

3

Methods of Microbiology

WE SAW IN THE last chapter that microbiology is a discipline that is unusually dependent on a distinctive set of methods; indeed, it is often defined by these methods. This dependency is a consequence of the very small size of microbes, which means that we cannot see them without microscopy, and the fact that we cannot do experiments with them without pure culture techniques. In this chapter, we examine in some detail these techniques.

These methods constitute a core of techniques common to all microbiology. In addition, almost all microbiologists use a wide variety of other techniques, especially those of biochemistry, genetics, and molecular biology.

Microscopy

Microscopes have improved immensely since Leeuwenhoek first observed microbes with a simple hand lens. Compound microscopes have become highly refined and now approach their theoretical limits. Optical methods of enhancing contrast are now in routine use; fluorescence microscopy allows the detection of specific molecules within cells, and the invention and improvement of electron microscopy have opened an entirely new world of submicroscopic observation.

3.1 Compound microscopes are used for observing microbes

The compound microscope is now routinely used for observing microbial cells. It gives a wider field of view, less distortion, and less eyestrain than the simple microscope, and it can be equipped for phase contrast, fluorescence, or other sophisticated optical refinements.

In the compound microscope, the objective lens forms a magnified **real image** inside the tube of the microscope. This image is real and can be seen if a piece of frosted glass or a piece of paper is placed into the tube at the right spot. The ocular lens then magnifies this image again. The ultimate magnification is the product of the individual magnifications: a 100× objective combined with a 10× ocular gives a total magnification of 1,000×.

Unlike the objective, the ocular lens does not produce an image. In conjunction with the eye, however, it produces the illusion of an image, which we call a **virtual image**. Whether a lens produces a real image or a virtual image depends on the relationship between the specimen and the focal point of the lens; you will learn about this in physics (Figures 3.1 and 3.2).

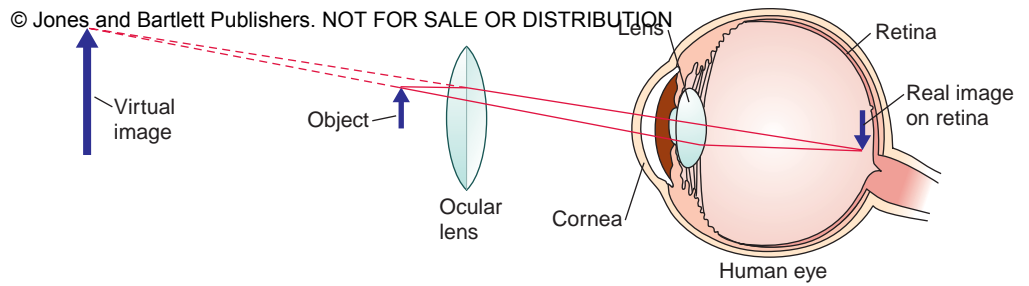


FIGURE 3.1 Virtual image formation.

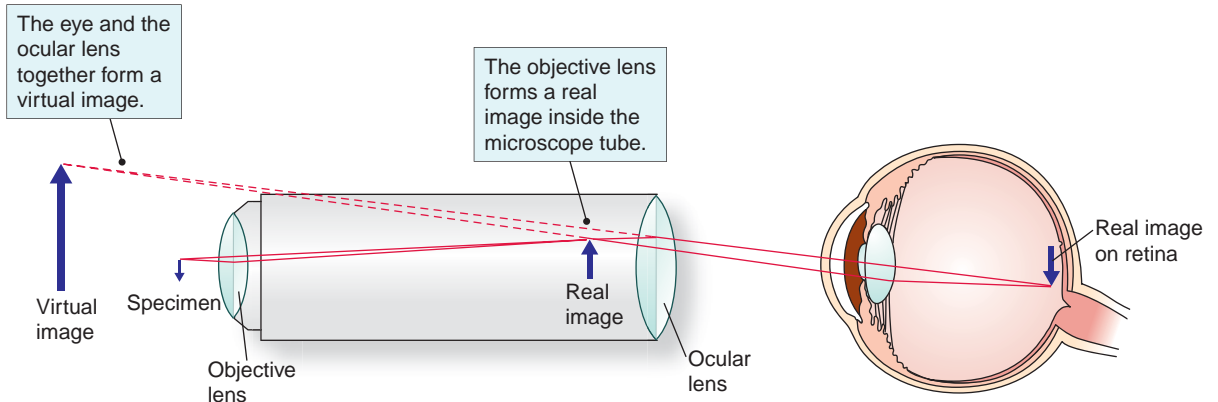


FIGURE 3.2 Image formation in the compound microscope.

3.2 Resolution of the light microscope is normally limited to about 0.2 μm

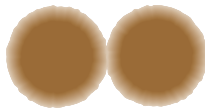


FIGURE 3.3

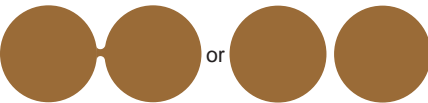


FIGURE 3.4

During normal use, fundamental physical properties limit the resolution of a light microscope. Resolution is the ability to distinguish between two closely spaced objects. For example, in Figure 3.3, are we looking at a single dumbbell-shaped object or two spheres very close together as in Figure 3.4?

The limit of resolution (d) is the distance separating the centers of two dots that can just barely be distinguished as separate.

The limit of resolution of a microscope is related to the wavelength of the light being used (λ) and the numerical aperture of the objective lens (NA) by this formula:

$$d = 0.5\lambda/\text{NA}$$

The numerical aperture is in turn a function of two physical properties of the objective lens (Figure 3.5): its diameter and its **working distance** (how close to the specimen it has to come to be in focus). This is expressed quantitatively as

$$\text{NA} = \eta \sin \alpha$$

Where η is the refractive index of the material in between the lens and the specimen (in air, $\eta = 1.0$). Because the physical properties of each lens are fixed and cannot be altered, the only way to improve resolution with a given lens is to increase the refractive index of the material between the lens and the specimen. This is normally done for only very high-power lenses (e.g.,

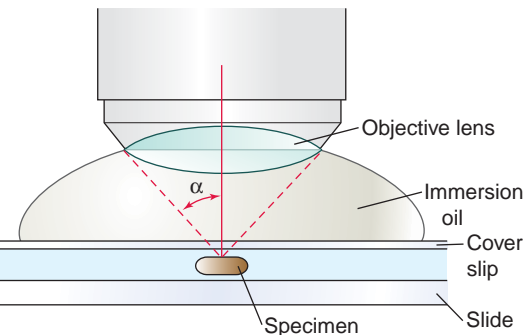


FIGURE 3.5 Geometry of the objective lens.

100× objectives), as it is only at these magnifications that the limit of resolution becomes a problem. Thus, very high-power objective lenses are made to be used with oil ($n = 1.5$) and are called **oil immersion** lenses.

Using about 500 nm as an approximation of the wavelength of visible light (actually it includes wavelengths from about 400 nm to about 800 nm) and a figure of about 1.3 for the numerical aperture of a modern, high-quality objective lens using immersion oil between the specimen and the lens, we obtain the following value for the limit of resolution for a modern research-quality microscope:

$$d = (0.5)(500 \text{ nm})/1.3 = 190 \text{ nm} = 0.19 \text{ } \mu\text{m}$$

Although this is the theoretical limit of resolution of the light microscope, it is not the smallest object that one can see; it is actually possible to see an object about half the size of the limit of resolution, but of course without any detail.

This limit is on the order of the size of a prokaryotic cell—typically about 1 μm in diameter. Thus, although cells can be easily seen, very little detail inside prokaryotic cells is visible with light microscopy; even in the larger eukaryotic cells, intracellular structures are often right at the limit of resolution, and little of their detail can be seen.

This limit of resolution applies during normal use; there are several circumstances under which significantly smaller objects can be seen clearly. One is using darkfield illumination, discussed later; the other is the use of videotaping of a microscopic image and then using sophisticated computer-based image enhancement techniques. With this latter approach, clear images of objects less than 20 nm can be obtained.

3.3 Brightfield microscopy is used for observing stained cells

When used in the traditional fashion, a microscopic specimen is flooded with light from below. After passing through and around the specimen, it enters the objective lens, which forms an image as described previously. The specimen looks darker than the background because it absorbs some light. Because many microbial cells are so small that they absorb very little light, they are often nearly transparent and very difficult to see in this way. Consequently, **brightfield microscopy**, as this traditional way of using the microscope is called, is rarely used anymore for observing living cells.

There is still a function for this type of microscopy, however: to observe stained cells. Originally, stains were developed to enhance contrast in brightfield microscopy, before the invention of the phase contrast microscope. Stains are rarely used this way anymore, as phase contrast is faster and allows living cells to be observed (staining normally kills cells), but **differential staining** is still used. Differential staining uses dyes that will stain some microbes and not others or some intracellular structures and not others. This allows different types of microbes and organelles to be distinguished by their staining reactions, even if they are morphologically indistinguishable. The most common differential stain in use today is the **Gram stain**, named after its inventor, the Danish physician Christian Gram who invented the stain in the 1880s. This stain differentiates one group of bacteria (the “gram-positive bacteria,” G+) from all other groups of bacteria (gram-negative, G-), based on the structure of their cell wall. It is not widely used outside the bacteria. We now know that almost all gram-positive bacteria fall into a single major sublineage, and thus, the Gram stain gives us evolutionary information (Figure 3.6).

Because phase-contrast microscopy, the other widely used method of light microscopy, distorts colors, it cannot be used when the object is to determine the

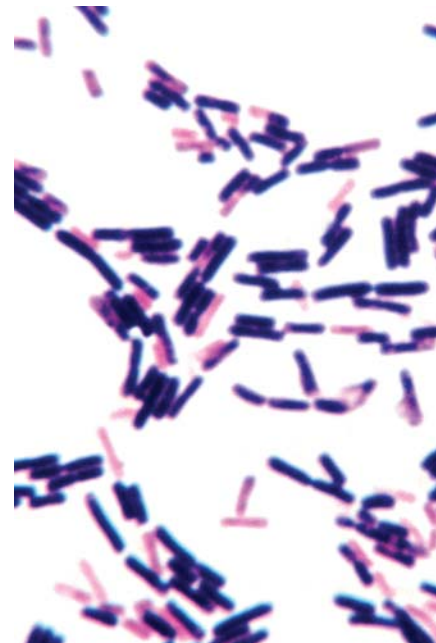


FIGURE 3.6 G+ (purple) and G- (red) cells.

