Lab 1: Introduction to the Equipment

Goal for the module:

Become familiar 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 particular camera on your microscope is a QICAM 12-bit mono fast 1394 cooled model.

Materials

A 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

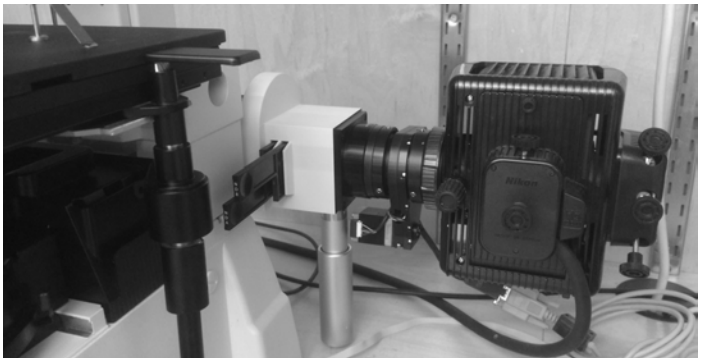
Task 1. Signing into the computer and the course Moodle site.

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

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.

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

1. Remove the microscope cover. Set aside on shelf or lab bench, not near the Epi-illuminator (black box to the right of scope), which gets very hot.



2. Turn on the transmitted light illuminator power box (the dia-illuminator)

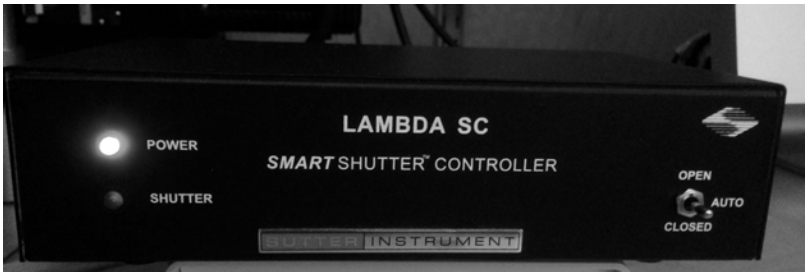
3. Turn on the lamp for transmitted light. The on/off switch is on the front left of microscope base.

4. If you plan to use the fluorescence epi-illuminator, turn on the epi-illuminator power supply (large box; green light on the left lights up). Push and hold in the start button until the red/yellow light on the power supply turns on.*

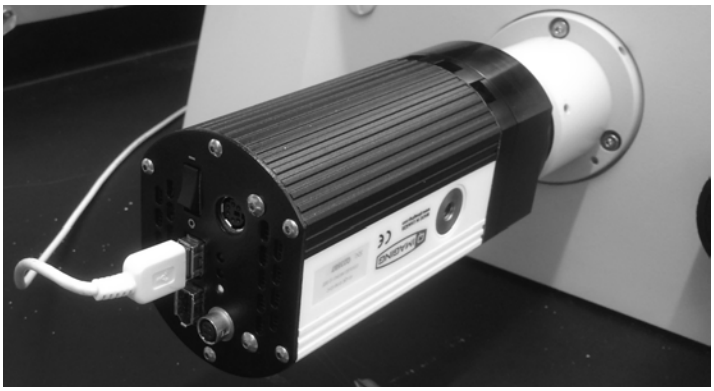
   This start procedure involves a high-voltage spark in the lamp. The sparking produces a voltage transient that can disrupt sensitive electronics. Keep your hands away from the microscope when you start the epi-fluorescence lamp.

* 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 30 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.

5. If you plan to use the fluorescence shutter, turn on the shutter controller (black box, toggle switch on rear).



6. If you plan to take digital images, turn on the camera. It has a toggle switch on the end.



When you are finished with the microscope, turn off BOTH lamp power supplies, the camera and shutter. The order in which you do that does not matter.

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).

