Microscopy and Imaging

2.1 Robert Hooke, 1635–1703, Described a Cell as the Basic Unit of Life by Studying the Bark of the Cork Oak Tree, *Quercus suber* – 47

2.2 Antoni Van Leeuwenhoek, 1632–1723, Was the First Scientist to Observe Microorganisms – 48

2.3 Nehemiah Grew, 1641–1712, Was the Father of Plant Anatomy – 50

2.4 Robert Brown, 1773–1858, Discovered the Nucleus of the Cell by Studying Orchid Petals – 51

2.5 Katherine Esau, 1898–1997, Advanced the Field of Plant Anatomy with Her Influential Textbooks – 52

2.6 Light Microscopy: The Most Useful Tool of the Plant Anatomist – 54

2.7 The Compound Light Microscope Uses Multiple Lenses to Form and Capture Images – 55

2.8 The Resolving Power of a Lens Places Limits on Resolution and Magnification – 56

2.9 The Confocal Microscope Allows for Sharper Detail, Computer Control, and 3-D Imaging with a Modified Compound Microscope – 58

2.10 Electron Microscopy Allows a View into the World of Cellular Ultrastructure – 61

2.11 The Transmission Electron Microscope Reveals Internal Cellular Detail – 63

The original version of this chapter was revised. The correction to this chapter can be found at https://doi.org/10.1007/978-3-319-77315-5_20

R. Crang et al., *Plant Anatomy*, https://doi.org/10.1007/978-3-319-77315-5_2

2.12 The Scanning Electron Microscope Resolves Surface Detail – 66

2.13 Different Microscopies Produce Different Images of the Same Specimen – 68

2.14 Chapter Review – 69

References and Additional Readings – 74

Introduction

Plant anatomy is typically regarded as the microscopic study of plant tissues and cells, and the development of light and electron microscopes has had a major impact on elucidating our knowledge of structure. It is, therefore, important that we have some understanding of the development, design, and use of these types of instruments. How did microscopy develop, and what has been its significance in the understanding of plant anatomy? This chapter will begin by getting to know some key individuals significant in the developing of microscopy and its relevant applications in plant biology. However, we begin this section, not by focusing on early microscopists, such as Zacharias Jansen who is credited with inventing the first **compound microscope**, but rather, we also explore the key individuals whose work ultimately led to advancements in the field of plant anatomy. Thus, we are starting with those scientists such as Hooke and van Leeuwenhoek who made discoveries and innovations with early microscopes.

2.1 Robert Hooke, 1635–1703, Described a Cell as the Basic Unit of Life by Studying the Bark of the Cork Oak Tree, *Quercus suber*

The Englishman, Robert Hooke (◘ Fig. 2.1a), first described biological matter, including plant material, from its microscopic perspective using an instrument of his own design (◘ Fig. 2.1b). His famous treatise, *Micrographia*, was published in 1665. He used the term "cellulae" to describe the tiny compartments of bottle cork (bark of the cork oak or *Quercus suber*), which reminded him of room-like compartments similar to the small, identical rooms of monasteries which were called cells (◘ Fig. 2.1c). The name "cell" has, of course, remained. A direct count of some 60 cells per one-eighteenth of an inch led him to calculate that there must be over a billion cells in a cubic inch of cork.

Hooke was a remarkable individual, but reportedly plagued with ill health and, according to all accounts, physically unattractive with long, untidy hair, with a stooping gait, and with a pallid complexion. However, besides microscopy, among his other accomplishments, Hooke studied problems in celestial mechanics; invented the vacuum pump, the first respirator, and the iris diaphragm; and developed an early balance spring used in watches. He was one of the most gifted inventors of his day, though not as well recognized as he might have been due to his antagonistic relationship with Sir Isaac Newton. Hooke's life and scientific contributions have received considerable attention by historians. See Jardine (2005) as an example.

Fig. 2.1 **a** A recent interpretation of the appearance of Robert Hooke by artist Rita Greer. The only known portrait of Hooke was destroyed or lost shortly after his death when Isaac Newton became president of the Royal Society of England. (Image courtesy of Rita Greer). **b** Drawing of a microscope designed and used by Hooke (public domain)

2.2 Antoni Van Leeuwenhoek, 1632–1723, Was the First Scientist to Observe Microorganisms

In the mid- and late 1600s, Antoni van Leeuwenhoek (Fig. 2.2a) published many letters to the Royal Society of London regarding his observations of different microorganisms observed with his hand-ground, single-lens microscopes (Fig. 2.2b). Van Leeuwenhoek's lens qualities were outstanding, and it is still not known how he designed them so perfectly, as he appeared to have closely guarded the technique.

Modern microscopes use multiple lenses to achieve maximum magnification and resolution. However, at lesser magnifications, the single-lens microscope of van Leeuwenhoek was quite successful. Thus, Leeuwenhoek made many pioneering discoveries of single cells from plants, animals, and microorganisms (Schierbeek 1959; Ford 1992).

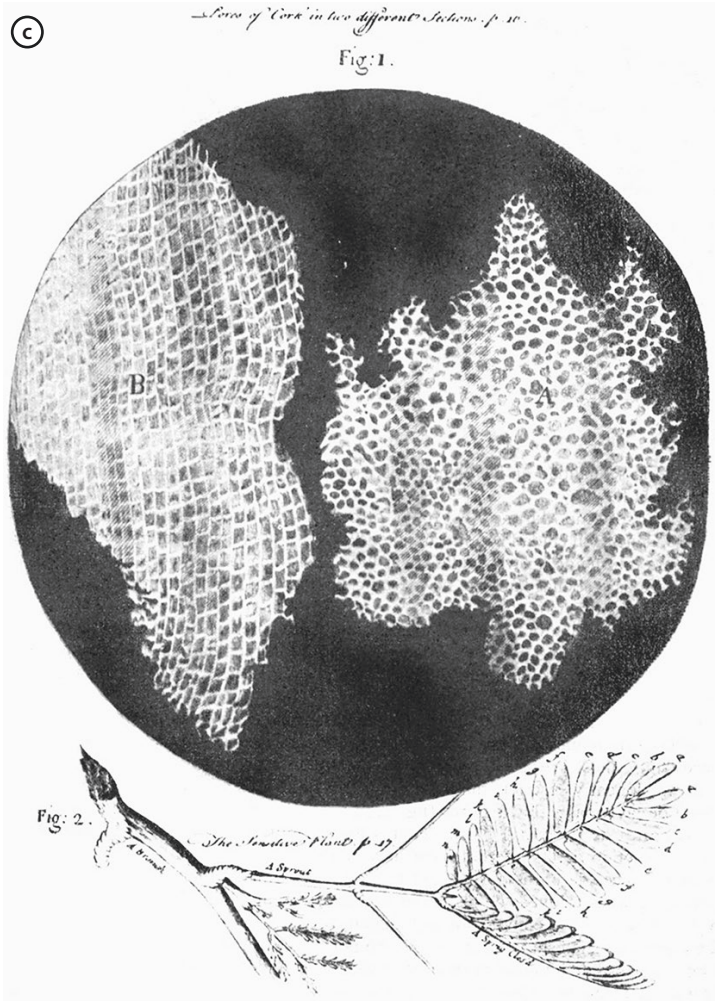


Fig. 2.1 **c** Hooke's most famous image of cork as he drew it from viewing with his light microscope. In it, he saw small compartments, which he referred to as "cells" (public domain)

Among the microorganisms first described were bacteria, protozoa, and rotifers. It is believed that van Leeuwenhoek made at least 500 microscopes during his career, but only nine are known to currently exist. The highest magnification obtainable was approximately 275×.

Fig. 2.2 **a** Antoni van Leeuwenhoek, a well-to-do Dutch draper, developed a strong interest in science and in the art of microscopy. His most famous instruments were designed to employ only a single lens. **b** A replica of Leeuwenhoek's single-lens microscope is shown above, with the single lens indicated by the red arrow. The microscope is quite small, with the brass back plate only being approximately 2 × 5 cm. (**a** RR Wise; Image **b** by Jeroen Rouwkema. Licensed under CC BY-SA 3.0 via Wikimedia Commons)

2.3 Nehemiah Grew, 1641–1712, Was the Father of Plant Anatomy

In 1682 Nehemiah Grew (Fig. 2.3a) published the first of two well-illustrated volumes with the Royal Society on the microscopic anatomy of plants entitled: *The Anatomy of Plants: With an Idea of a Philosophical History of Plants.* in which he described minute "vesicles" (the cells that were earlier described by Hooke). While some of his work was more morphology than anatomy, he did make considerable use of light microscopy in his investigations, and Grew was the first microscopist who limited his investigations to the anatomy

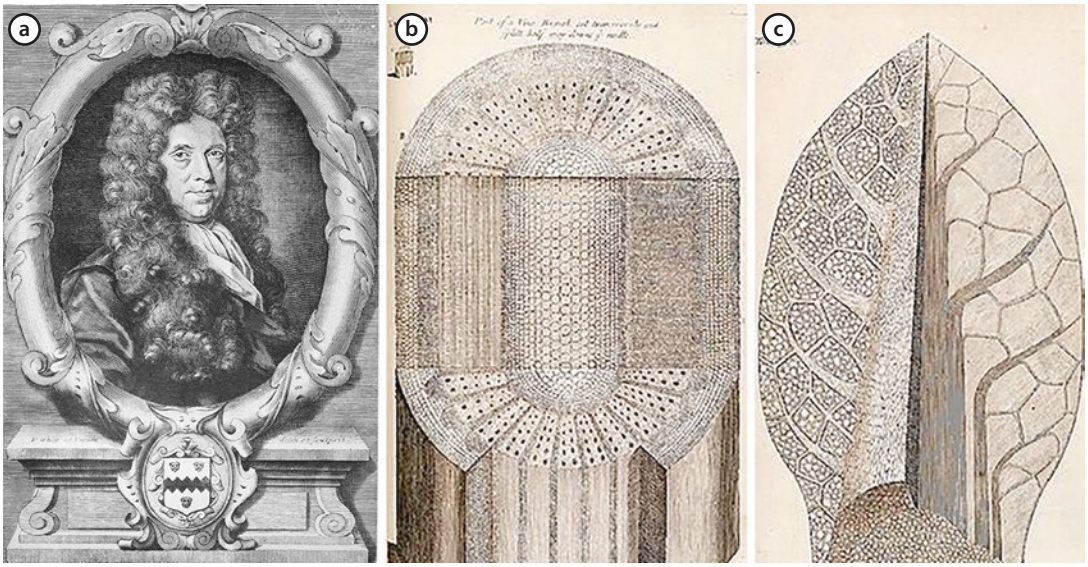


Fig. 2.3 **a**–**c** Nehemiah Grew, an English scientist and doctor, is often referred to as the "father of plant anatomy." **b**, **c** Two of the anatomical illustrations developed by Grew using an early compound microscope. **b** A eudicot stem and **c** different paradermal sections (cut parallel with the leaf surface) through a eudicot leaf (**a**–**c** public domain)

of plants (Fig. 2.3b, c). Although the compound light microscope had been around for over a half century, Grew and a few other individuals simultaneously described general plant microscopic structure for the first time during the end of the seventeenth century and the early eighteenth century.

