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Molecular Biology of the Cell

Chapter 9
Visualizing Cells

Looking at Cells in the Light Microscope

- A typical animal cell is 10–20 μm in diameter, which is about one-fifth the size of the smallest object that we can normally see with the naked eye.
- Only after good light microscopes became available in the early part of the nineteenth century did Schleiden and Schwann propose that all plant and animal tissues were aggregates of individual cells.
- Their proposal in 1838, known as the **cell doctrine**, marks the formal birth of cell biology.
- Animal cells are not only tiny, but they are also colorless and translucent.
- The discovery of their main internal features, therefore, depended on the development, in the late nineteenth century, of a variety of stains that provided sufficient **contrast** to make those features visible.
- Similarly, the **far more powerful electron microscope** introduced in the early 1940s required the development of new techniques for preserving and staining cells before the full complexities of their internal fine structure could begin to emerge.

Looking at Cells in the Light Microscope

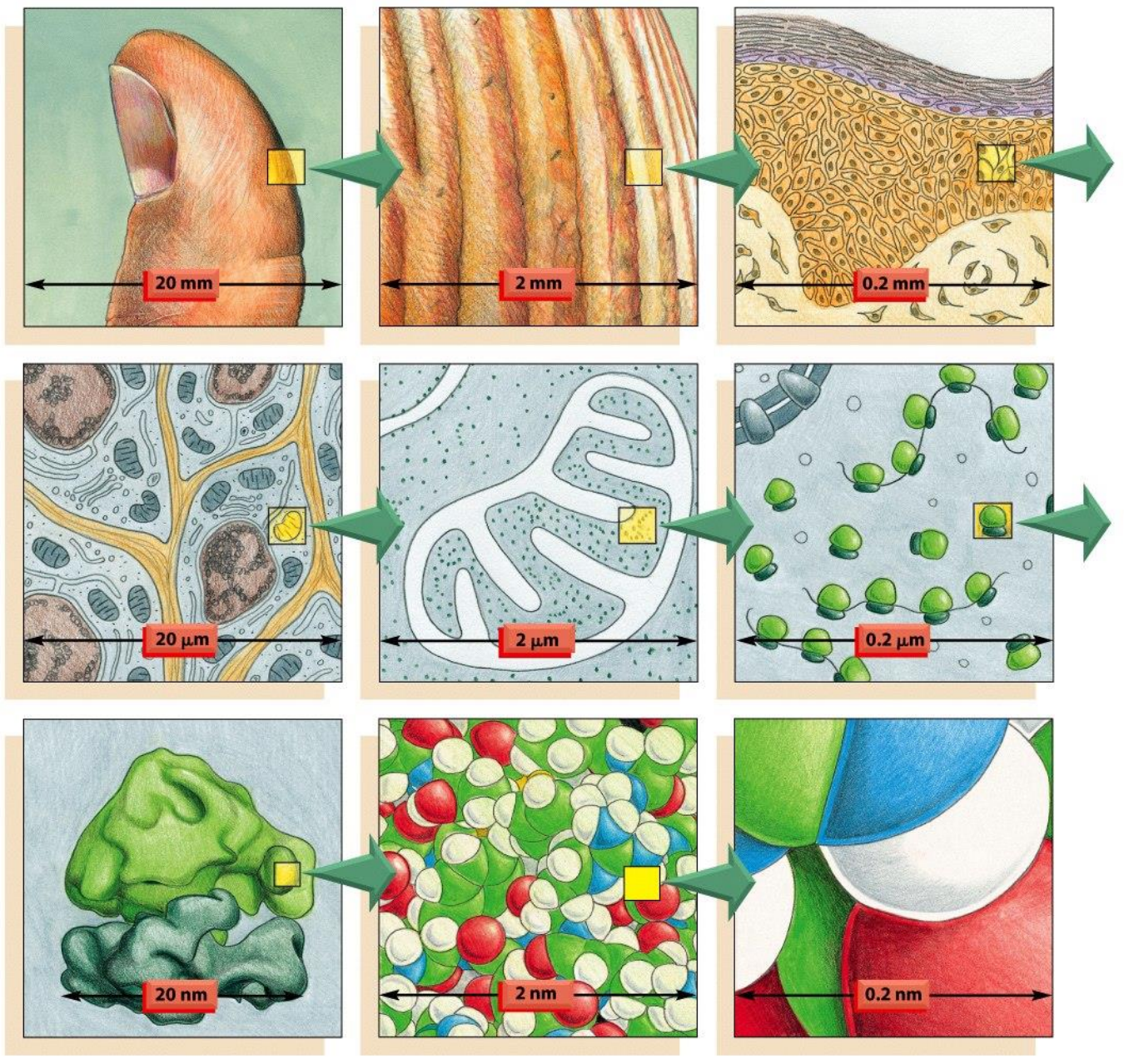
To this day, microscopy often relies as much on techniques for preparing the specimen as on the performance of the microscope itself.

In the following discussions, we therefore consider both **instruments** and **specimen preparation**, beginning with the light microscope.

The images in Figure 9–1 illustrate a stepwise progression from a thumb to a cluster of atoms.

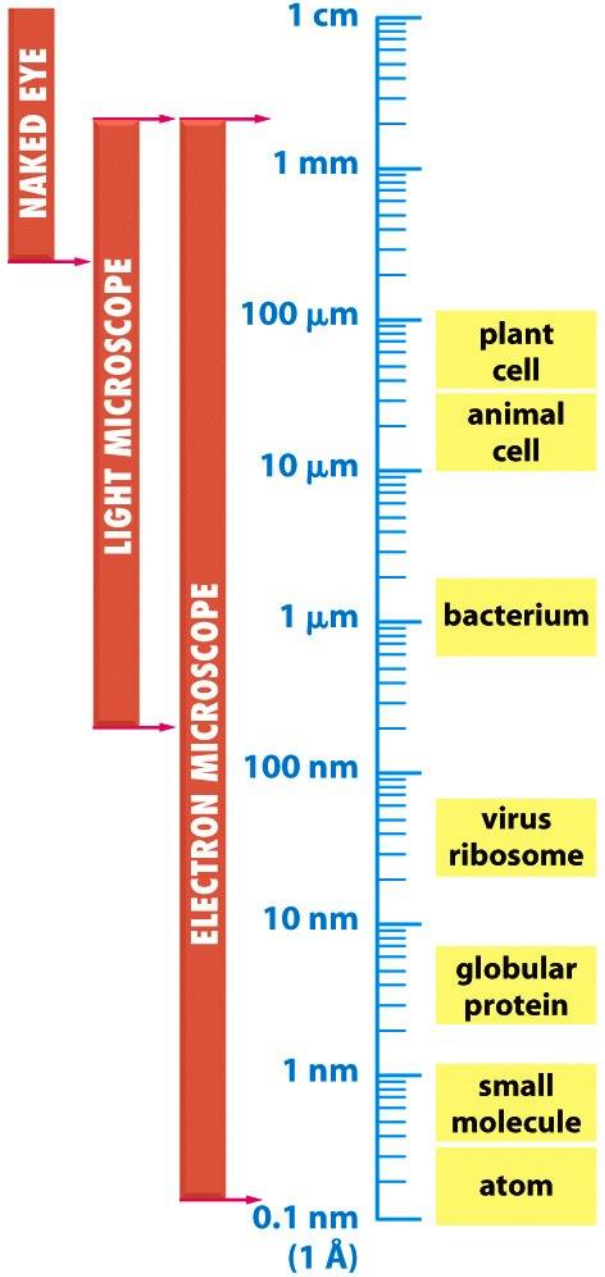
Each successive image represents a tenfold increase in magnification.

