Chapter 5

# Microscopy

## After reading this chapter, you should be able to:

- Define and differentiate between magnification and resolving power
- Describe the fundamental parts of a standard light microscope and how they manipulate light to magnify an image
- Compare the relative strengths and limitations of common forms of microscopy
- Discuss issues related to image preparation and analysis

## Techniques covered:

- **Light microscopy**: brightfield, phase-contrast, darkfield, differential interference contrast (DIC/Nomarski)
- **Fluorescence microscopy**: epifluorescence, confocal, two-photon laser scanning microscopy, superresolution microscopy, total internal reflection fluorescence (TIRF) microscopy, light sheet fluorescence microscopy
- **Electron microscopy**: transmission electron microscopy (TEM), scanning electron microscopy (SEM), electron tomography (ET)
- **Microscopy data**: image processing and interpretation

The first microscopes were used in the 17th century to expose the microscopic world of cells and single-celled organisms for the first time. Scientific pioneers such as Robert Hooke and Anton von Leeuwenhoek, often called the "fathers of microscopy," used homemade microscopes to study cell types from a variety of living organisms. In the late 19th and early 20th centuries, Santiago Ramón y Cajal used a microscope in combination with histology to produce highly detailed, seminal studies of nervous system structure.

The microscope remains an indispensable tool in neuroscience. Because organelles, glial cells, neurons, and even populations of neurons cannot be seen by the naked eye, a microscope is essential for examining the nervous system at the cellular level. Light microscopes can enlarge the image of an object up to 1000 times greater than normal, providing access to the structure of a cell and its local environment. Fluorescent microscopes provide an even greater ability to highlight individual subcellular structures. Electron microscopes can theoretically enlarge an image one million times greater than

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normal, providing unparalleled insight into the smallest structures including synapses, surface receptors, and even individual proteins.

The goal of this chapter is to provide a basic description of common forms of microscopy. First, we define the fundamental parameters and parts of a light microscope. Then, we survey the different forms of microscopy and why an investigator might choose one form over another. Finally, we examine issues related to processing and interpreting microscopy data. The information in this chapter will complement information in many other chapters, especially methods in Chapters 6 and 7 used to investigate neural structure and function.

## ESSENTIAL PRINCIPLES OF MICROSCOPY

While modern microscopes are undoubtedly more advanced than those used a few centuries ago, the fundamental parts of a microscope and the theory behind how they work together are essentially the same.

### Fundamental Parameters in Microscopy

There are two important values to consider in microscopy: magnification and resolution. **Magnification** refers to how much larger a sample appears compared to its actual size. Fig. 5.1 provides a sense of the relative size of various nervous system structures, from an entire brain down to individual atoms. The naked eye is able to perceive objects about 0.2 mm in size and larger. Therefore, it is possible to view entire brains and large neural structures, but not individual neurons or axons without additional magnification.

**Resolution** (or **resolving power**) refers to the minimum distance by which two points can be separated yet still be distinguished as two separate points. For example, imagine you are looking at the side of a building with a mosaic image made up of thousands of colored tiles. If you were standing a block away from the building, you would see the entire image but would be unlikely to make out the individual tiles themselves because the resolution would be too low to see that level of detail. If you move closer, the resolution becomes better and you can see the distinct tiles making up the image. Likewise, in microscopy, it can be difficult to perceive two structures as separate from one another—only by increasing the resolving power is it possible to make out distinct points in a specimen. Fig. 5.1 characterizes the resolution of commonly studied neural structures, demonstrating the range of objects that can be resolved by the naked eye and the increased resolving power provided by light and electron microscopy.

Though it may seem that the resolving power of a microscope is based on the ability of the microscope to magnify an object, this is not the case. Magnifying an object does not necessarily improve the clarity or resolution of the final image. The resolution of a magnified object depends on two

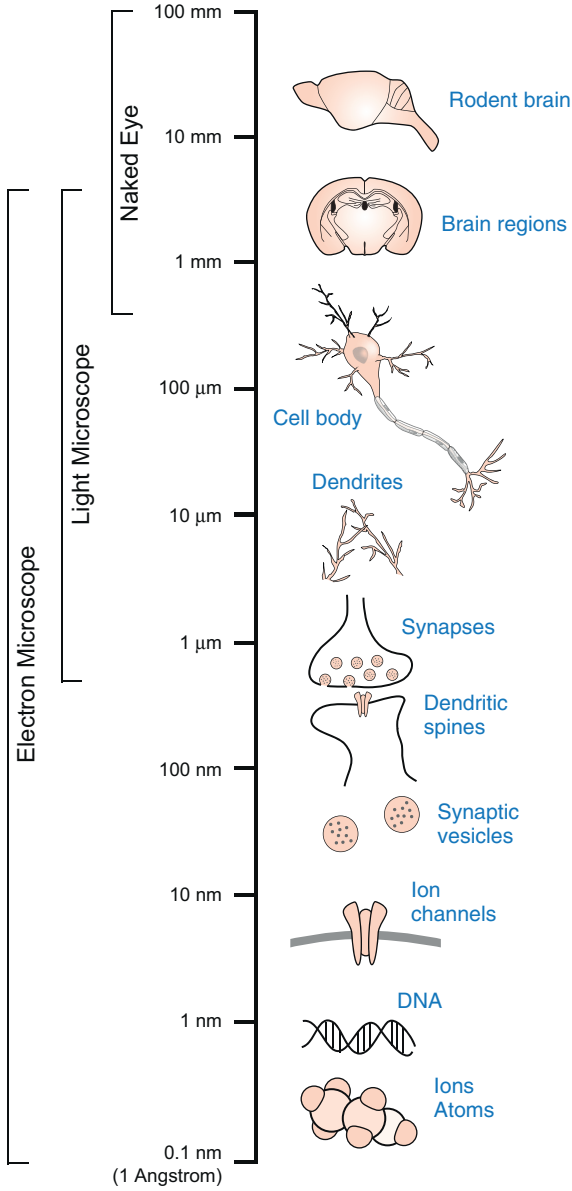


**FIGURE 5.1 Resolving power in microscopy.** The resolution required for distinguishing between neuroanatomical structures, from a rodent brain to individual atoms.

factors: numerical aperture and the wavelength of the light source. The **numerical aperture (NA)** is a measure of the light-collecting ability of the microscope objective, the lens that gathers and focuses light from the specimen.

An objective with a higher NA will collect more light rays, leading to better resolving power. The NA of an objective depends on the angle that light enters into the objective and the index of refraction of the medium in which the objective is working (Fig. 5.2). The **index of refraction** (or **refractive index**) indicates changes in the speed of light traveling through a particular medium (1.0 for air, 1.33 for water, and up to 1.55 for oils). The greater the index of refraction, the greater the NA. As light travels from one medium (e.g., air) to a medium with a higher index of refraction (e.g., oil), the angle of the light changes, allowing an objective to collect more light. This is the same principle that causes a straw in a glass of water to appear bent at the surface of the water. An objective lens immersed in oil has a higher NA than an objective in air, and thus the oil-immersed objective has a greater resolving power.

The other factor that determines the resolution of a magnified object is the wavelength of light either illuminating or emanating from the specimen (as in fluorescence microscopy). The wavelength of light is the distance between two repeating units of the light wave (Fig. 5.3A). Light rays with different wavelengths are perceived as different colors (Fig. 5.3B). The wavelength of light affects how broad a single point of light appears. Shorter wavelengths of light (UV through green) appear sharper than longer wavelengths (red through infrared), which tend to appear more spread out, decreasing resolving power.

In summary, the smallest object that can be observed using a microscope is a function of the magnification and resolving power used to image a specimen. Interestingly, while the ability of a microscope to magnify an object is essentially unlimited, the resolution is finite. A light microscope cannot resolve details of a specimen finer than 0.2 µm—about the size of a neural synapse—no matter what the magnification. This limitation is due to the minimum wavelength of visible light; wavelengths shorter than the UV to

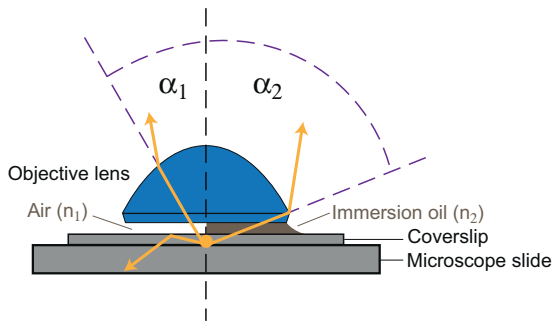


**FIGURE 5.2 Numerical aperture and the angle of light entering the objective.** An objective with a higher NA collects more light rays, leading to a higher resolving power. The NA depends on the angle that light enters the objective, $\alpha$, as well as the refractive index of the medium, $n$. When light travels through material with a different n, such as between glass and air ($n_1$), it refracts more and yields a smaller $\alpha$. Immersion, such as water or oil ($n_2$), reduces the difference and allows for a greater $\alpha$.

