Lab 4 Course Notes:
Köhler Illumination

Overview

The main goal this lab will be to set up a controlled illumination system for the collection optics you built last lab. Once you have done that, you will have an essentially complete microscope. We will explore the capabilities of this microscope configuration, and then move on to the second major part of the course in which we will exploit these capabilities to generate different modes of image contrast (such as darkfield and phase contrast).

Attention: in order to cover the material you will need to move fairly quickly in lab; to do this, everyone must have read the lab notes and know *ahead of time* what they are going to do that day. You will not be able to keep up if you are reading the notes for the first time during lab.

The effects of illumination on image quality should be quite obvious after Lab 3. For example, using a bare LED resulted in image containing many diffraction fringes, while trying to fix that problem with a diffuser resulted in very dim light and resultant poor images. This is an even bigger problem than it might first seem – the USAF sample is a very high contrast sample: the black lines block 100% of the light. Imagine how much worse things would look if the sample were small cells which absorb or scatter very little light (i.e., have low contrast to begin with).

There are several things we need to control in our microscope illumination:

1. Intensity (brightness)
2. Spectrum (color)
3. Location (what part of the sample is illuminated)
4. Uniformity (even illumination across the sample)
5. Angular distribution (from what angles the sample is illuminated)

Moreover, we want to be able to control these things independently – for example, when we change the intensity we want the color of the light to remain the same.

Unfortunately it would be too inefficient to have you walk through the development of this illumination by guided trial and error. Instead, we will cover the details of the design in the Course Notes and lecture, and you will build the complete system straightaway during lab. You will then explore the details of the system to gain an understanding of why it is put together the way it is.

Definitely Pay Attention: An excellent midterm or final exam might include having you set up a misaligned microscope for proper imaging and illumination.

More than that however, the first thing you should do each time you use a research microscope is to set it up for proper illumination. This typically takes less than five minutes, and is the difference between a novice getting poor images and an expert getting excellent images. At least one company has used it as an interview test when hiring technical personnel. Once you understand the basic design you will always find it easy to get it right, even on an unfamiliar microscope.

Köhler Illumination

The microscope illumination system is quite subtle and sophisticated, but also relatively simple. However, the simplicity is masked at first by the number of unfamiliar parts. In these notes, we will go through the various parts and the reasons for their arrangement in cursory detail. The supplementary reading and lecture will then fill in additional detail.

Let's return to the list of things we need to control in the illumination path:

1. Intensity (brightness)
2. Spectrum (color)
3. Location (what part of the sample is illuminated)
4. Uniformity (even illumination across the sample)
5. Angular distribution (from what angles the sample is illuminated)

The first two items are relatively straightforward and should be familiar. As you may have seen in the lab, for LEDs the light intensity can be adjusted by controlling the current through the LED, and the spectrum does not change much with this variation.

For an incandescent lamp, however, the spectrum does change with the temperature of the bulb filament – as you turn down the power, the filament gets cooler and the spectrum of the light emitted shifts toward the red. In order to control the intensity separately from the spectrum of the light, we will use "neutral density" (effectively, gray) filters to control the intensity without adjusting the power to (and hence the temperature and color of) the filament. Furthermore, since a hot filament emits light at all wavelengths, we can use a color filter to select only the wavelength region we want. As an example, a green color filter (like the one you have) is often used to limit the range of wavelengths one is imaging with and hence also to eliminate chromatic aberration. Green is usually chosen because it is near the peak sensitivity for the human eye. Conveniently many silicon-based cameras (e.g. CCD or CMOS) are also fairly sensitive in the green.

The remaining three properties – location, uniformity, and angular distribution, are more complicated. It makes sense to address these in order:

Location

There are actually two components to location: where the light source is and what part of the sample is illuminated. The location of the light source is simple – it is generally not convenient to put the actual light source up against the sample, especially if the light source is a hot filament. However, as you saw in the last lab, when the light source is far from the sample the light gets quite dim. As noted above, this problem is especially problematic for small or mostly transparent samples, which scatter very little of the incident light. The obvious thing to do is to use a lens to *collect* the light from the filament and focus it toward (or onto) the sample. Not surprisingly, the lens used to do this is called the "collector lens."

The simplest version of this type of limitation involves simply imaging the light source (e.g. the lamp filament) onto the sample, as shown in Figure 1.

