

Photomicroscopy

Photomicroscopy employs a variety of optical microscopes, some of which look through sectioned material (upright and compound light microscopes), and some of which allow views of the surface of samples (dissecting, or operating microscopes) or image light emitted from the specimen (epifluorescence or confocal laser scanning microscopes). Illumination of the sample may utilize focused light from an incandescent bulb or diffuse light from a fluorescent bulb. Epifluorescence microscopes employ arc lamps to produce high luminance levels and short-wavelength or ultra-violet light. Confocal microscopes generally use laser-derived illumination sources. Compound microscopes can be set up for standard bright-field viewing, dark-field viewing, phase-contrast microscopy, differential interference contrast (DIC) microscopy, epifluorescence, and polarization microscopy. Each type of microscope requires a variety of alignments and adjustments. Recording an image with modern photomicroscopes requires little more than focusing and pushing the shutter button, but producing an excellent photographic image requires considerable effort, whether using film or digital media for image storage. Producing a high-quality image is dependent on choosing proper optics, proper films, proper filtration or light temperatures (for color films), and making sure that Köhler illumination has been set up for the highest resolution and uniformity of illumination. This chapter provides an introduction to the various choices available for optics, lighting, and films and shows how to adjust a microscope to produce the best images. A large number of books and chapters in books have been written about light microscopy and photomicroscopy, but the texts by Bradbury (1991), Delly (1988), Rawlins (1992), Slayter (1976), and Slayter and Slayter (1992) provide a broad base of information and have served as the fundamental sources for this chapter.

I. LIGHT-MICROSCOPE OBJECTIVE LENSES

Objectives both magnify and focus the image of the specimen. They are described on the basis of their general optical design (bright-field, phase-contrast, DIC), numerical aperture, working distance, and degree of correction for spherical aberration and chromatic aberration (achromat, semiapochromat, apochromat). They may bring the specimen image to focus at a specific plane 160 mm from the nosepiece hole into which they are screwed, shown by the number 160 inscribed on the barrel of the objective, or they may contain a lens in between the objective and the ocular (a tube lens) that allows the image to be focused at any distance past the tube lens without the need for supplementary lenses, as found in older photomicroscopes. Some lenses are designed to be immersed in oil or water (immersion lenses), some need to have coverglasses over the sectioned sample, while others require no coverglasses. Finally, the best lenses for photomicroscopy are designed to give flat-field views from edge to edge and are usually designated as *plan* lenses if they have this feature. Each manufacturer has slightly different designations for the different classes of lenses they produce, so it is necessary to talk with their sales representatives to determine which group of lenses offers the features desired.

A. Numerical Aperture

The numerical aperture (NA) of a lens is a measure of its light-gathering ability and its capacity to capture image-forming rays of light that have been highly diffracted by the specimen. The latter ability makes high-resolution microscopy possible. The NA of a lens is described by the formula:

$$\text{NA} = n \sin \alpha$$

Thus, the NA of a lens is the product of the refractive index (Table 27) of the medium (n) and the sine of one half the angular aperture of the lens (α). The *resolving power* of a lens is described by the formula:

$$R = \frac{0.61\lambda}{\text{NA}}$$

With a given wavelength of an illumination source (λ), a higher NA will provide a higher resolution. Resolution is also dependent on the wavelength of the illumination source. An oil-immersion lens with an NA of 1.4, utilizing green light for illumination ($\lambda = 546 \text{ nm}$) will yield a theoretical resolution of 237.9 nm, or 0.24 μm :

$$\frac{0.61 \times 546 \text{ nm}}{1.4} = 237.9 \text{ nm}$$

Using the same lens with blue light provides a theoretical resolution of 190 nm, or 0.19 μm , indicating that shorter wavelengths of visible light will yield greater resolution:

$$\frac{0.61 \times 436 \text{ nm}}{1.4} = 189.97 \text{ nm}$$

B. Degree of Optical Correction

Spherical aberration is the inability of a lens to bring the peripheral portion of the specimen image to focus in the same plane as the axial portion of the specimen image. This is caused by greater refraction of the image at the edge of a curved lens than at the center of the lens (Fig. 249).

Chromatic aberration exists when light with different wavelengths is refracted by the curved lens as it passes through the same point on the lens (Fig. 250). Light of longer wavelengths, such as red light, comes to focus after refraction by a lens at a more distant point from the lens

Table 27. Refractive Indices of Various Media (Baldock and Graham, 2000)

Medium	Refractive Index	Maximum NA
Air	1.00	0.95
Water	1.33	1.2
Glycerin	1.44	1.3
Oil	1.52	1.4
Glass	1.52	1.4

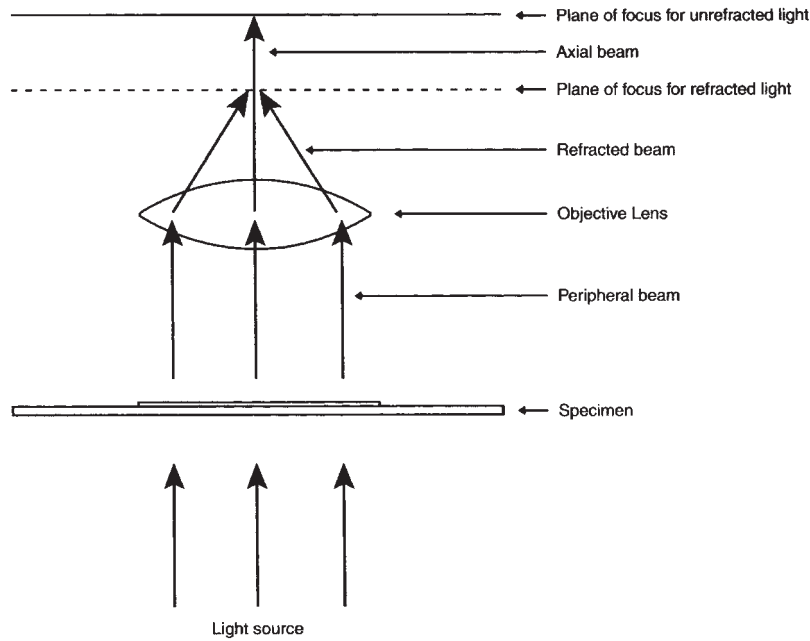


Figure 249. Spherical aberration causing the axial and peripheral portions of the light beam to focus at different planes.

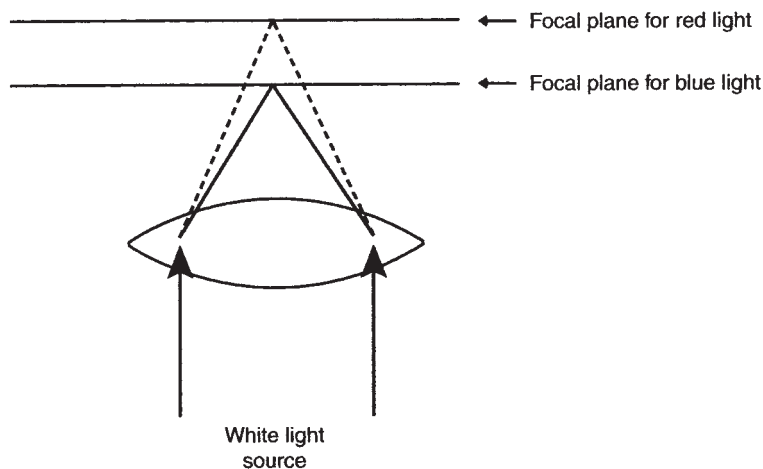


Figure 250. Chromatic aberration, where white light, containing blue and red light, passes through the same point of a curved lens. The blue light, with a shorter wavelength, comes to focus nearer the lens than the red light, which has a longer wavelength.

than blue light passing through the same point in the lens, since blue light has a shorter wavelength and is focused nearer the lens.

Both of these phenomena were addressed in the early 1800s by coupling so-called positive lenses (Fig. 251) and negative lenses (Fig. 252) together to form doublet lenses (Fig. 253) or triplet lenses. Coating lenses with a variety of materials also reduces chromatic aberration.

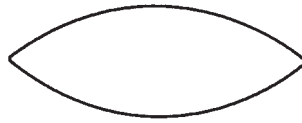


Figure 251. A positive lens.

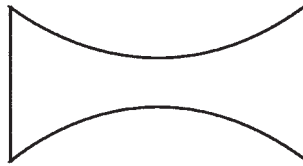


Figure 252. A negative lens.

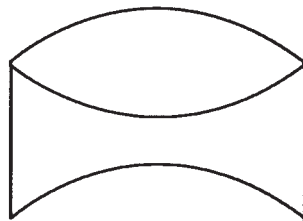


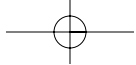
Figure 253. A doublet lens combining a positive and negative lens.

Flat-field or *plan* lenses made before the advent of infinity optics required matched compensating oculars to achieve the edge-to-edge flatness necessary for photomicroscopy. Modern flat-field infinity optics may be used with noncompensating oculars, so they can be easily adapted to various camera systems, without having to resort to specific oculars to project the image into the camera system.

C. Working Distance

Lenses from different microscope manufacturers exhibit different working distances, and lenses of different quality from the same manufacturer will also exhibit different working distances. This can be a critical feature when assembling a microscope, depending on the use to which it will be put. A microscope that will always be used to examine xylene-cleared histological slides with #1.5 coverglasses does not need great working distances. However, microbiologists who periodically examine Petri dish cultures with an upright compound microscope at relatively low magnifications (e.g., with $4\times$ or $10\times$ objectives) benefit from having microscopes with long-working-distance objectives. At the present time, the Nikon line of objectives offers the greatest working distances for each class of objectives in the industry.

