

### The Pathologist's Microscope

Rudolf Virchow, the father of Pathology, had available to him wonderful microscopes during the 1850's to 1880's, but the one you have now is far better. Your microscope is the most highly perfected of all scientific instruments. These brief notes on alignment, the objective lens, the condenser, and the eyepieces are what you need to know to get the most out of your microscope and to feel comfortable using it. Figure 1 illustrates the important parts of a generic modern light microscope.

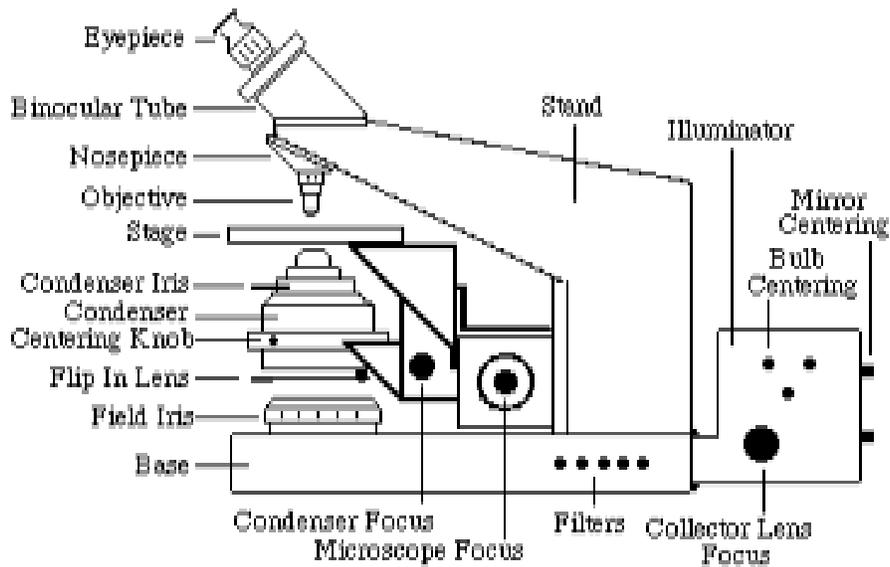


Figure 1 - Parts of the Microscope

## Alignment

August Köhler, in 1870, invented the method for aligning the microscope's optical system that is still used in all modern microscopes. To get the most from your microscope it should be Köhler aligned. Here is how:

1. Focus a specimen slide at 10X.
2. Open the **field iris** and the **condenser iris**.
3. Observe the specimen and close the **field iris** until its shadow appears on the specimen.
4. Use the **condenser focus knob** to bring the field iris into focus on the specimen. Try for as sharp an image of the iris as you can get. If you can't focus the field iris, check the condenser for a **flip-in lens** and find the configuration that lets you see the field iris. You may also have to move the field iris into the field of view (step 5) if it is grossly misaligned.
5. Center the field iris with the **condenser centering screws**.
6. Open the **field iris** until it is just out of the field of view.
7. Go to the highest magnification objective lens you normally use; focus the specimen; close the **condenser iris** until you see good contrast without fringes.

What good does alignment do? All of the optical corrections of the microscope are designed around Köhler alignment. Due to the effects of diffraction, a misaligned microscope produces false images that may not be recognized as false. A misaligned microscope is also very uncomfortable to use. A correctly aligned microscope produces its maximum resolution and contrast, and is a pleasure to use.

### Tips:

1. When you first get started, focus on the specimen slide looking through the fixed eyepiece only, then focus the adjustable eyepiece. This will prevent eyestrain.
2. For the very best image with a given objective lens, after focusing the specimen, close the **field iris** just to the edge of the field of view; pull out an eyepiece, look down the empty tube and adjust the **condenser iris** to cover just the outer edge of the field.
3. If the alignment is good but the image is lousy, the optics are dirty.

## Objective Lenses

The most important thing to know about your objective lenses is that they must be clean to function properly. Clean them and you will be amazed at the difference! Take them off the nosepiece and clean the end lens with a little glass cleaner on a cotton swab.

In addition to magnification, objective lenses have other characteristics. The most important is numerical aperture (N.A.). It's inscribed on the lens right after magnification. N.A. indicates resolution: larger N.A. means better resolution. The resolution of an objective lens, in nanometers, is approximately  $300 / \text{N.A.}$ . The largest N.A. for dry lenses is 0.95 and for oil immersion lenses about 1.4.

An inscription such as  $160 / 0.17$  or  $\infty / 0.17$  indicates the mechanical tube length - the distance from the back of the objective lens to the top of the eyepiece - and the cover glass thickness for which the objective is corrected. Modern microscopes have infinity corrected optics. An auxiliary lens near the eyepieces focuses these objectives' image in the eyepieces. Cover glass thickness really matters. All modern lenses are corrected for a 0.17 mm thick (i.e. a # 1.5) cover glass, unless they have NCG written on them to indicate no cover glass. Some high dry objectives have a cover glass correction collar that is used to correct for slight variations in cover glass thickness.

An abbreviation on the objective indicates how well it is corrected for spherical, chromatic and other aberrations. Achromats (Achro) are good, Fluorites (Flu) are better, Apochromats (Apo) are best and CFs (chromatic aberration free) are superior. Any of these might be Plan which means they are in focus from edge to edge.

