

Bi177 - Lecture 13 **Microscopy Outside the Box**

Fluorescence Nanoscopy

TIRF

4-pi

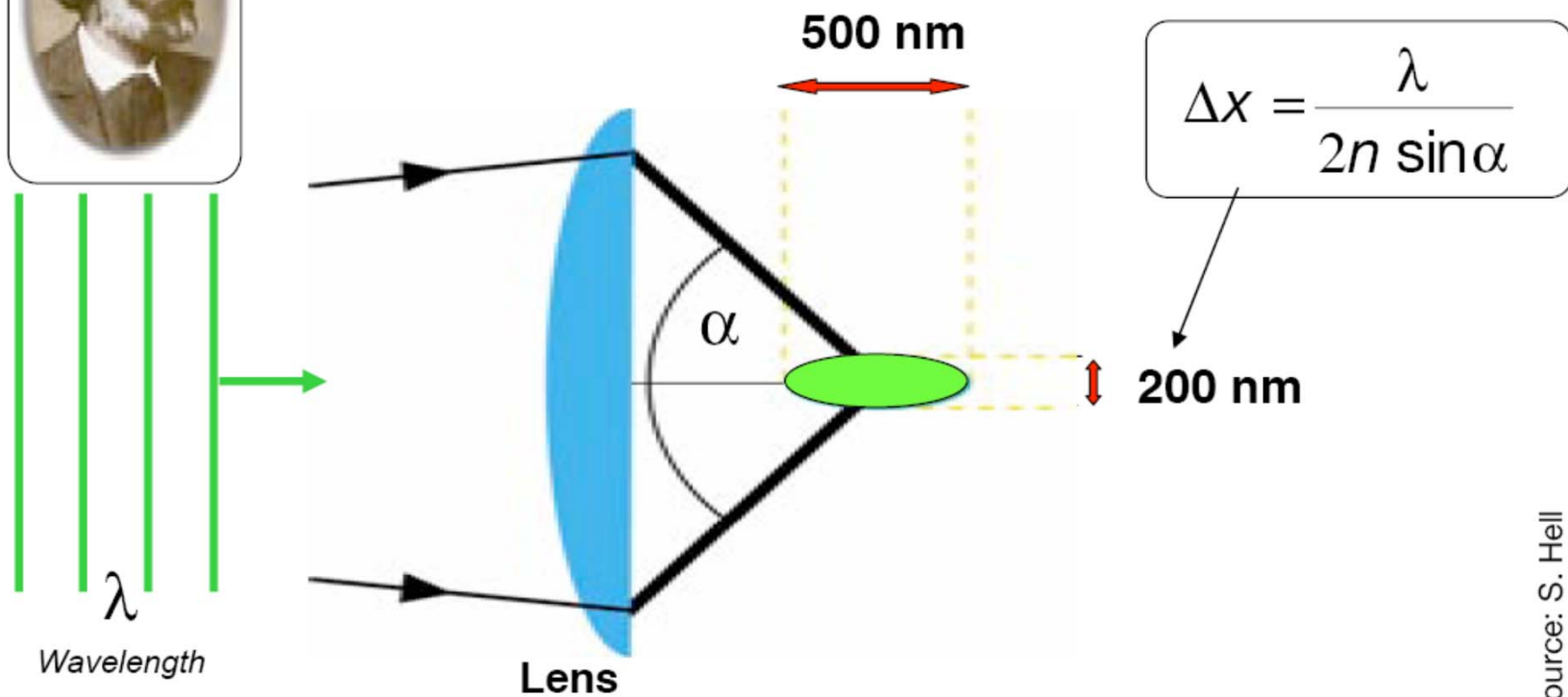
STED

STORM/PALM

The diffraction limit: Abbe's law



E. Abbe (1873), Arch. Mikroskop. Anat. 9, 413.

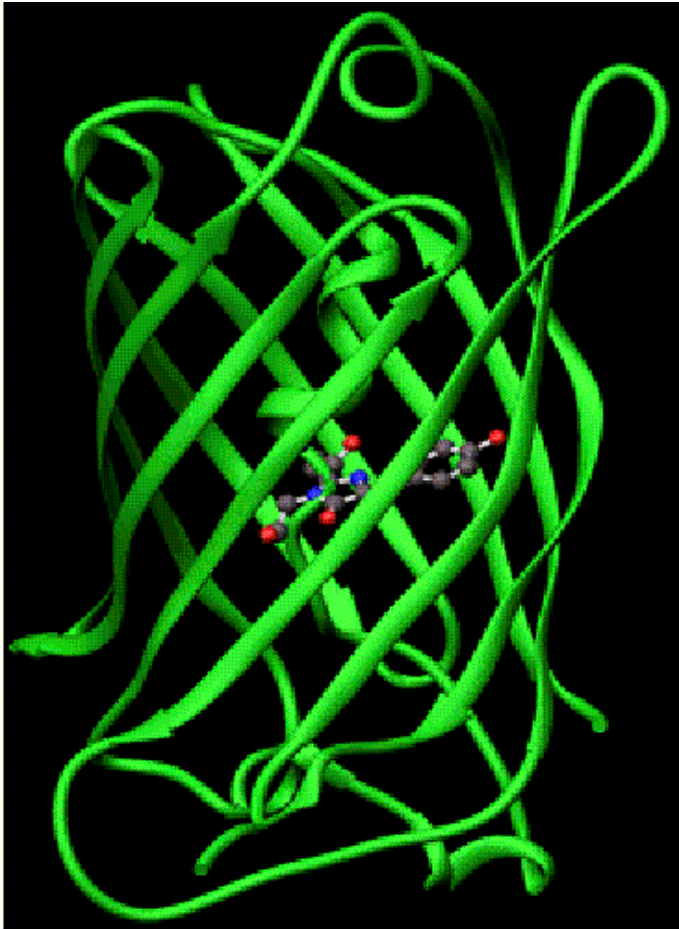


Source: S. Hell

The Problem...

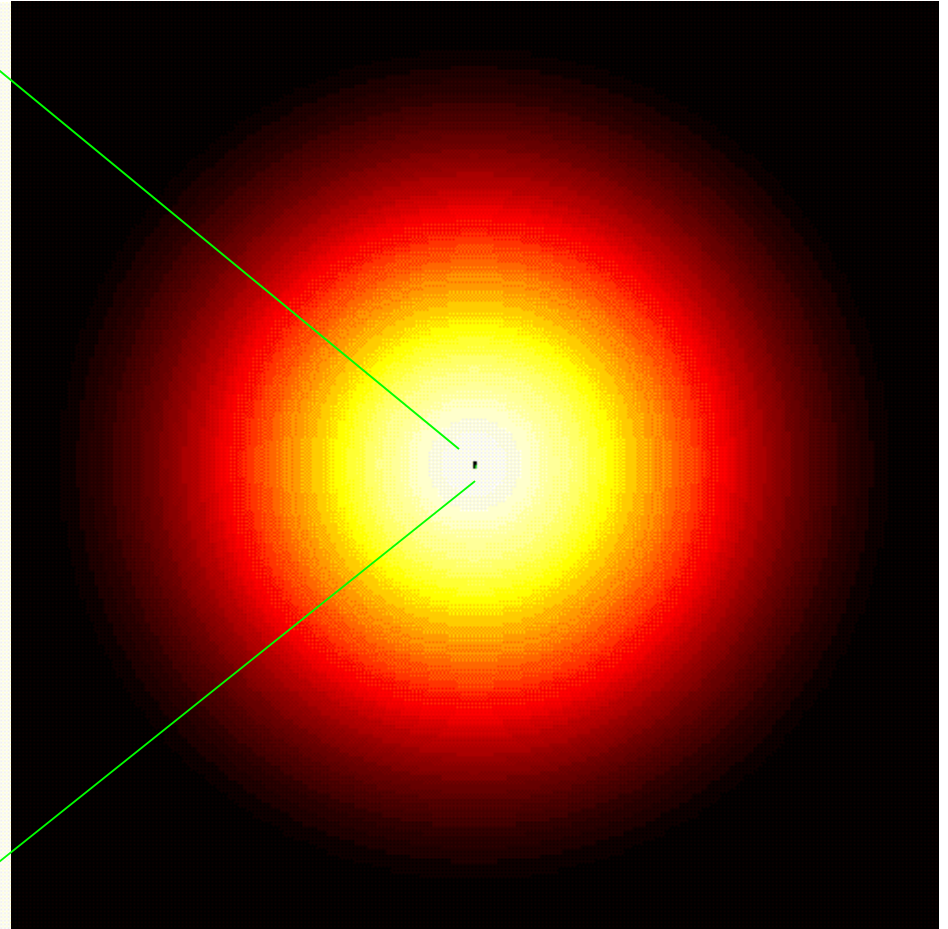
Diffraction limit 100x larger than molecular scale!

