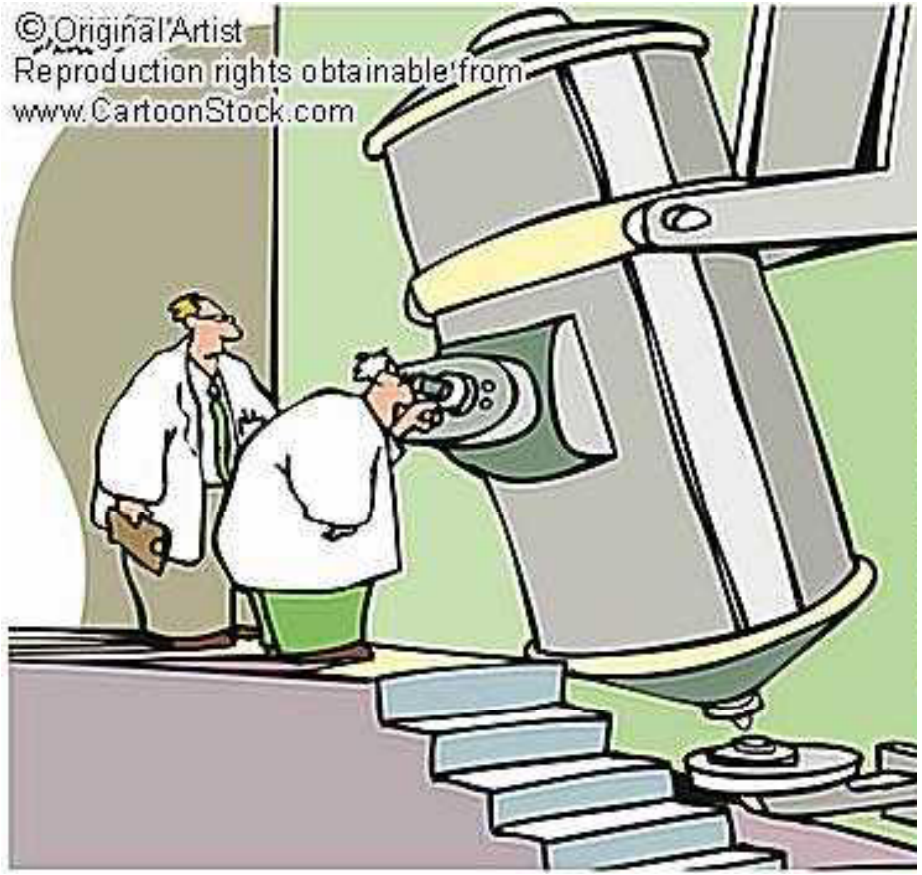
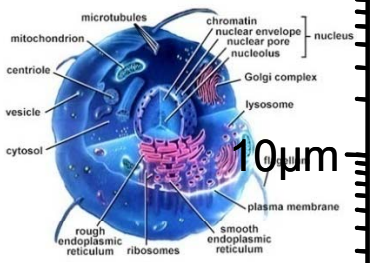
Super-Resolution Optical Microscopy

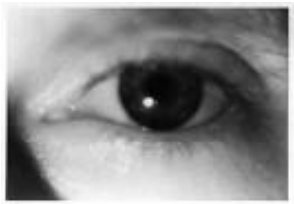


Bo Huang

Light Microscopy

May 10, 2010





Naked eye: ~ 50-100

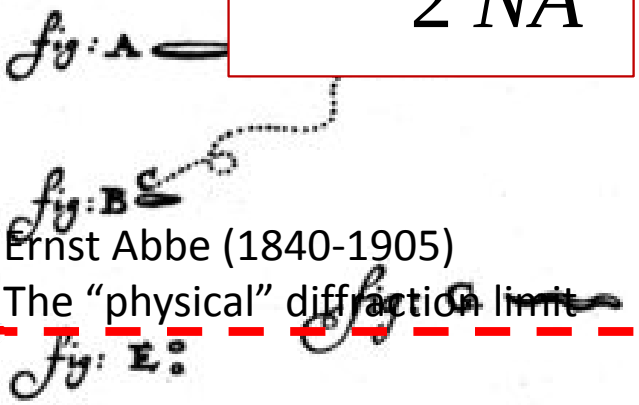
PLATE XXIV

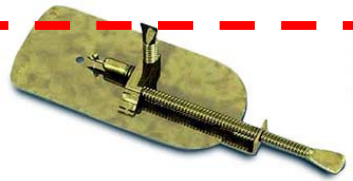
$$d \approx \frac{\lambda}{2\,NA}$$

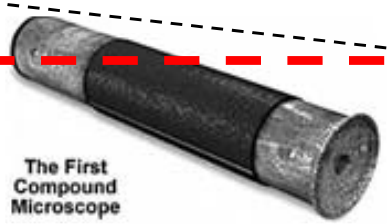
1595, Zaccharias and Hans Janssen
First microscope, 9x magnification

Antony Van Leeuwenhoek
(1632-1723), 200x

Ernst Abbe (1840-1905)
The "physical" diffraction limit

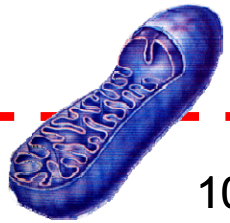


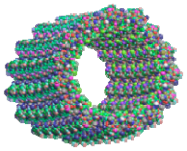


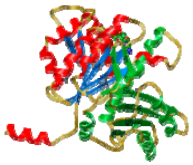


Compound microscope
>1000x

0.1mm
10μm
1μm
100nm
10nm
1nm
1Å

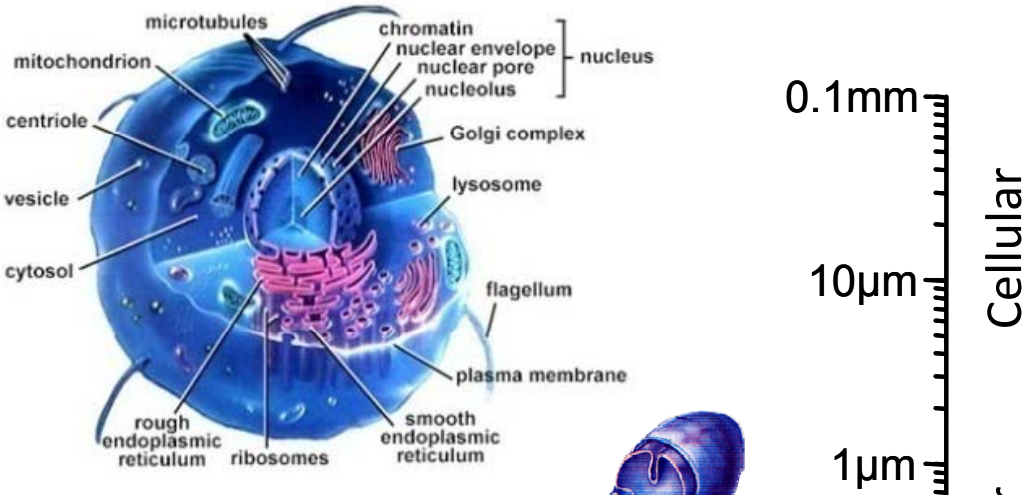


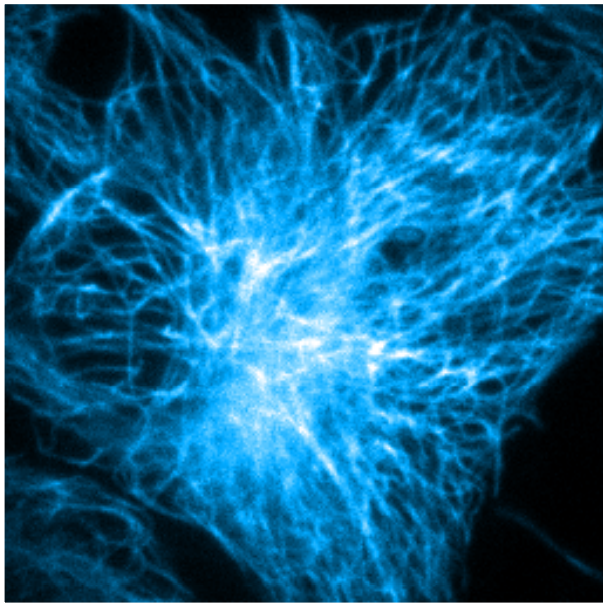




1600 1700 1800 1900 2000

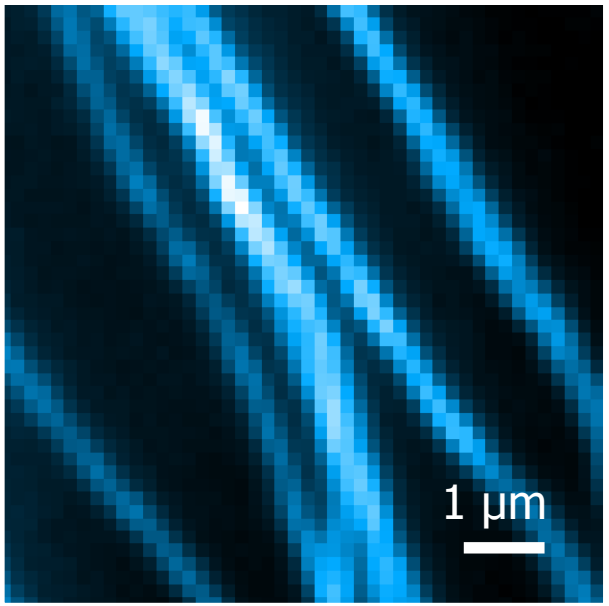
The diffraction barrier





Diffraction limit: ~ 250 nm lateral

~ 600 nm axial

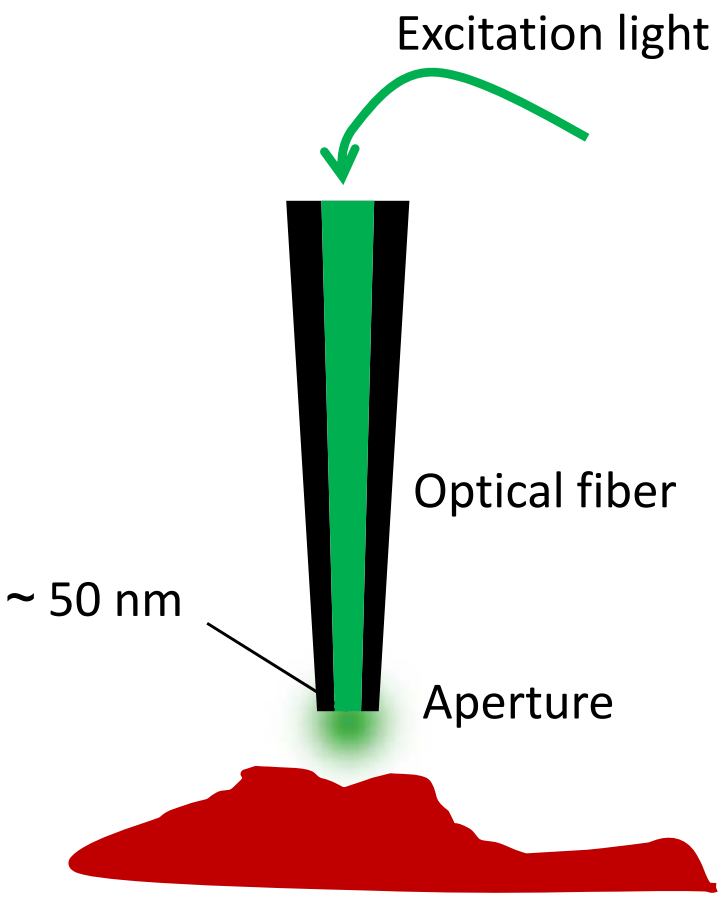


http://www.3dchem.com; http://cs.stedwards.edu; http://cvcweb.ices.utexas.edu; Fotin et al., *Nature* 2004; http://hrsbstaff.ednet.ns.ca; http://www.ebi.ac.uk

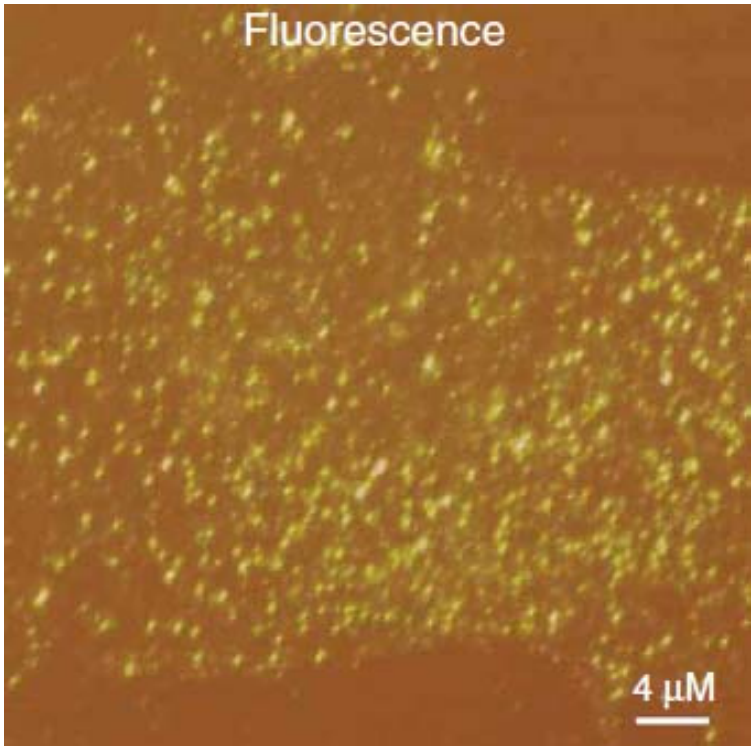
50 years to extend the resolution

- Confocal microscopy (1957)
- Near-field scanning optical microscopy (1972/1984)
- Multiphoton microscopy (1990)
- 4-Pi microscopy / I^5M (1991-1995)
- Structured illumination microscopy (2000)
- Negative refractive index (2006)

