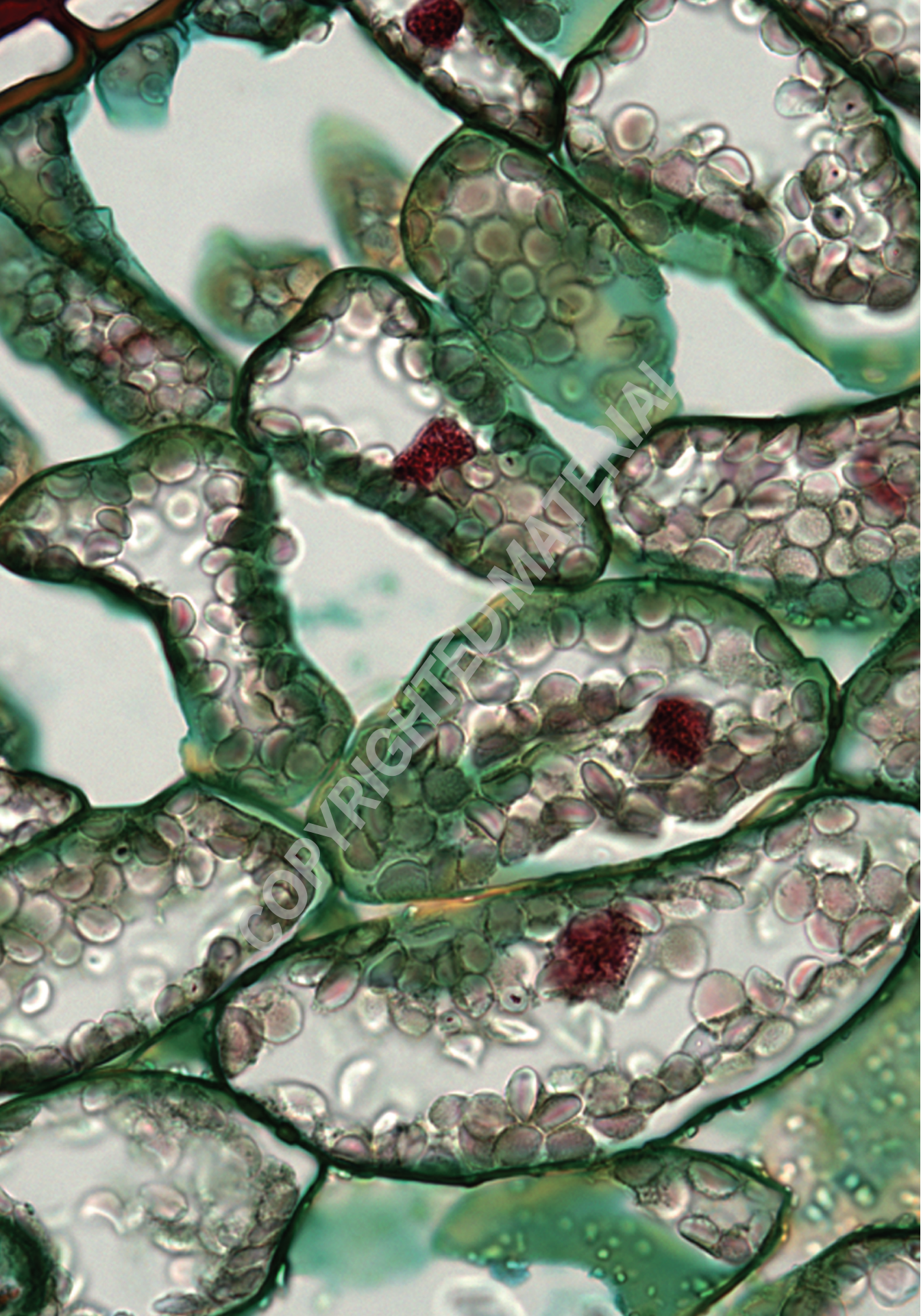


CHAPTER
1

FUNDAMENTALS OF LIGHT MICROSCOPY

OVERVIEW

In this chapter, we examine the optical design of the light microscope and review procedures for adjusting the microscope and its illumination to obtain the best optical performance. The light microscope contains two distinct sets of interlaced focal planes, eight planes in all, between the illuminator and the eye. All of these planes play an important role in image formation. As we will see, some planes are not fixed, but vary in their location depending on the focus position of the objective and condenser lenses. Therefore, an important first step is to adjust the microscope and its illuminator for Koehler illumination, a method of illumination introduced by August Koehler in 1893 that gives bright, uniform illumination of the specimen and simultaneously positions the sets of image and diffraction planes at their proper locations. We will refer to these locations frequently throughout the book. Indeed, microscope manufacturers build microscopes so that filters, prisms, and diaphragms are located at precise physical locations in the microscope body, assuming that certain focal planes will be precisely located after the user has adjusted the microscope for Koehler illumination. Finally, we will practice adjusting the microscope for examining a stained histological specimen, review the procedure for determining magnification, and measure the diameters of cells and nuclei in a tissue sample.

OPTICAL COMPONENTS OF THE LIGHT MICROSCOPE

A *compound light microscope* is an optical instrument that uses visible light to produce a magnified image of an object (or specimen) that is projected onto the retina of the

Brightfield microscopy of stained mesophyll cells in a leaf section.

Fundamentals of Light Microscopy and Electronic Imaging, Second Edition.
Douglas B. Murphy and Michael W. Davidson.

eye or onto the photosensitive surface of an imaging device. The word *compound* refers to the fact that two lenses, the objective and the eyepiece (or ocular), work together to produce the final magnification M of the image such that:

$$M_{\text{final}} = M_{\text{obj}} \times M_{\text{oc}}.$$

Two microscope components are of critical importance in forming the image: (1) the *objective*, which collects light diffracted by the specimen and forms a magnified real image at what is called the real intermediate image plane near the eyepieces or oculars, and (2) the *condenser*, which focuses light from the illuminator onto a small area of the specimen. (We define real vs. virtual images and examine the geometrical optics of lenses and magnification in Chapter 4; a real image can be viewed on a screen or exposed on a sheet of film, whereas a virtual image cannot.) The arrangement of these and other components in an upright stand research level microscope is shown in Figure 1.1, and for an inverted research microscope in Figure 1.2. Two lamps provide illumination for brightfield and interference (illumination from below: *diascopic*) and fluorescence (illumination from above: *episcopic*) modes of examination. Both the objective and condenser contain multiple lens elements that perform close to their theoretical limits and are therefore expensive. As these optics are handled frequently, they require careful attention. Other components less critical to image formation are no less deserving of care, including the tube lens and eyepieces, the lamp collector and lamp socket and its cord, filters, polarizers, retarders, and the microscope stage and stand with coarse and fine focus.

