Today your microscopy lab involves two parts. Part one is based on the use of epifluorescence microscopy to view live specimens. Part two focuses on the use of stains to enhance the use of bright-field microscopy.

### Part I: EPIFLUORESCENCE MICROSCOPY

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During your second week in lab, you learned how to use a microscope. In today’s lab, we are going to be using a microscope equipped for epifluorescence microscopy. The instrument you will be using today is an extremely sensitive and expensive piece of equipment and should be treated with care. In addition to following the rules for handling the microscope that were outlined in the previous lab, there are additional precautions that must be taken with epifluorescence microscopes. Please read and understand these additional rules BEFORE you approach the microscopes.

1. The mercury lamp connected to the microscope requires special handling, as gas is sealed under very high pressure inside the lamps. If not treated properly, the lamp can explode; if it explodes, leave the room immediately! (There is very little risk of explosion if these rules are followed carefully.)

   **First**, the mercury lamp MUST be turned on first, before the microscope can be turned on. This is because the lamp draws an enormous amount of voltage upon startup that can cause a spike in the line.

   **Second**, once the mercury lamp is turned on, it must be left on for at least half an hour. Turning it off prematurely will decrease the life of the bulb.

   **Third**, if you turn the mercury lamp off, you cannot turn it on again for at least 20 minutes. This is because you must let the bulb cool down to reduce the pressure before you ignite it again.

   **Fourth**, do not touch the mercury lamp house, as it gets extremely hot during use!

2. Excessive illumination will cause bleaching of your specimen, as well as wear on the filters, it is always good practice to keep the shutter closed (see picture below for location) whenever you are not actually looking through the binocular eyepiece. You can close the shutter by lowering the shutter knob; this blocks the optical path of the fluorescent light.

   You will notice that the microscope you will be using today is more complicated than the microscopes you have used previously. In fact, it is actually similar to a typical compound light microscope, but has some extra pieces attached to it for epifluorescence. A picture of an epifluorescence microscope with the component and operational parts labeled is on the next page (see Figure 1).
Today we will be examining fluorescent samples. Fluorescence is the property where a molecule (a chemical or a protein), when excited at one wavelength of light (the excitation wavelength), will emit a new wavelength of light of lower energy (the emission wavelength). We will be utilizing the intrinsic fluorescent properties of Green Fluorescent Protein (GFP) to examine the production of GFP in a specific subset of cells in an animal. The GFP is expressed in a specific subset of cells because it has been fused to a tissue-specific promoter that causes it to be expressed in certain types of cells. GFP has a maximal excitation wavelength of 470 nm and a maximal emission wavelength of 509 nm. GFP is only one of a number of fluorescent molecules that can be used for epifluorescence microscopy.

Figure 1. The parts of an epifluorescence microscope.

The type of microscopy we are doing today is called “epifluorescence” because it utilizes episcopic fluorescent light. Instead of the fluorescent light shining directly onto your specimen (which would result in direct fluorescence microscopy), the fluorescent light travels from the lamp house to a filter cube that is housed in the “Epi-fl attachment” at the front of the microscope. These filter cubes contain three types of optical components: an excitation filter, an emission filter, and a dichroic mirror (see Figure 2). (Note that you will not be able to see the filter cubes, as they are inside the microscope.) The excitation filter selectively allows the light within a certain range of wavelengths to pass through the filter. In general, you would want this filter to pass light close the maximal excitation wavelength for the fluorescent molecule you are using. The light that passes through the excitation filter is reflected by the
dichroic mirror, travels through the objective, and onto your sample. As the fluorescent molecule becomes excited and emits light, the emitted light is collected back through the objective, through the dichroic mirror to the emission filter. The emission filter is used to selectively allow only the light generated by the specimen to pass through the filter and blocks all other light reflected by the specimen, thus cutting out unnecessary background light. Light from the emission filter then passes to the eyepieces, for your visualization. The microscope you are using today has a filter designed for viewing GFP.

Another important part of the microscope is the fluorescent shutter. This knob controls a shutter, which is a physical barrier that blocks the light coming from the lamp house. When the shutter is closed, the light is prevented from reaching the filter block and the specimen. It is good practice to keep the shutter closed unless you are actually looking through the binocular eyepiece.

