Microscope Alignment

The light microscope will deliver optimal performance only if its various components are properly installed and aligned. For upright microscopes from any of the major manufacturers, proper manufacture and assembly will ensure that the following conditions are met.

1. The microscope stage is firmly mounted to the focusing mechanism and is level. The stage should travel straight up or down and remain level when either the coarse or fine focus knob is rotated.

2. The substage condenser is properly installed and secured. The substage condenser should travel straight up or down when its elevating knob is rotated.

3. The binocular or trinocular observation tube is properly seated, and each user has:
   - adjusted the interocular distance between the eyepiece tubes (the fields of view from each tube should “fuse” into a single, circular field of view) and
   - adjusted the eyepieces so that any difference in acuity between the two eyes has been properly compensated for (see Eyepiece Adjustment).

4. The nosepiece and its complement of objectives have been designed so that the objectives are:
   - parfocal (i.e., little, if any, refocusing is required when changing objectives) and
   - parcentric (i.e., an object in the center of the field of view remains centered after changing objectives).

Modern microscopes, including even moderately priced laboratory models with inexpensive achromatic objectives, are capable of producing excellent images providing that the optics are kept clean and free of obstructions.

In this unit, the two light paths of the microscope’s optical system will be discussed: the image-forming path and the illumination path. The components of these optical paths must be properly aligned to achieve optimal image quality. More comprehensive descriptions and discussion of the microscope’s optical system may be found in Leitz (1938), Mollring (1976), Spencer (1982), Bradbury (1984), Abramowitz (1985, 1987, 1993, 1994), Inoue (1986), Delly (1988), and UNIT 2.2.

THE IMAGE-FORMING SYSTEM

The optical components of the image-forming system (Fig. 2.7.1) include the objective and the eyepiece. When properly installed and undamaged, these components are pre-aligned—the user need only keep them clean and free from contaminants such as dust, fingerprints, and immersion oil. Routine observance of the following practices will go a long way toward keeping the optical components clean.

1. Keep fingertips away from all lenses and optical glass surfaces.

2. At the end of each day’s use, carefully and properly remove contaminants (e.g., immersion oil, fingerprints) from all optical surfaces using the manufacturer’s recommendations (see Maintenance of Optical Glass Surfaces).

3. Keep the microscope covered when not in use, using the plastic dust cover supplied by the manufacturer.
Figure 2.7.1  Image-forming ray paths in Köhler illumination are traced from two ends of the lamp filament. Conjugate planes are at the field diaphragm, specimen plane, intermediate image plane (entrance pupil of the eyepiece), and the retina of the eye. Modified with permission from Abramowitz (1985).
EYEPIECE ADJUSTMENT

The microscope’s eyepieces provide the port through which information, in the form of an image, is transferred from the microscope to the user. Differences in visual acuity between users, and even between the eyes of the same user, require that the eyepieces be adjusted to accommodate each individual’s interocular distance and each eye’s visual acuity. Otherwise, the observed image quality may suffer dramatically and the user may experience discomfort, caused by eye strain, when using the microscope.

To adjust the eyepieces, follow the procedure outlined below. It is intended for a typical microscope in which only the left eyepiece or eyepiece tube is adjustable, but is readily adaptable for microscopes with two adjustable eyepieces.

1. Select a low-magnification (e.g., 10×) dry objective and place a specimen slide on the stage.
2. With the left eye closed, look at the specimen through the right eyepiece, locate a well-defined target object near the center of the field of view, and bring it into sharp focus.
3. With the right eye now closed, rotate the diopter-adjustment ring on the left eyepiece alternately clockwise and counterclockwise until the target object is in sharp focus.
4. With both eyes open, touch up the focus of the left eyepiece using the diopter-adjustment ring.
5. Set the proper interpupillary distance for your eyes by grasping the bases of the eyepiece tubes (not the knurled adjustment ring) and moving them together or apart, as necessary.

THE ILLUMINATION SYSTEM

Proper adjustment of the microscope’s illumination system is a continuing and crucial requirement for observation, photomicrography, or electronic imaging. In typical modern microscopes the following components should be present and correctly aligned:

1. the light bulb
   • usually housed within a removable lamphouse; can be either precentered in the illumination path or centered by the user with knobs or screws built into the lamphouse
2. the collector lens
   • built into the base of the microscope and permanently aligned by the manufacturer
3. the variable field diaphragm
   • usually built into the base of the microscope, below the stage
4. the substage condenser
   • typically mounted beneath the stage in a bracket that can be raised or lowered independently of the stage by rotating a knurled knob
   • centered in the optical path using knobs or screws that extend from the housing
5. the variable aperture diaphragm
   • built into the substage condenser housing (optically, it is positioned at or near the front focal plane of the condenser)
   • opened or closed by means of a lever or knurled knob
Köhler Illumination

Köhler illumination is provided by all manufacturers of modern laboratory microscopes because it can provide specimen illumination that is uniformly bright and free from glare, thus allowing the user to realize the microscope’s optimum performance. The manufacturers have designed the microscope so that the collector lens and any other optical components built into the base of the microscope will project an enlarged and focused image of the lamp filament onto the plane of the aperture diaphragm of a properly positioned substage condenser. Closing or opening the condenser diaphragm controls the angle of the light rays emerging from the condenser and reaching the specimen from all azimuths. The setting of the condenser’s aperture diaphragm, along with the aperture of the objective, determines the realized numerical aperture (NA) of the microscope “system.” As the condenser diaphragm is opened, the working NA of the microscope increases, resulting in greater resolving power and light transmittance.

