

Excerpt from Chapter 1

C H A P T E R 1

Light Microscopy

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I N T R O D U C T I O N

The light microscope, often the symbol of research and scientific discovery, has evolved over the last 350 years from Antonie van Leeuwenhoek's simple magnifier to the more sophisticated instruments of today. Studies of biological structures and processes on both fixed and live specimens have advanced light microscopy into an indispensable tool for cell and molecular biologists.

This chapter provides an overview of light microscopy, including the principles and equipment as well as practical guidelines for achieving the best results. It will not replace the specific instructions provided for a given microscope. For more in-depth information, see the Reference list at the end of this chapter. Other aspects of and systems for microscopy are discussed elsewhere in this manual, for example, confocal microscopy (Chapter 2), preparation of cells and tissues for microscopy (Chapter 4), and scanning and transmission electron microscopy (Chapters 19–21).

The light microscope creates a magnified, detailed image of seemingly invisible objects or specimens, based on the principles of transmission, absorption, diffraction, and refraction of light waves. The various types of microscopes produce images of objects employing different strategies. In all instances (e.g., bright field, phase contrast, and fluorescence), production of a clear and informative image is dependent on the magnification of the object, its contrast with respect to its internal or external surroundings, and the ability to resolve structural details.

In the microscope, objects are enlarged or magnified with a convex lens that bends light rays by refraction. Diverging rays from points within the object (object points) are made to converge behind the convex lens and cross over each other to form image points (i.e., a focused image). The distance of the object from the lens divided into the distance of the focused image from the lens determines the magnification. In the compound microscope there are usually two magnifying systems in tandem, one defined by the objective and the other defined by the eyepiece. Another important property of a lens is its focal length, which is defined by the distance from the lens at which parallel rays of light are focused.

The visibility of the magnified object depends on contrast and resolution. In general, the contrast or differences in light intensity between an object and its background or surroundings render the object distinct. For colorless specimens, as is the case for most biological material, contrast is achieved in various ways. The object itself or selected portions of it may be stained, thus reducing the amplitude of certain light waves passing through the stained areas. However, this usually requires the killing or fixation and staining of cells. Such stained specimens are typically observed using bright-field microscopy (see p. 16). Alternatively, several kinds of specially developed microscope systems may be used that can enhance the contrast of live specimens. These systems, described in this section, include the following:

- Oblique illumination
- Dark field

- Phase contrast
- Polarized light
- Nomarski or differential interference contrast
- Reflection interference
- Fluorescence
- Video microscopy

Table 1.1 summarizes these various systems and their respective applications and Figure 1.1 illustrates the visualization of tissue (stained or unstained) using either bright-field or phase-contrast optics. The degree of structural detail revealed within a cell studied in the light microscope is determined by the “resolving power” of the entire microscope lens system. Resolution is defined as the limiting distance between two points at which they are perceived as distinct from one another. Superior quality objective lenses with high resolving power are critical for producing clear and precise images. The resolving power of a microscope also depends to a great extent on the condenser that delivers light to the specimen. These considerations are discussed in greater detail below.

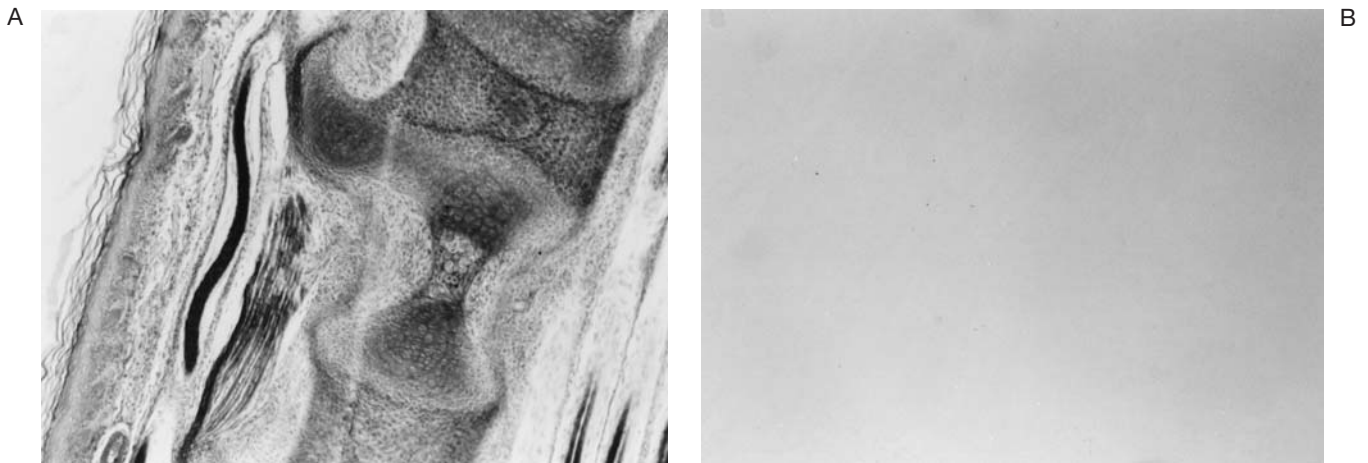
KÖHLER ILLUMINATION: PRINCIPLES OF LIGHT MICROSCOPY AND FACTORS RELATED TO RESOLUTION

The light microscope is a critical tool in studies ranging from subcellular structure and function to pathology, embryology, gene expression, and gene mapping. For many of these purposes, the limits of resolution of the light microscope must be exploited to the fullest potential. For optimal results in a given application, the microscope should be equipped with high-quality optics (objectives, eyepieces, and condensers), be precisely aligned, and make use of the appropriate light sources, filters, and contrast enhancement devices (e.g., phase contrast).

The first and most critical step in setting up a microscope for optimal resolution involves the mechanics of Köhler illumination. Köhler illumination was first described in 1893 by August

TABLE 1.1 A variety of microscopic techniques exploit light properties to enhance contrast

Contrast mode	Mechanism	Comments
Bright field	contrast depends on light absorption	usually used in conjunction with histological stains to boost contrast
Phase contrast	converts optical path differences to intensity differences	contrast proportional to local “phase dense” objects including mitochondria, lysosomes, chromosomes, nucleoli, and stress fibers
Differential interference contrast (DIC)	converts rate of change of optical path across specimen	cell and organelle edges where optical path abruptly changes stand out in relief
Dark field	scattered light observed	produces images of cell and organelle edges
Interference reflection (IRM)	contrast depends on interference between closely spaced surfaces	used to visualize zones of cell-substratum contact in cultured cells
Polarization	detects birefringence caused by supramolecular organization below optical resolution	used to study oriented arrays such as cytoskeletal structures (e.g., microtubules in the mitotic apparatus and stress fibers); also used to study membranes
Fluorescence	contrast depends on absorption of light by fluorophore and its quantum yield	limited only by appropriate fluorescent probes

**FIGURE 1.1**

(A) Bright-field microscope photomicrograph of a section of a paraffin-embedded late-stage mouse embryo. The section is through the proximal region of the tail. It has been deparaffinized and stained with hematoxylin and eosin. The skin is located on the left side where the stratum corneum is evident at the surface. Many cell types are evident and are readily observed because of the color-generated contrast. (B) A section that has been prepared exactly as in A through the same region of a mouse embryo. The only difference is that the section has not been stained. The skin is located in the same position at the left. The section and the various tissue cells are essentially invisible (without the color contrast generated by staining) when the microscope is arranged for optimal bright field with Köhler illumination (see below). (C) The same section as in B, but observed with phase-contrast optics (see

below). Even in the absence of color-generated contrast, the various regions of the tissue such as the stratum corneum of the skin (on the left side) are obvious. (Photos provided by R.D. Goldman, Northwestern University, and H.E. Keller, Carl Zeiss, Inc.)

