BCHEM 365
Lecture 13
December 10, 2018
Microscopy

INTRODUCTION

The study of tissues, cells and organelles is frequently accompanied by microscopic examination. Because cells are small and complex, it is hard to see their structure, hard to discover their molecular composition, and harder still to find out how their various components function. To understand contemporary cell biology, it is necessary to have knowledge about the methods used to study cells. A microscope is used to reveal the presence of a cell, its shape, internal structural organization and function by magnifying and resolving objects. Magnification is not however, the best measure of a microscope's usefulness, but rather, **resolution.** Resolution is the ability to distinguish between two closely spaced points in a specimen and is a much more reliable estimate of a microscope's utility.

Microscopic examinations are used in many different applications

1. To evaluate the integrity of samples during an experiment,
2. To map the fine details of the spatial distribution of macromolecules within cells,
3. To directly measure biochemical events within living tissues.

There are two fundamentally different types of microscopes:

1. Optical or light microscope
2. Electron microscope
3. Scanning probe microscopes: creates images of surfaces using a probe, example, Atomic Force Microscopy (AFM), and Scanning thermo-ionic microscope (STIM).

Fig. 13.1 shows a schematic of the two microscopes in comparison.

COMPARING OPTICAL MICROSCOPE AND THE ELECTRON MICROSCOPE

1. Light microscopes use a series of glass lenses to focus light in order to form an image whereas electron microscopes use electromagnetic lenses to focus a beam of electrons.

2. Light microscopes produce maximum magnification of approximately 1,000 times whereas electron microscopes are capable of magnifying to a maximum of approximately 200,000 times.
3. Light microscopes have a resolution limit of about 0.2 µm for routine analysis. In contrast, electron microscopes have a resolution of up to 1 nm (0.0002 µm).
4. Both living and dead specimens are viewed with a light microscope, and often in real color, whereas only dead specimens are viewed with electron microscope, and never in real color.

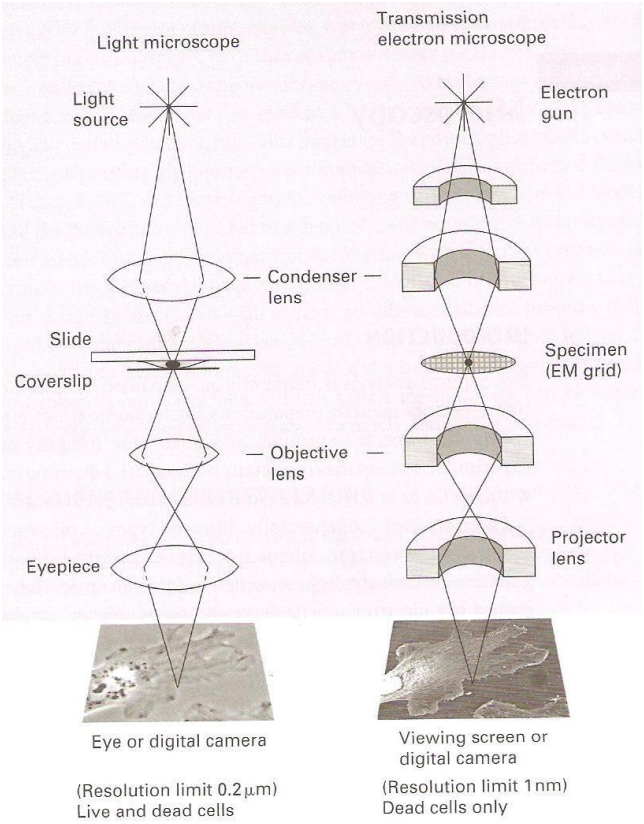


Fig. 13.1. Light and electron microscopy. Schematics that compare the path of light through a compound light microscope (LM) with the path of electrons through a transmission electron microscope (TEM). Light from a lamp (LM) or a beam of electrons from an electron gun (TEM) is focused at the specimen by a glass condenser lens (LM) or electromagnetic lenses (TEM). For LM, the specimen is mounted on a glass slide with a coverslip placed on top, and for TEM, the specimen is placed on a copper electron microscope grid. The image is magnified with a glass objective lens (LM) and an electromagnetic objective lens in TEM and projected onto a detector with eyepiece lens in LM or the projector lens in TEM. The detector can be the eye or a digital camera in the LM or phosphorescent viewing screen or digital camera in TEM.

In recent years optical microscopy has become even more important, owing to the development of methods for specific labeling and imaging of individual cellular constituents and the reconstruction of their three-dimensional architecture. An important advantage of optical microscopy is that light is

relatively nondestructive. By tagging specific cell components with fluorescent probes, such as intrinsically fluorescent proteins e.g., green fluorescent protein, GFP), we can watch their movement, dynamics, and interactions in living cells.

Optical microscopy is limited in resolution by the wavelength of visible light. By using a beam of electrons instead, electron microscopy can image the macromolecular complexes within cells at almost atomic resolution, and in three dimensions. Fig. 13.2 shows a series of images illustrating an imaginary progression from a thumb to a cluster of atoms. Each successive image represents a tenfold increase in magnification. The naked eye can see features in the first two panels, the resolution of the light microscope would extend to about the fourth panel, and the electron microscope to between about the seventh and eighth panel.

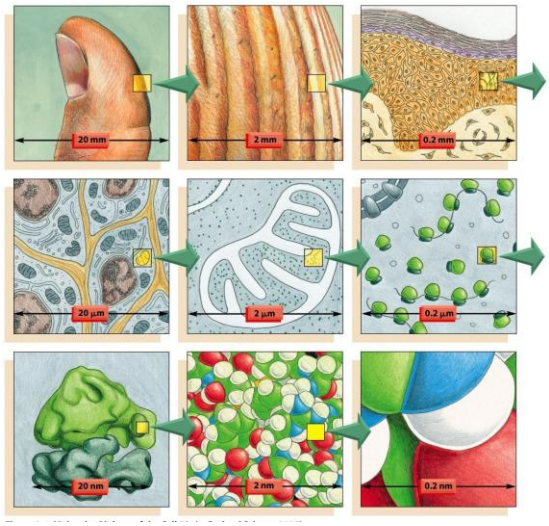


Fig. 13.2. **A sense of scale between living cells and atoms.** Each diagram shows an image magnified by a factor of ten in an imaginary progression from a thumb, through skin cells, to a ribosome, to a cluster of atoms forming part of one of the protein molecules in the human body. Atomic details of macromolecules as shown in the last panel are usually beyond the power of the electron microscope.

Fig. 13.3 shows the sizes of various cellular and subcellular structures and the range of sizes that different types of microscopes can visualize.