- The naked eye can see features in the first two panels,
- the light microscope allows us to see details corresponding to about the fourth or fifth panel,
- the electron microscope takes us to about the seventh or eighth panel.



Looking at Cells in the Light Microscope

Figure 9–2 shows the sizes of various cellular and subcellular structures and the ranges of size that different types of microscopes can visualize.



The Light Microscope Can Resolve Details 0.2 µm Apart

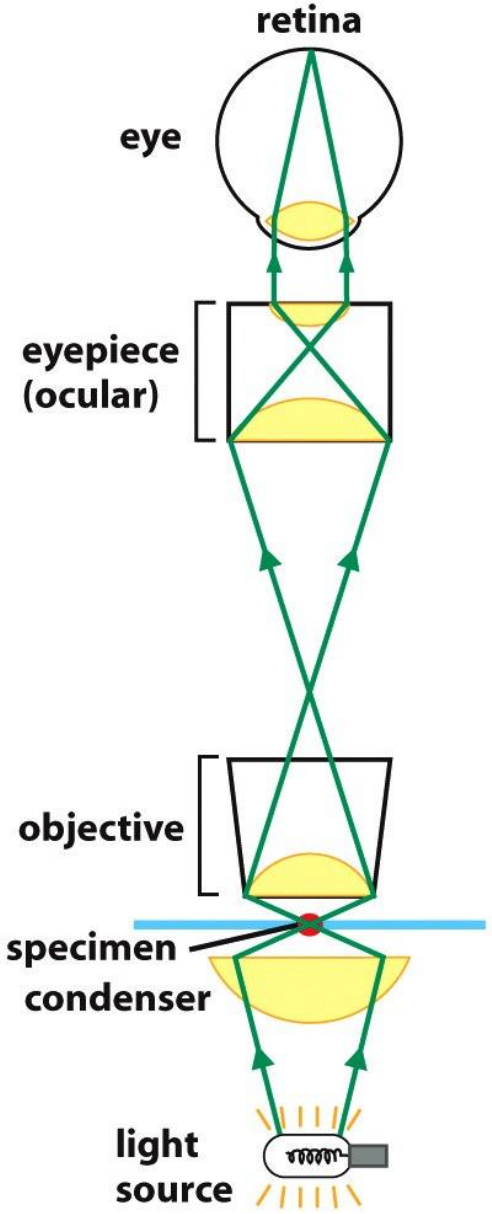
For well over 100 years, all microscopes were constrained by a **fundamental limitation**: **that a given type of radiation cannot be used to probe structural details much smaller than its own wavelength.**

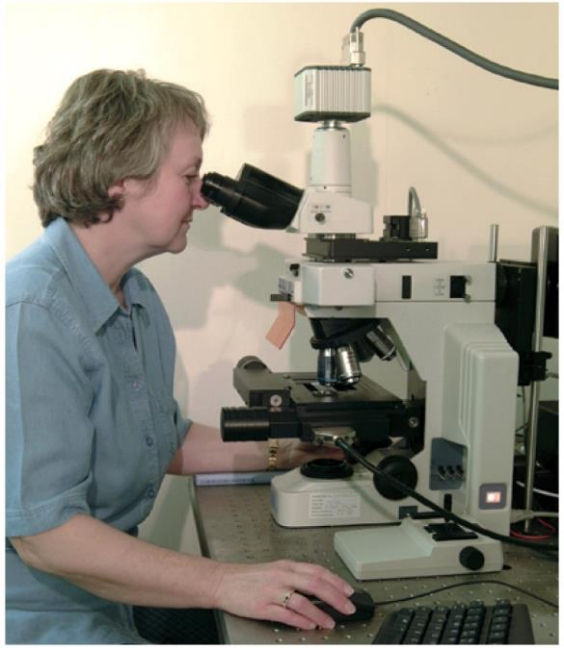
A limit to the resolution of a light microscope was therefore set by the wavelength of visible light, which ranges from about 0.4 µm (for **violet**) to 0.7 µm (for **deep red**).

In practical terms, bacteria and mitochondria, which are about 500 nm (**0.5 µm**) wide, are generally the smallest objects whose shape we can clearly discern in the light microscope;

Details smaller than this are obscured by effects resulting from the **wavelike nature of light**.

To understand why this occurs, we must follow the behavior of a beam of light as it passes through the lenses of a microscope.





A light microscope.

(A) Diagram showing the light path in a compound microscope.
Light is focused on the specimen by lenses in the condenser.

A combination of objective lenses, tube lenses, and eyepiece lenses is arranged to focus an image of the illuminated specimen in the eye.

