Alberts • Johnson • Lewis • Raff • Roberts • Walter

Molecular Biology of the Cell

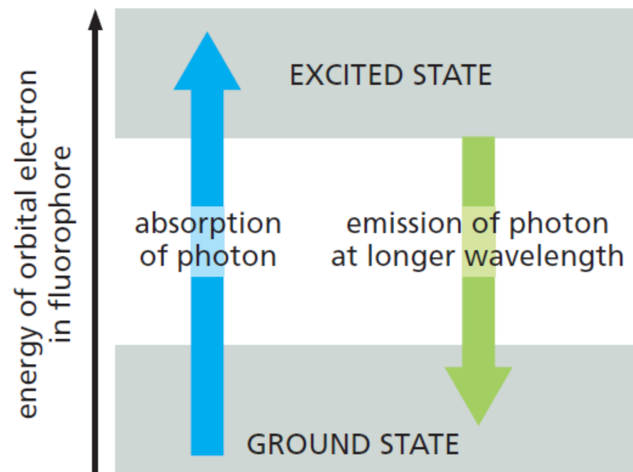
Chapter 9
Visualizing Cells

Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

Fluorescent molecules absorb light at one wavelength and emit it at another, longer wavelength (Figure below).

- ❑ If we illuminate such a molecule 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.



- An orbital electron of a fluorochrome molecule can be raised to an excited state following the absorption of a photon.
- Fluorescence occurs when the electron returns to its ground state and emits a photon of light at a longer wavelength.
- Too much exposure to light, or too bright a light, can also destroy the fluorochrome molecule, in a process called **photobleaching**.

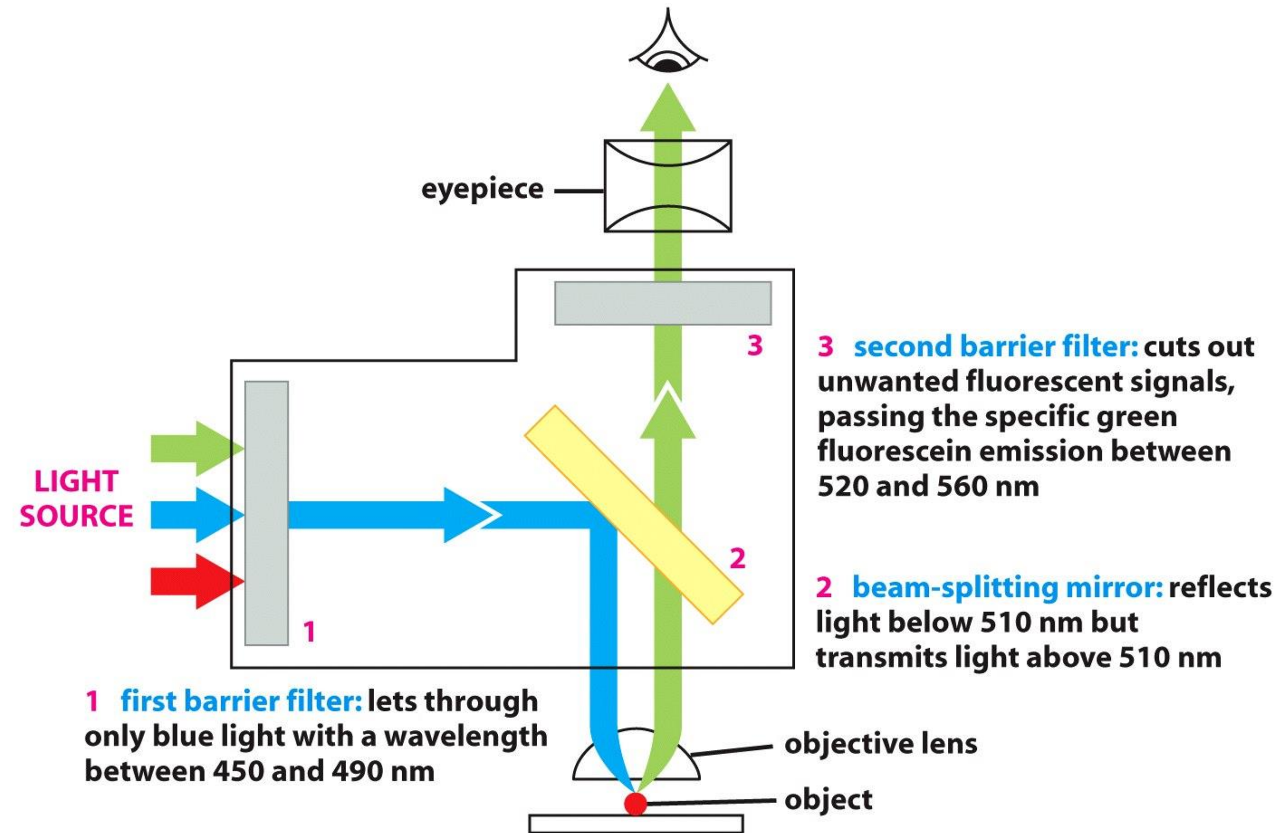
Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

In contrast, the same number of molecules of a nonfluorescent stain, viewed conventionally, would be practically indiscernible because the absorption of light by molecules in the stain would result in only the faintest tinge of color in the light transmitted through that part of the specimen.

The 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**, is passed through **two sets of filters**—one to filter the light before it reaches the specimen and one to filter the light obtained from the specimen:

The first filter passes only the wavelengths that excite the particular fluorescent dye, while the second filter blocks out this light and passes only those wavelengths emitted when the dye fluoresces.



