosselin, R.E. 1958. Influence of viscosity on metachronal rhythm of cilia. *Fed. Proc.* **17**: 372.
10. Gray, P. 1958. Handbook of Basic Microtechnique. McGraw-Hill Book Co., Inc., New York. pp.69.
11. Grimstone, A.V. & Skaer, R.J. 1972. A Guidebook to Microscopical Methods. Cambridge University Press, London. pp 13,16-17,8.
12. Holweck,F. & Lacassagne,A. 1931. Action de rayons a sur Polytoma uvella. Determination de "cibles" correspondent aux principles lésions observées. *Compt. Rend. Soc. Biol. Paris*,**107**:812-81.
13. Humason, G.L. 1967. Animal Tissue Techniques. 2nd ed. W.H.Freeman and Company, San Francisco, CA. pp.
14. Jahn, T.L & Bovee, E.C. 1967. Motile behavior of protozoa, *In*: Chen, T.T., (ed.), Research in Protozoology, Pergamon Press, New York **1**: 1-200.
15. King, R.L. & Beams, W.W. 1941. Some effects of mechanical agitation on Paramecium caudatum. *J. Morphol.* **68**: 149-159.
16. Kirby, H. 197. Methods in the Study of Protozoa. University of California Press, Berkeley, CA. pp.87.
17. Knudsen, J.W. 1966. Biological Techniques. Collecting, Preserving and Illustrating Plants and Animals. Harper & Row, Publishers, NY pp.106-107.
18. Koizumi, S. 1966. Serotypes and immobilization antigens in *Paramecium caudatum*. *J. Protozool.*,**13**:73-76.
19. MacKinnon, D.L. & Hawes, R.S.J. 1961. An Introduction to the Study of Protozoa. Clarendon Press, Oxford, England.pp.396.
20. Marsot, P. & Couillard, P.1973. The use of protamine-coated slides for immobilizing protozoa. *J. Protozool.*, **20**: 105-106.
21. Mugard, H. and Courtney, B. 1955. Paralysie des Infusories Ciliés au moyen des phosphates alcalins. *Bull. Soc. Zool. France,* **80**: 196-205.
22. Pantin, C.F.A. 1962. Notes on Microscopical Technique for Zoologists. Cambridge at the University Press, London. pp. 6-7.
23. Pigón, A. & Szarski, H. 1955. The velocity of the ciliary movement and the force of the ciliary beat in Paramecium caudatum. *Bull.Acad. Polon. Sci. Cl. II*, **3**: 99-102.
24. Preer,Jr., J.R. 1959a. Studies on the immobilization antigens of Paramecium. I. Assay methods. I *Immunol.*, **83**: 276-283.
25._____ 1959b. Studies on the immobilization antigens of Paramecium. II. Isolation. *J. Immunol.*,**83**: 378-38.
26._____ 1959c. Studies on the immobilization antigens of Paramecium. III. Properties. *J. Immunol.*,**83**: 385-391.

27. Repak, A.J. 1977. Protozoology Laboratory Manual. Quinnipiac College, Hamden, CT. pp.12.
28. Repak, A.J. & Levine, A.B. 1967. A new use for an old stain. *Turtox News*, **5**:227.
29. Stiles, K.A. & Hawkins, D.A. 197. Immobilizing paramecia for study in the introductory course. *Science,* **105**: 101-102.
30. Spoon, D.M. 1976. Use of thin, flexible plastic coverslips for microscopy, microcompression, and counting of aerobic microorganisms. *Trans. Amer. Micros. Soc.*, **95**(3): 520-523.
31. Spoon, D.M., Feise, C.O., & Sue Young, R. 1977. Poly(ethylene oxide), a new slowing agent for protozoa. *J. Protozool.*, **2**: 72-7.
32. Tartar, V. 1961. The Biology of Stentor. Pergamon Press, New York. pp. 22-23.
33. Tawada, K. & Miyamoto, H. 1973. Sensitivity of Paramecium thermotaxis to temperature change. *J. Protozool.*, **20**: 289-292.
34. Uhlig, G. 1972. Protozoa, *In*: Schlieper, C. (ed.) Research Methods in Marine Biology, University of Washington Press, Seattle, pp. 129-11.
35. von Gelei, J. 1935. Ni-Infusorien in Dienste der Forschung und des Unterrichtes. *Biol. Zentralbl.* **55**: 57-7.
36. Wolfe, J. & Colby, R.H. 1981. A method for the immobilization of living Tetrahymena. *Exp. Cell Res.*, **134**: 313-317.
37. Zimmerman, A.M. 196. The effects of mercaptoethanol upon form and movement of Amoeba proteus. *Biol. Bull.*, **127**: 538-56.

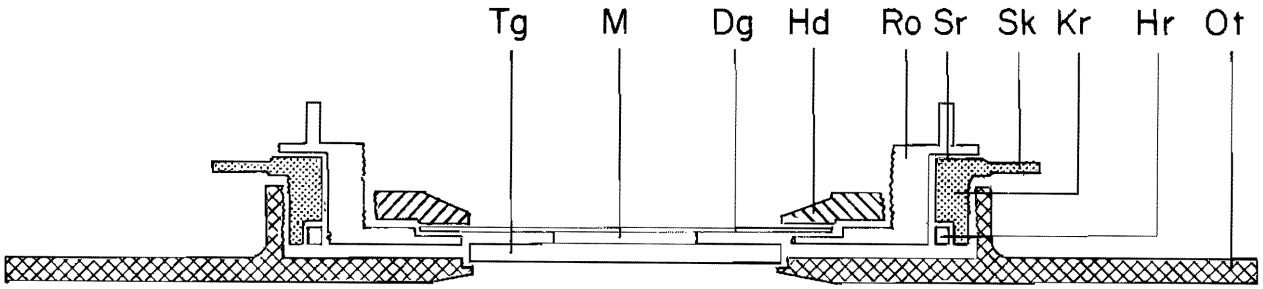

Fig.1. Roto-compressor chamber for immobilizing motile microorganisms for live observations for a number of hours. Dg = coverglass, Hd = support for coverglass, Hr = support for rotor, Kr = compressor ring, M = medium (object), Ot = slide, Ro= rotor, Sk = peg in the compressor ring, Sr = peg in rotor, Tg = supporting glass. (Manufactured originally by the Biological Institute of Philadelphia).

PHOTOMICROGRAPHY

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INTRODUCTION

Although there are many microscopes with built-in photomicrographic capabilities and expensive commercial accessaries for the purpose, financial considerations often dictate that practical alternatives be used. It is with the latter in mind that this protocol has been written. Photomicrography involves the taking of photographs through the lens of a compound microscope. The process requires the coupling of a camera, usually a 35 mm camera (8 mm and camcorders can be used) to a microscope and the effective use of illumination . To obtain satisfactory results one must deal with the quality of the equipment used (microscope, camera and film) as well as the skill of the photographer in the use of his equipment and, to a lesser extent, the dark room the darkroom phases of photomicrography. A knowledge of microscopy as well as photography is certainly a prerequisite for taking quality photomicrographs [1-5].

Any type of microscope can be utilized in making photomicrographs. The most common is "brightfield" illumination. Specimens may be dark or colored and appear against a bright, almost white background. More details and better contrast are often revealed if dark field, phase contrast, fluorescence, or interference microscopy is employed (5). Both scanning and transmission electron microscopy also employ photomicrography.

The choice of film for recording the image also depends on the objective in question. Color is good for presentations e.g. seminars and lectures. Black and white is preferred for publication and electron microscopic specimens. Whatever the purpose of a photomicrograph the recorded image is no better than the image produced in the microscope except that the contrast can be enhanced in the darkroom [3]. To obtain good photomicrographs two main problems must be solved: mounting the camera and establishing proper illumination.
Taking pictures through a microscope is actually a relatively simple, inexpensive and routine operation. Achieving correct focus at the film plane is a major concern. In contrast to a fix-focus camera, more expensive 35 mm cameras, especially a single lens- reflex (SLR), are more versatile and offer a range of shutter speeds, various distance settings, a variety of aperture settings and therefore more control of exposures.

C-2.2

PROTOCOL

Materials:

Any ordinary commercial camera or a single lens reflex camera with a removable 50 mm bayonet lens and a built-in exposure meter can be used for photomicrography.. A microscope with a trinocular body tube; binocular or monocular. An inclined ocular lens can be used if a vertical body tube is not available. Any illuminator built in to the microscope or an external illuminator can be used if the intentensity can be varied. In case these are not available the intensity can be varied by by inexpensive neutral density filters.

Methods:

A. Using a Conventional Camera or a SLR with lens Over a Microscope [3].

1. Mount camera body onto a tripod or fixed vertical stand (e.g. ring stand) with front surface of the camera lens at the eye point of the ocular lens of the microscope. See Fig.1. Mark the position of the stand so that you can return to the same spot.

2a. The position of the eyepoint can be found by holding a piece of paper right on the top of the eyepiece with the microscope focused on the subject on the stage and illuminated.

2b. A bright circle of light will appear on the paper. As you move the paper the circle becomes bigger or smaller. The position above the ocular lens at which the circle is smallest is the "eyepoint". The distance of this eyepoint varies with different ocular lens.

3. Set the distance scale of the camera at infinity.

4. Focus the image with the adjustment controls of the microscope.

5. To exclude light between the ocular lens and the camera lens, a piece of black cloth, tape or front-to-front mating sun shades can be used. Place one shade on the camera and the other on the microscope.

6. When image is sharp replace camera in position for exposure. Its recommended that the camera stand have some kind of positioning stop to bring the camera back to the same position each time.

COMMENTS

A problem associated with this kind of photomicrography is vignetting, i.e. if the camera shutter is too far above the eyepoint, a silhouette image of the shutter blade may be recorded as they open, particularly with fast shutter speeds (>1/60 sec)[2]. Because shutter speed is usually very short in simple, fixed focus cameras, the image in the microscope and the light source are very bright.

B. Using a Camera with Interchangeable Lens [3].

1. Remove lens of camera and replace with one or more extension tubes.

2. Fit a microscope adapter ring (an inexpensive accessary) with the microscope

ocular lens and then fasten to the front extension tube. Place onto the microscope fitting the ocular lens and adapter ring directly into the drawtube of the microscope. A rigid stand can be used to attach the assembly and independently support it.

3. View the image through the camera view finder.
4. At lower magnifications (<80X) focus image using the microscope focus knobs. At magnifications greater than 80X use the eye lens of an accessory photographic eyepiece if available. The accessory side telescope includes a beam splitter allows accurate focusing on the film plane. Critical focus can not be obtained on the ground screen of a camera view finder above 80X.

C. Illumination [2,3].

Good illumination is absolutely vital in photomicrography. If a microscope does not have built-in illumination, separate illuminators are available from various manufacturers. Whatever the light source it should provide sufficient intensity of light to allow reasonably short photographic exposure times.The lamp housing should allow easy access to the light source and contain those elements necessary for proper adjustment of the illumination furnished to the microscope. Heat should also be dissipated efficiently. Color film exposure requires that the light source have proper color temperature or allow suitable filtration to meet the requirements of the film. A source with a continuous visible spectrum is necessary. Most common light sources meet this requirement.

The most common system of illumination of a microscopic specimen is critical illumination. For photomicrography is Kohler illumination is best. In critical illumination, the image superimposed on the object is that of the light source itself(an image of the glowing filaments of the electric bulb). Kohler illumination allows a precise control of the light path. This preferred method of illumination involves the use of the field (lamp) diaphragm and microscope condenser to focus an image of the light source at the object plane. This provides a uniformly illuminated field.

1. Align the microscope illuminator with the plane mirror of the microscope so that an image of the spiral lamp filaments is focused on the substage iris with the aid of the field condenser.
    (a) First focus the filament of the lamp on a piece of paper laid on the microscope mirror.
    (b) Move the paper up until it rests against the underside of the substage condenser.
    (c) Adjust the focus with the field condenser until the filaments are again sharp. A small, hand held mirror will allow one to see clearly what is going on underneath the stage.
    (d) Tilt the lamp and move backward and forward with the mirror again tilted until the image of the filament, when focused twice, is centered both on the mirror and the substage.
2. Rack the condenser up to the top.
3. Remove an eyepiece from its tube.

4. Keep adjusting the mirror until the back of the objective is filled with light. It is advisable to dim the light by using a neutral-density filter.
5. Replace the eyepiece and focus the specimen with a 10X objective.
6. Close the iris diaphragm of the illuminator (field stop) almost completely and move its blurred image on the specimen into the center of the field of view by rotating and tilting the mirror. (For microscopes with built-in illuminators omit steps 1-5, centering is not accomplished with the aid of a mirror but by the centering screws of the condenser).
7. Focus the edge of the diaphragm image by slightly lowering the condenser. Both the specimen and the field stop should now be sharply defined.
8. Open the field stop until the entire field of view is just clear.
9. Adjust the lamp socket or condenser slightly until the field of view is illuminated.
10. Open the condenser iris completely and then closed it only far enough to eliminate glare in the most important image elements and render them with satisfactory contrast.(Do not use the iris diaphragm to reduce light intensity).
11. When using the low-power objectives, the front lens of the condenser (when present) may have to be swung out to illuminate the field fully. Open the condenser iris fully.
12. Use the lamp diaphragm for controlling contrast.
13. Remove the ocular lens and look down the barrel from at least 10 inches away.
14. Open or close the substage iris until approximately the outer tenth of the back lens of the objective is cut off.

COMMENTS

No further adjustment is necessary for this particular lens. Each lens used should be afforded the same adjustments. If oil immersion lens are used, the substage iris is opened to its fullest extent. Nothing else need be done since the maximum aperture of the substage is normally matched to the oil-immersion objective.

Since each glass slide varies in thickness, each time a different specimen is used start over with the 10 X objective and refocus the substage condenser to produce a sharp image of the field iris. See Fig. 2.

D. Exposure [2-4]

Exposure time can be determined by making an exposure test series or by using a sensitive exposure meter. Most quality 35 mm cameras today, as well as automatic photomicrography equipment, have built-in exposure meters, therefore eliminating the extra step of running an exposure series and keeping precision records of shutter speeds,etc. Light meters sensitive to low light levels are highly recommended because image brightness, particularly at higher magnifications, in photomicrography can be quite reduced.

1. Exposure Test Series

a. Set up a detailed record of all the shutter speeds available for your camera. Exposure times may range from a minimum of 1/1000 sec to 8 sec or more. Also include the specimen used, light source, filters, etc. This will allow for similar duplications in the future with similar specimens.

b. Run the exposure set in duplicate (i.e. two frames per shutter speed) so as to allow for mechanical problems or human error.

c. Following development of the film, one set of frames should be correct for the particular film and existing image brightness.

E. Film

This is an era of keen competition and rapid film development. A number of recently developed films have replaced the old photomicrographic standby black and white film, Kodak Panatomic-X, which is no longer manufactured. The replacements, available now, have outstanding qualities for photomicrography. Kodak T Max 100 and Kodak Pan professional 2415 are two excellent choices. The former, when developed with Kodak T Max developer, Kodak Microdol-X, Kodak HC-110 (dilution B),Kodak D-76, Rodinol, and several other developers, gives excellent quality negatives with a wide tonal range. Kodak Pan Professional 2415, when exposed at an ASA of 100 and developed with Kodak HC-110 (dilution B) produces very snappy contrasting negatives which are excellent for publications. Contrast can be raised by developing the film in Kodak D-19 and the range of contrast can be expanded by developing the film in Kodak Technidol, Kodak Microdol-X, Kodak D-76 and Rodinol. An electronic flash is recommended for moving protozoa, but sometimes good quality images can be captured on Kodak Tri-X Pan, Kodak T-Max 400, and Kodak T-Max P3200 Professional film (EI 3200) pushed to an ASA of 1000.

A wide choice of newly developed color films is now on the market . One of the best color print films is Kodak Ektar 25. It is a slow film but it has superb sharpness due to its exceedingly fine grain. Kodak Ektapress Pro 100 is a good second choice. It has a slight edge in sharpness over Kodacolor Gold 100, a film which is very widely distributed due to its popularity with photographers. Fujicolor super HR11 100 has a long shelf life and latent image retention, qualities which can be important to the occasional photomicrographer. Fuji Reala is an interesting and useful film which reproduces colors as you see them even under the artificial lighting and color temperature changes found in microscopes as you change light intensity. Agfacolor XRC 100 takes sharp, well saturated pictures with pleasing contrast and it tolerates a fairly wide range of under or over exposures. Automatic color print processing machines sometimes produce strange photomicrographic prints. The machines may be set to scan for flesh tones. It is important to let the processor know that there are no flesh tones in your photomicrographs. Kodachrome 200, Agfachrome 100 RS Professional, and Fujichrome 50

Professional, are new improved sharp, fine grained, vivid color slide films which are excellent for photomicrography.

LITERATURE CITED

1. Culling, C.F.A. 1974. Modern Microscopy: Elementary Theory and Practice. Butterworth Inc., Woburn, MA. pp.107-124.
2. Eastman Kodak Publication. 1985. Photography in Your Science Fair Project. Eastman Kodak Co., Kodak Publication No.AT-20. Rochester, NY. pp.16-18.
3. Eastman Kodak Publication. 1974. Photography through the Microscope. 6th ed. Eastman Kodak Co., Kodak Publication No.P-2. Rochester, NY. pp. 76.
4. Eastman Kodak Publication. 1970. Cinephotomicrography. Publication No. N-2. Rochester, NY pp. 40.
5. Smith, R.F. 1990. Microscopy and Photomicrography. Appleton-Century-Crofts, New York. pp.61-74.

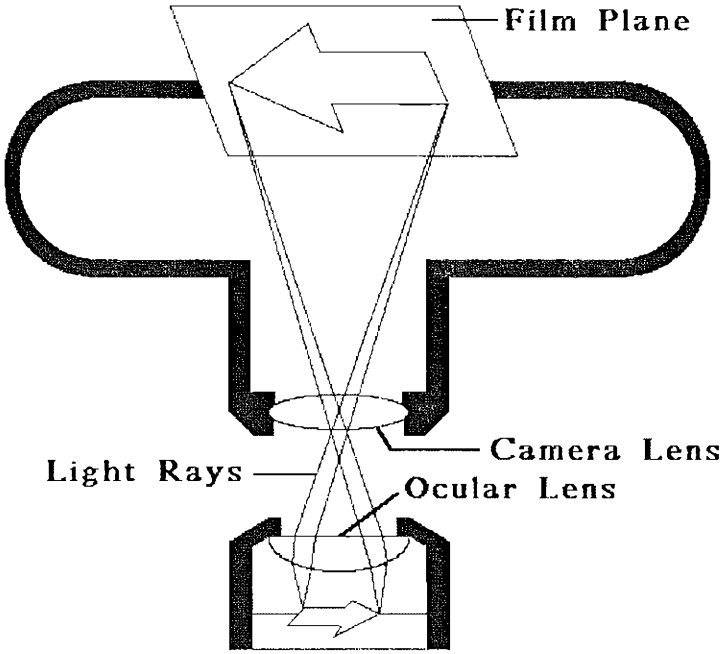

Figure 1. A camera with lens in place mounted over a microscope ocular.

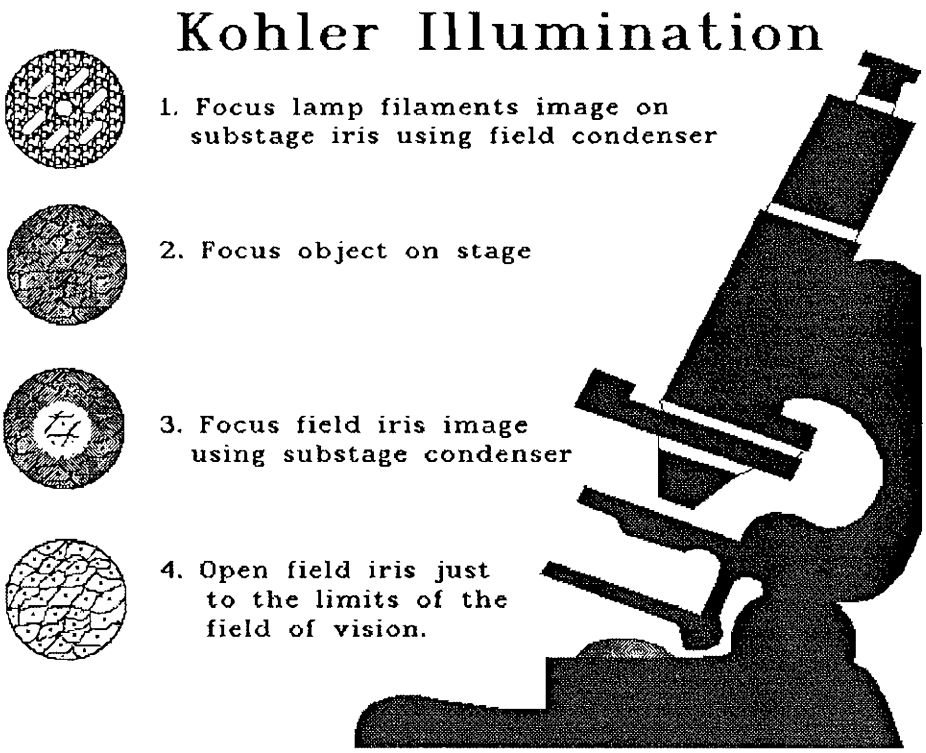

Figure 2. Steps in setting up Köhler illumination.

ATTACHMENT OF SESSILE AND THIGMOTATIC PROTOZOA TO COVERGLASSES

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INTRODUCTION

One of the greatest obstacles to the study of sessile or thigmotatic protozoa is difficulty in collecting enough organisms to undertake a complete study ot their morphology and morphogenesis. Present here is a procedure which minimizes this problem by allowing for the adhesion of sessile and thigmotatic protozoa directly onto coverglasses.

BASIC PROTOCOL

1. For sessile organisms, place clean coverglasses directly into a stock culture and allow several days for the organisms to attach.
   For thigmotatic organisms, carefully lower clean coverglasses onto the scum of stock cultures.

2. Lift the coverglasses from the medium with a minimum of culture fluid and place them organism-side up on paper toweling.

3. Pipet a drop of modified Perenyi's fluid onto the coverglass from a height of 2-3 cm.

Modified Perenyi's fluid	1% chromic acid	3.0 mL
	10% nitric acid	8.0 mL
	95% ethanol	0.5 mL
	t-butanol	5.5 mL
	distilled H_2O	3.0 mL

4. After 5 sec, flood the coverglass with additional drops of the modified Perenyi's fluid.

5. After 1 min, drain off excess fluid and place coverglasses back to back in formal alcohol (15 min).

6. Follow a normal Protargol staining procedure from this point
(i.e."A short procedure for Protargol staining" Wicklow & Hill this volume.)

C-3.2

COMMENTS

This procedure has given good results for sessile protozoa such as Vorticella and Heliophrya and thigmotatic species such as Aspidisca and Stephanopogon.

PROTARGOL STAINING

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INTRODUCTION

Protargol or silver proteinate staining methods have proved to be important procedures for revealing the cellular structures of a variety of protozoa, especially the flagellated and ciliated forms. Since Kirby's (2) description of its application to parasitic flagellates, it has been redescribed and modified many times (see Foissner [1] for a recent review). Protargol provides permanent stains of a variety of cellular structures: nuclei, extrusomes, and microtubular and microfilamentous constituents of cells (1, 6). Thus, it is extremely useful in the description of the subcellular structure of protozoa.

Three procedures will be described: (1) the "filter" procedure; (2) the "albumenized coverslip/smear" procedure; and (3) the "single cell" procedure. Each has its merits and applications.

1.Fixatives:

a) Standard Bouin's Fluid

Use this fixative when smears, single cells or cell cultures are to be fixed.
Buffer formalin by addition of excess calcium carbonate to a large volume. Agitate over several days prior to use in the fixative. Filter prior to use.
If poor fixation results, try adding the glacial acetic acid just prior to use.

Picric Acid, saturated aqueous solution	75	ml
Buffered Formalin (about 37% formaldehyde)	25	ml
Glacial Acetic Acid	5	ml

b)Concentrated Bouin's Fluid

Use this fixative when bulk samples of water are being fixed.

Saturate buffered formalin with moist picric acid crystals. Agitate over several days and add more picric acid crystals to saturate. Filter.

Dispense the picric acid-formaldehyde solution into the sample containers so that the volume of fixative to sample ranges from 1:10 for open ocean waters to 1:19 for brackish and fresh waters.

Add Glacial Acetic Acid to the sample containers, just prior to use, so that its final concentration will be 1%.

2. Solutions for Staining Procedure

It is generally best to use fresh solutions for each staining period. Store the stock solutions in the dark in a refrigerator. This ensures reproducibility of results once appropriate times have been determined and permits one to resolve problems with staining by varying only the times in various steps.

For coverslips, Columbia staining jars are used and are available only from A.H. Thomas, Inc., P.O. Box 779, Philadelphia, PA, U.S.A. 19105-0779. Unless stated otherwise, all reagents are placed in these jars from the stock solutions.

0.5% (w/v) Parlodion Solution

Dissolve a Parlodion strip in the appropriate volume of absolute methanol and store in tightly stoppered bottle.

a)Bleach

Any commercial hypochlorite bleach diluted 1:10 to 1:20 with distilled water. This dilution will vary depending upon the "opacity" of the species being stained.

0.5% (w/v) Potassium Permanganate

Make up stock solution by dissolving 0.5 g potassium permanganate ($KMnO_4$) in 100 ml distilled water.

5% (w/v) and 2% (w/v) Oxalic Acid

Make up 5% stock by dissolving 5 g oxalic acid in 100 ml distilled water and 2% stock by dissolving 2 g oxalic acid in 100 ml distilled water.

b)Protargol

Flame five pieces of coverslip-sized copper sheet or 0.5 g coiled, thin copper wire and quickly dip in 95% alcohol while still red hot and then transfer to a Columbia staining dish filled with 10 ml distilled water. Sprinkle 0.5 - 3% (w/v) protargol powder on the surface of the water and allow to dissolve without stirring. Adjusting the pH of the solution to about 8.8 using 0.1N HCl or 0.1N NaOH may improve staining, but is not absolutely essential. Prepare approximately 2-3 h before use.

c) Hydroquinone

Dissolve the chemicals in the following order in the distilled water.

Sodium carbonate, anhydrous	4	g
Sodium sulfite	5	g
Hydroquinone	1	g
Distilled water	100	ml

d)1% (w/v) Gold Chloride

Dissolve 1 g gold chloride in 100 ml distilled water. If this is too expensive, purchase at least 0.1 g gold chloride to provide 10 ml solution. This solution may be saved and reused many times. This step may be omitted although the final stains often do not look as fine.

e)5% (w/v) Sodium Thiosulfate

Make a 5% stock solution by dissolving 5 g sodium thiosulfate ($Na_2S_2O_3$) in 100 ml distilled water.

3. The "filter" procedure (after Montagnes and Lynn [4])

This procedure can be used to stain any small protists that are not easy to handle as single cells. It has been especially useful for handling samples of water that have been bulk-fixed for ecological research (Montagnes and Lynn [4]). A variety of protists has been stained. However, the filters do interfere with the maximum resolution obtained in the light microscope.

C-4.4

a)Fixation

For single cells or cultures, cells should be concentrated by centrifugation or some other means and rinsed in buffer or spring water prior to fixation in Bouin's fluid. For bulk water samples, proceed as described above (see Fixatives) using the appropriate dilution of fixative to bulk water sample. Water samples have been successfully stained after Lugol's fixation provided that the appropriate amount of concentrated Bouin's fluid is added to these Lugol's fixed samples at least 24 h prior to staining.

Samples should remain in fixative at least overnight.

b)Preparation of filter

1. Place the Bouin-fixed sample in a Millipore or similar filter holder with a fritted glass base for supporting filters. Use two 22 mm filters of mixed esters of cellulose: one filter for sample and the other as a backing filter. Use gridded filters if subsampling is desired. The pore size can be from 0.8 - 3.0µm depending upon the organisms being stained. Apply suction at no more than 100 mm Hg.
2. Suck sample from the column until it is just empty. Do not allow to go dry as the cells may rupture.
3. Remove the filter and place it, residue up, on a piece of warm glass (~ 50° C).
4. Immediately place a drop of liquid agar (2.5% (w/v)) on a 22 mm coverslip. Invert the coverslip, and keeping it horizontal, lower it gently but quickly onto the filter. Observe the residue to ensure that it is not resuspended. If resuspension occurs, allow the filters to dry longer on the heated glass prior to embedding. NOTE: The agar should be maintained in a liquid state in a water bath (~ 60° C).
5. Immediately apply pressure to the coverslip to create a thin layer of agar over the surface of the filter. Allow the agar to solidify (~ 3 - 7 min).
6. Trim two opposing sides of the filter that extend past the coverslip and peel off the filter.
7. Place filters as they are processed back to back in a Columbia staining jar containing tap water. Change the water several times until the water and filters are almost colorless.

The filters are now ready for staining.

c)Staining procedure

1. Transfer the filters to a 0.5% (w/v) solution of potassium permanganate for 5 min.
2. Wash in tap water until the excess permanganate is removed. Protists should be brown and the water clear after ~ 10 min. NOTE: For washes, unless stated otherwise, transfer the filters to a Columbia jar filled with tap water and completely immerse the jar in a constantly flushed water bath (~ 18-20° C).
3. Place filters in 5% (w/v) oxalic acid for 5 min. Change oxalic acid after every use.

4. Wash and drain, as in step 2, for 10 min.
5. Rinse in distilled water.
6. Place in 0.5-3.0% (w/v) protargol solution. Increase the concentration of protargol if the cells are understained. Leave the filters in protargol overnight (i.e., 12 - 24 h).
7. Remove filters from the protargol without draining and transfer them to hydroquinone solution for 5-10 min. Increase the duration of this step to darken the stain. When the stock solution of hydroquinone turns brown, discard.
8. Rinse through 5 changes of distilled water (~ 1 min each).
9. Place filters in 0.5% (w/v) gold chloride. The time in gold chloride varies with the cells being stained and may be seconds to minutes. A suggested initial time is 15 sec. The gold chloride solution may be used repeatedly and should be stored in the dark.
10. Place filters in 2% (w/v) oxalic acid for ~ 2 min. Over-exposure at this stage may hydrolyze structures and fade the stain. However, under-exposure may result in poor toning. Replace oxalic acid after each run.
11. Wash as in step 2 for 5 min.
12. Place filters in 5% (w/v) sodium thiosulfate for 5 min. Replace solution after each run.
13. Wash as in step 2 for 5 min.
14. Dehydrate in isopropanol (30-50-70-95-100-100-100%) for 5 min in each step. Replace the 100% alcohols every run and periodically change the others.
15. Transfer filters through three 100% xylene rinses for 5 min each. Change the xylene so that the final rinse is always new. If xylenes contain water, the filters may become opaque after mounting and hardening. CAUTION: The filters become very flexible at this stage. Be careful!
16. Place the filters in 20% xylene: 80% Permount solution for a minimum of 0.5 h. This solution may be reused.
17. Mount each filter with Permount on a slide by placing drops below and on the filter, then cover with a 22 X 40 mm coverslip. Ensure that no air is trapped under the filter or coverslip.
18. If filters become opaque, the Permount has not completely embedded the filter. To salvage the filter, soak the slide in xylene until the filter can be removed and repeat from 100% isopropanol in step 14.

4. The "albumenized coverslip/smear" procedure (after Lee et al. [3])

This procedure can be used for staining of any protists that are not easy to handle as single cells. If resolution is a concern, this is the procedure to use rather than the "filter" procedure (see above).

a)Fixation.
For single cells or cell cultures, the cells should be concentrated by centrifugation or by

micropipette (i.e., a finely drawn glass Pasteur pipette) and fixed in fresh fixative prior to embedding in albumen as described below. For protists that are found in blood or mucous secretions, a thin smear can be made on a slide or coverslip. This smear can be air-dried and then fixed in the case of flagellates or fixed immediately by placing smear-side down on fresh fixative and leaving for 10-15 min. Proceed directly to staining procedure after washing the fixed smears in several changes of water.

b)Preparation of albumenized coverslip.

1. Gently wash cells in tap water until most of the fixative is diluted out and the solution and cells are colorless. This may be carried out by recentrifuging the cells after each wash. If cell numbers are very small, a micropipette and spot depression should be used to transfer the cells from wash to wash.
2. Dehydrate in a graded isopropanol series (15-30-50-70-85%) for 5 min in each change. Alcohols should be freshly made up in distilled water just prior to the time of enrobement.
3. On a 22-mm square coverslip, place a very small drop (~5 mm diameter maximum) of Mayer's albumen (1 part glycerol: 1 part egg white; see Foissner [1] for one method for making this) and spread out with finger. Use only a small drop, as larger amounts of albumen will peel off more easily in subsequent steps. If the Mayer's albumen becomes cloudy, discard it and make up a new batch.
4. Allow albumen to dry until sticky (~1-5 min).
5. With a micropipette, add a concentrated drop of protists from 85% isopropanol to albumenized coverslip.
6. Allow most of the alcohol to evaporate but not enough to permit the desiccation of the cells.
7. Place a few drops of formol alcohol (3 parts 10% formalin: 1 part 95% ethanol) on each coverslip. Add drops slowly over center from a height of a few cm, allowing the formol alchohol to slowly spread over the coverslip. Allow to stand 3-4 min or until albumen turns opaque white. Quickly drain and carefully flood coverslip with 95% isopropanol.
8. Put 2 coverslips back to back and place in Columbia staining jar, filled with 95% isopropanol for 15 min.
9. Carefully drain coverslips by holding vertically in contact with absorbent paper so the liquid between coverslip pairs is also drained. Place the pairs in 2 changes of 100% isopropanol for a minimum of 5 min in each change.

   Carefully drain as described above for this and all subsequent steps in which solutions are changed.
10. Place in absolute methanol for 5 min. Drain.
11. Dip, with a deliberate and even movement without stopping, each coverslip pair into 0.5% (w/v) Parlodion solution. Drain excess Parlodion using absorbent paper.
12. With fine forceps, hold the coverslip pair in air until they just become hazy white. Do not dry completely!
13. Place in 70% isopropanol for 5 min. Drain.

14. Rehydrate through 50-30-15% isopropanol for 5 min in each. Drain at each step.
15. Wash in tap water 3 times for 30 sec in each wash. Best results are obtained when tap water is slightly alkaline.

c)Staining procedure.

Proceed from here to the first step (Step 1) in the protargol staining procedure for filters detailed above. Whenever filter is mentioned, treat the coverslip as a filter. Proceed until Step 15 after which the coverslips may be mounted on slides in Permount or any other mounting medium.

5. The "single cell" procedure (after Wilbert [5] and personal communication)

This procedure is best reserved for use with large cells or small numbers of very precious cells that may not be collected easily again. Small embryological staining dishes are ideal to use with this method. All steps are carried out under a dissecting or compound microscope.

a)Fixation

Fix cells by dropping them in Bouin's fixative and leaving for at least 10-15 min.

b)Staining proceedure

NOTE: Add solutions in all steps below by using a micropipette to deliver a small amount of the solution in the neighbourhood of the cells. Remove solutions by first withdrawing fluid from nearby the cells until most of the fluid has been removed. It is usually not advisable to transfer the cells from one solution to another because they may be lost during the pipetting.

1. Rinse with distilled water until the yellow color due to the Bouin's fluid has been removed.
2. Add diluted bleach and watch closely until the opacity of the cells approaches how they would appear when alive. If the bleach acts too quickly, discard these cells, dilute the bleach (e.g., from 1:10 to 1:20), and try again.
3. Rinse thoroughly with distilled water.
4. Add a pinch of protargol powder to the surface of 1-2 ml water in the embyrological dish and transfer the dish to a 40-60 C water bath for at least 1 h. This solution should be a "weak tea" color.
5. Add hydroquinone solution at the same temperature as the protargol and let develop. Examine the cells carefully at this stage. If necessary, transfer some to a slide and examine with the compound microscope to determine how the development is progressing.
6. When desired staining has been obtained, rinse with distilled water until clear.
7. Fix in 5% sodium thiosulphate solution for 5 min.
8. Rinse thoroughly with distilled water.

9. Add to previously dried albumenized coverslip or slide and then allow to dry completely.
10. Transfer to 80%-90%-100%-100%-100% ethanols for 5 min in each stage.
11. Transfer through three 100% xylenes rinses for 5 min in each.
12. Mount using Permount or other mounting medium.

LITERATURE CITED

1. Foissner, W. 1991 Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. *Eur. J. Protistol.* **27**:313-330
2. Kirby, H. 1945 The structure of the common intestinal trichomonad of man. *J. Parasitol.* **31**:163-174.
3. Lee, J J, Small, E B, Lynn, D H, & Bovee, E C. 1985. Some techniques for collecting, cultivating and observing protozoa. *In* Lee, J J, Hutner S H, & Bovee E C (eds.) *An Illustrated Guide to the Protozoa*, Society of Protozoologists, Allen Press, Lawrence, KS. pp. 1-7.
4. Montagnes, D J S, & Lynn D H. 1987 A quantitative protargol stain (QPS) for ciliates: method description and test of its quantitative nature. *Mar. Microb. Food Webs* **2**:83-93.
5. Wilbert, N. 1983 Eine neue Imprägnation der Basalkörper bei Wimpertieren.*Microkosmos* **7**: 193-197.
6. Zagon, I S. 1970 *Carchesium polypinum*: cytostructure after protargol silver deposition. *Trans. Amer. Microsc. Soc.* **89**:450-468.

C-5.1

A SHORT PROCEDURE FOR PROTARGOL STAINING

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INTRODUCTION

The Protargol staining procedure is a fundamental cytological method used in morphostatic and morphogenetic studies of protists. It is particularly useful in staining microtubular organelles. Most published methods however are lengthy and the results highly variable particularly when staining a mixed population of protozoa. Presented here is a short procedure (2-3 hours), modified from several sources [1-7] that gives consistently good results.

BASIC PROTOCOL

1. **Harvest and concentrate cells:** (e.g. centrifugation, filtration, Uhlig separation).

2. **Fix:** fix cells in Perenyi's solution (1-4 min).

Perenyi's fluid:	1% chromic acid	1.5 mL
	10% nitric acid	4.0 mL
	95% ethanol	3.0 mL
	distilled H_2O	1.5 mL

(for sensitive marine species add 0.3 g NaCl per 10mL fixative)

3. **Rinse and dehydration**: resuspend cells in 95% (3X) then 100% ethanol.

4. **Mount**: add 1 drop of concentrated cells to albuminized coverglass; allow most of the alcohol to evaporate being careful not to allow the cells to dry; add one drop of 100% ethanol. Stabilize cells and albumin by slowly adding formal alcohol to corners of coverglass until formal alcohol replaces ethanol and floods cover glass (2-3 min).

Formal alcohol: 10% aqueous formaldehyde 3 parts
95% ethanol 1 part

5. **Stabilize**: place cover glasses back to back in formal alcohol (15 min).

6. **Dehydrate:** 95%, 100% isopropanol (2 min each), then 100% methanol (3 min).

7. **Collodion coat:** dip briefly (1-2 sec) in 1% collodion in 100% methanol; allow to air dry briefly (5-10 sec). The number of coats of collodion can be varied for different cells - try

processing half cover glasses with 1 coat, half with 2 coats; in general thicker coats yield darker staining.

8. **Rehydrate**: 95%, 70%, 30% isopropanol (2 min each); DW (3X 2 min each).

9. **Oxidize**: 0.5% MnO_4 (3 min).

10. **Rinse**: DW (3X 3 min each).

11. **Bleach**: 5% Oxalic acid (5 min).
(prepare Protargol solution at this time, see step 13)

12. **Rinse**: DW several changes (total of 10 min).
(its important to remove all oxalic acid; I blot the edge of each coverglass pair between rinses)

13. **Protargol impregnation**: 1% Protargol, 20 min at 70° C.
Sprinkle 0.1 g Protargol on 10 ml DW (pH 5.0-6.0) in Columbia staining jar (don't mix); place Columbia jar in water bath (70° C). After the Protargol dissolves, add coverglasses.

14. **Develop**: transfer directly to developer (1% hydroguinone, 5% Na_2SO_3) (3-5 sec). I dip a coverglass pair in developer then observe using dissection microscope; when key structures (e.g. cirri, membranelles) darken, transfer to DW.

15. **Rinse**: DW (2 min).

16. **Reduce**: 2% oxalic acid (2-5 min).

17. **Rinse**: DW (2 min).

18. **Fix**: 5% sodium thiosulfate (5 min).

19. **Rinse**: DW (2 min).

20. **Dehydrate**: 30%, 70%, 95%, 100% (2x) isopropanol (2 min each); 100% methanol (3 min).

