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Chapter 5

Using Microbial Eukaryotes for Laboratory Instruction and Student Inquiry*

Donna M. Bozzone

Department of Biology
Saint Michael's College
Colchester, Vermont 05439
dbozzone@smcvt.edu

Donna Bozzone received her B.S. from Manhattan College (1978), M.A. (1980), and Ph.D. (1983) from Princeton University. From 1983-1987, she was a post-doctoral research associate in the Cell Biology Group at the Worcester Foundation for Experimental Biology. In 1987, she joined the faculty of Saint Michael's College where she is a Professor of Biology. In 2001, she was the recipient of the Saint Michael's College Joanne Rathgeb Teaching Award. A member of ABLE since 1991, she has served as member-at-large (1998-2002), and has presented several mini and major workshops. In addition to introductory biology, she teaches developmental biology, plant developmental physiology, science writing, and senior seminar in the history of biology. Her research interests include biology laboratory and curriculum development, the developmental biology of microbial eukaryotes, and the history of biology.

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Contents

Introduction

It can be a challenge to provide students with meaningful, open-ended laboratory experiences that allow them to ask their own testable questions and implement experiments of their own design. One way to meet this goal is by using microbial eukaryotes for laboratory instruction. Existing for all or part of their life cycles as single cells, microbial eukaryotes display many of the same basic biological phenomena observed in more complex organisms, but they can be cultured and handled like microorganisms. Cost-effective, microbial eukaryotes can be grown and manipulated easily. They don't require much space, and the relatively fast generation times and life cycles permit students to repeat or extend investigations. Microbial eukaryotes are student and instructor "friendly" allowing one to reduce the use of vertebrates, except for those biological problems that are particular to advanced organisms. Moreover, conceptualizing microbial eukaryotes as "teaching and learning models" to understand basic biological phenomena reinforces the concept of "model organisms" that has been so successful in basic research. It is important also to point out, however, that microbial eukaryotes are fascinating organisms in their own right.

Microbial eukaryotes are suitable for teaching at introductory to advanced levels. These organisms are also appropriate for student independent projects. Although not discussed here, some of the exercises presented in this workshop have also been adapted for use in middle school, high school, and science fairs. In our biology program at Saint Michael's, we focus our attention on four types of microbial eukaryotes: *Dictyostelium, Tetrahymena, Physarum*, and *Saccharomyces* (yeast). Our students first encounter these species in General Biology laboratory, and then revisit them for experimental work in an upper division course. We have found that the repeated exposure to and work with these organisms allows our students to more easily undertake independent laboratory work in their upper division courses.

The objective of this workshop is to provide information about two of these species: the ciliated protozoan, *Tetrahymena,* and the acellular slime mold, *Physarum*. Instructions for working with *Dictyostelium* were presented at ABLE 2003 and yeast work will be detailed at a future ABLE conference. The specific information described here includes background material about *Tetrahymena* and *Physarum*, "care and feeding" instructions, examples of introductory and advanced laboratories, and suggestions for further investigations.

Tetrahymena: Exercises and Experiments

Introductory Level: Culturing and Observations of *Tetrahymena*

Student Instructions

Introduction

The protozoan *Tetrahymena* is an excellent organism for teaching and learning about a wide range of topics including cell division, cell motility, cell behavior, cell population growth, and feeding. In addition, *Tetrahymena* is used in research labs throughout the world to study environmental toxicity, pharmacology, and basic research questions in cell and molecular biology, and ecology. In the instructions detailed here, you will learn how to culture *Tetrahymena*, and how

to manipulate the cells to make some very straightforward observations of cell morphology and behavior.

Background

Tetrahymena are common freshwater ciliated protozoa with a very wide geographical range. Although only 50-60 μm in length and 20 μm wide, it is likely that *Tetrahymena* were first described by Leeuwenhoek and since then, they have been isolated from locations all over the world and from various habitats including lakes, streams, rivers, thermal springs, soil, and the body cavities of aquatic insects. *Tetrahymena* species are generally free-living although many also live as facultative parasites. They can withstand a broad range of pH, temperature, nutrient availability, and oxygen concentration. Under good conditions, *Tetrahymena* populations grow quickly using asexual reproduction. In contrast, under certain environmental conditions, especially starvation, if opposite mating types are present together, they will initiate sexual development (conjugation).

Although *Tetrahymena* can be cultured similarly to the way one would grow a population of a less complex microorganism like bacteria, *Tetrahymena* has a more complex life cycle. After its sexual phase (conjugation), the offspring are sexually immature and need to divide a few times to become sexually mature. Once cells do become sexually mature, they can remain in this state over some time, and then become "senile" (one sign of which is selfing or mating within a clone). Ultimately the cells age and die.

Tetrahymena have been a model organism for studying many basic research questions. In fact, several "landmark" discoveries were first made using *Tetrahymena* including: dynein motors (the motor proteins that move the microtubules of cilia and flagella), RNA-mediated catalysis (RNA acting as an enzyme), and telomeres and telomerase (important for regulating cell lifespan). *Tetrahymena* have also been used to study isolated organelles, cilia, the cell cycle, chemotaxis, cell motility, chromosome rearrangement, gene mapping, and most recently, functional genomic analysis. Besides its importance in the world of basic research, *Tetrahymena* has also been and continues to be essential in applied research areas such as drug development and testing, screening for environmental pollutants, and biotechnology.

While there has been a great deal of important research done using *Tetrahymena*, many unsolved questions remain. In fact, many questions still need to be asked. As you make your initial acquaintance with *Tetrahymena*, observe the cells carefully with respect to their morphology and behavior. As you record your observations, consider also what questions you have about this organism. Use your imagination and you might come up with something no one else has thought of before you!

Methods

Culturing Instructions

1. Since the *Tetrahymena* culture you have is axenic (it contains *Tetrahymena* and no other organism), you need to use aseptic technique to grow a population of cells. To culture *Tetrahymena*, pipette 1 ml of a stock culture into 25 ml of 2% proteose peptone in a 125-ml Erlenmeyer flask. (You can use other volumes, but the ratio of medium volume to total flask volume should be 1:5 or less).
2. Incubate at room temperature, 20-22^{o} C. Set up enough cultures so that you will have an extra that can be used as your stock culture.
3. Transfer stock cultures once a week.

How to Make a Wet Mount

1. If you are not using your stock culture, it is not necessary to use aseptic technique. In general, it is best to never use your stock culture for experiments.
2. To make a wet mount, withdraw a small volume of a *Tetrahymena* culture into a pipette. Gently place a drop or two on a clean slide.
3. Take a coverslip and hold it perpendicular to the slide. Lower the cover slip, still in a perpendicular position, so that the edge touches the cell suspension on the slide.
4. When the cover slip is touching the edge of the cell suspension, gently lower it over the drop, trying to avoid air bubbles.
5. To observe *Tetrahymena*, place the slide on the stage of a compound microscope and using the lowest available magnification, focus on the edge of the coverslip. Once you have found the edge, adjust the fine focus and you should see *Tetrahymena* swimming actively.
6. Make observations at all magnifications available on your microscope, except don't attempt to use the 100x objective with a wet mount (it probably won't fit).
7. If the *Tetrahymena* are moving too rapidly to observe, try a second wet mount using Protoslo (methylcellulose). Make a ring of Protoslo on a microscope slide. The diameter of this ring should be similar to the width of a cover slip. Place a drop or two of *Tetrahymena* in the Protoslo ring and gently cover with a cover slip. Observations of cell structure will be easier because the viscosity of the Protoslo increases the resistance for ciliary beating. Consequently, cells still move, but do so more slowly.
8. Diagram and record a written description of your observations.

