Lecture 2

Visualization of cells

Outline

1. Light microscope
2. Fluorescence microscope
3. Confocal microscope
4. nanoscope
5. FRAP/FRET/Photoacivation
6. Electron microscope

Microscopes

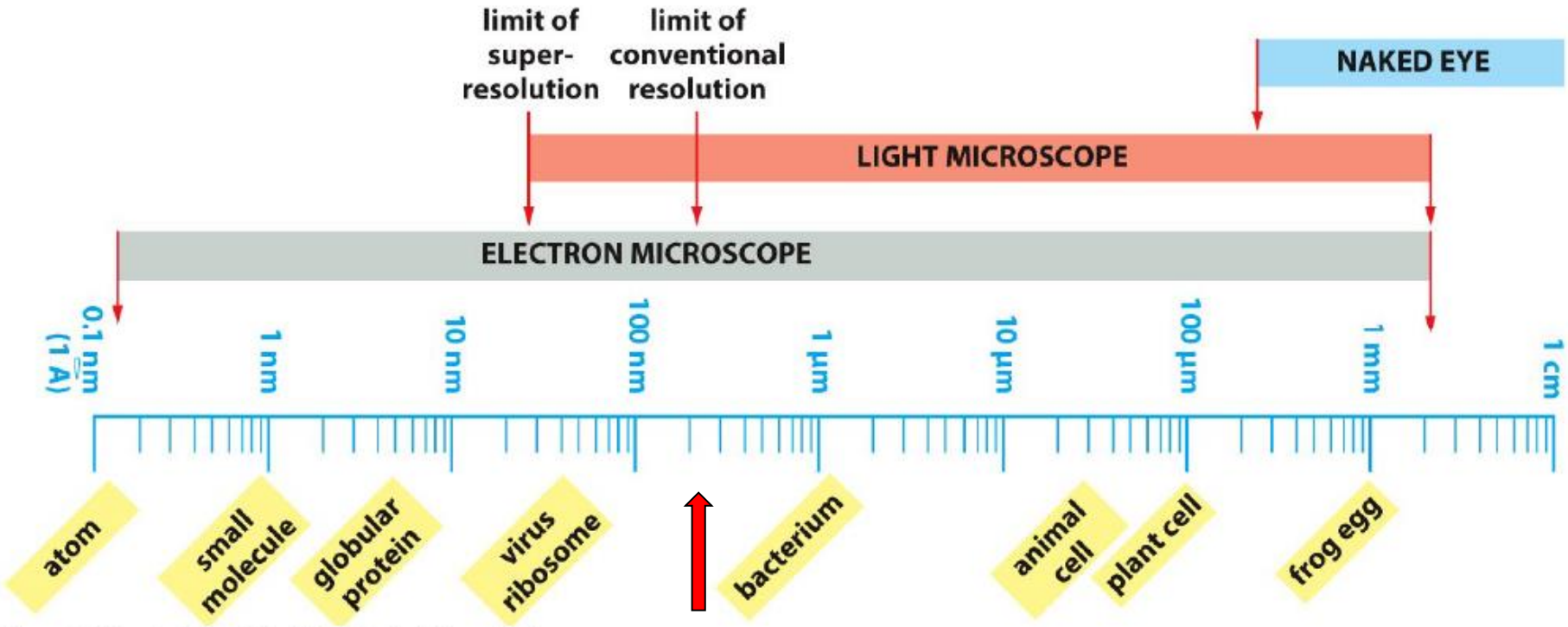


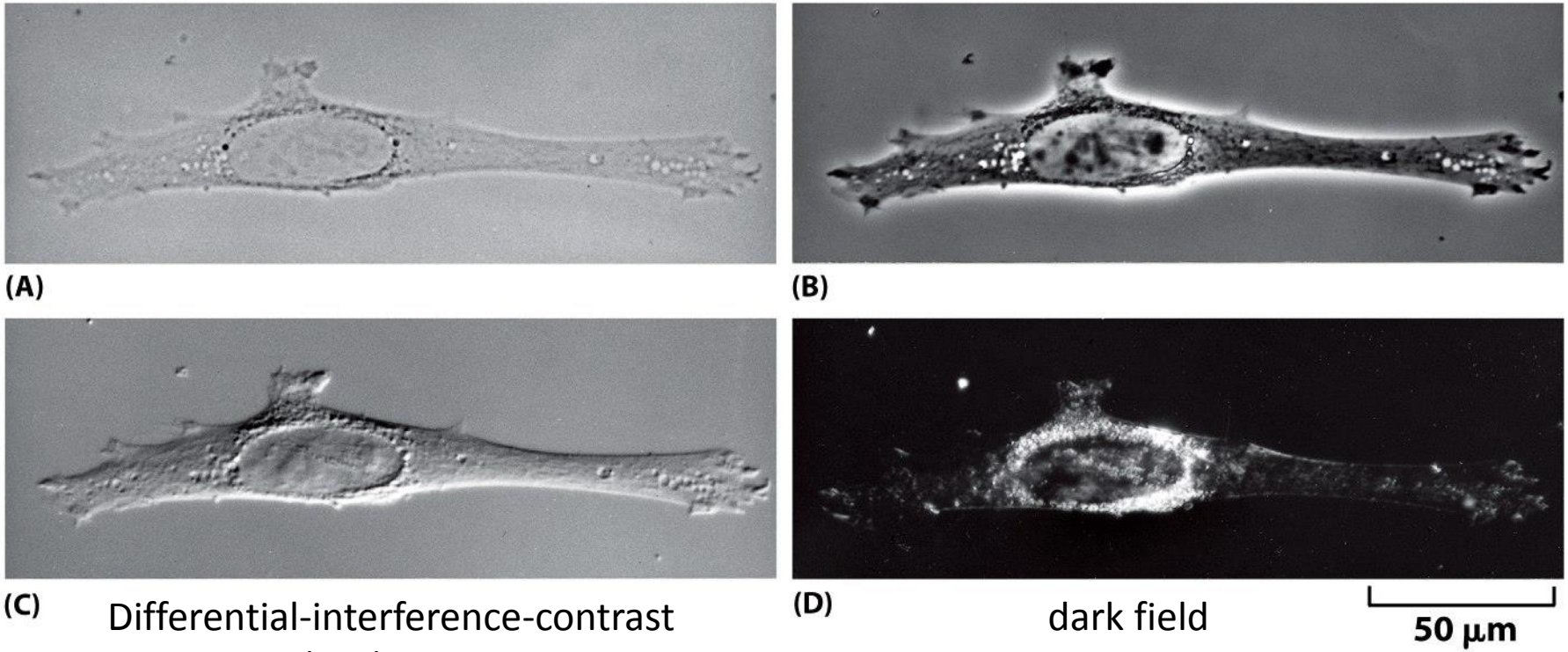
Figure 9-2 Molecular Biology of the Cell 6e (© Garland Science 2015)

0.2µm

1. Light microscope

Bright field

Phase contrast

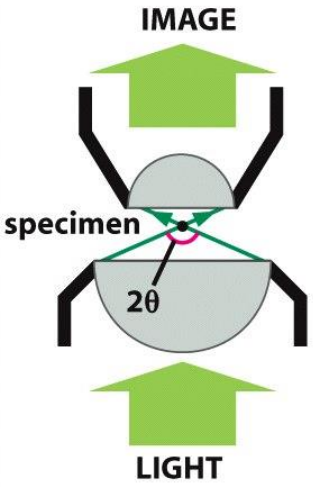


Differential-interference-contrast (DIC)

dark field

Conventional Light microscope has a resolution of 0.2µm

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

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

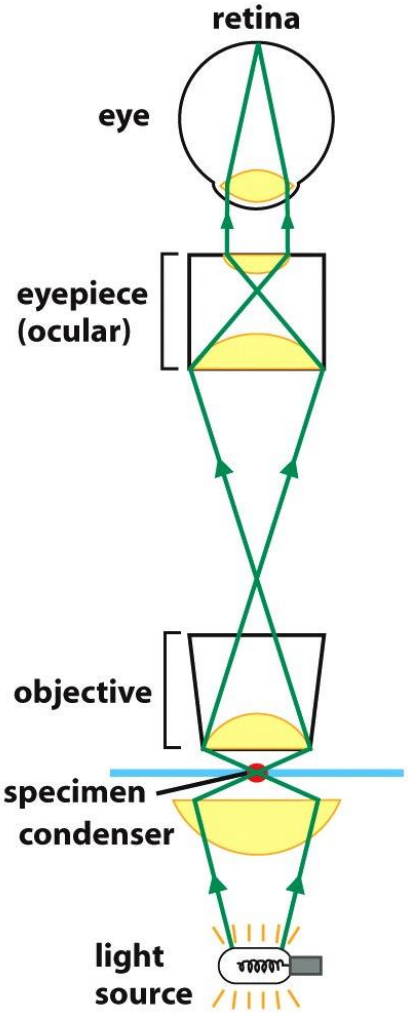
where:

- 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)

The smaller the number is, the bigger the resolution Is

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.

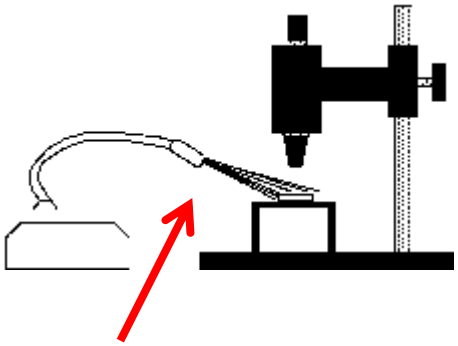
1.1. Bright field microscope



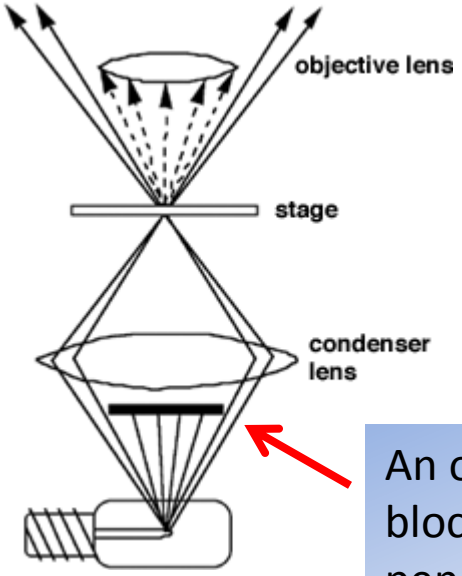
Magnification : up to 1000 fold
Eye piece: up to 10X
Objective: up to 100X

Figure 9-3a *Molecular Biology of the Cell* (© Garland Science 2008)

