MUNI
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C8116 Immunochemical techniques

Advanced microscopy III

Spring term 2024

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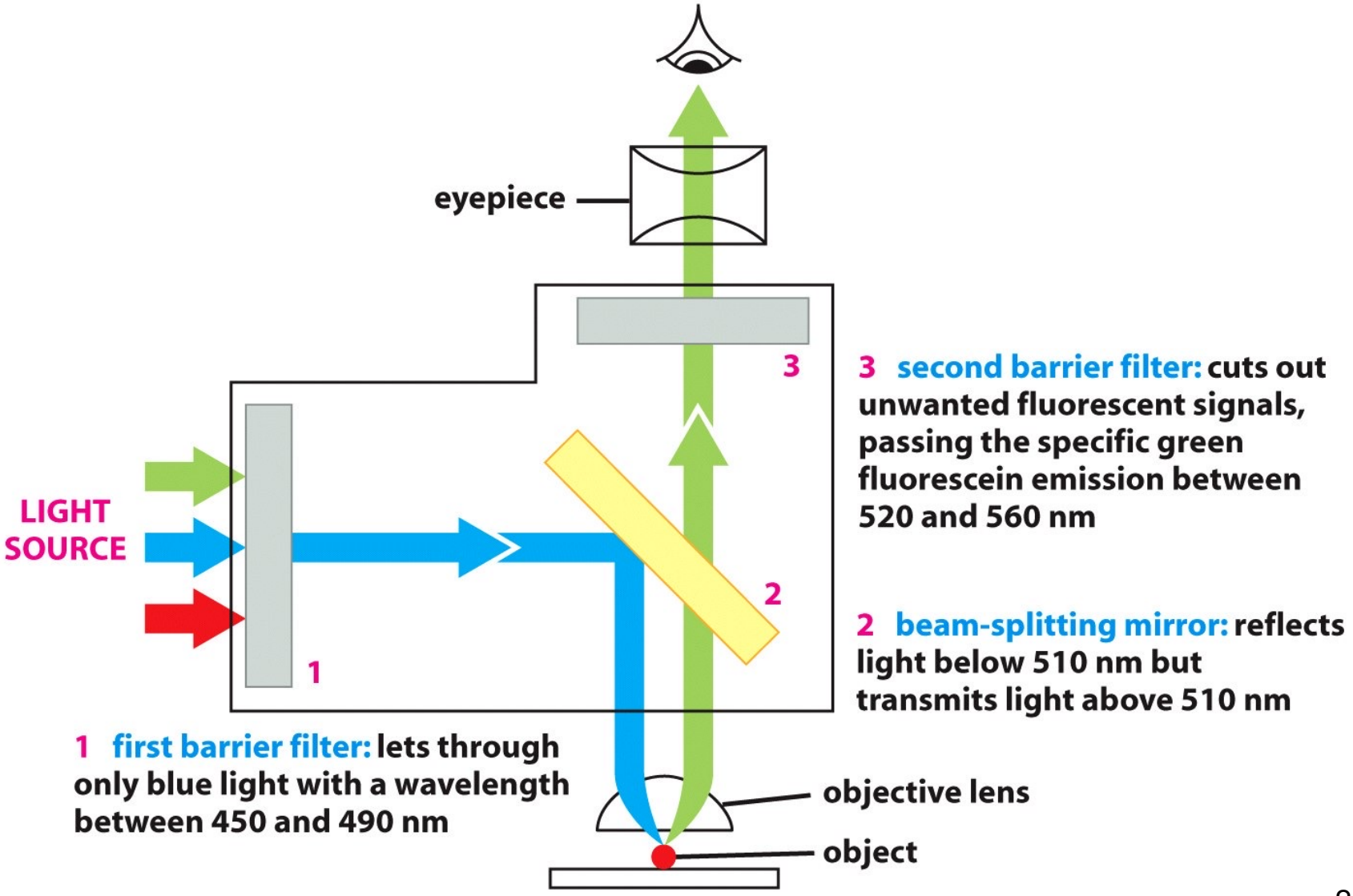
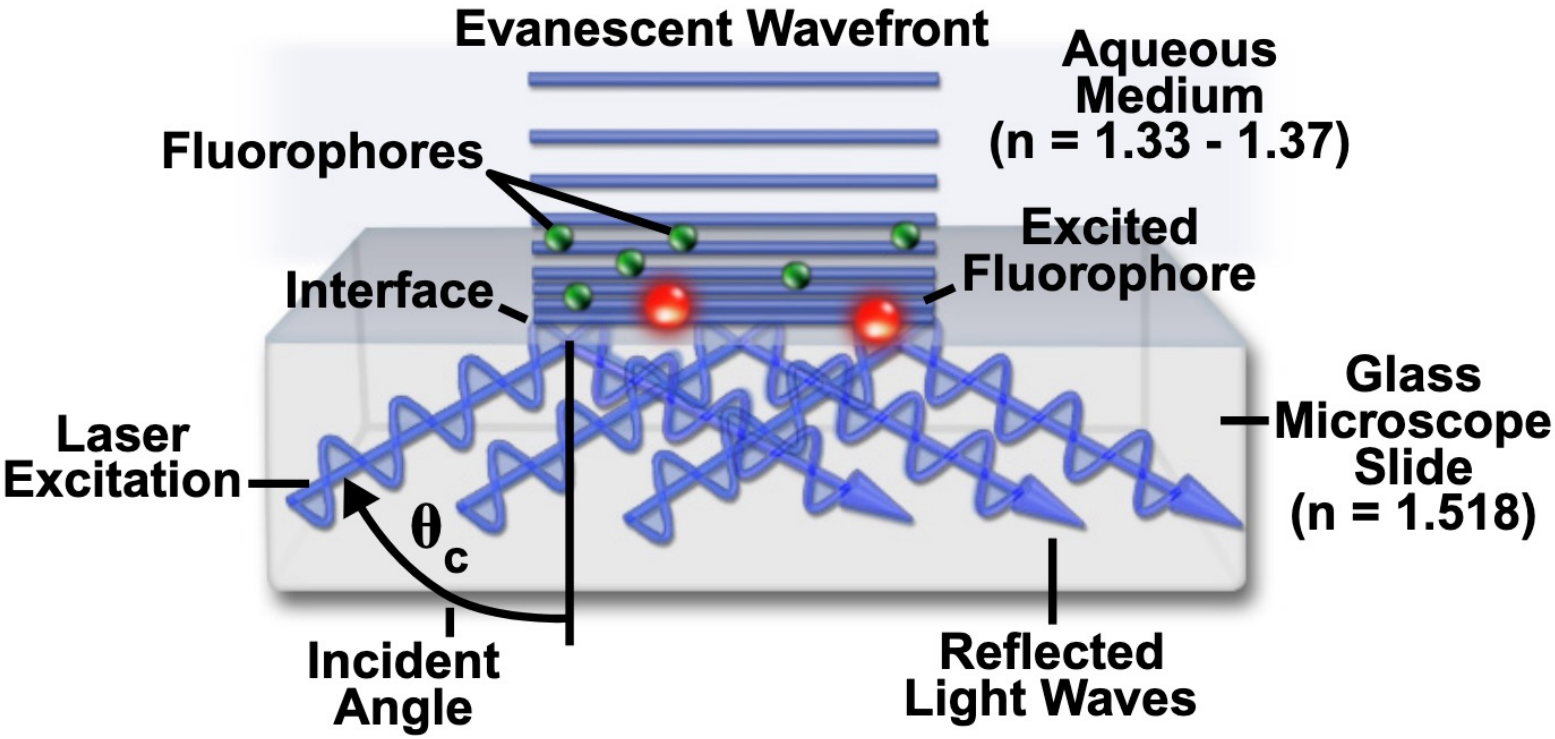
May 14th, 2024

Epifluorescence microscopy



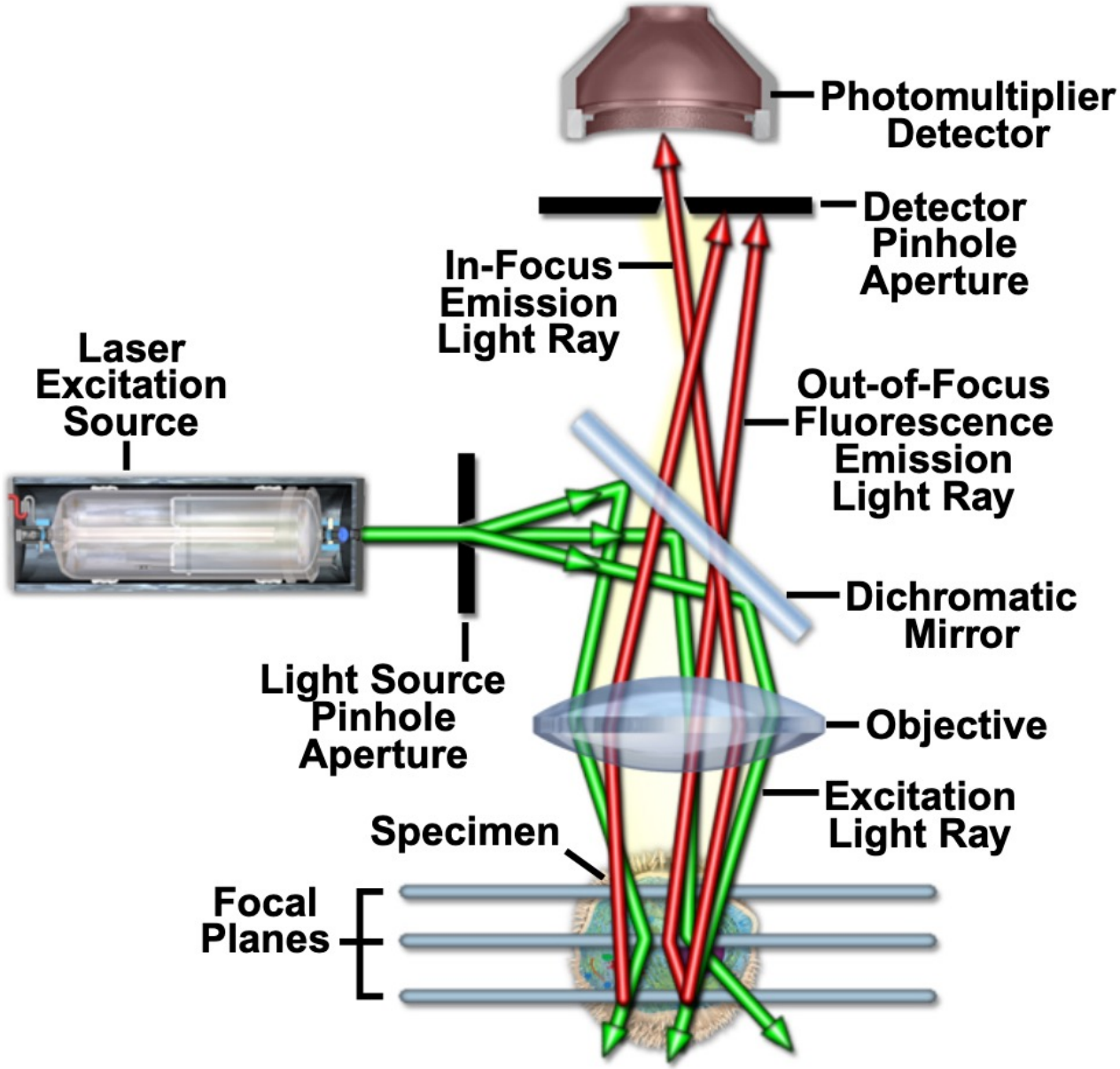
Total internal reflection fluorescence mic. (TIRF)

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



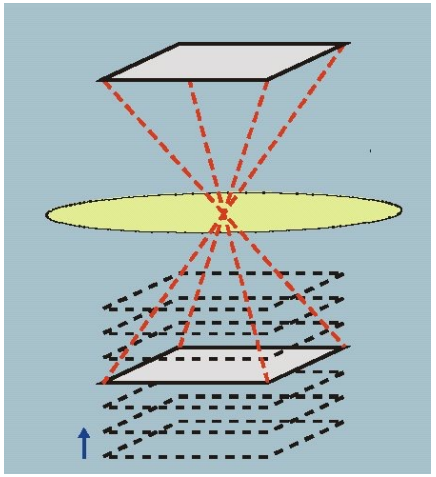
TIRF is suitable for investigating phenomena close to the glass slide
=> e.g. cell membranes

Confocal microscopy

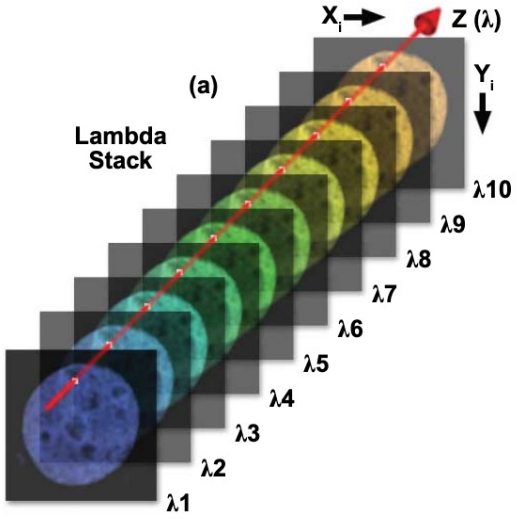


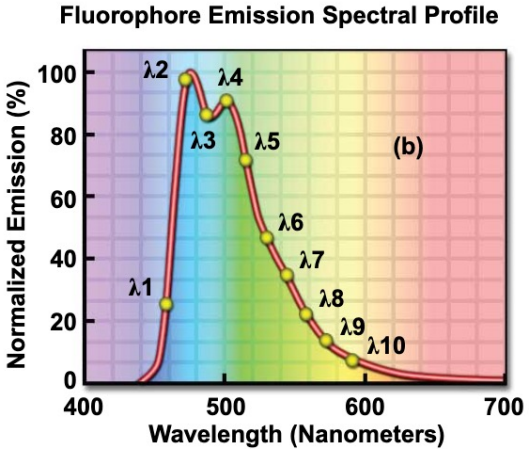
Additional features of confocal microscopy

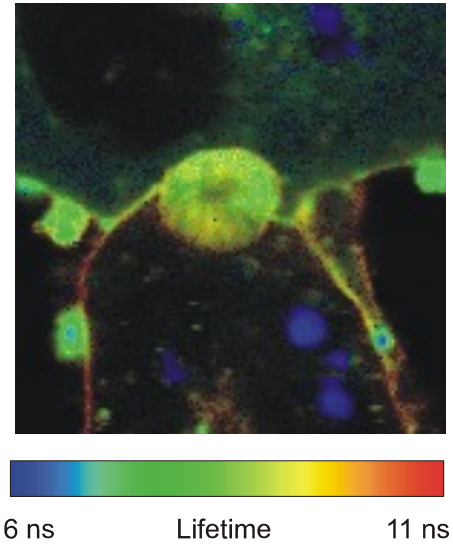
- improved contrast / multiphoton microscopy
- optical sectioning (z stacks)
- multiple fluorescence measurements can be performed in individual points (e.g. **spectra, lifetime, FRET, FCS**)



Spectral imaging: lambda stack anatomy

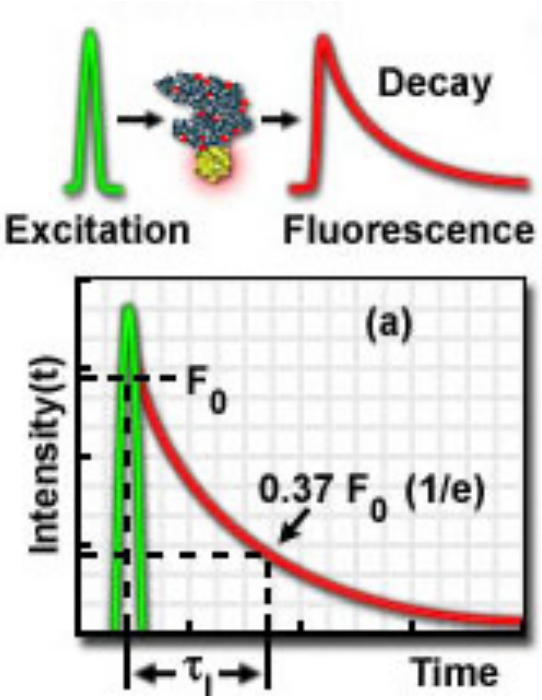






Fluorescence lifetime measurements (FLIM)

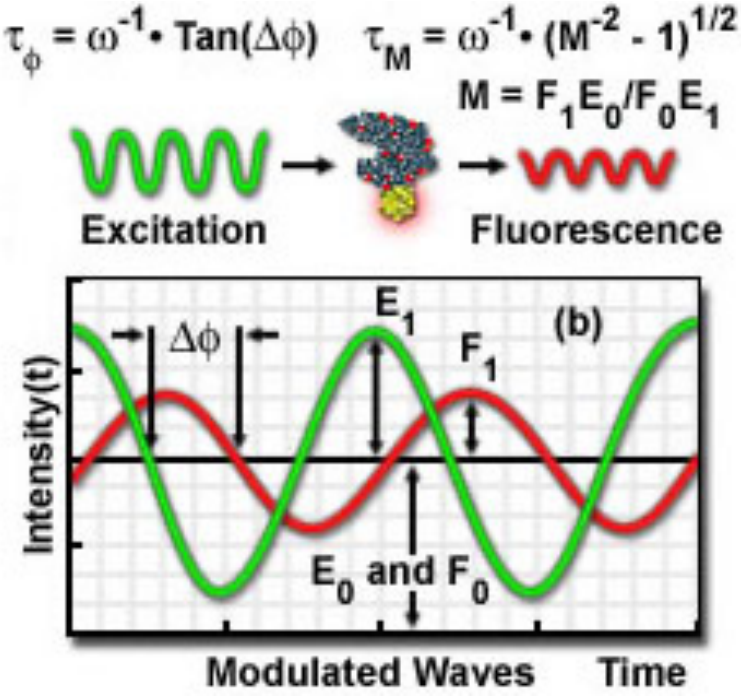
Time domain



Frequency domain

$\tau_\phi = \omega^{-1} \bullet \mathrm{Tan}(\Delta\phi)$ $\tau_M = \omega^{-1} \bullet (M^{-2} - 1)^{1/2}$

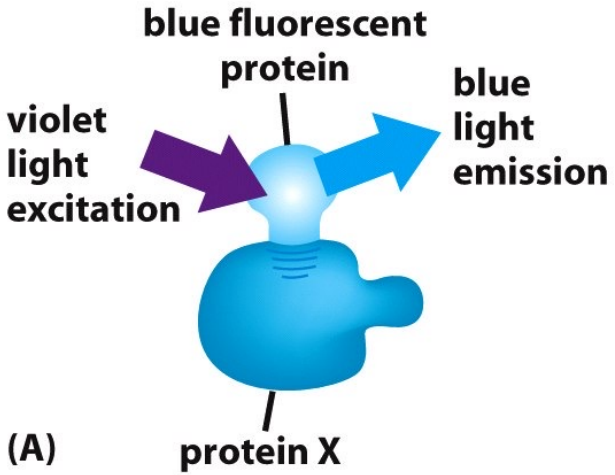
$M = F_1E_0/F_0E_1$

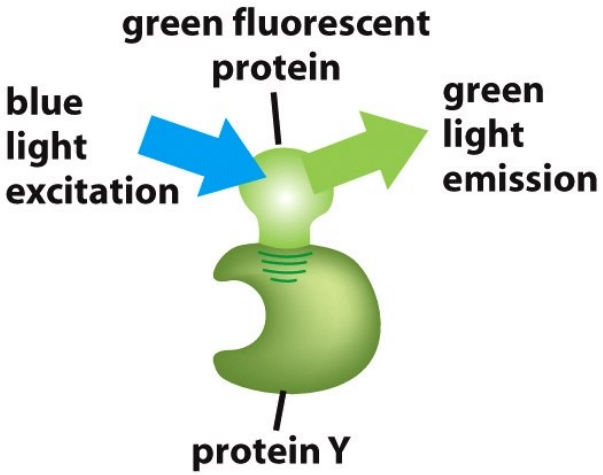


Advantages:

- Extremely low optical background
- independent of fluorophore concentration

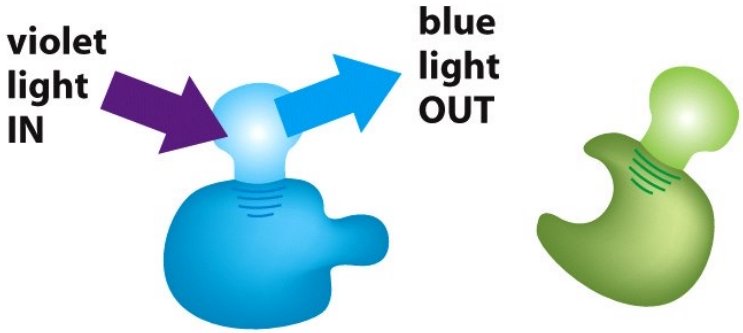
Analyzing protein-protein interactions by FRET





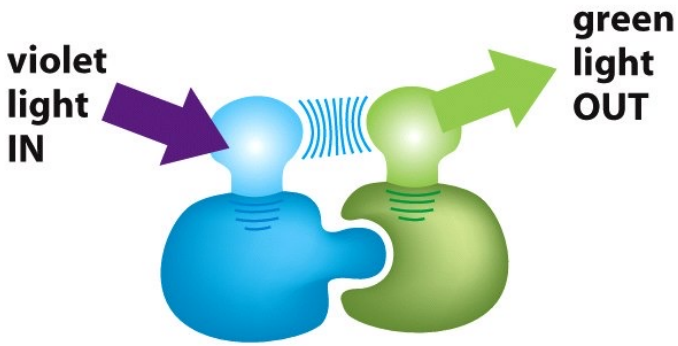
$$E_T = \frac{1}{1 + \left(\frac{R_{DA}}{R_0}\right)^6}$$