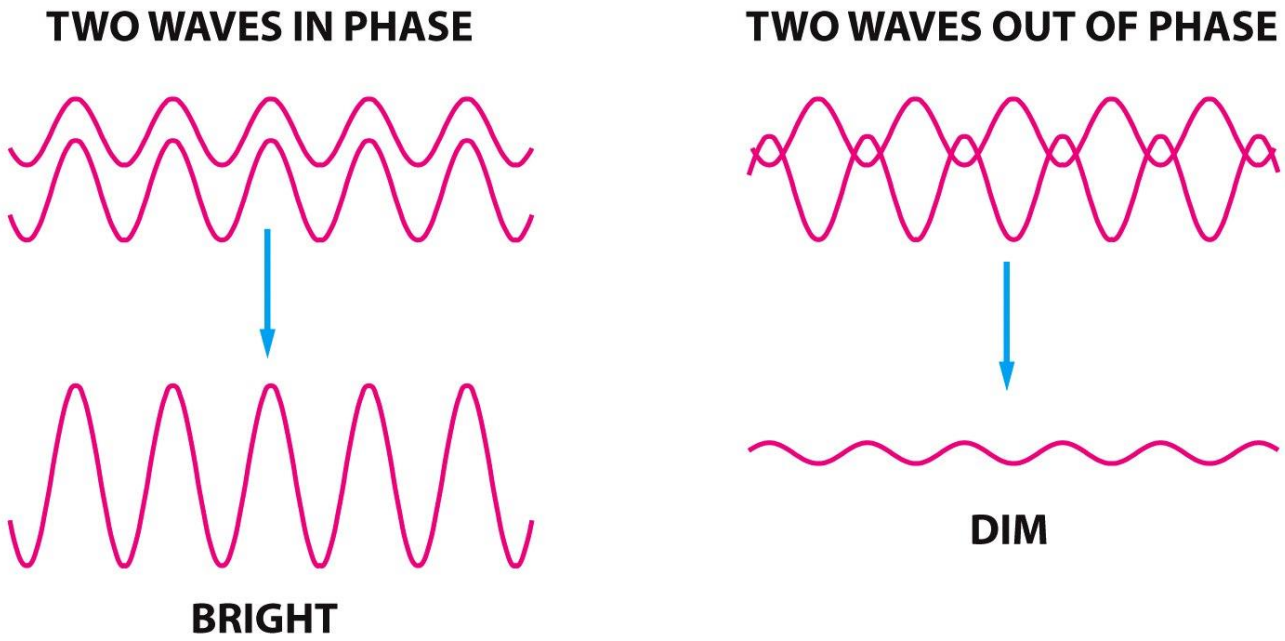
(B) A modern research light microscope.

The Light Microscope Can Resolve Details 0.2 µm Apart

Because of its **wave nature**, **light** does not follow the idealized straight ray paths that geometrical optics predicts.

Instead, light waves travel through an optical system by many slightly different routes, like **ripples in water**, so that they interfere with one another and cause **optical diffraction effects**.

- If two trains of waves reaching the **same point** by different paths are precisely in phase, with crest matching crest and trough matching trough, they will **reinforce each other so as to increase brightness**.
- In contrast, if the trains of waves are out of phase, they will interfere with each other in such a way as to cancel each other partly or entirely.



Interference between light waves.

When two light waves combine in phase, the **amplitude** of the resultant wave is **larger** and the **brightness** is **increased.**

Two light waves that are out of phase cancel each other partly and produce a wave whose **amplitude**, and therefore **brightness**, is **decreased**.

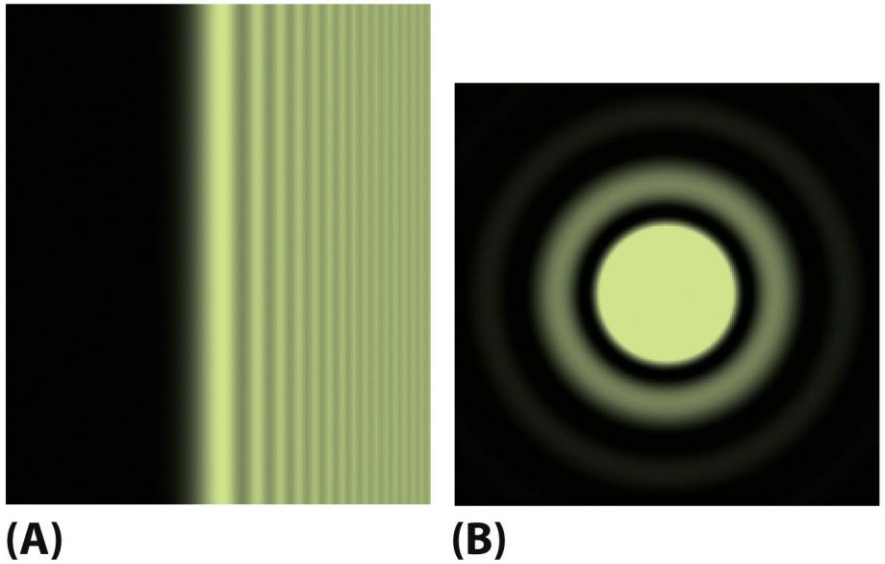
The Light Microscope Can Resolve Details 0.2 µm Apart

- The interaction of light with an object changes the **phase relationships of the light waves** in a way that produces **complex interference effects**.

At high magnification, for example, the shadow of an edge that is evenly illuminated with light of uniform wavelength appears as a set of parallel lines (Figure 9–5), whereas that of a circular spot appears as a set of concentric rings.

For the same reason, a single point seen through a microscope appears as a blurred disc, and **two points objects close together give overlapping images and may merge into one**.

Although no amount of refinement of the lenses can overcome the **diffraction limit** imposed by the wavelike nature of light, other ways of cleverly bypassing this limit have emerged, creating so-called **superresolution imaging techniques** that can even detect the position of single molecules.



Images of an edge and of a point of light.
(A) The interference effects, or **fringes**, seen at high magnification when light of a specific wavelength passes the **edge** of a solid object placed between the light source and the observer.
(B) The image of a **point source** of light.
Diffraction spreads this out into a complex, circular pattern, whose width depends on the numerical aperture of the optical system: the smaller the aperture, the bigger (more blurred) the diffracted image.
Two point sources can be just resolved when the center of the image of one lies on the first dark ring in the image of the other: this is used to define the limit of resolution.

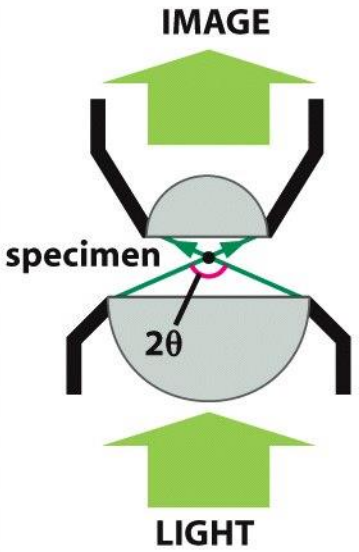
The Light Microscope Can Resolve Details 0.2 μm Apart

The limiting separation at which two objects appear distinct—the so-called **limit of resolution**—depends on both the **wavelength** of the light and the **numerical aperture** of the lens system used.