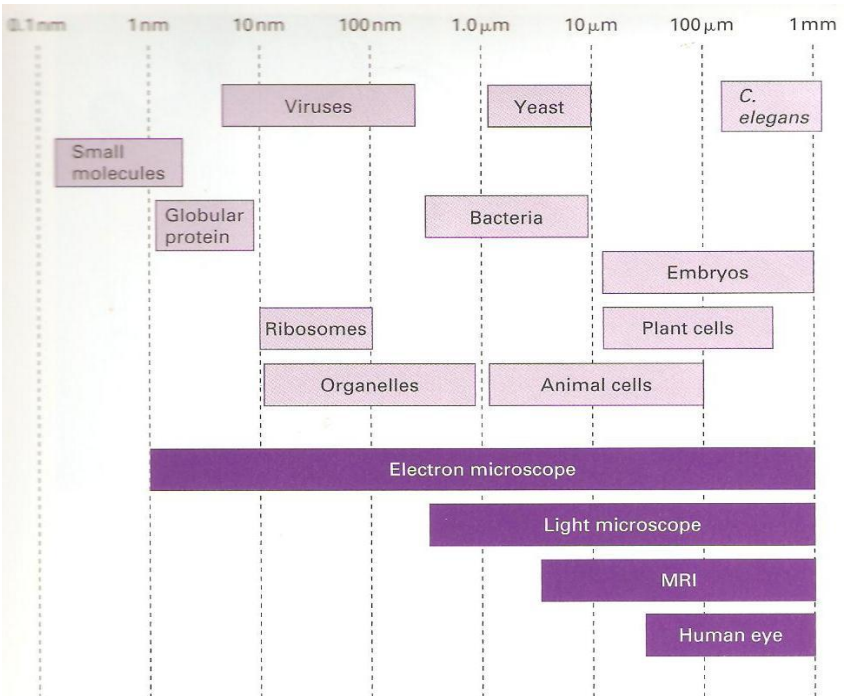


Fig. 13.3. **Resolving power**. The relative sizes of a selection of biological specimens and some devices used to image them. The following units of length are commonly employed in microscopy: μm (micrometer) = 10^{-6}m; nm (nanometer) = 10^{-9} m; Å (Ångström unit) = 10^{-10} m.

THE LIGHT MICROSCOPE

The resolving power of the light microscope under ideal conditions is about half the wavelength of the light being used. **Resolving power** is the distance that must separate two point sources of light if they are to be seen as two distinct images. With yellow light of a wavelength of 0.4 μm, the smallest separable diameters are thus about 0.2 μm. Compare with size of bacteria (0.2 to 2.0 μm) and eukaryotic cell (10 to 100 μm). The useful magnification of a microscope is the magnification that makes visible the smallest resolvable particles. A variety of light microscopes are commonly used in microbiology and cell culture studies: bright-field, dark-field, phase-contrast, fluorescence, and confocal microscopes, and stereomicroscopes.

The simplest form of light microscope consists of a single glass lens (simple microscope) mounted in a metal frame - a magnifying glass (Fig. 13.4). Here the specimen requires very little preparation, and is usually held close to the eye in the hand. Focusing of the region of interest is achieved by moving the lens and the specimen relative to one another. The source of light is usually the sun or ambient indoor

light. The detector is the human eye. The recording device is a hand drawing.

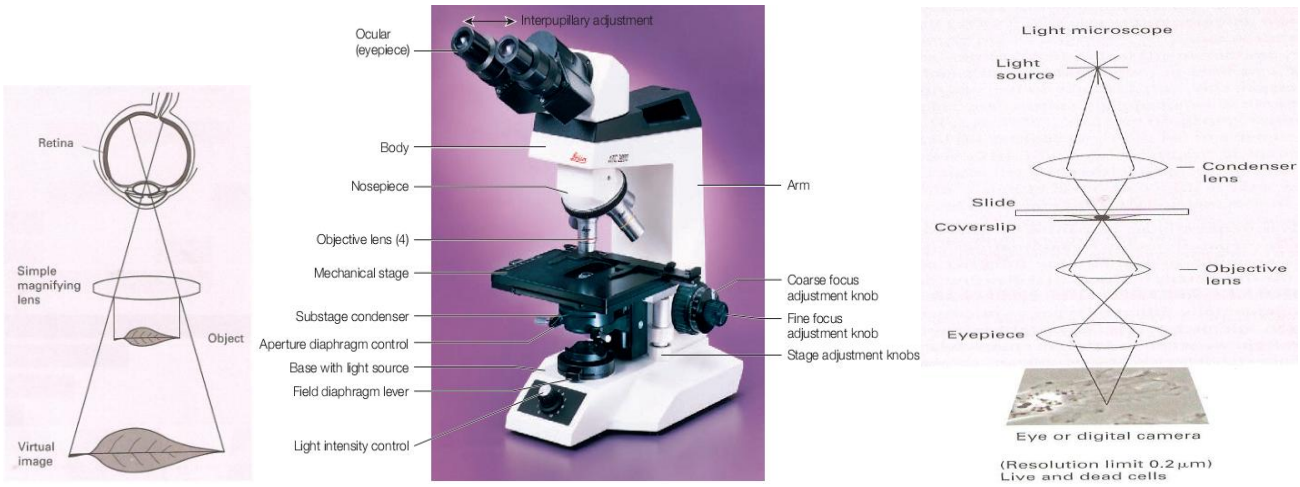


Fig. 13. 4. Left: Magnification in a simple microscope – a glass magnifying lens. Middle: parts of a bright-field compound microscope; Right: the path of light through a microscope. Source: Prescott, 2002.

Compound microscopes

Parts of a bright-field microscope

The microscope consists of a sturdy metal body or stand composed of a base and an arm to which the remaining parts are attached (Fig. 13.5). A light source, either a mirror or an electric illuminator, is located in the base. Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and can move either the stage or the nosepiece to focus the image. The stage is positioned about halfway up the arm and holds microscope slides by either simple slide clips or a mechanical stage clip and allows the operator to move a slide around smoothly during viewing by use of stage control knobs. Any movement or vibration will be detrimental to the final image. The stage enables the specimen to be moved and positioned in fine and smooth increments, both horizontally and transversely, in the X and the Y directions, for locating a region of interest.

The substage condenser is mounted within or beneath the stage and focuses light from the light source and illuminates the specimen with a parallel beam of light. Its position often is fixed in simpler microscopes but can be adjusted vertically in more advanced models. A correctly positioned condenser lens produces illumination that is uniformly bright and free from glare across the viewing area of the

specimen (Köhler illumination). Condenser misalignment and an improperly adjusted condenser aperture diaphragm are major sources of poor images in the light microscope.