R_0: E_T = 50%



(B) **NO PROTEIN INTERACTION**

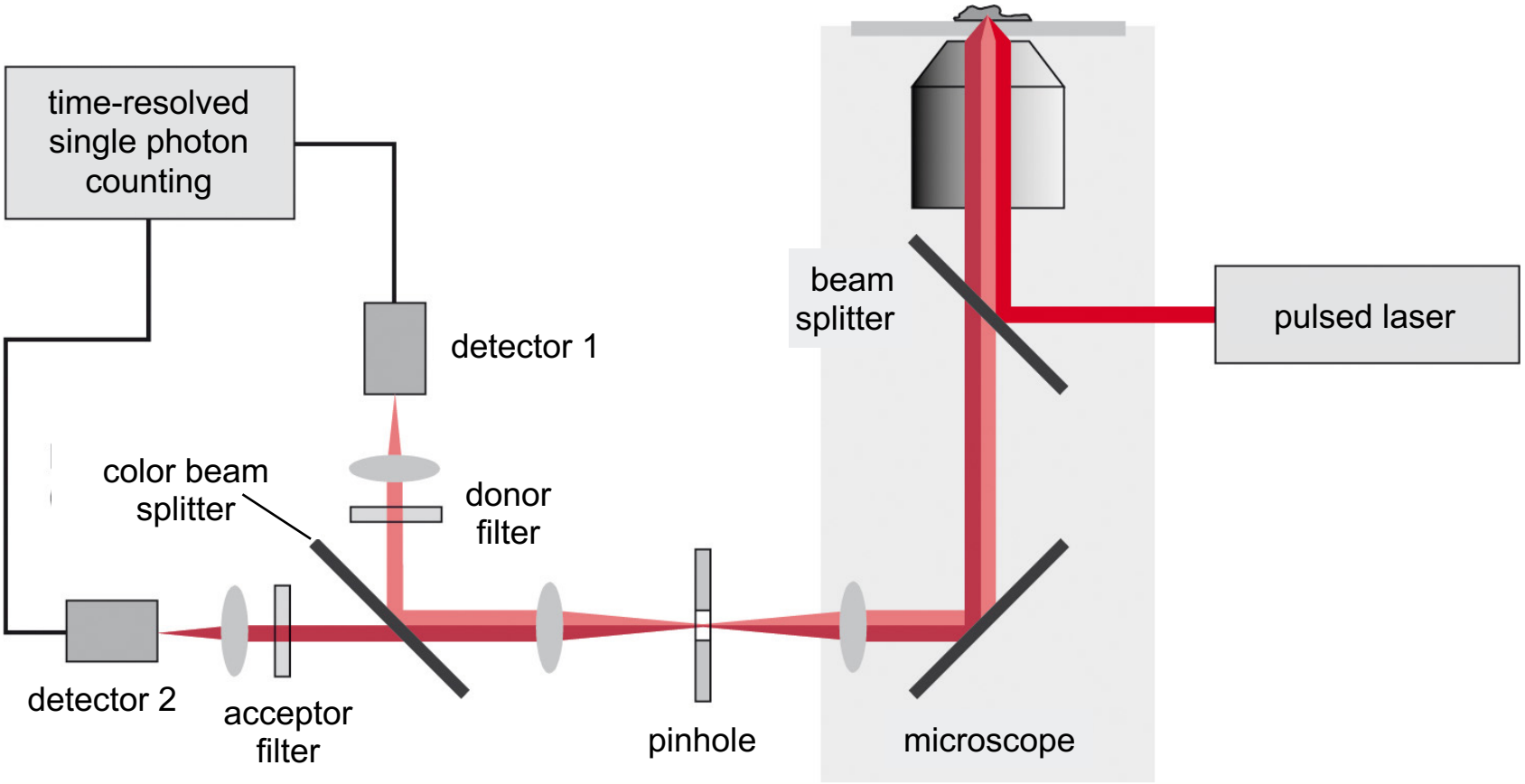
NO EXCITATION OF GREEN FLUORESCENT PROTEIN, BLUE LIGHT DETECTED



(C) **PROTEIN INTERACTION**

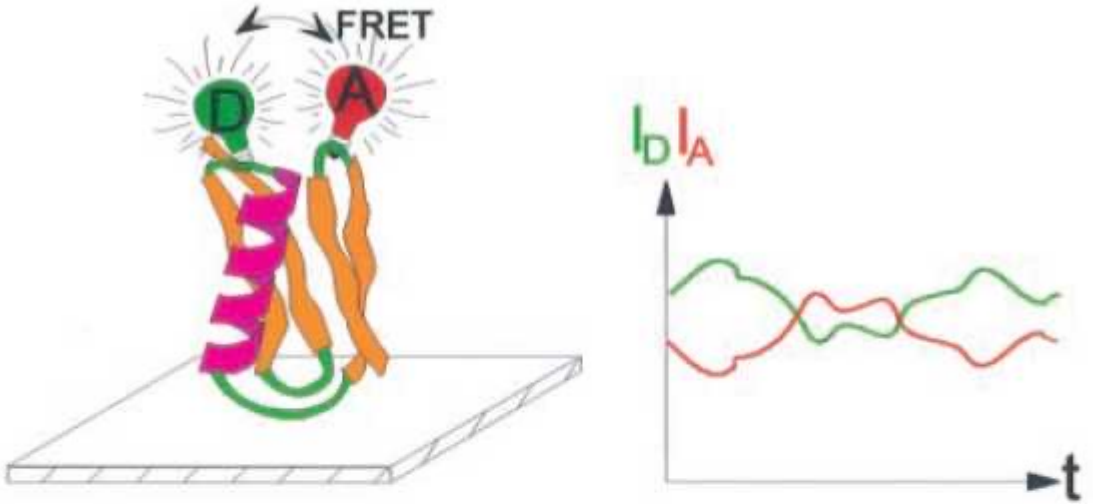
FLUORESCENCE RESONANCE ENERGY TRANSFER, GREEN LIGHT DETECTED

FRET microscopy: Experimental setup

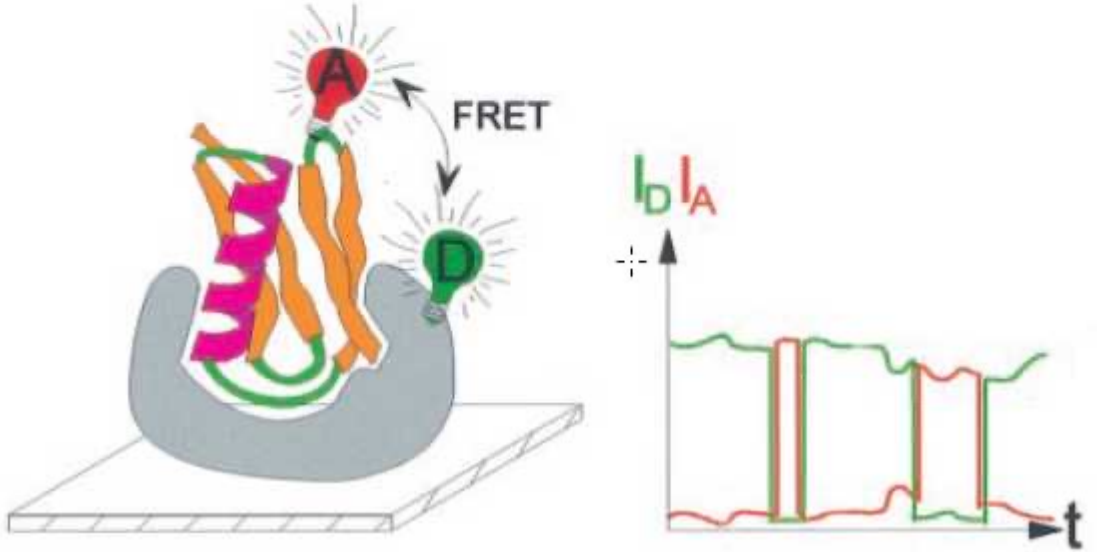


Single-molecule FRET

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

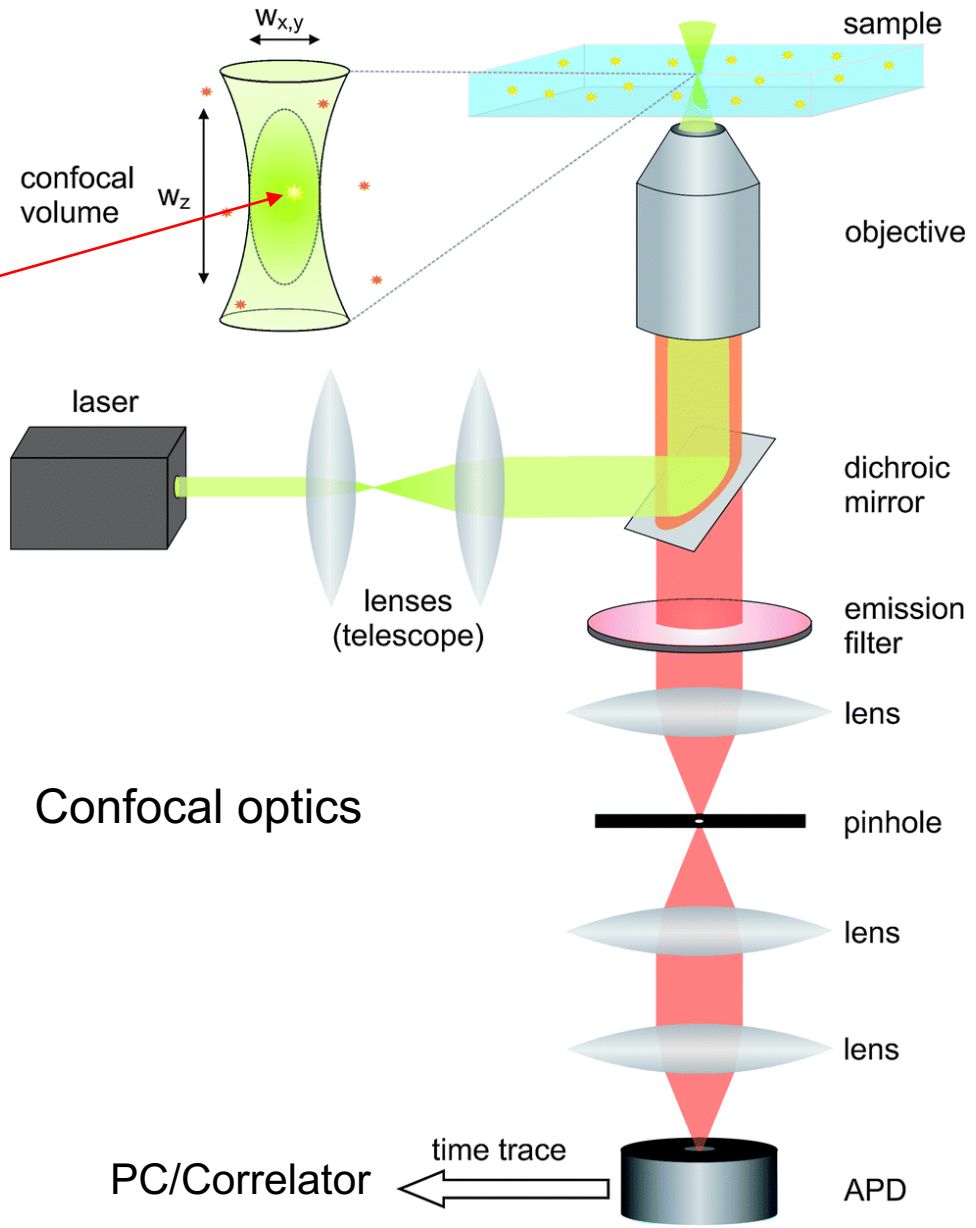
$\Rightarrow$ Sensitive cameras or avalanche photodiodes

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

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy

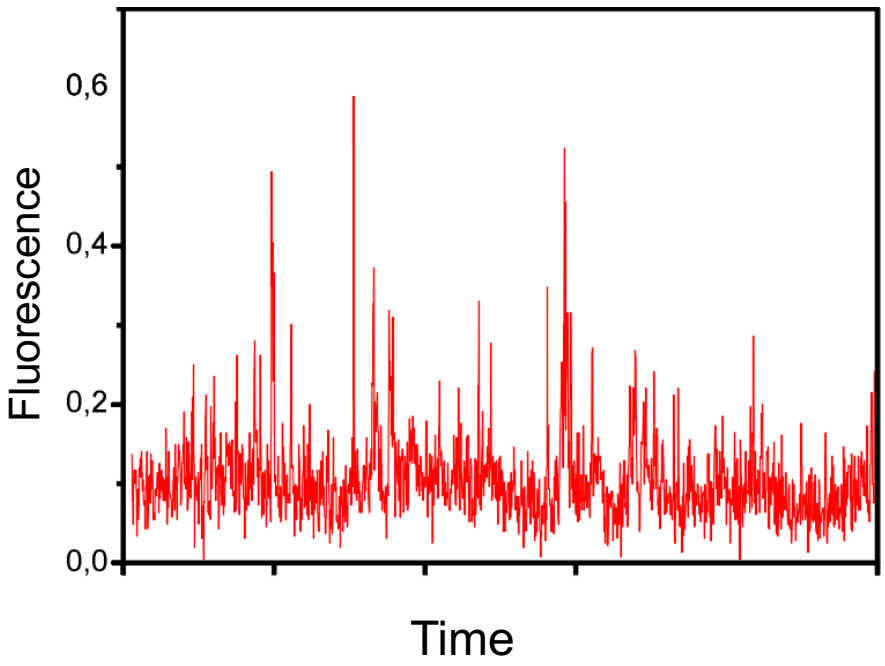
low fluorophore concentration
(~ 0.1 nM = 10^{-10} M)
+ very small focal volumen
(fL = 10^{-15} L)
=> single molecule in focal volume



=> Compare to
single molecule tracking

Fluorescence correlation spectroscopy

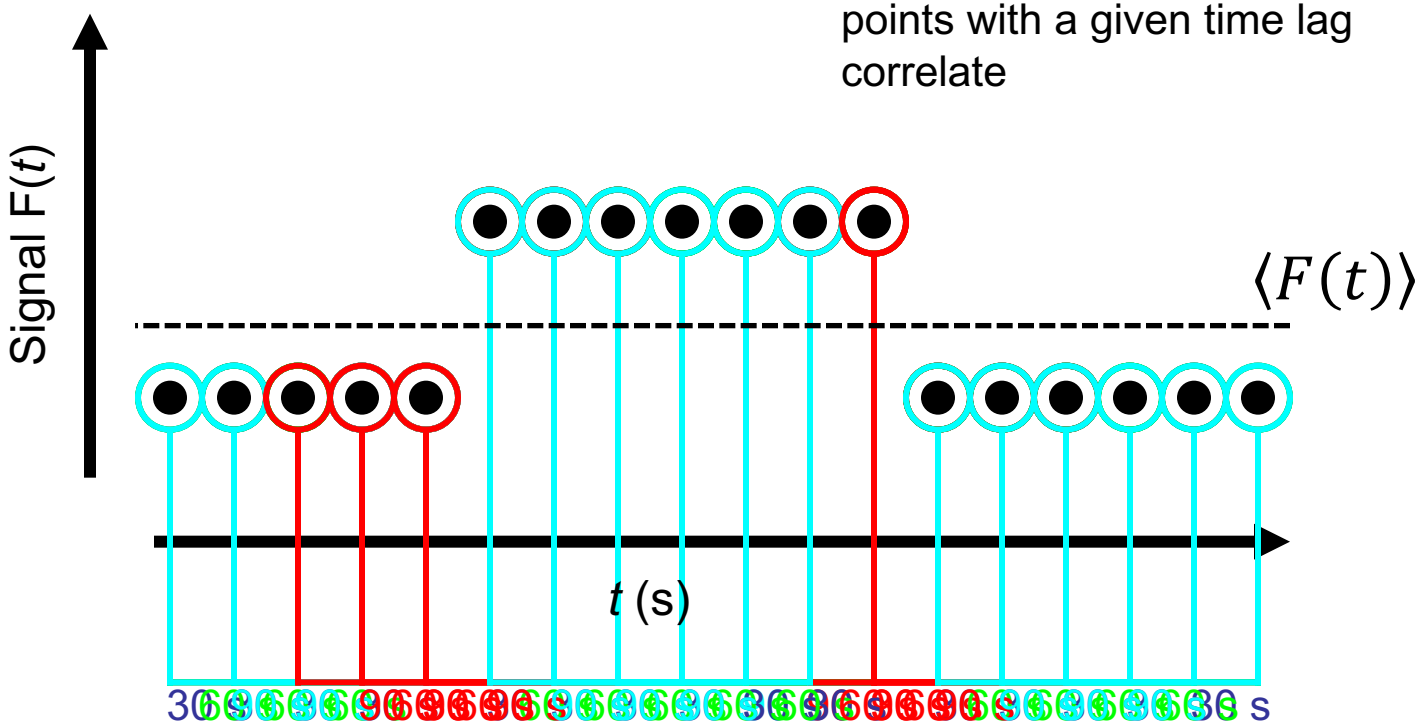
Raw data



Each time when a fluorescent molecule passes through the confocal volume, there is a **burst of light**

Data analysis: autocorrelation

Describes how strongly two data points with a given time lag correlate



Time lag ($\Delta\tau$)	correlation	no correlation
30 s	88 %	12 %
60 s	75 %	25 %
90 s	60 %	40 %

Fluorescence correlation spectroscopy

Calculation of increments:

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

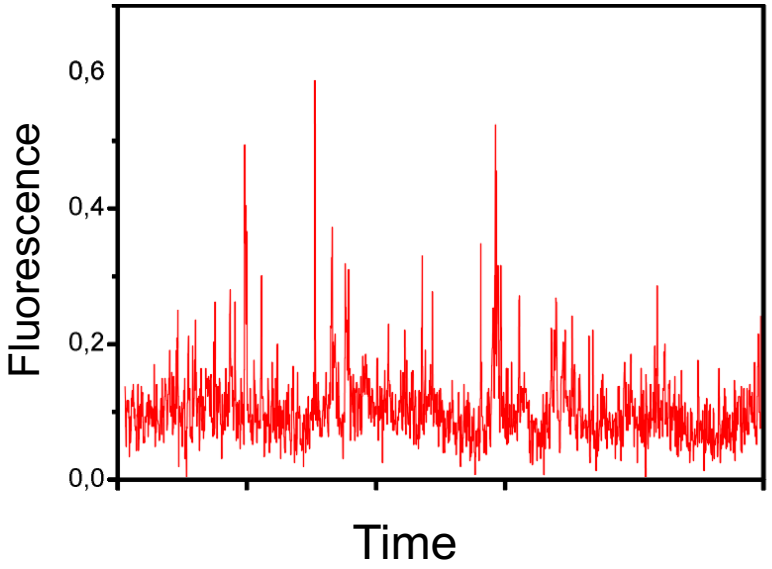
Calculation of G(τ) for the time series of the increments:

$$G(\tau) = \frac{\langle \delta F(t) * \delta(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

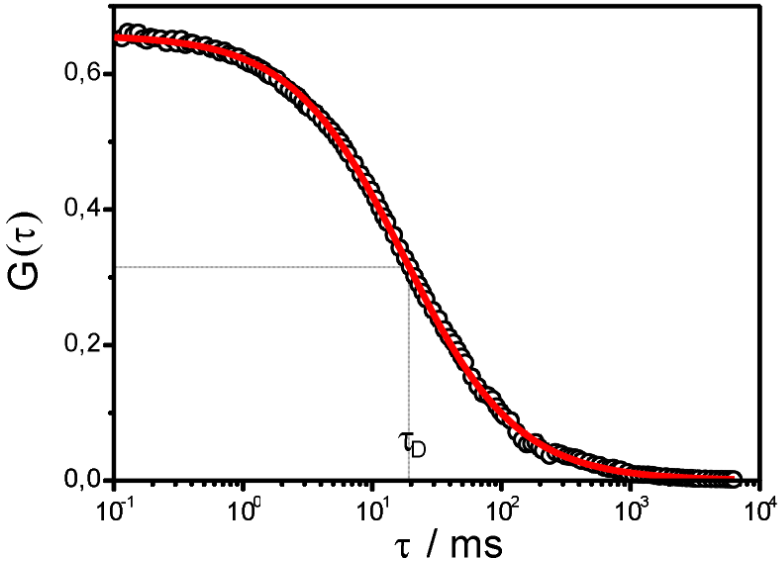
- brackets: averaging over time
- $F(t)$: fluorescence signal at time t
- $\delta F(t)$: deviation of the fluorescence signal at time t from the average fluorescence signal

Fluorescence correlation spectroscopy

Raw data



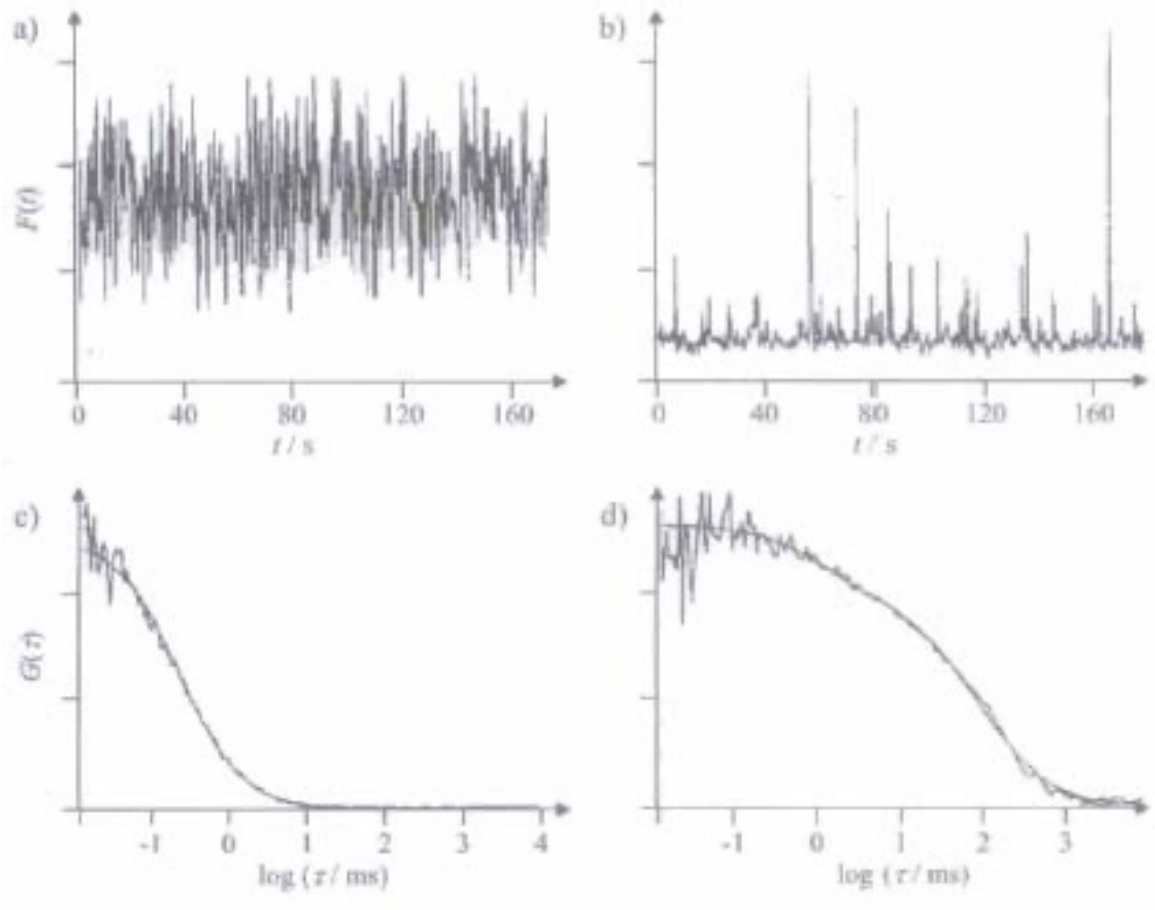
Autocorrelation function



τ_D: diffusion time through confocal volume

Investigating the mobilitiy of biomolecules

Proinsulin C-peptide (with fluorescent label)

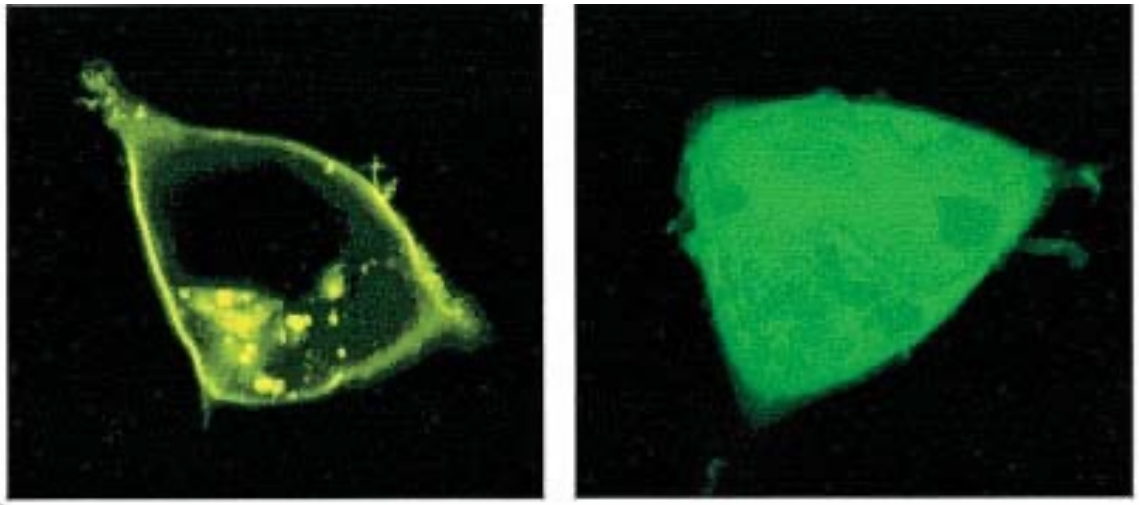


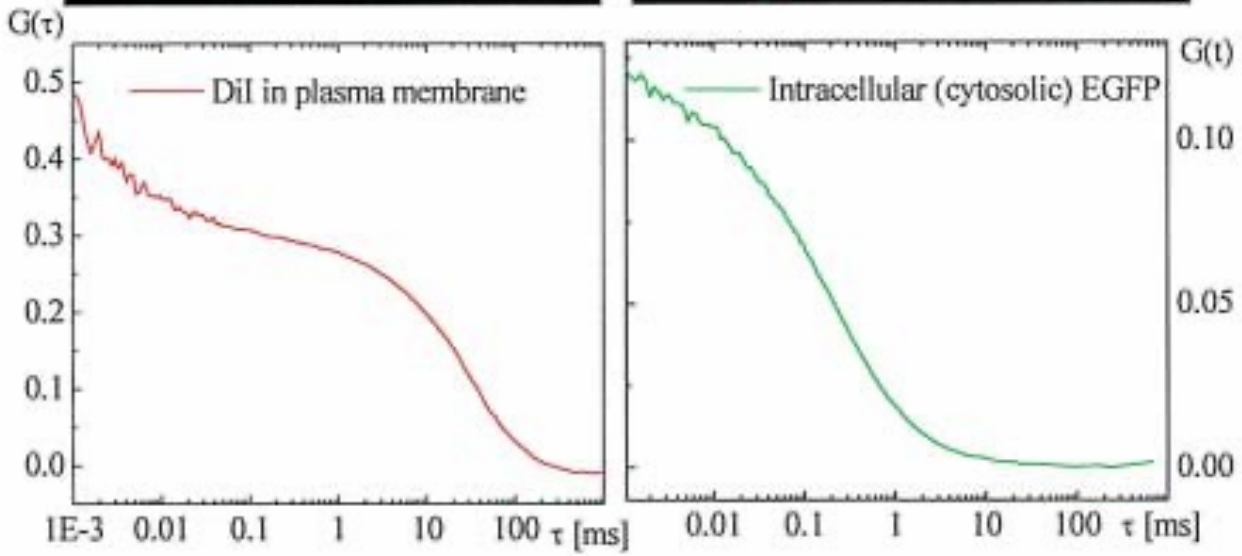
focal volume: in solution on membrane

Investigating the mobilitiy of biomolecules

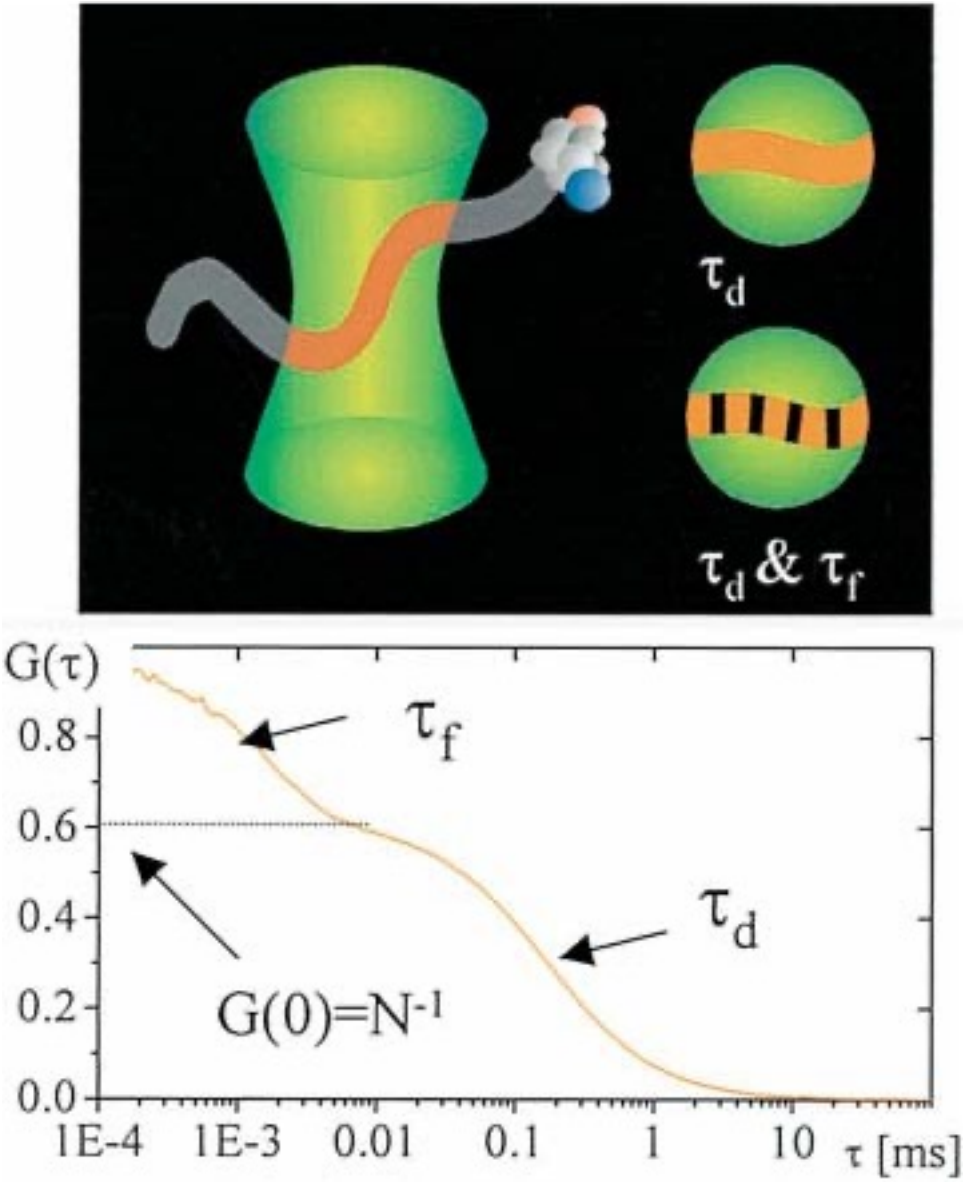
tetramethylindocarbocyanine (DIL) in cell membrane

