OPTICAL MICROSCOPY

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Introduction

The past decade has witnessed an enormous growth in the application of optical microscopy for micron and sub-micron level investigations in a wide variety of disciplines (reviewed in references 1-5). Rapid development of new fluorescent labels has accelerated the expansion of fluorescence microscopy in laboratory applications and research (6-8). Advances in digital imaging and analysis have also enabled microscopists to acquire quantitative measurements quickly and efficiently on specimens ranging from photosensitive caged compounds and synthetic ceramic superconductors to real-time fluorescence microscopy of living cells in their natural environment (2, 9). Optical microscopy, with help of digital video, can also be used to image very thin optical sections, and confocal optical systems are now in operation at most major research institutions (10-12).

Early microscopists were hampered by optical aberration, blurred images, and poor lens design, which floundered until the nineteenth century. Aberrations were partially corrected by the mid-nineteenth century with the introduction of Lister and Amici achromatic objectives that reduced chromatic aberration and raised numerical apertures to around 0.65 for dry objectives and up to 1.25 for homogeneous immersion objectives (13). In 1886, Ernst Abbe's work with Carl Zeiss led to the production of apochromatic objectives based for the first time on sound optical principles and lens design (14). These advanced objectives provided images with reduced spherical aberration and free of color distortions (chromatic aberration) at high numerical apertures.

Several years later, in 1893, Professor August Köhler reported a method of illumination, which he developed to optimize photomicrography, allowing microscopists to take full advantage of the resolving power of Abbe's objectives. The last decade of the nineteenth century saw innovations in optical microscopy, including metallographic microscopes, anastigmatic photolenses, binocular microscopes with image-erecting prisms, and the first stereomicroscope (14).

Early in the twentieth century, microscope manufacturers began parfocalizing objectives, allowing the image to remain in focus when the microscopist exchanged objectives on the rotating nosepiece. In 1824, Zeiss introduced a LeChatelier-style metallograph with infinity-corrected optics, but this method of correction would not see widespread application for another 60 years.

Shortly before World War II, Zeiss created several prototype ***phase contrast*** microscopes based on optical principles advanced by Frits Zernike. Several years later the same microscopes were modified to produce the first time-lapse cinematography of cell division photographed with phase contrast optics (14). This contrast-enhancing technique did not become universally recognized until the 1950s and is still a method of choice for many cell biologists today.

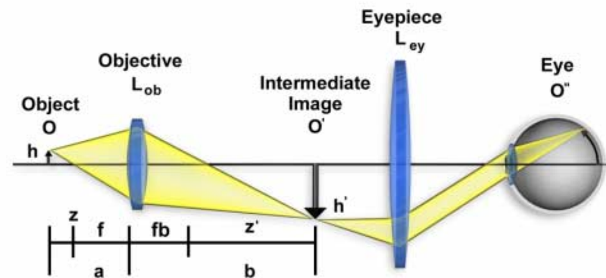
Physicist Georges Nomarski introduced improvements in Wollaston prism design for another powerful contrast-generating microscopy theory in 1955 (15). This technique is commonly referred to as ***Nomarski interference*** or ***differential interference contrast*** (DIC) microscopy and, along with phase contrast, has allowed scientists to explore many new arenas in biology using living cells or unstained tissues. Robert Hoffman (16) introduced another method of increasing contrast in living material by taking advantage of phase gradients near cell membranes. This technique is now termed Hoffman Modulation Contrast, and is available as optional equipment on most modern microscopes.

The majority of microscopes manufactured around the world had fixed mechanical tube lengths (ranging from 160 to 210 millimeters) until the late 1980s, when manufacturers largely migrated to infinity-corrected optics. Ray paths through both finite tube length and infinity-corrected microscopes are illustrated in Figure 1. The upper portion of the figure contains the essential optical elements and ray traces defining the optical train

of a conventional finite tube length microscope (17). An object (**O**) of height **h** is being imaged on the retina of the eye at **O''**. The objective lens (L_{ob}) projects a real and inverted image of **O** magnified to the size **O'** into the intermediate image plane of the microscope. This occurs at the eyepiece diaphragm, at the fixed distance **fb + z'** behind the objective. In this diagram, **fb** represents the back focal length of the objective and **z'** is the optical tube length of the microscope. The aerial intermediate image at **O'** is further magnified by the microscope eyepiece (L_{ey}) and produces an erect image of the object at **O''** on the retina, which appears inverted to the microscopist. The magnification factor of the object is calculated by considering the distance (**a**) between the object (**O**) and the objective (L_{ob}), and the front focal length of the objective lens (**f**). The object is placed a short distance (**z**) outside of the objective's front focal length (**f**), such that **z + f = a**. The intermediate image of the object, **O'**, is located at distance **b**, which equals the back focal length of the objective (**fb**) plus (**z'**), the optical tube length of the microscope. Magnification of the object at the intermediate image plane equals **h'**. The image height at this position is derived by multiplying the microscope tube length (**b**) by the object height (**h**), and dividing this by the distance of the object from the objective: **h' = (h x b)/a**. From this argument, we can conclude that the lateral or transverse magnification of the objective is equal to a factor of **b/a** (also equal to **f/z** and **z'/fb**), the back focal length of the objective divided by the distance of the object from the objective. The image at the intermediate plane (**h'**) is further magnified by a factor of 25 centimeters (called the ***near*** distance to the eye) divided by the focal length of the eyepiece. Thus, the total magnification of the microscope is equal to the magnification by the objective times that of the eyepiece. The visual image (virtual) appears to the observer as if it were 10 inches away from the eye.

Most objectives are corrected to work within a narrow range of image distances, and many are designed to work only in specifically corrected optical systems with matching eyepieces. The magnification inscribed on the objective barrel is defined for the tube length of the microscope for which the objective was designed.

The lower portion of Figure 1 illustrates the optical train using ray traces of an infinity-corrected microscope system. The components of this system are labeled in a similar manner to the finite-tube length system for easy comparison. Here, the magnification of the objective is the ratio **h'/h**, which is determined by the tube lens (L_{tb}). Note the ***infinity space*** that is defined by parallel light beams in every azimuth between the objective and the tube lens. This is the space used by microscope manufacturers to add accessories such as vertical illuminators, DIC prisms, polarizers, retardation plates, etc., with much simpler designs and with little distortion of the image (18). The magnification of the objective in the infinity-corrected system equals the focal length of the tube lens divided by the focal length of the objective.



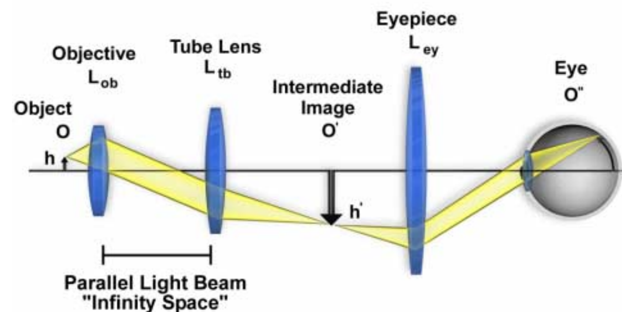


Figure 1. Optical trains of finite-tube and infinity-corrected microscope systems. (Upper) Ray traces of the optical train representing a theoretical finite-tube length microscope. The object (**O**) is a distance (**a**) from the objective (L**ob**) and projects an intermediate image (**O'**) at the finite tube length (**b**), which is further magnified by the eyepiece (**Ley**) and then projected onto the retina at **O''**. (Lower) Ray traces of the optical train representing a theoretical infinity-corrected microscope system.

Fundamentals of Image Formation

In the optical microscope, when light from the microscope lamp passes through the condenser and then through the specimen (assuming the specimen is a light absorbing specimen), some of the light passes both around and through the specimen undisturbed in its path. Such light is called direct light or undeviated light. The background light (often called the surround) passing around the specimen is also undeviated light.

Some of the light passing through the specimen is deviated when it encounters parts of the specimen. Such deviated light (as you will subsequently learn, called diffracted light) is rendered one-half wavelength or 180

degrees out of step (more commonly, out of phase) with the direct light that has passed through undeviated. The one-half wavelength out of phase, caused by the specimen itself, enables this light to cause destructive interference with the direct light when both arrive at the intermediate image plane located at the fixed diaphragm of the eyepiece. The eye lens of the eyepiece further magnifies this image which finally is projected onto the retina, the film plane of a camera, or the surface of a light-sensitive computer chip.

What has happened is that the direct or undeviated light is projected by the objective and spread evenly across the entire image plane at the diaphragm of the eyepiece. The light diffracted by the specimen is brought to focus at various localized places on the same image plane, where the diffracted light causes destructive interference and reduces intensity resulting in more or less dark areas. These patterns of light and dark are what we recognize as an image of the specimen. Because our eyes are sensitive to variations in brightness, the image becomes a more or less faithful reconstitution of the original specimen.

To help understand the basic principles, it is suggested that readers try the following exercise and use as a ***specimen*** an object of known structure, such as a stage micrometer or similar grating of closely spaced dark lines. To proceed, place the finely ruled grating on the microscope stage and bring it into focus using first a 10x and then the 40x objective (18). Remove the eyepiece and, in its place, insert a phase telescope so the rear focal plane of the objective can be observed. If the condenser aperture diaphragm is closed most of the way, a bright white central spot of light will appear at the back of the objective, which is the image of the aperture diaphragm. To the right and left of the central spot, a series of spectra (also images of the aperture diaphragm) will be present, each colored blue on the part closest to the central spot and colored red on the part of the spectrum farthest from the central bright spot (as illustrated in Figure 2). The intensity of these colored spectra decreases according to how far the spectrum is from the central spot (17,18).

Those spectra nearer the periphery of the objective are dimmer than those closer to the central spot. The diffraction spectra illustrated in Figure 2 using three different magnifications. In Figure 2(b), the diffraction pattern visible at the rear focal plane of the 10X objective contains two diffraction spectra. If the grating is removed from the stage, as illustrated in Figure 2(a), these spectra disappear and only the central image of the aperture diaphragm remains. If the grating is reinserted, the spectra reappear once again. Note that the spaces between the colored spectra appear dark. Only a single pair of spectra can be observed if the grating is examined with the 10x objective. In this case, one diffraction spot appears to the left and one appears to the right of the central aperture opening. If the line grating is examined with a 40x objective (as shown in Figure 2(c)), several diffraction spectra appear to the left and right of the central aperture. When the magnification is increased to 60x (and assuming it has a higher numerical aperture than the 40x objective), additional spectra (Figure 2(d)) appear to the right and left than are visible with the 40x objective in place.

Because the colored spectra disappear when the grating is removed, it can be assumed that it is the specimen itself that is affecting the light passing through, thus producing the colored spectra. Further, if the aperture diaphragm is closed down, we will observe that objectives of higher numerical aperture ***grasp*** more of these colored spectra than do objectives of lower numerical aperture. The crucial importance of these two statements for understanding image formation will become clear in the ensuing paragraphs.

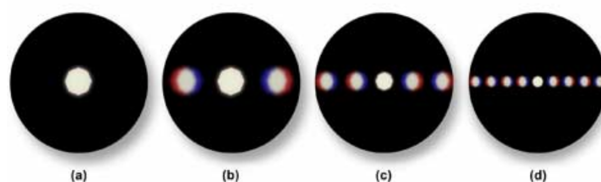


Figure 2. Diffraction spectra seen at the rear focal plane of the objective through a focusing telescope when imaging a closely spaced line grating. (a) Image of the condenser aperture diaphragm with an empty stage. (b) Two diffraction spectra from a 10x objective when a finely ruled line grating is placed on the microscope stage. (c) Diffraction spectra of the line grating from a 40x objective. (d) Diffraction spectra of the line grating from a 60x objective.

The central spot of light (image of the condenser aperture diaphragm) represents the direct or undeviated light passing through the specimen or around the specimen undisturbed (illustrated in Figure 3(b)). It is called the 0th or zeroth order. The fainter images of the aperture diaphragm on each side of the zeroth order are called the 1st, 2nd, 3rd, 4th, etc. orders respectively, as represented by the simulated diffraction pattern in Figure 3(a) that would be observed at the rear focal plane of a 40x objective. All the ***captured*** orders represent, in this case, the diffraction pattern of the line grating as seen at the rear focal plane of the objective (18).

The fainter diffracted images of the aperture diaphragm are caused by light deviated or diffracted, spread out in fan shape, at each of the openings of the line grating (Figure 3(b)). The blue wavelengths are diffracted at a lesser angle than the green wavelengths, which are diffracted at a lesser angle than the red wavelengths.

At the rear focal plane of the objective, the blue wavelengths from each slit interfere constructively to produce the blue area of the diffracted image of each spectrum or order; similarly for the red and green areas (Figure 3(a)). Where the diffracted wavelengths are 1/2 wave out of step for each of these colors, the waves destructively interfere. Hence the dark areas between the spectra or orders. At the position of the zeroth order, all wavelengths from each slit add constructively. This produces the bright white light you see as the zeroth order at the center of the rear focal plane of the objective (Figures 2, 3 and 4).

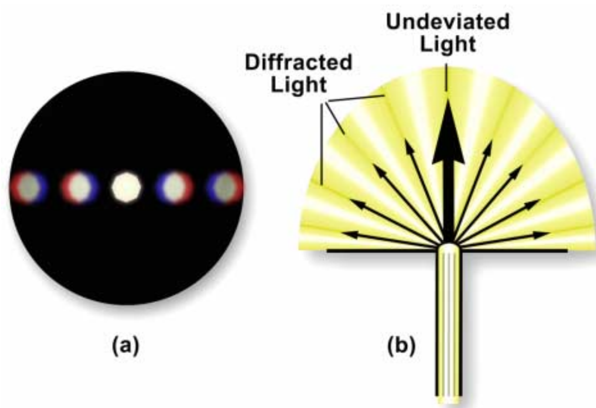


Figure 3. Diffraction spectra generated at the rear focal plane of the objective by undeviated and diffracted light. (a) Spectra visible through a focusing telescope at the rear focal plane of a 40x objective. (b) Schematic diagram of light both diffracted and undeviated by a line grating on the microscope stage.

The closer the spacing of a line grating, the fewer the spectra that will be captured by a given objective, as illustrated in Figure 4(a-c). The diffraction pattern illustrated in Figure 4(a) was captured by a 40x objective imaging the lower portion the line grating in Figure 4(b), where the slits are closer together (17, 18). In Figure 4(c), the objective is focused on the upper portion of the line grating (Figure 4(b)) where the slits are farther apart, and more spectra are captured by the objective. The direct light and the light from the diffracted orders continue on, being focused by the objective, to the intermediate image plane at the fixed diaphragm of the eyepiece. Here the direct and diffracted light rays interfere and are thus reconstituted into the real, inverted image that is ***seen*** by the eye lens of the eyepiece and further magnified. This is illustrated in Figure 4 (d-g) with two types of diffraction gratings. The square grid illustrated in Figure 4(d) represents the orthoscopic image of the grid (i.e. the usual specimen image) as seen through the full aperture of the objective. The diffraction pattern derived from this grid is shown as a conoscopic image that would be seen

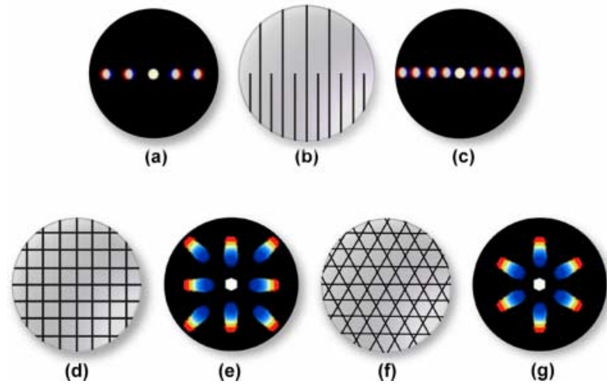


Figure 4. Diffraction patterns generated by narrow and wide slits and by complex grids. (a) Conoscopic image of the grid seen at the rear focal plane of the objective when focused on the wide slit pattern in (b). (b) Orthoscopic image of the grid with greater slit width at the top and lesser width at the bottom. (c) Conoscopic image of the narrow width portion of the grid (lower portion of (b)). (d) and (f) Orthoscopic images of grid lines arranged in a square pattern (d) and a hexagonal pattern (f). (e) and (g) Conoscopic images of patterns in (d) and (f), respectively.

at the rear focal plane of the objective (Figure 4(e). Likewise, the orthoscopic image of a hexagonally arranged grid (Figure 4(f)) produces a corresponding hexagonally arranged conoscopic image of first order diffraction patterns (Figure 4(g)).

Microscope specimens can be considered as complex gratings with details and openings of various sizes. This concept of image formation was largely developed by Ernst Abbe, the famous German microscopist and optics theoretician of the 19th century. According to Abbe (his theories are widely accepted at the present time), the details of a specimen will be resolved if the objective captures the 0th order of the light and at least the 1st order (or any two orders, for that matter). The greater the number of diffracted orders that gain admittance to the objective, the more accurately the image will represent the original object (2, 14, 17, 18).

Further, if a medium of higher refractive index than air (such as immersion oil) is used in the space between the front lens of the objective and the top of the cover slip (as shown in Figure 5(a)), the angle of the diffracted orders is reduced and the fans of diffracted light will be compressed. As a result, an oil immersion objective can capture more diffracted orders and yield better resolution than a dry objective (Figure 5(b)). Moreover, because blue light is diffracted at a lesser angle than green light or red light, a lens of a given aperture may capture more orders of light when the wavelengths are in the blue region of the visible light spectrum. These two principles explain

the classic Rayleigh equation often cited for resolution (2, 18-20):

$$d = 1.22 (l / 2NA) \quad (1)$$

Where **d** is the space between two adjacent particles (still allowing the particles to be perceived as separate), l is the wavelength of illumination, and **NA** is the numerical aperture of the objective.

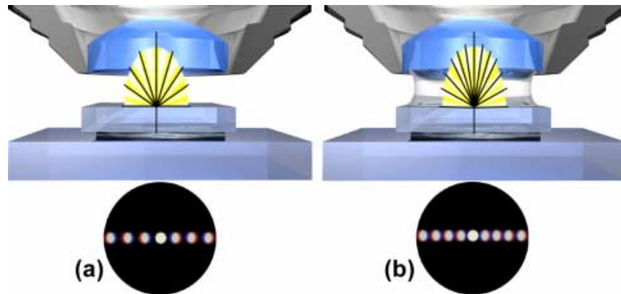


Figure 5. Effect of imaging medium refractive index on diffracted orders captured by the objective. (a) Conoscopic image of objective back focal plane diffraction spectra when air is the medium between the cover slip and the objective front lens. (b) Diffraction spectra when immersion oil of refractive index similar to glass is used in the space between the cover slip and the objective front lens.

The greater the number of higher diffracted orders admitted into the objective, the smaller the details of the specimen that can be clearly separated (resolved). Hence the value of using high numerical aperture for such specimens. Likewise, the shorter the wavelength of visible light used, the better the resolution. These ideas explain why high numerical aperture, apochromatic lenses can separate extremely small details in blue light.

Placing an opaque mask at the back of the objective blocks the outermost diffracted orders. This either reduces the resolution of the grating lines, or any other object details, or it destroys the resolution altogether so that the specimen is not visible. Hence the usual caution not to close down the condenser aperture diaphragm below the suggested 2/3 to 9/10 of the objective's aperture.

Failure of the objective to grasp any of the diffracted orders results in an unresolved image. In a specimen with very minute details, the diffraction fans are spread at a very large angle, requiring a high numerical aperture objective to capture them. Likewise, because the diffraction fans are compressed in immersion oil or in water, objectives designed for such use can give better resolution than dry objectives.

