

Lab 1: Introduction to the Equipment

Goal for the module

Familiarization with the hardware and software of a modern research microscope.

Introduction

Much research in the life sciences requires measurement of the localization and dynamics of biomolecules in cells. The characteristic size of most cells is too small for direct visualization, but is compatible with the resolution of the optical microscope. Innovations in microscopy and the introduction of new techniques have produced an explosion in the use of the optical microscope in modern life science research. By the end of this course you will be familiar with optical microscopy techniques and their application to cell biological systems.

The properties of the optical microscope are governed by the radiation that it manipulates. Recall that light is electromagnetic radiation that has a characteristic wavelength and frequency for each different color. The glass lenses in a microscope transmit visible light as well as light that is of somewhat shorter and longer wavelengths. This wavelength range has been selected because the microscope, through its history, has been used dominantly by humans viewing microscope images directly with their own retinas. In the past couple of decades, however, the wavelengths of light accessible to the optical microscopist have increased. This is due to the introduction of electronic detection methods and the broader wavelength sensitivity of these devices.

In this course we will use Nikon research-grade microscopes that can form brightfield, phase-contrast and fluorescence images. (These will be explained in much more detail in later lab modules.) A research-grade digital charge coupled device (CCD) camera from QImaging Corporation is connected to the microscope through a side port and can be used to collect images suitable for quantitation. (The workings of a CCD camera and the methods of quantitating digital images will be covered in later lab modules.) The particular camera on your microscope is a QICAM 12-bit mono fast 1394 cooled model. You can find out more about the camera by visiting the QImaging web site at <http://www.qimaging.com>.

Materials

Glass slide with a cover slip of fixed LLC-Pk1 pig kidney epithelial cells that have been stained with DAPI, a fluorescent marker for DNA.

Procedures

Task 1. Signing into the computer and the course web page.

Throughout the semester, you will be making, editing, analyzing, and storing, digital images, as well as uploading them to the course webpage. In this task, you will learn your way around the computer and our webpage.

1. Log into the computer with your BCRC ID and password. If you don't have a BCRC account,
 - a. Have someone else sign into the computer for you.
 - b. Open Firefox.
 - c. Click on "course accounts" on the BCRC homepage.
 - d. Follow the directions to set a BCRC password after authenticating with your OIT NetID and password.
2. Find the course website. You can get to all biology course websites from the Biology Department pages from the "Undergraduate" tab.
 - a. Log in with your BCRC username and password.
 - b. Tell an instructor you have done so, and you will be given uploading privileges.

Task 2. Turning on the microscope (without destroying it)

1. Remove the microscope cover.
2. If you plan to use the fluorescence epi-illuminator, turn on the epi-illuminator power supply (large box). Push the start button for a second or two until the lamp in the lamp housing on the back of the microscope lights (the red light on the power supply turns on)*. (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, and your hands away from the microscope when you start the epi-fluorescence lamp.)
3. Turn on the transmitted light illuminator and the microscope.
4. If you plan to use the fluorescence shutter, turn on the shutter controller (black box).
5. If you plan to take digital images, turn on the camera, and then the computer.
6. 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.

* The arc lamp in the epi-illuminator lamp housing gets very hot during operation. The lamp must cool between lamp lightings. Therefore, you must wait at least 15 minutes after the lamp is turned off before turning the lamp back on. Likewise, the lamp should be allowed to fully warm up before turning off, so leave it on at least 15 minutes after it is turned on.

Task 3. Observing cells

One of the most important aspects of optical microscopy is to locate a specimen of interest, to place it in the field of view, and to get its image in focus. Follow the steps below to observe fixed cells using phase contrast microscopy. Refer to Figure 1 (taken from the Nikon instruction manual available on the course web site).