In the 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

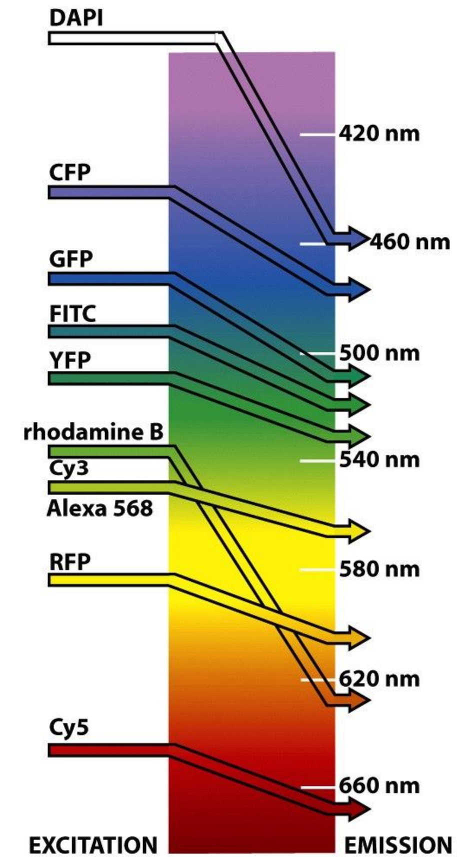
Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

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 deep red fluorescence when excited with green–yellow light.

By coupling one antibody to fluorescein and another to rhodamine, the distributions 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.



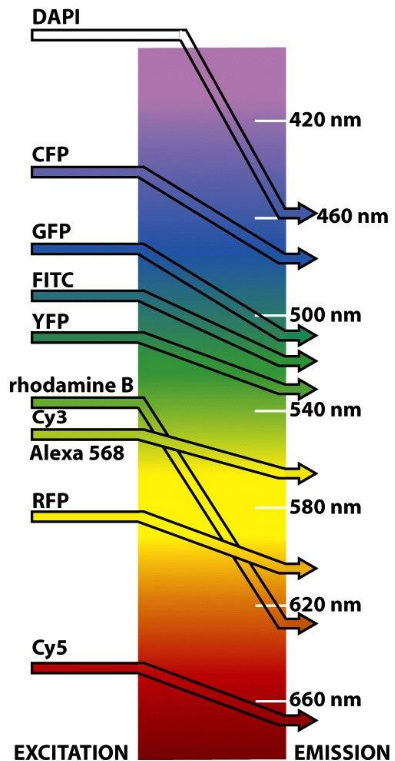
Fluorescent probes. The maximum excitation and emission wavelengths of several commonly used fluorescent probes are shown in relation to the corresponding colors of the spectrum.
The photon emitted by a fluorescent molecule is necessarily of **lower energy (longer wavelength)** than the absorbed photon and this accounts for the difference between the excitation and emission peaks.
CFP, **GFP**, **YFP**, and **RFP** are cyan, green, yellow, and red fluorescent proteins, respectively.
DAPI is widely used as a general fluorescent DNA probe, which absorbs ultraviolet light and fluoresces bright blue.
FITC is an abbreviation for **fluorescein isothiocyanate**, a widely used derivative of fluorescein, which fluoresces bright green.
The other probes are all commonly used to fluorescently label antibodies and other proteins.

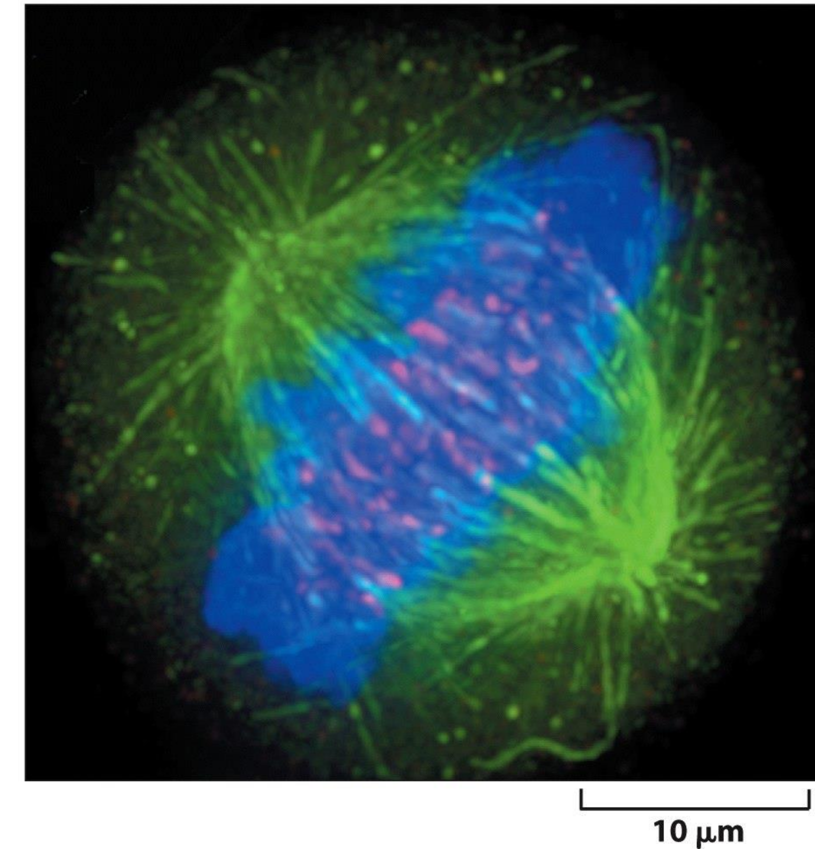
Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

As shown in Figure below, three fluorescent dyes can be used in the same way to distinguish among 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 but, like many organic fluorochromes, they fade fairly rapidly when continuously illuminated.

More stable fluorochromes have been developed based on inorganic chemistry.





