A number of standard and novel methods to optically image biological and other materials are discussed in this chapter to give the reader a sense of the large variety of tools available. We start with a survey of the arsenal of newer light microscopies available for the study of biological materials, in particular. Another wavelike property of light, its polarization, can be used in several optical polarization methods to study biomolecules. Earlier, we discussed the important imaging technique of MRI in Chapter 18; this chapter concludes with a discussion of two other wave-related imaging techniques: electron microscopy and x-ray diffraction/computed tomography (CT) imaging with x-rays.

1. THE NEW LIGHT MICROSCOPIES

Aside from the resolution needed to form an image of a microscopic object, discussed in the previous chapter for a standard compound microscope, a minimal amount of contrast is also needed to clearly detect an image. Contrast can be defined in terms of the visibility of a sample object compared to the background using the percent contrast,

\[ \text{% Contrast} = \frac{(I_{\text{bkgd}} - I_{\text{sample}})}{I_{\text{bkgd}}} \times 100, \]  

where the intensities are average values over those portions of the image. Contrast is determined both by the properties of the object and by those of the microscope. We can distinguish two fundamental types of contrast: amplitude and phase contrast.

Amplitude contrast is due to direct differences in the wave amplitude of the imaged sample and background light due to absorption or scattering from the sample. This is the basis for several types of microscopy including normal or bright-field microscopy discussed in the previous chapter. In this technique, the background appears bright white and objects are imaged by their darker or colored appearance due to absorption or scattering. Because most biological materials do not absorb much visible light, usually a colored stain that preferentially sticks to the sample and is washed from the background is used to enhance the contrast. Before defining phase contrast, we take a look at several microscopy methods that use amplitude contrast enhancing schemes.

Very small or thin objects are difficult to see in bright-field microscopy because of the light background and low contrast. If sufficient scattering occurs from an object, it can be better viewed using a variation known as dark-field microscopy in which the background light is blocked by a central stop and only the scattered light from the object is imaged. Figure 23.1 shows this microscope arrangement. A hollow cone of light from a special annular aperture is focused on the specimen and the...
collection optics are arranged so that only the scattered light, and not the directly transmitted cone of light, is collected and focused by the microscope. Figure 23.2 shows an example of a dark-field image.

*Fluorescence microscopy* is an important variation of amplitude contrast microscopy. Since most samples are not sufficiently fluorescent, usually fluorescent dyes are used to bind to specific sites on the sample and only fluorescent light is imaged in the microscope. To accomplish this, filters must be used to block other wavelengths of light. Unless a laser is used as a light source of the proper excitation wavelength, an excitation filter is used to limit the incident light to the shorter wavelengths capable of exciting the fluorescent dye. The incident light is used in either a dark-field microscope arrangement or in the arrangement shown in Figure 23.3 to direct excitation light onto the sample. Fluorescent light emitted by the sample is then collected and filtered using a barrier filter that passes only longer wavelength fluorescent light, blocking the incident light. In this way there is no background light except for a stray unwanted fluorescent signal from imperfections in the optics. In Figure 23.3, the dichroic mirror is specially coated to reflect only shorter wavelengths but to transmit only longer wavelengths of light, thereby acting both as two filters as well as a beamsplitter. Figure 23.4 shows an image of a multiply labeled fluorescent endothelial cell.

Recent developments of new multicolor fluorescent dyes for use in microscopy have been partly responsible for a revolution in fluorescent microscopy. Aside from advances in scientists’ ability to label specific molecules with a dye, many of the newer dyes have their fluorescence controllable by specific environmental changes. For example, certain dyes can serve as sensors of local pH, with their fluorescence properties depending on pH, whereas others can serve to monitor calcium ions Ca^{2+}, the important messenger and regulating ion in a cell, because their fluorescence is affected by the binding of...
calcium. An even newer class of fluorescent dyes can be used as optical biosensors to detect conformational changes in macromolecules or binding of ligands (small molecules with specific binding sites) to those molecules. In this way, not only can the locations of specific macromolecules to which the dyes are bound be monitored, but so can their physiological state (Figure 23.5).

As already mentioned, most biological samples for microscopy are essentially completely transparent to visible light, absorbing and scattering very little light, and therefore having very poor contrast (hence, the use of stains and fluorescent dyes). However, all such samples do have somewhat different refractive indices than the surrounding solvent and are therefore called phase objects. These produce a phase shift in the light waves they transmit relative to those through the background, more or less as shown in the last chapter in Figure 22.2. If light is simply allowed to pass through the sample and be imaged, the relative phase shifts will not change the intensity of the light and the objects will be invisible. However, encoded phase information in the light passing through the sample can be used to provide phase contrast in several types of microscopies. We discuss two major types: phase contrast and differential-interference-contrast (DIC) microscopy. In both cases the crux of the technique is to separately change the relative phase of the light that interacts with the sample and the undeviated light so that when they are recombined, there will be intensity differences in the images due to interference effects.

Phase contrast microscopy is similar to dark field microscopy in that a hollow cone of light is focused onto the sample but now that light is collected by the objective lens (Figure 23.6). In the absence of a sample, the objective lens produces an image of the annulus used to produce the cone of light at a plane known as the “source image plane.” However, light that interacts with the sample will be diffracted from that path (dotted lines in Figure 23.6) with a small phase shift from passing through the more optically dense sample as well. The intensity of this diffracted light will be much less than that of the undeviated light and it will be brought to a focus at a different plane (because

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**FIGURE 23.4** Three-color fluorescence image of an endothelial cell showing the tubulin (green), nucleus (blue), and actin cytoskeleton (red).

**FIGURE 23.5** A wave of increase in calcium ion concentration sweeps across an egg cell just after fertilization as monitored by the green fluorescence from a Ca-sensitive dye attached to small dextran molecules. The images are taken 5 s apart and show the Ca wave starting around the 1:30 o’clock position and spreading across the cell.

