A Quick, Simple, and Accurate Chemotaxis Bioassay

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Biography

Andrew Catalano is a PhD student in the cell and developmental laboratory of Dr. Danton H. O'Day. His thesis consists of characterizing two novel calmodulin-binding proteins, cmbB and nucleomorphin, from the model organism *Dictyostelium*. He has recently reviewed the calmodulin-binding proteins in *Dictyostelium* (Catalano and O'Day, 2008) and presented a poster entitled, "Peptides from the novel calmodulin-binding protein, cmbB, enhance the rate of migration and chemotaxis in the model organism *Dictyostelium*," at the Canadian Institutes of Health Research National Poster Competition in 2008.

Danton H. O'Day is a full professor who studies the cell and developmental biology of *Dictyostelium* with particular emphasis on the function of calmodulin-binding proteins. He conducts research on the pedagogical value of animations in teaching biology. His teaching includes Advanced Cell Biology (BIO315) and Human Development (BIO380).

Introduction

Chemotaxis is the directed movement of a cell towards or away from a chemical source. It occurs during neurogenesis, metastasis, inflammatory and immune response, fertilization, and embryogenesis, and is used by several unicellular organisms to find food. *Dictyostelium* is one of the most widely used organisms for the study of chemotaxis (Manahan *et al*., 2004). This is mainly because the pathways involved are very similar to those in mammalian leukocyte chemotaxis (Manahan *et al*., 2004). As well, *Dictyostelium* amoebae lend themselves easily to genetic manipulation, are small, cheap, and multiply quickly (Manahan *et al*., 2004).

The asexual life cycle of *Dictyostelium discoideum* is presented in Figure 1. Before development starts, the amoebae phagocytose bacteria for food. They locate their food source via chemotaxis to folic acid, a substance secreted by the bacteria. When bacteria become depleted the amoebae starve, triggering entry into their developmental phase, during which they are no longer chemoresponsive to folic acid but rather to cyclic adenosine 3',5' monophosphate (cAMP). Because the amoebae also secrete cAMP they chemoattract toward each other to form aggregations. These aggregations will eventually form fruiting bodies, consisting of spore cells supported atop dead stalk cells. When spores are exposed to bacteria they germinate into amoebae to continue the life cycle.

Several methods are available that assess various aspects of chemotaxis. The Boyden chamber can be used to determine if a substance is acting as a chemoattractant, however it gives little information on the rate of chemotaxis (Chen, 2005). Several computer programs are capable of tracking the movements of individual cells giving accurate measurements of the rate of chemotaxis, however only one sample at a time can be tested (Debeir *et al*., 2004). The radial bioassay method however, assesses the effect of a substance on the rate of chemotaxis and is also relatively efficient, as multiple samples can be tested simultaneously (O'Day, 1979; Gauthier and O'Day, 2001). It provides accurate results reliable enough to publish yet easily performed by students.

The radial bioassay method is presented in Figure 2. A tiny spot of concentrated cells is placed on an agar-filled plate pretreated with the substance of interest. The cells are allowed to migrate for 4 hours, the diameter of the spot is measured before and after, and the rate of chemotaxis is calculated. Several plates should be used simultaneously which contain either different concentrations of the same substance or different substances, in order to compare the rate of chemotaxis among different treatments. One plate should contain no treatment in order to serve as a negative control.

It is important to note that the net distance migrated divided by time does not give the true rate of chemotaxis because the cells do not move in a straight line, but rather zig-zag out from the central spot. Therefore, these values must be compared to either those from different treatments or to the negative control, and the data must be presented as a measure of the relative rate of chemotaxis.

Student Outline

Materials

1. *Dictyostelium* (*D. discoideum, D. mucoroides, or Polysphondylium pallidum*)
2. *Escherichia coli* (or *Klebsiella aerogenes*)
3. SM agar (0.5g/L Yeast Extract, 5.0g/L Protease Peptone No. 3, 5.0g/L D-glucose, 18.0g/L agar, 17.0mM KH_2PO_4, 7.5mM K_2HPO_4, pH 6.5)
4. Inoculation loop
5. Cell spreader
6. Bunsen burner
7. aluminum foil
8. 100mm Petri dish
9. 35mm tissue culture dish
10. Shaker (150rpm)
11. 15mL conical tubes
12. 1.5mL centrifuge tubes
13. Flasks
14. Swing-bucket centrifuge for 15mL conical tubes
15. Microcentrifuge for 1.5mL centrifuge tubes
16. Pipettes and tips
17. Microscope with image capture software