At this point, take time to examine Figure 1.3, which shows how an image becomes magnified and is perceived by the eye. The figure also points out the locations of important focal planes in relation to the objective, the ocular, and the eye. The specimen on the microscope stage is examined by the objective, which produces a magnified real image of the object in the image plane of the ocular. When looking in the microscope, the ocular acting together with the eye's cornea and lens projects a still more magnified real image onto the retina, where it is perceived and interpreted by the brain as a magnified virtual image about 25 cm (10 in) in front of the eye. For photography, the intermediate image is recorded directly or projected as a real image onto a camera.

Microscopes come in both inverted and upright designs (Figs. 1.1 and 1.2). In both designs the location of the real intermediate image plane at the eyepiece is fixed, and the focus dial of the microscope is used to position the image at precisely this location. In most conventional upright microscopes, the objectives are attached to a nosepiece turret on the microscope body, and the focus control moves the specimen stage up and down to bring the image to its proper location in the eyepiece. In inverted designs, the stage itself is fixed, being bolted to the microscope body, and the focus dials move the objective turret up and down to position the image in the eyepieces. Inverted microscopes are rapidly gaining in popularity because one can examine living cells in culture dishes filled with medium using standard objectives and avoid the use of sealed flow chambers, which can be awkward. One also has better access to the stage, which can serve as a rigid working platform for microinjection and physiological recording equipment. Inverted designs also have their center of mass closer to the lab bench and are therefore less sensitive to vibration. However, there is some risk of physical damage, as objectives may rub against the bottom surface of the stage during rotation of the objective turret. Oil immersion objectives are also at risk, because gravity can cause oil to drain down and enter the crevice between the nose and barrel, potentially

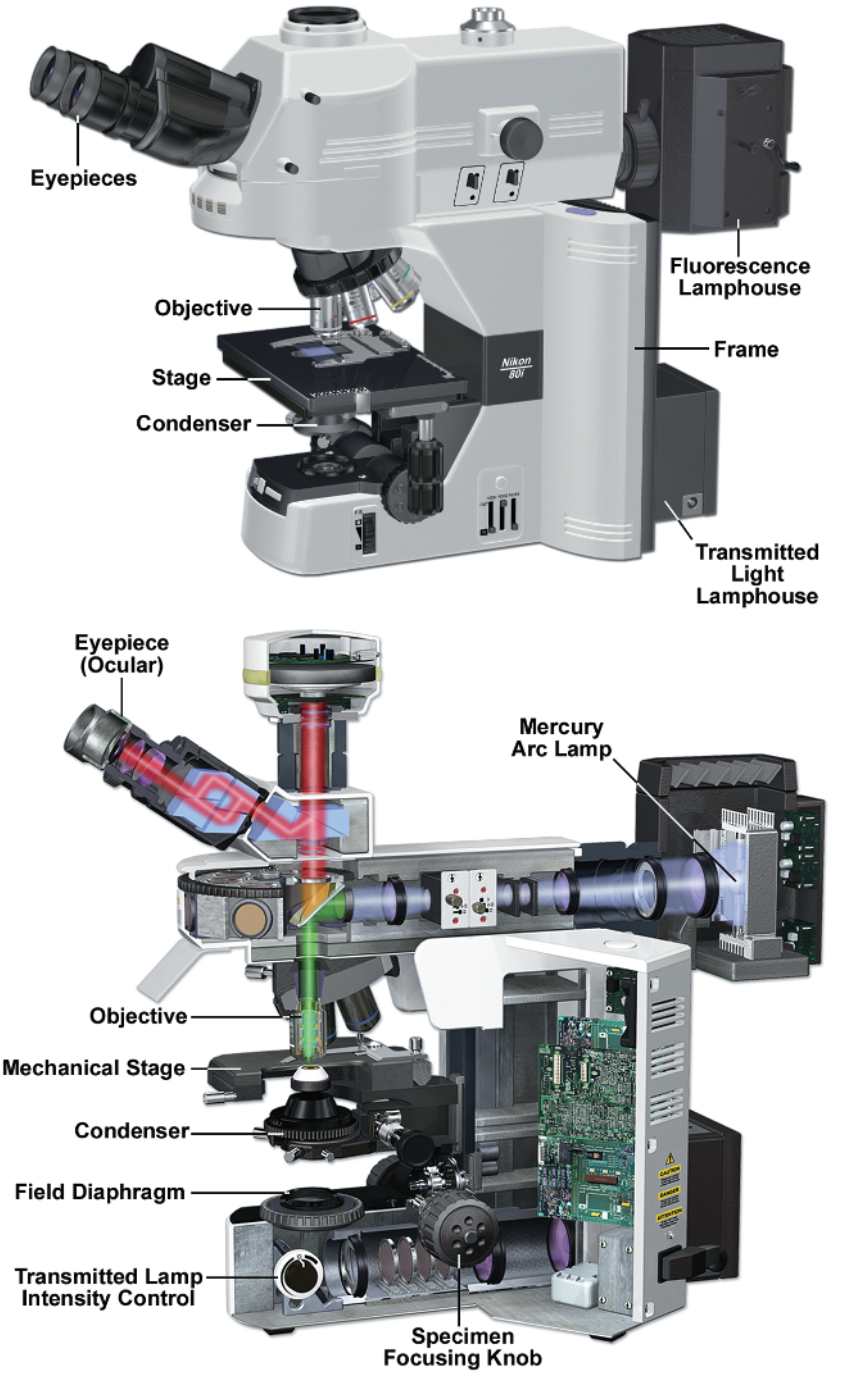


Figure 1.1

The research light microscope with upright stand. Two lamps provide transmitted and reflected light illumination. Note the locations of the knobs for the specimen and condenser lens focus adjustments. Also note the positions of two variable iris diaphragms: the field diaphragm near the illuminator, and the condenser diaphragm at the front aperture of the condenser. Each has an optimum setting in a properly adjusted microscope. Above: Nikon Eclipse 80i upright microscope; below: Olympus BX71 upright microscope.