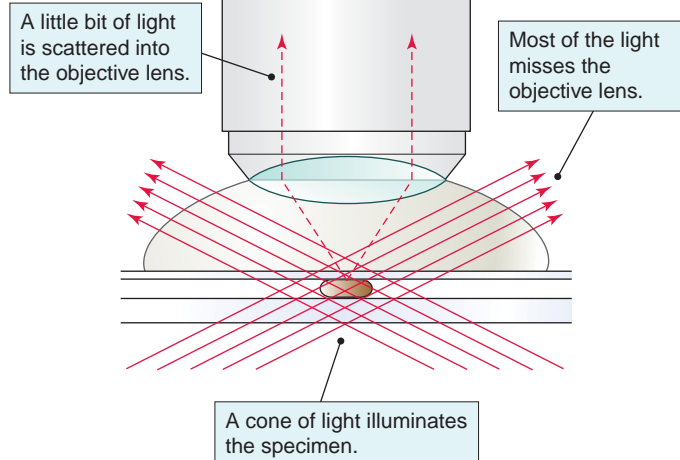


FIGURE 3.7 Light path in the darkfield microscope.

color of a stained object (as it is in the Gram stain and other differential stains). Hence, brightfield is routinely used for observing stained preparations.

3.4 Darkfield microscopy can resolve very small objects

We have all had the experience of being in a darkened room into which a beam of sunlight shines. In that beam we can see dust motes clearly shining against the dark background, yet when the room is fully illuminated, we see nothing in the air. The reason that we can see the tiny motes (which can be under $100\ \mu\text{m}$) is that they scatter light, making a bright spot against the black background. The same principle can be used in microscopy. If the condenser is set such that it illuminates the specimen with a cone of light that never enters the objective lens, the field of view will be black because no light is directly entering the lens. If there are objects in the field that scatter light, however, some of that light will enter the lens (Figure 3.7). If there is enough of it to register in the eye, we will see the object as bright against a dark background. With sufficiently intense light, extremely small objects can be seen—for instance, bacterial flagella, which are less than $20\ \text{nm}$ thick (Figure 3.8).

Darkfield microscopy is difficult to use routinely, and, thus, it is generally used only by those with a specific need for it, such as microbiologists working with the very smallest microbes. Some spirochetes (e.g., *Treponema*, the causative agent of several human diseases, including syphilis) are less than $0.2\ \mu\text{m}$ in diameter, and they are barely visible in phase contrast. They are routinely observed with darkfield microscopy.

3.5 Phase-contrast is routinely used for observing live microbial cells

Because of their very small size, most microbial cells absorb and scatter little of the light that passes through them; thus, they are nearly transparent and are very difficult to see with transmitted light. One of the great advances in microscopy in the 20th century was the development of sophisticated optical means of enhancing contrast by manipulating the light inside the microscope. The most common type of contrast instrument is the **phase-contrast microscope**; considerably less common, but equally effective, is the **interference-contrast microscope**. Most microbiologists now routinely use one of these means for observing live microbes suspended



FIGURE 3.8 Darkfield view showing bacterial flagella. The cell is about the size of the dark oval. It is surrounded by a halo because the light source is very bright.

in water. We describe the phase-contrast microscope in detail in the next section. Interference contrast is used; interference contrast operates on the same basic physical principles, although the detailed mechanisms are different, and hence, so is the image.

3.6 Phase-contrast is achieved by modifications to the condenser and the objective lens

In the brightfield microscope, the condenser functions to illuminate the specimen evenly and brightly, and the objective lens produces a magnified image. In a phase-contrast microscope, both of these elements are modified—the condenser by the addition of an annular diaphragm and the objective by the addition of a phase plate (Figure 3.9).

The phase-contrast microscope, like the darkfield microscope, illuminates the specimen with a cone of light, rather than an even flood of light. This is because the condenser has an **annular diaphragm**—a transparent ring in an otherwise opaque plate. Thus, a ring of light enters the condenser lens and is then bent into a hollow cone, with the specimen at its apex. Unlike the darkfield microscope, however, in

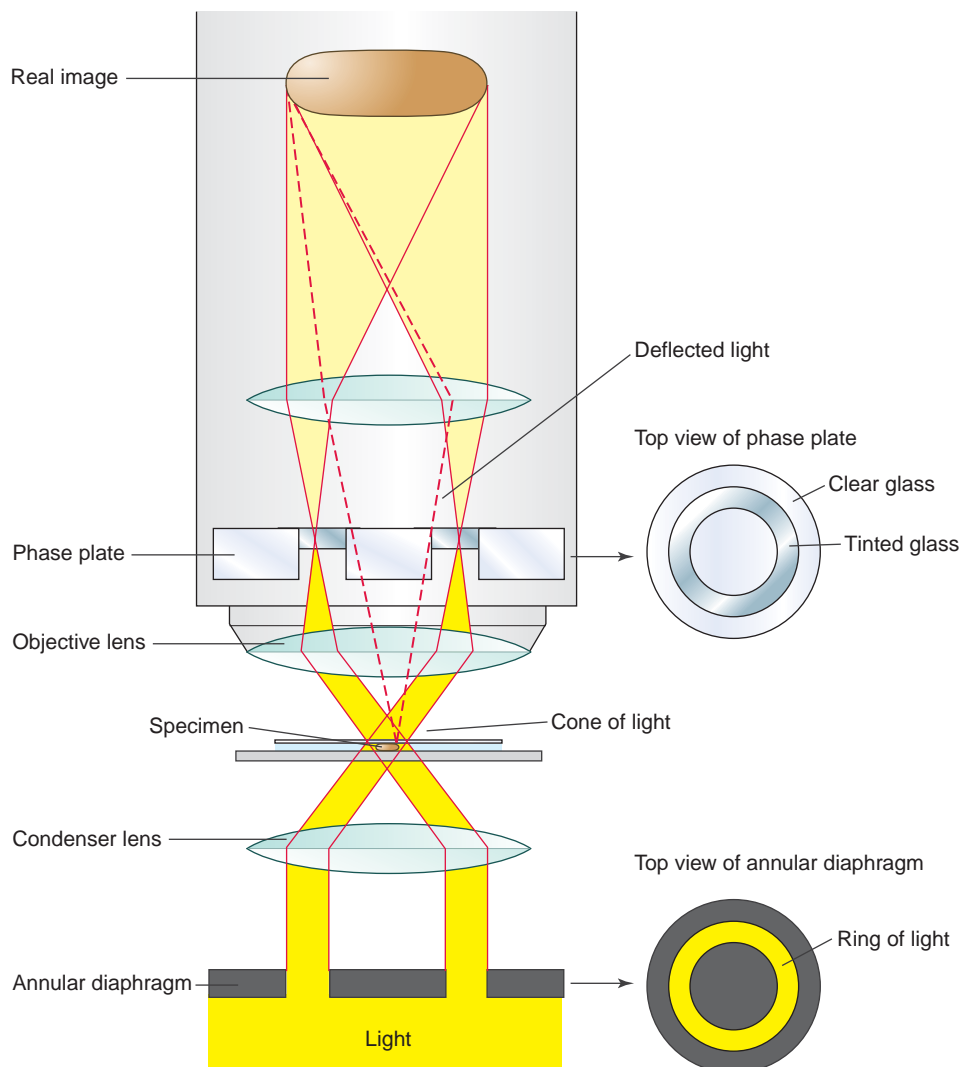


FIGURE 3.9 The light path in the phase-contrast microscope.

As this light passes through the specimen, some of it gets deflected, particularly from the edges of cells and organelles. Some of it gets absorbed. The great majority of it, however, passes straight through. Ultimately, the lens systems of the microscope will bring the deflected rays and the transmitted rays to a focus at the same point. The image is thus made up of light that came straight through the specimen and light that was deflected by the specimen.

After passing through the specimen, the cone of undeviated light enters the objective lens and passes through a ring of tinted glass on the **phase plate**. This **phase ring** is sized and positioned such that all of the light in the illuminating cone goes through it. Because the ring is tinted, like dark sunglasses, the light that passes directly through the specimen is dimmed by more than 70%. Of course, the background light is dimmed the same amount because it too goes through the phase ring.

The deflected light, although a minor component of the light leaving the specimen, is not dimmed because it passes randomly through any portion of the phase plate; most of it thus misses the tinted phase ring. Even though it is not tinted, however, the phase plate other than the ring is made of thicker glass; it thus shifts the phase of the deflected light. The total phase shift is one half wavelength. The deflected light is now 180° out of synchrony with the direct light, and when they combine in the image plane, destructive interference reduces the brightness of the object, contributing to contrast. The MicroTopic box on pages 32 and 33 gives a more detailed view of what is happening.

3.7 Fluorescence microscopy can locate specific molecules within cells

One of the most widely used forms of microscopy in biological research today is fluorescence microscopy because it can be used to visualize the location of specific molecules in a cell. For instance, specific proteins, or specific DNA sequences, can be visualized by this kind of microscopy (Figure 3.10).

Fluorescence microscopes are very complex instruments, but their principle is simple. There are three basic steps to fluorescence microscopy. First, a fluorescent molecule (a **fluorophore**) is linked to the molecule to be visualized. Second, the specimen is illuminated with a beam of light with a wavelength that will be absorbed by the fluorophore. Third, the specimen is observed through filters that cut out most of the light except the wavelengths that are emitted by the excited fluorophore.

Fluorescent compounds are ones that absorb light of one wavelength and then release the absorbed energy by emitting light of a longer wavelength. Many different fluorescent compounds are used in fluorescence microscopy; some are small organic compounds that are chemically reactive and can bind to certain cellular structures. Others are fluorescent proteins or short single-stranded DNA sequences to which a fluorescent dye has been covalently attached. The details of how specific fluorescent tags are attached to specific molecules in cells are complex, and we do not discuss it here.

