Introduction to *Dictyostelium* and Chemotaxis

1) Let's begin by finding out something about the asexual life cycle of *Dictyostelium discoideum*. Look at the Summary figure on the next page. Then read the first three subsections (The Life Cycle of *Dictyostelium*, Aggregation of *Dictyostelium* Cells, and Cell Adhesion Molecules in *Dictyostelium*) of Reading #1.

2) Reading #1 introduced the concept of chemotaxis and its role in *Dictyostelium* development. Further information about this process is provided in Reading #2.

3) Readings #3, #4, and #5 describe some examples of chemotaxis in other cell types. **Do not panic about the details in Reading #5!** Concentrate on the general concepts that are presented about what chemotaxis is and what cells must do as part of the chemotactic process.

4) As you read (or once you've finished), work on answering the following questions:

    - What environmental condition causes *Dictyostelium* cells to switch from the uni-cellular, cell division mode to the developmental phase of their life cycle?

    - What happens during the process of aggregation?

    - What role does extracellular cAMP play in the aggregation process?

    - How do *Dictyostelium* cells sense extracellular cAMP?

    - How would you define chemotaxis?

    - What are some other examples of situations in which chemotaxis plays an important biological role?

    - Imagine that you are designing a cell that will undergo chemotaxis. What does this cell need to be able to do? (Goofy analogy: Imagine that you have been blindfolded and placed in the center of a large room. There are lots of tables around the edge of the room. One of these tables has a plate of freshly-baked chocolate chip cookies on it. What steps do you need to go through to get to the cookies? Taking off the blindfold is not allowed!)

      Note: This is a theoretical question to get you thinking about chemotaxis. You will not find this question answered directly in any of the readings.

**Please see back for list of the sources for Readings #1 - #5.

Sources of Readings

- **Reading #1:** Gilbert, S. F. 1997. *Developmental Biology*. Sinauer Associates, Inc., Sunderland, MA, USA.

- **Readings #2 & #4:** Wolpert, L. 1998. *Principles of Development*. Oxford University Press, New York, USA.

- **Reading #3:** Gilbert, S. F. The Molecular Basis of Migratory Specificity. http://zygote.swarthmore.edu/cell5.html. 15 January 2002.

- **Reading #5:** Condliffe, A. M. and P. T. Hawkins. 2000. Moving in Mysterious Ways. *Nature* 404:135-137.

Chemotaxis Lab

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'll be using the cellular slime mold *Dictyostelium discoideum* as a model system for studying chemotactic movement. For this initial assay, we'll be focusing on one chemoattractant, cAMP. (*Dictyostelium* can also sense folic acid. See "Potential Jumping Off Points" document for additional information.) 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'll not be following the process that far in our experiments.

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. Folic acid 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 folic acid 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.

Note: Do not get *extracellular* cAMP (which is what triggers the chemotactic response) mixed up with *intracellular* cAMP (which activates the catalytic activity of PKA, the cAMP-dependent protein kinase). It is the same molecule, but a separate pool, due to the inability of cAMP to freely diffuse across the membrane. (The cAMP that cells secrete in response to binding cAMP at the cell surface is exported from the cell via an unknown transport mechanism.) In fact, one eventual effect of binding cAMP at the cell surface is often activation of PKA, but that effect is more important for triggering development-specific genes. The only role of PKA that is likely to concern us for purposes of this experiment is its role in up-regulating the transcription of the adenylyl cyclase gene as part of the starvation response.

The assay in brief:

The chemotactic assay that we'll 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. The chemoattractant is placed in a small well made in the center of a petri dish that's filled with a buffered agar matrix. The chemoattractant diffuses outward from the well, thereby creating a gradient. Cells are placed at varying distances from the well and, after a period of about 3 hours, the pattern of their movement is observed.

For your information, here are the structures of the molecules that we're testing as potential chemoattractants:

***** On each Wednesday it will be necessary to return to the lab approximately 3-4 hours after the cells are plated to score the assays. Please plan ahead! *****

Week 1:

During the first week you'll be familiarizing yourself with the assay and assessing the specificity of the cAMP receptors. We'll be using cells that have been starved for a period of approximately 6 hours. (Why might this starvation step be necessary?). The potential chemoattractants will include buffer, AMP, and varying concentrations of cAMP. You will need to come up with some sort of semi-quantitative system to score your assays. In scoring your assay you should keep track of both movement of cells towards the well and any aggregation that occurs within the original spot of cells. You and your lab partners should also give some consideration as to how you wish to proceed in weeks 2 & 3, in preparation for your presentation next Monday.