Köhler, a young zoologist in Giessen, Germany, who later joined Carl Zeiss. It provides efficient, bright, and even illumination in the specimen field, minimizes internal stray light, and allows for control of contrast and depth.

A look at the components of the microscope and at the path of light rays helps in understanding the underlying principle and assists in the alignment of the instrument for best performance. The basic components and image locations of the typical modern microscope, from light source to final image formation in either the eye, camera, or other detector, are displayed in Figure 1.2. The two geometric optical ray paths, the imaging and illuminating paths, shown in Figure 1.3, are depicted for Köhler illumination in both transmitted and reflected or incident light systems.

For the illumination ray path, the angle of radiation is depicted from a single point on the light source (Fig. 1.3A, L1) that is received by the lamp collector, which then images this point from the source onto the front focal plane of the condenser (location of condenser aperture diaphragm; see L2). From here, the source point is projected by the condenser to infinity and evenly illuminates the specimen. The objective receives the parallel, infinity-projected source rays and forms an image of the source in its back focal plane (exit pupil; L3). This image of the light source is then transferred to the exit pupil of the eyepiece, also called the eyepoint (L4). Therefore, from original light source to eyepoint, there are four images of the light source (“source-conjugated” images). The final source image in the exit pupil of the microscope eyepiece is located in the same plane as the entrance pupil of the observer’s eye.

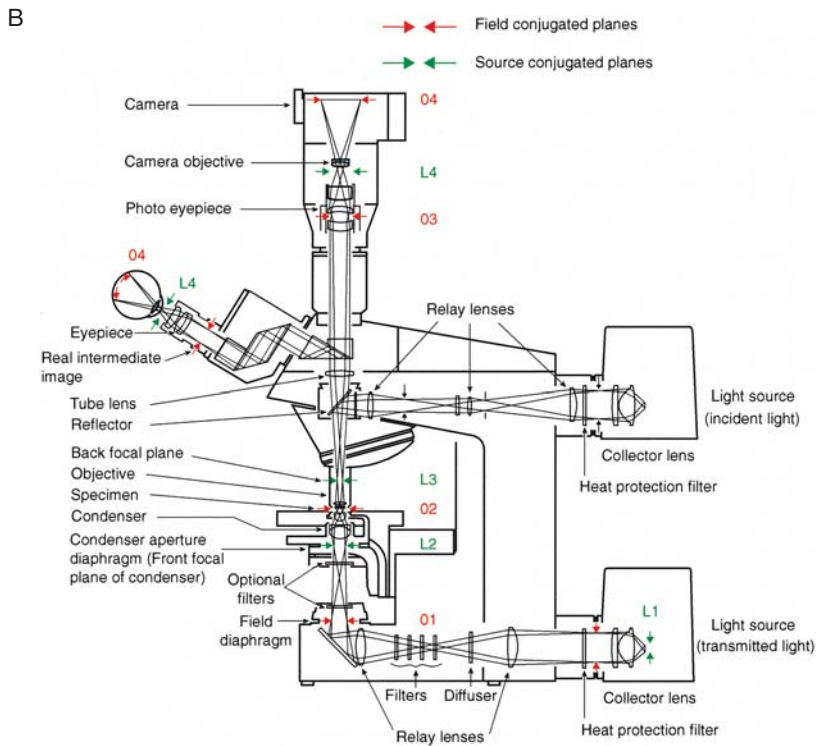
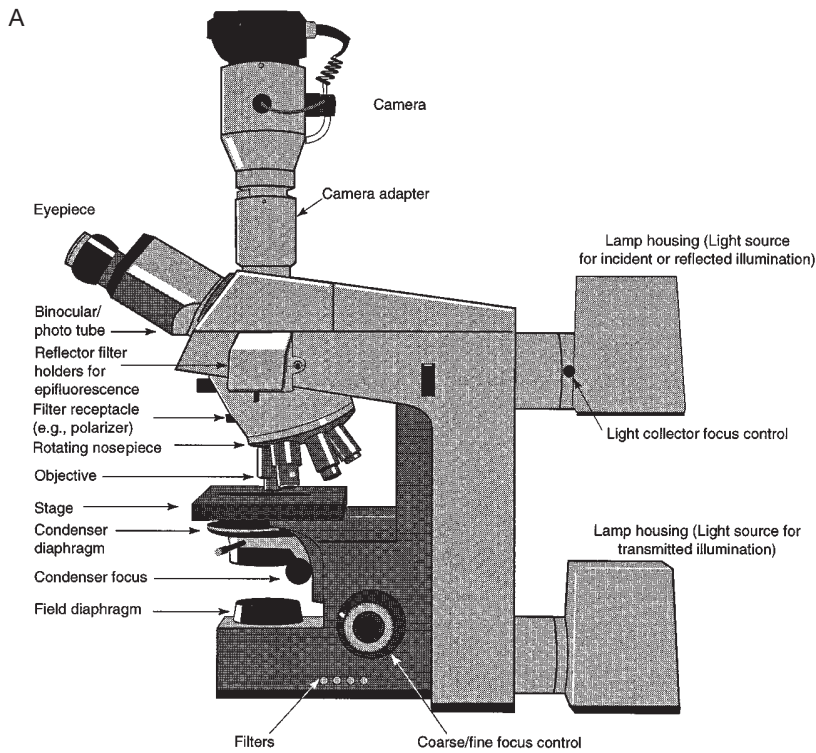
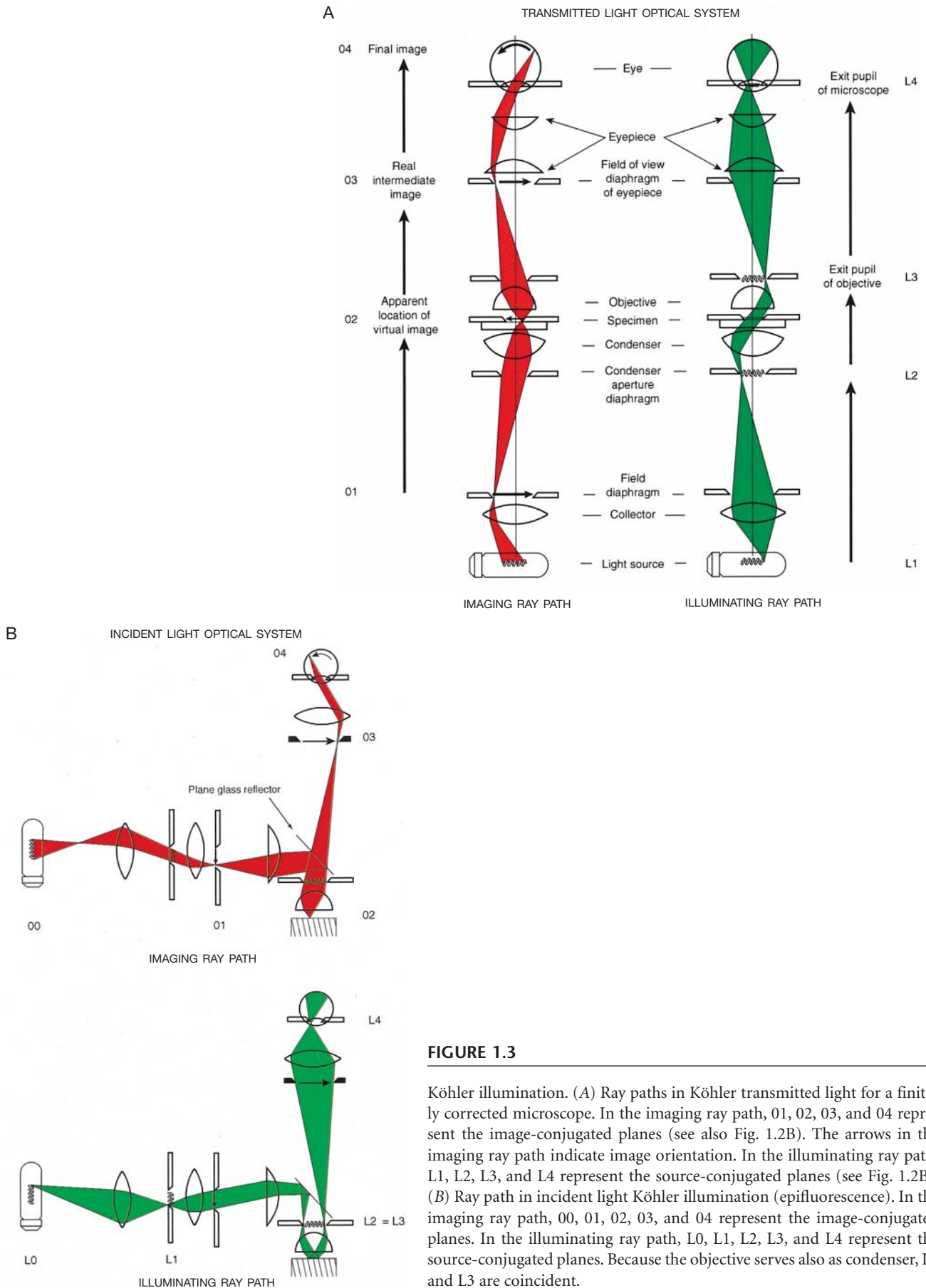


