Chapter 2

Observing the Microbial Cell

SUMMARY

This chapter introduces microbial observation. Optics are discussed in general, as are the principles of all forms of microscopy.

Types of microscopy are discussed with examples of what has been observed using each technique. The physics behind the use of electromagnetic radiation and lenses is discussed in each case.

This introduction to visualizing cells and cellular components will progress to Chapter 3, which describes how visual observation was used to reveal details about cell structure and function.

2.1 Observing Microbes

Learning Objectives

- 2.1.a. Explain how the structure of the human eye dictates the resolution of objects.
- 2.1.b. Differentiate between resolution and detection.
- 2.1.c. Describe and identify bacterial morphologies (e.g., bacilli, cocci, and spirochetes).
- 2.1.d. Identify the type(s) of microscopy needed to view a particular specimen.

This section introduces all the generic terms used in microscopy. It is imperative that students understand detection, resolution, and magnification as well as how they are related. It is also important to understand the size ranges of organisms. It is here that the major forms of observation, from light microscopy to X-ray crystallography, can be introduced.

Discussion Points

- Resolution is an extremely important concept. Using a real-life example to illustrate resolution, discuss how you may be able to see a person at a distance in a photo, but you will not be able to resolve enough features to determine the person's identity, even if you enlarge the photo. This concept is further illustrated in Figure 2.3.
- Figure 2.6 shows three examples of the use of light microscopy (LM) and three examples of the use of scanning electron microscopy (SEM). The difference in the level of resolution and detail between the two forms is very evident.
- Figure 2.7 serves to reinforce this concept by showing size ranges observable with each technique and a photograph to illustrate the point. Combine the discussion of this figure with a discussion of Figure 2.4

so that the students will understand which microscopy techniques are potentially appropriate for observing different cells.

2.2 Optics and Properties of Light

Learning Objectives

- 2.2.a. Identify what conditions must exist for electromagnetic radiation to resolve an object from neighboring objects or the surrounding medium.
- 2.2.b. Explain the properties of light.
- 2.2.c. Differentiate among absorption, reflection, refraction, and scattering.
- 2.2.d. Explain how lenses magnify images.

This section continues to introduce the physical properties of light, its interaction with objects, and optics. Visible light is one portion of the spectrum of electromagnetic radiation, which includes shorter wavelengths (such as ultraviolet and X-rays) as well as longer wavelengths (such as microwaves and radio waves). For the processes discussed in this chapter, the focus will be on the portion of the spectrum from visible light toward the ultraviolet end of the spectrum.

Light travels as a wave, which can be absorbed, reflected, refracted, or scattered by an object. Each of these processes plays a role in one or more forms of microscopy. In particular, the role that refraction plays in magnification should be explained.

Discussion Points

- Figure 2.8 presents the electromagnetic spectrum, which is useful for discussing the relationship between wavelength and frequency.
- Figures 2.9 and 2.10 show the interaction of light with matter. This can lead into discussions of magnification.
- Figure 2.11 illustrates how to use a lens and the principle of how diffraction leads to magnification.
- Figure 2.12 gives a good explanation of the optics of resolution.

2.3 Bright-Field Microscopy

Learning Objectives

- 2.3.a. State the factors that influence image quality in bright-field microscopy.
- 2.3.b. Label the parts of a compound microscope.
- 2.3.c. Explain the function of the components of a compound microscope.
- 2.3.d. Describe the advantages and disadvantages of a wet mount.

- 2.3.e. Explain the advantages and disadvantages of fixing and staining specimens.
- 2.3.f. Classify stains as simple or differential.
- 2.3.g. Explain how the structure of the bacterial cell wall relates to its Gram-stain status.

Bright-field microscopy is the method that most students will have the opportunity to use in introductory labs. The physics and operation of the microscope should be discussed at some level. With this should come an explanation of the reason behind the use of oil (Fig. 2.14) with the 100X lens and how to calculate total magnification.

