

Microscope and Köhler Illumination on Rails

Optics Basics

Goals: The overall goal of the lab is to build a microscope on the bench from scratch. In so doing, you will not only learn how the microscope works, but you will also gain skills in optical design and construction that will allow you to create novel microscopies in your future scientific research.

Basics of Handling Optics:

NEVER touch the optical surface of any component, lens, mirror, etc.

Only handle components by their edges or mountings.

USE cloth gloves or finger coverings, if possible.

NEVER lay lenses or mirrors on the metal bench with the optical surface touching the bench!

DO NOT leave optical components lying horizontally and uncovered for a long period of time.

If you see dirt or oil on an optical component, DO NOT try to rub it off with a tissue.

DO NOT wash optical components with any solvent such as alcohol or acetone. You may severely damage the surface.

Basics of Cleaning Optics:

If you optics does get dirty, here is what you can do.

1) If the optic is dusty, blow it off with clean air from a can.

DO NOT use house air or air from a tank unless you know if is filtered.

BE CAREFUL not to lose the optic or let it go flying off as you blow on it.

2) If the optic has oil on it – like a fingerprint – you need to use a very gentle soap solvent.

Using a sterile Q-Tip, that has the solvent on it, you carefully swipe the surface of the optic working from one side to the other.

ONLY USE lens paper to wipe off any excess liquid from the optical surface. Wipe from one edge to the other.

If that does not work the first time, you must repeat it again.

Optomechanics (Mounting Optics):

Optical components need to be held stationary so that the light can consistently and reproducibly move through the optics. String and bubble gum will not work. We need to use “opto-mechanics” to hold the optics stationary in the system. Today you will learn about different types of mounts, learn how to place optical components in the mounts, and learn how to affix the mounts onto posts.

Optical Components:

Today we will describe the types of optics we will use: **mirrors**, **lenses**, and **filters**. Most of our optics are 1-inch in diameter. There are a variety of methods to mount optics. I suggest you look at a few types on the ThorLabs website when you have time:

http://www.thorlabs.com/navigation.cfm?Guide_ID=50

Lenses, Focal Length, Creating an Image

Background: The goal of this set of hands-on activities is to give you some basic understanding of how to manipulate light using lenses. Lenses, made of glass or plastic have a different index of refraction from air, so that the light bends at the surface by the principle of SNELL'S LAW.

By creating interfaces of particular curvatures, we can direct light where we want it to go. Thus, lenses were created!

Microscopes have a number of lenses each designed and spaced to control the angle of light to create images where and how desired to ultimately create magnified, aberration-free images.

Goals: The goal of this activity is for you to: 1. Learn how to find the focal point of a unknown lens. 2. Learn how to create an image with a lens. 3. Understand how to collimate light from a point source with a lens. 4. Use a series of lenses to expand/magnify an image. 5. Increase your comfort level with lenses and how they work to make images such as those used in a microscope.

Finding the Focal Length:

You can find the focal length of any converging lens using the principle that collimated rays will form an image at the focal length. Rays that come from a source at infinity (ie. Very far away) will converge at the focal point of the lens. Using this knowledge, you can find the focal point by creating images of objects or light sources that are very far away.

Step 1: Use a source that is far away from the lens, so that the light coming from the source is basically collimated. We recommend using the room lights above you, or the light coming from outside the window.

Step 2: Use a card or paper as a "screen" to create an image. We recommend one person of the group hold the lens and the screen and vary the distance until you create an image.

Step 3: Measure the distance from the lens to the paper. We recommend a second person use the ruler to measure the distance from the lens (about center) to the screen. That measured distance is the focal distance.

Hands-on Work: You have 4 different lens types marked A, B, C, D. Using the method above, measure the focal lengths of the 4 lenses.

$$F_A =$$

$$F_C =$$

$$F_B =$$

$$F_D =$$

Check at the front to find the known focal lengths. Were you able to measure it correctly?

The focal lengths of the lenses we have are 30 mm, 50 mm, 100 mm and 150 mm. The measurements will be a bit off because the lenses aren't really "thin." After they are close, you can give them the real focal distances for later exercises.

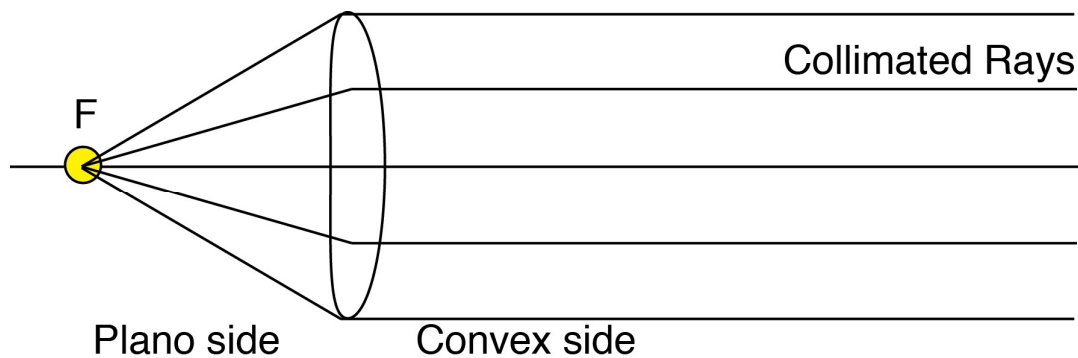
Collimating Light:

In optics, the light rays could run forward or backward. Using the same principle as above, if you take the two lenses and place the source at the focal point, the light will come out collimated. The rays should run parallel and an image will not be created.

Hands-on Work: Use the 50 mm focal length lens and the LED as a point source to collimate the light. Place the LED lamp a distance of 1 focal length (50 mm) away from the lens. The lenses we use a Plano-Convex (Flat on one side and bulging on the other). Make sure the flat side is facing the LED, and the bulging side faces toward the collimated light. That helps limit aberrations. The LED is a very small square in x-y, with a rounded glass top. When on, it is very bright. DO NOT look at the LED directly while it is on.

What does the light look like coming out of the lens?

Collimated light should be evenly illuminated in a circle. It will converge or diverge slightly, because the lenses are not perfect. But, it should be fairly good for several focal lengths (i.e., over 100-200 mm away from the lens). If you get an image of the LED, then it is not collimated.



Over what distance does the light stay collimated?

Should be OK for a couple of focal lengths.

At what distance can you observe an image of the LED lamp? Why do you think that happens?

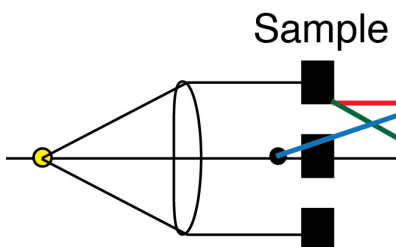
When it loses its collimation, you will see images of the LED (square or some other fine details). This occurs at “infinity” for the systems, which is a couple of focal lengths.

Creating an Image, Real and Virtual:

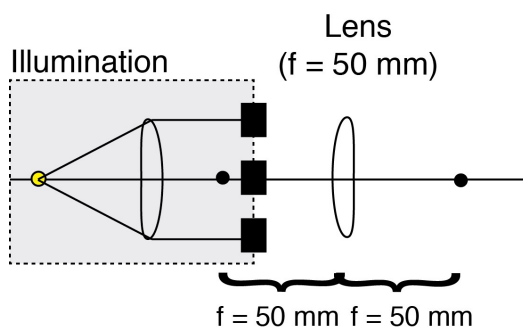
To find the focal length of the lenses, you created REAL images that can be projected onto a screen. You can also create virtual images. A reflection of yourself in a mirror is a virtual image of you. When you use a lens as a magnifying glass, the image you observe with your eye is a virtual image. (Your eye lens creates a real image of the virtual image on your retina, so you can “see” the virtual image.)

Hands-on Work: First, we will set-up a system to use in order make images. We need a sample to image. We will use a clear, plastic ruler as a “sample.” Use the holder with the two clips to hold the ruler.

Next, we need to illuminate the sample. We will illuminate the sample with collimated light. You already made collimated light above, and we will use that light to illuminate the sample.



After the sample, add **another** 50 mm lens. Mount and place the lens a distance **closer than** 1 focal length away from the sample (<50 mm). In other words, make the distance between the sample and the second 50 mm lens less than 50 mm.



Measure the distance from the sample to the lens and record it:

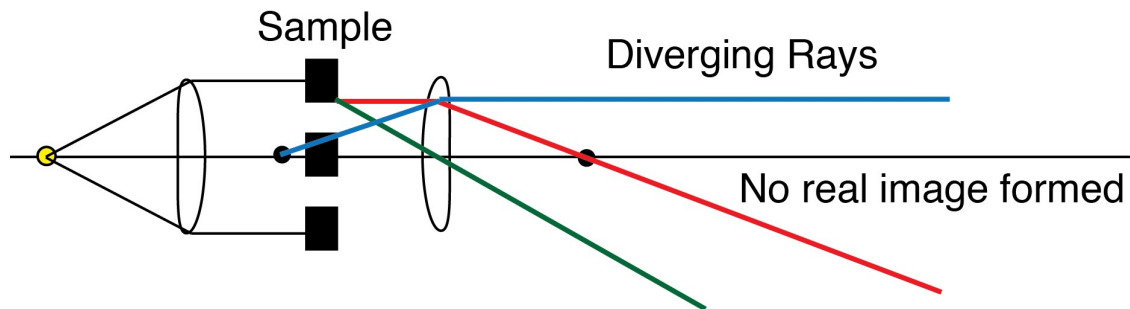
$$S = (<50 \text{ mm}) \underline{\hspace{2cm}}$$

What does the light look like coming out of the lens? Does it create an image of the ruler? If so, how far away from the lens is the image? Record it:

$i =$ **Cannot see an image.**

Is the image real? Or virtual? How do you know? Draw a diagram of the system with rays that emanate from the **SAMPLE**.

