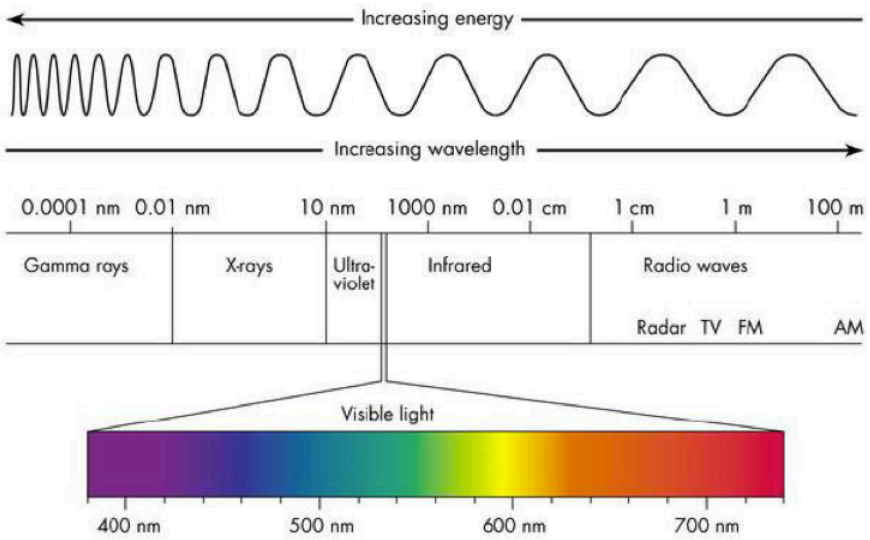
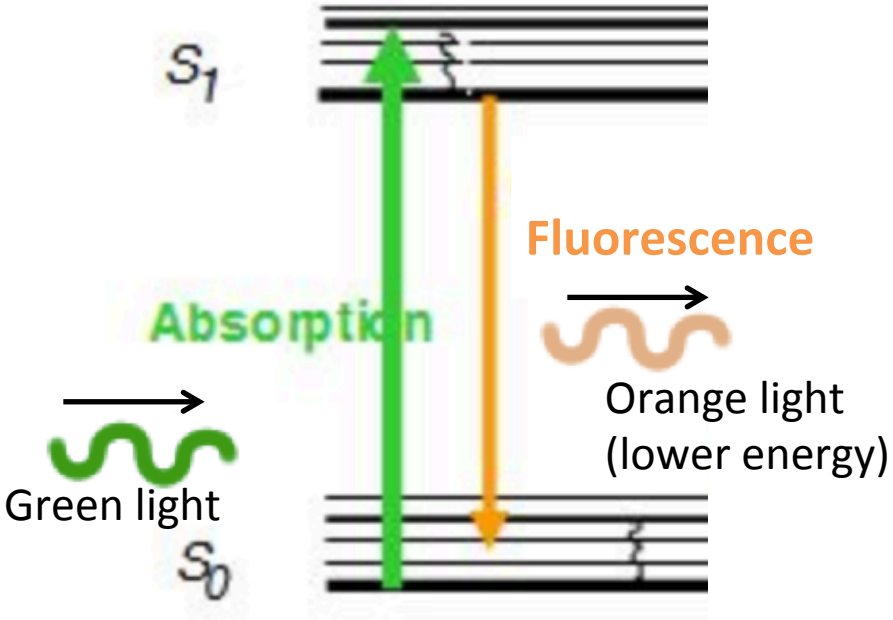
VISUALISING THE NANOWORLD:

OPTICAL MICROSCOPY
AND SUPER RESOLUTION MICROSCOPY

Fluorescence

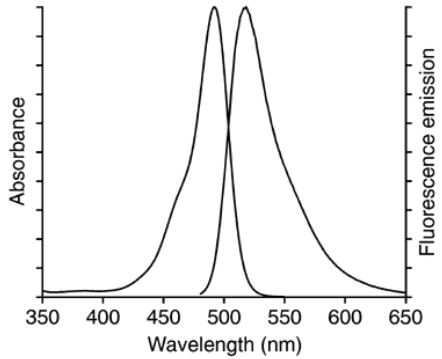
Emission of light by a substance as the results of the absorption of light of lower wavelength.





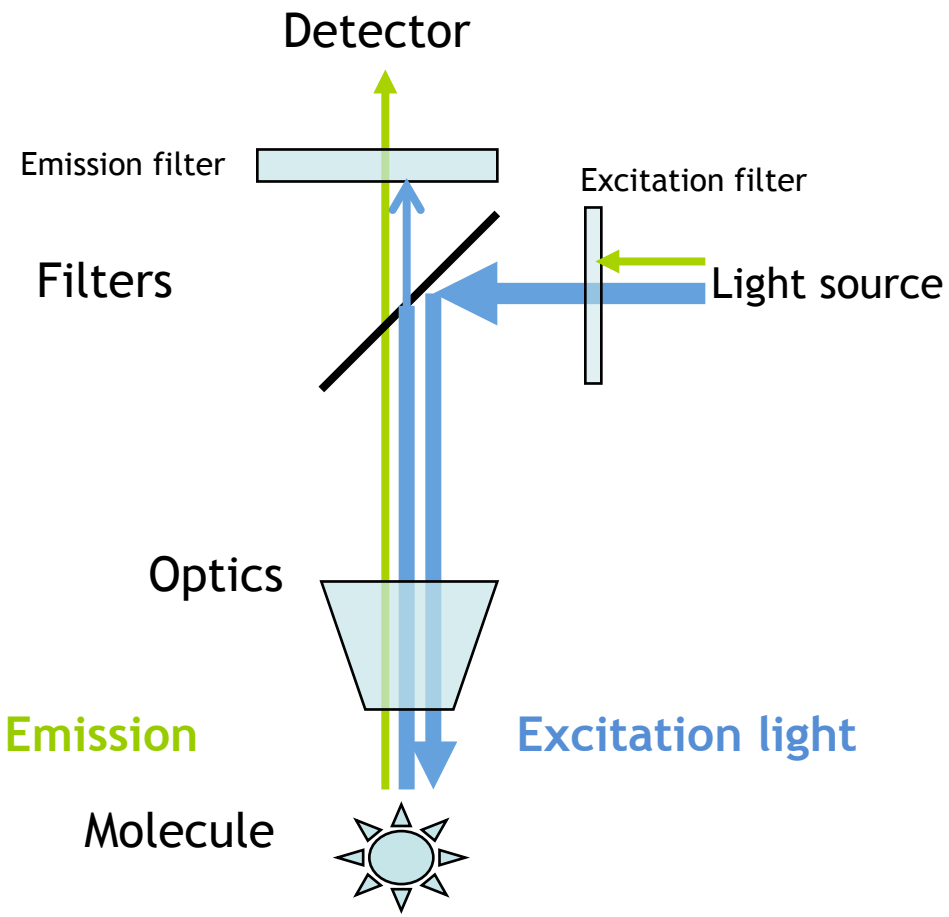
$$E = \frac{hc}{\lambda}$$

http://www.bristol.ac.uk/
http://quarknet.fnal.gov



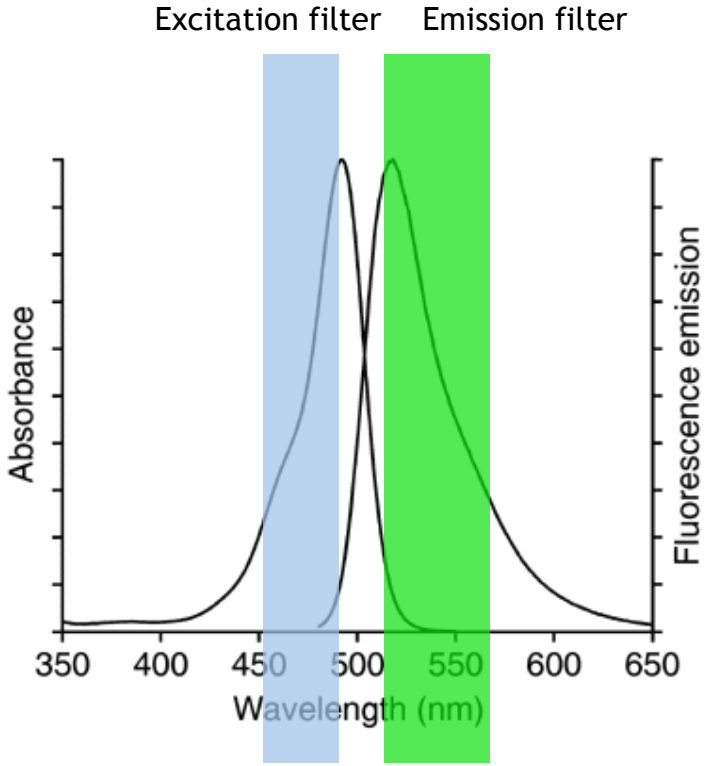
Example of a fluorescent dye excitation and emission spectra

Basic experimental setup – the optical microscope



Excitation filter to cut out any unwanted excitation light, essentially to cut out light from the excitation source that overlaps with the transmission window of the emission filter.

Emission filter to cut out the excitation light.