Green Fluorescent Protein



1 nm

Diffraction Limited Spot



100 nm

Can we improve the resolution when focusing light with an objective?

First, improving z resolution?

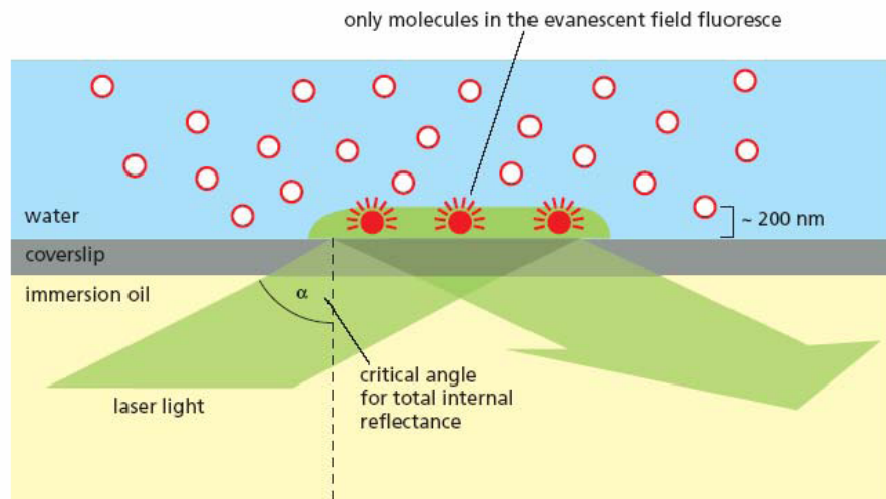
Why $\Delta_z \ll \Delta_{xy}$?

Because the process of focusing is not optimal with an obvious asymmetry resulting from the main direction of light propagation.

2 ways to improve z-resolution:

- near-field approach: evanescent wave microscopy or TIRF
- far-field approach: with an optimal illumination (4π solid angle): $\Delta_z = \Delta_{xy}$

Total Internal Reflection



(A)

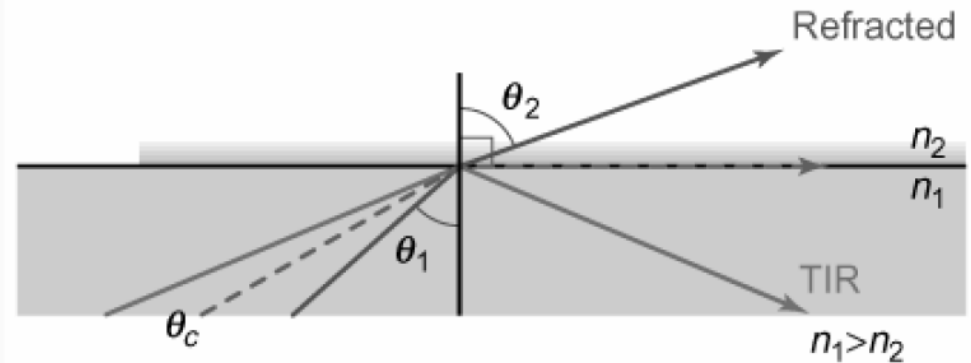


Fig. 1.

TRENDS in Cell Biology

Snell's law:

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$

θ = angle of incidence, n = refractive index

Critical angle:

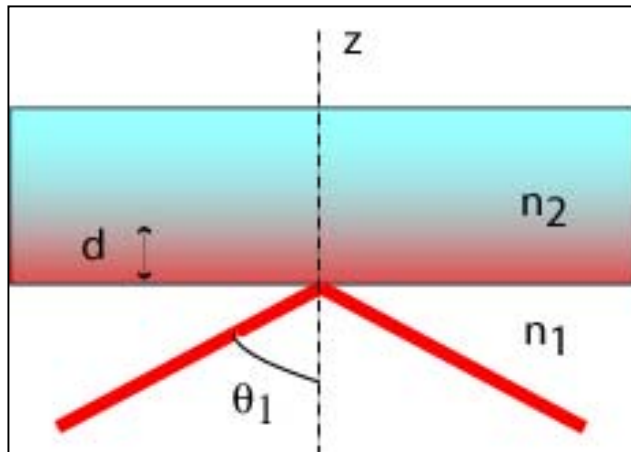
At the critical angle, θ_c , $\theta_2 = 90^\circ$; $\sin 90^\circ = 1$;

$$n_1 \sin \theta_c = n_2$$

$$\theta_c = \sin^{-1} (n_2 / n_1)$$

$$\text{If } n_1 = 1.515 \text{ and } n_2 = 1.36, \theta_c = \sin^{-1} \left(\frac{1.36}{1.515} \right) = 63.85^\circ$$

How far does the evanescent wave propagate?



$$I(z) = I_0 e^{-z/d}$$

$$\frac{1}{d} = \frac{4\pi}{\lambda} \sqrt{n_1^2 \sin^2 \theta_1 - n_2^2}$$

Angle critique : $\theta_c = \arcsin(n_2 / n_1)$

$$n_1 = 1.5 \quad \text{et} \quad n_2 = 1.0 \rightarrow \theta_c = 41.8^\circ$$

θ	d
41.8	∞
50	84,4 nm
60	57,6 nm
65	51,8 nm

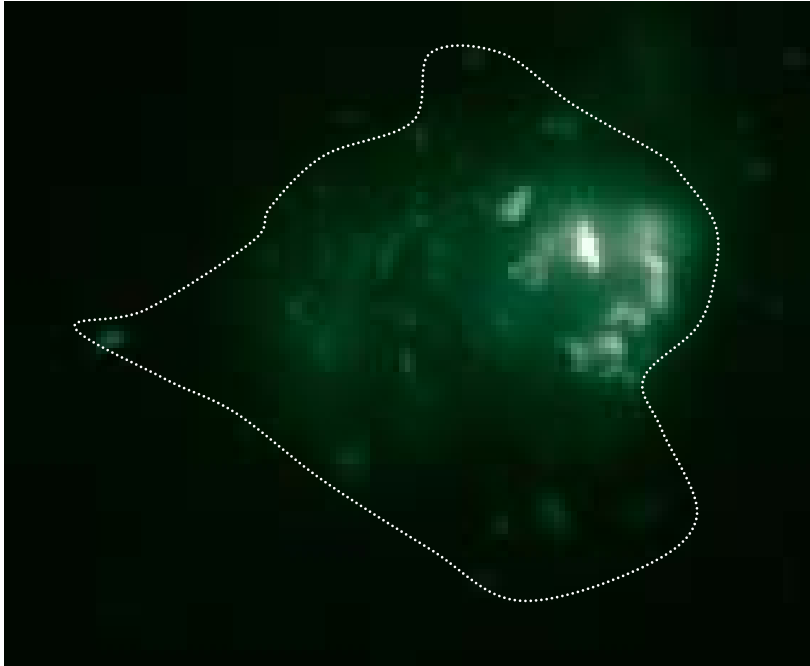
$$n_1 = 1.5 \quad \text{et} \quad n_2 = 1.33 \rightarrow \theta_c = 62.5^\circ$$

