Chapter 9

Microscopic Techniques

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9.1 HISTORY OF MICROSCOPY

Despite the increasing use of advanced genomic and proteomic techniques, microscopy still plays an integral role in the study of microorganisms. Microscopy had its origins in the seventeenth century when the Dutch discovered the ability to magnify objects by combining convex and concave glass lenses. However, who actually invented the first Dutch microscope is not clear. In 1611, Johannes Kepler, a German mathematician and astronomer, found that magnification could be achieved with the use of a convex ocular with a convex objective lens and created the Kepler ocular. Giovanni Faber coined the word "microscope" in 1625 in reference to the ability to see small things. The first use of the microscope to see microorganisms was by the Dutch merchant Antonie van Leeuwenhoek (1632–1723). At the age of 40, van Leeuwenhoek began experimenting with glass lenses, and he eventually made over 400 different microscopes. With these microscopes, van Leeuwenhoek was the first to see "animalcules," which today are known as microorganisms.

The Dutch physicist and astronomer Christiaan Huygens made a crucial discovery in the seventeenth century. The Huygens eyepiece consists of two convex lenses, each with the convex side facing the objective. The lower lens provides a brighter, smaller image from the objective lens, and the upper Huygens lens then focuses the image. The Huygens design is still used today in eyepieces with magnifications of 10× or less.

As the quality of lenses and understanding of resolution and magnification have improved, different types of microscopies have been developed. The first light microscope in the 1600s was followed by the development of the first electron microscope in the 1920s, and the confocal laser scanning microscope and atomic force microscope followed in the 1980s. In this chapter, we describe the basic techniques used in microscopy, introduce advances and show how microscopy is still fundamental to the field of environmental microbiology.

9.2 THEORY OF MICROSCOPY

Regardless of the microscope, microscopy relies heavily on user interpretation. While this makes it a highly subjective tool, microscopy can provide extremely useful information about microorganisms. The human eye alone can resolve about 150 μm between two points. The objective of the microscope is to increase the resolution of the human eye. Resolution is the smallest distance between

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two points visible to the eye, aided or unaided by a microscope. Similarly, resolving power, a function of the wavelength of light and the aperture of the objective lens used in viewing a specimen (see Information Box 9.1), is the ability to distinguish two points as separate. The resolving power of most light microscopes is 0.2 μm, a 750-fold improvement over what we can actually see.

An aberration in a microscope refers to the inability to image a point in an object as a point. The light microscope has five kinds of aberrations: spherical, coma, astigmatism, curvature of field and distortion. Aberrations are functions of the lenses in an optical system, and severe aberrations result in decreased resolution. However, in the light microscope, corrective lenses eliminate aberrations so that the theoretical resolving power can be achieved. For example, spherical aberration is the most common aberration. Spherical aberration results when light rays pass through a lens at different points on the lens, resulting in light rays of different focal lengths (Figure 9.1A). Recall that the wavelength of light determines resolution in the light microscope. Thus, light of varying focal planes or wavelengths results in poor resolution of two points in an object. The use of a light diaphragm corrects spherical aberrations by focusing light rays to a single focal plane (Figure 9.1B). The ability to stack on correcting lenses in the light microscope has eliminated aberrations, allowing the theoretical resolution to be achieved. The electromagnetic lenses in electron microscopes have the same aberrations as the glass lenses of light microscopes. However, there are no glass lenses in the electron microscope and so these aberrations are not as easy to correct. Consequently, although the theoretical resolution of the electron microscope is 0.0002 nm, the actual working resolution is only 0.2 nm.

Perhaps the most important aspect of microscopy is illumination of the sample. Without illumination, the specimen cannot be visualized. In light microscopy, transmitted light or reflected light may be used. The source of illumination can be white light or ultraviolet light. Realize, however, that all microscopic techniques rely on the manipulation of light (or electrons in electron microscopy) to influence the resolution of a specimen. In Köhler illumination, for example, a series of condenser lenses and diaphragms are used to focus light rays onto the specimen, increasing not only illumination but also resolution (Figure 9.2).

Magnification is the ability to enlarge the apparent size of an image, and useful magnification is a function of the resolving power of the microscope and the eye, which can be stated as:

$$\frac{\text{Limit of resolution by eye}}{\text{Limit of resolution of microscope}} = \frac{150\ \mu m}{0.2\ \mu m} = 750\times$$

Note that the ability to magnify an image is almost infinite, but most of this magnification is blurred because

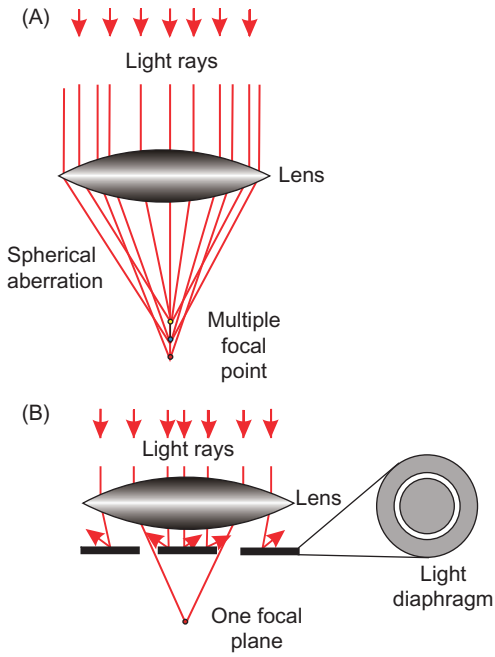

FIGURE 9.1 (A) Spherical aberrations are inherent in any lens, resulting in multiple focal planes of light. (B) The addition of a light diaphragm eliminates spherical aberrations in light microscopes by focusing the light onto one focal plane.

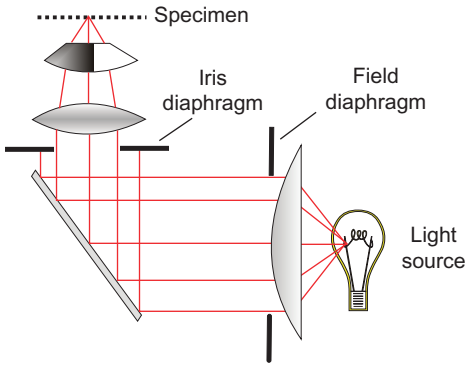

FIGURE 9.2 Schematic of Köhler illumination. Multiple diaphragms inserted between the light source and the condenser focus light directly on the specimen. Adapted from Zieler (1972), McCrone Research Institute, Microscope Publications Division.

Information Box 9.1 Theoretical Resolving Power

The resolving power (RP) is the minimum distance that an optical system can distinguish:

$$RP = \frac{\text{wavelength of light}}{2 \times \text{numerical aperture of objective lens}}$$

For example, for the light microscope

$$RP = \frac{500\ nm}{2 \times 1.25} = 200\ nm = 0.2\ \mu m$$

resolution is limited by the wavelength of the light, and so it becomes empty magnification.

Contrast refers to the ability to distinguish an object from the surrounding medium, and without it, both resolution and magnification become unimportant. More specifically, two points in an object that are resolved separately are not seen separately unless their images are contrasted against their surrounding medium. Because of their small size, bacteria, for example, provide little retardation of the light passing through the cell. The result is low contrast, and the color of the bacterial cell will be similar to its surrounding medium making visualization of the cell difficult. Many of the advances in microscopy have been for the sole purpose of increasing contrast, e.g., phase-contrast microscopy. Dyes and stains, such as methylene blue and safranin, are also used to increase contrast with all types of microscopes.

9.3 VISIBLE LIGHT MICROSCOPY

9.3.1 Types of Light Microscopy

Optical microscopes, also known as light microscopes, have multiple lenses, including ocular, objective and condenser lenses (Figure 9.3). By varying these lenses and light sources, five types of light microscopy can be defined: bright-field, dark-field, phase-contrast, differential interference and fluorescence. Characteristics of each of these types of microscopy are given in Table 9.1.

9.3.1.1 Bright-Field Microscopy

In bright-field microscopy, images are the result of light being transmitted through a specimen. The specimen absorbs some of the light, and the rest of the light is transmitted up through the ocular lens. The specimen will