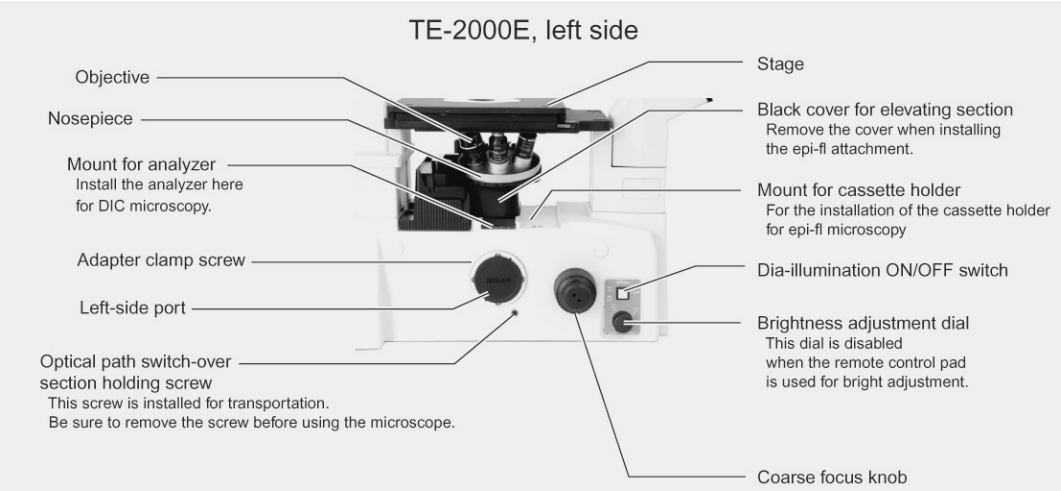
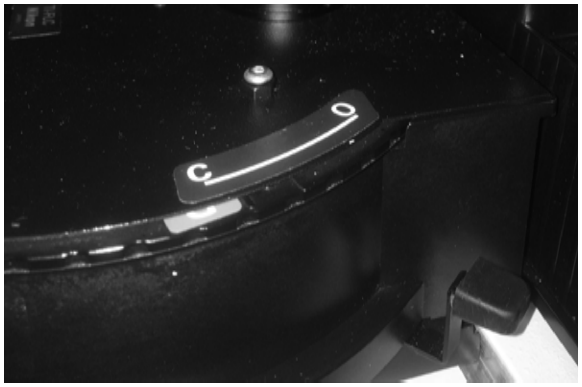


Figure 1. Photograph of the left side of the microscope base. (Image from Inverted Microscope Eclipse TE2000E Instructions, reprinted with permission from Nikon.)

1. If you have not already turned on your microscope, do so now. Follow the turn-on procedure on page 9. Locate the epi-illumination field block near the fluorescence excitation and emission selector wheel on the right side underneath the nosepiece. Be sure that it is closed ("C").



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.

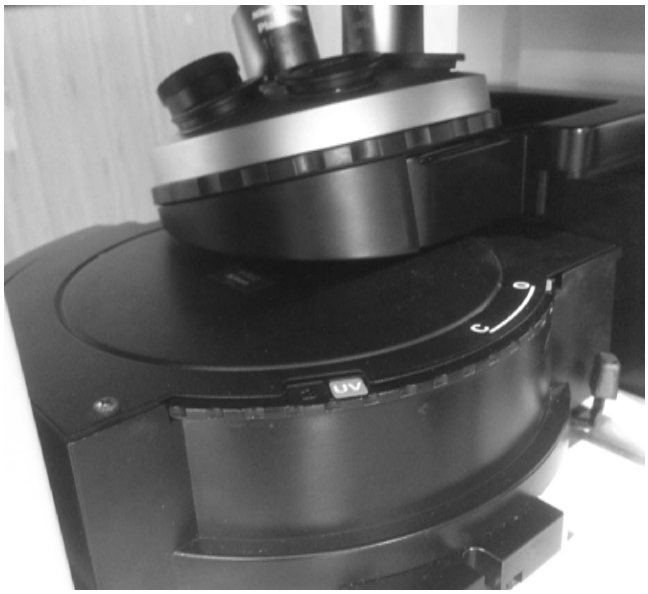
3. Place your glass slide with cell cover slip side DOWN on the stage. (The slide always should be put so that the cover slip, or the surface with the object to be examined, is closer to the objective lens. This is an inverted microscope, so the glass slide is "inverted", too.)
4. Turn on the transmitted light condenser illuminator (Nikon calls this the Dia-illuminator), adjust it for brightness, and find some cells. (See page 10.)
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. Histology slides, stained with bright dyes, are available for you to practice focusing on the sample.
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. After you successfully find the cells, take note of the height of the lens relative to the slide/coverslip. This can help you focus on your sample in the future.
7. Change to the Plan Fluor DLL 40× objective and find cells. Can you find the same cells that you observed at 10×? Again, take note of the height of the lens relative to the sample.