θ	d
62.5	∞
65	170 nm
70	100 nm
75	83 nm

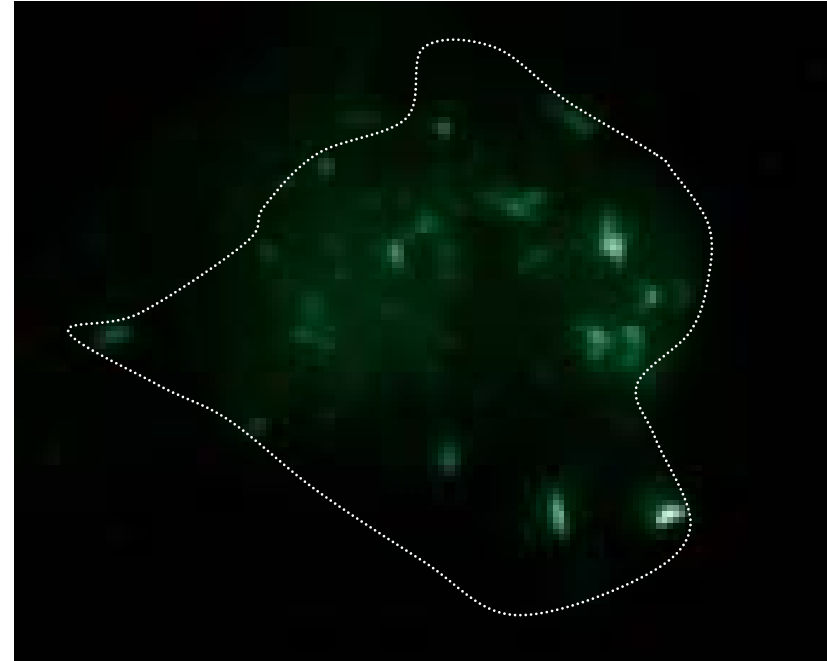
$$(\lambda = 600 \text{ nm})$$

Total Internal Reflection Fluorescence (TIRF) imaging

Epifluorescence



Evanescent only

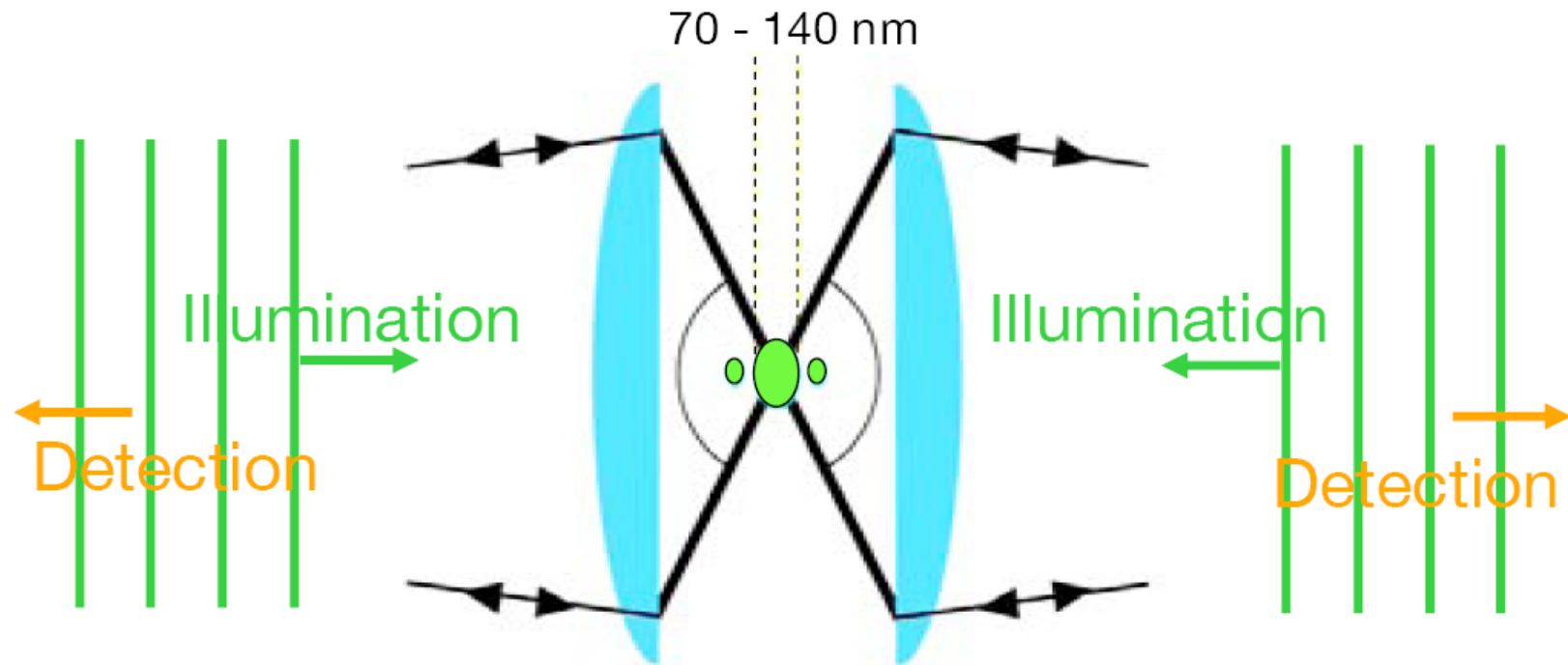


Like other near-field imaging: limited to the surface, no 3D imaging!

Problem 2: “Frustrated TIRF” (higher cell index of refraction → deeper)

4Pi microscopy: the principle

Coherent Illumination and/or Fluorescence Detection

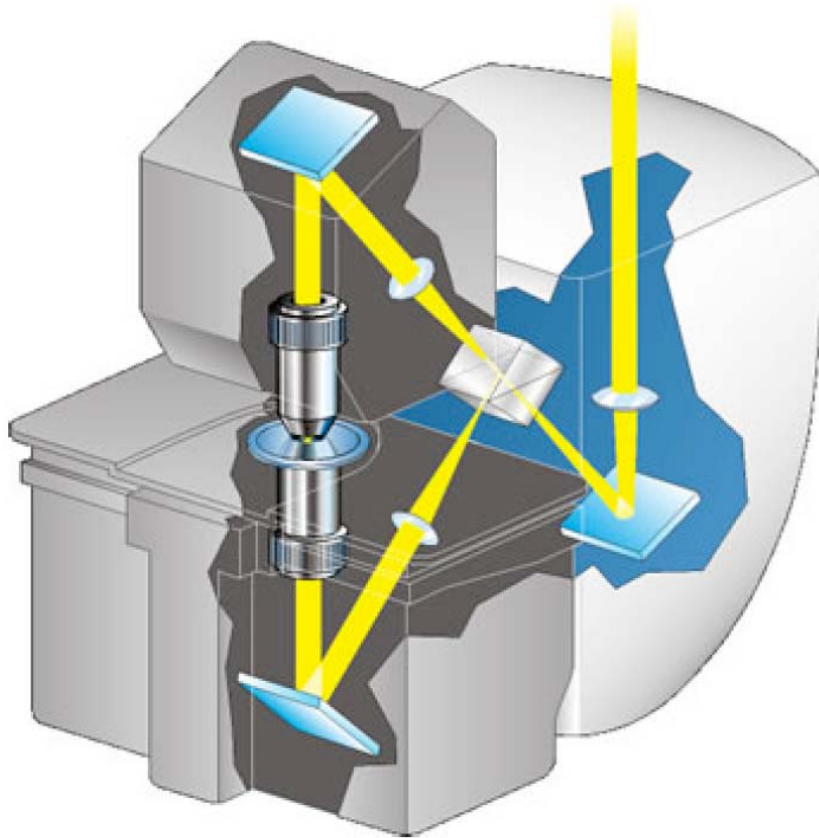


$$\vec{E}^{4\text{Pi}}(r, z, \varphi) = \vec{E}_1(r, z, \varphi) + \vec{E}_2(r, -z, \varphi)$$

S.W. Hell (1990), Europ. Patent OS 0491289. S.W. Hell, et al. (1992), Opt. Commun. 93, 277. M. Schrader, et al. (1998), Biophys. J. 75, 1659. H. Gudel, et al. (2004), Biophys. J. 87, 4146.

Engineering the focal volume using interference

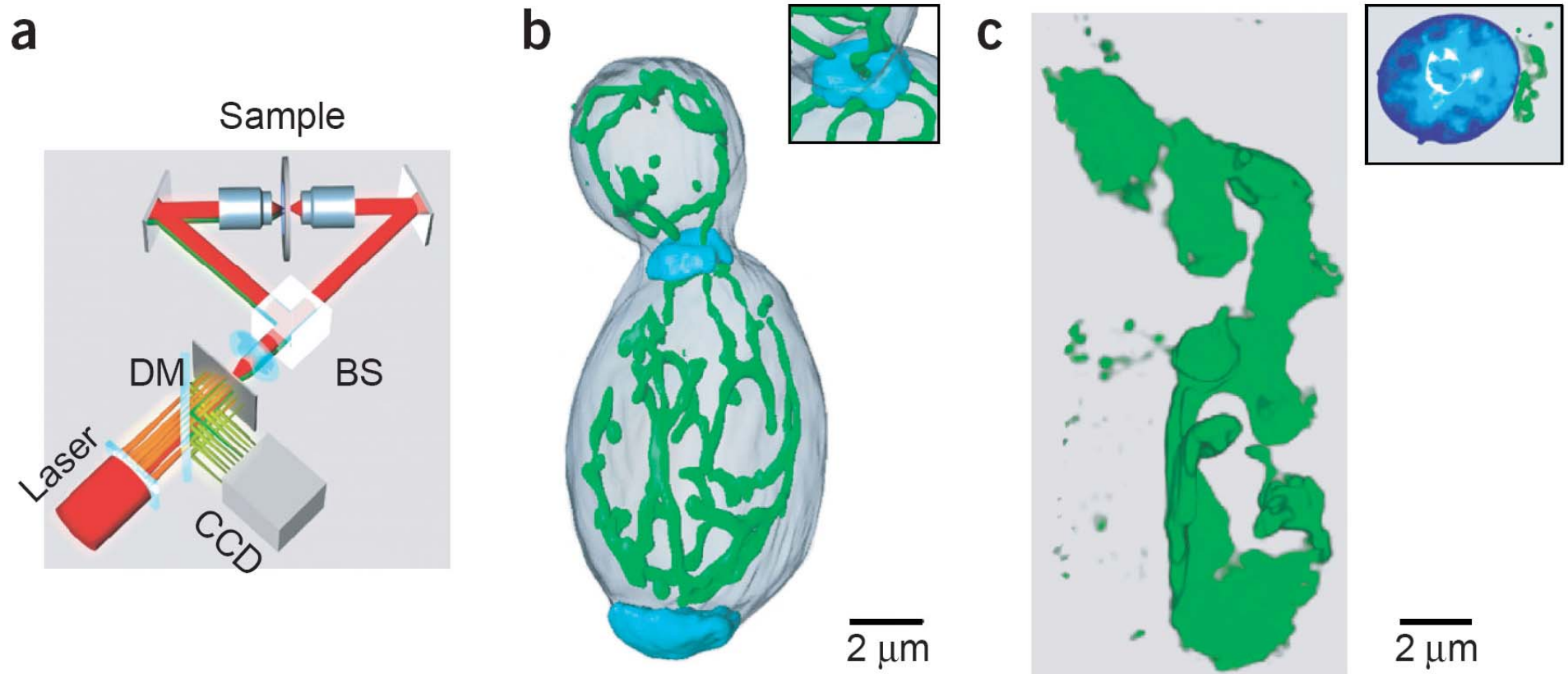
4Pi microscopy: the setup



Up to 7x better axial resolution
eg: 4Pi-TPLSM up to 80nm z-
resolution!