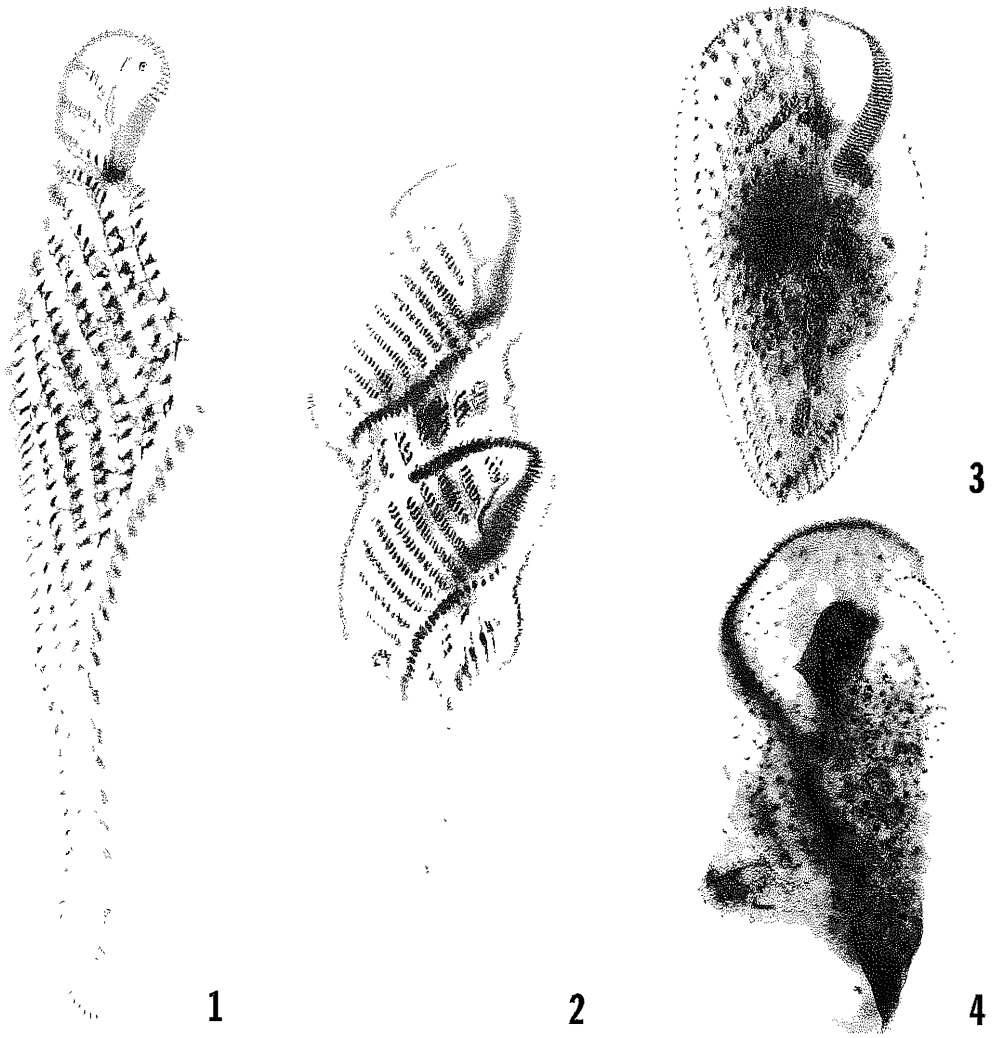
21. **Clear and mount**: Hemo-sol clearing agent (2 changes 5 min each); permount

COMMENTS

This procedure stains microtubular structures such as microtubular ribbons and bundles, kinetosomes, and ciliary structures such as cirri and membranelles. The macronucleus and micronuclei are also stained. Additional structures including extrusomes and euplotine interalveolar septa are also sometimes stained. The method was developed for hypotrichs but also gives excellent results for a wide range of fresh and marine psammolittoral species of protozoa.

LITERATURE CITED

1. Aufderheide, K. J. 1982. An improvement in the Protargol technique of Ng and Nelsen. Trans. Amer. Microsc. Soc., **101**: 100-104.
2. Dragesco, J. 1962. L'orientation actuelle de la systématique des ciliés et la technique d'imprégnation au protéinate d'argent. Bull. Micros. Appl., (ser. 2), **12**:49-58.
3. Hill, B. F. 1981. The cortical morphogenetic cycle associated with cell division in Diophrys Dujardin, 1841 (Ciliophora, Hypotrichida). J. Protozool., **28**: 215-221.
4. Jerka-Dziadosz, M. & Frankel, J. 1969. An analysis of the formation of ciliary primordia in the hypotrich ciliate Urostyla weissei. J. Protozool., **16**: 612-637.
5. Montagnes, D. J. & Lynn, D. H. 1987. A quantitative protargol stain (QPS) for ciliates: method description and test of its quantitative nature. Mar. Microb. Food Webs, **2**: 83-93.
6. Ng, S. F. & Nelsen, E. M. 1977. The Protargol staining technique: an improved version for Tetrahymena pyriformis. Trans. Amer. Microsc. Soc., **96**: 369-376.
7. Lee, J. J., Small, E. B., Lynn, D. H. & Bovee, E. C. 1985. Some techniques for collecting, cultivating and observing protozoa. In: Lee, J. J., Hunter, S. H. & Bovee, E. C. (eds.), An Illustrated Guide to the Protozoa. Society of Protozoologists and Allen Press, Lawrence, Kansas, pp. 1-7.
8. Tuffrau, M. 1967. Perfectionnements et pratique de la technique d'imprégnation au Protargol des infusoires ciliés. Protistologia, **3**: 91-98.



FIGURES

Figs. 1-4. Examples of cells stained using the short Protargol staining procedure. Fig. 1. Ventral view of the marine hypotrich *Epiclintes ambiguus* showing cirral bases and associated microtubular bundles. x700. Fig. 2. *E. ambiguus* showing cortical development in late cell division. x675. Fig. 3. Ventral view of the freshwater hypotrich *Onychodromus quadricornutus* showing cirri, cirral bases, and nuclei. x275. Fig. 4. Dorsal view of *O. quadricornutus* showing microtubule supported spines and dorsal bristles. x300.

PROTARGOL METHODS

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INTRODUCTION

Protargol methods are indispensable for descriptive research of ciliates. The first procedures were provided by Kirby (4), Moskowitz (7), Dragesco (2) and Tuffrau (9, 10) and many more modifications were subsequently proposed (1, 5, 6, 8, 11, 12, 13). Here, the three variations which produce good results in our laboratory are described. These procedures work well with most ciliate species (some, however, only rarely impregnate well, e. g. *Loxodes*, *Paramecium*) but require at least 20 specimens. Contrary to the silver carbonate method, a single specimen cannot usually be handled successfully. Depending on the procedure used, protargol can reveal many cortical and internal structures, such as basal bodies, cilia, various fibrillar systems, nuclear apparatus. The silverlines, however, do never impregnate. The shape of the cells is usually well preserved in permanent slides, which is an advantage for the investigation but makes photographic documentation more difficult. However, pictures as clear as those taken from wet silver carbonate impregnations can be obtained with the Wilbert modification if the cells are photographed prior to embedding in the albumen glycerol. Examples: Fig. 1 - 7.

PROTOCOL 1

Most of our preparations are done using this procedure (3). The quality of the slides is usually adequate but frequently not as good as with the Wilbert modification. The latter demands more material and experience; inexperienced workers may easily lose all the material. As in all protargol methods, the procedure is rather time consuming and complicated. Experiments with students showed that beginners have a fair chance of obtaining good slides. A centrifuge may be used for step 2; staining jars are necessary for steps 6-16.

1. Fix organisms in Bouin's or Stieve's fluid for 10-30 minutes.
   Remarks: The fixation time has little influence on the quality of the preparation within the limits given. Ratio fixative:sample fluid should be at least 2:1. Pour ciliates into fixative using a wide-necked flask in order to bring organisms in contact with the fixative as quickly as possible. Both fixatives work well but may provide different results with certain organisms. Stieve's fluid may be supplemented with some drops of 2 % osmium tetroxide for better fixation of very fragile ciliates, e. g. the hypotrich *Urosoma*. This increases the stability of the cells but usually reduces their impregnability.
2. Concentrate by centrifugation and wash organisms 3-4 times in distilled water.
   Remarks: There are now 2 choices: either to continue with step 3 or transfer the material through 30-50-70 % alcohol into 70 % alcohol (isopropanol or ethanol) where it remains stable for several years. Transfer preserved material back through the graded alcohol series into distilled water prior to continuing with the next step. Impregnation may be slightly modified in preserved material.
3. Clean 8 slides (or less if material is very scarce) per sample. The slides must be grease-free (clean with alcohol and flame). Insufficiently cleaned slides may cause the albumen to detach. Mark slides on back if several samples are prepared together. I use staining jars with 8 sections so that I can work with 16 slides simultaneously by putting them back

to back.

4. Put 1 drop each of albumen-glycerol and concentrated organisms in the centre of a slide. Mix drops with a mounted needle and spread over the middle third.
   Remarks: Use about equally sized drops of albumen-glycerol and suspended (in distilled water) organisms to facilitate spreading. The size of the drops should be adjusted so that the middle third of the slide is covered after spreading. Now remove sand, grains, etc. The thickness of the albumen layer should be equal to that of the organisms. Some thicker and thinner slides should however also be prepared because the thickness of the albumen layer greatly influences the quality of the preparation. Cells may dry out and/or shrink if the albumen layer is too thin; if it is too thick it may detach or the cells become impossible to study with the oil immersion objective.
5. Allow slides to dry for at least 12 hours (overnight) at room temperature.
   Remarks: Slides may be allowed to dry for up to 48 hours but no longer if quality is to be maintained. Oven-dried (2 hours at 60 °C) slides are usually also of poorer quality.
6. Place slides in a staining jar filled with 95 % alcohol (isopropanol or ethanol) for 20-30 minutes. Place a staining jar with protargol solution into an oven (60 °C).
   Remarks: Slides should not be transferred through an alcohol series into concentrated alcohol as this causes the albumen layer to detach! Decrease hardening time to 20 minutes if albumen is already rather old and/or not very sticky.
7. Rehydrate slides through 70 % alcohol and 2 distilled water steps for 5 minutes each.
8. Place slides in 0.2 % sodium permanganate solution. Remove first slide (or pair of slides) after 60 seconds and the others at 15 second intervals. Collect slides in a staining jar filled with distilled water.
   Remarks: Bleaching is by permanganate and oxalic acid (step 9). The procedure described above is necessary because each species has its optimum bleaching time. The sequence in which slides are treated should be recorded as the immersion time in oxalic acid must be proportional to that in the permanganate solution. The albumen layer containing the organisms should swell slightly in the permanganate solution and the surface should become uneven. If it remains smooth, the albumen is too sticky and this could decrease the quality of the impregnation. If the albumen swells strongly, it is possibly too weak (old) and liable to detach. Use fresh $KMnO_4$ solution for each series.
9. Quickly transfer slides to 2.5 % oxalic acid. Remove first slide (or pair of slides) after 160 seconds, the others at 20 second intervals. Collect slides in a staining jar filled with distilled water.
   Remarks: Same as for step 8! Albumen layer becomes smooth in oxalic acid.
10. Wash slides 3 times in distilled water for 3 minutes each.
11. Place slides in warm (60 °C) protargol solution and impregnate for 10-15 minutes at 60 °C.
    Remarks: Protargol solution can be used only once.
12. Remove staining jar with the slides from the oven and allow to cool for 10 minutes at room temperature.
    Remarks: In the meantime organize 6 staining jars for developing the slides - distilled water - distilled water - fixative (sodium thiosulfate) - distilled water - 70 % alcohol - 100 % alcohol (isopropanol or ethanol).
13. Remove the first slide from the protargol solution and drop some developer on the layer of albumen. Move slide gently to spread developer evenly. As soon as the albumen turns yellowish, pour off the developer, dip slide into the first 2 distilled water steps for about 2 seconds each and stop development by submerging the slide in the fixative (sodium thiosulfate), where it can be left for 5-10 minutes.
    Remarks: Now control impregnation with the compound microscope. The impregnation intensity is sufficient if the infraciliature is just recognizable. The permanent slide will be too dark if the infraciliature is distinct at this stage of the procedure! The intensity of the impregnation can be controlled by the concentration of the developer and the time of

development. 5-10 seconds usually suffice for the diluted developer! Some species (e. g., most microthoracids) must be treated with undiluted developer. Development time increases with bleaching time. Therefore commence developing with those slides which were in the bleaching solutions for 60 and 120 seconds, respectively. The thinner the albumen layer, the quicker the development.

14. Collect slides in the fixative (sodium thiosulfate) and transfer to distilled water for about 5 minutes.
    Remarks: Do not wash too long; the albumen layer is very fragile and detaches easily!
15. Transfer slides to 70 % - 100 % - 100 % alcohol for 5 minutes each.
16. Clear by two 10 minute transfers through xylene.
17. Mount in synthetic neutral mounting medium.
    Remarks: Do not dry slides between steps 16 and 17! Mounting medium should be rather viscous to avoid air-bubbles being formed when solvent evaporates during drying. If air-bubbles develop in the mounted and hardened slide, re-immerse in xylene for some days until the coverslip drops off. Remount using a more viscous medium and remove possible sand grains protruding from the albumen. Usually, some air-bubbles are found immediately after mounting; these can be pushed to the edge of the coverslip with a finger or mounted needle. The preparation is stable.

REAGENTS

a) Bouin's fluid (prepare immediately before use; components can be stored)
   - 15 parts saturated, aqueous picric acid ($C_6H_3N_3O_7$; preparation: add an excess of picric crystals to, e. g. 1 litre of distilled water; shake solution several times within a week; some undissolved crystals should remain; filter before use).
   - 5 parts formalin (HCHO; commercial concentration, about 37 %)
   - 1 part glacial acetic acid (=concentrated acetic acid; $C_2H_4O_2$)

b) Stieve's fluid (slightly modified; prepare immediately before use; components can be stored)
   - 38 ml saturated, aqueous mercuric chloride (dissolve 60 g $HgCl_2$ in 1 litre of boiling water)
   - 10 ml formalin (HCHO; commercial concentration, about 37 %)
   - 3 ml glacial acetic acid (=concentrated acetic acid; $C_2H_4O_2$)

c) Albumen-glycerol (2-4 month stability)
   - 15 ml egg albumen
   - 15 ml concentrated (98%-100%) glycerol ($C_3H_8O_3$)

   Pre-treatment of the egg albumen and preparation of the albumen-glycerol: Separate the white carefully from the yolk and embryo of 3 eggs (free range eggs are preferable to those from battery chickens, whose egg white is less stable and sticky). Shake the white, by hand (do not use a mixer!) for some minutes in a narrow-mouthed 250 ml Erlenmeyer flask until a stiff white foam is formed. Allow the flask to stand for about 1 minute. Pour the viscous rest of the egg white in a second Erlenmeyer flask and shake again until it is stiff. Repeat until most of the egg white is either stiff or becomes watery; usually 4-6 Erlenmeyer flasks of foam are obtained. Leave all flasks undisturbed for about 10 minutes. During this time a glycerol-like fluid percolates from the foam. This fluid and the white from the last flask are collected and used. Add an equal volume of concentrated glycerol and a small thymol crystal ($C_{10}H_{14}O$) for preservation to the mixture. Mix by shaking gently and pour mixture into a small flask. Leave undisturbed for 2 weeks. A whitish slime settles at the bottom of the flask. Decant the clear portion, discard slime and thymol crystal. A "good" albumen-glycerol drags a short thread when touched with a needle. The albumen is too thin (not sticky enough) or too old if this thread is not formed. Fresh albumen which is too thin may be concentrated by leaving it open for some weeks so that water can evaporate. If the albumen is too sticky, which may cause only one side of the organisms to impregnate well, it is diluted with distilled water or old,

less sticky albumen to the appropriate consistency. The preparation of the albumen-glycerol must be undertaken with great care because much depends on its quality. Unfortunately, all commercial products which I have tried detach during impregnation.

d) 0.2 % potassium permanganate solution (stable for about 1 day)
   0.2 g potassium permanganate ($KMnO_4$)
   ad 100 ml distilled water

e) 2.5 % oxalic acid solution (stable for about 1 day)
   2.5 g oxalic acid ($C_2H_2O_4 \cdot 2H_2O$)
   ad 100 ml distilled water

f) 0.4 % protargol solution (stable for about 1 day)
   100 ml distilled water
   ad 0.4 g protargol
   Remarks: Use light-brown "protargol for microscopy" (e. g., Merck's "Albumosesilber für die Mikroskopie" or "Proteinate d'Argent", Roques, Paris, France). Some dark-brown, cheaper products do not work! Sprinkle powder on the surface of the water and allow to dissolve without stirring; use a wide-mouthed bottle for solving the protargol.

g) Developer (mix in sequence indicated; sodium sulfite must be dissolved before hydroquinone is added)
   95 ml distilled water
   5 g sodium sulfite (Na_2SO_3)
   1 g hydroquinone ($C_6H_6O_2$)
   Remarks: This recipe yields the stock solution which is stable for some weeks and should be used undiluted for certain ciliates (step 13). Usually, however, it must be diluted with tap water in a ratio of 1:20 to 1:50 to avoid too rapid development and one-sided impregnation of the organisms. Freshly prepared developer is usually inadequate (the albumen turns greenish instead of brownish). The developer should thus be prepared from equal parts of fresh and old (brown) stock solutions. Take great care with the developer as its quality contributes highly to that of the slides. If the developer has lost its activity (which is not always indicated by a brown colour!) the silver is not or only insufficiently reduced and the slides stain too faintly. A fresh developer should therefore be prepared for each "impregnation week" and some old developer kept. Fresh developer can be artificially aged by adding some sodium carbonate (Na_2CO_3). However, better results are obtained with air-aged solutions, i. e. by a developer which has been kept uncovered for some days in a wide-mouthed bottle. It first turns yellowish, then light brown (most effective) and later dark brown and viscous (at this stage the developer has lost most of its activity but is still suitable for artificial aging of fresh developer = 1:1 mixture mentioned above).

h) Fixative for impregnation (stable for several years)
   25 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)
   ad 100 ml distilled water

PROTOCOL 2

This modification produces excellent results but demands much experience. I manipulate large cells with micropipettes in a watch-glass, whereas the centrifuge is used for steps 1-4, 7, 8 if cells are smaller than about 150 µm. The watch-glass method is used when there are only a few specimens of larger cells; thus an attempt is worthwhile even if only 20 cells are available. The organisms are very soft after development and fixation and are thus easily be compressed between slide and coverslip, which greatly facilitates photographic documentation.

1. Fix organisms as described in the first protargol procedure (Foissner's modification).
2. Wash and, if so desired, preserve organisms as described in the first protargol method (Foissner's modification).

Remarks: Wash cells either in the centrifuge (small species) or in a watch-glass. To change fluids allow cells to settle on bottom of watch-glass and remove supernatant with a micropipette under the dissecting microscope; concentrate cells in the centre of watch-glass by gentle swirling.

3. Transfer organisms with a small amount of distilled water to an at least tenfold quantity of sodium hypochlorite (NaClO) and bleach for about 3 minutes.
   Remarks: This is the critical step in this modification. If bleaching is too strong or too weak all is lost: cells either dissolve or do not impregnate well. Systematic investigations showed that not the bleaching time but the amount of active chloride in the sodium hypochlorite and the pre-treatment of the cells (fixation method, fresh or preserved material) are decisive for the quality of the preparation. Different species need different concentrations. Unfortunately, the concentration of active chloride in the commercial products varies (10-13 %) and is dependent on the age of the fluid. It is thus impossible to provide more than only a few guidelines: 100 ml distilled water + 0.2-0.4 ml NaClO (if product is fresh and cells were not stored in alcohol) or 100 ml distilled water + 0.5-1.6 ml NaClO (if product is older and cells were stored in alcohol). The transparency of the cells under the dissecting microscope may serve as a further indicator: fixed, unbleached cells appear dark and opaque, whereas accurately bleached cells are almost colourless and rather transparent (depends, however, also on size and thickness of the cell). Thus, increase the concentration of sodium hypochlorite stepwise if cells appear too dark with the recommended concentrations. We routinely start with 3 different hypochlorite concentrations if enough material is available.
4. Wash organisms at least 3 times with distilled water and finally once in the protargol solution.
   Remarks: Wash thoroughly, especially when fluids are changed with micropipettes, because even the slightest traces of the sodium hypochlorite disturb the impregnation.
5. Transfer to 1 % protargol solution and impregnate for 10-20 minutes at 60 °C.
   Remarks: This and the next step should be carried out in a watch-glass even for material which is otherwise manipulated with the centrifuge. The impregnation time depends on the kind of material and the degree of bleaching. Check the progress of impregnation every 3-4 minutes under the compound microscope by picking out a few cells with the micropipette under the dissecting microscope; add these to 1 drop of developer. Dilute developer and/or interrupt development by adding a little fixative (sodium thiosulfate) if impregnation is strong enough.
6. Remove most of the protargol solution with a micropipette and add some drops of developer to the remainder containing the organisms.
   Remarks: Fresh, undiluted developer is usually used (but see step 5). Control development in compound or dissecting microscope. As soon as the infraciliature becomes faintly visible, development must be stopped by adding a few drops of sodium thiosulfate. Judging the right moment is a question of experience; the permanent slide will be too dark if the infraciliature is very distinct at this stage of the procedure!
7. Stabilize the impregnation by 2 approximately 5 minute transfers through sodium thiosulfate.
   Remarks: The developer need not be removed before fixation. For small species this and the next step can be carried out in a centrifuge. Larger species must be manipulated with micropipettes because cells become very fragile and would be damaged in a centrifuge. Cells are very soft at this stage and can thus be easily compressed and photographed. Transfer some of the more darkly impregnated specimens with a very small amount of the fixative onto a clean slide using a micropipette and cover with a coverslip. Organisms are usually flattened by the weight of the coverslip; excess fluid may be removed from the edge of the coverslip with a piece of filter paper.
8. Wash very thoroughly in distilled water (3 times with the centrifuge; 7-10 times in watch-glass with micropipettes). Finally remove as much of the water as possible.

Remarks: Even the slightest traces of the fixative destroy the impregnation within a few days or weeks.

9. Smear a moderately thick layer of albumen-glycerol on a clean slide with a finger. Drop impregnated, washed cells on the albumized slide with a large-bore pipette (opening ~ 1 mm) and dry preparation for at least 2 hours.
   Remarks: The cells are too fragile to be spread with a needle. With much care it is possible to orientate cells using a mounted eyelash. Commercial albumen-glycerol can be used.
10. Harden albumen by two 10 minute transfers through concentrated alcohol (isopropanol or ethanol).
    Remarks: This and the next step are best carried out in staining jars. The albumen layer turns milky and opaque.
11. Clear by two 5 minute transfers through xylene.
    Remarks: The albumen layer turns transparent.
12. Mount in synthetic neutral mounting medium.
    Remarks: Same as for step 17 of the first protocol!

REAGENTS

If not stated otherwise, the same reagents like in the first protargol procedure (Foissner's modification) are to be used.

PROTOCOL FOR FEW SPECIMENS

I learned this simple modification in Dr P. Didier's laboratory (Clermont-Ferrand University). It sometimes produces excellent impregnations, especially with species having a firm pellicle (e. g. microthoracids). It also demands little material because the specimens are mounted on the slide without washing.

1. Collect specimens with a micropipette and place them at the centre of a grease-free slide. Remove excess fluid as far as possible.
2. Fumigate cells with 4 % aqueous osmium tetroxide for about 2 minutes.
   Remarks: Hold inverted slide close to the osmium tetroxide. Carry out procedure in a fume hood as osmic acid fumes are highly toxic.
3. Add an equal sized drop of albumen-glycerol, mix thoroughly but gently with a mounted needle and spread mixture in a moderately thin layer over the middle third of the slide.
   Remarks: Albumen-glycerol must be prepared as described in the first protargol procedure (Foissner's modification). Cells are very fragile and frequently break or dissolve.
4. Allow to dry for about 4 hours.
5. Proceed with steps 6 (coagulation of albumen in concentrated alcohol) to 17 of the first protargol procedure (Foissner's modification). Bleaching times are usually about 50 % shorter than with my modification.

LITERATURE CITED

1. Aufderheide, K J. 1982. An improvement in the protargol technique of Ng and Nelsen. *Trans. Am. microsc. Soc.* **101**:100-104.
2. Dragesco, J. 1962. L'orientation actuelle de la systématique des ciliés et la technique d'imprégnation au protéinate d'argent. *Bull. Micr. appl.* **11**:49-58.
3. Foissner, W. 1991. Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. *Europ. J. Protistol.* **27**:313-330.

4. Kirby, H. 1945. The structure of the common intestinal trichomonad of man. *J. Parasit.* **31**:163-174.
5. McCoy, J W. 1974. New features of the tetrahymenid cortex revealed by protargol staining. *Acta Protozool.* **13**:155-160.
6. Montagnes, D J S & Lynn, D H. 1987. A quantitative protargol stain (QPS) for ciliates: method description and test of its quantitative nature. *Mar. Microb. Food Webs* **2**:83-93.
7. Moskowitz, N. 1950. The use of protein silver for staining protozoa. *Stain Technol.* **25**:17-20.
8. Ng, S F & Nelsen, E M. 1977. The protargol staining technique: an improved version for *Tetrahymena pyriformis*. *Trans. Am. microsc. Soc.* **96**:369-376.
9. Tuffrau, M. 1964. Quelques variantes techniques de l'imprégnation des ciliés par le protéinate d'argent. *Archs Zool. exp. gén.* **104**:186-190.
10. Tuffrau, M. 1967. Perfectionnements et pratique de la technique d'imprégnation au protargol des infusoires ciliés. *Protistologica* **3**:91-98.
11. Wilbert, N. 1975. Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos* year 1975, Heft 6:171-179.
12. Wilbert, N. 1976. A standardized method for identifying and counting the vagile and sessile periphyton. *Oecologia (Berlin)* **24**:343-347.
13. Zagon, I S. 1970. *Carchesium polypinum*: cytostructure after protargol silver deposition. *Trans. Am. microsc. Soc.* **89**:450-468.

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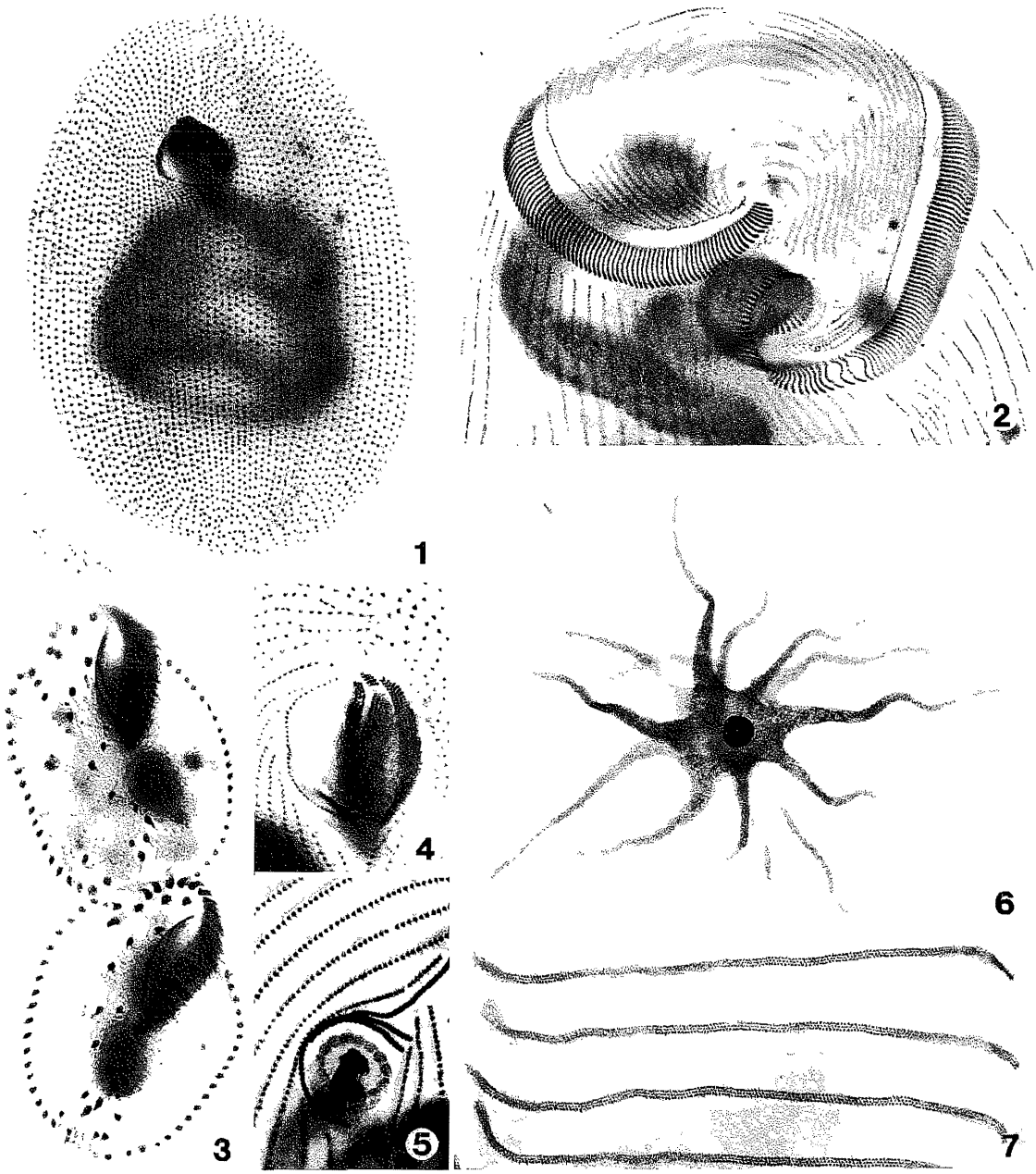

Fig. 1 - 7. Protists prepared with protargol protocols 1 (figure 6) and 2 (figure 1 - 5, 7). **1, 4.** *Epenardia myriophylli*, a tetrahymenid ciliate; ventral view and detail of oral apparatus, length about 100 µm. **2.** *Stentor roeselii*, a heterotrich ciliate; detail showing oral apparatus. **3.** *Sterkiella histriomuscorum*, a hypotrich ciliate; ventral view, length about 100 µm. **5.** *Trithigmostoma steini*, a cyrtophorid ciliate; detail showing oral structures. **6.** A naked soil amoeba, diameter about 50 µm. **7.** *Bursaria truncatella*, a colpodid ciliate; detail of adoral zone of organelles.

THE SILVER CARBONATE METHODS

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INTRODUCTION

Because of the large quantity of cells needed for the basic Fernández-Galiano (2) technique, Augustin et al. (1) proposed a modification which requires only few specimens and may also yield permanent slides. Although the results are highly variable with all modifications, the method is worthwhile because it works very quickly and often produces excellent impregnations, especially with hymenostomes (e. g., *Tetrahymena*, *Paramecium*), prorodontids (e. g., *Prorodon*, *Urotricha*), nassulids (e. g., *Colpodidium*, *Nassula*), colpodids (e. g., *Colpoda*, *Bryometopus*, *Platyophrya*) and heterotrichs (e. g., *Stentor*). Fixation is by formalin, which means that the shape of the cells is poorly preserved and even destroyed (cell bursts) in some species (e. g., most hypotrichs). The cells swell strongly during the preparation process but become very soft and are thus easily flattened between the slide and the coverslip. This makes photographic documentation easy but may result in interpretation errors. The silver carbonate methods reveal the infraciliature and certain cortical and cytoplasmic structures, especially the kinetodesmal fibres and the nuclear apparatus. Several other modifications have been suggested (3, 4). The silverlines in most cases do not stain. Examples: Fig. 1 - 6.

PROTOCOL

1. Place 1 droplet (about 0.05 ml) of a rich ciliate culture or even single specimens on a slide.
   Remarks: Slide need not be grease-free. Its middle third should be delimited by lines drawn with a greasy finger-tip or a wax crayon to prevent solutions from spreading over the whole slide.
2. Add 1-2 drops of formalin (about 4 %) and fix for 1-3 minutes. Mix organisms with formalin by circular motions of the slide.
   Remarks: The duration of this step may greatly influence the results. Species with a firm pellicle (or resting cysts) usually need to be fixed longer (3 minutes or more) than those with a more fragile pellicle (1 minute or less). Some species cannot be fixed well with formalin and cells may even burst. For these fixation with osmium tetroxide vapours (place inverted slide with ciliates for about 1 minute over a 4 % osmium tetroxide solution in a fume hood) is sometimes useful. Fix as usual with formalin after osmium treatment.
3. Add 1-3 drops of Fernandez-Galiano's fluid to the fixed ciliates, without first washing out the formalin, and mix by circular motions of the slide for 10-60 seconds.
   Remarks: The amount of Fernandez-Galiano's fluid needed depends on many unpredictable factors (e. g., amount and concentration of fixative, size of drops, kind of species, composition of sample fluid). 1-3 drops usually work well. The same holds for the reaction time (10-60 seconds). The trial and error method must frequently be used to obtain best results!
4. Place slide on a pre-heated (60 °C) hot-plate and leave until the drop, which will be rather large, turns golden brown (like cognac). This usually takes 2-4 minutes and the slide must be kept in constant circular motion during this time. As soon as the drop appears cognac-coloured, check impregnation with the compound microscope. Replace the slide on the hot-plate if impregnation is still too faint; if it is already too dark repeat procedure,

starting with step 1, but vary amount of Fernandez-Galiano's solution and/or impregnation time etc.

Remarks: The correct impregnation time depends on many factors which are difficult to control (size of drops, temperature, kind of species...). The amount of pyridine and silver carbonate in the Fernandez-Galiano fluid is especially important. Add some drops of pyridine and/or silver carbonate solution to the Fernandez-Galiano fluid if impregnation is repeatedly too faint, i. e. cannot be intensified by prolonged heating. Fix ciliates in 2-3 drops of formalin instead of 1-2 drops if impregnation is too faint. Ciliates from old cultures, ion-rich fluids (e. g., sewage, soil) or anaerobic biotopes frequently impregnate poorly. For these impregnation sometimes improves if they are washed prior to fixation (fluid from sample and distilled water 1:1).

5. Interrupt impregnation by removing the slide from the hot-plate and by adding 1 drop of fixative (sodium thiosulfate).

   Remarks: The preparation is now ready. Augustin et al. (1) describe a method for obtaining permanent slides. Their quality is, however, often not as good as with wet (fresh) preparations, which are thus usually preferred for investigation and photography. Pick out the well impregnated specimens with a micropipette, place them on a clean slide and cover with a coverslip. For good pictures the drop with the selected specimens should be very small so that cells are compressed between the slide and the coverslip. Excess fluid may be removed from the edge of the coverslip using a piece of filter paper. The impregnation need not be fixed with sodium thiosulfate if the investigation is undertaken immediately. The impregnation is stable for some hours when stored in a moist chamber. I recommend that the cells be compressed between the slide and coverslip immediately after the impregnation since silver precipitates may occur with time in the reaction fluid.

REAGENTS

a) Fixative for organisms (stable for a long time)
   0.1 ml formalin (HCHO; commercial concentration, about 37 %)
   ad 10 ml distilled water

b) Fernandez-Galiano's fluid (prepare immediately before use; components can be stored and must be mixed in the sequence indicated. The mixture must be slightly milky. If stored in brown flask it can be used for some hours. Keep away from sunlight. Make up a fresh fluid when no more impregnation can be achieved)
   0.3 ml pyridine (C_5H_5N; commercial concentration)
   2-4 ml Rio-Hortega ammoniacal silver carbonate solution
   0.8 ml proteose-peptone solution
   16 ml distilled water

c) Fixative for impregnation (stable for several years)
   2.5 g sodium thiosulfate ($Na_2S_2O_3$)
   ad 100 ml distilled water

d) Rio-Hortega ammoniacal silver carbonate solution. Preparation (the ratios are important!):
   50 ml of 10 % aqueous silver nitrate solution are placed in a flask; 150 ml of 5 % aqueous sodium carbonate (Na_2CO_3) are added little by little under constant stirring; add 25 % ammonia (NH_3), drop by drop, until the precipitate dissolves, being careful not to add an excess; finally add distilled water up to a total volume of 750 ml. The solution is stable for several years.

e) Proteose peptone solution (long term stability if not colonized by bacteria and/or fungi; discard dull solutions)
   96 ml distilled water
   4 g proteose-peptone (bacteriological; sprinkle powder on the surface of the water and allow to dissolve without stirring).
   0.5 ml formalin (HCHO; for preservation)

LITERATURE CITED

1. Augustin, H, Foissner, W & Adam, H. 1984. An improved pyridinated silver carbonate method which needs few specimens and yields permanent slides of impregnated ciliates (Protozoa, Ciliophora). *Mikroskopie* **41:**134-137.
2. Fernández-Galiano, D. 1976. Silver impregnation of ciliated protozoa: procedure yielding good results with the pyridinated silver carbonate method. *Trans. Am. microsc. Soc.* **95:**557-560.
3. Wilbert, N. 1983. Eine neue Imprägnation der Basalkörper bei Wimpertieren. *Mikrokosmos* year 1983, Heft 7:193-197.
4. Wolska, M. 1966. Application of the ammonium-silver impregnation method to the investigation of ciliates from the rumen of herbivorous mammals. *Acta Protozool.* **4:**105-108.

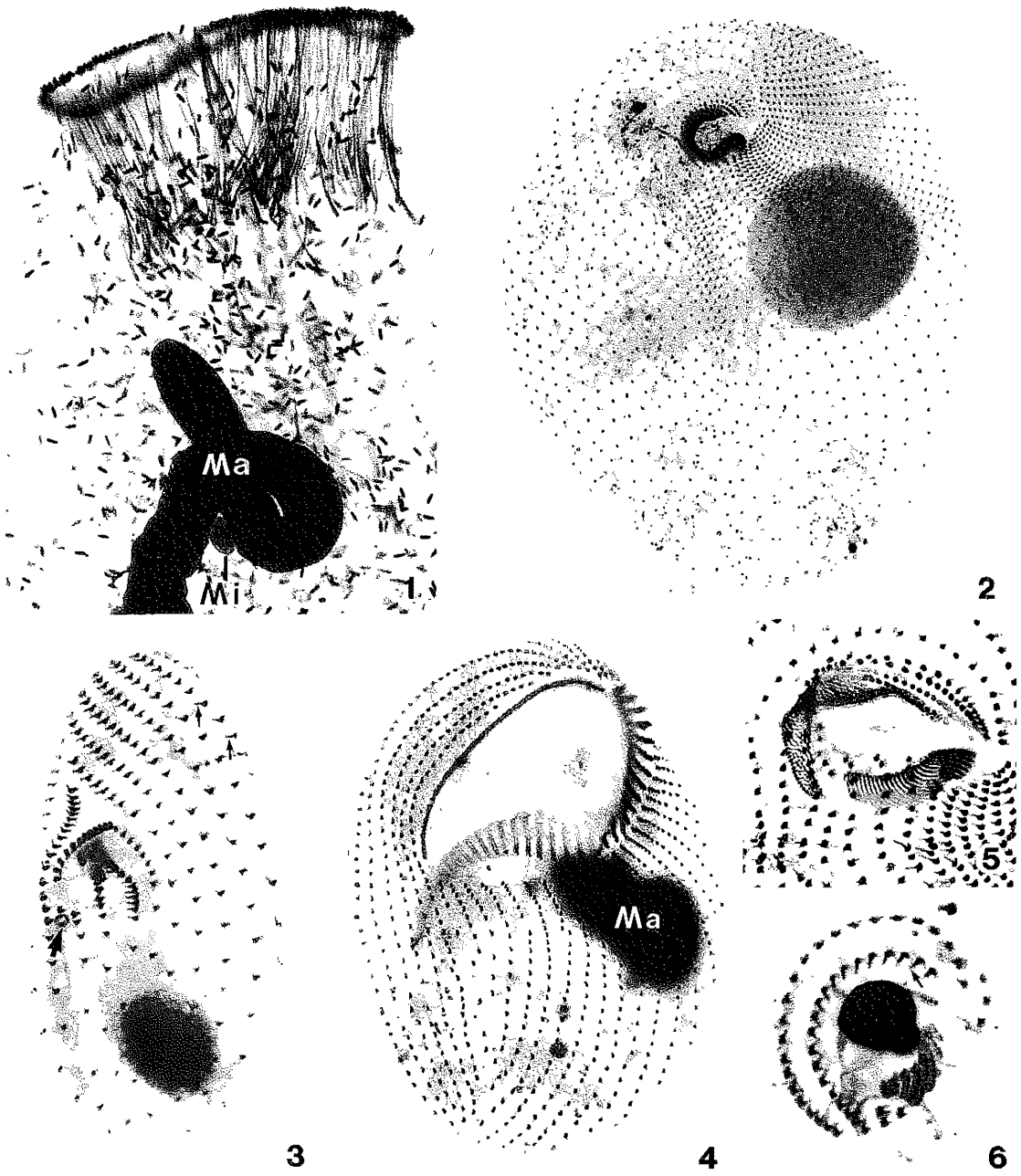

Fig. 1 - 6. Ciliates prepared with the silver carbonate protocol described. **1.** *Epispathidium terricola*, a haptorid ciliate; oral region showing many short and long extrusomes. **2, 5.** *Colpoda cavicola*, a colpodid ciliate; ventral view and detail of oral apparatus, length about 150 µm. **3.** *Colpodidium caudatum*, a nassulid ciliate; ventral view, length about 40 µm. Small arrows mark well impregnated kinetodesmal fibres; large arrow points to the excretory pore of the contractile vacuole. **4, 6.** *Bryometopus sphagni* and *Kreyella minuta*, bryometopid colpodids; ventral views, length about 100 µm and 20 µm. Arrow in figure 6 marks well impregnated transverse fibre. Ma, macronucleus; Mi, micronucleus.

THE "WET" SILVER NITRATE METHOD

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INTRODUCTION

The first wet ("wet" because cells are chemically fixed before being treated with silver nitrate) method was described by Chatton & Lwoff (2, 3). The technique became well known after Corliss (4) published the version in use in the Paris laboratory of Fauré-Fremiet. It works well with many different kinds of ciliates, especially with hymenostomes (e. g., *Tetrahymena*, *Paramecium*, *Cyclidium*), prorodontids (e. g., *Prorodon*, *Urotricha*), most colpodids (e. g., *Colpoda*, *Bresslauides*) and some hypotrichs (e. g., *Euplotes*). Less convincing results are usually obtained with peritrichs (e. g., *Vorticella*), heterotrichs (e. g., *Spirostomum*, *Metopus*), oligotrichs (e. g., *Halteria*) and most hypotrichs (e. g., *Oxytricha*, *Urostyla*). The wet methods provide valuable information on the somatic and oral infraciliature as well as the silverlines, which are, however, often rather faintly stained. The shape of the cells is usually well preserved, which is of advantage to the investigation but makes photographic documentation difficult. As with the dry methods, only cortical structures are revealed. Several modifications have been described (e. g., 1, 5, 6). Roberts & Causton (7) investigated the variables of this method in detail. Examples: Fig. 1 - 9.

PROTOCOL

Several slides should be prepared simultaneously from the same material. If only few specimens are available, these must be handled with micropipettes during steps 1-7 (difficult task!); for ample material a centrifuge may be used. Until dehydration (step 15), keep all solutions cold (about 5 °C) as warming detaches the gelatin layer from the slide. The method is not simple and requires experience. Since some steps must be done very quickly it is necessary to be well organized.

