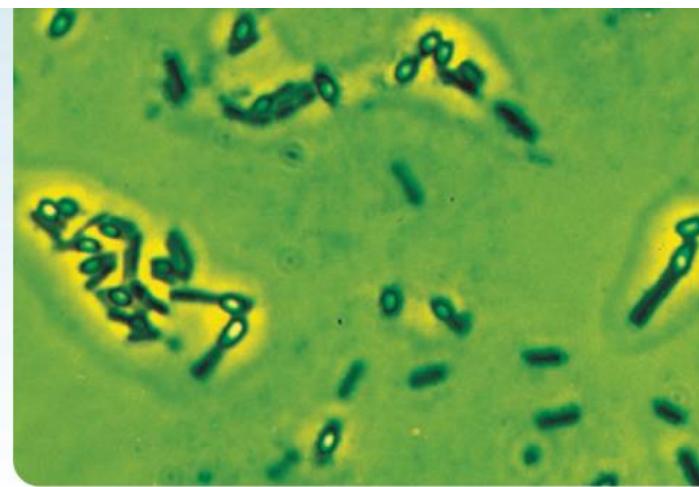


2

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.

PREVIEW

- Light microscopes use glass lenses to bend and focus light rays to produce enlarged images of small objects. The maximum resolution of a light microscope is about $0.2 \mu\text{m}$.
- Many types of light microscopes have been developed, including bright-field, dark-field, phase-contrast, and fluorescence microscopes. Each yields a distinctive image.
- Bright-field microscopy requires the application of stains to microorganisms for easy viewing. Stains are also used to determine the nature of bacterial cell walls or to visualize specific prokaryotic structures such as flagella and capsules.
- The useful magnification of a light microscope is limited by its resolving power. The resolving power is limited by the wavelength of the illuminating beam.
- Electron microscopes use beams of electrons rather than light to achieve very high resolution (up to 0.5 nm) and magnification.
- 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.

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.

In this chapter we begin with a detailed treatment of the standard bright-field microscope and then describe other common types of light microscopes. Next we discuss preparation and staining of specimens for examination with the light microscope. This is followed by a description of transmission and scanning electron microscopes, both of which are used extensively in cur-

rent microbiological research. We close the chapter with a brief introduction to two newer forms of microscopy: confocal microscopy and scanning probe 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; the direction and magnitude of bending is determined by the refractive indices 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

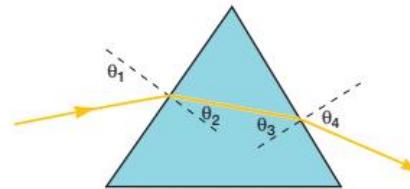


Figure 2.1 The Bending of Light by a Prism. Normals (lines perpendicular to the surface of the prism) are indicated by dashed lines. As light enters the glass, it is bent toward the first normal (angle θ_2 is less than θ_1). When light leaves the glass and returns to air, it is bent away from the second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

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

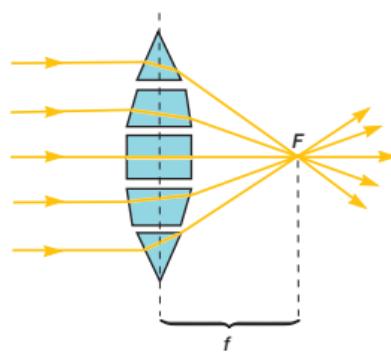


Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point F . The focal point lies a distance f , the focal length, from the lens center.

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	\AA	10^{-10} meter

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.

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.

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 micro-

scopes 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 **ocular lenses** 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 **objective lenses** of differing magnifying power and can be rotated to position any objective beneath the body assembly. Ideally a microscope should be **parfocal**—that is, the image should remain in focus when objectives are changed.

The image one sees when viewing a specimen with a compound microscope is created by the objective and ocular lenses working together. Light from the illuminated specimen is focused by the objective lens, creating an enlarged image within the microscope (figure 2.4). The ocular lens further magnifies this primary image. The total magnification is calculated by multiplying the objective and eyepiece magnifications together. For example, if a $45\times$ objective is used with a $10\times$ eyepiece, the overall magnification of the specimen will be $450\times$.

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.

Resolution is described mathematically by an equation developed in the 1870s by Ernst Abbé, a German physicist responsible for much of the optical theory underlying microscope design. The **Abbe equation** states that the minimal distance (d) between two

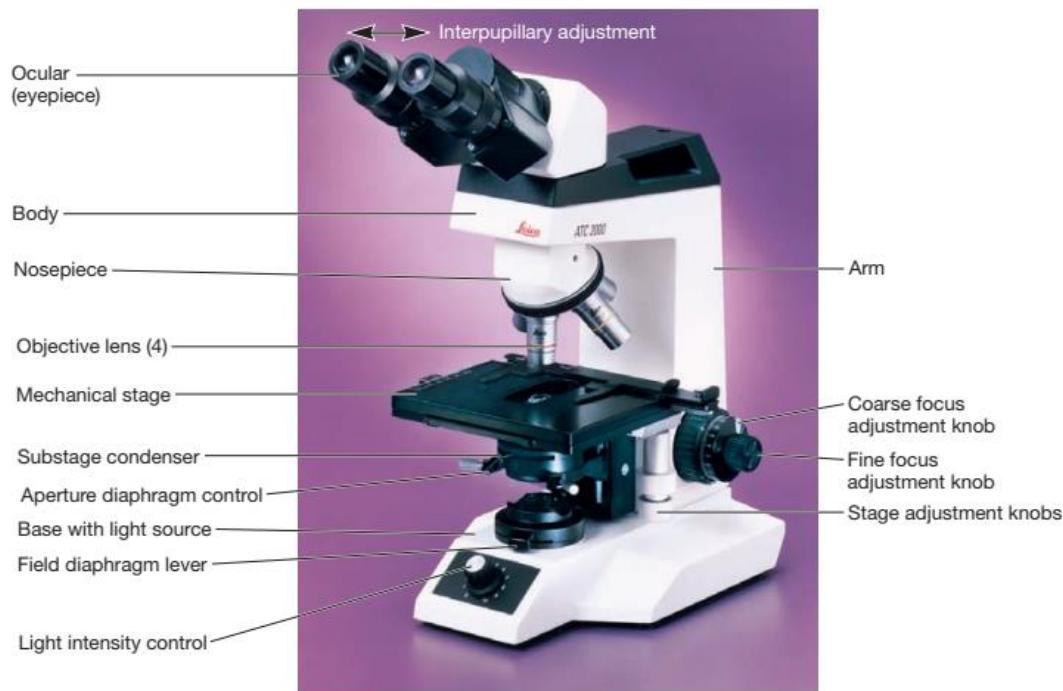


Figure 2.3 A Bright-Field Microscope. The parts of a modern bright-field microscope. The microscope pictured is somewhat more sophisticated than those found in many student laboratories. For example, it is binocular (has two eyepieces) and has a mechanical stage, an adjustable substage condenser, and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

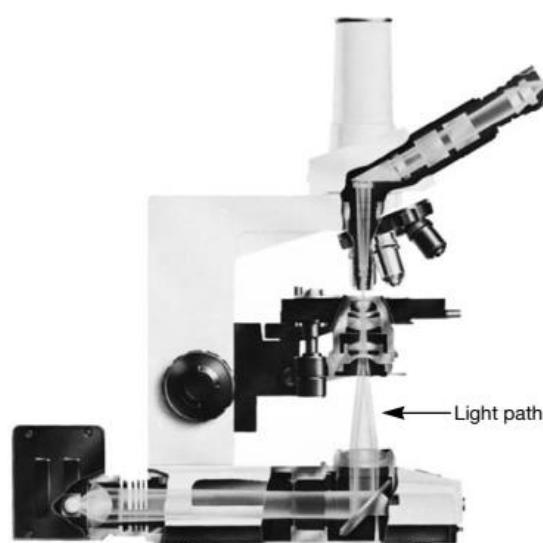


Figure 2.4 A Microscope's Light Path. The light path in an advanced bright-field microscope (see also figure 2.19).

objects that reveals them as separate entities depends on the wavelength of light (λ) used to illuminate the specimen and on the **numerical aperture** of the lens ($n \sin \theta$), which is the ability of the lens to gather light.

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

As d becomes smaller, the resolution increases, and finer detail can be discerned in a specimen; d becomes smaller as the wavelength of light used decreases and as the numerical aperture (NA) increases. Thus the greatest resolution is obtained using a lens with the largest possible NA and light of the shortest wavelength, light at the blue end of the visible spectrum (in the range of 450 to 500 nm; see figure 6.25).

The numerical aperture ($n \sin \theta$) of a lens is a complex concept that can be difficult to understand. It is defined by two components: n is the refractive index of the medium in which the lens works (e.g., air) and θ is 1/2 the angle of the cone of light entering an objective (figure 2.5). 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. 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 and $\sin \theta$ cannot be greater than 1 (the maximum θ is 90° and $\sin 90^\circ$ is 1.00). Therefore 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.

Numerical aperture is related to another characteristic of an objective lens, the working distance. 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 (table 2.2).

The preceding discussion has focused on the resolving power of the objective lens. The resolution of an entire microscope must

take into account the numerical aperture of its condenser as is evident from the equation below.

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

The condenser is a large, light-gathering lens used to project a wide cone of light through the slide and into the objective lens. 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.

Although the resolution of the microscope must consider both the condenser and the objective lens, in most cases the limit of resolution of a light microscope is calculated using the Abbé equation, which considers the objective lens only. 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 about 0.2 μm apart (the same size as a very small bacterium).