1.2. Dark field microscopy



Light illuminates on the surface of the specimen



An opaque blocker to block lights directly penetrating specimen

Dark field is especially useful for finding cells in suspension

Comparison for Dark-field and Bright-field microscopes

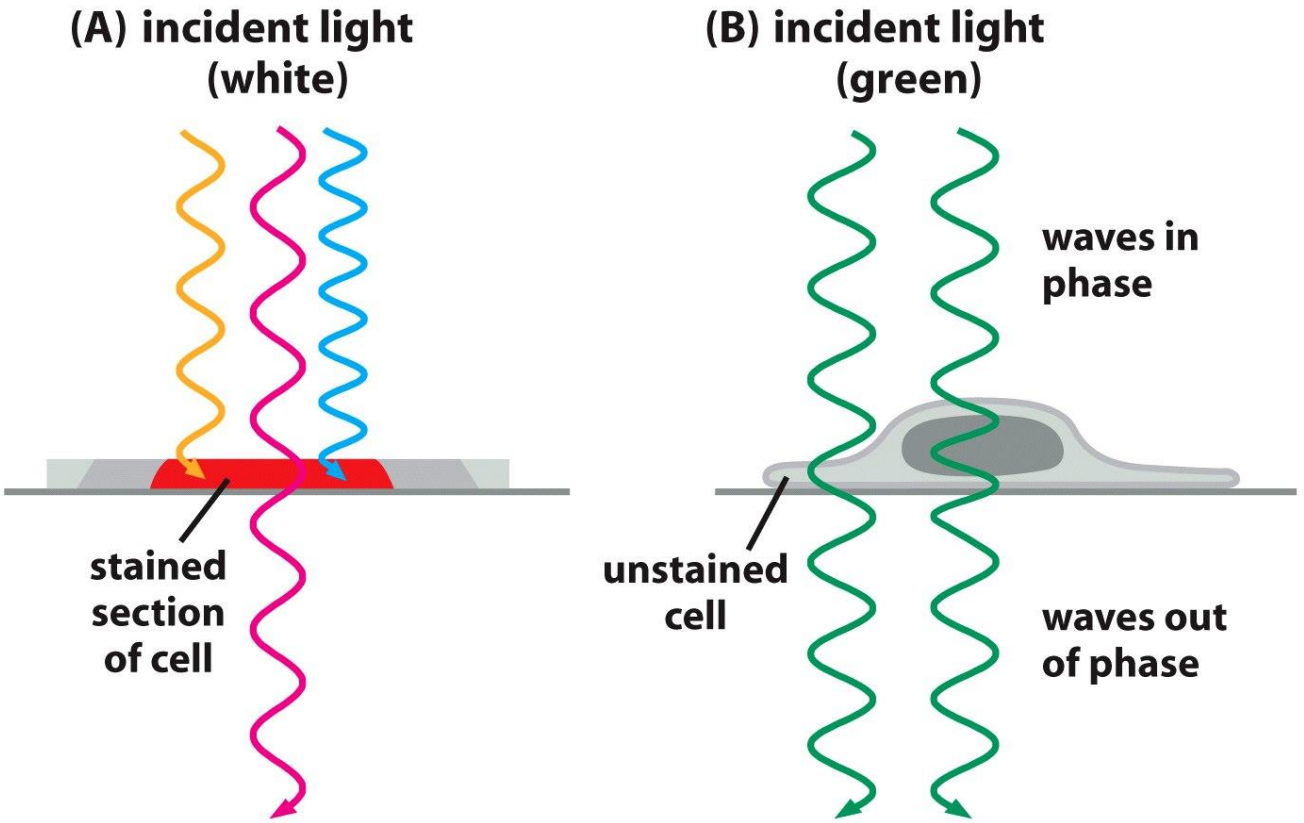
- Dark field: cells bright, background dark.

  Light illuminates from the side and only scattered light enter lenses

- Bright field: cells dark, background bright.

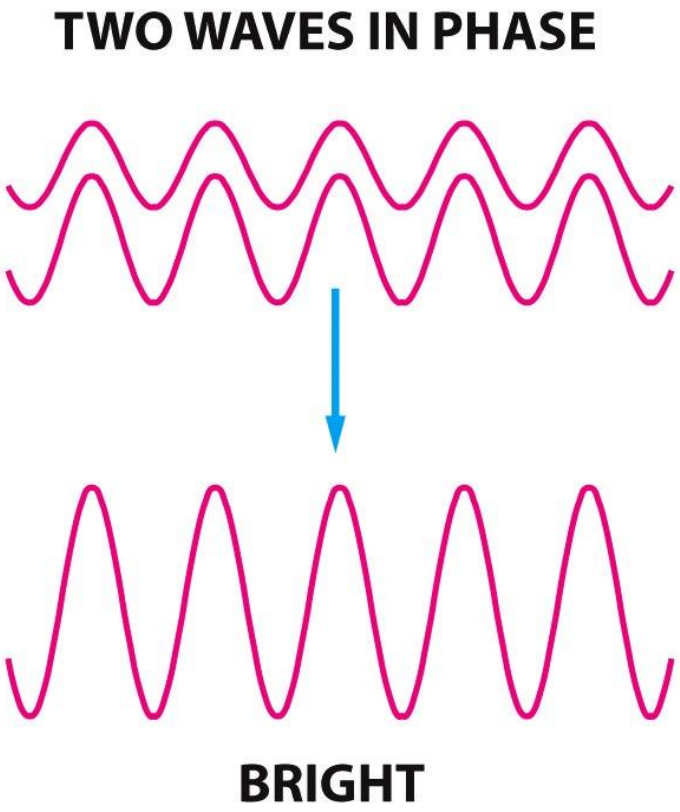
  light pass through a cell to form image directly

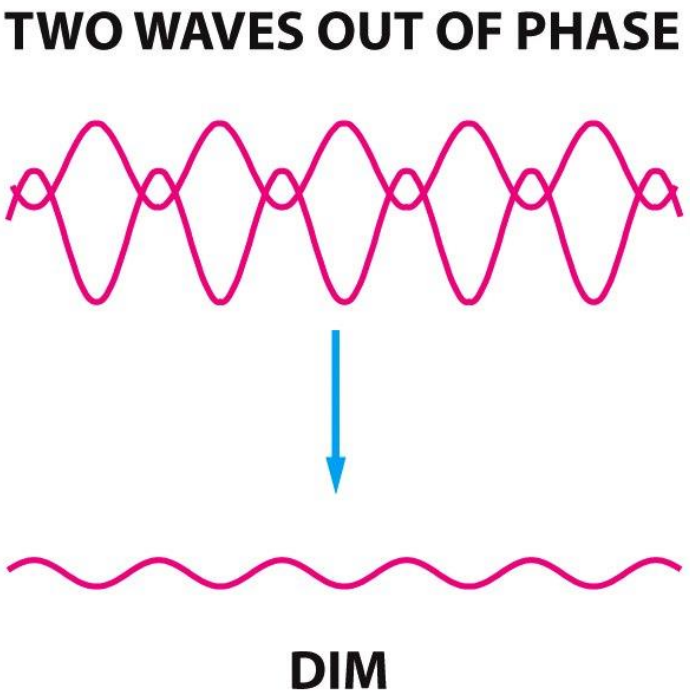
1.3. Phase contrast



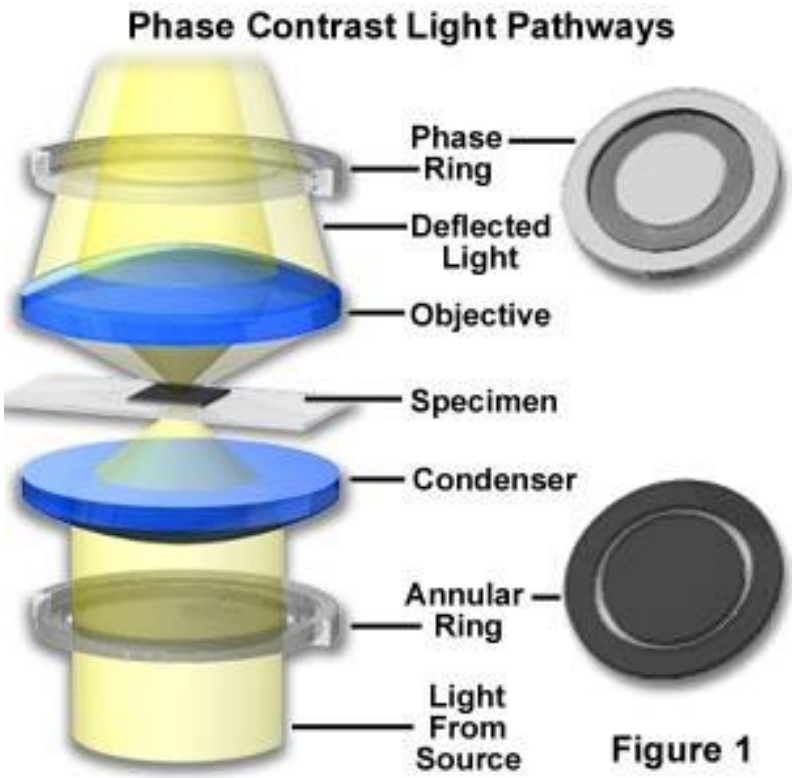
Phase contrast estimates phase changes after light passes through parts of a cell with different density, and then convert the change of phase into different brightness. Phase contrast microscope is frequently used in Cell Biology

Phase difference results in difference in brightness





Structure of a Phase Contrast Microscope



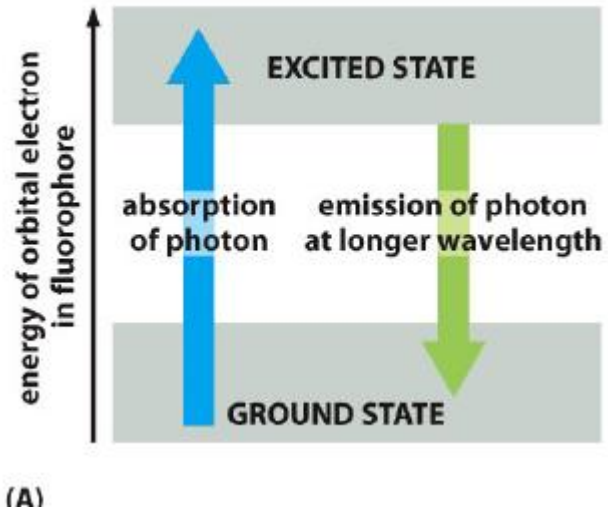
Phase ring

Annular diaphragm

1.4. Differential-Interference Contrast (DIC)

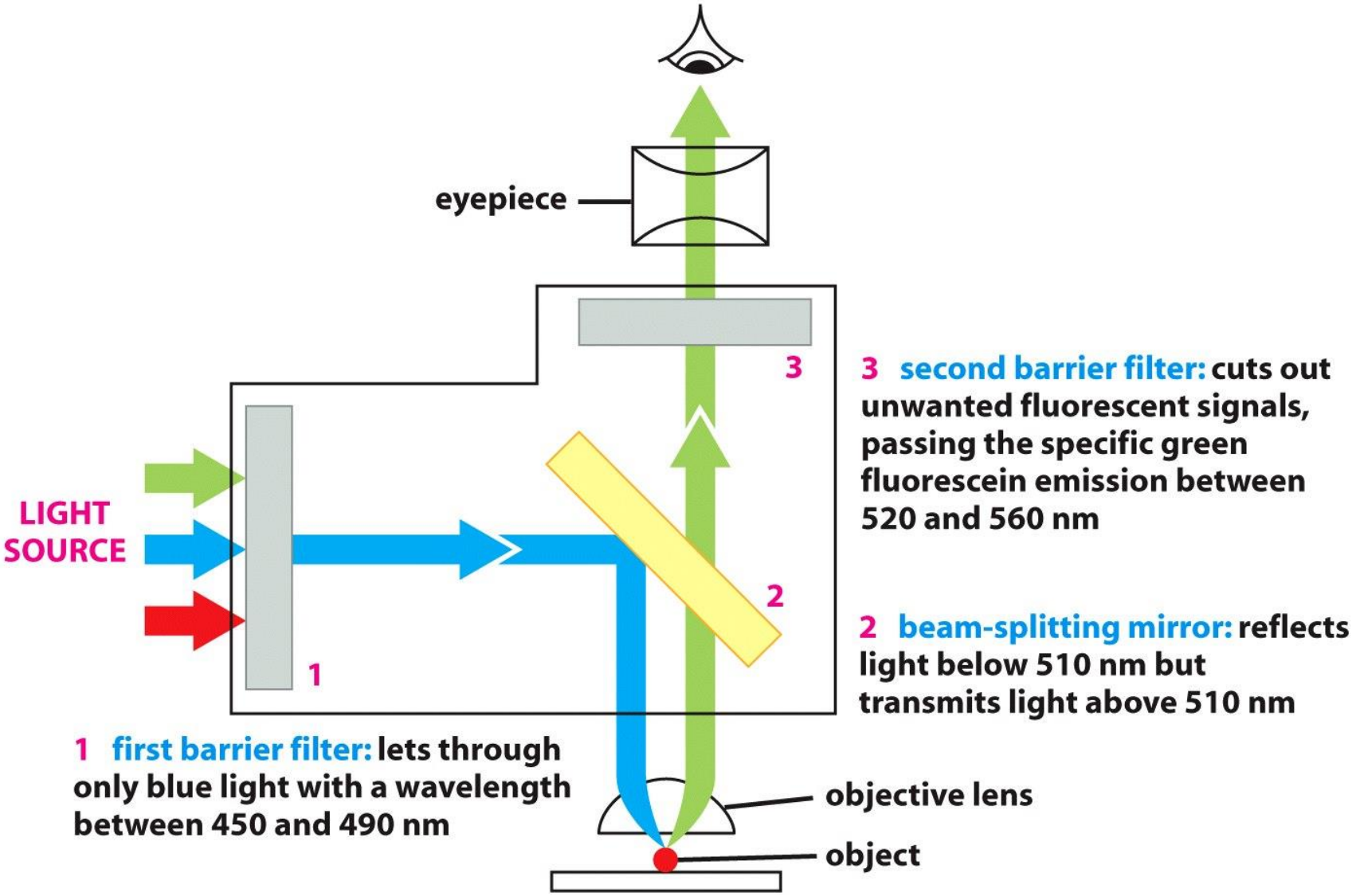
- Analyze the optical path length **gradients** (the rate of change for refractive light).
- No annular diaphragm and phase plate needed
- Produce shadow-cast image.
- Elimination of halo artifacts
- Excellent images with relatively thick specimens