-> Observe biomolecules, cell components…

Fluorescent markers (dyes)

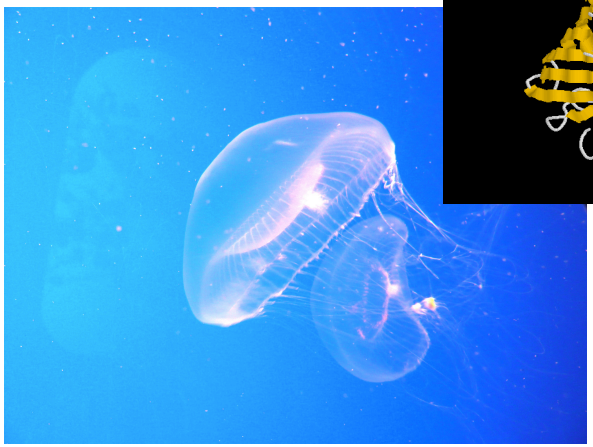
- GREEN FLUORESCENT PROTEIN

- ORGANIC FLUOROPHORES

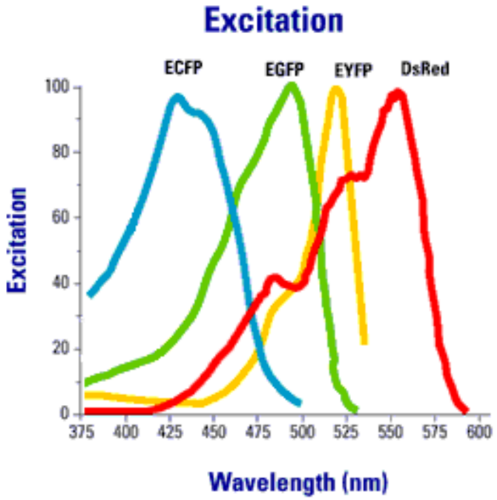
- QUANTUM DOTS

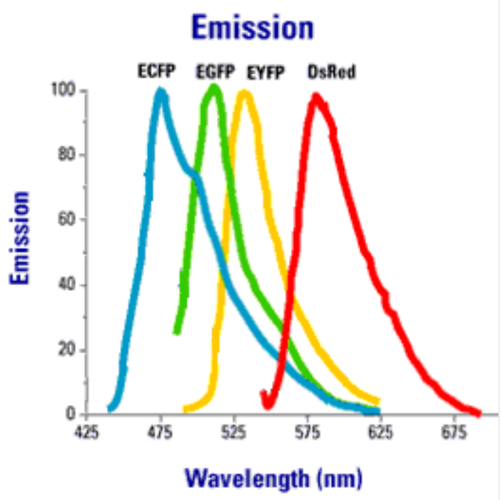
Fluorescent markers

GREEN FLUORESCENT PROTEIN



- 1960s: isolated from jellyfish Aequorea victoria.
- Since then: many engineered mutants have been developed with better spectral characteristics compared to the wild type GFP (increased fluorescence, better photostability) .
- Nobel Prize in Chemistry 2008
- They are produced biologically. Their sequence is known and their DNA can be combined with other proteins to form fusion proteins.





http://microscopy.duke.edu

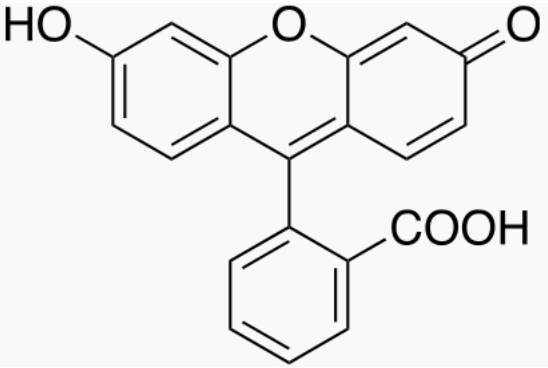
There are also other variants with other colors:
CFP, YFP, RFP

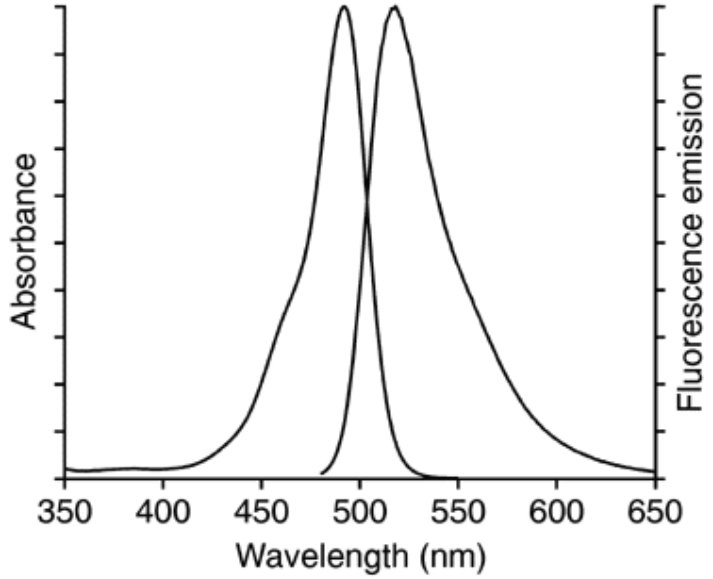
DsRed: protein from *Discosoma* (coral)

Fluorescent markers

ORGANIC FLUOROPHORES

- Ex: Fluorescein, Rhodamine, Cy3, Cy5, TRITC, FITC etc…)

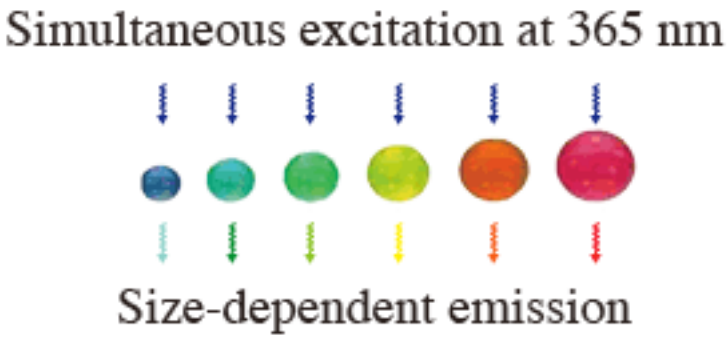


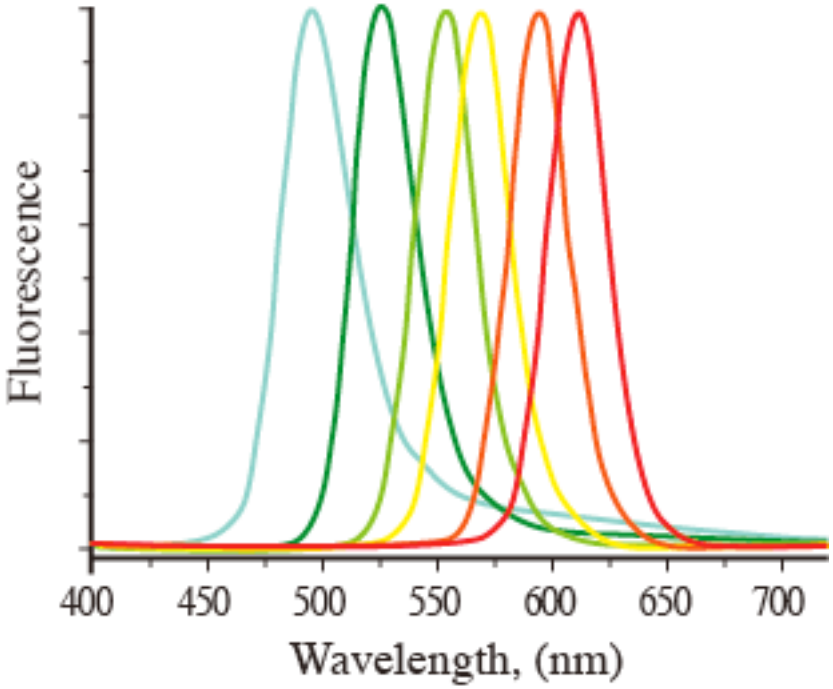


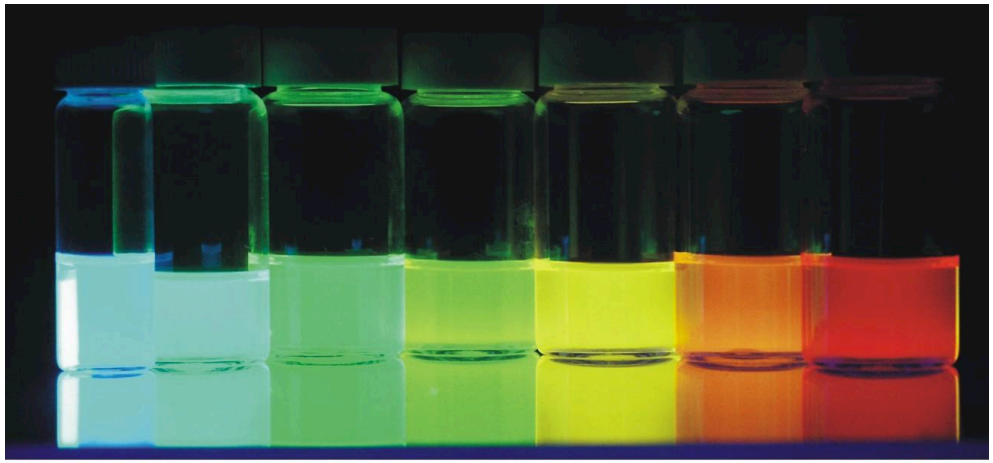
- They can be attached to proteins (including antibodies)

Fluorescent markers

QUANTUM DOTS: semiconductor nanocrystals-excitons confined in 3 dimensions.







Usually made in toxic materials but coatings are preventing any material leakage/ dissolution in bio media (in addition to improving the optical properties of the QD such as yield and stability.)

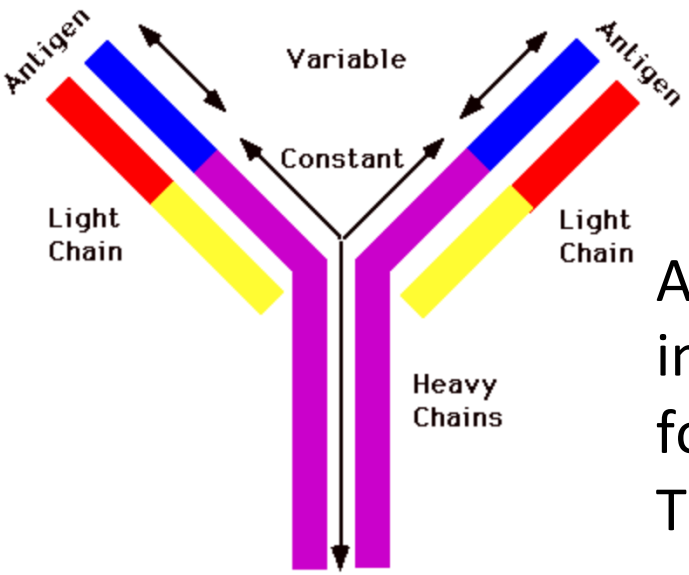
Suitable for long term studies

How to label specifically certain parts (proteins, biomolecules) of the cell fluorescently???