1. If possible, concentrate ciliates by gentle centrifugation (the fixative is expensive) or collect individual ciliates and drop them into the fixative.
2. Drop ciliates into Champy's fluid and fix them for 1-30 minutes.
   Remarks: The ratio of material to fixative should be at least 1:1, better 1:2. The fixation time apparently does not influence the results greatly. I usually fix for about 10 minutes. Fixation should be carried out in a fume cupboard since osmic acid fumes are highly toxic.
3. Remove fixative by centrifugation or micropipette and postfix in Da Fano's fluid for at least 5 minutes. Continue this replacement until the solution is the colour of Da Fano's fluid (2-3 times are usually enough).
   Remarks: Material can be stored in Da Fano's fluid for years.
4. Place a very clean, grease-free slide on a hot-plate (35-45 °C).
   Remarks: The slides must be absolutely grease-free (clean with alcohol and flame); even commercial pre-cleaned slides must be cleaned with an alcohol-moist cloth.
5. Place a small piece (about 2-4 mm in diameter) of gelatin in centre of the warmed slide and allow to melt.
   Remarks: Gelatin should have been stored in the refrigerator for at least one week before use. Fresh gelatin often causes cloudy silver precipitates.

6. Quickly add an equal sized or smaller drop of concentrated specimens to the molten gelatin and remove slide from hot plate.
   Remarks: Mix organisms thoroughly into the gelatin using a mounted needle.
7. Quickly remove excess fluid under the dissecting microscope with a warmed micropipette until ciliates remain just nicely embedded in a *thin* gelatin layer.
   Remarks: Steps 6 and 7 must be done quickly to avoid hardening and/or desiccation of the gelatin; if gelatin solidifies during the procedure return the slide to the hot-plate for a few seconds. Excess fluid can be removed only if ciliates are large. For small (< 100 µm) species it is more convenient to spread the drop over the slide until the gelatin layer has the appropriate thickness. If drop does not spread well the slide is not grease-free. The gelatin layer must be very thin to allow the silver nitrate to pass through. Material should be well concentrated. If too much Da Fano's fluid has been used or remains, precipitations develop or the gelatin detaches.
8. Immediately transfer slide to a cold, moist chamber (e. g., a covered petri dish with damp filter paper covering its bottom). Leave for about 5 minutes until gelatin has hardened.
   Remarks: Gelatin must be hardened (check with the tip of mounted needle under dissecting microscope if in doubt) but must not desiccate and/or freeze. Desiccated or frozen slides are of poor quality. Harden gelatin in refrigerator or by placing the moist chamber on an ice block.
9. Flush slide in cold distilled water for 3-10 seconds.
   Remarks: This step is essential and determines the quality and intensity of the impregnation. If the gelatin is washed too long, the impregnation may become too faint; if it is insufficiently washed coarse silver precipitations cover the gelatin. It is recommended that at least 4 slides, washed 3, 5, 7 and 10 seconds, respectively, be prepared.
10. Immediately transfer slide to cold silver nitrate solution for 30-60 minutes.
    Remarks: Keep silver nitrate solution cold, as warming melts and detaches the gelatin from the slide. 30 minute impregnation usually suffice. Prolonged immersion intensifies impregnation only slightly and may cause darkening of cytoplasmic inclusions. Gelatin layer becomes slightly milky in the silver nitrate solution. A distinct milky coat indicates that too much Da Fano's fluid has been used and/or remains (step 9!).
11. Flush slide thoroughly with cold distilled water for 1-3 minutes.
12. Immediately submerge slide in 1-4 cm cold distilled water in a white-bottomed dish, usually a large petri dish lined with white paper is used. Irradiate for 10-30 minutes using sunlight or an ultraviolet source (< 254 nm) placed 10-30 cm above slides until gelatin turns golden brown.
    Remarks: Tilt dish gently back and forth and change water after 2-3 minutes of irradiation to avoid silver precipitation. Take care that water remains cold, especially when reduction is performed with sunlight. Reduction with sunlight often produces clearer slides.
13. Check the intensity of impregnation after about 10 minutes of irradiation using a compound microscope. Continue irradiation for another 10-20 minutes if impregnation is still too faint.
    Remarks: The infraciliature should stand out dark brown against the light brown coloured gelatin and the unstained cytoplasm. A rusty brown coloured gelatin indicates that too much Da Fano's fluid remained (step 9!).
14. Transfer slides to chilled 30 % and then 70 % alcohol (isopropanol or ethanol) for 10 minutes each.
    Remarks: If necessary continue irradiation.
15. Complete dehydration by 2 transfers at least 10 minute long through 100 % alcohol (isopropanol or ethanol) at room temperature.
    Remarks: Gelatin hardens, the alcohol need not be chilled. Dehydrate thoroughly to avoid milky "water spots" in the mounted slides.

16. Clear by 2 at least 10 minutes transfers through xylene.
    Remarks: A prolonged stay in xylene (e. g., 2 days) sometimes produces extremely clear preparations.
17. Mount in synthetic neutral mounting medium.
    Remarks: Do not dry slides between steps 16 and 17! Mounting medium should be rather viscous to avoid air-bubbles being formed when solvent evaporates during drying. If air-bubbles develop in the mounted and hardened slide, re-immerse in xylene for some days until the coverslip drops off. Remount using a more viscous medium and remove possible sand grains protruding from the gelatine. Usually, some air-bubbles are found immediately after mounting; these can be pushed to the edge of the coverslip with a finger or mounted needle. The preparation is stable.

REAGENTS

a) Champy's fixative (prepare shortly before use; 9 ml of the fluid usually suffice for 1-2 fixations; use fume hood)
   7 parts (3.5 ml) 1 % aqueous chromic acid (CrO_3)
   7 parts (3.5 ml) 3 % aqueous potassium dichromate ($K_2Cr_2O_7$)
   4 parts (2.0 ml) 2 % aqueous osmium tetroxide (OsO_4)
b) Da Fano's fluid (stable for several years; large amounts can thus be prepared)
   900 ml distilled water (or sea-water, without additional NaCl, for marine ciliates)
   10 g cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$)
   100 ml formalin (HCHO; commercial concentration, about 37 %)
   10 g sodium chloride (NaCl)
c) Gelatin (may be used as long as not colonized by bacteria or fungi; fresh molten gelatin must be clear and yellowish in colour).
   2 g powdered gelatine
   0.005 g sodium chloride (NaCl)
   20 ml distilled water
   Mix these components and melt gelatin in a water bath, stirring frequently. Pour mixture into sterilized flask and store at least one week in refrigerator before use.
d) Silver nitrate solution (may be used for several preparations, i. e. for about 40 slides if these are made on the same day; used solutions which are older than 1 day may cause problems)
   3 g silver nitrate ($AgNO_3$)
   ad 100 ml distilled water

The following materials must be prepared on the day preceding the preparation:
a) Salinated gelatin (see also remarks at step 5!)
b) Osmium tetroxide (takes about 10 hours to dissolve)
c) Chill a moist chamber, a large petri dish (step 12), the silver nitrate solution, distilled water and alcohol (30 %, 70 %) in appropriate amounts.

LITERATURE CITED

1. Chatton, E. 1940. Sur une méthode rapide d'imprégnation a l'argent réduit par l'hydroquinone. *C. r. Séanc. Soc. Biol.* **134**:229-232.
2. Chatton, E & Lwoff, A. 1930. Imprégnation, par diffusion argentique, de l'infraciliature des ciliés marins et d'eau douce, après fixation cytologique et sans dessiccation. *C. r. Séanc. Soc. Biol.* **104**:834-836.
3. Chatton, E & Lwoff, A. 1936. Techniques pour l'étude des protozoaires, spécialement de leurs structures superficielles (cinétome et argyrome). *Bull. Soc. fr. Microsc.* **5**:25-39.
4. Corliss, J O. 1953. Silver impregnation of ciliated protozoa by the Chatton-Lwoff technic. *Stain Technol.* **28**:97-100.
5. Frankel, J & Heckmann, K. 1968. A simplified Chatton-Lwoff silver impregnation procedure for use in

experimental studies with ciliates. *Trans. Am. microsc. Soc.* **87**:317-321.
6. Lynn, D H, Suriano, D M & Beverley-Burton, M. 1981. Chatton-Lwoff silver impregnation: an improved technique for the study of oncomiracidia (Platyhelminthes: Monogenea) chaetotaxy. *Syst. Parasit.* **3**:21-23.
7. Roberts, D McL & Causton, H. 1988. Silver nitrate impregnation of ciliated protozoa. *Arch. Protistenk.* **135**:299-318.

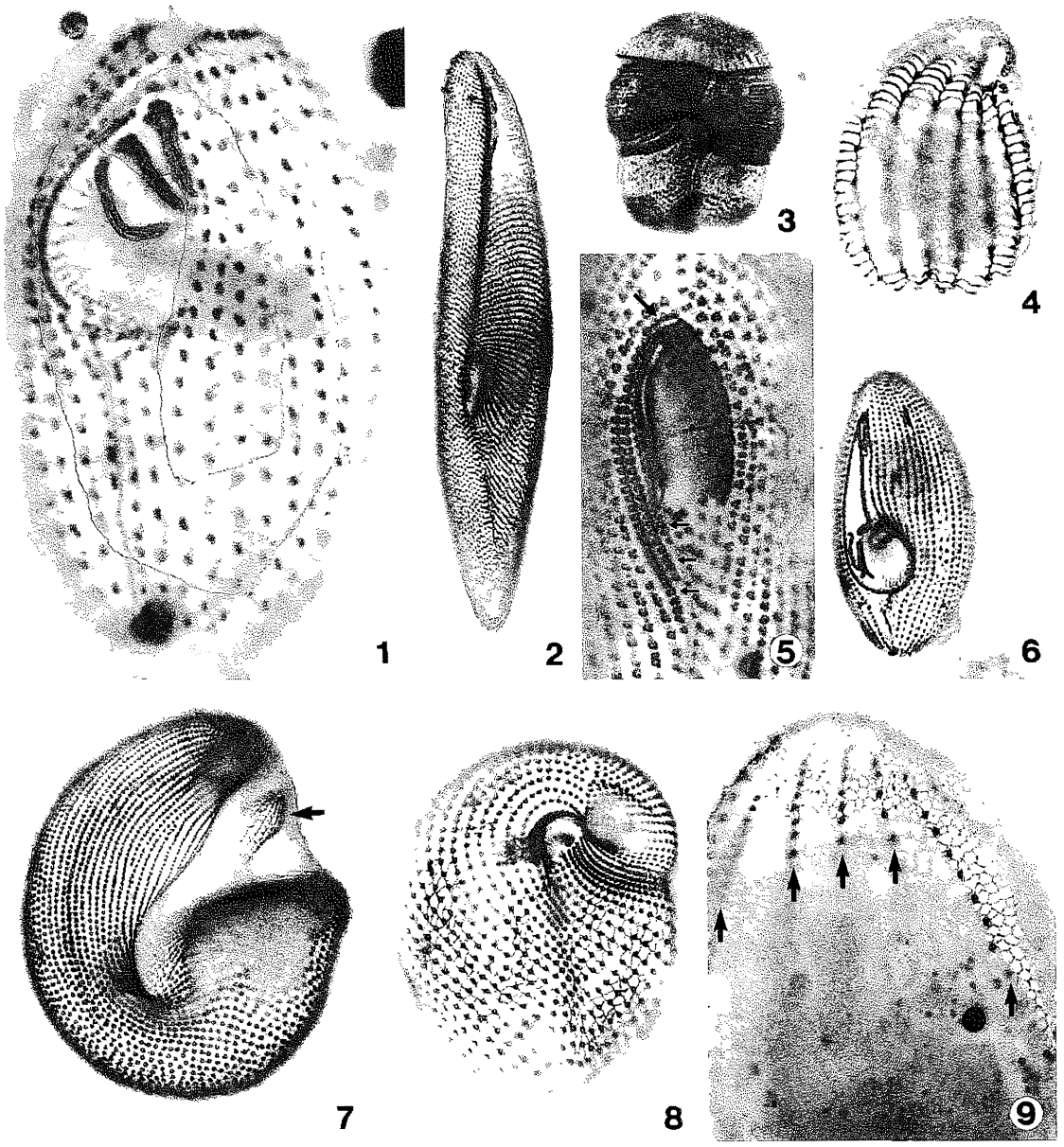

Fig. 1 - 9. Ciliates prepared with the wet silver nitrate protocol described. **1, 6.** *Sathrophilus muscorum* and *Pleuronema coronatum*, scuticociliatid ciliates; ventro-lateral views, length about 35 µm and 90 µm. **2, 3, 5.** *Paramecium caudatum, Urocentrum turbo*, and oral apparatus of *Frontonia depressa*, peniculinid ciliates; ventral views, length about 250 µm and 90 µm. Arrows in figure 5 mark vestibular kineties. **4, 7, 8.** *Cosmocolpoda naschbergeri, Bresslauides discoides*, and *Colpoda cavicola*, colpodid soil ciliates; ventro-lateral views, length about 50 µm, 250 µm and 100 µm. Arrow in figure 7 marks a *Tetrahymena* cell captured in the huge vestibulum. **9.** *Histriculus erethisticus*, a hypotrich ciliate; dorsal view showing the fine-meshed silverline system and the dorsal kineties (arrows).

SUPRAVITAL STAINING WITH METHYL GREEN-PYRONIN

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INTRODUCTION

This simple method was described by Foissner (1). It is an excellent technique for revealing the mucocysts of most ciliates (those of tetrahymenids, however, usually do not stain). Mucocysts are stained deeply and very selectively blue or red, and can be observed in various stages of explosion because the cells are not killed instantly. The nuclear apparatus is also stained. Examples: Fig. 1 - 4.

PROTOCOL

1. Pick out desired ciliates with a micropipette and place the small drop of fluid in the centre of a slide.
2. Add an equal sized drop of methyl green-pyronin and mix the two drops gently by swivelling the slide.
   Remarks: If ciliates were already mounted under the coverslip then add a drop of the dye at one edge of the coverslip and pass it through the preparation with a piece of filter paper placed at the other end of the coverslip.
3. Place a coverslip with vaselined corners on the preparation.
   Remarks: Observe immediately. Cells die in the stain within 2 minutes. Mucocysts stain very quickly and many can be observed at various stages of explosion. To reveal the nuclear apparatus, cells should be fairly strongly squashed (=flattened). The preparation is temporary. After 5-10 minutes the cytoplasm often becomes heavily stained and obscures other details.

REAGENTS

1 g methyl green-pyronin (Merck)
ad 100 ml distilled water
This solution is very stable and can be used for years

LITERATURE CITED

1. Foissner, W. 1979. Methylgrün-Pyronin: Seine Eignung zur supravitalen Übersichtsfärbung von Protozoen, besonders ihrer Protrichocysten. *Mikroskopie* **35**:108-115.

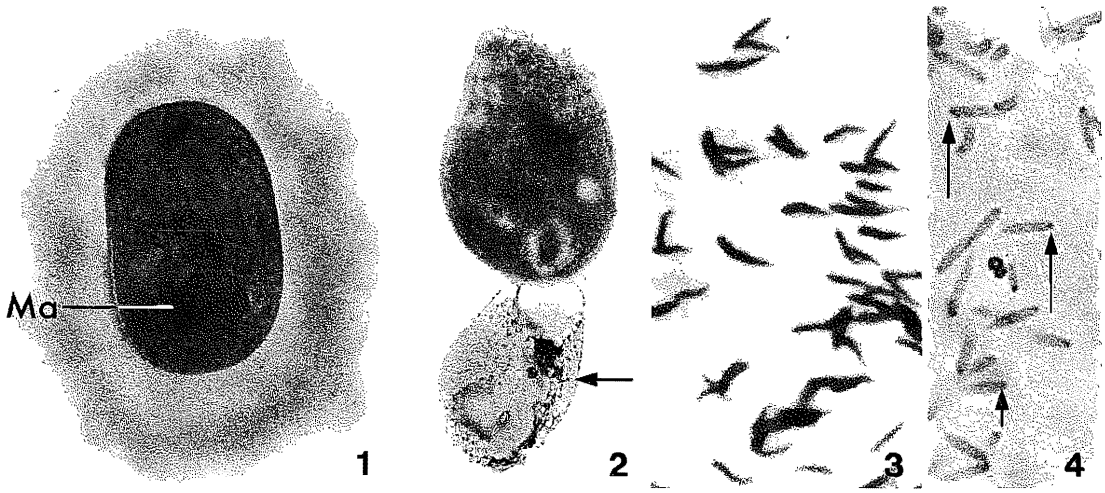

Fig. 1 - 4. Extrusomes stained with methyl green-pyronin. **1.** *Nassula picta*, a nassulid ciliate, secretes a slimy, voluminous, structureless coat when the stain is applied; Ma, macronucleus. **2.** *Phascolodon vorticella*, a cyrtophorid ciliate, secretes a membranous envelope (arrow) when the stain is added. **3.** Mucocysts of *Bursaria truncatella*, a colpodid ciliate. **4.** *Urotricha farcta*, a prorodontid ciliate, discharges trichocyst-like extrusomes which have a darker stained apical granule (arrows).

OBSERVING LIVING CILIATES

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Many physical and chemical methods have been described for retarding the movement of ciliates in order to observe structural details [for literature see (1)]. Chemical immobilization (e. g., nickel sulfate) or physical slowing down by increasing the viscosity of the medium (e. g., methyl cellulose) are, in my experience, usually unsuitable. These procedures often change the shape of the cell or cause premortal alterations of various cell structures. The following simple method is therefore preferable (Fig. 1A - D): place about 0.5 ml of the raw sample on a slide and pick out (collect) the desired specimens with a micropipette under a compound microscope equipped with a low magnification (e. g., objective 4:1, ocular 10X). If specimens are large enough they can be picked out from a petri dish under a dissecting microscope. Working with micropipettes, the diameter of which must be adjusted to the size of the specimens, requires some training. Transfer the collected specimens, which are now in a very small drop of fluid, onto a slide. Apply small dabs of vaseline (Petroleum jelly) to each of the four corners of a coverslip. Place this coverslip on the droplet containing the ciliates. Press on the vaselined corners with a mounted needle until ciliates are held firmly between slide and coverslip. As the pressure is increased the ciliates gradually become less mobile and more transparent. Hence, first the location of the main cell organelles (e. g., nuclear and oral apparatus, contractile vacuole) and then the details (e. g., extrusomes, micronucleus) can easily be observed under low (100 - 300X) and high (oil immersion objective) magnification.

The shape of the cells is of course altered by this procedure. Therefore, specimens taken directly from the raw culture with a large-bore (opening ≈ 1 mm) pipette must first be investigated under low magnification (100 - 400X). Many species are too fragile to withstand handling with the micropipette and coverslip trapping without deterioration. Investigation with low magnification also requires some experience but it guarantees that undamaged cells are recorded. Video-microscopy is very useful at this point of investigation, especially for the registration of the swimming behaviour.

A compound microscope equipped with differential interference contrast is best for observing ciliates. If not available, use bright-field or phase-contrast; the latter is only satisfactory for very flat species.

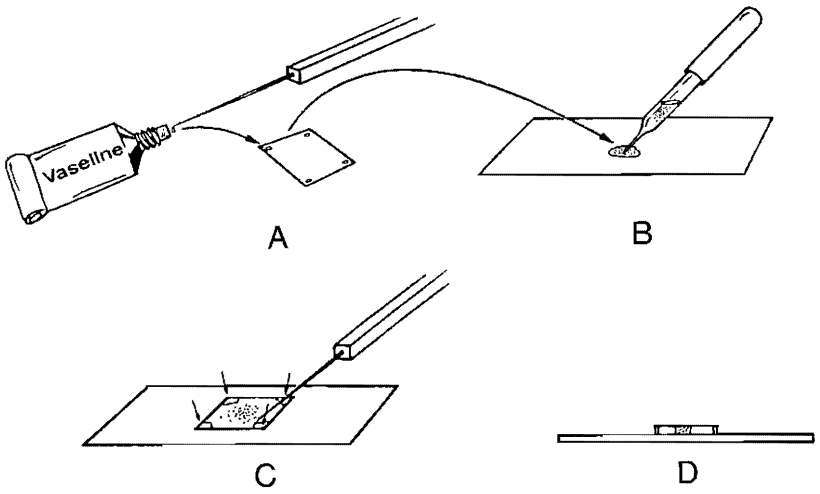

Fig. 1A - D. Preparation of slides for observing living ciliates [from (2)].

LITERATURE CITED

1. Foissner, W. 1991. Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. *Europ. J. Protistol.* **27**:313-330.
2. Dragesco, J & Dragesco-Kernéis, A. 1986. Ciliés libres de l'Afrique intertropicale. *Faune tropicale* **26**:1-559.

THE "DRY" SILVER NITRATE METHOD

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INTRODUCTION

Because of the numerous problems with the basic dry Klein (3, 4) technique, Foissner (1) and others (e. g., 2, 5) introduced some improvements. The dry methods ("dry" because cells are air-dried and not chemically fixed before being treated with silver nitrate) provide preliminary information on the somatic and oral infraciliature (=ciliary pattern) and are often best for revealing the silverline system (= lines revealed by silver nitrate and connecting basal bodies and other cortical organelles such as extrusomes and the cytopyge). Although the results vary highly, the method is worthwhile because it is quick and often produces excellent preparations, which can be well documented since the cells are flattened during dehydration. Only cortical structures are revealed. Examples: Fig. 1 - 4.

PROTOCOL

1. Take 5-10 clean slides and spread a very thin layer of albumen over the middle third of each with a finger-tip. Dry for at least 1 minute.
   Remarks: The egg-albumen (remove germinal disk! do not add glycerol) must have kept open in a wide-necked flask for at least 20 hours; fresh albumen is often less satisfactory. It can be used for 2-3 days if the flask is subsequently sealed; do not, however, stir before use, but skim the albumen from the surface with a finger-tip. To facilitate spreading breathe on slide so that a film of condensation is produced on which the albumen can glide. The albumen layer must be very thin and uniform and should not cover cells.
2. Place a drop of fluid containing the ciliates on the albumized slide, spread with a needle (do not touch albumen layer!) and dry preparation at room temperature.
   Remarks: Even single specimens can be placed on the albumized slide with a micropipette. If necessary enrich ciliates by gentle centrifugation or by leaving sample to settle for a few hours, after which time oxygen depletion induces many ciliates to move to the water surface. The amount and chemical composition of the fluid with which the ciliates are air-dried as well as temperature and humidity greatly influence the results. Therefore, 5-10 slides should usually be prepared simultaneously to vary these parameters, e. g. by washing cells with different amounts of distilled water or fresh culture medium. Washing cells with distilled water or spreading the drop to a very thin film is especially recommended with saline fluids, e. g. seawater, sewage, and soils. Temperature and humidity are easily varied using an ordinary hair-dryer.
3. Apply some drops of silver nitrate to the dried material for about 1 minute.
   Remarks: Do not touch albumen layer with the pipette. The reaction time does not influence the results; a few seconds are adequate.
4. Wash slides for about 3 seconds under distilled water and re-dry.
   Remarks: Wash gently! Apply water current from the top third of the tilted slide so that water runs gently over the dried material. Leave slides tilted during drying.
5. Pre-develop dried slide by exposing it for 5-60 seconds to a 40-60 watt electric bulb at a distance of 3-10 cm.
   Remarks: Time and distance influence intensity of impregnation (see also next step).

6. Apply a few drops of developer to the dried preparation for about 30-60 seconds.
   Remarks: The pre-development (step 5), the composition of the developer, and the material itself influences impregnation intensity, and quality. The best ratio of these parameters must be evaluated in pilot experiments. If the developer is well adjusted, the albumen around the dried fluid turns brownblack; if the developer is too strong, the albumen appears black (add some component A [see Reagents] and/or reduce pre-developing time); if the developer is too weak, the albumen appears brownish (add some components B and/or C and/or increase pre-development time).
7. Pour developer off slide, rinse gently in tap water for 5-10 seconds and immerse in fixative (sodium thiosulfate).
8. Remove slide from fixative, rinse gently in tap water for 5-10 seconds and immerse in 96-100 % ethanol.
   Remarks: Fixative must be thoroughly removed, otherwise crystals are formed in the alcohol and remain on the slide, causing the impregnation to fade with time. Do not wash too long and do not use distilled water, otherwise cells swell and eventually detach from the slide! Use ethanol as denaturated alcohol may contain substances which cause fading of preparations. Preparations usually fade within a few weeks when the silver nitrate is reduced only by sunlight or a UV-lamp.
9. Transfer slides to fresh 100 % alcohol for 3 minutes and air-dry again. Mount in synthetic neutral mounting medium (e. g., Eukitt, Euparal).
   Remarks: Slides should be tilted during drying. Mounting medium should be of medium viscosity. The preparation is stable.

REAGENTS

a) Silver nitrate solution (long term stability in brown flask)
   1 g silver nitrate ($AgNO_3$)
   ad 100 ml distilled water

b) Developer (stable for about 1-3 days; must be renewed as soon as it turns dark brown or shows crystals; mix components in the sequence indicated)
   20 ml solution A
   1 ml solution B
   1 ml solution C

   Solution A (this is an ordinary developer for negatives; dissolve ingredients in the sequence indicated; stable for years in brown bottle)
   1000 ml hot tap water (about 40 °C)
   10 g boric acid (H_3BO_3)
   10 g borax ($Na_2B_4O_7$)
   5 g hydroquinone ($C_6H_6O_2$)
   100 g anhydrous sodium sulfite (Na_2SO_3)
   2.5 g metol = methylamino-phenol-sulfate = $(CH_3NHC_6H_4OH)_2 \cdot H_2SO_4$

   Solution B (this is a concentrated photographic paper developer; dissolve ingredients in the sequence indicated; stable for about 6 months in brown bottle; soon turns brown [oxidizes], which, however, does not influence its activity)
   100 ml distilled water
   0.4 g metol = methylamino-phenol-sulfate = $(CH_3NHC_6H_4OH)_2 \cdot H_2SO_4$
   5.2 g anhydrous sodium sulfite (Na_2SO_3)
   1.2 g hydroquinone ($C_6H_6O_2$)
   10.4 g sodium carbonate (Na_2CO_3)
   10.4 g potassium carbonate (K_2CO_3)

0.4 g potassium bromide (KBr)

Solution C (stable for several years)
10 g sodium hydroxide (NaOH)
ad 100 ml distilled water

c) Fixative for impregnation (stable for several years)
25 g sodium thiosulfate ($Na_2S_2O_3$. $5H_2O$)
ad 1000 ml distilled water

LITERATURE CITED

1. Foissner, W. 1976. Erfahrungen mit einer trockenen Silberimprägnationsmethode zur Darstellung argyrophiler Strukturen bei Protisten. *Verh. zool.-bot. Ges. Wien* **115**:68-79.
2. Gelei, J v. 1935. Eine neue Abänderung der Klein'schen trockenen Silbermethode und das Silberliniensystem von *Glaucoma scintillans*. *Arch. Protistenk.* **84**:446-455.
3. Klein, B M. 1926. Über eine neue Eigentümlichkeit der Pellicula von *Chilodon uncinatus* Ehrbg. *Zool. Anz.* **67**:1-2.
4. Klein, B M. 1958. The "dry" silver method and its proper use. *J. Protozool.* **5**: 99-103.
5. Ruzicka, F K. 1966. Eine neue Methode zur Darstellung des Silberliniensystems bei Wimpertieren. *Mikrokosmos* year 1966, Heft 6:180-183.

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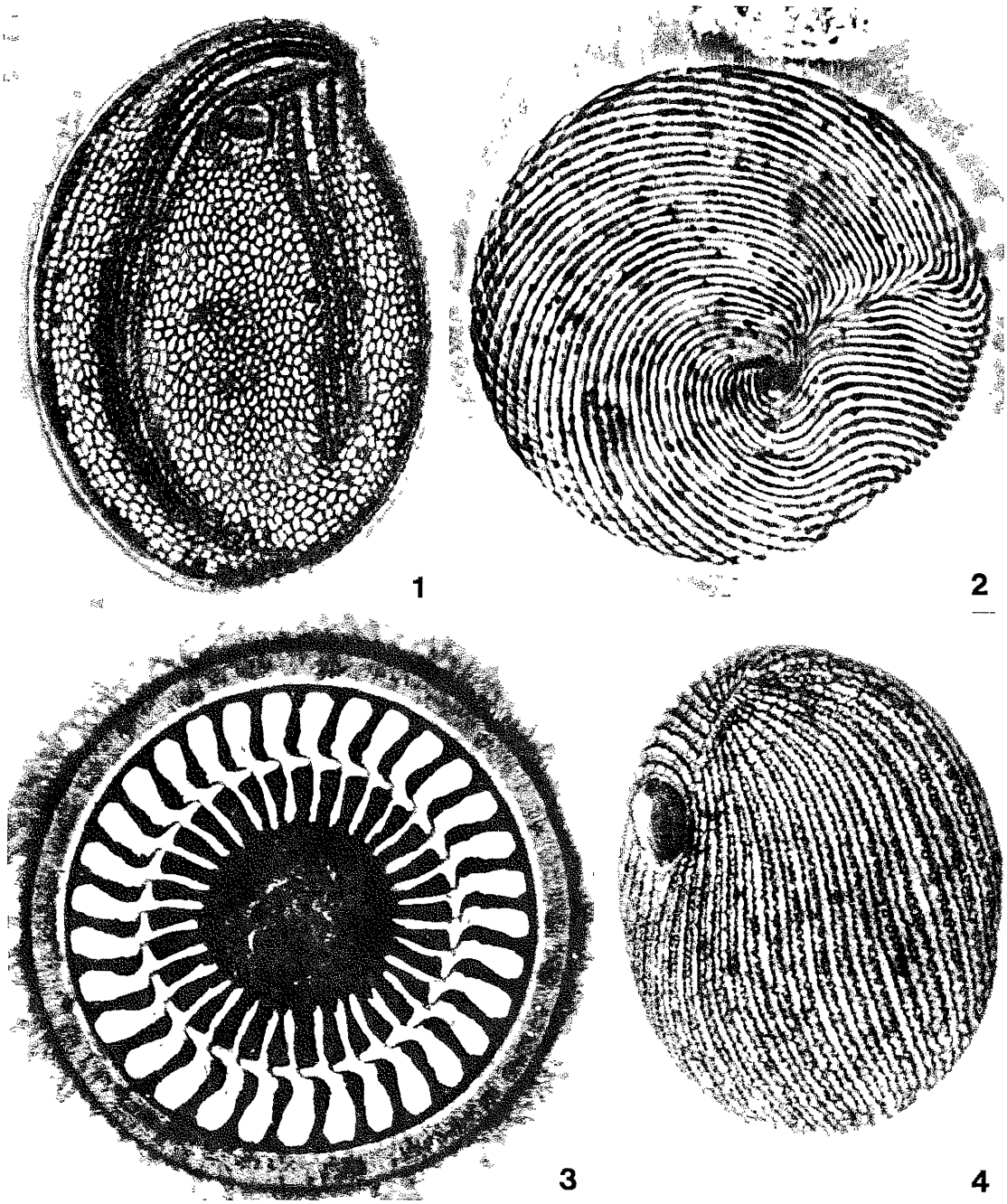

Fig. 1 - 4. Protists prepared with the dry silver nitrate protocol described. **1.** *Chilodonella uncinata*, a cyrtophorid ciliate; ventral view, length about 45 µm. **2.** *Peranema trichophorum*, a heterotrophic euglenoid flagellate; contracted specimen, diameter about 20 µm. **3.** *Trichodina mutabilis*, a mobiline peritrichous ciliate; adhesive disc, diameter about 50 µm. **4.** *Glaucoma scintillans*, a tetrahymenid ciliate; ventro-lateral view, length about 50 µm.

FEULGEN STAINING THE NUCLEI OF FORAMINIFERA

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Introduction

Some of the foraminifera have very thin and diffuse chromosomes which are very hard to stain and visualize by standard methods. The fixation methods used by Grell (2) followed by the Feulgen staining modifications of Rafalko (5) give generally give quite good results. Often it is useful to grow foraminifera on microscope slides placed on the bottom of large (150 mm) petri dishes. This facilitates removal of specimens without injury from the culture for examination in a compound microscope, with water emersion lenses, provided the test is translucent. If the foraminifera are small enough (e.g.,*Rotalliella elatiana*), the foraminifera can be attached to the slides with the aid of collodion and stained through their tests without the additional effort of embedment and sectioning (4).

Protocol

1. Of the several different fixatives which have been succesfully employed to fix foraminifera: Bouin's(1); Bouin-Dubosq(2); and Zenker's(3), the Bouin-Dubosq fixatiative seems the best starting point for a previously unstudied organism. A fixation time of 30 minutes at room temperature seems to work well with this fixative.
2. Prepare the bleaching solution as follows: 12 N HCL 1.0ml; $Na_2S_2O_5$ 0.4g/100ml distilled water.
3. Prepare the Schiff's reagent as follows:
   a)Boil 200 ml of water;
   b)Add 1 g basic fuchsin(C.I. 42500);
   c)Shake thoroughly;
   d)Cool to 50°;
   e)Add $Na_2S_2O_5$ 1 g;
   f) Add 1 N HCl 20 ml;
   g)If the solution is not water white, add 200 mg activated charcoal; shake for several minutes;
   h)Filter the solution;
   i)Place in a glass stoppered reagent bottle with a minimum of air space above the solution;
   j)Store in the refrigerator.
4. Deparaffinize embedded,sectioned, and mounted slides if the foraminifera have been prepared this way, or wash out the fixative of small, collodion enrobed, foraminifera.
5. Rinse in N HCl for 2 minutes at room temperature .
6. Hydrolyze in N HCl for 15 minutes at 60°C. (30 minutes at 50° also works well[4]).
7. Rinse in N HCl at room temperature.
8. Rinse in distilled water.

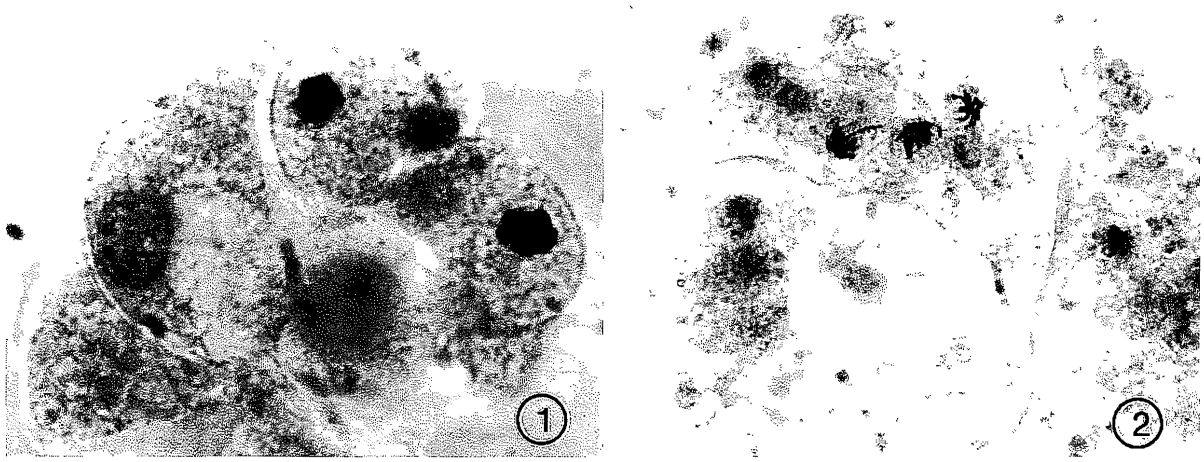
9. Stain in Schiff's reagent for 2 hours in the dark.
10. Drain, blot, and transfer quickly into the first bleaching solution.
11. After 2 minutes, blot and transfer to fresh bleaching solution.
12. Repeat step 11.
13. Rinse in distilled water.
14. Dehydrate in a graded series of ethyl alcohol to 95% alcohol.
15. Counterstain in fast green for 0.5-2 minutes. (Fast green FCF, C.I. 42053, 50mg% in 95% ethanol).
16. Continue dehydration and mount in a medium with a high refractive index (e.g., Permount,Fisher Scientific Co.)Use a number 0 coverglass so that high resolution lenses, with short working distances, can be used on the very thin chromosomes.

COMMENTS

The chromosomes of some foraminifera are so thin that they are not immediately apparent to the inexperienced observer, however, phase contrast makes observation easier. The use of a green filter also enhanses contrast and improves the quality of photographs. Depending upon the species studied, it has sometimes been necessary to vary the hydrolysis time to improve the quality of the preparations.

LITERATURE CITED

1. Arnold, Z. 1955. Life history of the foraminifer *Allogromia Laticollaris. Univ. Calif. Publ. Zool.,* **61**:167-252.
2. Grell, K G. 1954 Der Generationswechsel des polythalamen Foraminifere *Rotaliella heterocaryotica. Arch. Protistemk.*,**100**:268-269.
3. Lee, J J & McEnery,M E. 1970. Autogamy in *Allogromia laticollaris* (Foraminifera). *J. Protozool.,* **17:** 184-195.
4 Pawlowski, J & Lee, J J. 1992. The life cycle of *Rotaliella elatiana* N. Sp.: a tiny macroalgavorus foraminifer from the Gulf of Elat. *J. Protozool.*, **39**: 131-143.
5. Rafalko, J S. 1946. A modified Feulgen technic for small and difuse chromatin elements. *Stain Techn.*, **21**: 91-93.



Figures of agamonts of *Rotaliella elatiana* from (4). Both stained following the Feulgen protocol outlined above. A green filter was used to enhanse contrast. Photographed using Kodak Technical Pan 2415, at ASA 100, and developed with Kodak HC-110 (dilution B). Fig. 1. Specimen with 3 small generative and one somatic nucleus in the optical plane. Fig 2. Agamont with nuclei in second meiotic divisions.

FIXATION FOR IMMUNOLABELLING OF CELLS AND TISSUE FOR TEM

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INTRODUCTION

The process of fixation of cells or tissue for immuno-transmission electron microscopy requires a trade-off between preservation of antigenic sites and ultrastructural preservation. At the light microscopical level using immunofluorescence techniques this is usually not a problem since cells or tissues will not be examined at high resolution; therefore, fixation and extraction procedures used to reveal antigenic sites are not as critical. Cells and tissues are usually permeabilized to get the antibodies in and this essentially punches holes in cells, which at the TEM level, would be objectionable. However, at the EM level, resolution and preservation of antigens are important parameters. The conventional electron optical procedures used for fixation, such as glutaraldehyde followed by post fixation in osmium tetroxide are not adequate due to the loss of antigens and as a consequence, immunoreactivity, although cytoplasmic preservation is in most cases excellent. In most instances, optimal fixation for immunocytochemistry has relied on fixation with paraformaldehyde as the major component of fixatives with addition of low percentages of glutaraldehyde, generally less than 1% (i.e. 0.1-0.2% the most common). Needless to say, there are hundreds of fixation recipes that have emerged over the past 20 years. We initially started with the fixation protocol of Osborn and Weber (1, see also 2) which incidentally used 2.5% glutaraldehyde as the primary fixative followed by treatment with Sodium Borohydride to block exposed aldehyde sites. However, recently we are employing a paraformaldehyde-glutaraldehyde cocktail using concentrations of paraformaldehyde from 1-4% and glutaraldehyde concentrations of 0.1 - 0.2%. Higher concentrations of paraformaldehyde (3-4%) are used for mammalian tissues, other solid tissues rather than cell suspensions or protozoa.

It is beyond the purview of this author to provide an extensive literature review. The reader is referred to several texts on the topic of immunolabelling for TEM and SEM, especially a text by Polak and Varndell (3) and the 3 volume series by Hayat (4,5,6). Aikawa and Atkinson (7) have also published a review paper on immunoelectron microscopy of parasites in which is appended a complete protocol for processing of cells for immunoelectron microscopy. The current fixative we are using for immunocytochemistry for a wide variety of organisms, e.g. trypanosomes, ciliates, mycoplasmas, tissue culture cells and mammalian tissue is presented below.

PROTOCOL

A. Fixation

Fix tissue in 2.0% paraformaldehyde, 0.2% gluataraldehyde in 0.1M buffer, pH 7.4 (Sorensen's Phosphate buffer is fine). For tissues you can use up to 4% paraformaldehyde.

1. To make fixative mix 2 grams (or more) of paraformaldehyde to 45 ml of double distilled water, cover and boil on hot plate for 30 minutes. This will dissolve the paraformaldehyde and the mixture will appear milky white.
2. Add 4-6 drops of 1N-NAOH; the fixative should clear.
3. Add 50 ml of 0.2M phosphate buffer pH 7.4.

4. Add 2.5 ml of 8% glutaraldehyde solution or 0.8 ml of 25% glutaraldehyde.
5. Check pH and increase volume to 100 ml.
6. Fix tissue for 10-30 min. at room temperature. Depending on the density of the material, it has been found that tissues generally require longer times, sometimes overnight. However, prolonged fixation in paraformaldehyde promotes cross-linking which may be detrimental to preserving antigenicity. Here trial and error are necessary to insure optimal fixation times.

For example, Aikawa & Atkinson use 1.0% paraformaldehyde and 0.2% glutaraldehyde for fixing Plasmodium-infected erythrocytes (10 min. at room temperature) while Williams et al. (8) use 2% paraformaldehyde with 0.5% glutaraldehyde for 1 hr. at 4°C to fix the ciliate Euplotes.

LITERATURE CITED

1. Osborn, K H & Weber, K. 1982 Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin containing structures. *Meth. Cell Biol.* **24**:98-132.
2. Paulin, J J, Keith, C H & Tarleton, R. 1987 A monoclonal antibody to alpha tubulin recognizes host cell and Trypanosoma cruzi tubulins. *J. Protozool.* **35**(1):123-129.
3. Polak, J M & I M Varndell. 1984 Immunolabelling for Electron Microscopy. *Elsevier Science Publications,* Amsterdam, The Netherlands, and New York, N.Y.
4. Hayat, M A. 1989 Colloidal Gold: Principles, Methods and Applications. *Academic Press,* San Diego and London, Vol. 1, 536 pp.
5. Hayat, M A. 1989 Colloidal Gold: Principles, Methods and Applications. *Academic Press,* San Diego and London, Vol. 2, 484 pp.
6. Hayat, M A. 1991 Colloidal Gold: Principles, Methods and Applications. *Academic Press,* San Diego and London, Vol. 3, 421 pp.
7. Aikawa, M & Atkinson, C. 1990 Immunoelectron microscopy of parasites. *Adv. Parasitol.* **29**:151-214.
8. Williams, N E, Honts, J E, Qun, L, Olson, C L & Moore, K C. 1989 Identification and localization of major cortical proteins in the ciliated protozoan, Euplotes eurystomus. *J. Cell Sci.* **92**:433-439.