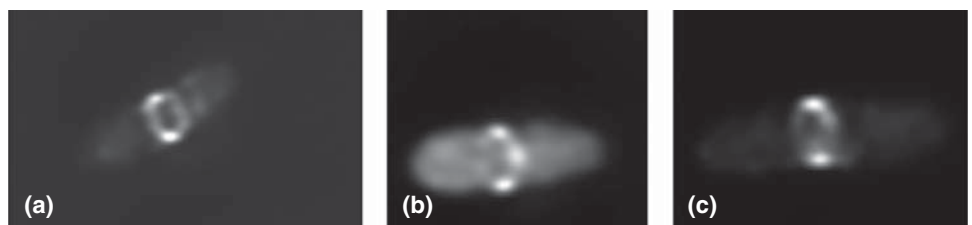


FIGURE 3.10 Fluorescence micrograph of *E. coli* showing location of a specific protein in an *E. coli* cell.

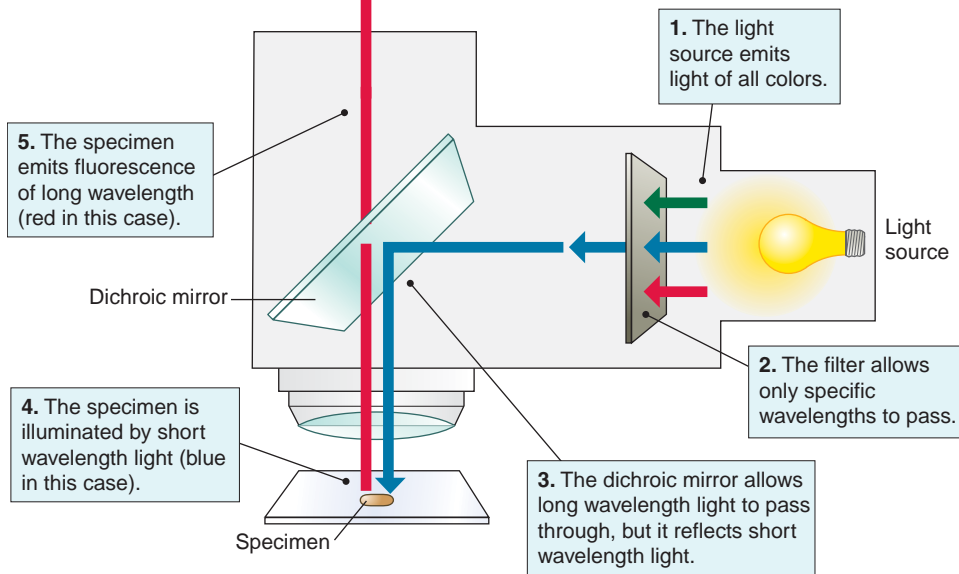


FIGURE 3.11 Light path in the fluorescence microscope.

Most fluorescence microscopes are constructed around a set of filters and a **dichroic mirror**, a piece of glass that acts as a mirror for some wavelengths, but is transparent to others. The illuminating light is passed through a filter that filters out all wavelengths except those in the region that will excite the fluorophore. The filters can be changed to allow different fluorophores to be used. The dichroic mirror then reflects the exciting light to the specimen. Fluorescence emitted by the specimen passes back through the dichroic mirror and to the image plane (Figure 3.11).

The image formed by a fluorescence microscope can be pretty fuzzy. This happens when the fluorescent molecules are distributed in a specimen through a wide zone above and below the plane on which the microscope is focused (which can be as narrow as 100 nm, one tenth of the thickness of a typical prokaryotic cell). Because all of the fluorophores are fluorescing, but only some are in focus, the image is fuzzy. A modification of the fluorescence microscope, termed the **confocal microscope**, removes light that is out of focus. The optical mechanism by which this is done is too complex for us to describe here, but it is important to know of the technique, as it is becoming widely used.

3.8 The electron microscope uses electromagnetic lenses to bend a beam of electrons

One of the insights of the quantum mechanical revolution in physics in the first third of the 20th century was the understanding of the dual nature of elementary particles. Thus, visible light, normally considered to be composed of waves, was realized to have many properties of a stream of particles (termed *photons*). The same was true of electrons—normally considered elementary particles, they have many of the properties of an electromagnetic wave. Thus, the fundamental nature of these elementary particles was understood to be different from both a pure wave and a pure stream of particles but, rather, to have properties of both.

One of the practical applications of this understanding was that if a way could be found to bend a beam of electrons, similar to the way glass lenses bend a beam

When light rays pass through a wet mount containing a suspension of bacterial cells, we can think about three different sets of rays (Figure 3B.1). One set goes through the water alone—it will be brought to a focus in the image plane and will form the bright background. We will call these rays number 1. Second, some light goes through the cells—some of this light is absorbed, but most goes right through the very small cells and is brought to a focus to form the image of the cells. This image is slightly darker than the background because of the light that was absorbed. We will call these rays number 2. Finally, some light is deflected by the cells. Much of the deflected light is lost; however, some is deflected at a small enough angle that it still enters the objective lens, and it too is brought to a focus and contributes to the formation of the image of the cells. We will call these rays number three. For physical reasons that we do not discuss here, this deflected light is retarded by one fourth of a wavelength, but that does not prevent it from contributing to image formation (Figure 3B.2).

In the brightfield microscope, rays 2 and 3 are added together to form the image of the cells. In the phase-contrast microscope, however, rays 3 are *subtracted* from rays 2, thus making the

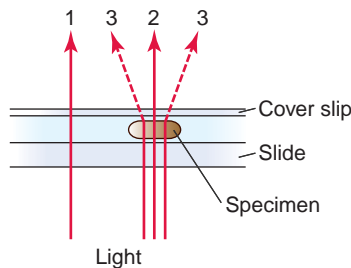


FIGURE 3B.1 Light rays in the phase-contrast microscope.

image darker. This by itself would make very little difference, as so little light is deflected that subtracting it instead of adding it makes a negligible difference in the image. Thus, a second manipulation of the light reduces the intensity of rays 1 and 2 dramatically; now, when rays 3 are subtracted from what is left of rays 2, the difference is dramatic.

These manipulations are the result of the undeviated light all passing through the objective as a ring. The deviated light, rays 3, however, passes through the entire surface of the objective. Thus, the two types of light can be treated separately. The way this is done is to construct the objective with a **phase plate** built

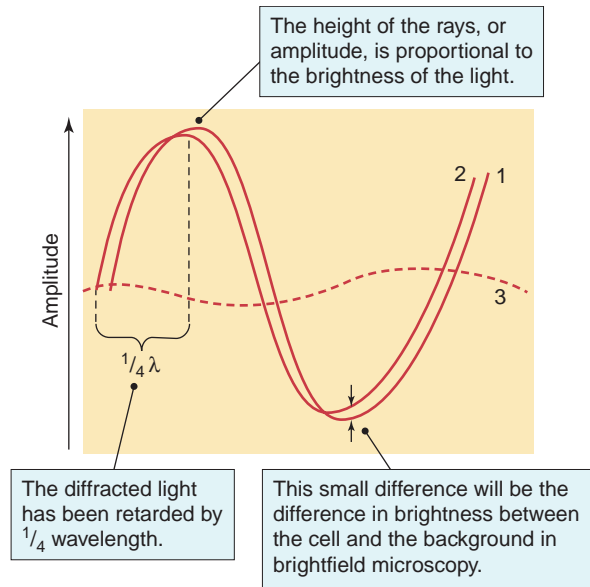


FIGURE 3B.2 Amplitude of light rays entering the objective lens.

of photons, then a microscope could be constructed using an electron beam instead of a light beam, and it would have vastly improved resolving power because the wavelength would be so much smaller. Work to construct such an instrument began in the 1930s, but it was not until the 1950s that they were sufficiently well developed to become useful in biology. Since then, however, their use has revolutionized our understanding of the architecture of the cell. This ability to resolve the fine details of internal cell structure has been coupled to the techniques of biochemistry and molecular biology to study the chemical composition of subcellular structures so that we now have an immensely detailed understanding of the structure and function of cells. This is one of the great achievements of modern biology.

In an electron microscope, the illuminating electron beam is generated by an **electron gun**. This produces electrons by passing a current through a thin filament, which ejects electrons into the vacuum within the gun. The electron gun generates a powerful electrical field that accelerates the electrons toward the positive electrode. Some pass through a small hole in the positive electrode to form a beam directed into the condenser lens.

into it. The phase plate is a relatively thick piece of glass with a thinner ring etched into it. The ring, or **conjugate area** of the phase plate, is positioned so that all of the undeviated light, rays 1 and 2, pass through it. It is also tinted very dark, and thus, it reduces the intensity of rays 1 and 2 significantly.

The rest of the phase plate, or the **complementary area**, is where the deviated light, rays 3, passes through. It is not tinted, and thus, these rays are not reduced in intensity. It is thicker than the conjugate area, however, and thus, the light that passes through it is slowed down briefly. The thickness is set so that light is retarded by one fourth of the wavelength. Because the deviated light is already retarded by one fourth of the wavelength, the total retardation is one half of wavelength. The deviated light rays are thus exactly out of phase with the undeviated light (Figure 3B.3).

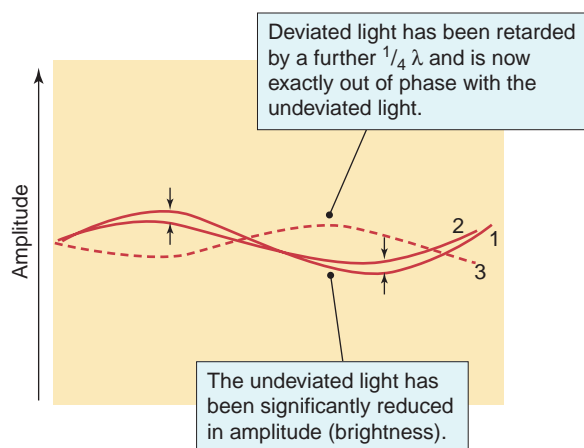


FIGURE 3B.3 Amplitude of light rays passing through the phase plate.

When these three sets of light rays reach the focal plane, rays 1 form the background. The image of the cells is the result of rays 2 and 3 combined. Because rays 2 and 3 are 180° out of phase, however, they interfere destructively with each other. The result is that rays 3 are subtracted from rays 2, making the image of the cells darker than it would otherwise have been (Figure 3B.4).