Observations of Living *Tetrahymena* in India Ink

1. Place a drop of *Tetrahymena* on a slide. Next to it, place a drop of 1% India ink. Mix with a pipette or toothpick. Gently cover the drops with a cover slip.
2. Observe *Tetrahymena*'s swimming behaviors. Notice especially how the cells rotate and how the ink particles move in response to the ciliary beating of the cells. (You can also try to observe the ciliary motion by mixing a drop of *Tetrahymena* with a drop of 5% India ink).
3. Notice also the formation of large black vacuoles in the cells as *Tetrahymena* "eats" the ink. Observe the formation of the vacuoles for a few minutes to note the intracellular paths they take.
4. You can also try to observe a "slower" version of this behavior by using Protoslo as described above.
5. Diagram and record a written description of your observations.

Observations of "Fixed" Cells

1. *Tetrahymena* can also be observed in a stationary state after exposure to fixative (a chemical that kills cells without distorting structure).
2. Take a drop or two of *Tetrahymena* and place it on a slide. Next to it, add a drop of Lugol's solution. Mix.
3. Gently place a coverslip on the drops and observe the slide. Notice the staining of the cytoplasm and the positions of the cilia.
4. You can also use Lugol's solution to fix cells that have been "eating" India ink so that you can get a better look at the vacuoles. To do this, simply place a drop of cells and a drop of

1% ink on a slide. Wait at least 5 minutes (longer is fine) and add a drop of Lugol's' solution. Gently add a coverslip.

5. Diagram and record a written description of your observations.

Analysis

You have made a series of basic observations of *Tetrahymena* morphology and behavior that you recorded both in diagrams and written descriptions. Using these observations and other information you know, pose at least one question suitable for further investigation.

Suggestions and Ideas for Observations

1a. Stain *Tetrahymena* with neutral red by mixing a drop of cells and a drop of stain together on a microscope slide. Gently place a coverslip on the drops. Examine the slide using a microscope. What do you observe? Explain.

1b. Now add a drop of 1% India ink to the edge of the coverslip and watch the *Tetrahymena* as the drop diffuses in their direction. What do you observe? How can you explain your observation?

2. Use a toothpick and Vaseline to draw three sides of a rectangle on a microscope slide. Place a couple of drops of *Tetrahymena* on the slide within this "three-sided Rectangle". Lower a coverslip on top and press gently to seal the three edges. Observe the behaviors of *Tetrahymena* for the next few minutes. How do you explain your observations?

3. Try some stains to visualize cell structure:
   - Lugol's solution: stains starch. Add one to two drops of stain to 1 ml of cells in a test tube. Make a wet mount and observe.
   - Neutral red: red at acidic pH and yellow at basic pH. Combine one drop of cells and one drop of stain on a microscope slide, add a coverslip, and observe with a microscope.
   - Janus Green B: stains mitochondria. Combine one drop of cells and one drop of stain on a slide, add a cover slip, and observe with a microscope.
   - Methyl green-pyronine: stains nuclei green and cytoplasm pink to red. Add one drop of cells and one drop of stain on a slide, add a coverslip, and observe with a microscope.
   - Nigrosin: stains surface details of cells. Combine one drop if cells and one drop of stain on a slide. Mix with a toothpick and spread into a thin layer. Let it dry. Observe with a microscope.

Introductory to Advanced Levels: Phagocytosis and Vacuole Formation in *Tetrahymena*

Student Instructions

Introduction

Many single-celled organisms and some specialized cells in multicellular organisms have the ability to "eat" relatively large materials such as microorganisms or cell debris. The general process whereby cells ingest materials and enclose them in membrane bound vesicles or vacuoles is called endocytosis. Phagocytosis ("cell-eating") refers to those cases of endocytosis where a "large" item is eaten, while smaller substances can enter through pinocytosis ("cell-drinking").

Phagocytosis is an essential cell behavior for many organisms. In protozoa, it is a type of feeding. Single-celled organisms, such as *Tetrahymena*, eat bacteria, other small organisms, or organic debris, and form special vacuoles called phagosomes. These vacuoles fuse with lysosomes, and the food is digested. Undigestible material is egested from the cell's cytoproct.

In more complex multicellular organisms, phagocytosis is used to protect the body from infection. Phagocytic cells of the mammalian immune system limit the spread of invading microbes. When microorganisms get into the body, through a cut in the skin for example, the damaged cells secrete chemical signals to attract white blood cells called neutrophils. The neutrophils crawl through the body to the site of the wound, and engulf and destroy bacteria present there. Also attracted to the wound is another type of white blood cell, macrophages, which also eat bacteria. In addition to their role in wound response, macrophages also reside in certain organs such as lungs, liver, kidney, connective tissue, lymph nodes, and spleen. Macrophages are vigilant in these organs, keeping them clear of bacteria and cellular debris.

Phagocytic cells in higher organisms also scavenge dead and damaged cells, and cellular debris. In some cases, the damaged cells come from the normal cellular turnover. For example, macrophages eat more than 100 billion worn out red blood cells each day. In other cases, the damaged cells come from the normal programmed cell death that is part of embryogenesis. For example, during metamorphosis in amphibians, the tails of tadpoles shorten and disappear. This event occurs because the cells of the tail undergo programmed cell death and macrophages digest the cellular debris thus produced. Similarly, programmed cell death is responsible for the loss of webbing between the fingers of the developing human hand. Again, phagocytic macrophages remove the resulting cellular debris.

As you can see, phagocytosis is an important eukaryotic cell behavior that is evident in both single-celled organisms as well as in the specialized cells of advanced organisms, such as vertebrates. While we seek to understand how complex organisms such as ourselves function biologically, many research scientists choose to study basic biological phenomena in simpler "model" organisms because of experimental advantages such as ease of culturing, handling and manipulation, and lower costs. With respect to phagocytosis, *Tetrahymena* is a popular choice both for basic research and for testing drugs and environmental toxins. In keeping with this research approach, you will explore phagocytosis and vacuole formation using the ciliated protozoan *Tetrahymena*. As described below, you will learn how to quantify phagocytosis, do a guided experimental exploration, and then have the opportunity to consider an experimental avenue of your own.

Background

Tetrahymena is ideal for studying phagocytosis and vacuole formation. This cell behavior is quite dramatic and easy to observe directly with a compound microscope. When hungry *Tetrahymena* encounter food, use their moving cilia to maintain a current of extracellular fluid and sweep material into each cell's buccal cavity ("mouth"). The receiving vacuole fills up with food particles and fluid, and pinches off. *Tetrahymena* will ingest a wide variety of materials including but not limited to India ink, carbon particles, carmine, yeast, bacteria, latex beads, polystyrene beads, and heat-coagulated egg albumin.

The ability of *Tetrahymena* to phagocytose and form vacuoles is influenced by a number of factors. The process is sensitive to calcium concentration, metabolic inhibitors, the viscosity of the medium, pH, temperature, inhibitors of the cytoskeleton, and the quality of the particulates being fed to them. The ability of *Tetrahymena* to phagocytose and form vacuoles is also dependent upon the state of the cells. For example, starved cells and well-fed cells behave differently as do mitotic and non-mitotic cells.

Phagocytosis and vacuole formation in *Tetrahymena* in particular, and in eukaryotic cells in general, continue to be important basic research topics. These processes are also being studied as tools to screen drugs for function and toxicity, as well as for their presence. For example, scientists have exposed *Tetrahymena* to two drugs used for cancer chemotherapy to see if they are toxic to eukaryotic cells. Other studies have shown that *Tetrahymena* phagocytosis can be used to see if opioid drugs are present as a byproduct of food production. Finally, studies conducted to understand the physiological and biochemical effects of drugs on eukaryotic cells have revealed that the rate of vacuole formation in *Tetrahymena* is increased by exposure to amphetamines, and decreased after exposure to cocaine, morphine, or hashish. The methods that you will be using to do your experiments are based on those used in research labs.

In the instructions that follow, you will learn how to observe phagocytosis and quantify vacuole formation. You will also be able to do one or more of the experiments detailed for you. Finally, there are suggestions for possible avenues of inquiry for you to explore in experiments of you own design.