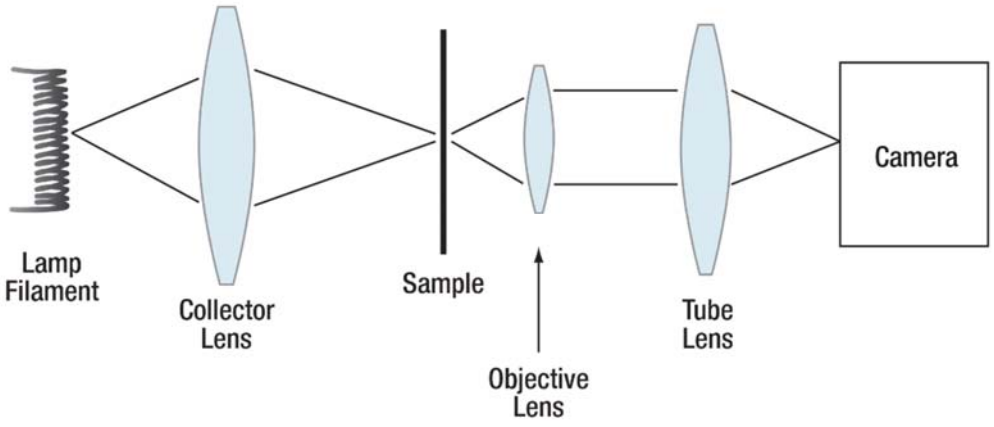

Figure 1: Critical Illumination

Note that we have also shown the collection path – the objective, tube lens, and camera. In addition, the collector lens is positioned such that it images the lamp filament onto the sample. Of course the sample is imaged onto the camera (by the objective and tube lens), so the filament must ***also*** be imaged onto the camera.

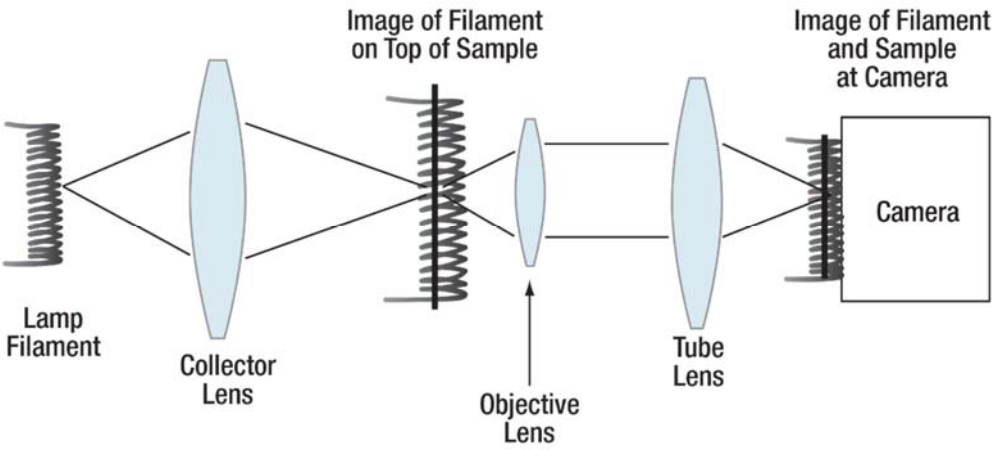

Figure 2: Conjugate Planes: The filament, sample, and camera face are all imaged onto each other, i.e. are "conjugate" (though the magnifications may vary).

The term for two planes that are imaged onto each other is "conjugate." Conjugate planes are very important in microscopy, and we will discuss them often. The most obvious conjugate planes are the sample plane and the camera – if the image is in focus then those two planes are conjugate. This concept can be extended further: for instance, if you look through a microscope eyepiece and see the sample, then your retina and the sample plane are conjugate.

In Figure 2 you can see both some advantages and a disadvantage of critical illumination: on the plus side, we can use the lens to magnify the filament so it illuminates the whole sample, and the lens has allowed us to collect more light from the filament. On the minus side however, all the structure in the filament (the coils) will be immediately visible in the image; the light on the sample is not at all *uniform*.

Before we move on it is worth introducing an additional concept: the field stop. Usually in microscopy the light source is much bigger than the sample you are interested in looking at. In addition, there is a limit to how small one can make the image of the filament – we will return to this, but typically one cannot make the image of the filament much smaller than its actual size. In the last lab, we turned off the room lights to reduce the amount of background scattered light polluting your images. For exactly the same reason, we will not want to illuminate more of the sample than the area we are specifically interested in (i.e. the "field") – any additional illuminated area will simply allow more background scatter into our optical system and that will reduce our image contrast (i.e., make the image look "washed out").