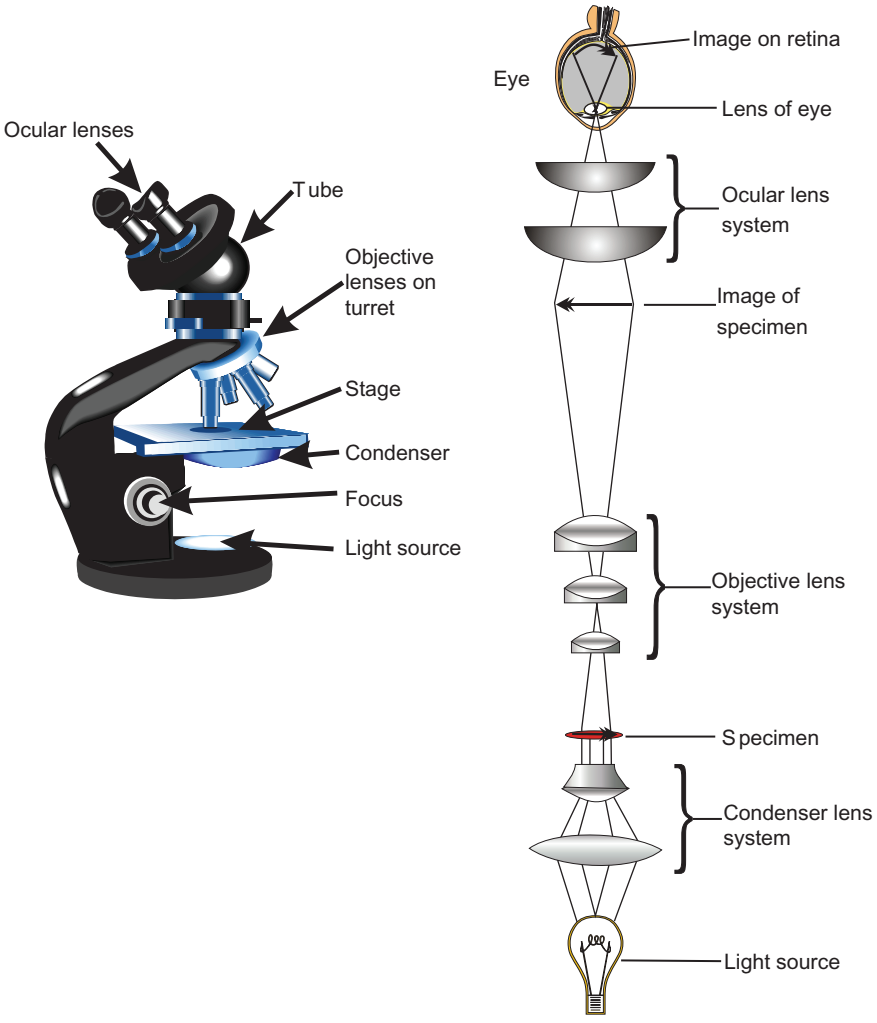

FIGURE 9.3 A typical compound light microscope and its optics.

TABLE 9.1 Comparison of Types of Microscopy

Microscope	Maximum Practical Magnification	Resolution	Important Features
Visible Light as Source of Illumination			
Bright-field	2000 ×	0.2 μm (200 nm)	Common multipurpose microscope for live and preserved stained specimens; specimen is dark, field is white; provides fair cellular detail
Dark-field	2000 ×	0.2 μm	Best for observing live, unstained specimens; specimen is bright, field is black; provides outline of specimen with reduced internal cellular detail
Phase-contrast	2000 ×	0.2 μm	Used for live specimens; specimen is contrasted against gray background; excellent for internal cellular detail
Differential interference	2000 ×	0.2 μm	Provides brightly colored, highly contrasting, three-dimensional images of live specimens
Ultraviolent Rays as Source of Illumination			
Fluorescence	2000 ×	0.2 μm	Specimens stained with fluorescent dyes or combined with fluorescent antibodies emit visible light; specificity makes this microscope an excellent diagnostic tool
Electron Beam Forms Image of Specimen			
Transmission electron microscope (TEM)	1,000,000 ×	0.5 nm	Sections of specimen are viewed under very high magnification; finest detailed internal structure of cells and viruses is shown; used only on preserved material
Scanning electron microscope (SEM)	100,000 ×	10 nm	Whole specimens are viewed under high magnification; external structures and cellular arrangement are shown; generally used on preserved material
Surface Forces Forms Image of Specimen			
Atomic force microscope (AFM)	1,000,000 ×	0.5 nm	Can examine live or preserved specimens. Provides surface detail at very high resolution

appear darker than the surrounding brightly illuminated field. Bright-field microscopy is most commonly used to examine morphology; however, due to the small size of microorganisms, in particular the bacteria, bright-field microscopy often requires staining to increase contrast in order to achieve the desired magnification. Other types of light microscopy (described below) use manipulation of light to increase contrast.

Many different types of stains are available, but in general they can be classified as basic dyes, which have a positive charge, or acidic dyes, with a negative charge. Cell components that are negatively charged such as nucleic acids attract basic dyes. In contrast, those that are positively charged, for example some cell-associated proteins, attract acidic dyes. Stains with positive charge attach more readily to the specimen, giving it color, while the background remains unstained. The most important types of positive stains are the simple stains, which involve a single dye such as methylene blue or Rose Bengal. These dyes stain the entire cell so that it takes on the color of the stain used (e.g., pink for Rose Bengal) against an unstained background. Simple stains are useful for size and morphological assessments, as well as for cell enumeration. Negative stains such as the acidic dye, and India ink, are less common. They are repelled by the negatively charged surface of the cell and so stain the background, which results in the highlighting of the specimen as a silhouette

Differential stains utilize two different dyes designated as the primary dye and the counterstain. The Gram stain, developed by Hans Christian Gram, is the most important differential stain. The Gram stain utilizes crystal violet and safranin dyes to classify bacteria into one of two major categories. Gram-positive bacteria stain purple, whereas Gram-negative bacteria stain red. In both cases, the differential staining is due to differences in cell wall components (see Section 2.2.1). For many environmental isolates, the Gram stain may be inconclusive, and such isolates are designated as Gram variable. In this case, both red and purple cells may be seen, as in the case of *Arthrobacter* spp., which are common soil organisms.

Finally, there are a number of special stains. These special stains are used to identify specific cell components, such as bacterial capsules and spores. One such stain is the acid-fast stain, which was developed to identify difficult-to-stain bacteria. These organisms do not

stain with commonly used dyes, such as those used in the Gram stain. Acid-fast bacteria are those that when stained with carbolfuchsin cannot be destained, even with acid. This property is typical of *Mycobacterium* spp., which have mycolic acids on their cell surface. Mycobacteria are of particular interest because they are causative agents of several serious human diseases, including tuberculosis and leprosy. They are also common soil isolates that are slow growing, with many of them having the ability to degrade organic contaminants.

9.3.1.2 Dark-Field Microscopy

Dark-field microscopy can be used to increase the contrast of a transparent specimen. By inserting a central stop before the condenser, some but not all of the light from the condenser is prevented from reaching the objective (Figure 9.4). Only light that is scattered from the edges of the specimen is viewed. Thus, the specimen appears as a bright image against a dark background. Dark-field microscopy is often used to visualize live specimens that have not been fixed or stained. For example, dark-field microscopy has been used to quantify the motility of bacteria and protozoa and to monitor the growth of bacterial microcolonies (Korber *et al.*, 1990). Although gross morphology can be delineated, internal details are not revealed. Murray and Robinow (1994) and Hoppert (2003) describe the nature of dark-field microscopy and its applications.

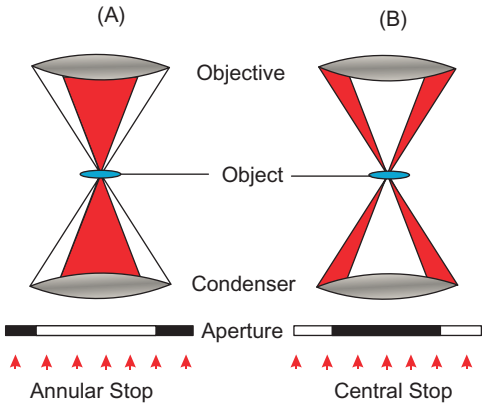

FIGURE 9.4 Differing from bright-field microscopy which uses an annular stop (A), phase-contrast microscopy uses a central stop (B), allowing some but not all of the light from the condenser to reach the objective. Adapted from Rochow and Tucker (1994).

9.3.1.3 Phase Contrast Microscopy

Phase-contrast microscopy enhances specimen contrast which aids viewing of high-contrast images of transparent specimens, such as living cells. This technique takes advantage of the fact that although many internal cell components are transparent, they have different densities. Different densities interact differently with light, thereby creating contrast between internal cellular components and the surrounding medium (Figure 9.5). Phase-contrast microscopy uses a series of diaphragms for separating and recombining direct versus diffracted light rays (Figure 9.6). Köhler illumination is used to focus the light source on one focal plane. Light rays through the Köhler diaphragm are focused as a hollow cone onto the specimen. In the back focal plane of the objective, there is an annular diaphragm or a diffraction plate. The phase of light rays entering the diffraction plate, also called a phase plate, is altered. The degree of retardation of light through the plate results in either lightening or darkening of the specimen.

9.3.1.4 Differential Interference Contrast Microscopy

Differential interference contrast (DIC) microscopy provides brightly colored, highly contrasting three-dimensional images of live specimens. In DIC microscopy, the illuminating beam is split into two separate beams. One beam passes through the specimen, creating a phase difference between the sample beam and the second or reference beam. The two beams are then combined so that they interfere with each other. DIC can allow the detection of small changes in depth or elevation in the sample, thus giving the perception of a three-dimensional image (Figure 9.7).

9.3.1.5 Polarization

Anisotropic light, light that depends on the angle of observation, originates from specimens that have asymmetry in their crystal lattice properties. Anisotropy is observable in liquid and solid crystals; stained glasses; stressed plastic materials; crystallized resins and

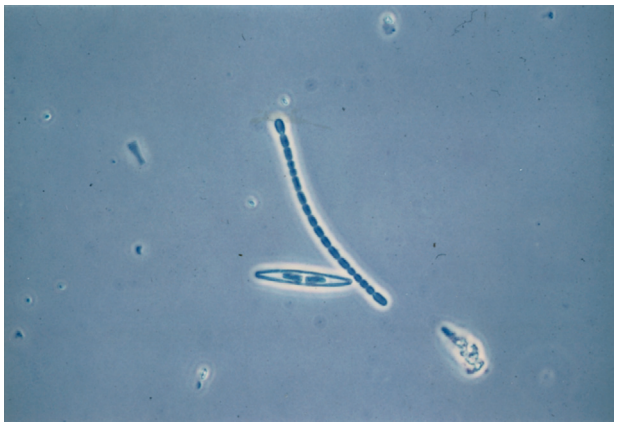

FIGURE 9.5 Phase-contrast image of a free-living nitrogen-fixing cyanobacterium (40 μm length) and the algal cell known as a diatom (12 μm length). Photo courtesy P. Rusin.