The phase-contrast microscope was a brilliant invention that transformed the practice of microscopy. For the first time scientists could clearly observe living microbial cells, with good contrast without using stains. The invention was the work of the Dutch physicist Frits Zernicke in the 1930s, for which he received the Nobel Prize in physics in 1953.

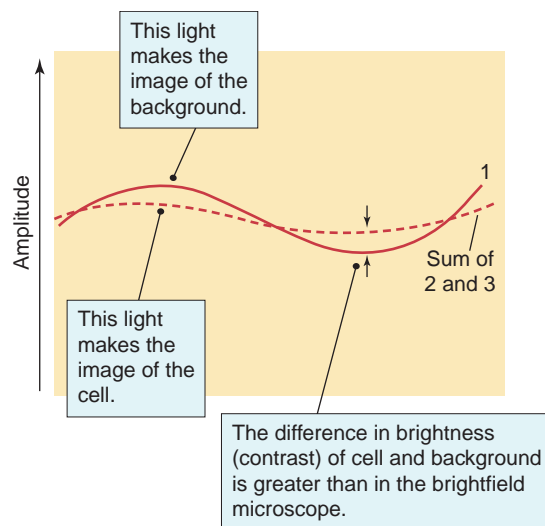


FIGURE 3B.4 Amplitude of light rays on the image plane.

To bend a beam of electrons, necessary to form an image, electromagnetic lenses are used. These consist of a powerful magnetic field shaped like a lens. This field is created by passing an electric current through a fine wire wound hundreds of times around a central hole. The current creates a magnetic field in the hole, whose strength is proportional to the current that creates it. Thus, unlike the glass lenses of a light microscope, the power of an electromagnetic lens can be varied by just turning a knob (Figure 3.12).

Because the human eye is not sensitive to electrons, the electron microscope has a **projector lens** instead of an ocular lens. The projector lens produces a second real image on a screen, which has a phosphorescent coating that glows when it is hit by the beam. We thus see the image on the screen.

3.9 The resolution of the electron microscope is a thousand-fold better than the light microscope

The same basic equation that predicts the limit of resolution for the light microscope works for the electron microscope, and thus, we can predict the resolution of

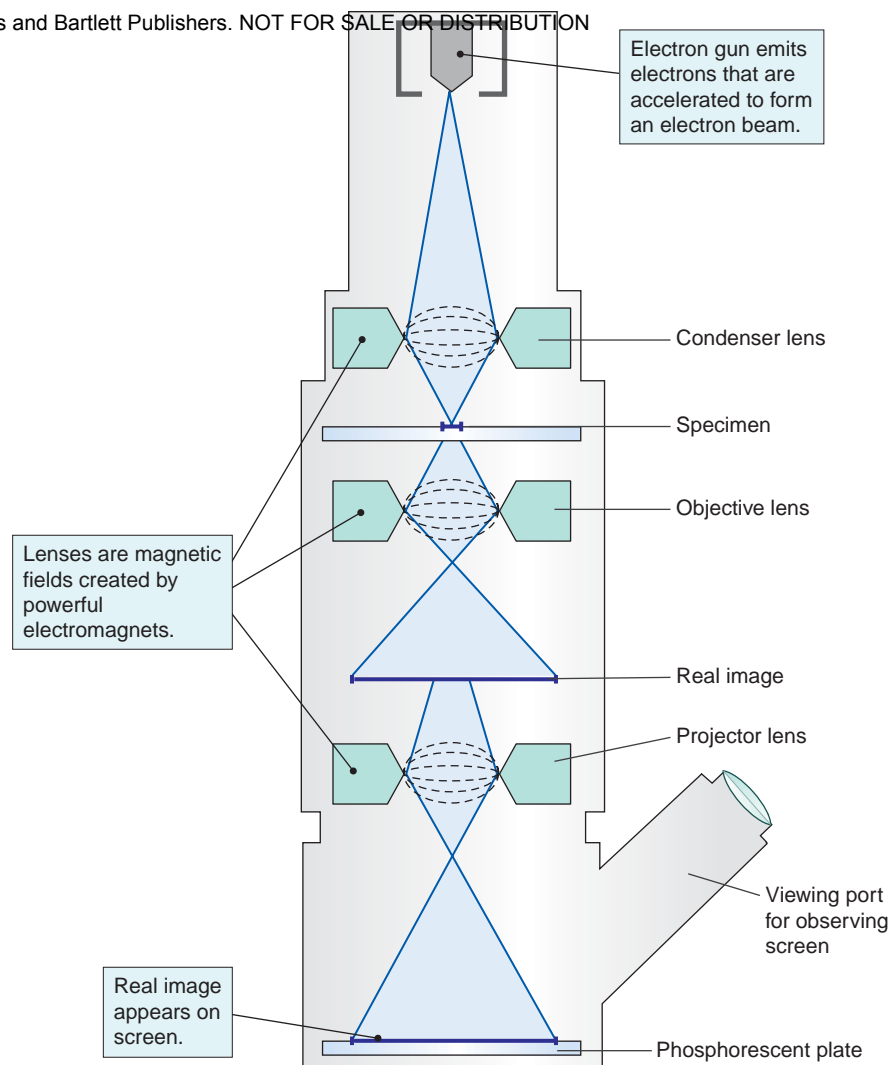


FIGURE 3.12 Image formation in the electron microscope.

the latter by knowing the wavelength of the electron beam and the geometry of the objective lens.

Physical theory indicates that an electron's wavelength is a function of its speed—the faster it goes, the lower the wavelength (and hence the better the resolution). Speed, in turn, is a function of the accelerating voltage (V). Skipping a lot of algebra and using known values of physical constants, we can calculate that the wavelength of a beam of electrons is

$$\lambda \approx 1.2/\sqrt{V} \text{ nm}$$

If we substitute this into the equation for resolution, we get

$$d \approx 0.7/\sin \alpha \sqrt{V} \text{ nm}$$

If we substitute values for a typical electron microscope ($\sin \alpha \approx 10^{-2}$, $V \approx 10^5$ volts), we get a theoretical resolution of close to 0.2 nm (2 Å), a thousand times smaller than the light microscope, easily able to resolve even the finest details of intracellular structure.

3.10 Specimens in the electron microscope need to be dehydrated

Although the beam of electrons in an electron microscope is very intense and has high velocity, the electrons are so light that they are easily deflected by collision with air molecules. Thus, the interior of the electron microscope is kept under vacuum. This is a problem for biological specimens, however, which can be severely distorted if simply placed in a vacuum.

The distortion is due to the evaporation of water and dissolved gases from cells; because this is a major fraction of the volume of the cell, its loss can result in collapse, shrinkage, and distortion of structures. Consequently, one of the first challenges to face electron microscopists was to develop a way of **fixing** the structures of the cell and then slowly dehydrating it before putting it into the microscope.

Typically, fixation involves the use of chemical agents that **cross-link** the macromolecules of the cell, covalently linking them to their neighbors. Thus, even when the water is removed, they retain their relative positions. Nevertheless, the preparation of specimens for the electron microscope remains a real art.

3.11 Thin sectioning slices through cells shows interior structures

Probably the most common form of sample preparation for electron microscopy is **thin sectioning**, in which the specimen is embedded in a small block of plastic after fixation and dehydration and then cut into very thin slices (typically about 100 nm, or approximately one-tenth of the thickness of a typical prokaryotic cell). This allows a view of the interior of the cell, and a large amount of our knowledge of the fine structure of cells comes from thin sectioning (Figure 3.13).

Thin sections have to be stained before viewing in the electron microscope because organic material is nearly transparent to an electron beam. Some fixatives also act as stains; osmium tetroxide (OsO_4), for example, acts both as a fixative by virtue of its four reactive oxygens and as a stain because its heavy metal atom easily deflects the electron beam. It is wise to remember that what we see in a thin section is not the cellular material itself, but the stain bound to it. If the binding is not equal to all cell components, we can be misled about the nature of cell structure. Much of the history of electron microscopy has been occupied with trying to determine what is real and what is an artifact due to distortions caused by fixation, drying, or staining.

3.12 Negative staining reveals the texture of the outside of cells and isolated subcellular organelles

One of the earliest techniques of electron microscopy, still widely used for very small objects, like virus particles and protein molecules, is **negative staining**. The fixed but still wet specimen is placed in a drop of stain (usually a heavy metal salt), which then precipitates on and around the specimen. More stain accumulates at the edges of objects and in surface depressions. This technique thus is particularly useful for showing surface structures (Figures 3.14 and 3.15).

3.13 Shadow casting shows the surface of very small isolated organelles

Shadow casting also uses the deposition of a heavy metal around a specimen to show surface structure; however, in this case, the fixed, dried specimen is placed in a vacuum chamber, and the metal is evaporated from an electrode. This results in deposition of



FIGURE 3.13 Thin section of a bacterial cell.

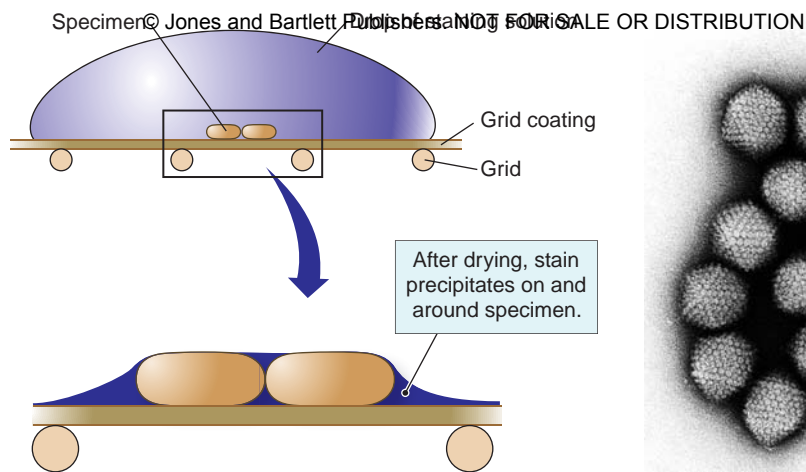


FIGURE 3.14 Negative staining technique.