FREEZE DRYING OF CELLS FOR SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

Although the drying of cells for SEM by the critical point method of Anderson (1) has been used extensively, it is not necessarily optimal. Leaching of cytoplasmic elements during processing through ethanols and swelling of cells associated with the use of intermediate fluids and/or transitional fluids are common artifacts associated with the application of the critical point method (2). Freeze drying is a good alternative, especially in laboratories where the apparatus for drying by the critical point method is not available.

PROTOCOL

1. Concentrate organisms either by light centrifugation to obtain a pellet or pipetting organisms into a depression slide.
2. Rinse organisms in several changes of a balanced physiological buffer, phosphate buffered saline or Sorensen's phosphate buffer, pH 7.0. Buffers used in the culture media of axenically or monoxenically grown protists are equally good as well as filtered seawater. The purpose of this step is to remove foreign particulates from the surface of the cells (i.e. debris, bacteria, diatoms, etc.) while the cells retain their normal shape. Ideally, the rinse buffer should be isotonic with the cells to prevent shrinking or swelling. Two to three washes are sufficient, either by centrifugation procedures or pipetting.
3. Fix organisms in a fixative of your choice either that used for TEM - Glutaraldehyde followed by Osmium tetroxide or Parducz's fixative (6 parts 2% aqueous osmium tetroxide, 1 part saturated mercuric chloride). We routinely use Parducz's for all sorts of cell types (e.g. bacteria, protists and tissue culture cells).
4. Rinse cells briefly in distilled water, 2-3 changes (1 minute each) to eliminate salts that may precipitate on the surface of the cells during the freeze drying process. Prolonged soaking of cells in distilled water will cause cells to swell. Your physiological buffer can be used here, but you run the risk of having, for example, a sodium chloride crystal sitting on your organism after drying.
5. Quick freeze the specimens. This can be accomplished in several ways. Float an aluminum weighing pan 5 cm is dia or less on the surface of liquid nitrogen. Pipette drops of cells in suspension on the aluminum surface, drops of 200-400 μl are about the right size. Keep the tip of the pipettes well above the cold aluminum surface; otherwise, the cells will freeze in the pipette! An alternative method is to place the cells in small drops on the aluminum weighing pan and then transfer the pan to the surface of the liquid nitrogen. This avoids the possible precooling of the specimen in the pipette before they are actually frozen.
6. Freeze drying is accomplished by transferring cells to a precooled brass or copper block that has been precooled to liquid nitrogen temperature. We use a brass block six centimeters in diameter and three centimeters high. The block has six holes drilled in it to accept the peg of an SEM stub. Before cooling the block, 6 scanning stubs are cleaned and placed on the block. We use a round styrofoam container (ID 16m x 7 cm deep) about 3/4 full of liquid nitrogen to cool the block and stubs. The block acts as a heat sink. We have found that the mass of this block is sufficient to allow cells to sublime in about 8

hours. The sublimation rate is critical, if it is too rapid, the cells tend to collapse. The precooled block with the stubs mounted should be placed on a glass petri dish or other insulator on the stage of a vacuum evaporator, again to prevent the block from heating rapidly via being in direct contact with the vacuum evaporator. This is done immediately before transferring cells to the stubs to avoid frost buildup.

7. Transfer of cells is accomplished by removal of frozen droplets of cells from weighing pans onto the stubs. This should be done quickly with a pair of forceps (EM type) cooled to liquid N_2 temperature. Two to three drops can be placed on a stub. After all stubs are loaded the bell jar is lowered and high vacuum obtained. In most instances we allow the material to sublime over night. The following day, the stubs are removed and coated before viewing in the SEM.
8. We have had good results with ciliates as large as Stentor (3) and small flagellates like Crithidia sp.

Note: The number of cells in a drop should not be excessive, otherwise you will have cells piled on top of each other after drying. Additionally, if the frozen droplets are allowed to stand in the aluminum pan floating on LN_2 for extended periods of time, liquid air will condense in the pan. This can be a nuisance. We routinely use a styrofoam box half filled with LN_2 to float our pans on. This can be moved to the vacuum evaporator so that the distance of transfer of cells to the stubs is minimized. We have also found that the Pearse tissue dryer is not sufficient for good preservation of cells, due to the rapid rate of sublimation at -50°C and the poor vacuum obtained.

The opening of the air inlet valve to the vacuum evaporator should be done slowly, otherwise you will blow some cells of the stub if the vacuum release valve is opened wide open. We have a baffle in our air inlet to avoid the big gush of air from entering the bell jar when the air inlet valve is opened.

LITERATURE CITED

1. Anderson, T F. 1951 Techniques for the preservation of three dimensional structure in preparing specimens for the electron microscope. *Trans. N.Y. Acad. Sci. Ser. II,* **13**:130.
2. Boyde, A. 1974 Freezing, freeze fracturing and freeze drying in biological specimen preparation for SEM. *Scan. Elec. Micro., IITRI/SEM,* 1043-1046.
3. Paulin, J J & Bussey, J. 1971 Oral regeneration in the ciliate Stentor coeruleus: a scanning and transmission electron optical study. *J. Protozool.*, **18**:201-213.

RAPID FREEZING AND FREEZE SUBSTITUTION OF PROTOZOA FOR TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

One of the greatest problems in preparing protozoa for examination in the electron microscope involves arresting cellular processes quickly before artificial changes can occur. Such artifacts may be as minor as a slight swelling of the cell or an extraction of some cellular components (e.g. lipids) or as drastic as the shedding of flagella or even total disintegration of the cell. Usually these changes are a direct result of the time it takes for a chemical fixative to act and cross-link cellular structures. For this reason ultrarapid freezing and freeze substitution have been used with great success in the preservation of protozoa (1, 2). The technique is successful for several reasons: 1) cellular processes are arrested on the order of milliseconds whereas chemical fixatives act much more slowly, 2) under ideal freezing conditions the nucleation of ice crystals is kept to a minimum so that these do not result in observable damage, 3) structural preservation is often superior to chemically fixed material (3) (e.g. smoother membranes, better retention of cytoplasmic components), and 4) damage resulting from osmotic shock is eliminated.

PROTOCOL

1. Collect cells in as concentrated form as possible either through centrifugation or filtration onto acetone resistent filters with a 2 μm pore size or smaller.
2. While keeping liquid volume to a minimum rapidly freeze cells using any of a variety of methods (4) (i.e. contact "slam" freezing, liquid propane or nitrogen slush plunge freezing, atomization onto a pre-cooled surface, propane jet freezing). Store frozen samples in liquid nitrogen until ready for further processing.
3. Replace the aqueous component of the cell with an organic solvent. Usually this is done by substituting the cells for 24-72 hours at -80°C in a solution of 1.0% osmium tetroxide dissolved in 100% acetone*.
4. Gradually warm cells according to the following schedule:
   4 hours at -40°C
   2 hours at -20°C
   2 hours at 0°C
   2 hours at 20°C
5. Rinse cells three times in 100% acetone at 20°C.
6. If the cells are to be examined using scanning electron microscopy they should be critical point dried, mounted, and coated with gold-palladium prior to viewing in the SEM (5).
7. If cells are to be examined using the transmission electron microscope they should be infiltrated in embedding resin and polymerized as usual.

* Osmium tetroxide should be dissolved in pre-cooled -80°C acetone as osmium tetroxide tends to oxidize quickly in room temperature acetone. For cytochemical studies osmium tetroxide should be avoided and cells should be substituted in 100% -80°C acetone (6).

LITERATURE CITED

1. Farmer, M A & Roberts, K R. 1989 Comparative analysis of the dinoflagellate flagellar apparatus. III. Freeze substitution of *Amphidinium rhynchocephalum*. *J. Phycol.* 25:280-292.

2. Lokhorst, G M & Segaar, P J. 1989 Ultrastructure of zoosporogenesis in the alga *Botrydiopsis alpina* (Tribophyceae), as revealed by cryofixation and freeze substitution. *Europ. J. Protistol.* **24**:260-270.
3. Aldrich, H C. 1989 Practical aspects of freeze-substitution. *EMSA Bull.* **19**:53-59.
4. Gilkey, J C & Staehelin, A. 1986 Advances in ultrarapid freezing for the preservation of cellular structure. *J. Elec. Microsc. Tech.* **3**:177-210.
5. Barlow, D I & Sleigh, M A. 1979 Freeze substitution for preservation of ciliated surfaces for scanning electron microscopy. *J. Microsc.* **115**:81-95.
6. Monaghan, P & Robertson, D. 1990 Freeze-substitution without aldehyde or osmium fixatives: ultrastructure and implications for immunocytochemistry. *J. Microsc.* **158**:355-363.

GENERAL COMMENTS ON FIXATION OF PROTOZOA FOR TRANSMISSION ELECTRON MICROSCOPY

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Because of the various ecological niches protozoa occupy, the electron microscopist is confronted with designing a fixative protocol that best suits the organism and what the expected results are to be used for, e.g. morphological analysis, immunocytochemistry or other cytochemical techniques. There are literally hundreds of recipes in the literature. Their selection and application are often bewildering to the novice and the seasoned microscopist alike. Experience however has its merits. The investigator should not look upon a fixative as a single entity, rather it is the sum of its ingredients, namely the fixative proper (glutaraldehyde, osmium tetroxide, etc.), the buffering vehicle (phosphate(s), sodium cacodylate, Pipes) and additives (NaCL, sucrose, $MgCL_2$, etc.). All of these play an important role and contribute to the overall tonicity of the fixative (1). Some will argue that the tonicity as measured in milliosmols by freezing point depression of primary fixatives and buffering systems is not of prime importance (2). However, even in the classic paper of Maser et al. (3) and more recently in excellent reviews on fixation by Bullock (4) and Coetzee and van der Merwe (5). Emphasis is placed on the importance of the buffer vehicle and knowledge of the osmolarity of the total fixative complement. Karnovsky's fixative (6) is frequently cited as a fixative of over 2000 milliosmols that still gives good preservation of tissue even though the tonicity of rat plasma is about 310-340 milliosmols which has usually been used as baseline for computation of fixative solutions for mammalian tissue. The Karnovsky fixative unless modified by dilution of the aldehydes has not been very successful as a fixative for protozoa. In mammalian tissues several factors play a role in the Karnovsky fixative, namely the rate of penetration of formaldehyde as opposed to the slower penetrating glutaraldehyde, the dilution factor of intercellular fluids and the buffer vehicle.

The trend in the use of glutaraldehyde since its introduction almost 30 years ago has been to decrease the percentage (7). Perusal of micrographs published a decade or so ago reveals shrinkage of cells when used in concentrations of 4-6 percent. In our hands we have found concentrations of 2-2.5% to be workable for most flagellates and ciliates, exceptions being the sand dwelling ciliates and some marine flagellates in which Osmium vapor is used as a prefix. Small amoebae, i.e. Entamoeba and dictyostellid amoebae fix well in 2% glutaraldehyde and 0.1 M cacodylate buffer (unpublished). We have also found that in using tables of calculated osmolarities for primary fixatives and buffering solutions as published (see 3,5) that the osmolarity of the fixative solution in most instances is greater than that of media the cells are taken from. For example, a fixative we use for trypanosomes is 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2. This translates into contributions of 210 milliosmols from the buffer at pH 7.2, 165 milliosmols from 2% glutaraldehyde for a grand total of 365 milliosmols which is slightly higher than rat blood plasma. This concentration for free living ciliates is also adequate even though the osmolarity of freshwater is considerably less. The take home message when thinking about using a known fixative recipe or formulating one, look up your components and see what the approximate osmolarity is before working with your cells. If you have access to an osmometer, you can measure by freezing point depression the osmolarity of the media your cells are bathed and adjust your fixative and buffer system accordingly. In the past we have found that fixation is improved if the osmolar concentration of the fixative is greater than the fluids cells are bathed in. Additionally, Hassel and Hand (8) have introduced some new diimidoesters that cross-link proteins and may be used in place of glutaraldehyde.

LITERATURE CITED

1. Hayat, M A. 1989 Principles and Techniques of Electron Microscopy. CRC Press, Boca Raton, FL, USA, pp. 4-68.
2. Gonzáles-Aguilar, F. 1982 Cell volume preservation and reflection coefficient in chemical fixation. *J. Ultra. Res.* **80**:354-362.
3. Maser, M, Powell, E & Philpott, C. 1967 Relationships among pH, osmolality and concentration of fixative solutions. *Stain Technol.* **42**:175-182.
4. Bullock, G R. 1984 The current status of fixation for electron microscopy: a review. *J. Micros.* **133**:1-15.
5. Coetzee, J & van der Merwe, C F. 1987 Some characteristics of the buffer vehicle in glutaraldehyde based fixatives. *J. Micros.* **146**:143-155.
6. Karnovsky, M J. 1965 A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137a.
7. Sabatini, D D, Bensch, K & Barrnett, J. 1963 The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19-58.
8. Hassel, J & Hand, A R. 1974 Tissue fixation with diimidoesters as an alternative to aldehydes. I Comparison of crosslinking and ultrastructure obtained by dimethylsuberimidate and glutaraldehyde. *J. Histochem. Cytochem.* **22**:223-239.

FIXATION OF PROTOZOA WITH COMBINATIONS OF GLUTARALDEHYDE, FORMALDEHYDE AND OSMIUM TETROXIDE

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INTRODUCTION

In 1966, Trump and Bulger (1) formulated a fixative "cocktail" containing glutaraldehyde and osmium tetroxide to take advantage of the excellent preservation of proteins by glutaraldehyde and the ability of osmium tetroxide to fix lipids. Karnovsky (2) introduced the combination of glutaraldehyde and formaldehyde to take advantage of the rapid penetration of formaldehyde and the cross-linking characteristics of glutaraldehyde. The Karnovsky fixative was formulated to preserve cells for histochemical studies while the Trump and Bulges techniques provided excellent overall fixation of cells for morphological studies since the osmium tetroxide would negate any histochemical applications. Chang (3) compared both techniques on Hela cells and concluded the combination of glutaraldehyde and osmium tetroxide preserved cellular detail better than Karnovsky's fixative and glutaraldehyde followed by post-fixation in osmium tetroxide. Shigenaka et al. (4) found that combinations of glutaraldehyde and osmium tetroxide provided excellent fixation of hypotrichous ciliates. In our laboratory, we have found this a very useful fixative for hemoflagellates and other ciliates. Recently, Schrenk and Bardele (5) have also demonstrated the excellent preservation of this fixative as applied to the ciliate, Saprodinium.

We have found that Karnovsky's original formulation does not work well with most protozoa. The fixative has an osmolarity of about 2,010 milliosmols and in most cases will shrink cells. Soyer (6) reported excellent preservation of the nuclear structures of dinoflagellates by modifying the original Karnovsky's recipe (see below).

PROTOCOL 1

Glutaraldehyde/osmium tetroxide fixative according to Shingenaka et al. (4).

1. Prepare stock solutions:

Solution A: M/15 phosphate buffer (pH 7)	7.2ml
1mM $MgSO_4$	0.2ml
0.1M Sucrose	0.2ml
25% ^{v}/v Glutaraldehyde	2.4ml
Solution B: 2% OSO_4 (Aqueous)	10ml

2. Mix solutions A and B just before using (room temperature). If solutions are allowed to stand, the fixative will turn a blackish/blue color due to the reduction of osmium tetroxide. The final concentration of the mixture above will be 3% glutaraldehyde ^{v}/v; 1% osmium tetroxide ^{w}/v: $MgSO_4$ 0.01mM and sucrose, 1mM. Fixation time, 30 min.
3. In some instances, we have found that if fixation is done at 4°C, better preservation is achieved and the deteriation of the fixative will not occur. We routinely cool both A & B solutions.

4. Sodium cacodylate buffer (0.2M) can also be substituted for the phosphate buffer. We have not used phosphate buffers for routine TEM because cytoplasmic structures have a grainey appearance of high magnifications.

PROTOCOL 2

Karnovsky's original Glutaraldehyde-Paraformaldehyde formulation:

1. Dissolve 2g of paraformaldehyde powder in 25ml distilled water, heat to 60-70° and stir. This generally takes about 30 minutes. The solution will appear milky white.
2. Add 1-3 drops of 1N NaOH while stirring until solution clears in a matter of seconds. If not, the mixture needs to be heated longer to dissolve paraformaldehyde.
3. Cool solution and add 5ml of 50% glutaraldehyde.
4. Bring total volume to 50ml with 0.2M cacodylate buffer.
5. Add 25mg of $CaCL_2$ (anhydrous).
6. Adjust pH to 7.2.
7. Add fixative to cells.
8. If you are preparing cells for histochemical work, dehydrate and embed as usual. For morphological work, cells can be post-fixed in osmium tetroxide with subsequent dehydration and embedding.

PROTOCOL 3

Soyer modification of Karnovsky's Fixative:

1. 8% paraformaldehyde (prepare as above and cool before using) 12.5 ml
   12.5% glutaraldehyde 5 ml
   Distilled H_2O (double distilled) 7.5 ml
   0.2M piperazine buffer (Pipes), pH 7.0 25 ml
   To prepare, dissolve 6.047g piperazine in 25ml distilled H_2O, adjust pH to 7.0 with 1N NaOH; bring volume to 50ml with distilled H_2O
2. Fix cells for 60 minutes.
3. Wash cell for 60 minutes in the Pipes buffer.
4. Post-fix in 2% osmium tetroxide in Pipes buffer.
5. Wash in buffer.
6. Dehydrate and embed as usual.

LITERATURE CITED

1. Trump, B F & Bulger, R E. 1966 New ultrastructural characteristics of cells fixed in a glutaraldehyde-osmium tetroxide mixture. *Lab. Invest.* **15**:368-379.

2. Karanosky, M J. 1965 A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137A.

3. Chang, J H. 1972 Fixation and embedment, in situ, of tissue culture cells for electron microscopy. *Tissue and Cell* **4**:461-574.

4. Shigenaka, Y, Watanabe, K & Kaneda, M. 1973 Effects of glutaraldehyde and osmium tetroxide on hypotrichous ciliates and determination of the most satisfactory fixation methods for electron microscopy. *J. Protozool.* **20**:414-420.

5. Schrenk, H-G & Bardele, C F. 1991 The fine structure of Saprodinium dentatum Louterborn, 1908 as a representative of the Odontostomatida (Ciliophora). *J. Protozool.* **38**:278-293.

6. Soyer, M-O. 1977 Une modification de la technique de Karnovsky pour la préservation optimale des structures nucléaires chez les dinoflagellés. *Bio. Cellu.* **30**:297-300.

POLY-L-LYSINE ADHESIVE FOR CELL SUSPENSION AND REFERENCE TO OTHER ADHESIVES USED IN SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

Poly-L-Lysine has been used with success in our laboratory for affixing various cell types to grids and coverslips during processing specimens for TEM and SEM. Two methods have been employed and described below. The reader is also referred to the papers by Whitcomb (2,3) in which approximately 169 adhesive materials have been evaluated.

PROTOCOL 1

I. As described by Mazia et al. (1)
   A. 0.1% Poly-L-Lysine (80,000-100,000 daltons) is dissolved in water.
   B. The polylysine is dispersed over the surface of a coverslip.
   C. The coverslip is washed with running water.
   D. The coverslip is washed with a medium appropriate for the cell suspension.
   E. The cell suspension is placed on the coverslip and the cells allowed to settle.
   Once the cells are attached to the coverslip, fixation, dehydration and critical point drying may be carried out without risk of loosing the cells.

PROTOCOL 2

II. Modified version
   A. Poly-L-Lysine (MW 115,000) is prepared in phosphate buffered saline, pH 7.2.
   B. A drop of polylysine is placed in the center of a 12 mm round coverslip (a size which conveniently fits aluminum specimen stubs) and allowed to stand for one hour at room temperature.
   C. Meanwhile, the cell suspension is fixed and rinsed in buffer.
   D. The polylysine-coated coverslip is rinsed in running water.
   E. A drop of washed cell suspension is placed at the point where the drop of polylysine had been.
   F. The coverslip is then stored in a moist chamber for two hours or overnight allowing the cells time to settle.
   G. The coverslip is dehydrated and critical point dried.

LITERATURE CITED

1. Mazia, D G, Schatten, G & Sale, W. 1975 Adhesion of cells to surfaces coated with polylysine. *J. Cell Biol.* **66**:198-200.

2. Witcomb, M J. 1980 The suitability of various adhesives as mounting media for scanning electron microscopy. *J. Micros.* **121**:289-308.

3. Witcomb, M J. 1985 The suitability of various adhesives as mounting media for scanning electron microscopy. II

C-18.2

General purpose glues. *J. Micros.* **139**:75-114.

PREPARATION OF CELLS FOR SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

Since the introduction of the first commercially available scanning electron microscope in the late 1960's, a voluminous literature has accumulated on the subject of specimen preparation. The greatest single source of information on this topic is found in the volumes of papers and tutorials published in the proceedings of Scanning Electron Microscopy". The reader is also referred to the review of techniques used to prepare a variety of fresh water invertebrates including protozoa published by Maugel et al. (1).

Unlike specimen preparation for transmission electron microscopy of protozoa, samples for SEM are prepared to preserve surface features rather than internal structures, unless samples cracked open to reveal internal features. In instances where TEM is to be used on the same samples viewed in the SEM, fixation protocols used for TEM are used. These protocols are not necessarily optimum for SEM. For SEM, special precautions must be taken to prepare clean surfaces, free of artifacts (e.g. detritus, mucous and other foreign objects) and yet maintain good preservation of delicate features such as cilia, flagella, pseudopodia and membrane topographical features.

In our laboratory, we have relied primarily on the Parducz's fixative (2) as initially used by Marszalek and Small (3). The fixative as used by Parducz was designed to stop ciliary motion instantaneously in situ. We have used the fixation protocol outlined below to fix a wide variety of freshwater, marine and parasitic ciliates and flagellates, amoebae, apicomplexan sporozoites, gregarines, fungi, bacteria, tissue culture cells, the brush boarder of intestinal epithelia and a wide variety of plant and animal tissues too numerous to mention. Combinations of glutaraldehyde and osmium tetroxide are also useful, especially if TEM is to be used on the same specimen. Specimens prepared for TEM after fixing in Parducz's and re-embedded after viewing in the SEM reveal well preserved cortical features and in most instances mitochondria, and nuclei are identifiable but the cytoplasm shows vacuolation and clumped endoplasmic reticulum and in general poor intracellular preservation (personal observations).

PROTOCOL 1

A. Cleaning cells of surface artifacts is the first step in preparing samples for SEM. Regardless of the niche cells are taken from, they must be gently cleaned.
   1. Cells are concentrated from their source either by light centrifugation, hand picking or whatever means are used to concentrate cells.
   2. Wash cells in a good physiological buffer such as phosphate buffered saline, Pipes, the buffer used to formulate the media cells are grown in, etc. Usually 1 or 2 washes are sufficient. The duration of the wash should be short, less then one minute each. With protozoa, you want to keep the cells close to isotonic conditions. These steps can be conveniently done in a centrifuge tube or depression slide.

B. Fixation
   1. Stock fixative solution consists of 1 part saturated mercuric chloride to 6 parts 2% osmium tetroxide (aqueous) made just before using.
   2. The fixative can be poured on the cells after the buffer wash has been removed. The volume of fixative should be at least 4 X the concentrated cell volume.
   3. The time of fixation is critical. We have found the fixation should be completed within 2 minutes. Prolonged fixation over 5 minutes is detrimental, cells tend to swell, cilia and flagella may bleb and the delicate pseudopods of tissue culture cells or slime molds maybe destroyed. Trial and error to determine the optimum fixation time may be necessary to suit the organism you are working with.

C. Washing
   1. Cells are transferred to distilled water. Usually 2 washes, 1 minute each is sufficient. This is to remove fixative and also salts that may precipitate out during the drying process. Again, avoid prolonged washing to prevent swelling.

D. Attaching cells to coverslips or other surfaces for processing
   1. Suspensions of cells can be transferred to polylysine coated coverslips as outlined by Mazia et al. (4).
   2. See Protocol on application of polylysine.
   3. Round glass coverslips (12mm dia) the same size as the aluminum specimen stubs used in a wide variety of SEMs can be coated with polylysine and the cells placed on them with a micropipette. After the cells adhere the coverslips can be passed through an ascending series of ethanols for dehydration and eventually for drying by the critical point method.
   4. Round coverslips can also be placed in culture media containing organisms that are sessile, suctorians, peritrichs, chonotrichs, etc. Additionally, amoebae and motile stages of Mycetozoa will also adhere. Tissue culture cells can also be grown on sterile coverslips. Cells grown on coverslips can be carried through steps A-C above and then dehydrated and dried by the critical point method. Care must be taken to never expose cells on coverslips to air. Evaporation of water or alcohols will cause cells to collapse.
   5. Coverslips can be attached with a small square of double stick tape or other adhesive.
   6. To make good electrical contact between the aluminum stub and coverslip, we ring the coverslip with carbon or silver paste before sputter coating.
   7. Stubs are sputter coated with gold or gold/palladium.

E. Freeze drying of cells
   1. After washing cells in distilled water, they can be freeze dried as described elsewhere in these protocols.

PROTOCOL 2

Fixation with glutaraldehyde and osmium tetroxide in combination or Glutaraldehyde alone (see 3 below)

A. Fixative solutions
   1. 3 or 4% Glutaraldehyde in 0.2 M phosphate buffer (Sorensens, pH 7.2) or 0.2 M cacodylate buffer
   2. 2% OSO_4 in distilled H_2O

B. Mix immediately before use.
   1. 1 part Glutaraldehyde buffer mixture to 2 parts osmium tetroxide; if cells tend to swell, use 1 part v/v of each solution.
   2. Fix for 10-15 minutes.

C. Wash cells briefly in distilled water and dehydrate and dry as necessary.

PROTOCOL 3

A. Use glutaraldehyde at concentrations of 2 or 3% in appropriate buffer, omit osmium tetroxide.
Note: In our hands we have found that fixing protozoa with glutaraldehyde alone does not provide "rigidity" of the cells, cilia and flagella tend to wilt with loss of metachronal wave patterns, etc. The combination of glutaraldehyde and osmium tetroxide is far superior because of the preservation of lipids and also the osmium provides a means for a portion of the surface charge built up by exposure to the scanning beam to be discharged. Osmium is a reasonable conductor.

LITERATURE CITED

1. Maugel, T K, Bonar, D B, Creegan, W J & Small, E B. 1980 Specimen preparation techniques for aquatic organisms. *Scan. Elec. Micros.*, II AMF O'Hare, 57-77.
2. Parducz, B. 1966 Ciliary movement and coordination in ciliates. *Int. Rev. Cytol.* **21**:91-128.
3. Marszalek, D S & Small, E B. 1969 Preparation of soft biological materials for scanning electron microscopy. Proc. 2nd Ann. Scan. Elec. Micro. Symp., IIT Res. Inst., Chicago, Illinois.
4. Mazia, D, Schatten, G & Sale, W. 1975 Adhesion of cells to surfaces coated with polylysine. *J. Cell Biol.* **66**:198-200.

Footnote:
* The publication, Scanning Electron Microscopy, first appeared in April of 1968 and was published under that title until March 1, 1987. At that time, the journal name was changed to Journal of Scanning Microscopy and the first issue (Vol. 1, No. 1) appeared in March of 1987. The format of the Journal remained the same but the cover was changed from hardback to softback.

PREPARATION OF SAMPLES FOR SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

Ciliate species cannot usually be identified solely by scanning electron microscopy because only a limited number of characters are revealed. However, SEM is useful for the beginner by allowing a three-dimensional view of the object and for the specialist in documenting details which are difficult to reveal with other methods.

PROTOCOL

1. Pour ciliates into Parducz's fixative and leave for about 30 minutes. Remarks: Concentrate and clean material as thoroughly as possible (see step 2). Ratio of sample: fixative should be at least 1:1, better, 1:2. Add some drops of 5n HCl if fixative becomes milky when the material is added. Parducz's fluid fixes most ciliates very well. However, the cirri of the hypotrichous ciliates usually disintegrate into their components, i.e. cilia. Hypotrichs should thus be fixed either in concentrated sublimate (dissolve 60 g $HgCl_2$ in 1 litre hot distilled water) or in a mixture composed of 4 parts concentrated sublimate and 1 part 2% osmium tetroxide.
2. Wash ciliates at least 5 times with 0.05 M Na-cacodylate buffer. Remarks: Ciliates in the buffer may be refrigerated for years. Washing must be done in a watch-glass and a micropipette should be used to remove bacteria and detritus. This cleaning of the material is essential but rather difficult and laborious, especially with small species (≪ 100 μm) and field material; thus cultures and/or pre-cleaned material (see below) should be used. The cleaning is performed as follows: Ciliates settle at the bottom of the fixation tube after 30 minutes (cp. step 1). Remove as much supernatant as possible with a pipette (do not centrifuge!). Then transfer the material to a watch-glass and allow to settle for about 5 minutes (use fume hood). Quickly remove most of the fixative with a micropipette under the dissecting microscope. Now wash the ciliates with the buffer by several passages through a large-bore (diameter about 1 mm) pipette. Bacteria and detritus adhering to the ciliates are mechanically removed. Again allow to settle, but control sedimentation with the dissecting microscope; remove supernatant containing bacteria and detritus with a micropipette as soon as ciliates settle. This procedure must be repeated until the material is clean, i.e. the detritus is removed. Use fractionated sedimentation if the sample contains several species differing in size and/or mass.
   Field material: Larger species (≫ 100 μm) are picked out with a micropipette and sprinkled into the fixative. Several hundred specimens must be collected because loss of material may be considerable during the following steps. Small species can be prepared by this method only if abundant material is available. Accumulation can often be achieved by the following simple method: leave a freshly collected sample containing ample mud to stand for some hours at room temperature. Due to oxygen depletion the ciliates usually move to the surface where they can be skimmed off with a teaspoon.
3. Transfer cleaned ciliated with a small drop of buffer to the preparation chamber (see figure below). Remarks: Place the drop on the bottom plankton net of the chamber which is weighed with washer 3. The net must be dry to avoid spreading of the drop to the chamber margin and the washer. Place the top plankton net carefully on the drop, i.e. on washer 3, using forceps. Weight top net with washer 2, close chamber with lid 1 and immediately transfer into 30% ethanol. The plankton net must have a mesh-size ≪ 12 μm and should be used only once. It should fit exactly into the chamber, which is best achieved using an appropriate punch.

4. Dehydrate chamber with ciliates in ethanol series (30-50-70-90-100-100%) for 5 minutes each.
5. Transfer chamber with ciliates to an ethanol:amylacetate series (2:1, 1:1, 1:2) for 5 minutes each.
6. Transfer chamber with ciliates to pure amylacetate ($C_7H_{14}O_2$) for about 12 hours.
7. Dry chamber with ciliates in a critical-point drying apparatus. Remarks: We use CO_2 and change the amylacetate at least 10 times. Sometimes good results are achieved by transferring the ciliates directly from the last ethanol step to CO_2.
8. Open chamber and place ciliates on the prepared SEM stub. Assist with a mounted eye lash if ciliates do not fall from the net. Remarks: The dried ciliates usually form a lump at the bottom plankton net. This lump is carefully transferred (by holding the net over the stub and stripping off the ciliates with the eyelash) to the SEM stub where it is dispersed under the dissecting microscope with the mounted eyelash. The ciliates spread easily if drying was sufficient. Preparation of the SEM stub: We use commercial aluminum SEM stubs. To get a black, homogenous background the stub is covered with a very thin layer of Mixtion à Dorer Clarifiée (Lefranc & Bourgeois, Le Mans) 1.5-3 hours before use. This fluid is used by artists to attach gold foil and is available in ordinary stores. It dries slowly (whereby its surface becomes smooth) and adheres the ciliates to the stub. The mixture may be diluted with turpentine. Note that small species sink into the lacquer more easily than large ones and the mixture should thus be allowed a longer drying time (2-3 hours).
9. Harden the lacquer for a least 48 hours at room temperature. Remarks: This drying is not essential but prevents the development of cracks when the preparation is placed in the vacuum of the scanning electron microscope.
10. Sputter with gold.
11. Stock Solutions

    Parducz's fixative (prepare immediately before use)

    4 ml aqueous 2% osmium tetroxide (OsO_4)

    1 ml concentrated aqueous sublimate solution ($HgCl_2$; preparation see protargol impregnation, Foissner's method)

    0.05M Na-cacodylate buffer (can be stored for several months in the refrigerator; adjust to pH 7 with HCL)

    10.7 g Dimethylarsinacid-Na salt ($C_2H_6AsNaO_2$-$3H_2O$)

    add 1000 ml distilled water

    Amylacetate ($C_7H_{14}O_2$). Use commercial product.

SPECIMEN PROCESSING CHAMBER

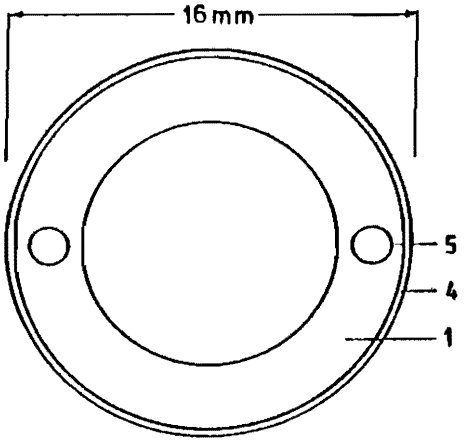


Fig. A

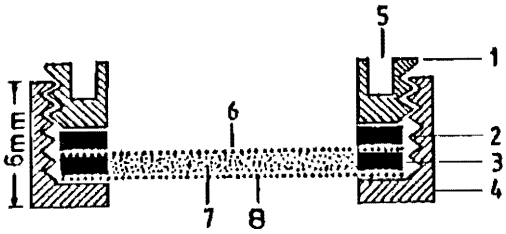


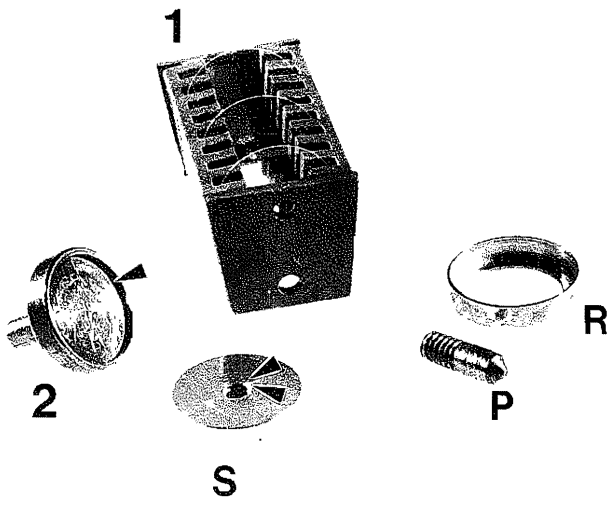
Fig. B

Legend: Figure A Top View of Chamber

Figure B Side View

Numerical Key

1	Threaded clamping ring (lid)
2-3	Washers to hold netting in place and act as spacers to create chamber
4	Base piece into which clamping ring threads
5	Holes partitially drilled into clamping ring (lid) to fasciliate tightening of the clamping ring. A stout forceps or spanner wrench can be used for tightening
6	Top net
7	Protozoa or other material to be processed
8	Lower Net



LEGENDS TO FIGURES 1 & 2

Fig. 1 Slotted carrier for processing coverslips for SEM. The carrier is 3 cm in length by 1.5 cm wide. There are 8 slots for coverslips. The end pieces are not square, but trapezoid, measuring 1.5 cm across the front (slotted side), 1.3 cm across the back and 1.5 cm deep. The end pieces being trapezoids hold the coverslips. Each slot is 1 mm thick and the carrier is made of plastic glued together with superglue. Two holes are bored on the end to facilitate handling with a forceps. The whole carrier can be loaded with coverslips and processed through the dehydration steps and finally placed in the critical point dryer. We generally use 15-25 ml beakers for our dehydrating series. Three coverslips are shown in the carrier.

Fig. 2 Specimen stub modified for SEM. The stub consists of three parts. The stub proper with a depression 1 mm deep (arrow), the peg (P) which is threaded so it can be removed during processing, and the clamping ring (R) which secures a filter to prevent specimens for floating out of the stub reservoir during processing. The clamping ring is made of aluminum and fits over the stub. We use nuclear pore filters (8 μ) or large pore size nets or screening depending on the size of the organisms we are working with. The cells are placed in the reservoir while in 100% ETOH and are never exposed to air. They are then transferred to the critical point drying apparatus. The peg (P) is screwed into the underside of the stub (S, double arrow) before or after sputter coating of specimen. The stub is standard size, 1.3 cm across the top and 1.3 cm from the base of the peg to top of stub.

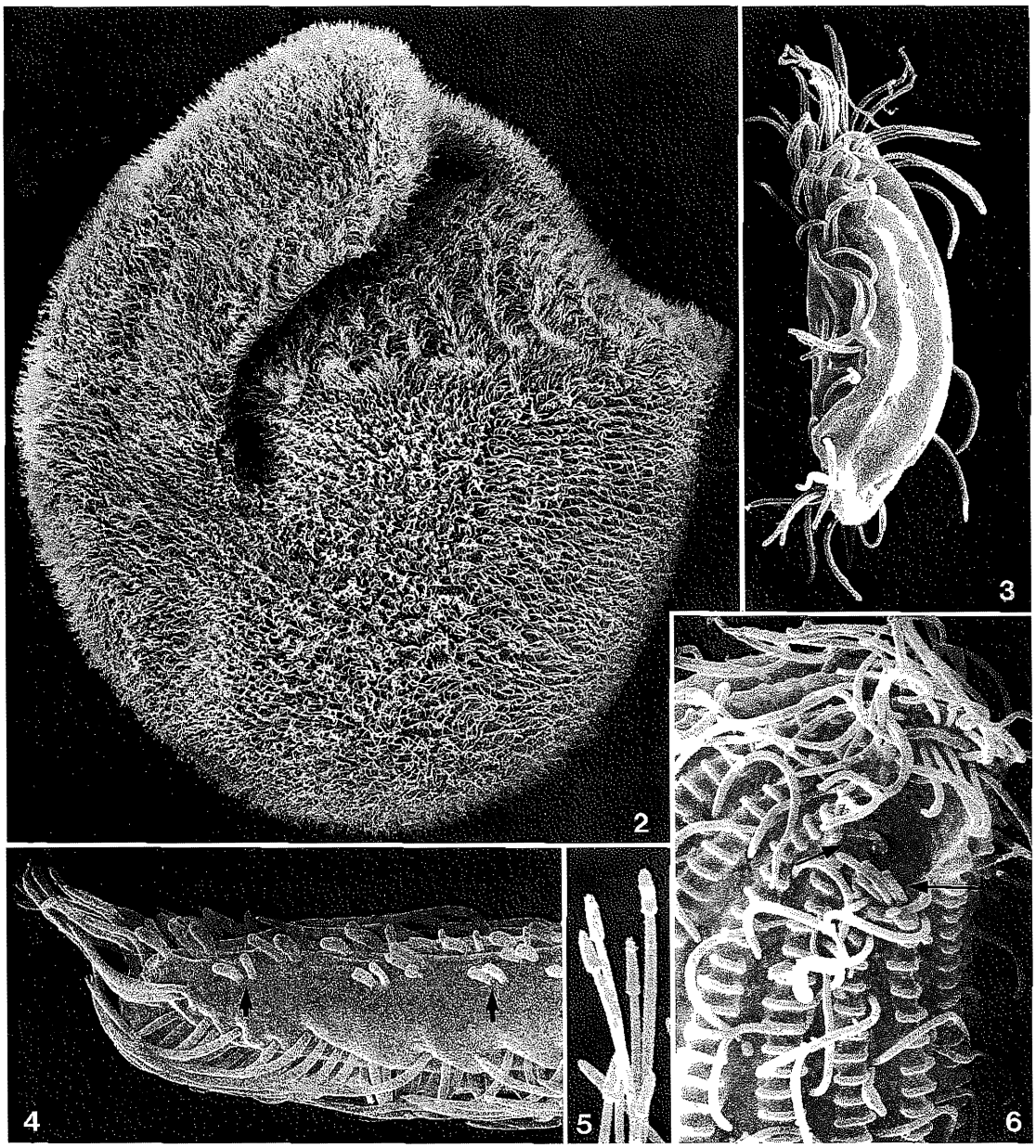


Fig. 2 - 6. Ciliates prepared with the SEM-protocol described. **2.** *Bresslauides discoides*, a huge colpodid ciliate from soil; right lateral view, length about 310 µm. **3.** *Drepanomonas exigua*, a distinctly furrowed microthoracid ciliate; ventral view, length about 30 µm. **4.** *Dileptus mucronatus*, a haptorid ciliate; left lateral view of distal portion of proboscis. Arrows mark the specialized cilia of the dorsal brush. **5.** Distal end of extruded trichocysts of *Paramecium*. **6.** *Cosmocolpoda naschbergeri*, a colpodid ciliate; ventro-lateral view showing strongly ribbed cortex and small oral organelles (arrows).

RUTHENIUM RED AS A STAIN

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INTRODUCTION

The use of Ruthenium red for cytological staining has been extensively studied by Luft (1). Ruthenium red will react with and stain acid mucopolysaccharides, acid substituted aliphitic polymers and acidic polypeptides. The stain will reveal surface coats of a variety of cell types including bacteria and in our laboratory we have used it to demonstrate the surface coat of several species of trypanosomes and Giardia (2) and suctorians (3,4). Aside from the staining of microtubules and microfilaments (2). Although not designed for this purpose, microtubule staining is equal to that obtained using tannic acid in the fixative. It is expected that although the ruthenium molecule is large, prolonged soaking in glutaraldehyde fixative containing ruthenian red and in post fixation with osmium tetroxide penetration is enhanced. Feria-Velasco and Arauz-Contreras (5) indicated Ruthenium red binds to osmium tetroxide, therefore, in the presence of OSO_4 the Ruthenium red may be bound to the microtubules and microfilaments via osmium.