It is virtual because the rays diverge and the image can be seen INSIDE the lens. Have the students look through the lens at the ruler. It should be magnified.



Hands-on Work: Using the same ruler sample and same second 50 mm lens, move the lens away from the sample, so that the distance between them is **farther than** 1 focal length away from the lens (bigger than 50 mm).

Measure the distance from the LED lamp to the lens and record it:

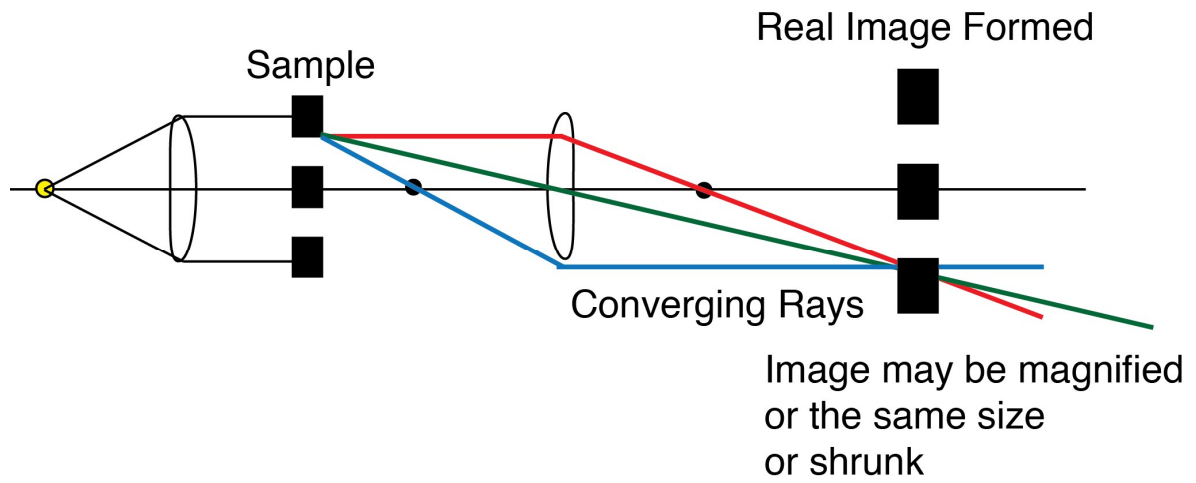
$s =$ **>50 mm**

What does the light look like coming out of the lens? Does it create an image? If so, how far away from the lens is the image? Record it:

$i =$ **>100 mm**

Is the image real? Or virtual? How do you know? Draw a diagram of the system with rays that emanate from the **SAMPLE**.

REAL. Because you can see it on the screen.



For the recorded measurements, does the distance to the source, s , and the distance to the image, i , fit the equation for image formation:

$$\frac{1}{f} = \frac{1}{s} + \frac{1}{i}$$

Two lenses:

The microscope is a professionally constructed system of lenses that control the light to create images with specific magnifications. The objective itself can have numerous lenses that are all different focal lengths both converging and diverging to magnify and correct aberrations. We will use two lenses to create a simple multi-lens system to magnify an image.

Hands-on Work: Start with the same system you just used above. Move the second 50 mm lens, the one that is making the image, so that it is exactly 50 mm away from the ruler/sample.

Think about this:

What does the light look like coming from the lens?

Should be collimated coming from the lens.

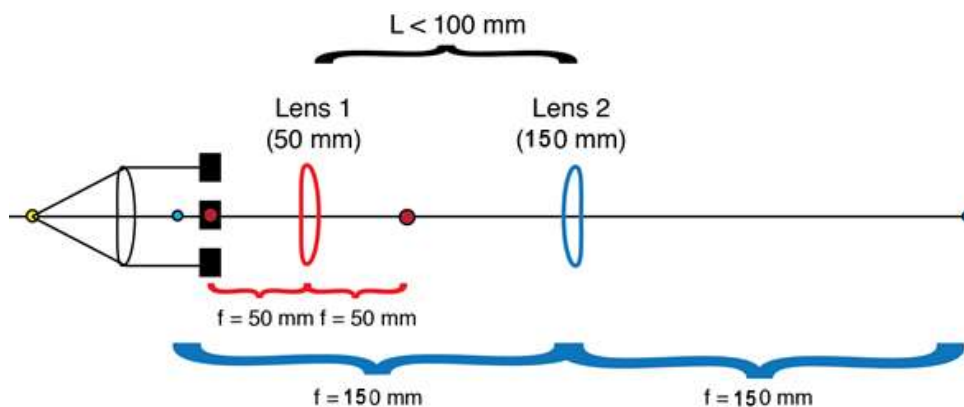
Can you make an image?

Maybe. The image should be at infinity, so several focal lengths away, you might see an image of the ruler.

Where is it?

Far away.

Put a second 200 mm lens 50 mm from the first lens. The second lens should be placed within the collimated space of the first lens.



Can you make an image of the ruler on the screen?

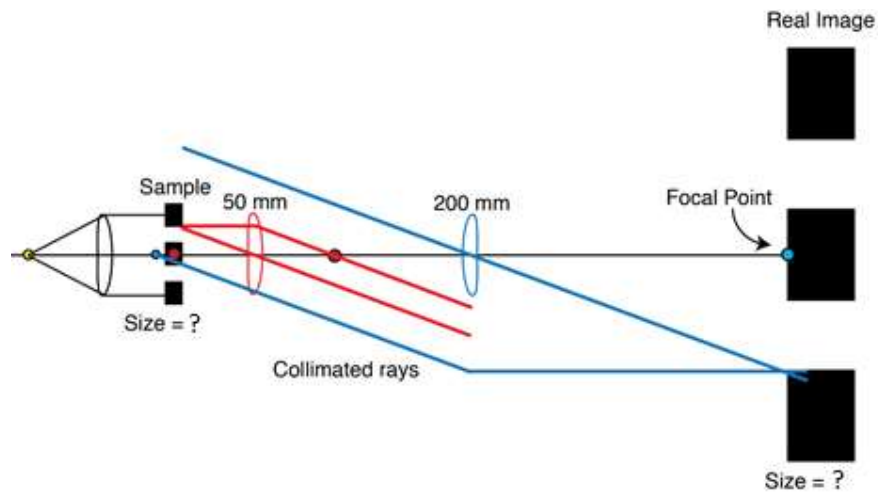
Yes. You should be able to make an image 200 mm away from the 200 mm lens (see schematic below).

If so, where is the image of the ruler located? (How far from the 200 mm lens?)

Should be about 200 mm.

Is the image magnified? How do you know? Draw the ray diagram:

Yes, it should be magnified. See the schematic.



Measure the size of the ruler spacing in the image, and call it I_{xy} . Use it to find the magnification, M , of the system you created.

$$M = \frac{I_{xy}}{S_{xy}}$$

Where S_{xy} is the size of the ruler spacing in the sample.

How does the magnification relate to the focal lengths of the two lenses?

$$M = I/S = 305/75 = 4 \text{ (Assuming } I=305, S=75)$$

Magnification is 4x.

$$4 = 200/50$$

Congratulations! You have built a 4 x objective! (Where the 4 is the magnification.)

Apertures and Field Stops

Background: In the last activity, you used lenses to bend light, but we can also use irises to block light. In this way, irises can also manipulate the light. In combination with lenses, irises can change the shape and intensity of light. Microscopes use irises to control the light going into the sample, and they are particularly used in condenser systems in both transmitted and epi-illumination schemes. The combination of iris and lens takes advantage of the wave-nature of light and the fact that the lens is a Fourier transformer of those waves.

The optical system of the condenser of the microscope has two irises that act as a field stop and an aperture. When the condenser is in Köhler Illumination, the field stop is in focus with sharp edges and the aperture dims the light.

Goals: The goal of this set of hands-on activities is to give you some basic understanding of how to manipulate light using irises. This set of activities will acquaint you with field stops and apertures as well as give you some. The learning goals are: 1. Demonstrate the differences between field stops and apertures. 2. Have you build a system with a field stop and aperture. 3. Discuss the connection between the aperture size and the *numerical aperture* of a lens system.

Field Stops:

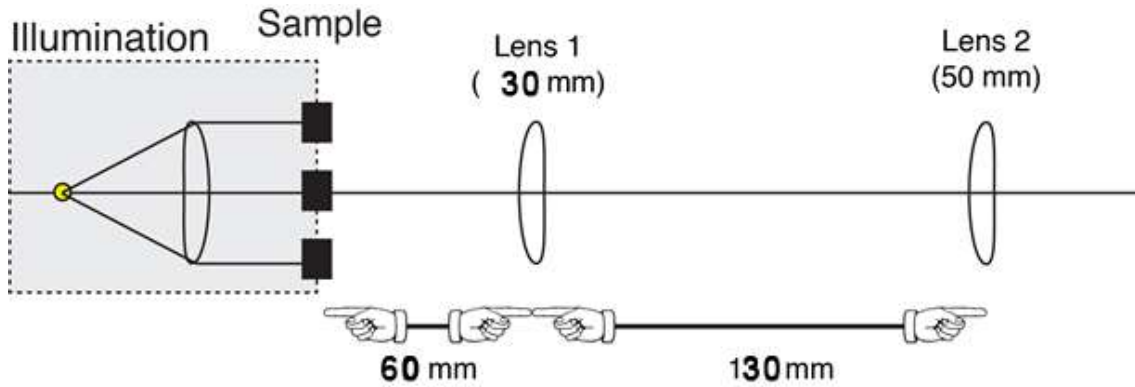
The field stop is an iris that acts like a curtain on a stage to block the light you do not want from the edges. It defines the field of view. Thus, field stops control the LOCATION of the light. For a microscope, the field stop controls the illumination region.