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. 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 (FITC)	B-2E/C
G	G (560 nm)	Red (630 nm)	mCherry 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.

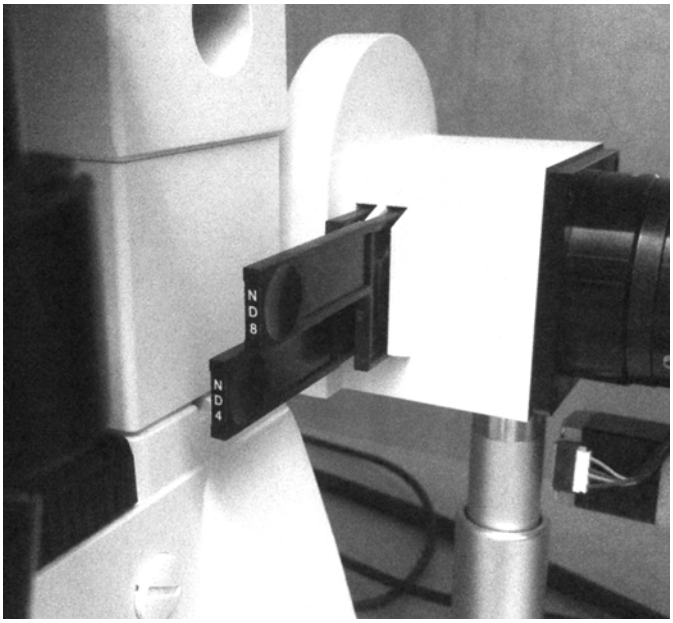
   a) **Sutter controller:** The epi-illuminator shutter is manually opened by switching the toggle switch on the controller box to "Open" and is manually closed by switching the toggle to "Close". With the toggle in the middle "Auto" position, the computer controls the shutter. The shutter should be set 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. Turn off the transmitted light if it is on. The DNA in the cells should fluoresce bright blue against a dark 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, 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 insert the ND 4, the ND 8 or both filters into the light path. 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.

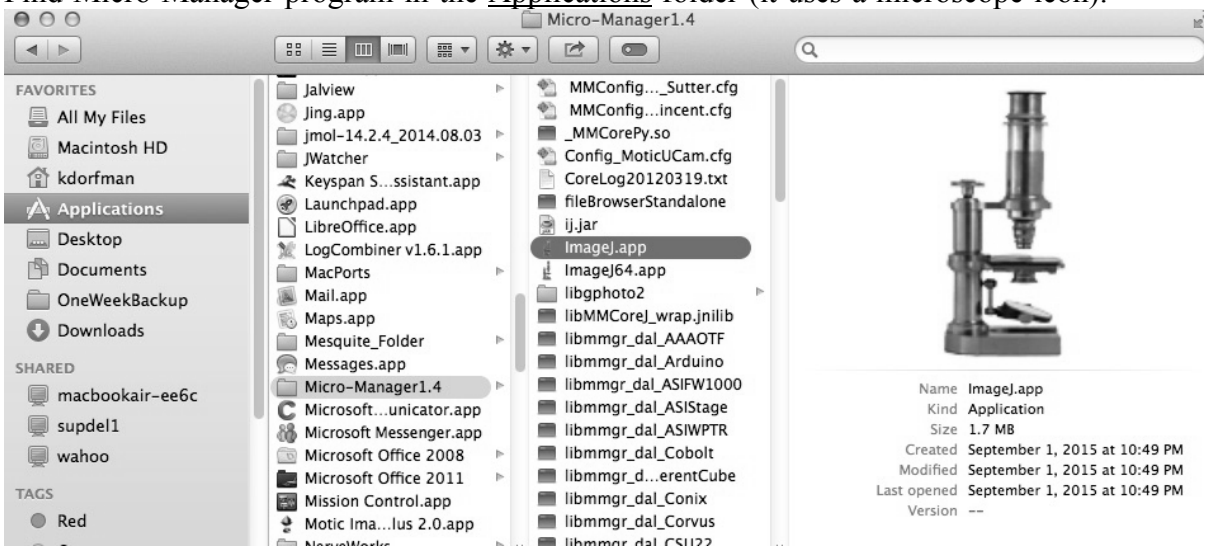


6. You can also try simultaneous phase and fluorescence imaging (i.e., having both the transmitted light illuminator and the fluorescence illuminator turned on at the same time). Keep the transmitted illumination low, so that the fluorescence can be observed.