PROTOCOL

A. Stock solutions
   1. Dissolve 0.15 grams of Ruthenium red w/v in 100 ml of distilled water. Mix just prior to use and keep in dark, i.e. brown bottle or bottle wrapped in aluminum foil. Primary and post fixations should also be done in dark. Otherwise, Ruthenium red will be reduced to a mixture of Ruthenium violet and Ruthenium red.
   2. 6% glutaraldehyde
   3. 0.2 M cacodylate buffer, pH 7.2
   4. 4.0% Osmium tetroxide (Aqueous)

B. Primary Fixative (fix for 1 hr. at 4°C in dark)

6.0% Glutaraldehyde	5.0 ml
0.2 M Cacodylate buffer	5.0 ml
0.15% Ruthenium Red	5.0 ml

C. Wash in 0.1 M Cacodylate buffer
   pH 7.2 with 5% sucrose, 3 X
   10 minutes each, 4°C, in dark

D. Post Fixation (Fix 1 hr. at 4°C in dark)

4.0% Osmium Tetroxide	1.0 ml
0.2M Cacodylate buffer, pH 7.2	2.0 ml
0.15% Ruthenium Red	2.0 ml

E. Dehydrate through ascending series of ethanols and embed as usual.

LITERATURE CITED

1. Luft, J. 1971 Ruthenium red and violet. I Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Rec.* **171**:347-368.
2. Paulin, J J. 1990 Past presidential address: the dance of electrons entices protists to reveal their innermost secrets. *J.*

C-21.2

Protozool. **37**(6):593-604. (See micrographs of Giardia, Figs. 22-23.)

3. Henk, W G. 1979 Ruthenium red staining of surface structures of Discophrya collini (Root). *Eur. J. Cell Biol.* **19**:83-88.

4. Sundermann, C & Paulin, J J. 1985 An ultrastructural and cytochemical study of the cell surface of a suctorian ciliate. *J. Protozool.* **32**:723-729.

5. Peria-Velasco, A & Arauz-Contreras, J. 1981 Ruthenium red-mediated osmium binding for examining uncoated biological material under the scanning electron microscope. *Stain Technol.* **56**:71-78.

CYTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE (LYSOSOMAL AND DIGESTIVE ACTIVITY MARKER ENZYME)

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INTRODUCTION

A variety of enzymes are secreted into regions of digestive activity including those that degrade proteins, fat esters, nucleic acids, other biological macromolecules, and a range of organo-phosphate esters. Classically, digestive vacuoles and lysosomes have been identified cytochemically by the presence of acid phosphatase activity. While it has been shown that acid phosphatase is, in general, a good marker for digestive activity, it is not always present with all digestive enzymes [6]. Therefore, it cannot be used as a universal indicator of digestive activity. Its wide occurrence, however, and relatively stable properties during fixation and preparation of material for microscopic examination make it a useful, though limited, indicator of lysosomal and general digestive activity. It has been widely used to detect digestive activity in eukaryotic micro-organisms [e.g., 1, 2, 4].

As implied by the name, acid phosphatase is an enzyme that cleaves phosphate from an organic substrate with optimum activity in the acid pH range, typically pH = 5.0. The substrate can be beta-glycerophosphate, naphtholphosphate, or other arylphosphates. Typically, when the substrate is beta-glycerophosphate, the enzyme is designated acid beta-glycerophosphatase. When the substrate is an arene derivative, the enzyme is designated acid arylphosphatase. The same enzyme, however, is probably active with all of these substrates.

For light microscopic localization of acid phosphatase activity [3], alpha-naphthylphosphate is typically used as a substrate. In some cases a better reaction product is obtained if the naphthylphosphate has other substituents (e.g., alpha-naphthol AS-BI phosphate). The dye base links covalently to naphthol, a hydrolytic cleavage product of the enzyme. A colored diazo compound is produced. Usually a dye base is chosen that yields a red product. Some dye bases are Fast Red Violet LB and Fast Garnet GBC.

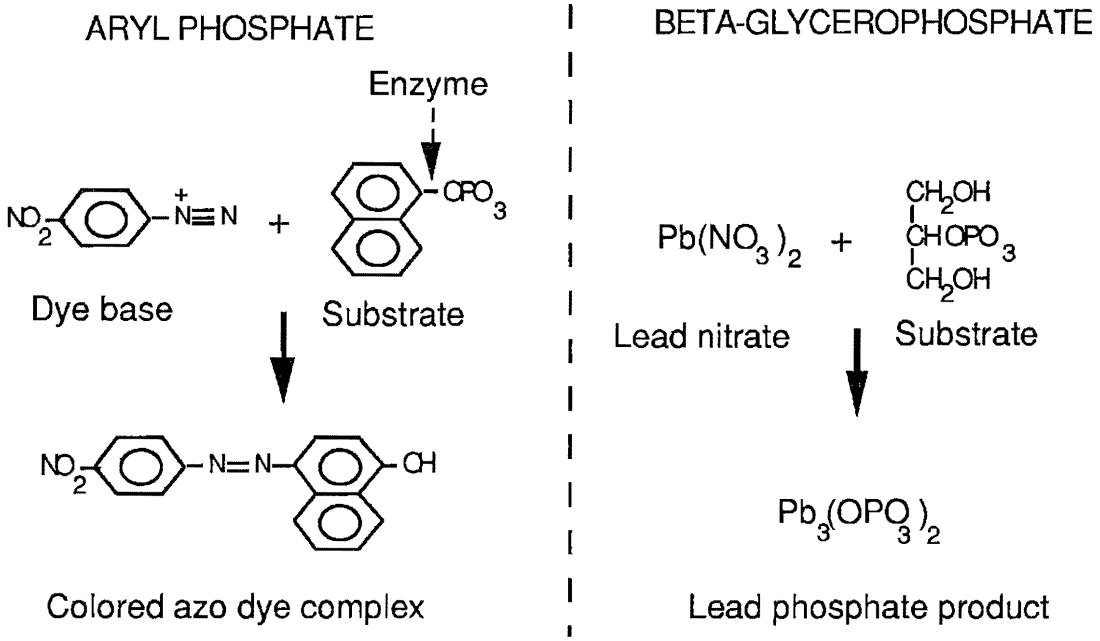
C-22.2

For electron microscopy [5], a salt of a heavy metal (e.g., lead nitrate) is added to the reaction mixture in place of the dye base. The lead ions react with the phosphate product of the enzyme activity to produce a lead phosphate precipitate. Typically, beta-glycerophosphate can be used as the substrate for electron microscopy. It is easily obtained and is less toxic than the arylphosphate substrates. The electron-dense lead product is readily visualized in ultrathin sections observed with the transmission electron microscope (e.g., Figs. 1 & 2). For light and electron microscopy, the finest possible fixatives should be used to preserve enzyme activity. In general, electron microscopic grade glutaraldehyde (sealed under nitrogen in ampoules) is suitable.

A valid cytochemical method for localization of enzyme activity must meet at least the following criteria:

1. The visualized product must be specifically deposited at the site of enzyme activity, and not elsewhere.
2. The product should be sufficiently stable and non-diffusible to remain visible and localized during subsequent preparation steps for microscopic observation.
3. In the absence of enzyme activity (e.g., when an inhibitor is added), no visible product should be produced.

The fundamental chemistry of the enzyme-specific products is shown for naphthol phosphate substrate on the left and beta-glycerophosphate substrate on the right. The latter is for electron microscopic visualization.



A number of variations have been published on the basic procedure for light microscopic localization of acid aryl phosphatase. A relatively standard procedure is described here. Also, there are commercial kits that make the procedure much more convenient. Among these is a "Leukocyte acid phosphatase" kit distributed by Sigma Chemical Co., St. Louis, MO. The required reagents are prepackaged in the correct concentrations for staining fixed tissue for light microscopy. After fixing the sample, it is immersed in the staining mixture as described in the procedure sent with the kit. For electron microscopy, a method using beta-glycerophosphate as a substrate is presented.

MATERIALS

Fixative

1. Glutaraldehyde fixative, 2% v/v (freshly prepared in buffer)
2. Buffer for fixative, 0.05 mol/L (phosphate or cacodylate, or other as appropriate)

For Light Microscopy:

1. Acetate buffer, 2.5 mol/L (pH 5.0 to 5.2), Cat. No. 386-3 Sigma Chem. Co.
2. Naphthol AS-BI Phosphoric Acid Solution in N,N'-dimethyl formamide, 12.5 mg/mL w/v, Cat. No. 386-4 Sigma Chem. Co.
3. Fast Garnet GBC Salt, 15 mg, Cat. No. 386-15 Sigma Chem. Co.
4. Acid Hematoxylin solution, 1g/L (pH 3.3), Cat. No. 285-2 Sigma Chem. Co.
5. 0.01 mol/L sodium fluoride solution in acetate buffer (enzyme inhibitor, poisonous)

For Electron Microscopy:

1. Acetate buffer, 0.05 mol/L (pH 5.0)
2. Beta-glycerophosphate solution, 0.1 mol/L, 3.15 g/100 mL
   (Note adjust pH = 5.0 with conc. hydrochloric acid)
3. Lead nitrate solution, 0.06 g/50 mL of acetate buffer
4. Concentrated hydrochloric acid
5. 0.01 mol/L sodium fluoride solution in acetate buffer (enzyme inhibitor, poisonous)

PROTOCOLS

1. Light Microscopic Localization of Acid Phosphatase Activity.

Prepare the staining mixture containing substrate and dye base in a 100 ml beaker as follows:

A. Water (37° C)	46.0 mL
B. Acetate buffer	2.0 mL
C. Naphthol AS-BI phosphoric acid	2.0 mL
D. Fast Garnet GBC salt	15.0 mg

NOTE: After step C, thoroughly mix the solutions, and then add the Fast Garnet powder. Stir for 30-60 seconds using a magnetic mixer.
Avoid all contact with these reagents. They are irritants and can be toxic. Where appropriate, check first with your health safety officer before proceeding.

Rapidly filter the solution through Whatman No. 54 or equivalent filter paper. It is preferable to prepare the mixture immediately before use. This can be done while the samples are being fixed.

C-22.4

Prepare the fixed sample for staining using high-grade glutaraldehyde in appropriate buffer, and at a pH that gives optimum cytoplasmic preservation. Fix at 3° to 5° C to preserve enzyme activity as much as possible. Typically, fixation at pH = 7.0 to 7.2 for periods of 15 to 20 minutes is adequate. Wash the fixed sample in ice-cold, deionized water or in isotonic aqueous solution (sucrose solutions of appropriate molarity are suitable) to remove the fixative. It is essential that the sample be rinsed free of the fixative that otherwise may interfere with the enzyme reaction. Immerse the fixed and washed sample in cold acetate buffer (pH 5.0) for approximately 10 minutes to bring the internal pH of the sample to pH = 5.0.

Separate the fixed sample into two aliquots. One will be used as a control to check for the specificity of staining. The control sample should be immersed in sodium fluoride solution to inhibit acid phosphatase activity for 5 min. before proceeding to the staining step. Remove most of the sodium fluoride solution before adding the substrate. Carefully label the two samples so you can keep track of each one throughout the succeeding steps. Also, scrupulously avoid contaminating the standard treatment preparation with the fluoride. If you prefer, an alternative control uses an incubation medium that lacks the substrate. The control incubation medium is identical to the staining medium except 2.0 mL of deionized water are added in place of the 2.0 mL of naphthol AS-BI phosphoric acid solution. Where possible, it is advisable to use both of these controls to more clearly establish that the red reaction product is indeed produced by the enzyme.

Incubate the standard treatment sample and the fluoride control sample for 1 hour at 37° C in separate vessels containing the staining solution prepared in steps A-D above. If the reaction product is too dark, or appears to invade the surrounding cytoplasm from the site of initial deposition, reduce the temperature and/or the incubation time. Some protozoa have high acid phosphatase activity, and it is wise to make a trial run with subsamples at varying incubation times to find the most appropriate one. Prepare a small aliquot as a wet mount and examine it by light microscopy to determine the degree of staining.

Remove the stained sample and wash in deionized water for 3 or more minutes.

If a counter stain is needed, stain in acid hematoxylin solution for 5 minutes. Rinse in deionized water for at least 3 minutes.

Some small protozoa can be examined directly using light microscopy by gently flattening the preparation with a cover glass before inspection.

For thin sectioning, rapidly dehydrate and embed the sample according to standard procedures. Where possible, it is preferable to use a water-soluble embedding medium, and avoid dehydration that may elute some of the dye product.

2. Electron Microscopic Localization of Acid Phosphatase Activity

Prepare the substrate by mixing 1 part of beta-glycerophosphate solution to 10 parts of lead nitrate solution. as follows:

A. Beta-glycerophosphate solution	5.0 mL
B. Lead nitrate solution	50.0 mL

See the Materials section above. Incubate the solution at 37° C for 1 hour. A light precipitate may form. Chill and filter through Whatman No. 2 filter paper.
Note: Keep the beta-glycerophosphate salt in the freezer and prepare the solution immediately before use.

Fix the sample in high-grade, electron microscopic glutaraldehyde using standard procedures that produce optimum preservation of fine structural features. It is particularly important for cytochemical visualization of enzyme activity that the enzyme remains localized. Therefore, great care must be taken to ensure that all membrane-enclosed structures are intact. Adjust the molarity of the fixative to give the best osmotic balance for preservation of internal structures. Isotonic sucrose solutions can be used to prepare the fixative, but avoid additives that may inhibit the enzyme. Carefully check to be certain that the sucrose solution in the buffer has the correct pH for fixation. Typically, fixation at pH = 7.0 to 7.2 for periods of 15 to 20 minutes at 3° to 5° C is adequate. Wash the fixed sample in ice-cold, deionized water or in isotonic aqueous solution to remove the fixative. It is essential that the sample be rinsed free of the fixative that otherwise may interfere with the enzyme reaction. Immerse the fixed and washed sample in cold acetate buffer (pH 5.0) for approximately 10 minutes to bring the internal pH of the sample to pH = 5.0.

Incubate subsamples of the fixed preparation in the substrates for staining (containing both beta-glycerophosphate and lead nitrate) and for control (lead nitrate solution only). A sodium fluoride control can be prepared as described in the foregoing protocol on light microscopy. The fluoride-treated sample is incubated in the staining medium in the same way as the standard preparation.

Incubation in the staining media at 37° C for 30 to 60 minutes is usually adequate. It is possible to take a small aliquot at intervals during the incubation to determine the degree of staining. A wet mount is prepared, and the residual staining solution is fully washed out by repeatedly drawing deionized water through the preparation using a piece of bibulous paper on one side and adding water on the other side. Thereafter, ammonium sulfide solution is added. A brown to black lead sulfide precipitate appears at the site of the lead phosphate deposition.

The stained preparation is washed thoroughly with deionized water to remove residual staining solution. The washed preparation is post-fixed and stained for transmission electron microscopy (TEM) in a 2% w/v osmium tetroxide solution containing buffer (pH = 7.2) at 3°-5° C for two to four hours. The preparation is washed, dehydrated, and embedded for sectioning using standard TEM procedures.

It is preferable to examine the ultrathin sections without additional stain first, to more clearly visualize the lead phosphate deposits. Thereafter, a light stain with Reynold's alkaline lead citrate can be used to highlight fine structural features and provide a clearer cytoplasmic context for the sites containing acid phosphatase reaction product. Always examine the control preparations at the same time and under the same preparative conditions. There should be no electron dense products in the control preparations. Compare Figs. 1 & 2. If the controls contain electron dense deposits in the same locations as in the standard stained material, this may indicate false localization. Alternatively, you may want to investigate if the enzyme was fully inhibited in your fluoride control. Likewise, be certain to clarify that no other organo-phosphate substrate was present in your other controls. In general, it is wise to carefully repeat your procedures, and to make many sections to ensure that any electron dense product is

enzyme produced, not a false localization. It is better to error on the side of rejecting a positive result than to uncritically accept a false localization as valid!

In some cases, a light nuclear precipitation product may form. It is important to have the proper concentration of lead nitrate in the staining medium or excessive nuclear or other sites of non-enzyme-produced deposition may occur.

COMMENTS

It is essential that the pH of the incubation medium be maintained at pH = 5.0 or in the acid range. Otherwise, reaction product may form by action of alkaline phosphatases, if they are present. Check the pH of the staining solution before you begin incubation. A good grade pH paper is sufficiently accurate. The fixation and subsequent preparations of the samples must be done with care to ensure that the enzyme is not denatured. In general, it is wise to keep the sample cold (c. 3^{o} to 5^{o} C) until the incubation in the staining solution is begun. Presence of electron dense reaction product under carefully controlled conditions is good evidence that the enzyme is present. Conversely, absence of reaction product does not necessarily indicate absence of the enzyme. Changes in the enzyme during preparation, inadequate diffusion of the stain mixture to the site of the enzyme, or other uncontrolled factors may account for absence of reaction product, even when the enzyme is present. Consult some of the major references [3, 5] if you encounter difficulty. Formulas are available for preparation of buffers in the appendix of many sources [3].

LITERATURE CITED

1. Anderson, O R & Botfield, M 1983 Biochemical and fine structure evidence for cellular specialization in a large spumellarian radiolarian, *Thalassicolla nucleat*a. Mar. Biol. **72**: 235-241.

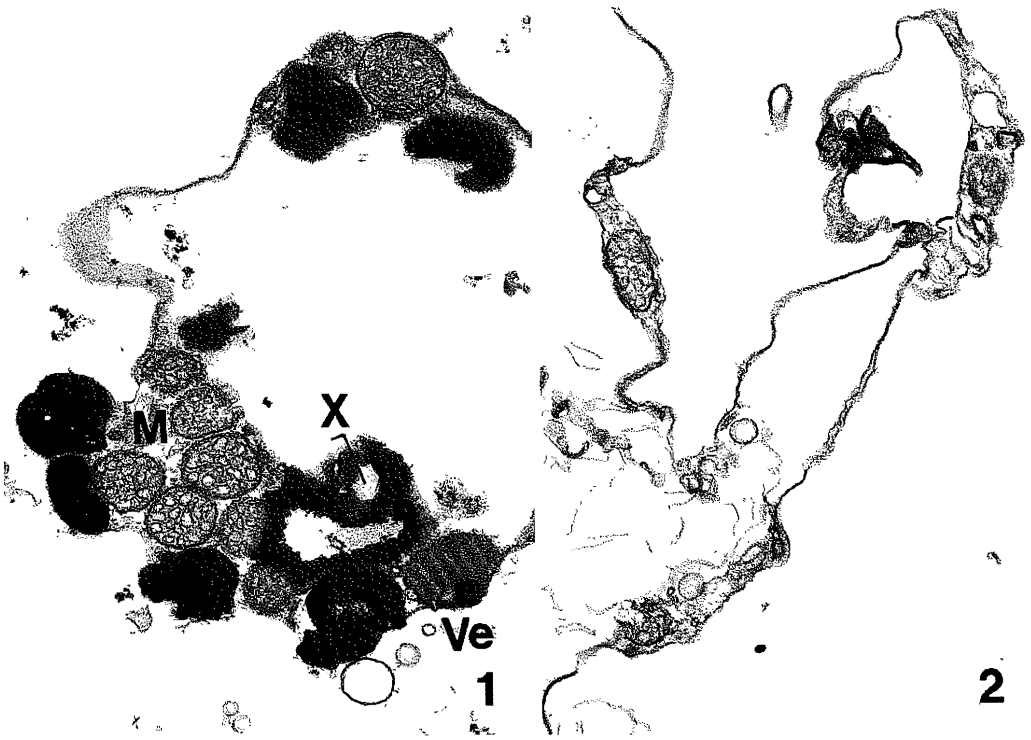
2. Anderson, O R 1975 The ultrastructure and cytochemistry of resting cell formation in *Amphora coffeaeformis* (Bacillariophyceae). J. Phycol. **11**: 272-281.

3. Barka, T & Anderson, P J 1965 Histochemistry: Theory, Practice, and Bibliography. Harper & Row, Publishers, Inc, New York.

4. Fok, A K & Paeste, R M 1982 Lysosomal enzymes of *Paramecium caudatum* and *Paramecium tetraurelia*. Exp. Cell Res. 139: 159-169.

5. Gomori, G 1952 Microscopic Histochemistry: Principles and Practice. Univ. of Chicago Press, Chicago. P. 189.

6. Holtzman, E 1989 Lysosomes. Plenum, New York.



Figs. 1 & 2 Acid phosphatase reaction product (X, Fig. 1) and a non-stained control (Fig. 2). Note that the mitochondria (M) and fibrillar vesicles (Ve) do not contain reaction product. X 15,600.

D. MOLECULAR BIOLOGICAL AND GENETIC METHODS

1. Isolating tubulin-free pellicles from protozoa. Norman E. Williams
2. Isolating the cortical cytoskeleton from Tetrahymena. Norman E. Williams
3. DNA purification from polysaccharide-rich cells. C. Graham Clark
4. Riboprinting: A molecular approach to the identification and taxonomy of protozoa. C. Graham Clark
5. Use of antibodies as probes in the study of amoebae. Kee J. Kim, Eui Y. Choi and Kwang W. Jeon
6. Cloning and sequencing a myosin gene of *Amoeba proteus*
7. Purification of ammoeba mtDNA using the unset procedure. E. R. Hugo, V. J. Stewart, R. J. Gast and T. J. Byers
8. Detection of human serum antibodies reactive with *Acanthamoeba polyphaga* by use of an indirect fluorescent antibody (IFA) test. Pamela B. Sheets, Anthony L. Newsome & Stephen D. Allen
9. Detection of animal serum antibodies reactive with *Acanthamoeba polyphaga* by use of an enzyme-linked immunosorbent assay (ELISA)

APPENDIX FOR PHYSIOLOGICAL AND MOLECULAR METHODS---A. T. Soldo

Commonly used stock solutions and buffers
Concentrated acids and bases
Amino acids
Molecular weight and some optical properties of nucleic acid derivatives
Conversion factors for radioactivity
Characteristics of radionuclides
Common abbreviations

ISOLATING TUBULIN-FREE PELLICLES FROM PROTOZOA

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Introduction

This method involves extracting cells in 1% Triton X-100 at high ionic strength (1.5 M KCl). It is rapid, easy to do, and yields intact pellicles from a variety of protozoa. The pellicles are devoid of membranes and microtubules, yet retain all proteins necessary to preserve cell shape. The method has been applied to Tetrahymena (1), Paramecium (2), Euplotes (3), and to Didinium ,Vorticella, and Peranema (unpublished). It has not been successful with ciliates lacking a prominent epiplasm (Stentor, Blepharisma, and Dileptus were tested). This procedure is useful for experimental studies, an also for identifying and comparing strains and species when accompanied by gel electrophoresis.

Protocol

Stock Solutions

1) 55.92 gm KCl (= 1.5 M)
   50 ml 10% Triton X-100
   179 mg EDTA (= 1 mM)
   H_2O to 500 ml
2) 55.92 gm KCl (= 1.5 M)
   50 ml 10% Triton X-100 (= 1%)
   190 mg EGTA (= 1mM)
   H_2O to 500 ml
3) 4 gm NaCl (= 137 mM)
   0.1 gm KCl (= 2.7 mM)
   0.1 gm KH_2PO_4 (= 1.5 mM)
   0.57 gm Na_2HPO_4 (= 8 mM)
   179 mg EDTA (= 1 mM)
   190 mg EGTA (= 1 mM)
   H_2O to 500 ml
4) 100 mg leupeptin in 10 ml cold water and freeze immediately (-20°C or -70°C)

Procedure

1. Make up THS (Triton X-100/high salt)-EDTA working solution (will not store) by adding 88 μl β-mercaptoethanol (= 25 μM final) and 25 μl leupeptin stock (=10μM final) to 50 ml of the THS-EDTA stock solution. Do this for each sample be processed.

be processed.

2. Make up THS-EGTA working solution (will not store) by adding 88 μl β-mercaptoethanol and 25 μl leupeptin stock as above, this time to 50 ml of the THS-EGTA stock solution. Do this for each 2 samples to be processed.

3. Add 88 μl β-mercaptoethanol to 50 ml of the phosphate buffered saline plus chelators described as stock solution #3 above.

4. Pellet the cells in their own medium using a 50 ml conical polycarbonate centrifuge tube. Tetrahymena may be washed first in 10 mM Tris-HCl, pH 7.4, at room temperature. Use 2 x 10^7 Tetrahymena, 10^6 Paramecium, or 10^5 Euplotes cells for the quantities specified in the following steps.

5. Add 40 ml cold THS-EDTA working solution to the cell pellet. Transfer to a beaker and mix 3 minutes with a stirring bar. Observe microscopically at this point. (The preparation can be dried down for staining (see Comments))

6. Transfer to a 50 ml round-bottomed polycarbonate centrifuge tube and centrifuge at 10,000 x g for 20 minutes.

7. Remove as much of the supernate as possible (some pellets are "slippery"), add 20 ml THS-EGTA working solution, mix well for several minutes, then centrifuge at 10,000 x g for 15 minutes.

8. Remove supernate, take up pellet in 3 - 6 ml of phosphate-buffered saline solution, transfer to a 125 ml tube and centrifuge at 10,000 x g for 10 minutes.

9. For electrophoresis, remove the supernate and boil the pellet 2 minutes in Laemmli sample buffer. Use 600 μl for Tetrahymena, 400 μl for Paramecium, and 200 μl for Euplotes.

Comments

Other protease inhibitors may be added to the THS solution. Pepstatin is stored at 1 μg /ml in methanol and used at 0.7 μg/ml. PMSF (phenylmethysulfonyl fluoride) is stored in 2-propanol at 100 mM and used at a final concentration of 0.2 mM. The pellicles can be used for immunofluorescence studies, but only prior to centrifugation. Take 2.5 ml of the initial lysate in THS-EDTA, dilute to 15 ml with water, spot on clean slides and air dry. Follow the standard procedures for immunofluorescence.

LITERATURE CITED

1. Williams, NE, Honts, JE, & Kaczanowska, J. 1990. The formation of basal body domains in the membrane skeleton of Tetrahymena. Develop. **109**:935-42.
2. Williams, NE, Honts, JE & Stuart, KR. 1989. Properties of microtubule-free cortical residues isolated from Paramecium tetraurelia. J. Cell Sci. **92**:427-32.
3. Williams, NE, Honts, JE, Lu, Q, Olson, CL, & Moore, KC. 1989. Identification and localization of major cortical proteins in the ciliated protozoan, Euplotes eurystomus. J. Cell Sci. **92**:433-39.

ISOLATING THE CORTICAL CYTOSKELETON FROM TETRAHYMENA

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Introduction

This method has been used mainly with Tetrahymena, but it can easily be adapted for use with other protozoa. The pellicles obtained are devoid of membranes but retain microtubules. It will be presented here as developed and used for Tetrahymena (1-4). though used primarily in experimental studies, cytoskeletal protein "fingerprints" can be produced in polyacrylamide gels that are diagnostic for individual strains and species (3).

Protocol

Stock Solutions

1) 0.25 M sucrose
2) 171 gm sucrose (= 1 M)
   175 mg EDTA (= 1 mM)
   500 μl β-mercaptoethanol (= 0.1%)
   to 500 ml in 10 mM Tris.HCl, pH 9.0
3) 10% Triton X-100
4) 10 mM sodium phosphate buffer, pH 6.9
   0.1% β-mercaptoethanol

Procedure

1. Chill 5 x 10^6 to 1 x 10^7 Tetrahymena cells in an ice bath.
2. Collect the cells in one 50 ml conical polycarbonate centrifuge tube by low speed centrifugation (300-400 x g) for 3-5 minutes at 5°C.
3. Aspirate the supernate and add 5 ml of cold 0.25 M sucrose. Mix gently with a spatula.
4. Transfer to a pre-cooled 100 ml beaker with a stirring bar.
5. While mixing at moderate speed, add 15 ml cold solution #2 (SEMT) and continue mixing for 30 seconds.
6. Add 2.5 ml of 10% Triton X-100 and mix 30 seconds more. Observe microscopically at this stage.
7. Transfer to a cold 50 ml round-bottomed polycarbonate centrifuge tube and centrifuge at 4000 x g for 20 minutes at 5°C.
8. Remove supernate, leaving the pellet in 1-2 ml of solution. Mix and add 10 ml of the phosphate buffer solution (#4). Transfer to a cold 15 ml tube and centrifuge at 15,000 x g for 20 minutes at 5°C.
9. Aspirate the supernate, drain and wipe the inside walls of the tube. For electrophoresis, solubilize the pellet in 500 μl Laemmli sample buffer in the standard manner.

Comments

In early studies, the cortical cytoskeletons were washed by centrifuging them into a sucrose cushion (1,2), but this is usually not necessary. Mechanical shear using something like a Logeman homogenizer after step 6 above will free the oral apparatuses, and these can be harvested separately (2). As a precaution, one or more protease inhibitors can be added to the lysate and subsequent washes. A stock solution of 10 mg/ml of leupeptin can be made in water (store frozen) and used at 10 μM. PMSF (phenymethylsulfonyl fluoride) can be stored in 2-propanol at 10 mM and used at a final concentration of 0.2 mM.

LITERATURE CITED

1. Vadaux, P. 1976. Isolation and identification of specific cortical proteins in Tetrahymena pyriformis strain GL. J. Protozool. **23**:458-64.
2. Williams, NE, & Bakowska, J. 1982. Scanning electron microscopy of cytoskeletal elements in the oral apparatus of Tetrahymena. J. Protozool. **29**:382-89.
3. Williams, NE, Buhse, HE Jr., & Smith, M. 1984. Protein similarities in the genus Tetrahymena and a description of Tetrahymena leucophrys, n.sp. J. Protozool. **31**:313-21.
4. Williams, NE, Honts, JE, & Jaeckel-Williams, RF. 1987. Regional differentiation of the membrane skeleton in Tetrahymena. J. Cell Sci. **87**:457-63.

DNA PURIFICATION FROM POLYSACCHARIDE-RICH CELLS

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Introduction

Polysaccharides interfere with many enzymes used in DNA analysis, and their removal is thus imperative. The cationic detergent, cetyltrimethylammonium bromide (CTAB) has been used in DNA purification since the 1950s. Originally used in bacterial DNA isolation, it has proven useful in plant (1) and protozoan DNA purification. The protocol presented below was adapted from a bacterial purification procedure (2). Specifically developed for rapid isolation of Entamoeba DNA (3), it has also successfully been used for kinetoplastid, Isonema, trichomonad and Blastocystis DNA purification. CTAB can also be used to extract polysaccharides from DNA prepared by other methods.

Protocol

The volumes given here allow for the entire procedure to be carried out in a microcentrifuge tube but may be scaled up with no problems.

Reagents needed: a) Lysis buffer - 0.1 M EDTA, pH 8.0, 0.25% SDS
b) 3.5 M NaCl
c) 10% CTAB in 0.7 M NaCl
d) Chloroform/isoamyl alcohol (24/1 v/v)
e) Phenol saturated with 10 mM Tris-HCl, pH 8.0
f) Proteinase K
g) 100% Ethanol

1. Pellet cells in a 1.5 ml capacity microcentrifuge tube (≤ 50 µl packed cell volume).
2. Disperse pellet in 0.25 ml lysis buffer.
3. Add Proteinase K to 0.1 mg/ml. Mix and incubate at 55°C for 1 h.
4. Add NaCl to 0.7M (75 µl) and mix well.
5. Add CTAB TO 1% (42 µl) and mix well. Incubate at 65°C for 15 min.
6. At room temperature, extract with 1 volume (420 µl) chloroform/isoamyl alcohol.
7. Spin in microfuge at 14,000 x g 10 min.
8. Take supernate and extract with an equal volume of phenol:chloroform (1:1; 420 µl).
9. Spin in microfuge at 14,000 x g 10 min.
10. Take supernate and precipitate with 2.5 volumes (1 ml) ethanol.
11. Mix and stand at room temperature 10 min.
12. Spin in microfuge at 14,000 x g 10 min.
13. Wash pellet once with 70% ethanol and spin 5 min.
14. Decant supernate and air dry the pellet. Resuspend in a small volume of sterile water (50 µl).

Comments

1. To remove polysaccharides from existing DNA preparations, start at step 4.
2. The interface at step 8 can be quite large. This can be reextracted with 0.7 M NaCl. The small amount of carried-over interface will not cause problems, because it is removed in the next extraction. Extraction with CTAB can also be repeated.
3. To dissolve 10% CTAB, heating to 65°C will probably be necessary. This solution is very viscous and reheating each time will make aliquoting (step 5) much easier.
4. The NaCl concentration is critical. If it falls much below 0.7 M a DNA-CTAB insoluble salt forms and the DNA will be lost. This is actually another purification method - precipitation of the CTAB-DNA complex in 0.35 M NaCl followed by dissociation in 0.7 M NaCl. This may help remove different types of enzyme inhibitors.
5. Non-polysaccharide inhibitors can often be removed by passing the resuspended DNA obtained in step 14 over a spin column containing, for example, 0.5 ml packed Sephacryl S400 (Pharmacia). DNA recovery is usually quantitative and most small molecules and salts are removed from the preparation.
6. Instead of ethanol, 0.6 volume of isopropanol can also be used for DNA preparation.

LITERATURE CITED

1. Murray, MG & Thompson, WF. 1980. Rapid isolation of high-molecular-weight plant DNA. Nucl. Acids Res. 8:4321-4325.
2. Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA, & Struhl, K.,eds 1987. Current protocols in molecular biology, Volume 1, Wiley and Sons.
3. Clark, CG & Diamond, LS. 1991. The Laredo strain and other Entamoeba histolytica-like amoebae are Entamoeba moshkovskii. Mol. Biochem. Parasitol. **46**:11-18.

RIBOPRINTING: A MOLECULAR APPROACH TO THE IDENTIFICATION AND TAXONOMY OF PROTOZOA

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Introduction

Ribosomal RNA gene sequences are among the most highly conserved molecules in nature and have proven very useful in determining phylogenetic relationships among organisms. However, obtaining these data is labor intensive, involving DNA cloning and sequencing. This often results in only a single strain of a species being studied, and frequently only a single species in any genus. Within-species studies usually rely on restriction fragment length polymorphisms, with mitochondrial DNA being the molecule of choice. The technique described here combines the two approaches to allow rapid identification of isolates and quantitation of divergence within and among related species. Polymerase chain reaction amplified small subunit ribosomal RNA gene restriction fragment length polymorphism analysis, or "riboprinting" (1), makes use of highly conserved sequences at either end of the small subunit ribosomal RNA gene (SSU-rDNA) and the polymerase chain reaction (PCR) to generate microgram quantities of SSU-rDNA specific product (first described in Ref. 2) from a small number of cells of each strain. The PCR product is digested with a battery of restriction enzymes and the resulting DNA fragments are separated electrophoretically. The pattern of bands thus obtained is usually diagnostic. The data can be further analyzed to estimate degrees of divergence between species.

Protocol

PCR is a recent and rapidly evolving method. The conditions listed here are essentially those supplied by kit manufacturers.

Reagents required: a) 10X PCR buffer- 0.1M Tris-HCl, pH 8.3 (at 25°C)
0.5M KCl
15 mM $MgCl_2$
0.01% (w/v) gelatin
b) 10 mM solutions of dATP, dGTP, dTTP & dCTP, pH 7.0
c) 20 μm solution of each primer (see comment 2)
d) Thermostable DNA polymerase (eg. Taq DNA polymerase (5 U/μl)

1. The optimal amount of DNA must be determined empirically by testing several dilutions (try 50 to 100 ng per 100 μl).
2. For each 100 μl reaction, combine: 61.5 μl sterile dH_2O
10 μl 10X PCR buffer
2 μl each dNTP
0.5 μl DNA polymerase (2.5 U)
5 μl each primer
10 μl DNA template

Mix gently to avoid bubbles.

3. Overlay with 100 μl sterile light mineral oil.
4. Place in a thermal cycler and cycle 30 to 35 times using the following schedule:
   94°C 1 minute
   55°C 1.5 minutes
   72°C 2 minutes
5. At room temperature, extract with 1 vol $CHCl_3$ to remove the oil.
6. The yield is estimated by running an aliquot of the product (2 to 5 μl) on an analytical agarose gel (0.8%).
7. Precipitate the product by the addition of 2 volumes of absolute ethanol and 0.3M sodium acetate and resuspend in 40 μl dH_2O.
8. Pass the product over a spin column to remove excess primers and salt (1 ml packed volume of Sephacryl S400 (Pharmacia), for example).
9. Digest PCR product with an array of restriction enzymes and separate the fragments on a 2.6% agarose gel (3:1 Nu-Sieve:Seakem LE agarose;FMC) in Tris-borate buffer.
10. Stain the gel with ethidium bromide and photograph in long wave (301 nm) UV.

Comments

1. Many modifications of PCR amplification methods exist and may prove beneficial for increasing the yield and/or specificity of product from certain DNAs. These include: adding 0.05% Tween 20, 1.25% to 5% formamide, 2.5% to 15% DMSO and/or 10^{-4} to 10^{-5} tetramethylammonium chloride to the reaction buffer; varying the $MgCl_2$ concentration; replacing the gelatin with 100 μg/ml of BSA or leaving it out all together; increasing the length and/or the temperature of the melting stage for the first few cycles. Several books are now available on the subject of PCR (eg. Ref. 3).

2. The sequences of the primers used in the amplification are ideally taken from the known SSU-rDNA sequence of a close relative to the organism of interest. The following were derived specifically for the amplification of Entamoeba rDNA but also successfully amplify kinetoplasmid, Blastocyctis, ciliate, Isonema, and Toxoplasma rDNAs:

   5'end specific - CCAGCTGCAGATCTGGTTGATCCTGCCAGT;
   3'end specific - CCGCTGCAGGATCCTTCCGCAGGTTCACCT.

   The underlined portions are added to help in the subsequent cloning of the PCR product and are not necessary for the amplification. These primers are eukaryote specific; bacterial or organellar rDNA will not amplify. Axenic cultures are therefore unnecessary, but cultures with a eukaryotic food source will present problems.

3. Removal of the primers after amplification is not strictly necessary, but the excess primers may conceal additional small DNA fragments upon restriction digest electrophoresis.

4. It is best and most convenient when concurrently amplifying several DNAs to make a "master mix" of everything except the template DNA before aliquoting into each tube.

5. The optimization of DNA concentration can be done in smaller volumes, such as 25 or 50 μl. Theoretically, a single cell should provide sufficient DNA. In practice, 10^5 to 10^6 target molecules per 100 μl reaction has been recommended as optimal. For

many protozoa this is equivalent to about 50 ng DNA for a single copy target. Ribosomal genes are usually multicopy, so that less DNA will also work well.

6. Under optimum conditions one 100 μl reaction will provide enough product for at least 12 restriction digests, the number routinely used in this laboratory.

7. A permanent record for each strain can be made by photographing a stained gel with all the restriction digests on it. However, for comparative purposes, it is often useful to run the DNA from several strains digested with the same restriction enzyme in adjacent lanes of a single gel.

8. A good size marker for riboprints is a combination of a) bacteriophage lambda DNA digested with EcoR I followed by Hind III and b) pBR322 DNA digested with Msp I. This marker covers the complete range of fragments sizes obtained.

9. Restriction enzymes with 4-base recognition sequences provide the most information. Those in use in this laboratory are Hae III, Hha I, Hinf I, Rsa I, Msp I, Alu I, Taq I, Dde I, Sau 96 I, Sau 3A, Bst U I and Scr F I. The more enzymes used, the more accurate the determination. Twelve restriction enzymes should give information on approximately 10% of the gene sequence.

Data Analysis

Although primarily an identification technique, riboprinting can be extended to quantitate the degree of divergence between related species. The reliability of this process depends on the patterns being sufficiently similar and a substantial number of restriction sites being available for analysis. The number of shared restriction sites is proportional to the degree of genetic relatedness of a pair of organisms. This value can be estimated either by counting the number of co-migrating restriction fragments, or by first constructing restriction maps and then counting the shared sites. The latter is more accurate but more laborious. The bare bones of the necessary calculations are given below. (For the theory behind and derivation of the equation, see Nei, M., 1987. Molecular Evolutionary Genetics, Columbia University Press.) The calculations are much simpler if all the restriction enzymes used recognize the same number of bases, and this is assumed to the case in the equations below.

Estimating divergence from fragment co-migration:

Calculate: m_x = Total number of restriction fragments obtained for 'x'.
m_y = Total number of restriction fragments obtained for 'y'.
m_{xy} = Total number of restriction fragments shared by 'x' and 'y'.
F = Expected proportion of shared fragments ($\approx 2m_{xy}/(m_x + m_y)$).
G = Probability that a site remains unaltered.

('G') is calculated by an iterative formula, i.e. the first estimated value of 'G' obtained is put back into the formula again and the second estimate calculated. This is repeated until the value of 'G' stabilizes to 3 significant figures, usually within a few rounds of calculation.

$G_{n+1} = [F(3-2G_n)]^{1/4}$; the first estimate of 'G' is taken as $G_o = F^{1/4}$
d = Estimated number of substitutions per site (degree of divergence between 'x' and 'y')
$= -(2/_r) \ln G$

where r = number of bases in a restriction site (usually four). This method is only accurate when d ≤ 0.05 (5% divergence).

Estimating divergence from restriction maps:

If d ≥ 0.05, restriction maps must be constructed to obtain an accurate estimate of relatedness. This is easiest if the complete SSU-rDNA sequence of a close relative is known.