Near-field scanning optical microscopy



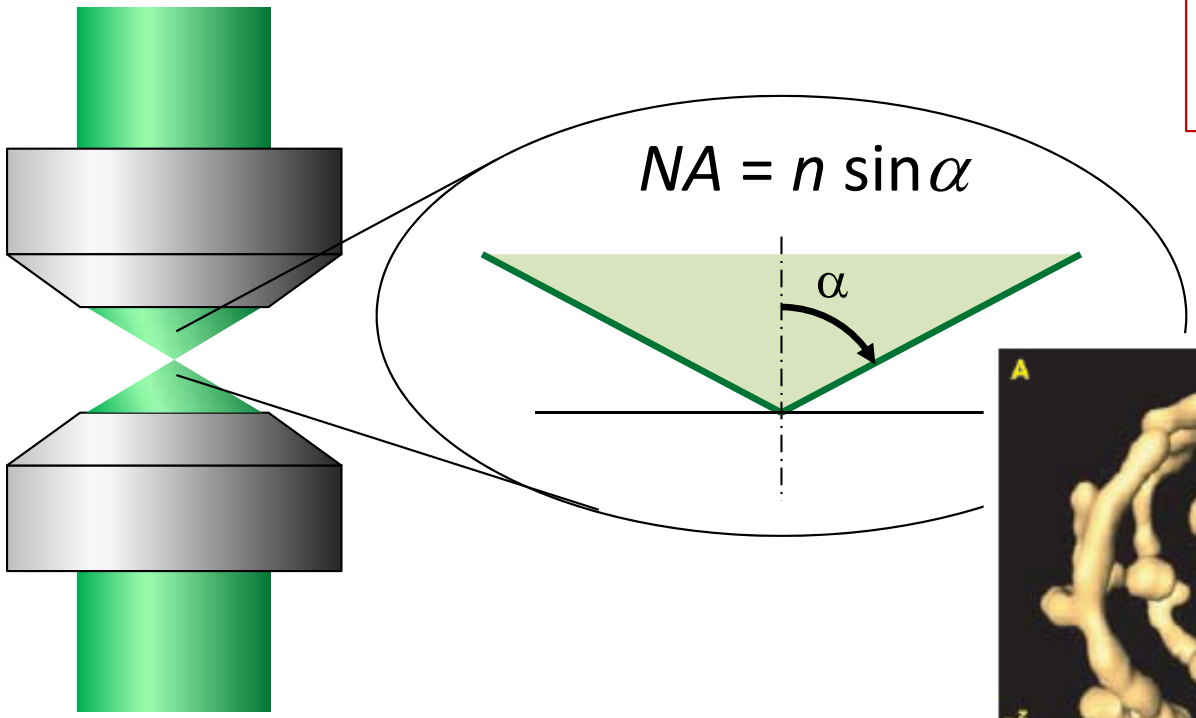
β_2 adrenergic receptor clusters on the plasma membrane

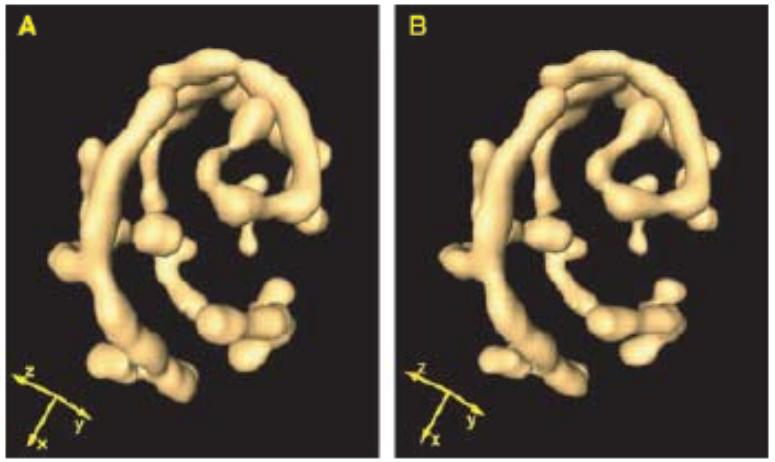


Ianoul et al., 2005

4-Pi / I^{5}M

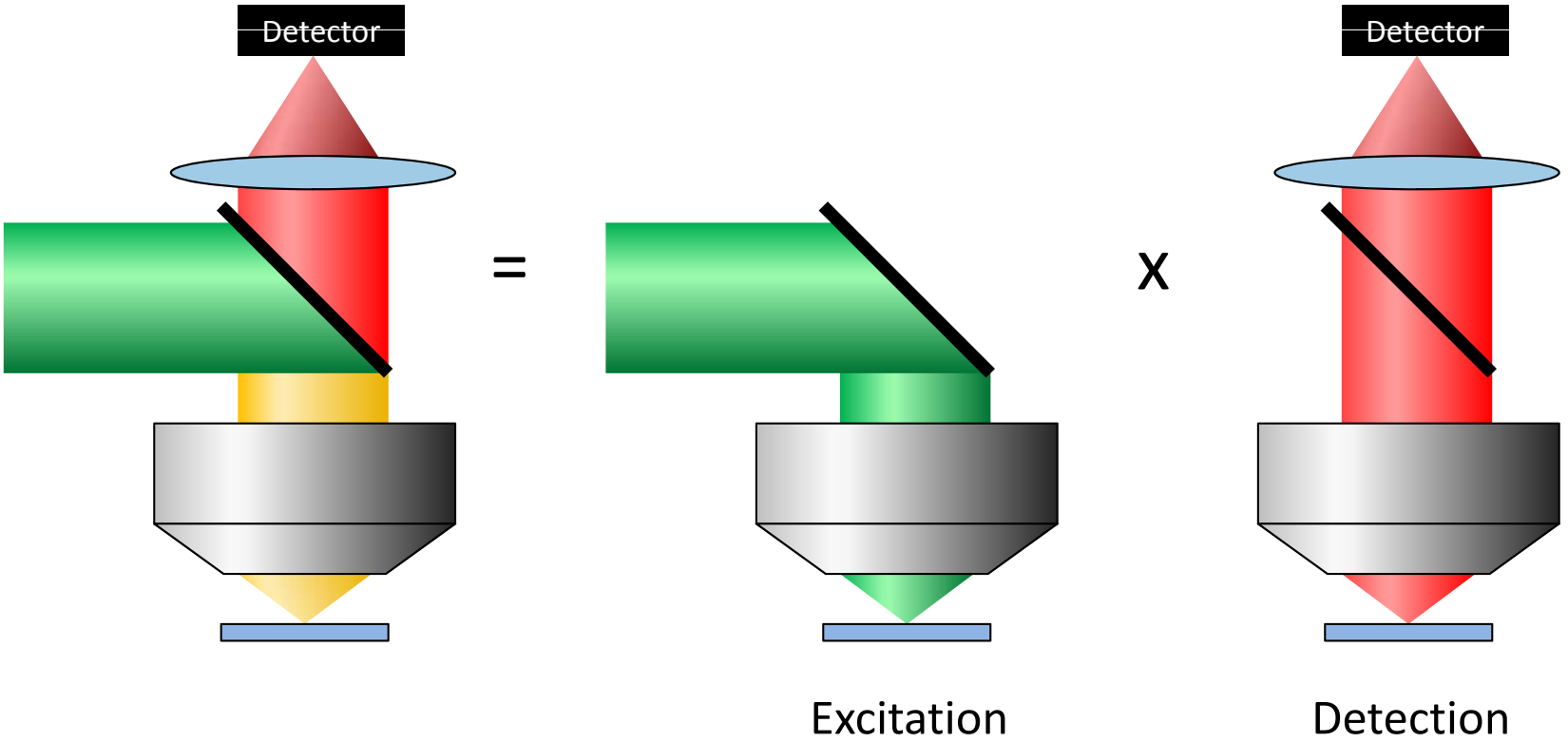
$$d \approx \frac{\lambda}{2\,NA}$$



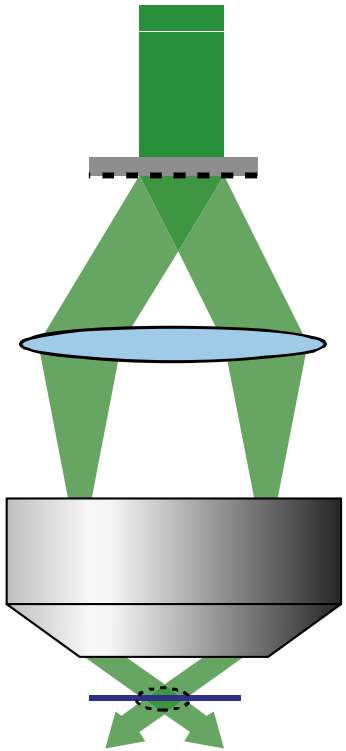


Major advantage:
Similar z resolution as x-y resolution

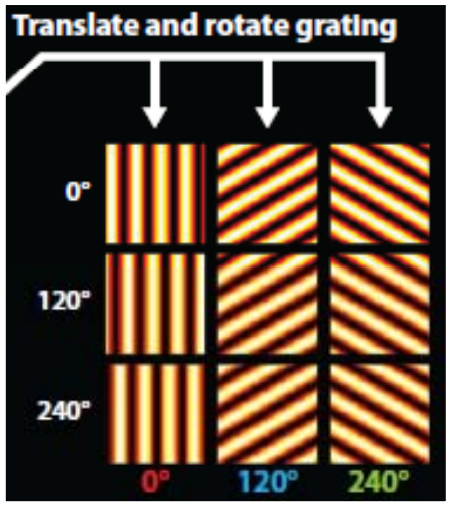
Patterned illumination



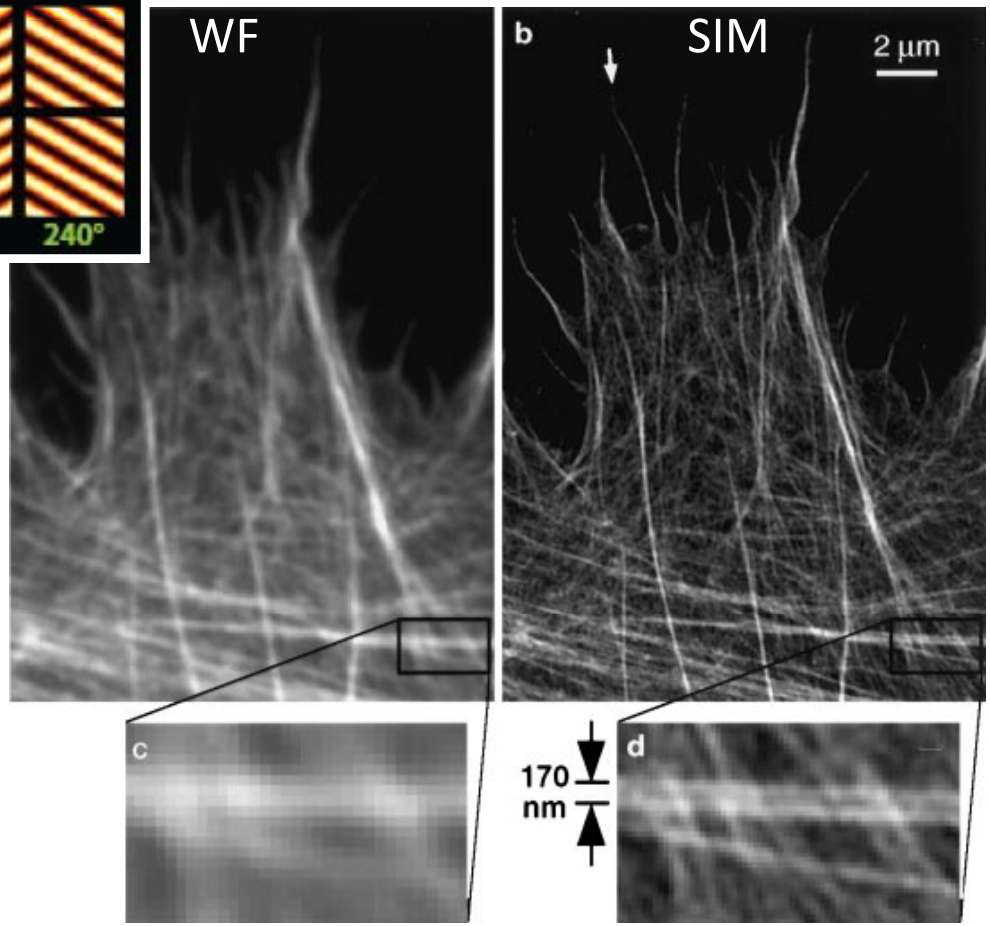
Structured Illumination Microscopy (SIM)