The **numerical aperture** affects the light-gathering ability of the lens and is related both to **the angle of the cone of light** that can enter it and to **the refractive index of the medium** the lens is operating in;

the wider the microscope opens its eye, so to speak, the more sharply it can see (Figure 9–6).

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.

The Light Microscope Can Resolve Details 0.2 µm Apart

- The **refractive index** is the ratio of the speed of light in a vacuum to the speed of light in a particular transparent medium.

For example, for water this is 1.33, meaning that light travels 1.33 times slower in water than in a vacuum.

- Under the best conditions, with violet light (wavelength = 0.4 µm) and a numerical aperture of 1.4, the basic light microscope can theoretically achieve a limit of resolution of about **0.2 µm**, or **200 nm**.

- Some microscope makers at the end of the nineteenth century achieved this resolution, but it is routinely matched in contemporary, factory-produced microscopes.

- Although it is possible to enlarge an image as much as we want—for example, by projecting it onto a screen—it is not possible, in a conventional light microscope, 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.

- It is important, however, to distinguish between **resolution** 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.

- In a similar way, we can see the stars in the night sky, even though their diameters are far below the **angular resolution** of our unaided eyes: they all appear as similar, slightly blurred points of light, differing only in their **color** and **brightness**.

Photon Noise Creates Additional Limits to Resolution When Light Levels Are Low

- Any image, whether produced by an electron microscope or by an optical microscope, is made by **particles**—**electrons or photons**—striking a detector of some sort.

- But these particles are governed by **quantum mechanics**, so the numbers reaching the detector are predictable only in a statistical sense.

- Finite samples, collected by imaging for a limited period of time (that is, by taking a snapshot), will show random variation: successive snapshots of the same scene will not be exactly identical. Moreover, every detection method has some level of background signal or noise, adding to the statistical uncertainty.

- **With bright illumination, corresponding to very large numbers of photons or electrons, the features of the imaged specimen are accurately determined based on the distribution of these particles at the detector.**

- However, with smaller numbers of particles, the structural details of the specimen are obscured by the statistical fluctuations in the numbers of particles detected in each region, which give the image a speckled appearance and limit its precision.

- The term noise describes this **random variability**.

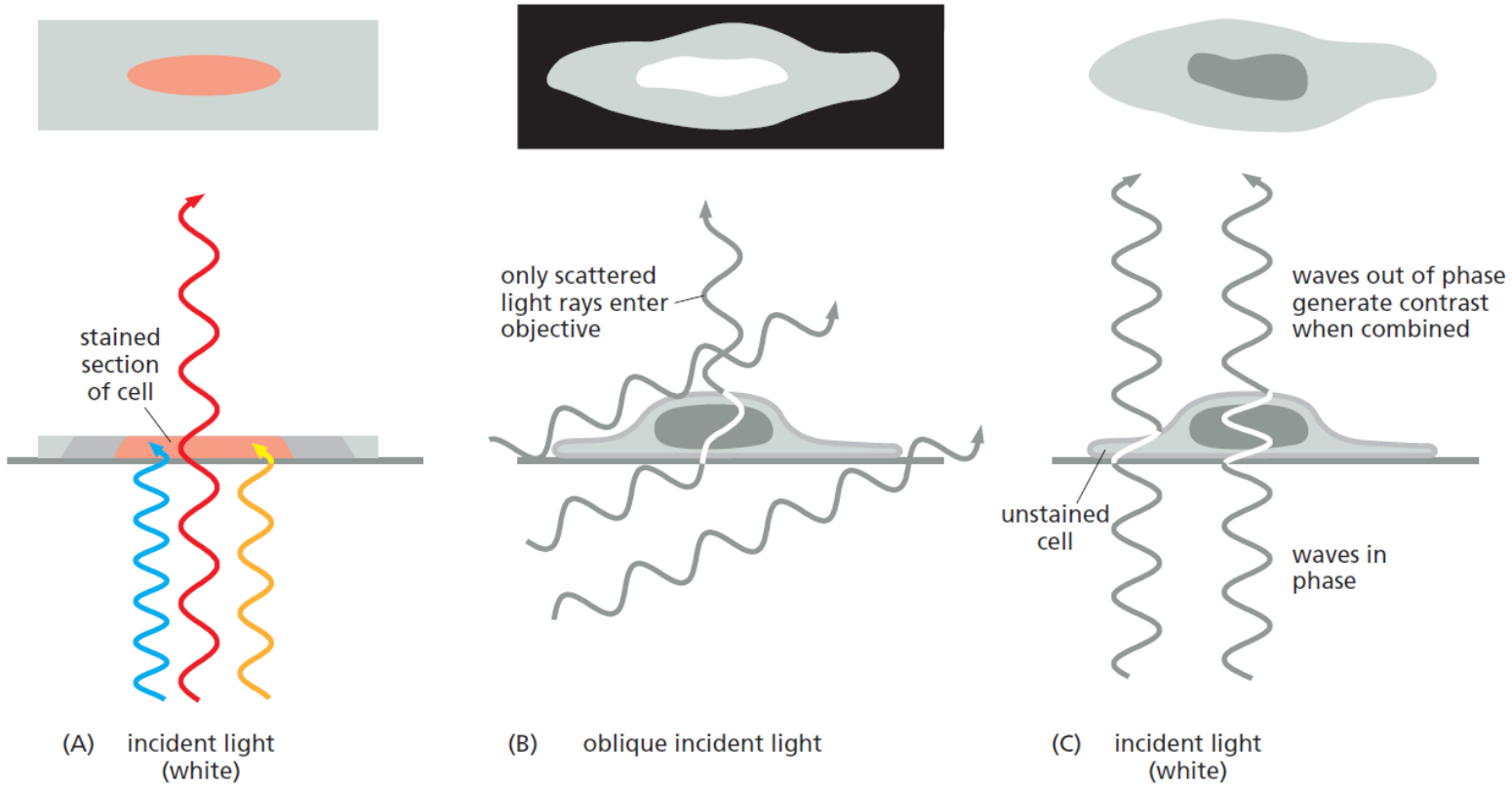
Living Cells Are Seen Clearly in a Phase-Contrast or a Differential-Interference-Contrast Microscope

There are many ways in which **contrast** in a specimen can be generated (Figure 9–7A).

While fixing and staining a specimen can generate contrast through color, 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.