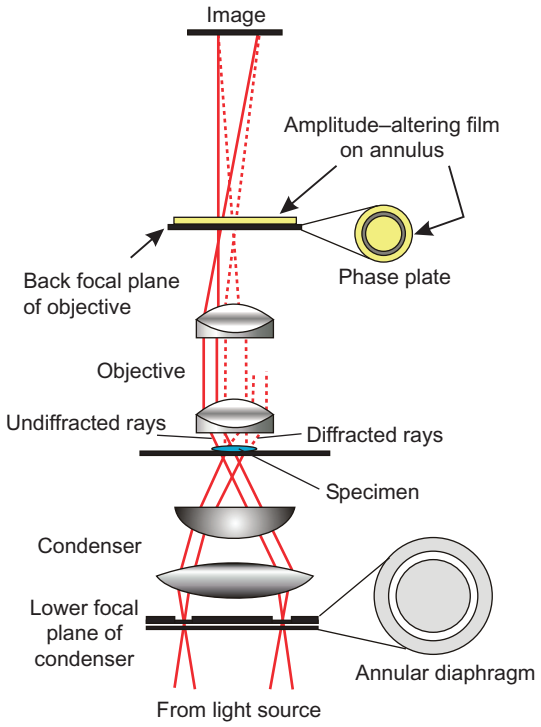

FIGURE 9.6 A phase-contrast microscope equipped with an amplitude-altering film on the phase plate to increase specimen contrast. *Handbook of Chemical Microscopy,* Vol. 1, 4th ed. C.W. Mason. Copyright © 1983. Adapted by permission of John Wiley & Sons, Inc.

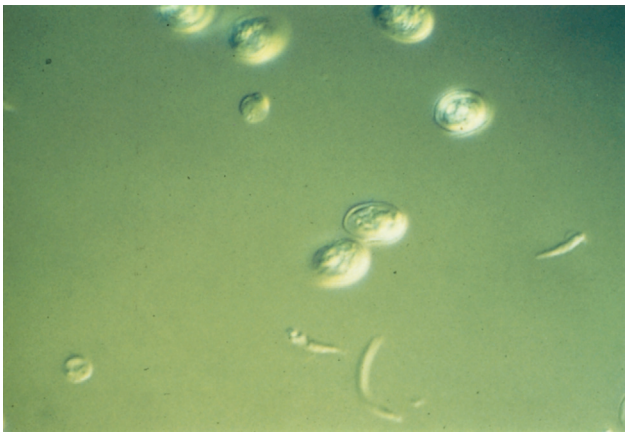

FIGURE 9.7 A differential interference contrast (DIC) image of *Cryptosporidium* with associated sporozoites. Photo courtesy P. Rusin.

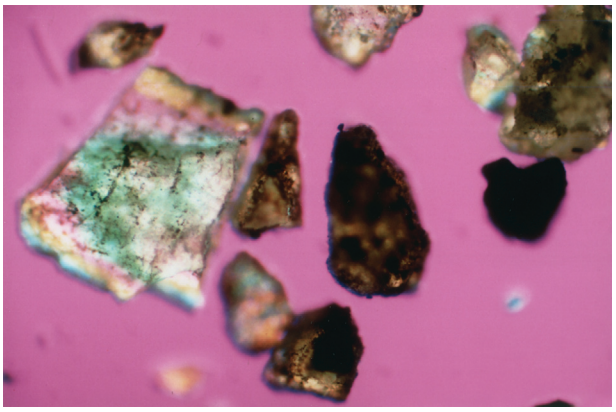

FIGURE 9.8 Polarization microscopy used to view sand grains in a sandy loam soil. The various colors are the result of light interference, which can be used to identify individual minerals. Magnification 400 ×. Photo courtesy T.M. Roane.

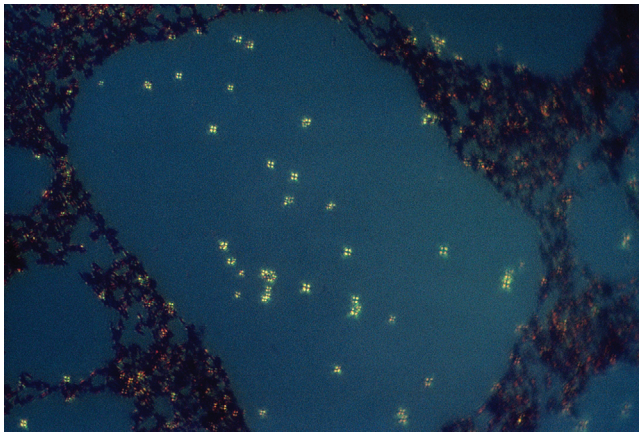

FIGURE 9.9 Polarization microscopy used to view bacteria producing extracellular polymeric substances. EPS producing cells show a cross-hatched illumination due to the resulting diffraction pattern as polarized light passes through the cell-surrounding EPS layer. Magnification 1000 ×. Photo courtesy T.M. Roane.

polymers; refracting surfaces; synthetic filaments; and biological fibers, cells and tissues. Polarized light is light in one plane that can be used to examine anisotropy in sample materials. Polarization microscopy is traditionally used to determine the optical properties of soil minerals to aid in their identification (Figure 9.8). The optical anisotropy of individual crystals reflects the bonding patterns of units, e.g., molecules or elements, and usually involves differences in at least two crystallographic directions (at least two directions of polarized light). Multiple anisotropic crystals have optical characteristics above and beyond those of individual crystals. Anisotropy observed in a sample can provide more information about the sample than ordinary unpolarized light.

For example, a result of light polarization is molecular birefringence. Molecular birefringence is manifested by long or flat molecules, especially polymeric macromolecules, and is particularly applicable in the examination of microbially produced extracellular polymers (Figure 9.9). In molecular birefringence, when polarized light encounters a series of atomic dipoles arranged in chains, as in long molecules, the strength of the dipoles causes the light to vibrate lengthwise along the chain, resulting in greater polar anisotropy at the poles. However, side

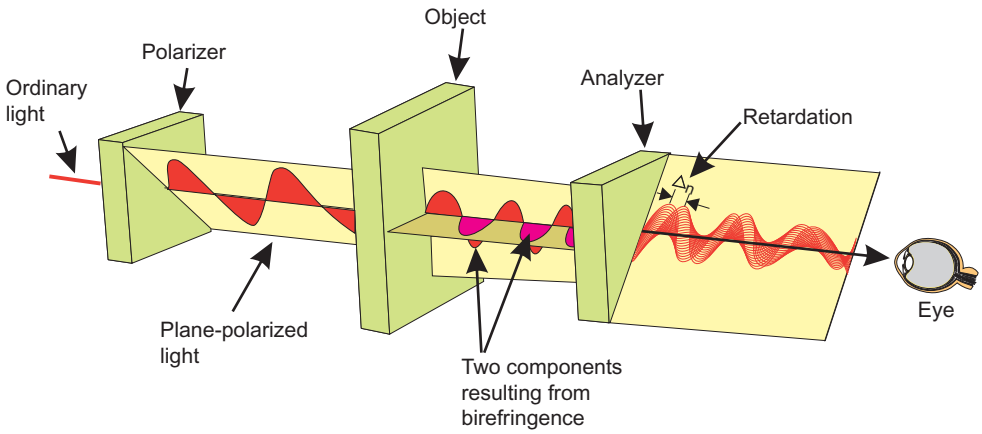

FIGURE 9.10 Schematic showing the function of the polarizer and the analyzer in polarization microscopy. Adapted from Mason (1983).

chains on the molecules tend to reduce the strength of birefringence in the main chain of the molecule, resulting in less polar anisotropy. The patterns and strengths of anisotropy evident in a sample can give indications of the purity and elemental structure of the sample.

The resolution of a specimen's effects on polarized light depends on producing plane polarized light with a polarizer and examining the effects with an analyzer. Two polarizers are used in polarization microscopy: a polarizer and an analyzer (Figure 9.10). For transmitted light, the polarizer is placed between the light source and the substage condenser lens. The analyzer is placed between the objective and ocular lenses. When polarized light from the first polarizer vibrates in a direction that allows it to pass through the analyzer, the field of view in the microscope will be black as the polarizers are crossed with respect to their directions of vibration of light. Thus, in a polarizing microscope, contrast is the result of various interference phenomena throughout the sample. Light interference or retardation at each point in a crystal results in contrast and color on a dark background. In accordance with the Michel-Levy interference spectra based on light retardation through varying sample thicknesses, light interference gives first order gray, high order white and color to the sample image.

9.3.2 Sample Preparation

9.3.2.1 Preparation from Liquid Samples

Sample preparation for microscopy can be as simple as placing a drop on a glass slide, or as complex as the thin sectioning and mounting on a copper grid as with transmission electron microscopy. In general, sample preparation varies with the type of microscopy used and the goal of the microscopic analysis.

Viable microorganisms are generally viewed via wet mounts. Here, cells are suspended in water, saline or some other liquid medium. The liquid maintains viability and allows locomotion. Wet mounts can be done on a simple glass slide with a coverslip or on specially constructed slides. For example, in the latter case, a drop containing the specimen can be placed on a glass coverslip and then a slide with a concave depression is placed on top of the coverslip. Upon inversion of both coverslip and slide, the drop hangs from the coverslip. The drop is not affected by the glass slide, due to the concave depression, thereby creating a hanging drop. Hanging drop slides are useful in monitoring bacterial motility. Wet mounts are often viewed with phase-contrast or DIC microscopes to maximize specimen contrast.