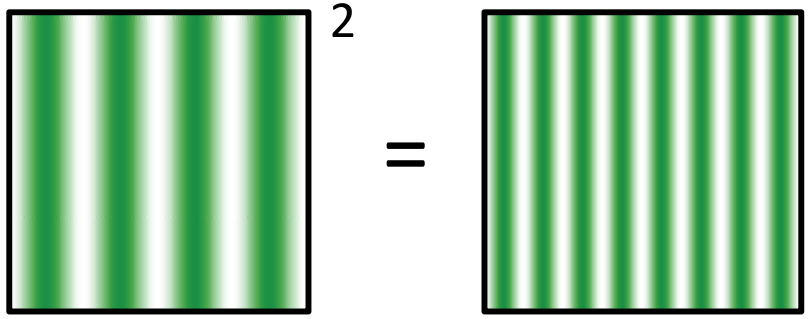


9 images



Reconstruction

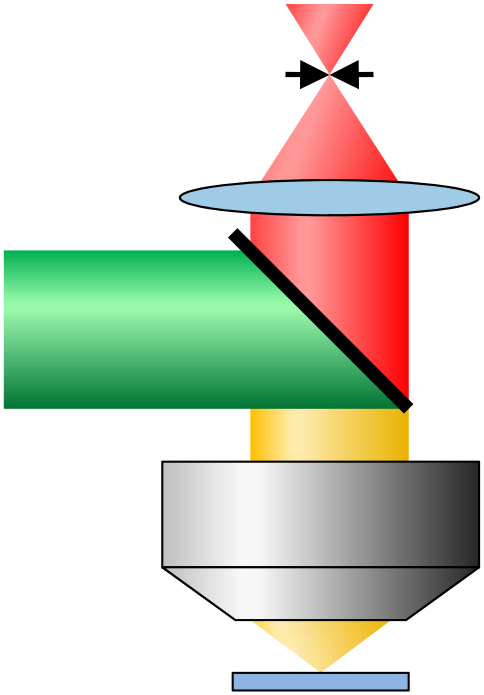




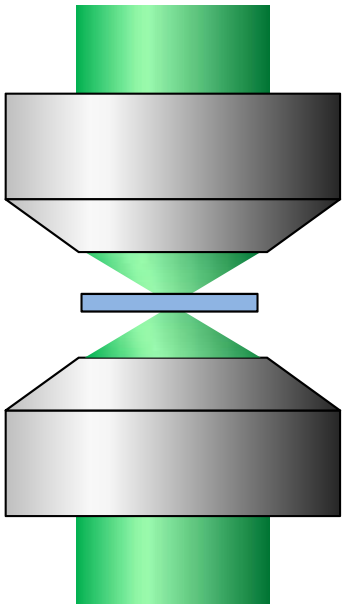
Gustafsson, J Microscopy 2000

The diffraction limit still exists

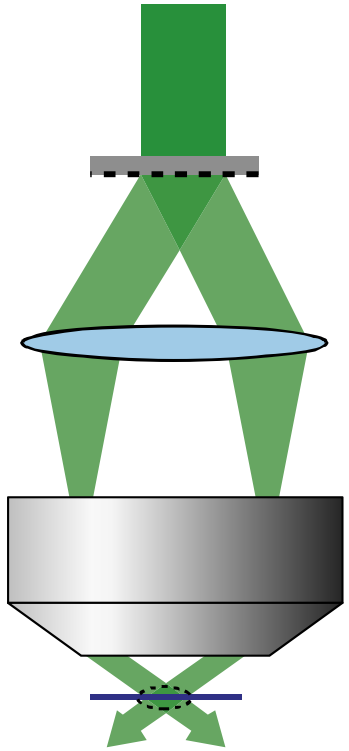
$$d \geq \frac{1}{2} \cdot \frac{\lambda}{2NA}$$



Confocal



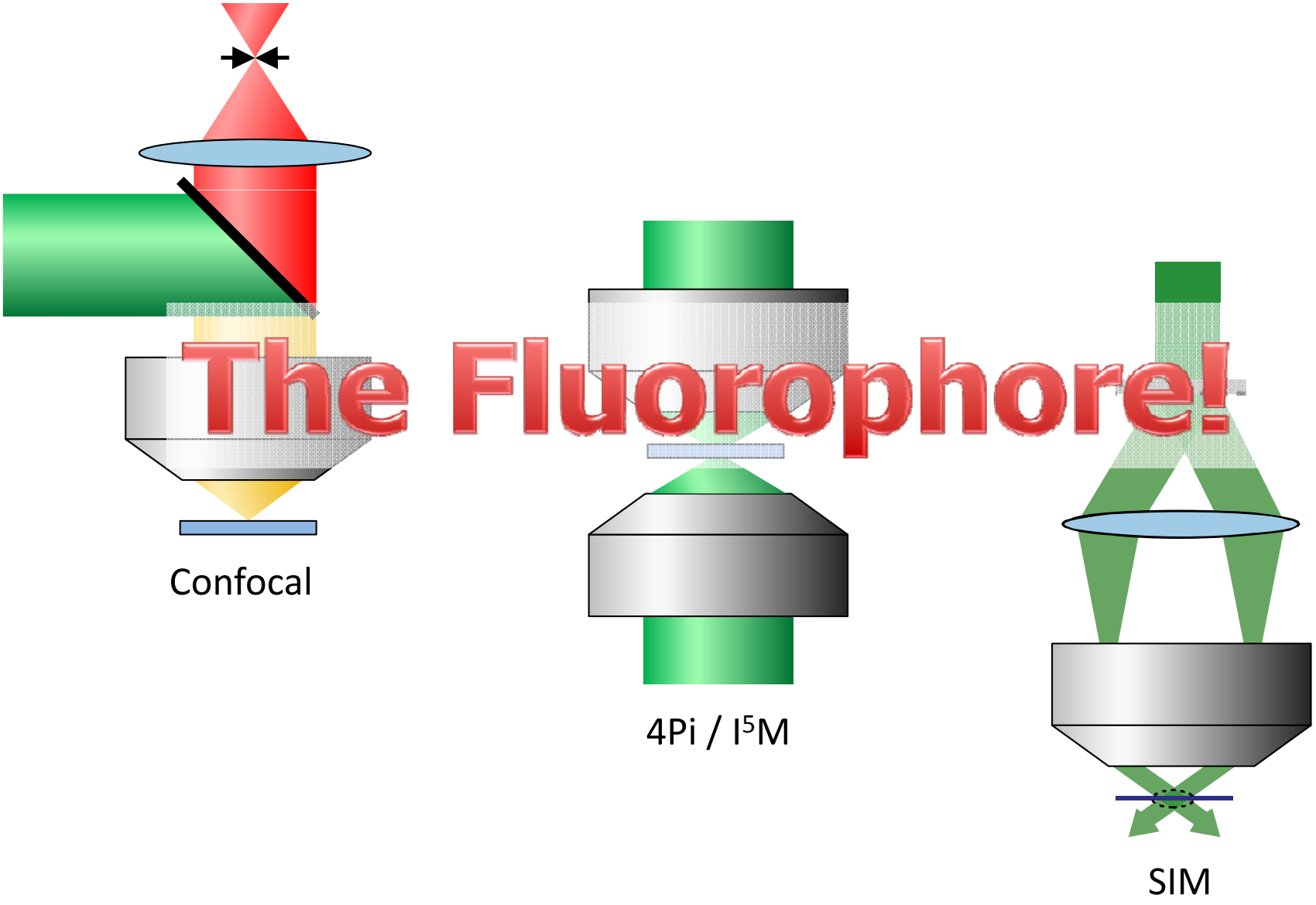
4Pi / I^{5}M



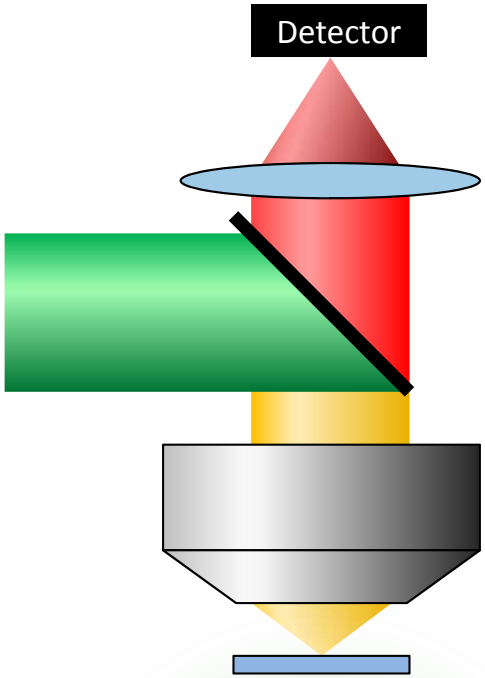
SIM

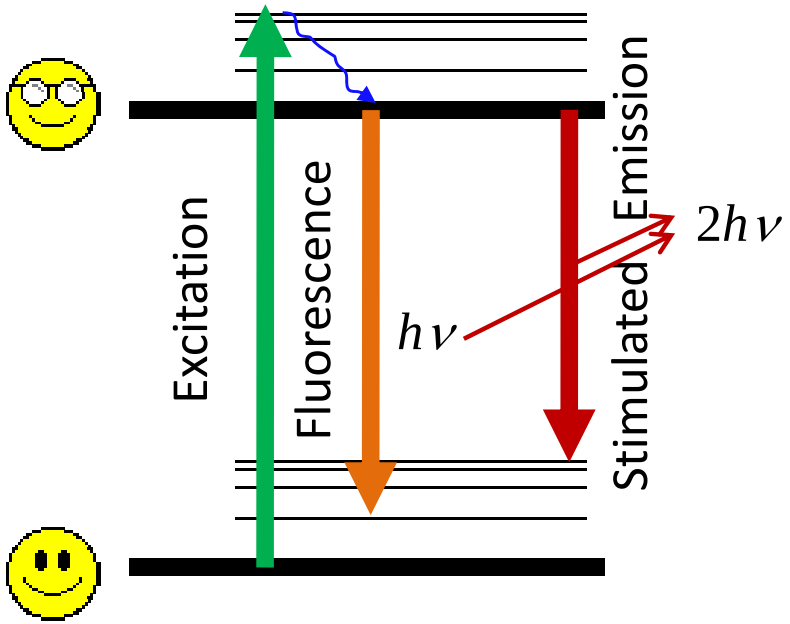
Breaking the diffraction barrier

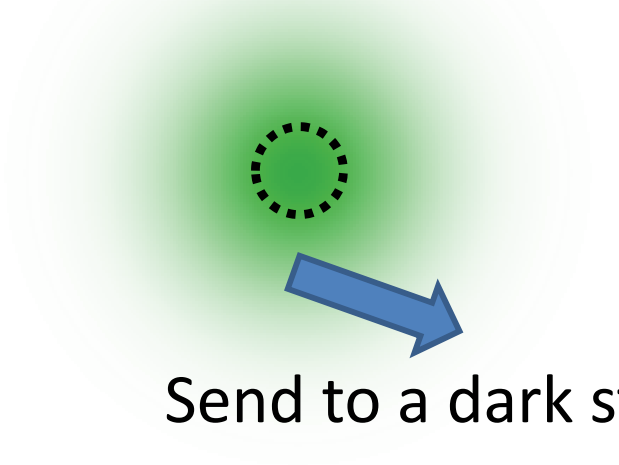
Breaking the diffraction barrier



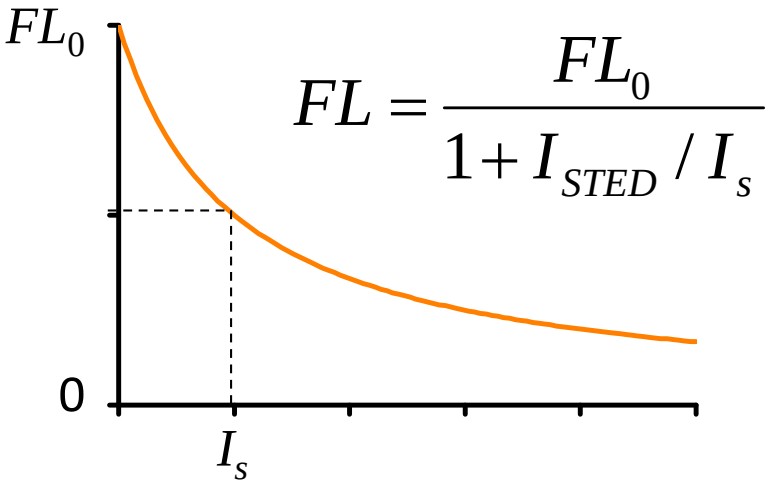
Stimulated Emission Depletion (STED)