If alternate diffracted orders are blocked out (still assuming the grating as our specimen), the number of lines in the grating will appear doubled (a spurious resolution). The important caveat is that actions introduced at the rear of the objective can have significant effect upon the eventual image produced (18). For small details in a specimen (rather than a grating), the objective projects the direct and diffracted light onto the image plane of the eyepiece diaphragm in the form of small, circular diffraction disks known as Airy disks (illustrated in Figure 6). High numerical aperture objectives capture more of the diffracted orders and produce smaller size disks than do low numerical aperture objectives. In Figure 6, Airy disk size is shown steadily decreasing from Figure 6(a)

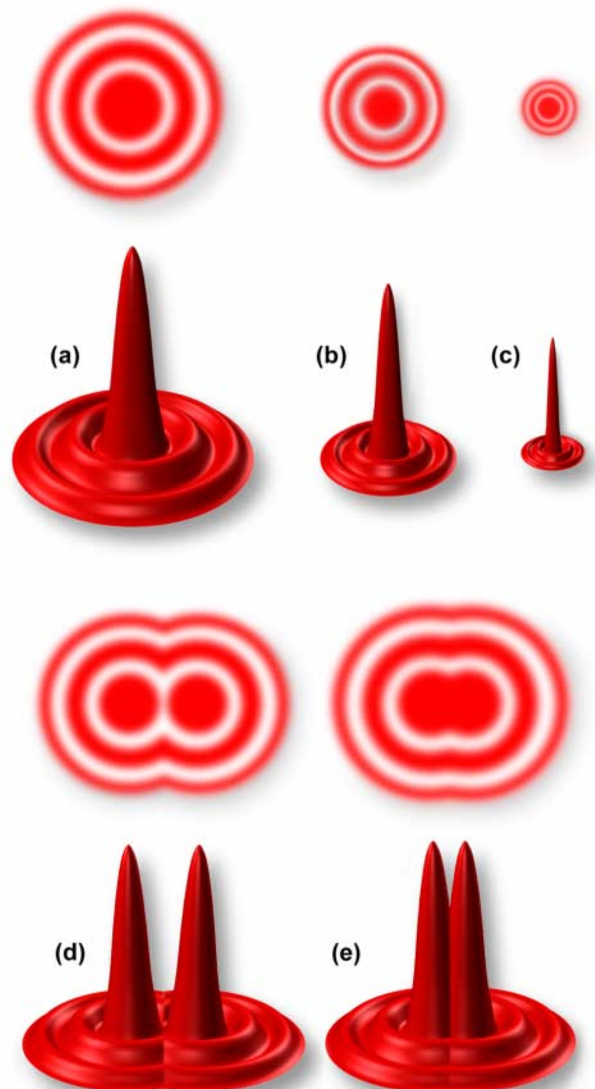


Figure 6. Airy disks and resolution. (a-c) Airy disk size and related intensity profile (point spread function) as related to objective numerical aperture, which decreases from (a) to (c) as numerical aperture increases. (e) Two Airy disks so close together that their central spots overlap. (d) Airy disks at the limit of resolution.

through Figure 6(c). The larger disk sizes in Figures 6(a) and (b) are produced by objectives with lower numerical aperture, while the very sharp Airy disk in Figure 6(c) is produced by an objective of very high numerical aperture (2, 18).

The resulting image at the eyepiece diaphragm level is actually a mosaic of Airy disks which are perceived as light and dark regions of the specimen. Where two disks are so close together that their central black spots overlap considerably, the two details represented by these overlapping disks are not resolved or separated and thus appear as one (illustrated in Figure 6(d)). The Airy disks shown in Figure 6(e) are just far enough apart to be resolved.

The basic principle to be remembered is that the combination of direct and diffracted light (or the manipulation of direct or diffracted light) is critically important in image formation. The key places for such manipulation are the rear focal plane of the objective and the front focal plane of the substage condenser. This principle is fundamental to most of the contrast improvement methods in optical microscopy (18, and see the section on **Contrast Enhancing Techniques**); it is of particular importance at high magnification of small details close in size to the wavelength of light. Abbe was a pioneer in developing these concepts to explain image formation of light-absorbing or ***amplitude*** specimens (2, 18-20).

Köhler Illumination

Proper illumination of the specimen is crucial in achieving high-quality images in microscopy and critical photomicrography. An advanced procedure for microscope illumination was first introduced in 1893 by August Köhler, of the Carl Zeiss corporation, as a method of providing optimum specimen illumination. All manufacturers of modern laboratory microscopes recommend this technique because it produces specimen illumination that is uniformly bright and free from glare, thus allowing the user to realize the microscope's full potential.

Most modern microscopes are designed so that the collector lens and other optical components built into the base will project an enlarged and focused image of the lamp filament onto the plane of the aperture diaphragm of a properly positioned substage condenser. Closing or opening the condenser diaphragm controls the angle of the light rays emerging from the condenser and reaching the specimen from all azimuths. Because the light source is not focused at the level of the specimen, illumination at specimen level is essentially grainless and extended, and does not suffer deterioration from dust and imperfections on the glass surfaces of the condenser. The opening size of the condenser aperture diaphragm, along with the aperture of the objective, determines the realized numerical aperture of the microscope system. As the condenser diaphragm is opened, the working numerical aperture of the microscope increases, resulting in greater light transmittance and resolving power. Parallel light rays that pass through and illuminate the specimen are brought to focus at the rear focal plane of the objective, where the image of the variable condenser aperture diaphragm and the light source are observed in focus simultaneously.

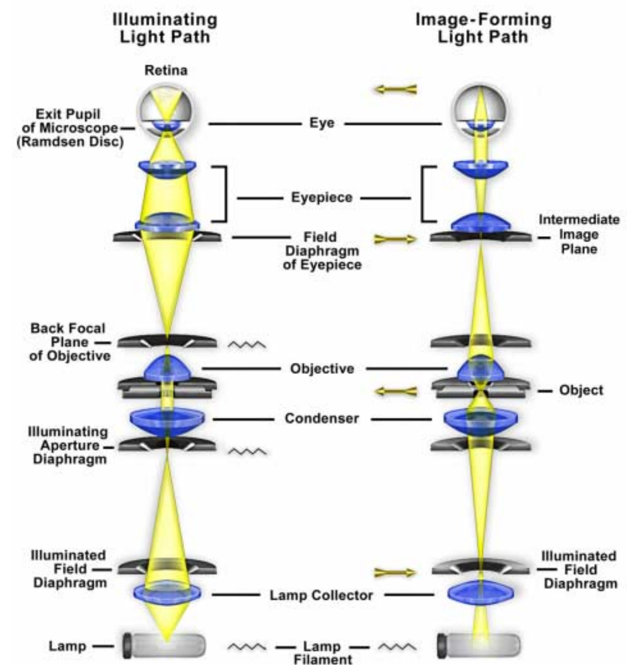


Figure 7. Light paths in Kohler illumination. The illuminating ray paths are illustrated on the left side and the image-forming ray paths on the right. Light emitted from the lamp passes through a collector lens and then through the field diaphragm. The aperture diaphragm in the condenser determines the size and shape of the illumination cone on the specimen plane. After passing through the specimen, light is focused at the back focal plane of the objective and then proceeds to and is magnified by the ocular before passing into the eye.

Light pathways illustrated in Figure 7 are schematically drawn to represent separate paths taken by the specimen-illuminating light rays and the image forming light rays (17). This is not a true representation of any real segregation of these pathways, but a diagrammatic representation presented for purposes of visualization and discussion. The left-hand diagram in Figure 7 demonstrates that the ray paths of illuminating light produce a focused image of the lamp filament at the plane

of the substage condenser aperture diaphragm, the rear focal plane of the objective, and the eyepoint (also called the ***Ramsden disk***) above the eyepiece. These areas in common focus are often referred to as conjugate planes, a principle that is critical in understanding the concept of Köhler illumination (2, 17-21). By definition, an object that is in focus at one plane is also in focus at other conjugate planes of that light path. In each light pathway (both image forming and illumination), there are four separate planes that together make up a conjugate plane set.

Conjugate planes in the path of the illuminating light rays in Köhler illumination (left-hand diagram in Figure 7) include the lamp filament, condenser aperture diaphragm (at the front focal plane of the condenser), the rear focal plane of the objective, and the eyepoint of the eyepiece. The eyepoint is located approximately one-half inch (one centimeter) above the top lens of the eyepiece, at the point where the observer places the front of the eye during observation.

Likewise, the conjugate planes in the image-forming light path in Köhler illumination (right-hand diagram in Figure 7) include the field diaphragm, the focused specimen, the intermediate image plane (i.e., the plane of the fixed diaphragm of the eyepiece), and the retina of the eye or the film plane of the camera. The presence of conjugate focal planes is often useful in troubleshooting a microscope for contaminating dust, fibers, and imperfections in the optical elements. When such artifacts are in sharp focus, it follows that they must reside on or near a surface that is part of the imaging-forming set of conjugate planes. Members of this set include the glass element at the microscope light port, the specimen, and the graticule (if any) in the eyepiece. Alternatively, if these contaminants are out of focus, then they occur near the illuminating set of elements that share conjugate planes. Suspects in this category are the condenser top lens (where dust and dirt often accumulate), the exposed eyepiece lens element (contaminants from eyelashes), and the objective front lens (usually fingerprint smudges).

In Köhler illumination, light emitted from the tungsten-halide lamp filament first passes through a collector lens located close to the lamp housing, and then through a field lens that is near the field diaphragm. A sintered or frosted glass filter is often placed between the lamp and the collector lens to diffuse the light and ensure an even intensity of illumination. In this case, the image of the lamp filament is focused onto the front focal plane of the condenser while the diffuser glass is temporarily removed from the light path. The focal length of the collector lens must be carefully matched to the lamp filament dimensions to ensure that a filament image of the appropriate size is projected into the condenser aperture. For proper Köhler illumination, the image of the filament should completely fill the condenser aperture.

The field lens is responsible for bringing the image of the filament into focus at the plane of the substage condenser aperture diaphragm. A first surface mirror (positioned at a 45-degree angle to the light path) reflects focused light leaving the field lens through the field diaphragm and into the substage condenser. The field diaphragm iris opening serves as a virtual light source for the microscope, and its image is focused by the condenser (raised or lowered) onto the specimen plane. Optical designs for the arrangement of these elements may vary by microscope manufacturer, but the field diaphragm should be positioned at a sufficient distance from the field lens to eliminate dust and lens imperfections from being imaged in the plane of the specimen.

The field diaphragm in the base of the microscope controls only the width of the bundle of light rays reaching the condenser—it does not affect the optical resolution, numerical aperture, or the intensity of illumination. Proper adjustment of the field diaphragm (i.e., focused by adjusting the height of the condenser and centered in the optical path, then opened so as to lie just outside of the field of view) is important for preventing glare, which can reduce contrast in the observed image. The elimination of unwanted light is particularly important when attempting to image specimens with inherently low contrast. When the field diaphragm is opened too far, scattered light originating from the specimen and light reflected at oblique angles from optical surfaces can act to degrade image quality.

The substage condenser is typically mounted directly beneath the microscope stage in a bracket that can be raised or lowered independently of the stage. Control of the aperture diaphragm opening size occurs with either a swinging arm, a lever, or by rotating a collar on the condenser housing. The most critical aspect of achieving proper Köhler illumination is correct adjustment of the substage condenser. Condenser misalignment and an improperly adjusted condenser aperture diaphragm are the main sources of image degradation and poor quality photomicrography (19).

When properly adjusted, light from the condenser will fill the rear focal plane of the objective and project a cone of light into the field of view. The condenser aperture diaphragm is responsible for controlling the angle of the illuminating light cone and, consequently, the working numerical aperture of the condenser. It is important to note, with respect to the size and shape of condenser light cones, that reducing the size of the field diaphragm only serves to slightly decrease the size of the lower portions of the light cone. The angle and numerical aperture of

the light cone remains essentially unchanged with reduction in field diaphragm size (21). Illumination intensity should not be controlled through opening and closing the condenser aperture diaphragm, or by shifting the condenser up and down or axially with respect to the optical center of the microscope. It should only be controlled through the use of neutral density filters placed into the light path or by reducing voltage to the lamp (although the latter is not usually recommended, especially for photomicrography). To ensure the maximum performance of the tungsten-halide lamp, refer to the manufacturer's instrument manual to determine the optimum lamp voltage (usually 5-10 volts) and use that setting. Adding or removing neutral density filters can then easily control brightness of the illumination without affecting color temperature.

The size of the substage condenser aperture diaphragm opening should not only coincide with the desired numerical aperture, but also the quality of the resulting image should be considered. In general, the diaphragm should be set to a position that allows 2/3 to 9/10 (60 to 90 percent) of the entire light disc size (visible at the rear focal plane of the objective after removal of the eyepiece or with a Bertrand lens). These values may vary with extremes in specimen contrast.

The condenser aperture diaphragm should be set to an opening size that will provide a compromise of resolution and contrast that depends, to a large degree, on the absorption, diffraction, and refraction characteristics of the specimen. This adjustment must be accomplished without overwhelming the image with artifacts that obscure detail and present erroneous enhancement of contrast. The amount of image detail and contrast necessary to produce the best photomicrograph is also dependent upon refractive index, optical characteristics, and other specimen-dependent parameters.

When the aperture diaphragm is erroneously closed too far, resulting diffraction artifacts cause visible fringes, banding, and/or pattern formation in photomicrographs. Other problems, such as refraction phenomena, can also produce apparent structures in the image that are not real (21). Alternatively, opening the condenser aperture too wide causes unwanted glare and light scattering from the specimen and optical surfaces within the microscope, leading to a significant loss of contrast and washing out of image detail. The correct setting will vary from specimen to specimen, and the experienced microscopist will soon learn to accurately adjust the condenser aperture diaphragm (and numerical aperture of the system) by observing the image without necessarily having to view the diaphragm in the rear focal plane of the objective. In fact, many microscopists (including the authors) believe that critical adjustment of the numerical aperture of the microscope system to optimize image quality is the single most important step in photomicrography.

The illumination system of the microscope, when adjusted for proper Köhler illumination, must satisfy several requirements. The illuminated area of the specimen plane must no larger than the field of view for any given objective/eyepiece combination. Also, the light must be of uniform intensity and the numerical aperture may vary from a maximum (equal to that of the objective) to a lesser value that will depend upon the optical characteristics of the specimen. Table 1 contains a list of objective numerical apertures versus the field of view diameter (for an eyepiece of field number 22 with no tube lens present – see discussion on field number) for each objective, ranging from very low to very high magnifications.

Many microscopes are equipped with specialized substage condensers that have a swing-out top lens, which can be removed from the optical path for use with lower power objectives (2x through 5x). This action changes the performance of the remaining components in the light path, and some adjustment is necessary to achieve the best illumination conditions. The field diaphragm can no longer be used for alignment and centering of the substage condenser and is now ineffective in limiting the area of the specimen under illumination. Also, much of the unwanted glare once removed by the field diaphragm is reduced because the top lens of the condenser produces a light cone having a much lower numerical aperture, allowing light rays to pass through the specimen at much lower angles. Most important, the optical conditions for

Table 1 Viewfield Diameters (FN 22) (SWF 10x Eyepiece)

Objective Magnification	Diameter (mm)
1/2x	44.0
1x	22.0
2x	11.0
4x	5.5
10x	2.2
20x	1.1
40x	0.55
50x	0.44
60x	0.37
100x	0.22
150x	0.15
250x	0.088

[a] Source: Nikon

Köhler illumination no longer apply.

For low power objectives (2x to 5x), alignment of the microscope optical components and the establishment of Köhler illumination conditions should always be undertaken at a higher (10x) magnification before removing the swing-out condenser lens for work at lower (5x and below) magnifications. The height of the condenser should then not be changed. Condenser performance is radically changed when the swing-out lens is removed (18, 21). The image of the lamp filament is no longer formed in the aperture diaphragm, which ceases to control the numerical aperture of the condenser and the illumination system. In fact, the aperture diaphragm should be opened completely to avoid vignetting, a gradual fading of light at the edges of the viewfield.

Contrast adjustment in low magnification microscopy is then achieved by adjustment of the field diaphragm (18, 19, 21). When the field diaphragm is wide open (greater than 80 percent), specimen details are washed out and a significant amount of scattering and glare is present. Closing the field diaphragm to a position between 50 and 80 percent will yield the best compromise on specimen contrast and depth of field. This adjustment is now visible at the rear focal plane of the objective when the eyepiece is removed or a Bertrand lens is inserted into the eye tube. Objectives designed for low magnification are significantly simpler in design than their higher magnification counterparts. This is due to the smaller angles of illuminating light cones produced by low magnification condensers, which require objectives of lower numerical aperture.

Measurement graticules, which must be in sharp focus and simultaneously superimposed on the specimen image, can be inserted into any of several conjugate planes in the image-forming path. The most common eyepiece (ocular) measuring and photomicrography graticules are placed in the intermediate image plane, which is positioned at the fixed diaphragm within the eyepiece. It is theoretically possible to also place graticules in any image-forming conjugate plane or in the plane of the illuminated field diaphragm. Stage micrometers are specialized ***graticules*** placed on microslides, which are used to calibrate eyepiece graticules and to make specimen measurements.

Color and neutral density filters are often placed in the optical pathway to reduce light intensity or alter the color characteristics of the illumination. There are several locations within the microscope stand where these filters are usually placed. Some modern laboratory microscopes have a filter holder sandwiched between the lamp housing and collector lens, which serves as an ideal location for these filters. Often, neutral density filters along with color correction filters and a frosted diffusion filter are placed together in this filter holder. Other microscope designs provide a set of filters built internally into the body, which can be toggled into the light path by means of levers. A third common location for filters is a holder mounted on the bottom of the substage condenser, below the aperture diaphragm, that will accept gelatin or glass filters.

It is important not to place filters in or near any of the image-forming conjugate planes to avoid dirt or surface imperfections on the filters being imaged along with the specimen (22). Some microscopes have an attachment for placing filters near the light port at the base (near the field diaphragm). This placement is probably too close to the field diaphragm, and surface contamination may be either in sharp focus or appear as blurred artifacts superimposed onto the image. It is also not wise to place filters directly on the microscope stage for the same reasons.

Microscope Objectives, Eyepieces, Condensers, and Optical Aberrations

Finite microscope objectives are designed to project a diffraction-limited image at a fixed plane (the **intermediate** image plane) that is dictated by the microscope tube length and located at a pre-specified distance from the rear focal plane of the objective. Specimens are imaged at a very short distance beyond the front focal plane of the objective through a medium of defined refractive index, usually air, water, glycerin, or specialized immersion oils. Microscope manufacturers offer a wide range of objective designs to meet the performance needs of specialized imaging methods (2, 6, 9, 18-21, and see the section on **Contrast Enhancing Techniques**), to compensate for cover glass thickness variations, and to increase the effective working distance of the objective.

All of the major microscope manufacturers have now changed their design to infinity-corrected objectives. Such objectives project emerging rays in parallel bundles from every azimuth to infinity. They require a tube lens in the light path to bring the image into focus at the intermediate image plane.

The least expensive (and most common) objectives are the achromatic objectives, which are corrected for axial chromatic aberration in two wavelengths (red and blue) that are brought into the same focus. Further, they are corrected for spherical aberration in the color green, as described in Table 2. The limited correction of achromatic objectives leads to problems with color microscopy and photomicrography. When focus is chosen in the red-blue region of the spectrum, images will have a green halo (often termed ***residual color***). Achromatic

Table 2 Objective Lens Types and Corrections [a]

Type	Spherical	Chromatic	Flatness Correction
	Corrections for Aberrations		
Achromat	* [b]	2 [c]	No
Plan Achromat	* [b]	2 [c]	Yes
Fluorite	3 [d]	< 3 [d]	No
Plan Fluorite	3 [d]	< 3 [d]	Yes
Plan Apochromat	4 [e]	> 4 [e]	Yes

[a] Source: Nikon Instrument Group
[b] Corrected for two wavelengths at two specific aperture angles.
[c] Corrected for blue and red - broad range of the visible spectrum.
[d] Corrected for blue, green and red - full range of the visible spectrum.
[e] Corrected for dark blue, blue, green and red.

objectives yield their best results with light passed through a green filter (often an interference filter) and using black and white film when these objectives are employed for photomicrography. The lack of correction for flatness of field (or field curvature) further hampers achromat objectives. In the past few years, most manufacturers have begun providing flat field corrections for achromat objectives and have given these corrected objectives the name of plan achromats.

The next higher level of correction and cost is found in objectives called fluorites or semi-apochromats illustrated by the center objective in Figure 8. This figure depicts three major classes of objectives: The achromats with the least amount of correction, as discussed above; the fluorites (or semi-apochromats) that have additional spherical corrections; and, the apochromats that are the most highly corrected objectives available. Fluorite objectives are produced from advanced glass formulations that contain materials such as fluorspar or newer synthetic substitutes (5). These new formulations allow for greatly improved correction of optical aberration. Similar to the achromats, the fluorite objectives are also corrected chromatically for red and blue light. In addition, the fluorites are also corrected spherically for two colors. The superior correction of fluorite objectives compared to achromats enables these objectives to be made with a higher numerical aperture, resulting in brighter images. Fluorite objectives also have better resolving power than achromats and provide a higher degree of contrast, making them better suited than achromats for color photomicrography in white light.

The highest level of correction (and expense) is found in apochromatic objectives, which are corrected chromatically for three colors (red, green, and blue), almost eliminating chromatic aberration, and are corrected spherically for two colors. Apochromatic objectives are the best choice for color photomicrography in white light. Because of their high level of correction, apochromat objectives usually have, for a given magnification, higher numerical apertures than do achromats or fluorites. Many of the newer high-end fluorite and apochromat objectives are corrected for four colors chromatically and four colors spherically.

