Microscopy Bootcamp

Goal: Learn to use the light microscope.

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! 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 lots 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.***

The 10X and 40X objective lenses that do not require oil. You should not apply any immersion oil to these lenses. For certain exercises you will use the 100X oil immersion lens; follow instructions in the proper use of the immersion oil.

Task 1. Turn-on the microscope and the computer

TURN ON PROCEDURE

1. Remove the microscope cover or open the cabinet.

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.

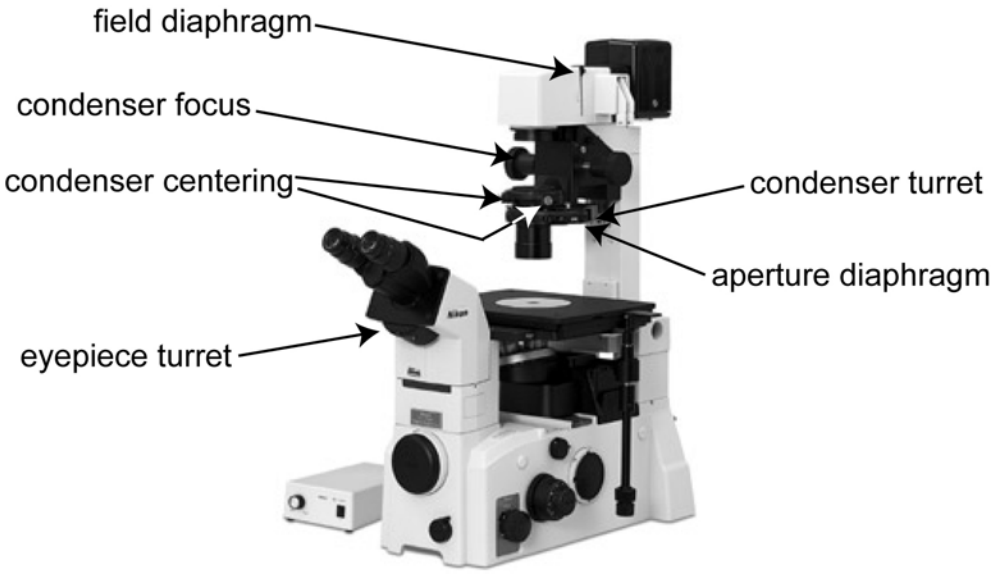
(This start procedure involves a high-voltage spark in the lamp. The sparking produces a voltage transient that can disrupt sensitive electronics. The computer should be turned off when you start the epi-fluorescence lamp. You should keep hands off the microscope while starting the lamp.)

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.



Nikon TE2000; image from inverted microscopy TE2000 instructions.

Task 2. Viewing cells in the Microscope

First we will view cells with transmitted light and then we will image cell nuclei that have been stained with a fluorescent dye, DAPI ((4',6-diamidino-2-phenylindole; http://en.wikipedia.org/wiki/DAPI), which binds tightly to double stranded DNA.

1. The nosepiece contains all the lenses; we will begin with the 10X lens. Rotate the nosepiece so that the lens with the yellow ring faces up, toward the stage opening. When rotating the nosepiece, use the knurled part at the base, not the lenses.

2. 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.

3. 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).

4. Find the cells. To do this, gradually move the objective lens toward the slide while observing through the eyepieces. You can simultaneously move the slide back and forth 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, you can look around the slide. Try to identify individual cells and nuclei.

5. To image the cells in fluorescence, you need to check two things: first 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 UV. Turn off the transmitted light. To illuminate the cells with the fluorescent light, open the epi-illumination field block (move the lever away from you (right side of filter box); open "O", closed "C". You will see UV light.

6. Look at the cells. What do you see? Close the block when you are finished with the observations. Leaving the fluorescent light on the sample will photobleach the sample.

Your microscope has four filter sets, each used for particular fluorophores. We will make use of these in subsequent labs.

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 pass green — red	chlorophyll and GFP simultaneously	B-2A

Using the Neutral Density filters: 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. If in an experiment the fluorescence is too bright, you should use the neutral density filters in the excitation beam.

Task 3: Aligning the optical system for Köhler illumination

Although a poorly aligned microscope can produce an image of a specimen, the best possible image can only be obtained when the microscope is properly aligned. In this task, you will align the transmitted light portion of the microscope optical path. The proper alignment of the light source and imaging lenses is said to follow Köhler illumination.