The field diaphragm in the base of the microscope controls only the width of the bundle of light rays reaching the condenser—it does not affect the optical resolution (NA) or the intensity of illumination. Proper adjustment of the field diaphragm (i.e., centered in the optical path and opened so as to lie just outside of the field of view) is important for preventing glare that can reduce contrast in the observed image.

To achieve Köhler illumination, proper adjustment of the condenser and field diaphragms is critical. These adjustments must be made by the microscopist each time the microscope is used and each time the objective is changed.

Steps in setting up Köhler illumination
1. Open the field and aperture diaphragms all the way.
2. Focus a typical specimen using a 10× objective, and adjust the transformer voltage so that the light intensity is comfortable for viewing.
3. Close down the field diaphragm most of the way.
4. While looking through the eyepieces, carefully raise or lower only the substage condenser (not the entire stage) until the polygon-shaped edge of the field diaphragm is inside the field of view and is sharply focused.
5. Using the small condenser-centering screws at the base of the substage holder, center the image of the field diaphragm in the field of view. Then open the field diaphragm until it just disappears from view.
6. Lift out one of the eyepieces and look down the observation tube at the back of the objective.
7. Slowly close the aperture diaphragm of the substage condenser and observe that the image of the condenser diaphragm is clearly visible at the back of the objective. If the illumination system does not have a frosted filter inserted in the light path, an image of the lamp filament will be visible. The filament image should be centered in the back aperture of the objective, either by the manufacturer (with precentered bulbs) or by utilizing a set of centering screws located on the lamphouse. The filament image should also fill, or nearly fill, the back aperture of the objective.
8. Adjust the aperture diaphragm so that it is open two-thirds to three-quarters of the way. This adjustment may vary according to the specimen: the aperture diaphragm might be opened nine-tenths of the way for a well-stained specimen.
9. Replace the eyepiece.
This completes the process of setting up Köhler illumination with the 10× objective. When the objective is changed during normal use of the microscope, the field diaphragm and the condenser diaphragm must be readjusted according to the procedure outlined above. For example, when switching from a 10× to a 20× objective:

a. the field diaphragm should be closed down somewhat, because the specimen area being viewed is smaller,

b. it should be recentered, and

c. the aperture diaphragm should be opened somewhat to increase the numerical aperture of the condenser to match the higher NA of the 20× objective.

**NOTE:** The substage condenser is properly aligned when the edge of the field diaphragm is sharpest. At this position, the edge may exhibit a slight blue tint, which should not vary in color around the edge. A highly corrected, achromatic-aplanatic condenser will yield an essentially colorless edge. Variation in color along the edge of the field diaphragm (e.g., from blue to red) indicates either that the diaphragm is tilted or, more likely, that some components in the illumination path are not properly aligned. For routine work this may not matter, but prior to critical work the microscope should be serviced.

### Conjugate planes

To better understand Köhler illumination, it is helpful to separate the image-forming light path and the illumination light path, and to look at the various planes, or levels, in each of these paths. This is referred to as the analysis of the conjugate planes of these paths. By definition, an object that is in focus at one plane is also in focus at the other conjugate planes of that light path.

Conjugate planes in the image-forming light path in Köhler illumination include:

1. the field diaphragm,
2. the focused specimen,
3. the intermediate image plane (i.e., the plane of the fixed diaphragm of the eyepiece), and
4. the retina of the eye or the film plane of the camera.

Conjugate planes in the path of the illuminating light rays in Köhler illumination include:

1. the lamp filament,
2. the condenser aperture diaphragm (at the front focal plane of the condenser),
3. the back focal plane of the objective, and
4. the eyepoint (also called the Ramsden disk) of the eyepiece, which is located ~1/2 in. (~1 cm) above the top lens of the eyepiece, at the point where the observer places the front of the eye during observation.

### What are the advantages of Köhler illumination?

1. The image appears bright and evenly lighted, providing ideal illumination for observation and photomicrography. In older methods of illumination, the image of the lamp filament was focused at the specimen plane, thus partially obscuring the image of the specimen and also heating the specimen excessively.

2. Proper adjustment of the field diaphragm is readily achievable, which is important in controlling the width of the illuminating light beam to minimize glare.