All three types of objectives suffer from pronounced field curvature and project images that are curved rather than flat. To overcome this inherent condition, lens designers have produced flat-field corrected objectives that yield flat images. Such lenses are called plan achromats, plan fluorites, or plan apochromats, and although this degree of correction is expensive, these objectives are now in routine use due to their value in photomicrography.

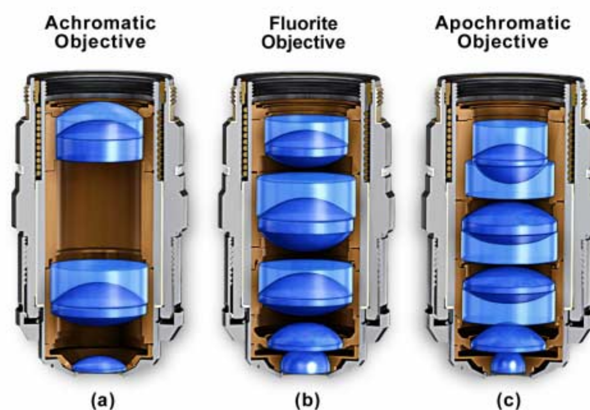


Figure 8. Levels of optical correction for aberration in commercial objectives. (a) Achromatic objectives, the lowest level of correction, contain two doublets and a single front lens; (b) Fluorites or semi-apochromatic objectives, a medium level of correction, contain three doublets, a meniscus lens, and a single front lens; and (c) Apochromatic objectives, the highest level of correction, contain a triplet, two doublets, a meniscus lens, and a single hemispherical front lens.

Uncorrected field curvature is the most severe aberration in higher power fluorite and apochromat objectives, and it was tolerated as an unavoidable artifact for many years. During routine use, the viewfield would have to be continuously refocused between the center and the edges to capture all specimen details. The introduction of flat-field (plan) correction to objectives perfected their use for photomicrography and video microscopy, and today these corrections are standard in both general use and high-performance objectives. Correction for field

curvature adds a considerable number of lens elements to the objective, in many cases as many as four additional lenses. This significant increase in the number of lens elements for plan correction also occurs in already overcrowded fluorite and apochromat objectives, frequently resulting in a tight fit of lens elements within the objective barrel (4, 5, 18).

Before the transition to infinity-corrected optics, most objectives were specifically designed to be used with a set of oculars termed ***compensating eyepieces***. An example is the former use of compensating eyepieces with highly corrected high numerical aperture objectives to help eliminate lateral chromatic aberration.

There is a wealth of information inscribed on the barrel of each objective, which can be broken down into several categories (illustrated in Figure 9). These include the linear magnification, numerical aperture value, optical corrections, microscope body tube length, the type of medium the objective is designed for, and other critical factors in deciding if the objective will perform as needed. Additional information is outlined below (17):

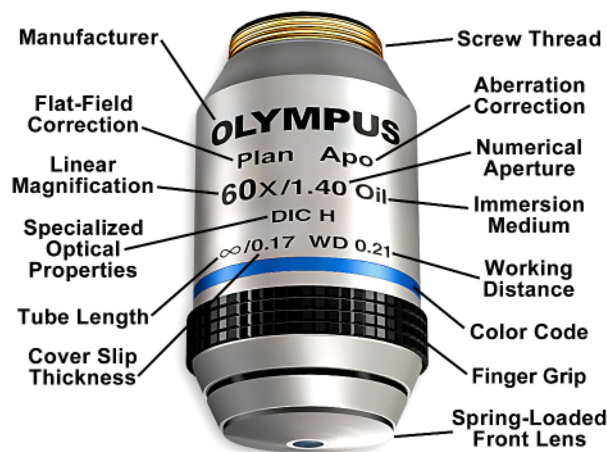


Figure 9. Specifications engraved on the barrel of a typical microscope objective. These include the manufacturer, correction levels, magnification, numerical aperture, immersion requirements, tube length, working distance, and specialized optical properties.

· **Optical Corrections:** These are usually abbreviated as **Achro** (achromat), **Apo** (apochromat), and **Fl, Fluar, Fluor, Neofluar, or Fluotar** (fluorite) for better spherical and chromatic corrections, and as **Plan, Pl, EF, Acroplan, Plan Apo** or **Plano** for field curvature corrections. Other common abbreviations are: **ICS** (infinity corrected system) and **UIS** (universal infinity system), **N** and **NPL** (normal field of view plan), **Ultrafluar** (fluorite objective with glass that is transparent down to 250 nanometers), and **CF** and **CFI** (chrome-free; chrome-free infinity).

· **Numerical Aperture:** This is a critical value that indicates the light acceptance angle, which in turn determines the light gathering power, the resolving power, and depth of field of the objective. Some objectives specifically designed for transmitted light fluorescence and darkfield imaging are equipped with an internal iris diaphragm that allows for adjustment of the effective numerical aperture. Designation abbreviations for these objectives include **I, Iris, W/Iris**.

· **Mechanical Tube Length:** This is the length of the microscope body tube between the nosepiece opening, where the objective is mounted, and the top edge of the observation tubes where the oculars (eyepieces) are inserted. Tube length is usually inscribed on the objective as the size in number of millimeters (160, 170, 210, etc.) for fixed lengths, or the infinity symbol (∞) for infinity-corrected tube lengths.

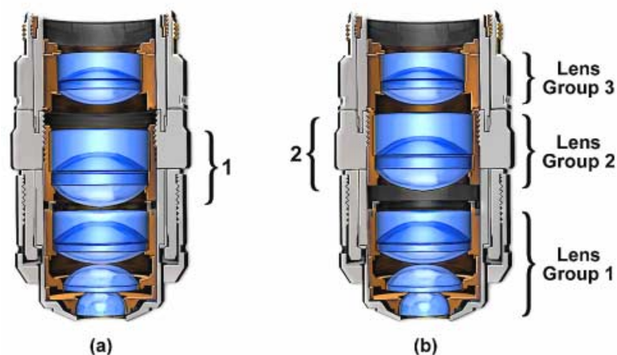


Figure 10. Objective with three lens groups and correction collar for varying cover glass thicknesses. (a) Lens group 2 rotated to the forward position within the objective. This position is used for the thinnest cover slips. (b) Lens group 2 rotated to the rearward position within the objective. This position is used for the thickest coverslips.

· **Cover Glass Thickness:** Most transmitted light objectives are designed to image specimens that are covered by a cover glass (or cover ***slip***). The thickness of these small glass plates is now standardized at 0.17 mm for most applications, although there is some variation in thickness within a batch of cover slips. For this reason, some of the high numerical aperture dry objectives have a correction collar adjustment of the internal lens elements to compensate for this variation (Figure 10). Abbreviations for the correction collar adjustment include **Corr, w/Corr,** and **CR,** although the presence of a movable, knurled collar and graduated scale is also an

indicator of this feature.

· **Working Distance:** This is the distance between the objective front lens and the top of the cover glass when the specimen is in focus. In most instances, the working distance of an objective decreases as magnification increases. Working distance values are not included on all objectives and their presence varies depending upon the manufacturer. Common abbreviations are: **L, LL, LD,** and **LWD** (long working distance); **ELWD** (extra-long working distance); **SLWD** (super-long working distance), and **ULWD** (ultra-long working distance).

· **Objective Screw Threads:** The mounting threads on almost all objectives are sized to standards of the Royal Microscopical Society (RMS) for universal compatibility. This standard specifies mounting threads that are 20.32 mm in diameter with a pitch of 0.706, which is currently used in the production of infinity-corrected objectives by manufacturers Olympus and Zeiss. Leica and Nikon have broken from the standard with the introduction of new infinity-corrected objectives that have a wider mounting thread size, making Leica and Nikon objectives usable only on their own microscopes. Abbreviations commonly used are: **RMS** (Royal Microscopical Society objective thread), **M25** (metric 25-mm objective thread), and **M32** (metric 32-mm objective thread).

· **Immersion Medium:** Most objectives are designed to image specimens with air as the medium between the objective and the cover glass. To attain higher working numerical apertures, many objectives are designed to image the specimen through another medium that reduces refractive index differences between glass and the imaging medium. High-resolution plan apochromat objectives can achieve numerical apertures up to 1.40 when the immersion medium is special oil with a refractive index of 1.51. Other common immersion media are water and glycerin. Objectives designed for special immersion media usually have a color-coded ring inscribed around the circumference of the objective barrel as listed in Table 3 and described below.

· **Color Codes:** Many microscope manufacturers label their objectives with color codes to help in rapid identification of the magnification. The dark blue color code on the objective illustrated in Figure 9 indicates the linear magnification is 60x. This is very helpful when you have a nosepiece turret containing 5 or 6 objectives and you must quickly select a specific magnification. Some specialized objectives have an additional color code that indicates the type of immersion medium necessary to achieve the optimum numerical aperture. Immersion lenses intended for use with oil have a black color ring, while those intended for use with glycerin have an orange ring. Objectives designed to image living organisms in aqueous media are designated ***water immersion*** objectives with a white ring and highly specialized objectives for unusual immersion media often are engraved with a red ring. Table 3 lists current magnification and imaging media color codes in use by most manufacturers.

Table 3 Color-Coded Rings on Microscope Objectives

Immersion color code [a]	**Immersion type**
Black	Oil immersion
Orange	Glycerol immersion
White	Water immersion
Red	Special
Magnification color code [b]	**Magnification**
Black	1x, 1.25x
Brown	2x, 2.5x
Red	4x, 5x
Yellow	10x
Green	16x, 20x
Turquoise blue	25x, 32x
Light blue	40x, 50x
Cobalt (dark) blue	60x, 63x
White (cream)	100x

[a] Narrow colored ring located near the specimen end of objective.
[b] Narrow band located closer to the mounting thread than the immersion code.

· **Specialized Optical Properties:** Microscope objectives often have design parameters that optimize performance under certain conditions. For example, there are special objectives designed for polarized illumination (signified by the abbreviations **P, Po, Pol,** or **SF,** and/or having all barrel engravings painted red), phase contrast (**PH**, and/or green barrel engravings), differential interference contrast (**DIC**), and many other abbreviations for additional applications. The apochromat objective illustrated in Figure 9 is optimized for DIC photomicrography and this is indicated on the barrel. The capital H beside the DIC marking indicates that the objective must be used with a specific DIC prism optimized for high-magnification applications.

There are some applications that do not require objectives designed to be corrected for cover glass thickness. These include objectives used to observe uncovered specimens in reflected light metallurgical specimens, integrated circuit inspection, micro machinery, biological smears, and other applications that require observation of uncovered objects. Other common abbreviations found on microscope objective barrels, which are useful in identifying specific properties, are listed in Table 4 (17).

Table 4 Specialized Objective Designations

Abbreviation	Type
Phase, PHACO, PC, Ph 1,2, 3, etc.	Phase contrast, using phase condenser annulus 1, 2, 3, etc.
DL, DM, PLL, PL, PM, PH, NL, NM, NH	Phase contrast: dark low, dark medium, positive low, positive low, positive medium, positive high contrast (regions with higher refractive index appear darker); negative low, negative medium, negative high contrast (regions with higher refractive index appear lighter)
P, Po, Pol, SF	Strain-free, low birefringence, for polarized light
U, UV, Universal	UV transmitting (down to approx. 340 nm), for UV-excited epifluorescence
M	Metallographic (no coverslip)
NC, NCG	No coverslip
EPI	Surface illumination (specimen illuminated through objective lens), as contrasted to dia- or transillumination
TL	Transmitted light
BBD, HD, B\D	For use in bright or dark field (hell, dunkel)
D	Dark field
H	Designed primarily for heating stage
U, UT	Designed to be used with universal stage (magnification/NA applied for use with glass hemisphere; divine both values by 1.51 when hemisphere is not used)
DI; MI; TI Michelson	Interferometry; noncontact; multiple beam (Tolanski)

[a] Many of the designation codes are manufacturer specific.

Optical Aberrations

Lens errors or aberrations in optical microscopy are caused by artifacts arising from the interaction of light with glass lenses (2-5, 19-24). There are two primary causes of aberration: (i) geometrical or spherical aberrations are related to the spherical nature of the lens and approximations used to obtain the Gaussian lens equation; and (ii) chromatic aberrations that arise from variations in the refractive indices of the wide range of frequencies found in visible light.

In general, the effects of optical aberrations are to induce faults in the features of an image being observed through a microscope. These artifacts were first addressed in the eighteenth century when physicist John Dollond discovered that chromatic aberration would be reduced or corrected by using a combination of two different types of glass (flint and crown) in the fabrication of lenses (13). Later, during the nineteenth century, achromatic objectives with high numerical aperture were developed, although there were still geometrical problems with the lenses. Modern glass formulations coupled with advanced grinding and manufacturing techniques have all but eliminated most aberrations from today's microscope objectives, although careful attention must still be paid to these effects, especially when conducting quantitative high-magnification video microscopy and photomicrography (23).

Spherical Aberration: These artifacts occur when light waves passing through the periphery of a lens are not brought into identical focus with those passing closer to the center. Waves passing near the center of the lens are refracted only slightly, whereas waves passing near the periphery are refracted to a greater degree resulting in the production of different focal points along the optical axis. This is one of the most serious resolution artifacts because the image of the specimen is spread out rather than being in sharp focus. Spherical aberrations are very important in terms of the resolution of the lens because they affect the coincident imaging of points along the optical axis and degrade the performance of the lens, which will seriously affect specimen sharpness and clarity. These lens defects can be reduced by limiting the outer edges of the lens from exposure to light using diaphragms and also by utilizing aspherical lens surfaces within the system. The highest-quality modern microscope objectives address spherical aberrations in a number of ways including special lens-grinding techniques, additional lens elements of different curvatures, improved glass formulations, and better control of optical pathways.

Chromatic Aberration: This type of optical defect is a result of the fact that white light is composed of numerous wavelengths. When white light passes through a convex lens, the component wavelengths are refracted according to their frequency. Blue light is refracted to the greatest extent followed by green and red light, a phenomenon commonly referred to as dispersion. The inability of the lens to bring all of the colors into a

common focus results in a slightly different image size and focal point for each predominant wavelength group. This leads to color fringes surrounding the image.

Lens corrections were first attempted in the latter part of the 18th century when Dollond, Lister and others devised ways to reduce longitudinal chromatic aberration (13). By combining crown glass and flint glass (each type has a different dispersion of refractive index), they succeeded in bringing the blue rays and the red rays to a common focus, near but not identical with the green rays. This combination is termed a lens doublet where each lens has a different refractive index and dispersive properties. Lens doublets are also known as achromatic lenses or achromats for short, derived from the Greek terms a meaning without and ***chroma*** meaning color. This simple form of correction allows the image points at 486 nanometers in the blue region and 656 nanometers in the red region to now coincide. This is the most widely used objective lens and is commonly found on laboratory microscopes. Objectives that do not carry a special inscription stating otherwise are likely to be achromats. Achromats are satisfactory objectives for routine laboratory use, but because they are not corrected for all colors, a colorless specimen detail is likely to show, in white light, a pale green color at best focus (the so-called secondary spectrum).

A proper combination of lens thickness, curvature, refractive index, and dispersion allows the doublet to reduce chromatic aberration by bringing two of the wavelength groups into a common focal plane. If fluorspar is introduced into the glass formulation used to fabricate the lens, then the three colors red, green, and blue can be brought into a single focal point resulting in a negligible amount of chromatic aberration (23). These lenses are known as apochromatic lenses and they are used to build very high-quality chromatic aberration-free microscope objectives. Modern microscopes utilize this concept and today it is common to find optical lens triplets made with three lens elements cemented together, especially in higher-quality objectives. For chromatic aberration correction, a typical 10x achromat microscope objective is built with two lens doublets (Figure 8(a)). Apochromat objectives usually contain two lens doublets and a lens triplet (Figure 8(c)) for advanced correction of both chromatic and spherical aberrations.

Despite longitudinal (or axial) chromatic aberration correction, apochromat objectives also exhibit another chromatic defect. Even when all three main colors are brought to identical focal planes axially, the point images of details near the periphery of the field of view, are not the same size; e.g., the blue image of a detail is slightly larger than the green image or the red image in white light, thus causing color ringing of specimen details at the outer regions of the field of view (23). This defect is known as lateral chromatic aberration or chromatic difference of magnification. It is the compensating eyepiece, with chromatic difference of magnification just the opposite of that of the objective, which is utilized to correct for lateral chromatic aberration. Because this defect is also found in higher magnification achromats, compensating eyepieces are frequently used for such objectives, too. Indeed, many manufacturers design their achromats with a standard lateral chromatic error and use compensating eyepieces for all their objectives. Such eyepieces often carry the inscription **K** or **C** or **Compens**. As a result, compensating eyepieces have build-in lateral chromatic error and are not, in themselves, perfectly corrected.

Coverslip Correction: It is possible for the user to inadvertently introduce spherical aberration into a well-corrected system (2, 23). For example, when using high magnification and high numerical aperture dry objectives (NA = 0.85-0.95), the correct thickness of the cover glass (suggested 0.17 mm) is critical; hence the inclusion of a correction collar on such objectives to enable adjustment for incorrect cover glass thickness. Similarly, the insertion of accessories in the light path of finite tube length objectives may introduce aberrations, apparent when the specimen is refocused, unless such accessories have been properly designed with additional optics. Figure 10 illustrates how internal lenses operate in an objective designed for coverslip correction.

Other Geometrical Aberrations: These include a variety of effects including astigmatism, field curvature, and comatic aberrations, which are corrected with proper lens fabrication.

Curvature of field in the image is an aberration that is familiar to most experienced microscopists. This artifact is the natural result of using lenses that have curved surfaces. When visible light is focused through a curved lens, the image plane produced by the lens will be curved. When the image is viewed in the eyepieces (oculars) of a microscope, it either appears sharp and crisp in the center or on the edges of the viewfield but not both. Normally, this is not a serious problem when the microscopist is routinely scanning samples to observe their various features. It is a simple matter to use the fine focus knob to correct small deficiencies in specimen focus. However, for photomicrography, field curvature can be a serious problem, especially when a portion of the photomicrograph is out of focus.

Modern microscopes deal with field curvature by correcting this aberration using specially designed flat-field objectives. These specially corrected objectives have been named plan or plano and are the most common type of objective in use today. Plan objectives are also corrected for other optical artifacts such as spherical and

chromatic aberrations. In the case of a plan objective that also has been mostly corrected for chromatic aberration, the objective is referred to as a plan achromat. This is also the case for fluorite and apochromatic objectives, which have the modified names: plan fluorite and plan apochromat.

Adding field curvature lens corrections to an objective that has already been corrected for optical aberrations can often add a significant number of lens elements to the objective. For example, the typical achromat objective has two lens doublets and a hemispherical lens, making of total of five lens elements. In contrast, a comparable plan achromat objective has three doublets and three single lenses for a total of nine lens elements, making it considerably more difficult to fabricate. As we have seen, the number of lens elements increases as lenses are corrected for spherical errors as well as chromatic and field curvature aberrations. Unfortunately, as the number of lens elements increases so does the cost of the objective.

Sophisticated plan apochromatic objectives that are corrected for spherical, chromatic, and field curvature aberrations can contain as many as eighteen to twenty separate lens elements, making these objectives the most expensive and difficult to manufacture. Plan apochromatic objectives can cost upward of $3,000 to $5,000 each for high-magnification units that also have a high numerical aperture. For most photomicrography applications, however, it is not absolutely necessary to have the best correction, although this is heavily dependent upon the purpose, the specimen, and the desired magnification range. When cost is important (when isn't it?), it is often wise to select more modestly priced plan fluorite objectives that have a high degree of correction, especially the more modern versions. These objectives provide crisp and sharp images with minimal field curvature, and will be sufficient for most photomicrography applications.

Field curvature is very seldom totally eliminated, but it is often difficult to detect edge curvature with most plan-corrected objectives and it does not show up in photomicrographs (19, 23). This artifact is more severe at low magnifications and can be a problem with stereo microscopes. Manufacturers have struggled for years to eliminate field curvature in the large objectives found in stereo microscopes. In the past ten years, companies like Leica, Nikon, Olympus, and Zeiss, have made great strides in the quality of optics used to build stereo microscopes and, while the artifacts and aberrations have not been totally eliminated, high-end models are now capable of producing superb photomicrographs.

