Fundamentals of Light Microscopy and Electronic Imaging

Douglas B. Murphy

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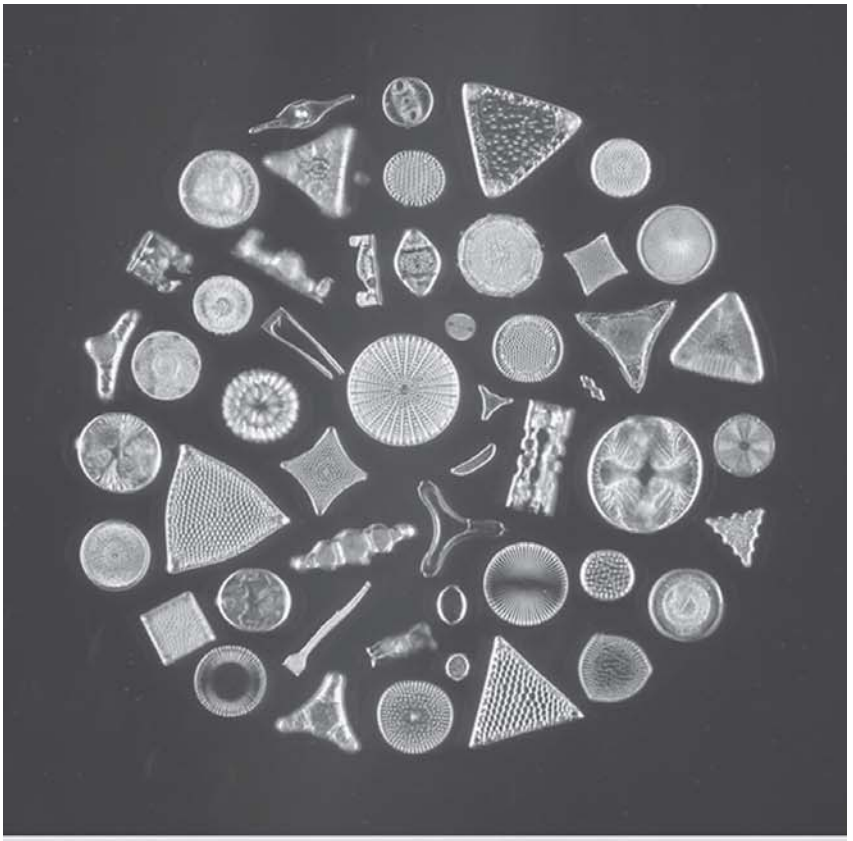
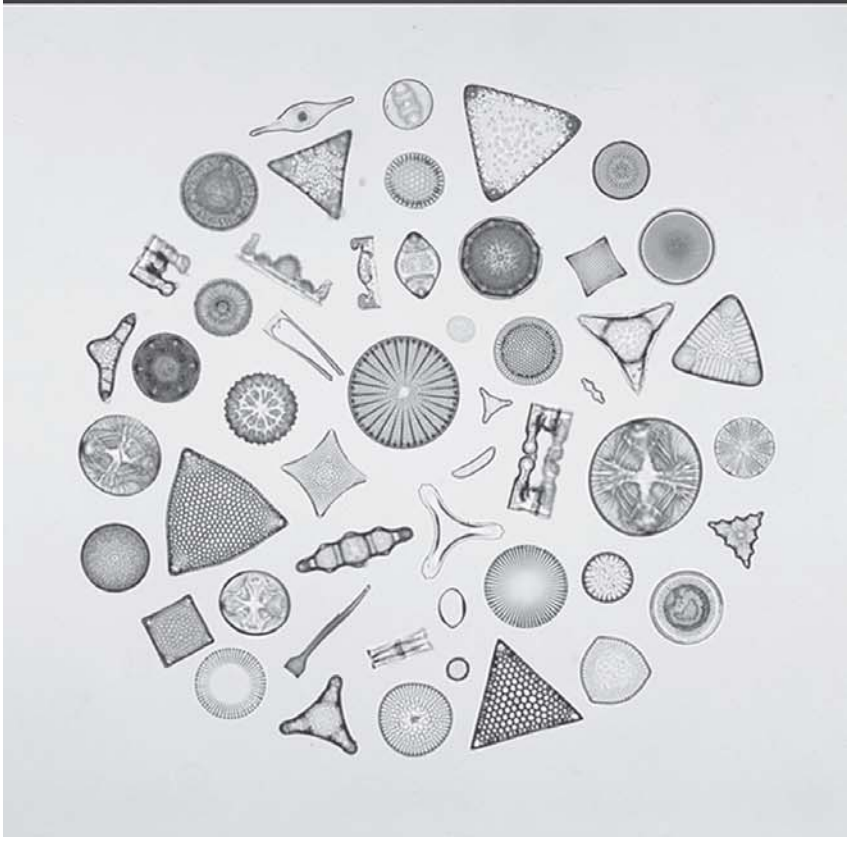
FUNDAMENTALS OF LIGHT MICROSCOPY AND ELECTRONIC IMAGING





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The cover image is an optical path in the Zeiss Axiophot upright microscope. For details, see the legend to the related Color Plate 1-2. (Courtesy Carl Zeiss, Inc.)

Frontispiece. Diatom exhibition mount, bright-field and dark-field microscopy. (This striking exhibition slide for the light microscope was prepared by Klaus Kemp, Somerset, England.)

This book is printed on acid-free paper. ♾

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PREFACE

Throughout the writing of this book my goal has been how to teach the beginner how to use microscopes. In thinking about a cover, my initial plan was to suggest a silhouette of a microscope under the title Practical Light Microscopy. However, the needs of the scientific community for a more comprehensive reference and the furious pace of electronic imaging technologies demanded something more. Practitioners of microscopy have long required an instructional text to help align and use a microscope one that also reviews basic principles of the different optical modes and gives instructions on how to match filters and fluorescent dyes, choose a camera, and acquire and print a microscope image. Advances in science and technology have also profoundly changed the face of light microscopy over the past ten years. Instead of microscope and film camera, the light microscope is now commonly integrated with a CCD camera, computer, software, and printer into electronic imaging systems. Therefore, to use a modern research microscope, it is clear that research scientists need to know not only how to align the microscope optics, but also how to acquire electronic images and perform image processing. Thus, the focus of the book is on the integrated microscope system, with foundations in optical theory but extensions into electronic imaging. Accordingly, the cover shows the conjugate field and aperture planes of the light microscope under the title Fundamentals of Light Microscopy and Electronic Imaging.

The book covers three areas: optical principles involved in diffraction and image formation in the light microscope; the basic modes of light microscopy; and the components of modern electronic imaging systems and the basic image-processing operations that are required to prepare an image. Each chapter is introduced with theory regarding the topic at hand, followed by descriptions of instrument alignment and image interpretation. As a cell biologist and practitioner of microscopy rather than a physicist or developer of new microscope equipment and methods, the reader will notice that I have focused on how to align and operate microscopes and cameras and have given somewhat abbreviated treatment to the physical theory and principles involved. Nevertheless, the theory is complete enough in its essentials that I hope even experienced microscopists will benefit from many of the descriptions. With the beginner microscopist in mind, each chapter includes practical demonstrations and exercises. The content, though not difficult, is inherently intricate by nature, so the demonstrations are valuable aids in absorbing essential optical principles. They also allow time to pause and reflect on the

economy and esthetic beauty of optical laws and principles. If carried out, the demonstrations and exercises also offer opportunities to become acquainted with new biological specimens that the reader may not have confronted or seen before by a new mode of light microscopy. Lists of materials, procedures for specimen preparation, and answers to questions in the problem sets are given in an Appendix. A basic glossary has also been included to aid readers not already familiar with complex terminology. Finally, because the text contains several detailed descriptions of theory and equipment that could be considered ancillary, an effort has been made to subordinate these sections so as to not obscure the major message.

Special thanks are due to many individuals who made this work possible. Foremost I thank profoundly my wife, Christine Murphy, who encouraged me in this work and devoted much time to reading the text and providing much assistance in organizing content, selecting figures, and editing text. I also thank the many students who have taken my microscope courses over the years, who inspired me to write the book and gave valuable advice. In particular, I would like to thank Darren Gray of the Biomedical Engineering Department at Johns Hopkins, who worked with me through every phrase and equation to get the facts straight and to clarify the order of presentation. I would also like to thank and acknowledge the help of many colleagues who provided helpful criticisms and corrections to drafts of the text, including Drs. Bill Earnshaw (University of Edinburgh), Gordon Ellis (University of Pennsylvania), Joe Gall (Carnegie Institution, Department of Embryology), Shinya Inoué (Marine Biological Laboratory), Ernst Keller (Carl Zeiss, Inc.), John Russ (North Carolina State University), Kip Sluder (University of Massachusetts Medical School), and Ken Spring (National Institutes of Health). Finally, I wish to thank many friends and colleagues who provided facts, advice, and much encouragement, including Ken Anderson, Richard Baucom, Andrew Beauto, Marc Benvenuto, Mike Delannoy, Fernando Delaville, Mark Drew, David Elliott, Vickie Frohlich, Juan Garcia, John Heuser, Jan Hinsch, Becky Hohman, Scot Kuo, Tom Lynch, Steven Mattessich, Al McGrath, Michael Mort, Mike Newberry, Mickey Nymick, Chris Palmer, Larry Philips, Clark Riley, Ted Salmon, Dale Schumaker, and Michael Stanley.

I also give special acknowledgment and thanks to Carl Zeiss, Leica Microsystems Nikon Corporation, and Olympus America for providing the color plates that accompany the book.

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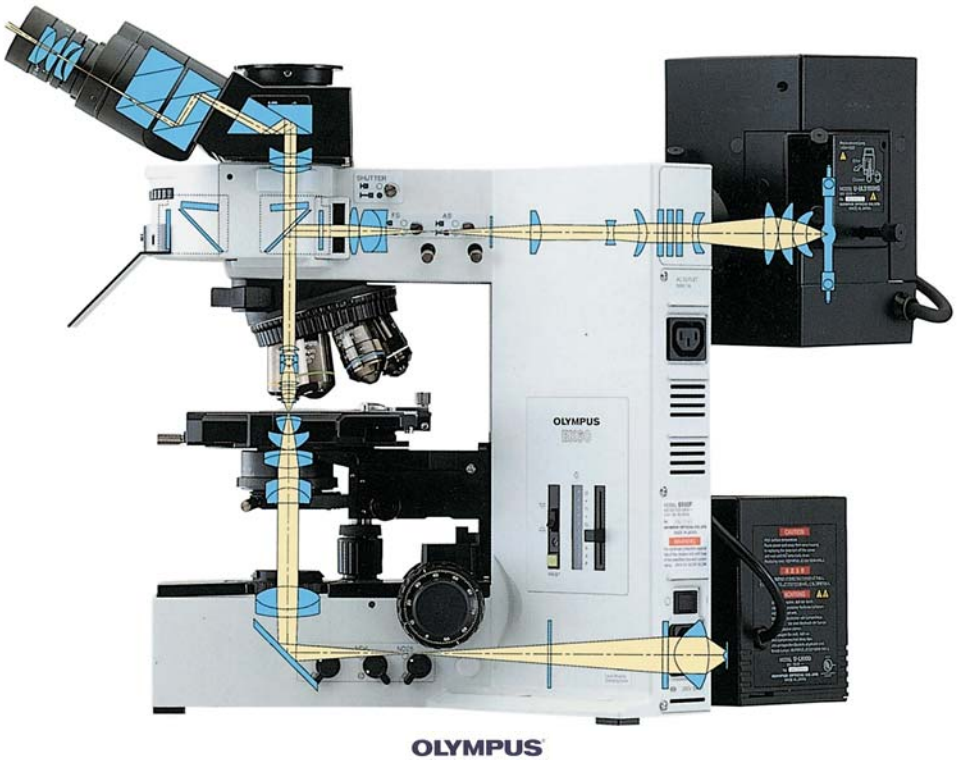
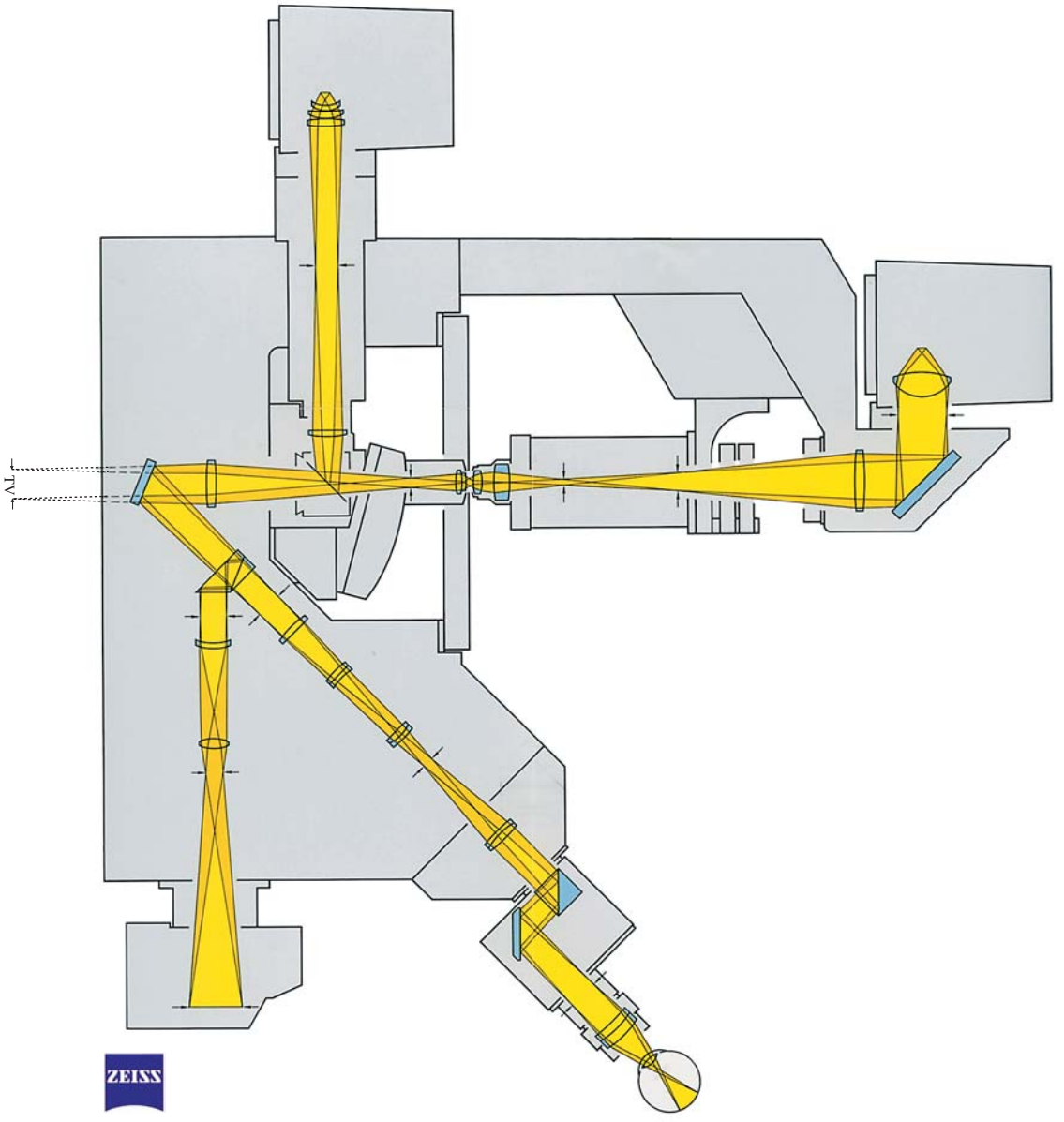
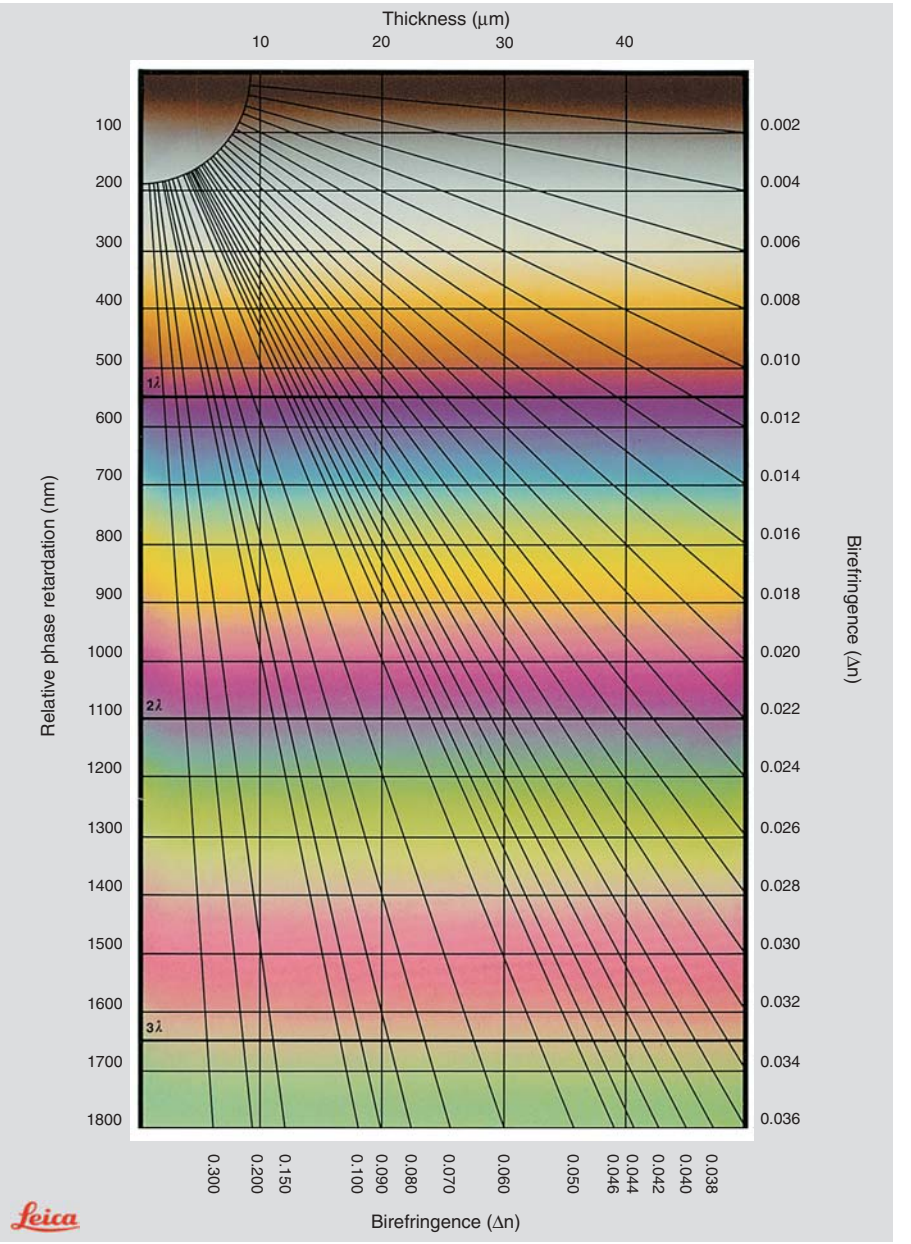
Baltimore, Maryland



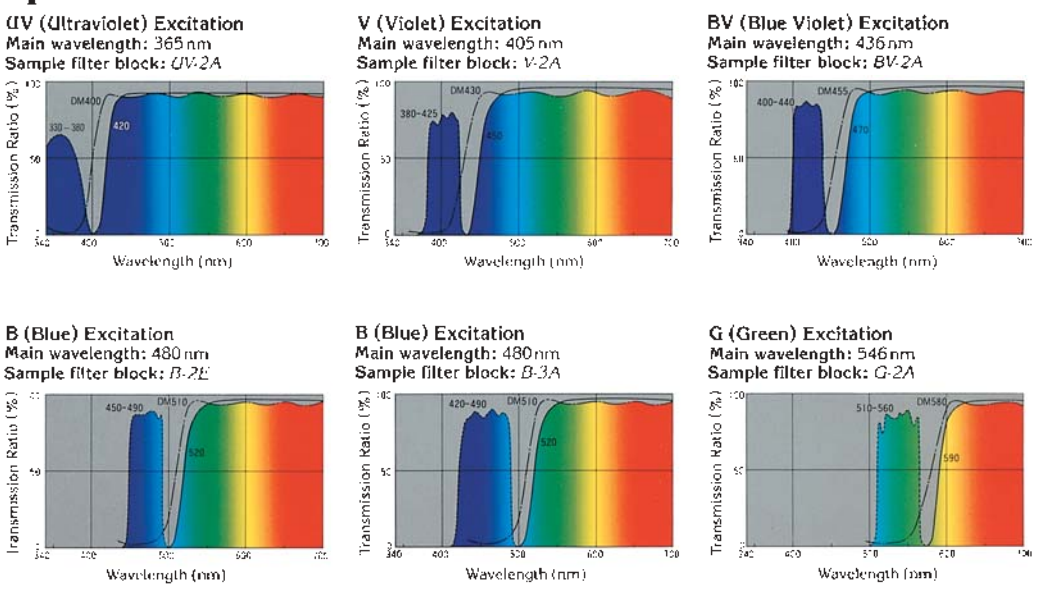
Color Plate 4-1. Optical path in the Olympus BX60 upright microscope. The microscope is fitted with a transilluminator (bottom) and epi-illuminator (top) and has infinity-corrected optics. Lenses, filters, and prisms are light blue. Light passing through the objective lens emerges and propagates as a parallel beam of infinite focus, which is collected by an internal tube lens (Telan lens) as an aberration-free image in the real intermediate image plane. The Telan lens is located where the black trinocular headpiece joins the white microscope body. The infinity space between objective and Telan lens allows insertion of multiple optical devices (fluorescence filter sets, waveplate retarders, DIC prisms, analyzer, and others) without altering the magnification of the image. This color plate was provided by Olympus America, Inc.

