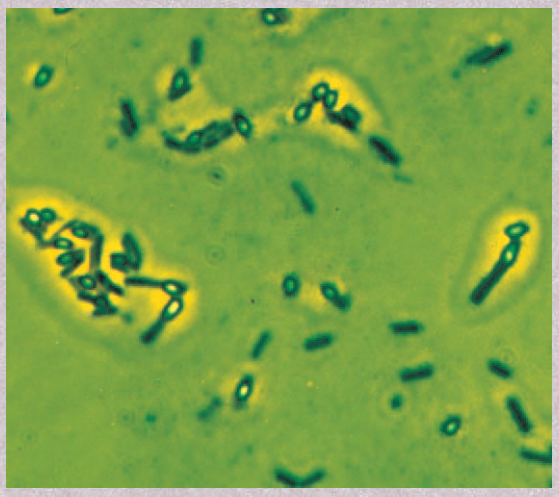
CHAPTER 2

The Study of Microbial Structure: Microscopy and Specimen Preparation



Clostridium botulinum is a rod-shaped bacterium that forms endospores and releases botulinum toxin, the cause of botulism food poisoning. In this phase-contrast micrograph, the endospores are the bright, oval objects located at the ends of the rods; some endospores have been released from the cells that formed them.

Outline

Concepts

1. Light microscopes use glass lenses to bend and focus light rays and produce enlarged images of small objects. The resolution of a light microscope is determined by the numerical aperture of its lens system and by the wavelength of the light it employs; maximum resolution is about 0.2 μm.
2. The most common types of light microscopes are the bright-field, dark-field, phase-contrast, and fluorescence microscopes. Each yields a distinctive image and may be used to observe different aspects of microbial morphology.
3. Because most microorganisms are colorless and therefore not easily seen in the bright-field microscope, they are usually fixed and stained before observation. Either simple or differential staining can be used to enhance contrast. Specific bacterial structures such as capsules, endospores, and flagella also can be selectively stained.
4. The transmission electron microscope achieves great resolution (about 0.5 nm) by using electron beams of very short wavelength rather than visible light. Although one can prepare microorganisms for observation in other ways, one normally views thin sections of plastic-embedded specimens treated with heavy metals to improve contrast.
5. External features can be observed in great detail with the scanning electron microscope, which generates an image by scanning a fine electron beam over the surface of specimens rather than projecting electrons through them.
6. New forms of microscopy are improving our ability to observe microorganisms and molecules. Two examples are the confocal scanning laser microscope and the scanning probe microscope.

There are more animals living in the scum on the teeth in a man's mouth than there are men in a whole kingdom.

—Antony van Leeuwenhoek

Microbiology usually is concerned with organisms so small they cannot be seen distinctly with the unaided eye. Because of the nature of this discipline, the microscope is of crucial importance. Thus it is important to understand how the microscope works and the way in which specimens are prepared for examination.

The chapter begins with a detailed treatment of the standard bright-field microscope and then describes other common types of light microscopes. Next preparation and staining of specimens for examination with the light microscope are discussed. This is followed by a description of transmission and scanning electron microscopes, both of which are used extensively in current microbiological research. The chapter closes with a brief introduction to two newer forms of microscopy: scanning probe microscopy and confocal microscopy.

2.1 Lenses and the Bending of Light

To understand how a light microscope operates, one must know something about the way in which lenses bend and focus light to form images. When a ray of light passes from one medium to another, **refraction** occurs—that is, the ray is bent at the interface. The **refractive index** is a measure of how greatly a substance slows the velocity of light, and the direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface. When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface (**figure 2.1**). As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal. Thus a prism bends light because glass has a different refractive index from air, and the light strikes its surface at an angle.

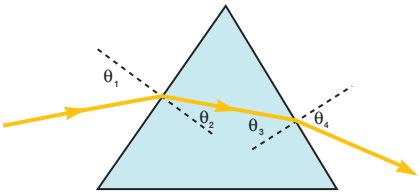


Figure 2.1 The Bending of Light by a Prism. Normals (lines perpendicular to the surface of the prism) are indicated by dashed lines. As light enters the glass, it is bent toward the first normal (angle θ_2 is less than θ_1). When light leaves the glass and returns to air, it is bent away from the second normal (θ_4 is greater than θ_3). As a result the prism bends light passing through it.

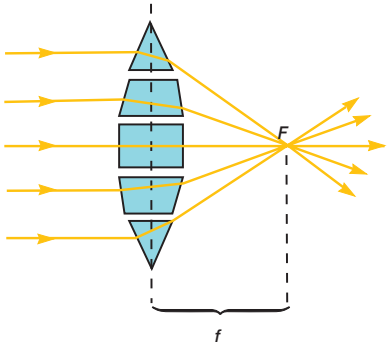


Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point *F.* The focal point lies a distance *f,* the focal length, from the lens center.

Lenses act like a collection of prisms operating as a unit. When the light source is distant so that parallel rays of light strike the lens, a convex lens will focus these rays at a specific point, the **focal point** (*F* in **figure 2.2**). The distance between the center of the lens and the focal point is called the **focal length** (*f* in figure 2.2).

Our eyes cannot focus on objects nearer than about 25 cm or 10 inches (**table 2.1**). This limitation may be overcome by using a convex lens as a simple magnifier (or microscope) and holding it close to an object. A magnifying glass provides a clear image at much closer range, and the object appears larger. Lens strength is related to focal length; a lens with a short focal length will magnify an object more than a weaker lens having a longer focal length.

Table 2.1 Common Units of Measurement

Unit	Abbreviation	Value
1 centimeter	cm	10^{-2} meter or 0.394 inches
1 millimeter	mm	10^{-3} meter
1 micrometer	μm	10^{-6} meter
1 nanometer	nm	10^{-9} meter
1 Angstrom	Å	10^{-10} meter

1. Define refraction, refractive index, focal point, and focal length.
2. Describe the path of a light ray through a prism or lens.
3. How is lens strength related to focal length?

2.2 The Light Microscope

Microbiologists currently employ a variety of light microscopes in their work; bright-field, dark-field, phase-contrast, and fluorescence microscopes are most commonly used. Modern microscopes are all compound microscopes. That is, the magnified image formed by the objective lens is further enlarged by one or more additional lenses.

The Bright-Field Microscope

The ordinary microscope is called a **bright-field microscope** because it forms a dark image against a brighter background. The microscope consists of a sturdy metal body or stand composed of a base and an arm to which the remaining parts are attached (**figure 2.3**). A light source, either a mirror or an electric illuminator, is located in the base. Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and can move either the stage or the nosepiece to focus the image.

The stage is positioned about halfway up the arm and holds microscope slides by either simple slide clips or a mechanical stage clip. A mechanical stage allows the operator to move a slide around smoothly during viewing by use of stage control knobs. The **substage condenser** is mounted within or beneath the stage and focuses a cone of light on the slide. Its position often is fixed in simpler microscopes but can be adjusted vertically in more advanced models.

The curved upper part of the arm holds the body assembly, to which a nosepiece and one or more **eyepieces** or **oculars** are attached. More advanced microscopes have eyepieces for both eyes and are called binocular microscopes. The body assembly itself contains a series of mirrors and prisms so that the barrel holding the eyepiece may be tilted for ease in viewing (**figure 2.4**). The nosepiece holds three to five **objectives** with lenses of differing magnifying power and can be rotated to position any objective beneath the body assembly. Ideally a microscope should

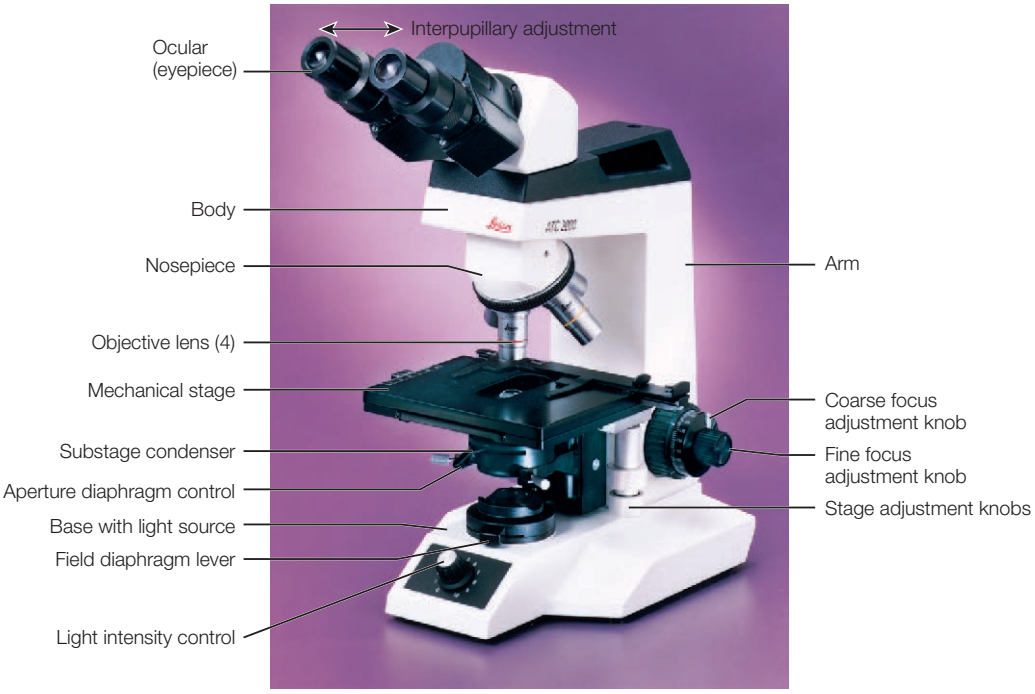


Figure 2.3 A Bright-Field Microscope. The parts of a modern bright-field microscope. The microscope pictured is somewhat more sophisticated than those found in many student laboratories. For example, it is binocular (has two eyepieces) and has a mechanical stage, an adjustable substage condenser, and a built-in illuminator.

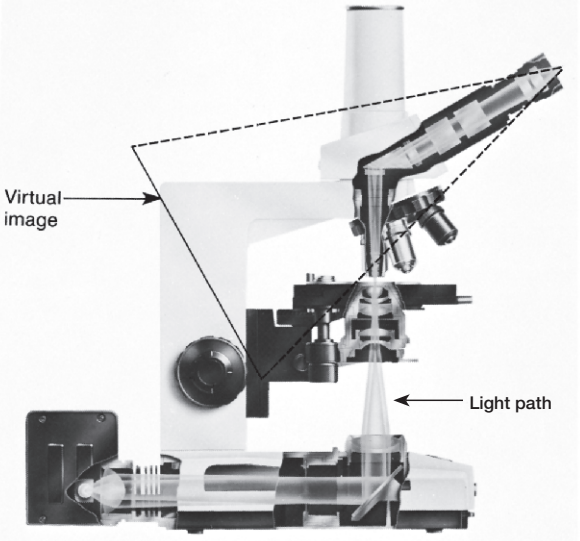


Figure 2.4 A Microscope's Light Path. The light path in an advanced bright-field microscope and the location of the virtual image are shown. (See also figure 2.23.)

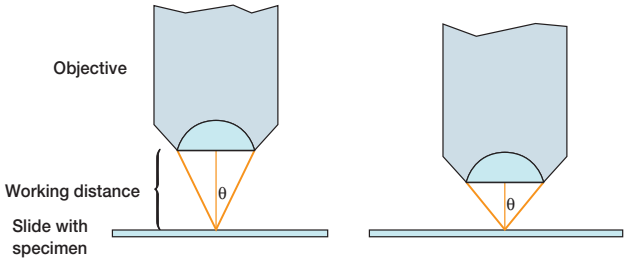


Figure 2.5 Numerical Aperture in Microscopy. The angular aperture θ is $\frac{1}{2}$ the angle of the cone of light that enters a lens from a specimen, and the numerical aperture is n sin θ. In the right-hand illustration the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

be **parfocal**—that is, the image should remain in focus when objectives are changed.

The path of light through a bright-field microscope is shown in figure 2.4. The objective lens forms an enlarged real image within the microscope, and the eyepiece lens further magnifies this primary image. When one looks into a microscope, the enlarged specimen image, called the virtual image, appears to lie just beyond the stage about 25 cm away. The total magnification is calculated by multiplying the objective and eyepiece magnifications together. For example, if a 45× objective is used with a 10× eyepiece, the overall magnification of the specimen will be 450×.

Microscope Resolution

The most important part of the microscope is the objective, which must produce a clear image, not just a magnified one. Thus resolution is extremely important. **Resolution** is the ability of a lens to separate or distinguish between small objects that are close together. Much of the optical theory underlying microscope design was developed by the German physicist Ernst Abbé in the 1870s. The minimum distance (d) between two objects that reveals them as separate entities is given by the Abbé equation, in which lambda (λ) is the wavelength of light used to illuminate the specimen and n sin θ is the numerical aperture (NA).

$$d = \frac{0.5\lambda}{n \sin \theta}$$

As d becomes smaller, the resolution increases, and finer detail can be discerned in a specimen.