Hands-on Work: Turn on the transmitted light on the commercial microscope at your station. Locate the field stop on the commercial microscope. Put a card at the sample plane (where you normally put the sample). Open and close the field stop. Is the field stop in focus? If not, move the condenser up and down until it is in sharp focus. Use the card to inspect the light above and below the condenser with the field stop open or closed to see how the field stop alters the light as it travels through the system.

Sketch a diagram of the condenser of the microscope pointing out the fieldstop

Commercial faculty, please help the students find the field stop in the condenser.

Build the following system using a 30 mm lens (Lens 1) and a 50 mm lens (Lens 2). Try to space apart the lenses about the distances listed (obviously, you won't get them perfect, and it doesn't matter too much).



Trace the rays through the system you built using an index card.

Where are real images of the sample formed?

Draw the ray diagram of the system and locate (with distances in millimeters) where the real images are formed in the system.

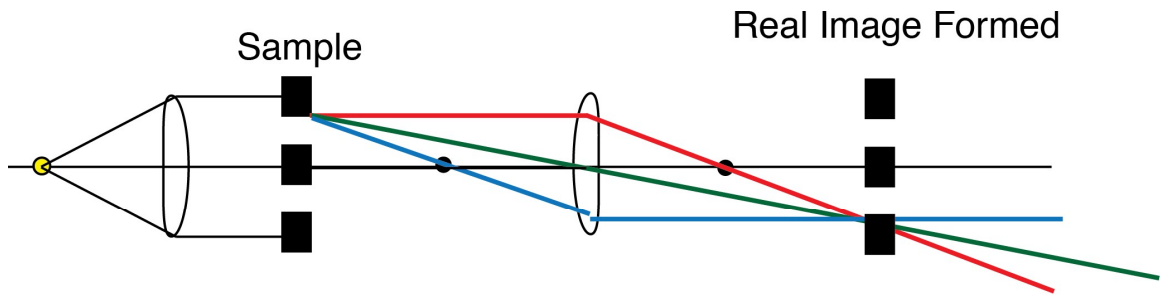


Image will be the same size

Can you make an image of the ruler on the other side of the 30 mm lens?

Yes. It should be a real image.

If so, where is it?

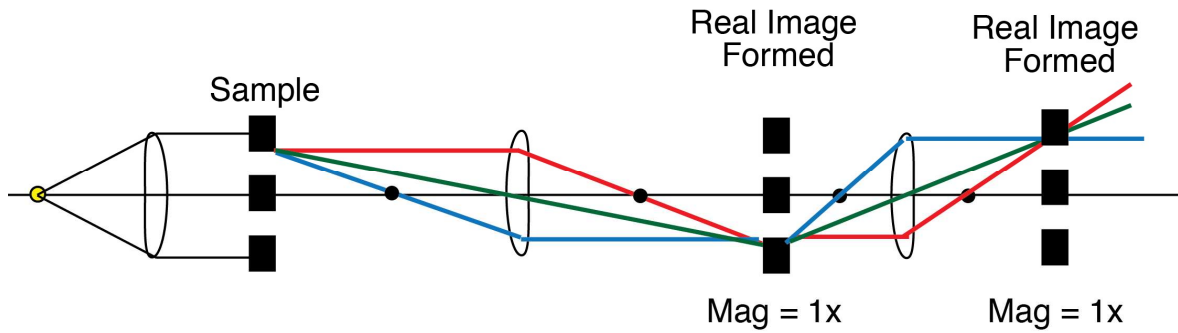
It should be 50 mm away from the second lens.

Describe the image you create. Draw a picture of it.

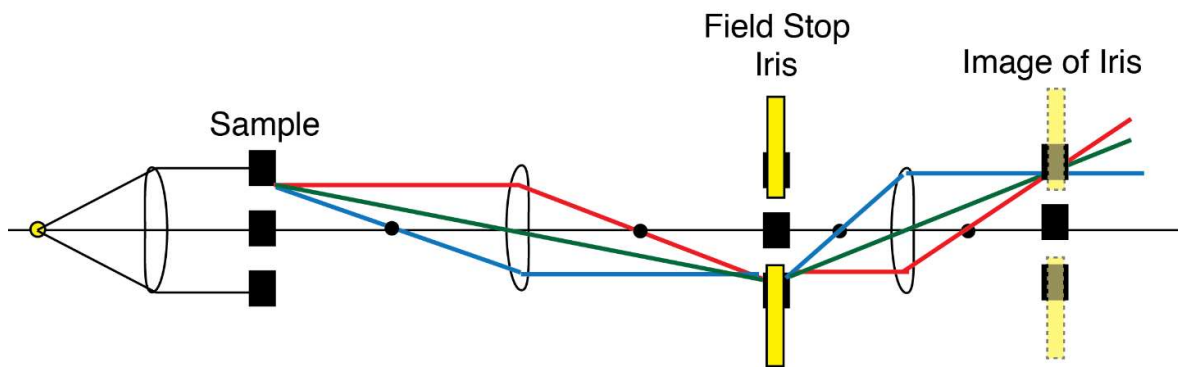
It should again not be magnified (1x). It should be upright.

Draw the lens system. Include rays traced from the SAMPLE.

Schematic of the final system.



Use one of the irises to make a field stop by placing the iris **AT** the intermediate image that is created *between* the two lenses (100 mm from the 50 mm lens and 50 mm from the 30 mm lens). Add the iris to your lens system drawing above. **See schematic.**



As you open and close the iris, what happens to the image of the ruler after the 30 mm lens? Describe what happens in words.

This iris is a field stop, so it will create an image of the iris at the final image plane. You will see the iris in focus getting larger and smaller.

Close the iris all the way, and draw the image you see:

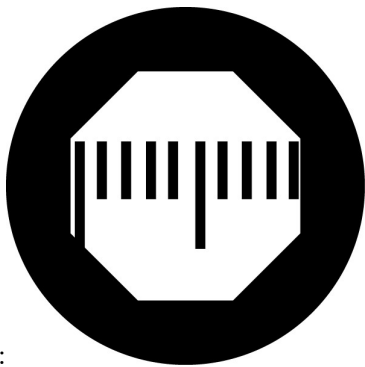
Should look like this:



Closed:

Open the iris all the way and draw the image you see:

Should look like this:



Open:

Apertures:

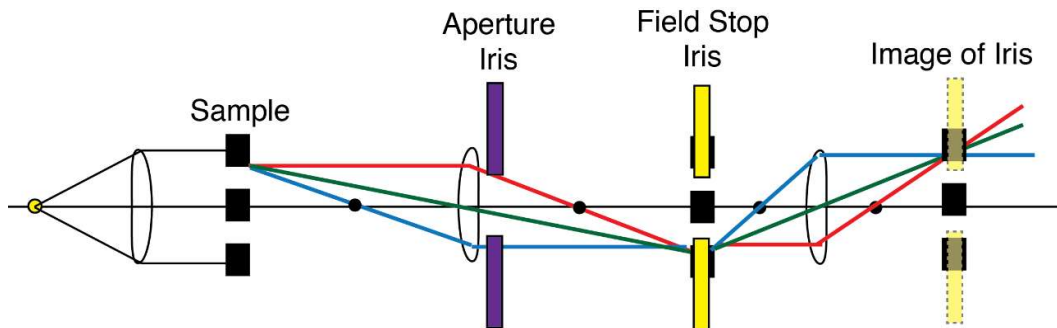
An aperture is an iris that acts like a dimmer switch. Apertures control the INTENSITY of the light. It works by cutting out the angles of light that go into making the illumination pattern at the image plane. For a microscope, the aperture controls the illumination intensity.

Hands-on Work: Turn on the transmitted light on the commercial microscope at your station. Locate the aperture on the commercial microscope. Put a card at the sample plane (where you normally put the sample). Open and close the aperture. Use the card to inspect the light above and below the condenser with the aperture open or closed to see how the aperture alters the light as it travels through the system.

Commercial faculty, please help the students find the aperture in the condenser.

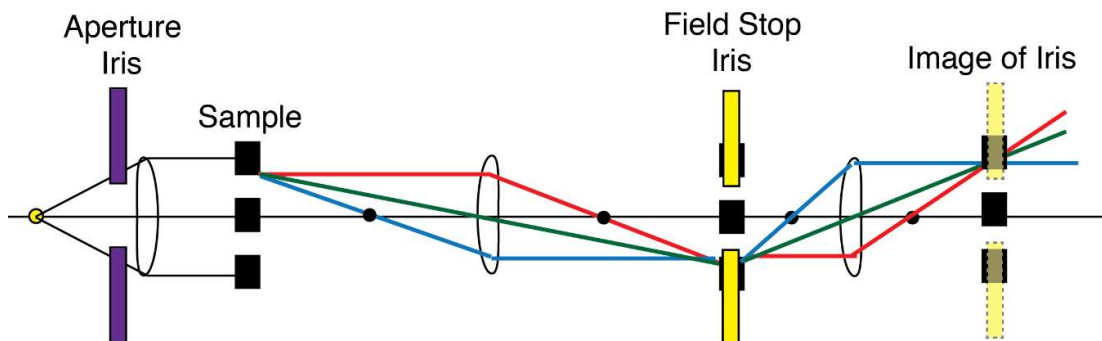
Use the same system you built above with the 50 mm and 30 mm lenses. Place an iris near the 50 mm lens to act as an aperture. What happens at each of the image planes (one intermediate image between the lenses and the final image after the 30 mm lens) as you close and open the iris? Describe it in words. Draw a picture of your lens system and trace rays coming from the SAMPLE.

If the iris is acting as an aperture, then the images should be dimmed at both the location where the field stop is and at the final image.



Place an iris directly in front of the LED. What happens at each of the image planes (one intermediate image between the lenses and the final image after the 30 mm lens) as you close and open the iris? Describe it in words. Why does this happen?