Weeks 2 & 3

During these weeks your group will have the chance to set up its own variation on the chemotaxis experiment. See the "Potential Jumping-Off Points" document for some ideas to get you started. You may plan to use up to a dozen small petri dishes. Be sure to think about what might make some sensible controls. Do you wish to set up some or all plates in duplicate? Do you wish to limit the number of concentrations of cAMP you use based on week 1's data? How will your results from week 2 inform your experimental design for week3?

Reference:

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.

Chemotaxis Assay – Week 1

1) **Already done:** harvest *Dictyostelium* cells via centrifugation, wash with KK2 buffer to fully remove medium (thereby inducing starvation), and resuspend at 10^7 cells per ml in KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2). Shake at 22°C for 6 hrs. Prior to assay, harvest cells via centrifugation and resuspend at 2.5 x 10^8 cells per ml in KK2.

2) While you're waiting for the starvation period to elapse, set up your chemotaxis plates as described below (and in the prelab lecture). As a lab group, think about how you will score the results of your assay (remember to keep track of both chemotactic movement towards the well and aggregation within the cell spot). Devise some sort of scoring system that you all agree upon. Finally, discuss with your other group members what possibilities intrigue you for your independent work.

3) Before preparing the chemotaxis plates, use the large plates & cork borer to practice your well forming technique.

4) Once you feel confident of your ability to make a well without destroying the plate in the process, make wells in the center of 10 of the small plates.

5) Put the lid back on the plate and turn the plate over. Using your marker & a ruler, make dots at 3, 4, 5, 6, and 7 mm from the *edge* (not center!!) of the well closest to the dot. Give some thought to how to configure your dots so that all the cell spots do not end up on top of each other – remember that the cell spots will occupy a larger spot that the dot that you make.

6) 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, 100μM AMP, 100μM cAMP, 10μM cAMP, and 1 μM cAMP. You will need **two each** of these 5 different plate types (*e.g.,* two plates labeled KK2, 2 labeled AMP, and so on). While you're in labeling mode, get a bit of masking tape from the front table and use it to make a label with your group's name on it for the large square plastic dish – we'll use this dish to hold the plates during the incubation period.

   Helpful hints about plate labeling: (i) There is a small frosted region around the lower edge of the plate to which ink will adhere better than the shiny surface of the plate. (ii) 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's easy to confuse two plates if you happen to remove their lids at the same time.

7) Using the KK2 buffer provided, dilute the 10mM AMP and 10mM cAMP stocks to make the necessary solutions for the assay (100μM AMP, 100μM cAMP, 10μM cAMP, and 1 μM cAMP). Place your diluted solutions in well-marked microfuge tubes (the small plastic snap-cap tubes).

   Notes about dilution: a 10mM stock is 100X as concentrated as a 100μM 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 -- 150μl is plenty. So, don't come up with a dilution scheme that will make 40L of whatever the solution is!

8) Once the cells are nearing readiness, you can carefully fill the wells in your chemotaxis assay plates. You may wish to practice this with some KK2 & the large practice plate. In my experience 25 - 30μl is sufficient, but this will vary with the size of your wells. Whatever volume you choose, use the same one for all the plates. (Why is this important?)

9) Practice making 1μl dots of cells on the large plate to get a sense of about how much such a dot spreads. Then, after mixing your cells well (in case they've begun to settle out), carefully set up the assay plates, dotting the cells on so that the edge of the spot is approximately in line with the marking that you placed at the various intervals.

10) Get out the dissecting microscopes and 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 & mirror will also probably help.

11) Place your plates in the large square plastic boxes and put the boxes in the 22^{o}C incubator.

12) In about 3 hours (possibly 2.5 – 5 hr window is OK; I know that 3-4 hrs is OK) return and score your plates. If you've not done so already, develop some semi-quantitative scoring system (and keep a record of it!).

