CHAPTER EIGHT RR2

Principles of Optics and Microscopy

from; Berlyn, G.P. and J.P. Miksche, 1976
Botanical Microtechnique and Cytochemistry.
Iowa St. Univ. Press. Ames, Iowa

LIGHT

In optical methods light is truly the medium and therefore the message. However, in order to read this message effectively we need some understanding of the nature and properties of the medium. The first question we might ask is, What is light? This is about as difficult to answer as, What is life? The physicists have left us with the intuitively unsatisfying answer that some properties of light may best be explained if light is considered a *wave* phenomenon while other properties are only accounted for if light is considered a *particulate* phenomenon; thus, we are left with particle-wave duality. The photon (particulate) theory of light is thought to be the fundamental one, and the wave theory represents a special case of this quantum theory at a more macroscopic level. In other words, in those phenomena where enormous numbers of photons are involved (i.e., the bulk of light phenomena) one observes the wave properties as a statistical consequence of the behavior of the large population of photons involved. This is akin to radioactive decay where it is impossible to predict which particular atom will decay at a certain time, yet if you consider a large group of atoms it is possible to write a precise mathematical expression which will very nicely fit the observed decay.

Wave Theory

The modern electromagnetic wave theory of light was first propounded by John Clerc Maxwell in 1873 and accounts for such properties as amplitude, frequency, phase, and polarization. In this theory light is considered to be composed of transverse waves (as opposed to sound waves which are pure longitudinal waves). Figure 8.1 is a pictorial representation of a transverse wave showing amplitude A, wavelength λ, and ray path RP; Figure 8.2 illustrates a longitudinal wave. Note that the direction of vibration of the transverse wave is in the plane of the paper and perpendicular to the direction of propagation. The wave normal (WN) is perpendicular to the direction of

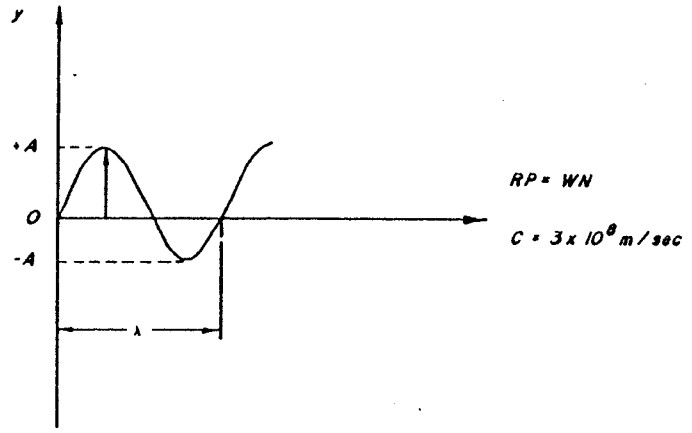


FIG. 8.1. Transverse wave (light): λ = wavelength, A = amplitude, RP = ray path, WN = wave normal, C = velocity of light.

vibration and in this case coincides with the ray path. The velocity of light C is 3×10^8 m/sec. Visible light is only a small part of the electromagnetic spectral continuum which ranges from gamma rays to radio waves (and maybe even gravity waves) (Tables 8.1, 8.2). In this Maxwellian viewpoint light is considered a continuous stream of electromagnetic energy and can be thought of as an example of physical symmetry.

What do we mean by the term electromagnetic energy? Consider the situation in Figure 8.3. A moving magnetic field B generates an electric field E at right angles to B. The moving electric flux E generates a perpendicular magnetic field B', and B' generates a perpendicular electric field E'. In the case of light $B = B'$, $E = E'$, and so on continuously (Fig. 8.3). The velocity, V, of these moving fields in a vacuum can be shown to equal $\sqrt{1/\mu_0 \epsilon_0}$, where μ_0 = vacuum permeability constant (12.57×10^{-7} webers/amp. m) and ϵ_0 =

FIG. 8.2. Undirected longitudinal wave (sound) emitting from telephone receiver.

Table 8.1. Types of Electromagnetic Radiation and Respective Wavelengths

Type	Wavelength (A)
Gamma rays	10^{-4}–1.0
X-rays	10^{-2}–10^{3}
Ultraviolet	1000–3800
Visible	3800–7800
Infrared	7800–10^{5}
Radio waves	10^{6}–10^{17}

Table 8.2. Wavelengths of Light and Corresponding Colors

	Visible Spectrum					
λ in A	3800–4360	4360–4950	4950–5660	5660–5890	5890–6270	6270–7800
Color	violet	blue	green	yellow	orange	red

vacuum dielectric constant (8.85×10^{-12} coulomb^2/n.m^2). The values of the constants are established through standard electromagnetic measurements; when this velocity is evaluated, it turns out $V = C = 3 \times 10^8$ m/sec. Thus light is a phenomenon that stems from rapidly varying (and mutually perpendicular) electric and magnetic fields propagating in a vacuum at 3×10^8 m/sec. In other materials (glass, calcite, fluorite) the velocity of light will be a specific function of the dielectric constant of the material through which the light is passed. The index of refraction, N, of a material is defined as the ratio of the speed of light in a vacuum to the speed of light in the material. It is therefore always greater than unity for a material substance.

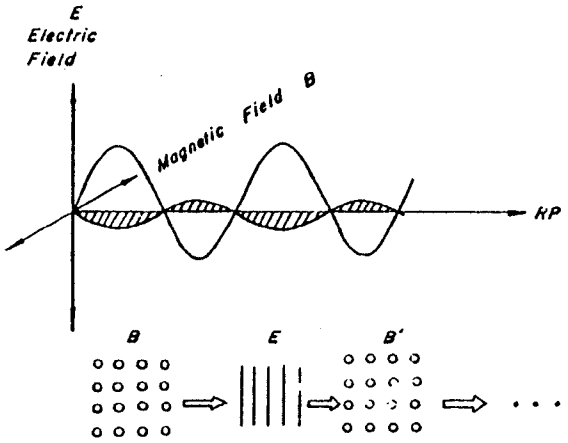


FIG. 8.3. Electric and magnetic field components of light.

Particulate (quantum) Theory

The particulate or quantum theory of optics is due to Einstein (1905) and conceives of light as a shower of photons: each photon contains a quantum of energy, $\epsilon = hf$, where h = Planck's constant (1.58×10^{-34} cal sec) and f = the frequency of the light = c/λ. Note that the photon has a wave component; a wavelength is assigned to each photon. Even matter itself is thought to have a wave component, and it was Einstein's work on the properties of light that led de Broglie to this conclusion. Only the quantum theory can account for the interactions of light with matter such as photoelectric emission and fluorescence. Oil drop experiments by Millikan (1909-1913) showed that the kinetic energies of photoelectrons were in exact agreement with the formulas proposed by Einstein. Compton also observed the motion of a photon and a single electron both before and after collision and found that both behaved like elastic material bodies having kinetic energy and momentum, both of which were conserved in collision. Note that the energy of a photon is proportional to its frequency; the smaller the wavelength of the light, the greater the energy per photon. A mole of photons (6×10^{23}) is called an *einstein* and is the modern unit for light energy calculations. Amount of energy in an einstein of blue light:

$$E = \frac{(1.58 \times 10^{-34} \text{ cal sec/photon})(3 \times 10^{10} \text{ cm/sec})(6 \times 10^{23} \text{ photons/einstein})}{436 \times 10^{-7} \text{ cm}}$$

$$= 65{,}447 \text{ calories/einstein}$$

Amount of energy in an einstein of red light:

$$E = \frac{(1.58 \times 10^{-34} \text{ cal sec/photon})(3 \times 10^{10} \text{ cm/sec})(6 \times 10^{23} \text{ photons/einstein})}{780 \times 10^{-7} \text{ cm}}$$

$$= 36{,}583 \text{ calories/einstein}$$

There is almost twice as much energy in a photon of blue light as in a photon of red light. This explains why ultraviolet light is so much more energetic than visible light; it can burn out the retina of a human eye very rapidly, and thus extreme care must be taken in fluorescent and ultraviolet microscopy.

The index of refraction is probably the most widely used parameter in microscopy. As mentioned previously, the index of refraction of substance i is given by:

$$N_i = C/C_i$$

where N_i = index of refraction of substance i
C = speed of light in a vacuum (3×10^{10} cm/sec)
C_i = speed of light in substance i.

Consider Figure 8.4 where we have two substances, A and B, of different indices of refraction. Let us imagine a beam of monochromatic light incident upon these two blocks of material. As the light passes through sub-

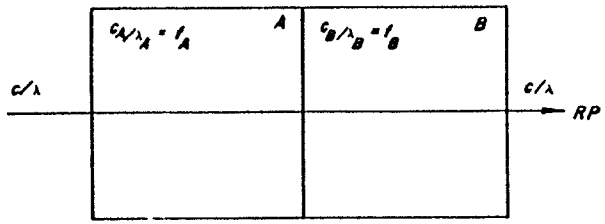


FIG. 8.4. The invariant nature of the frequency. As monochromatic light of wavelength λ enters substance A, it assumes velocity C_A and wavelength λ_A. When this light enters substance B, it assumes velocity C_B and wavelength λ_B. Upon leaving substance B the light again assumes the velocity C and the wavelength λ that it had before entering substance A.

stance A it will assume a velocity C_A and a wavelength λ_A characteristic of substance A. Similarly with substance B we have C_B and λ_B. Does anything remain constant about the monochromatic light? The answer is yes, and the invariant property is the frequency, f.

This implies the following:

$$f_A = f_B$$
$$C_A/\lambda_A = C_B/\lambda_B$$
$$C_A\lambda_B = C_B\lambda_A \qquad \text{(Divide both sides by } C\text{)}$$
$$C_A\lambda_B/C = C_B\lambda_A/C$$
$$\lambda_B/N_A = \lambda_A/N_B$$
$$\lambda_B/\lambda_A = N_A/N_B$$

Previously we mentioned that the color of light was a function of its wavelength. This is common usage, but it should now be apparent that this is not quite so, and that frequency is really the fundamental color parameter.

Properties of Waves

Since most of the properties of light dealt with in microscopy can best be considered from a wave standpoint, it behooves the microscopist to have at least an elementary understanding of waves.

Frequency

For reasons stated above, frequency, $f = c/\lambda$, is the fundamental property of waves. The inverse of frequency is called the period, and its units are seconds per cycle. In other words, frequency is the number of waves per unit of time (cycles/sec), while the period T is the time it takes for one wavelength to pass a given point. In every second f waves go past a particular

point; since each wave is a certain linear distance long, λ, a wave must travel $f\lambda$ per second, which is equal to the velocity of the wave ($V = f\lambda$).

A familiar example of waves is water waves. Let us take a closer look at them in order to better understand the wave phenomenon. The first thing to observe is that it is the disturbance that travels and not the wave itself. This can be demonstrated with the use of a fishing pole, a line, a cork, and a quiet pool. Suspend the cork to the fishing pole by means of the line and lower it into the center of the pool, jiggling it up and down slightly. In this situation the cork has no tendency to move from the center; only the disturbance moves.

If we could tag the individual water molecules and follow them as the wave motion passes, we would find that the molecules make a roughly circular orbit around their initial position with an average velocity of approximately zero. The wave disturbance moves much faster than the molecules move in their circular orbits. This can be illustrated in the following way.

Imagine yourself in a boat being tossed by waves. You note that the distance between successive wave crests is 180 ft and a wave passes underneath you every 4 seconds. From these observations we compute the following:

$$\lambda = 180 \text{ ft}; \qquad T = 4 \text{ sec}$$
$$V = f\lambda = 1/4 \times 180 = 45 \text{ ft/sec}$$

Thus we have the velocity of the wave motion (the disturbance), but what about the molecular motion? If the waves are 3 ft high ($A = 3$ ft) you know that in 4 seconds the molecules must move around the circumference of a circle 3 ft in radius; i.e., $S = 2\pi r = 18.85$ ft. The speed of the molecules in them is 18.85 ft ÷ 4 sec = 4.71 ft/sec. Therefore, it is clear that the disturbance is traveling approximately 9 times the speed of the molecules; i.e., energy can move faster than direct mass motion in this situation. Of course these calculations are not independent of the magnitude of the individual parameters, but the conclusion is a general one.

Polarization

Polarization refers to waves whose vibration directions are in a common plane. To illustrate this you can use a piece of rope and a fence post. Tie the rope to the post and stretch it out. If you shake the rope up and down, you have an analogy to a polarized light wave. Imagine a large number of posts in a row, all with vibrating ropes. This is analogous to a beam of polarized light. If we looked at a cross section of an unpolarized light beam we would see not just one ray vibrating in one plane but many rays traveling along the same axis and vibrating in many planes (Fig. 8.5A). This results in "chaotic motion." To polarize light means to bring order out of the chaos.

In polarized light all the light vibrates in a single plane—that dictated by the polarizing medium (Fig. 8.5B). The polarizing medium can be a crystal (calcite, herapatite) or an H-sheet of aligned polymeric iodine molecules. By analogy we can think of these crystals as a picket fence. Only if the light

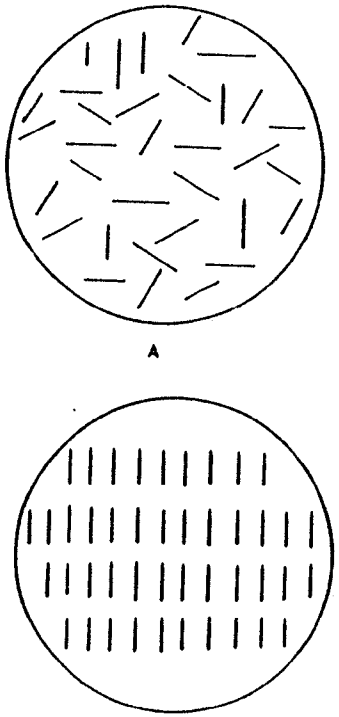


FIG. 8.5. A, Cross section of a beam of statistically unpolarized light. B, cross section of a beam of polarized light.

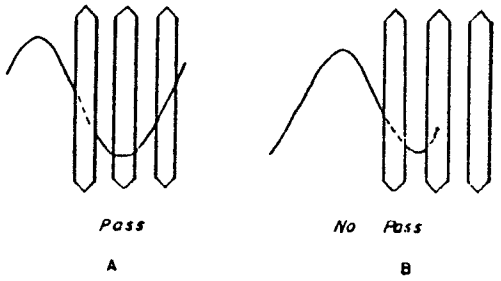


FIG. 8.6. Picket fence analogy for polarizing medium.

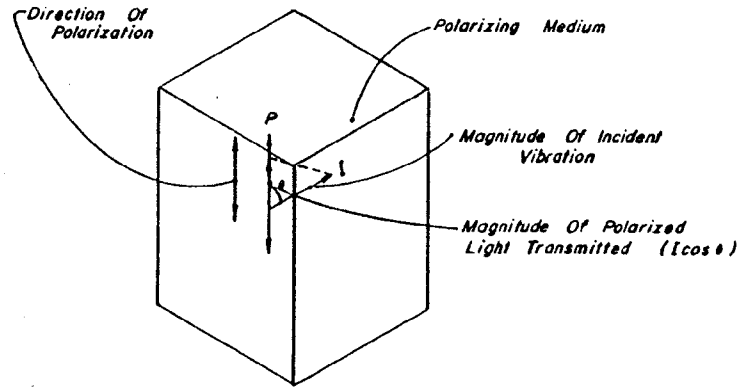


FIG. 8.7. Transmission of polarized rays by a polaroid sheet.

vibrations are parallel to the pickets will they be permitted to pass through the crystals (Fig. 8.6). The fence is analogous to the polarizer and the direction of the pickets is analogous to the plane of polarization. If a vibration is in some plane other than the ones the polarizer will completely transmit (parallel) or reject (at right angle), the trigonometric component or projection of this incident vibration on the plane of the polarizer will be transmitted (Fig. 8.7).

Phase

The meaning of phase is best understood in the context of two waves propagating from a common source (Fig. 8.8). Consider the case of two waves that are exactly out of phase, e.g., waves *A* and *B* (Fig. 8.8B). This means that when wave *A* peaks in the positive direction, wave *B* is peaking in the negative direction and vice versa. These waves will "interfere" with each other and in actuality only the resultant wave *R*, the algebraic sum of waves *A* and *B*, would be transmitted. This is a case of destructive interference, and the amplitude of the resultant wave is lower than that of the larger wave. If *A* and *B* had the same amplitude, the resultant wave *R* would have an amplitude of zero, and complete darkness would result. On the other hand, in constructive interference (8.8A) there is enhancement of the light. The amplitude of the resultant wave is again equal to the algebraic sum of the component waves, but in this case the resultant amplitude is greater than that of either of the component waves.

Often the difference in phase between waves is expressed in terms of fractions of wavelengths. If wave *A* differs from wave *B* by a whole wavelength the waves will be in phase (Fig. 8.9A). In fact, if the waves differ by any integral number of wavelengths (0λ, 1λ, 2λ, $\ldots n\lambda$) they will still be in phase. However, if wave *A* differs from wave *B* by a half wavelength the waves will be exactly out of phase (Fig. 8.9B). This applies to odd numbers of half wavelengths, e.g. $1/2\lambda$, $3/2\lambda$ $5/2\lambda$, $\ldots (2n + 1)/2\lambda$. Intermediate

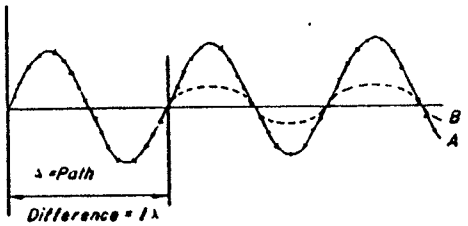


FIG. 8.8A. Constructive interference–in-phase waves.

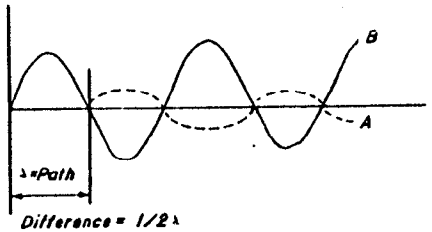


FIG. 8.8B. Destructive interference–out-of-phase waves.

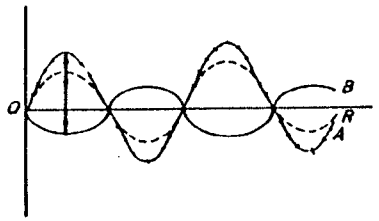


FIG. 8.9A. Waves out of phase.