fluorescent protein (EGFP) in cytoplasm





Investigating the mobilitiy & emission fluctuation

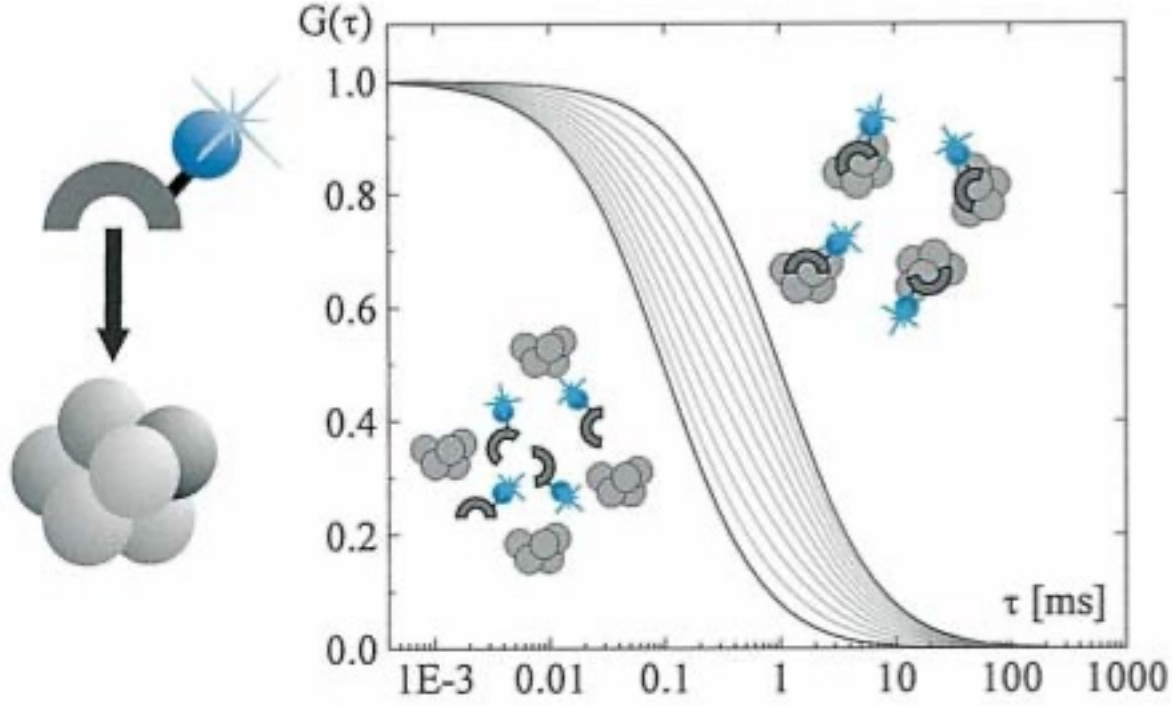


τ_d: diffusion time through confocal volume

τ_f: fluctuation time in confocal volume

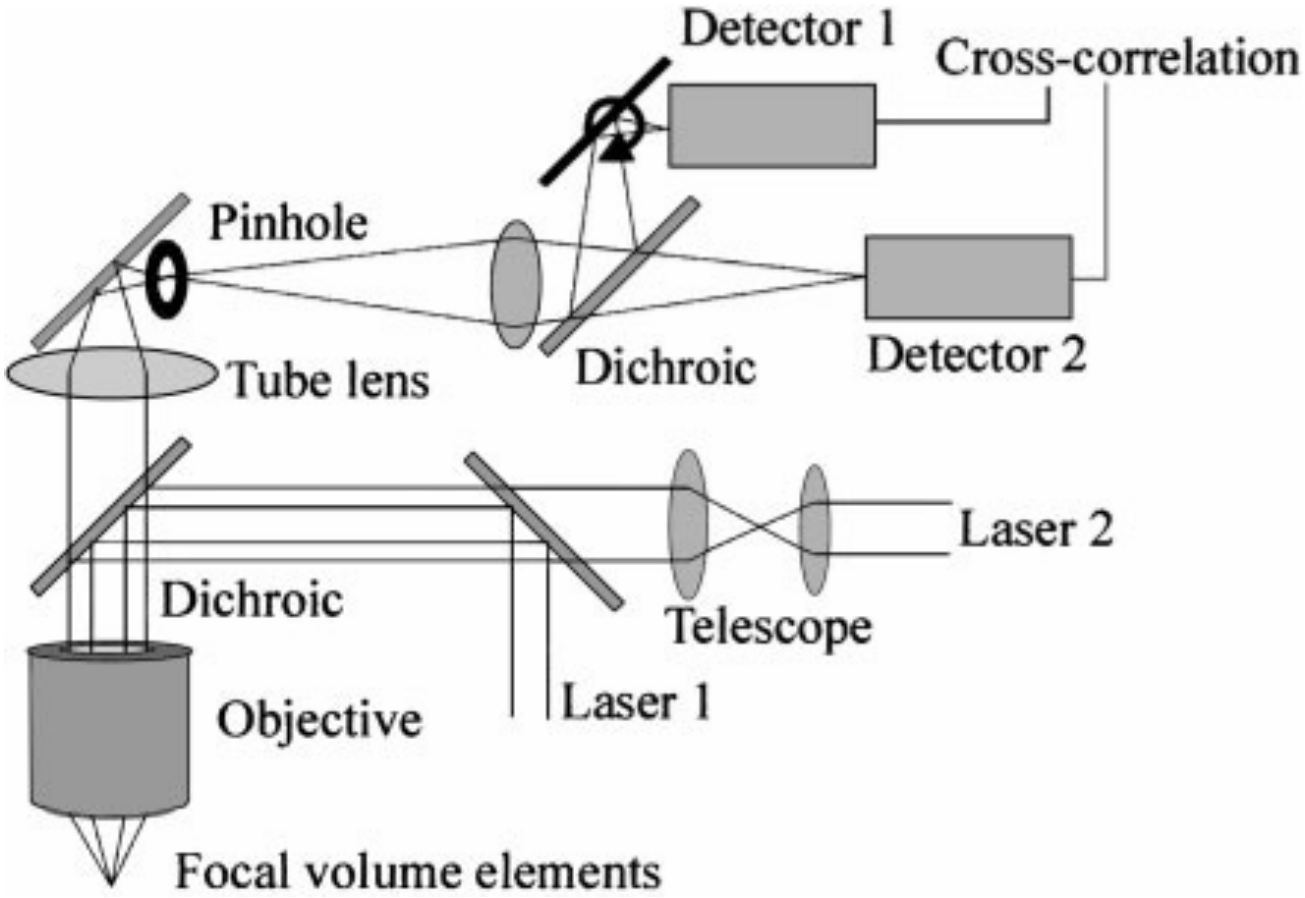
Interaction analysis

The size of a molecular complex changes the diffusion time

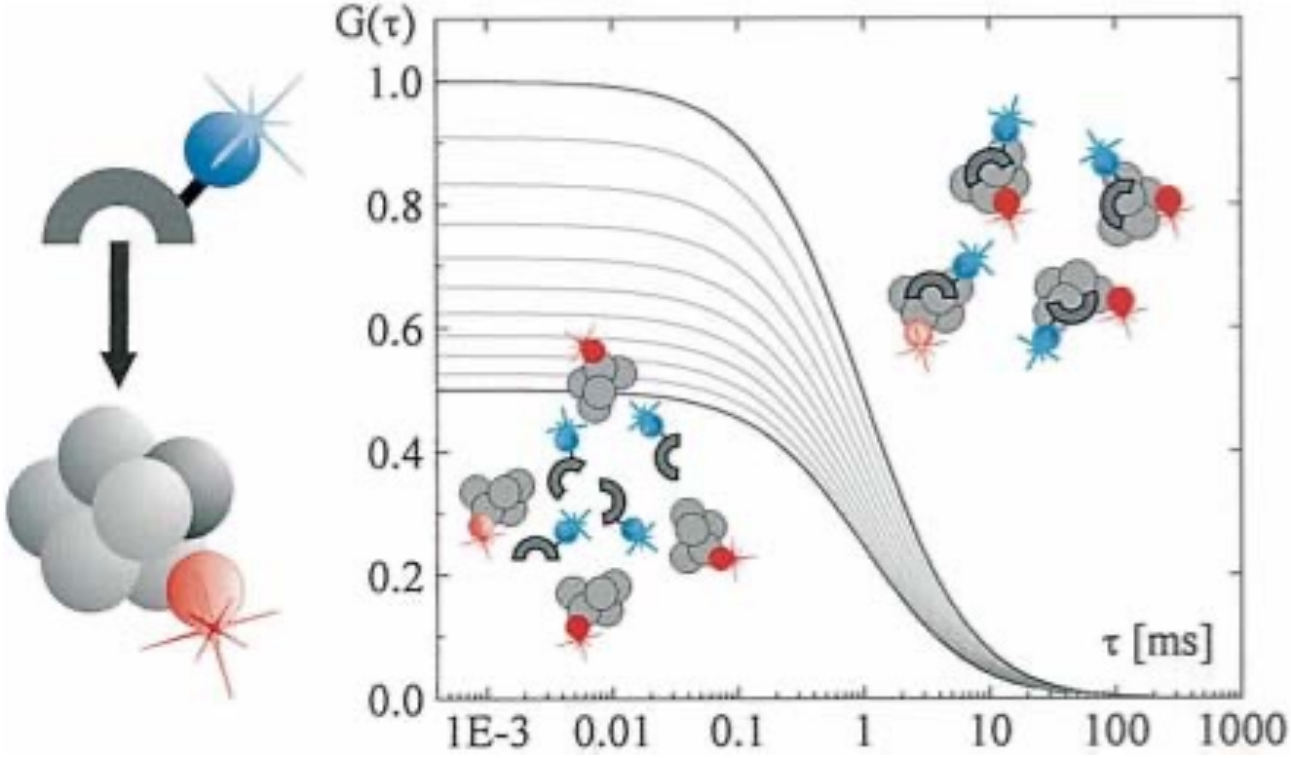


Cross correlation spectroscopy

Interaction analysis with two fluorophores



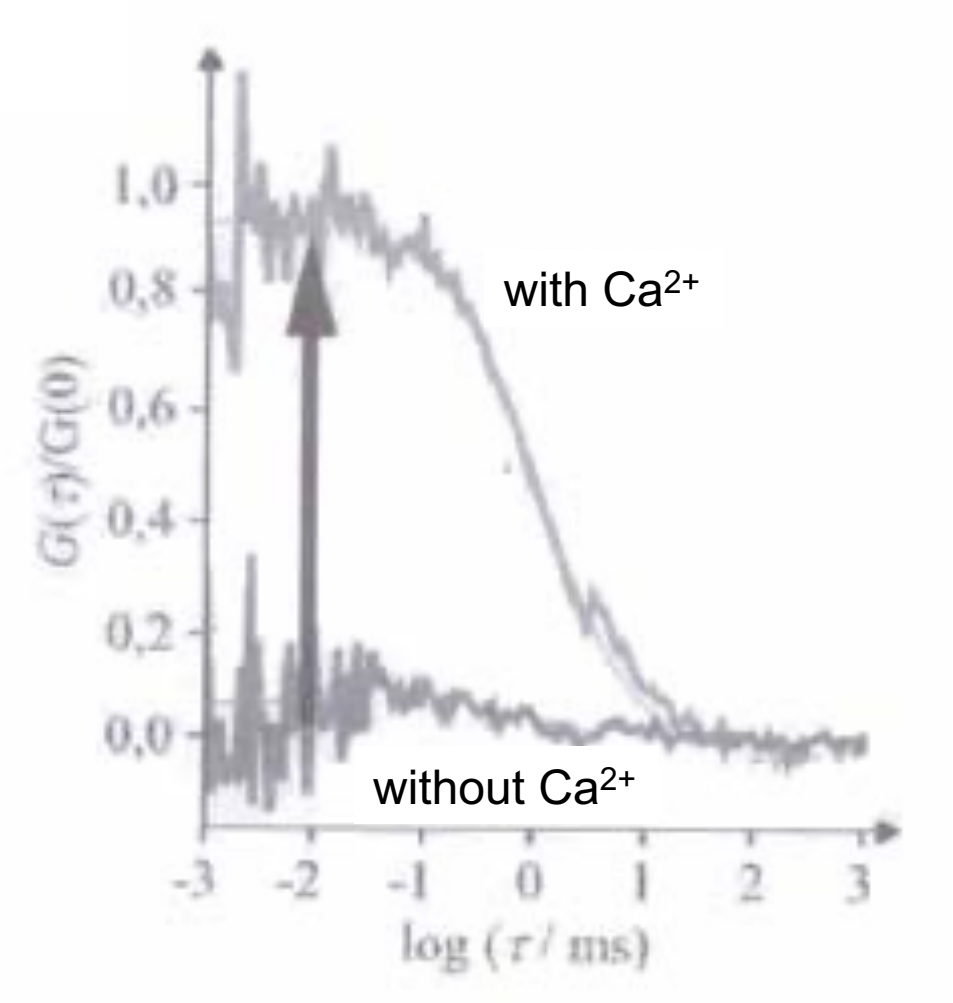
Interaction analysis with two fluorophores



Both binding partners carry a fluorescent label

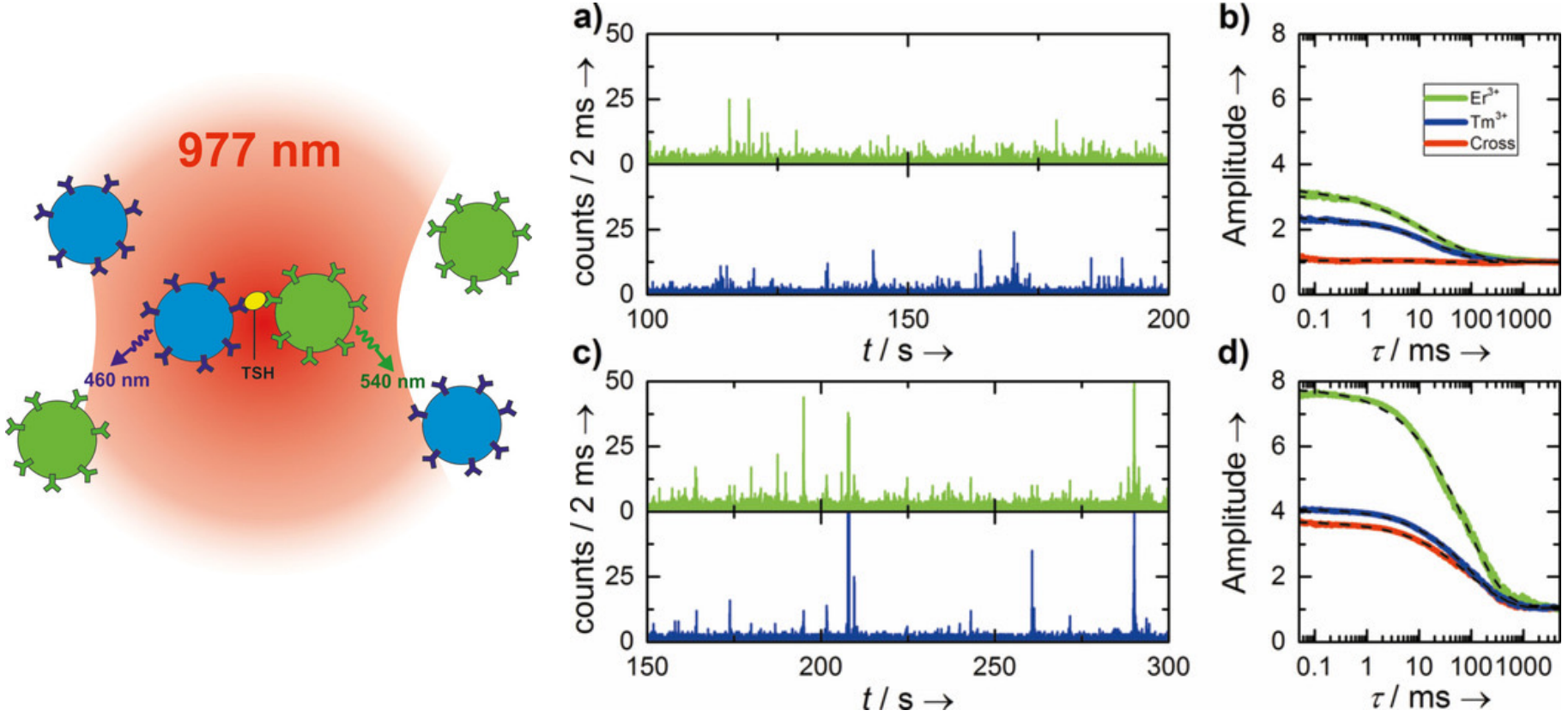
Interaction analysis with two fluorophores

Binding of calmodulin to CaM-dependent protein



Cross-correlation spectroscopy: immunoassay

The size of a molecular complex changes the diffusion time



Limitations of fluorescence microscopy

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

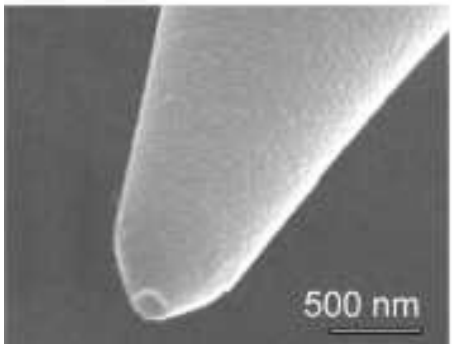
Numerical apperture (NA)

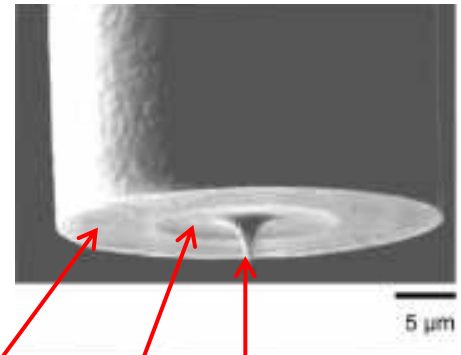
Near-field optical microscopy (NSOM)

Near-field scanning optical microscopy (NSOM)

Diffraction only occurs in far-field imaging, where spherical wave-fronts leaving the aperture can be regarded locally as plane waves

=> “Simple“ solution: avoid diffraction in the first place



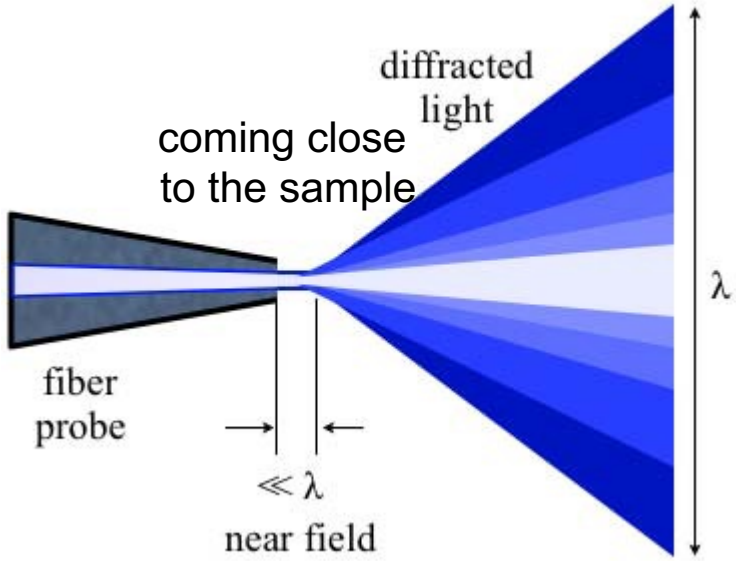


Usually metal coating to avoid stray light

Coating

Core

Tip

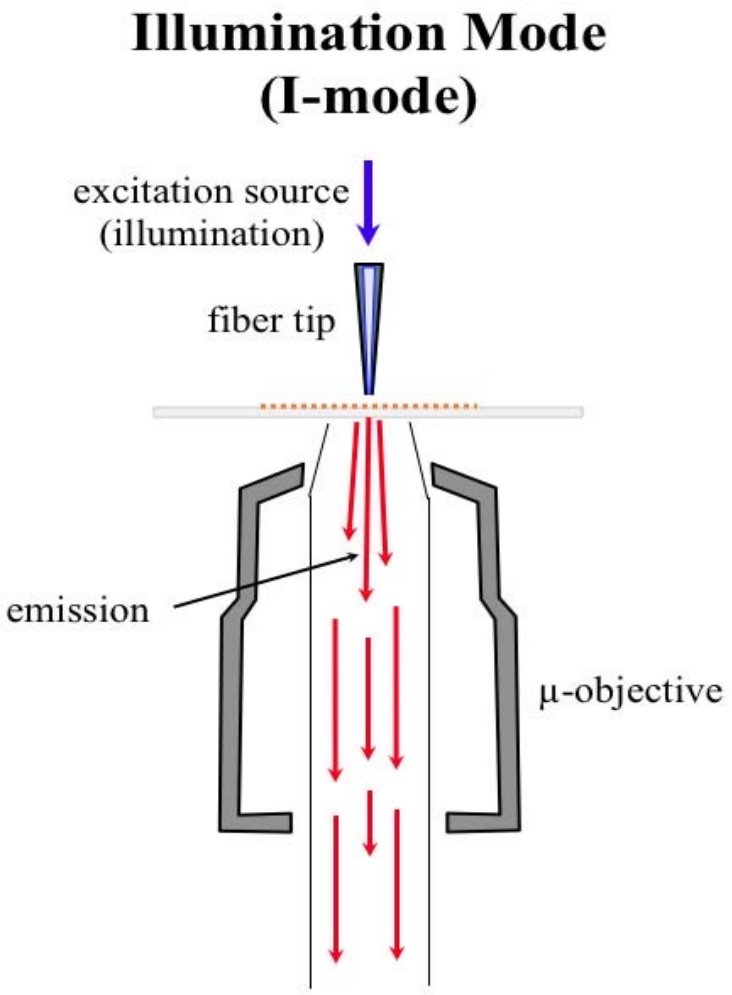


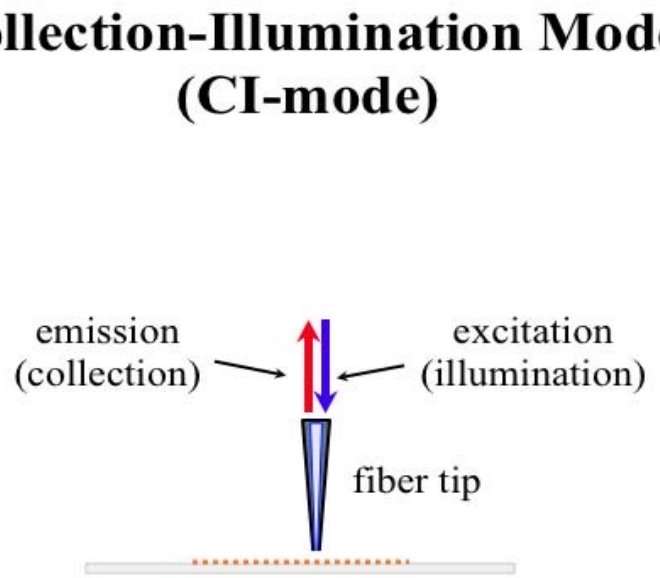
not diffraction limited

=> Near field illumination (evanescent field)

Near-field scanning optical microscopy (NSOM)

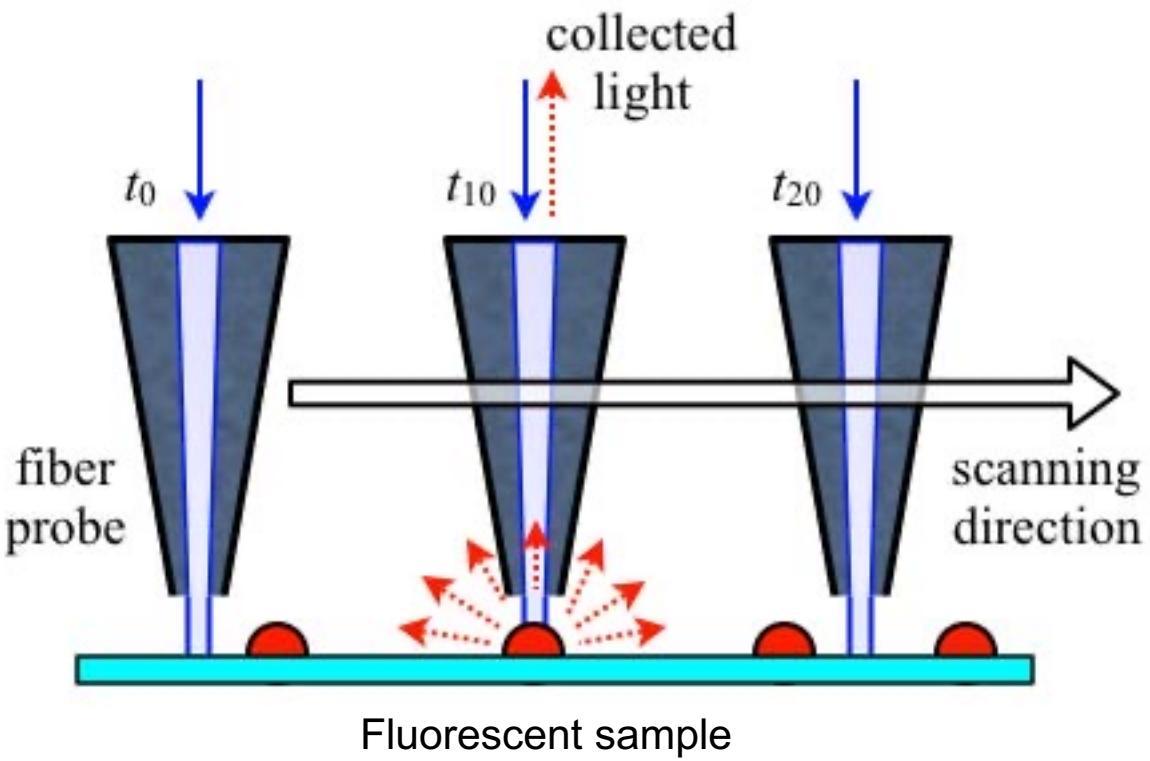
various operation modes – purely near-field or combining near-/far-field excitation/emission or vice versa



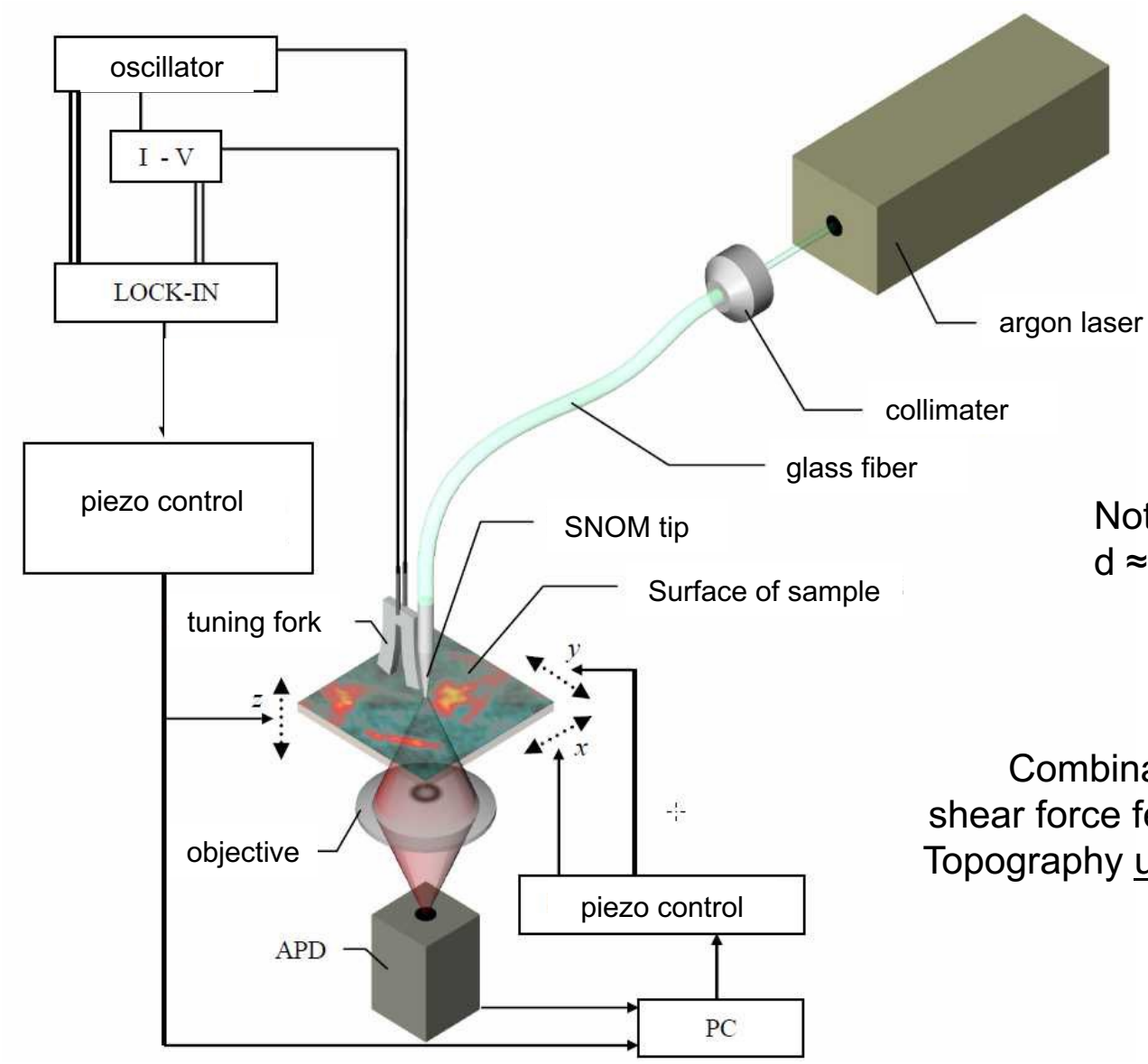


Near-field scanning optical microscopy (NSOM)

Scanning approach



Near-field scanning optical microscopy (NSOM)

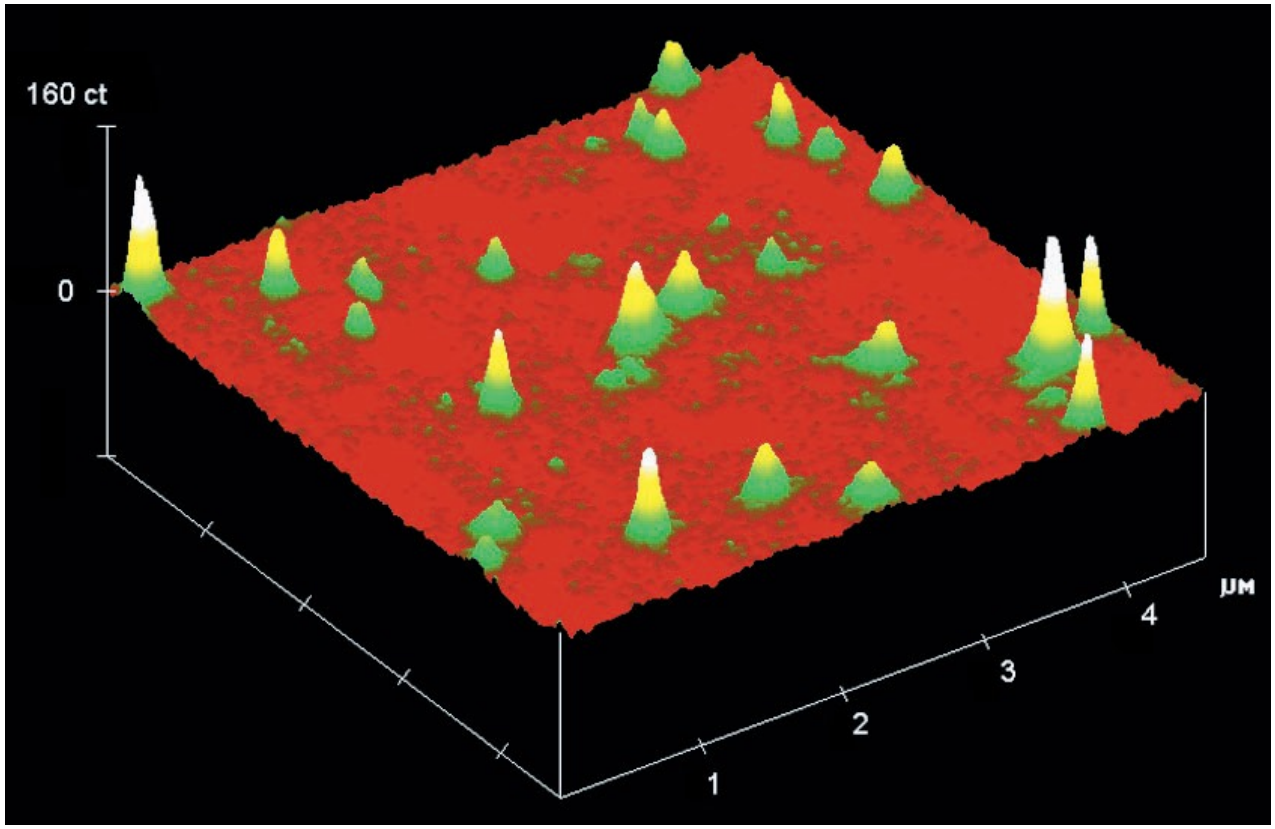


Not diffraction limited:
d ≈ 30 nm

Combination: NSOM and
shear force feedback (tuning fork):
Topography und optical information

Near-field scanning optical microscopy (NSOM)

Detection of single fluorescent molecules



Near-field fluorescence image (4.5 mm by 4.5 mm) of single oxazine 720 molecules dispersed on the surface of a poly(methylmethacrylate) film. Each subdiffraction peak (full width at half maximum, 100 nm) comes from a single molecule (X. S. Xie, *Acc. Chem. Res.* 29, 598 (1996)).