If the iris is acting as an aperture, then the images should be dimmed at both the location where the field stop is and at the final image. In this location, it is very clear that you are cutting the angles of the light coming from the LED light source, and that will dim the light.

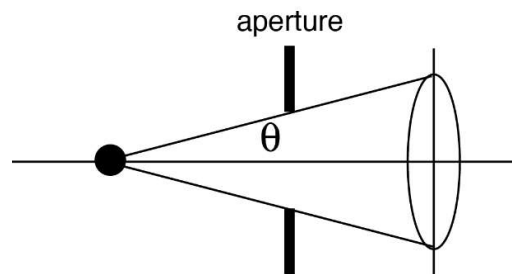


Numerical Aperture:

The numerical aperture (NA) of a lens or multi-lens system is a quantification of the aperture size. It is a direct measure of the angles over which the light is collected through the system.

$$NA = n \sin \theta$$

where n is the index of refraction of the lens material and θ is the maximum angle over which the light is captured. For optical systems, such as objectives, the angles are limited by the radius of the inlet or lens size itself.



Hands-on Work: Using the lens system that you constructed above, calculate the numerical aperture when the iris is set to the smallest diameter (0.8 mm is the smallest diameter).

Using the system in the last schematic, you can see that the angles of the light are being cut as they go to the first 50 mm lens. The angles can be found from the diameter of the aperture ($D = 0.8$ mm, $r = 0.4$ mm) and the distance from the LED to the aperture (ie. $d = 50$ mm).

$$\sin \theta = r/d = (0.4 \text{ mm})/(50 \text{ mm}) = 0.008$$

$$n = 1.4 \text{ (index of refraction of the glass)}$$

$$NA = 1.4 * \sin \theta = 1.4 * 0.008 = 0.011$$

Calculate the numerical aperture of the two-lens system when the iris is set to the largest diameter (12 mm is the largest diameter).

$$\sin \theta = r/d = (6 \text{ mm})/(50 \text{ mm}) = 0.12$$

$$n = 1.4 \text{ (index of refraction of the glass)}$$

$$NA = 1.4 * \sin \theta = 1.4 * 0.12 = 1.68$$

The closer the iris is to the LED, the higher the NA.

If $d = 20$ mm,

$$\text{Smallest NA} = 0.03$$

$$\text{Largest NA} = 0.42$$

What is the numerical aperture of the commercial microscope condenser system?

Find the numerical aperture on the condenser.

Conjugate Focal Planes, Front and Back Focal Planes

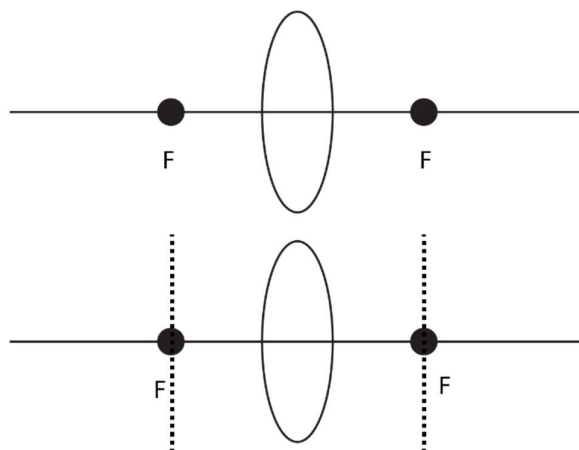
Background: In the last activity, you created a multi-lens system and placed irises in different locations to make field stops and apertures that cut out the light in different ways. There are a number of specific locations in the optical system that have specific properties. Here, we are going to discuss various focal planes including front and back focal planes, conjugate focal planes, and Fourier Transforms.

When people discuss optical systems and microscopes in particular, they often refer to the image plane and conjugate image planes or back focal planes and conjugate back focal planes. We will discuss what those focal planes are, where you can find them in a commercial microscope, and how to create them with a multi-lens system.

Goals: The goal of this set of hands-on activities is to give you some basic understanding of creating conjugate focal planes and what the light is doing at those planes. how to manipulate light using irises. This set of activities will acquaint you with focal planes as well as give you some physical intuition about the wave-nature and Fourier optics that can be used in optics and microscope design. The learning goals are: 1. Demonstrate the locations of front and back focal planes. 2. Demonstrate how to create conjugate focal planes and what the properties of these focal planes are. 3. Demonstrate that the back focal plane and front focal plane are *Fourier transform conjugates* of each other.

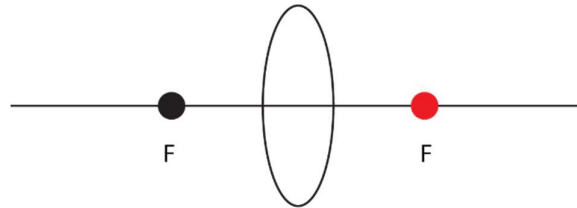
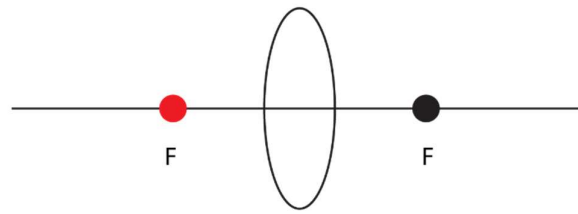
Focal Planes:

A focal plane is the two-dimensional plane where an image focuses due to a lens of multi-lens system. The focal plane is located a distance away from the lens that equals the focal point. You already tested several lenses to find the focal point. There are focal points on both sides of the lens, and light is time reversible. That means that there is another focal point and focal plane on the other side of the lens, too. It looks like this:

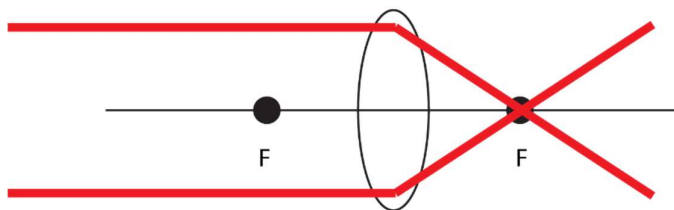
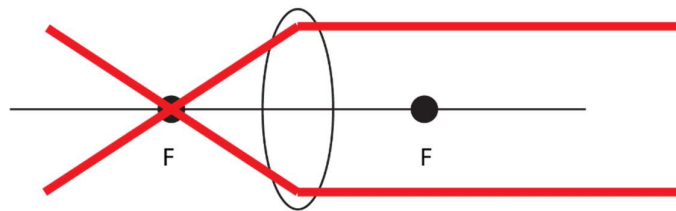


These two focal planes are the front and back focal planes. For a single lens, there is not much of a difference between the two focal planes. We call them the front and back focal planes. Light can propagate from left to right or from right to left. As we saw previously, a sample/source sitting at the focal point is collimated on the other side. This is true for both focal points – not matter what side of the lens the propagation is coming from.

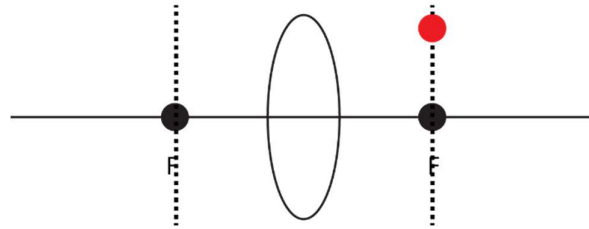
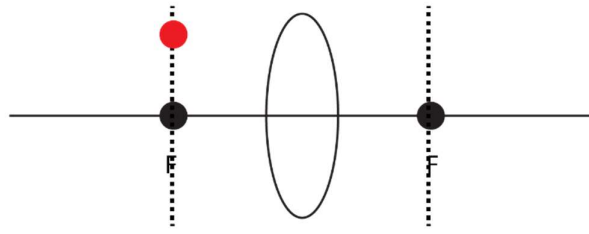
Group Work: Together as a group, draw the rays emanating from the red point for each lens below:



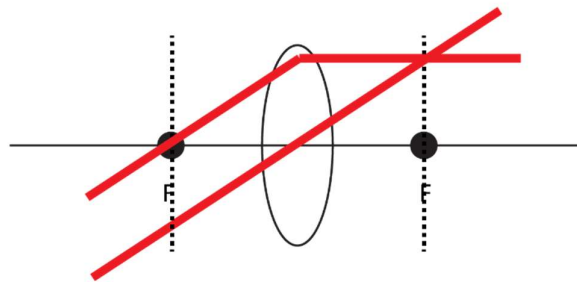
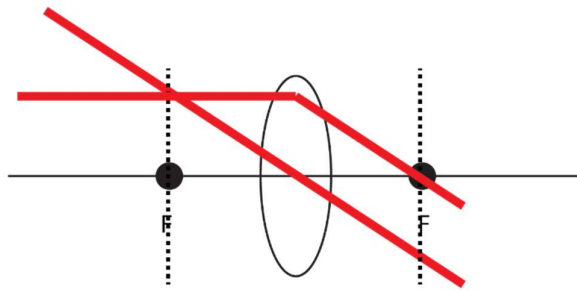
Solutions:



Group Work: For the lenses below, trace the rays from the red dots in these lenses. Is the light collimated? How is it the same or different from the situations above?