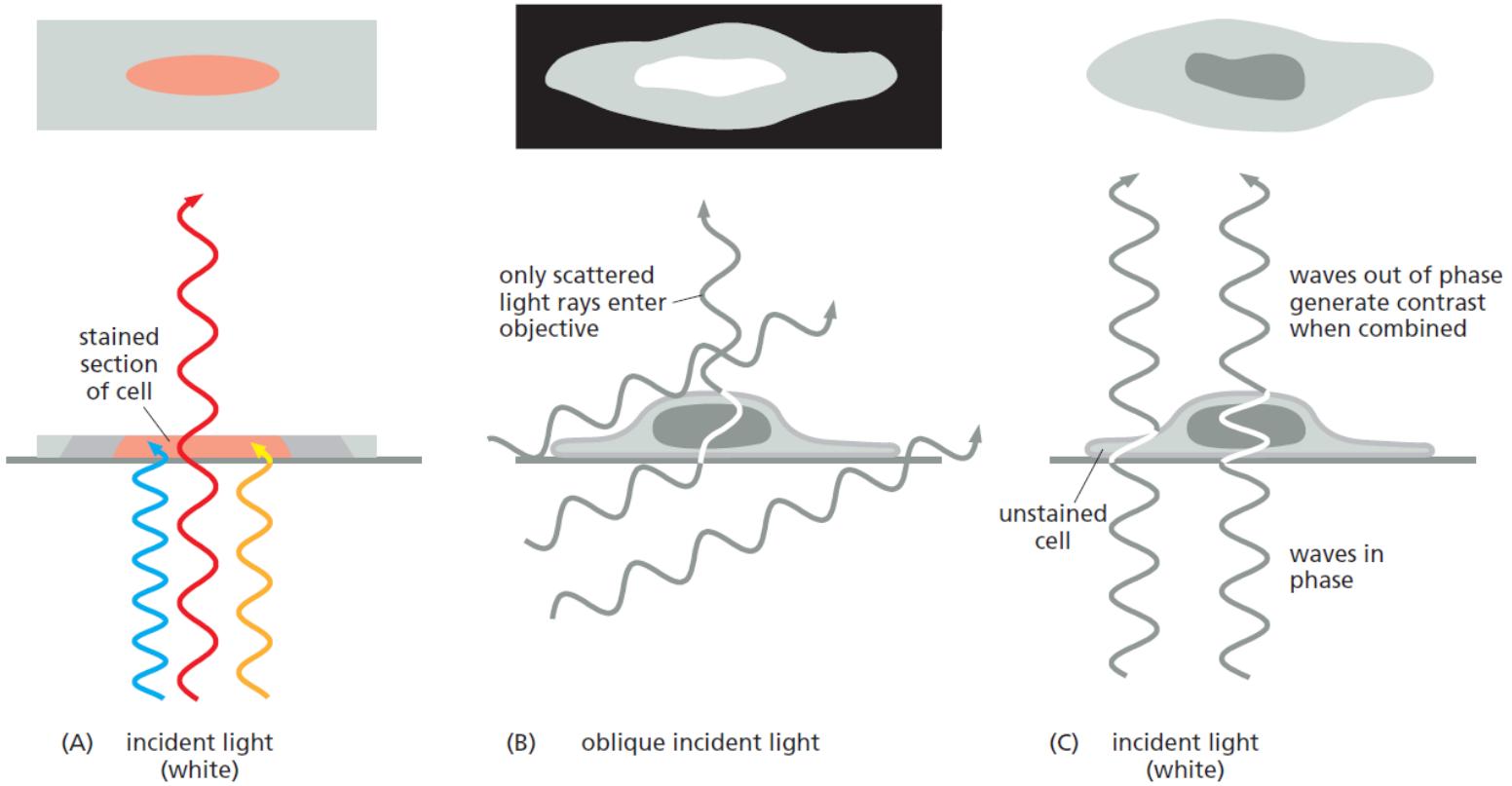
Contrast in light microscopy.
(A) The stained portion of the cell will absorb light of some wavelengths, which depends on the stain, but will allow other wavelengths to pass through it. A colored image of the cell is thereby obtained that is visible in the normal bright-field light microscope.

Living Cells Are Seen Clearly in a Phase-Contrast or a Differential-Interference-Contrast Microscope

- In the **normal bright-field microscope**, light passing through a cell in culture forms the image directly.

- Another system, **dark-field microscopy**, exploits the fact that light rays can be scattered in all directions by small objects in their path.

→ If **oblique lighting** from the condenser is arranged, which does not directly enter the objective, **focused but unstained** objects in a living cell can scatter the rays, some of which then enter the objective to create a bright image against a black background (Figure 9–7B).