Calculate: n_x = Number of sites on the restriction map of organism 'x'.
n_y = Number of sites on the restriction map of organism 'y'.
n_{xy} = Number of restriction sites shared by 'x' and 'y'.
S = Expected proportion of shared bases
$\approx 2_{xy}/(n_x + n_y)$.
P = Proportion of base differences
$= 1 - S^{1/r}$.
d = Estimated number of substitutions per site
$= -3/4[\ln (1 - (4/3)P)]$
The standard error of the estimate of 'd' = $SE_d = \sqrt{V_d}$
$V_d = 9P(1 - P)/[(3 - 4P)^2 n]$
n = number of bases compared
$= r(n_x + n_y)/2$

The method is only accurate when d = ≤ 0.25.

Both estimates of 'd' can be used to produce phlylogenetic trees by UPGMA or other methods. If restriction maps are used, the standard error of the branch point (where applicable) becomes $SE_b = \sqrt{(V_d/4)}$. These formulae are easily adapted to a spreadsheet to facilitate calculation. If the size of the PCR product differs between two organisms there is little chance that divergence estimation based on fragment co-migration will be possible. Although length variation can be detected on restriction maps and allowances made, it is usually indicative of a significant degree of sequence divergence and even restriction maps may not prove helpful in estimating values of 'd'.

LITERATURE CITED

1. Clark, CG & Diamond, LS. 1991. The Laredo strain and other Entamoeba histolytica-like amoebae are Entamoeba moshkovskii. Mol. Biochem. Parasitol. **46**:11-18.
2. Medlin, L, Elwood, HJ, Stickel, S, & Sogin, ML. 1988. The characterization of enzymatically amplified eukaryotic 16 S-like rRNA coding regions. Gene **71**:491-499.
3. Innis, MA, Gelfand, DH, Sninsky, JJ & White, TJ (eds). 1990. PCR protocols. A guide to methods and applications. Academic Press, Inc. San Diego, CA.

USE OF ANTIBODIES AS PROBES IN THE STUDY OF AMOEBAE

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INTRODUCTION

In studying the structure and physiology of amoebae, antibodies can be used as useful probes either directly conjugated with fluorescent dyes or indirectly labeled by using tagged secondary antibodies against the primary antibodies. Fluorescein and rhodamine are the most widely used dyes to label antibodies. Fluorochrome-labeled antibodies can be localized and visualized on or within the cell under a fluorescence microscope. This technique takes advantage of the sensitivity and specificity of antigen-antibody interaction that can be detected under the light microscope. Thus, for example, the method can be used for rapid identification of infective agents in cells and for the localization of cellular antigens. Double-labeling immunofluorescence can also be performed by using two antibodies tagged with different fluorochromes, whereby two different antigens can be identified at the same time. If antibodies are tagged with electron-dense labels, they can be detected under the electron microscope.

Here we describe two methods that have been used to immunostain amoebae. In the first method, amoebae with normal shapes in suspension are processed in groups, while in the second method amoebae are first fixed, attached and processed for immunostaining. Preparations of amoebae by the second method are thinner, producing better images under the microscope. A routine procedure for immuno-electron microscopy is also given.

PROTOCOL

A. Materials

1. Monoclonal antibodies against amoeba antigens (produced in the lab or purchased)

2. Secondary antibody against mouse IgG conjugated with FITC or rhodamine (available from several commercial suppliers)

3. Mounting solution

90%	Glycerol
1 mg/ml	p-Phenylenediamine
10%	PBS

4. 0.1 M Sodium phosphate buffer, pH 7.4

5. 3% Paraformaldehyde in cacodylate buffer, pH 7.4

6. 1% Glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4

7. Diaminobenzidine solution

0.1 M	Tris-HCl, pH 7.4
0.2 mg/ml	Diaminobenzidine
0.001%	Hydrogen peroxide

8. Osmium tetroxide solution

1%	Osmium tetroxide
1%	Potassium ferricyanide
0.1 M	Cacodylate buffer, pH 7.4

B. Method 1

1. Collect amoebae in a Syracuse watch glass and remove medium by aspiration.

*When localizing specific intracellular antigens in vivo, such as those on nuclear membranes or vesicle membranes, amoebae could be injected with purified primary antibody using the usual set up [1] before proceeding with fixation.

2. Fix amoebae with cold methanol (-20°C) for 5 min.

*Fixing amoebae in cold methanol also permeabilizes the plasma membrane and antibodies will enter the cell, reacting with intracellular antigens.

3. Wash amoebae 3 times with PBS.

4. Incubate amoebae in the first antibody solution diluted with PBS for 30 min at room temperature.

*1:1 Dilution for supernatants of hybridoma cell cultures, and 1:50 dilution for ascites fluids

5. Wash 3 times with PBS and incubate in the secondary antibody (1:1000 - 1:500 dilution in PBS) for 30 min at room temperature.

6. Wash 3 times in PBS and mount with a mounting solution on a glass slide.

7. Observe under a fluorescence microscope.

C. Method 2

1. Place amoebae on a glass slide using a fine-tipped mouth pipette under a dissecting microscope.

* Amoebae may be fixed and attached onto a coverslip. Also, the standard printed microscope slides with varying number of wells (Carlson Scientific, Peotone, IL) can be conveniently used for processing a large number of samples on a single slide at a time.

2. Remove as much medium as possible.

3. Add a drop of 45% acetic acid solution at the center of a siliconized coverslip (40 x 22 mm for convenience), invert and place the coverslip over the slide with amoebae at a right angle, and quickly place on a flat surface of dry ice block.

*Cell organelles are not well preserved when acetic acid is used as a fixative, which is necessary for fixing and attaching amoebae on glass by freezing on dry ice [2]. If liquid nitrogen is available, 70% ethanol can be used as the primary fixative [3] to preserve cell structure better.

4. After a few minutes, flip off the coverslip.

5. Fix amoebae on the slide in cold methanol (-20°C) for 5 min and air dry.

*For antigens located within cells, further permeabilize cells in cold acetone (-20°C) for 5 min.

6. Hydrate cells in PBS for 5 min, and process them as in Method 1 (Steps 4 - 7).

D. Immuno-Electron Microscopy

1. Collect amoebae in a Syracuse watch glass and remove medium by aspiration.

2. Fix amoebae in 3% paraformaldehyde in cacodylate buffer for 1 hr and wash with PBS.

3. Permeabilize cells in cold methanol (-20°C) for 5 min and wash with PBS.

4. Incubate amoebae in the first antibody solution diluted with PBS for 30 min at room temperature.

5. Wash with PBS and incubate in the secondary antibody solution for 30 min at room temperature.

6. Post-fix amoebae in 1% glutaraldehyde in cacodylate buffer for 30 min.

7. Wash with 0.1 M cacodylate buffer.

8. Add diaminobenzidine substrate solution and develop color, while watching under a dissecting microscope.

9. Wash in 0.1 M cacodylate buffer and fix in 1% osmium tetroxide and 1% potassium ferricyanide in 0.1 M cacodylate buffer.

10. Examine under the electron microscope after routine processing, sectioning and staining.

LITERATURE CITED

1. Jeon, K.W. and Lorch, I.J. 1968. New simple method of micrurgy on living cells. Nature 217:463.

2. Prescott, D.M. and Carrier, R.F. 1964. Experimental procedures and cultural methods for *Euplotes eurystomus* and *Amoeba proteus*. Methods Cell Biol. 1:85-95.

3. Rabinovitch, M.D. and Plaut, W. 1962. Cytoplasmic DNA synthesis in Amoeba proteus. I. On the particulate nature of the DNA-containing elements. J.Cell Biol. 15:525-534.

CLONING AND SEQUENCING A MYOSIN GENE OF *AMOEBA PROTEUS*

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INTRODUCTION

The first step in cloning and sequencing a specific cellular gene is the construction of a DNA library and various methods have become available for the purpose. It is known that the large, free-living amoebae have a small amount of DNA distributed among numerous (reported to be about 1000 [10]) chromosomes, but very little is known about the properties of amoeba DNA. Since it is not known if amoeba DNA contains many introns, it appeared to be more reasonable to construct a cDNA library from isolated mRNA than to construct a genomic library for the purpose of isolating a gene. Therefore, in cloning and sequencing the amoeba gene for myosin, we decided to construct a cDNA library and to screen it with a monoclonal antibody (mAb) against myosin.

In this chapter, we describe procedures for 1) the isolation of mRNA, 2) construction of a cDNA library, 3) cloning and immunoscreening DNA containing the myosin gene, and 4) nucleotide sequencing of the cloned gene as we have used in our study [5]. Where the vendors' instructional manuals give detailed, step-by-step procedures that can be used with no modifications, we have simply referred to them.

BASIC PROTOCOL

I. ISOLATION OF RNA

A. General

It is essential to minimize RNase activities during RNA purification to obtain good preparations of eukaryotic mRNA. One source of RNase contamination is the labware and solutions, and they should be prepared with utmost care to avoid contamination. All labware items, including glass and plastics, should be soaked in diethyl pyrocarbonate (DEPC) solution (0.1% in water) for at least 2 hr at 37°C and subsequently sterilized to inactivate DEPC. All solutions should be prepared in glass-distilled water containing 0.1% DEPC and autoclaved except Tris buffers. Since the hand is another major source of RNase contamination, disposable gloves should be worn during RNA preparation, with frequent change of gloves.

Another way to minimize RNase activities is to choose an extraction method that is least affected by RNase activities from endogenous sources, such as tissues or cells. The most commonly used method is to isolate total RNA in the presence of guanidium salts. By combining guanidium salts and ß-mercaptoethanol or organic solvents, RNase can be inactivated even in a tissue known to be rich in RNases. Cytoplasmic RNA can be prepared from cultured cells using RNase inhibitors, such as vanadyl complexes [1]. Cytoplasmic polysomes can also isolated for enriching a specific RNA species using antibodies [8].

For the isolation of RNAs from amoebae, established procedures for the isolation of cytoplasmic RNAs of cultured cells can be used with a slight modification. Since amoebae are phagocytic cells and hence contain numerous lysosomes rich in nucleases, it is desirable to separate the cytosol from lysosomes early [3]. Amoebae are lysed by a gentle homogenization in the presence of an RNase inhibitor and lysosomes are separated from the cytosol by centrifugation. Then, mRNA is further purified by poly(A)-affinity chromatography. Since we were able to isolate a cDNA clone that was about 6 kb long and contained almost the entire myosin gene from a library constructed from mRNA isolated by the procedures described here, it appears that there was minimal degradation of RNA during its isolation. For the isolation of RNAs from other protozoan cells which can be lysed by homogenization, our RNA-isolation procedure might work as well, with modifications if necessary.

Usually, most of the isolated cytoplasmic RNAs are rRNA and tRNAs, and mRNAs represent only a small portion of the RNA pool. Thus, the separation of mRNAs by additional procedures, such as affinity chromatography, is essential in the construction of cDNA libraries.

B. Materials

1) DEPC solution (0.1% in water) (Sigma Chemicals, St. Louis, MO.)

2) RNA Extraction buffer

150 mM	NaCl
1 mM	$MgCl_2$
10%	Sucrose
1 mM	Dithiothreitol
20 mM	Vanadyl-ribonucleoside complexes (BRL)
20 mM	Tris-HCl (pH 7.4)

3) Proteinase-K digestion buffer

0.6 M	NaCl
4%	SDS
50 mM	EDTA
0.4 M	Tris-HCl, pH 8.0

4) Proteinase K

20 mg/ml in distilled water

5) Oligo(dT)-cellulose column for mRNA separation

A Pasteur pipette plugged with glass wool, treated with DEPC and autoclaved.

6) mRNA-column-binding buffer (1X)

10 mM	Tris-HCl (pH 7.5)
0.5 M	NaCl
1 mM	EDTA
0.1%	Sodium lauryl sarcosinate

7) mRNA-elution buffer

10 mM	Tris-HCl (pH 7.5)
1 mM	EDTA

8) mRNA-column-washing buffer

10 mM	Tris-HCl (pH 7.5)
0.1 M	NaCl
1 mM	EDTA

9) 3 M Sodium acetate (pH 5.2)

C. Protocol for the isolation of cytoplasmic RNA from amoebae

1. Collect amoebae by centrifugation for 1 min at 170 *g*. (All procedures are carried out at 4°C, unless indicated otherwise.)

2. Wash amoebae (about 3-ml packed at a time for convenience) several times with DEPC-treated Chalkley's solution by centrifugation.

3. Transfer cells into a cooled glass homogenizer, and add equal volume of RNA-extraction buffer.

4. Lyse cells by 5 strokes of gentle homogenization, transfer the contents into 1.5-ml microcentrifuge tubes as quickly as possible, and centrifuge for 2 min at 10,000 *g*.

5. Transfer the supernatant into a 30-ml Corex tube and add 2.5 ml of Proteinase-K digestion buffer.

6. Cover the tube with Parafilm, mix the contents by inversion, add Proteinase K to a final concentration of 50 μg/ml, and incubate for 30 min at 37°C.

7. Extract the solution once with 7.5 ml of phenol:chloroform (1:1) and centrifuge for 3 min at 10,000 *g* at room temperature.

8. Collect the aqueous phase into a fresh Corex tube, add an equal volume of isopropanol, mix by inversion, and keep the tube on ice for 10 min.

9. Collect RNA by centrifugation for 10 min at 10,000 *g*, wash the pellet once with 70% ethanol, and air dry.

10. Suspend the pellet in sterile distilled water, and estimate the RNA concentration and purity by measuring OD at 260 and 280 nm. (With amoebae, the yield was 200 - 500 μg.)

* The cuvettes should be treated first with HCl:methanol (1:1) for 1 hr, washed extensively with DEPC-treated water and autoclaved.

D. Protocol for the isolation of mRNA

1. Wash the oligo(dT)-cellulose column for mRNA separation several times with 1X mRNA-binding buffer.

2. Suspend 0.1 - 0.2 g of oligo(dT) cellulose in 1 ml of mRNA-column-binding buffer and pour into the prepared column.

3. Mix the isolated RNA solution with an equal volume of 2 column-binding buffer, heat to 65°C for 5 min, and cool quickly on ice.

4. Apply RNA solution to the column, collect the flow-through and reapply.

5. Wash the column sequentially with 5 - 10 vol of column-binding buffer and 5 vol of column-washing buffer.

6. Elute the poly(A) mRNA with 2 - 3 vol of elution buffer. Measure OD_{260} and OD_{280} of the eluted solution and determine the RNA concentration.

7. Add 0.1 vol of 3 M Sodium acetate and 2.5 vol of ethanol to the solution, and keep on ice for 30 min.

8. Centrifuge for 15 min at 10,000 *g*, wash the pellet once with 70% ethanol, recentrifuge for 1 min, and air dry.

9. Dissolve RNA in 10 μl of water and use 5 μl for electrophoresis in a gel. The rest is used to construct a cDNA library.

*mRNA can be isolated faster by using a commercially available, prepacked column; e.g., Poly(A) Quick mRNA Isolation Kit (Stratagene Cat. #200348/9), instead of an oligo(dT)-cellulose column.

II. CONSTRUCTION OF cDNA LIBRARIES

For the purpose of constructing cDNA libraries from isolated poly(A) mRNA of amoebae, we used the Lambda ZAP-cDNA synthesis kit from Stratagene (La Jolla, CA) and followed protocols in the vendor's Instruction Manual with no modification. The major steps are listed in Fig. 1.

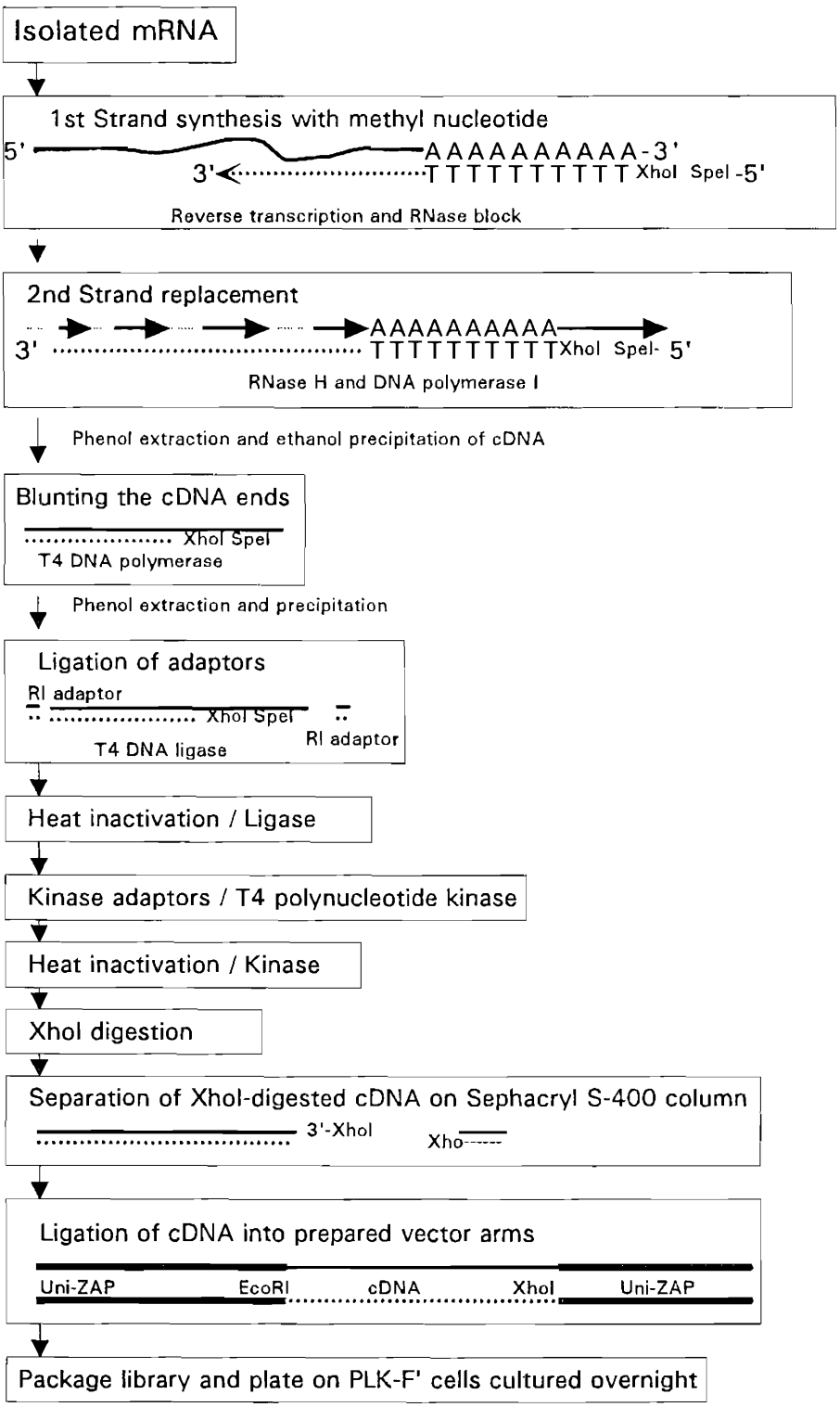
The list of materials and procedures are given in the Instruction Manual (Stratagene Cat. #200400).

III. CLONING THE DNA SEGMENT CONTAINING THE AMOEBA MYOSIN GENE

A. General

Successful isolation of a gene by immunoscreening an expression library depends on several factors. Here the quality of the expression library is of primary importance. It is not easy to determine whether a library is good or not until the expression library is tested with several antibodies that recognize different proteins. However, it is possible to evaluate a gene library indirectly. For example, since actin is present in all eukaryotes and is one of the most abundant and highly conserved

Flow Chart for cDNA Synthesis



Adapted from the Instruction Manual for ZAP-cDNA Synthesis Kit
Stratagene, 1990 (La Jolla, CA)

Fig. 1

proteins regardless of cell types, immunoscreening of the actin gene may give an indication as to how good a library is. If only a very small number of positive signals were detected after screening for the actin gene from $1x10^6$ plaques, for example, there would be little chance of obtaining any cloned genes whose products are much less abundant than actin. When the amoeba's expression library was screened with an anti-amoeba actin antibody, there were about 10 - 20 positive signals from each plate ($2x10^4$ plaques), i.e., 500 - 1,000 positives from $1x10^6$ plaques.

B. Materials

1) Host strains BB4 and XL1-Blue cells

2) LB (Luria-Bertani) medium (per liter)

10 g	Bacto-tryptone
5 g	Bacto-yeast extract
10 g	NaCl

Adjust pH to 7.5 with NaOH.

3) SM buffer (per liter)

5.8 g	NaCl
2 g	$MgSO_4.7H_2O$
50 ml	1 M Tris.HCl (pH 7.5)
5 ml	2% Gelatin

4) Isopropylthio-ß-D-galactose (IPTG)

10 mM in distilled water

5) PBS

10 mM	Sodium phosphate (pH 7.3)
150 mM	NaCl

6) PBS-Tween 20

Add 3 ml of Tween-20 in 1,000 ml of PBS.

7) TBS

10 mM	Tris-HCl (pH 8.0)
150 mM	NaCl

8) Blocking solution

5% Non-fat dry milk in PBS

9) 2 M Sodium azide solution (stock)

10) AlkPase buffer

100 mM	Tris-HCl (pH 9.5)
100 mM	NaCl
5 mM	$MgCl_2$

11) AlkPase substrate solution

50 μg/ml Nitro-blue tetrazolium (NBT) in 70% dimethyl formamide(DMF)
25 μg/ml bromo-chloro-indoyl-phosphate (BCIP, toluidine salt) in 100% DMF

12) Horse-radish peroxidase (HRP) substrate solution

Dissolve 30 mg of 4-chloro-1-naphtol in 10 ml of methanol
Add 50 ml of PBS.

C. Protocol for the phage titer determination

1. Streak out XL1-Blue cells for single-colony isolation on an LB plate containing 12.5 μg/ml tetracycline and grow overnight at 37°C.

2. Inoculate a single colony in 10 ml of LB medium supplemented with 0.2% maltose.

3. Grow cells overnight at 37°C with vigorous shaking, collect them by centrifugation for 10 min at 2,000 *g* at room temperature, and suspend in one-half volume of 10 mM $MgSO_4$ to give $OD_{600} = 1$.

* Maltose induces the maltose operon, which contains the bacteriophage lambda receptor gene (*lamB*). The cells grown in the presence of maltose absorb phage lambda efficiently.

4. To 0.2 ml each of the host XL1-Blue cell suspension placed in 13 x 100-mm tubes, add 20 μl of lambda serially diluted in the SM buffer.

*Here, the phage titer is assumed to be $1x10^{10}$ pfu/ml. The titer of phage stocks decreases with time. For example, when we first determined the titer of the amoeba cDNA library, it was $>1x10^{11}$ pfu/ml. The second check after 6 months showed a drastic decrease in phage titer to about $1x10^9$ pfu/ml.

5. Absorb phage to the cells for 20 min at 37°C with gentle shaking.

6. Add 2.5 ml each (for 90-mm plates) of molten top agar (47°C) to the mixture, invert the tube once for mixing, and pour the contents onto prewarmed, 2 - 3-day-old LB plates as quickly as possible. Leave the plates for 5 min at room temperature.

*Slightly dry, 2 - 3-day-old and prewarmed LB plates are used. Otherwise, the poured molten agar will not spread evenly and the top agar may peel off from the bottom agar when nitrocellulose filter is removed later.

7. Incubate the plates overnight at 37°C.

8. Count the plaque number and calculate the titer of phage stocks by multiplying by the dilution factors.

D-6.8

D. Protocol for immunoscreening libraries

1. Streak out XL1-Blue cells for the single-colony isolation on an LB plate containing 12.5 μg/ml tetracycline and grow overnight at 37°C.

2. To 0.2 ml of the XL1-Blue cell suspension (see above) in a tube, add 20 μl of SM buffer containing 2.5 x 10^4 pfu for each plate (90-mm plates). For 150-mm plates, mix 0.6 ml of the cell suspension with 1x10^5 pfu.

3. Let the cells absorb the phage for 20 min at 37°C.

4. Add 2.5 ml of top agar to the mixture and spread onto an LB plate as above.

5. Incubate the plate for about 3.5 hr at 42°C until tiny phage plaques can be visually identified.

*If the plaques become too large, the boundaries among plaques will disappear. The intensity of the color after chromogenic reaction is strongest at the edge of each plaque. Thus, the plaques should not be allowed to become too large.

6. Number NC filter discs with a pencil, and soak them in a Petri dish containing IPTG (10 mM in distilled water) for 5 min. Take out the filters with a pair of forceps and dry them on clean paper towels.

7. Take out the culture plates from the incubator and place one dried filter disc each on the plates.

*In placing a filter disc, air bubbles should not be trapped between the filter and agar. Each filter disc and the agar plate should be marked for later comparison: It is convenient to mark three locations asymmetrically with an 18-gauge hypodermic needle. The underside of a Petri dish may be marked with a water-proof marking pen.

*The plates should not be taken out all at once. The growth of phage will be inhibited when the plates cool down.

8. Incubate the plates for 4 hr at 37°C.

*Under good conditions the phage may grow faster than expected. Check the plaque size after 3 hr. Since the filter discs are slightly smaller than the plates, plaques can be seen on the edges of plates which are not covered by filters.

9. Remove plates from the incubator and cool them in a refrigerator.

10. Remove filter discs from plates and place them in a large volume of PBS-Tween 20 (or TBS-Tween 20). Wash out any remnant of agar by shaking the filters.

*When processing many (more than 20) filter discs at a time, it is advisable to use a large container to hold 500 ml of the washing buffer. Otherwise, filters may adhere together and may not be washed well.

11. Change the PBS-Tween 20 solution twice at 10-min intervals.

12. Block filter discs in blocking solution for 1 hr.

*Some protocols recommend the use of 20% fetal calf serum as a blocking agent. However, the serum may not be more effective in blocking filters. Since the serum is much more expensive, we prefer to use 5% dry-milk solution. If any problem of nonspecific binding of the primary antibody arises, calf serum may be used instead.

13. Wash filters with PBS once and treat them with the primary antibody with gentle shaking for 2 hr at room temperature or overnight at 4°C.

*If the supply of the antibody is not limited, use as much antibody as possible at the optimum concentration. It is convenient to incubate 20 90-mm filters in a jar (25 cm in diameter) containing 250 ml of antibody solution. When filters are incubated in a small volume of antibody solution, the filters may adhere to one another and the antibody may not be equally accessible to the filters. If the antibody supply is limited, change the position of filters in stack as frequently as possible, for example, by taking out the innermost filters and placing them on top of the filter stack at certain time intervals. In this way, filters will be exposed to the antibody evenly. The antibody solution may be stored at 4°C and reused several times.

*For a mAb, dilute the culture supernatant and ascites fluid 3 - 10 times and 1,000 times, respectively, with PBS. For antisera, dilute 200 - 1,000 times with PBS containing 1% bovine serum albumin. Since each antibody has a different specificity and affinity for an antigen, the dilution factor should be determined empirically. Results of Western blotting may be a good measure in determining how much an antiserum should be diluted.

14. Wash filters with PBS-Tween 20 (or TBS-Tween 20) 3 times at 10-min intervals.

15. Incubate filters in 100 ml of a secondary antibody solution, prepared in PBS-Tween 20, for 2 hr at room temperature with gentle shaking. If the primary antibody has a low titer, incubate filters overnight at 4°C.

*The working concentration of a secondary antibody conjugated with an enzyme is generally 0.5 - 1 μg/ml. The concentration of commercially available secondary antibodies is usually 1 mg/ml, and so dilute them 2,000 times. For ^{125}I-labeled secondary antibodies, use 1 μCi per filter.

*For immunoscreening, the alkaline phosphatase (AlkPase)-conjugated secondary antibody is a good choice. Although the chromogenic reaction of AlkPase is less sensitive than that of a ^{125}I-labeled secondary antibody, it is usually sensitive enough for the detection of most antigens by immunoscreening. The AlkPase-conjugated antibody can be stored for a longer period, at least 6 months, and it is safer to use without involving radiation hazards.

*The HRP-conjugated secondary antibody can also be used for immunoscreening of a gene product. The chromogenic reaction of HRP is even less sensitive than the AlkPase reaction, but the secondary antibody conjugated with HRP and the corresponding substrates are much less expensive than those for AlkPase. If the primary antibody has a high titer, the HRP-detection system would work as well as AlkPase phosphatase system in immunoscreening of genes.

*It is advisable to purchase secondary antibodies from companies specializing in immunochemicals, such as Cappel, Jackson ImmunoResearch, and Accurate Chemicals, rather than purchasing immunoscreening kits. The kits usually contain smaller quantities of antibodies and are much more expensive. All other items are available from Sigma Chemicals (St. Louis, MO).

16. Wash filters twice with PBS-Tween 20 at 10-min intervals.

17. Wash filters once with PBS (without Tween 20).

18. For AlkPase, rinse filters with AlkPase buffer briefly and develop color with a gentle shaking in the AlkPase buffer containing the substrate.

*If the signal is strong, the color would start to appear in minutes. The reaction may be continued for a few hours, but the background would become darker with longer reaction time. So, 10 - 20-min reaction time would be suitable. If color does not develop even after a 1-hr incubation, use a ^{125}I-labeled secondary antibody as a probe.

19. When the color reaction has progressed to a desired intensity, stop the reaction by rinsing filters with distilled water several times.

20. Dry filters in a dark place. Usually the color will bleach after drying, but it will reappear when the filter discs are placed in water.

*For HRP, start the color development by incubating filters in 100 ml of PBS-Tween-20 containing 50 mg of diaminobenzidine and 30 μl of H_2O_2 (30%).

*If 4-chloro-1-naphtol is used as a substrate for HRP, wash filters twice with PBS without Tween-20 for 5 min and initiate the color reaction by adding the substrate solution. Tween-20 tends to facilitate the precipitation of the substrate.

*When using a ^{125}I-labeled antibody, air dry and cover filters with a plastic wrap and expose them to an X-ray film overnight.

*Since it takes only 2 hr to prepare a ^{125}I-labeled secondary antibody, it is convenient to iodinate the secondary antibody by the chloramine-T method [2], which is easy and straightforward. The isotope and unlabeled secondary antibody are purchased separately.

*It is possible to obtain falsely positive signals, and the putative positive plaques should be retested. For this purpose, remove an agar plug from the region of the plate corresponding to the signal on the membrane: Using a sterile Pasteur pipette with a rubber bulb, pick up an agar plug by stabbing the pipette through the bottom agar. Place the plug in 1 ml of SM solution containing one drop of chloroform and leave it for 2 hr at room temperature. The phage particles will be released into the solution during incubation. Keep the phage stock at 4°C. Repeat the testing until all the phage plaques turn out to have positive signals (usually 3 times).

E. Subcloning

We have used the Stratagene's Lambda ZAP II vector system, taking advantage of the ease with which the subcloning can be done fast and conveniently. Most of the time-consuming steps in subcloning are unnecessary in the vector system. For instance, purification of bacteriophage lambda carrying the cloned gene and next subcloning the gene into a different cloning vector (e.g., M13, PUC19, or pBluescript) is omitted. The Bluescript plasmid carrying the cloned gene can be excised in vivo in the presence of a helper bacteriophage, and hence many of the DNA manipulation steps usually used in vitro are not needed.

The detailed procedures are described in the vendor's instructional manual (Stratagene Cat. #236211) for the "Predigested Lambda ZAP II/EcoRI Cloning Kit".

IV. DNA SEQUENCING

A. General

If a cloned gene is more than a kilobase long, it is difficult to read the whole nucleotide sequence of the gene in a single set of sequencing reaction, although both ends can be read. There are two approaches in determining the DNA sequence that is many kilobases in length: one is the shotgun sequencing and the other directional sequencing. Of the two, the directional approach is usually used for a cloned gene. In directional sequencing, a nested set of deletion mutants is generated, which begins at a common point and penetrate various distances into the target region. Among enzymes used for directional deletion, exonuclease III [4] is thought to be the best and is most commonly used.

B. Materials

1) 10X Mung Bean Buffer

0.3 M	Sodium acetate, pH 5.0
0.5 M	NaCl
10 mM	$ZnCl_2$
50%	Glycerol

2) 2X Exonuclease III Buffer

100 mM	Tris-HCl, pH 8.0
10 mM	$MgCl_2$
20 μg/ml	tRNA

3) 1X Mung Bean nuclease dilution buffer

10 mM	Sodium acetate, pH 5.0
0.1 mM	Zinc acetate
0.1 mM	Cysteine
0.1%	Triton X-100
50%	Glycerol

4) TB medium

1.2%	Bacto-tryptone
2.4%	Bacto-yeast extract
0.04%	Glycerol
0.17 M	KH_2PO_4
0.072 M	K_2HPO_4

5) Lysis buffer

50 mM	Glucose
25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA

6) Alkaline solution (prepared freshly)

0.2 N	NaOH
1%	SDS

7) TY medium

1.6%	Bacto-tryptone
1%	Bacto-yeast extract
0.5%	NaCl

8) TYP medium

1.6%	Bacto-tryptone
1%	Bacto-yeast extract
0.5%	NaCl
0.25%	K_2HPO_4

C. Exo III/Mung Bean Nuclease Deletion

1. For the unidirectional deletions, digest 20-50 μg DNA in a 100-μl reaction mixture with a restriction enzyme generating a unique 5' overhang or blunt end that lies between the DNA-sequencing primer and the 5' overhang restriction site.

2. Digest the DNA with a second restriction enzyme which produces a unique 3' overhang restriction site. Neither enzyme should cleave elsewhere in the plasmid.

*It is important to start the directional deletion with clean DNA. Also, the cleavage site for 5' overhang enzyme should be between the insert and the cleavage site for the 3' overhang enzyme.

*If there is no unique 3' overhang site, digest DNA with a 5' overhang reaction enzyme and fill in the single strands with α-thiophosphate dNTP with Klenow fragment. Then, digest the DNA with an enzyme that produces a unique 5' overhang restriction site.

*The amount of DNA would depend on how big the insert is. The number of base pairs that can be read confidently from sequencing gel is about 250 bp. Thus, if the insert is 2.5 kb, one would need about 10 time-points. So, start the deletion with 50 μg of DNA.

3. Check the completion of enzyme digestion by agarose gel electrophoresis.

*The DNA should be completely digested. Otherwise, the colonies that have undeleted DNA will be selected predominantly after transformation, and it will be necessary to screen a large number of colonies. If it is difficult to obtain complete digestion, proceed to the next deletion steps and isolate deleted DNA fragments from an agarose gel for ligation following deletion.

4. Extract DNA with phenol : Chloroform (1:1)

5. Add 0.1 vol of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol, and incubate for 30 min at -20°C or for 15 min at -70°C.

6. Centrifuge for 15min at 12,000 *g*, wash the pellet with 70% of ethanol, and air dry.

7. Dissolve the pellet in 20-50 μl of sterile distilled water.

8. Start exonuclease reactions for all time points in a single tube.

9. At each time point, remove 25 μl of the deleted DNA solution and mix with 175 μl of the Mung Bean buffer in the tubes as quickly as possible.

The stop solution is prepared for each Exo III time point. Dilute 20 μl of 10X Mung Bean buffer into 155 μl of sterile water in an Eppendorf tube for each time interval and place the tubes on ice.

*For each time point, the reaction solution contains:

5 μl	double-digested DNA
12.5 μl	2X Exonuclease III Buffer
2.5 μl	100 mM ß-mercaptoethanol
100 U	Exonuclease

Sterile water to a final volume of 25 μl.

*The given reaction rates of Exo III at different temperatures are:

400 bp/min at 37°C
375 bp/min at 34°C
230 bp/min at 30°C
125 bp/min at 23°C

10. When all aliquots have been removed, heat tubes at 68°C for 15min, and place them on ice.

11. Add 15U of mung bean nuclease diluted in 1X Mung Bean nuclease dilution buffer to each tube and incubate for 30min at 30°C.

12. Add 10 μl of 1M Tris-Cl (pH 9.5), 20 μl of 8 M LiCl, 4 μl of 20% SDS, and 250 μl of phenol:chloroform (1:1).

13. Vortex and spin for 1 min in a microfuge.

14. Remove upper aqueous phase and extract with chloroform once.

15. Add 0.1 vol of 3 M sodium acetate (pH 7.0) to the aqueous phase.

16. Add 2.5 vol of cold ethanol and keep for 30 min at -20°C.

17. Spin in a microfuge for 20 min, wash the pellet with 70% ethanol, and air dry the pellet.

18. Dissolve the pellet in 15 μl of sterile water, and use 7 μl to check deletion by electrophoresis in agarose gel.

19. Ligate deleted DNAs overnight at 4°C in the following mixture:

2.0 μl	Exo/Mung Bean nuclease-treated DNA
2.0 μl	10X Ligation buffer
2 μl	2U T4 ligase
14 μl	Sterile water

20. Transform competent cells with deletion sets according to the transformation protocol with $CaCl_2$.

21. Pick up several colonies from each plate and grow cells in LB medium containing ampicillin.

22. Isolate plasmid and determine the sizes of deleted DNA after cutting with an appropriate restriction enzyme in an agarose gel.

D. Mini preparation of double-stranded plasmid DNA

The pBluescript is a phagemid from which the single strand DNA can be rescued in the presence a helper phage. In E. coli containing F' episome, Bluescript plasmids are secreted as single-stranded plasmids when the bacteria are infected by help phages. Thus, both single- and double-stranded DNA sequencing can be performed with the Bluescript plasmid. Usually, a single-stranded DNA template gives better sequencing results than with double-stranded plasmids. However, it takes more time and energy to prepare single-stranded DNA. Thus, double-stranded templates [7] are used more often as templates in DNA sequencing. There are several different protocols used in isolating plasmids for double-strand DNA sequencing. We compared those protocols and found the following procedure to be most suitable in our work. The plasmid prepared by the protocol gave us the best results. One problem of the method was its low yield. Often, the DNA pellet was not visible after purification. However, since the purity of DNA is more important than the amount in obtaining good results, we have adopted the procedure in our routine work.

1. Inoculate a single bacterial colony in 2 ml of TB medium containing appropriate antibiotics and grow for 20 - 22 hr.

2. Harvest cells by centrifugation, resuspend the pellet in 200 μl lysis buffer and vortex for 5 sec.

3. After 5 min at room temperature, add 400 μl of alkaline solution, invert several times, and place on ice.

4. Add 300 μl of ice-cold 7.5 M ammonium acetate (pH 7.6), invert to mix, and place on ice for 5 min.

5. Centrifuge for 5 min in a microfuge at room temperature, transfer the supernatant to a new centrifuge tube, add 0.6 vol of isopropanol, and keep for 2 hr at -20°C (or for 20 min -70°C.)

6. Centrifuge for 10 min in a microfuge, discard the supernatant and add 100 μl of 2 M ammonium acetate (pH 7.4) to the pellet, vortex, and keep for 5 min on ice.

7. Centrifuge for 5 min in a microfuge at room temperature and collect the supernatant.

8. Add 100 μl isopropanol, and keep for 2 hr at -20°C.

9. Centrifuge for 10 min in a microfuge and discard the supernatant.

10. Wash the pellet with 70% ethanol, air dry, and dissolve in 50 μl of TE buffer (pH 8.0). Add 1 μl of RNase mixture.

11. Incubate for 15 min at 37°C and add 5 μl of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol to the mixture.

12. After keeping for 15 min at -70°C, centrifuge for 10 min in a microfuge and wash the pellet with 70% ethanol. (The pellet may not be visible.)

13. Dissolve the pellet in 25 μl of TE buffer.

E. Preparation of single-stranded DNA templates

As mentioned above, it takes more time to prepare single-stranded DNA. However, it is possible to read more DNA bases when single-stranded DNA is used as the template.

1. Pick up a single colony from a tetracycline-containing LB plate, inoculate in 2 ml of TY medium with ampicillin and grow overnight at 37°C.

2. Next morning, inoculate 10 ml of TYP medium (with no antibiotics) with 500 μl of the overnight culture, and grow bacteria for 1 hr at 37°C with vigorous shaking.

3. Infect bacteria with the helper phage R408 at a 20 multiplicity of infection; i. e., add 100 μl of a phage stock, at $1x10^{11}$ pfu. Continue incubation for 7 hr with shaking.

4. Centrifuge cells in a 15-ml Corex tube for 15 min at 8,000 *g* and transfer the supernatant into a 30-ml Corex tube.

5. Precipitate DNA by adding 0.25 vol of phage-precipitating buffer to the supernatant. Cover the tube with Parafilm, invert to mix, and let stand for 15 min at room temperature.

6. Centrifuge for 15 min at 12,000 *g* and remove the supernatant as much as possible.

7. Suspended the pellet in 700 μl of TE buffer (pH 8.0) and transfer into a 1.5-ml microfuge tube. Add 700 μl of phenol-chloroform (1:1), vortex for 30 seconds, and spin for 3 min at 1500 *g*.

8. Repeat the phenol extraction two more times.

9. Transfer the aqueous portion into a microfuge tube, add 500 μl of chloroform, vortex for 30 seconds, and spin for 2 min in a microfuge.

10. Repeat chloroform extraction once more, and transfer 400 μl of the aqueous phase into a fresh tube.

11. Add 200 μl of 7.5 M ammonium acetate, pH 7.5 and 800 μl of cold ethanol. Invert to mix and leave for 30 min at 20°C or for 15 min at -70°C.

12. Spin for 15 min in a microfuge, rinse with 70% ethanol, dissolve the residue in 20 μl of distilled water.

13. Take 1 μl of the DNA solution and run agarose-gel electrophoresis to check the amount and purity of DNA.

F. Growth of Phage R408

The helper phage is used in the self excision of the cloned gene during subcloning and in obtaining single-stranded DNA templates.

1. Pick up a single colony (XL1-Blue cells) from a tetracycline LB plate and grow in 2 ml of LB medium overnight.

2. Next morning, add 1 ml of the culture in 15 ml of TY medium and grow for 1 hr with shaking.

3. Inoculate 100 μl of a phage stock ($1x10^{11}$ pfu/ml) to the culture and grow for 8 hr with vigorous shaking.

4. Pellet cells by centrifugation for 15 min at 8,000 *g* and transfer the supernatant into a sterile 50 ml-size polystyrene tube.