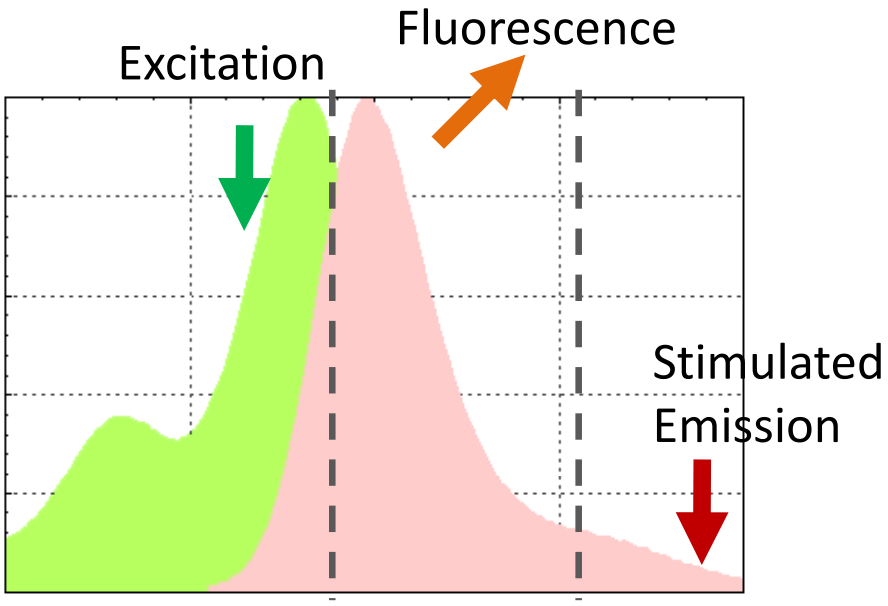


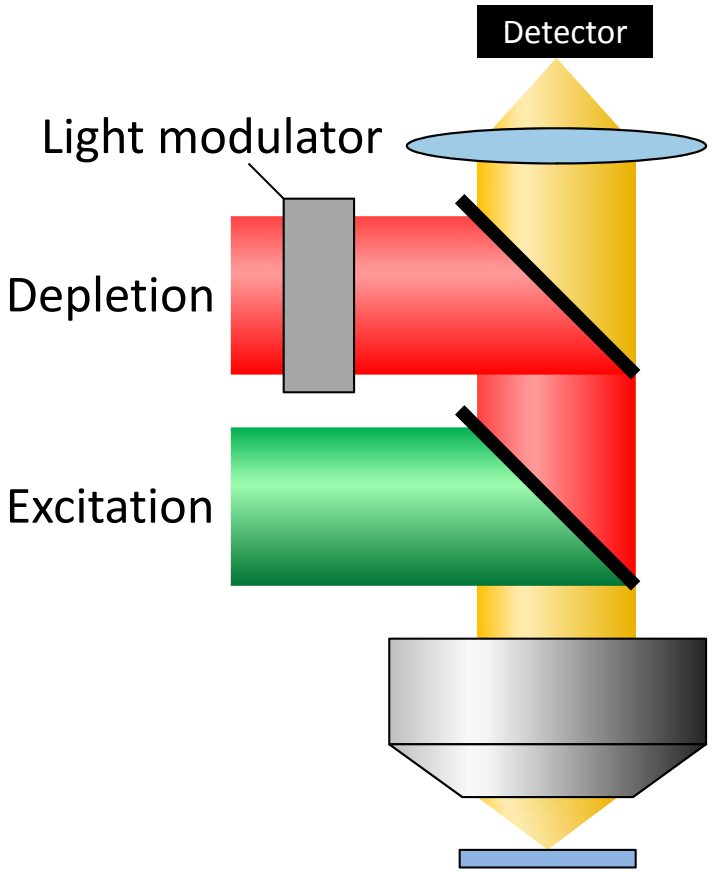
Send to a dark state

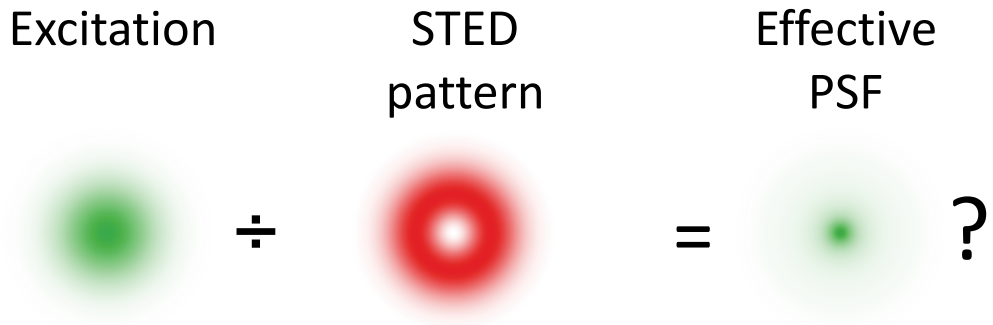


$$FL = \frac{FL_0}{1 + I_{STED} / I_s}$$

STED microscopy

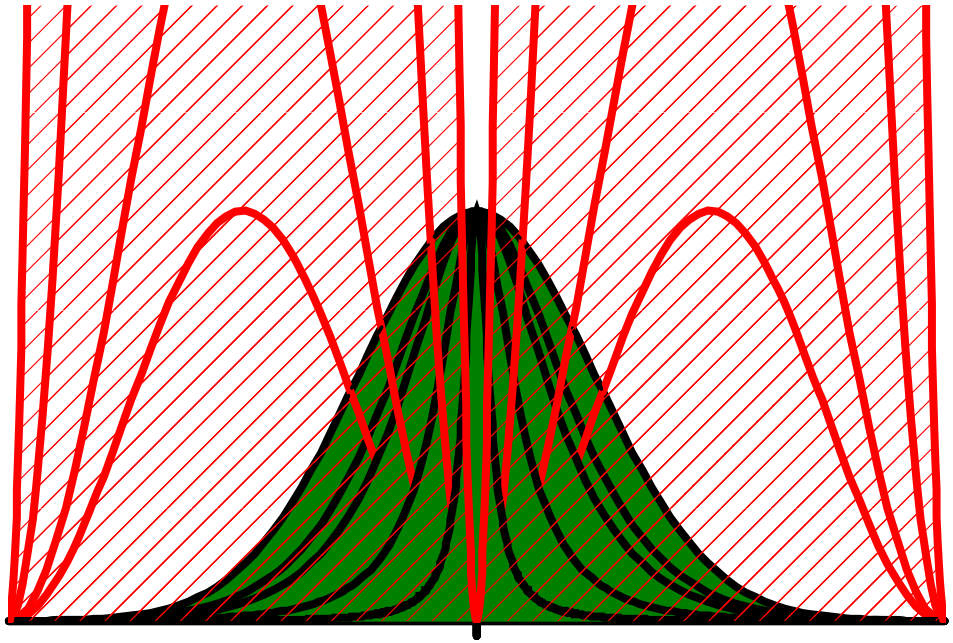






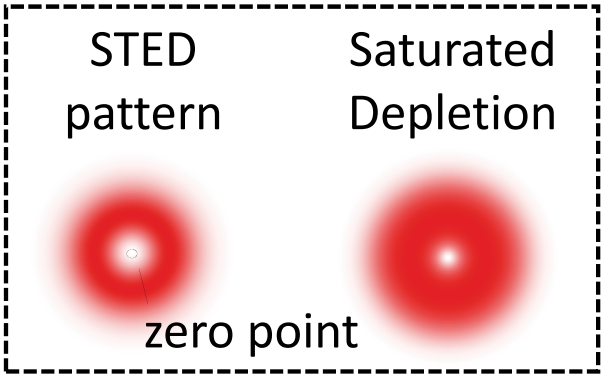
Hell 1994, Hell 2000

Saturated depletion

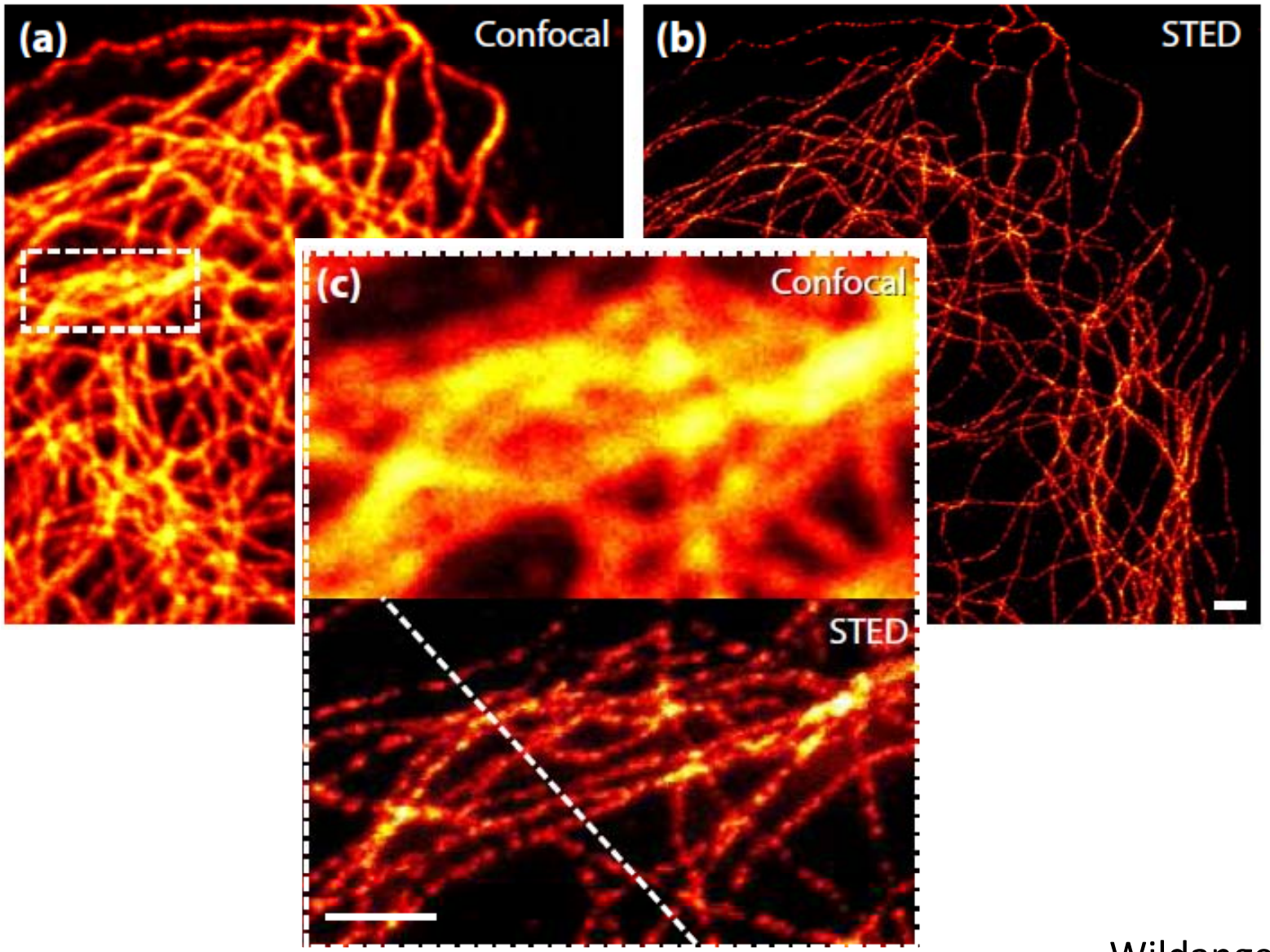


$$I_{\mathrm{STED}} = 100 I_S$$

$$d = \frac{1}{\sqrt{1 + I / I_s}} \cdot \frac{\lambda}{2NA}$$

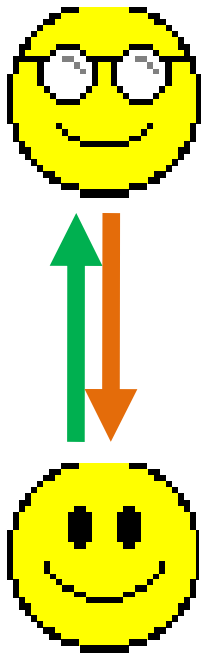


STED images of microtubules

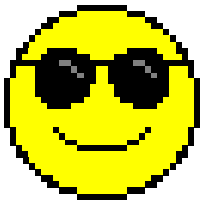


Wildanger et al., 2009

The “patterned illumination” approach



Multiple cycles



- Ground state
- Triplet state
- Isomerization

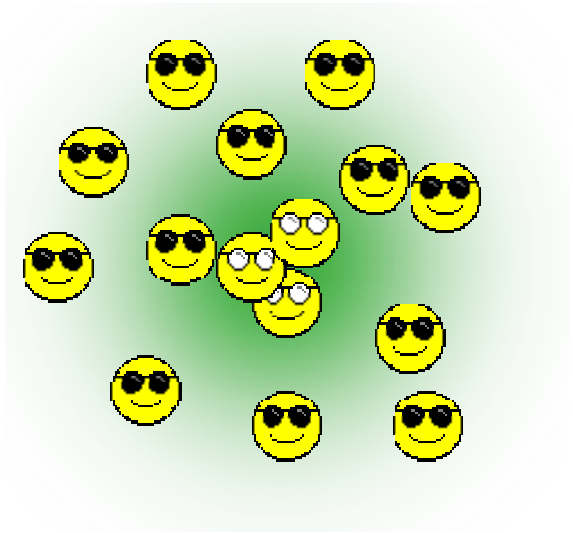
etc.

Excitation

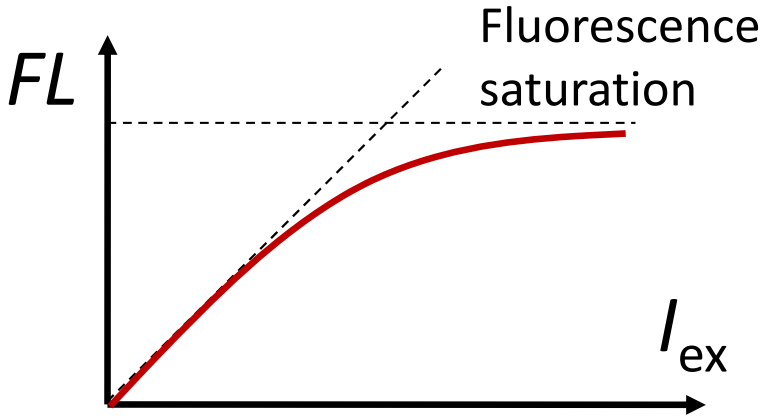
Depletion pattern

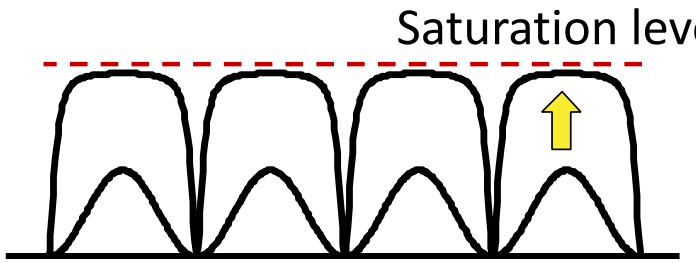
÷

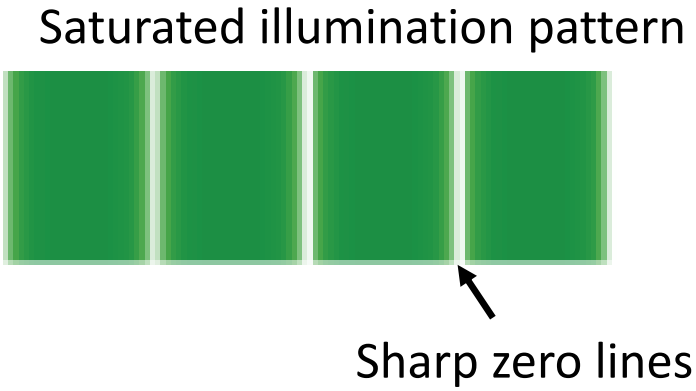
=

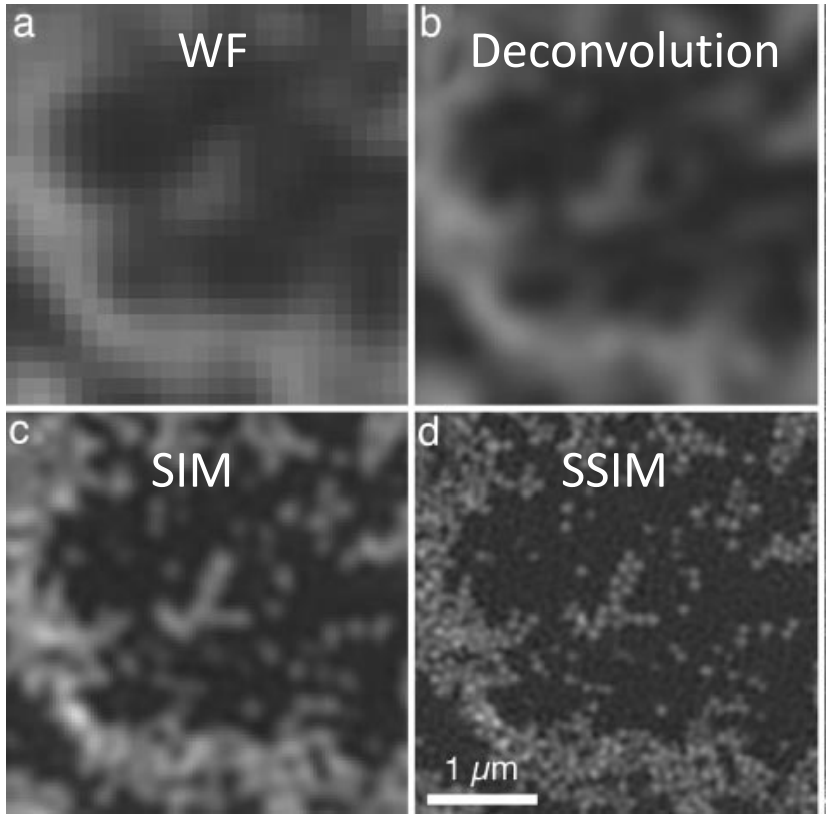


Saturated SIM









50 nm resolution

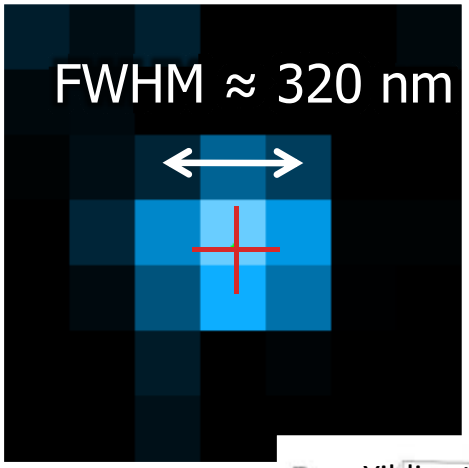
Suffers from fast photobleaching under saturated excitation condition