Comatic aberrations are similar to spherical aberrations, but they are mainly encountered with off-axis objects and are most severe when the microscope is out of alignment (23). In this instance, the image of a point is asymmetrical, resulting in a comet-like (hence, the term coma) shape. The comet shape may have its ***tail*** pointing toward the center of the field of view or away depending upon whether the comatic aberration has a positive or negative value. Coma may occur near the axial area of the light path, and/or the more peripheral area. These aberrations are usually corrected along with spherical aberrations by designing lens elements of various shapes to eliminate this error. Objectives that are designed to yield excellent images for wide field of view eyepieces, have to be corrected for coma and astigmatism using a specially-designed multi-element optic in the tube lens to avoid these artifacts at the periphery of the field of view.

Astigmatism aberrations are similar to comatic aberrations, however these artifacts depend more strongly on the obliquity of the light beam (23). This defect is found at the outer portions of the field of view of uncorrected lenses. The off-axis image of a specimen point appears as a line instead of a point. What is more, depending on the angle of the off-axis rays entering the lens, the line image may be oriented in either of two different directions, tangentially or radially. Astigmatism errors are usually corrected by design of the objectives to provide precise spacing of individual lens elements as well as appropriate lens shapes and indices of refraction. The correction of astigmatism is often accomplished in conjunction with the correction of field curvature aberrations.

Eyepieces (Oculars)

Eyepieces work in combination with microscope objectives to further magnify the intermediate image so that specimen details can be observed. Ocular is an alternative name for eyepieces that has been widely used in the literature, but to maintain consistency during this discussion we will refer to all oculars as eyepieces. Best results in microscopy require that objectives be used in combination with eyepieces that are appropriate to the correction and type of objective. Inscriptions on the side of the eyepiece describe its particular characteristics and function.

The eyepiece illustrated in Figure 11 is inscribed with **UW** (not illustrated), which is an abbreviation for the ultra wide viewfield. Often eyepieces will also have an **H** designation, depending upon the manufacturer, to indicate a high-eyepoint focal point that allows microscopists to wear glasses while viewing samples. Other common inscriptions often found on eyepieces include **WF** for wide field; **UWF** for ultra wide field; **SW** and **SWF** for

super wide field; **HE** for high eyepoint; and **CF** for eyepieces intended for use with CF corrected objectives (2, 3, 5, 19, 23, 24). As discussed above, compensating eyepieces are often inscribed with **K, C, Comp**, or **Compens**, as well as the magnification. Eyepieces used with flat-field objectives are sometimes labeled **Plan-Comp**. Magnification factors of common eyepieces range from 5x to 25x, and usually contain an inscription, such as **A/24**, which indicates the field number is 24, in reference to the diameter (in millimeters) of the fixed diaphragm in the eyepiece. Many eyepieces also have a focus adjustment and a thumbscrew that allows their position to be fixed. Manufactures now often produce eyepieces having rubber eye-cups that serve both to position the eyes the proper distance from the front lens, and to block room light from reflecting off the lens surface and interfering with the view.

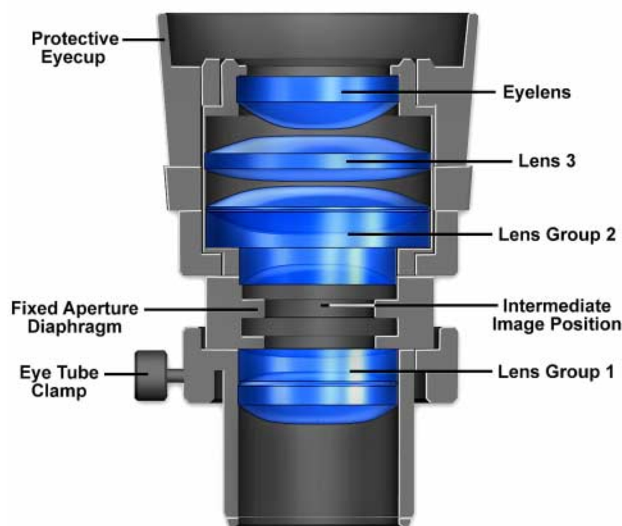


Figure 11. Cutaway diagram of a typical periplan eyepiece. The fixed aperture diaphragm is positioned between lens group 1 and lens group 2, where the intermediate image is formed. The eyepiece has a protective eyecup that makes viewing the specimen more comfortable for the microscopist.

There are two major types of eyepieces that are grouped according to lens and diaphragm arrangement: the **negative** eyepieces with an internal diaphragm between the lenses, and **positive** eyepieces that have a diaphragm below the lenses of the eyepiece. Negative eyepieces have two lenses: the upper lens, which is closest to the observer's eye, is called the eye-lens and the lower lens (beneath the diaphragm) is often termed the field lens. In their simplest form, both lenses are plano-convex, with convex sides facing the specimen. Approximately mid-way between these lenses there is a fixed circular opening or internal diaphragm which, by its size, defines the circular field of view that is observed in looking into the microscope. The simplest kind of negative eyepiece, or Huygenian eyepiece, is found on most routine microscopes fitted with achromatic objectives. Although the Huygenian eye and field lenses are not well corrected, their aberrations tend to cancel each other out. More highly corrected negative eyepieces have two or three lens elements cemented and combined together to make the eye lens. If an unknown eyepiece carries only the magnification inscribed on the housing, it is most likely to be a Huygenian eyepiece, best suited for use with achromatic objectives of 5x-40x magnification.

The other main type of eyepiece is the positive eyepiece with a diaphragm below its lenses, commonly known as the **Ramsden** eyepiece. This eyepiece has an eye lens and field lens that are also plano-convex, but the field lens is mounted with the curved surface facing towards the eye lens. The front focal plane of this eyepiece lies just below the field lens, at the level of the eyepiece fixed diaphragm, making this eyepiece readily adaptable for mounting graticules. To provide better correction, the two lenses of the Ramsden eyepiece may be cemented together.

Simple eyepieces such as the Huygenian and Ramsden and their achromatized counterparts will not correct for residual chromatic difference of magnification in the intermediate image, especially when used in combination with high magnification achromatic objectives as well as fluorite or apochromatic objectives. To remedy this in finite microscopy systems, manufacturers produce compensating eyepieces that introduce an equal, but opposite, chromatic error in the lens elements. Compensating eyepieces may be either of the positive or negative type, and must be used at all magnifications with fluorite, apochromatic and all variations of plan objectives (they can also be used to advantage with achromatic objectives of 40x and higher).

In recent years, modern microscope objectives have their correction for chromatic difference of magnification either built into the objectives themselves (Olympus and Nikon) or corrected in the tube lens (Leica and Zeiss), thus eliminating the need for compensation correction of the eyepieces.

Compensating eyepieces play a crucial role in helping to eliminate residual lateral chromatic aberrations inherent in the design of highly corrected objectives. Hence, it is preferable that the microscopist uses the compensating eyepieces designed by a particular manufacturer to accompany that manufacturer's higher-corrected objectives. Use of an incorrect eyepiece with an apochromatic objective designed for a finite (160 or

170 millimeter) tube length microscope results in dramatically increased contrast with red fringes on the outer diameters and blue fringes on the inner diameters of specimen detail. Additional problems arise from a limited flatness of the viewfield in simple eyepieces, even those corrected with eye-lens doublets.

More advanced eyepiece designs resulted in the Periplan eyepiece (Figure 11), which contains seven lens elements that are cemented into a doublet, a triplet, and two individual lenses. Design improvements in periplan eyepieces lead to better correction for residual lateral chromatic aberration, increased flatness of field, and a general overall better performance when used with higher power objectives.

Modern microscopes feature vastly improved plan-corrected objectives in which the primary image has much less curvature of field than older objectives. In addition, most microscopes now feature much wider body tubes that have accommodated greatly increased the size of intermediate images. To address these new features, manufacturers now produce wide-eyefield eyepieces that increase the viewable area of the specimen by as much as 40 percent. Because the strategies of eyepiece-objective correction techniques vary from manufacturer to manufacturer, it is very important (as stated above) to use only eyepieces recommended by a specific manufacturer for use with their objectives.

Our recommendation is to carefully choose the objective first, then purchase an eyepiece that is designed to work in conjunction with the objective. When choosing eyepieces, it is relatively easy to differentiate between simple and more highly compensating eyepieces. Simple eyepieces such as the Ramsden and Huygenian (and their more highly corrected counterparts) will appear to have a blue ring around the edge of the eyepiece diaphragm when viewed through the microscope or held up to a light source. In contrast, more highly corrected compensating eyepieces with have a yellow-red-orange ring around the diaphragm under the same circumstances. Modern non-compensating eyepieces are fully corrected and show no color. Most of the modern microscopes have all corrections done in the objectives themselves or have a final correction in the tube lens. Such microscopes do not need compensating eyepieces.

The properties of several common commercially available eyepieces are listed according to type in Table 5 (19, 23). The three major types of eyepieces listed in Table 5 are finder, wide field, and super wide field. The terminology used by various manufacturers can be very confusing and careful attention should be paid to their sales brochures and microscope manuals to ensure that the correct eyepieces are being used with a specific objective. In Table 5, the abbreviations that designate wide field and super widefield eyepieces are coupled to their design for high eyepoint, and are **WH** and **SWH**, respectively. The magnifications are either 10x or 15x and the Field Numbers range from 14 to 26.5, depending upon the application. The diopter adjustment is approximately the same for all eyepieces and many also contain either a photomask or micrometer graticule.

Light rays emanating from the eyepiece intersect at the exit pupil or eyepoint (Ramsden disc) where the front of the microscopist's eye should be placed in order to see the entire field of view (usually 8-10 mm above the eye lens). By increasing the magnification of the eyepiece, the eyepoint is drawn closer to the upper surface of the eye lens, making it much more difficult for microscopists to use, especially if he or she wears eyeglasses. Specially designed high eyepoint eyepieces have been manufactured that feature eyepoint viewing distances approaching 20-25 mm above the surface of the eye lens. These improved eyepieces have larger diameter eye lenses that contain more optical elements and usually feature improved flatness of field. Such eyepieces are often designated with the inscription "**H**" somewhere on the eyepiece housing, either alone or in combination with other abbreviations, as discussed above. We should mention that high-eyepoint eyepieces are especially useful for microscopists who wear eyeglasses to correct for near or far sightedness, but they do not correct for several other visual defects, such as astigmatism. Today, high eyepoint eyepieces are very popular, even with people who do not wear eyeglasses, because the large eye clearance reduces fatigue and makes viewing images through the microscope much more comfortable.

At one time, eyepieces were available in a wide range of magnifications extending from 6.3x to 30x and sometimes even higher for special applications. These eyepieces are very useful for observation and photomicrography with low-power objectives. Unfortunately, with higher power objectives, the problem of empty magnification (magnification without increased clarity) becomes important when using very high magnification eyepieces and these should be avoided. Today most manufacturers restrict their eyepiece offerings to those in the 10x to 20x ranges. The diameter of the viewfield in an eyepiece is expressed as a ***field of view number*** or ***field number*** (**FN**), as discussed above. Information about the field number of an eyepiece can yield the real diameter of the object viewfield using the formula (23):

$$\textbf{Viewfield Diameter} = \textbf{FN} / (\textbf{M}_O \times \textbf{M}_T) \quad \textbf{(2)}$$

Where **FN** is the field number in millimeters, $\mathbf{M_O}$ is the magnification of the objective, and $\mathbf{M_T}$ is the tube lens

Table 5 Properties of Commercial Eyepieces

Eyepiece Type	Finder Eyepieces			Super Wide Field Eyepieces	Wide Field Eyepieces		
Descriptive Abbreviation	PSWH 10x	PWH 10x	35 SWH 10x	SWH 10x H	CROSSWH 10x H	WH 15x	WH 10x H
Field Number	26.5	22	26.5	26.5	22	14	22
Diopter Adjustment	-8 ~ +2	-8 ~ +2	-8 ~ +2	-8 ~ +2	-8 ~ +2	-8 ~ +2	-8 ~ +2
Remarks	31/4 x "41/4" photo mask	31/4 x "41/4" photo mask	35mm photo mask	diopter correction	diopter correction crossline		diopter correction
Diameter of Micrometer Graticule	—	—	—	—	—	24	24

magnification factor (if any). Applying this formula to the super wide field eyepiece listed in Table 5, we arrive at the following for a 40x objective with a tube lens magnification of 1.25:

Viewfield Diameter = 26.5 / 40 x 1.25 = 0.53 mm (3)

Table 1 lists the viewfield diameters over the common range of objectives that would occur using this eyepiece.

Table 6 Range of Useful Magnification (500-1000 x NA of Objective)

Objective (NA)	Eyepieces 10x	12.5x	15x	20x	25x
2.5x (0.08)	—	—	—	x	x
4x (0.12)	—	—	x	x	x
10x (0.35)	—	x	x	x	x
25x (0.55)	x	x	x	x	—
40x (0.70)	x	x	x	—	—
60x (0.95)	x	x	x	—	—
100x (1.42)	x	x	—	—	—

Care should be taken in choosing eyepiece/objective combinations to ensure the optimal magnification of specimen detail without adding unnecessary artifacts. For instance, to achieve a magnification of 250x, the microscopist could choose a 25x eyepiece coupled to a 10x objective. An alternative choice for the same magnification would be a 10x eyepiece with a 25x objective. Because the 25x objective has a higher numerical aperture (approximately 0.65) than does the 10x objective (approximately 0.25), and considering that numerical aperture values define an objective's resolving power, it is clear that the latter choice would be the best. If photomicrographs of the same viewfield were made with each objective/eyepiece combination described above, it would be obvious that the 10x eyepiece/25x objective duo would produce photomicrographs that excelled in specimen detail and clarity when compared to the alternative combination.

Numerical aperture of the objective/condenser system defines the ***range of useful magnification*** for an objective/eyepiece combination (19, 22-24). There is a minimum magnification necessary for the detail present in an image to be resolved, and this value is usually rather arbitrarily set as 500 times the numerical aperture (500 x NA). At the other end of the spectrum, the maximum useful magnification of an image is usually set at 1000 times the numerical aperture (1000 x NA). Magnifications higher than this value will yield no further useful information or finer resolution of image detail, and will usually lead to image degradation. Exceeding the limit of useful magnification causes the image to suffer from the phenomenon of ***empty magnification*** (19), where increasing magnification through the eyepiece or intermediate tube lens only causes the image to become

more magnified with no corresponding increase in detail resolution. Table 6 lists the common objective/eyepiece combinations that fall into the range of useful magnification.

Eyepieces can be adapted for measurement purposes by adding a small circular disk-shaped glass graticule at the plane of the fixed aperture diaphragm of the eyepiece. Graticules usually have markings, such as a measuring rule or grid, etched onto the surface. Because the graticule lies in the same conjugate plane as the fixed eyepiece diaphragm, it appears in sharp focus superimposed on the image of the specimen. Eyepieces using graticules usually contain a focusing mechanism (helical screw or slider) that allows the image of the graticule to be brought into focus. A stage micrometer is needed to calibrate the eyepiece scale for each objective.

Condenser Systems

The substage condenser gathers light from the microscope light source and concentrates it into a cone of light that illuminates the specimen with parallel beams of uniform intensity from all azimuths over the entire viewfield. It is critical that the condenser light cone be properly adjusted to optimize the intensity and angle of light entering the objective front lens. Each time an objective is changed, a corresponding adjustment must be performed on the substage condenser aperture iris diaphragm to provide the proper light cone for the numerical aperture of the new objective.

A simple two-lens Abbe condenser is illustrated in Figure 12. In this figure, light from the microscope illumination source passes through the aperture or condenser diaphragm, located at the base of the condenser, and is concentrated by internal lens elements, which then project light through the specimen in parallel bundles from every azimuth. The size and numerical aperture of the light cone is determined by adjustment of the aperture diaphragm. After passing through the specimen (on the microscope slide), the light diverges to an inverted cone with the proper angle (2q in Figure 12) to fill the front lens of the objective (2, 18-24).

Aperture adjustment and proper focusing of the condenser are of critical importance in realizing the full potential of the objective. Specifically, appropriate use of the adjustable aperture iris diaphragm (incorporated into the condenser or just below it) is most important in securing correct illumination, contrast, and depth of field. The opening and closing of this iris diaphragm controls the angle of illuminating rays (and thus the aperture) which pass through the condenser, through the specimen and then into the objective. Condenser height is controlled by a rack and pinion gear system that allows the condenser focus to be adjusted for proper illumination of the specimen. Correct positioning of the condenser with relation to the cone of illumination and focus on the specimen is critical to quantitative microscopy and optimum photomicrography. Care must be taken to guarantee that the condenser aperture is opened to the correct position with respect to objective numerical aperture. When the aperture is opened too much, stray light generated by refraction of oblique light rays from the specimen can cause glare and lower the overall contrast. On the other hand, when the aperture is closed too far, the illumination cone is insufficient to provide adequate resolution and the image is distorted due to refraction and diffraction from the specimen.

Abbe Condenser/Objective Combination

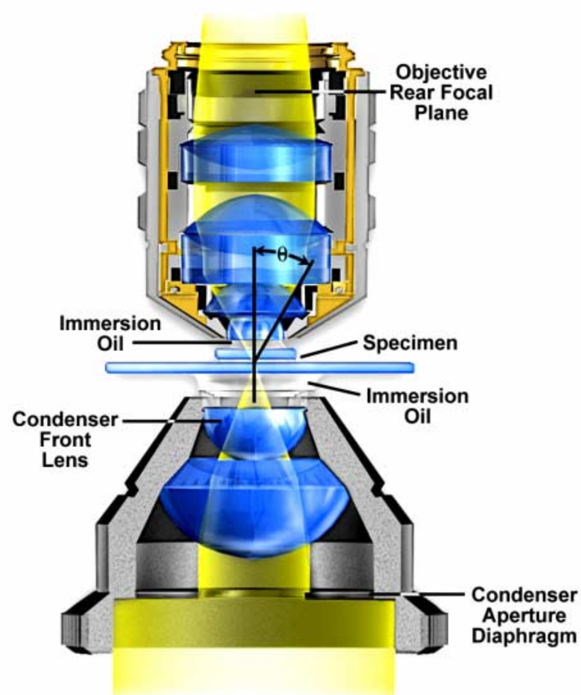


Figure 12. Condenser/objective configuration for optical microscopy. An Abbe two-lens condenser is illustrated showing ray traces through the optical train of the microscope. The aperture diaphragm restricts light entering the condenser before it is refracted by the condenser lens system into the specimen. Immersion oil is used in the contact beneath the θ underside of the slide and the condenser top lens, and also between the objective and cover slip. The objective angular aperture ( ) controls the amount of light entering the objective.

The simplest and least corrected (also the least expensive) condenser is the Abbe condenser that, in its simplest form, has two optical lens elements which produce an image of the illuminated field diaphragm that

is not sharp and is surrounded by blue and red color at the edges. As a result of little optical correction, the Abbe condenser is suited mainly for routine observation with objectives of modest numerical aperture and magnification. The primary advantages of the Abbe condenser are the wide cone of illumination that the condenser is capable of producing as well as its ability to work with long working distance objectives. The manufacturers supply most microscopes with an Abbe condenser as the default and these condensers are real workhorses for routine laboratory use.

Table 7 Condenser Aberration Corrections

Condenser Type	Aberrations Corrected	
	Spherical	Chromatic
Abbe	—	—
Aplanatic	x	—
Achromatic	—	x
Aplanatic-achromatic	x	x

The next highest level of condenser correction is split between the aplanatic and achromatic condensers that are corrected exclusively for either spherical (aplanatic) or chromatic (achromatic) optical aberrations.

Achromatic condensers typically contain four lens elements and have a numerical aperture of 0.95, the highest attainable without requiring immersion oil (5). This condenser is useful for both routine and critical laboratory analysis with dry objectives and also for black and white or color photomicrography.

A critical factor in choosing substage condensers is the numerical aperture performance, which will be necessary to provide an illumination cone adequate for the objectives. The condenser numerical aperture capability should be equal to or slightly less than that of the highest objective numerical aperture. Therefore, if the largest magnification objective is an oil-immersion objective with a numerical aperture of 1.40, then the substage condenser should also have an equivalent numerical aperture to maintain the highest system resolution. In this case, immersion oil would have to be applied between the condenser top lens in contact with the underside of the microscope slide to achieve the intended numerical aperture (1.40) and resolution. Failure to use oil will restrict the highest numerical aperture of the system to 1.0, the highest obtainable with air as the imaging medium (2, 5, 17-24).

Aplanatic condensers are well corrected for spherical aberration (green wavelengths) but not for chromatic aberration. These condensers feature five lens elements and are capable of focusing light in a single plane. Aplanatic condensers are capable of producing excellent black and white photomicrographs when used with green light generated by either a laser source or by use of an interference filter with tungsten-halide illumination.