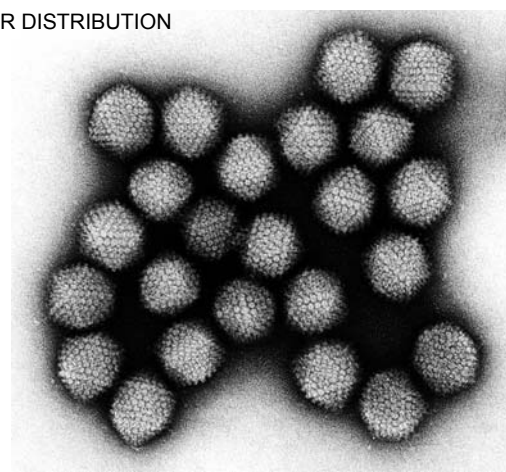


FIGURE 3.15 Negative stain of virus particles.

metal on the side toward the electrode, with little stain deposited on the side away from it. When such a preparation is examined in the electron microscope, it looks as if it is being illuminated from a low angle—hence the name (Figures 3.16 and 3.17).

3.14 Freeze etching does not require fixation or dehydration

The one method of sample preparation for electron microscopy that does not require the fixation and dehydration steps is **freeze etching**. It has thus been of great importance in evaluating whether things seen by thin sectioning are real or are artifacts caused by the stresses of fixing and drying.

In freeze etching, the specimen is frozen very rapidly, usually by immersing it in liquid nitrogen at -190°C (rapid freezing prevents the formation of large ice crystals during the freezing process, which can cause a lot of damage to cell structures). The

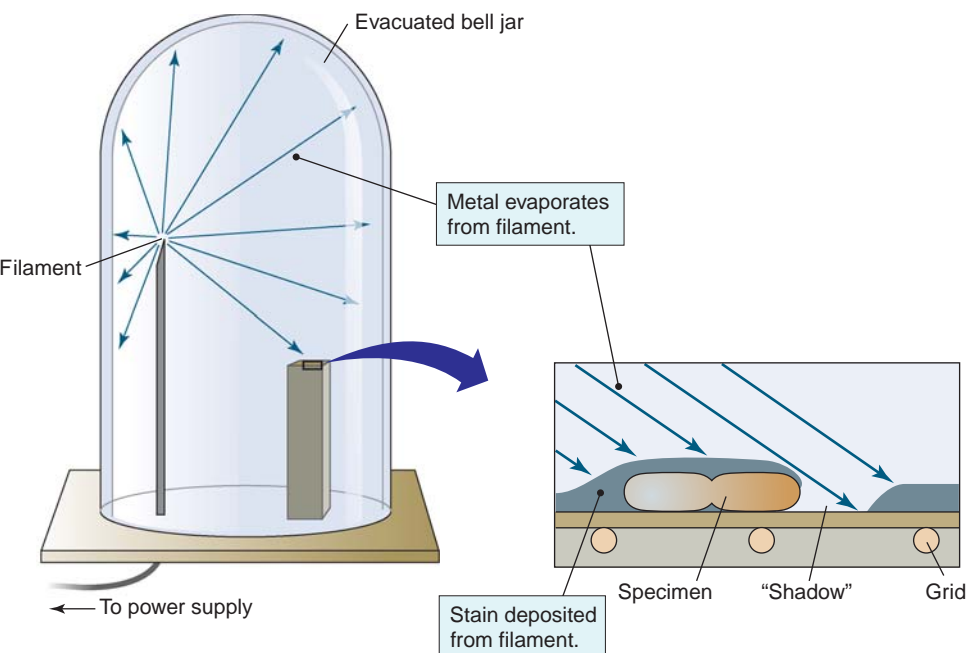


FIGURE 3.16 Shadow casting technique.

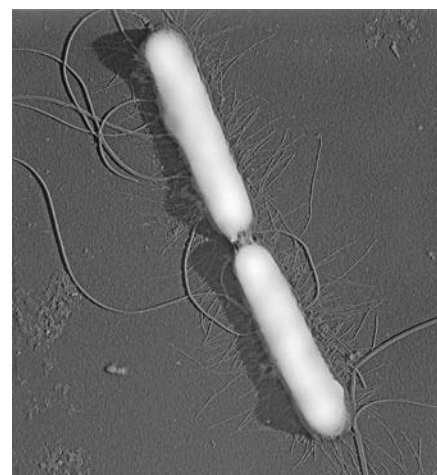


FIGURE 3.17 Shadow-cast electron micrograph of a bacterial cell.

frozen specimen is then fractured along the plane along which the specimen splits ahead of the knife is the plane of least resistance. With biological specimens, this is often the interface between a membrane and the ice or down the middle of the membrane (because the hydrophobic forces holding membranes together are weaker than the hydrogen bonds holding the water molecules together in ice).

The specimen is then usually put under vacuum to allow some of the ice to sublimate. This lowers the surface level of the ice, revealing some of the structure that was hidden before etching and giving a more three dimensional view. The fractured, etched specimen is then shadowed just as in shadow casting, and a layer of carbon is deposited on top of the shadow. This **replica** is then lifted off the specimen, taking the stain with it, and observed in the electron microscope (Figures 3.18 and 3.19).

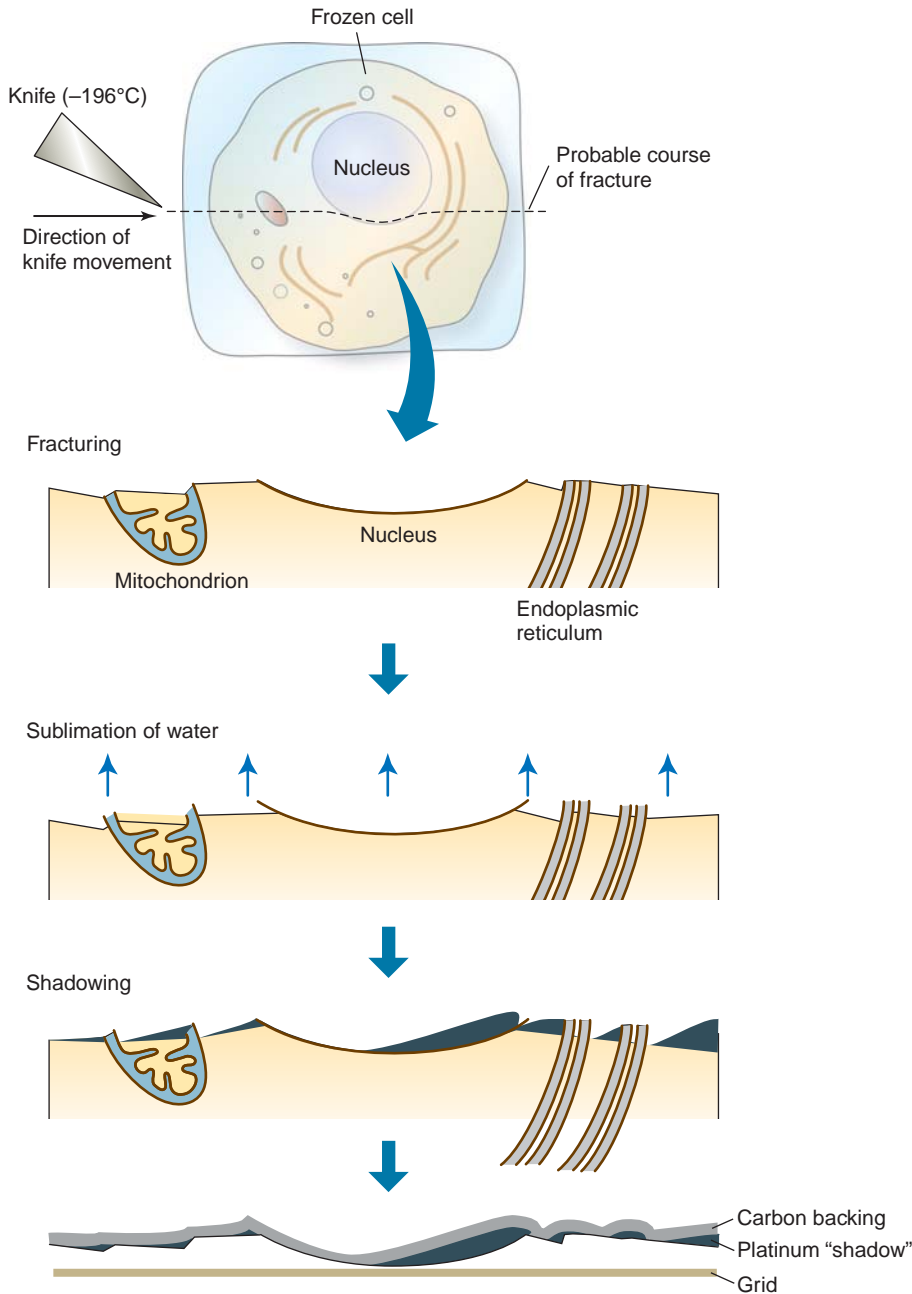


FIGURE 3.18 Freeze etching technique.

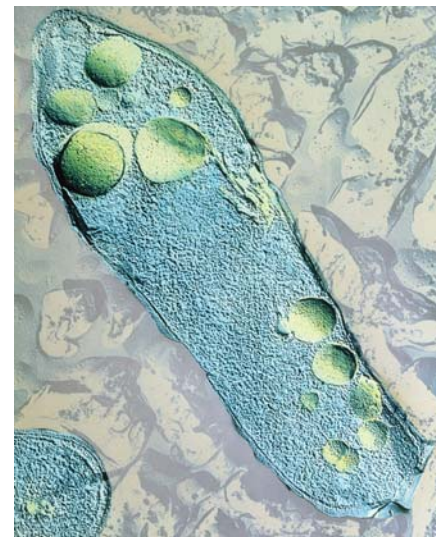


FIGURE 3.19 Freeze etch electron micrograph.

© Jones and Bartlett Publishers, not for sale outside of the United States. Cells are quickly frozen and thus protected from the distorting effects of fixing and dehydrating. Thus, freeze etching is often used to help decide whether structures seen in other techniques, like thin sectioning, are real or artifacts. If real, they should also be seen in freeze-etched preparation; if an artifact, they should not.

Freeze etching is also an excellent way to visualize membranes. It is especially good for detecting membrane proteins because when a membrane fractures down the middle, the proteins either stay with the specimen and are seen as bumps or are torn away, leaving a visible hole. In either case, their number and distribution is obvious.