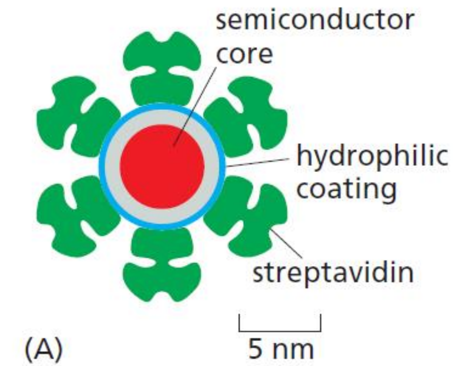
Different fluorescent probes can be visualized in the same cell.
In this composite micrograph of a cell in **mitosis**, three different fluorescent probes have been used to label three different cellular components:
The **spindle microtubules** are revealed with a green fluorescent antibody,
centromeres with a red fluorescent antibody,
and the **DNA of the condensed chromosomes** with the blue fluorescent dye DAPI.

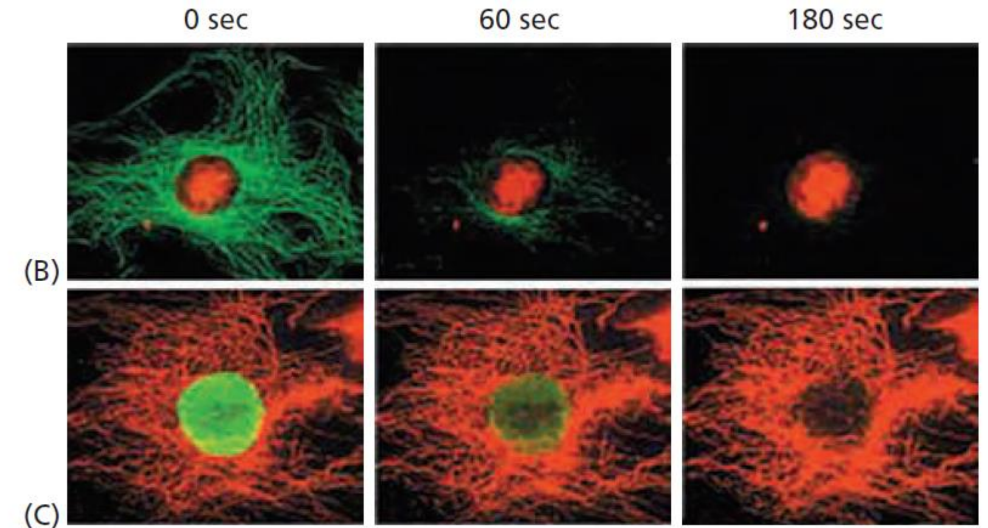
Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

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**, between 2 and 10 nm in diameter, and additionally the fluorescence fades only slowly with time.

These nanoparticles, when coupled to other probes such as antibodies, are therefore **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.





Fluorescent nanoparticles or quantum dots.
(A) Quantum dots are tiny particles of **cadmium selenide**, a semiconductor, with a coating to make them water-soluble.
They can be coupled to protein molecules such as antibodies or streptavidin and, when introduced into a cell, will bind to a target protein of interest.
Different-sized quantum dots emit light of different colors—the larger the dot, the longer the wavelength—but they are all excited by the same blue light.
Quantum dots can keep shining for weeks, unlike most fluorescent organic dyes.

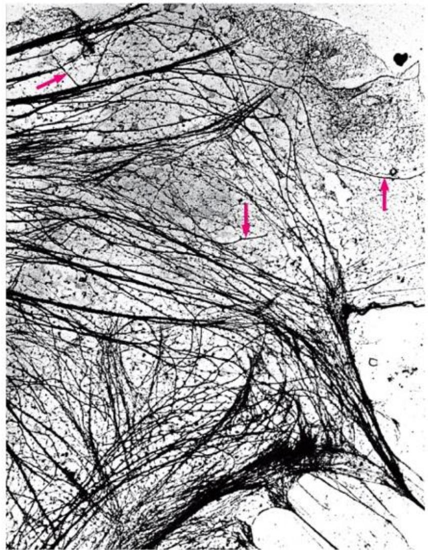
(B) In this cell, **microtubules** are labeled (green) with an organic fluorescent dye (Alexa 488), while a **nuclear protein** is stained (red) with quantum dots bound to streptavidin.
On continuous exposure to strong blue light, the fluorescent dye fades quickly while the quantum dots continue to shine.
(C) In this cell, the labeling pattern is reversed; a **nuclear protein** is labeled (green) with an organic fluorescent dye (Alexa 488), while **microtubules are labeled** (red) with quantum dots.

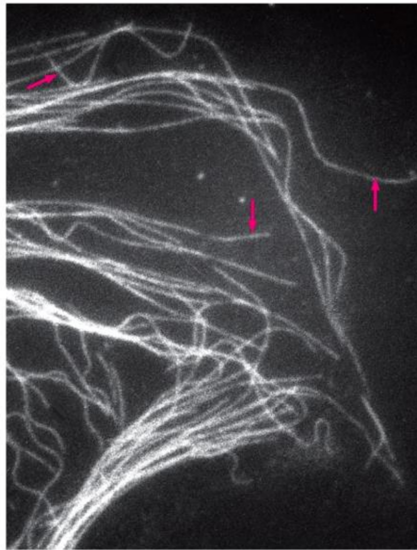
Antibodies Can Be Used to Detect Specific Molecules

- Antibodies are proteins produced by the vertebrate immune system as a defense against infection.
They are **unique among proteins** in that they are made in billions of different forms, each with a different binding site that recognizes **a specific target molecule (or antigen).**

- The precise antigen specificity of antibodies makes them powerful tools for the cell biologist.

- When labeled with fluorescent dyes, antibodies are invaluable for **locating specific molecules in cells by fluorescence microscopy**;
- labeled with **electron-dense particles such as colloidal gold spheres**, they are used for similar purposes in the electron microscope.
- The antibodies employed in microscopy are commonly either purified from antiserum so as to remove all nonspecific antibodies, or they are specific monoclonal antibodies that only recognize the target molecule.