⟶ Immunofluorescence

Immunofluorescence

Using the molecular recognition between an antibody and an antigen to label fluorescently specific biomolecules



http://www.apsubiology.org

Antigen=**Anti**body **Gen**erator

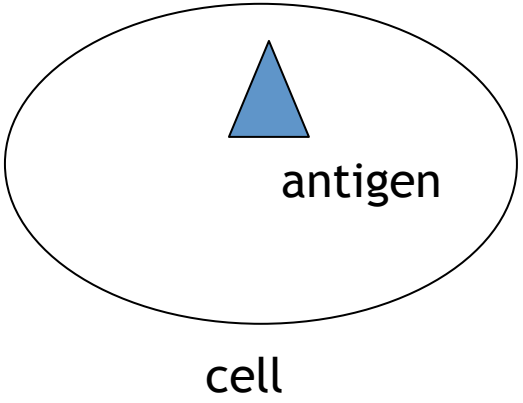
Epitope = part of the antigen that is recognized by the immune system

Antibodies are proteins synthesized by immune cells: they are used to identify (label) foreign objects in the body.

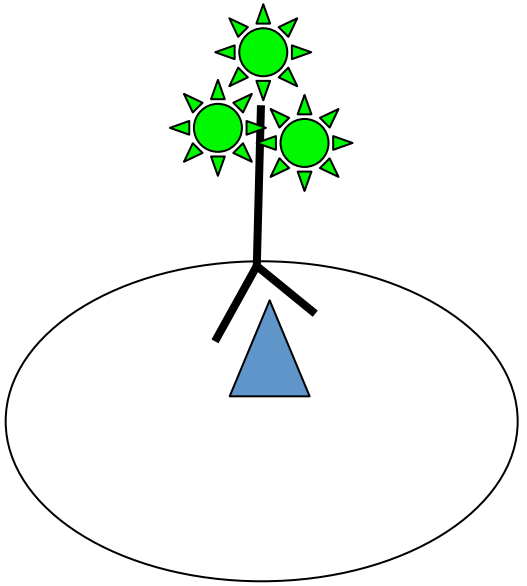
They can be made against nearly all molecules: Animals produce antibodies in response to antigens recognized as foreign by their immune system.

Antibodies can be labelled with fluorophores and be used to visualize subcellular structures.

Direct immunofluorescence



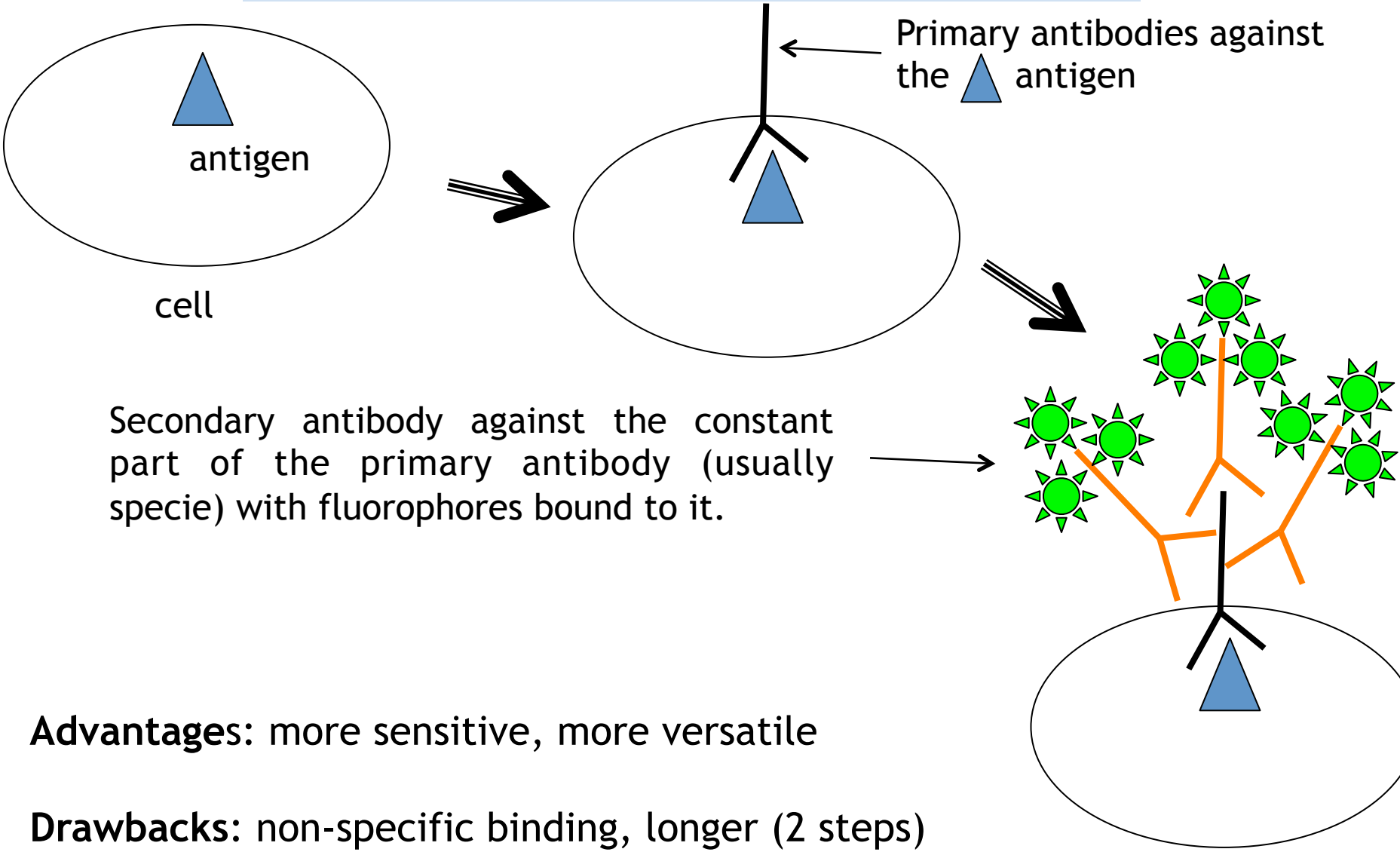
Primary antibodies against the  antigen
with fluorophores bound to it



Advantages: fast method, less non specific binding

Drawbacks: low sensitivity- limited by the number of fluorophore that can be bound to the primary antibody

Indirect immunofluorescence



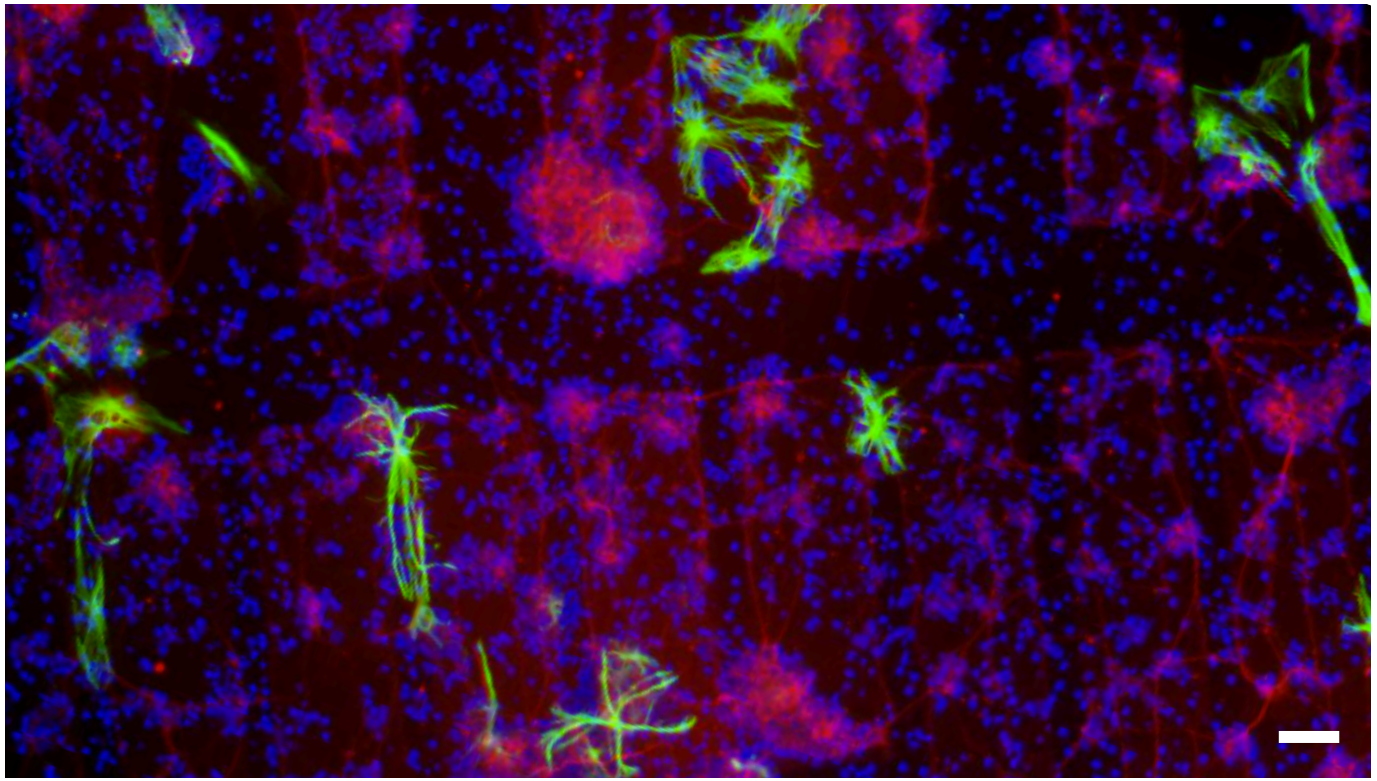
Secondary antibody against the constant part of the primary antibody (usually specie) with fluorophores bound to it.

