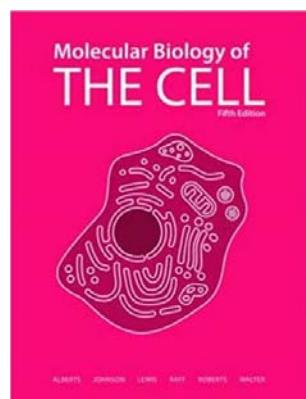
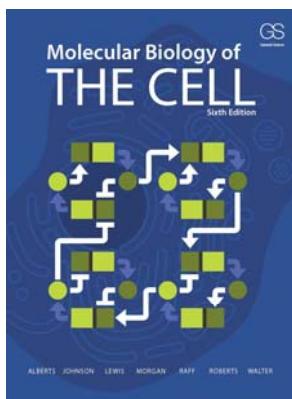


Optical analysis of cells: Techniques – requirements - results

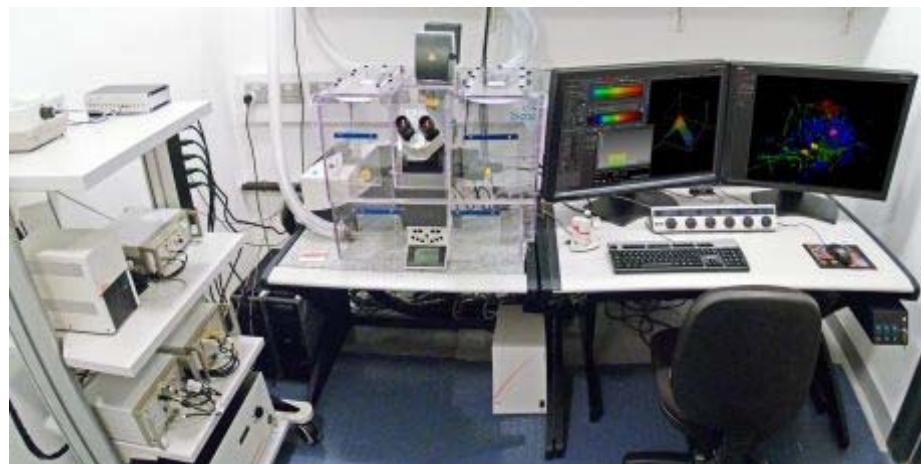
Outline

1. Light microscopy
2. Fluorescence microscopy
3. Confocal microscopy
4. The nanoscope
5. Advanced techniques in microscopy:
FRET, FLIM, FRAP, Photoactivation,
6. Electron microscopy (SEM/TEM)



Chapter 9: Visualizing Cells

Microscopy: different types of microscopes



Light microscopy from 1688 -2018



Leica TCP SP8 – FLIM (2018)
5 million RMB

Antoni van Leeuwenhoek's
single lens microscope (1688)

2,7 million RMB
(Christie's London, 2009)

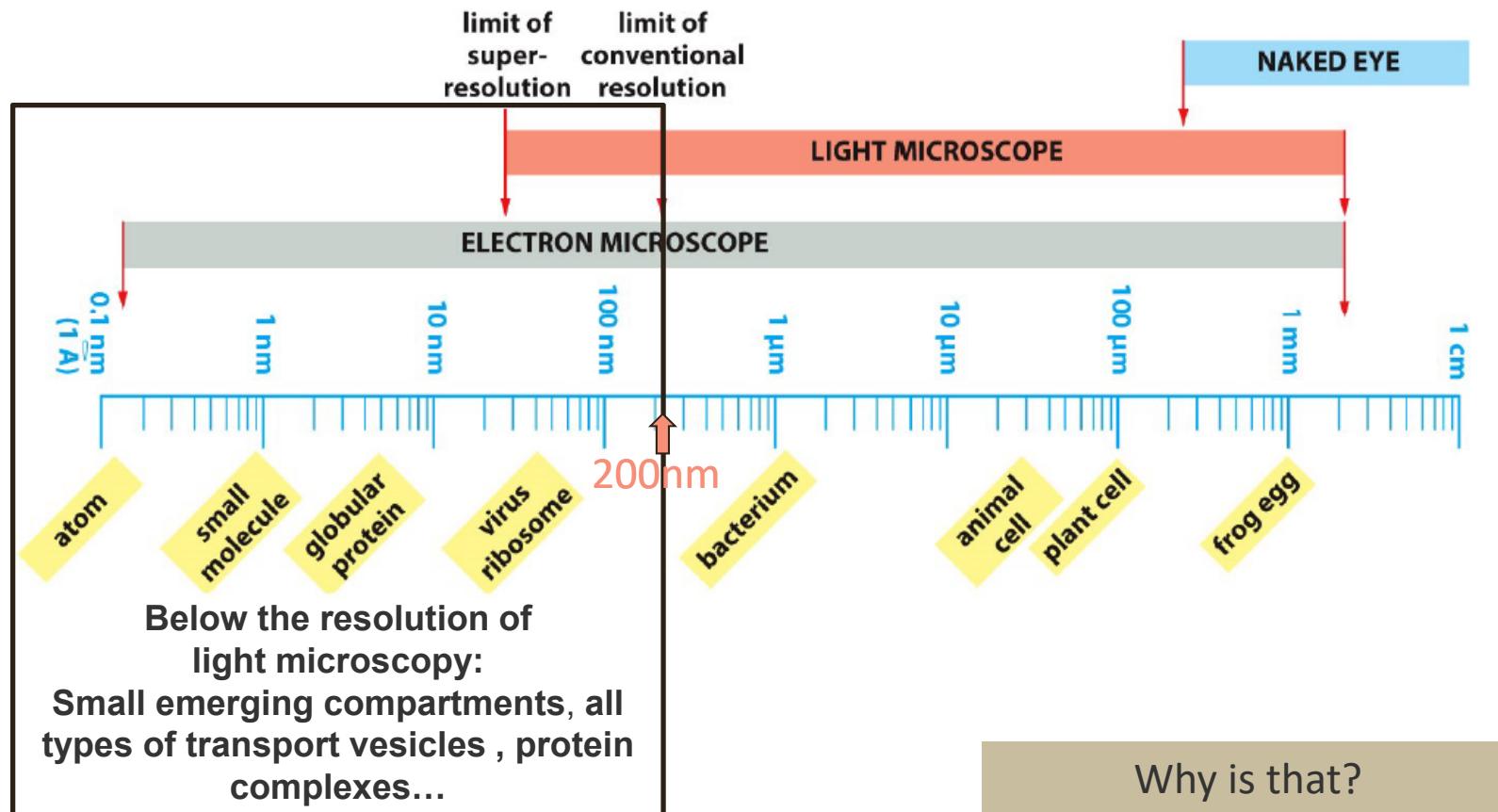
Sometimes its better so spend a little bit more....

Two major concepts: Light microscopy & electron microscopy

Light microscopy versus Electron microscopy:

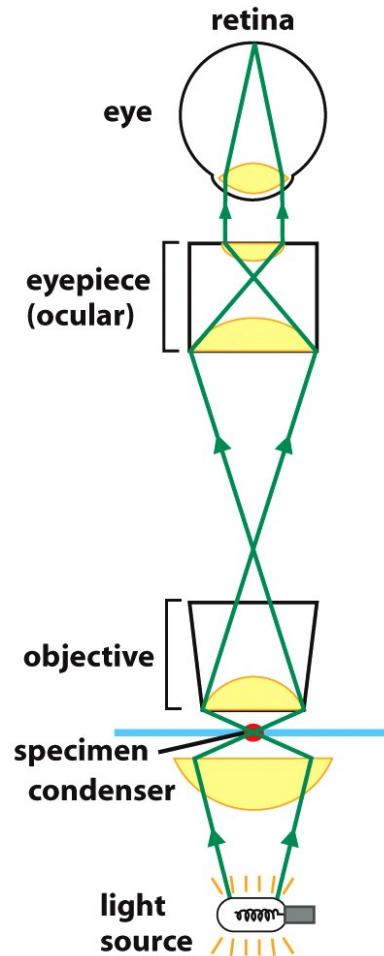
Resolution of light microscopy: 200 nanometer

Resolution of electron microscopy: 0.1 nanometer



The principle of light microscopy

Light path of a microscope



The combination of objective lenses and eye piece lenses are arranged to focus an image of the illuminated specimen in the eye

A cone of light rays is focused onto the **specimen** by the condenser

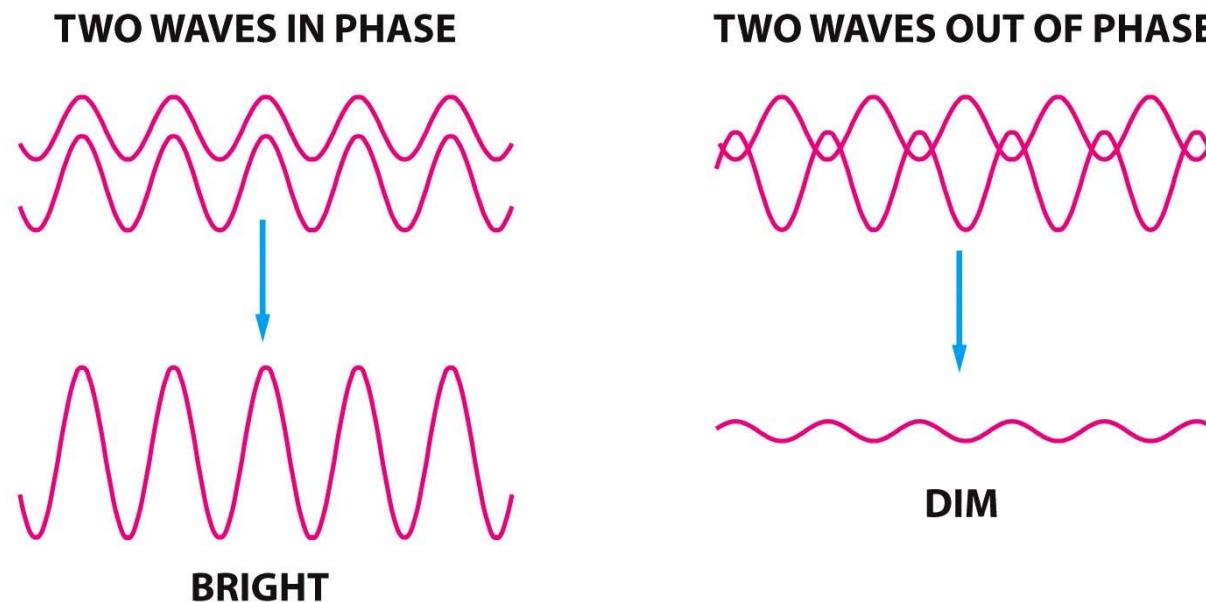


Magnification : up to 1000 fold
Eye piece: up to **10X**
Objective: up to **100X**

What is resolution and why is it important?

First of all, light has a wave nature: light waves

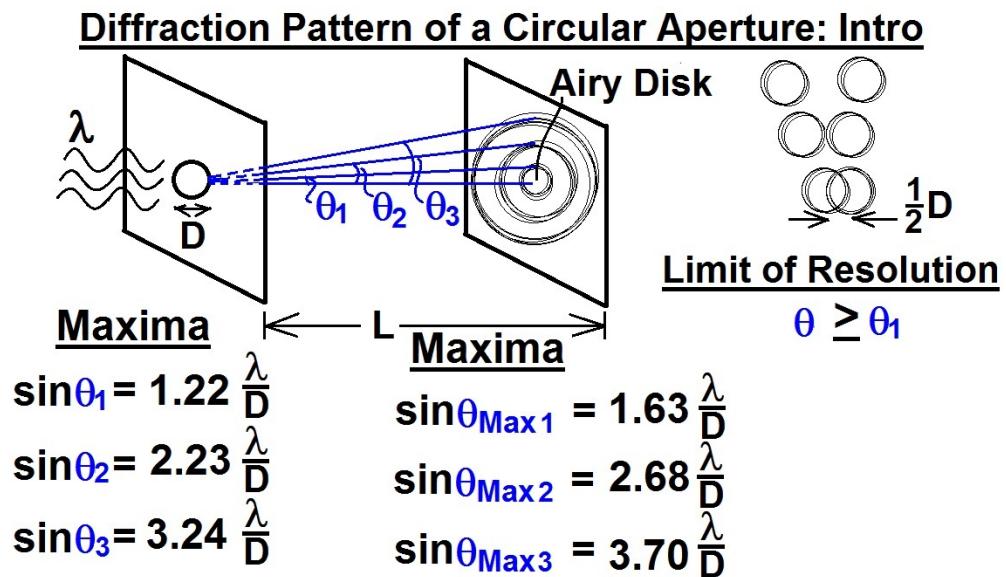
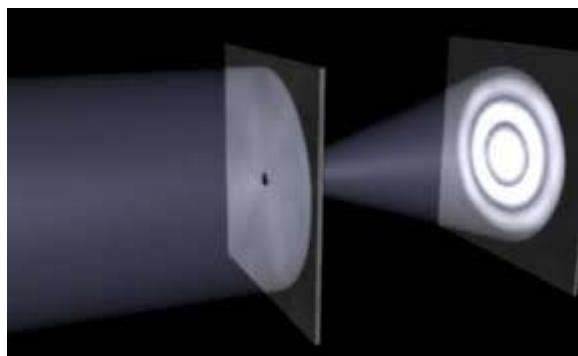
- All light waves transit optical systems with slightly different routes, thereby interfering with each other and cause optical diffraction effects



What is resolution and why is it important?

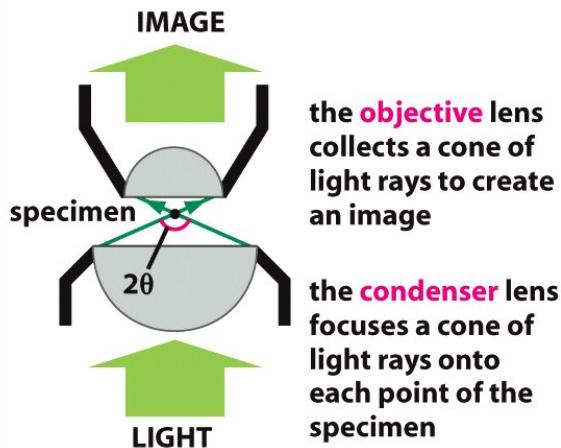
Next, interaction of light with objects:

- **Interaction of light with objects** alters the phase relationships of the light waves, which **results in interference effects**.
- **At high magnification**, the shadow of a circular spot appears as set of concentric rings. That's why a single point - seen through a microscope - appears as a blurred disk and **two points close together merge into one**
- This **smallest distance** at which two objects appear distinct is the **limit of resolution**



What is resolution and why is it important?