FIGURE 1.2

The light microscope. (A) Basic components of the light microscope arranged for transmitted and incident illumination. (B) Diagrammatic representation of the transmitted and incident light paths. Light from the source to final image either in the camera or on the human retina is shown. Four field-conjugated planes (represented by red arrows) and four source-conjugated planes (represented by green arrows) are within the optical system of the microscope. The last field-conjugated plane is the final image in the camera or on the retina. (For definitions of 01, 02, 03, 04 and L1, L2, L3, L4, see Fig. 1.3A.)



The imaging path shows four “image-conjugated” planes, starting with the luminous field diaphragm (field stop; Fig. 1.3A, 01), next the specimen (02), and then the real intermediate image formed by the objective (03). This real intermediate image is located in the focal plane of the eyepiece, from which it is projected into infinity and received by the relaxed and infinity-adjusted eye of the observer (04). Thus, from field stop to final image, there are again four “image- or specimen-conjugated” planes.

In visual observation through the microscope, an enlarged virtual image apparently suspended in space can be seen. This virtual image is formed by extension of the image-forming rays from the eyepiece and appears ~10 inches below the eyepiece, just below the microscope stage. For further details of this phenomenon, see p. 11, The Finite versus the Infinity-corrected Objective.

In the incident light ray path (e.g., the path used in epifluorescence; see Figs. 1.2 and 1.3B), the only difference is the addition of a “source-conjugated” plane in which an aperture diaphragm permits control of the illumination aperture (L1) outside of the imaging ray path. This is necessary because in incident light, the objective becomes its own condenser.

The advantages of Köhler illumination are listed below.

- Only the specimen area viewed by a given objective/eyepiece combination is illuminated; no stray light or “noise” is generated inside the microscope.
- Even, uniform illumination of the specimen area is achieved by distributing the energy of each source point over the full field.
- Full control of the illumination aperture (condenser iris diaphragm; see below) provides for best resolution, best contrast, and optimal depth of field.

Adjusting the microscope for Köhler illumination is explained in the section on bright-field microscopy (see p. 16).

The Objective

Although illumination of the specimen is important, the *microscope objective* is the single most critical component of the microscope. Its properties largely determine depth of focus, resolution, and contrast of the specimen. The eyepiece and/or other so-called transfer optical devices simply magnify the resolved detail in the real intermediate image formed by the objective, which permits a detector (eye or camera) to record what has been resolved.

To understand the resolution limits of an objective, it is helpful to review the “wave concept” of light. Electric and magnetic vectors oscillate as a sine wave around the direction of propagation, defined by the light “ray.” The wavelength in a vacuum or the frequency of light determines the color of light, whereas the amplitude (height) of the wave determines its intensity. Thus, the wavelengths of light in the visible spectrum range from about 400 nm for blue light to about 700 nm for red light; white light represents a mixture of all wavelengths. With light that is “coherent” or coming from the same source point, these waves can constructively or destructively interfere with each other. Wavefronts traveling at the speed of light are diffracted when they strike an object in their path or pass through a very small opening. When this occurs, Huygens’ “wavelets,” contained in the wavefront, form new spherical wavefronts at the obstruction. Rays of light are also bent by refraction, which occurs when light encounters an object of different density at an angle (as when light passes from air into water). The ratio of the speed of light traveling through a vacuum to its velocity through a particular object or medium is known as the refractive index.