Methods

Observing Phagocytosis

1. You will be provided with *Tetrahymena* grown in 2% proteose peptone for 48-72 hours.
2. Pipette 1 ml of *Tetrahymena* and 1 ml of 1% India ink into a test tube. Mix gently.
3. Make a wet mount of the resulting cell-ink suspension and using a compound microscope, observe the behaviors of the cells. Watch how the cells swim and eat. Record you observations.

The Effect of Ink Concentration on the Rate of Vacuole Formation

1. Place 1 ml of *Tetrahymena* into each of three test tubes. Label the tubes 1%, 5%, and 10% India ink.
2. To the "1%" tube, add 1 ml of 1% India ink. Immediately pipette 20 µl of cell and ink suspension in to a centrifuge into which you have placed 10 µl of 1% glutaraldehyde (or another suitable fixative). Exposing cells to glutaraldehyde, a fixative, will kill the ells. Consequently, you can work at your own pace once the cells have been sampled.
3. Sample cells from the cell+ India ink suspension at 5, 10, and 20 minutes as described in step 2.

4. Add 1 ml of 5% India ink to the cells in the "5%" tube. Sample cells immediately, and at 5, 10 and 20 minutes as described in steps 2 and 3.
5. Add 1 ml of 10% India ink to the cells in the "10%" tube. Sample cells immediately, and at 5, 10, and 20 minutes as described in steps 2 and 3.
6. To quantify your samples, place a drop of the fixed cells on a microscope slide and using a compound scope at a total magnification of 100-200X, count the number of vacuoles containing ink for at least 20 cells at each time point.
7. Determine the average number of vacuoles per cell at each time point for the 1%, 5%, and 10% ink treatments.
8. Prepare a table and graph to display your results. Interpret your results fully and pose a testable follow-up experimental question.

You can use the same basic protocol of feeding 1% India ink to cells, sampling cells immediately, and at 5, 10, and 20 minutes, and quantifying the number of vacuoles per cell, to do the next two experiments detailed below and to undertake any of the suggested investigations.

The Effect of Temperature on the Rate of Phagocytosis and Vacuole Formation

1. Place 1 ml of *Tetrahymena* into each of 7 test tubes.
2. Incubate one test tube containing *Tetrahymena* at each of the following (or comparable) temperatures: 5, 10, 15, 20, 25, 30, 35^{o} C. Also place a test tube containing 1% India ink at each of these temperatures.
3. After allowing 15 minutes for the cells and ink to equilibrate to the various temperatures, add 1 ml of 1% India ink to cells (of the same temperature), and immediately remove 20 µl of the cell and ink suspension and place it into a centrifuge tube containing 10 µl of 1% glutaraldehyde. You will do this for cells and ink at each of the 7 temperatures being tested.
4. Continue to incubate the cell+ink suspensions at their specific temperatures. Remove and fix samples at 5, 10, and 20 minutes.
5. Determine the number of vacuoles per cell for each time point and temperature.
6. Prepare a table and graph to display your results. Interpret your results fully and propose a testable follow-up experimental question.

The Effect of Starvation on Phagocytosis and Vacuole Formation

1. To starve *Tetrahymena*, centrifuge cells that have been grown 48-72 h in 2% proteose peptone, 1000 rpm, for 4 minutes, in a tabletop centrifuge. Discard the supernatant and resuspend the cell pellet in dilute salt solution.
2. Centrifuge and wash the cells one or two more times. Resuspend the cell pellet in a volume of dilute salt solution equal to the volume of medium that you initially removed.
3. Incubate the resuspended cells at room temperature for at least 15 hours.
4. To compare phagocytosis and vacuole formation in well-fed and starved cells, place 1 ml of starved cells into a test tube and 1 ml of *Tetrahymena* grown in 2 % proteose peptone for 48-72 h into another test tube. To each, add 1 ml of 1% India ink. Remove and fix samples for each cell+ink suspension at 0, 5, 10, and 20 minutes. Determine the average number of vacuoles per cell for each time point for both well-fed and starving cells.
5. Prepare a table and graph to display your results. Interpret your data fully and propose a testable follow-up experimental question.

More Suggestions and Ideas for Experiments

Using the basic methods described in these instructions, you can do any of the experiments suggested below, as well as pursue questions of your own.

- Test other concentrations of India ink
- Test other non-food particulates such as carbon particles, carmine, latex beads, polystyrene beads
- Test whether cells exhibit a preference for edible food particles and if so, whether that preference depends on the cell's nutritional state (starving or well fed)
- If you observed a response to temperature, see if the effect is permanent or reversible by transferring the cells back to room temperature and observing whether the rate of phagocytosis and vacuole formation changes
- Try starving cells for different time periods (24, 48, and 72 hrs)
- Starve cells for 24 hrs but then feed them and determine how long it takes for cells to recover from any "starvation effect"
- To determine if oxygen levels matter, alter the surface area to volume ratio of the medium in the culture flasks. (The greater the surface area/volume ratio, the greater the exposure of cells to oxygen)
- Agitate culture flasks on a shaker table to determine whether cultures need to be stationary for cells to feed effectively
- Test the effect of metabolic toxins, pollutants, or over-the-counter drugs
- Test the effect of pH
- Test the effect of medium viscosity (you can do this by mixing in various amounts of methylcellulose)
- Test to see whether bacteria or other foods influence whether ink is eaten in well fed and starved cells
- Test the effect of inhibitors of the cytoskeleton
- Test the effect of calcium concentration
- Feed the cells carmine particles for 5-10 minutes and then add ink to visualize the intracellular fates of specific vacuoles

Introductory to Advanced Level: Cell Population Growth

Student Instructions

Introduction and Background

Understanding what controls or influences the growth of cell populations is a central research problem in biology because of the importance of this cell behavior with respect to human health, as well as for the appropriate functioning of biologically balanced ecosystems. Many diseases involve losing control of cell population growth, with cancer as the most notable example. In natural ecosystems, unchecked growth of one species can cause the decay of entire communities. For example, cultural eutrophication, the dramatic increase in algal populations caused by nutrient overenrichment of freshwater, can lead to loss of fish and other organisms from the ecosystem.

In your laboratory work today, you will attempt to address experimentally how cell population growth is regulated by studying this question in *Tetrahymena*. Cell division and cell population growth have been and continue to be well studied in this ciliated protozoan. Besides the opportunity to understand the basic biology of cell division and the cell cycle, *Tetrahymena* growth is an important research area for scientists studying toxicity of environmental pollutants and wastewater treatment, where a balance between bacteria and protozoa must be maintained. Your specific objective will be to learn several methods for measuring *Tetrahymena* population growth, and then design and implement an experiment to address some aspect of how this cell behavior is influenced or regulated.

Methods: General Techniques

Measuring Cell Population Growth Spectrophotometrically

1. Inoculate new cultures of *Tetrahymena* as appropriate for your experimental design.
2. Turn on the spectrophotometer (Spec 20) at least 10 minutes before use. Set the wavelength to 450 nm or 600 nm (either will work for measurements of cell density). Do not change the setting for the rest of your experiment.
3. Prepare a blank tube by adding 5 ml of sterile 2% proteose peptone to a Spec 20 tube. Refrigerate your sterile 2% proteose peptone between readings.
4. Zero the Spec 20 according to your instructor's directions.
5. Take a 5 ml sample of your culture and place it in a Spec 20 tube.
6. Once you have zeroed the Spec 20 with your blank, measure and record % Transmittance of your cell sample.
7. Repeat these steps for each culture in your experiment.
8. Using the method detailed above, measure and record the % Transmittance of your cultures twice a day, once in the morning and once in the evening. Record the times. Prepare a new blank for each data collection time. Continue to make measurements for 3 to 4 days.