LENSES



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)

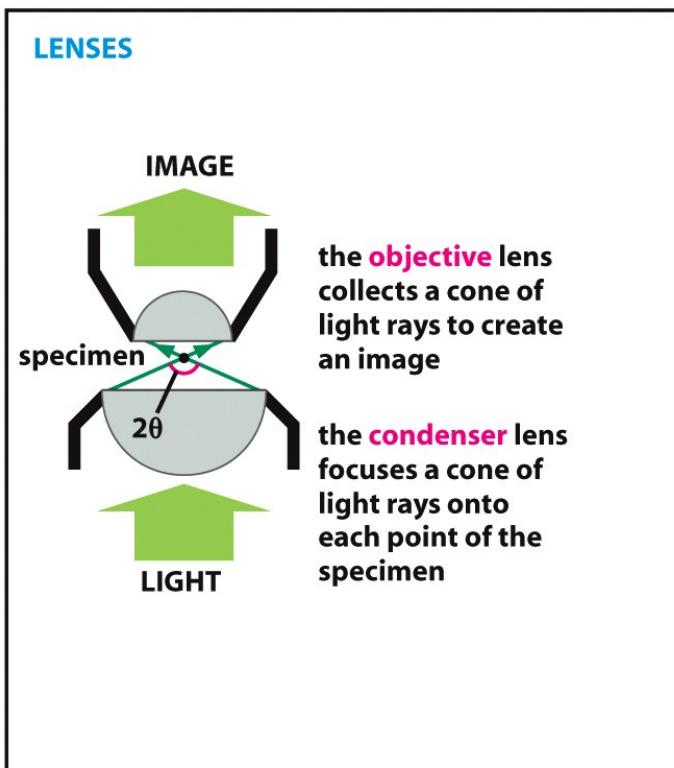
n = the refractive index of the medium (usually air or oil) separating the specimen from the objective and condenser lenses

λ = the wavelength of light used (for white light a figure of $0.53 \mu\text{m}$ 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.

Resolution is limited by wave length (λ) and numerical aperture



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

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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 $0.53 \mu\text{m}$ is commonly assumed)

The smaller the number or the shorter the wavelength, the bigger/higher is the resolution!

That's why the resolutions are so different:
LM: 200nm
EM: 0.1nm

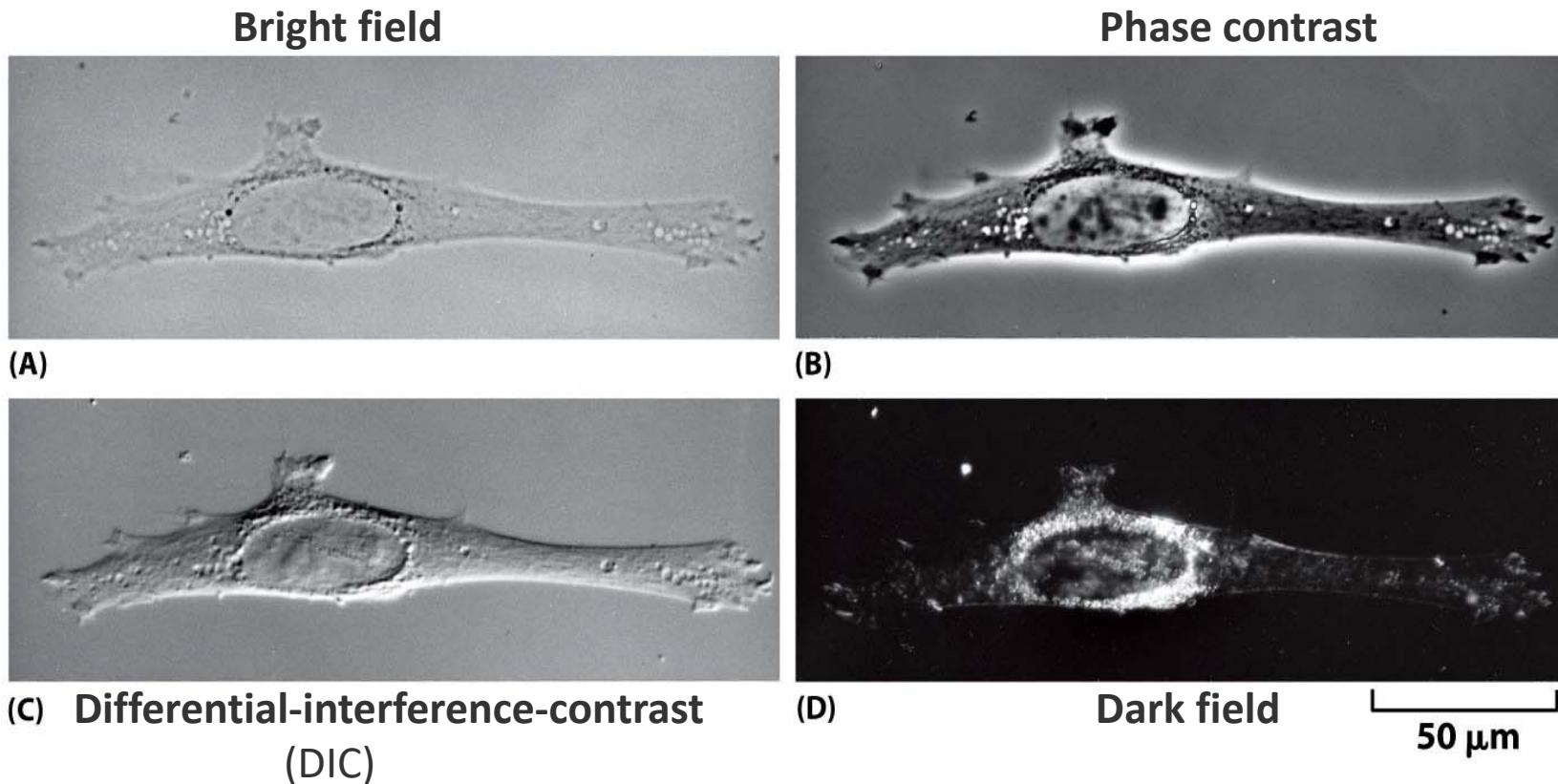
NUMERICAL APERTURE: $n \sin \theta$ in the equation above is called the numerical aperture of the lens (NA) and is a function of its light-collecting ability. For dry lenses this cannot be more than 1, but for oil-immersion lenses it can be as high as 1.4. The higher the numerical

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.

Light microscopy: Different techniques, different results...

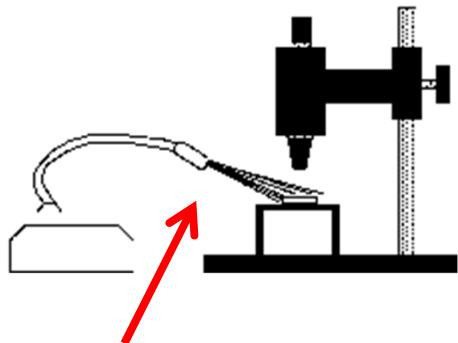
Problem: cells are usually colorless and translucent...

Solution : exploitation of interference effects of the light waves
(Phase contrast & Differential-interference -contrast)

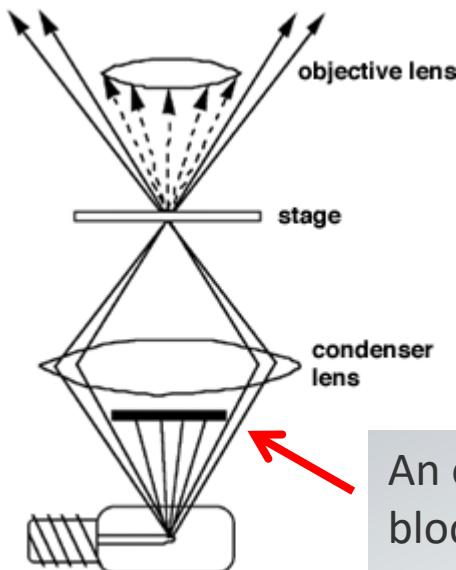


Dark field microscopy

Illumination from the side: only scattered light enters the objective



Light illuminates on the surface of the specimen



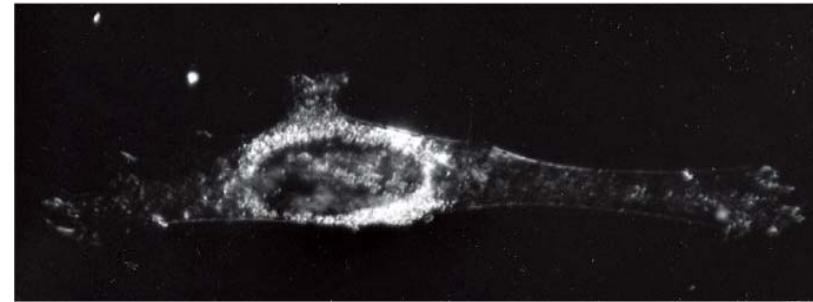
An opaque blocker to block lights directly penetrating specimen

Dark field is especially useful for finding cells in suspension

Comparison of dark-field and bright-field microscopy

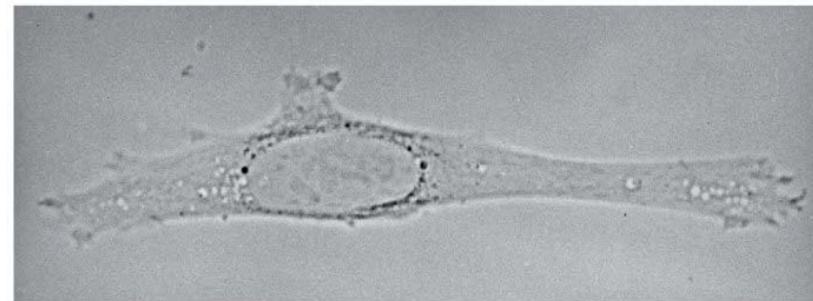
Dark field:

- **Cells appear bright**, background appears dark
- Light illuminates the specimen from the side ;
only scattered light enters the lenses

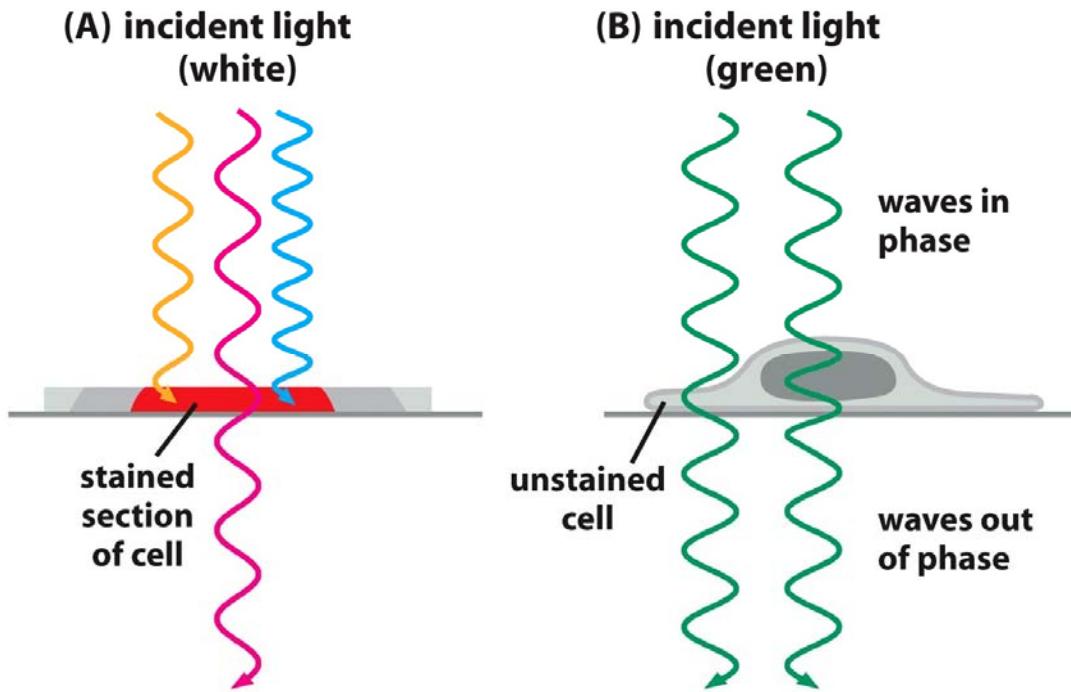


Bright field:

- **Cells appear dark**, background appears bright.
- light passes through a cell to form image directly



Phase contrast microscopy

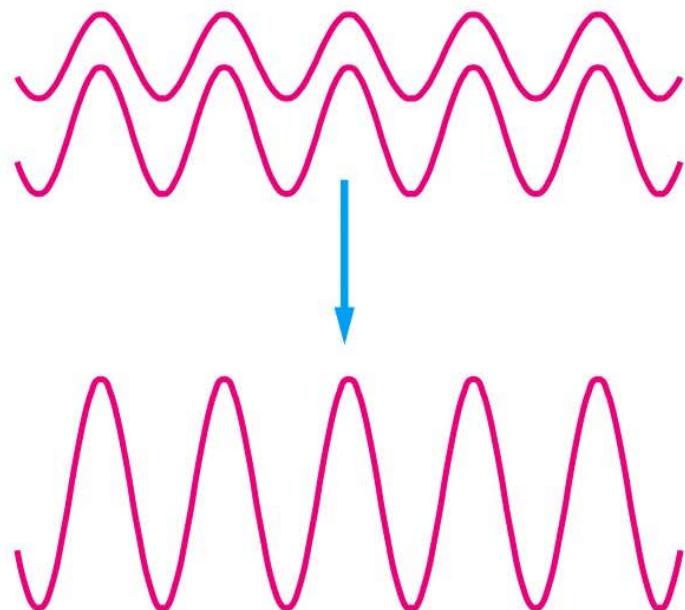


Phase contrast estimates phase changes after light passes through parts of a cell with different density, and then convert the change of phase into different brightness.