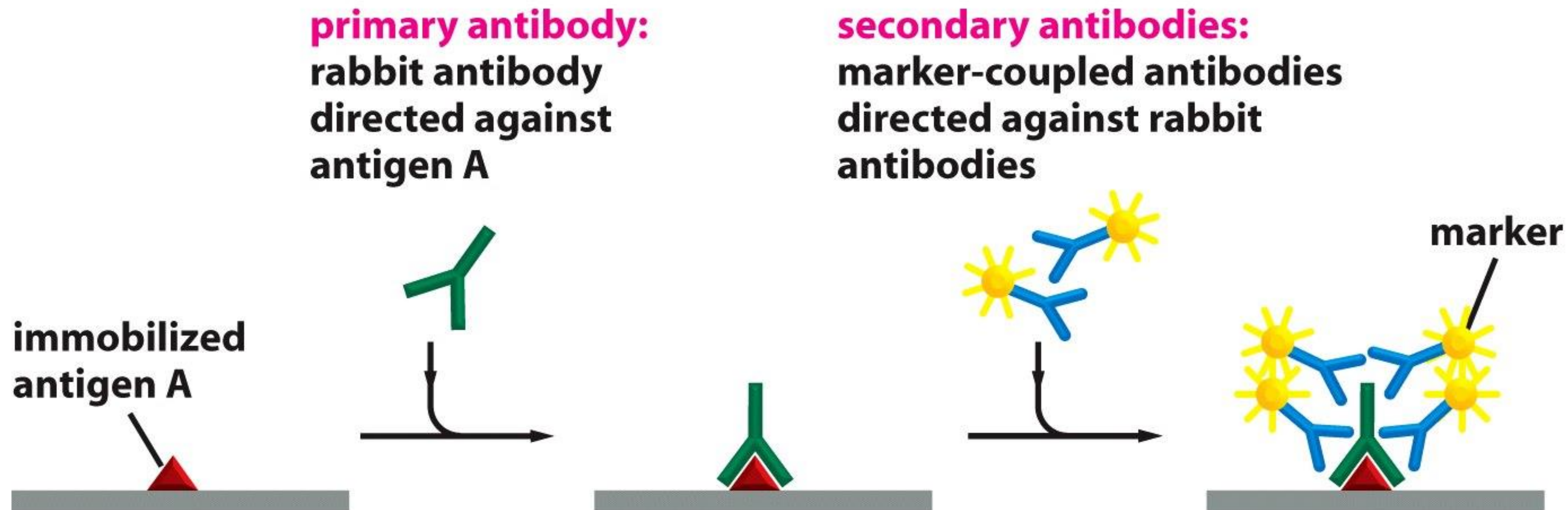


Immunofluorescence.
(A) A transmission electron micrograph of the periphery of a cultured epithelial cell showing the distribution of microtubules and other filaments.
(B) The same area stained with fluorescent antibodies against tubulin, the protein that assembles to form microtubules, using the technique of indirect immunocytochemistry.
Red arrows indicate individual microtubules that are readily recognizable in both images.
Note that, because of **diffraction effects**, the microtubules in the light microscope appear 0.2 µm wide rather than their true width of 0.025 µm.

Antibodies Can Be Used to Detect Specific Molecules

When we use antibodies as probes to detect and assay specific molecules in cells, we frequently use chemical methods to amplify the fluorescent signal they produce.

For example, although a marker molecule such as a fluorescent dye can be linked directly to an antibody—the primary antibody—a stronger signal is achieved by using an **unlabeled primary antibody** and then detecting it with a group of **labeled secondary antibodies** that bind to it. This process is called indirect ***immunocytochemistry***.



Indirect immunocytochemistry.

- This detection method is very sensitive because many molecules of the secondary antibody recognize each primary antibody.

- The secondary antibody is covalently coupled to a marker molecule that makes it readily detectable.

Commonly used marker molecules include:

- **fluorescent probes** (for fluorescence microscopy),
- **the enzyme horseradish peroxidase** (for either conventional light microscopy or electron microscopy),
- **colloidal gold spheres** (for electron microscopy), and
- **the enzymes alkaline phosphatase or peroxidase** (for biochemical detection).

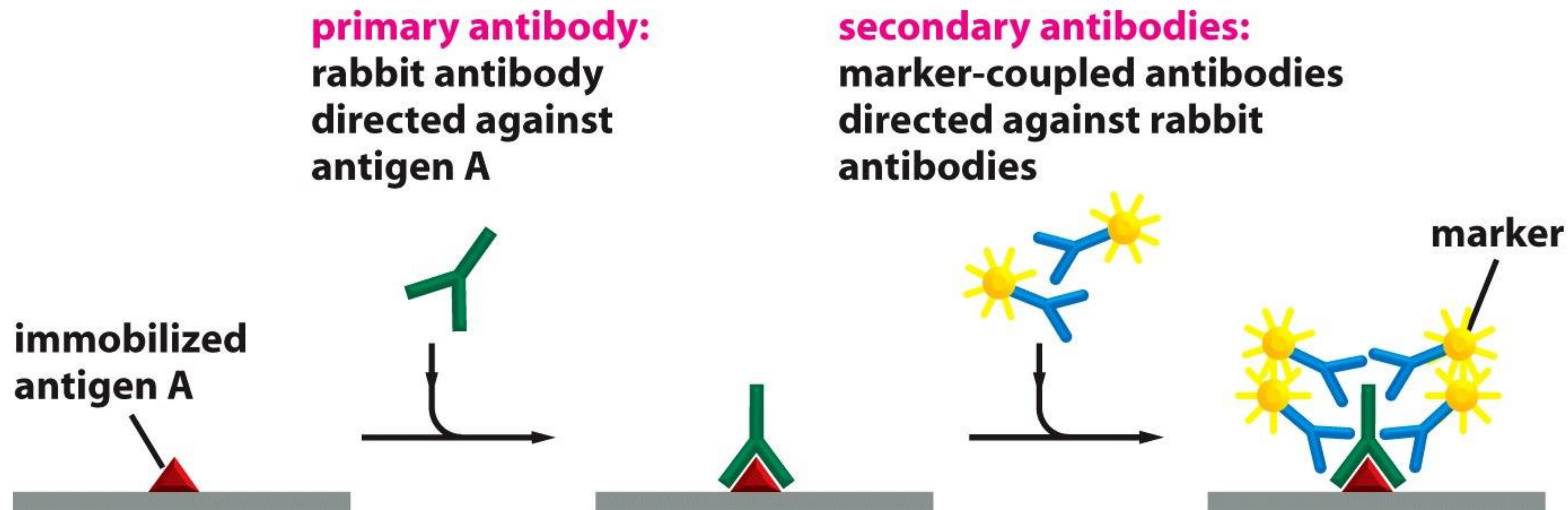
Antibodies Can Be Used to Detect Specific Molecules