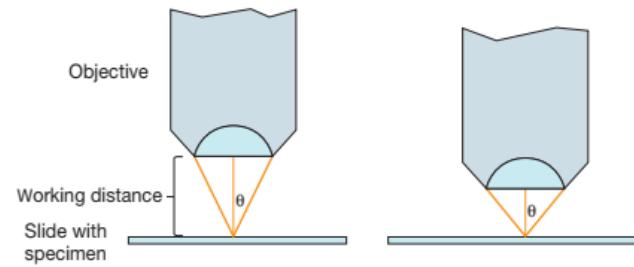


Figure 2.5 Numerical Aperture in Microscopy. The angular aperture θ is $1/2$ the angle of the cone of light that enters a lens from a specimen, and the numerical aperture is $n \sin \theta$. In the right-hand illustration the lens has larger angular and numerical apertures; its resolution is greater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

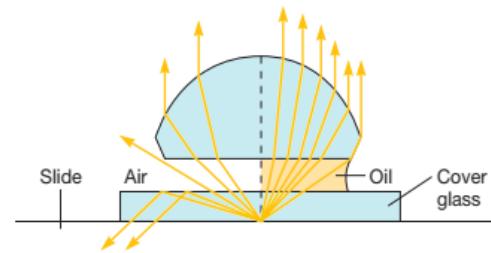


Figure 2.6 The Oil Immersion Objective. An oil immersion objective operating in air and with immersion oil.

Table 2.2 The Properties of Microscope Objectives

Property	Objective			
	Scanning	Low Power	High Power	Oil Immersion
Magnification	4 \times	10 \times	40–45 \times	90–100 \times
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

Given the limit of resolution of a light microscope, the largest useful magnification—the level of magnification needed to increase the size of the smallest resolvable object to be visible with the light microscope—can be determined. 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 \times eyepieces and have an upper limit of about 1,000 \times with oil immersion. A 15 \times eyepiece may be used with good objectives to achieve a useful magnification of 1,500 \times . Any further magnification does not enable a person to see more detail. Indeed, a light microscope can be built to yield a final magnification of 10,000 \times , but it would simply be magnifying a blur. Only the electron microscope provides sufficient resolution to make higher magnifications useful.

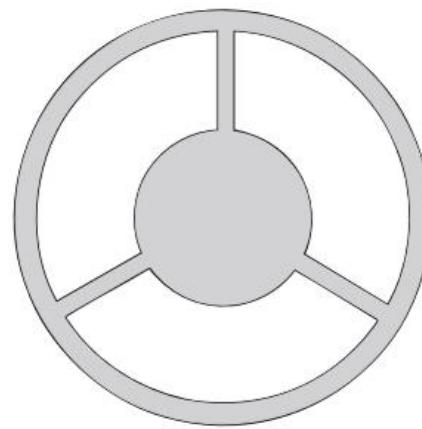
The Dark-Field Microscope

The **dark-field microscope** allows a viewer to observe living, unstained cells and organisms 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

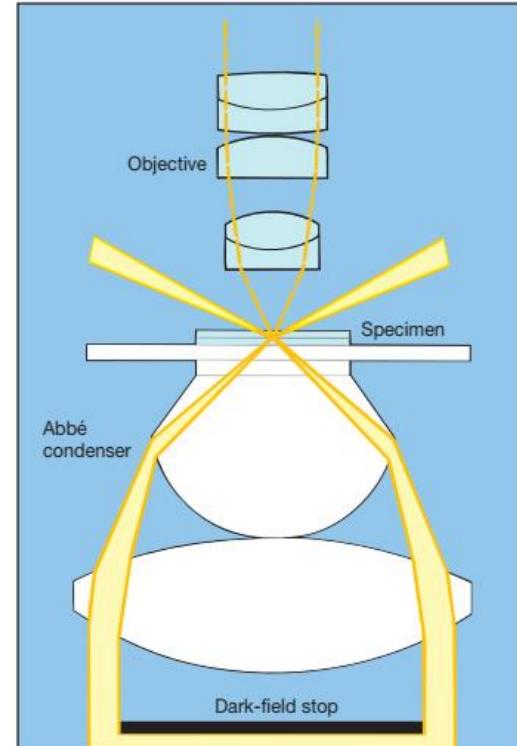
refracted by the specimen forms an image (**figure 2.7**). The field surrounding a specimen appears black, while the object itself is brightly illuminated (**figure 2.8a,b**). The dark-field microscope can reveal considerable internal structure in larger eucaryotic microorganisms (**figure 2.8b**). It also is used to identify certain bacteria like the thin and distinctively shaped *Treponema pallidum* (**figure 2.8a**), 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. As will be discussed in section 2.3, one solution to this problem is to kill and stain cells before observation to increase contrast and create variations in color between cell structures. But what if an investigator must view living cells in order to observe a dynamic process such as movement or phagocytosis? Phase-contrast microscopy can be used in this situation. 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 2.8c–e**).



(a)



(b)

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.

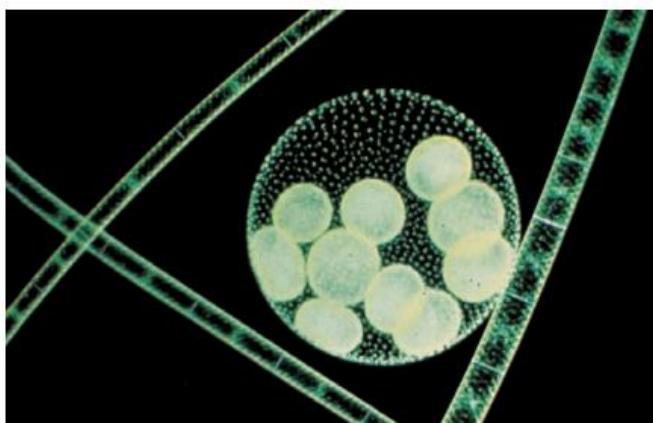
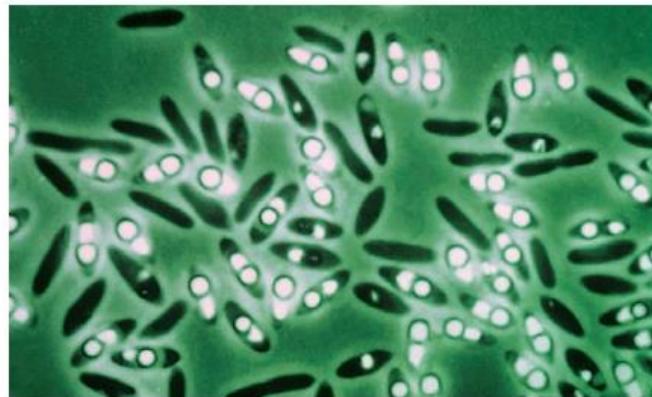
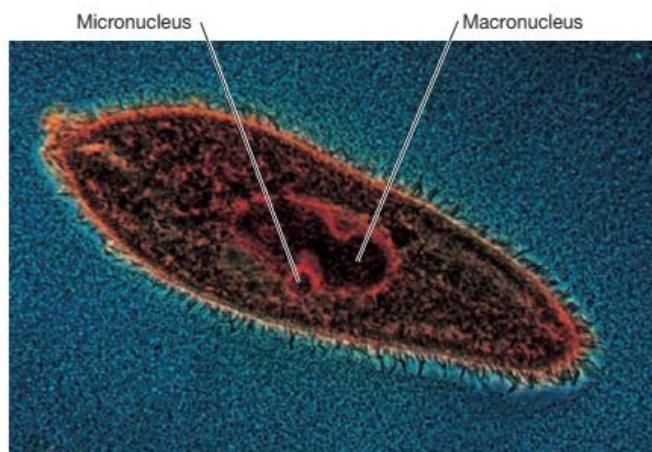
(a) *T. pallidum*: dark-field microscopy(c) *Pseudomonas*: phase-contrast microscopy(b) *Volvox* and *Spirogyra*: dark-field microscopy(d) *Desulfotomaculum acetoxidans*: phase-contrast microscopy(e) *Paramecium*: phase-contrast microscopy

Figure 2.8 Examples of Dark-Field and Phase-Contrast Microscopy. (a) *Treponema pallidum*, the spirochete that causes syphilis; dark-field microscopy. (b) *Volvox* and *Spirogyra*; dark-field microscopy ($\times 175$). Note daughter colonies within the mature *Volvox* colony (center) and the spiral chloroplasts of *Spirogyra* (left and right). (c) A phase-contrast micrograph of *Pseudomonas* cells, which range from $1\text{--}3 \mu\text{m}$ in length. (d) *Desulfotomaculum acetoxidans* with endospores; phase contrast ($\times 2,000$). (e) *Paramecium* stained to show a large central macronucleus with a small spherical micronucleus at its side; phase-contrast microscopy ($\times 100$).

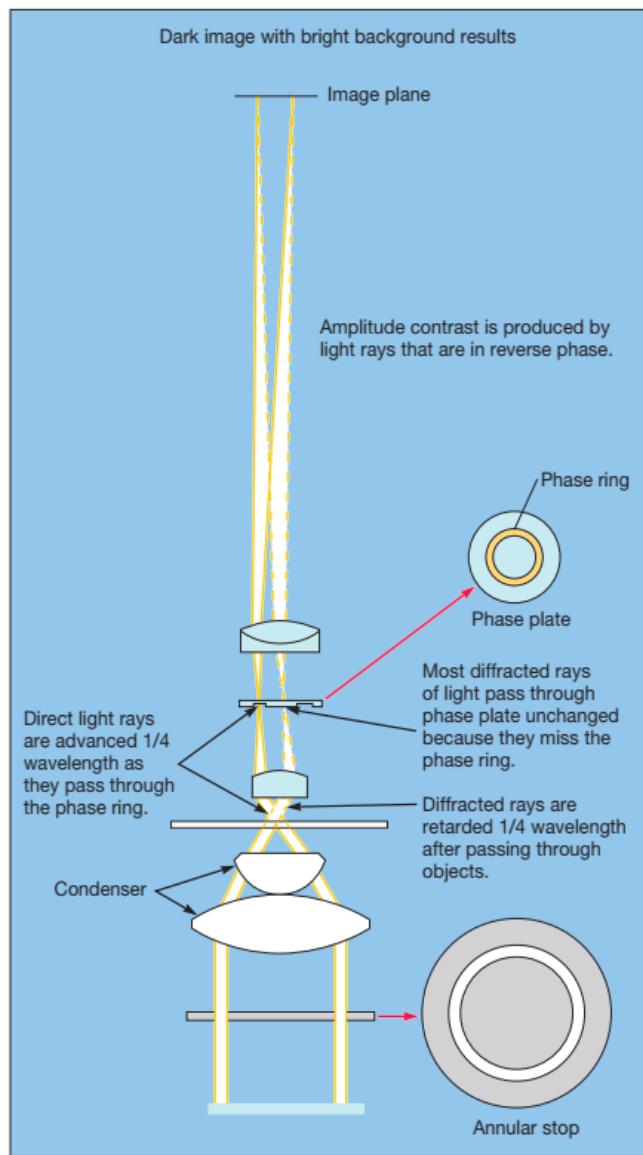


Figure 2.9 Phase-Contrast Microscopy. The optics of a dark-phase-contrast microscope.