**FIGURE 23.6** Optics of the phase contrast microscope. A cone of light is produced by the annulus and focused on the sample. The undeviated beam is focused by the objective onto a groove in a phase plate located at the source image plane. The groove both attenuates the undeviated beam intensity and shifts its phase with respect to the diffracted light, most of which passes through the rest of the phase plate and is brought to focus at the object image plane. This image is then further magnified by the eyepiece (not shown).
the object distance is much less than the light source distance from the objective), known as the “object image plane.”

In the phase contrast microscope a device known as a phase plate is inserted at the source image plane to improve the contrast. A groove in the phase plate aligned with the image of the annulus is used to shift the phase of the undeviated light relative to the diffracted light. An absorption coating in the groove also decreases the intensity of the undeviated beam, so that it is closer to matching the intensity of the diffracted light in order to provide even better contrast. Phase plates are usually built into objective holders and matched pairs of condenser and objective lenses are used to ensure proper alignment. The total phase difference between undeviated and diffracted portions results in intensity variations in the image that are directly proportional to optical path differences between the sample and background regions. Depending on whether the phase plate gives an additional positive or negative phase shift with respect to the diffracted light, the background can be made dark or bright (Figure 23.7).

In differential-interference-contrast (DIC) microscopy there is a complete physical separation of the incident light into two closely spaced beams that probe adjacent portions of the sample. These beams are then used to generate an interference pattern that produces intensity differences in the object image plane. Two special prisms, known as Wollaston prisms, are used both to produce two in-phase beams from one and to recombine them after passing through the sample into one final beam with a 180° phase shift introduced between the two (Figure 23.8). In the absence of a sample and with a uniform background, the two beams completely cancel after recombination due to the 180° phase difference. With a sample present in one beam but not the other, the extra phase differences between the two beams give rise to bright interference light. In this case the image intensities are not proportional to optical path differences, but rather, because of the two spatially separated beams, to the rate of change of optical path transversely (in the direction of the separation of the two beams) across the object. That’s the reason for the term “differential interference”. Because the rate of change, rather than the absolute optical path difference, is important in DIC microscopy, edge contrast is greater and thinner samples can be better imaged (Figure 23.9).

Wollaston prisms function by spatially separating the two different polarization components of light. They are able to do this because the calcite crystal of which they are made has different refractive indices along two different crystal axes as discussed further in the next section. After the two beams of light travel through the sample this process is reversed in a second matched prism and the two beams recombine after a 180° phase shift introduced by an asymmetric placement of the second prism. At this point, even though the beams are out of phase and overlapping, they cannot interfere with each other because their polarization directions are orthogonal and hence independent. A polarizer oriented at a 45° angle between these directions serves to analyze that portion of each beam and to allow them to subsequently interfere and produce the image. Thus, the Wollaston prisms are serving solely as a beamsplitter and recombines, whereas the polarization properties of the beams are not used to produce the DIC image. Polarized light can be used in microscopy in the polarization microscope discussed in the next section.

Most current versions of the above microscopic techniques use modern methods of digital recording and computers to further increase the resolution and contrast of images. Developments in detector technology have made use of CCDs (charge-coupled devices) and image intensifiers very commonplace in microscopy. CCD video cameras, based on arrays of discrete light-sensitive detectors, allow digital recording of time-dependent processes in two-dimensional arrays of picture elements, or pixels. These arrays are now relatively inexpensive and are widely used in digital
cameras, whose pictures can then be printed out on ordinary computer printers. Digitally stored video frames from microscopy can be computer-enhanced and manipulated to allow improved resolution, contrast, and quantitative measurements using special software.

Within the last ten years or so many new microscope techniques have been developed that use laser illumination, including confocal microscopy and multiphoton microscopy. Laser-scanning confocal microscopy focuses a laser beam to an extremely small spot within the sample and images light only from that spot onto the detector. A pinhole in front of the detector serves to eliminate out-of-focus light from other regions of the sample, only allowing light from the focused spot to be collected. The spot is then scanned over the sample, by moving either the microscope stage or laser beam, in a raster pattern to map out the sample image, having remarkable depth and lifelike appearance (Figure 23.10).

Multiphoton microscopy uses a pulsed laser to provide an intense beam of low-energy photons that is scanned across the sample similar to confocal microscopy. When two (for two-photon microscopy) or more (for three- or multiphoton microscopy) of these photons with identical energy are simultaneously absorbed by a fluorescent molecule they can provide the same total energy that a single photon would in the usual fluorescence microscope. The incident photon beam is tuned to the proper wavelength so that two or three or more photons, when combined, give an energy resonant with the fluorescent material, producing subsequent fluorescence emission. Quantum mechanics allows this additive resonance only when the multiple photons are absorbed nearly simultaneously, requiring very high laser intensities. One important advantage of this method is that there is virtually no absorption of these lower-energy photons at any other location in the sample where the beam is not focused and the density of photons is not sufficient to allow multiphoton absorption. Thus instead of using high-energy photons that can damage the sample to produce fluorescence, one can use much lower energy photons and excite the fluorescent molecules through the combined energy of several photons only where the beam is focused. This technique is sensitive enough to image the intrinsic or autofluorescent light from the amino acid tryptophan and other fluorescent macromolecular groups within the sample itself without the addition of fluorescent dyes. High-resolution, high-contrast, three-dimensional images can be obtained using these methods even with samples as thick as 0.5 mm (Figure 23.11).

**FIGURE 23.9** DIC image of a deer tick. Note the sharp edges and high contrast.

**FIGURE 23.10** Laser-scanning confocal microscopic images of mouse oocytes showing microtubules in red and actin filaments in green.