In bright-field microscopy the cell is observed as a dark object (absorbing light) against a bright background (transmitting light), hence the name. The contrast between a cell, which is predominantly water, and its environment, which is commonly water, is usually minimal. Consequently, the contrast has to be enhanced by fixing and staining the specimen prior to observation. Many different stains are used, each providing distinct information about a specimen. The most widely used staining procedure used in microbiology is the Gram stain. A more detailed discussion of cell envelope differences appears in Chapter 3.

Discussion Points

- The microscope and its optics are diagrammed in Figure 2.15. Students should be introduced to the operation and terms relevant to bright-field microscopy.
- Figure 2.13 is an excellent illustration for relating the optics of resolution (from Figure 2.12) to the increased resolution generated by greater magnification.
- Figure 2.14 illustrates the importance of using immersion oil with the 100X lens.
- It is important to emphasize the difference between a simple stain and a differential stain.
- Different staining processes are mentioned, but it is most important to discuss the Gram stain. This type of staining is illustrated in Figures 2.20, 2.21, and 2.22.

2.4 Fluorescence Microscopy and Super-Resolution Imaging

Learning Objectives

- 2.4.a. Differentiate between the excitation and emission wavelengths.
- 2.4.b. Recall the factors that determine the cell specificity of a fluorophore.
- 2.4.c. Describe the advantages of super-resolution imaging.
- 2.4.d. State the function of chemical imaging microscopy.

In fluorescence microscopy, the object absorbs light at one wavelength and then emits the light at a longer, visible wavelength. The wavelength that is emitted determines the color that is observed. A fluorophore is the fluorescent molecule used to stain the specimen. Some fluorophores have affinity for a certain component

of a cell. Fluorophores can be attached to antibodies or DNA for use in microscopic analysis.

In confocal microscopy a laser beam is used to excite a fluorophore and generate a three-dimensional image.

Discussion Points

- Figure 2.24A shows a fluorophore at the molecular level, and Figure 2.24B shows the absorption and emission spectra for a fluorophore.
- Discuss the specificity and usefulness of chemical affinity, immunofluorescence, gene fusion, and DNA hybridization. Understanding these techniques is necessary before studying Chapter 3.
- Figure 2.27B illustrates the use of fluorophores to observe the replisome and origin of DNA replication in bacterial cells.
- Figure 2.28 shows how super-resolution imaging of fluorescent microscope images may be used to observe the movement of molecules within cells.

2.5 Dark-Field and Phase-Contrast Microscopy

Learning Objectives

- 2.5.a. Describe the conditions under which dark-field microscopy is the best choice for viewing specimens.
- 2.5.b. Explain the principles behind phase-contrast microscopy.
- 2.5.c. State the types of images obtained with differential interference contrast microscopy.

Dark-field microscopy allows the detection of entities that are too small to be resolved with bright-field microscopy. You should emphasize the difference between detection of an object and resolution of an object's precise shape and size. Scattered light from the object is detected by the use of a condenser lens containing a "spider light stop." It can be used in the study of motility because it allows detection of bacterial flagella, which are too narrow to be resolved by bright-field microscopy.

Phase-contrast microscopy allows observation based on differences in refractive indexes between the cytoplasm, the medium, and subcellular entities. It employs an annular ring in the optics. This produces dramatic visual differences between objects having only a small difference in refractive index. Therefore, no stains are needed, and hence we can observe living cells.

Discussion Points

- The physics of the spider light stop condenser system in dark-field microscopy is illustrated in Figure 2.32.
- Studying motility is possible using dark-field microscopy. An excellent illustration of this is shown in

Figure 2.33. You can show this figure and follow up with Thought Question 2.7 to provoke class discussion.

- Figure 2.35 shows how the specimen and phase plate each shift the light wave by one-fourth of a wavelength, resulting in a total difference of one-half wavelength. This increases the contrast, enabling the visualization of live microbes with phase-contrast microscopy. A resulting image can be seen in Figure 2.34.

2.6 Electron Microscopy, Scanning Probe Microscopy, and X-Ray Crystallography

Learning Objectives

- 2.6.a. Compare and contrast light microscopy and electron microscopy.
- 2.6.b. Identify images obtained via scanning electron microscopy and transmission electron microscopy.
- 2.6.c. Propose applications for scanning probe microscopy.
- 2.6.d. State what kind of information is obtained from X-ray crystallography analysis.
- 2.6.e. Explain the methods and benefits of using cryo techniques in cryo-electron microscopy and cryocrystallography.