The highest level of correction for optical aberration is incorporated in the aplanatic-achromatic condenser (2, 5). This condenser is well corrected for both chromatic and spherical aberrations and is the condenser of choice for use in critical color photomicrography with white light. A typical aplanatic-achromatic condenser features eight internal lens elements cemented into two doublets and four single lenses.

Engravings found on the condenser housing include its type (achromatic, aplanatic, etc.), the numerical aperture, and a graduated scale that indicates the approximate adjustment (size) of the aperture diaphragm. As we mentioned above, condensers with numerical apertures above 0.95 perform best when a drop of oil is applied to their upper lens in contact with the undersurface of the specimen slide. This ensures that oblique light rays emanating from the condenser are not reflected from underneath the slide, but are directed into the specimen without deviation. In practice, this can become tedious and is not commonly done in routine microscopy, but is essential when working at high resolutions and for accurate photomicrography using high-power (and numerical aperture) objectives.

Another important consideration is the thickness of the microscope slide, which is as crucial to the condenser as coverslip thickness is to the objective. Most commercial producers offer slides that range in thickness between 0.95 and 1.20 mm with the most common being very close to 1.0 mm. A microscope slide of thickness 1.20 mm is too thick to be used with most high numerical aperture condensers that tend to have a very short working distance. While this does not greatly matter for routine specimen observation, the results can be devastating with precision photomicrography. We recommend that microscope slides be chosen that have a thickness of 1.0 ± 0.05 mm, and that they be thoroughly cleaned prior to use.

When the objective is changed, for example from a 10x to 20x, the aperture diaphragm of the condenser must also be adjusted to provide a light cone that matches the numerical aperture of the new objective. There is a small painted arrow or index mark located on this knurled knob or lever that indicates the relative size of the aperture when compared to the linear gradation on the condenser housing. Many manufacturers will synchronize this gradation to correspond to the approximate numerical

aperture of the condenser. For example, if the microscopist has selected a 10x objective of numerical aperture 0.25, then the arrow would be placed next the value 0.18-0.20 (about 80 percent of the objective numerical aperture) on the scale inscribed on the condenser housing.

Often, it is not practical to use a single condenser with an entire range of objectives (2x to 100x) due to the broad range of light cones that must be produced to match objective numerical apertures. With low-power objectives in the range 2x to 5x, the illumination cone will have a diameter between 6-10 mm, while the high-power objectives (60x to 100x) need a highly focused light cone only about 0.2-0.4 mm in diameter. With a fixed focal length, it is difficult to achieve this wide range of illumination cones with a single condenser (18).

In practice, this problem can be solved in several ways. For low power objectives (below 10x), it may be necessary to unscrew the top lens of the condenser in order to fill the field of view with light. Other condensers are produced with a flip-top upper lens to accomplish this more readily. Some manufacturers now produce a condenser that flips over completely when used with low power objectives. Other companies incorporate auxiliary correction lenses in the light path for securing proper illumination with objectives less than 10x, or produce special low-power and low-numerical aperture condensers. When the condenser is used without its top lens, the aperture iris diaphragm is opened wide and the field diaphragm, now visible at the back of the objective,

Table 8 Substage Condenser Applications

Condenser Type	Brightfield	Darkfield	Phase Contrast	DIC	Polarizing
Achromat / Aplanat N.A. 1.3	• [10x~100x]				
Achromat Swing-out N.A. 0.90	• [4x~100x]				
Low-Power N.A. 0.20	• [1x~10x]				
Phase Contrast Abbe N.A. 1.25	•	• [up to N.A. 0.65]	• [10x~100x]		
Phase Contrast Achromat N.A. 0.85	•	• [up to N.A. 0.70]	• [4x~100x]		
DIC Universal Achromat / Aplanat	•	• [up to N.A. 0.70]	• [10x~100x]	• [10x, 20x, 40x, 100x]	
Darkfield, dry N.A. 0.80~0.95		• [4x~40x]			
Darkfield, oil N.A. 1.20~1.43		• [4x~100x]			
Strain-Free Achromat Swing-out N.A. 0.90	•				• [4x~100x]

Table 9 Depth of Field and Image Depth [a]

Magnification	Numerical Aperture	Depth of Field (M)	Image Depth (mm)
4x	0.10	15.5	0.13
10x	0.25	8.5	0.80
20x	0.40	5.8	3.8
40x	0.65	1.0	12.8
60x	0.85	0.40	29.8
100x	0.95	0.19	80.0

[a] Source: Nikon

serves as if it were the aperture diaphragm. Flip-top condensers are manufactured in a variety of configurations with numerical apertures ranging from 0.65 to 1.40. Those condensers having a numerical aperture value of 0.95 or less are intended for use with dry objectives. Flip-top condensers that have a numerical aperture greater than 0.95 are intended for use with oil-immersion objectives and they must have a drop of oil placed between the underside of the microscope slide and the condenser top lens when examining critical samples.

In addition to the common brightfield condensers discussed above, there are a wide variety of specialized models suited to many different applications. Substage condensers have a great deal of interchangeability among different applications. For instance, the DIC universal achromat/aplanat condenser is useful for brightfield, darkfield, and phase contrast, in addition to the primary DIC application. Other condensers have similar interchangeability.

Depth of Field and Depth of Focus

When considering resolution in optical microscopy, a majority of the emphasis is placed on point-to-point resolution in the plane perpendicular to the optical axis. Another important aspect to resolution is the ***axial resolving power*** of an objective, which is measured parallel to the optical axis and is most often referred to as depth of field (2, 4, 5, 22). Axial resolution, like horizontal resolution, is determined by the numerical aperture of the objective only, with the eyepiece merely magnifying the details resolved and projected in the intermediate image plane.

Just as in classical photography, depth of field is determined by the distance from the nearest object plane in focus to that of the farthest plane also simultaneously in focus. In microscopy depth of field is very short and usually measured in terms of microns. The term ***depth of focus***, which refers to image space, is often used interchangeably with depth of field, which refers to object space. This interchange of terms can lead to confusion, especially when the terms are both used specifically in terms of depth of field.

The geometric image plane might be expected to represent an infinitely thin section of the specimen, but even in the absence of aberrations, each image point is spread into a diffraction figure that extends above and below this plane (2, 4, 17). The Airy disk, discussed in the section on **Image Formation**, represents a section through the center of the image plane. This increases the effective in-focus depth of the Z-axis Airy disk intensity profile that passes through slightly different specimen planes.

Depth of focus varies with numerical aperture and magnification of the objective, and under some conditions, high numerical aperture systems (usually with higher magnification power) have deeper focus depths than do those systems of low numerical aperture, even though the depth of field is less (4). This is particularly important in photomicrography because the film emulsion or digital camera sensor must be exposed at a plane that is in focus. Small errors made to focus at high magnification are not as critical as those made with very low magnification objectives. Table 9 presents calculated variations in the depth of field and image depth in the intermediate image plane in a series of objectives with increasing numerical aperture and magnification.

Reflected Light Microscopy

Reflected light microscopy is often referred to as incident light, epi-illumination, or metallurgical microscopy, and is the method of choice for fluorescence and for imaging specimens that remain opaque even when ground to a thickness of 30 microns (25). The range of specimens falling into this category is enormous and includes most metals, ores, ceramics, many polymers, semiconductors (unprocessed silicon, wafers, and integrated circuits), slag, coal, plastics, paint, paper, wood, leather, glass inclusions, and a wide variety of specialized

materials (25-28). Because light is unable to pass through these specimens, it must be directed onto the surface and eventually returned to the microscope objective by either specular or diffused reflection. As mentioned above, such illumination is most often referred to as episcopic illumination, epi-illumination, or vertical illumination (essentially originating from above), in contrast to diascopic (transmitted) illumination that passes through a specimen. Today, many microscope manufacturers offer models that permit the user to alternate or simultaneously conduct investigations using vertical and transmitted illumination. Reflected light microscopy is frequently the domain of industrial microscopy, especially in the rapidly growing semiconductor arena, and thus represents a most important segment of microscopical studies.

A typical upright compound reflected light (illustrated in Figure 13) microscope also equipped for transmitted light has two eyepiece viewing tubes and often a trinocular tube head for mounting a conventional or digital/video camera system. Standard equipment eyepieces are usually of 10x magnification, and most microscopes are equipped with a nosepiece capable of holding four to six objectives. The stage is mechanically controlled with a specimen holder that can be translated in the X- and Y-directions and the entire stage unit is capable of precise up and down movement with a coarse and fine focusing mechanism. Built-in light sources range from 20 and 100 watt tungsten-halide bulbs to higher energy mercury vapor or xenon lamps that are used in fluorescence microscopy. Light passes from the lamp house through a vertical illuminator interposed above the nosepiece but below the underside of the viewing tube head. The specimen's top surface is upright (usually without a cover slip) on the stage facing the objective, which has been rotated into the microscope's optical axis. The vertical illuminator is horizontally oriented at a 90-degree angle to the optical axis of the microscope and parallel to the table top, with the lamp housing attached to the back of the illuminator. The coarse and fine adjustment knobs raise or lower the stage in large or small increments to bring the specimen into sharp focus.

Another variation of the upright reflected light microscope is the inverted microscope—of the Le Chatelier design (25). On the inverted stand, the specimen is placed on the stage with its surface of interest facing downward. The primary advantage of this design is that samples can be easily examined when they are far too large to fit into the confines of an upright microscope. Also, the only the side facing the objectives need be perfectly flat. The objectives are mounted on a nosepiece under the stage with their front lenses facing upward towards the specimen and focusing is accomplished either by moving the nosepiece or the entire stage up and down. Inverted microscope stands incorporate the vertical illuminator into the body of the microscope. Many types of objectives can be used with inverted reflected light microscopes, and all modes of reflected light illumination may be possible: brightfield, darkfield, polarized light, differential interference contrast, and fluorescence. Many

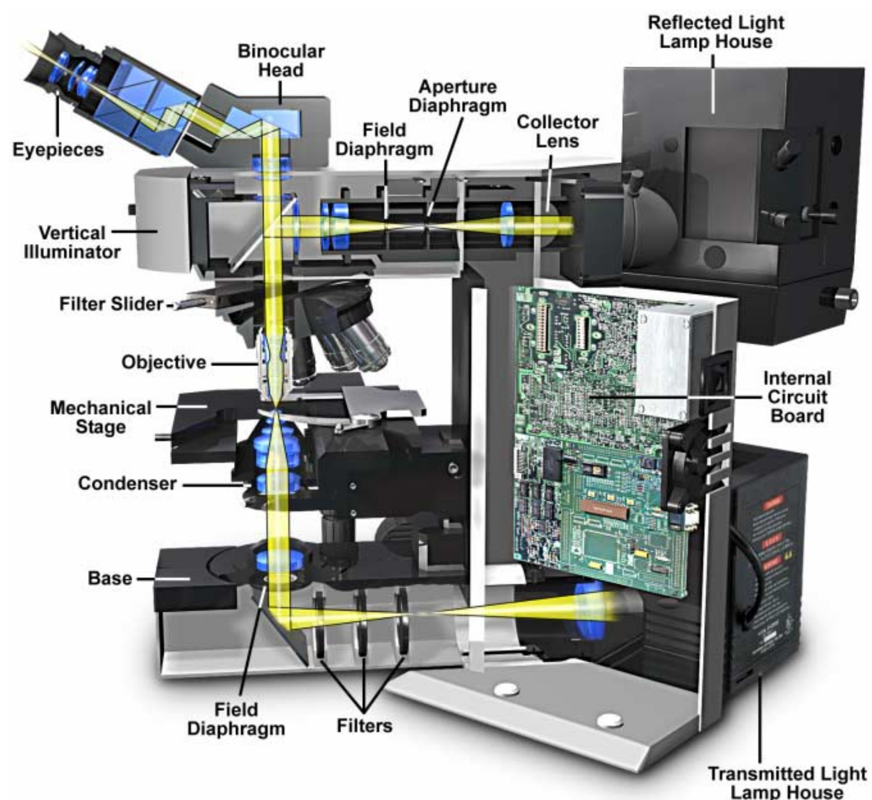


Figure 13. Components of a modern microscope configured for both transmitted and reflected light. This cutaway diagram reveals the ray traces and lens components of the microscope's optical trains. Also illustrated are the basic microscope components including two lamp houses, the microscope built-in vertical and base illuminators, condenser, objectives, eyepieces, filters, sliders, collector lenses, field, and aperture diaphragms.

of the inverted microscopes have built-in 35 millimeter and/or large format cameras or are modular to allow such accessories to be attached. Some of the instruments include a magnification changer for zooming in on the image, contrast filters, and a variety of reticules. Because an inverted microscope is a favorite instrument for metallographers, it is often referred to as a metallograph (25, 27). Manufacturers are largely migrating to using infinity-corrected optics in reflected light microscopes, but there are still thousands of fixed tube length microscopes in use with objectives corrected for a tube length between 160 and 210 millimeters.

In the vertical illuminator, light travels from the light source, usually a 12 volt 50 or 100 watt tungsten halogen lamp, passes through collector lenses, through the variable aperture iris diaphragm opening and through the opening of a variable and centerable pre-focused field iris diaphragm. The light then strikes a partially silvered plane glass reflector (Figure 13), or strikes a fully silvered periphery of a mirror with elliptical opening for darkfield illumination. The plane glass reflector is partially silvered on the glass side facing the light source and anti-reflection coated on the glass side facing the observation tube in brightfield reflected illumination. Light is thus deflected downward into the objective. The mirrors are tilted at an angle of 45 degrees to the path of the light travelling along the vertical illuminator.

The light reaches the specimen, which may absorb some of the light and reflect some of the light, either in a specular or diffuse manner. Light that is returned upward can be captured by the objective in accordance with the objective's numerical aperture and then passes through the partially silvered mirror. In the case of infinity-corrected objectives, the light emerges from the objective in parallel (from every azimuth) rays projecting an image of the specimen to infinity (25). The ***parallel*** rays enter the body tube lens, which forms the specimen image at the plane of the fixed diaphragm opening in the eyepiece (intermediate image plane). It is important to note, that in these reflected light systems, the objective serves a dual function: on the way down as a matching well-corrected condenser properly aligned; on the way up as an image-forming objective in the customary role of an objective projecting the image-carrying rays toward the eyepiece.

Optimal performance is achieved in reflected light illumination when the instrument is adjusted to produce Köhler illumination. A function of Köhler illumination (aside from providing evenly dispersed illumination) is to ensure that the objective will be able to deliver excellent resolution and good contrast even if the source of light is a coil filament lamp.

Some modern reflected light illuminators are described as universal illuminators because, with several additional accessories and little or no dismantling, the microscope can easily be switched from one mode of reflected light microscopy to another. Often, reflectors can be removed from the light path altogether in order to perform transmitted light observation. Such universal illuminators may include a partially reflecting plane glass surface (the half-mirror) for brightfield, and a fully silvered reflecting surface with an elliptical, centrally located clear opening for darkfield observation. The best-designed vertical illuminators include condensing lenses to gather and control the light, an aperture iris diaphragm and a pre-focused, centerable iris diaphragm to permit the desirable Köhler illumination (2, 25).

The vertical illuminator should also make provision for the insertion of filters for contrast and photomicrography, polarizers, analyzers, and compensator plates for polarized light and differential interference contrast illumination. In vertical illuminators designed for with infinity-corrected objectives, the illuminator may also include a body tube lens. Affixed to the back end of the vertical illuminator is a lamphouse, which usually contains a tungsten-halide lamp. For fluorescence work, the lamphouse can be replaced with one containing a mercury burner. The lamp may be powered by the electronics built into the microscope stand, or in fluorescence, by means of an external transformer or power supply.

In reflected light microscopy, absorption and diffraction of the incident light rays by the specimen often lead to readily discernible variations in the image, from black through various shades of gray, or color if the specimen is colored. Such specimens are known as amplitude specimens and may not require special contrast methods or treatment to make their details visible. Other specimens show so little difference in intensity and/or color that their feature details are extremely difficult to discern and distinguish in brightfield reflected light microscopy. Such specimens behave much like the phase specimens so familiar in transmitted light work. Such objects require special treatment or contrast methods that will be described in the next section.

Contrast Enhancing Techniques

Some specimens are considered amplitude objects because they absorb light partially or completely, and can thus be readily observed using conventional brightfield microscopy. Others that are naturally colored or artificially stained with chemical color dyes can also be seen. These stains or natural colors absorb some part of the white light passing through and transmit or reflect other colors. Often, stains are combined to yield contrasting

colors, e.g. blue haemotoxylin stain for cell nuclei combined with pink eosin for cytoplasm. It is a common practice to utilize stains on specimens that do not readily absorb light, thus rendering such objects visible to the eye.

Contrast produced by the absorption of light, brightness, or color has been the classical means of imaging specimens in brightfield microscopy. The ability of a detail to stand out against the background or other adjacent details is a measure of specimen contrast. In terms of a simple formula, contrast can be described as (18):

$$\textbf{Percent Contrast} = ((B_I - S_I) \times 100)/B_I \qquad (4)$$

Where B_I is the intensity of the background and S_I is the specimen intensity. From this equation, it is evident that specimen contrast refers to the relationship between the highest and lowest intensity in the image.

For many specimens in microscopy, especially unstained or living material, contrast is so poor that the object remains essentially invisible regardless of the ability of the objective to resolve or clearly separate details. Often, for just such specimens, it is important not to alter them by killing or treatment with chemical dyes or fixatives. This necessity has led microscopists to experiment with contrast-enhancing techniques for over a hundred years in an attempt to improve specimen visibility and to bring more detail to the image. It is a common practice to reduce the condenser aperture diaphragm below the recommended size or to lower the substage condenser to increase specimen contrast. Unfortunately, while these maneuvers will indeed increase contrast, they also seriously reduce resolution and sharpness.

An early and currently used method of increasing contrast of stained specimens utilizes color contrast filters, gelatin squares (from Kodak), or interference filters in the light path (18, 19). For example, if a specimen is stained with a red stain, a green filter will darken the red areas thus increasing contrast. On the other hand, a green filter will lighten any green stained area. Color filters are very valuable aids to specimen contrast, especially when black and white photomicrography is the goal. Green filters are particularly valuable for use with achromats, which are spherically corrected for green light, and phase contrast objectives, which are designed for manipulation of wavelength assuming the use of green light, because phase specimens are usually transparent and lack inherent color. Another simple technique for contrast improvement involves the selection of a mounting medium with a refractive index substantially different from that of the specimen. For example, diatoms can be mounted in a variety of contrast-enhancing mediums such as air or the commercial medium Styrax. The difference in refractive indices improves the contrast of these colorless objects and renders their outlines and markings more visible. The following sections describe many of the more complex techniques used by present-day microscopists to improve specimen contrast.

Darkfield Microscopy

Darkfield illumination requires blocking out of the central light rays that ordinarily pass through or around the specimen and allowing only oblique rays to illuminate the specimen. This method is a simple and popular method for imaging unstained specimens, which appear as brightly illuminated objects on a dark background. Oblique light rays emanating from a darkfield condenser strike the specimen from every azimuth and are diffracted, reflected, and refracted into the microscope objective (5, 18, 19, 28). This technique is illustrated in Figure 14. If no specimen is present and the numerical aperture of the condenser is greater than that of the objective, the oblique rays cross and all such rays will miss entering the objective because of the obliquity. The field of view will appear dark.

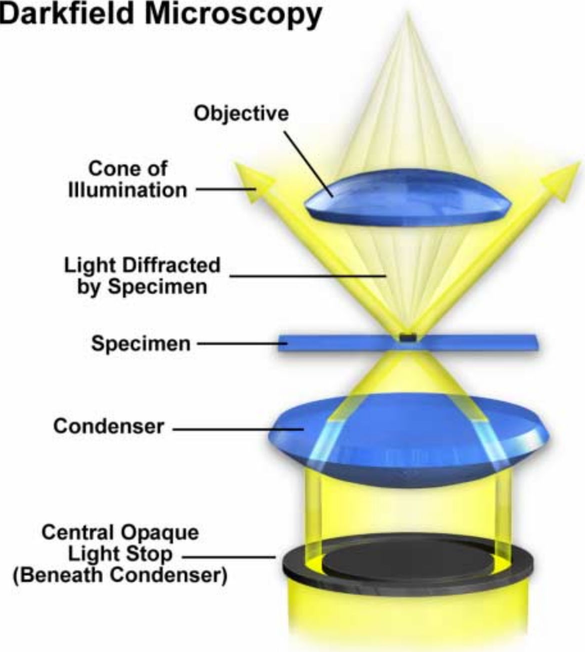


Figure 14. Schematic configuration for darkfield microscopy. The central opaque light stop is positioned beneath the condenser to eliminate zeroth order illumination of the specimen. The condenser produces a hollow cone of illumination that strikes the specimen at oblique angles. Some of the reflected, refracted, and diffracted light from the specimen enters the objective front lens.