Diffraction and Resolution: The Rayleigh Limit and the Airy Disk

Diffraction on the objective’s aperture converts infinitely small self-luminous points within an object into so-called Airy disks within the image. The Airy disk is a bright disk, surrounded by concentric rings, that has a negative impact on resolution (see Fig. 1.4). The diameter (D) of this disk

translated into the object itself is described as

$$D = \frac{1.22 \times \lambda}{n \sin \alpha} \quad \text{or} \quad D = \frac{1.22 \times \lambda}{(\text{NA})}$$

where λ = wavelength of light

α = half of collection angle of objective

n = refractive index of medium between object and objective

NA = numerical aperture = $n \sin \alpha$

The numerical aperture (NA) of the lens is a function of the light-collecting ability of the lens, or a measure of the “cone of light” entering the objective from a fixed object distance. The refractive index (n) of a material represents the optical density (e.g., the speed of propagation of light rays) between materials such as glass and air. Typically, the space between the objective lens and the specimen is air, which has a refractive index of about 1. Special lenses called oil immersion lenses function with oil rather than air in this space. Immersion oil has a refractive index of about 1.5. Therefore, with oil immersion lenses, resolution is increased (see below). Furthermore, because glass and immersion oil have the same refractive index, no light is lost through reflection from the surface of the lens and the cover glass. Therefore, the higher the refractive index, the smaller the resolvable distance between two points and the better the resolution. This can be explained as follows. In Figure 1.4, the dark ring around the central bright disk represents a zone of destructively interfering diffracted wavefronts and defines the diameter of the disk. Using this diameter, Rayleigh set the limit for the smallest resolvable distance between two points at

$$d = \frac{1.22 \times \lambda}{2 \times \text{NA}}$$

This resolution limit for self-luminous objects (fluorescence) can be exceeded somewhat by confocal microscopes (see Chapter 2) and by electronic image processing, but it provides a good rule of thumb for visual observation and photomicrography.

The Abbé Limit of Resolution for Illuminated Objects

Diffraction also takes place within the object when it is illuminated in the microscope. Constructive interference between two diffracted wavefronts from adjacent points within the object, separated by a distance d (Fig. 1.5A), generates new plane waves (diffraction orders) at the diffraction angle (Fig. 1.5B). The following relationship develops from this phenomenon:

$$\text{NA} = \frac{\lambda}{d} \quad \text{or} \quad d = \frac{\lambda}{\text{NA}}$$

This relationship assumes illumination parallel to the optical axis.

Abbé postulated that for two points to be resolved, at least two adjacent orders of diffracted light (see Fig. 1.5) produced by their spacing d must be collected by the objective. The NA of an objective directly determines its ability to collect diffracted wavefronts that emanate from the object.

Either diffracted and nondiffracted wavefronts or two orders of diffracted light are required to resolve structural detail. It is the constructive or destructive interference between the two in the intermediate image plane that permits the specimen detail to be resolved (see Fig. 1.6).

If a condenser is placed in the optical path and is to illuminate fully the objective's aperture, it effectively doubles the diffraction angle an objective can receive, especially when compared to the situation without a condenser (see Fig. 1.7).

