Chapter 15

Revisiting "A Practical Guide to the Use of Cellular Slime Molds for Laboratory Exercises and Experiments"

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Introduction

One of the challenges faced by laboratory instructors is to find organisms suitable for teaching a wide array of concepts, and amenable to open-ended exploration and experimentation by students. *Dictyostelium* and other cellular slime molds are ideal for the instruction of students ranging from the introductory level to advanced.

Easily cultured and maintained in the laboratory, this microbial eukaryote undergoes many of the same fundamental biological, biochemical, and molecular phenomena during its life cycle that other complex organisms do during their development. Consequently, cellular slime molds have been used to study a wide range of biological problems including gene expression, cell motility, cell signaling and communication, cell recognition and adhesion, differentiation, and morphogenesis.

Dictyostelium has an unusual life cycle. Under conditions where ample food is available, amoebae exist as independently feeding cells. Upon depletion of food, these amoebae initiate the social phase of the life cycle. Two developmental pathways are available to cells. During asexual development, starving amoebae aggregate and form multicellular fruiting bodies. Under appropriate conditions, sexual development may occur. Here, starving amoebae of opposite mating type fuse and then attract hundreds of other amoebae to produce multicellular structures called macrocysts.

Students can explore many aspects of *Dictyostelium* biology including the life cycle itself, how cells communicate to form multicellular structures, how cells choose between developmental pathways, the behavior and biology of the multicellular stages, how different species of cellular slime mold interact, and many other questions. In addition, because of the relatively short life cycle (starved amoebae will produce fruiting bodies within 24 hours under

appropriate conditions), students can repeat experiments or pursue new lines of inquiry and follow up experiments all within a reasonable time frame. Students undertaking research projects can make significant progress in even only one semester of work.

The goals of this chapter are to provide instructors with the information necessary for the "care and feeding" of cellular slime molds, and to present several examples of laboratory exercises using this organism. I also suggest possible avenues for additional student designed investigations.

Equipment and Materials Required

For routine handling and maintenance of cellular slime mold cultures, and for many experiments, the equipment and materials needed are fairly modest. Listed below are basic needs. I will detail any additional specific requirements for individual experiments when they are presented later in this paper.

Materials: Basic

- sterile petri dishes (various sizes depending on the experiment)
- sterile pipettes (1-, 5-, 10-ml)
- autoclave or pressure cooker for sterilization
- reagents for media and solutions (see recipes)
- bacteriological transfer loop
- source of a hot flame (Bunsen burner or other gas jet or an alcohol lamp)
- ice
- table top clinical centrifuge (preferably with a swinging bucket rotor)
- sterile test tubes with screw caps (15-ml or 50-ml)

Materials: Helpful but not essential

- incubator with lights (incubation of cultures in a lighted room works)
- transfer hood (bench top transfers done carefully will work too)
- automatic pipettors and sterile tips (to measure small volumes used in some experiments; however, sterile capillary tubes with a tiny plunger work fine)
- haemocytometer (to measure cell density)
- vortexer (to resuspend cell suspensions; however, pipetting up and down carefully will also work)

Media and other Solutions: Basic

- Lactose-peptone agar (LP)
- Sussman's Medium (SM/2)
- Non-nutrient agar (NNA)
- Bonner's Salt Solution (BSS)

Recipes for each of the above are provided in Appendix B.

General Methods: Cell Culture

Since *D. discoideum* is a microorganism, it can be cultured and maintained using relatively standard methods. This is particularly helpful to the instructor because preparation of live material is fairly straightforward and stock cultures can be stored over long periods of time. This is also helpful to the student because failed experiments can be repeated without too much extra work and more importantly, new experimental questions can be pursued without delay. The methods described in this section detail how to: (a) culture and maintain stock cultures, (b) grow and harvest cells for experimental work, (c) prepare "aggregation competent" cells, (d) culture cells for development, and (e) clean contaminated cultures.

Stock Cultures

For establishment and subculture of stock cultures, it is essential that aseptic technique be used throughout these procedures. Stock cultures of *D. discoideum* and *Escherichia coli* are maintained on lactose-peptone agar (LP). *E coli* is a bacterium that is used as a source of food for the cellular slime mold amoebae. One to three days before stocks are to be transferred, a broth culture of *E. coli* is prepared; approximately 50 ml of Sussman's Medium (SM/2) broth is inoculated with a loopful of *E. coli* (maintained as a stock on LP). Incubation is at 20 to 24° C.

To prepare stock plates of *D. discoideum*, two successive inoculations are done. First, 0.5 ml *E. coli* of suspension is pipetted onto LP agar. Second, a loopful of *D. discoideum* spores is added to the plate. In order to disperse the spores and bacteria, the suspension is spread over the agar using a glass elbow (a glass rod bent at a 90° angle). Stacks of plates should be taped to retard drying. Incubation is at 20 to 24°C in the light. Stock cultures can be stored at 20 to 24°C for up to 1 month (although it is best to subculture every 2 weeks), in the refrigerator for 4 to 6 months, or desiccated on silica gel for years; lyophilized samples are essentially immortal (Raper, 1984).

Growing and Harvesting Cells for Experiments

To prepare cells for use in experiments, one uses the same culturing technique employed for the preparation of stock cultures. Incubation is at 20 to 24°C for 34 to 38 hours, in the light for experiments involving fruiting body development, and 20 to 22 hours in the dark for those concerned with macrocyst production. Culturing and harvesting 22 hours later is obviously not a problem but a 34- to 38-hour incubation can be quite a challenge. Happily, one can put the cultures into the refrigerator for hours so that cell harvest can be done at a more convenient time. For example, I will inoculate cultures at 4 p.m. on Day 1. On Day 2, I will incubate these cultures in the refrigerator for 8 hours; as far as the cells are concerned, this time does not count. Incubation at 20 to 24°C is resumed and cells can be harvested at 12 p.m. on Day 3. The number of cultures prepared depends on the requirement of a particular experiment. For planning, you can estimate that every petri dish (diameter = 8.5 cm) will yield 1 x 10^7 to 5 x 10^7 cells depending upon the length and temperature of incubation.