In terms of Fourier optics, darkfield illumination removes the zeroth order (unscattered light) from the diffraction pattern formed at the rear focal plane of the objective (5). Oblique rays, now diffracted by the specimen and yielding 1st, 2nd, and higher diffracted orders at the rear focal plane of the objective, proceed onto the image plane where they interfere with one another to produce an image of the specimen. This results in an image formed exclusively from higher order diffraction intensities scattered by the specimen. Specimens that have smooth reflective surfaces produce images due, in part, to reflection of light into the objective (18, 19). In situations where the refractive index is different from the surrounding medium or where refractive index gradients occur (as in the edge of a membrane), light is refracted by the specimen. Both instances of reflection and refraction produce relatively small angular changes in the direction of light allowing some to enter the objective. In contrast, some light striking the specimen is also diffracted, producing a 180-degree arc of light that passes through the entire numerical aperture range of the objective. The resolving power of the objective is the same in darkfield illumination as found under brightfield conditions, but the optical character is the image is not as faithfully reproduced.

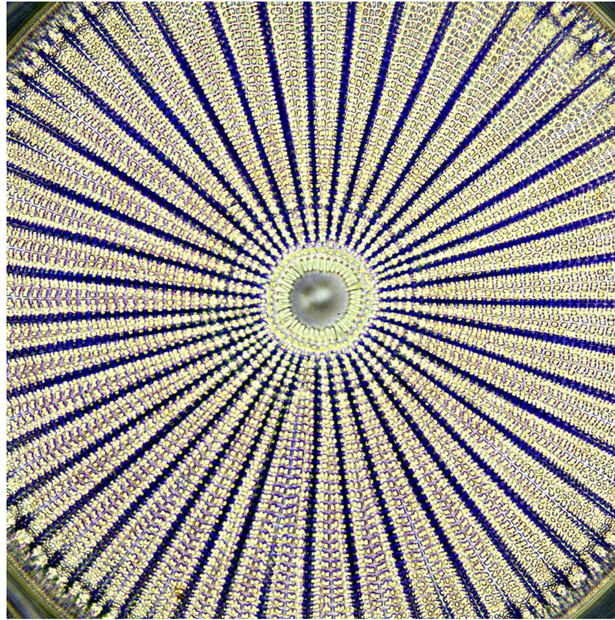


Figure 15. Darkfield photomicrograph of the diatom *Arachnoidiscus ehrenbergi* taken at high magnification using oil immersion optics and a 100x objective.

There are several pieces of equipment that are utilized to produce darkfield illumination. The simplest is a ***spider stop*** (Figure 14) placed just under the bottom lens (in the front focal plane) of the substage condenser (5, 18, 19, 28). Both the aperture and field diaphragms are opened wide to pass oblique rays. The central opaque stop (you can make one by mounting a coin on a clear glass disk) blocks out the central rays. This device works fairly well, even with the Abbe condenser, with the 10x objective up to 40x or higher objectives having a numerical aperture no higher than 0.65. The diameter of the opaque stop should be approximately 16-18 millimeters for a 10x objective of numerical aperture 0.25 to approximately 20-24 millimeters for 20x and 40x objectives of numerical apertures approaching 0.65.

For more precise work and blacker backgrounds, use a condenser designed especially for darkfield, i.e. to transmit only oblique rays (28, 29). There are several varieties including dry darkfield condensers with air between the top of the condenser and the underside of the slide. Immersion darkfield condensers are also available. These require the use of a drop of immersion oil (some are designed to use water instead) establishing contact between the top of the condenser and the underside of the specimen slide. The immersion darkfield condenser has internal mirrored surfaces and passes rays of great obliquity and free of chromatic aberration, producing the best results and blackest background.

Darkfield objects are quite spectacular to see and objects of very low contrast in brightfield shine brilliantly in darkfield. Such illumination is best for revealing outlines, edges, and boundaries. A high magnification darkfield image of a diatom is illustrated in Figure 15.

Rheinberg Illumination

Rheinberg illumination, a form of optical staining, was initially demonstrated by the British microscopist Julius Rheinberg to the Royal Microscopical Society and the Quekett Club (England) nearly a hundred years ago (18, 28). This technique is a striking variation of low to medium power darkfield illumination using colored gelatin or glass filters to provide rich color to both the specimen and background. The central opaque darkfield stop is replaced with a transparent, colored, circular stop inserted into a transparent ring of a contrasting color (illustrated in Figure 16). These stops are placed under the bottom lens of the condenser. The result is a specimen rendered in the color of the ring with a background having the color of the central spot. An example of photomicrography using Rheinberg illumination is illustrated in Figure 17 with the spiracle and trachea of a silkworm larva.

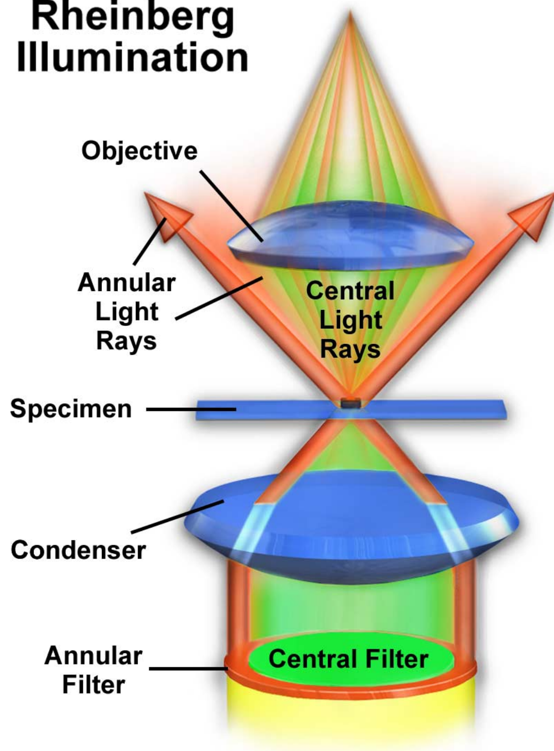


Figure 16. Schematic microscope configuration for Rheinberg illumination. Light passes through the central/annular filter pack prior to entering the condenser. Zeroth order light from the central filter pervades the specimen and background and illuminates it with higher order light from the annular filter. The filter colors in this diagram are a green central and red annular.

Phase Contrast Microscopy

Research by Frits Zernike during the early 1930s uncovered phase and amplitude differences between zeroth order and deviated light that can be altered to produce favorable conditions for interference and contrast enhancement (30, 31). Unstained specimens that do not absorb light are called phase objects because they slightly alter the phase of the light diffracted by the specimen, usually by retarding such light approximately 1/4 wavelength as compared to the undeviated direct light passing through or around the specimen unaffected. Unfortunately, our eyes as well as camera film are unable to detect these phase differences. To reiterate, the human eye is sensitive only to the colors of the visible spectrum or to differing levels of light intensity (related to wave amplitude).

In phase specimens, the direct zeroth order light passes through or around the specimen undeviated. However, the light diffracted by the specimen is not reduced in amplitude as it is in a light-absorbing object, but is slowed by the specimen because of the specimen's refractive index or thickness (or both). This diffracted light, lagging behind by approximately 1/4 wavelength, arrives at the image plane ***out of step*** (also termed ***out of phase***) with the undeviated light but, in interference, essentially undiminished in intensity. The result is that the image at the eyepiece level is so lacking in contrast as to make the details almost invisible.

Zernike succeeded in devising a method—now known as Phase Contrast microscopy—for making unstained, phase objects yield contrast images as if they were amplitude objects. Amplitude objects show excellent contrast when the diffracted and direct light are out of step (display a phase difference) by 1/2 of a wavelength (18). Zernike's method was to ***speed up*** the direct light by 1/4 wavelength so that the difference in wavelength between the direct and deviated light for a phase specimen would now be 1/2 wavelength. As a result, the direct and diffracted light arriving at the image level of the eyepiece would be able to produce destructive interference (see the section on **image formation** for absorbing objects previously described). Such a procedure results in the details of the image appearing darker against a lighter background. This is called dark or positive phase contrast. A schematic illustration of the basic phase contrast microscope configuration is illustrated in Figure 18.

Another possible course, much less often used, is to arrange to ***slow down*** the direct light by 1/4 wavelength so that the diffracted light and the direct light arrive at the eyepiece in step and can interfere constructively (2, 5, 18). This arrangement results in a bright image of the details of the specimen on a darker background, and is called negative or bright contrast.

Phase contrast involves the ***separation*** of the direct zeroth order light from the diffracted light at the rear focal plane of the objective. To do this, a ring annulus is placed in position directly under the lower lens of the condenser at the front focal plane of the condenser, conjugate to the objective rear focal plane. As the hollow cone of light from the annulus passes through the specimen undeviated, it arrives at the rear focal plane of the objective in the shape of a ring of light. The fainter light diffracted by the specimen is spread over the rear focal plane of the objective. If this combination were allowed, as is, to proceed to the image plane of the eyepiece, the diffracted light would be approximately 1/4 wavelength behind the direct light. At the image plane, the phase of the diffracted light would be out of phase with the direct light, but the amplitude of their interference would be almost the same as that of the direct light (5, 18). This would result in very little specimen contrast.

To speed up the direct undeviated zeroth order light, a phase plate is installed with a ring shaped ***phase shifter*** attached to it at the rear focal plane of the objective. The

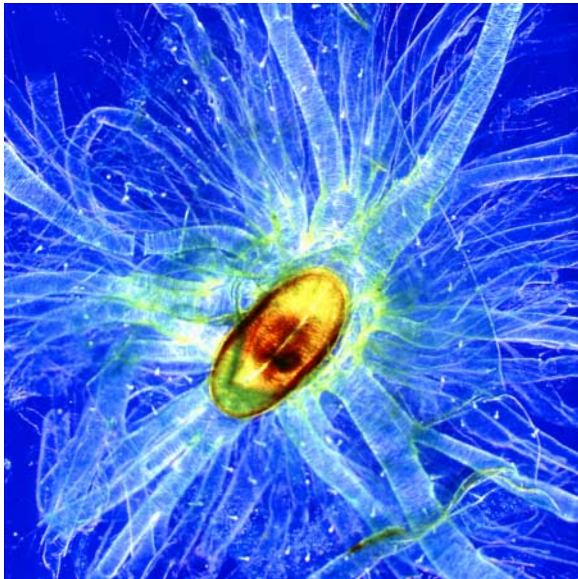


Figure 17. Spiracle and trachea from silkworm larva photographed at low magnification under Rheinberg illumination using a blue central and yellow annulus filters and a 2x objective.

narrow area of the phase ring is optically thinner than the rest of the plate. As a result, undeviated light passing through the phase ring travels a shorter distance in traversing the glass of the objective than does the diffracted light. Now, when the direct undeviated light and the diffracted light proceed to the image plane, they are 1/2 wavelength out of phase with each other. The diffracted and direct light can now interfere destructively so that the details of the specimen appear dark against a lighter background (just as they do for an absorbing or amplitude specimen). This is a description of what takes place in positive or dark phase contrast.

If the ring phase shifter area of the phase plate is made optically thicker than the rest of the plate, direct light is slowed by 1/4 wavelength. In this case, the zeroth order light arrives at the image plane in step (or in phase) with the diffracted light, and constructive interference takes place. The image appears bright on a darker background. This type of phase contrast is described as ***negative*** or ***bright contrast*** (2, 5, 18, 19).

Because undeviated light of the zeroth order is much brighter than the faint diffracted light, a thin absorptive transparent metallic layer is deposited on the phase ring to bring the direct and diffracted light into better balance of intensity to increase contrast. Also, because speeding up or slowing down of the direct light is calculated on a 1/4 wavelength of green light, the phase image will appear best when a green filter is placed in the light path (a green interference filter is preferable). Such a green filter also helps achromatic objectives produce their best images, because achromats are spherically corrected for green light.

The accessories needed for phase contrast work are a substage phase contrast condenser equipped with annuli and a set of phase contrast objectives, each of which has a phase plate installed. The condenser usually has a brightfield position with an aperture diaphragm and a rotating turret of annuli (each phase objective of different magnification requires an annulus of increasing diameter as the magnification of the objective increases). Each phase objective has a darkened ring on its back lens. Such objectives can also be used for ordinary brightfield transmitted light work with only a slight reduction in image quality. A photomicrograph of a hair cross sections from a fetal mouse taken using phase contrast illumination is illustrated in Figure 19.

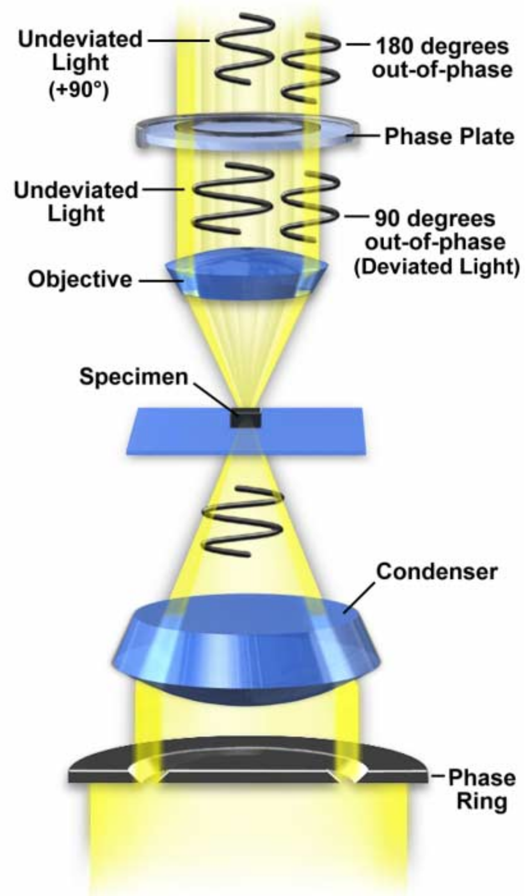


Figure 18. Schematic configuration for phase contrast microscopy. Light passing through the phase ring is first concentrated onto the specimen by the condenser. Undeviated light enters the objective and is advancedd by the phase plate before interference at the rear focal plane of the objective.

Polarized Light

Many transparent solids are optically isotropic, meaning that the index of refraction is equal in all directions throughout the crystalline lattice. Examples of isotropic solids are glass, table salt (sodium chloride), many polymers, and a wide variety of both organic and inorganic compounds (32, 33).

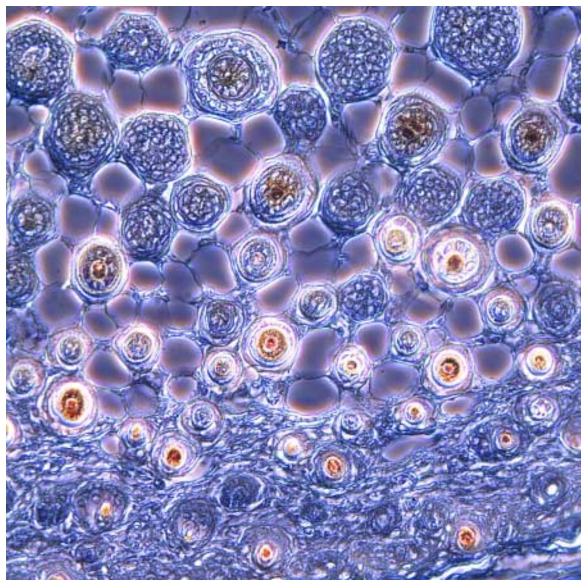


Figure 19. Photomicrograph of hair cross sections from a fetal mouse taken using phase contrast optics and a 20x objective.

Crystals are classified as being either isotropic or anisotropic depending upon their optical behavior and whether or not their crystallographic axes are equivalent. All isotropic crystals have equivalent axes that interact with light in a similar manner, regardless of the crystal orientation with respect to incident light waves. Light entering an isotropic crystal is refracted at a constant angle and passes through the crystal at a single velocity without being polarized by interaction with the electronic components of the crystalline lattice.

Anisotropic crystals, on the other hand, have crystallographically distinct axes and interact with light in a manner that is dependent upon the orientation of the crystalline lattice with respect to the incident light. When light enters the optical axis of anisotropic crystals, it acts in a manner similar to interaction with isotropic crystals and passes through at a single velocity. However, when light enters a non-equivalent axis, it is refracted into two rays each polarized with their vibration directions oriented at right angles to one another, and traveling at different velocities. This phenomenon is termed ***double*** or ***bi-refraction*** and is seen to a greater or lesser degree in all anisotropic crystals (32-34).

When anisotropic crystals refract light, the resulting rays are polarized and travel at different velocities. One of the rays travels with the same velocity in every direction through the crystal and is termed the ordinary ray. The other ray travels with a velocity that is dependent upon the propagation direction within the crystal. This light ray is termed the extraordinary ray. The retardation between the ordinary and extraordinary ray increases with increasing crystal thickness. The two independent refractive indices of anisotropic crystals are quantified in terms of their birefringence, a measure of the difference in refractive index. Thus, the birefringence (**B**) of a crystal is defined as:

$$\mathbf{B} = |\mathbf{n}_{high} - \mathbf{n}_{low}| \qquad (5)$$

Where $\mathbf{n}_{high}$ is the largest refractive index and $\mathbf{n}_{low}$ is the smallest. This expression holds true for any part or fragment of an anisotropic crystal with the exception of light waves propagated along the optical axis of the crystal. As mentioned above, light that is doubly refracted through anisotropic crystals is polarized with the vibration directions of the polarized ordinary and extraordinary light waves being oriented perpendicular to each other. We can now examine how anisotropic crystals behave under polarized illumination in a polarizing microscope.

A polarizer place beneath the substage condenser is oriented such that polarized light exiting the polarizer is plane polarized in a vibration direction that is east-west with respect to the optic axis of the microscope stand. Polarized light enters the anisotropic crystal where it is refracted and divided into two separate components vibrating parallel to the crystallographic axes and perpendicular to each other. The polarized light waves then pass through the specimen and objective before reaching a second polarizer (usually termed the analyzer) that is oriented to pass a polarized vibration direction perpendicular to that of the substage polarizer. Therefore, the analyzer passes only those components of the light waves that are parallel to the polarization direction of the analyzer. The retardation of one ray with respect to another is caused the difference in speed between the ordinary and extraordinary rays refracted by the anisotropic crystal (33, 34). A schematic illustration of microscope configuration for crossed polarized illumination is presented in Figure 20.

Now we will consider the phase relationship and velocity differences between the ordinary and extraordinary rays after they pass through a birefringent crystal. These rays are oriented so that they are vibrating at right angles to each other. Each ray will encounter a slightly different electrical environment (refractive index)

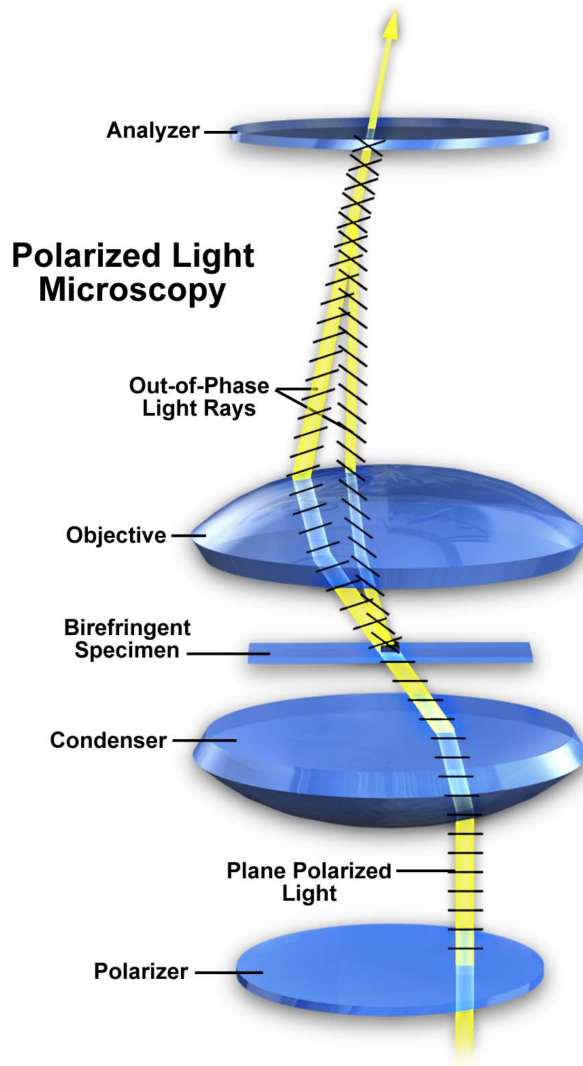


Figure 20. Schematic microscope configuration for observing birefringent specimens under crossed polarized illumination. White light passing through the polarizer is plane polarized and concentrated onto the birefringent specimen by the condenser. Light rays emerging from the specimen interfere when they are recombined in the analyzer, subtracting some of the wavelengths of white light, thus producing a myriad of tones and colors.

as it enters the crystal and this will affect the velocity at which ray passes through the crystal (32, 33). Because of the difference in refractive indices, one ray will pass through the crystal at a slower rate than the other ray. In other words, the velocity of the slower ray will be retarded with respect to the faster ray. This retardation can be quantified using the following equation:

$$\textbf{Retardation (G) = thickness (t) x Birefringence (B)} \quad (6)$$

or

$$\Gamma = t \times |n_{high} - n_{low}| \quad (7)$$

Where Γ is the quantitative retardation of the material, **t** is the thickness of the birefringent crystal (or material) and **B** is birefringence as defined above (33). Factors contributing to the value of retardation are the magnitude of the difference in refractive indices for the environments seen by the ordinary and extraordinary rays and also the sample thickness. Obviously, the greater the difference in either refractive indices or thickness, the greater the degree of retardation. Early observations made on the mineral calcite indicated that thicker calcite crystals caused greater differences in splitting of the images seen through the crystals. This agrees with the equation above that states retardation will increase with crystal (or sample) thickness.