Working distance can be a factor in lens selection because it is associated with the quality (and cost) of the different objectives. From a given manufacturer, inexpensive achromat lenses of a given magnification will have the greatest working distance, while apochromats have the least, and semiapochromats (fluorite) lenses are in between.



The greater the magnification of an objective, the shorter the working distance, and the more optical sectioning will take place when examining anything but the thinnest sections. What this means in practical terms is that a 40 \times lens will be capable of focusing on several different planes within a specimen. This can be a problem with epifluorescence microscopy, since out-of-focus specimen fluorescence above and below the plane of section will still be seen, but will not be from the plane of section being viewed and thus will produce extraneous noise.

D. Types of Objectives

As already mentioned, there are three major classes of objectives, with different degrees of correction for chromatic and spherical aberration, differing numerical apertures, working distances, and cost.

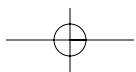
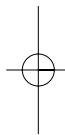
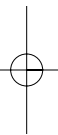
Achromats are the least expensive and have the greatest working distances, in general. This type of lens is typically found on student microscopes and is not recommended for photomicroscopy. They have the highest degree of chromatic and spherical aberration. Chromatic aberration is corrected for red and blue, while spherical aberration is corrected for yellow-green, which is the range of greatest spectral sensitivity for the human eye. Color fringes may appear at the margins with white light, which can decrease the resolution with black-and-white and color recording media. Using a green filter with black-and-white film can improve images, since both chromatic and spherical aberrations are minimized by using monochromatic light.

Semiapochromats or *fluorite* lenses are made from calcium fluorite, rather than glass, and provide a refractive index not possible with glass. They are generally two to three times more expensive than achromats, tend to have a shorter working distance, and are coupled with compensating oculars for the best image quality. They are corrected for two colors for chromatic aberration like achromats but have been corrected for two colors for spherical aberration, unlike the single color characteristic of achromats. A final advantage of this class of lens is that they transmit ultraviolet light more efficiently than glass lenses. If using the fluorescent dye DAPI, which is excited by ultraviolet light, fluorite lenses are recommended (Rawlins, 1992). With dyes such as FITC, which are excited by other wavelengths of light, glass lenses work well.

Apochromats are the most expensive type of objectives, and generally cost five to six times more than achromats. They are used with compensating oculars, and have chromatic correction for red, green, and blue light and spherical correction for red and blue light. Apochromats have the shortest working distance of the three types of objectives, the highest numerical apertures, and provide the least contrast.

Flat-field or *plan* lenses are produced for all three classes of objectives. The addition of this capability raises the price of a given objective considerably due to their added complexity and number of lens elements. For example, a 100 \times achromat lens may contain six glass elements, while a 100 \times planapochromat lens may contain 18 or more elements (Delly, 1988). Plan lenses are necessary for photomicroscopy to ensure edge-to-edge flatness needed for sharp photomicrographs but are not necessary for microscopes used merely for observational purposes.

Even though the maximum resolving capability for glass lenses was reached in the 1880s, manufacturers have continued to improve the contrast, the width of field of view, and the image brightness by improving glass composition, lens coatings, light sources, and glass element manufacturing methods, particularly since the advent of computer-design and manufacturing methods. In recent years, the development of so-called infinity optics has made it easier for manufacturers to produce photomicroscopes with multiple ports to which images can be sent without the



addition of various internal supplementary lenses to refocus the specimen image to the different focal planes represented by the various ports. The addition of various adapters to fit a variety of film and digital cameras has been simplified by the use of these optics. Carefully matched compensating oculars are no longer necessary to preserve the qualities of chromatic and spherical aberration correction, as well as flatness of field, that have been engineered into infinity objectives. As mentioned above, once the image is focused by the objective and projected through the tube lens, the image is focused at any further point in the system. For example, planapochromat and planfluorite (semiapochromat) infinity optic (UIS) objectives currently provided on Olympus microscopes not only offer excellent flatness of field, width of view, and contrast but are corrected for longitudinal chromatic aberration for four wavelengths (violet, blue, yellow–orange, and red) as explained by Abramowitz (1994). These lenses are also corrected for four wavelengths for spherical aberration. Achromat lenses in the UIS line are corrected for three wavelengths for chromatic and spherical aberration.

1. Objectives with focusing collars: When examining thick samples such as bone sections or gelatin-mounted immunostained preparations with standard high-dry objectives (40 \times , 50 \times , or 100 \times), the images frequently appear fuzzy or out of focus. This may also be a problem with coverglasses of unknown thickness. A standard high-dry objective is meant to be used with a flat, 4- to 10- μ m-thick section under a #1.5 coverglass (nominally 0.15–0.18 μ m thick). If these conditions are not met, a sharp image cannot be obtained because the narrow focal length of the lens at this high a magnification range produces a very narrow focal depth. When faced with routine work utilizing slides from various sources, such as received by diagnostic laboratories where section thickness and coverglass thickness cannot be controlled, it is wise to spend the extra money required to buy a high-dry objective with a focusing collar.

2. Objectives with internal diaphragms: Some oil-immersion objectives have adjustable collars that control an internal diaphragm but do not change the focus of the objective. We prefer using the substage condenser diaphragm (explained below) to increase the contrast and depth of field slightly, rather than using the diaphragm on the objective, which we leave wide open at all times.

3. Proper use of oil with oil-immersion lenses: In our experience, the proper use of oil and condenser lenses for high-resolution, high-magnification light microscopy seems to be fairly confusing to many light-microscope users. The first concept that comes into play concerns the purpose of immersion oil.

If a stick is pushed down into a clear pond, the part of the stick below the surface of the water appears to bend away from the straight line defined by the part of the stick above the water. This is a result of the phenomenon of refraction, which results in the image being bent as light passes from a medium of one refractive index (air, at 1.00) into a medium with another refractive index (water, at 1.33).

The glass of slides and coverglasses has a refractive index of 1.52, which is significantly different than the refractive index of air at 1.00. As shown in Table 27, the maximum numerical aperture possible with light passing through air is 0.95, while the maximum numerical aperture of glass is 1.4. The lowest numerical aperture is the limiting step in determining the potential resolution available with an optical system.

The highest resolution achievable with a light microscope (1.4, with an oil immersion lens) is thus available in a system where the light leaving the condenser assembly passes through nothing with an effective numerical aperture less than 1.4. Thus, the numerical aperture of the uppermost element of the condenser lens assembly must be 1.4. In addition, the light leaving the condenser assembly must not pass through air (with a numerical aperture of only 0.95), because

refraction and image degradation would result. Since immersion oil has a refractive index of 1.52, as do the glass slide and coverglass, a numerical aperture of 1.4 can be maintained if oil fills the space between the upper element of a 1.4 NA condenser assembly and the glass slide, and if oil also fills the space between the upper surface of the coverglass and the front of a 1.4 NA objective.

Some lenses primarily designed for epifluorescence work are designated *water-immersion* objectives. These are limited to the numerical aperture of water (1.2) because of the refractive index of water (1.33). To achieve the highest resolution with these lenses, a substage condenser assembly with at least 1.2 NA must be used (typically a 1.4 NA assembly) and both the space between the condenser assembly and slide and the space between the slide and the objective must be filled with drops of water. Again, if the illuminating light or image-containing light passes through air at any point between the upper condenser lens and the lens at the tip of the objective, the effective numerical aperture will be limited to 0.95.

II. LIGHT-MICROSCOPE OCULARS

Oculars, or eyepieces, magnify the image previously magnified and focused by the objective lens, may further correct aberrations in the image caused by the objective lens (compensating oculars), and frequently serve as a place to house reticles for measuring or counting objects being viewed. There are two major types of eyepieces found on light microscopes: *Huygenian* and *Ramsden* oculars.

Huygenian oculars are found most frequently on inexpensive microscopes fitted with achromat objectives. They contain two planoconvex lenses, a field lens closest to the objective, and an eye lens closest to the eye (Fig. 254). The diaphragm that limits the field of view and also is the plane at which a reticle may be placed is between the two lenses, both of which have their curved surfaces facing the objective.

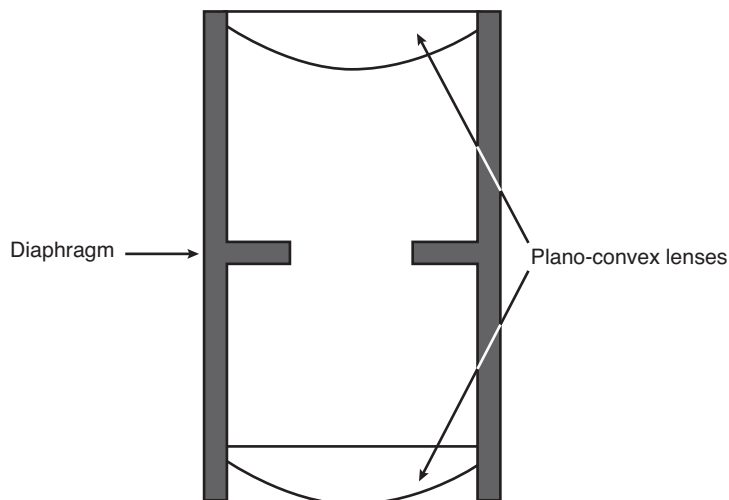


Figure 254. A Huygenian ocular consisting of two plano-convex lenses, each with the curved surface facing the objective. The diaphragm in the center is the point where an ocular micrometer would be placed.

Ramsden oculars are found on microscopes equipped with semiapochromat or apochromat objectives. They contain two sets of lenses like the Huygenian oculars, but the field lens closest to the objective has the curved surface facing away from the objective, while the eye lens has the curved surface pointing away from the eye (Fig. 255). Highly corrected Ramsden oculars have eye lenses constructed as doublet lenses. The limiting diaphragm where a reticle may be installed is located below the field lens.

