MUNI
SCI

C8116 Immunoaffinity techniques

Advanced microscopy II

Spring term 2024

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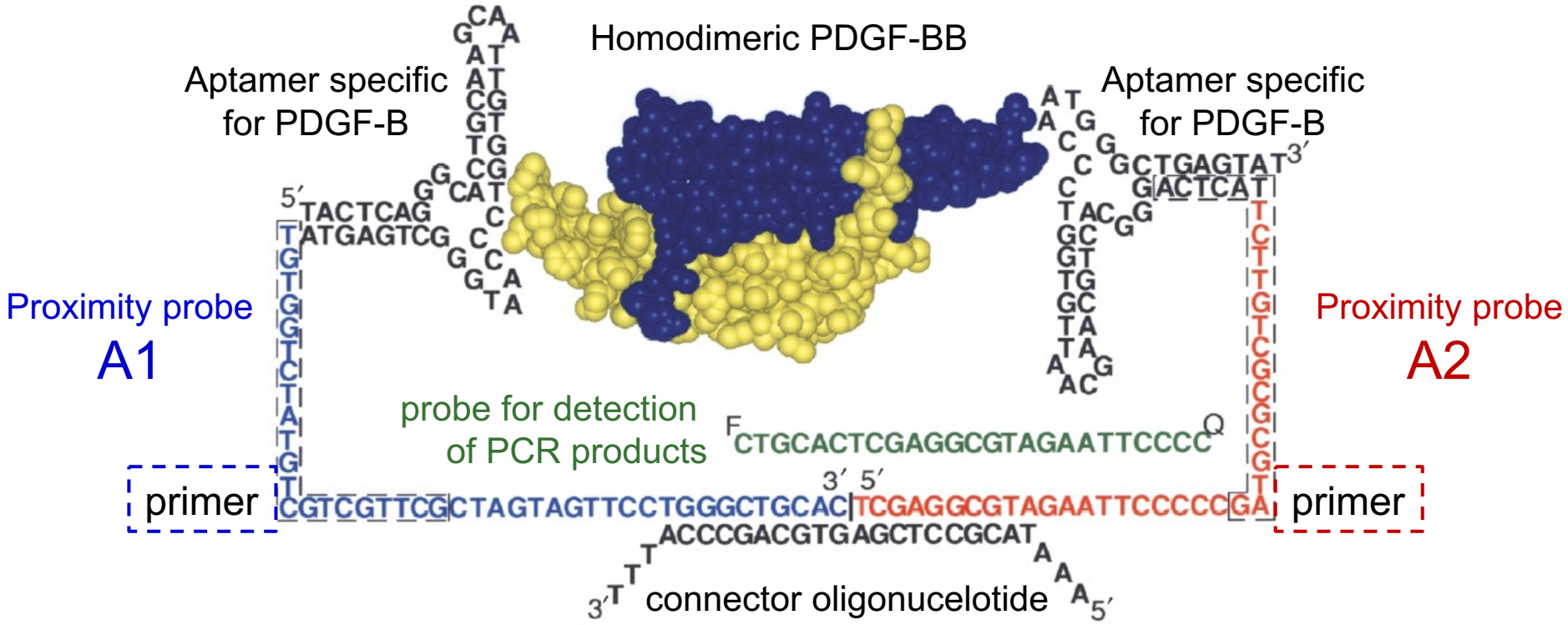
Department of Biochemistry
May 7th, 2024

Proximity ligation



F: fluorescent dye fluorescein
Q: quencher TAMRA

Fredriksson (2002): Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20:** 473-477

Preparation of recombinant proteins

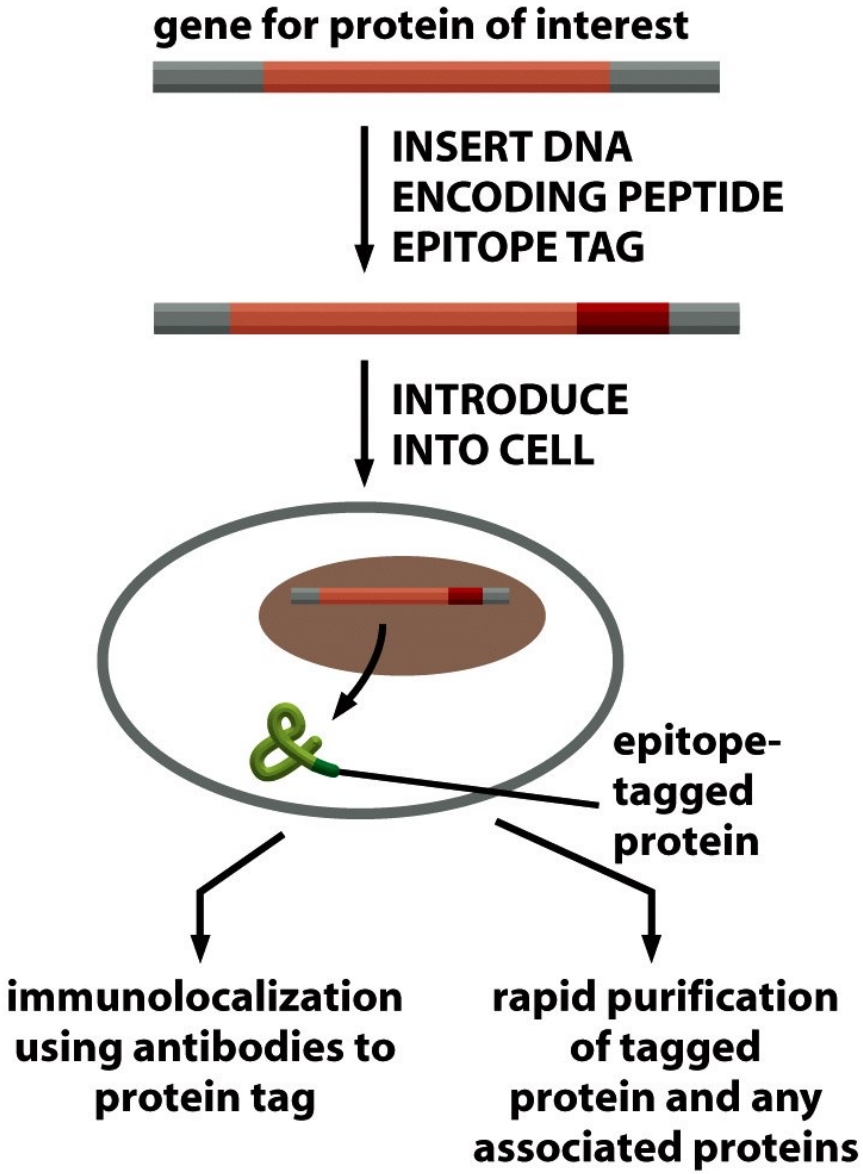
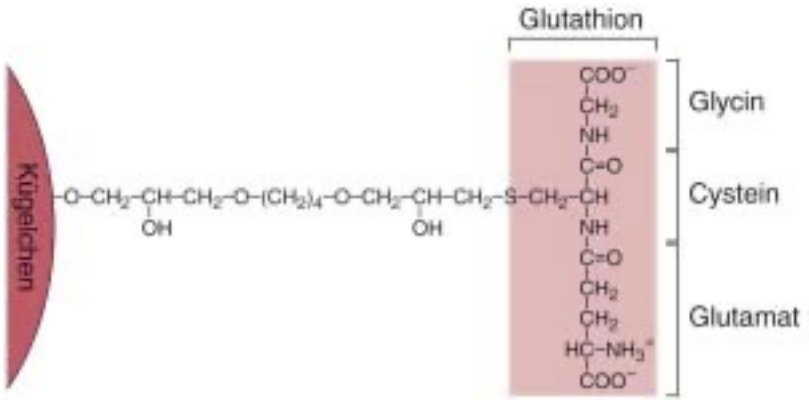


Figure 8-15 Molecular Biology of the Cell 5/e (© Garland Science 2008)

GST pulldown assay

Structure of glutathione beads



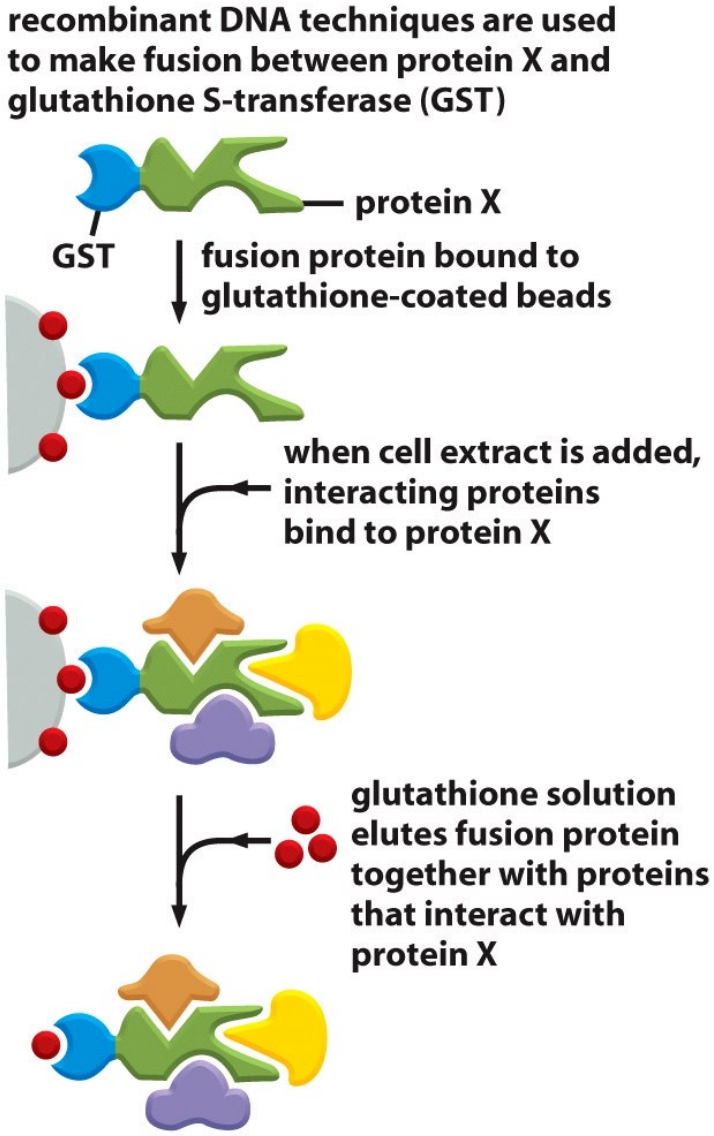
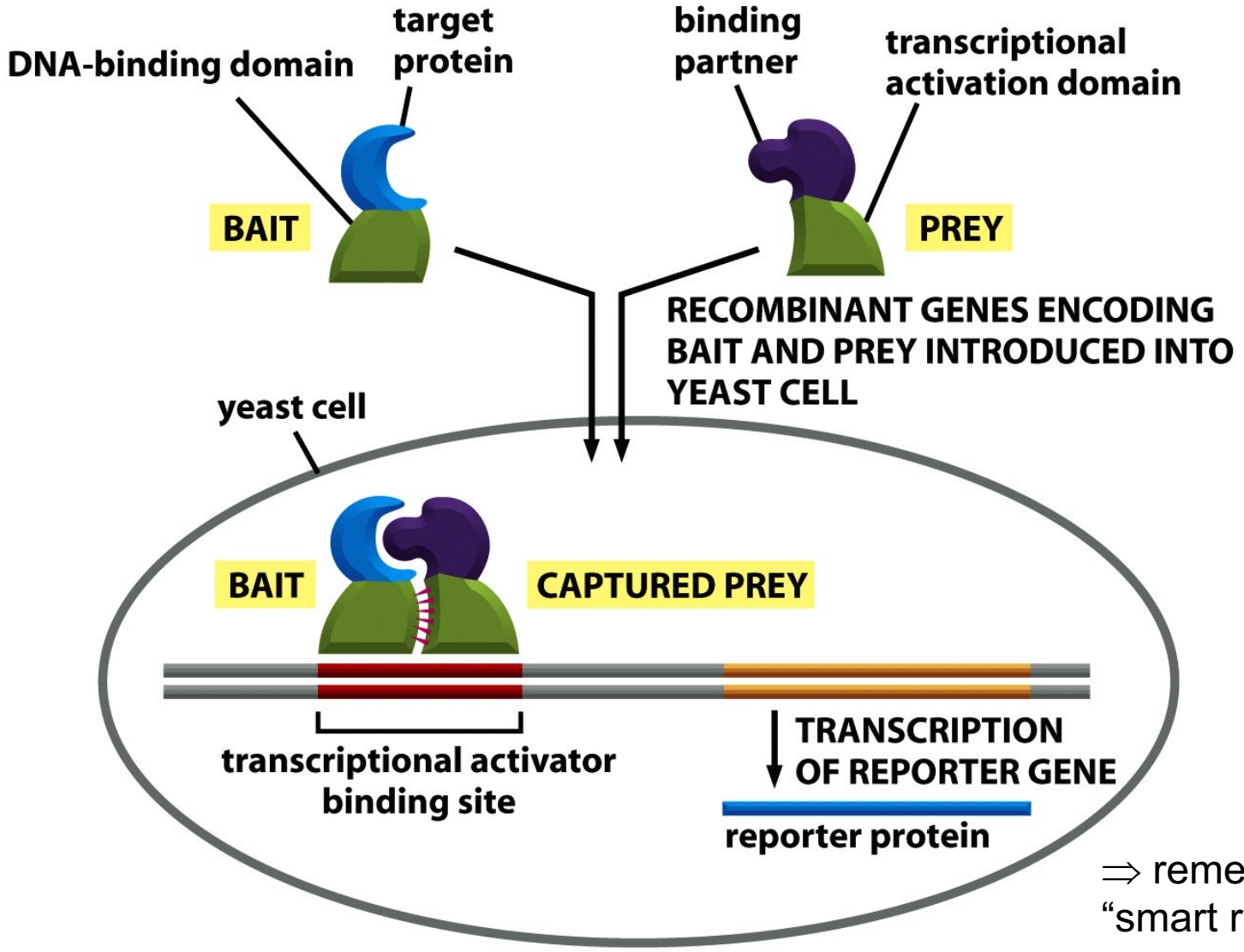


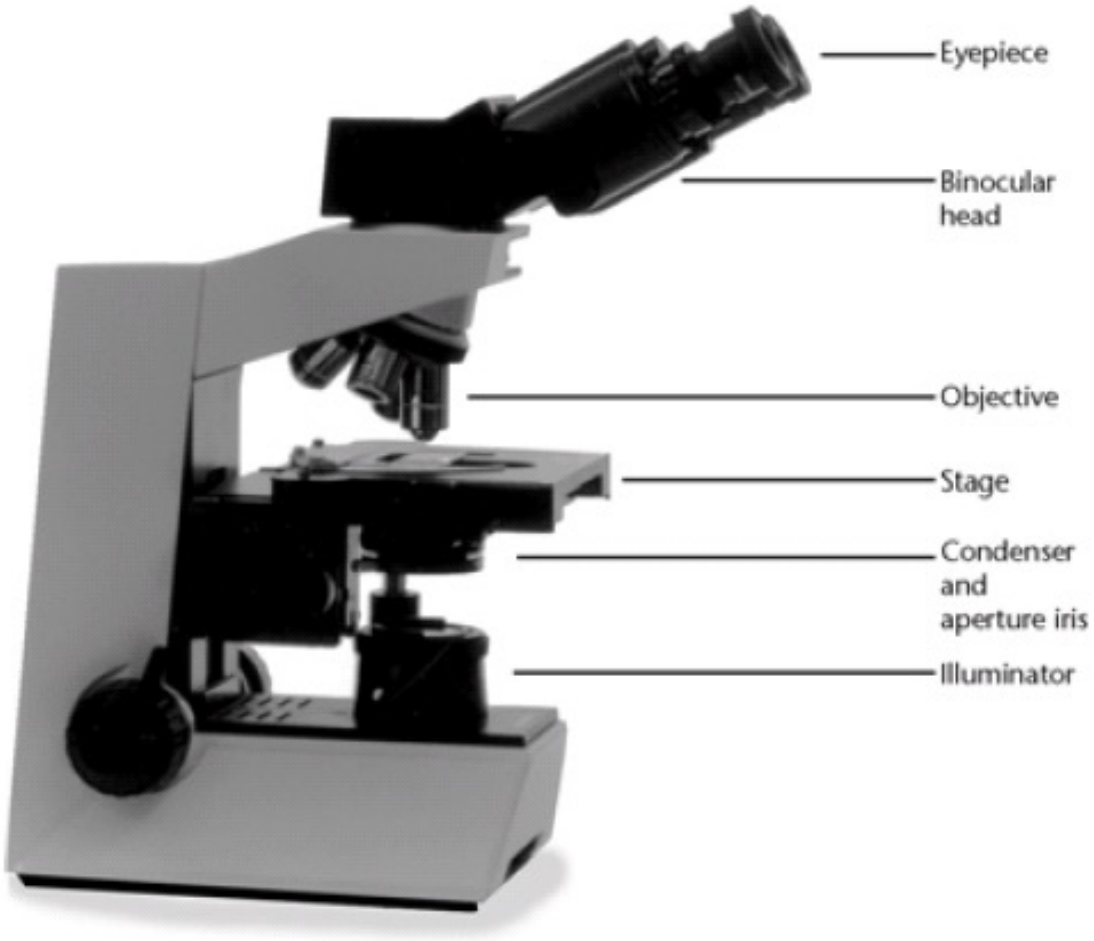
Figure 8-16 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Y2H: Protein fragment complementation assay

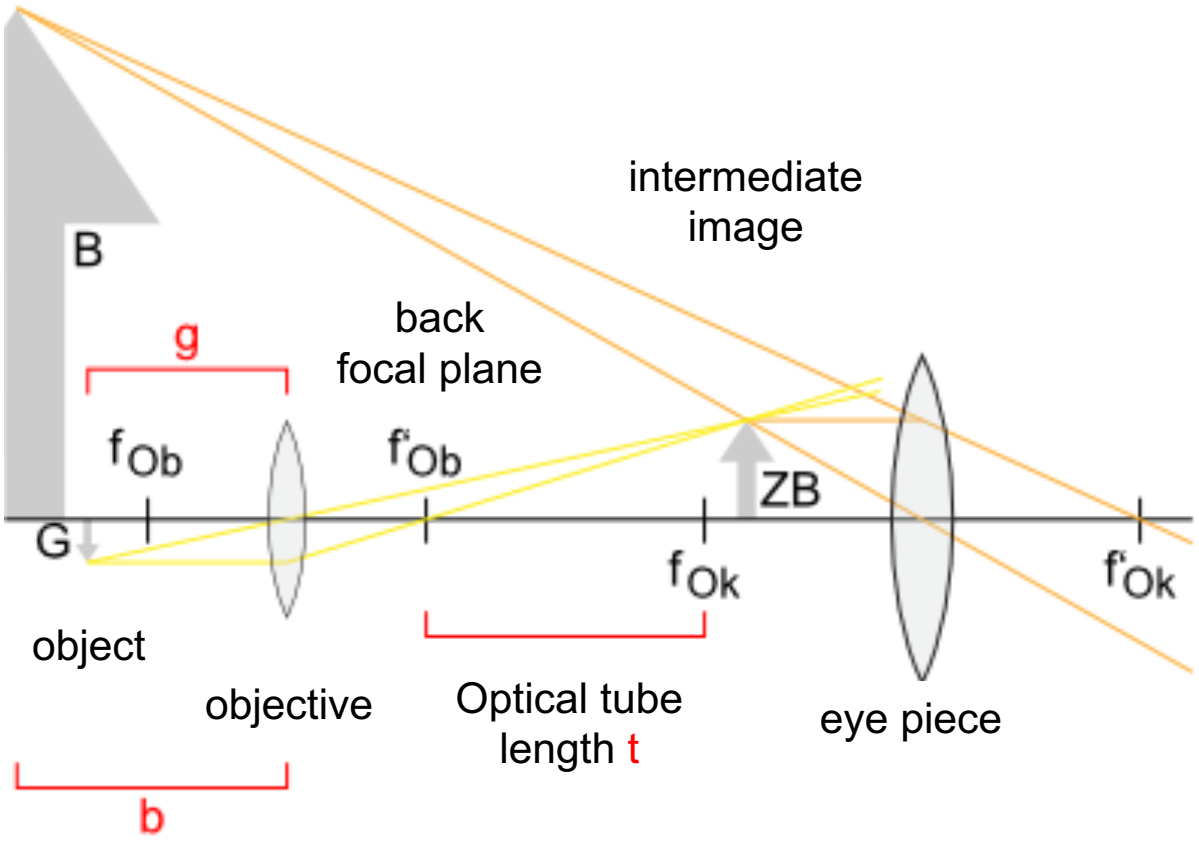


⇒ remember:
“smart reporters”

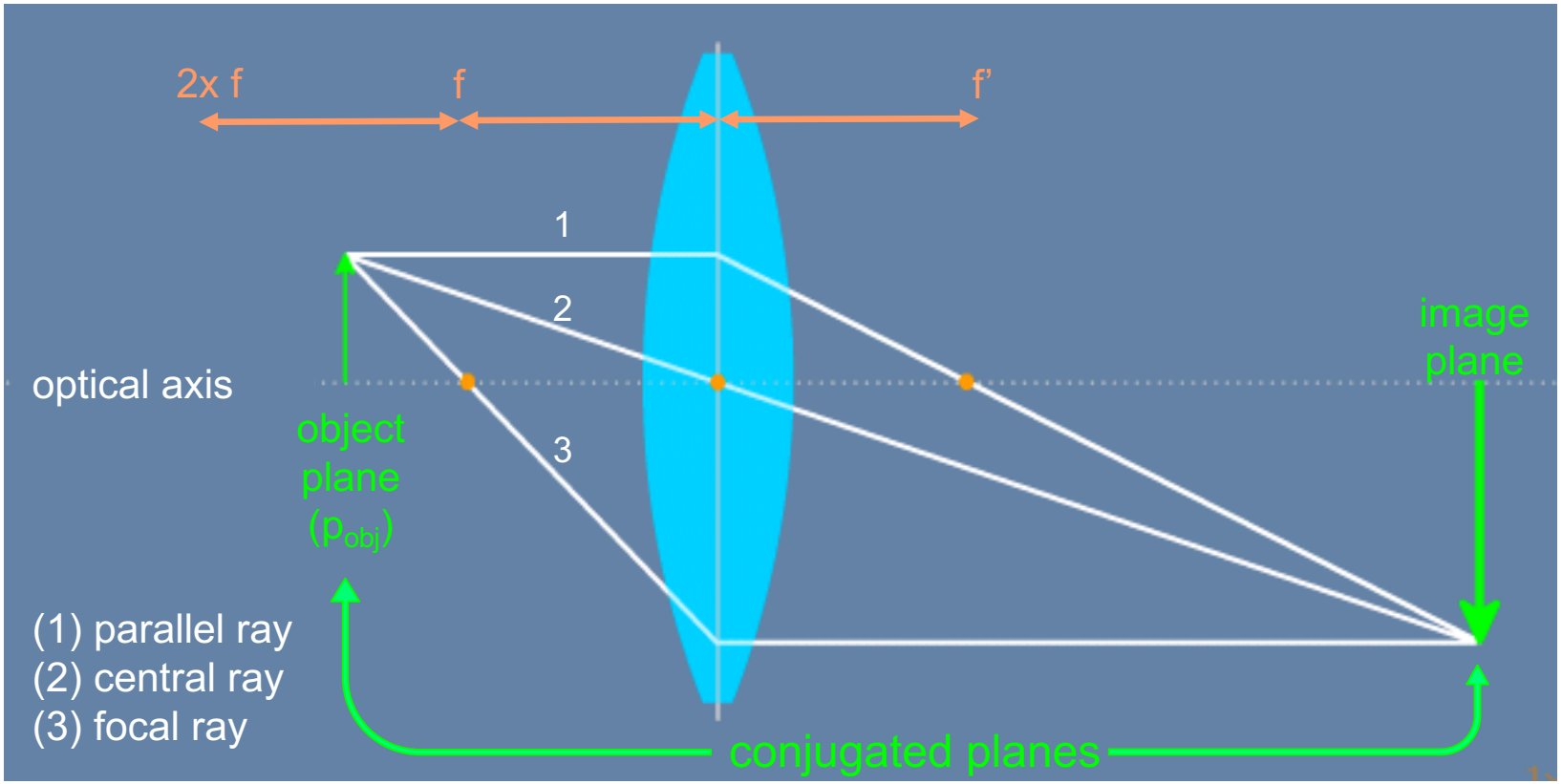
Light microscopy: Upright microscope



Imaging light path of an optical microscope



Conjugated planes



We obtain a real image (upside down) if object is placed:

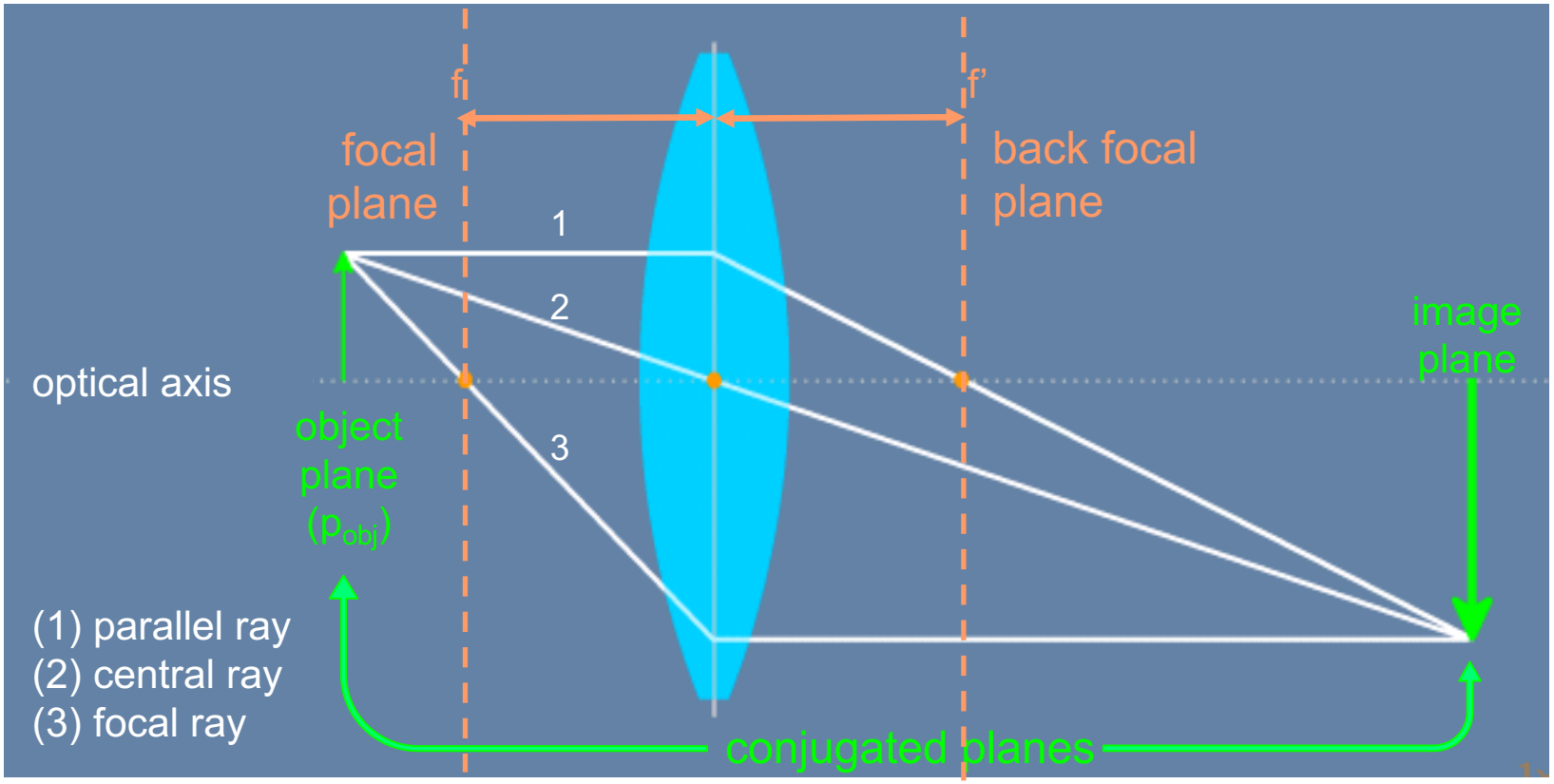
(A) in focal plane (p_{obj} = f): parallel rays emerge after lens; i.e. image is not focused