Advantages: more sensitive, more versatile

Drawbacks: non-specific binding, longer (2 steps)

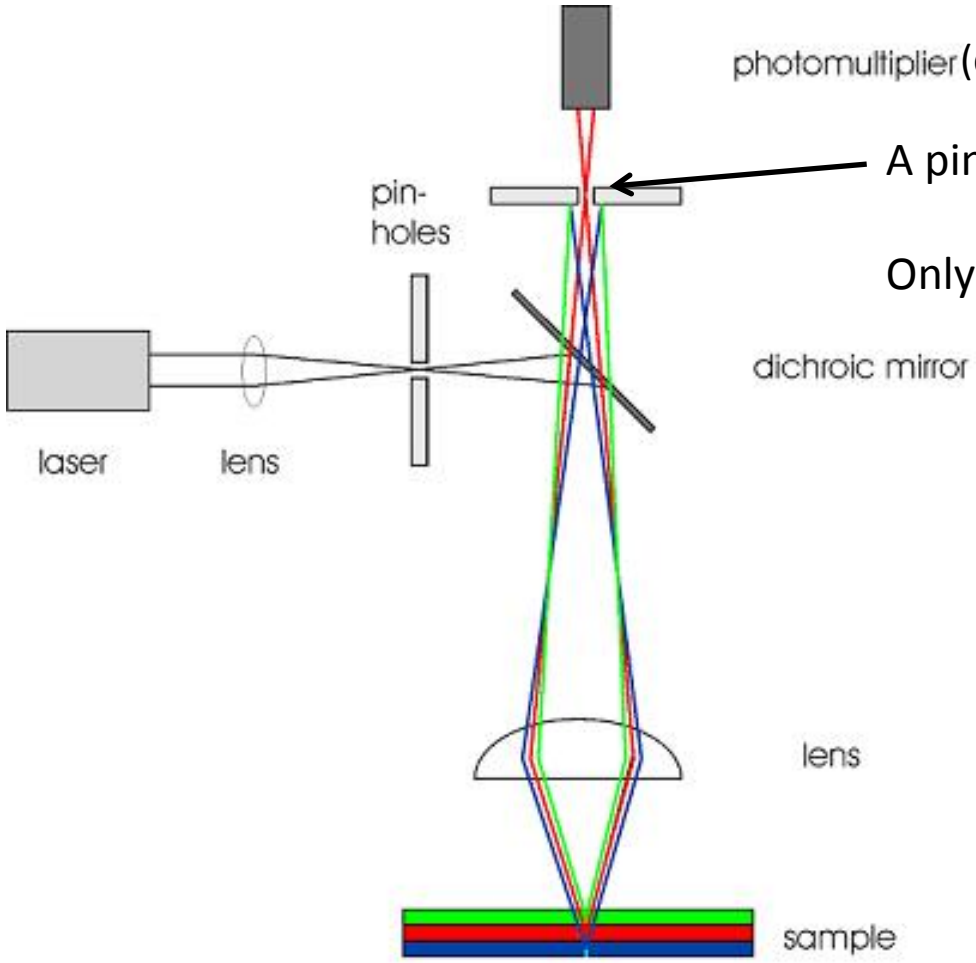
Fixate cells using paraformaldehyde (preserve the tissues by crosslinking proteins)
Permeabilize the cells (detergeant)
Prevent non-specific binding (main source = hydrophobic interactions):

-> Add bovine serum albumine or serum (not from the AB target species!) before and during incubation with antibodies.



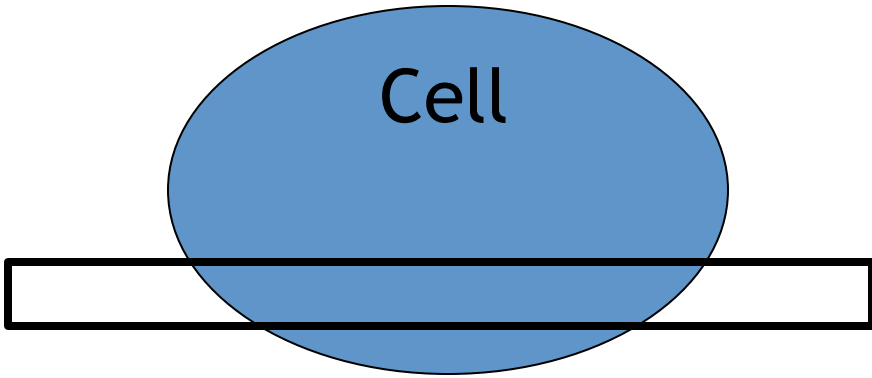
Retinal cells stained using DAPI (blue),
GFAP (rabbit polyclonal green) and DyLight-488 donkey anti-rabbit
β-tubulin III (mouse monoclonal, red) and DyLight-549 donkey anti-mouse

Confocal microscopy



A pinhole eliminates the out-of-focus light.

Only a thin slice of the sample is imaged at a time.



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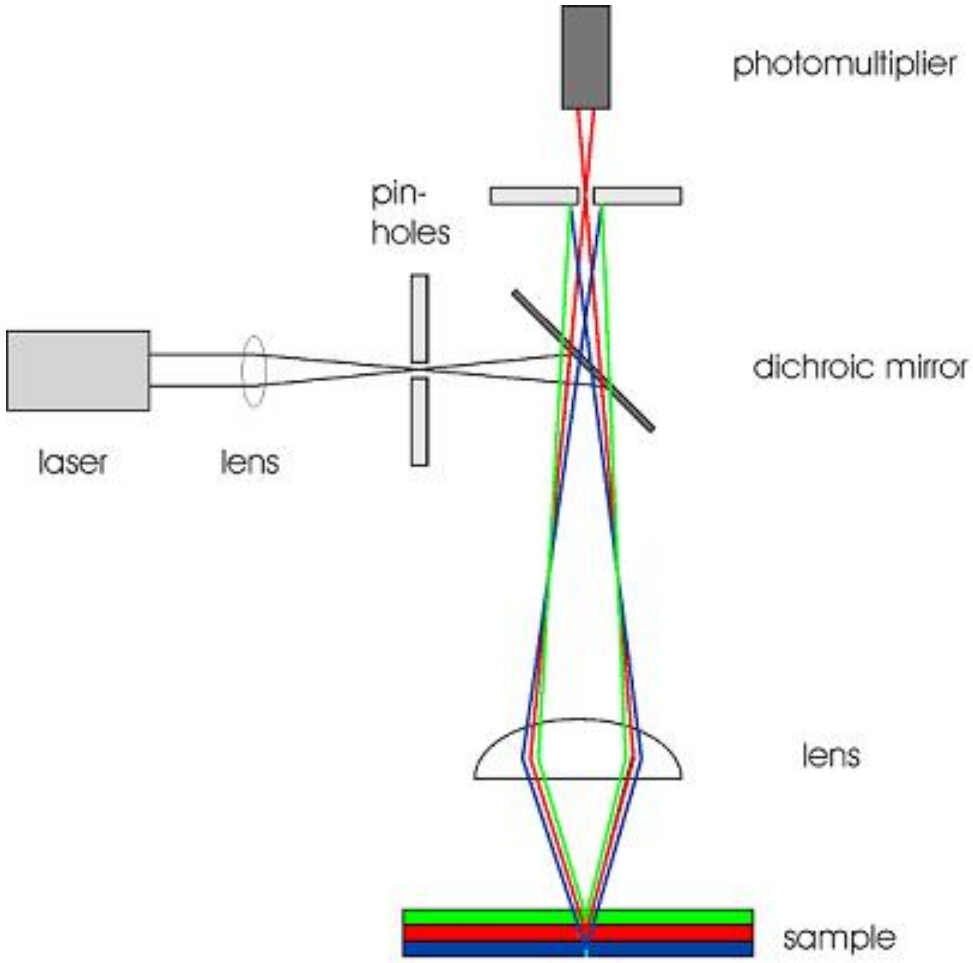
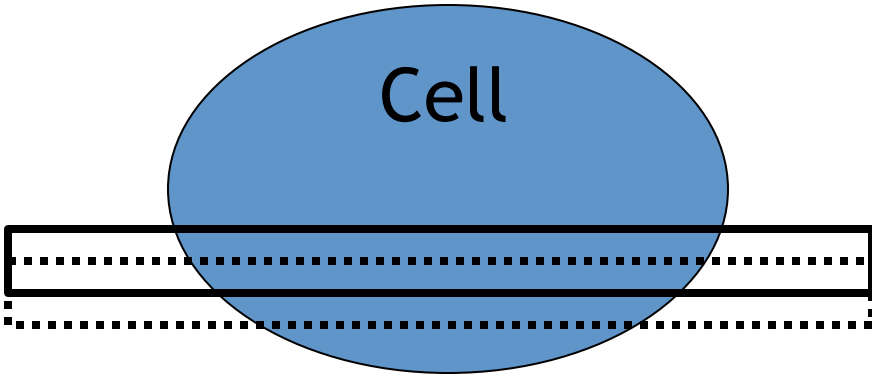
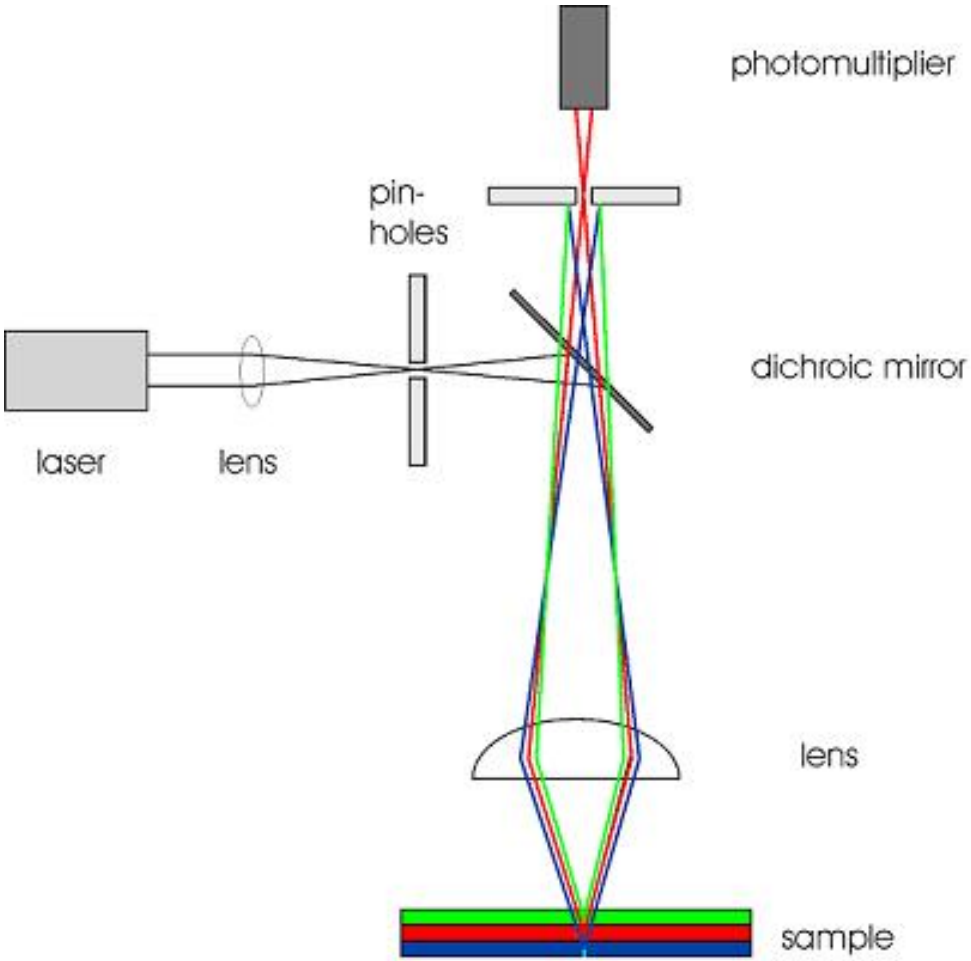
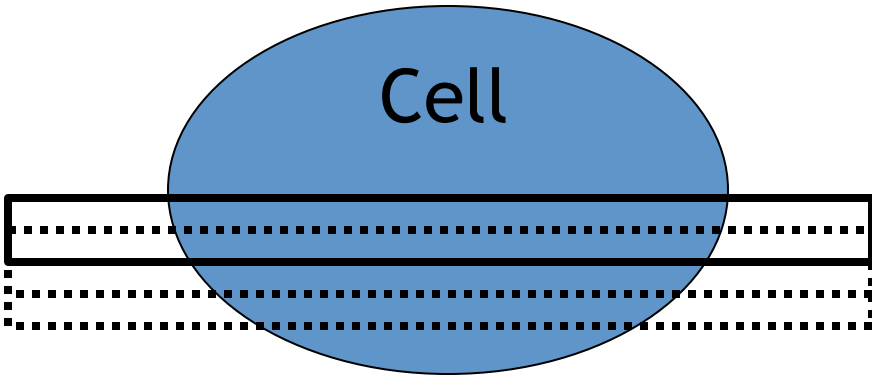