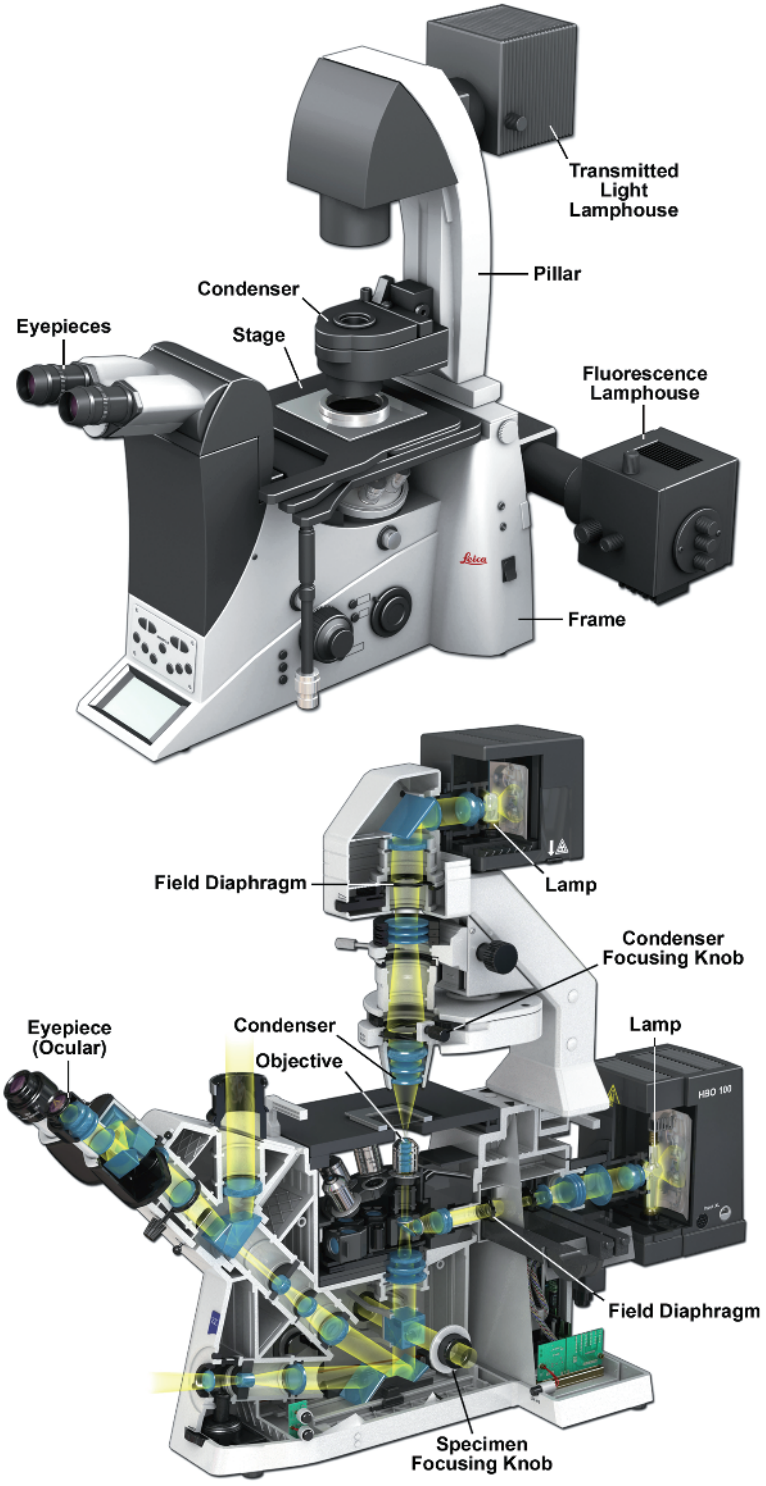


Figure 1.2

The research light microscope with inverted stand. As in upright designs, two lamps provide transmitted and reflected light illumination. Note the locations of the knobs for the specimen and condenser lens focus adjustments, which are often in different locations on inverted microscopes. Also note the positions of two variable iris diaphragms: the field diaphragm near the illuminator, and the condenser diaphragm at the front aperture of the condenser. Each has an optimum setting in a properly adjusted microscope. Above: Leica Microsystems DMI6000 B inverted microscope; below: Zeiss Axio Observer inverted microscope.

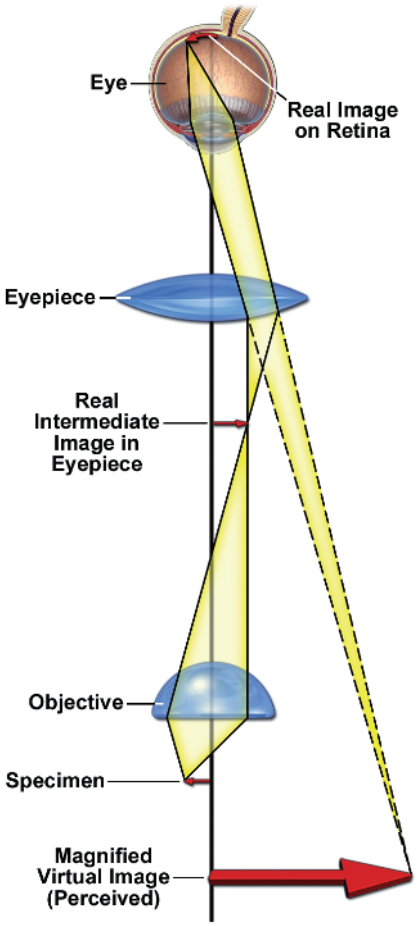


Figure 1.3

Perception of a magnified virtual image of a specimen in the microscope. The objective forms a magnified image of the object (called the real intermediate image) in the eyepiece; the intermediate image is examined by the eyepiece and eye, which together form a real image on the retina. Because of the perspective, the retina and brain interpret the scene as a magnified virtual image about 25 cm in front of the eye.

contaminating internal lens surfaces, ruining the optical performance and resulting in costly lens repair. This can be prevented by wrapping a pipe cleaner or hair band around the upper part of the lens to catch excess drips of oil. Therefore, despite many advantages, inverted research microscopes require a little more attention than do standard upright designs.

APERTURE AND IMAGE PLANES IN A FOCUSED, ADJUSTED MICROSCOPE

Principles of geometrical optics show that a microscope has two sets of interlaced conjugate focal planes, a set of four *object or field planes*, and a set of 4 *aperture or*

diffraction planes, that have fixed, defined locations with respect to the object, optical elements, the light source, and the eye or camera. Each plane within a set is *conjugate* with the other planes, with the consequence that all of the planes of a given set can be seen simultaneously when looking in the microscope. The field planes are observed in normal viewing mode using the eyepieces. This mode of viewing is called the normal, or object, or orthoscopic mode, and the real image of an object is called an *orthoscopic* image. Viewing the aperture or diffraction planes requires using an eyepiece telescope or Bertrand lens, which is focused on the rear aperture of the objective (see Note). This mode of viewing is called the aperture, or diffraction, or conoscopic mode, and the image of the diffraction plane viewed at this location is called the *conoscopic* image. In this text, we refer to the two viewing modes as the *normal* and *aperture viewing modes* and do not use the terms orthoscopic and conoscopic, although these terms are common in other texts.

Note: Objectives, Eyepieces, and Eyepiece Telescopes

An *aperture* is a hole or opening in an opaque mask designed to eliminate stray light from entering the light path, and most field and aperture planes of a microscope contain them. A fixed circular aperture is found at or near the rear focal plane of the objective (Fig. 1.4). (The precise location of the rear focal plane is a function of the focal length of the lens; for objectives with short focal lengths, the focal plane may be located inside the lens barrel.) The aperture mask is plainly visible at the back surface of the objective. This aperture marks one of the key aperture planes of the microscope, and we refer to this site frequently in the text.

The *eyepiece telescope* (not shown), sometimes called a phase or centering telescope, is a special focusable eyepiece that is used in place of an ocular to view the *rear aperture of the objective* and other aperture planes that are conjugate to it. To use the telescope, remove an eyepiece, insert the eyepiece telescope, and focus it on the circular edge of the objective rear aperture. Some microscopes contain a built-in focusable telescope lens called a *Bertrand lens* that can be conveniently rotated into and out of the light path as required.