Required to squeeze the sample
between two objectives... thin sample
only!

4Pi microscopy: applications



(a) MMM-4Pi microscopy

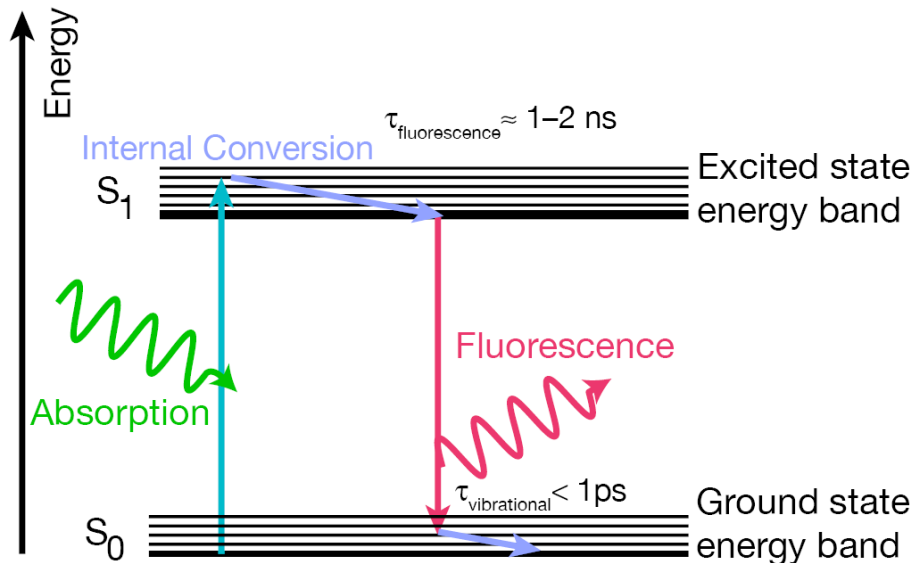
(b) GFP labeled compartments of *Saccharomyces cerevisiae*

(c) Golgi apparatus in a live Vero cell

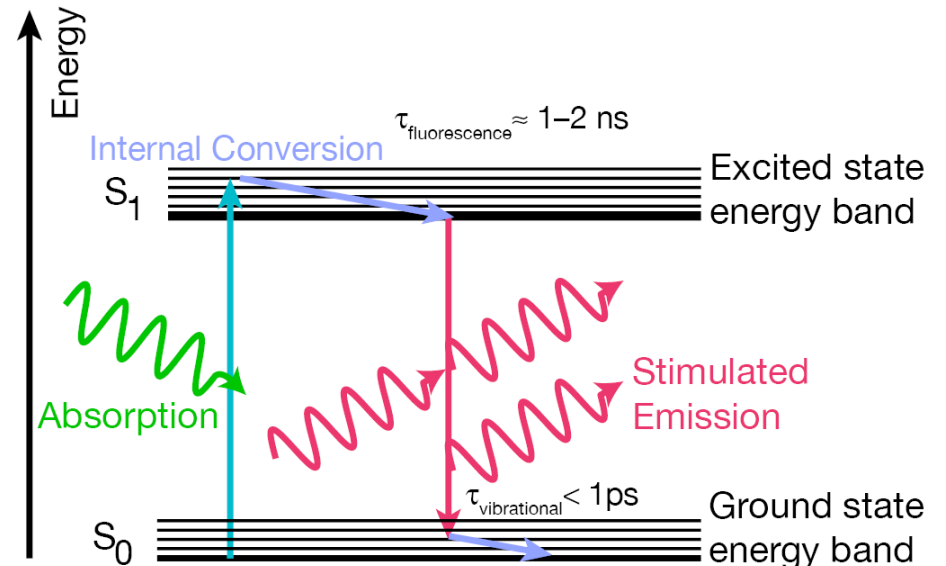
Hell SW, *Nature Biotechnology* (2003)

