APh 162 Week 1 Day 1

Objectives:

- Optical resolution
- Light path of the microscope
- Kohler illumination
- Lens aberrations

The microscope has been central to the practice of biophysics or physical biology. Beyond needing lenses of some sort to document phenomena, we use microscopes to look at stuff that is smaller than what we can resolve with our own eyes. Thus, an understanding of resolution is critical to choosing what sort of microscope we would like to use. Additionally, in this lab we will get used to using the microscopes. Though the number of exercises is small, it will still take a bit of time as you learn how to set up microscopes (Micromanager), acquire data, etc.

In the required sections below, there are questions which you are expected to answer. If there are questions as to how to do certain things, or what the answer should be, please consult the TAs. We have been as specific as possible, but there may still be confusion.

Lab 1 – Getting to know your microscope (all required)

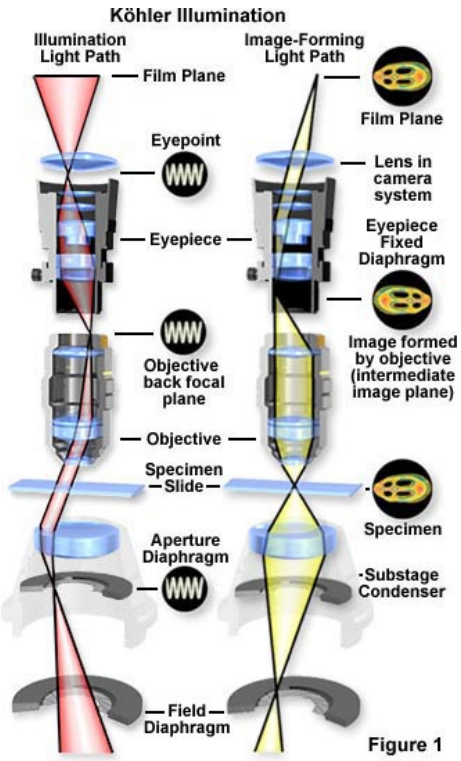


Figure 1

Brightfield microscopy. Undoubtedly, in the coming weeks, there will be times where the microscope seems to be not "working." A good way to diagnose an overly dark image, or a non-uniformly illuminated sample, or trouble-shooting in general, is to think about what's going on in the lightpath. The diagram on the left displays the brightfield illumination pathway of the microscope.

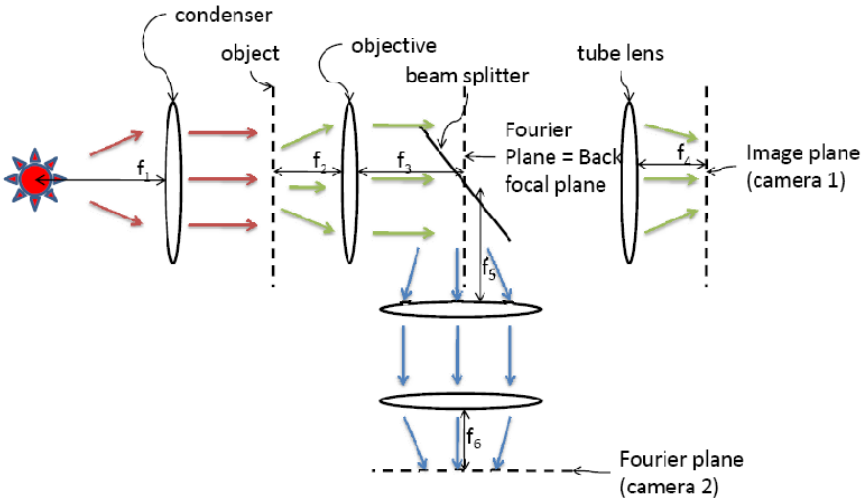
What is constant between all the brightfield techniques we will be dealing with is the use of Koehler illumination. Koehler illumination means that the sample is illuminated uniformly, so any structure or hot spots in the lamp filament will be minimized; as well, in Koehler, the full numerical aperture of the condenser lens is utilized, which boosts the resolution of the microscope (see below).

1. Identify the brightfield pathway of one of the microscopes with your TA.
2. Align the microscope in Koehler.
3. Become familiar with Micromanager, which runs most of the microscopes in the lab.