The identities of the sets of conjugate focal planes are listed in Table 1.1, and their locations in the microscope under conditions of Koehler illumination are shown in Figure 1.5. The terms *front aperture* and *rear aperture* refer to the openings at the front and rear focal planes of a lens from the perspective of a light ray traveling from the lamp to the retina. Knowledge of the location of these planes is essential for adjusting the microscope and for understanding the principles involved in image formation. Indeed, the entire design of a microscope is based around these planes and the user's need to have access to them.

The *exit pupil* of the eyepiece, one of the microscope's aperture planes, is the disk of light that appears to hang in space a few millimeters above the back lens of the eyepiece; it is simply the image of the illuminated rear aperture of the objective. Normally, we are unaware that we are viewing four conjugate field planes when looking

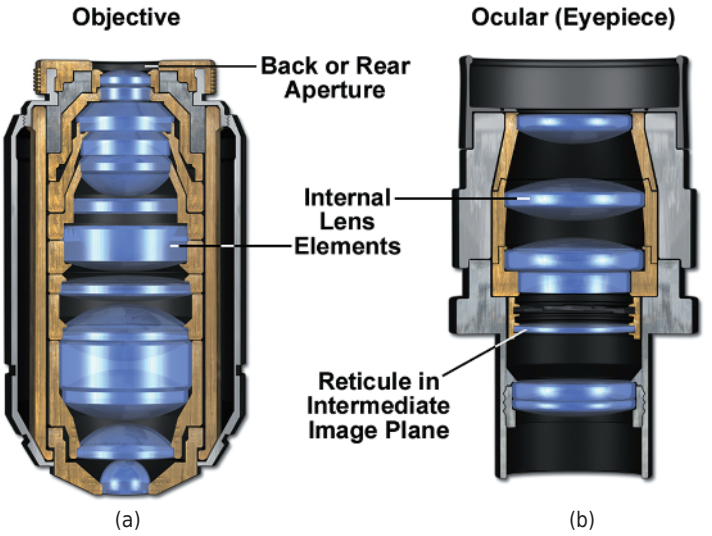


Figure 1.4
Objective and eyepiece diagrams. (a) Cross section of an objective showing the location of the back or rear aperture. (b) Cross sectional view of a focusable eyepiece, showing the location of the real intermediate image, in this case, containing an eyepiece reticule. Notice the many lens elements that make up these basic optics.

TABLE 1.1 Conjugate Planes in Optical Microscopy

Field Planes	Aperture Planes
(Normal view through the eyepieces)	(Aperture view through the eyepiece telescope)
Lamp (field) diaphragm	Lamp filament
Object (specimen) or field plane (diaphragm)	Front aperture of condenser
Real intermediate image plane (eyepiece field stop)	Rear aperture of objective
Retina or camera sensor face	Exit pupil of eyepiece (coincident with pupil of eye)

through the eyepieces of a microscope. As an example of the simultaneous visibility of conjugate focal planes, consider that the image of a piece of dirt on the focused specimen could lie in any one of the four field planes of the microscope: floaters near the retina, dirt on an eyepiece reticule, dirt on the specimen itself, and dirt on the glass plate covering the field diaphragm. With knowledge of the locations of the conjugate field planes, one can quickly determine the location of the dirt by rotating the eyepiece, moving the microscope slide, or wiping the cover plate of the field diaphragm. Before proceeding, you should take the time to identify the locations of the field and aperture planes on your microscope in the laboratory.

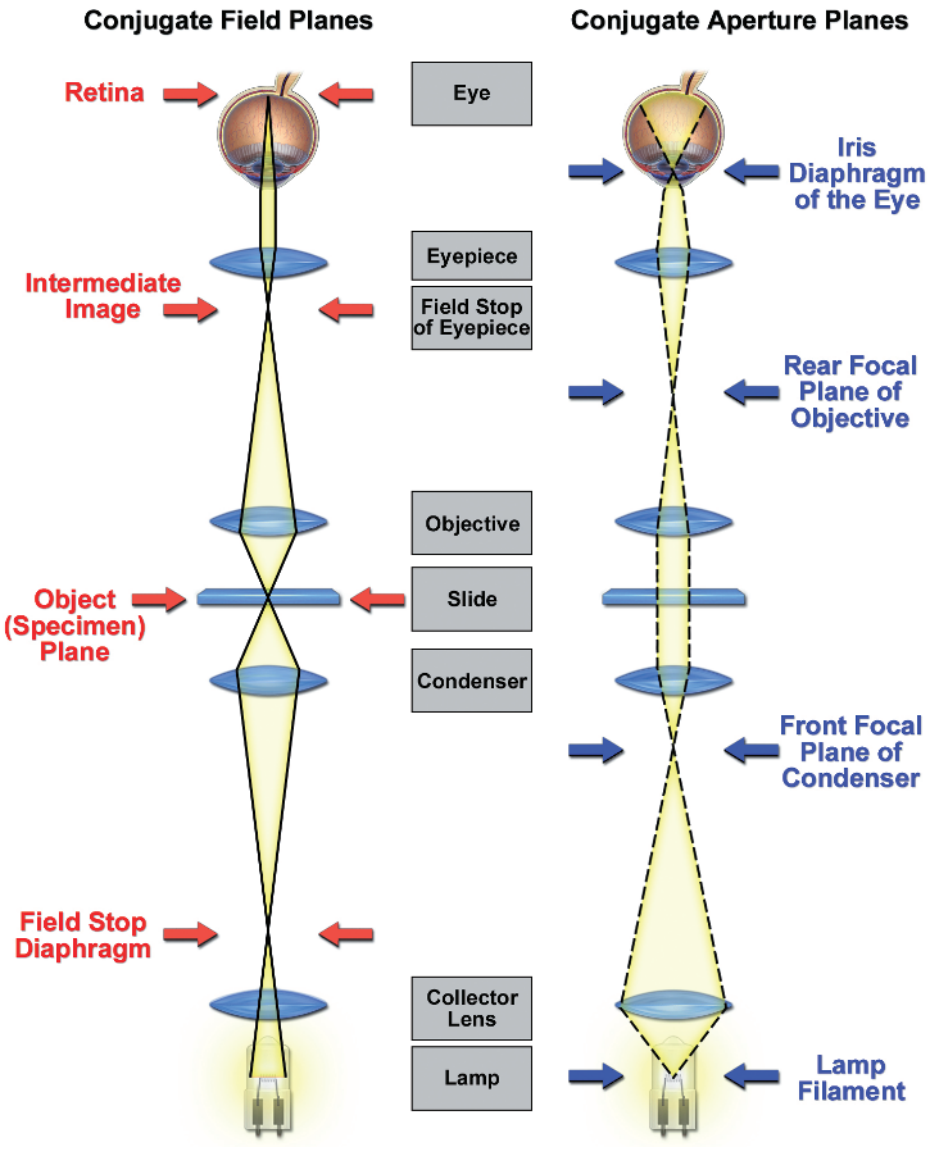


Figure 1.5

Conjugate and aperture planes in Koehler illumination. Arrows mark the conjugate focal planes. Note the locations of four conjugate field planes (red arrows; left) and four conjugate aperture planes (blue arrows; right) indicated by the crossover points of rays in the diagrams. The left-hand diagram shows that the specimen or object plane is conjugate with the real intermediate image plane in the eyepiece, the retina of the eye, and the field stop diaphragm between the lamp and the condenser. The right-hand drawing shows that the lamp filament is conjugate with aperture planes at the front focal plane of the condenser, the rear focal plane of the objective, and the pupil of the eye. The two sets of conjugate planes interdigitate with one another.