Task 5. Digital imaging

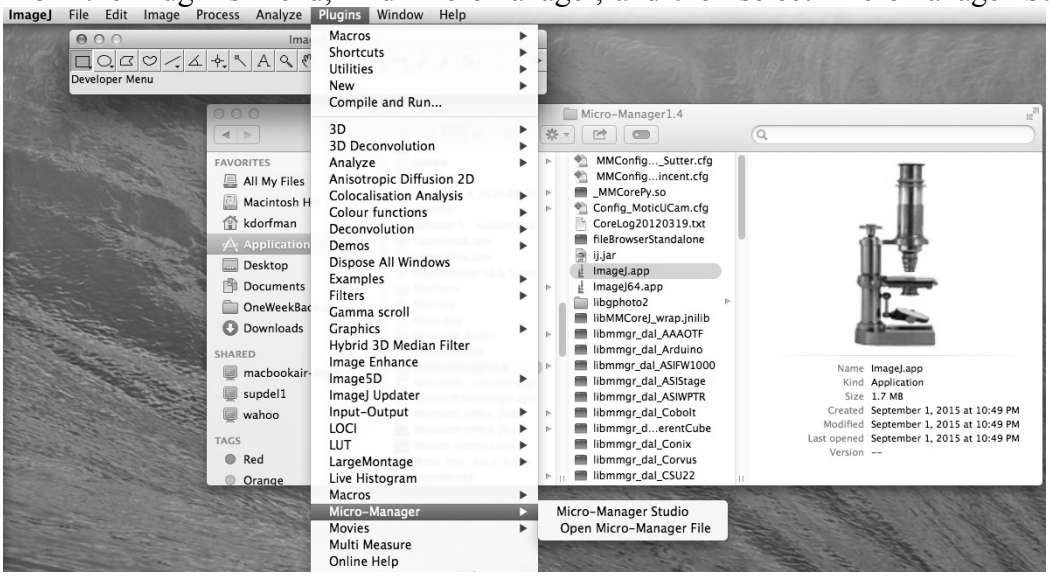
It is fine to observe directly with your eyes. However, it is difficult to quantitate the image you see. For example, can you tell what the average dimension of nuclear DNA is? Is there more DNA in one nucleus versus another? By taking and preserving digital images, you can use image analysis software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

1. Use the 40 X lens. 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. From the Plug-ins menu, find Micromanager, and then select Micromanager Studio



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. Gently 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 ("EYE) to the camera ("SIDE"). The image in the eyepieces will disappear. Please exercise caution when using this dial: be gentle because you are positioning a glass mirror that can break and also be sure that the knob "clicks" into position.

f. To see an image on the computer, click 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. Experiment 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 paired 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. Close the shutter and set it for computer control (Automatic). Turn 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. Save your images as Tiffs (Tagged Image File Format). Tiff images are not compressed and can be used for analysis. Other file formats use compression (jpeg, gif) and are therefore not suitable for quantification due to data loss.

   d. Make a folder with a your name and date. Name your images with the date and other identification (cell 001; experiment 001 etc). While you are working, it may be easiest to keep this folder on the desktop. Later you can move the file to a folder on Wahoo in the folder for your microscope..

   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 and save it..
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 12^{th} 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 to the 8^{th} 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.

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. Keep your files here. You can access these files from anywhere that has an internet connection. You can also copy the files to another storage space for access (Google drive, Box, Drop box, thumb drive).

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., "Date, Lab 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: /data/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. Sharing Images

At various times you may be asked to post images to Moodle or put images in a certain folder on Wahoo to share with the class.

1. Convert one of your ImageJ images (stored as TIFF image files) to JPEG. 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.

Task 10. Printing an image or file

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.