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 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 1/4 wavelength, the deviated

and undeviated waves will be about 1/2 wavelength out of 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 2.8d**).

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 -hydroxyalkanoates (e.g., poly- β -hydroxybutyrate), polymetaphosphate, sulfur, or other substances. These are clearly visible (**figure 2.8d**) because they have refractive indices markedly different from that of water. Phase-contrast microscopes also are widely used in studying eucaryotic cells. [The cytoplasmic matrix: Inclusion bodies \(section 3.3\)](#)

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** exposes a specimen to ultraviolet, violet, or blue light and forms an image of the object with the resulting fluorescent light. The most commonly used fluorescence microscopy is epifluorescence microscopy, also called incident light or reflected light fluorescence microscopy. Epifluorescence microscopes employ an objective lens that also acts as a condenser (**figure 2.12**). A mercury vapor arc lamp or other source produces an intense beam of light that passes through an exciter filter. The exciter filter transmits only the desired wavelength of excitation light. The excitation light is directed down the microscope by a special mirror called the dichromatic mirror. This mirror reflects light of shorter wavelengths (i.e., the excitation light),

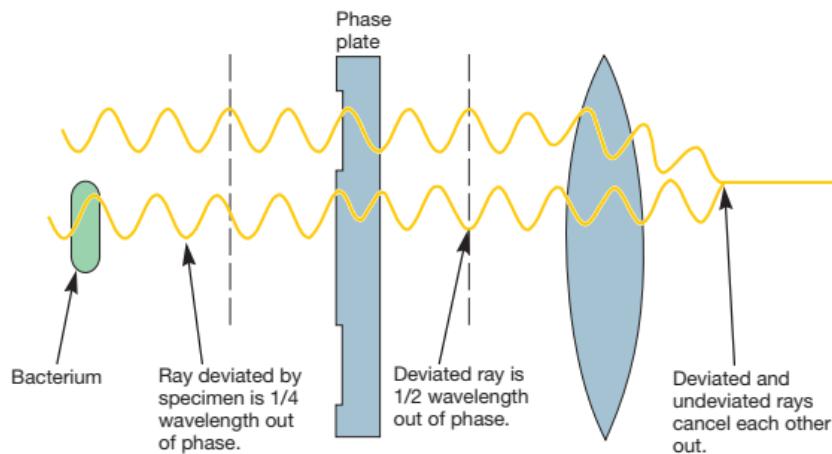


Figure 2.10 The Production of Contrast in Phase Microscopy. The behavior of deviated and undeviated or undiffracted light rays in the dark-phase-contrast microscope. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background.

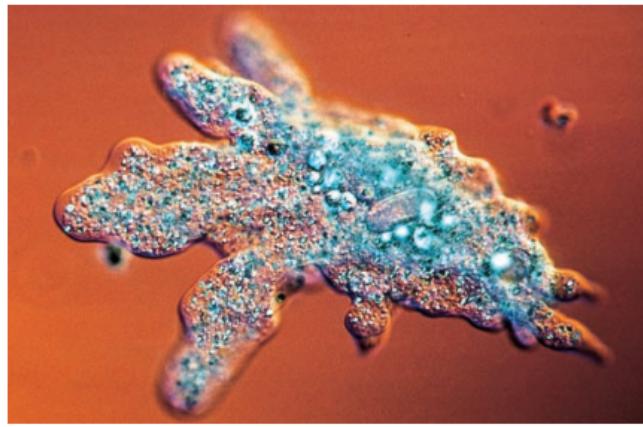


Figure 2.11 Differential Interference Contrast Microscopy. A micrograph of the protozoan *Amoeba proteus*. The three-dimensional image contains considerable detail and is artificially colored ($\times 160$).

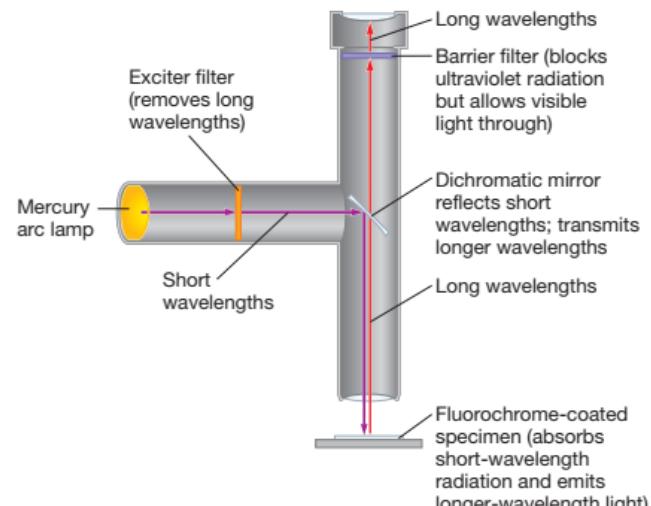


Figure 2.12 Epifluorescence Microscopy. The principles of operation of an epifluorescence microscope.

but allows light of longer wavelengths to pass through. The excitation light continues down, passing through the objective lens to the specimen, which is usually stained with special dye molecules called **fluorochromes** (table 2.3). The fluorochrome absorbs light energy from the excitation light and fluoresces brightly. The emitted fluorescent light travels up through the objective lens into the microscope. Because the emitted fluorescent light has a longer wavelength, it passes through the dichromatic mirror to a barrier filter, which blocks out any residual excitation light. Finally, the emitted light passes through the barrier filter to the eyepieces.

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 that bind specific cell constituents (table 2.3). In addition, microbial ecologists use epifluorescence microscopy to visualize photosynthetic microbes, as their pigments naturally fluoresce when excited by light of specific wavelengths. It is even possible to distinguish live bacteria from dead bacteria by

Table 2.3 Commonly Used Fluorochromes

Fluorochrome	Uses
Acridine orange	Stains DNA; fluoresces orange
Diamidino-2-phenyl indole (DAPI)	Stains DNA; fluoresces green
Fluorescein isothiocyanate (FITC)	Often attached to antibodies that bind specific cellular components or to DNA probes; fluoresces green
Tetramethyl rhodamine isothiocyanate (TRITC or rhodamine)	Often attached to antibodies that bind specific cellular components; fluoresces red

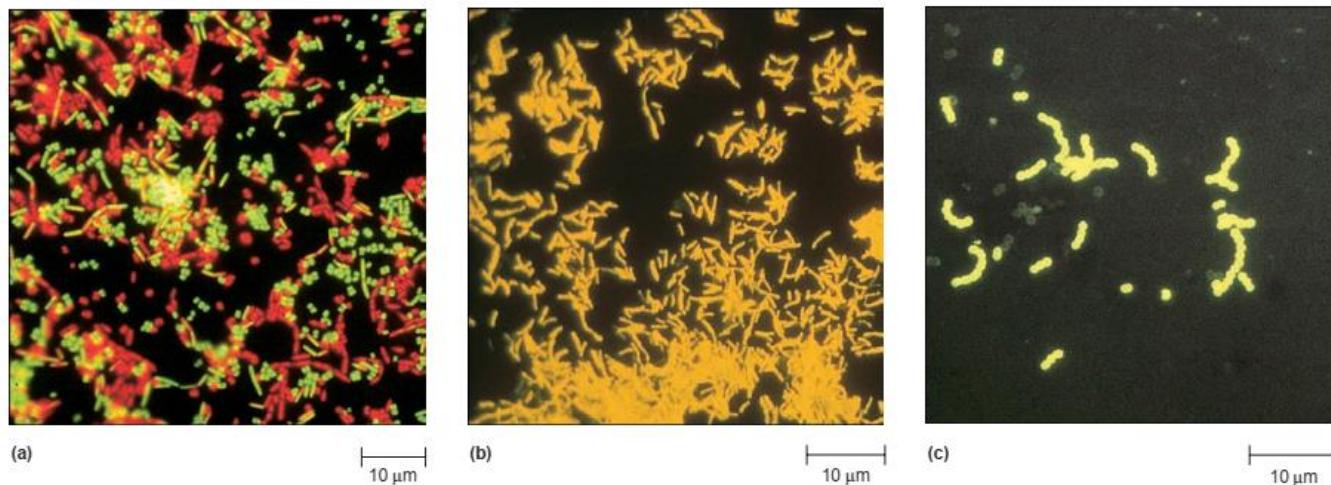


Figure 2.13 Fluorescent Dyes and Tags. (a) Dyes that cause live cells to fluoresce green and dead ones red; (b) Auramine is used to stain *Mycobacterium* species in a modification of the acid-fast technique; (c) Fluorescent antibodies tag specific molecules. In this case, the antibody binds to a molecule that is unique to *Streptococcus pyogenes*.

the color they fluoresce after treatment with a special mixture of stains (**figure 2.13a**). Thus the microorganisms can be viewed and directly counted in a relatively undisturbed ecological niche. **Identification of microorganisms from specimens: Immunologic techniques (section 35.2)**

1. List the parts of a light microscope and describe their functions.
2. Define resolution, numerical aperture, working distance, and fluorochrome.
3. If a specimen is viewed using a 5X objective in a microscope with a 15X eyepiece, how many times has the image been magnified?
4. How does resolution depend on the wavelength of light, refractive index, and numerical aperture? How are resolution and magnification related?
5. What is the function of immersion oil?
6. Why don't most light microscopes use 30X ocular lenses for greater magnification?
7. Briefly describe how dark-field, phase-contrast, differential interference contrast, and epifluorescence 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. **Heat fixation** is routinely used to observe prokaryotes. Typically, a film of cells (a smear) is gently heated as a slide is passed

through a flame. Heat fixation preserves overall morphology but not structures within cells. **Chemical fixation** is 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: they have **chromophore groups**, groups with conjugated double bonds that give the dye its color, and they can bind with cells by ionic, covalent, or hydrophobic bonding. Most dyes are used to directly stain the cell or object of interest, but some dyes (e.g., India ink and nigrosin) are used in **negative staining**, where the background but not the cell is stained; the unstained cells appear as bright objects against a dark background.