When the ordinary and extraordinary rays emerge from the birefringent crystal, they are still vibrating at right angles with respect to one another. However, the component vectors of these waves that pass through the analyzer are vibrating in the same plane. Because one wave is retarded with respect to the other, interference (either constructive or destructive) occurs between the waves as they pass through the analyzer. The net result is that some birefringent samples (in white light) acquire a spectrum of color when observed through crossed polarizers.

Polarized light microscopy requires strain-free objectives and condensers to avoid depolarization effects on the transmitted light (32-34). Most polarized

Figure 21. Photomicrograph of high-density columnar-hexatic liquid crystalline calf thymus DNA at a concentration of approximately 450 milligrams/milliliter. This concentration of DNA is approaching that found in sperm heads, virus capsids, and dinoflagellate chromosomes. The image was recorded using a polarized light microscope and the 10x objective.

microscopes are equipped with a centerable stage that has free 360-degree rotation about the optical axis of the microscope. A Bertrand lens is commonly inserted into the light path so that conoscopic images of birefringence patterns can be observed at the back of the objective. Manufacturers also offer a wide range of compensators and light retarders (full-wave and quarter-wave plates) for quantitative birefringence measurements and for adding color to polarized light photomicrographs. Birefringent DNA liquid crystals photographed using crossed polarized illumination are illustrated in Figure 21.

Hoffman Modulation Contrast

The Hoffman Modulation Contrast system is designed to increase visibility and contrast in unstained and living material by detecting optical gradients (or slopes) and converting them into variations of light intensity. This ingenious technique was invented by Dr. Robert Hoffman in 1975, and employs several accessories that have been adapted to the major commercial microscopes (16, 18). A schematic illustration of microscope configuration for Hoffman modulation contrast is presented in Figure 22.

An optical amplitude spatial filter, termed a ***modulator*** by Hoffman, is inserted at the rear focal plane of an achromat or planachromat objective (although higher correction can also be used). Light intensity passing through this system varies above and below an average value, which by definition, is then said to be modulated. Objectives useful for modulation contrast can cover the entire magnification range of 10x to 100x. The modulator has three zones: a small, dark zone near the periphery of the rear focal plane which transmits only one percent of light; a narrow gray zone which transmits 15 percent; and the remaining clear or transparent zone, covering most of the area at the back of the objective, which transmits 100 percent of the light (5, 16, 18). Unlike the phase plate in phase contrast microscopy, the Hoffman modulator is designed not to alter the phase of light passing through any of the zones. When viewed under modulation contrast optics, transparent objects that are essentially invisible in ordinary brightfield microscopy take on an apparent three-dimensional appearance dictated by phase gradients. The modulator does not introduce changes in the phase relationship of light passing through the system, but influences the principal zeroth order maxima. Higher order diffraction maxima are unaffected. Measurements using a Michelson interferometer confirm that the spatial coherency of light passed through a Hoffman-style modulator varies (if any) by a factor of less than l/20 (5).

Below the stage, a condenser with rotating turret is utilized to hold the remaining components of the Hoffman

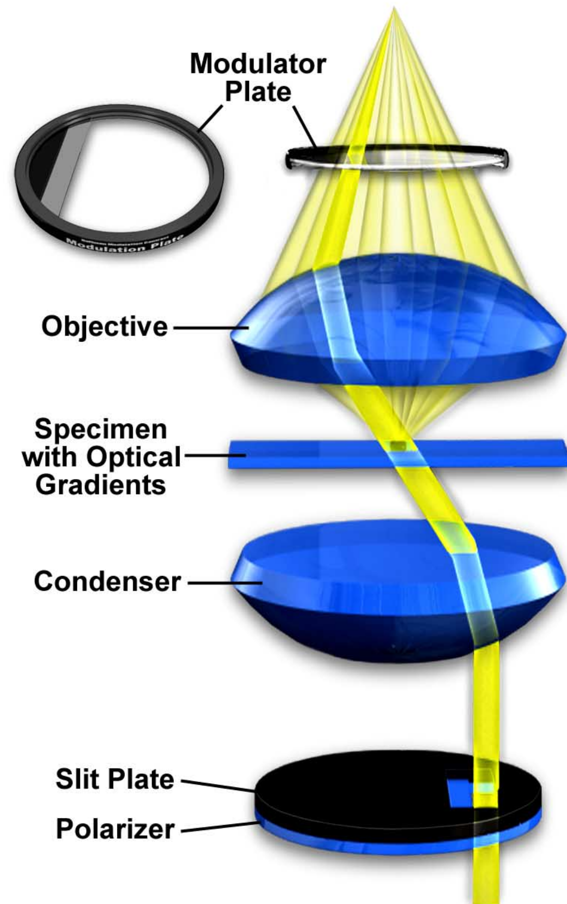


Figure 22. Schematic illustration of microscope configuration for Hoffman modulation contrast. Light passing through the polarizer and slit is concentrated onto the specimen by the condenser. After passing through the specimen light enters the objective and is filtered by the modulator plate in the rear focal plane of the objective.

Modulation Contrast system. The turret condenser has a brightfield opening with an aperture iris diaphragm for regular brightfield microscopy and for alignment and establishing proper conditions of Köhler illumination for the microscope. At each of the other turret openings, there is an off-center slit that is partially covered with a small rectangular polarizer. The size of the slit/polarizer combination is different for each objective of different magnification; hence the need for a turret arrangement. When light passes through the off-axis slit, it is imaged at the rear focal plane of the objective (also termed the Fourier plane) where the modulator has been installed. Like the central annulus and phase ring in phase contrast microscopy, the front focal plane of the condenser

containing the off-axis slit plate is optically conjugate to the modulator in objective rear focal plane. Image intensity is proportional to the first derivative of the optical density in the specimen, and is controlled by the zeroth order of the phase gradient diffraction pattern.

Below the condenser, a circular polarizer is placed on the light exit port of the microscope (note that both polarizers are below the specimen). The rotation of this polarizer can control the effective width of the slit opening. For example, a ***crossing*** of both polarizers at 90 degrees to each other results in ***narrowing*** the slit so that its image falls within the gray area of the modulator (16, 18). The part of the slit controlled by the polarizer registers on the bright area of the modulator. As the polarizer is rotated, contrast can be varied for best effect. A very narrow slit produces images that are very high in contrast with a high degree of coherence. Optical section imaging is also optimized when the slit is adjusted to its narrowest position. When the circular polarizer is oriented with its vibration direction parallel to that of the polarizer in the slit, the effective slit width is at a maximum. This reduces overall image contrast and coherence, but yields much better images of thicker objects where large differences in refractive index exist.

In modern advanced modulation contrast systems, both the modulator and the slit are offset from the optical axis of the microscope (18). This arrangement permits fuller use of the numerical aperture of the objective and results in good resolution of specimen detail. Shapes and details are rendered in shadowed, pseudo three-dimensional appearance. These appear brighter on one side, gray in the central portion, and darker on the other side, against a gray background. The modulator converts optical phase gradients in details (steepness, slope, rate of change in refractive index, or thickness) into changes in the intensity of various areas of the image at the plane of the eyepiece diaphragm. Resulting images have an apparent three-dimensional appearance with directional sensitivity to optical gradients. The contrast (related to variations in intensity) of the dark and bright areas against the gray gives a shadowed pseudo-relief effect. This is typical of modulation contrast imaging. Rotation of the polarizer alters the contrast achieved and the orientation of the specimen on the stage (with respect to the polarizer and offset slit) may dramatically improve or degrade contrast.

There are numerous advantages as well as limitations to modulation contrast. Some of the advantages include fuller use of the numerical aperture of the objective yielding excellent resolution of details along with good specimen contrast and visibility. Although many standard modulation contrast objectives are achromats or planachromats, it is also possible to use objectives with a higher degree of correction for optical aberration. Many major microscope manufacturers now offer modulation contrast objectives in fluorite-correction grades, and apochromats can be obtained by special order. Older objectives can often be retrofitted with a modulator made by Modulation Optics, Inc., the company founded by Dr. Robert Hoffman specifically to build aftermarket and custom systems.

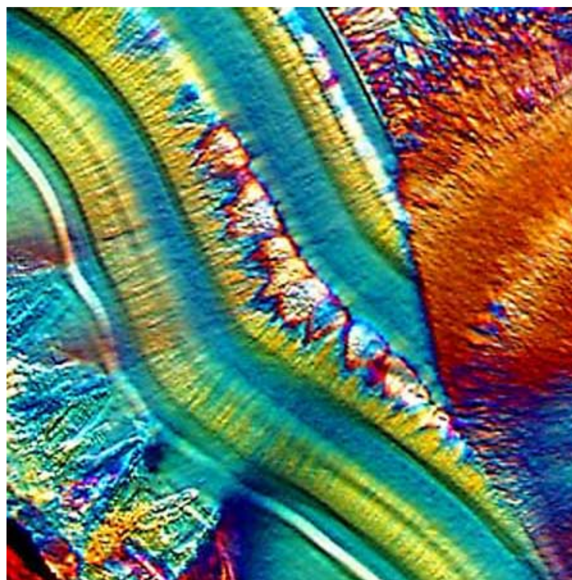


Figure 23. Duck-billed dinosaur bone thin section photographed using a combination of Hoffman modulation contrast and polarized light illumination using a 20x objective. Note the pseudo three-dimensional relief evident throughout the photomicrograph as a result of amplitude gradients (Hoffman modulation contrast). The vivid colors are due to interference of white light at the analyzer (polarized light).

In addition to the advantages of using higher numerical apertures with modulation contrast, it is also possible to do ***optical sectioning*** with this technique (16, 18). Sectioning allows the microscopist to focus on a single thin plane of the specimen without interference from confusing images arising in areas above or below the plane that is being focused on. The depth of a specimen is measured in a direction parallel to the optical axis of the microscope. Focusing the image establishes the correct specimen-to-image distance, allowing interference of the diffracted waves to occur at a pre-determined plane (the image plane) positioned at a fixed distance from the eyepiece. This enables diffracting objects that occur at different depth levels in the specimen to be viewed separately, provided there is sufficient contrast. The entire depth of a specimen can be optically sectioned by sequentially focusing on each succeeding plane. In this system, depth of field is defined as the distance from one level to the next where imaging of distinct detail occurs,

and is controlled by the numerical aperture of the objective (5, 16, 18). Higher numerical aperture objectives exhibit very shallow depths of field and the opposite holds for objectives of lower numerical aperture. The overall capability of an objective to isolate and focus on a specific optical section diminishes as the optical homogeneity of the specimen decreases.

Birefringent objects (rock thin sections, crystals, bone, etc.), that can confuse images in DIC, can be examined because the specimen is ***not*** situated between the two polarizers (18). Further, specimens can be contained in plastic or glass vessels without deterioration of the image due to polarization effects, because such vessels are also above both polarizers, not between them. This allows the Hoffman system to be far more useful than DIC in the examination and photomicrography of cell, tissue, and organ culture performed in plastic containers.

Hoffman modulation contrast can be simultaneously combined with polarized light microscopy to achieve spectacular effects in photomicrography. Figure 23 illustrates a dinosaur bone thin section photographed with a combination of Hoffman modulation contrast and plane polarized illumination. Note the pseudo three-dimensional relief apparent throughout the micrograph and the beautiful coloration provided by polarized light.

Differential Interference Contrast

In the mid 1950s a French optics theoretician named Georges Nomarski improved the method for detecting optical gradients in specimens and converting them into intensity differences (15). Today there are several implementations of this design, which are collectively called differential interference contrast (DIC). Living or stained specimens, which often yield poor images when viewed in brightfield illumination, are made clearly visible by optical rather than chemical means (2, 4, 5, 15, 18-22).

In transmitted light DIC, light from the lamp is passed through a polarizer located beneath the substage condenser, in a manner similar to polarized light microscopy. Next in the light path (but still beneath the condenser) is a modified Wollaston prism that ***splits*** the entering beam of polarized light into two beams traveling in slightly different direction (illustrated in Figure 24). The prism is composed of two halves cemented together (36). Emerging light rays vibrate at 90 degrees relative to each other with a slight path difference. A different prism is needed for each objective of different magnification. A revolving turret on the condenser allows the microscopist to rotate the appropriate prism into the light path when changing magnifications.

The plane polarized light, vibrating only in one direction perpendicular to the propagation direction of the light beam, enters the beam-splitting modified Wollaston prism and is split into two rays, vibrating perpendicular to each other (Figure 24). These two rays travel close together but in slightly different directions. The rays intersect at the front focal plane of the condenser, where they pass traveling parallel and extremely close together with a slight path difference, but they are vibrating perpendicular to each other and are therefore unable to cause interference. The distance between the rays, called the shear, is so minute that it is less than the resolving ability of the objective (5, 18, 36).

The split beams enter and pass through the specimen where their wave paths are altered in accordance with the specimen's varying thickness, slopes, and refractive

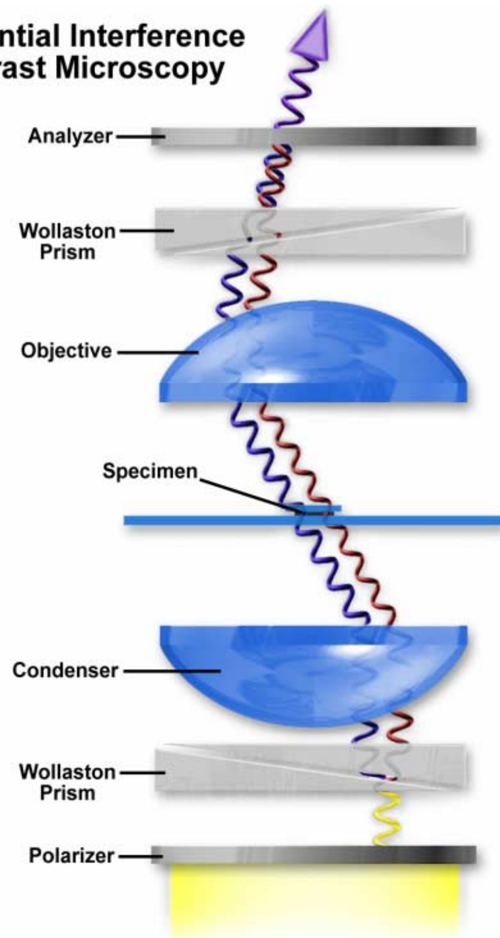


Figure 24. Schematic illustration of microscope configuration for differential interference contrast. Light is polarized in a single vibration plane by the polarizer before entering the lower modified Wollaston prism that acts as a beam splitter. Next, the light passes through the condenser and sample before the image is reconstructed by the objective. Above the objective, a second Wollaston (Nomarski) prism acts as a beam-combiner and passes the light to the analyzer, where it interferes both constructively and destructively.

(a)

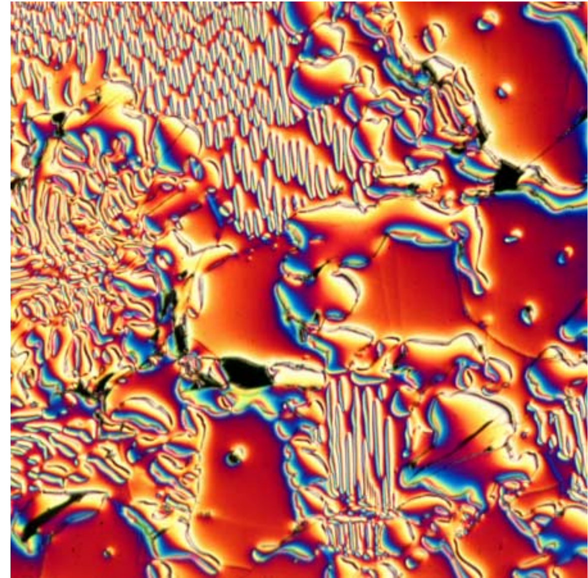

(b)

Figure 25 (a) Nomarski transmitted light differential interference contrast (DIC) photomicrograph of mouthparts from a blowfly. **(b)** Reflected light differential interference contrast photomicrograph illustration defects on the surface of a ferro-silicon alloy. Both images were captured using the 10x objective and a first-order retardation plate.

indices. These variations cause alterations in the wave path of both beams passing through areas of the specimen details lying close together. When the parallel beams enter the objective, they are focused above the rear focal plane where they enter a second modified Wollaston prism that combines the two beams. This removes the shear and the original path difference between the beam pairs. As a result of having traversed the specimen, the paths of the parallel beams are not of the same length (optical path difference) for differing areas of the specimen.

In order for the beams to interfere, the vibrations of the beams of different path length must be brought into the same plane and axis. This is accomplished by placing a second polarizer (analyzer) above the upper Wollaston beam-combining prism. The light then proceeds toward the eyepiece where it can be observed as differences in intensity and color. The design results in one side of a detail appearing bright (or possibly in color) while the other side appears darker (or another color). This shadow effect bestows a pseudo three-dimensional appearance to the specimen (18).

In some microscopes, the upper modified Wollaston prism is combined in a single fitting with the analyzer incorporated above it. The upper prism may also be arranged so it can be moved horizontally. This allows for varying optical path differences by moving the prism, providing the user a mechanism to alter the brightness and color of the background and specimen. Because of the prism design and placements, the background will be homogeneous for whatever color has been selected.

The color and/or light intensity effects shown in the image are related especially to the rate of change in refractive index, specimen thickness, or both. Orientation of the specimen can have pronounced effect on the relief-like appearance and often, rotation of the specimen by 180 degrees changes a ***hill*** into a ***valley*** or visa versa. The three-dimensional appearance is not representing the true geometric nature of the specimen, but is an exaggeration based on ***optical thickness***. It is not suitable for accurate measurement of actual heights and depths.

There are numerous advantages in DIC microscopy as compared particular to phase and Hoffman modulation contrast microscopy. With DIC, it is also possible to make fuller use of the numerical aperture of the system and to provide optical staining (color). DIC also allows the microscope to achieve excellent resolution. Use of full objective aperture enables the microscopist to focus on a thin plane section of a thick specimen without confusing images from above or below the plane. Annoying ***halos***, often encountered in phase contrast, are absent in DIC images, and common achromat and fluorite objectives can be used for this work. A downside is that plastic tissue culture vessels and other birefringent specimens yield confusing images in DIC. Also, high-quality apochromatic objectives are now designed to be suitable for DIC. Figure 24 presents transmitted and reflected light DIC photomicrographs of the mouthparts of a blowfly

(transmitted DIC) and surface defects in a ferro-silicate alloy (reflected DIC). Both photomicrographs were made using a retardation plate with a 10x objective.

Fluorescence Microscopy

Fluorescence microscopy is an excellent tool for studying material which can be made to fluoresce, either in its natural form (primary or autofluorescence) or when treated with chemicals capable of fluorescing (secondary fluorescence). This form of optical microscopy is rapidly reaching maturity and is now one of the fastest growing areas of investigation using the microscope (6-8).