Some amplification methods use an enzyme as a marker molecule attached to the secondary antibody.
The enzyme **alkaline phosphatase**, for example, in the presence of appropriate chemicals, produces **inorganic phosphate** that in turn leads to the local formation of **a colored precipitate**.

This reveals the location of the secondary antibody and hence the location of the **antibody–antigen complex**.

Since each enzyme molecule acts catalytically to generate many thousands of molecules of product, even tiny amounts of antigen can be detected.

Although the enzyme amplification makes enzyme-linked methods sensitive, **diffusion** of the colored precipitate away from the enzyme **limits the spatial resolution** of this method for microscopy, and **fluorescent labels are usually used for the most sensitive and precise optical localization.**



Imaging of Complex Three-Dimensional Objects Is Possible with the Optical Microscope

For ordinary light microscopy, as we have seen, a tissue has to be sliced into thin sections to be examined; the thinner the section, the crisper the image.

❖ Since information about the third dimension is lost upon sectioning, how, then, can we get a picture of the three-dimensional architecture of a cell or tissue, and how can we view the microscopic structure of a specimen that, for one reason or another, cannot first be sliced into sections?

Although an optical microscope is focused on a particular focal plane within a three-dimensional specimen, all the other parts of the specimen, above and below the plane of focus, are also illuminated and the light originating from these regions contributes to the image as "**out-of-focus**" blur.

✓ This can make it very hard to interpret the image in detail and can lead to fine image structure being obscured by the out-of-focus light.

❖ Two distinct but complementary approaches solve this problem: one is **computational**, the other **optical**.

These three-dimensional microscopic imaging methods make it possible to focus on a chosen plane in a thick specimen while rejecting the light that comes from out-of-focus regions above and below that plane.

→ Thus one sees a crisp, thin ***optical section***.

Imaging of Complex Three-Dimensional Objects Is Possible with the Optical Microscope

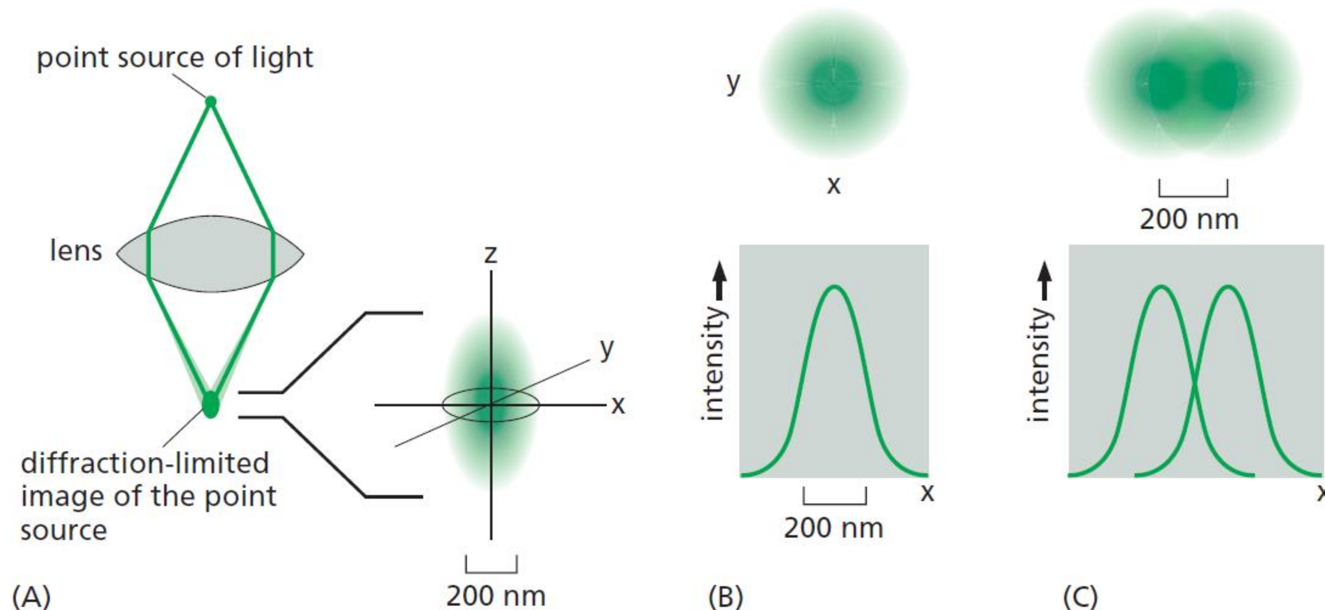
- From a series of such optical sections taken at different depths and stored in a computer, a three-dimensional image can be reconstructed.

The methods do for the microscopist what the computed tomography (CT) scanner does (by different means) for the radiologist investigating a human body: both machines give detailed sectional views of the interior of an intact structure.

- The computational approach is often called ***image deconvolution***.

To understand how it works, remember that the wavelike nature of light means that the microscope lens system produces a small blurred disc as the image of a point light source (see Figure 9–5), with increased blurring if the point source lies above or below the focal plane.

This blurred image of a point source is called **the point spread function.**



The point spread function of a lens determines resolution. (A) When a point source of light is brought to a focus by a lens system, diffraction effects mean that, instead of being imaged as a point, **it is blurred in all dimensions**.

