Appendix 1: Lab Protocols

BIO 218 – Cell and Molecular Biology Lab **B.F.Mitchell**
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CELL MOTILITY

One of the universal characteristics of eukaryotic cells is motility. The field of cell motility, or cell movement, encompasses several types of motion. The most obvious of these is, of course, movement of an entire cell, such as can be observed in a sperm cell swimming through liquid or a macrophage crawling through tissues and engulfing foreign bacteria. In cells which are anchored to a solid surface (e.g. cells within your respiratory tract), the beating of cilia or flagella cause movement of fluid past the cells, which are themselves stationary. Cell motility also includes movement within a cell, including chromosome movement during mitosis, movement of vesicles containing neurotransmitters along nerve axons, and muscle contraction. Although there are a variety of ways in which eukaryotic cells move, virtually all such movement involves one of two groups of **cytoskeletal proteins**, each of which includes a structural/scaffolding component (generally in the form of thin fibers) and a motor component (which transduces the chemical energy of ATP to mechanical energy). The first group of cytoskeletal proteins uses fibers made of the protein **actin** (microfilaments) and motors composed of proteins in the **myosin** family. Actin and myosin-based movement is seen in muscle contraction, amoeboid movement, cytoplasmic streaming, and cytokinesis (division of the cell body following mitosis or meiosis). The second set of motility proteins consists of the protein **tubulin**, which assembles into fibers known as microtubules, and motor proteins of the **dynein** and **kinesin** families. Ciliary and flagellar beating, chromosomal movement during mitosis, and several other types of cell movement are microtubule-based. For further information on these and other types of motility, refer to your text book.

In this laboratory you will use both brightfield and phase-contrast microscopy to observe a variety of living specimens. The types of motility you will see include examples of both major groups of cytoskeletal proteins, as well as one form of motility not yet well understood.

Procedures:

The instructor will demonstrate how to make a wet mount using vaseline to create ridges that will support a cover slip. All of the samples you will observe today should be set up as wet mounts. It is important to use the vaseline ridges so that the cells will not be squashed by the cover slip and unable to move! Be sure to look at all samples with both brightfield and phase-contrast microscopy, and note the differences in what you can see. **Do not** use your oil immersion lens to observe any of the wet mounts.

Part I: Actin-based motility

Amoeba proteus and/or ***Chaos carolinensis***

These two types of amoeba both move by a process known, not surprisingly, as amoeboid motion (some texts refer to it as 'cell crawling'). This process occurs in many other cell types, including macrophages in the mammalian immune system. Amoeboid movement results from the formation of **pseudopodia** (singular = pseudpod), or extensions of the cell in which directed cytoplasmic streaming occurs. This process, which involves both actin-myosin interactions and the polymerization/depolymerization of actin, is easy to observe in either of these amoebae. The cells of *C. carolinensis* are larger than those of *A. proteus*, and the former are also multinucleate. You may observe one or both types of amoebae; if you look at both, be sure to note any differences between them.

First prepare to make a wet mount by putting vaseline ridges on a slide. In order to observe either amoeba, you must locate one to put on your slide. Use a dissecting microscope to look in a small jar

containing one of the amoeba species. You will need to focus on the bottom of the jar in order to find any live amoebae, which will look like grainy, grayish blobs with finger-like projections. If you are unable to locate any amoebae, ask the instructor for assistance. Once you have located a specimen, you must work **carefully** to capture it without losing it. Take a Pasteur pipet and squeeze the bulb **before** you put it into the jar. While looking through the dissecting microscope, slowly and gently bring the tip of the pipet near the amoeba, then release the bulb to suck it into the pipet. Bear in mind that it is easy to literally "blow it away" by squeezing the bulb while it's in the liquid! Once the amoeba is in the pipet, you should put in on your prepared slide. Since amoebae are fairly large, they are visible (just) to the naked eye. You should therefore be able to see whether your amoeba is actually on the slide, or still in the pipet; you can confirm this by looking at the slide (before putting on a cover slip) under the dissecting microscope. Once you have a specimen on the slide, add a cover slip and observe the cell with a light microscope.

Using low power, observe the movement of an amoeba over a 10 min period, sketching an outline of the cell every 2 min. Be sure to draw each sketch with the same size and put an arrow in the diagram to indicate the direction of cell movement. Each sketch scale should be labeled with the magnification used for your observations, as well as the time.

Under both low power and high-dry (NOT oil immersion), observe the cytoplasmic streaming in different regions of one pseudopod until the organism changes direction, then describe any differences you observe in the cytoplasmic streaming seen in the new and the old pseudopodia. When the cell changes direction, is the change in cytoplasmic streaming seen first in the old or the new pseudopod? In which part of the pseudopod is streaming first observed?

Elodea

Make a wet mount of an *Elodea* leaf with the upper surface of the leaf facing up. Scan the leaf under low power and look for **cyclosis**, or cytoplasmic streaming. It is easiest to see by looking for movement of chloroplasts. Switch to high-dry to observe cyclosis more carefully. Describe the movement of the chloroplasts which occurs during cyclosis.

Part II: Microtubule-based motility

Paramecium caudatum

This organism is a ciliated protozoan which swims by beating its numerous cilia in a coordinated pattern known as a **metachronal wave**. The cells can change swimming direction by changing the orientation of their cilia. To observe this organism, prepare a slide and add a drop of the *Paramecium* culture. These cells are much smaller and more numerous than the amoebae, and you do not need to use the dissecting microscope to get a sample. To place two samples (and coverslips) on the same slide, place the first set of vaseline ridges near one end of the slide, as demonstrated by the instructor. First observe the cells swimming in your sample; this will show you what their normal swimming speed and patterns are like. Next you should observe some *Paramecium* which you have slowed down by using a **small** amount of Protoslo® or Detain® (a very viscous solution). To observe the slowed *Paramecium*, add a second drop of the culture to your slide (you will need additional vaseline ridges). Then put a **small** drop of Protoslo® or Detain® **near** the drop of *Paramecium* culture, **but not touching it**. Use the tip of a clean toothpick to mix a small amount into the drop of cells. If you add too much, the cells will be totally unable to swim because the solution will be too viscous.

Observe the *Paramecium* swimming immediately after adding the Protoslo® or Detain®, then again after about 10 minutes. Record any differences you see. When the cells are swimming more slowly, you can observe the ciliary beating better. Do all the cilia appear to beat identically? Explain why or why not.