Grew was a doctor of medicine, having studied at Cambridge University in England. Not unlike other botanists of his time, although his training was in medicine, his interest in plant structure developed from microscopic work in his medical practice.

2.4 Robert Brown, 1773–1858, Discovered the Nucleus of the Cell by Studying Orchid Petals

In 1831 Robert Brown (Fig. 2.4a, b) described the cell nucleus during a study of orchids. He is also credited with first observing **Brownian movement** (named for him and illustrating molecular motion he observed by studying pollen grains) and the process of **cytoplasmic streaming**. Although he practiced medicine as a surgeon for 5 years, he later abandoned this and turned all his efforts toward botanical science, publishing dozens of books and articles. He was distinguished in making a series of anatomical studies involving the process of reproduction and in the study of pollen and reproduction of gymnosperms (Brown 1828).

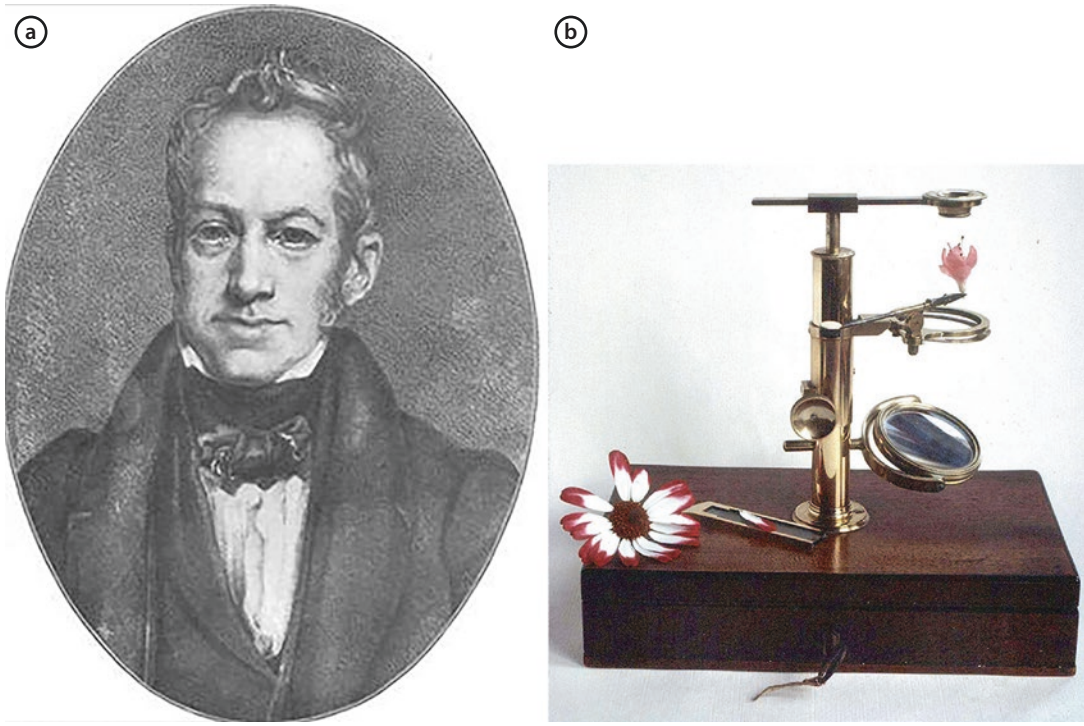


Fig. 2.4 **a** Robert Brown, a Scottish surgeon and botanist. **b** The microscope with which he made many of his observations that was made prior to 1820. It is a compound light microscope because it uses both objective and ocular lenses. (**a** public domain; Image **b** courtesy of Dr. Brian Ford)

2.5 Katherine Esau, 1898–1997, Advanced the Field of Plant Anatomy with Her Influential Textbooks

Born in the Ukraine, a daughter of the mayor of Yekaterinoslav, Katherine Esau (Fig. 2.5a) was educated at the Women's Agricultural College in Moscow, Russia. In 1917 her studies were interrupted due to the revolution, and she returned to her home, which had not been occupied by the Bolsheviks. She remained there until the end of the First World War when her family immigrated to Germany and later (1922) to America. They settled in Reedley, California, near Fresno.

In California, she was hired by a sugar company to develop a sugar beet that would be resistant to curly top disease caused by a virus. Her work drew the attention of researchers at the University of California, Davis, where she was recruited to study botany. Her dissertation research discovered that the curly top virus was spread within plants via the phloem. Upon graduation, she was offered the position of instructor of botany in the College of Agriculture at Davis where she taught plant anatomy, systematic botany, morphology of crop plants, and microtechnique (Fig. 2.5b, c). She achieved the rank of full

Fig. 2.5 **b** A transmission electron micrograph by Katherine Esau showing a portion of a tobacco leaf infected by the tobacco mosaic virus (V). Scale bar = 10 μm. **c** A longitudinal section of a Pinus sp. needle seen in the light microscope and prepared by Katherine Esau. Scale bar = 500 μm. (**b**, **c** Cheadle Center for Biodiversity and Ecological Restoration, UC Santa Barbara)

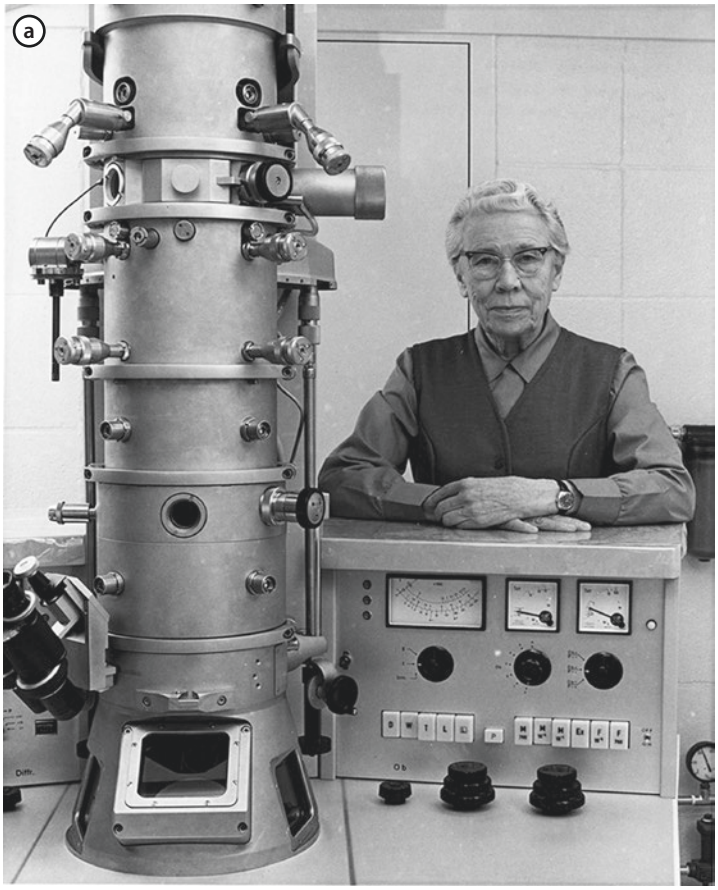
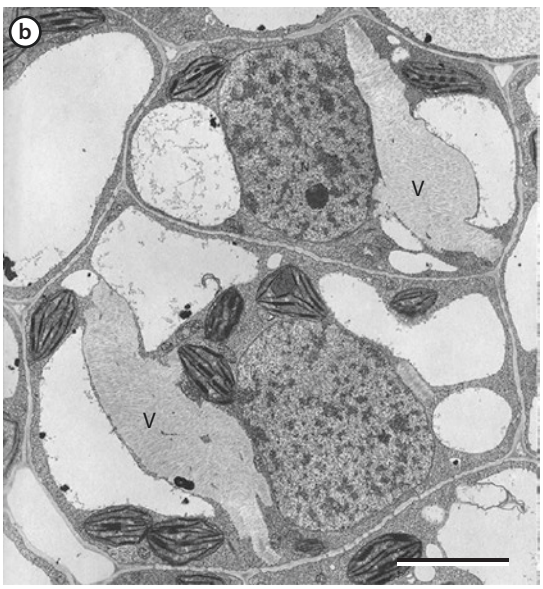
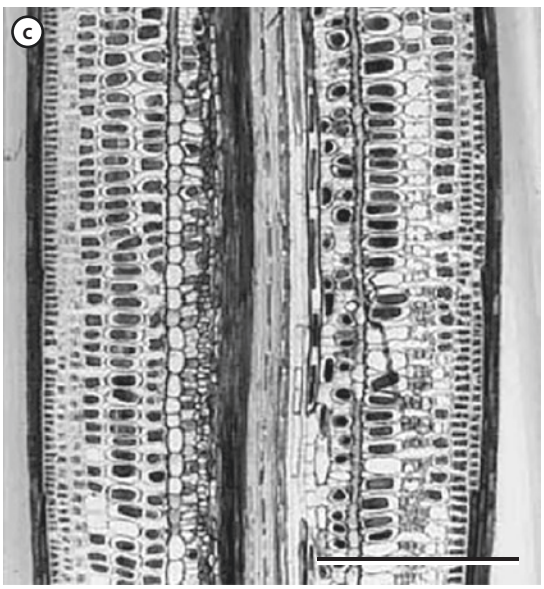


Fig. 2.5 **a** Katherine Esau, a Ukrainian botanist, plant pathologist, and plant anatomist. (Image courtesy of Cheadle Center for Biodiversity and Ecological Restoration, UC Santa Barbara)





professor in 1949 at the age of 51. Her research became increasingly involved with the anatomy of phloem, as she was concerned about plant pathways in disease, and for chemicals in weed control.