If we cannot do it by demagnifying the size of the filament to illuminate only the area we care about, then we could introduce an iris (or aperture) right before the sample, which serves to limit the area of the sample that gets illuminated. In many cases it is inconvenient to have an iris right next to the sample, but we can use the concept of conjugate planes to solve a problem like this:

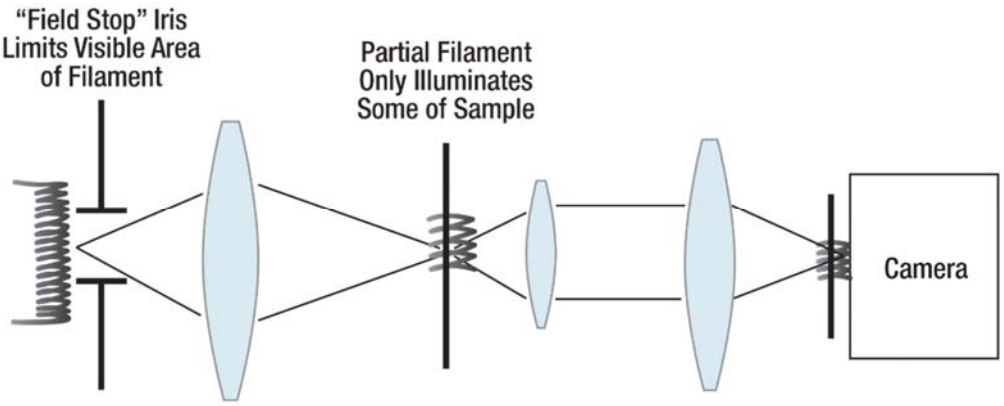

Figure 3: Field stop limits area of sample that gets illuminated. Compare this image to Figure 2.

We could achieve the same effect by putting the field stop next to the filament, next to the sample, or at the camera face. It is worth noting that by putting the field stop at the filament we block any excess light at the source – which is to say, far from the rest of the optical train. If we put the field stop down by the camera then any scattered light would already have polluted the rest of the image.

Important Note: The field stop is in a conjugate plane to the sample and the detector. (This is worth memorizing).

You may have noticed one problem in Figure 3: the filament of an incandescent light is actually enclosed in a lightbulb, so we cannot really put an iris there. One way to solve this would be to use yet another lens to make an image of the filament somewhere where we can put a field stop and then use a lens to image that onto the sample. In practice we will do this a little differently, but the principle is similar.

Important Note: New Terms to Learn (Memorize These):

- **Collector lens: the lens next to a light source that collects the light from it.**
- **Conjugate planes: planes which are images of each other.**
- **Field stop: an aperture which limits the illuminated field on the sample. By necessity, the field stop must be conjugate to both the sample plane and the camera plane.**

Uniformity

The biggest problem with imaging the lamp filament directly onto the sample (known as "critical illumination") is that any nonuniformities in the light source – e.g. the coils of the helical hot filament – are then very apparent in the image of the sample.

In the last lab, you positioned the LED some distance away from the sample in order to get more even illumination – imagine what the sample would have looked like if you slid the LED right up next to it (you could even try this in the next lab). If you wanted the illumination to be even more uniform you could slide the LED (or light bulb) even farther away from the sample. In fact, if the light source were infinitely far away, then the illumination would be extremely uniform. That last sentence should definitely have given you an idea for how we could make the light source more uniform.

➔ Stop and think about that for a moment if it is not already obvious to you.

A good way to make the filament look like it is infinitely far away without having to put it a long distance off (and so losing a lot of intensity) would be to put the filament one focal length behind a lens so that the filament would be focused at infinity on the other side. Even better, if we put the sample exactly one focal length on the *other side* of the lens then each point in the filament would illuminate the entire sample resulting in complete uniformity.

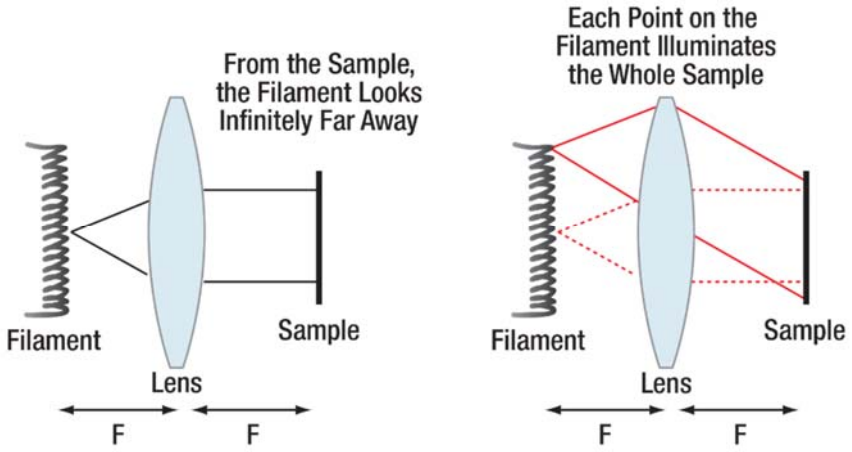

Figure 4: Placing the filament in one focal plane of a lens and the sample in the other focal plane generates good illumination uniformity, since each point in the filament illuminates the whole sample, and from the sample position the filament looks "infinitely far away" (or, equivalently, "totally out of focus").

By now it should already be occurring to you that we do not have to put the filament *itself* in the front focal plane of this lens, but rather could (equivalently) put *an image* of the filament in that focal plane.