Although morphology can be determined in a wet mount, this is often difficult because of the lack of contrast and detail between the specimen and the surrounding medium, and also because the microorganisms are moving. Thus, morphology and internal structure are better examined by fixing and staining specimens on glass slides, a process which kills the cells. Fixation of cells involves spreading a thin film of a liquid suspension of cells onto a slide and air drying it, producing a smear. The smear is then fixed on the slide by gently heating it over a flame for a few seconds. The smear is normally stained by the addition of a dye that enables cellular detail to be seen, as in bright-field and fluorescence microscopy.

9.3.2.2 Preparation from Soil Samples

Microorganisms in soil samples can be examined microscopically much like microorganisms from liquid samples, following microbial extraction from the soil. Microorganisms in soil can be ionically bound to soil and soil-associated particulates. To help release bound microorganisms, a combination of physical disruption, e.g., via mechanical mixing, and chemical neutralization can be used. Chemical neutralizers, such as sodium pyrophosphate, help homogenize ionic charges, causing repulsion between soil surfaces and microorganisms. Soil

particulates can be removed from the resulting suspension through filtration or centrifugation. The microbial suspension can then be processed similarly to a liquid sample.

Historically, direct examination of microorganisms *in situ*, or within their environment, has been an important tool for microbiologists. Both light and electron microscopies allow direct examination of the form and arrangement of microorganisms in their environments. However, quantitation of actual microbial numbers has been difficult because interfering colloids and soil particles potentially mask large numbers of organisms. An example of direct examination is the buried slide technique.

Rossi *et al*. (1936) first introduced the buried slide. In this technique, a glass microscope slide is embedded in a soil or sediment sample. After a period of incubation, the slide is carefully removed with minimal disturbance, and soil particles with attached microbes can be viewed directly under the light microscope (Figure 4.22 shows an example of a buried slide). Although this method is more than 60 years old, it is still useful in illustrating the abundance of microorganisms in soil and their relationship to each other and to soil particles. Details of this technique can be found in Pepper and Gerba (2004). A variation of the buried slide technique is the pedoscope technique. Here, optically flat capillary tubes (the tubes are square so that all light passing through the tube has the same distance to travel) are buried in soil. Because soil microorganisms grow in pores or within soil aggregates, the relationships seen on the surface of a typical flat glass slide may not be truly representative of the natural state. The pedoscope capillary tubes overcome this by resembling soil pore spaces.

9.4 FLUORESCENCE MICROSCOPY

Fluorescence microscopy is technically a type of light microscopy but it differs in that it utilizes ultraviolet (UV) light sources. This type of microscopy is used in combination with fluorescent dyes, such as acridine orange or fluorescein, which are used to directly stain samples and perform direct counts. More powerfully, fluorescence microscopy can be used to detect specific probes which have been hybridized with the sample to detect the presence of a target molecule such as an antibody (immunolabeling) or a nucleic acid sequence (fluorescence *in situ* hybridization).

9.4.1 Direct Counts

Microbiologists are often interested in determining numbers of microorganisms associated with a given environment or process. There are two main methods for determining microbial numbers. The first involves culture-based assays as discussed in Chapter 10. The second method, known as the direct count, involves direct microscopic observations. Direct count procedures usually provide numbers that are one to two orders of magnitude higher than culturable counts because direct counts include viable, dead and viable but nonculturable (VBNC) organisms (see Section 3.3).

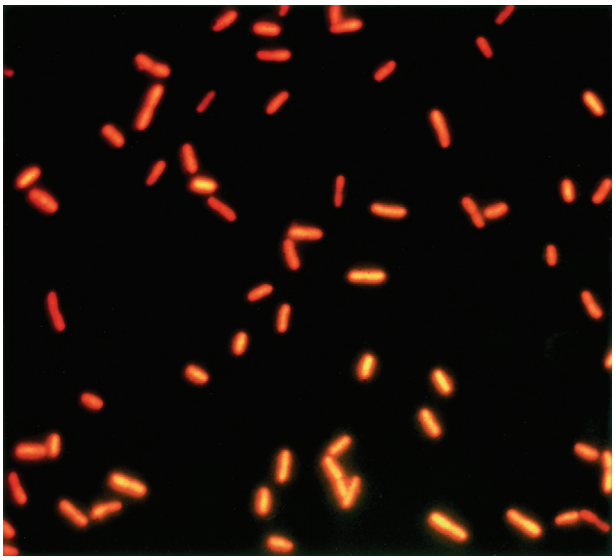

FIGURE 9.11 Acridine orange direct count. Photo courtesy K.L. Josephson.

For direct counts, fluorescent stains are used rather than simple stains. A widely used stain for direct microscopy of bacteria is acridine orange (AO), used in obtaining acridine orange direct counts (AODC). Acridine orange intercalates with nucleic acids, and bacteria stained with AO appear either green (high amounts of RNA) or orange (high amounts of DNA) (Figure 9.11). Originally, it was thought that the green or orange color correlated with the viability of the organism; however, this has not been established. Two other important stains used in direct counting are 4,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC). More recently, specialty stains have become available such as the LIVE/DEAD® *Bac*Light™ stain from Molecular Probes®. This stain can help differentiate the proportion of live and dead cells in the preparation (Berney *et al*., 2007) (Figure 9.12). The *Bac*Light™ stain uses a mixture of SYTO® 9 green-fluorescent nucleic acid stain and a red-fluorescent nucleic acid stain, propidium iodide. These stains differ in their ability to penetrate healthy bacterial cells. The SYTO 9 stain will label all bacteria in a population, but the propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, live bacteria appear green (SYTO 9) and dead bacteria appear red (propidium iodide).

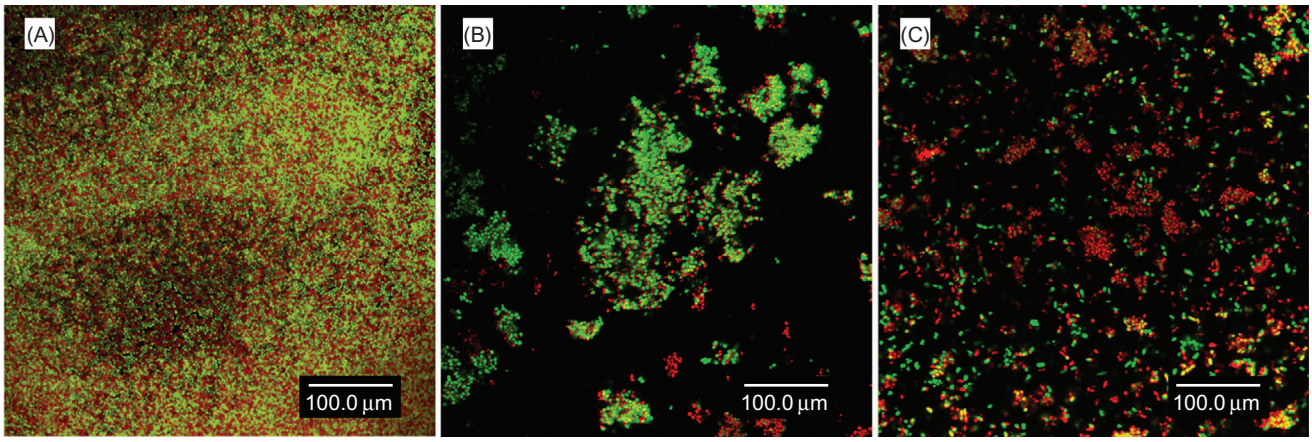

FIGURE 9.12 Bacterial cells stained with LIVE/DEAD BacLight bacterial viability stain to directly visualize the effects of an added antibiotic, vancomycin. These are confocal scanning laser microscope images of *Staphylococcus epidermidis* (SE6) intact biofilms (A), disrupted biofilm (B) and planktonic cells (C) on plastic coverslips after incubation for 24 h with 500 µg/ml of the vancomycin. These images suggest that the antibiotic is more effective on planktonic cells and disrupted biofilms than it is on an intact biofilm. From El-Azizi *et al*. (2005).

Sample preparation for direct stains depends on the type of sample. For pure cultures, samples can be centrifuged and resuspended in a known sample volume, stained and placed on a slide or in a counting chamber that holds a specific volume, such as a hemocytometer or a Petroff–Hauser chamber. Direct microscopy of water samples involves collecting the sample on a Nuclepore filter, staining and counting (Hobbie *et al*., 1977). Direct microscopy of soil microorganisms usually involves first separating the organisms from soil particles. Soils are treated with a dispersing agent, such as Tween 80 or sodium pyrophosphate, and vortexed or sonicated to remove organisms from soil particles and to disrupt soil aggregates. A known volume of the resulting soil suspension is then stained for counting (Ipsilantis and Sylvia, 2007). In soil suspensions, regardless of the fluorescent stain, there are problems associated with the presence of clay colloids, which can either autofluoresce or nonspecifically bind the fluorescent stain. Colloids also mask the presence of soil microorganisms, a phenomenon known as colloidal interference. At high dilutions, colloidal interference decreases; however, the numbers of organisms are also diluted, which may result in low counts that are not statistically valid. At low dilutions, the numbers of organisms increase, but colloidal interference also increases.

9.4.1.1 Estimating Biomass

Direct counts can be used to estimate the microbial biomass in a sample. Estimates can be calculated in terms of bacterial or fungal biomass as carbon as shown in Table 9.2. To calculate bacterial biomass, some assumptions must be made. Approximate bacterial volumes must be determined using average cell lengths and diameters. Approximate bacterial numbers are determined using direct count microscopy. For fungi, an estimate of fungal hyphal lengths per gram of sample must be known. In addition, estimates of the solids content for each organism have to be made. It should be noted that biomass estimates can also be made using chemical fumigation methods (see Section 11.4.2.3) or DNA content (see Chapter 13). The obvious limitations associated with estimating biomass are in estimating organism numbers. This problem is exacerbated in soils with high clay content resulting in colloidal interference.