(B) In the plane of the image, the distribution of light approximates a Gaussian distribution, whose width at half-maximum height under ideal conditions is about 200 nm.

(C) Two point sources that are about 200 nm apart can still just be distinguished as separate objects in the image, but if they are any nearer than that, their images will overlap and not be resolvable.

Imaging of Complex Three-Dimensional Objects Is Possible with the Optical Microscope

An image of a complex object can then be thought of as being built up by replacing each point of the specimen by a corresponding blurred disc, resulting in an image that is **blurred overall:**

- For ***deconvolution***, we first obtain **a series of (blurred) images**, usually with a cooled CCD camera or more recently a CMOS camera, focusing the microscope in turn on a series of focal planes—in effect, a (blurred) three-dimensional image.

- **Digital processing of the stack of digital images** then removes as much of the blur as possible.

In essence, the computer program uses the measured ***point spread function of a point source of light from that microscope*** to determine what the effect of the **blurring** would have been on the image, and then applies an equivalent "**deblurring**" (**deconvolution**), turning the blurred three-dimensional image into a series of **clean optical sections**, albeit still constrained by the diffraction limit.

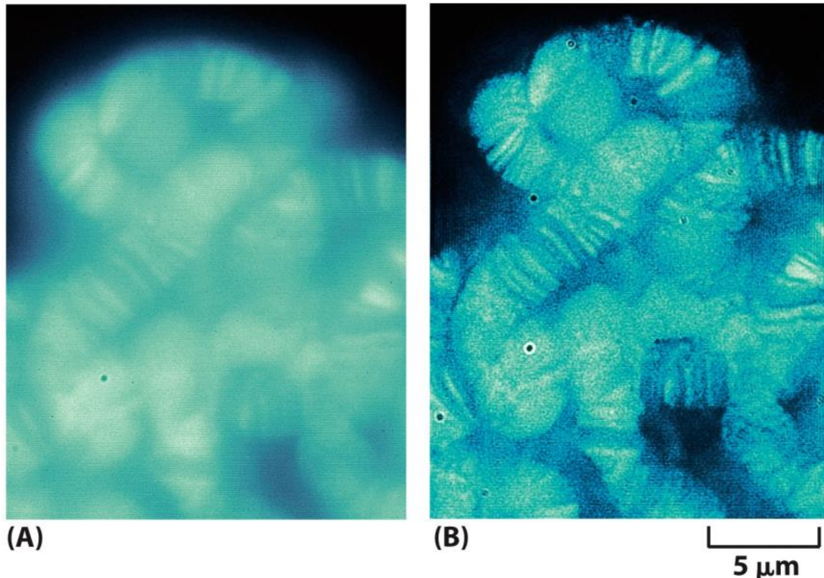
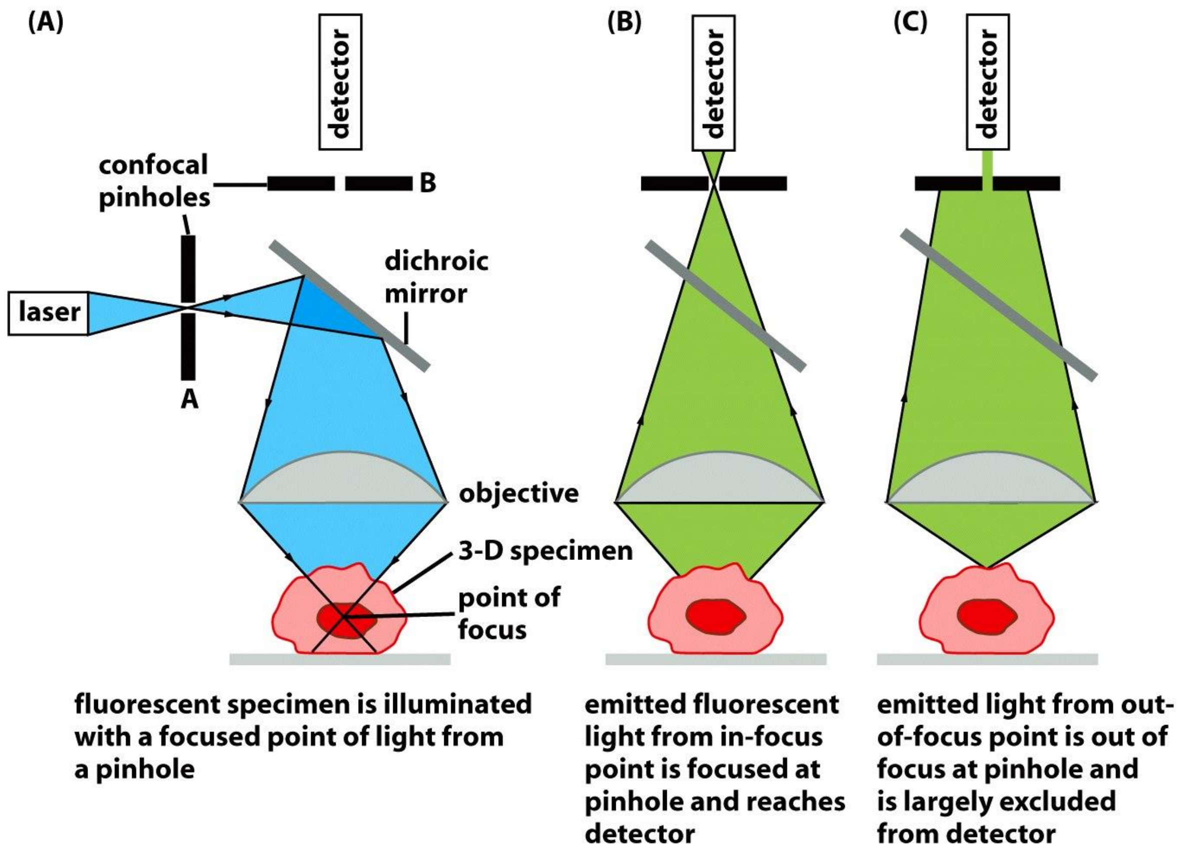


Image deconvolution.
(A) A light micrograph of the large polytene chromosomes from Drosophila, stained with a fluorescent DNA-binding dye.
(B) The same field of view after image deconvolution clearly reveals the banding pattern on the chromosomes.
Each band is about 0.25 µm thick, approaching the diffraction limit of the light microscope.