STED is based on stimulated emission

Spontaneous fluorescence emission

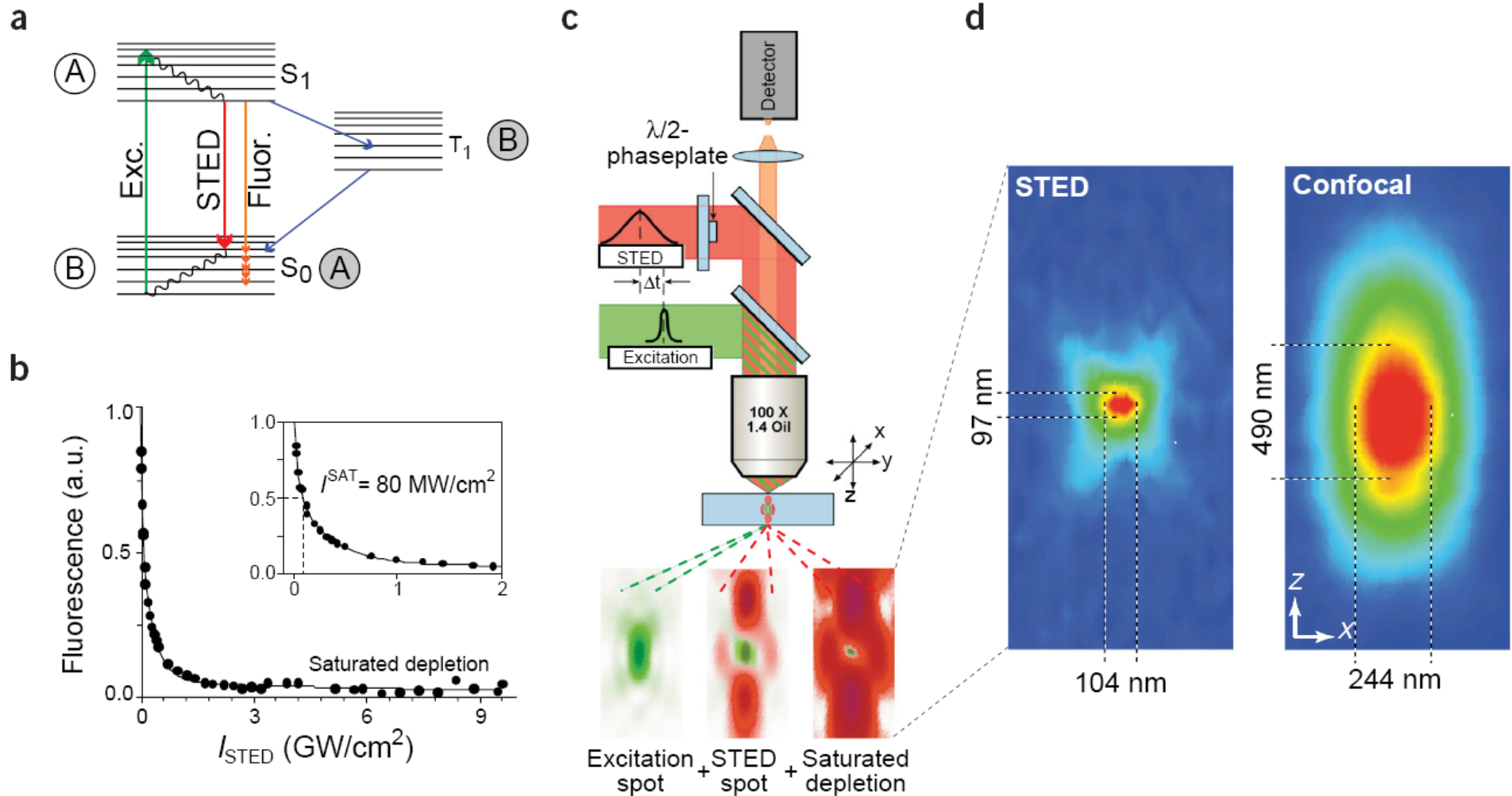


Stimulated fluorescence emission

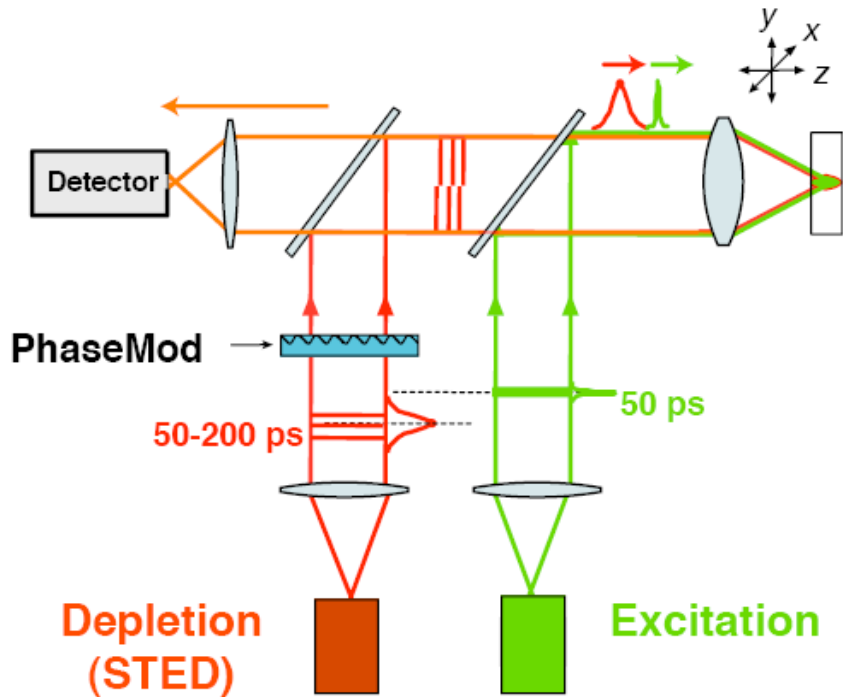


The perturbing photon with the correct energy is seemingly unchanged in the process, and the stimulated emission generate a second photon with the same phase, frequency/energy, polarization, and direction of travel as the first one.

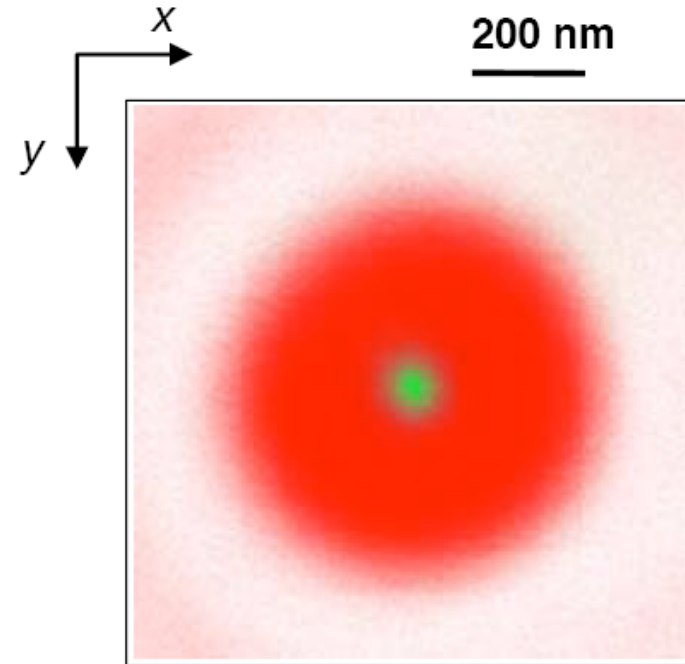
STED: breaking the resolution limit by shaving the PSF



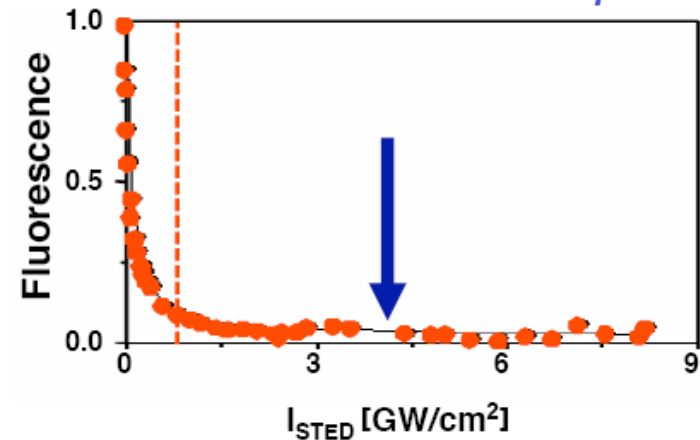
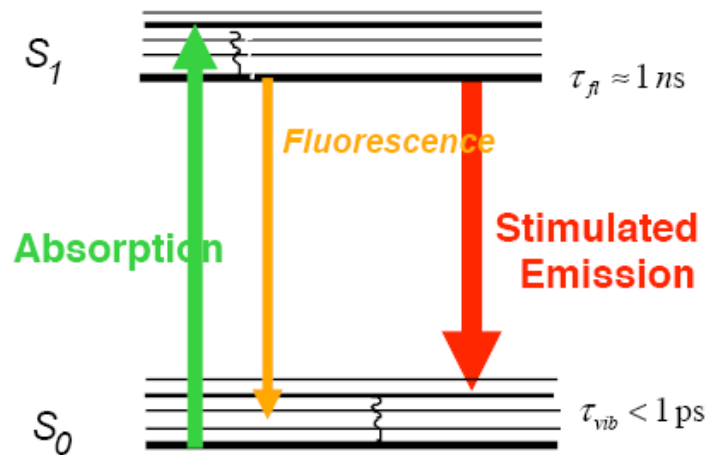
STED: breaking the resolution limit by shaving the PSF



S.W. Hell & J. Wichmann (1994), Opt. Lett. 19, 780.

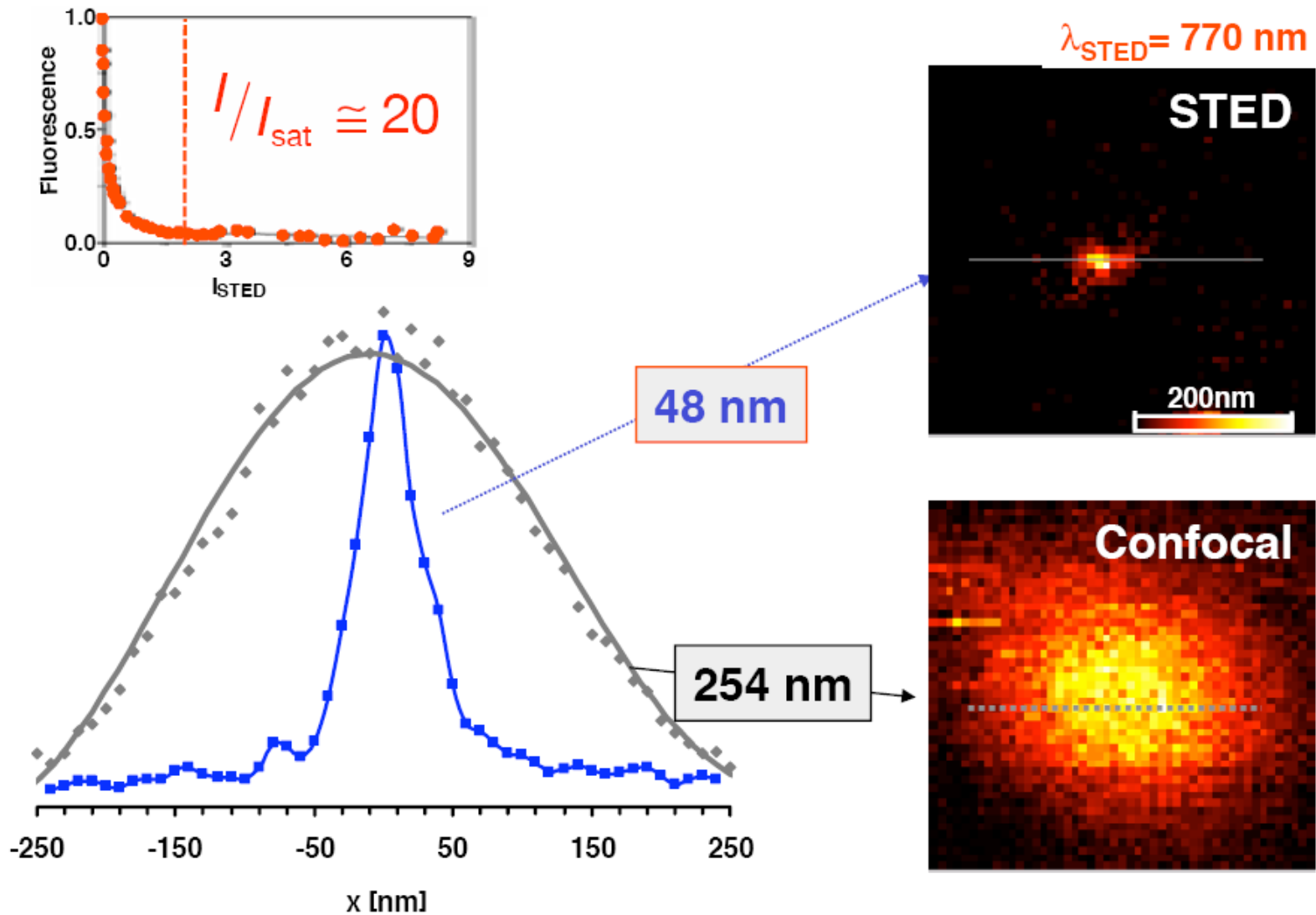


The stronger the STED beam the narrower the fluorescent spot!