Dr. Esau was elected to the National Academy of Sciences in 1957. In the early 1960s she became interested in electron microscopy involving the study of phloem tissue—an area of study she pursued throughout the rest of her career. In 1989, US President George H.W. Bush awarded her the National Medal of Science. She is most noted for her highly read textbooks that established a modern foundation for the field of plant anatomy (Esau 1953, 1961, 1969, 1977).

2.6 Light Microscopy: The Most Useful Tool of the Plant Anatomist

Resolution is the ability to distinguish a gap between two adjacent lines or objects. The smaller that gap, the higher the resolution, which allows for greater detail at higher magnifications. Light microscopy enables us to resolve structures of about 0.2 μm and achieve magnifications of about 2000×, which is not fine enough to visualize cell membranes and many subcellular units. However, in the range in which it works, and given the use of color and special imaging techniques which are possible, it has been the most valuable tool of the plant anatomist for almost four centuries. Realizing that, it is useful to understand some basics of light microscopy, starting with image formation.

How a compound light microscope works can be related to some simple optical principles. To start with, we may imagine making simple images, even without a glass lens. Prior to the popularity of digital photography, many students in elementary or secondary school designed and built pinhole cameras (◘ Fig. 2.6). These are very simple devices which have a small circular pinhole in a piece of foil through which light rays pass and are projected onto the back of a light-tight box where there is a piece of photographic film attached. An image of a well-lighted scene can be projected onto the film through the pinhole due to the rectilinear propagation of light (that means that light travels in straight lines). The size of image points produced on the film depends on the diameter of the pinhole and the distance between the hole and the film. The larger the hole, the more blurred is the image. If the hole is too small, brightness and diffraction become limiting factors.

Now suppose that you are photographing a tree at some distance. The light travels from any point on the plant in all directions, but a certain solid angle of the light is intercepted by the pinhole (or lens) of the camera, refracted (bent), and recombined at the film plane. Normally, the image on the film plane will be much smaller than the original object, and the distance from the center of the pinhole (or lens) to the image is much shorter than the distance from the object to the pinhole (or lens) center. Actually, this sets up a consistent ratio in which we can say that A/a = B/b where A is the height of the object (the tree) and where a is the distance to the pinhole (or lens) from the object. B is the height of the image on the film plane, and b is the distance from the center of the pinhole (or

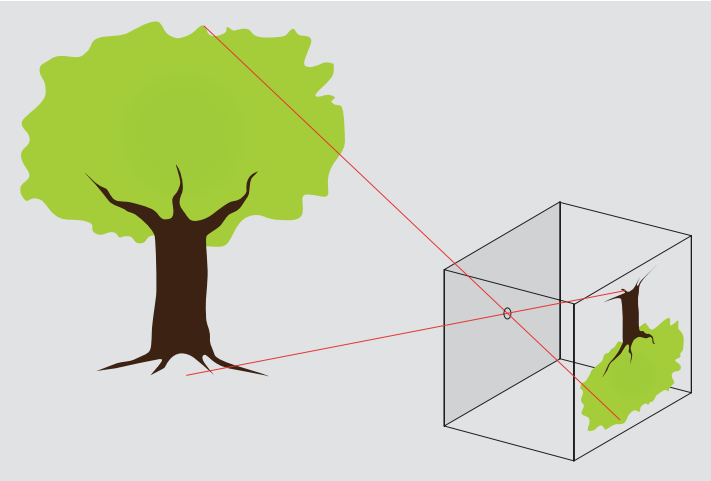


Fig. 2.6 An example of how images are formed using a pinhole camera (Redrawn from Crang and Vassilyev 2003)

lens) to the film plane. Thus, the ratio of A to a is always equal to the ratio of B to b when the image is in focus.

The image produced on the piece of film can be greatly improved by using a glass lens in place of the pinhole. A lens has the extremely valuable property of refracting light, thus counteracting the principle of the rectilinear propagation of light. It also can be of much larger diameter than a pinhole and thereby capable of collecting much more light to make a much shorter exposure time on the film.

2.7 The Compound Light Microscope Uses Multiple Lenses to Form and Capture Images

In the case of the single-lens microscope, such as the early instruments designed by van Leeuwenhoek, greater magnification is obtained by increasing the curvature of the surface of the lens. In theory, the greatest magnification would result from a perfectly spherical lens. However, the focal length of the lens would be on the surface of the lens, and the field of view would be infinitely small, making the device impractical.

There are several advantages to using a compound microscope over a single-lens instrument. First, using two or more lenses in tandem enables magnification to be viewed as the product of the combined magnifications of the individual lenses. Second, it is easy to obtain variable magnification by simply changing objective lenses. Third, the compound microscope allows for a relatively wide field of view at all magnifications. Additional advantages in using a compound scope include brighter imaging as well as the ability to work greater distances from a specimen than when using a single lens.

The modern compound light microscope (Fig. 2.7) possesses two or more magnifying lenses. Light travels from a light source in

Fig. 2.7 The basic components of a modern compound light microscope (Olympus CX23). (Image courtesy of Jennifer Reed, Olympus Instruments)

the base of the microscope, through a condenser underneath the microscope stage, through the specimen, objective lens, and then to the ocular lenses. Various magnifications are selected by rotating the revolving nosepiece and inserting a different objective lens into the light path. Unlike the single-lens microscope, it also permits a greater working distance from a specimen by allowing for finite focal lengths outside of the lens itself.

2.8 The Resolving Power of a Lens Places Limits on Resolution and Magnification

The physicist Ernst Abbé, working with the master lens maker Carl Zeiss in Jena, Germany, during the latter part of the eighteenth century, defined the rules of light optics and determined the theoretical limits of resolution. To their amazement, it was found in the 1880s that Zeiss had already produced (through his own skills) microscopes that had essentially reached the limits of resolution (approximately 0.2 μm). Abbé later founded the lens-making company named after Carl Zeiss, which continues today.

Resolution, introduced above, is usually meant to represent the unitless concept of the amount of detail available at high magnifications. The **resolving power**, or R.P., on the other hand, is the mathematical expression of the resolution and is determined by the equation shown below. Lambda (λ) is the average wavelength of light used in imaging, and N.A. represents the numerical aperture of the objective lens ($N.A._{obj}$) plus that of the condenser lens ($N.A._{cond}$) in the microscope.

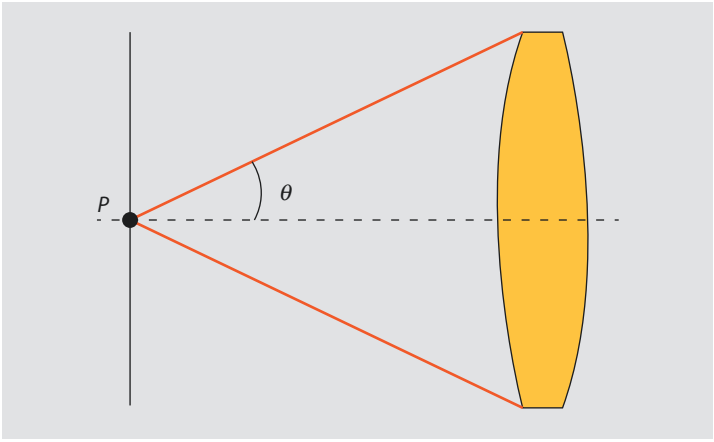
$$\text{R.P.} = \frac{\lambda}{\text{N.A.}_{\text{obj}} + \text{N.A.}_{\text{cond.}}}$$

The **numerical aperture** is a function of n (the index of refraction of the medium being used) and of the sine of the half angle that light rays would take from a specimen in focus through the objective lens (◘ Fig. 2.8). It is represented by the formula shown here.

$$\text{N.A.} = n \sin \theta$$

The index of refraction (n) is the ratio of the speed of light in a vacuum to the speed of light in a medium such as air, water, or oil. The value is always equal to or greater than 1.0. Air has a **refractive index** of 1.0, thus in this case, the numerical aperture is dependent upon the half angle of the light cone (θ).

Numerical apertures are values that can be found inscribed on the housing of the objective lens or on the condenser lens. Some representative values for objective lenses are as follows (there may be variation due to different manufacturers). A 4× lens may have a N.A. = 0.1, a 9× lens may have a N.A. = 0.25, a 40× lens may have a N.A. = 0.65, and an oil immersion 100× lens may have a N.A. = 1.3. There is a rule of thumb, which says that the maximum magnification obtainable that yields good resolution is not greater than 1000× the N.A. of an objective lens. Thus, this sets a limit on how much magnification can be obtained by the corresponding ocular lens of the microscope. Therefore, a microscope with a 10× ocular lens and a 4× objective lens would have a total magnification of 40×. This is lower than 100× magnification (1000 * 0.1) and thus, would be expected to yield a high-resolution image.