5. Heat the tube for 15 min at 65°C.

6. Determine the titer of the phage stock and store it at 4°C. The titer should be about $1x10^{11}$ pfu/ml.

G. Sequencing reaction

There are several DNA sequencing kits available from different suppliers, and we have used Sequenase 2.0 from United States Biochemical Corporation (USB; Cleveland, OH). The sequencing kit comes with a detailed reaction protocol that can be followed with no modification. One item not included in the kit is the radioactive precursor. Two types of isotopes are used in DNA-sequencing reactions, ^{35}S and ^{32}P. Of the two, we prefer ^{35}S-labeled dATP, which gives a better resolution and can be kept for a longer period of time. In our experience, the isotope can be used for more than 6 months, if kept at -70°C. From our experience, we can read 100 bp more in a ^{35}S gel.

H. DNA-Sequencing gel system

We have used Lang and Burger's [6] DNA-sequencing gel system with slight modifications and obtained good results. From a standard-sized 45-cm gel, we could read 300 bases with confidence. By using a longer, 60-cm gel, we could read up to 500 bases. When using glass plates wider than 30 cm, we found it helpful to place another spacer in the middle to make the entire gel of even thickness.

LITERATURE CITED

1. Berger, S.L. 1987. Isolation of cytoplasmic RNA: Ribonucleoside-vanadyl complexes. Method. Enzymol. 152:227-234.

2. Bolton, A.E. 1986. Comparative method for the radiolabeling of peptides. Method. Enzymol. 124:18-29.

3. Choi, E.Y., Kim, K.J. & Jeon, K.W. 1991. Lysosome-phagosome fusion in amoebae, as studied using lysosome-membrane-specific monoclonal antibodies. J.Cell Biol. 115:229a.

4. Henikoff, S. 1984. Unidirectional digestion of exonuclease III creates targeted break points for DNA sequencing. Gene 28:351-359.

5. Kim, H.J., Choi, E.Y. & Jeon, K.W. 1991. The presence of myosin in *Amoeba proteus*: Isolation and characterization of cDNA encoding myosin heavy chain. J. Cell Biol. 115:330a.

6. Lang, B.F. & Burger, G. 1990. A rapid, high resolution DNA sequencing gel system. Anal.Biochem 188:176-180.

7. Lee, S. & Rasheed, S. 1990. A simple procedure for maximum yield of high-quality plasmid DNA. Biotechnique 9:676-679.

8. Lynch, D.C. 1987. Use of antibodies to obtain specific polysomes. Method. Enzymol. 152:248-253.

9. MacDonald, R.L., Swift, G.H., Prazybyla, A.E. & Chirgwin, J.M. 1987. Isolation of RNA using guanidinium salts. Method. Enzymol. 152:219-227.

10. Ord, M.J. 1971. *Amoeba proteus* as a cell model in toxicology. In "Mechanism of Toxicity" (W. N. Aldridge, ed.), pp. 175-186. London, Macmillan.

PURIFICATION OF AMOEBA mtDNA USING THE UNSET PROCEDURE

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INTRODUCTION

The isolation of intact mitochondrial DNA (mtDNA) from *Acanthamoeba* is difficult due to extensive degradation of the DNA, which has been attributed to high levels of endogenous endonucleases. The UNSET technique was originally developed by Garriga et al. (1) to purify intact mRNA from *Neurospora crassa* mitochondria. R. A. Akins proposed the use of the procedure in our lab and we developed it for use with *Acanthamoeba*. Bisbenzimide is a fluorescent dye that intercalates preferentially into AT-rich helical DNA and decreases the buoyant density. This allows enhanced separation of the nuclear and mitochondrial DNAs because mtDNA is often higher in AT content than nuclear DNA. Mitochondrial DNA isolated by this procedure is intact, uncontaminated by nuclear DNA, and readily digested by restriction endonucleases to reveal restriction fragment length polymorphisms of potential taxonomic value. The mitochondrial origin of the DNA can be confirmed by extraction from isolated mitochondria. The method has been used extensively in our lab for *Acanthamoeba* (2) and by Milligan and Band (3) for *Naegleria* and *Vahlkampfia*.

PROTOCOL

1. Pellet log phase amoebae from culture medium at 1000 X g for 15 minutes at 4° C. Discard supernatant. 1 X 10^8 amoebae of the Neff strain contain about 20 µg of mtDNA.
2. Resuspend amebas in cold 0.15M KCl. Use 5 ml per liter of original culture medium and keep the amoebae on ice.
3. Pellet cells at 1000 X g for 10 minutes at 4° C. Discard supernatant.
4. Repeat steps 2 and 3 two more times. Keep the amoebae cold. Store at -20° C or continue.
5. Lyse cells by adding 5 ml of UNSET Lysis Buffer (8M Urea, 2% sodium dodecyl sulfate, 0.15M NaCl, 0.001M EDTA, 0.1M Tris pH 7.5) per 1 X 10^8 amoebae at room temperature. The lysis buffer is viscous, so use a wide bore pipet. Immediately add an equal volume of phenol-CIA (phenol:chloroform:isoamyl alcohol 25:24:1) and shake gently at room temperature for 10 minutes. Make sure that the tubes you are using are resistant to phenol.
6. Centrifuge at 3000 X g for 10 minutes at room temperature. Urea and phenol crystallize at lower temperatures.

7. Remove the upper aqueous phase using a wide bore pipet. Do not disturb the protein interface between the aqueous and organic phases. Discard the phenol-CIA mixture. Check with your hazardous waste office for correct disposal procedures. Repeat the extraction using fresh phenol-CIA until the interface is gone (2-3 times).
8. Add 3M NaCl to bring the NaCl concentration to 0.3M.
9. Add 2-3 volumes of cold 95% ethanol, mix gently by inverting and freeze at -20° C overnight, or at -70°C for 15-30 minutes.
10. Centrifuge at 12,000 X g for 30 minutes at 4° C to sediment the nucleic acids (NA).
11. Wash the nucleic acid pellet with 70% ethanol and repeat step 10.
12. Dry the pellet under vacuum.
13. Dissolve the pellet in TE (10mM Tris pH 7.6, 1mM EDTA) at 0.2 - 0.3 mg nucleic acid/ ml TE (from a rough estimate of NA recovery). We use 5 ml for 1 X 10^8 amoebae. Add 1.25 g of CsCl per ml TE, and 10 µg of bisbenzimide per ml of TE. The solution should have a refractive index between 1.3944 and 1.3947 or a density of 1.55 gm/ml.
14. Dispense the solution into ultracentrifuge tubes. Vertical or fixed angle rotors may be used. Centrifuge for 48 hours at 130,000 X g at 20° C.
15. Remove the upper band, which can be visualized as a yellow/green fluorescent band by using a UV light source. See Sambrook et al. (4) for a description of this method.
16. Extract the removed solution five times using isopropanol saturated with 20 X SSC (175.3 gm NaCl, 88.2 g sodium citrate per liter of H_2O) to remove the bisbenzimide. The bottom layer is saved.
17. Dialyze in three changes of 1 X STE (10mM Tris pH 7.6, 100mM NaCl, 1mM EDTA) for 24 hours in the cold (4° C).
18. Ethanol precipitate and dry the pellet as in steps 9 - 12.
19. Dissolve the pellet in TE. Start with a small volume, such as 200µl. Determine the concentration of a diluted sample spectrophotometrically. The nucleic acid can then be diluted further if desired.

COMMENTS

If you have questions about the gradient portion of this protocol, reference (4), has a detailed section on ethidium bromide/CsCl gradients.

LITERATURE CITED

1. Garriga, G., Bertrand, H. & Lambowitz, A. 1984. RNA splicing in *Neurospora* mitochondria: nuclear mutants defective in both splicing and 3' end synthesis of the large rRNA. *Cell*, **36**:623-634.
2. Byers, T. J., Hugo, E. R. & Stewart, V. J. 1990. Genes of *Acanthamoeba*: DNA, RNA and protein sequences. *J. Protozool.*, **37**(4):17S-25S.
3. Milligan, S. M. & Band, R. N. 1988. Restriction endonuclease analysis of mitochondrial DNA as an aid in the taxonomy of *Naegleria* and *Vahlkampfia*. *J. Protozool.*, **35**(2):198-204.
4. Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular Cloning, Vol. 1, Cold Spring Harbor Laboratory, sec. 1.42-1.45.

DETECTION OF HUMAN SERUM ANTIBODIES REACTIVE WITH *ACANTHAMOEBA POLYPHAGA* BY USE OF AN INDIRECT FLUORESCENT ANTIBODY (IFA) TEST

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INTRODUCTION

This protocol describes an indirect fluorescent antibody (IFA) test designed to detect human and animal serum antibody reactive with *Acanthamoeba polyphaga* whole antigen. The aim of this writing is to present a detailed, controlled, and standardized protocol that can be adapted for use in detecting serum antibodies, both human and animal, directed against many different whole-cell microorganisms. One need only to substitute the organism of interest for *Acanthamoeba polyphaga* when preparing antigen slides.

The use of serum controls when performing IFA tests is important. An extensive explanation of the selection and use of controls follows the basic IFA procedure.

BASIC PROTOCOL

I. REAGENTS AND SUPPLIES

1. TSB: trypticase soy broth
   30 g powder in one liter distilled H2O, autoclaved
2. formalin (37% W/W formaldehyde solution)
3. PAS: Page's amoeba saline
   0.120 g/l NaC1
   0.004 g/l $MgSO_4 \cdot 7H_20$
   0.004 g/l $CaC1_2 \cdot 2H_20$
   0.142 g/l $Na2HPO_4$
   0.136 g/l $KH2PO_4$
   dissolve in one liter distilled H2O and adjust pH to 6.8-7.0; store at 4°C
4. gelatin powder

5. chromalum: chromium potassium sulfate, $CrK(SO_4)2 \cdot 12H_2O$
6. PBS: phosphate buffered saline, pH 7.6; store at 4°C
7. FITC conjugates: Sigma anti-human IgG (gamma chain) conjugated to FITC (affinity isolated, developed in goat)
   Sigma anti-rabbit IgG (whole molecule) conjugated to FITC (developed in goat)

Note:The IFA protocol described hereafter gives satisfactory results when these particular conjugates are used. Other FITC conjugates may be used to detect antibodies of other classes or antibodies to other species; however, minor adjustments may be necessary in test parameters in order to obtain optimal results.

8. 0.1% Evan's blue counterstain: 0.1 g Evan's blue powder in 100 ml PBS pH 7.6; store at 4°C
9. IFA slides: 10-well, black teflon-coated, glass immunology slides

II. PREPARATION OF ANTIGEN SLIDES

A. COATING SLIDES WITH GELATIN

The purpose of coating immunology slides with gelatin is to provide a protein covering on the slides with which formalin-fixed amoebae will readily bind. Formalin-fixed amoebae will not adhere well to uncoated slides.

1. Into a heat-proof pan designed to hold microscope slides, place:
   1 liter distilled H2O
   0.06 g chromalum
   6 g gelatin powder, tightly sealed inside a cheesecloth bag
2. Heat the pan on moderate heat until the liquid boils. Remove the pan from the heat immediately to prevent boiling over.
3. Remove the cheesecloth bag and skim the froth from the surface of the liquid.
4. Place the slides into a slide holder and place the holder into the hot liquid. Leave the slides immersed for two minutes.
5. Remove the holder containing the slides from the liquid. Tilt the holder slightly to drain off excess liquid.
6. Place the holder containing the slides into a 60°C oven. Bake for 30 minutes.
7. Cool the slides. Store the slides at room temperature (RT).

B. BINDING AMOEBAE TO SLIDES

1. Using aseptic technique, add 0.5 ml of a stock culture of axenically grown *Acanthamoeba polyphaga* to a culture tube containing 4 ml of TSB. Incubate the amoebae on a slant at RT for two days. Two-day incubation results in a large trophozoite yield, with a minimum amount of cysts and cellular debris.
2. (From this point on, aseptic technique and the use of sterile equipment and supplies is unnecessary.) After two days, add 0.5 ml formalin to the tube culture, making a 1:10 dilution. Adding this much formalin fixes organisms rapidly and enhances retention of shape, size and pseudopodia. Let the tube stand for 18-24 hrs. at RT. (Tubes may be stored at 4°C for later use.)
3. Vortex the tube gently to dislodge any amoebae from the sides of the tube.
4. Centrifuge the tube at 1500 g for 15 minutes. Aspirate off the supernatant.

5. Add 5 ml PAS to the tube. Resuspend the amoebae. Determine the concentration of the suspension (# amoebae/ml) using a hemacytometer.
6. Centrifuge the tube and aspirate the supernatant as in step 4. Adjust the suspension to approximately 10^6 amoebae/ml using fresh PAS. (NOTE: Use of PBS instead of PAS will result in crystal deposition on the slides.)
7. Mix the amoeba suspension and apply 10 µl suspension to each well of properly labelled gelatin-coated immunology slides, spreading the mixture evenly over the entire well. Allow the slides to barely air dry.
8. When the slides are barely dry, place them into a 60°C oven for two minutes.
9. Cool the slides at RT, place them into an airtight container, such as a plastic ziplock™ bag, and store them at -20°C or below. Slides prepared and stored as directed retain amoeba morphology and serum reactivity for at least two months.

III. SPECIMEN COLLECTION

1. Collect animal or human blood in an aseptic manner, without anticoagulant.
2. Allow the blood to clot and centrifuge the blood tube as soon as possible.
3. Remove the serum, and place it into a properly labelled plastic vial.
4. For maximum antibody reactivity, store the serum at 4°C for up to one week or at -20°C or below indefinitely.

IV. IFA TESTING

1. Draw a diagram showing which dilution of each specimen or control will go onto each well of the slides. Plan to place a PBS control onto one well of every slide. Plan to test the unimmunized animal serum control, the immunized animal serum control, and the pooled normal human sera control (at dilutions as described in Section VI) at least once per run.
2. Remove antigen slides from the freezer, allow them to reach RT, and label them appropriately.
3. In properly labelled tubes, prepare serial doubling dilutions of control and test sera in PBS.
4. Layer 12 µl of diluted serum (or PBS, for the PBS control) onto appropriate wells, using the diagram as a guide.
5. Incubate the slides in a covered, moist chamber for 30 minutes at 37°C.
6. Using three different washes, rinse the slides a total of 10 minutes in PBS. After removing the slides from the last wash, stand them on end to dry. Add conjugate (described below) when the area between wells has just become dry but before the wells themselves have dried. If wells dry out, fluorescence may be adversely affected.
7. With PBS as diluent, dilute the chosen FITC conjugates according to the manufacturer's recommendation. Prepare only as much volume as is needed for the run, and protect both the stock conjugate and the diluted conjugate from the light.
8. Layer 12µl of diluted conjugate onto the appropriate wells.
9. Repeat step 5, incubating the slides in the dark.
10. Using PBS in a squirt bottle, run a stream of PBS over the wells, to rinse off the conjugate. Direct the stream at a portion of the slide above the wells so that

organisms are not dislodged.

11. Immerse slides in 0.1% Evan's blue counterstain for 5 minutes at RT, in the dark. This stain quenches any background fluorescence.
12. Rinse and dry the slides as in step 6, in the dark.
13. Place IFA mounting medium onto each slide and then coverslip. At this point, slides may be stored, if kept in the dark at 4°C; but for best results, read slides immediately.
14. Read each well using a fluorescence microscope with filters appropriate for detecting FITC fluorescence. Grade the fluorescence of organisms on a scale 0 to 4+.

V. INTERPRETATION

Each laboratory must establish internal guidelines for grading fluorescence. Because interpretation of IFA tests is subjective, the following parameters should be standardized: the time elapsed from test completion to slide reading; the amount of time the observer takes to adjust to darkness before reading; the amount of external light present in the room; the magnification used when reading. A limited number of people should read the slides, and the readers should confer often so that their grading criteria become consistent. Even when these guidelines are followed, a reproducibility difference of one doubling dilution is acceptable, due to the subjectivity inherent in IFA test interpretation. When acute and convalescent sera, collected two to six weeks apart, are tested with anti - IgG conjugate, results showing a four-fold rise or more in titre are consistent with a host antibody response to antigens of the test organism.

VI. SELECTION AND USE OF CONTROLS

A. POOLED NORMAL HUMAN SERA CONTROL

This control has two functions: it allows for monitoring of run-to-run variations by repeated use of the same specimen; and it provides a standard normal titre with which the titres of unknown human specimens can be compared and consequently placed into interpretative categories.

1. Collect sera from healthy individuals who are age- and gender-matched to the test population group.

2. Using the IFA protocol described above, determine the endpoint titre of each specimen in the control group. Each lab should establish its own criteria for endpoint determination, deciding what percentage of organisms must fluoresce and at what intensity. Doubling dilutions from 1:5 to 1:80 are recommended for this screening step. If one or more individuals have significantly increased titres, consider omitting them from further testing.

3. Prepare a pool of equal amounts of all acceptable sera from the control group. This pool is referred to as the "pooled normal human sera control".

4. Determine the endpoint titre of this pool.

5. In all subsequent testing of unknown specimens, test the pooled normal human sera control at its endpoint dilution and at one dilution above and one dilution below the established endpoint.

B. TESTING OF HUMAN SERUM SPECIMENS:

Human serum specimens whose antibody titre is to be determined may be tested in one of two ways:

1. Each individual human serum specimen may be diluted over a wide range of doubling dilutions and each dilution tested in one run. The endpoint of the unknown serum is compared to the endpoint of the pooled normal human sera control and determined to be normal or above normal in antibody reactivity.

2. Or, each individual serum may be tested at a screening dilution defined as one doubling dilution (or two) above the endpoint dilution of the pooled normal human sera control. Those sera that react at the screening dilution are considered to have above normal antibody reactivity and may be titred further in a subsequent run. Those that do not react at the screening dilution are considered to have normal antibody reactivity.

C. ANIMAL SERUM CONTROLS:

It may not be possible to obtain highly reactive serum from a human with a diagnosed case of acanthamoebiasis. In order to ascertain and monitor the sensitivity of reagents in the IFA test, it is desirable to test a serum which reacts strongly at a very high dilution. The immunized animal control provides such a serum. This control cannot, however, monitor the anti-human Ig-FITC reagent, because that reagent must be substituted with the proper anti-species Ig-FITC reagent when testing animal serum.

1. Collect serum from a healthy, unimmunized animal. (We use rabbits.) This provides the "unimmunized animal serum control".

2. Immunize the animal with the organism being investigated: Grow amoebae for 2 days at 37°C in tubes containing 5 ml TSB. Shake tubes, then centrifuge tubes and remove supernatant. Resuspend amoebae in PBS centrifuge the tubes, remove the supernatant, and wash the pellet again in PBS. Using PBS, adjust the suspension to a final concentration of $2x10^5$ amoebae/ml. Freeze and thaw suspension five times. Inject two ml of suspension into the marginal ear vein of a rabbit at weekly intervals, for three weeks. One week after the last injection, collect serum from the rabbit for the "immunized animal serum control".

3. Dilute the unimmunized animal serum control from 1:5 to 1:80 and determine its endpoint titre by using the same IFA protocol as described previously for human serum, substituting the proper anti-species Ig-FITC for the anti-human Ig-FITC.

4. Dilute the immunized animal serum control from low to very high dilutions and test as described for the unimmunized animal serum.

5. In all subsequent IFA runs, test:

a) the unimmunized animal serum control at its endpoint dilution and at one dilution above and one dilution below the endpoint. This control allows for monitoring of run-to-run variations.

b) the immunized animal serum control at a dilution that reads approximately 3+ in strength. This control monitors run-to-run variations and also monitors functioning of the microscope.

D. PBS CONTROL:

This control differs from other controls in that no serum is placed in the well. The control allows one to determine whether fluorescence is a direct result of factors present in test serum.

1. Substitute an equal volume of PBS for serum on one well of each IFA slide and continue testing as outlined, using FITC conjugate when indicated.

2. This well should always be negative for fluorescence. If organisms fluoresce in this well, results of the entire slide are invalid. Fluorescence of this control can result when FITC conjugate which is not affinity-isolated binds directly to the surface of the organism.

LITERATURE CITED

1. Culbertson, C G, & Harper, K. 1984 Pathogenic free-living amoebae-Immunocytologic demonstration and species identification. *Am. J. Trop. Med. Hyg.* **33**:851-856.
2. Nairn, R C, ed. 1969 Fluorescent Protein Tracing, Third Edition, The Williams and Wilkins Company, Baltimore.
3. Page, F C. 1967 Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *J. Protozool.*,**14**:499-521.

DETECTION OF ANIMAL SERUM ANTIBODIES REACTIVE WITH *ACANTHAMOEBA POLYPHAGA* BY USE OF AN ENYZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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INTRODUCTION

This protocol describes an enzyme-linked immunosorbent assay (ELISA) designed to detect animal serum antibody reactive with *Acanthamoeba polyphaga* whole antigen. This is an indirect method, which measures antibody in serum by allowing serum antibodies to be captured by whole cell antigens which have been passively adsorbed to wells of microtiter plates. Enzyme-labelled anti-species immunoglobulin conjugate is then added to the wells, where it reacts with the captured serum antibodies. Lastly, enzyme substrate is added to the wells. The substrate is selected for its ability to develop color as it is degraded by the enzyme. The amount of color change is proportional to the amount of reactive antibody in the serum.

In general, the ELISA method allows detection of antibody at a much higher dilution than is possible using the indirect fluorescent antibody (IFA) method. In certain experimental situations, such increased test sensitivity may be required. At these times, making minor modifications in the following protocol (adjusting for the particular organism and class of antibody being studied, and for the experimental animal being immunized) may provide a highly sensitive test method capable of measuring small amounts of antibody.

This protocol is optimized to detect the IgG class of antibody in the serum of rabbits experimentally immunized with *Acanthamoeba polyphaga* antigen. Absorption of the immune rabbit serum with homologous antigen removes much of the measured antibody activity; therefore, we conclude that the ELISA test method may be fairly specific. The ELISA method detects antibody activity at a much higher immune rabbit serum dilution than the IFA method does; therefore, we conclude that the ELISA method is highly sensitive.

The following protocol is based on studies which measure reactive antibody in experimentally immunized animals only. We recommend that absorption studies be performed to validate test specificity.

D-9.2

BASIC PROTOCOL

I. REAGENTS AND SUPPLIES

1. TSB: trypticase soy broth
   30g powder in one liter distilled H_2O(autoclaved)
2. formalin (37% W/W formaldehyde solution)
3. PBS: phosphate buffered saline, pH 7.6 and pH 7.4; store at 4°C
4. PBS-Tween 20:add 0.5 ml Tween 20 to one liter PBS pH 7.4;
5. BLOTTO: add 25 g powdered nonfat dry milk to 500 ml PBS-Tween 20; prepare fresh daily, store at 4°C
6. carbonate/bicarbonatebuffer:add one capsule carbonate/bicarbonate buffer powder(Sigma catalog #C-3041) to 100 ml deionized H_2O; this is a 0.05 M solution with pH 9.6; use immediately.
7. substrate buffered diluent: 0.1 M diethanolamine buffer; add 0.525 g diethanolamine and 5 mg $MgCl_2 \cdot 6H_2O$ to 50 ml deionized H_2O; adjust pH to 9.8; prepare fresh daily
8. substrate: p-nitrophenyl phosphate (p-NPP), supplied as 5mg tablets (Sigma catalog #N-9389); add one tablet to every 5 ml of substrate buffered diluent immediately prior to use.
9. alkaline phosphatase conjugate: Sigma anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase (developed in goat)
10. ELISA microtiter plates:96-well, flat bottom, sterile polystyrene ELISA plates, with covers

II. PREPARATION OF ELISA ANTIGEN PLATES

1. Add a 5 ml TSB culture of axenically grown *Acanthamoeba polyphaga* (or similar organism) to a large, sterile flask containing 50 ml TSB. Incubate the flask for 2 days at 35°C or for 3 days at room temperature (RT).

2. Decant the TSB from the flask. Add 50 ml PBS pH 7.6 to the flask.
3. Place the flask in an ice water bath for 30 minutes, so that amoebae will release from the inner surface of flask.
4. After 30 minutes, inspect the flask to ensure that amoebae are free from the surface. Shake the flask and pour the contents into a 50 ml conical centrifuge tube.
5. Centrifuge the tube for 10 minutes at 2500 g. Aspirate off the supernatant.
6. Add 9.9 ml PBS pH 7.6 and 0.1 ml formalin to tube, to make a 1% formalinized suspension. Shake the tube gently to disperse the amoebae. (From

this point on, aseptic technique and the use of sterile reagents and supplies is unnecessary).

7. Let the tube stand at RT for several hours. (Formalinized amoebae may be stored at 4°C until time of use. Satisfactory results are obtained with preserved amoebae stored for several months).

8. Fill the tube with PBS pH 7.6. Centrifuge the tube 10 minutes at 2500 g. Aspirate the supernatant and wash once more.

9. While the amoebae are spinning, prepare 100 ml carbonate/bicarbonate buffer (see Reagents section).

10. After the amoebae are washed, resuspend them in 20 ml fresh PBS. Determine the concentration of the suspension (# amoebae/ml) using a hemacytometer.

11. Centrifuge the tube as in step 8 and aspirate the supernatant.

12. Add a sufficient volume of carbonate/bicarbonate buffer to the amoebae to achieve the pre-determined optimal coating concentration*. Verify the concentration of the antigen suspension before loading the plates.

> * NOTE:The optimal coating concentration must be determined by trial and error for each particular organism. This is done by measuring the absorbance value obtained using various concentrations of antigen suspension with various dilutions of antiserum, in a checkerboard pattern. Choose the concentration which gives a relatively high absorbance value and above which the increase in absorbance value is minimal compared to the corresponding increase in concentration of antigen suspension. We determined the optimal coating concentration of *A. polyphaga* when tested with immune rabbit antiserum to be 10^5 organisms/ml.

13. Draw a diagram of the plate layout. (Refer to section on controls.) Plan to leave at least seven wells on each plate with no antigen suspension in them and plan to test each dilution of each specimen in triplicate. Add 100 µl of antigen suspension to all wells (except those for controls), and incubate covered plates at 35°C for one hour. At this point, plates may be stored at 4°C overnight for testing the following day, or testing may be done immediately.

III. SPECIMEN COLLECTION

1. Collect blood from the experimental animal (both before and after immunization) in an aseptic manner, without anticoagulant.
2. Allow the blood to clot and centrifuge the blood tube as soon as possible.
3. Remove the serum and place it into a properly labelled plastic vial.

4. For maximum antibody reactivity, store the serum at 4°C for up to one week or at -20°C or below indefinitely.

IV. ELISA TESTING

A. ADDING BLOCKER TO PLATES

1. On the day of testing, remove excess antigen from the plates by washing them three times with PBS-Tween 20, in the following manner (this wash procedure should be used throughout the test when plate washing is indicated): Invert the plates over a waste container wider and longer than an ELISA plate, capable of holding a liter of liquid, and containing a small amount of bleach diluted in water. Shake the liquid from the plates into the waste container. Blot the plates, upside down, on paper towels. Place the plates right side up on a counter and fill all wells with PBS-Tween 20, using a squirt bottle and directing the stream onto the sides of each well, so as not to dislodge any organisms. Let the wash solution remain in the wells for two minutes. Invert the plates over the waste container and shake out the PBS-Tween 20. Blot the plates, upside down, on paper towels. One wash step has been described. Repeat twice for a total of three washes.
2. To prevent non-specific binding of serum or conjugate proteins to the organisms or to the plate itself, use of a blocker both to coat wells and also as the diluent for both serum and conjugate is recommended. Our experience shows that the most effective blocking agent is 5% nonfat dry milk powder dissolved in PBS-Tween 20, termed "BLOTTO". After washing the plates, add 200 µl BLOTTO to each well (except the "blank" well, described later).
3. Incubate the plates with BLOTTO at RT for 90 minutes. Wash the plates only once.

B. ADDING SERUM TO PLATES

1. While the plates are incubating with the BLOTTO blocker, dilute the animal serum in BLOTTO. For immune serum, recommended dilutions for initial testing are 1:100, 1:200, 1:400, 1:800, etc., on up to 1:10,240 or more. Serum drawn from the animal prior to immunization should be tested as well. It is unnecessary to carry out dilutions of this serum past 1:400.
2. After the plates are washed, and omitting the blank and conjugate control wells, add 100 µl of serum dilutions to all appropriate wells.

3. Add 100 µl BLOTTO to the conjugate control wells. Add nothing at this step to the blank well.
4. Cover the plates and incubate them at 35°C for one hour.
5. Wash the plates three times in PBS-Tween 20.

C. ADDING CONJUGATE TO PLATES

1. While the plates are incubating with serum, dilute the conjugate according to the manufacturer's recommendation, using BLOTTO as diluent. Prepare only as much volume as is needed for the run.
2. After the serum is washed from the plates, add 100 µl diluted conjugate to all wells but the blank well.
3. Cover the plates and incubate at 35°C for one hour.
4. Wash the plates three times in PBS-Tween 20.

D. ADDING SUBSTRATE TO PLATES

1. While the plates are incubating with the conjugate, prepare the substrate buffered diluent, as described above in reagents section. Just before washing the conjugate from the plates, add p-NPP (substrate) to the buffered diluent at a concentration of one mg substrate to one ml diluent. Keep this solution in the dark. Prepare only as much substrate as is needed for the run.
2. After the conjugate is washed from the plates, add 100 µl diluted substrate to all wells, including controls and blank.
3. Incubate the plates at 35°C in the dark. The time of incubation may vary, depending upon the rate of conversion of substrate, which is controlled by the amount of antibody bound to the organisms during the serum incubation step. Observe the plates at ten minutes intervals and read them when color appears strong in wells with low serum dilutions but is only slightly apparent or not visible in wells with higher serum dilutions.

E. READING PLATES

1. When alkaline phosphatase is the enzyme used, there is no need to add an acid or a base to stop the reaction, as long as the plates are read immediately. Not adding a stop solution allows one to read the same plates at various time intervals as the substrate is progressively converted.

NOTE: If this procedure is modified for use with a different

converting enzyme, it may be necessary to use a stop solution at this point. Refer to standard texts for details.

2. The plates may be read qualitatively, by observing color development visually and assigning grades dependent upon color intensity. The ELISA test reaches its greatest potential, however, when the plates are read by a spectrophotometer (ELISA reader) specially designed to detect and display the change in absorbance in each separate well. Results are then quantitative and data can be analyzed more accurately. To read plates prepared by the above protocol:

a. Warm up the ELISA reader at least 10 minutes before use.

b. Set the reader to a 405 nm wavelength setting (for alk phos/p-NPP system).

c. Keeping the ELISA plates protected from the light, place the plates, one at a time, in the reader. Use the blank well to blank the reader - this sets the absorbance of the blank well at zero, cancelling out any absorbance due to unreacted substrate.

d. Read and record the absorbance values of all wells.

e. If the plates are covered, protected from the light, and placed back into a 35°C incubator, they may be read again after any time interval. If color development does not occur within one to two hours, adjustments may be made, one at a time, in antigen suspension concentration, serum dilution, conjugate dilution, or incubation times and temperatures.

V. SELECTION AND USE OF CONTROLS

The ELISA test can be a highly sensitive, quantitative test. It must be tightly controlled, however, because it is not possible to look at an ELISA plate and differentiate whether reactivity is occurring specifically with an organism or nonspecifically with the plate surface. In either case, the end result, color development, occurs.
The following is a chart describing those controls which we recommend be used:

CONTROL CONJUGATE NAME	ANTIGEN IN WELL?	BLOTTO PRE-COAT IN WELL?	SUBSTANCE USED IN SERUM STEP IN WELL?
Blank	no	no	none
no			
Conjugate	no	yes	BLOTTO
yes			
control			

Nonspecific serum control	no	yes	reactive serum
Diluent control	yes	yes	BLOTTO

Substrate is added to all the wells, including the blank. Test each control in triplicate, except for the blank.

The purpose of each control is as follows:

1. Blank - Blanks out any change in absorbance value due to unconverted substrate.

2. Conjugate control - Detects nonspecific binding of conjugate to plates coated with BLOTTO but having no antigen.

3. Nonspecific serum control - Detects nonspecific binding of a reactive serum to plates coated with BLOTTO but having no antigen.

4. Diluent control - Detects a change in absorbance value contributed by anything in the system except serum. Provides a "background absorbance value" for comparison with absorbance values of serum wells.

If any of the above controls has a high absorbance value, results for that plate are suspect and tests done on that plate should be repeated.

VI. TESTINTERPRETATION-QUANTITATIVE

1. Check to ensure that all control wells have absorbance values of less than 0.100. Ideally, they should read 0.050 or less.
2. Check the reproducibility of the results of each specimen tested in triplicate:

Determine the mean absorbance value of the three replicates. The standard deviation from the mean of each replicate result should be 10% of the mean. If so, the mean absorbance value for the three replicates is used as the absorbance value for that specimen. If only one replicate is out of range, it can be ignored, and the mean recalculated using the remaining two readings. If results do not fall into either of the above two categories, results for that specimen are invalid, and that specimen must be repeated in a future run.
3. To obtain a final absorbance value for each specimen, subtract the absorbance value of the diluent control (which represents the "background absorbance value") from the mean

absorbance value for that specimen.
4. Absorbance values can be used, as calculated above, for analyzing the strength of the immune response elicited in immunized animals. If many ELISA runs will be done and many distinct sera will be tested and compared, the following additional tests and calculations may be helpful in standardizing results:

a. Choose a serum at a certain dilution that is known to give a fairly high absorbance value. Test this specimen in triplicate in the same wells on every plate with every run. This is the calibrator.

b. After performing the above calculations and determining the final absorbance value of the calibrator, convert all absorbance values of all specimens to ELISA units/ml (EU/ml) as follows:

1. Arbitrarily assign a value of 100 EU/ml to the absorbance value of the calibrator.
2. Divide the final absorbance value of each test specimen by the absorbance value of the calibrator and then multiply by the EU/ml value of the calibrator (i.e., by 100). The result is the EU/ml value of the test specimen.

COMMENTS

This protocol was developed as the first step toward designing an ELISA method capable of measuring human serum antibodies reactive with *A. polyphaga* whole cell antigen. To date, our studies suggest that the general principles developed for the ELISA using rabbit immune serum and anti-rabbit conjugate may be applicable to measurement of human serum antibodies using anti-human conjugate. The studies demonstrate not only differences in ELISA reactivity using adult human sera, but also minimum reactivity using infant sera. However, these studies are preliminary, in that tests to determine sensitivity and specificity of the ELISA method using human serum and *Acanthamoeba* antigen need to be performed. Therefore, use of the protocol as described for analysis of human serum antibodies is not recommended. The book *Antibodies Volume II - A Practical Approach* (1) may be purchased from Sigma Chemical Company and is highly recommended reading if one plans to develop an ELISA procedure. It contains information on all aspects of ELISA, including detailed instructions on how to determine optimal antigen concentrations and optimal serum and conjugate dilutions.

LITERATURE CITED

1. Catty, D (ed). 1989 *Antibodies Volume II - a practical approach.* IRL Press, Oxford University Press, Oxford.
2. Diamedix Corporation. 1988 Package insert: Amebiasis microassay.
3. Johnson, D A, et al. 1984 Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Techn.* 1:3-8.
4.Voller, A, Bidwell, D, and Bartlett, A. 1980 Enzyme-linked immunosorbent assay, in: *Manual of clinical immunology, 2nd edition*, American Society for Microbiology, Washington, D.C.

APPENDIX

COMMONLY USED STOCK SOLUTIONS AND BUFFERS

STOCK SOLUTIONS

10 M Ammonium acetate
385.4 gm ammonium acetate in 500 ml H_2O

1 M CaCl
147 gm $CaCl_2.2H_2O$ in 1 liter H_2O

100X Denhardt solution, 500 ml
10 gm Ficoll 400
10 gm polyvinylpyrrolidone
10 gm bovine serum albumin (Pentex Fraction V, Miles Labs)
Filter and store at -20°C in 25 ml aliquots

1 M dithiothreitol (DTT)
15.45 gm DTT in 100 ml H_2O
Store at -20°C

0.5 M EDTA (ethylenediamine tetraacetic acid)
186.1 gm $Na_2EDTA.2H_2O$ in 700 ml H_2O
Adjust pH to 8.0 with 10 M NaOH (≈ 50 ml)
Add H_2O to 1 liter

10 mg/ml ethidium bromide
0.2 gm ethidium bromide in 20 ml H_2O
Store at 4°C in the dark
Ethidium bromide is a mutagen. Handle with care.

1 M HCl, 1 liter
913.8 ml H_2O plus 86.2 ml concentrated HCl.
Add acid slowly with stirring

1 M KCl
74.6 gm in 1 liter H_2O

1 M $MgCl_2$
20.3 gm $MgCl_2.6H_2O$ in 100 ml H_2O

1 M $MgSO_4$
24.6 gm $MgSO_4.7H_2O$ in 100 ml H_2O

5 M NaCl
292 gm NaCl in 1 liter H_2O

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10 M NaOH

400 gm NaOH (pellets) in 1 liter H_2O

3 M sodium acetate

408 gm sodium acetate.$3H_2O$ in 800 ml H_2O
Adjust pH to 5.2 with acetic acid. Dilute to 1 liter

1 M Tris.Cl [tris(hydroxymethyl)aminoethane]

121 gm Tris base in 800 ml H_2O
Adjust to desired pH with concentrated HCl
Dilute to 1 liter

BUFFERS

Phosphate-buffered saline (PBS)

10X Stock solution, 1 liter	Working solution, pH ≈ 7.3
80 gm NaCl	137 mM NaCl
2 gm KCl	2.7 mM KCl
11.5 gm $Na_2HPO_4.7H_2O$	4.3 mM $Na_2HPO_4.7H_2O$
2 gm KH_2PO_4	1.4 mM KH_2PO_4

20x SSC

3 M NaCl (175 gm/liter)
0.3 M Na_3citrate.$2H_2O$ (88 gm/liter)
Adjust pH to 7.0 with 1 M HCl

TAE electrophoresis buffer

50X stock solution, 1 liter	Working solution:
242 gm Tris base	0.04 M Tris-acetate
57.1 ml glacial acetic acid	0.002 M EDTA
37.2 gm $Na_2EDTA.2H_2O$	
pH 8.5	

TBE electrophoresis buffer

10X stock solution, 1 liter	Working solution:
108 gm Tris base	0.089 M Tris base
55 gm boric acid	0.089 M boric acid
40 ml 0.5 M EDTA, pH 8.0	0.002 M EDTA

TE buffer

10 mM Tris.Cl (pH 7.5 or 8.0)
1 mM EDTA, pH 8.0

TABLE I

CONCENTRATED ACIDS AND BASES

Acid/ Base	Molecular weight	% by Weight	Molarity (approx)	1 Molar (ml/l)	Specific gravity
Acetic acid (gl)	60.05	99.6	17.4	57.5	1.05
Ammonium hydroxide	35.0	28	14.8	67.6	0.90
Formic Acid	46.03	90 98	23.6 25.9	42.4 38.5	1.205 1.22
Hydrochloric acid	36.46	36	11.6	85.9	1.18
Nitric acid	63.01	70	15.7	63.7	1.42
Perchloric acid	100.46	60 72	9.2 12.2	108.8 82.1	1.54 1.70
Phosphoric acid	98.0	85	14.7	67.8	1.70
Sulfuric acid	98.07	98	18.3	54.5	1.835

TABLE II

AMINO ACIDS

Amino Acids	3-Letter Code	1-Letter Code	Molecular Weight (gram/mol)
Alanine	Ala	A	89.1
Arginine	Arg	R	174.2
Asparagine	Asn	N	132.1
Aspartate	Asp	D	133.1
Cysteine	Cys	C	121.2
Glutamate	Glu	E	147.1
Glutamine	Gln	Q	146.2
Glycine	Gly	G	75.1
Histidine	His	H	155.2
Isoleucine	Ile	I	131.2
Leucine	Leu	L	131.2
Lysine	Lys	K	146.2
Methionine	Met	M	149.2
Phenylalanine	Phe	F	165.2
Proline	Pro	P	115.1
Serine	Ser	S	105.1
Threonine	Thr	T	119.1
Tryptophan	Trp	W	204.2
Tyrosine	Tyr	Y	181.2
Valine	Val	V	117.1

D-ap-v

TABLE III

MOLECULAR WEIGHT AND SOME OPTICAL PROPERTIES OF NUCLEIC ACID DERIVATIVES

Nucleic acid derivative	Mol. Wt. (gm/mol)	λ_{max}	λ_{min}	A_{max}/A_{min}
ATP	507.2	259	227	0.15
ADP	427.2	259	227	0.16
AMP	347.2	259	227	0.16
Adenosine	267.2	260	227	0.14
Adenine	251.2	260	225	0.15
CTP	483.2	271	249	0.97
CDP	403.2	271	249	0.98
CMP	323.2	271	249	0.98
Cytidine	243.2	271	250	0.93
Cytosine	227.2	271	250	0.97
GTP	523.2	253	223	0.66
GDP	443.2	253	224	0.66
GMP	363.2	252	224	0.66
Guanosine	283.2	253	223	0.67
Guanine	267.2	254	223	0.68
UTP	484.2	262	230	0.38
UDP	404.2	262	230	0.39
UMP	324.2	262	230	0.39
Uridine	244.2	262	230	0.35
Uracil	112.1	260	227	-
TTP	482.2	267	-	0.73
TMP	322.2	267	234	0.73
Thymidine	242.2	267	235	0.7
Thymine	126	265	233	-

TABLE IV

CONVERSION FACTORS FOR RADIOACTIVITY

Measurement of Radioactivity

The unit of measurement of radioactivity is the Becquerel:
1 Becquerel (Bq) = 1 disintegration per second

More commonly, the Curie (Ci) is used:
1 Ci = $3.7\ 10^{10}$ Bq
= 2.22×10^{12} disintegrations per minute (dpm)

1 millicurie (mCi) = 3.7×10^{7} Bq = 2.22×10^{9} dpm

1 microcurie (μCi) = 3.7×10^{4} Bq = 2.22×10^{6} dpm

Measurement of Dose

The unit for energy absorbed from radiation is the Gray (Gy)
1 Gy = 1 joule/kgm
The previous units of absorbed energy were the rad (r) and Roentgen (R).
1 r = 100 ergs/gm = 10^{-2} Gy
1 R = 0.877 r in air
=0.93 - 0.98 r in water and tissue

The unit for radiation dosage is the Sievert (Sv) (This unit empirically takes into account the relative biological effectiveness (RBE) of a given form of radiation)
Dosage (Sv) = RBE x dosage (Gy)
RBE = (biological effect of a dose of standard radiation [Gy])/(biological effect of a dose of other radiation [Gy])
RBE = 1 for commonly encountered radionuclides.