Solutions:



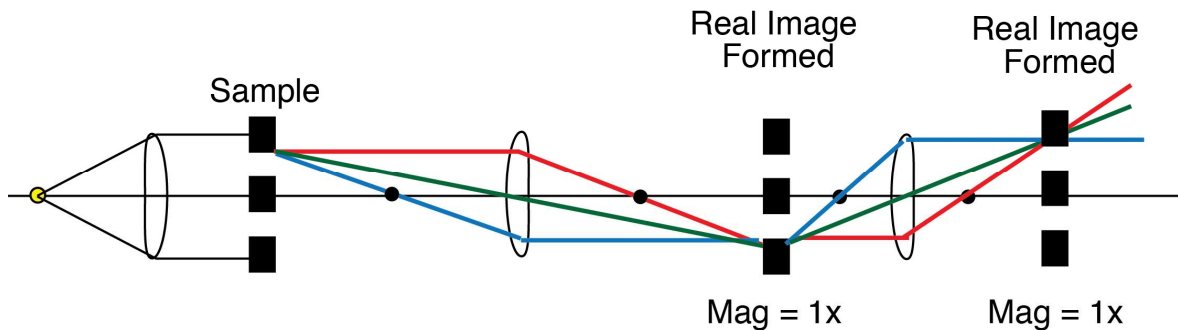
Conjugate Focal Planes:

A conjugate focal plane is a focal plane that has the same optical properties as another focal plane. These regions are optically similar. As you saw in the last worksheet, there are multiple focal planes in a multi-lens system. Previously, we used those locations to place irises to make field stops of apertures. Here, we are going to highlight the properties of the focal planes themselves.

Hands on Work: Using the same two lens system from the last set-up, identify the locations where the sample is in focus. You can do this empirically by finding the image of the ruler (sample) at various locations. These locations are conjugate focal planes to the sample plane.

Draw the optical system and draw in the conjugate focal planes:

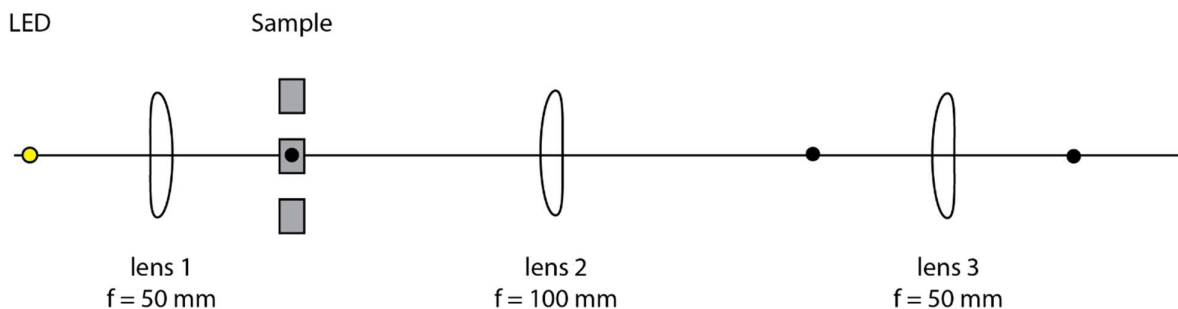
Solution:



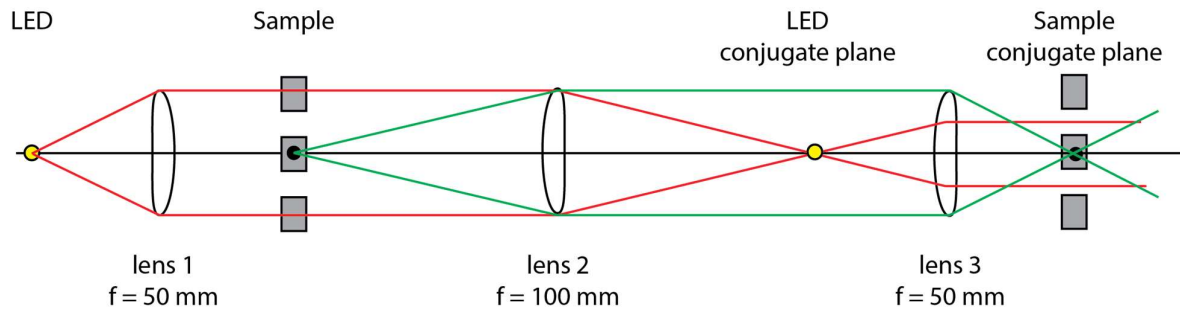
The locations where there are real images are the conjugate focal planes. They are conjugate to the sample plane.

There are also focal planes that are conjugate to the LED/lamp. Find them empirically using a card to trace the light and theoretically using ray tracing by tracing the rays from the LED (not the sample). Are these good locations for field stops? Or apertures? Or neither?

Draw the optical system and draw in the conjugate focal planes:



Solutions:



Planes that are conjugate to the LED focal plane have an image of the LED and thus have converging rays from the LED at those locations.

Planes that are conjugate to the sample focal plane have an image of the sample and thus have converging rays from the sample at those locations.

Rays from the LED are collimated at conjugate sample planes.

Rays from the sample are collimated at conjugate LED planes.

Building a Microscope Condenser

Goal: Our goal for this portion is to design and build a microscope condenser in Köhler Illumination. Through building the condenser, you will learn how it works and why it has the components that it does. The purpose of the condenser is to control the light onto the sample for transmitted light microscopy. The condenser has a field stop and an aperture to control the location and intensity of the light on the sample. You will design and build a condenser that will evenly illuminate the sample and the intensity and size of the illumination area can be controlled.

Hands-on Work: You will use the LED light as the illumination source.

You will need to use lenses and two irises to control the light at the sample.

Use the knowledge you just gained from the lenses and imaging work and the apertures work to help you.

Notes: You want the light at the sample to be *evenly illuminated*.

Most importantly, you do **NOT** want to create an image of the LED onto the sample. Use the concepts you learned with the lenses to help determine how to collect the light and use multiple lenses to illuminate the sample.

Use the irises to control the light by using one as a field stop and the other as an aperture. Use the concepts and ideas you learned before along with trial and error to determine the best location for each of the irises.

There are unlimited correct and workable ways to design the condenser (that is why optics is SO COOL!).

Compare your condenser designs to the condenser of the commercial microscope at your station. How are they the same? How are they different? Can you identify all the parts and components of each?

Draw out your final condenser design and label the components.

As the students make their condensers, they should be checking that the location of the irises can do the correct functions. One should control the intensity of the light at the sample (aperture). The other should control the region of the light (field stop).

We recommend, and it will be easiest, if:

The light is mostly collimated when it hits the sample.

The aperture should be in a region where the rays are focusing or defocusing.

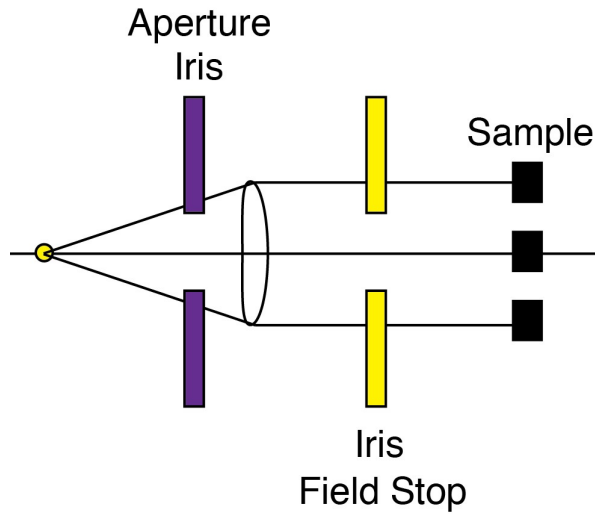
The field stop should be in a region of collimated light.

After they build it, the students should check that:

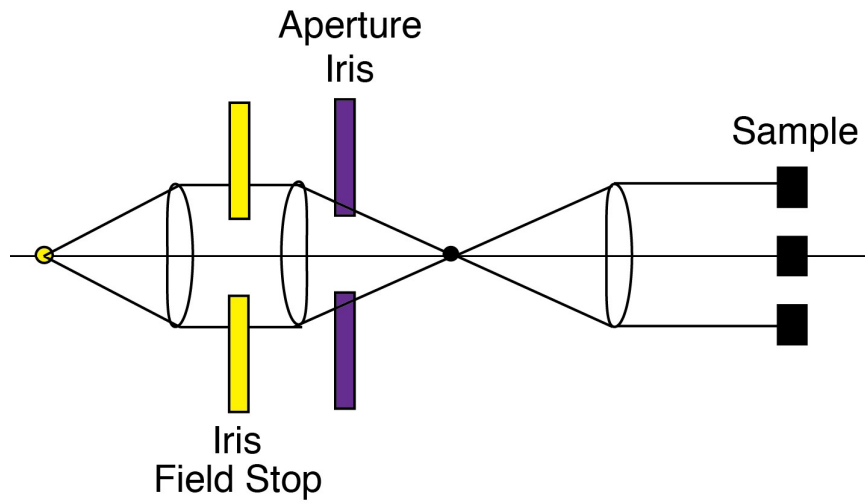
Opening the field stop changes the region on the sample plane that can be illuminated and the field stop is in focus at the “sample plane”

Opening the aperture causes the illuminated region at the sample to get brighter and closing the aperture causes the illuminated region to get dimmer.

