Department of Biological Sciences
Animal and Plant Physiology 2nd Year
November – December 2002

Chemotaxis Practical

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Introduction:

Chemotaxis is defined as the oriented movement of cells (or an organism) in response to a chemical gradient. Many sorts of motile cells undergo chemotaxis. For example, bacteria and many amoeboid cells can move in the direction of a food source. In our bodies immune cells like macrophages and neutrophils can move towards invading cells. Other cells, connected with the immune response and wound healing, are attracted to areas of inflammation by chemical signals. In addition, many of the morphogenetic (shape altering) movements that occur during the course of development are associated with cell migration in response to chemical gradients.

In our case we will be using the cellular slime mold *Dictyostelium discoideum* as a model system for studying chemotactic movement. For this initial assay, we will be focusing on two chemoattractants, cAMP. and folate. Extracellular cAMP is the chemoattractant that attracts starving cells to one another to begin the developmental phase of the life cycle. As well as moving towards cAMP, cells that encounter cAMP also secrete cAMP, thereby amplifying the chemoattractant gradient. This relay system increases the distance over which starving cells can be recruited to an aggregate. Later in development, cAMP plays an important role in pattern formation, but we will not be following the process that far in our experiments. On the other hand, folate is normally secreted by bacteria and yeasts on which *Dictyostelium* feeds. Therefore, vegetatively growing cells can sense a gradient of folateand move towards its source.

The process of chemotaxis requires that the cell be capable of several tasks. First, it must have a mechanism to sense the signal. Generally this involves some sort of membrane-bound receptor. This receptor, in turn, must be linked in some way to intracellular machinery so as to elicit the appropriate response. In the case of cAMP, the cAMP receptor (of which *Dictyostelium* has several versions, cAR1 – cAR4, that are active at different times and/or cell types during development) interacts with a heterotrimeric G protein. The subunits of this G protein, once activated in response to cAMP binding, dissociate into α and $\beta\gamma$ subunits that go off to activate yet more pathways. One result is an increase in the intracellular levels of cGMP. This cGMP appears to be necessary for eliciting a response from the cytoskeleton (actin & myosin, in this case), which eventually results in cellular movement. Folate interacts with a different cell surface receptor than cAMP, but activates many of the same intracellular pathways to produce directed movement. Although neither cAMP nor folate is a common chemoattractant in mammalian systems, many of the same intracellular signaling molecules and regulators of cytoskeletal assembly do appear. Hence *Dictyostelium* is a popular model system for analysis of the chemotactic response.

The assay in brief:

The chemotactic assay that we will be using is based on one developed by Wallace and Frazier (1979). While it is only semi-quantitative, it will at least allow us to make some determination of binding specificity and the effect of varying concentrations of cAMP and folate. The chemoattractant is placed in a small well made in a petri dish that is filled with a buffered agar matrix. The chemoattractant diffuses outward from the well, thereby creating a gradient. Cells are placed at a small distance from the well and, after a period of about 4 hours, the pattern of their movement is observed. You will also observed *Dictyostelium* cells at characteristic stages of their developmental cycle.

Ref: Wallace, L. J. and W. A. Frazier. 1979. Photoaffinity labeling of cyclic-AMP and AMP-binding proteins of differentiating *Dictyostelium discoideum* cells. *Proc. Natl. Acad. Sci. USA* 76:4250-4254.