The previous unit for dosage was the rem (Roentgen-equivalent-man):
1 rem = 0.01 Sv

TABLE V

CHARACTERISTICS OF RADIONUCLIDES

Nuclide	Half Life	Emission	Energy,max (Mev)	Range of Emission, max
^{3}H	12.43 yrs	β	0.0186	0.42cm (air)
^{14}C	5370 yrs	β	0.156	21.8cm (air)
^{32}P	14.3 days	β	1.71	610cm (air) 0.8cm (water) 0.76cm (plexiglass)
^{35}S	87.4 days	β	0.167	24.4cm (air)
^{125}I	60 days	γ	0.27-0.35	0.2mm (lead)
^{131}I	8.04 days	β	0.606	165cm (air)
		γ	0.364	2.4cm (lead)

COMMON ABBREVIATIONS

A_{260} absorbance at 260 nm
A adenine or adenosine; one letter code for alanine
Ab antibody
ADP adenosine 5'-diphosphate
AEX anion exchange
Ag antigen
AMP adenosine 5'-monophosphate
ATP adenosine 5'-triphosphate
β-gal β-galactosidase
BAP bacterial alkaline phosphatase
bis, bisacrylamide N,N'-methylene bisacrylamide
bp base pair
BSA bovine serum albumin
C cytosine or cytidine; one letter code for cysteine
cAMP adenosine 3',5'-cyclic-monophosphate
cDNA complementary deoxyribonucleic acid
CDP cytidine 5'-diphosphate
CEX cation exchange
Ci curie
CMP cytidine 5'-monophosphate
cpm counts per minute
CTAB cetyltrimethylammonium bromide
CTP cytidine 5'-triphosphate
dA deoxyadenosine
Da dalton
dAMP deoxyadenosine monophosphate
DAPI 4'6-diamidino-2-phenylindole
dATP deoxyadenosine triphosphate
dC deoxycytosine
dCMP deoxycytidine monophosphate
dCTP deoxycytidine triphosphate
ddATP dideoxyadenosine triphosphate
ddCTP dideoxycytidine triphosphate
ddGTP dideoxyguanosine triphosphate
ddNTP dideoxynucleoside triphosphate
ddTTP dideoxythymidine triphosphate
DEA diethyl amine
DEAE diethyaminoethyl
dG guanosine
dGTP deoxyguanosine triphosphate
DMF dimethylformamide
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
Dnase deoxyribonuclease
dNTP deoxynucleoside triphosphate
ds double stranded
dT deoxythymine or thymidine
DTT dithiothreitol
dTTP deoxythymidine triphosphate
dUMP deoxyuridine monophosphate
dUTP deoxyuridine triphosphate
ECTEOLA epichlorohydrin triethanolamine
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA enzyme-linked immunosorbent assay
exo exonuclease
F Farad
g gravity
G gauge;guanine or guanosine;one letter code for glycine
GDP guanosine 5'-diphosphate
GF gel filtration
GMP guanosine monophosphate
GTP guanosine 5'triphosphate
HEPES N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HPCF high-performance chromatofocusing
HPLC high-performance liquid chromatography
IEF isoelectric focusing
IEX ion exchange
Ig immunoglobulin
IPTG isopropyl-1-thio-β-D-galactoside
K_m Michaelis constant
mAB monoclonal antibody

MES 2-(N-morpholino)ethanesulfonic acid
MOPS 3-(N-morpholino)propane sulfonic acid
mp melting point
M molecular weight
mRNA messenger RNA
NTP nucleoside triphosphate
OD_{260} optical density at 260 nm
PAGE polyacrylamide gel electrophoresis
PB phosphate buffer
PBS phosphate-buffered saline
PCR polymerase chain reaction
PEG polyethylene glycol
PFA paraformaldehyde
pfu plaque-forming units
pI isoelectric point
PIPES piperazine-N,N'-bis(2-ethane sulfonic acid)
PMFS phenylmethylsulfonyl fluoride
poly(A) polyadenylic acid or polyadenylate
poly(A)$^+$ polyadenylated (mRNA)
RE restriction endonuclease
RFLP restriction-fragment-length polymorphisms
RIA radioimmunoassay
RNA ribonucleic acid
RNase ribonuclease
rRNA ribosomal ribonucleic acid
RT reverse transcriptase
Sarkosyl N-lauroylsarcosine
SDS sodium dodecyl sulfate
ss single stranded
SSC sodium chloride/sodium citrate (buffer)
T thymine or thymidine; one-letter code for threonine
TAE Tris/acetate (buffer)
Taq Thermus aquaticus DNA (polymerase)
TBE Tris/borate electrophoresis (buffer)**TBS** Tris-buffered saline
TCA Trichloracetic acid
TE Tris/EDTA (buffer)
TEAE triethylaminoethyl
TEMED N,N,N',N'-tetramethyl ethylenediamine
TFA trifluoroacetic acid
TLC thin-layer chromatography
T_m melting (or mid-point) temperature; thermal denaturation
Tris (hydroxymethyl)aminoethane
Tris-Cl Tris hydrochloride
tRNA transfer ribonucleic acid
TTP thymidine 5'-triphosphate
U unit; uracil or uridine
UDP uridine 5'-diphosphate
UMP uridine 5'-monophosphate
UTP uridine 5'-monophosphate
UV ultraviolet
X-gal 5-bromo-4-chloro-3-indoyl-β-D-galactoside

E. EDUCATIONAL EXPERIMENTS AND DEMONSTRATIONS

1. Sources of strains for research and teaching. Pierre-Marc Daggett and Thomas A. Nerad
2. Conjugation in *Tetrahymena thermophila.* Linda A. Hufnagel
3. Special techniques for viewing living protozoa. Karl A. Aufderheide

SOURCES OF STRAINS FOR RESEARCH AND TEACHING

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INTRODUCTION

Other than directly isolating strains there are two other sources by which strains needed can be obtained. One source is to obtain a strain from an investigator who has published on research done with the desired organism. A review of recent literature is an easy method to determine where an organism may be obtained since most journals require an investigator to make available strains to those inquiring. A second source is from organized culture collections and companies providing teaching materials. An annotated partial listing of sources is provided here.

SOURCES

ATCC
American Type Culture Collection
12301 Parklawn Drive, Rockville, Maryland 20852
USA
Telephone: 301-881-2600
FAX: 301-231-5826
Telex: 908-768 ATCCROVE
Maintains a diverse collection of freshwater and marine protists including algae and both free-living and parasitic protozoans. All strains are maintained in the cryopreserved state. A total of 1156 strains available. Fee charged. Seventeenth Edition 1991 Catalogue available.

Carolina Biological Supply Company
2700 York Road
Burlington, North Carolina 27215
USA
Telephone: 919-584-0381 FAX: 919-584-3399 Telex: 574354
An educational supply company selling both photosynthetic and non-photosynthetic protozoans for teaching purposes. Annual Catalogue available.

CCAP
Culture Collection of Algae and Protozoa*. With laboratories at two locations.
CCAP at Freshwater Biological Association
The Ferry House, Ambleside,
Cumbria LA22 0LP
UNITED KINGDOM

Telephone: (09662) 2468, (09662) 2469
Telex: 8950511 ONE ONE G REF16173001
FAX: (Group 2 or 3): 6914
Maintains a diverse collection of freshwater and marine free-living photosynthetic and non-photosynthetic protozoans.
CCAP at Scottish Marine Biological Association (SMBA)
Dunnstaffnage Marine Research Laboratory
P.O. Box 3 Oban, Argyll PA34 4AD
SCOTLAND
Maintains a collection of marine algae and photosynthetic protozoa.
A total of about one-third of the strains at CCAP are maintained in the cryopreserved state. Total number of strains available = ~2000. Fifth Edition, 1988 Catalogue available. *Formerly the Culture Centre of Algae and Protozoa located in Cambridge, England.

CCMP
Culture Collection of Marine Phytoplankton
Bigelow Laboratory for Ocean Sciences
McKown Pt., West Boothbay Harbor, Maine 04575
USA
Telephone: 207-633-2173 (8:00 AM - 4:30 PM)
: 207-633-2175 (other times)
Fax: 207-633-6584
Telex: 757567 CCMPUD
OMNET: Bigelow.lab
Maintains a collection of marine algae and photosynthetic protozoans. None of the strains are cryopreserved. Total number of strains available = ~1300. Fee charged. 1991 Catalogue of Strains available.)

Connecticut Valley Biological
82 Valley Road, P.O. Box 326
SouthHampton, MA 01073
Telephone: (413) 527-4030
An educational supply company selling both photosynthetic and non-photosynthetic protozoans for teaching purposes. Annual Catalogue available.

NEPCC
The Northeast Pacific Culture Collection
Department of Oceanography
University of British Columbia
Vancouver, British Columbia V6T 1W5
CANADA
Telephone: 604-228-4378
Maintains a marine phytoplankton collection (mostly photosynthetic dinoflagellates) mostly isolated from local waters. None of the strains are cryopreserved. Total number of strains available = 342. Fee charged. Listing available.

NIES
NIES Collection of Microalgae and Protozoa
NO.1 Service Division
Research Association for Environmental Science
c/o The National Institute for Environmental Sciences
Yatabe-cho, Tsukuba-shi, Ibaraki 305
JAPAN
Telephone: 0298-51-6111

Cable: KOGAIKENTSUKUBA
Maintains a collection of protists, mostly algae but including a some photosynthetic and a few non-non-photosynthetic protozoans isolated primarily in Japan. Some strains cryopreserved. Total number of strains available = 510. Fee charged. Third Edition, 1991 Catalogue available.

NIVA
Culture Collection of Algae
Norwegian Institute for Water Research
P.O. Box 333 - Blindern, 0314 Oslo 3 NORWAY
Telephone: 47.2.235380 Telex: 74190 niva n Telegrams: NIVA, Oslo
(Maintains a collection of algae and blue-green bacteria mostly isolated in Norway. Total number of strains available = ~300. Catalogue available.)

SAG
Sammlung von Algenkulturen
Pflanzephysiologisches Institut der Universität Göttingen
Nikolausberger Weg 18, D-3400 Göttingen, GERMANY
Maintains a collection of algae, including photosynthetic protozoans. Fee charged. Listing available.

UTCC
University of Toronto Culture Collection
Department of Botany, University of Toronto,
Toronto, Ontario M5S 3B2, CANADA
Telephone: 416-978-3641
Fax: 416-978-5878
E-Mail: UTCC @VM.UTCS.UTORONTO.CA on NETNORTH(BITNET)
Maintains a collection of algal strains (including photosynthetic protozoans) and blue-green bacteria. Listing available. Total number of strains available = 167.

UTEX
The Culture Collection of Algae at the University of Texas at Austin
Department of Botany, The University of Texas at Austin, Austin, Texas 78713-7640, USA
Telephone: 512-471-4019
Maintains a collection of freshwater algal strains (including photosynthetic protozoans). Strains are not cryopreserved. Total number of strains available = ~2000. Fee Charged. Catalogue available.

Wards Natural Science Establishment
P.O. Box 92912, 5100 West Henrietta Road, Rochester, New York 14692-9012, USA (West coast USA: 815, Fiero Lane, Saint Luis Obsipo, CA 9341)
Telephone: 1-800-962-2660 (world-wide) 1-800-672-7289; 1800-672-7289 (West coast USA)
FAX: 716-334-6174
An educational supply company selling living protists for teaching. Annual Catalogue available.

COMMENTS

The World Data Center on Microorganisms (address listed below) maintains a directory of collections and their holdings of algae and photosynthetic protozoans. The 2nd edition of the World Catalogue of Algae [1] is currently avaliable for a fee.

E-1.4

World Data Center on Microorganisms
World Data Center, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-01, Japan
Telephone: 0484-62-1111 ext. 6023, 6024; 0484-66-6181
FAX: 0484-62-1554
Telex: 2962818 RIKEN J

LITERATURE CITED

1. Miyachi, S, Nakayama, O, Yokohama, Y, Hara, Y, Ohmori, M, Komagata, K, Sugawara, H, Ugawa, Y ed. 1989. World Catalogue of Algae, 2nd edition, Japan Scientific Societies Press, 329pp.

CONJUGATION IN TETRAHYMENA THERMOPHILA

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INTRODUCTION

Conjugation in Tetrahymena is a transient sexual interaction of cells, leading to the exchange of haploid genomes. Conjugation results in the replacement of the old macronuclei and micronuclei with new nuclei, all having the same combination of alleles obtained from both parents. Conjugation can be viewed as a developmental process (1). The first stage in this process ("initiation") takes place when well-fed but mating-incompetent cells are starved in a low ionic strength, non-nutrient medium. Starvation induces them to become mating-competent. When mating-competent cells of complementary mating types are mixed, they then undergo further developmental changes (see Fig. 1), including inductive interactions known as "costimulation" (2), shape changes known as "tip transformation"(3), changes in adhesive properties resulting in pair formation (4; see Fig 2) and the development of a "conjugation junction" (5) which tightly joins cells of a pair. Their micronuclei meanwhile undergo meiosis (see Fig 3) and haploid pronuclei are then exchanged through the conjugation junction (6). Following this, each "migratory pronucleus" fuses with a "stationary pronucleus" to form a diploid zygotic nucleus in each conjugating cell (7, 8). These then give rise to new micronuclei by mitosis and to new macronuclei by a unique gene editing and amplification process (9). Paired cells then disassemble their shared junction and separate.

Tetrahymena can be induced to conjugate synchronously in large numbers suitable for biochemical analysis, under physiological conditions. With careful control of conditions, better than 80% of the cells will be in pairs by 2 hrs (see Fig 4). The growth medium and other reagents needed are inexpensive. Thus, conjugation in Tetrahymena provides an attractive model system in which to analyze a variety of developmental processes of eucaryotic cells, including cell-to-cell signaling, formation of an intercellular junction, meiosis, and gene processing.

The following protocol is suitable for a class of 6-18 students, in a laboratory course in experimental cell or developmental biology, given at the advanced undergraduate or graduate level.

E-2.2

BASIC PROTOCOL

MATERIALS:

1. Enriched proteose peptone (EPP) medium (10):

Proteose peptone (Difco) ----------	20.0	gm
Glucose----------	2.0	gm (Optional, for increased yield)
Yeast extract----------	1.0	gm
Sequestrine (Iron-EDTA; Ciba-Geigy,P.O. Box 19103, Greenville, NC, 27419)---------	0.03	gm (Optional, for increased yield)

Add 1 liter distilled water, heat briefly to dissolve ingredients and dispense 100 ml into each of four 250 ml erlenmeyer flasks. Plug with cheese cloth-wrapped cotton and cover with aluminum foil. Dispense the remaining medium in 10 ml aliquots into 15 ml screw-cap tubes. Cap loosely. Autoclave 20 min at 20 lb pressure. Cool; tighten caps on tubes. Can be stored at room temperature for several weeks.

2. Two clonal cultures of *T. thermophila*, inbred strain B, of different mating type. These may be obtained from the American Type Culture Collection, Rockville, MD (ATCC#s: 30007-30307), or from various individuals who work with this species, including the author; P. Bruns, Dept. of Genetics and Development, Cornell University, Ithaca, New York; and Paul Doerder, Department of Biology, Cleveland State University, 1983 E. 24th St., Cleveland, Ohio, 44115. Maintain on EPP medium in 15 ml screw-cap tube cultures under aseptic conditions, at 20-25°C. Subculture by loop transfer at least once a month and prepare fresh subcultures about 4-7 days prior to starting this procedure.
3. 10 mM Tris buffer, pH 7.4, about 500 ml, sterile (autoclave or filter to sterilize).
4. 250 ml flasks, 2, plugged with cheese cloth-wrapped cotton, covered with aluminum foil and sterilized.
5. Two dozen Pasteur pipettes, 5.25 in, plugged with cotton, wrapped in a paper towel and aluminum foil (or in a pipette canister) and sterilized.
6. One package 5.25 in Pasteur pipettes, unsterile.
7. Twelve 15 ml conical glass centrifuge tubes, plugged with cheesecloth-wrapped cotton, covered with aluminum foil and sterilized (or use sterile, disposable plastic centrifuge tubes).
8. Four 15 ml conical centrifuge tubes, glass or plastic, not sterile.
9. One box glass microscope slides.
10. Glass vials, snap cap, 20-30.
11. Sterile, dispo graduated pipettes, 1 ml, 1 pkg.
12. Table-top clinical centrifuge with rotor for 15 ml conical tubes.
13. Shaker incubator or water bath set at 30°C (a second bath or incubator without shaking capabilities may also prove convenient.
14. For each student or pair of students:
    a. One 50 ml flask.
    b. One package Drummond microcaps, 4 ul.
    c. Lugol's Iodine in small dropping bottle with narrow-tipped dropper.
    d. Hand tally.
    e. Dissecting microscope.
    f. Compound microscope with 40X and 100X objectives, preferably phase contrast or Nomarski interference.

PROCEDURES:

Day 1: Two days prior to lab period, early in the morning, the lab instructor or assistant should inoculate one flask of EPP Medium with 1 ml of a 4-7 day tube culture of one of the mating types, and inoculate a second flask with 1 ml of the second mating type, using aseptic technique. Incubate the flasks with shaking at 30°C for 30-36 hr.

Day 2: By late afternoon, the cultures should have reached the late exponential stage of growth and the cells should be at a density of between 8×10^5 and 2×10^6 cells/ml. At this density the cultures appear quite cloudy. To ensure that the cultures are dense enough, the instructor or assistant should do a cell count on each (11):

Swirl the flask to distribute the cells evenly and remove 0.1 ml cells using a sterile graduated pipette. Add to a vial containing 2.0 ml Lugol's Iodine. Swirl the vial to distribute the cells evenly and remove 4 ul using a Drummond Microcap. Be sure that the capillary is full. Distribute entire contents of capillary as a series of small drops (8-10) on a clean glass slide. Rapidly count cells under a high power of the dissecting scope using the hand tally. If there are less than 20 or more than 75 cells in 4 ul, prepare a fresh dilution of cells in Lugol's to give about 50-75 cells/4 ul. Count enough 4 ul aliquots to give counts of at least 250 cells. Do not allow the drops to dry while counting and adjust the light to optimum for distinguishing between cells and debris. Use the following formula to calculate the number of cells per ml in the original culture:

$$\text{cells/ml} = \frac{\text{total cells counted}}{\text{total ul cells counted}} \times 1000 \times \text{dilution factor}$$

Keeping mating types separate and conditions as aseptic as possible, wash the cells 4 times with sterile 10 mM Tris buffer, as follows: Pour 10 ml from each flask into each of two sterile centrifuge tubes and centrifuge in a table-top clinical centrifuge for 1 min at half maximum speed (~1288g). (To wash the entire culture, pear-shaped tubes holding 100 ml and a specially designed centrifuge to hold them are recommended). Carefully pour off the supernatants; it will be necessary to retain some of the liquid, to avoid losing cells. After the fourth wash, resuspend the cells in Tris buffer to a density of about 5×10^5 cells/ml. Put up to 100 ml of each mating type in separate 250 ml sterile flasks, keeping the two mating types separate and discarding any excess cell suspensions. Incubate overnight with shaking at 30°C, to starve the cells.

Day 3: At the beginning of the lab period, each student should do a cell count as described above on a 0.1 ml aliquot of starved cells. Half the students should do one mating type and half the other. Pool the results for each mating type and on the basis of the results, centrifuge the cells to concentrate (sterile centrifuge tubes are not necessary) and, using a pasteur pipette, remove enough starvation medium so that when resuspended, cells of each mating type will be at a concentration of 1-2 $\times 10^6$ cells/ml and both mating types will be at the same concentration. Each student or pair of students should then mix equal volumes of cells of the two mating types in a 50 ml flask (2.5 ml of each mating type), to give a thin layer of cells with a large surface to volume ratio. The mixtures should then be incubated without shaking in a 30°C water bath or incubator. At 15 min or half hr intervals over a period of at least 2 hrs, gently swirl flask to distribute cells evenly (do not shake too hard or loosely paired cells will separate!) and remove 1 drop with a pasteur pipette. Put on a clean glass slide and immediately add one drop of Lugol's Iodine. Cover with a coverslip and view under a compound microscope using a 40 or 100X objective. To determine % cells in pairs, count at least 250 cells at random and calculate as follows:

$$\%\ \text{cells in pairs} = \#\ \text{pairs} \times 2 / \text{total}\ \#\ \text{cells}$$

E-2.4

Record your results for each time point in the following table:

Time	Total # cells	# cells in pairs	% cells in pairs
15 min			
30 min			
45 min			
60 min			
75 min			
90 min			
105 min			
120 min			

Plot your results on graph paper, plotting % cells in pairs against time.

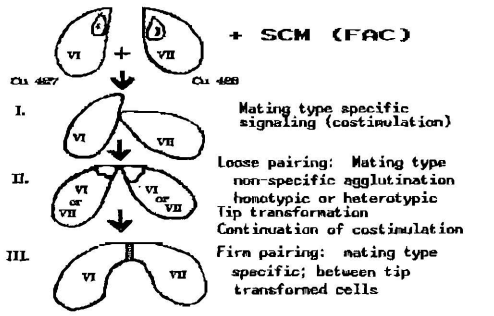
COMMENTS

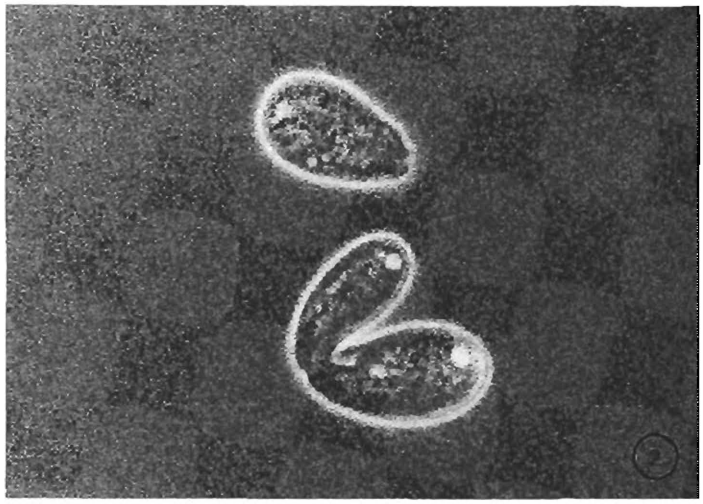
1. To get the best mating efficiency, the following factors are important: (a) Cultures should be harvested at late exponential phase and should not be contaminated with bacteria; (b) glassware must be extremely clean and free of detergent; (c) Cells should be starved at a lower concentration but must be at 1 x 10^6 cells/ml or higher when mixed for mating; (d) mating mixtures must be well oxygenated but physically disturbed as little as possible; (e) Centrifugation to collect cells must be gentle and brief and cell resuspension should be gentle, (f) exactly equal numbers of cells of the two mating types must be mixed and (g) mixed cells should be incubated at 30°C (however, for less quantitative results, slightly lower or higher temperatures [from 27 to 33°C] are adequate to get pairing within a reasonable time.
2. To get tip transformation without pairing, after mating types are mixed and placed at 30°C, shake the flask vigorously by hand every 10 min. To detect the distinctive, truncated tip, cells must be viewed after flattening under a coverslip. Count only those cells with clearly truncated end.
3. For cell densities greater than 1 X 10^5 cells/ml, a Bausch and Lomb Spectronic 20 colorimeter can be used to measure cell density (12): At 550 nm, 1.0 O.D. unit = 0.75 X 10^6 cells/ml.
4. For a less quantitative lab exercise, students can mix several drops of each mating type on a slide or in a deep depression dish and place in a moist chamber made from a standard petri dish. Pair formation can be followed under a dissecting microscope and samples taken out at 1/2, 1, 1 1/2 and 2 hrs to observe changes in cell shape, behavior and nuclear structure, using a phase contrast or Nomarski interference microscope with 40X and 100X objectives.
5. The effects of adverse environmental chemicals can be easily tested in this lab exercise by adding dilute amounts of the chemicals to duplicate flasks at the time the two mating types are mixed.
6. Nuclear events show remarkable synchrony. These can be followed by use of conventional stains such as aceto-orcein or Feulgen's (7, 8, 13). If a fluorescence microscope and suitable filters are available, DAPI staining (14, 15) is simple and gives spectacular results.
7. Triplets are occasionally seen. These are due to the common presence of doublet cells with two junction-forming regions. The possible genetic consequences of matings between singlet and doublet cells can be explored with students.

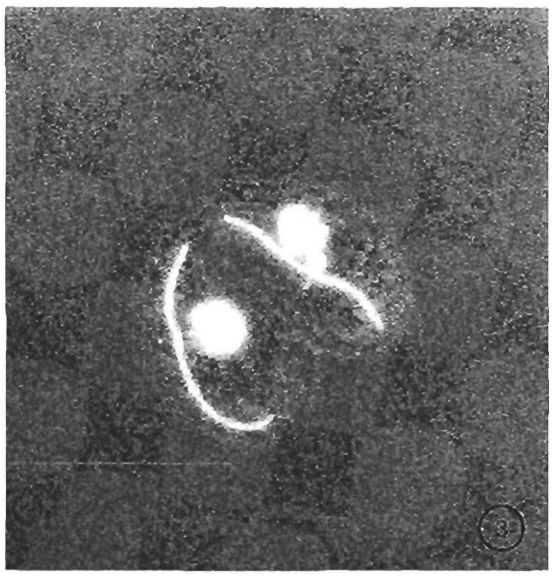
LITERATURE CITED

1. Bruns, P J, & Brussard, T B. 1974. Pair formation in *Tetrahymena pyriformis,* an inducible developmental system. *J Exp Zool* **188**:337-344.
2. Finley, M J & Bruns, P J. 1980. Costimulation in *Tetrahymena.* II. A nonspecific response to heterotypic cell-cell interactions. *Devel Biol* **79**:81-94.
3. Wolfe, J & Grimes, G W. 1979. Tip transformation in *Tetrahymena*: a morphogenetic response to interaction between mating types. *J Protozool* **26**:82-89.
4. Ofer, O, Levkovitz, H, & Loyter, A. 1976. Conjugation in *Tetrahymena pyriformis.* The effect of polylysine, concanavalin A, and bivalent metals on the conjugation process. *J Cell Biol* **70**:287-293.
5. Wolfe, J. 1982. The conjugation junction of *Tetrahymena*: Its structure and development. *J Morphol* **172**:159-178.
6. Orias, J D, Hamilton, E P, & Orias, E. 1983. A microtubule meshwork associated with gametic pronucleus transfer across a cell-cell junction. *Science* **222**:181-184.
7. Ray, C R. 1956. Meiosis and nuclear behavior in *Tetrahymena pyriformis. J Protozool* **3**:88-96.
8. Sugai, T, & Hiwatashi, K. 1974. Cytologic and Autoradiographic studies of the micronucleus at meiotic prophase in *Tetrahymena pyriformis. J Protozool* **21**:542-548.
9. Yao, M-C. 1986. Amplification of ribosomal RNA genes, in *The Molecular Biology of Ciliated Protozoa,* J G Gall, ed. Academic Press, Inc, New York.
10. Gorovsky, M, & Woodard, J. 1969. Studies on nuclear structure and function in *Tetrahymena pyriformis* I. RNA synthesis in macro-and micronuclei. *J Cell Biol* **42**:673-682.
11. Lin, R-Y, & Hufnagel, L A. Unpublished observations.
12. Gorovsky, M A, Hattman, S, & Pleger, G L. 1973. [^{6}N] Methyl adenine in the nuclear DNA of a eucaryote, *Tetrahymena pyriformis. J Cell Biol* **56**:697-701.
13. Lee, JJ, Hutner, SH, & Bovee, EC. 1985. *An Illustrated Guide to the Protozoa*. Soc Protozool, PO Box 368, Lawrence, Kansas.
14. Porter, KG, & Feig, YS. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* **25**:943-948.
15. Coleman, AW. 1980. Enhanced detectgion of bacteria in natural environments by fluorochrome staining of DNA. *Limnol Oceanogr* **25**:948-951.

E-2.6







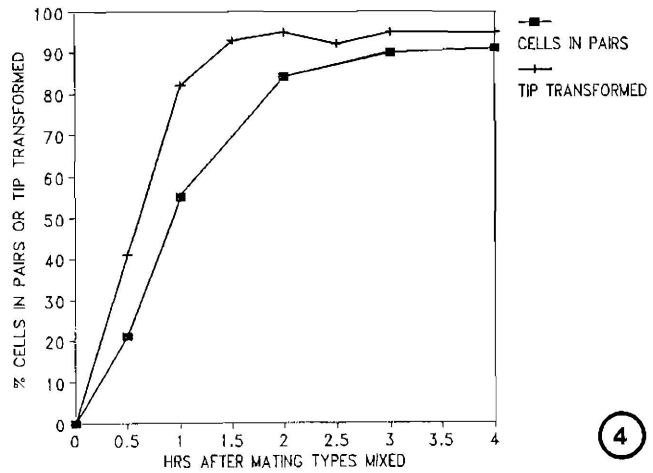


Figure 1. A THREE-STAGE MODEL FOR MATING INTERACTIONS IN TETRAHYMENA. When previously starved cells of complementary mating type (here illustrated by strain Cu427/mtVI and strain Cu428/mtVII) are mixed in the presence of conditioned starvation medium (SCM) containing unknown factors required for mating (FAC), they immediately interact through direct contact (Stage I). Mating type-specific inductive signaling (costimulation) occurs during this stage, which last at least 1/2 hr. Cells next form loose pairs (Stage II), which may be homotypic or heterotypic, by adhering via cilia near the anterior ends of the cells. The anterior ends of the cells gradually change shape, from pointed to truncated (tip transformation), resulting in the development of firm pairs (stage IV), in which cells of complementary mating type become joined by a characteristic conjugation junction.

Figure 2. PAIR FORMATION IN MATING TETRAHYMENA. A phase-contrast micrograph of cells from a mating mixture of T. thermophila, showing a pair of cells at the firm pair stage (about 2.5 hr) in comparison with a single cell. X1,250.

Figure 3. NUCLEAR EVENTS DURING CONJUGATION. This mating pair of T. thermophila is stained with DAPI to reveal DNA-containing nuclei. The cells are in prophase of micronuclear meiosis. In this stage of micronuclear morphological changes (Stage IV, as defined by Sugai and Hiwatashi [8]), the micronucleus elongates into a thin thread. This occurs between 3 and 4 hrs after complementary mating types are mixed. X1,250.

Figure 4. KINETICS OF PAIR FORMATION AND TIP TRANSFORMATION. Two different mating types were starved separately and mixed in equal numbers at a concentration of 1 X 10^6 cells/ml at T=0. Counts were made at 1/2 hr intervals on 250-300 randomly selected cells. Each point is the average of three separate experiments. + - tip transformed; • - pairs.

SPECIAL TECHNIQUES FOR VIEWING LIVING PROTOZOA

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Although various staining techniques show specific structures in exquisite detail, most such techniques require fixed (i.e., dead) cells as materials. Several venerable techniques use "vital stains" which do not kill the cells immediately [2, 6, & 9]. These techniques seem to be limited in their specificity and use. Many "vital staining" techniques are not really vital and will kill the cells in time.

The advent of phase contrast and differential interference contrast optical systems permitted the microscopist to observe structures within cells without the need for staining or fixing. The advantages for observations of dynamic processes within living cells are obvious. Furthermore, except perhaps for the intense light that might be focused on the cell during observation, contrast enhancement optical techniques are less fraught with potential artifacts than other microscopic techniques. A major problem in viewing living protozoa is their motility--they frequently refuse to hold still long enough to be photographed. This problem is especially acute for those studying the swimming flagellates and the ciliates. Therefore, a range of techniques have been developed for immobilization or slowing a living cell so that they can be viewed for longer periods of time and be photographed.

Immobilization techniques depend on two basic approaches: chemical techniques and physical techniques. These are summarized and discussed briefly below:

Chemical Techniques

Most chemical means of immobilization are not really "vital" in their action and appear to affect the cell adversely unless they are used very carefully. I have not had good results with techniques using metal ions, but I have had success with the antibody immobilization approach, provided a good homologous antiserum is available.

Simple chemical immobilization protocols involve the use of various metal ions. A good example would be the Nickel Ion Immobilization technique [11,12]. Sikora uses the following solution: $NiCl_2$, 0.125 - 0.25 mM, $CaCl_2$ 1 mM, KCl 1 mM, Tris-HCl 5 mM, pH 7.2. He suggests trying a range of nickel ion concentrations and also trying other similar metals such as copper.

The technique of antibody immobilization exploits the ability of multivalent antibodies to bind the cilia of a cell together, thereby preventing swimming. Kúznicki and Sikora [7] used this technique to observe cyclosis in living paramecia. Homologous antiserum of an appropriate dilution is applied to the cells. The cells are monitored with the dissecting microscope until their swimming ceases. They can then be carefully placed on a standard microscope slide and observed with higher magnifications. Because higher concentrations of antiserum can be toxic, one must determine the lowest possible concentration of serum which will still immobilize.

E-3.2

Physical Techniques

These techniques in general depend on alterations of the viscosity of the medium or on some means of capturing and holding cells.

A number of compounds, usually large, non-osmotic polymers, can be used to make the medium more viscous. Protozoa swim slowly or not at all in these media and thus can be observed. Some of these compounds are somewhat toxic and will eventually kill the cells. Methocel™ (15 centipoise viscosity) is an old reliable polymer [6]. A 1 to 2% solution has the viscosity of honey. A droplet of methocel can be mixed on a microscope slide with a droplet of cells. Protoslo™ is a substance sold by Carolina Biological Supply Co. and works much the same way. Spoon, et al. [15] discuss the advantages and disadvantages of various thickening agents.

Methods of capturing and holding protozoa for microscopic observations can be simple or complex, depending on the degree of control one desires. These techniques involve squeezing the cell between a coverglass and a slide until the cell is caught and held.

If the protozoan is on a slide and under a coverglass, one may simply apply a slip of filter paper or bibulous paper to the edge of the coverglass and dry the mount until the cell is trapped. One usually has a few minutes to observe the cell before further drying causes so much distortion that the cell is crushed. This technique is useful for quick peeks at internal organization but not for long term studies. Spoon [13] suggests the use of small 1 cm squares of Handiwrap on a side as flexible coverglasses. The mount can be dried with filter paper and sealed around the edges with silicon grease. Spoon claims that gas exchange can occur through the film of Handiwrap but that water evaporation is inhibited. He claims that such mounts can be kept viable for 10 days or more. However, Handiwrap may distort the polarized light beams essential for Nomarski Differential Interference Contrast optics.

More complex capture techniques depend upon the use of a special instrument called a rotocompressor or microcompressor [18]. These instruments are basically a coverglass attached to a micrometer so that a cell may be trapped and squeezed with considerable precision. The original design for a rotocompressor was developed by Asa Schaeffer in the 1930's. This apparatus was available for many years but eventually became unavailable some time before Dr. Schaeffer's death. People who have the Schaeffer model generally hoard them, so they are hard to find today. The Schaeffer model has been used not only for simple observations but also for microbeam surgery on protozoa [e.g., 5 & 4] and for quantitative measurements of biomass of microorganisms [3]. An additional advantage of a rotocompressor is the ability to recover the cell after it has been observed or otherwise manipulated.

There are three more modern designs of rotocompressors: the model of Spoon [14], the model of Uhlig and Heimberg [17], and the model of Aufderheide [1]. Spoon's model is not commercially available at this time. It is difficult to manufacture, containing some 22 precision metal parts, but it uses commercial glass slides and coverglasses. The Uhlig model is commercially available (Hydro-Bios Apparatebau GmbH, P.O.B. 8008, 2300 Kiel 17, Germany), but is expensive, and is more specialized for use on inverted microscopes. It is quite large and heavy and requires the use of special large round coverglasses. Both the Spoon and Uhlig models require that the coverglasses be cemented onto the appropriate metal surfaces. If the coverglass is broken, replacement will obviously require removal of the cement as well as the broken glass. The Aufderheide model is a highly modified Schaeffer unit with several design improvements. It is not commercially available at this time, although I can arrange for special order manufacture. It does require the use of special glass slides and coverglasses which are available, but somewhat difficult to find. The Schaeffer and Spoon models do not permit one to establish Köhler illumination on an upright microscope because the bottom plate of glass is offset from the plane of the microscope stage by about 1 mm. The Uhlig and Aufderheide units permit one to adjust for Köhler illumination (even double oil immersion) on

a upright instrument, and the Uhlig unit works equally well on an inverted microscope. Using the Aufderheide unit, I have been able to capture small flagellates or bacteria and hold them for observation. The sensitivity of optical images possible with a rotocompressor is illustrated by Aufderheide [1], in which individual basal bodies in a living *Paramecium* are made visible.

In all rotocompressors, the cells to be observed are placed in a very small droplet (about 1 mm diameter) in the center of the viewing area of the unit. The top is applied and the unit is placed on the stage of the compound microscope. Under low power (about 100x) the unit is screwed down until the cells are initially immobilized. One may then switch to higher magnifications for critical observations. Further adjustments of the distance between the slide and the coverglass may be made at any time. For *Paramecium*, the gap between the slide and the coverglass required for secure, long-term immobilization is about 10μm. After one is done with observations, careful release of the pressure will usually permit the cell to be recovered alive and intact. One develops a "touch" for how much compression and how much time of compression a particular species will tolerate. All rotocompressors must be kept scrupulously clean (especially the glass surfaces) and properly lubricated.

Although rotocompressors are extremely useful for observations of living cells, one must remember that the cell is rather severely distorted by the capture technique. *Paramecium* tolerates this compression rather well, but it usually ceases cyclosis and feeding while it is captured. Other dynamic processes (e.g., morphogenesis and division) also appear to be sensitive to the distortion. Other species of small organisms may have very different expressions of tolerance to the compression. One must be aware that observations of rotocompressed cells may express possible artifacts from the compression process.

A number of unusual capture techniques have been described. One involves "gluing" cells to a glass surface. Protamine sulfate (0.015 to 0.05%) has been used to coat slides for the attachment of protozoa [8]. Polylysine coating may be of similar use [16]. Sikora [10] describes a technique for immobilization using powdered iron particles. These are fed to the protozoan before transfer of the cell to a slide. The cells are immobilized by application of a magnetic field to the slide so that the cells are held by the iron particles in their food vacuoles. A simple bar magnet placed in the vicinity of the microscope stage is supposed to suffice.

Ultimately, the researcher must be encouraged to try a number of immobilization techniques and to select the ones which seem to be the best for the organism used and the viewing or manipulation techniques involved. Because all immobilization techniques are bound to alter the physiology of the cell to some degree, it may be advisable to use more than one technique to document a particular phenomenon.

LITERATURE CITED

1. Aufderheide, K. J. 1986. Identification of the basal bodies and kinetodesmal fibers in living cells of *Paramecium tetraurelia* Sonneborn, 1975 and *Paramecium sonneborni* Aufderheide, Daggett & Nerad,1983. *J. Protozool.* **33**:77-80.

2. Ball, G. H. 1927. Studies on Paramecium. III. The effects of vital dyes on *Paramecium caudatum*. *Biol. Bull.* **52**:68-78.

3. Fenchel, T. 1967. The ecology of marine microbenthos. I. The quantitative importance of ciliates as compared with metazoans in various types of sediments. *Ophelia* **4**:121-137.

4. Grimes, G. W. 1976. Laser microbeam induction of incomplete doublets of *Oxytricha fallax*. *Genet. Res. Camb.* **27**:213-226.

5. Hanson, E. D. and Ungerlieder, R. M. 1973. The formation of the feeding organelle in *Paramecium aurelia*. *J. Exp. Zool.* **185**:175-188.

6. Jahn, T. L., Bovee, E. C. and Jahn, F. F. 1979. How to Know the Protozoa, 2nd edn. Wm. C. Brown

Co., Dubuque, Iowa pp. 11-16.

7. Kúznicki, L. and Sikora, J. 1971. Cytoplasmic streaming within Paramecium aurelia. I. Movements of crystals after immobilization by antiserum. *Acta. Protozool.* **8**:439-446.

8. Marsot, P. and Couillard, P. 1973. The use of protamine-coated slides for immobilizing protozoa *J. Protozool.* **20**:105-106.

9. Mohr, J. L. 1981. Methods for protozoa. *In*: Staining Techniques, 4th edn., G. Clark (ed.). (Biological Stain Commission: Williams & Wilkins, Baltimore & London) pp. 281-309.

10. Sikora, J. 1975. A new method of locomotion arresting in some ciliates without ciliary immobilization. *Acta Protozool.* **13**:421-424.

11. Sikora, J. 1981. Cytoplasmic streaming in Paramecium. *Protoplasma* **109**:57-77.

12. Sikora, J. and Wasik, A. 1978. Cytoplasmic streaming within Ni2+ immobilized *Paramecium aurelia. Acta Protozool.* **17**:389-397.

13. Spoon, D. M. 1976. Use of thin, flexible plastic coverslips for microscopy, microcompression, and counting of aerobic microorganisms. *Trans. Amer. Micros. Soc.* **95**:520-523.

14. Spoon, D. M. 1978. A new rotary microcompressor. *Trans. Amer. Micros. Soc.* **97**:412-416.

15. Spoon, D. M., Feise, C. O. and Youn, R. S. 1977. Poly(ethylene oxide), a new slowing agent for protozoa. *J. Protozool.* **24**:471-474.

16. Steinbiss, H.-H. and Stabel, P. 1983. Protoplast derived tobacco cells can survive capillar microinjection of the fluorescent dye Lucifer Yellow. *Protoplasma* ,**116**:223-227.

17. Uhlig, G. and Heimberg, S. H. H. 1981. A new versatile compression chamber for examination of living microorganisms. *Helgoländer Meeresunter.* **34**:251-256.

18. Wichterman, R. 1953. The Biology of *Paramecium.* (Blakiston, New York).

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