General and Cell Biology

Visualizing Cells

Dr.Rasime Kalkan

Chapter Outline

- Describe the principal microscopy methods used to study cells
- Looking at cells in the light microscope
- Looking at cells and molecules in the electron microscope

- The microscopy choice is depending on the:

-cell type

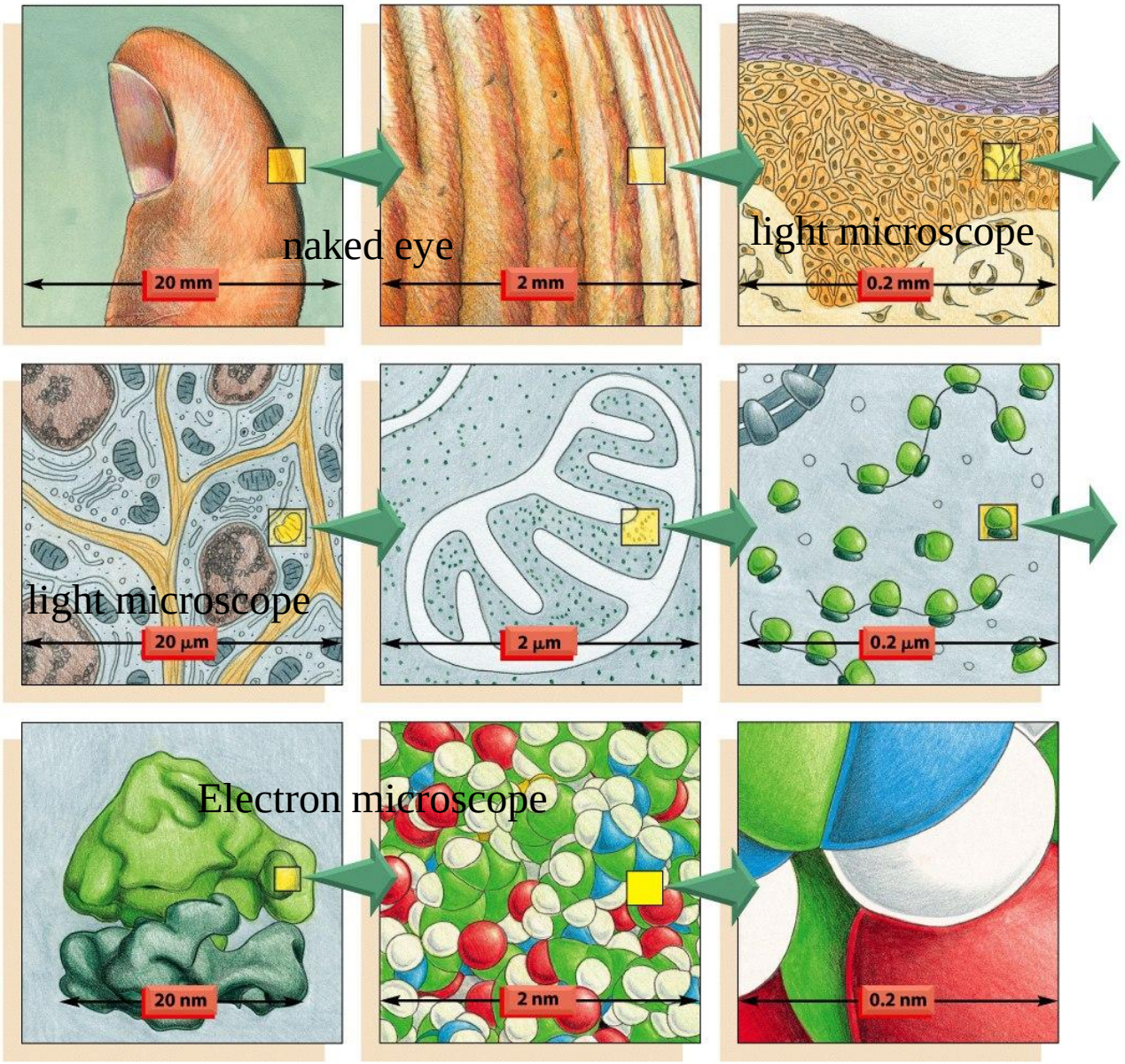
*Live or fixed cell

-Depending on the dye: fluorescent or antibody

Looking at cells in the light microscope

- Typical animal **cell** is 10–20 μm in diameter.

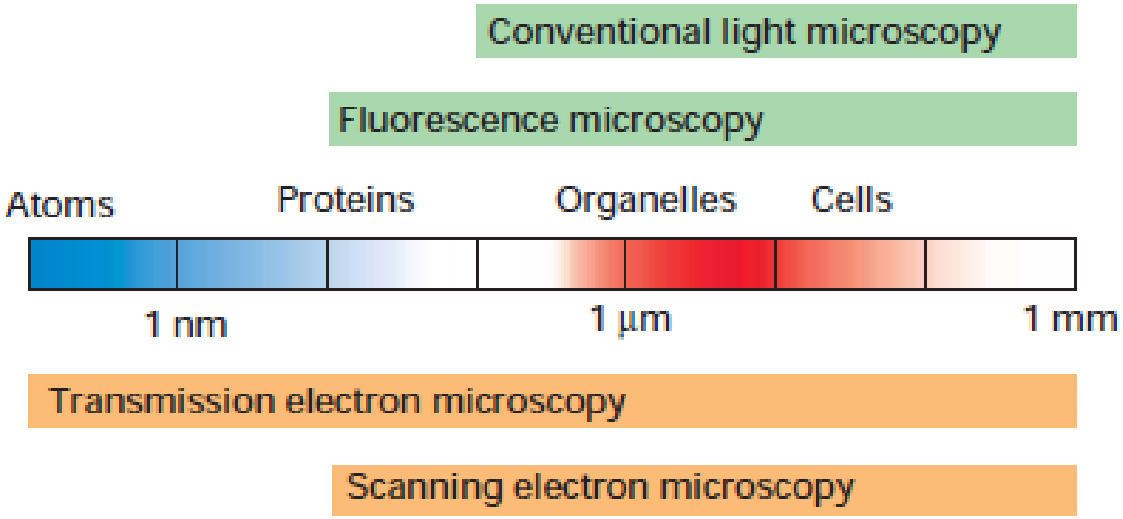
*one-fifth the size of the smallest particle visible to the naked eye.



A sense of scale between living cells and atoms

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter.(2008) Molecular Biology of the Cell, 5th edition

The range in sizes of objects imaged by different microscopy techniques.



The smallest object that can be imaged by a particular technique is limited by the resolving power of the equipment and other factors.

A Microscope Detects, Magnifies, and Resolves Small Objects

Lodish H, Berk A, Zipursky SL, et al. (2003).Molecular Cell Biology 5th edition

The Light Microscope Can Resolve Details 0.2 μm Apart

- Fundamental limitation of all microscopes is that a given type of radiation cannot be used to probe structural details much smaller than its own wavelength.
- The ultimate limit to the resolution of a light microscope is therefore set by the wavelength of visible light, which ranges from about 0.4 μm (for violet) to 0.7 μm (for deep red).

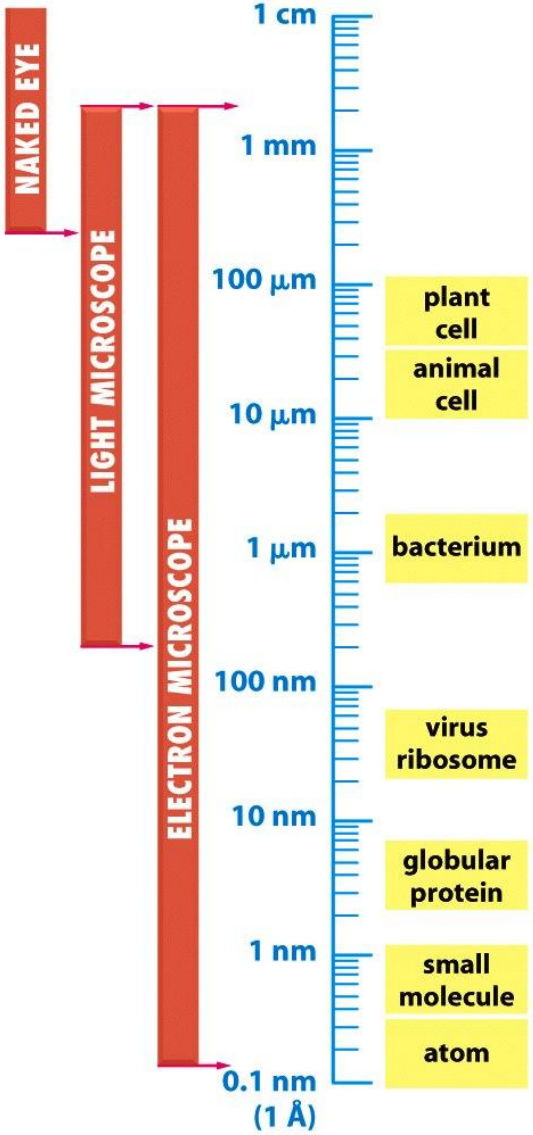
Resolution

- Resolution is the ability to see two images as separate and discrete.
- The wavelengths of visible light from 420 to 620 prevent resolution of two points closer than 220 nm
- By using the light emitted from an electron it is possible to resolve two points that are 2nm apart

- The most important property of microscope is not its magnification but its **resolving power**, or **resolution**, the ability to distinguish between two very closely positioned objects.

Specialized Microscopy

- Dark field
- Phase Contrast
- Differential interference
- Fluorescence
- Confocal
- Digital



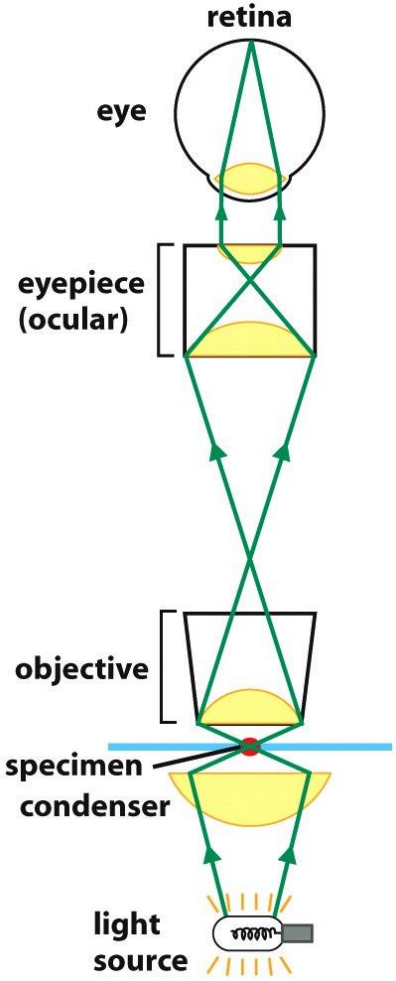
The following units of length are commonly employed in microscopy:
µm (micrometer) = 10^{-6} m
nm (nanometer) = 10^{-9} m
Å (Ångström unit) = 10^{-10} m

Resolving power

Sizes of cells and their components

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter.(2008) Molecular Biology of the Cell, 5th edition

The light microscope can resolve details 0.2 μm apart

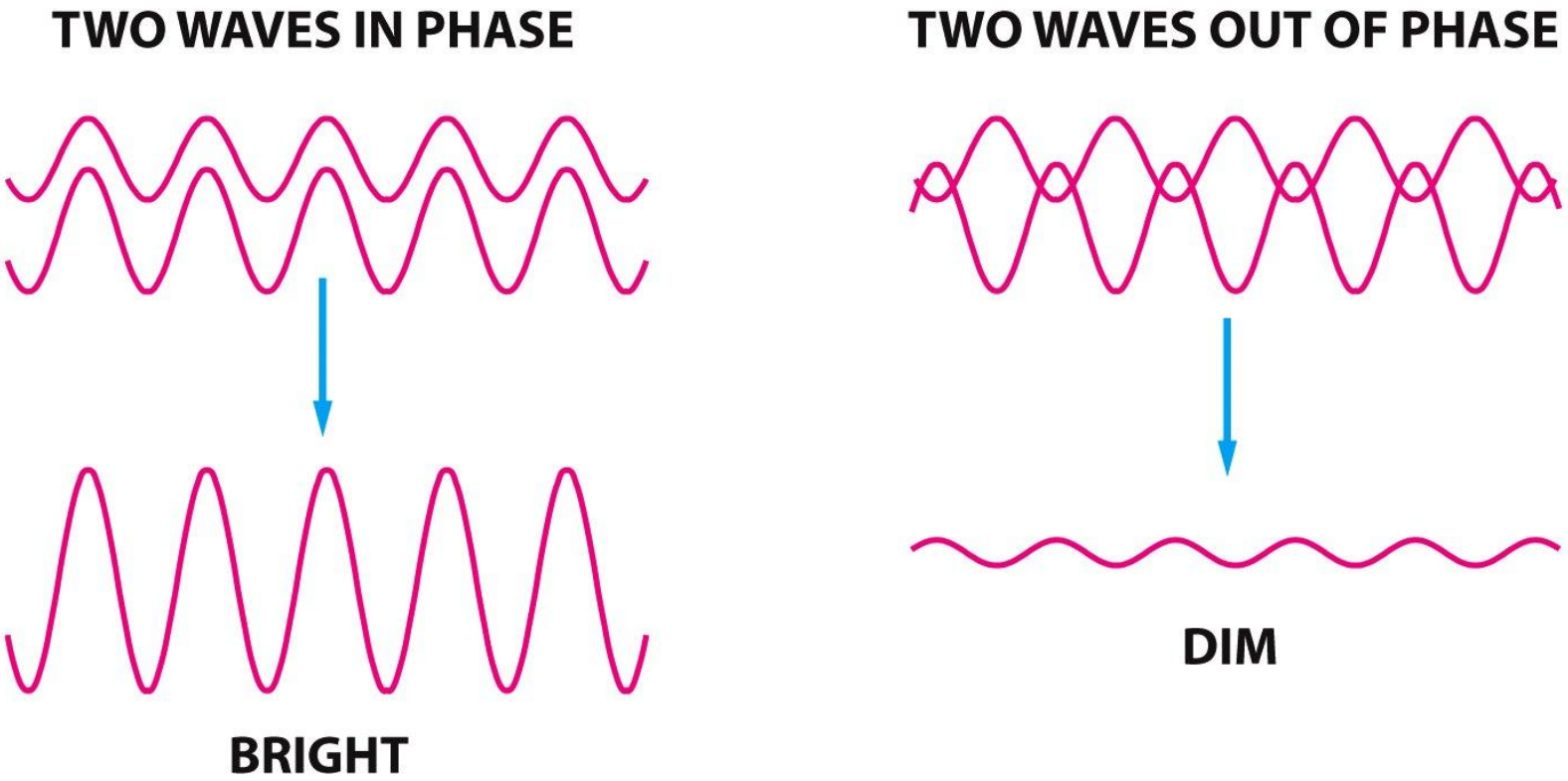


The limit of resolution of a light microscope is set by the wavelength of visible light from about 0.4 μm (for violet) to 0.7 μm (for deep red). Under the best conditions, with violet light and a numerical aperture of 1.4, a limit of resolution of 0.2 μm can be obtained.

Limit of resolution is depend on the wavelength of the light and the numerical aperture of the lens system used.

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter.(2008) Molecular Biology of the Cell, 5th edition

The wave nature of light causes optical diffraction effects



When two light waves are *in phase*, the amplitude of the resultant wave is larger and the brightness is increased. When two light waves are *out of phase*, they cancel each other partly and produce a wave whose amplitude, and therefore brightness, is decreased.