To harvest cells, pipette or pour approximately 10 ml of cold Bonner's Salt Solution (BSS) onto a culture. Gently dislodge the cells, using the glass elbow, and pour the resulting cell suspension into the next culture. Dislodge the cells in that culture with the glass elbow, pour the suspension into the next plate; repeat until all plates have been scraped. The cell suspension is poured into a sterile centrifuge tube and placed on ice. Next, wash the culture plates that have

just been harvested by pipetting approximately 5 ml of BSS onto one of the cultures. Again, gently dislodge any cells that might remain and pour the suspension into the next dish. Repeat until all plates have been washed and combine this suspension with the one collected above. It is important to keep this cell suspension on ice as much as possible during these procedures. The number of tubes needed will depend upon the number of plates to be harvested. Plates are best harvested in groups of 10 to 15, each group producing 15 ml of crude cell suspension.

The cell suspension collected still has too many bacteria in it for use in an experiment. In order to separate the amoebae and bacteria, the cell suspension is subjected to differential centrifugation. Using a clinical, table-top centrifuge (or whatever you like), spin the cell suspensions at 1500 to 2000 rpm for 4 minutes. Discard the supernatant and resuspend the cell pellet in cold BSS. Vortex gently. Spin again. Discard supernatant, resuspend the pellet; repeat this 3 to 5 times until the supernatant is fairly clear. Resuspend cells in cold BSS, determine cell density (using a haemocytometer or Coulter Counter) and adjust the cell density to whatever you require. At the end of the cell harvest, you will have a suspension of clean, vegetative amoebae that when cultured on a solid substrate, will initiate development and complete the life cycle in 24 to 36 hours.

A summary of the procedure for cell harvest and quantitation of cell density is as follows:

1. Inoculate bacteria and spores.
2. Incubate 34 to 38 hours.
3. To harvest:
   (a) Wash cells off plates.
   (b) Centrifuge, discard supernatant, resuspend cells, ..., until clean.
4. To count:
   (a) Dilute a small aliquot.
   (b) Count using a haemocytometer.
   (c) Resuspend cells to appropriate density.

Aggregation-Competent Cells

Newly starved amoebae must go through some very important molecular and biochemical changes before they are able to commence aggregation and hence, morphogenesis. This interval is called interphase (Wier, 1977). For some experiments, you may not want to wait for cells to complete interphase, you might prefer to start your experiment with cells that are closer to beginning morphogenesis; these are called "aggregation-competent" cells. The usefulness of aggregation-competent cells is especially great in any experiment studying chemotaxis, morphogenesis, or any particular multicellular stage of the life cycle.

To prepare aggregation-competent cells, *D. discoideum* spores are grown and harvested using the methods described above. The resulting cell suspension is adjusted to a cell density of 2×10^8 cells/ml and 1 ml is pipetted onto a petri dish containing non-nutrient agar (NNA). The cells are spread over the plate using a glass elbow. The cultures are incubated at 20 to 24°C in the light for 2 to 4 hours. They are placed in the refrigerator overnight (15 to 18 hours) and restored to room temperature approximately 2 to 3 hours before the cells are to be used. Such cells will begin aggregation in 1 to 4 hours.

The procedure to prepare aggregation competent cells is as follows:

1. Grow and harvest cells.
2. Adjust cell density.
3. Place cells on non-nutrient agar.
4. Incubate 2 to 4 hours at room temperature.
5. Refrigerate overnight (15 to 18 hours).
6. Return to room temperature.

Development Cultures

There are two principle methods used to develop amoebae in culture. Both permit clear observation and study of morphogenesis as well as investigation of the effects of various chemical parameters on development.

With the first method, vegetative or aggregation-competent cells are deposited onto petri dishes containing NNA. An optimal number of cells to deposit on a standard size petri dish would be 1×10^8. To test the effects of a specific chemical, dissolve it in the agar before the plates harden (see the instructions for Chemotaxis Experiment). To see whether the effects of a particular chemical are reversible, harvest the cells from the dish and replate them on fresh NNA.

The second method uses a different solid substrate. Black nuclepore filters are placed on top of two supporting filter pads and all are placed in a small sterile petri dish. The pads are wetted with approximately 0.5 ml of BSS. Gently pipette 250 µl of a suspension of vegetative or aggregation competent cells (2×10^8/ml) onto the nuclepore filter; use the pipet tip to spread the cells to cover the filter. After the cells have soaked into the filter, add more BSS to the petri dish so that the filter pads are thoroughly soaked and the bottom of the dish is covered with liquid. To test the effects of a particular chemical, substitute it for the BSS. To examine the timing of sensitivity to a chemical or the reversibility of the effect, you can transfer the nuclepore filter to a new dish containing filter pads soaked with BSS.

Interesting chemicals to test include transcriptional inhibitors, translation inhibitors, metabolic poisons, drugs that affect the cytoskeleton, and certain ions.

Clearing Contaminated Cultures

It is a good idea to maintain several stock plates of each strain or species of cellular slime mold that you carry. Even so, sometimes a culture can become contaminated. When this happens, the first thing to attempt is a subculture from a non-contaminated region of the plate. If this fails, there is one last trick that you can try before you reorder stocks. This method takes advantage of the fact that the slug stage of the life cycle is phototactic.

Inoculate one edge of a LP plate with approximately 50 µl of *E. coli* suspension. Deposit a small loop of spores from your contaminated culture right into the *E. coli* on the plate; do not spread. Tape the culture shut and very carefully cover the plate with aluminum foil. On the side of the dish, directly opposite the position where you inoculated the plate (180° from the inocula) make a small hole in aluminum foil. The hole should be about the size of the head of a pin and it must be on the side and not on top. When the spores you inoculated germinate and the amoebae deplete the *E. coli* available, the life cycle will be initiated. The slug stage will migrate in response to the light (your contaminant will not). Consequently, the slugs will migrate to the pinhole, and you will have uncontaminated fruiting bodies. This works best if you give the culture one week to clean itself.