The curved upper part of the arm holds the body assembly, to which a **nosepiece** and one or more **eyepieces** or **oculars** are attached. More advanced microscopes have eyepieces for both eyes and are called binocular microscopes. The body assembly itself contains a series of mirrors and prisms so that the barrel holding the eyepiece may be tilted for ease in viewing. The nosepiece holds three to four objective lenses which often have specific magnifying power (Table 13.1) and can be rotated to position any objective beneath the body assembly.

Table 13.1. Magnification of lenses in a microscope

Magnification of objective lens	Magnification of eyepiece	Total magnification
4×	10×	40×
10×	10×	100×
40×	10×	400×
100×	10×	1000×

The path of light through a bright-field microscope is shown in Fig. 13.5B. The objective lens forms an enlarged real image within the microscope, and the eyepiece lens further magnifies this primary image. When one looks into a microscope, the enlarged specimen image, called the virtual image, appears to lie just beyond the stage about 25 cm away. The image is either viewed directly by eye in the eyepiece or is most often projected onto a detector, for example photographic film or, more likely, a digital camera. The images are displayed on the screen of a **computer imaging system**, stored in a digital format and reproduced using digital methods.

Lenses

All modern light microscopes are made up of more than one glass lens in combination. The major components are the condenser lens, the objective lens and the eyepiece lens, and, such instruments are therefore called compound microscopes (Fig. 13.4). Each of these components is in turn made up of combinations of lenses, which are necessary to produce magnified images with reduced artifacts and aberrations, such as chromatic aberration.

Chromatic aberration is a type of distortion in which there is failure of a lens to focus all wavelengths of light to the same convergence point. It is common with single lenses made of conventional glass. It occurs because lenses have different refractive index for different wavelengths of light (the dispersion of the lens). The refractive index decreases with increasing wavelength. Since the focal length, f , of a lens is inversely related to the refractive index n, different wavelengths of light will be focused at different positions.

Chromatic aberration manifests itself as "fringes" of color or rainbow colors around the edges of the image, because each color in the optical spectrum cannot be focused at a single common point (Fig. 13.5). This problem was encountered in the early microscopes of Antonie Van Leeuwenhoek and Robert Hooke. All modern lenses are corrected to some degree in order to avoid this problem.

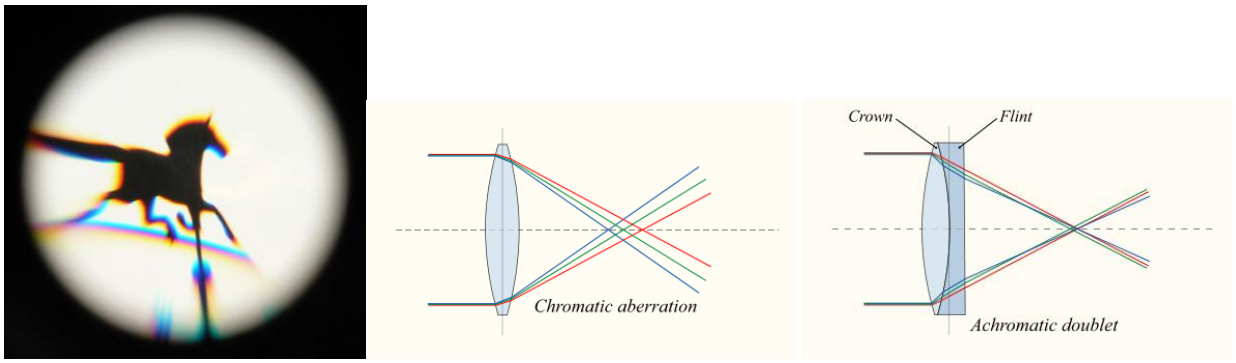


Fig. 13.5. Chromatic aberration and its minimization

Chromatic aberration can be minimized by using an achromatic lens or '*achromat*', in which different glass materials having low dispersion are assembled together to form a compound lens. The most common type is an 'achromatic doublet', with elements made of **crown** glass and **flint** glass (soda lime silica with lead oxide), and not conventional glass (silica). This reduces the amount of chromatic aberration over a certain range of wavelengths, though it does not totally eliminate aberration. By combining more than two lenses of different composition, the degree of correction can be further increased, as seen in an '*apochromatic*' lens or '*apochromat*'. Note that "achromat" = combination of two lenses that corrects focusing of two wavelengths; "apochromat" refers to combination of more than two lenses and corrects focusing of three or more wavelengths sharply with less error. Many types of glass have been developed to reduce chromatic aberration. These are low dispersion glass, most notably,

glasses containing **fluorite**. These hybridized glasses have very low level of optical dispersion. A combination of two such lenses can produce a high level of correction (Fig. 13.5).
Crown glass (soda-lime-silica; Na_2O- CaO-SiO_2) and containing approximately 10% potassium oxide is one of the earliest low dispersion glasses. It has relatively low refractive index (≈1.52) and low dispersion. Flint glass (soda-lime-silica; Na_2O- CaO-SiO_2) plus additives such as titanium dioxide, lead oxide, and zirconium dioxide without significantly altering the optical properties of the glass. It has relatively high refractive index and high dispersion. A concave lens of flint glass is commonly combined with a convex lens of crown glass to produce an achromatic doublet lens because of their compensating optical properties, which reduces chromatic aberration (color defects).

The objective lens produces a magnified image, and can be the most expensive component of the light microscope. Objectives are available in many different varieties, and have some information inscribed on each one. These may include: manufacturer's name, e.g., *Zeiss*, magnification (e.g., 4×, 100×, etc), immersion requirements (air, oil or water), coverslip thickness (usually 0.17 mm) and often with more-specialized optical properties of the lens.
In addition, lens corrections for optical artifacts such as chromatic aberration and flatness of field may also be included in the lens description. For example, words such as fluorite, the least corrected (often shortened to 'fluo'), or 'plan apochromat', the most highly corrected (often shortened to 'plan' or 'plan apo'), may appear somewhere on the lens.

Lenses can either be dry or immersion lenses, and as a rule of thumb, most objectives below 40× are air (dry) objectives and those of 40× and above are immersion objective (oil, glycerol or water). Should the objective be designed to operate in oil it will be labeled 'OIL' or 'OEL'. The lens will be marked to indicate which immersion must be used. Many lenses are color coded to a manufacturer's specifications.