The preceding equation indicates that a major factor in resolution is the wavelength of light used. The wavelength must be shorter than the distance between two objects or they will not be seen clearly. Thus the greatest resolution is obtained with light of the shortest wavelength, light at the blue end of the visible spectrum (in the range of 450 to 500 nm). The electromagnetic spectrum of radiation (p. 130).

The **numerical aperture** (n sin θ) is more difficult to understand. Theta is defined as $\frac{1}{2}$ the angle of the cone of light entering an objective (**figure 2.5**). Light that strikes the microorganism after passing through a condenser is cone-shaped. When this cone has a narrow angle and tapers to a sharp point, it does not spread out much after leaving the slide and therefore does not adequately separate images of closely packed objects. The resolution is low. If the cone of light has a very wide angle and spreads out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved. The angle of the cone of light that can enter a lens depends on the refractive index (n) of the medium in which the lens works, as well as upon the objective itself. The refractive index for air is 1.00. Since sin θ cannot be greater than 1 (the maximum θ is 90° and sin 90° is 1.00), no lens working in air can have a numerical aperture greater than 1.00. The only practical way to raise the numerical aperture above 1.00, and therefore achieve higher resolution, is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass (**table 2.2**). If air is replaced with immersion oil, many light rays that did not enter the objective due to reflection and refraction at the surfaces of the objective lens and slide will now do so (**figure 2.6**). An increase in numerical aperture and resolution results.

Table 2.2 The Properties of Microscope Objectives

	Objective			
Property	**Scanning**	**Low Power**	**High Power**	**Oil Immersion**
Magnification	4×	10×	40–45×	90–100×
Numerical aperture	0.10	0.25	0.55–0.65	1.25–1.4
Approximate focal length (*f*)	40 mm	16 mm	4 mm	1.8–2.0 mm
Working distance	17–20 mm	4–8 mm	0.5–0.7 mm	0.1 mm
Approximate resolving power with light of 450 nm (blue light)	2.3 μm	0.9 μm	0.35 μm	0.18 μm

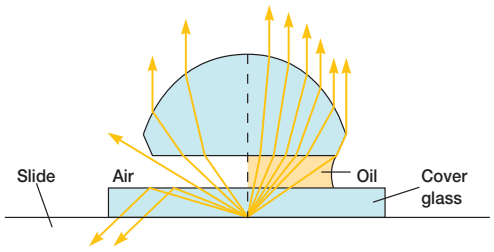


Figure 2.6 The Oil Immersion Objective. An oil immersion objective operating in air and with immersion oil.

The resolution of a microscope depends upon the numerical aperture of its condenser as well as that of the objective. This is evident from the equation describing the resolution of the complete microscope.

$$d_{\text{microscope}} = \frac{\lambda}{(\text{NA}_{\text{objective}} + \text{NA}_{\text{condenser}})}$$

Most microscopes have a condenser with a numerical aperture between 1.2 and 1.4. However, the condenser numerical aperture will not be much above about 0.9 unless the top of the condenser is oiled to the bottom of the slide. During routine microscope operation, the condenser usually is not oiled and this limits the overall resolution, even with an oil immersion objective.

The limits set on the resolution of a light microscope can be calculated using the Abbé equation. The maximum theoretical resolving power of a microscope with an oil immersion objective (numerical aperture of 1.25) and blue-green light is approximately 0.2 μm.

$$d = \frac{(0.5)(530\text{ nm})}{1.25} = 212\text{ nm or }0.2\ \mu\text{m}$$

At best, a bright-field microscope can distinguish between two dots around 0.2 μm apart (the same size as a very small bacterium).

Normally a microscope is equipped with three or four objectives ranging in magnifying power from 4× to 100× (table 2.2). The **working distance** of an objective is the distance between the front surface of the lens and the surface of the cover glass (if one is used) or the specimen when it is in sharp focus. Objectives with large numerical apertures and great resolving power have short working distances.

The largest useful magnification increases the size of the smallest resolvable object enough to be visible. Our eye can just detect a speck 0.2 mm in diameter, and consequently the useful limit of magnification is about 1,000 times the numerical aperture of the objective lens. Most standard microscopes come with 10× eyepieces and have an upper limit of about 1,000× with oil immersion. A 15× eyepiece may be used with good objectives to achieve a useful magnification of 1,500×. Any further magnification increase does not enable a person to see more detail. A light microscope can be built to yield a final magnification of 10,000×, but it would simply be magnifying a blur. Only the electron microscope provides sufficient resolution to make higher magnifications useful.

Proper specimen illumination also is extremely important in determining resolution. A microscope equipped with a concave mirror between the light source and the specimen illuminates the slide with a fairly narrow cone of light and has a small numerical aperture. Resolution can be improved with a substage condenser, a large light-gathering lens used to project a wide cone of light through the slide and into the objective lens, thus increasing the numerical aperture.

The Dark-Field Microscope

Living, unstained cells and organisms can be observed by simply changing the way in which they are illuminated. A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective. Only light that has been reflected or refracted by the specimen forms an image (**figure 2.7**). The field surrounding a specimen appears black, while the object itself is brightly illuminated

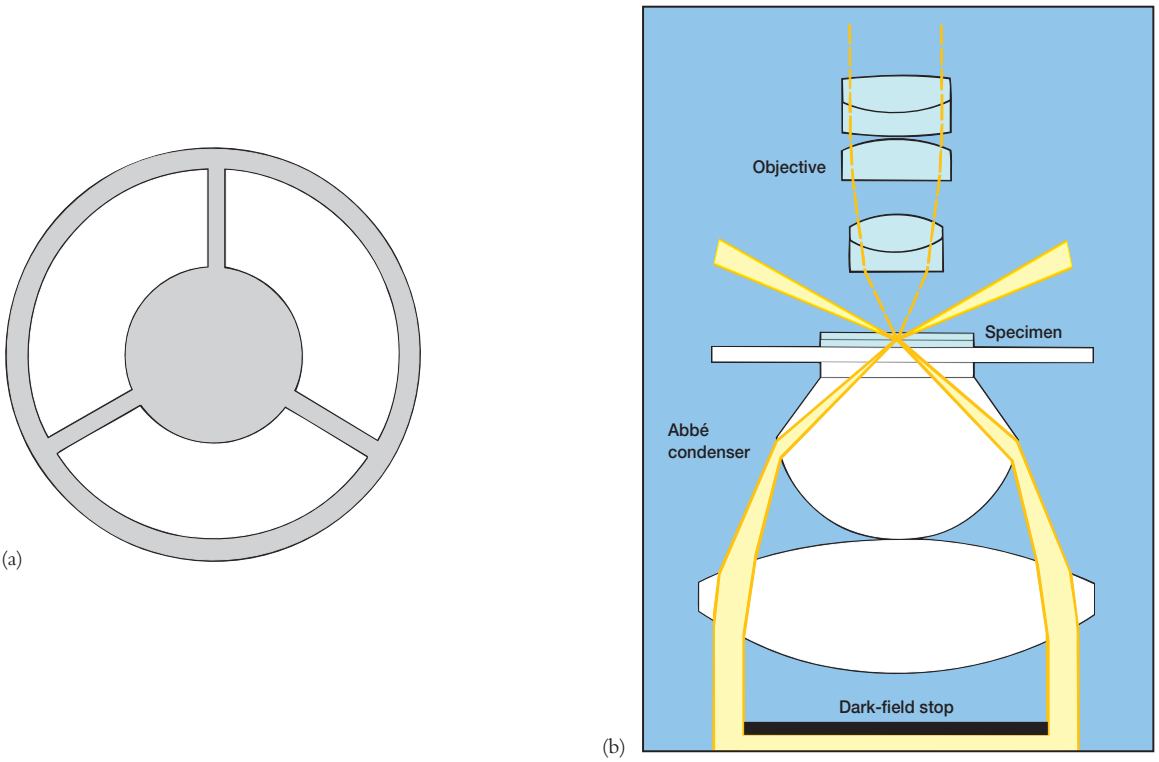


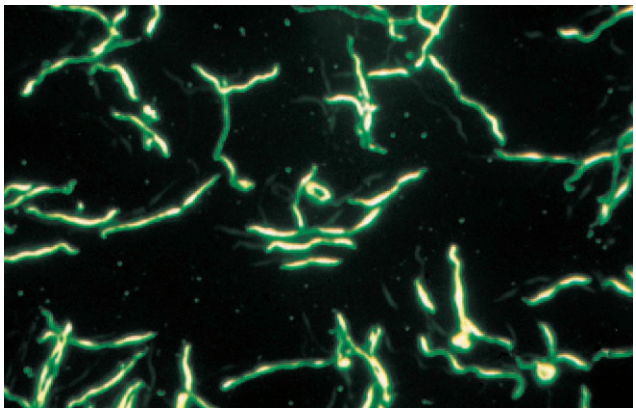
Figure 2.7 Dark-Field Microscopy. The simplest way to convert a microscope to dark-field microscopy is to place (**a**) a dark-field stop underneath (**b**) the condenser lens system. The condenser then produces a hollow cone of light so that the only light entering the objective comes from the specimen.

(**figure 2.8*a,b***); because the background is dark, this type of microscopy is called **dark-field microscopy.** Considerable internal structure is often visible in larger eucaryotic microorganisms (figure 2.8*b*). The dark-field microscope is used to identify bacteria like the thin and distinctively shaped *Treponema pallidum* (figure 2.8*a*), the causative agent of syphilis.

The Phase-Contrast Microscope

Unpigmented living cells are not clearly visible in the bright-field microscope because there is little difference in contrast between the cells and water. Thus microorganisms often must be fixed and stained before observation to increase contrast and create variations in color between cell structures. A **phase-contrast microscope** converts slight differences in refractive index and cell density into easily detected variations in light intensity and is an excellent way to observe living cells (figure 2.8*c–e*).

The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light (**figure 2.9**). As this cone passes through a cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about $\frac{1}{4}$ wavelength. The deviated light is focused to form an image of the object. Undeviated light rays strike a phase ring in the phase plate, a special optical disk located in the objective, while the deviated rays miss the ring and pass through the rest of the plate. If the phase ring is constructed in such a way that the undeviated light passing through it is advanced by $\frac{1}{4}$ wavelength, the deviated and undeviated waves will be about $\frac{1}{2}$ wavelength out of



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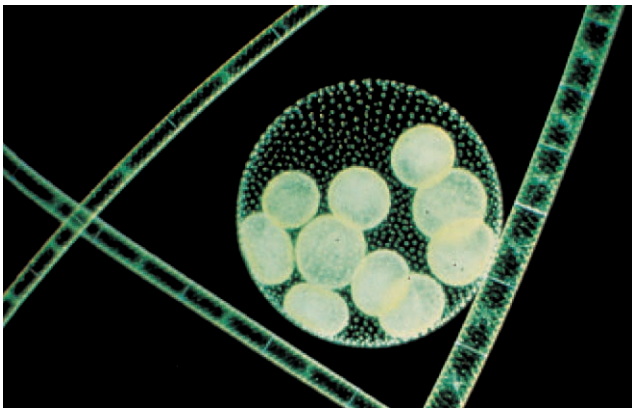

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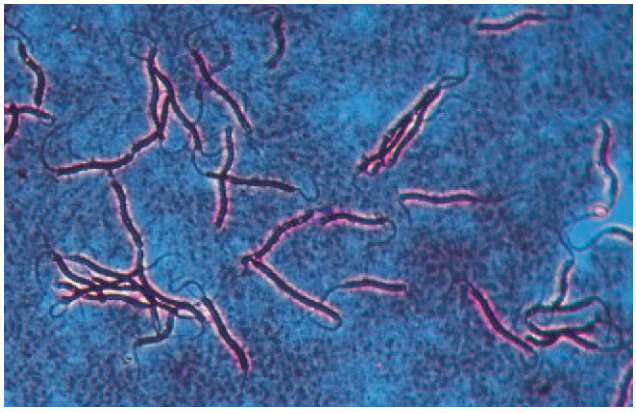