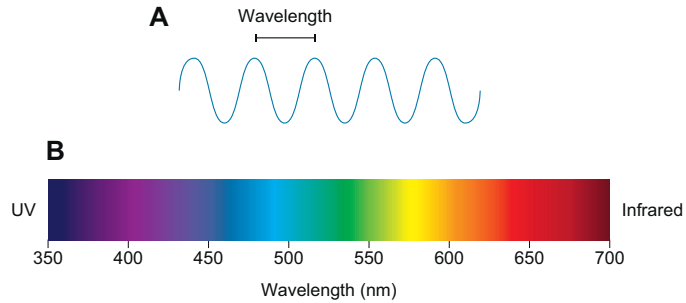


**FIGURE 5.3 The visible spectrum of light.** (A) The wavelength of light is the distance between two repeating units of a propagating light wave. (B) Light rays of different wavelengths appear as different colors to the human eye. Shorter wavelengths appear violet-blue, while longer wavelengths appear more red.

green portion of the spectrum are invisible to the human eye. This means that the resolving power of any light microscope has a maximal value. The unaided human eye is limited to seeing details about 0.2 mm in size. A typical light microscope can only resolve details of about 0.2 μm, so magnifying those details more than 1000× (1000 × 0.2 μm = 200 μm = 0.2 mm) would not be useful. Increasing the magnification higher than 1000× is like using the zoom feature on a computer to enlarge a digital picture of a fixed size: you will not get any finer details with a digital zoom—you simply make the pixels bigger. Forms of microscopy that do not use visible light to image a specimen, such as electron microscopy, have much higher resolving powers and can image much smaller structures than light microscopes. Additionally, recent advances in **super resolution fluorescence microscopy** combine sophisticated optics with properties of fluorescent molecule kinetics to resolve details finer than 0.2 μm in fixed tissues.

Now that we have discussed magnification and resolution, we examine the fundamental parts and design of two categories of microscopes: compound microscopes and stereomicroscopes.

## The Design of a Compound Microscope

The simplest form of microscopy, one that has been used for centuries, is the magnifying glass. A magnifying glass is a convex lens that enlarges nearby objects (Fig. 5.4). It works by refracting the light scattered by an object to a focal point on the other side of the glass. What appears is a larger image of the object behind the lens.

A single lens has limited magnification ability, but multiple lenses can be arranged, one behind the other to multiply the magnification of each lens. This arrangement is the basis of the **compound microscope**. The term *compound* refers to the two or more lenses used in concert to magnify an object.

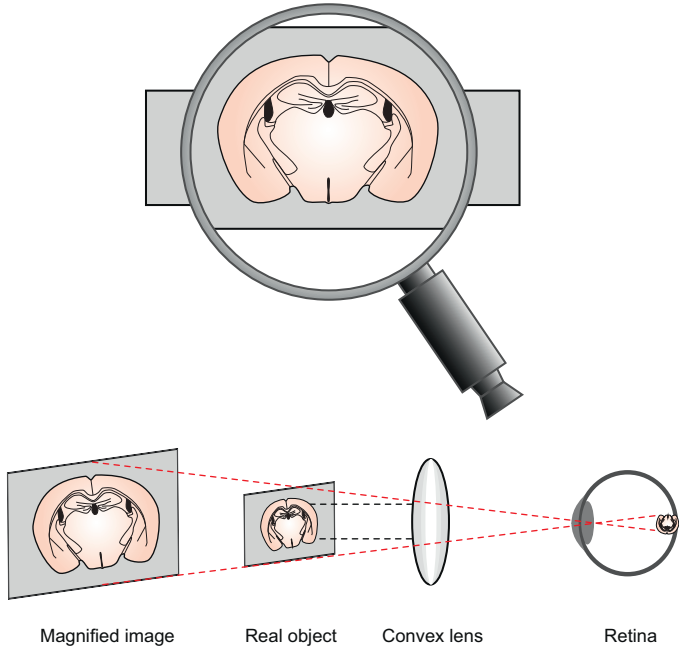


**FIGURE 5.4 The magnifying glass.** A magnifying glass is a convex lens that refracts light to make an object appear larger.

Most light microscopes use at least two lenses. The first lens is called the **objective lens** and is placed adjacent to the specimen (Figs. 5.5 and 5.6). Investigators can choose from multiple objective lenses to use a desired degree of magnification, usually 4×, 10×, 20×, 40×, or 100×. The second lens is located in the eyepiece and is referred to as the **ocular lens**. This lens is often set at 10×. The total magnification of the microscope is multiplicative, so if the objective lens is set at 40× and the ocular lens is set at 10×, the total magnification achieved is 400×.

A **condenser** focuses light from a light source onto a specimen (Figs. 5.5 and 5.6). Light transmitted through the specimen is then collected and magnified by the objective lens. This magnified image is focused onto the ocular lens, which essentially acts like a magnifying glass to further enlarge the image. Although each manufacturer designs light microscopes with unique features, the conceptual design of a compound microscope remains the same.

Compound microscopes are classified as being either upright or inverted. In an **upright microscope**, the specimen is placed just below the objective (Fig. 5.7A). This type of microscope is ideal for examining a specimen mounted on a glass slide. However, there is little space between the objective

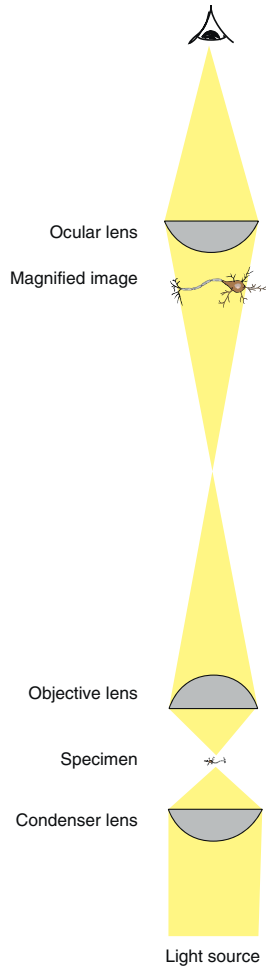


**FIGURE 5.5 Compound microscopes use multiple lenses to magnify a specimen.** A condenser lens focuses light onto a specimen, which then transmits (or emits) light through the objective and ocular lenses that magnify the image that reaches the eye.

and specimen, making it impractical for viewing living cells in a thick cell culture dish or inserting an electrode in electrophysiology experiments. For cell culture experiments, it is more optimal to use an **inverted microscope** in which the objective is located below the specimen and the light source and condenser are located relatively far above the sample (Fig. 5.7B).

The other parts of a basic compound microscope (Fig. 5.6) allow the investigator to adjust the magnification, focus, and light level of the microscope, as well as the placement of the specimen.

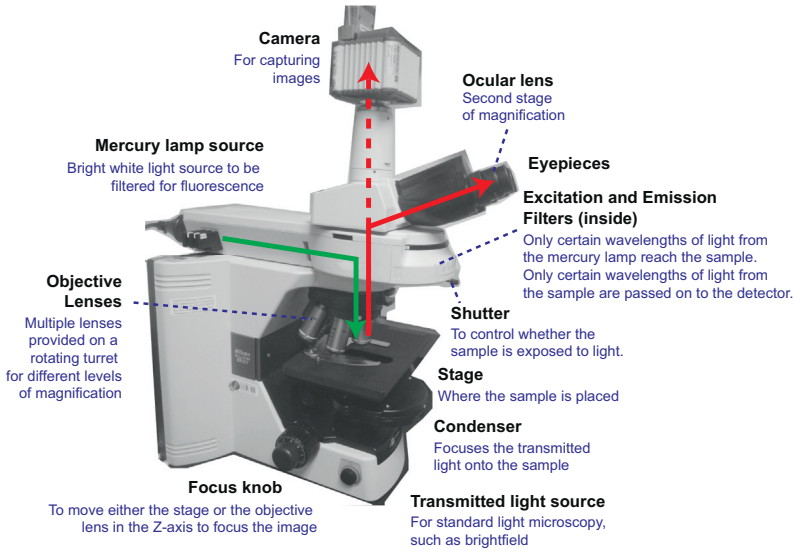


**FIGURE 5.6** **Components of a compound microscope.** In brightfield microscopy, the light comes from the transmitted light source. In fluorescent microscopy, the light comes from a mercury lamp (*green arrow*). Light is transmitted through the specimen or excited from the specimen and travels to the eyepieces or camera (*red arrow*).