2. Fluorescence microscope

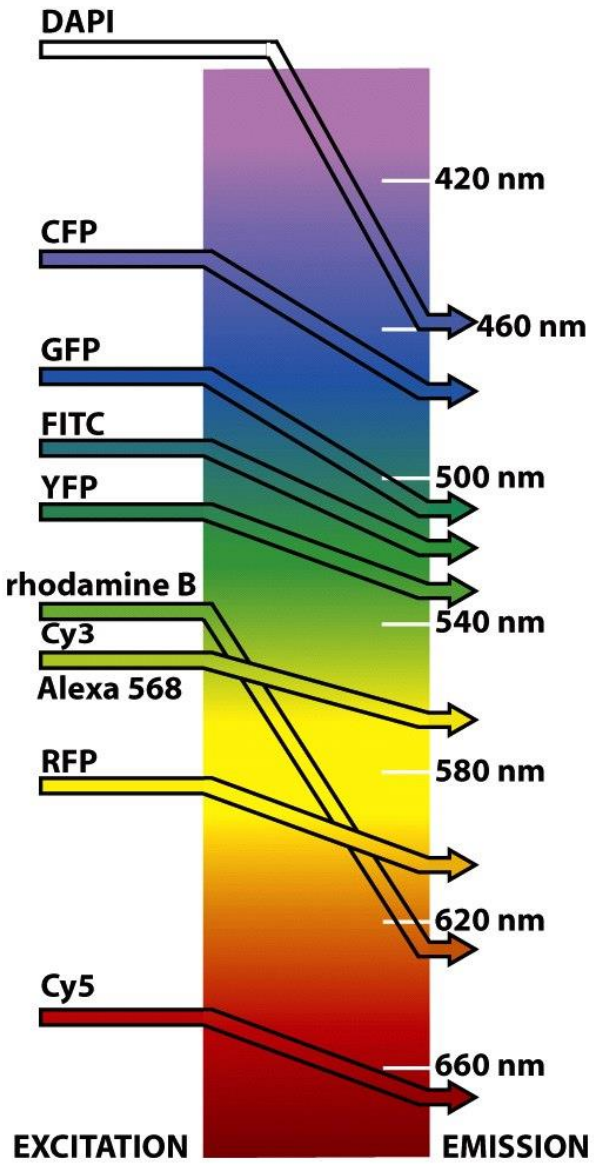


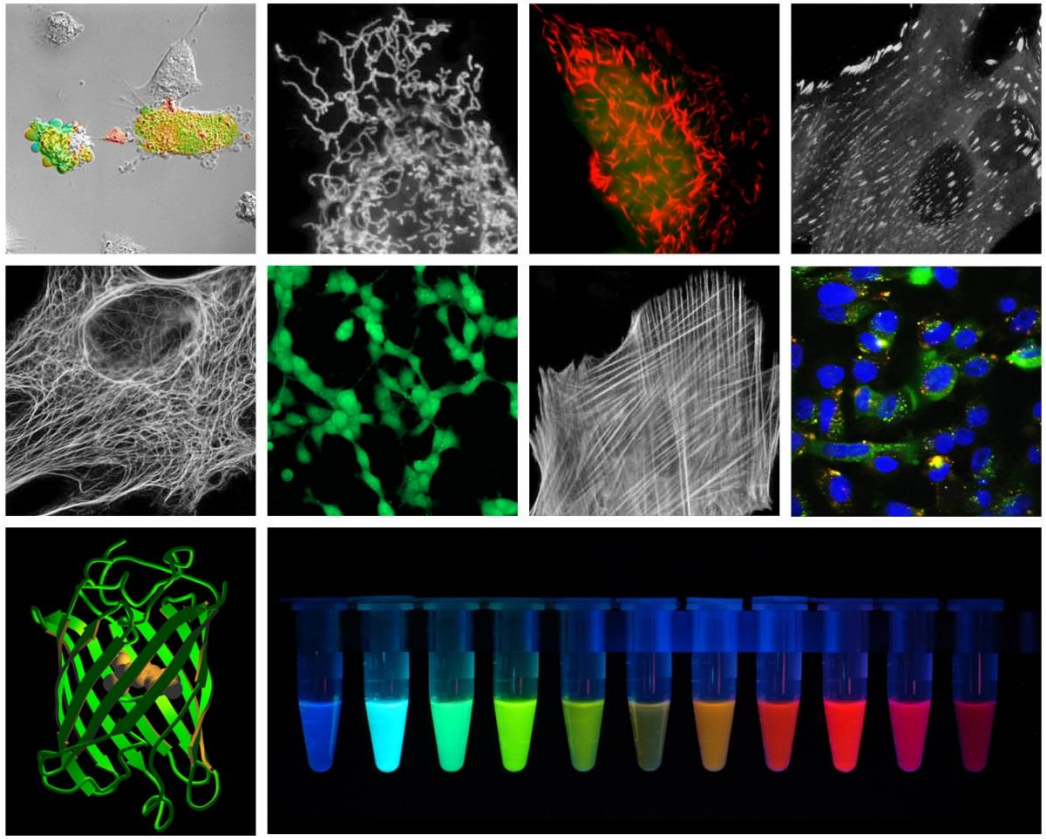
- A fluorophore absorbs a photon to be excited
- It is not stable and undergoes conformational change to emit a photon
- After it emits, it goes back to the ground state.
- The cycle can be repeated.
- The energy of excitation light is higher than the energy of emission light.
- Lower energy = longer wavelength
- Stokes shift: the difference between excitation energy and the emission energy for a given fluorophore

How does the fluorescence microscopy work?



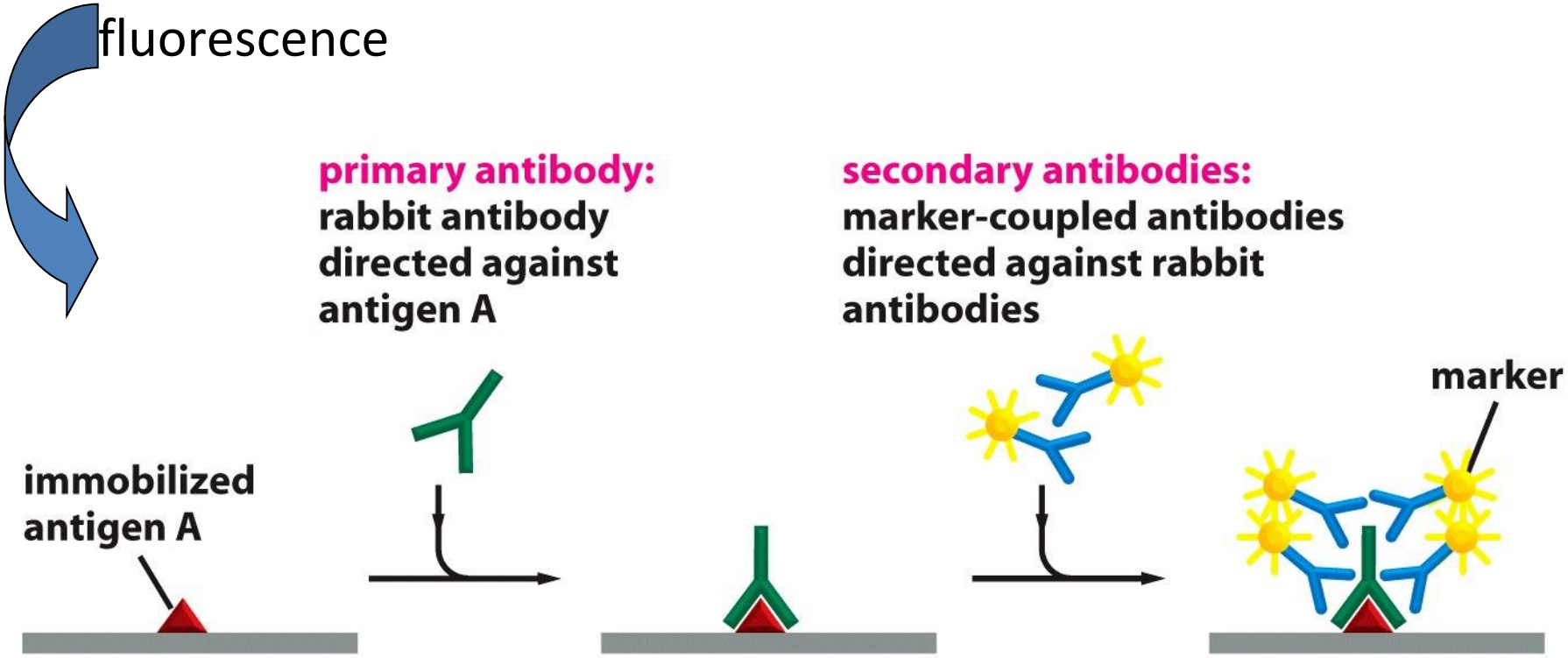
2.1. Fluorescence probes



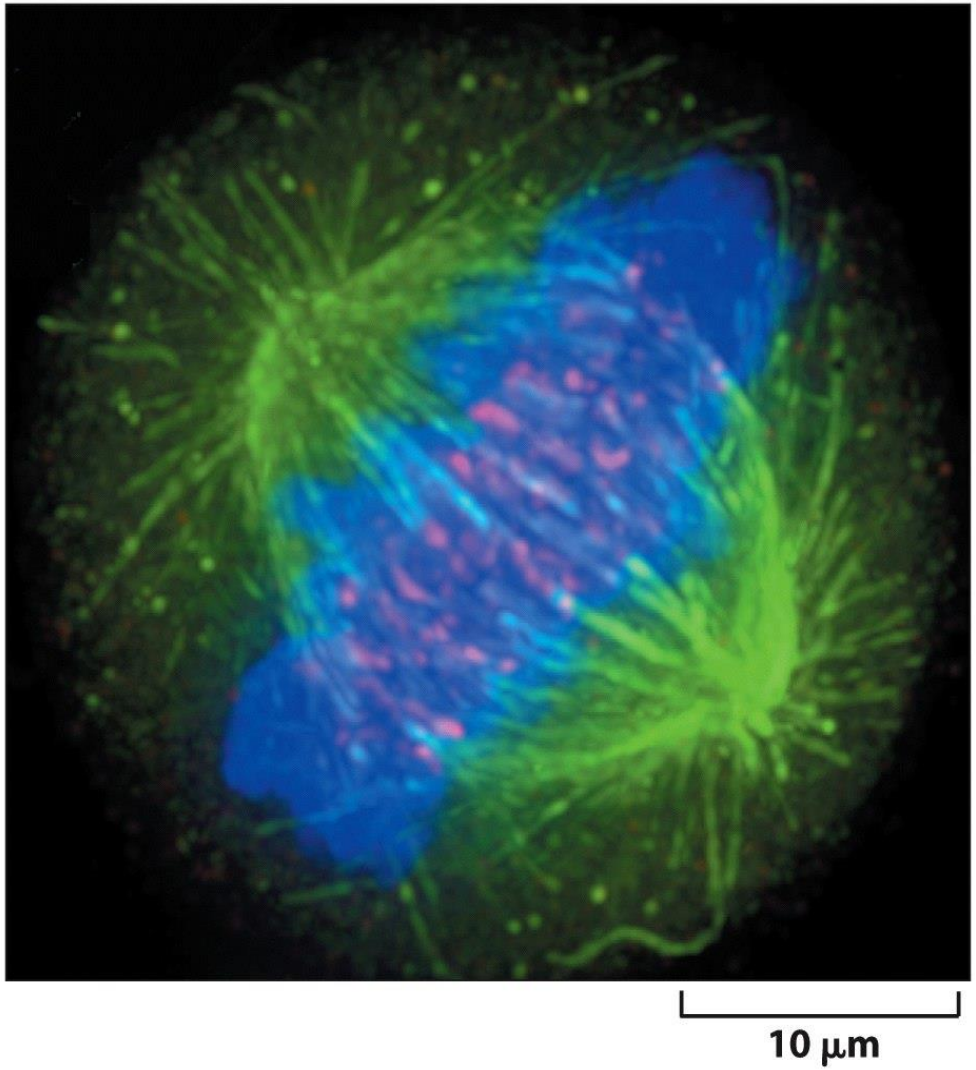


2.2. Categories for Fluorescence microscope

- Direct immunofluorescence primary antibody-conjugated fluorescence
- Indirect immunofluorescence secondary antibody-conjugated fluorescence



