Our naked eye is unable to resolve two objects that are separated by less than 70 µm. Perhaps we are fortunate that, without a microscope, our eyes are unable to resolve small distances. John Locke (1690) wrote in *An Essay Concerning Human Understanding*:

> We are able, by our senses, to know and distinguish things,... if that most instructive of our senses, seeing, were in any man a thousand or a hundred thousand times more acute than it is by the best microscope, things several millions of times less than the smallest object of his sight now would then be visible to his naked eyes, and so he would come nearer to the discovery of the texture and motion of the minute parts of corporeal things; and in many of them, probably get ideas of their internal constitutions: but then he would be in a quite different world from other people: nothing would appear the same to him and others: the visible ideas of everything would be different. So that I doubt, whether he and the rest of men could discourse concerning the objects of sight, or have any communication about colours, their appearances being so wholly different. And perhaps such a quickness and tenderness of sight could not endure bright sunshine, or so much as open daylight; nor take in but a very small part of any object at once, and that too only at a very near distance. And if by the help of such microscopical eyes (if I may so call them) a man could penetrate further than ordinary into the secret composition and radical texture of bodies, he would not make any great advantage by the change, if such an acute sight would not serve to conduct him to the market and exchange; if he could not see things he was to avoid, at a convenient distance; nor distinguish things he had to do with by those sensible qualities others do. He that was sharp-sighted enough to see the configuration of the minute particles of the spring of a clock, and observe upon what peculiar structure and impulse its elastic motion depends, would no doubt discover something very admirable: but if eyes so framed could not view at once the hand, and the characters of the hour-plate, and thereby at a distance see what o’clock it was, their owner could not be much benefited by that acuteness; which, whilst it discovered the secret contrivance of the parts of the machine, made him lose its use.

Alexander Pope (1745) considered the same question in *An Essay on Man*:

> Why has not Man a microscopic eye?  
> For this plain reason, Man is not a Fly.  
> Say what the use, were finer optics given,  
> T’inspect a mite, not comprehend the heaven.

The eye is a converging lens that produces a minified image of the object on the retina. The dimensions of the cells that make up the light-sensitive retina limit the ability of the human eye to resolve two dots that are closer than 70 µm from each other. The retina contains approximately 120 million rods and 8 million cones packed into a single layer. In the region of highest resolution, known as the fovea, the cones are packed so tightly that they are only 2 µm apart. Still, this distance between the cones limits the resolving power of our eyes. If a point of light originating from one object falls on only one cone, that object will appear as a point. If light from two objects that are close together fall on one cone, the two objects will appear as one. When light from two points fall on two separate cones separated by a third cone, the two points can be clearly resolved. The resolving power of the eye can be increased slightly by eye movements that vary the position of the cones.

The numerical value of the limit of resolution of the human eye was first discovered by Robert Hooke in 1673. Birch (1968) wrote:

> Mr. Hooke made an experiment with a ruler divided into such parts, as being placed at a certain distance from the eye, appeared to subtend a minute of a degree; and being earnestly and curiously viewed by all the persons present, it appeared, that not any one present, being placed at the assigned distance, was able to distinguish those parts, which appeared of the bigness of a minute, but that they appeared confused. This experiment he produced, in order to shew, what we cannot by the naked eye make any astronomical or other observations to a greater exactness than that of a minute, by reason, that whatever object appears under a lens angle, is not distinguishable by the naked eye; and therefore he alleged, that whatever curiosity was used to make the divisions of an instrument more nice, was of no use, unless the eye were assisted by other helps from optic glasses.

In order for two points to appear as separate points, light from those points must enter the eye forming an angle greater than one minute of arc. Thus the object must be brought very close to the eye. However, due to the limitation of our eye to focus at close distances, a specimen can be brought up only to the near point of the eye, which is about 25 cm from our eye (see Chapter 2, Figure 2-40). A microscope makes it possible to increase the visual angle, so that light, emanating from two near, but separate points, can enter the eye, forming an angle that subtends more than one minute of arc such that the light from the two separate points fall on separate cones (Figure 4-1; Gage, 1908). The eye of the observer plays an integral role...
as the optical interface between the microscope and the effigy of the specimen produced by the brain.

To make a very simple microscope, place an index card that contains a minute pinhole, smaller than your pupil, between your eye and an object closer than the near point of your eye. Then look at an object that would be blurry at this distance without the very simple pinhole microscope. An object that was blurry at this close distance will appear clear through the pinhole. Moreover, since it is close to your eye, it also appears magnified, compared to how it would look when it was placed at the distance where it would look sharp without the pinhole. The pinhole acts as a microscope by reducing the angle of the cone of light that comes from each point in the object without decreasing the visual angle of an object placed so close to your eye. Since, with the pinhole, the image on your retina is formed by cones of light with smaller zones of confusion, objects closer than the near point of your eye that would have formed blurry images on the retina now form sharp images.

Thus, microscopes are necessary to create large visual angles that allow us to resolve microscopic objects. We need a microscope if we want to see a mite, or any other microscopic aspect of the natural world. Indeed the microscope opened up a whole new world to seventeenth and eighteenth century microscopists (Hooke, 1665; Leeuwenhoek, 1674–1716; Malpighi, 1675–1679; Swammerdam, 1758). Augustus de Morgan (1872) wrote:

Great fleas have little fleas upon their backs to bite e’em,  
And little fleas have lesser fleas, and so on ad infinitum.

According to Emily Dickinson (1924):

Faith is a fine invention  
When gentlemen can see,  
But microscopes are prudent  
In an emergency.

The word microscope comes from the Greek words μικρος (small) and σκοπεω (to see). The word microscope was coined by Giovanni Faber on April 13, 1625. The bright-field microscope is, perhaps, one of the most elegant instruments ever invented, and the first microscopists used the technologically advanced increase in the resolving power of the human eye to reveal that the workmanship of the Creator can be seen at the most minute dimensions (Hooke, 1665; Grew, 1672, 1682; Leeuwenhoek, 1674–1716; Malpighi, 1675–1679; Swammerdam, 1758). The bright-field microscope also has been instrumental in revealing the cell as the basic unit of life (Dutrochet, 1824; Schwann, 1847; Schleiden, 1849), the structural basis for the transmission of inherited characteristics (Strasburger, 1875; Flemming, 1880), and the microscopic basis of infectious diseases (Pasteur, 1878, 1879; Koch, 1880; Dobell and O’Connor, 1921). Color plates 1 and 2 show examples of cork cells and chromatin observed with bright-field microscopy.

The bright-field microscope also has had a tremendous influence in physics and chemistry in that it made it possible for Robert Brown (1828, 1829) to discover the incessant movement of living and nonliving particles, now known as Brownian motion or Brownian movement (Deutsch, 1991; Ford, 1991, 1992a, 1992b, 1992c; Rennie, 1991; Bown, 1992; Wheatley, 1992; Martin, 1993). In 1905, Albert Einstein (1926) analyzed Brownian motion and concluded that the movement occurred as a result of the statistical distribution of forces exerted by the water molecules surrounding the particles. Jean Perrin (1909, 1923) confirmed Einstein’s hypothesis by observing Brownian motion under the microscope and used his observations, along with Einstein’s theory, to calculate Avogadro’s number, the number of molecules in a mole. Ernst Mach and Wilhelm Ostwald, who were the last holdouts to accept the reality of atoms and molecules, became convinced in the reality of molecules from the work done on Brownian motion. These influential scientists were held back from accepting the evidence of the existence of molecules from other kinds of physicochemical data because of their positivist philosophy, which could be summed up by the phrase “seeing is believing.”

