BP210: Biological Light Microscopy
Lab 2: Kohler Alignment of a Research Microscope and Introduction to Brightfield Contrasting Techniques

Goal: Learn how the brightfield light path of a research microscope is laid out and how to properly focus and align the condenser for Kohler illumination. Be introduced to phase contrast and differential interference contrast (DIC). Look at the effect of illumination and detection NA on resolution.

Samples:

H&E stained samples

Diatoms

Cheek cells

Protocol:

1. Identify the microscope condenser and brightfield lamp. Examine the objectives on the microscope. What are their magnifications and numerical apertures? What immersion media do they use?
2. Examine the condenser. What is its NA? Identify the field and aperture diaphragms and the condenser focus.
3. To set up Kohler illumination, first focus on an easy-to-find sample (an H&E stained pathology specimen works great).
4. Kohler illumination requires that the condenser be focused to the same focal plane as your sample. You do this by focusing the condenser so that the field diaphragm is in focus at the same time as your sample. Close down the field diaphragm and focus the condenser so that you can see it in focus, superimposed on your sample. Make sure it is as well focused as you can get it and that it is centered in the field of view.
5. Have someone misalign the scope and then realign it yourself to figure out how to get everything back in alignment and proper Kohler illumination when starting from a badly aligned scope (as is often the case!).
6. Now look at the effect of the aperture diaphragm. How does the depth of field and contrast change with the position of the aperture diaphragm? This may be easier to see with a lower contrast specimen.
7. Identify the parts of the microscope used for phase contrast and differential interference contrast microscopy. Are all the lenses on your microscope capable of DIC and phase contrast? If not, which lenses can do which contrasting technique? What part of the lens allows it to do Phase and/or DIC?
    a. Phase Contrast: There should be a phase ring in the illumination path and in the

detection path of the microscope; where are they located?

b. DIC: Similarly, there should be illumination and detection polarizers and illumination and detection Wollaston prisms; where are they located?

Phase Contrast

8. Set up your microscope for Kohler illumination in brightfield. You will not be able to get good DIC or phase without it. Put on a diatom slide or other sample easy to find in brightfield.
9. Now switch to a phase objective, observing first without the illumination phase ring in place. Then rotate the illumination phase ring in place and observe how the contrast changes.
10. Looking at the back focal plane, verify that the illumination and detection phase rings are aligned.
11. If the phase rings are movable, move them out of alignment and observe how the contrast changes as they are misaligned.

DIC

12. Now set up DIC. Switch to a DIC objective, and make sure both Wollaston prisms are out of the light path. If the polarizers are adjustable, adjust them for maximum extinction. Now reinsert the Wollaston prisms and examine the resulting image. Adjust the Wollaston prism or the illumination polarizer (de Senarmont DIC) to get the best image.
13. Now that you've successfully set up phase contrast and DIC, try a more difficult sample to image: human cheek cells. These are easily obtained by scraping the inside of your cheek with the wooden end of a cotton swab or other blunt object. They can then be spread on a slide and a coverslip placed on top. They're very hard to see in brightfield alone, so you should first find them in phase contrast or DIC. Once you've found some cells, image them in brightfield, phase contrast, and DIC to see how the images differ using these imaging techniques. See what organelles you can identify and how they appear using these different contrasting techniques.
14. Look at the cheek cells at high resolution 60 or 100x, 1.4NA objective. There are very small ridges on the cell surface that can be seen in high-resolution DIC.

Resolution

15. Diatoms make great resolution test samples, as different species have characteristic spacings between the holes in their frustrule and these spacings are on the order of the resolution limit of the microscope. Put a diatom sample on the microscope. Figure out what the smallest frustrule spacing you can see with a given objective. Close down the aperture diaphragm and see if you can see the decrease in resolution. Ideally, you should able to find a diatom / objective combination where you can clearly resolve the hole spacing with the condenser diaphragm open, but not with it closed.
16. Fill a 35 mm petri dish with water and a few drops of milk or milk powder to see the light as it passes through. Put the dish on the microscope stage. Don't spill it! Focus the field diaphragm about halfway into the dish of milky water. Then look at it from the side and open and close the **field diaphragm** and the **condenser diaphragm**. You can also look at excitation light coming out of objective lenses with different numerical apertures.