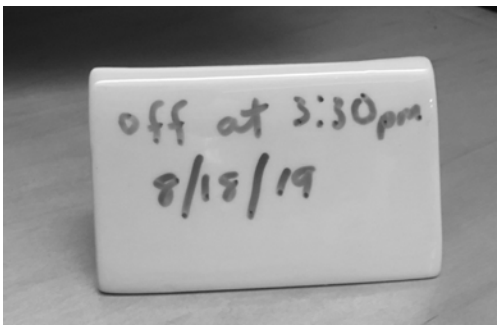
Lens surfaces are coated with antireflective materials that can be scratched. ***Never use any abrasive materials to wipe them***. Only lens tissues are soft enough to be used. Dust should be blown off of optical surfaces, not wiped off.

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.***

Tell the next user when you turned off your epi-illuminator.



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.

Lab 2: Image Formation in the Microscope

Goal for the module

Learning the basics of image formation in the microscope.

Pre-lab homework

Watch the iBio seminar on Kohler illumination (https://www.ibiology.org/talks/set-up-koehler-illumination/)

For additional information, visit Nikon's MicroscopyU (http://www.microscopyu.com/sitemap.html). Look over the contents to get a feel for what's there and read the sections on refractive index, the optical train, Köhler illumination and phase contrast.

Introduction

The principles that allow the visualization of cells and cell structures in the optical microscope are based on so-called geometric optics. This is the field that describes the redirection of light by lenses. The ability of a lens to divert light arises from one fundamental aspect of the electromagnetic radiation that we call light: the speed of the light wave decreases when the light enters a medium from a vacuum. You probably remember the relationship between light's wavelength (λ), frequency (υ) and speed (c):

$$\lambda\nu = c = 3.0\times10^{8}\ {}^{m}\!/_{\text{sec}}$$

This equation is valid when the light passes through a vacuum. When it passes through a specimen, the speed of the light drops and the wavelength decreases, both by the same amount. The degree to which the speed drops and the wavelength decreases is called the index of refraction (n). Therefore, when the light is in a medium, its wavelength is

$$\lambda_{med} = \frac{\lambda_{vac}}{n}$$

and its speed is

$$c_{med} = \frac{c_{vac}}{n} = \frac{3.0\times10^{8}\ {}^{m}\!/_{\text{sec}}}{n}\ .$$

This relationship is important because it shows why curved lenses with high refractive index bend light coming from air with a low refractive index. In essence, the light takes longer to travel through a thick piece of lens as compared to a thin piece, and so the wave is retarded in the thick section of lens. See Figure 2 and the tutorial at MicroscopyU (http://www.microscopyu.com/articles/formulas/formulasri.html).

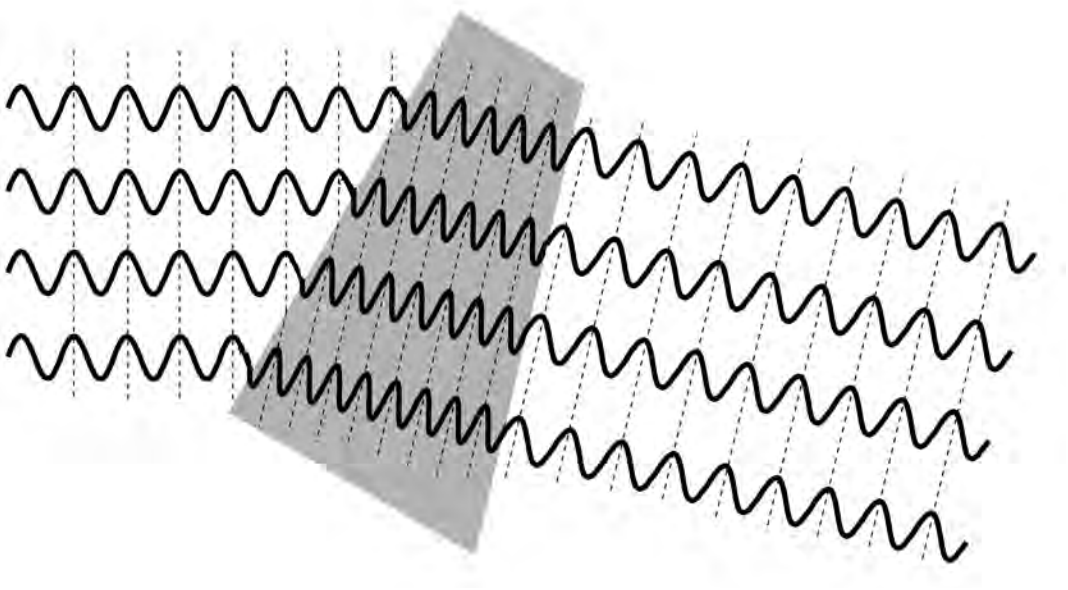


Figure 2. Light rays entering a medium of higher index of refraction at an angle have their wave fronts bent toward the normal of the medium due to the slowing of the rays by the high index medium. If the medium is wedge-shaped, the waves are bent.