(B) between simple and double focal length (f < p_{obj} < 2f): magnified image

(C) in double focal length (p_{obj} = 2f): image has the same size as object

(D) beyond double focal length (p_{obj} > 2f): demagnified image

Conjugated planes



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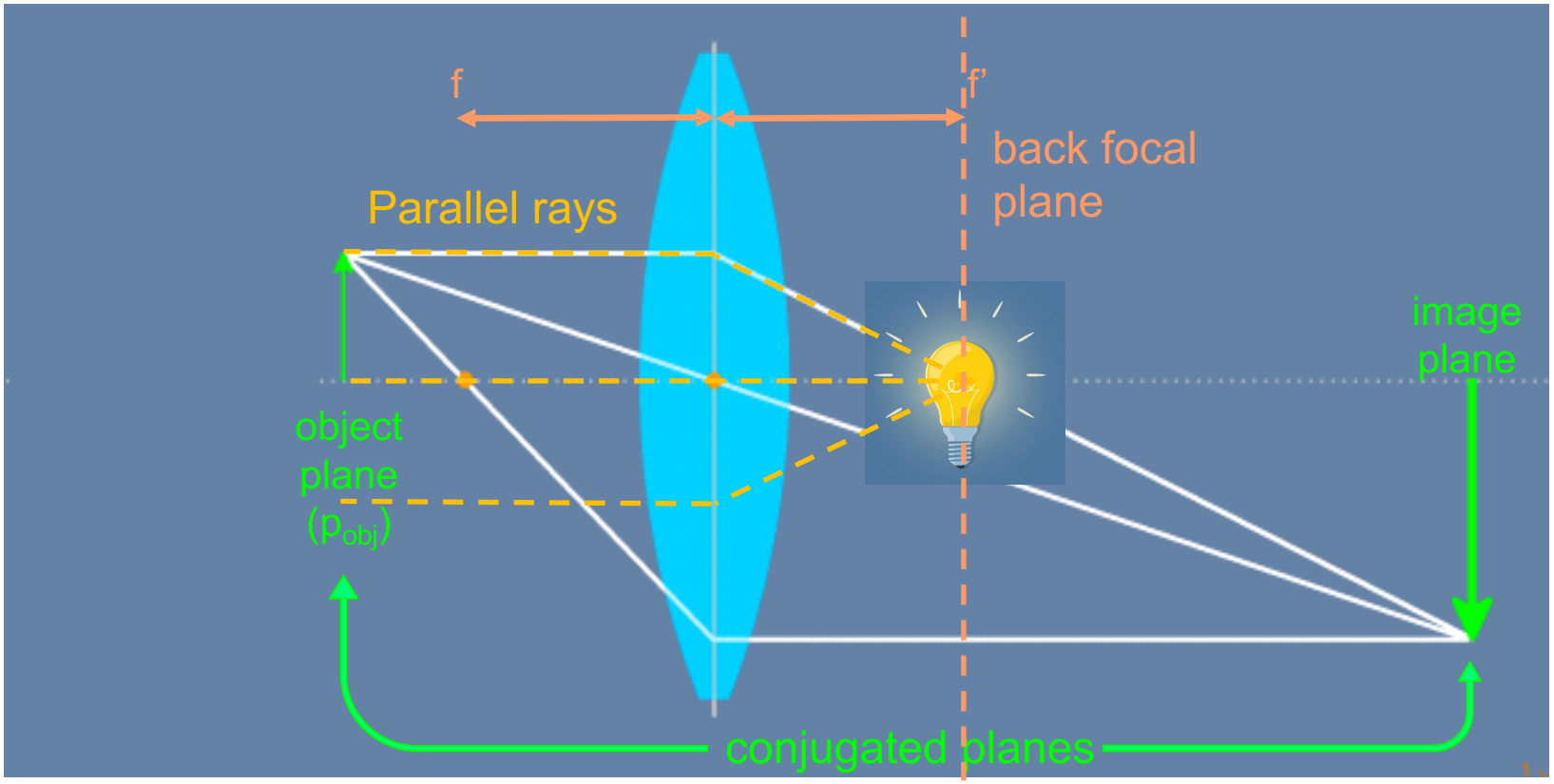
Conjugated planes



We obtain a real image (upside down) if object is placed:

(A) in focal plane ($p_{obj} = f$): parallel rays emerge after lens; i.e. image of light bulb is not focused

(B) between simple and double focal length ($f < p_{obj} < 2f$): magnified image

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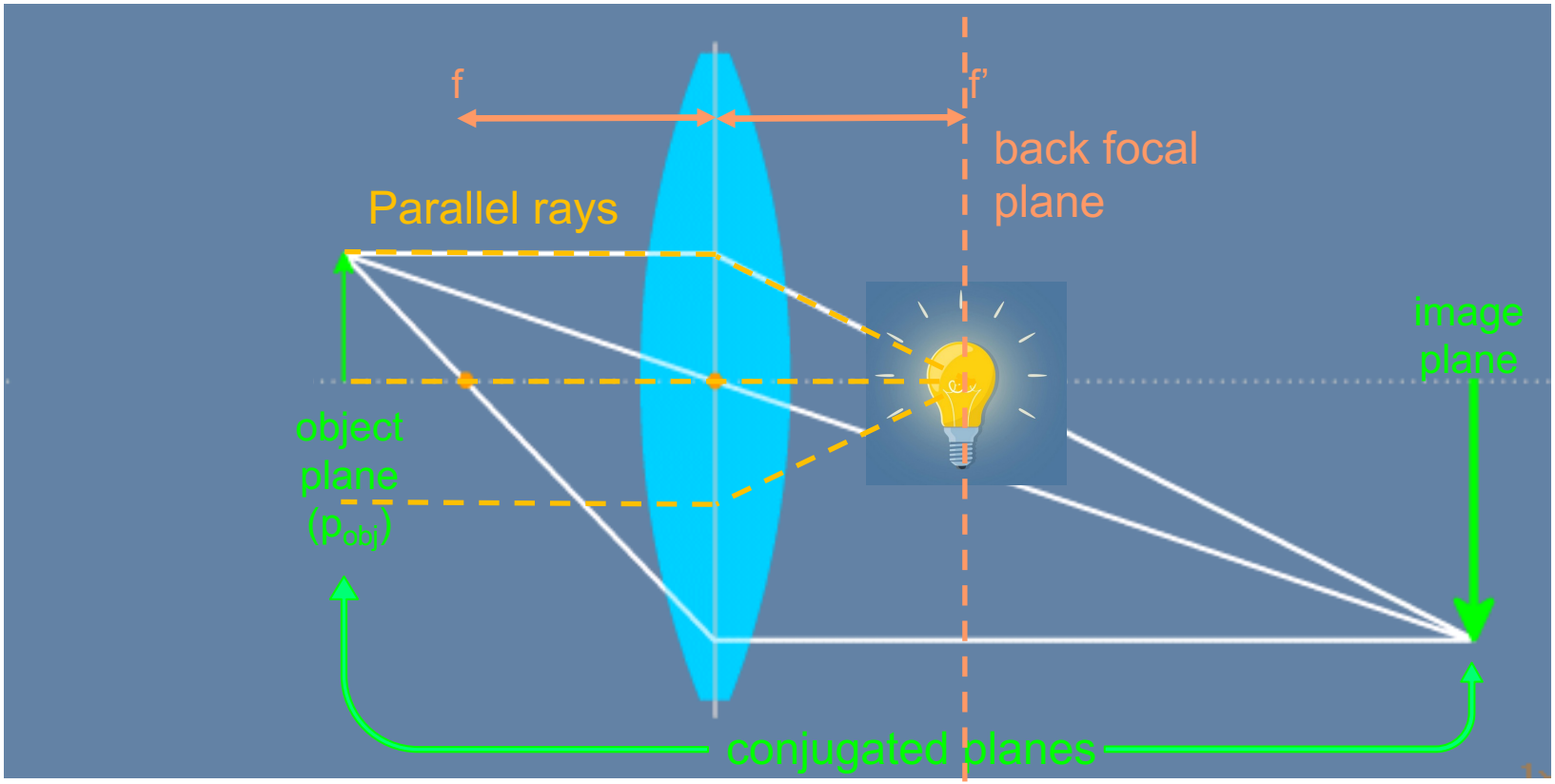
Conjugated planes

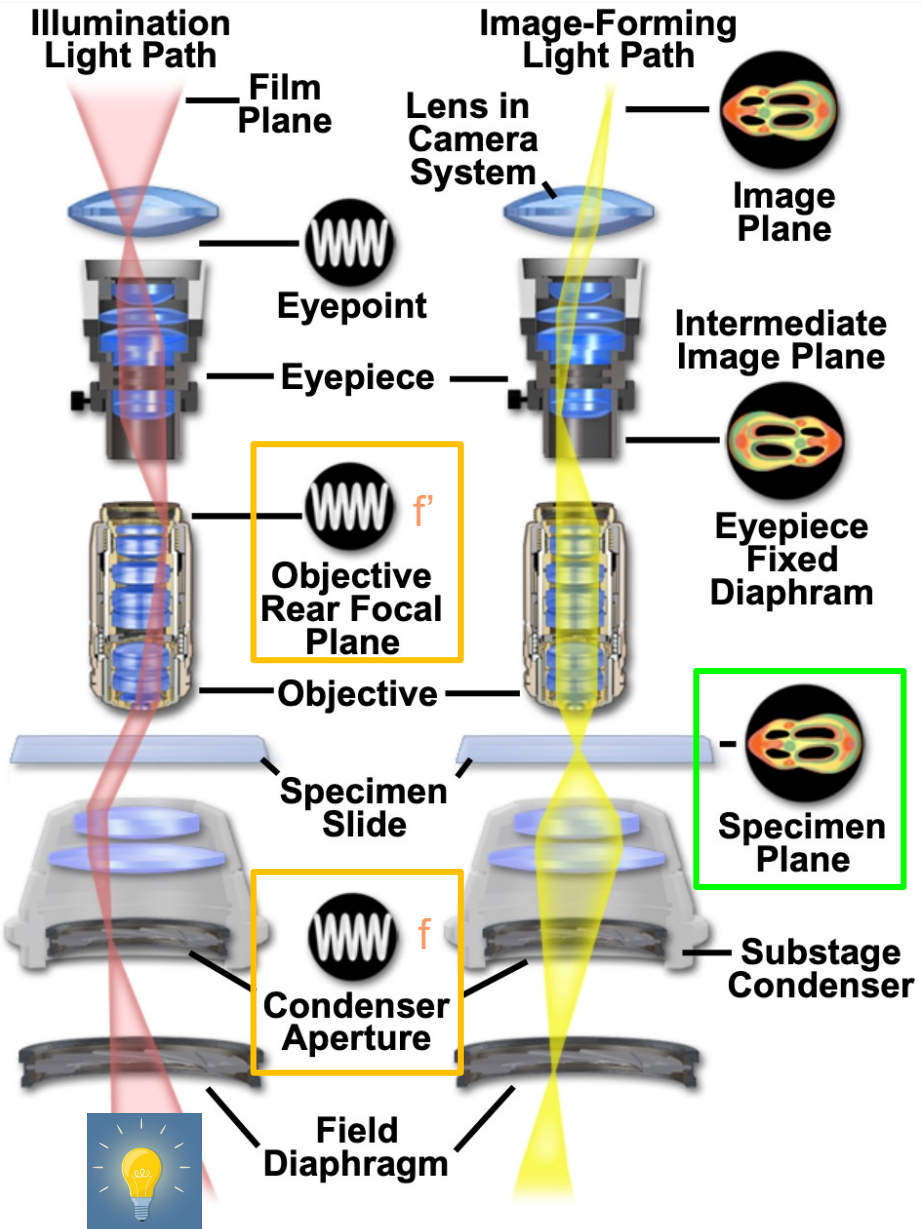


We must distinguish between:
(1) Imaging light path
(2) Illumination light path

=> First implemented in microscopy in 1893
by August Köhler (Zeiss company)

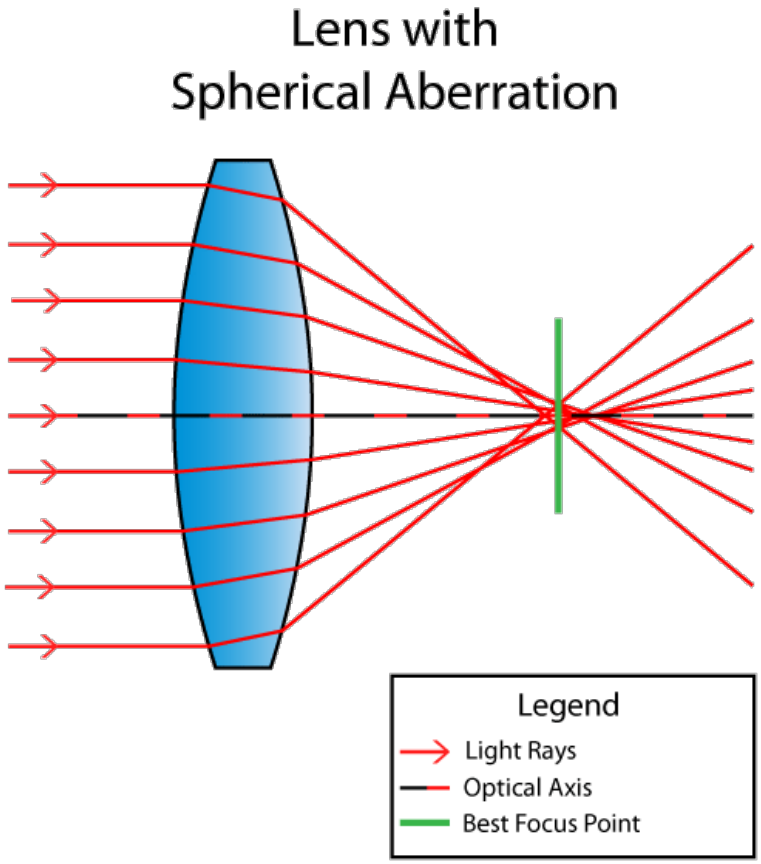
Conjugate planes in an optical microscope

=> Köhler Illumination in an upright microscope



As the light source is **not** focused at the level of the specimen, the light at specimen level is essentially grainless and extended, and does not suffer deterioration from dust and imperfections on the glass surfaces of the condenser.

Optical defects in lens systems (1)



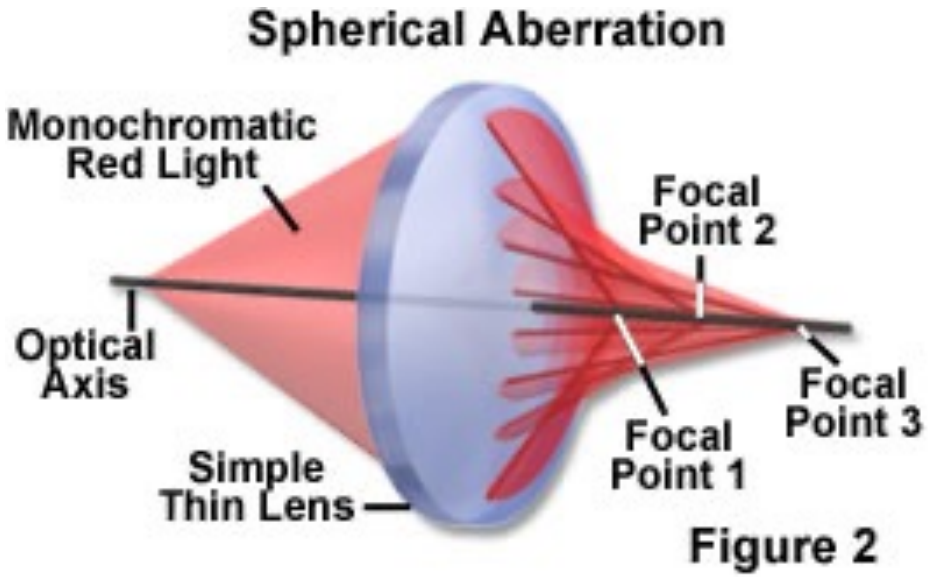
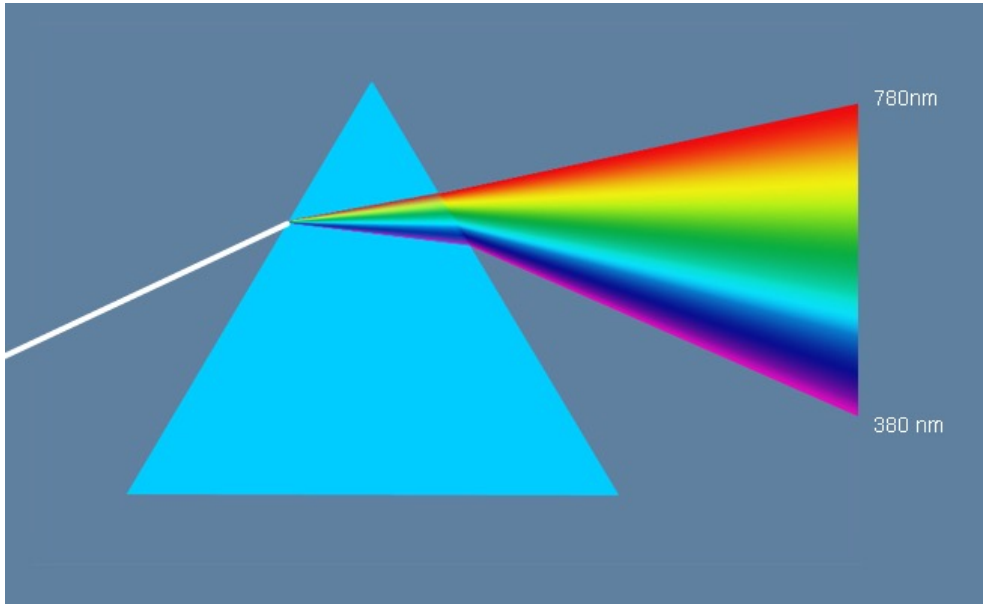


Figure 2

Optical defects in lens systems (2)



Axial Chromatic Aberration

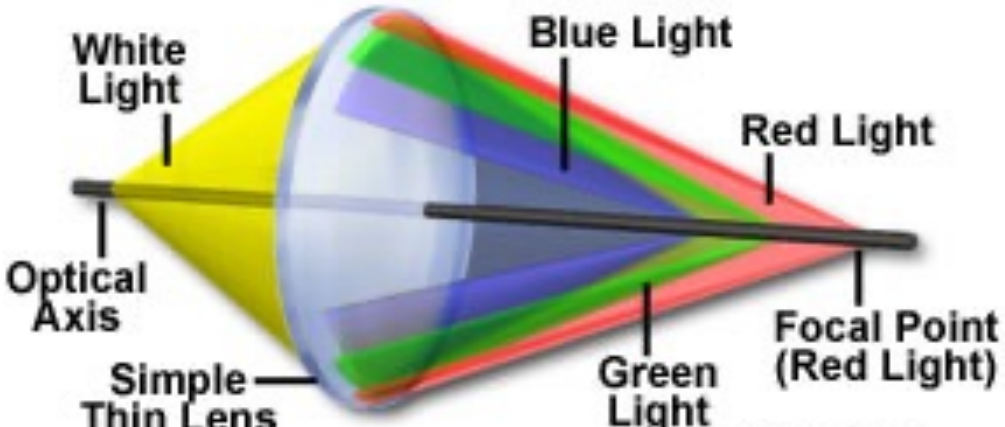
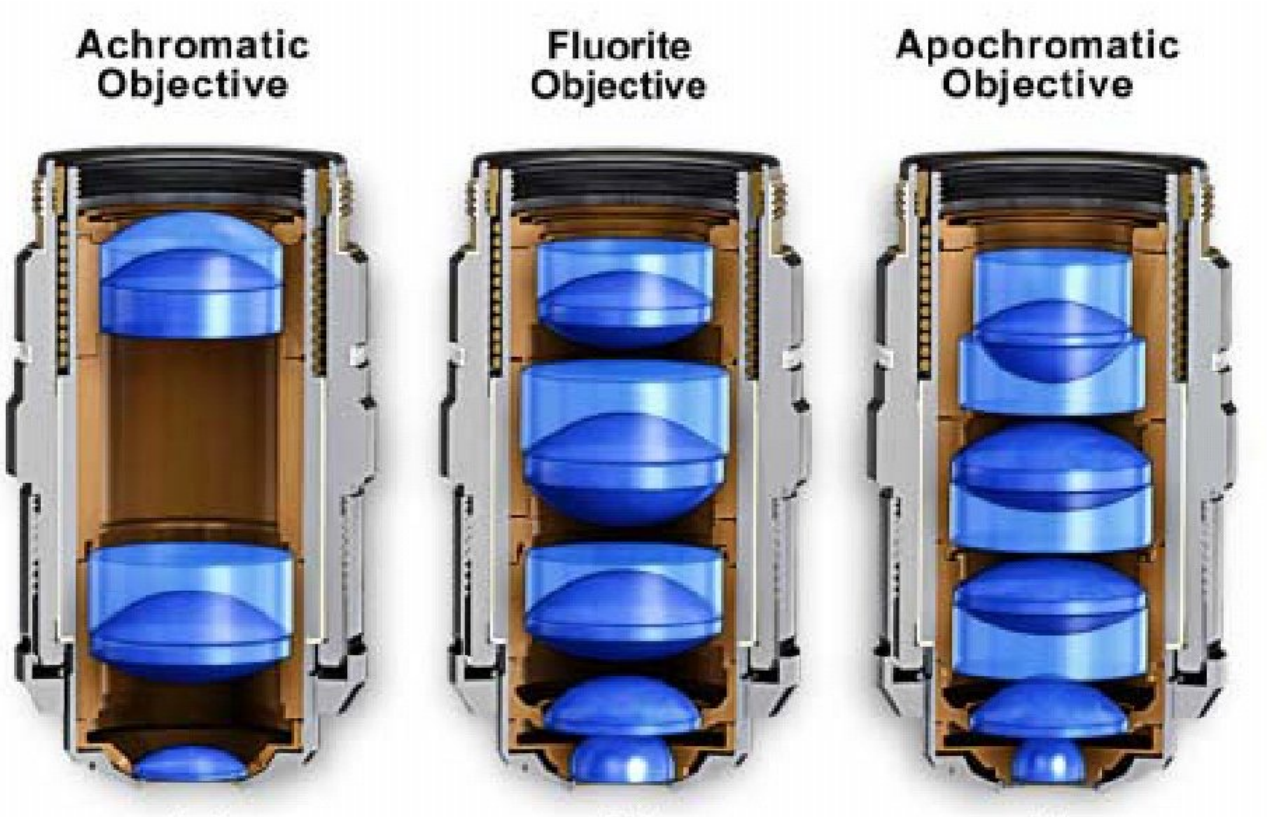


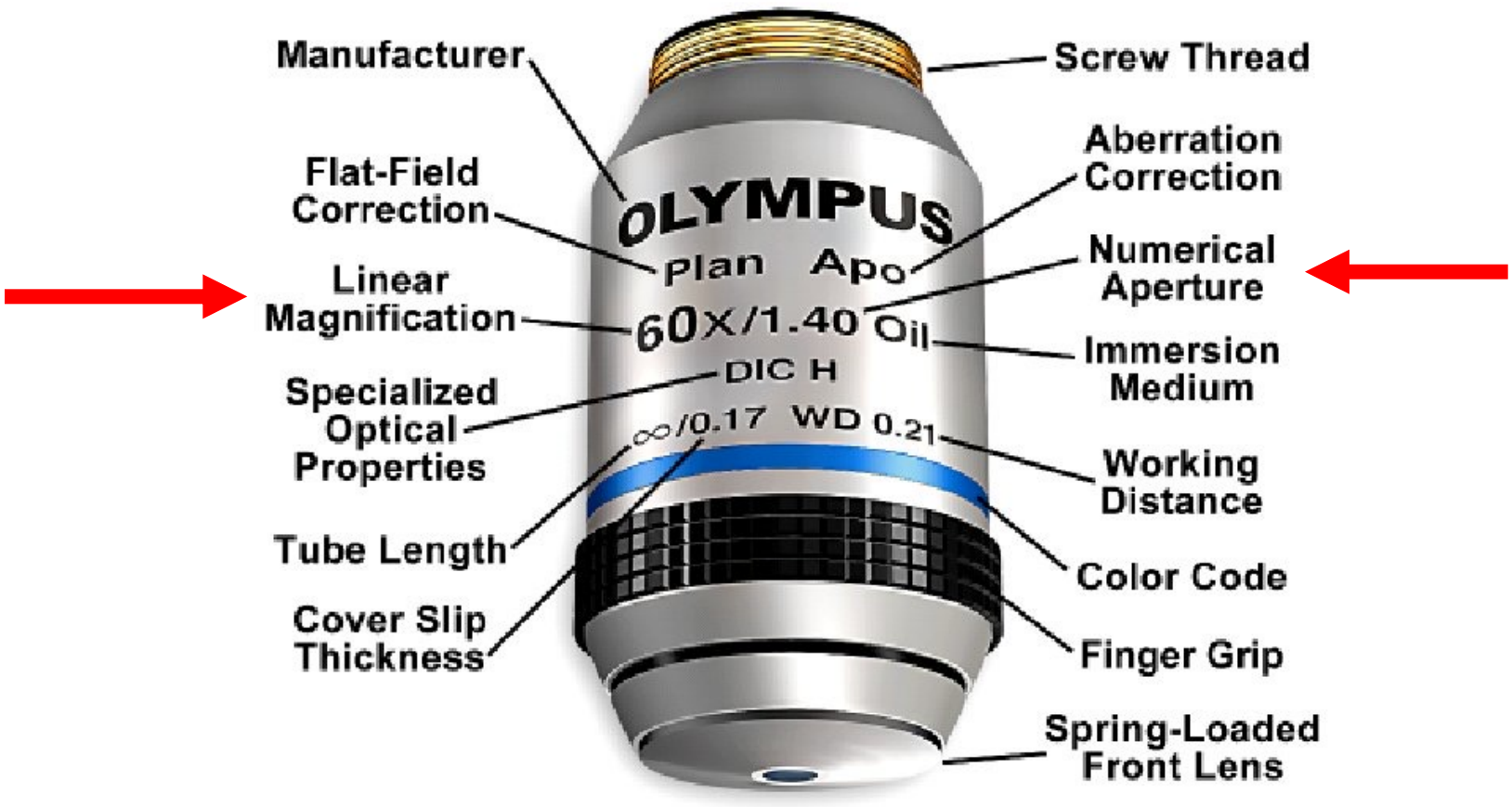
Figure 1

Objective



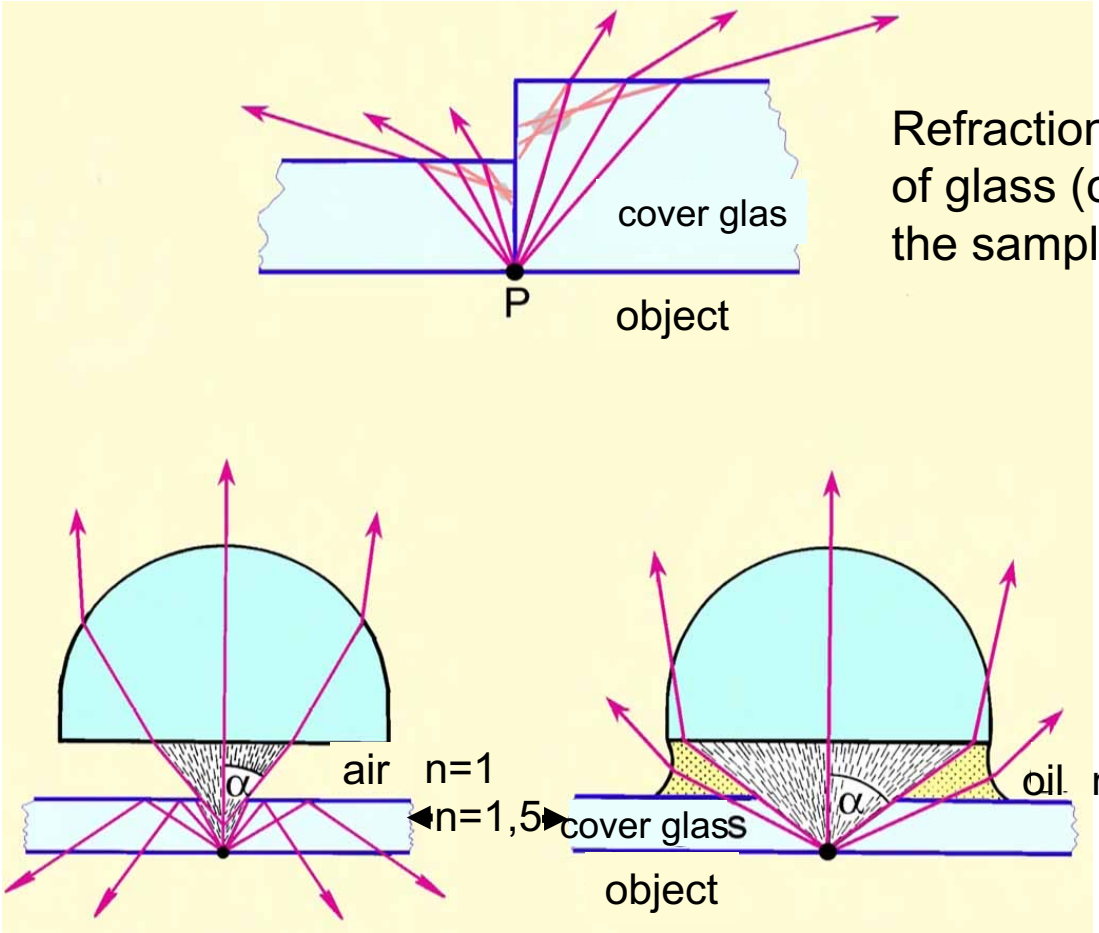
2 color correction	3 color correction	4 color correction