Near-field scanning optical microscopy (NSOM)

Advantages:

- resolution ~ 20 nm in lateral (depending on tip size) and ~ 2-5 nm in axial direction
- optical and topological information

Limitations:

- only applicable to surfaces
- tip may break in contact with specimen (scanning)

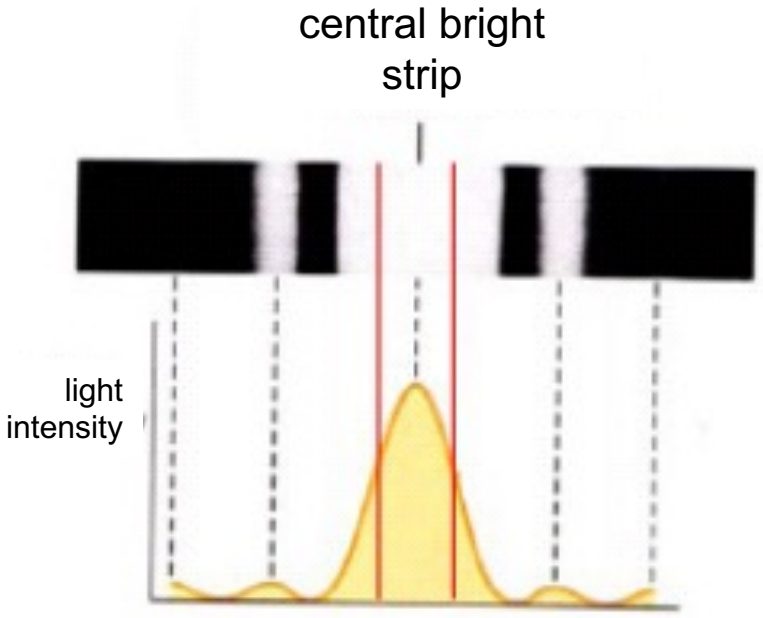
- far-field microscopy has many advantages (except the diffraction limit)

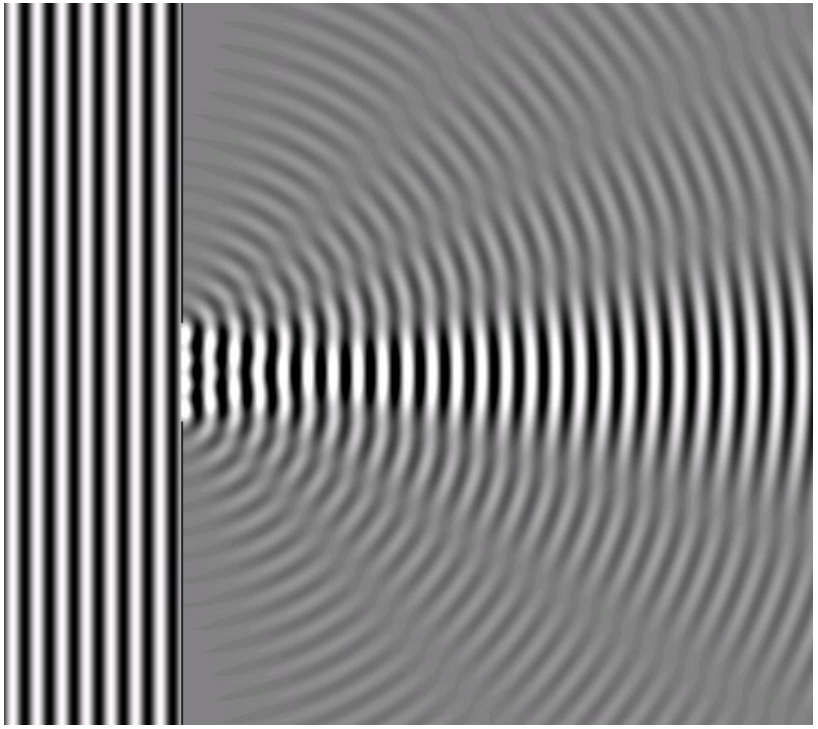
Far-field optical microscopy

=> Using freely propagating light waves

Optical resolution of light microscopy

Ernst Abbe: Diffraction of waves at a cleft



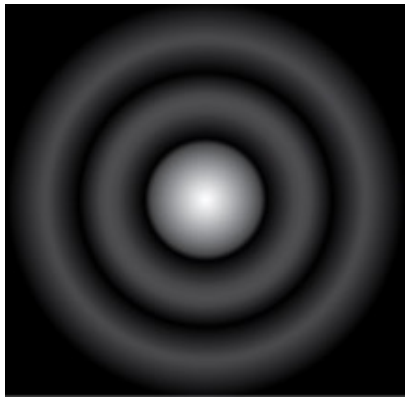


$$d = \frac{\lambda}{2n\sin\alpha}$$

Optical resolution of light microscopy

Rayleigh criterion: when are two objects visible as separate points

Light from a point source (e.g. a fluorophore) is diffracted by the inner rim of the objective and forms an **Airy disc**.



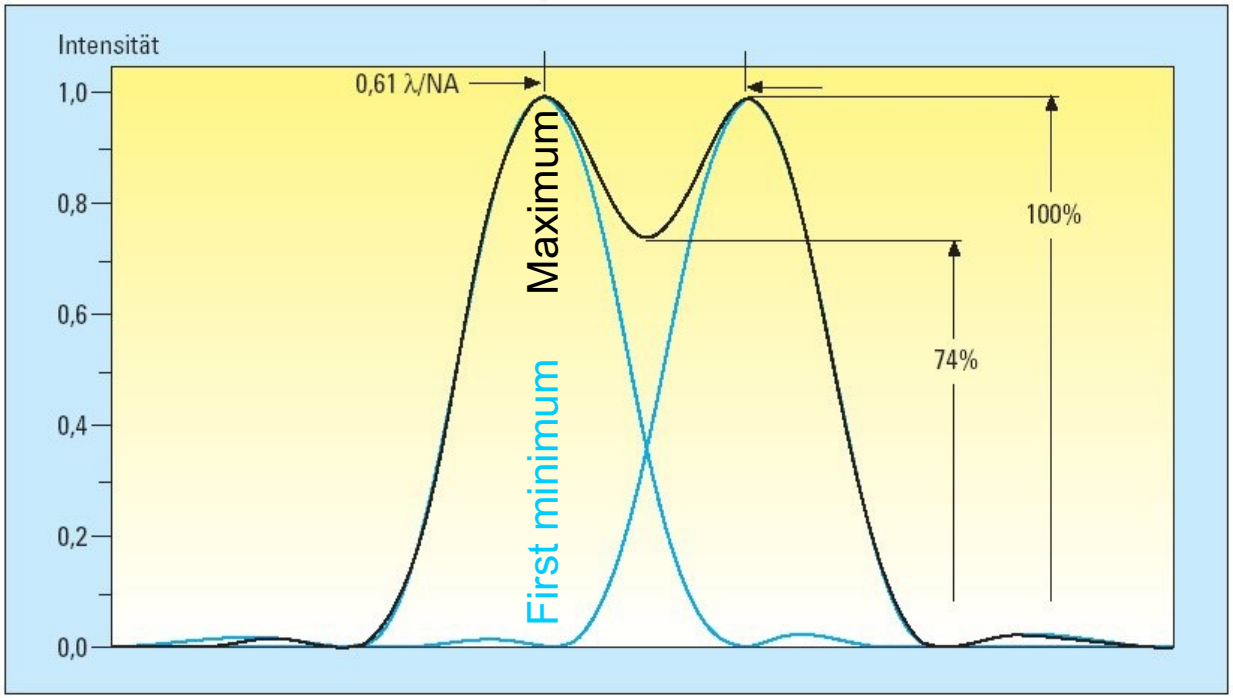
Airy disc

Airy pattern

The size of the **Airy disc** depends on λ and NA of the objective:

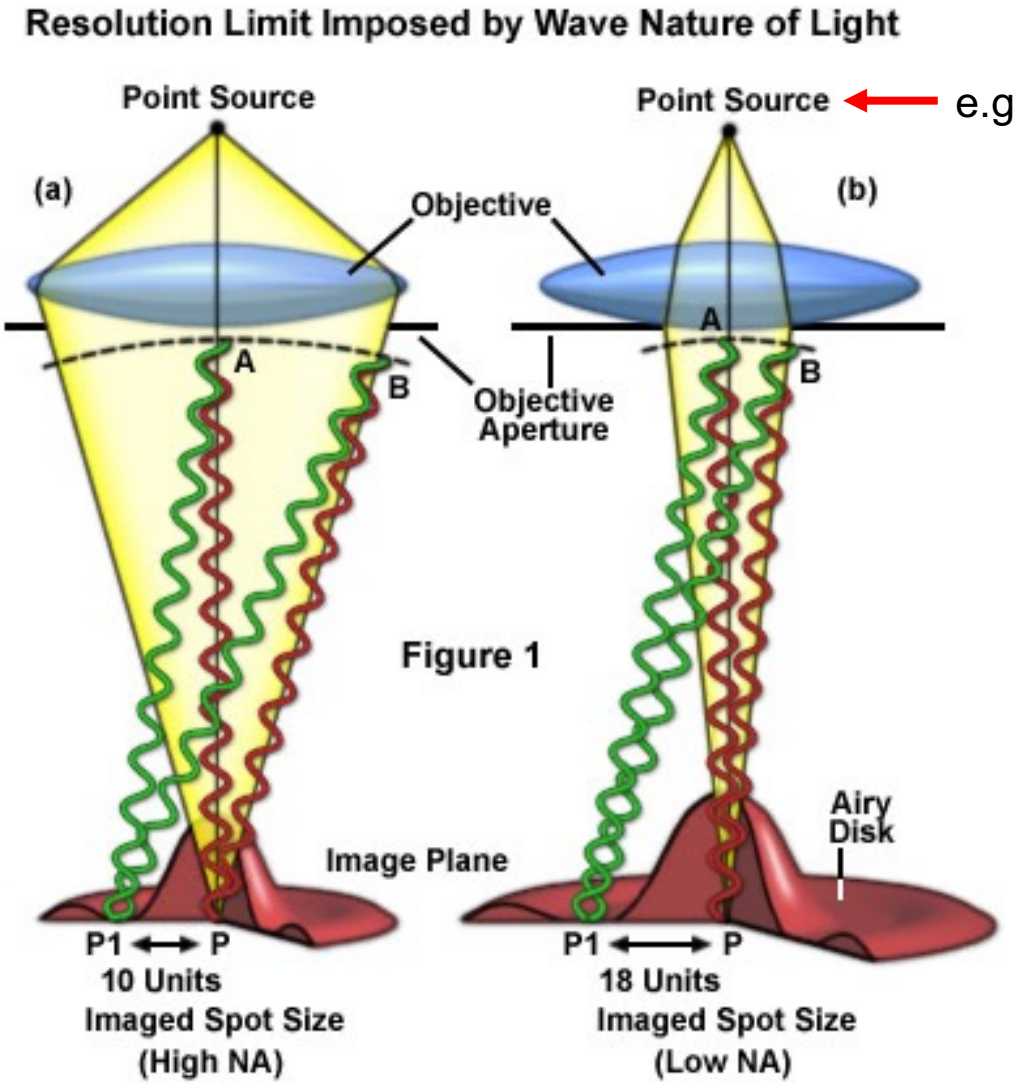
$$d = \frac{0.61\lambda}{n \sin \alpha}$$

Corresponding intensity profile



=> 2 points are resolvable if the maximum of one Airy disc coincides with the first minimum of the next Airy pattern.

Optical resolution of light microscopy



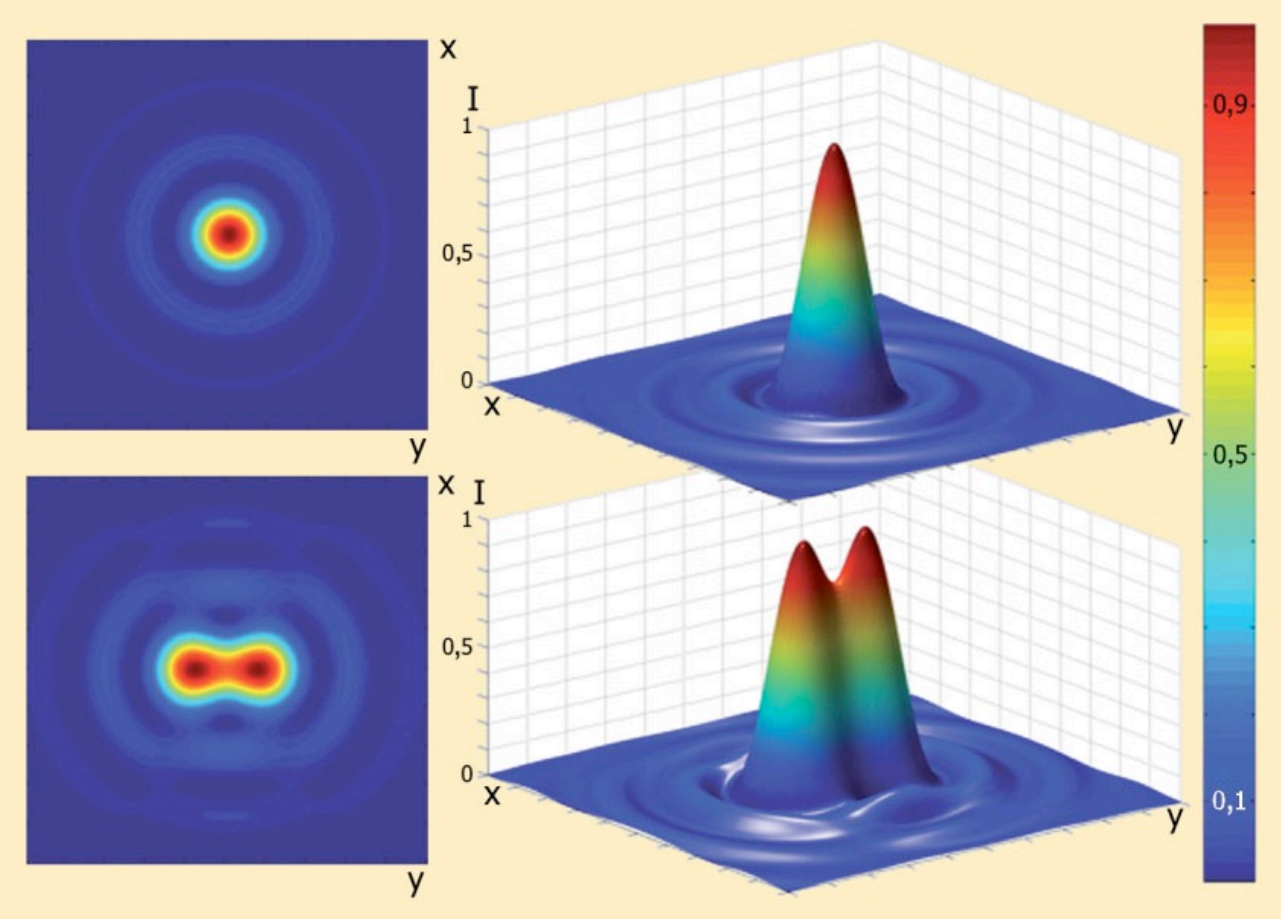
red waves:
positive interference

green waves:
negative interference

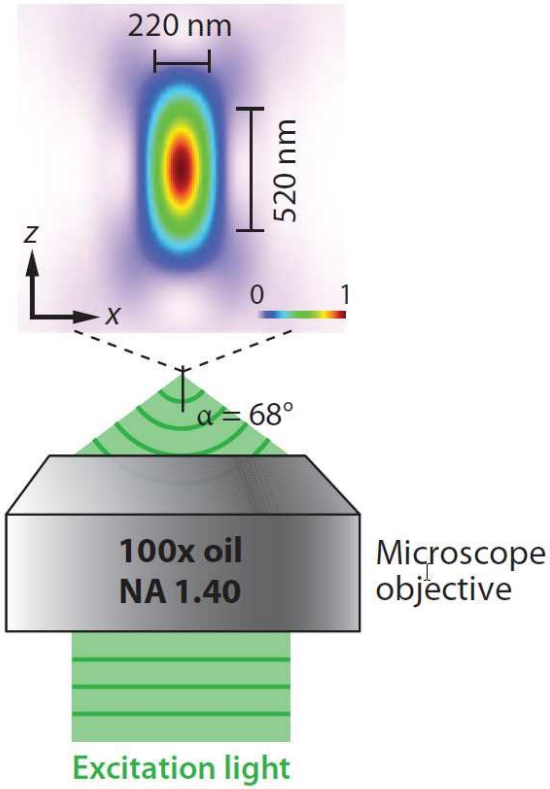
Source Nikon: http://www.microscopyu.com

Optical resolution of light microscopy

Diffraction limited spot: Point spread function



Point spread function:
Max. axial und
lateral resolution



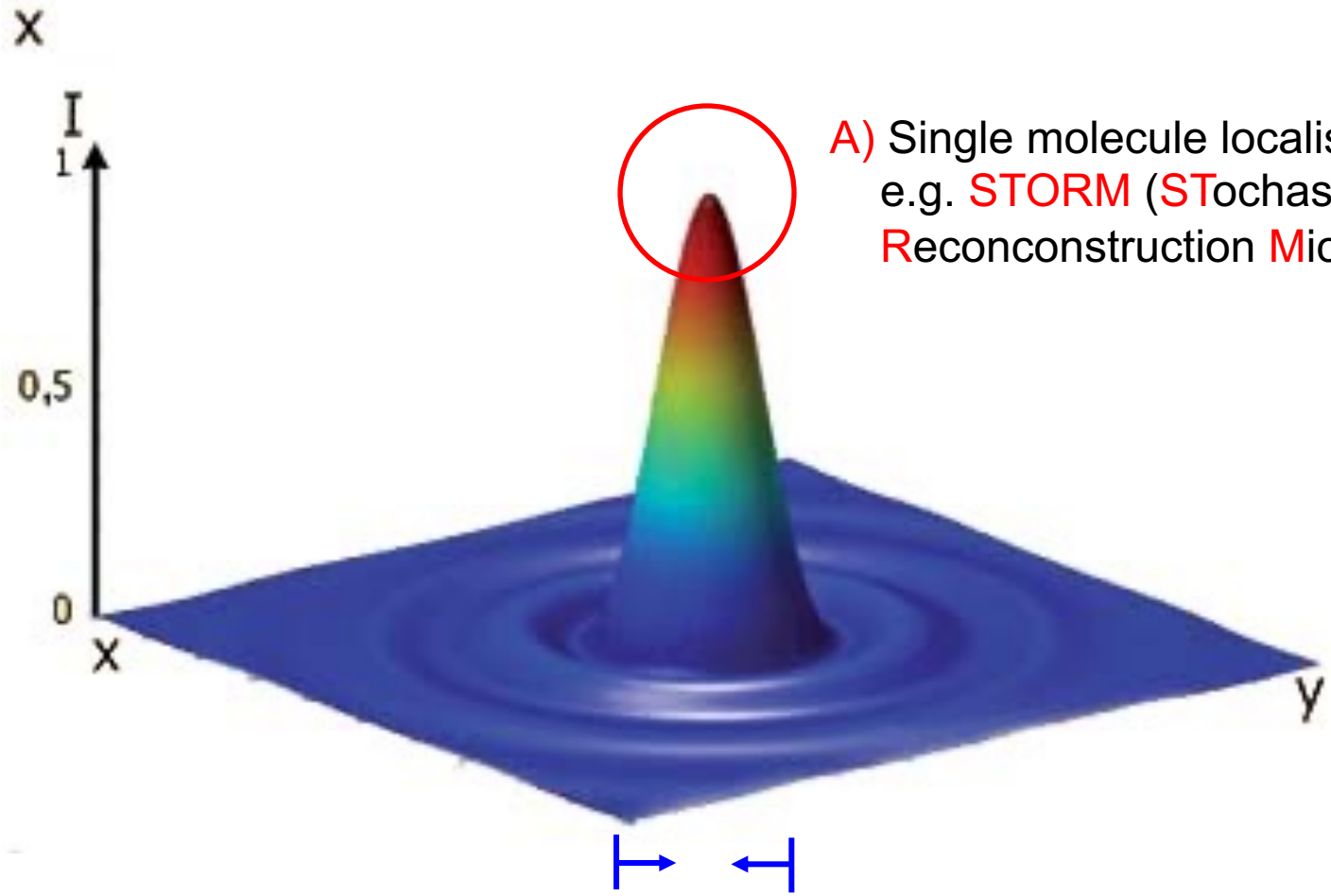
(Far-field) microscopy beyond the diffraction limit

Nobel prizce for Chemistry in 2014

Stefan Hell	William Moerner	Eric Betzig
STED	- Detection of single fluorescent molecules - switchable fluorophores	- Near field microscopy - STORM

Microscopy beyond the diffraction limit

Using non-linear optical processes



A) Single molecule localisation (2006)
e.g. STORM (STochastic Optical Reconconstruction Microscopy)

B) Structured illumination (1994/1999)
z.B. STED (STimulated Emission Depletion)

STochastic Optical Reconconstruction Microscopy

STORM

=> based on wide-field microscopy
(frequently in combination with TIRF)

STORM microscopy

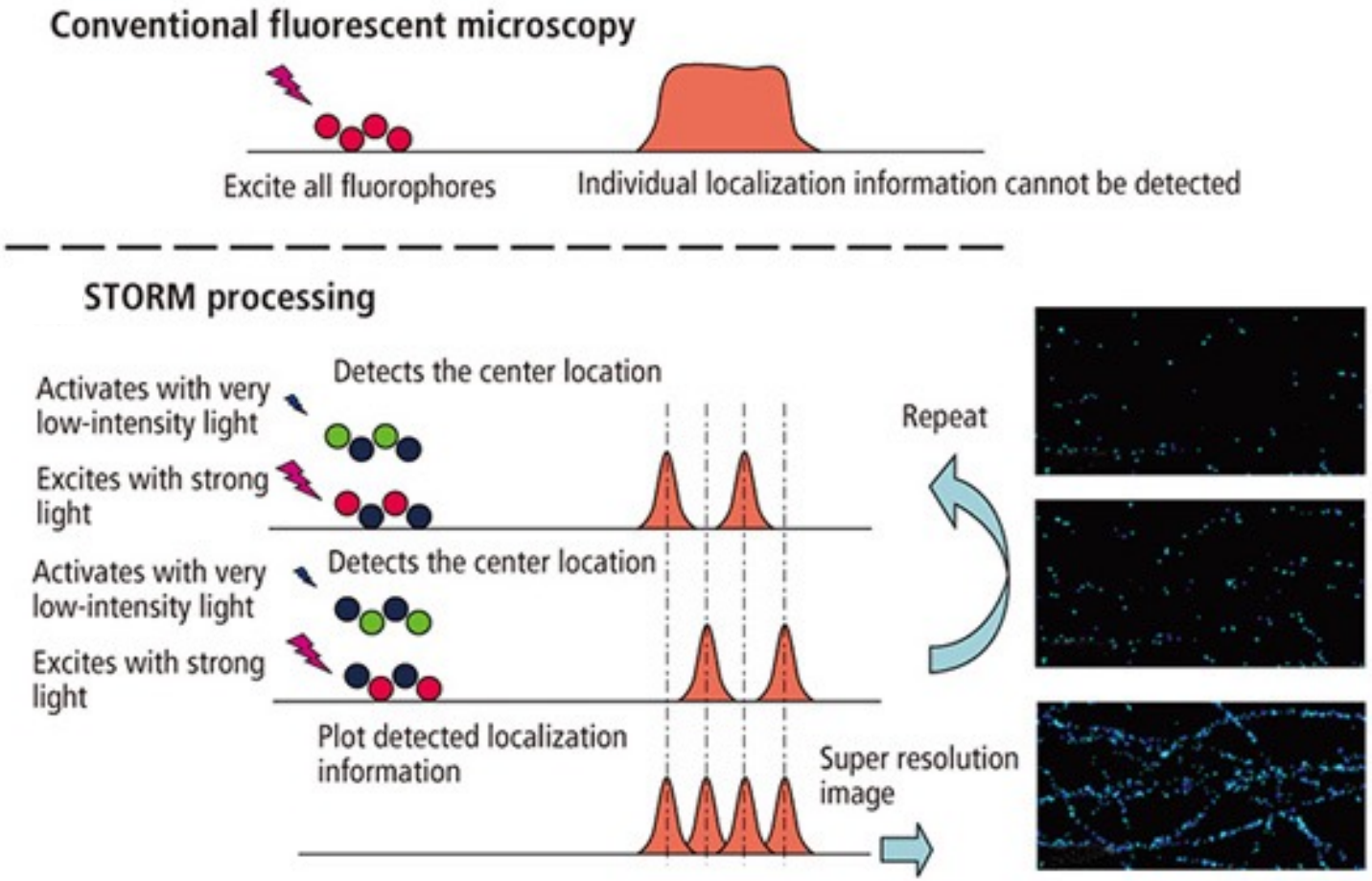
Single Molecule Tracking

⇔

Imaging (STORM)