Source: Stefan Hell

STED can break the diffraction limit!

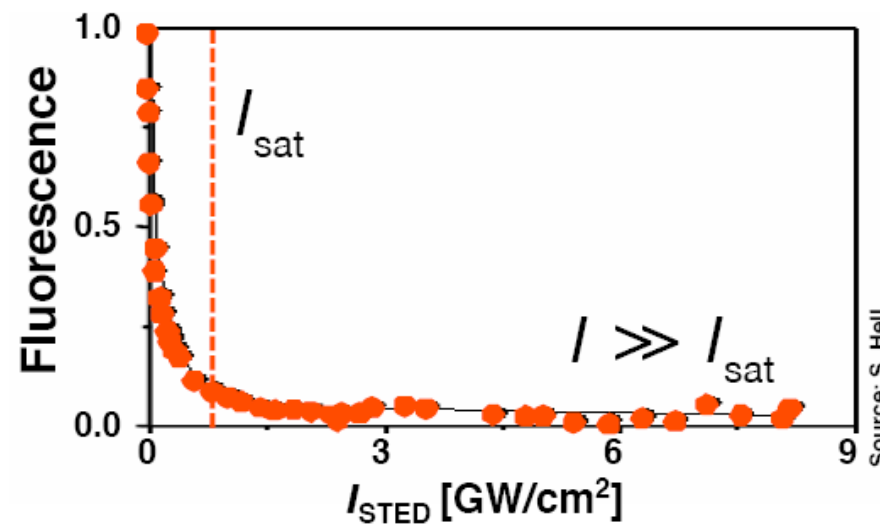


V. Westphal & S.W. Hell (2005), Phys. Rev. Lett. 94, 143903.

STED microscopy summary

- Resolution is not limited by the wavelength of light!
- Resolution just depends on the level of fluorescence depletion.
- Resolution at the molecular scale is possible with visible light and regular lenses!
- Resolution follows a new law; a modification of Abbe's law:

$$\Delta x \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + I/I_{\text{sat}}}}$$



STochastic Optical Reconstruction Microscopy
(STORM)

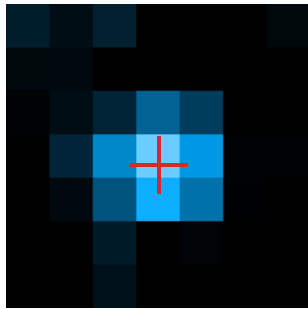
and/or

Photo-Activated Localization Microscopy (PALM)

and/or

Fluorescent Photo-Activated Localization Microscopy
(FPALM)

Finding out the position of a molecule



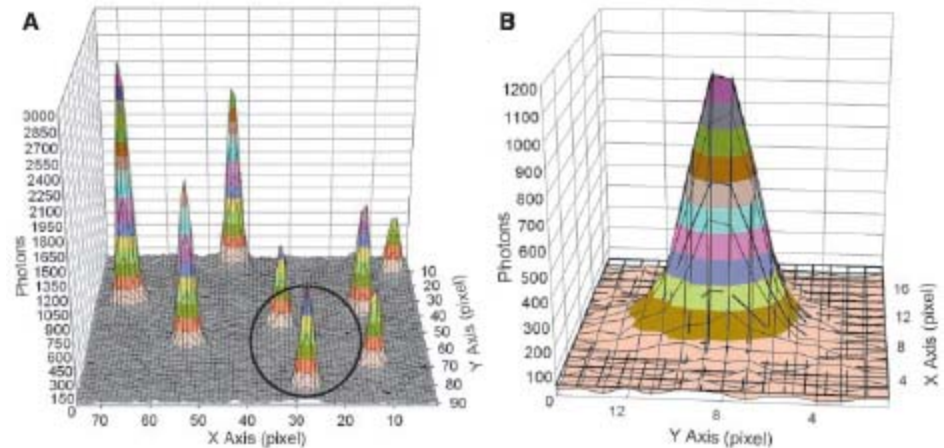
$$\sigma \approx \sigma_{\text{PSF}} / N^{1/2}$$

Thompson et al., *Biophys. J.*, 2002

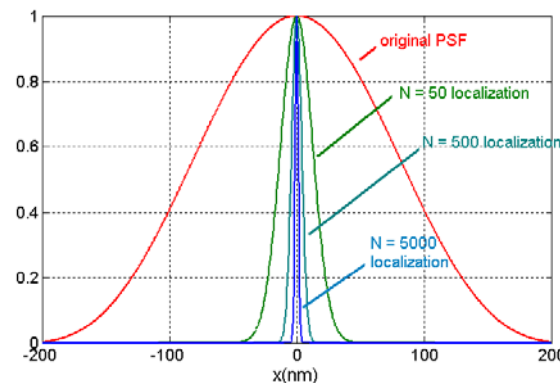
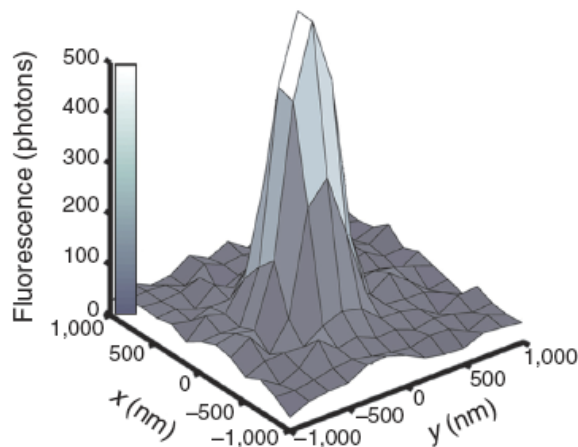
By fitting the signal profile from a single molecule, its position can be estimated with an accuracy higher than the resolution of the microscope!

Note: resolution $\neq$ localization precision

Fluorescence Imaging with One-Nanometer Accuracy (FIONA)
= single molecule detection

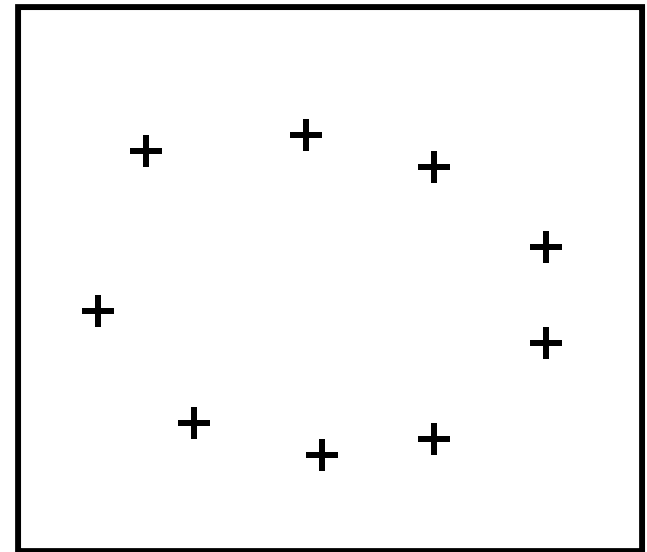
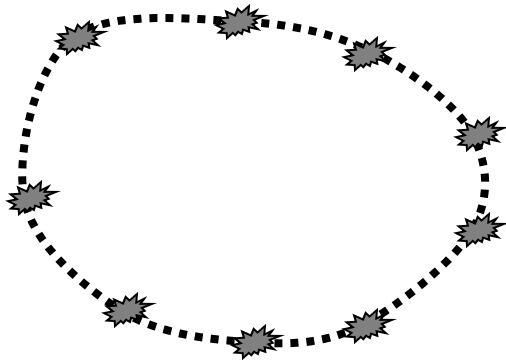


