**Lab 1: Learn to use the fluorescence light microscope.**

***Learning Goal:*** *Familiarize with the use of a modern research microscope.*

***First thing first: Microscope Care***

You should treat your microscope like the delicate instrument that it is. Your microscope and camera cost over $35,000. The objective lenses cost between $780 and $2300 each. Lens surfaces are coated with antireflective materials that can be scratched. Do not touch the top lens element with your dirty fingers, or anything else for that matter. If the lens appears to be dirty, we can use air to blow dust off of optical surfaces. We will use the 100x oil immersion objective in this class, so you will need to clean oil off of the objective lens when you are finished for the day. Let the TA or instructor know if you think an objective is dirty.

When moving parts of the microscope system, be gentle. If a part of the microscope doesn’t seem to work properly, *don’t try to force it to move.* Ask an instructor if you have a question. Learn the parts of the microscope and how they function so that you’ll know if there is a problem with something.

The microscope should be covered when you are finished for the day. Never put the cover on when power is applied to any part of the microscope. The lamps and electronics produce significant amount of heat that can cause damage if not properly ventilated. *Always double check that all power is turned OFF before you cover the microscope. This is very important.*

*For this module we will use the 100X objective lens that will require immersion oil. Please make sure you ask which is the appropriate immersion oil to use.*

***Using the Microscope (Lights! Camera! Action!)***

During the first 3 labs of this module, we will make extensive use of the microscope to observe living, fluorescently-labeled yeast cells. Today, you will learn to place the slide on the microscope stage, find the cells, focus, and take a picture.

The microscopes will be ON and the objective that you will need to use will already be in place.

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| ***Information for Turning on Microscope***TURN ON PROCEDURE**[It is already turned ON for you today]**1. Remove the microscope cover.2. If you plan to use the fluorescence epi-illuminator, turn ON the epi-illuminator power supply (large black box) using the toggle switch on the front, and then push and hold the small start button until the yellow light is illuminated. 3. Turn on the transmitted light illuminator (small gray box) and the light switch on the microscope (lower left side).4. If you plan to use the fluorescence shutter, turn on the shutter controller (black box labeled Sutter or Vincent). Switch on back.5. If you plan to take digital images, turn on the camera (toggle on far end), and then the computer (power switch on back).When you are finished with the microscope, turn off all of the lamp power supplies and electronics. The order in which you do that does not matter. |

***Find, view, and focus in transmitted light***

First we will view cells with transmitted light and then we will learn to capture an image with the camera.

1. The nosepiece contains all the lenses; we will only need to use the 100X lens. This objective should have been rotated to the right position, facing up, toward the stage opening. [Note: when rotating the nosepiece, use the knurled part at the base, not the lenses.]
2. Gently apply a drop of immersion oil onto your slide, the coverslip side, on the region where you could see the spread yeast cells on the agarose pad.
3. Place the glass slide on the stage with the coverslip DOWN, facing the objective lens. This is an inverted microscope, so the slide is also inverted. Use the stage clips to hold the slide in place.
4. Turn on the transmitted light (the controller box should be on, and the button on the lower left of the microscope needs to be ON as well; the dial on the left side of the microscope controls the light intensity). Set the light intensity at a level appropriate for visualization through the eyepieces (i.e. not too bright). Too much light may heat the yeast cells on the agarose pad.
5. Find the cells. To do this, gradually turn the focusing knob, raising the objective until you can visually see that it touches the immersion oil drop on the slide. Then, with your eyes looking through the eyepieces, slowly turn the focusing knob, moving the objective lens toward the slide. You can simultaneously move the slide left and right (and north and south) with the stage x-y position controller – this allows you to identify the sample even when it is still out of focus. If you can’t find the cells, ask for assistance.

Once you have found the cells in transmitted light, you can look around the slide. Try to identify individual yeast cells. See if you could identify the mother and bud compartments of a yeast cell.

***Capturing an image with digital camera***

Most research microscopes are equipped with cameras to record the image that you see. In addition, a major advantage of using a digital camera is the ability to quantify your images using image analysis software. Although the details of digital imaging are beyond the scope of this class, you will learn how to capture an image with your camera, and how to use the shutter to control exposure time or minimize photobleaching (more on photobleaching below).