Exercises and Experiments

Described on the following pages are protocols for eight experiments, each of which illustrates an important biological phenomenon. For each experiment, information relevant to the instructor is presented and the student instructions are delineated. In addition, several possible variations or extensions of each of these experiments are provided along with the instructional level intended for each experiment.

Life-Cycle Plates

The life cycle of *D. discoideum* consists of two possible developmental pathways, each with a very different outcome: asexual development results in the formation of a fruiting body and sexual development produces macrocysts. Development starts when independently feeding amoebae run out of food. As long as nutrients are available, amoebae will continue to eat and divide. When food is depleted, cell division ceases and development begins (reviewed in Bonner, 1982).

During asexual development, starving amoebae aggregate to central collection points thus producing a multicellular structure. This aggregate undergoes a series of shape changes resulting in the formation of a fruiting body, a ball of spores on top of a cellular stalk. If food becomes available, these spores germinate to release amoebae thereby beginning the cycle again (Bonner, 1982).

During sexual development, starving amoebae of opposite mating type fuse to form a giant cell. These giant cells attract other amoebae and eat them. Protective walls are laid down around the giant cell, thus producing a macrocyst. Eventually, the giant cell, within the macrocyst, undergoes meiosis and then several rounds of mitosis so that upon germination of the macrocyst, a new population of haploid amoebae is released (Erdos *et al*., 1973; Filosa and Dengler, 1972).

When people first learn about cellular slime molds, they find the asexual life cycle a particularly intriguing aspect of the biology of this organism. This was true for Albert Einstein (Bonner, 1993) and it will undoubtedly be the case for your students too. For introductory labs in which one might want to demonstrate this life cycle and for advanced labs where students may need to gain skill in the recognition of various morphological stages, it is very helpful to prepare "life cycle" plates.

Two procedures for preparing life-cycle plates are as follows:

Procedure 1

1. Inoculate plate with bacteria.
2. Spread bacteria with glass elbow.
3. Add a loop of spores to center of plate.
4. Tape shut.
5. Incubate.

Procedure 2

1. Inoculate plate with one streak of bacteria across the diameter of the dish.
2. Add a loop of spores to the end of the streak.
3. Tape shut.
4. Incubate.

Procedure 1

Approximately 3 to 5 days before the plates are needed, inoculate LP with 0.5 ml of *E. coli* cell suspension prepared as described above. Using a glass elbow spread the bacterial suspension on the agar. Next, deposit a loopful of spores into the center of the plate; do not spread them around. Tape the plate shut and incubate at 20 to 24°C in the light. The spores deposited in the center of the plate will germinate, and the resulting amoebae will feed on the bacteria. As food becomes scarce, amoebae will migrate out as a ring towards the greater quantity of bacteria present on the rest of the plate. Any amoebae left behind will starve and thus initiate their development. Successive rings of feeding amoebae will continue to migrate out towards the edge of the dish and other groups of amoebae will be left behind to develop. The result is that the amoebae that have developed for the longest amount of time will be in the center of the dish and the vegetative, feeding amoebae will be at the periphery. In between, stages of the entire life cycle will be present. The life cycle plates work best if the feeding amoebae have moved to a position approximately 2 cm from the edge of the petri dish. If the cultures seem to be developing too fast for your needs, simply put the plates in the refrigerator and they will go into "suspended animation." You can retrieve them before your scheduled lab. For the preparation of life cycle plates, it is a good idea to maintain aseptic technique.

Procedure 2

This method is very similar to the one above with respect to timing and incubation instructions. The principal difference is that an *E. coli* suspension is deposited as a single streak either with a bacteriological loop or by slowly releasing the suspension from a sterile 1ml pipette. Spores are deposited on the end of the bacterial streak. Amoebae will feed on the bacteria and a feeding front of amoebae will advance down the streak. Behind these feeding amoebae will be starving cells that are undergoing development. Ultimately, fruiting bodies will be present at one end of the streak and feeding amoebae will be at the other. All other stages of the life cycle will be in between, in order.

Choice Between Developmental Pathways: Macrocysts vs. Fruiting Bodies

(For a more complete presentation of this experiment, see Bozzone, D. M. 1994. J. College Science Teaching 23: 363-366.)

Instructor's Information

One of the most important, unanswered questions in biology is how do embryonic cells choose particular developmental pathways. One aspect of this question is to ask: What are the cues or signals that influence the choice between, for example, becoming liver vs. brain, flower vs. root, or spore vs. amoeba? This experiment addresses the question of how do cellular slime mold amoebae decide whether to mate and produce a macrocyst or develop asexually and construct a fruiting body. While not very much is known about how *D. discoideum* chooses mating vs. fruiting, experiments have shown that certain physical conditions are important. For instance, light, lack of moisture, presence of phosphate ions, absence of a compatible mate and warmer temperatures favor fruiting body formation while the converse enhances macrocyst production (reviewed in Raper, 1984).

The protocol outlined below provides instructions for preparing cultures and suggests some ideas for experiments. Students can set up their experiments using stock plates of *D. discoideum*

NC4 and V12 as sources of spores or you may provide them with spore suspensions.

To prepare spore suspensions, harvest the fruiting bodies from stock plates, within 1 or 2 hours before the beginning of your lab. For a class of 20, with students working in five teams of four, you will need three stock plates of *D. discoideum* NC4 and three of V12. To harvest the spores, pipette approximately 15 ml of BSS onto a plate of spores. Gently dislodge the fruiting bodies with the glass elbow and pour the suspension into the third plate of the same strain. After dislodging the fruiting bodies from this third plate, pour the spore suspension into a sterile beaker or flask. Pipette another 15 ml of BSS onto the first harvested plated, gently swirl with the glass elbow, and pour into the second plate and into the third. This wash should be combined with the spore suspension. Finally, bring the spore suspension volume up to approximately 50 ml with BSS. Repeat the same washing and harvesting steps for the other *D. discoideum* strain.