The alteration of wave speed and wavelength in media of differing indices of refraction also affects the movement of light through a microscope specimen. Although the differences in index of refraction between the surrounding aqueous medium and a cell are small, they can be exploited to produce image contrast.

Materials

Glass slide with a cover slip of fixed LLC-Pk1 pig kidney epithelial cells, stained with DAPI.

A ruled stage micrometer.

Procedures

Task 1. 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. 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 (see Figure 3.) Rotate the condenser turret to the "A" position (the one facing forward). Both of these settings leave the optical path fully open.

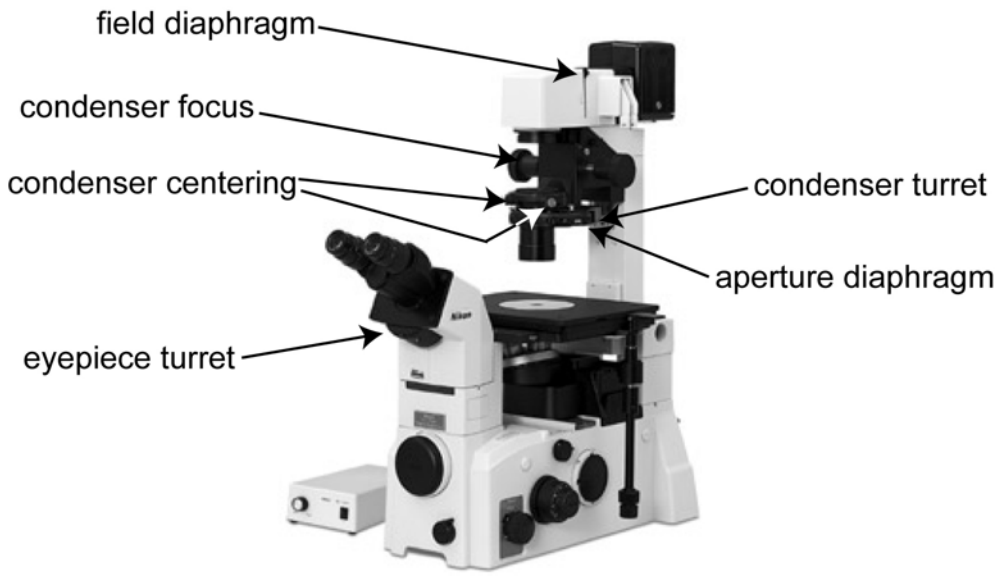
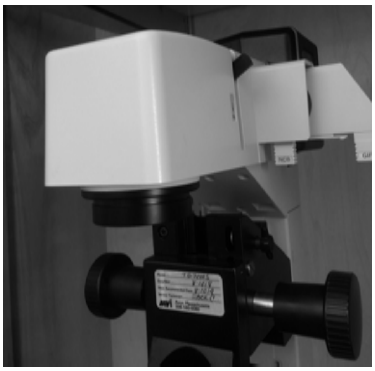


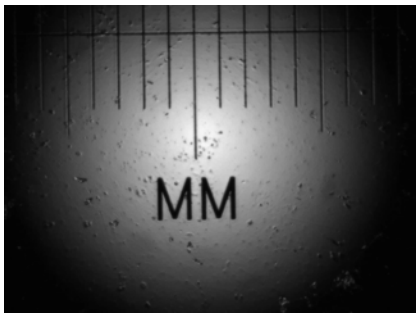
Figure 3. Adjustment points on the Nikon TE2000. (Image from Inverted Microscope Eclipse TE2000E Instructions, reprinted with permission from Nikon.)