3.15 Scanning electron microscopy shows surfaces with great depth of field



FIGURE 3.20 Scanning electron micrograph.

The scanning electron microscope is actually an electron probe, rather than a microscope, but it can be used to form an image. Images in the scanning electron microscope typically have great depth of field and look very realistic (Figure 3.20).

As in the transmission electron microscope, a sample preparation for the scanning electron microscope requires fixing and dehydration. There is an additional step, however: the sample is coated with a very thin (several atoms thick) coating of gold by using a shadowing apparatus adapted such that the specimen is continuously turned, evenly spreading the coating over the entire surface.

In the scanning electron microscope, the electron beam is squeezed by magnetic lenses down to a very tiny spot only a few square nanometers in area. This spot is then scanned back and forth across the specimen; as it moves across the surface, the high-intensity electron beam hits atoms in the gold coating, ejecting electrons from their orbits. Most of these ejected electrons are recaptured by other atoms in the gold coating, and they are eventually carried away (the specimen is grounded so that these electrons, as well as electrons from the beam itself, can flow out of the specimen).

Some electrons, however, are completely ejected from the specimen, and these are captured by an electron collector, forming a very weak electrical current. This electrical current is proportional to the number of electrons ejected from the specimen, which is in turn proportional to the shape of the surface. When the electron beam hits a high point, more electrons are ejected than when it hits a low spot (Figure 3.21).

The current that is generated by the electron beam and the specimen then is used to form an image on a cathode ray tube (just like in a television set). The electron beam in the tube is scanned back and forth in a pattern exactly in register with the beam that is scanning across the specimen (Figure 3.22). Thus, when the beam passes over a high

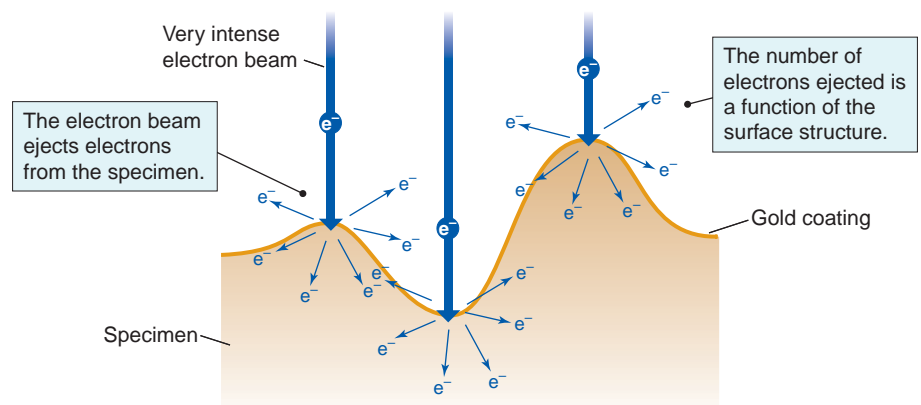


FIGURE 3.21 Secondary electrons in the electron microscope.

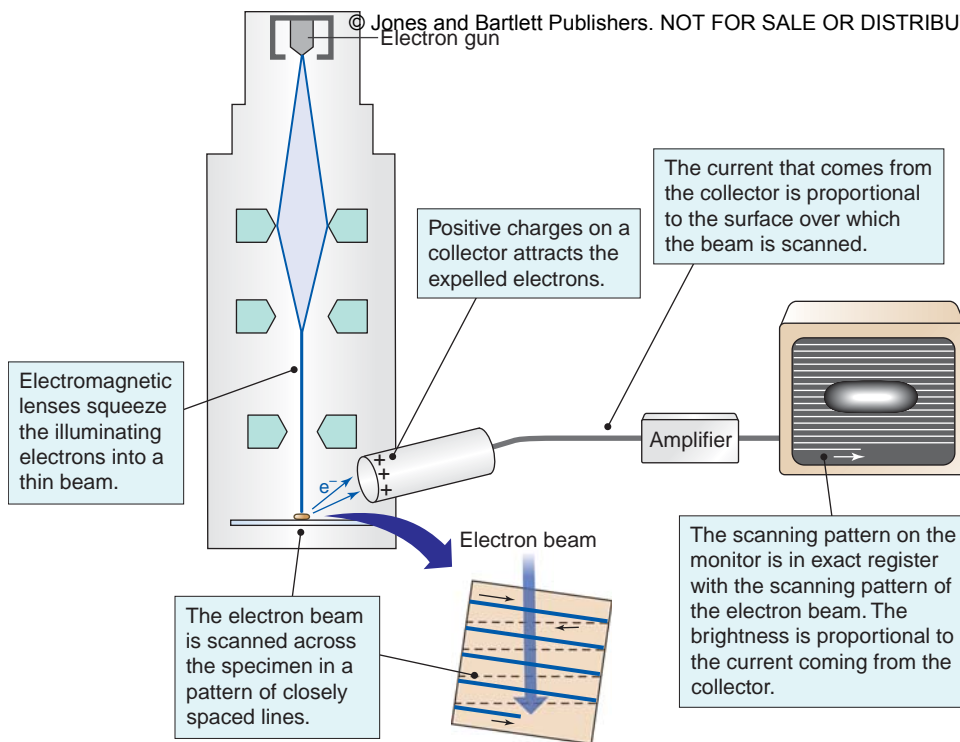


FIGURE 3.22 Image formation in the electron microscope.

spot in the specimen, more electrons than average are ejected, and the beam in the tube makes a bright spot on the screen; when a depression in the specimen is encountered, few electrons are ejected, and the screen shows a dark spot. This shading produces an image on the screen with a pronounced three-dimensional feeling.

Magnification in the scanning electron microscope is a simple matter of the ratio of the size of the pattern that the electron beam scans and the size of the pattern on the screen. For instance, if the screen is 10 cm on a side and the microscope beam is scanning a square that is 100 μm on a side, the magnification is $100,000 \mu\text{m}/100 \mu\text{m}$, or 1,000 \times . Magnification is controlled simply with a dial, which controls magnets that scan the beam back and forth.

Pure Culture Technique

Although individual microbes may be observed in the microscope and their morphology and behavior observed, the study of their biochemistry and genetics requires that populations (called **cultures**) be studied rather than individuals. Obviously, for experimental results to mean anything, all of the cells in the population must be essentially the same; such populations are called **pure cultures**. A set of techniques, mostly developed in the late 19th century by Koch, Pasteur, and their collaborators, allows the routine isolation, cultivation, and study of pure cultures. Probably more than anything else, it is these techniques that define microbiology.

3.16 Pure cultures do not consist of identical cells

Unfortunately, the concept of pure cultures is more complex than it appears. Pure cultures do not consist of identical cells for several reasons. One is that an actively

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cells in different stages of the cell cycle: small “baby” cells that were just “born,” large “mother” cells about to divide, and everything in between. In most cases this does not matter; the activities that a microbiologist measures (i.e., the activity of a particular enzyme) are simply the average of all the different size classes of cells.

Pure cultures are also heterogeneous genetically. The spontaneous mutation rate—that is, the rate at which spontaneous chemical reactions cause one base pair in the DNA to change to another, or one or more base pairs to be deleted from or inserted into the DNA—is very low. There are so many base pairs, however, even in the prokaryotic chromosome that each cell in a pure culture is likely to have at least one base pair difference from all of the others. Of course, the cells in a pure culture are still nearly identical; because an average bacterial chromosome contains several million base pairs, a single difference between two cells means that their DNA is 99.9999% identical. Of course, the more generations the culture goes through, the more variable it becomes as new mutations continue to accumulate.

These complications make it very difficult to define a pure culture. Practically speaking, a pure culture is normally considered to consist of a **clone** of cells—a population all derived from a single ancestral cell not very far in the past. A careful microbiologist will normally work with populations derived from a single cell within about 100 or so generations in the past. How microbiologists do this is explained later.

3.17 Pure culture technique consists of three interrelated techniques

Pure culture technique requires reliable methods for (1) sterilizing growth media and glassware, (2) introducing desired cells into sterile growth media or removing samples from pure cultures without accidentally introducing other contaminating microbes, and (3) isolating single cells, or their progeny, to obtain pure cultures.

Sterility means the absence of living organisms, and it is in principle an all or nothing phenomenon—something is either sterile or it is not; nothing is “90% sterile.” However, sterility can be expressed as a probability—something may have a 90% probability of being sterile after a certain treatment, such as boiling for 15 minutes. This would mean that if you boiled 100 samples for 15 minutes, you would expect about 90 of them to be sterile, and the rest would not be sterile; each individual sample would either be sterile or not.

3.18 The most common method of sterilizing is autoclaving

As Tyndall discovered, even prolonged heating at 100°C is insufficient to kill some bacterial spores. Thus, sterilizing by heat is routinely done at higher temperatures; 121°C is standard. Because this is over the boiling point of water at atmospheric pressure, it is done in a fancy pressure cooker called an **autoclave**. The autoclave maintains a pressure of 15 lb/in² (1.06 kg/cm²), at which pressure the boiling point of water is 121°C. To prevent boiling of liquids, the atmosphere in an autoclave is saturated with water vapor; autoclaving is sometimes termed “steam sterilizing” for this reason.

3.19 Heat-sensitive solutions are sterilized by filtration

Many chemical compounds that microbiologists sometimes need to place in media are heat sensitive and decompose at the temperature of the autoclave. Thus, media

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common is to pass them through a filter in which the pore size is so small that most cells cannot go through. Although smaller pore size filters sterilize more effectively, they are slower, and they clog more rapidly than larger pore size filters. Hence, we usually use the largest pore size filter that experience indicates is usually effective. In most cases, a pore size of 0.4 μm is sufficient (Figure 3.23).