Yildiz et al., *Science*, 2003



Hari Shroff, Janelia Farm Research Campus, HHMI

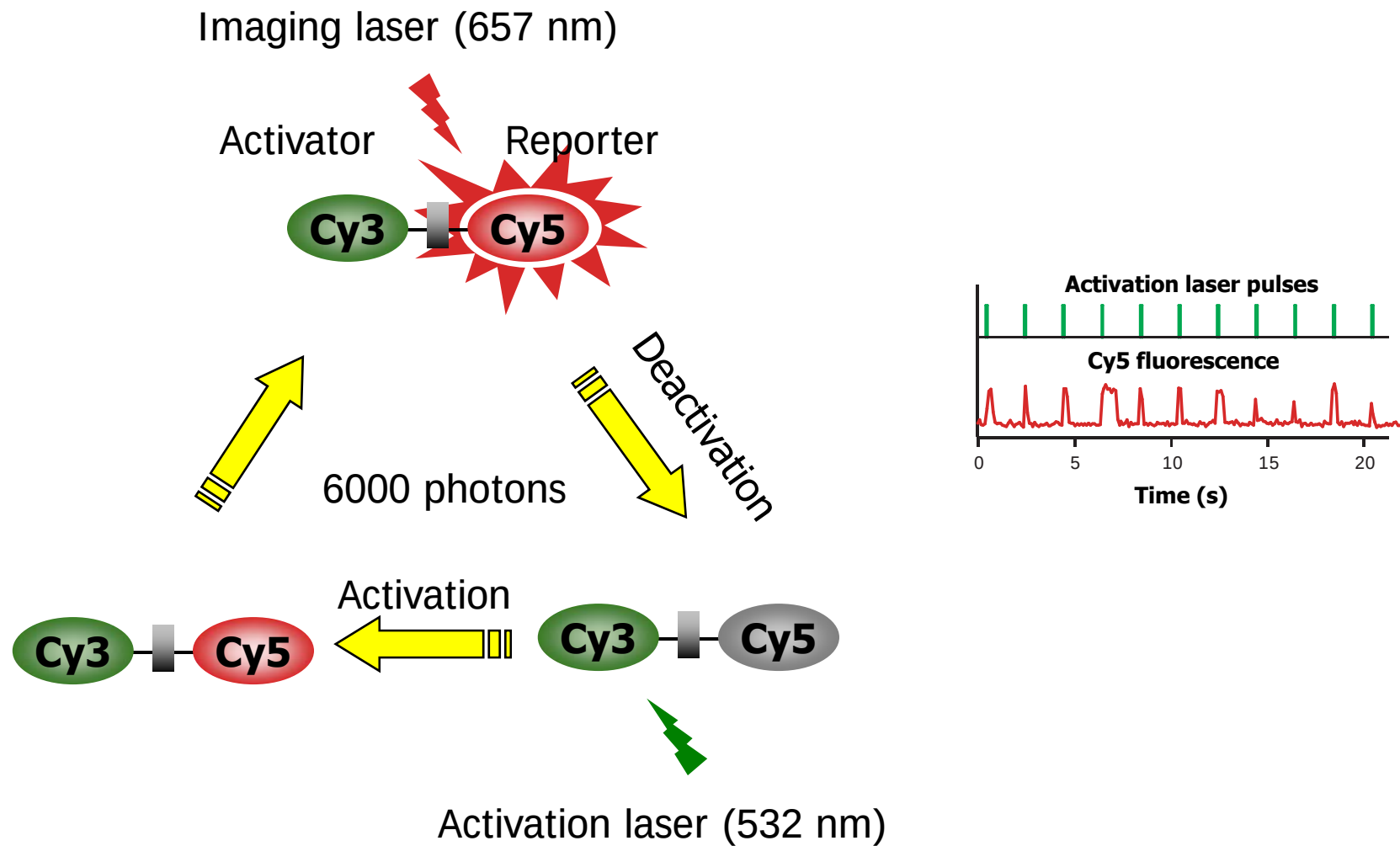
The principle of STORM



How to excite only a few fluorophores at a time???

By using low level photo-activation = ability to turn molecules on/off

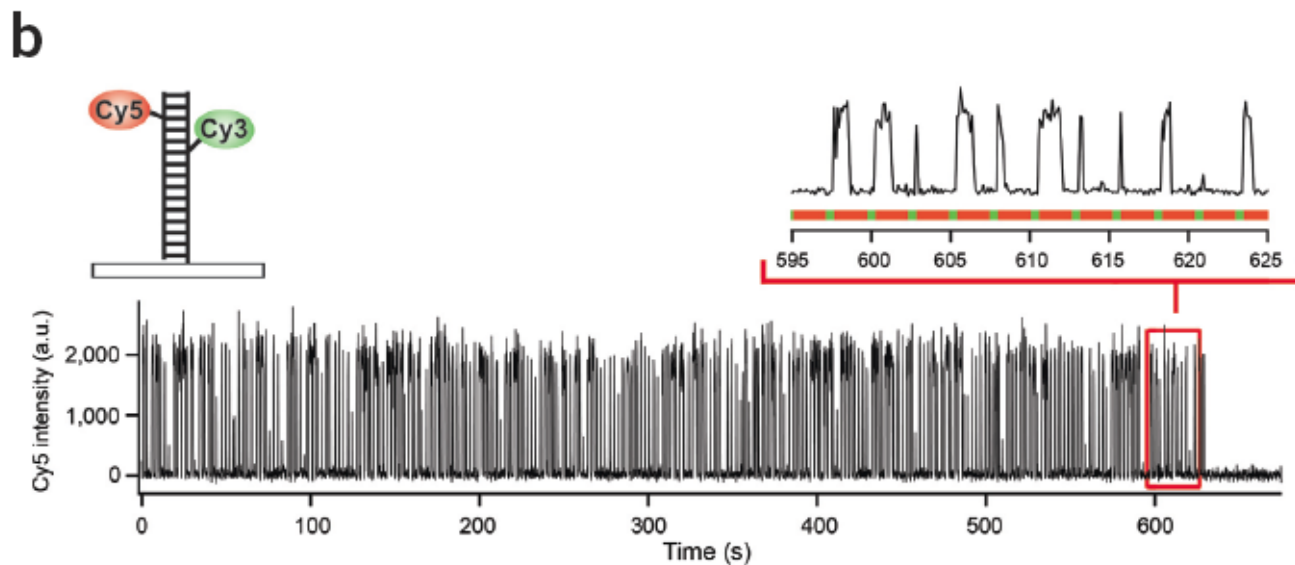
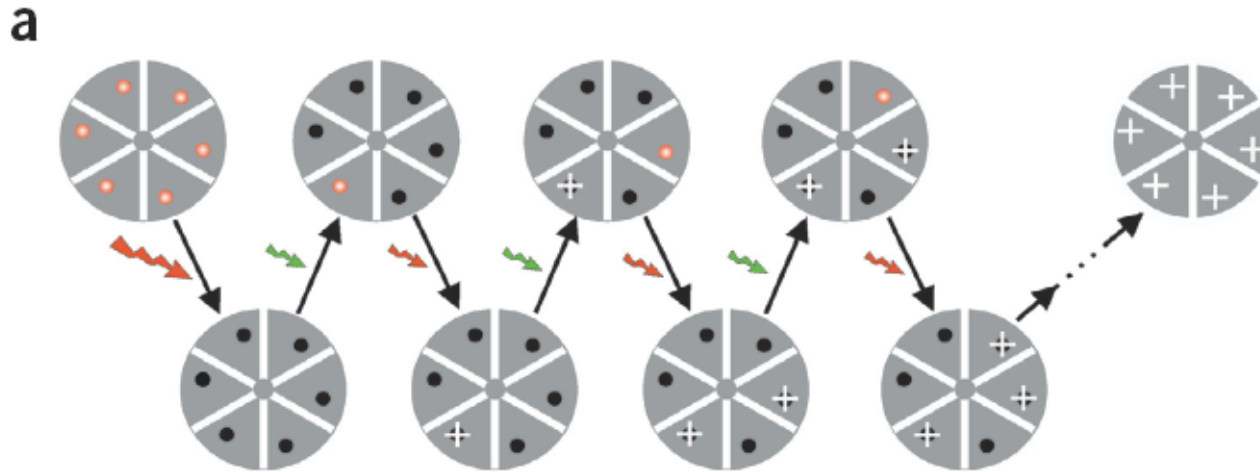
STORM requires photo-switchable probes