### Tips:

1. For easy focusing, start at low magnification and work to high; low mag lenses have a long working distance and large depth of field thus aiding specimen location. And the lens series is parfocal so the next lens up should be nearly in focus.
2. To adjust a cover glass correction collar: open the condenser iris, focus with the microscope focus knob, blur the image with the correction collar, refocus with the focus knob. See if the image is more or less sharp. Repeat until you get the sharpest image; reset the condenser iris.
3. Check the cleanliness of your objective lenses by taking them off the nosepiece and examining them with an eyepiece turned backward and used as a hand lens.
4. To achieve highest **resolution** with your **oil** lenses, connect the condenser to the slide with oil (and align for Köhler). Condenser N.A. must be equal to or better than the objective's N.A.
6. Resolution in nanometers (nm) is calculated by  $0.61 \lambda / \text{N.A.}$  where  $\lambda$  is the wavelength of light in nm. Using a green filter, this is  $0.61 \times 500 / \text{N.A.}$  or about  $300 / \text{N.A.}$
7. Under ideal conditions, the best resolution you can get is about 250 nm or 0.25  $\mu\text{m}$  using the highest N.A. objective and condenser and homogenous immersion.

## Condenser

The condenser controls the microscope. It is probably the most underappreciated part of all microscope optics. Used incorrectly, it can choke off light and create false images. Used correctly, it can bring all microscope systems to their ultimate perfection.

With Köhler alignment, the condenser illuminates the specimen with a beautiful, homogeneous cone of light that perfectly matches the objective's angular aperture, maximizing resolution. (Angular Aperture is the largest angle at which a lens can converge or conversely accept light). The circle of light on the slide is no greater in diameter than the objective's field of view. This eliminates light scatter and flare from objects outside the field that would reduce contrast. The condenser's iris, called the aperture iris, is used to increase or decrease **contrast** and **depth of field**. If it is closed too far, it will choke off the light, produce artificial fringes around objects, and ruin resolution. If the condenser is not properly focused on the field iris, fine specimen structures will appear to be wider than they are and often more numerous than they are.

The condenser's N.A. is written on it. This should be as large or larger than the largest objective N.A.; otherwise, the condenser cannot match the angular aperture of the high N.A. objectives. This would reduce resolution. If "Abbe" is written on the condenser, it has no corrections for chromatic or spherical aberrations. Aplanat-Achromat types are corrected for these aberrations.

To get the full N.A. of an oil immersion objective, the objective and the condenser must be oiled to the slide and the condenser's N.A. must be at least as large as the objective's. This is called **homogenous immersion**. It requires an **oil immersion condenser**. If your condenser's N.A. is not larger than 0.95 it is not an oil immersion condenser. Your oil immersion condenser will work fine with your dry lenses as long as it is either clean and oil free or connected to the slide with oil. Your oil lenses will work fine with a dry condenser. In that case, you will have their magnification but not their resolution.

### Tips:

1. If the condenser has a flip-in lens, the lens' correct position for a particular objective lens is that in which the field iris can be brought into focus using the condenser focus knob. It is usually in for lenses 10X and higher.
2. If you still cannot focus the field iris, your glass slide is too thick (or your slide is upside down). Condensers are designed for a standard slide of 1.2 mm thickness.
3. Look out for bubbles when oiling a slide to the condenser and the objective. Bubbles can ruin the image. Check for bubbles by removing an eyepiece and looking down the tube. You may see the bubble's image in the objective lens's back focal plane. Try to dislodge bobbles by gently sweeping the objective lens back and fourth through the oil. If this doesn't work you will have to wipe the oil off the objective and the condenser and try again.

## Eyepieces

One or both of your eyepieces may be focusable and the binocular tube they fit into is adjustable. Use the following procedure to set up your eyepieces and binocular tubes:

1. View a specimen and set the interocular distance to match your eyes. With both eyes open you should see one large field of view.
2. Focus on the specimen with the eye that looks through the fixed (non focusable) eyepiece. If both eyepieces are focusable set them both to the middle focus position and pick one eye for this step.
3. Focus on the specimen using only your other eye by adjusting its eyepiece.

Magnification may be the only inscription on eyepieces; but, their field number or field of view (e.g. WF for wide field or /22 for 22 mm diameter), their correction (e.g. K or C or Com for compensating), and their eyepoint (e.g. an eyeglasses symbol for a high eye point) may also appear. Eyepieces have optical corrections in them that interact with the objective's corrections making them matched sets.

### Tips:

1. Total magnification in your eye is the objective magnification multiplied by the eyepiece magnification. Total magnification to your camera is the objective magnification multiplied by the projection lens magnification – this might be different from your eyepiece. Taking a picture of a stage micrometer is a good way to determine the magnification to your camera.
2. You can make counting and measuring of a specimen easier by placing a reticule in your eyepiece that provides some lines or a grid and that is calibrated in real units using a stage micrometer.
3. **Abbe's Rule** states that total magnification greater than 1000 times the objective's N.A. is empty of further resolution. This is called **empty magnification**. Thus, changing your eyepieces to higher magnification ones generally does not improve resolution.

## Conclusion

While Rudolf Virchow was inventing the science of Pathology, Ernst Abbe was inventing modern microscopy. Abbe created the concept of numerical aperture, the compensating eyepiece, the apochromatic objective, and oil immersion lenses. Most important of all, Abbe developed the diffraction theory of microscopy that is the basis of all modern light microscopy.

If you would like to know more, visit our web site:

<http://www.med.unc.edu/microscopy>

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