Objective



Objektive descriptions

Objective: refractive index mismatch



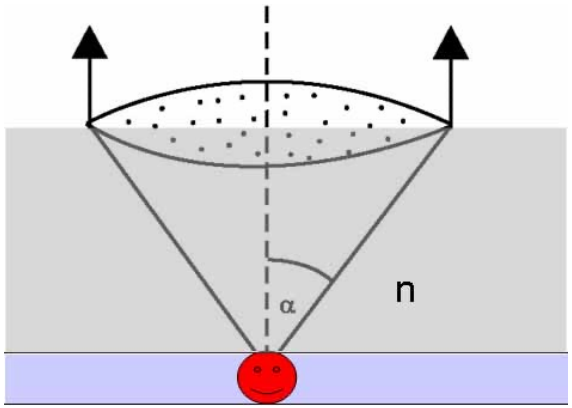
Refraction at the interface of glass (cover glass of the sample) and air

=> Immersion liquid reduces the refractive index mismatch

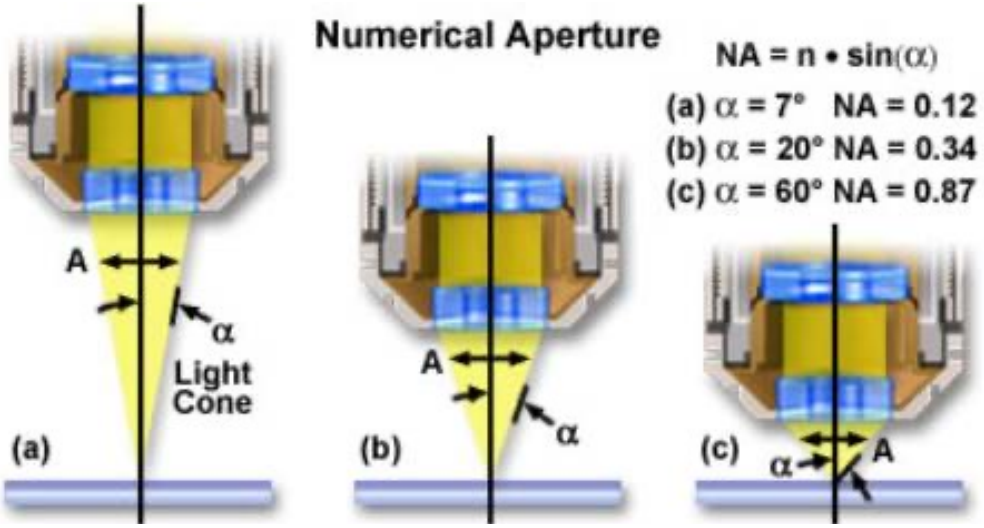
Objective: numerical aperture

It is not the magnification but rather the numerical aperture (NA) of the objective that determines the quality of on image.

$NA = n \times \sin\alpha$



n: refractive index
α: acceptance angle
of the objective

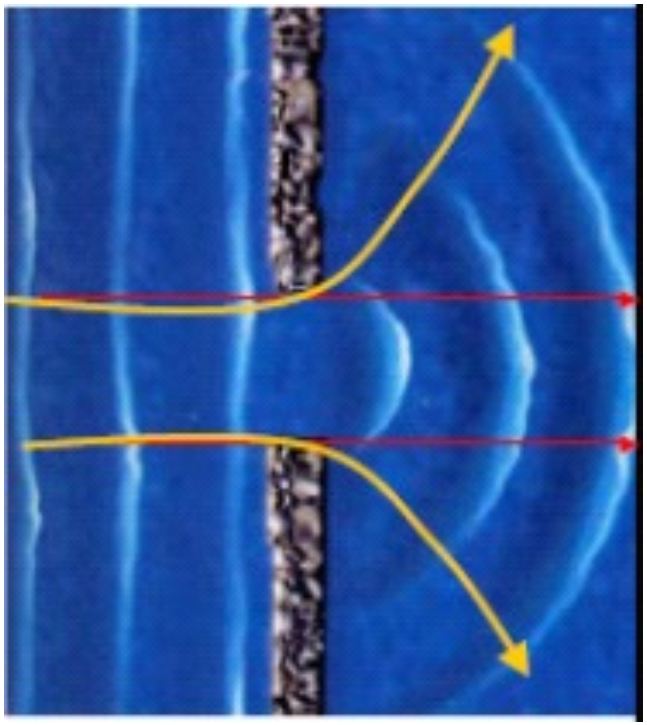


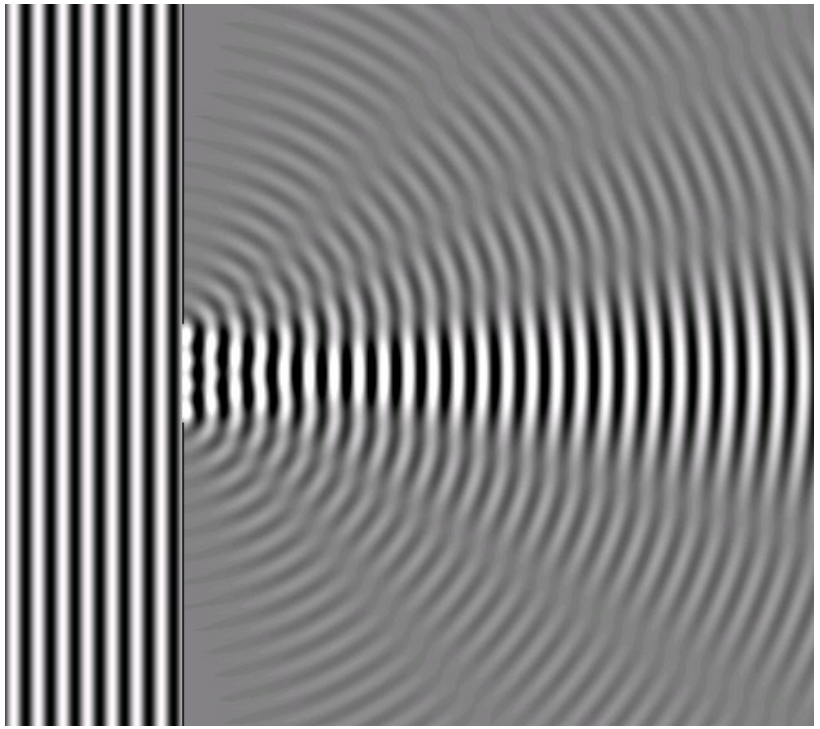
Width of the acceptance cone
=> How much light can be focused?

High NA improves
1. Resolution
2. Brightness (also contrast)

Optical resolution of light microscopy

Wave-optical explanation: Diffraction of rays at a cleft

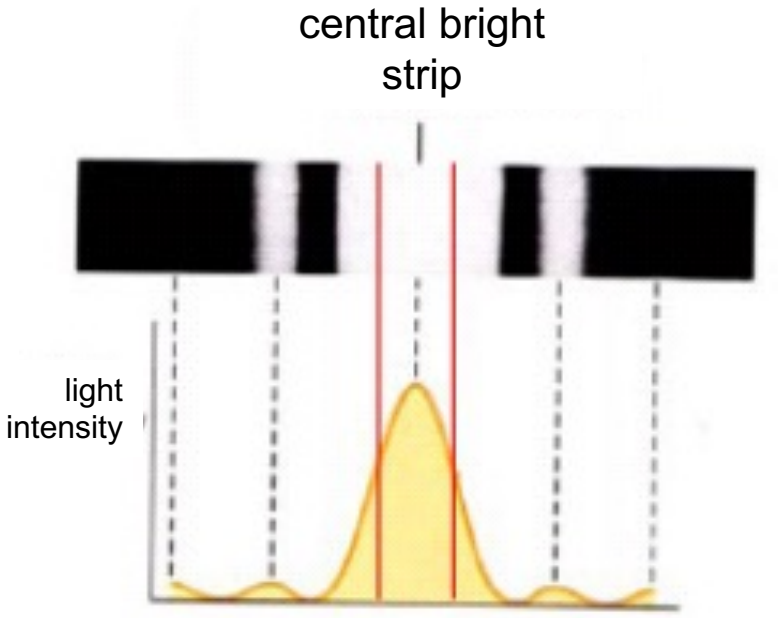


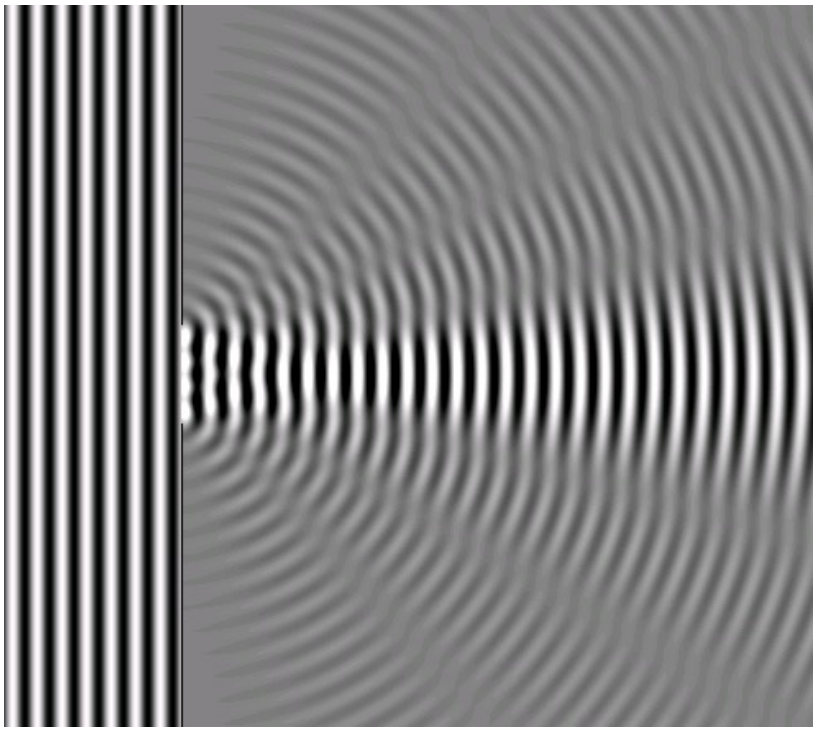


Requirement for an objective with a wide acceptance cone (NA)
to focus diffracted light efficiently
=> high-resolution objective
Diffraction increases with wavelength!

Optical resolution of light microscopy

Wave-optical explanation: Diffraction of rays at a cleft





$$d = \frac{\lambda}{2n\sin\alpha}$$

Resolution is diffraction limited!

Possibilities to attain a higher resolution?

=> d ≈ λ / 2 ≈ 200 nm

Bright-field microscopy

Light from the condenser passes through sample (transmission mode), is attenuated by absorbing materials and collected by the objective

Total magnification (M_{tot}) = $M_{objective}$ x $M_{eyepiece}$

- but there is a fundamental limit of resolution depending only on the objective:
  $\lambda/(2n*\sin\alpha)$ – note: M does *not* appear in this equation!

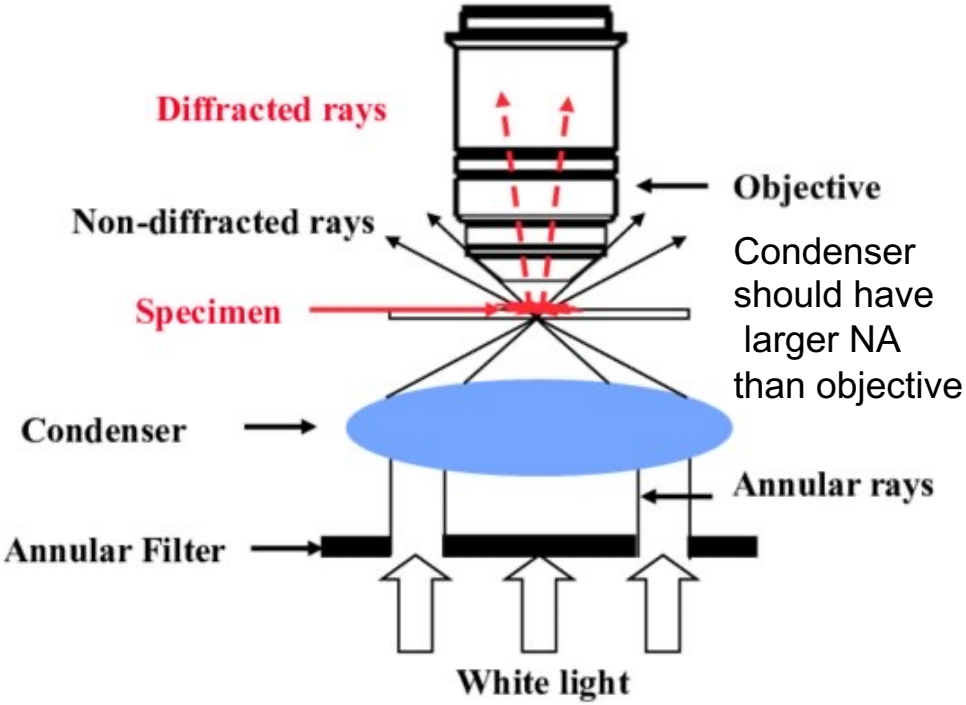
  with λ: wavelength of light
  n: refractive index
  α: half of acceptance cone

- higher magnifications are called empty magnification

- The objective forms an image in the the intermediate image plane that contains all information on the specimen accessible by the microscope! Any further image magnification by eyepiece or camera lenses only changes the size for easier observation or to fit the camera chip, but does not add any information.

=> The resolution and brightness/contrast of an objective are essential

Dark-field microscopy

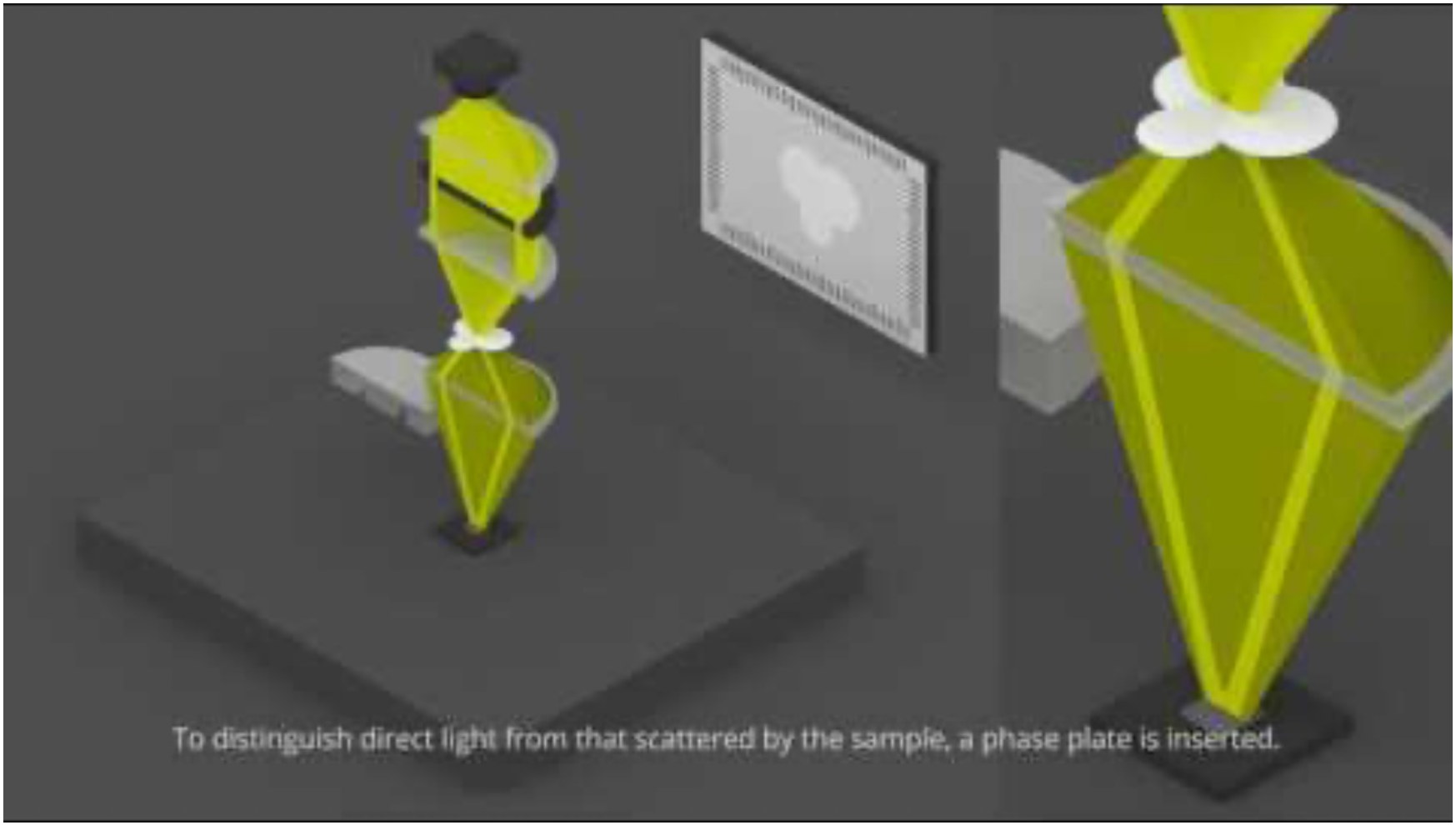


Amphipod crustacean (25x magnification)

Dark-field microscopy prevents non-diffracted light from entering the objective. Only light rays diffracted by the specimen are collected by the objective. Thus, a bright image appears against a **dark background**, resulting in a much better image contrast compared to bright-field microscopy.
=> Enables observation of living cells/organisms.

In biology, dark-field microscopy has been replaced by improved techniques, but it has recently reemerged for the analysis of strongly light scattering (plasmonic) nanomaterials.

Dark-field and phase contrast microscopy

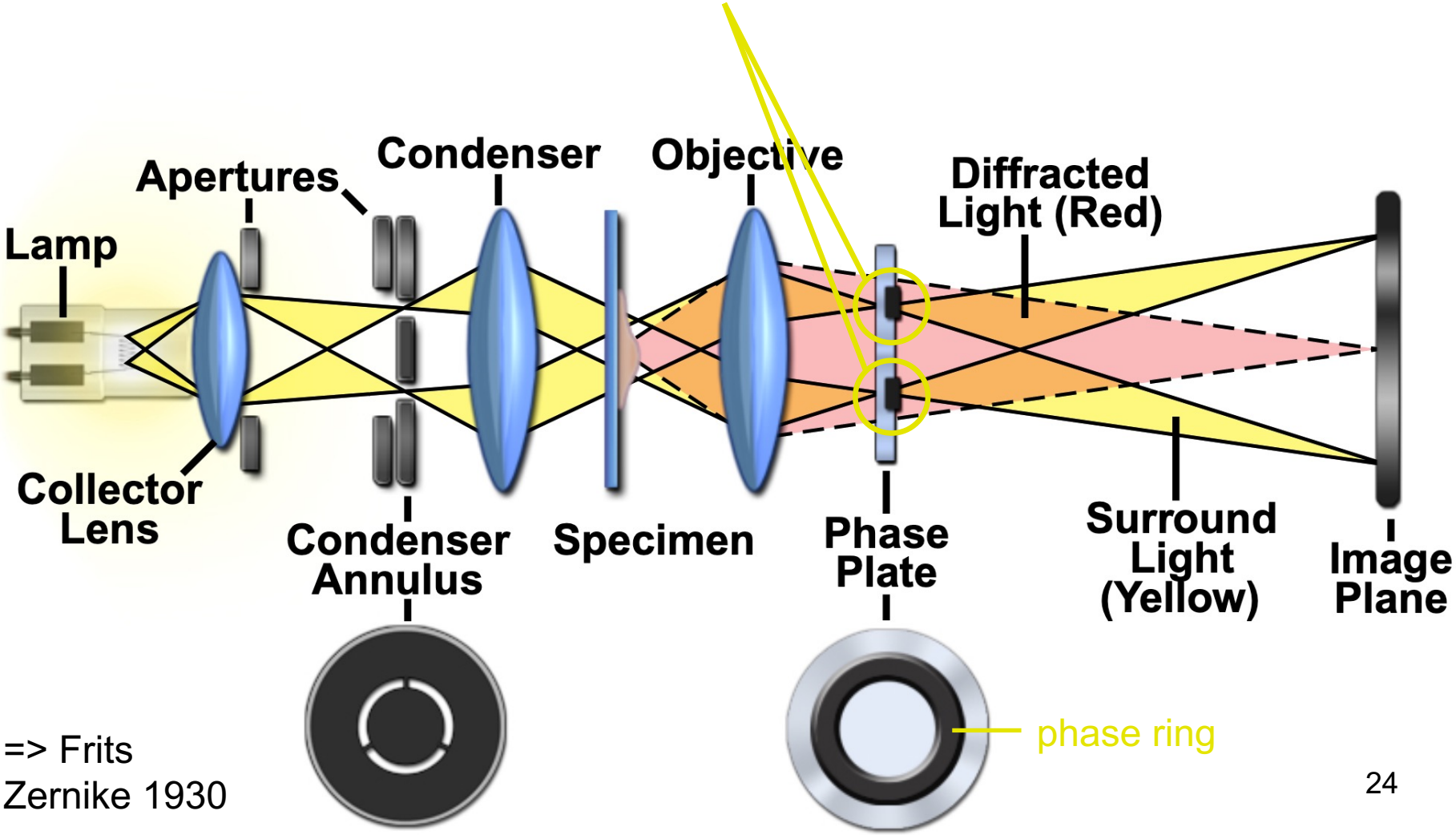


Source: https://toutestquantique.fr/en/dark-field-and-phase-contrast/

Phase contrast microscopy

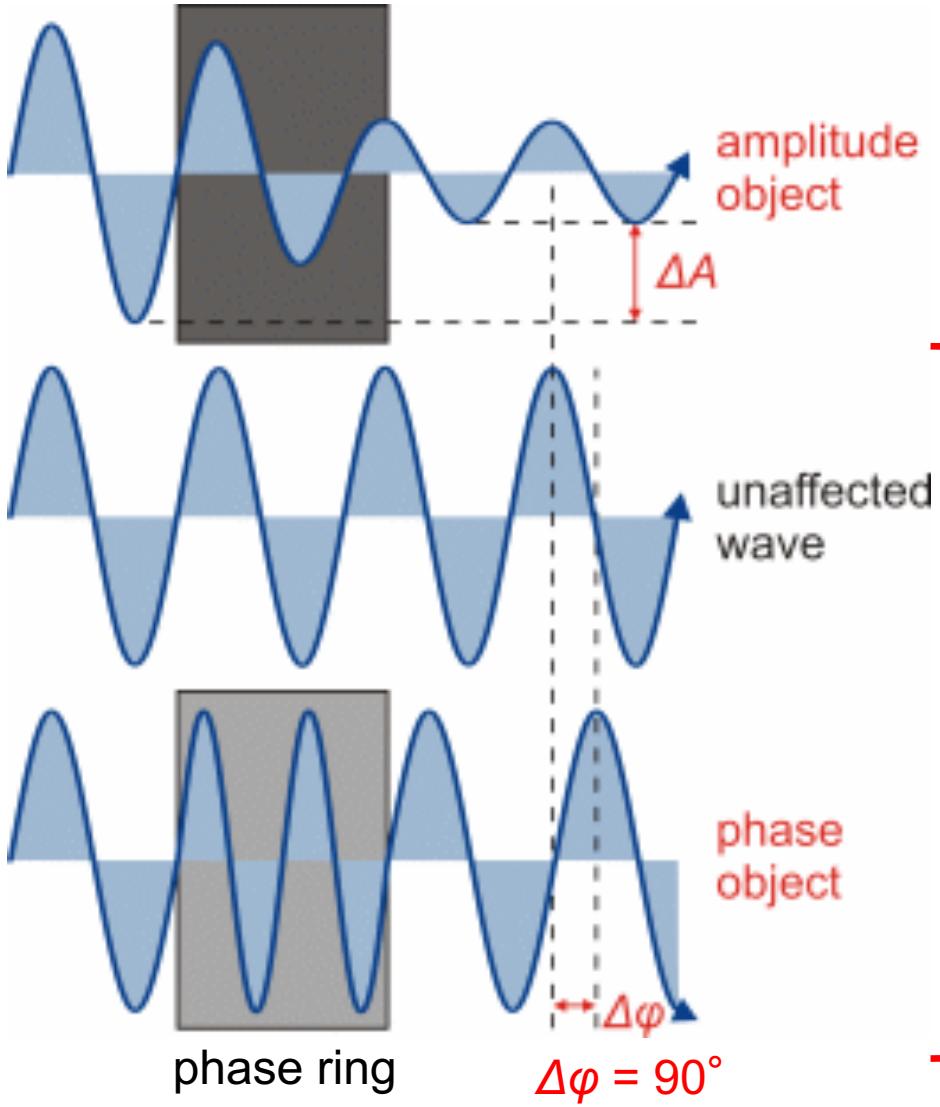
Improved cellular contrast by shifting the phase of light

=> in the phase ring, light is retarded (or advanced) by ¼ wavelength (Δφ = 90°)



=> Frits Zernike 1930

Phase contrast microscopy



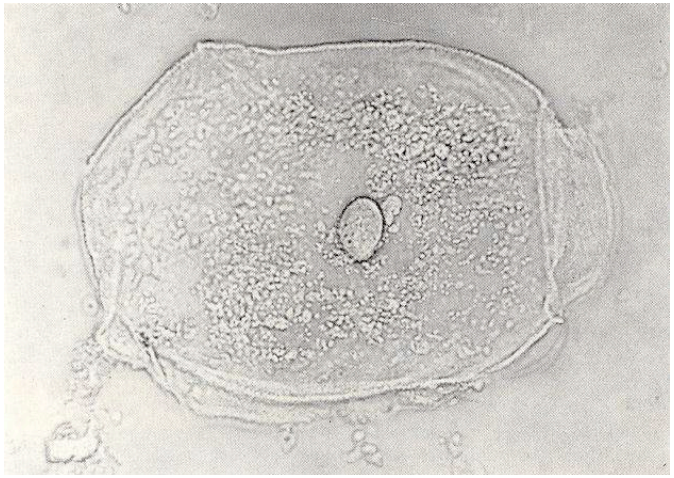
diffracted light (from specimen)

non-diffracted light (from light source)