◘ **Fig. 2.8** Diagrammatic representation of numerical aperture. The numerical aperture of a microscope lens in alignment with a point (P) depends on the half-angle (θ) of the maximum cone of light that either enters or exits the lens. The N.A. has no units of measurement. The orange object to the right represents a lens, which parallel light rays enter from the right (Redrawn from Crang and Vassilyev 2003)

Box 2.1 How Low Can You Go? Achieving Maximum Image Quality by Post-imaging Processing

Light and electron microscopes are some of the plant anatomist's most basic tools. Both use lenses to manipulate a beam of photons or electrons. Strict manufacturing tolerances allow for the production of high quality lenses. However, there are technological and physical limits to maximum magnification and resolution, and the image produced by even the finest lens will always have a certain amount of built-in "aberration." The advent of digital cameras, coupled with inexpensive and powerful personal computers, has allowed microscope manufacturers to partner with computer scientists to develop software-based approaches to markedly improve images generated by microscopists. While theoretical resolution is limited by the physics of the light or electron beam, sophisticated mathematically-based methods such as energy loss spectroscopy and image deconvolution are being used to greatly enhance the quality, and scientific value, of modern imaging techniques.

References: Ramasse (2017) and Storath et al. (2017).

2.9 The Confocal Microscope Allows for Sharper Detail, Computer Control, and 3-D Imaging with a Modified Compound Microscope

During the mid-to-late 1980s, commercial microscopes began to appear that made use of the principle of confocal imaging (◘ Fig. 2.9a).

◘ **Fig. 2.9** **a** A confocal laser scanning microscope (Nikon A1R CLSM) with computerized functions and display. (Image courtesy of Eric Flem, Nikon Instruments, Inc.)

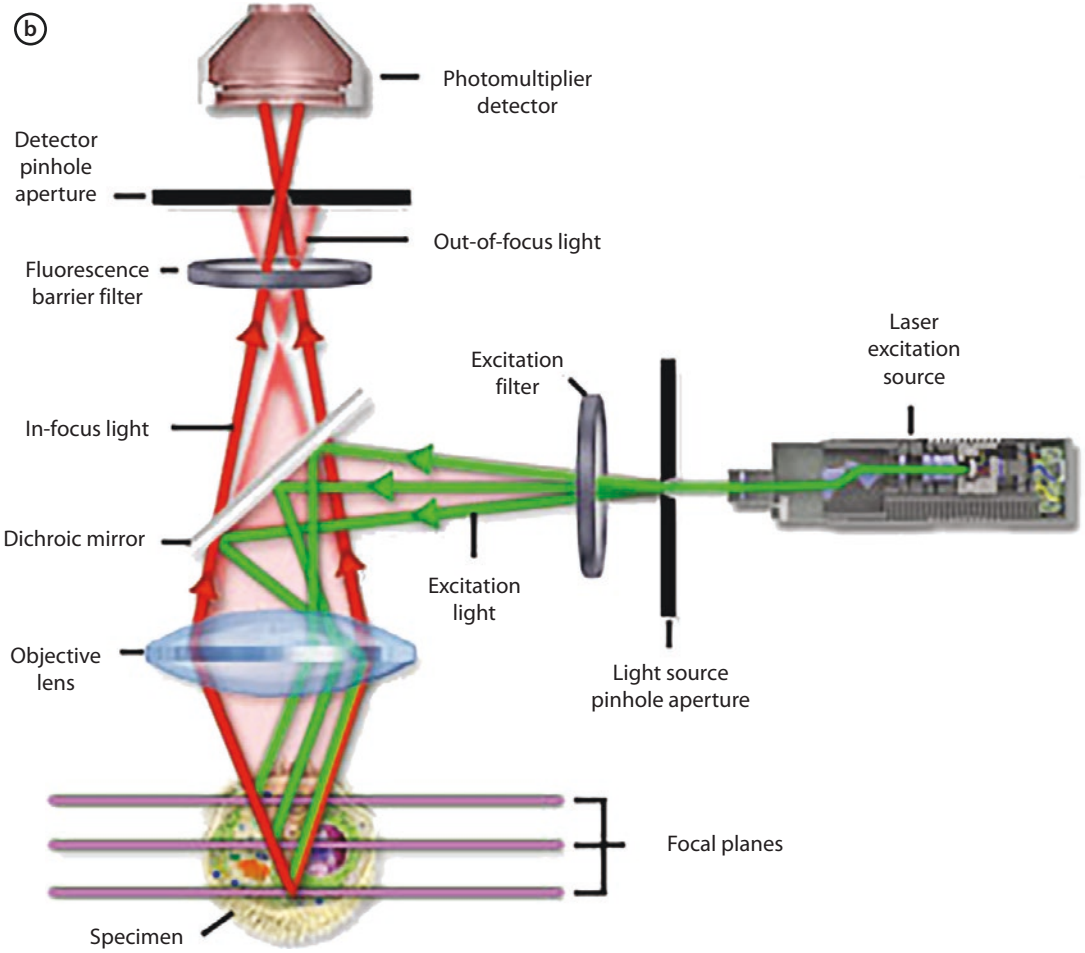


Fig. 2.9 **b** Schematic diagram of a confocal microscope. A rastered light pathway is projected onto a specimen by epiluminescence. Reflected or fluoresced light is imaged back through the objective lens. (Image courtesy of Carl Zeiss Microscopy, LLC, with modification)

This type of microscopy utilizing the confocal laser scanning microscope (CLSM) is very powerful in that it is a versatile instrument that allows scientists to study gene expression and protein movements within specific plant structures. By fluorescently tagging genes, plant geneticists can observe protein expression as well as how substances move within plants. Thus, while the CLSM isn't necessarily used for classic plant anatomy per se, it is a powerful tool for plant cell biologists and geneticists.

Both full-color visible light instruments and laser instruments have been designed and are useful in different applications. Both have similar principles of design, however (Fig. 2.9b). A beam of light is passed through a small opening and may be deflected in a series of lines that constitute a raster which is projected from above the objective lens of a light microscope, through that lens, and onto the surface of a specimen located in the front focal plane of the lens. The pathway of the light will be at its smallest diameter as determined by the point of light source and its crossover on the surface of the specimen. Since the excitation light is coming from above the specimen, it is said to represent **epiluminescence**.

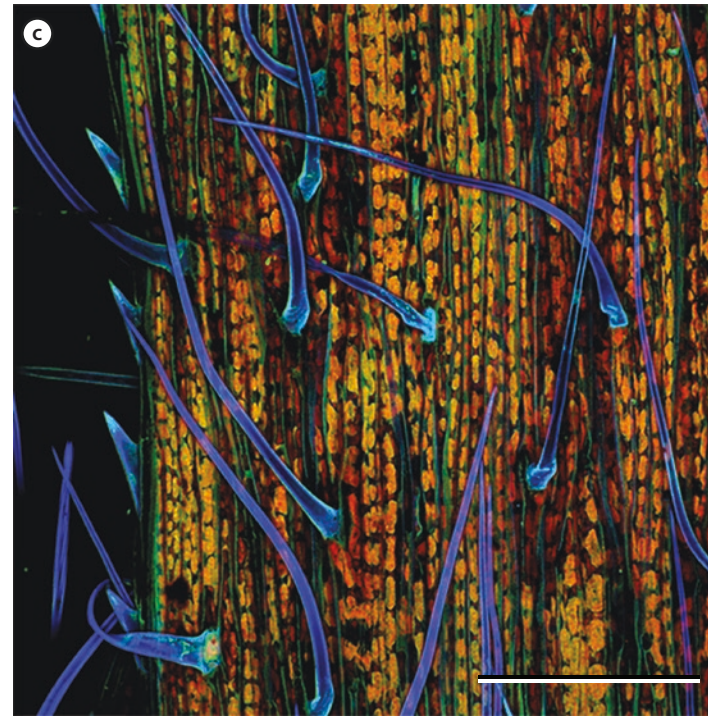


Fig. 2.9 **c** A CLSM image of autofluorescence of a blade of grass. Trichomes are shown in blue, and epidermal cells appear mostly orange. Scale bar = 200 μm. (Image courtesy of Dr. Donna Stolz University of Pittsburgh School of Medicine)

The object being illuminated may be a section or, more often, a layer (or multiple layers) of the specimen. The crossover light, being in a raster, will be projected one point at a time in rapid sequence. Either reflected or fluoresced light can be captured by the objective lens and be conveyed back up the image tube of the light microscope to a dichroic mirror (a glass surface coated with a special metal film that reflects certain colors of light while allowing others to pass through), that, in turn, projects the radiant image toward a pinhole aperture plate. Any light that was received from either above or below the focal plane of the objective lens will strike the aperture plate away from the pinhole and will be blocked. Only those light paths that have come from the exact focal plane on the specimen will be focused through the aperture. Such light is then picked up by a photomultiplier tube and used to create a point of some brightness (depending on the amount of light captured through the aperture) on a cathode ray tube or computer monitor screen. The position of each point of light displayed will correspond with the position of the rastered laser beam on the specimen. Thus, a confocal laser scanning image can be generated (Fig. 2.9c).

Since the operation of the confocal laser scanning microscope is controlled by computer inputs, and its images are electronically stored, it can then assemble the various images from the specimen and reconstruct a three-dimensional image, which can be rotated and/or analyzed for structural composition.

2.10 Electron Microscopy Allows a View into the World of Cellular Ultrastructure

It remained well into the twentieth century before resolution greater than that of the light microscope could be achieved. This depended upon employing illumination with a vastly shorter wavelength than that possible in the light optical range. Electrons, discovered by the British physicist, J.J. Thompson, in 1898, were shown by L. de Broglie in 1924 to possess wave properties nearly a thousand times shorter than that of visible light. Since it was recognized that the resolution of the light microscope was limited mostly by the wavelength of visible light, electrons seemed to offer an outstanding possibility for vastly improved resolution (and therefore also magnification).