**COMPONENTS OF THE MICROSCOPE**

A simple bright-field microscope, like that used by Leeuwenhoek (1674–1716), Jan Swammerdam, Robert Brown, and Charles Darwin consists of only one lens, which
forms a magnified, erect, virtual image of the specimen (Ford, 1983, 1985). By contrast, the compound microscope uses two lens systems to form an image. The primary lens system (object glass or objective lens) captures the light that is diffracted by the object and then forms a real intermediate image that is further magnified by a second lens system, known as the eyepiece or ocular.

There are currently two standard optical configurations. Traditional objectives are designed to produce a real, magnified, inverted image 16 or 17 cm behind the objective lens when the specimen is placed in front of the front focal plane of the objective (Gage et al., 1891). Most microscope objectives manufactured today produce an image of an object at infinity when the object is placed at the front focal plane of the objective (Lambert and Sussman, 1965). When infinity-corrected objectives are used, an intermediary lens, known as the tube lens, is placed behind the objective lens. The tube lens focuses the parallel rays leaving the objective lens, and produces a real image at the back focal plane of the tube lens. This arrangement, by which parallel rays pass from the objective lens, minimizes the introduction of additional aberrations introduced by rays coming from every conceivable angle going through extra optical pieces placed between the objective lens and the ocular. Objectives that produce an intermediate image 160 mm or 170 mm behind the lens are marked with 160 or 170, respectively. Objectives that produce the intermediate image at infinity are marked with ∞.

Each objective lens is labeled with a wealth of information (Figure 4-2). The most prominent number signifies the transverse magnification \( m_r \) of the intermediate image. Remember from Chapter 2, that

\[
m_r = \frac{y_i}{y_o} = -s_i/s_o
\]

The objective lenses often are surrounded by a thin band whose color represents the magnification of the objective.

From the beginning, microscopists and inventors realized that microscopes do not magnify objects faithfully. In fact the microscope itself introduces fictions or convolutions into the image that are not part of the object itself. Over the years, lenses were developed that were as free as possible from spherical aberrations (Kepler, 1604; Descartes, 1637; Molyneux, 1692, 1709; Gregory, 1715, 1735; Smith, 1738; Martin, 1742, 1761, 1774; Adams, 1746, 1747, 1771, 1787, 1798; Baker, 1742, 1743, 1769; McCormick, 1987) and chromatic (Dollond, 1758; Amici, 1818; Lister, 1830; Beck, 1865; Abbe, 1887; Cheshire, 1905; Disney et al., 1928; Clay and Court, 1932; von Rohr, 1936; Payne, 1954; Feffer, 1996)—aberrations that were once thought to be absent in the human eye (Paley, 1803).

Due to dispersion, which is the wavelength-dependence of the refractive index (see Chapter 2), a single lens will not form a single image, but a multitude of images, each of which is a different color and each of which is offset axially and laterally from the others. For example, when we use an aspheric objective that is not color-corrected, the microscopic image will go from bluish to reddish as we focus through the object. This chromatic aberration can be mitigated, but not eliminated by using an achromatic doublet, which was invented by Chester Moor Hall, John Dolland, and James Ramsden. The achromatic doublet is made by cementing a diverging lens made of high-dispersion flint glass to the converging lens made of low-dispersion crown glass. The flint glass mostly cancels the dispersion due to the crown glass, while only slightly increasing the focal length of the lens.

When we plot the focal length of an aspheric lens as a function of wavelength, we get a monotonic plot, where wavelengths in the blue range experience shorter focal lengths, wavelengths in the red range experience longer focal lengths, and wavelengths in the green range experience intermediate focal lengths (Figure 4-3). When we plot the focal length of an achromatic lens as a function of wavelength, we

![FIGURE 4-2](image120x94 to 211x236) Each objective is labeled with an abundance of useful information.

![FIGURE 4-3](image189x226 to 286x328) Chromatic aberration means that the focal length of an objective lens is wavelength dependent. There is a large variation in focal lengths with wavelength in aspheric objectives, a smaller variation in achromatic objectives, an even smaller variation in apochromatic objectives, and the smallest variation in superachromatic objectives.
get a parabolic plot, where the focal length for a wavelength in the blue region (spectral line F) is similar to the focal length for a wavelength in the red region (spectral line C).

Ernst Abbe developed an objective lens that introduced less chromatic aberration than the achromats and coined the term apochromat, which means “without color,” to characterize his lens design (Haselmann, 1986; Smith, 1987). He defined apochromats as lenses that had the same focal length for three widely spaced wavelengths in the visible range. Apochromats can be defined as objective lenses whose differences in focal lengths do not exceed \( \lambda/4 \) throughout the spectral range from 486.1 nm (spectral line F), through 546.1 nm (spectral line E) to 656.3 nm (spectral line C). However, this definition remains fluid as microscope lens makers are striving to make “super” apochromatic objective lenses that each have a single wavelength-independent focal length all the way from the ultraviolet to the infrared wavelengths.

More lens elements are required to effectively reduce chromatic aberration and this increases the cost of the objective dramatically. Consequently, apochromats are the most expensive lenses and achromats are the least expensive objective lenses available in good microscopes. Microscope makers make a variety of lenses that are more corrected than achromats, but less corrected than apochromats. These semi-apochromats and fluorites, which go by a variety of manufacturer-specific names, are intermediate in cost.

A single lens also introduces spherical aberrations because the rays that enter the peripheral region of the lens experience a shorter focal length than do the paraxial rays. Spherical aberration often is corrected at the same time as chromatic aberration so that spherical aberration in aspheric objective lenses is absent only for green wavelengths. Green light was chosen, not because it produces diffraction-limited images with the highest resolution, but because it is the color that people can see with the least amount of eye strain. Spherical aberration is nearly absent for all wavelengths for apochromatic objective lenses. Again, the spherical aberration is intermediate in achromatic and semi-apochromatic objective lenses. Objectives can be readily tested for chromatic and spherical aberration by using a homemade Abbe-test plate (Sanderson, 1992).

The resolving power of the objective lens is characterized by its numerical aperture (NA; Chapter 3). The numerical aperture of the lens describes the ability of the lens to resolve two distinct points. The numerical apertures of objectives typically vary from 0.04 to 1.40. Objectives with NAs as high as 1.6 were made over a century ago (Spitta, 1907), but were not popular as a result of their requirement for special immersion oils and cover glasses. However, objectives with NAs between 1.49 and 1.65, which are used for Total Internal Reflection Fluorescence Microscopy (TIRFM; Chapter 12), are being reintroduced. The NA of an objective is given by the small print number that either follows or is printed under the magnification.

The brightness of the image, in part, is proportional to the square of the numerical aperture and inversely proportional to the square of the transverse magnification (Naegeli and Schwendener, 1892; Beck, 1924).

\[
\text{Brightness} \propto (\text{NA}/m^2)^2
\]

In order to produce a maximally bright image we may want to use an objective lens with the highest possible NA and the lowest possible magnification. However, the brightness of the image does not only depend on the geometry of the lens but also on the transparency of the optical glasses used to construct the lenses (Hovestadt, 1902). In the past there has been a tradeoff between the transparency of an objective lens and the number of corrections. For example, the fluorites, which are composed of lens elements made of calcium fluoride, were more transparent to the ultraviolet light used for fluorescence microscopy than were the apochromats made with glass lens elements. Manufacturers are striving to make highly corrected lenses that are transparent throughout the spectrum from ultraviolet to infrared.