Direct Cell Counting

Two methods for the direct counting of cells are presented below. In both cases, you will set up *Tetrahymena* cultures according to your experimental design. At 0 hr and every 8-12 hr thereafter, remove 200 µl of cells from your cultures and place them into microfuge tubes containing 100 µl of either 1% glutaraldehyde or Lugol's solution. Take samples for 3-4 days. These fixed cell samples can be stored in the refrigerator for later counting. Fixed cells should be observed and quantified within a week of collection.

Counting with a Haemocytometer:

1. Vortex your fixed cell sample to make a uniform cell suspension. Using a Pasteur pipette, remove a drop, and place it properly on a haemocytometer.
2. Follow your instructor's guidelines regarding how to use a haemocytometer.
3. If you need to make dilutions to count dense samples, use 2% proteose peptone as your diluent.

Counting with a Capillary Pipette

1. Vortex your fixed cell sample to make a uniform cell suspension. Using a wiretrol plunger, and capillary pipette, remove 5 µl of fixed cell suspension from the tube.
2. Depress the plunger to place 1 µl of fixed cell suspension on to a microscope slide. Using a compound microscope, count the number of cells present.
3. If you need to make dilutions to count dense samples, use 2% proteose peptone as your diluent.

Suggestions and Ideas for Experiments

Test whether any of the following parameters affect cell population growth:

- Incubation temperature
- pH
- salt concentration
- viscosity of the medium
- presence of bacteria
- presence or absence of particulates in the medium (filter sterilize medium to remove particulates)
- presence of other protozoa species
- presence of bacteria

- concentration and species of bacteria present
- proteose peptone concentration of the medium
- presence of sugars and other nutrients in the proteose peptone medium
- oxygen concentration (alter the surface area-to-volume ratio of the medium to affect oxygen availability)
- stationary versus shaking incubation
- presence of ammonia and other culture wastes
- incubation of cell in "conditioned medium," that is, medium in which other cells have already grown but were then removed
- age of the cells at the start of culturing
- nutritional state of the cells at the start of culturing (to starve cells, see the instructions in "Phagocytosis and Vacuole Formation in *Tetrahymena*")
- cell density at the start of culture
- metabolic inhibitors
- pollutants
- over the counter drugs

Advanced Level Experiments: *Tetrahymena* Chemokinesis

Student Instructions

Introduction and Background

Cells have an amazing capacity to respond to their environment in ways that enhance their survival. The signals to which cells react may come from abiotic factors such as light, temperature, and oxygen, or from chemicals produced by other organisms. In some cases, these chemical signals emitted by other cells elicit, in the responsive cell, dramatic movement toward or away from the chemical. This chemosensory behavior is evident in bacteria, where it is used for finding food and avoiding noxious chemicals. Similarly, microbial eukaryotes such as *Tetrahymena*, display attraction to nutrients and repulsion from harmful chemicals. Chemosensory behavior is also observed in specialized cells of more complex organisms, such as vertebrates. For example, leukocytes, a type of immune system cell, respond to chemical signals in the body to crawl to sites of infection and inflammation.

Tetrahymena is an especially attractive organism for studying chemosensory behaviors. In *Tetrahymena*, the specific mechanisms used to located or avoid a chemical signal is chemokinesis, a temporal chemosensory response. When in the presence of an attractant, Tetrahymena decrease the rate at which they turn away or avoid the chemical. Sometimes, they may also increase the rate at which they swim; therefore, cells collect at the site of the attractant. In contrast, cells that encounter a repellent increase the rate at which they turn away from it.

Tetrahymena is a valuable model for basic chemokinesis research. In addition, because *Tetrahymena* is responsive to certain anilines and phenols, some scientists have proposed that it is an appropriate test organism for the screening of industrial water pollution. Evidently, *Tetrahymena* has an important place in both basic and applied research.

Your objectives today are to learn the basic method for assaying chemokinesis in *Tetrahymena*, described below, and to design and carry out an experiment to answer a question you have about this interesting cell behavior.

Methods: General Technique

1. Prepare *Tetrahymena* cultures in 2% proteose peptone according to your experimental design. Incubate for 48 hr at room temperature.
2. Starve *Tetrahymena* by centrifuging to remove growth media and resuspending in dilute salt solution. (See the specific instructions in the "Phagocytosis and Vacuole Formation in *Tetrahymena*" section). Starve cells for 15-24 hr at room temperature.
3. Take an 8-ounce Styrofoam cup and using scissors or a scalpel, cut off the bottom of the cup, approximately 3-4 cm from the bottom. Discard the top of the cup.
4. Use a 20 g needle or a pin of similar diameter to poke 2 holes, on opposite sides of one another, around the circumference of the shortened cup. The holes should be close to the bottom of the cup. Repeat for more cups as appropriate for your experimental design.
5. Fill capillary pipettes with either 2% proteose peptone or distilled water. Seal one end of the pipette with clay.
6. Insert a capillary pipette containing 2% proteose peptone into one hole in the Styrofoam cup. Insert another capillary pipette containing distilled water into the hole on the other side of the cup. The unsealed, open ends of the capillary pipettes should be inside the cup. This experimental set-up will allow you to determine what cells will do when offered a "choice" between water and a potential chemoattractant. Another way to do this experiment is to present cells with two 2% proteose peptone capillaries in one cup and two water capillaries in another cup. This design will let you determine whether cells exhibit an attraction to a particular chemical rather than showing relative preferences.
7. Place 5 ml of starved *Tetrahymena* cell suspension into the cup.
8. After 30 minutes, remove each capillary pipette, unseal the ends, and using a wiretrol plunger, empty the contents of each into an individual microfuge tube containing 20 µl of fixative (either 1% glutaraldehyde or Lugol's solution).
9. Vortex the sample to suspend cells evenly. Either place 1 µl directly on to a microscope slide and count the number of cells present, or count the sample using a haemocytometer.
10. Samples can be counted immediately or stored in the refrigerator and counted within a week.
11. Use the basic protocol detailed in steps 1-10 to address one of the suggestions listed below or a question of your own design.

Suggestions and Ideas for Experiments

Test whether any of the following parameters affect *Tetrahymena* chemokinesis:

- Incubation temperature during growth
- Incubation temperature during starvation
- Incubation temperature during the chemokinesis test
- Cell density
- Composition of the starvation solution
- Length of starvation
- Oxygen availability (vary the surface area to medium volume ratio to affect oxygen availability)
- Growth state of the cell (log phase versus stationary, for example)
- Salt concentration
- Concentration of the chemoattractant or chemorepellent
- pH

And some other suggestions:

- Test to see if cells are attracted to amino acids, sugars, or nucleosides (alone and in combination)
- Determine how cells will respond if you expose them to chemoattractants and chemorepellents, simultaneously
- Use inhibitors such as cycloheximide to see whether protein synthesis is needed for chemokinesis
- Use inhibitors such as Actinomycin D to see whether RNA synthesis is need for chemokinesis
- Determine the relative rates of chemokinesis by collecting data at various time intervals (0, 5, 10, 20, and 30 min, for example)
- Test whether *Tetrahymena* are repelled by water pollutants

Materials (for *Tetrahymena labs*)

The list below includes *all* of the equipment and materials you would need to do *all* of the *Tetrahymena* exercises and experiments detailed in this chapter. Everything is available from standard laboratory supply sources such as Carolina Biological, Sigma, Aldrich, Fischer, and VWR.