4. *Edge-based resolution test*: get one of the targets that has small bars on it. These targets have edges that have sub-wavelength sharpness. Estimate how sharp the edges are using a 10-90% criterion. Since the edges are sub-diffraction, you won't be able to see a perfect corner. Measure the degree of imperfection of the edge. How wide is it? To get the width, you will need to figure out how to a) mount the slide onto a microscope, b) how to take an image of the slide, c) locate the image on the hard disk, d) import the image into Matlab, e) figure out how many pixels corresponds to the edge, and f) how to convert pixels to a meaningful dimension, such as "micron" or "nanometer". How does this value match what you expect from the response of the microscope? In your website, post an image of your resolution test. List all the parameters necessary for your measurement (objective NA, magnification, pixel size, etc.). Use λ = 550 nm. Post also a line scan of the edge that you used. Your precision must have an error bar.
5. *Depth of field*: Find a sample that has some depth to it. Hairs on a horse fly are convenient. Now stop down the condenser aperture. What happens to the image? One of the 60x objectives also has a variable numerical aperture. What happens to the image if you reduce the NA of this lens, keeping the condenser aperture size constant? Take a picture before and after you stop down the condenser. Post a picture of each, listing the objective and condenser used, and the NA of each. In some cases (see exercise 6) you may need to estimate the condenser NA.
6. *Koehler illumination resolution*: Below there is a formula for the expected resolution of a brightfield microscope, knowing the numerical aperture of the objective and condenser. Using 550 nm as the wavelength of light, does the formula work? That is, open the condenser aperture all the way and find a diatom with a pattern that is barely resolvable. Take a picture and measure the distance between the pattern lines. Now stop down the condenser all the way and increase the bulb intensity (you might also need to increase the integration time of the camera) to get a decent image. Is the pattern resolvable? Estimate the numerical aperture of the condenser that you have just stopped down (there's helpful formulas below).
7. Become familiar with the *fluorescence light path*.
8. *Point-spread function (see also Lab 3)*: We can characterize the optical response of a microscope by its "point-spread function". Basically, the PSF tells us how the microscope images a point object – i.e., if we had a diffraction-limited object, then imaged that object through the microscope, the result would be the PSF. The PSF is a 3 dimensional object. Get some diffraction-limited fluorescent beads and take a z-stack of the beads. Reconstruct the PSF in 3D (a y-z or x-z plant will suffice). Why is there out-of-focus light? What are the limits of resolution in x, y, and z? Post your image of the PSF. Include measurements on the width of the PSF and the length (in z) of the PSF in real units. In this case, the wavelength of light is the wavelength of the fluorescence. If you don't know this, consult a TA.

Lab 2 - Fourier Optics

In this set up, we have "blown up" a conventional microscope to reveal its innards. With this, we will be able to visualize the 2D Fourier transform of some objects, and to understand what a microscope is doing. By blocking or passing some of the Fourier components, you will be able to modify the characteristics of the resulting image. Below is a simplified diagram of the instrument:



A laser beam, collimated by the condenser lens, illuminates (red arrows) a transparency placed at the object plane, one focal length distance from the objective (f_2). Light is diffracted (green arrows) off the object which has the electric field distribution $E_0(x,y)$ and is collected by the objective. An image of the object is formed by the tube lens, which interferes the light at its focus (f_4). The same principle holds for imaging the Fourier transform of the object: the Fourier transform (with electric field distribution $E_{FT}(x,y)$) appears behind the objective at the Fourier plane, or back focal plane.

$$E_{FT}(X,Y) \propto \int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty} E_0(x,y)\exp\left[-i2\pi\left(X/f\lambda\right)x\right]\exp\left[-i2\pi\left(Y/f\lambda\right)y\right]dxdy$$

X and Y are the spatial coordinates of the Fourier plane. We subsequently image the Fourier transform (blue arrows) on the Fourier plane (camera 2).

Objects in real space (x) are transformed according to $X/f\lambda$, where f is the focal length of the objective (200 mm), and λ the wavelength of light (660 nm). The dimensions of the Fourier transform are hence m^{-1}. We will illustrate with a few samples.