Chlamydomonas reinhardtii

Chlamydomonas reinhardtii is a single-celled green alga with two anterior flagella. It is a widely used model organism (see your text for a definition of "model organism") for studies of flagellar function, as well as other cell biological problems. Over the next few weeks you will be using this organism for a series of experiments on flagellar composition and assembly. Today you will be studying the function of this organelle, by looking at several strains of *C. reinhardtii* which exhibit differences in motility. All the *C. reinhardti* samples can be viewed as wet mounts prepared with a small drop of the correct culture. You will want to look at these cells under phase contrast as much as possible, especially when viewing gliding in *pf* cells. **Note**: Please be careful to avoid cross-contamination of the *C. reinhardtii* strains. Do NOT use the same pasteur pipette in more than one tube!

137c is a wild-type strain of *C. reinhardti* which swims normally. Wild-type cells swim rapidly, beating their flagella at a frequency of about 60 Hz (60 times per second). Again, set up for more than one coverslip per slide. After observing the wild-type cells swimming, you should try to determine how the cells use their flagella. In order to view these cells more closely, you will want to add a **small** amount of Protoslo® or Detain® to a drop of *137c* cells in order to slow them down. You do not want to add a drop of Protoslo® directly to the cells, as it is difficult not to add too much...in which case the cells will be attempting to swim through the equivalent of molasses and you won't be able to observe anything. Put a drop of cells on a prepared slide, then near, but not touching the cells, put a small drop of Protoslo®, and use a toothpick to mix a little of this with the cells before adding a cover slip.

What swimming stroke does the motion of the two *C. reinhardti* flagella resemble?

After observing the cells under phase contrast, switch to bright-field microscopy to see the eyespot, which should appear as a reddish patch near the anterior end of the cell. The eyespot, which is important in phototaxis (see below) contains a pigment related to the rhodopsin in your retina; in other words, the "vision" of this alga is evolutionarily related to your own!

pf-1 is a strain of *C. reinhardti* with a mutation which results in the absence of radial spoke heads in the flagella (see your text for a diagram of flagellar structure). These cells have paralyzed flagella (hence the designation *pf*) and are unable to swim. Although *pf-1* cells cannot swim, they do exhibit a different form of motility which is still dependent on their flagella. If you look carefully at your slide of *pf-1* you should find a few cells whose flagella appear to be stuck to the slide or cover slip; the flagella in these cells are held at a 180° angle. If you watch such a cell carefully, you should be able to observe **gliding**, an alternate form of flagellar motility in which the cells glide along on their flagella. This behavior is not limited to this strain; all *C. reinhardti* strains can glide. While it may be easier to observe in cells that can't swim, you may also observe gliding in wild-type or other cells if you are unable to see it in the *pf-1* cells.

Do the cells appear to be pulled by the leading flagellum, or pushed by the trailing one? Are the gliding cells able to switch directions, such that the leading flagellum becomes the trailing one and vice versa?

Sup-pf-1 is a strain of *C. reinhardti* with a mutation in the outer dynein arms of the flagella. This mutation results in an altered swimming pattern, with a beat frequency about half that of wild-type cells. This mutation is interesting not only in its own right, but also because it can act as a bypass suppressor for the effects of a second mutation. *Sup-pf-1, pf-1* cells are double mutants, carrying both the *pf-1* and the *sup-pf-1* mutations. Although these cells still lack the radial spoke heads (due to the *pf-1* mutation), the altered outer dynein arms (due to the *sup-pf-1* mutation) allow these cells to swim. In other words, the *sup-pf-1* mutation allows the cell to bypass the effect of the *pf-1* mutation. Prepare a slide with three wet mounts, and examine samples of *137c*, *sup-pf-1*, and *sup-pf-1, pf-1*. Go back and forth between the three samples and describe the differences in swimming and gliding motility that you observe for these **three**

strains of *C. reinhardti*. Now prepare a slide with two wet mounts, one of *sup-pf-1* (an outer dynein arm mutant) and one of *ida-2* (an inner dynein arm mutant. Note any differences in the motility of these two strains.

Phototaxis

Both single-celled organisms and individual cells within a multicellular organism often show directed movement. In other words, movement is not random, but is aimed at moving a cell toward (or away from) a particular location. The cues for such directed cell movement, which is known as **taxis** (tak'·sis), include stimuli such as light, heat, and specific chemicals. A stimulus can result in a cell moving toward it (positive taxis) or away from it (negative taxis) and the type of stimulus is generally indicated in the term used to describe the movement. Chemotaxis refers to directed movement in response to chemical gradients; the movement of white blood cells during an infection is an example of chemotaxis. Sometimes, cells can exhibit either positive **or** negative taxis in response to the same signal, depending on stimulus strength. *Chlamydomonas*, for example generally exhibit positive **phototaxis**, an unsurprising fact given that they are photosynthetic. However, when exposed to very high intensity light, *Chlamydomonas* exhibits negative phototaxis, a phenomenon known as photoshock.

In the following experiment you will observe positive phototaxis in *Chlamydomonas*. Get four clean 60 mm plastic petri dishes, three (3) with lids half-covered with black tape and one (1) without tape and label them as follows: label the plate without tape "*WT* C" (wild-type control), label the three plates with tape "*WT* Exp" (experimental), "*sup-pf-1"*, and "*ptx-1"*. Pipette 5 ml of each strain of *Chlamydomonas* into the appropriate petri dish. Put each dish except the *WT* C on the paper such that ½ the dish is over the black paper and ½ is over the white paper. Put the *WT* C so that the entire dish is over white paper; this dish has a clear lid and will be compared to the experimental plates which will be only partially exposed to light. Observe the distribution of cells prior to the beginning of the experiment. For your three experimental plates (NOT the control), place the lid on the petri dish so that that the dark/light interface is perpendicular to the black/white line on the paper (in other words, the tape line should be at right angles to the paper line under the dish). Shine a light onto the petri dishes, and note the time. Observe the dishes for a total time of at least 20 minutes, **checking them every 3-5 minutes**. **NOTE**: 5 minutes between observations is the MAXIMUM time you should allow. DO NOT MOVE the petri dishes when you check them, since it is very hard to move the plates without changing the distribution of the cells! Each time you look at your plates, record your observations on the distribution of the cells in each petri dish.