Confocal microscopy

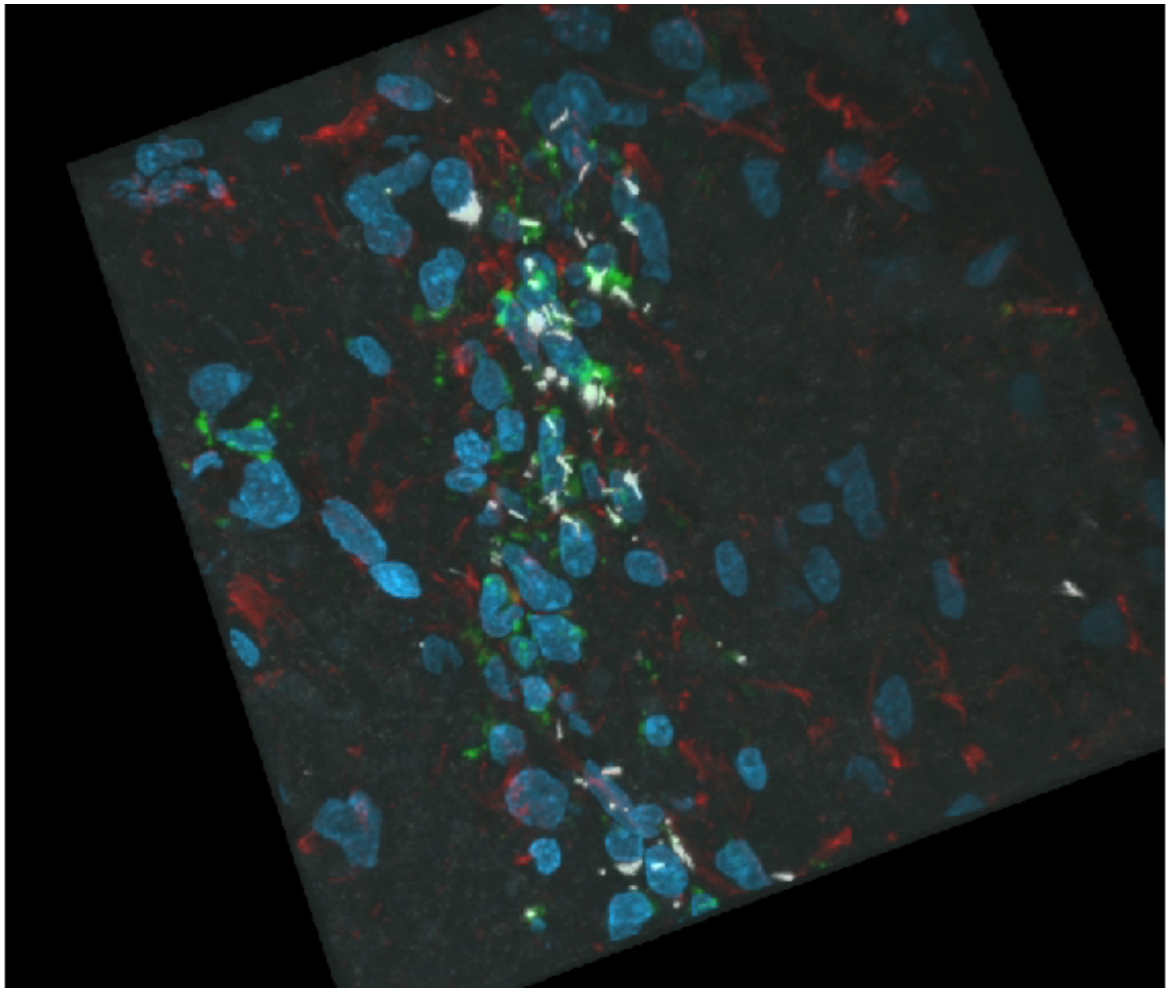




http://www.uni-mainz.de/FB/Chemie/AK-Janshoff/Illustrationen/scheme_confocal_microscopy.jpg

3D reconstruction from confocal images

Nanowires in the rat brain
(green: ED1, red: astrocytes, white: nanowires, blue: cell nuclei)



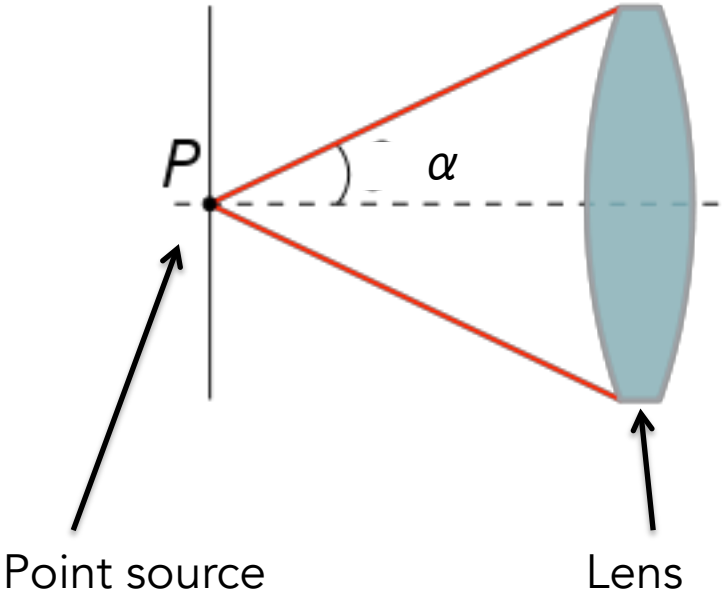
Linsmeier et al. Nano Lett. 2009

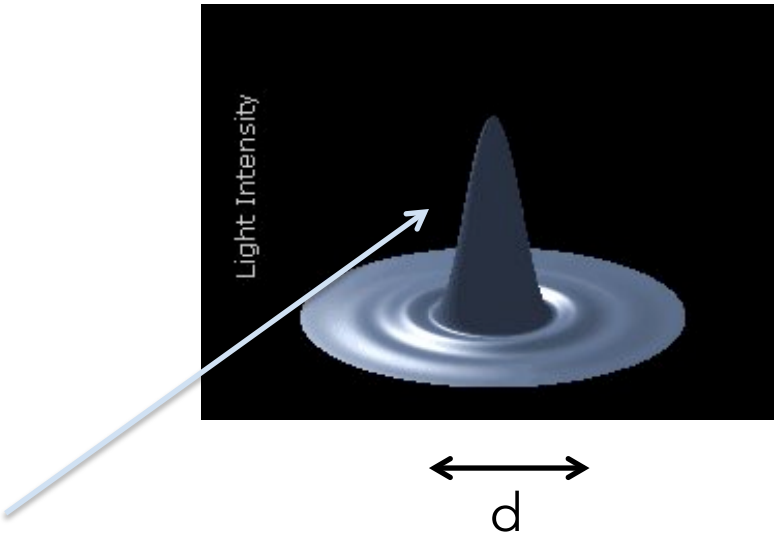
Optical microscopy: Abbe resolution limit

Due to diffraction a point source of light appears as a finite object described by the point-spread function (PSF).

$$d \sim \frac{\lambda}{2n\sin\alpha} = \frac{\lambda}{2NA} \sim 250nm$$

NA: numerical aperture of the objective
n=c/v: refractive index of the medium
α: half-angle of the maximum cone of light that can enter the lens.





PSF: three-dimensional diffraction pattern of light

Abbe resolution limit

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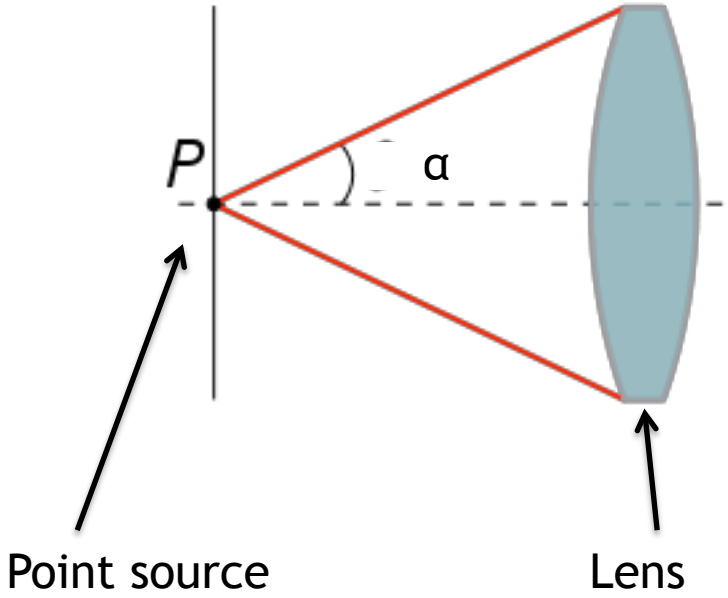
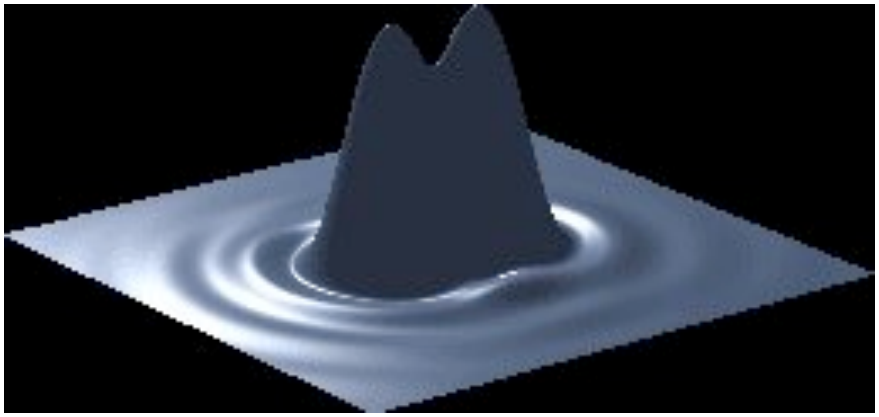




It can be difficult to resolve 2 objects that are close to one another

Super resolution light microscopy

STORM "Stochastic Optical Reconstruction Microscopy"

= Randomly switch on and off individual fluorophores and localize their position.