**FIGURE 23.11** Confocal microscopic image of anaphase in a cultured epithelial cell showing chromosomes (blue), spindle apparatus (green), and actin (red).
2. OPTICAL ACTIVITY; APPLICATIONS OF LIGHT POLARIZATION

In Chapter 19, we introduced the concept of polarization of a light beam and discussed linearly polarized light as well as the use of Polaroid as a polarizing device to preferentially absorb light with its electric field oriented along one direction. Here, we further discuss the notion of circularly and elliptically polarized light and the use of polarization methods in the study of biomolecules.

Consider two light waves with the same frequency linearly polarized along perpendicular directions as shown in Figure 23.12. If the amplitudes and phases of the two waves are equal, then the superposition of the two waves results in a linearly polarized wave along the vertical direction in (a). With different amplitudes for the two waves, the resultant wave will still be linearly polarized so long as the phases are equal (b). If two waves of equal amplitude are 90° (π/2 rad or π/4) out of phase then when one component is at a zero the other will be at a maximum or minimum. The superposition of those two waves will describe a helical path as the tip of the electric field vector executes circular motion in the transverse wavefront plane itself traveling along at speed $c$ in a vacuum (c). Depending on the relative phases, the circular polarization can be left- or right-handed. Handedness is defined in terms of an observer looking back at the source and the light is right-handed if rotates clockwise.

We can make these ideas quantitative by writing out expressions for the two linearly polarized electric fields (say, along $x$- and $y$-axes) as

$$
E_x = E_{ox} \cos(\omega t) \\
E_y = E_{oy} \sin(\omega t)
$$

(23.2)

where we have assumed that $E_x$ leads $E_y$ by 90° (at time 0, $E_x$ is at a maximum and $E_y$ is zero; after 1/4 of a period, $E_x$ is now zero and $E_y$ has increased to a maximum, etc.), and $E_{ox}$ and $E_{oy}$ are the amplitudes of the fields. By using the trigonometry identity $\cos^2\theta + \sin^2\theta = 1$, we find that the components of the vector $E$ satisfy

$$
\left(\frac{E_x}{E_{ox}}\right)^2 + \left(\frac{E_y}{E_{oy}}\right)^2 = 1,
$$

(23.3)

which is the equation of an ellipse. If the two amplitudes are equal (so that $E_{ox} = E_{oy} = E_o$) then Equation (23.3) becomes the equation of a circle ($E_x^2 + E_y^2 = E_o^2$, with radius $E_o$), the case shown in Figure 23.12c. In the transverse plane the tip of $E$ will describe these closed ellipses or circles, but the light wave is actually propagating at the speed of light along the $z$-direction and the tip of $E$ actually describes a helical path in space. The projection of the helix in the $x$–$y$ plane will be a circle or an ellipse, depending on the amplitudes of the $x$- and $y$-components of $E$. In a similar way one can show that linearly polarized light can be considered to be the sum of in-phase right- and left-handed circularly polarized light. For example, if the left-handed circularly polarized beam shown in Figure 23.12c is added to its mirror image right-handed beam, the resulting beam has an $E$ that is vertically polarized (imagine the summation in the figure: the horizontal components will always cancel with the mirror-image beam). This idea is used below in a discussion of optical activity.

Circularly polarized light can be produced most easily by sending linearly polarized light through a special device known as a quarter-wave plate, or $\lambda/4$ plate. These are made from a birefringent (double-refracting) material, one having...
two crystal axes with different refractive indices, as mentioned in the last section in connection with a Wollaston prism. When linearly polarized light passes through such a material, the different polarization components along either axis travel at different speeds, because \( v = \frac{c}{n_1} \) or \( \frac{c}{n_2} \), and will develop phase differences. Furthermore, one beam, called the “ordinary” beam, will be transmitted undeviated, whereas the other, called the “extraordinary” beam, will be refracted and physically separated from the ordinary beam (see Figure 23.8). By adjusting the thickness of the material, a quarter-wave phase difference can be introduced between the two beams, producing fields governed by Equations (23.2). In general this will produce elliptically polarized light but if the wave plate is adjusted to have its axis at 45° to the incident polarization direction then circularly polarized light is produced.

**Example 23.1** Suppose that a vertically polarized beam of 500 nm light is incident on a birefringent crystal of mica with a mean index of refraction of 1.552 and which has its crystal axes making a 45° angle with respect to the vertical. If the birefringence of the crystal is \( \Delta n = 0.006 \), find the minimum thickness of the crystal along the transmission direction of beam so as to produce circularly polarized light.

**Solution:** If we call the unknown thickness \( t \), then the optical path difference of the two equal components of the vertical polarization along the two crystal axes will be \( t\Delta n \) (see Equation (22.2)). In order to produce circularly polarized light, this difference should be set equal to \( 1/4 \) wavelength of the light, so that, as in the Figure 23.12c, after leaving the crystal there will be two equal components of electric field that are 90° out of phase, combining to produce a circularly polarized beam. We therefore require \( t\Delta n = \frac{1}{4} \) (500 nm), so that \( t = 2.1 \times 10^{-5} \) m = 0.021 mm. Mica can be cleaved and polished to produce such quarter-wave plates designed for different wavelengths.

Many biological systems contain components that are anisotropic. These are ordered structures that look different in different directions; for example, the fibrils within a muscle fiber or the crystal-like proteins of the lens of the eye. Polarized light will interact with electrons in such a material in different ways depending on relative orientations and can be used to gain information about such structures. Because of the anisotropy there will be changes in the polarization of transmitted light. Polarization microscopy is yet another way to get images of such anisotropic structures. Linearly polarized light is used as a light source and the imaged light through the objective is passed through a crossed-polarizer. In the absence of any sample, the background light is completely extinguished by the crossed-polarizer. Any resolvable structures that produce some depolarization of the incident light will then produce a bright image (Figure 23.13).
Most individual biological macromolecules are asymmetric, meaning that they appear different from their mirror image. Most simple molecules are symmetric. Water, carbon dioxide, and many more complex molecules look the same as their mirror images. Biopolymers tend to be formed, at least partially, from helical arrays of molecules, and these will have a handedness. Handedness is a property that changes when viewed in a mirror. As shown in Figure 23.14, a right-handed coiled spring will appear to be a left-handed spring when viewed in a mirror.