Preparation of cells

Dictyostelium amoebae must be grown using *E. coli* (or similar bacteria such as *K. aerogenes*) as a food source. Once enough amoebae are present they must be harvested and separated from the *E. coli* by centrifugation. They should then be starved to trigger entry into the developmental stage thus maximizing the cAMP-mediated chemotaxis response. Follow the steps below to obtain cAMP-mediated chemotaxis-competent cells:

1. Inoculate spores from newly developed fruiting bodies of *Dictyostelium* with *E. coli* on a new SM agar plate.
2. Using a cell spreader, spread/mix both cell types together evenly across the surface of the plate.
3. Wrap plate in aluminum foil (or place in darkness) and allow to incubate at 21°C (or room temperature if you do not have access to an incubator).
4. The student should look for small circular clearings which begin to occur after 40-42 hours of incubation. These clearings will appear to be void of cells and are not to be mistaken for cell aggregations. When clearings are first observed the plate is ready to be harvested.

5. Add 2-3 mL of KK_2-phosphate buffer to the surface of the agar and harvest cells into a 15 mL conical tube using a cell spreader. Perform this step twice to harvest as many cells as possible.
6. Fill the remainder of the tube with KK_2-phosphate buffer and spin at 1,000 rpm for 30 seconds in a swing-bucket centrifuge. Make sure to balance the centrifuge before spinning. A light brown pellet of *Dictyostelium* cells should be visible. Carefully pour off supernatant (containing *E. coli*) making sure to minimize disruption to the pellet. Fill up the tube with new buffer and resuspend the pellet. Perform this step twice to remove as much *E. coli* as possible.
7. Resuspend pellet at 5.0×10^6 cells/mL in KK_2-phosphate buffer in a flask and shake for 2-4 hours at 150 rpm.

Preparation of agar-filled plates

Dictyostelium require a surface to translate cytoskeletal rearrangements into cell movement. Agar gel provides an excellent surface for them to move across. Treatments such as peptides, inhibitors, or other chemicals can be placed in the agar prior to solidification in order to test the effect of the treatment on the rate of chemotaxis. It is important to use fresh agar-filled plates for the assay. While the cells are shaking, follow the steps below to create treated agar:

1. Create a stock solution of the substance to be tested. For example, 1 mM cAMP in distilled water.
2. Prepare 0.4 % w/v agar in KK_2-phosphate buffer in a flask and boil, being careful to not let overflow. The volume used will depend on the number of treatments, keeping in mind that some liquid will evaporate during boiling and some agar might stick to the inside of the pipette tip. Therefore, it is recommended to add about 10-20 % more volume than what is needed.
3. After boiling, place flask in kettle containing warm water to prolong solidification.
4. Place treatment (x µL) and boiled KK_2-agar (1,000-x µL) into a new small flask and mix (x should be no more than 50 µL to avoid altering the osmotic potential of the agar). For example, to obtain a final concentration of 10 µM cAMP in 1 mL of agar, add 10 µL of 1 mM cAMP to 990 µL of KK_2-agar.
5. Place the 1mL solution into a 35 mm cell culture plate and swirl to spread solution evenly throughout the plate. Let sit for 5-10 minutes until solid.
6. Prepare the rest of the plates in the same way being sure to make a negative control plate containing only KK_2-agar and no treatment.

Spotting, imaging, and calculations

Once the cells and the agar plates are ready, cells will be placed in a small spot on the agar surface and allowed to migrate radially outwards. By measuring the spot diameter before and after, the migration rate can be calculated. Image capture software must be used for measurements. Follow the steps below to obtain the chemotaxis rate:

1. After the cells have been spinning for 2-4 hours (as long as the spinning time is consistent for each treatment) place 1 mL of cell suspension into a 1.5 mL centrifuge tube and spin at 7,000 rpm for 1 second. Resuspend cells at 1.0×10^8 cells/mL in KK_2-phosphate buffer.
2. Carefully place 0.2 µL of cell suspension on the agar surface being careful not to puncture the agar with the pipette tip. These spots can also be made by pressing out a tiny amount of cell suspension from the pipette tip and carefully touching the cell suspension on the surface of the agar. Up to four spots can be made on each plate for endoreplication.
3. Place the plate under a light microscope and take a picture of each spot using a 4 or 5x objective lens.
4. Take another picture 4 hours later (Figure I).
5. Calculate the difference in spot diameter (before and after) and divide by two to obtain the difference in spot radius. Divide this value (µm) by time (minutes) to obtain a migration rate in µm/minute.

