
Working with Protozoa

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Introduction

Protozoa are among the most fascinating organisms that can be studied in the classroom or laboratory. The Protista kingdom has seven groups that are divided into fifteen phyla. These subdivisions show the wide range of morphology and function that demonstrate the basic properties of living matter. This diversification is one of the reasons that students seem to be instantly fascinated by the study of these organisms.

The different phyla are distinguished from one another by such features as structure, means of locomotion, and formation of spores, although the locomotor organelles are the primary distinguishing feature. There are three main locomotor organelles found in the different classes of protozoa, and they are pseudopodia, cilia, and flagella.

A pseudopodium is generally not considered to be a separate organelle, but rather is formed by the extension of protoplasm outward from the main body. The pseudopodium then anchors itself while the rest of the organism flows into the pseudopodium. Pseudopodia are also used to surround and ingest food particles. Unlike the pseudopodium, both cilia and flagella are considered to be discrete organelles. Both structures move the organism by beating in a rhythmic or random pattern, and structurally are almost the same. The main difference between these two organelles is that flagella are usually found singly or in small groups near the leading end of the body, while cilia are found in large numbers in longitudinal rows on the body or near the mouth.

Most protozoa reproduce through a process called fission, which is simply the organism dividing into two cells by mitosis. In addition to fission, some ciliates are able to reproduce sexually through conjugation. In this process two cells unite and exchange genetic material.

Collection

Collection of protozoa is possible from almost any conceivable habitat. Free-living species occur wherever there is water, while parasitic species occur in most metazoa. A hay infusion set up in the lab will yield many different types of microorganisms. Mud and vegetation collected from ponds, streams, and transient bodies of water will yield microscopic life after being kept for a short time in the laboratory.

Care and Feeding

Receiving

All packages containing live materials should be opened immediately and all jars, vials, etc. should be checked for breakage.

Loosen (do not remove) the caps on the jars and tubes containing cultures to permit air exchange. (A frozen culture is not necessarily ruined. Thaw slowly, then follow instructions below.)

Inspect all cultures microscopically in the culture container using a low-power binocular microscope (Know what you are looking for). One can also pipet a bit of the liquid which contains the protozoan onto a slide and examine under a conventional microscope.

If the organism is not visible, allow the culture to rest undisturbed at room temperature for one-half to one hour and re-examine. Also refer to the particular organisms in the section entitled Comments in the chart below.

Handling

Common sense should apply here. Most Protozoans are extremely fragile; therefore all manipulations must be performed gently. Pipetting should be done slowly; cultures should not be handled roughly.

Short-Term Maintenance

Keep loosened caps on all culture-containing jars and tubes when not in use, and be sure to keep out of direct sunlight. Most protozoans do best at a temperature of 20°C, however the temperature may be anywhere between 18°C and 22°C.

Culturing

The following chart covers most of the basic knowledge needed to work with each listed protozoan as well as some suggestions of methods to try in working with others. The pH of the media should be as close to 7 as possible and all glassware used should be clean, sterilized, and free from chemical contamination.

Organism	Culture Containers	Recommended Medium	Recommended Subculture Frequency	Comments
Mastigophora				
Chilomonas	250 ml flask capped or plugged with cotton.	Distilled water with two wheat grains	2 – 3 weeks	Organisms collect on surface.
Euglena	1 L flask capped or plugged with cotton.	Split Pea	2 – 3 weeks	Organisms distributed throughout.
Peranema	250 ml flask capped or plugged with cotton.	Hay	2 – 3 weeks	Keep culture in indirect light. Organisms distributed throughout.