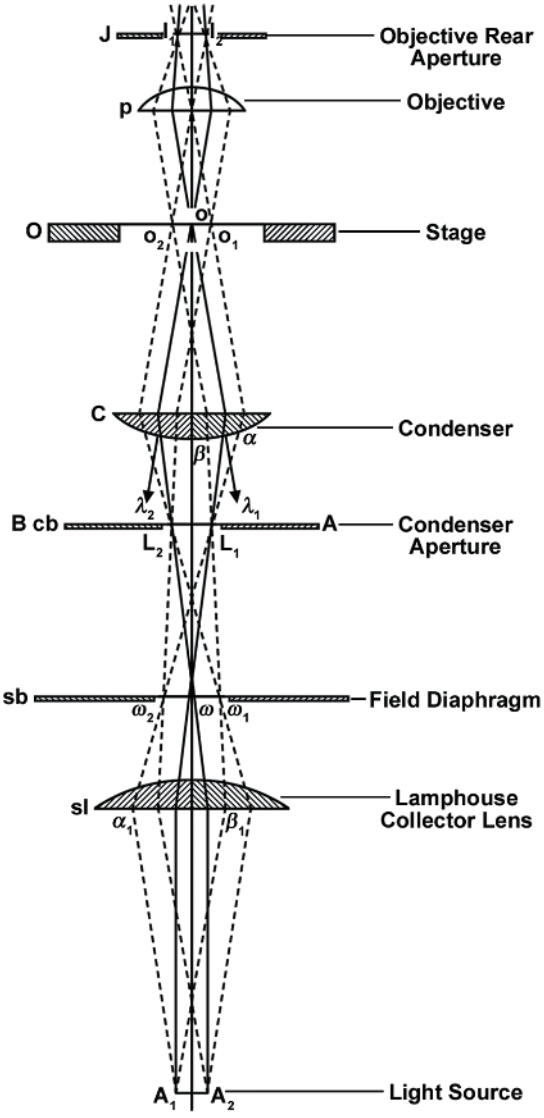
KOEHLER ILLUMINATION

Illumination is a critical determinant of optical performance in light microscopy. Apart from the intensity and wavelength range of the light source, it is important that a large cone of light emitted from each source point be collected by the lamp collector and that the source be imaged onto the front aperture of the condenser. From there, each point of the source image is projected through the specimen and to infinity as a parallel collimated pencil of light. The size of the illuminated field at the specimen is adjusted so that it matches the specimen field diameter of the objective being employed. Because each source point contributes equally to illumination in the specimen plane, variations in intensity in the image are attributed to the object and not to irregular illumination from the light source. The method of illumination introduced by August Koehler in the late nineteenth century fulfills these requirements and is the standard method of illumination used in light microscopy (Fig. 1.6). Under the conditions set forth by Koehler, a *collector lens* on the lamp housing is adjusted so that it focuses an image of the lamp filament at the front focal plane of the condenser while completely filling the aperture with light. Under this condition, illumination of the specimen plane is bright and even. Achieving this condition also requires focusing the condenser using the condenser focus knob, an adjustment that brings two sets of conjugate focal planes into precise physical locations in the microscope, a requirement for a wide range of image contrasting techniques that are discussed later in Chapters 7–11. The main advantages of Koehler illumination in image formation are:

- *Bright and even illumination in the specimen plane and in the conjugate image plane*. Even when illumination is provided by an irregular light source, such as a lamp filament, illumination of the object-specimen is remarkably uniform across an extended area. Under these conditions of illumination, a given point in the specimen is illuminated by every point in the light source, and conversely, a given point in the light source illuminates every point in the specimen.
- *Positioning of two different sets of conjugate focal planes at specific locations along the optical axis of the microscope*, a strict requirement for maximal spatial resolution and optimal image formation for a variety of optical modes. As we will see, stage focus and condenser focus and centration position the focal planes correctly, while correct settings of the field diaphragm and the condenser aperture diaphragm give control over resolution and contrast. Once properly adjusted, it is easier to locate and correct faults, such as dirt and bubbles that can degrade optical performance.

ADJUSTING THE MICROSCOPE FOR KOEHLER ILLUMINATION

Review Figure 1.5 again to familiarize yourself with the locations of the two sets of focal planes, one set of four field planes, and one set of four aperture planes. You will need an eyepiece telescope or Bertrand lens to examine the aperture planes and to make certain adjustments. In the absence of a telescope lens, one may simply remove an eyepiece and look straight down the optical axis at the objective aperture; however, without a telescope, the aperture diameter is small and difficult to see clearly. The adjustment procedure is given in detail below. You will need to check your alignment every time you change a lens to examine the specimen at a different magnification.



August Koehler
1866-1948

Figure 1.6

August Koehler introduced a new method of illumination that greatly improved image quality and revolutionized light microscope design. Koehler introduced the system in 1893 while he was a university student and instructor at the Zoological Institute in Giessen, Germany, where he performed photomicrography for taxonomic studies on limpets. Using the traditional methods of critical illumination, the glowing mantle of a gas lamp was focused directly on the specimen with the condenser, but the images were unevenly illuminated and dim, making them unsuitable for photography using slow-speed emulsions. Koehler's solution was to reinvent the illumination scheme. He introduced a collector lens for the lamp and used it to focus the image of the lamp on the front aperture of the condenser. A luminous field stop (the field diaphragm) was then focused on the specimen with the condenser focus control. The method provided bright, even illumination, and fixed the positions of the focal planes of the microscope optics. In later years, phase contrast microscopy, fluorescence microscopy with epi-illumination, differential interference contrast microscopy, and confocal optical systems would all utilize and be critically dependent on the action of the collector lens, the field diaphragm, and the presence of fixed conjugate focal planes that are inherent to Koehler's method of illumination. The interested reader should refer to the special centenary publication on Koehler by the Royal Microscopical Society (see Koehler, 1893).

Note: Summary of Steps for Koehler Illumination

1. Check that the lamp is focused on the front aperture of the condenser.
2. Focus the specimen.
3. Focus the condenser to see the field stop diaphragm.
4. Adjust the condenser diaphragm using the eyepiece telescope.