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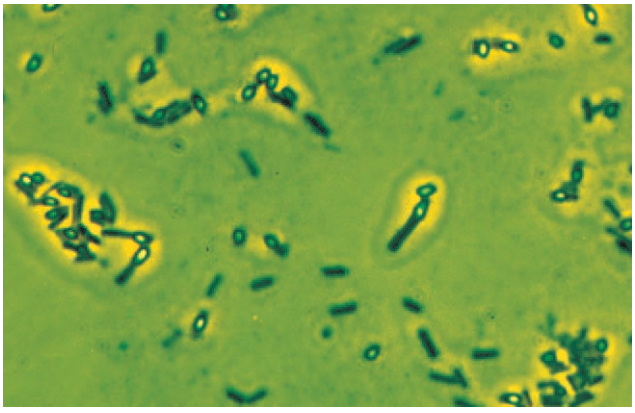
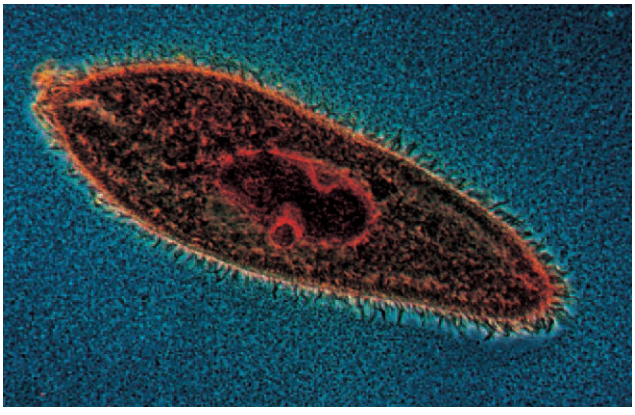


(d)



(e)

Figure 2.8 Examples of Dark-Field and Phase-Contrast Microscopy. (**a**) *Treponema pallidum,* the spirochete that causes syphilis; dark-field microscopy (×500). **(b)** *Volvox* and *Spirogyra;* dark-field microscopy (×175). Note daughter colonies within the mature *Volvox* colony (center) and the spiral chloroplasts of *Spirogyra* (left and right). **(c)** *Spirillum volutans,* a very large bacterium with flagellar bundles; phase-contrast microscopy (×210). **(d)** *Clostridium botulinum,* the bacterium responsible for botulism, with subterminal oval endospores; phase-contrast microscopy (×600). **(e)** *Paramecium* stained to show a large central macronucleus with a small spherical micronucleus at its side; phase-contrast microscopy (×100).

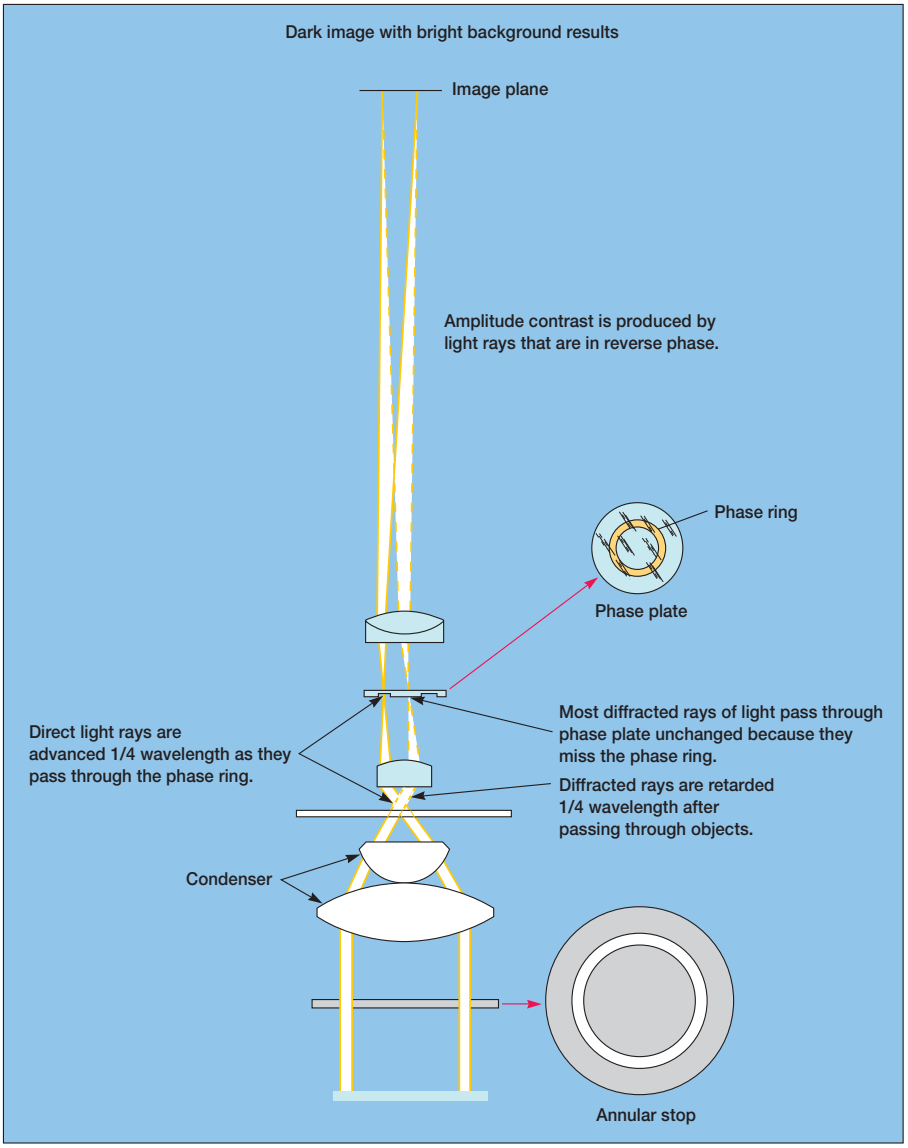


Figure 2.9 **Phase-Contrast Microscopy.** The optics of a dark-phase-contrast microscope.

phase and will cancel each other when they come together to form an image (**figure 2.10**). The background, formed by undeviated light, is bright, while the unstained object appears dark and well-defined. This type of microscopy is called **dark-phase-contrast microscopy.** Color filters often are used to improve the image (figure 2.8*c,d*).

Phase-contrast microscopy is especially useful for studying microbial motility, determining the shape of living cells, and detecting bacterial components such as endospores and inclusion bodies that contain poly-β-hydroxybutyrate, polymetaphosphate, sulfur, or other substances (*see chapter 3*). These are clearly visible (figure 2.8*d*) because they have refractive indexes markedly different from that of water. Phase-contrast microscopes also are widely used in studying eucaryotic cells.

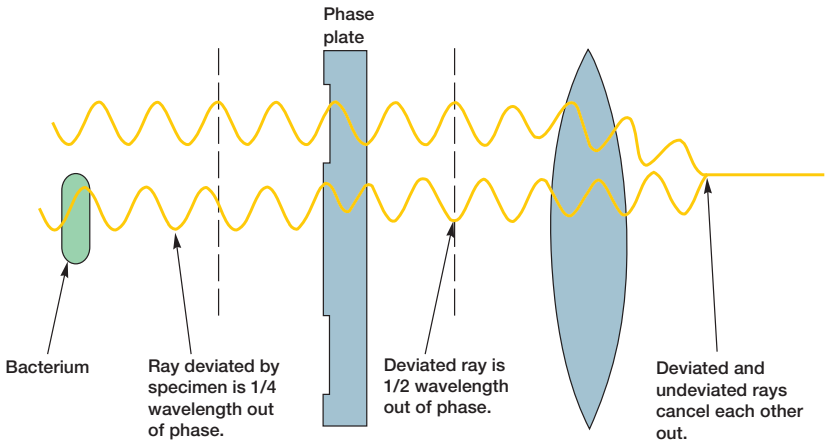


Figure 2.10 The Production of Contrast in Phase Microscopy. The behavior of deviated and undeviated or undiffracted light rays in the dark-phase-contrast microscope. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background.

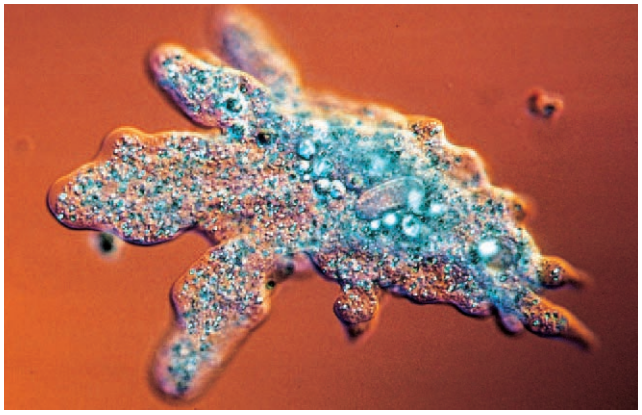


Figure 2.11 Differential Interference Contrast Microscopy. A micrograph of the protozoan *Amoeba proteus.* The three-dimensional image contains considerable detail and is artificially colored (×160).

The Differential Interference Contrast Microscope

The **differential interference contrast (DIC) microscope** is similar to the phase-contrast microscope in that it creates an image by detecting differences in refractive indices and thickness. Two beams of plane polarized light at right angles to each other are generated by prisms. In one design, the object beam passes through the specimen, while the reference beam passes through a clear area of the slide. After passing through the specimen, the two beams are combined and interfere with each other to form an image. A live, unstained specimen appears brightly colored and three-dimensional (**figure 2.11**). Structures such as cell walls, endospores, granules, vacuoles, and eucaryotic nuclei are clearly visible.

The Fluorescence Microscope

The microscopes thus far considered produce an image from light that passes through a specimen. An object also can be seen because it actually emits light, and this is the basis of fluorescence microscopy. When some molecules absorb radiant energy, they become excited and later release much of their trapped energy as light. Any light emitted by an excited molecule will have a longer wavelength (or be of lower energy) than the radiation originally absorbed. **Fluorescent light** is emitted very quickly by the excited molecule as it gives up its trapped energy and returns to a more stable state.

The **fluorescence microscope** (**figure 2.12**) exposes a specimen to ultraviolet, violet, or blue light and forms an image of the object with the resulting fluorescent light. A mercury vapor arc lamp or other source produces an intense beam, and heat transfer is limited by a special infrared filter. The light passes through an exciter filter that transmits only the desired wavelength. A dark-field condenser provides a black background against which the fluorescent objects glow. Usually the specimens have been stained with dye molecules, called **fluorochromes,** that fluoresce brightly upon exposure to light of a specific wavelength, but some microorganisms are autofluorescing. The microscope forms an image of the fluorochrome-labeled microorganisms

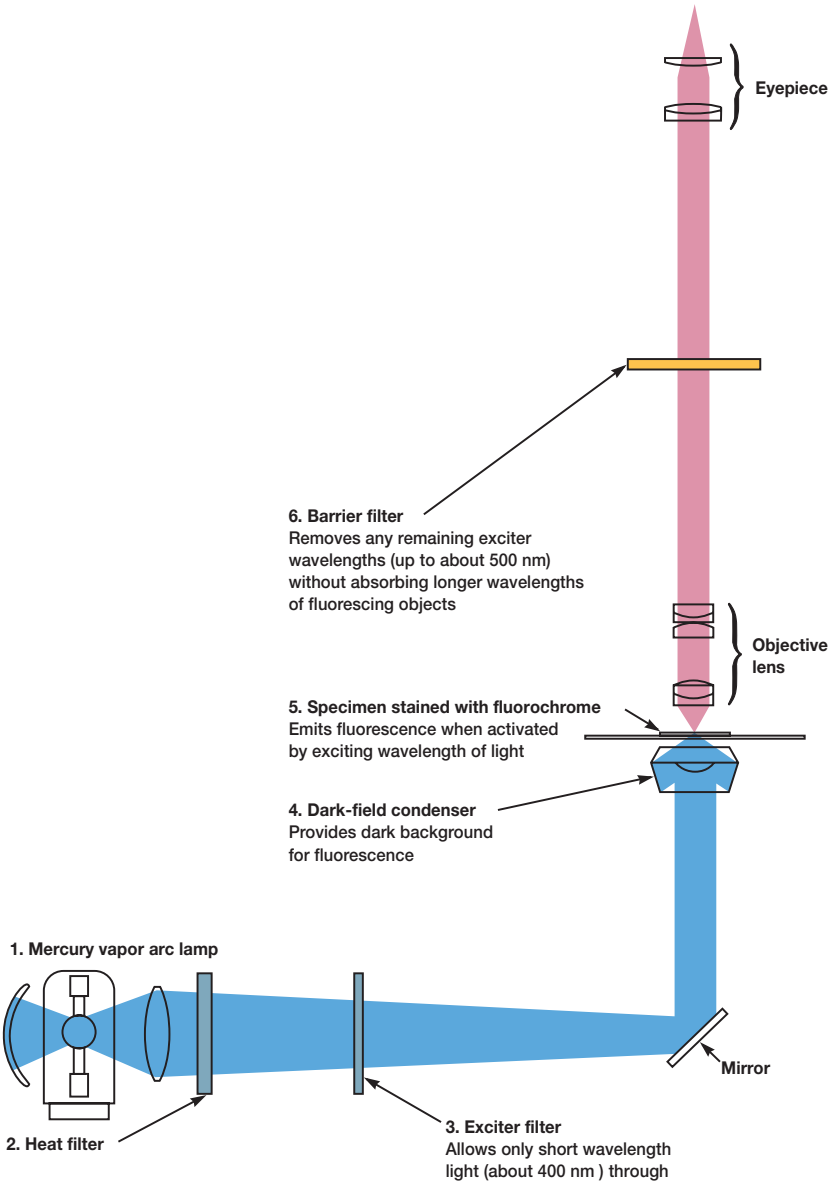
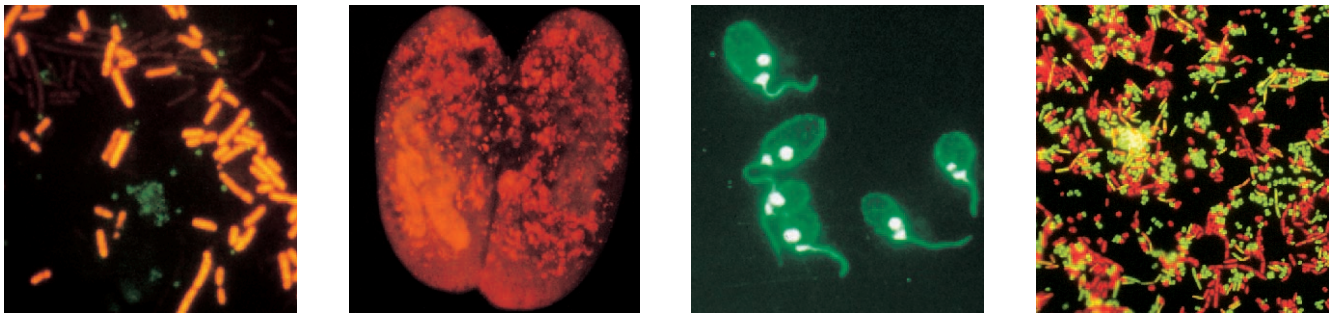