Dyes that bind cells by ionic interactions are probably the most commonly used dyes. These 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, many proteins, and the surfaces of prokaryotic cells.
2. **Acidic dyes**—eosin, rose bengal, and acid fuchsin—possess negatively charged groups such as carboxyls ($-COOH$) and phenolic hydroxyls ($-OH$). Acidic dyes, because of their negative charge, bind to positively charged cell structures.

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

Dyes that bind through covalent bonds or because of their solubility characteristics are also useful. For instance, DNA can be stained by the **Feulgen procedure** in which the staining compound (Schiff's reagent) is covalently attached to its deoxyribose sugars. 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 dye is used (figure 2.14a,b). Simple staining's value lies in its simplicity and ease of use. One covers the fixed smear with stain for a short period 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 in simple staining to determine the size, shape, and arrangement of prokaryotic cells.

Differential Staining

The **Gram stain**, developed in 1884 by the Danish physician Christian Gram, is the most widely employed staining method in bacteriology. It is an example of **differential staining**—procedures that are used to distinguish organisms based on their staining properties. Use of the Gram stain divides *Bacteria* into two classes—gram negative and gram positive.

The Gram-staining procedure is illustrated in figure 2.15. In the first step, the smear is stained with the basic dye crystal violet, the primary stain. This is followed by treatment with an iodine solution functioning as a **mordant**. 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 (figures 2.14c and 2.15b). [The bacterial cell wall \(section 3.6\)](#)

Acid-fast staining is another important differential staining procedure. It is most commonly used to identify *Mycobacterium tuberculosis* and *M. leprae* (figure 2.14d), the pathogens responsible for tuberculosis and leprosy, respectively. These bacteria have cell walls with high lipid content; in particular, mycolic acids—a group of branched-chain hydroxy lipids, which prevent dyes from readily binding to the cells. However, *M. tuberculosis* and *M. leprae* can be stained by harsh procedures such as the **Ziehl-Neelsen method**, which uses heat and phenol to drive basic fuchsin into the cells. Once basic fuchsin has penetrated, *M. tuberculosis* and *M. leprae* are not easily decolorized by acidified alcohol (acid-alcohol), and thus are said to be acid-fast. Non-acid-fast bacteria are decolorized by acid-alcohol and thus are stained blue by methylene blue counterstain.

Staining Specific Structures

Many special staining procedures have been developed to study specific structures with the light microscope. One of the simplest is **capsule staining** (figure 2.14f), a technique that reveals the presence of capsules, a network usually made of polysaccharides that surrounds many bacteria and some fungi. Cells are mixed with India ink or nigrosin dye and spread out in a thin film on a slide. After air-drying, the cells appear as lighter bodies in the midst of a blue-black background because ink and dye particles cannot penetrate either the cell or its capsule. Thus capsule staining is an example of **negative staining**. The extent of the light region is determined by the size of the capsule and of the cell itself. There is little distortion of cell shape, and the cell can be counterstained for even greater visibility. [Components external to the cell wall: Capsules, slime layers, and S-layers \(section 3.9\)](#)

Endospore staining, like acid-fast staining, also requires harsh treatment to drive dye into a target, in this case an endospore. An endospore is an exceptionally resistant structure produced by some bacterial genera (e.g., *Bacillus* and *Clostrid-*

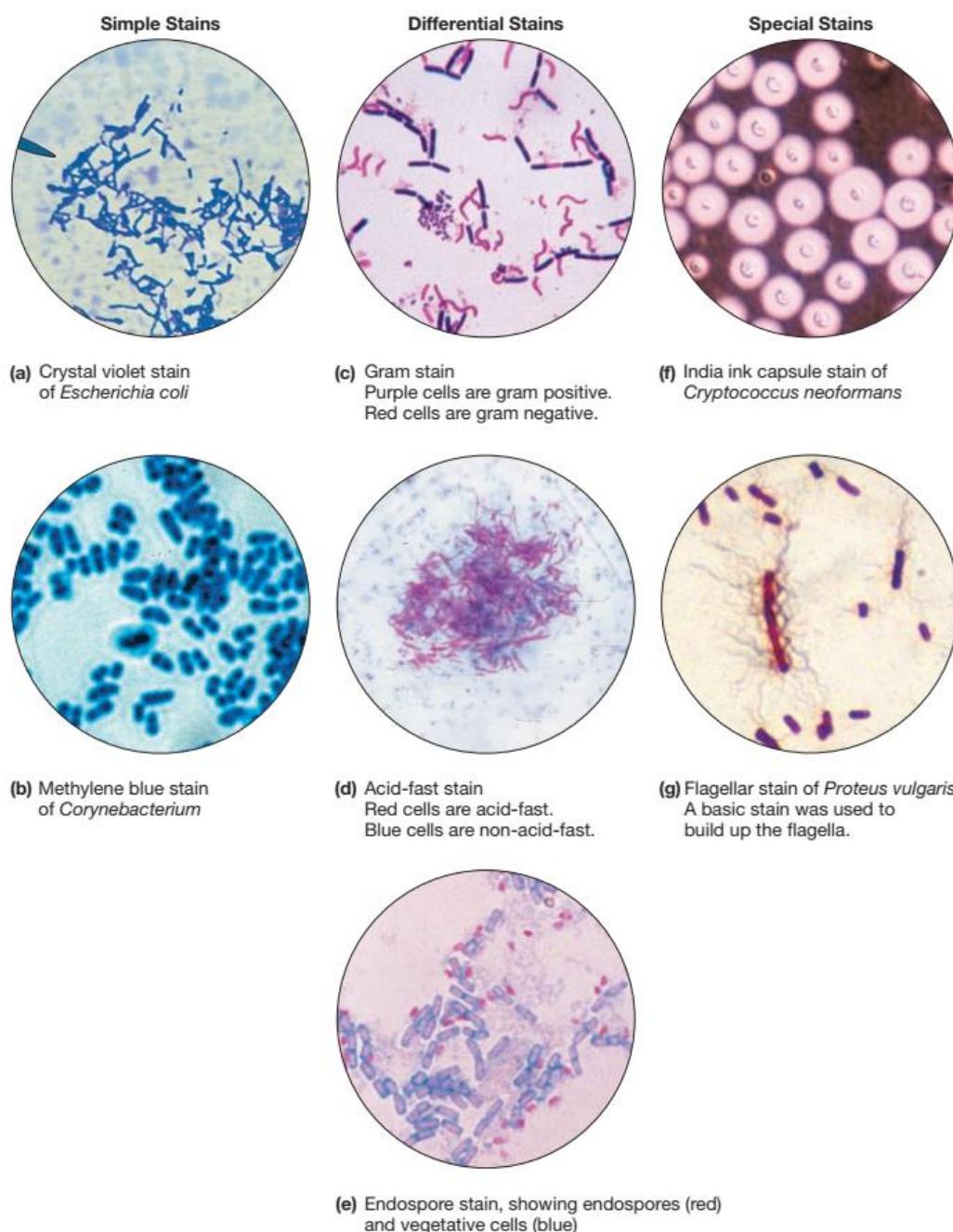


Figure 2.14 Types of Microbiological Stains.

ium). It is capable of surviving for long periods in an unfavorable environment and is called an endospore because it develops within the parent bacterial 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. Endospores are

not stained well by most dyes, but once stained, they strongly resist decolorization. This property is the basis of most endospore staining methods (figure 2.14e). 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