Gonium pectorale, Pandorina morum, and ***Volvox aureus***

The last series of organisms you will examine today are all evolutionary descendants of *Chlamydomonas*. In fact, all of these algae are colonial organisms; that is they are multicellular aggregates on the evolutionary path to true multicellular organisms such as higher plants. These three species are all colonies of cells; each individual cell of the colony is closely related to *Chlamydomonas*, so that these organisms look like groups of *Chlamydomonas* cells stuck together by their posterior ends, with their flagella pointing outward. The three species are listed above in order of evolutionary lineage and complexity. *Gonium pectorale* are flattened colonies composed of 4-32 individual cells. *Pandorina morum* are ellipsoidal colonies made up of 16-32 cells. *Volvox* are the most complex cells of this lineage. *Volvox aureus* form large hollow colonies and possess two types of differentiated cells: somatic cells, which form the bulk of the colony, and germ cells, which are involved in reproduction. Most *Volvox* colonies contain one or more daughter colonies within the main colony. Eventually the mother colony ruptures and the daughter colonies are released.

Make wet mounts of *Gonium, Pandorina,* and *Volvox*. When making wet mounts of *Volvox aureus,* be sure to use enough vaseline that the colonies are not crushed beneath the cover slip. Examine

all three colonial algae, and compare their motility to that of *Chlamydomonas*. Why do these colonial algae move as they do?

LAB REPORT:

This lab report will **not** be in the format of a scientific paper, but should consist of a brief introduction* (between ½ and 1 page, typed) and a combined results and discussion section. The latter should include all observations made during the lab, any drawings you were directed to make, and answers to the questions posed throughout the lab. All lab reports must be **typed** (apart from drawings) in a font size between 10 and 12 points and **proofread**.

Specifically, your report should include:

---A brief description each procedure you did. Generally this should be 1-2 sentences stating what you did, so that any subsequent observations are read in an appropriate context. [e.g. "A wet mount was prepared of a specimen of *Felis domesticus*. The organism was observed for 24 hours with electron microscopy, and all changes in size were noted."] This is not a real Materials & Methods, as it lacks detail, but even in this short lab report, some information is necessary. The example above shows the sort and level of information you should give prior to stating your observations and answering any questions raised in the lab handout. If you just tell what you saw, the reader is at a loss to know what you were attempting to look for. Immediately following the description of what you did, you should state any relevant observations and answer any questions. Thus, unlike a formal lab report, this one will be broken into sections in the same manner as the lab handout. Any discussion of your results and observations should follow the latter and the questions should be incorporated into this. The grade sheet that will be used by instructors is attached. It may also be found on the course website.

The questions, etc. raised in the lab procedure that should appear your report include the following:

--- your drawings of the movement of an amoeba and answers to the following questions:
When the cell changes direction, is the change in cytoplasmic streaming seen first in the old or the new pseudopod? In which part of the pseudopod is streaming first observed?

---description of the movement of the chloroplasts which occurs during cyclosis in *Elodea*.

---observations of *Paramecium* swimming immediately after making the slide and after 10 min. including any differences you see. Answers to the questions: Do all the cilia appear to beat identically? Explain why or why not.

---What swimming stroke does the motion of the two *Chlamydomonas* flagella resemble?

---Do gliding *Chlamydomonas* appear to be pulled by the leading flagellum, or pushed by the trailing one? Are the gliding cells able to switch directions, such that the leading flagellum becomes the trailing one and vice versa?

--- descriptions of the differences in motility that you observe for the strains of *Chlamydomonas* (*137c sup-pf-1; sup-pf-1, pf-1; ida-2;* and *pf-1)*.

* You should cite at least two references in your introduction. Please refer to McMillan for correct citation format using the "author-year" system.

--- observations on the distribution of the *Chlamydomonas* cells of different strains in the petri dishes at each time point during the phototaxis experiment and explanation of your results.

--- observations of motility in the three colonial algae and comparison to *Chlamydomonas*. Answer to question: Why do these colonial algae move as they do?

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FLAGELLAR REGENERATION IN *Chlamydomonas*

Eukaryotic cells exhibit numerous types of motility, but the vast majority of eukaryotic motility is based on one of two types of cytoskeletal filaments: microtubules or microfilaments. This experiment will enable you to study microtubule polymerization and assembly of an organelle (the flagellum) in a living cell. The eukaryotic flagellum (or cilium) is a useful system for studying microtubule-based motility both because it is composed of a highly organized array of microtubules and because it is relatively easy to remove only this structure without otherwise damaging the cell. Single microtubules consist of a hollow tube having walls composed of 13 rows of subunits. Each row, or protofilament, is made up of pairs of globular subunits, i.e. dimers. Biochemical analysis has shown that each dimer is actually a heterodimer composed of two related but non-identical polypeptides, known as α-tubulin and β-tubulin. Both polypeptides have similar molecular weights (approx. 55,000 daltons) and similar amino acid sequences, suggesting that they arose from a gene duplication event early in evolutionary history. The microtubules comprising the axoneme, or structural core, of a flagellum consist of a central pair of singlet microtubules surrounded by nine doublet microtubules (the "9 plus 2" configuration). Although proteins other than tubulin, for example the large dynein motor proteins (which have molecular weights in the range of 300,00-500,000 daltons) are also present in flagella, they are considerably less abundant, since the major structural components of flagella are the microtubules.

Microtubule assembly and disassembly both occur preferentially at one end of a microtubule. The microtubule therefore has a polarity to its structure, and microtubules within the cell are oriented in a polarity-dependent manner. While cytoplasmic microtubules, as well as those studied *in vitro*, are highly dynamic structures, undergoing constant polymerization and depolymerization, flagellar microtubules are quite stable. In a typical flagellum, there is little net depolymerization observed, except at certain well-defined periods (e.g. just before mitosis). On the other hand, cells whose flagella have been experimentally removed can regenerate their flagella. Experimental evidence has shown that during the process of regeneration, tubulin heterodimers add on at the distal end of the growing flagellum (the end farthest from the cell body; Rosenbaum et al., 1969). For more information on microtubule structure and assembly, as well as further detail on flagellar structure, please consult your textbook.