Organism	Culture Containers	Recommended Medium	Recommended Subculture Frequency	Comments
Sarcodina				
Actinospherium	Culture dish, 2 1/4" x 4 1/2"	Hay	2 – 3 weeks	Organisms collect on bottom, gathering around rice, wheat, or hay in culture. Extremely fragile, use care when handling.
<i>Amoeba proteus</i>	Culture dish, 2 1/4" x 4 1/2"	Amoeba Medium	Monthly	Organisms collect on bottom, gathering around rice, wheat, or hay in culture. Very sensitive to higher temperatures.
Arcella	Culture dish, 2 1/4" x 4 1/2"	Hay	2 – 3 weeks	Organisms collect on bottom in debris.
Chaos	Culture dish, 2 1/4" x 4 1/2"	Distilled Water and Active Paramecium Culture Grown in Hay Medium	2 – 3 weeks	Organisms collect on bottom, gathering around rice, wheat, or hay in culture.
Diffugia	Culture dish, 2 1/4" x 4 1/2"	Soil Water and Spirogyra with a Pinch of Fine Sand	2 – 3 weeks	Organisms collect on bottom in debris and on Spirogyra
Sporozoa				
Gregarina		Parasite in Tenebrio Larvae		Found in the intestines of Mealworm Larvae
Ciliophora				
Blepharisma	250 ml flask capped or plugged with cotton.	Hay	2 – 3 weeks	Organisms collect at sides and on bottom of container.
Colpidium	250 ml flask capped or plugged with cotton.	Hay	1 – 2 weeks	Organisms collect on surface.
Didinium	Culture dish, 2 1/4" x 4 1/2"	Active Paramecium Culture	As needed	Organisms distributed throughout. If cysts are found on bottom of culture, they may be activated by adding fresh medium and/or Paramecium.

Organism	Culture Containers	Recommended Medium	Recommended Subculture Frequency	Comments
Ciliophora (cont.)				
Euplotes	250 ml flask capped or plugged with cotton.	Hay	1 – 2 weeks	Organisms distributed throughout.
Paramecium				
<i>P. aurelia</i>	1 L flask capped or plugged with cotton.	Hay	As needed	Organisms distributed throughout.
<i>P. bursaria</i>	1 L flask capped or plugged with cotton.	Hay	2 – 3 weeks	Organisms collect on surface and are distributed throughout. Keep culture in indirect light.
<i>P. caudatum</i>	1 L flask capped or plugged with cotton.	Hay	2 weeks	Organisms collect on surface and are distributed throughout.
<i>P. multi-micronucleatum</i>	1 L flask capped or plugged with cotton	Hay	2 weeks	Organisms collect on surface and are distributed throughout.
Spirostomum	Culture dish, 2 ¼" x 4 ½"	Hay	Monthly	Organisms collect on bottom in debris.
Stentor	Culture dish, 2 ¼" x 4 ½"	Active Euglena Culture	Monthly	Organisms collect on surface, at sides of container, and on bottom in debris.
Tetrahymena	Bacteriological culture tube with screw cap or plugged with cotton.	Tetrahymena Medium	As Needed	Organisms collect on surface.
Vorticella	Culture dish, 2 ¼" x 4 ½"	Hay	Weekly	Organisms collect on surface and on bottom in debris.

Media

Amoeba Medium: To 100 ml of distilled water add two rice grains.

Note: *Chalkey's medium can be used instead of distilled water as a more refined medium. This consists of 0.1 g of NaCl, 0.004 g of KCl, and 0.006 g CaCl₂ in one liter of distilled water. Proceed as above.*

Diffugia: Soil-water medium is the basis. Place 5–10 cm of garden or muck soil free from chemical fertilizers and pesticides in the bottom of a culture container and fill three-quarters full with distilled water. Plug and steam for one hour on two consecutive days. Filter the soil water and dilute 1:4 with conditioned tap water. To 100 ml of the dilute soil water, add a pinch of extremely fine sand and a few strands of spirogyra.

Hay medium: Put 15 g of Timothy hay into one liter of distilled water. Autoclave at 15 PSI for 15 minutes. After cooling, filter and store in refrigerator. Dilute the hay medium 1:4 with distilled water. Dispense approximately 100 ml per culture container. Then add three boiled wheat grains.

Chaos: To 100 ml of distilled water, add two rice grains. Then add *Paramecium caudatum* grown in hay medium. Replenish *P. caudatum* as needed.

Split Pea: Add forty split peas to one liter of distilled water and autoclave at 15 PSI for 15 minutes, or boil for approximately 10 minutes. Cool and use immediately.