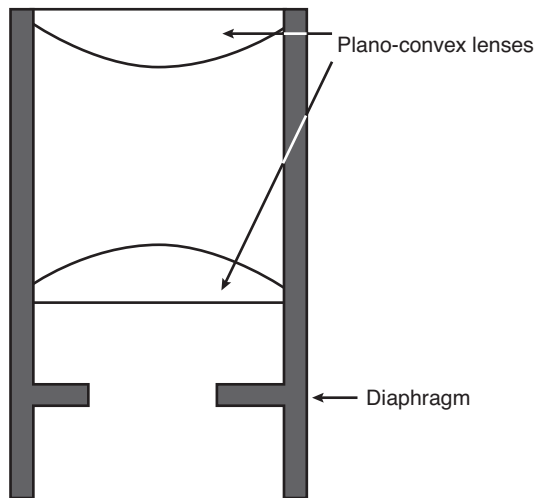


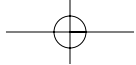
Figure 255. A Ramsden ocular consisting of two plano-convex lenses. The field lens, which is closest to the objective, has its flat side facing the objective. The eye lens, which is farthest from the objective, has its curved side facing the objective. The diaphragm below the field lens is the point where an ocular micrometer would be placed.

Some oculars of both types are designed as *high-eyepoint* eyepieces, suitable for users who wear their glasses while using microscopes. Oculars designed for wide-field viewing are ideal for surveying large areas quickly and are useful for laboratories doing diagnostic light microscopy, such as medical laboratories screening blood smears.

There are several reasons why it is useful to identify the type of ocular found on a given microscope. Highly corrected Ramsden oculars have compensating functions, as mentioned above, and are often carefully matched by the manufacturer to a type of objective in their line of microscope lenses. It is rare that using a compensating ocular from one manufacturer with the objectives from another manufacturer will produce good results. Properly placing reticles so that they can be focused sharply in the field of view necessitates placing them in the proper position, which may be in the middle of the ocular (Huygenian), or below the field lens (Ramsden). The Ramsden type of ocular produces sharper reticle images because the focal plane of the eyepiece lies below the field lens (Bradbury, 1991). A compensating eyepiece can be identified by looking through it toward a white light source. A red–orange color will be noted at the edge of the ocular field of view. A noncompensating (simple) ocular will show a blue ring around the edge of the field of view.

III. LIGHT-MICROSCOPE CONDENSER ASSEMBLIES

A typical substage condenser assembly contains the condenser lens, a substage condenser diaphragm (aperture assembly) and either an additional lens below the condenser that can be



brought into the light path to spread the beam of light for low-magnification objectives, or a mechanism for swinging the uppermost element of the condenser lens assembly out of the illumination field for the same purpose. The condenser assembly also has some means to move it up and down in relationship to the sample, for purposes of focusing the light beam at the plane of the specimen to produce the most even lighting and best resolution, as described by August Köhler in 1893. Finally, the condenser assembly may contain annular rings for dark-field or phase-contrast microscopy.

Condenser assemblies are designed for different purposes and must be matched with appropriate objectives to achieve the highest image quality. Abbe condensers are the simplest and least expensive. They typically contain two or three glass elements and are uncorrected for spherical or chromatic aberration. Aplanatic condensers are corrected for spherical aberration, and aplanatic-achromatic condensers are corrected for both spherical and chromatic aberration.

Dark-field condensers are designed to deliver only the light scattered by the specimen to the objective. The substage condenser diaphragm must be opened to its maximum diameter, and the numerical aperture of the condenser assembly must be greater than that of the objective being used to avoid direct light entering the image. The upper element of the dark-field condenser must have oil between it and the bottom of the glass slide.

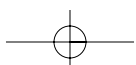
The substage condenser diaphragm can be used to increase depth of field and specimen contrast, at some cost to resolution. At low magnification, where resolution is usually not an issue, the substage condenser can be closed down so that it intrudes approximately one quarter to one third into the field of view. This is monitored by removing an ocular and looking down the ocular tube. If the diaphragm is closed down too far, the image will exhibit unacceptable graininess due to diffraction, causing each point in the specimen to become artificially enlarged. The substage condenser diaphragm also can be used to increase contrast at higher magnifications ($40\times$ to $100\times$), but there will be a decrease in ultimate resolution due to the numerical aperture being reduced by the intrusion of the aperture edges into the cone of light from the condenser assembly.

IV. SLIDE THICKNESS

Glass slides may be purchased from a variety of sources, in a variety of configurations. Slides may have chamfered edges to decrease the risk of cuts from handling them, and they may have frosted ends to make them easy to label. Another often-overlooked variable is the thickness of a slide. Except for special purposes, glass slides should have a nominal thickness of 1 mm (0.96–1.06 mm thick). Because condensers of high numerical aperture suitable for high-resolution microscopy with high numerical aperture objectives have short working distances, it may be impossible to focus the light source on the image plane as described by Köhler with a slide over 1 mm thick (Delly, 1988).

V. LIGHT SOURCES

Older microscopes have either 6 or 12 V incandescent bulbs with a glass globe and a tungsten filament. Both of these suffer from an aging phenomenon, whereby the slowly evaporating tungsten of the heated filament condenses on the relatively colder part of the bulb, the inside of



the glass globe. Over time, the globe becomes increasingly grayer because of tungsten deposition, and the color temperature of the light that started out at $3,200^{\circ}\text{K}$ slowly shifts to increasingly lower temperatures. This results in the light source becoming increasingly redder as the filament ages. Color films such as Kodak Ektachrome T-64 produce proper color rendition when used with a new tungsten bulb adjusted to its maximum rated voltage of either 6 or 12 V. As the bulb ages, images captured on T-64 film will be more yellow–red than those produced with a new bulb. When a Kodak 80 A color-conversion filter, or equivalent, is used to boost the color temperature of the light source to the $5,500^{\circ}\text{K}$ needed for daylight-balanced films such as Kodachrome, daylight Fujichrome, or daylight Ektachrome, excellent color rendition will be possible with a new bulb but will suffer the same color shifts noted above as the bulb ages.

Modern halogen light sources found on newer photomicroscopes avoid the aging phenomenon associated with old tungsten bulbs, and consequent color temperature shifts are not a problem with halogen bulbs. The quartz envelope of a halogen bulb operates at a much higher temperature than does the glass envelope of a standard tungsten bulb. Thus, the evaporating tungsten does not readily coat the inside of the bulb envelope, essentially eliminating color changes as the bulb ages. A halogen bulb produces $3,200^{\circ}\text{K}$ when first installed, and continues to do so until the filament blows, requiring bulb replacement. *A halogen bulb should not be touched with bare hands.* The oil from fingertips will adhere to the surface of the bulb, and the high operating temperature of the bulb will cause the residual oil to degrade the surface of the bulb, resulting in premature bulb failure. If the bulb is touched with bare fingers, carefully remove the fingerprint oils from the surface with an alcohol-soaked tissue and let the bulb dry thoroughly before installation.

Fluorescent microscopes typically use mercury arc lamps that have a recommended bulb life of several hundred hours. The power supplies for these bulbs have elapsed time counters to help guide the user to change the bulb prior to the recommended time limit for the bulb, since a shattered bulb would disperse vaporized mercury into the room housing the microscope, creating a health hazard.

Finally, laser light sources of several types are coupled to confocal scanning microscopes. They produce an intense monochromatic light, which is necessary to produce sufficient signal when collecting images point by point from specimens. True color cannot be recorded by these microscopes, but false colorization of the computer-derived images is often used to produce color images.

VI. TYPES OF OPTICAL SYSTEMS

Compound light microscopes used to look through specimens are available as upright microscopes traditionally used to examine histological slides and as inverted microscopes designed to look through the bottom of culture dishes and flasks. Bright-field microscopes that can perform dark-field analyses with a simple condenser change are the most commonly encountered light microscopes. Phase-contrast and DIC microscopes are utilized extensively by microbiologists and other investigators looking at unstained materials with little inherent contrast. Most microscopes can be easily adapted to polarization sufficient to demonstrate oriented fibers, crystals, or other anisotropic substances within samples by the addition of a polarizing filter in the light path preceding the specimen, and another polarizing filter located above the objectives. Epifluorescence microscopy capabilities can be coupled to most bright-field microscopes with the addition of an arc lamp assembly with a dedicated power supply, a light tube to direct light through the light microscope objectives, and a set of filters to narrow the bandwidth of the light needed to

Photomicroscopy

excite various naturally fluorescent materials in specimens or fluorochromes incorporated into the samples. Finally, confocal scanning light microscopes that scan a beam of light across a sample can be used to collect images from a very narrow plane of section within a specimen and achieve levels of resolution with fluorescence microscopy techniques that are not possible with standard epifluorescence microscopes.

A. Bright-Field Microscopes

These microscopes are the most widely used in biological light microscopy and can be extremely inexpensive instruments if they are used only to view specimens, without any intention of recording images. An inexpensive basic bright-field microscope used to observe, but not record images of, samples can be purchased for approximately \$2,000. However, a bright-field photomicroscope with a motorized condenser assembly and nosepiece, along with autofocus and multiple output ports, can cost upwards of \$75,000. The difference in cost between these two extremes represents increasing complexity in mechanical design and increased complexity in the optical components such as objectives, condensers and oculars, and filtration systems necessary to produce high-contrast, high-resolution images that are easily recorded through automated exposure and focusing systems standard with the high-end microscopes. In addition, the more expensive microscope stands are easily upgraded with the addition of a variety of different lighting systems, optical systems, and recording devices.