Then the point-to-point resolution becomes

$$\frac{\lambda}{\text{NA}_{\text{Obj.}} + \text{NA}_{\text{Cond.}}}$$

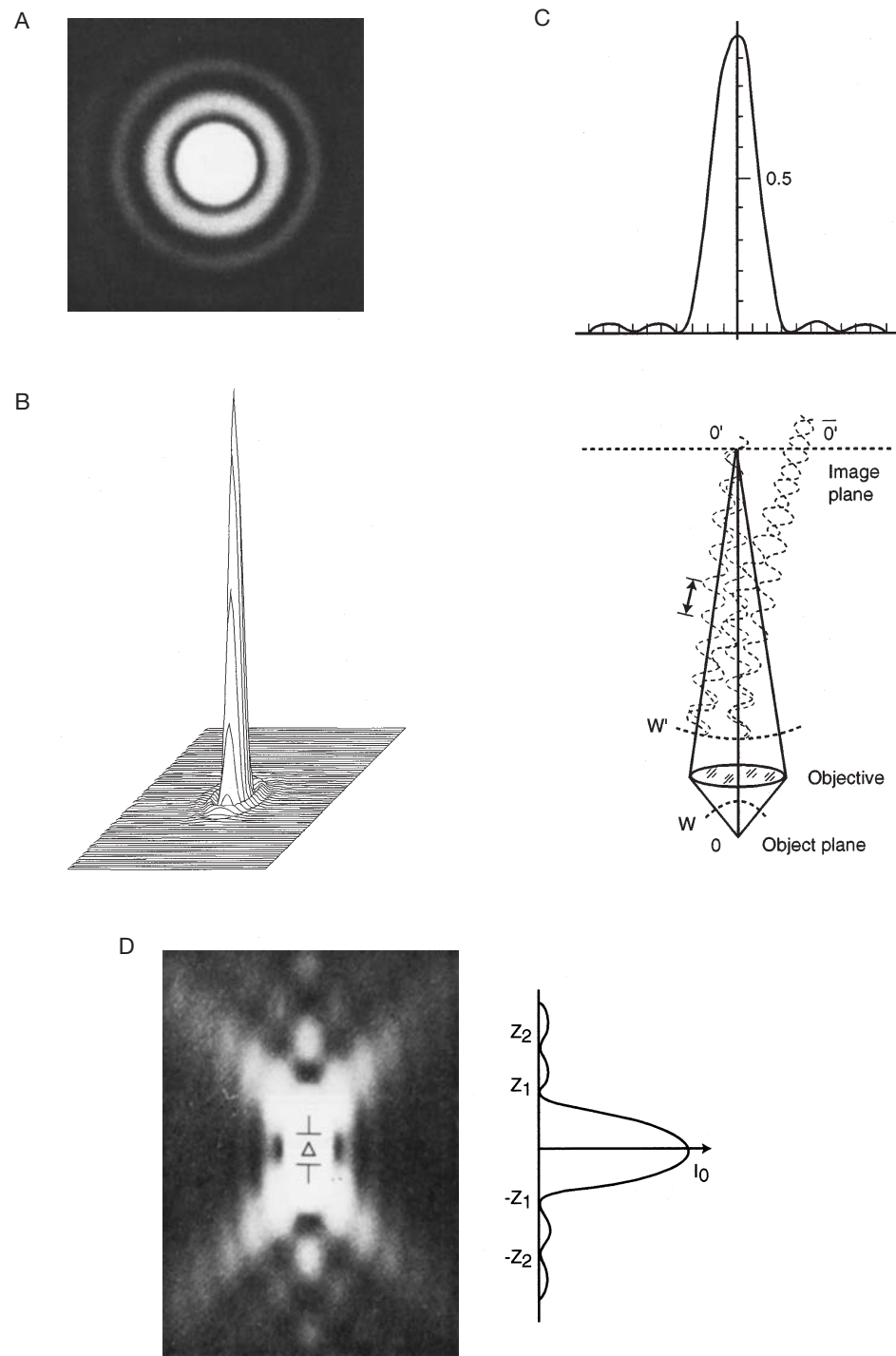


FIGURE 1.4

Generation of the Airy disk. It is the Airy disk phenomenon that limits resolution in light microscopy. With the highest numerical aperture objective (e.g., 1.4), the Airy disk phenomenon limits resolution to $\sim 0.2\ \mu\text{m}$. (A) Micrograph of the Airy disk generated by a $0.2\text{-}\mu\text{m}$ pinhole. This represents a cross-sectional view through the Airy body in the image plane. (B) Light intensity distribution across an Airy disk. (C) Diagrammatic representation of wavefronts diffracted by the objective aperture and their constructive ($0'$) and destructive ($\bar{0}'$) interference. This results in the dark and light concentric rings seen in A. The light intensity plot across the image plane is seen above the dotted line. (D) This diagram represents a section through the Airy body in the optical (Z) axis (perpendicular to the image plane), and its intensity distribution.

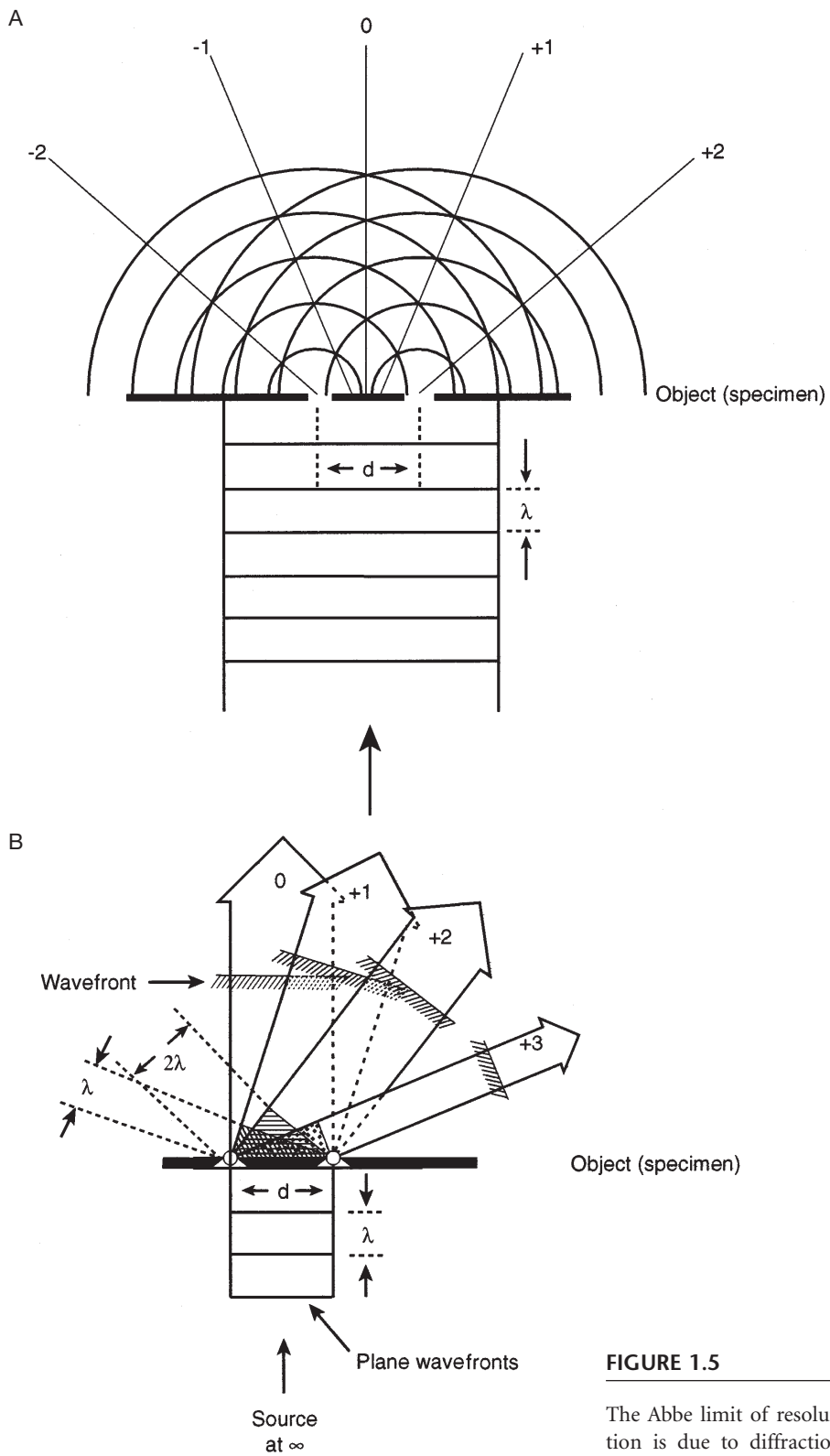


FIGURE 1.5

The Abbe limit of resolution. The limit of resolution is due to diffraction in the object and the numerical aperture of the lens. (A) Diffraction orders (+1, +2, and -1, -2) generated by two points (slits) separated by d . (B) Three diffraction orders (+1, +2, and +3) shown in the plus direction only.

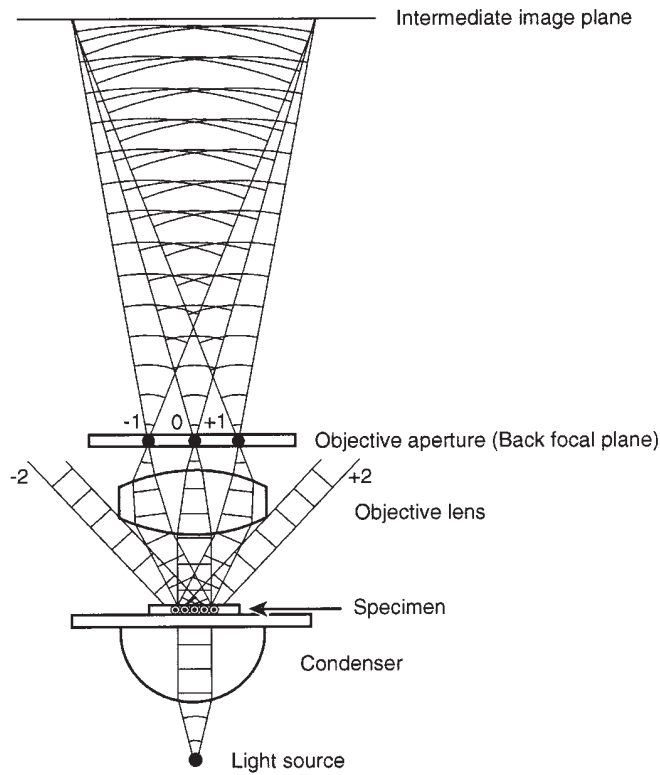


FIGURE 1.6

Nondiffracted (0 order) and diffracted (+1 and -1, etc., orders) wavefronts interfere in the image plane to resolve structural spacing in the specimen. Either 0 and +1 or 0 and -1 are the two orders of diffraction required to resolve the detail within the object (specimen) in the intermediate image plane. Note that the diffracted orders +2 and -2 do not contribute to the resolution of structures within the object (d in Fig. 1.5).