Once you have located cells with your eyes (through the eyepieces) using the **transmitted light**, you will then look at the cells with the digital camera. To do this:

1. Open the Micro-Manager program (double click on the microscope icon). Select the correct configuration file (Vincent or Sutter config file, depending on your shutter) in the Micro-Manager window to tell the software about your hardware. Note that the camera must be turned ON for the software to properly initialize.
2. Rotate the optical path switch dial on the bottom right front of the microscope. This places a mirror in the optical path that moves the image from the eyepieces to the camera. The image in the eyepieces will disappear. Do this GENTLY, but completely turn the lever so that it clicks into place.
3. Activate the camera using Micro-Manager by clicking the “Live” button. If the image is too dark or too bright, adjust the light intensity knob in the microscope. You want to aim for a sharp image with good contrast. You would need to adjust the focusing knob to get a sharp image, which in this case occurs at the equator of the yeast cells.
4. To take a picture, turn OFF the “live” imaging. Click “Snap”. If the exposure is too dim or too bright, adjust it by changing the “Exposure (ms)” value. Try “Snap” again.
5. Save the image using the “Save” at the button of the image window. Make a folder (name it with something you can remember, such as the date and/or your name(s). For example: 20140121\_Lee group).



***View and focus in fluorescence light***

The yeast cells on your microscope slide are expressing a chromosomally-tagged GFP-tubulin fusion protein. They are alive and growing on the agarose pad prepared in non-fluorescent synthetic growth medium! GFP-tubulin will label the microtubules in the cells, thus allowing you to observe the mitotic spindles in the cells. We will learn to view cells in fluorescence today. To do this:

1. Select the appropriate filter. The filters are located beneath the objective lens, in a black box. Filters are selected by turning the knurled ring of the filter box; each filter is labeled (UV, G, B, top right side). Select “B”.

Your microscope is equipped with four filter sets, each used for a particular fluorophore.

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| **Label on filter wheel** | **excitation λ** | **emission** **λ** | **Used for** | **Label on filter** |
| UV | UV | blue | DAPI | UV 2E-C |
| B | B | green | GFP, fluorescein | B-2E/C |
| G | G | red | Texas red | Y-2E/C |
| LP | B | long passgreen — red | chlorophyll and GFP simultaneously | B-2A |

1. Turn off the transmitted light, then open the epi-illumination field block (move the lever away from you (right side of filter box); open “O”, closed “C”). You will see a bright blue-ish light illuminating your sample when the epi-illumination field block is open.
2. Look at the cells through the eyepieces. Close the block when you are finished with the observations. Illuminating the sample with strong excitation light for extended period of time will photobleach the sample.

What do you see? You may need to adjust the focusing knob while looking through the eyepieces.

1. Try ND filters: The more intense the excitation beam, the faster the photobleaching of your fluorescence, resulting in loss of fluorescence. Try reducing the excitation beam intensity. Locate the epi-illuminator neutral density (ND) filters at the back of the microscope. They are on the right hand side, just before the light path from the arc lamp housing to the far right makes a turn into the microscope. Turn the shutter on again and play with these to see how they control the fluorescence excitation light level. The ND 4 filter reduces illumination intensity by a factor of ¼. The ND 8 reduces intensity by a factor of 1/8. Together they reduce the intensity by a factor of 1/32. Sometimes, if in an experiment the fluorescence is too bright, you may use the neutral density filters in the excitation beam.

***Capturing an image in fluorescence light***

1. Turn off the transmitted light. Your system has an electronic shutter to control the fluorescent light. The electronically-controlled shutter is handy because it prevents photobleaching of the fluorophores in the sample when the camera is not capturing images.