- Axenic *Tetrahymena* culture
- 2% proteose peptone medium (see "Recipes")
- Dilute salt solution (see "Recipes")
- Slides
- Cover slips
- Pipettes
- Pipette bulbs
- Protoslo (methylcellulose)
- India Ink (see Recipes")
- Lugol's solution
- Vaseline
- Toothpicks
- 1% glutaraldehyde
- carmine
- neutral red
- Janus green B
- Carbon particles
- Methyl green-pyronine
- Nigrosin
- Capillary tubes that measure 1-5 µl
- Wiretrol plunger
- Heparinized capillary pipettes
- Parafilm
- wax
- Erlenmeyer flasks
- Microfuge tubes
- Centrifuge tubes (conical, 15 ml with screw caps)
- Test tubes (at least 5 ml in volume)
- Automatic pipettors and tips
- Autoclave
- Compound microscopes
- Incubators
- Ice and/or refrigerator
- Milton Roy Spectrophotometer 20 ("Spec 20")
- Vortexer

Tetrahymena Exercises: Teacher's Instructions, General Methods, and Recipes

Microbial eukaryotes offer excellent opportunities for student instruction at many educational levels. Because these organisms can be cultured and manipulated easily, it is possible for students to do directed work or to pursue their own lines of inquiry. Moreover, microbial eukaryotes are an economical choice for laboratory instruction and a suitable alternative for many types of experiments done with animals.

As described above, *Tetrahymena* is a versatile ciliated protozoan that can be used to teach many important biology concepts. A good place to start is to incorporate some of the exercises described in these instructions into your "Introduction to the Microscope" lab. Having the chance to observe living, motile organisms, engaged in interesting behaviors, enhances student enthusiasm for microscope use. More important, the observations that students make can help them generate their own questions many of which can be pursued.

Culturing *Tetrahymena*

It is easy to grow *Tetrahymena* for your class. To maintain your stock culture, inoculate 25 ml of fresh medium once a week. Incubate the cultures at room temperature (20-22^{o} C). It is fine to incubate at lower temperatures, although cell population growth will slow. It is also safe to incubate at a higher temperature; in this case, the cell population growth rate will increase. Don't exceed 29^{o} C for stock culture incubation. Each team of 2-4 students can work from one culture. Students do not need to maintain aseptic technique when working with their cultures since you have the "clean" stock culture.

India Ink

The brand of India ink used for phagocytosis matters because some companies put detergent in their inks. Detergent lyses cells. I use Hunt-Speedball (no.3398). Other brands will work but you should test them before use in class. India ink in the bottle, from an art store, is considered "100%." To make dilute suspensions, use distilled water as the diluent. Possible substitutes for India ink are to make suspensions of carbon particles or carmine particles, and feed these to cells. (Note: if you want to see what happens to neutral red staining when cells are feeding, you must use India ink or a carbon particle suspension in order to see the red staining. Since carmine is red, it is not suitable for this exercise).

Fixation Methods

Lugol's solution is an excellent fixative but if you wish to see fixed cells without staining the cytoplasm, a drop of 1% glutaraldehyde or a drop of 95% ethanol added to a couple of drops of cells will do the job. You should be aware that the ethanol will eventually evaporate from a microscope slide, but not before you can get some very good observations. (Note: if you wanted to observe fixed cells that have eaten carmine, you will need to use glutaraldehyde or ethanol as your fixative because the staining from Lugol's will obscure observation of the red vacuoles).

Recipes

2% Proteose peptone

- 10 g proteose peptone
- 500 ml distilled water

Place 25 ml in each of 20 Erlenmeyer flasks. Plug the tops of the flasks with cotton or a foam plug. Cover the tops with aluminum foil. Autoclave 20 minutes on slow exhaust (liquid cycle).

After cooling, this medium should be refrigerated and used within two months. You can inoculate *Tetrahymena* directly into medium taken out of the refrigerator.

Neutral Red (1% stock)

- 100 mg neutral red
- 10 ml distilled water

Dilute to 0.1% or 0.05% for use.

Janus Green B (1 % stock)

- 100 mg Janus Green B
- 10 ml distilled water

Dilute to 0.01% for use.

Nigrosin

- 1 g Nigrosin
- 10 ml distilled water

Use as formulated.

Methyl Green-Pyronine

- 1 g methyl green –pyronine
- 100 ml distilled water

Use as formulated.

Physarum Exercises and Experiments

Introductory to Advanced Levels: Culturing, Observations, Growth and Development of *Physarum*

Students Instructions:

Introduction

While all organisms have unique characteristics that make them fascinating to study, the slime mold *Physarum polycephalum* exhibits an especially unusual feature during part of its life cycle – it exists as a crawling, slimy, gigantic, multinucleated "cell" that responds to the environment as it actively seeks food. This gigantic "cell" or plasmodium is only one part of the life cycle of *Physarum*, but it is a stage that provides great opportunity for you to study many fundamental biological problems in an experimental system simple enough for you to make observations, do experiments, and design and pursue your own research projects. In the instructions presented here, you will learn how to culture and handle *Physarum*, and do observations of cytoplasmic streaming, locomotion, and plasmodium fusion. You will also have the opportunity to take on two experimental questions: 1) What factors influence plasmodial growth, and 2) What factors influence whether the plasmodium forms the resistant structures, called sclerotia. Finally, I have suggested some possible directions for further inquiry.

Background

Physarum polycephalum and other slime molds have been popular organisms for research for many years for a variety of reasons. First, they seem unusual to us because they possess characteristics that appear "fungal" and "protozoan." In fact, in 1859, deBary suggested the name mycetozoa for these organisms to capture this observation (mycet= fungal, zoa= animal). With respect to "protozoan" features, the slime mold life cycle starts with haploid, independently feeding, soil amoebae. Under appropriate conditions, amoebae fuse and form an amoeboid zygote that undergoes mitosis without cell division. This event produces a giant, multinucleate, diploid plasmodium. Plasmodia are motile and crawl in search of food. As plasmodia eat, they continue to grow and can reach enormous sizes—more than 30 cm in diameter in lab cultures and more than a meter in diameter in nature! Actively avoiding the dark while feeding, plasmodia will move toward the light when starved. With respect to "fungal" characteristics, under appropriate conditions, the plasmodium stops moving and forms fruiting bodies, each of which possess thousands of resistant spores. When conditions are favorable again, the spores germinate, each releasing an amoeba, and the life cycle can start again. In addition to spores, slime molds can form two other types of resistant structure; amoebae can encyst and plasmodia produce sclerotia. Second, as evidenced by this description of the life cycle, slime molds display many of the same important eukaryotic features such as cell differentiation, motility, and mitosis, which are seen in more complex organisms such as vertebrates, yet they can be grown and handled like microorganisms.

Modern research using slime molds was initiated in the 1940's by Harold Rusch, a cancer researcher. Dr. Rusch wished to develop a simple "model" system to study the biochemistry of growth and differentiation. Rusch's motivation was interesting; he had developed an allergy to the mice and rats he had been using in his cancer research and therefore was unable to continue this specific work. He also realized that cancer tissue itself was quite complex and variable as an experimental material. After some searching, Rusch decided that *Physarum* was exactly what he

needed, and he established the methods and approaches that lay the foundation for research that continues actively to this day.

Studying how growth, development, and cell differentiation are controlled in *Physarum* continue to be important research questions. Because of the great size that plasmodia can attain, these organisms have been great sources of material for biochemical and molecular analyses of these and other questions. For example, biochemical investigations have been especially fruitful in the analysis of the cytoplasmic streaming and motility that are dramatically displayed by the plasmodia. Scientists have learned that proteins similar to the actin and myosin that powers our own muscle contraction are responsible for motion in the plasmodium too.

Another important area of research in *Physarum* concerns the cell cycle. The giant plasmodium possesses millions of nuclei and division (mitosis) of these nuclei within a plasmodium, no matter how big, is synchronous, occurring every 8-10 hours. Consequently, plasmodia have been particularly valuable for studies regarding how mitosis and the cell cycle are controlled and how internal "timers" work in cells. Because all cell cycle events are synchronized, scientists have also been able to study the regulation and control of DNA synthesis, RNA synthesis, and protein synthesis in *Physarum* plasmodia.

Physarum research has also revealed information about how tissue compatibility is regulated. Compatible Plasmodia will fuse to form a single plasmodium, whereas when incompatible plasmodia fuse, there is a lethal reaction and one or both plasmodia will die, reminiscent of what is observed if a transplanted organ or skin graft is rejected.