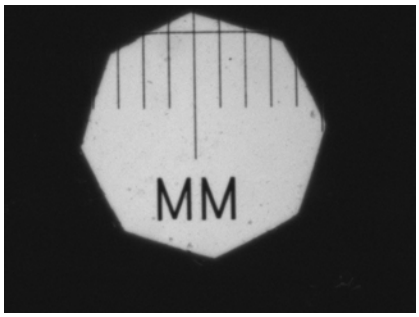
3. ***Focus the sample***. For all subsequent steps, the sample must remain in focus. Place the stage micrometer on the stage, turn on the transmitted light condenser illuminator, adjust it for brightness, and focus the stage micrometer. Notice which side the stage micrometer is on. That side faces the 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.*** (See Figure 3 for a key to the adjustable elements on the microscope.)

   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 (as shown in Figure 3), 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 large black 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 silver 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.*

Task 2. Bright field imaging

In this task you will use unstained cells to explore the ability of the microscope to generate contrast using transmitted light.

1. Leave the microscope set up as in Task 1. Place a slide with a coverslip of fixed mammalian tissue culture cells on the microscope. Adjust the stage and focus to get cells in view. This will be difficult. The reason is that most animal cells have little in the way of pigments or coloring to produce image contrast that is necessary to provide the brain a way to interpret the image.
2. Contrast can be gained at the expense of resolution (a topic to be covered in a later lab). To do this, reduce the size of the *condenser aperture diaphragm*. This is not the field diaphragm that you just centered. The condenser aperture diaphragm control is on the front of the condenser module, just below the two knurled nuts that you used to center the field diaphragm.

   You may need to increase the illumination level because reducing the condenser aperture will decrease the amount of light entering the specimen. What is happening physically is that the diameter of the column of parallel rays is changing as the condenser diaphragm is changing. This causes the cone of light emanating from the specimen to decrease, and this, in turn, reduces the resolution in the image, increases the depth of focus, and increases the contrast partly because the amount of background light in the image decreases. Observe your cells as you open and close the iris. How does the image change?
3. Open the condenser aperture diaphragm as far as possible and still see cells. You want to operate this way so as to keep the image resolution as high as possible, unless you need to increase contrast.
4. Brightfield imaging is used for viewing stained samples, for example, histological specimens which are often stained with hematoxylin and eosin. View an "H & E" stained slide, and notice the increase in visible structures.

Task 3. Phase contrast

As you saw in Task 2, 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 method 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 4). 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 4) 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.

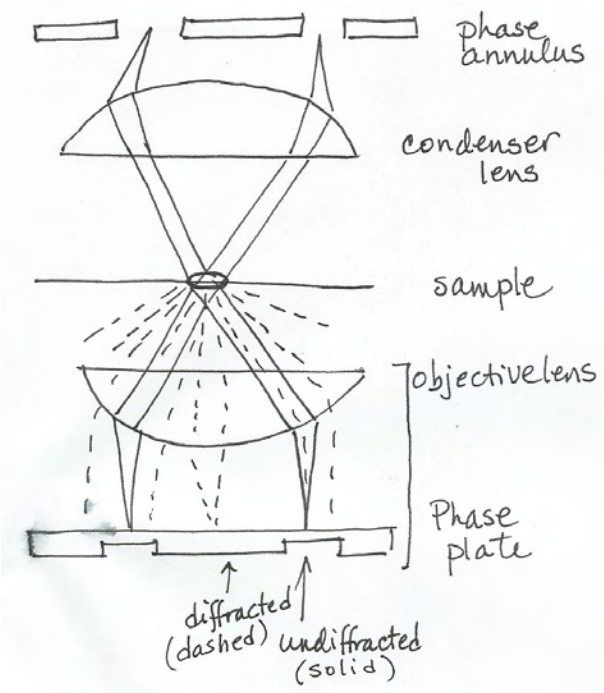


Figure 4. Main features of the phase contrast microscope. The Illumination is restricted to a ring by the annulus. The object (sample) diffracts light, giving rise to diffracted (dashed lines) and unaffected ("zero order") light. These beams recombine in the image (not shown). The phase of the zero order 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.

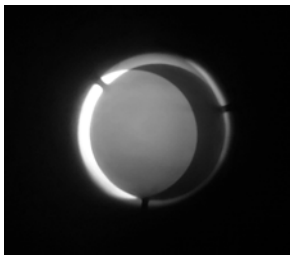
In this task you will set up the microscope for phase contrast imaging.