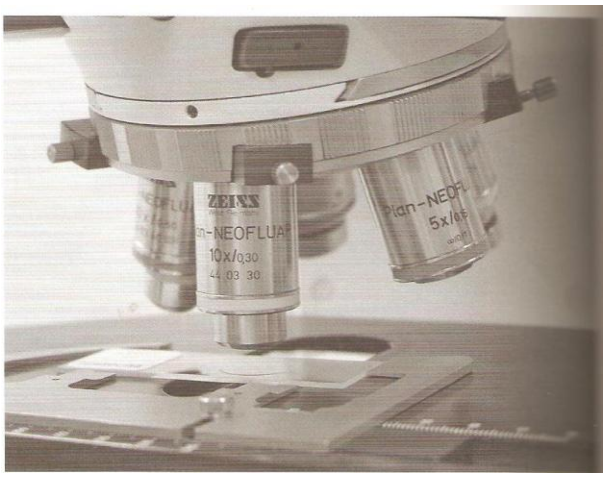


Fig. 13.6. The objective lens. A selection of objective lenses mounted on the upright research grade compound light microscope. The inscriptions present on the lenses are
Magnification of 10× and 5×; Numerical aperture of 0.3 and 0.16; both lenses are Plan NeoFluor, which means they are relatively well corrected for chromatic aberration. The 10× lens is directly above a specimen mounted on a slide and coverslip and held in place on the coverslip.

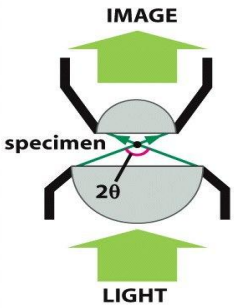
Limit of resolution

It is the limiting separation at which two objects appear distinct. It depends on both the wavelength of the light and the **numerical aperture** (NA*)* of the lens system used. The numerical aperture is a property of lens that determines the quantity of light that can enter. It is driven by the angle of the cone of light (theta) entering the objective. It is scaled according to its distance from the object; the wider the microscope opens its eye, so to speak, the more sharply it can see.

$$\mathbf{NA = n\sin\theta}$$

where *n*=refractive index of the imaging medium between the front lens of the objective and the specimen cover glass (could be air, oil, etc). Its value ranges from 1.00 for air to 1.51 for specialized immersion oils. Theta, θ, is half the angular aperture.

LENSES



the **objective** lens collects a cone of light rays to create an image

the **condenser** lens focuses a cone of light rays onto each point of the specimen

RESOLUTION: the resolving power of the microscope depends on the width of the cone of illumination and therefore on both the condenser and the objective lens. It is calculated using the formula

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin\theta}$$

where:

- θ = half the angular width of the cone of rays collected by the objective lens from a typical point in the specimen (since the maximum width is 180°, $\sin\theta$ has a maximum value of 1)
- n = the refractive index of the medium (usually air or oil) separating the specimen from the objective and condenser lenses
- λ = the wavelength of light used (for white light a figure of 0.53 μm is commonly assumed)

NUMERICAL APERTURE: $n \sin\theta$ in the equation above is called the numerical aperture of the lens (NA) and is a function of its light-collecting ability. For dry lenses this cannot be more than 1, but for oil-immersion lenses it can be as high as 1.4. The higher the numerical aperture, the greater the resolution and the brighter the image (brightness is important in fluorescence microscopy). However, this advantage is obtained at the expense of very short working distances and a very small depth of field.

Under the best conditions, with violet light (wavelength =0.4 μm) and a numerical aperture of 1.4, the light microscope can theoretically achieve a limit of resolution of just under 0.2 μm. This limit of resolution was achieved by microscope makers at the end of the nineteenth century and since then an improvement above this has not been achieved.

Although it is possible to achieve any desired magnification, it is never possible to resolve two objects in the light microscope that are separated by less than about 0.2 μm; they will appear as a single object and look blurred.

Notice, there is a difference between **resolution**, discussed above, and **detection**. If a small object, below the resolution limit, itself emits light, then we may still be able to see or detect it. Thus, we can see a single fluorescently labeled microtubule even though it is about ten times thinner than the resolution limit of the light microscope. Diffraction effects, however, will cause it to appear blurred and at least 0.2 μm thick.

Using sensitive detection methods, we can detect and follow the behavior of even a single fluorescent protein with a light microscope. We can exploit interference and diffraction to study unstained cells in the living state.

The **numerical aperture** (NA) is always marked on the lens. This is a number usually between 0.04 and 1.4. The NA is a measure of the ability of a lens to collect light from the specimen. Lenses with a low NA collect less light than those with a high NA. Resolution varies inversely with NA, which infers that higher NA objectives yield the best resolution. Generally speaking, the higher power objectives have a higher NA and better resolution than the lower power lenses with lower NAs. For example, 0.2 μm limitresolution can only be achieved using a 60× or a 100× plan-apochromat oil immersion lens with a NA of 1.4. Should there be a choice between two lenses of the same magnification, then it is usually best to choose the one of higher NA.

The objective lens is also the part of the microscope that can most easily be damaged by mishandling. Many lenses have a protective coating but, even so, one scratch on the lens can result in serious image degradation. Therefore great care should be taken when handling objective lenses. Objective lenses must be cleaned using a protocol recommended by the manufacturer, and only by a qualified person. A dirty objective lens is a major source of poor images.

Light source – chosen to provide correct illumination of specimen in order to achieve high quality images and photomicrographs. Light sources are mercury lamps, xenon lamps or lasers.

Bright-Field Microscope

The ordinary microscope is called a bright-field microscope because it forms a dark image against a brighter background. The bright-field microscope is most commonly used in microbiology work and consists of two series of lenses **-objective** and **ocular/eyepiece lens,** which function together to resolve the image. The magnified image rendered by the objective lens is further magnified by the ocular lens. These microscopes generally employ a 100 × objective lens with a 10 × ocular lens, thus magnifying the specimen 1,000 times. Particles 0.2 μm in diameter are therefore magnified to about 0.2 mm (200 μm) and so become clearly visible. Further magnification would give no greater resolution of detail and would reduce the visible area **(field).**

With bright-field microscopes, specimens are rendered visible by staining with dyes to bring contrast between cells or their organelles and surrounding medium and hence be easily seen in the bright-field microscope.

A disadvantage of bright-field microscopy is their inability to visualize living cells because specimen preparation kills cells. Microscopists have always been challenged by the possibility that some components of the cell may be lost or distorted during specimen preparation. The only certain way to avoid the problem is to examine cells while they are alive, without fixing or freezing. For this purpose, light microscopes with special optical systems are especially useful.

Dark-Field Microscope

The dark-field microscope is a light microscope in which the lighting system has been modified to reach the specimen from the sides only. This is accomplished through the use of a special condenser that both blocks direct light rays and deflects light off a mirror on the side of the condenser at an oblique angle, thus, directing light from the side only and permitting scattered light to enter the microscope lenses. This creates a "dark field" that contrasts against the highlighted edge of the specimen and results when the oblique rays are reflected from the edge of the specimen upward into the objective of the microscope. In dark-field mode, contrast is introduced in the living cell by observing the light that is scattered by its various components. Consequently, the cell appears as a bright object against a dark background (Fig. 13.7).