Today you will receive several live specimens expressing GFP. In these animals, GFP has been fused to a promoter that causes the protein to be expressed in cells where that promoter normally functions. For example, if you had a promoter that normally expresses in the eyes of an animal and you use molecular biology techniques to fuse the eye promoter DNA with the DNA that encodes for GFP and put this fusion gene back into an animal, the animal should now express GFP in its eyes (see Figure 3).

Figure 3. Tissue-specific promoters can drive GFP in specific body parts of animals.

**Materials:**
- *C. elegans* containing GFP under the control of a *pha-2* promoter on a petri plate
- *melanogaster* containing GFP under the control of the *hml* promoter, the *krüppel* promoter, and the *elav* promoter
- PBS (phosphate buffered saline) and pipettes to transfer PBS
- slides, coverslips
- bent spatulas to scoop out flies
- pointed probes to manipulate flies
- epifluorescence equipped microscopes

**Procedures:**
1. Familiarize yourself with the microscope. BE SURE TO READ THE RULES FOR OPERATING THE MICROSCOPE OUTLINED ON PAGES 59 AND 60. Find the lamp house (do not touch; it is hot), the shutter, and the lever on the epifluorescence attachment that changes the filter cube.
2. Look at your *C. elegans* strain using the 4x objective. You can look at these worms directly on the plate. First, be sure the fluorescence shutter is closed. Using the bright light, find the *C. elegans* under the microscope. Now, turn off the bright light, move the filter cube selector such that the correct filter cube is in place, and open the fluorescence shutter. What do you see? Using the picture of *C. elegans* below, identify the structure being labeled with GFP.

![Diagram of C. elegans](image)

3. Take your vials containing the three *D. melanogaster* strains. Today we will be looking at the third instar larvae, which can be recognized as the white larvae crawling on the sides of the vial. The brown creatures on the side of the vial are pupae, which are what the third instar larvae will turn into later in development; these animals are too old for us to visualize the GFP well.

The three strains you are looking at contain GFP under the control of the *hml* promoter, the *krüppel* promoter, and the *elav* promoter. One of these promoters drives the expression of GFP in the nervous system, one of these promoters drives the expression of the GFP in the fat bodies, and one of these promoters drives the expression of GFP in blood cells. Remember that the nervous system includes the fly brain (which can be found in the head). GFP expressed in the nervous system should also label the axonal projections from neurons, which can run the length of the animal. The fat bodies contain fat reserves and may look greasy, as they are filled with lipid droplets. Flies have an open circulatory system and therefore you should be able to see some of the blood cells moving around in the animal.

Using your bent spatula, pick up some third instar larvae from one of the strains. Put the larvae on a slide with about 40µl of PBS and place a cover slip on top of it. Set your microscope to the 4x objective, and using the bright light (be sure the fluorescence shutter is closed and the GFP filter cube is not in place), find the larvae under the microscope. Now, turn off the bright light, move the filter cube selector such that the correct filter cube is in place, and open the fluorescence shutter. What do you see? Once you have the specimen in focus, change the magnification by rotating the nose-piece using the ring. Go to 10x and 40x, you may need to fine focus as you change objectives. Do you think the GFP expression you are looking at is consistent with expression in the nervous system, the fat bodies, or the blood? Be sure to look at all three strains.

4. Once you identify the fly strain expressing GFP in blood cells, obtain a third instar larvae from that vial, put it in about 20µl of PBS, pull it apart using a toothpick such that the blood can
come out of the animal. Remove the larvae and put a coverslip on top of the PBS. Using epifluorescence, look in the microscope at the blood cells. Draw what you see.

Part II:

**CHROMOSOME STAINING AND STRUCTURE**

**CHROMOSOME STRUCTURE OF *DROSOPHILA* SALIVARY GLAND CHROMOSOMES**

In *Drosophila* the chromosomes of the germ cells and most somatic cells are very small. The larval salivary glands, however, contain very large nuclei. In these nuclei there are giant (polytene) chromosomes. Usually chromosomes contain one continuous double helix of DNA. Each giant chromosome is actually many copies of this DNA, all perfectly aligned side by side, to form a much thicker chromosome than normal. Genes on one copy of a chromosome are aligned with those of every other copy of the same chromosome. Thus, each polytene chromosome has a characteristic pattern of cross bands (banding pattern) due to perfect alignment of all the many DNA copies of the chromosome. These bands are particularly striking when stained, but may even be observed in unstained, living nuclei.