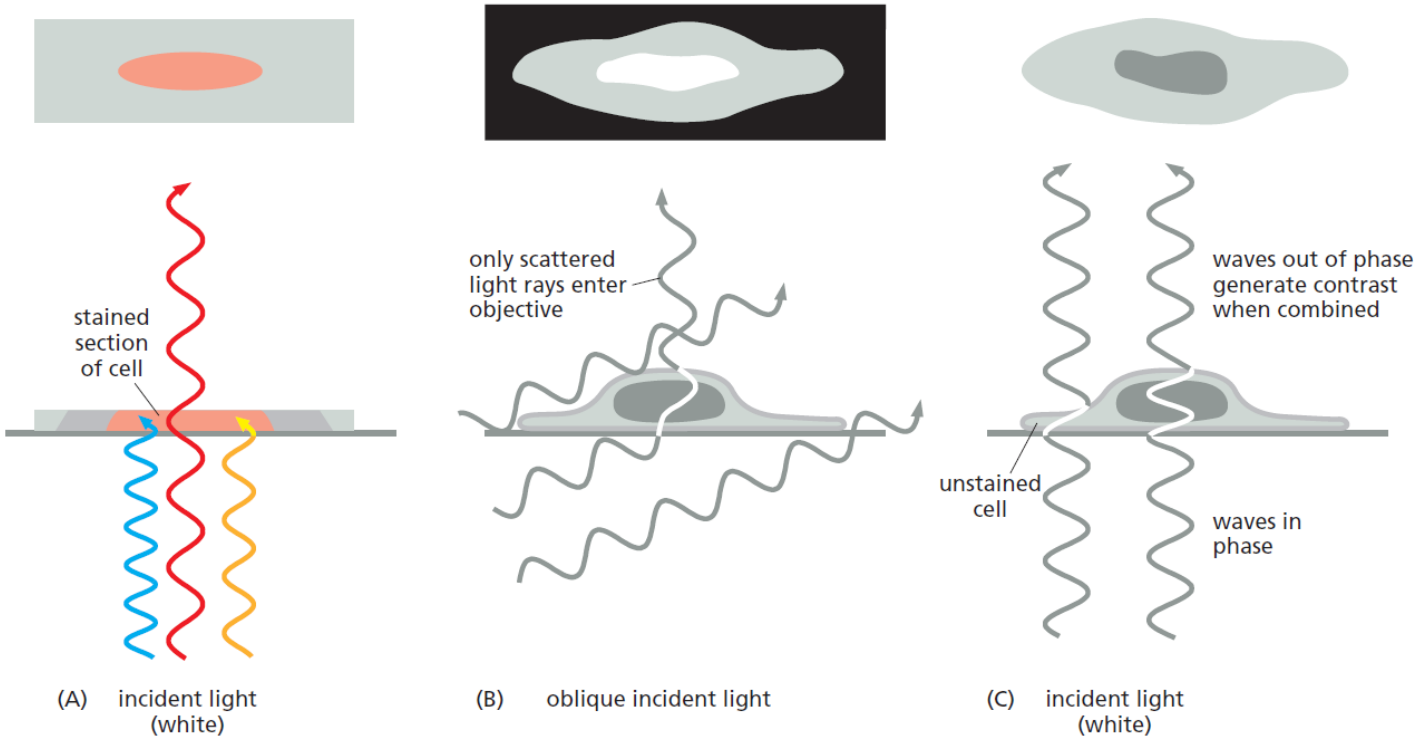
Contrast in light microscopy.
(B) In the dark-field microscope, oblique rays of light focused on the specimen do not enter the objective lens, but light that is scattered by components in the living cell can be collected to produce a bright image on a dark background.

Living Cells Are Seen Clearly in a Phase-Contrast or a Differential-Interference-Contrast Microscope

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 the cell, such as a nucleus, slows the 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 (Figure 9–7C).
The **phase-contrast microscope** and, in a more complex way, the **differential-interference-contrast microscope** increase these **phase differences** so that the waves are more nearly out of phase, producing **amplitude differences** when the sets of waves recombine, thereby creating an image of the cell's structure.

Both types of light microscopy are widely used to visualize living cells.



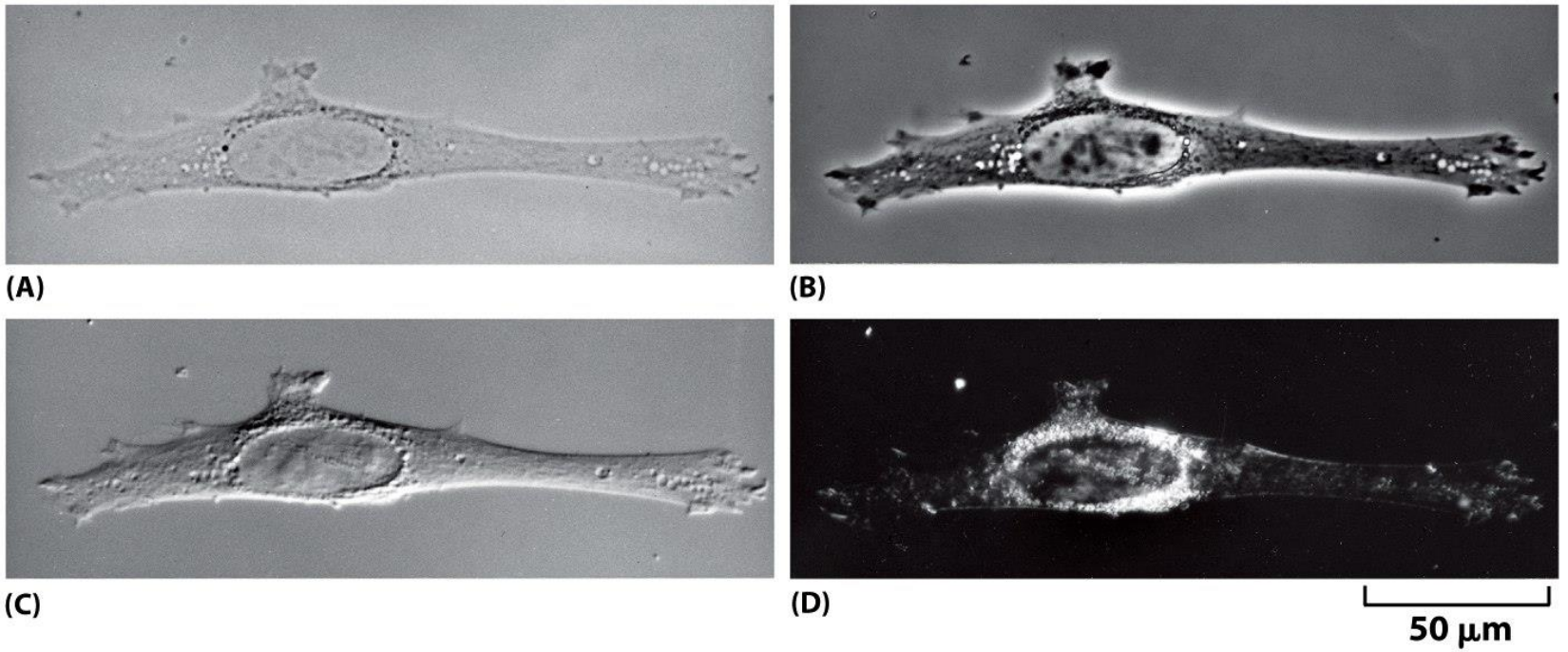
Contrast in light microscopy.
(C) Light passing through the unstained living cell experiences very little change in amplitude, and the structural details cannot be seen even if the image is highly magnified. The phase of the light, however, is altered by its passage through either thicker or denser parts of the cell, and small phase differences can be made visible by exploiting interference effects using a phase-contrast or a differential-interference-contrast microscope.

Living Cells Are Seen Clearly in a Phase-Contrast or a Differential-Interference-Contrast Microscope

Figure 9–8 compares images of the same cell obtained by four kinds of light microscopy.

Phase-contrast, differential-interference-contrast, and dark-field microscopy make it possible to watch the movements involved in such processes as **mitosis and cell migration**.

Since many cellular motions are too slow to be seen in real time, it is often helpful to make **time-lapse movies** in which the camera records successive frames separated by a short time delay, so that when the resulting picture series is played at normal speed, events appear greatly speeded up.