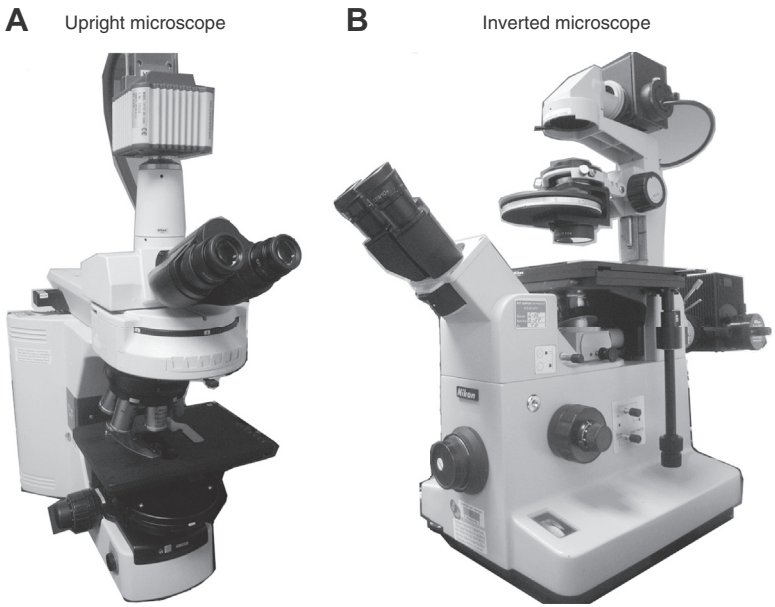


**FIGURE 5.7** **Upright versus inverted microscopes.** (A) In an upright microscope, the specimen is placed just below the objective. (B) In an inverted microscope, the specimen is placed just above the objective, allowing for experimental access from above.

## The Design of a Stereomicroscope

A **stereomicroscope**, also called a **dissecting microscope**, serves a different purpose than a compound microscope and works in a different way (Fig. 5.8). The main purpose of a stereomicroscope in neuroscience is to examine the three dimensional surface of brains, tissue slices, or large neural structures. It is especially useful for the fine manipulation needed during dissections, surgeries, or the fabrication of small tools such as electrodes or implants. Rather than passing through a single objective and ocular lens system, light in a stereomicroscope goes through two separate lens systems. While a compound microscope directs light to both eyes from a single lightpath, a stereomicroscope directs light to each eye from two independent lightpaths. Because light from a single point on the specimen travels independently through two different paths to reach each eye, the specimen appears three dimensional.

In addition to the appearance of the specimen, there are at least two other major differences between a stereomicroscope and compound microscope. First, stereomicroscopes typically use light that is reflected off a specimen, while compound microscopes often use light transmitted through a specimen. Therefore, stereomicroscopes are useful for examining specimens that are too

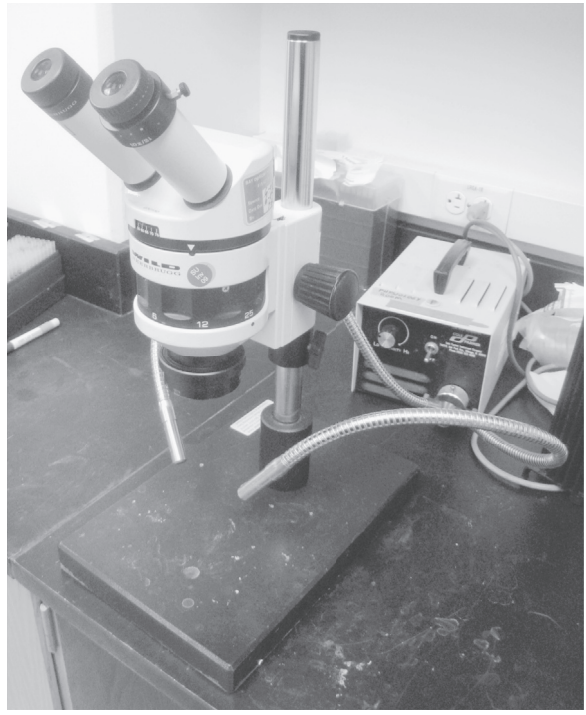

FIGURE 5.8 **A standard stereomicroscope.**

thick for light to pass through. Second, the magnification power of a stereomicroscope is often less than a compound microscope. The ocular lenses of a stereomicroscope are usually fixed at 10× and the objective lenses typically range from 0.1 to 8×. Therefore, a stereomicroscope is useful for examining gross neural structures, while a compound microscope is better for examining microscopic structures such as single neurons or fiber tracts.

Now that we have examined the basic concepts of microscopy, we survey the various categories of light microscopy and why each may be particularly useful for a given experiment.

## LIGHT MICROSCOPY

A **light microscope** is any microscope that uses visible light to illuminate and image a specimen. This definition includes white light composed of all wavelengths, as well as light of a specific wavelength used in fluorescent microscopy. Usually when people refer to light microscopy, they refer to nonfluorescent microscopy, even though fluorescent microscopy does, of course, use light.

The most common and general form of light microscopy is **brightfield microscopy**, in which light passes directly through or is reflected off a specimen. Most cells and tissues are transparent due to their high water content. Unless naturally pigmented or artificially stained, distinct structures are difficult to differentiate using brightfield microscopy. Thus, a variety of histological procedures have been developed to preserve and stain specimens to enhance contrast among different microscopic structures (Chapter 6). However, most of these procedures result in the death of cells to preserve the specimen.

For many experiments, working with preserved cells is not a problem. However, when an investigator wishes to magnify living, unpreserved cells or tissues, such as in cell culture experiments, it is necessary to provide contrast without killing the specimen. Thus, methods of manipulating light to enhance contrast have been developed to make resolvable details stand out to the eye without requiring the application of special dyes (Fig. 5.9). For example, variations in density within cells cause tiny differences in the way distinct regions scatter light. Therefore, different cellular structures have different indices of refraction. **Phase-contrast microscopy** takes advantage of these slight differences, amplifying them into larger intensity differences with high contrast that can be more easily discerned. Because this form of microscopy does not require histological staining of the specimen, phase-contrast is often used to examine cultured cells. **Darkfield microscopy** (or **darkground microscopy**) illuminates a specimen using oblique rays of light directed from the side so that only scattered light enters the objective lens. The regions of the specimen that do not scatter much light, such as the cytoplasm of cells, will appear dark. In contrast, highly refractive organelles that scatter large amounts of light appear bright. **Differential interference contrast (DIC) microscopy**, also known as **Nomarski microscopy**, exaggerates changes in the light-scattering

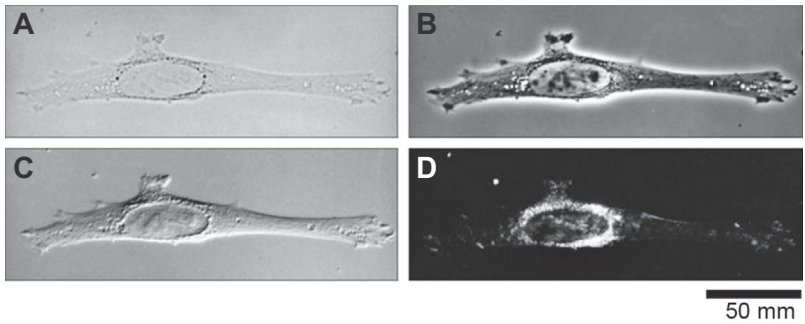


**FIGURE 5.9 Four categories of light microscopy.** Various methods of light microscopy are used to enhance contrast and visualize details in unstained tissue preparations. (A) Standard brightfield microscopy. (B) Phase-contrast microscopy. (C) Differential interference contrast (DIC/Nomarski) microscopy. (D) darkfield microscopy. *Reprinted from Alberts, B., et al., 2002. Molecular Biology of the Cell, fourth ed., Fig. 9.8, with permission from Garland Science: New York, NY).*

properties of cellular structures, highlighting the edges around structures to create a textured, three-dimensional appearance. Taken together, these different forms of light microscopy enhance different features of living cells depending on the specific needs of the investigator.