Objective lenses must also be corrected so that the specimen is in sharp focus from the center to the edge of a flat image plane. Objectives that are designed to produce flat fields are labeled with F- or Plan-; for example, F-achromat or Plan-Apochromat. The Plan-objectives are more highly corrected for curvature of field than the F-objectives. It is the nature of optics that it takes one lens element to produce a magnified image of the object and many lens elements to eliminate or perform a deconvolution on the aberrations or convolutions introduced by the “imaging” lens element. Following are some examples of the lens combinations used to make highly corrected objective lenses (Figure 4-4).

Objectives are made with features that are useful for doing specific types of microscopy. For example, some objectives have an iris at the back focal plane, which can reduce the NA of the objective. These objectives may have the word Iris printed on them. This feature, which increases contrast at the expense of resolution, is useful when doing dark-field microscopy (see Chapter 6).

Some objectives are made out of completely homogeneous glass, known as strain-free glass. A lens made out of strain-free glass does not depolarize linearly polarized

![FIGURE 4-4 Chromatic aberration is reduced by building an objective lens with additional achromatic doublets and triplets.](image-url)
light that passes through the lens. These objectives may have the word Pol printed on them. They are useful when doing polarization microscopy (see Chapter 7).

The working distance of an objective lens is defined as the distance between the front of the lens and the top of a cover glass (Gage, 1917). Typically, the working distances, which vary between 0.1 and 20 mm, are inversely proportional to the magnifications and the numerical apertures of objective lenses. However, some long working distance objectives are made that also have high magnifications and numerical apertures. These objectives, which may be marked with an LD, are especially useful for doing micromanipulation.

When doing micromanipulation, sometimes it is helpful to immerse the lens directly in the dilute aqueous solution that bathes the specimen. Water immersion objectives, which are marked with a W, are good for this use. Objectives also are made that can be used in solutions with higher refractive indices, like glycerol. Glycerol immersion objectives are marked with a Glyc. Lastly, immersion oils are used to increase the resolving power of the microscope. Objective lenses that are made to be used with oil are marked with the word Oil. Some objective lenses can be immersed in media with refractive indices from 1.333 to 1.515. Depending on the manufacturer, these objectives are marked with Imm or W/Glyc/Oil.

Objectives are designed so that the cover glass acts as the first lens in objectives that are corrected for spherical aberration. Most objectives used in transmitted light microscopy are marked with 0.17, which means it is corrected for use with a 0.17 mm (1/12) cover glass. Some objectives are made for use without cover slips and are marked with /0. Objectives, which are insensitive to cover glass thickness, are marked with /-. Some objectives can be used with a variety of cover glasses. These objectives have a correction collar and may be marked with korr.

The cover glass introduces an increase in the optical path length of the diffracted rays that pass through it (Figure 4-5). The magnitude of the increase depends on the angle or order of the diffracted rays. The more oblique the rays are; the greater the increase in optical path length. The thicker the cover glass, the greater the difference between rays emanating at different angles from the same point. The highly diffracted rays appear to come from a nearer object than the rays that are diffracted from a smaller angle. Consequently, the different diffraction order rays will be focused at different distances from the image plane. This results in a zone of confusion instead of a single point, and contributes to spherical aberration. The manufacturers of the objective lenses design the objectives to compensate for the increase in the optical path induced by a given cover glass thickness. The Abbe Test Plate can be used to determine the effect of cover glass thickness on spherical aberration.

One of the characteristics of objective lenses is their cost; and unfortunately cost will probably play the biggest part in your choice of objectives. As I discussed in the last chapter, resolution and contrast are often competing qualities. However, as I will discuss in Chapter 14, we can use analog or digital image processing to enhance the contrast in electronically captured images. Thus, in our constant tug of war between contrast and resolution, we can opt for an objective that will provide the highest resolution and/or the greatest brightness at the expense of contrast and then enhance the contrast of the high resolution image using image processing techniques.

A real image of the specimen formed by the objective lens falls on the field diaphragm between the front focal plane of the ocular and the eye lens of the ocular itself. The ocular-eye combination forms a real image of the intermediate image on the retina, which appears as a magnified virtual image 25 cm in front of our eyes. Since the intermediate image is inverted with respect to the specimen, the virtual image also is inverted with respect to the specimen. Oculars typically add a magnification of 5x to 25x to that produced by the objective. Moreover, a turret that contains a series of low magnification lenses can be inserted into the microscope just under the oculars. These ancillary magnification lenses increases the magnification of the virtual image by one or two times. Most oculars used in binocular microscopes can be moved laterally to adjust for your interpupillary distance. One or both oculars will have a diopter adjustment ring, which can be moved to compensate for difference in magnification between your two eyes. When the interpupillary distance and the diopter adjustment are set correctly, it is actually relaxing to sit in front of a microscope all day.

When correcting aberrations in microscopes, designers take into consideration the objectives, tube lens, and oculars. Depending of the objective lens, special matching

![FIGURE 4-5](Image)

The cover glass introduces spherical aberration. The higher-order diffracted rays are refracted more than the lower-order diffracted rays, resulting in the point being imaged as a zone of confusion. This occurs because the lower order-diffracted rays appear to come from a position close to the object and the higher order-diffracted rays, when they are traced back through the cover glass, seem to come from a position between the real object position and the objective lens. The greater the cover glass thickness, the greater the amount of spherical aberration that will be introduced by the cover glass. A correction for the spherical aberration introduced by an objective lens also corrects the spherical aberration introduced by a cover glass of a certain thickness (e.g., 0.17 mm). Objectives with correction collars can correct for the spherical aberration introduced by a range of cover glass thicknesses.
oculars may have to be used with it in order to obtain the best image. There are several common types of oculars in use, and they fall into two categories: negative and positive (Figure 4-6). The Huygenian eyepiece, which was designed by Huygens for a telescope ocular, is composed of two plano-convex lenses. The upper lens is called the eye lens and the lower lens is called the field lens. The convex sides of both lenses face the specimen. Approximately midway between the two lenses there is a fixed circular aperture that defines the field of view and holds an ocular micrometer. This is where the intermediate image formed by the objective lens is found. Since the object focus is behind the field lens, the Huygenian eyepiece is an example of a negative ocular. Huygenian oculars are found on relatively routine microscopes with achromatic objectives.

The Ramsden eyepiece is an example of a positive ocular, whose object focal plane is in front of the field lens. The Ramsden eyepiece consists of two plano-convex lenses where the convex side of both lenses face the inside of the eyepiece. The circular aperture that defines the field of view and holds the ocular micrometer is below the field lens.

Compensating eyepieces, which can be either negative or positive, contain a number of lens elements. Compensating oculars are important for correcting the residual chromatic aberration inherent in the design of some objective lenses from the same manufacturer. As digital imaging techniques develop (see Chapters 13 and 14), fewer and fewer people are looking at microscopic images through the oculars and more and more people are looking at the images displayed on a monitor. Consequently, optical corrections to mitigate aberrations are no longer included in the oculars. The corrections are completed in the objective lenses or the objective lens-tube lens combination.

Oculars may be labeled with a field of view number, which represents the diameter (in millimeters) of the field that is visible in the microscope when using those oculars. The diameter of the field can be obtained by dividing the field of view number by the magnification of the objective lens and any other lenses between the objective and the ocular. This is helpful in estimating the actual size of objects. The field of view numbers vary from 6.3 to 26.5.

The total transverse magnification of the compound microscope is given by the product of the magnification of the objective lens (obj), the ocular (oc), and any other intermediate pieces (int), including the optivar.

\[
m_{\text{total}} = (m_{\text{obj}})(m_{\text{int}})(m_{\text{oc}})
\]

In microscopy, there is a limit to the amount of magnification that is useful, and beyond which, the image quality does not improve. This is reached when two points in the image appear to send out rays that subtend one minute of arc, which is the resolution of the human eye.