With the work of physicists, such as H. Busch, M. Knoll, and E. Ruska from 1925 through 1934, strong electromagnetic lenses were developed that enabled electron beams to be focused in much the same manner as glass lenses direct the pathway of visible light. In fact, the basic design of early transmission electron microscopes (and even today's instruments) follows similar optical pathways as that of light microscopes. Electrons, however, can be deflected by the presence of air; hence the electron microscope needed to have a vacuum system for the pathway of the electron beam.

There are two basic types of electron microscope (and many variations thereof). The transmission electron microscope (TEM) was invented in the early 1930s by the German physicist Ernst Ruska (◘ Fig. 2.10a), for which he shared the 1986 Nobel Prize in Physics (Hawkes 1990). The first electron micrograph of a biological sample followed almost immediately and coincidentally in the field of plant anatomy. L.L. Marton published a shadowy outline of a sundew leaf in 1934 (◘ Fig. 2.10c). However, the TEM did not become commercially available until the late 1940s after the end of the Second World War. In addition to the interruption caused by the war, the TEM was invented by physicists, not biologists, and it took the 1940s and 1950s for biologists to develop the sample preparation techniques necessary to prepare and image biological samples to the level of resolution with which we are familiar today. The invention of those techniques and protocols, coupled with the resolving power of the TEM, revolutionized cellular biology in the mid-twentieth century (Rasmussen 1997). The micrograph atlas of plant cell ultrastructure produced by Myron Ledbetter and Keith Porter remains a classic to this day (Ledbetter and Porter 1970).
► Section 2.11 covers the TEM in more detail.

True to its name, the TEM works by transmitting a beam of electrons through a thin slice of tissue. Organelles and molecules in the tissue section selectively block the incident electrons, which projects a pattern on a viewing screen or film negative much like a slide projector sends an image to a viewing screen. Thus, cellular internal detail is revealed. The image can be collected directly on film.

The scanning electron microscope (SEM) was developed in 1937 (◘ Fig. 2.12b) by Manfred von Ardenne, another German physicist. Just as there was a 20–30-year lag between the invention of the TEM and the generation of useful micrographs of biological specimens,

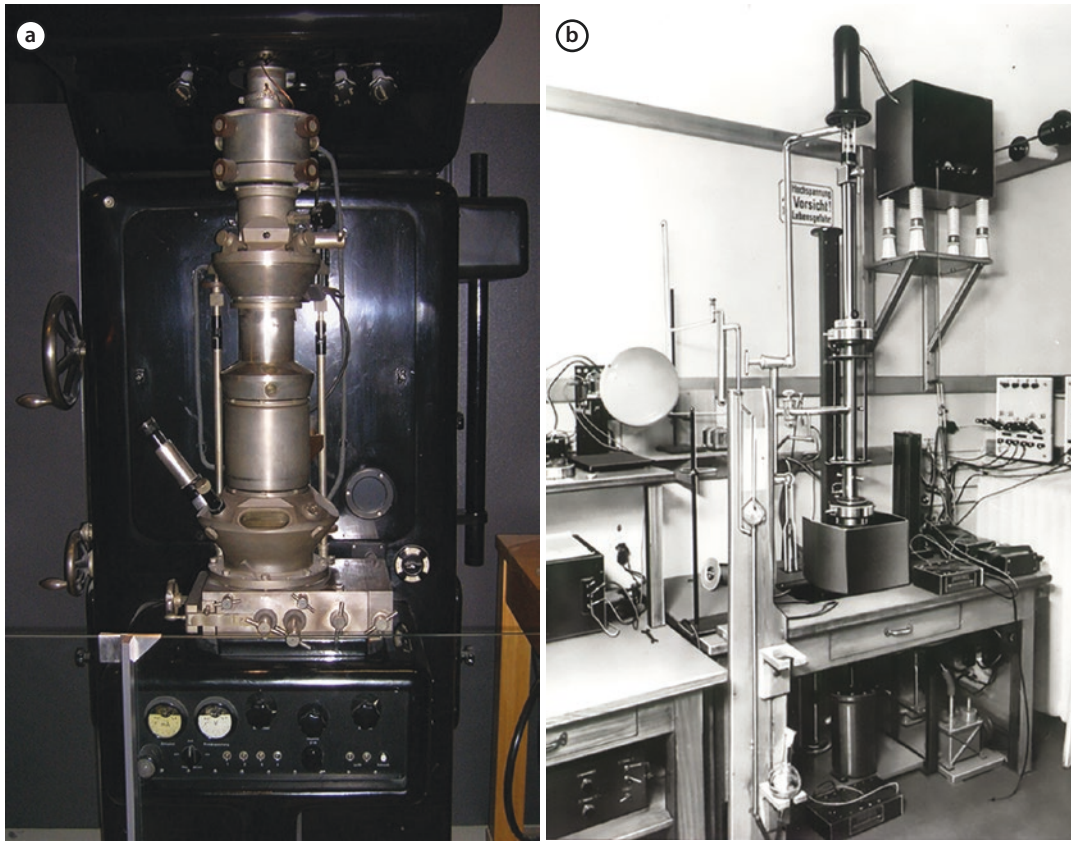


Fig. 2.10 Early electron microscopes. **a** A prototype transmission electron microscope built in 1933 by German physicist Ernst Ruska. (J Brew, CC BY-SA 3.0). **b** The first SEM capable of high-magnification imaging, built in 1937 by Manfred von Ardenne. (A von Ardenne, CC BY-SA 3.0). (Image **a** by J Brew, licensed under CC BY-SA 3.0 via Wikimedia Commons. Image **b** by Rechteinhaber: Dr. rer. nat. Alexander von Ardenne, by inheritance from his father Prof. Dr. h. c. mult. Manfred von Ardenne, C licensed under C BY-SA 3.0)

the SEM was not immediately made available to the scientific community either. In the case of the TEM, the lag was largely due to the development of suitable sample preparation techniques. SEMs were not commercially available until the mid 1960s because of the need for high quality electronics and a viable method for collecting images. Cambridge Scientific Instrument Company of Cambridge, England, sold the first SEM in 1965, the so-called Steroscan model.

A SEM scans a very narrow beam of electrons over the outside of a specimen to reveal surface detail point by point (a pattern referred to as a raster), not internal detail. The interaction of the beam with the specimen can produce reflected electrons, electrons knocked off the surface, photons, X-rays, and other signals. Different detectors pick up those signals and produce an image on a computer monitor screen that replicates the signal from each point of the raster. Notable SEM plant anatomy reference volumes include O'Brien and McCully (1969) and Lott (1976).

Compared to the maximum resolution and magnification achievable by light microscopes (about 0.2 μm and 2000×), electron microscopes can achieve theoretical resolutions of roughly 50 picometers (0.00005 μm) and magnifications of up to 10 million-fold. While

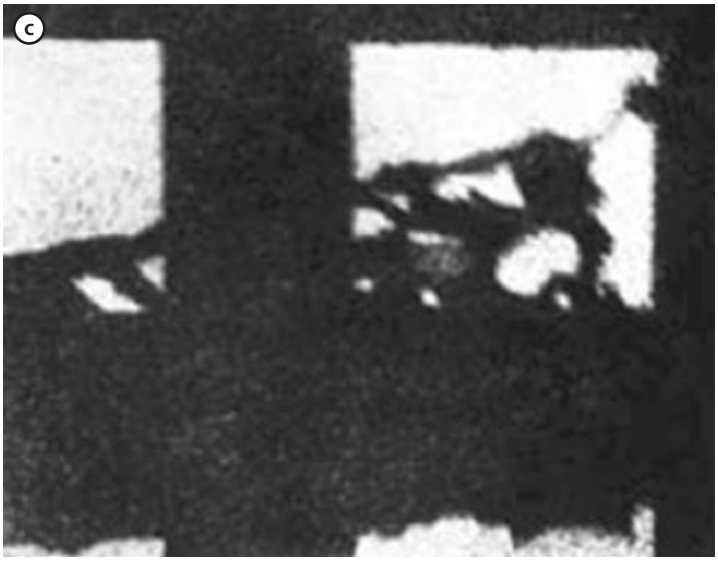


Fig. 2.10 **c** The first transmission electron micrograph of a biological specimen ever published was a hand-cut section of sundew (*Drosera intermedia*) leaf prepared by L.L. Marton 1934, in Belgium. Dark areas illustrate where the electrons could not penetrate the screen within the TEM. (Image from Marton (1934), reprinted in Süsskind (1985))

such extremes are useful for physicists and materials scientists, biological electron microscopists typically operate in the 100× to 10,000× range because biological structures, even viruses, are fairly "large" as compared to crystals and atoms.