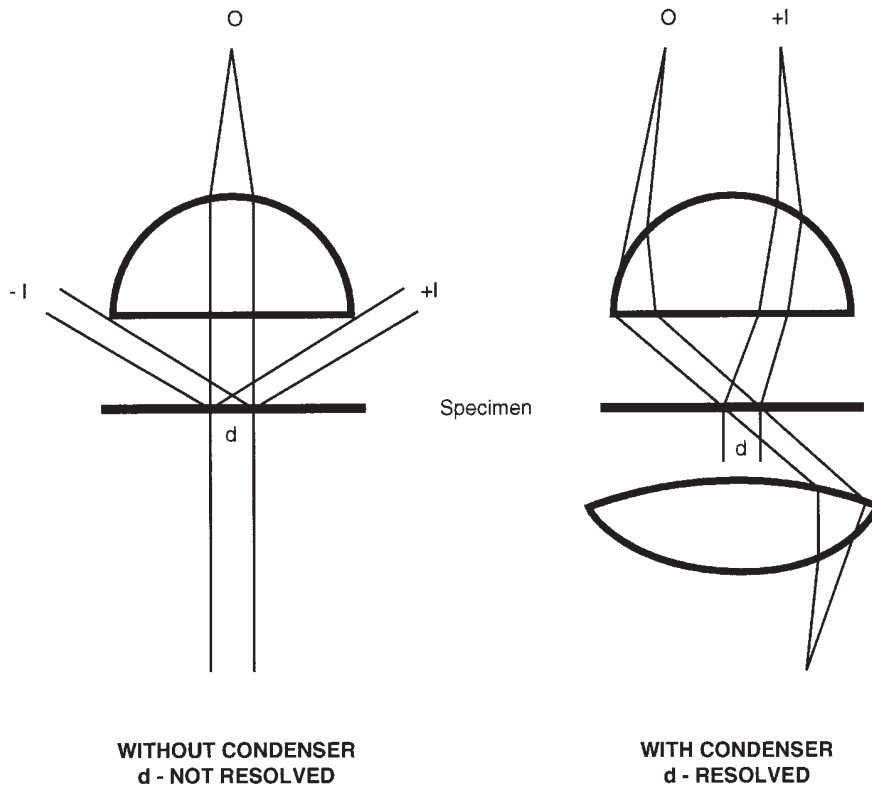


FIGURE 1.7

How the condenser enhances resolution. (Left) Without condenser, the first order of diffraction (-1 and +1) for spacing d is not collected by the objective. (Right) With condenser, the zeroth order enters obliquely and the first order (+1) of diffraction is collected.

Other Parameters of the Microscope Objective

The use of high-quality objective lenses is necessary for obtaining the maximum amount of information while studying a specimen. The ideal objective lens system must have both a high resolving power and effective correction for spherical and chromatic lens aberrations. Spherical aberration is caused by the spherically curved surfaces of the lens. This latter aberration can also be due to the use of cover glasses of the incorrect thickness or refractive index mismatch (e.g., the use of improper immersion oil or aqueous medium in which cells are immersed).

Microscopes used in the early 1800s, with either single or compound lens systems, were unable to resolve fine detail because of chromatic aberration: White light, broken up into its constituent colors, resulted in halos of colors around small objects. After about 1820, simple achromatic objective lenses were developed that corrected for spherical aberrations in the middle of the light spectrum while imaging blue and red into the same plane. The more recently developed and more complex Plan-achromatic objectives produce much less curvature of field aberration than ordinary achromatic objectives (see Table 1.2). The sophisticated Plan-apochromatic objectives are complex flat-field objectives that provide the best correction for all aberrations. From Achromats to Fluorites and Plan-apochromats, a wide range of basic performance criteria is always clearly marked on the body tube of objectives (Fig. 1.8). These include the magnification, the NA, finite or infinity designed, immersion properties (e.g., oil), correction collar (to compensate for differences in cover-glass thickness), Ph (phase contrast) or Pol (strain-free for polarized light), and occasionally an iris diaphragm to reduce the NA as required for dark-field microscopy and in instances where there is too much light for fluorescence microscopy (see Fig. 1.8). All of these properties should be understood in order to achieve the maximum resolution of a given lens.

The light microscope is typically equipped with 10× eyepieces. A low-power 10× or 20× dry objective is used to scan and locate the object or specimen (e.g., cells or chromosomes), an intermediate-power 40× to 63× objective is used to gather more detailed information, and a high-power 100× oil immersion objective provides maximum analysis of detail within a given specimen.

The objective magnification times the eyepiece magnification times possible magnification changers (e.g., optovars and projection lenses) results in the total magnification. For visual observation, this total magnification should not exceed 500–1000× the NA (useful magnification range). Below 500× NA, the eye will not be able to see resolved detail in the image; above 1000× NA, “empty magnification” develops, resulting in blurred images.

The Finite versus the Infinity-corrected Objective

The finitely corrected objective directly projects a real intermediate image within the microscope (see Fig. 1.3A), whereas the infinity-corrected objective sends this image to infinity, requiring a tube lens to form the intermediate image. The direct availability of “infinity space” offers advantages whenever elements, such as reflectors, DIC prisms, color filters, and filters composed of polaroid that are used as analyzers or compensators for polarized light and DIC optical systems,

TABLE 1.2 Common objective descriptions

Achromats	corrects chromatic aberration for blue and red wavelengths; also corrects spherical aberration for green
Fluorites	corrects chromatic aberration for blue and red wavelengths; also corrects spherical aberration for two colors
Apochromats	corrects chromatic aberration for blue, green, and red wavelengths; also corrects spherical aberration for two colors
Plan	corrected to provide flat field



FIGURE 1.8

Lenses with performance criteria. Typical lenses with their properties etched into the metal body tubes. (Photo provided by H.E. Keller, Carl Zeiss, Inc.)

need to be inserted into the observation beam path. The use of these infinity-corrected lenses reduces the number of lenses required in the optical path (e.g., making telan lenses superfluous; see Fig. 1.9), thereby yielding more light and fewer internal reflections, limiting lens aberrations, and enhancing contrast. In these cases, no axial or lateral image shift (resulting in image deterioration) occurs, as long as the components of these elements are plane parallel (Fig. 1.9).