Notes for Instructor

Maintaining cell stocks

Stock plates should be kept and maintained if experiments are to be performed on a regular basis or in the future. To create a stock plate, inoculate spores from newly developed fruiting bodies of *Dictyostelium* with *E. coli* on a new SM agar plate. Instead of harvesting the cells when clearing is first observed, allow the cells to develop into fruiting bodies. These fruiting bodies can be used to either start experiments or to create new stock plates. Stock plates can be stored at room temperature for up to two weeks, after which cell viability declines abruptly. To create permanent stocks, refrigerate plates containing fruiting bodies at 4°C. These cells will be viable for several months, although when brought out to room temperature they may have to go through their life cycle once or twice before being used for experiments.

Preparation of cells

Almost any strain of *Dictyostelium* or similar organism can be used. Some common examples are *D. discoideum* (AX3 or NC4), *D. mucoroides*, or *Polysphondylium pallidum*. Although some strains are axenic, this protocol is for bacterially-grown cells. Axenic strains grown in HL-5 liquid medium should be grown to $1.0\text{-}2.0 \times 10^6$ cells/mL, resuspended in KK_2-phosphate buffer via centrifugation (1,000 rpm for 30 seconds), and allowed to shake at 150 rpm for 2-4 hours. Cell spots can then be made on the agar surface.

Depending on seasonal variation and other uncontrollable factors, the time from incubation to the initiation of clearings could be anywhere from 25-50 hours. It is recommended that this timing be determined before starting the experiment. It is important to harvest the plates before aggregations have started to form in order to maximize the chemotaxis response.

Preparation of agar-filled plates

Two to four hour starved cells are chemoresponsive to 10 µM cAMP but not to folic acid, while vegetative cells (unstarved) are chemoresponsive to 50 µM folic acid but not to cAMP (Gauthier and O'Day, 2001). For folic acid-mediated chemotaxis follow the same protocol but do not starve the cells prior to plating. Cells spots must be placed on the agar immediately after harvesting, thus the agar plates must be prepared before harvesting the cells.

Spotting, imaging, and calculations

Any light microscope and image capture program will suffice. Some program examples are ImageJ and Northern Eclipse.