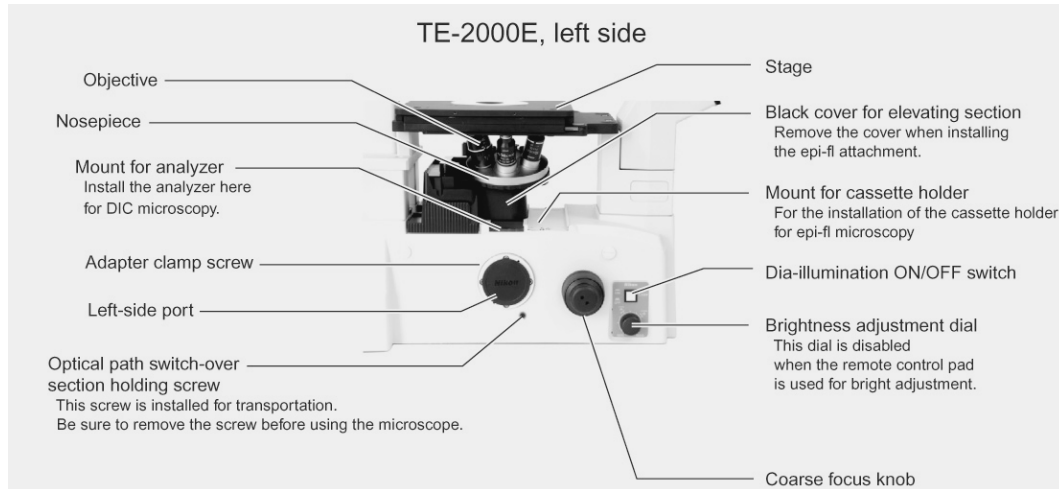


Figure 1. Photograph of the left side of the microscope base. (Image from Inverted Microscope Eclipse TE2000E Instructions, reprinted with permission from Nikon.)

1. If you have not already turned on your microscope, do so now. Follow the turn-on procedure on page 10. ***Be sure to turn off the computer before you turn on the epi-illumination arc lamp. The arc lamp emits an electromagnetic pulse that can damage electronic components.*** Locate the epi-illumination field block near the fluorescence excitation and emission selector wheel on the right side underneath the nosepiece (see figure above). Be sure that it is closed (pushed to the front).
2. Rotate the nosepiece (see Figure 1) so that the 10× lens is facing up, toward the stage opening. Use the side of the nosepiece for the rotation of the lenses so as to avoid fingerprints or other damage to the (expensive) microscope objective lenses.
3. Place your glass slide with cell cover slip side down on the stage. (The slide always should be put so that the cover slip, or the surface with the object to be examined, is on the side of the slip closer to the objective lens. This is an inverted microscope, so the glass slide is “inverted”, too.)
4. Turn on the transmitted light condenser illuminator (Nikon calls this the Dia-illuminator), adjust it for brightness, and find some cells.
5. This is often harder than it sounds. You might try to observe through the eyepieces while you move the stage from side to side using the stage x-y positioning knobs (on the extended bar coming down from the stage). The lateral movements allow you to find contrasting parts of the image, which you can then use to focus on the specimen. Focus as you move the stage. It is also helpful to partially close the condenser iris.
6. Can't find cells? Check the orientation of your slide, settings on the microscope and brightness of the illumination light. Still can't find cells? Ask for help.
7. Change to the Plan Fluor DLL 40× objective and find cells. Can you find the same cells that you observed at 10×?

Task 4. Fluorescence

The use of fluorescent probes for cellular structures is a powerful tool that allows high-contrast images of cellular components to be visualized. In this task you will observe the same cells from the previous step, but now in fluorescence mode. These cells have been treated with DAPI (4',6-diamidino-2-phenylindole; <http://en.wikipedia.org/wiki/DAPI>), a fluorescent probe that binds strongly to double-stranded DNA and, in doing so, shows the location of dsDNA in cells.

1. Leave the 40× lens and cells in place from 0. Locate the rotating disk below the objective lens nosepiece, in the location “Black cover” shown in Figure 1. The black cover has been removed and replaced with fluorescence filters and mirrors. ***The controls are on the right hand side as you face the microscope.*** Rotate the filter wheel until you find the “UV” click stop. This sets the fluorescence filters in the microscope for the ultraviolet excitation and blue emission needed to visualize DAPI.