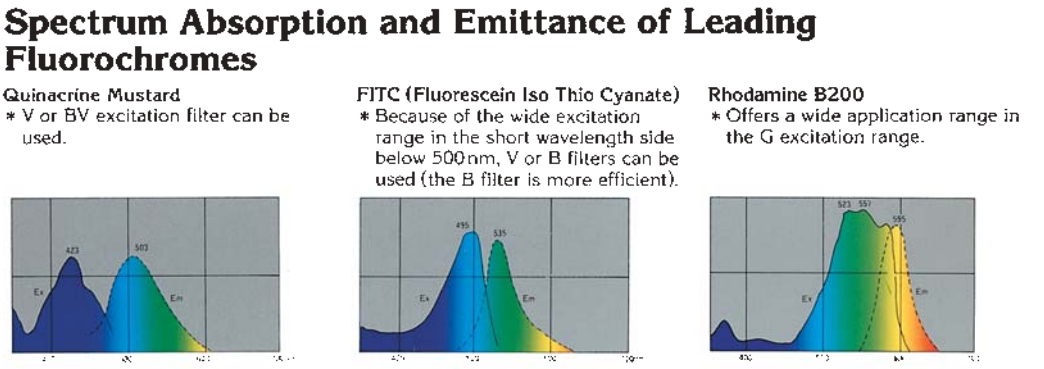


Color Plate 4-2. Optical path in the Zeiss Axiovert-135 inverted microscope. The microscope is fitted with a transilluminator (top) and epi-illuminator (bottom) and uses infinity-corrected optics. This plate shows the locations, marked by pairs of arrows, of multiple field planes (full beam diameter, bright yellow) and aperture planes (full beam diameter, dull gold.) Lens, mirror, and prism locations are shown in light blue. In this design, the stage is fixed to the microscope body and the specimen focus dial raises and lowers the objective lens. The black square outline at the site of intersection of the epi-illuminator beam with the microscope axis marks the position where filter sets are inserted for fluorescence microscopy. The identifications of conjugate sets of focal planes are described in Chapter 1. This color plate was provided by Carl Zeiss, Inc.



Color Plate 9-1. Michel Lèvy chart showing four orders of the interference color spectrum. Removal of the wavelengths shown on the left edge of the chart through destructive interference yields the indicated interference colors. The chart is used to determine the phase difference between O and E rays for birefringent specimens examined in a polarizing microscope equipped with a 1-plate compensator. The procedure for adjusting the compensator with white light illumination is described in Chapter 9. The Michel Lèvy chart also indicates the refractive index or thickness of a birefringent specimen if one of the two parameters is independently known. In geology, the chart is used to determine the identity, refractive index, or section thickness of birefringent crystals (indicated by the diagonal lines on the chart). Color plate courtesy Leica Microsystems Wetzlar GmbH.





Color Plate 11-1. Transmission curves of common fluorescence filter sets. TOP: Filter sets for excitation at UV, violet, blue violet, blue, and green excitation wavelengths are shown. Each set shows the transmission profiles of an excitation bandpass filter (left), a dichroic mirror (labeled DM) and an emission filter (right). BOTTOM: Absorption and emission spectra of some common fluorochromes; the wavelengths corresponding to spectral maxima are indicated. In selecting a filter set to excite fluorescence of a given dye, the excitation bandpass filter must cover the excitation peak of the dye. Likewise, dichroic mirror and emission filter profiles must cover the principal emission peak of the dye. Thus, filter blocks B-2E and B-3A are suitable for examining FITC fluorescence, and block G-2A is suitable for examining the fluorescence of Rhodamine B200 and TRITC. This color plate was provided by The Nikon Corporation, Inc.

CHAPTER

1

FUNDAMENTALS OF LIGHT MICROSCOPY

OVERVIEW

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

OPTICAL COMPONENTS OF THE LIGHT MICROSCOPE

A *compound light microscope* is an optical instrument that uses visible light to produce a magnified image of an object (or specimen) that is projected onto the retina of the eye or onto an imaging device. The word *compound* refers to the fact that two lenses, the objective lens and the eyepiece (or ocular), work together to produce the final magnification M of the image such that

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

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

At this point take time to examine Figure 1-2, which shows how an image becomes magnified and is perceived by the eye. The figure also points out the locations of important focal planes in relation to the objective lens, the ocular, and the eye. The specimen on the microscope stage is examined by the objective lens, which produces a magnified real image of the object in the image plane of the ocular. When looking in the microscope, the ocular acting together with the eye s cornea and lens projects a second real image onto the retina, where it is perceived and interpreted by the brain as a magnified virtual image about 25 cm in front of the eye. For photography, the intermediate image is recorded directly or projected as a real image onto a camera.

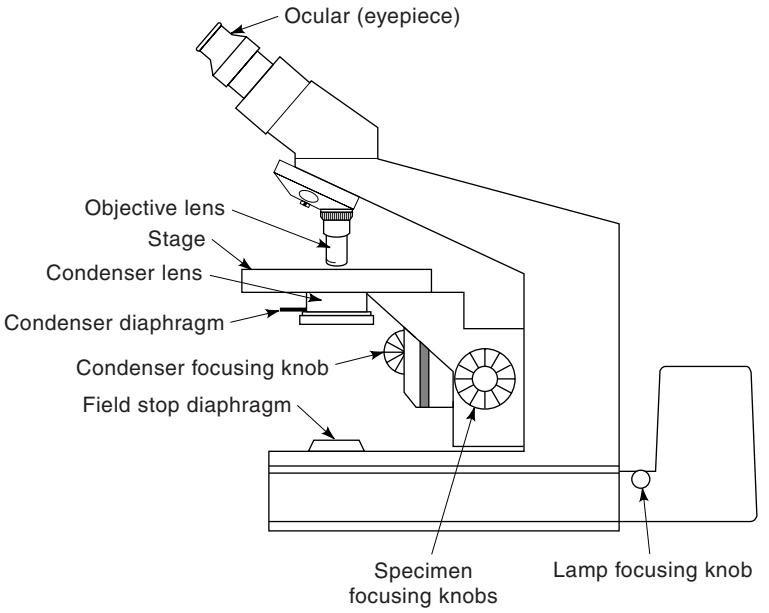


Figure 1-1

The compound light microscope. Note the locations of the specimen focus dials, the condenser focus dial, and the focus dial of the collector lens on the lamp housing. Also note the positions of two variable iris diaphragms: the field stop diaphragm near the illuminator, and the condenser diaphragm at the front aperture of the condenser. Each has an optimum setting in the properly adjusted microscope.

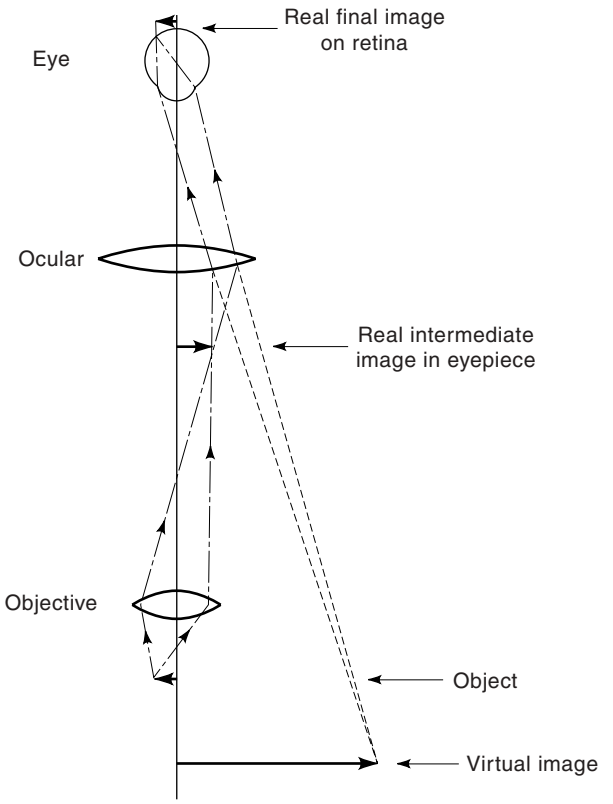


Figure 1-2

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

Microscopes come in both inverted and upright designs. In both designs the location of the real intermediate image plane at the eyepiece is fixed and the focus dial of the microscope is used to position the image at precisely this location. In most conventional upright microscopes, the objectives are attached to a nosepiece turret on the microscope body, and the focus control moves the specimen stage up and down to bring the image to its proper location in the eyepiece. In inverted designs, the stage itself is fixed to the microscope body, and the focus dials move the objective turret up and down to position the image in the eyepieces.

Note: Inverted Microscope Designs

Inverted microscopes are rapidly gaining in popularity because it is possible to examine living cells in culture dishes filled with medium using standard objectives and avoid the use of sealed flow chambers, which can be awkward. There is also better access to the stage, which can serve as a rigid working platform for microinjection and physiological recording equipment. Inverted designs have their center of

mass closer to the lab bench and are therefore less sensitive to vibration. However, there is some risk of physical damage, as objectives may rub against the bottom surface of the stage during rotation of the objective lens turret. Oil immersion objectives are also at risk, because gravity can cause oil to drain down and enter a lens, ruining its optical performance and resulting in costly lens repair. This can be prevented by wrapping a pipe cleaner (the type without the jagged spikes found in a craft store) or by placing a custom fabricated felt washer around the upper part of the lens to catch excess drips of oil. Therefore, despite many advantages, inverted research microscopes require more attention than do standard upright designs.

APERTURE AND IMAGE PLANES IN A FOCUSED, ADJUSTED MICROSCOPE

Principles of geometrical optics show that a microscope has two sets of conjugate focal planes a set of four *object or field planes* and a set of four *aperture or diffraction planes* that have fixed, defined locations with respect to the object, optical elements, light source, and the eye or camera. The planes are called *conjugate,* because all of the planes of a given set are seen simultaneously when looking in the microscope. The field planes are observed in normal viewing mode using the eyepieces. This mode is also called the orthosocopic mode, and the object image is called the orthoscopic image. Viewing the aperture or diffraction planes requires using an eyepiece telescope or Bertrand lens, which is focused on the back aperture of the objective lens (see Note and Fig. 1-3). This mode of viewing is called the aperture, diffraction, or conoscopic mode, and the image of the dif-

Figure 1-3
The back aperture of an objective lens and a focusable eyepiece telescope.

fraction plane viewed at this location is called the conoscopic image. In this text we refer to the two viewing modes as the *normal* and *aperture* viewing modes and do not use the terms *orthoscopic* and *conoscopic,* although they are common in other texts.

Note: Using an Eyepiece Telescope to View the Objective Back Aperture

An *aperture* is a hole or opening in an opaque mask designed to eliminate stray light from entering the light path, and most field and aperture planes of a microscope contain apertures. A fixed circular aperture is found at or near the rear focal plane of the objective lens. (The precise location of the back focal plane is a function of the focal length of the lens; for objectives with short focal lengths, the focal plane is located inside the lens barrel.) The aperture mask is plainly visible at the back surface of the objective lens (Fig. 1-3). We refer to this site frequently in the text.

The *eyepiece telescope* (sometimes called a phase or centering telescope) is a special focusable eyepiece that is used in place of an ocular to view the back aperture of the objective lens and other aperture planes that are conjugate to it. To use the telescope, remove the eyepiece, insert the eyepiece telescope, and focus it on the circular edge of the objective back aperture. Some microscopes contain a built-in focusable telescope lens called a *Bertrand lens* that can be conveniently rotated into and out of the light path as required.

The identities of the sets of conjugate focal planes are listed here, and their locations in the microscope under conditions of Koehler illumination are shown in Figure 1-4. The terms *front aperture* and *back aperture* refer to the openings at the front and back focal planes of a lens from the perspective of a light ray traveling from the lamp to the retina. Knowledge of the location of these planes is essential for adjusting the microscope and for understanding the principles involved in image formation. Indeed, the entire design of a microscope is based on these planes and the user s need to have access to them. Taken in order of sequence beginning with the light source, they are as follows:

Field Planes (normal view through the eyepieces)	**Aperture Planes (aperture view through the eyepiece telescope)**
lamp (field) diaphragm	lamp filament
object or field plane	front aperture of condenser (condenser diaphragm)
real intermediate image plane (eyepiece field stop)	back aperture of objective lens
retina or camera face plate	exit pupil of eyepiece (coincident with the pupil of the eye)

The *exit pupil* of the eyepiece, which occupies the location of one of the aperture planes, is the disk of light that appears to hang in space a few millimeters above the back lens of the eyepiece; it is simply the image of the back aperture of the objective lens. Normally we are unaware that we are viewing four conjugate field planes when looking through the eyepieces of a microscope. As an example of the simultaneous visibility of conjugate focal planes, consider that the image of a piece of dirt on a focused specimen could lie in any one of the four field planes of the microscope: floaters near the retina,

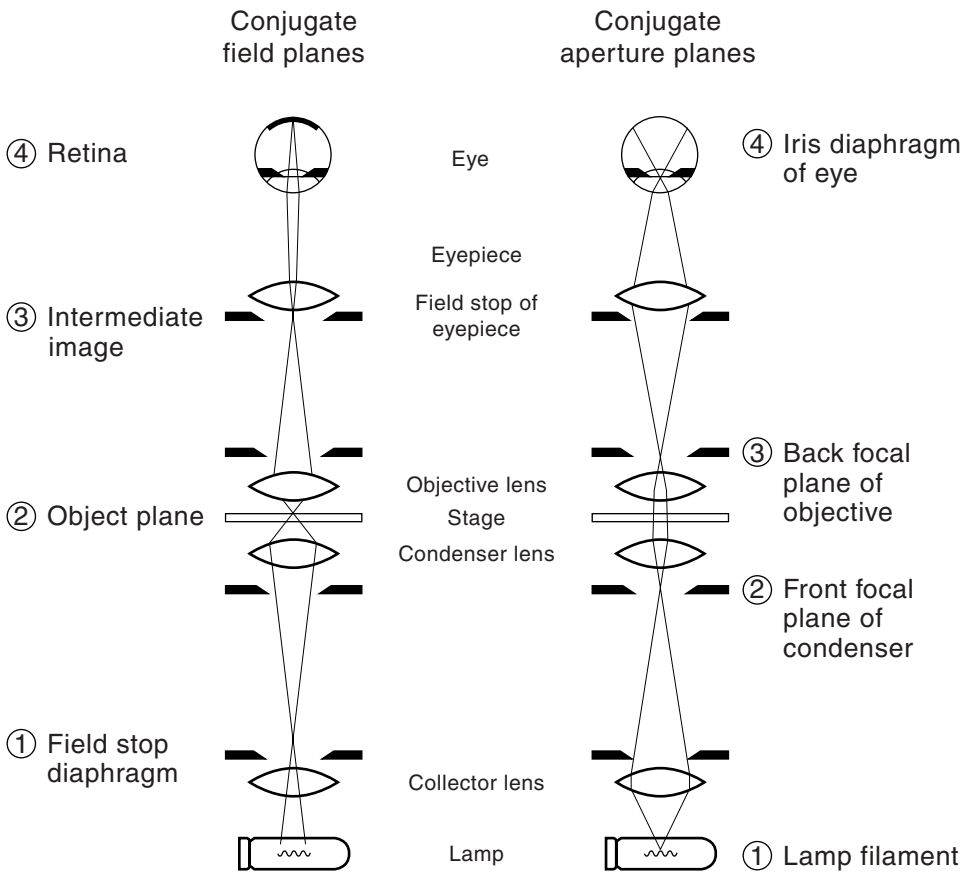


Figure 1-4

The locations of conjugate focal planes in a light microscope adjusted for Koehler illumination. Note the locations of four conjugate field planes (left) and four conjugate aperture planes (right) indicated by the crossover points of rays in the diagrams. The left-hand diagram shows that the specimen or object plane is conjugate with the real intermediate image plane in the eyepiece, the retina of the eye, and the field stop diaphragm between the lamp and the condenser. The right-hand drawing shows that the lamp filament is conjugate with aperture planes at the front focal plane of the condenser, the back focal plane of the objective, and the pupil of the eye.

dirt on an eyepiece reticule, dirt on the specimen itself, or dirt on the glass plate covering the field diaphragm. With knowledge of the locations of the conjugate field planes, the location of the dirt can be determined quickly by rotating the eyepiece, moving the microscope slide, or wiping the cover plate of the field diaphragm.

Before proceeding, take the time to identify the locations of the field and aperture planes on your microscope in the laboratory.

KOEHLER ILLUMINATION

Illumination is a critical determinant of optical performance in light microscopy. Apart from the intensity and wavelength range of the light source, it is important that the light emitted from different locations on the filament be focused at the front aperture of the

condenser. The size of the illuminated field at the specimen is adjusted so that it matches the specimen field diameter of the objective lens being employed. Because each source point contributes equally to illumination in the specimen plane, variations in intensity in the image are attributed to the object and not to irregular illumination from the light source. The method of illumination introduced by August Koehler fulfills these requirements and is the standard method used in light microscopy (Fig. 1-5). Under the conditions set forth by Koehler, a *collector lens* on the lamp housing is adjusted so that it focuses an image of the lamp filament at the front focal plane of the condenser while completely filling the aperture; illumination of the specimen plane is bright and even. Achieving this condition also requires focusing the condenser using the condenser focus dial, an adjustment that brings two sets of conjugate focal planes into precise physical locations in the microscope, which is a requirement for a wide range of image contrasting techniques that are discussed in Chapters 7 through 12. The main advantages of Koehler illumination in image formation are:

Bright and even illumination in the specimen plane and in the conjugate image plane. Even when illumination is provided by an irregular light source such as a lamp filament, illumination of the object is remarkably uniform across an extended area. Under these conditions of illumination, a given point in the specimen is illuminated by every point in the light source, and, conversely, a given point in the light source illuminates every point in the specimen.

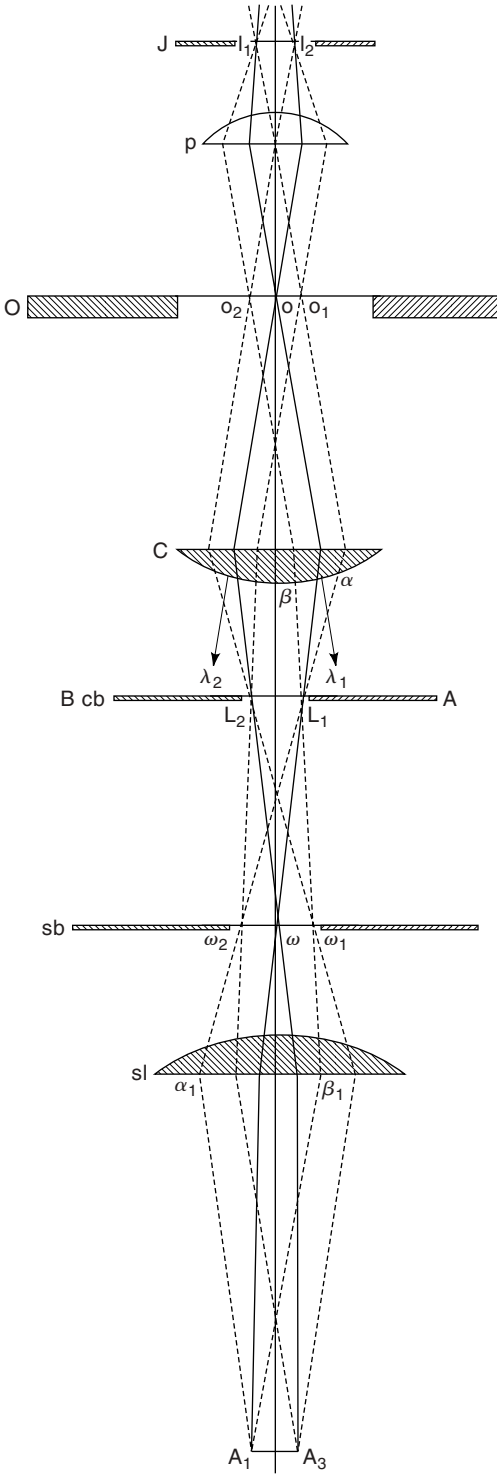
Positioning of two different sets of conjugate focal planes at specific locations along the optic axis of the microscope. This is a strict requirement for maximal spatial resolution and optimal image formation for a variety of optical modes. As we will see, focusing the stage and condenser positions the focal planes correctly, while adjusting the field and condenser diaphragms controls resolution and contrast. Once properly adjusted, it is easier to locate and correct faults such as dirt and bubbles that can degrade optical performance.