1. If you have not already turned on your microscope, do so now. Follow the turn-on procedure on page **Error! Bookmark not defined.**. You will not be using fluorescence in this lab, so do not turn on the epi-illuminator. The arc lamp used in the epi-illuminator has a limited lifetime, and so should be left turned off unless you need to use it.
2. Rotate the nosepiece so that the 10× lens is facing the stage opening. Use the side of the nosepiece so as to avoid fingerprints or other damage to the (expensive) microscope objective lenses. Rotate the eyepiece turret to the "O" position. Rotate the condenser turret to the "A" position (the one facing forward). Both of these settings leave the optical path fully open.
3. ***Focus the sample***. For all subsequent steps, the sample must remain in focus. Place the stage micrometer on the stage, turn on the transmitted light condenser illuminator, adjust it for brightness, and focus the stage micrometer. Notice which side the stage micrometer is on. That side faces the objective lens. If you are having difficulty finding the micrometer, try to center it over the objective first by looking at the micrometer from above.
4. ***Adjust the microscope for proper Köhler illumination.***
    a. With the stage micrometer still in focus, reduce the size of the *field diaphragm* by moving the lever. When the lever is in the up position the diaphragm is fully open. Moving it down will reduce its size. *You should be able to see an outline of the field diaphragm as you reduce its size.*
    b. Move the condenser focus knobs until the field diaphragm is in focus. ***Do not change the focus of the specimen (the stage micrometer).*** You will note that the edges of the field diaphragm appear to have a color gradient, making the edge a bit difficult to discern. This is due to what is called chromatic aberration in the lenses. Chromatic aberration causes light of different wavelengths (colors) to focus at slightly different focal planes.

       You can limit this problem by restricting the range of wavelengths of light that are being observed. One of the sliders on the right hand side of the microscope top between the field diaphragm and the light source is a so-called bandpass filter that passes a limited range of wavelengths in the green range of the spectrum. It is marked "G". Push it into the beam path and see how the field diaphragm image is improved.

c. Adjust the centering of the field diaphragm until it is nearly centered, using the two knobs on either side of the front of the condenser turret.

d. It can be somewhat difficult to determine the center of the field of view when the field diaphragm is fully stopped down. A trick to get perfect centering is this: Open the field diaphragm until it is nearly, but not quite out of the field of view. The edges of the field diaphragm will be close to the edges of the field of view. Tweak the centering until the field diaphragm is exactly concentric with the edge of the field of view. *Repeat b and c until you have a sharp field diaphragm that is centered.*

e. Open the field diaphragm a bit until the whole field of view is illuminated. Open it just to the point that it disappears. Opening farther than that will cause light to scatter inside the body of the microscope, thus degrading the image.

f. The microscope is now set up for Köhler illumination. What you have accomplished by this process is to direct light rays originating from the light source onto the focal point of the condenser (so that the light coming from the condenser is composed of parallel rays) and you have centered the condenser (so that the parallel illumination rays are also parallel to the optical axis of the microscope). These adjustments must be made any time that you change objective lenses. *You should do this every time you use the microscope.*

Summary steps from Video;

https://www.ibiology.org/talks/set-up-koehler-illumination/

1. Focus the objective lens on the specimen.

2. Close down the field diaphragm and *focus* the condenser lens.

3. *Center* the condenser lens with adjustment screws. Adjust the condenser diaphragm opening.

4. Open up the filed diaphragm just beyond the field of view.

Task 4. Adjusting the Microscope for Phase contrast

As you saw, strong contrast is not typically an inherent property of the specimen that is being observed. Good contrast is produced by exploiting the interaction of the illuminating light with the specimen, and using the changes produced by the specimen in clever ways. One such way is called *phase contrast*. This method was invented by Fritz Zernike, for which he received a Nobel Prize.
Phase contrast takes advantage of the fact that all microscopic samples diffract light and images are built up from the recombination of the un-diffracted (so-called *zero order*) and diffracted beams. Think of the zero-order beam as the background. Even absorbing (i.e., black) samples diffract light. You can see this by blocking the zero order beam and noting a dark object becomes bright (we can demonstrate this in

lab). Absorbing samples look dark in part because the diffracted light is 180˚ out of phase with the zero order light and hence destructive interference ensues. Transparent samples diffract light too, but introduce a phase shift that does not cause appreciable interference. Phase contrast is a trick to introduce a relative phase shift between the diffracted and zero-order beams that lets them interfere destructively and gives rise to an intensity difference we can see.
This is accomplished in the phase-contrast microscope by two circular, optical elements (Figure 1). In the front focal plane of the condenser, there is an annulus that restricts the illumination to a ring. In the rear focal plane of the objective, there is a phase ring (labeled phase *plate* in Figure 1) onto which the ring of illuminating light is focused. The illuminating light is the zero-order beam, un-deviated by diffraction; when it traverses the phase ring, its phase is advanced or delayed (depending on the instrument design) relative to light passing through the rest of the rear focal plane. It is also customary to reduce the intensity of the zero order beam by reducing the transmission of the ring. The annulus is built as an opaque sheet with an annular opening through which light passes; the phase ring is a flat plate, which is etched away or built up in a ring shape, at the appropriate diameter. Phase contrast requires the illuminating annulus and the objective phase ring to be superimposed precisely.