3. The condenser aperture diaphragm can be varied to control the angle of the illuminating rays reaching the specimen and the image-forming optics, and thus controls...
the working NA of the microscope. Leaving the condenser aperture wide open provides maximum theoretical resolution, but greatly reduces contrast and results in an inability to see fine detail. Closing the condenser diaphragm somewhat, to two-thirds or three-quarters of the area of the back focal plane of the objective (see discussion of steps in setting up Köhler illumination, above), strikes a reasonable compromise among resolution, contrast, and depth of field.

A Note About Reflected Light Microscopy

Köhler illumination is also important in reflected light brightfield microscopy (see Fig. 2.7.2) and epifluorescence microscopy. A good-quality reflected light illuminator has a field diaphragm and an aperture diaphragm. However, in a reflected light illuminator their positions are reversed—the aperture diaphragm is situated closest to the lamp filament, and the field diaphragm is closest to the specimen. In reflected light fluorescence, where the nature of the specimen often makes resolution less important than light capture, the aperture diaphragm is usually left wide open to permit capture of the most light. Conjugate planes are similar to those described for transmitted light. The microscope objective performs a dual role: that of condenser lens (for light traveling toward the specimen) and objective lens (for light returning from the specimen).

![Köhler illumination for brightfield reflected light](image)

**Figure 2.7.2** Köhler illumination for brightfield reflected light (diagrammatic for finite-tube-length objective system). F, filament light source; F', image of light source at condenser aperture diaphragm (CAD); F'', image of light source at back focal plane of objective; FD, field diaphragm; FD' and FD'', conjugate planes to FD; L1, L2, and L3, lenses of vertical illuminator. Reprinted with permission from Abramowitz (1990).
MAINTENANCE OF OPTICAL GLASS SURFACES

The microscope’s optical surfaces are its most important but also its most delicate feature. Routine care of the optical surfaces as part of regular use will greatly help to preserve the microscope’s image quality. In the next two sections, several recommended routine practices are outlined, and guidelines are given for cleaning external optical glass surfaces. Do not attempt to clean internal optical or other microscope surfaces, or to clean stubborn contaminants (e.g., dried immersion oil) from external surfaces—call your local microscope service dealer!

Routine Care

1. Keep the microscope covered when not in use.
2. Keep fingers and foreign materials away from optical surfaces.
3. Remove dust by blowing it from the surface of the lens using a duster can of compressed air or nitrogen (purified and filtered).
4. Regularly check the external surface of components such as eyepieces and filters for contaminants. Dust, makeup, and body oil are common; blow off dust and, if necessary, clean as described below.
5. For immersion objectives, use only immersion oil recommended by the microscope manufacturer, and clean immersion oil from the objectives at the end of each day (check all objectives—dry objectives can become contaminated accidentally).

Cleaning

While a detailed treatment of cleaning supplies and methods is beyond the scope of this section, some discussion is warranted. Consult your microscope’s manufacturer or local dealer for any booklets or written materials that might be available. Many service dealers can provide expert advice and tips, as well as proper cleaning supplies, so don’t hesitate to consult them.

Selection of the proper cleaning fluid is important, and there are many opinions about which cleaners and solvents are safe and effective. As noted above, the best place to start may be your local microscope service dealer. As a general rule, unless you are knowledgeable about their use and limitations, avoid toxic or dangerous (e.g., explosive) solvents such as acetone and ether, which may also damage painted and other microscope surfaces. Some references recommend a cleaning solution of 7 parts ether and 3 parts absolute ethanol, but substituting Freon for the ether is safer. Ethanol alone may also be used. While certain commercial cleaners such as Windex and Kodak Lens Cleaner can be used for the exposed surface of an eyepiece or objective, they may leave a slight film. In many cases, simply breathing onto the surface of the lens (followed by gentle wiping) provides a moisture layer that will lift off dust, dissolve organics, discharge static, and let the user see any dirt or imperfections on the surface.

To wipe, use only optical-quality soft lens tissue or lint-free cloth that has been stored in resealable plastic bags to avoid dust contamination. Wooden, cotton-tipped applicators (individually wrapped) can be ordered from hospital supply companies. Avoid consumer cotton swabs and tissues, commercial lab-wipes (which may contain glue, wax, or dust), and camera lens cleaners with unknown ingredients. Exert only very gentle pressure while cleaning—the weight of the materials is sufficient, and additional pressure may cause trapped dust particles to scratch the lens. To clean a lens surface:

1. Wrap a layer of lens tissue around a cotton-tipped applicator.
2. Moisten it with cleaning solution and shake off the excess, which could seep into microscope parts.
3. Rest the tip of the applicator in the center of the glass surface to be cleaned.

4a. *For an eyepiece or filter:* Gently wipe the surface in an outward spiral pattern, from the center to the edge.

4b. *For an objective lens:* Rotate the lens (i.e., on a table top) while moving the applicator from the center toward the periphery.

5. Discard the used applicator and repeat with fresh materials, if necessary.

Alternatively, moisten a piece of lens tissue with cleaning solution, cover the lens as if with a blanket, and gently pull the tissue along the surface and off of the lens. Gently remove any remaining fluid using fresh lens tissue.

**LITERATURE CITED**


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