## FLUORESCENCE MICROSCOPY

**Fluorescence microscopy** takes advantage of specialized fluorescent molecules called **fluorophores** that have the property of absorbing light at a specific wavelength and then emitting light at a different, longer wavelength. Fluorophores that absorb blue light typically emit green light; fluorophores that absorb green light emit red; fluorophores that absorb red emit infrared, and so on. Most fluorophores are small organic molecules or genetically encoded fluorescent proteins, such as GFP. There are dozens of commercially available and commonly used fluorophore molecules each with their own characteristic absorption (excitation) and emission wavelengths (Fig. 5.10). These molecules can be linked to antibodies or other molecular probes to indicate the presence of specific proteins, organelles, or other structures within a cell.

The principal use of fluorescence microscopy is to examine specimens labeled with these fluorescent reagents or expressing genetically encoded fluorescent proteins (Chapter 6). Light from an extremely bright source passes through an **excitation filter** that allows only light in a specific range of wavelengths through the objective to illuminate the specimen. That light is absorbed by and excites the fluorophores in the specimen, which react by emitting a longer wavelength of light. The emitted light is collected back through the objective and passed through a second filter, an **emission filter**. The emission filter blocks extraneous wavelengths of light, including the light

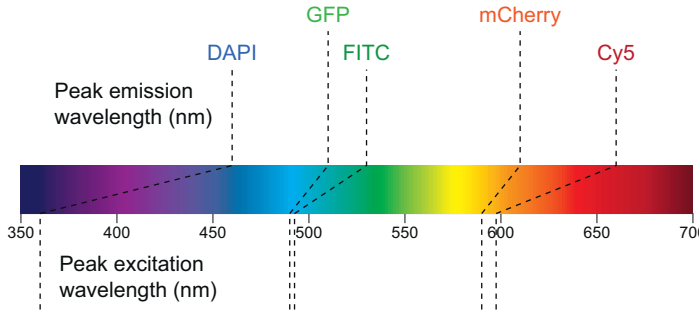


**FIGURE 5.10 Excitation and emission spectra of some common fluorophores.** DAPI, a chemical that intercalates with DNA and stains the nuclei of cells, is excited by UV light and emits blue light. Green fluorescent protein (GFP) and the FITC fluorophore are excited by blue light and emit green light. The mCherry reporter protein and Cy5 fluorophore are excited by green/yellow light and emit red light.

used to illuminate the specimen, but allows emitted light wavelengths to pass through to the eyepieces or a camera. The optical filters make it appear as if structures tagged with the fluorophores light up against a dark background. Because the background is dark, even a tiny amount of the glowing fluorophore is visible, making this form of microscopy a very sensitive technique.

A major appeal of fluorescence microscopy over conventional light microscopy is that structures and proteins within a cell can be labeled with a wide range of fluorophores. Because each fluorophore has its own characteristic excitation and emission spectra (the wavelengths of light they absorb and emit), it is possible to label different structures in the same sample and investigate their relative locations. For example, an investigator can use a fluorophore that emits green light to label one structure, and a fluorophore that emits red light to label another. Because the excitation and emission spectra of the green and red fluorophores do not overlap, they distinctly mark the locations of these different proteins within the same sample. This technique can even be used in living specimens to observe the dynamic interactions of multiple fluorescently labeled proteins and molecules.

Despite the advantages of fluorescence microscopy, there are some disadvantages. One major limitation is that fluorescent reagents cannot be illuminated indefinitely. The intensity of the light emitted from a fluorophore will decrease over time as it is continuously exposed to light, a process called **photobleaching**. This limitation makes it necessary to minimize the light exposure of fluorescently tagged specimens and capture images of them before fluorescence becomes too dim. A limitation in live cell imaging is **phototoxicity**, in which illumination leads to the death of cells expressing the fluorophore due to free-radical generation. Finally, while fluorescently labeled structures *can* be detected easily, in practice, background noise can mask the actual signal of interest. Background noise is caused by nonspecific

fluorescence that does not indicate true specific signal. One form of background noise is **autofluorescence**, in which certain structures and chemicals naturally fluoresce without the addition of a fluorophore. If this fluorescence has the same emission wavelength as the fluorophore, it can be difficult to determine true signal from noise.

Fluorescence microscopy has advanced every area of neuroscience. Fluorescent molecules have made breakthroughs possible in studying neural structure and function, identifying the spatial relationships of proteins within a cell, examining the fine branching of neural structures, and detailing the time course of a cell's functional response to chemical or electrical stimulation. Various forms of microscopy attempt to maximize the advantages and minimize the disadvantages of fluorescent reagents. We now survey the main categories of fluorescence microscopy.

## Epifluorescent Microscopy

The most fundamental form of fluorescence microscopy is **epifluorescent microscopy**, also known as **wide-field fluorescent microscopy**. Epifluorescent microscopes work as just described: specimens labeled with fluorophores or expressing fluorescent proteins are illuminated by light with a specific excitation wavelength (Fig. 5.11A). The specimen is then viewed using a second

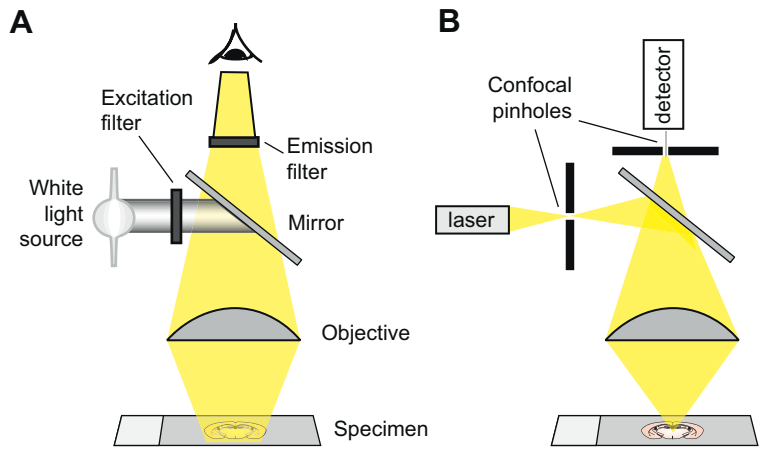


**FIGURE 5.11 Epifluorescent and confocal microscopy.** (A) Conceptual strategy of an epifluorescent microscope. Light from a bright lamp passes through a filter that only allows light of the excitation wavelength through to the specimen. Light emanates from the specimen and passes through a second filter that is opaque to the excitation wavelength of light but that transmits the longer wavelength of the emitted light. (B) Conceptual strategy of a confocal microscope. Light is focused from a pinhole aperture onto a single point on the specimen. Fluorescent light emanating from the specimen is transmitted through a second pinhole aperture before hitting a detector. The two apertures are *confocal* with each other, so out-of-focus fluorescence is avoided by the detector pinhole.

filter that is opaque to the excitation wavelength but transmits the longer wavelength of the emitted light. Thus, the only light transmitted through the eyepiece is the light emitted by the specimen. Most epifluorescent microscopes are placed on a solid surface in a dark room to allow for optimal visualization of neural specimens. However, advances in miniaturizing the components of microscopes have made it possible to create extremely small epifluorescent microscopes that can be mounted on the head of a freely moving rodent to visualize fluorescent biosensors in vivo (Chapter 7).

Epifluorescence is an excellent tool, but has one major disadvantage: light excites fluorophores throughout the entire depth of the specimen. Thus, fluorescence signals are collected not only from the plane of focus but also from areas above and below this plane. Such background fluorescence can lead to hazy, out-of-focus images that appear blurry and lack contrast. Thus, the value of epifluorescence in relatively thick specimens ($>15-30$ μm) can be limited. Alternative forms of fluorescent microscopy attempt to limit out-of-focus fluorescence to produce sharper, clearer fluorescent images.