Safety and Good Laboratory Practice: Working in a Cell Biology Laboratory

As you learnt in last year's practicals, the success of your work and the safe use of the laboratory depends on the attention to certain procedures. Please read these short notes and follow the procedures recommended:

General lab practice:

1. Always wear your saftey glasses and your laboratory coat fully buttoned up.
2. ALWAYS WASH YOUR HANDS BEFORE STARTING AN EXPERIMENT AND BEFORE LEAVING THE LABORATORY. A sink and paper towels are provided. This minimises contamination of your experiment by the environment and of the environment by your experiment. Do not eat, drink or chew in the laboratory. Keep your fingers away from your face and mouth.
3. Avoid unnecessary movement in the laboratory and do not walk about with glassware or cultures unless this is essential.
4. Always read through the protocol you are about to follow, and make sure you understand what you are meant to do before you begin.
5. Do not discard, flush or throw away cultures or contaminated material, but dispose of all pipette tips and plasticware in appropriate containers for appropriate disposal.
6. Label all plasticware and petri dishes needed for the following practicals and place them in the appropriate containers provided.
7. You MUST wear protective gloves and safety spectacles when you are advised to do so. You might be directed to use hazardous substances which must not come into contact with your skin or might be allergic to some. Remember to remove the gloves when leaving your work-place.
8. Many of the compounds you will be using in these practical, both chemicals and cells, are sensitive to temperatures. It is therefore importnat you keep them always on ice as directed.
9. Most of the procedures you will carry out will use very small volumes (e.g. microliters!). The components are expensive and large quantities are often unnecessary. You will have to take great care in handling such small volumes of liquid and it is advisable to practice dispensing small volumes of water: the demonstrators will help you to do this.
10. At the end of the session, leave your bench clean and tidy.

Practice for use of GM organism

1 *Dictyostelium discoideum* is a common soil amoeba that presents no risk to for human health. The observation of chemotaxis will make use of a genetically modified strain of *Dictyostelium discoideum*. By homologous recombination, the gene for the Gβ subunit of trimeric G proteins has been replaced with an expression cassette encoding the resistance to the antibiotics Geneticin. This GM organism is severely weakened and cannot likely survive in the environment. Standard desinfection practices are therefore sufficient.

2 In case of minor spills, mop up the liquid with paper towels and wipe with a solution of 70% ethanol.

3 All solid waste generated during the experiment have to be collected in appropriate containers and will be autoclaved.

4 The appropriate Risk Assessment has been conducted and approved by the local GM Safety Committee. It can be consulted during the practical, ask the demonstrators.

I-Chemotaxis Assay

1) **Already done by the demonstrators:** Preparation of vegetatively growing cells and starved cells. Harvest *Dictyostelium* cells via centrifugation, wash with phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) to fully remove medium (thereby inducing starvation), and resuspend at 10^7 cells per ml in phosphate buffer. Shake at 22°C for 6 to 8 hrs. Prior to assay, vegetatively growing cells and starved cells are harvested via centrifugation and resuspend at 2.5 x 10^8 cells per ml in phosphate buffer. The assay compares wild-type (AX2) cells and Gβ minus cells.

2) **Already done by the demonstrators:** Preparation of chemoatractant stock solutions. Both cAMP and folate stocks are prepared at 10 mM and kept cold in the dark.