What is the maximum useful magnification of a light microscope? Let us assume that the final image is formed 25 cm from the eye and the smallest detail visible in the specimen is given by the following equation: \( d = \frac{1.22 \lambda}{(2NA)} \), and \( d = 0.161 \mu m \). Since the eye can just resolve two points, 70 \( \mu m \) apart, the magnification necessary for the eye to resolve two points (i.e., useful magnification) is 70 \( \mu m / 0.161 \mu m = 435x \). It is uncomfortable to work at the limit of resolution of the eye, so we typically use a magnification two to four times greater than the calculated useful magnification, or up to 1740x. Higher magnifications can result in a decrease in image quality since imperfections of the optical system and vibration become more prominent at high magnifications. As a rule of thumb, the optimal magnification is between 500 (NA) and 1000 (NA). However, when working with good lenses and stable microscopes, it is possible to increase the magnification to 10,000x (Aist, 1995; Aist and Morris, 1999).

The specimen is illuminated by the sub-stage condenser (Wenham, 1850, 1854, 1856). There are a variety of sub-stage condensers that have different degrees of corrections (Figure 4-7). The Abbe condenser, which originally was designed to provide an abundance of axial rays, is neither

![FIGURE 4-6](image) A negative ocular (A) has the field diaphragm between the eye lens and field lens. A positive ocular (B) has the field diaphragm in front of the field lens.

![FIGURE 4-7](image) Lens elements in sub-stage condensers. An Abbe chromatic sub-stage condenser (A), an aplanatic sub-stage condenser (B), and an achromatic sub-stage condenser (C).
achromatic nor aplanatic (Martin, 1993). Other condensers designed to use oblique rays as well as axial rays are corrected for spherical aberration and/or chromatic aberration. The aplanatic condenser is corrected for spherical aberration and the achromatic condenser is corrected for both spherical and chromatic aberration.

A sub-stage condenser may contain a rotating ring with annuli, prisms, or slits at its front focal plane that allows us to do many forms of light microscopy, including dark-field and phase-contrast (see Chapter 6), differential interference contrast (see Chapter 9), and amplitude modulation contrast (see Chapter 10). Some condensers are made with long working distances, which are convenient to use when performing micromanipulations on inverted microscopes.

The objectives, oculars, and sub-stage condenser usually are supported by either an upright or inverted microscope stand. The typical microscope, which is on an upright stand, is ergonomically designed for most uses. However, inverted microscopes make manual manipulations, including micro-injection, microsurgery, and electrophysiological measurements much easier. The microscope stand often is overlooked as an important part of the microscope. It provides stability for photomicrography or video microscopy and flexibility to convert the stand for various types of optical microscopy. In the long run, it is worth getting the most stable, flexible, and, consequently, most expensive microscope stand. The stand contains the coarse and fine focus knobs that raise or lower the nosepiece that holds the objectives.

The stage is where the specimen is placed. Inexpensive glide stages let you position the specimen by sliding the stage plate over a greased plate placed below it. In order to exactly position a specimen, you need a stage that has controls to translate the specimen in the XY direction. Such a stage may be motorized and controlled by computers. Rotating stages are useful for polarized light microscopy (see Chapter 7) and other forms of microscopy that utilize polarized light or depend on the direction of shear (see Chapters 9 and 10).

Originally the specimen in a bright-field microscope was illuminated by sunlight and a heliostat, which rotated with the speed of the rotation of the earth so that the sunlight would remain stationary with respect to the microscope. Alcohol, kerosene, and gas lamps were used on bleak days or after the sun set at night. Eventually electric lights became standard (Beale, 1880; Bracegirdle, 1993). Currently a bright-field microscope is equipped with one or more electric light sources to illuminate the specimen (Davidson, 1990). The light source is usually a quartz halogen bulb, although mercury vapor lamps, xenon lamps, lasers, and light-emitting diodes may also be used in special cases.

The light source usually is placed between a parabolic mirror and a collector lens. The light source is placed at the center of curvature of the parabolic mirror so that the rays that go backward are focused back on the bulb. The collector lens is used to project an image of the filament onto the front focal plane of the condenser (Köhler illumination) or onto the specimen itself (critical or confocal illumination).

The diameter of the field illuminated by the light source is controlled by the field diaphragm, and the number and angle of the illuminating rays is controlled by the aperture diaphragm.

### THE OPTICAL PATHS OF THE LIGHT MICROSCOPE

I will briefly discuss two types of microscope illumination: Köhler and critical. In practice, Köhler illumination is used in most microscopes, and a specialized form of critical illumination is used in confocal microscopes. Köhler illumination provides a uniformly illuminated, bright field of view, which is important when using an uneven light source, like a coiled tungsten filament. At the end of the nineteenth century, microscopists used sunlight or oil lamps to illuminate their specimens, and very slow film to photograph them. The exposures needed to expose the film were as long as five hours. Thus August Köhler (1893) was motivated to find a way to obtain the brightest image possible so that he could continue his work investigating the taxonomic position of the mollusk, *Syphonaria*, by taking good photomicrographs of the taxonomically important gills.

Köhler devised a method in which an image of the source is formed by a converging lens, known as the collector lens, at the front focal plane of the sub-stage condenser, while an image of the field diaphragm is formed in the plane of the specimen by the sub-stage condenser. The sub-stage condenser produces collimated light beams, each of which originates from a point on the source. Each point on the source forms a collimated beam of light that illuminates the entire field of view. The points on the center of the source form a collimated beam that is parallel to the optical axis. The points farther and farther away from the optical axis make collimated beams that strike the object at greater and greater angles. Thus, the specimen is illuminated with a cone of light composed of both parallel and oblique illumination (Evennett, 1993; Gundlach, 1993; Haselmann, 1993).

In critical illumination, an image of the light source is focused in the plane of the specimen. The illumination is intense, but it is uneven unless a ribbon filament is used. Critical illumination does not require a sub-stage condenser. In critical illumination, each point in the object acts as a point source of light. If the light radiating from two nearby points is truly incoherent, it will form two overlapping images of Airy discs, the intensity of which will be the sum of the two intensities. Since light from two nearby points will be somewhat coherent and will interfere, the intensity of each point will not be exactly the sum of the two intensities, but will, in part, be described by the square of the sum of the amplitudes of the light radiating from both points.

When the microscope is set up for Köhler illumination, the following optical conditions result. The collector lens focuses an image of the light source onto the front focal
plane of the sub-stage condenser where the aperture diaphragm resides. The sub-stage condenser turns each point of light at its front focal plane into a beam of light whose angle is proportional to the lateral distance of the point from the principle axis. These beams of light are focused on the rear focal plane of the objective by the objective lens itself. The relative positions of the points of light on the back focal plane of the objective are identical to the relative positions they had when they originated at the front focal plane of the sub-stage condenser. The ocular makes a real image of this light disc at the eye point, also known as the exit pupil or Ramsden disc of the ocular. The eye point is where we place the front focal point of our eye. In Köhler illumination, light originating from each and every point of the light source illuminates our entire retina.

At the same time as the illuminating rays illuminate our retina, the sub-stage condenser lens focuses an image of the field diaphragm on the plane of the specimen. The objective lens forms an intermediate image on the field diaphragm of the ocular. Together, the ocular and the eye form an image of the specimen on the retina. In Köhler illumination, the light that falls on any point on the retina originated from every point of the filament.