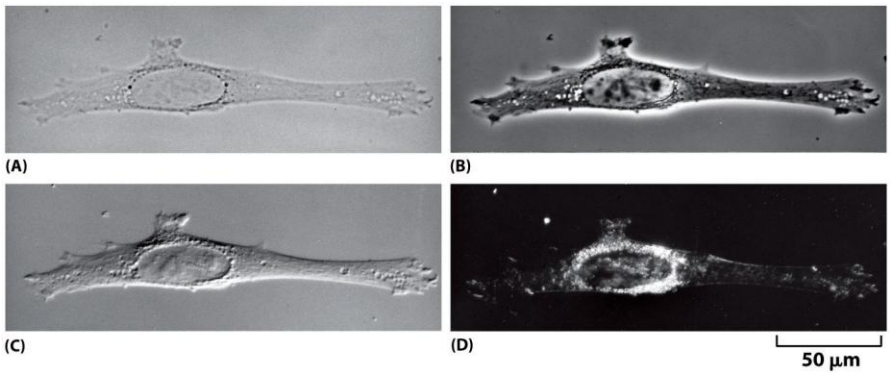


Fig. 13.7. Four types of light microscopy. Four images are shown of the same fibroblast cell in culture.

All images can be obtained with most modern microscopes by interchanging optical components. (A) Bright-field microscopy. (B) Phase contrast microscopy. (C) Normanski differential-interference-contrast microscopy. (D) Dark-field microscopy

This technique has traditionally been used for viewing the outlines of objects in liquid media such as living spermatozoa, microorganisms, **cells growing in tissue culture** or for a quick check of the status of a biochemical preparation. For lower magnifications, a simple dark-field setting on the condenser will be sufficient. For a higher magnification and better resolution, a dark-field condenser with a dark-field objective lens will be required. Resolution by dark-field microscopy is quite high. Thus, this technique has been particularly useful for observing organisms such as *Treponema pallidum*, the organism which causes syphilis, a spirochete which is less than 0.2 μm in diameter and therefore cannot be observed with a bright-field or phase contrast microscope (Fig. 13.8).

Fig. 13.8. *Treponema pallidum* in dark-field microscopy

Phase Contrast Microscope

The phase contrast microscope was developed to improve contrast differences between cells and the surrounding medium, making it possible to see living cells without staining them; remember, with bright-field microscopes, killed and stained preparations must be used. The phase contrast microscope takes advantage of the fact that light waves passing through transparent objects, such as cells, emerge in different phases depending on the refractive index of the materials through which they pass. When light passes through a living cell, the phase of the light wave is changed according to the cell's refractive index: a relatively thick or dense part of cell, such as a nucleus, retards light passing through it. The phase of the light, consequently, is shifted relative to light that has passed through an adjacent thinner

region of the cytoplasm (Fig. 13.9). The **phase-contrast microscope** and, in a more complex way, the **differential-interference-contrast microscope** exploit the interference effects produced when these two sets of waves recombine, thereby creating an image of the cell's structure (Fig. 13.7).
Both types of light microscopy are widely used to visualize living cells. It requires a specialized phase condenser and phase objective lenses (both labeled 'ph'). Each phase setting of the condenser lens is matched with the phase setting of the objective lens. These are usually numbered as phase 1, phase 2 and phase 3, and are found on both the condenser and the objective lens. The difference in interference produced is amplified by a special ring in the objective lens of a phase contrast microscope, leading to the formation of a dark image on a light background.

Phase contrast, DIC, and dark-field microscopy makes it possible to watch movements involved in processes such as mitosis and cell migration, however, because cellular motions are too slow to be seen in real time, special cameras which employ successive frames separated by short time delay, so that when the resulting picture series is played at normal speed, events appear greatly speeded.

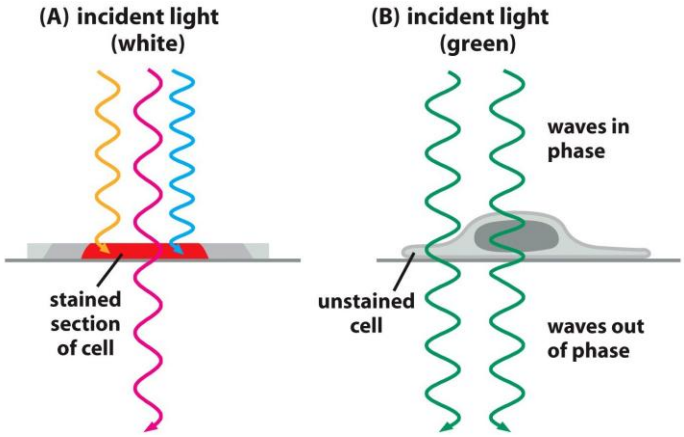


Fig. 13.9. Two ways to obtain contrast in light microscopy. (A) The stained portion of the cell will absorb light of some wavelengths, which depend on the stain, but will allow other wavelengths to pass through it, so that a colored image is obtained that is visible in normal bright-field light microscope. (B) Light passing through the unstained living cell experiences very little change in amplitude. The phase of the light is altered by its passage through either thicker or denser parts of the cell, and small phase difference can be made visible by exploiting interference effects using a phase contrast or differential-interference-contrast microscope.
Differential interference contrast (DIC) is a form of interference microscopy that produces images with a

shadow relief (Fig. 13.7). It is used for viewing unstained cells in tissue culture, eggs and embryos, and in combination with some stains.

Fluorescence microscopy is currently the most widely used contrast technique. Specific molecules can be located in cells by fluorescence microscopy. Fluorescent molecules absorb light at one wavelength and emit it a longer wavelength. If we illuminate such a compound at its absorbing wavelength and then view it through a filter that allows only light of the emitted wavelength to pass, it will glow against a dark background. Because the background is dark, even a minute amount of the glowing fluorescent dye can be detected. The same number of molecules of an ordinary stain viewed conventionally would be practically invisible because the molecules would give only the faintest tinge of color to the light transmitted through this stained part of the specimen.

The most commonly used fluorescence technique is called **epifluorescence** light microscopy, where 'epi' simply means 'from above'. Here, the light source comes from above the sample, and the objective lens acts as both condenser and objective lens. Fig. 13.10 shows the optical system of a fluorescence microscope.