9.4.2 Fluorescent Immunolabeling

A second common application of fluorescence microscopy is the detection of environmental microorganisms using antibodies that are labeled with fluorescent probes (see Chapter 12). The basis of this methodology is that a specific antibody can be designed and used as a probe for almost any target molecule including proteins, nucleic acids, polysaccharides and lipids. Once the target is determined, an antibody is constructed that will bind to the target with both selectivity and sensitivity. The antibody can be directly labeled with a fluorophore (primary detection agent). Fluorescein is most common but there are a number of alternative dyes such as Cy5 or biotin–avidin. Figure 9.13 shows an example of the use of a fluorescent antibody to detect rhizobia in a complex bacterial community.

Although many biomolecules bind selectively to a biological target, the result can be weak fluorescence necessitating the use of a secondary detection reagent, defined as a molecule that can be indirectly linked to the molecule of interest (see Figure 12.10). The labeled secondary detection reagent is designed to bind to the original

TABLE 9.2 Equations for Calculating Biomass

Calculation of Bacterial Numbers in Soil:

$$N_g = N_f \frac{A}{A_m} \frac{V_{sm}}{V_{sa}} D \frac{W_w}{W_d}$$

N_g = number of bacteria per gram dry soil
N_f = bacteria per field
A = area (mm^2) of smear (or filter)
A_m = area (mm^2) of microscope field
V_{sm} = volume (ml) of smear or filter
V_{sa} = volume (ml) of sample
D = dilution
W_w = wet weight soil
W_d = dry weight soil

Calculation of Bacterial Biomass as Carbon:

$$C_b = N_g V_b e\, S_c \frac{\%C}{100} \times 10^{-6}$$

C_b = bacterial biomass carbon (μg/g soil)
N_g = number of bacteria per gram soil
V_b = average volume (μm^3) of bacteria (r^2L; r = bacterial radius, L = length)
e = density (1.1×10^{-3} in liquid culture)
S_c = solids content (0.2 in liquid culture, 0.3 in soil)
%C = carbon content (45% dry weight)

Calculation of Fungal Biomass Carbon:

$$C_r = \pi r^2 L S_c \%C \times 10^{10}$$

C_r = fungal carbon (μg carbon/g soil)
r = hyphal radius (often 1.13 μm)
L = hyphal length (cm/g soil)
e = density (1.1 in liquid culture, 1.3 in soil)
S_c = solids content (0.2 in liquid culture, 0.25–0.35 in soil)

Adapted from Paul and Clark (1989).

antibody probe thereby linking the fluorescently labeled secondary detection reagent to the target.

9.4.3 Fluorescence *In Situ* Hybridization

Combining identification and visualization of cells in their natural environment is a task of considerable interest to environmental microbiologists. Fluorescence *in situ* hybridization (FISH) has been developed to meet that need. Commonly used in medical applications, it was first developed as a cultivation-independent means of identifying bacterial cells by DeLong *et al.* (1989). FISH involves the use of fluorescently labeled nucleic acid probes to target DNA or RNA sequences within an organism. Due to its high copy number and comparative accessibility, the 16S rRNA gene is by far the most popular target site in bacteria as is the 18S rRNA gene for eukaryotes

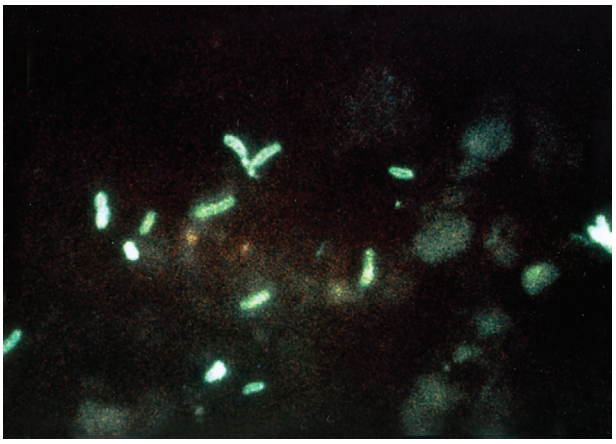

FIGURE 9.13 Use of fluorescent antibodies coupled to fluorescein isothiocyanate to detect antigens. Here rhizobia fluorescence in response to UV irradiation is shown. Photo courtesy I.L. Pepper.

(Zwirglmaier, 2005). The probe is designed to selectively target regions of rRNA that consist of evolutionarily conserved or variable nucleotide regions. Thus, by choosing the appropriate rRNA probe sequence, FISH can be used to detect all bacterial cells (a universal probe to a conserved region), or a single population of cells (a strain-specific probe to a variable region). Recent developments have made other nucleic acid sequences feasible targets such as a gene on a high copy number plasmid or stable mRNA transcripts (Amann and Ludwig, 2000).

FISH is a valuable tool for microbiologists interested in detecting otherwise unculturable bacteria, and understanding microbial diversity and complexity of microbial communities. FISH can also provide insight into how microorganisms interact with each other under varying environmental conditions. However, sensitivity can be problematic and is restricted if cells are not actively growing. This results because the number of target rRNA copies within a cell is dependent on metabolic activity. This technique can be useful in analyzing spatial distributions of microorganisms. For example, Maixner *et al.* (2006) used FISH to study the niche differentiation of *Nitrospira* (a nitrifying bacterium) populations in wastewater-associated biofilms. In this study, FISH helped show that the spatial distribution of different populations depends on the nitrite concentration. As a second example, Figure 9.14 shows how FISH can be used to examine bacterial colonization of plant roots grown in mine tailings. In this case, a universal bacterial FISH probe was used to detect all bacteria on the root surfaces. This experiment examined the effect of compost amendment on colonization (Iverson and Maier, 2009). The results show that there was extensive root colonization in the presence of compost (along with good plant growth), while in the absence of compost there was little bacterial colonization of the root and poor plant growth.

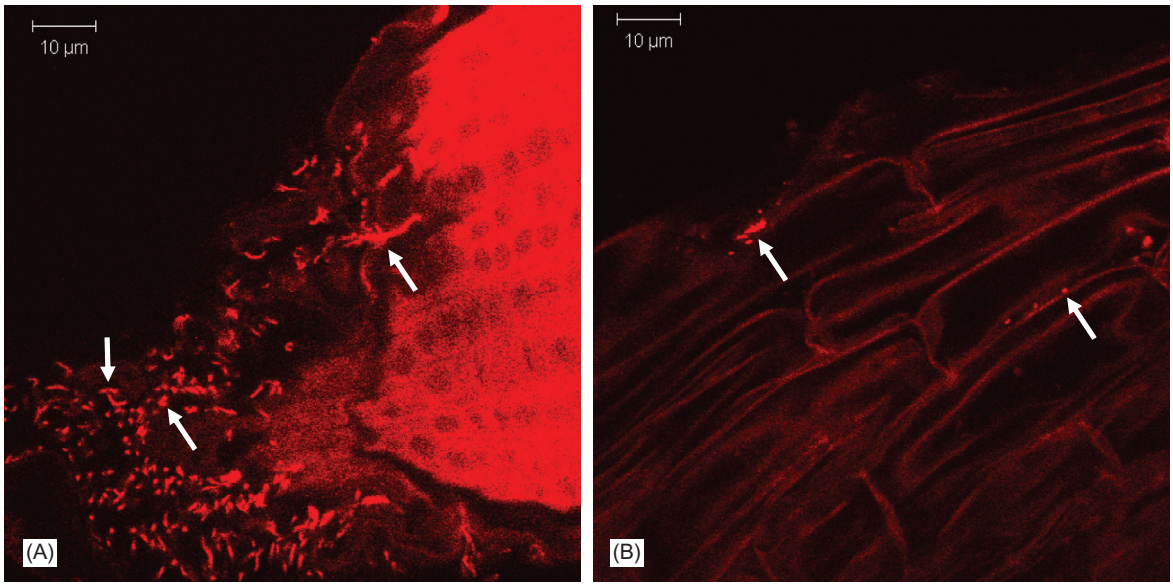

FIGURE 9.14 Use of FISH to compare root colonization of *Buchloe dactyloides* (buffalo grass) grown in mine tailings with either 15 or 0% compost amendment. The FISH probe used was a universal probe EUB338 labeled with the CY3 fluorophore. Samples were visualized on a Zeiss Confocal LSM 510 equipped with a 543 nm laser. Arrows point to bacterial colonies. (A) Heavy bacterial colonization on a root tip grown in mine tailings amended with 15% (w/w) compost. Optical slice is 1.0 μm thick. (B) Minimal colonization of a root grown in unamended mine tailings. Optical slice is 0.6 μm thick. Image courtesy S.L. Iverson.

9.4.4 Confocal Laser Scanning Microscopy

Most commonly used for imaging fluorescent specimens, the confocal laser scanning microscope (CLSM) is often used to document transects through a specimen, such as tissue sections. Computer software can be used to compile the images into a three-dimensional composite. Because nonfocused light is reduced in confocal microscopy, the confocal scanning microscope gives higher resolution, increased contrast and thinner planar views than other forms of light microscopy. Since three-dimensional views can be generated, the CLSM readily lends itself to digital processing, by which images of thin optical sections can be reassembled into a composite, three-dimensional image (Figures 9.12 and 9.14). These images may be viewed as a whole or as individual sections for greater detail. Confocal scanning microscopy is commonly used in bright-field, dark-field and fluorescence microscopies.