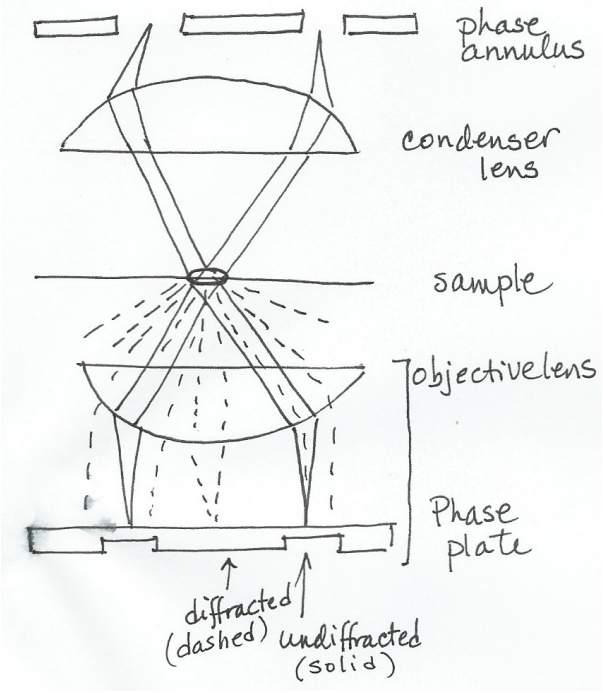


Figure 1. Main features of the phase contrast microscope. The Illumination is restricted to a ring by the annulus. The object (sample) diffracts light, giving rise to diffracted (dashed lines) and unaffected ("zero order") light. These beams recombine in the image (not shown). The phase of the zero order beam relative to the diffracted beam is altered by a quarter wavelength at the phase plate, which allows destructive interference to occur, making the object appear dark.

In this task you will set up the microscope for phase contrast imaging.
Leave the 10× objective lens and cells in place on the microscope. Confirm that cells are in focus and that the microscope is set up for Köhler illumination.

1. Observe the tube of the 10× objective. It has "Ph 1" printed on its side, which indicates that the objective has in its back focal plane a phase ring that partially obscures a circular annulus in the back focal plane. This phase ring is matched

to a clear annulus that can be placed at the focal plane of the condenser. Rotate the condenser turret selector until it says "Ph 1" (at the front). Observe the improved contrast in the image.

2. The phase ring in the objective lens is fixed in place. The condenser annulus can be moved, and needs to be aligned relative to the phase ring in the objective lens. To do this, move the eyepiece turret selector to "B", which stands for Bertrand. There is a small silver knob to the right and underneath of where it says B. Make sure the image of the annulus is sharp by rotating that knob. A Bertrand lens allows the eyepiece to image the condenser focal plane rather than the specimen focal plane. Because the condenser focal plane is the location of the condenser phase annulus (and is conjugate with the location of the objective lens phase plate), the Bertrand lens allows you to see the location of these two elements. If the condenser annulus is not concentric with the phase ring, use the red-handled tools on the side of the microscope to adjust it. There are two, recessed hex-head adjustment screws on either side of the marking on the condenser turret for this adjustment.
3. Return the eyepiece turret to the "O" position and observe the cells again.

Task 5: Digital Imaging.

Most 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 minimize photobleaching and control exposure time.

Turn on the camera if you haven't done that already. Locate cells with your eyes using the **transmitted light** (block the fluorescence for the time being). Now look at the cells with the 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 in the microscope. You are after a sharp image with good contrast.

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)).

Taking Pictures using Fluorescence

1. Turn off the transmitted light. Your system has an electronic shutter to control the fluorescent light. It is handy because it prevents photobleaching of the fluorophores in the sample.

Use of the electronic shutter:

a) **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. The shutter should be set for manual operation and be closed.

b) **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.

2. Open the 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).

3. Take a single fluorescence image by clicking the "Snap" button. The shutter should open for the duration of the exposure and then close. If the exposure is too dim or bright, adjust it 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.