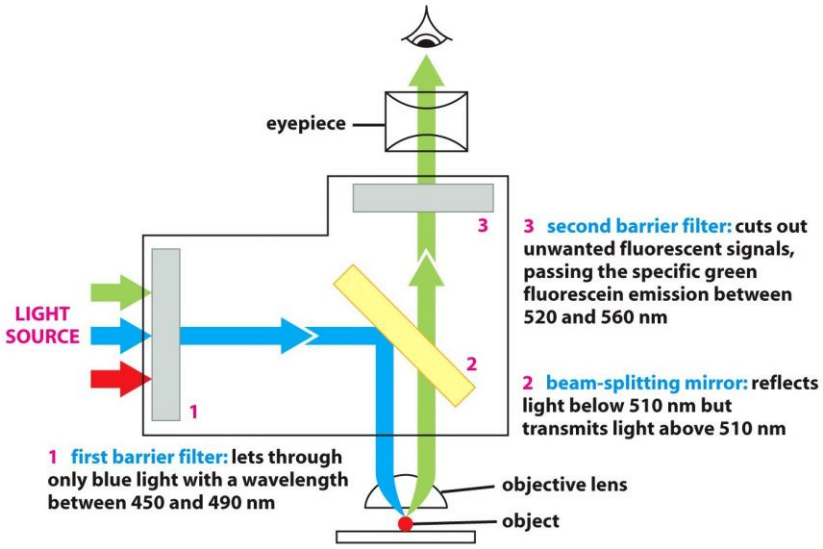


Fig. 13.10. The optical system of a fluorescence microscope. A filter set consists of two barrier filters (1 and 3) and a dichroic (beam-splitting) mirror (2).

This example shows the filter set for detection of the fluorescent molecule fluorescein. High-numerical-aperture objective lenses are especially important in this type of microscopy because, for a given magnification, the brightness of the fluorescent image is proportional to the fourth power of the numerical aperture.

Fluorescent dyes used for staining cells are visualized with a fluorescence microscope. This microscope is similar to an ordinary light microscope except that the illuminating light, from a very powerful source, usually a high pressure mercury or xenon vapor lamp, which emits from the ultraviolet into the red wavelengths is passed through two sets of filters- the first to filter the light before it reaches the specimen and the second to filter the light obtained from the specimen. The first filter permits only light of a specific wavelength that excites the particular fluorescent dye, while the second filter blocks out this light and passes only those wavelengths emitted when the dye fluoresces. The filters are mounted in a single housing above the objective lens.

For example, the commonly used fluorophore, fluorescein is optimally excited at a wavelength of 496 nm, and emits maximally at 518 nm (Table 13.1).

A set of glass filters for viewing fluorescein requires that all wavelengths of light from the lamp be blocked except for the 496 nm light. A filter is available that allows a maximum amount of 496 nm light to pass through it (the excitation filter). The 488 nm light is then directed to the specimen via the dichromatic mirror. Any fluorescein label in the specimen is excited by the 496 nm light, and the resulting 518 nm light that returns from the specimen passes through both the dichromatic mirror and the barrier filter to the detector. The emission filters only allow light of 518 nm to pass through to the detector, and ensuring that only the signal emitted from the fluorochrome of interest reaches it.

Table 13.1. A list of fluorescent dyes (fluorophores)

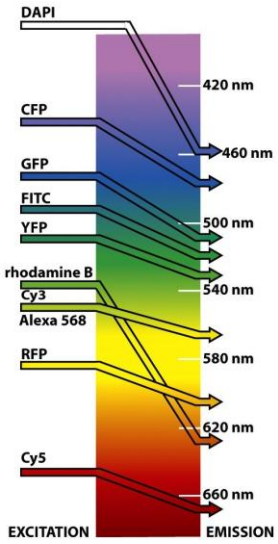
Dye	Excitation maximum (nm)	Emission maximum (nm)
Commonly used fluorophores		
Fluorescein (FITC)	496	518
Bodipy	503	511
CY3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
Texas Red	592	610
CY5	652	672
Nuclear dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine Orange	502	526
Propidium iodide	536	617
TOTO3	642	661
Ethidium bromide	510	595
Ethidium homodimer	528	617
Feulgen	570	625
Calcium indicators		
Fluo-3	506	526
Calcium Green	506	533
Reporter molecules		
Green fluorescent protein (GFP)	395/489	509
DsRed	558	583
Mitochondria		
JC-1	514	529
Rhodamine 123	507	529

DAPI, 4′,6′-diamidino-2-phenylindole.

Chromatic mirrors and filters can be designed to filter two or three specific wavelengths for imaging specimens labeled with two or more fluorochromes (multiple labeling). The fluorescence emitted from the specimen is often too low to be detected by the human eye or it may be out of the wavelength range of detection of the eye, for example in the far-red wavelengths. A sensitive digital camera easily detects such signals, for example a Charge-Coupled Device (CCD) or a photomultiplier tube.

Fluorescence microscopy is most often used to detect specific proteins or other molecules in cells and tissues. A very powerful and widely used technique is to couple fluorescent dyes to antibody molecules, which then serve as highly specific and versatile staining reagents that bind selectively to the particular macromolecules they recognize in cells or in the extracellular matrix. Two fluorescent dyes that have been commonly used for this purpose are **fluorescein**, which emits an intense green fluorescence when

excited with blue light, and **rhodamine**, which emits a deep red fluorescence when excited with green-yellow light (Fig. 13.11).



DAPI – a blue fluorescent nucleic acid stain which stains dsDNA by associating with AT-rich clusters in the minor groove. A powerful mutagen
CFP-Cyan fluorescent protein
Yellow Fluorescent Protein (YFP) is a genetic mutant of green fluorescent protein, derived from *Aequorea victoria*. Its excitation peak is 514 nm and its emission peak is 527 nm. Like green fluorescent protein (GFP), it is a useful tool in cell and molecular biology, usually explored using fluorescence microscopy.
Alexa Fluor (405-750 nm) family of fluorescent dyes is produced by Molecular Probes, a subsidiary of Invitrogen. Alexa Fluor dyes are frequently used as cell and tissue labels in fluorescence microscopy and cell biology. The excitation and emission spectra of the Alexa Fluor series cover the visible spectrum and extend into the infrared. The individual members of the family are numbered according roughly to their excitation maxima (in nm).
Alexa Fluor dyes are synthesized through sulfonation of coumarin, rhodamine, xanthene (such as fluorescein), and cyanine dyes. Sulfonation makes Alexa Fluor dyes negatively charged and hydrophilic. Alexa Fluor dyes are generally more stable, brighter, and less pH-sensitive than common dyes (e.g. fluorescein, rhodamine) of comparable excitation and emission, and to some extent the newer cyanine series. However, they are also more expensive.