2.11 The Transmission Electron Microscope Reveals Internal Cellular Detail

The designers of the transmission electron microscope used the same optical principles as used in light microscopy. However, electrons will not penetrate through any significant mass, including air, and therefore must be projected within a vacuum. This also means that glass lenses cannot be employed. Instead, hollow electromagnetic lenses are used in the electron microscope since electrons, being charged particles, can be refracted by circular magnetic fields in much the same way as glass lenses influence the pathway of light photons. In a transmission electron microscope, the design is similar to that of an inverted light microscope with the illumination source (an electron gun assembly) at the top of an optical column (Fig. 2.11a, b). The emitted and accelerated electrons are projected by one or two condenser lenses through a very thin specimen (remember that electrons cannot penetrate through very thick objects) and into the field of an objective lens where focusing of the electron beam takes place and projection of a real image some distance away occurs. Then, one or more projector lenses cast a final magnified image onto a sheet of film, electronic camera, or a viewing screen. When electrons strike objects, they give up their energy

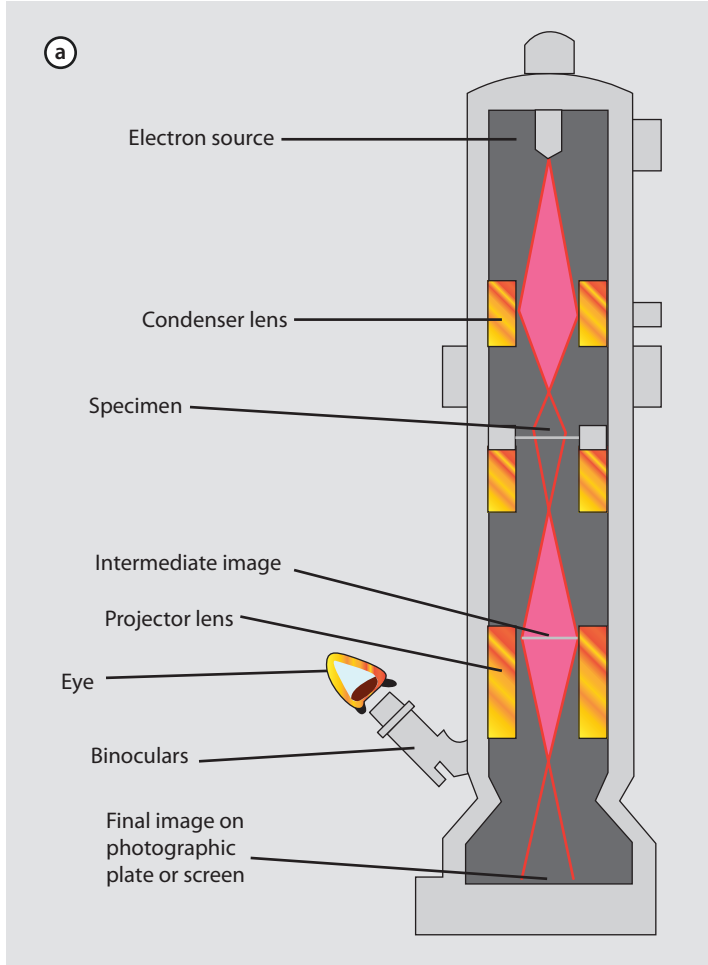


Fig. 2.11 **a** A diagrammatic representation of the basic function of the electron beam and the electromagnetic lenses in producing a shadow-type image on a fluorescent screen or electronic detection camera (Redrawn from Crang and Vassilyev 2003)

by generating X-rays that can be damaging to the operator. Thus, the operator must observe images through a thick leaded glass window, which also separates the vacuum of the column from the room outside of the microscope. Electrons cannot be directly visualized, so they can only be observed on a viewing screen (within the vacuum) that is coated with a phosphorescent paint that glows when excited by the energy of the electrons, on photographic film (again, within the vacuum) or, on modern instruments, with a digital image sensor (as in Fig. 2.11b).

Transmission electron microscopes form images based on the selective absorption of electrons by various parts of the specimen. The specimen lies above the fluorescent screen, and hence, the electrons that make a mark on the screen are the ones able to pass through the specimen. The parts that absorb the electrons prevent their passage to the screen and thus appear dark. The parts that allow the electrons to pass through appear bright (Fig. 2.11c).

Fig. 2.11 **b** A modern transmission electron microscope (Hitachi HT-7700) with many refinements is shown. Although appearing quite different, it uses essentially the same optical plan as the instrument from 1933. Note the lack of a viewing port. All imaging is done digitally and displayed on the computer monitor. (Image courtesy of Roger Teppert, Hitachi High Technologies America, Inc.)

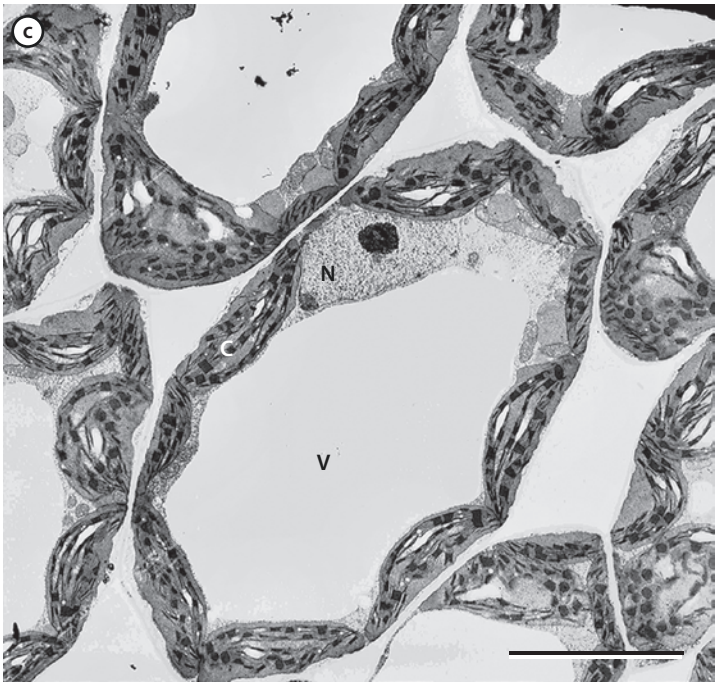


Fig. 2.11 **c** Transmission electron micrograph of a cucumber (*Cucumis sativus*) leaf cell. Note vacuole (V), nucleus (N) and chloroplasts (C). Scale bar = 20 μm (RR Wise)

2.12 The Scanning Electron Microscope Resolves Surface Detail

The scanning electron microscope has many features similar to the transmission electron microscope, up to a point. To a degree, it may be thought of as the "top half" of a transmission electron microscope column. In essence, the electron beam is reduced in size by one or more condenser lenses producing a very narrow beam diameter, and then it is electronically deflected in a raster pattern across a solid specimen surface in a series of lines, each of which is composed of many image points. The electron beam dwells for a very short time at each image point (e.g., a millionth of a second or less), during which time it excites outer shell electrons out of the specimen (called secondary electrons). These are low-energy electrons (typically <50 electron volts of energy), and are therefore capable of being attracted to a positively charged (usually ~ + 250 eV) detector, where they create an electrical signal that is proportional to their numbers from any given site on the specimen. The strength of this signal regulates the intensity

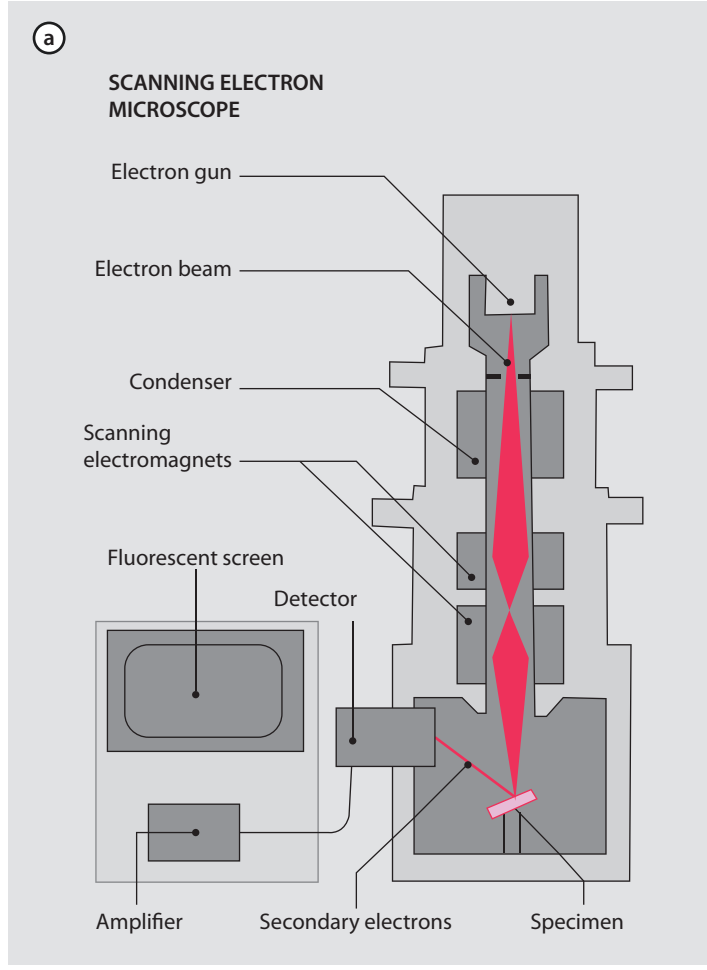


Fig. 2.12 **a** Diagram of the beam path in an SEM (Redrawn from Crang and Vassilyev 2003)

Fig. 2.12 **b** A modern scanning electron microscope with ultra-high-resolution capabilities (SU-3500). It is capable of resolving structures 0.8 nm at 15 eV beam acceleration. (Image courtesy of Roger Teppert, Hitachi High Technologies America, Inc.)

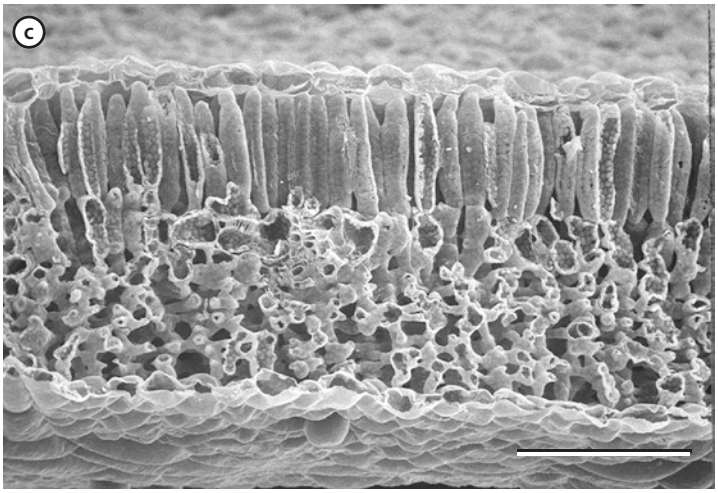


Fig. 2.12 **c** Upland cotton (*Gossypium hirsutum*) leaf in cross-section. The specimen was frozen and then fractured along a cross-sectional plane, which was then prepared for observation with the SEM. Scale bar = 100 µm (RR Wise)

(brightness) of a corresponding electron beam in a television or monitor-like screen. Thus, the number of electrons emitted from the surface of a specimen at any one point determines the intensity of the signal on the electronic viewing screen (Fig. 2.12a–c).