For photomicroscopy, a basic bright-field system with an automatic camera, a full set of semiapochromat plan lenses (4 \times , 10 \times , 20 \times , 40 \times and 100 \times), and an appropriate condenser assembly can be purchased for about \$10,000–\$15,000. The resolving power of such a system would be slightly lower than that available if equipped with plan apochromats. Microbiologists would probably need to add the expense of the plan apochromat objectives and matched substage condenser assembly to reach the level of resolution that they customarily need. One further point to consider is the fact that semiapochromat objectives actually exhibit higher contrast than apochromats. Unless a slightly higher resolution is a critical issue, semiapochromat lenses will probably do a better job than apochromats for most investigators. This represents a case where buying the best product may actually decrease the quality of the photographs produced, in terms of contrast.

Recommended options include a set of built-in neutral density filters that can decrease illumination intensity, primarily for viewing comfort, without changing color values in the specimen. Built-in color-balancing filters to bring the light source to the proper color temperature for daylight films (5,500° K) and tungsten films (3,200° K) are very useful if the light source is properly calibrated for their use. If a contemporary 12 V halogen light source is provided with 12 V (3,200° K), a Kodak 80A filter (blue), or equivalent, introduced into the light path allows the use of daylight color films. Tungsten-based films (T-64) can be used with no filtration. A green filter is recommended for black-and-white film work because it provides a monochromatic light in the middle of the visible spectrum that is in the most sensitive portion of the spectral sensitivity curve for most black-and-white films. In addition, the use of monochromatic green light eliminates any possibility of chromatic aberration and tends to build contrast in a typical hematoxylin- and eosin-stained histological sample because it accentuates its complementary color, red. Red areas (eosin-stained areas) in the specimen are shown in higher contrast to the bluish areas produced by the hematoxylin with the use of a green filter.

B. Dark-Field Microscopy

As mentioned above, dark-field microscopy can be achieved with a conventional bright-field microscope by adding a dark-field condenser assembly. On some microscopes, the substage condenser assembly is provided with a dark-field annulus that can be placed into the light path to allow dark-field work without changing condenser assemblies.

This optical method allows detection of objects such as bacterial flagella ($0.02\ \mu\text{m}$ in diameter) that are below the resolution limits of light optics (Slayter and Slayter, 1992). The dark-field stop occludes the central part of the cone of illumination (Fig. 256), such that the specimen is illuminated by a hollow cone of light. The oblique light rays that emerge from the condenser lens interact with the specimen, and those that are deviated by specimen structures then are collected by the objective lens and produce the dark-field image. Undeviated light rays cannot enter the objective lens and are subtracted from the specimen image. Thus, specimen structures appear as bright areas against a black background. For *dark-field microscopy*, *immersion oil needs to be put between the top lens of the condenser assembly and the bottom of the microscope slide*.

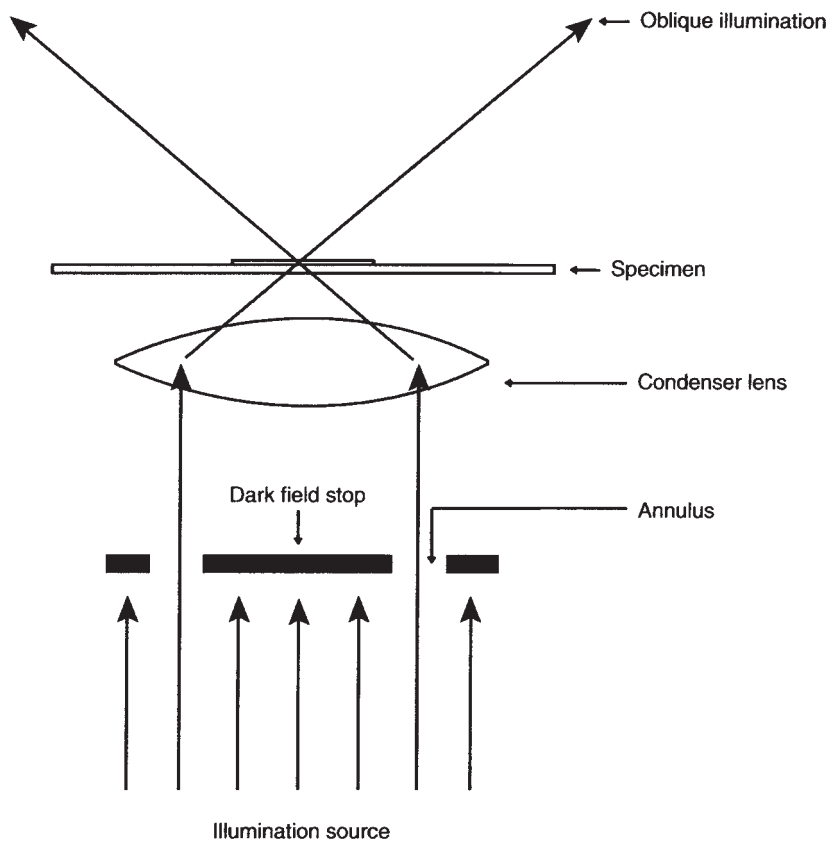


Figure 256. A dark-field condenser. The condenser has a central opaque disk, allowing only oblique rays of light passed by the annulus to interact with the specimen.

C. Phase-Contrast Microscopy

As explained by Delly (1988), phase-contrast microscopy provides contrast to specimens with little inherent contrast by producing interference between light rays. If a ray of light from a single point source is split into two light rays, and both are then passed through the same medium, they can be recombined without interference because the respective peaks and valleys of the waveform will coincide with each other. However, if the two light rays are passed through media of different refractive indices, their relative speeds will change. The difference in speed between the two rays may result in their being out of phase when recombined. When the phase differences are less than one wavelength, a decrease in illumination intensity is observed. If one light ray is exactly one half wavelength out of phase with the other, the peak and valleys of the two wave forms will cancel each other out, causing extinction of the illumination.

A set of annular apertures are located in the condenser assembly. These annular apertures must be matched to phase-contrast objectives containing complementary phase plates (Fig. 257). The phase plate in an objective has an annular groove that is visually superimposed over the annular aperture in the condenser assembly by looking through the microscope with an ocular removed and moving the condenser assembly adjusting screws until the groove and aperture are coincident (Fig. 258). Once that relationship is established, the oblique illumination provided by the annular aperture which is undeviated by the specimen passes through the annular groove, which typically retards the light rays one quarter of a wavelength. At the same time, the light rays deviated by interactions with the specimen will pass through the phase plate in locations other than at the annular groove. Because the rest of the phase plate is thicker and has different coatings on it than the annular groove, the light rays deviated by the specimen will be retarded more than those light rays that remain undeviated by specimen interactions and subsequently pass through the

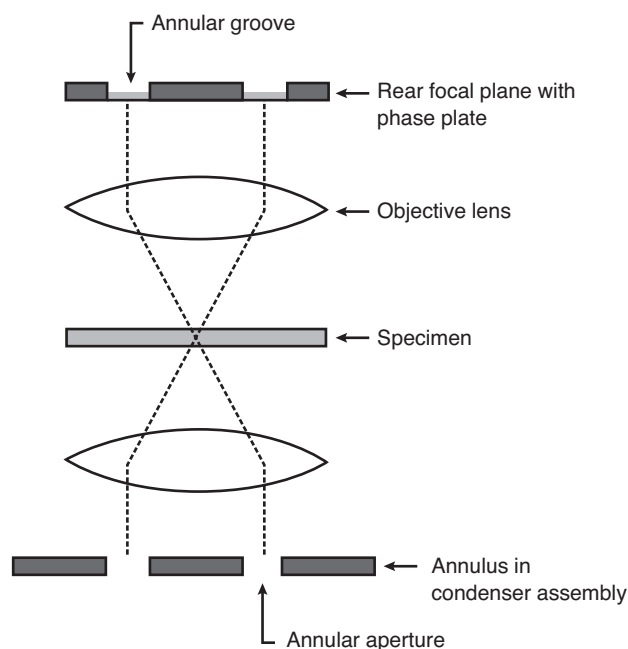


Figure 257. Optical elements in a phase-contrast microscope.

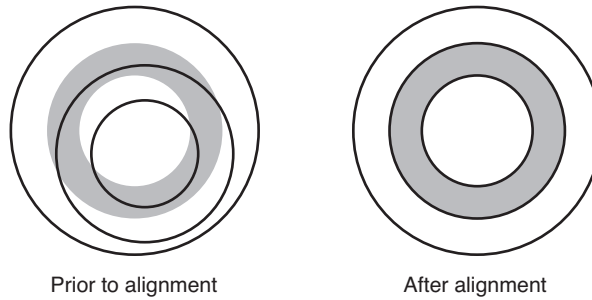


Figure 258. Alignment of the phase plate and the phase annulus with a phase-contrast microscope.

annular groove on the phase plate. Thus, the undeviated light ray speeds up relative to the deviated light rays that pass through the thicker portion of the phase plate after specimen interactions. When the difference between the deviated and undeviated light rays is 180° out of phase, the specimen details will appear dark due to extinction of the illumination, and the enhanced specimen contrast makes the detail of an unstained sample more apparent (see Slayter and Slayter, 1992). Spurious haloes around the specimen details are common (Fig. 259).

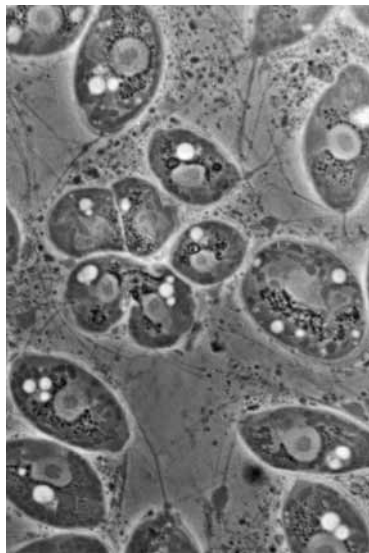


Figure 259. Phase-contrast photograph of the protozoan, *Sorodiplophorys stercorea* taken with a $100\times$ oil objective.