In confocal microscopy, a laser beam is used to focus light of a specific wavelength onto the specimen. The confocal scanning microscope has the ability to take optical sections at successive focal planes (known as a Z series). Pinhole apertures are used so that only a small area of the specimen is focused at any given time. Light from the plane of focus enters the detector, eliminating any scattered light, which has the tendency to blur images. The focused light beam moves across the specimen, scanning it, which is required because only a small volume is illuminated at any given time, and a number of these small volumes must be collected for a complete specimen image.

9.4.5 Flow Cytometry

In flow cytometry, microscopic detection of cells or other particles is required as the cells pass through a laser detector. Flow cytometry was first discovered in the 1950s and its uses include the detection of a variety of microorganisms, including bacteria and parasites. As a cell passes through the detector's laser beam, the amount of light scattered in the forward direction and a direction at a 90° angle is measured. These measurements respectively correlate with the size and internal complexity of the particle. The instrument can also measure the fluorescent light emitted by each particle. Data in the flow cytometer are collected as light energy, converted to electrical energy and then plotted on user-defined histograms.

In flow cytometry, particles are separated and flow singly through the detector. Flow cytometer cell sorters have the ability to detect target cells or particles among unwanted ones. The cell sorter vibrates the sample stream, causing it to break into droplets. Information about the particles of interest, such as light scattering and fluorescence criteria, is programmed into the cytometer computer so that when the particle is encountered, the instrument electrically charges the droplet

carrying that particle. Oppositely charged deflection plates pull the particles of interest out of the uncharged sample stream toward the charged plate, and ultimately deflect them onto a glass microscope slide or into a collection tube. The droplets containing unwanted particles flow into a waste collection tank.

Flow cytometry is commonly used in environmental microbiology. The FITC method relies on the binding of a fluorescein (FITC)-conjugated antibody to antigens present in the sample. For example, this technique has been used to quantitate *Cryptosporidium* and *Giardia* present in environmental samples (see Figure 8.10 and Section 22.3.1). The FITC-stained sample suspension is aspirated by the flow cytometer and each particle in the sample is examined in the instrument's laser beam. The fluorescein molecule, when excited by the 488 nm laser light, in return emits light at 525 nm. The light energy is detected in the flow cytometer and quantitated. The cysts and oocysts of these organisms are identified by their 90° light scattering and additional FITC fluorescence properties. Collection by the flow cytometer on a glass slide allows additional microscopic analysis for identification.

9.4.6 Developing Methods in Fluorescence Microscopy

Limited resolution is a challenge with most fluorescence microscopy studies. While providing high specimen contrast, cellular and subcellular visualization of structures is limited by the weak resolution. However, techniques have been developed to combine the contrasting ability of fluorescence microscopy with enhanced resolution.

Stimulated emission depletion (STED) fluorescence microscopy increases resolution through the use of lasers to excite very specific locations of specimen-associated fluorophore label. STED microscopy has been used to identify protein complexes in mitochondria (Donnert *et al.*, 2007) and synaptic vesicles in living cells (Westphal *et al.*, 2008). Another increasingly common technique is single-molecule fluorescence imaging. This approach uses a fluorescent microscope with digital detection to track the cellular location of a fluorescence emission (Biteen and Moerner, 2010). Based on the fusion of a protein of interest to a fluorophore label, this type of microscopy allows for investigation of live cells, a major advantage over other types of microscopies. The fluorescent protein fusion allows the cell to remain physiologically active while being microscopically monitored. Continuing developments of fluorescence techniques offer tremendous potential in increasing our understanding of the subcellular structure of microorganisms.

9.5 ELECTRON MICROSCOPY

The electron microscope produces high resolution detail by using electrons instead of light to form images. The extremely short wavelength and focusability of electron beams are responsible for the theoretically high resolving power of electron microscopes. The increased resolution allows a functional magnification of up to 1,000,000 × for the observation of fine structure and detail. Although electron microscopes are conceptually similar to light microscopes, there are some fundamental differences between using light versus electronic illumination (Table 9.3). In the electron microscope, an electron gun aims a beam of electrons at a specimen placed in a vacuum sample chamber. A series of coiled electromagnets are used to focus the beam. As in light microscopy, poor contrast is a problem in electron microscopes, so samples are often stained to increase contrast. Images produced in the electron microscope are in shades of gray, although computerized color may be added in some scopes. The two most common types of electron microscopy are scanning electron microscopy and transmission electron microscopy.

TABLE 9.3 Comparison of Optical Microscopes and Electron Microscopes

Characteristic	Optical	Electron
Illuminating beam	Light beam	Electron beam
Wavelength	7500 Å (visible)	0.086 Å (20 kV)
	2000 Å (ultraviolet)	0.037 Å (100 kV)
Medium	Atmosphere	Vacuum
Lens	Glass lens	Electrostatic lens
Resolving power	2000 Å	3 Å
Magnification	Up to 2000 ×	Up to 1,000,000 ×
Focusing	Mechanical	Electrical
Viable specimen	Yes	No
Specimen requires staining or treatment	Yes/no	Always
Colored image produced	Yes	No

9.5.1 Scanning Electron Microscopy

In the scanning electron microscope (SEM), an image is formed as an electron probe scans the surface of the specimen (Figure 9.15), producing secondary electrons,

backscattered electrons, X-rays, Auger electrons and photons of various energies. The SEM uses these signals to produce three-dimensional surface characteristics of specimens (Figure 9.16). There are several advantages of the SEM. These include a large depth of field and the ability to examine bulk samples with low magnification, and lifelike images.

In a typical SEM, an electron gun and multiple condenser lenses produce an electron beam whose rays are aligned through electromagnetic scan coils. Electron-accelerating voltages in the gun range from 60 to 100 kV (kilovolts). A tungsten filament, heated to approximately 2700K, is the illumination source within the gun. Heating the filament causes electrons to be released from the tip of the filament. An image of the surface topography of the specimen is generated by electrons that are reflected (backscattered) or given off (secondary electrons). Contrast in the SEM is enhanced by coating the sample with a thin layer of a conductive metal, e.g., gold or palladium, or even carbon. Image formation itself is the result of rastering the electron beam (from 2 to 200 Å in diameter) back and forth along the specimen surface (Figure 9.17). A visual image corresponding to the signal produced by the interaction between the beam spot and the specimen at each point along each scan line is simultaneously built up on the face of a cathode ray tube in the same way a television picture is generated. As with all microscopy, interpretation of SEM images is subjective, particularly because SEM images include high resolution, high contrast and varying depths of focus resulting in topography.

Sample preparation for the SEM is relatively straightforward. The general sequence involves: (1) sample fixation with an aldehyde solution; (2) dehydration of the sample (because the sample must be under vacuum in the SEM); (3) mounting of the specimen on a metal stub; and (4) coating of the specimen with a thin layer of electrically conductive material.

9.5.2 Transmission Electron Microscopy

In the SEM, electrons interacting with the surface of the specimen form the image. In transmission electron microscopy (TEM) (Figure 9.18), the image is formed by electrons passing through the specimen. Consequently, the specimens must be thin sectioned to allow the passage

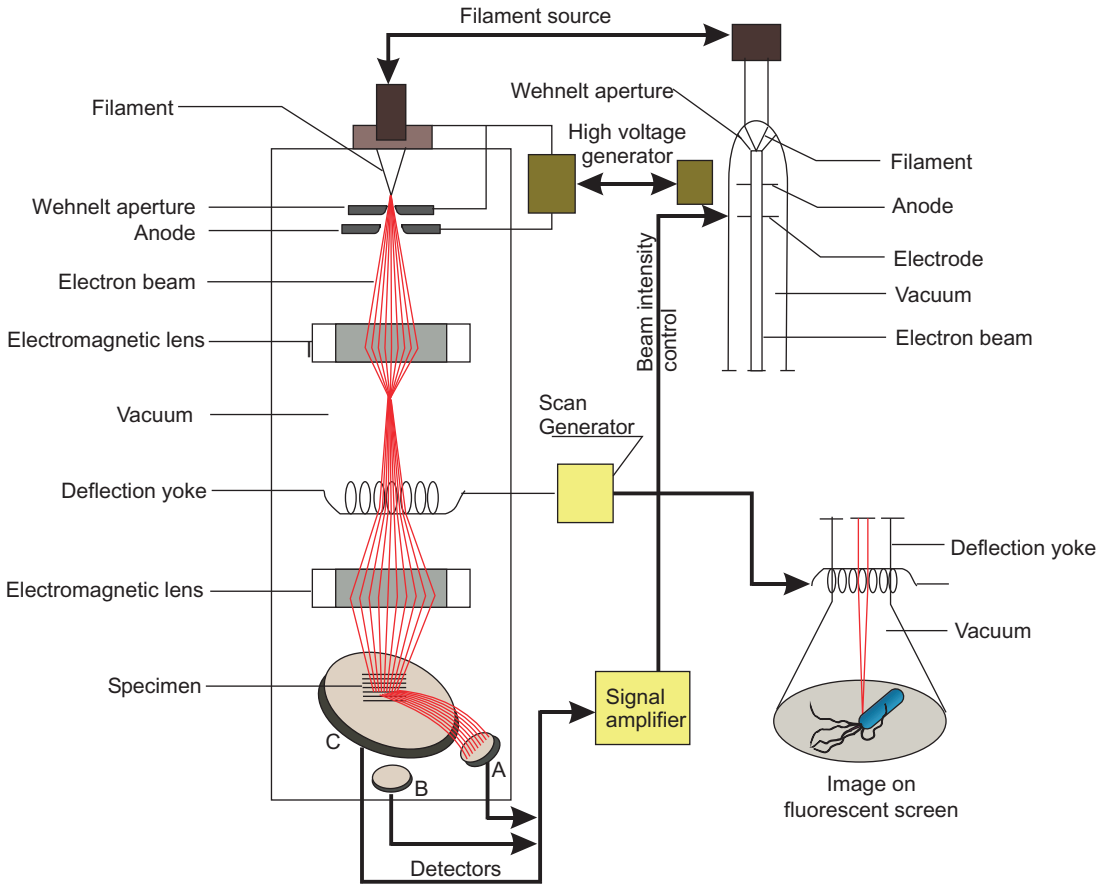

FIGURE 9.15 A typical scanning electron microscope (SEM) and its similarity to a television. They are based on the same principles. Courtesy FEI Company. Reproduced with permission.