A Multi-fluorescent cell



Step 1: cell fixation (2-4% paraformaldehyde,
acetone/methanol(1:1)
10% formalin/10%methanol)
Step 2: permeabilize with detergent
(0.3%triton-X-100)
Step 3: incubate with primary antibody
Step 4: incubate with secondary-Ab-
conjugated-fluorescence

1. Can be protein-specific
2. Detect cellular localization
3. Roughly quantify a biomolecule

Aequorin, a luminescent protein that glows blue in the presence of Ca2+

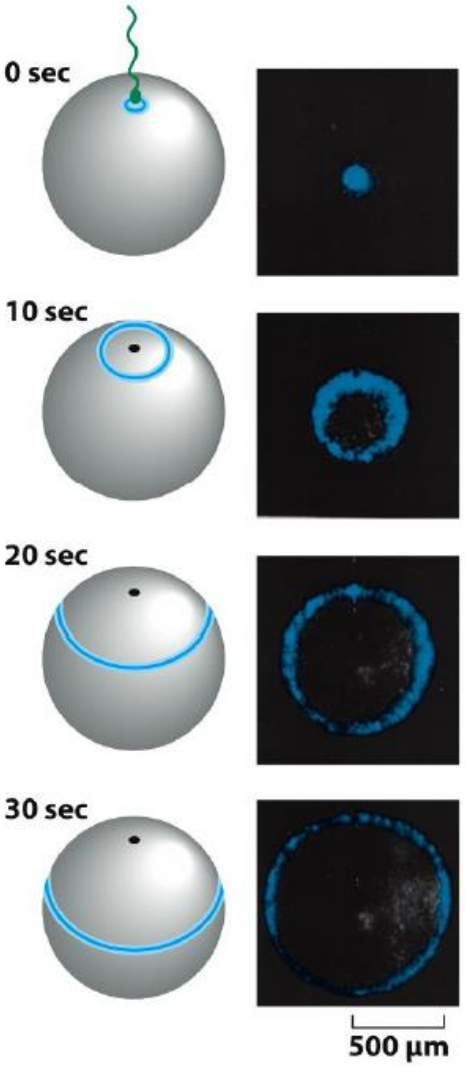
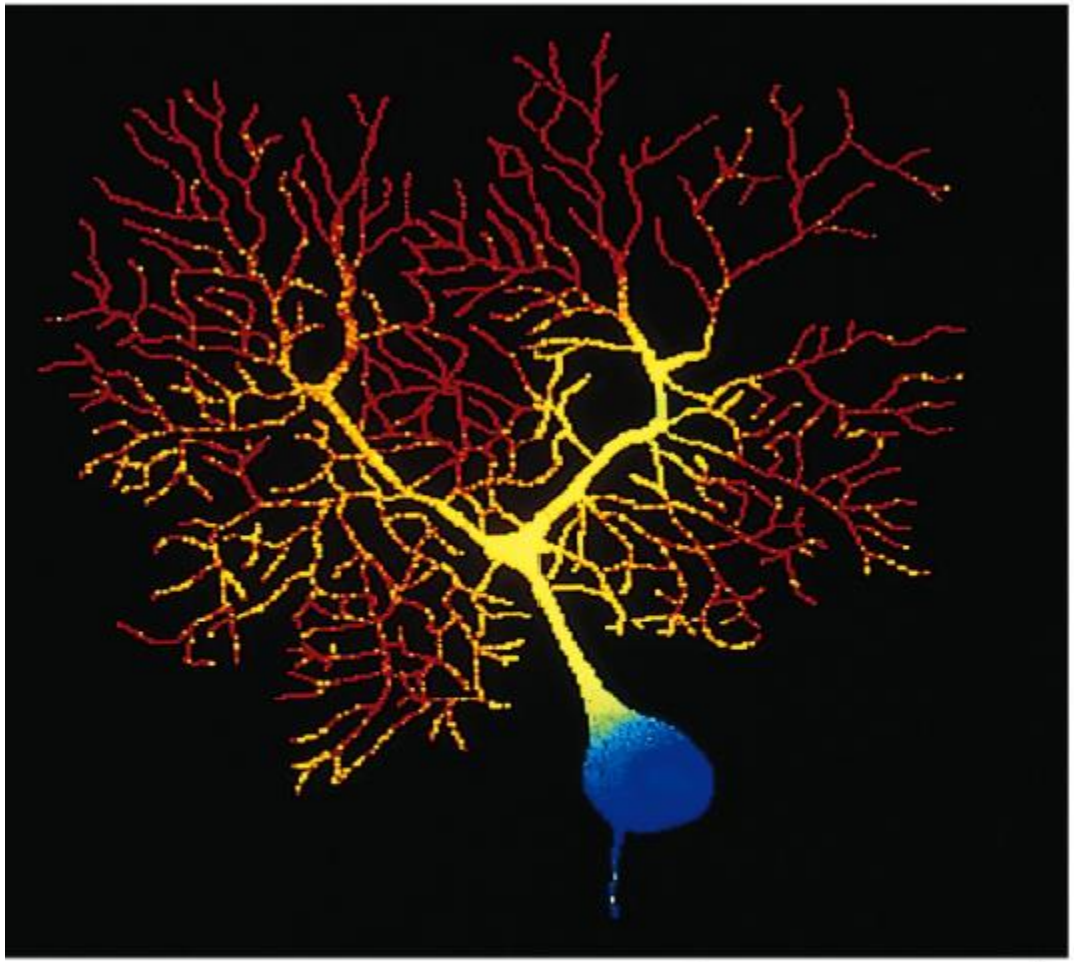


Figure 9-30 Molecular Biology of the Cell 6e (© Garland Science 2015)

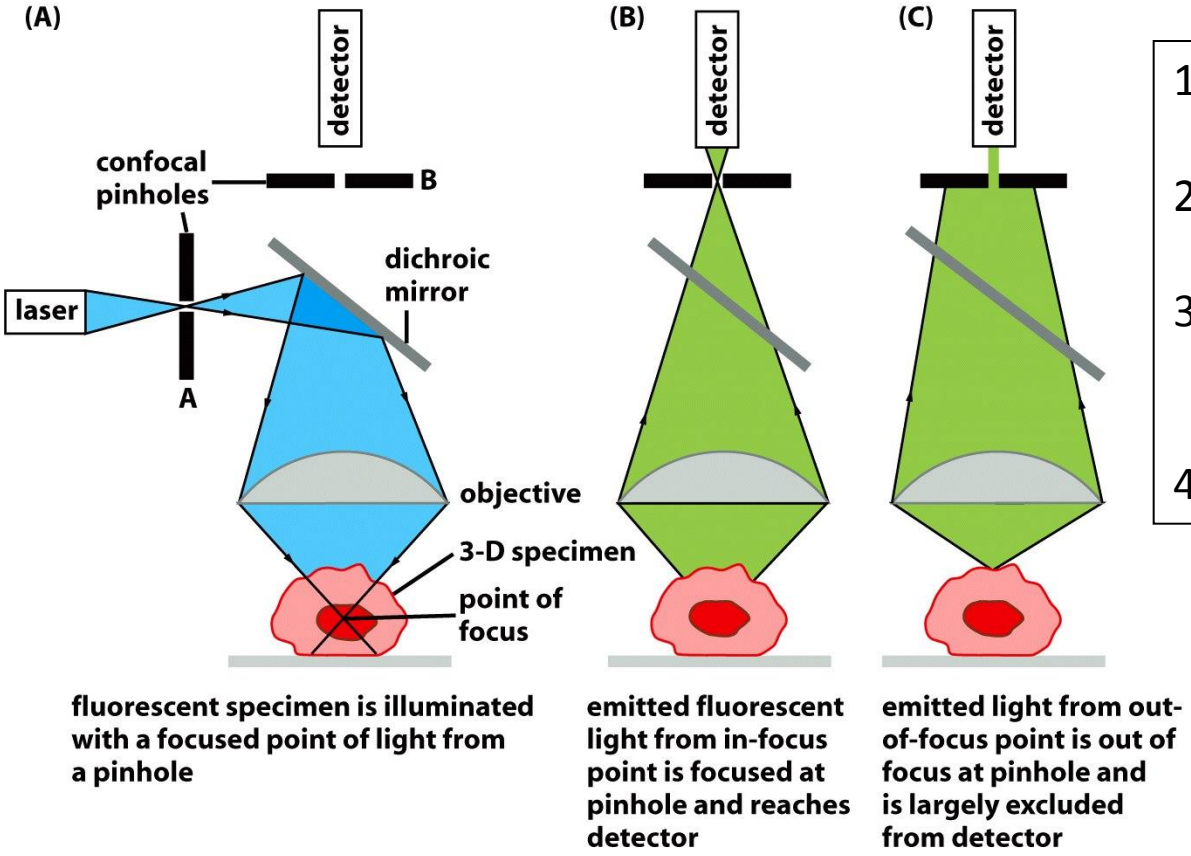
Fura-2 can sense free Ca2+ ions in cells



Red: higher Ca2+
Blue: lower Ca2+

Figure 9-31 Molecular Biology of the Cell 6e (© Garland Science 2015)