In the form presented here, this exercise is suitable for a course in introductory biology and, with minor variations, cell or developmental biology. Such variations might include (a) starting the experiment with washed amoebae of each mating type rather than with spores; and (b) designing more complex experiments to study this question. If you do start the experiment with washed amoebae, use NNA instead of LP for the experiment itself.

Student Methods

A general protocol for preparing your cu1tures is described below. You will undoubtedly deviate from this procedure according to the specific needs of your experiments but this description should serve as a guideline.

1. **Maintain sterile technique throughout this experiment.**
2. Each group will be provided with lactose peptone agar (LP; for growth and development of *D. discoideum*), a suspension of *E. coli* (for *D. discoideum* to eat), separate suspensions of spores of NC4 and V12 (opposite mating types), and Bonner's Salt Solution (BSS; for adjusting moisture level).
3. To prepare a culture of *D. discoideum* for development, put the following on an LP plate: 0.5 ml *E. coli*, 0.25 ml NC4 spores, 0.25 ml V12 spores, and 2 ml BSS. Gently rock plate back and forth to distribute liquid. Tape shut. Incubate in the light for incubation in the dark; cover the petri dish with aluminum foil.
4. For your experiments, you may choose one of the following ideas or you may pursue a question of your own:
    - Light vs. dark
    - Temperature
    - Different wavelengths of light (wrap the petri dishes in cellophanes of different colors)
    - Different moisture contents (adjust the amount of BSS added)
    - Relative importance of light and moisture
    - Effect of phosphate
    - pH
    - Amount of time in light vs. in dark
    - Requirement for presence of both mating types

5. Data collection and analysis:
   (a) Using a dissecting scope, observe your cultures and record the types of structures that developed: fruiting bodies, macrocysts, indeterminate.
   (b) Depending upon your instructor's assignment, either assess the relative amounts of each type of structure with the following scale: 0 = none; + = a few; ….. +++++ = lots.

   or

   Quantitate by counting the number of macrocysts, fruiting bodies, or indeterminate structures per field for at least 10 randomly chosen fields of view. To see macrocysts and indeterminate structures well, use a magnification of 80X. For fruiting bodies, use 20X or 40X.

   (c) Record any pertinent observations.

Cell Recognition: Effects of Lectins and Sugars on Mating in *Dictyostelium discoideum*

Instructor Information

In this experiment, students explore one of the most important functions of the cell membrane, namely molecular recognition. Cells display an amazing ability to bind specifically to other cells, molecules, and tissues. This cell binding can be virtually permanent or transient and can trigger a variety of responses. For example, the cell recognition between an egg and sperm results in the two cells fusing to create a zygote and ultimately, a new individual.

Dictyostelium discoideum can be used as a model system to study cell recognition. Specifically, one can focus on sexual reproduction, which in this organism produces a structure called a macrocyst. As I have described above, macrocyst formation is the outcome of a somewhat unusual developmental pathway. Cellular slime molds exist as solitary amoebae as long as adequate food is available. When food is depleted, starving amoebae initiate development. If environmental conditions are appropriate, amoebae of opposite mating type find each other and fuse to form a zygote called the giant cell. Next, the giant cell attracts hundreds of other amoebae towards it and then eats them! Consequently, the giant cell gets bigger and bigger and ultimately deposits several protective coverings around itself; this structure is now called a macrocyst. Cell recognition/binding is often mediated by membrane glycoproteins; students can test whether this is also true for sexual development by *D. discoideum*. During macrocyst development, there are at least two opportunities for cell recognition events: zygote formation and the attraction and phagocytosis of amoebae. Interference with either (or both) of these steps should prevent macrocyst formation.

In their experiments, students use lectins (carbohydrate binding proteins), and sugars as tools to determine whether membrane glycoproteins are involved in macrocyst development, and if so, which specific residues are most important. I provide students with stock solutions of various lectins such as *Concanavalin A*, wheat germ agglutinin, and *succinyl Con A* (all available from Sigma), and sugars such as glucose, mannose, and N-acetyl glucosamine. Because lectins are toxic, sometimes we run this experiment using only non-metabolizable sugars such as methyl glucopyranoside (Sigma M9376) and methyl mannopyranoside (M6882). In any case, lab teams

choose or are assigned a lectin or sugar and then prepare a dilution series in order to test for dose-response. Lectins should be tested at concentrations ranging from 0 to 200 micrograms per ml whereas appropriate concentrations for sugars are 0 to 0.05 M. By testing several lectins, as a class, student can get an idea not only whether glycoproteins are important for macrocyst development, but also what specific sugars are involved in the actual cell-cell event(s).

Student Methods

Cell Culture:

Cultures of opposite mating types, of *D. discoideum,* NC4 and V12, have already been prepared for you. Plates of lactose-peptone medium were inoculated with the bacterium *Escherichia coli* (the food source for *D. discoideum*) and also with spores of either NC4 or V12. Incubation was at 20 to 24° C for 20 to 22 hours in the dark.

Cell Harvest:

**You will begin here. Be sure to keep the mating strains apart for the harvesting procedure. Prepare a cell suspension of NC4 and a separate one of V12.

To harvest cells, pipette or pour approximately 10 ml of cold Bonner's Salt Solution (BSS) onto a culture. Gently dislodge the cells, using a sterile glass elbow, and pour the resulting cell suspension into the next culture. Dislodge the cells in that culture with the glass elbow, pour the suspension into the next plate; repeat until all plates have been scraped. The cell suspension is poured into a sterile centrifuge tube and placed on ice. Next, wash the culture plates that have been harvested by pipetting approximately 5 ml of BSS into one of the culture. Again, gently dislodge any cells that might remain and pour the suspension into the next dish. Repeat until all plates have been washed and combine this suspension with the one collected above. It is important to keep this cell suspension on ice as much as possible during these procedures. The number of tubes needed will depend upon the number of plates to be harvested. Plates are best harvested in groups of 10 to 15, each group producing 15 ml of crude cell suspension.