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter.(2008) Molecular Biology of the Cell, 5th edition

THE QUALITY OF LIGHT

- As an analytic probe used in light microscopy, we also describe the kind or quality of light according to the degree of uniformity of rays comprising an illuminating beam. The kinds of light most frequently referred to in this text include:
- *Monochromatic—waves having the same wavelength or vibrational frequency (the* same color).
- *Polarized—waves whose E vectors vibrate in planes that are parallel to one another*. The E vectors of rays of sunlight reflected off a sheet of glass are plane parallel and are said to be linearly polarized.
- *Coherent—waves of a given wavelength that maintain the same phase relationship* while traveling through space and time (laser light is coherent, monochromatic, and polarized).
- *Collimated—waves having coaxial paths of propagation through space—that is*, without convergence or divergence, but not necessarily having the same wavelength, phase, or state of polarization. The surface wavefront at any point along a cross-section of a beam of collimated light is planar and perpendicular to the axis of propagation

Living cells are seen clearly in a phase-contrast or a differential interference contrast microscope

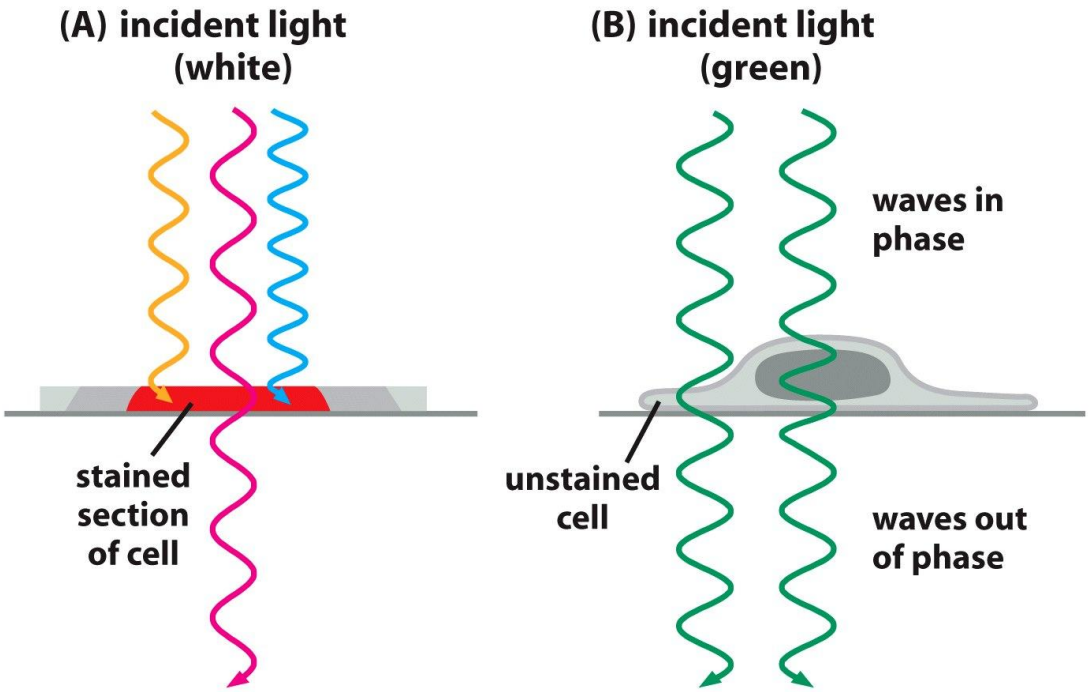
- When light passes through a living cell, the phase of the light wave is changed according to the cell's refractinve index.
- Types of light microscope that used to visualize living cells:

*Phase- contrast microscope

*differential inteference contrast microscope

- **Dark-field microscope**: The illuminating rays of light are directed from the side so only scattered lights enters the microscope lenses.
- **Bright-field microscope**:Light passing through a cell in cukture forms the image directly.

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. A colored image of the cell is obtained that is visible in the normal bright-field microscope.
(B) Light passing through an unstained cell undergoes 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.

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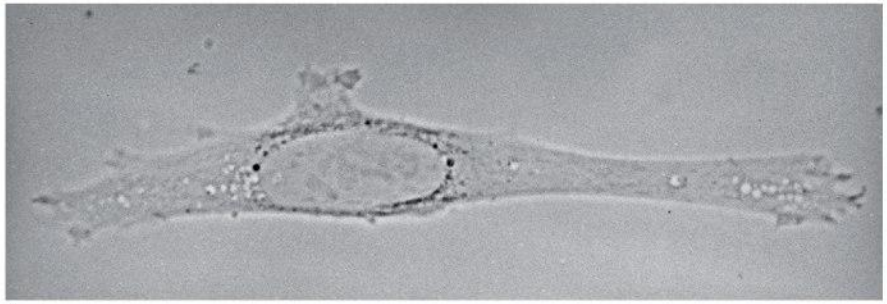
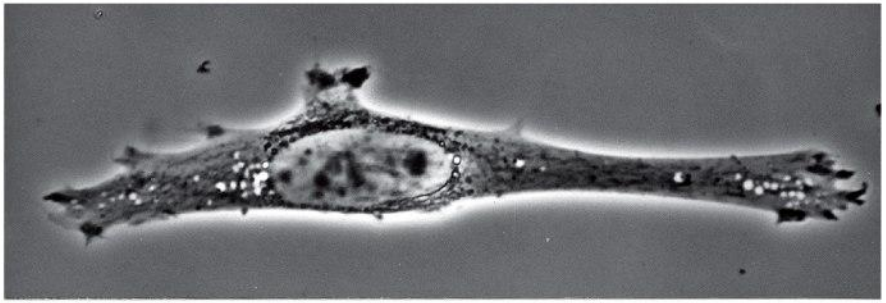
Four types of light microscopy

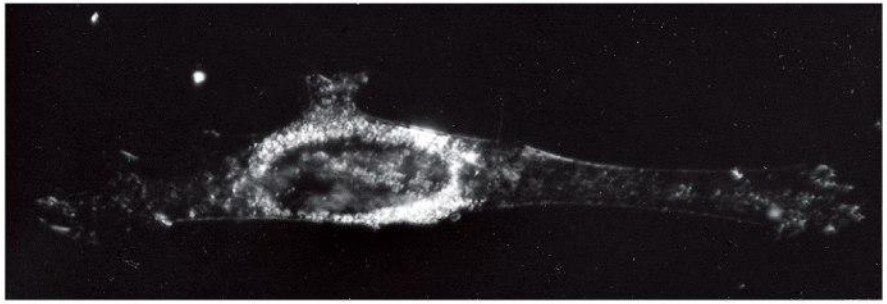


(A) Bright-field microscopy



(B) Bright-field microscopy

(C) Nomarski differential-interference-contrast microscopy



(D) Dark-field microscopy

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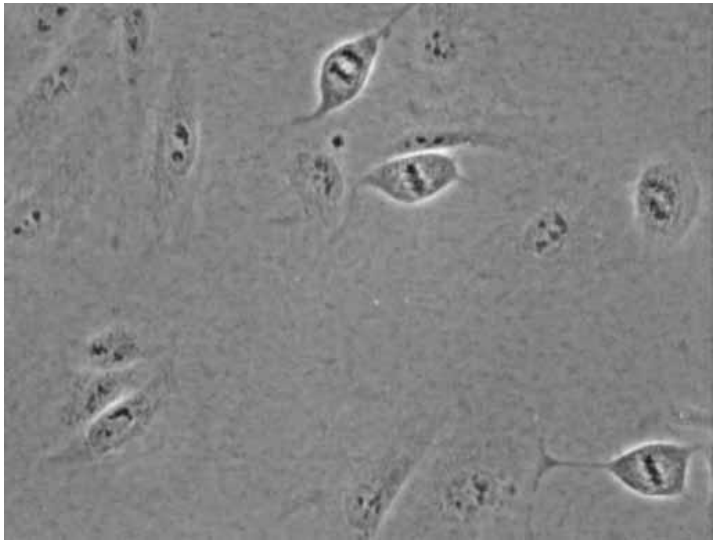
Phase Contrast Microscopy

- Accentuate small differences in the refractive index of the specimen
- More detail is apparent in living cells
- Assist in the visualization of cell structure in transparent cells



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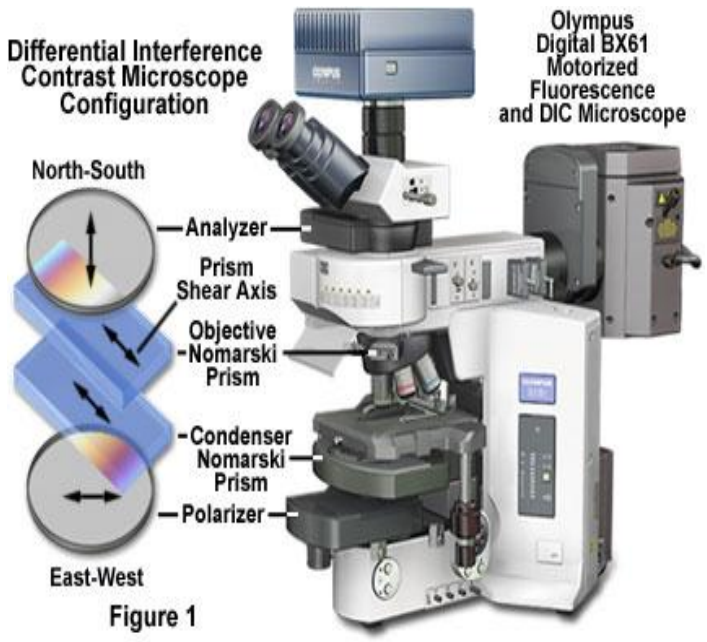
- In phase-contrast images, the entire object and subcellular structures are highlighted by interference rings—concentric halos of dark and light bands.
- This artifact is inherent in the method, which generates contrast by interference between diffracted and undiffracted light by the specimen. Because the interference rings around an object obscure many details, this technique is suitable for observing only single cells or thin cell layers but not thick tissues.

- It is particularly useful for examining the location and movement of larger organelles in live cells

Differential interference microscopy

- Produces higher resolution
- Depends on a gradient
- It can produce almost a three dimensional image

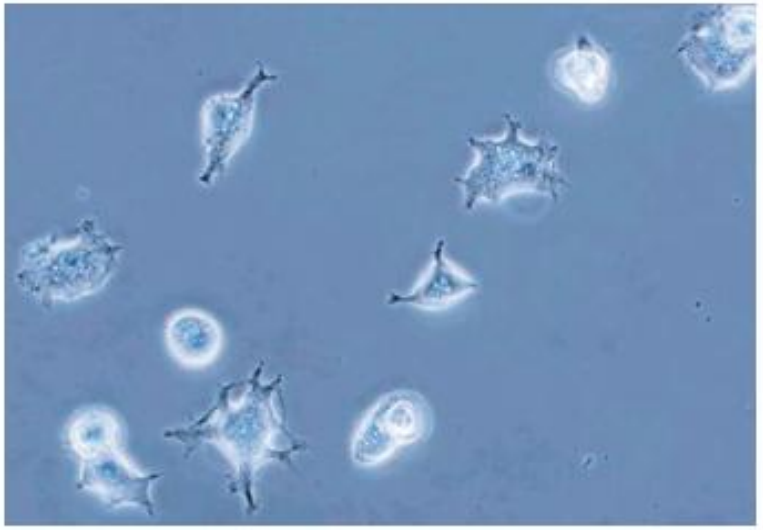


- DIC microscopy is based on interference between polarized light and is the method of choice for visualizing extremely small details and thick objects.

- A slightly more difficult modification
- Contrast obtained by difference in phase change
- – That is: optical gradients converted to intensity differences
- Produces fake 3D effect
- Orientation-dependen

Comparison of Phase Contrast and Differential Interference Contrast

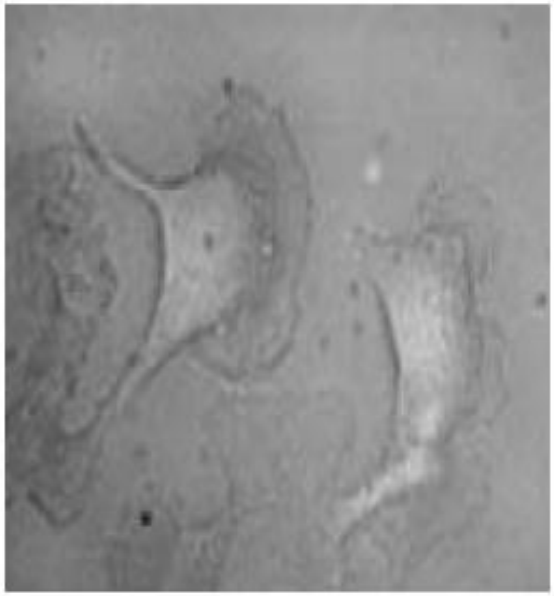
A) DIC image



B) Phase contrast image

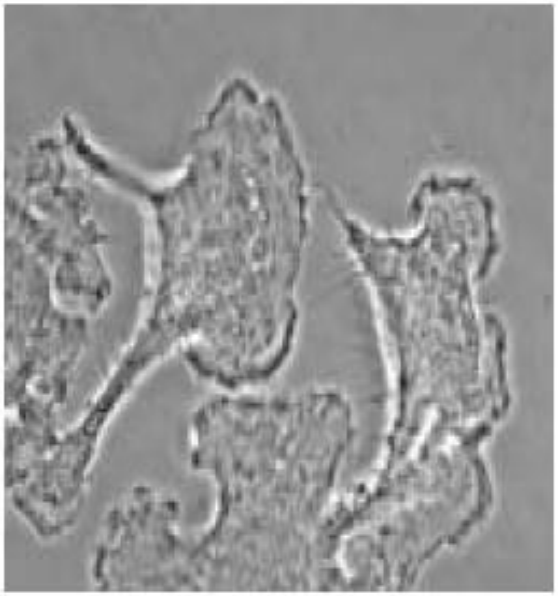
Comparison of DIC and phase contrast observation (neural cells, NG108-15)

Olympus, Basics of Inverted Microscope



bright-field microscopy

Phase contrast microscopy



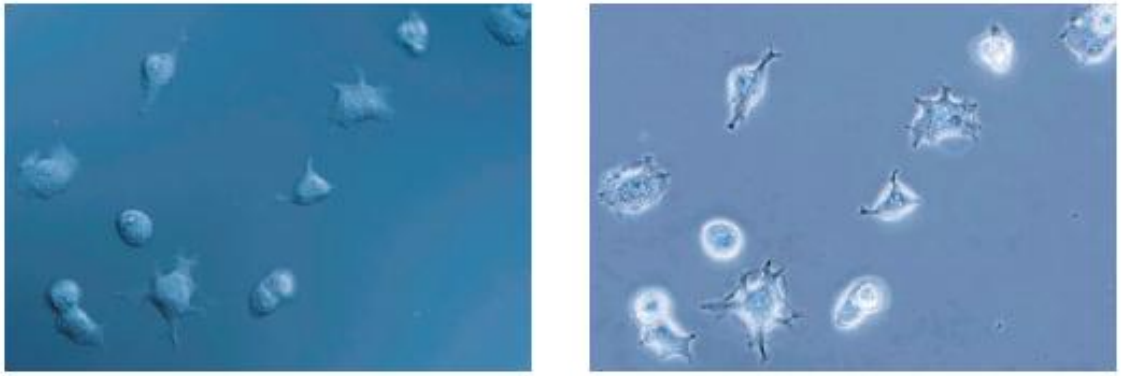
differential interference contrast (DIC) microscopy

Lodish H, Berk A, Zipursky SL, et al. (2003).Molecular Cell Biology 5th edition

DIC vs. Phase Contrast Microscopy

	DIC Observation	Phase Contrast Observation
How Contrast is Added	• Contrast added by gradients in sample thickness	• Contrast added at sample borders or points
Image Features	• Bright/dark or color contrast added, conveying a three-dimensional appearance • Shadows added depending on orientation	• Bright/dark contrast added • Pronounced halo around thick samples
Contrast Adjustment and Selection	• Fine adjustment of three-dimensional contrast possible	• Choice of negative or positive contrast
Resolution	• High	• Poor compared with DIC*
Suitable Samples	• Capable of observing structures with sizes ranging from minute to large • Sample thicknesses up to several 100 µm	• Useful for observing minute structures • Sample thickness up to 10 µm
Use of Plastic Containers	• No	• Yes

*In phase contrast observations, illumination is limited by the ring slit, and consequently its resolution is poor compared with DIC.



A) DIC image B) Phase contrast image

Comparison of DIC and phase contrast observation (neural cells, NG108-15)

Olympus, Basics of Inverted Microscope

- Electronic imaging systems have 2 limitations of th human eye:

*the eye cannot see well in extremly dim light

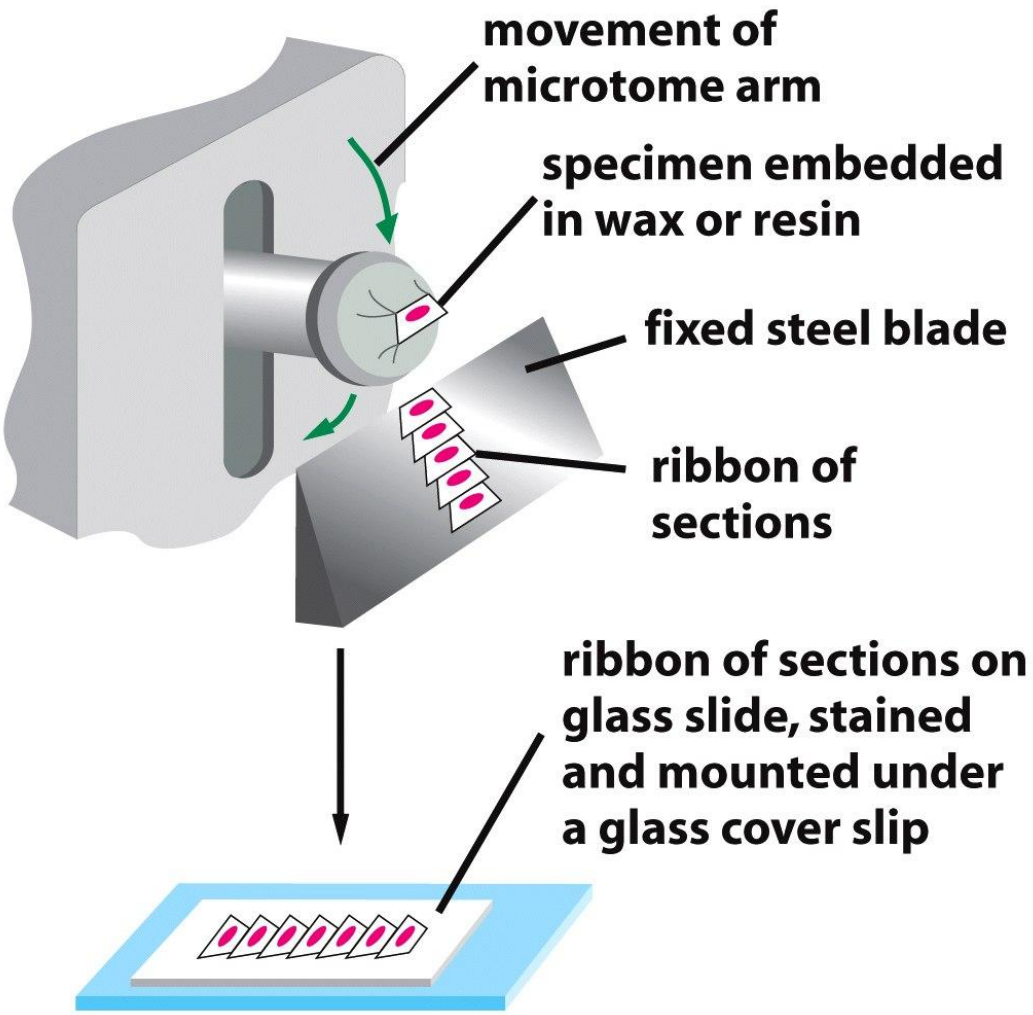
*it cannot perceive small differences in light intensity againist a bright background.