Phase contrast microscope is frequently used in Cell Biology

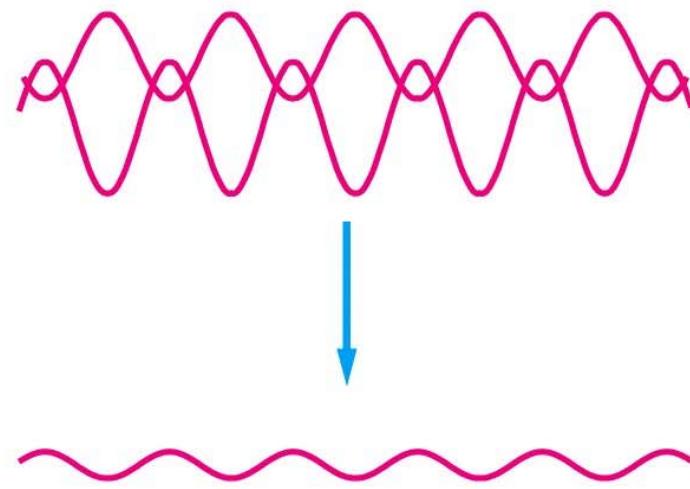
Phase difference results in difference in brightness

TWO WAVES IN PHASE



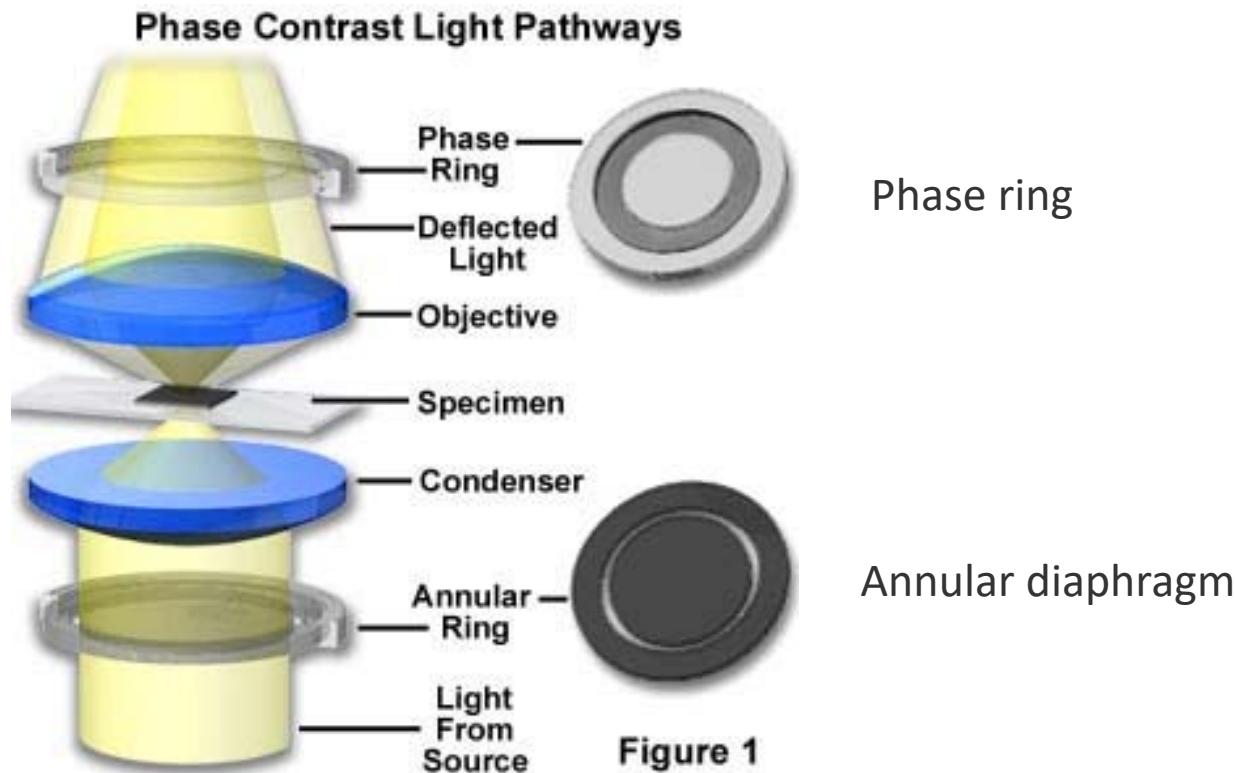
BRIGHT

TWO WAVES OUT OF PHASE



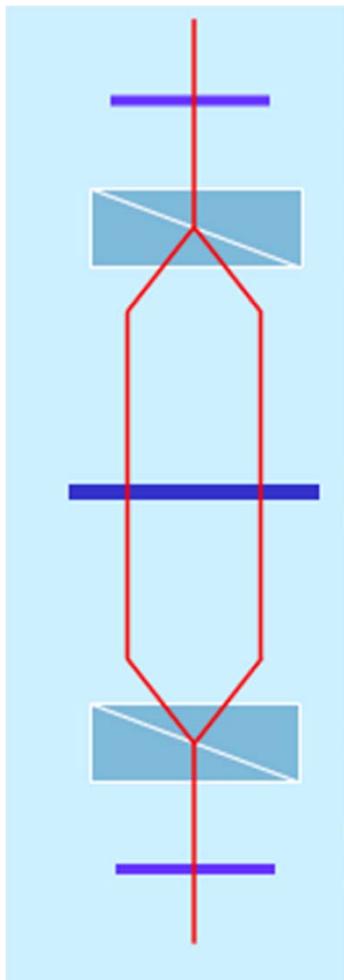
DIM

Components of a phase contrast microscope



Differential-Interference Contrast (DIC)

Requires linear polarized light



Interference analyzer

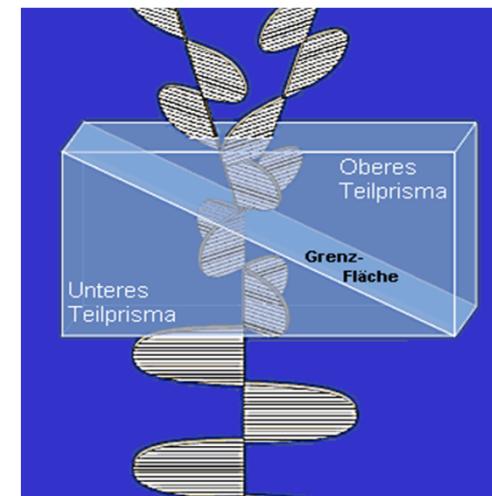
Recombining beams
using 2 Wollaston prisms

Specimen

Splitting beam
using 2 Wollaston prisms

Linear polarizer

2 Wollaston prisms
(splitting)



Differential-Interference Contrast (DIC)

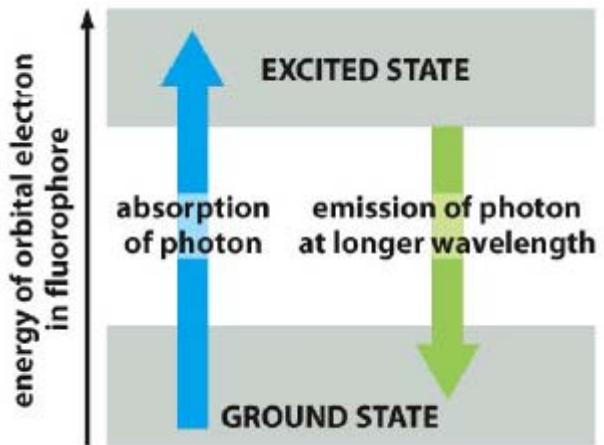
Advantages of DIC – high contrast and plasticity

- 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



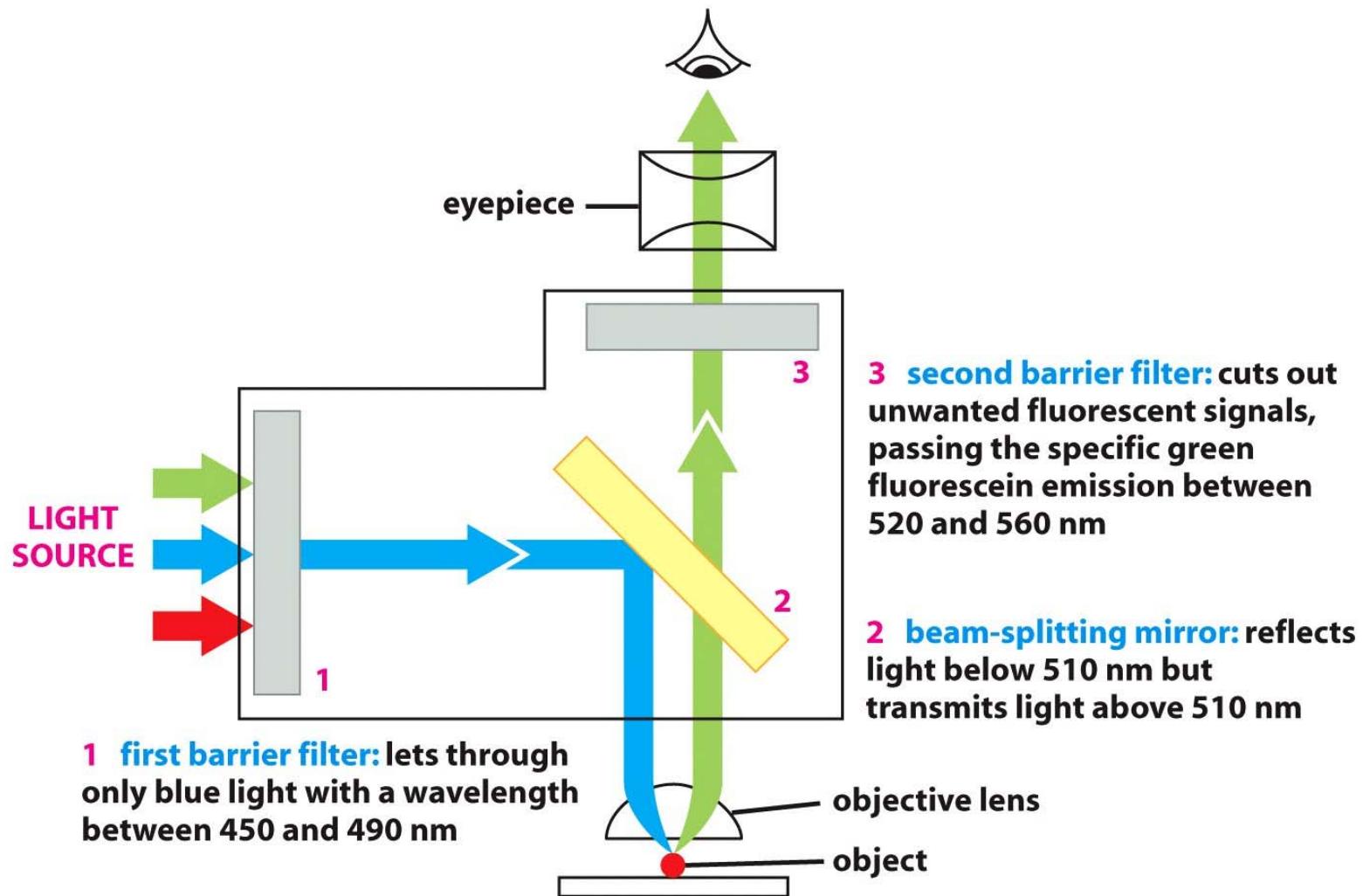
Light microscopy: Fluorescence microscopy

What is fluorescence?

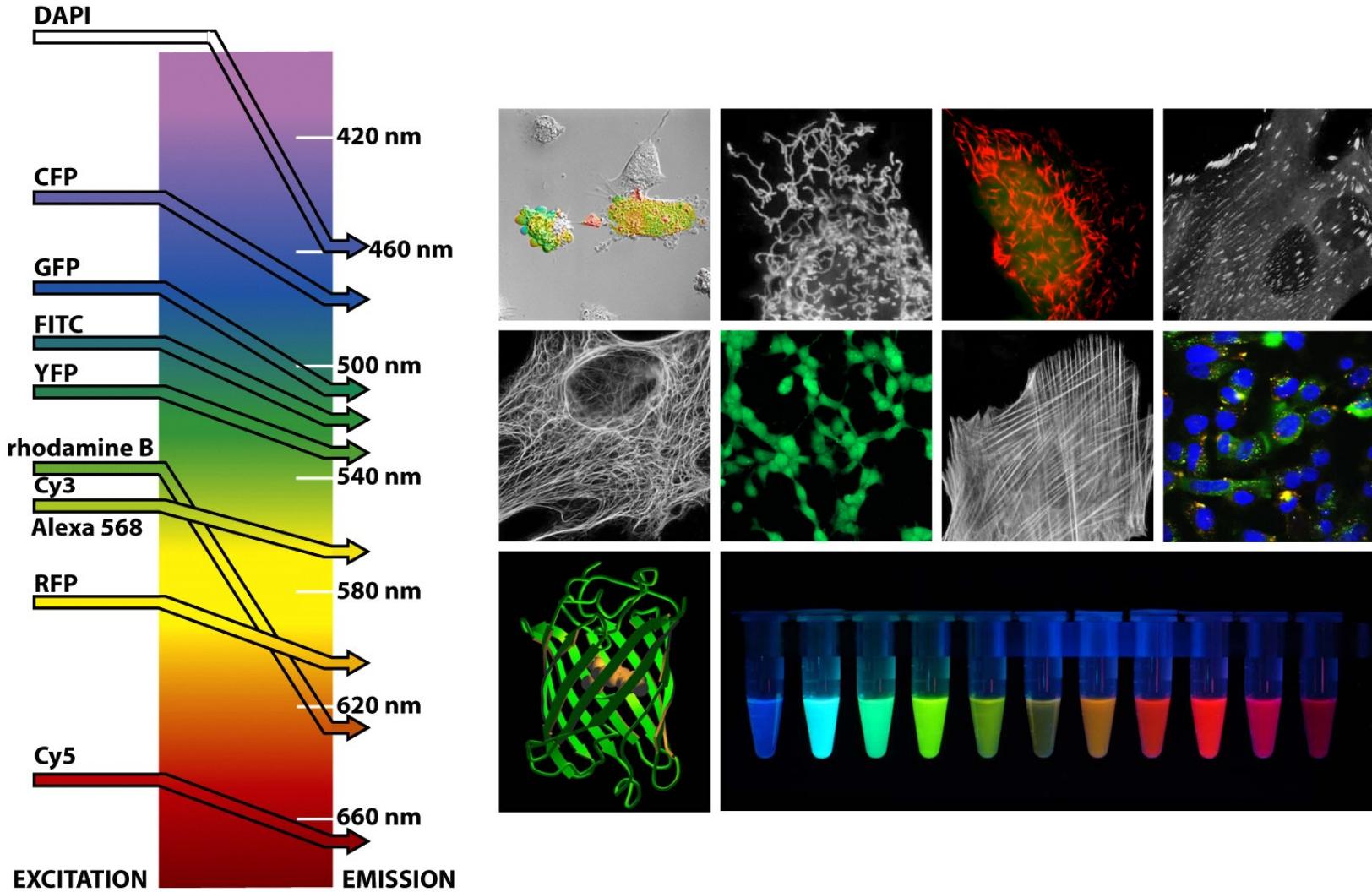


- A fluorophore absorbs a photon to be excited
- It is not stable and undergoes conformational change to emit a photon
- After it emits, it goes back to the ground state.
- The cycle can be repeated.
- The energy of excitation light is higher than the energy of emission light.
- Lower energy = longer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