The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

The confocal microscope achieves a result similar to that of deconvolution, **but does so by manipulating the light before it is measured**; it is an analog technique rather than a digital one.

The optical details of the confocal microscope are complex, but the basic idea is simple, as illustrated in below.



The confocal fluorescence microscope.

(A) This simplified diagram shows that the basic arrangement of optical components that a laser is used to illuminate a small pinhole whose image is focused at a single point in the three dimensional (3-D) specimen.

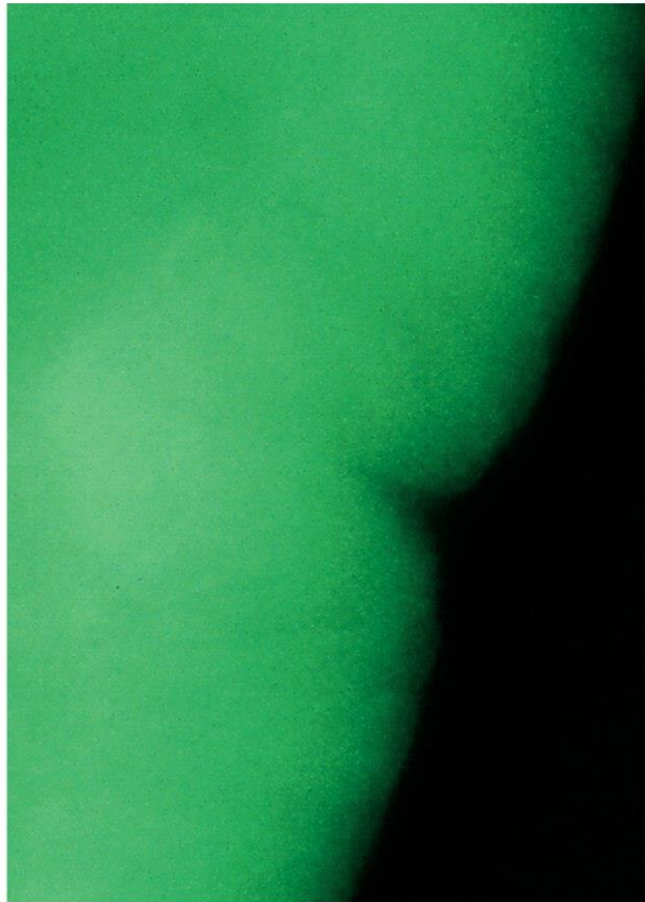
(B) Emitted fluorescence from **this focal point** in the specimen is focused at a second (confocal) pinhole.

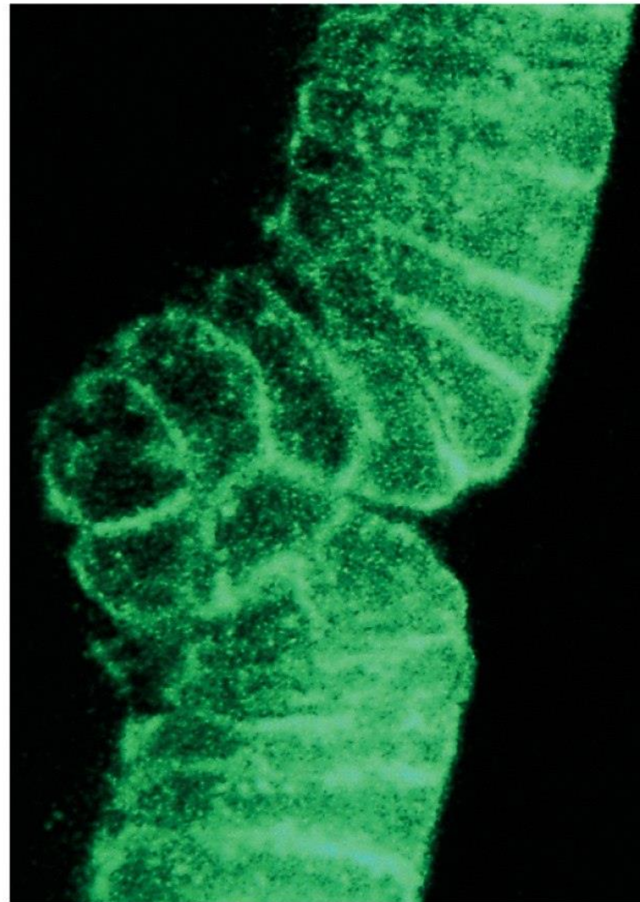
(C) Emitted light from elsewhere in the specimen is not focused at the pinhole and therefore does not contribute to the final image.

By scanning the beam of light across the specimen, a very sharp two-dimensional image of the exact plane of focus is built up that is not significantly degraded by light from other regions of the specimen.

The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

And the results are far superior to those obtained by conventional light microscopy.





Confocal fluorescence microscopy produces clear optical sections and three-dimensional data sets. These two micrographs are of the same intact gastrula-stage Drosophila embryo, which has been stained with a fluorescent probe for actin filaments.

(A) The conventional, unprocessed image is blurred by the presence of fluorescent structures above and below the plane of focus.

(B) In the confocal image, this out-of-focus information is removed, resulting in a **crisp optical section** of the cells in the embryo.