Köhler illumination gives rise to two sets of optical paths and two sets of conjugate image planes—the illuminating rays, which are equivalent to the zeroth-order diffracted light, and the image-forming rays, which are equivalent to the sum of the first-order and higher-order diffracted light (Figure 4-8). When the microscope is adjusted for Köhler illumination, we get the following advantages:

1. The field is homogeneously bright even if the source is inhomogeneous (e.g. a coiled filament).
2. The working NA of the sub-stage condenser and the size of the illuminated field can be controlled independently. Thus, glare and the size of the field can be reduced without affecting resolution.
3. The specimen is illuminated, in part, by a converging set of plane wave fronts, each arising from separate points of the light source imaged at the front focal plane of the condenser. This gives rise to good lateral and axial resolution. Good axial resolution allows us to “optically section.”
4. The front focal plane of the sub-stage condenser is conjugate with the back focal plane of the objective lens, a condition needed for optimal contrast enhancement.

To achieve Köhler illumination, the light source is placed a distance equal to twice the focal length of the parabolic mirror so that the rays that travel “backward” are focused back onto the filament (Figure 4-9). The collector lens produces a magnified, inverted, real image of the light source onto the front focal plane of the sub-stage condenser where the aperture diaphragm resides. That is, any given point of the filament is focused to a point at the aperture diaphragm. Light emanating from a point in the plane of the aperture diaphragm emerges from the sub-stage condenser as a plane wave (Figure 4-10). All together, the points in the front focal plane of the sub-stage condenser give rise to a converging set of plane waves. The angle of each member of the set of plane waves is related to the distance of the point to the center of the aperture. In order to produce radially symmetrical cones of light from the sub-stage condenser, the filament in the bulb should also be radially symmetrical.

The plane waves emerging from the sub-stage condenser traverse the specimen and enter the objective lens. The objective lens converts the plane waves to spherical waves, which converge on the back focal plane of the objective lens. Thus each point at the back focal plane of the objective lens is conjugate with a corresponding point in the plane of the sub-stage condenser aperture diaphragm as well as a point on the filament. The sub-stage condenser and the objective together form a real inverted image of the filament at the back focal plane of the objective (Figure 4-11).

The back focal plane of the objective lens and the front focal plane of the sub-stage condenser can be visualized by inserting a Bertrand lens between the oculars and the objective, or by replacing an ocular with a centering telescope. The ocular forms a real minified image of the uniformly illuminated back focal plane of the objective lens at the eye point. The eye point is located just beyond the back focal point of the ocular, and it is where the front focal point of the eye is placed. The object (i.e., the filament in this case) and the image (of the filament) lie in conjugate planes. In Köhler illumination, the light source, the aperture diaphragm, the back focal plane of the objective lens, and the eye point of the ocular lie in conjugate planes called the aperture planes. In each of these planes, the light that does not interact with the specimen, that is the zeroth-order light, is focused.

Now I will trace the path of the waves that interact with the specimen. These are called the image-forming waves and they represent the superposition of all diffracted waves. When the microscope is set up for Köhler illumination, the condenser lens forms a minified, inverted real image of the field diaphragm on the specimen plane. Each point on the field diaphragm is illuminated by every point of the filament (Figure 4-12). The specimen is then focused by the objective lens, which produces a magnified, inverted image of the specimen and the field diaphragm in the optical tube, past the back focal plane of the objective. This plane is just behind the front focal plane of the ocular (Figure 4-13).

The lenses of the ocular and the eye together form an image on the retina as if the eye were seeing the virtual image of the specimen (Figure 4-14). These four conjugate planes are called the field planes. With Köhler illumination there are two sets of conjugate planes, the aperture planes and the field planes (Figure 4-8). The two sets of conjugate planes are reciprocally related to each other.
To find illuminating rays between the light source and the specimen plane (Figure 4-15):

1. Draw two or three characteristic rays from each of three points on the filament. Find the image plane. For Köhler illumination, we move the filament and collector lens so that the image plane is on the aperture diaphragm.

2. Draw two to three characteristic rays from each of three points on the image of the filament on the aperture diaphragm. Since the aperture diaphragm is at the front focal plane of the sub-stage condenser, all rays from a single point come through the condenser as parallel pencils of light. Draw each pencil till it reaches the objective lens.

To find image-forming rays between the light source and the specimen plane:

3. Draw two or three characteristic rays from two or three points on the field diaphragm to the image plane, where the specimen is placed. For Köhler illumination, the sub-stage condenser is adjusted so that the image plane is identical to the specimen plane on the stage.

**FIGURE 4-8** Paths of the illuminating rays (A) and the image forming rays (B) in a microscope set up with Köhler illumination. The conjugate aperture planes are shown in (A) and the conjugate field planes are shown in (B).

**FIGURE 4-9** The lamp is placed at the center of curvature of a concave mirror to capture the backward-going light. The collecting lenses focus an image of the filament onto the aperture plane at the front focal plane of the sub-stage condenser.
As the aperture diaphragm is closed, points 1 and 3 and bundles 1 and 3 are eliminated. Thus, the angle of illumination and the NA of the condenser are decreased. As the field diaphragm is closed, the size of the viewable field is decreased.

**USING THE BRIGHT-FIELD MICROSCOPE**

When using a microscope, it is as important to prepare your mind and eyes as it is to prepare the specimen and the microscope (Brewster, 1837; Schleiden, 1849; Schacht, 1853; Gage, 1941). Chapters 1 through 3 set the foundation for preparing your mind. In order to prepare your eyes, make sure that you are comfortable sitting at the microscope. Adjust your seat to a comfortable height. Adjust the interpupillary distance of the oculars for your eyes. Set the diopter adjustment to correct for any optical differences between your two eyes. Make sure that the room is dark, and your eyes are relaxed. Focusing with a relaxed eye will prevent eyestrain and prolong your eyes’ ability to accommodate.

Place the specimen on the stage, and focus the specimen with the coarse and fine focus knobs using a low magnification objective. Then close down the field diaphragm, and adjust the height of the sub-stage condenser until the leaves of the diaphragm are sharply focused in the specimen plane. Center the sub-stage condenser if the
field diaphragm is not in the center of the field. Open the field diaphragm so that the light just fills the field. This will minimize glare, which is light that is out of place, just as dirt is matter that is out of place. Then adjust the aperture diaphragm to give optimal contrast and resolution. The cone of light that enters the objective typically is controlled by the aperture diaphragm. Determine which position gives optimal resolution and contrast. Repeat the process with higher magnification objectives. There is no need to raise the objectives before rotating them into the optical path, since all the objectives are parfocal and will give a nearly in-focus image at the position the lower magnification lens gave a focused image. Many excellent books describe the theory and practice of the microscope (Martin, 1966; Slayter, 1970; Zieler, 1972; Rochow and Rochow, 1978; Kallenbach, 1986; Spencer, 1982; Richardson, 1991; Oldfield, 1994; Murphy, 2001).

Abbe (1889, 1906, 1921), a physical (and social) experimentalist and theorist, recommended using small cones of light because "the resulting image produced by means of a broad illuminating beam is always a mixture of a multitude of partial images which are more or less different and dissimilar from the object itself." Moreover, Abbe did not see any reason for believing "that the mixture should come nearer to a strictly correct projection of the object ... by a narrow axial illuminating pencil" since the image of an object actually is formed by double diffraction. The image of an object illuminated with a cone of light will be formed from many different diffraction patterns, and the image will be "a mixture of a multitude of partial images."