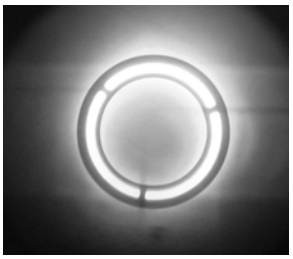
Leave the 10× objective lens and cells in place from Task 2. Confirm that cells are in focus and that the microscope is set up for Köhler illumination.

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 of 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.

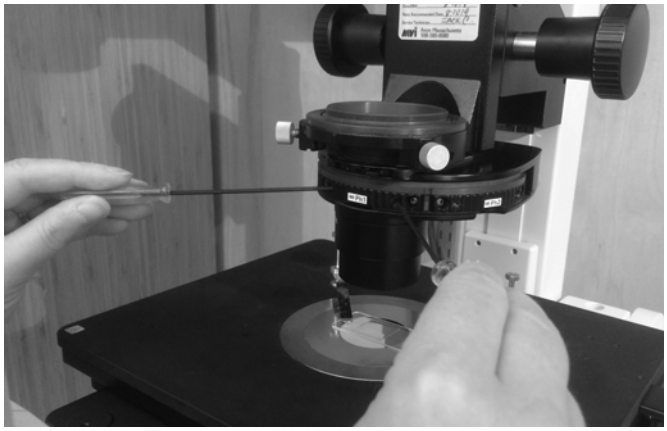
2. The phase ring in the objective lens is fixed in place. The condenser annulus can be moved, and needs to be aligned relative to the phase ring in the objective lens. To do this, move the eyepiece turret selector to "B", which stands for Bertrand. There is a small silver knob to the right and underneath 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.

3. 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



4. Return the eyepiece turret to the "O" position and observe the cells again.

Task 4. Exercise in adjusting the microscope

In this task you will take a misadjusted microscope and return it to proper alignment. When you are ready to do this, ask one of the instructors to misadjust the microscope. You then will readjust the microscope and document your steps by taking pictures during the process.

1. For this exercise, use the 10× lens. You should follow the procedures above to first set up the microscope for Köhler illumination and then for phase contrast. You should take note of the following as you align the scope. Document with images if you want and record your observations in your lab notebook.
   - What do the cells look like when *first focused* in the misaligned microscope?
   - What does the edge of the *unfocused* field diaphragm look like as you begin the adjustment for Köhler illumination? Compare it with the *focused* field diaphragm that has been centered.
   - Take an image of the *cells in brightfield* when the system has been properly adjusted for Köhler illumination, and another image of the cells when the phase annulus and phase ring are in place, but *misaligned*.
   - Finally, take an image of the cells when the phase annulus and phase ring are *properly aligned*.

   Save your images in a folder on Wahoo.

Task 5. Image spatial quantification

To begin this task, take some nice images of cells using phase contrast. Now you will calibrate your microscope so you can determine the sizes of cells and structures within them. To do this, you will use a calibrated stage micrometer, a slide that has a very fine ruler etched into it (Figure 5).

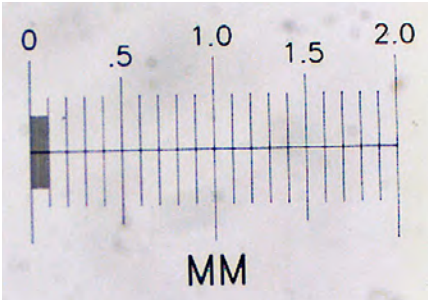


Figure 5. Stage micrometer from Ward's Science (part # 949910)

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 phase contrast, 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 near the center of the objective lens 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 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. (Why?)
6. 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. 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. Make this global.
7. Put a scale bar on one of the two cell images from Task 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, ***give the dimensions of the scale bar in the caption instead of adding that number to the image***. The scale bar is usually positioned in one corner of the image where it will not obscure any important image detail.
8. 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).

Further studies

You now have the basic tools to set up the microscope for Köhler illumination and phase contrast. You might want to examine other samples, comparing phase contrast with brightfield. Try a stage micrometer. Which imaging technique works best for a sample that has strong absorption contrast (like the stage micrometer)? Are the camera pixels really square? To what accuracy?

Clean-up

Save good slides for future use. Discard (in the glass trash) any slides that had bad specimens, got cracked, or are totally photobleached. Return the micrometer to its box.