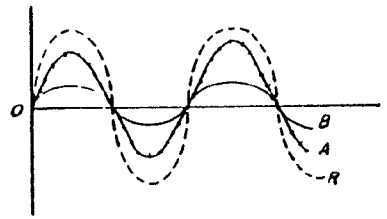


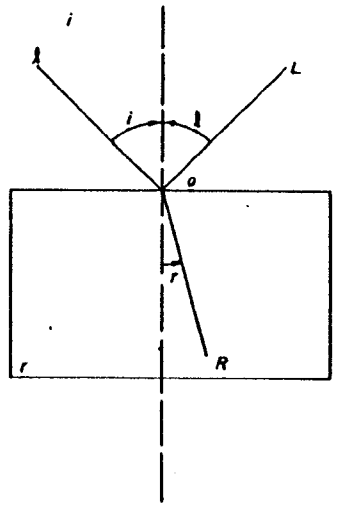
FIG. 8.9B. Waves in phase.

conditions occur, and these can also be calculated. The path difference Δ is a linear unit of difference and could be expressed in m, cm, or nm as well as in fractions of wavelength. In general, to find the amplitude R of the resultant wave when a path difference of $x\lambda$ exists between waves A and B (A and B also represent the respective amplitudes) the following relationship is used:

$$R^2 = A^2 + B^2 + 2AB\cos(x \cdot 360^\circ).$$

Refraction

When light travels from a medium of a certain index of refraction N_i into a medium of different index of refraction N_r, the light is bent (refracted). The extent of bending is expressed precisely in Snell's law: $N_i \sin i = N_r \sin r$. The angle of incidence, i, is equal to the angle of reflection, l. Note in Figure 8.10 that the angle of refraction r can only have values from 0° to 90°. For r values greater than 90° (calculated by Snell's law) there is total reflection. The angle of incidence at which total reflection occurs is called the critical angle. The critical angle from glass to air is 41.3° and from glass to water 61.7°. There is no critical angle from glass ($N = 1.515$) to immersion oil ($N = 1.515$), and this is one of the reasons for immersion.



∠o = Incident Light Ray
o L = Reflected Light Ray
o R = Refracted Light Ray
∠i = Angle Of Incidence In Medium i
∠r = Angle Of Refraction In Medium r
∠l = Angle Of Reflection In Medium i

$N_i \sin i = N_r \sin r$
$\angle i = \angle l$

FIG. 8.10. Diagrammatic representation of refraction.

Dispersion

The separation of a beam of white light into its component colors (wavelengths, frequencies) is termed dispersion, and the dispersing medium must have a different index of refraction for each wavelength that is dispersed. The phenomenon is commonly observed with prisms. For most substances the angle of refraction becomes larger as wavelength becomes longer. It is possible to determine the index of refraction N for any wavelength of a particular substance through use of the Cauchy equation: $N = A + B/\lambda^2 + C/\lambda^4 + \ldots$, where A, B, and C are the Cauchy constants of the substance which in practice are tabulated in various handbooks; they can also be calculated as needed using three equations to determine the three unknowns.

Diffraction

Diffraction refers to a systematic scattering that occurs when a light beam passes across an edge of an object. When light encounters an object, three things happen: (1) part of the light is diffracted by the margins of the object; (2) part of the light is diffracted by the internal structure of the object; and (3) part passes by the object without being diffracted at all and is termed the direct or background light. The diffracted waves interfere with each other giving rise to bands of maximum, minimum, and in-between light. The bands of enforcement (due to interference of exactly in-phase beams) are termed the first, second, third, etc. orders of diffraction. The beam along the ray path axis is termed the zero-order beam and is also a maximum enforcement beam. The finer the diffracting structure, the greater the angle of divergence of the diffracted orders. The diffracted beams register in their phase, intensity, and direction the minute features of the object. Groups of diffracted beams together with direct beams enter the microscope objective separately and are brought together at the image plane where their interference results in image formation. The more diffraction orders collected by the objective, the more exactly does the image resemble the object. Normally the image will be good enough to separate two small internal objects if the objective collects at least the first-order beam. Nevertheless, the advantage in using objectives of high numerical aperture (see Chapter 1) is obvious; they have greater ability to collect a widely spread diffraction pattern and give truer images of the object.

LENSES

In general there are two main types of lenses—thin-edged and thick-edged—each with three subtypes (Fig. 8.11). Thin-edged lenses when illuminated by monochromatic light traveling parallel to the lens axis (Fig. 8.12) converge all the light to a point on the axis known as the principle focus. On the other hand, thick-edged lenses under the same illumination (parallel) diverge all the parallel light rays (Fig. 8.13). However, if one traces back the diverged rays, one observes that they come back to a point on the axis in front of the lens. This point is also the principle focus. The distance along

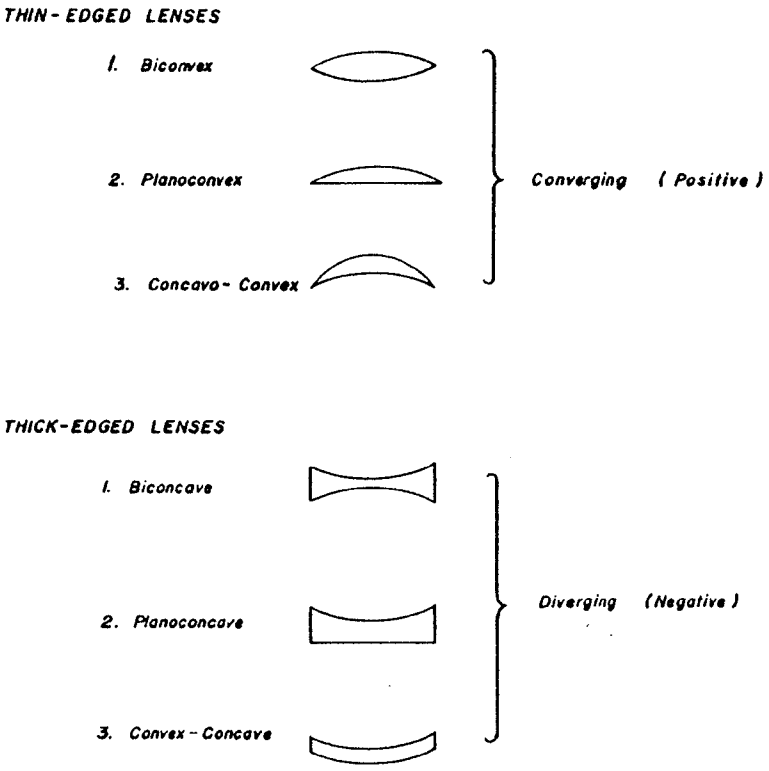


FIG. 8.11. General types of lens shapes.

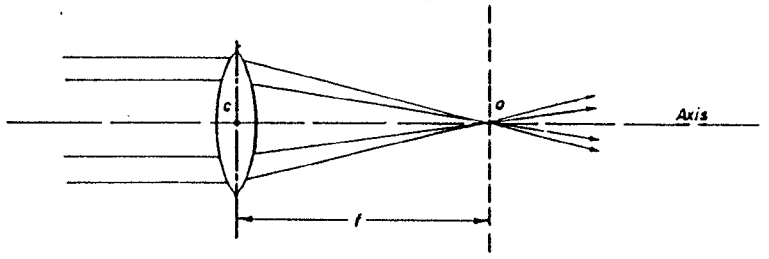


FIG. 8.12. Converging of parallel monochromatic light by a thin-edged lens: c = center of lens; o - principal focus; f = focal length.

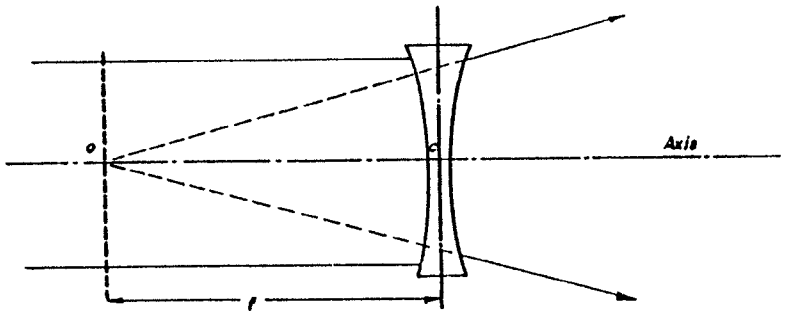
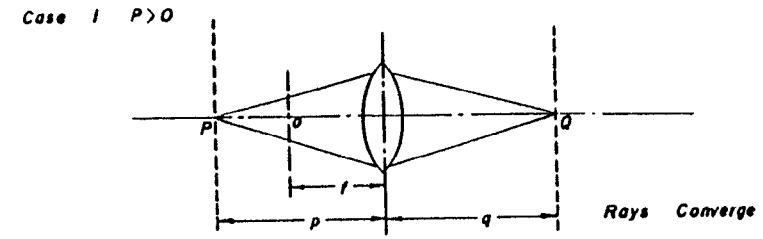
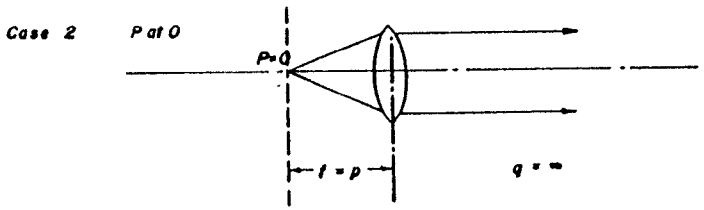


FIG. 8.13. Diverging of parallel monochromatic light by a thick-edged lens: c = center of lens; o = principal focus; f = focal length.





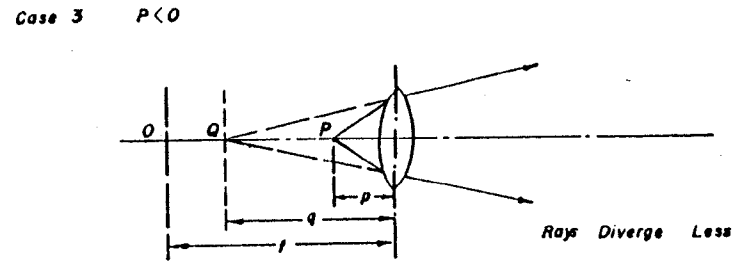
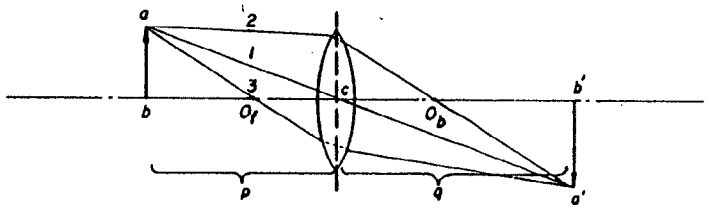


FIG. 8.14. Point source data for action of thin-edged lenses.

the axis from the center of the lens to the principle focus is termed the focal length, f.

For illustrative purposes we will concentrate on the action of thin-edged lenses. There are three possible situations or cases that can be set up with thin-edged lenses, as illustrated in Figure 8.14. In this example the lens is being illuminated by a point source P, and the image of the point source is Q. The distance from the center of the lens (note that a ray passes through the center of the lens undeviated) to the point source is called the object distance, p. Similarly the image distance is designated q. In Case 1 P is in front of the principle focus so the rays are converged forward to Q. This is the situation between a specimen and the front lens of a microscope objective. In Case 2 P is at the principle focus and no image is formed. In Case 3 P is located between the front principle focus and the lens; the thin-edged lens causes the light rays to diverge less, and a virtual image in front of the lens occurs. This is the situation with regard to the eyepiece of the microscope.

Out of the massive number of light rays entering a symmetrical lens let us select three to use as an aid to understanding the geometrical properties of image formation (Fig. 8.15). The first ray we select passes from the tip of the specimen (arrow point) through the center of the lens and is therefore undeviated. The second ray passes from the specimen to the lens parallel to the lens axis and therefore is refracted through the back focus of the lens. The third ray passes through the front focus on its way to the lens and is therefore projected parallel to the lens axis. These three rays cross at the image plane, as depicted in Figure 8.15. The distances p, q, and f are related by the classic lens makers' formula: $1/f = 1/p + 1/q$. Magnification is q/p, as shown in Figure 8.15; as the focal length decreases, magnification increases. These



$$\triangle abc \sim \triangle a'b'c'$$

$$\frac{a'b'}{b'c} = \frac{ab}{bc}$$

$$\frac{a'b'}{ab} = \frac{b'c}{bc} = \text{magnification} = \frac{q}{p}$$

FIG. 8.15. Relationship between image distance, object distance, and magnification: ab = object size; $a'b'$ = image size; O_f = front focus; O_b = back focus; c = center of lens; p = object distance; q = image distance.

formulas hold for virtual as well as real images; but in virtual images q has a negative value because it is on the same side of the lens as the object (left of the lens), and therefore magnification is also negative.

Human Eye

In any discussion of microscopy the human eye (Fig. 8.16) should also be considered, since it is part of the optical system. The glass lenses we have been discussing have fixed focal length. The eye, on the other hand, can accommodate; i.e., it can focus on both close and distant objects. It does this by actually changing the shape of the crystalline lens and therefore changing its focal length. The edge of this lens is encased by the ciliary muscle; when this muscle contracts the lens bulges out and its focal length is reduced, bringing near objects into focus. When the ciliary muscle relaxes the suspensory ligaments come under tension and the lens is flattened. This causes an increase in focal length which permits more distant objects to be focused. This is why the normal eye is most relaxed when viewing distant objects (parallel light). Ten inches (250 mm) is the distance of most distinct vision for most people, hence the image distance for the compound microscope. In the normal eye (Fig. 8.17) the rays are focused right on the retina (emmetropia); in the nearsighted eye (myopia) the rays are brought to focus in front of the retina; and in the farsighted eye (hypermetropia) the rays are brought

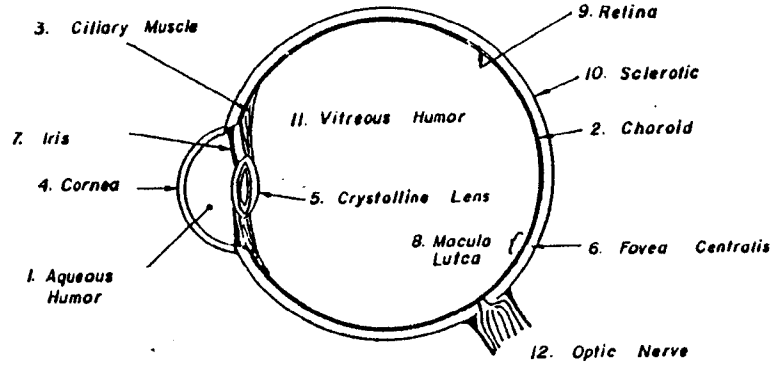


FIG. 8.16. Human eye: (1) fluid with $N = 1.33$; (2) dark pigmented cells that eliminate stray light; (3) muscle that effects accommodation; (4) transparent outer layer of front of eye, $N = 1.38$; (5) crystalline lens, $N = 1.41$ at center to 1.38 in outer layers; (6) center of macula lutea; (7) diaphragm that controls amount of light entering the eye; (8) special sensitive area of retina where vision is most distinct; (9) receiving screen upon which images are formed; (10) opaque and tough outer layer of eye; (11) transparent jellylike medium, $N = 1.33$; (12) optic nerve.

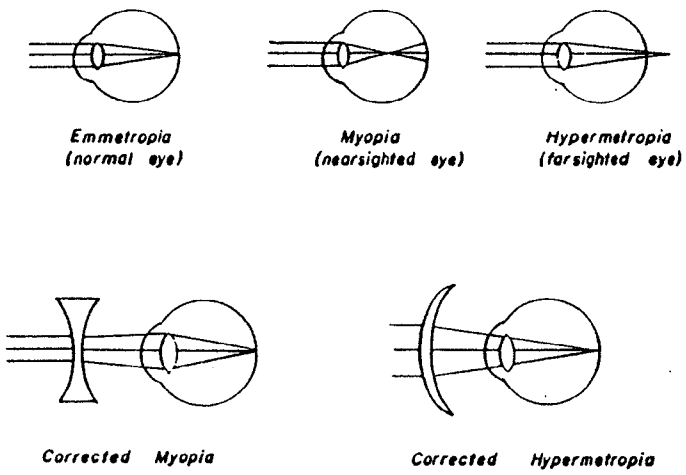


FIG. 8.17. Focus of the human eye.

to focus behind the retina. Thus myopia requires a negative lens (thick-edged) while hypermetropia requires a positive lens (thin-edged) for correction. With age many people tend to become farsighted because the ciliary muscle weakens and loses some of its elasticity and contractive power.

The crystalline lens of the eye functions as any simple lens (see Fig. 8.15) and inverts the image; that is, the retinal image is inverted, but the brain perceives it as erect. The noted student of vision, Erisman, demonstrated this very dramatically by wearing a pair of spectacles that inverted his normal vision. At first he clearly perceived everything upside down, but gradually his vision grew blurred and then began to clear again. At this point he began to see erect images. When he finally removed the spectacles everything was upside down again, but fortunately after a time his brain rereversed his image perception. This type of experiment takes a very brave experimenter, and the student is not urged to attempt to verify these results.

RESOLUTION

At this point we are able to consider resolution at a somewhat more sophisticated level. In Chapter 1 we presented a formula for calculating resolution in the microscope: $h = 0.61\lambda/\mathrm{NA}$, where h is the minimal distance by which two structural entities in the object may be separated and still perceived as two distinct structures, λ is the wavelength of light, and NA is the numerical aperture of the optical system. For the unaided human eye the minimal distance, h', is about 200 μm; for a research microscope h is about 0.2 μm. This gives an increase in resolution of about a thousandfold. For the electron microscope (which uses electrons instead of light and operates with electrical or magnetic lenses instead of glass lenses) the increase is about a

millionfold. These increases in resolution are accompanied by increases in magnification, but there is no cause-and-effect relationship between the two. There is, however, a fundamental limit to the amount of resolution that may be attained. There is no corresponding limit to the amount of magnification that may be attained, but there is a limit to the amount of *useful* magnification that can be employed. This useful magnification, designated M, is that amount of magnification necessary to allow the human eye ($h' = 200\ \mu m$) to see the resolution produced by the optical system

$$M \times h = 200\ \mu m$$

$$M = 200/0.2 = 1{,}000$$

In practice M is often taken as 1,000 NA in order to provide some extra magnification to take into account the variation in the resolution of the human eye. It also facilitates the viewing of resolved structures. Magnification beyond this level is called empty magnification and is associated with deterioration of the image.