Tetrahymena medium: Dissolve 1 g Proteose Peptone in 100 ml distilled water. Dispense, cap, and autoclave at 15 PSI for 15 minutes.

Observation

The majority of experiments done with protozoa require only a microscope and good powers of observation. It is important that all slides and coverslips be completely clean. Slides can be washed in a mild detergent solution, rinsed in distilled water, and allowed to dry before use. If new slides are not pre-cleaned, they should also be washed.

Wet Mounting

There are two techniques that provide the best viewing for organisms on a slide. For the first technique, draw an outline of a coverslip and then place the slide on top of the tracing. Draw a thin line of petroleum jelly within the tracing lines using a toothpick. Place a drop of liquid containing the organisms to be viewed in the center of the coverslip and carefully lower the slide onto the coverslip to form an airtight seal. Invert the preparation smoothly to keep the seal intact. If relatively thick objects are to be viewed, such as *Chaos*, a few pieces of broken coverslip should be included within the petroleum jelly border to avoid crushing the organism. (**Caution should be used, since the broken glass will be sharp.**)

The second technique utilizes silicone culture gum to form a chamber on the slide. Remove a small piece of the gum and roll it into a sphere in the center of a clean slide. Take a second slide that has been wetted and press it on the sphere until you have the desired thickness. The wetted slide can then be removed, since the silicone gum will not

stick to it. A tight bond will be formed between the silicone gum and the dry slide. Using a sharp cork borer, cut a hole through the gum to the slide and remove the plug. Fill the cell with the culture to be observed and cover with a coverslip, excluding as much air as possible. The silicone gum will allow carbon dioxide to escape and oxygen to enter. One can keep the prepared slide many days by placing it in a humidity chamber, taking it out only to study the culture. A simple humidity chamber may be made by placing the slide in a Petri dish lined with moist paper towel.

Once the organisms have been mounted on the slide, the most common behaviors to observe are feeding and sexual reproduction. Several different organisms' feeding behavior can be easily observed. Chaos and Didinium can be observed by placing a drop of medium containing several Chaos or Didinium on a slide, and then adding a drop of concentrated Paramecia. Observe the different methods used by the Chaos and the Didinium to capture, ingest, and digest the Paramecia. In a mixed protozoa culture, Euglena and similar green forms may be observed in the food vacuoles of larger protozoa. In the mixed protozoa culture you may also observe and compare the food gathering techniques of the Ciliophora, Mastigophora, and Sarcodina.

The mating of *Paramecium bursaria* can be observed by mixing equal quantities of the opposite mating types on a slide or in a watch glass. Evaporation must be prevented as the experiment takes about two days. The mixing should be done about midday, since time is an environmental factor in mating. The individuals will immediately start to form clumps. After 16 hours (depending on conditions) individual mating pairs may be observed. Large numbers of conjugants normally occur from 24 to 48 hours, after which time little conjugation occurs.

Sporozoan Demonstration

All sporozoans are parasitic, absorbing nutrients from their hosts. Plasmodium vivax is a sporozoan that causes malaria in humans. Sporozoans of the genus Gregarina are found in the guts of arthropods.

Gregarines are found in the gut of the mealworm, Tenebrio. Mealworms burrow into their food and defecate. This develops an environment in which they readily infect each other with the protozoan. A simple demonstration of the presence of gregarines utilizes the live Tenebrio larva. With a scalpel or razor cut off the head and the last somite of the mealworm. Pull out the digestive tract with a probe or fine forceps and remove any adhering tissue. Then place the gut in a drop of Frog Ringer Solution (6.5 g NaCl, 0.14 g KCl, 0.12 g CaCl₂, and 0.2 g MgSO₄ in one liter of distilled water) on a slide. Place a coverslip over the gut and press down gently. Scan the slide under low power. The gregarines should appear as clear to dark-colored objects, longer than they are wide. Sometime they appear as short chains. They may be flushed out of the gut with another drop of saline and examined under higher power for structural details.