The experiment you will carry out is adapted from an exercise of Bregman (1990) and is based on a study by Rosenbaum et al. (1969). You will be examining the effects of various drugs on flagellar regeneration in *Chlamydomonas reinhardtii*, a single-celled green alga. *Chlamydomonas* is an excellent model organism for studies of flagellar motility and it is used by numerous researchers. One useful feature of these cells is the ease with which they can be induced to either lose or regenerate their flagella, simply by modifying environmental conditions. One condition which induces **deflagellation** (loss of flagella) is an abrupt decrease in the pH of the culture medium, known as pH shock. Following pH shock, the flagella fall off into the medium and can be separated from the cell bodies by centrifugation. The cell bodies can then be resuspended in fresh medium, either with or without drugs, and flagellar regeneration followed over a period of time. This flagellar regeneration, which is an example of how cells can assemble complex organelles, depends upon the process of microtubule polymerization, as well as on a process called intraflagellar transport (IFT). In humans, mutations in the IFT system may produce cells that are unable to assemble flagella; such mutations result in a number of serious diseases, including polycystic kidney disease and some forms of blindness.

For today's experiment, each lab group will receive three aliquots (or samples of uniform size) of

deflagellated cells, each of which will then be resuspended in one of the following media:

1 - Minimal medium (also called Medium I and designated as "M")
This is the nutrient medium in which the cells are normally grown.
2 - Medium I containing 2 mg/ml colchicine ("colchicine")
3 - Medium I containing a specified concentration of an experimental drug, or other condition chosen from the list of possible drugs/conditions below.

Name of Drug	Function	Concentration
cycloheximide	inhibitor of translation	10 μg/ml
actinomycin D	inhibitor of transcription	50 μg/ml
other***		

***In addition to the drugs listed above there are two other experimental conditions you might consider. The first is to examine the effect of temperature (specifically of cold) on flagellar regeneration by allowing regeneration to occur both at room temperature and at 4°C (note that if you choose this condition you must actually measure room temperature). The second is to see whether or not the effect of colchicine is reversible. Prior to their assembly (polymerization) into a microtubule, previously synthesized tubulin dimers exist free in the cytoplasm. The drug colchicine binds to these cytoplasmic tubulin dimers. While such colchicine-bound dimers can add on to the growing ends of microtubules, they then block the addition of any more dimers. By this means, colchicine blocks microtubule polymerization. To determine whether the effects of colchicine are reversible, cells can be exposed to colchicine for 20 minutes, then washed free of the colchicine-containing medium and placed in fresh Medium I. If the binding of colchicine to tubulin dimers is irreversible, then removing the drug should have no effect.

Cycloheximide is an inhibitor of eukaryotic translation and actinomycin D is an inhibitor of eukaryotic transcription. These drugs thus inhibit protein synthesis at different steps and therefore might (or might not) block the synthesis of new α- and β-tubulin subunits.

Once your lab group has decided on a particular experimental condition (condition 3 above), you must develop a formal hypothesis for this condition. While there are a number of ways to describe a scientific hypothesis, it may help you to understand the *function* of a hypothesis by thinking of it as a *testable question*; you will be testing the validity of your hypothesis during this experiment. We will spend time in pre-lab discussing what a hypothesis is and how to formulate one.

During the lab session, cell samples will periodically be fixed with Lugol's iodine solution, which both kills the cells (thus arresting them at a particular stage of regeneration) and stains the flagella, making them easier to visualize with the microscope. This experimental procedure involves two distinct control conditions. Deflagellated cells (i.e. cells whose flagella have been removed) allowed to regenerate flagella in Medium I will serve as a control for measuring the normal rate of flagellar regeneration, while samples of non-deflagellated *Chlamydomonas* will allow you to determine the normal flagellar length at the beginning and end of the experimental period. Why is the latter an important control?

Procedure:

You will be working on this experiment in groups of two or three; for pairs, one person will be responsible for conditions 1 and 2 described above and the other person will be responsible for condition 3 above and the non-deflagellated control (see below). The instructor will carry out the deflagellation procedure, since the pH shock treatment will result in sufficient cells for the entire class.

1. Each individual should take one or two porcelain spot plate(s) and label it (using a wax pencil) with the condition (e.g. M) and their initials. You should then label the individual depressions in the spot plate with the sampling times: 0, 10, 20, 30, 40, 50, 60, 75, 90, and 105 min. The group member who is sampling the experimental-treated culture will also be responsible for sampling the non-deflagellated control culture at the beginning (0 min) and end (105 min) of the experiment.

2. To each depression in the spot plate, add 1 drop of Lugol's iodine and 2-3 drops of Medium I (M).

   When all groups have spot plates labeled and are ready to begin sampling, the instructor will proceed with the deflagellation as described below:

   A 150 ml aliquot of *Chlamydomonas* cells will be taken from an actively growing culture and placed in a 250 ml beaker with a magnetic stir bar. The remainder of the culture will be placed on an orbital shaker and used as the non-deflagellated control. The pH of the cells in the beaker will be monitored with a pH meter, and the cells subjected to constant stirring throughout the deflagellation procedure.

   The pH of the medium will be rapidly lowered to 4.5 (within 30 sec), by the dropwise addition of 0.5 M acetic acid. The instructor will immediately observe the cells using the phase contrast microscope to confirm that all cells have dropped their flagella. Following this, the pH of the medium will be returned to 6.8 by the dropwise addition of 0.5 M KOH (potassium hydroxide).

   The deflagellated cells will be poured into conical 15 ml centrifuge tubes, 5 ml per tube for experimental conditions and 10 ml for control and colchicine conditions, and spun at 1300 x g (top speed in a clinical centrifuge) for 5 min.

3. The instructor will aspirate the supernatant from all tubes, and give each lab group 1 tube of cells. To your tube you should add 5 ml of the correct medium (i.e. the appropriate medium for the condition you have chosen). **Each tube must be labeled** such that it is clear (a) which condition is in which tube (i.e. M, colchicine, etc.) and (b) to which group each tube belongs. After medium has been added to each tube, the cells should be resuspended using a pasteur pipet, then transferred to a 50 ml conical test tube which should be placed in the rack on an orbital shaker. The tubes containing cells exposed to conditions common to all groups (M, M+colchicine) will be shared between groups; these tubes will be set up by the lab instructor with 10 ml of the appropriate medium.

4. **IMMEDIATELY** after you have placed the tubes on the shaker, begin your sampling, since the first time point you should sample is 0 min. This means you must be READY to take your first sample as soon as the deflagellated cells have been resuspended.

