## Lab 4 - Flagellar regeneration in *Chlamydomonas*

**Introduction:**

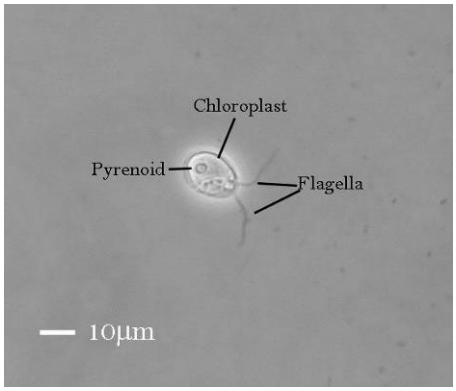


Figure 1: Phase contrast image of *C. reinhardtii*, showing locations of chloroplast, pyrenoid and flagella

*Chlamydomonas* are single-celled, motile green algae. They move by rotating two flagella that extend from the anterior end of the cell. Figure 1 shows a typical cell. These are photosynthetic eukaryotic cells that contain a nucleus, and a single large cup-shaped chloroplast. Within the chloroplast is a carbon-fixation region called the pyrenoid. The pyrenoid is encased in highly refractive starch molecules and so appears as a bright spot in phase microscopy.

The flagella contain cytoskeletal structures called microtubules which are in turn polymers of protein called tubulin (see Figure 2). The microtubules are arrayed into a cylindrical shape called an axoneme (see Figure 3), surrounded by an extension of the cell membrane.

*Chlamydomonas* loses its flagella just before dividing and after mating. The cell can regenerate its flagella by rebuilding the microtubule polymers of the axoneme. As the new flagellum grows, monomeric tubulins are transported to the distal end by molecular motors called kinesins, and added to the growing structure at this end. The process of regeneration is triggered by signals transduced through both Calcium ion and cAMP-activated pathways.

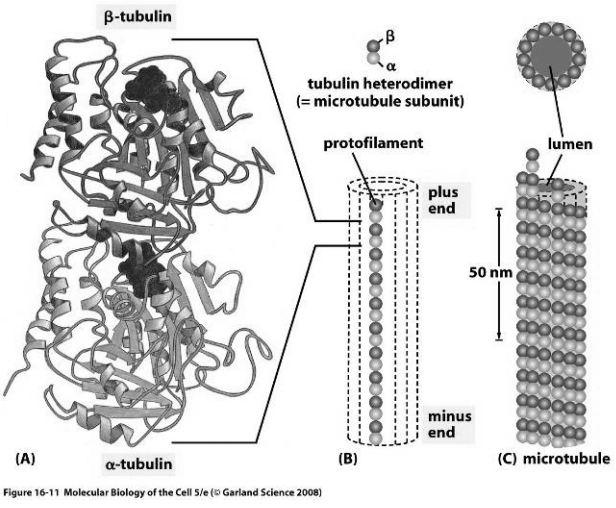


Figure 2: composition of microtubules showing tubulin monomers (adapted from Alberts et al, 2008)

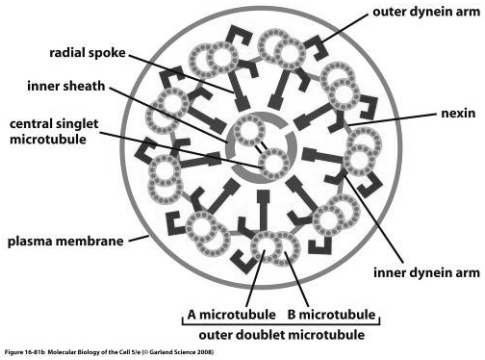


Figure 3: Cross-section of a flagellar axoneme, with microtubule polymers visible as rings (adapted from Alberts et al, 2008)

**The experiment**

1. Each lab group will start by familiarizing themselves with *Chlamydomonas reinhardtii* cells with intact flagella. This will include practice with immobilizing the motile cells and staining, visualizing and photographing the flagella.

2. Each group will then be provided with suspensions of *Chlamydomonas* cells that have been deflagellated by exposing them to a pH shock (pH 4.5 for 2 minutes, followed by returning the pH to approximately 7). You will be told what time was time zero. The flagellar length at time zero is 0µm.

3. You will monitor the length of flagella over time in your experimentally-treated and control cultures by taking photographs of flagella every 20-30 minutes over 90 minutes. Your lab instructors will provide you details about how your experimental cultures were treated.

4. As individuals, you will measure the lengths of the flagella from your photographs at each timepoint. You will pool the data from everyone in your group and plot the results. It should then be possible to reach some conclusions about the kinetics of flagellar formation and the effects of treatment with your experimental condition. Your measurements (raw data and calculated averages) only will be **due next week (April 2 for Dyneins, April 9 for Kinesins).**

**Sampling procedures**

There are many steps to keep track of during the lab. For example, it is important to monitor the time carefully so that you have good records of your timepoints. Keep careful records of the photographs you take and keep track of which timepoint and condition each photograph shows. Think about the data that you will be collecting and how to calculate and organize these results. Be sure that each group member plays a role, and that you each have a chance to photograph the cells.

*Important:* Many of the drugs used in these experiments are toxic, so handle anything that comes in contact with these solutions with care. Do not dump drug-treated cultures down the drain, a waste bottle will be available for their disposal. Used pipette tips and other waste will be collected in a plastic bag.