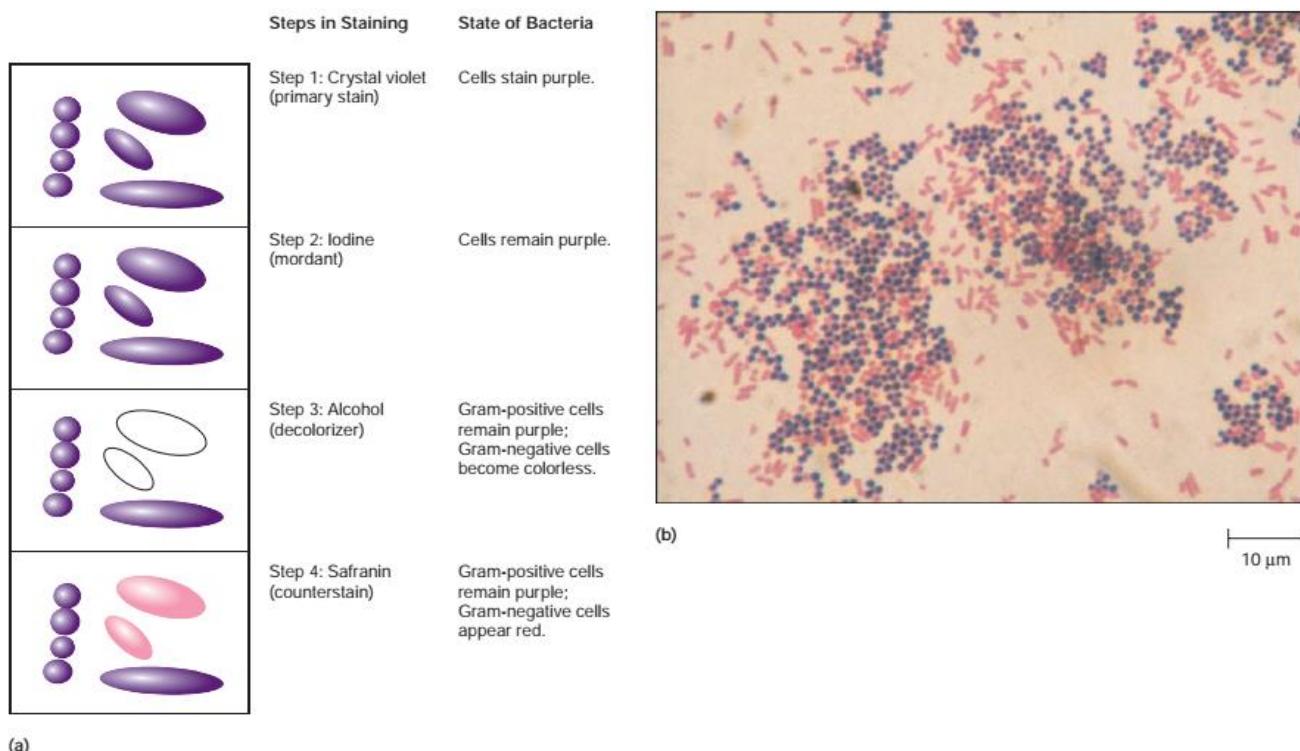


Figure 2.15 Gram Stain. (a) Steps in the Gram stain procedure. (b) Results of a Gram stain. The Gram-positive cells (purple) are *Staphylococcus aureus*; the Gram-negative cells (reddish-pink) are *Escherichia coli*.

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. [The bacterial endospore \(section 3.11\); Class Clostridia \(section 23.4\); and Class Bacilli \(section 23.5\)](#)

Flagella staining provides taxonomically valuable information about the presence and distribution pattern of flagella on prokaryotic cells (figure 2.14g; see also figure 3.39). Prokaryotic 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 then staining with pararosaniline (Leifson method) or basic fuchsin (Gray method). [Components external to the cell wall: Flagella and motility \(section 3.9\)](#)

1. Define fixation, dye, chromophore, basic dye, acidic dye, simple staining, differential staining, mordant, negative staining, and acid-fast staining.
2. Describe the two general types of fixation. Which would you normally use for prokaryotes? For protozoa?
3. Why would one expect basic dyes to be more effective under alkaline conditions?
4. Describe the Gram stain procedure and explain how it works. What step in the procedure could be omitted without losing the ability to distinguish between gram-positive and gram-negative bacteria? Why?
5. 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. However, even the very best light microscopes have a resolution limit of about 0.2 µm, which greatly compromises their usefulness for detailed studies of many microorganisms. Viruses, for example, are too small to be seen with light microscopes. Prokaryotes can be observed, but because they are usually only 1 µm to 2 µm in diameter, just their general shape and major morphological features are visible. 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. Electron microscopes have much greater resolution. They have 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

Electron microscopes use a beam of electrons to illuminate and create magnified images of specimens. Recall that the resolution of a light microscope increases with a decrease in the wavelength of the light it uses for illumination. Electrons replace light as the

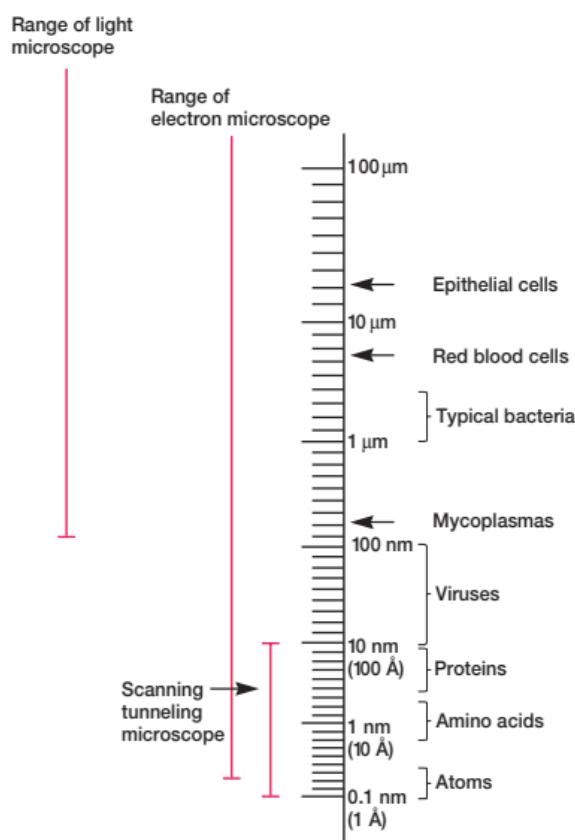


Figure 2.16 The Limits of Microscopic Resolution. Dimensions are indicated with a logarithmic scale (each major division represents a tenfold change in size). To the right side of the scale are the approximate sizes of cells, bacteria, viruses, molecules, and atoms.

illuminating beam. They can be focused, much as light is in a light microscope, but their wavelength is around 0.005 nm, approximately 100,000 times shorter than that of visible light. Therefore, electron microscopes have a practical resolution roughly 1,000 times better than the light microscope; with many electron microscopes, points closer than 0.5 nm can be distinguished, and the useful magnification is well over 100,000 \times (**figure 2.16**). The value of the electron microscope is evident on comparison of the photographs in **figure 2.17**, microbial morphology can now be studied in great detail.

A modern **transmission electron microscope (TEM)** is complex and sophisticated (**figure 2.18**), but the basic principles behind its operation can be readily understood. A heated tungsten filament in the electron gun generates a beam of electrons that is then focused on the specimen by the condenser (**figure 2.19**). 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 some electrons, but those that pass through are used to form an enlarged 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; these regions are said to be "electron dense." In contrast, electron-transparent regions are brighter. The image can also be captured on photographic film as a permanent record.

Table 2.4 compares some of the important features of light and transmission electron microscopes. The TEM has distinctive features that 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 deflected by air molecules and are easily absorbed and scattered by solid matter, only extremely thin slices (20 to 100 nm) of a microbial specimen can be viewed in the average TEM. 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.

As with bright-field microscopy, cells usually must be stained before they can be seen clearly. 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 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.

Two other important techniques for preparing specimens are negative staining and shadowing. In 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 objects (**figure 2.17c**). In **shadowing**, a specimen 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. In one commonly used imaging method, the area coated with metal appears dark in photographs, whereas the uncoated side and the shadow region created by the object is light (**figure 2.20**). This technique is particularly useful in studying virus morphology, prokaryotic flagella, and DNA.

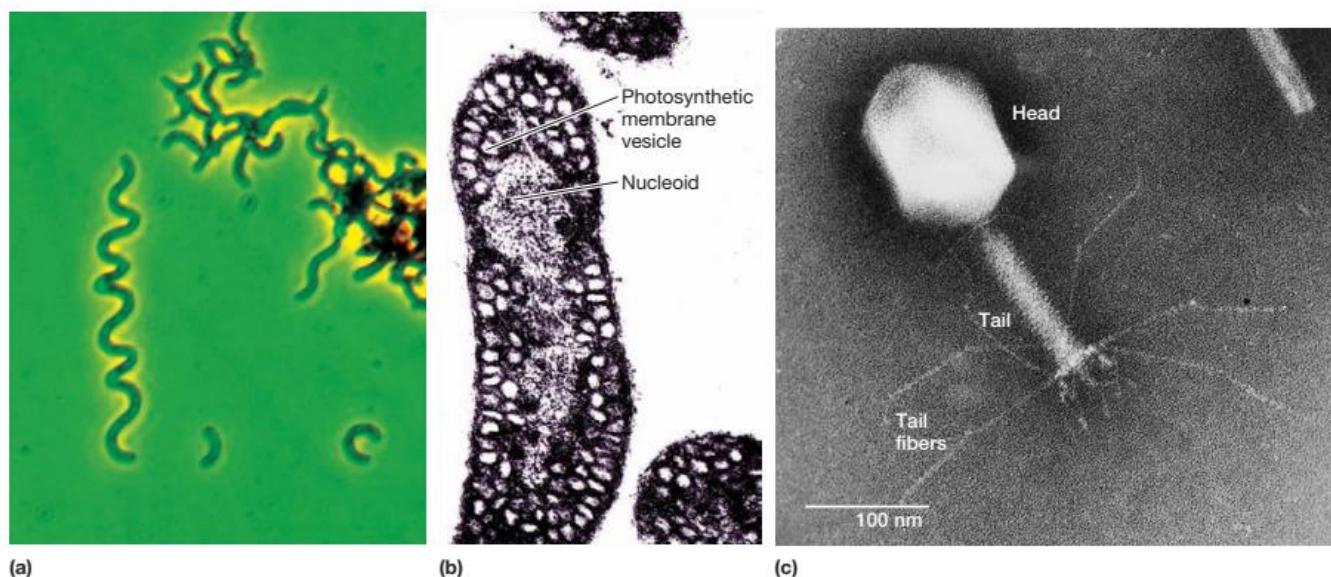


Figure 2.17 Light and Electron Microscopy. A comparison of light and electron microscopic resolution. (a) *Rhodospirillum rubrum* in phase-contrast light microscope ($\times 600$). (b) A thin section of *R. rubrum* in transmission electron microscope ($\times 100,000$). (c) A transmission electron micrograph of a negatively stained T4 bacteriophage.