Intact Tissues Are Usually Fixed and Sectioned before Microscopy

- Most tissue samples are too thick for their individual cells to be examined directly at high resolution, they must be cut into very thin transparent slices,or sections.

- To first immobilize, kill, and preserve the cells within the tissue they must be treated with a fixatiue.
- Fixatives: formaldehyde and glutaraldehyde.
- Form covalent bonds with the free amino groups of proteins, cross-linking them so they are stabilized and locked into position.

Tissues are usually fixed and sectioned for microscopy



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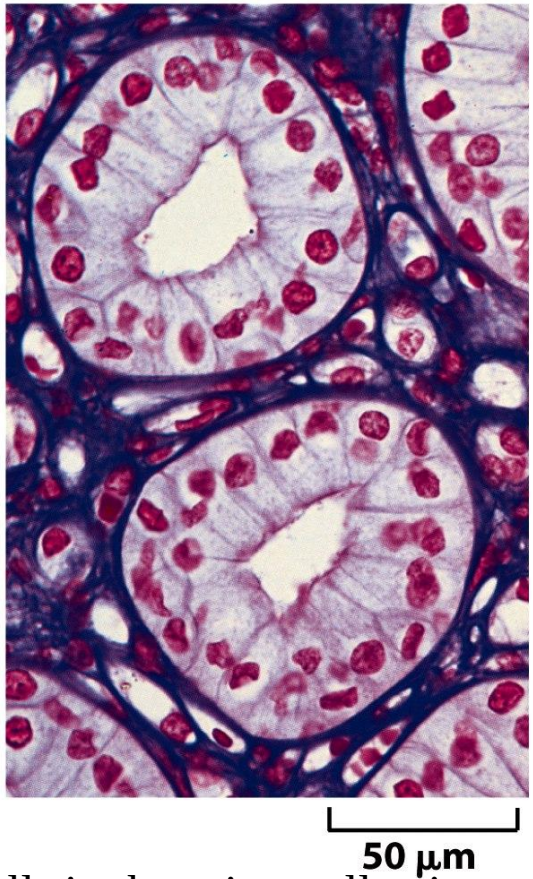
- Tissues are generally soft and fragile, even after fixation, they need to be embedded in a supporting medium before sectioning. The usual embedding media are waxes or resins.

•sections can be stained with organic dyes that have some specific affinity for particular subcellular components.

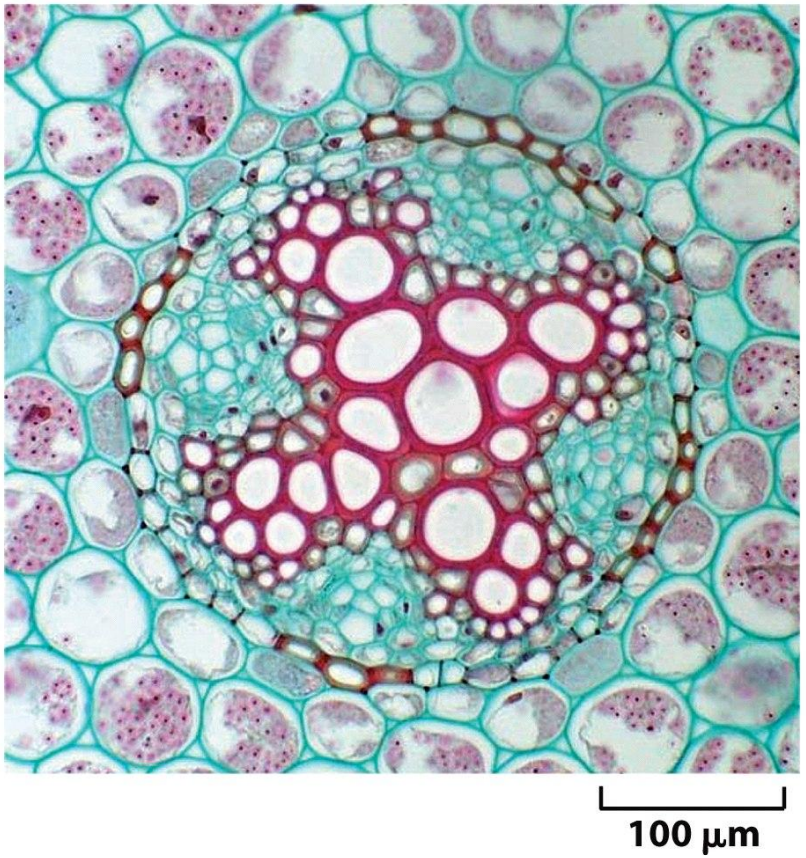
•The dye hematoxylin, has an affinity for negatively charged molecules and therefore reveals the distribution of DNA, RNA and acidic proteins in a cell .

- *Hematoxylin* binds to basic amino acids (lysine and arginine) on many different kinds of proteins
- *Eosin* binds to acidic molecules (such as DNA and side chains of aspartate and glutamate).
- Because of their different binding properties, these dyes stain various cell types sufficiently differently that they are distinguishable visually.

Different components of the cell can be selectively stained



Cells in the urine-collecting ducts of the kidney was stained with a combination of dyes, hematoxylin and eosin.



young plant root is
stained with two dyes, safranin and fast green

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Specific Molecules Can Be Located in Cells by Fluorescence Microscope

- 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.

- Two fluorescent dyes that have been commonly used for this purpose are;

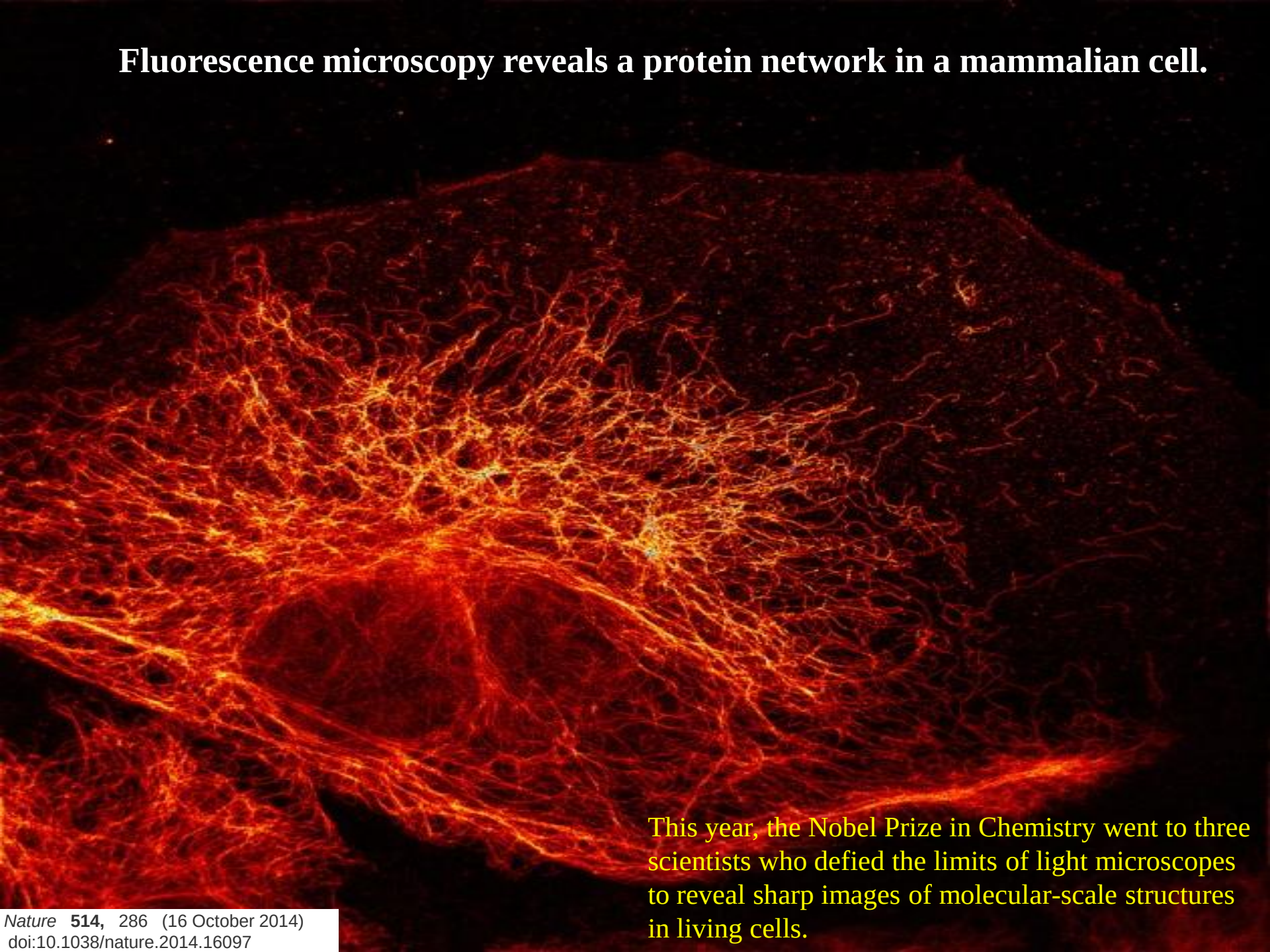
**fluorescein, which emits an intense green fluorescence when excited with blue light

** rhodamine, which emits a deep red fluorescence when excited with green- yellow light

- Many newer fluorescent dyes, such as Cy3, Cy5, and the Alexa dyes, have been specifically developed for fluorescence microscopy

Fluorescent Microscopy

Fluorescence microscopy reveals a protein network in a mammalian cell.



This year, the Nobel Prize in Chemistry went to three scientists who defied the limits of light microscopes to reveal sharp images of molecular-scale structures in living cells.

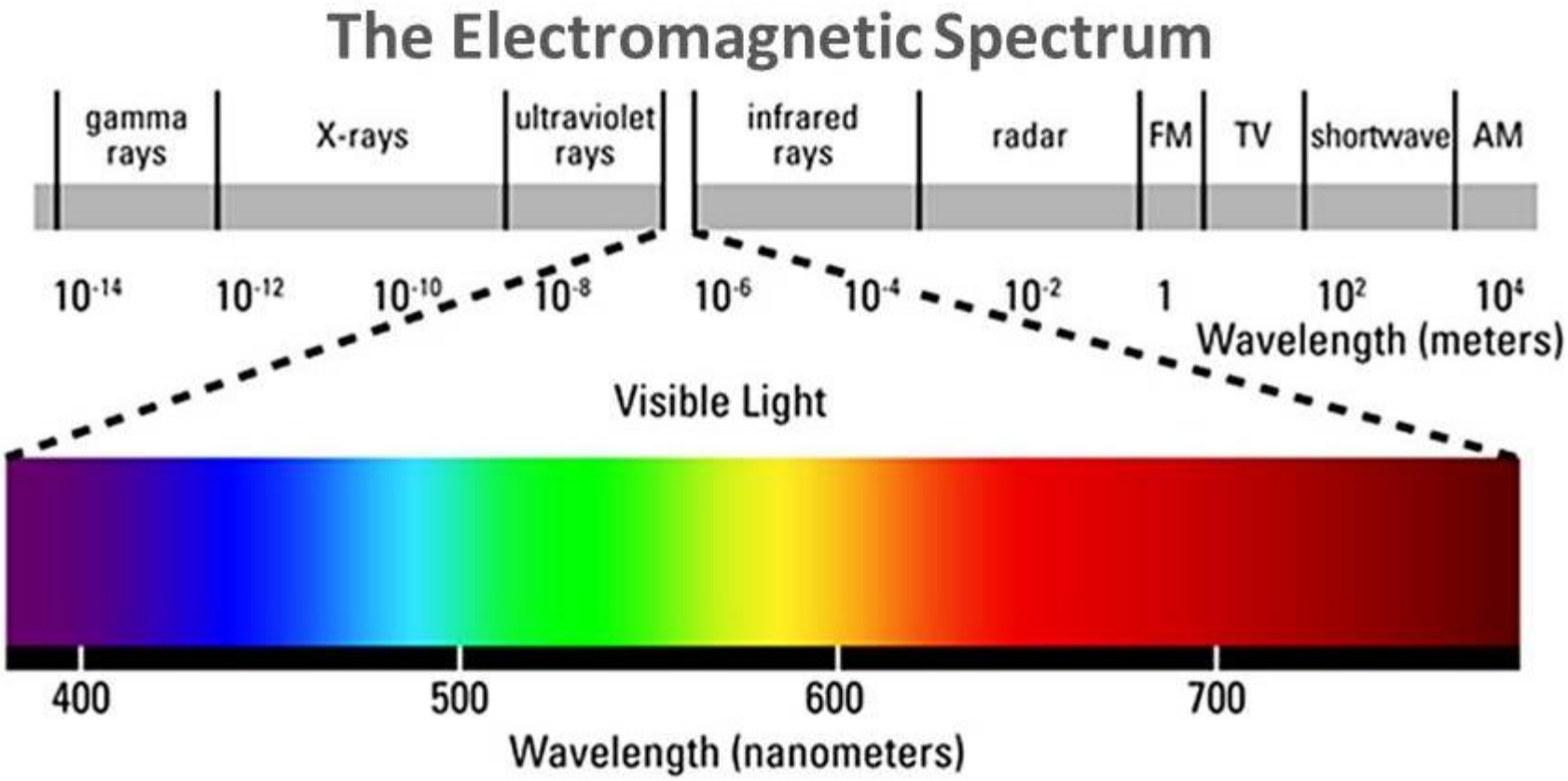
Nature **514,** 286 (16 October 2014)
doi:10.1038/nature.2014.16097

Fluorescence in situ hybridization (FISH)

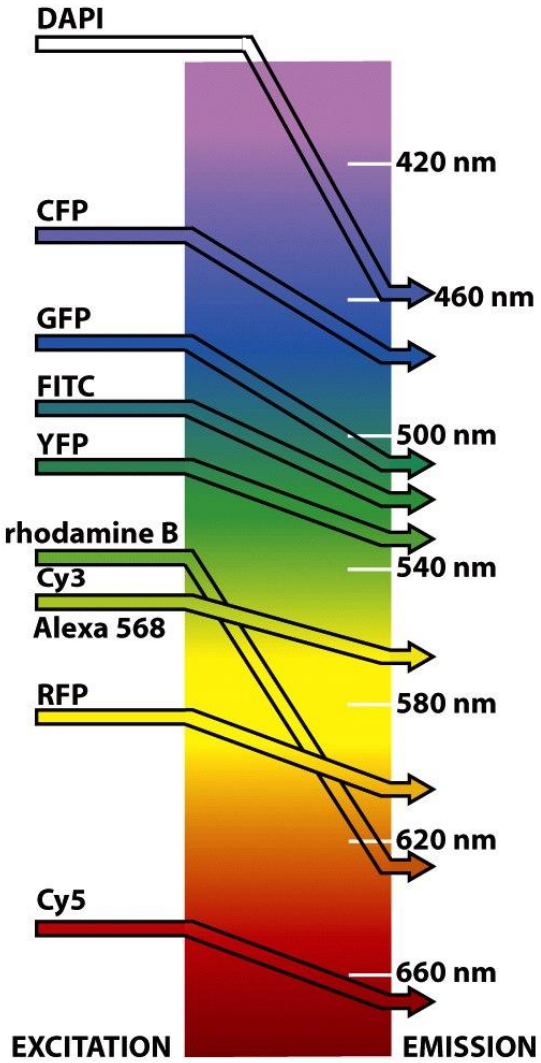
permits detection of selected acquired genetic changes in **dividing** (metaphase) and **nondividing** (interphase nuclei) cells

is useful in establishing the percentage of neoplastic cells at the time of diagnosis and after therapy