D. DIC Microscopy

As succinctly described by Abramowitz (1987), DIC systems as developed by Nomarski employ a polarizing filter to produce light vibrating only in a plane perpendicular to the direction of the light beam. This light is passed through a modified Wollaston prism that splits the light beam into two rays perpendicular to each other. The two light rays then pass through the condenser

lens and emerge as two parallel beams that are extremely close together but have a slight path difference. The shear, or distance between the two rays, is below the resolving capabilities of the objectives. The split beam then enters the specimen, and the two beams are deviated according to their interactions with specimen features. The two beams of light altered individually by their interactions with the specimen pass into the objective in use and then are recombined with the beam-combining modified Wollaston prism above the objectives. The light beams then pass through another polarizing lens (the analyzer). When the two beams of light with slightly different path lengths are recombined by the modified Wollaston prism and brought into the same plane and axis by the upper polarizing lens, differential interference occurs between the formerly independent beams of light. Differences in light intensity or color seen are dependent on variations in refractive indices and/or specimen thickness. When the upper prism is set to produce the grayest image, the most evident three-dimensional view of the specimen is provided (Fig. 260). Rotating the specimen 180° can turn areas perceived as raised to areas that seem to be depressed.



Figure 260. Nomarski interference (DIC) photograph of the protozoan, *Sorodiplophrys stercorea* taken with a $100\times$ oil objective.

E. Epifluorescence Microscopy

Epifluorescence microscopy utilizes a high-intensity light source to excite primary, or autofluorescent, materials in a specimen and areas containing fluorescent dyes such as fluorescein isothiocyanate (FITC), resulting in secondary fluorescence. Two types of light sources are used: high-pressure mercury vapor arc lamps that produce light in the ultraviolet to short blue wavelength area of the spectrum, and xenon arc lamps that produce light across the visible spectrum as well as ultraviolet light (Abramowitz, 1993). These high-intensity light sources are needed to produce adequate fluorescence for imaging, since the majority of the light impinging on the specimen is lost to the imaging process.

Modern epifluorescence microscopes (Fig. 261) conduct light from the light source through an excitation filter that limits the wavelength of light reaching the specimen (Table 28). The light passed by the excitation filter then passes down through the objective and interacts with the specimen. Those illuminated areas containing fluorochromes or exhibiting autofluorescence will emit fluorescent light in all directions, some of which will be collected by the objective and transmitted through a barrier filter that passes the emitted light, but not the original transmitted, or excitation, light. The filtered emitted light is delivered to the ocular that subsequently projects the image to the eye or a suitable recording device.

There are four fundamental problems for epifluorescence microscopy. First, specimens exhibiting significant autofluorescence can obscure the signal from introduced fluorochromes. Second, fluorescent emissions from the specimen are many orders of magnitude dimmer than the

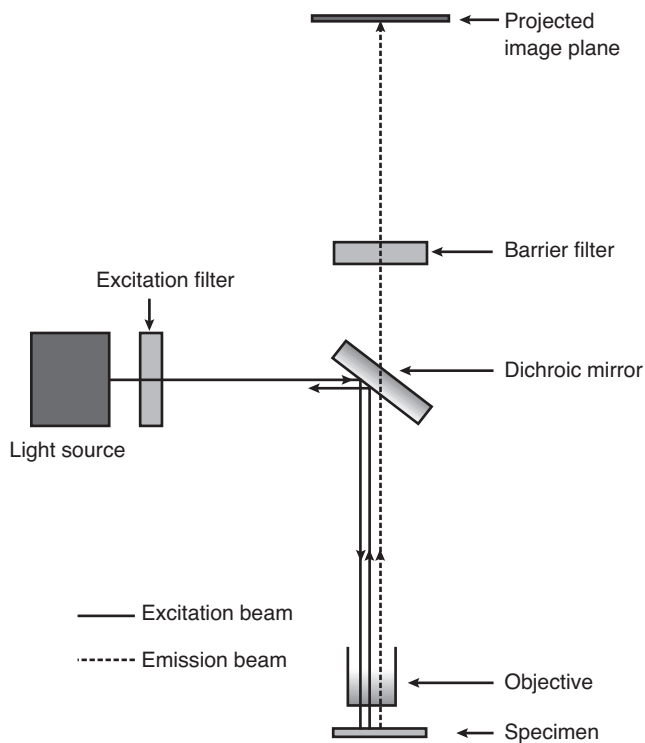


Figure 261. Optical elements of an epifluorescence microscope. (Redrawn from Rawlins, 1992, with permission of BIOS Scientific Publishers Ltd.)

Table 28. Common Excitation Filters and the Wavelengths Passed

Fluorochrome	Excitation (nm)	Emission (nm)	Color Observed
Fluorescein (FITC)	495	525	Green
Hoechst 33258	360	470	Blue
R-Phycoerythrin (PE)	488	578	Orange-red
Rhodamine (TRITC)	552	570	Red
Quantum Red	488	670	Red
Texas Red	596	620	Red
Cy3	552	570	Red

original illumination source, necessitating high-speed films and/or long exposure times for recording images. Modern cooled CCD digital cameras with high SNRs make recording of these low-luminance images easier in most cases. Third, fluorescent emissions in all directions from the source as well as emissions from areas of the specimen not in the plane of focus at higher magnifications can degrade the spatial resolution potential of the optical elements in the microscope. Confocal scanning microscopes have helped alleviate this problem by allowing very narrow planes of focus, eliminating the effects of out-of-focus fluorescence from above and below the imaged portion of the specimen. Fourth, many fluorochromes lose their capacity to fluoresce over time, particularly if illuminated with high levels of light typical of epifluorescent light sources. A variety of anti-quenching agents (Table 29) will lessen, but not eliminate, this phenomenon. In some cases, the rapid quenching of the signal (fluorescence) will make it difficult to record images from weakly emitting specimens because the length of time necessary to record sufficient signal will be hampered by the destruction of the fluorescence capacity of the specimen.

Table 29. Several Common Anti-Quenching Agents Available from Sigma Chemical Co. (St. Louis, MO)

Catalog Number	Name	Instructions for Use	Reference
P 3130	<i>n</i> -Propyl gallate	Used as a 0.1 M solution in 90% glycerol in PBS	
P 6001	<i>p</i> -Phenylenediamine, free base		<i>J. Immunol. Meth.</i> 43: 349–350 (1981)
P 1519	<i>p</i> -Phenylenediamine, dihydrochloride		<i>J. Immunol. Meth.</i> 43: 349–350 (1981)
D 2522	1,4-Diazabicyclo [2,2,2] octane (DABCO)	Added to mounting medium to 2.5% concentration	<i>Antibodies: A Laboratory Manual</i> , Harlow and Lane, Cold Spring Harbor (1988); <i>J. Histochem. Cytochem.</i> 33: 755 (1985)

Most epifluorescent microscopes are equipped with excitation filters capable of passing wavelengths of light (ultraviolet, blue, green) suitable for a variety of individual fluorochromes. Dichroic filters are interference filters that have coatings designed to reflect shorter wavelengths of light (e.g., ultraviolet light at 330–370 nm) used for excitation while transmitting longer wavelengths emitted by the excited fluorochromes, which are ultimately imaged. Most modern microscopes incorporate the exciter filter, dichroic filter, and barrier filter into single cubes for specified groups of fluorochromes.

F. Polarizing Microscopy

Polarizing filters are used to differentiate between isotropic substances with a single refractive index and anisotropic materials that have two or three principal refractive indices. When the azimuths of the polarized light being passed through two polarizing filters are parallel, the maximum amount of light is passed. When the azimuths of polarized light are perpendicular to each other, the polarizers are “crossed.” This is also known as a position of extinction, and no light is passed unless it interacts with an anisotropic substance. Viewing isotropic materials with crossed polarizing filters shows a dark field, while anisotropic materials are revealed as illuminated areas (they exhibit birefringence). Examples of isotropic substances would be water, unoriented polymers, and the cytoplasm of cells. Anisotropic materials would include bone containing

crystallized calcium, asbestos fibers in lung sections, and cellular components consisting of oriented fibers such as mitotic spindles and cellulosic cell walls.

Polarizing microscopes can detect and then measure the amount of birefringence. As described in detail in Slayter and Slayter (1992), polarizing microscopes are used by crystallographers to determine the sign and type of birefringence of materials for analytical purposes. Most biologists examine materials only to detect birefringent areas. The more complex and expensive analyzers and polarizers needed for crystallographic analyses are generally not needed for biological work, and the addition of one fixed polarizing filter (analyzer) above the specimen and a rotatable polarizing filter located below the specimen will usually suffice. Limited budgets have led some labs to buy a pair of cheap plastic polarized sunglasses from which two pieces of the lens material are cut to serve as the analyzer and polarizer, respectively. The former is inserted somewhere above the nosepiece, and the latter is placed on the exit pupil for the light tube on the base of the microscope and rotated in relationship to the stationary analyzer. These less than optically perfect lenses generally would be contraindicated for photographic purposes, but they certainly work well for the diagnostic detection of birefringence in a specimen.

G. Confocal Microscopy

As alluded to above, confocal microscopy has greatly improved fluorescence microscopy because of its ability to image an extremely narrow plane within a specimen, while eliminating any spurious out-of-focus information from above and below the plane of section being viewed (Fig. 262). Numerous books and papers have been published concerning the physics, instrumentation designs, and applications of confocal microscopy. The books by Diaspro (2002), Inoué and Spring (1997), Kriete (1992), Paddock (1999), Pawley (1995), and Shotten (1993) are devoted to confocal microscopy or provide chapters with good treatments of the subject. In particular, the two major types of confocal instruments, laser scanning and Nipkow disk-based tandem scanning reflected light microscopes, are discussed by Inoué (1995) and Kino (1995), respectively.