If there were no fundamental limit to resolution, we could design multistage magnification systems for the microscope that along with image intensifiers would be far more useful to man than even the electron microscope.

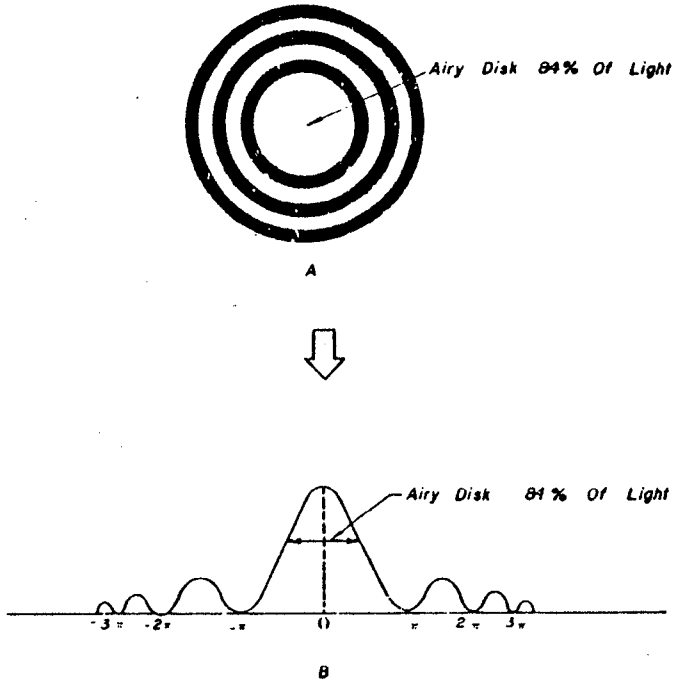


FIG. 8.18. Diagrammatic representation: A, Airy disk; B, energy distribution in Airy disk.

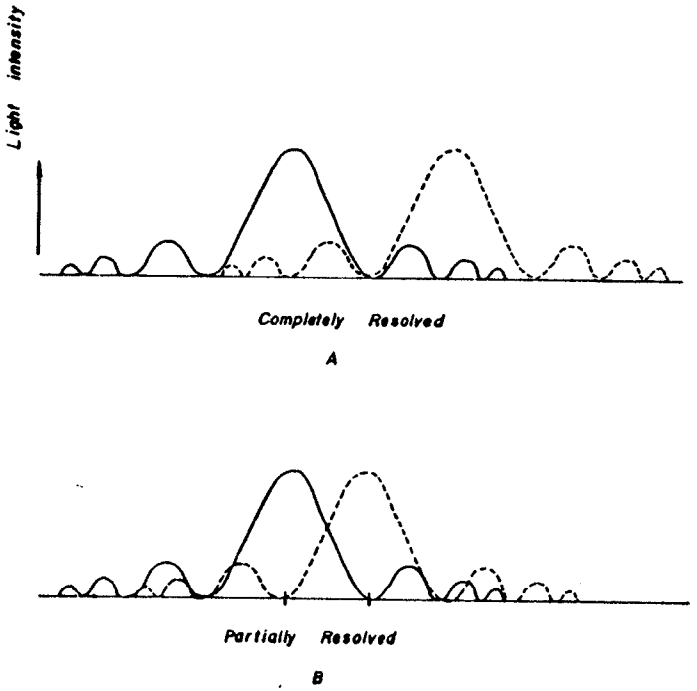


FIG. 8.19. Diagrammatic representation of Airy disk under conditions of (A) complete resolution; (B) partial resolution.

Unfortunately this cannot be, primarily because of the phenomenon of diffraction which causes the image of an object point not to be a point but instead to be diffracted into a small circular spot of light surrounded by rings of lesser light (Fig. 8.18). The central spot of light is called the Airy disk, after Sir George Airy (1834) who first elucidated its nature. The disk itself contains about 84% of the light, and the rest is distributed in the concentric light rings. An energy or intensity plot of this situation is illustrated diagrammatically in Figure 8.18B. If two structures in the object are so situated that their diffraction disks just touch (are tangent), the two object points are said to be completely resolved (Fig. 8.19A). Even if the disks overlap by one radius the structures may still be discerned as two separate structures (Fig. 8.19B).

Thus resolution is limited by the smallest object that will cause the light waves to be diffracted. If the object is too small to affect the light waves passing through it, there would be zero information coded in these light waves when they arrived at the image plane.

Let us consider the case of two closely spaced structural points, A and B in Figure 8.20. If we impinge light upon these structures, the diffracted wave-

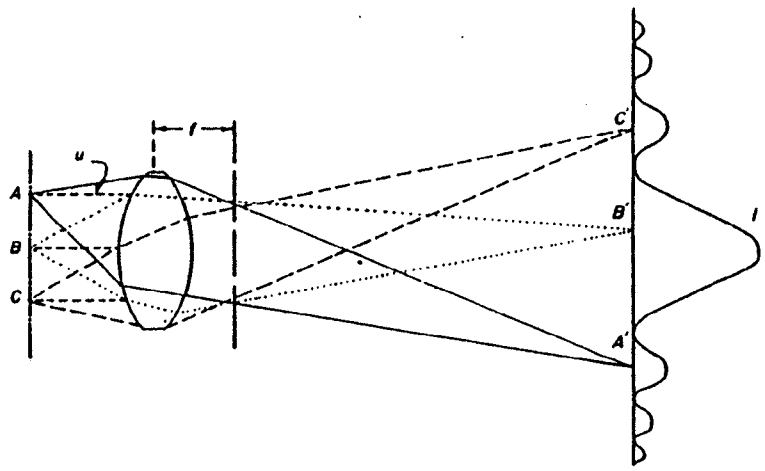


FIG. 8.20. Diagrammatic presentation of the role of interference in the resolution of two closely spaced object points A and B.

lets from each object will interfere with each other. Some of the resultant waves will be enhanced (constructive interference) and some will be diminished (destructive interference). This process results in beams of maximum light (exactly in phase, see p. 137), termed the first, second, third, etc. orders of diffraction. The beam along the central axis (also a maximum beam) is termed the zero-order beam. As previously stated, the greater the number of these diffracted orders that are collected by the objective, the more truly will the image resemble the object, and our two structures A and B will be resolved as separate entities if at least the first-order beam enters the objective.

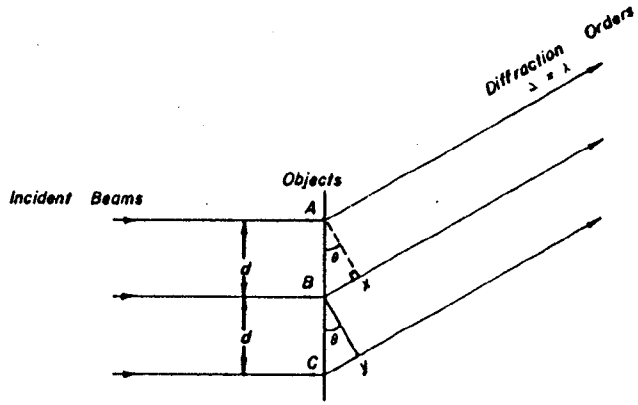
The diffracted beams are all maximum and differ from each other by whole wavelengths: 0λ, 1λ, 2λ, $\ldots n\lambda$. In other words, the path difference (Δ) between successive diffracted orders is one wavelength. Figure 8.21 illustrates the situation. Let Ax be the perpendicular from A to the ray path from B. The path difference between the two beams is $Bx = \lambda$; θ is the angle between the axis and the first diffracted beam; and d is the linear distance between structures A and B. Thus $\sin\theta = Bx/AB = \lambda/d$.

The closer together points A and B, the larger θ will become. The minimum condition for separation of A and B is met when the first-order beam just fits into the objective. This is when $\theta = \mu$, where 2μ = the angle subtended by the objective lens; therefore, in Figure 8.22 we see that:

$$\sin\mu = \lambda/h$$

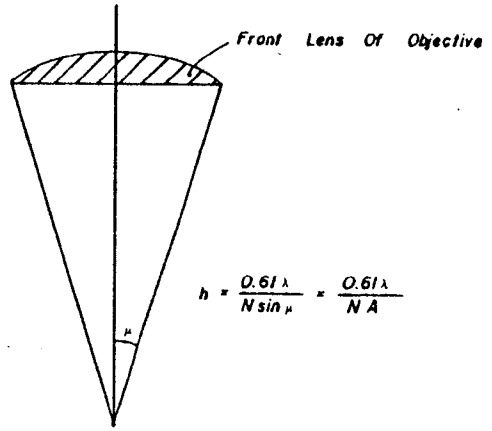
and

$$h = \lambda/\sin\mu$$



$$\sin\theta = \frac{Bx}{AB} = \frac{\lambda}{d}$$

FIG. 8.21. Diffraction pattern from three object points A, B, and C in specimen. The closer together points A and B, the larger θ will become. Minimal separation occurs when $\theta = \mu$ (see Fig. 8.22).



$$h = \frac{0.61\lambda}{N\sin\mu} = \frac{0.61\lambda}{NA}$$

FIG. 8.22. Diagram of angular relationship between specimen and front lens of the microscope objective: μ = half-angle subtended by the objective lens; N = index of refraction; NA = numerical aperture; h = minimal distance of resolution.

where μ = angular aperture
$\sin \mu$ = numerical aperture = NA
h = minimal distance under vertical illumination.

The angular aperture has a theoretical limit of 90°; in practice the limit is about 72°, giving a maximum numerical aperture in air of 0.95. The numerical aperture may be increased to 1.4 by using oil immersion on both objective and condenser. The use of the immersion oil reduces the wavelength by $1/N$, as can be deduced from the formula previously given on page 145:

$$\lambda_B = N_A \lambda_A / N_B$$

If N_A is the index of refraction in air, $\lambda_B = \lambda_A / N_B$. The formula for minimal distance that we have derived is for vertical illumination. However, if oblique illumination is used, higher orders of diffraction beams may be collected by the objective, albeit on one side of the central axis only. According to the Abbe theory of resolution, the maximum condition yields a minimal distance of $h = 0.5\lambda/\text{NA}$. The Rayleigh-Airy theory on the other hand takes a different approach and determines the diameter of the Airy disk to be $1.22\lambda/\text{NA}$, leading to a minimal distance formula of $h = 0.61\lambda/\text{NA}$. For the best commercially available microscopes the maximum resolution is around $1/3\lambda$ and is unfortunately an immutable limit for the light microscope.

CHAPTER NINE

Types of Microscopy

VOLUMES have been written on each of the various types of microscopes, and intensive coverage of every instrument is impossible within the confines of this chapter. Our aim is to provide an introduction to the instruments and to convey an elementary understanding of their principles and use. Readers requiring more detailed and specific information are referred to the specialized literature.

DARK-FIELD

The object under study appears self-luminous on a dark background in dark-field microscopy. The effect is achieved by focusing an oblique hollow cone of light at the specimen plane. The cone is so oblique (Fig. 9.1) that if no specimen is present, no light will be collected by the front lens of the objective. If a specimen is placed in the specimen plane, only light diffracted and scattered by the specimen will enter the objective.

Nearly any laboratory and research microscope can be used for dark-field microscopy. Basically the two methods of achieving dark-field illumination are by modification of a regular bright-field condenser or by using a special dark-field condenser. The former is the simplest and the method of choice for low-power dark-field microscopy (30X-200X). It is achieved by inserting a central dark-field stop into a filter holder below the condenser (Fig. 9.2). The central disk permits only a hollow cone of light to strike the specimen. Obviously, for optimum effects the diameter of the disk must be varied with the magnification to be used, depending on the ocular power as well as the objective magnification. Disk sizes range from 7 to 18 mm and can be cut out of cardboard and blackened with India ink. The numerical aperture of the objective must be less than that of the condenser for best results. It is most convenient to have objectives with built-in iris diaphragms; lacking this, funnel stops may be inserted inside the objectives in order to accomplish the required reduction of numerical aperture. Needless to say, numerical aperture is not reduced any more than necessary to eliminate glare and provide a homogeneous dark background. Observations are best made in a darkened room.

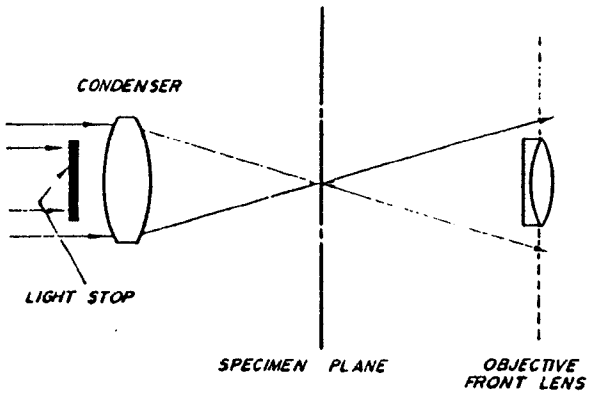


FIG. 9.1. Action of dark-field system with no specimen in the specimen plane.

For higher magnifications and resolution it is necessary to use special oil-immersion reflecting dark-field condensers. The paraboloid condenser was the first specifically designed for dark-field and has a single reflecting surface. It is based on the fact that parallel light rays incident upon a paraboloid reflector will all be focused at the focal point of the paraboloid. The condenser is in the shape of a paraboloid but cut so that the focal point is slightly above the condenser top. The oil film, the slide, and the specimen are necessary to complete the geometry of the system. Oil immersion is also necessary to avoid the total internal reflection that would otherwise occur, due to the fact that the angle of the hollow cone is greater than the glass-to-air critical angle of 41.3°. The paraboloid condenser can be used only with objectives of less than NA 0.85, so it is limited to intermediate magnification and resolution.

For smaller objects or where somewhat better resolution is required (objective NA 1.05 or less) the cardioid dark-field condenser is employed. This condenser has two reflecting surfaces, the second of which is usually in the shape of a cardioid of revolution. This design is one of several that are naturally aplanatic: i.e., the optical system is free from spherical aberration, coma, and chromatic aberration.

The dark-field condenser for high-aperture work is of the cassegrain or spot-ring type. These are also doubly reflecting condensers. They permit the

FIG. 9.2. Dark-field stop for insertion below bright-field condenser.

Table 9.1. Dark-field Condensers and Applications

Type	Oil	Condenser	Maximum Objective	Application
Dark-field stop	no	to 1.40	0.65	large objects, temporary aqueous mounts
Paraboloid	yes	1.00–1.40	0.85	large fibers, crystals
Cardioid	yes	1.20–1.33	1.05	smaller objects, permanent mounts
Cassegrain (spot ring)	yes	1.40–1.50	1.30	highest resolution, for very small specimen detail mounting medium ≥ 1.45

full use of objectives with NA 1.30 and are used for fine detail. Even objects below the resolution of the light microscope (less than 0.2 μm) can be seen as diffraction rings. Table 9.1 lists the principal types of dark-field condensers and their suggested usages. In selecting a dark-field condenser, the nature and size of the objects to be observed are of major import. The immersion condensers focus a great deal more light on the specimen so are greatly preferred for higher magnification work, but an intense light source is necessary for optimum results.

In practice, first set up the standard Kohler illumination system (with condenser annealed to the slide with oil if an immersion condenser is employed). As you focus the condenser, a dark spot will appear below the correct positioning of the condenser. Rack the condenser up until the center of the field is clear and uniform in light intensity, keeping the substage diaphragm wide open. Close the objective iris (or insert funnel stops) until glare is minimized. Most dark-field condensers are designed for a certain slide thickness, and this should be checked. The proper slide thickness can be determined with the aid of a polished piece of uranium glass, as shown in Figure 9.3. Older model condensers were often designed for thick slides, but

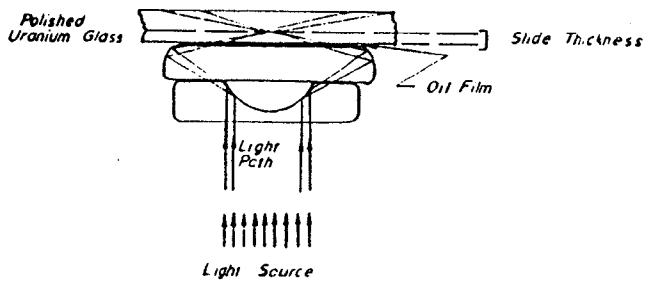


FIG. 9.3. Determination of slide thickness in dark-field microscopy and light path in a double reflecting dark-field condenser.

this can be compensated for by oiling coverslips onto the bottom of the slide. Slides sometimes contain internal inclusions that scatter light. Each slide should be checked before mounting the specimen by observing it under the same dark-field system to be employed in studying the mounted specimen.

PHASE-CONTRAST

The typical compound microscope allows visualization of object detail by means of amplitude differential. Thus maximum contrast is achieved by selective staining of the object detail; without staining most biological preparations are practically invisible under the microscope. Even with staining the specimen must be sectioned sufficiently thin so that it transmits light; fixation is usually necessary to preserve the structure during the preparatory procedures of sectioning, staining, and mounting. The stained object becomes visible because the stain absorbs some of the light so that the amplitude of the light passing through the specimen and the amplitude of the background light are different (Fig. 9.4). The human eye is sensitive to these intensity differences, and hence the stained structure appears darker than the background.

In contrast, when light passes through a thin unstained biological specimen there is very little amplitude differential between the background light and the light diffracted by the specimen. For this reason the specimen appears virtually invisible under normal bright-field microscopy. However, the specimen does induce some phase changes between the background and the diffracted light. It breaks up the incident portion of the light beam into discrete bundles of rays diverging from the specimen detail over a wide angle, and the objective lens collects these diffraction orders (within the limits of the objective aperture) plus the background light. These two forms of light are brought together at the image plane and interact to form the image (Fig. 9.5). The amount of phase change exerted by the object detail depends on the index of refraction of the structures:

$$\text{phase difference} = \phi = 360\ \Delta/\lambda$$

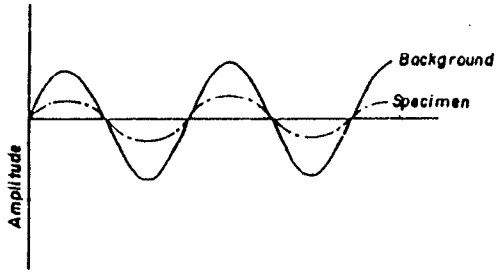


FIG. 9.4. Amplitude differential at image plane of background light and the light transmitted by a stained specimen.