As you have seen, *Physarum* is an excellent organism for research in important biological problems. Similarly, it is also a terrific material for you to study, observe, and with which to do experimentation. Your strategy can be the same as the one used so successfully by research scientists. Define your research question and then study it in an organism that is not too complex but offers biological versatility in a "user-friendly" form.

Methods

How to Culture

Physarum plasmodia can be cultured using a variety of straightforward methods. The method to choose will depend upon the exercise or experiment wish to do.

Non-nutrient agar + Oatmeal flakes

1. Pour sterile non-nutrient agar into petri dishes. Approximately 20 ml of agar per plate is adequate. Let the agar harden.
2. Sprinkle 25-30 sterile oatmeal flakes on to the surface of the agar.
3. If you are subculturing from a growing plasmodium, use a sterile scalpel or spatula, and cut a block of agar on which a piece of plasmodium is present. The block should measure approximately 1 cm^2. Place the agar block, plasmodium side down, on the non-nutrient agar + oatmeal flakes.
4. If you are subculturing from a sclerotium, use sterile forceps to transfer a piece of filter paper containing this resting stage, to the non-nutrient agar + oatmeal flakes plate. Wet the filter paper with a drop of sterile water.

5. Once your cultures are set up, seal the edges of each petri dish with parafilm, plastic, or electrical tape, and wrap the dishes in aluminum foil to keep out the light. Incubate at room temperature (20-22° C).
6. Transfer cultures started from plasmodia every 3-4 days, if you incubate at room temperature. You can incubate at a lower temperature to slow growth or even refrigerate cultures for up to several weeks (the plasmodia will go into "suspended animation").
7. If you start cultures from sclerotia, you will need an extra day or two of incubation before growth commences.

Oatmeal Agar

1. Prepare oatmeal agar as described in the "Recipes."
2. Culture plasmodia pieces or sclerotia as described above. Culture incubation is also as described above.

Growth on oatmeal agar is slower than on non-nutrient agar+oatmeal flakes. Consequently, subculturing does not need to be done as often. This is a good medium to use if you want slower growth with room temperature incubation.

Semi-defined Medium

1. Prepare semi-defined medium as described in the "Recipes."
2. Because this medium is nutrient rich, it is important to use aseptic technique when culturing plasmodia.
3. Culture plasmodia pieces or sclerotia as described above. Culture incubation is also as described above.
4. Growth on semi-defined medium is vigorous and observations of plasmodia are easily made with this medium.
5. It is better not to subculture from semi-defined medium because the nutrient richness of this substrate increases the risk of microbial contamination. The optimal medium for subculturing is non-nutrient agar + oatmeal flakes.

Semi-defined Medium + Filter

Plasmodia can be grown on top of sterile filters that have been placed on hardened semi-defined medium. This is a great method for growing plasmodia, and then being able to move them, intact, to another medium or environmental condition.

1. Prepare semi-defined medium as described in the "Recipes."
2. Pour this medium into petri dishes, and after the agar has hardened, place a sterile filter paper on top of the medium.
3. Use a sterile spatula or forceps to gently flatten the filter onto the medium. Make sure there are no air bubbles under the filter or dry spots on the filter.
4. Using aseptic technique, cut out an agar block with a piece of plasmodium on it and transfer it, plasmodium side down, on to the filter.
5. Incubate as described above.

Starving Plasmodia

For some experiments, it will be helpful to have plasmodia that are starving rather than actually feeding. To do this, transfer an agar block with a piece of plasmodium on it, to a petri dish

containing non-nutrient agar. Seal the edge of the dish and cover it with aluminum foil. Incubate at room temperature for at least 15 hours but not more than 48 hours before use. If you wish to starve plasmodia for a longer period, incubate in the light during starvation.

Observations and Investigations

Cytoplasmic (Shuttle) Steaming and Motility

1. Without removing the cover of the petri dish, examine the stock plate of *Physarum* provided to you. The plasmodium is the yellow, glistening material on the dish.
2. Again, without opening the dish, smell the sealed edge of the culture. Does this smell remind you of anything? If so, what?
3. Use a dissecting scope to examine the flow of cytoplasm in the plasmodium. Cytoplasmic streaming is present in many types of cells but it is particularly dramatic in *Physarum* plasmodia. Visualization of this process will be most clear if you use transmitted light (shine the light through the bottom of the culture).
4. What can you observe about this streaming? Is the flow directional? Is the rate constant? Does the rate vary depending on the size of the vein? Why or why not? Is the direction of cytoplasmic flow the same all over the plasmodium? Does the direction of flow change within individual veins? Is the plasmodium changing position in response to the direction of cytoplasmic flow? (This will take a while to tell since a "fast" plasmodium moves about 1 cm/hr. You can use a permanent marker to mark the location(s) of the plasmodium at various times on the bottom of the petri dish).
5. What is the probable function of the cytoplasmic streaming? What is the function of the plasmodium's motility?
6. Repeat your observations using a starved plasmodium. Reconsider all of the above questions for this starved plasmodium.

Comparison of Feeding and Starving Plasmodia

1. What were the similarities and differences between feeding and starving plasmodia with respect to cytoplasmic streaming?
2. What were the similarities and differences between feeding and starving plasmodia with respect to motility?
3. What were the similarities and differences between feeding and starving plasmodia with respect to the relationship between the direction of streaming and that of motility?
4. What are the similarities and differences with respect to the overall morphology of feeding and starving plasmodia?
5. Explain your observations and comparisons.

Fusing Plasmodial Pieces

1. Cut two blocks of agar containing plasmodia and place them on semi-defined medium, about 3-4 cm apart from each other.
2. Within 24 hours, the two plasmodia will have grown toward each other in fan-like shapes. When the plasmodia are a few mm apart, they should be observed regularly.
3. Note the details of cytoplasmic streaming and the movement of particles from one plasmodium to the other.

4. Do the soon-to-be fused plasmodia have similar cytoplasmic flow? Does the direction of flow remain the same? Does it reverse? Are the two plasmodia synchronized?
5. What happens to the structure of the network of veins where the plasmodia meet?
6. How many hours does it take to no longer be able to distinguish the plasmodia as separate?
7. Does the same type of fusion occur if you place pieces of plasmodia on non-nutrient agar rather than semi-defined medium?

Measuring Growth

1. Subculture a piece of plasmodium on each of several dishes of semi-defined medium.
2. Turn each petri dish over gently and use a permanent marker to trace, on the bottom of the petri dish, the agar block/plasmodium you placed there.
3. Incubate one set of cultures in the light and one in the dark.
4. Observe the growth of plasmodia at 24, 48, and 72 hours. For each dish at each time point, use a permanent marker to trace, on the bottom of the dish, the area covered by the plasmodium.
5. Measure the areas of growth for each day and determine the average rates of growth in the light and in the dark.
6. Prepare a table and graph to display your data. Also record any observations regarding the appearance of the plasmodia under these two incubation conditions. Explain your results fully and pose a testable follow-up experimental question.

Sclerotium Formation

Sclerotia are resting structures formed by starving plasmodia subjected to certain environmental stress. When plasmodia form sclerotia, veins empty and the cytoplasm all gathers into one location producing a yellow "blob." The method below describes a straightforward way to produce sclerotia.

1. Transfer agar blocks with pieces of feeding plasmodia onto 10 medium sized dishes of non-nutrient agar.
2. Incubate 5 of these cultures in the light and 5 in the dark. Wrap each dark dish separately.
3. After 24 hours, observe one culture incubated in the light and one incubated in the dark. Describe the morphology of the plasmodia. Is cytoplasmic streaming evident? Is there evidence of plasmodial motility (you will see slime tracks on the agar)? Are there any differences for any of these observations made for the light and dark incubated plasmodia?
4. Repeat step 3 for 48, 72, 96, and 120 hour cultures.
5. What changes do you see in the streaming behavior, motility, and the morphology of the plasmodia form the light and dark cultures? Explain your observations.
6. Pose a testable experimental question to explore what factors influence whether a plasmodium will form a sclerotium.