=> Rather than using a highly diluted solution of fluorophores, individual fluorophores are switched on/off in a sequential manner

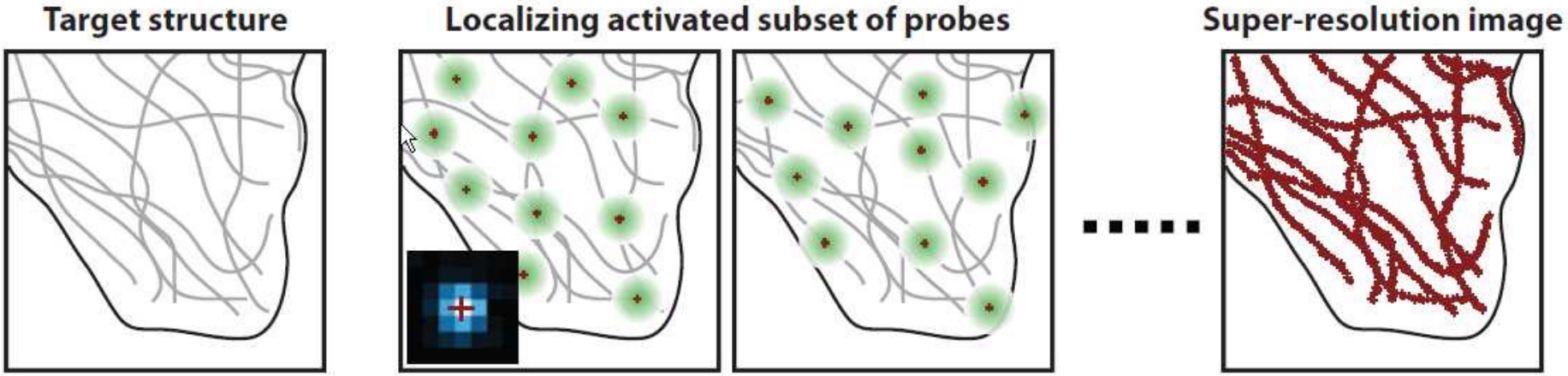
STORM microscopy



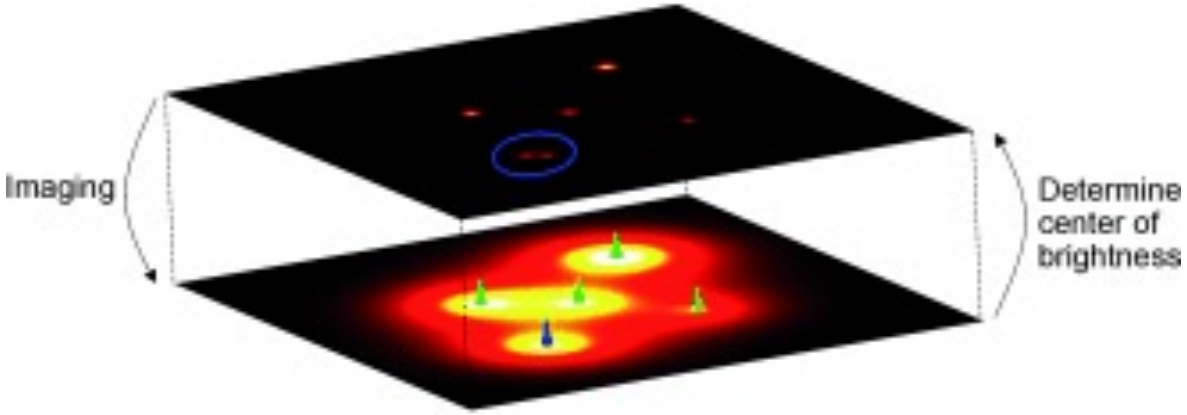
=> Maximum of the point spread function of a single fluor. molecule can be determined precisely
But: 1000-10.000 images required to put together a high-resolution image
=> Need for high computational power / appropriate „switchable“ fluorophores

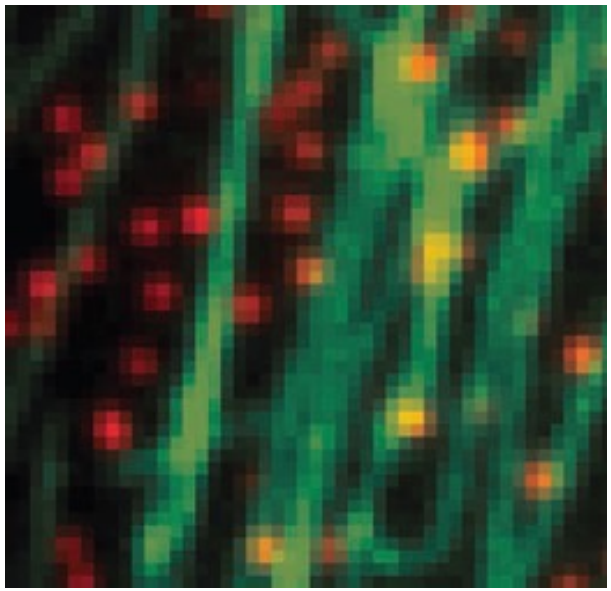
STORM microscopy

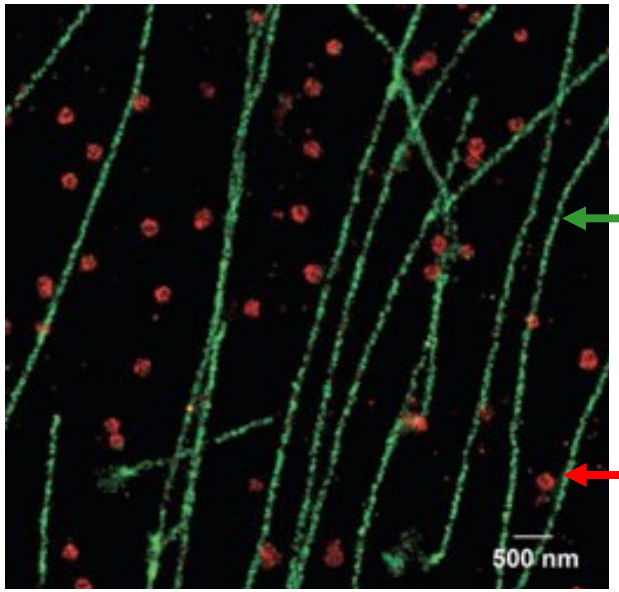
Pointillism in modern art



STORM microscopy



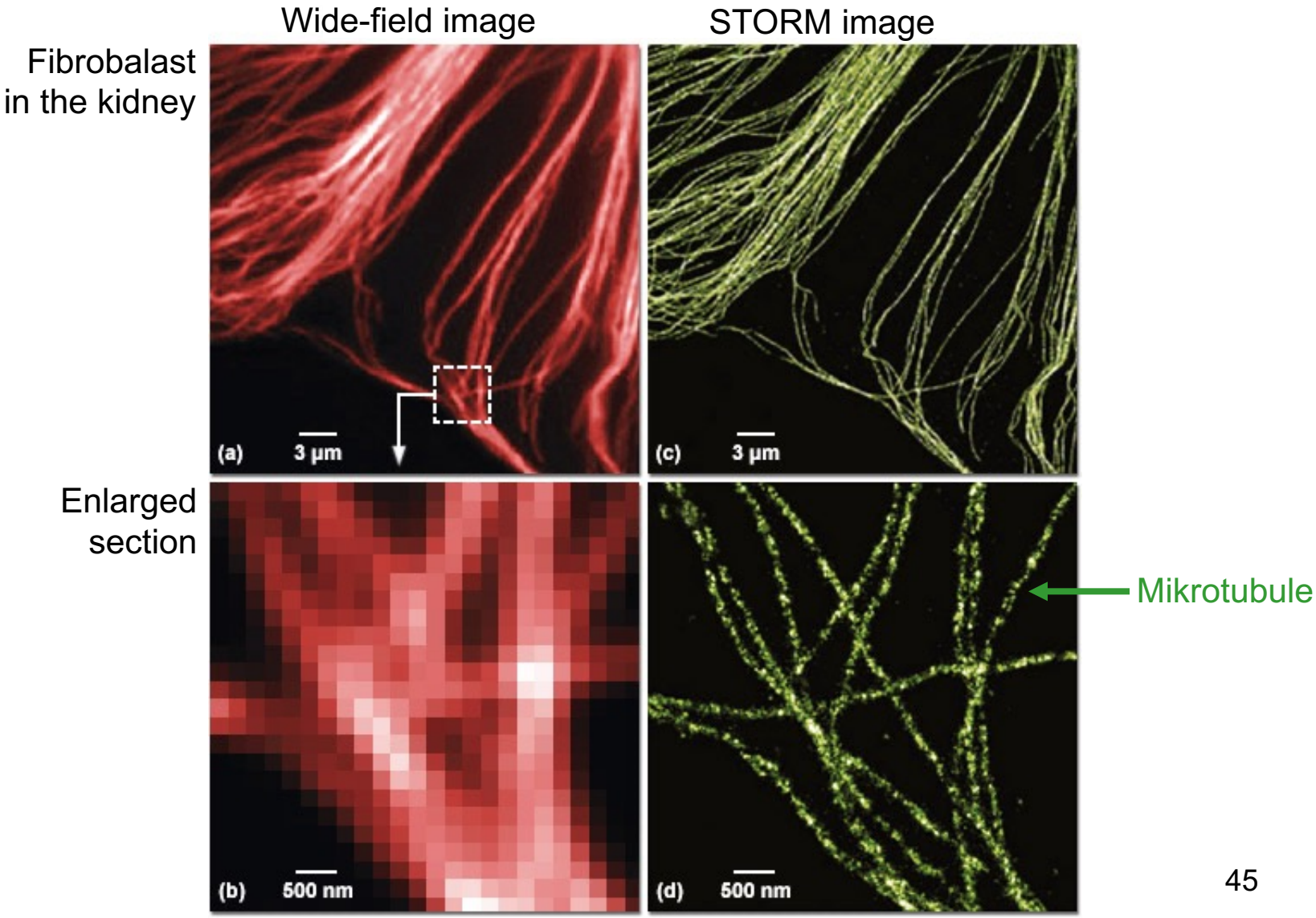




Microtubule

Clathrin-
coated pit

STORM microscopy: images



STORM microscopy

photoswitchable fluorophores are required:

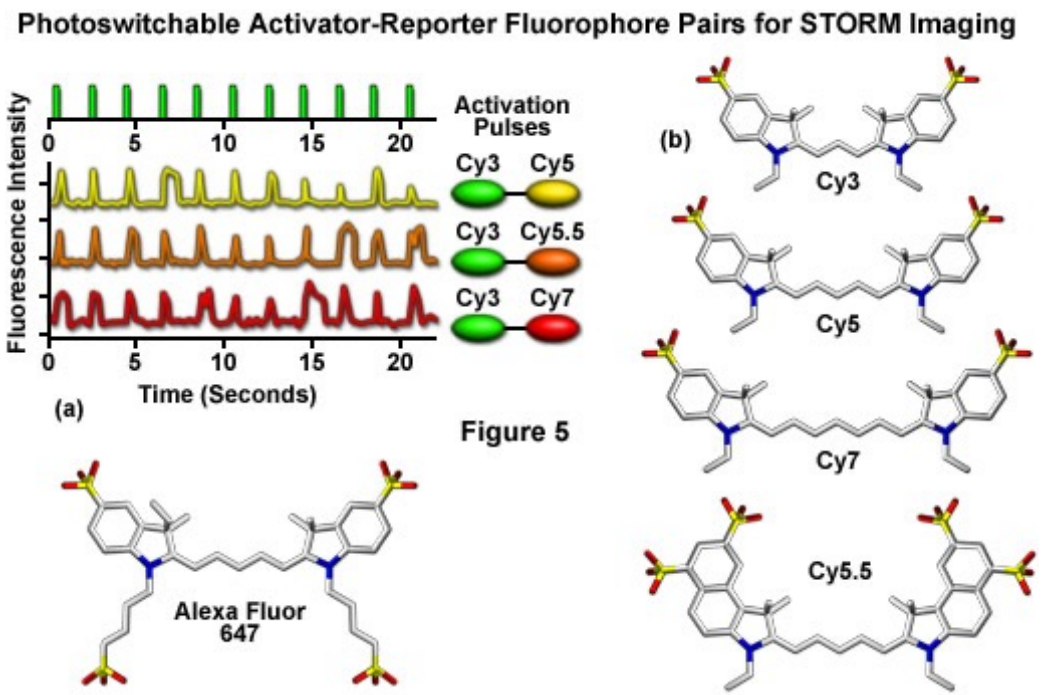


Figure 5

Switching on to fluorescent state by supporting dye (e.g. Cy3)
Switching off to dark state: spontaneously

Yield: 6000 photons per activated fluorescent molecule

Variation: PALM
(Photoactivated Localization Microscopy)
=> based on FP

Switching on by UV-Licht
Switching off by photobleaching
Yield: ~500 photons

STORM microscopy

Resolution:
2 points that can just be distinguished by using STORM:

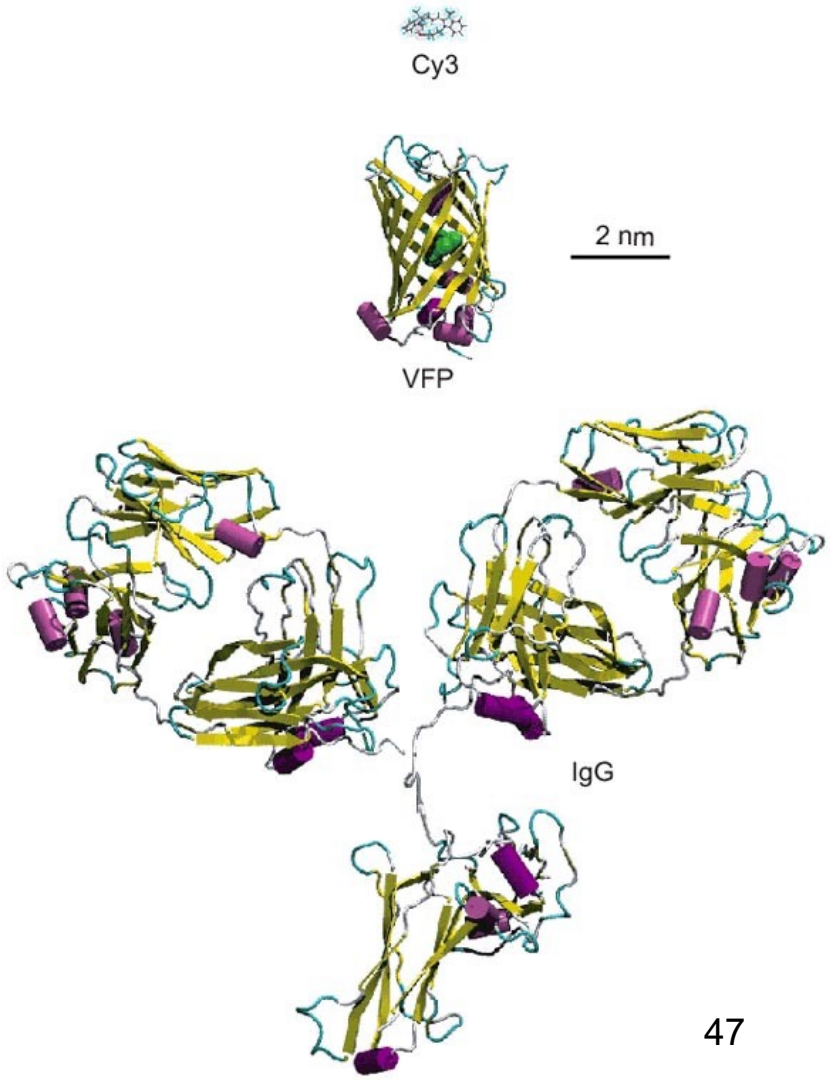
$$d = \frac{\lambda}{2n \sin \alpha \sqrt{N}}$$

N: Number of photons emitted by a single fluorophore molecule that can be detected

In praxis, resolution of 10 - 20 nm:
> factor 10!

Other factors are limiting:
e.g. antibodies have a diameter of 15 nm

Different sizes of molecules



STimulated Emission Depletion Microscopy

STED

=> is based on Confocal Microscopy

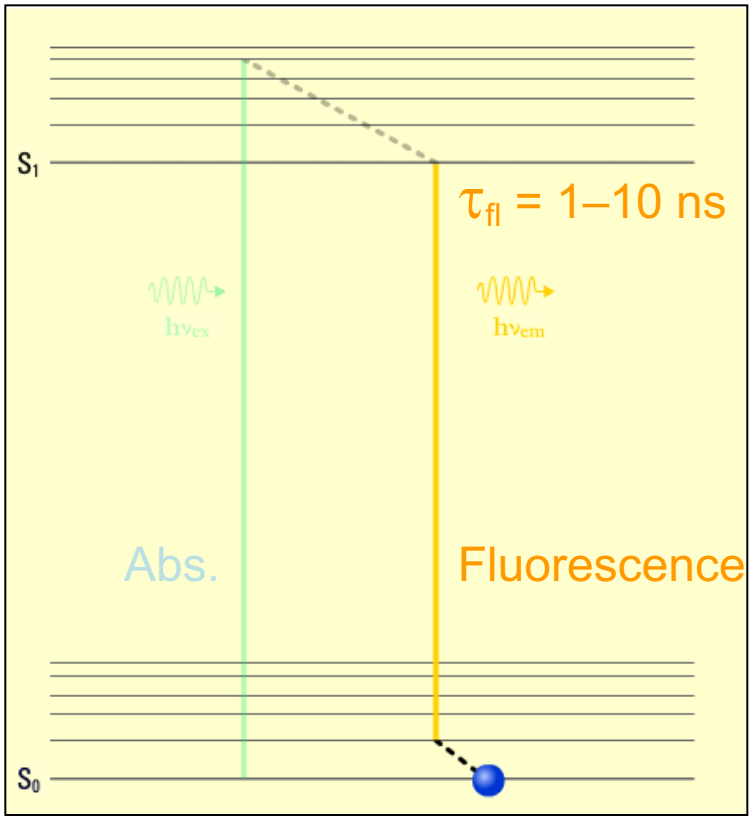
STED microscopy

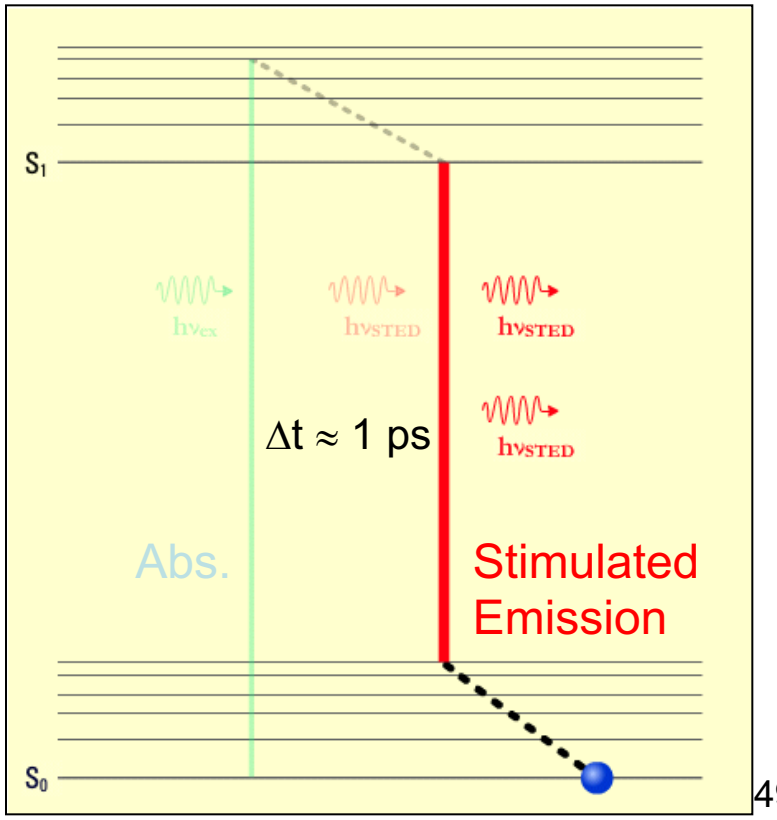
Light can interact with matter:

1. Absorption

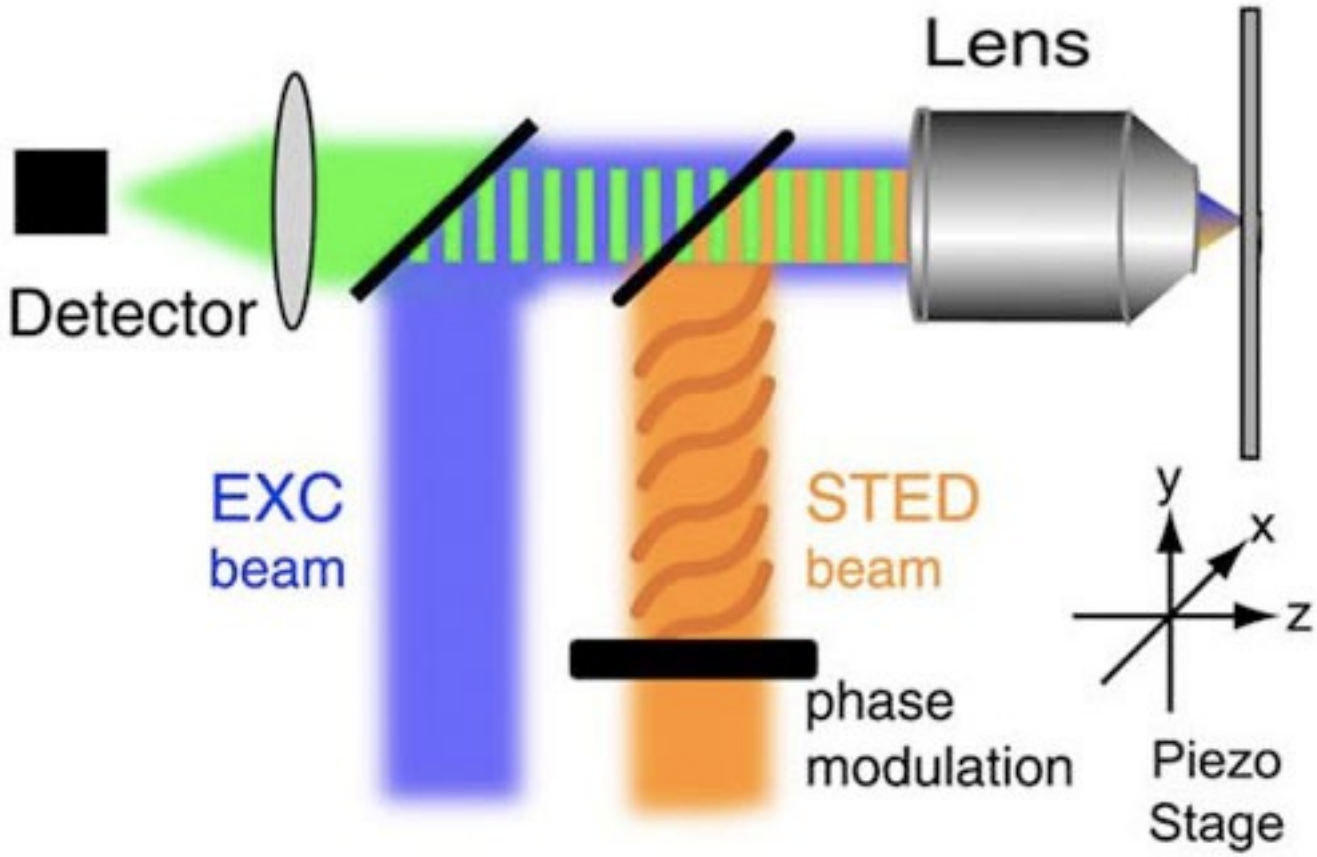
2. Spontaneous emission

3. Stimulated emission



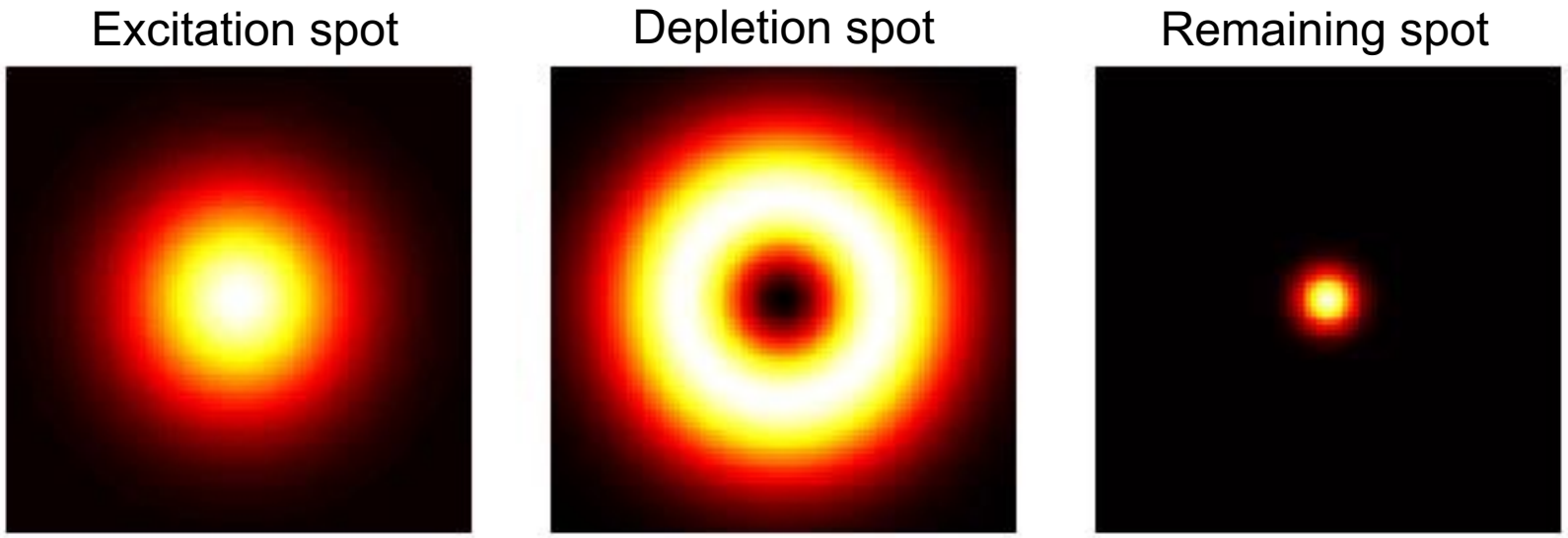


STED microscopy: instrumental setup

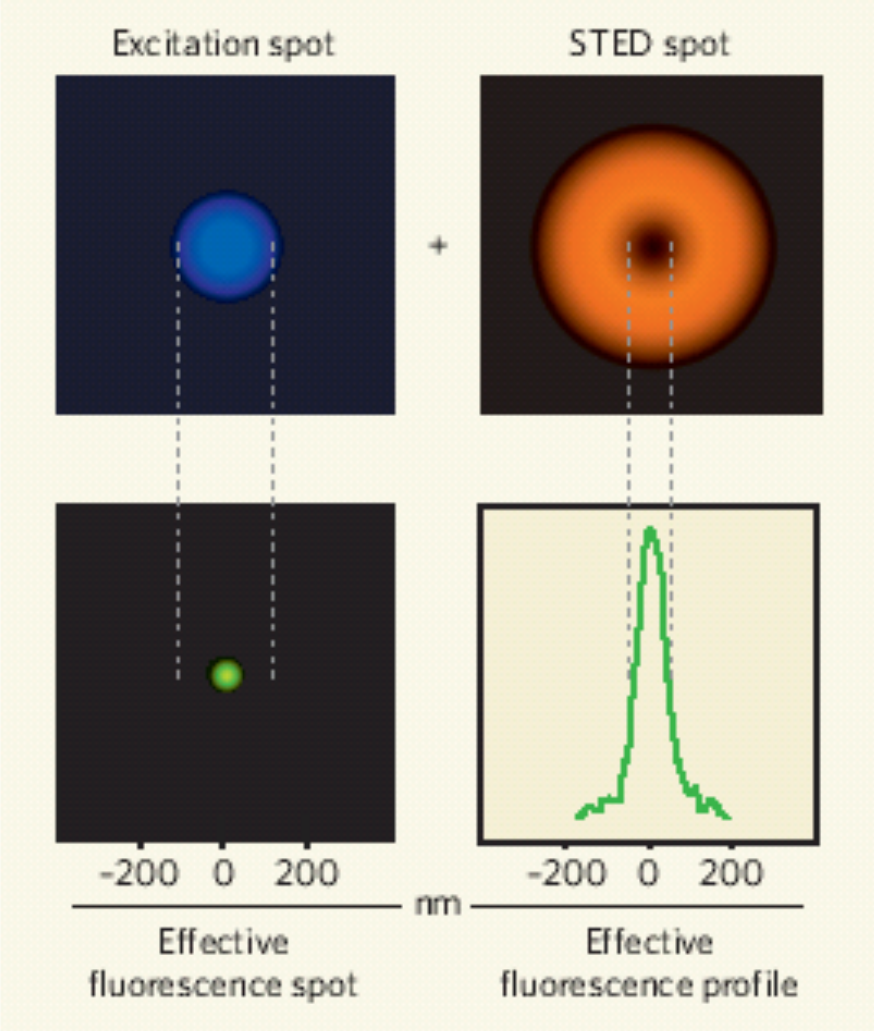


EXC und STED are pulsed lasers with defined timing of pulses

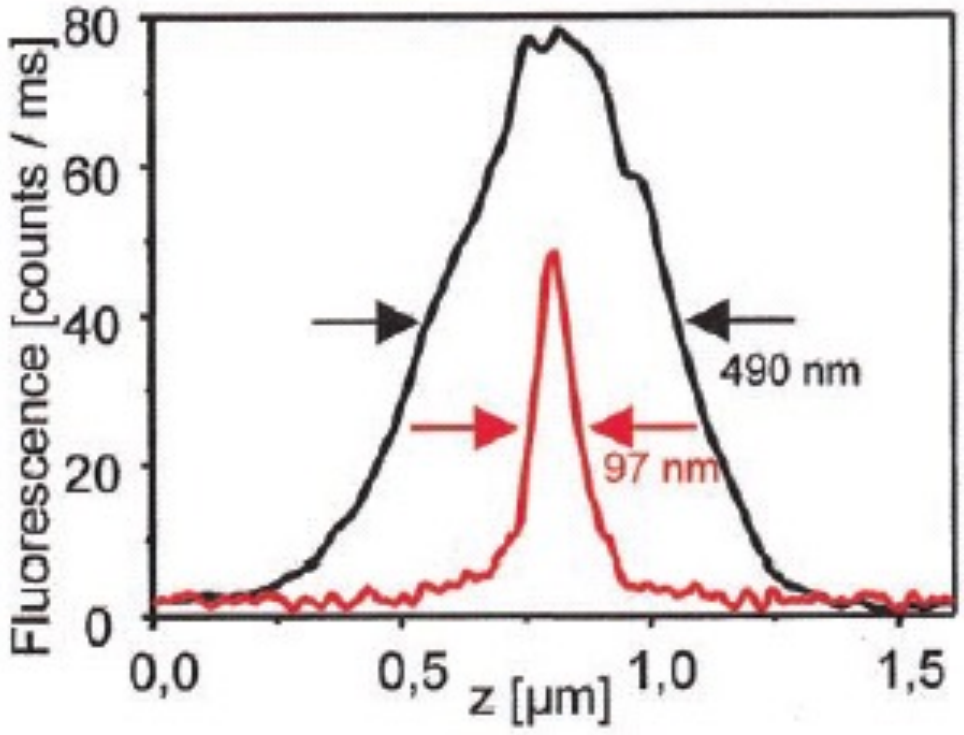
STED microscopy: improved lateral resolution