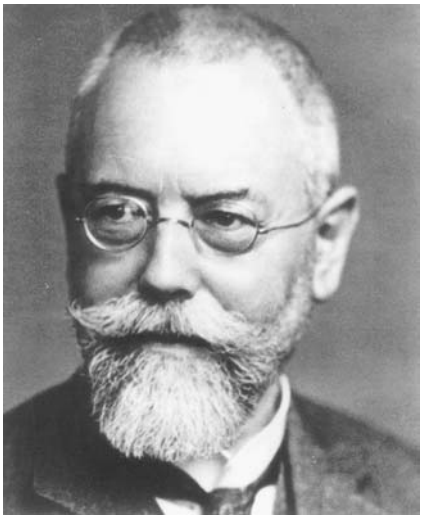
ADJUSTING THE MICROSCOPE FOR KOEHLER ILLUMINATION

Take a minute to review Figure 1-4 to familiarize yourself with the locations of the two sets of focal planes: one set of four field planes and one set of four aperture planes. You will need an eyepiece telescope or Bertrand lens to examine the aperture planes and to make certain adjustments. In the absence of a telescope lens, you may simply remove an eyepiece and look straight down the optic axis at the objective aperture; however, without a telescope the aperture looks small and is difficult to see. The adjustment procedure is given in detail as follows. You will need to check your alignment every time you change a lens to examine a specimen at a different magnification.

Note: Summary of Steps for Koehler Illumination

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





August Kohler
1866-1948

Preliminaries. Place a specimen slide, such as a stained histological specimen, on the stage of the microscope. Adjust the condenser height with the condenser focusing knob so that the front lens element of the condenser comes within ~1 2 mm of the specimen slide. Do the same for the objective lens. Be sure all diaphragms are open so that there is enough light (including the illuminator s field diaphragm, the condenser s front aperture diaphragm, and in some cases a diaphragm in the objective itself). Adjust the lamp power supply so that the illumination is bright but comfortable when viewing the specimen through the eyepieces.

Check that the lamp fills the front aperture of the condenser. Inspect the front aperture of the condenser by eye and ascertain that the illumination fills most of the aperture. It helps to hold a lens tissue against the aperture to check the area of illumination. Using an eyepiece telescope or Bertrand lens, examine the back aperture of the objective and its conjugate planes, the front aperture of the condenser, and the lamp filament. Be sure the lamp filament is centered, using the adjustment screws on the lamp housing if necessary, and confirm that the lamp filament is focused in the plane of the condenser diaphragm. This correction is made by adjusting the focus dial of the collector lens on the lamp housing. Once these adjustments are made, it is usually not necessary to repeat the inspection every time the microscope is used. Instructions for centering the lamp filament or arc are given in Chapter 3. Lamp alignment should be rechecked after the other steps have been completed.

Focus the specimen. Bring a low-power objective to within 1 mm of the specimen, and looking in the microscope, carefully focus the specimen using the microscope s coarse and fine focus dials. It is helpful to position the specimen with the stage controls so that a region of high contrast is centered on the optic axis before attempting to focus. It is also useful to use a low magnification dry objective (10 25×, used without immersion oil) first, since the *working distance* that is, the distance between the coverslip and the objective is 2 5 mm for a low-power lens. This reduces the risk of plunging the objective into the specimen slide and causing damage. Since the lenses on most microscopes are *parfocal*, higher magnification objectives will already be in focus or close to focus when rotated into position.

Figure 1-5

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

Focus and center the condenser. With the specimen in focus, close down (stop down) the *field diaphragm*, and then, while examining the specimen through the eyepieces, focus the angular outline of the diaphragm using the condenser s focusing knob. If there is no light, turn up the power supply and bring the condenser closer to the microscope slide. If light is seen but seems to be far off axis, switch to a low-power lens and move the condenser positioning knobs slowly to bring the center of the illumination into the center of the field of view. Focus the image of the field diaphragm and center it using the condenser s two centration adjustment screws. The field diaphragm is then opened just enough to accommodate the object or the field of a given detector. This helps reduce scattered or stray light and improves image contrast. The condenser is now properly adjusted. We are nearly there! The conjugate focal planes that define Koehler illumination are now at their proper locations in the microscope.

Adjust the condenser diaphragm while viewing the objective back aperture with an eyepiece telescope or Bertrand lens. Finally, the condenser diaphragm (and the built-in objective diaphragm, if the objective has one) is adjusted to obtain the best resolution and contrast, but is not closed so far as to degrade the resolution. In viewing the condenser front aperture using a telescope, the small bright disk of light seen in the telescope represents the objective s back aperture plus the superimposed image of the condenser s front aperture diaphragm. As you close down the condenser diaphragm, you will see its edges enter the aperture opening and limit the objective aperture s diameter. Focus the telescope so that the edges of the diaphragm are seen clearly. Stop when ~3/4 of the maximum diameter of the aperture remains illuminated, and use this setting as a starting position for subsequent examination of the specimen. As pointed out in the next chapter, the setting of this aperture is crucial, because it determines the resolution of the microscope, affects the contrast of the image, and establishes the depth of field. It is usually impossible to optimize for resolution and contrast at the same time, so the 3/4 open position indicated here is a good starting position. The final setting depends on the inherent contrast of the specimen.

Adjust the lamp brightness. Image brightness is controlled by regulating the lamp voltage, or if the voltage is nonadjustable, by placing neutral density filters in the light path near the illuminator in specially designed filter holders. *The aperture diaphragm should never be closed down as a way to reduce light intensity,* because this action reduces the resolving power and may blur fine details in the image. We will return to this point in Chapter 6.

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

Note: Focusing Oil Immersion Objectives

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

Begin focusing by bringing the lens in contact with the drop of oil on the coverslip. The drop of oil expands as the lens is brought toward focus, and at contact (essentially the desired focus position) the oil drop stops expanding. If overfocused, the microscope slide is pushed up off the stage by a small amount on an inverted microscope; on an upright microscope the spring-loaded element of the objective compresses a bit. Retract the lens to the true focal position and then examine the specimen. In normal viewing mode it should only be necessary to change the focus by a very small amount to find the specimen. It can help to move the specimen stage controls with the other hand to identify the shadows or fluorescence of a conspicuous object, which may serve as a guide for final focus adjustment. Notice that if focus movements are too extreme, there is a risk that the objective (on an upright microscope) or the condenser (on an inverted microscope) might break the microscope slide, or worse, induce permanent strain in the optics. Focusing with oil immersion optics always requires extra care and patience.

Before observing the specimen, examine the back focal plane of the objective with an eyepiece telescope to check for lint and oil bubbles. An insufficient amount of oil between the lens and coverslip can cause the entire back aperture to be misshapen; if this is the case, focusing the telescope will bring the edge of the oil drop into sharp focus. These faults should be removed or corrected, as they will significantly degrade optical performance. Finally, when using immersion oil, never mix oils from different companies since slight differences in refractive index will cause pronounced blurring.

PRECAUTIONS FOR HANDLING OPTICAL EQUIPMENT

Never strain, twist, or drop objectives or other optical components. Optics for polarization microscopy are especially susceptible to failure due to mishandling.

Never force the focus controls of the objective or condenser, and always watch lens surfaces as they approach the specimen. This is especially important for high-power oil immersion lenses.

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

Exercise: Calibration of Magnification

Examine a histological specimen to practice proper focusing of the condenser and setting of the field stop and condenser diaphragms. A 1 μm thick section of pancreas or other tissue stained with hematoxylin and eosin is ideal. A typical histological specimen is a section of a tissue or organ that has been chemically fixed, embedded in epoxy resin or paraffin, sectioned, and stained with dyes specific for nucleic acids, proteins, carbohydrates, and so forth. In hematoxylin and eosin (H&E) staining, hematoxylin stains the nucleus and cell RNA a dark blue or purple color, while eosin stains proteins (and the pancreatic secretory granules) a bright orange-pink. When the specimen is illuminated with monochromatic light, the contrast perceived by the eye is largely due to these stains. For this reason, a stained histological specimen is called an *amplitude specimen* and is suitable for examination under the microscope using bright field optics. A suitable magnification is 10 40×.

Equipment and Procedure

Three items are required: a focusable eyepiece, an eyepiece reticule, and a stage micrometer (Fig. 1-6). The eyepiece reticule is a round glass disk usually containing a 10 mm scale divided into 0.1 mm (100 μm) units. The reticule is mounted in an eyepiece and is then calibrated using a precision stage micrometer to obtain a conversion factor (μm/reticule unit), which is used to determine the magnification obtained for each objective lens. The reason for using this calibration procedure is that the nominal magnification of an objective lens (found engraved on the lens barrel) is only correct to within ± 5%. If precision is not of great concern, an approximate magnification can be obtained using the eyepiece reticule alone. In this case, simply measure the number of micrometers from the eyepiece reticule and divide by the nominal magnification of the objective. For a specimen covering 2 reticule units (200 μm), for example: 200 μm/10× = 20 μm.

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

To mount the eyepiece reticule, unscrew the lower barrel of the focusing eyepiece and place the reticule on the stop ring with the scale facing upward. The stop ring marks the position of the real intermediate image plane. Make sure the reticule size matches the internal diameter of the eyepiece and rests on the field stop. Carefully reassemble the eyepiece and return it to the binocular head. Next focus the reticule scale using the focus dial on the eyepiece and then focus on a specimen with the microscope focus dial. The images of the specimen and reticule are conjugate and should be simultaneously in sharp focus.

Examine the stage micrometer slide, rotating the eyepiece so that the micrometer and reticule scales are lined up and partly overlapping. The stage micrometer consists of a 1 or 2 mm scale divided into 10 μm units, giving 100 units/mm. The micrometer slide is usually marked 1/100 mm. The conversion factor we need to determine is simply the number of μm/reticule unit. This conversion factor can be calculated more accurately by counting the number of micrometers contained in *several* reticule units in the eyepiece. The procedure

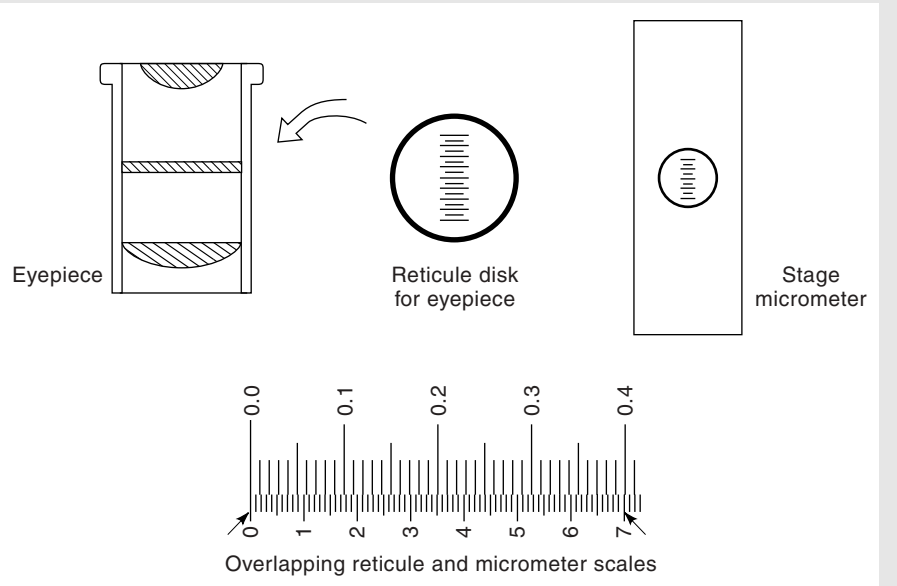


Figure 1-6

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

must be repeated for each objective lens, but only needs to be performed one time for each lens.

Returning to the specimen slide, the number of eyepiece reticule units spanning the diameter of a structure is determined and multiplied by the conversion factor to obtain the distance in micrometers.

Exercise

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

CHAPTER

2

LIGHT AND COLOR

OVERVIEW

In this chapter we review the nature and action of light as a probe to examine objects in the light microscope. Knowledge of the wave nature of light is essential for understanding the physical basis of color, polarization, diffraction, image formation, and many other topics covered in this book. The eye-brain visual system is responsible for the detection of light including the perception of color and differences in light intensity that we recognize as contrast. The eye is also a remarkably designed detector in an optical sense the spacing of photoreceptor cells in the retina perfectly matches the requirement for resolving the finest image details formed by its lens (Fig. 2-1). Knowledge of the properties of light is important in selecting filters and objectives, interpreting colors, performing low-light imaging, and many other tasks.

LIGHT AS A PROBE OF MATTER

It is useful to think of light as a probe that can be used to determine the structure of objects viewed under a microscope. Generally, probes must have size dimensions that are similar to or smaller than the structures being examined. Fingers are excellent probes for determining the size and shape of keys on a computer keyboard, but fail in resolving wiring patterns on a computer s integrated circuit chip. Similarly, waves of light are effective in resolving object details with dimensions similar to the wavelength of light, but generally do not do well in resolving molecular and atomic structures that are much smaller than the wavelength. For example, details as small as 0.2 μm can be resolved visually in a microscope with an oil immersion objective. *As an approximation, the resolution limit of the light microscope with an oil immersion objective is about one-half of the wavelength of the light employed.*

Visible light, the agent used as the analytic probe in light microscopy, is a form of energy called electromagnetic radiation. This energy is contained in discrete units or quanta called photons that have the properties of both particles and waves. Photons as electromagnetic waves exhibit oscillating electric and magnetic fields, designated E and

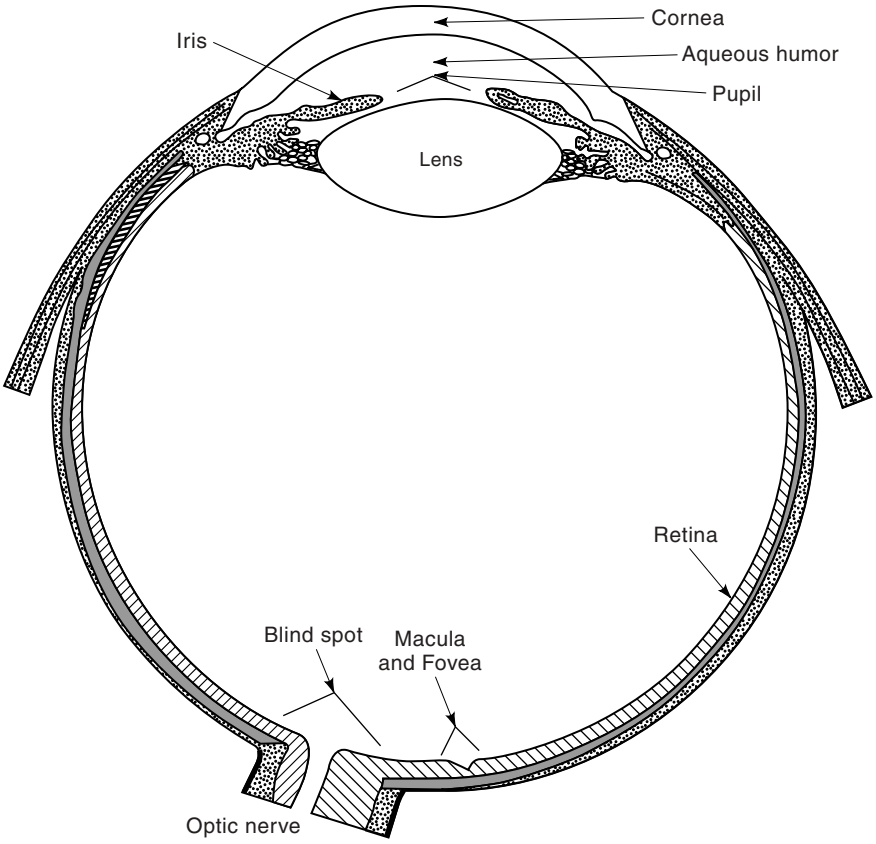


Figure 2-1

Structure of the human eye. The cornea and lens of the eye work together with the eyepiece to focus a real magnified image on the retina. The aperture plane of the eye-microscope system is located in front of the lens in the pupil of the eye, which functions as a variable diaphragm. A large number of rod cells covers the surface of the retina. The 3 mm macula, or yellow spot, contains a 0.5 mm diameter fovea, a depressed pit that contains the majority of the retina s cone cells that are responsible for color vision. The blind spot contains no photoreceptor cells and marks the site of exit of the optic nerve.

B, respectively, whose amplitudes and directions are represented by vectors that oscillate in phase as sinusoidal waves in two mutually perpendicular planes (Fig. 2-2). Photons are associated with a particular energy (ergs), which determines their wavelength (nm) and vibrational frequency (cycles/s). It is important to realize that the electromagnetic waves we perceive as light (400 750 nm, or about 10^{-7} m) comprise just a small portion of the entire electromagnetic spectrum, which ranges from 10^{4} m (radio waves) to 10^{-10} m (γ-rays) (Fig. 2-3). The figure also compares the sizes of cells, molecules, and atoms with the wavelengths of different radiations. See Hecht (1998) and Longhurst (1967) for interesting discussions on the nature of light.

Although it is frustrating that light cannot be defined in terms of a single physical entity, it can be described through mathematical relationships that depict its dual particle- and wavelike properties. The properties of energy, frequency, and wavelength are

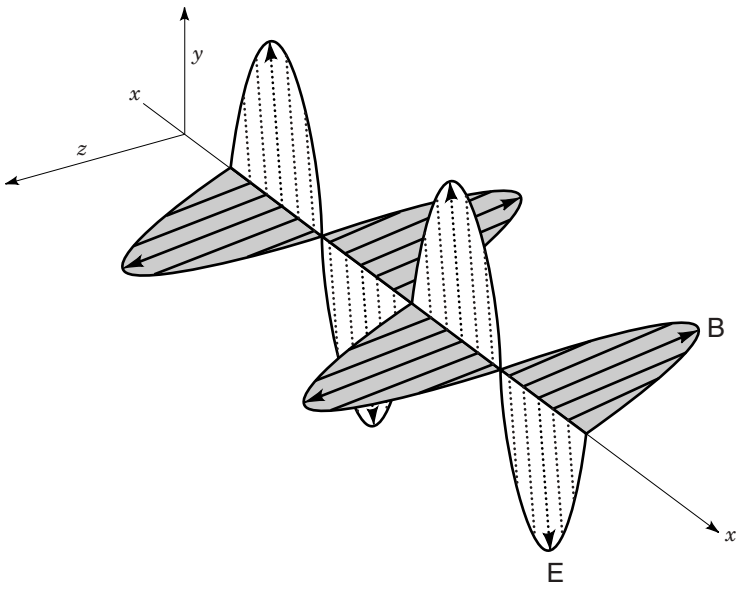


Figure 2-2

Light as an electromagnetic wave. The wave exhibits electric (E) and magnetic (B) fields whose amplitudes oscillate as a sine function over dimensions of space or time. The amplitudes of the electric and magnetic components at a particular instant or location are described as vectors that vibrate in two planes perpendicular to each other and perpendicular to the direction of propagation. However, at any given time or distance the E and B vectors are equal in amplitude and phase. For convenience it is common to show only the electric field vector (E vector) of a wave in graphs and diagrams and not specify it as such.

related through the following equations, which can be used to determine the amount of energy associated with a photon of a specific wavelength:

$$c = \nu\lambda,$$

$$E = h\nu,$$

and combining,

$$E = hc/\lambda,$$

where c is the speed of light (3×10^{10} cm/s), ν is the frequency (cycles/s), λ is the wavelength (cm), E is energy (ergs), and h is Plank s constant (6.62×10^{-27} erg-seconds). The first equation defines the velocity of light as the product of its frequency and wavelength. We will encounter conditions where velocity and wavelength vary, such as when photons enter a glass lens. The second equation relates frequency and energy, which becomes important when we must choose a wavelength for examining live cells. The third equation relates the energy of a photon to its wavelength. Since $E \sim 1/\lambda$, 400 nm blue wavelengths are twice as energetic as 800 nm infrared wavelengths.

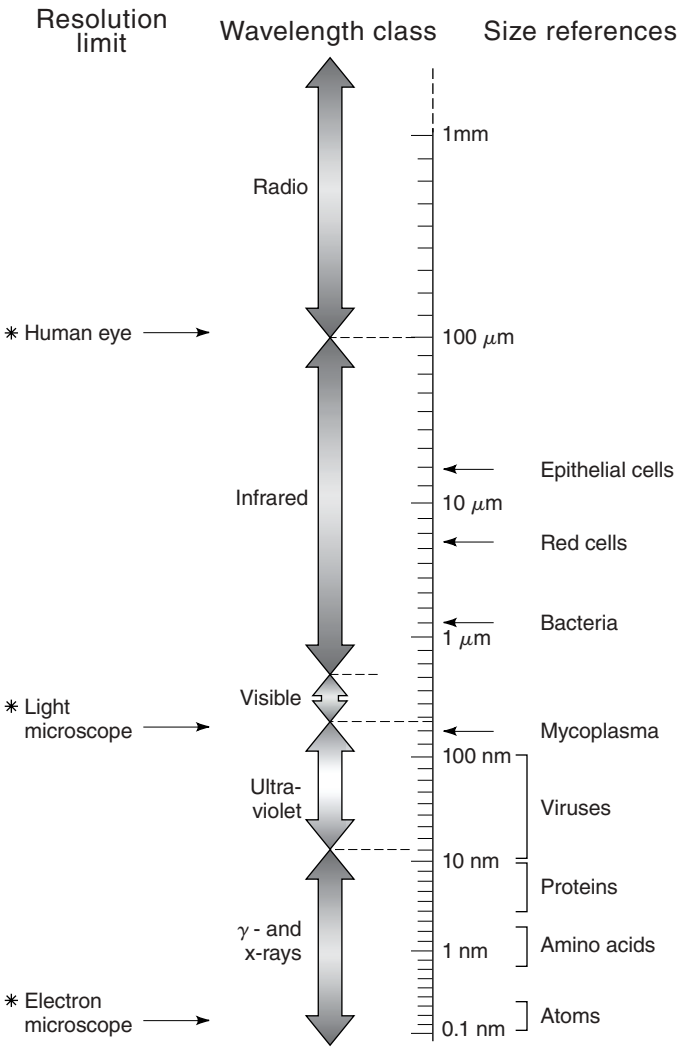


Figure 2-3

The electromagnetic spectrum. The figure shows a logarithmic distance scale (range, 1 mm to 0.1 nm). One side shows the wavelength ranges of common classes of electromagnetic radiation; for reference, the other side indicates the sizes of various cells and macromolecules. Thus, a red blood cell (7.5 μm) is 15 times larger than a wavelength of visible green light (500 nm). The resolution limits of the eye, light microscope, and electron microscope are also indicated. For the eye, the resolution limit (0.1 mm) is taken as the smallest interval in an alternating pattern of black and white bars on a sheet of paper held 25 cm in front of the eye under conditions of bright illumination. Notice that the range of visible wavelengths spans just a small portion of the spectrum.