Figure 2.12 **Fluorescence Microscopy.** The principles of operation of a fluorescence microscope.

from the light emitted when they fluoresce (**figure 2.13**). A barrier filter positioned after the objective lenses removes any remaining ultraviolet light, which could damage the viewer's eyes, or blue and violet light, which would reduce the image's contrast.

The fluorescence microscope has become an essential tool in medical microbiology and microbial ecology. Bacterial pathogens (e.g., *Mycobacterium tuberculosis,* the cause of tuberculosis) can be identified after staining them with fluorochromes or specifically labeling them with fluorescent antibodies using immunofluorescence procedures. In ecological studies the fluorescence microscope is used to observe microorganisms stained with fluorochrome-labeled probes or fluorochromes such as acridine orange and DAPI (diamidino-2-phenylindole, a DNA-specific stain). The stained organisms will fluoresce orange or green and can be detected even in the midst of other particulate material. It is even possible to distinguish live bacteria from dead bacteria by the color they fluoresce after treatment with a special mixture of stains (figure 2.13*d*). Thus the microorganisms can be viewed and directly counted in a relatively undisturbed ecological niche. Immunofluorescence and diagnostic microbiology (pp. 781, 831–32).



(a) (b) (c) (d)

Figure 2.13 Examples of Fluorescence Microscopy. (**a**) *Escherichia coli* stained with fluorescent antibodies (×600). The green material is debris. **(b)** *Paramecium tetraurelia* conjugating; acridine-orange fluorescence (×125). **(c)** The flagellate protozoan *Crithidia luciliae* stained with fluorescent antibodies to show the kinetoplast (×1,000). **(d)** A mixture of *Micrococcus luteus* and *Bacillus cereus* (the rods). The live bacteria fluoresce green; dead bacteria are red.

1. List the parts of a light microscope and their functions.
2. Define resolution, numerical aperture, working distance, and fluorochrome.
3. How does resolution depend upon the wavelength of light, refractive index, and the numerical aperture? What are the functions of immersion oil and the substage condenser?
4. Briefly describe how dark-field, phase-contrast, differential interference contrast, and fluorescence microscopes work and the kind of image provided by each. Give a specific use for each type.

2.3 Preparation and Staining of Specimens

Although living microorganisms can be directly examined with the light microscope, they often must be fixed and stained to increase visibility, accentuate specific morphological features, and preserve them for future study.

Fixation

The stained cells seen in a microscope should resemble living cells as closely as possible. **Fixation** is the process by which the internal and external structures of cells and microorganisms are preserved and fixed in position. It inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. A microorganism usually is killed and attached firmly to the microscope slide during fixation.

There are two fundamentally different types of fixation. (1) Bacteriologists heat-fix bacterial smears by gently flame heating an air-dried film of bacteria. This adequately preserves overall morphology but not structures within cells. (2) Chemical fixation must be used to protect fine cellular substructure and the morphology of larger, more delicate microorganisms. Chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble, and immobile. Common fixative mixtures contain such components as ethanol, acetic acid, mercuric chloride, formaldehyde, and glutaraldehyde.

Dyes and Simple Staining

The many types of dyes used to stain microorganisms have two features in common. (1) They have **chromophore groups,** groups with conjugated double bonds that give the dye its color. (2) They can bind with cells by ionic, covalent, or hydrophobic bonding. For example, a positively charged dye binds to negatively charged structures on the cell.

Ionizable dyes may be divided into two general classes based on the nature of their charged group.

1. **Basic dyes**—methylene blue, basic fuchsin, crystal violet, safranin, malachite green—have positively charged groups (usually some form of pentavalent nitrogen) and are generally sold as chloride salts. Basic dyes bind to negatively charged molecules like nucleic acids and many proteins. Because the surfaces of bacterial cells also are negatively charged, basic dyes are most often used in bacteriology.
2. **Acid dyes**—eosin, rose bengal, and acid fuchsin—possess negatively charged groups such as carboxyls (—COOH) and phenolic hydroxyls (—OH). Acid dyes, because of their negative charge, bind to positively charged cell structures.

The pH may alter staining effectiveness since the nature and degree of the charge on cell components change with pH. Thus anionic dyes stain best under acidic conditions when proteins and many other molecules carry a positive charge; basic dyes are most effective at higher pHs.

Although ionic interactions are probably the most common means of attachment, dyes also bind through covalent bonds or because of their solubility characteristics. For instance, DNA can be stained by the Feulgen procedure in which Schiff's reagent is covalently attached to its deoxyribose sugars after hydrochloric acid treatment. Sudan III (Sudan Black) selectively stains lipids because it is lipid soluble but will not dissolve in aqueous portions of the cell.

Microorganisms often can be stained very satisfactorily by **simple staining,** in which a single staining agent is used. Simple staining's value lies in its simplicity and ease of use. One covers the fixed smear with stain for the proper length of time, washes the excess stain off with water, and blots the slide dry. Basic dyes like crystal violet, methylene blue, and carbolfuchsin are frequently used to determine the size, shape, and arrangement of bacteria.

Differential Staining

Differential staining procedures divide bacteria into separate groups based on staining properties. The **Gram stain,** developed in 1884 by the Danish physician Christian Gram, is the most widely employed staining method in bacteriology. It is a differential staining procedure because it divides bacteria into two classes—gram negative and gram positive. Gram-positive and gram-negative bacteria (pp. 55–60, 440–41).

In the first step of the Gram-staining procedure (**figure 2.14**), the smear is stained with the basic dye crystal violet, the primary stain. It is followed by treatment with an iodine solution functioning as a **mordant.** That is, the iodine increases the interaction between the cell and the dye so that the cell is stained more strongly. The smear is next decolorized by washing with ethanol or acetone. This step generates the differential aspect of the Gram stain; gram-positive bacteria retain the crystal violet, whereas gram-negative bacteria lose their crystal violet and become colorless. Finally, the smear is counterstained with a simple, basic dye different in color from crystal violet. Safranin, the most common counterstain, colors gram-negative bacteria pink to red and leaves gram-positive bacteria dark purple (**figure 2.15**). Cell wall structure and the mechanism of the Gram stain (p. 60).

Acid-fast staining is another important differential staining procedure. A few species, particularly those in the genus *Mycobacterium* (*see chapter 24*) do not bind simple stains readily and must be stained by a harsher treatment: heating with a mixture of basic fuchsin and phenol (the Ziehl-Neelsen method). Once basic fuchsin has penetrated with the aid of heat and phenol, acid-fast cells are not easily decolorized by an acid-alcohol wash and hence remain red. This is due to the quite high lipid content of acid-fast cell walls; in particular, mycolic acid—a group of branched chain hydroxy lipids—appears responsible for acid-fastness. Non-acid-fast bacteria are decolorized by acid-alcohol and thus are stained blue by methylene blue counterstain. This method is used to identify *Mycobacterium tuberculosis* and *M. leprae* (**figure 2.16**), the pathogens responsible for tuberculosis and leprosy, respectively.

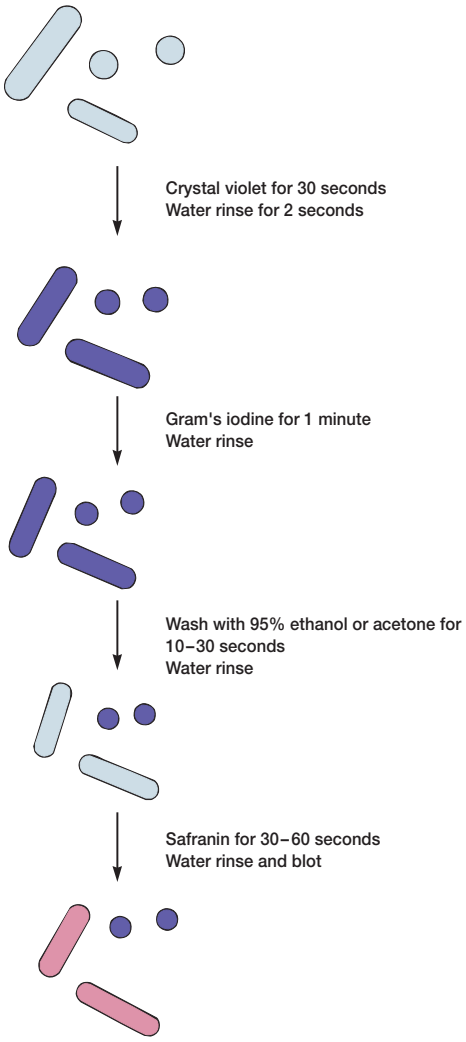
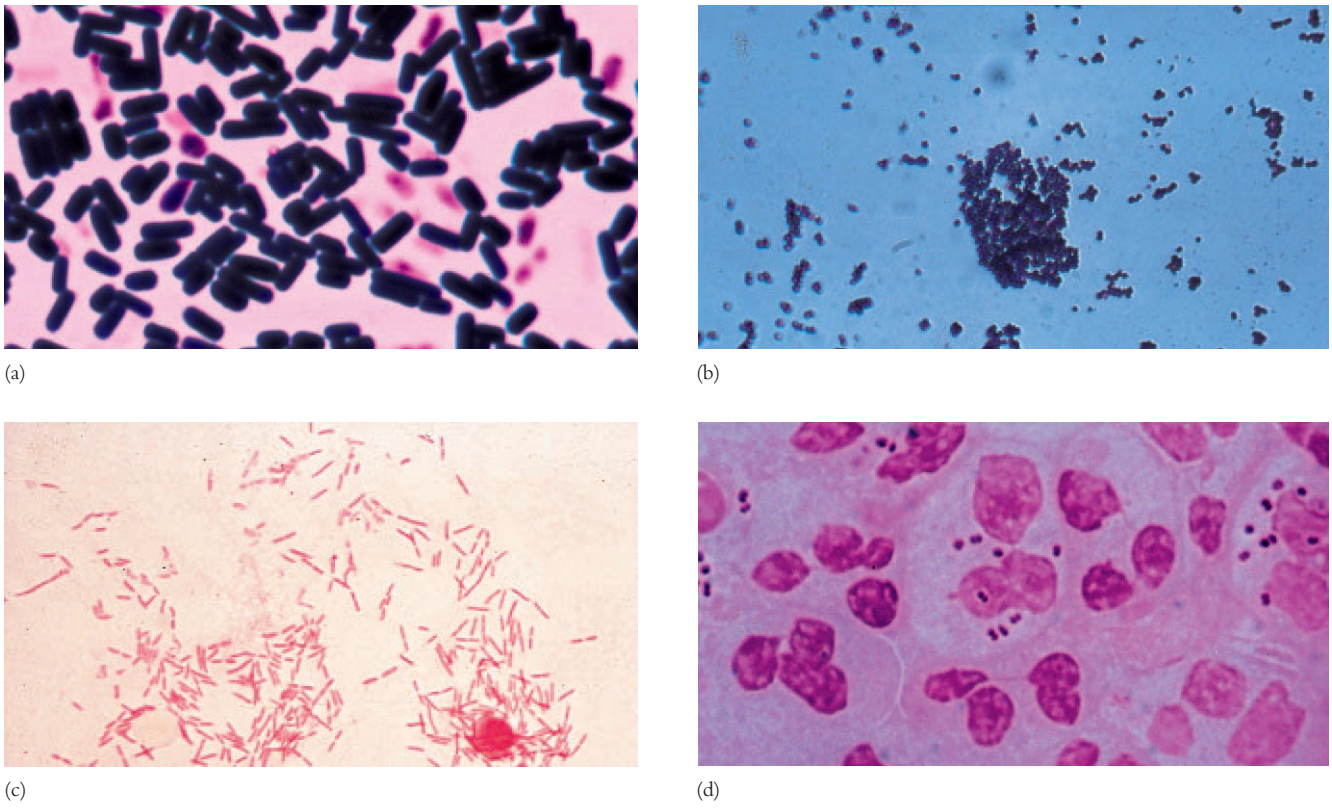


Figure 2.14 The Gram-Staining Procedure. Note that decolorization with ethanol or acetone removes crystal violet from gram-negative cells but not from gram-positive cells. The gram-negative cells then turn pink to red when counterstained with safranin.