Fig. 13.11. Some fluorescent probes

By coupling one antibody to fluorescein and another to rhodamine, the distribution of different molecules can be compared in the same cell; the two molecules are visualized separately in the microscope by switching back and forth between two sets of filters, each specific for one dye. As shown in Fig. 13.12, three fluorescent dyes can be used in the same way to distinguish between three types of molecules in the same cell. Many newer fluorescent dyes, such as Cy3, Cy5, and the Alexa dyes, have been specifically developed for fluorescence microscopy. These organic fluorochromes have some disadvantages. They are excited only by light of precise, but different wavelengths, and additionally they fade fairly rapidly when continuously illuminated.

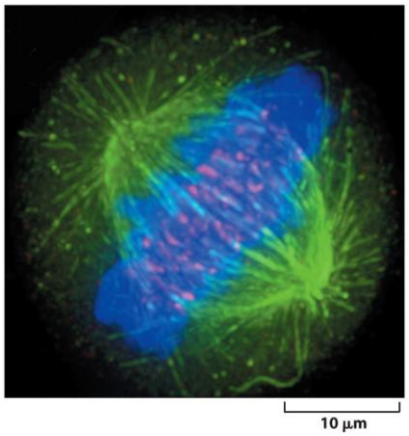
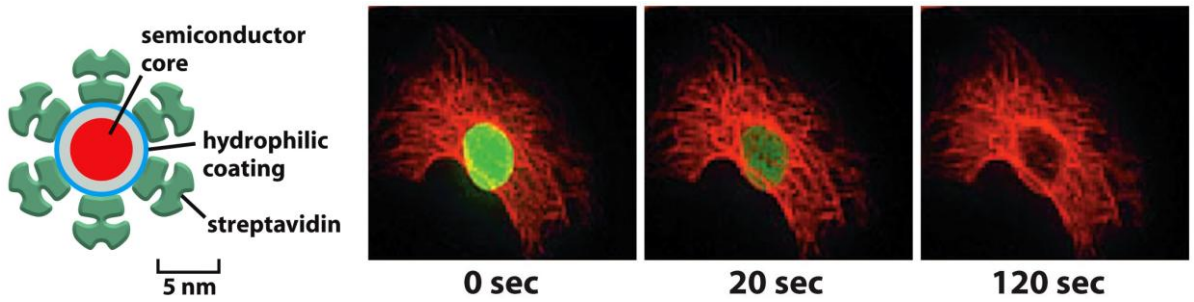


Fig. 13.12. Multiple fluorescent-probe microscopy. In this composite micrograph of a cell in mitosis, three different fluorescent probes have been used to stain three different cellular components. The spindle microtubules are revealed with a green fluorescent antibody, centromeres with a red fluorescent antibody, and the DNA of the condensed chromosomes with the blue fluorescent dye, DAPI (Source: Kevin Sullivan)

More stable inorganic fluorochromes have recently been developed. Tiny crystals of semiconductor material, called nanoparticles, or **quantum dots**, can be excited to fluoresce by a broad spectrum of blue light. Their emitted light has a color that depends on the exact size of the nanocrystal, usually between 2 and 10 nm in diameter, and the fluorescence fades only slowly with time (Fig. 13.13). These nanoparticles, when coupled to other probes such as antibodies, are ideal for **tracking molecules** over time. If introduced into a living cell, in an embryo for example, the progeny of that cell can be followed many days later by their fluorescence, allowing cell lineages to be tracked.



(A) (B)

Fig.13.13. Fluorescent nanoparticles or quantum dots. Quantum dots are tiny nanoparticles of cadmium selenide, a semiconductor, with a coating to make them water-soluble (A). They can be coupled to protein probes such as antibodies or streptavidin, and when induced into a cell, will bind to a protein of interest. Different-sized quantum dots emit light of different colors - the larger the dot, the longer the wavelength, though they are all excited by the

same blue light. (B) Quantum dots can keep shining for weeks, unlike most fluorescent organic dyes. In this cell, a nuclear protein is labeled (green) with an organic fluorescent dye (Alexa 488), while microtubules are stained (red) with quantum dots bound to streptavidin. On continuous exposure to blue light, the fluorescent dye fades quickly while the quantum dots continue to fluoresce. (Source: Wu et al. 2003. Nat. Biotechnol. 21:41-46)

Streptavidin is a 60 kDa protein purified from the bacterium *Streptomyces avidinii*. Streptavidin homo-tetramers have an extraordinarily high affinity for biotin (also known as vitamin B7). With a dissociation constant (K_d) on the order of $\approx 10^{-14}$ mol/L, the binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature. Streptavidin is used extensively in molecular biology and bionanotechnology due to the streptavidin-biotin complex's resistance to organic solvents, denaturants (e.g. guanidinium chloride), detergents (e.g. SDS, Triton), proteolytic enzymes, and extremes of temperature and pH.

Mode of operation of a microscope

Microscopes may be built in the following modes: upright, inverted, or dissecting. Fig. 13.14 shows upright and inverted microscopes.

Dissecting microscope

Dissection of small pieces of tissue, e.g., embryonic organs or tissues from smaller invertebrates require a dissecting microscope. The dissecting microscope is also useful for counting monolayer colonies and for counting and picking small colonies on agar.

Inverted microscopes

The inverted microscope is engineered so that the light source and the condenser lens are above the specimen stage, and the objective lenses are beneath it. This allows additional room for manipulating the specimen directly on the stage. A simple inverted microscope is vital for observing cultures regularly to detect morphological changes and possibility of microbiological contamination. It is required that the stage be large enough to accommodate large roller bottles, if required, between it and the condenser, in which case, the condenser has to be removed, because, if there, the bottle will not fit on the stage. So choose a microscope with sufficient stage accommodation. It is worth getting a microscope with a phototube for digital recording or viewing linked to a monitor (Fig. 13.14).When using culture flasks made of thick plastics, a phase-contrast condenser and objective lenses are required to compensate for the thickness.

CCD camera and monitor

Digital cameras and monitors are valuable aids to studies on cell and tissue cultures. You must choose a high-resolution but not high-sensitivity camera as high sensitivity my lead to over illumination. Black and white usually gives better resolution and is adequate for phase-contrast observation of living cultures. A still digital camera is sufficient to record shots, but a charge-couple-device (CCD) camera will allow a real time viewing and may be of use for time-lapse recording.