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**, in which light is transmitted straight through the specimen.
(B) **Phase-contrast microscopy**, in which phase alterations of light transmitted through the specimen are translated into brightness changes.
(C) **Differential-interference-contrast microscopy**, which highlights edges where there is a steep change of refractive index.
(D) **Dark-field microscopy**, in which the specimen is lit from the side and only the scattered light is seen.

Images Can Be Enhanced and Analyzed by Digital Techniques

In recent years, electronic, or digital, imaging systems, and the associated technology of image processing, have had a major impact on light microscopy.

Certain practical limitations of microscopes relating to imperfections in the optical system have been largely overcome.

Electronic imaging systems have also circumvented two fundamental limitations of the human eye:
the eye cannot **see well in extremely dim light**, and it cannot perceive **small differences in light intensity** against a bright background.

To increase our ability to observe cells in these difficult conditions, we can attach a **sensitive digital camera** to a microscope.

These cameras detect light by means of **charge-coupled devices (CCDs),** or high sensitivity **complementary metal-oxide semiconductor (CMOS) sensors**, similar to those found in digital cameras.

Such image sensors are 10 times more sensitive than the human eye and can detect 100 times more intensity levels.

- ✓ It is therefore possible to observe cells for long periods at very low light levels, thereby avoiding the damaging effects of prolonged **bright light** (and **heat**).

Such **low-light cameras** are especially important for viewing fluorescent molecules in living cells.

Images Can Be Enhanced and Analyzed by Digital Techniques

- Because images produced by digital cameras are in electronic form, they can be processed in various ways to **extract latent information**.

- Such image processing makes it possible to compensate for several optical faults in microscopes.

- Moreover, by digital image processing, **contrast can be greatly enhanced** to overcome the eye's limitations in detecting small differences in light intensity, and background irregularities in the optical system can be digitally subtracted.

- This procedure reveals **small transparent objects** that were previously impossible to distinguish from the **background**.

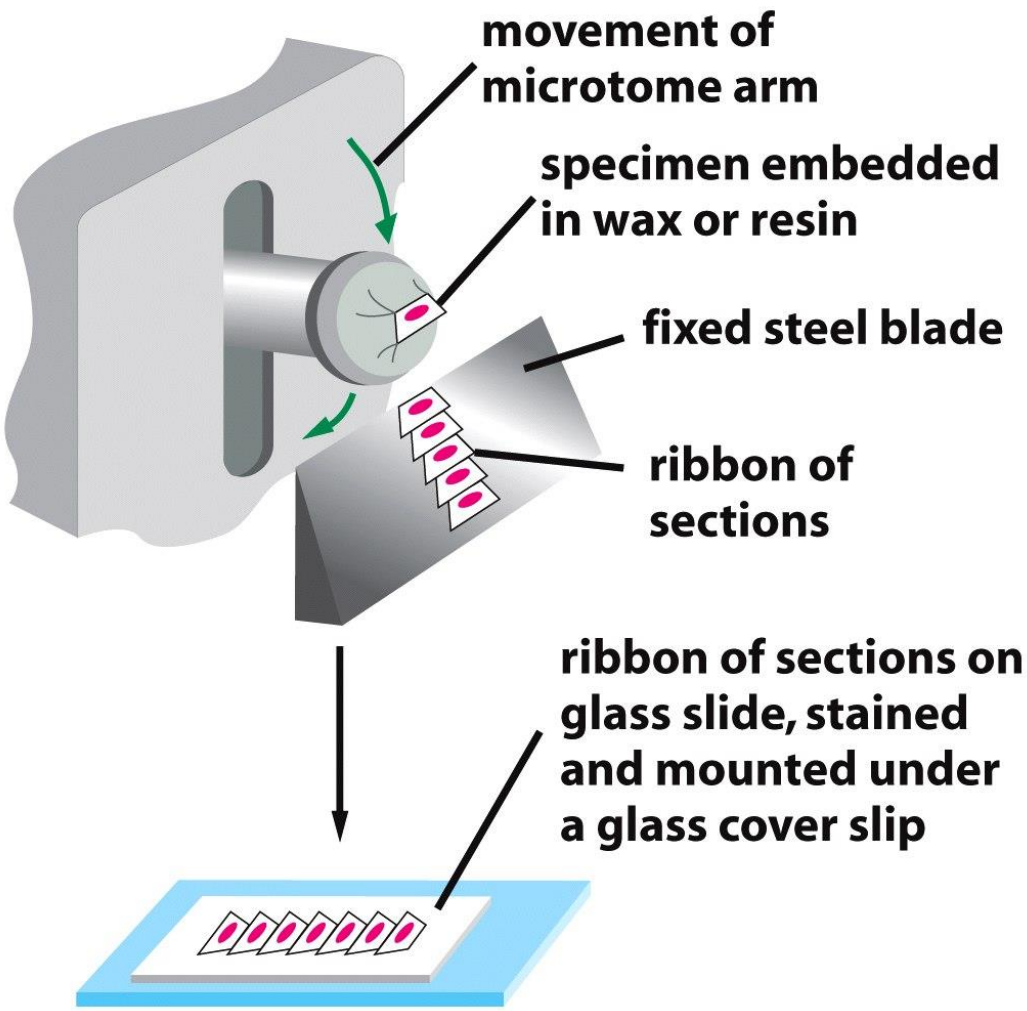
Intact Tissues Are Usually Fixed and Sectioned Before Microscopy

- Because most tissue samples are too thick for their individual cells to be examined directly at high resolution, they are often cut into **very thin transparent slices**, or **sections**.
- To preserve the cells within the tissue they must be treated with a **fixative**.

- Common fixatives include **glutaraldehyde**, which forms covalent bonds with the free amino groups of proteins, **cross-linking them so they are stabilized and locked into position.**

- Because tissues are generally soft and fragile, even after fixation, they need to be either **frozen** or **embedded in a supporting medium** before being sectioned.

- The usual embedding media are **waxes or resins**.
- In liquid form, these media both **permeate** and **surround the fixed tissue**; they can then be hardened (by cooling or by polymerization) to form a solid block, which is readily sectioned with a **microtome**.
- This is a machine with a **sharp blade**, usually of steel or glass, which operates like a meat-slicer.
- The sections (typically **0.5–10 µm thick**) are then laid flat on the surface of a glass microscope slide.