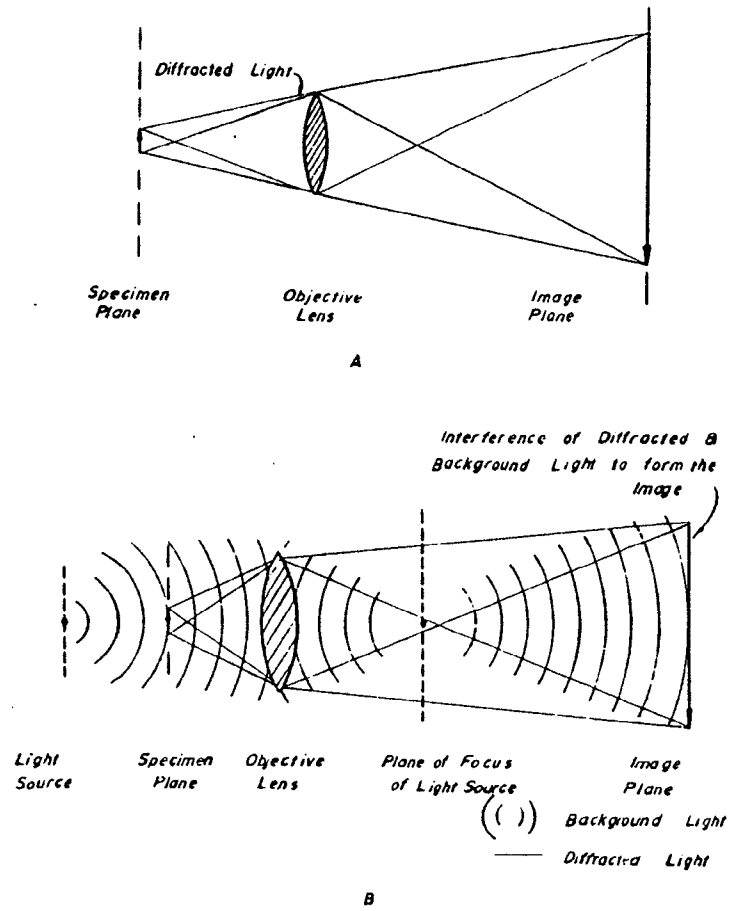


FIG. 9.5. Interaction of background and diffracted light in image formation.

where $\Delta = (N_s - N_m)\,\ell$ optical path difference
N_s = index of refraction of specimen
N_m = index of refraction of mounting medium
λ = wavelength of illuminating light
ℓ = thickness of specimen

Phase difference is often expressed in terms of fractions of wavelengths—e.g., 1/8 λ, 1/4 λ, 1/2 λ. A thin section of cytoplasm, for example, might give a 1/4 λ phase difference with respect to the background light.

In 1933 Fritz Zernike, a Dutch scientist, discovered a way to convert these phase differences into amplitude differences and thereby provide the

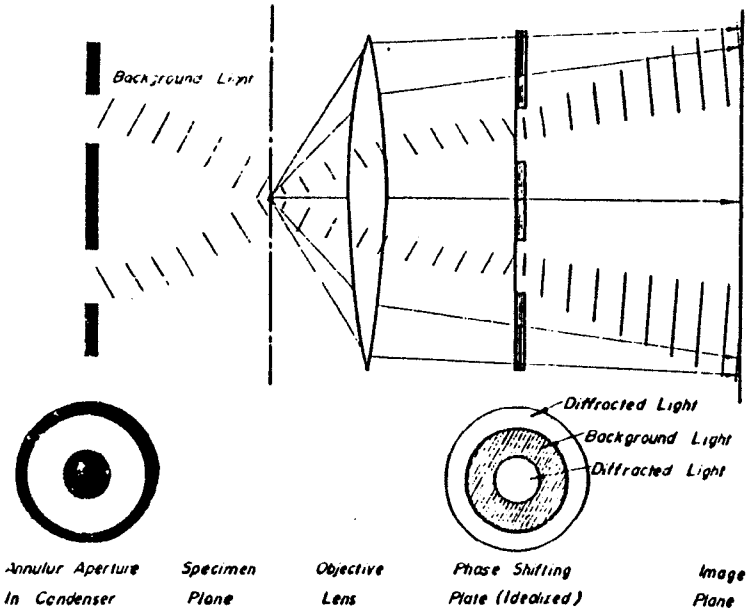


FIG. 9.6. Diagrammatic illustration of light train separation in phase-contrast microscopy.

human eye with a high-contrast image of unstained objects. Zernike reasoned that if he could alter the phase difference between the diffracted and background light, he could produce interference effects that would lead to amplitude changes in the image. For example if the cytoplasm retarded the phase by 1/4 λ and he could artificially retard the diffracted light by another 1/4 λ, the cytoplasm would then differ from background by 1/2 λ; as we noted in Chapter 8, this would cause destructive interference. The expected result is that the cytoplasm would now be visibly darker than the background. This is in fact the case. A critical problem facing Zernike was how to alter the phase difference between the background and diffracted light. This separation of the two light trains was accomplished by inserting an annular aperture (Fig. 9.6) similar to a dark-field stop in the condenser. This aperture is of such size that it provides a hollow cone of light that can be collected by the objective. The diffracted light is spread out all over the objective lens, but the background light is limited to the hollow cone. The separation is not complete, but the system works, although certain defects of the phase-contrast image have their origin in this circumstance. A phase shifting plate is placed on the back lens of the objective. In positive (dark) phase contrast the background light is channeled through an optically thin ring in the plate (Fig. 9.6). The relative thicknesses of the two areas of the phase shifting plate are such that the background light acquires another 1/4 λ lead over the diffracted light. This is the 90 + Ve phase plate system. In negative

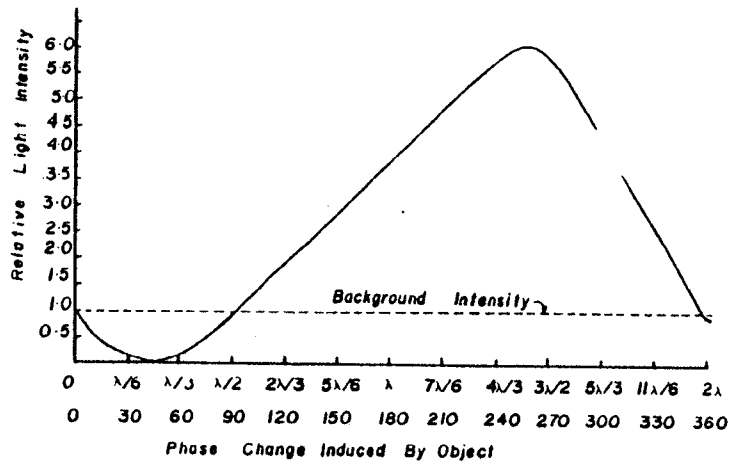


FIG. 9.7. Graphic illustration depicting the effect of phase difference induced by object on the intensity of the image in a positive phase-contrast microscope, zero phase plate absorption. (From Ross, 1967)

phase contrast the reverse of the above situation holds, and a thin object will appear light against a dark background (90 - Ve plate).

When you look into a positive phase-contrast microscope you see hues that may range from bright white to deep black. These gradations depend on two things: (1) the construction of the microscope, especially the amount of absorption of the phase ring, and (2) the phase changes induced in the diffracted light by the structures contained in the particular specimen being observed, i.e. the object detail. Figure 9.7 illustrates the effect of phase change variation induced by objects of varying phase difference. Note that there are three points where the specimen has the same intensity as the background light. These are called match points; with a positive phase-contrast microscope with zero phase plate absorption these match points occur at phase differences of 0 λ, λ/2, and 2 λ. As previously stated the phase difference, ϕ, is a function of the thickness of the object and the difference in index of refraction between the specimen and the mounting medium. Figure 9.7 represents the case where the phase plate in the objective has zero percent absorption. However, under this condition the phase plate is very difficult to see in the focusing telescope, and alignment of the system is painful if not impossible. Thus most manufacturers make phase plates with varying but rather high percentages of absorption. This produces a more visible phase plate, but the higher the phase ring absorption, the thinner (optically) the object that yields maximum image darkness. Additionally the range of object phase differences that will provide images darker than background is simultaneously restricted. These properties are illustrated in Figure 9.8. Most manufacturers supply phase-contrast microscopes with a phase plate absorp-

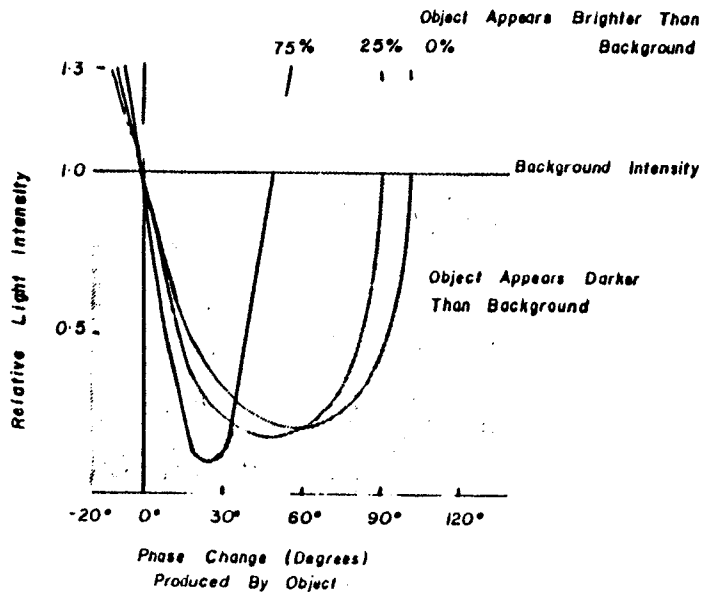


FIG. 9.8. Relationship between the phase change produced by an object and the intensity of its image when viewed under phase contrast with ordinary 90 + Ve phase plates with absorptions of 0, 25, and 75%. (Ross, 1967)

tion of 75% or more. This construction is particularly suitable for work with thin tissue sections (optically and geometrically) and a small range of phase difference. On the other hand a small percentage absorption is better for plant tissue, since plant tissues generally are not cut as thin as animal tissues and have cell walls of high phase difference plus inclusions such as starch grains that are optically thick. A 50% or even 25% absorption, depending on the application, could be advantageous. Also low absorption plates have fewer halo and shading-off defects, which can also be an advantage in the plant sciences.

Disadvantages

In addition to the nonlinear relationship between phase difference and image intensity two other complexities of phase-contrast microscopy are the halo and shading-off effects. The halo effect is noticeable in all phase-contrast images. Objects that appear darker than background are surrounded by a ring of light termed the *halo*. The shading-off defect becomes apparent when viewing a flat object of uniform thickness. In this case the outer boundaries appear darker than the center of the object.

Both the halo and shading-off artifacts are introduced in the image by the incomplete separation of the background and diffracted light. As we have shown, the background light is completely limited to the ring area of the phase plate, but the diffracted light is actually spread out over the entire phase plate area. This causes an unresolved reverse image of the object that is superimposed on the main images. This in turn results in the halo effect, which is particularly prominent at the boundaries of objects that have indices of refraction that differ greatly from the mounting medium. However, if the halo effect is not too intense, it provides a type of contrast that can be useful in some situations.

INTERFERENCE

Interference microscopy is an important quantitative tool for biological research. It can be used to measure the dry mass of the unstained cell (living or fixed) or of cellular components such as cell walls, nuclei, and nucleoli. It has a sensitivity of about 10^{-12} g and is especially good at measuring changes in dry mass like those that occur in enzymatic reactions. The instrument may also be used for qualitative observations of unstained cells where it can provide images of high contrast and striking topographic relief. In the qualitative mode of operation it is often complementary to phase-contrast microscopy in that it usually works better on subjects that are not well handled by phase, e.g., optically or geometrically thick specimens such as cell walls and certain plastids. Furthermore, it has the capacity to "optically section" thick subjects. Where it can be used, the interference microscope has four advantages over phase contrast: (1) it does not have the halo and shading-off artifacts; (2) the amount of contrast can be varied to suit the subject; (3) interference colors can be used to provide color contrast; and (4) higher numerical apertures may be employed, especially in the Nomarski differential interference contrast system.

There are numerous types of interference microscopes (Hale, 1958), but only three will be discussed here: the split-beam polarizing interference microscope, the Nomarski differential interference contrast microscope, and the Mach-Zehnder interference microscope (double-objective).

Polarizing Interference Microscope

This instrument is primarily a measuring device. Figure 9.9 is a diagram of the optical train. The specimen must occupy no more than a third of the microscope field. This means that many specimens must be double-sectioned before a small enough object for examination is attained. Thus, to obtain a cross section, a longitudinal section is cut first. Alternatively a suitable object may be mechanically separated from a section by means of a micromanipulator. The polarizing interference microscope is ideal for unicellular organisms or for examining macerations of multicellular tissues. With the improved enzymatic maceration techniques for living tissues and cell culture

$\Delta_{m.}$ and $\Delta_{n.}$ respectively. We can algebraically partition the optical path differences thusly: $\Delta_{m.} = \Delta_{m_1} + \Delta_{m_2}$. The observed optical path difference in medium m is the sum of the OPDs for substance 1 and substance 2, where

$$\Delta_{m_1} = (N_1 - N_m)\,\ell_1$$
$$\Delta_{m_2} = (N_2 - N_m)\,\ell_2$$

Similarly $\Delta_{n.} = \Delta_{n_1} + \Delta_{n_2}$. For solution of our problem we restate these equations:

$$\Delta_{m.} = \Delta_{m_1} + \Delta_{m_2} = [(M_1/A)\chi_{m_1}] + [(M_2/A)\chi_{m_2}]$$
$$\Delta_{n.} = \Delta_{n_1} + \Delta_{n_2} = [(M_1/A)\chi_{n_1}] + [(M_2/A)\chi_{n_2}]$$

Solving these equations simultaneously we arrive at the following equations

$$\frac{M_1}{A} = \frac{\Delta_{n.}\chi_{m_2} - \Delta_{m.}\chi_{n_2}}{n_1 m_2 - m_1 n_2}$$
$$\frac{M_2}{A} = \frac{\Delta_{m.}}{m_2} - \left(\frac{\chi_{m_1}}{\chi_{m_2}}\right)\left(\frac{M_1}{A}\right)$$

Also

$$\ell_1 + \ell_2 = \frac{\Delta_{m.} - \Delta_{n.}}{N_n - N_m}$$
$$F = 1 - \left[\frac{\Delta_{m.} - \Delta_{n.}}{(N_n - N_m)\,\ell}\right]$$

The masses of three or more substances may also be determined by algebraic extension of these methods, but the use of a computer is advised.

There are several assumptions and difficulties in the techniques presented in this section, and these have been reviewed in the literature (Davies, 1958; Krug et al., 1964; Lange, 1958; Ross, 1967). The reader is strongly advised to consult the literature before embarking on quantitative microscopical studies. It is a cardinal principle of quantitative microscopy that whenever possible, measurements are made in at least two different ways so the results can be checked. For example, the use of microspectrophotometry and interference microscopy constitutes an excellent combination.

Nomarski Differential Interference Contrast

Nomarski differential interference contrast (NDIC) is a qualitative mode of microscopy that produces high-contrast images of unstained transparent specimens in astounding three-dimensional array. It also provides excellent surface observations of opaque specimens with reflected light systems. A number of optical companies now include this equipment in their manufacturing programs; the optics can be designed as accessories that adapt to almost all models of bright-field, phase-contrast, or polarizing microscopes.

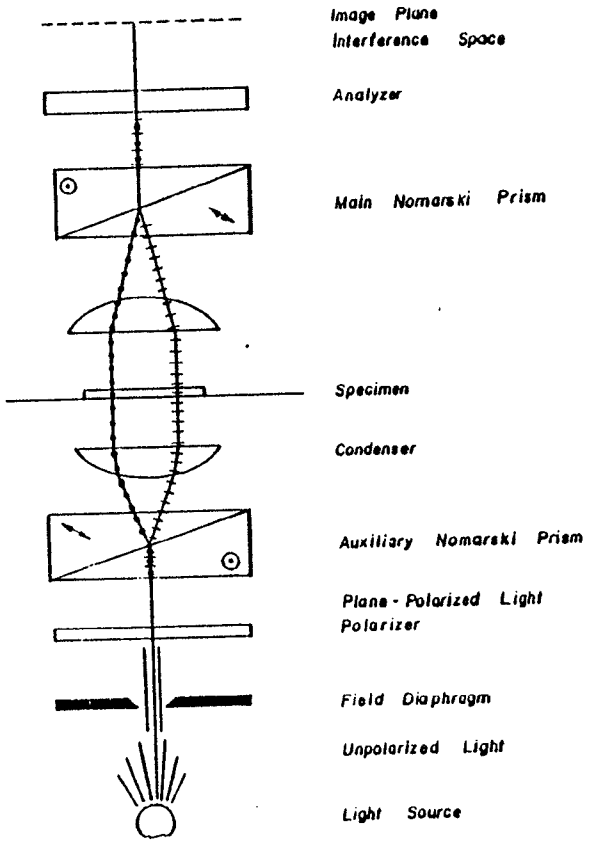


FIG. 9.11. Schematic diagram of light path in Nomarski differential interference contrast microscopy.

Basically the required accessories consist of a polarizing filter, an interference contrast condenser, and a prism-analyzer combination plate that effectively enters the optical train at the upper focal plane of the objective. The polarizer of the polarizing microscope may be used instead of the polarizing filter.

The microscope functions in the following manner (Fig. 9.11). The polarizer provides plane-polarized light incident on the NDIC condenser. In addition to the standard condenser elements the NDIC condenser contains a modified Wollaston prism (auxiliary prism) of a design first proposed by Nomarski (1954, 1955). The design is such that this prism functions as if it were located in the lower focal plane of the condenser; it divides the plane-polarized light into two rays—the ordinary ray (O-ray) and the extraordinary

ray (E-ray). These light rays are polarized at right angles to each other and travel parallel and very close to each other through the specimen. They are then recombined by the second Nomarski prism (main prism) which is functionally situated in the upper focal plane of the objective.

The beauty of the Nomarski prism is that its interference plane is outside the prism. The actual back focal plane of a high-aperture objective lies below the thread seat, and it would be impossible to position a prism there. By suitable orientation of the Nomarski prism, its interference plane can be made to coincide with the back focal plane of the objective even though the prism is physically located well above the objective. The same advantage holds for the auxiliary prisms in the condenser.

After being recombined by the main prism, the two light rays have vibration planes that are perpendicular and thus do not interfere with each other. The light is then transmitted to the analyzer which makes the two vibration planes parallel to each other and permits the interference of the two rays.