Suggestions and Ideas for Further Investigation

- Determine whether exposure of metabolic inhibitors affect cytoplasmic streaming or motility of plasmodia
- Determine whether cytoskeletal inhibitors affect cytoplasmic streaming or motility of plasmodia
- Determine whether temperature affects cytoplasmic streaming or motility
- Determine whether introduction of food (oatmeal flakes) will affect cytoplasmic streaming or motility

- Test the effects of light intensity, the wavelength of light, or time length of exposure to light, on the growth of plasmodia
- Test the effect of temperature on the growth of plasmodia
- Test whether plasmodia will grow under submerged conditions (oxygen will be limited)
- Determine whether plasmodia of different ages can fuse
- Test whether any of the following stressors will cause starving plasmodia to form sclerotia: incubation at low temperature, incubation at high temperature, drying, low or high pH, exposure to heavy metals or other pollutants, or exposure to high osmotic pressure (0.5 M sucrose)
- Determine how long starving plasmodia need to be incubated in the dark to trigger sclerotium formation. Test whether plasmodia starved in the light for various times need less incubation time in the dark to form sclerotia than do well fed, newly starved plasmodia

Introductory to Advanced Level Experiments: *Physarum* Chemotaxis

Student Instructions:

Introduction and Background

Both prokaryotic and eukaryotic cells need to be able to receive and process information from their environments, including other cells. Depending upon what cells learn from this information gathering, they will behave in some specific, appropriate manner. For example, many motile microorganisms optimize their locations with respect to light quality, humidity, temperature, or oxygen availability. As well, many microorganisms and certain specialized cells in complex multicellular organisms, respond to chemical cues and either move toward or away from thee signals. This ability to measure and move in response to gradients of chemical signals is called chemotaxis.

Bacteria and eukaryotic microbes such as protozoa and amoebae generally use their chemosensory behaviors to locate food and to avoid noxious substances. In more complex, multicellular organisms (such as us), this behavior is a part of our immune system arsenal. White blood cells recognize the chemical signals that emanate from sites of infection or inflammation and crawl there in response.

There are two principal mechanisms whereby cells display attraction to or repulsion from a chemical. Chemokinesis involves a temporal response. Cells "read" the presence of a chemical and then decrease their turning or avoidance in the case of a chemoattractant, or increase their turning, or avoidance, in the case of a chemorepellents. In some cases, swimming speeds may also be altered. The consequence of these behaviors is that cells concentrate around an attractant and disperse in response to a repellant. Bacteria and ciliated protozoa exhibit chemokinesis. Among eukaryotes, *Tetrahymena* and *Paramecium* chemokinesis have both been widely studied by researchers.

In contrast to chemokinesis, chemotaxis is a spatial response. Cells measure the relative concentrations of a chemical signal on different parts of the cell surface. Cells monitor the concentration gradients such that they move towards higher concentrations of attractants and towards lower concentrations of repellent. Chemotaxis is the specific mechanism used by white blood cells when they crawl towards sites of infection in the body.

Physarum polycephalum, a plasmodial slime mold, is an excellent organism for exploring chemotaxis. One of the amazing aspects of the biology of *Physarum* is that it exists, for part of its life cycle, as a plasmodium, which is a giant, multinucleated "supercell." The crawling plasmodium is very sensitive to its environment; somehow it is able to collect and integrate information and

respond to it by migrating as a coordinated whole "body." Plasmodia move towards food and other attractants and away from repellents. They also migrate in response to light quality, temperature, and humidity. Because plasmodia behave very much like giant amoebae, they are terrific material for you to use to study the phenomenon of chemotaxis. Your objective will be to propose and carry out an experiment that attempts to answer a question about *Physarum* chemotaxis. Three methods for measuring chemotaxis are detailed below. Choose the one most appropriate for your experiment. Also listed below are some suggestions for investigations. You may use one of these to start you off or propose a question of your own,

Methods: General Techniques

Agar Blocks and Solid Food Method

1. A plasmodium culture, several non-nutrient agar plates, test substances, and a scalpel or spatula will be provided for you.
2. Cut blocks of non-nutrient agar from one of the plates. These blocks should be approximately 1 $cm^{2.}$
3. Deposit one non-nutrient agar block onto the surface of a dish of non-nutrient agar. The block should be positioned approximately 1 cm from the edge of the dish. Prepare several dishes this way as called for by your experimental design.
4. If your test substance is dissolved in agar, place a 1-cm^2 block of this medium on the opposite side of these dishes, also approximately 1 cm from the edge. If your test substance is not in agar, sprinkle or place a small amount of food on the side of opposite of the plate approximately 1 cm from the edge.
5. Cut the plasmodium culture into 1-cm^2 blocks. Transfer a piece of plasmodium to each of your experimental plates. When transferring *Physarum*, try to select a thick vein or solid piece of "tissue" located near the edge of the plasmodium. Place the agar block, plasmodium side down, in the center of the dish. Do not transfer pieces of oatmeal from the stock culture.
6. Draw a line down the middle of the petri dish so that the plasmodium is in the middle, the block of non-nutrient agar is on one side, and the test material is on the other side.
7. Wrap the dishes in aluminum foil and incubate right side up at room temperature.
8. Observe plasmodium migration at 20-24 hr and record its position. A plasmodium located anywhere besides the center can be scored as '+' for that half of the dish.
9. If you run enough samples, or if the class runs the same experiment and data are pooled, your instructor will explain how to analyze the results using the chi-square test.

Paper Strip Method

1. Cut filter paper into strips approximately 8 cm by 1.5-2 cm. Soak some filter paper strips in a beaker containing distilled water and some in a beaker containing a solution of your test substance. Allow the filter paper strip to soak for at least 15 min.
2. Using forceps, take a filter paper strip from the distilled water beaker, allow excess fluid to drip, and place the wet strip on the bottom of a sterile petri dish. Similarly, remove a filter paper strip from the test substance beaker; allow excess fluid to drip, and place the strip right next to the distilled water strip, on the bottom of the petri dish. The two strips should abut against one another.

3. Use a scalpel or spatula to cut a plasmodium culture into blocks sized 0.5-1 cm^2. Transfer 3 or 4 such blocks, plasmodium side down, onto the junction of the two filter paper strips. When you select the agar block with *Physarum* to transfer, be sure that you get a thick vein or sheet of "tissue" located toward the edge of the plasmodium. Do not transfer pieces of oatmeal from the stock culture.
4. Wrap the dishes in aluminum foil and incubate right side up at room temperature.
5. Observe plasmodium movement every hour for several hours, and again the next day. The yellow plasmodia will be easily seen if they migrate onto the white filter paper.

Agar Drop or Chip Method

1. Take a petri dish of non-nutrient agar, turn it over and use a permanent marker to place a dot on the center of the bottom of the plate. Place dots, 2 cm away, on both sides of this central dot. Place dots 2 cm away on each side of the two dots you just made. When you are done, you should have two rows of three dots parallel to each other. Between these two rows should be the one center dot.
2. Open the petri dishes containing non-nutrient agar and pipette 0.1 ml of non-nutrient agar on to the surface right over the positions of each of the three dots in a row. Pipette 0.1ml of agar, containing your test solution, onto the surface right over the positions of the three dots in the other row. Alternatively, place a small chip of non-nutrient agar over each of the dots in one row and a small chip of agar containing your test substance on each of the dots in the other row.
3. After the agar drops have solidified or the agar chips are properly positioned, cut a plasmodium culture into 0.5-cm^2 blocks. Transfer the agar block, plasmodium side down, onto the center dot. Cover the plate with aluminum foil and incubate, right side up, at room temperature.
4. Set up the appropriate number of cultures for your experiment.
5. Observe cultures after 2, 4, and 6 hr, and again at 20-24 hr to determine whether plasmodia were attracted to or repelled by your test substance.