Staining Specific Structures

Many special staining procedures have been developed over the years to study specific bacterial structures with the light microscope. One of the simplest is **negative staining,** a technique that reveals the presence of the diffuse capsules surrounding many bacteria. Bacteria are mixed with India ink or Nigrosin dye and spread out in a thin film on a slide. After air-drying, bacteria appear as lighter bodies in the midst of a blue-black background because ink and dye particles cannot penetrate either the bacterial cell or its capsule. The extent of the light region is determined by the size of the capsule and of the cell itself. There is little distortion of bacterial shape, and the cell can be counterstained for even greater visibility (**figure 2.17**). Capsules and slime layers (pp. 61–62).



(a) (b) (c) (d)

Figure 2.15 Examples of Gram Staining. (**a**) Gram-positive *Clostridium perfringens* (×800). Some rods have stained pink rather than purple, as often happens when gram-positive cells age. **(b)** *Staphylococcus aureus.* Gram stain, bright-field microscopy (×1,000). The gram-positive cocci associate in grapelike clusters. **(c)** *Escherichia coli,* Gram stain (×500). **(d)** *Neisseria gonorrhoeae.* The diplococci are often within white blood cells (×1,000).

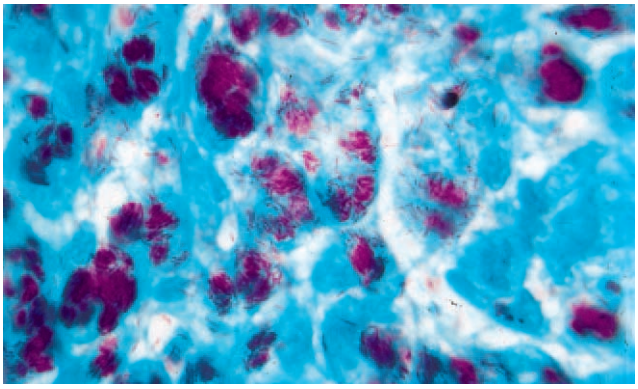


Figure 2.16 Acid-Fast Staining. *Mycobacterium leprae.* Acid-fast stain (×380). Note the masses of red bacteria within host cells.

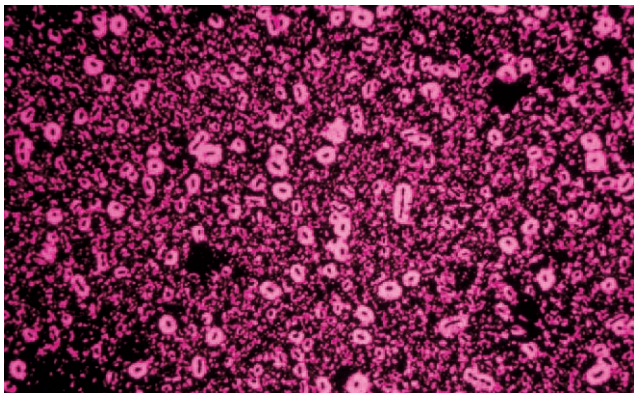


Figure 2.17 Negative Staining. *Klebsiella pneumoniae* negatively stained with India ink to show its capsules (×900).

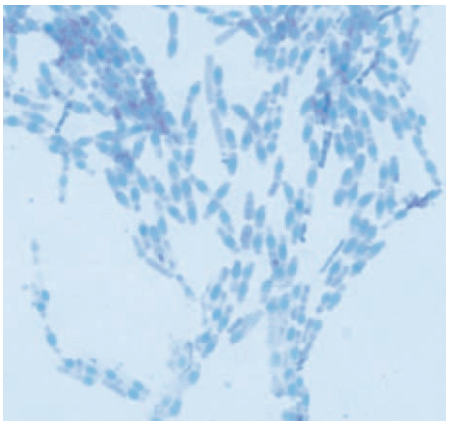


Figure 2.18 Spore Staining. *Bacillus cereus* stained with the Schaeffer-Fulton procedure. Note the central, elliptical blue to green spores within the red to purple cells (×1,000).

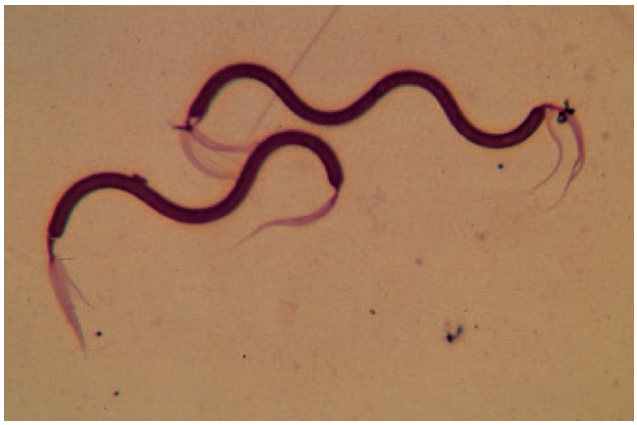


Figure 2.19 Example of Flagella Staining. *Spirillum volutans* with bipolar tufts of flagella (×400). (*See also figure 3.31.*)

Bacteria in the genera *Bacillus* and *Clostridium* (*see chapter 23*) form an exceptionally resistant structure capable of surviving for long periods in an unfavorable environment. This dormant structure is called an endospore since it develops within the cell. Endospore morphology and location vary with species and often are valuable in identification; endospores may be spherical to elliptical and either smaller or larger than the diameter of the parent bacterium. They can be observed with the phase-contrast microscope or negative staining. Endospores are not stained well by most dyes, but once stained, they strongly resist decolorization. This property is the basis of most **spore staining** methods (**figure 2.18**). In the Schaeffer-Fulton procedure, endospores are first stained by heating bacteria with malachite green, which is a very strong stain that can penetrate endospores. After malachite green treatment, the rest of the cell is washed free of dye with water and is counterstained with safranin. This technique yields a green endospore resting in a pink to red cell. Bacterial endospore structure (pp. 68–71).

Bacterial flagella are fine, threadlike organelles of locomotion that are so slender (about 10 to 30 nm in diameter) they can only be seen directly using the electron microscope. To observe them with the light microscope, the thickness of flagella is increased by coating them with mordants like tannic acid and potassium alum, and they are stained with pararosaniline (Leifson method) or basic fuchsin (Gray method). **Flagella staining** procedures provide taxonomically valuable information about the presence and distribution pattern of flagella (**figure 2.19;** *see also figure 3.31*). The bacterial flagellum (pp. 63–66).

1. Define fixation, dye, chromophore, basic dye, acid dye, simple staining, differential staining, mordant, negative staining, and acid-fast staining.
2. Describe the Gram-stain procedure and how it works.
3. How would you visualize capsules, endospores, and flagella?

2.4 Electron Microscopy

For centuries the light microscope has been the most important instrument for studying microorganisms. The electron microscope now has transformed microbiology and added immeasurably to our knowledge. The nature of the electron microscope and the ways in which specimens are prepared for observation are reviewed briefly in this section.

The Transmission Electron Microscope

The very best light microscope has a resolution limit of about 0.2 μm. Because bacteria usually are around 1 μm in diameter, only their general shape and major morphological features are visible in the light microscope. The detailed internal structure of larger microorganisms also cannot be effectively studied by light microscopy. These limitations arise from the nature of visible light waves, not from any inadequacy of the light microscope itself.

Recall that the resolution of a light microscope increases with a decrease in the wavelength of the light it uses for illumination. Electron beams behave like radiation and can be focused much as light is in a light microscope. If electrons illuminate the specimen, the microscope's resolution is enormously increased because the wavelength of the radiation is around 0.005 nm, approximately 100,000 times shorter than that of visible light. The transmission electron microscope has a practical resolution roughly 1,000 times better than the light microscope; with many electron microscopes, points closer than 5 Å or 0.5 nm can be distinguished, and the useful magnification is well over 100,000× (**figure 2.20**). The value of the electron microscope is evident on comparison of the photographs in **figure 2.21**; microbial morphology can now be studied in great detail.

A modern **transmission electron microscope (TEM)** is complex and sophisticated (**figure 2.22**), but the basic principles

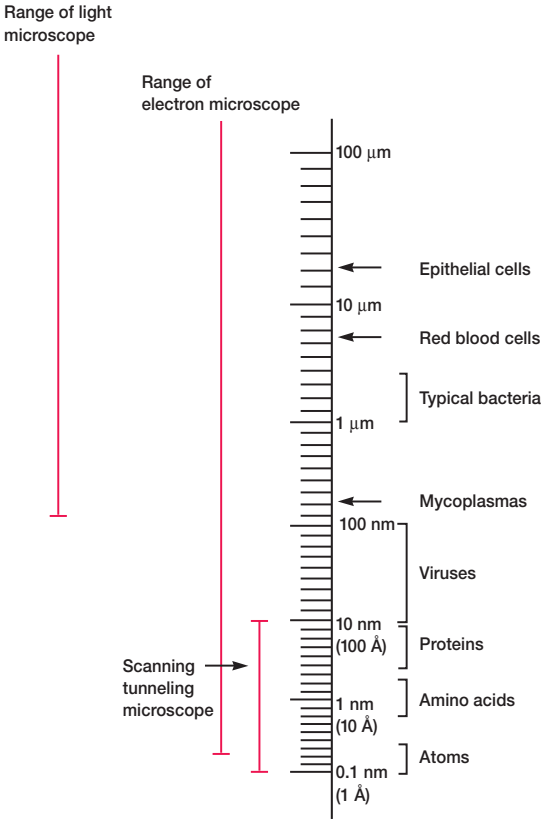
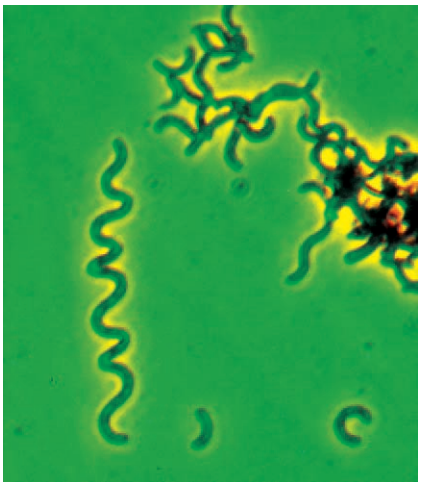
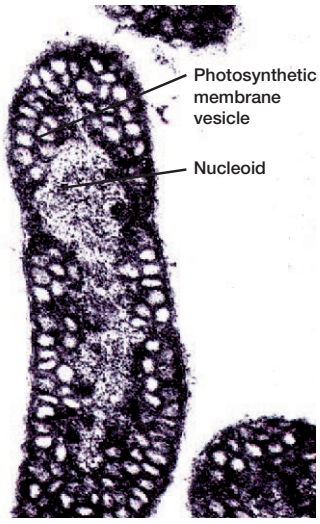


Figure 2.20 **The Limits of Microscopic Resolution.** Dimensions are indicated with a logarithmic scale (each major division represents a tenfold change in size). To the right side of the scale are the approximate sizes of cells, bacteria, viruses, molecules, and atoms.

Figure 2.22 **A Modern Transmission Electron Microscope.** The electron gun is at the top of the central column, and the magnetic lenses are within the column. The image on the fluorescent screen may be viewed through a magnifier positioned over the viewing window. The camera is in a compartment below the screen.