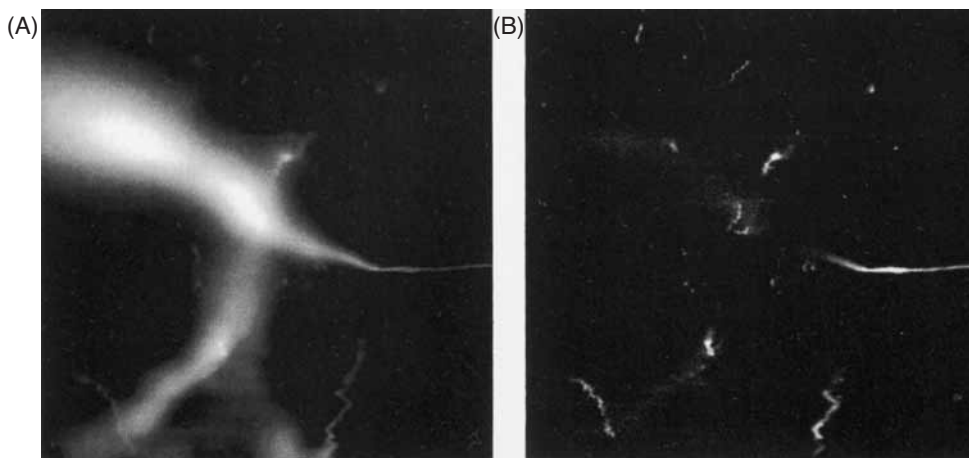


Figure 262. Comparison of an image of a fluorescently labeled neuron taken with an epifluorescence microscope (A) to an image of the same neuron taken with a confocal laser scanning microscope (B). In the epifluorescent image, both in-focus and out-of-focus areas of the specimen contribute to the image and degrade resolution. In the confocal image, only the in-focus areas are imaged, leading to greater resolution. (Image from Wallén *et al.*, 1992, with permission from UCH-Wiley Publishers.)

The original scanning reflected light microscope conceived by Nipkow in 1884 (Inoué, 1995) was based on a Nipkow disk with a series of square holes arranged in single series into an Archimedean spiral toward the center of the disk (Fig. 263). This was modified by Petran *et al.* (Kino, 1995) to produce a path consisting of interleaved Archimedean spirals (Fig. 264). Light is passed through holes in the spinning perforated disk and focused onto the specimen by the objective lens. The light reflected or scattered by the specimen is then focused by the objective lens onto a centro-symmetric portion of the disk (Inoué, 1995). The second set of pinholes exclude light originating from the specimen areas not illuminated by the first set of pinholes.

The real-time scanning optical microscope described by Xiao and Kino in 1987 (Kino, 1995) had a glass disk coated with a layer of specularly reflecting black chrome, which produced a narrow reflected light beam that was effectively eliminated with a light trap (Fig. 265). In addition, the input light was polarized, and then the light reflected by the specimen was passed through an analyzer (another polarizing filter) to further decrease illumination from the light source that was not contributing to image formation. The disk was rotated at 2,000 rpm, which produced up to 700 frames/sec containing 5,000 lines per image, if sufficient light intensity could be delivered. The images exhibited depth of focus characteristics comparable with the best mechanically scanned confocal laser images. Kino (1995) showed images of a rabbit eye examined with a $50\times$ water-immersion lens on his real-time scanning optical microscope that revealed endothelial cells $400\ \mu\text{m}$ below the tear film.

Nipkow disk-based microscopes produce real-time images in true color, but since approximately 99% of the disk is opaque (Kino, 1995), a very strong light source (mercury arc lamp) is needed. In addition, the critical nature of alignment of the disk and the various optical elements make for a mechanically complex instrument that is expensive and difficult to maintain, compared with a confocal laser scanning microscope.

The most significant aspect of the confocal laser scanning microscope is the short depth of focus, which makes it well suited for optical sectioning of specimens. These microscopes produce a high illumination intensity and a better transverse definition and contrast than that possible with a conventional light microscope. The precise optical sectioning produced, along with computer-assisted assembling of the optical sections, allows three-dimensional reconstructions of samples

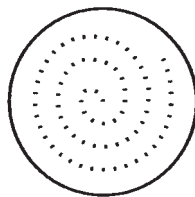


Figure 263. Nipkow disk. Note the single Archimedean spiral of holes.

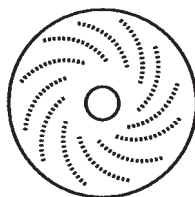


Figure 264. Modified Nipkow disk with interleaved Archimedean spirals of holes. (Redrawn from Kino, 1995 with permission from Kluwer Academic Plenum Publishers.)

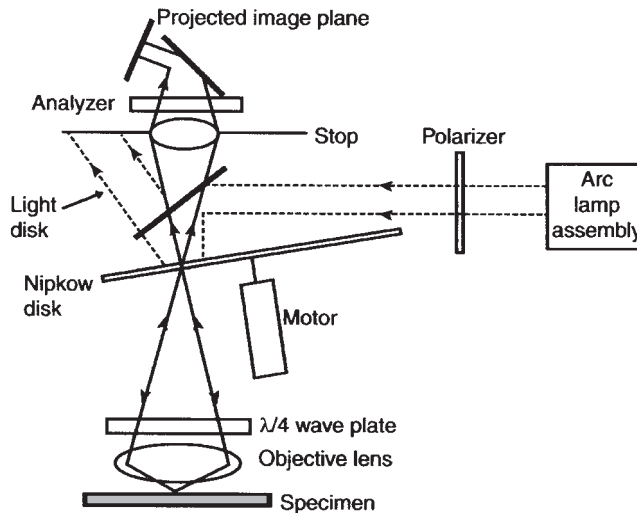


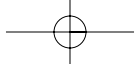
Figure 265. Optical components of a real-time scanning optical microscope. (Redrawn from Kino, 1995 with permission from Kluwer Academic Plenum Publishers.)

with a speed and accuracy previously unattainable. The major disadvantage for this instrument is that the point-by-point acquisition of the image results in a scan time too slow to produce an image allowing real-time viewing. In addition, the typical monochromatic laser light source prevents true color images of a specimen.

Confocal laser scanning microscopes either move the specimen stage relative to the light beam (stage or object scanning) or scan a light beam over the specimen (beam scanning). The latter type of instrument is the most commonly manufactured system on the market today because it can be adapted to virtually any microscope, is less expensive to manufacture than a stage-scanning system, and has fewer mechanical difficulties. With typical systems, the optical pathway is similar to that found in conventional epifluorescent microscopes. The scanned laser light source is delivered to the microscope via a fiber optic cable, and the images are stored as digital files that can be reassembled into three-dimensional images by software supplied by the manufacturer of the confocal laser scanning system.

In a typical confocal laser scanning microscope, a minute spot of laser light is scanned across the sample, with a pinhole placed conjugate to the spot being scanned, which eliminates any light originating from other areas on the specimen or from light scattered from within the optical system. The volume of the sample that is imaged point by point is around $0.02 \mu\text{m}^3$ (Diaspro and Sheppard, 2002). This reduces the impact of out-of-focus light scattering and fluorescence, leading to much crisper images than with standard epifluorescence microscopy (Inoué and Spring, 1997). As explained by Diaspro and Sheppard (2002), one of the disadvantages to standard confocal laser scanning microscopy is that the whole section thickness within an hourglass-shaped region is affected by each scan, even if no image is collected from the out-of-focus regions. In practical terms, this means that three-dimensional imaging may be compromised because of photo bleaching of the fluorescent label in the sample within this hourglass-shaped region.

A variety of different laser light sources are currently available, and their characteristics and applications are thoroughly discussed by Gratton and vandeVen (1995). As they explain, there are two major classes of lasers, continuous-wave and pulsed, with a number of subcategories



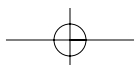
in each. These different types of lasers vary widely in cost, power, stability, maintenance requirements, and breadth of wavelength produced (Table 30). Consideration of all of these factors will help to determine which laser package is most appropriate for an individual laboratory.

Table 30. Wavelengths Produced from Lasers Commonly Used for Confocal Laser Scanning Microscopy (Gratton and vandeVen, 1995)

Wavelength (nm)	Type of Laser
325 or 442	Helium–cadmium laser
351	Water-cooled argon laser with UV option
364	Air-cooled argon-ion laser
457–514.1	Small-frame argon-ion laser
543.5	Green helium–neodymium laser
630	External cavity semiconductor laser
630	Colliding pulse dye laser
632.8	Helium–neodymium laser
700–1,100	Titanium–sapphire laser
1,152	Helium–neodymium laser