Immobilization

In order to view many types of fast-moving ciliates and flagellates under a compound microscope it is necessary to slow or immobilize them. There are three basic methods of immobilization: mechanical, chemical, and narcotic. Because of the unsuitability of narcotic means for classroom use and the versatility of other methods, this method will not be discussed.

Mechanical: If debris is present in the culture, remove some with the drop of culture to be examined. The organisms will eventually tend to come to rest near the debris and can be studied.

If no debris is present, a small amount of shredded filter paper or lens tissue may be placed in the drop on the slide; use only a few fibers.

Chemical: WARD'S Detain is a water-soluble, viscoelastic, nonionic resin solution used to slow motile protists. Detain is less toxic to protists than the traditional chemical slowing agents stated below. Detain will dissolve readily in fresh or salt water. Due to its high viscosity, Detain will support a coverslip, and after a few minutes will form a ring around the periphery, retarding evaporation. Protozoa have been observed to survive for periods of over 96 hours following introduction of Detain.

Methyl cellulose is an excellent agent for immobilization of protozoa. It can be used as a 1.5% liquid or as a 10% paste. To use the 1.5% liquid, add a drop to the side with the culture on it. As the methyl cellulose diffuses through the medium, the change in viscosity will slow the protozoan's movements. To use the 10% paste, follow the same procedure used for the petroleum jelly wetmount. As the methyl cellulose diffuses inward, it will immobilize the protozoans.

To prepare the 1.5% liquid, mix 1.5 g methyl cellulose powder with 50 ml boiling water. Cool, then stir 48.5 ml of cool water into the mixture. The liquid should have the consistency of syrup. The 10% paste is prepared in like man-

ner. Add 10 g of methyl cellulose powder to 50 ml of water, then add another 40 ml of water. The consistency of this concentration is like solidified gelatin.

Polyvinyl alcohol is used by adding one drop of 14% PVA solution to one or two drops of the culture on the slide. It may be more effective to place the drop of PVA at the margin of the culture drop, because this causes the PVA to diffuse slower. The immobilization is both mechanical (change in medium's viscosity) and chemical.

To prepare the 14% PVA solution, add 14g of the PVA powder to 36mL anhydrous ethyl alcohol. It may be necessary to heat in a water bath until the PVA dissolves. Stir and add 50mL of distilled water.

Vital and Supravital Staining

Many chemical stains can be applied to living protozoans to color various parts of the organism. If the stain is not harmful in the dilution used, it is a vital stain. If it is ultimately lethal, it is called supravital staining. For use, most stains must be dissolved and diluted. Percent solutions, for example a 1% methylene blue solution, will refer to only the percentage of solute weight to volume. To make a 1% aqueous solution of a stain, place 1 g of dry powder in a graduated cylinder and add enough distilled water to total 100 ml. This method may also be used for alcohol solutions, although it is not as accurate because 1 ml of alcohol is not 1 g as in the case of water. This method, however, will be sufficient for use in most cases. All water used for dilutions should be distilled. All alcohol used should be absolute (100%) ethanol. Other alcohols may be used, but the results may not be as satisfactory as those obtained with ethanol. The stains discussed are made up in absolute ethyl alcohol.

Stains

Bismarck Brown: Will stain cytoplasmic inclusions in living protozoa. Place a drop of 0.1% Bismarck Brown alcohol solution on a slide. Allow the drop to dry. Place a drop of protozoan culture on the slide and cover with a coverslip. If this concentration disrupts the protozoan cells, try a 0.05 – 0.01% dilution. Alternatively, a 1.0% Bismarck Brown alcohol solution diluted to 0.1% or 0.01% with water may be dropped directly on a drop of culture on a slide.

Brilliant Cresyl Blue: Will stain the nucleolus of a protozoan. It is used in similar concentrations as Bismarck Brown.

Bromothymol Blue: A pH indicator, which is yellow at pH 6.0 and blue at pH 7.6, it is used to study the food vacuoles of protozoans. It can be used as described for Bismarck Brown, or it may be used to stain yeast cells which are then fed to Paramecia in order to demonstrate the pH of food vacuoles.