On the other hand, many leading microscopists, including E. M. Nelson and the bacteriologist, Robert Koch, suggested that using a broad cone of light instead of axial illumination gives a more faithful image without ghosts. Here is what Nelson (1891) had to say:

The sub-stage condenser is nearly as old as the compound Microscope itself. The first microscopical objects were opaque, and in very early times a lens was employed to condense light upon them. It was an easy step to place the lens below the stage when transparent objects were examined.

On the Continent, where science held a much more important place, the real value of the Microscope was better understood, and it at once took an important place in the medical schools. But the increase of light due to the more perfect concentration of rays by achromatism enabled objects to be sufficiently illuminated by the concave mirror to meet their purposes. Therefore, we find that on the Continent the Microscope had no condenser.

England followed the Continental lead, and now the "foolish philosophical toy" has entirely displaced in our medical schools the dog-Latin text-book with its ordo verborum. But the kind of

![Diagram of microscope components](image1)

**FIGURE 4-13** The objective lens produces an intermediate image of the specimen and the field diaphragm at the field plane of the ocular. If the objective is marked with a 160 or 170, the field plane is 160 or 170 mm behind the objective. If the objective is marked with an \( \infty \), the objective lens produces an intermediate image at infinity and a tube lens is inserted so that the intermediate image is produced at the field plane of the ocular.

![Diagram of intermediate image](image2)

**FIGURE 4-14** The intermediate image is formed between the focal plane and the eye lens of a Ramsden ocular. Together, the eye lens and the eye produce a real image of the specimen, any reticle in the ocular and the field diaphragms on the retina. Without the eye lens, the visual angle of the intermediate image would be tiny. With the eye lens, the visual angle is large and we imagine that we see the specimen enlarged 25 cm from our eye.
Microscope adopted was not that of the English dilettanti, but the condenserless Continental. It may be said that the Microscope for forty years—that is, from the time it was established in the schools in, say, 1810 to 1880, has been without a condenser.

In 1880 a change came from two separate causes—first, the rise of bacteriology; secondly, the introduction of a cheap chromatic condenser by Abbe in 1873.

Taken by itself, the introduction of the Abbe condenser had not much effect, but as Zeiss’s Microscopes had for some time been displacing the older forms, and when the study of bacteriology arose, oil-immersion objectives of greater aperture than the old dry objectives (especially those of the histological series) were used, illumination by the mirror was soon discovered to be inefficient, so a condenser became a necessity. The cheap Abbe condenser was the exact thing to meet the case.

The real office of the sub-stage condenser being a cone-producer, the first question that arises is, What ought to be the angle of the cone?

This is really the most important question that can be raised with regard to microscopical manipulation. To this I reply that a 3/4 cone is the perfection of illumination for the Microscope of the present day. By this I mean that the cone from the condenser should be of such a size as to fill 3/4 of the back of the objective with light, thus N.A. 1.0 is a suitable illuminating cone for an objective of 1.4 N.A. (dark grounds are not at present under consideration). This opinion is in direct opposition to that of Prof. Abbe in his last paper on the subject in the December number of the R.M.S. Journal for 1889, where he says: ‘The resulting image produced by means of a broad illuminating beam is always a mixture of a multitude of partial images which are more or less different (and dissimilar to the object itself). There is not the least rational ground—nor any experimental proof—for the expectation that this mixture should come nearer to a strictly correct project to the object (be less dissimilar to the latter) than that image which is projected by means of a narrow axial illuminating pencil.’

This paper I consider to be the most dangerous paper ever published, and unless a warning is sounded it will inevitably lead to erroneous manipulation, which is inseparably connected with erroneous interpretation of structure.

If you intend to carry out his views and use narrow-angled cones, you do not need a condenser at all—more than this, a condenser is absolutely injurious, because it affords you the possibility of using a large cone, which, according to Prof. Abbe, yields an image dissimilar to the object. If there is the slightest foundation for Prof. Abbe’s conclusion, then a condenser is to be avoided, and when a mirror is used with low powers care must be exercised to cut the cone well down by the diaphragm.

Let me at the place state that I wish it to be distinctly understood that I am not, in this paper, attacking Prof. Abbe’s brilliant discovery that the image in the Microscope is caused by the reunion of rays which have been scattered by diffraction, neither do I question what I venture to think is his far more brilliant experiment, which exhibits the duplication of structure, when the spectra of the second order are admitted, while those of the first are stopped out.

I regard these facts as fundamental truths of microscopy.

What is a microscopist to do when the experts disagree? Trust experience. According to Spitta (1907):

The situation then is exceedingly difficult to deal with; for, when the result of direct experiment, conducted with all the refinement and skill of a master hand like that of Mr. Nelson, coupled with a full scientific appreciation of the situation, seems to point absolutely and directly in the opposite direction to the teaching of a mathematical expert and philosopher such as the late Professor Abbe, undoubtedly was, one who has never been surpassed, if ever equaled, in acuteness of thought coupled with resourcefulness of investigation in all matters concerning the microscope—we repeat, when these opinions are positively at variance, the onlooker is compelled, from shear inability, to wait and consider. We are bound to confess, however, after several years of attention to this difficult and far-reaching problem, the weight of evidence in our
DEPTH OF FIELD

Geometrical optics tells us that there is an image plane where the specimen is in focus. Physical optics tells us that even if we used aberration-free lenses, each point in the object plane is inflated by diffraction to a spheroid at the image plane, and consequently, the resolution that can be obtained at the image plane is limited. Putting these two ideas together, we see that there is a distance in front of and behind the image plane where we will not be able to resolve whether or not the image is in focus. The linear dimension of this region is known as the depth of focus.

The depth of field is a measure of how far away from either side of the object plane an object will be in focus at the true image plane. Thus, the numerical aperture will affect both the depth of focus and the depth of field (Slayter, 1970).

The depth of field (or axial resolution) is defined as the distance between the nearest and farthest object planes in which the objects are in acceptable focus (Delly, 1988). Here we derive the relationship between depth of field and the numerical aperture (NA) using Abbe’s criterion and simple trigonometry (Staves et al., 1995). According to Abbe’s criterion, the minimum distance (d) between two points in which those two points can be resolved is:

\[ d = \lambda/(2NA) \]

We make the assumption that d is the zone of confusion surrounding a point and represents the size of an object that will be in acceptable focus at the nearest and farthest object plane. The depth of field (Y) is the distance between the plane of nearest and farthest acceptable focus (Figure 4-16). Let x = d/2 and y = Y/2. Using the Abbe criterion for resolution, an object that has a linear dimension of d will appear as a point as long as 2x ≤ \lambda/(2NA). The largest that x can be is x = \lambda/(4NA). Given the definition of tangent (\tan \theta = x/y),

\[ x = y \tan \theta \]

After solving for y we get:

\[ y = \lambda/(4NA)(\tan \theta) \]

Since depth of field (Y) is equal to 2y,

\[ Y = \lambda/(2NA)(\tan \theta) \]

Remember that \tan \theta = (\sin \theta)/\cos \theta. Multiply the right side of the equation by (n/n):

\[ \tan \theta = (n \sin \theta)/(n \cos \theta) \]

Since NA = (n sin \theta), then

\[ \tan \theta = NA/(n \cos \theta) \]

Remember that \cos^2 \theta + \sin^2 \theta = 1, and \cos^2 \theta = (1-\sin^2 \theta). Thus,

\[ \cos \theta = \sqrt{(1-\sin^2 \theta)} \]

After substitution, we get

\[ \tan \theta = NA/\sqrt{(n^2(1-\sin^2 \theta))} \]

Since n = \sqrt{n^2},

\[ \tan \theta = NA/\sqrt{(n^2(1-\sin^2 \theta))} \]

Distribute the \sqrt{n^2} on the right side to get

\[ \tan \theta = NA/\sqrt{n^2} \]

Simplify, since \sqrt{n^2\sin^2 \theta} = (n \sin \theta)^2 = NA^2:

\[ \tan \theta = NA/\sqrt{n^2 - NA^2} \]

Substitute into \( Y = \lambda/(2NA)(\tan \theta) \):

\[ Y = \lambda/\sqrt{(n^2 - NA^2)}/(2NA) \]

Simplify:

\[ Y = \lambda/\sqrt{n^2 - NA^2}(2NA) \]
This equation relates the depth of field to the numerical aperture of the objective lens. This equation is based on the validity of the Abbe criterion, the assumption that the “zone of confusion” is equal to d, and the use of illumination where the full NA of the lens is utilized. This equation states that the depth of field is proportional to the wavelength of light and decreases as the numerical aperture of the objective lens increases. Thus for a narrow depth of field, as is prerequisite for the observation of a localized plane, we need an objective lens with a fairly high numerical aperture.