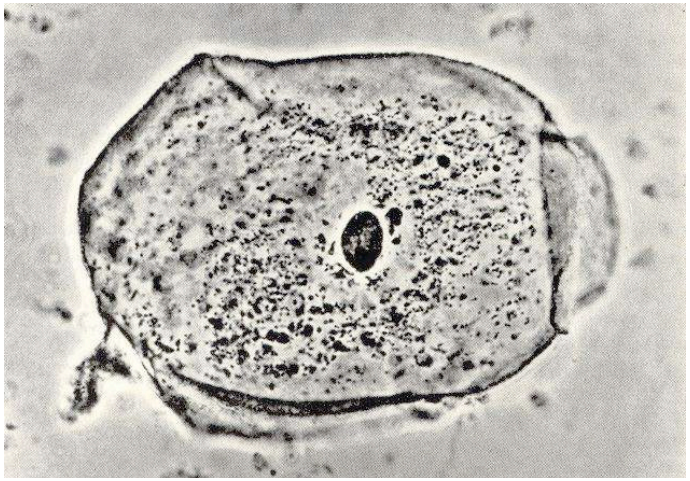
Interference: converts a phase difference into an amplitude difference (=> visible by eye)

Phase contrast microscopy

Bright-field image



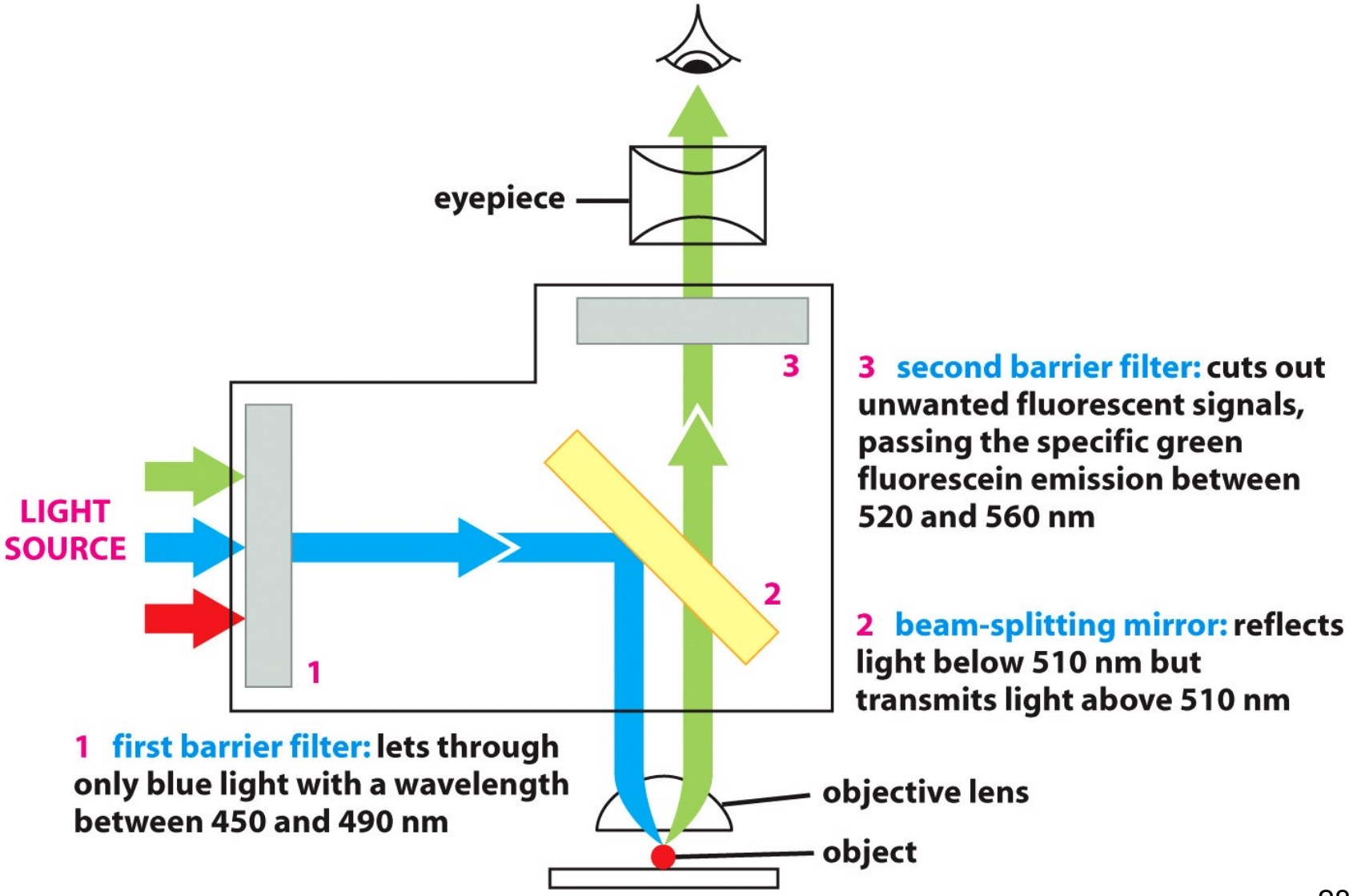
Phase contrast image



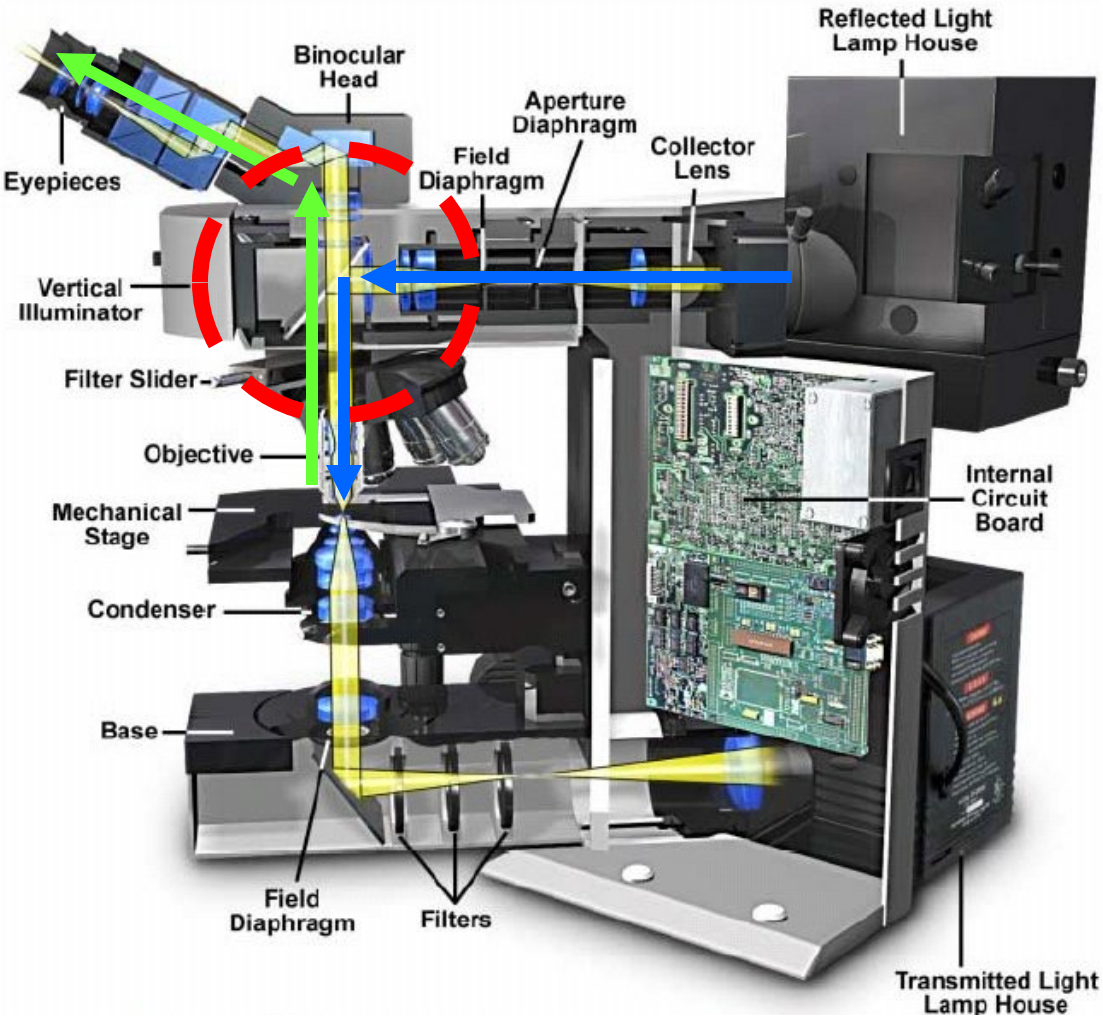
=> Phase contrast microscopy enables label-free detection of living cells

Fluorescence microscopy

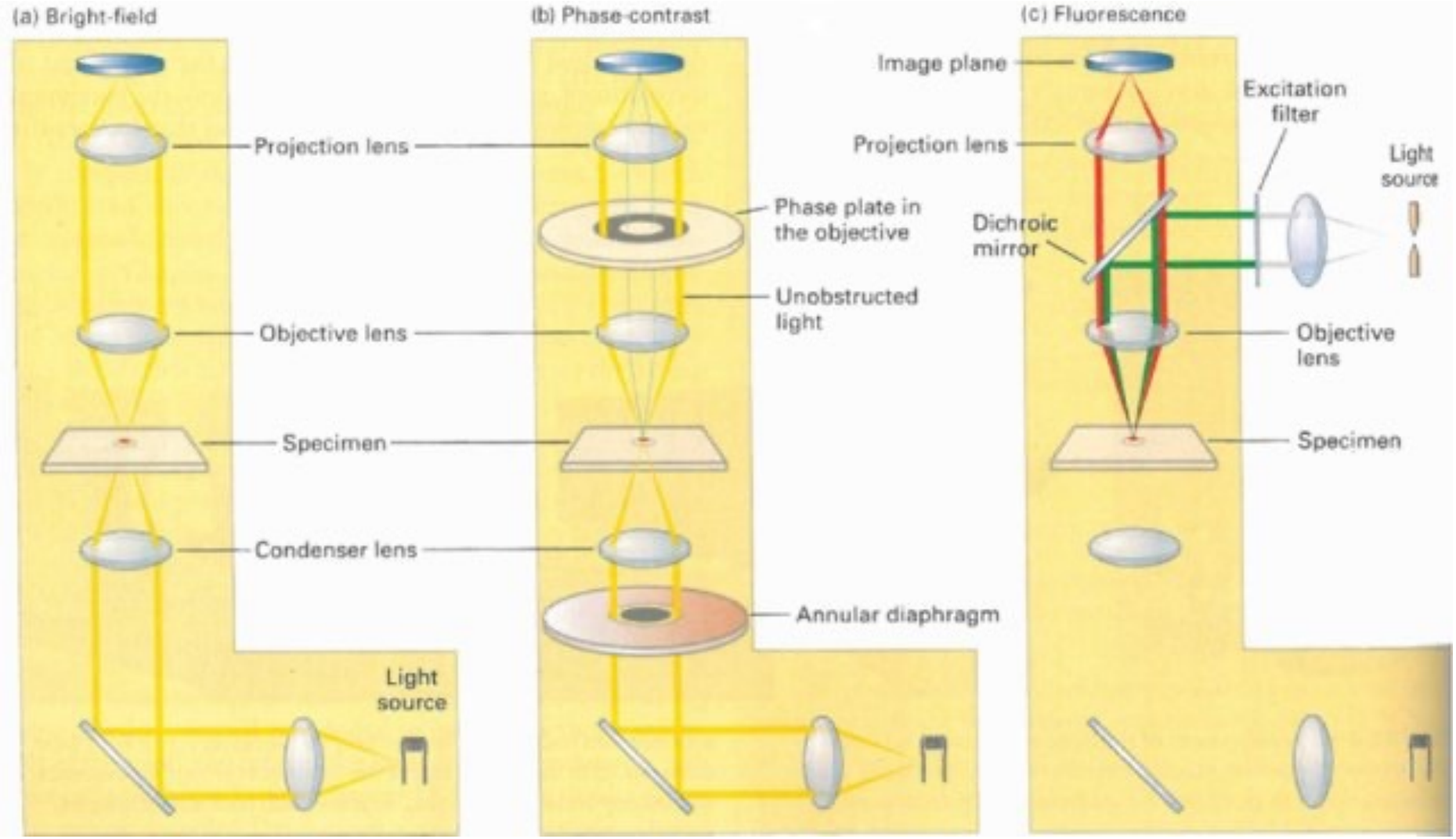
Epifluorescence microscopy



Setup of epifluorescence microscope



Comparision of microscopy in the life sciences



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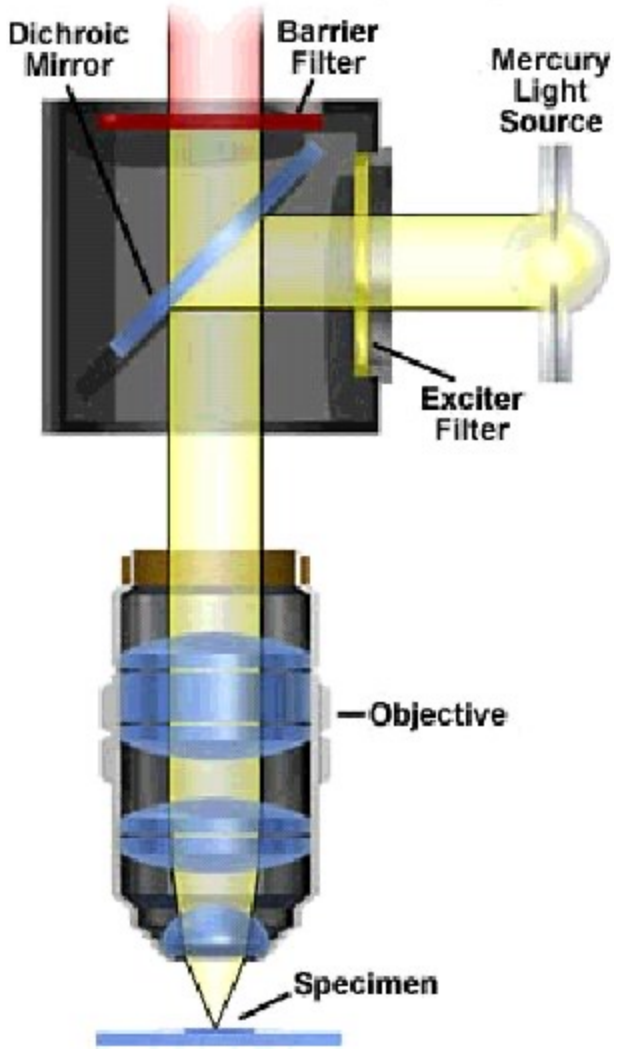
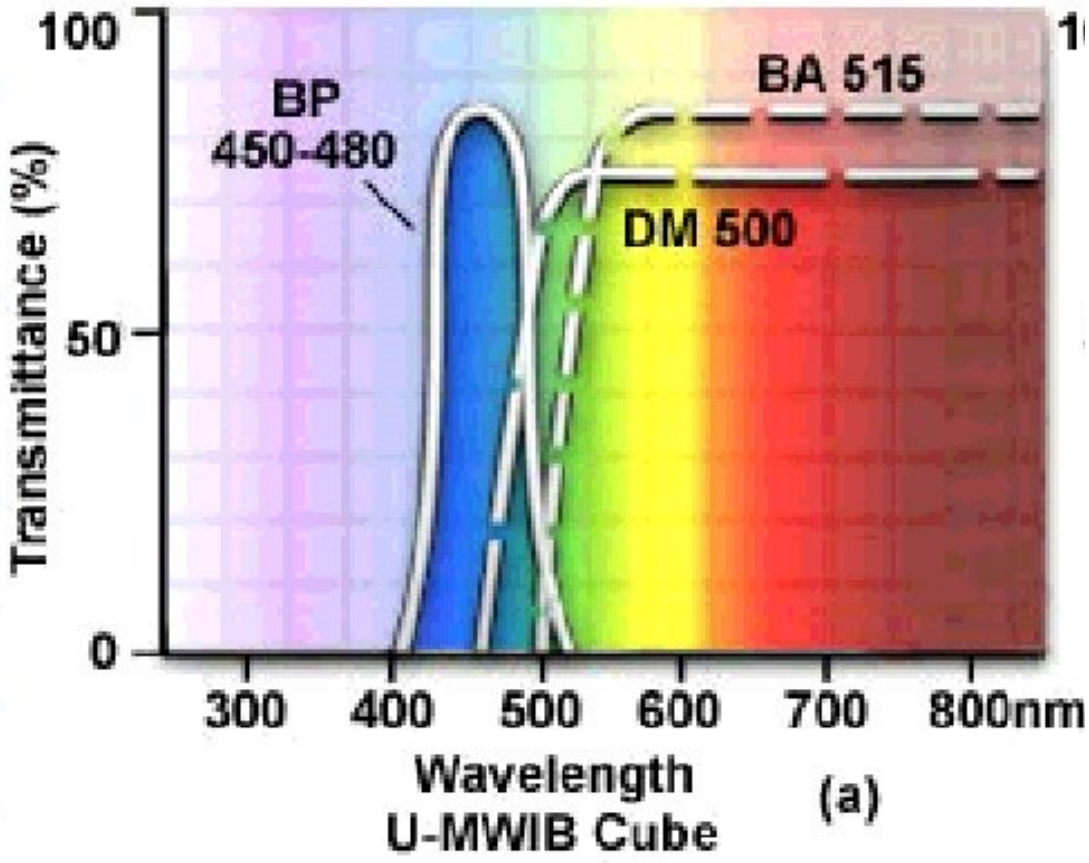
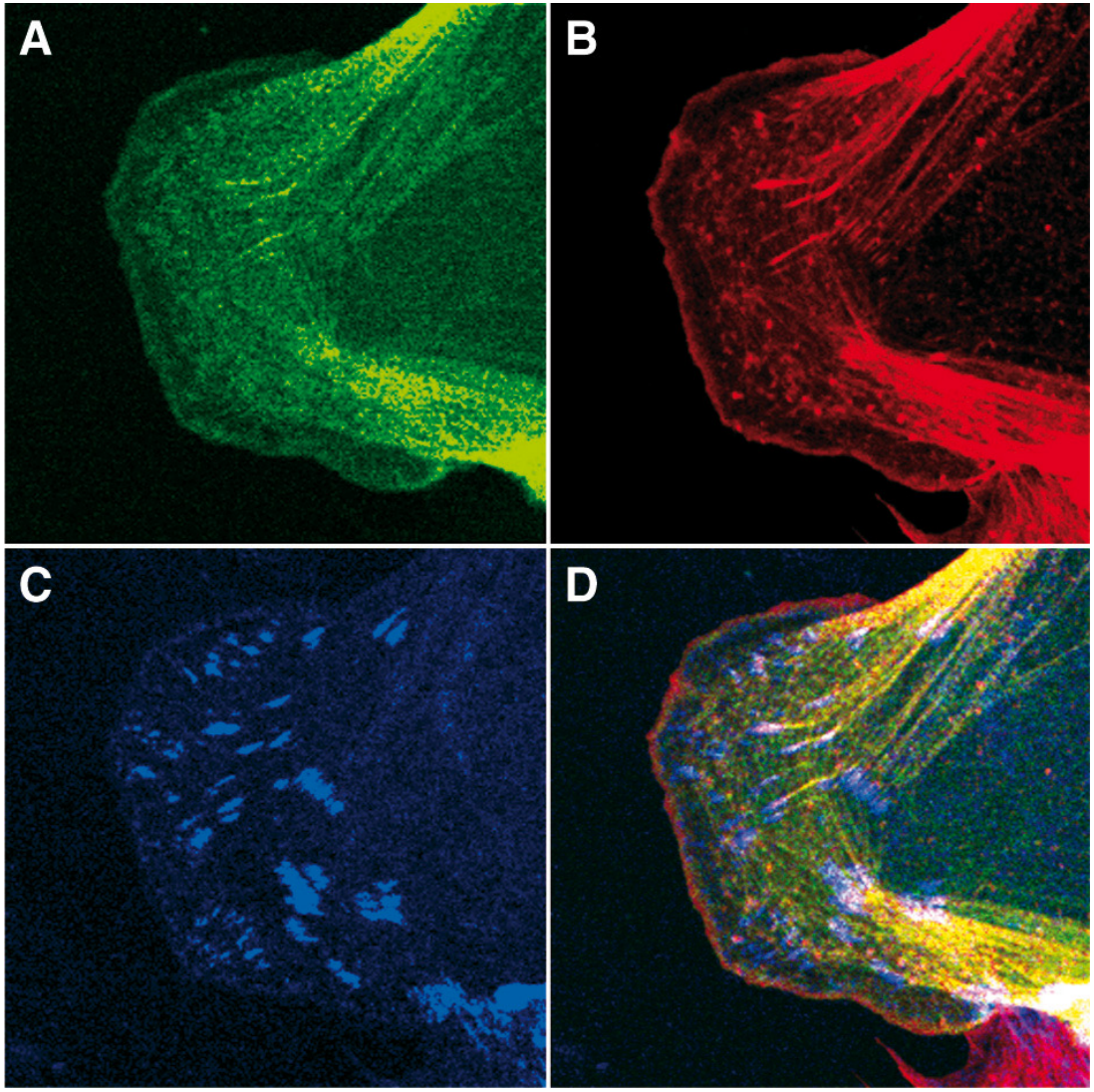




Fluorescence microscopy

3-fold fluorescence labeling of keratinocyte ➔ detection in 3 color channels



A) GFP-coupled pallidin
=> binds to actin
B) AlexaFluor546-phalloidin
=> binds to F-actin
C) Cy5-coupled antibody
=> binds to cell-substrate-adhesion protein
(immune fluorescence)
D) Overlay of three fluorescence signals

=> Sensitivity through dark background

Fluorescent dyes

natural fluorophores

Try, NADH, $FADH_2$ → UV excitation

GFP, EGFP, EYFP etc. → Excitation with UV or visible light

fluorescent labels

labeling of cell components that are non-fluorescent by themselves

proteins (directly or via antibodies): FITC, TRITC, Cy-3, Cy-5

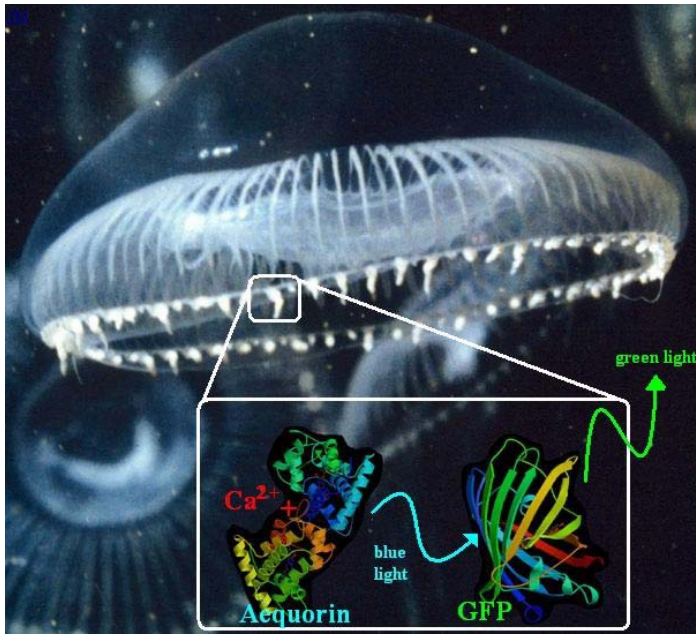
DNA, RNA: ethidium bromide, DAPI

lipids: DPH, Pyrenyl-PC

low molecular weight ions: Fluorescein (pH), Fura-2 (Ca^{2+})

problems:	reasons:	consequences:
- autofluorescence - light scattering	using short-wavelength light	high background
- photobleaching - cytotoxicity	strong excitation intensities	short imaging times
- labeling	non-specific binding	artifacts

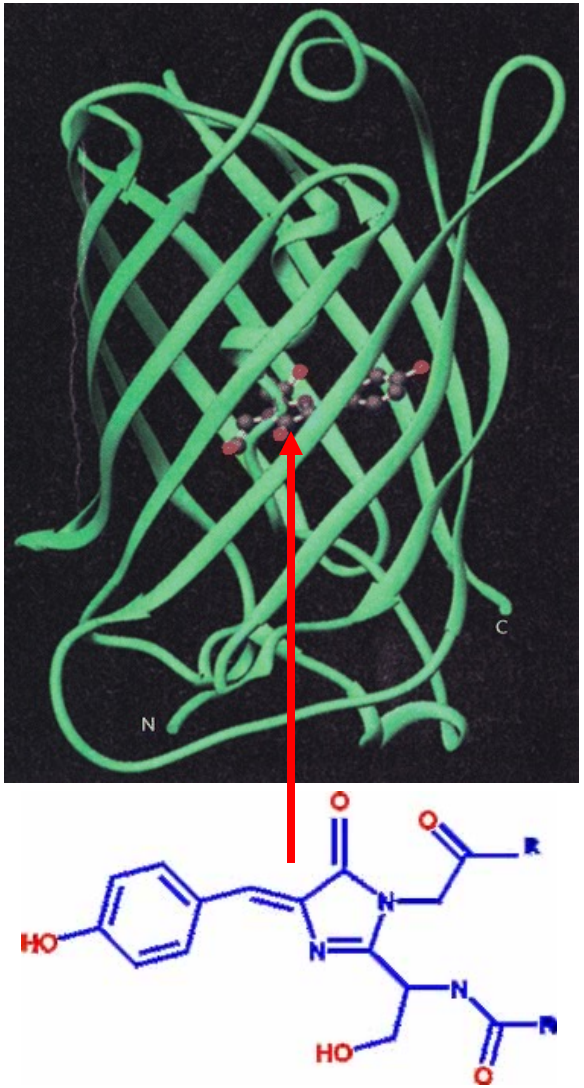
Green fluorescent protein (GFP)



Originally isolated from jellyfish.

=> Enormous importance via recombinant expression!