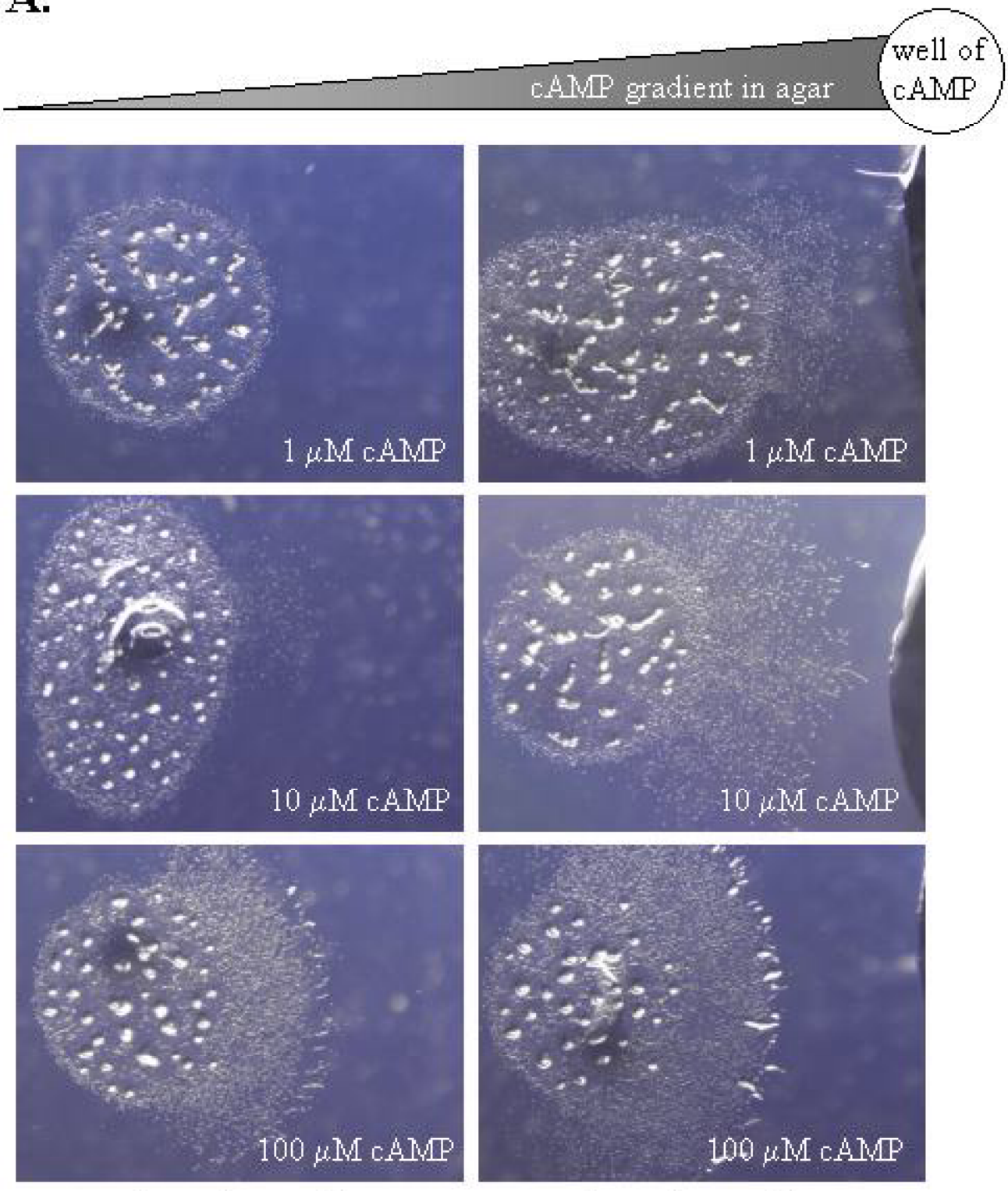
Figure 1: Underlying Principle of Chemotaxis Assay. A well is made in the center of a 35mm petri dish containing 2% KK2 agar. A solution of chemoattractant is placed in the central well, from which it diffuses outward, creating a gradient in the agar. Depending upon their distance from the well and the concentration of the chemoattractant, cells respond to varying degrees by moving towards the central well. See Figure 3 for sample data. [Based on an assay described in Wallace & Frazier (1979).]

Figure 2: Protocol for Basic Chemotaxis Assay. The instructor prepares a cell culture and induces starvation 6 hrs prior to cell plating. The students prepare the chemotaxis plates, place the cells on the plates, and score the assay (using a dissecting microscope) approximately 4-5 hrs later. See Figure 3 for sample data.