LIGHT AS PARTICLES AND WAVES

For the most part, we will be referring to the wave nature of light and the propagation of electromagnetic radiation as the movement of planar wavefronts of a specific wavelength through space. The propagation vector is linear in a homogeneous medium such

as air or glass or in a vacuum. The relatively narrow spectrum of photon energies (and corresponding frequencies) we experience as light is capable of exciting the visual pigments in the rod and cone cells in the retina and corresponds to wavelengths ranging from 400 nm (violet) to 750 nm (red). As shown in Figure 2-4, we depict light in various ways depending on which features we wish to emphasize:

As *quanta* (photons) of electromagnetic radiation, where photons are detected as individual quanta of energy (as photoelectrons) on the surfaces of quantitative measuring devices such as charge-coupled device (CCD) cameras or photomultiplier tubes.

As *waves,* where the propagation of a photon is depicted graphically as a pair of electric (E) and magnetic (B) fields that oscillate in phase and in two mutually perpendicular planes as functions of a sine wave. The vectors representing these fields vibrate in two planes that are both mutually perpendicular to each other and perpendicular to the direction of propagation. For convenience it is common to show only the wave s electric field vector (E vector) in graphs and diagrams and not specify it as such. When shown as a sine wave on a plot with x, y coordinates, the amplitude of a wave on the y-axis represents the strength of the electric or magnetic field, whereas the x-axis depicts the time or distance of travel of the wave or its phase relative to some other reference wave. At any given time or distance, the E and B field vectors are equal in amplitude and phase. Looking down the x-axis (the propagation axis), the plane of the E vector may vibrate in any orientation through 360° of rotation about the axis. The angular tilt of the E vector along its propagation axis and

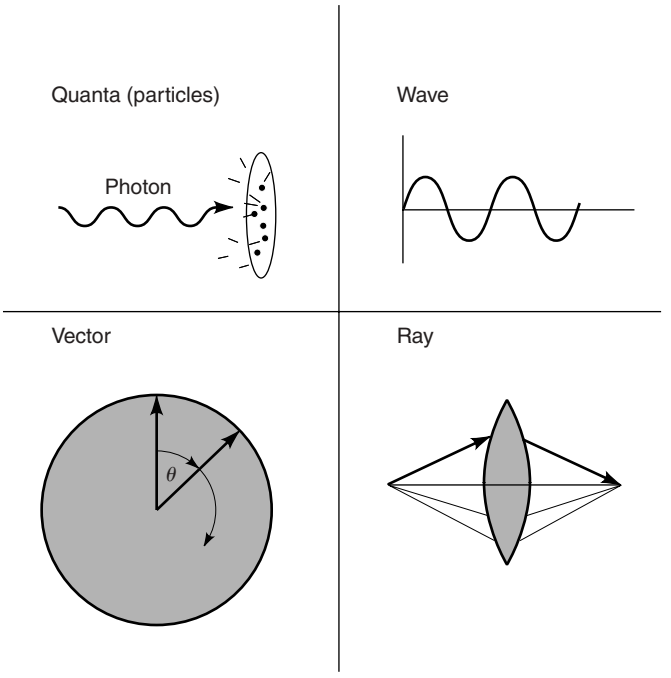


Figure 2-4
Light as quanta, waves, vectors, and rays.

relative to some fixed reference is called the *azimuthal angle* of orientation. Commonly, the sine waves seen in drawings refer to the average amplitude and phase of a beam of light (a light train consisting of a stream of photons), not to the properties of a single electromagnetic wave.

As *vectors,* where the vector length represents the amplitude, and the vector angle represents the advance or retardation of the wave relative to an imaginary reference. The vector angle is defined with respect to a perpendicular drawn through the focus of a circle, where 360° of rotation corresponds to one wavelength (2π radians).

As *rays* or *beams,* where the linear path of a ray (a light train or stream of photons) in a homogeneous medium is shown as a straight line. This representation is commonly used in geometrical optics and ray tracing to show the pathways of rays passing through lenses of an imaging system.

THE QUALITY OF LIGHT

As an analytic probe used in light microscopy, we also describe the kind or quality of light according to the degree of uniformity of rays comprising an illuminating beam (Fig. 2-5). The kinds of light most frequently referred to in this text include:

Monochromatic waves having the same wavelength or vibrational frequency (the same color).

Polarized waves whose E vectors vibrate in planes that are parallel to one another. The E vectors of rays of sunlight reflected off a sheet of glass are plane parallel and are said to be linearly polarized.

Coherent waves of a given wavelength that maintain the same phase relationship while traveling through space and time (laser light is coherent, monochromatic, and polarized).

Collimated waves having coaxial paths of propagation through space that is, without convergence or divergence, but not necessarily having the same wavelength, phase, or state of polarization. The surface wavefront at any point along a cross-section of a beam of collimated light is planar and perpendicular to the axis of propagation.

Light interacts with matter in a variety of ways. Light incident on an object might be absorbed, transmitted, reflected, or diffracted, and such objects are said to be opaque, transparent, reflective, or scattering. Light may be absorbed and then re-emitted as visible light or as heat, or it may be transformed into some other kind of energy such as chemical energy. Objects or molecules that absorb light transiently and quickly re-emit it as longer wavelength light are described as being phosphorescent or fluorescent depending on the time required for re-emission. Absorbed light energy might also be re-radiated slowly at long infrared wavelengths and may be perceived as heat. Light absorbed by cells may be damaging if the energy is sufficient to break covalent bonds within molecules or drive adverse chemical reactions including those that form cytotoxic free radicals. Finally, a beam of light may be bent or deviated while passing through a transparent object such as a glass lens having a different refractive index (*refraction*), or may be bent uniformly around the edges of large opaque objects (*dif-*

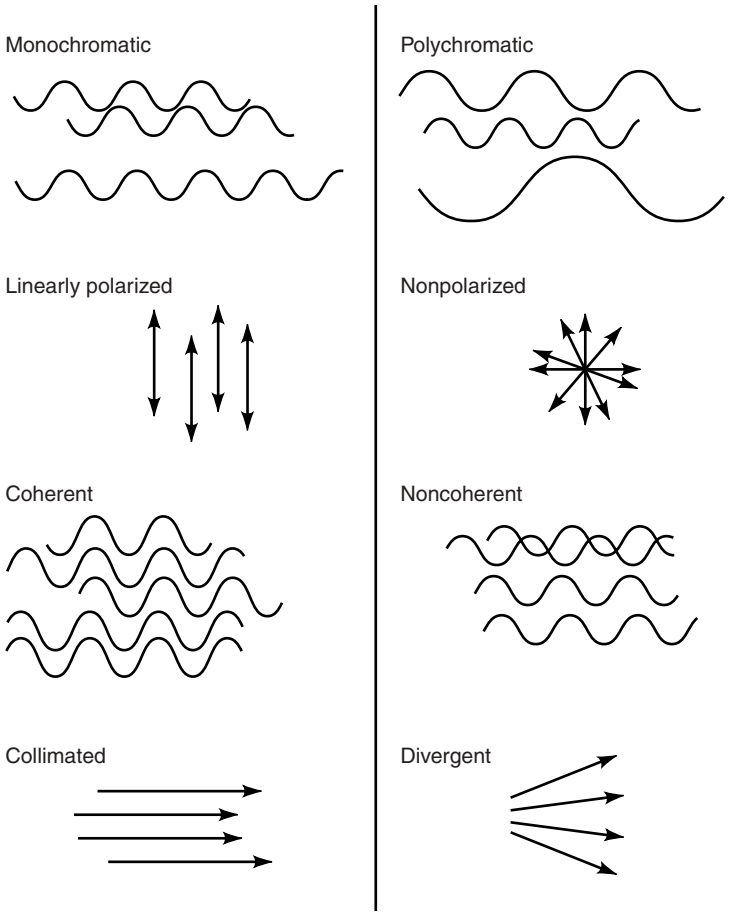


Figure 2-5
Eight waveforms depicting variations in the quality of light.

fraction), or even scattered by small particles and structures having dimensions similar to the wavelength of light itself (also known as diffraction). The diffraction of light by small structural elements in a specimen is the principal process governing image formation in the light microscope.

PROPERTIES OF LIGHT PERCEIVED BY THE EYE

The eye-brain system perceives differences in light intensity and wavelength (color), but does not see differences in the phase of light or its state of polarization. Thus, laser light, which is both coherent and polarized, cannot be distinguished from random light having the same wavelength (color). We will restrict our discussion here to the perception of light intensity, since the perception of color is treated separately in the following section.

The brightness of a light wave is described physically and optically in terms of the *amplitude* (A) of its E vector, as depicted in a graph of its sine function. Indeed, the amplitudes of sine waves are shown in many figures in the text. However, the nervous activity of photoreceptor cells in the retina is proportional to the light *intensity* (I), where intensity is defined as the rate of flow of light energy per unit area and per unit time across a detector surface. Amplitude (energy) and intensity (energy flux) are related such that the intensity of a wave is proportional to the square of its amplitude, where

$$I \propto A^2.$$

For an object to be perceived, the light intensity corresponding to the object must be different from nearby flanking intensities and thereby exhibit contrast, where *contrast (C)* is defined as the ratio of intensities,

$$C = \Delta I / I_b,$$

ΔI is the difference in intensity between an object and its background, and I_b is the intensity of the background. If $I_{obj} \sim I_b$, as it is for many transparent microscope specimens, $C = 0$, and the object is invisible. More specifically, visibility requires that the object exceed a certain *contrast threshold*. In bright light, the contrast threshold required for visual detection may be as little as 2 5%, but should be many times that value for objects to be seen clearly. In dim lighting, the contrast threshold may be 200 300%, depending on the size of the object. The term *contrast* always refers to the ratio of two intensities and is a term commonly used throughout the text.

PHYSICAL BASIS FOR VISUAL PERCEPTION AND COLOR

As we will emphasize later, the eye sees differences in light intensity (contrast) and perceives different wavelengths as colors, but cannot discern differences in phase displacements between waves or detect differences in the state of polarization. The range of wavelengths perceived as color extends from 400 nm (violet) to 750 nm (red), while peak sensitivity in bright light occurs at 555 nm (yellow-green). The curves in Figure 2-6 show the response of the eye to light of different wavelengths for both dim light (night or rod vision) and bright light (day or cone vision) conditions. The eye itself is actually a *logarithmic* detector that allows us to see both bright and dim objects simultaneously in the same visual scene. Thus, the apparent difference in intensity between two objects I_1 and I_2 is perceived as the logarithm of the ratio of the intensities, that is, as $\log_{10}(I_1/I_2)$. It is interesting that this relationship is inherent to the scale used by Hipparchus (160 127 B.C.) to describe the magnitudes of stars in 6 steps with 5 equal intervals of brightness. Still using the scale today, we say that an intensity difference of 100 is covered by 5 steps of Hipparchus stellar magnitude such that $2.512 \log_{10} 100 = 5$. Thus, each step of the scale is 2.512 times as much as the preceding step, and $2.512^5 = 100$, demonstrating that what we perceive as equal steps in intensity is really the log of the ratio of intensities. The sensitivity of the eye in bright light conditions covers about 3 orders of magnitude within a field of view; however, if we allow time for physiological adaptation and consider both dim and bright lighting conditions, the sensitivity range of the eye is found to cover an incredible 10 orders of magnitude overall.

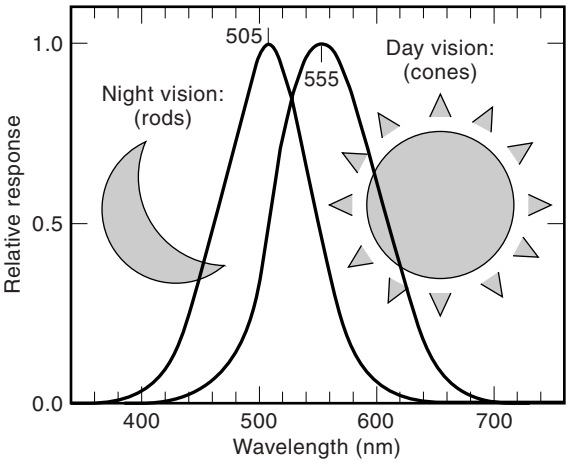


Figure 2-6

The spectral response of the eye in night and day vision. The two curves have been normalized to their peak sensitivity, which is designated 1.0; however, night (rod) vision is 40 times more sensitive than day (cone) vision. Rhodopsin contained in rod cells and color receptor pigments in cone cells have action spectra with different maxima and spectral ranges.

The shape and distribution of the light-sensitive rod and cone cells in the retina are adapted for maximum sensitivity and resolution in accordance with the physical parameters of light and the optics of the eye-lens system. Namely, the outer segments of cone cells, the cells responsible for color perception in the fovea, are packed together in the plane of the retina with an intercellular spacing of 1.0 1.5 μm, about one-half the radius of the minimum spot diameter (3 μm) of a focused point of light on the retina. The small 1.5 μm cone cell diameter allows the eye to resolve structural details down to the theoretical limit calculated for the eye-lens system. For an object held 25 cm in front of the eye, this corresponds to spacings of ~0.1 mm. It appears nature has allowed the light receptor cells to utilize the physics of light and the principles of lens optics as efficiently as possible!

Rod cell photoreceptors comprise 95% of the photoreceptors in the retina and are active in dim light, but provide no color sense. Examine Figure 2-1 showing the structure of the eye and Figure 2-7 showing the distribution of rod cells in the retina. Rods contain the light-sensitive protein, *rhodopsin,* not the photovisual pigments required for color vision, and the dim light vision they provide is called *scotopic vision.* Rhodopsin, a photosensitive protein, is conjugated to a chromophore, 11-cis-retinal, a carotenoid that photoisomerizes from a cis to trans state upon stimulation and is responsible for electrical activity of the rod cell membranes. The peak sensitivity of the rod photoreceptor cells (510 nm) is in the blue-green region of the visual spectrum. Rod cell vision is approximately 40 times more sensitive to stimulation by light than the cone cell receptors that mediate color vision. Bright light rapidly bleaches rhodopsin, causing temporary blindness in dim lighting conditions, but rhodopsin isomerizes gradually over a 20 30 min period, after which rod receptor function is largely restored. Full recovery may require several hours or even days ask any visual astronomer or microscopist! To avoid photobleaching your rhodopsin pigments and to maintain high visual sensitivity for dim specimens (common with polarized light or fluorescence optics), you

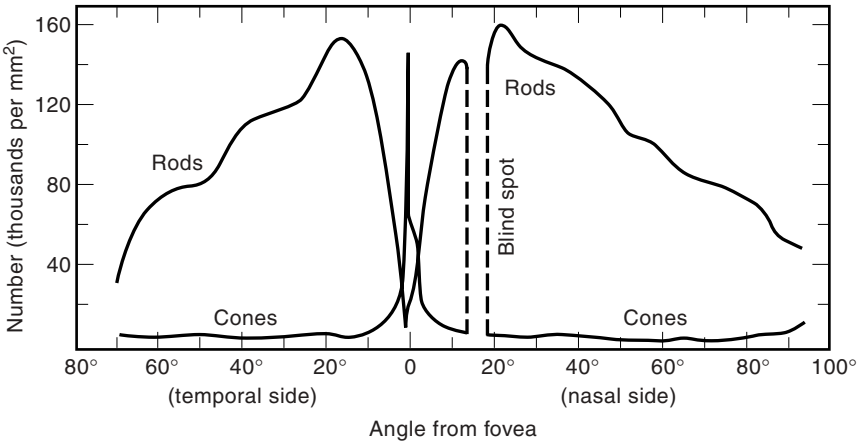


Figure 2-7

Distribution of rod and cone cells in the retina. The number of cells per mm^2 is plotted vs. the angle from the fovea as seen from the lens. The fovea is distinct in having a paucity of rods and an abundance of cones. The blind spot lacks photoreceptor cells.

should make observations in a darkened room. Red light illumination in the otherwise darkened microscope room is also commonly employed, because red wavelengths bleach the rhodopsin inefficiently (see Fig. 2-8 for differences in absorption spectra of visual pigments), yet allow you to see to operate equipment and take notes.

Cone cell photoreceptors comprise only 5% of the retinal photoreceptor cells and are contained nearly exclusively in the small central *fovea* of the retina, a 0.5 mm diameter spot that is responsible for color perception and visual acuity. Vision dominated by the function of cones under bright light conditions is called *photopic vision.* Cone cells contain red-, green-, or blue-sensitive pigment proteins that are also conjugated to 11-cis-retinal. The color photovisual pigments are highly homologous to each other and share about 40% amino acid sequence homology with rod cell rhodopsin (Nathans, 1984). Absorption spectra for purified rhodopsin and the three color pigments are shown in Figure 2-8.

POSITIVE AND NEGATIVE COLORS

As discussed in this section, color can be described as the addition or subtraction of specific wavelengths of light. Light is perceived as white when all three cone cell types (red, green, and blue) are stimulated equally as occurs when viewing a nonabsorbing white sheet of paper in sunlight. It was found over a century ago by James Clerk Maxwell (1831 1879) that color vision can be approximated by a simple tristimulus system involving red, green, and blue color stimulation. By varying the relative intensities of the three colors, all of the colors of the visual spectrum can be created, ranging from violet to red. *Positive colors* are created by combining different color wavelengths. A fine example of mixing wavelengths to create positive colors can be made using three slide projectors, each equipped with monochromatic red, green, and blue cellophane filters (the kind used for RGB color analysis) from a scientific supply house. The filters are mounted in slide holders and covered with an opaque aluminum foil mask containing a

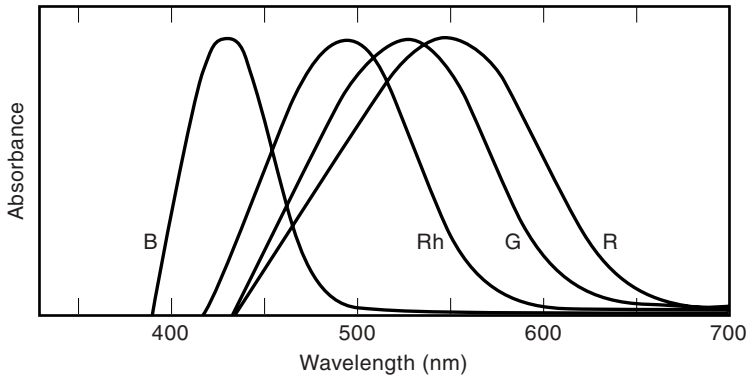


Figure 2-8

Absorption difference spectra of the four human visual pigments. The four pigment proteins were cloned, purified, and characterized with respect to their absorption spectra in vitro. Photobleaching difference spectra were obtained by subtracting an absorption spectrum measured after light exposure from one measured prior to light exposure. The pigments show maxima in the expected red, green, and blue regions of the visual spectrum. The values are close to those measured for rod and cone cells in vivo and confirm Maxwell s theory for RGB-based color vision over a century ago. (Courtesy of Jeremy Nathans, Johns Hopkins University.)

1 cm diameter hole in the center. Three color disks can be projected on a screen and made to overlap as shown in Figure 2-9. Try it and experience why mixing magenta and green light gives white. *Negative colors,* in contrast, are generated by the subtraction (absorption) of light of a specific wavelength from light composed of a mixture of wavelengths. A pigment that looks red, for example, absorbs blue and green wavelengths, but reflects red, so it is red by default. To appreciate this point, it is informative to examine colored objects (paints and pigments) with a handheld *spectroscope* under bright white light illumination. It is fascinating that yellow, cyan-blue, and magenta pigments are composed, respectively, of equal mixtures of red and green, green and blue, and blue and red wavelengths.

Thus, perception of the color yellow can arise in two ways: (1) by simultaneous stimulation of the red and green cone cells by a monochromatic yellow (580 nm) light source the red and green photovisual pigments exhibit broad excitation spectra that

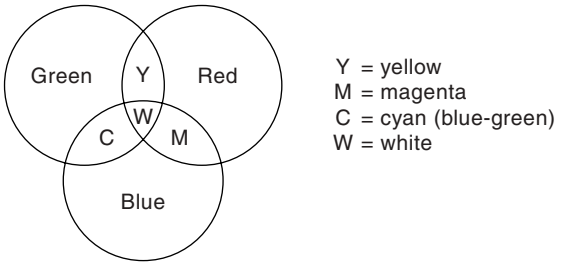


Figure 2-9

Addition colors of the red-green-blue tricolor system. This color display can be produced by projecting the colors of RGB color filters with three separate slide projectors on a screen as described in the text.

overlap significantly in the 580 nm band region and are both stimulated almost equally; or (2) by stimulating the red and green cones separately with a mixture of distinct red and green wavelengths, each wavelength selectively stimulating red and green cone cells in the retina. In either case, the color yellow is defined as the simultaneous stimulation of both red and green visual pigments. Perception of other colors requires stimulation of one, two, or all three cone cell types to varying degrees. The mixing of different colored paints to produce new colors, which is our common experience in producing colors, is actually a subtractive process. Consider why mixing yellow and blue paints produces a green color: Yellow pigment (reflects red and green, but absorbs blue) and blue pigment (reflects blue and green, but absorbs red) gives green because green is the only wavelength not absorbed by the mixture of yellow and blue pigments. Thus, combining blue and yellow wavelengths of light gives white, but mixing blue and yellow pigments gives green! Removal of a specific wavelength from the visual spectrum is also the mechanism for producing interference colors, and is discussed in Chapter 9. A useful overview on the perception of color when viewing natural phenomena is given by Minnaert (1954).