Notice the new terminology: "front focal plane" and "back focal plane." These are the planes one focal length in front and in back of a lens. Because in microscopy one usually follows the light starting from the illumination source and going step-by-step through all the optics to the detector, "front" is the side of a lens nearest the lamp, and "back" is the side farthest from the lamp. As an example, in the last lab you set up an iris in the back focal plane of the ½" achromat you are using as an objective.

Important Note: New Terminology (Worth Memorizing):

- **Front Focal Plane: The plane one focal length away from a lens and (in our case) *closest to the lamp.***
- **Back Focal Plane: The plane one focal length away from a lens and *farthest from the lamp.***

Angular Distribution

Angular distribution is the last of the things we would want to control about the illumination. Before getting into details of how we might control that, it is worth considering a simple example of why we would care: imagine trying to look at a faint fingerprint on a piece of glass. If you look straight through the glass at a light, the light from the light source will overwhelm any contrast from the fingerprint. However, if instead you look through the glass at something dark, with the light off to the side (out of your immediate field of view), then the fingerprint will be easily visible. Technically, this is called oblique illumination – and "oblique" refers to an angle; in this case the angle between the direction you are looking through the glass and the direction to the light source. By shifting the angle of the illumination, you have gone from being unable to see the fingerprint to being able to see it fairly clearly; that should help provide a sense of why it can be convenient to be able to manipulate the angle at which illumination is hitting the sample.

The manner in which we can manipulate the angular distribution of illumination is hinted at in Figure 5, where in the right-hand image it is apparent that light emitted from different parts of a filament is directed at the sample from different angles. This is a direct consequence of the fact that light collimated on one side of the lens is focused down to a point on the other side – so, conversely, light emitted from a point on a filament (if that filament is in a focal plane) will be collimated on the other side of the lens.

In fact, the height of a point above the axis in one focal plane of the lens corresponds directly to the angle of the parallel bundle of rays in the other focal plane.

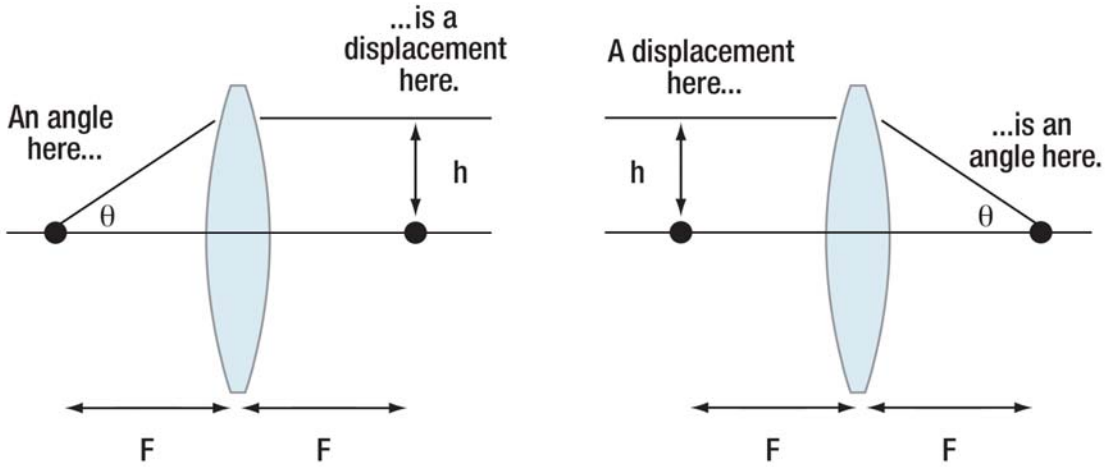

Figure 5: An angle in the front focal plane is a displacement in the back focal plane and vice-versa.

Important Note: In Figure 5, one can see from geometry that $\tan(\theta) = \frac{h}{F}$. Remember from the formula sheet that imaging lens systems are usually carefully designed so that the actual

relationship is given by $sin(\theta) = \frac{h}{F}$. This requires a lot of work (and systems of multiple lenses) but is necessary for proper imaging. We will discuss this further in the middle part of this course; for now, be sure to remember the sin(θ) relationship for microscope lens systems.

Figure 6 shows how we can use an aperture in front of the filament in the front focal plane of a lens to implement control over the angular distribution of the illumination at the sample. Note that since we are using a circular aperture centered on the optical axis, the illumination appears to converge ("condenses") in a conical path onto the sample. As a result, this lens is called the "condenser lens."