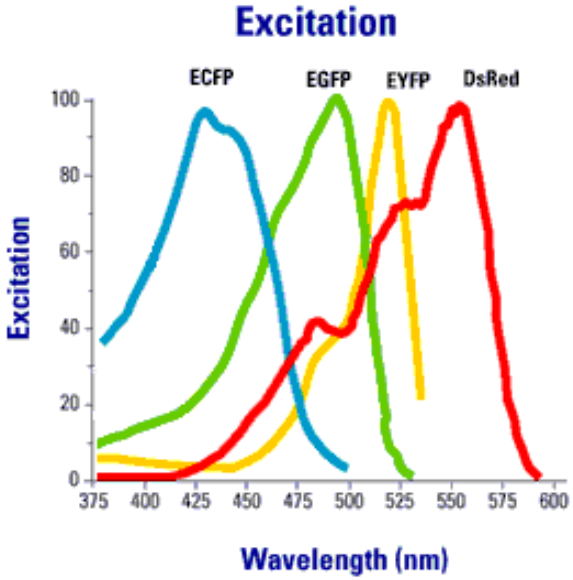
(Nobel Prize in 2008)

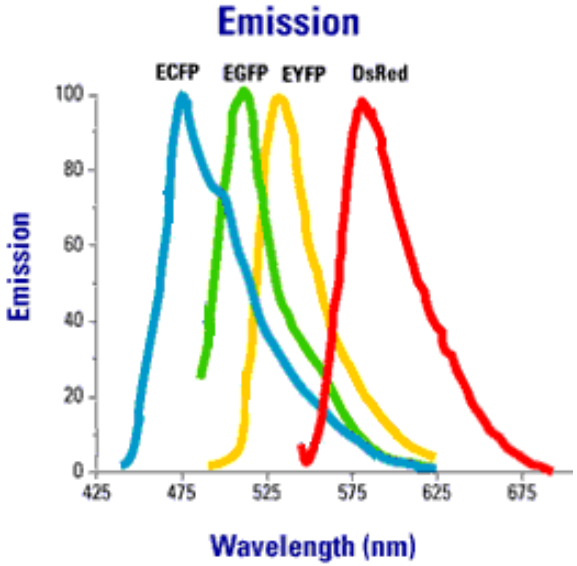


cyclic Ser_{65}-Tyr_{66}-Gly_{67}

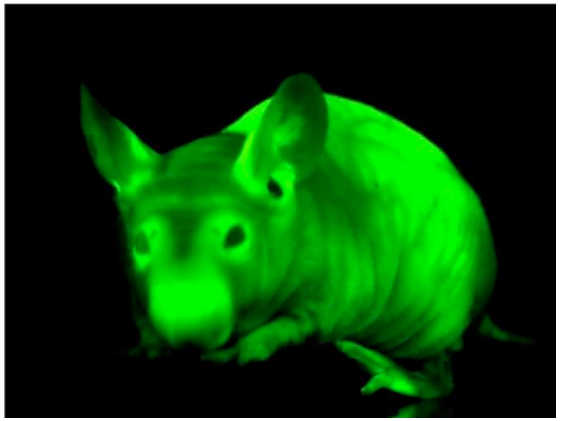
GFP and its derivatives

Enhanced *Green* Fluorescent Protein





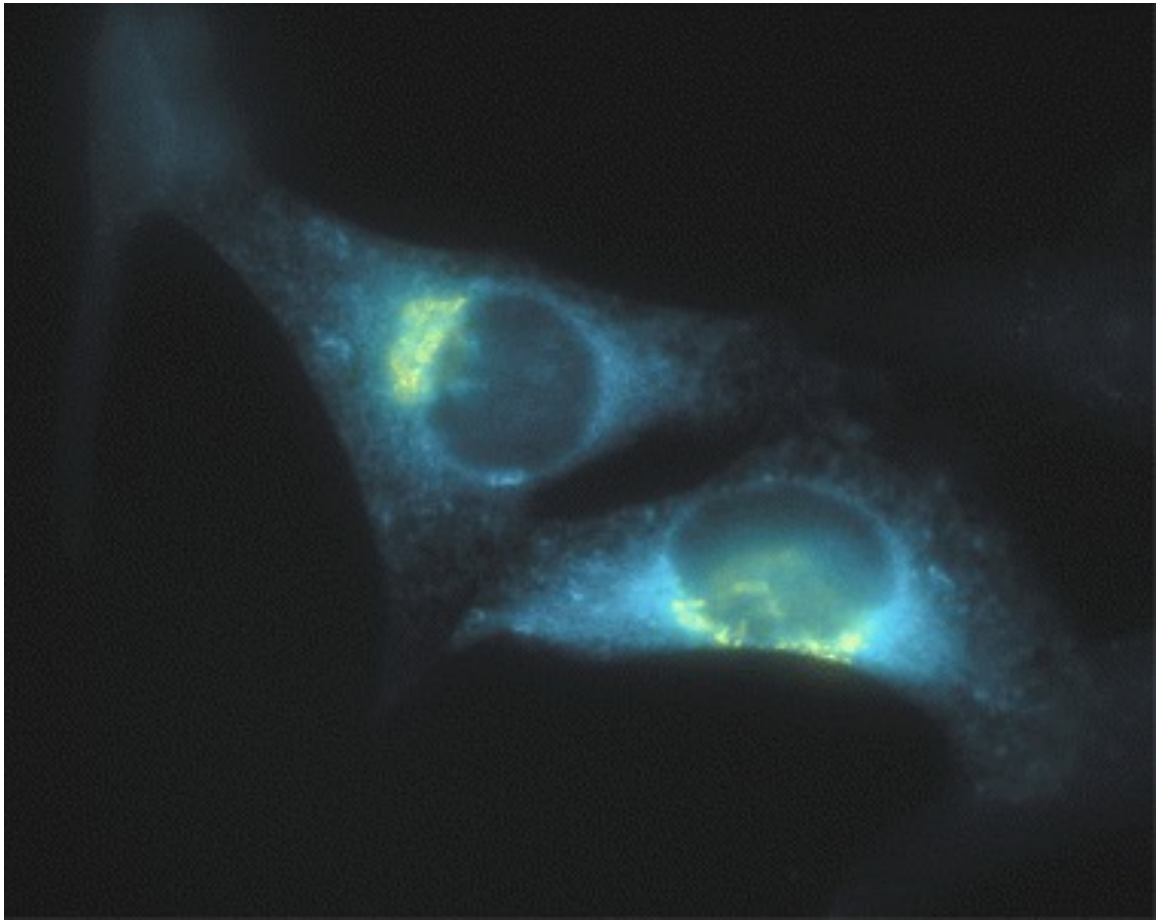
day light



under UV light

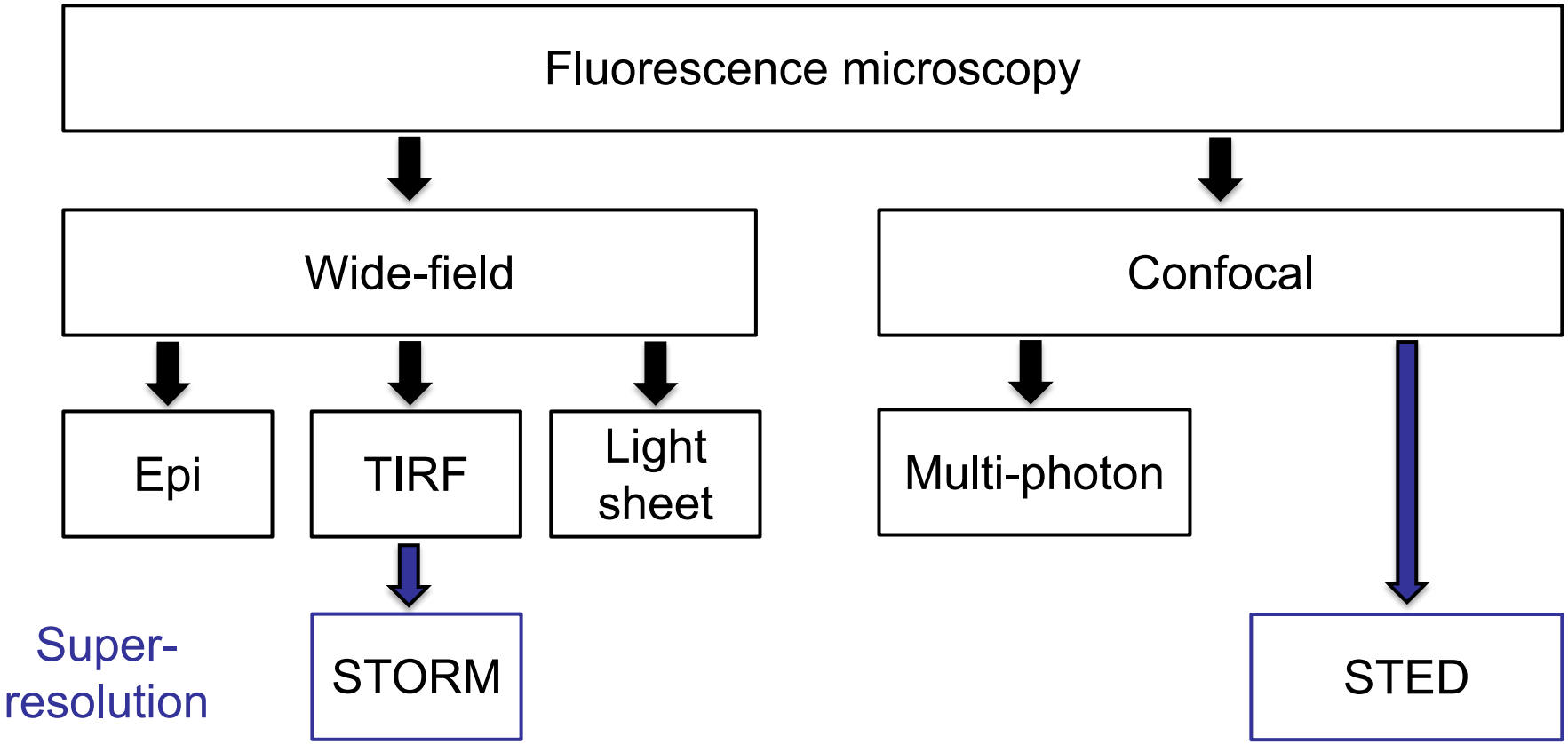
promoter	structural gen	EGFP

GFP chimera



ECFP - marker protein for endoplasmic reticulum
EYFP - marker for Golgi

Subcategories of fluorescence microscopy



Fluorescence microscopy: limitations

1. High background fluorescence:

Conventional wide field microscopy => A single fluorophore molecule cannot be detected (ultimate detection limit)

2. Diffraction limit of light:

The image resolution was defined by Ernst Abbe (1873):

$$d = \frac{\lambda}{2n \sin \alpha}$$

ca. 200 nm

=> Both problems have been solved over the last 30 years

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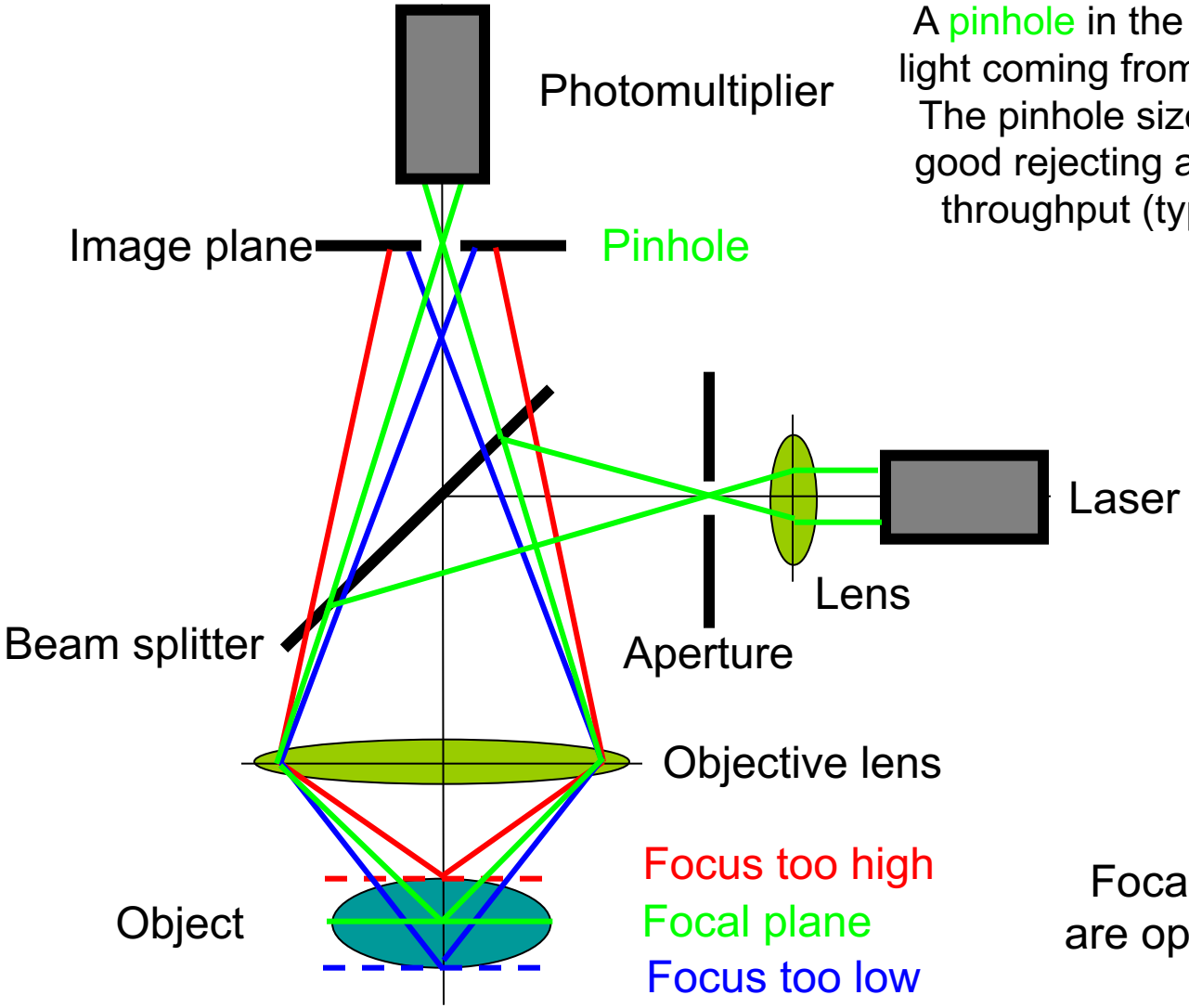
Avoiding autofluorescence and light scattering

State of the art microscope systems and cameras/photomultipliers are sensitive enough to visualize single fluorescent molecules
=> A single fluorophore can emit up to 1.000.000 photons before it photobleaches

Problem: Background signal
(Autofluorescence / Rayleigh scattering / Raman scattering)
Background can be reduced by reducing the excitation volume
- Confocal microscopy / Multi-photon microscopy
  (ellipsoid excitation volume of ca. 1 x 1.5 µm = 10^{-15} L = 1 Femtoliter)
  => contains 1 molecule of fluorophore but also 10^{10} solvent molecules
- Total internal reflection microscopy (TIRF)
  (planar excitation volume of ca. 100 nm depth, evanescent field)

Confocal laser scanning microscopy (CLSM)

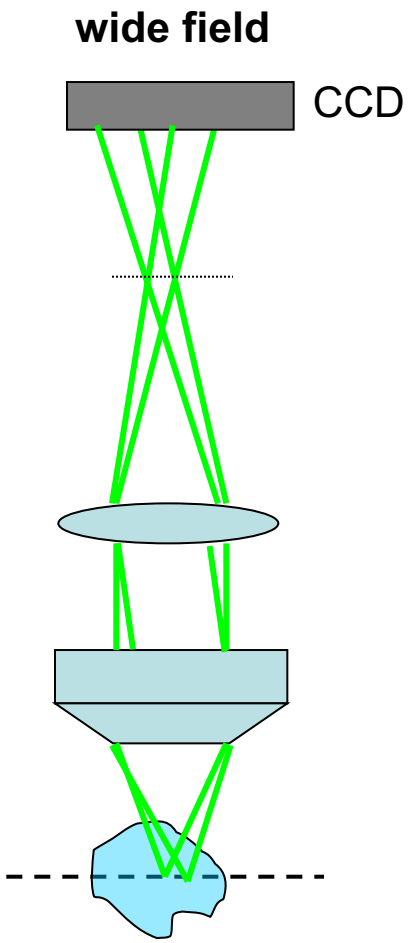


A pinhole in the image plane rejects the light coming from outside the focal plane. The pinhole size is a trade-off between good rejecting ability and sufficient light throughput (typically ~ 30 – 150 μm)

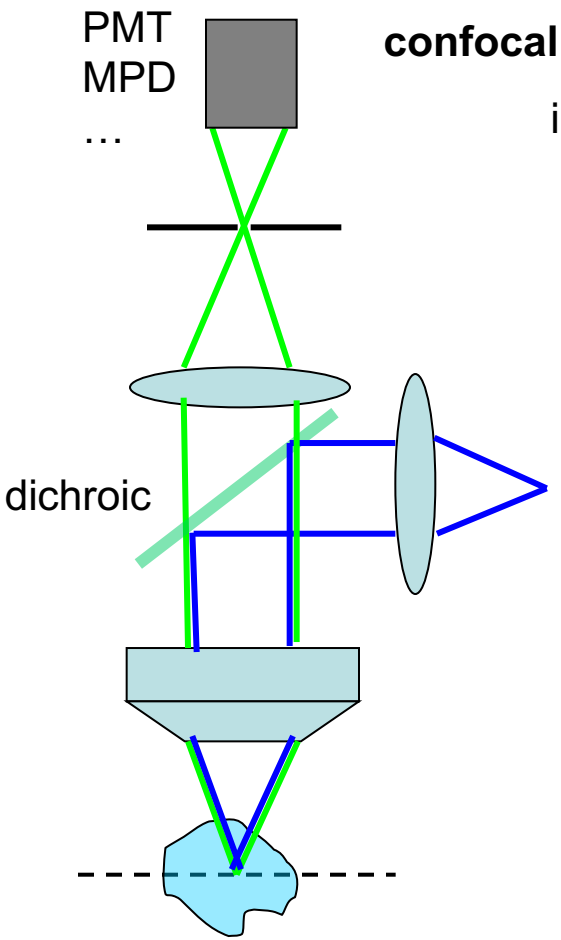
Focal plane + Pinhole are optically conjugated!

Confocal laser scanning microscopy (CLSM)

The pinhole restricts the observed volume of the sample to a single point (the size of which is restricted by the pinhole size). Excitation by a collimated beam (point source optically conjugated to the pinhole) focused to a diffraction limited spot

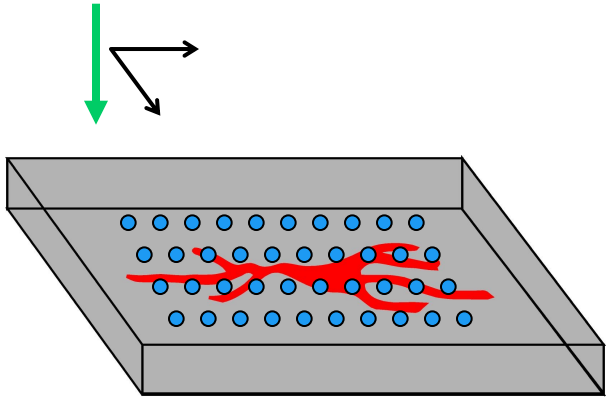


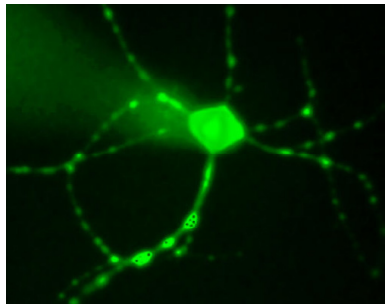
whole image at once



confocal

image is scanned point by point

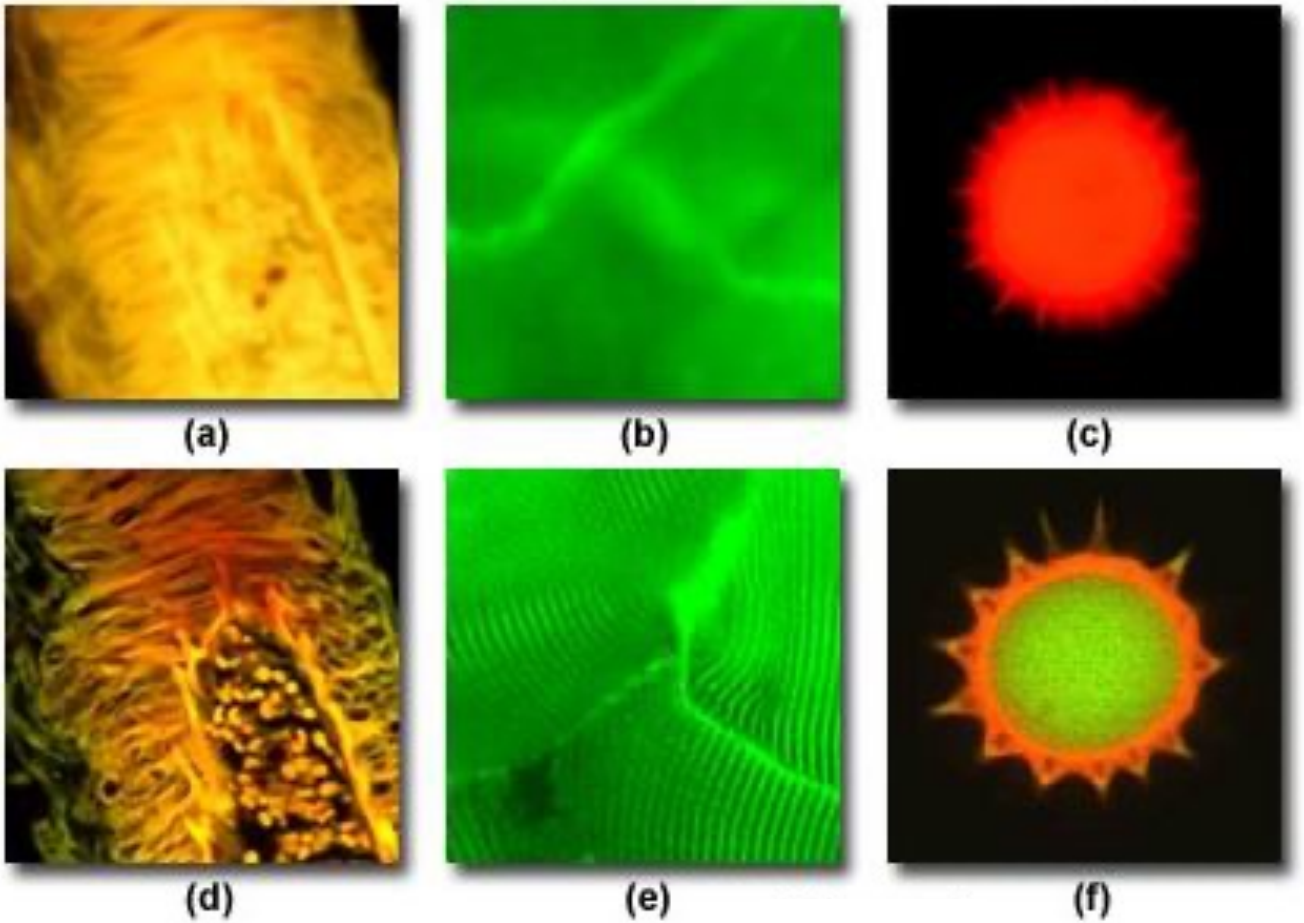




Confocal vs. wide-field microscopy

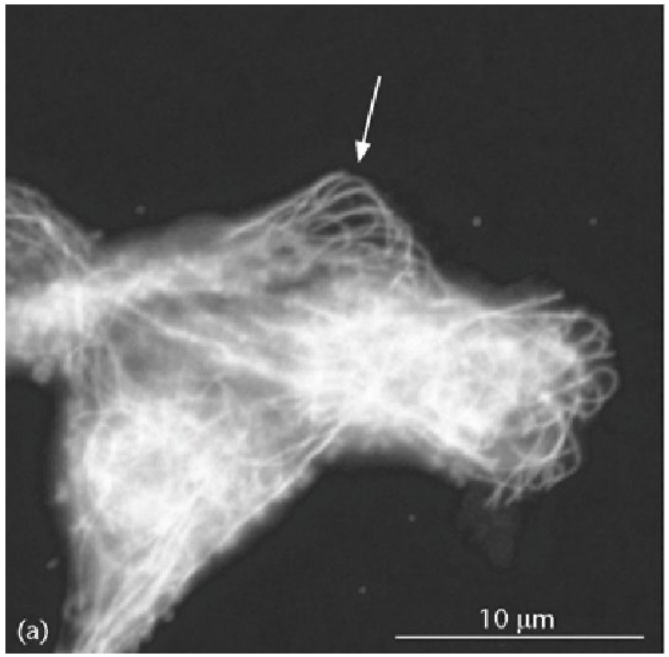
Wide-field

Confocal



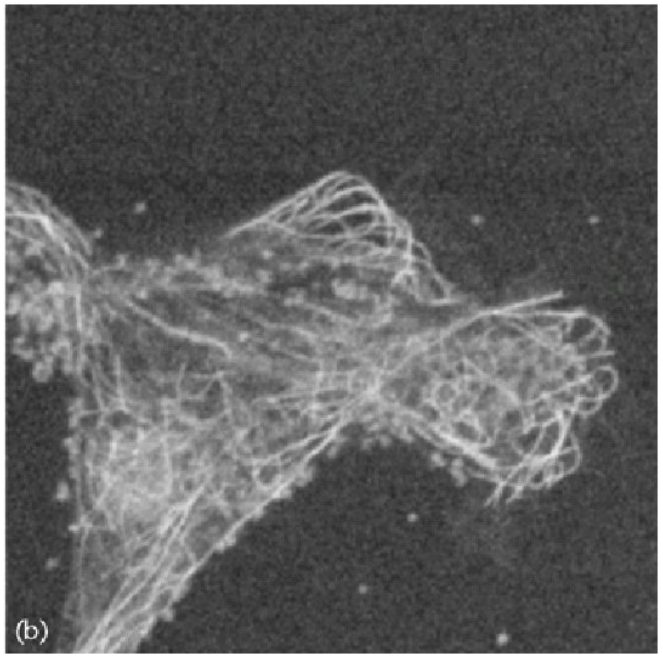
Elimination of out-of-focus light improves contrast and, thus, resolution

Confocal microscopy: improved lateral resolution



confocal aperture open

no depth of field (hloupka pole)