Although these nuclei are in interphase, meaning the DNA exists as chromatin rather than chromosomes, the interphase chromosomes are visible because of the many DNA copies. Although arguable, most researchers believe the dark bands are tightly coiled DNA, sometimes referred to as heterochromatin, and thought to be transcriptionally inactive. The several polytene chromosomes of *Drosophila* are attached, at a structure called the chromocenter. The chromocenter is believed to be composed of centromeres and heterochromatin adjacent to the centromere.

The bulbous, or puffed, regions are called chromosome puffs. They are regions where high levels of transcription are occurring. The many copies of DNA in that region have been separated from each other, sort of like a rope fraying at the end, to permit easy access to the DNA by the RNA polymerases. The puff regions have very high levels of transcription occurring; there are certainly many other areas along the chromosome where transcription is also occurring. These other areas may appear as smaller puffs, or they may not even be puffed. Their transcription levels are much less than in the large puffs. Each large puff consists of at least one gene, but more frequently consists of a group of genes, all of which have been turned on by the same signal (hormone, growth factor, etc.)

This laboratory exercise outlines the mechanism by which salivary glands are removed and prepared so that the polytene chromosomes may be observed. *Drosophila virilis* is used instead of *Drosophila melanogaster* because *D. virilis* is much larger and it is easier to dissect and remove the salivary glands from the larvae of this species. Do not become frustrated, this is difficult to do and may require a number of attempts. At the end of this lab section, there is an image of several larval body parts dissected from a *D. melanogaster* larva. All of these parts are located in the anterior part of the larva. Try to identify them as you are making your preparation (you are of course going for salivary glands!), because you will need this knowledge in the Cell Cycle Lab when you will have to do the dissections again to obtain larval brains.
Salivary Gland Preparation (Squash technique)

Materials
Fruit fly larva (*Drosophila virilis*)
Ringers insect saline
Fine forceps and probe
Microscope slides and coverslips
Dissecting and compound microscopes
Aceto-orcein
Insect pins
Lens paper and paper towels

Procedure
1. Select a third instar larva, for which the cuticle has not yet hardened, from a wild-type culture of *Drosophila virilis*. Place it into a drop of Ringer's saline solution on a slide. Periodically add a drop of ringers saline solution to your sample to keep it from drying out.

2. Place the slide on the stage of a dissecting microscope and view the larva with low power. Grasp the anterior of the larva with a fine point forceps and pin down the posterior portion with a probe. Gently pull the head off and discard the tail of the larva.

3. Locate the salivary glands and their attached fat bodies. (See figures a, b and c, and a photo at the end of this section) The glands are semitransparent and attached by ducts to the digestive system. The fat bodies are white and opaque. Tease away the fat bodies and discard.

4. Place a very small drop of aceto-orcein on the slide next to the Ringer's and move the salivary glands into the stain. Blot away any excess Ringer's.
5. Place a coverslip over the preparation and allow it to stand for 1-3 minutes (it will take a few trials to obtain properly stained chromosomes). Gently squash the gland preparation in the following manner:
   1. Gently press on the middle of the coverslip with a pencil eraser and carefully move the coverslip in a circular motion with the pencil eraser.
   2. Gently tap all around the coverslip, with the pencil eraser.
   3. Observe the stained salivary glands.

6. Examine the slide with the microscope using 40x magnification, find, center and focus on the chromosomes. Half rotate the 40x objective lens away, so that no objective is in place but the 100x is the next to rotate in. Add a small drop of immersion oil to the cover slip and rotate the 100x objective onto the coverslip. You cannot go back to lower magnification at this point without cleaning the oil off of the slide, so stay at 100x. Diagram the banding patterns that are observed on the chromosomes.

7. Compare your squash preparation to that of the prepared slides.

Results
- Examine the slides for the presence of bands. Select a single chromosome spread demonstrating all four chromosomes and draw the complete structure.
- Try to find and label each of the four chromosomes of the fruit fly, as well as the chromocenter of the connected chromosomes.
- Compare your drawings to the demonstration slides and the handout of the genetic map for Drosophila.