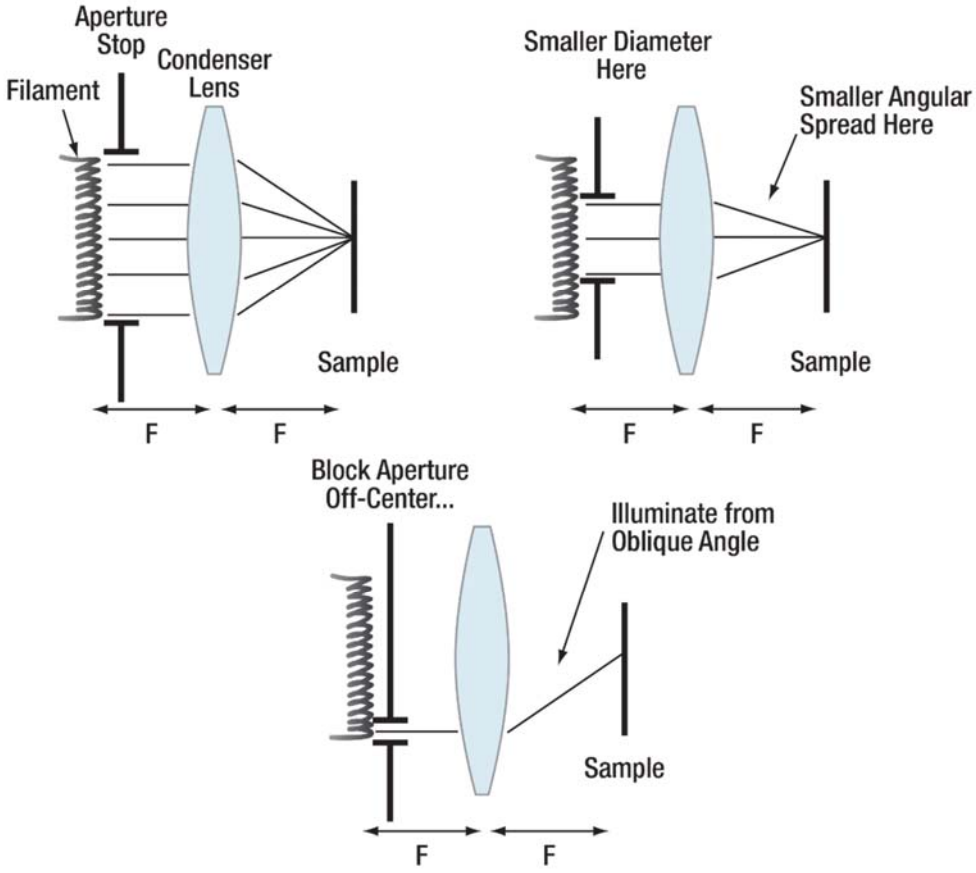

Figure 6: Using an aperture in the front focal plane of the condenser lens to control the angular distribution of the illumination on the sample.

The aperture placed in the front focal plane of the condenser lens determines the angle or spread of the rays hitting the sample; in fact, using the relation given earlier, the diameter of the aperture determines the numerical aperture (NA) of the rays hitting the sample. As a result, it is referred to as the "aperture stop."

Important Note: New Terminology (Worth Memorizing):

- **Condenser Lens: The lens which focuses the illumination down onto the sample.**
- **Aperture Stop: The iris which limits the illumination NA (cone of rays) onto the sample.**

Putting it All Together

From the above discussion, we know we want several things:

- To be able to put the lamp some distance from the sample without losing intensity
- A field stop (which needs to be imaged at the sample plane)
- A condenser system that involves an image of the filament in the front focal plane of the lens and an adjustable aperture stop at that same plane, and which focuses the illumination down onto the sample.

There are number of ways to accomplish this; however, typically one wants the total optical path to be reasonably short (~ 30 cm) such that the instrument is not too large, and in addition it is preferable not to require too many lenses (to keep costs down). Since optimizing this is somewhat complicated, and there is an accepted standard for how it is done in typical microscope systems, we will have you implement the usual system directly. The typical configuration is shown in Figure 7.

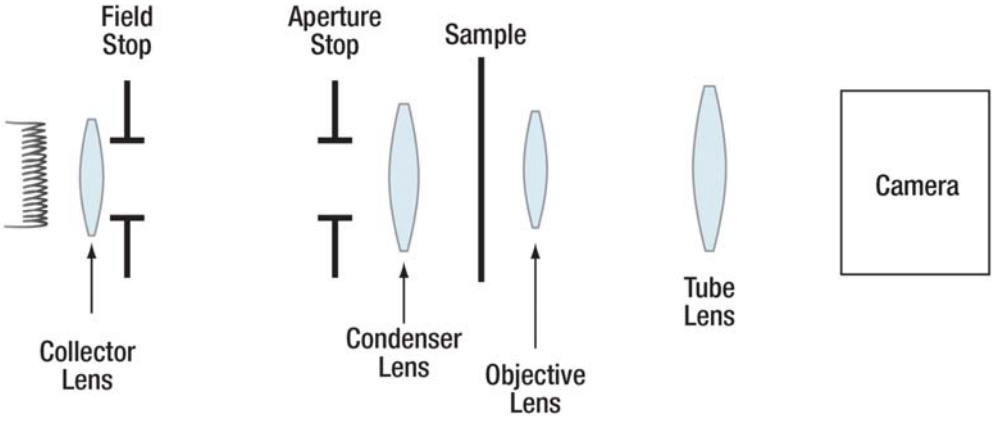

Figure 7: All the parts of the microscope, in order.