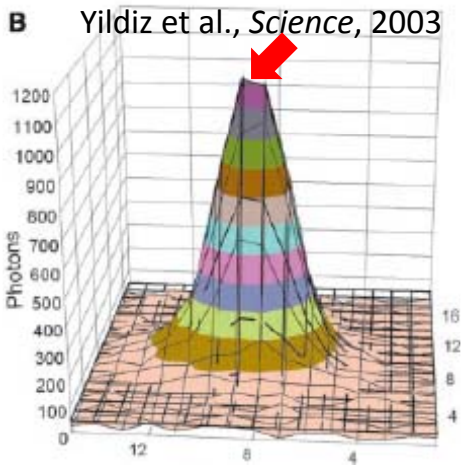
Gustaffson, PNAS 2005

The single-molecule switching approach

Single-Molecule Localization

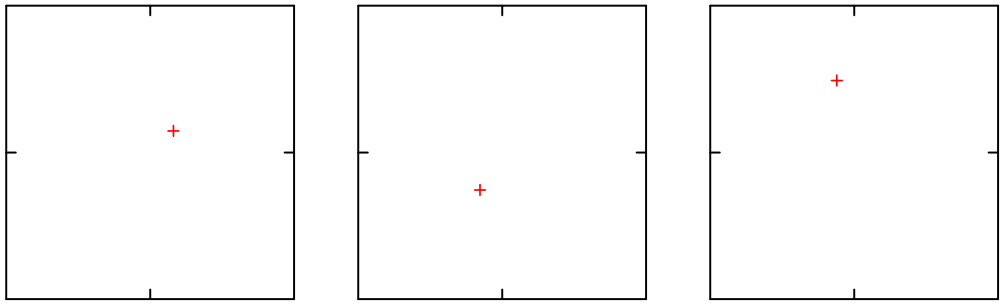
Image of one fluorescent molecule





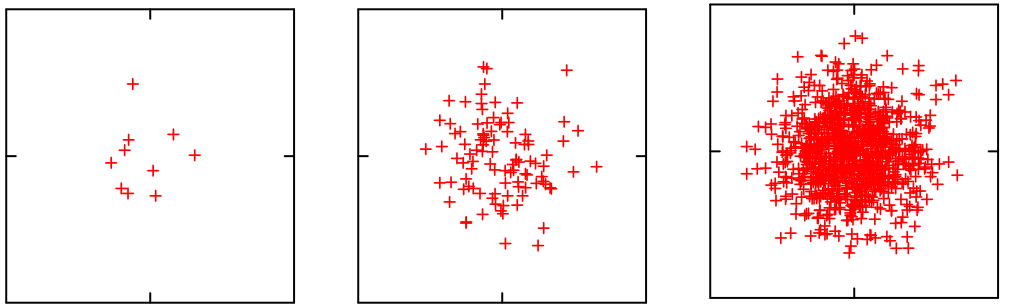
Yildiz et al., *Science*, 2003

Single-molecule localization precision

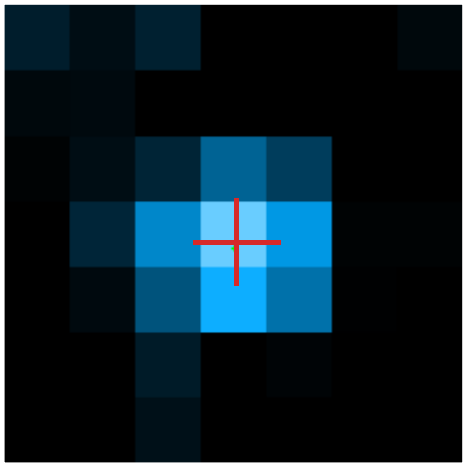


1 photon

$$d \approx \frac{\lambda}{2\,NA}$$



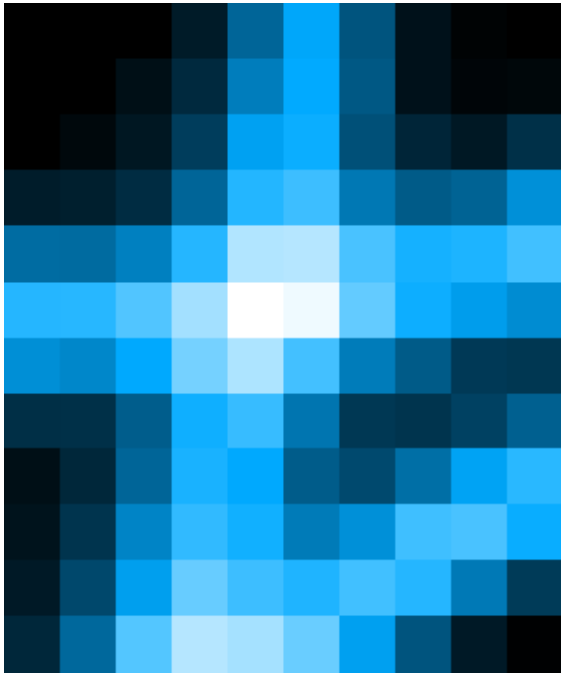
10 photons 100 photons 1000 photons



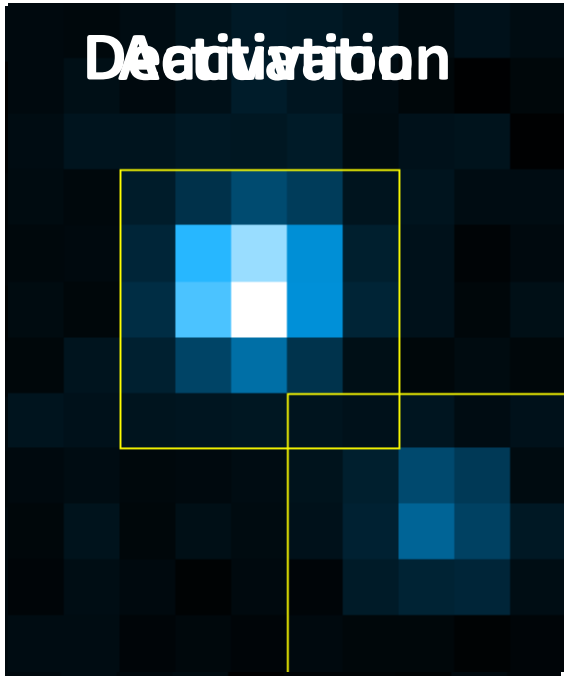
$$d = \frac{1}{\sqrt{N}} \cdot \frac{\lambda}{2NA}$$

Super-resolution imaging by localization

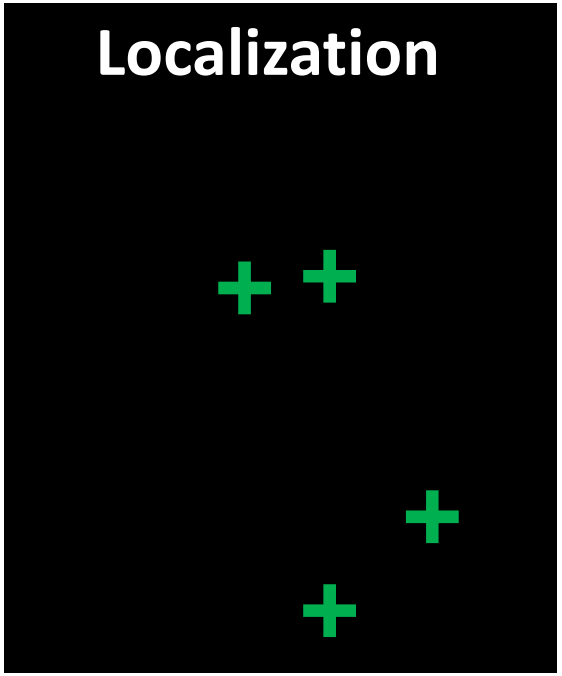
Conventional fluorescence



Raw images



STORM Image



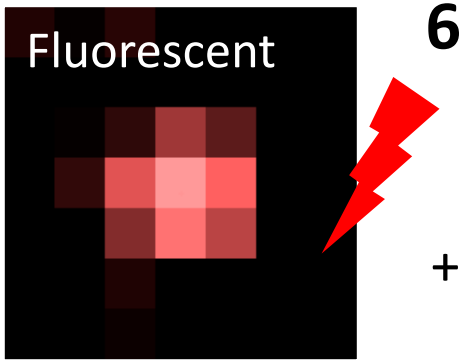
2x real time

Stochastic Optical Reconstruction Microscopy = STORM

Also named as PALM (Betzig et al., Science, 2006) and FPALM (Hess et al., Biophys. J. 2006)