On the other hand, a solution of randomly oriented asymmetric molecules will not produce an image in a polarization microscope because the solution as a whole is isotropic. However, asymmetric molecules do have an effect on the polarization properties of light that can be used to gain information about the macromolecules. Asymmetric molecules are said to have optical activity and are characterized by different refractive indices for left- and right-handed circularly polarized light. Asymmetric molecules will interact differently with left- and right-handed circularly polarized light because of their handedness.

A simple example may help to clarify this. Imagine a solution of small left-handed helical molecules. Because the electric field vector of the light interacts with the electrons of the helical molecule, left-handed circularly polarized light will allow a stronger interaction with the electrons of a left-handed helical molecule, with the ability to drive them around the helix, and therefore a larger fraction of such light will be absorbed than would be the case for right-handed circularly polarized light. This is somewhat similar to the reason why Polaroid film, with its oriented long polymers, preferentially absorbs light polarized along the polymers: the electric field can then interact more strongly with polymer electrons.

Because linearly polarized light can be considered a sum of left and right circularly polarized light, a solution of optically active molecules probed with linearly polarized light will interact differently with each of these components and affect the polarization of the transmitted light. If the sample absorbs no light, then the light remains linearly polarized, but has its direction of polarization rotated due to different effective optical paths for each polarization. Molecules that rotate the polarization in a left-handed sense are called levarotatory (L) and those that rotate the polarization in a right-handed sense are called dextrorotatory (D). It is a fact that all proteins and most other biological molecules are found only in the L form in nature.

When linearly polarized light is incident on an optically active solution, there can be both phase and amplitude changes associated with the equivalent left- and right-handed circular polarization components making up the incident linear polarization. These can be characterized by two quantities: the circular birefringence \( \Delta n \),

\[
\Delta n = (n_L - n_R),
\]

for the phase changes, where \( n_L \) and \( n_R \) are the refractive indices for left and right circularly polarized light; and the circular dichroism \( \Delta \varepsilon \),

\[
\Delta \varepsilon = \varepsilon_L - \varepsilon_R,
\]

for the amplitude changes, where \( \varepsilon_L \) and \( \varepsilon_R \) are the absorption coefficients for left and right circularly polarized light. Recall from Chapter 19 (Section 6) that the absorption coefficient is a measure of the intensity of light absorbed in a unit path length and per unit concentration of sample.

Both the circular birefringence and dichroism values depend on the wavelength of light used on a given optically active sample. Spectra showing the wavelength dependence of the birefringence (using the technique known as optical rotary dispersion or ORD experiments) and of the dichroism (using circular dichroism or CD experiments) can be used to characterize biological materials. These techniques are used most to probe the optically active regions of macromolecules, determining their helical content.
or following relatively slow kinetic changes that can occur from conformational changes due to environmental factors or to the binding of small ligands. Figure 23.15 shows an example CD spectrum for standards in particular conformations and for a real protein, myoglobin.

3. ELECTRON MICROSCOPY

In our discussion of the resolution possible in a microscope, the resolving power, or closest distance that two distinct objects can lie and still be distinguished under optimal conditions, was given by Equation (22.15) to be no less than $\lambda/4$. For visible light this limits the resolution under the best conditions to about 200 nm. Any further improvement on this limit requires that the wavelength of the probing radiation be decreased. Although ultraviolet microscopes have been developed, the most feasible method for improving resolution is to use electrons in place of light. We show in the next chapter that electrons have an associated wavelength that depends on their momentum (or, in turn, on their energy). Just as with photons, where higher-energy photons have a correspondingly shorter wavelength, we show that higher-energy electrons also have a shorter wavelength. Exactly what it means for an electron or another elementary “particle” to have a wavelength is explored further in the next chapter. For now, we can use the notion of a wave packet introduced in Chapter 19 (Section 5) to picture an electron as having wavelike properties.

Electrons accelerated through a potential difference of 50 kV, typical for an electron microscope (EM), have a wavelength of 0.005 nm, allowing a theoretical improvement in resolution over a light microscope by a factor of 40,000. Unfortunately, other problems limit the practical resolution of the EM, although using a particular variation of electron microscopy has allowed resolutions approaching 0.1 nm at which individual atoms can be directly imaged. The recently developed method of scanning tunneling microscopy (STM), described in the next chapter, allows even higher resolution of surface topography with a resolution of better than 0.1 nm.

The general plan of an EM is shown in Figure 23.16. An electron “gun,” or filament and anode combination, is the source of electrons boiled off a tungsten filament heated to very high temperature, similar to a light bulb. The electrons are accelerated through a large potential difference of typically 40–100 kV reducing the wavelength of the electron as it gains kinetic energy. The entire microscope column is evacuated to a fairly high vacuum, reducing energy losses of the electrons from collisions with air molecules. Because electrons can be steered in a magnetic field, a “magnetic condenser lens” is used to focus the electron beam at or near the sample plane down to a spot size of several microns. Samples are supported on copper grids with an array of typically 100 $\mu$m $\times$ 100 $\mu$m square holes coated with a thin uniform layer of a supporting material, such as carbon, that is essentially transparent to electrons. Copper is used...
because it is a good electrical and thermal conductor, carrying away any heat from the interaction with the beam, and also minimizing the distortion of the focusing magnetic field. The sample is mounted on a movable stage for positioning it in the focused electron beam.