You have 4 filter cubes in your filter wheel, each set up for particular fluorophores:

Label on filter wheel	excitation λ	emission λ	used for	Label on filter
UV	UV (360nm)	Blue (460 nm)	DAPI	UV 2E-C
B	B (480 nm)	Green (535 nm)	GFP, fluorescein	B-2E/C
G	G (560 nm)	Red (630 nm)	Texas red, rhodamine	Y-2E/C
LP	B (470 nm)	long pass green-red (≥ 500 nm)	chlorophyll and GFP simultaneously	B-2A

2. Your microscope will have either a Sutter Instruments or a Vincent Associates shutter controller. Determine which one you have.
 - 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 Vincent Associates 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.
3. Open the epi-illumination field block. This is the rotating slider just below the epi-illumination fluorescence filter selector wheel under the nosepiece. The closed position is forward, marked as “C”. The open position is back, marked as “O”. This will allow fluorescence excitation light to pass freely into the microscope and therefore be gated by the computer-controlled shutter. Open the shutter as directed above.
4. Observe your cells. The DNA in the cells should fluoresce bright blue against a dim background. Close the shutter when you are finished with the observations. Leaving the epi-illumination turned on will photobleach your specimen. It will also bleach optical filters. ***Always be sure to close the shutter whenever you are not imaging by eye or camera.***

5. 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 $\frac{1}{4}$. The ND 8 reduces intensity by a factor of $\frac{1}{8}$. Together they reduce the intensity by a factor of $\frac{1}{32}$. If in an experiment the fluorescence is too bright, you should use the neutral density filters in the excitation beam.
6. Play with simultaneous phase and fluorescence imaging (i.e., having both the transmitted light illuminator and the fluorescence illuminator turned on at the same time). Can you find cells in different stages of the cell cycle?

Task 5. Digital imaging

It is fine to observe directly with your eyes. However, it is difficult to quantitate the image you see. For example, can you tell what the average dimension of nuclear DNA is? Is there more DNA in one nucleus versus another? By taking and preserving digital images, you can use image analysis software to answer these questions. In this task you will capture images of the cells in phase contrast and fluorescence modes.

1. Leave the $40\times$ lens and cells in place from Task 4. Turn on your computer if it is not already on.
2. Turn on the camera and the shutter if you haven't done that already. Locate cells with your eyes. To look at the cells with the camera, do the following:
 - a. Find Micro-Manager program in the Applications folder (it uses a microscope icon).
 - b. Open ImageJ.
 - c. Find and open Micro-Manager Studio from the Plug-ins menu.
 - d. 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.
 - e. 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.
 - f. 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.
3. Play with your specimen and the camera. 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.)

4. Take individual phase and fluorescence images of an interesting group of cells. Try to get at least a few cells in the field of view. To do this,
 - a. Turn off the live image collection.
 - b. Assure that the shutter controller is set for computer control and that the shutter is closed (see Task 4.2). Block off the transmitted light by turning the lamp off with the button on the left bottom of the microscope.
 - c. Take a single fluorescence image by clicking the “Snap” button. 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.
 - d. Make a folder with a sensible name and use a sensible name for the image file. While you are working, it may be easiest to keep this folder on the desktop. Later you can move it to permanent storage. (If you come back in two weeks and want to find your fluorescence image from today, what would make it easy to locate?)
 - e. Take a single phase image by blocking off the fluorescence illumination light or turning the shutter controller to manual control and closed, which defeats the computer control of the shutter. Don’t forget to turn on the transmitted light. Save this image, too. You may need to adjust the exposure time and/or image illumination brightness.

Task 6. Controlling the image display

The Micro-Manager software that runs the camera is actually a software plugin for the freeware image-processing program called ImageJ. You will use these programs throughout the semester.

In this task you will use some of the features of ImageJ to get used to the program and to explore the properties of the camera attached to the microscope.

1. Get cells in the field of view using fluorescence illumination for DAPI. Snap a picture as you did in Task 5.
2. 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.
3. 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.
 - a. 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.
 - b. 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).

4. 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.
5. 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.)

6. 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.

7. 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 pull-down 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 pull-down 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 pull-down menu is set to “12bit”.

It is possible to save a lower resolution image by using the “Pixel type” pull-down 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 (2^8 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.

8. There’s one more important control to play with in the Micro-Manager window. The “Auto-stretch” box below the pull-down 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:
 - a. 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.
 - b. 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.

Task 7. Saving image files to Wahoo

Image files tend to be very large. The ISB server, Wahoo, has space set aside for storage of Bioimaging files. This is a very useful place for you to keep your files. You can access these files from anywhere that has an internet connection.

There should be a link on the desktop that takes you to the Wahoo Bioimaging space. Find the folder already set up there for your microscope. Make a new folder with an informative and unique identifying name e.g., “Unit 1 – Tom & Jerry” Save your image files there, creating identifying subfolders as needed, and keeping a record of what goes where.