## Confocal Microscopy

**Confocal microscopes** produce clear images of structures within relatively thick specimens by selectively collecting light from thin ($<1$ μm) regions of the specimen. It is the tool of choice for examining fluorescently stained neurons in brain slices or small, intact organisms such as *Drosophila* or embryonic zebrafish.

With epifluorescence, the entire specimen is illuminated at once such that all fluorophores emit light at the same time, reducing the signal-to-noise ratio and making it difficult to clearly perceive signals from a specific focal plane within a thick specimen. In a confocal system, light from a laser passes through a pinhole aperture that focuses the light at a specific depth in the specimen (Fig. 5.11B). Although this pinhole aperture restricts focused illumination to one plane, other regions receive dispersed, less intense illumination and emit out-of-focus fluorescence. However, all the emitted light passes through a second pinhole aperture before reaching a detector. The key aspect of confocal microscopy is that the pinhole aperture in front of the detector is at a position that is *confocal* with the illuminating pinhole—that is, the only light to reach the detector comes from the same plane in the specimen where the illuminating light comes to a focus, minimizing background fluorescence and maintaining sharp focus on a single plane. The detected light is digitized and sent to a computer for display and storage.

To generate a two-dimensional image, data from each point in the plane of focus is collected sequentially by scanning across the field. Confocal microscopes can focus the laser beam at precise intervals throughout the thickness of the specimen, producing optical sections at multiple focal planes. Computer image analysis can then be used to "stack" these sections to reconstruct a three-dimensional volume with optimal contrast and resolution.

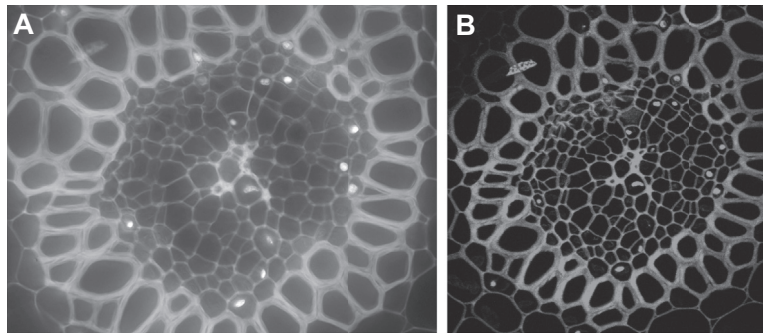


**FIGURE 5.12 Comparison of image quality in epifluorescence and confocal microscopy.** (A) A standard epifluorescent microscope captures fluorescence emitted from all planes, not just those in focus, which can cause a hazy background. (B) A confocal microscopy image of the same specimen appears sharper and in focus.

A major advantage of confocal microscopy over standard epifluorescence is the ability to produce sharp images of cells and cellular structures without background fluorescence (Fig. 5.12). However, there are still limitations to confocal microscopy. Because confocal lasers scan specimens point by point to form a complete image of the specimen, samples are exposed to intense light for a longer time period than epifluorescent microscopes, making photobleaching and phototoxicity more problematic. The longer time necessary to capture an image also makes it less desirable for live cell imaging of extremely fast events. In addition, though it is a great advantage to be able to create three-dimensional reconstructions by "stacking" multiple optical sections, the lateral X−Y resolution is finer than resolution in the Z (depth) dimension, so three-dimensional reconstructions may appear warped. Despite these disadvantages, confocal microscopy is a fantastic tool for imaging neural specimens, especially thick brain sections or even entire small invertebrate organisms.

## Two-Photon Microscopy

**Two-photon microscopy**, also referred to as **two-photon laser scanning microscopy**, is a further refinement of precision fluorescence microscopy. In epifluorescent or confocal microscopy, a single photon of light at a specific wavelength excites a fluorophore to emit light (Fig. 5.13A). In two-photon microscopy, a fluorophore is excited by absorbing two photons simultaneously. Each photon has a wavelength that is twice the usual excitation wavelength, and thus has half the energy level per photon. When a fluorophore absorbs the two lower-energy photons at the same time, each contributes half the needed excitation energy to interact with the fluorophore as if a single photon with more energy and a shorter wavelength had struck it (Fig. 5.13B).

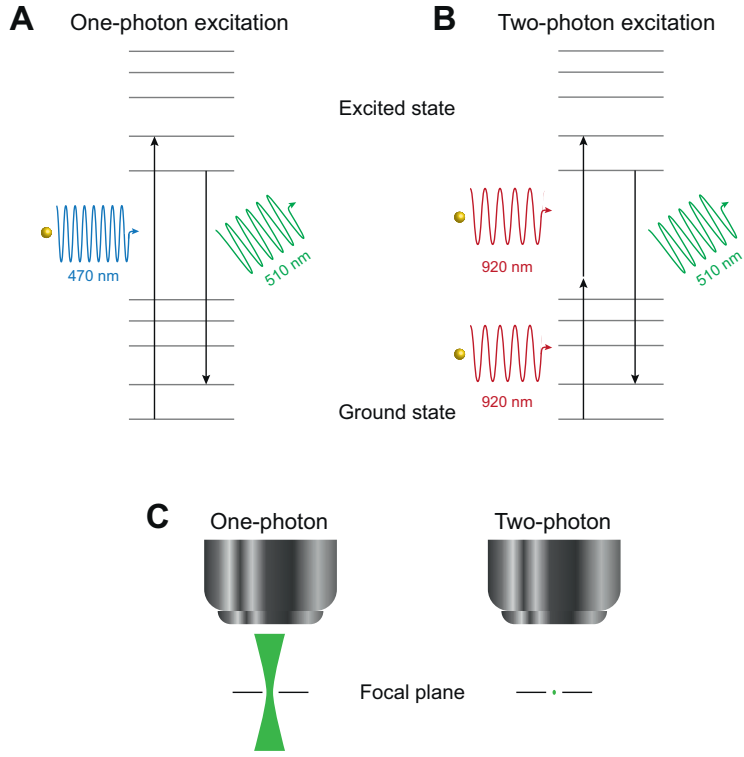


**FIGURE 5.13** **Two-photon imaging.** (A) In one-photon excitation, a single photon of light (in this case, 470 nm light) moves an electron from the ground state to an excited state. When the electron returns to the ground state, slightly longer wavelength light is emitted. (B) In two-photon excitation, the energy required to excite the electron comes from two photons instead of one. The excitation wavelength in two-photon microscopy is therefore twice that of single-photon microscopy. (C) Both single-photon and two-photon excitation focus a beam of light at the focal plane. In one-photon excitation, fluorescent molecules above and below the focal plane are excited by out-of-focus light and thus emit fluorescence. In two-photon excitation, only the fluorescent molecules that are at the focal plane receive enough photons for two to hit electrons at the same time. Thus, no excitation occurs above or below the focal plane.

The main advantage of this method is that the near-simultaneous arrival of two photons is an extremely rare event, so fluorescence excitation is restricted to a narrow plane of focus within the specimen. This process results in clearer images than traditional confocal microscopy, even in cases where relatively few fluorophores are present, because photons are only emitted from the excited fluorophores located at the focal plane. Thus, all emitted light can be collected. There is not enough energy to excite fluorophores above and below the focal plane to produce any background fluorescence (Fig. 5.13C).

Two-photon microscopy can penetrate deeper into tissue (500 μm−1 mm) than confocal or epifluorescence microscopy because the excitation

wavelength is twice as long as the usual wavelengths used. Light with longer wavelengths scatters less, allowing deeper penetration into tissue. Because excitation is restricted to a focal point, it also causes less photobleaching and phototoxicity, making two-photon microscopy particularly useful for extended observation of cells in undissected, living brain tissue in whole animals, or fine-scale neural structures in brain slices. For example, through glass cranial windows or implanted GRIN lenses (Chapter 7), two-photon microscopy can be used to record the activity of hundreds of neurons simultaneously while mice are exposed to a stimulus or performing a task. The resolution and stability over time has even allowed scientists to track subcellular structures such as dendritic spines as mice learn a task. Some laboratories have used three-photon microscopes that utilize even longer wavelengths of light to penetrate deeper into tissue with higher resolution.