The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

The confocal microscope is generally used with fluorescence optics, but instead of illuminating the whole specimen at once, in the usual way, **the optical system at any instant focuses a spot of light onto a single point at a specific depth in the specimen:**

- This requires a source of pinpoint illumination that is usually supplied by a laser whose light has been passed through a pinhole.
- The fluorescence emitted from the illuminated material is collected at a suitable light detector and used to generate an image.
- A pinhole aperture is placed in front of the detector, at a position that is confocal with the illuminating pinhole—that is, precisely where the rays emitted from the illuminated point in the specimen come to a focus.
- **Thus, the light from this point in the specimen converges on this aperture and enters the detector.**
- By contrast, the light from regions out of the plane of focus of the spotlight is also out of focus at the pinhole aperture and is therefore largely excluded from the detector.

- To build up a two-dimensional image, data from each point in the plane of focus are collected sequentially by scanning across the field from left to right in a regular pattern of pixels and are displayed on a computer screen.

Although not shown in Figure 9–19, the scanning is usually done by deflecting the beam with an oscillating mirror placed between the dichroic mirror and the objective lens in such a way that the illuminating spotlight and the confocal pinhole at the detector remain strictly in register.

❖ **The confocal microscope has been used to resolve the structure of numerous complex three-dimensional objects including the networks of cytoskeletal fibers in the cytoplasm and the arrangements of chromosomes and genes in the nucleus.**

The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

The relative merits of deconvolution methods and confocal microscopy for three-dimensional optical microscopy depend on the specimen being imaged.

Confocal microscopes tend to be better for thicker specimens with high levels of out-of-focus light.

They are also generally easier to use than deconvolution systems and the final optical sections can be seen quickly.

In contrast, the cooled CCD or CMOS cameras used for deconvolution systems are extremely efficient at collecting small amounts of light, and they can be used to make detailed three-dimensional images from specimens that are too weakly stained or too easily damaged by the bright light used for confocal microscopy.

Both methods, however, have another drawback; neither is good at coping with very thick specimens.

Deconvolution methods quickly become ineffective any deeper than about 40 μm into a specimen, while confocal microscopes can only obtain images up to a depth of about 150 μm.

Special microscopes can now take advantage of the way in which fluorescent molecules are excited, to probe even deeper into a specimen.

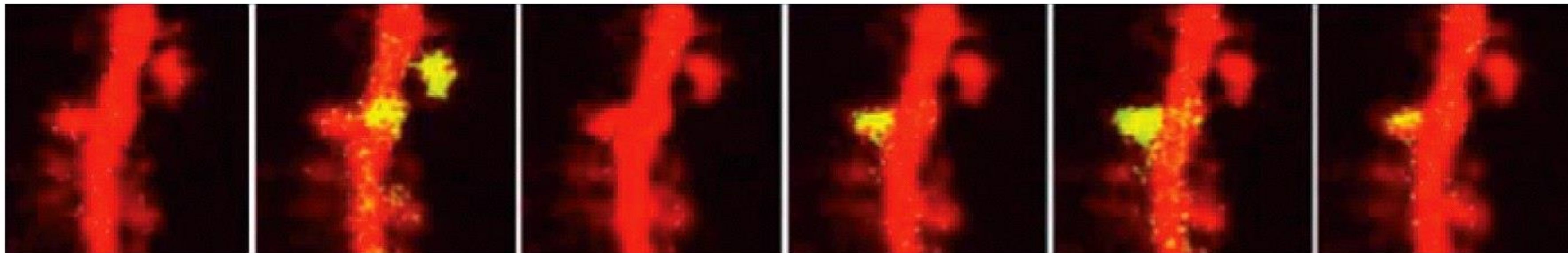
The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

Fluorescent molecules are usually excited by a single high-energy photon, of shorter wavelength than the emitted light, but they can in addition be excited by the absorption of two (or more) photons of lower energy, as long as they both arrive within a femtosecond or so of each other.

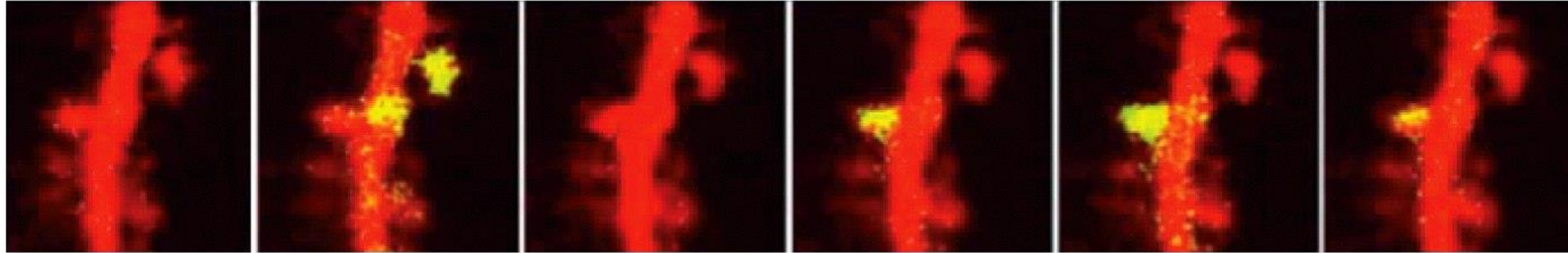
The use of this longer-wavelength excitation has some important advantages. In addition to reducing background noise, red or near-infrared light can penetrate deeper into a specimen.

Multiphoton microscopes, constructed to take advantage of this two-photon effect, can obtain sharp images, sometimes even at a depth of 250 μm within a specimen.

This is particularly valuable for studies of living tissues, notably in imaging the dynamic activity of synapses and neurons just below the surface of living brains (Figure 9–21).



The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light



Multiphoton imaging.
Infrared laser light causes less damage to living cells than visible light and can also penetrate further, allowing microscopists to peer deeper into living tissues.

The **two-photon effect**, in which a fluorochrome can be excited by two coincident infrared photons instead of a single high-energy photon, allows us to see nearly **0.5 mm** inside the cortex of a live mouse brain.
A dye, whose fluorescence changes with the calcium concentration, reveals active synapses (yellow) on the dendritic spines (red) that change as a function of time;

In this case, there is a day between each image.