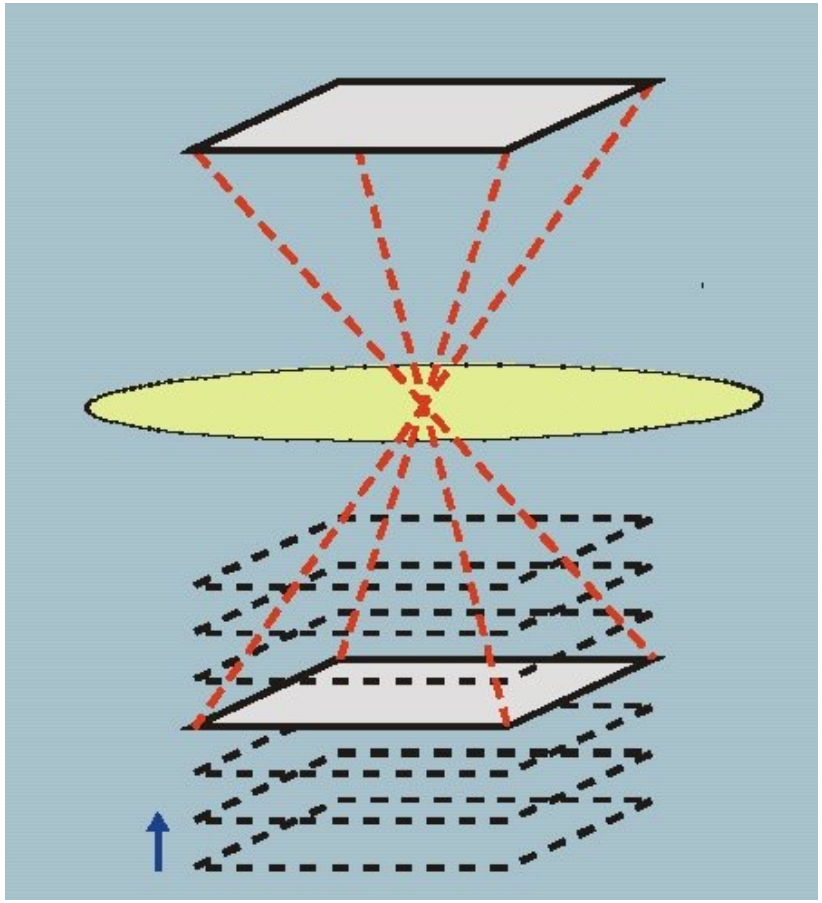


confocal apterture (optimal)

depth of field, z-resolution

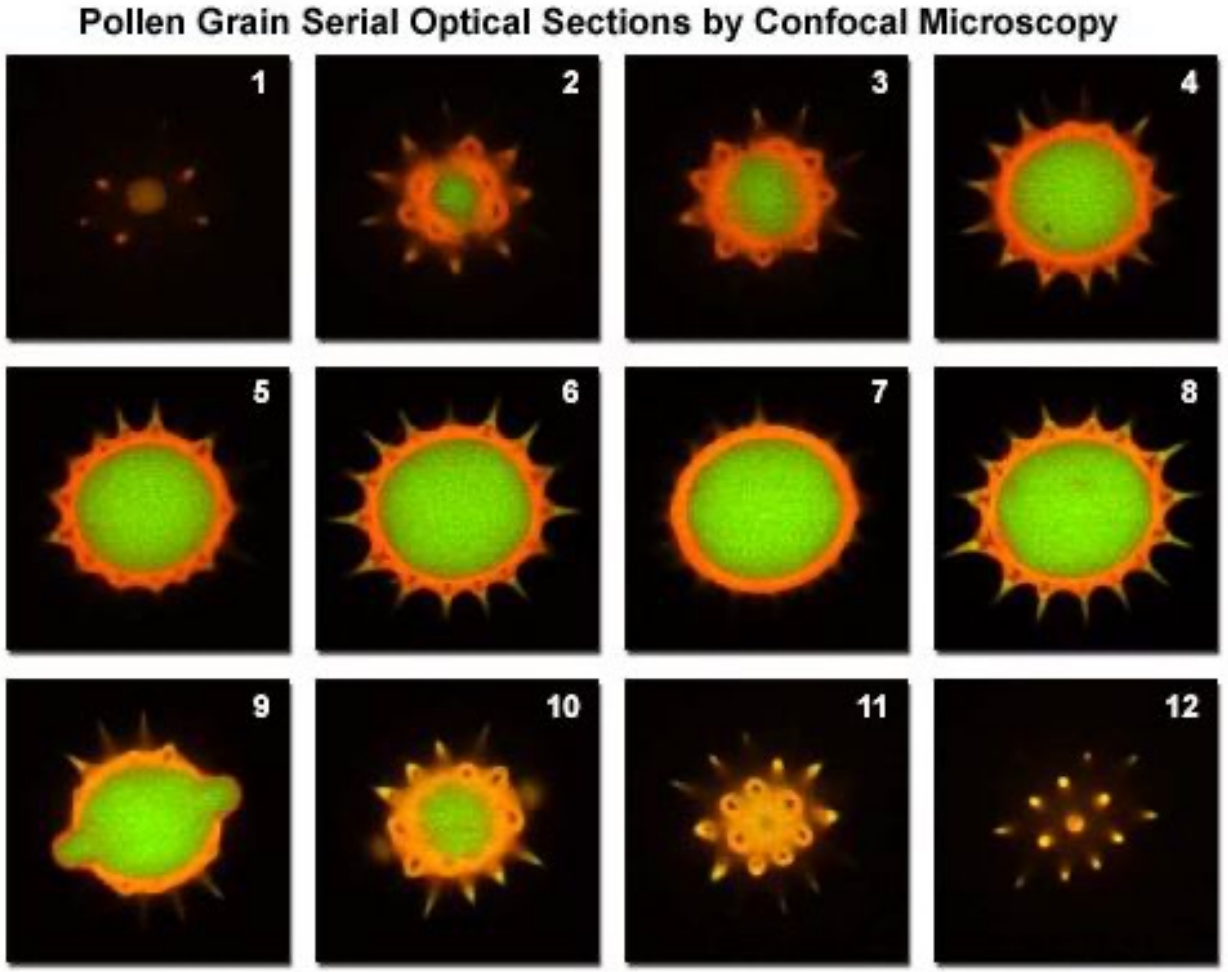
Confocal microscopy

Preparation of „optical sections“ through thick samples: z resolution



From the optical sections:
Calculation of side views
3D-reconstruction

Confocal microscopy



Focusing only in one plane → axial sectioning of the sample to ~ μm slices

Summary of confocal microscopy

Advantages

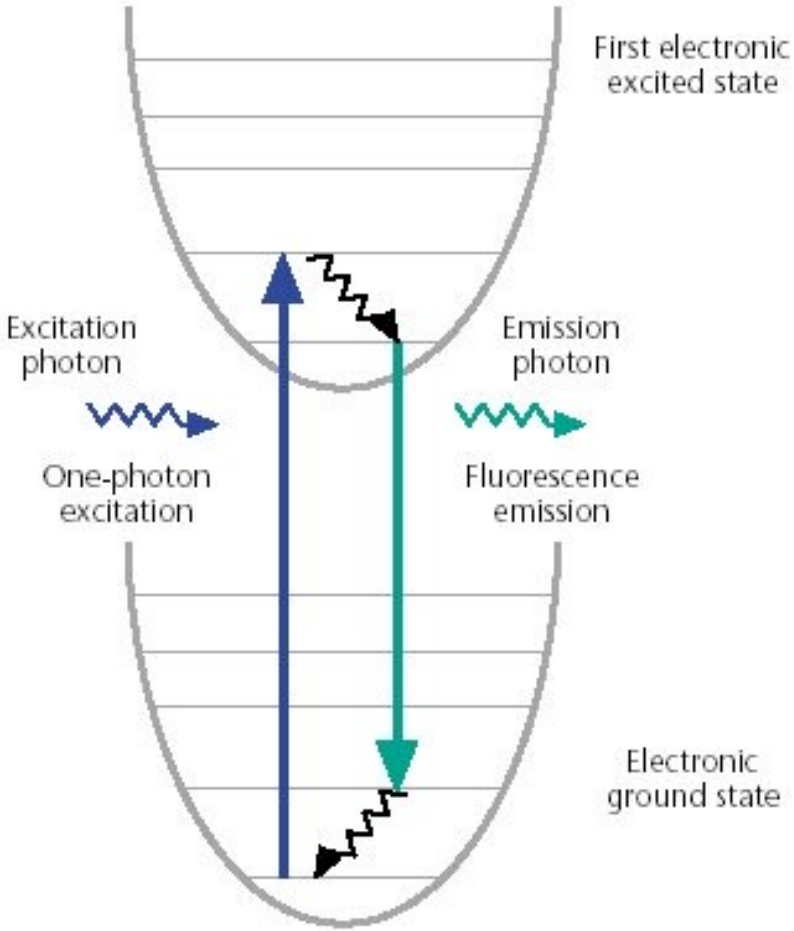
- improved contrast
- optical sectioning (z stacks)
- multiple fluorescence measurements can be performed in individual points (e.g. lifetime, spectra, fluorescence correlation spectroscopy)

Limitations

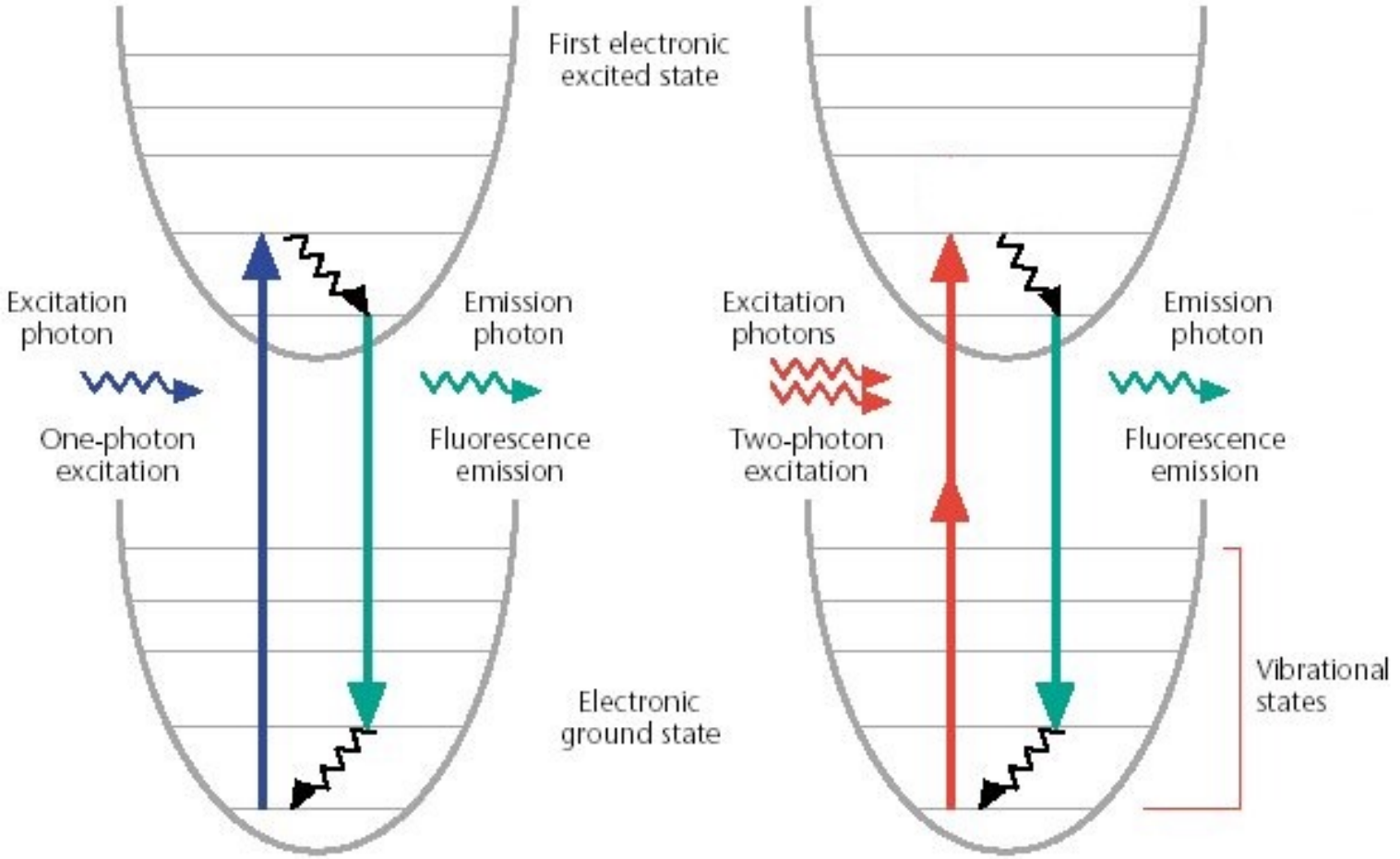
- more expensive and complicated setup
- slower than wide-field imaging
- longer imaging time needed
  => more photobleaching

Multi-photon microscopy (2p-, 3p-, 4p-microscopy)

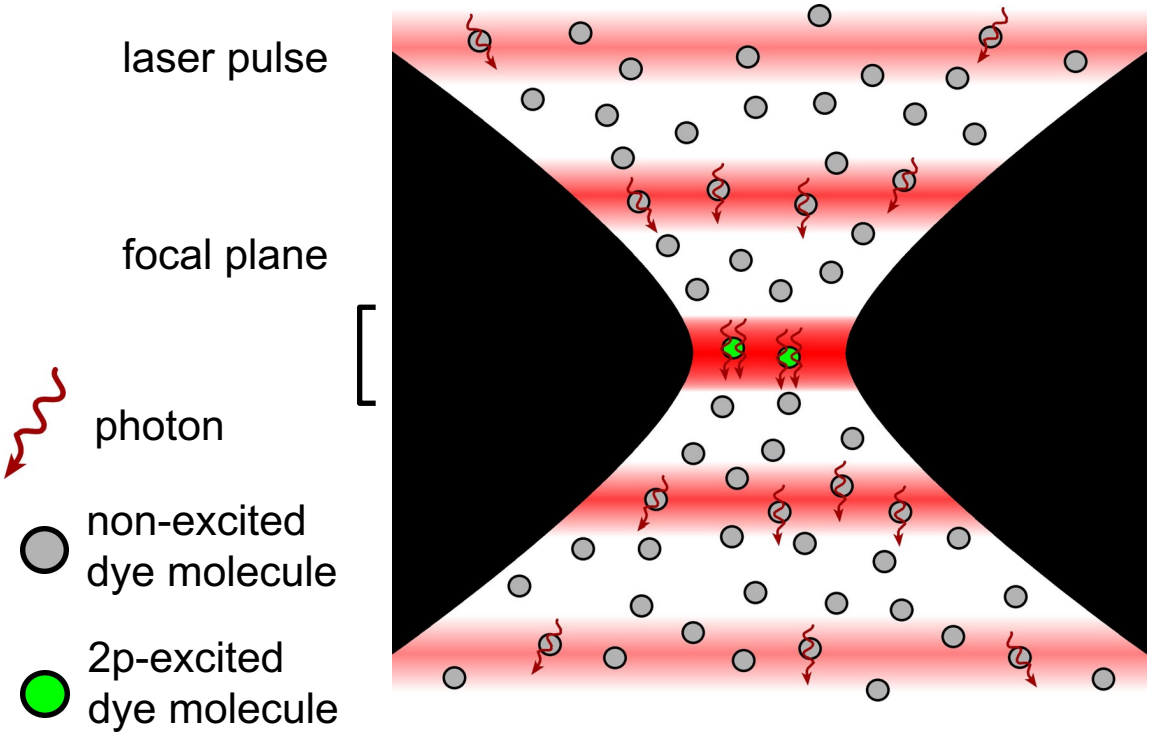
Two-photon microscopy



Two-photon microscopy



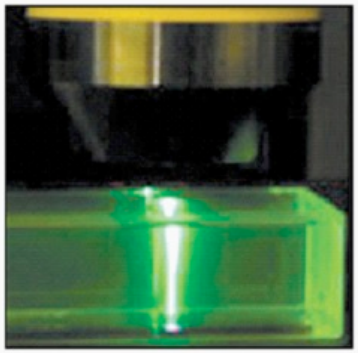
Two-photon microscopy: Axial resolution



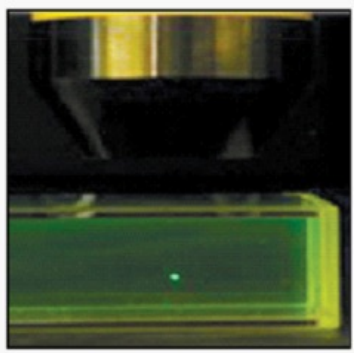
the required photon density for 2-photon excitation is established only in the focal plane

- no out-of focus fluorescence
- no pinhole needed

conventional 1p-excitation



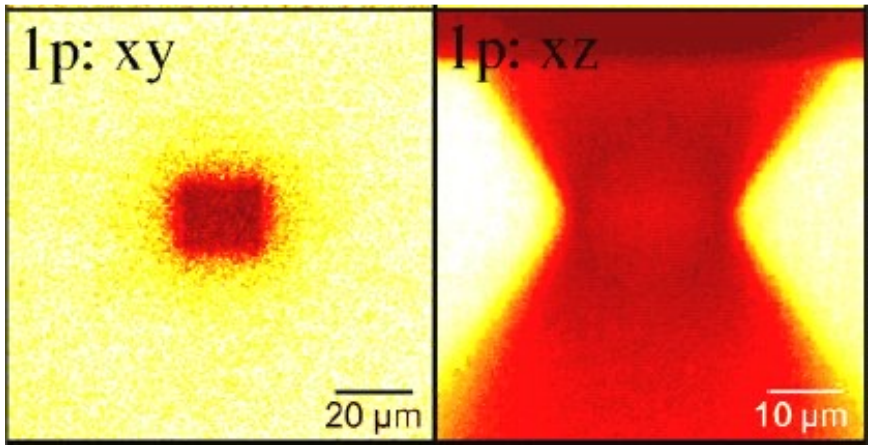
2p-excitation

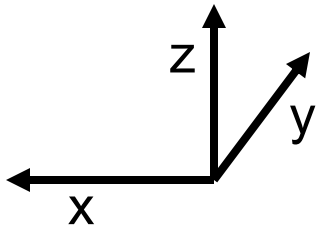


Two-photon microscopy

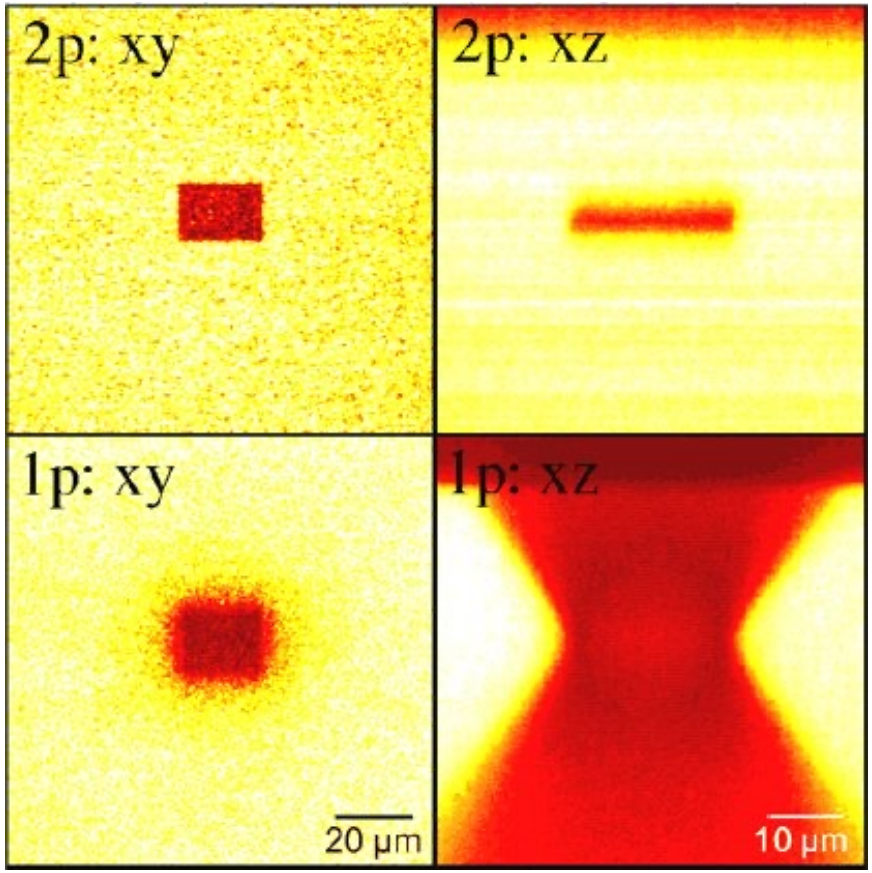
Comparison of emission profiles 1p vs 2p

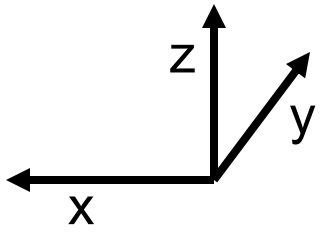
Illumination spot





Two-photon microscopy





Illumination spot

Summary of two-photon microscopy

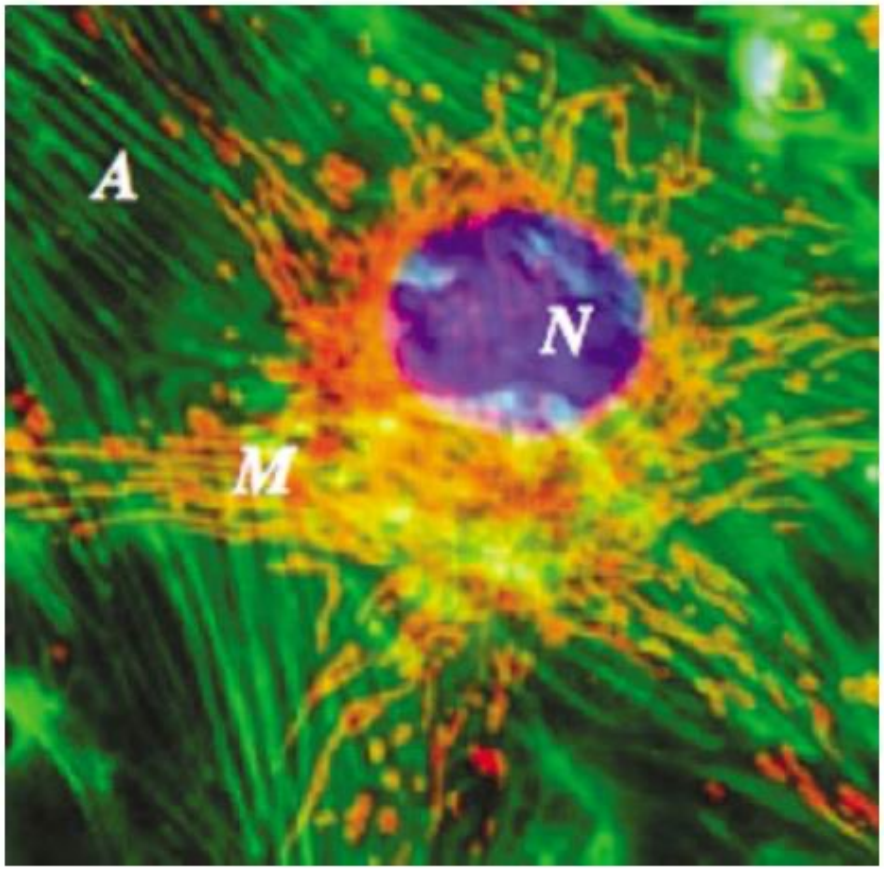


Fig. 5. Multiple fluorescence image. Nuclear DNA (N), mitochondrial distribution (M), and actin filaments (A) are visible after TPE at 720 nm. Sample is a bovine pulmonary artery endothelial cell (F-147780, Molecular Probes). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

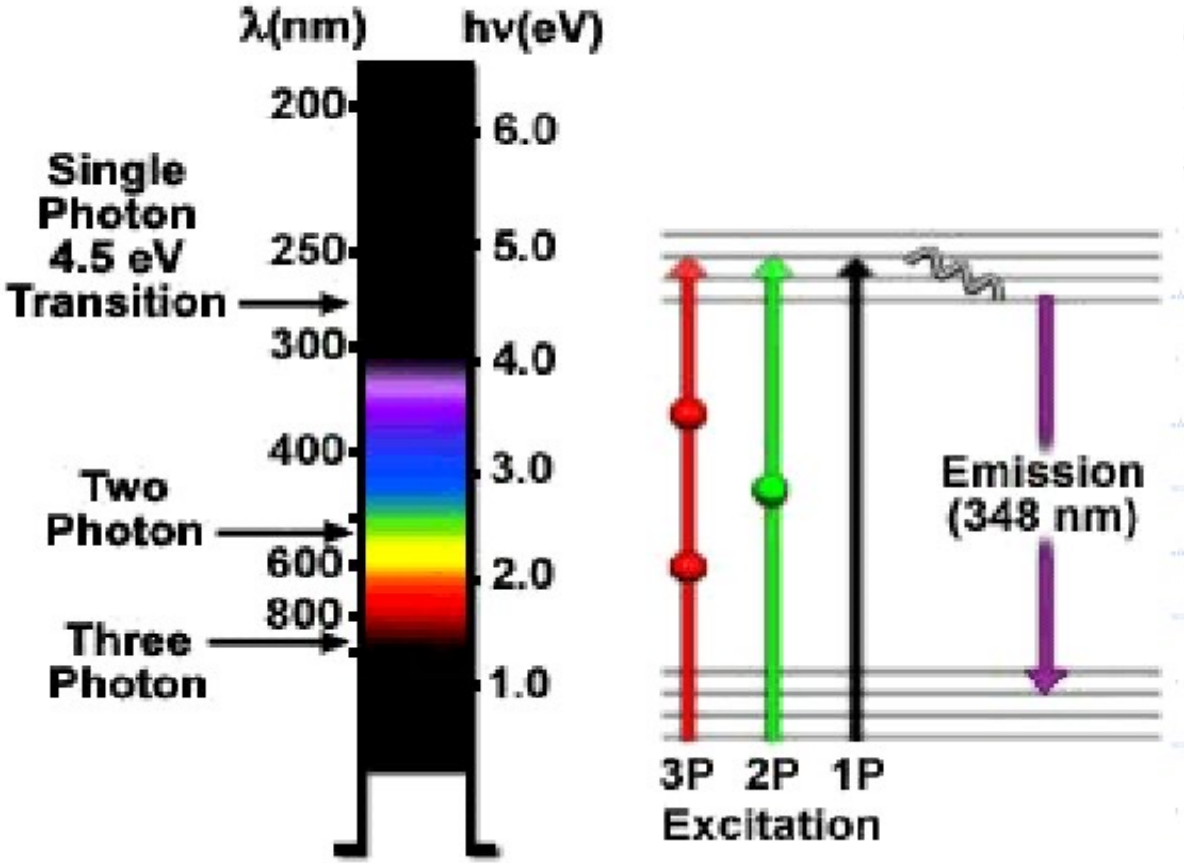
Advantages

- improved axial resolution
- reduced bleaching out of focus
- higher light collection efficiency (no pinhole)
- higher depth of light penetration (≈ 5 x)
- broader excitation spectra: simultaneous excitation of more dyes

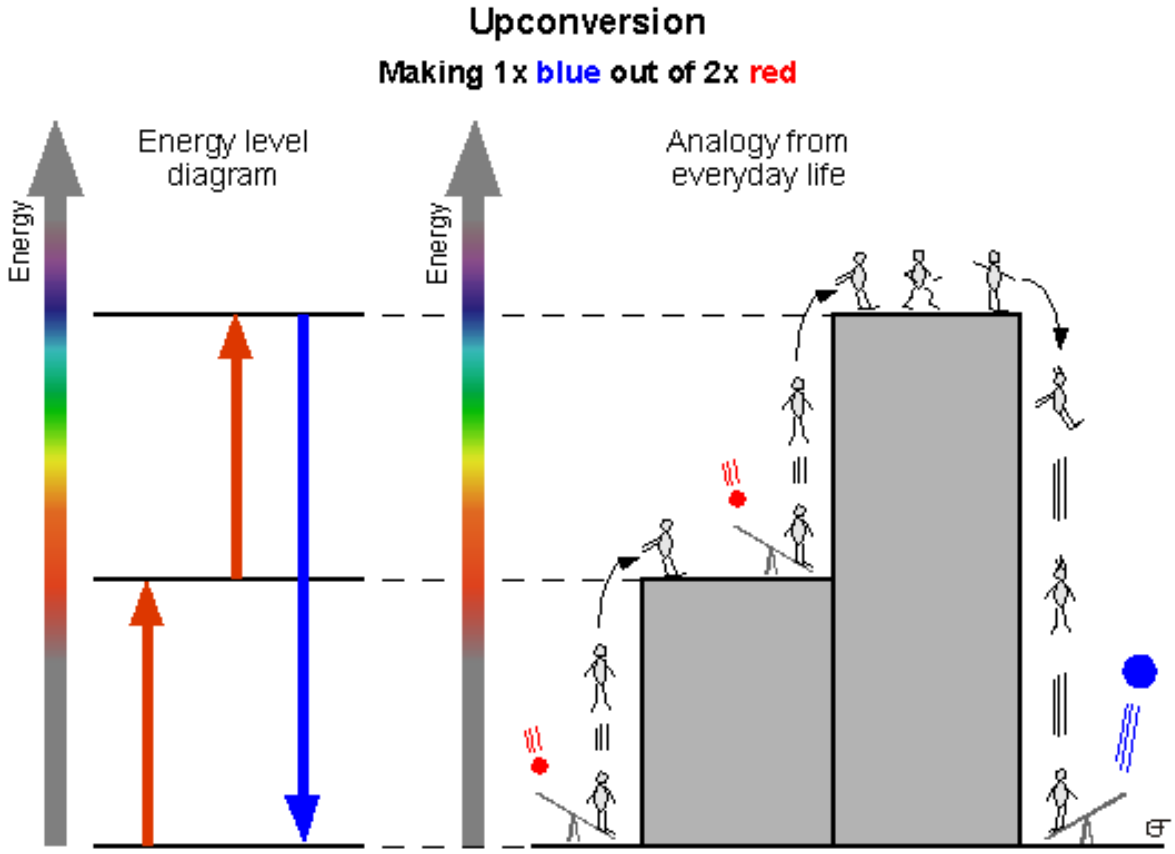
Limitations

- more expensive and complicated instrumental setup: pulsed (femtosecond) solid state lasers required for extremely high excitation powers (100 kW)
- higher bleaching in the focus
- broader excitation spectra: decreased selectivity of excitation
- scanning technique is slower (=> confocal microscopy)