## Light Sheet Fluorescence Microscopy

Like confocal and two-photon microscopy, **light sheet fluorescence microscopy** uses optical sectioning to image tissue in vivo or in vitro at cellular resolution. However, light sheet microscopy differs from confocal and two-photon in how it achieves optical sectioning. Both confocal and two-photon microscopy rely on point scanning, whereby a single point of focused light moves throughout the tissue to excite fluorophores. In contrast, light sheet microscopy uses a thin sheet of focused light to illuminate a two-dimensional plane of tissue (Fig. 5.14). There are two main advantages of light sheet microscopy over conventional point-scanning techniques. First, because a larger portion of tissue is imaged at once, there is less photobleaching and phototoxicity in the specimen. Second, light sheet microscopy is much faster than point-scanning techniques, allowing for imaging of much larger volumes of

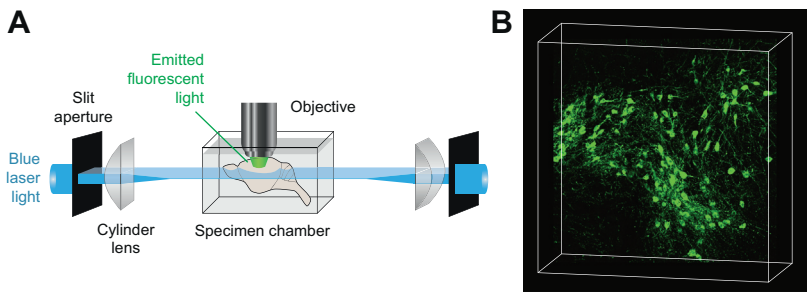


**FIGURE 5.14 Light sheet fluorescence microscopy.** (A) A light sheet microscope utilizes narrow slit apertures and cylinder-shaped lenses to focus light into a two-dimensional plane, which scans through a relatively thick specimen. Excited fluorescent molecules emit light that is captured by the objective and reconstructed into a three-dimensional image. (B) Example of a three-dimensional reconstruction of neurons within a relatively thick brain specimen.

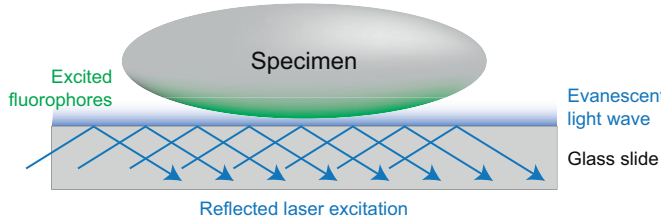


**FIGURE 5.15 TIRF microscopy.** Reflected laser light causes an evanescent wave of excitation at the interface between a specimen and a glass slide. The light penetrates only 100 nm, ensuring that all fluorescent excitation and emission occurs only at the surface of the specimen, usually the plasma membrane.

tissue. For example, light-sheet microscopy is often used in conjunction with tissue clearing and expansion (Chapter 6) to image an entire fixed mouse brain or even a whole worm or small fish.

### Total Internal Reflection Fluorescence Microscopy

**Total internal reflection fluorescent (TIRF) microscopy** is useful for investigating molecules and biochemical events that occur at the surface of a cell. For example, a fluorophore-tagged molecule of interest may be located on the plasma membrane as well as in the cytoplasm of the cell. Using an epi-fluorescent microscope, fluorescence emitted from the membrane can be overwhelmed by the fluorescence coming from the much larger population of molecules in the cytoplasm. A TIRF microscope allows an investigator to selectively excite and image the membrane-bound fluorophores. This kind of microscopy is often used to selectively visualize processes that occur at the plasma membrane in living cells with high resolution.

In TIRF microscopy, the thin section of detection is fixed at the interface between the specimen and the surface on which the specimen is mounted, usually a glass coverslip (Fig. 5.15). TIRF microscopes excite fluorophores with an evanescent wave of light exclusively at the interface between the specimen and its adjacent surface. The evanescent wave exponentially decays with distance and is typically lost at a depth of 100 nm into the sample. Therefore, light only effectively excites the plasma membrane ($\sim$7.5 nm thick) and the immediately adjacent cytoplasmic zone.

## ELECTRON MICROSCOPY

The resolving power of a light microscope is limited by the fixed minimal wavelength of visible light. However, an electron has a much shorter wavelength than a photon. By focusing a beam of electrons rather than a beam of photons to image a specimen, **electron microscopy (EM)** can increase the resolving power as much as 1000-fold to 0.2 nm, about the radius of a

glutamate molecule. Electron microscopy is essential for examining small neural structures such as synapses, synaptic vesicles, and ion channels. EM is also commonly used to visualize myelin and even has the resolution to visualize the number of times myelin wraps around an axon.

The major limitation to using EM is that specimens must be fixed, dehydrated, and chemically treated. Therefore, EM cannot be applied to living samples. Furthermore, artifacts could appear that do not exist in the living cell. To reduce these artifacts, investigators can rapidly freeze specimens (using techniques such as high pressure freezing) such that water and other components in cells do not have time to rearrange themselves or even crystallize into ice. These methods essentially "lock" ultrastructures into place to improve tissue preservation.

There are two major categories of electron microscopy: transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

## Transmission Electron Microscopy

In **transmission electron microscopy (TEM)**, a beam of electrons is aimed through a thin section of a specimen that has been chemically treated to enhance contrast. Extremely thin (<100 nm) sections of preserved tissue are stained with atoms of heavy metals, which are preferentially attracted to certain cellular components. These heavy metal stains are electron dense; therefore, when the electron beam hits the heavy metal atoms in the specimen, the electrons are absorbed or scattered, making electron-dense areas appear dark. Electromagnets focus and magnify the image by bending charged electron trajectories. Because it is impossible to *see* electrons, the variations in electron intensity are converted into photons by projecting the electrons into a special detector or onto a screen that fluoresces at intensities relative to the amount of electrons. TEM produces two-dimensional images of thin tissue sections that are useful for studying the fine detailed structure of cells (Fig. 5.16).

## Scanning Electron Microscopy

**Scanning electron microscopy (SEM)** is useful for detailed visualization of a specimen's surface. A specimen is first coated with a very thin film of gold or platinum. During imaging, a high-energy electron beam scans across the specimen. The electrons interact with the metal atoms coating the sample, producing various signals that contain information about the surface topography and composition of the sample. These electronic signals are collected, processed, and eventually translated as pixels on a monitor to form an image of the specimen's surface topography that appears three dimensional (Fig. 5.16). SEM images show striking details of extremely small (nanometer-scale) structures. Sometimes investigators apply false colors to distinctive elements within a SEM image to distinguish between different structures or to convey a sense of depth or composition.

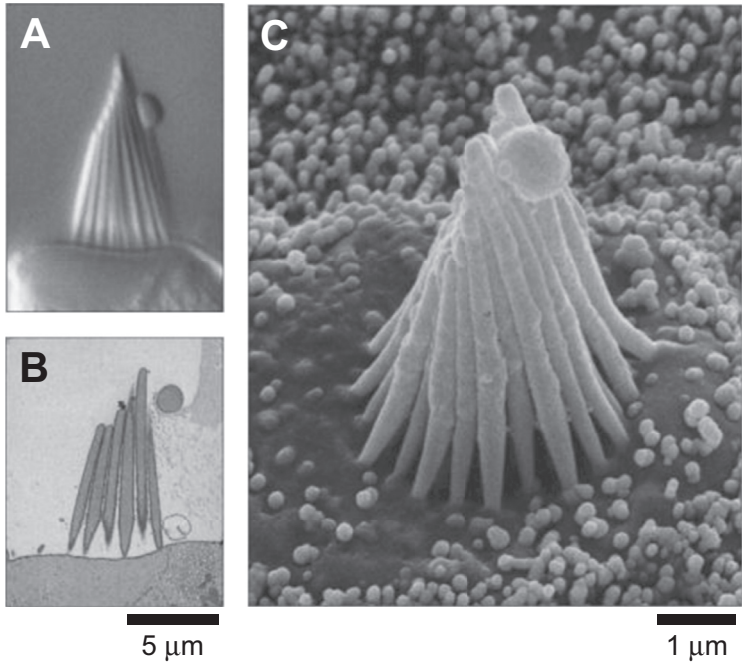


**FIGURE 5.16 Comparison of images taken by different electron microscope techniques.** (A) A DIC image of stereocilia projecting from a hair cell in the inner ear. (B) An image of the same structure taken with a transmission electron microscope and (C) a scanning electron microscope. *Courtesy of Drs. A.J. Hudspeth and Dr. R. Jacobs.*

### Electron Tomography

**Electron tomography**, also known as **electron microscope tomography**, is similar to computerized tomography described in Chapter 1. In a CT scan, the imaging equipment is moved around a patient to generate different images of a slice of the brain. In electron tomography, the specimen is tilted within the electron microscope to produce TEM images from many different perspectives to reconstruct a three-dimensional image. With a resolution of 2–20 nm, electron tomography can be used for determining both molecular structures and the three-dimensional ultrastructure of organelles. It has been especially informative for elucidating the organization of presynaptic vesicles in an axon terminal (Fig. 5.17).