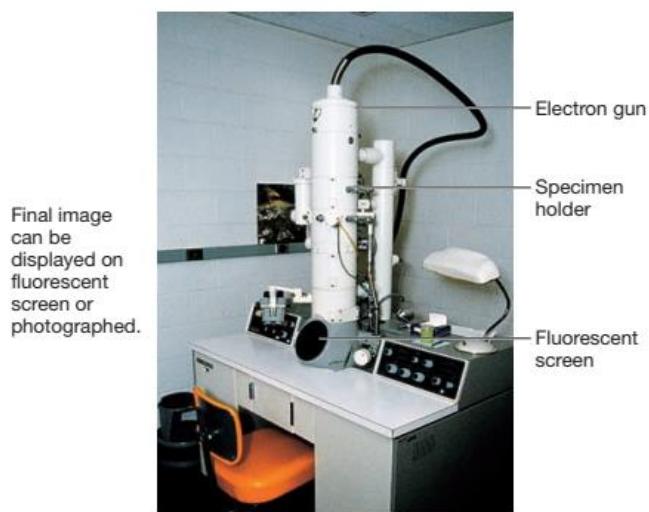


Figure 2.18 A Transmission Electron Microscope. The electron gun is at the top of the central column, and the magnetic lenses are within the column. The image on the fluorescent screen may be viewed through a magnifier positioned over the viewing window. The camera is in a compartment below the screen.

The TEM will also disclose the shape of organelles within microorganisms if specimens are prepared by the **freeze-etching** procedure. First, 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) frac-

tures the frozen cells, which are very brittle and break along lines of greatest weakness, usually down the middle of internal membranes (figure 2.21). The specimen is left in the high vacuum for a minute or more so that some of the ice can sublime away and uncover more structural detail. 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 2.22). 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.

The Scanning Electron Microscope

Transmission electron microscopes form an image from radiation that has passed through a specimen. The **scanning electron microscope (SEM)** works in a different manner. It produces an image from electrons released from atoms on an object's surface. The SEM has been used to examine the surfaces of microorganisms in great detail; many SEMs have a resolution of 7 nm or less.

Specimen preparation for SEM is relatively 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.

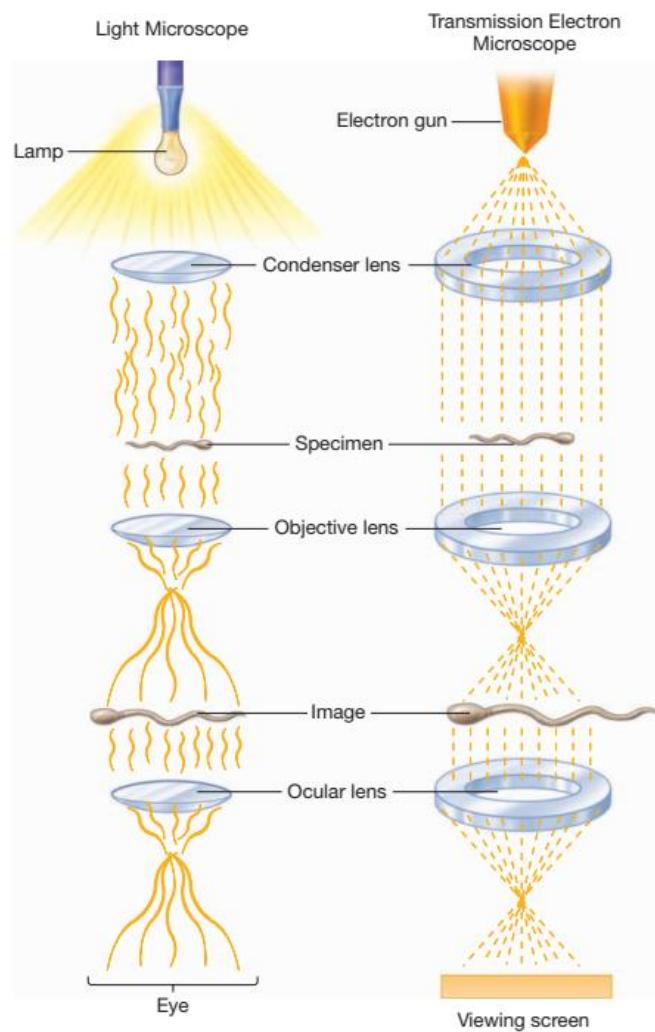


Figure 2.19 Transmission Electron Microscope Operation.
An overview of TEM operation and a comparison of the operation of light and transmission electron microscopes.

To create an image, the SEM scans a narrow, tapered electron beam back and forth over the specimen (**figure 2.23**). 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.

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 results (**figure 2.24**). 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.

1. Why does the transmission electron microscope have much greater resolution than the light microscope?
2. Describe in general terms how the TEM functions. Why must the TEM use a high vacuum and very thin sections?
3. 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?
4. Under what circumstances would it be desirable to prepare specimens for the TEM by use of negative staining? Shadowing? Freeze-etching?
5. 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

Like the large and small beads illustrated in **figure 2.25a**, biological specimens are three-dimensional. When three-dimensional objects are viewed with traditional light microscopes, light from

Table 2.4 Characteristics of Light and Transmission Electron Microscopes

Feature	Light Microscope	Transmission Electron Microscope
Highest practical magnification	About 1,000–1,500	Over 100,000
Best resolution*	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)

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

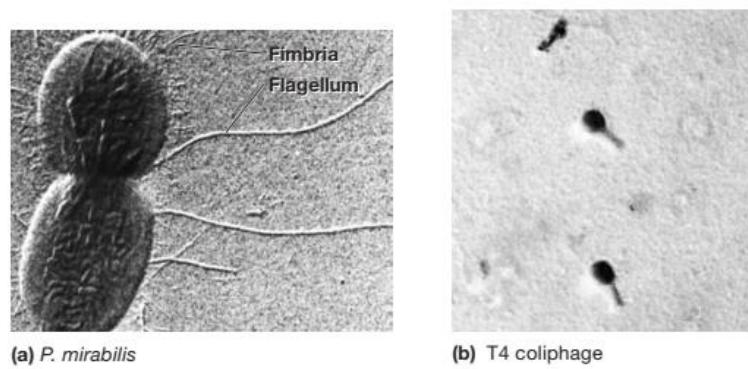


Figure 2.20 Specimen Shadowing for the TEM. Examples of specimens viewed in the TEM after shadowing with uranium metal. (a) *Proteus mirabilis* ($\times 42,750$); note flagella and fimbriae. (b) T4 coliphage ($\times 72,000$).

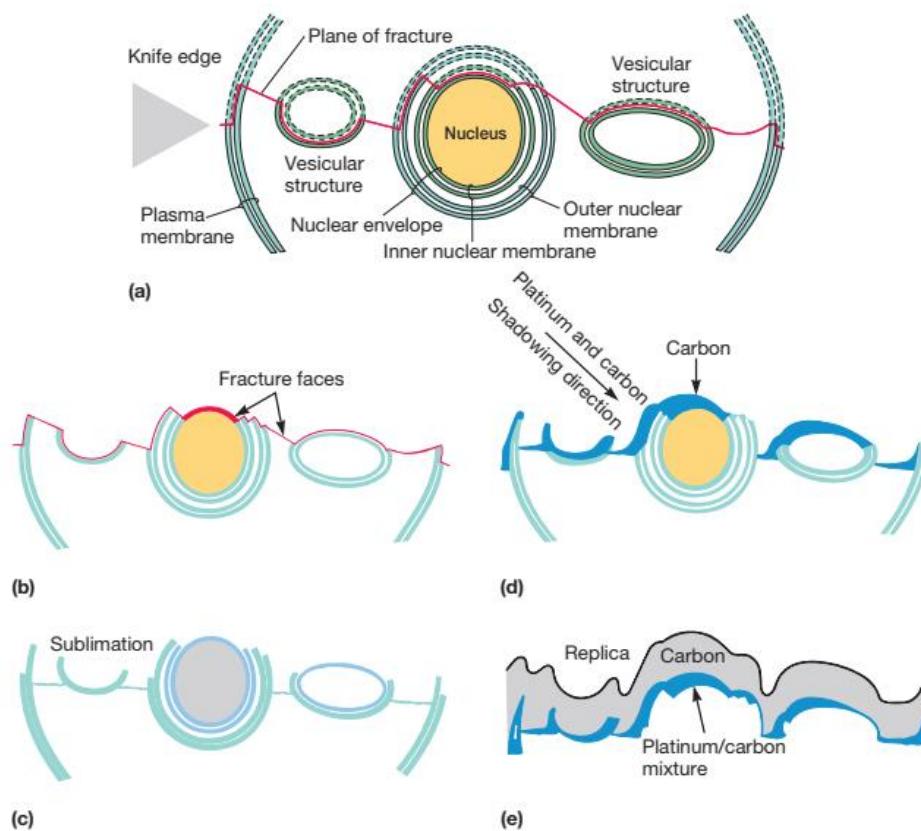


Figure 2.21 The Freeze-Etching Technique. In steps (a) and (b), a frozen eukaryotic cell is fractured with a cold knife. Etching by sublimation is depicted in (c). Shadowing with platinum plus carbon and replica formation are shown in (d) and (e). See text for details.

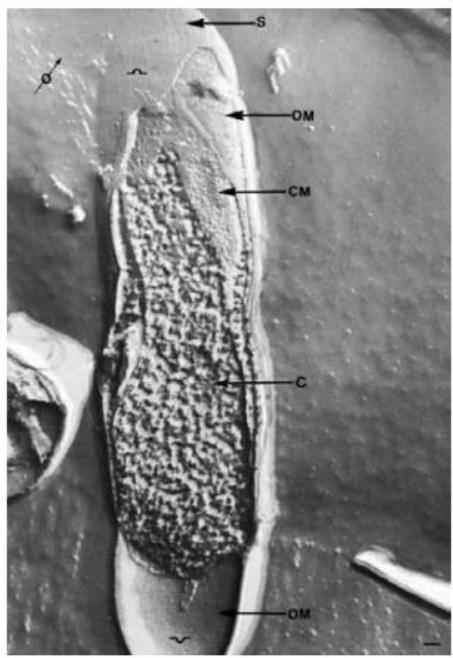


Figure 2.22 Example of Freeze-Etching. A freeze-etched preparation of the bacterium *Thiobacillus kabobis*. Note the differences in structure between the outer surface, S; the outer membrane of the cell wall, OM; the cytoplasmic membrane, CM; and the cytoplasm, C. Bar = 0.1 μm .