STED microscopy: improved lateral resolution



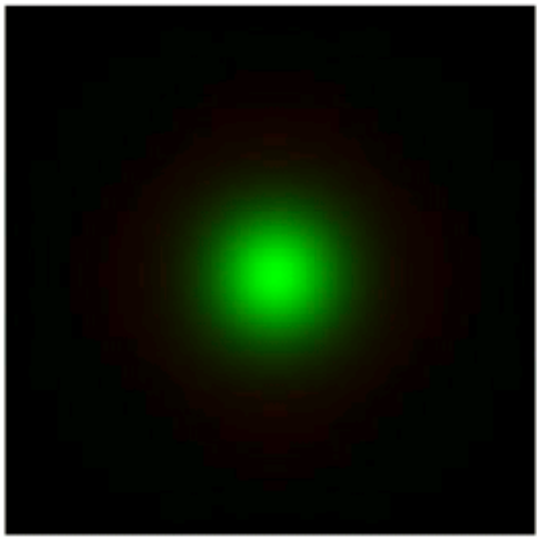
STED microscopy: improved lateral resolution

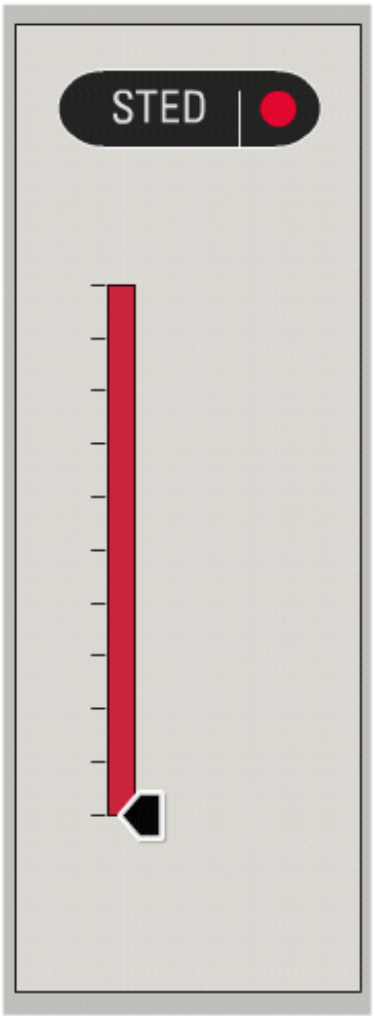


Conventional confocal microscopy

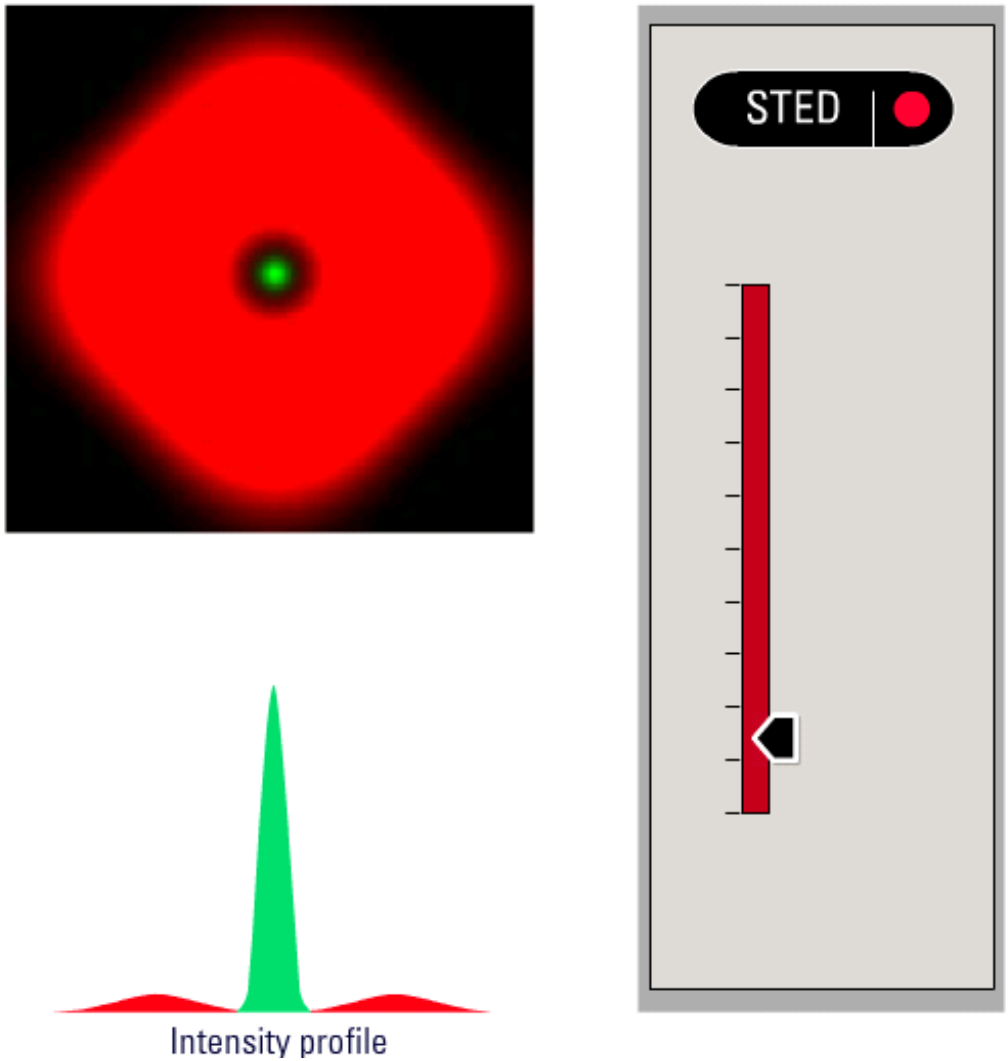
STED microscopy

STED microscopy: STED pulse intensity

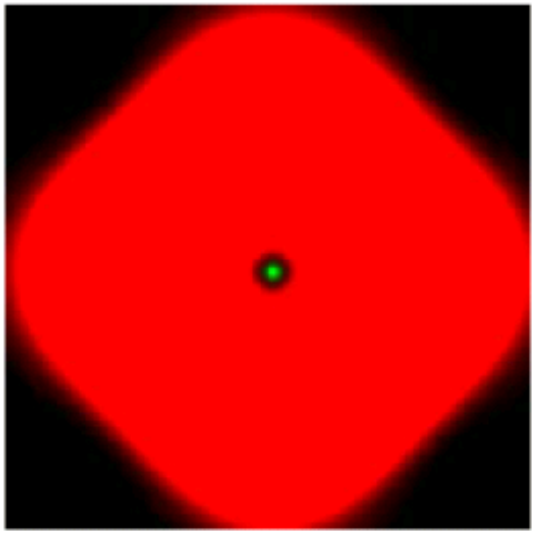


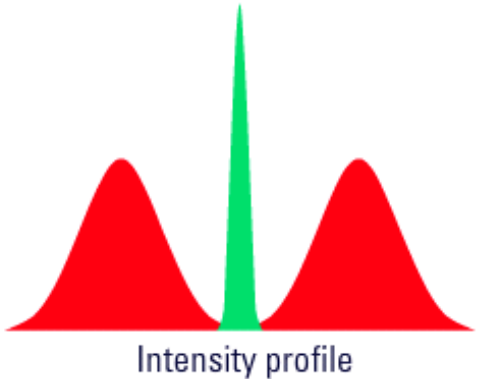


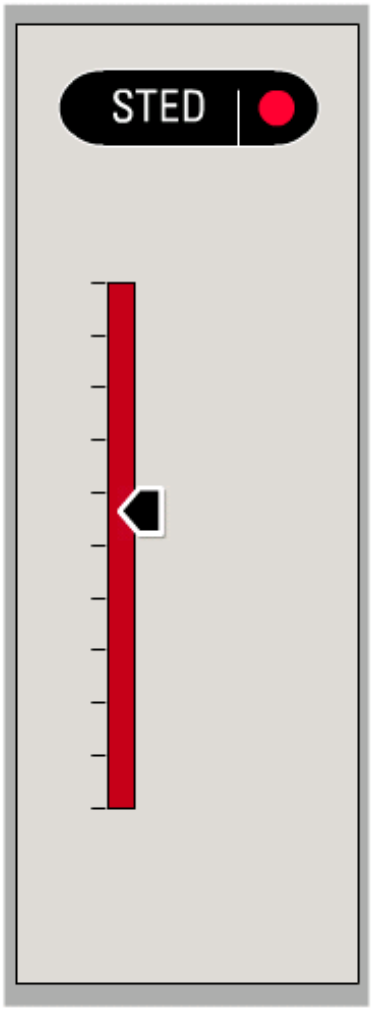
STED microscopy: STED pulse intensity



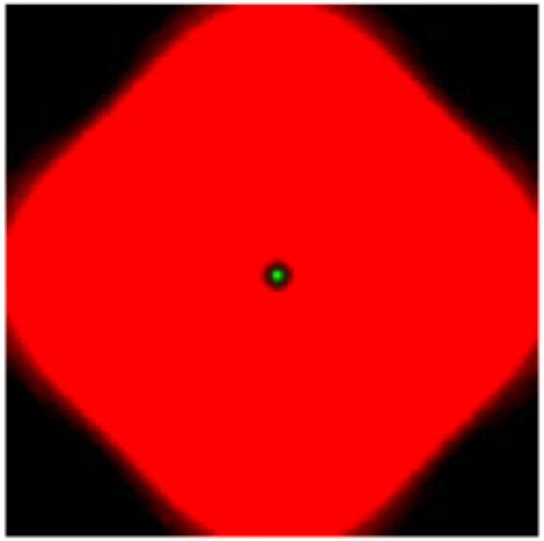
STED microscopy: STED pulse intensity

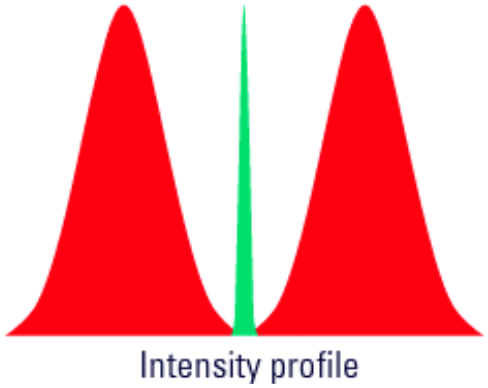


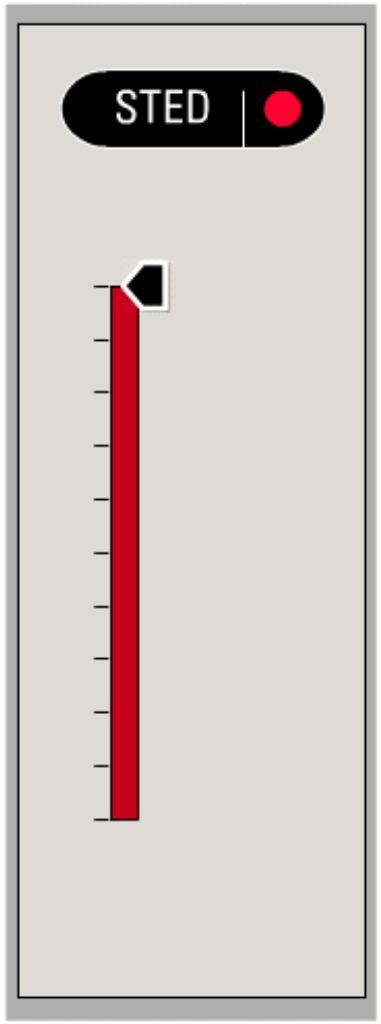




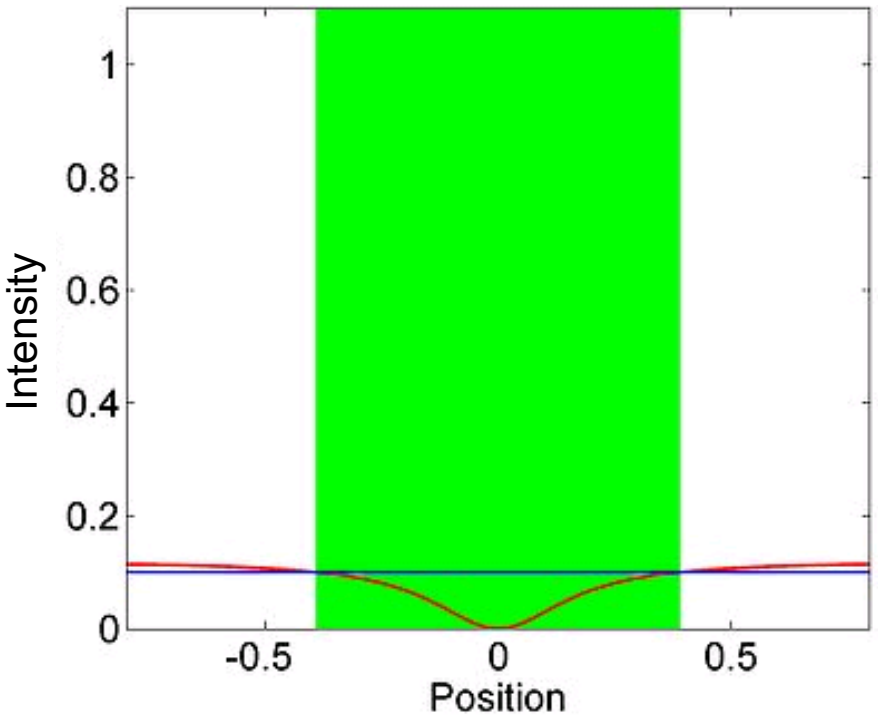
STED microscopy: STED pulse intensity

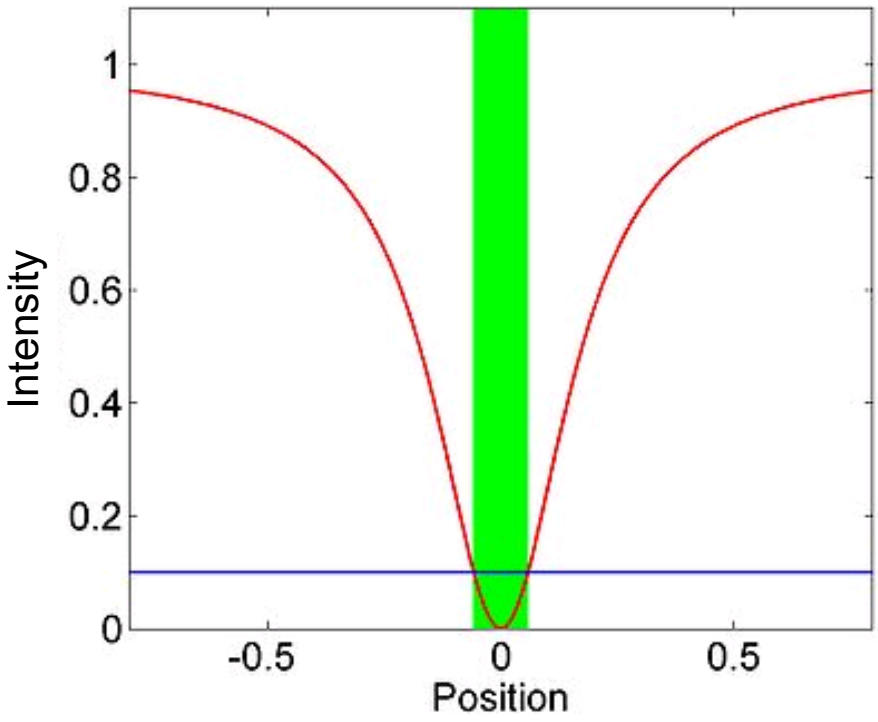






STED microscopy: STED pulse intensity

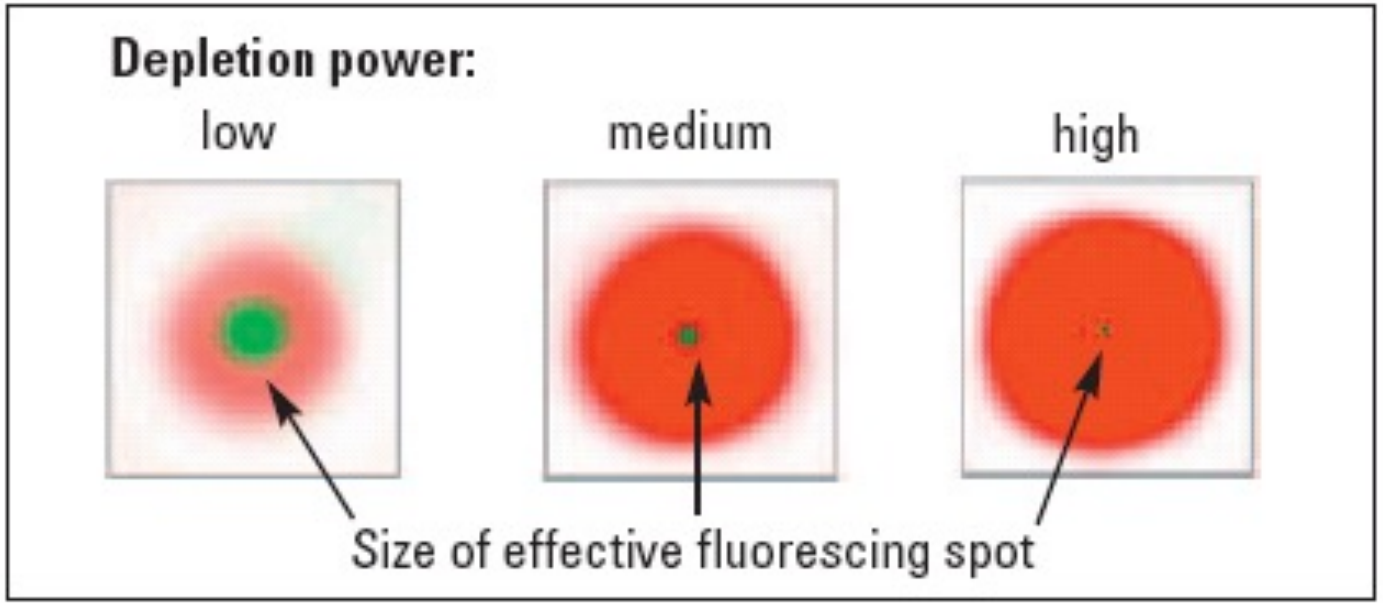




I: Intensity of the STED laser

I_s: Required intensity to completely deplete the excited state

STED microscopy: STED pulse intensity



$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_s}}}$$