(Xiaowei Zhuang- Harvard University)

STORM

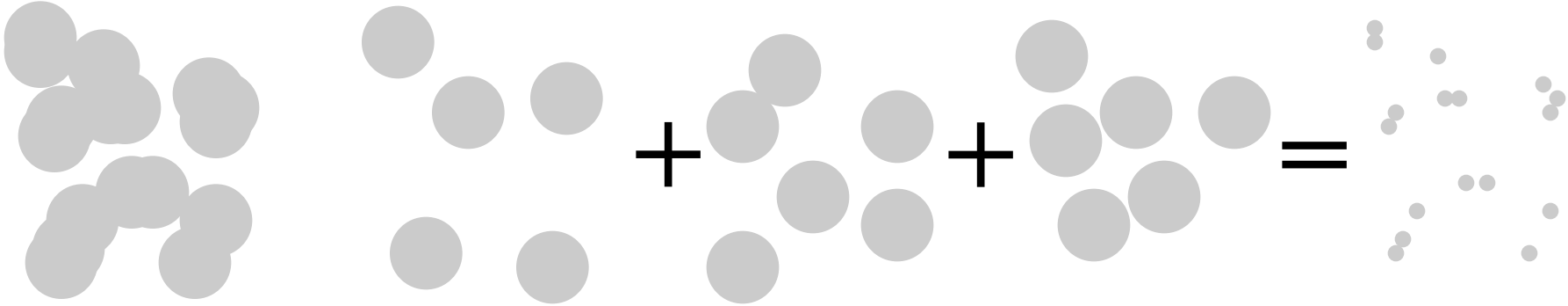
Poor resolution

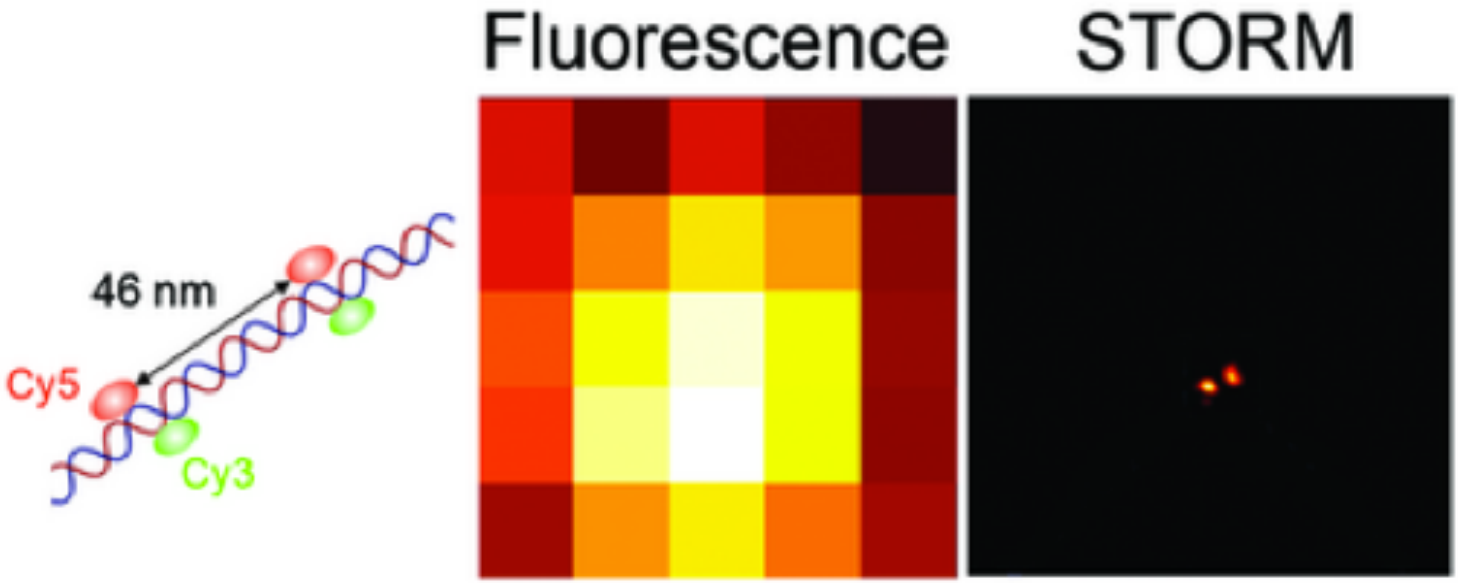
STORM

Poor resolution

Good *positioning* uncertainty

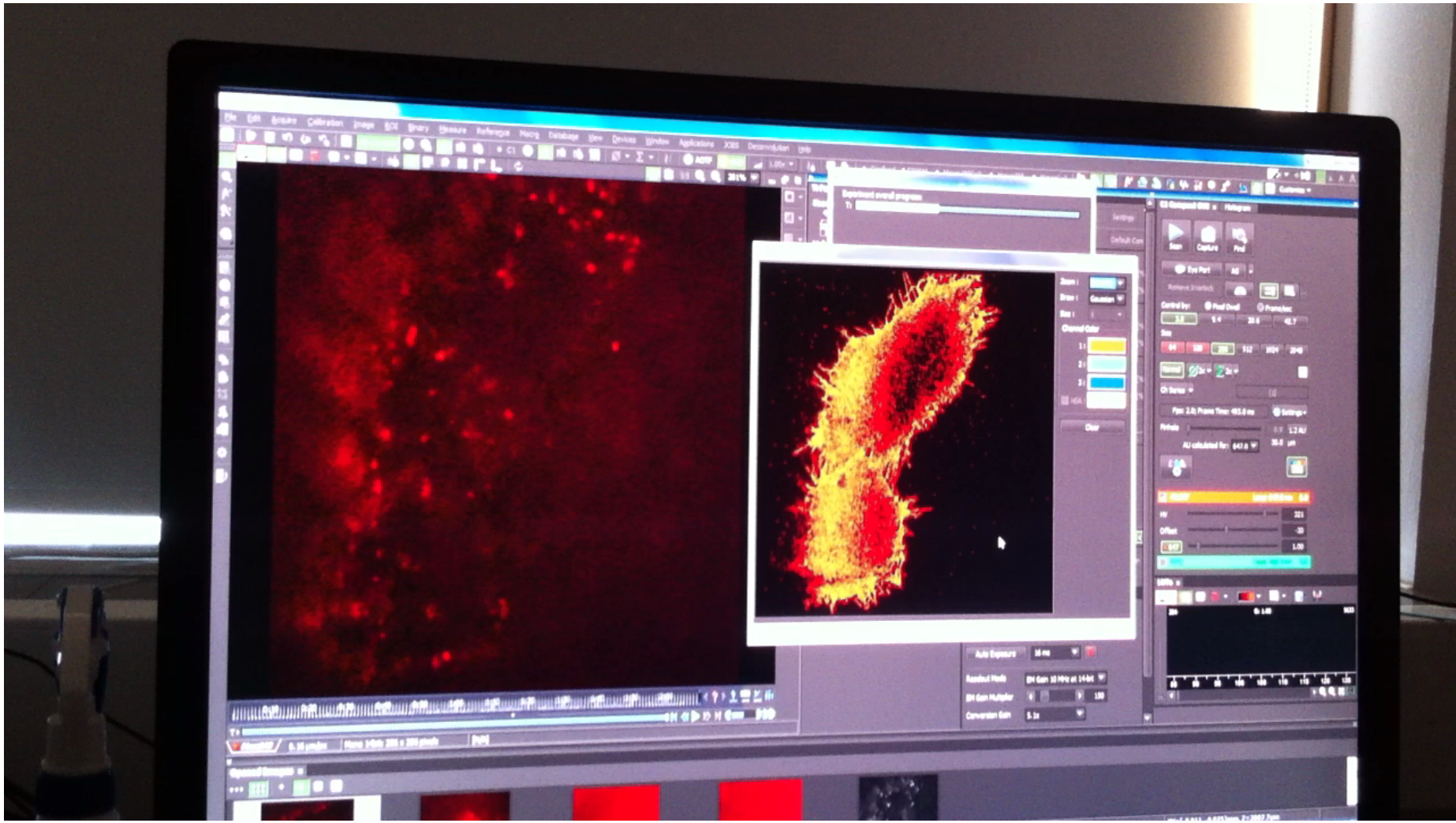
High *resolution*





Xiaowei Zhuang's group-Harvard University

STORM



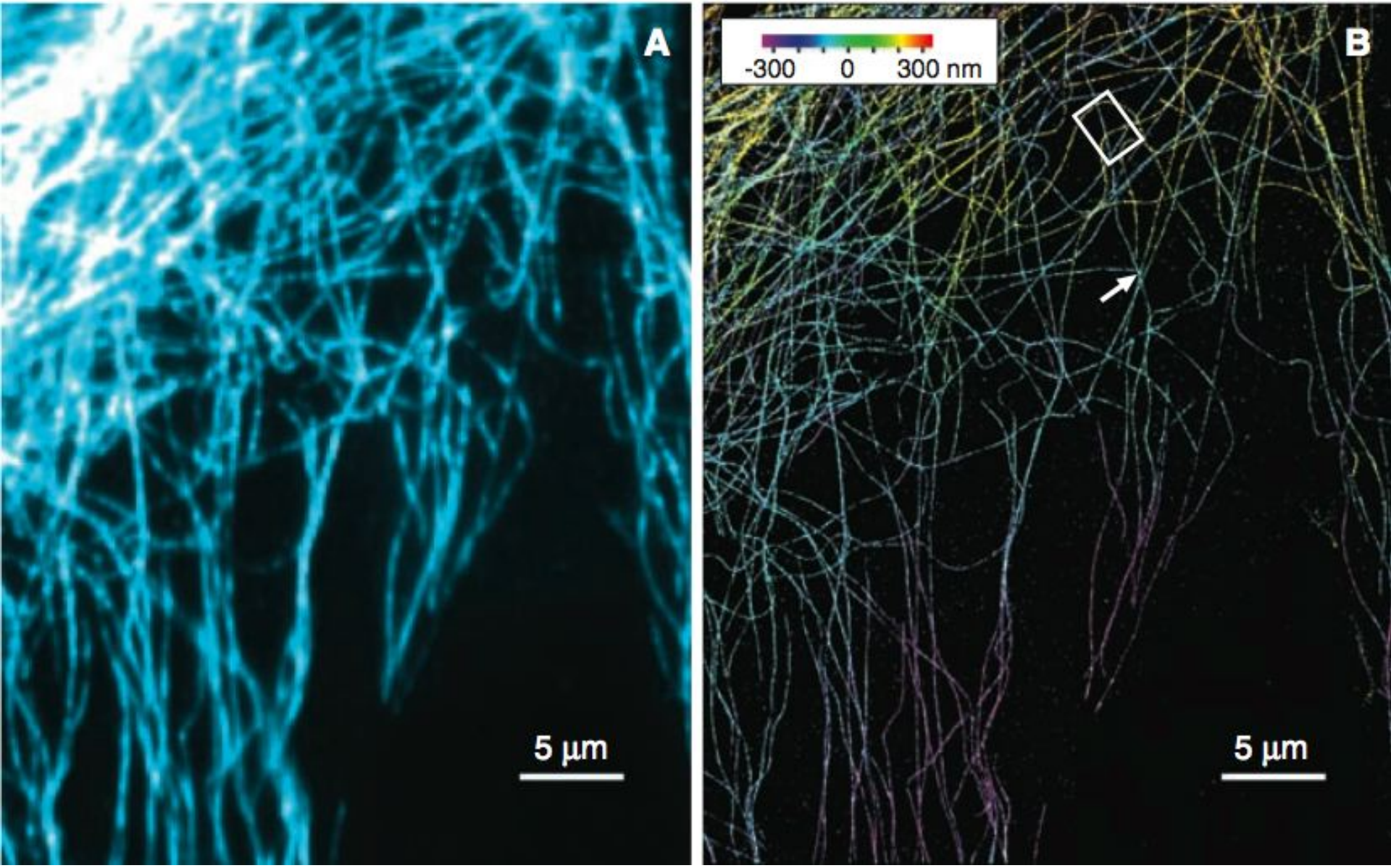
STORM

Confocal

~250nm resolution

STORM

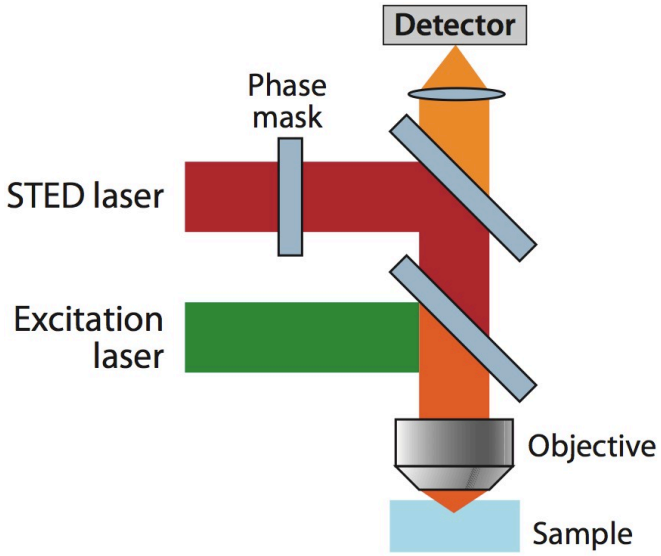
~25nm resolution



microtubules