After interacting with the sample, electrons are collected by a (magnetic) objective lens and a magnified image is projected onto a detector by a system of other lenses. Overall magnifications can range from 1000 to over 300,000 times, limited mainly by aberrations in the magnetic lenses. The simplest detector is a fluorescent screen that emits light when struck by the electrons and can be viewed directly by eye or with some further magnification using optical lenses. Other detectors include photographic film or image intensifiers that allow digitization and computer enhancement of images.

Three types of EMs can be distinguished: transmission (TEM), scanning (SEM), and the less common scanning transmission (STEM). Normal TEM, developed in the 1940s, basically creates a greatly enlarged shadow of the sample at the detector. Samples must be very thin for good resolution and thin sections or evaporated deposits of solutions are used. Biological materials are made of smaller atoms (mostly H, C, O, N, P, S) that do not strongly interact with the electron beam and so the contrast is very poor. In order to “see” the sample, some contrast improvement is needed in order to cast a shadow. The usual method is to deposit a heavy metal with high electron scattering power (such as osmium, platinum, gold, or uranium) to coat the structures of interest. This is done in a variety of ways including “shadowing” by direct deposit of heavy metals on the grid, or by negative staining in which heavy metal salt solution fills the region immediately around particles of interest producing a dark background edge around bright images of the transparent objects of interest. Figure 23.17 shows a TEM image of two virus particles with a closed loop of its single-stranded DNA.

SEM uses a tightly focused electron beam (spot size of ~10 nm) directed off-axis at a heavy metal-coated sample as shown schematically in Figure 23.18. The beam is made to scan along the sample in a raster, or TV-like, pattern by a set of scanning coils that steer the electron beam and are coupled to the detectors. Electrons or radiation “reflected” from the sample at each scanned point are collected and used to create an image on a TV screen as the electron beam is scanned across the sample. The spatial pattern of the scanned beam is reproduced in the spatial pattern displayed on the TV screen. A variety of different signals from the electron–sample interaction can be measured using different detectors in the SEM, including backscattered electrons and secondary electrons released from the sample itself, as well as x-rays and emitted light. Although the resolution of this method is much lower (~10 nm at best) than the TEM, the depth of focus is extremely large and the images are very three-dimensional and lifelike (Figure 23.19).

STEM was developed to try to collect not only the “reflected” electrons and radiation as in SEM, but also the transmitted electrons that have interacted with the sample. These transmitted electrons undergo two basic types of interactions, elastic and inelastic, aside from the bulk of the electrons that simply pass through without any interaction at all. Inelastically scattered electrons lose some energy to the sample through excitation of target atoms, whereas elastically scattered electrons, fewer in number, are simply deflected from their path through much larger angles by interaction with the nuclei of target atoms without a change in their energy. The ratio of the intensities of the elastic to inelastic electron scattering is a characteristic of the particular target atom and increases with the number of protons in the nucleus of the atom. STEM scans an even more tightly focused electron beam (~0.5 nm) across the sample simultaneously measuring the elastic and inelastic transmitted electron intensities. Furthermore, the inelastically scattered electrons can be energy-analyzed to determine their energy loss. STEM pictures are at very high resolution (Figure 23.20).
and can also determine the elemental composition of the sample from point to point. Unfortunately, the fundamental limitation of sample degradation in the electron beam has made STEM less useful in biological imaging than first expected when developed in the 1970s.

4. X-RAYS: DIFFRACTION AND COMPUTED TOMOGRAPHY (CT)

X-ray photons have wavelengths in the range from about 0.01–10 nm, short enough to provide atomic resolution according to the equation for resolving power. Unfortunately, until recently x-rays could not be easily focused and magnified images, such as have been made with light and electron beams, have not yet been produced with x-rays. (In 1996 scientists developed a simple and effective way to focus x-rays; this method is expected to lead to many new applications, particularly in microelectronics.) Even if we had the ability to focus x-rays, their interaction with biological tissue is so weak that there would be virtually no contrast seen in normal thin samples used in microscopy. However, x-rays have two properties that make them extremely useful in both medicine and science. First, because x-rays are a form of electromagnetic radiation, they diffract from objects of comparable dimension to their wavelength, similar to the diffraction of light. Because of their atomic-sized wavelength, x-ray diffraction effects can be used to probe the atomic structure of matter and have been used to determine the structure of many complex biological macromolecules at atomic resolution. Second, because x-ray energies are high, these photons are capable of passing through otherwise opaque materials and x-rays can be used to produce “shadow” pictures of internal structures within thick samples, for example, the human body.

Crystalline materials have a three-dimensional periodic array of their atoms that can diffract x-rays and produce a pattern of detected x-rays containing information about the spatial array of the atoms. In a similar way that a one-dimensional array of slits gives rise to a diffraction pattern with light, the crystalline array of atoms results in a more complicated pattern of diffracted x-rays. In this case the x-rays are scattered, or diffracted, in all directions from the crystalline array of atoms and interference effects result in a detected pattern of x-ray spots.
Consider a simple cubic crystal made of identical atoms in a periodic array, or lattice, with separation distance $d$ as shown in cross-section in Figure 23.21. The atoms form planes, known as Bragg planes, and the pattern of diffracted x-rays can be determined by imagining that the x-ray beam reflects from these planes in a process known as Bragg diffraction. This picture greatly simplifies the analysis but gives the correct general result. For the x-ray beams shown in the figure, there will be a path difference for beams reflecting from neighboring planes. From the figure, we see that this path difference will depend on the angle $\theta$ between the ray and the Bragg plane and is given by $2d \sin \theta$. (Note that $\theta$ is not the usual angle of reflection between the ray and the normal, but is the angle between the ray and the line of atoms in the plane of reflection.) Constructive interference will occur when this path difference is equal to a whole number of wavelengths and the Bragg equation,

$$m \lambda = 2d \sin \theta,$$

where $m$ is an integer called the order, defines the location of an interference maximum. X-rays incident at an angle given by Equation (23.6), known as a Bragg angle, will produce a diffraction peak, or spot, at some distant detector located at the “reflected” ray. In a noncubic crystal with three different repeat distances along different directions there will be two additional order numbers for the other directions and a generalized Bragg equation. In this case, the “unit cell,” or basic repeating structure, dimensions can be found by the location of the Bragg spots.