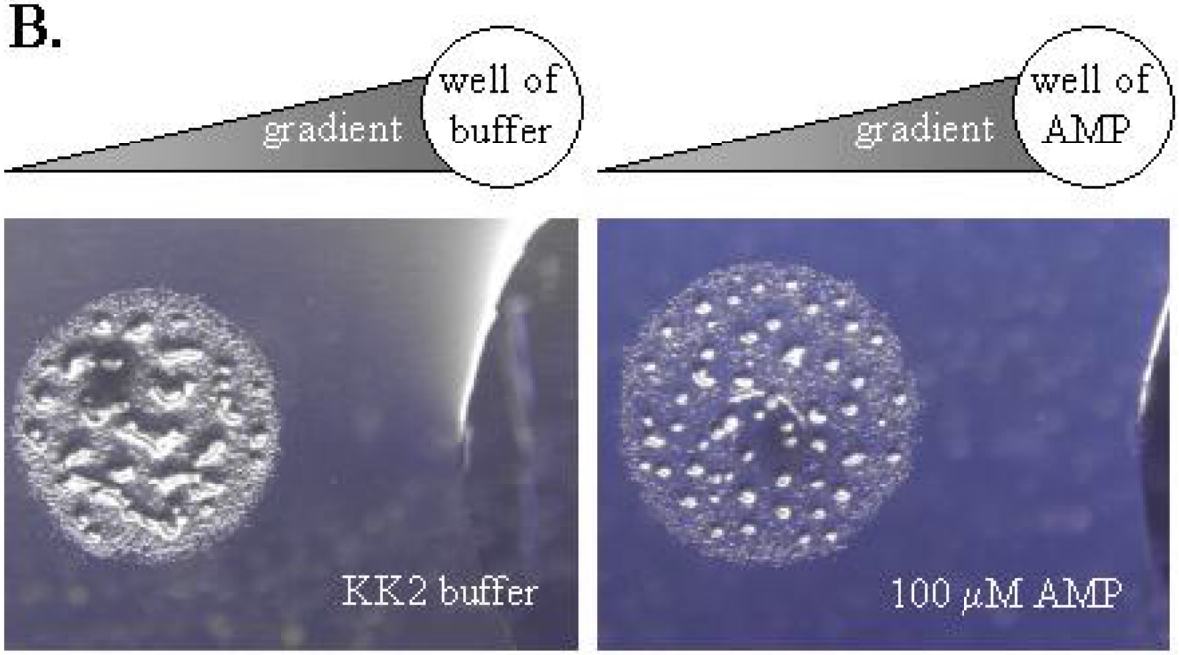
Figure 3: Sample Data from Basic Chemotaxis Assay. The schematic diagram above each set of photos shows the relative orientation of the cells and the central well. (A) Response of cells to differing concentrations of cAMP (1, 10, & 100 _M) at differing distances (4 & 6mm) from the well containing cAMP. (B) Negative control (KK2 buffer) and a test for receptor specificity (100 μM AMP), both shown at 3mm from the central well. [Samples were viewed under a Nikon SMZ1500 stereozoom microscope and photographed with an RT Slider digital camera & SPOT RT software (Diagnostic Instruments, Inc.).]

Figure 4: Sample Data from an Independent Experiment. The experiment was designed to assess the effect of varying starvation times (0, 4, & 6hr) on the ability of cells to respond to either cAMP (the normal chemoattractant during development) or folic acid (a bacterially secreted compound that attracts feeding *Dictyostelium* cells). The schematic diagram above each set of photos shows the relative orientation of the cells and the central well. [Samples were viewed under a Nikon SMZ1500 stereozoom microscope and photographed with an RT Slider digital camera & SPOT RT software (Diagnostic Instruments, Inc.).]

A.