Exercise: Complementary colors

A complementary color is a color that gives white light when mixed with its complement. Thus, yellow and cyan-blue are complementary colors as are the color-pairs green with magenta and red with cyan. Our perception of complementary colors is due to the red, green, and blue photovisual pigments in the cone cells of the retina. Note that mixing wavelengths of different colors is distinct from mixing colored pigments.

Combining Red, Green, and Blue Light. To experience the relationships among complementary colors, prepare 3 slide projectors each containing a red, blue and green color filter sandwiched together with masks containing a 1 cm diameter hole, and project three disks of red, green and blue color on a projection screen. Focus each projector so the edges of the circular masks are sharp. Move the projectors to partially overlap the colors so that it is possible to see that red plus green gives yellow, red plus blue gives magenta, and blue plus green gives cyan. The overlap of red, green and blue gives white. Thus, when all 3 color types of cone cells in the retina are saturated, we see white light.

Mixing Colored Pigments. As is known, mixing yellow and blue pigments gives green. The reason for this is that blue and yellow pigments reflect green light; all other wavelengths are absorbed by the blue and yellow dyes in the mixture. To demonstrate this, prepare 2 beakers with 500 mL water and add 8 drops of blue and yellow food coloring separately to each beaker. Display the beakers on a light box. The generation of green by mixing the yellow and blue pigmented solutions is different from the mixing of blue and yellow light, which gives white light, as was demonstrated above.

Removing Colors from a White Light Source. The relationship between complementary colors and subtraction colors can be demonstrated using a bright white light source, a slit, a diffraction grating and a large diameter magnifying glass to form the image of the slit on a projection screen. Set up the optical bench apparatus with a bright xenon light source, a 1 mm wide slit made from razor blades, an IR blocking filter, and a holographic diffraction grating as described in Figure 2-10. Intercept the dispersed color spectrum with a white card and exam-

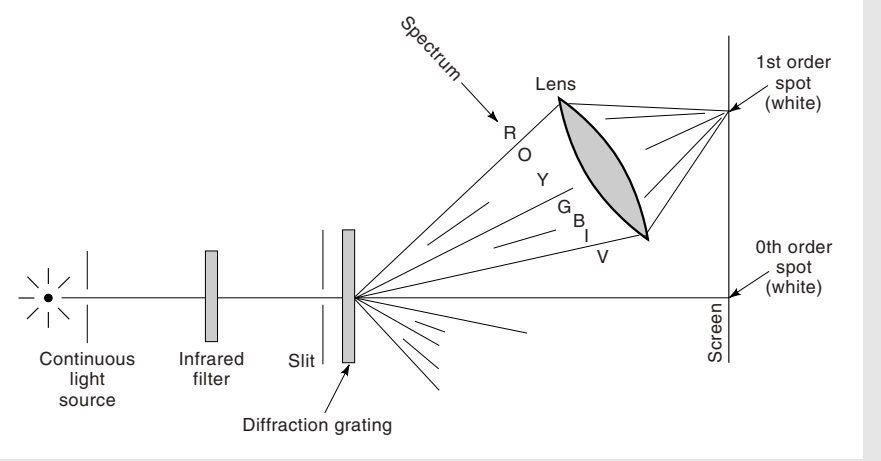


Figure 2-10
Optical bench setup for demonstrating complementary colors.

ine the spectral colors. With the help of a partner, examine the colors on the card with the spectroscope. Scan the spectroscope back and forth across the spectrum and confirm that each color is pure, monochromatic, and located in the spectrum according to its wavelength. Next intercept the spectrum between the grating and the projection screen with a 4 6 inch diameter magnifying glass and focus the image of the slit on a projection screen. Notice that the color of the focused image of the slit is white. It is clear that the individual spectral colors have been recombined to give white light. Next insert an opaque 1 cm wide white paper strip into the light path to remove a band of wavelengths such as red and orange from the spectrum. Note the corresponding color of the slit in the image plane. The color of the slit on the screen and the color of the blocked light are called complementary colors. What are the complementary colors for red, green, and blue?

Examine the Colors of After-images. Another way to examine complementary colors is to produce after images on the retina. Stare at a collection of large brightly colored objects for 30 60 seconds in a brightly illuminated area. Do not let your eyes wander, but focus steadily on a central spot in the visual field. Then shift your vision to stare at a blank white sheet of paper or brightly illuminated white wall. Do you see an after-image composed of complementary colors of the objects? The complementary colors are seen because the cones stimulated by certain colors become depleted and temporarily exhausted after prolonged viewing, so unstimulated cones in the same area of the retina provide the principal stimulus when you look at a white page. Cyan-blue, magenta-red, and yellow are the three complementary colors to red, green, and blue.

CHAPTER

3

ILLUMINATORS, FILTERS, AND THE ISOLATION OF SPECIFIC WAVELENGTHS

OVERVIEW

To obtain optimal imaging performance in the light microscope, the specimen must be properly illuminated. This requires proper selection of wavelength and intensity and correct alignment and focus of the lamp. The first objective is met by matching the particular application to the proper combination of illuminator and filters. Since research microscopes may be equipped with a variety of lamps, including quartz halogen lamps and other tungsten filament lamps, mercury, xenon, and metal halide arc lamps, we discuss the energy and spectral output of various illuminators. Filters that adjust light intensity and provide wavelengths of a particular color are also discussed. For example, if the microscope is equipped with a constant wattage power supply, the intensity must be controlled using neutral density filters; similarly, colored glass filters and interference filters are used to isolate specific color bandwidths. It is the combination of illuminator and filters that determines the quality of light directed to the condenser for illuminating the specimen. While all forms of light microscopy require selecting illuminators and filters, knowledge of their action and function becomes especially important in fluorescence and confocal fluorescence microscopy. We close the chapter by discussing how illuminators and filters must be carefully considered when examining living cells.

ILLUMINATORS AND THEIR SPECTRA

Successful imaging requires delivery to the condenser of a focused beam of light that is bright, evenly distributed, constant in amplitude, and versatile with respect to the range of wavelengths, convenience, and cost. Alignment and focus of the illuminator are therefore essential and are the first steps in adjusting the illumination pathway in the microscope. A number of incandescent filament lamps and arc lamps are available to meet the needs of various applications. The spectra of the principal lamps used in microscopy are shown in Figures 3-1 and 3-2 and are summarized here.

Incandescent lamps with tungsten wire filaments and inert argon gas are frequently used for bright field and phase contrast optics and are bright enough for certain applications requiring polarized light. Tungsten and quartz halogen lamps are convenient and

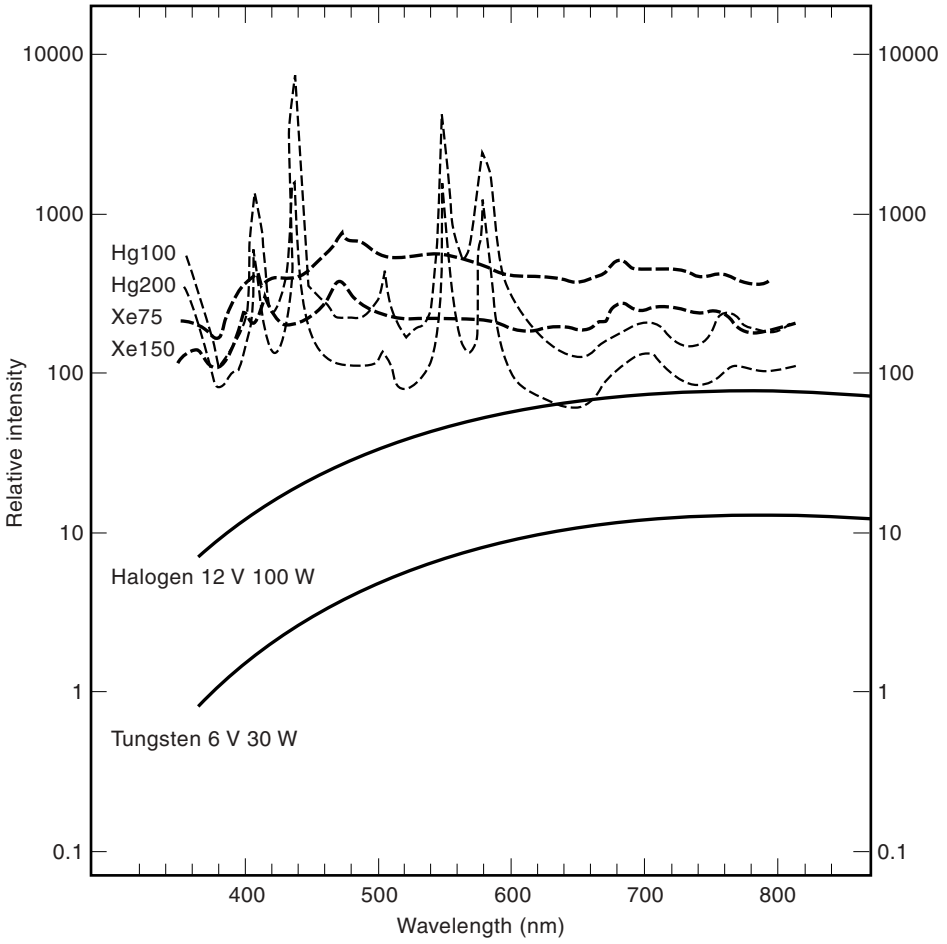


Figure 3-1

The spectra of various illuminators. Tungsten filament lamps give continuous emission, but their output is reduced at shorter wavelengths; mercury and xenon arc lamps are brighter, although mercury contains prominent emission lines in the visible range. Notice that over much of its range, the intensity of a 75 W xenon bulb (XBO) is several times greater than that of a 100 W mercury lamp (HBO). Although higher-wattage arc lamps generate a lower luminous flux (lumens/mm^2/s), they cover a much larger area and their total luminous output is considerably greater.

inexpensive, easy to replace, and provide bright, even illumination when used together with a ground glass filter; hence their popularity in nearly all forms of light microscopy. These lamps produce a continuous spectrum of light across the visual range, with peak output occurring in the red and infrared (IR) range and blue and ultraviolet (UV) output being the weakest. Excitation of the filament is regulated by a continuously variable power supply. As voltage and excitation are increased, brightness increases and the spectrum shifts to increasingly higher-energy photons with shorter wavelengths. Therefore, color balance of the light from an incandescent lamp varies depending on the voltage applied to the lamp. When producing color micrographs, a specific voltage is selected in order to obtain a consistent and predictable spectrum of wavelengths. Spe-

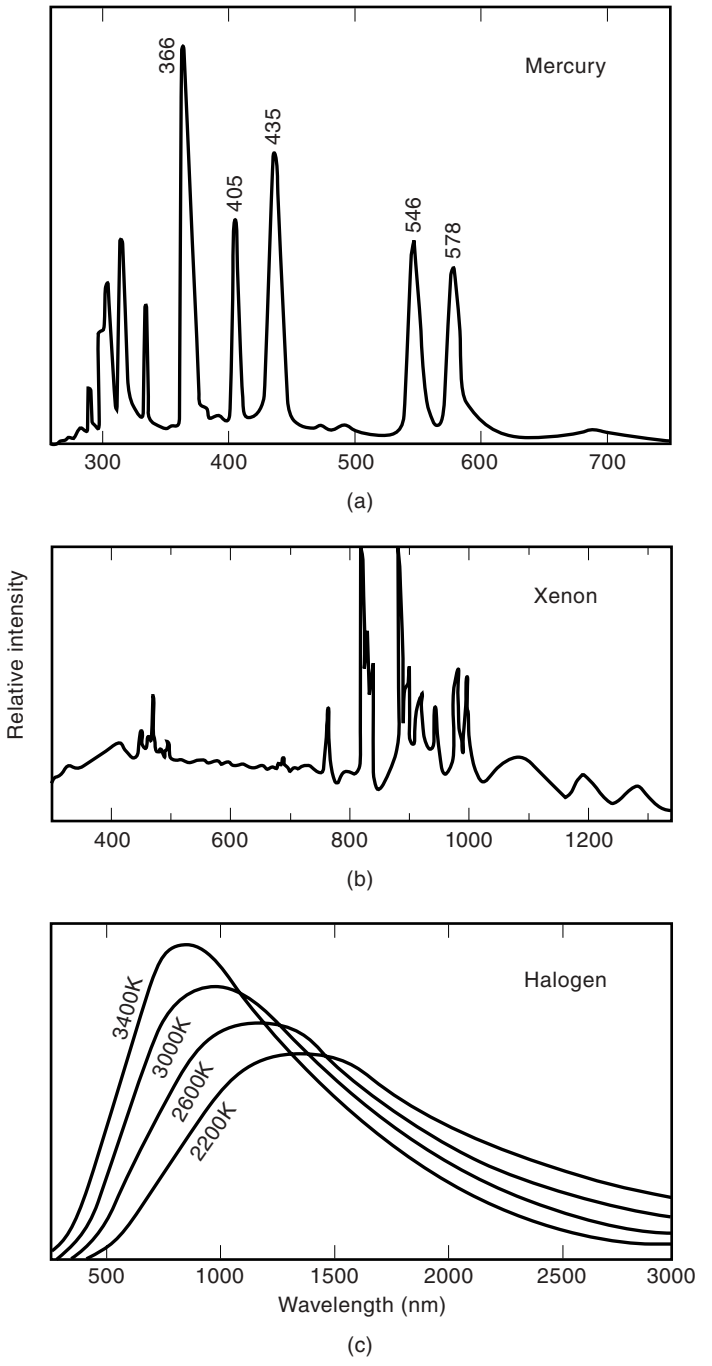


Figure 3-2

Detailed spectra of arc and tungsten filament lamps. (a) Mercury arc lamp. A continuous spectrum in the visible range is superimposed with bright emission lines, of which the most useful are at 366, 405, 435, 546, and 578 nm. Significant emission occurs in the UV (below 400 nm) and IR portions of the spectrum (not shown). (b) Xenon arc lamp. In the visible range, this lamp produces a continuous spectrum without major emission lines. Emission is significant in the UV and very large in the infrared. (c) Quartz halogen tungsten filament lamp. Much of the radiation is in the infrared range. Intensity increases and the peak of radiation intensity shifts to lower visible wavelengths as voltage is increased.

cial tungsten film and photographic filters are available to correct for the red-rich spectral output of these lamps.

Ion arc lamps are 10 100 times brighter than incandescent lamps and can provide brilliant monochromatic illumination when combined with an appropriate filter, but the increase in brightness comes with some inconveniences in mechanical alignment, shorter lifetime, and higher cost. Two types are commonly used: 75 W xenon and 100 W mercury arc lamps. Both lamps produce continuous spectra across the entire visible range from 400 750 nm and extending into the ultraviolet and infrared. In fact, only about a fifth of the output of these arc lamps is in the visible portion of the spectrum, the remainder being in the ultraviolet and infrared, so special blocking filters must be used when examining living cells, which are sensitive to UV and IR radiation.

Arc lamps tend to flicker due to subtle variations in power. This can be annoying, especially during time lapse recording, but stabilized power supplies are now available that minimize this problem. It is also common practice to avoid turning the lamp on and off frequently, as this poses a risk to nearby electronic equipment and shortens the life of the bulb. If there is a 20 30 min pause in the use of the microscope, it is better to leave the lamp on than to turn it off and reignite it. There are, however, new lamp designs that use a variable transformer to control light intensity. As the bulb is turned down, a heating mechanism keeps the lamp hot so that brightness increases immediately when the power is turned up again. The lifetime of mercury and xenon lamps is generally rated at 200 hours; however, the UV output of a mercury lamp weakens (sometimes considerably) with prolonged use, since metal vapors from the electrodes become deposited on the glass envelope. In addition, the arc becomes unstable and begins to flicker. Although arc lamps are expensive, the actual cost works out to be about 50 cents per hour, so it is advisable to replace them after their nominal lifetime has expired even if they are still working.

The *mercury arc lamp* is distinct in emitting several prominent emission lines, some of which are up to 100 times brighter than the continuous background: 254 (far UV), 366 (near UV), 405 (violet), 435 (deep blue), 546 (yellow-green), 578, 579 (yellow doublet band), plus several lines in the IR. The 546 nm green line of the mercury arc lamp is a universal reference for calibrating wavelengths in a number of optical devices and is a favorite among biologists for examining living cells. UV emission accounts for about half of the output of the mercury lamp, so care must be taken to protect the eyes and living cells that are illuminated by it. When changing and aligning a new lamp, avoid staring at the unattenuated beam; when examining live cells, use a green bandpass filter plus a UV-blocking filter such as a Schott GG420 glass filter. Since mercury lamps also emit in the IR, heat-cut filters are desirable to block these wavelengths as well.

The spectrum of the *xenon arc lamp* is largely continuous and lacks prominent emission lines. Its advantage is bright, uniform output across the entire range of visual wavelengths. At blue-green and red wavelengths it is significantly brighter than a 100 W mercury lamp, making it advantageous for certain applications in fluorescence microscopy. Since about half of the light emitted from a xenon lamp is in the IR, special IR-blocking filters, such as a Schott BG38 or BG39 glass filter and/or an IR-reflecting mirror, are used to attenuate and block these wavelengths and protect cells from excess heat. The detectors of electronic cameras, particularly those of CCD cameras, are also particularly sensitive to infrared light, which can fog the image. Although the intensity of a 75 W xenon lamp is high, the distance between the lamp electrodes is small only 0.75 mm which can make it difficult to obtain an even distribution of the light across the front aperture of the condenser and therefore across the specimen in the object plane.

Metal halide lamps, which have a spectral output similar to mercury, are becoming popular because they are bright (150 W), have a long bulb life (1000 hr), and have a large electrode gap (5 mm). Nikon Corporation promoted their use in combination with a liquid fiber bundle for delivering bright, homogeneous illumination for video microscopy.

Demonstration: Spectra of Common Light Sources

Please note: *Never look directly at unattenuated mercury or xenon beams, because they are, respectively, extremely UV- and IR-rich and potentially damaging to the eye!*

It is useful to become familiar with the spectra of common illuminators by indirect inspection of their light with a spectroscope or a diffraction grating. There are several ways to do this:

For display and discussion in a group, set up the I-beam/optical bench and project the diffraction pattern of a diffraction grating on a projection screen as shown in Figure 2-10.

For individual study, wrap a piece of aluminum foil containing a narrow slit aperture over the mouth of the illuminator and examine the slit at a distance of several feet with a handheld diffraction grating held close to the eye. A transparent holographic grating works best. To make a slit, cut a 1 cm long slit in the foil using a razor blade, while holding the foil placed against a sheet of stiff cardboard.

An inexpensive handheld spectroscope based on a sine-wave (holographic) diffraction grating is available from Learning Technologies, Cambridge, Massachusetts. Direct the unfiltered beam of the illuminator onto a white card or projection screen positioned several feet away and follow the instructions for using the spectroscope. The advantage of the spectroscope is that it permits you to determine the wavelengths of colors and emission lines from a built-in wavelength scale. You should perform these observations in a darkened room.

Examine the spectrum of a tungsten lamp or quartz halogen lamp first. The continuous, smooth nature of the spectrum and the relative falloff in brightness at the blue end of the spectrum are characteristic. Examine the spectrum with the power supply adjusted at the minimum and maximum permissible settings to see the shift in the peak spectral output. As power increases, the intensity of shorter bluer wavelengths increases. (The peak emission wavelength in the infrared also decreases, but this cannot be seen visually.)

Next, examine the spectrum of the xenon arc and notice the uniform intensity across the entire visible range. Careful inspection will show that the spectrum is not perfectly smooth, but rather has weak emission lines in the visible range near 470 nm (blue) and also at the red end of the spectrum near 680 nm. Fifty percent of the output of this lamp is in the IR, where prominent, though invisible, emission lines occur at $>$800 nm.

Finally, inspect the mercury arc spectrum with its continuous spectrum and superimposed prominent emission lines. Half of the output of this lamp is in the UV, with one of the most prominent (but invisible) emission lines being located at 366 nm. This wavelength is commonly used for photoactivation of caged fluorescent compounds, stimulation of UV-excitable fluorescent dyes, and conversion of colchicine to its photoisomer, lumicolchicine. This line and the 405 nm violet line can be visualized using the optical bench setup by holding a piece of fluorescent white paper in the proper location in the spectrum in a darkened room. A suitable piece of paper can be found using a handheld near-UV black light to select for sheets that exhibit brilliant bluish white fluorescence due to the presence of phenolic compounds in the paper. The 405 nm violet line and the 366 nm near-UV line suddenly leap into view when the white card is inserted into the blue end of the spectrum.