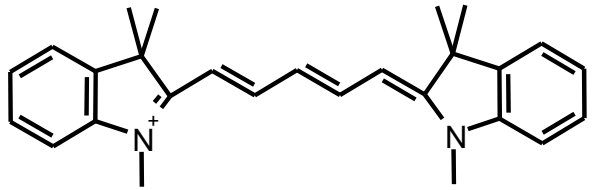
Huang et al., Annu Rev Biochem, 2009

Photoswitching of red cyanine dyes



650 nm

+ thiol



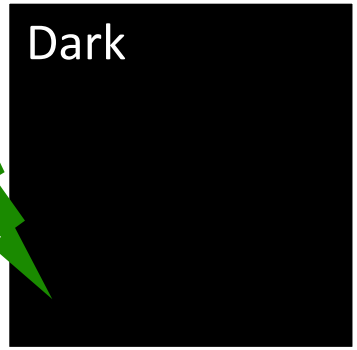
Cy5 / Alexa 647

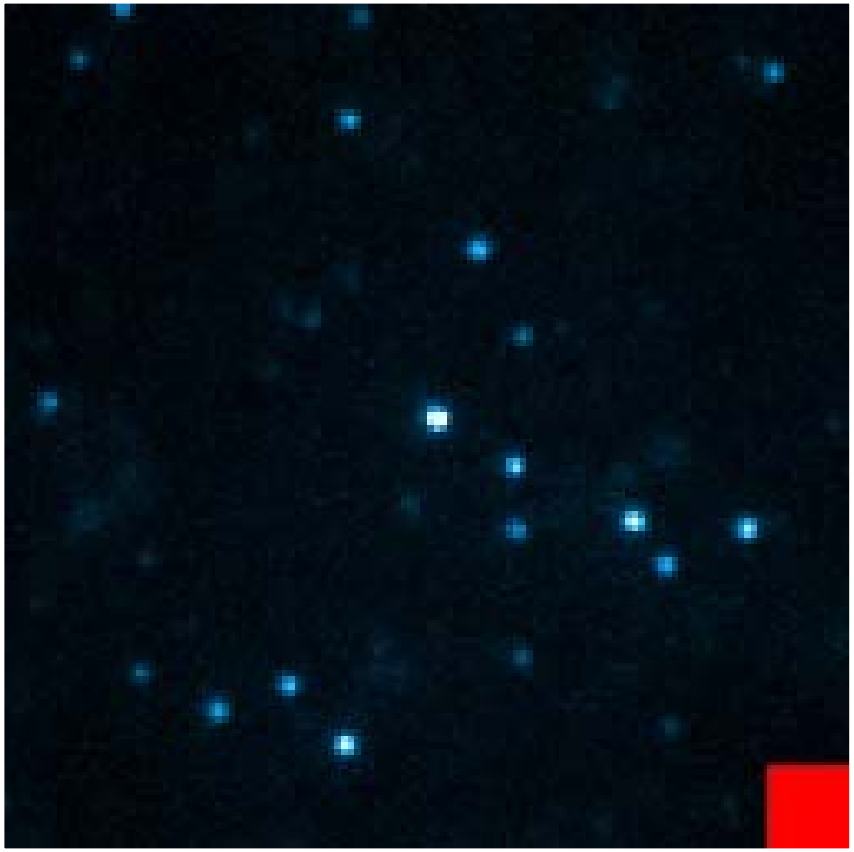
photoactivation

Deactivation

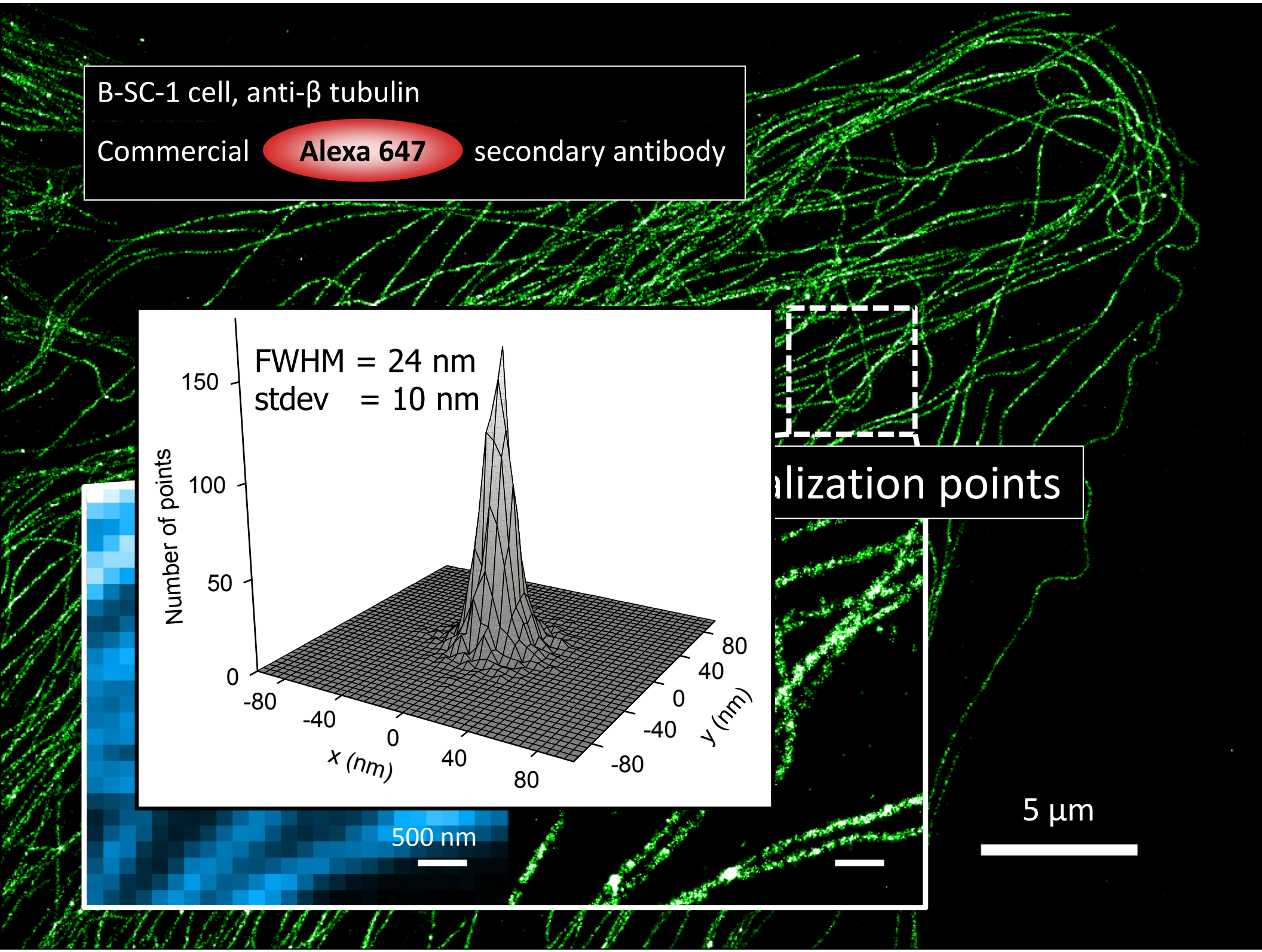
360 nm

650 nm

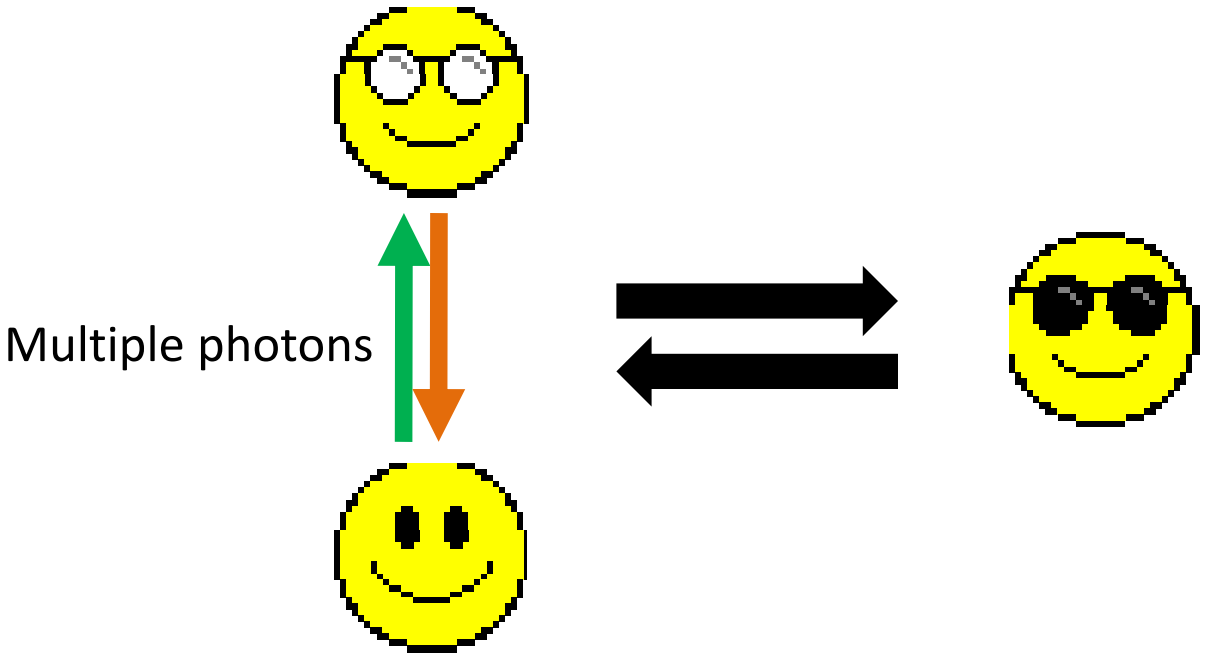




Bates eta l., *PRL* 2005, Bates et al., *Science* 2007, Dempsey et al., *JACS* 2009


B-SC-1 cell, anti-β tubulin
Commercial
Alexa 647
secondary antibody
FWHM = 24 nm
stdev = 10 nm
Number of points
150
100
50
0
-80
-40
0
40
80
x (nm)
y (nm)
lization points
500 nm
5 µm

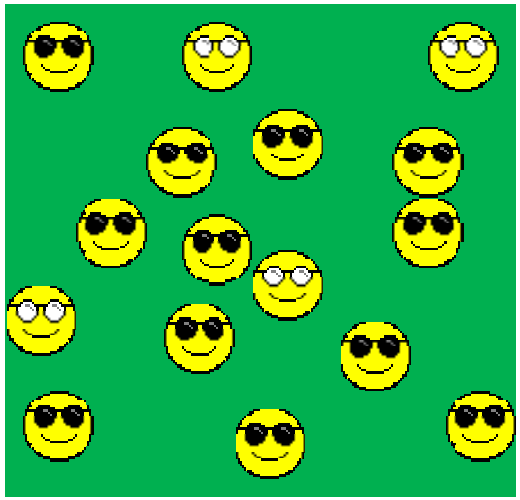
The “single-molecule switching” approach



- Photoswitching
- Blinking
- Diffusion
- Binding

  etc.

\+ Stochastic Switching =



STORM probes commercially available or already in your lab

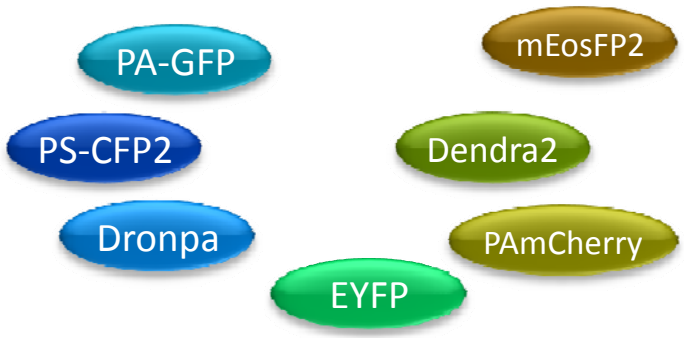
Cyanine dye + thiol system

Bates et al., 2005, Bates et al., 2007, Huang et al., 2008

Rhodamine dye + redox system

Heilemann et al., 2009

Photoactivatible fluorescent proteins

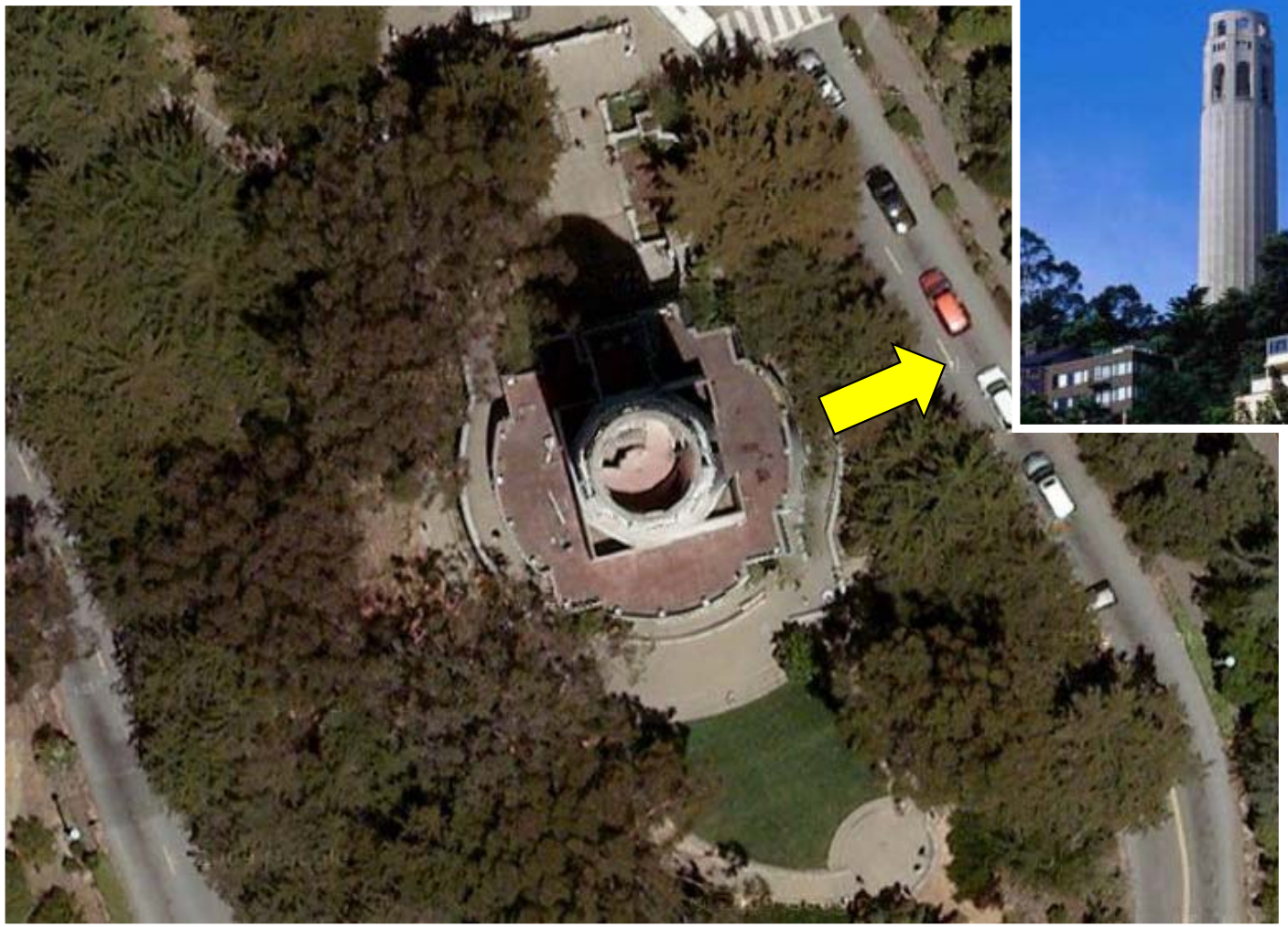


Reviews:
Lukyanov et al., Nat. Rev. Cell Biol., 2005
Lippincott-Schwartz et al., Trends Cell Biol., 2009

3D Imaging

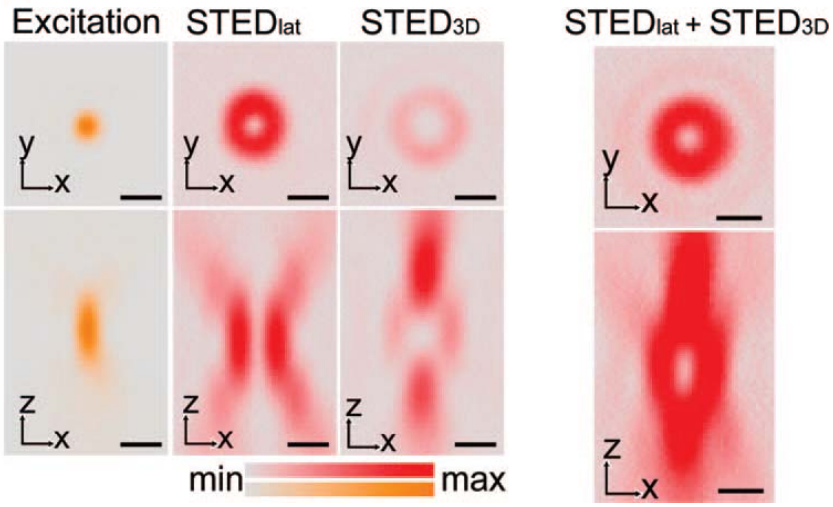
In a 2D world…

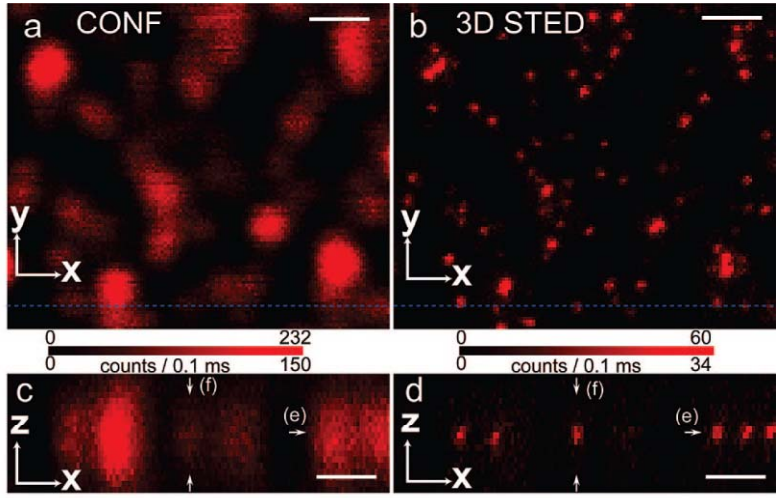
Satellite image of ???