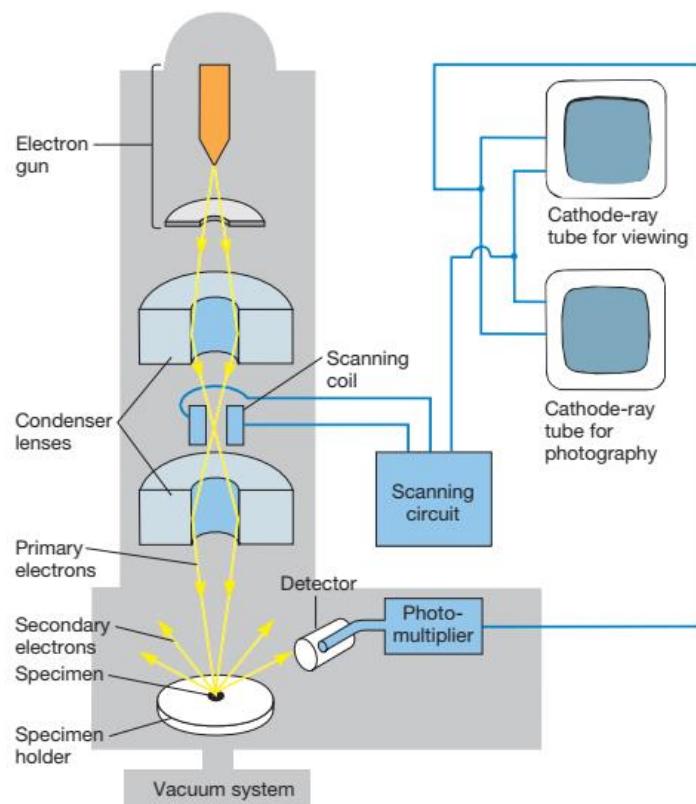
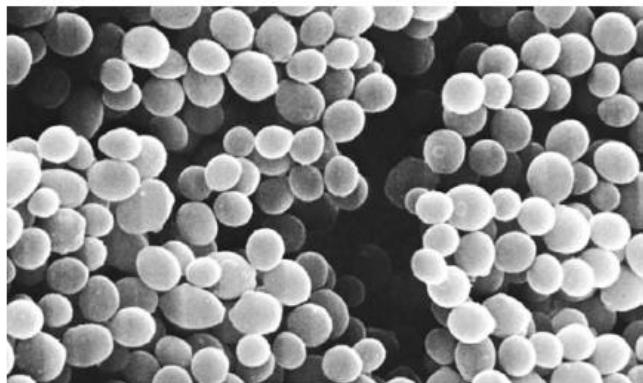


Figure 2.23 The Scanning Electron Microscope. See text for explanation.



(a) *S. aureus*



(b) *Cristispira*

Figure 2.24 Scanning Electron Micrographs of Bacteria. (a) *Staphylococcus aureus* ($\times 32,000$). (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 ($\times 6,000$).

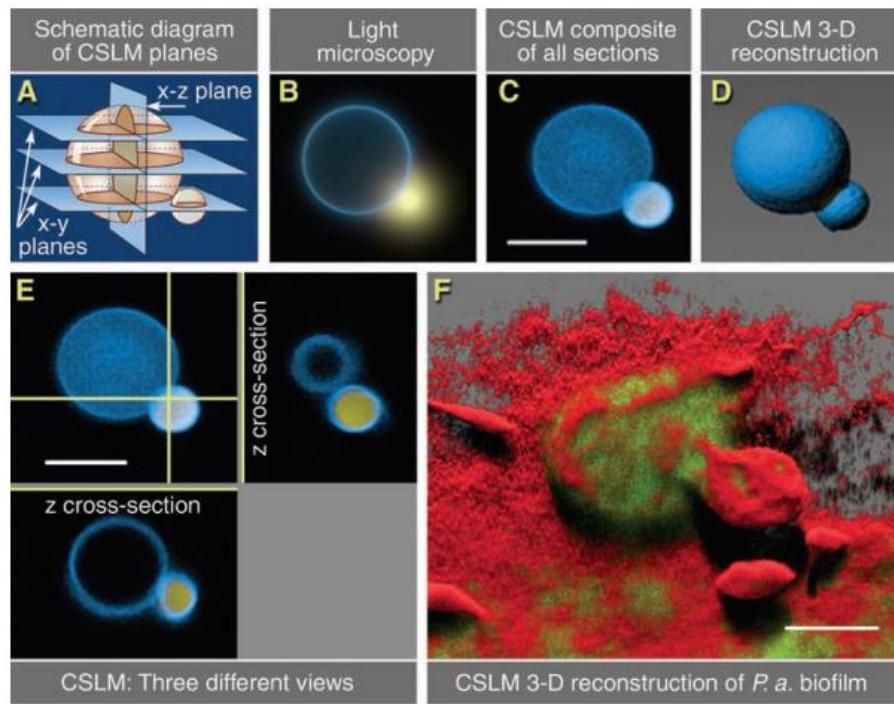


Figure 2.25 Light and Confocal Microscopy. Two beads examined by light and confocal microscopy. Light microscope images are generated from light emanating from many areas of a three-dimensional object. Confocal images are created from light emanating from only a single plane of focus. Multiple planes within the object can be examined and used to construct clear, finely detailed images. (a) The planes observable by confocal microscopy. (b) The light microscope image of the two beads shown in (a). Note that neither bead is clear and that the smaller bead is difficult to recognize as a bead. (c) A computer connected to a confocal microscope can make a composite image of the two beads using digitized information collected from multiple planes within the beads. The result is a much clearer and more detailed image. (d) The computer can also use digitized information collected from multiple planes within the beads to generate a three-dimensional reconstruction of the beads. (e) Computer generated views of a specimen: the top left panel is the image of a single x-y plane (i.e., looking down from the top of the specimen). The two lines represent the two x-z planes imaged in the other two panels. The vertical line indicates the x-z plane shown in the top right panel (i.e., a view from the right side of the specimen) and the horizontal line indicates the x-z plane shown in the bottom panel (i.e., a view from the front face of the specimen). (f) A three-dimensional reconstruction of *Pseudomonas aeruginosa* biofilm. The biofilm was exposed to an antibacterial agent and then stained with dyes that distinguish living (green) from dead (red) cells. The cells on the surface of the biofilm have been killed, but those in the lower layers of the biofilm are still alive.

all areas of the object, not just the plane of focus, enter the microscope and are used to create an image. The resulting image is murky and fuzzy (figure 2.25b). This problem has been solved by the development of the **confocal scanning laser microscope (CSLM)**, or simply, confocal microscope. The confocal microscope uses a laser beam to illuminate a specimen, usually one that has been fluorescently stained. A major component of the confocal microscope is an aperture placed above the objective lens, which eliminates stray light from parts of the specimen that lie above and below the plane of focus (figure 2.26). Thus the only light used to create the image is from the plane of focus, and a much clearer sharp image is formed.

Computers are integral to the process of creating confocal images. A computer attached to the confocal microscope receives

digitized information from each plane in the specimen that is examined. This information can be used to create a composite image that is very clear and detailed (figure 2.25c) or to create a three-dimensional reconstruction of the specimen (figure 2.25d). Images of x-z plane cross-sections of the specimen can also be generated, giving the observer views of the specimen from three perspectives (figure 2.25e). Confocal microscopy has numerous applications, including the study of biofilms, which can form on many different types of surfaces including in-dwelling medical devices such as hip joint replacements. As shown in figure 2.25f, it is difficult to kill all cells in a biofilm. This makes them a particular concern to the medical field because formation of biofilms on medical devices can result in infections that are difficult to treat. [Microbial growth in natural environments: Biofilms \(section 6.6\)](#)