Intact Tissues Are Usually Fixed and Sectioned Before Microscopy

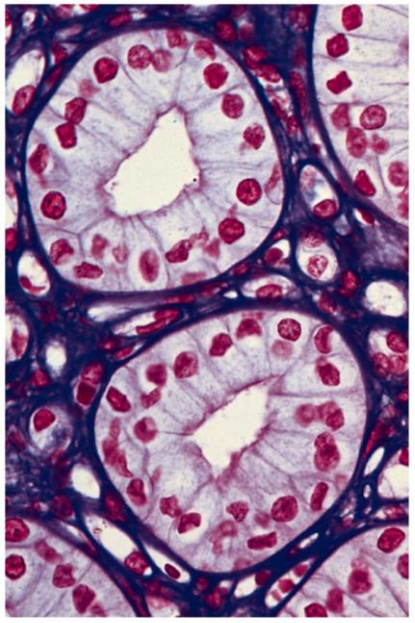
There is little in the contents of most cells (which are 70% water by weight) to impede the passage of light rays.

→ Thus, most cells in their natural state, even if fixed and sectioned, are almost invisible in an ordinary light microscope.

We have seen that cellular components can be made visible by techniques such as phase-contrast and differential-interference-contrast microscopy, but these methods tell us almost nothing about the underlying chemistry.

There are three main approaches to working with thin tissue sections that reveal differences in **types of molecules that are present**:

✓ First, and traditionally, sections can be stained with **organic dyes** that have some specific affinity for particular subcellular components. The dye **hematoxylin**, for example, has an affinity for **negatively charged molecules** and therefore reveals the distribution of **DNA**, **RNA**, and **acidic proteins** in a cell.



Staining of cell components.

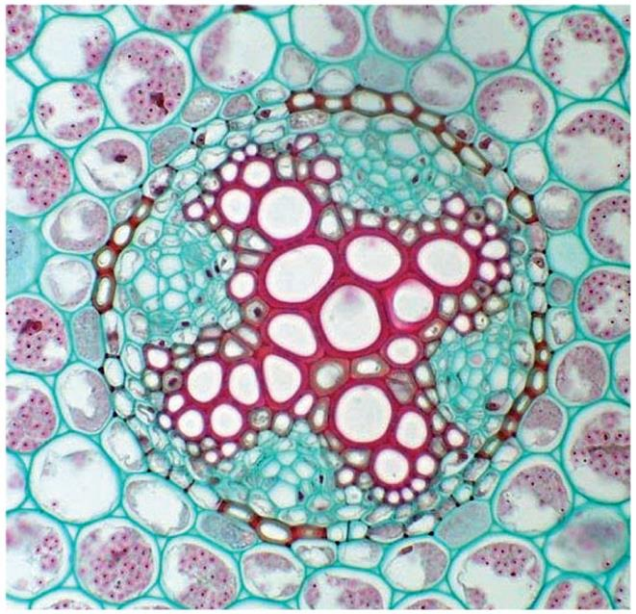
(A) This section of cells in the urine collecting ducts of the kidney was stained with **hematoxylin and eosin**, two dyes commonly used in histology.

Each duct is made of closely packed cells (with nuclei stained red) that form a ring.

The ring is surrounded by extracellular matrix, stained purple.

(B) This section of a young plant root is stained with two dyes, safranin and fast green.

The fast green stains the **cellulosic cell walls** while the safranin stains the **lignified xylem cell walls** bright red.



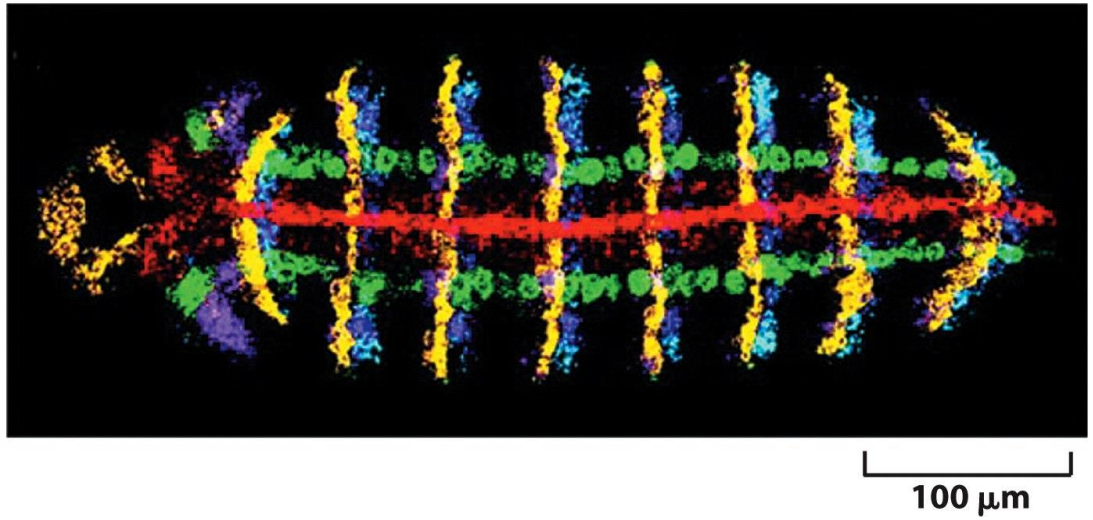
Intact Tissues Are Usually Fixed and Sectioned Before Microscopy

There are three main approaches to working with thin tissue sections that reveal differences in **types of molecules that are present**:

- ✓ Second, sectioned tissues can be used to visualize **specific patterns of differential gene expression**.

In situ hybridization, reveals the cellular distribution and abundance of specific expressed RNA molecules in sectioned material or in whole mounts of small organisms or organs.
This is particularly effective when used in conjunction with fluorescent probes.

- ✓ A third and **very sensitive approach**, generally and widely applicable for localizing proteins of interest, also depends on using **fluorescent probes and markers**, as we explain next.



RNA in situ hybridization. It is possible to visualize the distribution of different RNAs in tissues using in situ hybridization.
Here, the **transcription pattern** of five different genes involved in patterning the early fly embryo is revealed in a single embryo.
Each RNA probe has been fluorescently labeled in a different way, some directly and some indirectly; the resulting images are displayed each in a different color ("false-colored") and combined to give an image where different color combinations represent different sets of genes expressed.
The genes whose expression pattern is revealed here are **wingless** (yellow), **engrailed** (blue), **short gastrulation** (red), **intermediate neuroblasts defective** (green), and **muscle specific homeobox** (purple)