As your are learning to take images, notice 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.) Notice 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 is probably not quite parfocal with the eyepieces.) Get a feel for the response time of the camera, the way that the image changes with respect to changes in image brightness, and the camera's resolution compared to your eyes' resolution. (Eyes are nearly always better for resolution when light is bright enough.)

Take images at 40X

Repeat the steps that you just performed using the 10X lens with the 40X lens. To switch lenses, lower the 10X lens away from the sample with the coarse focus knob, and move the 40X (the silver lens that says pH2 on the side) into place. On the black condenser lens assembly above the stage, move the dial so that the setting pH2 faces you. This is necessary because the 40X lens uses the phase 2 setting. Now, refocus on the sample, by carefully moving the lens up toward the sample while looking in the eyepieces. Snap some pictures using transmitted light and fluorescence light. Save them in your folder.

Storing your images on your computer

1. If you have not already done so, create a login account on the course web site. Go to http://bcrc.bio.umass.edu/bioimaging/. Click on the "Create new account" link below the login button (or log in if you have already created an account, then skip to part 2).
2. For a username, please use your full name (first name nicknames are fine). The email address should be the one that you read regularly. Once you have successfully opened an account, tell one of the instructors. They will give you permission on the system to download and upload files.
3. When you have an account and the proper permissions you can navigate to shared server space on the computer. The server is called Wahoo. There will be folders for the class on the server.
4. The images that you collected will be in a format called TIFF (tagged image format file). Think of this as your Raw data. Be sure to save these files. IF you want to share images, for example put them on Moodle, you need to convert the TIF file to a jpeg file (or other). This conversion results in a loss of information. Do a SAVE AS, so you always have the TIFF images. Naming files: using the date is a convenient way to keep track of your images. For example, 20180123 would be a folder name for images collected on January 23, 2018. In that folder you can have sub folders such as 20180123Task1 etc. Calling images "picture 1" is not very helpful!
5.

Task 6: Determine the size of a cellular structure

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 cell or a subcellular structure. 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. Return the 10× objective as the active lens. Remove the cell slide and replace it with the calibrated stage micrometer slide. Notice which side the stage micrometer is on. That side faces the objective lens.
2. Using transmitted light, locate the stage micrometer by eye, center it, and get it into 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 again.
3. Get the camera image of the stage micrometer, optimize it and be sure it is in good focus.
4. Take a single image of the stage micrometer and save it.
5. Switch to the ImageJ window. Click on the
6. 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.
7. 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 = 10^4 μm.) Leave the aspect ratio set at 1. Your camera has square pixels, so the aspect ratio (height:width) is 1.
8. Put a scale bar on one of the two cell images from Aim 3. Use "Analyze > Tools > Scale Bar". Save this in a separate file, appropriately named, using the "Save as" button. Make another Word or Open Office file with this image and an appropriate caption. When a scale bar appears in an image you should always give the dimensions of the scale bar, in the caption instead of adding that number to the image.
9. Determine the size that a camera pixel represents when the 10× lens is used. You can do this by dividing the known distance between hash marks in part 6 by the distance in pixel units from part 6. 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 lenses (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. A small cell, like a RBC is about 7 microns, and large cells can be 50 microns or more in length.

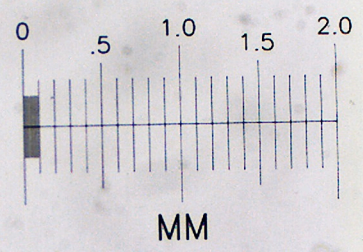


Image of the stage micrometer.

For curious students: Learning about the Image Processing Software

The Micro-Manager software that runs the camera is actually a software plugin for the freeware image processing program called ImageJ.

1. The Micro-Manager display shows several pieces of information. Next to the row of buttons at the top left is a list of "Camera settings". If you haven't played with them already, do so. Change the "Exposure [ms]" and "Binning" values and snap pictures to see what happens.
2. Snap another image with binning set at 1. The display at the bottom of the Micro-Manager window is a histogram of the brightness values (or "gray levels") contained in the image that you just took. The histogram shows the number of pixels in the image (the vertical part of the plot) versus the brightness in the pixel (on the horizontal part of the plot). More light falling on a pixel in the camera will give a larger signal that is translated into a brighter pixel on the displayed image. The plot shows the values contained in all the pixels of the image from full black (on the far left) to full white (on the far right). Peaks in the graph correspond to gray level values that are the most commonly found in the image. From your graph you should be able to see that there are a lot of dark pixels compared to the number of pixels that are bright (the DAPI-stained nuclei).
3. That "Log hist." box under the two display range control buttons changes the vertical axis on the plot to a logarithmic scale from a linear scale. This allows you to expand the small values on the vertical scale relative to the large values. Try it.