Suggestions and Ideas for Experiments

- Test various foods and spices for their abilities to attract or repel plasmodia
- Determine whether the rate of plasmodium movement changes during a chemotactic response
- Test whether starving plasmodia demonstrate an enhanced chemotaxis toward food than do well-fed plasmodia
- Test whether plasmodia are attracted to the organisms on which they feed such as bacteria or yeast
- Test the effectiveness of various sugars, vitamins, and amino acids, at different concentrations, alone and in combination
- Test a mixture of repellent and attractant
- Explore the relative response of plasmodia to light and chemoattractants
- Test metabolizable sugars and non-metabolizable analogues of the same sugars to see if molecules need to be nutritionally useful to be chemoattractants
- Determine at what distances plasmodia are capable of detecting and responding to chemical signals

Materials (for *Physarum* labs)

- Scalpels or spatulas
- Oatmeal flakes
- Sclerotia or plasmodium
- Petri dishes
- Non-nutrient agar
- Semi-defined medium
- Autoclave or pressure cooker
- Dissecting microscope
- Aluminum foil
- Filter paper
- Parafilm or plastic wrap or electrical tape

Teacher's instructions, General Methods, and Recipes

Physarum polycephalum is amenable for instruction of students from introductory to advanced levels. Because plasmodia are easy to culture and handle, students can focus attention on posing questions and designing studies and experiments to answer these questions. *Physarum* plasmodia offer the opportunity for students to connect their own work to the very active investigations being done all over the world.

Culturing

Although students can culture their own plasmodia, you may instead choose to prepare plasmodia for your class. If so, each student should have a culture to work from, to set up experiments and do observations. The best method to use is for you to subculture yellow, glistening, fresh plasmodia pieces on non-nutrient agar + oatmeal flakes four days before use. Incubate at room temperature (20-22°C).

As mentioned above, it is a good idea to subculture your stocks every 3-4 days when your class is actively studying *Physarum*. You can store plasmodia at a lower temperature in the refrigerator for weeks and then subculture from these plates if you wish. For long-term storage, it is best to make sclerotia.

How to Make Sclerotia

To make sclerotia for long-term storage (up to a year), culture a plasmodium on non-nutrient agar + oatmeal flakes. After 3-4 days, transfer pieces of the actively growing plasmodium onto sterile filter papers placed on top of nutrient agar. Incubate the plasmodia in the dark. After 24 hrs, remove the filter paper with the migrating plasmodia, and place it into a sterile petri dish. Incubate in the dark at room temperature. Once the filter has dried, you will see that the plasmodium has formed dry, crusty sclerotia. Cut the filter paper, with attached sclerotia, into approximately 1-cm squares and store them in a dark, dry place. Alternatively, transfer pieces of actively growing plasmodia to non-nutrient agar. Incubate in the dark. After 72-96 hours, the plasmodium will retract its "veins," cease cytoplasmic streaming, and gather into a yellow "blob." Cut out agar pieces bearing these "blobs" and transfer them to empty sterile petri dishes. Incubate in the dark. These "blobs" will dry out and form sclerotia. They should be stored in a dark, dry place.

How to Clean Contaminated Cultures

Physarum cultures maintained on non-nutrient agar + oatmeal flakes don't usually exhibit growth of microbial contaminants. Because of its nutrient richness, microbial contamination can be

more of a problem on semi-defined medium. If any of your cultures do become contaminated, try to subculture a piece of plasmodium from a "clean" area onto a new dish. Alternatively, take a piece of plasmodium and place it in a petri dish containing sterile non-nutrient agar. Position the plasmodium at the edge of the dish. Cover the dish with aluminum foil and poke a small hole in the foil on the side of the dish right next to the position of the plasmodium. The plasmodium will migrate away from the light but its contaminants won't. Usually, this will clear contamination after one journey away from the light, but if not, transplant the plasmodium to another dish of non-nutrient agar and let it migrate again. After this second round of migration, transfer the plasmodium to the growth medium of your choice.

Information about Cytoplasmic Streaming and Motility

If students are patient in their observations of cytoplasmic steaming and motility, they will be able to report a great deal. To help you guide them, here is some interesting information about these behaviors:

- Cytoplasmic streaming, which is also called shuttle streaming, depends upon the proteins actin and myosin. Although not identical to the actin and myosin of skeletal muscle, they operate similarly to the muscle proteins to produce contraction.
- Cytoplasmic streaming, in a given vein, goes in one direction, stops, and reverses flow. These reverses of flow occur at regular intervals, every 1-3 minutes. These reversals of cytoplasmic flow don't necessarily occur in adjacent channels at the same time.
- The advancing edge of the migrating plasmodium is composed of a continuous sheet of cytoplasm interspersed with small veins. Behind this leading edge, there is a network of larger veins. There are usually spaces between these veins.
- Motile plasmodia do not have a permanent morphology. The pattern of veins in a given area changes.
- Starving plasmodia will migrate for days, especially if incubated in the light. They leave a slime track when they migrate. Consequently, the path(s) taken by plasmodia can be easily observed.
- A close look at the veins reveals ectoplasm, the outer, clearer, more viscous cytoplasm, and endoplasm, the inner, flowing, grainy-looking cytoplasm. Within the endoplasm are nuclei, mitochondria, ribosomes, vacuoles, and pigment granules.
- The mechanism of plasmodial motility has similarities to amoeboid motion in that both involve endoplasm to ectoplasm interconversions, and ectoplasmic contractions.
- The rate of cytoplasmic flow can be as fast as 1 mm/sec.
- Plasmodia can migrate at a speed of up to 1 cm/hr.
- Cytoplasmic streaming mixes the contents of the entire cytoplasm.
- Cytoplasmic streaming and plasmodium migration pause for mitosis.

Recipes

Non-nutrient Agar

- 20 g agar
- 1 liter distilled water

Plug the top of the flask with cotton or a foam plug and cover the top with aluminum foil. Autoclave 20 min. on slow exhaust (liquid cycle).

Sterile Oatmeal Flakes

Place oatmeal flakes (not instant) into a beaker. Cover the top with aluminum foil and autoclave 20 min. on slow or fast exhaust.

Non-nutrient Agar + Oatmeal Flakes

Sprinkle 25 to 30 sterile oatmeal flakes onto petri dishes containing non-nutrient agar.

Oatmeal Agar

- 15 g agar
- 30 g oatmeal flakes
- 1 liter distilled water

Plug the top of the flask with cotton or a foam plug and cover the top with aluminum foil. Autoclave 20 min. on slow exhaust (liquid cycle).

Semi-defined Medium

- 10 g glucose
- 10 g peptone
- 1.5 g yeast extract
- 3.5 g citric acid
- 2 g KH_2PO_4
- 0.9 g $CaCl_2$-$6H_2O$
- 0.6 g $MgSO_4$-$7H_2O$
- 0.034 g $ZnSO_4$-$7H_2O$
- 0.06 g $FeCl_2$-$4H_2O$
- 15 g agar
- 1 liter distilled water

Plug the top of the flask with cotton or a foam plug. Cover the top with aluminum foil. Autoclave 20 min. on slow exhaust (liquid cycle). After autoclaving add 10 ml of sterile hemin stock solution per each liter of medium. To make this stock solution, dissolve 0.5 mg hemin per ml of 1% NaOH. Autoclave the stock solution 20 min. on slow exhaust.

Store the hemin stock solution and the semi-defined medium (with or without the hemin added) in the refrigerator.

Sterile Filters

Wrap filter papers, cut to fit petri dishes, in aluminum foil. Autoclave 20 min. on slow or fast exhaust.

Appendix: References

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