Each lab bench will have a 6-well plastic sample plate that you can label with a permanent marker. For each sample, including your preliminary practice with flagellated cells, follow these steps:

1. Immediately before sampling, transfer one drop of Lugol's solution (an iodine solution that will kill and stain the cells) from a *small pipettor* into a well of the sample plate. Using a *transfer pipette*, mix the cell culture sample by pipetting up and down in order to resuspend any cells

that may have settled to the bottom. Then *add one drop* of the cell culture to the drop of Lugol's solution; do not contaminate the pipette you use for cells with Lugol's solution so that you can use it for each sample timepoint.

2. Use a pipettor to mix the droplets if necessary and then take a 5 µl sample (you don't need to replace the pipette tip each time) and place it on a slide, immediately covering it with a coverslip. *You should be able to use one slide for **three separate samples** if you space them carefully (and label with care!).*

3. Examine the cells with phase contrast microscopy, starting with a lower objective and using the 40X objective for photography. Photograph 10 cells for each timepoint; since you will only measure one flagellum per cell, make sure that at least one flagellum is visible, note that the plane of focus for the cell body is not the same as the plane of focus for the flagellum. Ideally, you would want to choose 10 cells at random but in practice you will need to reject some cells if the flagellum is in knots or otherwise too tangled to measure. Cells that have undergone division recently may not have yet begun to regenerate their flagella.

4. Between each set of pictures, take some blanks to separate photographs from different timepoints.

5. Continue sampling at 20-30 minute intervals until you have reached 90 minutes from time zero.

6. Move the images to your computer (use the Scratch drive to move things to your U: drive). Be sure to copy an image of the micrometer calibration files along with your images. The calibration files needs to be taken from the same microscope that you used for these photographs.

**Using Image J to measure flagellar length**

The computers in lab and in LSB 428 have installed copies of ImageJ. (See appendix to install ImageJ on your computer)

Each of you will do this individually, you will hand in your data in discussion the week after lab.

1. Open ImageJ. Calibrate for your measurement by opening the micrometer image from your microscope.

2. Right click (Ctrl + click on Mac) on the **Lines Selection tool**, and select **"Straight Lines".** Click and drag a straight line across one or two segments (remember that each segment represents 10µm).

3. Then open **Analyze>Set Scale.** The number of pixels the line crosses is already calculated.

Enter the distance for the line you drew (10 or 20µm depending on the number of segments). Enter µm (or "um") as the unit, and check the "global" box so that this calibration holds for all the images you analyze in this session.

4. **Analyze>Set measurements.** Uncheck all the boxes and set decimal places to one.

5. Close the calibration image, and open your first sample image. Click on the **Magnifying Glass** and then click on the image to magnify it. Click on the **Scrolling tool** and drag the hand across the image to center it.

6. Right click on the **Lines Selection tool** again, and select **"Segmented lines"**. Click and drag the cursor to carefully trace along one of the flagella. Click every time you change direction; this allows you to follow a curved line more accurately. This takes a steady hand and some practice. *Double-click* to stop the line. If you are not happy with the line you drew, click somewhere else on the image to clear the line and start over.

7. When you are satisfied with the line, use **Analyze>Measure**. A small window will appear, showing the length of the line you drew (it will also have an "angle" column; ignore this). Move this window to a corner of the computer screen so it doesn't disappear behind an image.

8. Continue until you have measured one flagellum each from 10 different cells. You can either copy the lengths by hand or copy and paste them to an Excel file (**Edit>Select all>Edit>Copy;** open excel file and paste the results in).

9. Now, calculate a mean length from each sample timepoint and enter this into another table (sample and time in one column, mean flagellar length in the next).

10. Be sure to close the results window before starting the measurements from your next timepoint. Continue until you have measurements from all the timepoints from experimental and control samples.

Graph
After we have pooled all the data (one week after your lab), each of you should individually construct a single graph that shows experimental and control samples, with time after deflagellation on the X axis and mean flagellar length on the Y axis. If using Excel, make a **Scatter plot**, with one set of data as "Series 1" and the other as "Series 2" (you should give each series an appropriate name).

**The assignment:**
One week after lab (5 points): Bring printouts of your raw data and mean lengths to discussion.
Two weeks after lab (40 points): You will prepare a formal lab report from the pooled data of your group. More instructions about the lab report will be discussed in discussion group.

**Appendix: Downloading and installing Image J**

The computers in lab and in LSB 428 have installed copies of ImageJ. If you are using your own computer or one on campus that does not have ImageJ, you must download this software. It only takes a few moments.

1. For Windows:

a. Go to rsb.info.nih.gov/ij/download.html

b. Under "Windows", click on "Download ImageJ 1.41 bundled with Java 1.6.0_10"

c. Select "save to disk" and select the desktop as the destination.

d. It will take a few moments to download. When it is finished, double clock on the .exe installation file that now appears on your desktop. Follow the instructions for installation.

e. the ImageJ microscope icon should appear on your desktop. Double click on it to launch the program.

2. For Macs

a. Go to rsb.info.nih.gov/ij/download.html

b. Under Mac OS X, click on "ImageJ 1.41."

c. Select "save to disk."

d. An icon labeled "ij141.tar.gz" will appear on the desktop, double click on it, then double click on the ImageJ folder that appears.

e. the ImageJ microscope icon should appear on your desktop. Double click on it to launch the program.