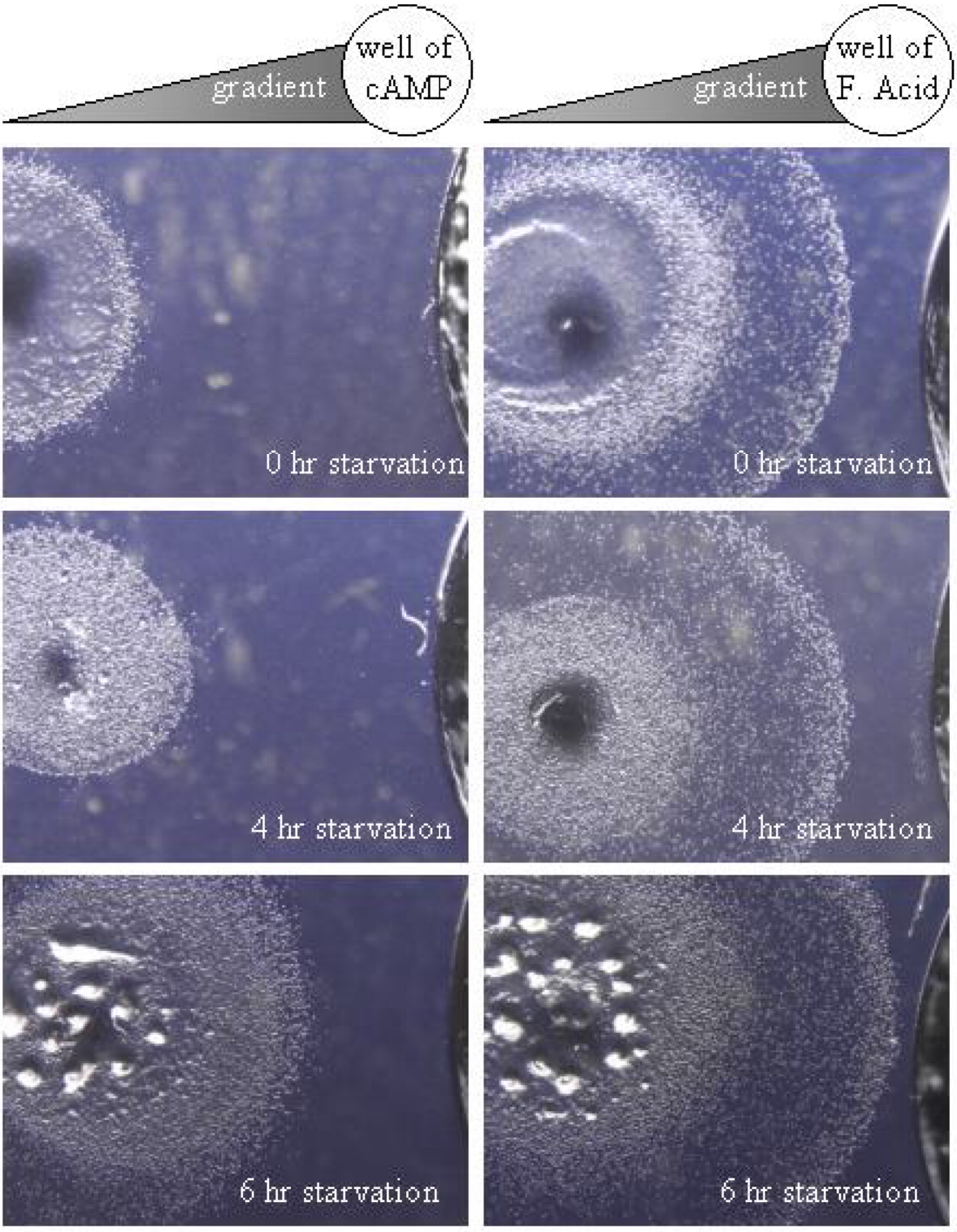

cAMP gradient in agar
well of cAMP
1 μM cAMP
1 μM cAMP
10 μM cAMP
10 μM cAMP
100 μM cAMP
100 μM cAMP
6 mm from well
4 mm from well

B.



3mm from well

3mm from well


gradient
well of cAMP
gradient
well of F. Acid
0 hr starvation
0 hr starvation
4 hr starvation
4 hr starvation
6 hr starvation
6 hr starvation
5mm from well
5mm from well

A Few Potential Jumping-Off Points for Further Experimentation:

- Our initial experiment used cells that had been starved for ~6 hours. Why was this time chosen? Are cells maximally responsive (as judged by our assay) at that time?

- In our initial experiment we compared the response of cells to three different concentrations of cAMP. What is the optimal concentration to achieve a successful response in our assay? Why might concentrations that are too high or two low be problematic?

- In our initial experiment, each cell drop contained 2.5 x 10^5 cells (1_l of a 2.5 x 10^8 cells/ml solution). How might changes in the number of cells in the cell drop influence a cell's "decision" to move either towards its neighbors or towards the central well?

- In our initial experiment, we compared the effectiveness of cAMP and AMP as chemoattractants. How well do the cells respond to another cyclic nucleotide (like cGMP) or to the non-hydrolyzable cAMP analogue 2'-deoxy-cAMP?

- The cAMP analogue 2'-deoxy-cAMP is resistant to hydrolysis by phosphodiesterase (PDE). Dithioerythritol (DTT) has been reported to inhibit PDE activity. How might chemotaxis be affected if hydrolysis of the chemoattractant was reduced or eliminated? (See background reading assigned for the first class + outline from first day of class for some info on the role of PDE in chemotaxis.)