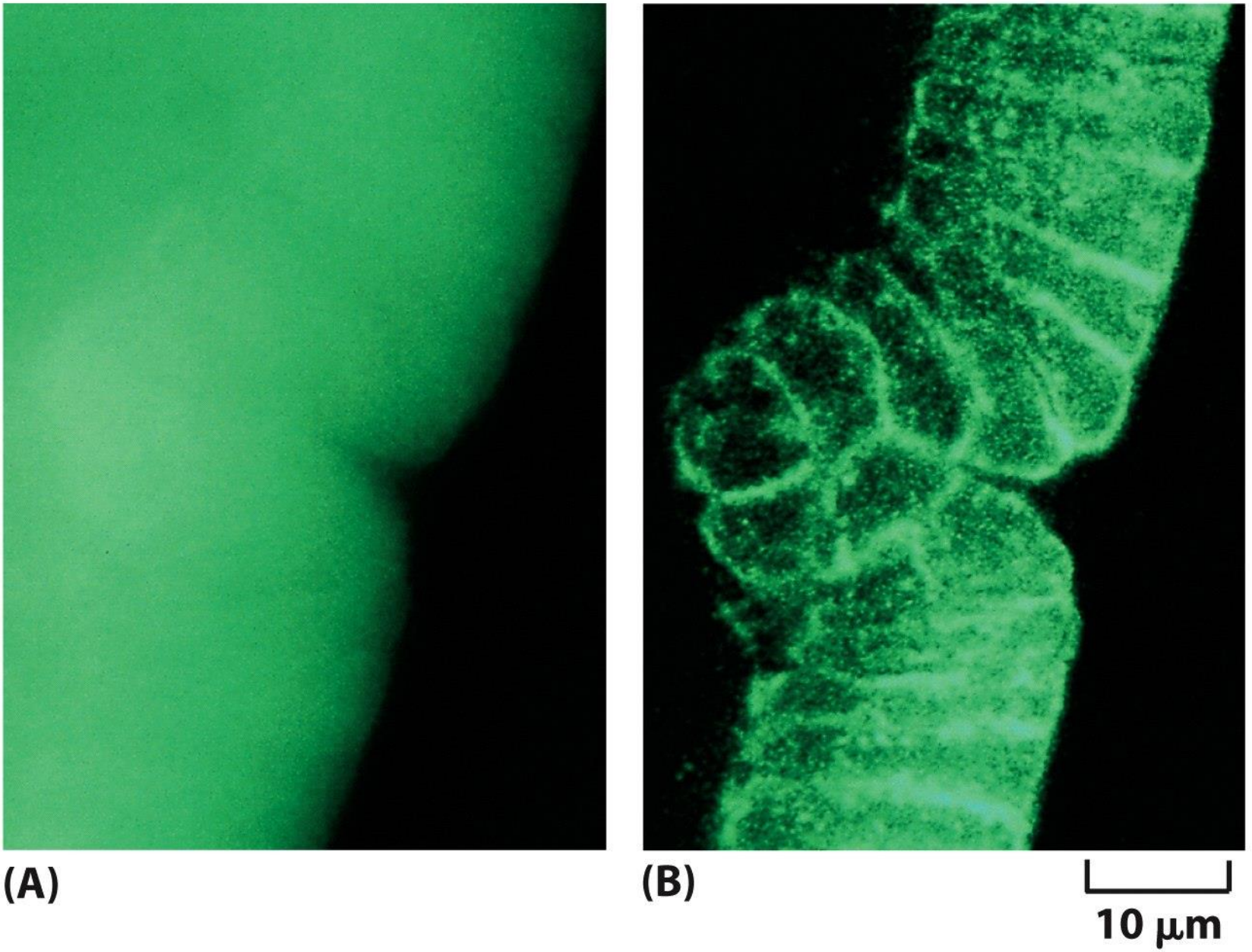
3. Confocal microscope



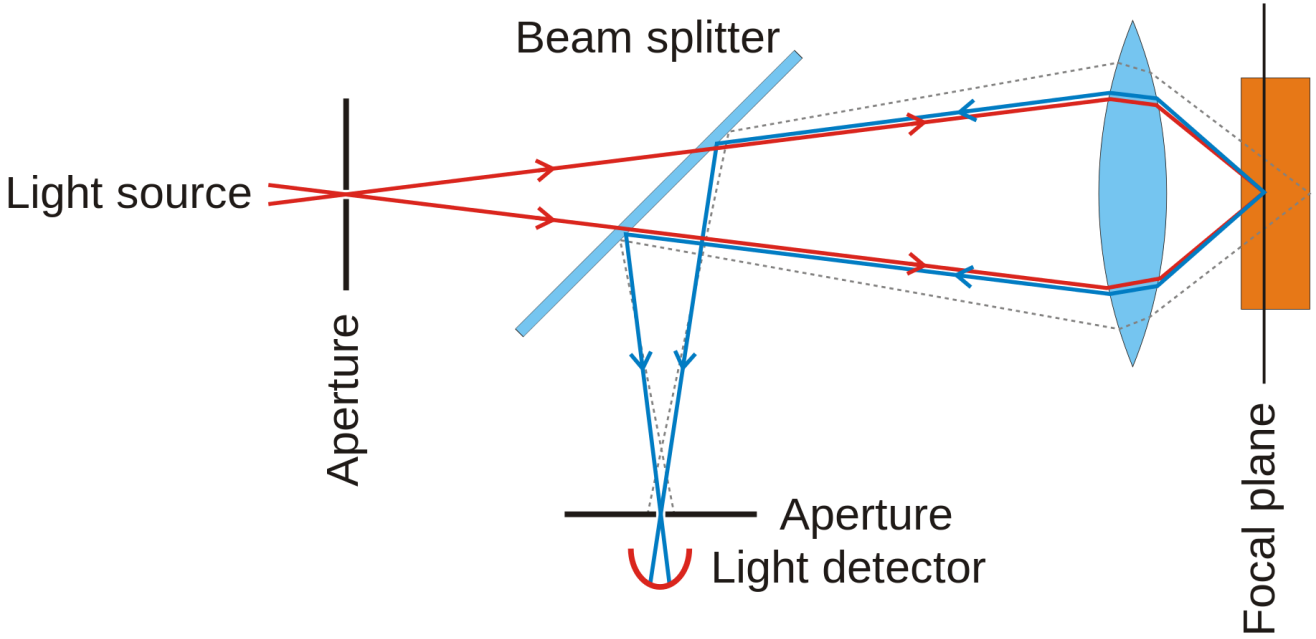
1. More accurately detect protein localization in a 3-D location.
2. Use laser beam as light source provide better resolution.
3. Use focused light from a pinhole to illuminate on a certain spot in specimen.
4. Our of focus light will be excluded

Conventional immunofluorescence

confocal microscope



3.1. Laser scanning confocal

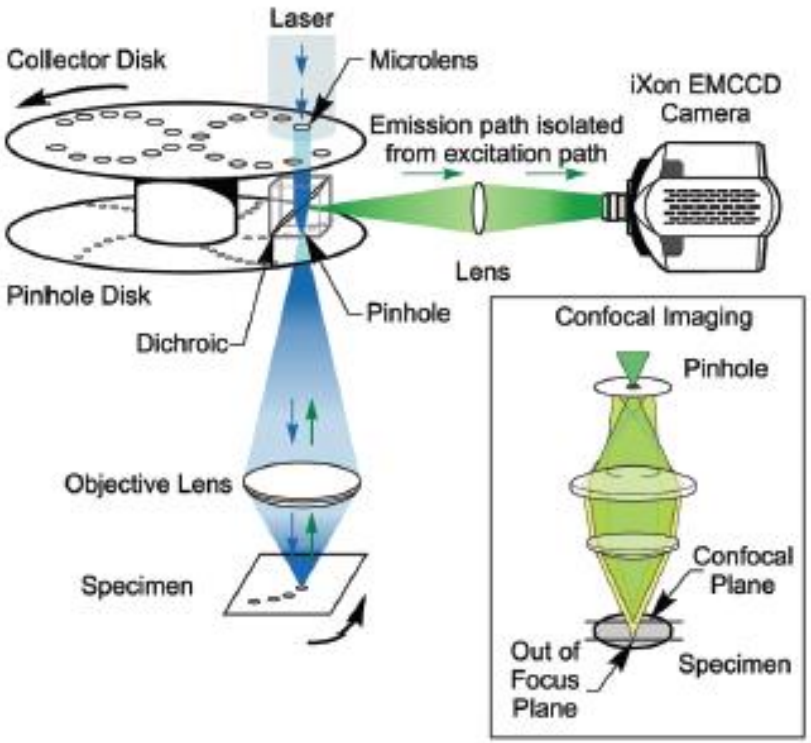


one pinhole

Good: better resolution

Bad: Illumination on specimen
for longer time, sometimes
cause bleaching for fluorescence signal

3.2. Spinning disk confocal

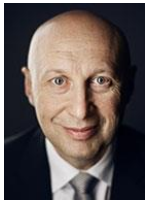


Multiple pinholes allow specimen
To be illuminated at multiple
Points simultaneously

Good: quick and dynamic imaging
Bad: lower resolution

4 Nanoscopy

Fluorescence microscopy that surpasses the limitation of 0.2µm



Stefan W. Hell

Prize share: 1/3

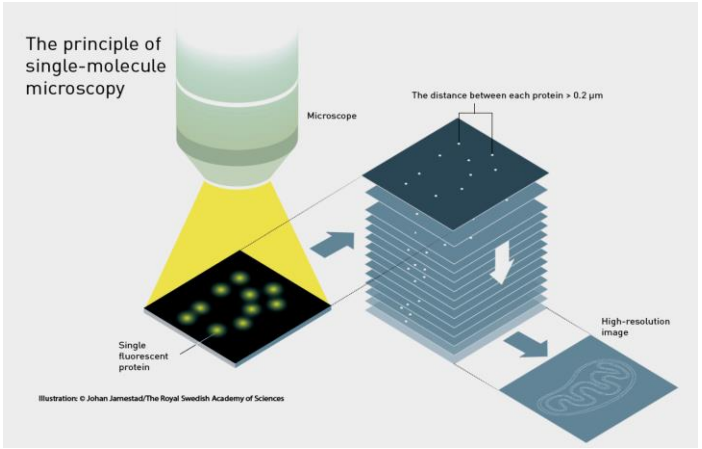
William E. Moerner

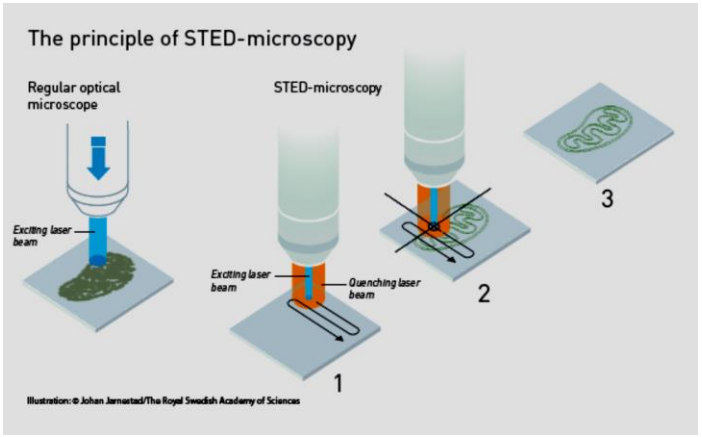
Prize share: 1/3

Eric Betzig

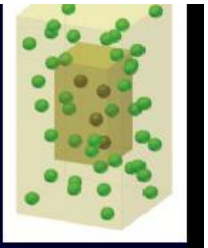
Prize share: 1/3

Nobel Laureates
in 2014 Chemistry





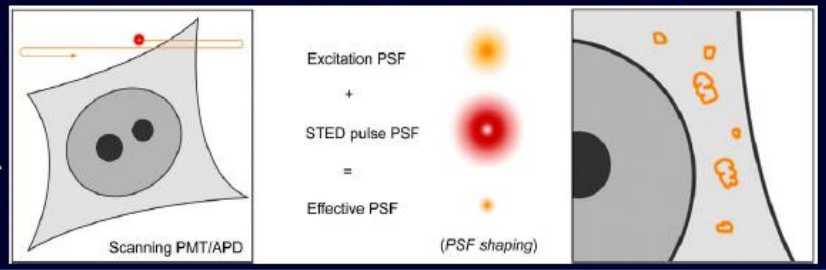
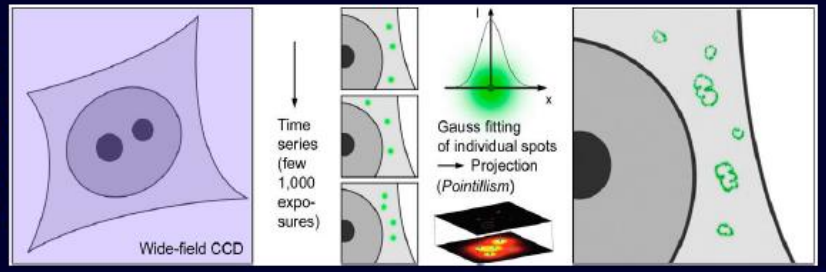
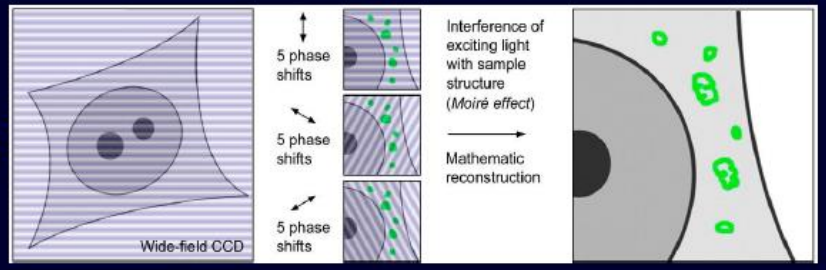
Caveats with Super-Resolution Microscopy: Live Cells



Nyquist criterion:

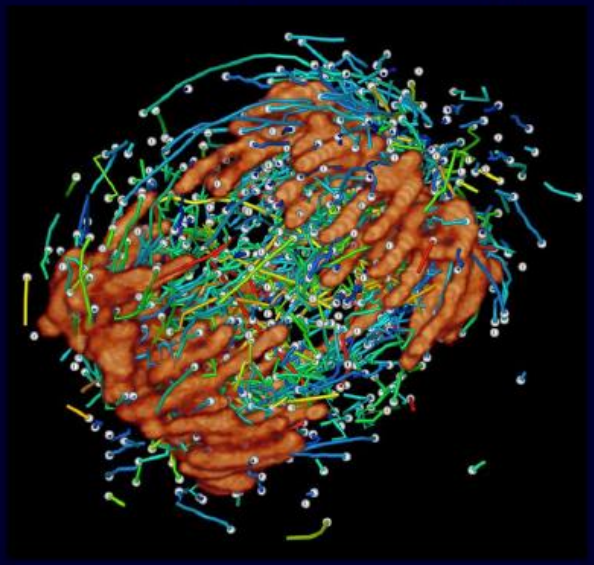
N -fold resolution increase in *D* dimensions → N^D -fold more photons collected