Optical system of a fluorescence microscope

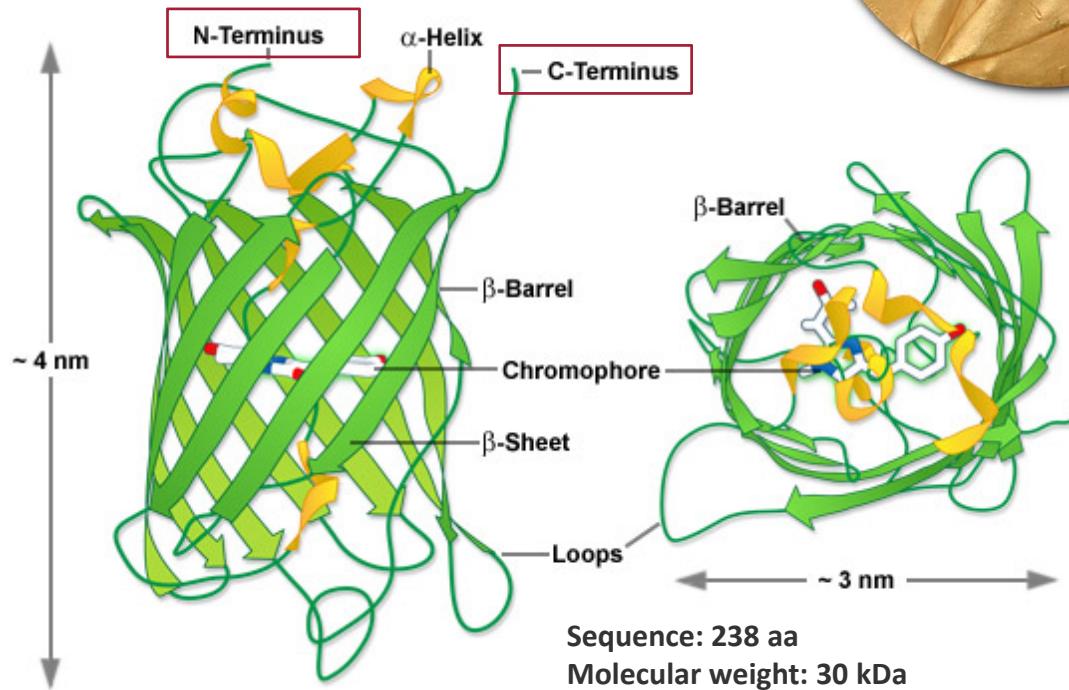


Fluorescence probes



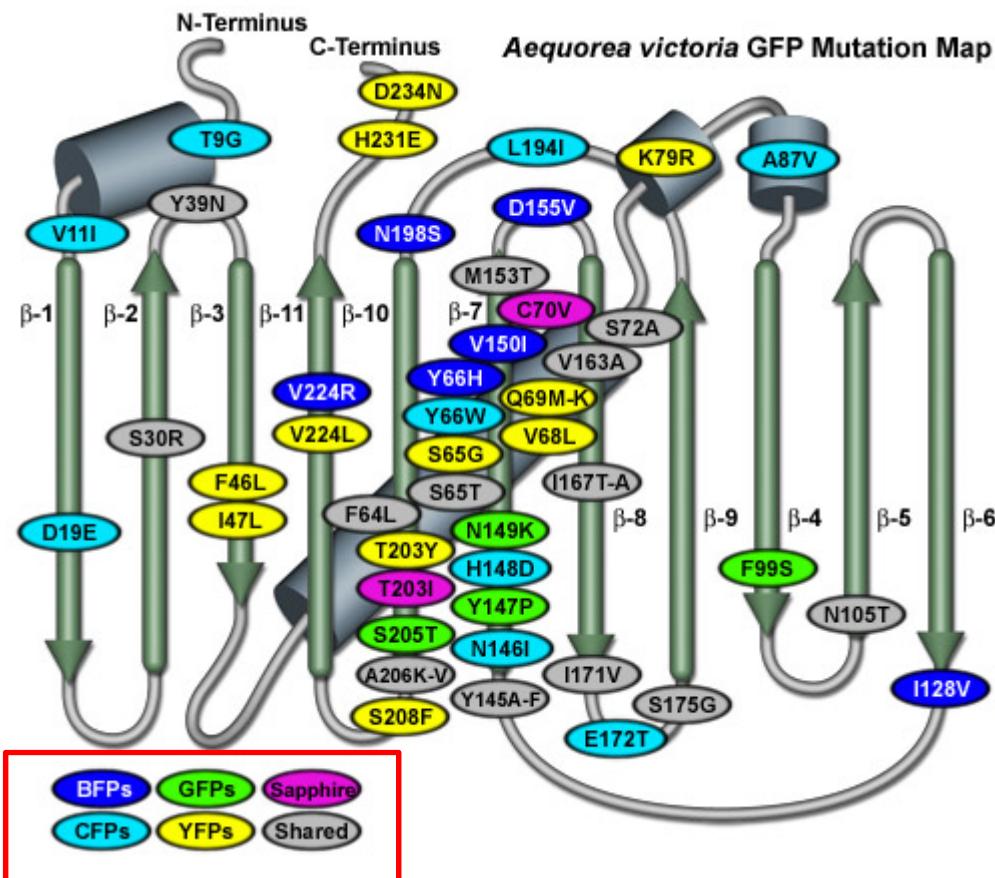
The green fluorescent protein GFP: a versatile tool for engineering reporter and marker proteins

Glowing proteins – a guiding star for biochemistry



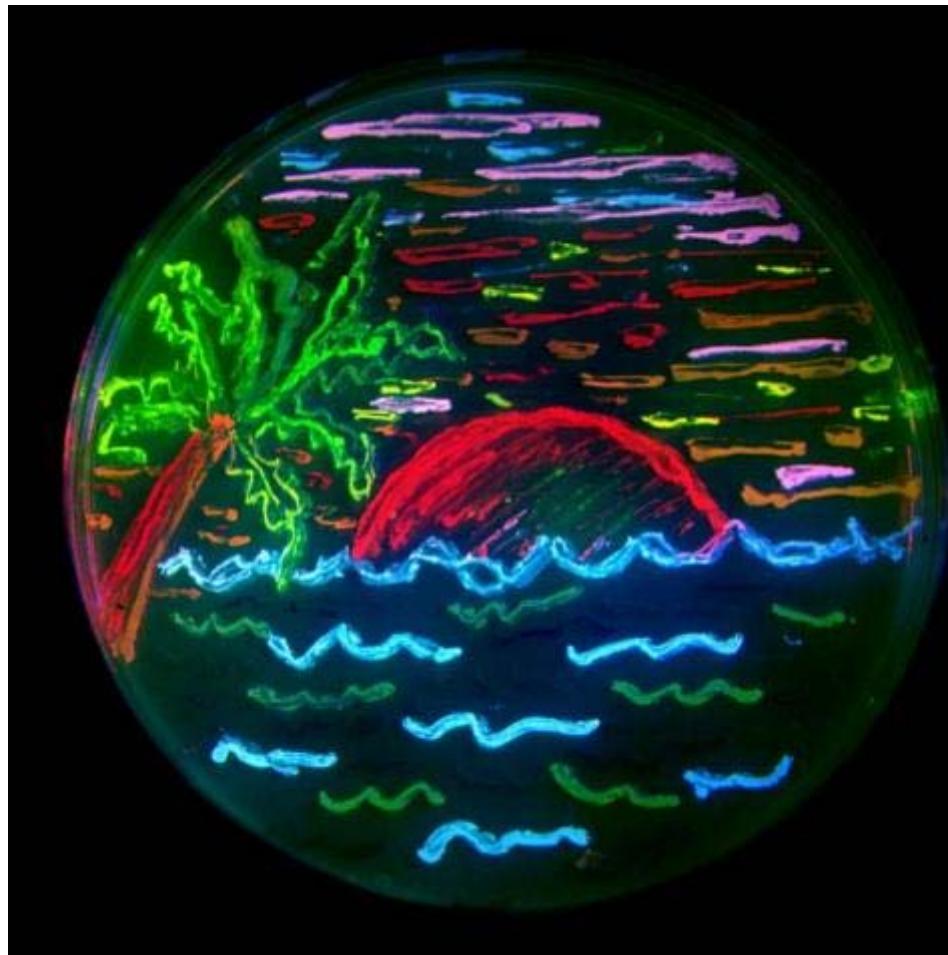
Many spectral variants of GFP are available...

All variants result from point mutations in the “original GFP” sequence



<http://zeiss-campus.magnet.fsu.edu>

... for multiple purposes

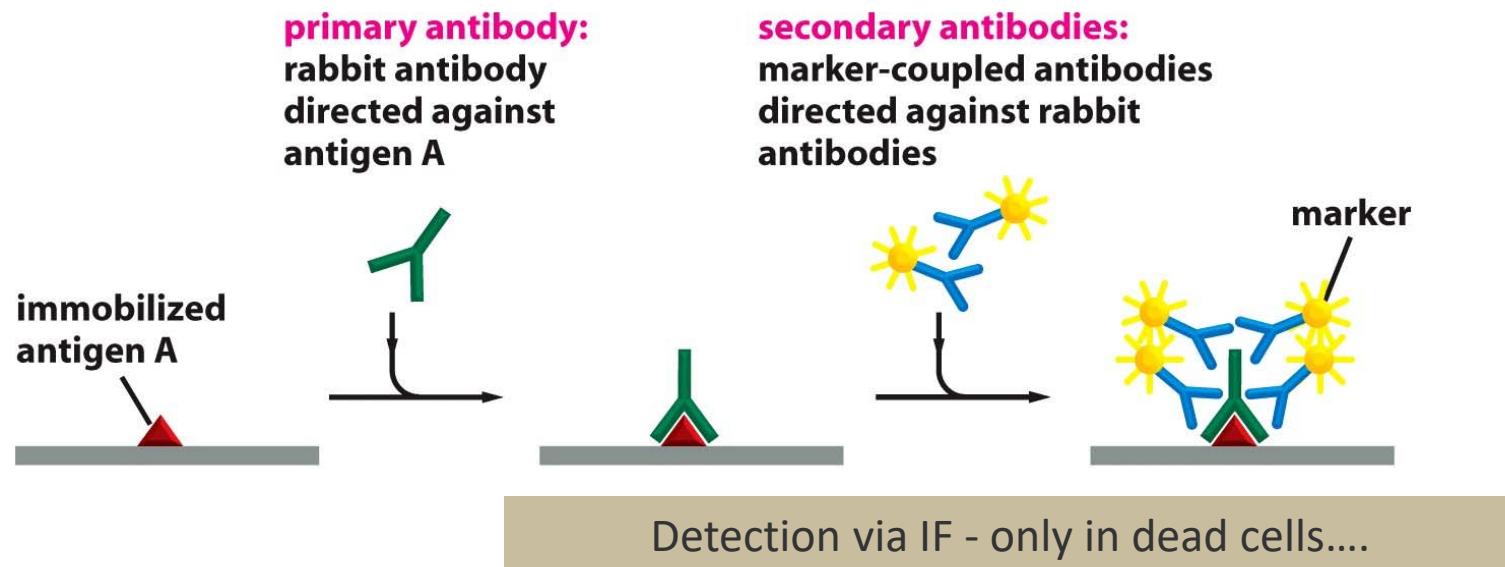


<http://www.tsienlab.ucsd.edu>

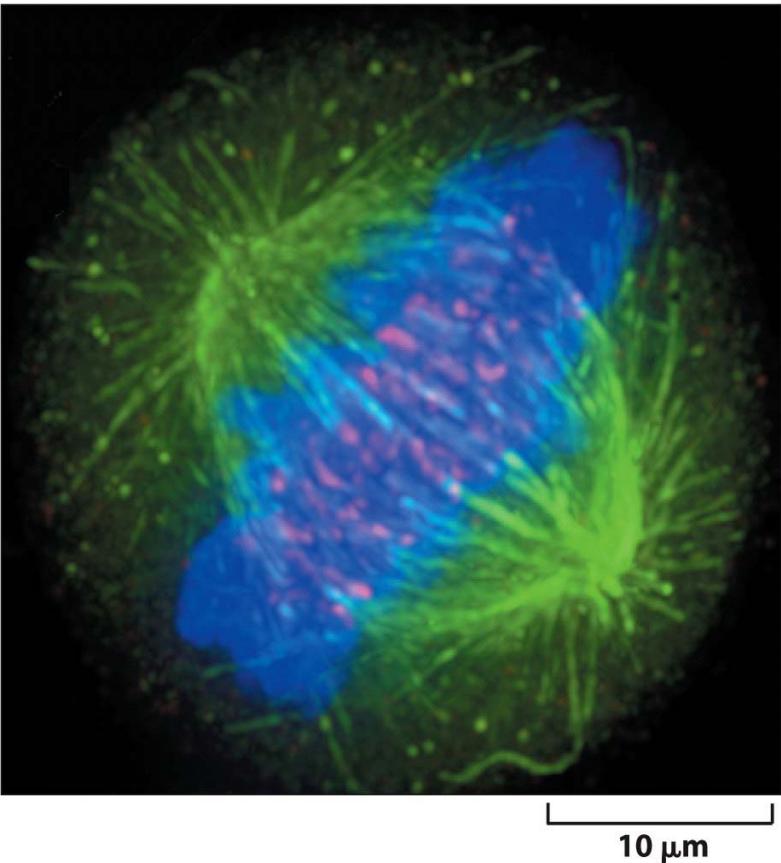
Fluorescence microscopy: Immunofluorescent microscopy (IF)

IF: specific detection rather than simple neutral observation...

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



IF: A Multi-fluorescent cell



Specimen preparation

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 antibody (conjugated-fluorescence)

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

Fluorescence microscopy for metabolite imaging

Aequorin, a luminescent protein
that glows blue in the presence of Ca^{2+}

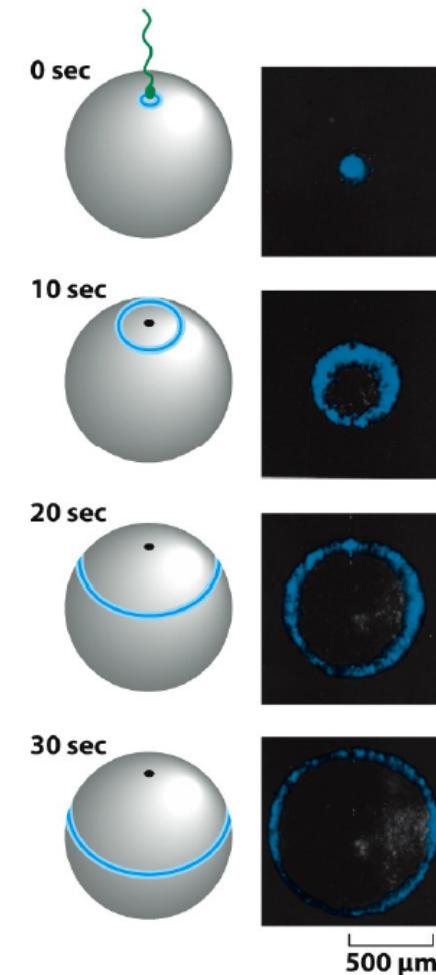


Figure 9-30 Molecular Biology of the Cell 6e (© Garland Science 2015)