(a)



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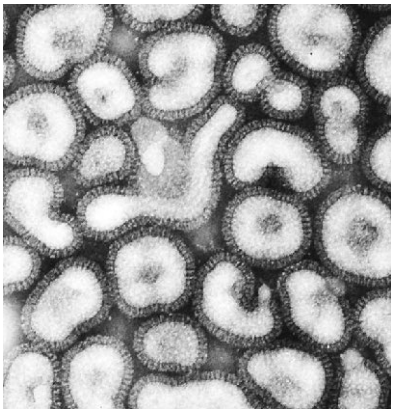


(c)

Figure 2.21 **Light and Electron Microscopy.** A comparison of light and electron microscopic resolution. **(a)** *Rhodospirillum rubrum* in phase-contrast light microscope (×600). **(b)** A thin section of *R. rubrum* in transmission electron microscope (×100,000). **(c)** A micrograph of human influenza viruses (×282,000). The particles are about 100 nm in diameter, much smaller than bacterial cells.

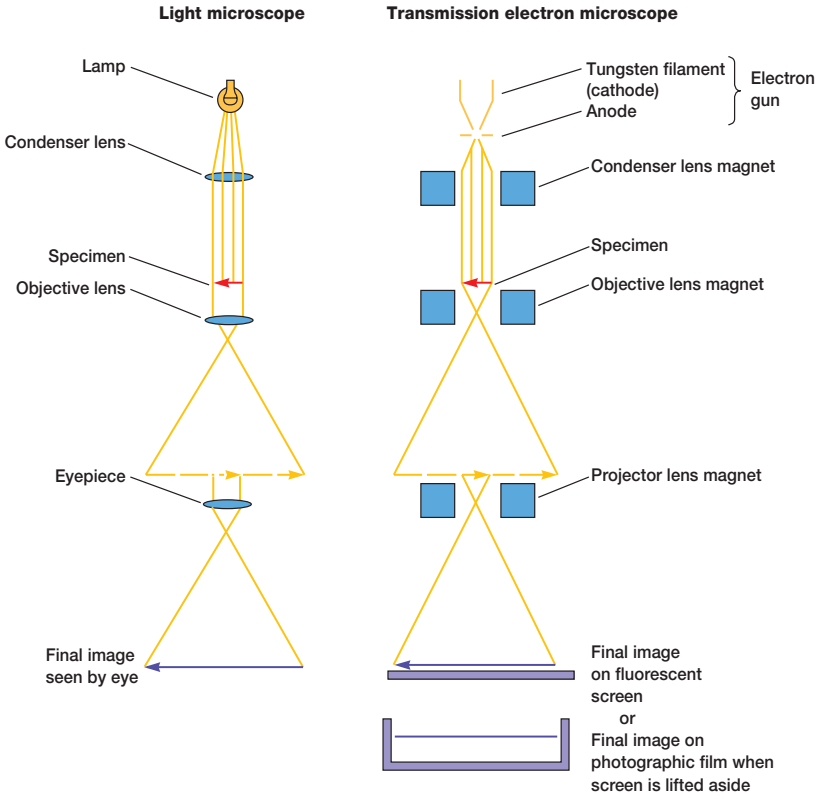


Figure 2.23 **Transmission Electron Microscope Operation.** An overview of TEM operation and a comparison of the operation of light and transmission electron microscopes.

behind its operation can be understood readily. A heated tungsten filament in the electron gun generates a beam of electrons that is then focused on the specimen by the condenser (**figure 2.23**). Since electrons cannot pass through a glass lens, doughnut-shaped electromagnets called magnetic lenses are used to focus the beam. The column containing the lenses and specimen must be under high vacuum to obtain a clear image because electrons are deflected by collisions with air molecules. The specimen scatters electrons passing through it, and the beam is focused by magnetic lenses to form an enlarged, visible image of the specimen on a fluorescent screen. A denser region in the specimen scatters more electrons and therefore appears darker in the image since fewer electrons strike that area of the screen. In contrast, electron-transparent regions are brighter. The screen can also be moved aside and the image captured on photographic film as a permanent record.

Specimen Preparation

Table 2.3 compares some of the important features of light and electron microscopes. The distinctive features of the TEM place harsh restrictions on the nature of samples that can be viewed and the means by which those samples must be prepared. Since electrons are quite easily absorbed and scattered by solid matter, only extremely thin slices of a microbial specimen can be viewed in the average TEM. The specimen must be around 20 to 100 nm thick, about 1/50 to 1/10 the diameter of a typical bacterium, and able to maintain its structure when bombarded with electrons under a high vacuum! Such a thin slice cannot be cut unless the specimen has support of some kind; the necessary support is provided by plastic. After fixation with chemicals like glutaraldehyde or osmium tetroxide to stabilize cell structure, the specimen is dehydrated with organic solvents (e.g., acetone or ethanol). Complete dehydration is essential because most plastics used for embedding are not water soluble. Next the specimen is soaked in unpolymerized, liquid epoxy plastic until it is completely permeated, and then the plastic is hardened to form a solid block. Thin sections are cut from this block with a glass or diamond knife using a special instrument called an ultramicrotome.

Cells usually must be stained before they can be seen clearly in the bright-field microscope; the same is true for observations with the TEM. The probability of electron scattering is determined by the density (atomic number) of the specimen atoms. Biological molecules are composed primarily of atoms with low atomic numbers (H, C, N, and O), and electron scattering is fairly constant throughout the unstained cell. Therefore specimens are prepared for observation by soaking

Table 2.3 Characteristics of Light and Transmission Electron Microscopes

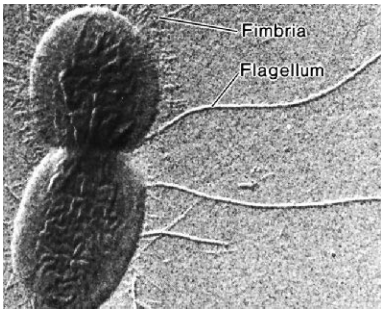
Feature	Light Microscope	Electron Microscope
Highest practical magnification	About 1,000–1,500	Over 100,000
Best resolution[a]	0.2 µm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Focusing mechanism	Adjust lens position mechanically	Adjust current to the magnetic lens
Method of changing magnification	Switch the objective lens or eyepiece	Adjust current to the magnetic lens
Specimen mount	Glass slide	Metal grid (usually copper)

[a]The resolution limit of a human eye is about 0.2 mm.

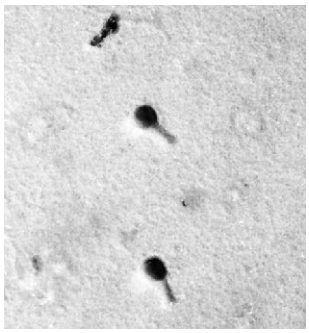
thin sections with solutions of heavy metal salts like lead citrate and uranyl acetate. The lead and uranium ions bind to cell structures and make them more electron opaque, thus increasing contrast in the material. Heavy osmium atoms from the osmium tetroxide fixative also "stain" cells and increase their contrast. The stained thin sections are then mounted on tiny copper grids and viewed.

Although specimens normally are embedded in plastic and thin sectioned to reveal the internal structure of the smallest cell, there are other ways in which microorganisms and smaller objects can be readied for viewing. One very useful technique is negative staining. The specimen is spread out in a thin film with either phosphotungstic acid or uranyl acetate. Just as in negative staining for light microscopy, heavy metals do not penetrate the specimen but render the background dark, whereas the specimen appears bright in photographs. Negative staining is an excellent way to study the structure of viruses, bacterial gas vacuoles, and other similar material. A microorganism also can be viewed after **shadowing** with metal. It is coated with a thin film of platinum or other heavy metal by evaporation at an angle of about 45° from horizontal so that the metal strikes the microorganism on only one side. The area coated with metal scatters electrons and appears light in photographs, whereas the uncoated side and the shadow region created by the object is dark (**figure 2.24**). The specimen looks much as it would if light were shining on it to cast a shadow. This technique is particularly useful in studying virus morphology, bacterial flagella, and plasmids (*see chapter 13*).

The TEM will also disclose the shape of organelles within microorganisms if specimens are prepared by the **freeze-etching** procedure. Cells are rapidly frozen in liquid nitrogen and then warmed to −100°C in a vacuum chamber. Next a knife that has been precooled with liquid nitrogen (−196°C) fractures the frozen cells, which are very brittle and break along lines of greatest weakness, usually down the middle of internal membranes (**figure 2.25**). The specimen is left in the high vacuum for a minute or more so that some of the ice can sublimate away and uncover more structural detail (sometimes this etching step is eliminated). Finally, the exposed surfaces are shadowed and coated with layers of platinum and carbon to form a replica of the surface. After the specimen has been removed chemically, this replica is studied in the TEM and provides a detailed, three-dimensional view of intracellular structure (**figure 2.26**). An advantage of freeze-etching is that it minimizes the danger of artifacts because the cells are frozen quickly rather than being subjected to chemical fixation, dehydration, and plastic embedding.



(a)



(b)

Figure 2.24 **Specimen Shadowing for the TEM.** Examples of specimens viewed in the TEM after shadowing with uranium metal. **(a)** *Proteus mirabilis* (×42,750); note flagella and fimbriae. **(b)** T4 coliphage (×72,000).

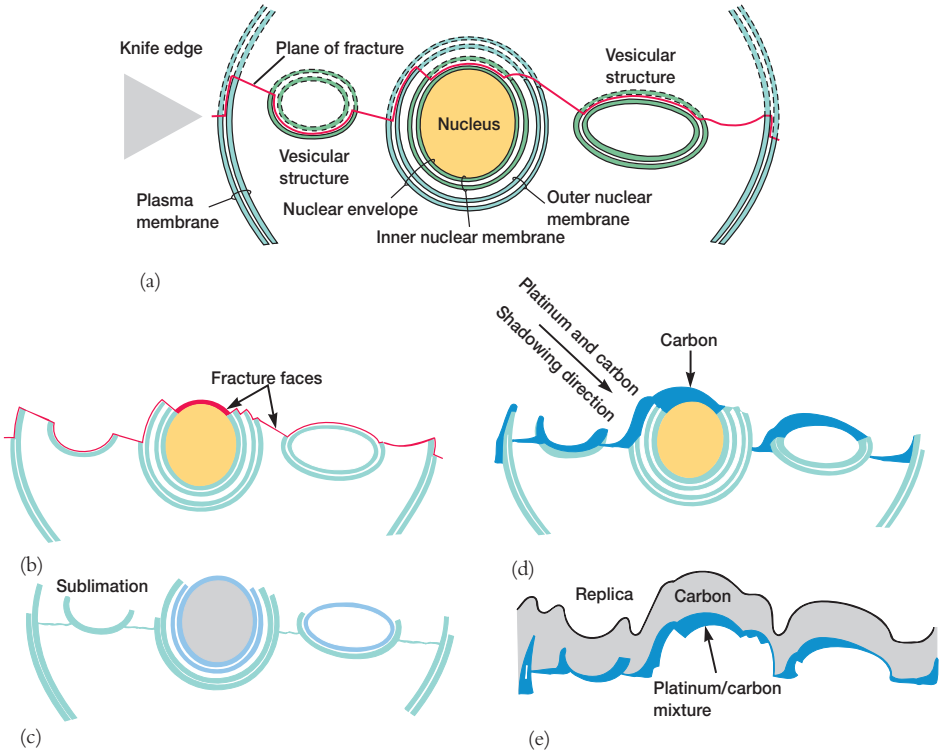


Figure 2.25 The Freeze-Etching Technique. In steps (**a**) and (**b**), a frozen eucaryotic cell is fractured with a cold knife. Etching by sublimation is depicted in (**c**). Shadowing with platinum plus carbon and replica formation are shown in (**d**) and (**e**). See text for details.

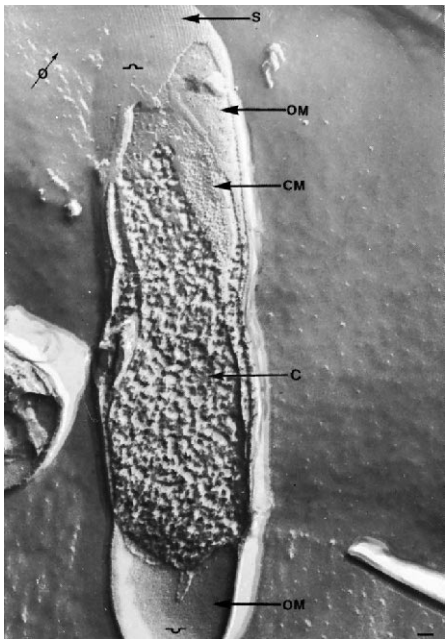


Figure 2.26 Example of Freeze-Etching. A freeze-etched preparation of the bacterium *Thiobacillus kabobis.* Note the differences in structure between the outer surface, S; the outer membrane of the cell wall, OM; the cytoplasmic membrane, CM; and the cytoplasm, C. Bar = 0.1 μm.