The pseudo-three-dimensional effect of NDIC arises from the fact that as the two beams (E-ray and O-ray) travel through the specimen they are very close to each other, resulting in a stereoscopic effect. The degree of "topographic" relief is a function of refractive index differences at the specimen's boundary surfaces. Although the NDIC optical technique does not require any special objectives or oculars, it is important that the optics be strain free in order to secure maximum contrast. This can be checked with an ordinary polarizing microscope. Cross the polarizer and analyzer of the instrument and view through the objective of interest. It should not affect the dark-field condition in any way if it is indeed strain-free. Usually the same main prism (beam-combining prism) is used for all objectives; three auxiliary prisms are necessary to accommodate the range of available objectives (usually one prism each for low-, intermediate-, and high-power objectives). The auxiliary prisms are most conveniently mounted on a revolving turret such as in Zernike phase-contrast; in fact, since the two methods are complementary, it is expedient to have a condenser with revolving disks that contain both phase-contrast annuli and Nomarski prisms.

A relatively systematic procedure for using NDIC equipment is as follows. First align the microscope for Kohler illumination on a suitable preparation. Remove the specimen and introduce the proper auxiliary prism for the selected objective into the optical train. Temporarily replace the desired objective with a 3.5X objective and insert a phase telescope into one of the eyepieces. Rotate the analyzer to the center of its excursion and insert into the system. Next focus the telescope and rotate the polarizer until the interference fringes are sharply focused in the back lens of the objective. Now rotate the analyzer until the center fringes are centered in the telescope field. Now turn to the proper objective and focus on the specimen. The instrument is set for minimal interference. At this point rotate the analyzer until the desired contrast is attained. The use of white light permits the use of color contrast, and interference filters provide shadow contrast. Additional compensators such as a red-one plate can be employed and provide dramatic color contrasts in some specimens.

The NDIC is generally more effective than phase-contrast on specimens of large path difference (up to 1 λ); the latter is best suited for thin sections with small path differences. In most cases it is advisable to try both systems and visually select the method that works best for a given specimen. The high apertures and shallow depth of field with NDIC permits "optical sectioning" of the specimen while the color contrasts provide "optical staining."

Double-Objective Interference Microscope

This microscope is based on the "round-the-square" interferometer system first suggested by Mach and Zehnder in 1891. The application of this system was suggested by Horn (1958) and is commercially manufactured by the Leitz optical firm. The schematic for this instrument is presented in Figure 9.12. The light from the light source is split into two coherent and equal beams by the beam splitter S. One beam is directed onto mirror M_2 where it is reflected through one microscope of this twin microscope system. This beam traverses the object. The second beam is directed onto mirror M_1 where it is projected through the second microscope. This beam serves as a reference beam and does not pass through the object. The measuring and reference beams are recombined by the beam combiner C (Fig. 9.12).

The Mach-Zehnder system achieves a wide separation between measuring and reference beams (62 mm), and thus measurements of phase difference can be made at any point in the microscopic field. The reference beam may be passed through an empty slide (slide + mounting medium + coverslip), or a

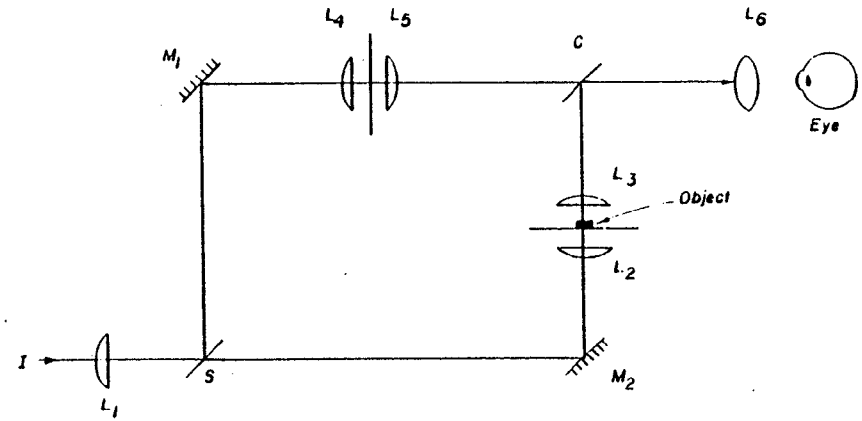


FIG. 9.12. Schematic diagram for Mach-Zehnder type of interference microscope: L_1, lamp condenser lens; L_2, condenser lens of microscope 1; L_3, objective of microscope 1; L_4 condenser of microscope 2; L_5, objective of microscope 2; L_6 ocular; S, beam splitter; C, beam combiner; M_1, mirror; M_2, mirror.

special device may be used to compensate for specimen thickness. With this instrument measurements of phase difference may be made in two ways: by the extinction transfer system or some variation (e.g. homogeneous field procedures) and by band deviation. In this second method a control band is not spread over the entire field (infinite band width) but several bands are allowed to cross the field in parallel. In the band field with monochromatic light the distance between two neighboring dark bands corresponds to a phase difference of one wavelength. If a specimen is placed in the measuring beam (and a suitable control in the reference beam), the bands will be deviated by the specimen structure; the amount of this deviation is equal to the phase difference, which can be measured directly with an ocular micrometer. However, a more accurate measuring procedure is to take a picture of the field and analyze the negative with a densitometer.

Anisotropic as well as isotropic specimens may be measured with this instrument, but as in the polarizing interference microscope an anisotropic specimen must be properly oriented before measurements are made. Push-in polarizing filters are used for this purpose, and the principal refractive index is set parallel to the polarizer. Measurements are then made with the analyzer removed from the light path.

POLARIZED LIGHT

For the following discussions of polarized light microscopy we are greatly indebted to a number of sources, especially to an excellent text on optical crystallography by F. Donald Bloss (1961), *Introduction to the Methods of Optical Crystallography*. The reader is referred to this eminently readable book for more extensive and detailed information on the topics covered here. Other valuable references are Hartshorne and Stuart (1960), Preston (1952, 1974), Wahlstrom (1969), and Winchell (1937).

The chief constituent of higher plant cell walls and the one that provides the bulk of the mechanical strength of the wall is cellulose. This substance is crystalline and thus can be effectively studied, both qualitatively and quantitatively, by means of the polarizing microscope. Cellulose has both intrinsic and form birefringence, which will not be considered here but which can be found in the texts on crystallography. Besides cellulose there are other important crystalline or partially crystalline substances in plants, such as nucleic acids and starch. Thus polarized light microscopy can be a powerful tool in studies of cell structure and function.

A polarizing microscope is almost identical to a normal bright-field microscope except that in addition it has a polarizer, an analyzer, and in some cases a slot for compensators used for specific measurements or effects. To be effective a polarizing microscope should also have a circular revolving stage. The following sections depend heavily on the reader's grasp of the material on the principles of optics covered in Chapter 8, and it may be helpful to reread that chapter before embarking on the discussions below.

Figure 9.13 is a diagram of a polarizing microscope illustrating the function and light flow in the instrument. Light is directed on the polarizer

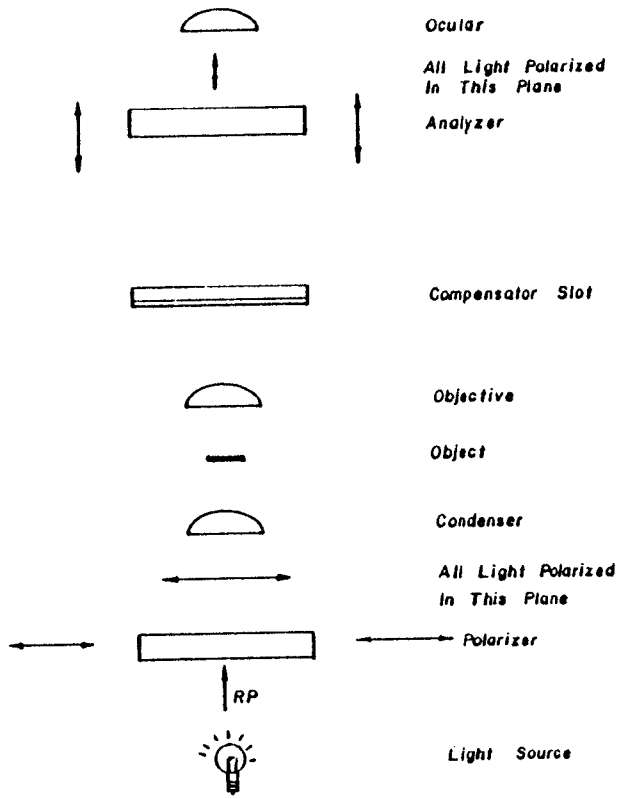


FIG. 9.13. Functional elements of the polarizing microscope.

which polarizes the light in a plane parallel to the paper with vibration direction perpendicular to the ray path (RP). The plane polarized light then successively passes through the condenser, object, and objective and is incident on the analyzer. In this diagram the analyzer is "crossed" with respect to the polarizer. The analyzer is really identical to the polarizer in every way except for position in the light train, and in this case its principal direction is positioned at right angles to that of the polarizer. This situation is termed crossed polaroids (or crossed nicols if nicol prisms are used); if the specimen is isotropic, the entire field of view will be dark (Fig. 9.14A). If the analyzer or polarizer is rotated (Fig. 9.14B), the trigonometric component from the polarizer in the plane of the analyzer will be transmitted by the analyzer (see Chapter 8). Similarly if a crystalline specimen observed between crossed polaroids rotates the plane polarized light (is anisotropic), the trigonometric component in the plane of the analyzer will be transmitted by the analyzer (Fig. 9.14C).

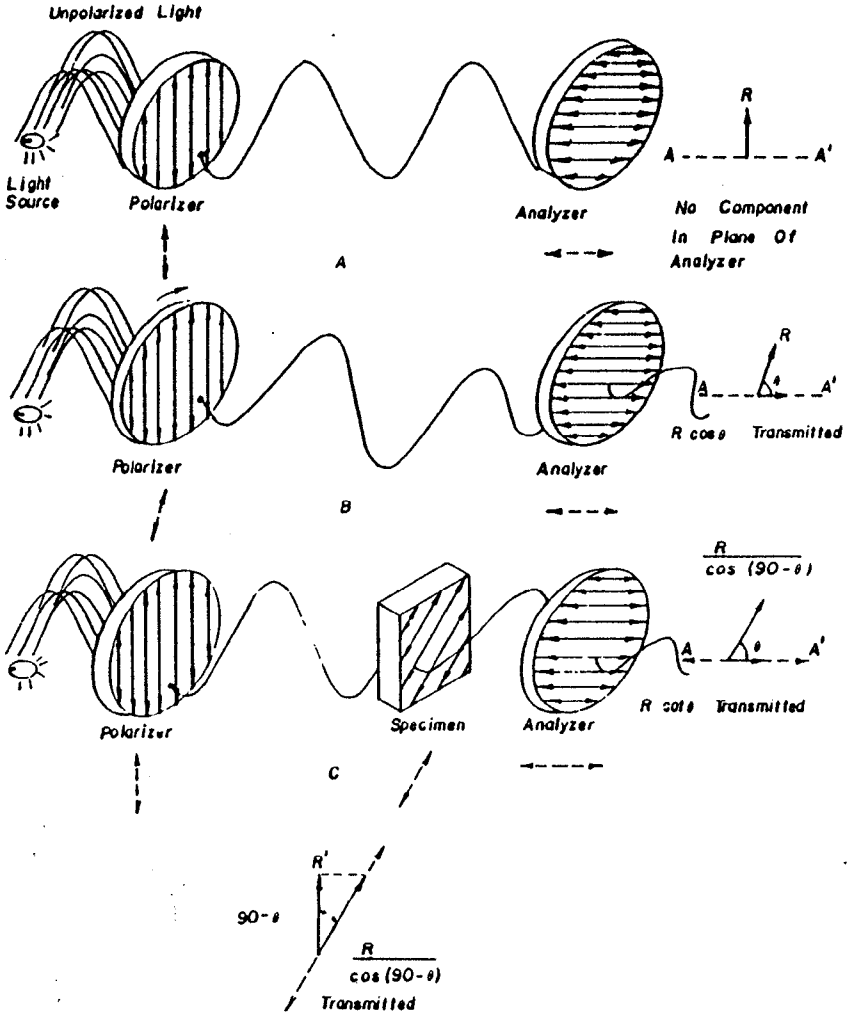


FIG. 9.14. Diagram of polarized light transmission by polarizer and analyzer: A, crossed polaroids. The field of view will be dark because the light transmitted by the polarizer (R) has no trigonometric component in the plane of the analyzer. B, The polarizer has been rotated so that the light transmitted by it (R) has a trigonometric component ($R \cos \theta$) in the plane of the analyzer. Thus the field of view will be light. C, The plane polarized light from the polarizer (R) is rotated by the crystalline specimen and a component $[R/\cos (90 - \theta)]$ is transmitted to the analyzer, whereupon another trigonometric component ($R \cot \theta$) is transmitted, producing a bright field of view.

If you rotate this anisotropic specimen by 360° there will be four dark-field conditions. If the specimen is aligned so that its principal direction is parallel to the polarizer, the analyzer will block out all the light transmitted by the specimen (this happens twice during a 360° rotation of the specimen). When the principal direction of the specimen is aligned parallel to that of the analyzer, the specimen blocks out all the light coming from the polarizer (this also happens twice during a 360° rotation).

There are two types of specimens observed with the polarizing microscope—isotropic and anisotropic. Isotropic materials have identical properties in all directions—that is, they have only one index of refraction. Anisotropic specimens, on the other hand, do not have identical properties in all directions, and these materials may be further subdivided into two types. The first type (called uniaxial) has two indices of refraction, and the second type (called biaxial) has three indices of refraction. It may seem somewhat surprizing that a substance can have more than one index of refraction, since Snell's law—the basic principle of optics—contains no provision for this. However, if one considers wave normals (Chapter 8) instead of ray paths, Snell's law still holds.

In polarized light microscopy we wish to determine the structural anisotropy that is revealed in the optical anisotropy. However, in order to extract and translate the information contained in the optical anisotropy, we must have a systematic method of analysis. In using the powerful polarized light techniques that have been developed for this purpose, we must forget about the actual aspect of the specimen and think only of symbols inscribed in a particular area of a specimen. The symbols we shall use are the indicatrix and its cross section, the index ellipse.

Isotropic Material

In an isotropic preparation there is only one index of refraction for light traveling in any direction through the specimen. Consider a specimen illuminated by a beam of light. From a central point think of arrows radiating out, with the length of each arrow a function of the index of refraction for light traveling through the specimen in that direction (Fig. 9.15A). By definition of an isotropic material the arrows must all be of the same length. If we connect a surface with the tips of these arrows (vectors), we obtain a sphere (Fig. 9.15B) which is termed the isotropic indicatrix. The isotropic index ellipse is thus a circle (Fig. 9.15C), which is just a special case of an ellipse where the major and minor axes are of identical length. An analysis of transparent glasses, liquids, and isometric crystals indicates that these substances fit the isotropic indicatrix concept. It should be noted that the sphere representing the isotropic indicatrix is not necessarily of constant size. That is, the index of refraction of a substance usually varies with the wavelength of light used; in general the smaller the wavelength, the greater the index of refraction, and thus the greater the diameter of the indicatrix. Furthermore, a crystalline substance displays isotropic optical properties along its optic axis. This fact can be used to determine the orientation of a crystal; e.g., one

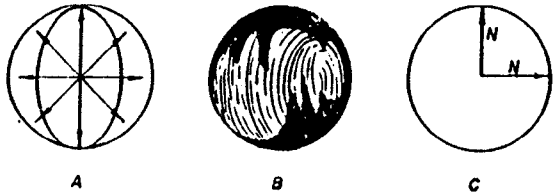


FIG. 9.15. Isotropic indicatrix: A, arrows of equal length representing equal indices of refraction in all directions; B, surface connecting the tips of the vectors forms a sphere; C, circular index ellipse for the isotropic indicatrix.

can use this property in determining the cellulose crystallite orientation within the plant cell wall.

Uniaxial Indicatrix

Plant cell walls behave optically as if they were a uniaxial material (see Berlyn, 1964, 1970). Thus we will consider the orthoscopic examination of these materials in some detail and give several methods of measurements, some of which (Senarmont method), will be applicable to interference microscopy as well. Orthoscopic examination refers to the "normal" microscope image as opposed to conoscopic examination, which refers to observation of interference figures in the back lens of the objective. Conoscopic observations are made with the use of a Bertrand lens; however, this mode of microscopy is not amenable to most plant material and finds its maximum use in mineralogy so will not be dealt with here.

The immersion refractometric measurements of index of refraction are extremely accurate, especially if a phase-contrast microscope is used. However, for discussion purposes we will consider an approximate evaluation procedure, often called the Duke de Chaulnes method. An understanding of this procedure provides insight into the basis of birefringence. Consider a flat isotropic specimen being observed in a microscope. If we first focus the 10X (0.25 NA) objective on top of the glass slide to one side of the specimen and then move the specimen into view and focus on its upper surface, racking the focus up through the specimen, we can determine a thickness, ℓ, from the difference in drum readings on the fine-focus knob. Racking down through the specimen and focusing on top of the glass slide just below the bottom of the specimen, we obtain a measurement, ℓ_a, the apparent thickness. It will be less than ℓ because the index of refraction of the specimen is normally greater than that of air. Figure 9.16 depicts the geometry of the situation. In this case the angle of incidence i occurs within the specimen, whose index of refraction is therefore designated N_i. The angle of refraction at the upper surface, S, of the specimen is r, and the index of refraction of air is $N_r = 1.0$.

$$\tan r = OS/O\ell'$$

$$\tan i = OS/O\ell$$

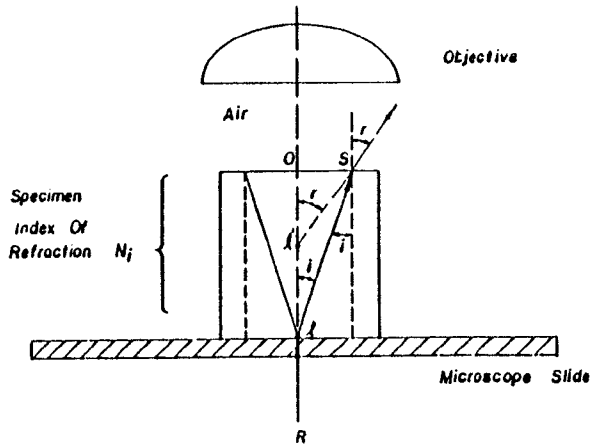


FIG. 9.16. Chaulnes method for determination of index of refraction.

where $O\ell' = \ell_a$ = apparent thickness
$O\ell = \ell$ = geometric or "true" thickness

Therefore:

$$\tan r = OS/\ell_a, \qquad \tan i = OS/\ell$$

$$OS = \ell_a \tan r, \qquad OS = \ell \tan i$$

therefore:

$$\ell_a \tan r = \ell \tan i$$

$$\ell/\ell_a = \tan r/\tan i, \text{ which for small angles} \cong \sin r/\sin i$$

From Snell's law

$$N_i \sin i = N_r \sin r$$

$$N_i = N_r \sin r/\sin i = \sin r/\sin i = \ell/\ell_a$$

where ℓ and ℓ_a are measured in drum units of the fine-focus knob.