ILLUMINATOR ALIGNMENT AND BULB REPLACEMENT

Microscope illuminators consist of a lamp housing with a lamp and concave reflector, a focusable collector lens, an electrical socket for holding the bulb, and an external power supply. The socket and power cord, in particular, deserve attention. Oxidized metal surfaces of the socket electrodes and the copper conducting wire in an arc lamp should be cleaned with an emery cloth each time the lamp is changed to assure good electrical contact. The socket s power cord should never be crimped (as occurs when the illuminator is shoved against a wall) as this action loosens wires, which can lead to inconvenient and expensive repair. The bulb, rear reflector, and front collector lens should be kept clean of all dirt, lint, and finger oils. At the time of changing a bulb and after the collector lens and metal housing have been removed, move the illuminator s adjustment screws with a screwdriver to observe their effect on the location of the bulb and the reflector. Some arc lamp housings only contain adjustment screws for the rear reflector, whereas others contain additional screws for adjusting the bulb itself. Arc lamp illuminators should be maintained in an upright position during use to preserve the life of the bulb. *Never ignite an arc lamp when it is outside its protective metal housing!*

After a bulb is changed and aligned, the image of the arc or filament should be focused in the front aperture plane of the condenser using the illuminator s collector lens focusing dial, which is located on the illuminator housing. On some microscopes it may be possible to place a lens tissue across the front of the condenser aperture or to stop down the condenser diaphragm in order to see the image of the focused filament. Alternatively, the focused image of the filament or arc may be viewed at its conjugate location at the objective back aperture using an eyepiece telescope or Bertrand lens. In this case, the light should be turned down to a minimum or attenuated with appropriate neutral density filters. To see the image clearly, it may be necessary to remove a ground glass diffusing screen, whose function is to remove the pattern of the filament from the image of the specimen.

Alignment of a new bulb is especially critical for mercury or xenon arc lamps, such as those mounted in epi-illuminators used for fluorescence microscopy, because the arc in the lamp is small (~1 mm) and the arc s image at the condenser aperture must be

positioned on the optic axis of the microscope. Light from an arc lamp can be safely examined after attenuation with a fluorescence filter set plus additional neutral density filters. It is easier on the eye to examine the green excitation light provided by a rhodamine filter set. A similar procedure can be applied for arc lamps used in transillumination mode.

Demonstration: Aligning a 100 W Mercury Arc Lamp in an Epi-illuminator

Always turn off the power supply and allow the lamp to cool completely before changing a failed bulb. Since arc lamps are under moderately high pressure when they are hot, an applied mechanical force can cause them to explode. After replacing a bulb, secure the lamp socket to the lamp housing and fasten the housing to the microscope before reigniting the lamp.

Place neutral density filters in the light path sufficient to block ~97% of the light and place a rhodamine fluorescence filter cube into position so that the 546 nm green line of the arc is reflected onto the specimen plane. Insert additional UV- and IR-blocking filters into the path to protect the eyes.

Tape a white card or paper on the microscope stage, focus an objective lens until you see an intense, focused dot on the card, and mark the location with a pen. The dot defines the position of the optic axis of the microscope (Fig. 3-3).

Without disturbing the card, rotate the objective turret to an empty position and observe an intense, extended spot of light on the card. Focus the collector lens of the lamp until the bright primary image of the arc is sharply defined. If the arc s image does not coincide with the dot, you will need to adjust the bulb using the adjustment screws on the illuminator housing.

There should also be a dimmer reflection image of the arc, which is focused and aligned using the reflector s adjustment screws on the lamp housing. Position the reflection image so that it is on top of or next to the primary image of the arc.

Slowly defocus the lamp collector lens and ascertain that the beam expands uniformly around the black dot on the card. This is an important test of

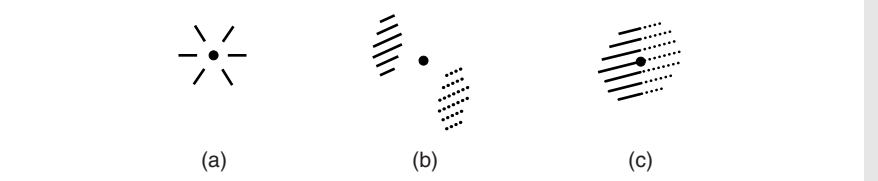


Figure 3-3

Alignment of an arc lamp. (a) The optic axis is marked on a white card with a pen as described in the text. (b) When the collector lens is properly adjusted, the direct image and reflection image of the arc are seen as two separate patches of light on the card. (c) Adjustment screws on the illuminator housing are moved to align the two spots on the optic axis.

alignment. If the arc s image does not expand symmetrically, you must make an additional round of adjustments. Sometimes it helps to expand the image slightly with the collector lens before making additional adjustments.

While looking in the microscope at a focused fluorescent specimen, adjust the collector lens of the illuminator until the image is bright and uniform from one edge of the field to the other and across all diameters of the field. At this position the arc is focused on the objective s back aperture. In epi-illumination, the objective lens functions as both a condenser and an objective.

For incandescent filament lamps the procedure is easier. After remounting the bulb, turn on the power supply and examine the lamp filament in the microscope using a telescope eyepiece or Bertrand lens. Focus the telescope to view the edge of the condenser diaphragm at the front aperture of the condenser. In the absence of a diffuser screen, the filament should be sharply focused; if not, adjust the lamp s collector lens. Notice the pattern of vertical lines representing the filament. Center the image of the filament, then center the image of the reflection so that the vertical lines of the primary and reflection images are interdigitated (see Fig. 3-4). Some illuminators do not contain adjustable collector lenses for low-power lamps. If the filament image seems off-center, try remounting the bulb.

FIRST ON LAST OFF : ESSENTIAL RULE FOR ARC LAMP POWER SUPPLIES

It is very important to understand the potential hazard of turning on and off an arc lamp power supply located near functioning electronic equipment. Arc lamps should be turned on and allowed to stabilize for a minute or two *before* turning on the other pieces of nearby electronic equipment. Although the power supply and cable are generally well shielded, a momentary 20,000 50,000 V surge passing between the DC power supply and the arc lamp generates magnetic fields that are strong enough to damage sensitive integrated circuits in nearby VCRs, electronic cameras, and computers. Upon turning

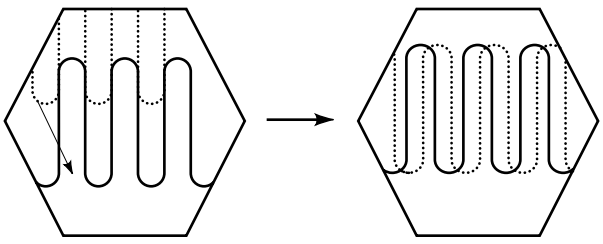


Figure 3-4

Alignment of a filament lamp. In the absence of a diffuser screen, the image of the lamp can be seen in the back aperture of the objective lens using an eyepiece telescope or Bertrand lens. The reflection image of the filament is adjusted to interdigitate with the filament loops seen in the direct image.

the lamp off, the reverse procedure should be used: First, turn off the accessory electronics, then turn off the arc lamp. It is advisable to post a simple warning on or near the arc lamp power supply that states *First on Last off* to act as a reminder to yourself and other microscope users. Second, when an arc lamp fails, remember that some power supplies try to continually reignite the lamp with a series of high-voltage pulses that can be heard as a rapid series of clicks. When heard, the supply should be shut off immediately; otherwise, electromagnetic fields might damage peripheral equipment as well as the power supply itself. There is always the risk that this may happen when you are out of the room and away from the microscope. To protect against this, and especially if time lapse studies are performed with no one at the microscope, be sure the power supply is protected with an *automatic trigger override switch.*

FILTERS FOR ADJUSTING THE INTENSITY AND WAVELENGTH OF ILLUMINATION

Selecting and adjusting the lamp for a particular application is important, but the actual control of the wavelength and intensity of illumination in a microscope requires the use of filters, so it is important to understand the fundamentals of their action and performance. The microscopist needs to know how to interpret the transmission spectra of filters, select the best among several possible filters for a given application, and explain differences in image quality, fluorescence quality, and cell behavior obtained with different filter combinations. This is particularly true in fluorescence microscopy, where filters must match the excitation and emission requirements of fluorescent dyes. Fortunately, the task is manageable, and filtering light to select a band of wavelengths from the illuminating beam presents many challenges and rewards.

Neutral density filters regulate light intensity, whereas *colored glass filters* and *interference filters* are used to isolate specific colors or bands of wavelengths. There are two classes of filters that regulate the transmission wavelength: edge filters and bandpass filters (Fig. 3-5). Edge filters are classified as being either *long-pass* (transmit long wavelengths, block short ones) or *short-pass* (transmit short wavelengths, block long ones), whereas bandpass filters transmit a band of wavelengths while blocking wavelengths above and below the specified range of transmission. Optical performance is defined in terms of the efficiency of transmission and blockage (% transmission), and by the steepness of the so-called cut-on and cut-off boundaries between adjacent domains of blocked and transmitted wavelengths. Edge filters are described by referring to the wavelength giving 50% of peak transmission; *bandpass* filters are described by citing the full width at half maximum transmission (FWHM), and by specifying the peak and central transmitted wavelengths. FWHM is the range of the transmitted band of wavelengths in nanometers and is measured as the distance between the edges of the bandpass peak where the transmission is 50% of its maximum value. For high-performance filters these boundaries are remarkably sharp, appearing on transmission spectra as nearly vertical lines.

In part, the resurgence of light microscopy as an analytic tool in research has been driven by the technologies used for depositing thin films of dielectric materials and metals on planar glass substrates. Companies now manufacture interference filters with transmission and blocking efficiencies approaching 100% and with bandwidths as narrow as 1 nm anywhere along the UV-visible-IR spectrum a truly remarkable accomplishment. This achievement has stimulated development of new research applications

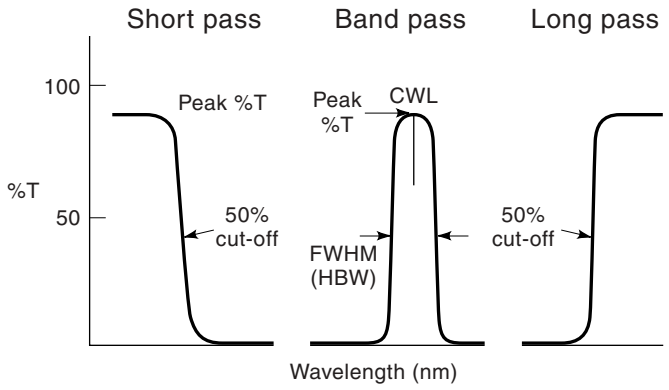


Figure 3-5

Filters for isolating the wavelength of illumination. Short-pass and long-pass filters, sometimes called edge filters, block or transmit wavelengths at specific cut-off wavelengths. Bandpass filters exhibit broadband or shortband transmission centered on a particular band of wavelengths. Filter performance is defined by the central wavelength (CWL) and by the full width at half maximum (FWHM). Another term for FWHM is halfbandwidth (HBW). A bandpass filter can also be created from two overlapping short-pass and long-pass filters.

involving laser-based illumination, new fluorescent dyes and molecules, and ratio imaging at multiple specific wavelengths.

Neutral Density Filters

Neutral density filters are used in microscopy to attenuate uniformly the intensity of light over the entire range of visible wavelengths. They are commonly employed in differential interference contrast (DIC), polarization, and fluorescence microscopy with high-intensity arc lamps that cannot be regulated with an adjustable power supply. In these circumstances, neutral density filters must be used. As discussed in Chapter 6, it is impermissible to reduce the intensity of illumination by closing down the condenser diaphragm, as this action affects resolution and contrast. A light-absorbing filter is the only solution.

Neutral density (ND) filters have a neutral gray color like smoked glass and are usually calibrated in units of *absorbance* or optical density (OD), where

$$OD = \log_{10}(1/T),$$

and T is the transmittance (intensity of transmitted light/intensity of incident light). Thus, a 0.1 OD neutral density filter gives 79% transmission and blocks 21% of the incident light. Other manufacturers indicate the transmittance directly. ND filters can be stacked in a series, in which case the total density of the combination is equal to the sum of the individual filter densities.

ND filters are either absorbing or reflecting. Absorbing filters contain rare earth elements throughout the glass, so there is no reflective coating that can be scratched off, and their orientation in the beam is not critical. Reflecting ND filters contain an evaporated coating of metal on one of the surfaces, so care must be taken not to scratch

them. These filters must be inserted into the beam with the reflective surface facing the light source. They can, however, be cheaper and thinner, and are the filter of choice for use with lasers.

Colored Glass Filters

Colored glass filters are used for applications not requiring precise definition of transmitted wavelengths. They are commonly used to isolate a broad band of colors or as long-pass filters to block short wavelengths and transmit long ones. Colored glass filters contain rare earth transition elements, or colloidal colorants such as selenide, or other substances to give reasonably sharp transmission-absorption transitions at a wide range of wavelength values across the visual spectrum. Since colored glass filters work by absorbing quanta of nontransmitted wavelengths, they can be heat sensitive and subject to altered transmission properties or even breakage after prolonged use. However, as the absorbent atoms are contained throughout the glass and are not deposited on its surface, colored glass filters offer major advantages: They are less resistant to physical abrasion and chemical attack from agents contained in fingerprints and other sources, and their optical performance is not sensitive to the angle of incidence of incoming rays of light. Colored glass filters are also less expensive than interference filters and are generally more stable and long-lived.

Interference Filters

Interference filters often have steeper cut-in and cut-off transmission boundaries than colored glass filters and therefore are frequently encountered in fluorescence microscopy where sharply defined bandwidths are required. Interference filters are optically planar sheets of glass coated with dielectric substances in multiple layers, each $\lambda/2$ or $\lambda/4$ thick, which act by selectively reinforcing and blocking the transmission of specific wavelengths through constructive and destructive interference (Fig. 3-6). Bandpass filters transmit a limited range of wavelengths that experience reinforcement through constructive interference between transmitted and multiple reflected rays; wavelengths that do not reinforce each other destructively interfere and are eventually back-reflected out of the filter.

Interference filters contain layers of *dielectric substances*, electrically nonconductive materials of specific refractive index, typically optically transparent metal salts such as zinc sulfide, sodium aluminum fluoride (cryolite), magnesium fluoride, and other substances. In some designs semitransparent layers of metals are included as well. The interface between two dielectric materials of different refractive index partially reflects incident light backward and forward through the filter, and is essential for constructive interference and reinforcement. The wavelength that is reinforced and transmitted depends on the thickness and refractive index (the optical path) of the dielectric layers. The coatings are built up in units called cavities, with 1 cavity containing 4 or 5 alternating layers of dielectric salts separated by a spacer layer (Fig. 3-7). The steepness of the transmission boundary and the definition of filter performance are increased by increasing the number of cavities. An 18-cavity filter may contain up to 90 separate dielectric layers. The deposition of salts is performed by evaporation of materials in a computer-controlled high-vacuum evaporator equipped with detectors for optical interference, which are used to monitor the thicknesses of the various layers. The final layer is covered

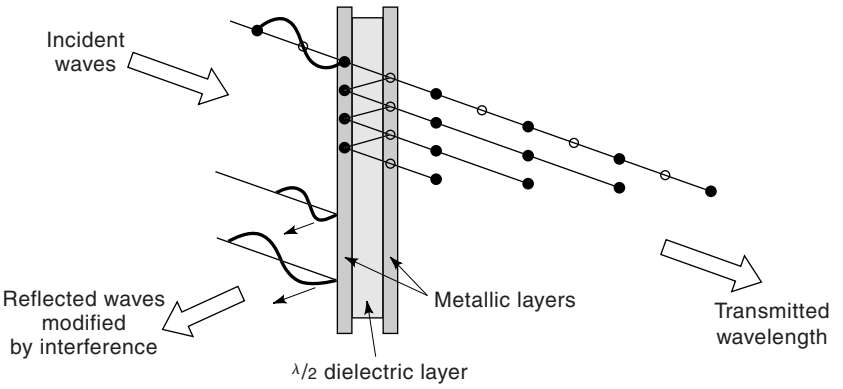


Figure 3-6

The action of an interference filter. An interference filter selectively transmits waves centered at a particular wavelength. For the filter shown, two thin films of metal cover a layer of dielectric material with an optical path of exactly λ/2 for a particular wavelength. The angle of the incident beam is usually perpendicular to the face of the filter, but is shown obliquely to reveal the behavior of transmitted waves. Since transmitted and multiply reflected waves of the designated wavelength are in phase, principles of constructive interference allow reinforcement and transmission through the filter. Shorter and longer wavelengths experience destructive interference and exit the filter as a back-reflection.

with glass or overcoated with a scuff-resistant protective coating of silicon monoxide or silicon dioxide (referred to as quartz) to guard against abrasion. The availability of computers and programs to model the behavior of multiple dielectric cavities has stimulated a revolution in thin film technology, allowing significant improvements in the selection of specific wavelengths and in the intensity and contrast of fluorescence images. The technology has also stimulated research for the production of new fluorescent dyes, optical variants of green fluorescent protein (GFP), fluorescent crystals, and other substances for use with a variety of illuminators and lasers. Because filter production is technology dependent and labor intensive, interference filters remain relatively expensive.

Interference bandpass filters for visible wavelengths frequently transmit wavelengths in the UV and IR range that may not be included in transmission spectra and documentation provided by the manufacturer. For microscopy involving live cell applications, it is safest to obtain the extended transmission spectra of all filters used and to employ efficient UV- and IR-blocking filters, particularly when UV- and IR-rich mercury or xenon arc lamps are used. Even with fixed fluorescent cells, an IR-blocking filter, such as a BG38 or BG39 glass filter, is frequently used as a heat-cut filter to protect optics and to prevent image fogging on IR-sensitive CCD cameras.

Interference filters gradually deteriorate upon exposure to light, heat, humidity, and especially exposure to abrasion, fingerprints, and harsh chemicals. Gently remove fingerprints and deposits from interference filters with a lens tissue and a neutral lens cleaner. Care must be taken not to rub interference coatings too hard, as this might scratch the surface, making the filter less efficient and shortening its life. Filters containing semitransparent metal coatings are usually mounted with the shiniest (silvery, nearly colorless) side of the filter facing the light source. The bindings at filter edges are usually inscribed with arrows to aid in the orientation and placement of filters in the light path of the microscope.

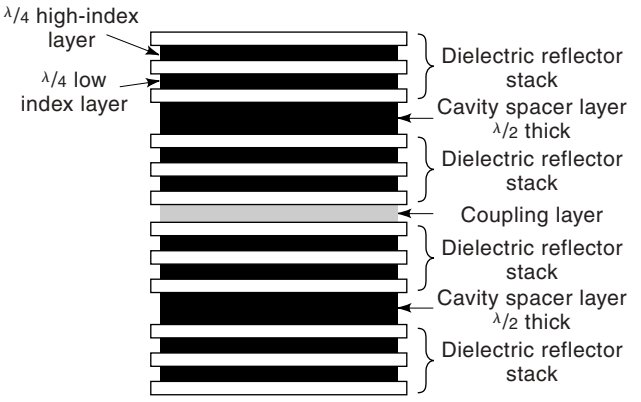


Figure 3-7

Structure of an all-dielectric interference filter. The revolution in thin film technology continues to drive the development of high-performance interference filters. The 2-cavity filter shown contains alternate layers of high- and low-refractive index dielectric materials, each λ/4 and λ/2 thick, with 5 such layers defining a cavity. Computers control the deposition of layers of dielectric materials in a vacuum evaporator while film thickness is determined by interference optics. Dozens of such layers are deposited during a single run in the evaporator. Three cavities are the industry standard, but 18-cavity filters with 90 separate layers that can produce bandwidths of less than 1 nm are now routinely produced.

EFFECTS OF LIGHT ON LIVING CELLS

Since the energy per quantum is related to wavelength ($E = hc/\lambda$), short wavelengths are more energetic than long ones. UV wavelengths flanking the blue end of the visual spectrum (200 400 nm) are particularly damaging to cells, because photons of ultraviolet light are energetic enough to break covalent bonds, thereby creating reactive free radicals that chemically alter and denature macromolecules such as proteins, nucleic acids, lipids, and small metabolites. Damage to membrane proteins, such as ion channels and gates, is a particular concern. Photons of infrared radiation (750 1000 nm) are less energetic than those corresponding to visible wavelengths, but are strongly absorbed by carbon bonds in macromolecules such as DNA and by water, leading to accumulation of kinetic energy (heat) and denaturation of molecules. Visible light itself is unique because it is absorbed relatively poorly by living cells, particularly at green and yellow wavelengths. For the most part, cellular absorption of visible light is considerably less than for the flanking UV and IR wavelengths. Since green light is relatively nontoxic and marks the peak sensitivity for human color vision, the 546 nm green line of the mercury arc lamp is commonly used for monochromatic illumination of living cells.

It is apparent that live cells must be protected from unwanted UV and IR radiation. IR- and UV-blocking filters, such as Schott filters BG38 (for IR) and GG420 (for UV), are especially useful, since the spectra generated by mercury and xenon arc lamps used in microscopy are rich in UV and IR radiation (for mercury, 30% UV, 40% IR, 30% visible; for xenon, 5% UV, 70% IR, and 25% visible). Phototoxicity in the microscope is recognized by the cessation of cell motility and the arrest of organelle movement; within 3 seconds of exposure to the full spectrum of a 100 W mercury arc, amoebae retract filopodia and freeze, their cytoplasm appearing to have gelled. Upon further exposure,

cells respond by blebbing and swelling, and particles contained in dilated vesicles begin to exhibit vibrational movements (Brownian movements) that are not as obvious in the living state. It is useful to observe cells without protective filters to become familiar with these effects.