The Scanning Electron Microscope

The previously described microscopes form an image from radiation that has passed through a specimen. More recently the **scanning electron microscope (SEM)** has been used to examine the surfaces of microorganisms in great detail; many instruments have a resolution of 7 nm or less. The SEM differs from other electron microscopes in producing an image from electrons emitted by an object's surface rather than from transmitted electrons.

Specimen preparation is easy, and in some cases air-dried material can be examined directly. Most often, however, microorganisms must first be fixed, dehydrated, and dried to preserve surface structure and prevent collapse of the cells when they are exposed to the SEM's high vacuum. Before viewing, dried samples are mounted and coated with a thin layer of metal to prevent the buildup of an electrical charge on the surface and to give a better image.

The SEM scans a narrow, tapered electron beam back and forth over the specimen (**figure 2.27**). When the beam strikes a particular area, surface atoms discharge a tiny shower of electrons called secondary electrons, and these are trapped by a special detector. Secondary electrons entering the detector strike a scintillator causing it to emit light flashes that a photomultiplier converts to an electrical current and amplifies. The signal is sent to a cathode-ray tube and produces an image like a television picture, which can be viewed or photographed.

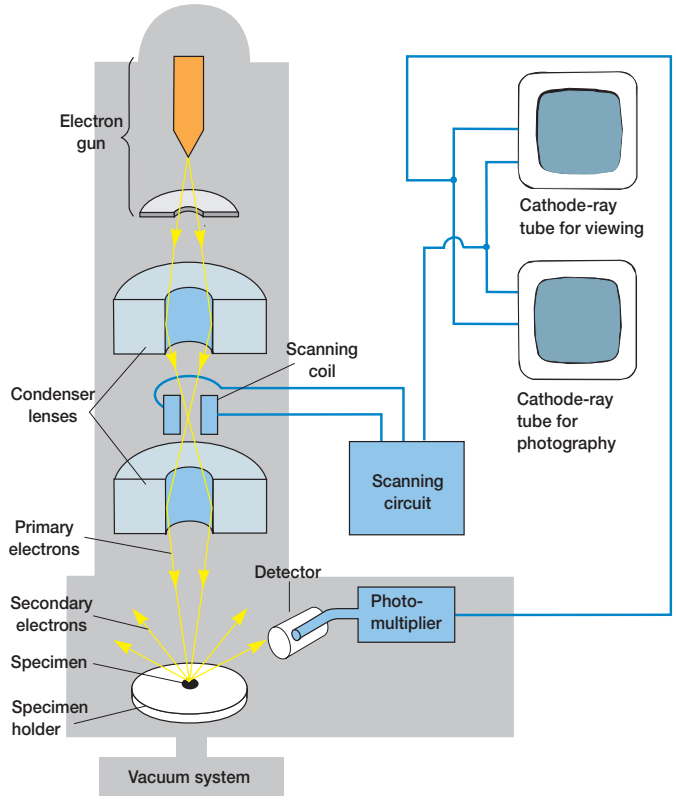
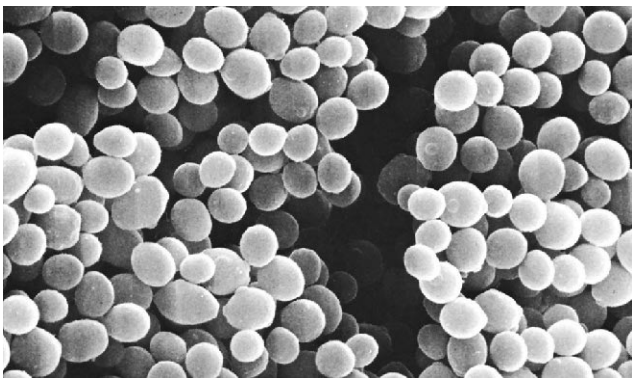


Figure 2.27 **The Scanning Electron Microscope.**



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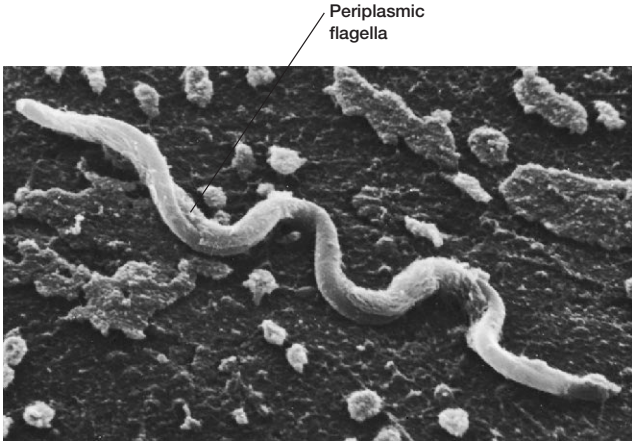


(b)

Figure 2.28 **Scanning Electron Micrographs of Bacteria.** (**a**) *Staphylococcus aureus* (×32,000). (**b**) *Cristispira,* a spirochete from the crystalline style of the oyster, *Ostrea virginica.* The axial fibrils or periplasmic flagella are visible around the protoplasmic cylinder (×6,000).

The number of secondary electrons reaching the detector depends on the nature of the specimen's surface. When the electron beam strikes a raised area, a large number of secondary electrons enter the detector; in contrast, fewer electrons escape a depression in the surface and reach the detector. Thus raised areas appear lighter on the screen and depressions are darker. A realistic three-dimensional image of the microorganism's surface with great depth of focus results (**figure 2.28**). The actual in situ location of microorganisms in ecological niches such as the human skin and the lining of the gut also can be examined.

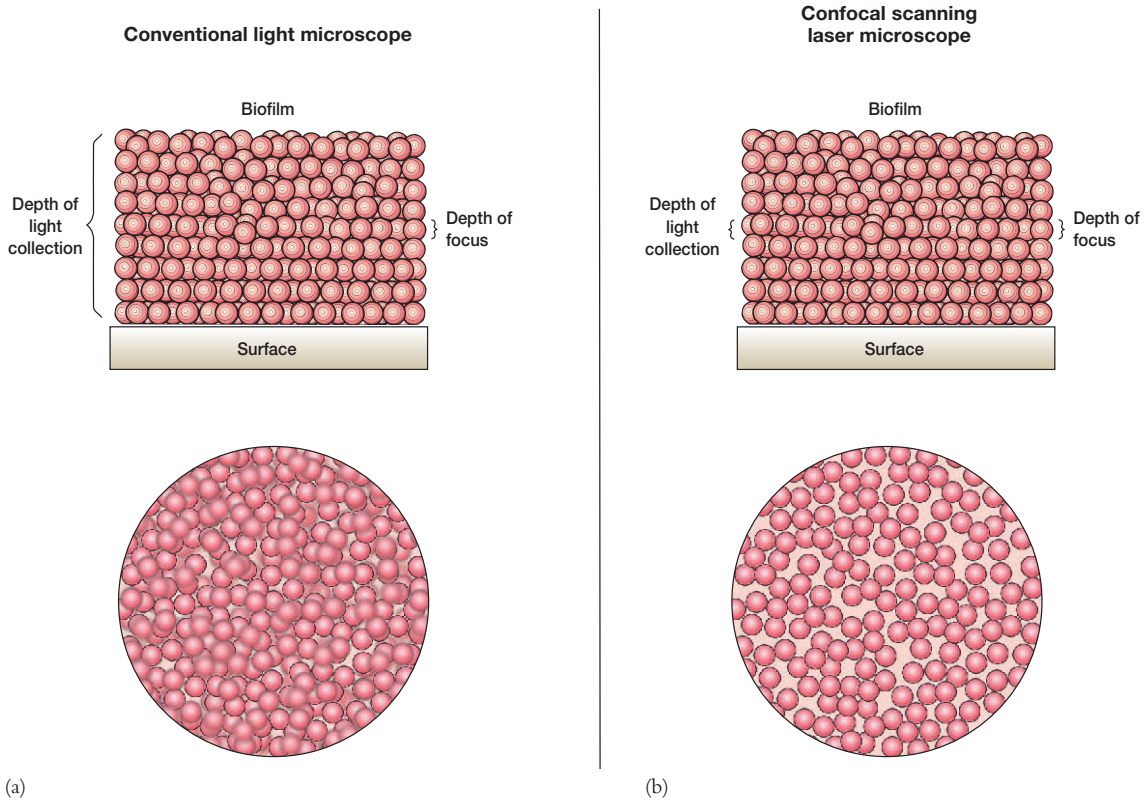


Figure 2.29 **Confocal Scanning Laser Microscopy: Light Collection Depth and Image Clarity.** (**a**) Conventional light microscopic observation. (**b**) Confocal scanning laser microscopic observation.

1. Why does the transmission electron microscope have much greater resolution than the light microscope? Describe in general terms how the TEM functions.
2. Describe how specimens are prepared for viewing in the TEM. How are sections usually stained to increase contrast? What are negative staining, shadowing, and freeze-etching?
3. How does the scanning electron microscope operate and in what way does its function differ from that of the TEM? The SEM is used to study which aspects of morphology?

2.5 Newer Techniques in Microscopy

Confocal Microscopy

A conventional light microscope, which uses a mixed wavelength light source and illuminates a large area of the specimen, will have a relatively great depth of field. Even if not in focus, images of bacteria from all levels within the field will be visible. These will include cells above, in, and below the plane of focus (**figure 2.29**). As a result the image can be murky, fuzzy, and crowded.

The solution to this problem is the **confocal scanning laser microscope (CSLM)** or confocal microscope. Fluorescently stained specimens are usually examined. A focused laser beam strikes a point in the specimen (**figure 2.30**). Light from the illuminated spot is focused by an objective lens onto a plane above the objective. An aperture above the objective lens blocks out stray light from parts of the specimen that lie above and below the plane of focus. The laser is scanned over a plane in the specimen (beam scanning) or the stage is moved (stage scanning) and a detector measures the illumination from each point to produce an image of the optical section. When many optical sections are scanned, a computer can combine them to form a three-dimensional image from the digitized signals. This image can be measured and analyzed quantitatively.

The confocal microscope improves images in two ways. First, illumination of one spot at a time reduces interference from light scattering by the rest of the specimen. Second, the aperture above the objective lens blocks out stray light as previously mentioned. Consequently the image has excellent contrast and resolution. A depth of 1 μm or less in a thick preparation can be directly observed. Special computer software is used to create high-resolution, three-dimensional images of cell structures and complex specimens such as biofilms (**figure 2.31**).