REFERENCES

- Abramowitz, M. 1987. *Contrast methods in microscopy. Transmitted light*. Olympus Corporation, Lake Success, NY.
- Abramowitz, M. 1993. *Fluorescence microscopy. The essentials*. Olympus America, Lake Success, NY.
- Abramowitz, M. 1994. *Optics, a primer*. Olympus America, Lake Success, NY.
- Baldock, R., and Graham, J. (eds.). 2000. *Image processing and analysis. A practical approach*. Oxford University Press, New York.
- Bradbury, S. 1991. *An introduction to the optical microscope*, rev. edn. Oxford University Press, Oxford.
- Delly, J.G. 1988. *Photography through the microscope*, 9th edn. Kodak, Rochester, NY.
- Diaspro, A. (ed.). 2002. *Confocal and two-photon microscopy*. Wiley-Liss, New York.
- Diaspro, A., and Sheppard, C.J.R. 2002. Two-photon excitation fluorescence microscopy. In: A. Diaspro (ed.), *Confocal and two-photon microscopy*. Wiley-Liss, New York.
- Gratton, E., and vandeVen, M.J. 1995. Laser sources for confocal microscopy. In: J.B. Pawley (ed.), *Handbook of biological confocal microscopy*, 2nd edn (pp. 69–97). Plenum Press, New York.
- Inoué, S. 1995. Foundations of confocal scanned imaging in light microscopy. In: J.B. Pawley (ed.), *Handbook of biological confocal microscopy*, 2nd edn (pp. 1–17). Plenum Press, New York.
- Inoué, S., and Spring, K.R. 1997. *Video microscopy. The fundamentals*. Plenum Press, New York.
- Kino, G.S. 1995. Intermediate optics in Nipkow disk microscopes. In: J.B. Pawley (ed.), *Handbook of biological confocal microscopy*, 2nd edn (pp. 155–165). Plenum Press, New York.
- Kriete, A. (ed.). 1992. *Visualization in biomedical microscopies. 3-D imaging and computer applications*. VCH, New York.
- Paddock, S.W. (ed.). 1999. *Confocal microscopy. Methods and protocols*. Humana Press, Totowa, NJ.
- Pawley, J.B. (ed.). 1995. *Handbook of biological confocal microscopy*, 2nd edn. Plenum Press, New York.
- Rawlins, D.J. 1992. *Light microscopy*. BIOS Scientific, Oxford.
- Shotten, D. (ed.). 1993. *Electronic light microscopy*. Wiley-Liss, New York.
- Slyater, E.M. 1976. *Optical methods in biology*. Krieger, Huntington, NY.
- Slyater, E.M., and Slyater, H.S. 1992. *Light and electron microscopy*. Cambridge University Press, New York.
- Wallén, P., Carlsson, K., and Mossberg, K. 1992. Confocal laser scanning microscopy as a tool for studying the 3-D morphology of nerve cells. In: A. Kriete (ed.), *Visualization in biomedical microscopies. 3-D imaging and computer applications* (pp. 109–143). VCH, New York.



CHAPTER 18 TECHNIQUES

Köhler Illumination

1. Applications and Objectives

To achieve the highest resolution, maximum illumination, and even illumination required for excellent photomicrographs, it is necessary to focus the light source carefully onto the plane of focus of the specimen. *This procedure must be performed for each objective prior to photomicroscopy on any nonmotorized light microscope.* Motorized microscopes may have different procedures specified by the manufacturer due to the complexity of the condenser assemblies supplied.

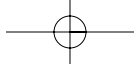
2. Procedure

1. Focus the ocular(s) on the built-in or projected reticle image by turning the ocular exit pupil in reference to the ocular barrel.
2. Choose the objective desired and focus it on the specimen.
3. Close the field diaphragm located somewhere near the light exit port in the microscope base until the edges of the diaphragm blades become visible.
4. Find the knob that raises and lowers the condenser assembly, and adjust it up or down until the edge of the field diaphragm blades are sharp.
5. Center the field diaphragm image with the adjusting screws on the substage condenser assembly.
6. Open the field diaphragm until it is just beyond the field of view. If it is opened any wider, noninformational, widely scattered light from the condenser assembly may be captured by the objective, leading to *flare*, which can decrease image contrast.
7. For low-power objectives, typically $10\times$ or below, it is necessary to swing the front element of the substage condenser assembly out of the light path or to remove the substage condenser assembly completely in the case of some low-magnification objectives. Some inexpensive microscopes may have a supplementary lens below the substage condenser assembly that is moved into the light path to spread the light for full coverage of the field of view. When this is done, it may not be possible to effect proper Köhler illumination.
8. If dust appears at the plane of focus for the specimen following Köhler illumination, either clean the optics in the illumination system or defocus the condenser assembly slightly until the dirt is no longer focused in the same plane as the specimen image.

Use of the Substage Condenser Diaphragm

1. Applications and Objectives

Proper adjustment of the substage condenser diaphragm at low objective magnifications will enhance the depth of field and contrast in the specimen. At higher objective magnifications ($40\times$ to $100\times$), the aperture should be adjusted so that it does not impinge on the cone of light leaving the condenser assembly, since it would decrease the numerical aperture of the optical system, leading to decreased resolution. With most microscopes, decreasing the aperture diameter with objectives below $25\times$ is recommended to increase contrast, since contrast is a more critical issue than resolution at lower magnifications. If the substage condenser is closed down to too small a diameter, diffraction will cause the specimen image to become unacceptably grainy.



2. Procedure

After Köhler illumination is set up as described above, remove an ocular and close the substage condenser diaphragm located in the condenser assembly until it is about a quarter to a third of the way into the field of view. Replace the ocular.

Focusing Using a Focusing Telescope (Bertrand Lens)

1. Applications and Objectives

At low magnifications, typically using a $10\times$ or lower objective, it can be difficult to see true focus. The human eye, particularly prior to the need for bifocal lenses, has a remarkably pliable lens that can be focused such that even out-of-focus images can be brought into satisfactory focus. At the same time, a camera attached to a photomicroscope has only one plane of focus. Thus, it is frequently useful to use a Bertrand lens to further magnify both the image of the reticle and the image of the specimen for critical focusing.

2. Procedure

1. Focus the Bertrand lens (focusing telescope) on some object at infinity, such as the bumps on concrete blocks at a distance of about 15–20 ft (4.6–6.1 m).
2. Place the focusing telescope on an ocular and refocus the ocular reticle image and then focus on the specimen. Avoid squinting, closing the nonfocusing eye, or focusing for too long a time, since straining can cause the eye to change focus, resulting in improperly focused photographs.
3. Take the photograph.

Using a Stage Micrometer and Ocular Scale to Measure Objects or to Calibrate Microscopes and Morphometry Programs

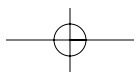
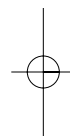
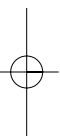
1. Applications and Objectives

It is often useful to be able to measure an object on a slide being examined, particularly in a diagnostic setting. For example, identifying narrow ($5\text{-}\mu\text{m}$ -diameter) versus wide ($10\text{--}20\text{-}\mu\text{m}$ -diameter) hyphae can help differentiate between “higher” and “lower” fungi seen in histological sections and thus help determine which type of antifungal therapy would be dictated to treat the infection in humans and other animals.

A second application allows calibration of the microscope so that the magnification on the film plane is positively known or to calibrate each objective for a morphometry program so that the program can provide accurate morphometric assessment of linear measurements.

2. Procedures

Determining Final Magnifications at the Film Plane. A typical stage micrometer obtained from scientific supply houses consists of a glass slide containing a 2-mm scale marked off in 0.1-mm units, with the terminal 0.1-mm unit further subdivided into 10 units of 0.01 mm. Place the stage micrometer on the photomicroscope stage, and photograph it with each objective and supplementary magnification lens on the microscope in all combinations possible. Develop the negatives and then use a loupe with a calibrated scale



(also available from scientific supply houses) to measure the photographed scale. Record the magnification factor for each lens combination so that all future negatives or 2×2 slides taken with specific lens combinations can be given a specific magnification factor from the chart you have created. For example, if the negative of the micrometer scale shows five markings that were originally 0.1 mm apart on the stage micrometer and the loupe scale shows the five marks to now span 1.0 mm, the magnification factor on film for that particular lens combination would be $2 \times$ and would be recorded for that specific set of magnification lenses on your table.

Calibration of an Ocular Scale for Measuring Objects Being Viewed Live. Place a stage micrometer on the microscope stage and insert a micrometer scale (available from scientific supply houses) into the proper location for the specific ocular you have, such that the scale is in sharp focus. Ramsden oculars will have a ring to hold the scale below the lowest glass element, while a Huygenian ocular will have a ring to locate the scale between the two glass elements of the ocular. For each objective lens, record the number of lines in the ocular scale that span a known distance on the stage micrometer. Divide the known distance on the stage micrometer by the number of lines in the ocular scale that covers the distance to determine the distance measured between two lines on the ocular scale. Thus, if six lines on the ocular scale fall on or between the beginning of the scale on the stage micrometer and the 0.03-mm mark, each of two adjacent lines in the ocular scale spans 0.005 mm and should be recorded as such. If a fungal spore is observed with the same lens combination, and the spores touch two lines of the ocular scale, the spores are $5\text{-}\mu\text{m}$ in diameter (0.005 mm).

Calibration of Morphometry Programs. Turn on the microscope, digital camera, and computer to which the camera is attached. Place a stage micrometer onto the microscope stage and open the morphometry software. Find the calibration utility, which typically will specify that you focus on the stage micrometer with a given objective, and then open the calibration utility. From there, select fixed end-points on the stage micrometer scale, put a calibrating line across them, and enter the distance spanned into the program, along with a designation for the objective in place when the measurement was performed. After this is done for all of the objectives on the microscope, the program will typically provide a look-up table from which you would select the appropriate objective being used (e.g., $10 \times$) before future morphometric measurements are taken or scale bars produced for inclusion in images stored by the morphometry program. Unless the software, camera, or objectives are replaced, these measurements will be accurate for all future morphometric work.

Reading an Objective Lens

1. Applications and Objectives

Manufacturers provide several critical pieces of information on the barrels of their objectives. Knowing how to decipher their inscriptions will ensure that an objective can be used to its best advantage.

2. Procedure

Look at the inscriptions on the lens barrel. The following information will be noted:

1. *Apochromat*, or *apo*, means that the lens is color-corrected for three or four colors. As mentioned in the main text of this chapter, these lenses are the most expensive and have the highest numerical apertures, the least working distance, and the lowest contrast. *Semiapochromat*, or

some designation with *fluor* incorporated, indicates fluorite lenses and means that the lens has an intermediate numerical aperture, working distance, and color correction, and gives good contrast. *Achromat*, or *achro*, identifies the least expensive category of objective, with the greatest working distance, least color correction, and the lowest numerical apertures.