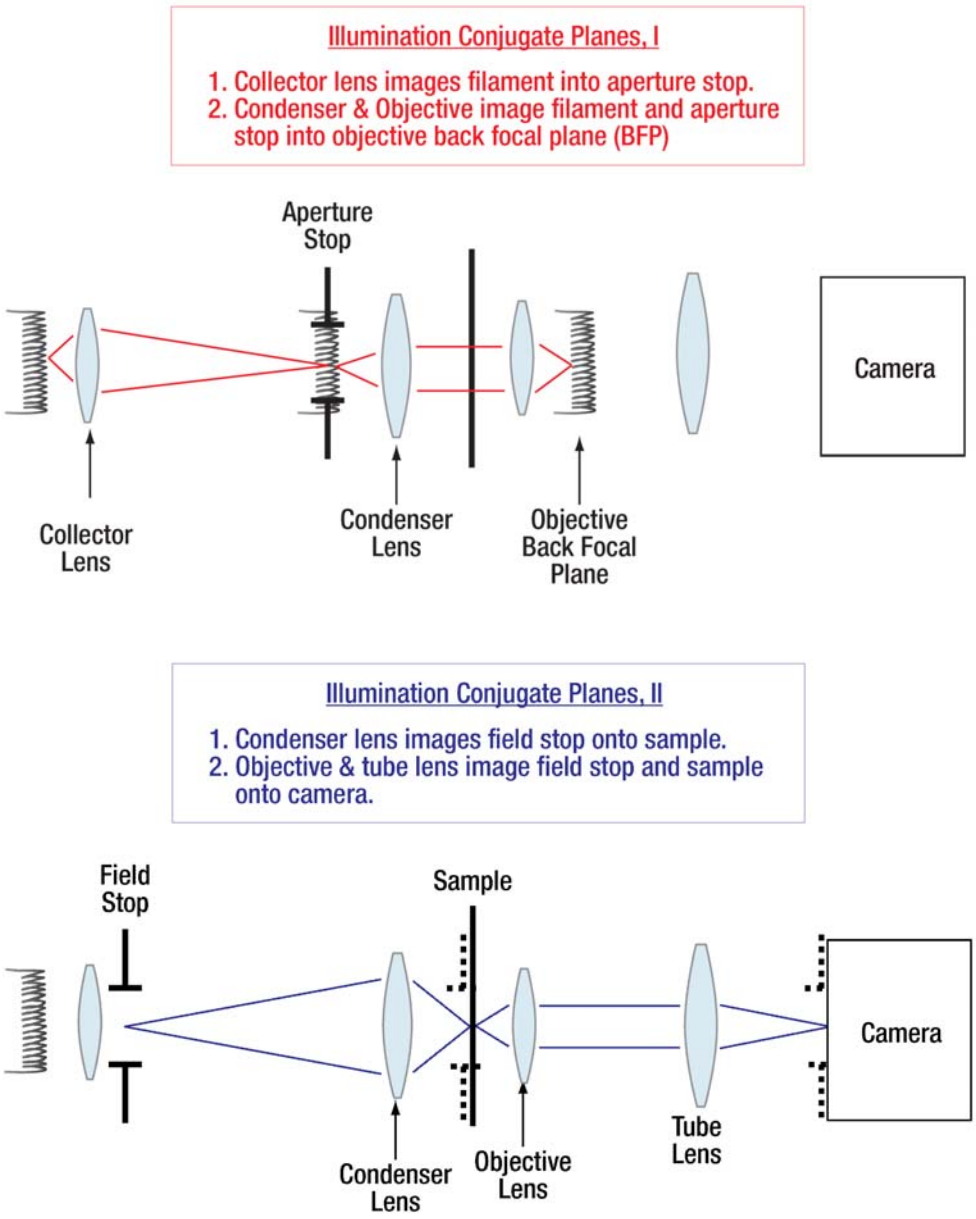

Figure 8: Conjugate planes:

Which lens is imaging what to where? Note that:

a. The filament is ***not*** imaged onto the sample or camera – so illumination is uniform.

b. The field stop ***is*** imaged onto the sample and camera, limiting the opportunity for stray/scattered light to pollute the image.

c. **The aperture stop (one focal length from the condenser lens, and so ***not*** imaged onto the sample) limits the size of the filament image in the condenser front focal plane, which in turn sets the angle of the cone of rays focused onto the sample (illumination NA).**

d. The iris in the objective back focal plane (not shown, but just like you built in the last lab) limits the cone of rays ***collected*** from the sample (i.e., limits the objective NA).