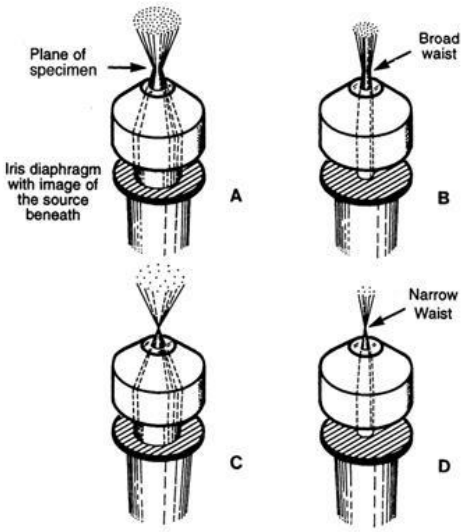
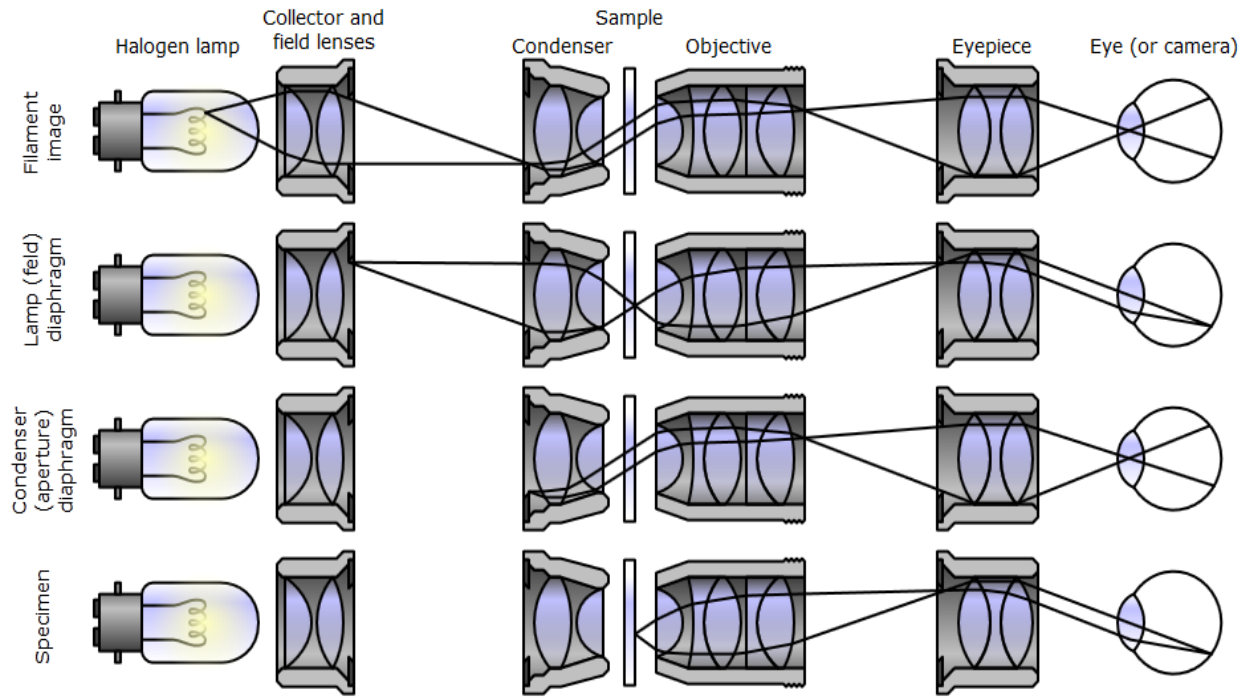


FIGURE 3-1. Effects of changing the field diaphragm and condenser iris diaphragm settings. A and C have identical condenser iris settings, as do B and D. Closing the condenser iris results in an illuminating cone of small angle (lower working NA; B and D); changing the field diaphragm results in a broad (A and B) or narrow (C and D) waist, without affecting cone angle. Effects such as these can be visualized by placing a fluorescent uranium-glass block on the condenser (or its conjugate above the ocular).

Video Microscopy; The Fundamentals second edition
1997 Shinya Inoué and Kenneth R. Spring p.124



References:

1. Molecular Expressions: Köhler Microscope Illumination *http://micro.magnet.fsu.edu/primer/anatomy/kohler.html*
2. MicroscopyU: Microscope Alignment for Köhler Illumination *http://www.microscopyu.com/tutorials/java/kohler/*
3. Wikipedia: Kohler Illumination http://en.wikipedia.org/wiki/K%C3%B6hler_illumination
4. Wikipedia Differential Interference Contrast Microscopy http://en.wikipedia.org/wiki/Differential_interference_contrast_microscopy
5. Wikipedia Phase Contrast Microscopy http://en.wikipedia.org/wiki/Phase_contrast_microscopy
6. Wikipeida Dark Field Microscopy http://en.wikipedia.org/wiki/Dark_field_microscopy
7. Frits Zernike, “How I discovered Phase Contrast”: http://www.nobelprize.org/nobel_prizes/physics/laureates/1953/zernike-lecture.pdf