Example 23.2 In an x-ray diffraction experiment on a cubic crystal with $\lambda = 0.40 \times 10^{-10}$ m, find the crystal plane spacing if the first-order maximum occurs at an angle of 6.4°. At what angle will the third-order maximum be found?

Solution: Using Equation (23.6) with $m = 1$, we have that $d = \lambda/(2 \sin \theta) = 1.79 \times 10^{-10}$ m. The third-order maximum will then be found at the angle given by $\sin \theta = 3\lambda/2d = 0.34$, so that $\theta = 19.6°$.

In the study of macromolecular structure, if a crystal of the macromolecule can be formed, then x-ray diffraction can often be used to determine the three-dimensional arrangement of all its atoms. In such a crystal, the individual scattering centers, or unit cells, may consist of thousands of individual atoms. In addition to the unit cell dimensions affecting the observed diffraction pattern, x-ray scattering from the molecules within the unit cell will affect the pattern due to its “structure factor.” In general, if there are $N$ atoms per unit cell, there will be $N^2$ peaks in the diffraction pattern from the atoms within the unit cell. As $N$ increases for larger molecular crystals, the diffraction patterns become extremely complex and rich with information. From detailed studies of such patterns, together with as much independent information on the macromolecular structure as possible, the detailed three-dimensional atomic arrangement has been determined for many macromolecules. Figure 23.22 shows the three-dimensional structure of myoglobin, a subunit of hemoglobin consisting of 153 amino acids with a total of...
1260 atoms. To obtain the current resolution of better than 0.2 nm, more than 9600 diffraction spots were measured and analyzed. These pictures of the structure conceal the fact that most macromolecules have extensive flexibility and motion. Because the x-ray pictures are obtained over relatively long times, the resulting 3-D structures represent average positions of the constituent atoms. X-ray diffraction is one of the best methods we have for determining macromolecular structure at an atomic resolution.

Not all biological materials can be made to crystallize so that they can be studied by x-ray diffraction. A large class of filamentous macromolecules can, however, be oriented into fibers and studied by x-ray diffraction even though they are not in regular crystalline arrays. Special techniques have been developed for helical proteins and nucleic acids that reveal the symmetries present even when neighboring oriented helices may not be “in register” along the axial direction. Such methods first led to the structural determination of the helical nature of DNA by Watson and Crick and to the basic ideas on how DNA transmits genetic information.

On the much larger dimensional scale of human organs and internal structures, x-rays penetrate though skin and other soft tissue and travel in straight lines without diffraction. In this geometrical optics limit, they can be used to produce shadow images of, for example, bones within the body, based entirely on differences in absorption of x-rays. In fact, when Roentgen first discovered x-rays in 1895, within a week he had obtained the first x-ray picture of a hand (Figure 23.23). The depth of penetration of x-rays depends on the density of the material; denser materials, such as lead, are more effective in absorbing x-rays. Medical technology uses x-rays to obtain pictures of such structures as bone and teeth in x-ray radiography. Softer tissues can be pictured best if a dense material is introduced to increase the contrast. The gastrointestinal tract can be imaged if it is filled with a dense barium solution that casts a shadow in an x-ray picture. Similarly water-soluble organic compounds with iodine are used to give contrast for pictures of the cardiovascular system, the urinary tract or the brain. Mammography can be done without a contrast agent using low-energy x-rays because these give the greatest contrast for soft tissues.

These pictures produce two-dimensional projection images, lacking resolution along the beam direction because the intensity of the x-rays at the detector is determined by an integration or sum through the body along the beam. Thus three-dimensional information is lost on conversion to a two-dimensional picture. Put another way, there is no depth information in an x-ray picture and doctors must infer relative depths of neighboring features in these pictures with much care. Furthermore, it is more difficult to detect small differences in x-ray absorption at neighboring points because there is no resolution along the beam and therefore many minor abnormalities in x-ray radiography are not detectable.

To improve this situation, computed tomography (CT; the Greek word tomo means cut or slice) is able to obtain three-dimensional information from a collection of x-ray pictures taken at different orientations. The original CT machines developed in the 1970s used a single x-ray source and detector held in precise register on opposite sides of a patient. These were translated across the sample region, rotated by 1° and scanned across the sample again, and so on, in steps all around the body, so that a sequence of many pictures was obtained in a few minutes that could then be used to reconstruct the depth information in a three-dimensional image. Today, CT machines use a wide fanlike beam and an array of several hundreds to a few thousand x-ray detectors to decrease the time required to a few seconds (Figure 23.24). The newest designed machines have stationary detector arrays with an x-ray beam made to sweep in a circular pattern around the patient with no moving parts. We show in Chapter 25 that x-rays are generated by
transitions of outer electrons to inner empty electron shells after the inner electrons have been ejected by bombardment with high-speed electrons. In modern CT machines a scanning high-energy electron beam that generates the x-rays is an integral part of this design. Projection data can then be obtained in about 50 ms, fast enough to image a beating heart without motion artifacts.