An important thing to note here is that in order for the condenser to image the field stop onto the sample, it is *impossible* for the sample to be in the back focal plane of the condenser (i.e., one focal length

away from the condenser). Ideally that is where we would like it to be; in practice we can get fairly close if we place the field stop as far as possible from the condenser (so the object distance S_o is large).

There is a certain amount of additional detail in choosing the focal lengths, diameters, and distances between the lenses; for this lab, you will simply copy the layout provided in the Lab Notes. As the course goes on we will discuss some of the reasons behind the choices involved in this implementation.

Because it may be useful to have the steps laid out in terms of the specific parts in your microscope (rather than a research microscope), the sequence for setting up proper illumination is laid out below.

Setting Up Köhler Illumination when Building a Microscope

1. With your camera focused at infinity, put the camera approximately one tube lens focal length behind the objective (the best distance depends on lens design; if in doubt, closer than a focal length is better than farther, to avoid clipping rays on the edge of the lens, called "vignetting").
2. Turn on your lamp so that there is some illumination on the sample.
3. Position a sample in front of the objective such that it is in focus on your camera (a marker drawn on a slide works fine; even a fingerprint on a slide can work well).
4. With the aperture stop and field stop wide open, position the condenser roughly one focal length from the sample.
5. Position the lamp, collector lens, and field stop at the far end of the optical rail.
    a. Make sure everything is at the same height as the tube lens; this is hardest to do with the lamp, so adjust lamp height as best you can.
6. Adjust the collector lens position so that the filament is in focus at the aperture stop
    a. Adjust the lamp height to get the filament vertically centered on the aperture stop.
7. Position the field stop conveniently close to the collector.
8. Close down the field stop and adjust the condenser position along the optical axis in order to bring the field stop into focus at the sample (and hence on the camera image).
9. Reposition the collector if necessary to bring the filament back into focus at the aperture stop.
10. Open the field stop as much as desired – usually so no more area is illuminated than what is being viewed.
11. Close down the aperture stop until contrast is optimized (this is usually ~ 70% of the objective back aperture; we will return to this later).

Note: Filters (e.g. ND filters, color filters) or diffusers should be positioned *away* from the field stop so that they are not in focus at the detector – that way the diffuser texture and/or scratches or dust on the filters will not be visible.

Setting Up Köhler Illumination on a Standard Research Microscope

This is usually fast (~1-2 minutes) once you are experienced, but slow the first time.

- ➔ **The information below does not apply to this course; rather, it is to help you make the connections between the material in this course and what you are doing when you set up illumination on a laboratory microscope.**
- ➔ The microscope manuals usually have a detailed 1-3 page procedure that is easy to follow, with a diagram of where the relevant parts are on their microscope. Finding the manual (e.g. online) and following it can be the easiest way to get started.

1. Turn on the lamp.
2. Put in a low-magnification objective (20X or less).
3. Open the field stop and aperture stop fully.
    a. Remove any phase rings, polarizers, etc. from the light path (sometimes this requires rotating the condenser turret).
    b. You can tell when you have got this right because you can see light from the condenser hitting the sample.
4. Position a sample on the sample stage, so the light is hitting it.
    a. Marker drawn on a slide or coverslip works fine; even a fingerprint on a slide can work well.
    b. If you are using a high-NA objective, the sample may need to be on a coverslip, not a slide – high NA objectives often cannot focus all the way through a slide.
5. Adjust the condenser height so the light spot on the sample is as small as possible – this will be (roughly) the correct height for the condenser.
    a. If the condenser is hard to move, check to see if someone locked down the positioning lock for it (some microscopes have these). Similarly, if you cannot get it to come down far enough, check that there is not a block preventing it from moving farther down (some microscopes have these too) – if it is a problem, release that.
6. Adjusting the lamp filament and collector: Usually you cannot do this.
    a. If you change a lamp, sometimes you do need to position the filament – in that case, check the manual for the particular scope to see where the screws that adjust filament (or arc) position are.
7. Use ND filters or reduce the lamp intensity so you do not hurt your eyes when focusing.
8. Focus on the sample.
    a. You may want to do this by looking at the sample by eye (not through the eyepieces, but staring right at it) while bringing the objective up – that way it's far less likely that you'll accidentally ram the very expensive objective into the sample. Then, look through the eyepieces, or at the monitor if using a camera, and adjust the focus **by moving the objective AWAY** from the sample, until things come into focus.

b. For low-NA objectives (magnification of 20X or lower, usually), the working distance can be quite long – sometimes you need to lower the objective a long way before things come into focus. Eventually you get a sense for this.