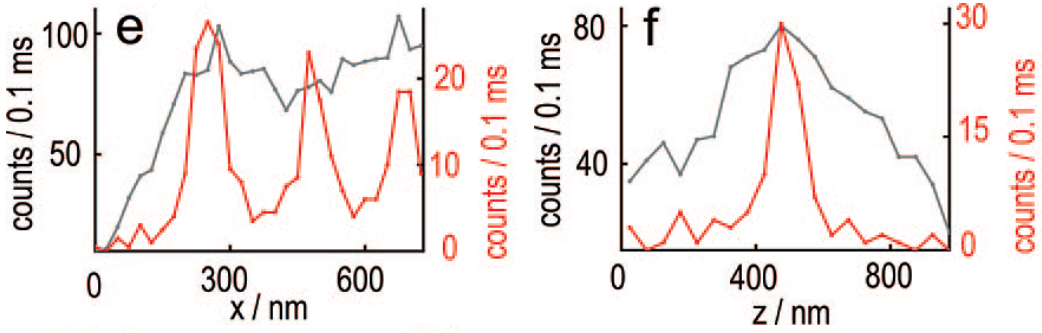


Google maps

3D STED

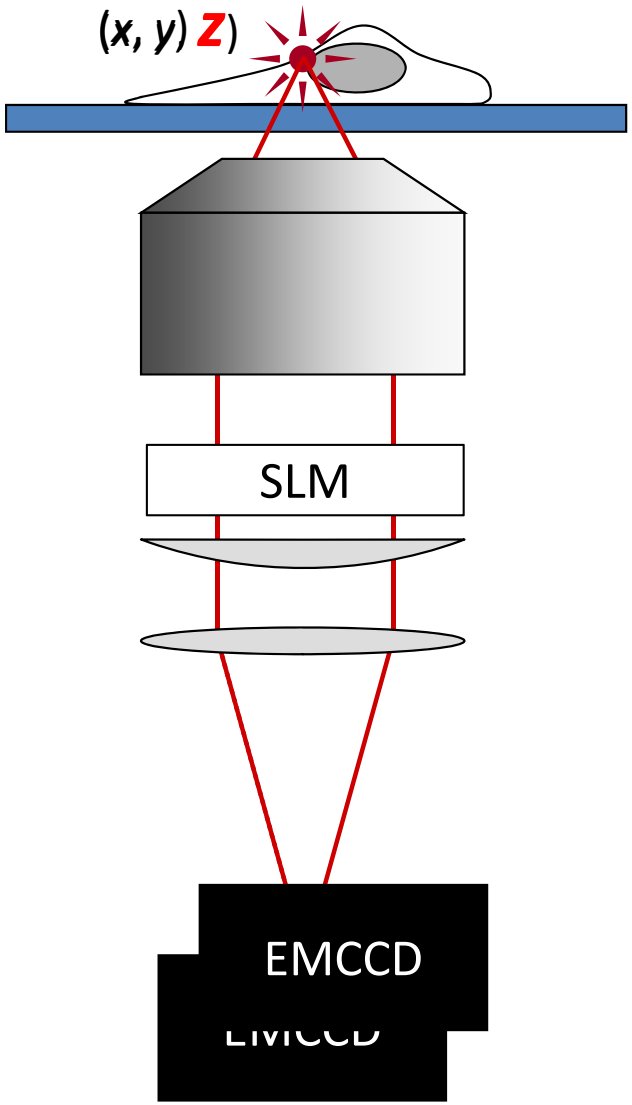




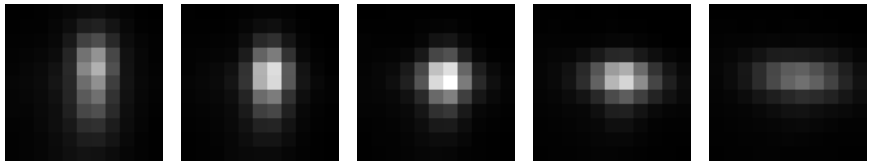


Harke et al., Nano Lett, 2008

3D STORM/PALM



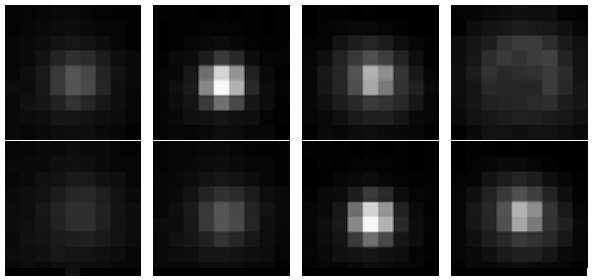
Astigmatic imaging



z (nm) -400 -200 0 200 400

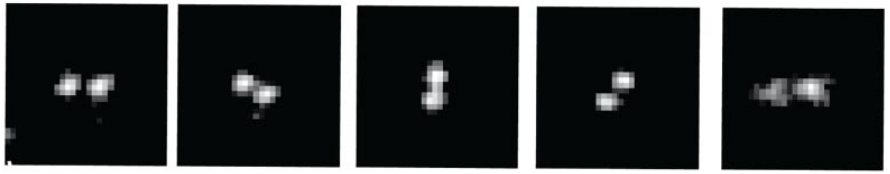
Huang et al., Science 2008

Bi-plane imaging



Juette et al., Science 2008

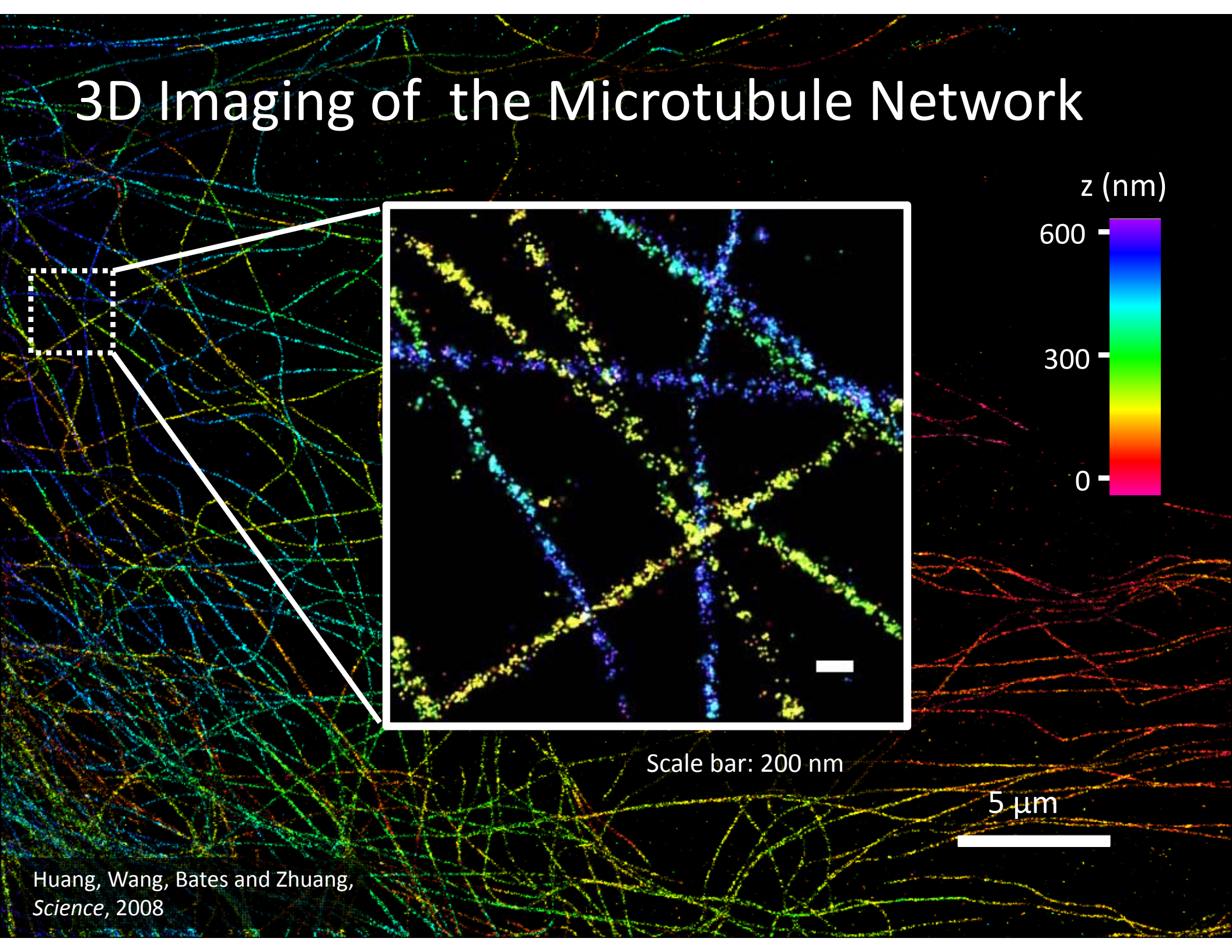
Double-helical PSF



z (nm) -900 -500 0 600 1400

Pavani et al., PNAS 2009

3D Imaging of the Microtubule Network



Huang, Wang, Bates and Zhuang,
Science, 2008

3D Imaging of the Microtubule Network

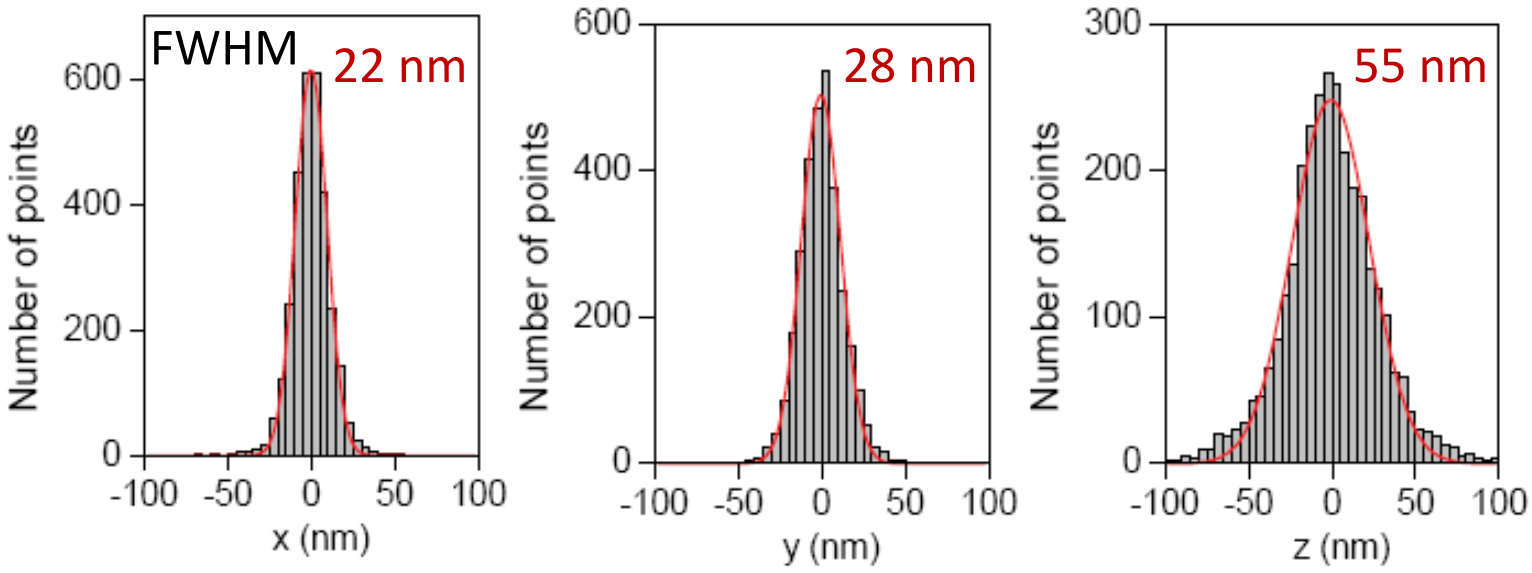
z (nm)

600

300

0

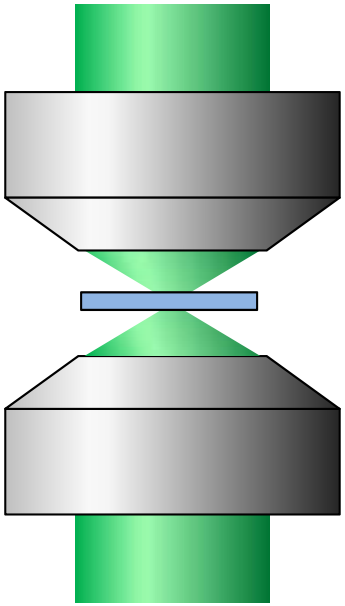
Small, isolated clusters



5 µm

Huang, Wang, Bates and Zhuang,
Science, 2008

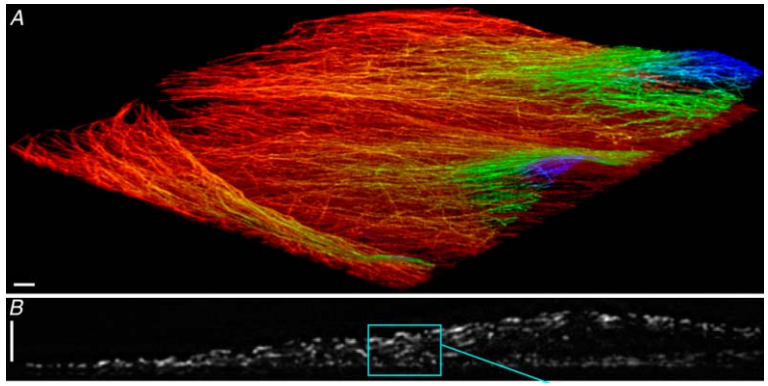
The use of two opposing objectives



4Pi scheme

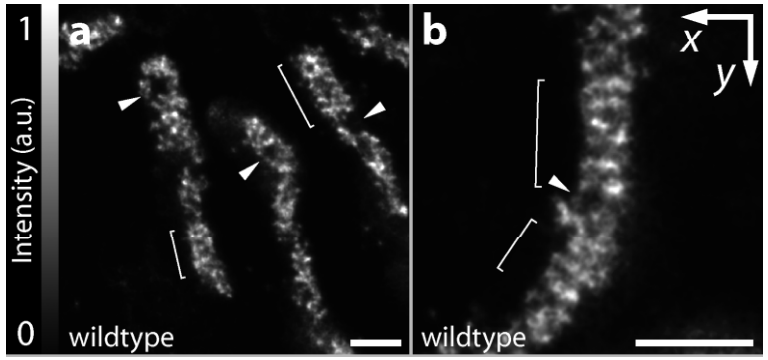
Near isotropic
3D resolution

I^{5}S



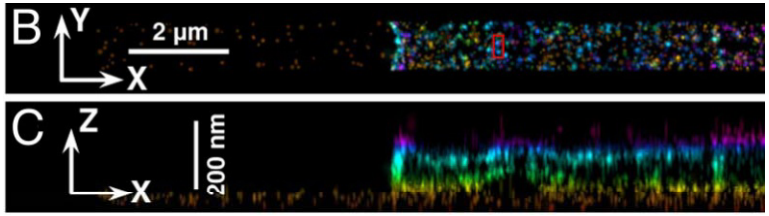
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