Fluorescence microscopy for metabolite imaging

Fura-2 can sense free Ca^{2+} ions in cells

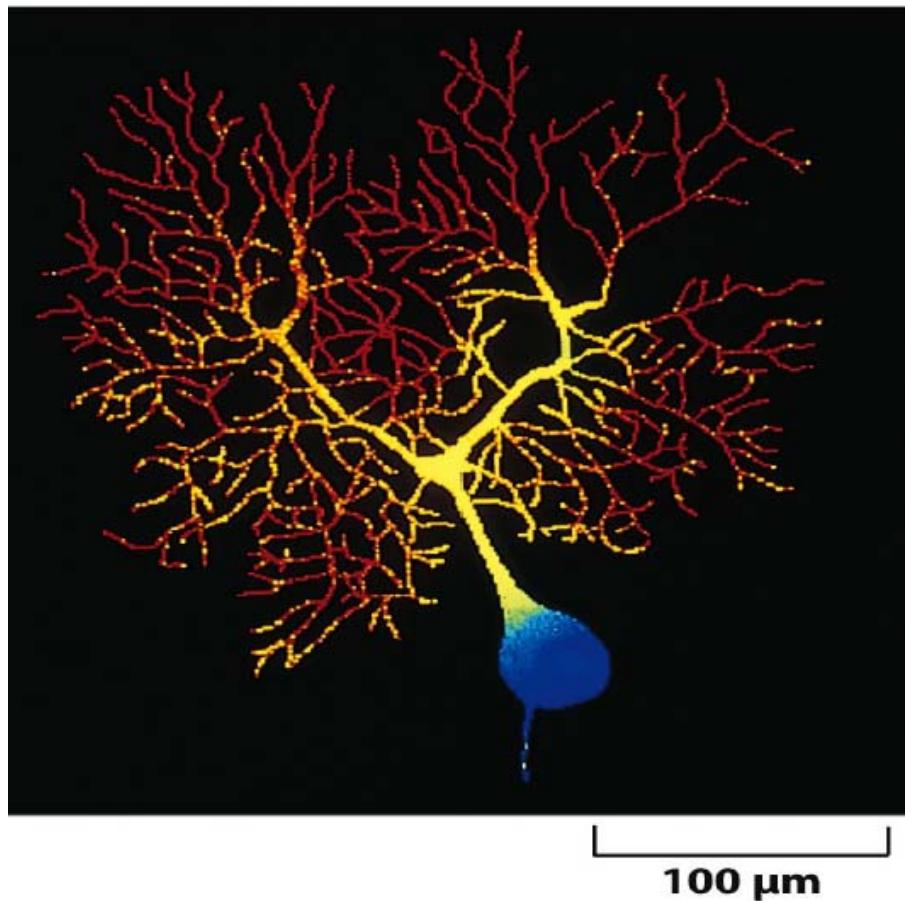
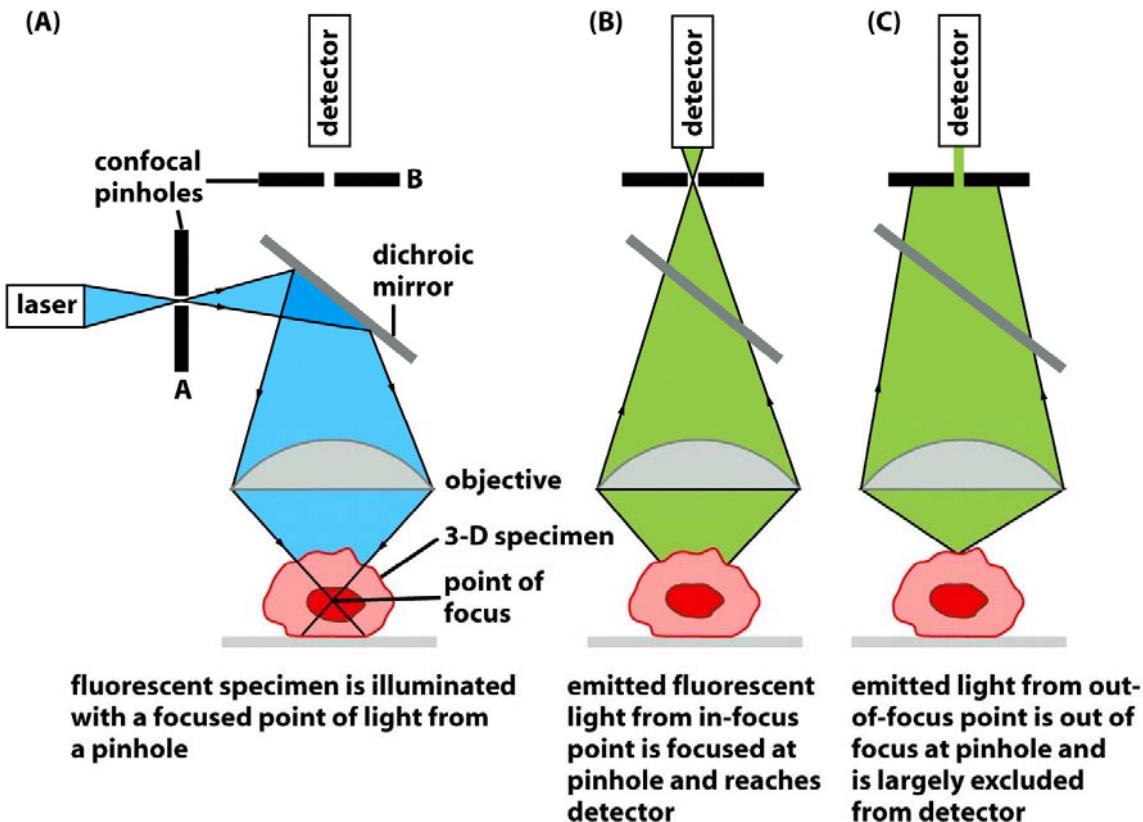


Figure 9-31 Molecular Biology of the Cell 6e (© Garland Science 2015)

Confocal microscopy

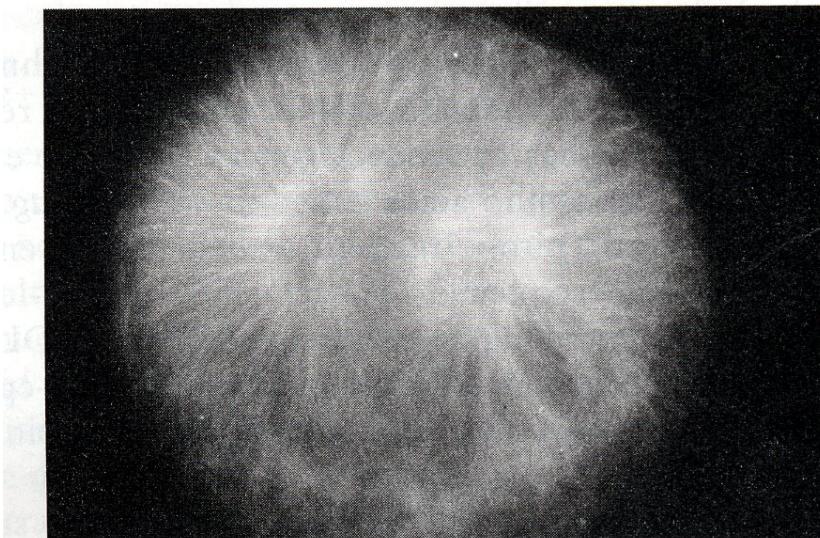


1. More accurately detection of protein localization in a 3-D location.
2. Use laser beam as light source provide better resolution.
3. Use focused light from a pinhole to illuminate on a certain spot in specimen.
4. The of focus light is excluded from detection!

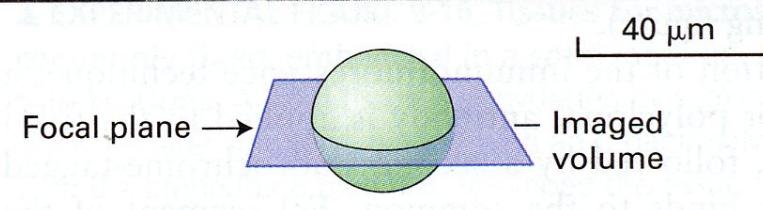
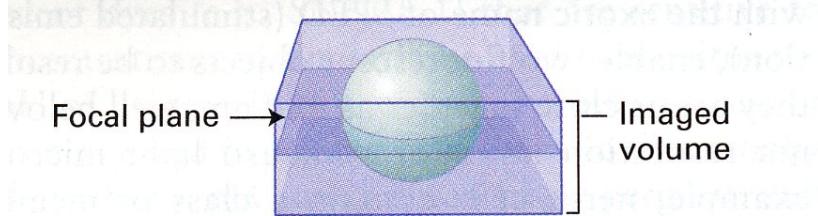
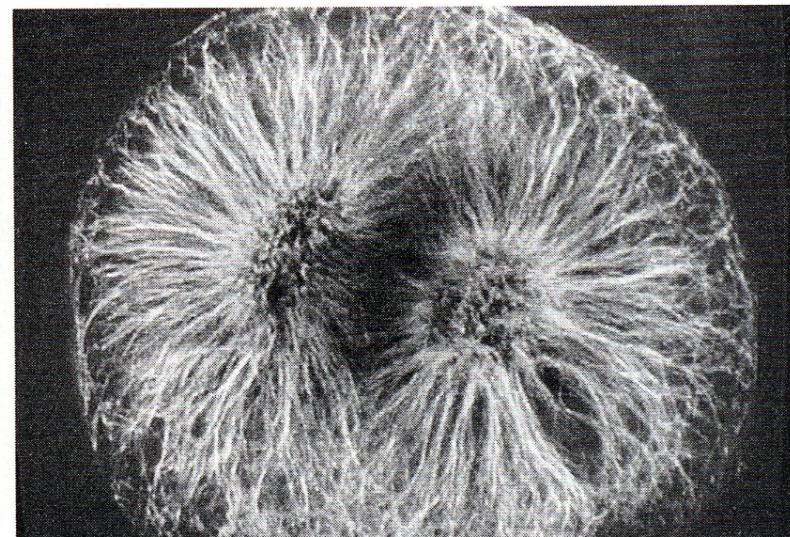
Comparison of conventional and confocal fluorescence microscopy

Advantage: removal of “background” fluorescence

Conventional microscope



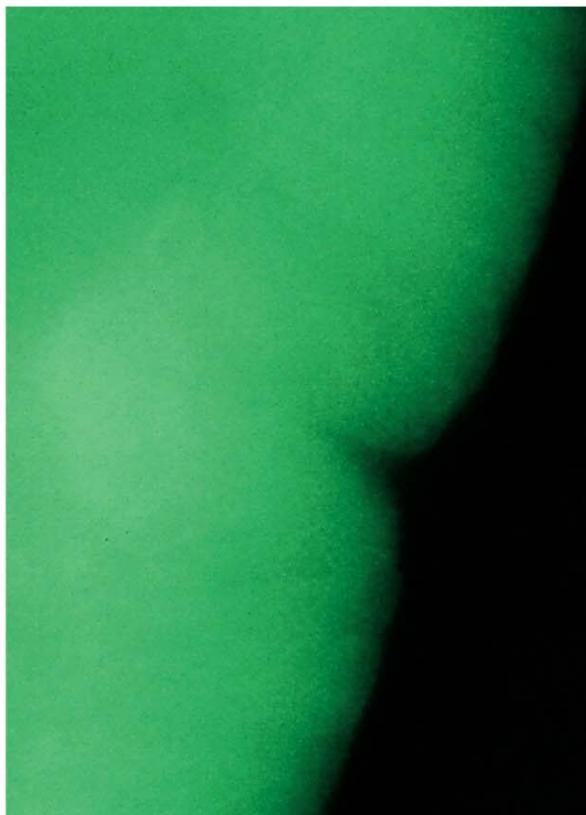
Confocal microscope



Comparison of conventional and confocal fluorescence microscopy

Advantage: removal of “background” fluorescence

Conventional microscope



(A)

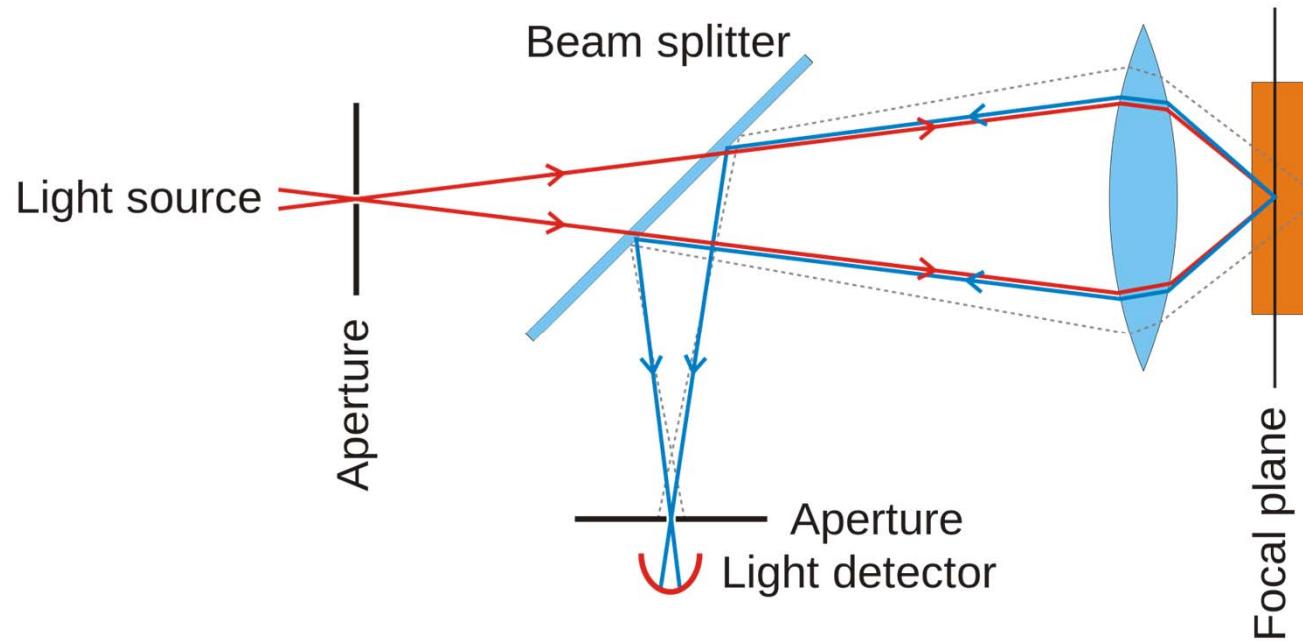
Confocal microscope



(B)