4. With the image window and the Micro-Manager window both in view, click the "Full" and "Auto" buttons alternately. Clicking the "Full" button makes the image appear black. Nothing has happened to the image data. Rather, what you are doing is changing the displayed brightness of the image by changing the image gray level values that correspond to displayed brightness levels on the screen.

   The image that is displayed on the computer screen is a representation of a data file of numbers. The usual way in which the numbers are converted to a screen image is to make the brightness of a particular image pixel proportional to the value in the data file for that pixel.
   The image produced by the camera has a limited gray level range, from zero to 4095. The "Full" button causes the gray level value of zero in the digital image to be displayed as black and a gray level value of 65,535 to be displayed as white, with intervening values displayed in proportion to the numerical value in each pixel. When you click the "Auto" button, the Micro-Manager software sets the minimum value in the image as black and the maximum as white. (Notice that these values are shown under the "Log hist." button on the left.)
5. Notice that the two slider controls underneath the plot change when you click the "Full" and "Auto" buttons. You can manually adjust these two slider controls. The top one sets the gray level value that is shown as black on the screen while the bottom one does the same thing for the gray value that is shown as white. Play with the controls to see how the image changes when you change these values. All pixels that are equal to or less than the top slider value will be shown as black in the display and pixels equal to or greater than the bottom slider value will be shown as white. By changing these values you can emphasize the parts of the image that you want to.

   Notice that the Min and Max values change as you change the sliders. Those Min and Max values give the exact values below which pixels are colored black and above which they are colored white.
6. The pull-down menu just below the "Min" and "Max" values changes the scaling on the plot at the right. The default value is "camera" which means that the maximum value on the plot is set to the maximum possible value of the image data file that the camera and its software will produce. Your camera can make so-called 12-bit images, which means that each pixel in the camera can discern up to 4096 (that's 2 to the 12th power) gray level values. The computer doesn't handle 12-bit files well, so the data is stored in a so-called 16-bit file (numbers can range up to 65,535). Because your camera images are created as 16-bit data files, the minimum for the "Full" button is zero and the maximum is 65,535. Use the pulldown menu to change the scale to some value. "16bit" will be the same as "camera", while other settings will be different. Play with them to see how they affect the plot and the displayed image. You should find that the image always behaves the same when you flip between "Full" and "Auto" while the plot looks a lot different. The reason is that the pulldown menu changes only the scale on the gray level plot, but doesn't affect the range of the lower sliders, which are tied to the data file. You should find that the plot gives the full range of the camera when the pulldown menu is set to "12bit".

It is possible to save a lower resolution image by using the "Pixel type" pulldown menu under "Camera settings" at the top middle of the window. As you have already seen, the default is to store the full 12-bit range of camera data in a 16-bit file. Change the "Pixel type" to "8bit". This means that the next picture that the camera takes will have a gray scale resolution of zero to 255 (2 to the 8th power is 256). There are fewer possible gray levels, so the image contains less information than an image taken with 12 bit gray scale resolution. The computer can handle 8-bit data files, so the image will be stored as an 8-bit file. The image file will take up less storage space because only 8 bits need to be stored for each pixel. This can be an issue in some applications, but we are using computers that have plenty of disk space, so we won't be using this feature in subsequent modules.

Take an image with the "Pixel type" set at "8bit". Then play with the histogram controls at the lower left of the window to compare how this lower resolution image is handled by the software.

7. There's one more important control to play with in the Micro-Manager window. The "Auto-stretch" box below the pulldown menu at the lower left of the window allows you to control the display of images as they are taken by the camera. The function is best seen by example. Get an image on the camera and look at it "Live" with the camera. If the "Auto-stretch" box is checked, the Micro-Manager software automatically sets the Min and Max values for each image that is collected so that Min is the lowest gray level in the image and Max is the highest. This will effectively "stretch" the image gray scale so that the display is from full black to full white. Notice that on the live image, when "Auto-stretch" is ticked on that Min and Max change constantly. This is because each image is slightly different than the previous image.

   Tick the "Auto-stretch" box off. Now adjust the slider bars. Notice that the live image display responds to your control. Notice also that the Min and Max remain fixed where you set them.

   This control can be quite useful when you want to set the image so that the brightest area in the image is just below the maximum gray level that the camera can register. It also can be useful when you want to follow changes in image brightness qualitatively as an experiment progresses. You will do this later on in the semester to follow fluorescence bleaching as a function of time.