The electron beam of the microscope and the raster display both move in synchrony. However, the ratio of the length of the electron

beam scan across the specimen, to the scan of the one across the viewing screen, determines image magnification. The smaller the sweep across the specimen, the greater the displayed magnification. Thus, this instrument is more like an image mapping system compared to the optical projection of the transmission electron microscope.

Box 2.2 An Atomic-Resolution Microscope Without Lenses

Light and electron microscopes use lenses, either glass or electromagnetic, to manipulate an illuminating beam of photons or electrons to produce the image of a specimen. Alternatively, an atomic force microscope (AFM), physically scans the surface of a specimen with a very small and highly sensitive probe capable of measuring atom-to-atom interactions. In effect, the AFM "feels" its way across the surface and generates a three-dimensional map of surface features. Resolutions of 30 nm (1×10^{-9} m) in the horizontal direction and 1 nm in the vertical direction can be achieved. The plant cuticle is a layer of lipids, largely wax, that coats the leaves and stems of terrestrial plants (refer to ► Chap. 9). By gently removing the cuticle from living plant leaves, and then using AFM to monitor the redeposition of waxes, researchers have been able to determine the timing and rate of various stages of cuticle formation. This information is useful in understanding the basic biology of cuticle formation at an extremely high "magnification."

Reference: Koch et al. (2004).

2.13 Different Microscopies Produce Different Images of the Same Specimen

Below are four images of the same specimen—stomata from cotton (*Gossypium hirsutum*). All have been adjusted to the same magnification, yet note the different kinds of visual information conveyed by each. The light micrograph (◘ Fig. 2.13a) shows a flat appearance and low resolution due to the very shallow depth of focus of the instrument. For the CLSM image (◘ Fig. 2.13b), the leaf was stained with Auramine O, which fluoresces only in the presence of lipids and waxes. It is used to highlight the layer of epicuticular wax that covers the exterior of the leaf and extends into the **substomatal cavity**. The transmission electron micrograph (◘ Fig. 2.13c) is of a section that shows internal structure of the guard cells. The scanning electron micrograph (◘ Fig. 2.13d) shows a three-dimensional like view, which quickly gives the observer a feel for the surface properties of the leaf and stomatal complex.

The transmission electron microscope, like the light microscope, projects an optical image on a viewing or recording plane from a thin specimen. On the other hand, the scanning electron microscope generates an image map of the surface of a specimen. It

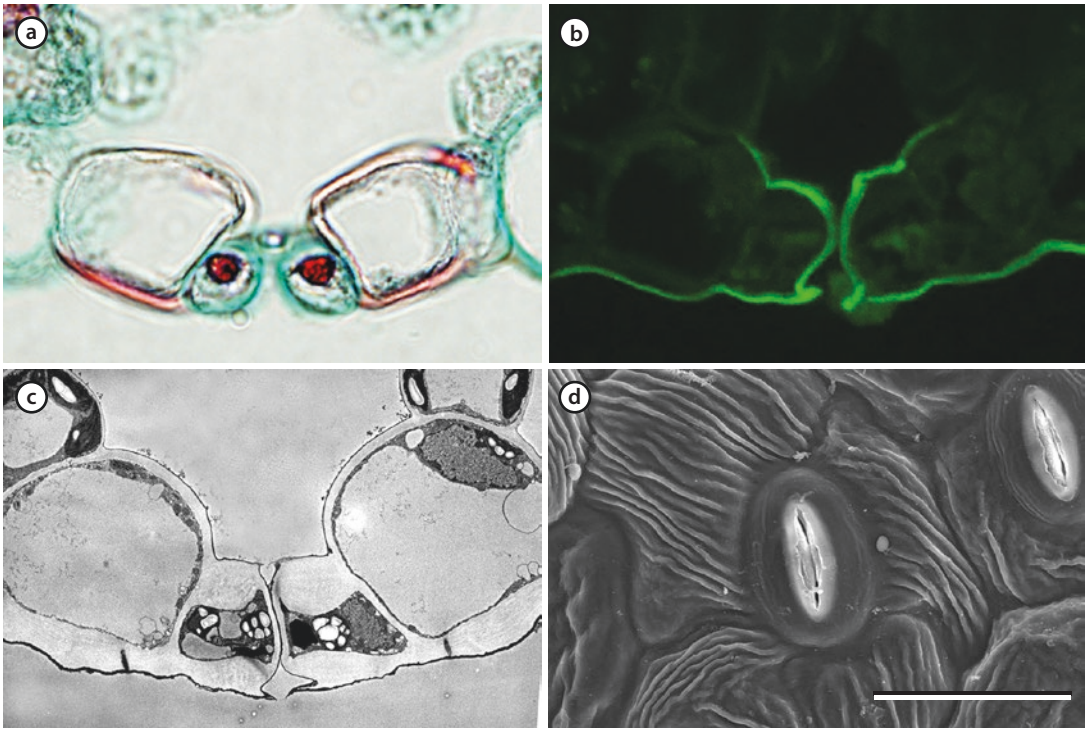


Fig. 2.13 Comparative micrographs of upland cotton (*Gossypium hirsutum*) stomata revealed in cross-sectional view using **a** light microscopy, **b** confocal laser scanning microscopy (stained green to reveal epicuticular wax), **c** transmission electron microscopy, and **d** in surface view using scanning electron microscopy. Scale bar in (**d**) = 20 μm and applies to all panels (**a**–**d** RR Wise)

can readily be calculated that the limit of resolution of the light microscope is approximately 0.2 μm (200 nm), that of the scanning electron microscope is approximately 1.0 nm, and that of the transmission electron microscope normally is approximately 0.1 nm. Thus, based on these resolution potentials, the highest useful magnification of the transmission electron microscope is about 500× greater than the light microscope and 10× greater than that of the scanning electron microscope.

2.14 Chapter Review

Concept Review

2.1 *Robert Hooke, 1635–1703, was the first to describe a cell as the basic unit of life*. Hooke was the first to describe biological materials under microscopes and discover the cell as the simplest unit of life.

2.2 *Antoni van Leeuwenhoek, 1632–1723, was the first scientist to observe microorganisms*. Van Leeuwenhoek had a deep interest in science and microscopy which lead to innovations in single-lens microscopes. He is also known for his work in describing microorganisms.

2.3 *Nehemiah Grew, 1641–1712, was the father of plant anatomy*. Grew is most noted for his pivotal work on two illustrated books focused solely on plant anatomy.

2.4 *Robert Brown, 1773–1858, discovered the nucleus of the cell*. While Brown's work is exciting for any cell biologist, his discovery of the nucleus in orchid cells earns him notoriety in the field of plant anatomy. Other notable discoveries include Brownian movement and cytoplasmic streaming.

2.5 *Katherine Esau, 1898–1997, advanced the field of plant anatomy with her influential textbooks*. Esau's work in plant anatomy is so thorough that no other books on plant anatomy have been as detailed or complete. Her life's work in plant anatomy led to many honors including the National Medal of Science.

2.6 *Light microscopy: The most useful tool of the plant anatomist*. Light microscopy provides relatively high magnification and resolution and continues to be a vital tool for the modern plant anatomist.

2.7 *The compound light microscope uses multiple lenses to form and capture images*. Compound light microscopes use two or more lenses, which allow for both variable and greater magnification of images, wider fields of view, and brighter images. It also allows the observer to work at greater distances from the specimen.

2.8 *The resolving power of a lens places limits on resolution and magnification*. The resolving power of a microscope is a function of the wavelength divided by the sum of the numerical aperture of the objective and condenser lenses.

2.9 *The confocal microscope allows for sharper detail, computer control, and 3-D imaging with a modified compound microscope*. Confocal microscopy provides greater detail in the anatomy, structure, function, and gene expression within plants using fluorescence.

2.10 *Electron microscopy allows a view into the world of cellular ultrastructure*. Using electron beams, scientists can view exceptionally small structures due to the greater resolution and magnification afforded by electron microscopy.