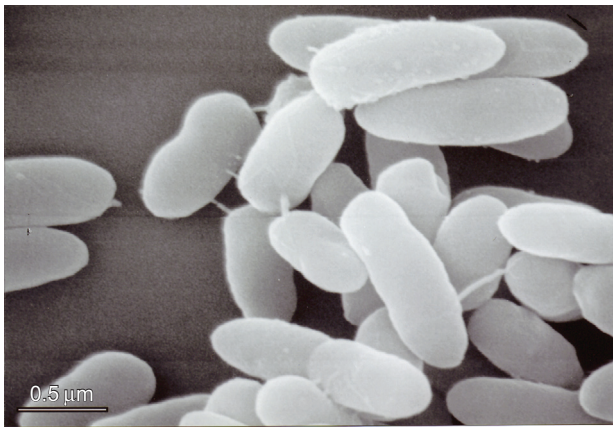

FIGURE 9.16 Scanning electron microscope image of *Pseudomonas aeruginosa* cells. The size bar is 5 µm. Photo courtesy A.A. Bodour.

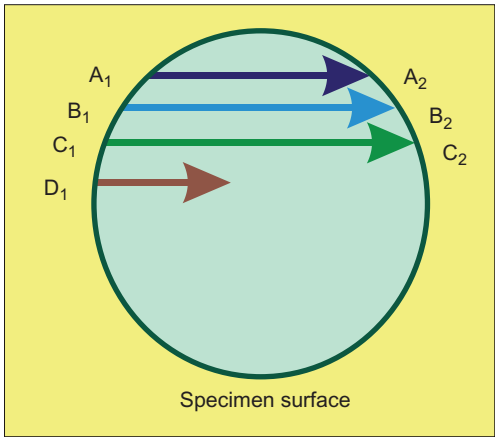

FIGURE 9.17 Direction of scanning of the electron beam on a sample surface in a scanning electron microscope. Diagram courtesy D. Bentley.

FIGURE 9.18 A typical transmission electron microscope (TEM). Image courtesy FEI Company.

of electrons. TEM is often used to view detail of fine structures (Figure 9.19) and internal cell structures (Figure 9.20), due to the selective absorption of electrons by different parts of the specimen.

Sample preparation for the TEM is much more extensive than for the SEM. A sample initially undergoes fixation in glutaraldehyde or formaldehyde to preserve structure. Fixation also protects the sample from damage that may occur during the rest of the preparation. Following fixation, the sample is dehydrated, most commonly by replacing water with ethanol. The ethanol acts as a solvent between the aqueous environment of the cell and the hydrophobic embedding medium. Embedding involves resin infiltration, where the ethanol is replaced by a highly miscible plastic embedding agent, and is later cured at a high temperature ($\approx 70^{\circ}$C). Curing causes the embedding medium to polymerize and become solid. A microtome equipped with either a glass or diamond knife is then used to make thin sections approximately 90 nm thick for viewing under the TEM.

A technique called cryo-TEM can be used to directly image small fluid structures in water (Figure 9.19). Cryo-TEM requires extremely rapid cooling (vitrification) of the sample to $-170-185^{\circ}$C. This technique has been valuable for examining biological molecules and their aggregation behavior in water solutions (Won, 2004). A related technique called electron cryotomography (ECT) provides three-dimensional imaging of intact cells at a resolution of ≈ 4 nm, allowing for subcellular examination of cells in their native state (Tocheva *et al.*, 2010). Increasing in use, ECT has revealed detailed information about septum formation in dividing bacterial cells, bacterial cytoskeletons and subcellular structures associated with motility and chemotaxis. The continued use of ECT will revolutionize our understanding of microbial cell structure.

All lenses are subject to aberrations, and the electromagnetic lenses used in electron microscopy are no exception. Unlike those in light microscopy, however, aberrations in the electron microscope are difficult to resolve because of the inability to add corrective lenses to the optics. Consequently, whereas in the light microscope the theoretical and achievable resolving powers are

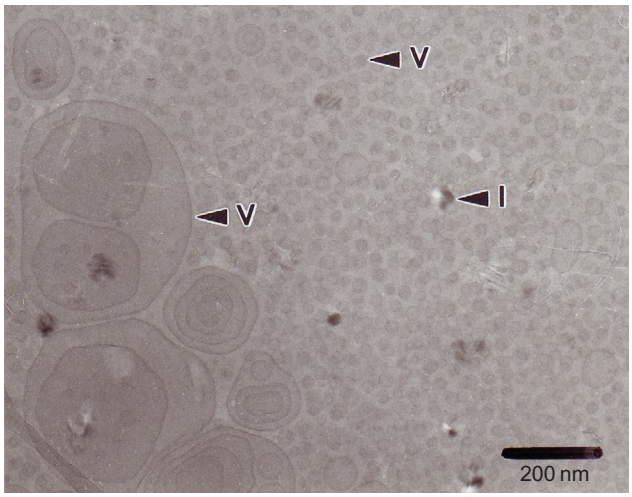

FIGURE 9.19 A cryo-TEM micrograph of the morphology of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* at pH 6.5. This micrograph shows bilayer surfactant vesicles (V) which range in size from 30 nm to several hundred nm. The size bar = 200 nm. Image courtesy J.T. Champion.

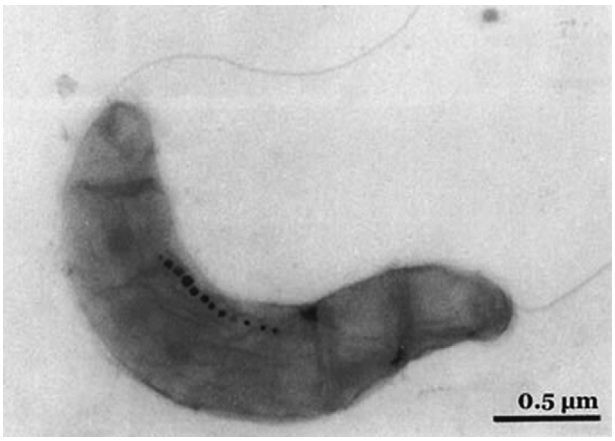

FIGURE 9.20 A TEM micrograph of a magnetotactic bacterium. A single bacterium in cross-section shows a chain of seven electron-dense magnetosomes. Each magnetosome contains a single crystal of a tiny magnet (iron oxide magnetite). Because the motility of this type of bacterium is directed by these magnets, they are referred to as being "magnetotactic." This sample was taken at a depth of 4 meters in the Pettaquamscutt River, Rhode Island. Magnification 116,000×. Taken by Paul Johnson.

similar, in the electron microscope the theoretical resolution is not reached.

9.5.3 Elemental Analysis

One of the advantages of electron microscopy is the ability to perform microanalysis X-ray spectrometry with an energy-dispersive spectrometer (EDS). In EDS, when an electron beam of sufficient energy encounters a surface, X-ray photons of characteristic energies may be emitted via inner-shell ionization (Figure 9.21A and B). The result is a fingerprint of X-ray energies specific for a particular element. By comparison with fingerprints of known elements, an unknown element in a sample can be identified and quantitated. Energy-dispersive spectrometry can be performed with both transmission and scanning electron microscopes. No additional sample preparation is needed beyond that necessary for the electron microscope. EDS applications in environmental microbiology are broad and are discussed in more detail in Chapter 21.

9.6 SCANNING PROBE MICROSCOPY

Scanning probe microscopy deals with imaging surfaces on a very fine scale, even to the level of molecules and groups of atoms. This technique uses an extremely sharp tip (3–50 nm radius of curvature) to scan across the surface of the sample. When the tip moves close to the sample surface, the forces of interaction between the tip and the surface of the sample can be measured. The most common types of scanning probe microscopy are atomic force microscopy, scanning tunneling microscopy and near-field scanning optical microscopy. Such microscopes have the ability to view single atoms with a magnification of 1,000,000×. We discuss the first of these below.

9.6.1 Atomic Force Microscopy

The atomic force microscope (AFM) measures surface contours with a probe or "tip" placed very close to the sample. The image is acquired when the probe is raster-scanned over the sample. Depending on the set-up, this measures either the contour height or the electric potential at any given site. The AFM does not use lenses, so the size of the probe tip rather than diffraction is the limiting factor in image resolution. The fine resolution offered by AFM is allowing scientists to begin to decipher interactions of biological molecules with surfaces (Figure 9.22).