Light



Fluorescent probes



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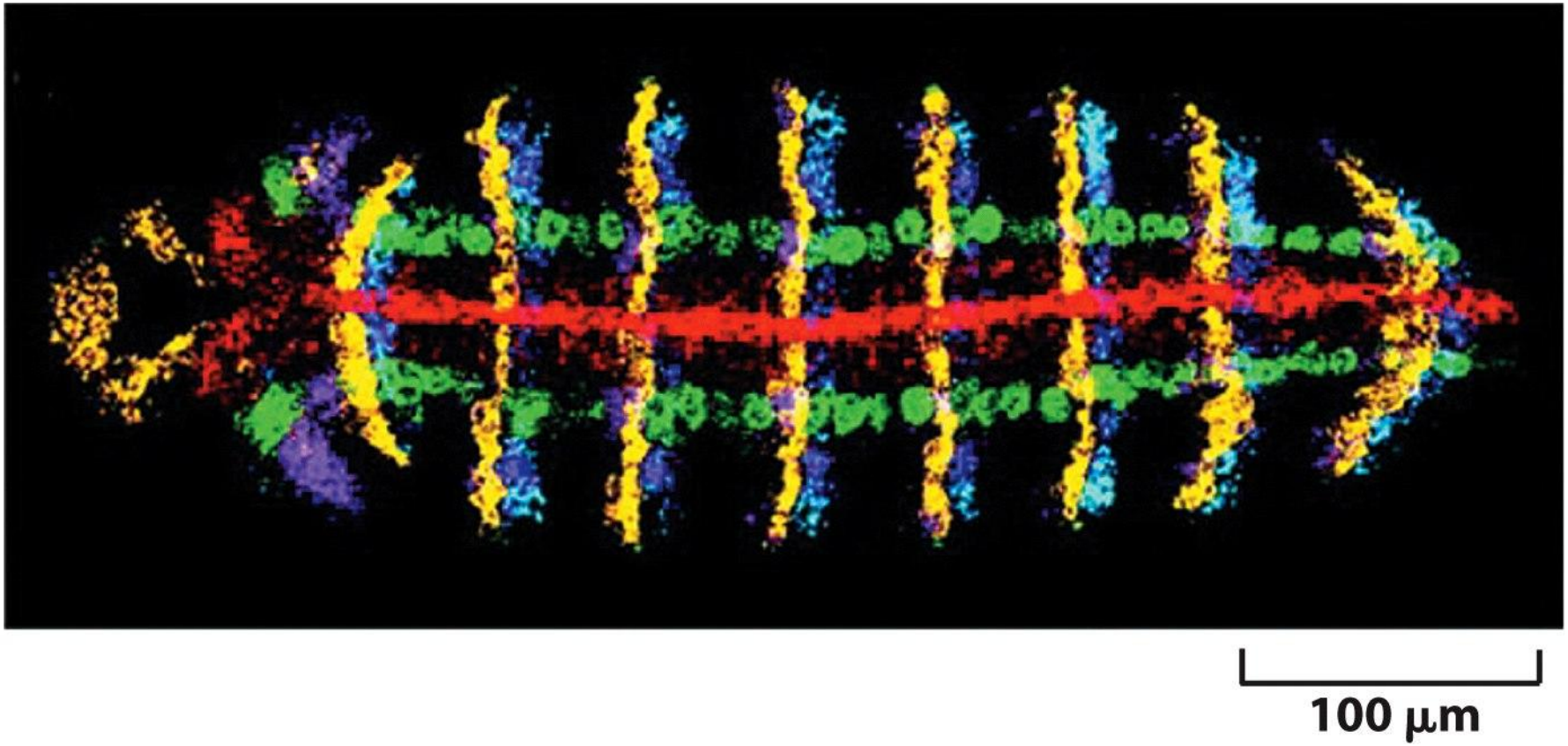
Sectioned tissue can be used to visualize specific patterns of differential gene expression



RNA *in situ* hybridization

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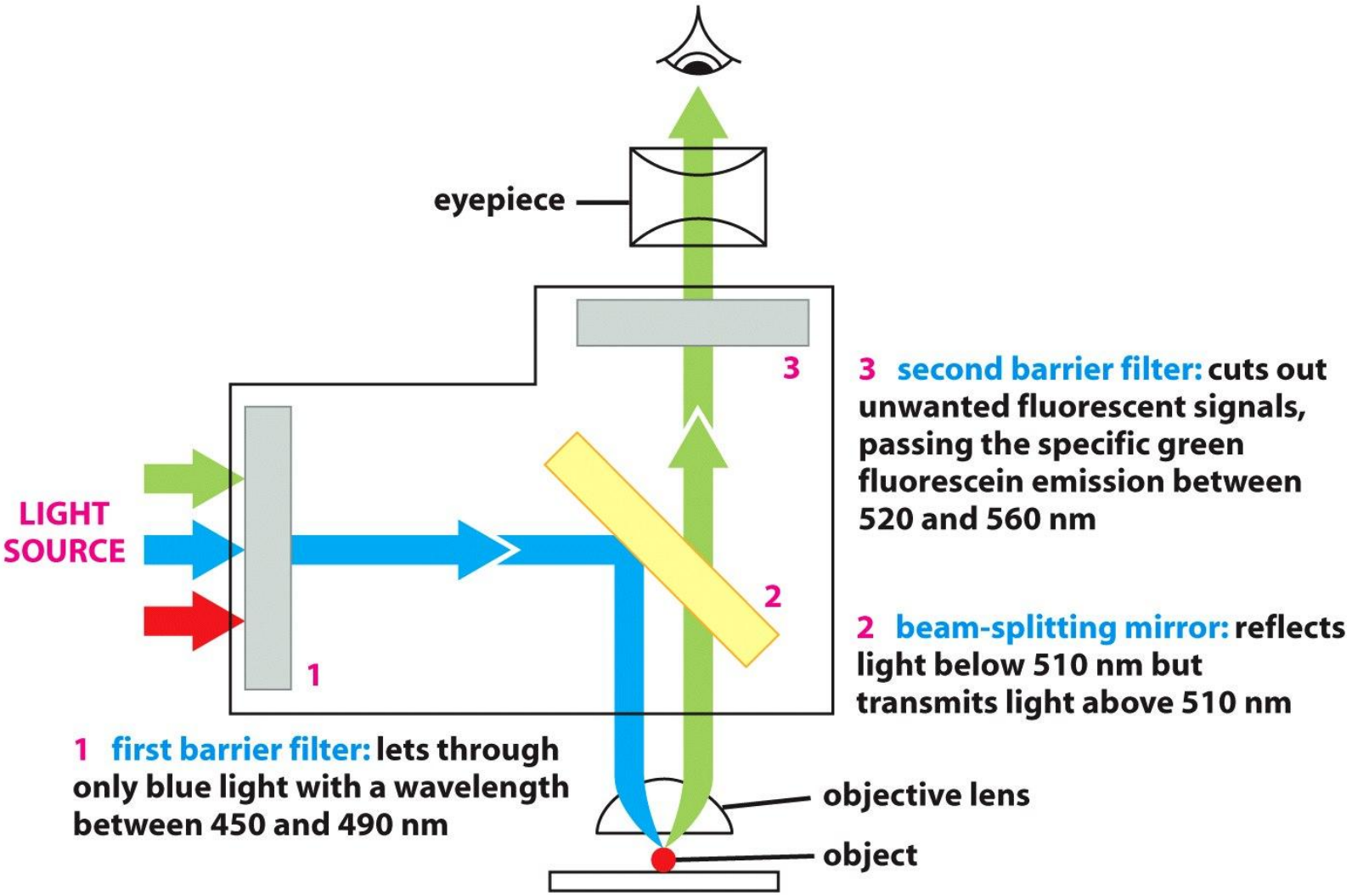
Specific molecules can be located in cells by fluorescence microscopy



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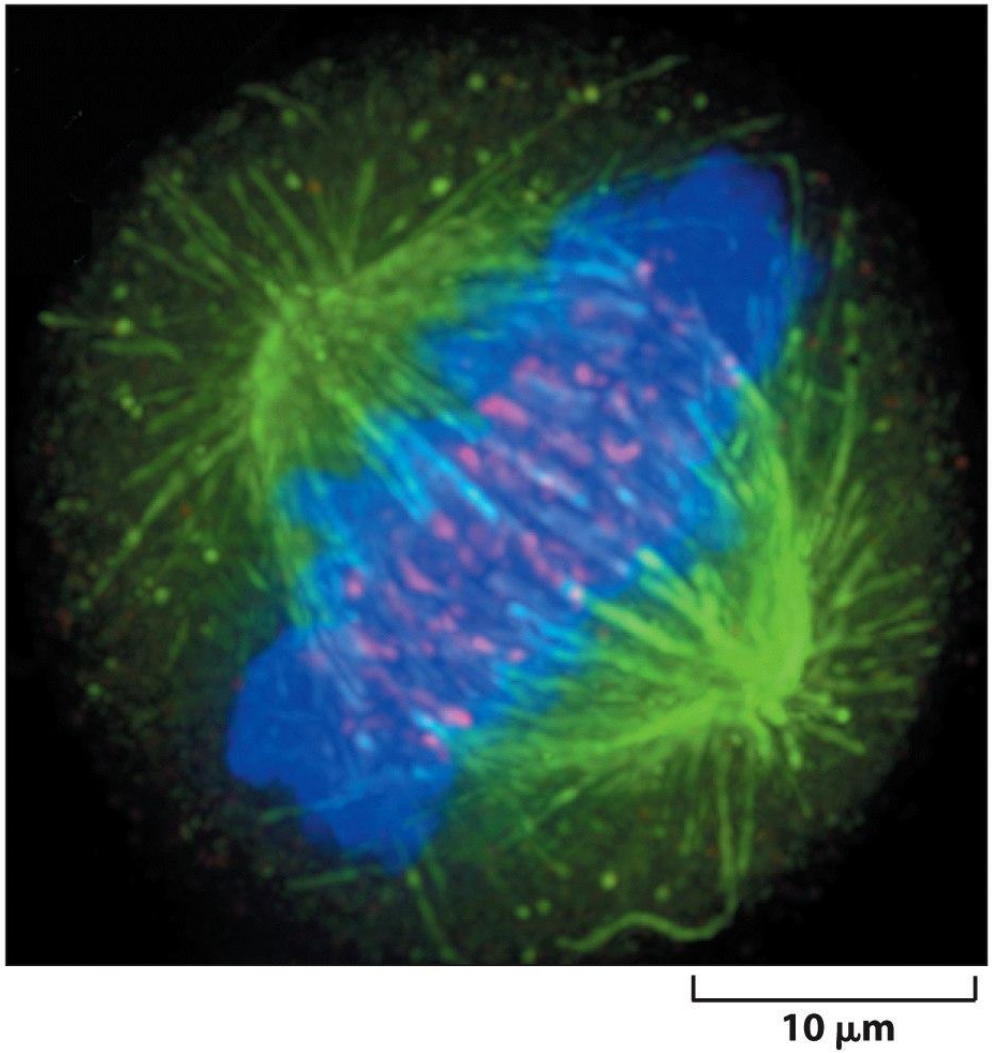
- Fluorescence microscopy methods, can be used to monitor changes in the concentration and location of specific molecules inside living cells
- Ex. GFP



Multiple-fluorescent-probe microscopy

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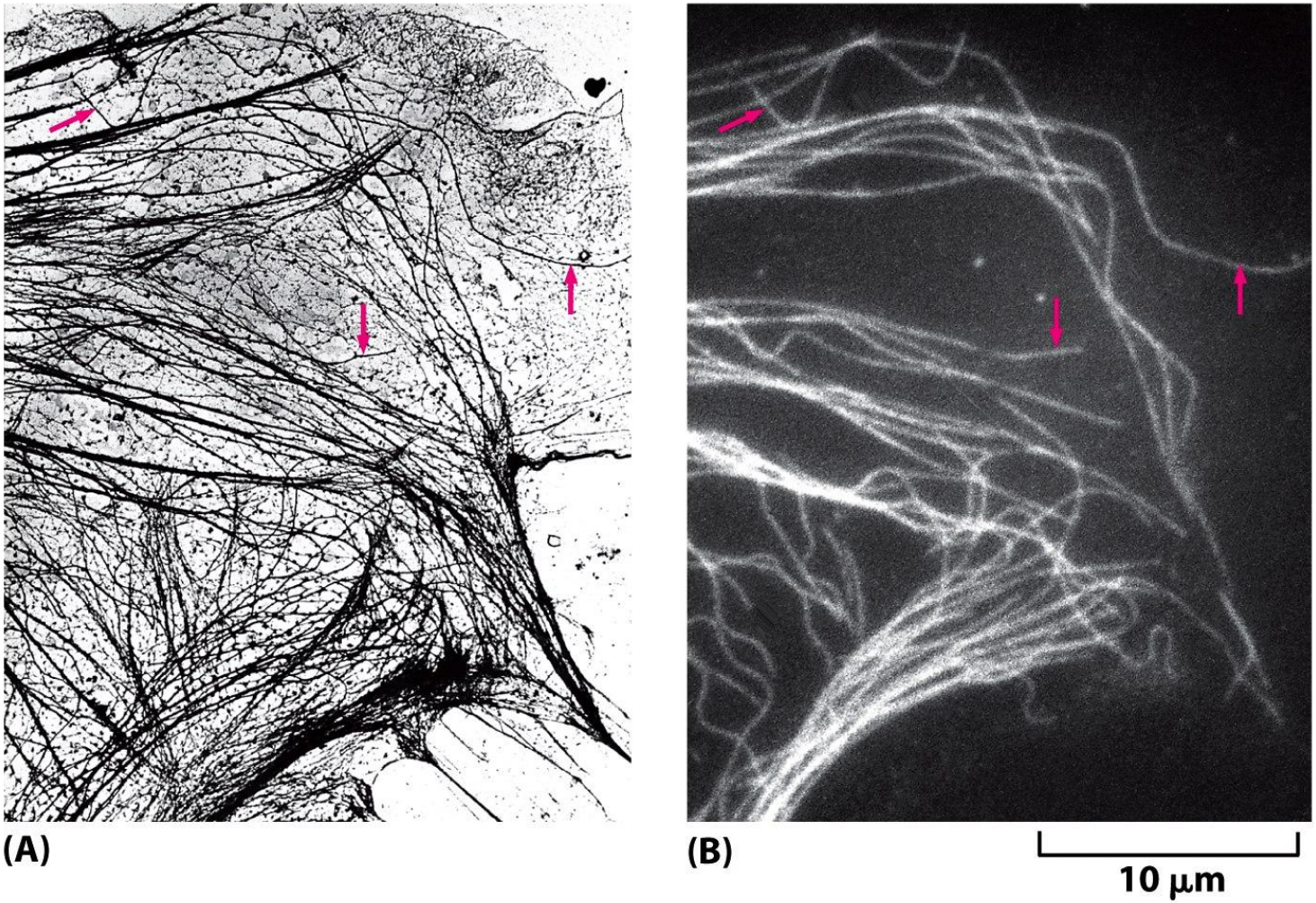
Antibodies Can Be Used to Detect Specific Molecules

- Antibodies are proteins produced by the vertebrate immune system as a defense
- against infection .
- They are unique among proteins because 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.

Antibodies can be used to detect specific molecules



Immunofluorescence

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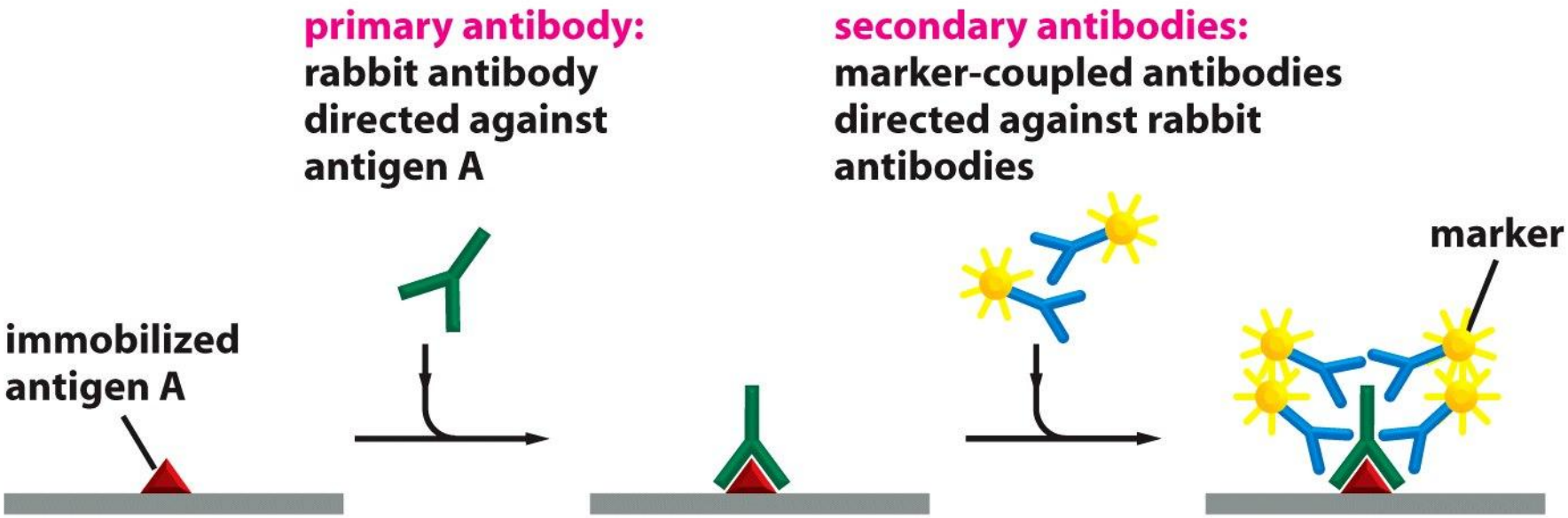