Filtration is less reliable than autoclaving as a means of sterilization, as some bacterial cells are very small and can pass through even very small-pore filters, and of course, most viruses can pass these filters. Fortunately, in most applications, viruses and very small bacteria are rarely encountered. One case in which this can be a serious problem is in the filter sterilization of media for the culturing of animal cells (called **tissue culture**). Tissue culture media often contain animal serum (blood from which the cells have been removed); this cannot be autoclaved because the proteins in serum coagulate when heated. Thus, tissue culture media are routinely filtered to sterilize them. Animals from which the serum is derived (usually cattle), however, may be infected with bacteria of the mycoplasma group, whose cells are very small (as small as 0.2 μm in diameter), and are very plastic because they lack a rigid cell wall. They can therefore pass easily through filters of 0.4 or 0.2 μm pore size, or even smaller. Mycoplasma contamination is known to have ruined many tissue culture experiments; antibiotics are now routinely included in the medium to kill any mycoplasmas that get through the filter.

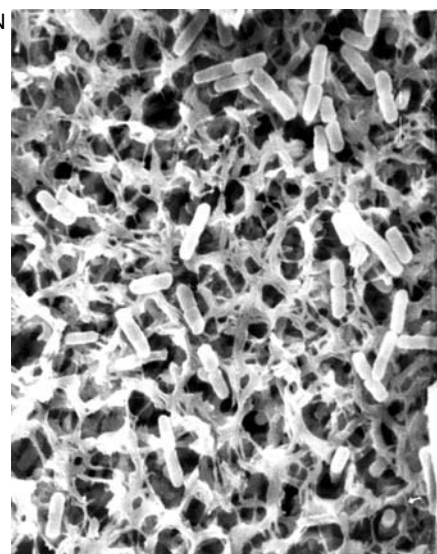


FIGURE 3.23 Bacterial cells on the surface of a filter.

3.20 Glassware is sterilized by dry heat

Reusable glassware, such as flasks and pipets, is usually sterilized in an oven, usually set to about 180°C. This temperature easily kills even the hardiest bacterial spores; however, laboratories increasingly are using plastic, disposable labware to replace reusable glass. This material is produced commercially at low cost, and it saves the effort of washing, drying, and sterilizing glassware. Sterile plastic ware is sterilized at the factory, either by exposure to gamma irradiation or to the toxic gas ethylene oxide.

3.21 Bunsen burner flames help to prevent contamination during transfer into or out of containers

Sterile media in flasks and other containers are kept sterile by stoppering the container so as to prevent any access by microbes to the interior. This can be by making the cap overlap the container by a significant amount so that any microbes would have to travel a convoluted route to enter. The same principle keeps solid media in a Petri plate sterile. Alternatively, the container may be plugged with a fibrous material such as cotton or foam rubber, which will trap particles in the mesh of fibers. In both cases, air can enter the container, but suspended particles, such as microbial cells, get trapped in the meshwork of cotton or rubber fibers.

Of course, the principal opportunity for unwanted microbes in air to gain access to a container is when it is opened to introduce a few cells of a pure culture, to withdraw a sample, or for any other reason. Thus, microbiologists very early developed techniques to allow them to maintain the container free of unwanted airborne organisms while they manipulated it. The principal tool here is a simple Bunsen burner flame. Whenever a flask is opened, its mouth is passed quickly through the flame. Even though the exposure to the flame is momentary (too brief to heat the

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of the flame to kill microbes on the surface, thus preventing them from falling into the container and contaminating it. Similarly, any instrument, such as a pipet, that is going to be used to add or remove material from the flask is also first flamed before being used. The mouths of containers are flamed again before the stopper is reinserted.

This simple technique is quite effective, and routine flaming of glassware as it is used helps keep contamination to a minimum.

3.22 Pure cultures are isolated by streaking on solid media

Solid media are prepared by adding agar, a complex polysaccharide derived from marine algae, to liquid media. The agar dissolves at the high temperature of the autoclave and remains liquid as it cools down to a temperature of about 45°C. Below that it solidifies into a firm, transparent gel, which will not melt again until the temperature is increased to nearly 100°C. It is resistant to hydrolysis by most bacteria.

Solid media are normally poured into *Petri plates*, whose overlapping halves work just like the caps on flasks to prevent airborne contaminants from reaching the agar after it has been poured into the plate and is cooling. After the agar has solidified, however, Petri plates are normally incubated upside down to prevent any condensation that might form on the lid from dripping onto the agar. In this position, although contaminants can reach the interior of the plate, they settle on the lid, rather than on the agar.

To obtain pure cultures, microbes are normally **streaked** onto solid media. A small amount of **inoculum** (material containing microbes to be placed onto solid media or into liquid media) is picked up on a sterile **inoculating loop**, made of wire. The loop is then drawn repeatedly across the surface of the plate, depositing microbes as it moves. The plate is then incubated at the desired temperature, and some time later (typically 24 hours, but sometime more for slowly growing organisms), **colonies** are visible wherever a microbial cell capable of growth on the particular medium was deposited on the agar surface. A colony is a mass of cells derived by the multiplication of a single cell; normally colonies contain about 10^7 to 10^8 cells (Figure 3.24).

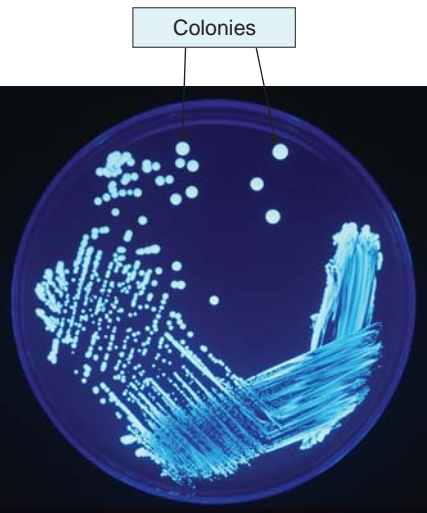


FIGURE 3.24 A streaked plate.

3.23 Different media are necessary for different microbes

All cells have to find in their medium all nutrients required for growth, or they cannot multiply. Different cells, however, have widely varying requirements, and thus, there is no single medium that satisfies all; every medium is to some extent selective.

Of course, some media will support the growth of more types of microbes than others. What we call **rich media**, or **complex media**, typically have a mixture of many different organic compounds, including all of the amino acids, purines, pyrimidines, vitamins (enzyme cofactors), etc. They are usually made by hydrolyzing natural products, such as meat, with enzymes to break down macromolecules and release the monomers (peptone and tryptone are examples), or they are made by extracting the small molecules from other cells (yeast extract is an example). Rich media will usually support growth of many different types, including ones that require **growth factors** (specific compounds required for growth, such as vitamins, amino acids, or other compounds). There are microbes that do not grow in rich media, however; they have more simple nutritional needs and find one or more components of rich media to be toxic.

Other media, called **minimal media** (sometimes **minimal media**), contain mineral salts of major bioelements, such as sulfur, nitrogen, and phosphorous. This is

supplemented, when necessary, with carbon and energy source. If we are culturing a photosynthetic microbe, we would not need such a supplement. Minimal media are much more restrictive, as no organism with a growth factor requirement can grow unless that factor is added separately, and only cells that have the enzymes to metabolize the particular carbon and energy source can grow.

3.24 Cultures are incubated under different conditions according to their relations with oxygen

Microbes fall into three major categories based on their relations to oxygen. Some are **obligate aerobes** and require oxygen to grow. These organisms use aerobic respiration as their only mode of metabolism and, hence, cannot grow when their terminal electron acceptor (oxygen) is absent. Others are **obligate anaerobes**, for which oxygen is toxic. These organisms are restricted to habitats where oxygen is absent and to modes of metabolism that do not require it (anaerobic respiration, fermentation, certain types of photosynthesis). Finally, some organisms are **facultative anaerobes** (the term facultative aerobes means the same thing, but is rarely used). These organisms can grow in the presence or absence of oxygen. Most facultative anaerobes switch between aerobic respiration and fermentation, according to the availability of oxygen.

Obviously, different methods of cultivation are needed for anaerobes and aerobes. Aerobes are often simply cultivated under a normal atmosphere of air, with no special measures taken to insure adequate supplies of oxygen (which is about 20% of air). In liquid cultures, however, microbes can multiply to huge numbers (in excess of 10^9 per ml), and they can use dissolved oxygen faster than it can diffuse into the medium unless special measures are taken. Most frequently, liquid cultures are shaken vigorously to insure that the medium is continuously exposed to the atmosphere.

On a Petri plate, aerobes can also use oxygen so rapidly that the interior of a colony becomes anaerobic. Thus, for obligate aerobes, colonies consist of an outer layer of growing cells and an inner core of nongrowing cells that are oxygen starved (Figure 3.25).

It is much more challenging to culture microbes under anaerobic conditions. This requires air to be excluded, which is a difficult task, especially for very sensitive anaerobes, which can be killed by even momentary exposure to oxygen. The most straightforward way of cultivating these organisms is to use an anaerobic glove box—a sealed box in which the atmosphere is composed so that there is no oxygen (i.e., 95% N_2 , 5% CO_2). In such an anaerobic chamber, materials are introduced and removed through an airlock so that the interior is never exposed to air, and materials are manipulated by gloves that are hermetically sealed to the chamber (Figure 3.26).

For less fastidious anaerobes, which can tolerate exposure to air but which cannot grow in its presence, and for anaerobic culturing of facultative anaerobes, simpler techniques suffice. Petri plates can be streaked, liquid cultures inoculated, and other manipulations performed under air, and then the cultures placed under anaerobic conditions. For Petri plates, this usually means that the plates, after being streaked, are placed in an **anaerobic jar** in which a commercially available “gas pack” has been placed just before sealing the jar (Figure 3.27). When water is added to the gas pack, it evolves H_2 gas, which then combines with the O_2 in the air on a platinum catalyst built into the lid of the jar. This chemically removes most of the oxygen from the air and allows many anaerobes to grow well. Liquid cultures of these aerotolerant anaerobes can be grown in sealed tubes or flasks that are filled to the brim to exclude air.



FIGURE 3.25 Colony of *Rhodobacter*; the red center indicates anaerobic conditions.



FIGURE 3.26 Anaerobic chamber.



FIGURE 3.27 Anaerobic jar.

3.25 Some pathogenic microbes require special containment facilities

Pure culture techniques were designed to prevent airborne microbes from contaminating microbial cultures; that is, they were designed to keep microbes out—not in. When the microbes being cultured are **pathogens** (microbes that cause disease), however, additional techniques may be necessary to prevent them from infecting the investigator or others in the vicinity. Of course, such accidental infections are an unavoidable hazard of working with pathogens. Accidents happen and cannot be completely prevented; dropped flasks, a slip with a hypodermic needle, or any of dozens of other accidents can infect laboratory workers. Such accidents can be minimized (e.g., unbreakable plastic flasks), but not completely prevented. Accidental infection is thus a fact of life for medical microbiologists, and dozens have died of laboratory infections during the 120-year history of microbiology.