Cells may require additional chemical protection from the buildup of high concentrations of damaging free radicals. For well chamber slides the simplest measures are to increase the volume of medium in the chamber to dilute the radicals or to use anti free radical reagents such as 10 mM ascorbate or succinate in the culture medium to neutralize free radicals as they form. Alternatively, the rate of free radical formation can be slowed by reducing the concentration of dissolved oxygen in the culture medium using a mixture of oxygen-scavenging enzymes, such as catalase, glucose oxidase, and D-glucose, or supplementing the medium with Oxyrase (Oxyrase, Inc., Mansfield, Ohio), which is a commercial preparation of respiratory particles of bacterial cell membranes that contains oxygen-removing enzymes. To maintain low oxygen concentrations, cells must be enclosed in specially designed flow cells. If well chambers are used, the medium should be covered with a layer of lightweight nontoxic mineral oil to prevent recharging of the medium with atmospheric oxygen. The presence of these agents is usually harmless to vertebrate somatic cells, since cells in most tissues exist in a low-oxygen environment.

CHAPTER

4

LENSES AND GEOMETRICAL OPTICS

OVERVIEW

In this chapter we discuss some essential principles of geometrical optics, the action of lenses on light as revealed by ray tracing and explained by principles of refraction and reflection (Fig. 4-1). With the help of a few simple rules and from studying examples, we can understand the process of magnification, the properties of real and virtual images, the aberrations of lenses, and other phenomena. We also examine the designs and specifications of condenser and objective lenses, review the nomenclature inscribed on the barrel of an objective lens that specifies its optical properties and conditions for use, and give some practical advice on the cleaning of optical components.

IMAGE FORMATION BY A SIMPLE LENS

To understand microscope optics, we begin by describing some of the basic properties of a thin, simple lens. A thin lens has a thickness that is essentially negligible, and by simple we mean consisting of a single lens element with two refracting surfaces. The *principal plane* and *focal plane* of a lens are defined as those planes, within the lens and in the focused image, respectively, where rays or extensions of rays intersect and physically reunite. Thus, for a simple positive lens receiving a collimated beam of light, the plane in the lens in which extensions of incident and emergent rays intersect is called the principal plane, and the plane in which rays intersect to form an image is the focal plane. The *focal length* of a lens is the distance between the principal plane and the focal plane. Lenses can be either positive or negative (Fig. 4-2). A *positive lens* converges parallel incident rays and forms a real image; such a lens is thicker in the middle than at the periphery and has at least one convex surface. (See the Note for definitions of real and virtual images.) Positive lenses magnify when held in front of the eye. A *negative lens* causes parallel incident rays to diverge; negative lenses are thinner in the middle than at the periphery, and have at least one concave surface. Negative lenses do not form a real image, and when held in front of the eye, they reduce or demagnify.

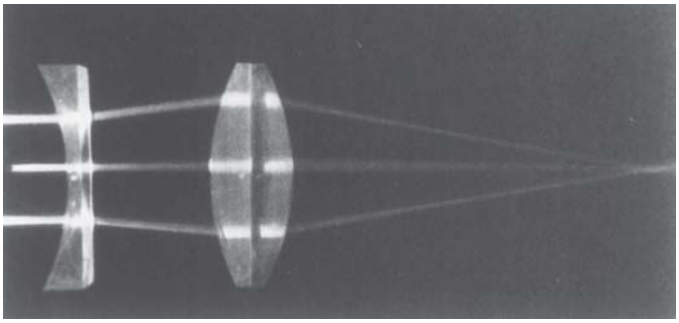


Figure 4-1
Geometrical optics of a positive lens. (From Hecht, 1998.)

For any given lens there are two principal planes: one each for the front and back surface of the lens. For the special case of a thin biconvex lens, the two principal planes are coincident in the middle of the lens. Microscope objectives contain multiple lens elements, some of which may be united with transparent sealing compound to make a complex *thick lens.* The principal planes of thick lenses are physically separated, but their locations can be determined by ray tracing. Most lens elements used in microscope optics are ground and polished with spherical curvatures.

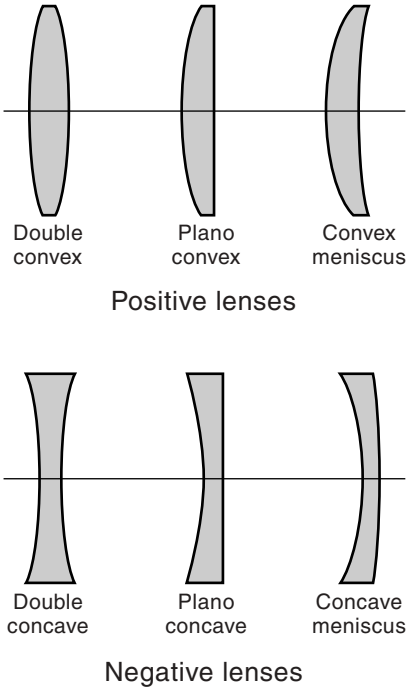


Figure 4-2
Examples of positive and negative lenses.

Note: Real and Virtual Images

Images can be defined as regions where rays, or the extensions of rays, become convergent as the result of refraction by a lens or reflection by a mirror. If the rays intersect and physically reunite, the image is said to be real. A *real image* can be seen on a viewing screen or recorded on a piece of film when a screen or film is placed in the image plane. If rays diverge, but the imaginary backward extensions of the rays become convergent and intersect, the image is said to be virtual. The plane occupied by a *virtual image* cannot be observed on a viewing screen or recorded on film. To be perceived, a real image must be formed on the retina of the eye. In the case of viewing an image in a microscope, a real image is formed on the retina but is perceived as a virtual image located some 25 cm in front of the eye. Lens configurations giving real and virtual images are described in this chapter.

The geometric parameters of a *simple thin lens* are described in Figure 4-3, where the vertical line represents the combined principal planes of a thin biconvex lens of focal length *f.* The object, an arrow on the left-hand side of the figure, is examined by the lens and imaged as a magnified real image (magnified inverted arrow) in the image plane on the right. The *focal length* is shown as the distance *f* from the principal plane of the lens to its *focal point F,* the front and rear focal lengths having the same value. The optic axis is shown by a horizontal line passing through the center of the lens and perpendicular to its principal plane. The *object distance a* (distance from the object to the principal plane of the lens) and *image distance b* (distance from the image to the principal plane of the lens) are also indicated.

The *focal length* of any simple lens can be determined by aiming the lens at a bright infinitely distant light source ($>$ 30 times the focal length) such as a lamp across the room or a scene outdoors; by focusing the image on a sheet of paper held behind the lens, the focal length is readily determined (Fig. 4-4). We will now examine the basic rules that determine the action of a simple convex lens.

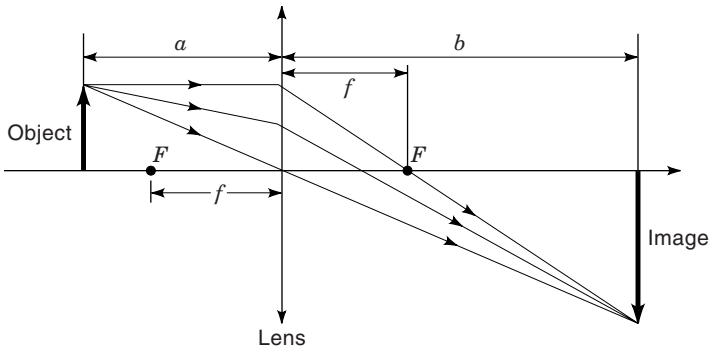


Figure 4-3

Geometrical optics of a simple lens. The focal length *f,* focal point *F,* object-lens distance *a,* and lens-image distance *b* are indicated.

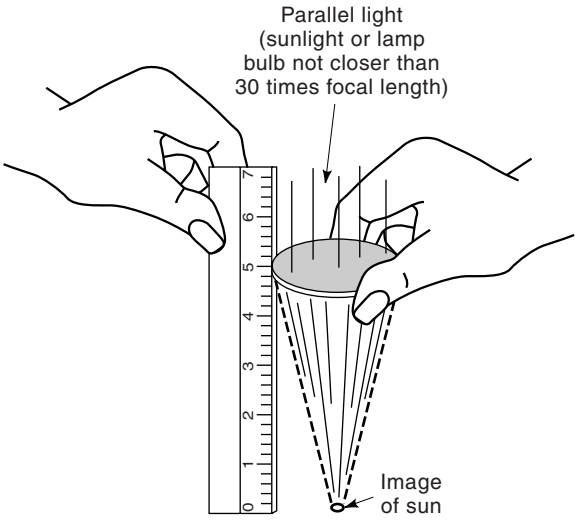


Figure 4-4
Determining the focal length of a simple lens. The image of a distant source is projected by the lens on a viewing surface; the focal length is the distance between the focal plane, and the lens as measured with a ruler.

RULES OF RAY TRACING FOR A SIMPLE LENS

The three rules governing ray tracing for a simple lens are depicted in Figure 4-5 and are listed as follows:

1. A light ray passing through the center of a lens is not deviated.
2. A light ray parallel with the optic axis will, after refraction, pass through the rear focal point.
3. A ray passing through the front focal point will be refracted in a direction parallel to the axis.

Notice that the intersection of any two of the three key rays just described identifies the location of the image plane.

OBJECT-IMAGE MATH

The well-known *lens equation* describes the relationship between focal length f and object and image distances, a and b:

$$1/f = 1/a + 1/b,$$

or

$$b = af/(a - f).$$

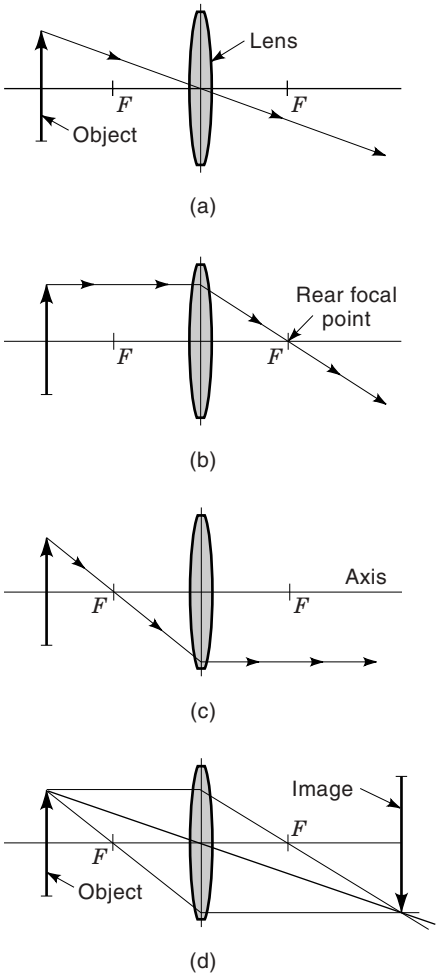


Figure 4-5

Principles governing ray tracing for a thin lens. (a) A ray, incident at any angle, that passes through the center of the lens remains undeviated from its original course on the other side of the lens. (b) Any ray traveling parallel to the optic axis and refracted by the lens always passes through the rear focal point *F*. (c) A ray passing through the front focal point of a lens at any angle is refracted and follows a path parallel to the optic axis. (d) The intersection of any two of the three rays described defines the location of the image plane.

Further, the magnification factor M of an image is described as:

$$M = b/a,$$

or

$$M = f/(a - f).$$

From these relationships the action of a lens on image location and magnification can be deduced. Depending on the location of the object relative to the focal point of the lens,

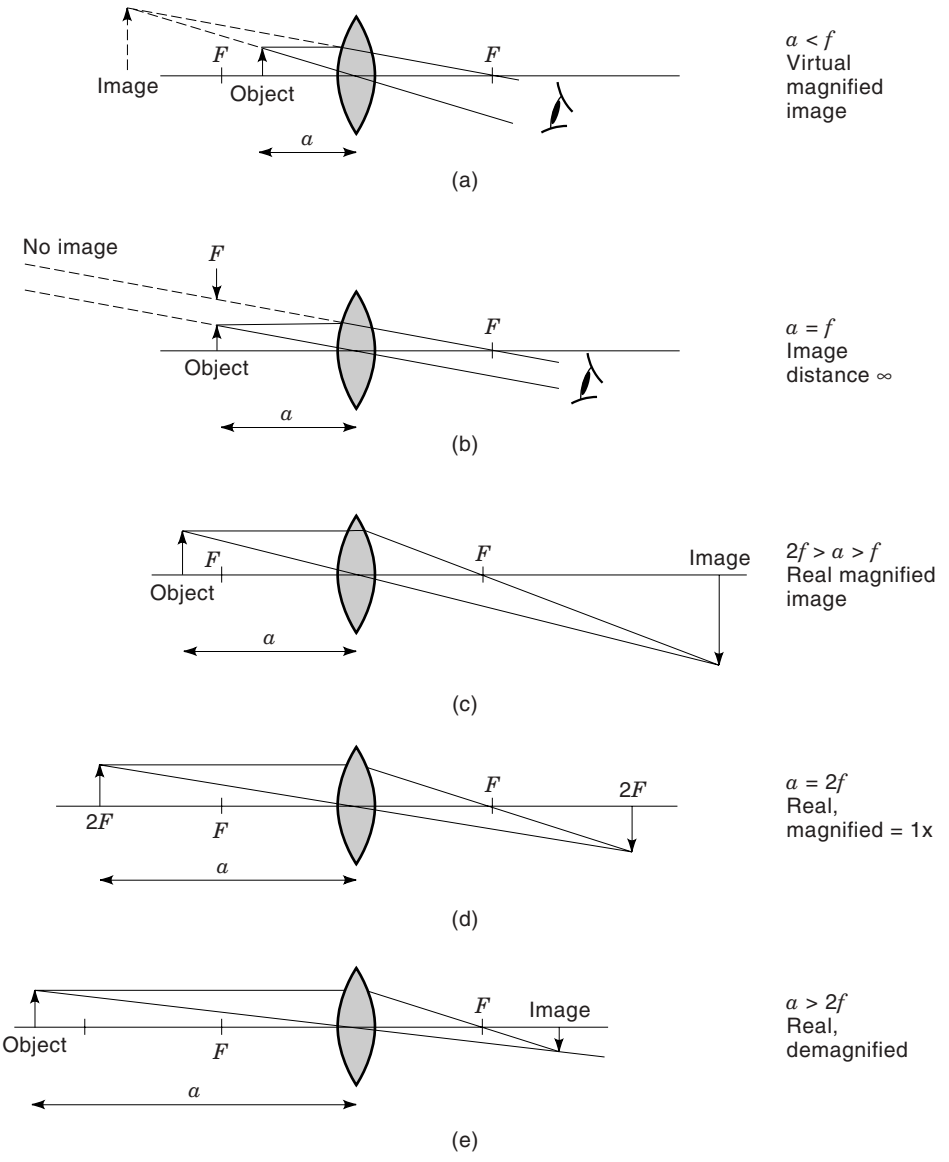


Figure 4-6

Object-image relationships. (a) $a < f$; (b) $a = f$; (c) $2f > a > f$; (d) $a = 2f$; (e) $a > 2f$.

the image may be real or virtual, and either magnified or demagnified (Fig. 4-6; Spencer, 1982). It is good practice to work through the following relationships using any handheld magnifier or simple biconvex lens such as the front lens of a binocular (10 50 mm is convenient), whose focal length has been determined by the procedure just described. The object should be self-luminous, moderately bright, and well defined. A 5 10 mm diameter hole cut in a large opaque cardboard sheet placed in front of a lamp works well as an object. Work in a partially darkened room. It is useful to remember the principal conditions describing object-image relationships for a simple positive lens:

$a < f$: No real image exists that can be projected on a screen. If the eye is placed behind the lens, a virtual image is perceived on the far side of the lens.

$a = f$: The image distance b is infinite, so no image exists that can be projected on a screen. We used this condition previously to determine the focal length of a lens, only in reverse: Parallel beams of light from an infinitely distant object converge at the focal length of the lens. This is the case for image formation in a telescope.

For the condition that $a > f$, a real image is always formed. The unique domains for this condition are as follows:

$2f > a > f$: A real *magnified* image is formed. This arrangement is used for producing the first real image in a microscope.

$a = 2f$: This is a specialized case. Under this condition, $b = 2f$ also. A real image is formed, but there is *no magnification* and $M = 1$.

$a > 2f$: A real *demagnified* image is formed and $M < 1$.

In the case of a microscope objective lens focused on a specimen, the image is both real and magnified, meaning that the object is located at a distance a between $1f$ and $2f$ ($2f > a > f$) (Fig. 4-7). Since the focused objective is very near the specimen, we deduce that the focal length of the objective must be very short, only a few millimeters. In the course of using the focusing dials of a microscope, the image comes into sharp focus when the correct object distance a has been obtained, and we obtain the correct adjustment without even thinking about object and image distances. In practice, focusing a microscope positions the image (the real intermediate image plane) at a fixed location in the front aperture of the eyepiece; when the microscope is defocused, there is still a real image nearby, but it is not in the proper location for the ocular and eye to form a focused image on the retina. Finally, notice that the image distance b is many centimeters long. The ratio b/a (the magnification M) usually ranges from <10 100. Thus, when a microscope with finite focus objectives is focused on a specimen, the specimen lies just outside

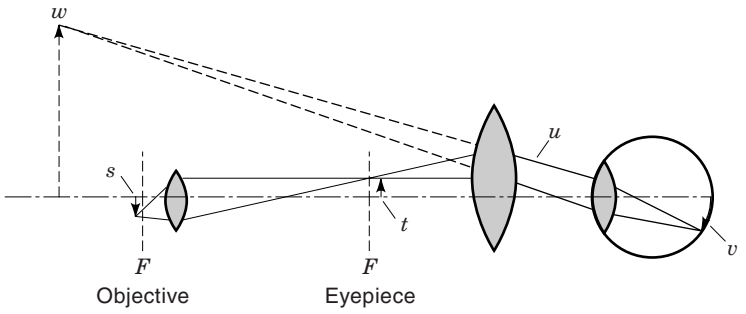


Figure 4-7

Location of real and virtual images in a light microscope marked s through w. Note that the specimen at *s* lies just outside the focus of the objective, resulting in a real, magnified image at *t* in the eyepiece. The primary image at *t* lies just inside the focus of the eyepiece, resulting in diverging rays at *u*. The cornea and lens of the eye form a real image of the object on the retina at *v*, which because of the diverging angle at *u* perceives the object as a magnified virtual image at *w*.

the front focal point of the objective, while the intermediate image is located at a distance 10 100 times the focal length of the objective in the eyepiece. For more detailed discussions on the topic, refer to Pluta (1988) or Hecht (1998).

Modern microscopes with *infinity focus objective lenses* follow the same optical principles already described for generating a magnified real image, only the optical design is somewhat different. For an objective with infinity focus design, the specimen is located at the focus of the lens, and parallel bundles of rays emerging from the back aperture of the lens are focused to infinity and do not form an image; it is the job of the *tube lens* in the microscope body to receive the rays and form the real intermediate image at the eyepiece. The advantage of this design is that it allows greater flexibility for microscope design while preserving the image contrast and resolution provided by the objective. Items such as waveplates, compensators, DIC prisms, reflectors, and fluorescence filter sets can be placed anywhere in the infinity space between the back of the objective and the tube lens. As long as these items have plane-parallel elements, their location in the infinity space region of the imaging path is not critical. If we consider the combination of objective plus tube lens as the effective objective lens, then the same optical rules pertain for generating a real magnified image and we observe that the relationship $2f > a > f$ is still valid. Sketches showing the infinity space region and tube lens in upright and inverted microscopes are shown in Color Plates 4-1 and 4-2.

The function of the eyepiece or ocular is to magnify the primary image another 10-fold, and together with the lens components of the eye, to produce a real magnified image of the intermediate image on the retina. Thus, the object of the eyepiece is the intermediate image made by the objective lens. Note that in the case of the ocular, $0 < a < f$, so the object distance is less than one focal length, resulting in a virtual image that cannot be focused on a screen or recorded on film with a camera. However, when the eye is placed behind the eyepiece to examine the image, the ocular-eye combination produces a real secondary image on the retina, which the brain perceives as a magnified virtual image located about 25 cm in front of the eye. The visual perception of virtual images is common in optical systems. For example, we also see virtual images when we employ a handheld magnifying glass to inspect small objects or when we look into a mirror.

THE PRINCIPAL ABERRATIONS OF LENSES

Simple lenses of the type already discussed have spherical surfaces, but a spherical lens is associated with many intrinsic optical faults called *aberrations* that distort the image in various ways. Of these faults, the major aberrations are chromatic aberration, spherical aberration, coma, astigmatism, curvature of field, and distortion (Fig. 4-8). Corrective measures include use of compound lens designs, use of glass elements with different refractive indexes and color dispersion, incorporation of aspherical lens curvatures, and other methods. The tube lens performs an additional important function in removing residual aberrations of the objective lens. In some microscopes the eyepieces also help perform this function. Objective lenses are designed to correct for aberrations, but can never completely remove them. It is common that a solution for correcting one fault worsens other faults, so the lens designer must prioritize goals for optical performance and then work toward the best compromise in correcting other aberrations. For these reasons, objective lenses vary considerably in their design, optical performance, and cost.