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| ***Use of the electronic shutter:**** 1. **Sutter controller:** The epi-illuminator shutter is manually opened by switching the toggle switch on the controller box to “Open” and is manually closed by switching the toggle to “Close”. With the toggle in the middle “Auto” position, the computer controls the shutter.
	2. **Vincent controller:** The epi-illuminator shutter is manually opened by switching the right toggle to “Local”. Then switching the left N.O./N.C. (normally open/normally closed) toggle on the controller box to “N.O.” opens the shutter and switching to “N.C.” closes it. With the right toggle in the “Remote” position, the computer controls the shutter. The key switch should be in the “Std” position.
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1. Open the epi-illumination field block (hereafter referred to as epi-block) for the fluorescent light. Now close and open the shutter and see what happens. Set the shutter for computer control (no light should be on the sample, even though the block is open).
2. Take a single fluorescence image by clicking the “Snap” button in the computer’s Micro-Manager program window. The shutter should open for the duration of the exposure and then close. If the picture is too dim or bright, adjust the exposure by changing the “Exposure [ms]” value. Save the image to disk by using the “Save” button at the bottom of the image window.

Whenever possible, use the electronic shutter when taking fluorescent images. The shutter is not in the transmitted light path, so for these images you should leave the shutter closed.

1. As you are learning to take images, take note of what part of the field of view is seen by the camera (this is due to a magnifying lens that sits in front of the camera). This will help you to correlate what is observed through the eyepieces with what will be captured by the camera.
2. As you are learning to take images, take note of any focal shift that occurs when you go between the eyepiece and the camera. When the camera is at the same exact focus as the eyepiece, it is said to be parfocal. Your camera may not be parfocal with the eyepieces.
3. As you are learning to take images, get a feel for the response time of the camera, and the camera’s resolution compared to your eye’s resolution.

Can you find out how many pixels make up one image that is captured by your camera?

***Determine the size of a yeast cell***

You now have several digital images of cells. What sorts of information can be gotten from these images? One obvious thing you might need to know is the size of a yeast cell. This can be done once you calibrate the imaging system. To do this, you will use a calibrated stage micrometer, a slide that has a very fine ruler etched into it.

1. Remove the yeast slide and replace it with a stage micrometer slide. Make a note of which side the stage micrometer is on. Gently apply a drop of immersion oil onto the micrometer side. This side faces the objective lens.
2. Using transmitted light, locate the stage micrometer by eye, center it, and get it into sharp focus. If you are having trouble locating the micrometer, look at it from above the microscope stage. You should be able to make it out with your eyes. Move it to as close to the center of the objective lens as you can and then try to find it in the microscope through the eyepieces.
3. Snap a camera image of the stage micrometer, optimize it and be sure it is in sharp focus. Save the image.
4. Switch to the ImageJ window. Click on the line symbol and draw a straight line from one stage micrometer hash mark to another. It will be most accurate if you draw the line from hash marks as far apart as possible.
5. From the “Analyze” pull down menu, select “Set scale…”. The window that pops up will show you the distance in pixels of your line. You need to type in the actual distance between the two hash marks that you used. The micrometer is divided into 10 micron divisions (smallest divisions) and 100 micron divisions (larger divisions). If you can’t figure this out, ask for help. Be sure to use the correct units of measurement in the “Set Scale…” box. (1 cm = 104 μm.) Leave the aspect ratio set at 1. Your camera has square pixels, so the aspect ratio (height:width) is 1.
6. Determine the size that a camera pixel represents when the 100X objective lens is used. You can do this by dividing the known distance between hash marks by the number of pixels from step 5 above. This value is the number of µm per pixel (or cm per pixel if you prefer). This tells you how much of the specimen area falls on each pixel in the camera. This value will remain the same for any future use of this objective lens and camera in this microscope. The value will be different for different lens (and for different cameras, if you happened to use a different one). Make note of this number – you need it to convert pixels in your images to microns.



**Example:** if the distance between two hashmarks is 40 microns and the number of pixels is 200, then 1 micron = 5 pixels and 1 pixel = 0.2 microns. To measure the size of a cell, draw a line using the tool in ImageJ, from one edge of the cell to the other. Using the conversion factor, you can now figure out how long the cell is in microns.

***Task: What is the average size of a yeast cell?***

Take multiple fields of cells. Measure the diameter of as many yeast cells as possible using ImageJ (with the line symbol selected in the tool bar, goto: Analyze>Measure). Ensure you have a significant sampling size (at least n > 50 cells). Plot your size distribution in a histogram. Write a caption (=Legend) describing the graph and indicating the average diameter (remember to specify the measurement unit) and standard deviation.

After you are done for today, please remember to discard (in the glass trash) your yeast slides.