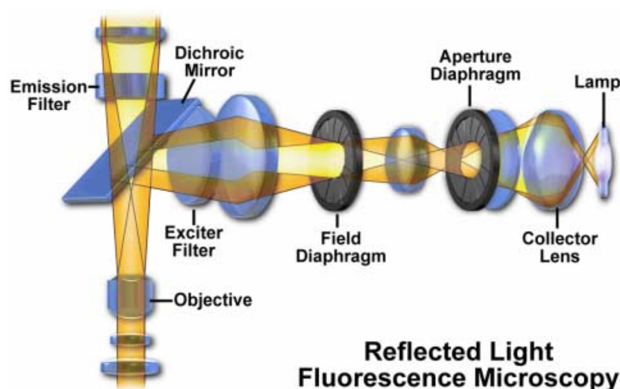


Figure 26. Schematic diagram of the configuration of reflected light fluorescence microscopy. Light emitted from a mercury burner is concentrated by the collector lens before passing through the aperture and field diaphragms. The exciter filter passes only the desired excitation wavelengths, which are reflected down through the objective to illuminate the specimen. Longer wavelength fluorescence emitted by the specimen passes back through the objective and dichroic mirror before finally being filtered by the emission filter.

The basic task of the fluorescence microscope is to permit excitation light to irradiate the specimen and then to separate the much weaker re-radiating fluorescent light from the brighter excitation light. Thus, only the emission light reaches the eye or other detector. The resulting fluorescing areas shine against a dark background with sufficient contrast to permit detection. The darker the background of the non-fluorescing material, the more efficient the instrument. For example, ultraviolet (UV) light of a specific wavelength or set of wavelengths is produced by passing light from a UV-emitting source through the exciter filter. The filtered UV light illuminates the specimen, which emits fluorescent light of longer wavelengths while illuminated with ultraviolet light. Visible light emitted from the specimen is then filtered through a barrier filter that does not allow reflected UV light to pass. It should be noted that this is the only mode of microscopy in which the specimen, subsequent to excitation, gives off its own light (6). The emitted light re-radiates spherically in all directions, regardless of the direction of the exciting light. A schematic diagram of the configuration for fluorescence microscopy is presented in Figure 26.

Fluorescence microscopy has advantages based upon attributes not as readily available in other optical microscopy techniques. The use of fluorochromes has made it possible to identify cells and sub-microscopic cellular components and entities with a high degree of specificity amidst non-fluorescing material. What is more, the fluorescence microscope can reveal the presence of fluorescing material with exquisite sensitivity. An extremely small number of fluorescing molecules (as few as 50 molecules per cubic micron) can be detected (6-8). In a given sample, through the use of multiple staining, different probes will reveal the presence of different target molecules. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of the respective objectives, the presence of fluorescing molecules below such limits is made visible.

Techniques of fluorescence microscopy can be applied to organic material, formerly living material, or to living material (with the use of in vitro or in vivo fluorochromes). These techniques can also be applied to inorganic material (especially in the investigation of contaminants on semiconductor wafers). There are also a burgeoning number of studies using fluorescent probes to monitor rapidly changing physiological ion concentrations (calcium, magnesium, etc.) and pH values in living cells (1, 7, 8).

There are specimens that fluoresce when irradiated with shorter wavelength light (primary or autofluorescence). Autofluorescence has been found useful in plant studies, coal petrography, sedimentary rock petrology, and in the semiconductor industry. In the study of animal tissues or pathogens, autofluorescence is often either extremely faint or of such non-specificity as to make autofluorescence of minimal use. Of far greater value for such specimens are the fluorochromes (also called fluorophores) which are excited by irradiating light and whose eventual yield of emitted light is of greater intensity. Such fluorescence is called secondary fluorescence.

Fluorochromes are stains, somewhat similar to the better-known tissue stains, which attach themselves to visible or sub-visible organic matter. These fluorochromes, capable of absorbing and then re-radiating light, are often highly specific in their attachment targeting and have significant yield in absorption-emission ratios. This makes them extremely valuable in biological

application. The growth in the use of fluorescence microscopes is closely linked to the development of hundreds of fluorochromes with known intensity curves of excitation and emission and well-understood biological structure targets (6). When deciding which label to use for fluorescence microscopy, it should be kept in mind that the chosen fluorochrome should have a high likelihood of absorbing the exciting light and should remain attached to the target molecules. The fluorochrome should also be capable of providing a satisfactory yield of emitted fluorescence light. Illustrated in Figure 27 is a photomicrograph of cat brain cells infected with Cryptococcus. The image was made using a combination of fluorescence and DIC microscopy, taking advantage of the features from both contrast enhancing techniques. Infected neurons are heavily stained with the DNA-specific fluorescent dye acridine orange, and the entire image is rendered in a pseudo three-dimensional effect by DIC.

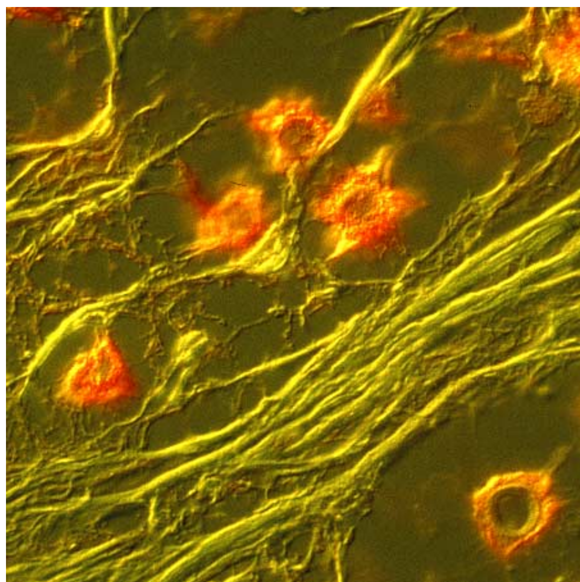


Figure 27. Fluorescence/DIC combination photomicrograph of cat brain tissue infected with Cryptococcus. Infected neurons are heavily stained with the DNA-specific fluorescent dye acridine orange. Note the pseudo three-dimensional effect that occurs when these two techniques are combined. The micrograph was recorded using a 40x objective.

Photomicrography and Digital Imaging

The use of photography to capture images in a microscope dates back to the invention of the photographic process (37). Early photomicrographs were remarkable for their quality, but the techniques were laborious and burdened with long exposures and a difficult process for developing emulsion plates. The primary medium for photomicrography was film until the past decade when improvements in electronic camera and computer technology made digital imaging cheaper and easier to use than conventional photography. This section will address photomicrography both on film and with electronic analog and digital imaging systems.

The quality of a photomicrograph, either digital or recorded on film, is dependent upon the quality of the microscopy. Film is a stern judge of how good the microscopy has been prior to capturing the image. It is essential that the microscope be configured using Köhler illumination, and that the field and condenser diaphragms are adjusted correctly and the condenser height is optimized. When properly adjusted, the microscope will yield images that have even illumination over the entire field of view and display the best compromise of contrast and resolution.

Photographic Films

Films for photography are coated with a light-sensitive emulsion of silver salts and/or dyes. When light is allowed to expose the emulsion, ***active centers*** combine to form a latent image that must be developed by use of photographic chemicals (19, 37-39). This requires exposure of the film in a darkened container to a series of solutions that must be controlled with respect to temperature, development time, and with the appropriate agitation or mixing of the solutions. The developing process must then be halted by means of a ***stop*** solution. Next, the unexposed emulsion material, which consists of silver salts and dyes, is cleared and the film fixed, then washed and dried for use. The development, stop, fixing, and clearing must be done under darkroom conditions or in light-tight developing tanks, and film must be handled in complete darkness. The rigors of temperature, duration, and agitation are usually dependent upon the film being used. For example, the Kodachrome K14 process is far more demanding than the E6 process used for Ektachrome and Fujichrome (19, 37). The emulsion speed determines how much light must be used to expose the film in a given time period. Films are rated according to their ASA or ISO number, which gives an indication of the relative film speed. Larger ISO numbers indicate faster films with an ISO of 25 being one of the slowest films available and ISO 1600 one of the fastest. Because the microscope is a relatively stable platform with good illumination properties, films in the 50-200 ISO range are commonly used for photomicrography.

Film is divided into a number of categories depending upon whether it is intended for black/white or color

photography. Color films are subdivided into two types: color films that yield positive transparencies (colors like those of the image being observed) and color negatives (colors complementary to those in the microscope image, e.g., green for magenta). The color films can be further subdivided into two groups: films designed to receive light of daylight quality and those designed to receive indoor or tungsten light. As a rule, but with some exceptions, transparency or positive films (slides) have the suffix ***chrome***, such as Kodachrome, Ektachrome, Fujichrome, Agfachrome, etc. Color negative films usually end with the suffix ***color***, such as Kodacolor, Ektacolor, Fujicolor, Agfacolor, etc. (37). Each of these film types is offered in a variety of film speeds or ISO ratings. Film packages usually display the ISO of the film and whether the film is intended for daylight or an indoor/tungsten balanced illumination. Modern film magazines have a code (termed the DX number) that allows camera backs to automatically recognize the film speed.

The color temperature of tungsten-halide lamps found in modern microscopes varies between 2900K and 3200K, depending upon the voltage applied to the lamp filament. Film manufacturers offer film balanced for this illumination, and usually indicate on the magazine that the film is intended for indoor or scientific use. Fuji offers a very nice transparency film, Fujichrome 64T, that has a rather slow emulsion speed intended for tungsten illumination, but is designed to perform well with ***push*** film processing. To push a film, it is first underexposed by one or several f-stops, then the development time in the first developer is increased to decrease film density. This technique often will increase the color saturation of the image (19, 37).

Daylight-balanced film, by far the most common film available at retailers at a wide variety of ISOs, can also be used with the microscope, provided an appropriate filter is added to the light path. Manufacturers usually add a daylight conversion light filter to their microscope packages as standard equipment and high-end research microscopes usually have this filter (called a daylight color temperature conversion filter) in a cassette housed in the base of the microscope. Almost any color print or transparency film can be used for microscopy, provided the daylight-balanced filter is in place for those films designed for a 5500K color temperature or removed if the film is balanced for tungsten illumination (3200K).

For many applications, securing the photomicrograph almost immediately is a necessity or a great advantage. Here the Polaroid films are unrivaled. These films for photomicrography are available in three sizes: 35 millimeter Polachrome (color transparency), and Polapan (black/white) in 3¼” x 4¼” film packs and 4” x 5” individual film packets (37, 39). Larger formats are available in color (ID numbers 668/669 or 58/59) or black/white (ID numbers 667, 52, 665, 55, etc.).

The Polaroid large format films produce a paper negative and a paper positive print. After the positive print has been made, the paper negative is peeled away and discarded. This can be accomplished within a matter of a few minutes. The color films, with the exception of the film pack 64T and the large format 64T, are balanced for daylight (5500 Kelvin) color temperature. All microscope manufacturers supply adapters for their photomicrographic cameras that accept large format Polaroid film sizes.

Polaroid black/white films 665 P/N and 55 P/N require special mention. These films produce a paper positive print and a polymer-based negative. If the negative is bathed in an 18% sodium sulfite solution for a few minutes, washed, and hung up to dry, it will produce a permanent negative of high quality and high resolution suitable for use in printing (37, 39).

The Polaroid 35mm films deserve more popular use. They are loaded into the usual 35mm camera back and exposed in the typical manner according to their rated ISO and color temperature requirements. When the film is purchased, the container includes the film cassette and a small box of developer. The film is processed in a tabletop processor that measures approximately 4" x 5" x 9" in size. All processing is carried out in daylight after the processor has been loaded and closed. The finished strip of positive color or positive black/white transparencies is removed and ready for examination and for cutting apart for mounting in frame holders for projection. The actual processing takes only five minutes and produces micrographs of quite accurate color and good resolution.

Recently, Polaroid has marketed a relatively inexpensive photomicrographic camera call the MicroCam. This camera is lightweight and can be inserted into one of the eyetubes or phototube of the microscope. It contains an exposure meter, an eyepiece and an automatically controlled shutter. The camera accepts 339 color film packs or 331 black/white film packs. These films, approximately 3" x 3" in size, are self-developing and do not require peeling apart. Although the films have only about half the resolution of the more common large format Polaroid films, the resulting print is easily obtained and may be quite adequate for many applications.

Digital Photomicrography

Over the past decade, the quality of digital cameras has greatly improved and today, the market has exploded with at least 40 manufacturers offering a wide variety of models to suit every application. The cameras operate by

capturing the image projected directly onto a computer chip without the use of film. In recent years, the number of pixels of information capable of being captured and stored by the best digital cameras is approaching, but still short of, the resolution available with traditional film (37). Digital images offer many opportunities for computer-controlled image manipulation and enhancement as well as the advantage of permanence of digital storage. The highest quality digital cameras can cost many thousands of dollars.

Selection of an electronic camera must be preceded by careful consideration of its proposed use. This includes examination of fixed specimens or live specimens, need for grayscale or true color images, sensitivity to low light levels as in fluorescence, resolution, speed of image acquisition, use in qualitative or quantitative investigations, and the video feed rate into a computer or VCR (1, 2, 9, 37).

The two general types of electronic cameras available for microscopy are the tube cameras (Vidicon family) or CCD (charge coupled device) cameras. Either type can be intensified for increased sensitivity to low light. The SIT (silicon intensified tube) or ISIT is useful for further intensification of tube cameras, while the ICCD is an intensified CCD camera. CCD cameras can also be cooled to increase sensitivity by giving a better signal to noise ratio. These kinds of cameras can be designed to respond to light levels undetectable by the human eye (2, 37).

The criteria for selection of an electronic camera for microscopy include sensitivity of the camera, quantum efficiency, signal to noise ratio, spectral response, dynamic range capability, speed of image acquisition and readout, linearity or response, speed of response in relation to changes in light intensity, geometric accuracy, and ready adaptability to the microscope. A very important criterion for the newer digital CCD cameras is resolution. Current chips range from as few as 64 x 64 pixels up to 2048 x 2048 and above for very specialized applications, but larger arrays are continuously being introduced and, at some not-to-distant time, digital image resolution will rival that of film.

The purchaser must also decide whether or not the camera will operate at video rate (therefore being easily compatible with such accessories as video recorders or video printers). Tube cameras and CCD cameras are available for video rate operation. CCD cameras can also deliver slow acquisition or high-speed acquisition of images. Scientific, rather than commercial grade, CCD cameras are the variety most suitable for research.

CCD cameras are usually of small size. They have low distortion, no lag, and good linearity of response. These cameras are also more rugged and less susceptible to mishandling than tube cameras. Each pixel of the CCD camera serves as a well for charge storage of the incoming photons for subsequent readout. The depth of these wells relates to the quality of the response. Binning of adjacent pixels by joining them together into ***super pixels*** can be employed to speed readout in a slow-scan CCD camera (2, 37).

An emerging technology that shows promise as the possible future of digital imaging is the active pixel sensor (APS) complementary metal oxide semiconductor (CMOS) ***camera on a chip***. Mass production of CMOS devices is very economical and many facilities that are currently engaged in fabrication of microprocessors, memory, and support chips can be easily converted to produce CMOS optical sensors. Although CCD chips were responsible for the rapid development of video camcorders, the technology has remained trapped as a specialized process that requires custom tooling outside the mainstream of integrated circuit fabrication. Also, the CCD devices require a substantial amount of support circuitry and it is not unusual to find five to six circuit boards in a typical video camcorder.

In the center of the APS CMOS integrated circuit is a large array of optical sensors that are individual photodiode elements covered with dyed filters and arranged in a periodic matrix. A photomicrograph of the entire die of a CMOS chip is illustrated in Figure 28. High magnification views of a single “pixel” element (Figure 28) reveal a group of four photodiodes containing filters based on the primary colors red, green, and blue. Each photodiode is masked by either a red, green, or blue filter in low-end chips, but higher resolution APS CMOS devices often use a teal (blue-green) filter in place of one of the green filters. Together, the four elements illustrated in Figure 28 comprise the light-sensitive portion of the pixel. Two green filter masks are used because visible light has an average wavelength of 550 nanometers, which lies in the green color region. Each pixel element is controlled by a set of three transistors and an amplifier that work together to collect and organize distribution of optical information. The array is interconnected much like memory addresses and data busses on a DRAM chip so that the charge generated by photons striking each individual pixel can be accessed randomly to provide selective sampling of the CMOS sensor.

The individual amplifiers associated with each pixel help reduce noise and distortion levels, but they also induce an artifact known as ***fixed pattern*** noise that arises from small differences in the behavior of individual pixel amplifiers. This is manifested by reproducible patterns of ***speckle*** behavior in the image generated by CMOS active pixel sensor devices. A great deal of research effort has been invested in solving this problem, and the residual

level of noise has been dramatically reduced in CMOS sensors. Another feature of CMOS active pixel sensors is the lack of column streaking due to pixel bloom when shift registers overflow. This problem is serious with the CCDs found in most video camcorders. Another phenomenon, known as ***smear***, which is caused by charge transfer in CCD chips under illumination, is also absent in CMOS active pixel sensor devices.

Assisting the CMOS device is a coprocessor that is matched to the APS CMOS to optimize the handling of image data. Incorporated into the co-processor is a proprietary digital video processor engine that is capable of performing automatic exposure and gain control, white balance, color matrixing, gamma correction, and aperture control. The CMOS sensor and co-processor perform the key functions of image capture, digital video image processing, video compression, and interfacing to the main computer microprocessor.

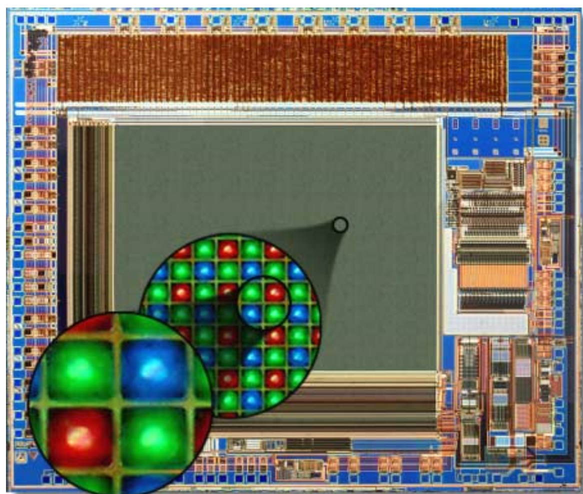


Figure 28. CMOS active pixel sensor used in new camera on a chip technology that is rapidly emerging. The photomicrograph captures the entire integrated circuit surface showing the photodiode array and support circuitry. The insets illustrate progressively higher magnification of a set of pixel elements and a single pixel element composed of four dyed photodiodes.

The primary concerns with CMOS technology are the rather low quality, noisy images that are obtained with respect to similar CCD devices. These are due primarily to process variations that produce a slightly different response in each pixel, which appears in the image as ***snow***. Another problem is that the total amount of chip area that is sensitive to light is less in CMOS devices, thus making them less sensitive to light. These problems will be overcome as process technology advances and it is very possible that CMOS devices will eclipse the CCD as the technology of choice in the very near future.

If you have prized transparencies and negatives collected over a long period of time, many of the better camera stores can take these and put them onto a Kodak Photo CD, which is the same size as an audio CD. The Photo CD can hold up to 100 images that are stored at several levels of resolution. Digital images recorded onto the Photo CD can be displayed on a good monitor by means of a Photo CD player. If you have a computer and a program such as Adobe Photoshop, Corel Photo Paint, or Picture Publisher and a CD drive, you can open the images on your computer screen, manipulate and/or enhance the images and then print the images using a digital printer—all without a darkroom! Kodak, Fuji, Olympus, Tektronix, and Sony market dye sublimation printers that can produce prints virtually indistinguishable from those printed with the usual color enlarger in a darkroom.

35mm negative and positive transparency scanners and flatbed scanners, available from such manufacturers as Nikon, Olympus, Polaroid, Kodak, Agfa, Microtek, Hewlett-Packard, etc., can directly scan transparencies or negatives or prints into your computer for storage or manipulation. Images can be stored on the hard drive of the computer or stored on floppy disks in JPEG or TIFF files. Because floppy disks have storage limited to 1.44 megabytes, many micrographers are now storing images on Zip disks or magneto-optical drive disks; these can hold many images to sizes of 10 megabytes or more. Another popular storage medium, quickly gaining widespread popularity, is the recordable CD-ROM. Magneto-optical disks or CD-ROMs can be given to commercial printers and then printed with stunning color accuracy and resolution.

A photomicrograph is also a photograph. As such, it should not only reveal information about the specimen, it should also do so with attention to the aesthetics of the overall image. Always try to compose photomicrographs with a sense of the balance of the color elements across the image frame. Use diagonals for greater visual impact, and scan the frame for unwanted debris or other artifacts. Select a magnification that will readily reveal the details sought. Remember to keep detailed records to avoid repeating mistakes and to help with review of images that are several years old. Excellent photomicrographs are within the capability of most microscopists, so pay attention to the details and the overall picture will assemble itself.

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