Bo Huang et al, Science 2008

STED (Stimulated emission depletion) microscopy

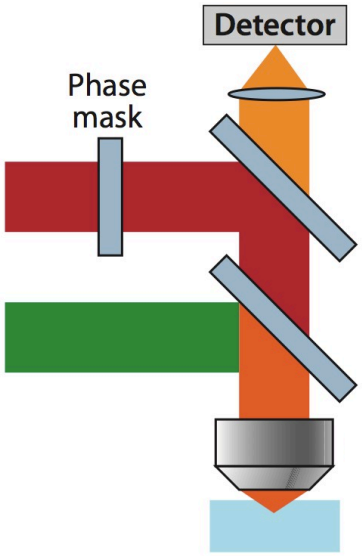


Confocal microscope with two parallel laser beams. The excitation beam excites the fluorophores and the other beam forces the emission to take place in a color that is not detected through stimulated emission. The result is a smaller point spread function and thereby an improved resolution.

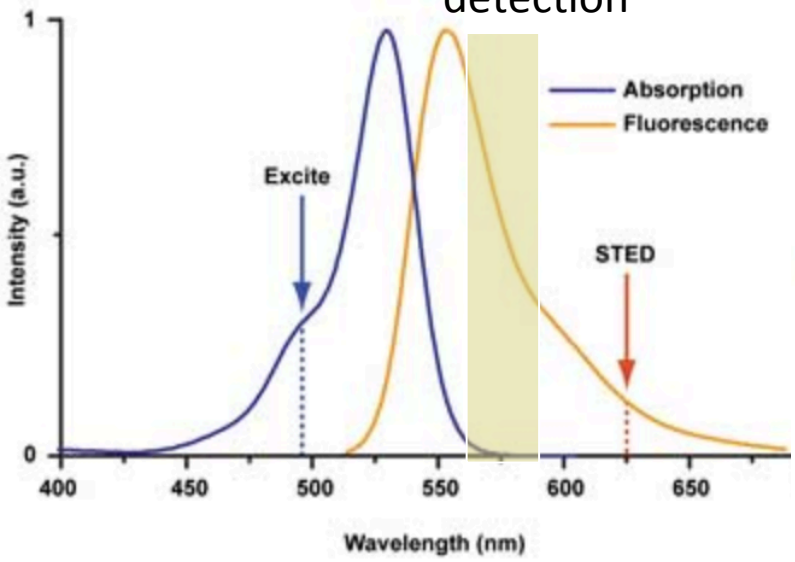
(Stefan Hell, Max Planck Institute, Göttingen)