Now let us consider what happens if we replace the isotropic specimen with an anisotropic one such as a calcite crystal. The light is divided into two rays by the specimen—the ordinary ray and the extraordinary ray. The ordinary ray is so termed because its ray path follows Snell's law as a consequence of the fact that the light of the ordinary ray vibrates perpendicular to the ray path. In this case the normal and ray path are identical. The extraordinary ray does not follow Snell's law, although as discussed previously its wave normal does. In the extraordinary ray (E-ray) the light vibrates at right angles to that of the ordinary ray (O-ray). Furthermore, the O-ray light vibrates at right angles to the plane containing the O-ray and the optic axis, while the E-ray vibrates within this plane.

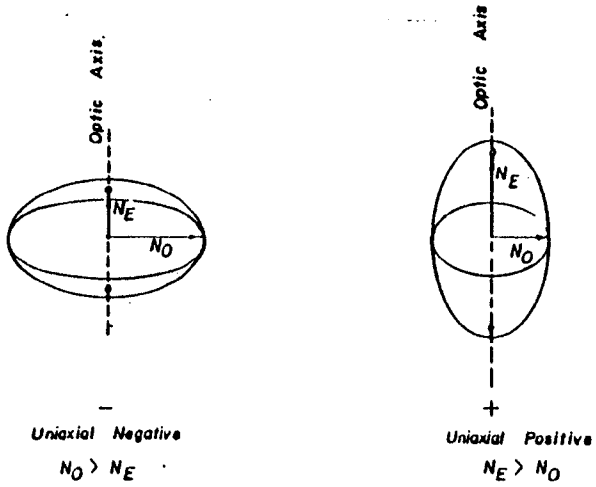


FIG. 9.17. Positive ($N_E - N_O > 0$) and negative ($N_E - N_O < 0$) uniaxial indicatrices. The optic axis of the indicatrix corresponds to the *c* axis, the vertical crystallographic axis.

If an India ink dot is placed on the upper surface of the slide just below the specimen at point ℓ in Figure 9.16, an image of two dots would appear within the calcite crystal. The dot image formed by the O-rays would appear to be closer to the upper surface (closer to point *O* in Fig. 9.16) than the dot imaged by the E-rays. Thus the apparent thickness (ℓ_O and ℓ_E) and therefore the index of refraction for the two rays is different. If N_E is the index of refraction of the E-ray and N_O is the index of refraction of the O-ray, we have the following relationships: $N_E = \ell/\ell_E$, and $N_O = \ell/\ell_O$. In the case described $N_O > N_E$.

This is a demonstration of the phenomenon of birefringence—the possession of two indices of refraction by a single specimen. With two major indices of refraction it should be obvious that the uniaxial indicatrix is not a sphere but an ellipsoid (Fig. 9.17). There are two types of uniaxial indicatrices—positive and negative. In the negative uniaxial indicatrix N_E is the minimum index of refraction and N_O is the maximum. Just the opposite is the case for the positive uniaxial indicatrix; N_E is the maximum and N_O is the minimum. Together N_E and N_O are termed the principal indices of the uniaxial indicatrix. The plane cut parallel to these indices is the principal plane and forms an ellipse with N_E and N_O as the semiaxes. Note that in Figure 9.18 there are three planes that can be passed through the uniaxial indicatrix: the principal plane, the circular plane, and a random plane. The principal plane contains the optic axis and thus will always have both the maximum (N_E) and minimum (N_O) indices of refraction as the semiaxes of the index ellipse. A section cut parallel to this plane will show the maximum birefringence. In the case of the circular plane, light traveling along the

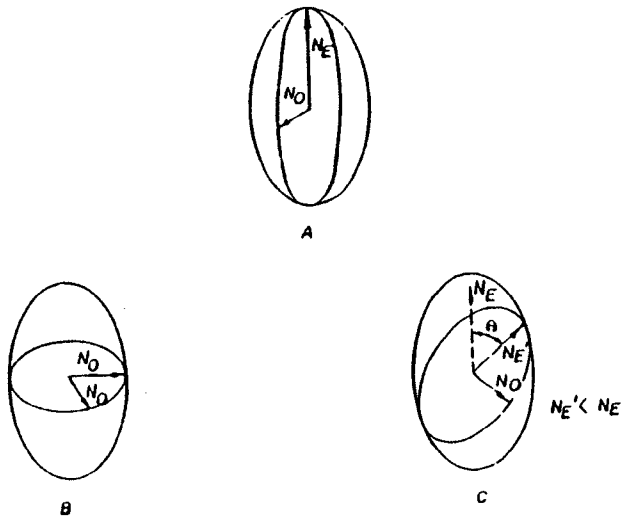


FIG. 9.18. Principal planes of the uniaxial indicatrix. A, principal plane; B, circular plane; C, random plane.

optic (its ray path coincident with the optic axis) vibrates along a radius of the circular section (its polarization direction is along a radius). All these radii are of equal length, N_O, so that there is no privileged direction and no birefringence results. The first two planes are unique; the random plane is one of many random planes that can be passed through the uniaxial indicatrix. In every case N_O remains an invariant; in the random elliptical plane the larger semi-axis is $N_{E'}$ and varies between N_O and N_E—that is, $N_O < N_{E'} < N_E$. $N_{E'}$ may assume all possible values between N_O and N_E but may not equal either limit and still be an axis of a random section. If $N_{E'} = N_O$, we have the circular plane; if $N_{E'} = N_E$ we have the principal plane. The angle between N_E and $N_{E'}$ is designated θ, and the following equation can be used to calculate $N_{E'}$: $N_{E'} = N_O N_E / \sqrt{N_O^2 \cos^2\theta + N_E^2 \sin^2\theta}$ (cf. Bloss, 1961, p. 74).

Remember that the index of refraction is inversely related to the velocity of light in the direction of the index; therefore, in negative uniaxial crystals the E-ray travels faster than the O-ray, while in positive uniaxial crystals the O-ray travels faster than the E-ray. Both light and the chemical bonds of a specimen are electrical in nature and thus interact with each other, producing the observed decrease in the velocity of the light that occurs within the specimen. For light vibrating parallel to the larger axis of a molecule, the charge separations induced by the light vibrations in consecutive atoms or molecules reinforce each other. However, if the light vibrates at right angles (orthogonally) to the larger axis, the induced dipole in one atom opposes that in the adjacent one. This dual sequence generates the two indices of refrac-

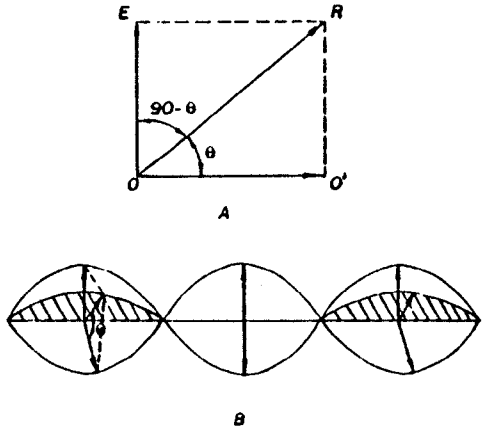


FIG. 9.19. Interference of light polarized in perpendicular planes: A, vector diagram; B, three-dimensional ray diagram. OE = E-ray, OO' = O-ray; OR = resultant ray; θ = angle between a ray and resultant.

tion, and a substance with two indices of refraction is said to be birefringent—mathematically defined as $N_E - N_O$.

Earlier in this chapter we considered the interference of light waves traveling in the same plane. Now let us consider the interference of two light waves polarized in perpendicular planes such as the E- and O-ray. The resultant ray produced can be defined by the vectorial addition of the component rays (Fig. 9.19). When a plane polarized light ray enters a uniaxial crystal along some direction other than the optic axis, the ray is immediately divided into a slow wave (On) and a fast wave (ON), with index of refraction N_α for the fast wave and N_γ for the slow wave where $N_\gamma > N_\alpha$. At the moment of incidence the waves are exactly in phase, but when they emerge at the upper surface of the specimen they may not be in phase at all. When we discussed interference microscopy we derived a path difference $\Delta = (N_s - N_m)\ell$ where N_s = index of refraction of the specimen, N_m = index of refraction of the mounting medium, and ℓ = thickness of the specimen. An analogous situation holds for polarized light, and the path difference is $\Delta = (N_\gamma - N_\alpha)\ell$. The path difference determines whether the resultant wave emerging from the upper surface of the crystal is linearly polarized—$\Delta = n\lambda, \ldots [(2n + 1)/2]\lambda$, circularly polarized—$\Delta = [(2n + 1)/4]\lambda$, or elliptically polarized (general case)—$\Delta \neq [(2n + 1)/2]\lambda$ or $[(2n + 1)/4]\lambda$.

Linear (Plane) Polarization

There are two cases of plane polarized resultants to consider. In the first, the path difference between the slow wave and fast wave at the point of emergence of the slow wave from the upper surface of the specimen is

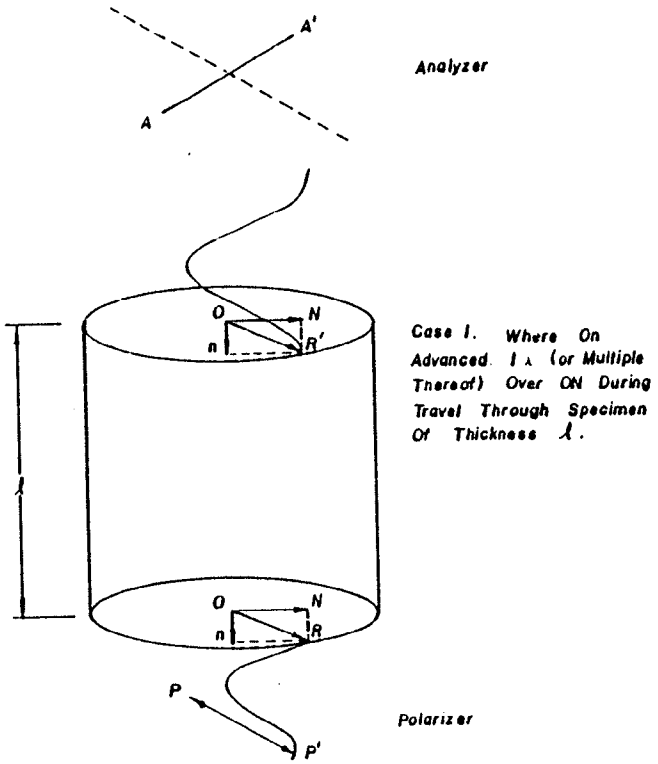


FIG. 9.20. Plane polarized resultant in phase. Crystal is set on rotating stage at 45° off extinction, which gives maximum darkness under crossed polaroids because OR is vibrating in same direction as light from the polarizer and will thus be extinguished by the analyzer.

$\Delta = 0\lambda, 1\lambda, 2\lambda, \ldots n\lambda$. In this situation ON and On have exactly the same orientation at the top of the specimen as they do at the bottom (Fig. 9.20). The ON and On depicted on top of the specimen are exactly superimposed over the ON and On depicted on the bottom of the specimen. The resultant ray R will be vibrating in exactly the same plane as the light from the polarizer and thus will not be transmitted by the analyzer.

In the second case the path difference is $\Delta = 1/2\lambda, 3/2\lambda, \ldots [(2n + 1)/2]\lambda$. In other words the fast ray and the slow wave are exactly out of phase at the top of the specimen. In this case ON at the top is superimposed over ON at the bottom while On at the top is reversed in direction from On of the bottom surface (Fig. 9.21). The resultant ray R in this case

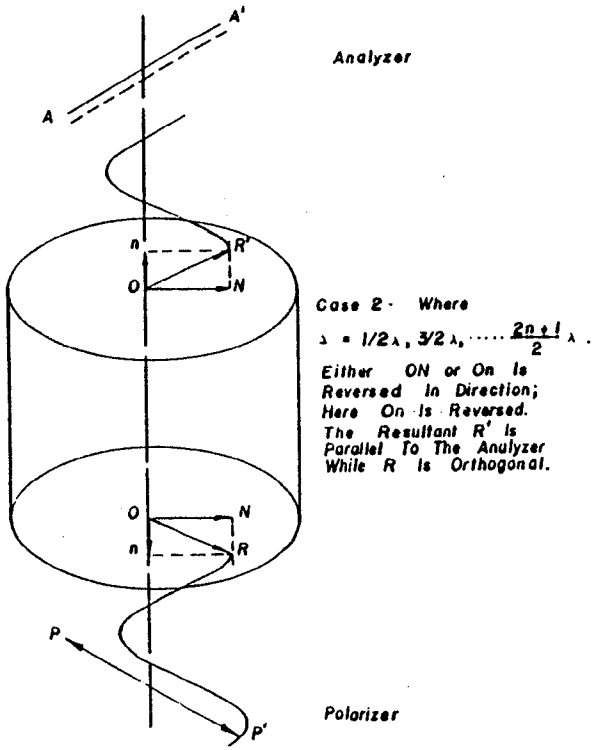


FIG. 9.21. Plane polarized resultant out of phase. Crystal is again at 45° off extinction, but in this case maximum brightness occurs because OR' is vibrating in the exact direction of the analyzer.

will be crossed with respect to the polarizer, i.e., will be transmitted by the analyzer.

Circular Polarization

This type of polarized light results when the path difference during the passage of light through the specimen is 1/4 λ, 3/4 λ, . . . [(2n + 1)/4] λ. The vibrations of the fast ray and slow ray interfere in space time in such a way as to result in vibration vectors of variable direction (azimuth) but equal magnitude (Fig. 9.22). Within the crystal there are two orthogonally polarized waves, but the figure shows only the resultant wave. Above the crystal where the waves are free to interfere you actually have this arching vibration pattern. If a surface is made by connecting the tips of the vectors, you would generate a circular cylinder.

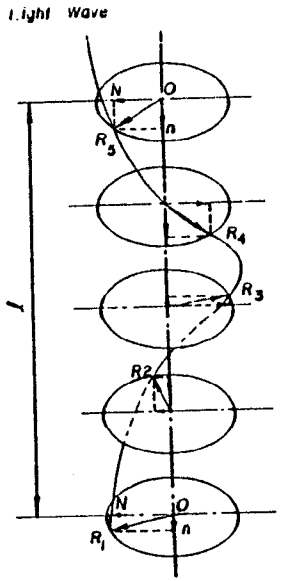


FIG. 9.22. Circular polarization of light. $R_1 = R_2 = R_3 = R_4 = R_5$; ON = slow wave, On = fast wave, ℓ = thickness of specimen.

Elliptical Polarization

This is the general case and is depicted in Figure 9.23. Here the vibration vectors change in both magnitude and azimuth. As the two light waves travel through the specimen of thickness ℓ, they develop a path difference between them that is unequal to 0 λ, 1/4 λ, 1/2 λ, 3/4 λ, 1 λ, etc. If the tips of the vectors are connected in this situation, an elliptical cylinder results.

It is possible to interconvert the various forms of polarized light by the use of compensators that alter the path difference.

We can summarize some of the elementary principles that should be kept in mind when interpreting observations made with the polarizing microscope: Points of darkness in the field of view may be:

1. Points where the specimen is isotropic
2. Points where the polarized light does not pass through the specimen—e.g., the lumina of vessels and tracheids
3. Points where the light travels along the optic axis of an anisotropic portion of the specimen
4. Points where the specimen is anisotropic but aligned parallel to the polarizer
5. Points where the specimen is anisotropic but aligned parallel to the analyzer

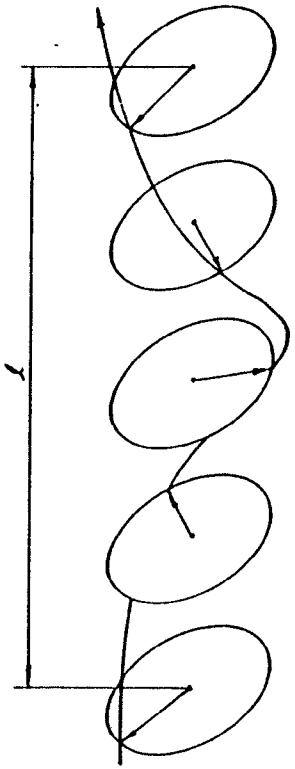


FIG. 9.23. Elliptical polarization of light.

To find the point of maximum brightness, place the specimen point between crossed polaroids and rotate the stage to maximum darkness. If the stage is then rotated 45°, the specimen point will be at maximum brightness. The true maximum brightness of the specimen is obtained only when you have a section cut parallel to the principal section so that you have the maximum mathematical birefringence ($N_E - N_O$).

Interference Colors

We mentioned in discussing the isotropic indicatrix that the index of refraction varies with the wavelength of light in a given substance. Suppose you are observing a point in a specimen under crossed polaroids using an interference filter to provide green monochromatic light of 546 nm. If the specimen point has a path difference of 273 nm, all this green light is imparted with a path difference of 273/546 = 1/2 λ. Thus the green light is rotated 90° from the plane of the polarizer and all of it will pass through the

analyzer. The specimen point will appear green. If you remove the filter and let white light in, the situation is changed. The specimen point still has a path difference of 1/2 λ for the green light, but all the other wavelengths in the white light will be retarded more or less than 1/2 λ and will not have maximum transmission through the analyzer. However, if the light has a strong output in the green, the specimen may well appear green as before. At another point in the specimen the situation might be different, and in this case yellow light might have a path difference of 1/2 λ. This is why precise quantitative work in polarized light often requires the use of monochromatic light.

In polarized light microscopy observations are often made between crossed polaroids with the specimen at 45° off extinction. In this case the percent of light transmitted by the analyzer is given by the formula: $L = 100 \sin^2 (\Delta/\lambda\ 180^\circ)$ . . . (Bloss, 1961). Interference colors are classified by order according to particular path differences that gives rise to them. The first-order colors arise from path differences of 0–550 nm, second-order from 550 to 1100 nm, third-order from 1100 to 1650 nm, and so forth. Thus a red color can be first-, second-, third-, or fourth-order red. However, as path differences increase beyond 2300 nm, the interference colors assume a milky color and are termed high-order white. When a specimen is rotated between crossed polaroids, the interference colors can change in intensity but not in hue.