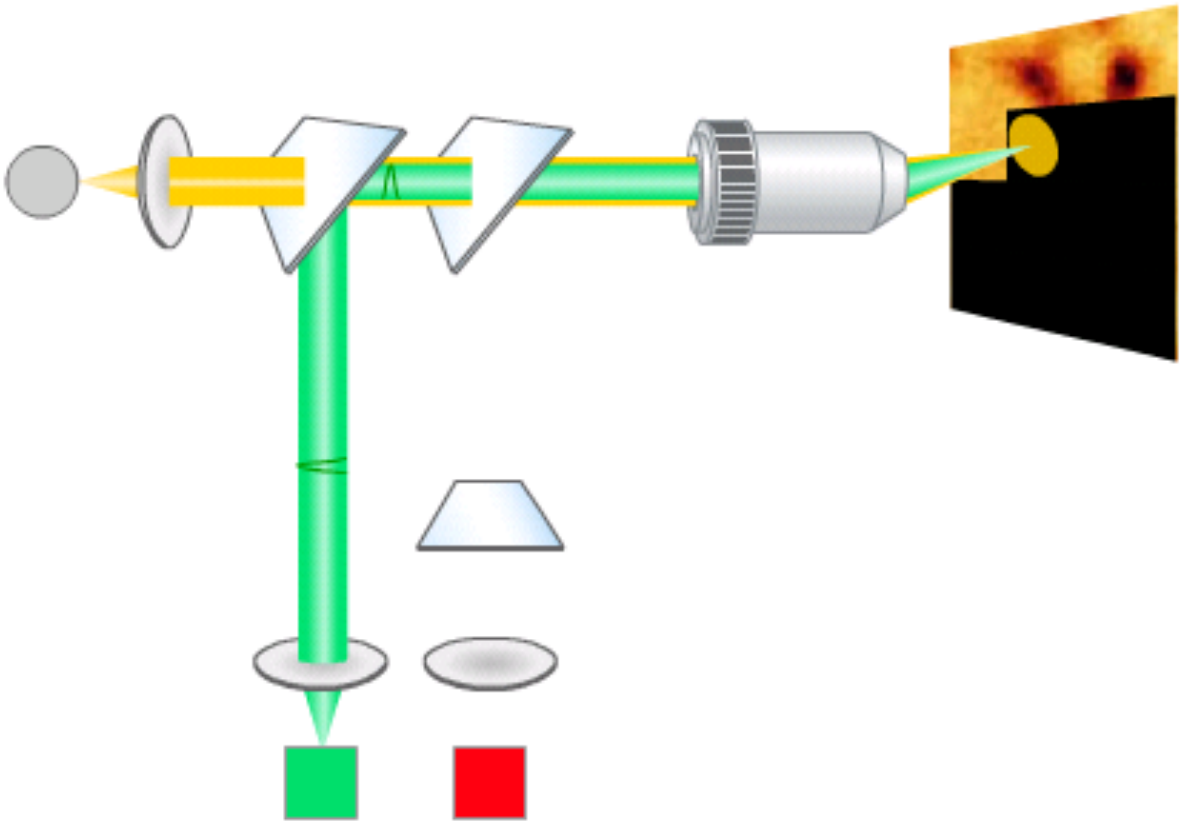
In praxis, resolution of < 10 nm

I: Intensity of the STED laser

I_s: Required intensity to completely deplete the excited state

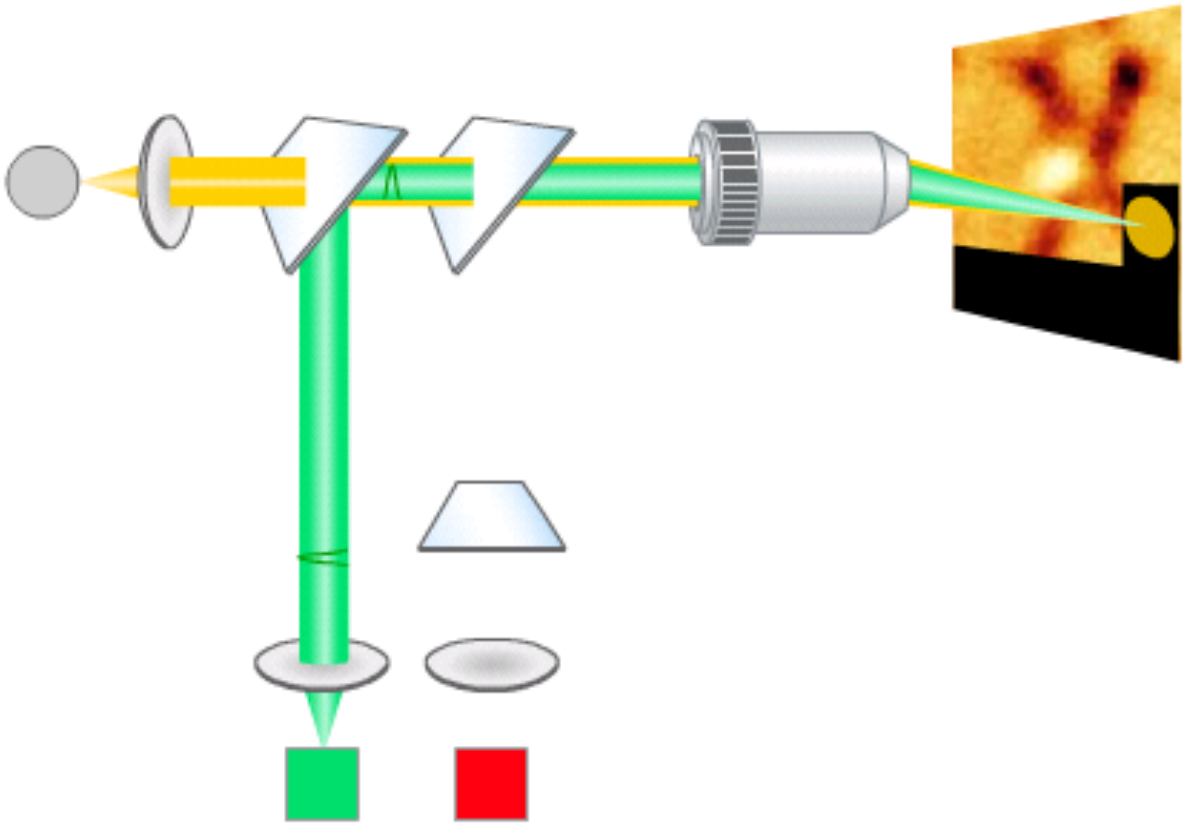
STED microscopy: scanning

Conventional CLSM



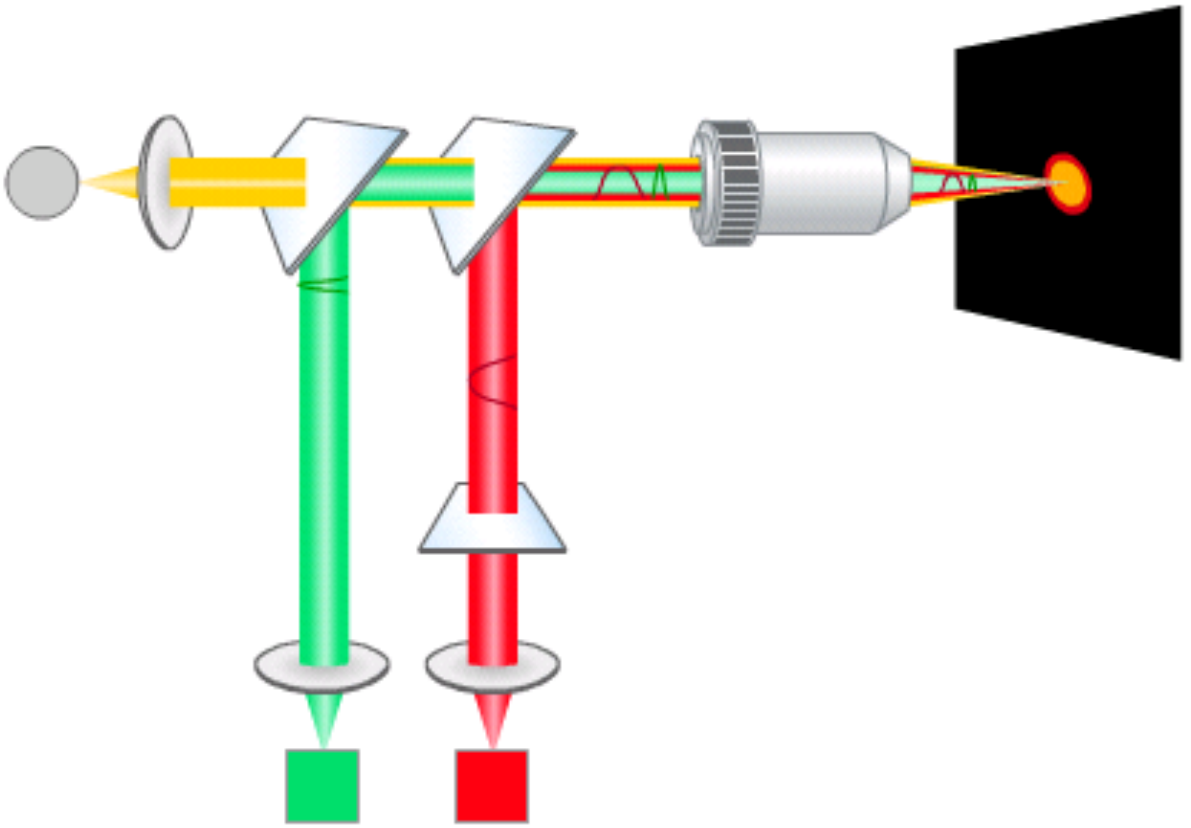
STED microscopy: scanning

Conventional CLSM



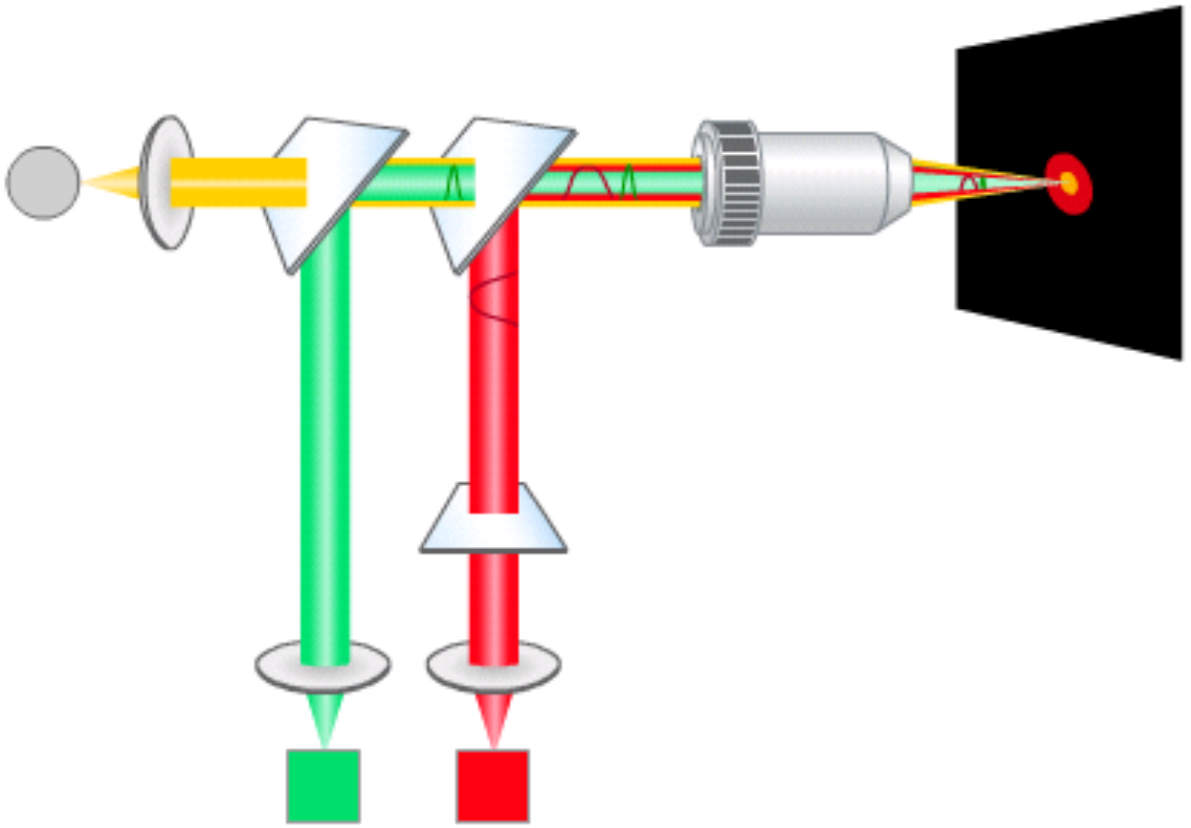
STED microscopy: scanning

STED-CLSM (low power STED)



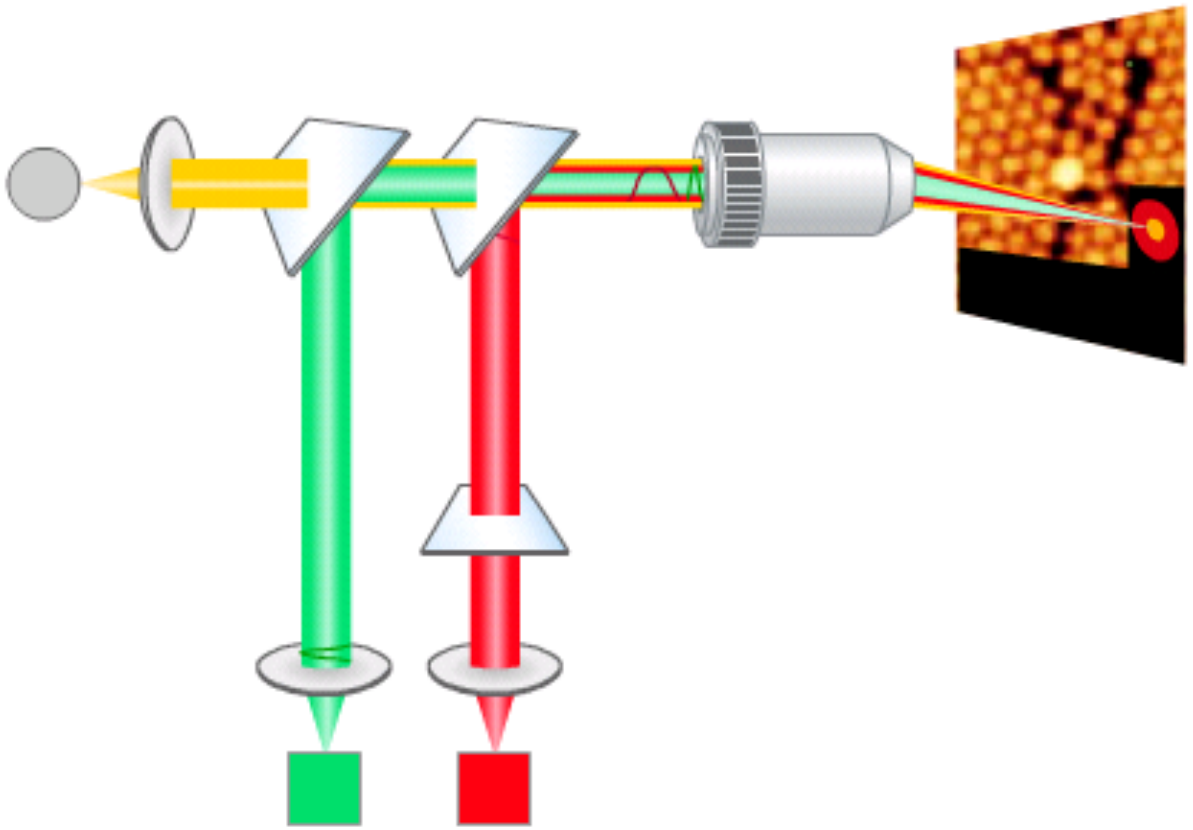
STED microscopy: scanning

STED-CLSM (high power STED)



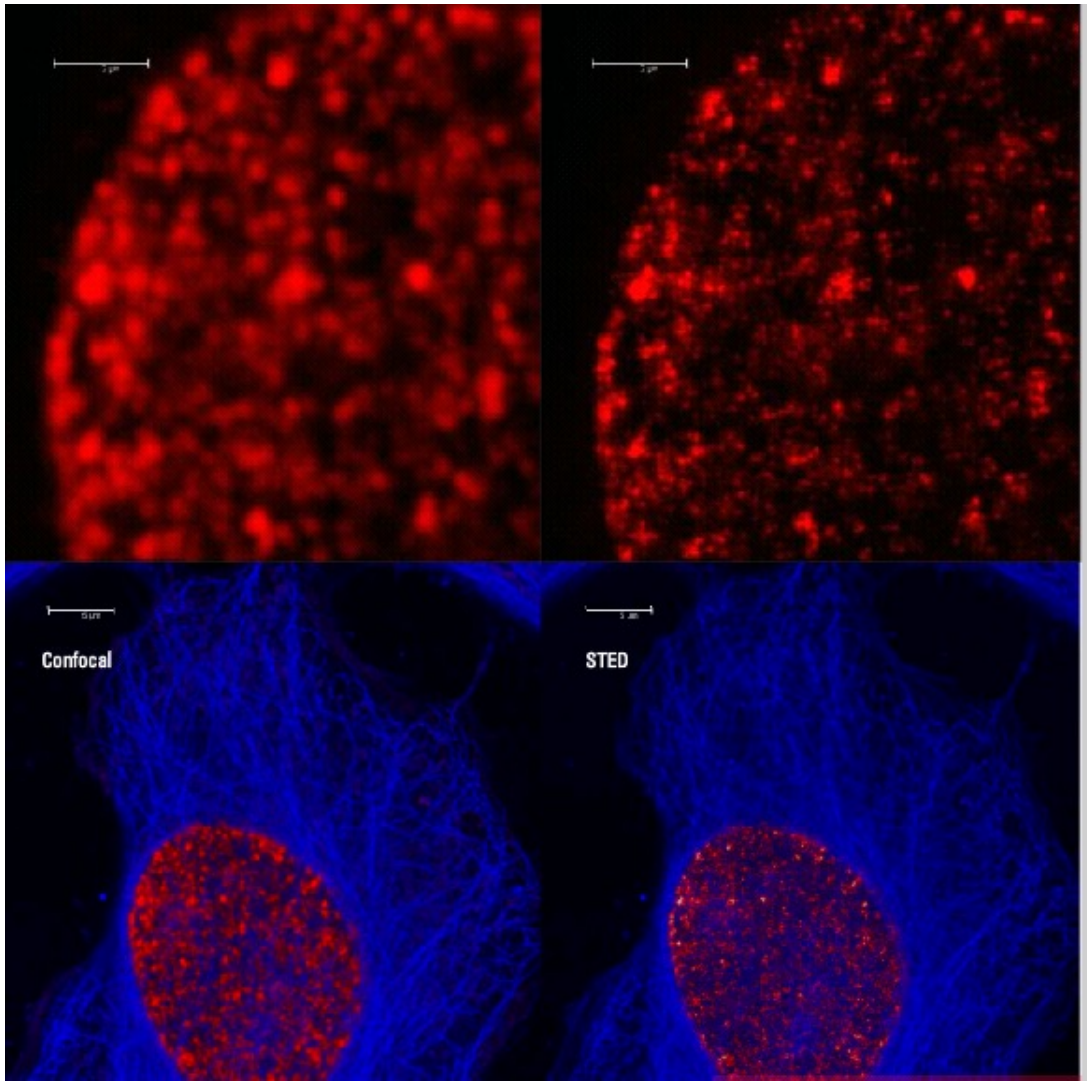
STED microscopy: scanning

STED-CLSM



=> A higher resolution requires more scanning steps

STED microscopy: images



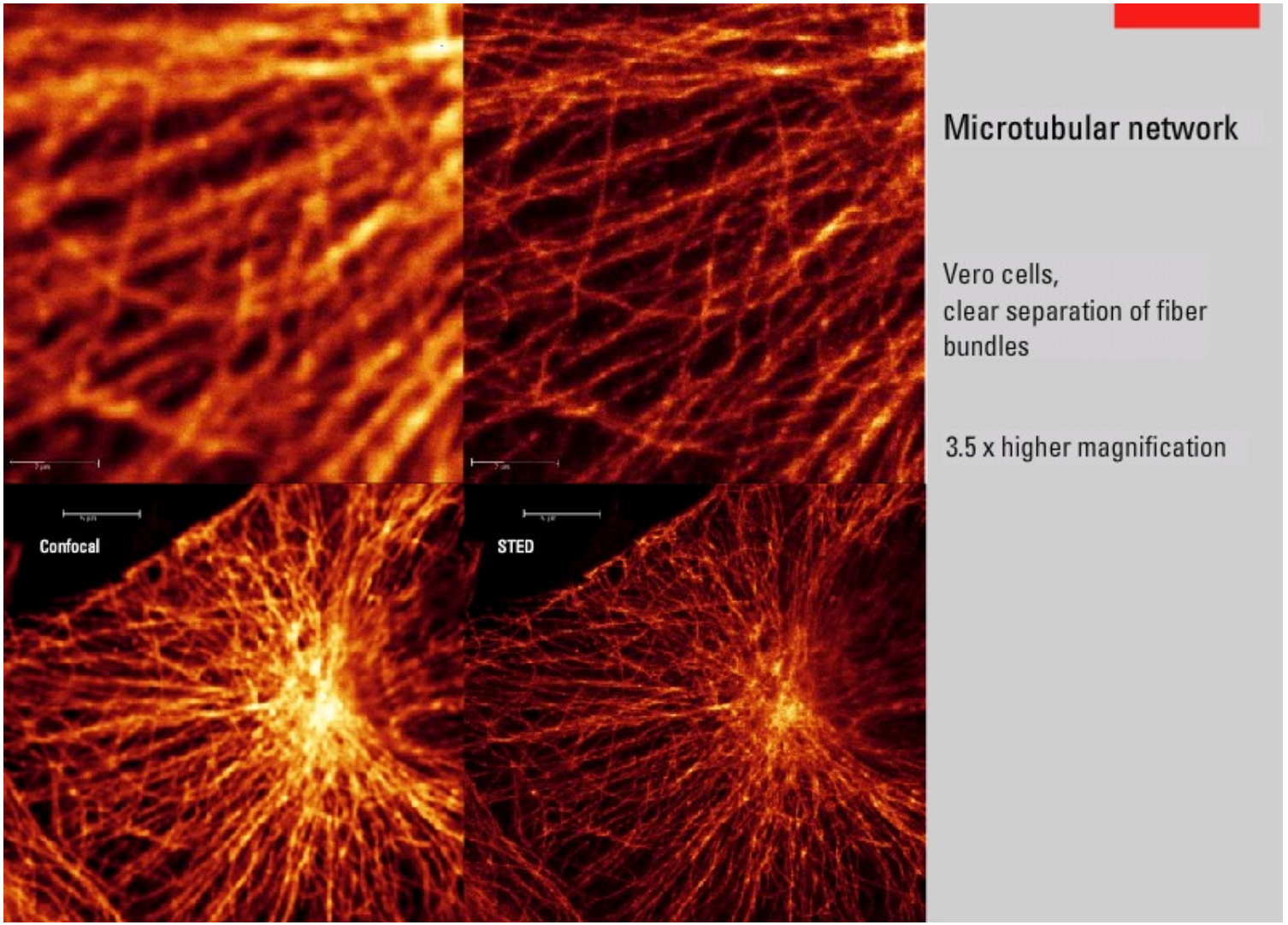
Histone distribution in the nucleus

HeLa cells,
blue: microtubular network (Oregon Green);
red: Histone H3 (ATTO 647N)

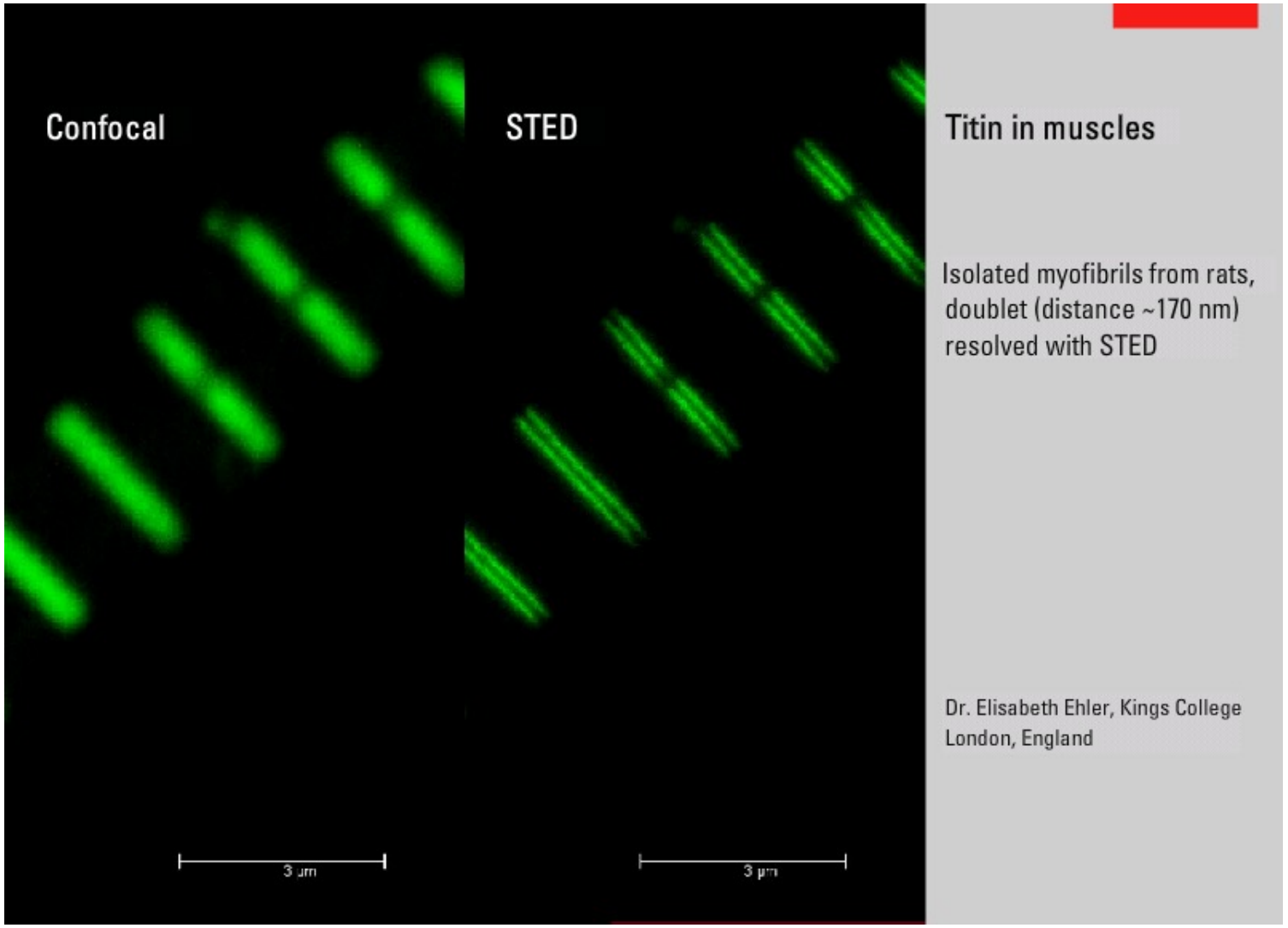
higher magnification

Dr. Brian Bennett, Lake Placid Biochemicals, NY, USA
Secondary antibody from LakePlacid Biochemicals