- *Preliminaries.* Place a specimen slide, such as a stained histological specimen, on the stage of the microscope. Adjust the condenser height with the condenser-focusing knob so that the front lens element of the condenser comes within ~1–2 mm of the specimen slide. Do the same for the objective. Be sure all diaphragms are open so that there is enough light (includes illuminator's field diaphragm, the condenser's front aperture diaphragm, and in some cases, a diaphragm in the objective itself). Adjust the lamp power supply so that the illumination is bright but comfortable when viewing the specimen through the eyepieces.
- *Check that the lamp fills the front aperture of the condenser.* Inspect the front aperture of the condenser by eye and ascertain that the illumination fills most of the aperture. It helps to hold a piece of lens tissue against the aperture to check the area of illumination (Fig. 1.7). Using an eyepiece telescope or Bertrand lens, examine the rear aperture of the objective and its conjugate planes, the front aperture of the condenser, and the lamp filament. Be sure the lamp filament is centered, using the adjustment screws on the lamp housing if necessary, and

Figure 1.7
Examining the area of illumination at the condenser front aperture.

confirm that the lamp filament is focused in the plane of the condenser diaphragm. This correction is made by adjusting the focus dial of the collector lens on the lamp housing. Once these adjustments are made, it is usually not necessary to repeat the inspection every time the microscope is used. Instructions for centering the lamp filament or arc are given in Chapter 3. Lamp alignment should be rechecked after the other steps have been completed.

- *Focus the specimen.* Bring a low power objective to within 1 mm of the specimen, and looking in the microscope, carefully focus the specimen using the microscope's coarse and fine focus dials. It is helpful to position the specimen with the stage controls so that a region of high contrast is centered on the optical axis before attempting to focus. It is also useful to use a low magnification "dry" objective (10–25×, used without immersion oil) first, since the *working distance*, the distance between the coverslip and the objective, is 2–5 mm for a low power lens. This reduces the risk of plunging the objective into the specimen slide and causing damage. Since the lenses on most microscopes are *parfocal* (see Chapter 4), higher magnification objectives will already be in focus or close to focus when rotated into position.
- *Focus and center the condenser.* With the specimen in focus, close down (stop down) the *field diaphragm* and then, while examining the specimen through the eyepieces, focus the angular outline of the diaphragm's periphery using the condenser's focusing knob (Fig. 1.8). If there is no light, turn up the power supply and bring the condenser closer to the microscope slide. If light is seen but seems to be far off axis, switch to a low power lens and move the condenser positioning knobs slowly to bring the center of the illumination into the center of the field of view. Focus the image of the field diaphragm and center it using the condenser's two centration adjustment screws (Fig. 1.9). The field diaphragm is then opened just enough to accommodate the object or the field of a given detector. This helps

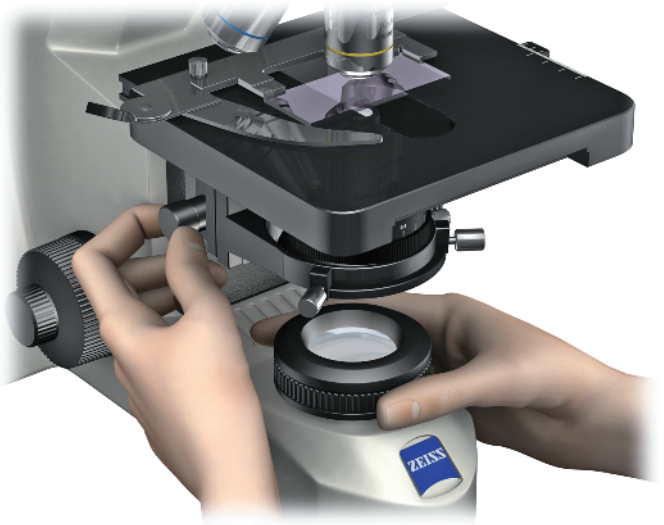


Figure 1.8
Adjusting the field diaphragm opening size and focusing the condenser.

Figure 1.9
Adjusting the condenser centering knobs during alignment of the microscope for Koehler illumination.

reduce scattered or stray light and improves image contrast. The condenser is now properly adjusted. We are nearly there! The conjugate focal planes that define Koehler illumination are now at their proper locations in the microscope.

- *Adjust the condenser diaphragm while viewing the objective rear aperture with an eyepiece telescope or Bertrand lens.* Finally, the condenser diaphragm is adjusted to obtain the best resolution and contrast, but is not closed so far as to degrade the resolution. In viewing the condenser front aperture using a telescope, the small bright disc of light seen in the telescope represents the objective's rear aperture plus the superimposed image of the condenser's front aperture diaphragm. As you close down the condenser diaphragm, you will see its edges enter the aperture opening and limit the objective aperture's diameter. Focus the telescope so the edges of the diaphragm are seen clearly. Stop when ~3/4 of the maximum diameter of the aperture remains illuminated and use this setting as a starting position for subsequent examination of the specimen (Fig. 1.10). As pointed out in Chapter 6, the setting of this aperture is crucial, because it determines the resolution of the microscope, affects the contrast of the image, and establishes the depth of field. It is usually impossible to optimize for resolution and contrast at the same time, so the 3/4 open position indicated here is a good starting position. The final setting depends on the inherent contrast of the specimen.
- *Adjust the lamp brightness.* Image brightness is controlled by regulating the lamp voltage, or if the voltage is nonadjustable, by placing neutral density filters in the light path near the illuminator in specially designed filter holders. *The aperture diaphragms should never be closed down as a way to reduce light intensity,* because this action reduces the resolving power and may blur fine details in the image. We will return to this point in Chapter 6.

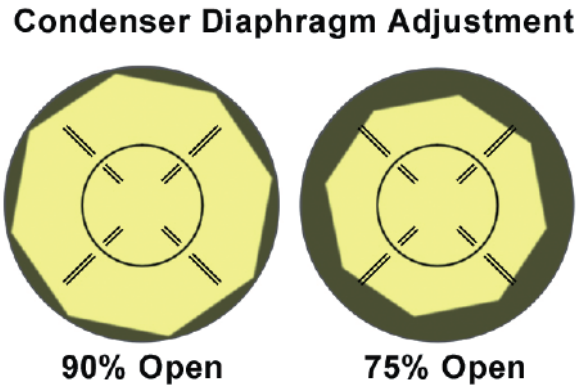


Figure 1.10
Adjusting the condenser diaphragm opening size to maximize contrast.

The procedure for adjusting the microscope for Koehler illumination seems invariably to stymie most newcomers. With so many planes and devices to think about, this is perhaps to be expected. To get you on your way, try to remember this simple two step guide: *Focus on a specimen and then focus and center the condenser*. Post this reminder near your microscope. If you do nothing else beyond this, you will have properly adjusted the image and aperture planes of the microscope, and the rest will come quickly enough after practicing the procedure a few times. Although the adjustments sound complex, they are simple to perform, and their significance for optical performance cannot be overstated. The advantages of Koehler illumination for a number of optical contrasting techniques will be revealed in the next several chapters.