10 μm

Laser scanning confocal microscope



one pinhole

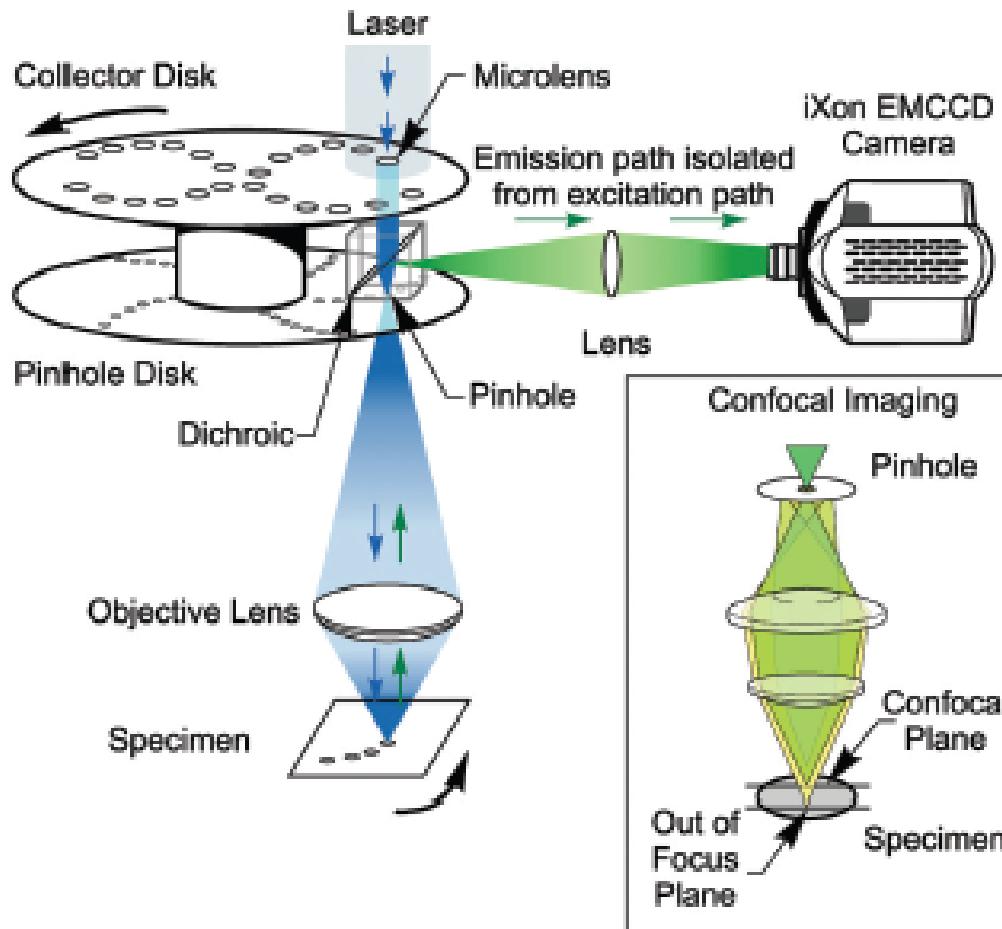
Good: better resolution

Bad: Illumination on specimen

for longer time, sometimes

cause bleaching for fluorescence signal

Spinning disk confocal microscope



Multiple pinholes allow specimen illumination at multiple points simultaneously
Good: quick and dynamic imaging
Bad: lower resolution

Nanoscopy

Fluorescence microscopy that surpasses the limitation of $0.2\mu\text{m}$

Stimulated emission depletion (STED) microscopy



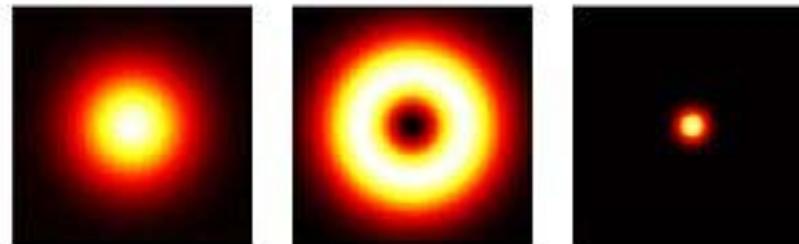
Stefan W. Hell



William E. Moerner



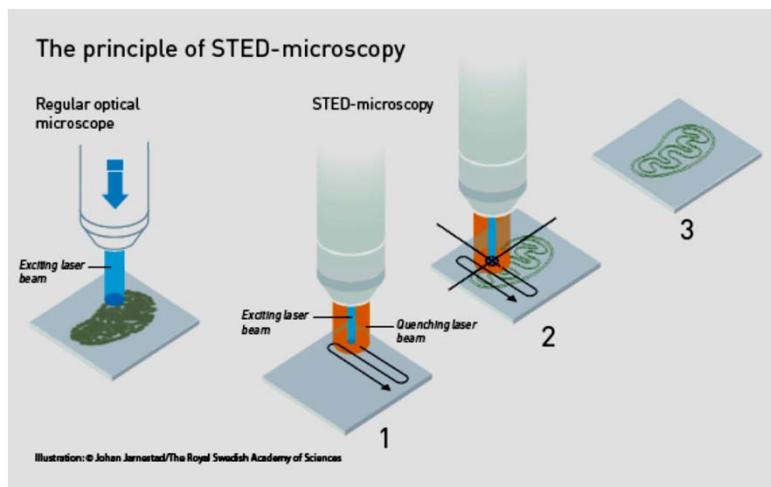
Eric Betzig



Excitation spot (2D, left), doughnut-shape de-excitation spot (center) and remaining area allowing fluorescence (right).

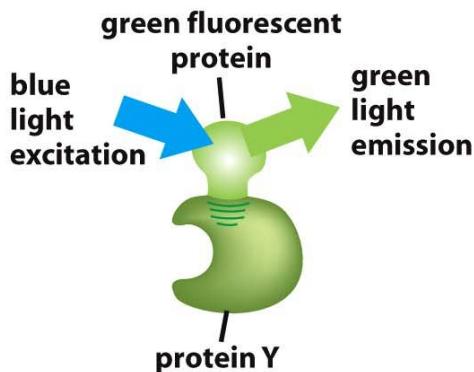
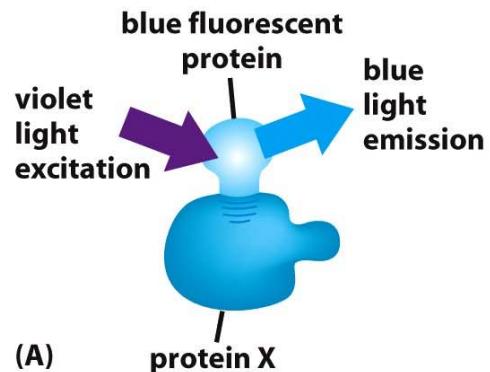


Nobel Laureates
in 2014 Chemistry

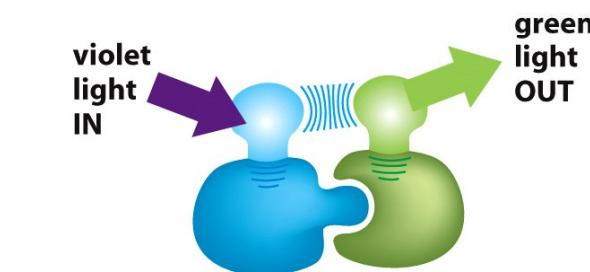


Förster-resonance energy transfer (FRET) microscopy

Detection of protein-protein interactions



(B) NO PROTEIN INTERACTION
NO EXCITATION OF GREEN FLUORESCENT PROTEIN, BLUE LIGHT DETECTED



(C) PROTEIN INTERACTION
FLUORESCENCE RESONANCE ENERGY TRANSFER, GREEN LIGHT DETECTED

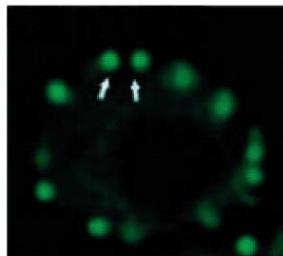
Two separate fluorescent dyes to label two putatively interacting proteins.

Interaction brings the fluorophores in **close proximity (<10nm)**. This triggers **energy transfer** from one fluorophore to the other, thereby causing the excitation of the other fluorophore and thus light emission of the second fluorophore!

Techniques: Förster-resonance energy transfer (FRET) microscopy

FRET occurs only in close distance (>10nm).

Sla1P-YFP



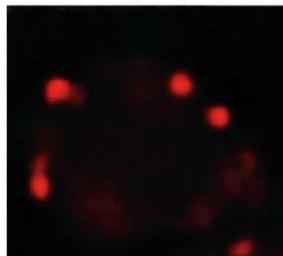
(A)

Abp1p-CFP

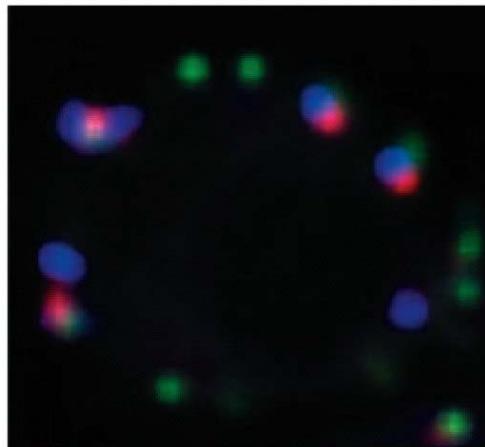


(B)

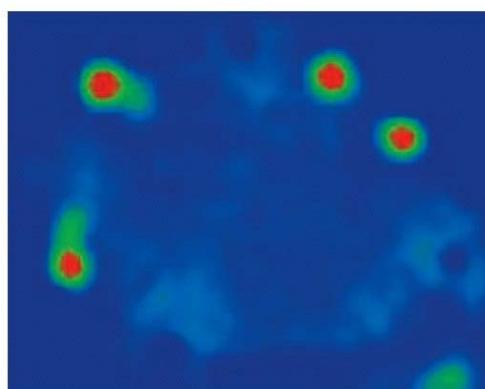
FRET signal
After Sla1p interacts
with Abp1p



(C)



(D)

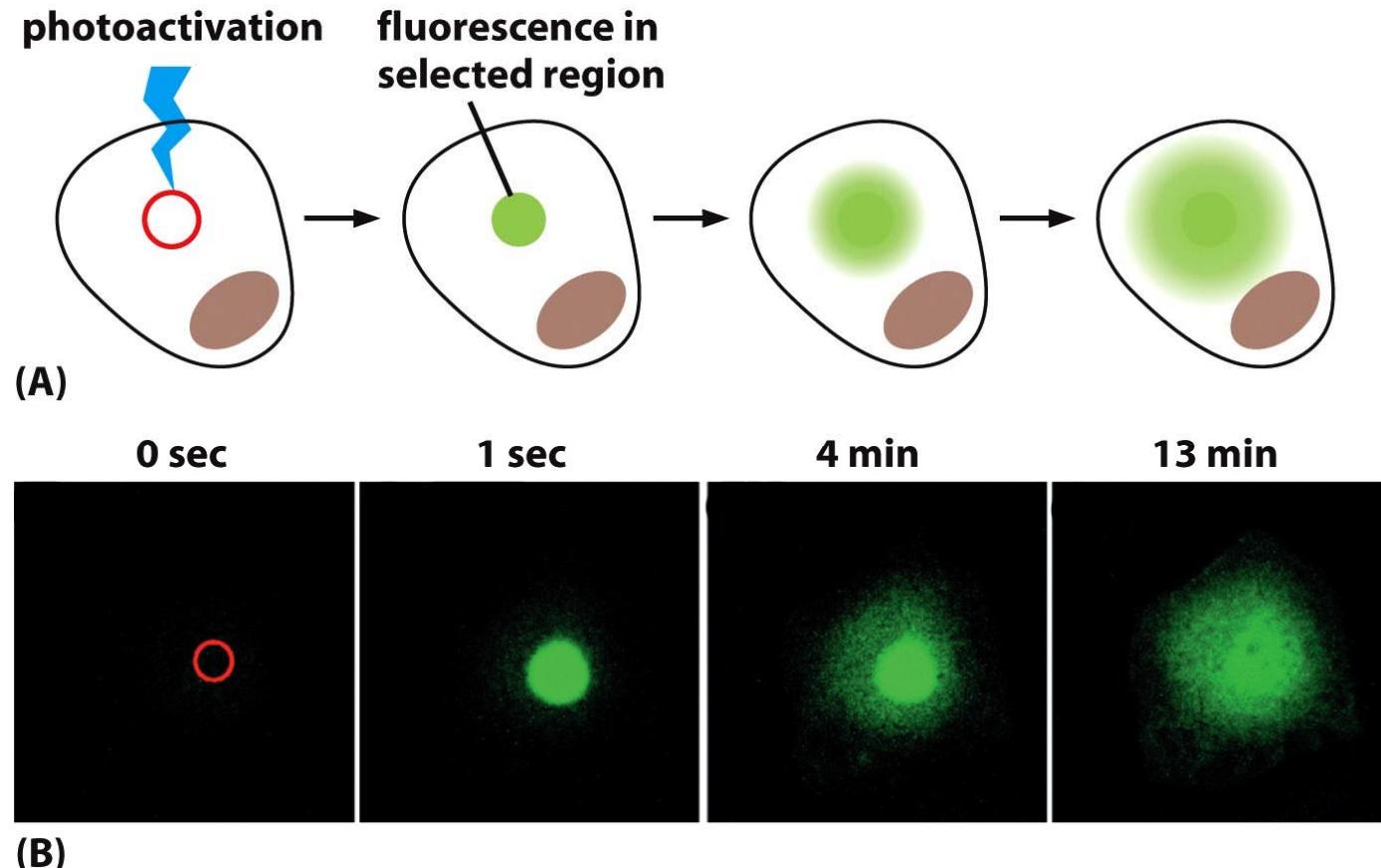


(E)

Rules for choosing the two fluorophores:

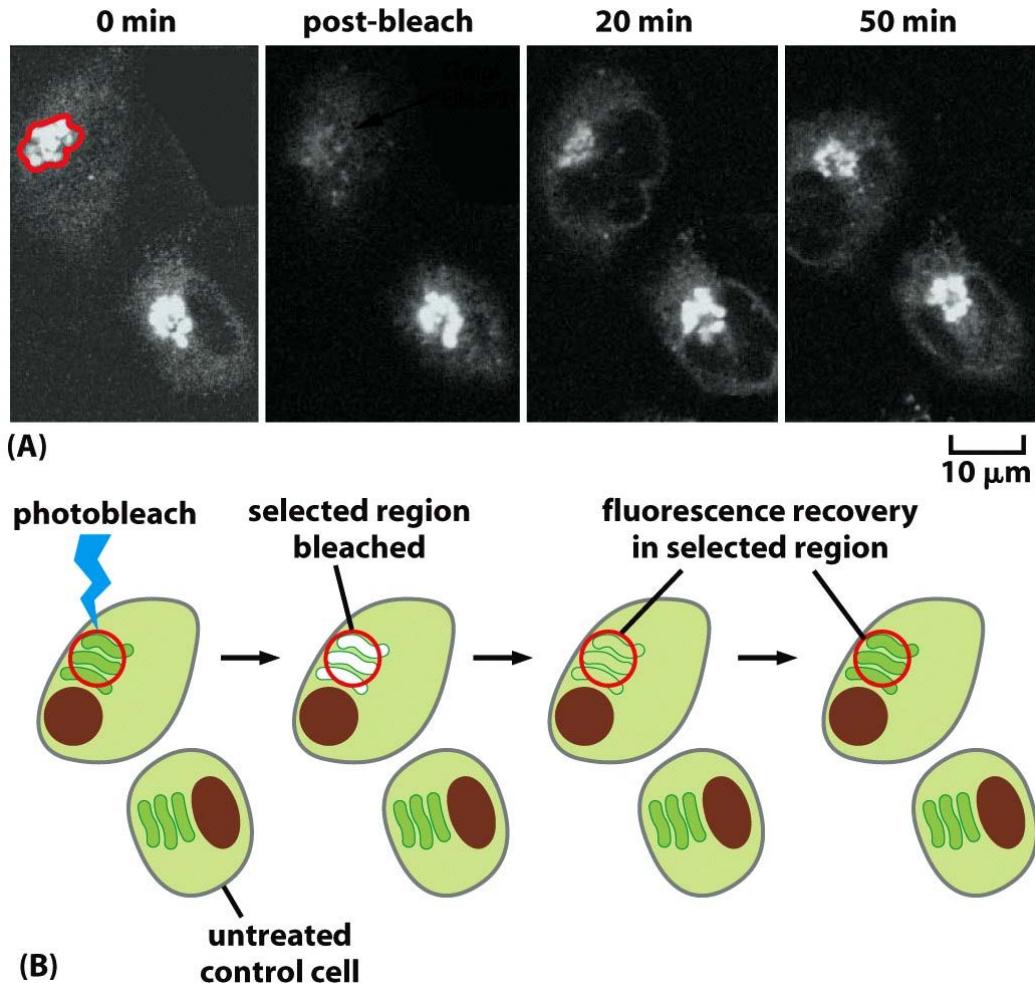
Emission spectrum of one must overlap with the excitation spectrum of the other.