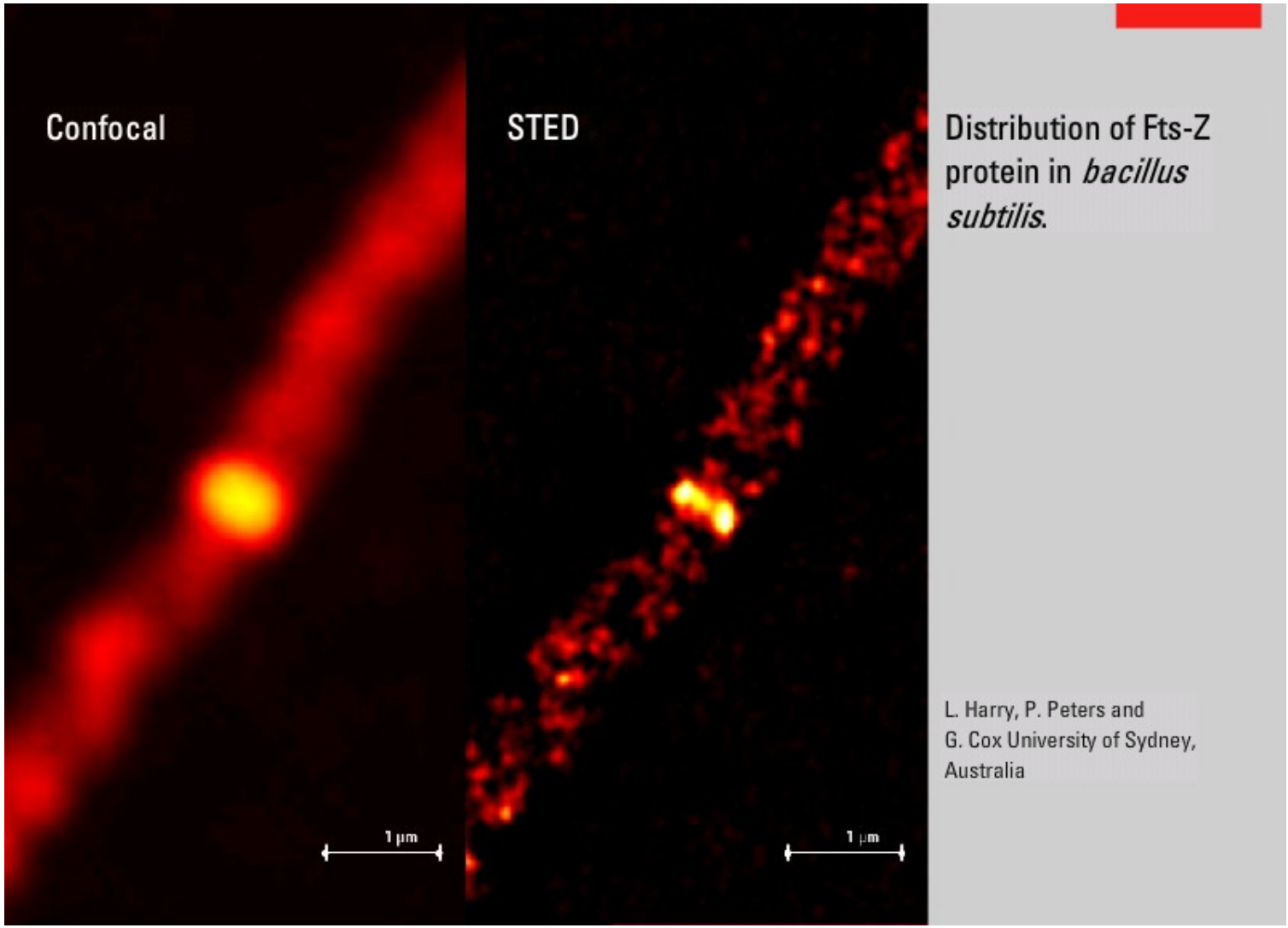
STED microscopy: images



STED microscopy: images

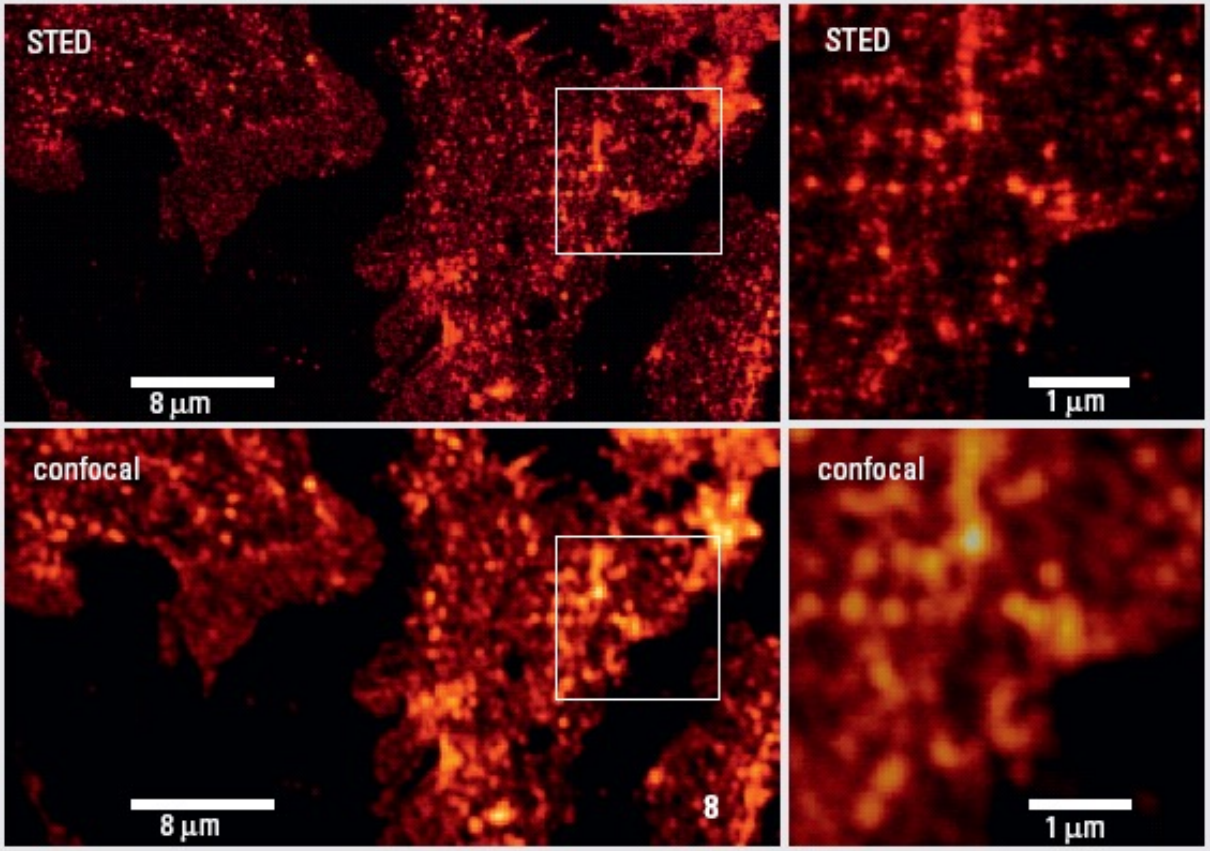


STED microscopy: images



STED microscopy: images

Membrane domains

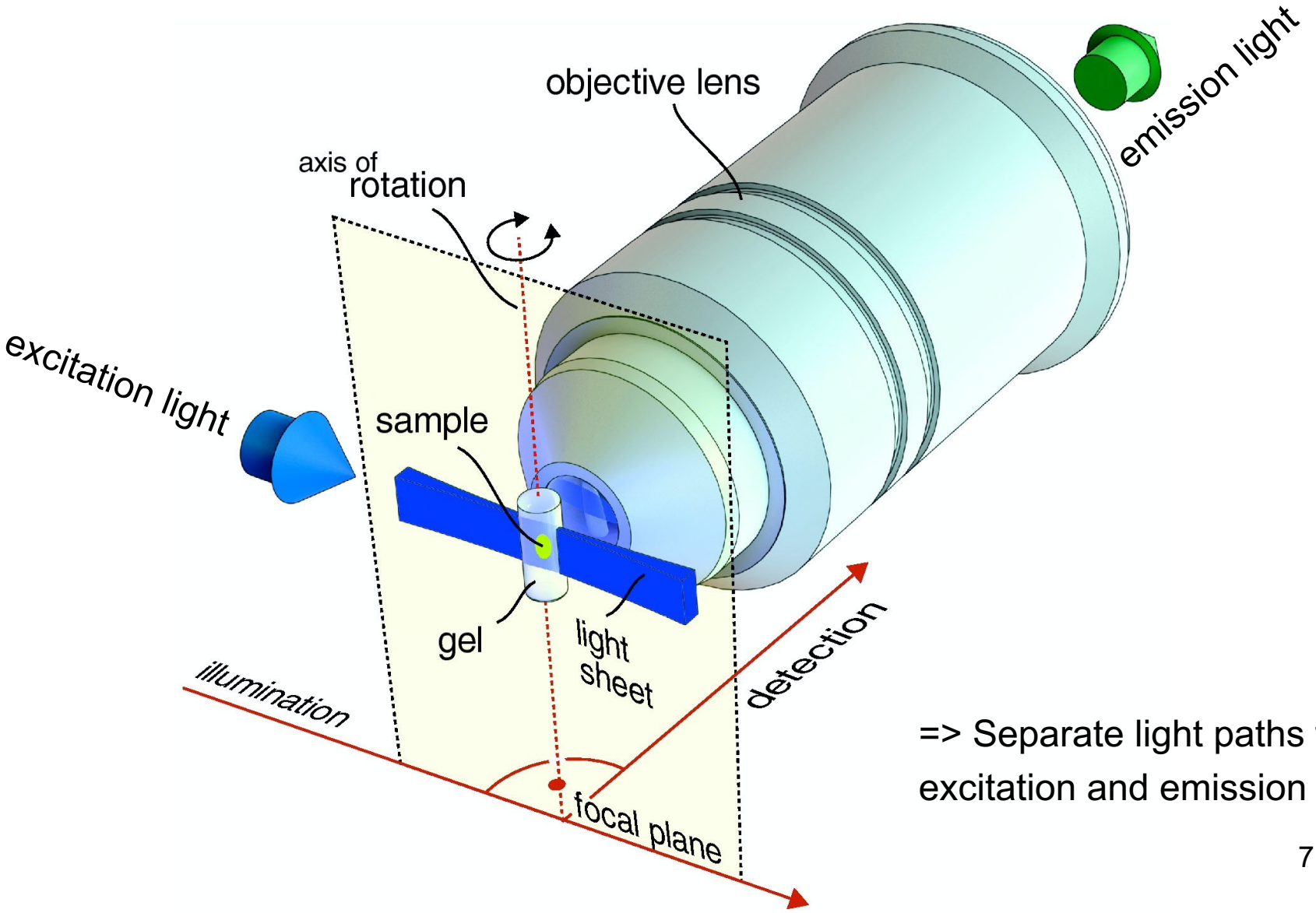


Analyis of the spatial distribution of syntax-in STED STED within the basal plasma membrane of PC12 cells. STED microscopy allowed the py investigation of cluster density and the determination of average cluster sizes of 50 – 60 nm. [Science, Sieber JJ., 2007]

Light Sheet Microscopy

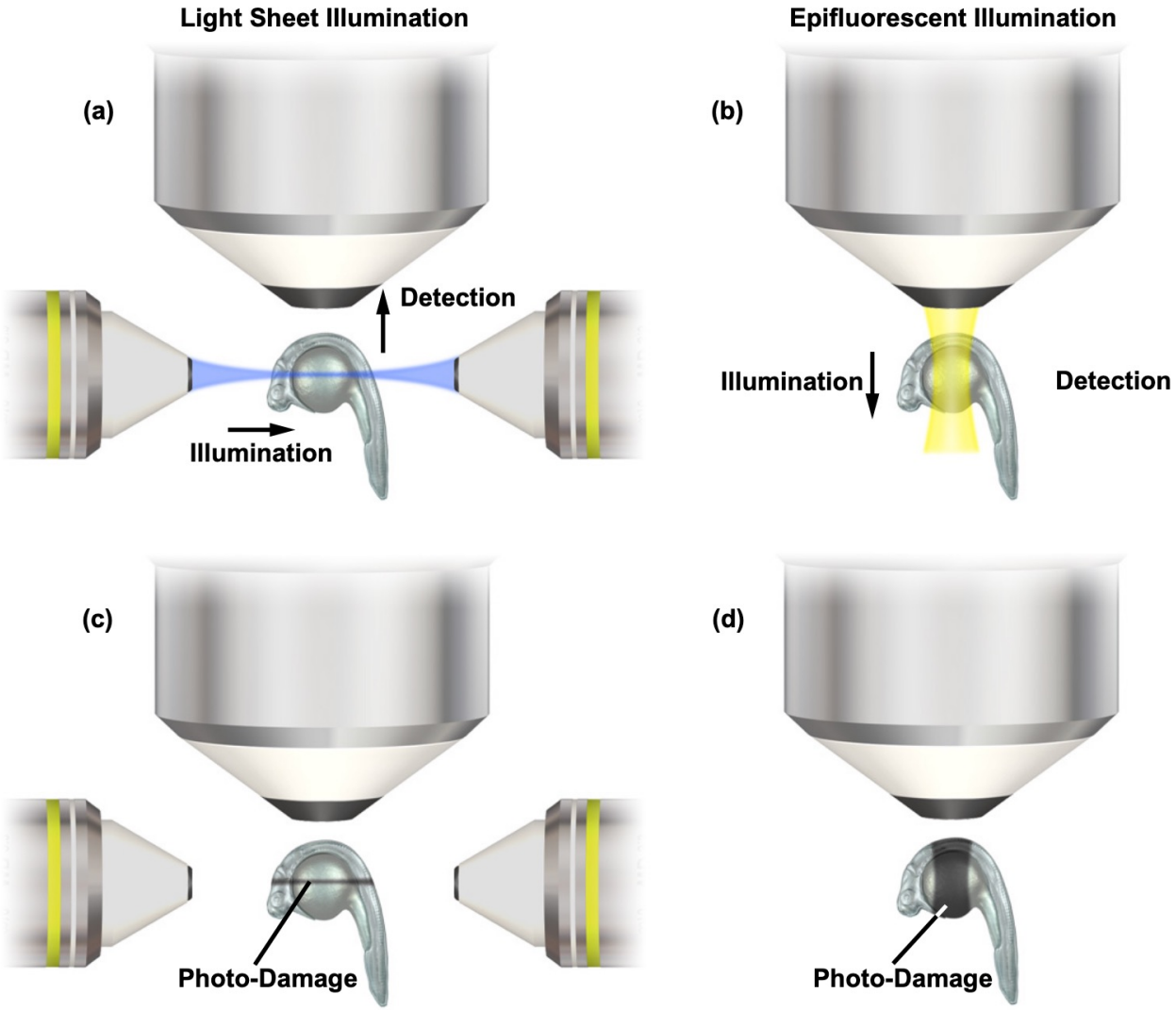
=> based on wide-field microscopy

Light sheet microscopy



=> Separate light paths for excitation and emission light

Light sheet microscopy: planar illumination



=> Separate light paths for excitation and emission light

Light sheet microscopy: advantages

Intrinsic optical sectioning

=> only the focal plane is illuminated

=> avoids photobleaching outside the sheet

Fast image acquisition

=> Whole image taken in a single exposure

(no scanning required, but scanning techniques also exist)

=> more than 100 full images can be taken per second

(depending on camera)

Applicable to larger biological samples

=> 3-D imaging

=> small living organisms

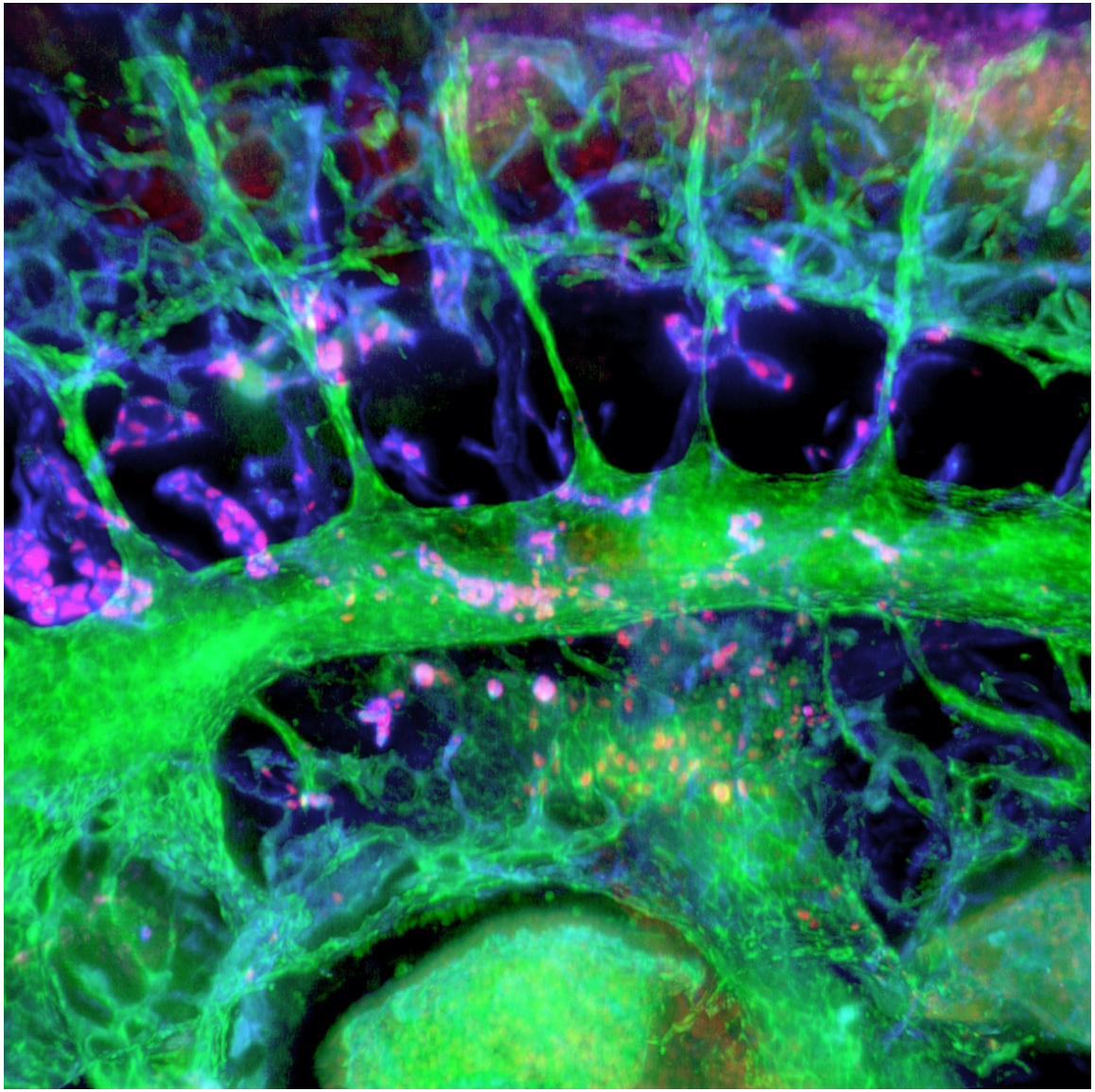
=> embryo development

Light sheet microscopy: images

A sea horse: detection of autofluorescence for imaging

Light sheet microscopy: images

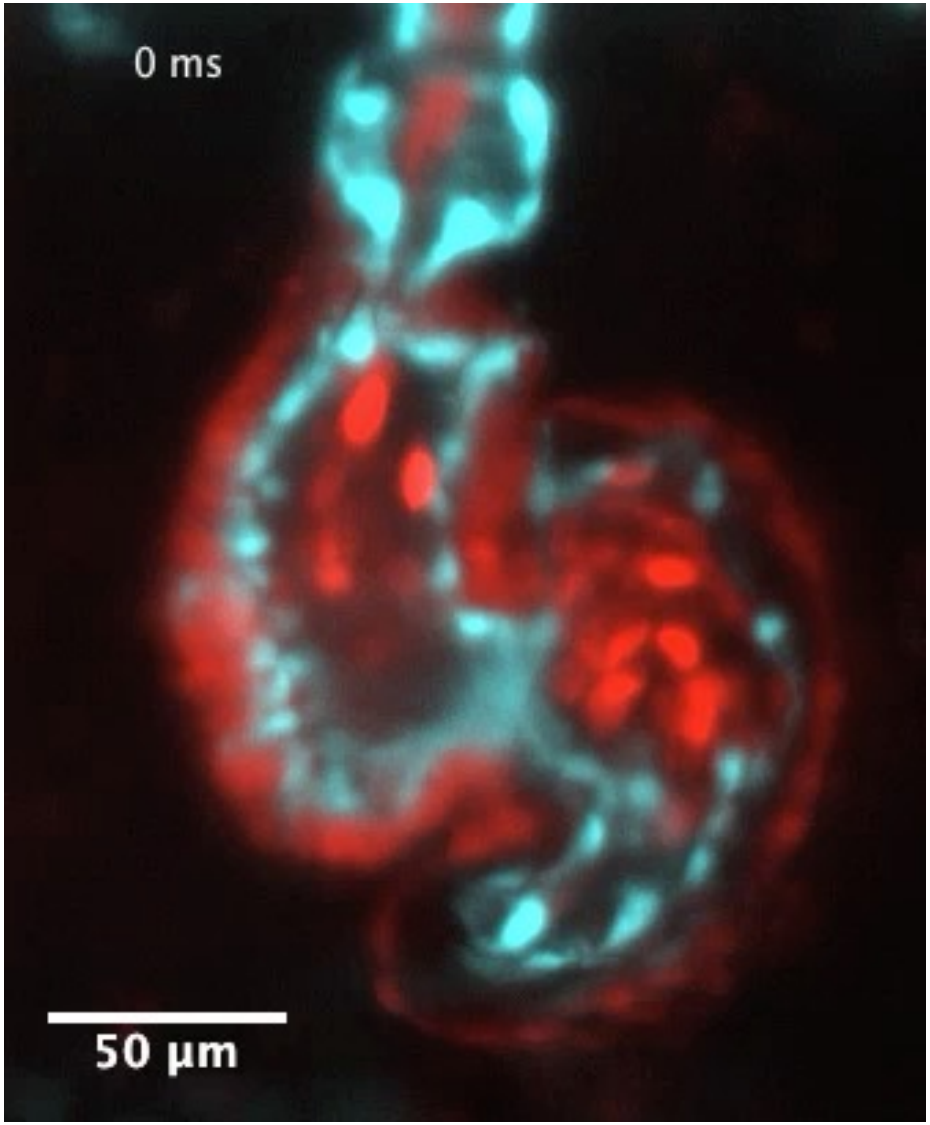
Formation of lymph vessels in a mouse embryo



pink: progenitor cells
green: aorta
blue: vein

Light sheet microscopy: video

Heartbeat of zebrafish



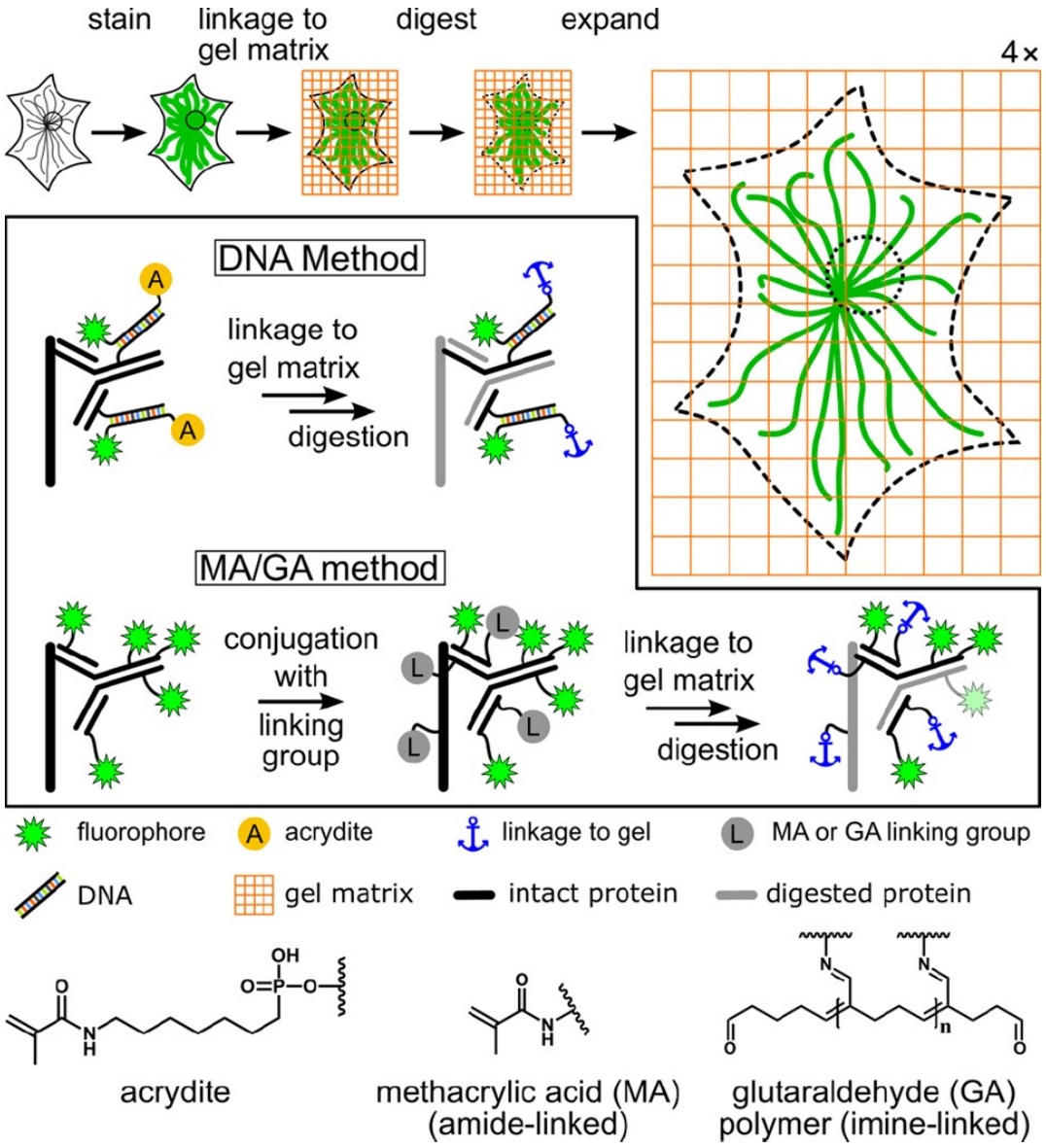
red:
(1) red blood cells
(2) myocard
(heart muscle)

cyan: endocard
(inner lining of heart)

Expansion Microscopy

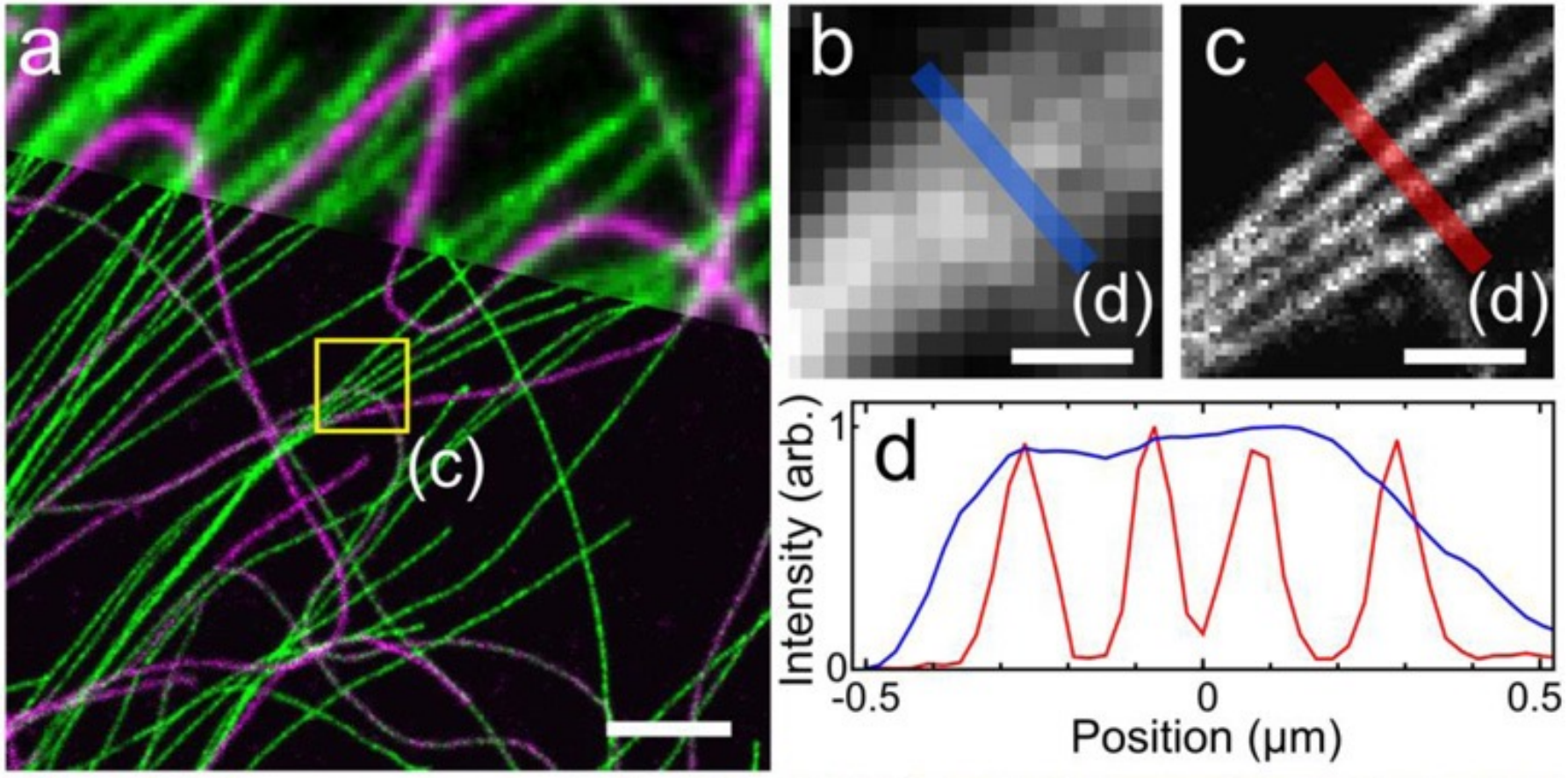
=> Blows up sample before imaging

Expansion microscopy



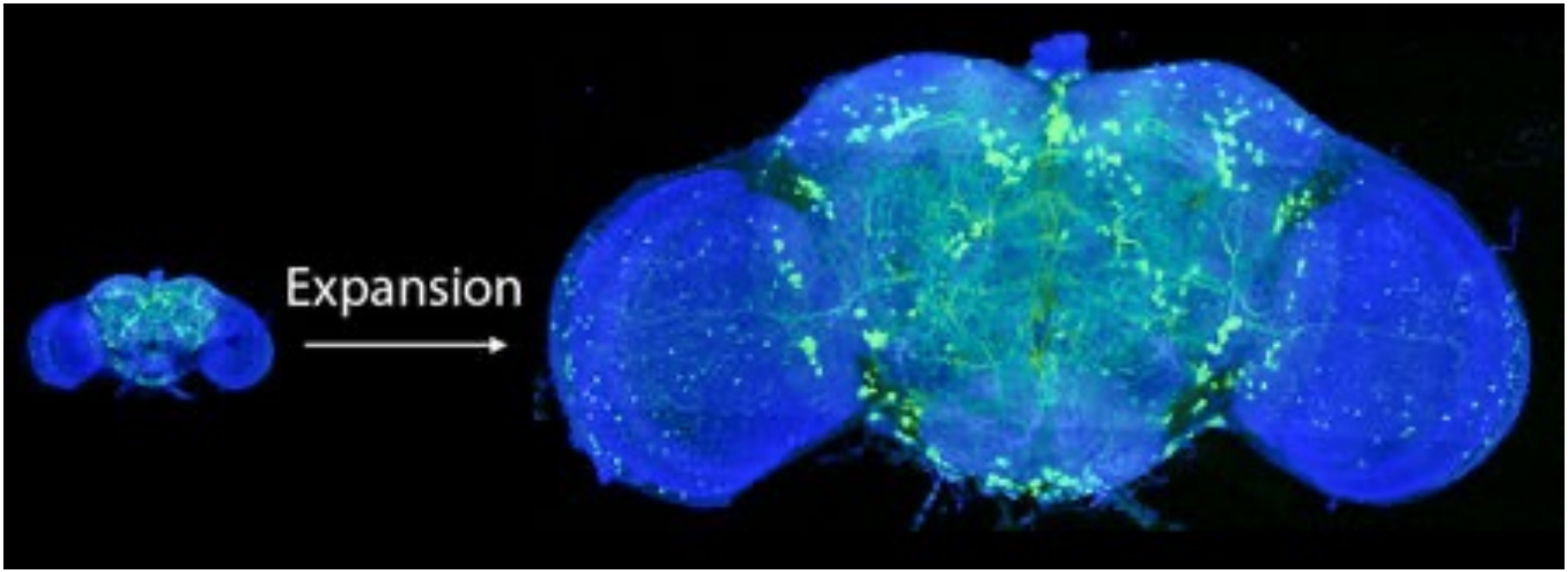
Expansion microscopy: images

Different types of microtubles



Expansion + light sheet microscopy: images

Brain of fruit fly *Drosophila*:
Mapping of more than 40 million synapses in 62 hours



4x expanded
=> 60 nm resolution

Gao et al. (2019) Science 363, 245

Expansion + light sheet microscopy: video

Labeling of neuronal cells in the brain