5. At each time interval noted on the data sheet, fix a sample of cells as follows: Using the pasteur pipet in the culture for which you are responsible (NOTE: The pasteur pipets should have tape which is color-coded to match the label on the 50 ml tube. DO NOT put the pasteur pipets in the

wrong tube!) gently resuspend the cells to be sure you have a representative sample, not just cells left over from the last time point. Remove one or two drops from the culture and add them to the appropriate well in your spot plate. Using a toothpick, mix the cells with the Lugol's iodine to fix them quickly.
NOTE: When you fix your 0 min sample, be sure to note the time so you can take all other samples at the correct intervals.

6. At any time after the cells have been fixed you may begin measuring flagellar length. You do not have to measure all 10 cells within the 10 min interval. It will not hurt them to sit for longer once they have been fixed, however, you should begin measuring immediately after fixing your 0 min sample, and continue to measure flagella between all subsequent time points. Do not wait to measure the flagella until all the sampling is done, or you will be in lab for much longer than necessary!

With a fresh pasteur pipet (or one which has been rinsed thoroughly in a beaker of distilled water, then dried on a kimwipe) place a small drop of fixed cells on a clean slide. Remember that once the cells are fixed (killed) they will settle to the bottom of the spot plate, so be sure to mix your sample before putting some on a slide. Place a coverslip on your sample (remember, if you use too large a drop, the coverslip will float) and examine the slide at 10X magnification. DO NOT use vaseline to make wet mount slides; simply put the coverslip directly on the **small** drop of sample. Locate an area with a number of cells and adjust the slide so at least one cell is exactly in the center of the field of view. Once you have located the cells this way, you can switch carefully to the oil-immersion objective. Using a calibrated ocular micrometer with the oil-immersion objective, measure the flagellar length in each of 10 cells per time point. Since the two flagella will be about the same length for a particular cell, measure only one flagellum per cell. It will be easier if you try to find cells that have at least one fairly straight flagellum, although you may have to extrapolate for some slightly curved flagella. Record your measurements on Data Sheet 1.

The data analysis for this experiment can be done outside of lab. Remember, however, that you are responsible for sharing your data not only with the other members of your team, but with the class as a whole. Therefore, **you must complete the data summary sheets as quickly as possible**.

For each sampling time in your culture(s), calculate the mean flagellar length. Do this in ocular micrometer units first, then convert this number to actual micrometers. Enter these values on **Data Sheet 1 (Individual Data)**. On Data Sheet 2 (**Group Data**) , enter the mean flagellar length in micrometers obtained by your group for all conditions, including the non-deflagellated control. **Copies of group data should be put onto CLASS DATA sheets, which will be available in lab next week during your lab period.** Each group will receive a copy of these data, and all students will have to generate class means for each condition tested by their group. Using the combined class data, plot a curve showing flagellar length (ordinate) vs time (abscissa). While you may use a computer program to plot your data points, we prefer you to draw a best-fit curve for each set of data. Unless you are a whiz with a computer, this is probably best done by hand, using both a ruler and a French curve template. There are French curves available in the lab; you may use them there, but may not take them out of the lab. The lab instructor will show you how to use the French curve if you have not used one before. If you think you can use a computer graphing program to get a good curve, you must have your graph approved by the lab instructor prior to handing it in. The final results of this experiment, based on class data, will be included in the first full-length lab report.

References

Bregman, A. 1990. Laboratory Investigations in Cell & Molecular Biology. John Wiley & Sons., NY.

Lefebvre, P.A. and Rosenbaum, J.L. 1986. Regulation of the synthesis and assembly of ciliary and flagellar proteins during regeneration. Ann. Rev. Cell Biol. 2:517-546.

Rosenbaum, J.L., Moulder, L.E., and Ringo, D.L. 1969. Flagellar elongation and shortening in *Chlamydomonas*: The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. J. Cell Biol. 41:600-619.

Data Sheet 1 FLAGELLAR REGENERATION - **INDIVIDUAL DATA**

Lab Section _____ Condition _____

SAMPLING TIME (MIN)	**FLAGELLAR LENGTH MEASUREMENTS (OCULAR MICROMETER UNITS)**										**MEAN**	**MEAN (μM)**
0												
10												
20												
30												
40												
50												
60												
75												
90												
105												

SAMPLING TIME (MIN)	**FLAGELLAR LENGTH MEASUREMENTS (OCULAR MICROMETER UNITS) IN THE NON-DEFLAGELLATED CONTROL CELLS**										**MEAN**	**MEAN (μM)**
0												
105												

Condition _____

SAMPLING TIME (MIN)	**FLAGELLAR LENGTH MEASUREMENTS (OCULAR MICROMETER UNITS)**										**MEAN**	**MEAN (μM)**
0												
10												
20												
30												
40												
50												
60												
75												
90												
105												

Data Sheet 2 FLAGELLAR REGENERATION -- **GROUP DATA**

Lab Section _____

SAMPLING TIME (MIN)	**MEAN FLAGELLAR LENGTH (μM)**					
	NON-DEFLAG.	**M**	**COLCHICINE**			
0						
10						
20						
30						
40						
50						
60						
75						
90						
105						

FLAGELLAR REGENERATION -- **CLASS DATA**

Lab Section ___________ Lab Group ___________________________________

SAMPLING TIME (MIN)	**MEAN FLAGELLAR LENGTH (μM)** **NON-DEFLAG.**	**M**	**COLCHICINE**			
0						
10						
20						
30						
40						
50						
60						
75						
90						
105						

Lab Section ___________ Lab Group ___________________________________

SAMPLING TIME (MIN)	**MEAN FLAGELLAR LENGTH (μM)** **NON-DEFLAG.**	**M**	**COLCHICINE**			
0						
10						
20						
30						
40						
50						
60						
75						
90						
105						

Lab Section ___________ Lab Group ___________________________________

SAMPLING TIME (MIN)	**MEAN FLAGELLAR LENGTH (μM)** **NON-DEFLAG.**	**M**	**COLCHICINE**			
0						
10						
20						
30						
40						
50						
60						
75						
90						
105						

BIO 218 – Cell and Molecular Biology Lab **B.F.Mitchell**
Le Moyne College Department of Biological Sciences

ISOLATION AND ELECTROPHORESIS OF FLAGELLAR PROTEINS

Part I: Isolation of flagella

In this laboratory you will be using biochemical extraction procedures and high speed centrifugation to isolate flagellar proteins from the unicellular green alga *Chlamydomonas reinhardtii*. At various stages during this procedure, samples will be saved to be used in the second part of the experimental protocol: electrophoretic separation of proteins. For background on cell fractionation and differential centrifugation, see your text.