Task 8. Retrieving files from Wahoo

From room 360

Obviously, you can readily locate these files from any computer in room 360.

On a networked Macintosh on campus

Open a finder window, and pull down the Go menu. Choose “connect to server” and type “smb://wahoo.cns.umass.edu/bioimaging”. Sign in with your BCRC username and password.

From anywhere else

Download and install FileZilla following the instructions on the OIT web-hosting support page (<http://www.oit.umass.edu/support/web-hosting>).

- Open FileZilla.
- Open Site Manager from the File menu, and enter the following:
 - Select Entry: New Site
 - Hostname: wahoo.cns.umass.edu
 - Port: 22
 - Protocol: SFTP – SSH File Transfer Protocol
 - Logon Type: Ask for password
 - User: your BCRC username
- Click Connect.
- Enter your BCRC password, and click OK
- **Note:** The first time you log in, a one-time screen warning you about an unknown host key may appear. Check the box next to **Always trust this host, add this key to the cache** and click **OK**.
- The remote site is something like this: /u1/home/bio/username (personalized with your username, of course).
- Clear the Remote site box, and type this: /export/bioimaging
- Enlarge the absurdly small window under the remote site bar and scroll to find your microscope folder. Move files between local and remote folders by drag-and-drop, or by right click and upload (local to remote) or download (remote to local). Voilá!

Task 9. Uploading an image to the course webpage

You will be putting images on the course webpage throughout the semester. Course information will be posted there as well. ***Check the webpage for changes in uploading procedure.***

1. When you have an account and the proper permissions for the web site, navigate to the “Image Galleries” link under “Course Resources”. You will see that there is a place to put images for the course. Image files should be JPG, PNG or GIF.
2. Convert one of your ImageJ images (stored as TIFF image files) to JPG. To do this, have an image displayed as you want it. Go to the ImageJ>File>Save as>Jpeg... pull-down menu, make the filename sensible to you and put the file where you can find it.
3. Now that you have a JPG image file, import it into the image gallery for the course. To do this, expand the “Create content” link on the course website menus and click on the “Image” link. Give your image an appropriate title, select the appropriate gallery for the course, browse to your JPG file, and write a very short image caption in the “Body:” box. You can preview your submission before you submit it. You must click the “Submit” button to upload the image.
4. You can now see your image along with any others posted by navigating through the Image Galleries link on the menu. When you have a pretty image to share any time during the semester, please post it here.

Task 10. Printing an image

First, spend some time making the file look the way you want. It’s hard enough to print without wasting time and effort printing something that looks ridiculous.

Warning: there are many steps to this process. Much frustration, heartbreak, and wasted paper can be avoided by mastering these steps now.

- Sign in with the print authorization widget on the desktop, using your BCRC username and password. This should open up the print queue page.
 - Return to your file and select “print” from the file menu. ***This does not send your job to the printer!***
 - Notice the name of the printer, as this will tell you where to look for your printout. (The printers in ISB biology space are named after Pacific islands.)

Printer name	room
Nifiloli	264
Nupani	360
Nukapu	364
Ngawa	368

- You may be able to get a menu that lets you change image quality by looking at your options under “copies and pages”.
- Use the preview function to make sure you will be printing the right thing. Check which pages will be included.
- Click print. ***This does not send your job to the printer!***

- You should be directed to the print queue page. (If not, you can find the ISB print release page here: <https://wahoo.cns.umass.edu/printrelease/>.) Refresh repeatedly until your job shows up. Check the box for your job and click “release”. (If you have made a mistake, this is a good time to cancel a job.) ***This does not send your job to the printer!***
- After you release your job, you get a confirm release page. Click “really print”, and ***this will finally send your job to the printer!***

Task 11. Microscope hygiene

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. Never use any abrasive materials to wipe them. Only lens tissues are soft enough to be used. Dust should be blown off of optical surfaces, not wiped off.

Use care when moving filters into the optical path, when switching from viewing by eye to viewing by camera and when switching fluorescent filters.

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, or if the lamp housing is hot. 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.***

Clean-up

Save good slides for future use (and note which slide you used). Discard (in the glass trash) any slides that had bad specimens, got cracked, or are totally photobleached.