Indirect immunocytochemistry

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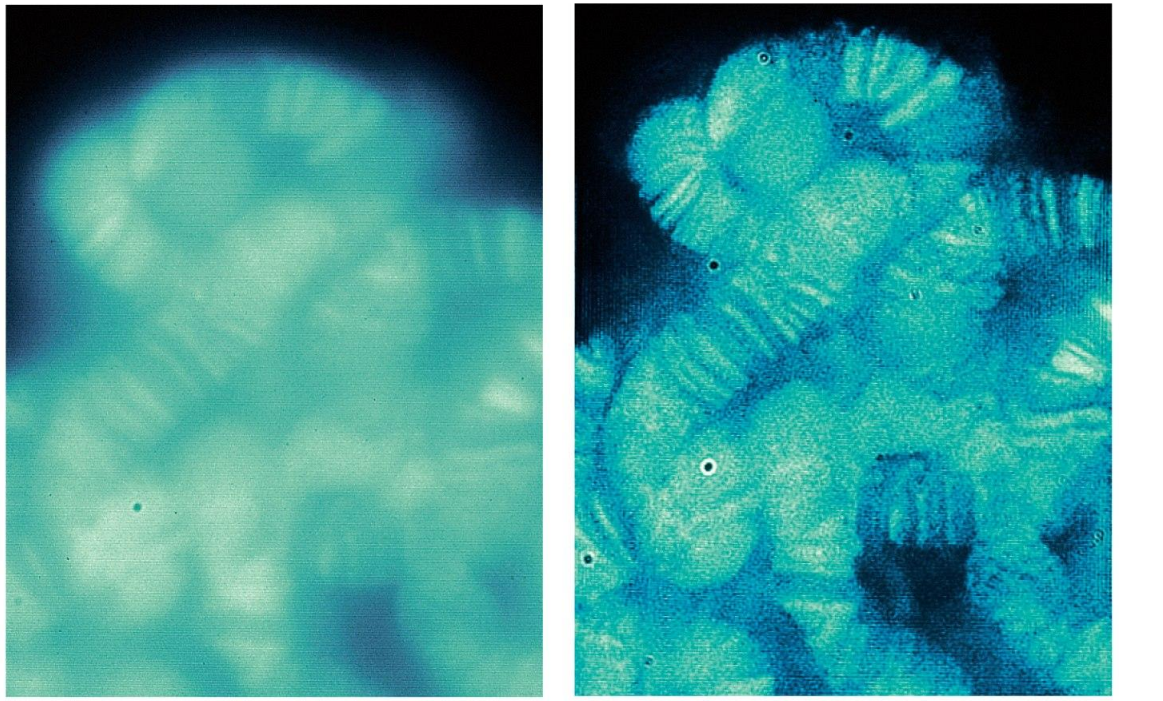
Imaging of Complex Three-Dimension Objects Is Possible it the Optical Microscope

Imaging of complex three-dimensional objects is possible with the optical microscope



(A) Light micrograph (B) After image deconvolution

Image deconvolution

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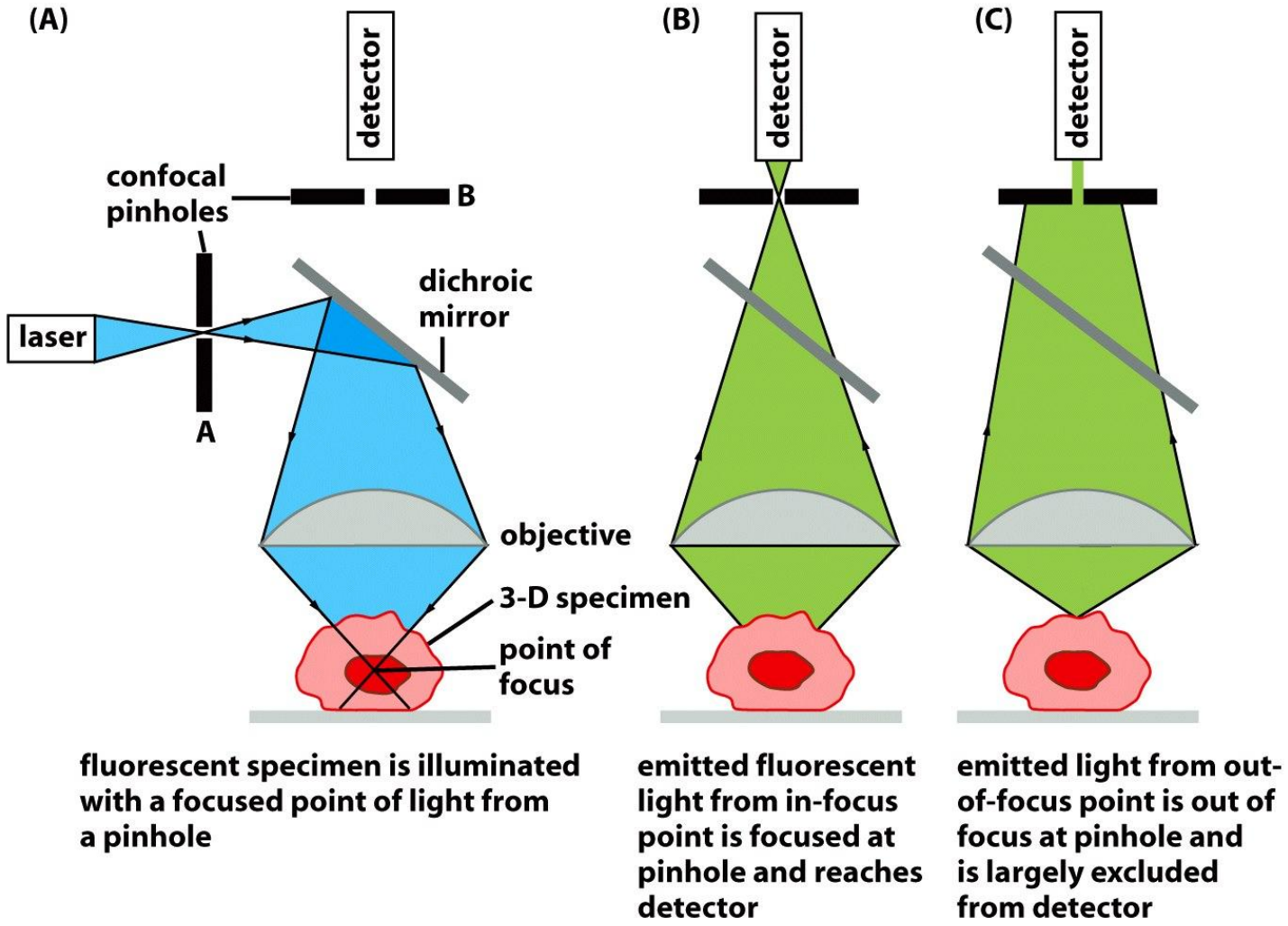
The confocal microscope produces optical sections by excluding out-of-focus light



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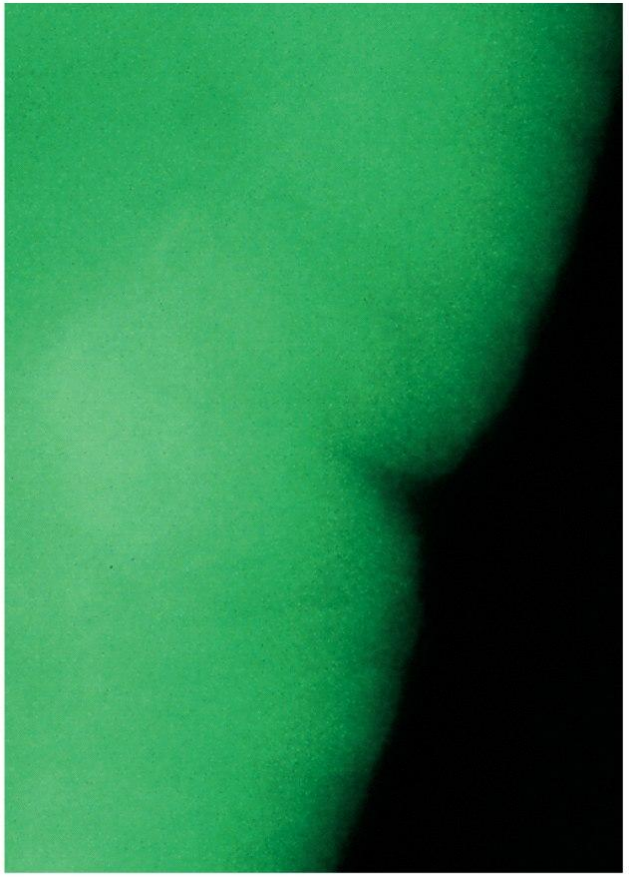
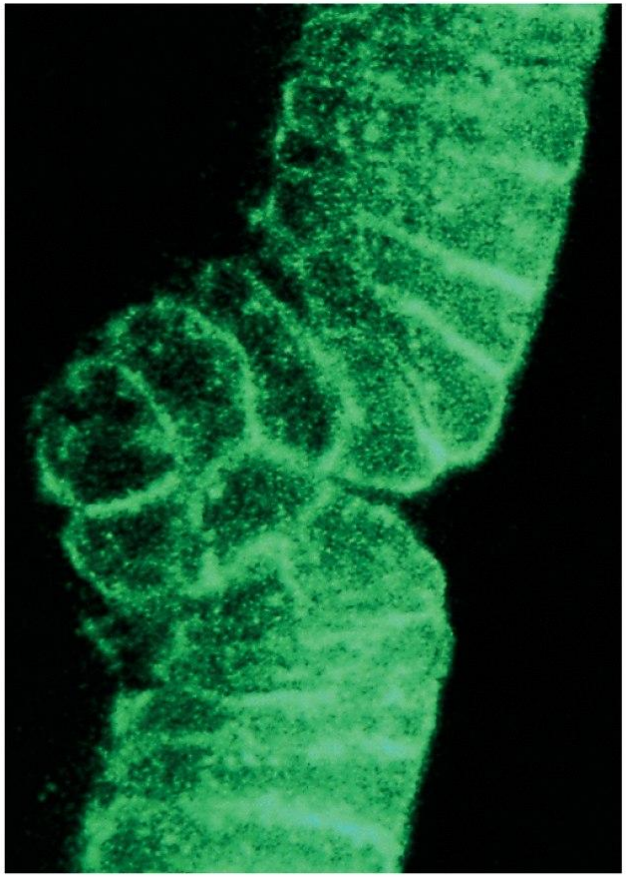
Comparison of conventional and confocal fluorescence microscopy



(A) The conventional unprocessed image



(B) In the confocal image, this out of-focus information is removed, resultingin a crispo pticals ectiono fthe cellsi n the embryo.

gastrula –stage of Drosophila embryo

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Confocal Microscopy

- Utilizes beams of ultraviolet light to excite fluorescent dye molecules.
- The exciting light is focused on the specimen with a thin optical fiber
- Resulting fluorescence is focused through a narrow aperture
- The light is detected and analyzed by a computer
- Very sharp focus
- For thick specimens an image is constructed in layers

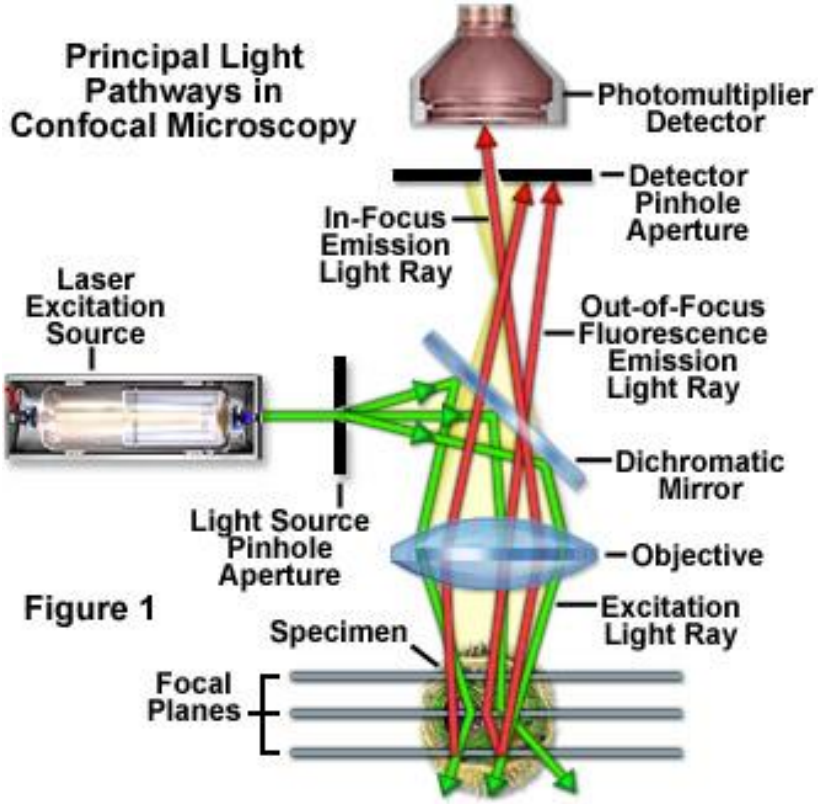


Figure 1

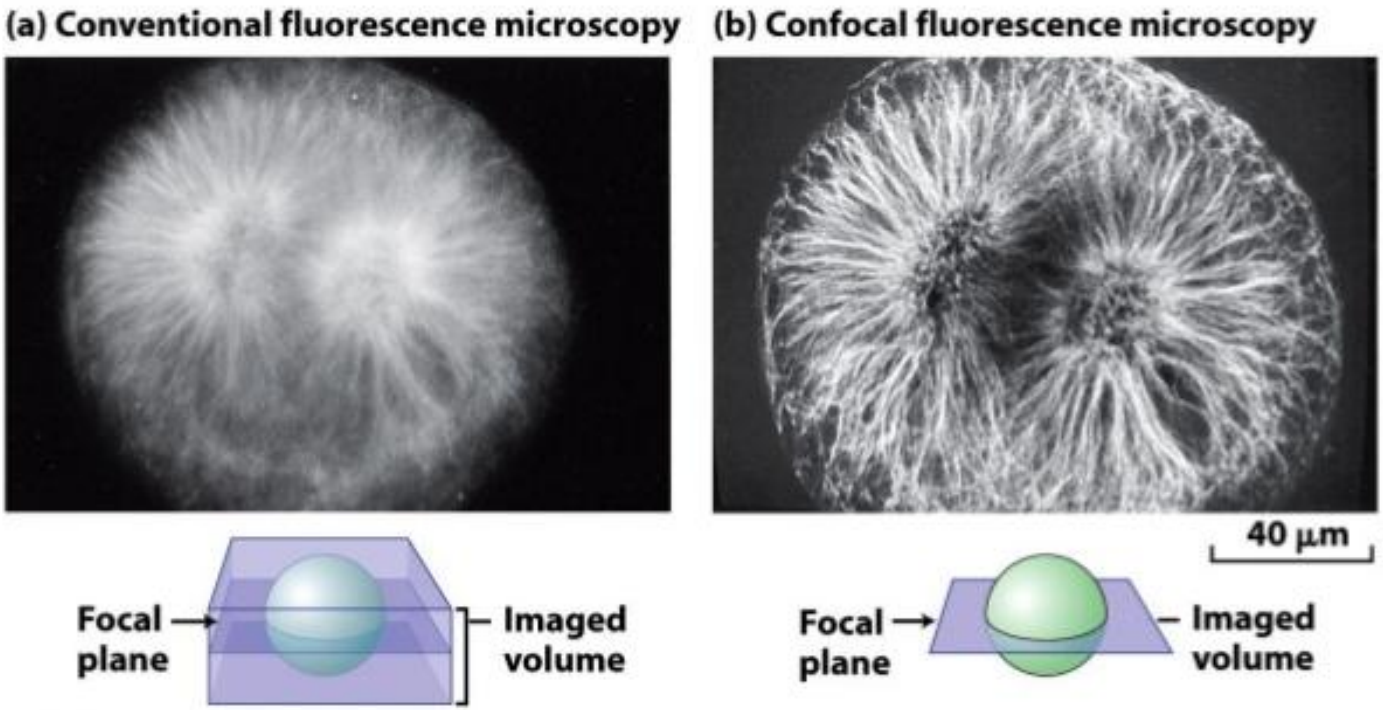
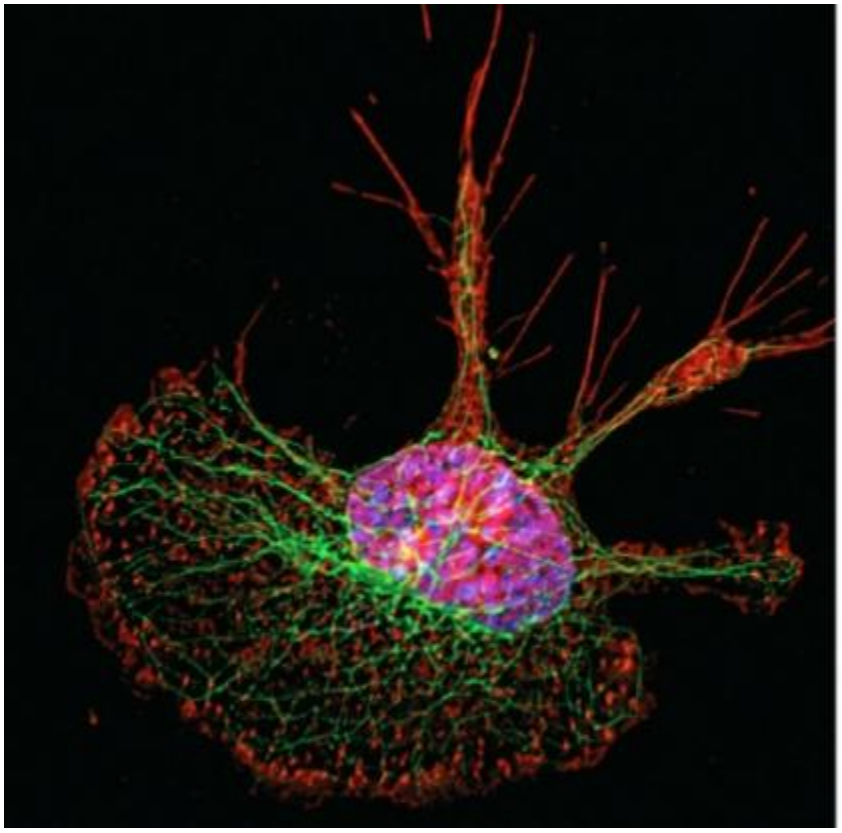