From two- to multi-photon microscopy



Sequential absorption of two or more photons

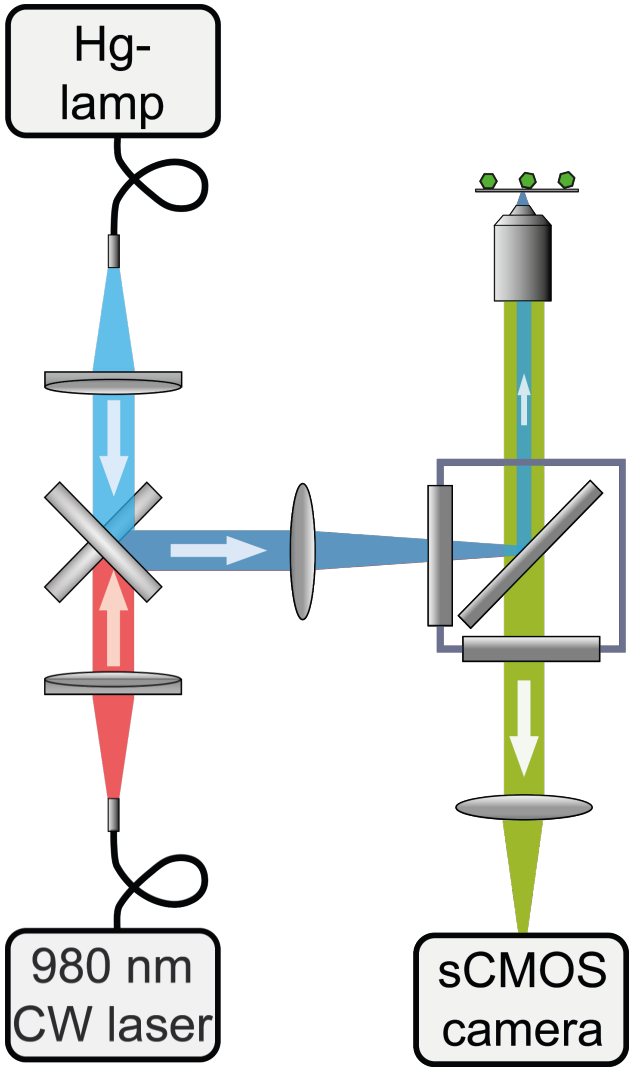


Sequential absorption of 2 or more photons via long-lived transition states
=> More time for absorbing a further photon

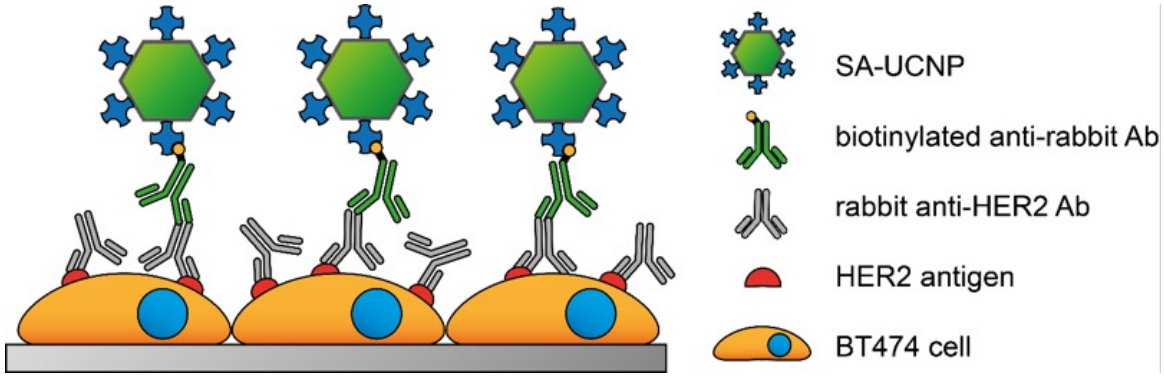
The process is ca. 1 million times more efficient than 2-photon excitation
=> a continuous wave (CW) laser diode can be used

Upconversion microscopy

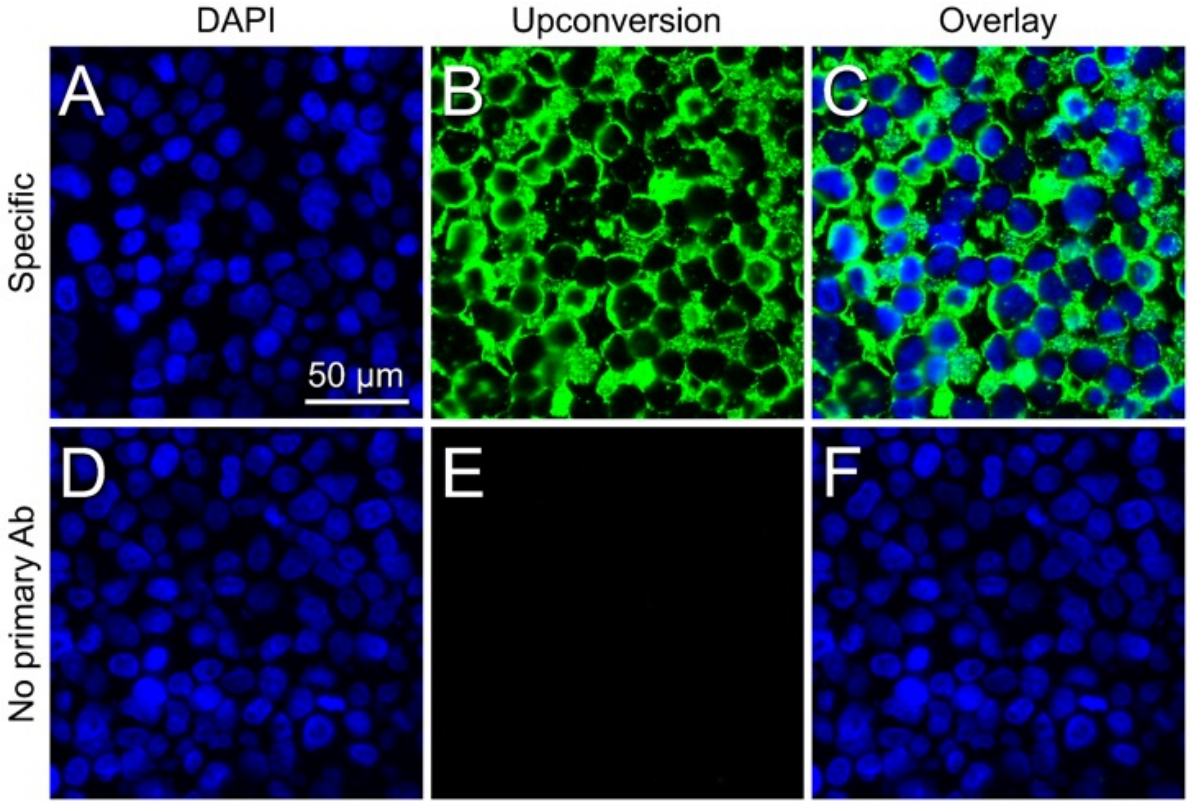
Wide-field microscopy

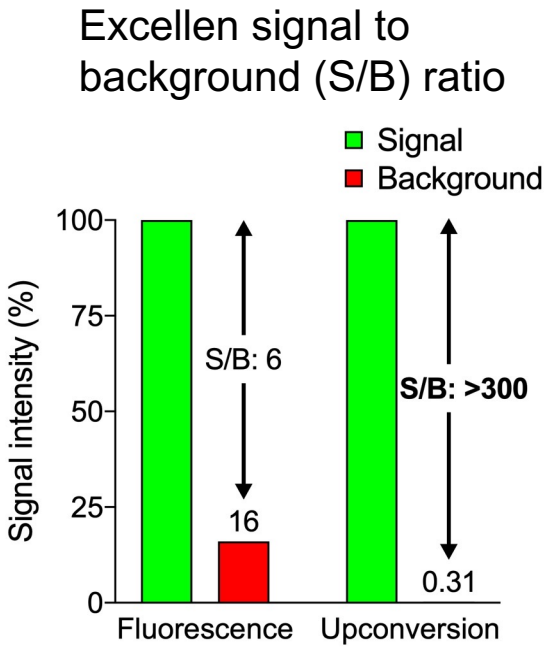


Labeling of cancer cells:



Upconversion microscopy





⇒ Small differences in protein expression levels can be dected.

Farka Z, Mickert MJ, Mikušová Z, Hlaváček A, Bouchalová P, Xu W, Bouchal P, Skládal, P, Gorris HH (2020) *Nanoscale 12*, 8303

Total Internal Reflection Fluorescence microscopy (TIRF)

Total internal reflection fluorescence mic. (TIRF)

Snell‘s law:

$n_1 \sin\alpha = n_2 \sin\alpha‘$

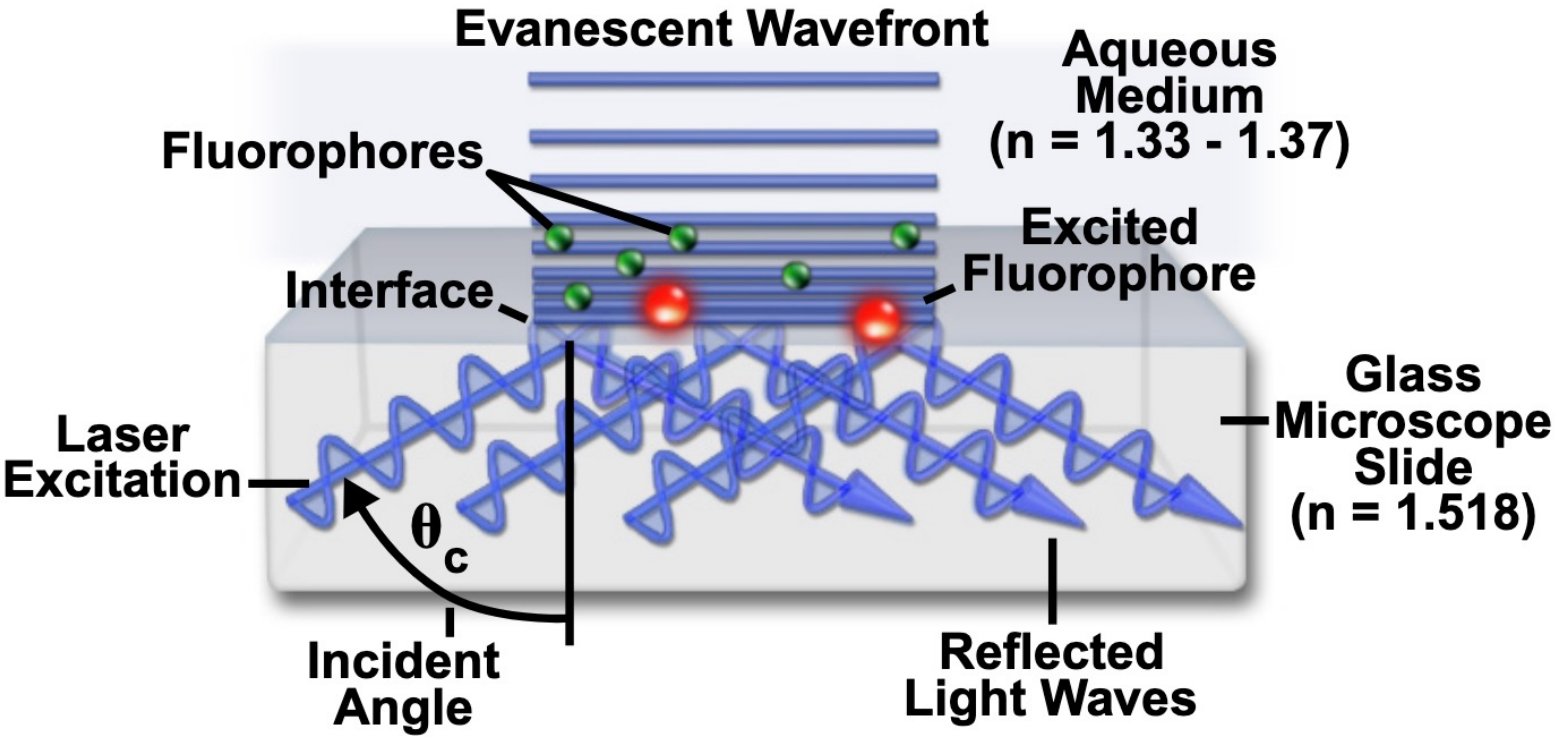
Holds until reaching the critical angle (θ), then: Total internal reflection

$$d = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2 \vartheta - n_2^2}}$$

d: depth of evanescent field
λ: wavelength of light
θ: critical angle

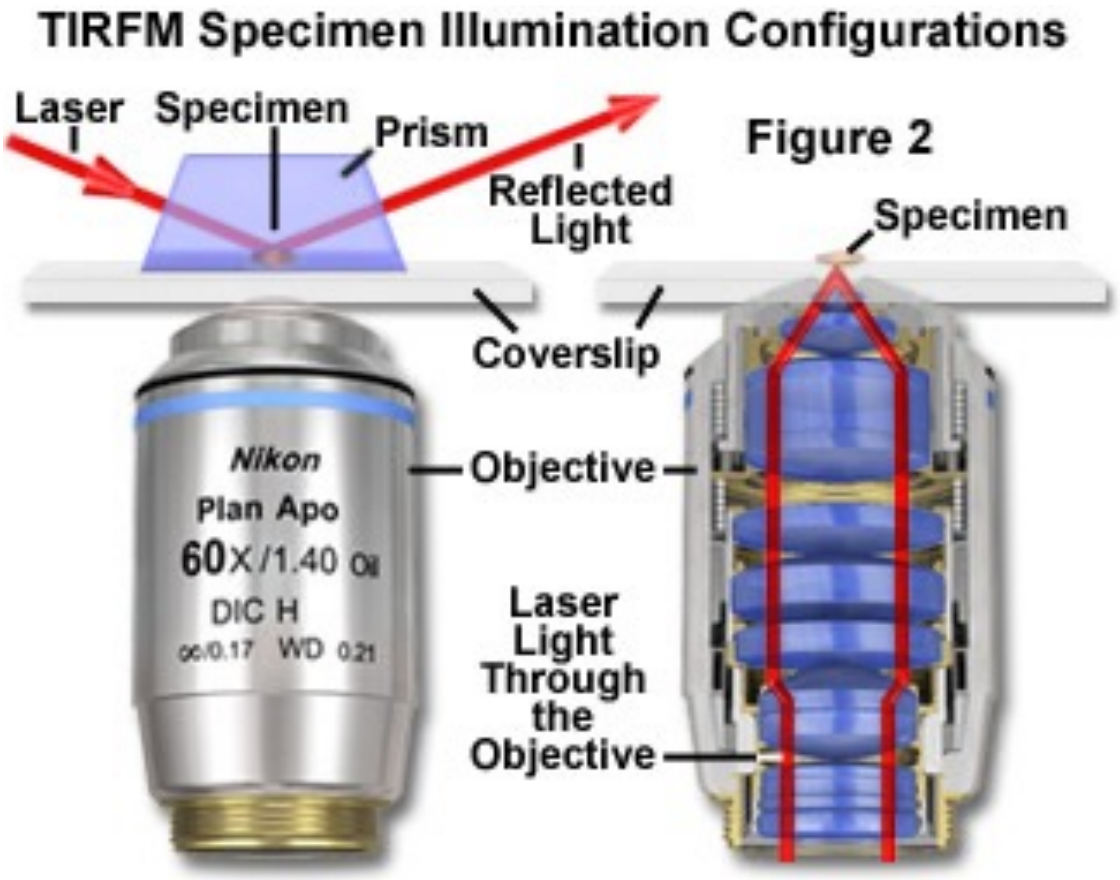
Total internal reflection fluorescence mic. (TIRF)

Total internal reflection leads to emergence of an evanescent field (with exponential decay of intensity):
=> reduces the excitation volume to a depth of ca. 100 nm



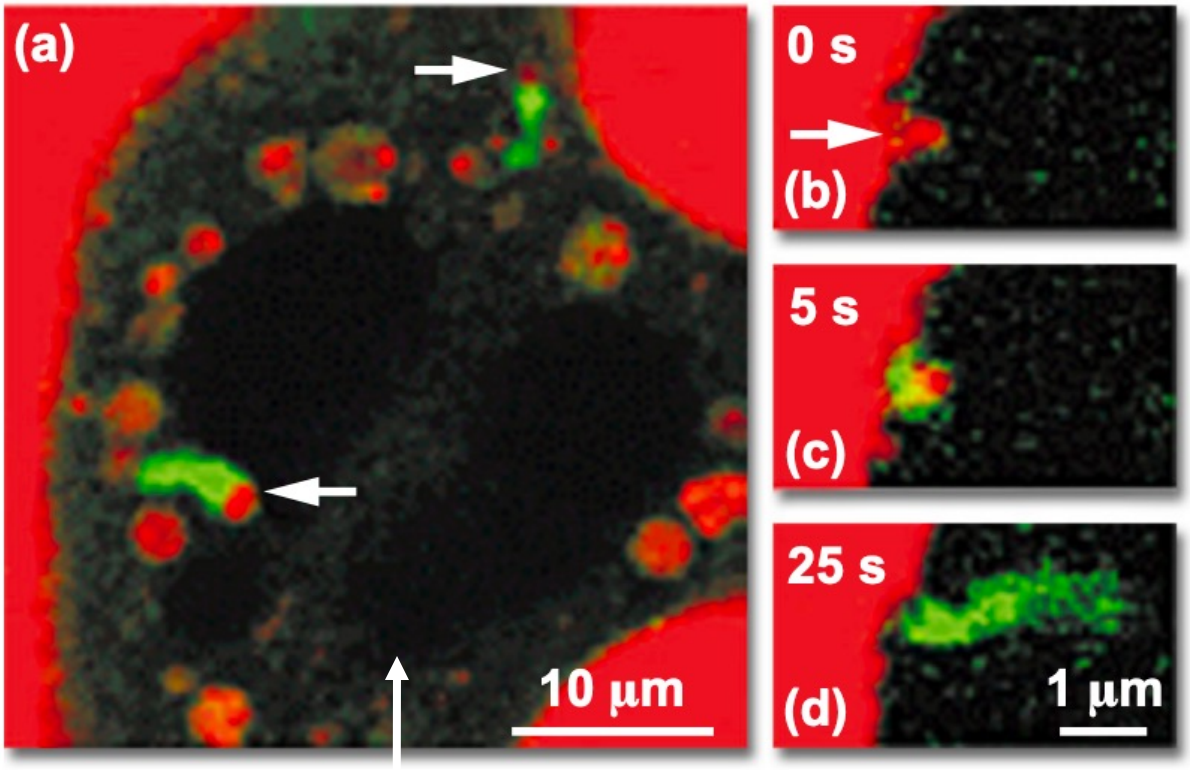
TIRF is suitable for investigating phenomena close to the glass slide
=> e.g. cell membranes

Total internal reflection fluorescence mic. (TIRF)



Total internal reflection fluorescence mic. (TIRF)

Vesicle budding by endocytosis

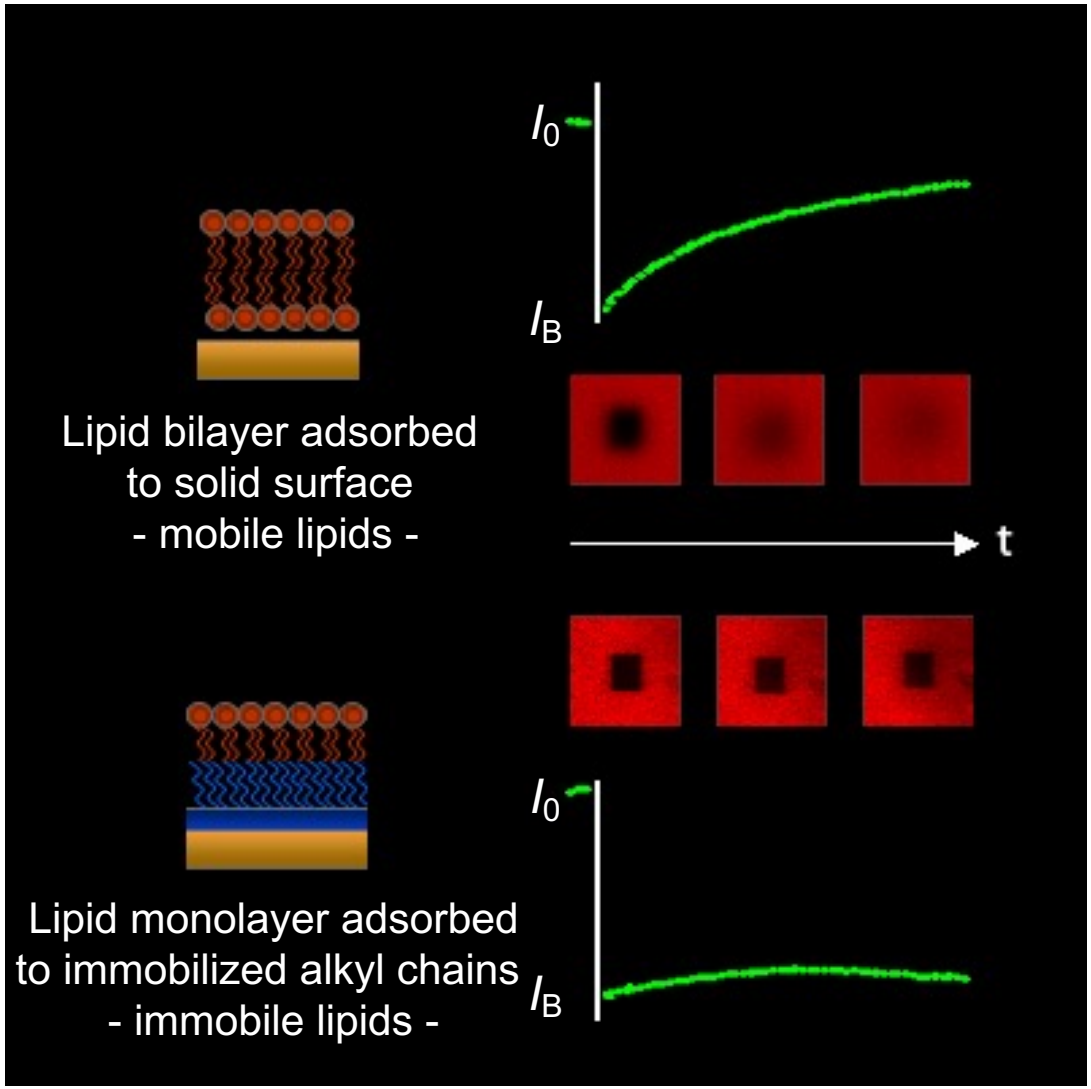


Cytoplasm

green: Staining of actin
red: Soluble dye rhodamine

Fluorescence recovery after photobleaching

How fast are photobleached fluorophores replaced by diffusion (D)?



D can be determined by fitting the recovery curve with a model accounting for the size and shape of the bleached area.

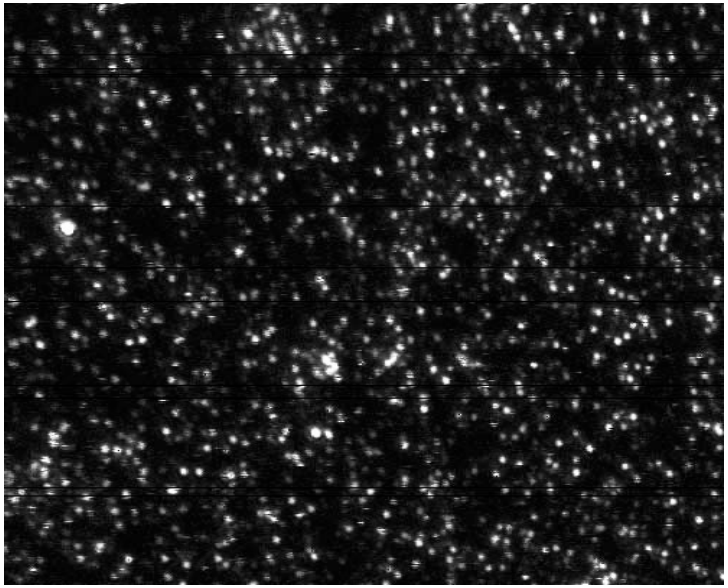
Single molecule fluorescence microscopy

(frequently in combination with TIRF)

Single-molecule fluorescence microscopy

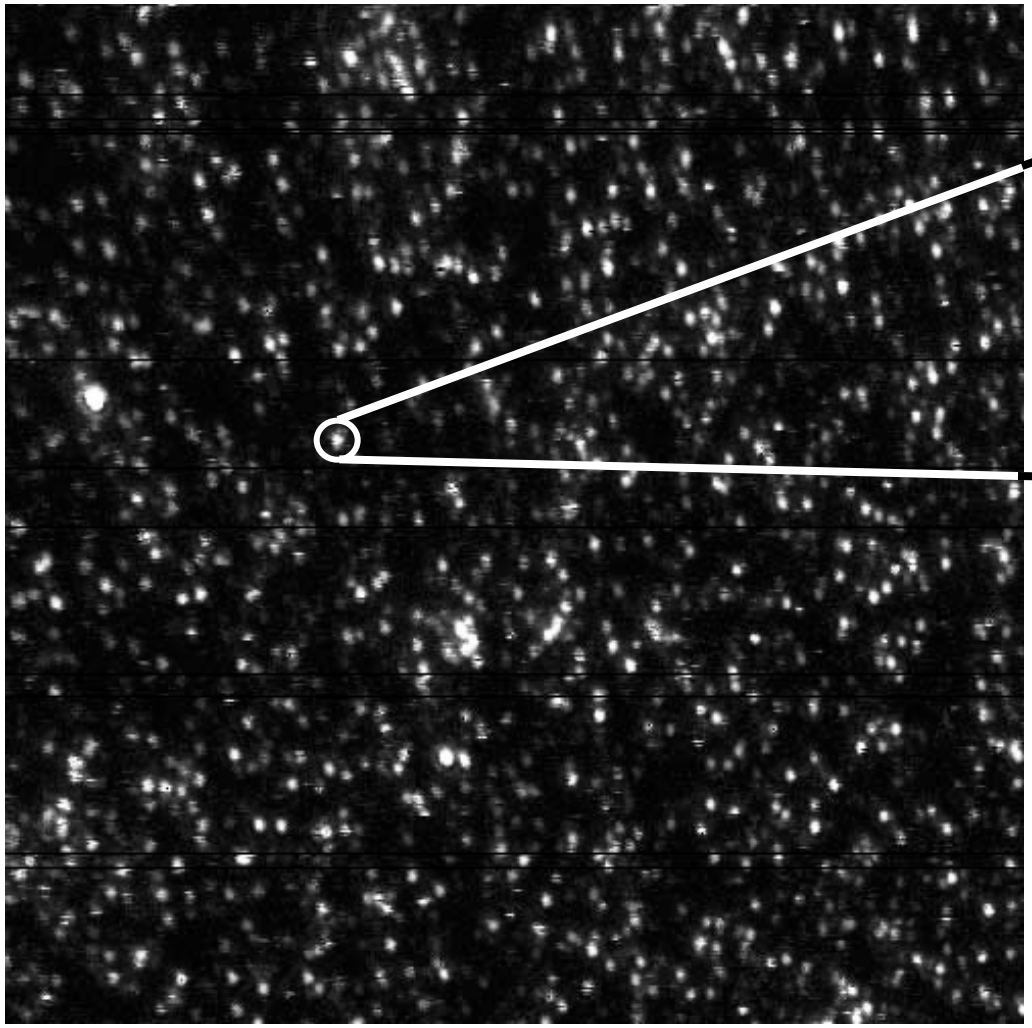
Conditions:

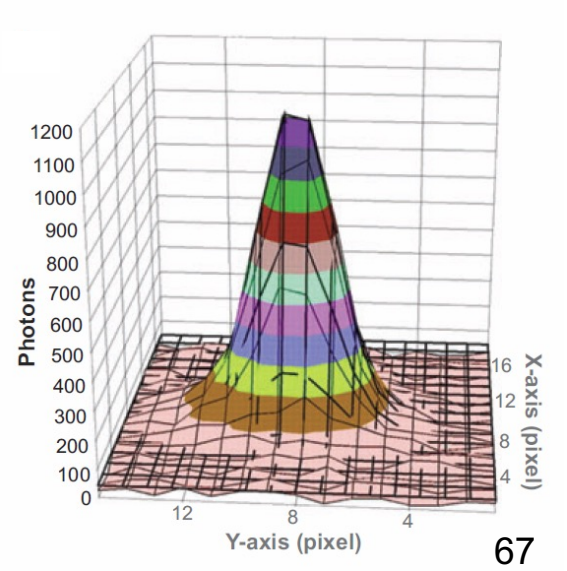
- Fluorescent probes with high quantum yield / low bleaching rates
  further problems can be avoided by the choice of buffer systems:
  e.g. „blinking“ by transition into a triplet state or oxidation by O_2
- Wide field epifluorescence microscopy with TIRF => very low background
- Wide field microscopy provides a much better time resolution compared to scanning techniques (video rates = up to 100 images / sec)



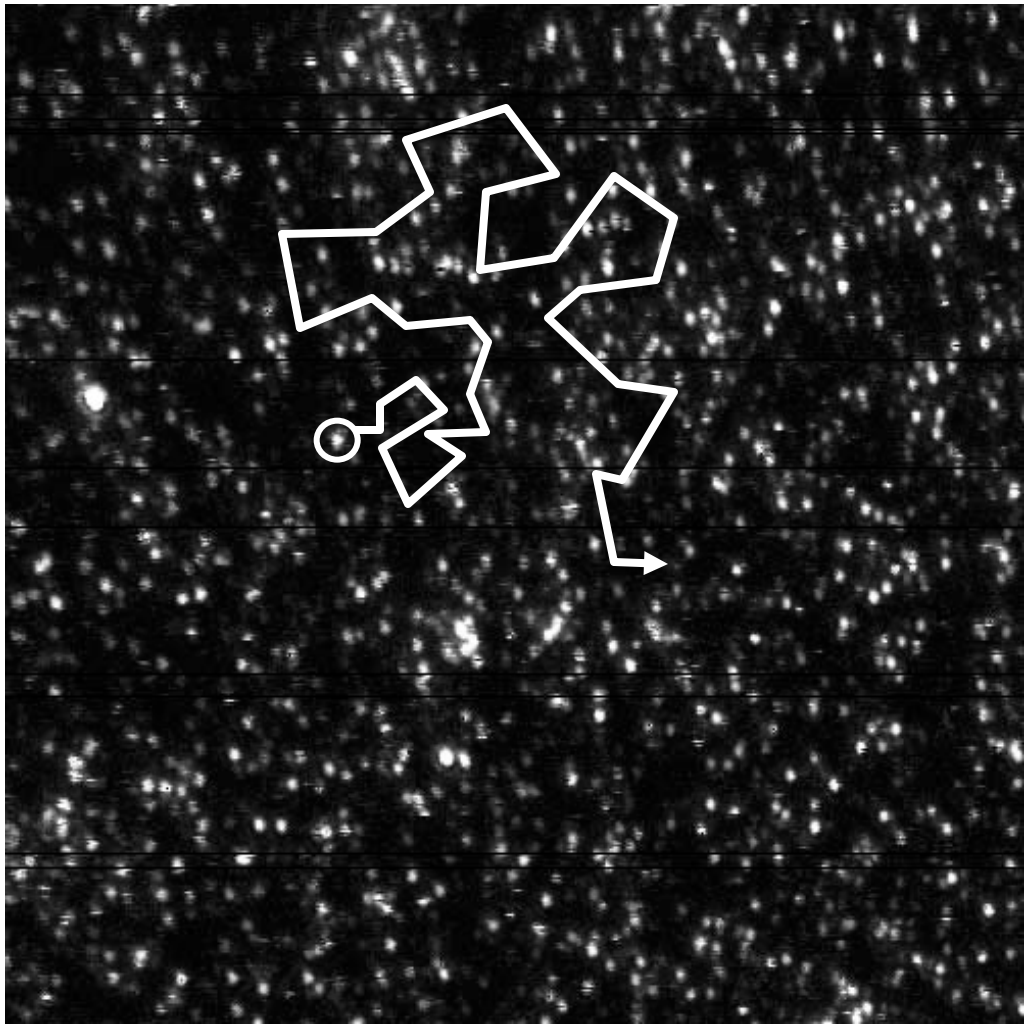
=> Each fluorophor molecule
is visible as a diffraction limited spot.