The cell suspension collected still has too much bacteria in it for use in an experiment. In order to separate the amoebae and bacteria, the cell suspension is subjected to differential centrifugation. Using a clinical, table-top centrifuge spin the cell suspensions at 1500 to 2000 rpm for 4 minutes. Discard the supernatant and resuspend the cell pellet in cold BSS. Vortex gently. Spin again. Discard supernatant, resuspend the pellet; repeat this 3 to 5 times until the supernatant is fairly clear. Resuspend cells in 6 ml cold BSS.

Setting Up Your Experiment

You will be provided with 24 tiny petri dishes (35 mm) containing 2% agar, a stock solution of lectin and a stock solution of sugar. You will need to prepare a dilution series for your lectin and/or sugar (we'll discuss this together) and to design your experiment to test your lectin's or sugar's effect on macrocyst developments (we will also discuss experimental design). A general protocol for preparing your cultures is described below. Remember: MAINTAIN STERILE TECHNIQUE THROUGHOUT THIS EXPERIMENT.

1. To prepare a culture of *D. discoideum* for macrocyst development put the following on a NNA plate: 100 µl NC4 cell suspension, 100 µl V12 cell suspension. Gently rock plate back and forth to distribute liquid.

2. To your cultures, add 100 µl of the appropriate concentration of lectin or sugar and 100 µl of BSS. Be sure to prepare a control (200 µl of BSS). You will test five lectin or sugar concentrations in addition to the control. All plates will be set up in duplicate.
3. Tape plates together in stacks of four and cover with aluminum foil. Incubate, right-up side, at room temperature.
4. Data collection and Analysis - This will be done after 5 to 7 days of incubation.
    a. Using a dissecting scope, observe your cultures and record your observations.
    b. Quantify macrocyst development for each experimental treatment. Adjust the dissecting scope to the highest magnification (80X) and count 12 randomly selected fields of view on the culture. Record the number of macrocysts per field. Average the 12 fields. Repeat for the replicate plate. Calculate the average number of macrocysts/field for the two replicates.
    c. Present your results in a Table and graphically.

Developmental Choice: Microcyst Formation in *Polysphondylium pallidum*

Instructors Information

The life cycle of the cellular slime mold *Polysphondylium pallidum* consists of three possible developmental pathways, each with a very different outcome; asexual development results in the formation of fruiting bodies, sexual development produces macrocysts, and certain conditions lead to the encystment of individual amoebae thus forming microcysts. The objective of this experiment is for the students to examine some factor that influences the choice of amoebae to make microcysts.

Microcysts seem to form in response to certain environmental stimuli. For example, high concentrations of particular salts can induce encystment and other ions can modulate this osmotic response. Starvation is a necessary prerequisite and dark enhances microcyst production. Finally, ammonia also induces microcyst formation.

Although we know some information about individual extrinsic factors that trigger microcyst development, we understand little about either the relationships between these various factors, or the intrinsic factors important for this pathway. For example, is protein synthesis or transcription necessary for microcyst formation?

Students are provided with feeding amoebae of *P. pallidum*. To culture *P. pallidum*, use the same cell cultures methods described above for *Dictyostelium*. After inoculation of spores and bacteria on LP, incubate the cultures for 36 to 38 hours at 22 to 24°C. Since 36 to 38 hours is not a convenient time interval, I inoculate my cultures approximately 44 hours before I need to harvest the cells. Next, I incubate overnight (15 to 17 hours), refrigerate the cultures for 6 to 8 h the next day and then incubate at room temperature overnight again.

Once provided with cultures of *P. pallidum* amoebae, students harvest the cells, wash them free of bacteria, and design and implement an experiment to address either one of the following questions or one of their own choosing:

- Is the response of amoebae to salt dose dependent?
- Salt type specific?

- Is the response of amoebae to ammonia dose dependent?
- Are the salt and ammonia responses additive?
- Does pH modulate the response to salt or ammonia?
- Do calcium and/or magnesium modulate the salt or ammonia response?
- Temperature effects?
- What happens if you add food to cultures forming microcysts?
- Is protein synthesis required? Are there sensitive time periods?
- Is transcription required? Are there sensitive time periods?

Student Methods

Cell Culture

Cultures of *P. pallidum* PN200 have already been prepared for you. Plates of lactose-peptone medium were inoculated with the bacterium *Escherichia coli* (the food source for cellular slime molds) and also with *P. pallidum* spores. Incubation was at 20 to 24° C in the light.

Cell Harvest (You will begin here.)

1. To harvest cells, pipette or pour approximately 10 ml of cold Bonner's Salt Solution (BSS) onto a culture.
2. Using a sterile glass elbow, gently dislodge the cells and pour the resulting cell suspension into the next culture.
3. Dislodge the cells in that culture with the glass elbow and pour the suspension into the next plate; repeat until all plates have been scraped. Pour the cell suspension into a sterile centrifuge tube and place the tube on ice.
4. Next, wash the culture plates that have just been harvested by pipetting approximately 5 ml of BSS onto one of the cultures.
5. Gently dislodge any cells that might remain and pour the suspension into the next dish. Repeat until all plates have been washed and combine this suspension with the one collected above.
6. The cell suspension collected still has too many bacteria in it for use in an experiment. In order to separate the bacteria and amoebae, the cell suspension will be subjected to differential centrifugation.
7. Using a clinical tabletop centrifuge, spin the cell suspension at 1500 to 2000 RPM for 4 minutes. Discard the supernatant and suspend the cell pellet in cold BSS. Vortex gently.
8. Spin again, discard the supernatant, resuspend the pellet; repeat this 3 to 5 times until the supernatant is fairly clear. The cell pellet will be resuspended in an appropriate solution depending upon your individual experiment.