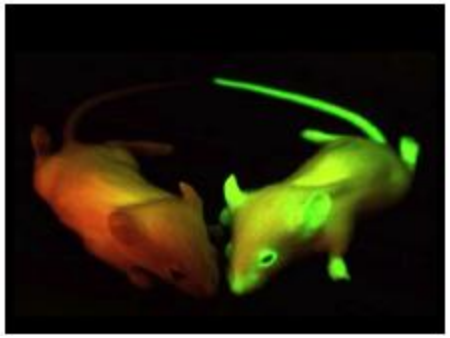
Figure 9-18
Molecular Cell Biology, Sixth Edition

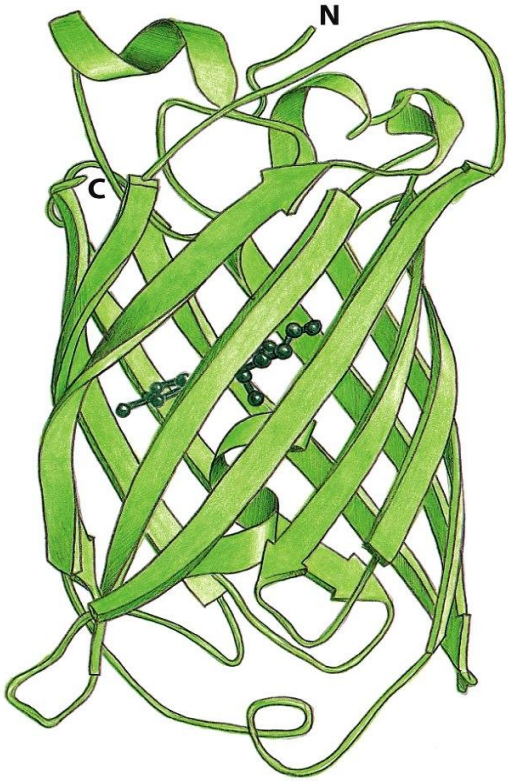
Lodish H, Berk A, Zipursky SL, et al. (2003).Molecular Cell Biology 5th edition



Lodish H, Berk A, Zipursky SL, et al. (2003).Molecular Cell Biology 5th edition

Fluorescent proteins can be used to tag individual proteins in living cells and organisms





The structure of GFP.

Green fluorescent protein (GFP) – the structure shows the eleven β strands that form the staves of a barrel. Buried within the barrel is the active chromophore that is formed post-translationally from the protruding side chains of three amino acid residues

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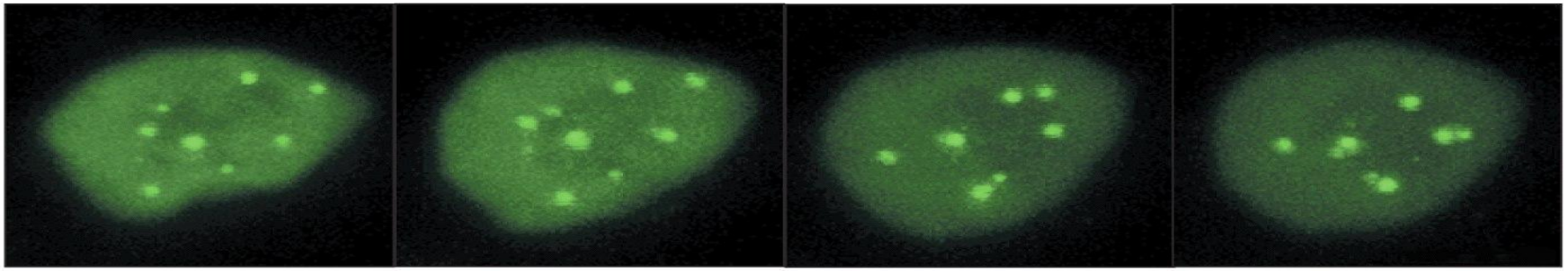
Isolated from the jellyfish *Aequoria Victoria*

This protein is encoded in the normal way by a single gene that can be cloned and introduced into cells of other species.

The freshly translated protein is not fluorescent, but within an hour or so it undergoes a self-catalyzed post-translational modification to generate an efficient and bright fluorescent center, shielded within the interior of a barrel-like protein.



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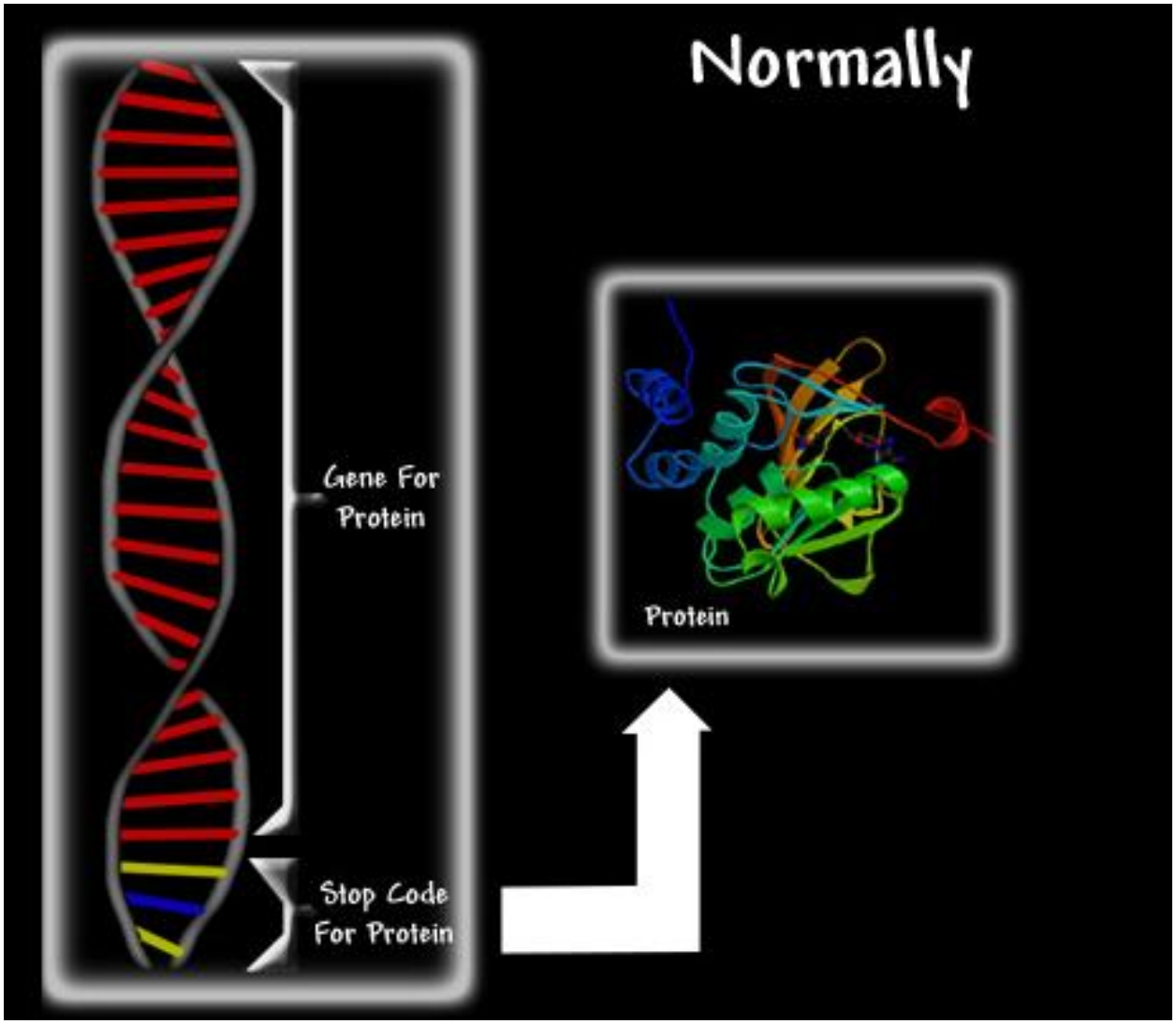
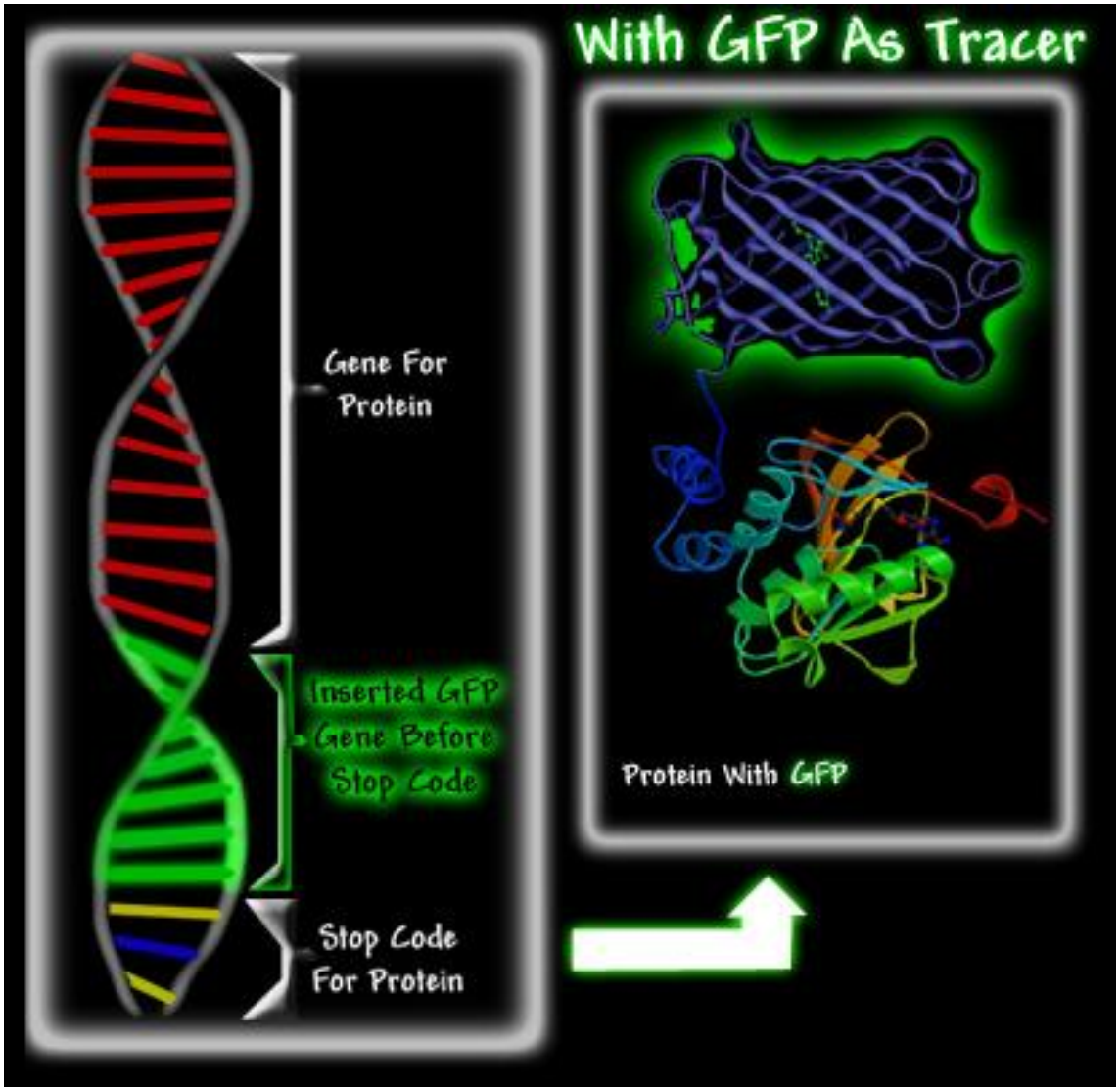
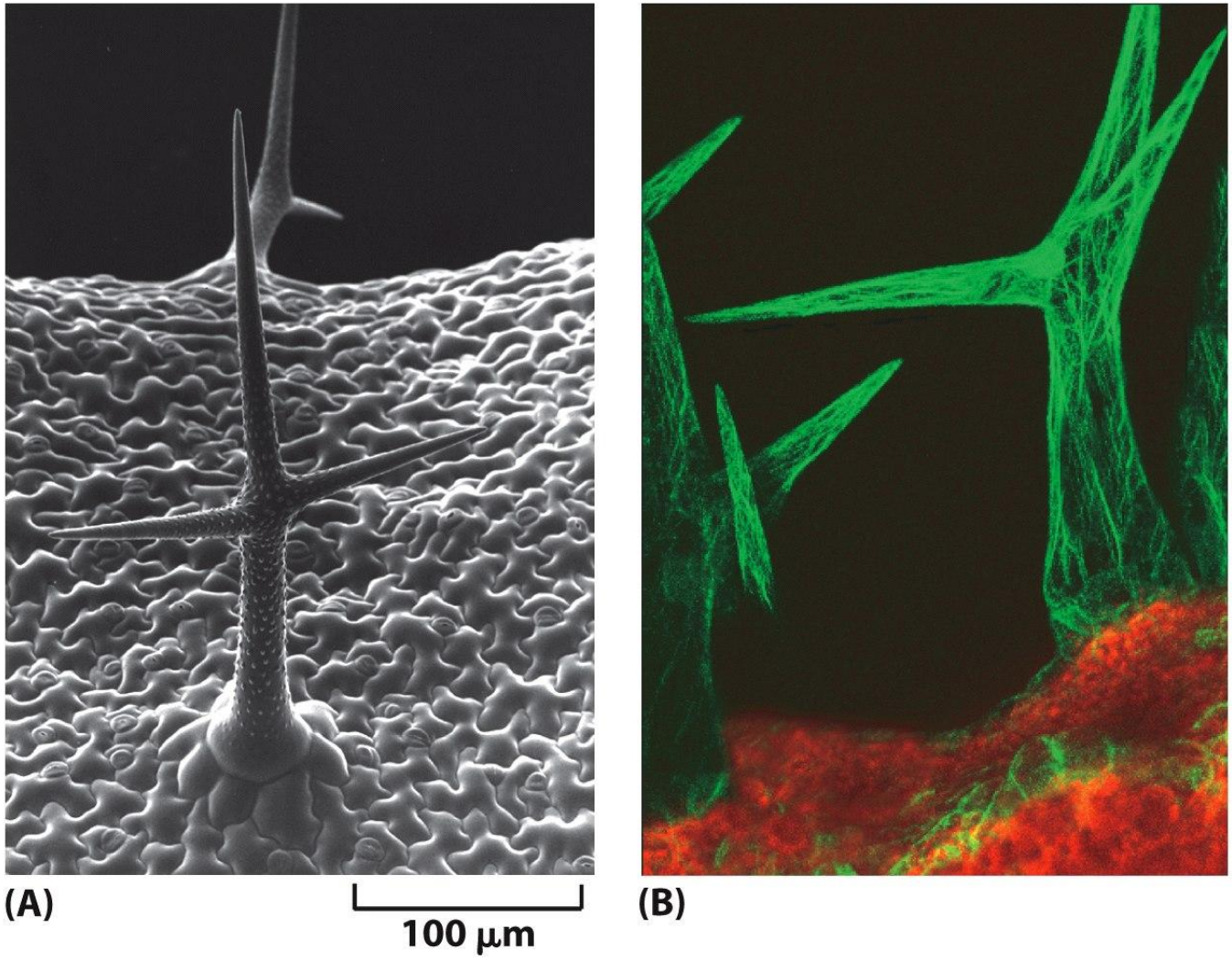
- GFP is used for as a reporter molecule, a fluorescent probe to monitor gene expression

• A peptide location signal can be added to the GFP to direct it to a particular cellular compartment, such as the endoplasmic reticulum or a mitochondrion, lighting up these organelles so they can be observed in the living state.