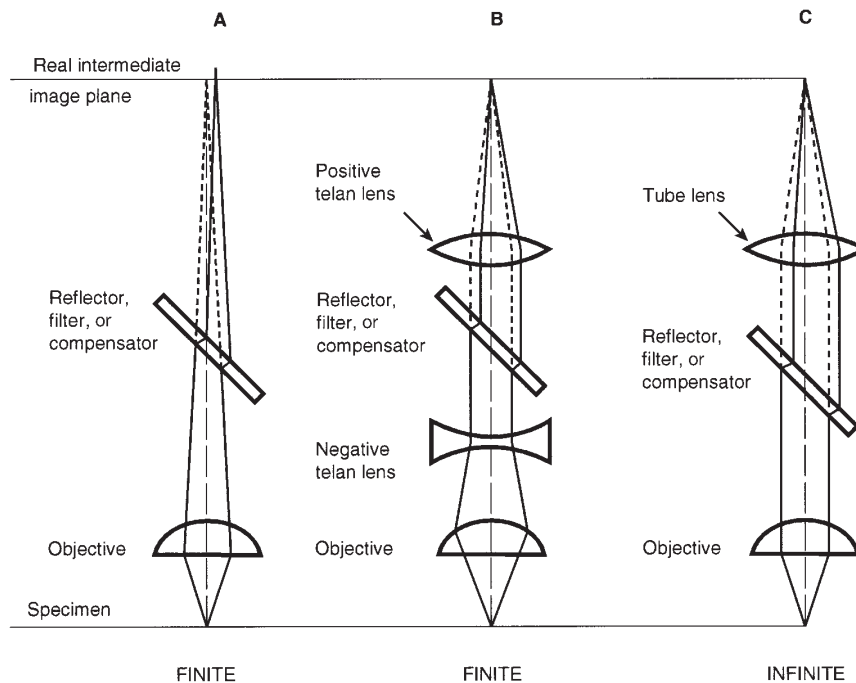


FIGURE 1.9

Benefits of infinity space. (A) Insertion of reflector or filter causes lateral and axial shift. (B) Two telan lenses generate infinity space to eliminate shift. (C) Objective directly provides infinity space.

More about Aberrations

“Refractive” optics can have wavelength-independent monochromatic aberrations as well as chromatic deficiencies (Figs. 1.10–1.14). Among the monochromatic aberrations are flatness of field, astigmatism, coma (radial distortion of the image point), and distortion. Chromatic aberrations are composed of longitudinal as well as lateral chromatic distortions. Of all of the possible aberrations, spherical aberration, which has a monochromatic as well as a chromatic component, is the most critical (see Fig. 1.10). Even the most highly corrected Plan-apochromat can render fuzzy images because of spherical aberration if coverslip thickness, immersion medium, mounting medium, and tube length, or the axial location of the intermediate image, do not meet the objective’s design specifications. Therefore, it is essential to provide conditions that meet the specifications of an objective.

Dry objectives of high NA are designed for use with cover glasses of 0.17-mm thickness (#1.5) to render optimal images. Bear in mind that cover-glass tolerances vary greatly for different optical systems. For critical work, cover-glass thickness should be measured, for example, by a micrometer or by using the calibrated fine-focus control of the microscope, i.e., going from the top of the coverslip to the substrate (slide) on the edge of the cover glass through air. Using coverslips of the proper thickness, cultured cells attached to their surface can be optimally resolved. However, live specimens in aqueous media several micrometers below the cover glass may render poor images even with a high NA oil objective because of refractive index mismatches. The refractive indices and dispersions of all media between object and objective influence the size and intensity distribution in the Airy disk and consequently the contrast, resolution, and sharpness of the image.

For the best performance, all optical components from collector to eyepiece must be clean. Lens paper, cotton, lens cleaner, distilled water, and so forth are all acceptable tools and solvents for cleaning these components. The less rubbing needed, the better. As the whole microscope is a precision tool, so is each component. The objective, in particular, where top performance can depend on precise axial and lateral alignment of all lenses to submicron tolerances, must be treated carefully, gently, and with great respect.

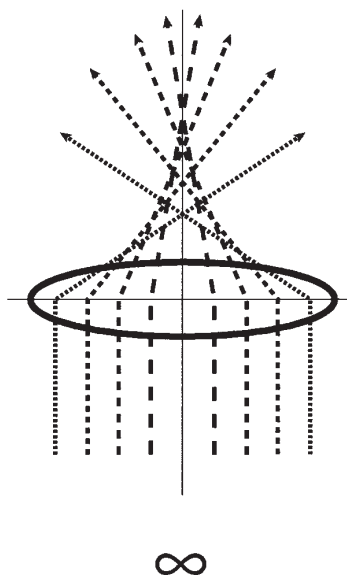


FIGURE 1.10

Spherical aberration. Peripheral rays (dotted lines) passing through a spherical lens are focused closer to the lens than are paraxial rays (those rays entering the objective closest to the optical axis). Nowhere can a sharply focused image be found.