Electron microscopy is based on magnification using a beam of electrons as the radiation source. Electrons traveling in a voltage potential exhibit a wave property, analogous to the wave property of light rays. The wavelength of the electron beam is much smaller than that of light; for this reason, much smaller dimensions can be resolved by electron microscopy than by light microscopy.

The two major forms of electron microscopy are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The electron microscope has parts analogous to those of a light microscope. In electron microscopy, the radiation source is an electron beam rather than visible light, and the lenses are magnets rather than glass. In both TEM and SEM, samples can be stained with heavy metal. In TEM, the electron beam is transmitted through the thin section of a stained specimen, revealing internal structure. In SEM, the electron beam is reflected off the surface of the stained specimen, and a picture of the surface of the specimen is obtained.

In cryo-electron microscopy and cryo-electron tomography, the flash-frozen samples are unstained, resulting in high-resolution images.

Atomic force microscopy is a method that measures van der Waals forces between the electron shells of adjacent atoms on the cell surface and the tip of the probe. It allows the study of surfaces of live bacteria in water solution.

X-ray crystallography allows studies of structures at the molecular level. X-ray crystallography has revealed the structure of many molecules. The X-ray crystallography of DNA by Rosalind Franklin, which helped reveal the double-helical nature of DNA, is probably the most widely discussed example.

Discussion Points

- Discuss sample preparation for TEM and SEM microscopy and the possibility of artifact introduction in the process.
- Discuss cryo-EM and how sample preparation leaves the specimen in a form that should closely resemble the viable form.
- Use Figure 2.38 to compare and contrast light microscopy and TEM.
- Discuss sample preparation for TEM, including fixation, embedding, sectioning, and staining.
- Figure 2.39A, which illustrates the structure of the SEM, can be used as an aid in the discussion of the relevant physics.
- Discuss the need for shadowing a surface of a specimen for SEM.
- Figure 2.40 contains an image of flagellar motors generated by TEM. This might help students understand the kinds of structures that could be visualized with TEM as opposed to light microscopy.
- Figures 2.43 and 2.44 describe cryo-electron tomography and include a viral structure inferred from the use of this technique.
- Figure 2.45 illustrates the principle of atomic force microscopy.
- Figure 2.46 shows the principle behind X-ray diffraction and the data observed in a particular case.
- X-ray crystallography requires a crystallized specimen and therefore amounts to looking at a static picture.
- X-ray data analysis can provide information about model molecular structure, as illustrated by Figure 2.48.

PROCESS ANIMATIONS

The following process animation expands upon the art from the textbook. The process animations can be accessed through the Digital Resources website, digital.wwnorton.com/microbio4, as well as through the ebook and in the coursepacks, which are provided for download on the instructor's resource site, wwnorton.com/instructors.

- **Microscopy: Optics and Properties of Light**

This animation depicts the concepts of magnification and resolution that are described in this chapter using the figures from the text. The structure and functioning of compound microscopes are also discussed at some length.

Animation Discussion Question 1: Discuss Airy discs and how they are related to resolution.

ANS: Airy discs are formed by the alternating pattern of constructive and destructive interference of light waves due to refraction through a lens. The size of the Airy disc is determined by the quality of the lens and the wavelength of light, with shorter wavelengths producing

smaller discs. Structures may only be distinguished from one another by our eyes if their Airy discs do not overlap.

Animation Discussion Question 2: What is the purpose of the condenser lens of a compound microscope?

ANS: The condenser lens focuses the light on a small area of the specimen slide. This allows for more efficient illumination of the sample.

eTOPICS

eTopics are supplementary, stand-alone sections that explore additional material in depth. They are available to students within the ebook and within the coursepacks, which are provided for download on the instructor's resource site, wwnorton.com/instructors.