Note that we are actually observing the intensity of light at the Fourier plane, that is, the magnitude of the Fourier transform, and not the actual transform itself. We are actually measuring the *power spectrum* of the object, or, how much optical power is within each spatial frequency of the object:

$$I \propto EE^* = |E|^2$$

1. Identify the components of the "microscope" with your TA. How does the spatial filter work, given your knowledge about diffraction?
2. **(not required)** What is the magnification of the system (optical and digital)? Knowing this, calibrate the camera on the television screen(s). What do you suppose the smallest object you can see is? How does this compare with d_{min} (see below)?
3. Use a grating pattern in the object plane. Do the Fourier space coordinates match the spatial frequency seen at the image plane? (That is, if you have a pattern that is 3 cycles/mm, where is

the corresponding peak in Fourier space?) Use both a high frequency and a low frequency grating. Please describe the measured results in your report. What is the central spot? Why is it always there?

4. **(not required)** Use a grating pattern with symmetry in more than one direction. Does the power spectrum make sense? What happens if you rotate the object? What about a grating with variable line density?
5. **(not required)** Use the various patterns; see if you can predict its Fourier transform!
6. **(no need to write up this question)** Place a mask in the path of the Fourier space to filter the Fourier transform. What happens if you use a high frequency grating and block off the Fourier components furthest from the center? How does this relate to resolution?

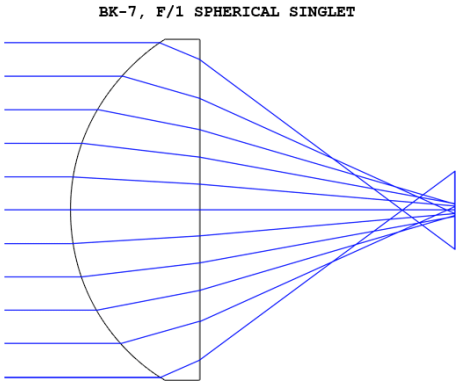
Now we know that an object with spatial extent *d* placed one focal length away from the objective has as its transform components which are approximately *1/d* in Fourier space. Thus as the object becomes smaller, the Fourier component moves further and further from the origin. However, a lens is only of finite diameter, and cannot therefore capture all Fourier components of a diffracted object.

The objective of a microscope, which serves to collect the light diffracted by an object, thus serves as a low-pass filter. The maximum transmitted spatial frequency is $f_{max} \sim D/\lambda f_{obj}$, where D is the diameter of the objective lens. Thus the minimum size object that can be imaged is approximately $d_{min} \sim \lambda f_{obj}/D$. What this means is that any object smaller than d_{min} will look like d_{min}. Using the Rayleigh criterion for distinguishability, we define resolution as $d_{min}/2$.

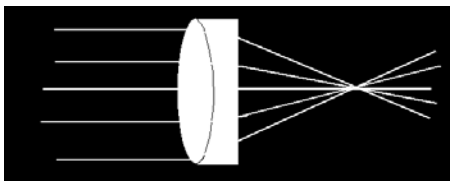
Note that in photography, the f/# of a lens is nothing more than f_{obj}/D. The equivalent quantity in microscopy is the numerical aperture, which is defined as: $NA = n \sin\theta \sim n\, D/(2f_{obj})$, where n is the index of refraction, and θ is the half angle of the collected light. For a point radiating light in a microscope, it will produce an image of finite diameter with the minimum resolution $d_{min} = 1.22\, \lambda/NA_{obj}$. The situation is slightly more complex for brightfield images, since we must take into account the condenser numerical aperture: $d_{min} = 2.44\, \lambda/(NA_{obj} + NA_{condenser})$.

Lab 3 – Aberrations (not required except for part 5)

Aberrations are the result of certain approximations used with designing lenses. Basically, it is physically easier to grind glass as sections of a sphere. However, the result is that lenses only focus light near the center of the lens to its focal point; at the margins of the lens, parallel rays will tend to over focus. This is called “spherical aberration”. Please read through this. There is only 1 required exercise, #5 below.