Note: Focusing Oil Immersion Objectives

The *working distance*, the distance between the front lens element and the first surface of the coverslip of an oil immersion lens is so small (~60 μm for some oil immersion lenses) that the two optical surfaces nearly touch each other when the specimen is in focus. Since the focal plane at the specimen (the depth of field) is also very thin (0.1 μm for a 100×, 1.4 NA objective), focusing on a thin, transparent specimen can be a real challenge. Due to such close tolerances, it is unavoidable that the lens and coverslip will occasionally make contact, but this is usually of little consequence. The outermost lens elements are mounted in a spring-loaded cap, so that the lens can be compressed a bit by the specimen slide without damaging the optics. The lens surface is also recessed and not coplanar with the surface of the metal lens cap, which prevents accidental scratching and abrasion.

Begin focusing by bringing the lens in contact with the drop of oil on the coverslip. The drop of oil will expand as the lens is brought towards focus, and at contact (essentially the desired focus position), the oil drop stops expanding. If overfocused, the microscope slide is pushed up off the stage by a small amount on an inverted microscope; on an upright microscope the spring-loaded element of the objective compresses a bit. Retract the lens to the true focal position and then examine the

specimen. In normal viewing mode, it should only be necessary to change the focus by a very small amount to find the specimen. It can help to move the specimen stage controls with the other hand to identify the shadows or fluorescence of a conspicuous object, which may serve as a guide for final focus adjustment. Notice there is a risk, in cases where focus movements are too extreme, that the objective (of an upright microscope) or the condenser (on an inverted microscope) might break the microscope slide, or worse, induce permanent strain in the optics. Focusing with oil immersion optics always requires extra care and patience.

Before observing the specimen, examine the rear focal plane of the objective with an eyepiece telescope to check for lint and oil bubbles. Small bubbles and lint produce bright foci of scattered light whose identity can be confirmed by focusing the telescope. An insufficient amount of oil between the lens and coverslip can cause the entire rear aperture to be misshapen; if this is the case, focusing the telescope will bring the edge of the oil drop into sharp focus. These faults should be removed or corrected, as they will significantly degrade optical performance.

FIXED TUBE LENGTH VERSUS INFINITY OPTICAL SYSTEMS

Until the late 1980s, most microscopes had a fixed tube length with a specified distance between the nosepiece opening, where the objective is attached, and the eyepiece seat in the observation tubes. This distance is known as the *mechanical tube length* of the microscope. The design assumes that when the specimen is placed in focus, it is a few micrometers further away than the front focal plane of the objective (Fig. 1.11a). Finite tube lengths were standardized at 160 mm during the nineteenth century by the Royal Microscopical Society (RMS), and were in use for over 100 years. Objectives designed to be used with a microscope having the industry standard tube length of 160 mm are inscribed with "160" on the barrel.

Adding optical accessories into the light path (between the microscope frame and observation tube head) of a fixed tube length microscope increases the effective tube length to a value greater than 160 mm. Therefore, inserting auxiliary components, such as a reflected light or fluorescence illuminator, polarizers, filters, and DIC prisms, can introduce spherical aberration and "ghost images" into an otherwise perfectly corrected optical system. During the period when most microscopes had fixed tube lengths, manufacturers were forced to place additional optical elements into these accessories to reestablish the effective 160-mm tube length of the microscope. The optical cost was an increase in magnification and reduced light intensity in resulting images. To circumvent these artifacts, the German microscope manufacturer Reichert pioneered the concept of *infinity optics*. The company started experimenting with infinity-corrected optical systems as early as the 1930s, but this concept did not become standard equipment for most manufacturers until 50 years later.

Infinity optical systems have a different objective design that produces a flux of parallel light wavefronts imaged at infinity, which are then brought into focus at the intermediate image plane by a special optic termed a *telan* or *tube* lens. The region between the objective rear aperture and the tube lens is called *infinity space*, where auxiliary components can be introduced into the light path without producing focus artifacts or optical aberrations (Fig. 1.11b). Correction for optical aberration in infinity

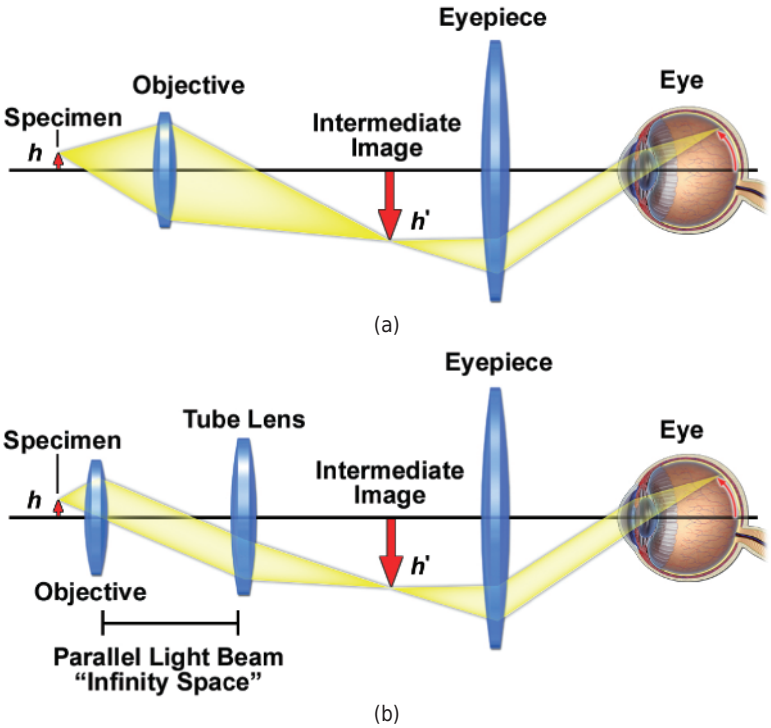


Figure 1.11

Finite and infinity corrected microscope optical configuration. (a) Finite microscope optical train showing focused light rays from the objective at the intermediate image plane. (b) Infinity-corrected microscope with a parallel light beam between the objective and tube lens. This is the region of the optical train that is designed for auxiliary components, such as DIC prisms, polarizers, and filters.

microscopes is accomplished by modifying either the tube lens or the objective, or both. Infinity microscopes can maintain parfocality between objectives even when auxiliary components are introduced, and these components are designed to produce exactly 1× magnification to enable comparison of specimens using a combination of several optical techniques, such as phase contrast and DIC with fluorescence. This is possible because optical accessories (such as DIC prisms) placed in the infinity space do not shift the location or focal point of the image. A note of caution: objectives designed for older 160-mm fixed tube length microscopes are not interchangeable with newer infinity-corrected microscopes.

PRECAUTIONS FOR HANDLING OPTICAL EQUIPMENT

- *Never strain, twist, or drop objectives or other optical components*. Optics for polarization microscopy are especially susceptible to failure due to mishandling.
- *Never force the focus controls* of the objective or condenser, and always watch lens surfaces as they approach the specimen. This is especially important for high power, oil-immersion lenses.

- *Never touch optical surfaces*. In some cases, just touching an optical surface can remove unprotected coatings and ruin filters costing hundreds of dollars. Carefully follow the procedures for cleaning lenses and optical devices.