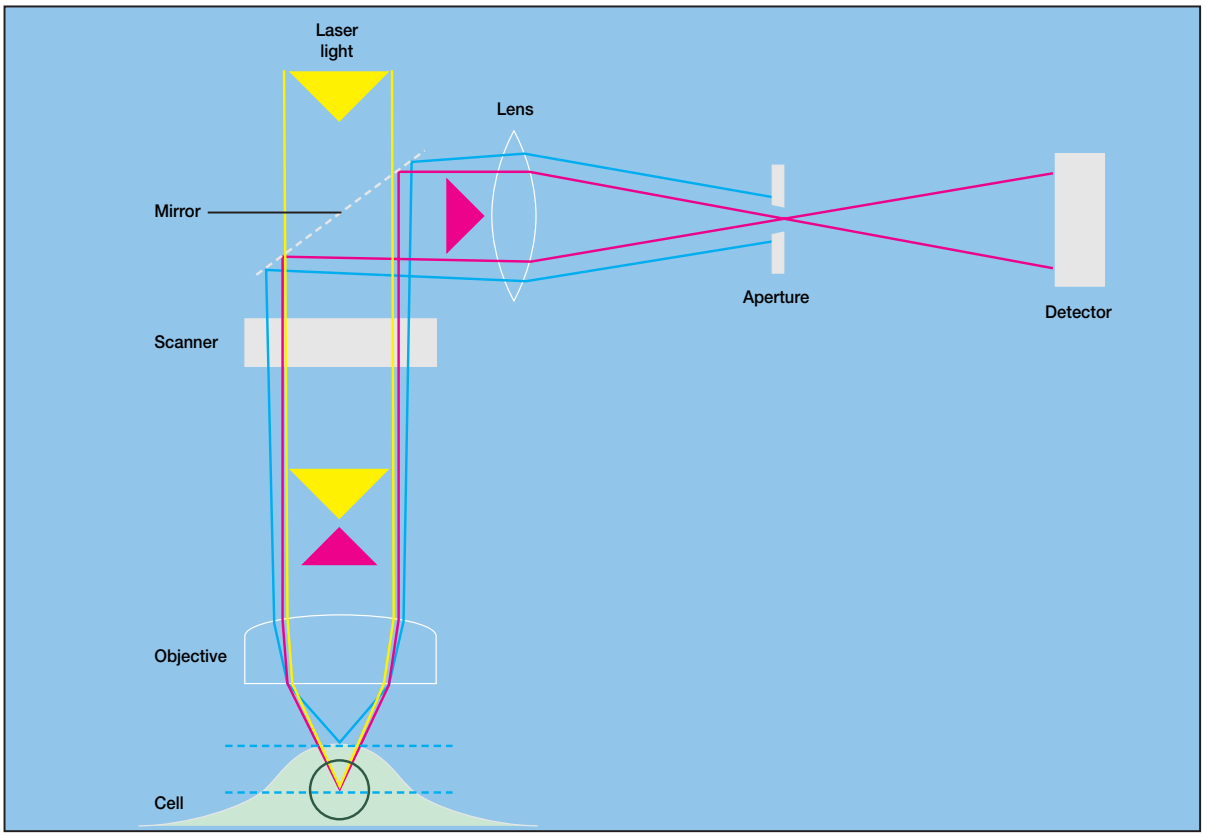
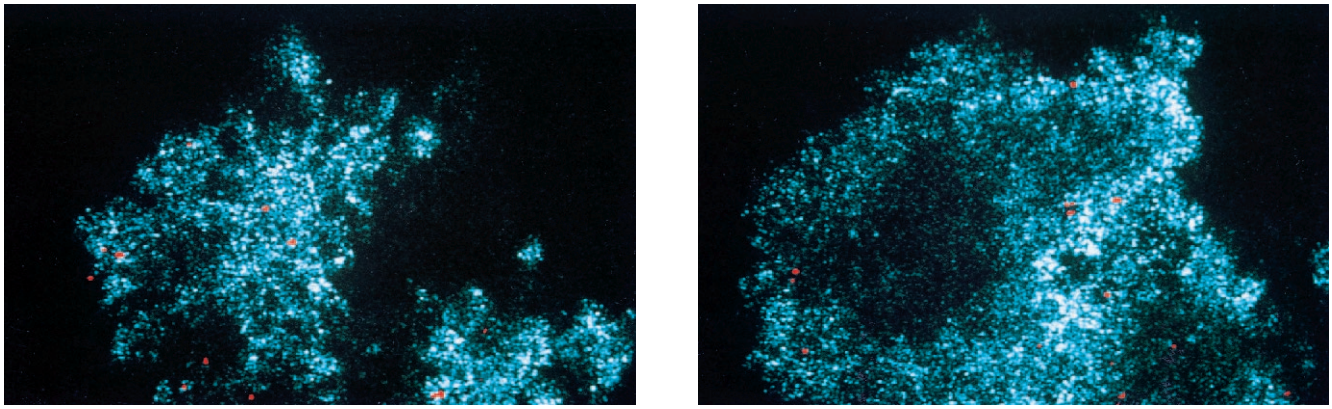


Figure 2.30 A Ray Diagram of a Confocal Laser Scanning Microscope. The yellow lines represent laser light used for illumination. Red lines symbolize the light arising from the plane of focus, and the blue lines stand for light from parts of the specimen above and below the focal plane. See text for explanation.



(a) (b)

Figure 2.31 Confocal Images at Various Depths below the Top of a Biofilm. **(a)** 20 μm. **(b)** 40 μm. Each of these confocal images—which combines fluorescent and reflection images—has a depth of 2 μm and shows red-colored fluorescent tracer beads.

Scanning Probe Microscopy

Although light and electron microscopes have become quite sophisticated and reached an advanced state of development, powerful new microscopes are still being created. A new class of microscopes, called **scanning probe microscopes,** measure surface features by moving a sharp probe over the object's surface. The **scanning tunneling microscope,** invented in 1980, is an excellent example of a scanning probe microscope. It can achieve magnifications of 100 million and allow scientists to view atoms on the surface of a solid. The electrons surrounding surface atoms tunnel or project out from the surface boundary a very short distance. The scanning tunneling microscope has a needlelike probe with a point so sharp that often there is only one atom at its tip. The probe is lowered toward the specimen surface until its electron cloud just touches that of the surface atoms. If a small voltage is applied between the tip and specimen, electrons flow through a narrow channel in the electron clouds. This tunneling current, as it is called, is extraordinarily sensitive to distance and will decrease about a thousandfold if the probe is moved away from the surface by a distance equivalent to the diameter of an atom.

The arrangement of atoms on the specimen surface is determined by moving the probe tip back and forth over the surface while keeping it at a constant height by adjusting the probe distance to maintain a steady tunneling current. As the tip moves up and down while following the surface contours, its motion is recorded and analyzed by a computer to create an accurate three-dimensional image of the surface atoms. The surface map can be displayed on a computer screen or plotted on paper. The resolution is so great that individual atoms are observed easily. The microscope's inventors, Gerd Binnig and Heinrich Rohrer, shared the 1986 Nobel Prize in Physics for their work, together with Ernst Ruska, the designer of the first transmission electron microscope.

The scanning tunneling microscope will likely have a major impact in biology. Recently it has been used to directly view DNA (**figure 2.32**). Since the microscope can examine objects when they are immersed in water, it may be particularly useful in studying biological molecules.

More recently a second type of scanning probe microscope has been developed. The **atomic force microscope** moves a sharp probe over the specimen surface while keeping the distance between the probe tip and the surface constant. It does this by exerting a very small amount of force on the tip, just enough to maintain a constant distance but not enough force to damage the surface. The vertical motion of the tip usually is followed by measuring the deflection of a laser beam that strikes the lever holding the probe. Unlike the scanning tunneling microscope, the atomic force microscope can be used to study surfaces that do not conduct electricity well. The atomic force microscope has been used to study the interactions between the *E. coli* GroES and GroEL chaperonin proteins, to map plasmids by locating restriction enzymes bound to specific sites, and to follow the behavior of living bacteria and other cells.

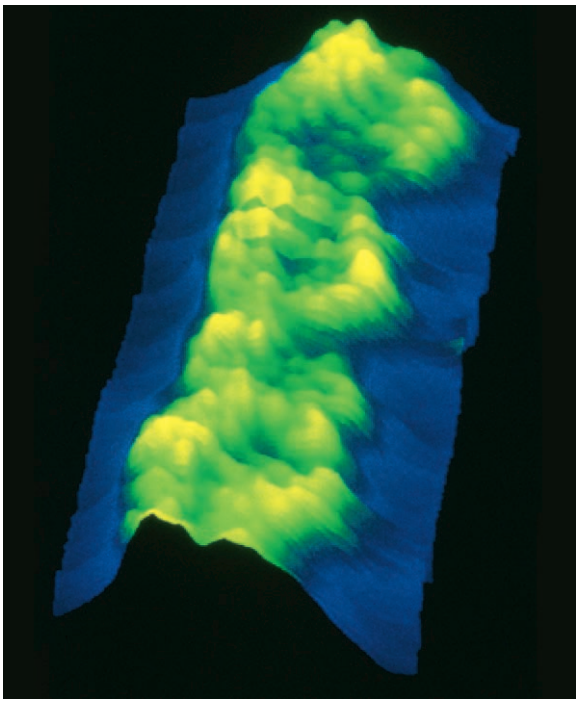


Figure 2.32 Scanning Tunneling Microscopy of DNA. The DNA double helix with approximately three turns shown (false color; ×2,000,000).

1. How does a confocal microscope operate and why does it provide better images of thick specimens than the standard compound microscope?
2. Briefly describe the scanning probe microscope and its most popular versions, the scanning tunneling microscope and the atomic force microscope. What are these microscopes used for?

Summary

1. A light ray moving from air to glass, or vice versa, is bent in a process known as refraction. Lenses focus light rays at a focal point and magnify images (**figure 2.2**).
2. In a compound microscope like the bright-field microscope, the primary image is formed by an objective lens and enlarged by the eyepiece or ocular lens to yield a virtual image (**figure 2.3**).
3. A substage condenser focuses a cone of light on the specimen.
4. Microscope resolution increases as the wavelength of radiation used to illuminate the specimen decreases. The maximum resolution of a light microscope is about 0.2 μm.
5. The dark-field microscope uses only refracted light to form an image (**figure 2.7**), and objects glow against a black background.
6. The phase-contrast microscope converts variations in the refractive index and density of cells into changes in light intensity and thus makes colorless, unstained cells visible (**figure 2.9**).
7. The differential interference contrast microscope uses two beams of light to create high-contrast, three-dimensional images of live specimens.

8. The fluorescence microscope illuminates a fluorochrome-labeled specimen and forms an image from its fluorescence (**figure 2.12**).
9. Specimens usually must be fixed and stained before viewing them in the bright-field microscope.
10. Most dyes are either positively charged basic dyes or negative acid dyes and bind to ionized parts of cells.
11. In simple staining a single dye mixture is used to stain microorganisms.
12. Differential staining procedures like the Gram stain and acid-fast stain distinguish between microbial groups by staining them differently.
13. Some staining techniques are specific for particular structures like bacterial capsules, flagella, and endospores.
14. The transmission electron microscope uses magnetic lenses to form an image from electrons that have passed through a very thin section of a specimen (**figure 2.23**). Resolution is high because the wavelength of electrons is very short.
15. Thin section contrast can be increased by treatment with solutions of heavy metals like osmium tetroxide, uranium, and lead.
16. Specimens are also prepared for the TEM by negative staining, shadowing with metal, or freeze-etching.
17. The scanning electron microscope (**figure 2.27**) is used to study external surface features of microorganisms.
18. The confocal scanning laser microscope (**figure 2.29**) is used to study thick, complex specimens. Scanning probe microscopes can visualize molecules and cells.

Key Terms

acid dyes *27*
acid-fast staining *28*
atomic force microscope *38*
basic dyes *27*
bright-field microscope *19*
chromophore groups *27*
confocal scanning laser microscope (CSLM) *36*
dark-field microscopy *22*
dark-phase-contrast microscopy *24*
differential interference contrast (DIC) microscope *25*
differential staining procedures *28*
eyepieces *19*
fixation *27*
flagella staining *30*
fluorescence microscope *25*
fluorescent light *25*
fluorochromes *25*
focal length *18*
focal point *18*
freeze-etching *33*
Gram stain *28*
mordant *28*
negative staining *28*
numerical aperture *20*
objectives *19*
oculars *19*
parfocal *20*
phase-contrast microscope *22*
refraction *18*
refractive index *18*
resolution *20*
scanning electron microscope (SEM) *34*
scanning probe microscope *38*
scanning tunneling microscope *38*
shadowing *33*
simple staining *28*
spore staining *30*
substage condenser *19*
transmission electron microscope (TEM) *30*
working distance *21*

Questions for Thought and Review

1. How are real and virtual images produced in a light microscope? Which one is a person actually seeing?
2. If a specimen is viewed using a 43× objective in a microscope with a 15× eyepiece, how many times has the image been magnified?
3. Why don't most light microscopes use 30× eyepieces for greater magnification?
4. Describe the two general types of fixation. Which would you normally use for bacteria? For protozoa?
5. Why would one expect basic dyes to be more effective under alkaline conditions?
6. What step in the Gram-stain procedure could be dropped without losing the ability to distinguish between gram-positive and gram-negative bacteria? Why?
7. Why must the TEM use a high vacuum and very thin sections?
8. Material is often embedded in paraffin before sectioning for light microscopy. Why can't this approach be used when preparing a specimen for the TEM?
9. Under what circumstances would it be desirable to prepare specimens for the TEM by use of negative staining? Shadowing? Freeze-etching?
10. Compare the microscopes described in this chapter—bright-field, dark-field, phase-contrast, DIC, fluorescence, TEM, SEM, confocal, and scanning probe—in terms of the images they provide and the purposes for which they are most often used.
11. Describe briefly how the scanning probe microscope operates. For what is it used? Distinguish between the two types of scanning probe microscopes with respect to their mechanism of operation.
12. Prepare a summary table showing the advantages of each type of microscope described in the chapter.

Critical Thinking Questions

1. If you prepared a sample of a specimen for light microscopy, stained with the Gram stain, and failed to see anything when you looked through your light microscope, list the things that you may have done incorrectly.
2. In a journal article, find an example of a light micrograph, a scanning or transmission electron micrograph, or a confocal image. Discuss why the figure was included in the article and why that particular type of microscopy was the method of choice for the research. What other figures would you like to see used in this study? Outline the steps that the investigators would take in order to obtain such photographs or figures.

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