Example of a 1-lens condenser:

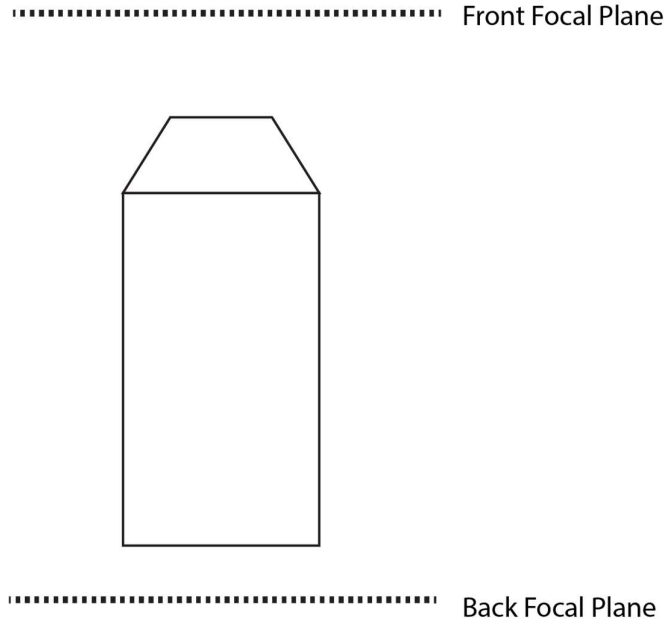


Example of a 3-lens condenser:

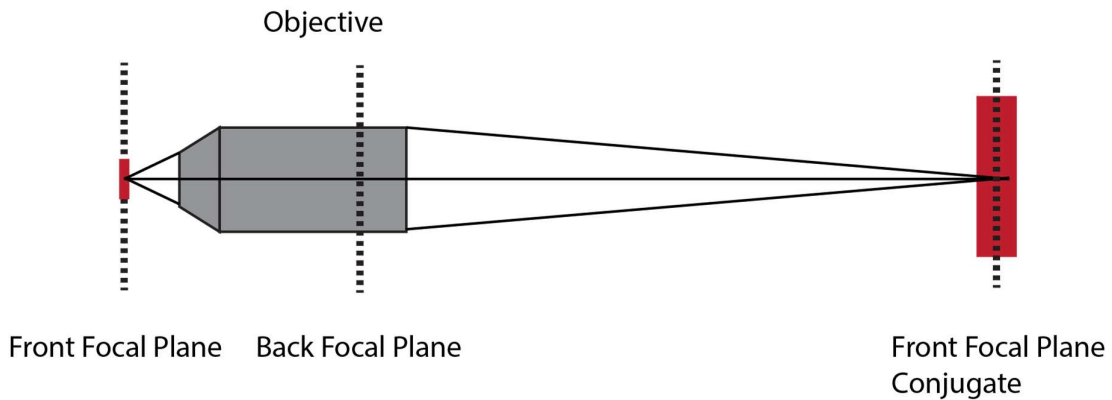


Microscope Objectives:

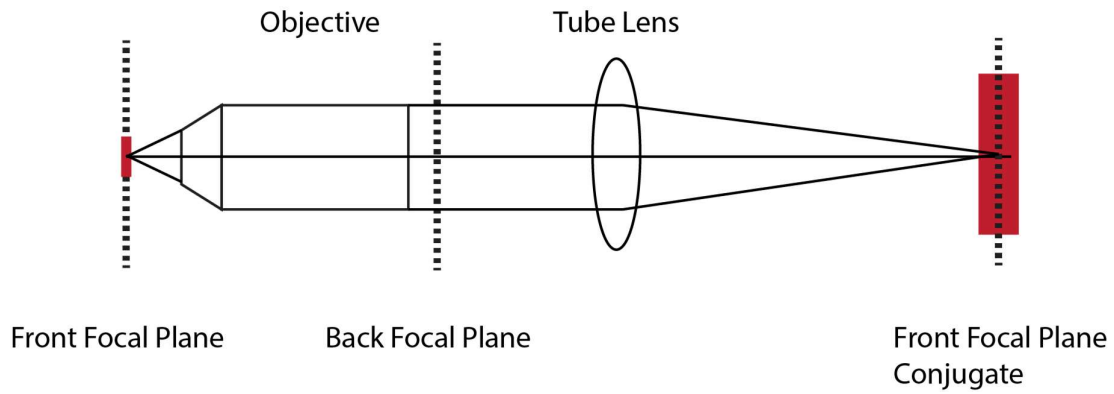
Although the front and back focal planes for a single lens are similar, that is not true to a system of lenses. For an objective, which is a multi-lens system, the front focal plane is where we put the sample. The back focal plane is behind the back of the objective.



There are two types of objectives that you can use. The first is a $f = 160$ mm focal length objective. These are older objectives that you might come across, but will likely not see on a modern microscope. This type of objective focuses the light from the front focal plane 160 mm away from the back of the objective. Thus, the sample plane has a conjugate focal plane 160 mm from the back of the objective:



The modern type of objective is an infinity-corrected objective. Infinity-corrected objectives have an infinity space behind the objective. Thus, the light is collimated out of the objective, like a single lens. These objectives require a tube lens in order to focus the light and create a conjugate focal plane. These lenses are preferred because the back focal plane is accessible to place other optical components that can manipulate the light such as phase rings or prisms.



When you take the eyepiece out of the microscope, you can visualize the back focal plane of the objective. Using a commercial microscope, remove the eyepiece and describe what you can see. What are you looking at? How can you change what you see?

Solutions:

If there are anything in position in the back focal plane of the objective, like a phase ring, the students will see it. They may also see the aperture of the condenser or the transmitted light source, if the condenser is Kohler.

Building a Microscope Imaging Path

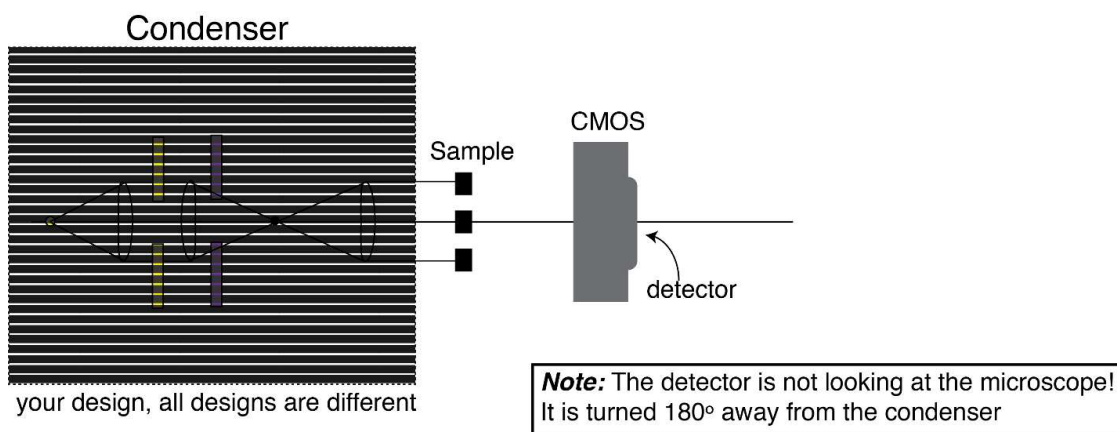
Goal: Our goal for this portion is to finish the microscope by adding the objective and the camera for recording the sample. The imaging path consists of the sample plane/holder, the objective system, and the camera. You will decide on the objective type and magnification, align the system, and make an image with the camera. Tomorrow, you will test the system's magnification and resolutions and make adjustments based on those tests.

Making an Image on the Camera:

Before we build the rest of the microscope, we need to make sure we understand how to make an image on the camera.

Hands-on Work: Turn on the computer hooked to the CMOS camera for the microscope system. Click on the camera recording program on the desktop.

Place the CMOS camera pointing away from the condenser system you created yesterday. See the schematic:

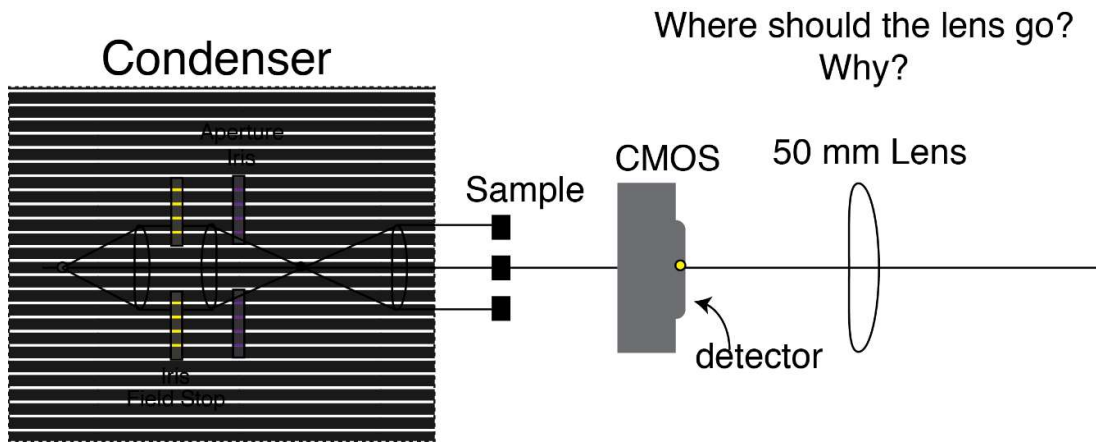


Open the camera software on the computer. Make sure everything is plugged in and getting power and sending signals! Using the software, image using the camera. Can you see anything?

If you wave your hand in front of the detector, you should see something on the screen. Is it making focused images?

Why or why not?

Mount a 50 mm lens in front of the camera, like in this schematic:



Move the lens back and forth until you make an image on the CMOS that shows up on the computer screen. It is best to image something very far away (at least 500 mm away). It is even better if you can image something across the room.

When you make an image, take a picture with the camera and save it. How far away from the camera did you have to place the lens in order to get the image in focus?

Assuming the source is at “infinity,” and knowing the focal length of the lens you used, where is the image?

$$\frac{1}{f} = \frac{1}{s} + \frac{1}{i}$$

Build the Imaging Path with an objective:

Pick an objective from the front of the room. Note what type of objective it is and record the properties:

Type:

Manufacturer:

Magnification:

Numerical Aperture:

Working Distance:

Other important information:

Mount the objective in the mount with the adapter from RMS to the screw-type of the tube system. There should be one available for each group.

Use the plastic ruler as a sample, or make another easily visible sample (sharpie on a slide, for instance) to use as a sample for aligning. Place the objective in front of the sample, between the sample and the CMOS camera. Make sure to point the detector toward the objective. Turn on the lamp for the condenser to illuminate the sample. Use a card to see if you can make an image from the objective of the sample.