However the various projection data are recorded, a computer will have a record of digitized intensities that needs to be processed to reconstruct the image of a cross-sectional slice in the body. The image consists of a large number of two-dimensional spots, or pixels, each having some grayscale level, a digital value representing the brightness. Grayscale displays are sometimes converted to false color images where the colors represent the brightness level but have nothing to do with the color of the original tissue being imaged. These brightness scales are normally set according to the absorption coefficient of the tissue \( \varepsilon \), compared to that of water, \( \varepsilon_w \), by the CT number

\[
\text{CT number} = 1000 \frac{\varepsilon - \varepsilon_w}{\varepsilon_w}. \quad (23.7)
\]

Table 23.1 shows the CT numbers for different tissue and media for 60 keV x-rays. The absorption coefficients of tissue depend on the x-ray beam energy and corrections usually need to be made for this fact. Note that negative CT numbers indicate that there is less absorption of x-rays than in water.

<table>
<thead>
<tr>
<th>Material</th>
<th>CT Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0</td>
</tr>
<tr>
<td>Air</td>
<td>−1000</td>
</tr>
<tr>
<td>Bone</td>
<td>808</td>
</tr>
<tr>
<td>Striated muscle</td>
<td>−48</td>
</tr>
<tr>
<td>Fat</td>
<td>−142</td>
</tr>
</tbody>
</table>

* Using 60 keV x-rays.

We briefly try to give the reader a sense of how projection data can be used to determine the CT numbers for an array of pixels in order to generate a cross-sectional
picture of the body based on x-ray contrast. In our context, the absorption coefficient is in the relation

\[ I = I_0 e^{-ex} \quad \text{or} \quad \log \frac{I_0}{I} = ex, \quad (23.8) \]

where \( I \) and \( I_0 \) are the transmitted and incident intensity on a tissue thickness \( x \) and the log is to base \(!e\). Each x-ray beam can be imagined to have traveled through a distance \( x \) in the body and the transmitted intensity detected. We imagine that each of \( N \) such neighboring parallel beams (the rows) is divided into \( N \) intervals of length \( x/N \) (the columns), forming a two-dimensional cross-sectional grid of \( N \) rows by \( N \) columns, with \( N \) typically in the range 256–1024. In the pixel display of this slice, the term \( ex \) in Equation (23.8) for the \( i \)th row, for example, is given by the sum

\[ e_{ix} = \sum_{j=1}^{N} e_{ij} \Delta x, \]

where we have labeled the \( e_{ij} \) values according to the pixel number (ith row and jth column) and have assumed that the pixel width, \( \Delta x = x/N \), is the same in any direction. In the simplest case, imagine that two sets of parallel x-ray beams are used to define a square grid as shown in Figure 23.25 (left) and that the projected (transmitted) intensity is measured for each beam. Using values for the projected intensities, computer algorithms can determine the \( e_{ij} \) for the \( N \times N \) pixels, giving a two-dimensional absorption image.

In general, more complex patterns of beams can be used (Figure 23.25 right). Because a set of \( N \times N \) pixels is needed to image a given plane, a minimum of \( N^2 \) values for \( e_{ij} \) are needed. These can be obtained from at least that many data points for \( \log I_0/I \), or \( ex \), obtained by imaging the same region of the body at many, many different orientations. Large numbers of equations must be simultaneously solved on a computer; with \( N = 256 \), there are at least \( N^2 = 65,500 \) equations to solve. Various computational techniques have been developed to do these calculations rapidly.

With current technology, multiple cross-sectional images can be rapidly obtained and computer techniques allow these to be superposed to produce 3-D images (Figure 23.26). These same tomography methods can be applied to other types of imaging, including ultrasonic (Chapter 11), magnetic resonance (Chapter 18), and to such nuclear decay imaging as positron emission tomography (PET; discussed in Chapter 26). The quality of images from CT and MRI scans are often comparable and the choice of method depends on the type of tissue to be imaged.

**CHAPTER SUMMARY**

Contrast is the other major factor, in addition to resolution discussed in the previous chapter, that determines whether an object can be imaged in a microscope. We can distinguish two types of contrast: amplitude and phase. Microscopes that use amplitude contrast include the standard bright-field compound microscope discussed in the previous chapter, as well as the dark-field and fluorescence microscopes. Phase contrast and DIC (differential interference contrast) microscopes use phase contrast to image objects. Newer microscopes use laser-scanning methods to do point-by-point imaging. These include confocal and multiphoton microscopes.

Optical activity refers to the effect of anisotropic molecules on the circular polarization of light. Such

(Continued)
QUESTIONS

1. Compare image contrast with resolution for a bright-field microscope. How does each enter into producing an image?

2. What is the function of the dichroic mirror in a fluorescent microscope? (See Figure 23.3.)

3. What are the origins of phase and amplitude contrast? Are both always present to some extent?

4. Describe the main differences, in your own words, between phase contrast and differential interference contrast microscopy.

5. What is the function of the Wollaston polarizing prisms in DIC optics? Is the fact that the two beams have different polarizations important in the final image seen?

6. What are the advantages of multiphoton microscopy over single-photon methods?

7. Discuss the superposition of two linear polarized light beams of the same frequency and equal amplitude, one polarized along the x-axis and one along the y-axis. What is the result if the two are in phase? 90° out of phase? 180° out of phase?

8. Because a plane mirror reverses left and right, but does not reverse up and down, if you hold a coiled right-handed spring and look at its image in a mirror is there an orientation of the spring that results in a right-handed image?

9. Simple molecules produced in chemical reactions, even if they have a handedness, are usually produced in nearly equal quantities of left- and right-handed molecules. Biological molecules, on the other hand, are nearly always found in pure left-handed form. What benefits might be derived from only having one form in living materials?

10. A linearly polarized light beam passes through a birefringent material and two beams emerge. If the beams are each made to pass through one slit of a double-slit experiment, will a standard double-slit interference pattern be produced on a distant screen?

11. What is the difference between circular birefringence and circular dichroism?

12. As the accelerating voltage in an electron microscope is increased, what happens to the theoretical magnification? To the sample degradation? To the magnetic field needed to focus the electron beam?