IT IS IMPORTANT TO KEEP THE CELL SUSPENSION ON ICE DURING THESE PROCEDURES.

Setting Up Your Experiment

1. A general protocol for setting up your cultures is described below. You will undoubtedly deviate from this procedure according to the specific needs of your experiment but this description should serve as a guideline.
2. Each group will be provided with ten plates of *P. pallidum* for cell harvesting using the procedure described above.
3. To prepare amoebae for microcyst development, transfer 0.1 ml of the cell suspension into 5 ml of 120-mM KCl in a 50-ml flask. Incubate the flask on a rotary shaker (at 200 rpm).
4. Set up other cultures as appropriate to your experiment. In all cases, 0.1 ml of amoebae will be transferred to 5 ml of a test solution.
5. To collect data, observe the cultures after 24 hours and count the number of amoebae and the number of microcysts present. For each flask, you should count at least 200 amoebae or spores. Calculate % microcyst formation for each flask.

There are a variety of materials available for you to use for your experiment. In all cases, you should run a BSS control and a 120-mM KCI control and at least 3 more cultures. Be sure to run replicates for both your controls and experimentals. If you have a very big idea, you can combine your efforts with more than one group.

Available materials:

- 240 mM KCl (for dilution series)
- 240 mM NaCl (for dilution series)
- 240 mM $CaCl_2$ (for dilution series and as a cation)
- 240 mM $MgCl_2$ (for dilution series and as a cation)
- Suspension of *Escherichia coli* (to test whether starvation is necessary)
- Actinomycin D (inhibits transcription)
- Cycloheximide (inhibits protein synthesis)
- Ammonium chloride and NaOH (to generate ammonia)
- Aluminum foil (for dark incubation)
- Different types of plugs for the flasks (to test for volatility of the signal)
- Chemicals for other solutions that you need....

Food Preferences and Interactions Between Different Species of Cellular Slime Amoebae
(For a more complete presentation of this experiment, please see Bozzone, D.M. 1997. American Biology Teacher 59: 565-572.)

Instructor's Information

Examination of soil samples collected from a variety of locations reveal that different species of cellular slime mold exist very close together in nature and in all probability, inhabit the same physical space (Buss, 1982; Kuserk, 1980). The question arises then, how do these organisms partition their niches? At least for some cases, it has been determined that different species of cellular slime mold feed on different soil bacteria (Horn, 1971). Therefore, the

similarity of the physical space occupied is not important; the different species avoid direct competition by having different food preferences. The experiment outlined below describes a simple method to study this behavior.

This experiment is appropriate for an introductory biology lab or, with minor modifications, a course in ecology. Such modifications include increasing the number of bacterial species and cellular slime mold species tested, or examining the results of direct competition by mixing cellular slime mold species. Suitable bacterial species to use include any soil bacteria. Cooperative cellular slime mold species include *D. discoideum*, *D. mucoroides*, *D. rosarium*, *D. giganteum*, *Polysphondylium violaceum*, and *P. pallidum*.

Student Methods

1. On Day 1 of the experiment, you will prepare the bacterial streaks. Pipet 1 ml of sterile SM/2 into a sterile test tube. Inoculate the SM/2 with 2 to 3 loops of bacterial Species 1. Vortex to suspend the cells. Using a sterile loop, "paint" a thin line of the bacterial suspension on a petri dish of LP agar. Prepare two plates in this manner.
2. Repeat step 1 for bacterial Species 2 and 3.
3. On Day 2 or 3, deposit, at one end of the streak of bacteria Species 1, 25 µl of a suspension of cellular slime mold spores (1 x 10^7/ml) or starved vegetative amoebae (1 x 10^7/ml). Repeat for bacteria Species 2 and 3.
4. Repeat step 3 using a different species or strain of cellular slime mold.
5. Determine the rate of feeding by measuring the movement of the feeding front. Measurements should be taken daily for 7 to 10 days. Class data can be pooled.

Chemotaxis

Instructor's Information

One of *D. discoideum's* main claims to fame is that the process of cell aggregation is mediated by chemotaxis to cyclic AMP (cAMP; Konijn *et al*., 1967). The protocol outlined below describes a straightforward quantitative assay. Aggregation-competent amoebae are deposited as drops onto agar containing various concentrations of cAMP. The assay takes advantage of the fact that aggregating *D. discoideum* amoebae release an extracellular phosphodiesterase, which degrades cAMP. This degradation of cAMP will be greatest in the positions where amoebae are located. The amoebae will migrate away, in a concentric ring, from the spot where they were initially deposited. The amoebae will degrade the cAMP present in this new area and will migrate out further. The consequence is that if cells are placed on agar containing a concentration of cAMP appropriate to elicit a chemotactic response, the cells will migrate out in a ring and onto the agar. One can measure the distance migrated as a function of time to quantitate the chemotactic response.

This experiment is suitable for cell biology or developmental biology courses. Related questions include testing cAMP analogues and related molecules, determining the chemotactic response of various developmental stages (e.g., vegetative amoebae, slugs, etc.), or investigating the species specificity of the chemotactic response (not all cellular slime molds use cAMP as a chemoattractant; reviewed in Raper, 1984).