Techniques: Photoactivation of fluorescent probes



Activate a target molecule by **photoactivation**, as time goes, the fluorescence signal changes. It can be used to monitor **dynamic processes** like protein trafficking, turnover, degradation, diffusion, etc.

Fluorescence recovery after photobleaching (FRAP)



FRAP is used to detect movement of molecules. Fluorescence is destroyed (bleached) in a selected region first. Then it is observed whether and how fast the fluorescence reappears. This is then due to arrival of new fluorescent molecules in that selected region.

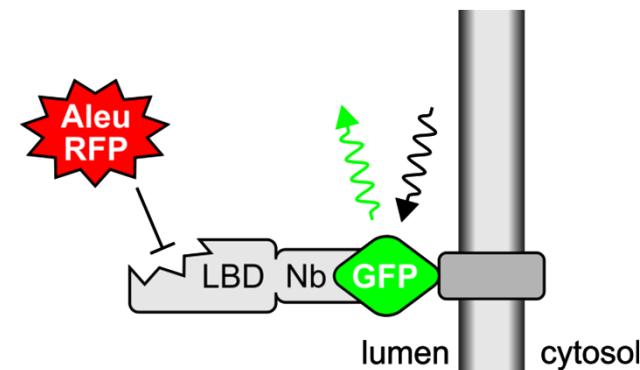
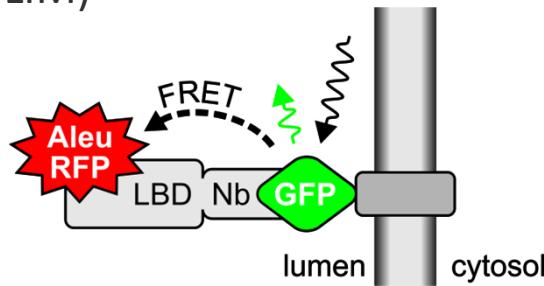
Detect molecular movement

Fluorescence lifetime imaging microscopy (FLIM)

FRET-FLIM analysis to detect VSR-ligand interaction

**Fluorescence lifetime microscopy (FLIM) to detect
Förster-resonance energy transfer (FRET) between GFP-Sensor and RFP-Ligand**

Fluorescence lifetime imaging microscopy
(FLIM)



Binding of the RFP-ligand triggers
Förster-resonance energy transfer
(FRET) from the GFP in the sensor

- reduced fluorescence lifetime

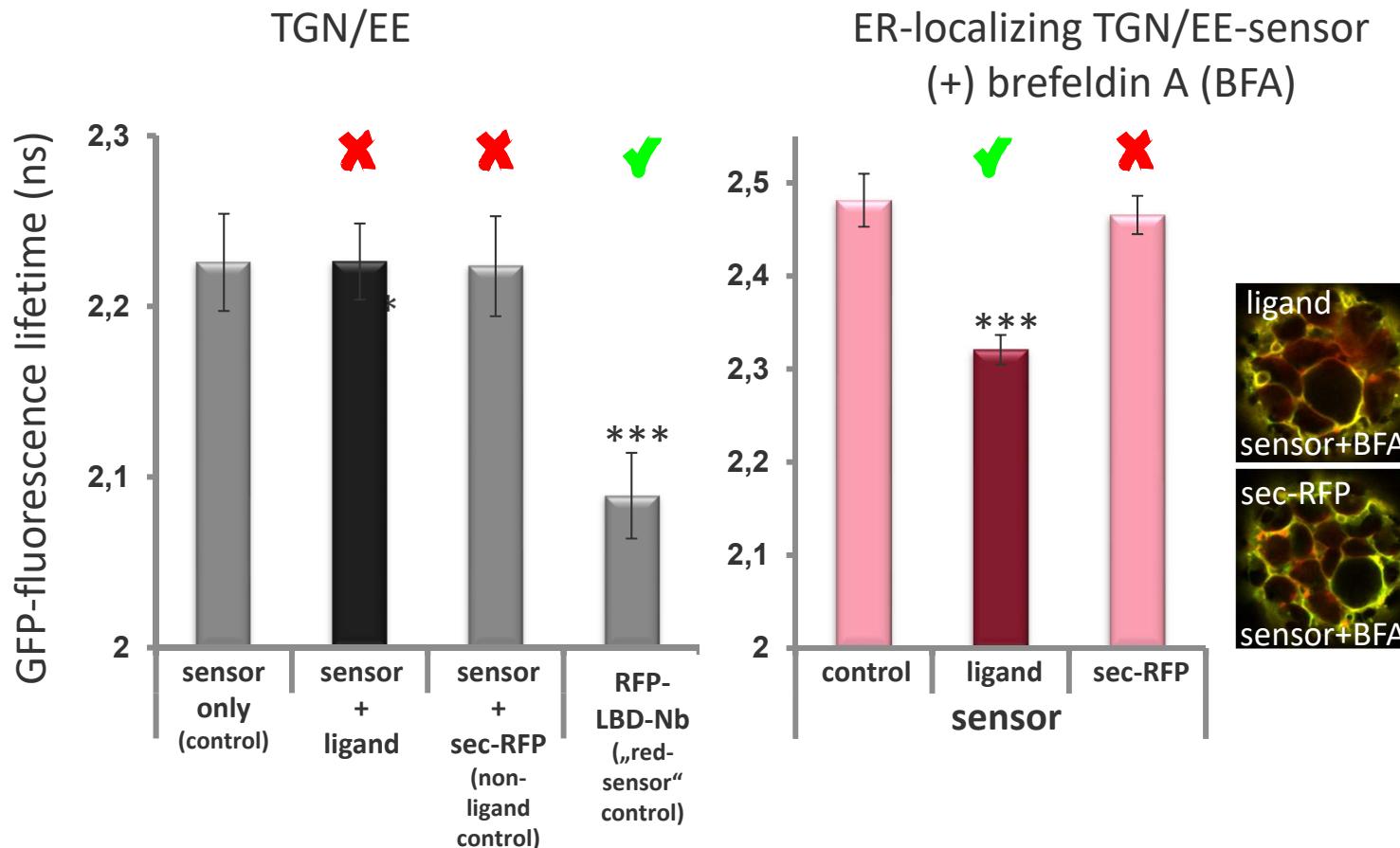
Unbound RFP-ligands don't trigger
Förster-resonance energy transfer
(FRET) from the GFP in the sensor

- no change of fluorescence lifetime

Fluorescence lifetime imaging microscopy (FLIM)

FRET-FLIM reveals that VSR don't bind ligands in the TGN/EE

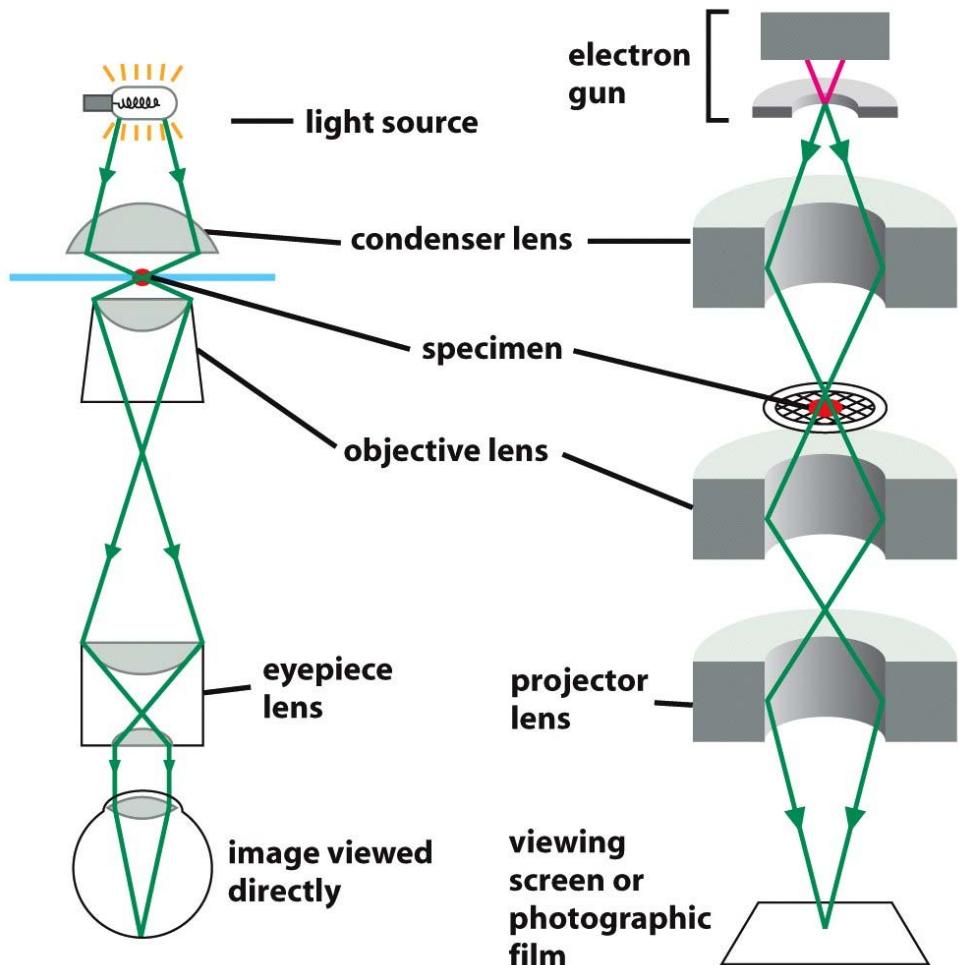
RFP-ligands do not reduce the fluorescence lifetime of the sensors in the TGN/EE



Künzl et al., 2016, *Nature Plants* 2

6. Electron microscopy

Transmission electron microscopy (TEM)



Resolution: 0.1nm-1nm

Transmission electron microscopy

Principle & features:

- Electrons penetrate the specimen
- Produce high resolution images
- Observed subcellular organelles, virus structure, etc.
- More expensive (no-not true anymore)
- Special preparation of the sample required

TEM requires special sample preparation

Always: Dehydrated specimen (no live cell imaging possible)

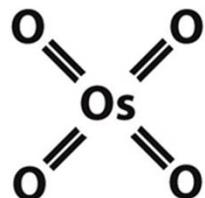
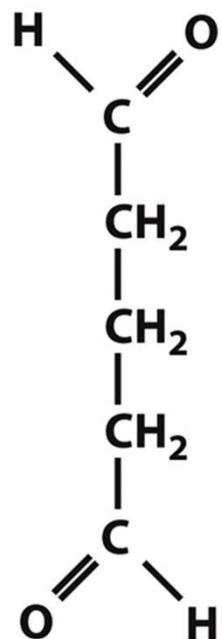
- Sample has to be dehydrated and ultra thin sliced, 50-100nm thick(**penetrating power for electron is very weak**)
- Sample is usually **rapidly frozen** to preserve its original state and to prevent formation of hexagonal ice crystals by **high-pressure freezing (HPF)**.
- Sometimes, chemical fixation with Glutaraldehyde (**to crosslink proteins**) and osmium tetroxide (**to stabilize lipid membranes**)
- Contrast can be enhanced by using heavy metals (uranyl-acetate and lead-citrate)
- Dehydration of the sample
- Resin-embedding

Fixatives for specimen preparation EM are extremely dangerous!!!

All chemicals that are used to fix the cells or
to enhance the contrast are very toxic!

ALWAYS:

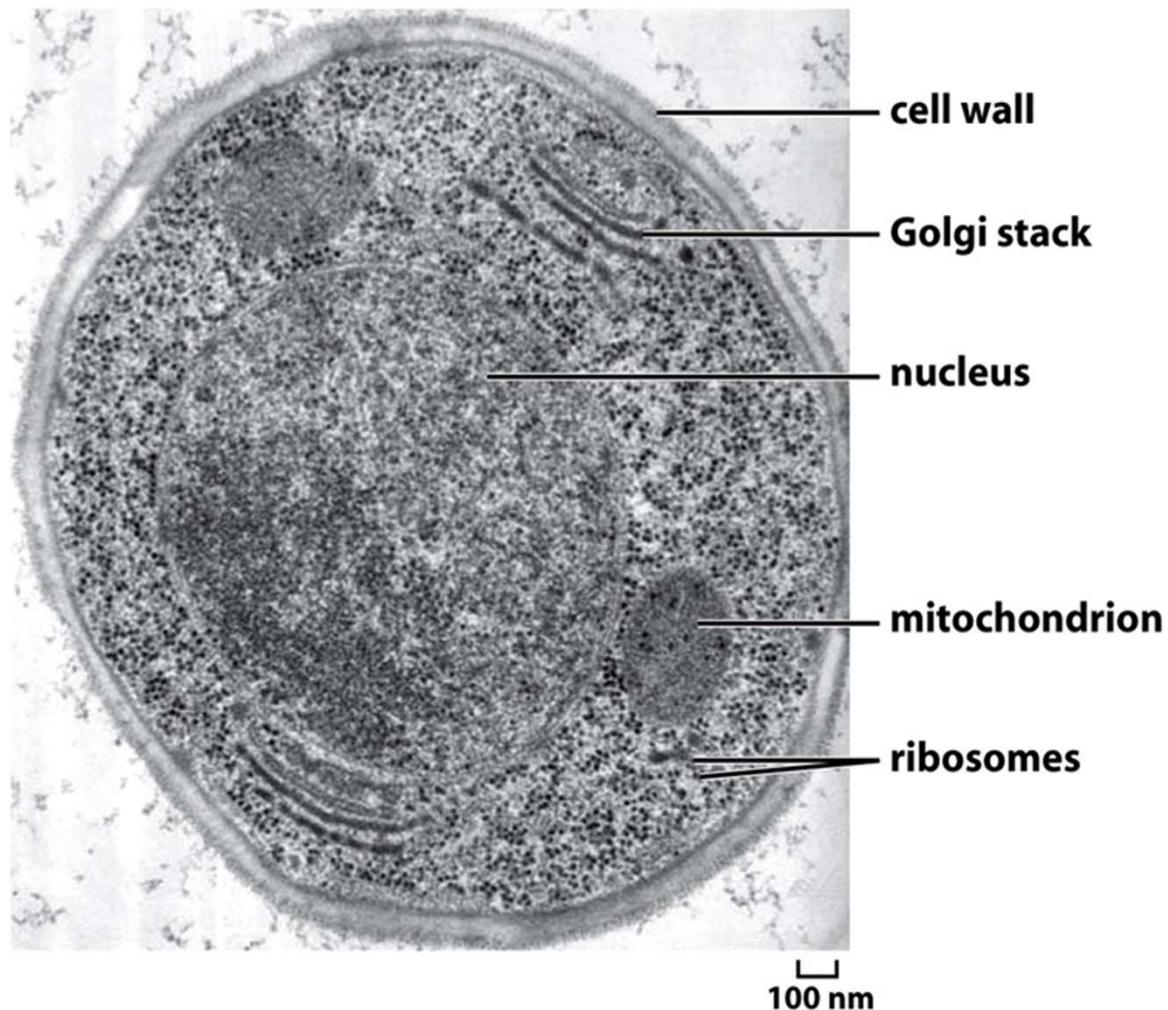
Carefully check all instructions and procedures