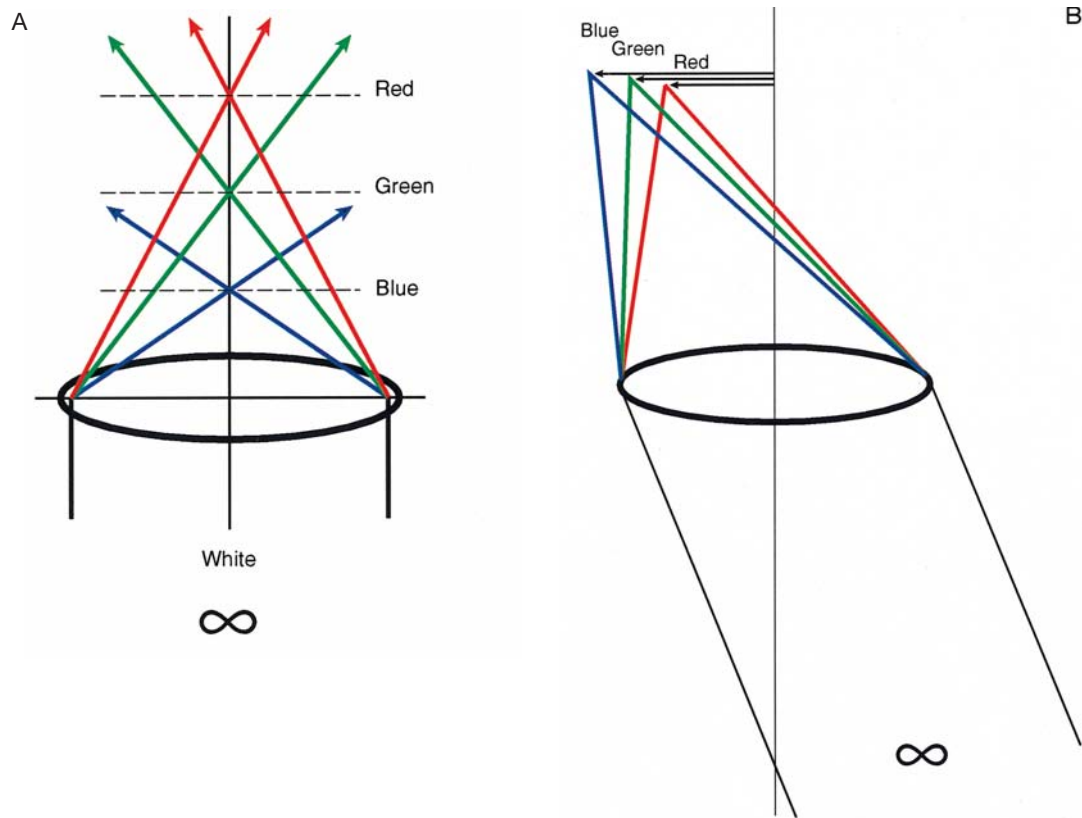


FIGURE 1.11

Chromatic aberration. (A) Longitudinal chromatic aberration: Blue light passing through a lens is focused closer to the lens than are green and red light. (B) Lateral chromatic aberration: The blue image is larger than the red image. Peripheral color fringing occurs.

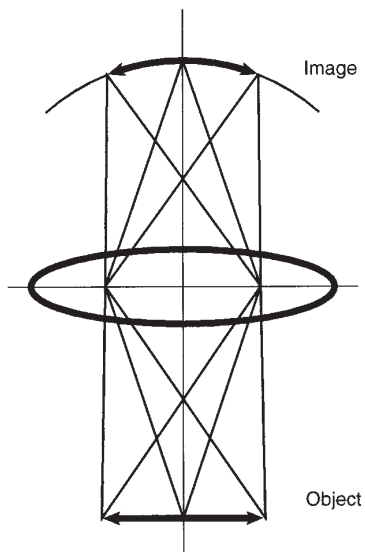


FIGURE 1.12

Curvature of field. Peripheral object points are focused closer to the lens than are paraxial ones. An image “dish” results.

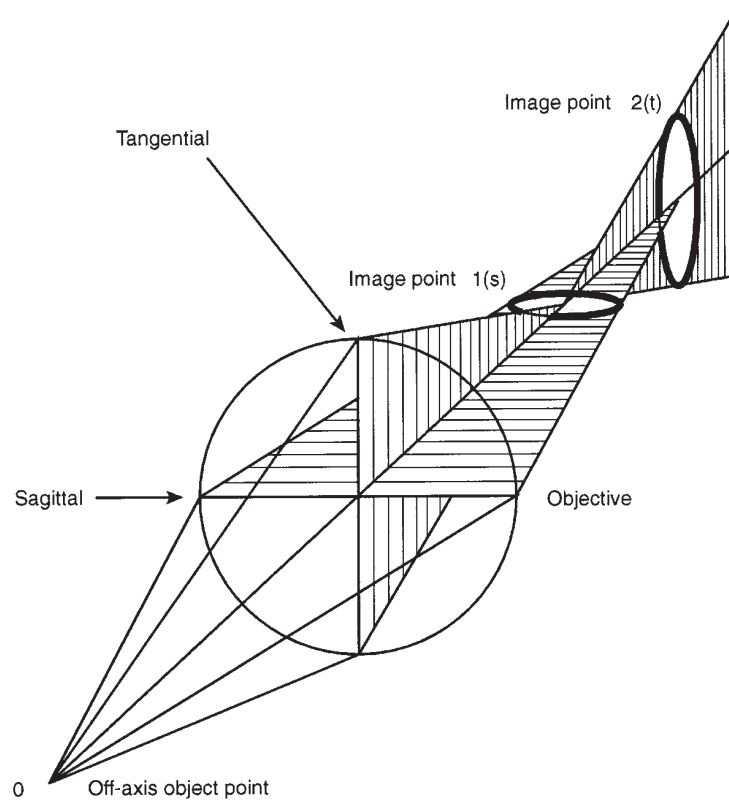


FIGURE 1.13

Astigmatism. Tangential (t) and sagittal (s) cross sections through the objective form different image locations from the optical axis for off-axis points. Depending on the focus, the point becomes tangentially or radially distorted. Note that the further off axis an object point is, the more distorted its image becomes.

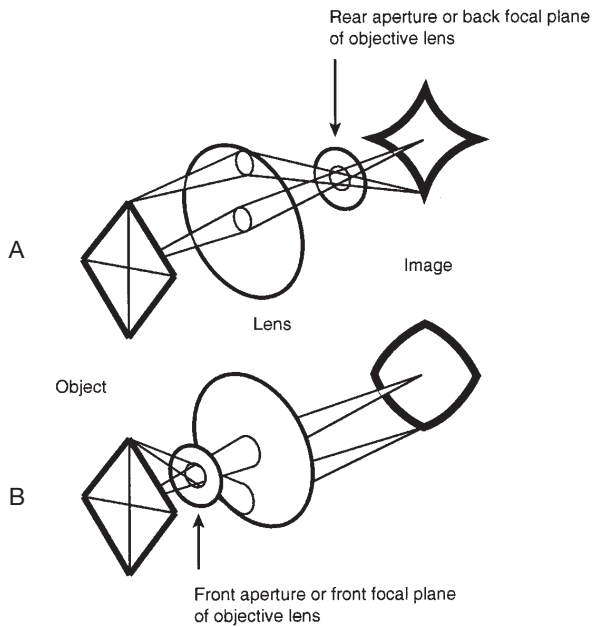


FIGURE 1.14

Distortion. Nonlinear magnification from center to edge of field can result in (A) pincushion or (B) barrel distortion.

Below is a summary of useful formulas†:

Numerical aperture: $NA = n \sin \alpha$

Resolution: $d = \frac{\lambda}{NA_{Obj.} + NA_{Cond.}}$

Useful magnification = $500\text{--}1000 \times NA$

Field of view (mm in specimen) = $\frac{\text{field of view number}^* \text{ of eyepiece}}{\text{Magn. obj.} \times \text{mag. Ch' ger (e.g., optovar)}}$

Depth of field: $T \text{ (mm)} = \frac{1000}{7 \times NA_{Obj.} \times \text{mag. total}} + \frac{\lambda}{2 \times NA_{Obj.}^2}$

†See p. 7 for definitions of n , α , and λ .

*The diameter of the field-limiting fixed stop in the eyepiece in millimeters is usually marked on the eyepiece after the magnification.