Compensation

There are two compensators commonly used in cytological work—the red-one plate (1° red, first-order red) and the Senarmont compensator (1/4 λ mica plate with its slow direction of vibration parallel to the vibration direction of the polarizer). These compensators are usually placed in the compensator slot, which in modern polarizing microscopes is positioned between the objective and the analyzer.

Red-one Plate

The red-one plate is usually constructed of gypsum (selenite) but may also be made of birefringent plastic or quartz. The birefringence and thickness of the plate are so designed that it produces a path difference (Δ) of 550 nm. That is, if two orthogonal in-phase light waves are incident on the bottom of the plate, they will be divided into a fast wave and a slow wave; after passage through the plate the fast wave vibrations will lead the slow wave vibrations by 550 nm. The usual use of this crystal plate is to locate the major and minor indices of refraction of a crystalline specimen point. It can also be used to determine signs of crystals and for the average fibril angle of tracheids and fibers (Preston, 1952).

In the usual use of the red-one plate, the specimen is placed between crossed polaroids and the red-one compensator is inserted into the system. At this point the background field will be red (tint of passage). The largest

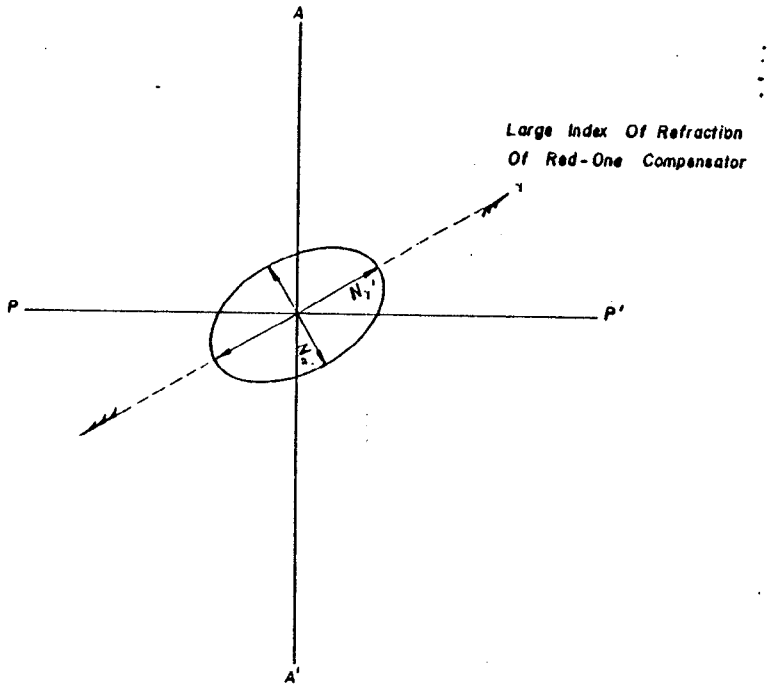


FIG. 9.24. Specimen with its largest index of refraction ($N\gamma'$) lined up with the largest index of refraction of the red-one compensator (γ).

index of refraction of the compensator is at 45° from that of the polarizer and analyzer—usually northeast to southwest as you look into the microscope. If you rotate the stage until the specimen is the same color as the background, you are at one of the extinction positions where the specimen's indices of refraction are lined up with those of the compensator (Fig. 9.24). However, the problem is to determine which index of the specimen is lined up with which index of the compensator. If the larger index of the compensator is directly above the larger index of refraction of the specimen, the slow wave will be even more retarded behind the fast wave after passage through the compensator. Say the specimen has a path difference of 100 nm; i.e., after passage through the specimen the fast wave is leading the slow wave by 100 nm. After passing through the compensator, the fast wave will lead the slow wave by 100 + 550 nm = 650 nm. This is known as addition, and if the specimen is rotated clockwise 45° off extinction it will appear as a second-order blue. If, on the other hand, the large index of refraction of the red-one compensator is directly above the smallest index of refraction of the specimen, the fast wave coming out of the specimen will be changed into the slow wave by the compensator and the path difference will be 100 - 550 = -450 nm. This is called subtraction, and if the specimen is rotated 45° off extinction it will show a yellow subtraction color.

To illustrate the use of the red-one compensator we can employ macerated tracheids from a coniferous wood such as spruce (a procedure suggested by Preston, 1952, and modified by Beckwith, 1964). Slides are prepared by coating a suitable portion with a thin film of Epon 812 resin (50% A plus 50% B) (Luft, 1961) and curing it first for 2 hours in a 50 C oven and then for 20 hours at 25 C. Precured slides can be stored by freezing but must be allowed to return to room temperature before use. Before mounting the tracheids an additional 15-minutes cure at 80 C is necessary to insure that the tracheids are adhered rather than embedded. Tracheids may be positioned on the slide from an aqueous (or better an alcoholic) solution in a single layer. This is done under the dissecting microscope. The slides are then placed in a 60 C oven for several hours or overnight. After cooling, the upper surface of the tracheids is cut away by drawing a microtome knife across the slide by hand in the same manner as if using a sharpening stone. The angle at which the knife is held is adjusted to result in the best slicing and least scraping of the cell wall material. It is necessary to remove the upper portion of the wall; otherwise the cellulose microfibrils wind around the wall, and their effects on polarized light would not be observed because the effects of the bottom layer of the wall would be cancelled by the upper layer. (Alternatively you can determine the fibril angle across the wall by embedding the individual tracheids in paraffin and serial-sectioning them.) The macerated preparations are scanned between crossed polaroids with the red-one compensator inserted. A well-sliced tracheid is located and brought to the center of the ocular cross hair. The stage is then rotated clockwise until the color of the observed wall just matches the color of the red background—and until the tracheid will turn green or blue (addition) upon further clockwise rotation. The angular reading on the vernier of the rotating stage at this point is recorded. The stage is then rotated through the smallest angle that is required to align the longitudinal axis of the tracheid with the perpendicular component of the ocular cross hair. The stage angle is again observed, and the difference between the larger and smaller angle is the crystallite orientation relative to the longitudinal axis of the cell.

Senarmont Plate

By this method it is possible to very accurately measure path differences up to one wavelength in magnitude. The Senarmont compensator is a $\lambda/4$ mica plate which differs from the usual $\lambda/4$ plates in that it is inserted into the compensator slot with its vibration direction parallel to the polarizer instead of at 45°. This compensator may be used in the polarizing microscope and in the polarizing interference microscope. Measurements of path difference can be used to calculate a number of specimen parameters such as thickness, index of refraction, and dry mass (as outlined in the sections on interference microscopy). It is also the compensator of choice for the determination of microfibrillar angle in each layer of the cell wall.

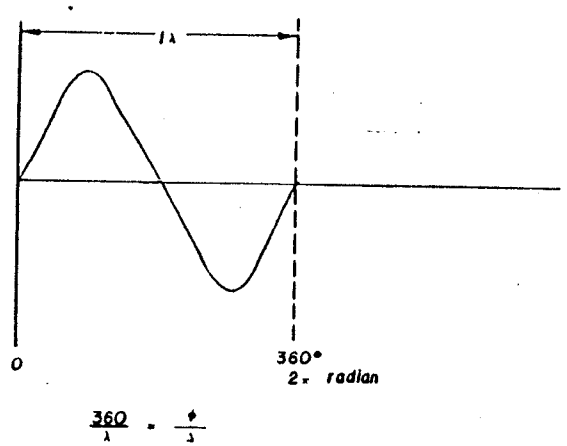


FIG. 9.25. Relation of phase angle (ϕ) to path difference (Δ).

The Senarmont compensator can still be used if the path difference to be measured is greater than one wavelength, but then it is necessary to know the number of complete wavelengths involved. This can be done by observations in white light and/or by using a rotary compensator of the Eringhaus or Berek type.

The Senarmont compensator operates on the principle of converting elliptically polarized light into linearly polarized light. The azimuth of the linearly polarized light is used to calculate the phase difference of the specimen structure of interest. A complete discussion of this compensator and its use may be found in Hartshorne and Stuart (1960), Wahlstrom (1969), Manwiller (1966), and Mark (1967).

To understand the function of the Senarmont compensator it is necessary to comprehend the relationship between phase difference and path difference (Fig. 9.25). Considering light as a sine wave, it is apparent that a phase difference (ϕ) of 360° (2π radian) corresponds to a path difference (Δ) of one wavelength (λ). Therefore, $360/\lambda = \phi/\Delta$, and $\phi = 360\ \Delta/\lambda$.

If the specimen is positioned in the optical system so that its slow wave is parallel to the vibration direction of the polarizer, the monochromatic polarized light coming from the polarizer strikes the specimen and is divided into two component waves (O-rays and E-rays) of equal amplitude. Upon passage through the specimen the two waves acquire a path difference $\Delta = N'_\gamma - N'_\alpha$, as previously defined. After emergence from the specimen the resultant vibration is in the general case elliptically polarized (Fig. 9.26). The semiaxes of the ellipse ($a/2$ and $b/2$) are diagonal to the vibration directions of the specimen. The phase difference between the ellipse axes is 90°, but the axes are of unequal magnitude unlike the equal amplitude vibrations that are the true source of the elliptically polarized light.

In practice the specimen is placed on the stage so that its slow wave

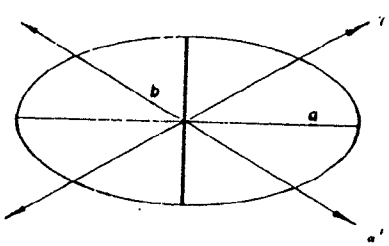


FIG. 9.26. Elliptical vibration caused by path difference of specimen ($N\gamma' - N\alpha'$). The ellipse is generated by two vibrations of equal magnitude but can be conceived of as being generated by two waves of unequal magnitude ($a/2$, $b/2$) of phase difference of 90°

(N'_γ) is parallel to the vibration direction of the polarizer, accomplished by means of the red-one compensator. When inserted, the red-one compensator has its slow vibration direction running in the first and third quadrant. Now a linear vibration results from two complementary vibrations with a phase difference of 0° or 180° ($\Delta = 0\ \lambda$ or $\Delta = 1/2\ \lambda$). Considering the elliptical vibration of semiaxes $a/2$ and $b/2$ with constant phase differences of 90°, it should be obvious that this elliptical vibration can be transformed into a linear vibration by introducing a supplementary phase difference of 90° ($\lambda/4$) which can either be subtracted or added to provide the required phase difference of 0 or 180°. This is what the Senarmont compensator ($\lambda/4$ plate) does.

The specimen is rotated clockwise until at 45° off extinction it shows a

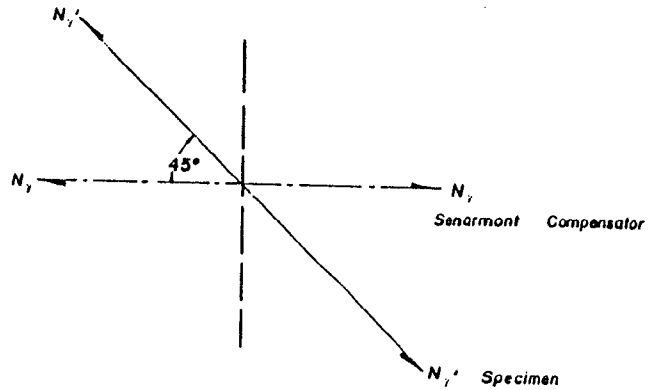


FIG. 9.27. Orientation of specimen slow direction $N\gamma'$ in relation to Senarmont slow direction $N\gamma$ when analyzer is rotated clockwise. In this position the specimen would show a subtraction color with the red-one compensator.

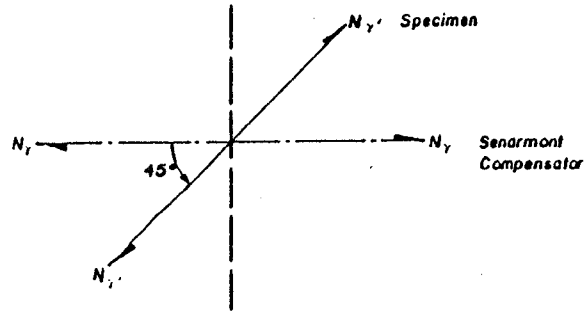


FIG. 9.28. Orientation of specimen slow direction $N\gamma'$ in relation to Senarmont slow direction $N\gamma$ when analyzer is rotated counterclockwise. In this position the specimen would show an addition color with the red-one compensator.

subtraction color. Then the red-one compensator is removed and the Senarmont compensator is inserted. Monochromatic light of the designated wavelength for the compensator must be used. This wavelength is customarily engraved on the compensator and is usually 589 nm or 546 nm. The 45° angle between the slow wave of the compensator and the slow wave of the specimen is clockwise (Fig. 9.27), and the analyzer is rotated in the clockwise direction until the specimen is at extinction (maximum darkness). This analyzer rotation, V, is equal to one-half the phase difference. If one positions the specimen point so that it shows an addition color, the direction of analyzer rotation will be counterclockwise (Fig. 9.28). Caution! The direction of analyzer rotation is not necessarily the same as the direction of rotation of the analyzer control knob!

FLUORESCENCE

Over the past decade fluorescence microscopy has probably shown more growth than any other single area of microscopy in terms of usage, primarily as the result of: (1) increased medical applications of the techniques in cytodiagnosis, (2) development of fluorescent protein (antibody, antigen) staining methods, (3) improved instrument design, (4) the provision for quantitative measurement (fluorometry), and (5) the extreme sensitivity of the method. The last reason probably accounts for the other four; e.g., the minimum concentration for a detectable fluorescent substance is about one in 10^4, and as little as 10^{-18} g may be detected. This extreme sensitivity of fluorescence detection together with the extreme specificity of immunological techniques (such as in fluorescent antibody work) provides a very powerful microtechnique.

Theory

Whenever a substance absorbs light, it takes up energy in the form of quanta. Since by the first law of thermodynamics we know that energy cannot be destroyed but can only be changed in form, we must be able to account for the fate of this energy. For one thing it can be turned into heat and eventually lost to entropy or perhaps used as activation energy for a chemical reaction. When a molecule absorbs light it is transformed into an activated state, and thus the potential energy of the system is increased. Some of this energy can be captured into chemical bonds which can be utilized for cellular work, as in photosynthesis. A number of processes may be involved in energy dissipation such as resonance radiation, Rayleigh scattering, and Raman effects (Udenfriend, 1962). Another possible fate for the absorbed light energy—the one we are concerned with here—is in reemission as luminescent light. Luminescence is a general term for light emission caused by electrical, chemical, and biological means as well as by light absorption (photoluminescence). The process of photoluminescence may be subdivided into fluorescence and phosphorescence on the basis of the time delay between the absorption and emission of light (as a consequence of specific types of electronic transitions). If this period, δ, is less than 10^{-4} seconds, we describe the photoluminescence as fluorescence; if it is greater than 10^{-4} seconds, we term it phosphorescence. Phosphorescence, unlike fluorescence, may continue for some time after the excitation source is shut down. The absorption of light is a highly specific event, and certain molecular configurations are required for the absorption of energy of specific frequency. Light energy is absorbed in discrete units or quanta, and only those absorbing molecules transformed into an excited state (an energy level above that of the "ground" level for the molecule) are capable of fluorescing. A quantum is absorbed in about 10^{-15} seconds, and the excited state persists for about 10^{-18} seconds. Just as the transition of a substance to an excited state requires energy absorption, the transition from the excited to the ground state requires energy emission. In almost all cases the wavelength of the fluorescent light is longer than that of the exciting light, because some energy is lost during absorption to other processes of energy dissipation. This observed wavelength increase is known as Stokes's law (1852). Both light and chemical bonds are electrical in nature, and the oscillating electrical field of the light sets the electrons and atomic nuclei into oscillation, which causes them to act as an antenna through which the fluorescent light can be emitted. Thus, in explanation of Stokes's law, we can say that some energy is necessarily lost in setting the antenna system into oscillation, and for this reason the emitted quanta have a lower frequency.

For fluorescence to occur the absorbing molecule must be excited to an electronic change; i.e., an electron must be raised to a higher energy level. The absorption of light quanta is a discrete process and whole quanta are absorbed, meaning that the energy levels in a molecule assume quantized values. However, the fluorescence does not result from this single jump to a higher energy level followed by a single jump down to the ground level. The molecule usually returns to the ground state by falling in several jumps from

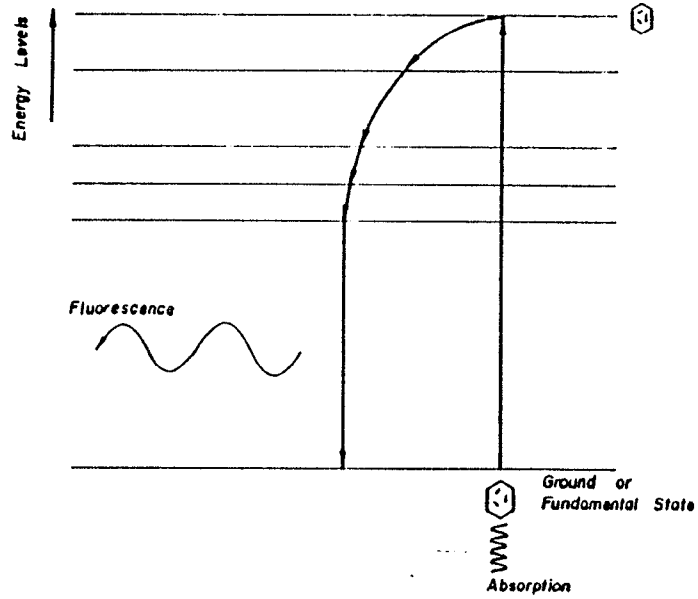


FIG. 9.29. Hypothetical absorption, excitation, vibrational deactivation, and fluorescence of a model substance.

higher to lower energy levels, and fluorescence occurs only in the jump from the lowest elevated energy level to the ground level (Fig. 9.29).