## PREPARING AND INTERPRETING MICROSCOPY DATA

Microscopy data are one of the most frequently presented forms of data in neuroscience, and possibly all life sciences. Microscopy data may also be the most subjective form of data to interpret. Therefore, it is important to

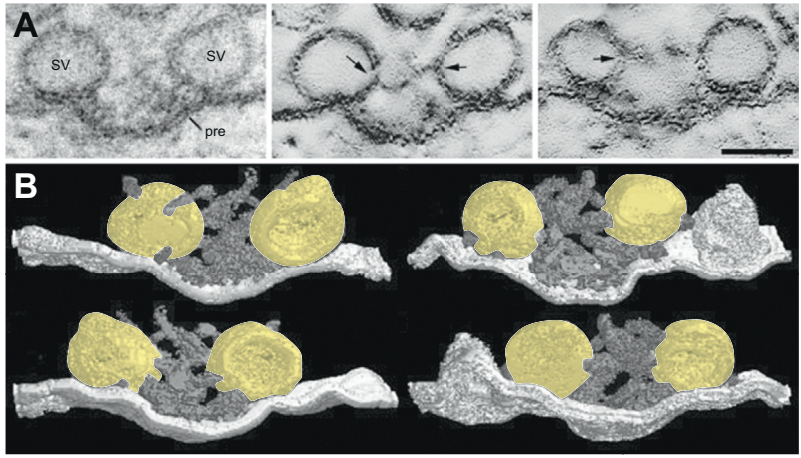


**FIGURE 5.17 Example of an electron tomography image: synaptic vesicles docking at release sites in the neuromuscular junction.** (A) Two-dimensional TEM images used in the ET volume reconstruction of an active zone at a frog neuromuscular junction. *sv*, docked synaptic vesicles; *pre*, presynaptic membrane. Scale bar, 50 nm. (B) Three-dimensional surface-rendered active zone, docked vesicles and presynaptic membrane that allow the shape, size, and associations of different components to be examined. *Reprinted by permission from Harlow, M.L., et al., 2001. The architecture of active zone material at the frog's neuromuscular junction. Nature: Macmillan Publishers, Ltd.*

become familiar with standard practices used to prepare and analyze microscopy data to evaluate published findings and to better interpret your own results.

## Image Processing

The digital images taken at the microscope are generally not the exact same images that appear in publications. Raw images are usually processed by computer software accompanying the microscope or by secondary software applications such as Adobe Photoshop or NIH ImageJ. Image processing is appropriate and often necessary to extract meaningful information. For example, processing may help to facilitate quantitative measurements and accurate analysis, to highlight meaningful aspects of an image, and to improve aesthetic appearance when it does not interfere with accurately interpreting the results. The guiding principle of image processing is to faithfully represent reproducible results without altering the inherent information contained in the image.

The most common form of image processing is creating pseudocolor representations of fluorescent microscopy images taken in different channels (different emission wavelengths) to overlay into a single image. Most microscope cameras capture light intensity in grayscale rather than color. If multiple fluorophores are imaged in a single specimen, different channels can

be selected by changing the excitation and emission filters on the microscope. This process allows different fluorophores to be imaged independently, but they will all appear in grayscale. The investigator can then assign a different color to each signal and merge the separate images of each fluorophore into a single image with multiple colors. This processing helps visualize relative intensity differences and spatial relationships for each fluorescently labeled molecule (Fig. 5.18).

Other common forms of image processing include digitally enhancing contrast or reducing background fluorescence. Although this processing can aid in elucidating useful information from an image, it must not introduce false elements into the image to provide support for what an investigator *wishes* to see. Many journals provide guidelines for the types of image processing allowed and require that all processing be reported in the Materials and Methods section.

In general, there are two important guidelines to follow when processing microscopy images for analysis and publication: (1) Any processing applied to one image of a set should be applied to *all* images within the set. For example, if altering the contrast between the lightest and darkest aspects of an image, it is important that all remaining images receive the exact same digital enhancements. This consistency is especially important when comparing images between experimental groups. (2) An investigator should process and analyze images blind to the identity of the samples between experimental and control groups. Even with the best of intentions, scientists can make mistakes in analysis or introduce subconscious bias. When working with microscopy data, as with most data, blind processing and scoring is critical to prevent potential bias.

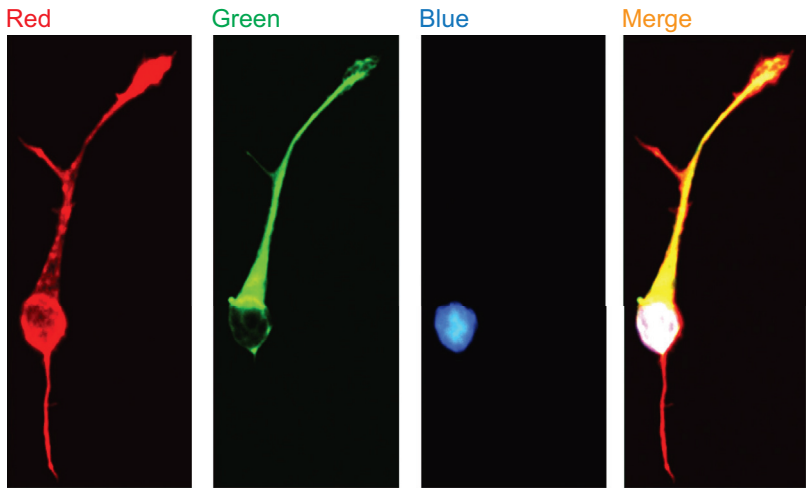


**FIGURE 5.18 Merging images of different fluorophores into a single image.** Photomicrographs of the same specimen using filters for (from left to right) rhodamine (red), FITC (green), and DAPI (blue). Because these spectra do not overlap, the images can be overlayed to compare the spatial relationship of fluorescent signals.

## Interpreting Images

When evaluating your own or published microscopy images, it can be helpful to ask some critical questions:

- **Does it look right?** Familiarity with other work in the field can provide an intuition and expectation for standard images. If the image is supposed to be of neurons, do the structures look like neurons? If the image is supposed to represent a specific brain region, can you identify that region based on the image presented? Is the image taken at an appropriate scale to make a judgment? If the author claims that expression of a gene, protein, or lesion is localized to a specific structure, does the image show that structure in the context of neighboring regions?
- **Does the signal-to-noise ratio allow for proper examination of the data?** Nonspecific background staining can potentially mask a specific signal. Is the contrast between the background and the signal substantial enough to produce a consistent, observable result?
- **Is the exposure and brightness optimal?** An overexposed image (Fig. 5.19A) can mask details or improperly convey a high concentration of the measured signal. An underexposed image (Fig. 5.19B) can make important details impossible to perceive. An investigator can affect the proper exposure of an image during both the capture of an image and during secondary image processing. A properly exposed image allows for maximum possible visual detail to be discerned (Fig. 5.19C).