When θ approaches 90 degrees and NA approaches n, the objective lens tends to form an image at a single plane. This is known as optical sectioning. The higher the NA of the objective, the smaller the depth of field and the more we are able to optically section. As the NA increases, the contrast and depth of field decrease, which makes it more difficult to see the specimen. When you first observe a specimen, it is good to close down the aperture diaphragm and get the maximal contrast and depth of field. As you get to know a specimen, you should aim for the greatest axial and transverse resolution.

As we will discuss later in the book, the depth of field can be decreased using illumination methods, such as two-photon confocal microscopy (see Chapter 12), and image processing methods (see Chapter 14).

OUT-OF-FOCUS CONTRAST

I have been discussing the observation of specimens that show amplitude contrast or sufficient scattering-contrast. However, highly transparent objects would be almost invisible in a perfectly focused, aberration-free microscope that captures most of the diffracted rays. However, the bright-field microscope can detect pure phase objects when you defocus the specimen (Figure 4-17). The contrast arises because waves that originate from nearby points in the specimen are partially coherent and can interfere before and behind the image plane. The degree of interference and the intensity of the light at these other planes depend on the relative phase and amplitude of the interfering waves. Since the relative amplitude and phase of these waves depend on the nature of the points from which they originate, they contain some information about the object. So slightly defocusing allows us to see a pure phase object in a bright-field microscope. In the next chapter, I will discuss ways of viewing perfectly focused images of phase objects.

USES OF BRIGHT-FIELD MICROSCOPY

The bright field microscope can be used to characterize chemicals (Chamot, 1921; Schaeffer, 1953), minerals (Smith, 1956; Adams et al., 1984), natural and artificial fibers in textiles (Schwarz, 1934), food (Winton, 1916; Vaughan, 1979; Flint, 1994), microorganisms (Dobell, 1960), and cells and tissues in higher organisms (Lee, 1921; Chamberlain, 1924; Kingsbury and Johannsen, 1927; Conn, 1933; McClung, 1937; Johansen, 1940; Jensen, 1962; Berlyn and Miksche, 1976; Harris, 1999). It has been used in the study of The Shroud of Turin, and in the identification of art forgeries (McCrone, 1990; Weaver, 2003).

Bright-field microscopy has long been used to furnish strong evidence in criminal trials (Robinson, 1935). Typically hair and fibers are identified with a light microscope to see, for example, if the hair of the accused is at the crime scene or if the hair of the victim or a fiber from the victim’s house carpet can be found on the accused (Smith and Glaister, 1931; Rowe and Starrs, 2001). Moreover, since plants have indigestible cell walls, the food that an autopsied homicide victim last ate can readily be identified (Bock et al., 1988). Light microscopes are becoming useful in the United States’ counterterrorism program (Laughlin, 2003).

CARE AND CLEANING OF THE LIGHT MICROSCOPE

First of all, try to keep the microscope and the area around it clean, but let’s face it, we live in a world of dirt and dust. When dirt and dust do fall on the microscope, localize the surface that contains the dirt or dust by rotating or raising or lowering the various components of the microscope and looking through the microscope. The surface that has the dirt on it is the one, which when moved, causes the dirt to move. Remove what dust you can with a dust blower. The dirt can be removed by breathing on the lens, or wetting the lens surface with distilled water of a lens cleaning solution used for cleaning camera lenses. Then gently wipe the lens using a spiral motion moving from the center.
of the lens toward the edge with “no-glue” cotton-tipped cleaning sticks or lint-free lens paper. Immersion oil can be removed the same way. Be careful if you use organic solvents to clean the objectives because the cement that holds a given lens together may dissolve in that solvent. Each microscope manufacturer has its own recommendations on which solvents to use. You can easily check how clean the front surface of the objective lens is by inspecting them by looking through the wrong end of the ocular.

WEB RESOURCES

Molecular Expressions. Exploring the world of optics and microscopy: http://micro.magnet.fsu.edu/

Nikon U: http://www.microscopyu.com/
Olympus Microscopy Resource Center: http://www.olympusmicro.com/
Carl Zeiss Microimaging: http://www.zeiss.com/micro
Leica Microsystems: http://www.leica-microsystems.com/
The Moody Medical Library’s Collection of Microscopes can be viewed online at: http://ar.utmb.edu/areas/informresources/collections/blocker/microscopes.asp
Microscopy UK: http://www.microscopy-uk.org.uk/index.html
McCrone Research Institute: www.mcri.org
Southwest Environmental Health Sciences Center Microscopy & Imaging Resources on the web: http://swehsc.pharmacy.arizona.edu/exppath/micro/index.html
Methods of Generating Contrast

The resolving power attainable with the bright-field microscope is meaningless when we look at invisible, transparent, colorless objects typical of biological specimens. We can make such transparent specimens visible by closing down the aperture diaphragm; but when doing so, contrast is gained at the expense of resolving power. A goal of the light microscopist is to find and develop methods that increase contrast while maintaining the diffraction-limited resolving power inherent in the light microscope. In this chapter I will describe four methods (dark-field, Rheinberg illumination, oblique, and annular illumination) that can increase contrast in the light microscope by controlling the quality and/or quantity of the illuminating light when the microscope is set up for Köhler illumination. When using these four methods, the illumination is controlled by the aperture diaphragm situated at the front focal plane of the sub-stage condenser. I will also describe a method, known as phase-contrast microscopy, which can increase contrast and maintain resolving power by manipulating the light at the back focal plane of the objective lens as well as at the front focal plane of the sub-stage condenser (McLaughlin, 1977). Color plates 3 through 6 give examples of specimens observed with dark-field illumination, oblique illumination, and phase-contrast microscopy.

DARK-FIELD MICROSCOPY

All that is required for dark-field microscopy is to arrange the illuminating system so that the deviated (first- and higher order diffracted light) rays, but not the illuminating (zeroth-order diffracted light) rays enter the objective lens (Gage, 1920, 1925). Dark-field microscopy is as old as microscopy itself, and Antony von Leeuwenhoek, Robert Hooke, and Christiaan Huygens all used dark-field microscopy in the seventeenth century. Leeuwenhoek (in Dobell, 1932) wrote, “… I can demonstrate to myself the globules in the blood as sharp and clean as one can distinguish with one’s eyes, without any help of glasses, sandgrains that one might bestrew upon a piece of black taffety silk.”

Hooke wrote (in Martin, 1988), “If the flame of the candle were directly before the microscope, then all those little creatures appeared perfectly defined by a blackline, and the bodies of them somewhat darker than the water; but if the candle were removed a little out of the axis of vision all those little creatures appeared likewise so many small pearls or little bubbles of air, and the liquid in which they swam appeared dark.”