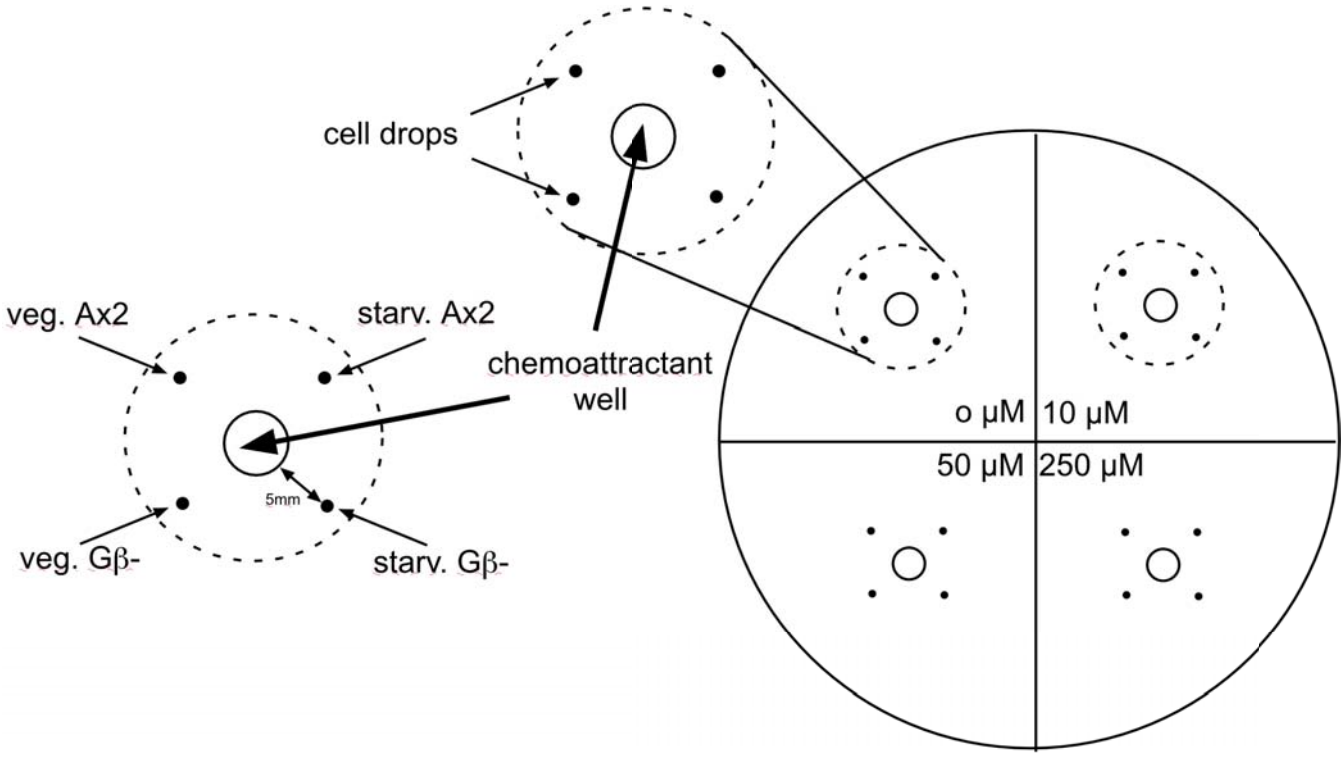
3) Using the phosphate buffer provided, dilute the 10mM cAMP and 10mM folate stocks to make the necessary solutions for the assay (10, 50 and 250µM of each, in a serial dilution, max 500 µl of each solution). Place your diluted solutions in well-marked microfuge tubes (the small plastic snap-cap tubes). A tube of "buffer alone" containing a dye will be provided for the control well.

   Notes about dilution: a 10mM stock is 40X as concentrated as a 250µM one. Remember that the smallest volume that you can accurately measure is 1 µl. Also, think about the fact that you will need a relatively small volume of each in the end -- 500µl is plenty. So, don't come up with a dilution scheme that will make 40L of whatever the solution is!

4) The Agar plates need to be fully hydratred before starting the experiment. Therefore, they were overlayed with phosphate buffer that now needs to be removed, thoroughly drained and wiped from the plate side.

5) Before preparing the actual chemotaxis plates, use one plate and the specially prepared plastic pipette to practice your well forming technique. Then, once you feel confident of your ability to make a well without destroying the plate in the process, make 4 wells in the center of each of the quadrant as described below (and in the prelab lecture). One plate is reserved to observe chemotaxis to cAMP, the other to folate.

Shematic drawing of the Petri dish, with quadrants, wells and drops of cells



6) Put the lid back on the plate and turn the plate over. Using your marker & a ruler, make 4 small dots at 5 mm from the *edge* (not center!!) of each well. Remember that the cell spots will occupy a larger area than the dot that you make.

7) Label the plates (in some way that will not get in the way of seeing cell movement) using some sensible code to correspond to: buffer alone, 10, 50 and 250μM cAMP, on one plate and buffer alone, 10, 50 and 250μM folate on the other plate. Each group of 4 dots will be used to spot AX2 and Gβ minus cells, both vegetatively growing and starved. Each group of dots should have the cells in the same configuration !