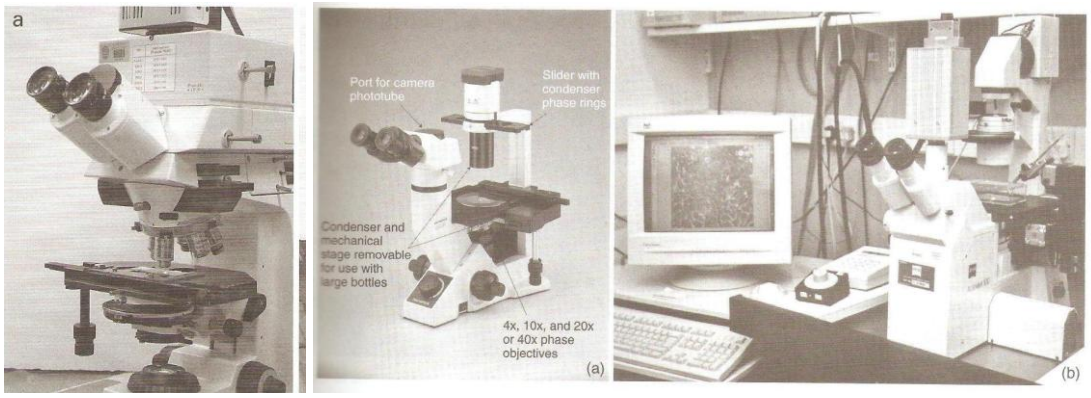


Fig. 13.14. Upright (left) and inverted microscope (middle and right). Middle; Olympus CKX41 inverted microscope fitted with phase contrast optics and binocular head with port for attaching a digital camera. Right: CCD camera attached to Zeiss Axiovert inverted microscope. Can be used for direct printing or for time-lapse studies when linked to a video recorder.

The electron microscope

Electron microscopy is used when the greatest resolution is required. Image produced in an electron microscope (EM) reveals the ultrastructure of cells. There are two types of electron microscopes:

1. Transmission electron microscope (TEM)
2. Scanning electron microscope (SEM).

Design of TEM

In overall design the transmission electron microscope (TEM) is similar to a light microscope, although it is much larger and "upside down" (Fig. 13.15).

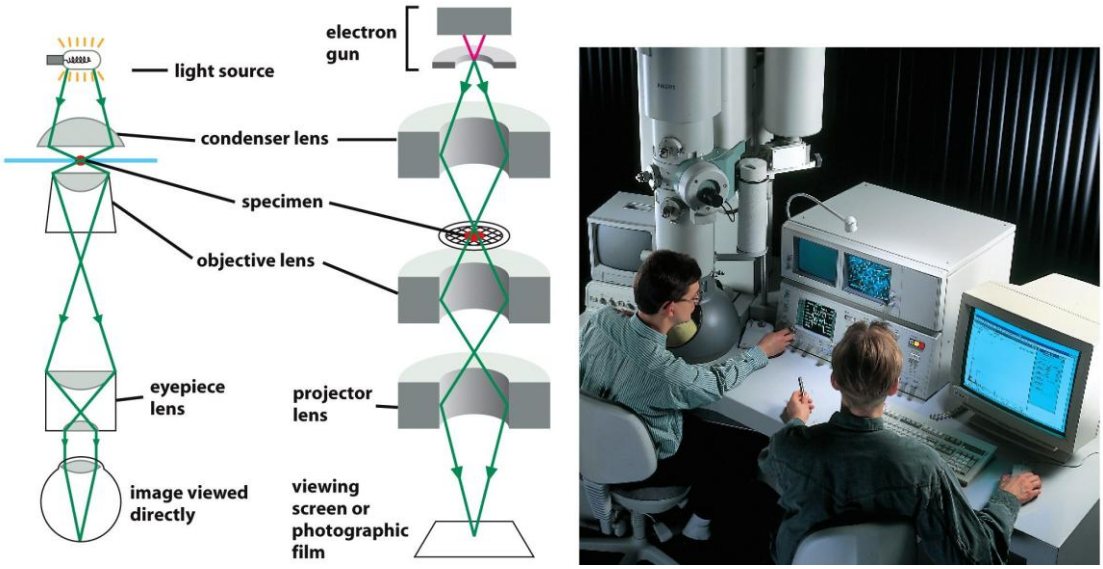


Fig. 13.15. The principal features of light and transmission electron microscope.

The equivalent of the light source in an electron microscope is the **electron gun** positioned at the top of a cylindrical column about 2 m high. The electron gun is made up of a tungsten filament which serves as a cathode and placed in a tube under high voltage of between 40,000 and 100,000 V. Under this voltage, electrons are ejected from the filament and accelerated toward the anode. The negatively charged electrons pass through a hole in the anode forming an electron beam. The beam of electrons passes through a stack of electromagnetic lenses (the column). Focusing of the electron beam is achieved by changing the voltage across the electromagnetic lenses just as glass lenses focus the light in a light microscope. Since electrons are scattered by collisions with air molecules, air must first be pumped out of the column to create vacuum.

The specimen is placed in the vacuum, through an airlock, into the path of the electron beam. As in light microscopy, the specimen is usually stained-in this case, with *electron-dense* material. When the electron beam passes through the specimen some of the electrons are scattered by structures stained with the electron-dense material; the remainder are focused by the projector lens onto a phosphorescent screen to form an image, in a manner analogous to the way an image is formed in a light microscope. The image can be observed on a phosphorescent screen or recorded, either on a photographic film or with a high-resolution digital camera. Because the scattered electrons are lost from the beam, the dense regions of the specimen show up in the image as areas of reduced electron flux, which look dark.

The electrons have limited penetration power, which means that a specimen must be thin (50-100 nm) to allow them to pass through. In TEM, electrons that pass through the specimen are imaged. In the SEM, electrons that are reflected back from the specimen (secondary electrons) are collected, and the surfaces of specimens imaged.

The electron microscope resolves the fine structure of the cell

The relationship between limit of resolution and wavelength of the illuminating radiation is true for any form of radiation, whether it is a beam of light or a beam of electrons. With electrons, however, the limit of resolution can be made very small. The wavelength of an electron decreases as its velocity increases. In an electron microscope with an accelerating voltage of 100,000 V, the wavelength of an electron is 0.004 nm. In theory the resolution of such a microscope should be about 0.002 nm, which is 100,000 times that of the light microscope. Because the aberrations of an electron lens are considerably harder to correct than those of a glass lens, the practical resolving power of most modern electron microscopes is, at best, 0.1 nm. This is because only the very center of the electron lenses can be used, and the effective numerical aperture is tiny. Furthermore, problems of specimen preparation, contrast, and radiation damage have generally limited the normal effective resolution of biological objects to 1 nm. This is nonetheless about 200 times better than the resolution of the light microscope. Moreover, in recent years, the performance of electron microscopes has been improved by the development of electron illumination sources called **field emission guns**. These very bright and coherent sources can substantially improve the resolution.