- When *Dictyostelium* are in the "vegetative" (feed & divide as uni-cellular amoebae) stage of their life cycle, they sense bacteria via folic acid/folate, which is secreted by bacteria. At what stage of their life cycle are cells most sensitive to folic acid? Is this sensitivity affected by starvation? If given the choice of cAMP or folic acid, which chemoattractant will the cells choose?

- Caffeine has been reported to inhibit the function of adenylyl cyclase. How might the presence of caffeine affect a cell's "decision" to move towards its neighbors or towards the central well?

- RegA is an intracellular phosphodiesterase that acts to lower intracellular cAMP levels. The FbxA protein affects the stability of the RegA protein. Cells lacking the FbxA protein (fbxA- cells) have unusually large amounts of RegA in them, whereas cells with extra FbxA protein ($FbxA^{++}$ cells) would presumably have extra low levels of RegA. (The former has been experimentally demonstrated; the latter has not been.) Do fbxA- or $FbxA^{++}$ cells behave any differently than normal cells in our chemotaxis assay? (See outline from first day of class for potential importance of PKA activity in up-regulating expression of adenylyl cyclase & other genes.)

- Unlike cGMP, the cGMP analogue 8-Br-cGMP diffuses freely through cell membranes. How would the presence of 8-Br-cGMP in our assay affect the response of the cells?

- Unlike cAMP, the cAMP analogue 8-Br-cAMP diffuses freely through cell membranes. How would the presence of 8-Br-cAMP in our assay affect the response of the cells?

Basics of *Dictyostelium* Chemotaxis & Associated Intracellular Events

I. Chemotaxis – The Big Picture

A. Minimal Requirements for Chemotaxis

1. some means to **detect** (cell surface receptor; cAR 1 in this case)
2. some means to determine **which direction to move** (gradient of chemoattractant helpful; see Parent & Devreotes 1999 review for further info about events at molecular level)
3. some means to **move cell** (actin/myosin cytoskeleton)

B. Added Bells & Whistles

1. "relay" response – secretion of cAMP by cells → increased potential size of aggregation territory
2. repeated rounds of receptor adaptation/deadaptation as well as the activity of a phosphodiesterase (PDE) that degrades cAMP to AMP → pulsatile patterns of movement

II. Basic Categories of Cellular Responses to Extracellular cAMP

A. Secrete cAMP = Relay Response (requires activity of adenylyl cyclase)

B. Move up cAMP Gradient (involves stimulation of actin/myosin cytoskeleton by a cGMP-dependent process)

C. Up-Regulate Expression of Genes Associated with Aggregation & Development

1. PKA-mediated events (requires increase in **intracellular** cAMP concentration)
2. Other signaling pathways we won't worry about

III. Events at the Molecular Level

A. **Extracellular** cAMP binds cell-surface receptor (cAR1 major player during chemotaxis)

1. cARs are G-protein linked receptors
2. cAMP binding causes conformational change in associated heterotrimeric G-protein, so that the _ subunit can bind GTP
3. _ subunit dissociates from __ complex; both _ and __ can now activate other proteins in the cell
4. we'll focus on activation of guanylyl cyclase and adenylyl cyclase

B. Guanylyl cyclase

1. catalyzes the synthesis of cGMP from GTP
2. cGMP, in turn, is required to activate the actin/myosin cytoskeleton to achieve cell movement

C. Adenylyl cyclase (aca; now known to be one of 3 adenylyl cyclases in *Dictyostelium*)

1. catalyzes the synthesis of cAMP from ATP
2. export of cAMP through membrane (by as-yet unknown means) increases **extracellular** cAMP concentration – part of relay response

3. intracellular cAMP levels also rise, which activates PKA (cAMP-dependent protein kinase)

D. PKA

1. activates transcription of adenylyl cyclase
2. activates transcription of numerous genes involved in aggregation and development

Additional Information on Cells, Cell growth conditions, Plate composition
(See lab protocol for information on starvation step, etc.)

Strain of *Dictyostelium* used: Ax2 cells

Growth conditions: HL5 medium; shaking cultures at 22^0C; grown to a density of 1 x 10^7 cells/ml to 3 x 10^7 cells/ml prior to starvation step

Chemotaxis plates: 2% agar in KK2 buffer (see lab protocol for composition of KK2); petri dish sizes were either 35mm (ones we used the first week), 100mm (if you used the "large" ones), or 60mm (for the one group that used the medium sized plates in week 2)

Microscopes: stereozoom dissecting microscopes