Single-molecule fluorescence microscopy





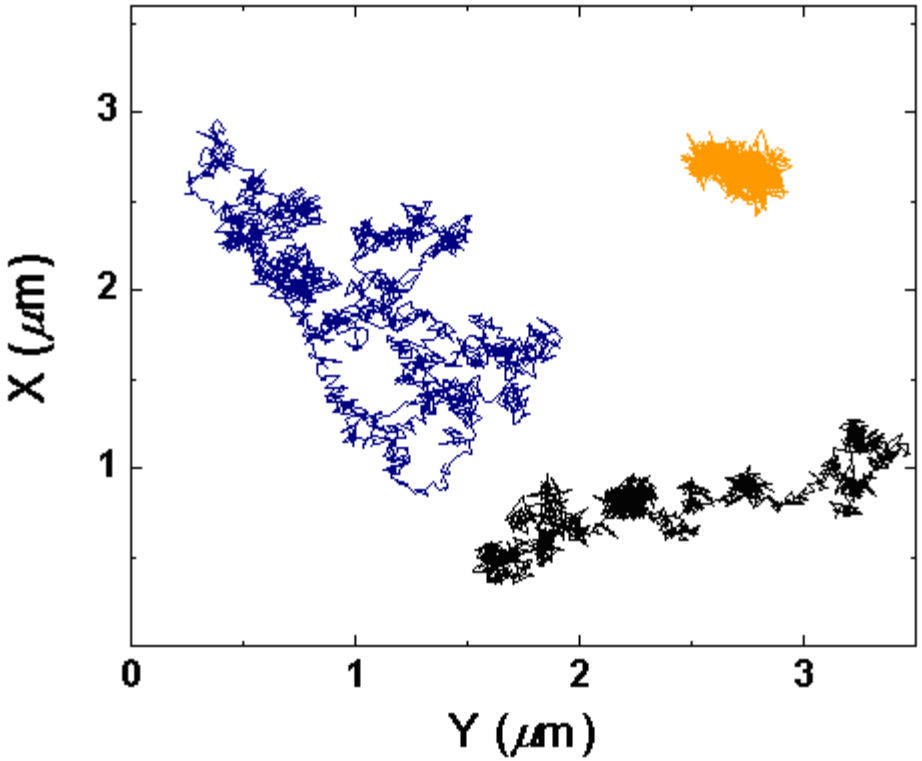
Single-molecule fluorescence microscopy



=> Single Particle Tracking (SPT)

Single-molecule fluorescence microscopy

Analysis of trajectories: *Random Walk*



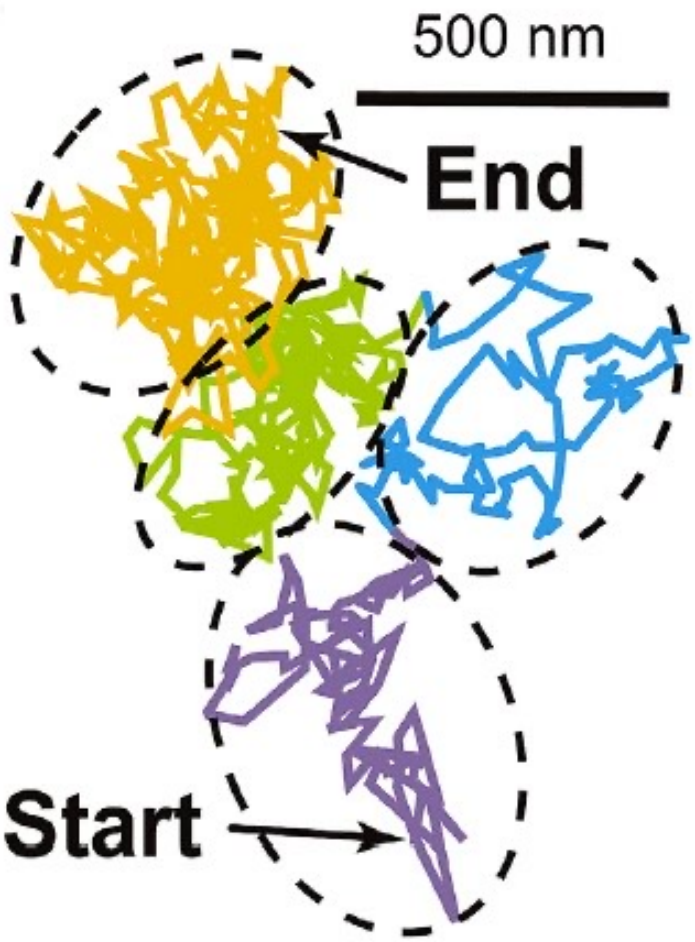
Diffusion coefficient (D) given by Stokes Einstein equation:

$$D = \frac{k_b * T}{3 * \pi * \eta * d}$$

=> Diffusion coefficients of single molecules (single molecules vs. ensemble)

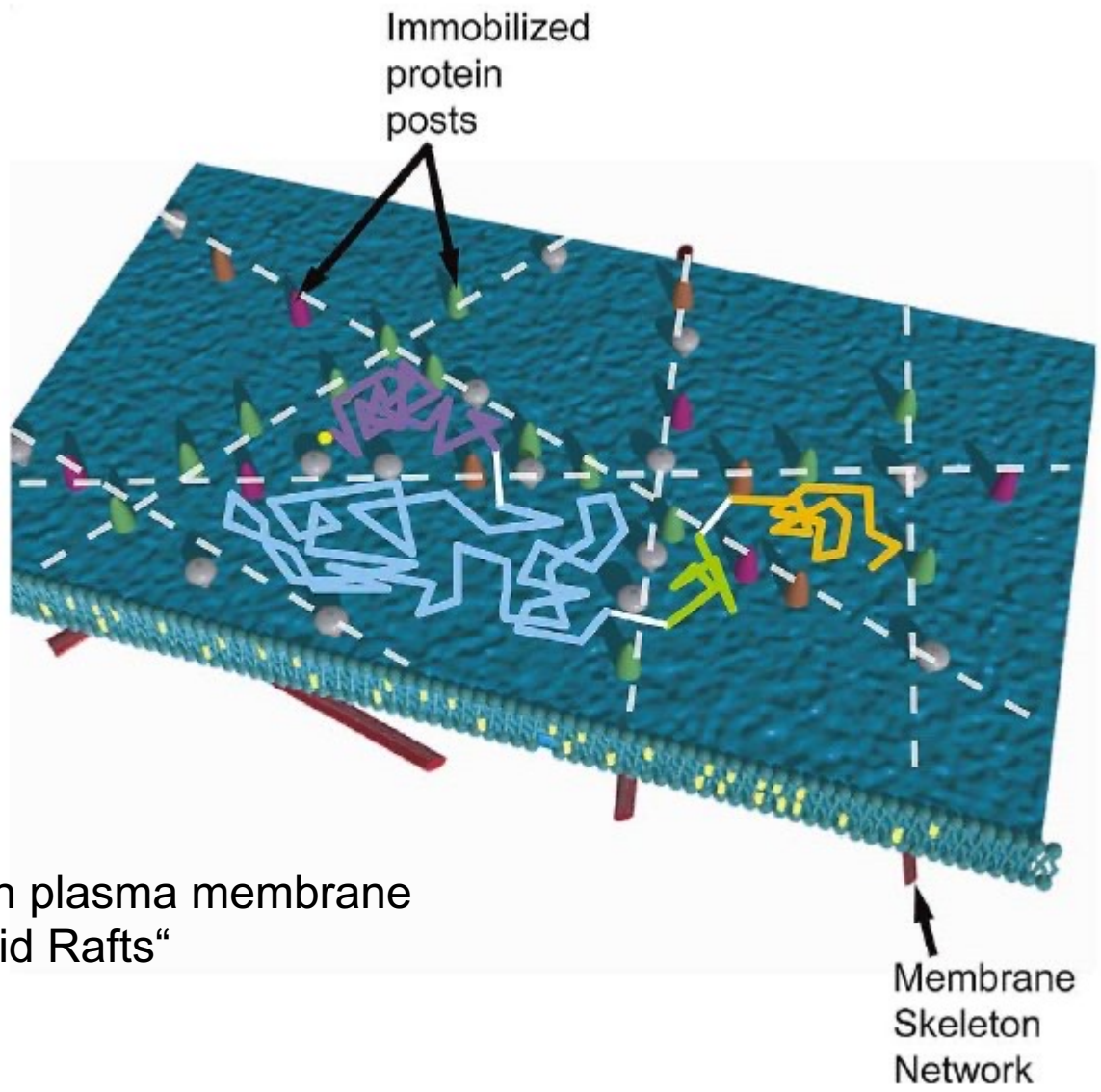
Single-molecule fluorescence microscopy

Dynamics of biological membranes on a molecular level



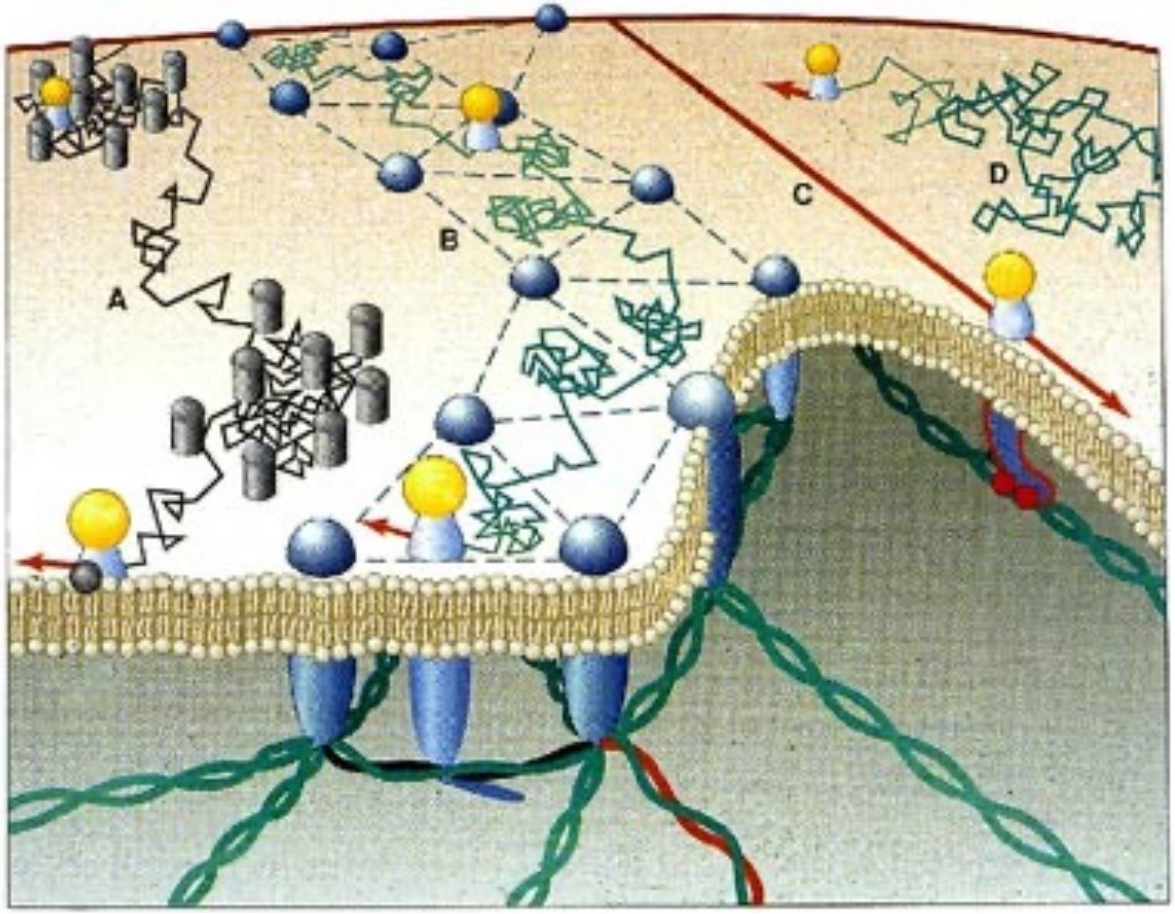
Trajectory of a single molecule temporal sequence: violet, blue, green, yellow

Single-molecule fluorescence microscopy



Microdomains in plasma membrane
„Lipid Rafts“

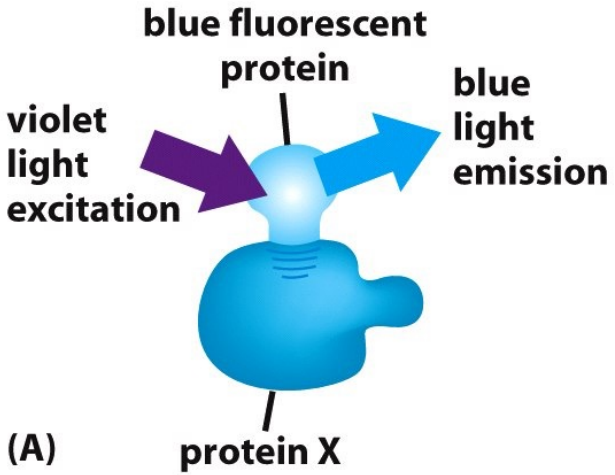
Single-molecule fluorescence microscopy

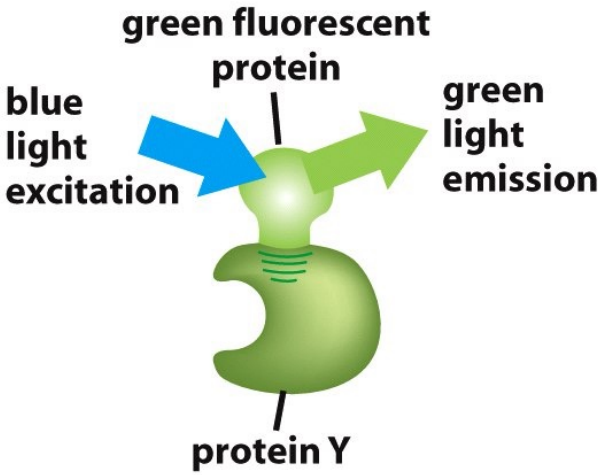


=> Fluorophores in membrane can be well excited in evanescent field

Fluorescence resonance energy transfer (FRET) microscopy

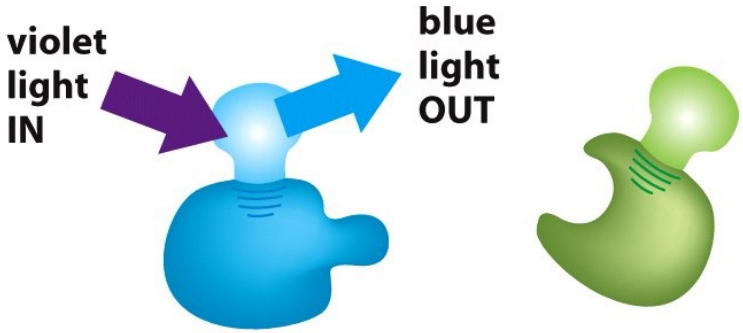
Analyzing protein-protein interactions by FRET





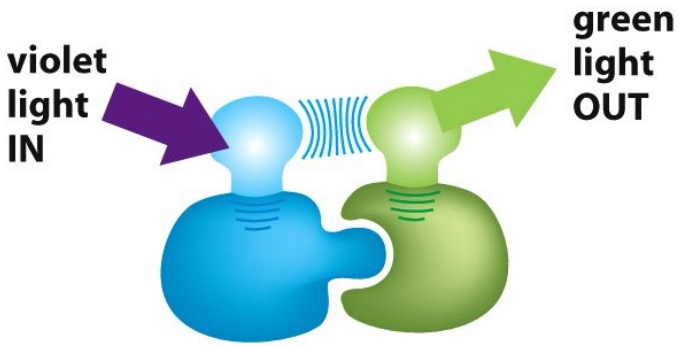
$$E_T = \frac{1}{1 + \left(\frac{R_{DA}}{R_0}\right)^6}$$

R_0: E_T = 50%



(B) **NO PROTEIN INTERACTION**

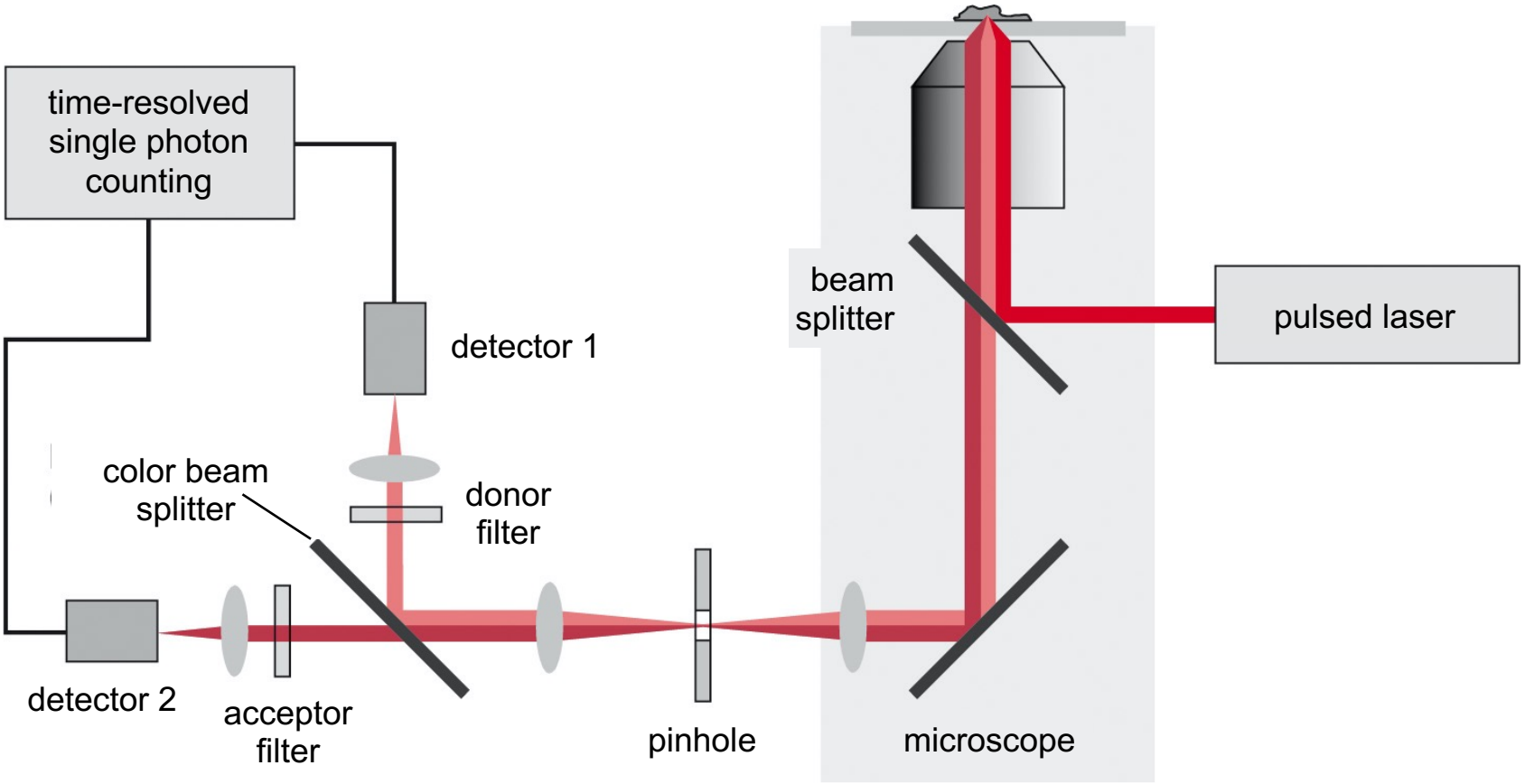
NO EXCITATION OF GREEN FLUORESCENT PROTEIN, BLUE LIGHT DETECTED



(C) **PROTEIN INTERACTION**

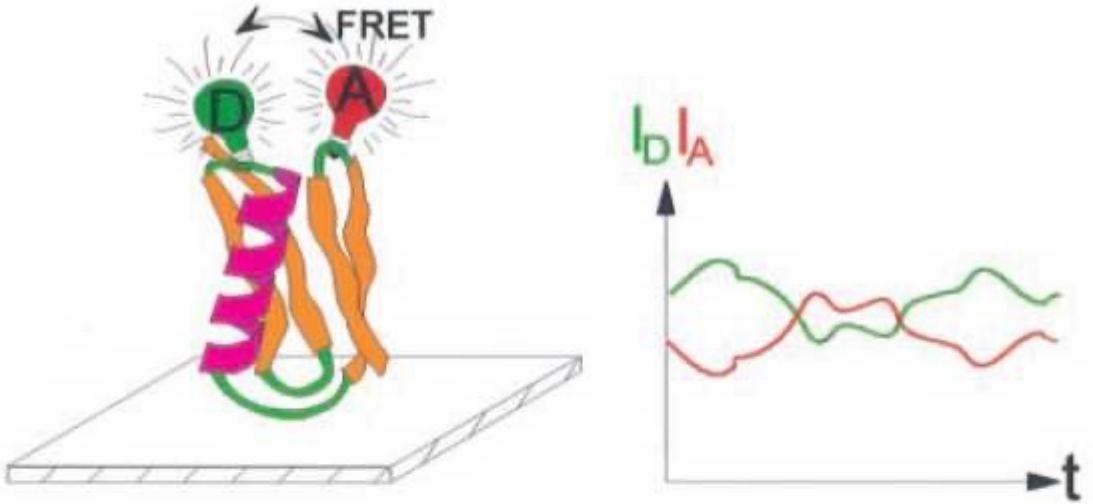
FLUORESCENCE RESONANCE ENERGY TRANSFER, GREEN LIGHT DETECTED

FRET: Experimental setup

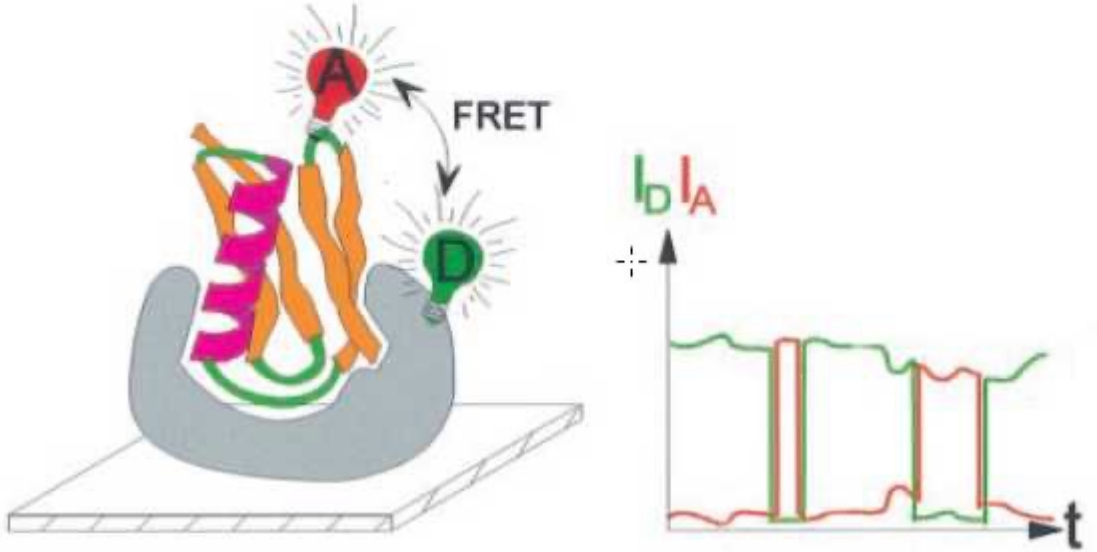


Single-molecule FRET

Intermolecular FRET



Intramolecular FRET



Single-molecule FRET *in vivo*

- Protein-protein interactions are investigated in their natural environment
- Fusion with fluorescent proteins (e.g. GFP) are used
- The location of the interaction can be determined (=> super-resolution microscopy)
- Real-time imaging
- Heterogeneous and dynamic biological processes can be observed

Requires dedicated equipment:

$\Rightarrow$ Strong background reduction (autofluorescence): confocal microscopy or TIRF

$\Rightarrow$ Sensitive cameras or avalanche photodiodes

$\Rightarrow$ Reduction of photobleaching (GFP is not very photostable)