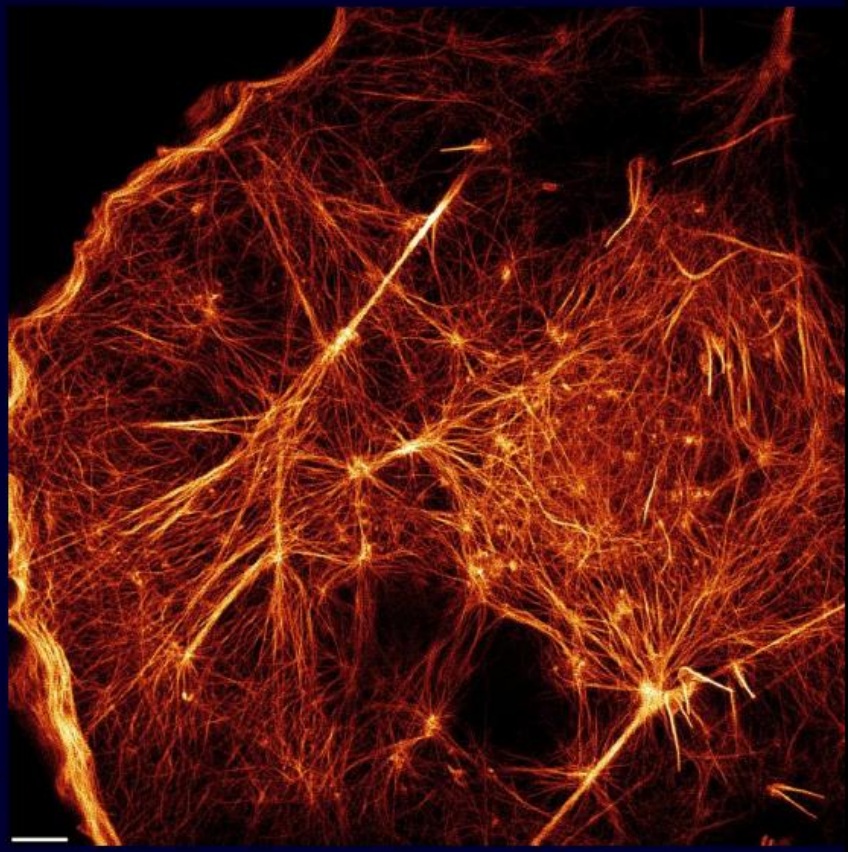
L. Schermelleh, R. Heintzmann, *J. Cell Biol*. (2010)

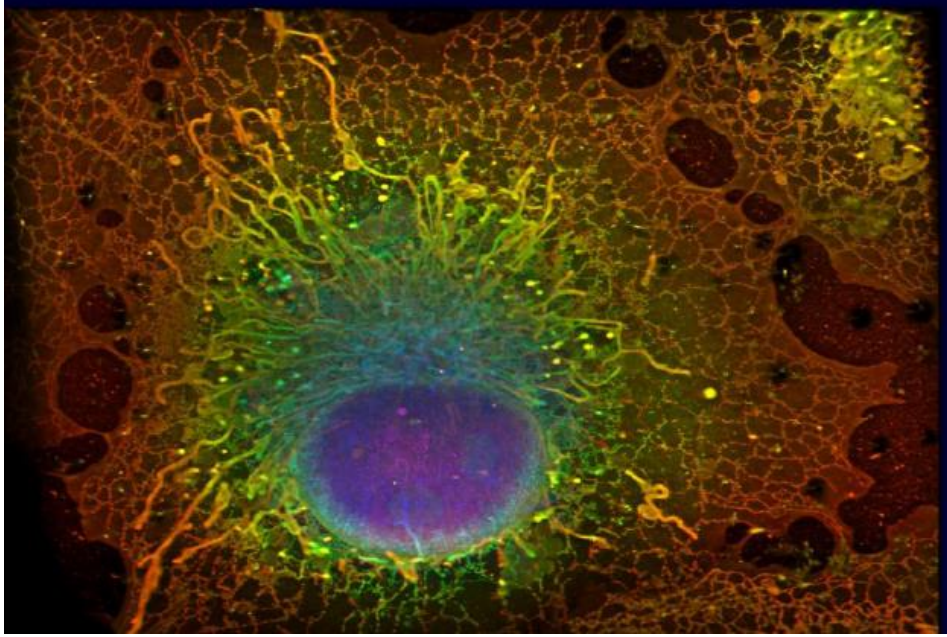
		reported resolution (nm)	photon increase required	intensity (W/cm²)	acquisition time (sec)
STED / RESOLFT		*xy*: 20 nm	100	10^4 - 10^9	> 60
		xyz: 30 nm	1,070		~1,000
Localization		*xy*: 20 nm	100	10^3 - 10^4	>20
		xy: 10 nm, *z*: 20 nm	14,400		1,500
SIM		*xy*: 100 nm	4	10 - 10^2	0.1 - 1
		xy: 100 nm, *z*: 370 nm	8		~10

--- Nobel prize website

The Beauty and Complexity of Living Systems

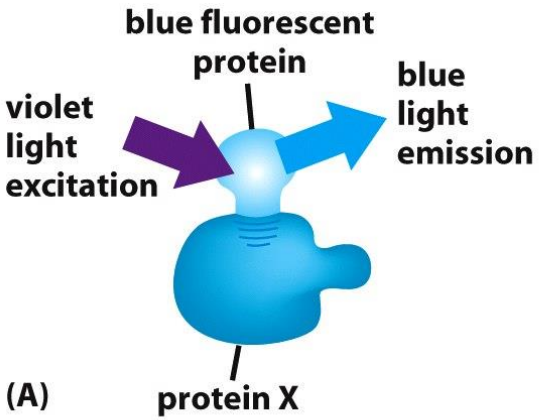


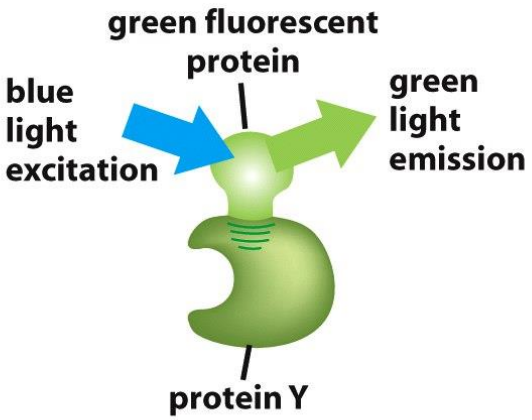




--- Nobel prize website

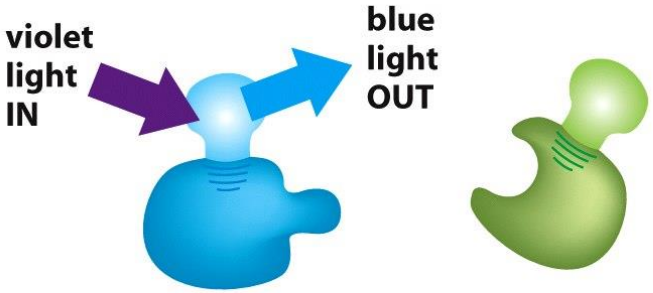
5.1 FRET (fluorescence resonance energy transfer) to detect protein-protein interaction .





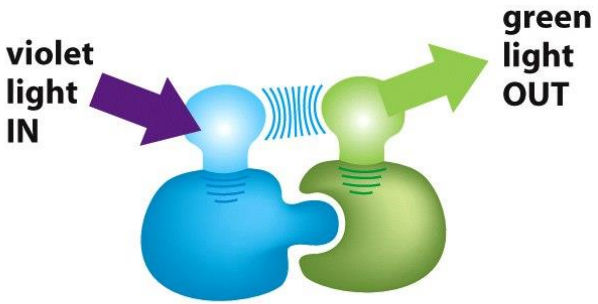
Two separate dyes to label two interesting molecules, respectively.

Upon direct interaction, The energy from one dye will cause the excitation of the other dye and to emit light. The signal can be Recorded.