Can you make an image? Why or why not? Work with your group members and instructors until you can make an image. Remember about the different needs of the different types of objectives. Does your objective have everything it needs? This part will take some trial and error, but you will be learning a lot along the way.

Once you are able to get an image on the computer, make sure it is focused by moving the sample and objective relative to each other. When the image is sharply in focus, record it, and save the image.

If you are done early, you can move to the testing phase (see next hand out).

Test Your Microscope Magnification and Resolution

Goal: Last time, you built a microscope with an objective and a camera. You were able to capture images, but how do you know that the microscope is set up properly? We need to test the microscope to check that the magnification and the resolution are as specified. These are determined mostly by the objective, but improper placement and alignment can alter the magnification and resolution significantly.

Measure the Magnification:

We need to **quantify** the magnification we are getting on the screen in order to determine if the objective and camera are placed properly.

Hands-on Work: Try to use the plastic ruler as the sample and get an image of it on the camera, as you did yesterday. Make sure that you can observe two markings of the ruler. If you can see two marks of the ruler, take an image. If not, adjust the sample so that you can see two marks. If you can never observe two marks, get a different sample with a known spacing between marks. There are diffraction gratings available and other samples around. You can also make a sample.

Take an image of the sample and save it. Start Fiji/ImageJ on the computer. Open your image in Fiji/ImageJ. Measure the number of pixels between the lines of your sample:

Select the line tool on the toolbar.

Draw a line between the two lines. Make sure you are being consistent. Are you drawing from middle to middle? Or right side to right side. The placement of the line may alter your measurement (try it).

Select **Analyze/Measure** to determine the length of the line you drew in pixels.

Record the number of pixels between the markings. We will call this the *Image Size* (I_{xy})

$I_{xy} =$ _____

You can find the physical size of the camera pixel using the documentation from the camera (online). Check to make sure the physical size of a single pixel is $5.2 \mu\text{m} \times 5.2 \mu\text{m}$. You need this information to measure your magnification.

Before we go on, let's think about magnification, because it can be confusing. Consider this:

If your objective is 1x, then the distance between two marks that are $100 \mu\text{m}$ apart would be 19 pixels. (Check that this is true.)

If your objective is 2x, then the distance between two marks $100 \mu\text{m}$ apart would be 38 pixels. (Check that this is true.)

What is the distance in millimeters between the markers in your sample? This is the size of your source in millimeters (S),

$$S(\text{mm}) = \underline{\hspace{4cm}}$$

So, we know that magnification is the ratio of the image size to the source size. Unfortunately, the units of these two lengths are in different units. The Image size is in pixels and the source size is in millimeters. We need to convert the source size to pixels. How many pixels ($5.2 \mu\text{m} \times 5.2 \mu\text{m}$) is the size of the source? This is the size of the source in pixels (S_{xy}).

$$S_{xy} = \underline{\hspace{4cm}}$$

Now that the image size and the source size are in the same units, we can take the ratio to find the magnification. You can calculate the magnification as:

$$M = \frac{I_{xy}}{S_{xy}}$$

Is the magnification you measure the same as the magnification written on the side of the objective? If not, why?

Correct the objective and camera placement to get the correct magnification and re-measure it to be sure.

Measure the Resolution:

The resolution is a measure of how well you can separate two nearby objects. High resolution measures smaller separation distances. We will theoretically estimate the resolution of your microscope and then test it using samples with known spacing (graticules and diffraction gratings).

Theory: Resolution comes from the phenomenon of diffraction, which is the bending of light around the edge of an aperture. In the case of objectives, the aperture we care about is characterized by the numerical aperture (NA) of the objective. The smallest discernable distance between two objects in the sample plane is given by:

$$d = 1.22 \frac{\lambda}{2NA}$$

where, d is the smallest distance, λ is the wavelength of the light, and NA is the numerical aperture of your objective. Calculate the smallest resolvable distance for your microscope using this equation.

Hands-on Work: Use several diffraction gratings and take images of them.

Which diffraction gratings can you resolve (actually distinguish) the different lines of the gratings?

Which have the lines too closely spaced to make out the lines?

Does this match you're the predictions of your theoretical resolution?

If yes, can you alter the resolution? How?

If your predictions were not met, why not? What could be changed?

Comparing 160-mm and Infinity-Corrected Objectives:

If you have time, change the objective you started with to a new objective. If you used a 160-mm focal length objective, try an infinity-corrected objective and vice versa.

Older microscopes had 160 mm objectives. These objectives are designed to make an image of the correct magnification 160 mm behind the objective. Getting the right magnification amounts to getting the right distance from the objective to the camera. The focus is achieved by moving the sample relative to the objective. This makes focusing harder, since the distance between the objective and the camera must be fixed.

Modern microscopes use infinity-corrected objectives. Infinity-corrected objectives have a collimated “infinity space” at their back focal plane. When a sample is at the front focal plane, there is not an image at the back focal plane, but rather the Fourier Transform of the image. The back focal plane of the infinity corrected objective is where optical components can be placed (phase rings, prisms, and analyzers) to manipulate the light, as you will learn in coming parts of the course.

Hands-on Work: Pick a new objective from the front of the room.

Note what type of objective it is and record the properties:

Type:

Manufacturer:

Magnification:

Numerical Aperture:

Working Distance:

Other important information:

Mount the objective. Are you able to focus onto the camera? What is different or needed? Change things one at a time until you are able to get an image of the correct magnification for the new objective.

Fourier Transforms:

Lenses are two-dimensional Fourier Transformers. Whatever is at the front focal plane will be Fourier transformed at the back focal plane. The equation for a Fourier Transform is:

$$F(k) = \int_{-\infty}^{\infty} f(x) e^{-2\pi i k x} dx$$

where x is the displacement, k is the wave number ($2\pi/\lambda$), $f(x)$ is a function of x that is a particular illumination pattern, and $F(k)$ is the Fourier transform of the function $f(x)$.

Because $e^{ix} = \cos x + i \sin x$, and the integral is an infinite sum, you can think of the Fourier transform as a form that pulls out the spatial frequencies of a pattern.

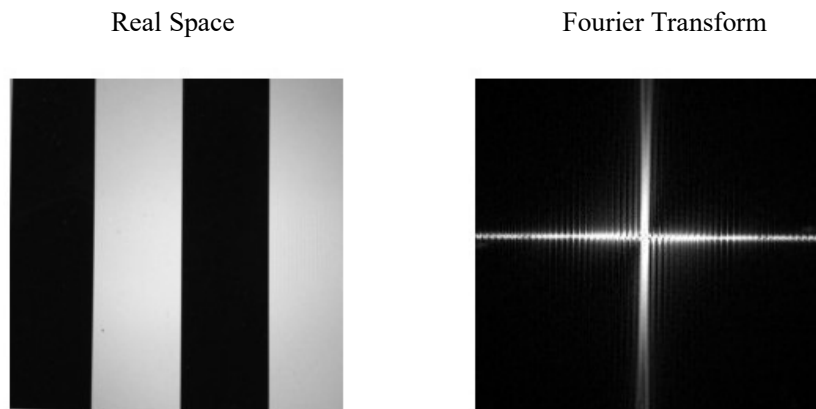
(See <http://mathworld.wolfram.com/FourierSeries.html>

<http://mathworld.wolfram.com/FourierTransform.html>

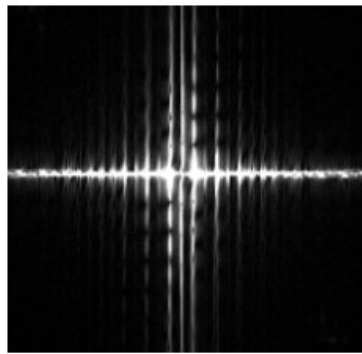
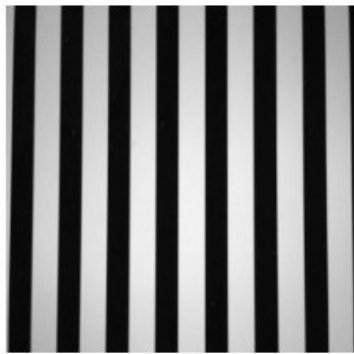
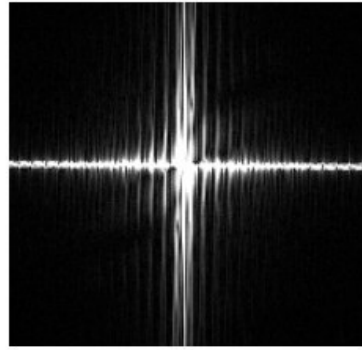
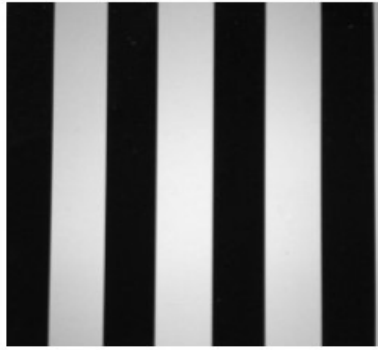
<http://mathworld.wolfram.com/FourierTransformDeltaFunction.html> for more information)

For instance, the Fourier Transform of a bright dot is an evenly illuminated region. What does this mean? It means that a bright source at the focal point will be collimated at the back focal point. The Fourier Transform of a pattern convolves the spatial components of the pattern.

Here are some examples of spatial patterns and their Fourier transforms to help you build some intuition.



When the spatial frequency increases, the peaks in the Fourier transform spread apart.



Köhler Illumination, Back Focal Plane Imaging, Fourier Optics

Background: We are making the transition from our open-air, training microscopes to the commercial microscope stands. The commercial stands are designed to have fewer moving parts, but there are still many locations where you can alter the system to manipulate the light and allow you to create contrast and see different structures. Before being able to do phase or DIC imaging, you need to put the microscope in Köhler illumination. This is a specific location of the condenser that makes the front focal planes of the objective and condenser at the same location, they become “conjugate planes.”