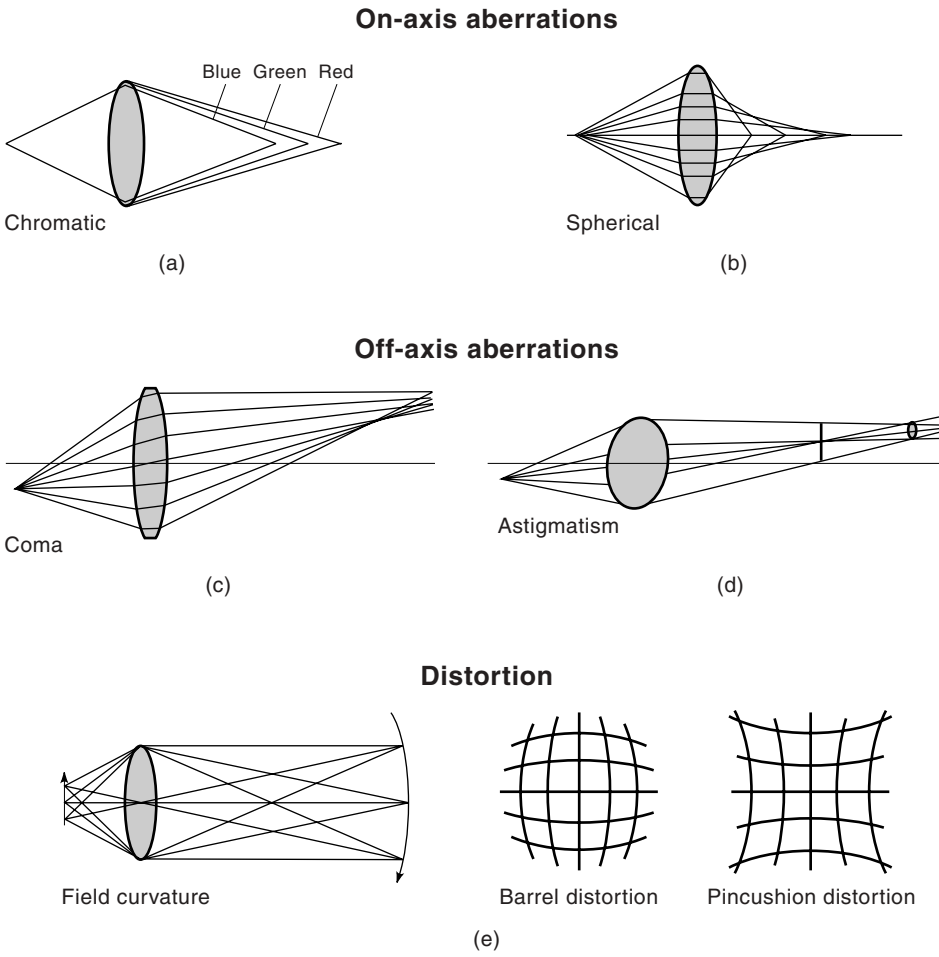


Figure 4-8

Aberrations of a simple lens. (a) Chromatic aberration: Parallel incident rays of different wavelength are focused at different locations. (b) Spherical aberration: Incident rays parallel to the optic axis and reaching the center and the periphery of the lens are focused at different locations. (c) Coma: Off-axis rays passing through the center and periphery of the lens are focused at different locations. (d) Astigmatism: An off-axis aberration causes waves passing through the vertical and horizontal diameters to focus an object point as a streak. (e) Distortion and field curvature: The image plane is curved and not planar. So-called barrel and pincushion distortions produce images that are not high in fidelity compared to the object.

Chromatic aberration occurs because a lens refracts light differently depending on the wavelength. Blue light is bent inward toward the optic axis more than red light. The result is disastrous: Blue wavelengths are focused in an image plane closer to the lens than the image plane for red wavelengths. Even at the best focus, point sources are surrounded by color halos, the color changing depending on the focus of the objective, the image never becoming sharp. Since each wavelength is focused at a different distance from the lens, there is also a difference in magnification for different colors (chromatic magnification difference). The solution is to make compound lenses made of glasses

having different color-dispersing properties. For example, glass types known as crown and flint are paired together to make an achromatic doublet lens that focuses blue and red wavelengths in the same image plane.

Spherical aberration is the undesirable consequence of having lenses figured with spherical surfaces, the only practical approach for lens manufacture. Parallel rays incident at central and peripheral locations on the lens are focused at different axial locations, so there is not a well-defined image plane and a point source of light at best focus appears as a spot surrounded by a bright halo or series of rings. For an extended object, the entire image is blurred, especially at the periphery. One common solution is to use a combination of positive and negative lenses of different thicknesses in a compound lens design. Lenses corrected for spherical aberration are intended for use under a specific set of working conditions. These include the coverslip thickness, the assumption that the focal plane is at or near the coverslip surface, the refractive index of the medium between the lens and coverslip, the wavelength of illumination, and others. Thus, users employing well-corrected lenses can unknowingly induce spherical aberration by using coverslips having the wrong thickness or refractive index. Special lenses are available with adjustable correction collars so that spherical aberration can be minimized for specimens distant from the coverslip, or when it is desirable to be able to use various immersion media (Brenner, 1994).

Coma refers to a streak of light with the shape of a comet s tail that appears to emanate from a focused spot at the periphery of an image. Coma occurs for object points that are off the optic axis that is, when object rays hit the lens obliquely. It is the most prominent off-axis aberration. Rays passing through the edge of the lens are focused closer to the optic axis than are rays that pass through the center of the lens, causing a point object to look like a comet with the tail extending toward the periphery of the field. Coma is greater for lenses with wider apertures. Correction for this aberration is made to accommodate the diameter of the object field for a given lens.

Astigmatism, like coma, is an off-axis aberration. Rays from an object point passing through the horizontal and vertical diameters of a lens are focused as a short streak at two different focal planes. The streaks appear as ellipses drawn out in horizontal and vertical directions at either side of best focus, where the focused image of a point appears as an extended circular patch. Off-axis astigmatism increases with increasing displacement of the object from the optic axis. Astigmatism is also caused by asymmetric lens curvature due to mistakes in manufacture or improper mounting of a lens in its barrel.

Curvature of field is another serious off-axis aberration. Field curvature indicates that the image plane is not flat, but has the shape of a concave spherical surface as seen from the objective. Different zones of the image can be brought into focus, but the whole image cannot be focused simultaneously on a flat surface as would be required for photography. Field curvature is corrected by the design of the objective and additionally by the tube or relay lens and sometimes the oculars.

Distortion is an aberration that causes the focus position of the object image to shift laterally in the image plane with increasing displacement of the object from the optic axis. The consequence of distortion is a nonlinear magnification in the image from the center to the periphery of the field. Depending on whether the gradient in magnification is increasing or decreasing, the aberration is termed pincushion or barrel distortion after the distorted appearance of a specimen with straight lines such as a grid or reticule with a pattern of squares or rectangles. Corrections are made as described for field curvature.

DESIGNS AND SPECIFICATIONS OF OBJECTIVE LENSES

Achromats are red-blue corrected (meaning for wavelengths at 656 and 486 nm). Spherical correction is for midspectrum yellow-green light at 540 nm. These objectives give satisfactory performance in white light and excellent performance in monochromatic light, and are quite suitable for low magnification work at 30 40× and lower. They are also much less expensive than more highly corrected lens designs (Fig. 4-9).

Fluorite or *semiapochromat* lenses contain elements made of fluorite or fluorspar (CaF_2) or synthetic lanthanum fluorite materials giving low color dispersion (Fig. 4-9). Corrections for color dispersion and curvature of field are easily applied. The combination of good color correction, extremely high transparency (including to near UV light) and high contrast makes them favorites for immunofluorescence microscopy, polarization and differential interference contrast (DIC) microscopy, and other forms of light microscopy. The maximum obtainable numerical aperture (NA) is about 1.3.

Apochromats are expensive, highly color-corrected designs suitable for color photography using white light (Fig. 4-9). These lenses are red-, green-, blue-, and dark blue corrected for color, and are corrected for spherical aberration at green and blue wavelengths. This design tends to suffer some curvature of field, but is corrected in *plan-apochromatic* lenses. The high degree of color correction makes them desirable for fluorescence microscopy, since various fluorescence wavelengths emitted from a multiple-stained specimen are accurately focused in the same image plane. It is also possible to obtain very large NAs (up to 1.4) with this lens design, making them desirable for low light applications such as dim fluorescent specimens. Newer designs are now transparent to near UV light, making them suitable for fluorescence microscopy involving UV-excitable dyes.

A summary of the characteristics of some commonly used objective lenses is provided in Table 4-1.

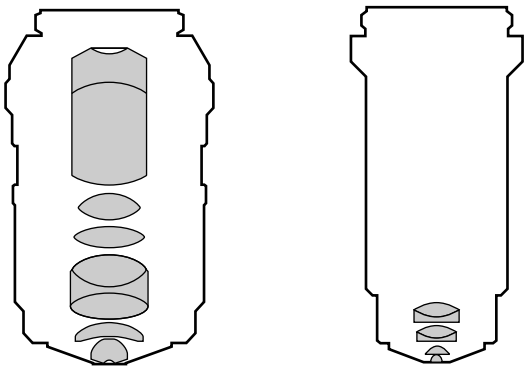


Figure 4-9

Objective lens designs. Two popular lenses for fluorescence microscopy are shown. Apochromatic lenses may contain 12 or more lens elements to give bright, flat images with excellent color correction across the visual spectrum. Fluorite lenses have fewer lens components and produce sharp, bright images. These lenses exhibit excellent color correction and transmit UV light.

TABLE 4-1. Characteristics of Selected Objective Lenses[a]

M	Type	Medium (n)	WD (mm)	NA	d_{min} (μm)	DOF (μm)	B
5	Achromat	1	9.9	0.12	2.80	38.19	0.1
10	Achromat	1	4.4	0.25	1.34	8.80	0.4
20	Achromat	1	0.53	0.45	0.75	2.72	1.0
25	Fluorite	1.515	0.21	0.8	0.42	1.30	6.6
40	Fluorite	1	0.5	0.75	0.45	0.98	2.0
40	Fluorite	1.515	0.2	1.3	0.26	0.49	17.9
60	Apochromat	1	0.15	0.95	0.35	0.61	2.3
60	Apochromat	1.515	0.09	1.4	0.24	0.43	10.7
100	Apochromat	1.515	0.09	1.4	0.24	0.43	3.8

[a]The magnification (M), type of lens design, refractive index (n) of the intervening medium (air or immersion oil), working distance (WD), numerical aperture (NA), minimum resolvable distance (d), depth of field (DOF), and brightness (B) are indicated. Terms are calculated as: wave-optical depth of field, $n\lambda/NA^2$; brightness in epi-illumination mode, $10^4\ NA^4/M^2$. Resolution and depth of field are discussed in Chapter 6.

Special Lens Designs

Other performance characteristics such as working distance, immersion design, and UV transparency are optimized in special lens designs:

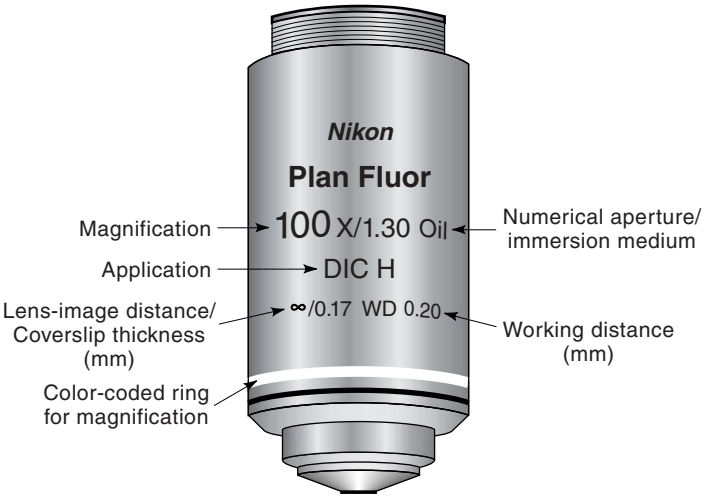
Long working distance lenses allow focusing through thick substrates (microscope slides, culture dishes) or permit the introduction of devices such as micropipettes between the specimen and the lens. The *working distance* is the distance between the surface of the front lens element of the lens and the surface of the coverslip. In contrast, conventional oil immersion lenses have short working distances that may be as small as 60 μm.

Multi-immersion and *water immersion lenses* are used for examination of specimens covered with medium in a well chamber, and in some cases, can be placed directly on the surface of a specimen such as a slice of tissue. These lenses usually contain a focusable lens element to correct for spherical aberration.

UV lenses made of quartz and other UV-transparent materials support imaging in the near-UV and visible range (240 700 nm).

Markings on the Barrel of a Lens

The engraved markings on the barrel of the objective describe the lens type, magnification, numerical aperture, required coverslip thickness if applicable, and type of immersion medium (Fig. 4-10).



Mag.	1X	2X	4X	10X	20X	40X	50X	60X	100X
Color code	Black	Gray	Red	Yellow	Green	Light blue	Light blue	Dark blue	White

Figure 4-10

Key for interpreting the markings on the barrel of an objective lens. Markings on the lens barrel indicate the type of lens and correction, initial magnification, immersion medium, numerical aperture, lens-image distance, and required coverglass thickness. For quick reference, the color-coded ring, near the thread, denotes the initial magnification, while the color-coded ring near the front lens denotes the type of immersion medium (black-immersion oil, white-water, orange-glycerin, yellow-methylene iodide, red-multi-immersion).

Image Brightness

Notice in Table 4-1 that the ratio of numerical aperture to magnification determines the light-gathering power of a lens and hence the image *brightness B*. *B* is defined through the relationships

$$B \propto (\mathrm{NA/M})^2 \text{ (transillumination mode)}$$

and

$$B \propto (\mathrm{NA}^4/\mathrm{M}^2) \text{ (epi-illumination mode)}$$

where M is the magnification, and NA is the numerical aperture, a geometric parameter related to the light-gathering power of an objective lens. Numerical aperture as a primary determinant of the spatial resolution of an objective is discussed in Chapters 5 and 6. The values for magnification and NA are indicated on the lens barrel of the objective. A 60×/1.4 NA apochromatic objective lens gives among the brightest images, and because its image is color corrected across the entire visual spectrum and is flat and substantially

free of common aberrations, it is popular in fluorescence microscopy. The 40×/1.3 NA fluorite lens is significantly brighter, but is less well corrected.

CONDENSERS

Imaging performance by a microscope depends not only on the objective lens but also on the light delivery system, which includes the illuminator and its collector lens, and of particular importance, the condenser. High-performance condensers are corrected for chromatic and spherical aberrations and curvature of the focal plane (field curvature). However, most of these aberrations are still apparent when using the Abbe condenser, a very common condenser that is based on a two-lens design (Fig. 4-11). The three-lens aplanatic condenser (indicating correction for spherical aberration and field curvature) is superior, but still exhibits chromatic aberration. The highly corrected achromatic-aplanatic condenser has five lenses including two achromatic doublet lenses, provides NAs up to 1.4, and is essential for imaging fine details using immersion-type objectives. These condensers are corrected for chromatic aberration at red and blue wavelengths, spherical aberration at 550 nm, and field curvature. Such a condenser can be used dry for numerical apertures up to ~0.9, but requires immersion medium for higher NA values, although the condenser is commonly used dry even with oil immersion objectives. Note, however, that for maximal resolution, the NA of the condenser must be equal to the NA of the objective, which requires that both the condenser and the objective should be oiled.

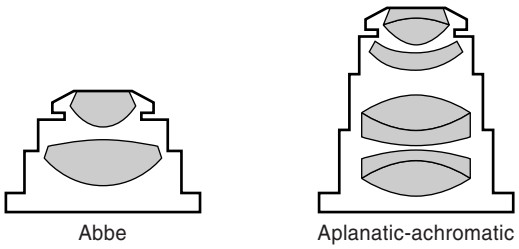


Figure 4-11

Two common microscope condensers. The Abbe condenser contains two achromatic doublet lenses and gives very good performance for dry lenses of low to medium power. The achromatic-aplanatic condenser is useful for lenses with NA > 0.5, and is essential for oil immersion lenses with high numerical apertures. For low NA performance, the top element of this condenser can be removed. This condenser focuses light in a flat focal plane and is highly corrected for the significant lens aberrations.

OCULARS

Oculars or eyepieces are needed to magnify and view the image produced by the objective. To make optimal use of the resolution afforded by the objective, an overall magnification equal to 500 1000 times the NA of the objective lens is required. More magnification than this gives empty magnification, and the image appears highly

magnified but blurry. For most applications, 10× eyepieces work well. When higher magnifications are required for a specific objective, a magnifying booster lens (in Zeiss microscopes, an Optovar lens magnification system) can be rotated into the optical path. Alternatively, a different set of higher-magnification eyepieces can be employed. If the eyepiece is examined when the microscope is focused and the lamp turned on, a bright disk can be seen floating in space a few millimeters outside the eyepiece. The disk is called the *exit pupil* or *Ramsden disk* and represents the aperture at the back focal plane of the objective lens. When viewing a focused specimen, the exit pupil of the eyepiece will be found to be coincident with the *entrance pupil* of the eye, an adjustment that occurs automatically as we focus the specimen.

Eyepiece specifications are engraved on the lens barrel to indicate their magnifying power and field of view. An eyepiece engraved 10×/20 indicates a 10× magnification and 20 mm diameter field of view. The field of view marking also provides a quick reference for determining the diameter of the field in the specimen plane as seen with a given objective. For example, when combined with a 100× objective lens, this eyepiece would give 20 mm/100, or 200 μm for the diameter of the visible object field. Other special design features of the ocular are designated by letter codes, the most common of which indicate high eyepoint (distance between ocular surface and Ramsden disk) for glasses wearers, additional corrections for color and flatness of field, and wide field or wide angle of view. Eyepieces also come with focusable and nonfocusable eye lenses. At least one focusable eyepiece should be included on the microscope to allow parfocal adjustment of the optics so that the same focal plane examined by the eye will be in focus on a camera mounted on the microscope. Oculars are based around a general design containing two achromatic doublet lenses (the field and eye lenses) and a *field stop,* a raised ridge or flange along the inside wall of the ocular that marks the site of the intermediate image plane. In oculars of so-called Huygenian design, the field stop and image plane are located between the field and eyepiece lenses; in Ramsden designs, the focal plane and field stop are located in front of the field lens below the eyepiece. To use an eyepiece reticule, the eyepiece is unscrewed and the reticule is placed in the image plane and rests on the flange comprising the field stop.

MICROSCOPE SLIDES AND COVERSLIPS

Many objectives are designed to be used with standard (1.1 mm thick) glass slides and coverslips of a certain thickness, usually 0.17 mm, which corresponds to thickness grade 1.5. Other coverslip thicknesses induce spherical aberration and give poorer performance, especially when used with high, dry lenses above 40×. For lenses with an NA < 0.4, coverslip thickness is not particularly important. *Remember the thickness of your slides and coverslips counts!* Refer to the following chart when ordering coverslips:

Grade Number	Thickness (mm)
0	0.083 0.13
1	0.13 0.16
1.5	0.16 0.19 (standard)
2	0.19 0.25

THE CARE AND CLEANING OF OPTICS

Maintenance and care are required to protect an expensive optical instrument and to guarantee that optimal high-contrast images will be obtained from it. Neglect, such as not removing immersion oil, forgetting to cover open ports and apertures, or accidentally twisting or dropping an objective lens can ruin optical performance. Even if the microscope is left unused but unprotected on the lab bench, image quality can deteriorate rapidly due to the accumulation of dust from the air. James (1976) and Inoué and Spring (1997) provide detailed descriptions on the effect of dirt on the microscope image and the cleaning of optical surfaces. Following are a few tips for maintaining the performance and image quality of your microscope.

Dust

Keep the microscope protected with a plastic or cloth cover. Wipe dust off the microscope body and stage with a damp cloth. Keep the objective lens turret spotless and free of dust, immersion oil, spilled culture medium, and salt solutions. Hardened thread grease, or additionally on inverted microscopes, dried immersion oil, buffers, and media can weld objectives onto the rotating objective turret, making them difficult to remove or exchange. If an objective is frozen fast, place a drop of water (if salts) or oil-penetrating agent (if oil) at the lens-turret interface to loosen the material before trying to remove the objective. Keep all openings covered with caps so that dust cannot enter the microscope and soil inaccessible lenses, mirrors,and prisms in the microscope body. Make use of the plastic caps designed to cover all objective ports, eyepiece sleeves, and camera ports that are unoccupied and empty. The internal optical pathway should always be completely protected from airborne dust.

Immersion Oil

When finished with an observing session, gently wipe off and clean away excess oil with a high-quality lens tissue and then clean the lens surface with an agent designed for cleaning microscope optics. Immersion oil is a slow-acting solvent that can weaken the cementing compounds that act as a seal between the front lens element and the metal lens cap of the objective. Residual oil should be removed with a lens tissue wetted with a mild lens cleaner such as the solution sold by Edmund Scientific Company (Barrington, New Jersey). Commercial glass cleaners such as Sparkle and Windex are also effective in removing immersion oil, but these generally contain an acid or base that has the potential to erode the antireflection coating on the front lens element and should be used only if a neutral cleaner is not available. For more tenacious deposits, try, in order, ethanol or ethyl ether. However, do not use toluene or benzene, as these solvents used over time will eventually dissolve the front lens sealing compounds. Generally, it is advisable to remove immersion oil and contaminating liquids with the objective lens still mounted on the microscope, as this will avoid mishandling or dropping, the worst accidents that can befall a valuable lens.

Scratches and Abrasions

Never wipe the surfaces of objectives with papers or cloths that are not certified to be free of microscopic abrasives. All objectives contain an exposed optical surface that must be