13. What is the purpose of heavy metal deposition in TEM? How does it affect resolution?

14. Can you argue why the backscattered electrons in SEM allow the images to appear much more three-dimensional than the images transmitted electrons produce in TEM?

15. Fill in the details in the derivation of the Bragg equation, Equation (23.6), using Figure 23.21.

16. Why, when you have a dental x-ray taken, are you covered with a heavy lead-coated gown?

17. Contrast how a CT image is obtained with how you perceive depth with two eyes.
MULTIPLE CHOICE QUESTIONS
1. In dark-field microscopy (a) the sample images darker than the background, (b) an annular aperture is inserted between the sample and the objective lens, (c) the image contrast is usually better than that of bright-field, (d) the samples must be stained to show up.

2. Fluorescent dyes can be used for all but which of the following? (a) Imaging calcium concentration variations, (b) imaging pH variations, (c) localizing specific molecules, (d) high-resolution imaging of molecules.

3. Which of the microscopic techniques usually requires that the sample be stabilized? (a) Phase contrast, (b) bright field, (c) DIC, (d) polarizing microscopy.

4. In DIC microscopy, the edges of microscopic objects are sharp because (a) that’s where the most stain is, (b) that’s where there is an extra π phase shift, (c) that’s where there is the greatest change in index of refraction, (d) that’s where the greatest polarization difference occurs.

5. In three-photon microscopy, to excite a fluor at 450 nm the incident wavelength of light should be (a) 150 nm, (b) 450 nm, (c) 900 nm, (d) 1350 nm.

6. In laser-scanning confocal microscopy all of the following are true except (a) the beam is focused to a very small spot, (b) the beam is moved across the sample, (c) two or more photons are absorbed at the same time, (d) the images appear three-dimensional.

7. A circularly polarized beam of light (a) travels in a spiral around its magnetic field, (b) travels in a spiral around its propagation direction, (c) has an electric field vector whose tip rotates in a closed circle, (d) has an electric field vector whose tip travels in a spiral.

8. Which is not true of a birefringent material? (a) It must be a solid because it has different indices of refraction along two different directions, (b) it can produce two beams of light from one, (c) it can produce circularly polarized light, (d) light can travel through it with two different speeds.

9. Which of the following is not true of an optically active molecule? (a) It produces a circular birefringence signal, (b) it produces a circular dichroism signal, (c) it must be asymmetric, (d) a solution of them can always be imaged in a polarizing microscope.

10. A typical accelerating voltage used in an electron microscope is (a) 100 kV, (b) 1 kV, (c) 10 MV, (d) 100 V.

11. Electron microscope samples must be stained or metal-coated because (a) the atoms are too small to detect otherwise, (b) the samples are not colored otherwise, (c) the samples do not interact with electrons otherwise, (d) the samples would evaporate from the grid otherwise.

12. All of the following are consequences of using high accelerating voltages and small focused spot sizes in scanning electron microscopy except (a) higher resolution, (b) decreased heating of the sample, (c) increased backscattered electrons, (d) more accurate elemental analysis.

13. Which of the following is not true? 60 keV x-rays are absorbed by (a) water more than fat, (b) water more than air, (c) bone more than striated muscle, (d) fat more than striated muscle.

14. The intensity remaining in a beam after traveling 10 cm through a sample with an absorption coefficient of 0.2 cm⁻¹ is (a) 1%, (b) 1.4%, (c) 14%, (d) 20%.

PROBLEMS
1. With a compound microscope adjusted poorly, the % contrast for a certain sample is only 5%. If the microscope is adjusted and the sample intensity is reduced by 10% and the background intensity is increased by 20%, what is the new % contrast?

2. In three-photon microscopy, if the peak in the absorption band of a fluorescent molecule to be imaged is at 360 nm, what incident frequency of light should be used?

3. Show that the tip of the electric field vector produced by the superposition of equal amplitude electric fields given in Equation (23.2) rotates in a circle. Viewed from a location at which the beam is approaching you, does the E vector rotate clockwise or counterclockwise?

4. A birefringent crystal has a birefringence given by Δn = n₁ - n₂ = 0.01, where n₁ and n₂ are the indices of refraction along its two transverse crystal axes at right angles with each other. Suppose a linearly polarized wave with 550 nm wavelength, is polarized at 45° to the crystal axes. If the crystal has a thickness of 1 cm, what will be the path difference between the two waves polarized along the crystal axes when they exit the crystal? What will be the net phase difference (as a fraction of 2π rad, or modulo 2π rad) of the two waves?

5. Suppose that the spot size in an SEM is 10 nm and that the beam is scanned over a region of 100 μm × 100 μm in a raster pattern, producing a single-scanned image in 10 ms. If the overall region is digitized into a 200 × 200 pixel area, (a) What sample area is represented by 1 pixel? (b) How long is the beam exposure in each pixel? (This determines resolution time of the detector.)

6. X-rays with a 0.12 nm wavelength produce a first-order diffraction peak at a Bragg angle of 24°. What crystal spacing gave rise to this diffraction?
for example, in Figure 23.21. Using simple trigonometry, draw a two-dimensional square lattice projection of the crystal (as in Figure 23.21) and find two other crystal plane spacings in terms of \( d \).

9. If an x-ray beam is incident on a 1.5 cm thick sample and 98% of the beam is transmitted what is the average absorption coefficient of the material in units of \( \text{m}^{-1} \)?

10. Two samples for an x-ray absorption experiment have the same thickness. With the same incident intensity one has a 95% transmission and the second has an 85% transmission. What is the ratio of their absorption coefficients?

11. Suppose that an x-ray beam is directed on a tissue sample and suppose that 99.3% of the beam is transmitted. If a dummy blank sample of water is used 99.5% of the x-rays are transmitted using exactly the same geometry and beam. What is the CT number of this sample?