Acknowledgements

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Graphics

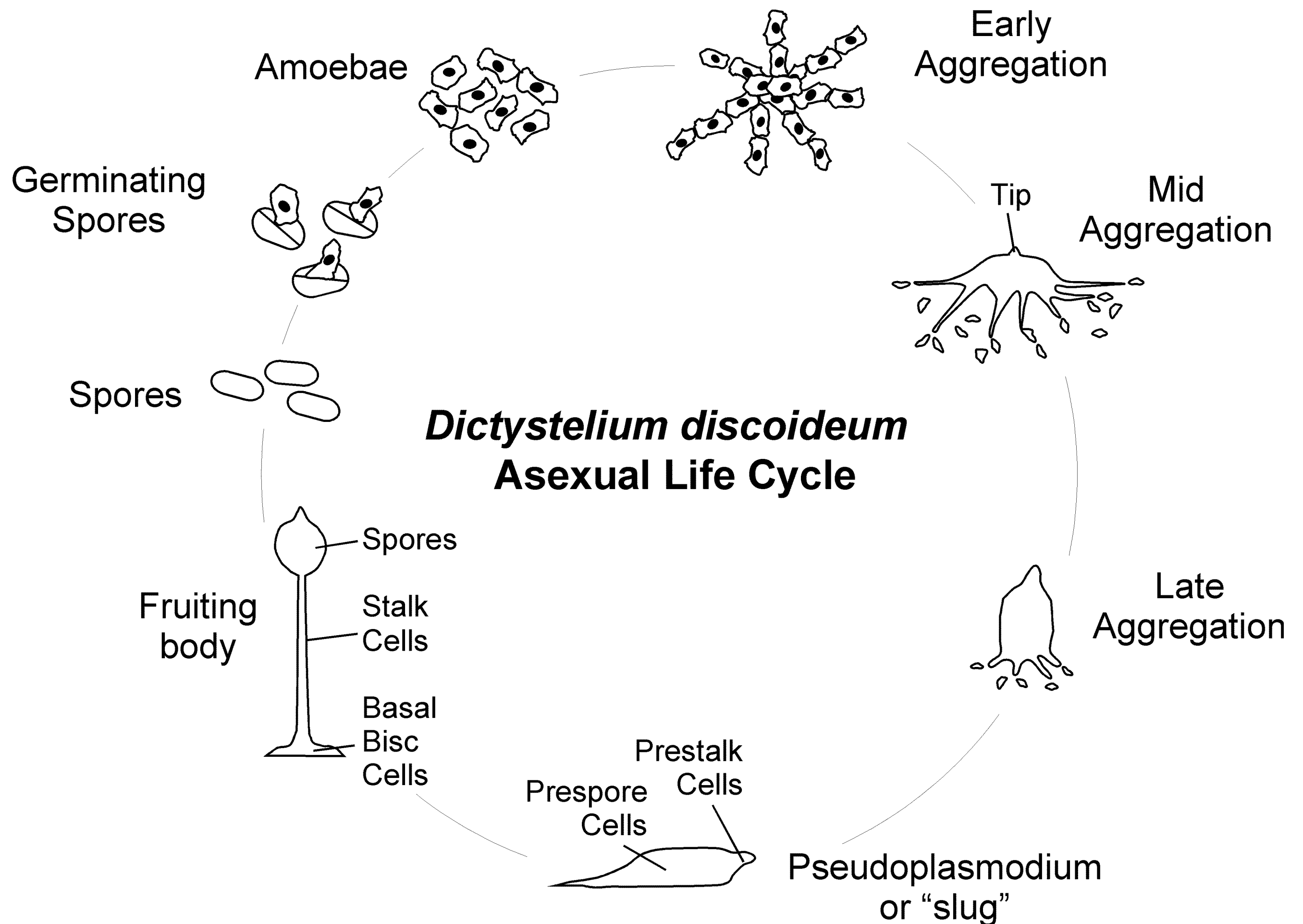

Figure 1. The asexual, multicellular life cycle of *Dictyostelium discoideum*. Amoebae feed on bacteria in the soil, increasing in number until the local food supply is exhausted. The starved cells then aggregate in response to cAMP forming early aggregates. As aggregation continues, a tip appears at the top of the mid-aggregate which serves as a control center for further development. The late aggregate falls over producing a migrating multicellular pseudoplasmodium, commonly called a “slug”. The future stalk cells (prestalk cells) begin to develop at the front end of the slug, while the future spores (prespore cells) reside at the back end. When conditions are favorable, the slug stops moving and culminates to form a supportive stalk, with its basal disc, atop which sits a fluid droplet containing spores. Under the proper conditions the spores germinate, each releasing a single haploid amoebae to restart the cycle.

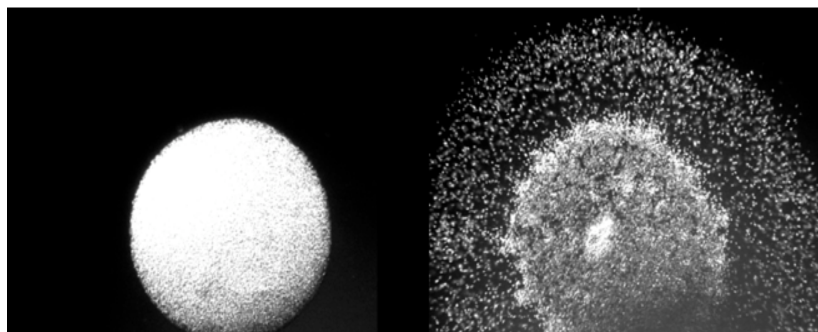

Figure 2. Image of initial cell spot (left) and same spot after four hours (right). 0.2 µL of 1.0 x 10^8 cells/mL was placed on the surface of agar pretreated with a chemoattractant. Spot diameters were measured and used to calculate the rate of chemotaxis.