PROCEDURES

Students will work in teams to isolate flagellar proteins, as well as to carry out SDS gel electrophoresis. Since different teams may be working with different strains of *Chlamydomonas* (e.g. some of the motility mutants examined previously in the lab on motility), be sure to record what strain of cells your group has. The strain should be indicated on the flask in which the cells are grown (e.g. "137c", which is a wild-type strain or "sup-pf-1", which is a mutant strain).

1. Two groups will start with one 1 L culture of *Chlamydomonas*. Divide the cells evenly between 4 250ml centrifuge bottles (two centrifuge bottles for each group). Spin the cells in the Sorvall centrifuge (GSA rotor) at 3000 rpm (1000 x g) for 6 minutes at room temperature. Each group should work independently from this point onward. Discard the supernatant (medium) from two centrifuge bottles by immediately and **CAREFULLY** pouring most of the supernatant into the sink near the Sorvall centrifuge. Do **NOT** pour quickly, or shake the centrifuge bottle, as the cell pellet is very soft and easily dislodged. After decanting most of the supernatant, remove as much of the remaining supernatant as possible by aspiration (the lab instructor will demonstrate how to do this). Since the pellet is soft, it will be easy to aspirate and you must exercise caution not to suck up cells as well as medium. At this point it is more important to retain as many cells as possible than to get the pellet absolutely dry. After aspirating the supernatant, resuspend the cells (pellet) from the two centrifuge bottles in a total volume of 20 ml of 10 mM HEPES buffer, pH 7.4. This is best done by dividing the 20 ml of HEPES between both of your bottles, individually resuspending the pellets by ***trituration*** (this consists of repeatedly sucking the pellet up and blowing it out with the pipet; the instructor will demonstrate this), and then pooling them in a single 50 ml conical centrifuge tube. Each group should save a 1 ml aliquot of the culture to be used later for protein analysis and electrophoresis. This sample should be placed in a microfuge tube and kept on ice.

2. Spin the cells, being sure to balance the centrifuge, for 8 minutes at top speed (1300 x g) in a clinical centrifuge. Remove the supernatant by aspiration and resuspend the washed cells in 5 ml of ice cold HMDS (10 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 4% sucrose, pH 7.4). From here on the samples should be kept cold at all times. This means that ***both your solutions and your centrifuge tubes*** should be kept on ice.

3. Add 0.5 ml of 50 mM dibucaine to the cells (final concentration: 5mM) and immediately mix by swirling. Dibucaine is a drug (used as a local anesthetic) which causes the cells to lose their flagella. **After addition of dibucaine, a sample of the cells should be observed with the phase**

contrast microscope to be sure they are really deflagellated. If flagella are still attached to the cells, add another 0.5 ml of dibucaine and check again.

4. Add 5 ml ice cold HMDES (HMDS containing EGTA, aprotinin, and PMSF [1]), to your cell suspension and mix gently, but thoroughly. (final concentrations: 2 mM EGTA, 5 mM aprotinin, and 1 mM PMSF). Gently mix the cells with a 5 ml pipet, then transfer this solution to a **chilled** 16 ml round bottom tube. Spin the cells in the Sorvall (SS34 rotor) at 4000 rpm (2000 x g) for 5 minutes at 4°C. Using a 10 ml plastic pipet, carefully transfer the supernatant to a clean 16 ml tube and repeat this spin (5 min., 4000 rpm) to remove any remaining cell bodies. Save the pellet from the first spin, which contains the cell bodies, to use for the second half of the experiment (resuspend the pellet in 2 ml of ice-cold HMDEK [30 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA, 25 mM $KC_2H_3O_2$, 5 mM aprotinin, 1 mM PMSF, pH 7.4] and keep it on ice).

5. Using a 10 ml plastic pipet, carefully transfer the supernatant, which contains the flagella, into a clean, chilled 16 ml tube. Spin the tubes containing the supernatant in the Sorvall (SS34) at 10,000 rpm (9,000 x g) for 12 minutes at 4°C to pellet the flagella.

6. Carefully remove the supernatant from the flagellar pellet by aspiration (the instructor will demonstrate). Resuspend the flagella in 250 µl HMDEK.

7. Run a protein assay on samples of the following:
   a. whole cells
   b. deflagellated cells
   c. isolated flagella

The purpose of this assay (or test) is to determine the concentration of protein in your various samples, so you can determine appropriate volumes for loading onto an SDS polyacrylamide gel in next week's lab. What you will do is to run the protein assay on both your own samples and a set of "standards" of known protein concentration. This set of known concentrations will be used to plot a **standard curve** which will allow you to determine the protein concentration of your samples. The standard curve is plotted based on knowing two dependent parameters for the standards, that is

(1) the mass of protein (in mg or µg)
and
(2) the Absorbance of light at 595nm (the greater the amount of protein, the darker the solution and hence the more light absorbed as it passes through the cuvette)

Since you will determine one of these parameters (Absorbance at 595nm, or A_{595}) for your isolated cell fractions, you can use the standard curve to determine the other parameter (µg of protein).

You will be using bovine serum albumin (BSA, 1µg/µl) as a standard for the protein assay. Before you set up your protein assay, turn on the spectrophotometer, set the wavelength for 595 nm, and allow it to warm up. ***Run all protein assay samples (except blank) in duplicate*** (i.e. set up two identical tubes).

IMPORTANT: Immediately before taking an aliquot of any of your samples for use in the protein assay, be sure the contents of the sample are uniformly suspended. This is particularly important for your whole cell sample which has been sitting on ice for several hours so all the cells will have settled to the bottom of the tube. The best way to be sure the contents are evenly suspended is to finger vortex the tube; ask the instructor how to do this.

[1] EGTA is a divalent metal ion chelator which helps to inhibit proteolysis during the extraction procedure; aprotinin, pepstatin and PMSF are protease inhibitors; DTT (dithiothreitol) is a reducing agent to prevent oxidation of proteins.

7.a. Label and set up a series of tubes as follows:

Tube	dist.H2O(µl)	BSA(µl)	sample(µl)
1(blank)	25	--	--
2	20	5	--
3	15	10	--
4	10	15	--
5	5	20	--
6	--	25	--
WC	5	--	20 (whole cells)
CB	15	--	10 (cell bodies)
F	15	--	10 (flagella)

7.b. **AFTER** adding all the solutions in the table THEN add 2 ml of BioRad protein reagent to each of these tubes. Allow tubes to sit at room temperature for 5 minutes, then read their absorbance at 595nm. **Note**: the readings for all your unknown samples must be within the range of your standards. If they are not, see your instructor immediately for further instructions. Record your data in Table I.