Normally
Gene For Protein
Stop Code For Protein
Protein


With GFP As Tracer
Gene For Protein
Inserted GFP Gene Before Stop Code
Stop Code For Protein
Protein With GFP



GFP-tagged proteins

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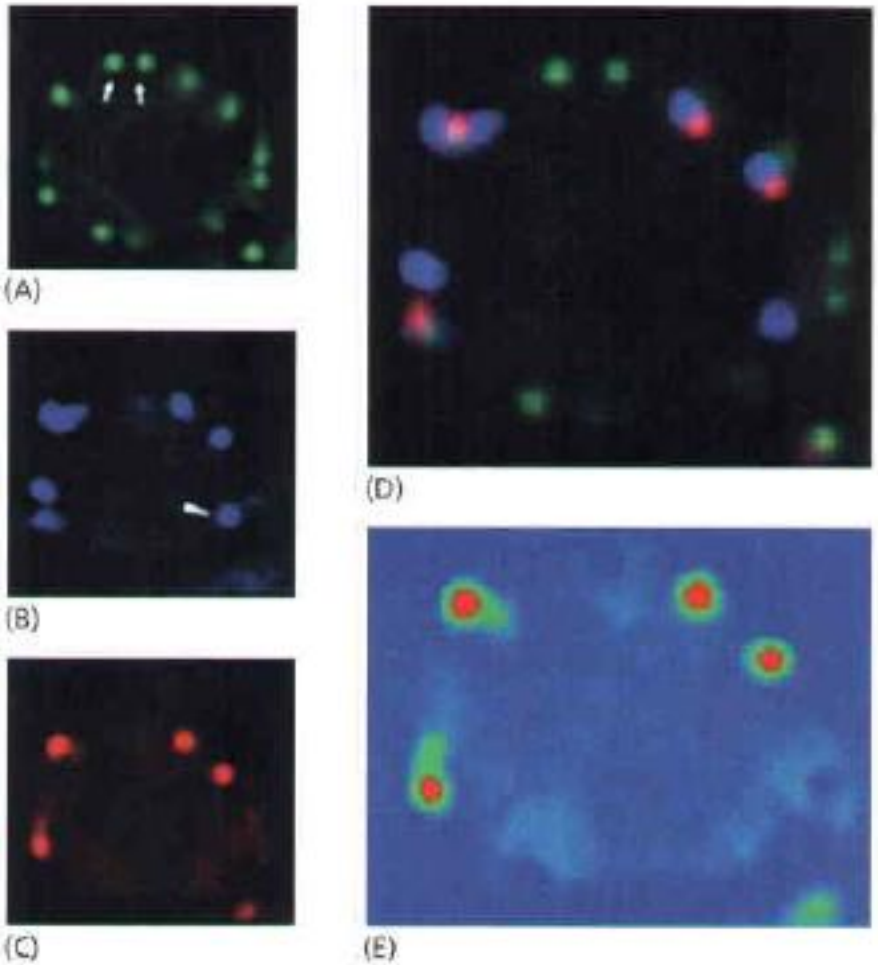
Protein dynamics can be followed in living cells

1- The monitoring of interactions between one protein and another by fluorescence resonance energy transfer (FRET).

FRET: In this technique, the two molecules of interest are each labeled with a different fluorochrome, chosen so that the emission spectrum of one fluorochrome overlaps with the absorption spectrum of the other. This method can be used with two different spectral variants of GFP as fluorochromes to monitor processes such as the interaction of signaling molecules with their receptors, or proteins in macromolecular complexes.



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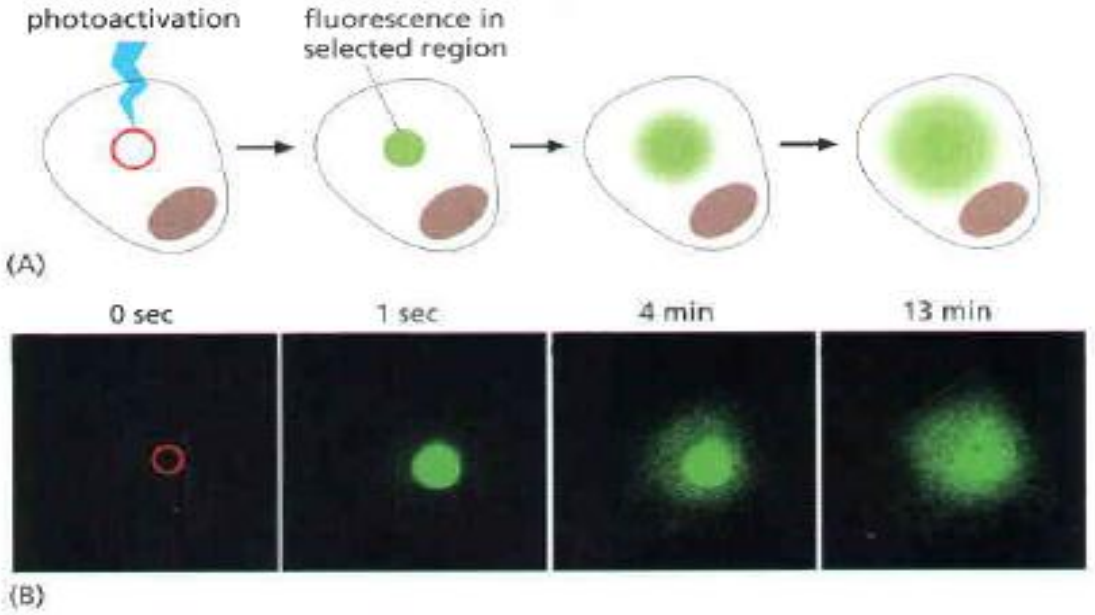
2-Photoactivation:Inactive photosensitive precursors of this type, often called caged molecules, have been made for many fluorescent molecules. A microscope can be used to focus a strong pulse of light from a laser on any tiny region of the cell, so that the experimenter can control exactly where and when the fluorescent molecule is photoactivated.



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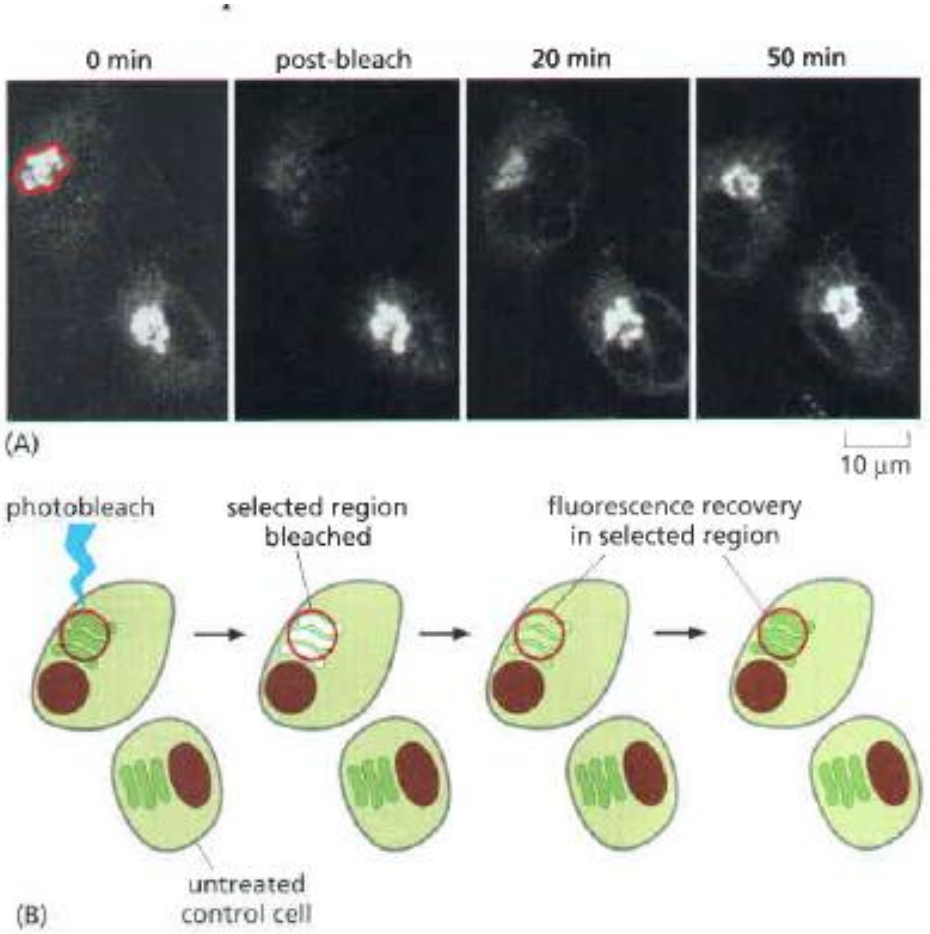
3- GFP fused to a protein of interest is to use a strong focussed beam of light from a laser to extinguish the GFP fluorescence in a specified region of the cell.

FRAP, Fluorescence recovery after photobleaching,



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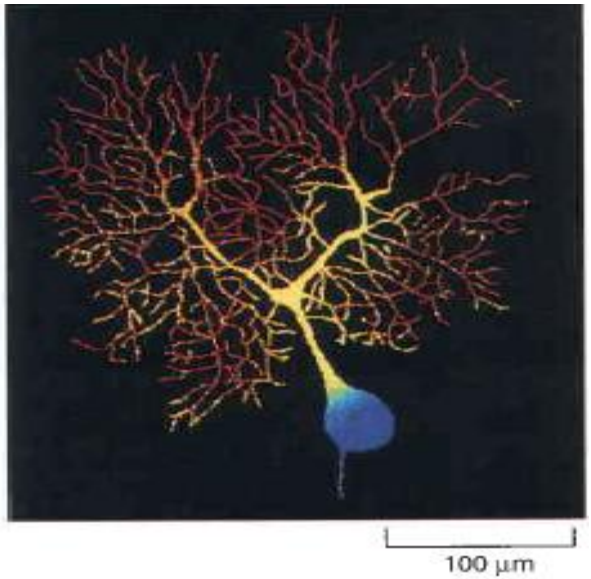
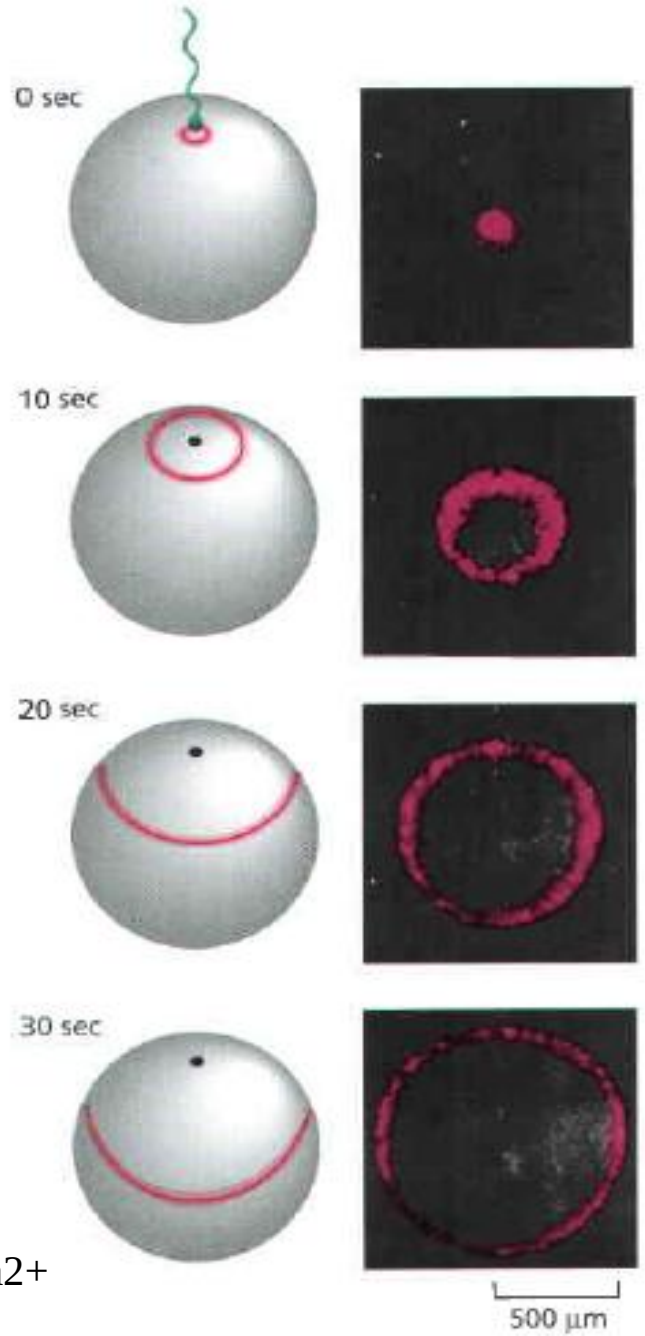
Light emitting indicators can measure rapidly changing intracellular ion concentrations

- To study the chemistry of a living cell is to insert the tip of a fine, glass,ion sensitive microelectrode directly into the cell interior through the plasma membrane.
- This technique used to measure the intracellular concentrariopns of common inorganic ions, such as H+,NA+,K+,Cl-,Ca2+.

- Aequorin , is aluminescent potein. İsolateed from a marine jellyfish.
- It emits the light in the presence of free Ca2+ and respond to changes in Ca2+ concentrarion in the range of 0,5-10μM.



Visualizing in tracellular Ca2+ Concentrations by using a Fluorescent indicator.



Egg of the medaka fish

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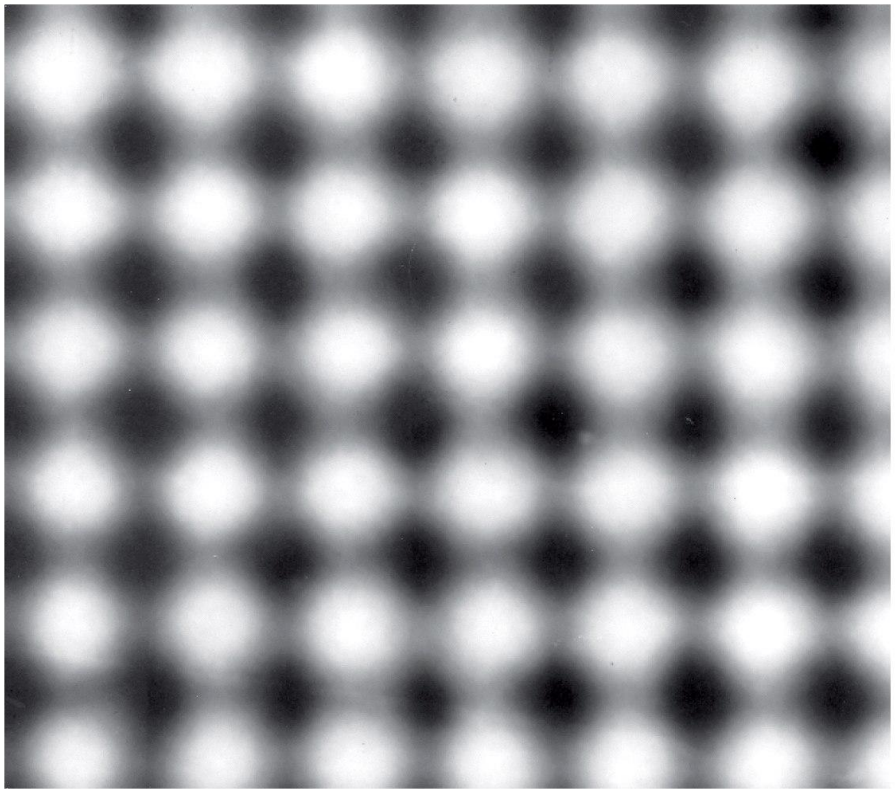
LOOKING CELLS AND MOLECULES IN THE ELECTRON MICROSCOPE

- The relationship between the limit of resolution and the wavelength of the illuminating radiation holds 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

The limit of resolution of the electron microscope



Individual files of gold atoms resolved by transmission electron microscope. The distance between adjacent files of gold atoms is about 0.2nm.

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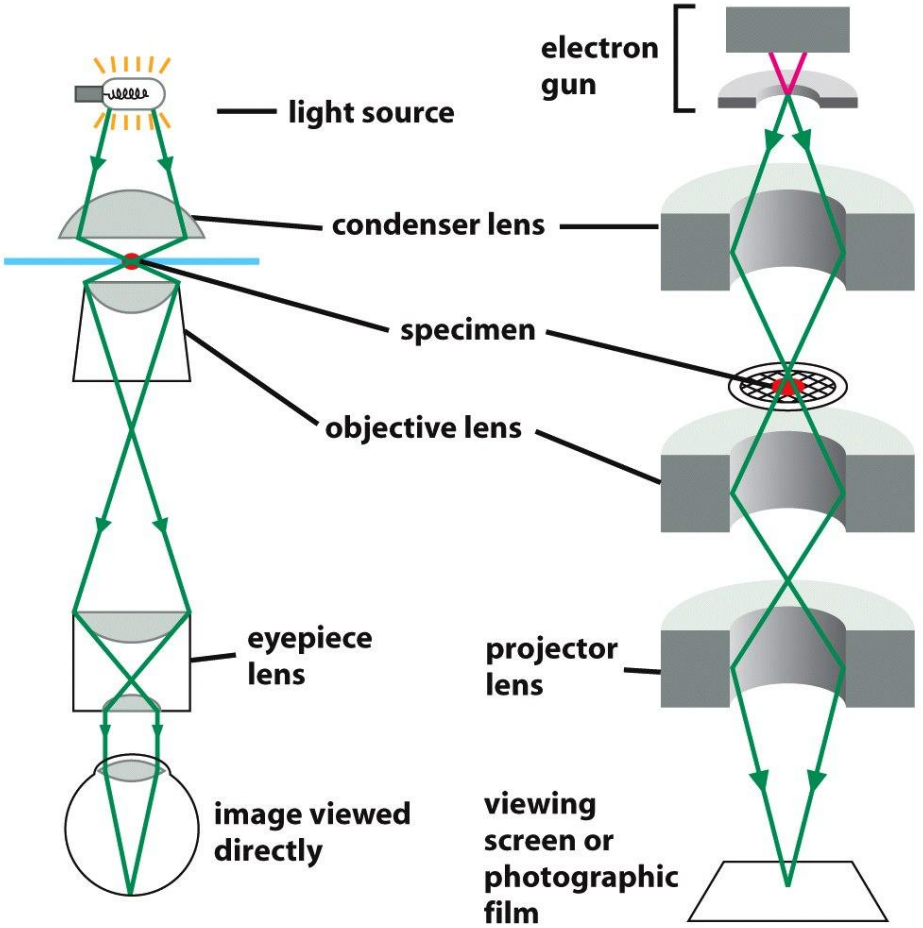
The principal features of a light microscope and a transmission electron microscope

- The lenses in the light microscope are made of glass, those in the electron microscope are a magnetic coils.
- The electron microscope requires that the specimen be placed in a vacuum.
- The inset shows a transmission electron microscope in use.