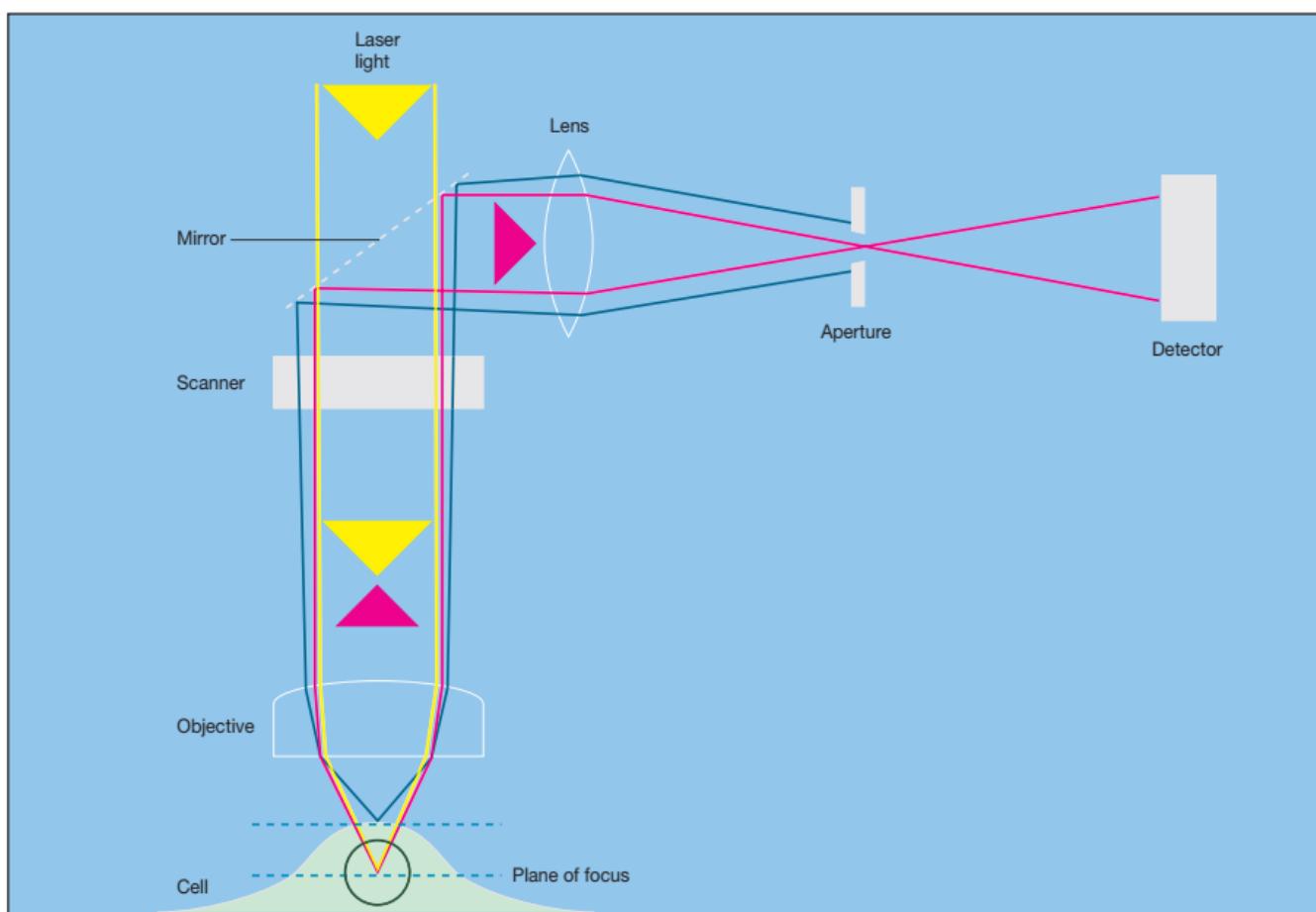


Figure 2.26 A Ray Diagram of a Confocal Laser Scanning Microscope. The yellow lines represent laser light used for illumination. Red lines symbolize the light arising from the plane of focus, and the blue lines stand for light from parts of the specimen above and below the focal plane. See text for explanation.

Scanning Probe Microscopy

Although light and electron microscopes have become quite sophisticated and have 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 needle-like 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 tun-

neling 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 above the specimen 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 is already having a major impact in biology. It can be used to directly view DNA and other biological molecules (**figure 2.27**). 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 be-

tween 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 (**figure 2.28**). 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

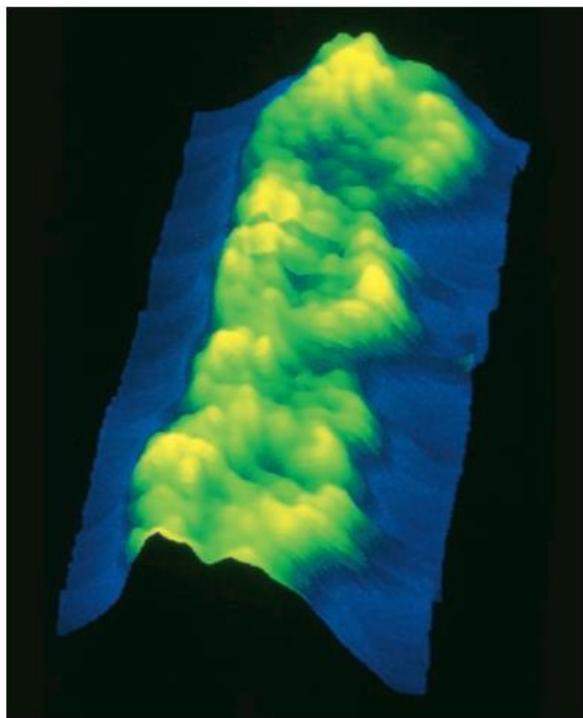


Figure 2.27 Scanning Tunneling Microscopy of DNA. The DNA double helix with approximately three turns shown (false color; $\times 2,000,000$).

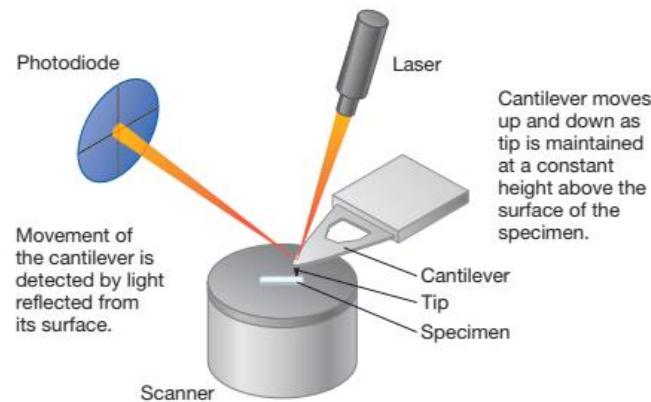
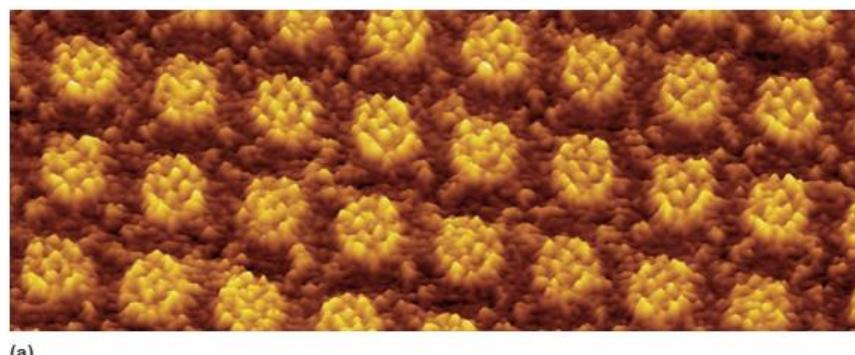
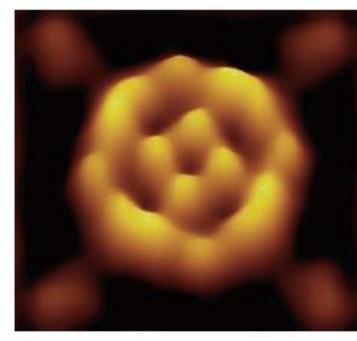


Figure 2.28 Atomic Force Microscopy—The Basic Elements of an Atomic Force Microscope. The tip used to probe the specimen is attached to a cantilever. As the probe passes over the “hills and valleys” of the specimen’s surface, the cantilever is deflected vertically. A laser beam directed at the cantilever is used to monitor these vertical movements. Light reflected from the cantilever is detected by the photodiode and used to generate an image of the specimen.



(a)



(b)

Figure 2.29 The Membrane Protein Aquaporin Visualized by Atomic Force Microscopy. Aquaporin is a membrane-spanning protein that allows water to move across the membrane. (a) Each circular structure represents the surface view of a single aquaporin protein. (b) A single aquaporin molecule observed in more detail and at higher magnification.

has been used to study the interactions between the *E. coli* GroES and GroEL chaperone proteins, to map plasmids by locating restriction enzymes bound to specific sites, to follow the behavior of living bacteria and other cells, and to visualize membrane proteins (**figure 2.29**).

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

Summary

2.1 Lenses and the Bending of Light

- a. A light ray moving from air to glass, or vice versa, is bent in a process known as refraction.
- b. Lenses focus light rays at a focal point and magnify images (**figure 2.2**).

2.2 The Light Microscope

- a. 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 the final image (**figure 2.3**).
- b. A substage condenser focuses a cone of light on the specimen.
- c. 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.
- d. The dark-field microscope uses only refracted light to form an image (**figure 2.7**), and objects glow against a black background.
- e. 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**).
- f. The differential interference contrast microscope uses two beams of light to create high-contrast, three-dimensional images of live specimens.
- g. The fluorescence microscope illuminates a fluorochrome-labeled specimen and forms an image from its fluorescence (**figure 2.12**).

2.3 Preparation and Staining of Specimens

- a. Specimens usually must be fixed and stained before viewing them in the bright-field microscope.

- b. Most dyes are either positively charged basic dyes or negative acidic dyes and bind to ionized parts of cells.
- c. In simple staining a single dye is used to stain microorganisms.
- d. Differential staining procedures like the Gram stain and acid-fast stain distinguish between microbial groups by staining them differently (**figure 2.15**).
- e. Some staining techniques are specific for particular structures like bacterial capsules, flagella, and endospores (**figure 2.14**).

2.4 Electron Microscopy

- a. 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 2.19**). Resolution is high because the wavelength of electrons is very short.
- b. Thin section contrast can be increased by treatment with solutions of heavy metals like osmium tetroxide, uranium, and lead.
- c. Specimens are also prepared for the TEM by negative staining, shadowing with metal, or freeze-etching.
- d. The scanning electron microscope (**figure 2.23**) is used to study external surface features of microorganisms.

2.5 Newer Techniques in Microscopy

- a. The confocal scanning laser microscope (**figure 2.25**) is used to study thick, complex specimens.
- b. Scanning probe microscopes reach very high magnifications that allow scientists to observe biological molecules (**figures 2.27 and 2.29**).

Key Terms

acidic dyes 26	differential staining 26	Gram stain 26	resolution 18
acid-fast staining 26	endospore staining 26	heat fixation 25	scanning electron microscope (SEM) 30
atomic force microscope 36	eyepieces 18	mordant 26	scanning probe microscope 35
basic dyes 26	fixation 25	negative staining 26	scanning tunneling microscope 35
bright-field microscope 18	flagella staining 28	numerical aperture 19	shadowing 29
capsule staining 26	fluorescence microscope 23	objective lenses 18	simple staining 26
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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.

Learn More

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