2.1 Molecular "Snapshots": Chemical Imaging

eTopic 2.1 discusses nanoscale secondary ion mass spectrometry (NanoSIMS). The chemical and physical bases of this technique are discussed along with some examples of its utility for answering biological questions. This technique is also discussed in Special Topic 2.1.

2.2 Confocal Microscopy

eTopic 2.2 discusses confocal laser scanning microscopy. The physics underlying this technique are discussed and some example images are shown.

RECOMMENDED READINGS

The following readings are presented at the end of the textbook chapter as resources for further exploration of the topics discussed in Chapter 2.

Altindal, Tuba, Suddhashil Chattopadhyay, and Xiao-Lun Wu. 2011. Bacterial chemotaxis in an optical trap. *PLoS ONE* **6**:e18231.

Chiu, W., M. L. Baker, W. Jiang, and Z. H. Zhou. 2002. Deriving folds of macromolecular complexes through electron cryomicroscopy and bioinformatics approaches. *Current Opinion in Structural Biology* **12**:263–269.

Graumann, Peter L., and Richard Losick. 2001. Coupling of asymmetric division to polar placement of replication origin regions in *Bacillus subtilis*. *Journal of Bacteriology* **183**:4052–4060.

Jiang, W., J. Chang, J. Jakana, P. Weigele, J. King, et al. 2006. Structure of epsilon15 bacteriophage reveals genome organization and DNA packaging-injection apparatus. *Nature* **439**:612–616.

Komeili, A., Z. Li, D. K. Newman, and G. J. Jensen. 2006. Magnetosomes are cell membrane invaginations organized by the actin-like protein MamK. *Science* **311**:242–245.

Lucic, Vladan, Friedrich Förster, and Wolfgang Baumeister. 2005. Structural studies by electron tomography: From cells to molecules. *Annual Review of Biochemistry* **74**:833–865.

Matias, Valério R. F., Ashruf Al-Amoudi, Jacques Dubochet, and Terry J. Beveridge. 2003. Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Bacteriology* **185**:6112–6118.

Murphy, Douglas B. 2001. *Fundamentals of Light Microscopy and Electronic Imaging.* Wiley-Liss, Hoboken, NJ.

Popescu, Aurel, and R. J. Doyle. 1996. The Gram stain after more than a century. *Biotechniques in Histochemistry* **71**:145–151.

Ptacin, Jerod L., Steven F. Lee, Ethan C. Garner, Esteban Toro, Michael Eckart, et al. 2010. A spindle-like apparatus guides bacterial chromosome segregation. *Nature Cell Biology* **12**:791–798.

Tocheva, E., Z. Li, and G. Jensen. 2010. Electron cryotomography, p. 213–232. *In* Lucy Shapiro and Richard M. Losick (eds.), *Cell Biology of Bacteria: A Subject Collection from Cold Spring Harbor Perspectives in Biology.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ANSWERS TO REVIEW QUESTIONS (P. 73)

1. What principle defines an object as microscopic?

   ANS: An object is microscopic if we cannot see it clearly without magnification. The fact that it is microscopic to us is based on our eyes' inherent properties. The size at which something becomes visible depends on the resolution of our eyes.

2. Explain the difference between detection and resolution.

   ANS: Detection of an object simply means that it can be observed. Resolution means the smallest distance at which objects become distinguishable from one another. We can observe a bacterial colony containing thousands of bacteria, but we cannot resolve each bacterium. To resolve or distinguish the individual cells requires magnification with an instrument having increased resolution.

3. How do eukaryotic and prokaryotic cells differ in appearance under the light microscope?

   ANS: Eukaryotic cells are generally larger, and their internal compartmentalized structure can be resolved. Prokaryotic cells tend to be smaller so they can be detected, but internal details are generally too small to be resolved.

4. Explain how electromagnetic radiation carries information and why different kinds of radiation can resolve different kinds of objects.

   ANS: Electromagnetic radiation is a form of energy propagated as waves associated with electrical and magnetic fields. Visible light, ultraviolet light, X-rays, and gamma rays all travel as waves; visible light has the longest wavelength and gamma rays have the shortest. The shorter the wavelength of the energy, the greater is the resolving power.