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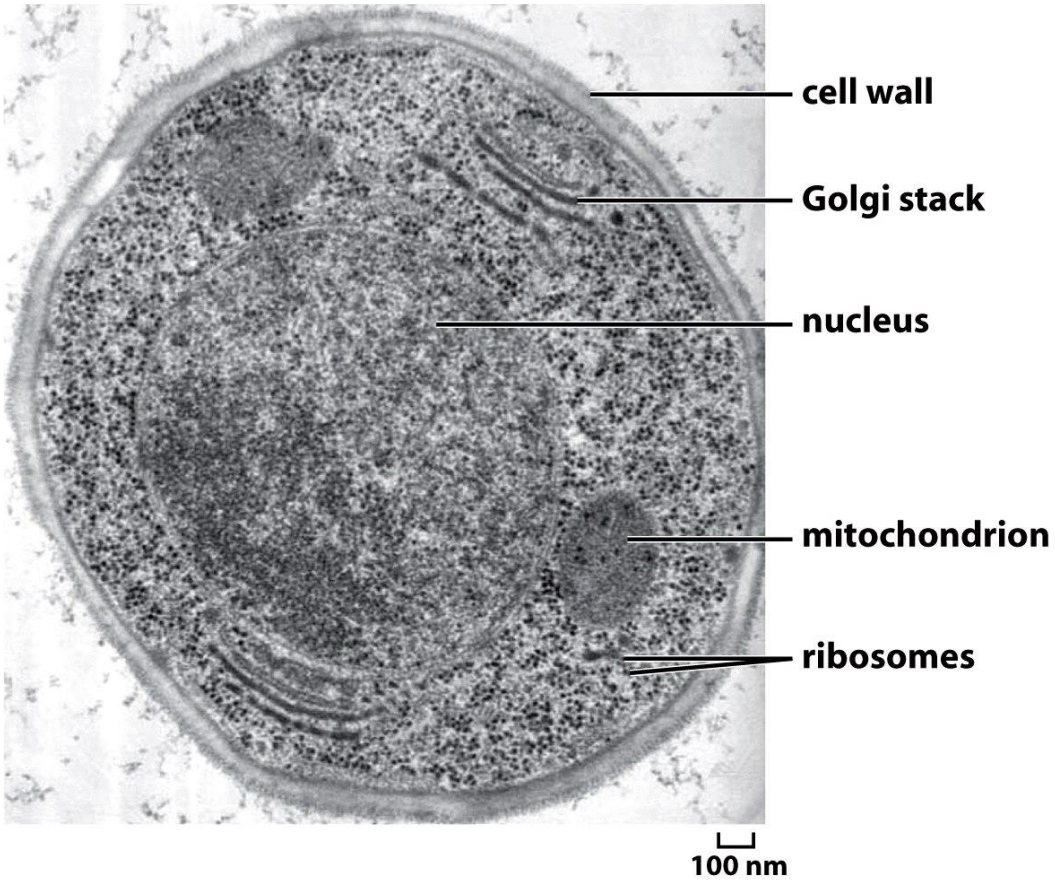
The electron microscope resolves the fine structure of the cell



Thin section of a yeast cell showing the nucleus, mitochondria, cell wall, Golgi stacks, and ribosomes in a state that is presumed to be as life-like as possible

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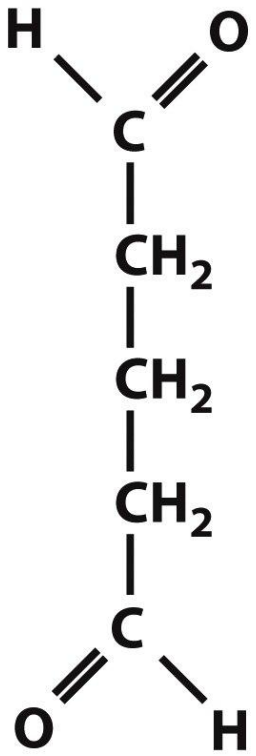
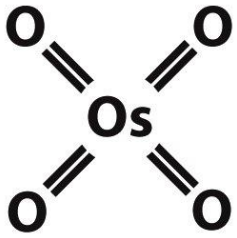
Biological specimens require special preparation for the electron microscope



glutaraldehyde



osmium tetroxide

Two common chemical fixatives used for electron microscopy

Optical vs. Electron Microscopy

Why electrons?

- Resolution
- Depth of Focus

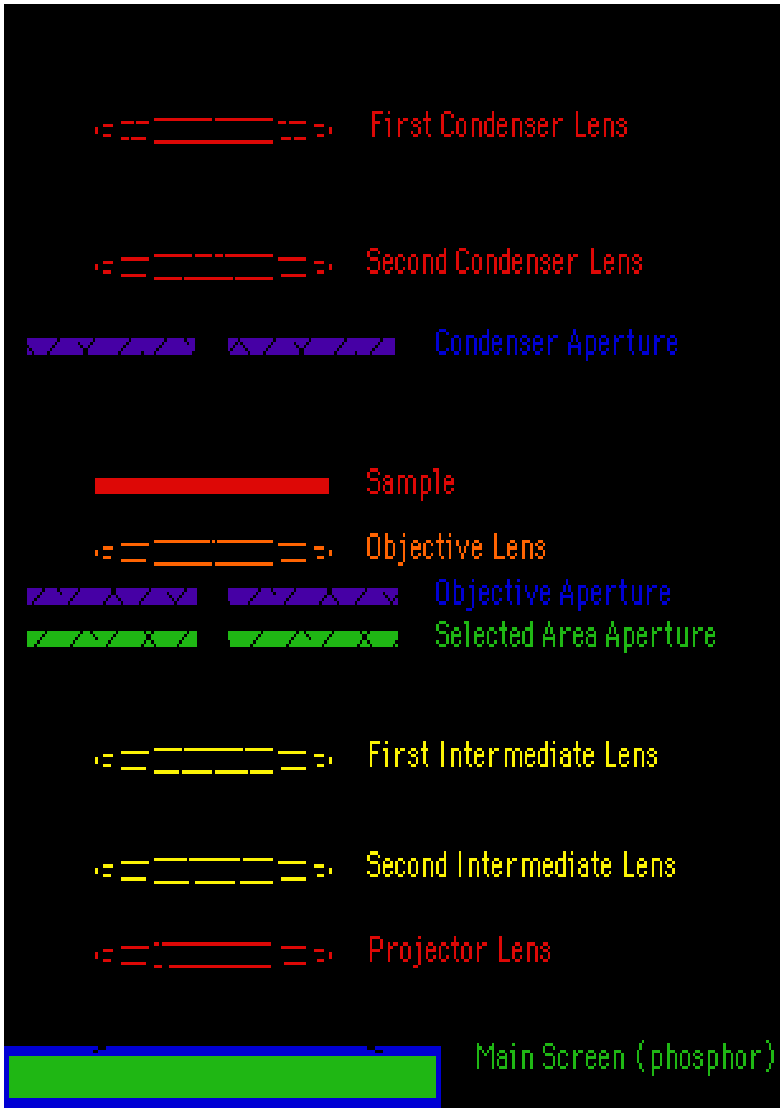
Resolution:

- ❑ Our eyes: 0.1-0.2 mm
- ❑ Optical microscope: 400-700nm, resolution?
- ❑ Electron microscope:100-1000 keV, resolution?

"The best possible resolution that can be resolved with a light microscope is about 2,000 Angstroms" --Slayter, Elizabeth. Microscope. *Grolier Multimedia Encyclopedia Online*. Grolier, 1998.

Transmission Electron Microscopy

- Electrons are used as the source of light
- Produced by a high voltage current running through a tungsten filament



Transmission Electron Microscope

- The lenses are electromagnetic
- They act on the negatively charged electrons to focus them in a concentrated path through the specimen
- The image is magnified by additional lenses and visualized on a screen

- In transmission electron microscopy (TEM), a beam of highly focused electrons are directed toward a thinned sample (<200 nm). Normally no scanning required --- helps the high resolution, compared to SEM.
- These highly energetic incident electrons interact with the atoms in the sample producing characteristic radiation and particles providing information for materials characterization.
- Information is obtained from both deflected and non-deflected transmitted electrons, backscattered and secondary electrons, and emitted photons.

TEM images

- Images produced display high resolution
- Staining with heavy metals that interact with the electrons
- Gradations of black, gray, and white contrast areas of greater density that absorb the stain

Advantages and Disadvantages of TEM

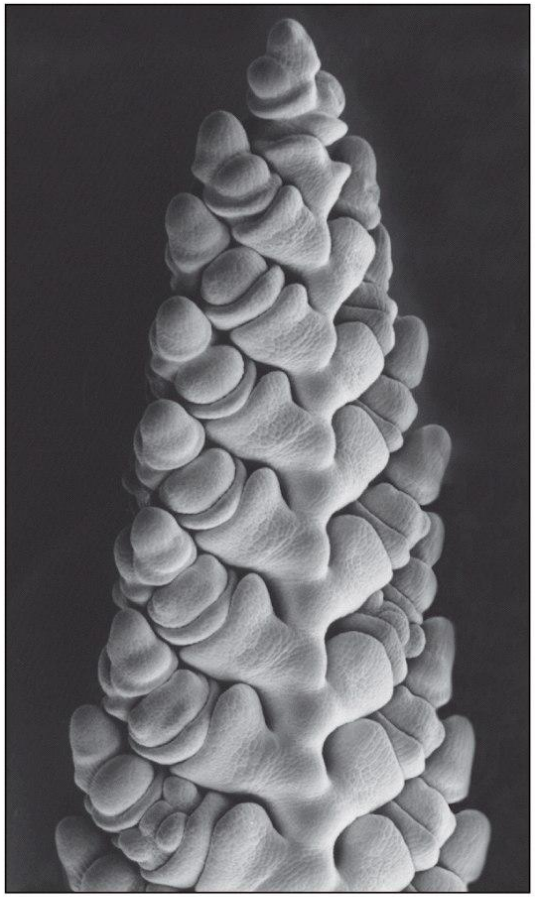
Advantages:

- High resolution, as small as 0.2 nm.
- Direct imaging of crystalline lattice.
- Delineate the defects inside the sample.
- No metallic stain-coating needed, thus convenient for strucutral imaging of organic materials,
- Electron diffraction technique: *phase identification, structure and symmetry determination, lattice parameter measurement, disorder and defect identification*

Disadvantages:

- To prepare an electron-transparent sample from the bulk is difficult (due to the conductivity or electron density, and sample thickness).

Images of surfaces can be obtained by scanning electron microscopy



•A scanning electron microscope (SEM) directly produces an image of the three dimensional structure of the surface of a specimen.

• The SEM is usually a smaller, simpler, and cheaper device than a transmission electron microscope

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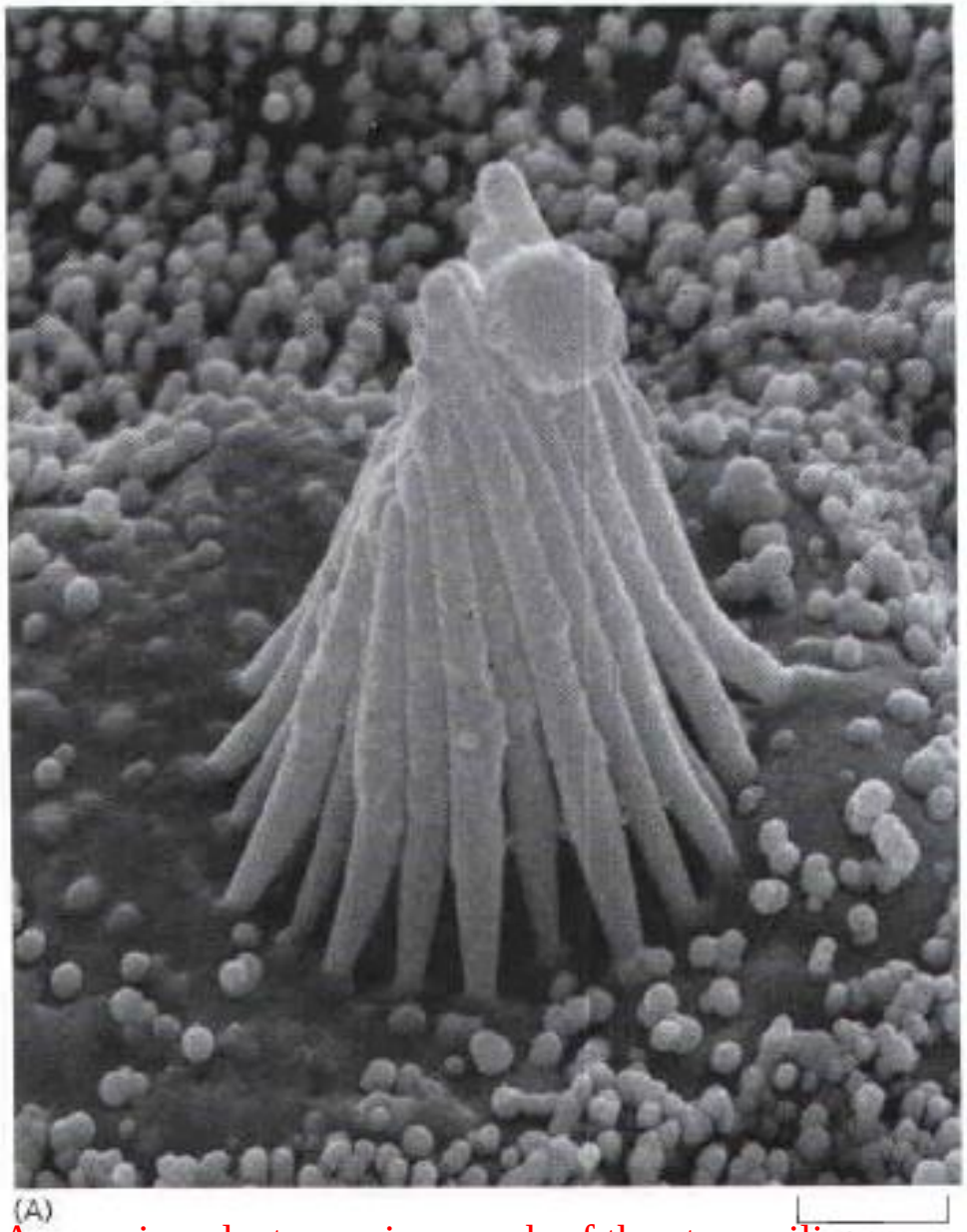
Scanning Electron Microscopy

- Electrons are reflected and collected off of the surface of a cell

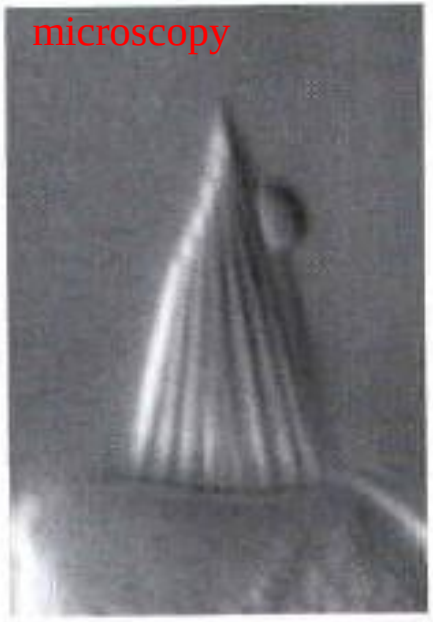
- Images show surface contours
- Three dimensional image

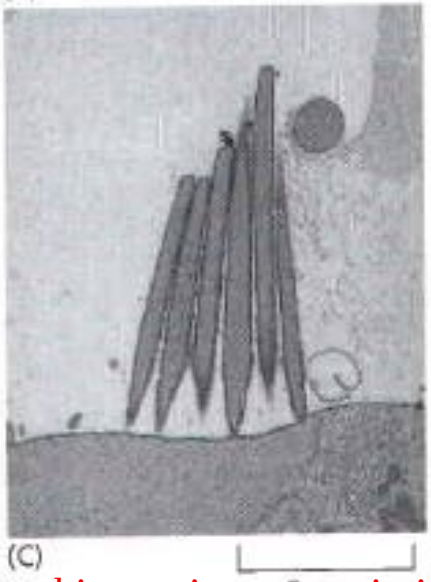
- In scanning electron microscopy (SEM) an electron beam is focused into a small probe and is rastered across the surface of a specimen.
- Several interactions with the sample that result in the emission of electrons or photons occur as the electrons penetrate the surface.
- These emitted particles can be collected with the appropriate detector to yield valuable information about the material.
- The most immediate result of observation in the scanning electron microscope is that it displays the shape of the sample.
- The resolution is determined by beam diameter



A scanning electron micrograph of the stereocilia projecting from a hair cell in the inner ear of a bullfrog

differential-interference-contrast light microscopy





thin-section transmission electron microscopy

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Advantages and Disadvantages of SEM

Advantages:

- Almost all kinds of samples, conducting and non-conducting (stain coating needed);
- Based on surface interaction --- no requirement of electron-transparent sample.
- Imaging at all directions through x-y-z (3D) rotation of sample.

Disadvantages:

- Low resolution, usually above a few tens of nanometers.
- Usually required surface stain-coating with metals for electron conducting.