   Helpful hints about plate labeling: It is better practice to label the agar-containing portion of the plate in addition to/instead of the lid. If only the lid is labeled, it is easy to confuse two plates if you happen to remove their lids at the same time.

8) Carefully fill the wells in your chemotaxis assay plates. You may wish to practice this with some phosphate buffer on the practice plate. About 15 - 30μl is sufficient, but this will vary with the size of your wells. Whatever volume you have to use, **do not overfill the wells**, as spillage on the surface of the agar will disturb the assay. Leave the plates about 10-15 min to dry without lid.

9) In the meantime, practice making 1μl dots of cells on the practice plate to get a sense of about how much such a dot spreads. Then, after mixing your cells well by flicking the tube (in case they have begun to settle out), carefully set up the assay plates, dotting the cells right onto the marking that you placed. Allow some time to absorb excess liquid from the spot.

10) Place your two chemotaxis assay plates in the Tupperware box with a few humid towels, close it and place it on a safe, leveled surface of the bench.

11) Get out the dissecting microscopes and, using the practice plate, practice finding the wells, spots and cells under them. You will probably eventually wish to use the highest magnification possible. However, you may find it easier to locate things initially if you begin at a lower magnification and work your way up. Adjusting the light source will also probably help.

12) Now, take a 1h break and return to undertake section II (see below) of the practical. In about 3-4 hours you should be able to score your plates and complete this section I. If you have not done so already, develop a semi-quantitative scoring system (and keep a record of it!).

13) Score your results in a way that allows you to compare the 4 types of cells, vegetative and starved, wild-type and Gβ minus cells. Organise them in a tabular form. Also, draw what you observe and describe it briefly in words. Answer the questions on the Report sheet.

II-Observation of *Dictyostelium* development stages.

1) **Already done by the demonstrators:** Preparation of Agar dishes with starved cells undergoing their development cycle. Harvest *Dictyostelium* cells via centrifugation, wash with phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) to fully remove medium (thereby inducing starvation), and resuspend at 10^8 cells per ml in phosphate buffer. Plate wild-type (AX2) cells, wait until the excess liquid has been absorbed, leaving a film of cells on the Agar and incubate the plates for up to 24 h upside down in a humid chamber, at room temperature.

2) Take the plates, turn them gently right side up and observe under the dissection microscope.

3) Make drawings of what you see on the three different plates. Compare to the documentation, pictures and films available through the demonstrators and estimate the timing and stages of development you can observe on each plate. If time allows, ask the demonstrators to show you the plates and the individual cells at higher magnification under a phase contrast microscope.

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Chemotaxis Practical, December 2nd

To be handed in at 2 pm on Thursday December 5th at the latest.

Your name:

The names of your colleagues in the group:

Questions to Section I

1) Briefly describe what happens with the chemoattractant concentration over time, as it diffuses into the agar (less than 50 words)

2) Do you observe a difference between wild-type and Gβ minus cells, both for vegetative and starved cells ? If yes, what do you think the starvation does to the cells ? Is the length of the starvation period critical, and why ? (less than 200 words)

3) Estimate the maximal speed of cell motility (in μm/min) towards folate and cAMP.

4) How would the cells behave if you were to fill a well with 5 mM of cAMP ? (less than 50 words)

5) Describe what you think will happen to the cells in each of the "drops", for the four cell types (vegetative and starved wild-type and Gβ null cells) on both plates if you further incubate them overnight in the same conditions. What would happen if you left the plates in the fridge ? (less than 200 words)

6) Based upon what you learned at the lecture on chemotaxis, suggest two genes, ablation of which should disrupt chemotaxis to folate only, and chemotactic motility to any chemoattractant, respectively. Briefly explain your logical arguments. (less than 200 words)