Place about $\frac{1}{4}$ cake of fresh yeast or 0.5 g of dry yeast in 10 ml of distilled water. Add 0.1 ml of 1% Bromothymol Blue Solution. Boil in a water bath 5–10 minutes to kill the yeast cells. Cool and place a small amount of the suspension (using a toothpick) in a drop of paramecium culture on a slide. Observe the pH in the food vacuoles over a period of time.

Carmine Powder: Used to demonstrate the feeding in paramecia and other ciliates. Place a drop of the protozoan culture on a slide and dip the end of a toothpick into the Carmine powder and transfer a bit of it to the slide. Cover with a coverslip. Observe the action of the cilia in sweeping the Carmine into the "mouth". Notice the formation of food vacuoles and the circulation and discharge of Carmine.

Janus Green B: Will stain the mitochondria a greenish blue. It will also stain the Golgi apparatus. It can be used at the same concentration as Bismarck Brown. Janus Green B may be combined with neutral red to give a multiple stain. This is done by mixing one part Janus Green B solution (fifteen parts absolute alcohol and one part 1% Janus Green B solution in absolute alcohol) and two parts Neutral Red solution (ten parts absolute alcohol and one part 1% Neutral Red in absolute alcohol).

Methylene Blue: Will stain the nucleus, cytoplasmic granules, and cytoplasmic processes of protozoa. It may be used in a 0.05% alcohol solution or in lesser concentrations as used with Bismarck Brown. Methylene Blue may also be used to demonstrate the discharge of trichocysts in Paramecia. Place a drop of Paramecium culture on a slide and cover with a coverslip. Put a drop of 0.1% methylene blue solution at the edge of the coverslip and observe the trichocysts discharge as the Paramecia encounter the stain.

Neutral Red: pH indicator. It is red below pH 6.8 and yellow above 8.0. It will also stain the macronucleus slightly. It can be used in the same manner as Bismarck Brown. Observe the pH changes in the food vacuoles as they form and circulate.

Fixation and Staining of Protozoa

When permanently fixing a contractile organism for observation, it may be necessary to relax the organism in order to prevent the organism from being fixed in an abnormal shape. If direct fixation does not destroy or distort the protozoan, the relaxation is not necessary. A 3% solution of copper acetate will immobilize and relax most ciliated protozoans.

Other chemicals which may be used include the following: 10% methyl alcohol, 1% hydrochloride, and 1% magnesium sulfate. These chemicals are added drop by drop until the absence of any contraction is observed when the protozoan is under stimuli. Then the protozoans are fixed.

Glutaraldehyde: Suitable for fixation of some ciliates and other protozoans. To use add one to two drops of 25% glutaraldehyde to one drop of the protozoan culture on a slide. If this method causes distortion or disintegration of the protozoan the following method using glutaraldehyde may be effective.

Place a drop of the protozoan culture on a slide and invert it over a watch glass containing a few drops of glutaraldehyde. Leave the slide in contact with the fumes approximately 30 seconds, then remove and proceed to the staining of the protozoan.

Schaudinn's Fluid: A broad-spectrum fixative for protozoa. To use, the protozoan culture to be fixed should have as little culture fluid as possible. The fixative should then be added slowly. For use in the fixation of a drop of protozoan culture on a slide, add one part Schaudinn's Fluid to three parts water. Then add one drop of the diluted fixative to one drop of the protozoan culture on the slide. When the turbidity clears the protozoan may be stained.

Preparation of Schaudinn's is as follows: add two parts saturated aqueous solution of mercuric chloride to one part absolute alcohol (ethanol). Just before use add 1 ml glacial acetic acid to 99 ml of the fixative.

Staining

Toluidine Blue: used to stain cilia, cirri, flagella, and membranelles of protozoans. It may be used in a 0.1–0.001% aqueous solution. Experience will determine the best dilution for the protozoans to be stained.

To use, add one drop to the fixed material on the slide. In order to be the most effective, the stain should just tint the liquid slightly. Water mounted protozoa have many limitations when it comes to staining because it is difficult to remove the stain from the liquid. For fine work and to produce permanent slides one should research into the literature on protozoa and microtechnique.