(B) **NO PROTEIN INTERACTION**

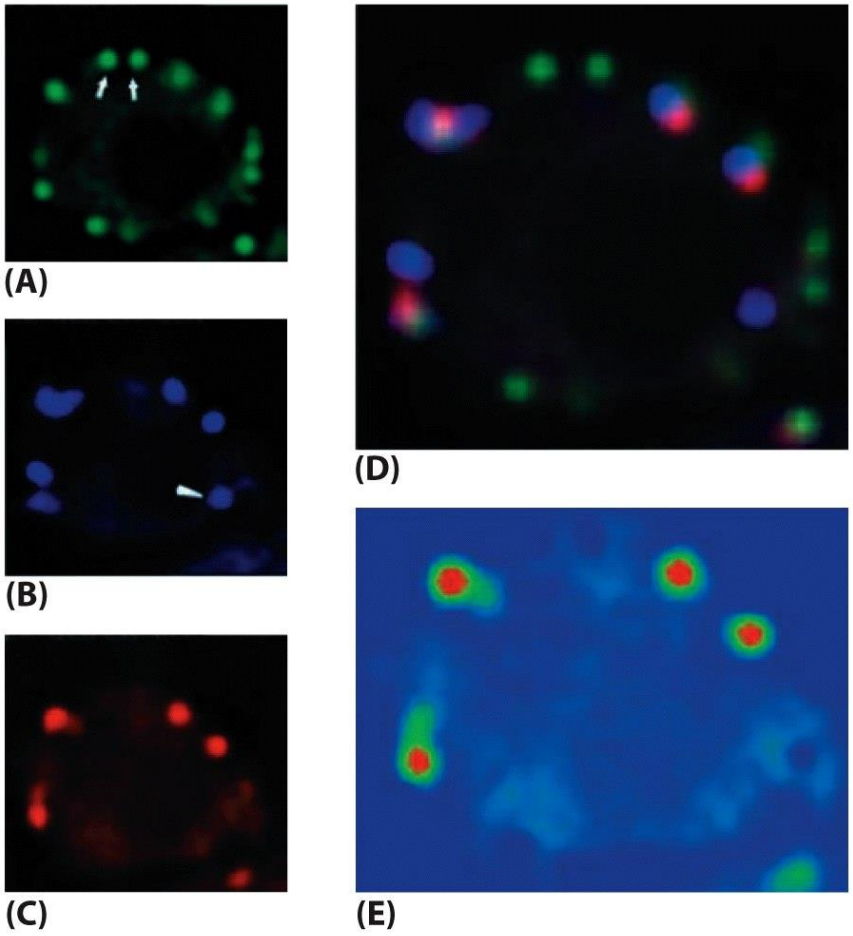
NO EXCITATION OF GREEN FLUORESCENT PROTEIN, BLUE LIGHT DETECTED



(C) **PROTEIN INTERACTION**

FLUORESCENCE RESONANCE ENERGY TRANSFER, GREEN LIGHT DETECTED

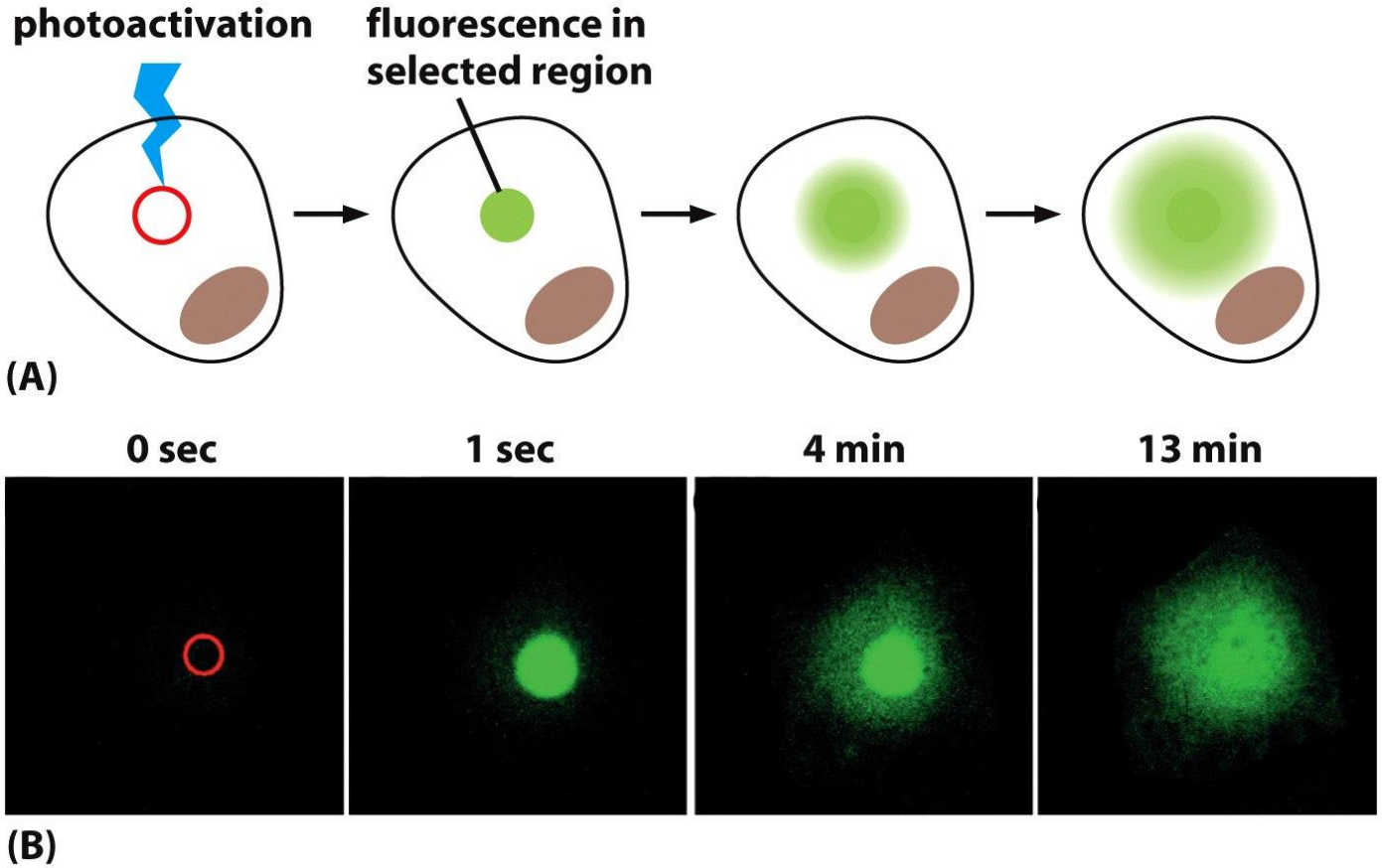
e.g. FRET



FRET occurs in close distance ~5nm.

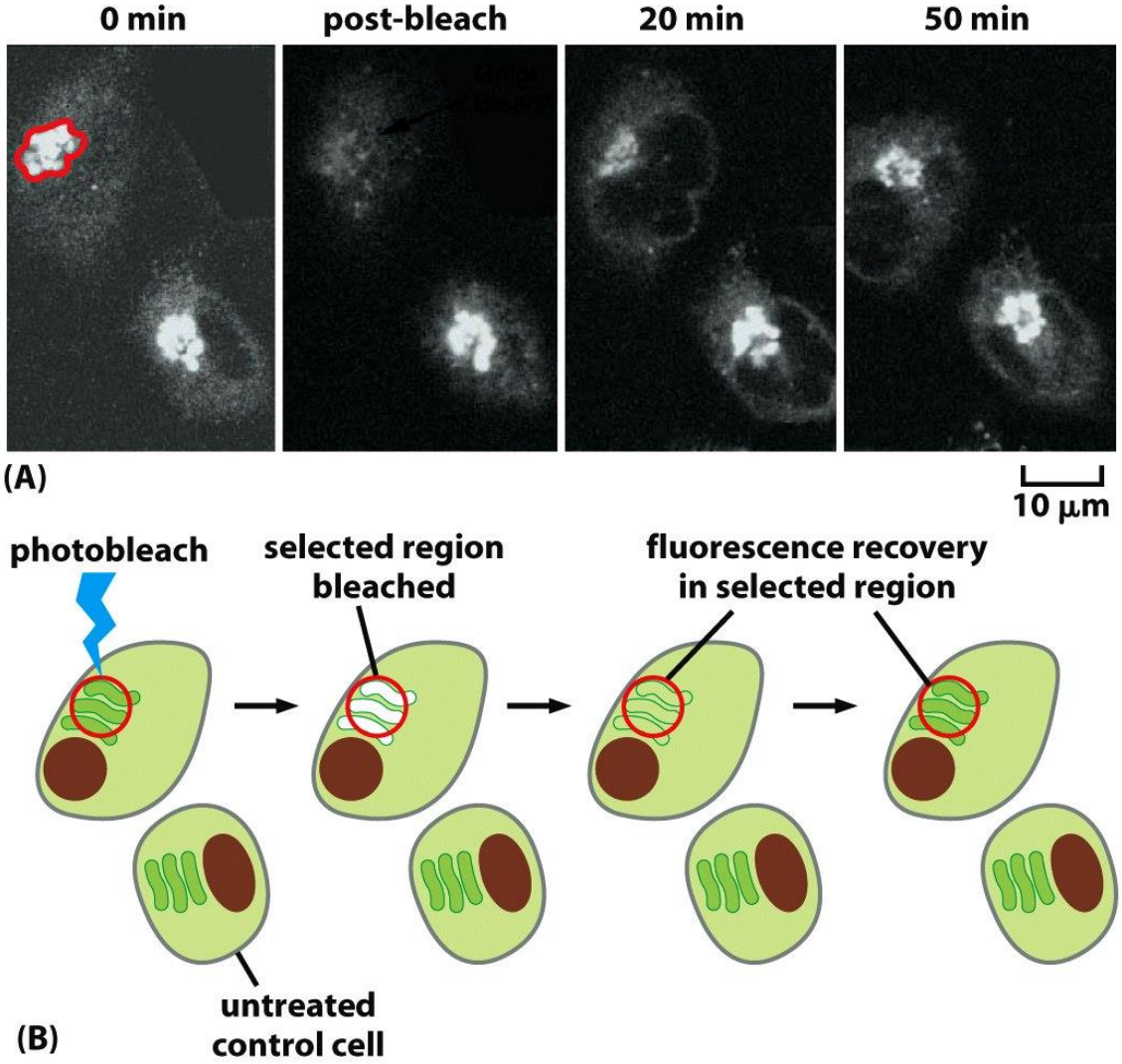
Rules for two fluorochromes:
Emission spectrum of one should overlap with the absorption spectrum of the other.

5.2 Photoactivation



Activate a target molecule by photoactivation, as time goes, the fluorescence signal changes
It can measure protein trafficking, turnover, degradation, diffusion, etc.

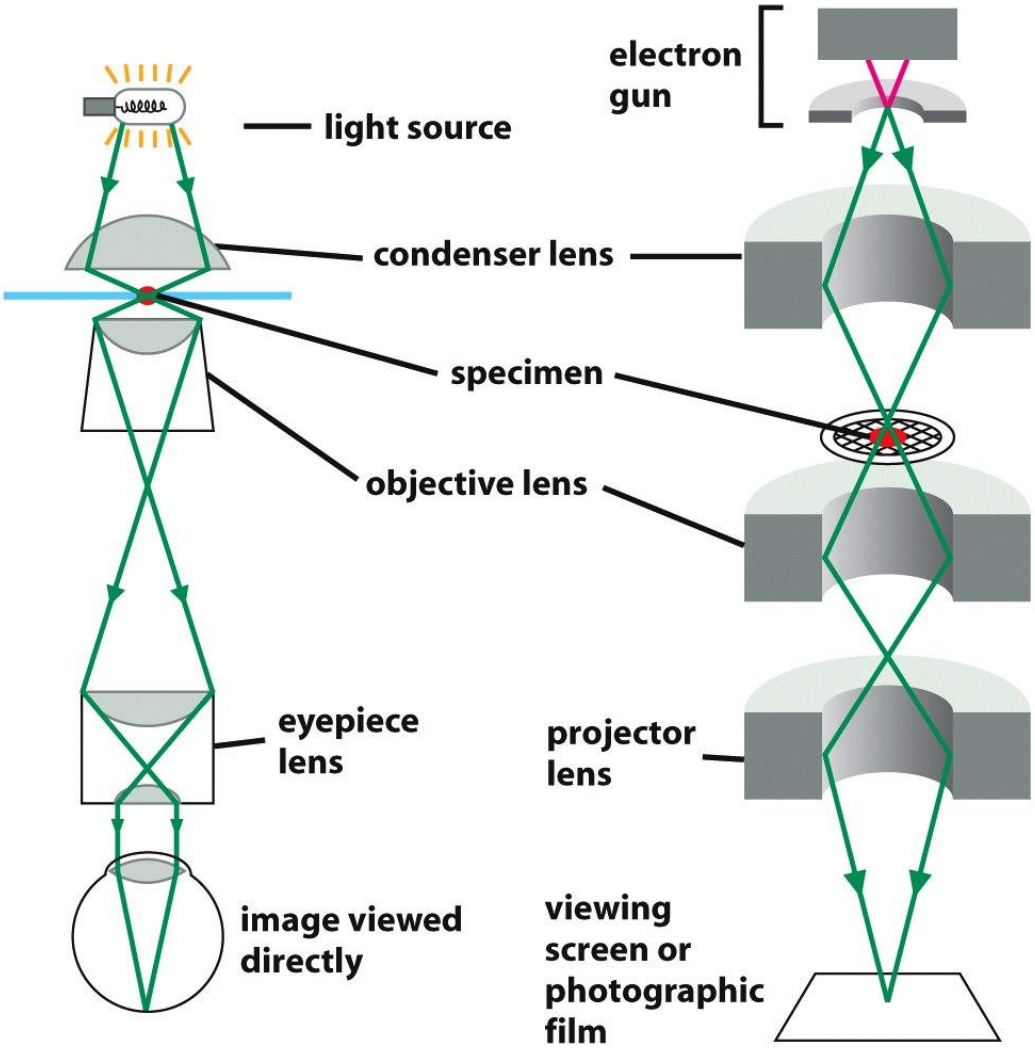
5.3 Fluorescence recovery after photobleaching (FRAP)



Detect molecular movement

10.6-FRAP.mov

6. Electron microscope

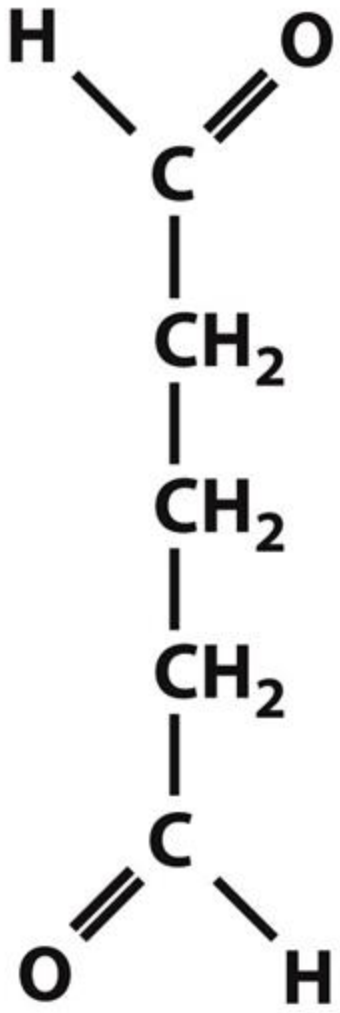


Resolution: 0.1nm-1nm

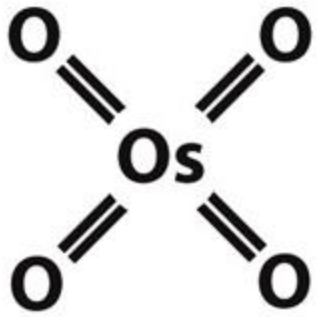
Transmission microscope

Special sample preparation

- Sample very thin: 50-100nm thick(penetrating power for electron is very weak)
- Sample rapidly frozen to preserve its original state
- Glutaraldehye(to crosslink proteins) and osmium tetroxide (to stabilize lipid membrane)
- Sample dehydrated, resin-embedded