2. *Plan*, or *plano*, designates a flat-field objective suitable for photomicroscopy, since the edge-to-edge flatness of the field of view is critical to good photomicrographs. This designation can be associated with all three classes of lenses above.
3. An objective inscribed with *160/-* or *160 NC* indicates a lens meant for a microscope with a 160-mm mechanical tube length that focuses the image of the specimen 160 mm from the nosepiece surface to which the objective is attached. The “-” or NC designation indicates that a coverglass is not needed to produce a good image. The marking *160/0.17* means that the lens is meant for a microscope with a 160-mm tube length and that a #1.5 coverglass (nominally 0.15–0.18 mm thick) is needed over the specimen to produce sharp images. Finally, an inscription of an infinity symbol indicates an infinity optic that focuses the image at any distance beyond the point where the tube lens focuses the image.
4. *OEL*, or *oil*, indicates that the lens should be immersed in immersion oil, while *W* indicates the lens is intended for immersion in water. Some lenses meant for dedicated fluorescence microscopy have the latter designation.
5. The numbers *1*, *10*, *25*, *40*, *100*, and so on, with or without *X* indicate the magnification provided by the objective. Remember that the final magnification of the image is dependent on the objective, any tube lenses present, any internal “photo” lenses, and the specific ocular, if any, projecting the image onto the recording instrument. *The magnification factor of viewing oculars has no effect on the magnification of recorded images in the microscope.*
6. Designations such as *PH* or *Phaco* indicate a lens containing a phase plate for phase-contrast microscopy. Reading the objective shown in Fig. 266 indicates that it is a phase-contrast objective meant to be used with the #3 phase annulus in the substage condenser assembly (PHACO 3). In addition, the lens is a semiapochromat lens (FLUOTAR), is meant to be used on a microscope with a 160-mm tube length and with a #1.5 coverglass (160/0.17), and magnifies 100× with a numerical aperture of 1.32 and must be used with immersion oil (100/1.32 OEL).
7. Finally, numbers such as *0.32*, *1.25*, and *1.4* indicate the numerical aperture of the lens, which helps the user determine how critical coverglass thickness will be to image quality. High-numerical-aperture dry lenses demand the coverglass thickness designated on the lens barrel. Knowing the numerical aperture of a high-resolution objective helps a user to make sure that the condenser assembly is properly matched to the objective. *If the numerical aperture of the objective lens is greater than that of the front element of the condenser assembly, the resolution will be limited by the lowest numerical aperture found in the optical system.* In other words, if a 0.9 NA condenser lens is used with a 1.4 NA objective, the actual numerical aperture for the optical system would be 0.9.
8. A high-dry lens with a movable collar and numbers from approximately 0.11 to 0.23, with a line scribed onto the stationary portion of the objective barrel, can be adjusted for various coverglass or specimen thicknesses. Figure 267 shows a variable-focus flat-field 40× apochromat (SPlanApo 40) objective with a numerical aperture of 0.95 (0.95), meant for a microscope with a 160-mm tube length and coverglass thicknesses from 0.11–0.23 mm (160/0.11–0.23).

Use of Focusing Collars on High Dry (40×) Objective Lenses

1. Applications and Objectives

The proper use of these objectives allows adjustment of depth of focus for differing coverglass and/or specimen thickness.



Figure 266. A 100 \times semiapochromat phase-contrast objective intended for use on a microscope with a 160-mm tube length. The numerical aperture of the oil-immersion lens is 1.32, and the lens is meant to be used with a #1.5 coverglass.

2. Procedure

Examine the focusing collar on a lens so equipped and note that it will have a range of numbers printed on the barrel, with a reference mark above them. Typically, the range will be from about 0.1 to 0.2. These numbers refer to the thickness, in millimeters, of the coverglass to be used on the slide. A typical H&E preparation with a #1.5 coverglass attached with a Permount™ type of mounting medium over a xylene-cleared section should yield the sharpest images with the focusing collar set at about 0.16–0.17, since the #1.5 designation refers to a coverglass of approximately 0.15-mm thickness. However, some thicker preparations, such as a glycerin-mounted immunostained slide or a bone section will be significantly thicker. Using a 40 \times dry lens without a focusing collar will make it unlikely that a sharp image can be obtained. With the focusing collar adjusted to somewhere around 0.2, a noticeably sharper image will be seen. *In addition, if the substage condenser diaphragm is closed down until diffraction is seen (the image becomes grainy) and then opened slightly until the graininess disappears, the image will potentially appear even sharper because this adjustment further increases contrast in the specimen, as well as increasing the depth of field.*

Using Oil- or Water-Immersion Objectives

1. Applications and Objectives

The purpose of immersion media is to decrease refraction of the light beam as it passes from one medium (air) into another (the glass slide), and then back into air before entering the glass of the objective



Figure 267. A flat-field 40 \times apochromat objective intended for use on a microscope with a 160-mm tube length. The numerical aperture of the lens is 0.95, and the high-dry lens may be used with #1, #1.5, and #2 coverglasses, as well as for specimens of various thickness. The focusing collar should be adjusted empirically for various specimens, until the image is as sharp as possible.

lens. This phenomenon decreases resolution. In addition, dark-field observation requires immersion oil between the condenser lens and the slide.

2. Procedure

A lens designated for use with water or oil, as described above, should have the appropriate liquid placed on the surface of the slide, with or without a coverglass, based on the designation after the mechanical tube length on the objective. The objective lens is then brought into contact with the liquid. *Do not allow these liquids to come into contact with objectives designated for dry work.* Any lens with a NA above 0.95 should also have the immersion medium placed between the front element of the condenser lens and the bottom of the glass slide. If the condenser assembly has a numerical aperture of 0.95, it is not useful to put immersion media on the front element of the condenser assembly, since air limits the numerical aperture to 0.95.

Use of Filters with Black-and-White Films

1. Applications and Objectives

Filters can be used to increase the overall contrast in an image or to accentuate or diminish certain colors in the original polychromatic specimen as well as to improve the image quality by eliminating the effects of chromatic aberration.

2. Procedure

Filters are customarily placed over the exit port of the light tube in the base of a photomicroscope but may be placed in slots near the light housing and could be introduced above the objectives if a suitably shaped filter holder is available. When possible, filters should be placed slightly out of the plane of focus after Köhler illumination adjustments so that any minor imperfections, scratches, or dust are not focused in the plane of the image.

Green Filter. This filter delivers monochromatic light to the film, eliminating the possibility of chromatic aberration causing decreased resolution. Black-and-white films are typically most sensitive in the middle of the visible light spectrum (green). In addition, hematoxylin and eosin (H&E) preparations will develop added contrast with the use of this filter, since the complementary red of the eosin-stained areas will be enhanced with a green filter.

Filters of Other Colors. Colored filters diminish their own color in the specimen and accentuate the complementary color. In other words, a red filter will diminish the eosinophilic, or reddish areas in an H&E preparation, while enhancing the basophilic, or bluish areas. Semithin plastic sections stained blue with Toluidine Blue O will exhibit a greater contrast if photographed with complementary orange or yellow filters in place.

Neutral Density Filters. Neutral density filters will diminish illumination without altering the colors of a specimen. They are primarily used to diminish light levels to comfortable viewing levels for the microscopists. Most modern camera metering systems can take perfectly exposed photographs at essentially any illumination level set up on a microscope.

Use of Filters with Color Films

1. Applications and Objectives

Three classes of filters are used with color films: light balancing filters, color compensating (CC) filters, and neutral density filters.

2. Procedure

Light Balancing (Color Temperature) Filters. Unless the photomicroscope comes with specific filters designated for use with tungsten- and daylight-balanced films, it will often be necessary to expose several test rolls of film with various filter combinations until perfect exposures are obtained. Once the standard filter package that delivers images with proper color rendition is identified, it should produce consistent results with a halogen-based illuminator. As mentioned previously, glass light bulbs darken over time, dropping the color temperature of the light source, with resulting shifts in color rendition. If using an older photomicroscope, the state of the light bulb should be monitored regularly, and any darkening of the globe due to tungsten deposition should suggest that it is time to change the bulb.

Tungsten-balanced films such as Kodak T-64 Professional film are designed to be exposed with lights exhibiting a color temperature of 3,200° K, which is produced by a new glass tungsten bulb at its maximum rated voltage (6 or 12 V), or a halogen bulb of any age that is set at its maximum rated voltage (6 or 12 V). No filtration is required under such conditions. However, if this film is used under daylight or stroboscopic flash illumination conditions (5,500° K), an amber color must be added by a Kodak 85B filter, or equivalent, to decrease the color temperature to 3,200° K.

Daylight-balanced films such as Fujichrome 100 daylight, Ektachrome daylight, or Kodachrome are meant to be exposed under daylight or stroboscopic light conditions (5,500° K). If they are used with tungsten light (3,200° K), a blue Kodak 80 A filter, or equivalent, must be used to shift the color temperature from 3,200° K up to 5,500° K.

CC Filters. These filters come in varying densities of red, green, blue, cyan, magenta, and yellow. They can be used to compensate for minor color variations associated with lens coatings and other optical pieces (mirrors, prisms) within photomicroscopes. One Leitz microscope that was in our lab 20 years ago, and was probably 15–20 years old at the time, had optics that imparted a slightly greenish cast to color photographs. To correct the problem, a CCM (magenta) filter was used to diminish the green cast to the photographs.

Neutral Density Filters. As mentioned above, these filters are generally used to reduce illumination to comfortable levels for the microscopists, since the camera metering systems on modern photomicroscopes can cope with a wide variety of illumination levels and still take consistently exposed photographs. We have found that all neutral density filters are not created equal. Neutral density filters designed for photomicroscopy typically cost over \$50 each, while neutral density filters to be screwed onto camera lenses can be purchased for less than half that amount. Unfortunately, our experience has shown us that inexpensive neutral density filters that appear to work well on cameras produce slight color shifts when used with photomicroscopes.