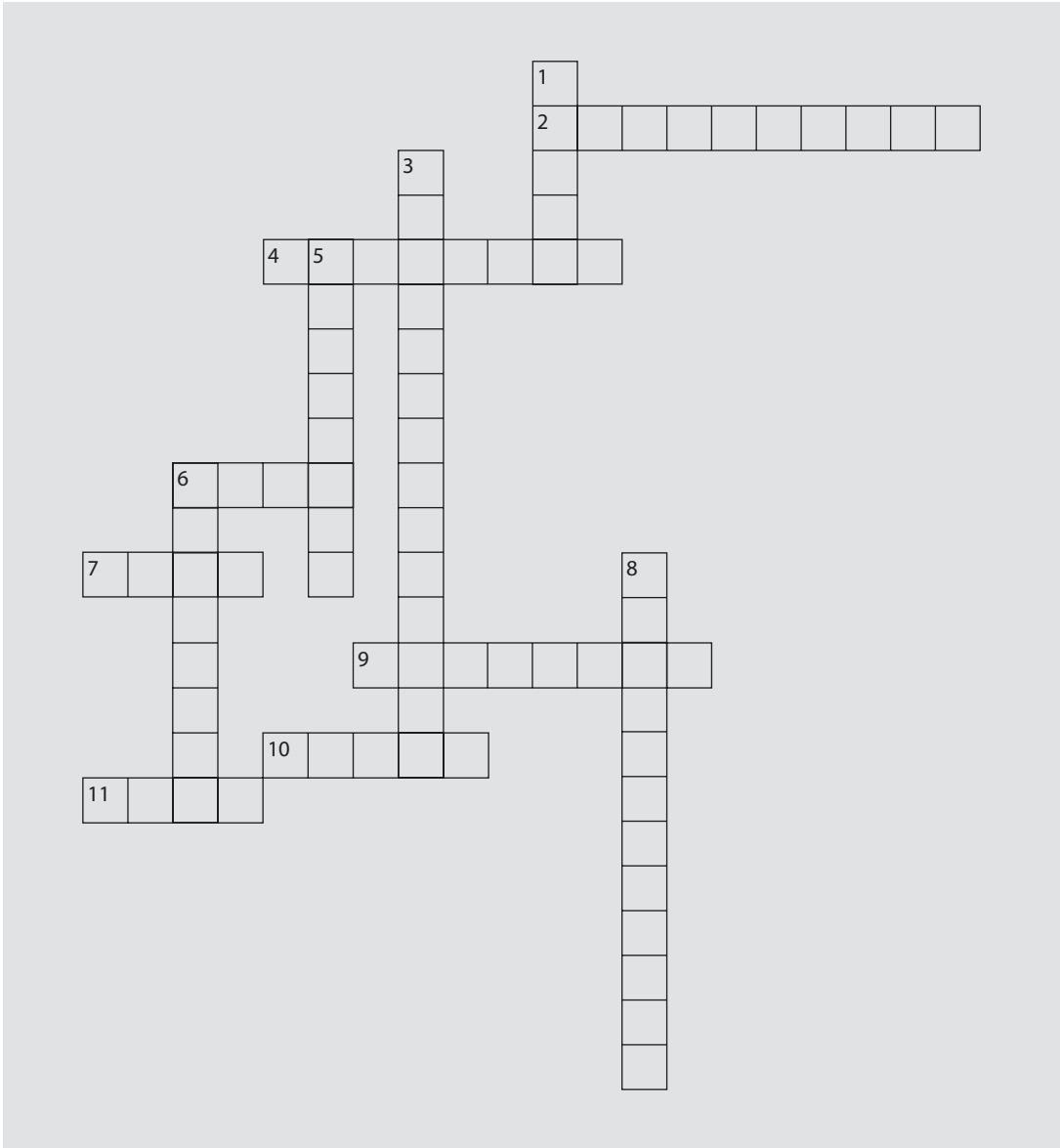
2.11 *The transmission electron microscope reveals internal cellular detail*. Electrons have a very limited ability to penetrate substances. When looking at a TEM image, the parts that are dark represent those areas where the electrons were absorbed, while the parts that allowed the passage.

2.12 *The scanning electron microscope resolves surface detail*. SEM scans electrons over the outside surface of a specimen. When internal structures are to be visualized, the specimens are frozen and fractured to allow for the surfaces of the internal components of the cell to be imaged.

2.13 *Different microscopies produce different images of the same specimen*. This chapter discusses a variety of different microscopy techniques, which are all valuable at the level of detail that they can capture from light microscopy to the exceptionally high resolution of the electron microscopes.

■ Concept Connections

1. Complete the crossword puzzle with the most appropriate term.



Across

2. When this is high, gaps between adjacent objects are small
4. Type of electron microscopy that details surfaces
6. Famous plant anatomist who began studying how viruses travel through phloem
7. Father of plant anatomy
9. Uses fluorescence to build 3-D plant structures
10. First described the cell as cellulae
11. Magnifies a specimen

Down

1. Determined that a nucleus was in a cell
3. An excellent lens maker
5. Type of light microscope that has more than a single lens
6. Type of microscopy that can provide highly magnified images using electron beams
8. Type of electron microscopy that can detail the inner details within a cell

■ Concept Assessment

2. _____ is credited with the discovery of the cell.
   a. Robert Hooke.
   b. Robert Brown.
   c. Katherine Esau.
   d. Nehemiah Grew.
   e. Zacharias Jansen

3. Antoni van Leeuwenhoek was very important in the field of microscopy. What advances he credited with?
   a. high-quality single-lens microscopes.
   b. cytoplasmic streaming.
   c. initial descriptions of many types of microorganisms.
   d. both a and c.
   e. a, b, and c.

4. The most noteworthy plant anatomist of the twentieth century was
   a. Zacharias Jansen.
   b. Robert Hooke.
   c. Katherine Esau.
   d. Nehemiah Grew.
   e. Robert Brown.

5. One primary advantage of a lens in a camera over a pinhole is
   a. greater light-gathering power.
   b. a fixed focal length.
   c. a small numerical aperture.
   d. greater empty magnification.
   e. lower cost.

6. The physical principles of microscope resolution were first determined by
   a. Ernst Abbé.
   b. Katherine Esau.
   c. J. J. Thompson.
   d. Robert Brown.
   e. Carl Zeiss.

7. Resolution in a microscope is primarily determined by which lens?
   a. ocular.
   b. condenser.

c. objective.
d. projector.
e. intermediate.

8. The compound microscope is more powerful than a single-lens microscope because
   a. it reduces the amount of light necessary to view the specimen.
   b. it allows for a narrow field of view to enhance magnification.
   c. two lenses allow for increased magnification of the specimen.
   d. the multiple lenses allow the microscopist to work closer to the specimen.
   e. the multiple lenses have little to no effect on the brightness of the image.

9. In its optical design, the transmission electron microscope is most like a (n)
   a. scanning electron microscope.
   b. atomic force microscope.
   c. single-lens microscope.
   d. confocal microscope.
   e. compound light microscope (brightfield).

10. How does the scanning electron microscope differ from the transmission electron microscope? It
    a. uses electromagnetic lenses.
    b. operates with a vacuum.
    c. uses an electron beam.
    d. maps images rather than optically projecting them.
    e. produces monochrome images.

11. Which instrument is best used to view the structure of viruses?
    a. single-lens microscope.
    b. confocal microscope.
    c. scanning electron microscope.
    d. transmission electron microscope.
    e. compound light microscope (brightfield).

■ Concept Applications

12. If you want to investigate the expression of a gene in the root of an oak tree, what type of microscopy would you use? Why? Could you use this same type of microscopy in a leaf, why or why not?

13. You have been charged with understanding the leaf anatomy of a plant in the desert in contrast to a plant from a tropical rain forest. What types of microscopy would you use to investigate similarities or differences associated with the leaves?

References and Additional Readings

Brown R (1828) A brief account of microscopical observations made on the particles contained in the pollen of plants. Philosophical Mag 4:161–173

Crang RFE, Vassilyev A (2003) Electronic plant anatomy. McGraw-Hill, New York

Cutler DF, Botha T, Stevenson D (2008) Plant anatomy: an applied approach. Blackwell Publishing, Malden

Dickinson WC (2000) Integrative plant anatomy. Hardcourt, Inc., Orlando

Esau K (1953) Plant anatomy. Wiley, New York

Esau K (1961) Plants, viruses, and insects. Harvard University Press, Cambridge, MA

Esau K (1969) The Phloem (Handbuch der Pflanzenanatomie, Histologie Band 5, Teil 2). Gebrüder Borntraeger, Berlin

Esau K (1977) Anatomy of seed plants. Wiley, New York

Evert RF (2006) Esau's plant anatomy: meristems, cells and tissues of the plant body – their structure, function and development, 3rd edn. Wiley & Sons, Hoboken

Fahn A (1990) Plant anatomy, 4th edn. Pergamon Press, New York

Ford BJ (1992) From dilettante to diligent experimenter: a reappraisal of Leeuwenhoek as microscopist and investigator. Biol Hist 5:3

Grew N (1682) The anatomy of plants: with an idea of a philosophical history of plants. W Rawlins, London

Hawkes PW (1990) Ernst Ruska. Phys Today 43:84–85

Hooke R (1665) Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses. With observations and inquiries thereupon. The Royal Society, London

Jardine L (2005) The curious life of Robert Hooke: the man who measured London. Harper Collins Pub, New York

Koch K, Neinhuis C, Ensikat H-J, Barthlott W (2004) Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). J Exptl Bot 55:711–718

Ledbetter MC, Porter KR (1970) Introduction to the fine structure of plant cells. Springer, Berlin

Lott JNA (1976) A scanning electron microscope study of green plants. CV Mosby Company, Saint Louis

Marton LL (1934) La microscopie électronique des objects biologiques: I. Bull Acad Roy Belgique 20:439

Mauseth JD (1988) Plant anatomy. Benjamin/Cummings, Menlo Park

Metcalfe CR, Chalk L (1979) Anatomy of the Dicotyledons: Vol 1, Systematic anatomy of the leaf and stem, 2nd edn. Oxford University Press, New York

Metcalfe CR, Chalk L (1983) Anatomy of the Dicotyledons: Vol 2, Wood structure and conclusion of the general introduction, 2nd edn. Oxford University Press, New York

O'Brien TP, McCully ME (1969) Plant structure and development: a pictorial and physiological approach. Macmillan Co, New York

Ramasse QM (2017) Twenty years after: how "aberration correction in the STEM" truly placed a "a synchrotron in a microscope". Ultramicroscopy 180:41–51

Rasmussen N (1997) Picture control: the electron microscope and the transformation of biology in America, 1940–1960. Stanford University Press, Stanford

Schierbeek A (1959) Measuring the invisible world: the life and works of Antoni van Leeuwenhoek. Abelard-Schuman, London

Solereder H (1908a) Systematic anatomy of the Dicotyledons: a handbook for laboratories of pure and applied botany, vol 1. Clarendon Press, Oxford

Solereder H (1908b) Systematic anatomy of the Dicotyledons: a handbook for laboratories of pure and applied botany, Monochlamydea, addenda, and concluding remarks, vol Vol. 2. Clarendon Press, Oxford

Storath M, Rickert D, Unser M, Weinmann A (2017) Fast segmentation from blurred data in 3D fluorescence microscopy. IEEE Trans Image Process 26:4856–4870

Süsskind C (1985) LL Marton, 1901–1979. In: Hawkes PW (ed) The beginnings of electron microscopy. Advances in Electronics and Electron Physics, vol 16. pp 501–524