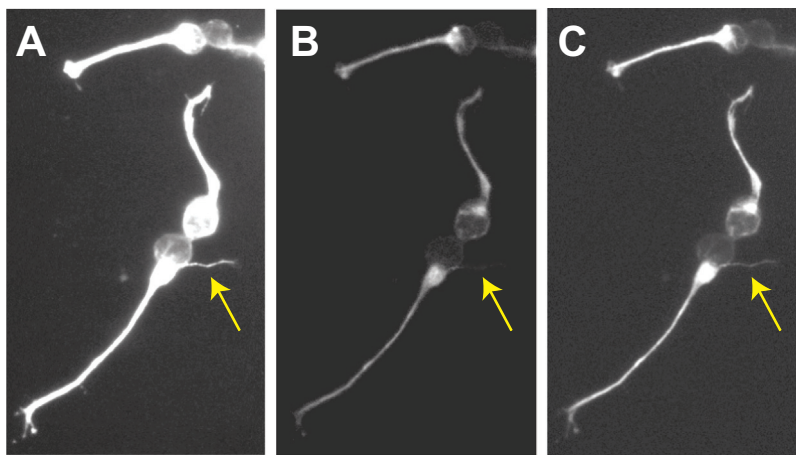


**FIGURE 5.19 Over- and underexposure of an image.** (A) An overexposed image of neurons that lacks subcellular details because there is too much brightness. (B) An underexposed image of the same specimen that lacks details because there is not enough brightness. (C) An image of the same specimen at an optimal exposure. The *yellow arrows* point to a neural process that is lost when underexposed. The brightness and levels of an image can be optimized to allow for the visualization of weaker fluorescent signals without oversaturating more intense regions.

- **If this is the most convincing example, what do the other samples look like?** For obvious reasons, authors tend to publish images that best represent and support their conclusions. Usually, these images represent one of many individual data points. Therefore, a critical review of a study should assume that the other images are of an equal or *lesser* quality than the images presented. If the image is suboptimal or difficult to interpret, the validity of conclusions reached from unpublished images should be questioned.
- **Is there quantitative analysis of the images?** Though a picture is worth a thousand words, subjective interpretation of data is not as strong as objective, quantifiable results. Therefore, the strongest evidence to support a conclusion presents images alongside quantification of the data that demonstrates the reproducibility of the results from image to image. Common types of quantitative information calculated from images include relative cell counts, size and shape distributions, variation in fluorescence intensity to indicate relative concentrations of the measured signal, and colocalization of independently labeled proteins.
- **Does the illumination look uniform?** Improper microscope alignment can cause differences in signal intensity that might lead to inappropriate conclusions. If the entire field of view is not evenly illuminated, important information and resolution can be lost.
- **Could fluorescent images represent poor quality control issues, such as photobleaching or autofluorescence?** Photobleaching could lead an investigator to believe there is no signal in a sample when the signal has actually faded. Alternatively, autofluorescent substances in a sample might lead an investigator to believe there is signal where there is none. Investigators can avoid inappropriate conclusions caused by photobleaching or autofluorescence by processing all samples in the same way and by performing the appropriate positive and negative control experiments.
- **Could fluorescent images represent bleed-through of different emission spectra?** Investigators usually assume that signals from fluorophores of different wavelengths are independent of each other. However, some fluorophores have overlapping excitation or emission spectra and may be detected when not expected. **Bleed-through**, also known as **cross-talk**, occurs when the signal for one fluorophore is present when examining the signal for a different fluorophore: the signal of one fluorophore has "bled-through" the filters that are set for the other fluorophore. To control for this situation, specimens can be labeled with a single fluorophore and the signal recorded in each channel. Bleed-through of fluorophores becomes especially pertinent when an investigator claims that two signals overlap, as when two proteins colocalize. It is rare that the signal from two different fluorophores will be exactly identical, no matter how strong the colocalization may be. Therefore, if two different channels appear exactly the same, despite use of different detection agents, the image could indicate an artifact.

## CONCLUSION

Whether using photons or electrons, microscopes provide the ability to explore structures much smaller than can be viewed by the naked eye. This chapter has served as an introduction to microscopy and a survey of various techniques (Table 5.1). Most microscope manufacturers (Nikon, Leica, Zeiss) provide detailed instructions in the proper use and theory behind their products. These guides can provide further information about the specific microscopes used in a laboratory. While optical methods can be used to provide enhanced contrast to make out individual structures within cells, histological preparations are utilized to examine specific processes and structures. The next two chapters present methods on preparing samples to visualize neural structure and function: Chapter 6 describes methods of characterizing neural morphology and neuroanatomy, and Chapter 7 discusses imaging neural activity. Both topics depend on good microscopes and good microscopy skills.

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**TABLE 5.1** Comparison of Different Forms of Microscopy.

| | Description | Advantages | Disadvantages | Utility in neuroscience |
|---|---|---|---|---|
| Brightfield | Light is transmitted through specimens. Contrast generated by natural pigmentation or added dyes. | Simple and inexpensive. Differentcolored dyes can be used to stain structures. | Most cells and tissues are transparent, so they can be difficult to see. Adding dyes to visualize structures usually requires fixation and sectioning. | Fixed samples processed using detection reagents that produce colored by-products (Chapter 6). |
| Phase-contrast | Contrast generated by changes in index of refraction because of variation in organelle densities. | Can be used with living cells. Does not require added chemicals or tissue processing. | Difficult for the entire specimen to appear in focus. | Examining cell culture specimens. |
| Darkfield | Specimen illuminated from the side at an oblique angle. Only scattered light is imaged. | Can be used on living cells. High signal-to-noise ratio. No added chemicals necessary. | Cannot see structures that do not scatter light. | Samples processed using in situ hybridization using radioactivity (Chapter 6). |
| DIC/Nomarski | Optical methods used to exaggerate changes in index of refraction, enhancing contrast at the edges of objects. Has a 3D appearance. | Can be used on living cells. Can create thin optical sections. Can create 3D reconstructions from thin optical sections. | Can only image single, thin focal plane at a time. | Examining cell culture specimens. |

*Continued*

**TABLE 5.1** Comparison of Different Forms of Microscopy.—cont'd

| | Description | Advantages | Disadvantages | Utility in neuroscience |
|---|---|---|---|---|
| Epifluorescent | Specimen is illuminated by excitation wavelengths and emits light from excited fluorophores throughout entire thickness. | As with all forms of fluorescent microscopy, can be used to detect fluorescently labeled molecules. Multiple fluorophores can be imaged in same sample. High signal-to-noise ratio. | Out-of-focus fluorescence can cause blurry images that make structures difficult to resolve. | Thin physical sections of fluorescently stained tissue or cells. Time-lapse fluorescence imaging in cells. |
| Confocal | Out-of-focus illumination is eliminated by the use of pinhole apertures, so only in-focus light is collected. | Optical sectioning ability creates sharp, in-focus images. | Intense laser illumination can cause photobleaching and phototoxicity. Long scanning time. | Detecting fluorescence in thick tissues or small organisms (e.g., *Drosophila*). Time-lapse fluorescence imaging in thicker tissues (e.g., slice cultures) or for small structures (e.g., synaptic vesicles). |
| Two-photon Microscopy | Fluorophores in a thin focal plane are selectively excited by absorbing the combined energy of two photons that cannot excite fluorophores on their own. | Longer wavelengths of laser illumination allow deeper penetration of fluorescence excitation. Reduced photobleaching and phototoxicity. | Expensive equipment. | In vivo imaging of intact organisms. Long-term fluorescence imaging. |

| | | | | |
|---|---|---|---|---|
| Total internal reflection fluorescence (TIRF) microscopy | Used to image the surface (membrane) of cells. An evanescent wave of illumination rapidly decays after passing the interface between a cell membrane and the surface it is on. | Eliminates out-of-focus fluorescence from regions more than 100 nm away from surface. High resolution. | Only fluorophores at the interface between the membrane surface and the glass coverslip are illuminated; cannot image an arbitrary region within the cell. | Imaging protein dynamics at the plasma membrane in live cells. |
| Transmission electron microscopy (TEM) | Electron beams transmitted through ultrathin sections. | Nanometer resolution. | Cannot be used on live cells. Harsh processing conditions can cause artifacts. Requires specialized equipment. | Ultrastructure of cells. Synapse structure. |
| Scanning electron microscopy (SEM) | Detects electrons scattered off surface of sample. | Provides 3D topological information. | Cannot be used on live cells. Harsh processing conditions can cause artifacts. Requires specialized equipment. | Three-dimensional topography of cells and tissues. |
| Electron tomography | A specimen is rotated to take TEM images from multiple perspectives and create 3D reconstruction. | Provides 3D organization information. | Cannot be used on live cells. Requires intensive computation to reconstruct TEM views. | 3D cellular ultrastructure and organization. |