5. Describe how light interacts with an object through absorption, reflection, refraction, and scattering.

   ANS: *Absorption:* A photon's energy is absorbed by the object and usually converted to a different form of electromagnetic radiation. In bright-field microscopy, when a specimen absorbs light, it is observed as a dark spot against a bright field.

   Reflection: Reflection occurs when a wavefront is redirected from the surface of an object at an angle equal to its incident angle. It is used in the optics of a microscope.

   Refraction: Light bends when it enters an object (such as glass) with a higher refractive index than air. The speed and direction of the light change, resulting in a wider emerging wavefront.

   Scattering: This occurs when a portion of the wavefront is converted to a spherical wave originating from the object. Special optics can use scattered light to detect microbial shapes smaller than the wavelength of light (dark-field microscopy).

6. Explain how refraction enables magnification of an image.

   ANS: When light passes through a refractive material that is shaped to spread the light waves, the image is magnified. When an object is placed within the focal plane of a lens, the light rays from the object are bent by the lens and converge at the opposite focal point. The light rays continue from the focal point and generate an inverted but magnified image of the object.

7. Explain how magnification increases resolution and why empty magnification fails to increase resolution.

   ANS: When an image is magnified by lenses with increased resolution, the distances between parts of the image are enlarged, enabling us to resolve finer details. Empty magnification occurs when details of an image are enlarged in proportion to the entire object. An example of empty magnification is enlarging a pixelated photo. No more detail will be gained; each pixel will simply be enlarged in proportion to the overall picture. Nothing will be gained except size.

8. Explain how angle of aperture and resolution change with increasing lens magnification.

   ANS: The greater the angle of aperture of the lens, the better is the resolution. With a lower magnification lens, the angle of aperture is small, the specimen is farther away, and there is a wide Airy disk. With a higher magnification lens, the angle of aperture is larger, the specimen must be closer to the lens, and there is a narrow Airy disk.

9. Summarize the optical arrangement of a compound microscope.

   ANS: A compound microscope has a light source at the bottom; light passes through the diaphragm, the condenser lens, the specimen, the objective lens, the ocular lens, and then ultimately reaches the eye.

10. Explain how to focus an object and how to tell when the object is in or out of focus.

    ANS: Most microscopes are parfocal, so it is easiest to focus with a low-power objective first, since it generates a greater depth of field. It is possible then to rotate the higher-power lens into view and then perform only a minor adjustment of focus. An object is in focus when its edge appears sharp and distinct

from the background.

11. Explain the relative advantages and limitations of wet mount and stained preparations for observing microbes.

    ANS: The advantage of wet mount preparation is that the specimens may be observed in their natural state without any introduced artifacts. A major disadvantage, however, is that most cells are transparent and there is little or no contrast between the specimen and its background. For this reason, detection and resolution are minimal. To stain a specimen, it must be fixed and then stained. Fixing kills the cells and the stains introduce contrast, allowing the microbe to be observed. However, the fixation or the staining process can introduce artifacts.

12. Explain the significance (and limitations) of the Gram stain for bacterial taxonomy.

    ANS: The Gram stain is a key tool for chemical identification of species in the clinical laboratory. It is used to categorize cells as either Gram-positive, if they retain the crystal violet stain, or Gram-negative, if they do not. The nature of their cell walls determines which category they fall into. The Gram stain differentiates between two major bacterial taxa, Proteobacteria (Gram-negative) and Firmicutes (Gram-positive). Some organisms have very different cell walls and cannot be distinguished by the Gram stain.

13. Explain the basis of dark-field, phase-contrast, and fluorescence microscopy. Give examples of applications of these advanced techniques.

    ANS: Dark-field microscopy uses a "spider light stop" in the condenser lens to detect light scattered by an object. This allows objects to be observed as spots of light in a dark background. This is exceptionally useful for studying bacterial motility. Samples must be very clean because even small dust particles will be observed.

    In phase-contrast microscopy, an annular ring is used. This allows both refracted light from the specimen and the outer cone of transmitted light to be detected. The waves are out of phase, which ultimately results in regions of darkness within the specimen. This technique is particularly useful for eukaryotic organisms, which contain many intracellular components.