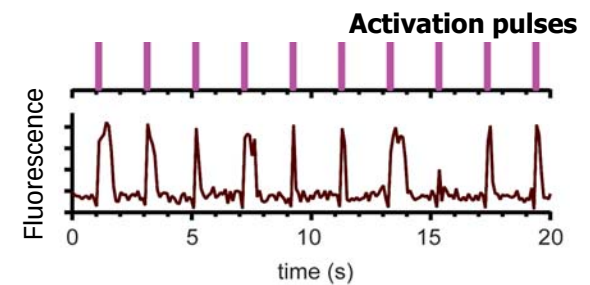
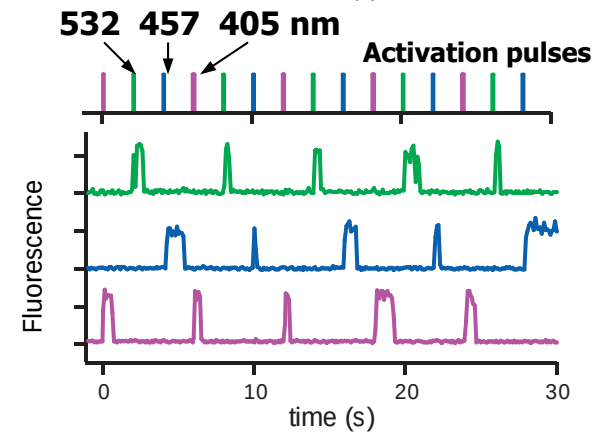
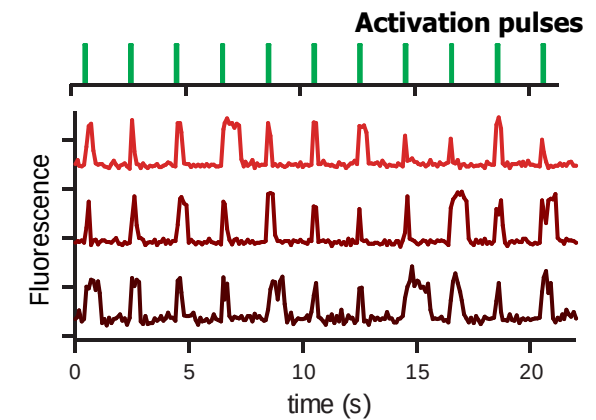
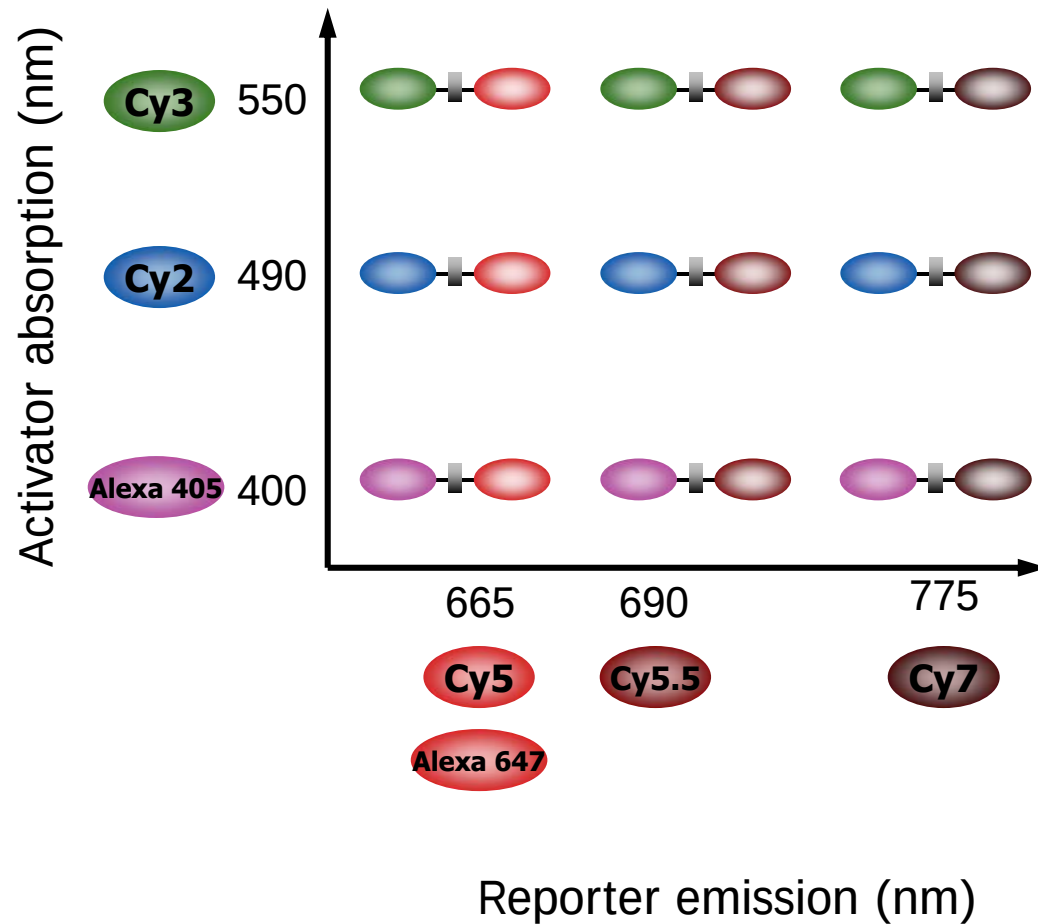
STORM use Cy3 to rejuvenate Cy5

Cycles of low level activation / imaging / localization / desactivation

At each cycle, only a few molecules are activated within the field of view: the density is low enough to avoid overlap between fluorophores



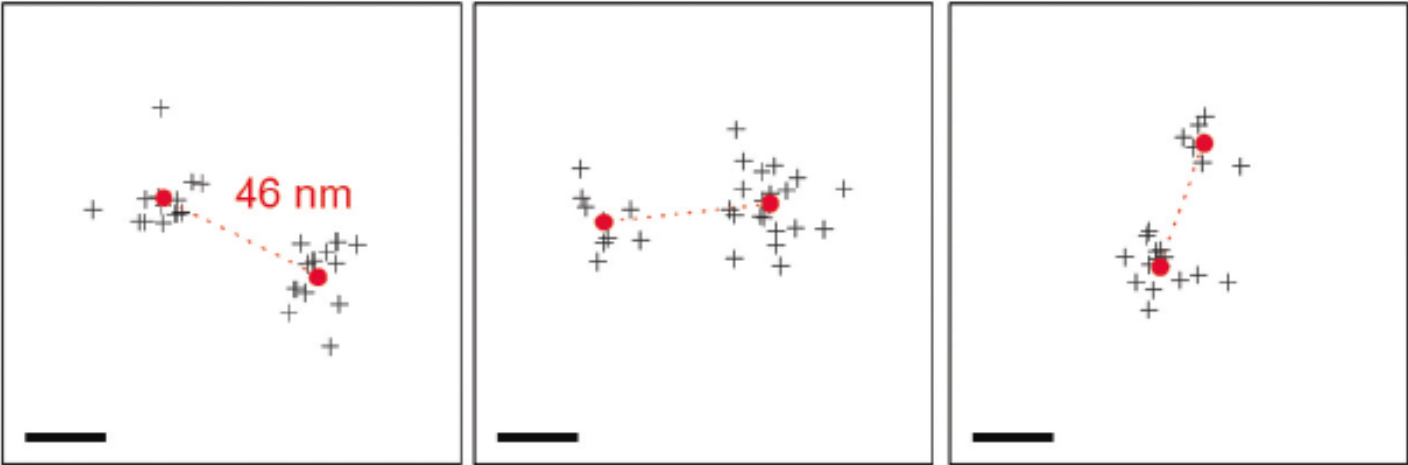
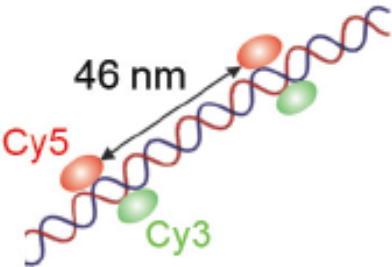
More colors?



Bates et al, *Science* **317**, 1749 – 1753 (2007)

STORM resolution validated by DNA yardstick

a



STORM-PALM-FPALM imaging

PALM: Photo-Activated Localization Microscopy

FPALM: Fluorescent Photo-Activated Localization Microscopy

Exactly the same concept but using photo-activated fluorescent proteins

Good: genetically encoded approach

Bad: photo-activation is not reversible! It requires to bleach the molecule after each cycle (photo-toxicity, loss of signal,...)

...but reversible photo-switchable proteins can be used now!

PALM

Betzig E, et al: Imaging intracellular fluorescent proteins at nanometer resolution, ***Science* (2006)**

FPALM

Hess ST, et al: Ultra-high resolution imaging by fluorescence photoactivation localization microscopy, ***Biophysical J.* (2006)**

STORM:

Rust MJ, et al: Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM), ***Nature Methods* (2006)**

STORM-PALM-FPALM imaging

Conclusions:

Statistical approaches to reconstruct images: these techniques permit to reconstruct the distribution of fluorophores within an image with ultra-high accuracy ($\ll$ resolution of the microscope).

They require many cycles of activation/localization (several thousands of images acquired for one reconstruction!)

= low time resolution!