9. Close down the field stop to a small diameter and adjust the condenser height until the field stop is as sharply focused in the image as you can get it.
    a. Especially with high-NA objectives, the field stop may not come into very sharp focus; just do the best you can.
    b. If you cannot see the sample, open up the field stop until you can see the edge, and focus on that.
        i. If there are centering knobs on the condenser, adjust them so the field stop is centered in the field of view.
10. Flip in the Bertrand lens, or take off the eyepiece (usually they pull straight out of the tube) and look in to see the objective back focal plane.
    a. Adjust the aperture stop so it is ~ 70% of the size of the objective back aperture.
        i. The objective back aperture will look like a disk of light; close the aperture stop until it makes that disk about 2/3 as wide as it was when fully open).
    b. Note: if you are setting up Phase, you need the aperture stop all the way open! Then put in the phase ring and make sure the ring of light is centered over the phase ring in the objective back focal plane (this is a bit darker and easy to see). If any light is not hitting the objective phase ring, you need to adjust the position of the phase rings; this cannot be done using the condenser adjusters, so do not try those. Get the microscope manual; usually there are little setscrews you can adjust on the phase ring housing, easy to confuse with the screws that hold the housing on.
    c. Sometimes there is a focus knob on the Bertrand lens to allow you to get the aperture stop in best focus; this is a convenience but not necessary, since normally the Bertrand lens is not in the optical path.
11. If you will be doing microscopy by eye (and not with a camera), adjust the eyepieces for your eyes.
    a. One eyepiece usually has an adjustment (the "Diopter adjustment" ring on it. Use a business card to block your vision through that eyepiece (closing one eye perturbs the muscles around the other eye, so try use a card to block your vision instead).
    b. Looking with the unblocked eye, adjust the focus knob so the sample is sharpest.
        i. The best way to do this is to move the objective *a bit too far* from the sample, and to bring the sample slowly into focus. This way your eye will be focused on infinity (its most natural / comfortable state) when the image comes into focus. This also guarantees that the sample will be at the plane of best aberration adjustment for the objective when you are looking at it.
            1. To avoid ramming the objective into the sample, start by moving the objective close to the sample while watching the objective itself. Then while using the eyepieces moving the objective away from the sample until things come into focus, then go a bit farther away until you lose focus, wait for your eye to relax, then move the objective back to focus.

ii. If you focus by bringing the objective *away* from the sample, then your eye can adjust to focus when the objective is still too close, and the sample will not be in the plane where the objective is best corrected, and your eye will be stressed and you will get a headache after long viewing.

c. DO NOT touch the focus knob! Now block the other eye, and adjust the Diopter Ring on the eyepiece until the sample is again in sharpest focus.

i. As with focusing, there is a best direction to go: adjust the diopter ring to the highest "plus" position (e.g. +4), usually fully clockwise / all the way in, and then rotate it back out until the sample hits sharpest focus.

d. Adjust the spacing between the eyepieces (if they move at all, you can grab them and adjust the spacing by pushing/pulling) until they are best for your eyes.

i. Adjust them a bit too wide, then, while looking through the eyepieces with both eyes, slowly bring the eyepieces closer together. At a certain point, the two separate disks of light you see will merge and you will see them become one. Keep going until they look like a single disk of light, but no farther. If viewing is uncomfortable, obviously adjust further.

Notes:

1. Adjusting the eyepieces is really worth it – it is like going from a normal theater to 3-D IMAX. Very impressive and cool when properly set up, and it usually takes only a minute or less once you know how to do it.
2. A good / easy sample for setting up complicated contrast methods (phase or DIC) is cheek epithelial cells: use a (clean!) pipette tip to gently scrape the inside of your cheek, and then touch the tip to a slide (try to get a little spit onto the slide too). Put on a coverslip, and seal the edges with wax or nail polish if you have it to keep the small volume of liquid from drying out. I like to put a marker stripe on the slide before putting on the cells, to have something easy to focus on.
3. Setting the eyepiece diopter can sometimes be a bit tricky; here are a few tips:
    a. If you have astigmatism, keep your glasses on – the eyepieces will not adjust for that. In general, if your glasses prescription is very powerful, chances are you should keep them on.
    b. [Rare issue – listed here only for completeness of coverage] Sometimes microscopes have diopter adjustments on **both** eyepieces, what then? Use the fact that depth of focus is smaller for a high-NA objective than a low-NA one:
        i. Set the diopter adjustments to the middle of their range.
        ii. Focus on the sample using a moderate NA (say, 20X 0.4 NA) objective.
        iii. Switch to a lower NA objective (say, 10X or 5X).
        iv. Adjust the diopters to the highest setting (usually the twisted all the way in / clockwise).
        v. Blocking one eye at a time, rotate the diopter rings back out until focus is best. Do this for each eye.
        vi. It is probably best to iterate this procedure by going back to the higher-NA objective, refocus using the focus knob, then repeating the steps above.