Student Methods

1. You will each receive two plates of NC4 aggregation-competent amoebae. These cells have already been washed clean of bacteria and will be ready for action. Each plate contains 2 x 10^8 cells.
2. Place 3 ml of cold BSS (Bonner's Salt Solution) onto one plate. Gently dislodge the cells with a glass spreader (remember to dip in alcohol and flame first). Pour the cell suspension into the second plate, gently dislodge these cells and decant into the conical, screw cap centrifuge tube. You should have 3 ml of a cell suspension of 1.5 x 10^8 cells/ml. Place 1 drop on a slide and make sure you have plenty of cells. Keep the cells on ice.
3. Meanwhile, someone should prepare a cAMP dilution series. You will be given 1.5 ml of 2 x 10^{-3} M cAMP. You will use this to make a set of cAMP dilutions.
   (a) Take 0.5 ml 2 x 10^{-3} M cAMP and place in one of the seven small test tubes. Add to it 4.5 ml of BSS. This will result in a 1:10 dilution (= 2 x $l0^{-4}$ M camp).
   (b) Take 0.5 ml 2 x 10^{-4} M cAMP and place it in a second test tube. Add to it 4.5 ml of BSS. This will result in another 1:10 dilution (1:100 overall) (= 2 x 10^{-5} cAMP).
   (c) Repeat these steps to make 2 x 10^{-6}, 2 x 10^{-7}, 2 x 10^{-8}, 2 x $l0^{-9}$, and 2 x 10^{-10} M concentrations of cAMP. (You will eventually have eight different cAMP concentrations.)
4. Once the dilutions are done, you can prepare your cAMP agar. In the water bath in the lab, there will be flasks of 3% agar (one per group). To prepare your cAMP agar do the following:
   Take your nine tiny petri dishes and prepare as follows:
   (a) 1 ml agar + 1 ml BSS = BSS Agar (no cAMP control)
   (b) 1 ml agar + 1 ml 2 x 10^{-3} M cAMP = 10^{-3} M cAMP agar
   (c) 1 ml agar + 1 ml 2 x 10^{-4} M cAMP = 10^{-4} M cAMP agar
   And so on, using all 8 different cAMP concentrations to make 8 different cAMP agar preparations.

Hints: Add cAMP to petri plates first starting with 0 cAMP (BSS) and working up to higher concentrations; if you do this you will only need one pipet.
Add agar after you have obtained cAMP into all the plates. Do not get the agar until you are ready for it because you don't want it to solidify too soon. Measure the agar with a 5 or 10 ml pipet so you can get it into the dishes quickly. Be sure to mix plates gently by swirling or rocking.

5. After the agar has hardened, add 20 µl of cells to each dish. Let this drop of cells dry a little before you move the dish or cover it. When the drop has dried (it no longer runs), mark the border of the drop by scratching a line on the petri dish bottom.
6. You will follow the movement of the cells across that border and, using an ocular micrometer, measure it at appropriate time intervals. Measuring every 1 to 2 hours for the next 8 hours would be ideal. You are trying to determine the optimal [cAMP] for chemotaxis.

7. Also, you will record what the development of the cultures looks like at each cAMP concentration

8. In order to test whether the response to cAMP is specific, follow steps 3 through 7 substituting AMP for cAMP.

Phototaxis and Slug Migration

Instructor's Information

Another interesting cell behavior exhibited by cellular slime molds is phototaxis. While individual amoebae are phototactic during various periods of development (reviewed in Loomis, 1982; Raper, 1984), this phenomenon is easiest to demonstrate and study in slugs. After aggregation of amoebae to form the pseudoplasmodium, the aggregate undergoes morphogenesis to form a migratory, multicellular structure called a slug. Migrating slugs will travel in search of light, optimal temperature and dryness; it is thought that this stage is important for locating an appropriate site for fruiting body development (Bonner *et al*., 1982). The simple experiment outlined below demonstrates the phototaxis of *D. discoideum* slugs to white light.

This experiment is suitable for introductory biology as described below. It is also a good exercise to do along with the chemotaxis experiment to make a cell behavior unit for courses in cell or developmental biology. Related questions include determining the optimal wavelength for phototaxis, studying the species specificity of the response, and examining the effects of relevant physical or chemical parameters on this behavior (e.g., pH, ammonia, moisture; Bonner *et al*., 1982, 1988).

Student Methods

1. You will each receive one plate of NC4 aggregation competent amoebae. These cells have already been washed clean of bacteria. Each plate contains 2 x 10^8 cells.

2. Place 2 ml of cold BSS (Bonner's Salt Solution) onto the plate. Gently dislodge the cells with a glass spreader (remember to dip in alcohol and flame first). Decant the cell suspension into a conical, screw cap centrifuge tube. Keep the cells on ice.

3. Take six non-nutrient agar plates and set-up duplicates as follows:
   (a) 50 µl cells on one side of the dish. Tape shut Incubate in the light.
   (b) 50 µl cells on one side of the dish. Tape shut. Incubate in the dark (wrap plate in foil).
   (c) 50 µl cells on one side of the dish. Tape shut. Wrap plate in foil but make a small hole (size of the head of a pin) in the foil on the side of the dish directly opposite the cells. This hole should be on the side and not on the Lid so that cells perceive light from the side and not above.

4. Observe in 18 to 20 hours for slug development. Measure (a) the distance migrated for at least 10 slugs per dish and (b) determine the slug orientation (which way are they pointing).

This experiment can also be done using *Dictyostelium purpureum*. In this species, the slug produces a cellular stalk during its migration. Consequently, it is easy to retrace the specific paths followed by individual migrating slugs.

Regulation and Reorganization of Slugs

Instructor Information

As detailed in Raper, 1940, *D. discoideum* slugs are excellent subjects for investigations concerning the organization of tissues and commitment to developmental fate. In the experiments described below, students bisect slugs into fragment, or dissociate the slug entirely, and then observe the behavior and development of the pieces.

In order to provide slugs for your class, see the instructions in the Phototaxis of Slugs section of this paper. The operations on the slugs are most successful if the slugs are cooled in a refrigerator for 10 to 15 min. and if the bisections are done with and "eyelash probe". Eyelash probes are made by taking a Pasteur pipette and dipping the narrow end into melted paraffin. Next take an eyelash (from yourself or a willing donor) and insert one end of it into the paraffin on the pipette. It will stick there. Let the wax harden. Now you can use it.