9.7 IMAGING

Micrography, taking an image using a microscope, provides a means of permanently recording an image for both artistic and scientific purposes. Historically, micrographs consisted of images on photographic film, but the use of digital imaging via a digital camera attached to a microscope is now standard. With the development of more sophisticated computers and imaging systems, images can be digitized; this process has higher resolution than traditional photographic methods and creates a more accurate reproduction of a microscopic image. The use of

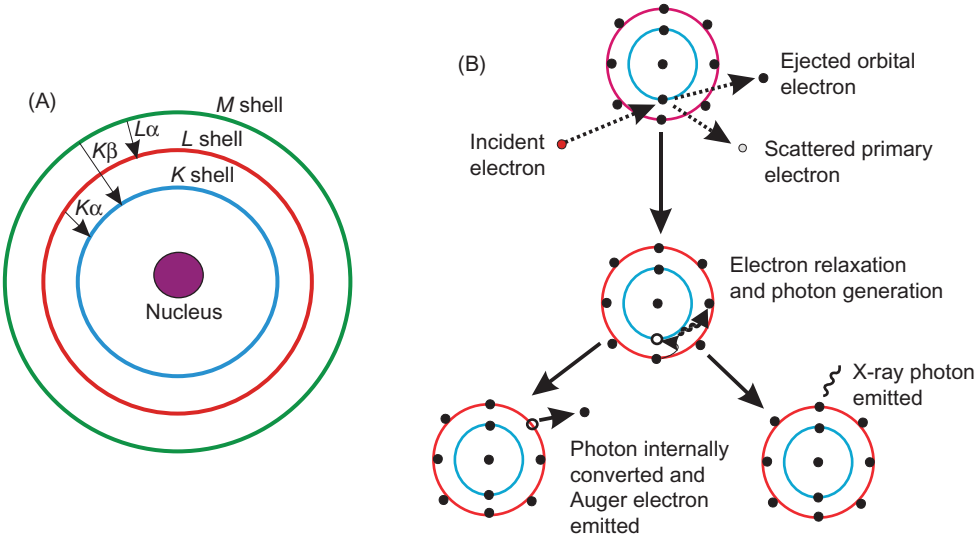

FIGURE 9.21 (A) Electronic transitions in an atom and (B) electron interactions as an electron from the electron beam encounters an atom. X-ray generation is used in electron dispersive spectrometry. Adapted from Goldstein and Yakowitz (1975).

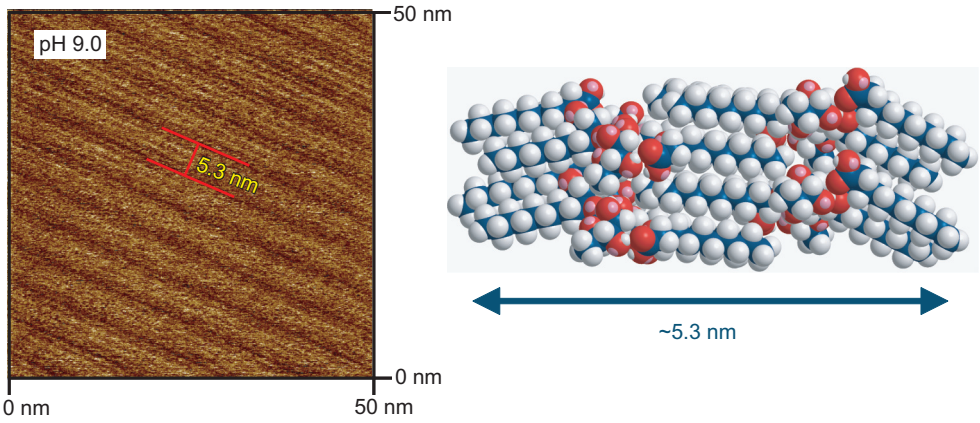

FIGURE 9.22 (Left) An AFM image of monorhamnolipid adsorbed on graphite at pH 4.3. (Right) The information from this image can be used to make a precise molecular model of how rhamnolipid interacts with the surface. Image and model courtesy V. Ochoa and J.E. Pemberton.

computers in the digital processing of microscopic images has allowed automated image processing. Manipulation of images into three dimensions or overlaying images of multiple fluorescent probes is now possible with digital micrographs. The development of increasingly sophisticated imaging is a necessary component of advancing microscopic techniques used in microbiology.

QUESTIONS AND PROBLEMS

1. List four possible applications in environmental microbiology for each of the following microscopic techniques: bright-field, fluorescence, electron and *in situ* microscopy.
2. Direct microscopic counts using acridine orange (AODC) are often two to three orders of magnitude greater than viable counts. Why?
3. If you wanted to determine whether or not a specific membrane protein was being produced by a microorganism, which microscopic techniques might you use and why?
4. List at least four things about a microorganism you can learn from viewing it with bright-field microscopy.
5. Which is most important in microscopy—resolution, contrast or magnification? Why?
6. List two prokaryotic structures that would stain in response to (i) an acidic dye. (ii) A basic dye.

REFERENCES AND RECOMMENDED READING

Recommended Microscopy Texts

Goldstein, J. I., and Yakowitz, H. (1975) "Practical Scanning Electron Microscopy," Plenum, New York.

Hayat, M. A. (2000) "Principles and Techniques of Electron Microscopy Biological Applications," Cambridge University Press, Cambridge, UK.

Hoppert, M. (2003) "Microscopic Techniques in Biotechnology," Wiley-VCH Verlag, Weinheim.

Mason, C. W. (1983) "Handbook of Chemical Microscopy", vol. 1, 4th ed. John Wiley & Sons, New York.
Murphy, D. B. (2001) "Fundamentals of Light Microscopy and Electronic Imaging," Wiley-Liss, New York, NY.
Rochow, T. G., and Tucker, P. A. (1994) "Introduction to Microscopy by Means of Light, Electrons, X-Rays or Acoustics," Plenum, New York.

Chapter References

Amann, R., and Ludwig, W. (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev.* **24**, 555–565.
Berney, M., Hammes, F., Bosshard, F., Weilenmann, H. U., and Egli, T. (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Appl. Environ. Microbiol.* **73**, 3283–3290.
Biteen, J. S., and Moerner, W. E. (2010) Single-molecule and superresolution imaging in live bacteria cells. *Cold Spring Harb. Perspect. Biol.* **2**, a000448.
DeLong, E. F., Wickham, G. S., and Pace, N. R. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* **243**, 1360–1363.
Donnert, G., Keller, J., Wurm, C. A., Rizzoli, S. O., Westphal, V., Schonle, A., *et al*. (2007) Two-color far-field fluorescence nanoscopy. *Biophys. J.* **92**, L67–L69.
El-Azizi, M., Rao, S., Kanchanapoom, T., and Khardori, N. (2005) In vitro activity of vancomycin, quinupristin/dalfopristin, and linezolid against intact and disrupted biofilms of staphylococci. *Ann. Clin. Microbiol. Antimicrob.* **4**, 2.
Goldstein, J. I., and Yakowitz, H. (1975) "Practical Scanning Electron Microscopy," Plenum, New York.
Hobbie, J. E., Daley, R. J., and Jasper, S. (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**, 1225–1228.
Hoppert, M. (2003) "Microscopic Techniques in Biotechnology," Wiley-VCH Verlag, Weinheim.
Ipsilantis, I., and Sylvia, D. M. (2007) Abundance of fungi and bacteria in a nutrient-impacted Florida wetland. *Appl. Soil Ecol.* **35**, 272–280.
Iverson, S. L., and Maier, R. M. (2009) Effects of compost on colonization of roots of plants grown in metalliferous mine tailings, as examined by fluorescence in situ hybridization. *Appl. Environ. Microbiol.* **75**, 842–847.
Korber, D. R., Lawrence, J. R., Zhang, L., and Caldwell, D. E. (1990) Effect of gravity on bacterial deposition and orientation in laminar flow environments. *Biofouling* **2**, 335–350.
Maixner, F., Noguera, D. R., Anneser, K. S., Wegl, G., Wagner, M., and Daims, H. (2006) Nitrite concentration influences the population structure of *Nitrospira*-like bacteria. *Environ. Microbiol.* **8**, 1487–1495.
Mason, C. W. (1983) "Handbook of Chemical Microscopy", vol. 1, 4th ed. John Wiley & Sons, New York.
Murray, R. G. E., and Robinow, C. F. (1994) Light microscopy. In "Methods for General and Molecular Bacteriology" (P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, eds.), American Society for Microbiology, Washington, DC, pp. 8–20.
Paul, E. A., and Clark, F. E. (1989) "Soil Microbiology and Biochemistry," Academic Press, San Diego.
Pepper, I. L., and Gerba, C. P. (2004) "Environmental Microbiology Laboratory," Academic Press, San Diego.
Rochow, T. G., and Tucker, A. (1994) "Introduction to Microscopy by Means of Light, Electrons, X-Rays or Acoustics," Plenum, New York.
Rossi, G., Riccardo, S., Gesue, G., Stanganelli, M., and Wang, T. K. (1936) Direct microscopic and bacteriological investigations of the soil. *Soil Sci.* **41**, 53–66.
Tocheva, E. I., Li, Z., and Jensen, G. J. (2010) Electron cryotomography. *Cold Spring Harb. Perspect. Biol.* **2**, a003442.
Westphal, V., Rizzoli, S. O., Lauterbach, M. A., Kamin, D., Jahn, R., and Hell, S. W. (2008) Video-rate far-field optical nanoscopy dissects synaptic vesicle movement. *Science* **320**, 246–249.
Won, Y.-Y. (2004) Imaging nanostructured fluids using cryo-TEM. *Korean J. Chem. Eng.* **21**, 296–302.
Zieler, H. W. (1972) "The Optical Performance of the Light Microscope", Part 1. Microscope Publications, Chicago.
Zwirglmaier, K. (2005) Fluorescence in situ hybridisation (FISH)—the next generation. *FEMS Microbiol. Lett.* **246**, 151–158.