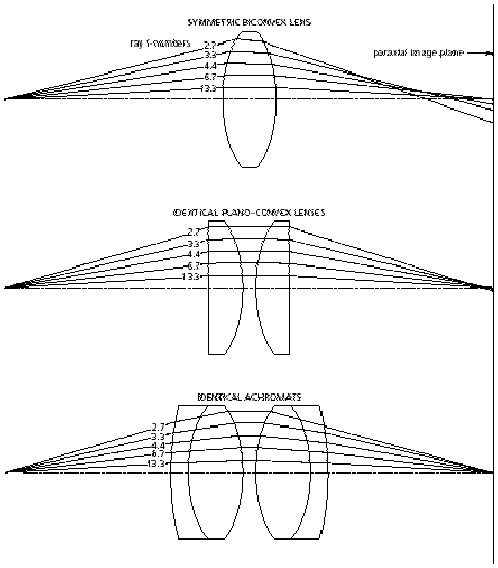


These types of lenses are called “singlets” (left) since they have a single surface that bends light. In order to “unbend” rays of light near the margins of the lens, a negative lens is added to the singlet, forming a “doublet” (right). We will explore spherical aberrations, coma,

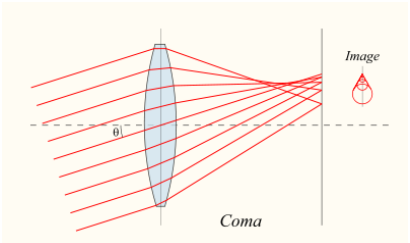


and chromatic aberrations.

The following lens pairs will help to explain why the Fourier transform in the "exploded" microscope looks so shoddy. Since we have used two identical plano-convex lenses to image the Fourier plane, due to spherical aberration, the image does not form right, giving us undesired flares.



1. Using the free-space optics setup, place a mask in front of the objective lens to create a "line" beam. Substitute the objective lens (which is a doublet) for a singlet lens of the same focal length. Try and figure out a method for measuring the focal length. Does the line focus everywhere at the same time? Draw a diagram of what's happening. At the lens, place an iris. How much should you "stop down" the lens until everything focuses at the true focus? What is the f/#? Can you now explain how pinhole cameras work? Hint: explain the principle of "depth of focus".
2. Now tilt the single lens. What happens to the focus? This phenomenon is called "coma" (see left).



3. Do you expect the spherical aberration to result from the orientation of the lens? Try flipping the singlet around and see what happens. Which surface of the lens should face the collimated light?
4. Replace the singlet with the original doublet. Check the resultant spherical aberrations. What is the optical f/#?
5. **(required)** Chromatic aberrations. Go back to the diffraction-limited multi-colored beads. Image their PSF using different filters. Is there an offset in the x-y plane? In z? Microscope objectives are often optimized for green light and so red and blue will be at a slightly different plane. Offsets in the x and y plane are generally due to slight angular deviations in the filters used, and not the lenses. In your report, please post images of at least 2 colours of the beads. Measure what the chromatic aberrations in x, y, and z are. Describe how you performed the measurement.

Homework:

Write up the results of the exercises above, and use Matlab for the analysis of images. You and your partner(s) in crime may turn in 1 write-up together (post it on the web, or email to us and we'll post it). Read online the principles of phase contrast microscopy.

Matlab homework: You may collaborate with each other, but write down with whom you collaborated with. If you have troubles with Matlab, see the tutorial posted online, or ask your TA for help. Turn in your code, naturally, online.

1. Figure out how to import images into Matlab.
2. Automatically identify the beads in your bead images. The “image processing toolbox” is useful for this.
3. Find the centroids (calculate the center of mass) of your diffraction limited beads (sub-pixel resolution). What plane in z do you use? What is the error on the position calculation? How much is each colour off-set from each other (use the data obtained from the multiple-coloured beads)?
4. In a paragraph, explain why magnification is a poor measure of image quality. Explain resolution in your answer. If we wanted a microscopy objective with a working distance of 10 mm from the sample with an NA of 1.0, how large is the lens?
5. For the extra-motivated (**not required**): fit the diffraction limited spots to a 2D Gaussian. What is the position error? (see Thompson et al., Biophys J., **82**:2775+, 2002)
6. Read up on cyanobacteria
   http://www.genoscope.cns.fr/spip/Synechococcus-ubiquitous-marine.html