Fluorescence intensity can be derived from the following considerations: $dI/d\ell = \alpha I$. This says that the change in light intensity, dI, that occurs as light of initial intensity I passes through a specimen thickness of $d\ell$ is equal to a constant, α, times the initial intensity I. Thus

$$\int_{I_0}^{I} \frac{dI}{I} = -\int_{0}^{\ell} \alpha d\ell$$

Note that a minus sign is required because light intensity decreases as it goes from the initial intensity I_0 to the transmitted intensity I (going from 0 to ℓ through the specimen). Thus

$$\text{Ln } I - \text{Ln } I_0 = -\alpha\ell,$$

where Ln = natural logarithm

$$\text{Ln } I/I_0 = -\alpha\ell$$

$$T = I/I_0 = e^{-\alpha\ell} \quad \text{(Lambert-Bouguer Law)}$$

where T = transmittance. According to Beer's law,

$$\alpha = \mu c$$

where μ = an extinction coefficient
c = concentration
$T = e^{-\mu c\ell}$

Changing to base 10, $T = 10^{-kc\ell}$, where $k = 0.4343\,\mu$. Let us define quantum efficiency of fluorescence as ϕ_f = number of quanta fluoresced divided by the number of quanta absorbed.

The proportion of light absorbed by the specimen is $(1 - T)$. Thus the amount of fluorescence $\mathfrak{F}$ is given by: $\mathfrak{F} = I_0(1 - T)\phi_f$ or $\mathfrak{F} = I_0(1 - 10^{-kc\ell})\phi_f$. However, if the amount of light absorbed is very small, the first term of the series expansion of $e^{-\mu c\ell}$ may be used and substituted in the above equation, yielding a simplified formula of $\mathfrak{F} = I_0\phi_f\,(2.3\,kc\ell)$. In this case the fluorescence is directly proportional to the concentration of the fluorescing substance. If the fluorescence response is not linear with concentration, the simplified formula cannot be used.

The quantum efficiency must be calculated with the aid of standards, and to do this the following relationship is used:

$$\mathfrak{F}_i/\mathfrak{F}_s = \phi_{fi}/\phi_{fs} \times E_i/E_s$$

where $\mathfrak{F}_i$ and $\mathfrak{F}_s$ are the fluorescence intensity of specimen i and the standard respectively; ϕ_{fi}, ϕ_{fs} = the respective quantum efficiencies; and E_i, E_s = the respective optical densities, log I_0/I. This equation can be used without correction only if the absorption and fluorescence maxima of the two substances are identical. Otherwise corrections have to be made for differential sensitivity of detector and differential intensity of excitation.

Porro and co-workers (1963, 1965) have published fluorescence and absorption spectra of a great number of biologically important dyes, and these are useful in developing microscopical systems for specific purposes utilizing these dyes. Quantum efficiency is influenced by many factors including temperature, viscosity, pH, ion concentration, dye concentration, chemical composition of the tissue, and mounting media. However, under optimal conditions at least, the spectral distribution of the fluoresced light is independent of the spectral distribution of the exciting light. In fact, if the quantum efficiency varies with the wavelength of the exciting light, additional chemical species or molecular rearrangements should be considered as a possible cause. Figure 9.30 illustrates the fact that no matter what part or fraction of the absorption spectrum is used for excitation, the fluorescence spectrum remains unchanged. Note (Fig. 9.30A) that the fluorescence spectrum is a mirror image of the long wavelength absorption peak. This relates to the fact that emission occurs only on the final energy fall (Fig. 9.29). In Figure 9.30B only the short wavelength light has been used for excitation, but the fluorescence spectrum is unchanged; in Figure 9.30C only the long wavelength portion of the absorption spectrum has been used for excitation, and again we see that the fluorescence spectrum is unchanged.

Quantum efficiencies vary from 0.95 to less than 0.01. Generally low temperature and high viscosity increase the quantum efficiency by mininiz-

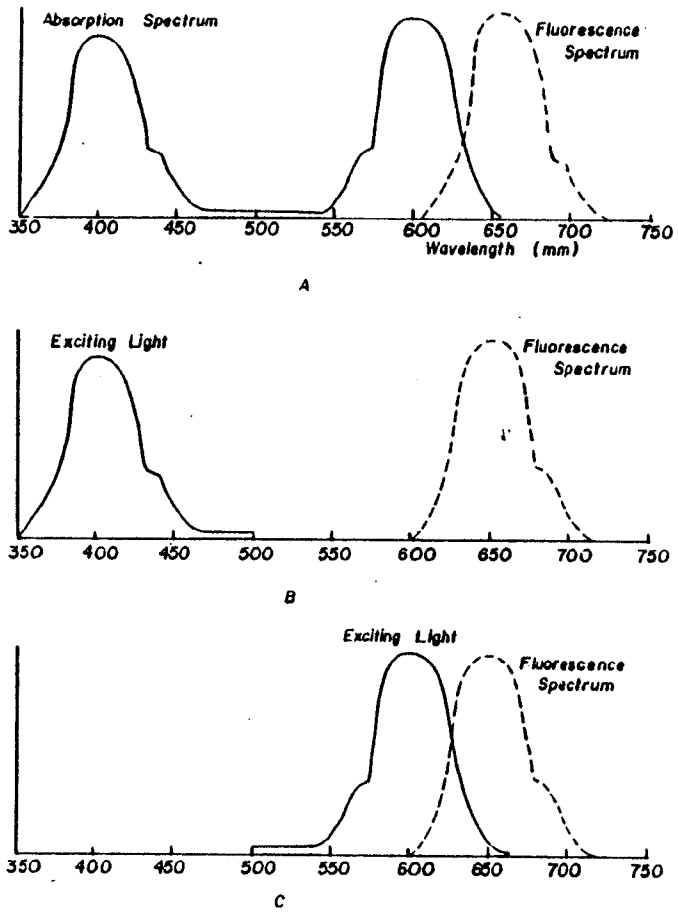


FIG. 9.30. Illustration of the independence of the fluorescence spectrum from the absorption spectrum. Absorption is shown in solid lines and fluorescence in dashed lines.

ing energy loss through heat transfer to solvent or neighboring molecules. Certain functional chemical groups present in the milieu may increase quantum efficiency–e.g., –OH, $-OCH_3$, $-NH_2$, and $-N(CH_3)_2$; others may depress quantum efficiency–e.g., –CO, –COOH, –CN, –CH=, $-NO_2$, and –CH=CH– (Bartels, 1961). Hydrogen ion (pH) is often a critical factor, and optima trials should always be enacted in developing or using a particular technique. Hydrogen ion concentration is often controlled with the use of buffers, but the presence of buffers can cause quenching of the fluorescence so this approach must be used with caution. Overoptimal concentrations of a

fluorescent dye can cause self-quenching, so this factor must also be controlled. Specific procedures will not be discussed in this section, but it should be noted that specimens should be mounted in a nonfluorescing medium. Many of the xylene-soluble resins are adequate, and Fluormount (E. Gurr, Ltd., London) is specifically compounded for use in fluorescence microscopy. This same firm also supplies a nonfluorescing water-soluble mount, Uvak. Spurr (1954 and personal communication) has also developed nonfluorescent aqueous mounting media:

	Medium I	Medium II
distilled water	68 cc	40 cc
cadmium iodide	2.0 g	34 g
polyvinyl alcohol (Elvanol 51-05)	27.0 g	18 g
sodium thiocyanate (NaSCN)	1.5 g	0 g
fructose	0 g	8 g

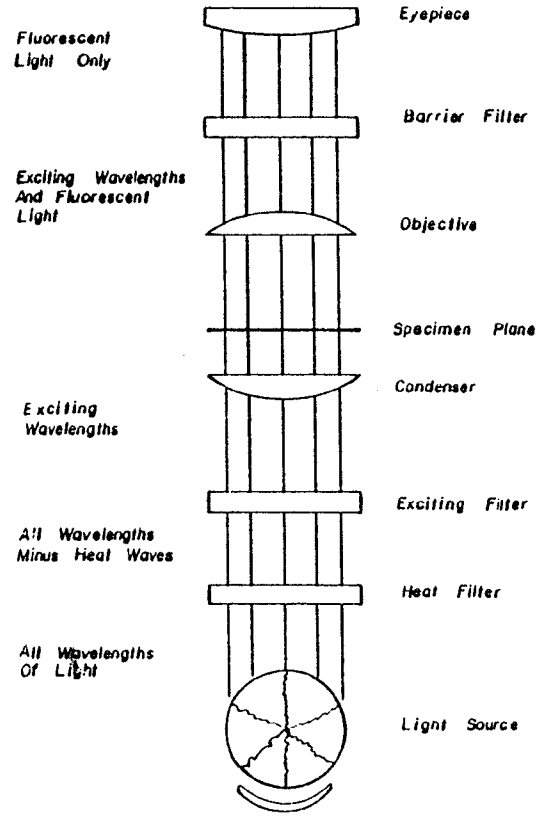


FIG. 9.31. Schematic diagram of a transmitted light fluorescence microscope.

Instrumentation

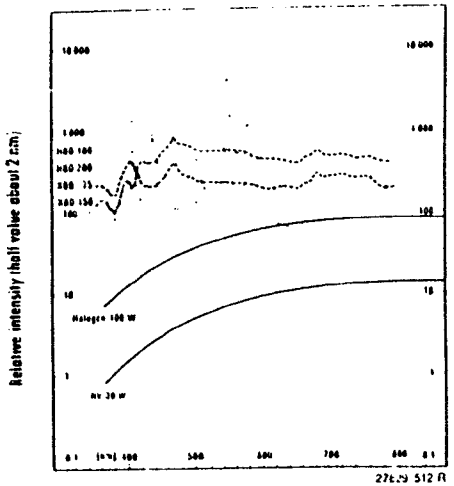
Most research model microscopes have the capacity to be converted into efficient fluorescence microscopes (at least of the qualitative type) upon addition of accessories. The essential components of a fluorescence microscope are diagramed in Figure 9.31. The light source must have a high intensity output in the required exciting wavelengths. Since these wavelengths are often near the UV and blue range (350–450 nm), mercury, xenon, halogen, and deuterium lamps are usually used as light sources. The Osram HBO 200 high-pressure mercury arc is probably the most common light source for fluorescence microscopy. It comes in two types–L_1 and L_2–and it is essential that the proper power supply is used for each type; i.e., an L_1 should always be replaced by an L_1 if the same power supply is to be used. This lamp has a life of about 200 hours and *must* be replaced at that time, because when it is allowed to burn out it has a tendency to explode. This will usually ruin the lamp condenser, and the mercury vapor is, to say the least, hazardous to human health. It is also important to have a heat sink (now supplied by reputable manufacturers) attached to the upper electrode to prolong lamp life and diminish the possibilities of an explosion. The spectral distribution of light intensity of the various light sources is given in Figure 9.32. Note that the 200-watt high-pressure mercury arc (e.g. HBO 200) does not have uniform intensity over the spectral range. It also lacks constancy of emission intensity and for these reasons is not the light source of choice for quantitative work. The halogen and xenon light sources are much better for this purpose. In the future the tunable dye laser may become an important light source for fluorescence microscopy. The dye laser has a lasing medium consisting of an organic dye mixed with a liquid solvent. This laser is wavelength tunable from ultraviolet to infrared. It can deliver extremely high excitation energy (up to 0.5 megawatt), and this is propagated in short pulses of 0.4 μsec. In some cases a single pulse can be utilized for photomicrography; even when multiple pulses are required, little or no photodecomposition of the fluorochrome is reported (Anon, 1972).

Filters

In Figure 9.31 we see that the first filter is a heat filter. This usually filters out the long wavelength (red and infrared) heat waves and serves the dual purposes of protecting the subsequent filters and the specimen from heat damage. These heat filters must have good transmission characteristics in the UV, and in some systems both a heat filter (e.g. KG1) and a red-suppression filter (BG 38) are used.

The next filter is used to isolate the exciting wavelength and is often termed the exciting or excitation filter. It is necessary to avoid complications in image interpretation that would ensue; if the high-intensity extraneous light were not eliminated, it would completely mask the lower-intensity fluorescent light. We can separate excitation filter systems into three types: broad-band, narrow-band, and selective. *Broad-band excitation* refers to the use of filters that are dimensioned to provide an excitation peak of wide

Intensity comparison of various light sources suitable for fluorescence microscopy (referred to 6 v 30 W low voltage lamp)



Spectral energy distribution of the 200 W ultra-high pressure mercury lamp as example of a suitable fluorescence light source

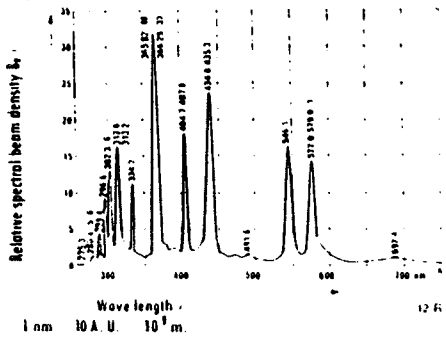


FIG. 9.32. Data on light sources for fluorescence microscopy. (Courtesy E. Leitz, Inc., Rockleigh, N.J.)

breadth, e.g., the BG 12 blue light filter. This method provides maximum excitation energy but can only be used where the excitation and fluorescence maxima are widely separated (e.g. 100 nm) in wavelength. *Narrow-band excitation* is used to deal with the situation where the excitation and fluorescence maxima are not so widely separated. Very often a combination of filters is used to achieve this narrow width excitation peak. *Selective excitation* is just the extreme of narrow-band excitation. In this case a monochromator or asymmetric interference filters are used to provide very narrow

aperture, for good dark-field illumination. Lacking an iris diaphragm, funnel stops may be used, but these are less convenient and less adjustable.

For quantitative work the Ploem vertical illuminator has many advantages. Furthermore, it can be used in combination with other methods of microscopy. The Ploem vertical illuminator was developed by J. S. Ploem (1967) and employs a dichromatic mirror as a beam splitter (Fig. 9.34). Light from the source is directed to the dichromatic mirror which reflects the short wavelength light down onto the specimen surface and transmits the long wavelength light away from the optical axis. The short wavelength light excites fluorescence from the specimen, and both short wavelength and fluorescent light are reflected from the specimen surface back up to the dichromatic mirror. Here the short wavelength light is reflected back toward

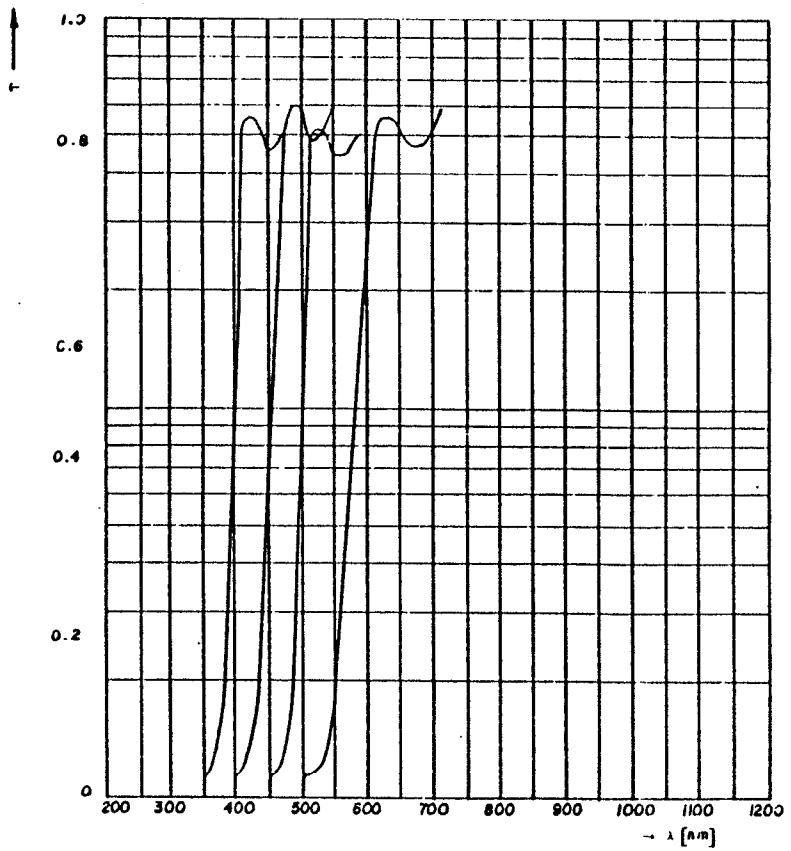


FIG. 9.35. Characteristics of Leitz dichromatic beam splitters TK 400, TK 455, TK 510, and TK 580. The beam splitters reflect on the left side of the curves and transmit on the right side. (Courtesy E. Leitz, Inc., Rockleigh, N.J.)

the light source while the long wavelength light is transmitted straight through the mirror toward the ocular and/or photomultiplier tube. Note that the dichromatic beam splitter transmits long wavelengths and reflects short wavelengths at a 90° angle. A good dichromatic mirror will reflect over 90% of the short wavelength light to the specimen and transmit away from the specimen the longer wavelength and infrared heat radiation; on the way back virtually all of the fluorescent light will be transmitted to the observational system. The cutoff point between reflection and transmission can be changed by varying the interference coatings on the dichromatic mirror. The characteristics of four different dichromatic mirrors are illustrated in Figure 9.35. The function of these beam splitters must be augmented by both excitation and barrier filters as described previously. This combination of barrier filters and dichromatic mirror provides great versatility in both excitation and suppression. We can now excite with green light, which for some fluorochromes would be at the excitation maximum (e.g., fluorescein isothiocyanate—FITC). This results in high fluorescence intensity and excellent contrast.

The Ploem system permits imaginative combination optical systems to be developed. For example, two fluorochromes can be used and the specimen observed both in incident and transmitted fluorescent light so that both fluorochromes will be localized at the same time. The Ploem illuminator offers the following advantages over previous systems:

1. It is better suited for quantitative work (cytofluorometry) because the fluorescence intensity is not critically dependent on a substage condenser; the objective itself serves as both condenser and objective.
2. The surface of the specimen provides the fluorescence; this minimizes measuring error due to such things as variation in specimen thickness and light loss due to absorption or scatter within the specimen.
3. In some cases specimen preparation is simplified if only the surface is viewed; i.e., microtoming of this section can sometimes be eliminated.
4. The full aperture of objectives may be employed, which is not the case in dark-field.
5. The Ploem system can be combined with transmitted light, dark-field, fluorescence, phase-contrast, or polarized light microscopy.

MICROSPECTROPHOTOMETRY

Prior to the middle 1950s, microspectrophotometric instrumentation had to be fabricated by the investigator; now instruments are available from several optical manufacturing firms.

Quantitative optical absorption spectroscopy at the tissue and cellular levels can be performed with a microspectrophotometer. The absorption laws presented for fluorescence microscopy are applicable to microspectrophotometry.

Microspectrophotometers are composed of the items shown in Figure 9.36. The light source emits energy at UV or visible wavelengths. The light passes through an interference filter or a monochromator which filters out all light except a narrow band of monochromatic light. The monochromatic