Questions
1. Can you detect the banding pattern?
2. Can you determine the chromosome number for D. virilis from the slide preparation you have made?
3. Would you expect the salivary gland cells to possess the haploid or diploid number of chromosomes for this species? Why or why not?
The following image may be useful for finding things inside the larva. Left to right, the following organs were dissected:

- Larval brain with two spherical brain lobes (top left), one brain stem (bottom), and two leg imaginal discs (top right). Imaginal discs are the larval precursors of the adult body parts.
- One salivary gland, somewhat transparent (this is the part you want!) with a whitish attachment on the top which is the fat body. You should trim away the fat body as much as you can because it does not contribute to the prep. In the larva, the salivary glands are paired with a common stalk (duct). Sometimes during dissection both salivary glands come out together, still attached by the duct. In fact, you can use this duct to grab the glands and move them around.
- Wing imaginal disc – a precursor of the fly’s wing.
- Eye-antennal imaginal disc – these are the precursors of both the eye and the antenna of the fly and are frequently attached to the brain lobes.
Part II: COMPUTER INTERFACED MICROSCOPY

The objective of this part of today’s lab is to familiarize yourself with the imaging software ImageJ as well as a camera interfaced microscopy system used to capture images of cells. In upcoming labs, you will be expected to know how to use ImageJ remotely for measuring digital images, in particular protein bands on the digital photos of your SDS-PAGE. Today you will first become acquainted with digital microscopy imaging, and then learn to use ImageJ to measure cells and organelles.

Modern microscopy often incorporates computers, cameras and imaging software. Many benefits exist: photo-documentation of a specimen or results, precision measurements, developmental videos, the list goes on. Most useful is its role in teaching, displaying a specimen on a monitor for a group to see allows both the instructor and student to recognize what each is seeing. An added bonus, is comfort for the eyes, neck and shoulders for those working long hours on microscopes, not to mention the increase in the speed of measuring large quantities of specimens.

Today you will have time in lab to become familiar with the imaging set ups we have in lab. We have 4 stations, each consisting of a computer and a stereoscope with a built in camera. The software on the computer for capturing a photo is called Motic Images, it is also the maker of the cameras we have. After capturing photos and saving them, you will use a different program to measure images. Though Motic has measurement capacities, we would like to show you an excellent and widely used, free version that you can download onto your own computer. It is available from the National Institute of Health’s (NIH) web site and is called ImageJ, and you will be using it to make measurements today and in later labs within this course. (See the appendix, ImageJ, to download this software at home)

Your group will work at one station. You can each take turns on the microscope, and still be able to all look at the monitor to see what is being magnified. The computers will be turned on already, and the software you will use, will be open and ready to use. The cameras will also be turned on and hitched up. Your task is to get comfortable with the scopes and software. These computers are not online. If you want to save images beyond use in lab today, transfer them off of the computer onto your CD, thumb-drive or email it to yourself.

Procedure Part A : Capture the image using Motic Images software
1. Get both a prepared slide to look at and a stage micrometer from the tray on your table, bring them to your station, and place the micrometer on the stage of the microscope you will be using.
2. Turn on the lamp, look through and focus on the finest divisions on the micrometer.
3. Now turn to the computer monitor. When Motic Images is started you should see the main interface window:
4. Using the mouse, move the cursor to the top center of the menu bar. Find the capture window button, with the image of a video camera on it and click on it.

It will open up what is called the capture window, it is real time video of your specimen or in this case the micrometer, sent from your microscope to the monitor with the capacity to capture still images and save them.

5. You should now see an image of your specimen (the micrometer) in that window. You can increase the magnification, move the slide around or alter the light and be able to see it on the screen just as you do if you are looking through the oculars of the microscope.

6. When you are ready to capture and save and image, simply click on the green camera icon (the capture still image button) it should be the second to the bottom on the vertical tool bar. Or go to the menu bar of that window, to Capture, click and select still image, move down and select. Give it a few seconds, you should see a second larger image appear in the window. Your active, real time capture frame should be smaller than the image just captured.