glutaraldehyde

osmium tetroxide

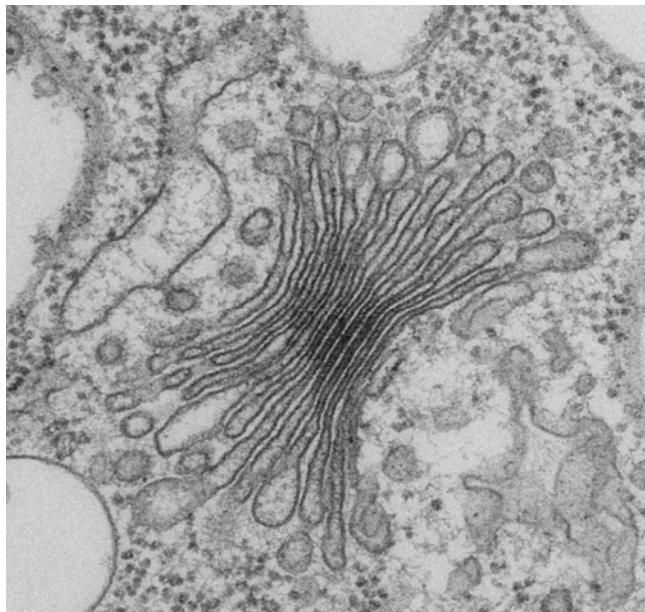
Ultrastructural analysis (TEM)



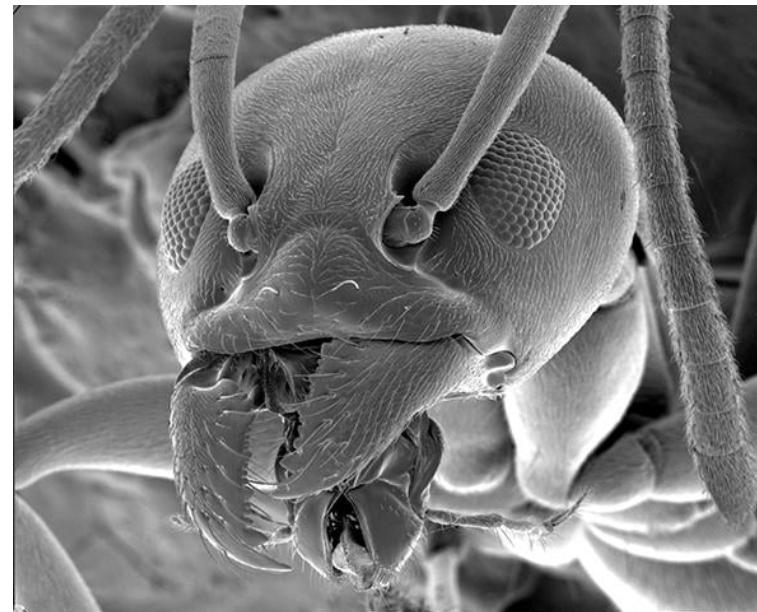
Electron microscopy

Different concepts for different purposes

Transmission EM (TEM)



Scanning EM (SEM)



Immunogold electron microscopy (IEM)

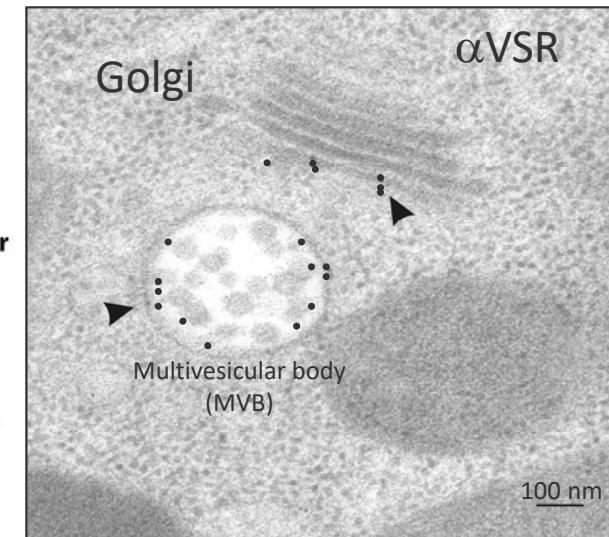
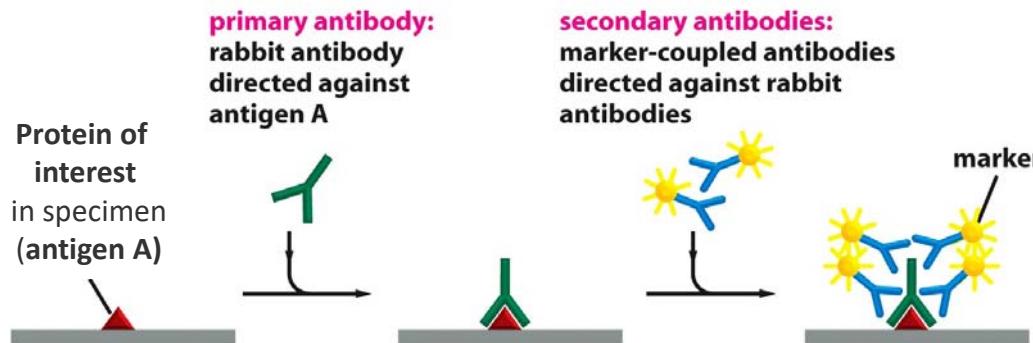
Immunodetection of proteins via antibodies

- Specimen is incubated with primary antibody (the antigen has to be present on the surface of the sectioned sample)
- Gold particle-conjugated secondary antibody is then incubated to label the primary antibody with a gold particle.
- Gold is electron-dense and appears as black dot.

Immunogold electron microscopy (IEM)

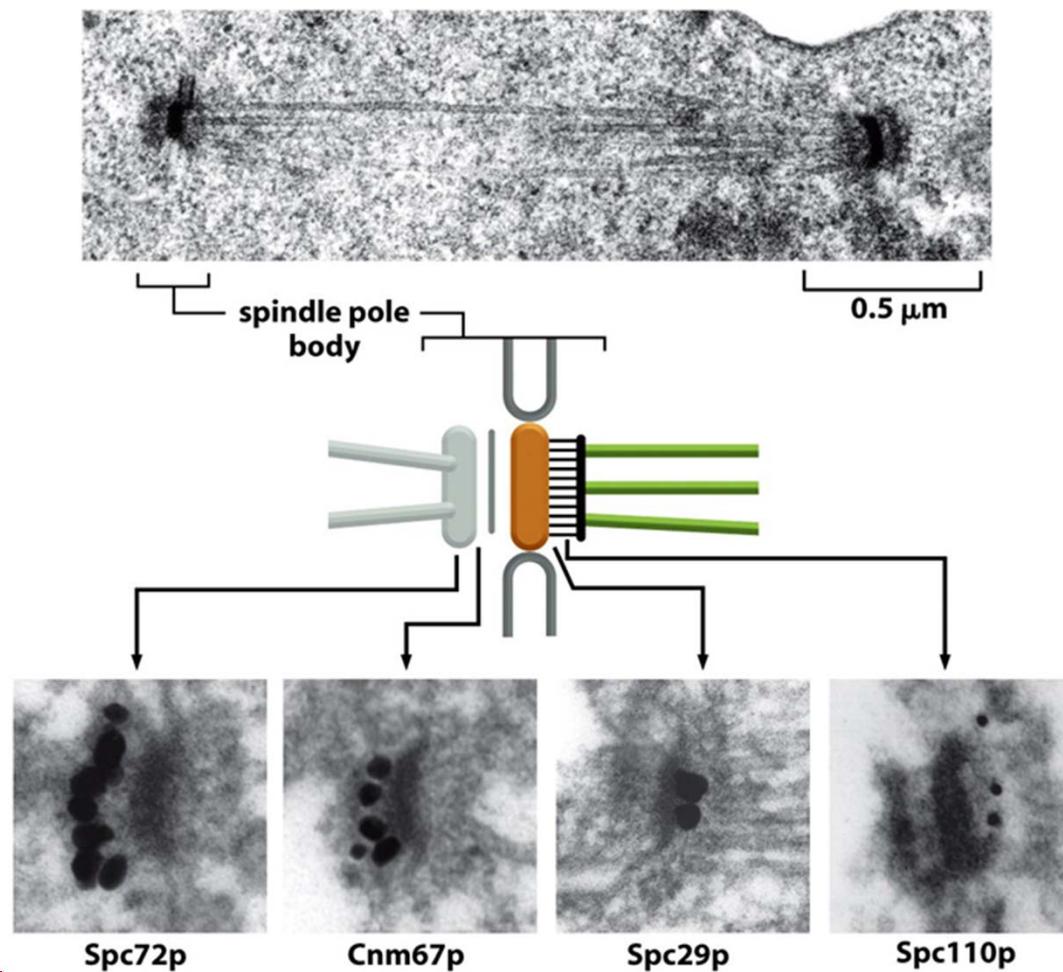
Immunodetection of proteins via antibodies

- Special protein is incubated with primary antibody
- Gold particle-conjugated secondary antibody is incubated to label the primary antibody with a gold particle.
- Gold is electron-dense and appears as black dot



Niemes et al., 2010, *Plant Journal* 61

Images of immunogold electron microscope

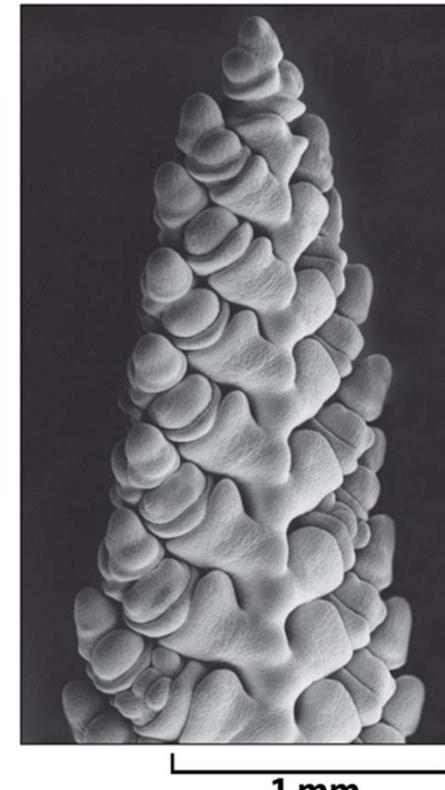
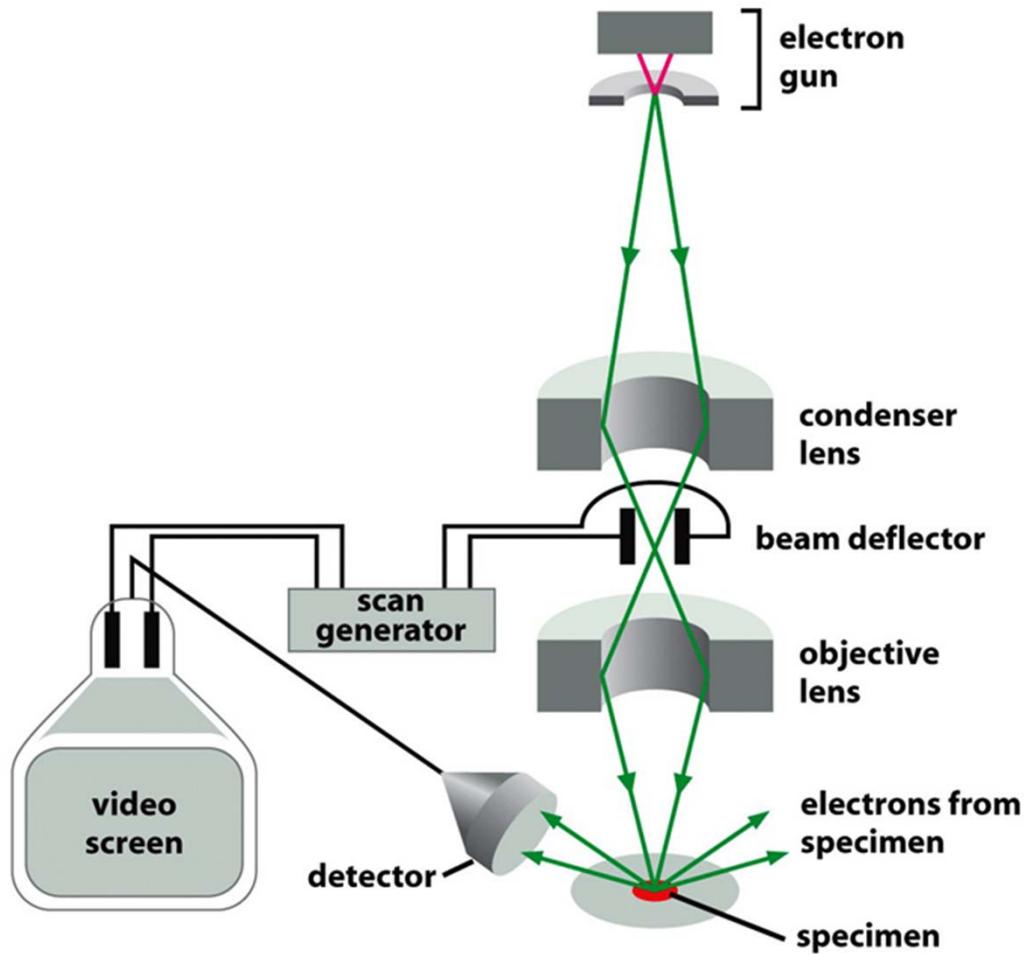


Scanning electron microscope (SEM)

Principles and advantages:

- Produces 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.

Scanning electron microscope (SEM)



Scanning electron microscope (SEM)