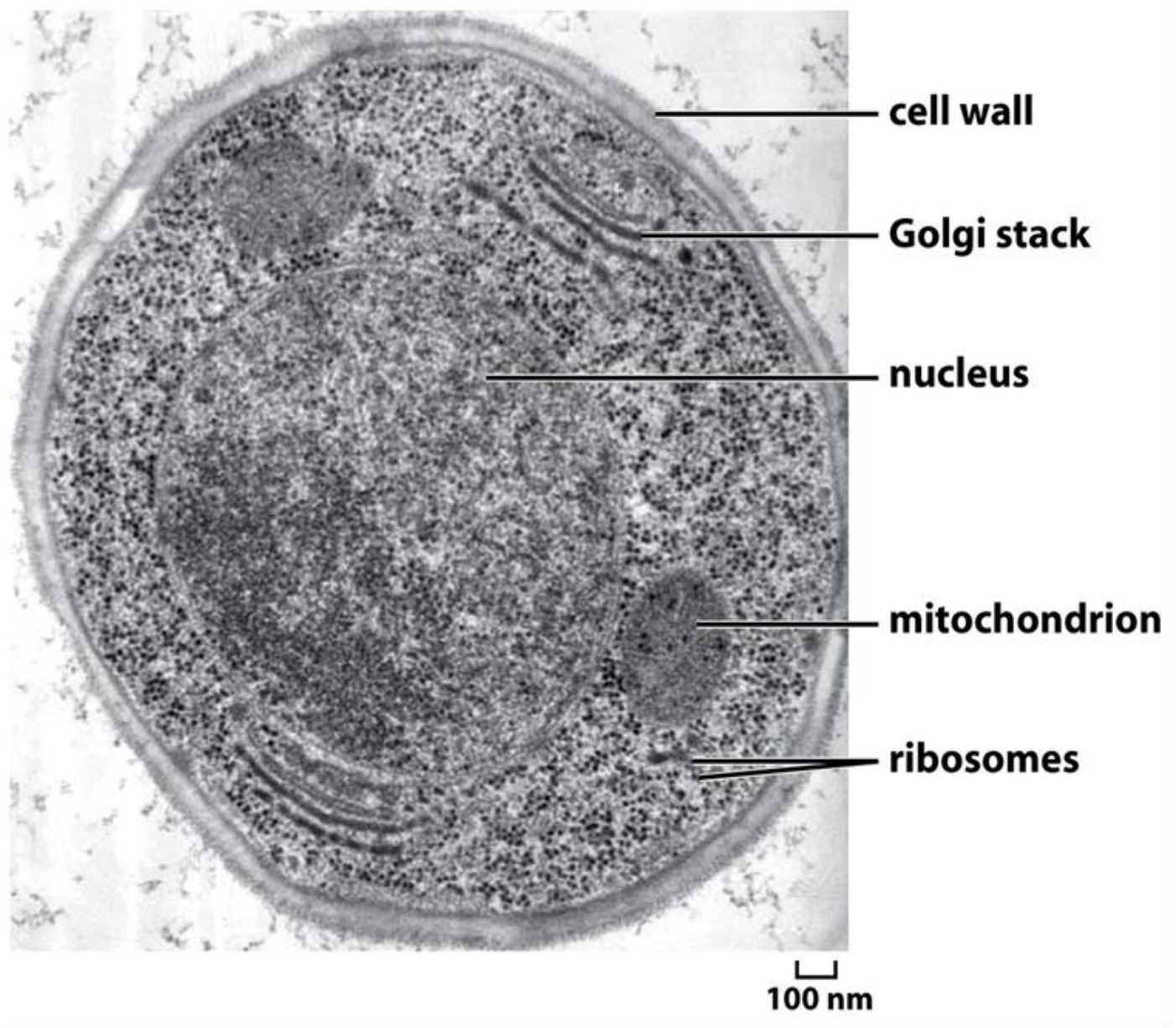


glutaraldehyde



osmium tetroxide

An image of a cell under electron microscope



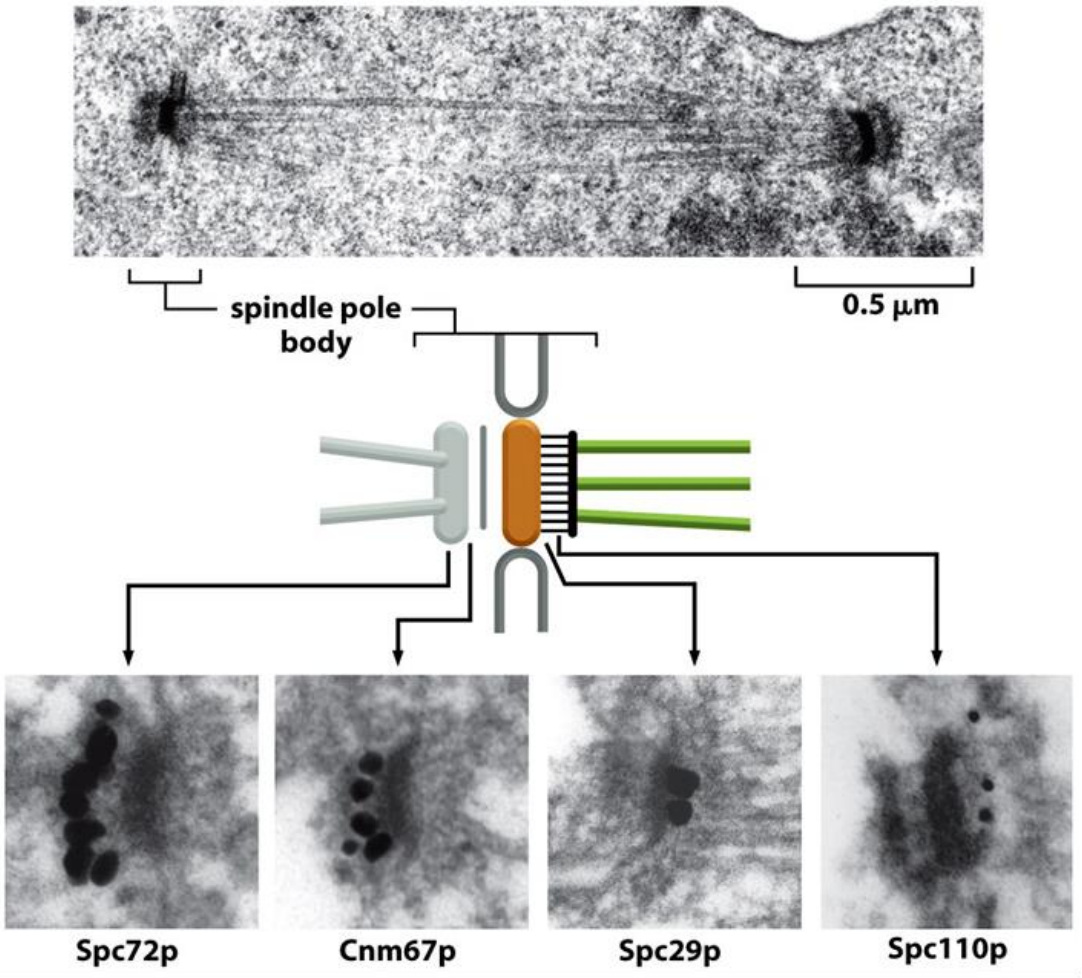
Electron microscope

- Immunogold electron microscope
- Scanning electron microscope (SEM)
- Transmission electron microscope (TEM)

6.1. Immunogold electron microscope

- Special protein is incubated with primary antibody
- Gold particle attached secondary antibody is incubated to label the protein with a gold paritcle.
- Gold is electron-dense and can be seen under the electron microscope

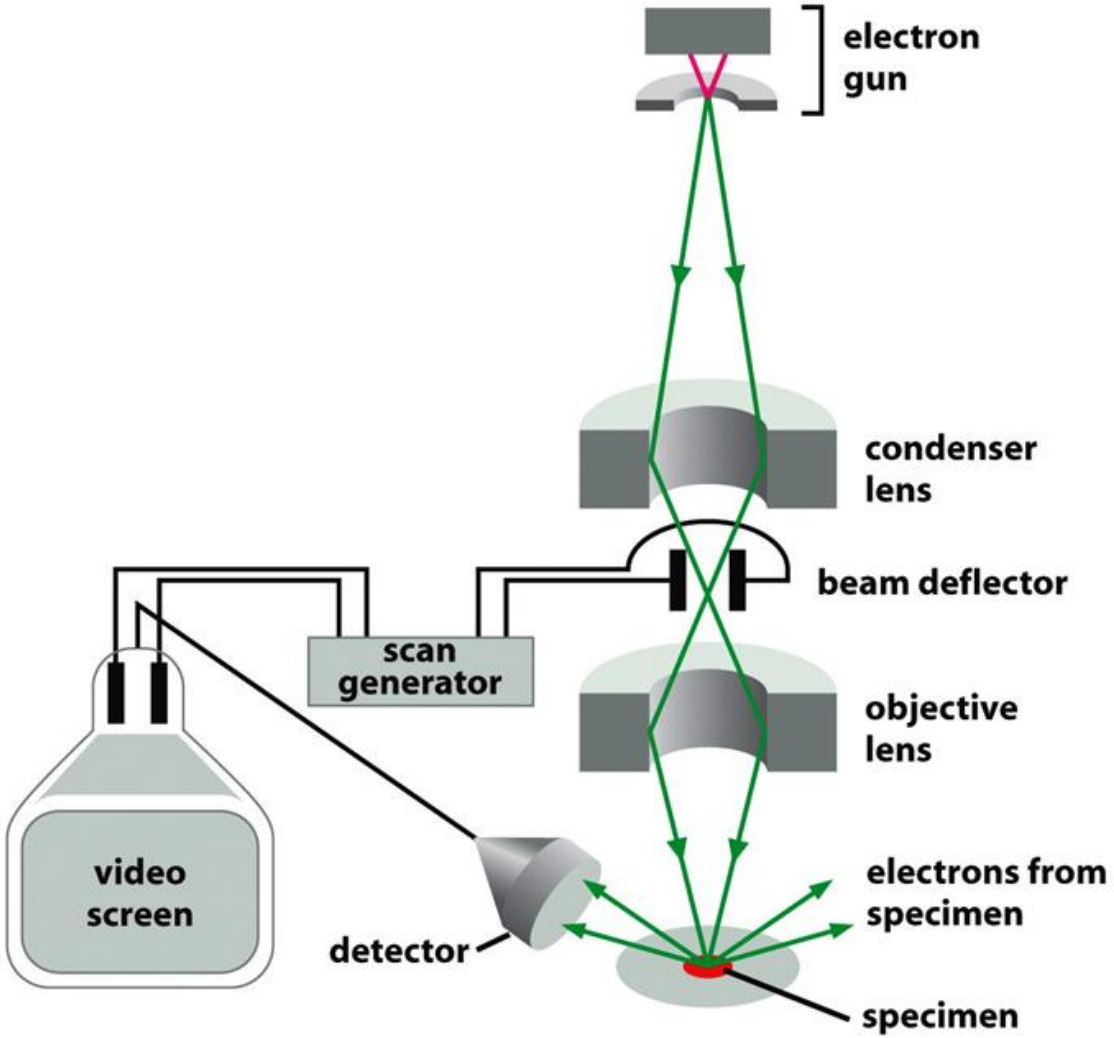
Images of immunogold electron microscope



6.2. Scanning electron microscope

- Produce 3-D structure for the surface of a specimen.
- Uses electrons that are scattered or emitted from the specimen’s surface.
- Lower resolution ~ 10nm
- Usually whole cells or tissues, not subcellular organelles.
- Less expensive

SEM


electron gun
condenser lens
beam deflector
scan generator
objective lens
video screen
electrons from specimen
detector
specimen


1 mm

6.3. Transmission electron microscope

- Electrons penetrate through the specimen
- Produce high resolution images
- Observed subcellular organelles, virus structure, etc.
- More expensive

TEM