7. The program has captured and given the image a number, you need to save it and name it, otherwise it will not be saved and if you do not name it, you will not find it again.
   - To Save and name it, click on the image it captured, go to “file” on the menu bar of your captured image. (Keep in mind the micrometer is for calibration, use that in the name, and the objective used)
Select “Save As,” a save as dialog box will appear with three things for you to fill in:

- **Save in**: where you save it; select desk top, or your disk’s name
- **File name**: what you call it; type in an informative name you will recognize
- **File type**: select Jpeg, they are smaller files (use the arrow on the right to see the list)

8. Now, turn back to the microscope, increase the magnification, and capture another photo of the stage micrometer, name it, save it as a Jpeg and to the desktop. Then repeat at yet another higher magnification. Name it, save it as a Jpeg and to the desktop. You should now have 3 micrometer images, each at a different magnification. These will be for calibration, just like when you calibrated your microscopes in the first microscope lab, only this time you are using a computer, and a digital image for calibration of size at each magnification.

9. **Now you can take additional images of some interesting biological samples**. There are specimens on the instructor’s table, choose four different types to focus on. On each slide, there may be a variety of different cell types that make up that tissue or a lot of the same type of cell. Don’t forget to indicate magnification used within the names of images you save.

**Procedure Part B: Measure a Specimen or a Gel image using ImageJ software**

1. Minimize the Motic Images screen by clicking on the minimizing button, the first of the three buttons in the upper right corner of the window, it looks like a dash. The window should disappear, but notice that a Motic Images tab is on the lower left corner of your screen and if you click on it the screen will maximize and reappear. It is not closed.

2. There should be a small gray toolbar that says ImageJ on it, on your screen. Go to file on that menu/toolbar, and scroll down and select open file, select from the desktop or your disk, depending on where you saved your images. Look for the name of the images you captured; select the lowest magnification calibration image first, the stage micrometer.

3. Once you see your image, click on the straight line selections drawing icon on the toolbar, it is the fifth box in from the left as seen above. Use it to draw a line of known length along the image of the stage micrometer or if you are looking at a gel, along the ruler in the photo. (If you click on a button, the name of it appears on the bottom of the toolbar.) To draw a line: point the crosshair where you want to begin the line and click, drag it to where you want to end the line and release.
4. If you now go to the menu bar and select analyze, select set scale. You will see a dialog box like the one below, with the length of the line in pixels highlighted, and other boxes to check or fill in. To turn pixels into a meaningful and proper scale, you need to calibrate by equating that pixel number to the distance you know to be true, the known length of the line you drew.

![Set Scale dialog box](image)

4. Use the mouse to move the cursor to the known distance area and click inside the box to fill in what you know to be true. Fill in the units they may need to be changed to microns if using a micrometer (cm or mm if looking at a gel image). Then select global, so it will apply your calibration to your other images as well. This is only accurate if all images were taken at the same magnification. Double check your scale by drawing a new line of a length you can estimate, go to analyze and select measure. It should now give you readings in your known units. *(If you are looking at a gel image, draw a line the distance from the well to a band and then go to analyze, measure to see the mm migrated.)*

5. Now you can open other images of specimens/prepared slides that you took at that magnification and measure the diameter of cells and organelles, or the area. Before you move on to images taken at a different magnification, you must reset the scale using an image of the stage micrometer. Make measurements of the different cells on the images you captured across the 4 different tissue types and record in your notebook some average cell sizes for each tissue type.

6. Get two prepared slides of larger whole specimens and use the stereoscope next to your computer set up to photograph the specimens. You simply need to go to source select and switch from the compound camera to the stereoscope, your TA can help you with this. When using the stereoscope put some cubic centimeter graph paper beneath the slide, the fine lines are one millimeter apart. This way you will be including a known scale in your images, you can set the scale for each image created on the stereomicroscope. Just be sure to capture images with visible lines, they will be a bit out of focus, since your specimen should be sharp in focus. Then use imageJ to open and measure what you photographed.

**By the end of class each group should have:**
- Reviewed how to use ImageJ to analyze SDS-PAGE.
- Digital images taken at various magnifications with Motic software, of cells typical of four different types of prepared slides.
- Made measurements of typical cell size for the specimens photographed.

*If you wish to download ImageJ at home see the appendix for additional information.*