In addition to accident, laboratory infections can occur for some microbes by the aerosol route. Certain laboratory manipulations create **aerosols**—suspensions of fine droplets or particles in air—and these can cause infection when breathed into the lungs. Not all pathogens can cause infection by the aerosol route, but for those that can, normal laboratory manipulations can cause dangerous and invisible aerosols. For these organisms, special containment techniques or equipment is used to reduce the chance of aerosol infection.

In normal practice, any aerosols produced by laboratory manipulation are very low concentration; that is, the number of particles or droplets released is low. Thus, the hazard is only to laboratory workers in the immediate vicinity; normally, there is no danger to anyone outside the laboratory. Thus, special containment techniques are normally needed only to protect laboratory workers rather than the general public, which is not at risk. If high concentration aerosols are deliberately created (e.g., to study the process of airborne infection), however, then those outside may possibly be at risk.

Generally, four categories of hazard are recognized, usually referred to as **biosafety levels (BSL)** (Table 3.1). Biosafety level 1 (BSL 1) is for nonpathogenic microbes.

Table 3.1 Summary of Recommended Biosafety Levels for Infectious Agents

BSL	Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	Not known to consistently cause disease in healthy adults	Standard microbiological practices	None required	Open bench top sink required
2	Associated with human disease, hazard = percutaneous injury, ingestion, mucous membrane exposure	BSL-1 practice plus: Limited access Biohazard warning signs “Sharps” precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies	Primary barriers = class I or class II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials; PPEs: laboratory coats; gloves; face protection as needed	BSL-1 plus: Autoclave available
3	Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences	BSL-2 practice plus: Controlled access Decontamination of all waste Decontamination of lab clothing before laundering Baseline serum	Primary barriers = class I or class II BSCs or other physical containment devices used for all open manipulations of agents; PPEs: protective lab clothing; gloves; respiratory protection as needed	BSL-2 plus: Physical separation from access corridors Self-closing, double-door access Exhausted air not recirculated Negative airflow into laboratory
4	Dangerous/exotic agents which pose high risk of life-threatening disease, aerosol-transmitted lab infections; or related agents with unknown risk of transmission	BSL-3 practices plus: Clothing change before entering Shower on exit All material decontaminated on exit from facility	Primary barriers = All procedures conducted in class III BSCs or class I or class II BSCs in combination with full-body, air-supplied, positive-pressure personnel suit	BSL-3 plus: Separate building or isolated zone Dedicated supply and exhaust, vacuum and decon systems

“Sharps precautions” refers to procedures designed to reduce accidents with hypodermic needles or broken glass. BSC stands for biological safety cabinet. PPE stands for personal protective equipment (such as lab coats and gloves). “Baseline serum” refers to the taking of a blood sample from each worker before they enter the facility for the first time to be used as a reference in case of suspected laboratory infection. “Negative airflow into laboratory” means that the laboratory is maintained at a lower pressure than the outside air so that any air leakage will be into the facility, not out of it.

Source: Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. Washington, DC: U.S. Government Printing Office, 1999.

BSL 2 is for those pathogens that do not readily transmit by the aerosol route. BSL 3 is for pathogens that transfer readily by the aerosol route and that pose a serious health threat. The highest category, BSL 4, is for pathogens that transmit readily by the aerosol route and that cause fatal diseases for which there is no cure.

One of the primary pieces of protective equipment in most BSL 2, BSL 3, and BSL 4 laboratories is the **biological safety cabinet (BSC)**, a chamber that is constructed so as to minimize the chance of any aerosol particles escaping into the laboratory air.

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Researcher. The cabinet is closed, and workers access the interior through gloves sealed to the front panel. Air is exhausted through very fine filters termed **HEPA filters** (for high-efficiency particulate air filters). Class I and II BSCs have an opening in the front through which workers insert their hands. Fans create air currents that enter the BSC through this opening, preventing particles inside the cabinet from leaving this way. Exhaust air is filtered through HEPA filters (Figure 3.28).

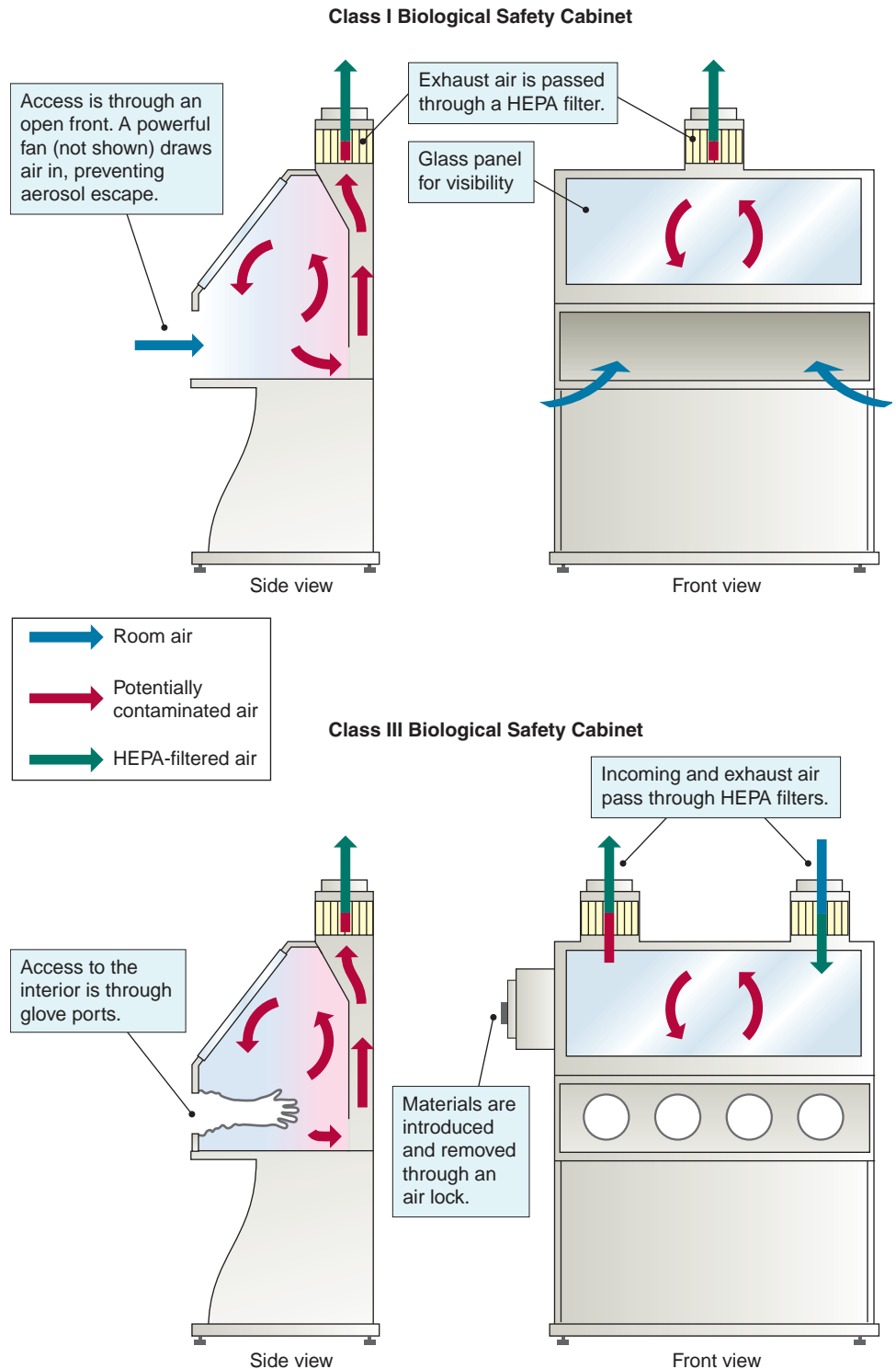


FIGURE 3.28 Diagram of Class I and III biological safety cabinets.

Even with these features, reduced rates compared with the period before the introduction of formal biosafety systems. For instance, in 2003, the virus that causes the serious disease SARS (see section 20.8) infected laboratory workers in high-containment laboratories in three different countries (fortunately, none of the three infected workers started epidemics of SARS, which they easily could have done).

Summary

Microbiology as a field is characterized by the special techniques that are required to study organisms too small to be seen—microscopy to visualize them and pure culture techniques to allow large populations of essentially identical organisms to be prepared free of other microbes. The basic elements of both of these technologies were established in the late 19th century. In the 20th century, the principal innovations were the development of electron microscopy and the application of biochemistry and genetic techniques (developed for the study of plants and animals) to the study of microbes. Now, at the start of the 21st century, genomics and associated techniques are again enriching microbiology. Microbiology continues to evolve in intellectually exciting and practically important ways; nevertheless, it remains ultimately dependent on our ability to see microbes and to obtain pure cultures of specific ones.

Study questions

1. Compare and contrast image formation in the light and electron microscopes.
2. Describe the contemporary uses of brightfield, phase-contrast, and dark-field light microscopy.
3. Calculate the limit of resolution in micrometers of a light microscope using green light (wavelength about 400 nm) and an objective lens with a numerical aperture of 1.2.
4. What would be the maximum useful magnification of such a microscope, assuming that your eye has a limit of resolution of 0.2 mm? (Maximum useful magnification is the magnification at which the maximum amount of information is obtained; further magnification enlarges the image but yields no more information.)
5. If you wished to design a phase-contrast microscope that made the specimen brighter than the background, rather than darker, what modifications would you make?
6. Summarize the principal techniques of specimen preparation for the electron microscope and the principal uses of each.
7. Discuss the concept of a pure culture.
8. Why do microbiologists commonly use cultures rather than individual cells?
9. What are the principal elements of pure-culture technique?
10. How are media and materials sterilized?
11. Discuss the chemical composition and uses of different kinds of media.
12. Summarize the principal features of the biosafety level system—the kinds of organisms worked with at each level and the kinds of containment measures applied.