CARE AND MAINTENANCE OF THE MICROSCOPE

Microscopes are sophisticated instruments that require periodic maintenance and cleaning to guarantee satisfactory performance. When neglected by continuous exposure to dust, lint, pollen, dirt, and failure to remove immersion oil after use, the optical performance can deteriorate to the point that images are negatively affected. Likewise, regular maintenance of the microscope's mechanical and electrical components is equally important to prevent a gradual degradation in the operation of the focusing rack, stage translation mechanism, adjustable diaphragms, filter sliders, and auxiliary electronics. Dust covers are usually provided with microscopes when they are purchased and should be installed whenever the instrument is not in use to prevent contamination from airborne particles drifting through the laboratory. However, even when the microscope is routinely covered during periods of inactivity, those instruments that are used on a daily basis are still likely to experience a slow buildup of contaminants over time.

Particulates, such as dust, lint, fibers, and general debris (collectively referred to as dirt), can seriously affect the quality of an image if they land on a glass surface in a plane near the specimen or the camera sensor. Critical areas to examine for dirt contamination are the objective front lens element, the surface of the camera sensor (and its protective glass cover), both surfaces of the cover slip, the surface of the microscope slide, camera adapter optical surfaces, the upper lens of the condenser, the eyepiece lenses, both surfaces of the reticule, and other glass surfaces in the light path, including lamps, filters, beamsplitters, collector lenses, and heat filters. Cleaning of the microscope optical components is discussed in Chapter 4. Problems with the focusing rack or mechanical stage controls should be left for qualified microscope technicians. External painted surfaces on most microscopes are extremely durable. However, they can be cleaned when needed using a lightly moistened microfiber cloth. Remove loose dust and dirt using a soft hairbrush or ear syringe (available in drugstores). Avoid using compressed air as the propellant can leave unwanted deposits on painted and glass surfaces.

Exercise: Calibration of Magnification

Examine a histological specimen to practice proper focusing of the condenser and setting of the field stop and condenser diaphragms. A 10-μm thick section of pancreas or other tissue stained with hematoxylin and eosin (H&E) is ideal. A typical histological specimen is a section of a tissue or organ that has been chemically fixed, embedded in epoxy resin or paraffin, sectioned, and stained with dyes specific for nucleic acids, proteins, carbohydrates, and so on. In H&E staining, hematoxylin stains the nucleus and cytoplasmic RNA a dark blue or purple color, while eosin stains proteins (and the pancreatic secretory granules) a bright orange-pink. When the specimen is illuminated with monochromatic light, the contrast perceived by the eye is largely due to these stains. For this

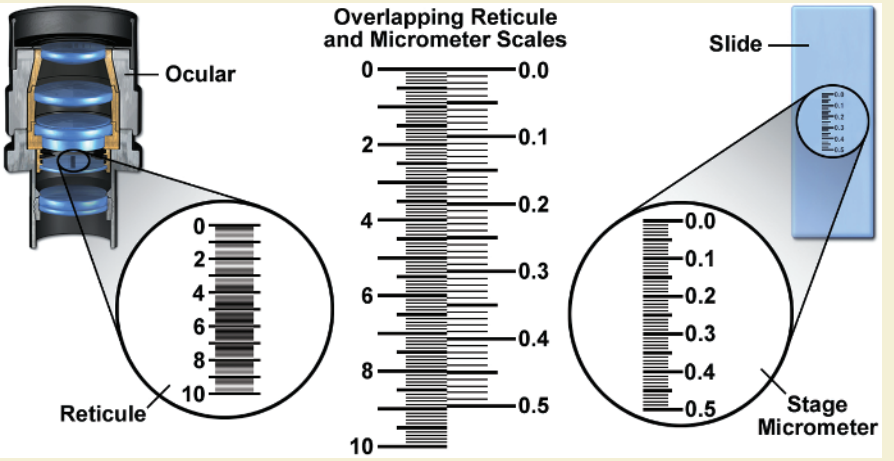


Figure 1.12
The eyepiece reticule and stage micrometer used for determining magnification. The typical eyepiece reticule is divided into 1/100 cm (100 μm unit) divisions, and the stage micrometer into 1/100 mm (10 μm unit) divisions. The appearance of the two overlapping scales is shown in the middle of the figure.

reason, a stained histological specimen is called an *amplitude specimen*, and is suitable for examination under the microscope using "brightfield" optics. A suitable magnification is 10–40×.

Equipment and procedure

Three items are required: a focusable eyepiece, an eyepiece reticule, and a stage micrometer (Fig. 1.12). The eyepiece reticule is a round glass disk usually containing a 10-mm scale divided into 0.1 mm (100 μm) units. The reticule is mounted in an eyepiece and is then calibrated using a stage micrometer to obtain a conversion factor (μm/reticule unit) that is used to determine the magnification obtained for each objective. The reason for using this calibration procedure is that the nominal magnification of an objective (found engraved on the lens barrel) is only correct to within ±5%. If precision is not of great concern, however, an approximate magnification can be obtained using the eyepiece reticule alone. In this case, simply measure the number of micrometers from the eyepiece reticule and divide by the nominal magnification of the objective. For a specimen covering 2 reticule units (200 μm), for example: 200 μm/10× Mag = 20 μm length.

The full procedure, using the stage micrometer, is performed as follows:

- To mount the eyepiece reticule, unscrew the lower barrel of the focusing eyepiece and place the reticule on the stop ring with the scale facing upwards. The stop-ring marks the position of the real intermediate image plane. Make sure the reticule size matches the internal diameter of the eyepiece and rests on the field stop. Reticules can be obtained from the microscope vendor or companies, such as Edmund Scientific Company or Klarmann Rulings.

Carefully reassemble the eyepiece and return it to the binocular head. Next, focus the reticule scale using the focus dial on the eyepiece and then focus on a specimen with the microscope focus dial. The images of the specimen and reticule are conjugate and should be simultaneously in sharp focus.

- Examine the stage micrometer slide, rotating the eyepiece so that the micrometer and reticule scales are lined up and partly overlapping. The stage micrometer consists of a 1- or 2-mm scale divided into 10-μm units, giving 100 units/mm. The micrometer slide is usually marked 1/100 mm. The conversion factor we need to determine is simply the number of μm/reticule units. This conversion factor can be calculated more accurately by counting the number of micrometers contained in several reticule units in the eyepiece. The procedure must be repeated for each objective, but only needs to be performed one time.
- Returning to the specimen slide, the number of eyepiece reticule units spanning the diameter of a structure is determined and multiplied by the conversion factor to obtain the distance in micrometers.

Exercise

1. Calibrate the magnification of the objective/eyepiece system using the stage micrometer and an eyepiece reticule. First determine how many micrometers are in each reticule unit.
2. Determine the mean diameter and standard deviation of a typical cell, a nucleus, and a cell organelle, where the sample size, n, is 10. Examination of cell organelles requires a magnification of 40–100×.
3. Why is it wrong to adjust the brightness of the image using either of the two diaphragms? How else (in fact, how should you) adjust the light intensity and produce an image of suitable brightness for viewing or photography?