    Fluorescence microscopy takes advantage of the fact that some compounds fluoresce. Chlorophyll, for example, is a cellular compound that fluoresces, so chlorophyll-containing microbes may be observed directly with a fluorescence microscope. Most of the time, however, it is necessary to use some sort of stain that fluoresces (a fluorophore) to observe cells or cellular components. DAPI, for example, binds to DNA and fluoresces. One can also attach a fluorophore to an antibody for immunofluorescence studies.

14. Explain how super-resolution imaging enables tracking of intracellular molecules.

    ANS: When a fluorophore emits light, the light emission is not uniform. There is a peak intensity surrounded by light of less intensity. Using computer techniques, the location of peak light emission can be estimated to a much greater precision than is possible with the naked eye. This allows for the tracking of something like a molecule, which is too small to resolve with light microscopy and without the use of

these super-resolution techniques.

15. Explain the difference between transmission and scanning electron microscopy, including the different applications of each.

    ANS: In TEM, the specimens are fixed, embedded, and then cut into thin sections prior to staining. The electron beam is transmitted through the thin sections and the stains increase the contrast within the cell. One can compile data from sequential sections to obtain a composite like a three-dimensional (3D) model of a specimen. In SEM, the specimen is shadowed with a heavy metal. The electron beam then is deflected off the surface of the specimen, allowing observation of peaks and valleys. This generates a type of relief map of a surface. It is also possible to look at an inner surface by subjecting the specimen to freeze-fracture prior to shadowing.

ANSWERS TO END-OF-CHAPTER THOUGHT QUESTIONS (P. 74)

1. Explain what features of bacteria you can study by: (a) light microscopy; (b) fluorescence microscopy; (c) scanning EM; (d) transmission EM.

   ANS: Light microscopy shows the overall shape of bacterial cells. In a stained specimen, light microscopy can reveal a particular aspect of a cell, such as a Gram-positive cell wall. Fluorescent microscopy can show the position of subcellular parts, even a single molecule such as a protein bound to a DNA origin sequence. The shape of the protein, however, is only detected, not resolved. Scanning EM resolves details of the surface contours of bacteria. Transmission EM reveals many subcellular structures, such as ribosomes and DNA fibers, as well as isolated cell parts such as the flagellar motor.

2. Explain how resolution is increased by magnification. Why can't the details be resolved by your unaided eye? Explain why magnification reaches a limit. Why can it not go on resolving greater detail?

   ANS: The resolution of the human eye is limited by the distance between photoreceptor cells in the retina. As an image is magnified, the distance between light rays originating from the object increases. The rays carry information, including the distance between points of an object. Eventually the rays diverge enough that they can be resolved by the retina. Magnification, however, is limited by the wavelength of light. If the details of an object are smaller than the wavelength of light, then the width of the Airy disk interference pattern exceeds the size of the details (Fig. 2.14). As the light rays diverge, the Airy disk only expands and fails to show any smaller details.

3. Explain why artifacts appear in microscopic images, even with the best lenses. Explain how you can tell the difference between an optical artifact and an actual feature of an image.

   ANS: Every lens has an edge. At the edge, the light rays deviate from the parabolic focus. Thus, all lenses cause artifacts arising from aberrations. In addition, some parts of the specimen are always out of focus. An object outside the focal plane may look blurred, or it may appear as a ring with a bright center. To tell whether the appearance of an image is characteristic of the object, or whether it arises from the optics, try focusing up and down. If a ring-shaped feature disappears as the object appearance sharpens,

then the ring shape was an artifact of the optical system.

4. How can "detection without resolution" be useful in microscopy? Explain with specific examples.
   ANS: Detection without resolution is useful in dark-field microscopy. In dark-field, the curve of a flagellum can be detected even though the protein filament is narrower than the wavelength of light, so it is possible to see the flagella rotating on a living bacterium. In fluorescence microscopy, the position of tiny subcellular structures such as the DNA-replicating apparatus can be detected without resolution. While the fluorescent "blob" appears much larger than the structure, its position within the cell is nonetheless accurate and can show how the structure functions within the cell.