8. **Mix 100 µl of each of your isolated *Chlamydomonas* fractions (WC, CB, and F, *NOT* your protein assay samples)** with an equal volume (100 µl) of 2X (double strength) SDS PAGE sample buffer in a **labeled** microfuge tube. Be sure to designate your lab group as well as which sample is in the tube. The samples may now be placed in the freezer until the next laboratory period.

9. **BEFORE next week's lab**, plot a standard curve, putting absorbance (A_{595}) on the ordinate and µg protein (BSA) on the abscissa. Using your standard curve, determine the protein concentration of each of your three samples. Decide what volume of each protein sample to load on a gel, given the following information:
   a. You have mixed your sample with an equal volume of 2X (double strength) sample buffer before loading it on the gel.
   b. You will be using "mini-gels", which can be run quickly and with minimal protein loads. You should (ideally) load 5-25 µl of sample (after it has been mixed with sample buffer) containing 2-50 µg of protein. If you do not have enough protein in some or all of your samples, then you should try to load **the maximum volume** of your **least** concentrated sample, and load close to the same mass of protein for your other samples, if that is feasible. Check your calculations with the lab instructor prior to loading samples onto the gel next week.
   c. You will be running duplicate lanes on your gel for each sample, so the maximum volume you can load in any one lane is half the total sample volume.

10. We will go over the use of the standard curve and the calculations for determining gel loads in pre-lab, however you should attempt to do these prior to coming to next week's pre-lab. You may work on this with your lab partners or individually.

References

Witman, G.B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. Methods Enzymol. 134:280-290.

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Table I: Protein Assay Data

Tube number	μg BSA	A_{595}	A_{595}	Average A_{595}	μg protein
1					----
2					----
3					----
4					----
5					----
6					----
WC	----				
CB	----				
F	----				

Data Table II

Sample	protein concentration (μg/μl)	mass to load on gel (μg)	volume to load on gel (μl)	total yield (μg)	
WC				-----	
CB					
F					

BIO 218 – Cell and Molecular Biology Lab **B.F.Mitchell**
Le Moyne College Department of Biological Sciences

ISOLATION AND ELECTROPHORESIS OF FLAGELLAR PROTEINS

PART II: Electrophoretic Separation of Flagellar Proteins by SDS-PAGE

The term electrophoresis refers to a group of techniques which allow for the separation of biomolecules on the basis of charge and mass. Samples are applied to a supporting medium such as paper, or agarose or polyacrylamide gel, and exposed to an electric current. Cationic molecules move toward the negative pole (cathode) and anions move toward the anode. The rate of movement is dependent upon both the charge and the mass of the particular molecule. See your text for additional information on protein separation and gel electrophoresis).

The technique of SDS polyacrylamide gel electrophoresis, or SDS-PAGE, uses a polyacrylamide gel as a supporting medium. The porosity of the gel can be controlled by regulating the extent of cross-linking. As the concentration of acrylamide is increased, say from 5% to 10%, the porosity of the gel is decreased and any given protein will travel more slowly when exposed to the same electrical current. In SDS-PAGE, the presence of the detergent SDS (sodium dodecylsulfate) in the acrylamide alters the properties of the system. SDS denatures proteins by disrupting non-covalent bonds and coats the polypeptides, resulting in a uniform negative charge for all polypeptides. The reducing agent β-mercaptoethanol is also added to disrupt any disulfide bridges present in the protein, either within a single polypeptide chain or between two polypeptide chains composing a multi-subunit protein. Since all proteins are now present as individual polypeptide chains with equivalent charge, the electrophoretic separation in this case is due solely to differences in mass. In other words, SDS-PAGE separates polypeptides on the basis of molecular weight: larger polypeptides migrate more slowly than smaller ones in a uniform electrical field. If a number of proteins of known molecular weights are run on a gel, a standard curve can be generated by plotting distance of migration vs. molecular weight. If a protein of unknown molecular weight is electrophoresed in parallel with such molecular weight standards, then its molecular weight can be determined.

The resolution of this system can be improved by using two layers of polyacrylamide gel with different degrees of cross-linking. The upper layer, or stacking gel, contains relatively little cross-linking, so polypeptides move through this gel quite rapidly. The lower or separating gel is more highly cross-linked, as well as having a very different pH, so the polypeptides encountering this layer are suddenly slowed down. The result of this sudden slow-down is that all the polypeptides in the sample pile up and get condensed into a tight band so that they all enter the separating gel at essentially the same time.

Another modification of SDS-PAGE involves using a gradient of polyacrylamide such that the percentage of polyacrylamide (and therefore the porosity of the gel) increases linearly from the top of the gel to the bottom. The use of such gradient gels can not only increase the resolution of individual protein bands, but also allows separation of proteins with a much larger range of molecular weights than is possible with a straight percentage gel (i.e. a uniform acrylamide concentration throughout the gel).

In this laboratory you will be running protein samples on slab minigels composed of a 4-15% linear gradient of acrylamide The samples you will run will include a set of molecular weight standards and your 3 samples from last week's lab.

Procedure

1. Prepare SDS Electrode buffer as follows. Mix 100 ml of 10X Tris-glycine electrode buffer and 10 ml of 10% SDS with 890 ml distilled water to make 1 liter of 1X electrode buffer. **BE SURE TO ADD THE SDS AFTER YOU HAVE ADDED NEARLY ALL THE WATER TO AVOID EXCESSIVE BUBBLE FORMATION.**

2. Prepare sample. **NOTE**: you will have done part of this procedure in the preceding lab period. Make sure you do not repeat what has already been done. The entire sequence of sample preparation is stated here so you can see how all the steps are related.
Each sample will be mixed 1:1 with a 2X (double strength) sample buffer (you did this last week), and placed in a boiling water bath for five (5) minutes. The sample buffer contains the following components:

   a) a tracking dye for monitoring the progress of the electrophoresis (in this case, Bromphenol Blue).

   b) glycerol to make the sample solution dense so it won't disperse into the buffer in the upper chamber of the electrophoresis apparatus.

   c) SDS and β-mercaptoethanol (or dithiothreitol-DTT) to denature the proteins.