Goals: The goals of this activity are for you to: 1. Compare your home-made microscope to the commercial stand. 2. Master placing the condenser of a commercial microscope in Köhler Illumination. 3. Visualize the back focal plane of a microscope. 4. Begin to understand Fourier Optics and how the back and front focal planes are “conjugate” to each other.

Köhler Illumination, Back/Front Focal Plane Conjugates:

You already built your home-made microscope in Köhler illumination, but the commercial microscope’s condenser settings can be adjusted and must be aligned. You will work with your commercial faculty on two different microscope stands to place the microscope in Köhler illumination. Commercial microscopes are set up such that, when the condenser is in Köhler, the field stop is in focus and that places the optical components in a focal plane that is conjugate to the back-focal plane of the objective.

Hands-on Work: Identify the condenser system, the field stop, and aperture. Draw a picture of the system:

Locate the location in the condenser where optical components would be to manipulate the incoming light for phase or DIC imaging. This is the back-focal plane of the condenser and it needs to be located at the conjugate back focal plane to the objective. Add this location to your drawing above.

Work with the faculty to place two different microscopes in Köhler. After you get the microscope in Köhler, do the next activity to visualize the back focal plane through the eyepieces. You need to:

1. Focus on the sample.
2. Close down the field stop. Move the condenser up and down to make the field stop focused on the sample plane (conjugate planes). The image of the field stop should be sharply in focus.
3. Adjust the location of the condenser to center the field stop using the pressure screws within the condenser.

(Each microscope may be slightly different. Listen to the faculty for specific instructions on their microscopes.)

Challenge: If you are waiting for your turn on the microscope or you already had your turn, do this challenge: Identify the back and front focal plane conjugates on the home built and commercial microscopes in the pictures on page 1. Draw them in and distinguish them in some way.

Visualizing the Back Focal Plane:

Once you have your microscopes in Köhler, they are set such that the back focal plane of the objective is a conjugate focal plane with the back focal plane of the condenser. This allows us to modulate the light hitting the sample. We can modulate the phase, polarization, and intensity, etc... When you place optical components in this back focal plane conjugate, they can be visualized if you can image in the back focal plane. Most microscopes allow this by removing one of the eyepieces.

Hands-on Work: Work with the commercial faculty to remove the eyepiece to visualize the back focal plane of the objective.

What does the back focal plane look like? Draw it:

Place a phase ring into the back focal plane using the phase rings on the condenser. What does the back focal plane look like? Draw it:

If you put a sample onto the stage, does it change what you see in the back focal plane? How?

Fourier Optics:

This part is optional, if you get to it. You can come back to it if you have down time or are truly interested.

The front and back focal planes of a microscope are Fourier conjugates of each other. What you do in the back focal plane does affect the image of the front focal plane, but it gets Fourier transformed. The Fourier transform is the spatial frequency spectrum of the pattern of light. This is similar to the frequency of sound waves you might visualize on a synthesizer. I have included a lot of notes about Fourier Transforms as extra pdfs for you, if you are inclined to look at them.

Hands-on Work:

Since the microscope is already all set up in Köhler, it is the perfect opportunity to look at the Fourier Transforms of the front and back focal planes. Using the one eyepiece that is off and the one that is on, you can visualize both the front and back focal planes of the same optical configuration.

Take out all phase rings in the condenser and open the aperture and field stop. This is the open/reference state. Draw the Front and Back Focal planes that you see in the eyepieces:

Front Focal Plane:

Back Focal Plane:

Close the field stop all the way and draw what you see.

Front Focal Plane:

Back Focal Plane:

Open the field stop and put in a diffraction grating as the sample. Draw what you see.

Front Focal Plane:

Back Focal Plane:

Add a phase ring. Draw what you see.

Front Focal Plane:

Back Focal Plane:

Add a prism. Draw what you see.

Front Focal Plane:

Back Focal Plane:

The image below represents a system where the spatial frequencies of two images have been convolved with different weights to the high and low spatial frequencies that compose the image. When you look at them close up, the high frequency images are obvious. When you view them from far away, the low frequency images are more obvious. Instead of going in and out, you can also squint. Squinting reduces your eye's resolution by reducing the aperture.



Introduction to Transmitted Light Microscopy on Compound Microscope

Transmitted Light Path, Köhler, Resolution

Items needed for the lab: diatom slides, slides of diffraction gratings, filter paper and scissors, red filter or red transparent paper.

First examine your microscope to identify components. If you finish your exercise on your assigned microscope, we also recommend going around to other microscopes from different company to identify components again and see how they are operated.

Transmitted Light Path

- 1) Identify transmitted light source (and any adjustments to lamp position if they exist).
- 2) What is between the light source and objective lens?

Take apart light housing, look at bulb and collecting lens.

Find diffuser glass element, neutral density, other filters,

Polarizer (used for polarization microscopy and DIC).

Field diaphragm.

Condenser focusing knob.

Condenser aperture diaphragm adjustment.

Condenser turret-identify bright field position.

Condenser lens.

Objective Lens (Look at the lens specs).

- 3) What is between the objective lens and the eye piece/camera?

DIC prism (may not be there, but find its position and we will use this space for this lab).

Dichroic filter changer (use an “open” position for this lab).

Analyzer (another polarizing filter-we will use it later; for now make sure it is out).

Optivar.

Mirror to direct light to camera or oculars.

Ocular and camera port.

Next Set Up Köhler Illumination

First-review the conjugate image planes in the microscope (in Köhler illumination) all the way from the light source to the eye or detector (light source plane and the specimen plane).

- 1) Put a stained tissue section slide on the microscope.
- 2) Adjust for Köhler.

- a. Focus with a 20X or 40X objective (make a note on the objective goes up and down when you turn the focusing knob in one direction or the other). Adjust the intensity of the light source.
- b. Adjust eyepiece, width and focus.
- c. Close down field diaphragm aperture.
- d. Focus condenser so that the field diaphragm is in focus.
- e. Center the condenser in the field of view.
- f. Put in the Bertrand Lens to look at the BFP. Take the diffuser out of the light path (if possible) and look at the image of the light source at the BFP.
- g. Open the field diaphragm to the field of view (eye or camera).
- h. Adjust the condenser diaphragm so that it fills the BFP.

When you switch objectives, you will have to refocus both objective and condenser for Koehler.

What does the condenser and field diaphragms do?

- 1) Condenser diaphragm controls the illumination NA (cone angle).

Open and close the condenser and look at the angle emerging from the condenser (can see this by rotating the objective out; also can be seen in the BFP).

- 2) Field diaphragm controls the area of view illuminated (also look at the BFP; changes the intensity but not the area illuminated in the BFP)

Diffraction, Resolution

- 1) We have 2 test specimens-diatoms with different spacing (see last page) and slides with lines with defined spacing.
- 2) Start with slides- examine the image and the BFP. How does the spacing and orientation of the diffraction pattern relate to the image that you see?
- 3) Next look at the diatoms. The goal is to resolve the repetitive frustules spacing and understand how we are able to image them using the bright field microscopy.

View them with Köhler illumination with the 40X air (if you have a hard time finding them use the 20X or 10X first).

- 4) Close down the condenser aperture. Look at the back focal plane (BFP) of the objective. What does the BFP look like when you are focused on a diatom in field of view versus background? Do the diffraction patterns of the different diatoms look different and why? Which is the “zero order” and diffracted light? Why is there a spectrum of colors?
- 5) Resolution versus contrast. With the 40X air lens, open the condenser aperture slowly.

Look at the diatoms and the BFP.

What happens without the 0th order and 1st order light?

What happens to image contrast and resolution? Discuss what is happening.

- 6) The effect of oil. Use oil immersion 40X or 60X lens with 1.3NA or 1.4 Na. (Make sure that the diatoms are first centered with a lower mag lens). Establish Köhler illumination again. Can you see more details in the diatom? What is the effect of closing down the condenser aperture?

What is the effect of closing down the field aperture?

(If you switch back to air objective, make sure that you clean oil from the slide)

- 7) Calculating of NA. With any objective lens, find the diatom where you can barely distinguish the spacing. With this illumination, can you estimate the NA of that objective? Do the calculation.

Experiments with the Back Focal Plane.

- 1) With the diatoms and a 20X air objective, set up Köhler illumination. Close down the condenser aperture. Look at the BFP with the Bertrand lens.
- 2) Remove the DIC slide above the objective (if your microscope has this slot!). Cut a long narrow piece of filter paper and slide it into this position into BFP. Stop when you just block out the zero order light but leave one side of the diffracted light coming through. What does the image and background look like and why?
- 3) Now slide out the filter paper slightly, so that the zero order and only 1 side of the diffracted light come through.
- 4) What does the image look like? Do you need both 1st order spots to see an image?
- 5) Now cut a piece of filter paper with a tiny circular hole (may be punch through with a pen). Slide it into the DIC position above the objective. Looking at the BFP. Position the filter paper such that just zero order light makes it through the hole but the diffracted light is blocked. What does the image look like and why? What kind of microscopy is this?
- 6) Find a diatom where the spacing is barely visible. Now place the red filter over the field diaphragm. What happens to the image of the diatom? Better or worse? Why? Look at the BFP with and without the red filter in place and see what is happening.

Diatoms

Source: <http://glinda.Irsm.upenn.edu/diatom.html>

Specimen Approximate length (microns) Frustule spacing (nm)

Amphipleura pellucid	80-140,270
Frustulia rhomboids	50,300
Pleurosigmaangulatum	150,525
Surirellagemma	100,500
Nitzschia sigma	200,440
Stauroneisphoenocenteron	150,720
Naviculalyra	160, 1250
Gyrosigmabalticum	280,670