Huygens wrote (in Martin, 1988), “I look at these animals not directly against the light but on turning the microscope a little which makes them appear on a black ground. One can best discover by this means the smallest animals living and can also distinguish best the parts of larger ones.”

When dark-field illumination is desired, the specimen usually is illuminated with a hollow cone of light. In the absence of a specimen, the illuminating light does not enter the objective lens because the numerical aperture of the sub-stage condenser is larger than the numerical aperture of the objective (Lister, 1830; Reade, 1837; Queckett, 1848; Carpenter, 1856). Special sub-stage condensers are made for dark-field microscopy; however, for low magnifications, a clever and frugal person can create the hollow cone of light by inserting a “spider stop” or a black circular piece of construction paper in the front focal plane of a sub-stage condenser designed for the bright-field microscope. A clever or frugal person with a phase-contrast microscope can create a dark-field microscope by using the 100x phase-contrast annular ring in combination with a 10x or 20x objective. A high-contrast dark-field image can also be obtained by removing the undeviated, zeroth-order diffracted light by inserting an opaque stop in the central region of any plane that is conjugate with the aperture plane, including the back focal plane of the objective and the eye point (Figure 6-1). A dark-field image can also be produced by a bright-field microscope connected to a digital image processor that removes the low-frequency components of the Fourier spectrum in a process known as spatial filtering (Pluta, 1989; Chapter 14).

In order to ensure that the rays emanating from the sub-stage condenser are as oblique as possible, we must raise the sub-stage condenser as high as it goes and open the aperture diaphragm to its full capacity. We can also put water or immersion oil between the sub-stage condenser and the glass slide. Water is used for convenience; immersion oil is used for better resolution.
or eliminate the refraction that takes place at the air-glass interface. When refraction occurs at the air-microscope slide interface, the illuminating rays are refracted toward the optical axis of the light microscope. This means that an objective lens will capture more of the illuminating rays when there is air between the top lens of the sub-stage condenser and the microscope slide than when there is water or oil there. Thus when we place water or oil on top of the sub-stage condenser, we get better contrast with an objective lens with a low numerical aperture or can use an objective lens with a higher numerical aperture to get better resolution (Figure 6-2).

Sub-stage condensers that are especially made for dark-field microscopy are designed to transmit only the most oblique rays (Wenham, 1850, 1854, 1856). Dry dark-field condensers are made to be used with objectives with numerical apertures up to 0.75, and the oil immersion dark-field condensers are made to be used with objectives with numerical apertures up to 1.2. The dry dark-field condensers use prisms that are cut in such a manner that the light that enters the prism is reflected internally so that the light leaving the prism exits at a very oblique angle. Some oil immersion dark-field sub-stage condensers use a convex mirror to bring the light to a concave parabolic mirror, which acts as the main lens (Figure 6-3). Mirrors, unlike glass lenses and prisms, have virtually no chromatic aberration.

Since the dark-field condition requires that the numerical aperture of the objective be smaller than the numerical aperture of the sub-stage condenser, an objective with a variable aperture or iris is very useful for dark-field microscopy. A variable iris in the objective lens lets us adjust the numerical aperture of the objective so that it is “just” smaller than the numerical aperture of the sub-stage condenser and thus obtain optimal resolution and contrast.

Once an object is inserted into the dark-field microscope, the illuminating light that interacts with the specimen is deviated by refraction and/or diffraction. The refracted and/or the first- and higher-order diffracted rays are the only rays that are able to enter the objective. These rays recombine to make the image, and the specimen appears bright on a dark background. Dark-field microscopy is best suited for revealing outlines, edges, and boundaries of objects. It is less useful for the revealing internal details of cells unless there are a lot of highly refractile bodies in a relatively transparent cytosol.

The more oblique the illuminating rays are, the easier it is to detect the presence of very minute objects. Of course the upper limit of obliquity is having the illuminating rays pass perpendicular to the optical axis of the microscope. Microscopes, known as ultramicroscopes, have the dark-field condenser set so that the illuminating rays emerge from the condenser at a 90 degree angle with respect to the optical axis. Typical dark-field microscopes can detect cilia, which are 250 nm in diameter, and single microtubules, which are 24 nm in diameter (Koshland et al., 1988); the ultramicroscopes can detect particles as small as 4 nm (Siedentopf and Zsigmondy, 1903; Hatschek, 1919; Chamot, 1921; Ruthmann, 1970). The volume of a particle is estimated by counting the number of particles in a solution containing a preweighed amount of substance of a known density (Kruyt and van Klooster, 1927; Weiser, 1939). The linear dimensions can then be ascertained by making an assumption about the shape of the particle.

Dark-field microscopes often are used to visualize particles whose size is much smaller than the limit of resolution. How is this possible? Doesn’t the wavelength of light limit the size of a particle that we can see? No, this is a very important point; the limit of resolution is defined as the minimal distance between two object details that can just be recognized as separate structures. Resolving power is truly limited by diffraction. But the concept of the limit of resolution does not apply to the minimal size of a single particle whose presence can be detected because of its light scattering ability. The limit of detection in a dark-field microscope is determined by the amount of contrast attainable between the object and the background. To obtain maximal contrast, the rays that illuminate the object must be extremely bright, and the zeroth-order rays must not
be collected by the objective lens. Moreover to obtain the maximal contrast it is important to have scrupulously clean slides, since every piece of dust will act as a glaring beacon of light. Conrad Beck (1924) says that dirt is “matter out of place” and glare is “light out of place.”

We can infer the presence of minute objects using the naked eye. In a dark room, pierced by a beam of sunlight, we can detect the scattering of light by tiny motes of dust in the beam as long as we do not look directly into the beam. In fact, John Tyndall made use of this optical phenomenon to determine whether or not the room in which he was working while he was performing his experiments on the refutation of the theory of spontaneous generation was dust-free. For this reason, the phenomena of scattering by microscopic objects and the ability to detect minute objects by scattering is often referred to as the Tyndall Effect and Tyndall scattering, respectively (Gage, 1920, 1925).

**RHEINBERG ILLUMINATION**

Rheinberg illumination is a variation of dark-field microscopy first described by Julius Rheinberg in 1896. Rheinberg discovered this method when he accidentally placed colored glass filters in the sub-stage ring (Spitta, 1907). Rheinberg called this type of illumination “multiple or differential color illumination.” It is also known as optical staining since when we use Rheinberg illumination, the image of a normally colorless specimen is made to appear colored without the use of chemical stains. Zeiss introduced Rheinberg illumination under the name of Mikropolychromar in 1933.

When Rheinberg illumination is desired, the central opaque stop of the dark-field microscope is replaced with a transparent, colored circular stop inserted into a transparent ring consisting of a contrasting color (e.g., a red annulus surrounding a green circle). The Rheinberg stop is placed in the front focal plane of the sub-stage condenser. With Rheinberg illumination, the illuminating rays that pass through the annular ring are too oblique to pass through the objective lens and consequently, the background in the image plane is formed only from the illuminating rays that pass through the central area of the circular stop. In order to get the color of the annular ring in the image plane, the light originating from the annular ring must be deviated by refraction and/or diffraction so that it passes into the objective lens. Since the deviated rays originate from the illuminating light passing through the annular ring, the specimens appear to be the color of the annular ring. If we were to observe a protist like *Tetrahymena* with a microscope equipped with a Rheinberg filter with a green central stop inside a red annulus, the protist would appear red swimming in a green background (see Strong, 1968). If we were to use a yellow annulus around a blue central stop, the protist would appear yellow in a sea of blue (Figure 6-4).