Student Methods

Bisection of Slugs

1. Open the petri dish and use a dissecting scope to locate a field of view in which there is a single or a relatively small number of slugs.
2. Using an eyelash probe, slice the slug into two fragments. Make sure that the pieces are not touching each other.
3. Using a scalpel or some other sharp pointy object, trace a circle into the agar to surround the area in which your slug is located so that you can find it again.
4. Repeat this operation on at least 5 more slugs.
5. Incubate your culture in the light at room temperature.
6. Observe the development of the slug fragments periodically through tomorrow. Record your observations both descriptively and in diagrams.
7. Interpret your results fully.
8. Repeat steps 1 through 7 except that after you have cut the slug into two fragments, push the pieces together so that they touch each other.
9. Repeat steps 1 through 7 but cut the slug into more than two segments.
10. Repeat steps 1 through 7 but instead of bisecting the slug, dissociate it into a pile of cells.

Behavior of Intact Slugs and Slug Fragments in Response to Light

An interesting behavior exhibited by cellular slime molds is phototaxis. While individual amoebae are phototactic during various periods of development, this phenomenon is easiest to demonstrate and study in slugs. The simple protocol outlined below demonstrates the phototaxis of *D. discoideum* slugs to white light and will allow you to also test whether fragments of slugs are able to orient similarly to the intact slugs.

1. Take an intact slug, and gently position it on one side of a fresh petri dish containing non-nutrient agar.
2. Repeat this for 9 more slugs.
3. Cut 5 of these slugs into two pieces (see above), and leave the other 5 intact.
4. Tape the dish shut.
5. Incubate the dish in directional light by covering the dish in aluminum foil and then making a tiny hole (no bigger than the size of the head of a pin) in the foil on the side of the dish directly opposite the cells. This hole should be low on the side and not on the lid; cells should perceive the light from the side and not above.
6. Observe these cultures regularly for slug and/or "slug-fragment" migration.
7. Record your observations carefully both as descriptions and diagrams. Interpret your result fully.

Commitment to Cell Fate

1. Isolate a slug. Pipette 0.05 ml of *E. coli* suspension on to the slug. Repeat this for 4 more slugs.
2. Bisect a slug and pipette 0.05 ml *E. coli* on the fragments. Repeat this for 4 more slugs.
3. Dissociate a slug (see above) and pipette 0.05 ml of *E. coli* on the pile of cells. Repeat this for 4 more slugs.
4. Observe the development of all of these individuals. Record your observations carefully both as descriptions and diagrams. Interpret your results fully.

Summary

This paper presents methods to enable you to use cellular slime molds as an experimental system for laboratory instruction. I have outlined several examples of experiments that have been tested with students (and which work most of the time!). All information regarding recipes and suggested sources of materials are found in the appendices.

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APPENDIX A: Additional References

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APPENDIX B: Recipes

Bonner's Salt Solution (BSS)

$CaCl_2$	0.3 g
KCl	0.75 g
NaCl	0.6 g

Add distilled or deionized H_2O up to 1 liter. Autoclave 20-25 minutes, slow exhaust (liquid cycle).

Lactose-Peptone Agar (LP)

Lactose	1 g
Peptone	1 g
KH_2HPO_4	0.28 g
Na_2HPO_4	0.28 g
Agar	15 g

Add distilled or deionized H_20 up to 1 liter. Autoclave 20-25 minutes, slow exhaust (liquid cycle). One liter of agar is sufficient for 30 petri dishes (8.5 cm in diameter).

Non-Nutrient Agar (NNA)

Agar	20 g

Add BSS up to 1 liter. Autoclave 20-25 minutes, slow exhaust (liquid cycle).

Sussman's Medium (SM/2)

Glucose	0.5 g
Peptone	0.5 g
Yeast extract	0.05 g
$MgSO_4.7H_2O$	0.05 g
KH_2P0_4	0.1 g
K_2HP0_4	0.05 g

Add distilled or deionized H_20 up to 100 ml. Autoclave 20-25 minutes on slow exhaust (liquid cycle).

APPENDIX C: Sources of materials

Cultures

Dictyostelium discoideum, strains NC4 and V12 and other cellular slime mold species, can be obtained from:

American Type Culture Collection (ATTC)
12301 Parklawn Dr.
Rockville, MD 20852
(301) 881-2600

Dictyostelium discoideum can also be purchased from:

Carolina Biological Supply Co.
2700 York Rd.
Burlington, NC 27215
(919) 584-0381

Klebsiella aerogenes (Enterobacter aerogenes) and *Escherichia coli* B/r (another suitable food source for *D. discoideum*) are available from: American Type Culture Collection (ATTC)

K. aerogenes and *E. coli* (non-mucoid) can be purchased from: Carolina Biological Supply Co.

Supplies

Petri dishes: I generally use standard size petri dishes (8.5 cm in diameter, e.g., Falcon 1029) for stock cultures, life-cycle plates, growing and harvesting cells, preparation of aggregation-competent cells, and for the following experiments: Choice Between Developmental Pathways, Food Preferences, and Phototaxis. For the Chemotaxis experiment and for Cell Development on filters, I use small petri dishes (3.5 cm in diameter, e.g., Falcon 1008) so that I can conserve chemicals. Suggested sources: VWR, Fisher, or Falcon. All of these experiments can be done on smaller dishes provided the volumes of cell suspension applied are adjusted taking into account the differences in the surface area of the variously sized petri dishes.

Filters: Nuclepore, Polycarbonate Membrane Filter, Black.
Pore size: 0.2 or 0.4 pm. Size: 25 mm.
Suggested sources: VWR, Fisher.

Pads: Filter Paper. 3-mm Whatman.
Size: 2.5 cm
Suggested sources: VWR, Fisher.

Glass Elbows: You can make your own or purchase Bacti-Spreader from Carolina Biological Supply

Loops: Nichrome Wire Inoculating Loop.
Suggested source: Carolina Biological Supply Co.

Conical Centrifuge Tubes: VWR 15-ml sterile centrifuge tubes with sterile, polypropylene caps. (These can also be purchased from other sources.)