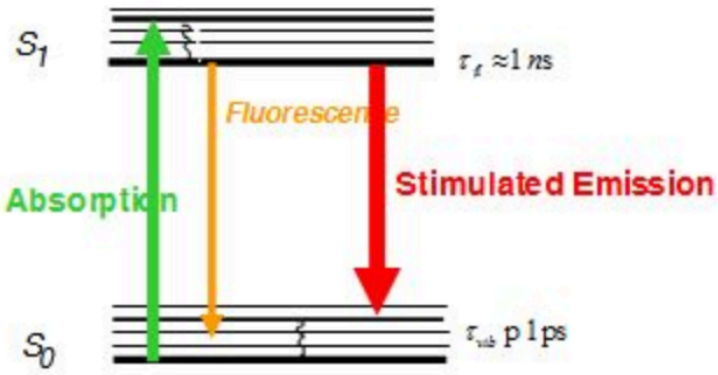
Nobel Prize in Chemistry 2014

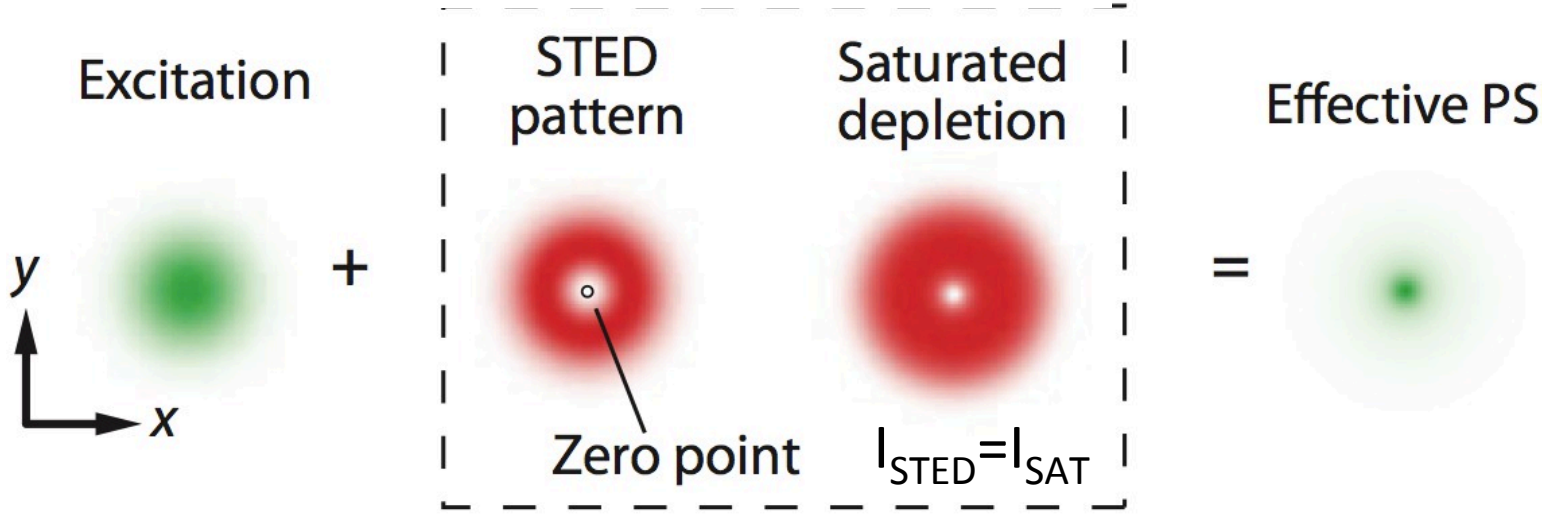
STED – Stimulated Emission Depletion



detection

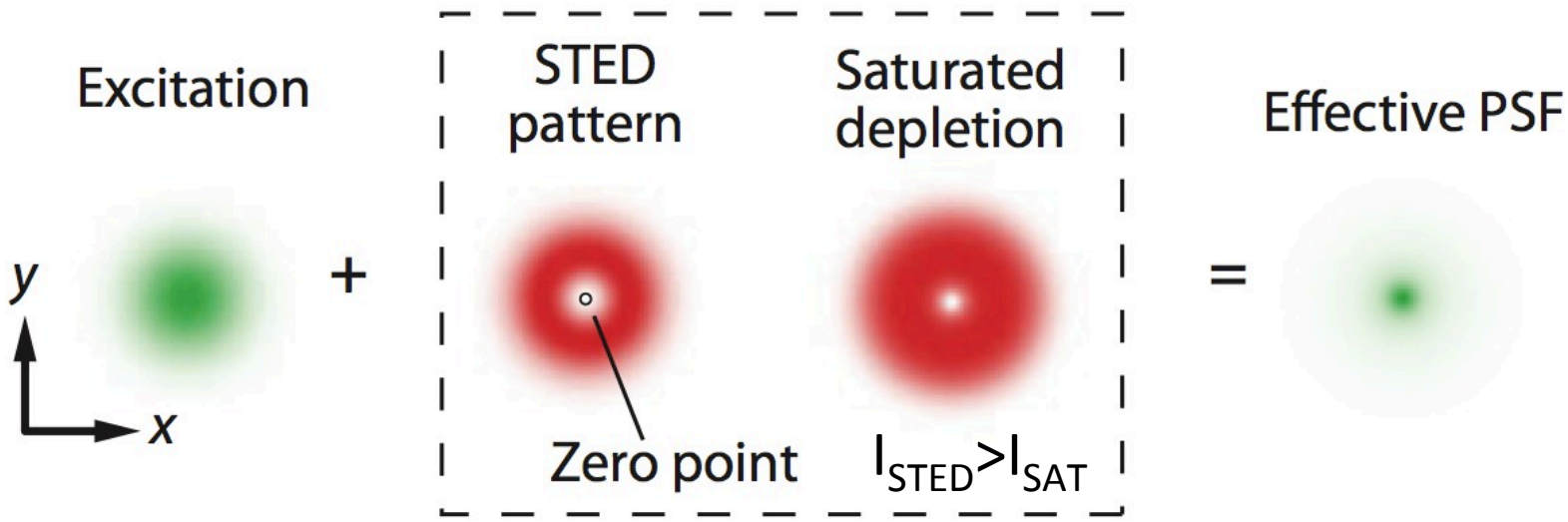


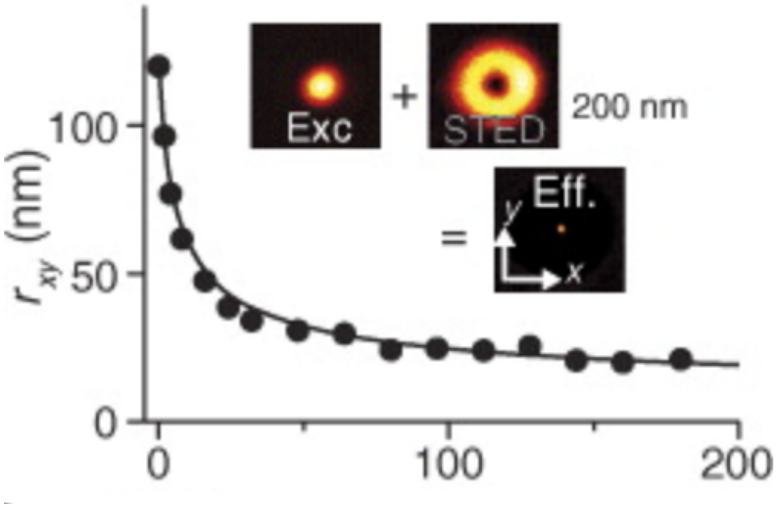




$$d_{STED} \sim \frac{\lambda}{2n\sin\alpha\sqrt{1+I_{STED}/I_{SAT}}} \sim < 50nm$$

STED – Stimulated Emission Depletion





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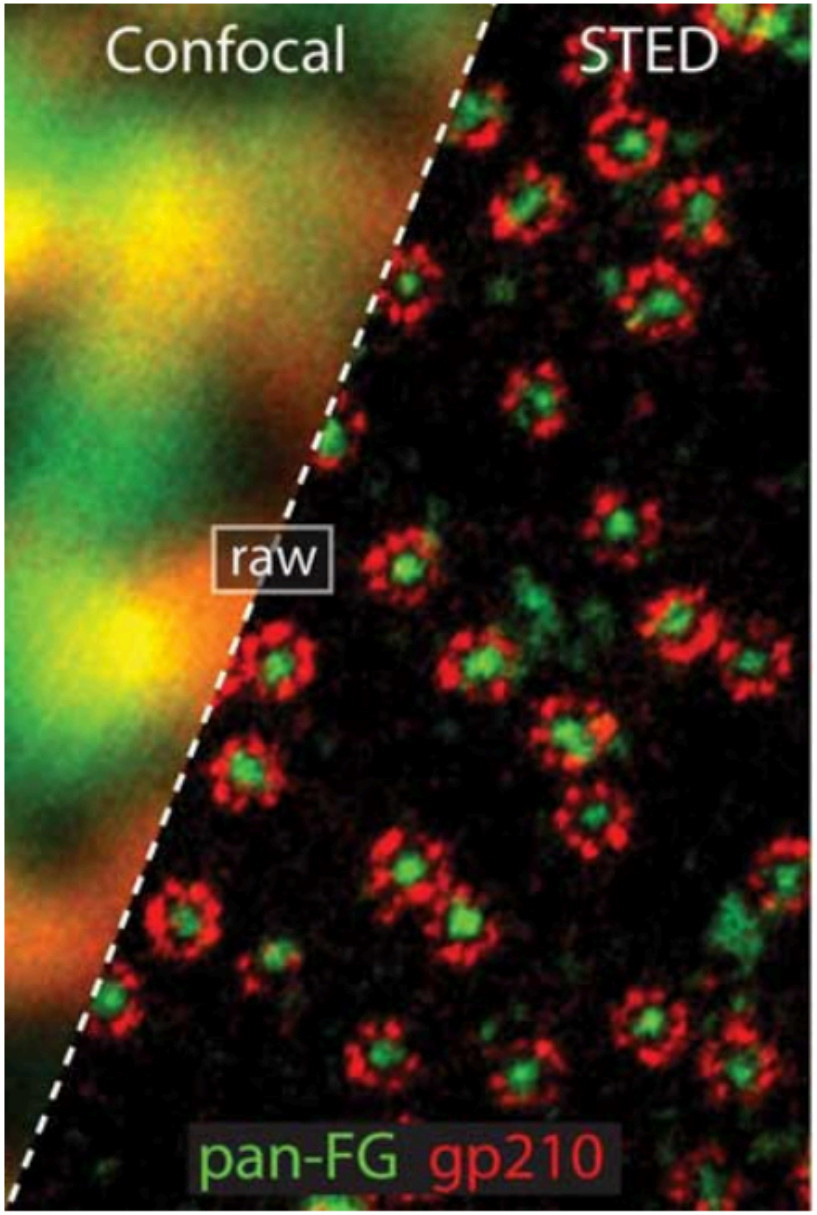
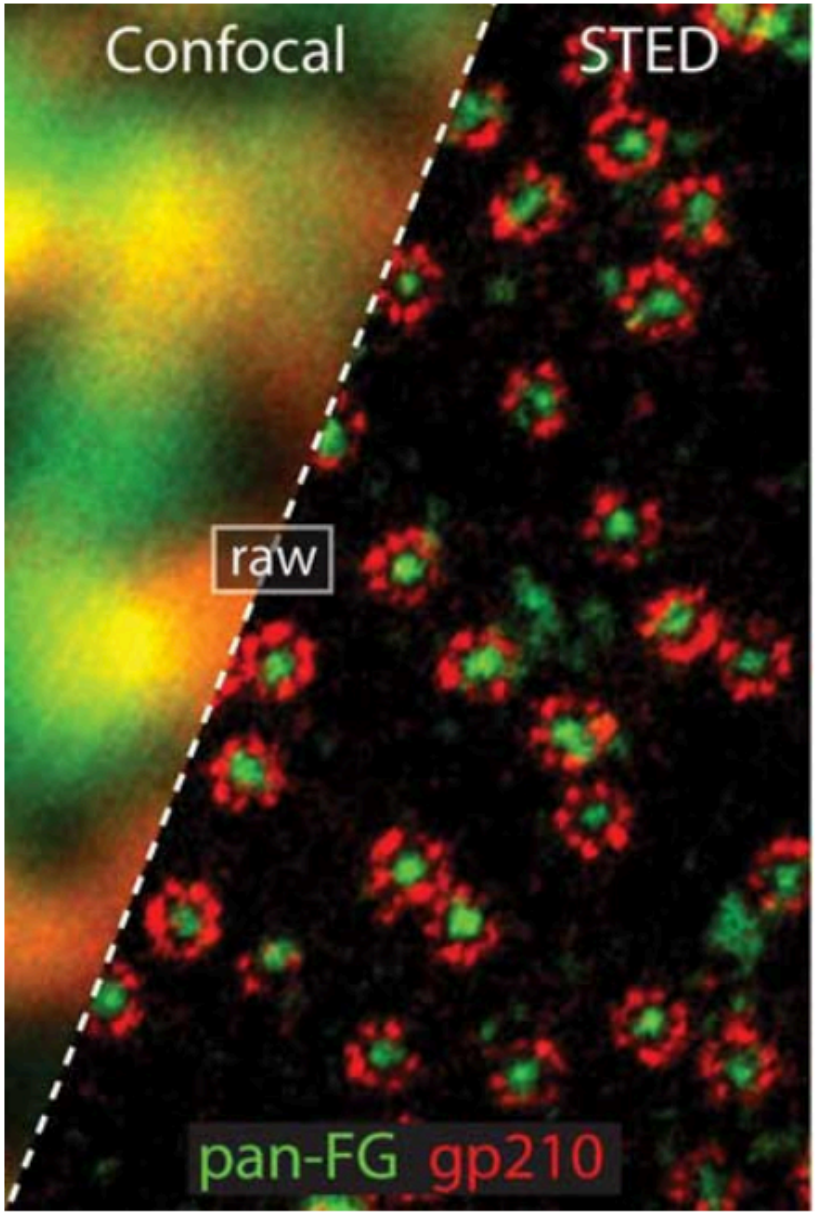


Willig et al Science 2006
Schmidt Nat Methods 2008

STED – Stimulated Emission Depletion



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