   Remove the three samples you prepared last week from the freezer. **Be sure all the tubes are tightly closed**, then incubate them in the boiling water bath for 5 minutes, by inserting them gently, but firmly into the spaces in the floating tube rack. Gently swirl the contents at the end of this incubation. **Keep an eye on the tubes when they are in the water bath to be sure they don't pop open (when the contents heat up and expand), and fill with water!**

   Remove the microfuge tubes from the water bath, place in a rack, and allow to cool to room temperature. Obtain a microfuge tube containing molecular weight standards (in sample buffer) from the instructor. DO NOT boil the molecular weight standards.

3. Using a Sharpie, mark the bottoms of the wells (i.e. draw a line on the glass plate at the level of the tip of each "tooth" of the teflon comb). The instructor will demonstrate how to do this. The instructor will show you how to insert the gel into the electrophoresis chamber. **Immediately before inserting gel plates in the apparatus,** slowly and carefully remove the teflon comb. Do NOT do this ahead of time, or the gel will dry out.

4. Add SDS electrode buffer to both the top and bottom chambers of the electrophoresis apparatus.

5. Apply sample to wells in the following order. Samples will be applied using a pipetman. This is a very expensive and delicate piece of equipment and its correct use will be demonstrated by the instructor.

   Lanes 1 and 10 – 1X SDS sample buffer – 10µl
   Lanes 2 and 6 - molecular weight standards - 5µl
   Lanes 3 and 7 - whole cells - volume determined prior to lab
   Lanes 4 and 8 - cell bodies - " " " " "
   Lanes 5 and 9 - flagella - " " " " "

6. Put the lid on the electrophoresis apparatus and plug the leads into the power source. Electrophorese at 200 Volts until the dye front is within about 1 cm of the bottom of the gel (~30-45 min.). If your group is unable to run the gel for that long, discuss with the lab instructor the possibility of stopping the electrophoresis earlier...in any case, you need to run the gel as long as possible. The bottom line is: the closer you get the dye front to the 1 cm ideal, the better your results will be.

7. Remove gel from glass plates as demonstrated by the instructor, and stain gel as described below. Be sure to notch the bottom right corner of your gel (instructor will demonstrate) to keep the correct orientation during and after staining.

   The staining solution contains Coomassie Brilliant Blue, which binds specifically to protein. The destain will remove the Coomassie Brilliant Blue that is not bound to protein (i.e. it will eliminate non-specific staining) leaving a gel with one or more blue bands corresponding to polypeptides of various molecular weights. Each lab group is responsible for staining and destaining their own gel, and for measuring the distance of migration for the various protein bands.

8. <u>Staining gel</u>

   a. Be sure to wear gloves when handling your gel. This will not only prevent your hand from being stained blue, but more importantly will prevent you from leaving fingerprints on the gel. Remove the gel from the glass plates and immerse it in approximately ¼ to ½ inch of staining solution (10% isopropyl alcohol, 10% acetic acid, 0.025% Coomassie blue) in an airtight plastic container; set container on the platform shaker. Incubate for two hours or longer. (Incubation overnight or for several days is OK, but make sure that the box is tightly sealed so that evaporation of the solvents and precipitation of the stain do not occur.) The instructor will describe how to pour off the stain into the sink (be sure to flush well with water).

   b. After staining, place the gel in ~ ½ to 1 inch of destain (10% acetic acid; remember to wear gloves) for at least two hours on the platform shaker, then carefully pour off the destain and replace it with fresh destain solution. The gel should have a clear background by the second or third change. Sometime during the day following this laboratory **care-fully** remove your gel from destain (pour this down the sink and <u>flush well</u> with water). Be sure to pick up your gel from the bottom corners (the bottom has the highest percent acrylamide and is stronger than the top); handle it gently to prevent tearing. Lay the get flat on the light box and measure the distance (in mm) from the <u>top</u> of the gel (the bottom of the wells into which you loaded samples) to the leading edge (i.e. the bottom) of the protein bands in all lanes.
   - Measure <u>all bands</u> in both lanes containing your molecular weight standards.
   - For each of the lanes containing *Chlamydomonas* samples, choose three (3) prominent bands and measure the distance of migration for these (you may measure more than three if you wish). Note that you must use the same three bands for duplicate lanes, but not for lanes containing different samples. We will also photograph your gel and give each student in the group a copy of the photo. The instructor will assist you in photographing your gel. You should use the photo for further analysis of your gel (see questions at end of this protocol) and if necessary, you can redo the measurements used for your standard curve.

9. Determine the average distance of migration for each of your samples using your duplicate lanes. Use these averages for your graphing and data analysis.

10. Using the known molecular weight values for your standards, prepare a graph of mobility (R_f: in this case, calculated as distance migrated from origin of gel, in mm, divided by total length of gel, in mm) on the abscissa (x-axis) vs. molecular weight (in kilodaltons) on the ordinate (y-axis). Draw a best fit line for the points. Be sure to use semi-log graph paper (this can be obtained from the instructor).

The sample of molecular weight standards that you received contains the following proteins:

Component	molecular weight (Da)
Myosin	200,000
β-galactosidase	116,000
Phosphorylase B	97,400
Bovine Serum Albumin	66,200
Ovalbumin	45,000
Carbonic Anhydrase	31,000
Soybean Trypsin Inhibitor	21,500
Lysozyme	14,400
Aprotinin	6,500

11. Using your standard curve, determine the approximate molecular weight of three major bands in each of your samples: flagellar proteins, cell body proteins and whole cell proteins. How do the total numbers of protein bands (not just the ones you measured) compare in the various samples you loaded on the gel? What can you conclude from this regarding the procedure you followed to isolate flagellar proteins? **NOTE**: ***The identity of the proteins in the set of molecular weight standards is irrelevant***. They are listed only to let you know what proteins are being used for size comparisons. The only important information for you, in terms of identifying any unknown proteins, is that the molecular weight standards allow you to create a standard curve relating how big a protein is (i.e. what is its molecular weight) with how far it moved in the gel. Once you have the standard curve, you can use it to determine the molecular weight of unknown proteins run on the same gel. For these unknown samples, all you can determine directly from the gel is how far they have migrated. ***Since there are many possible proteins which could have the same or very similar molecular weights, a molecular weight close to one of the standards is NOT sufficient evidence to guess that your unknown is that protein, unless you have other reasons for expecting that particular protein to be in your unknown sample. For example, bovine serum albumin is a cow blood protein, and will not be found in Chlamydomonas. What proteins*** **might** ***you expect to see in flagellar samples?***