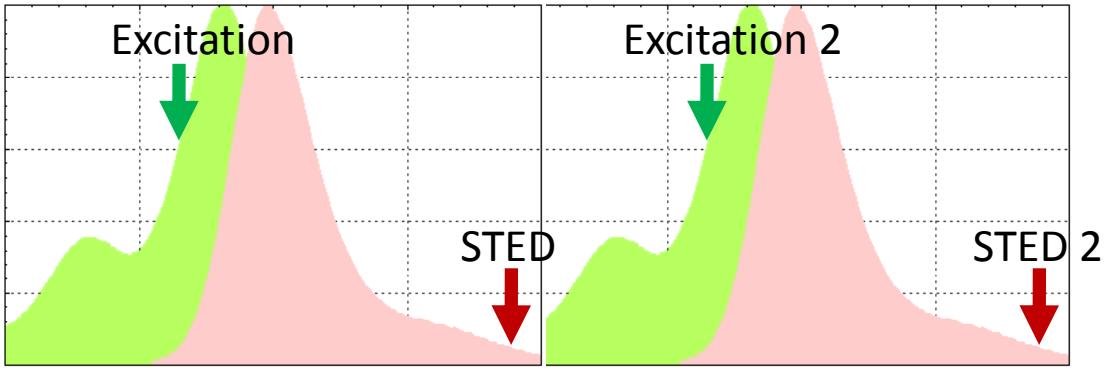
Shtengel et al., PNAS 2009

3D resolution of super-resolution methods

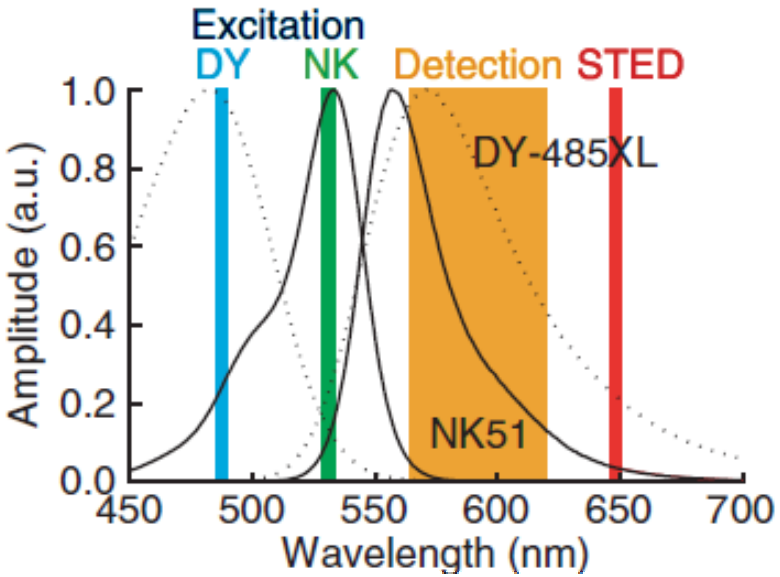
	x-y (nm)	z (nm)	Opposing objectives (nm)	Two-photon
Conventional	250	600	4Pi: 120	
SIM	100	250	I^5S: 120 xyz	
STED	~30	~100	isoSTED: 30 xyz	100 µm deep
STORM/PALM	20-30	50-60	iPALM: 20 xy, 10 z	

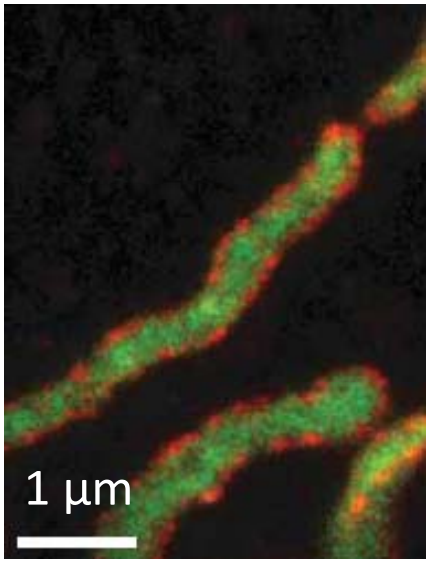
Multi-color Imaging

Muticolor STED



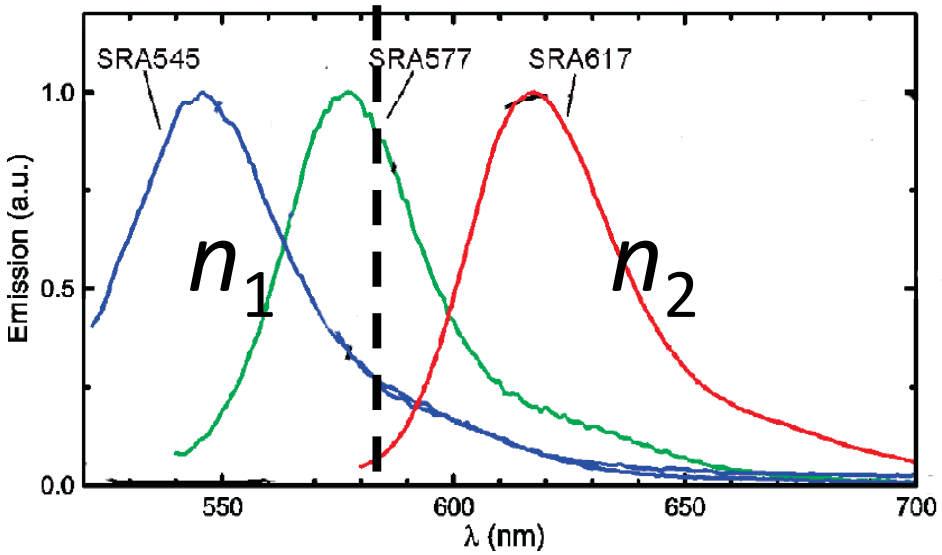
2 color isoSTED resolving
the inner and outer membrane
of mitochondria





Schmidt et al., Nat Methods 2008

Multicolor STORM/PALM: Emission

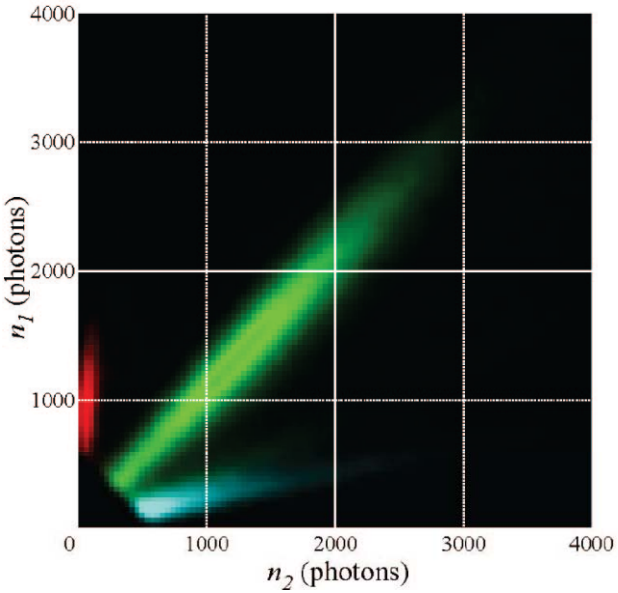


$n_1 = n_2$

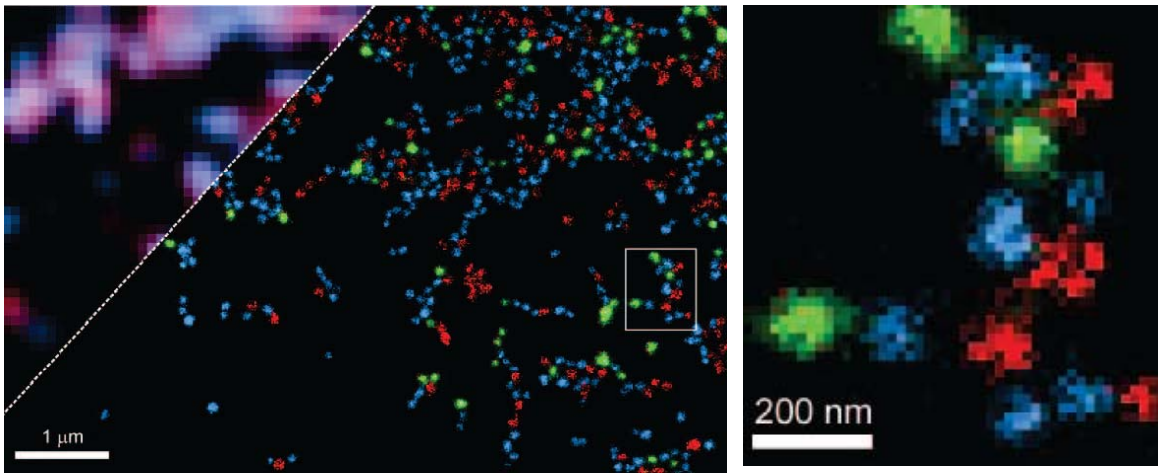
➔ 50% SRA545 + 50% SRA617?

➔ 100% SRA577?

Single-molecule detection!

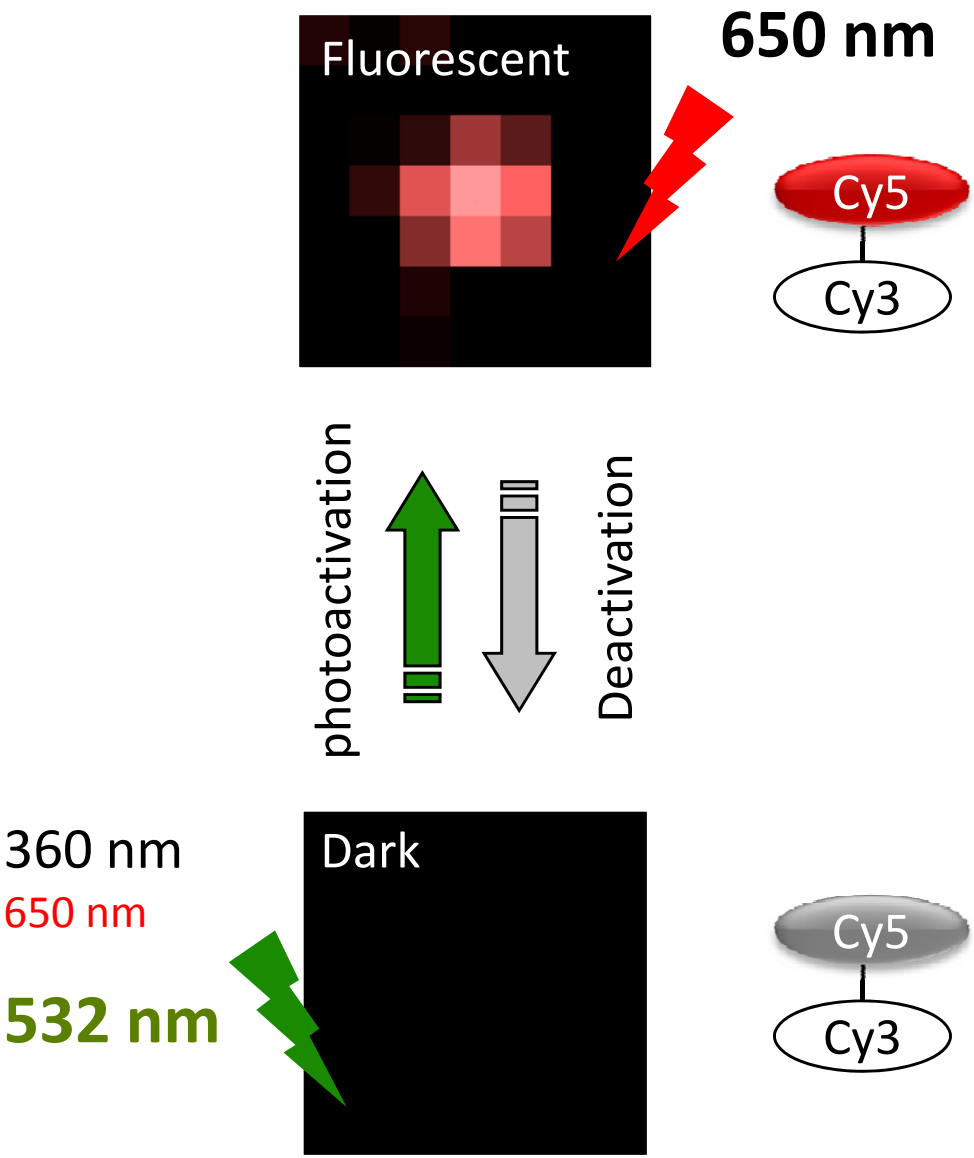


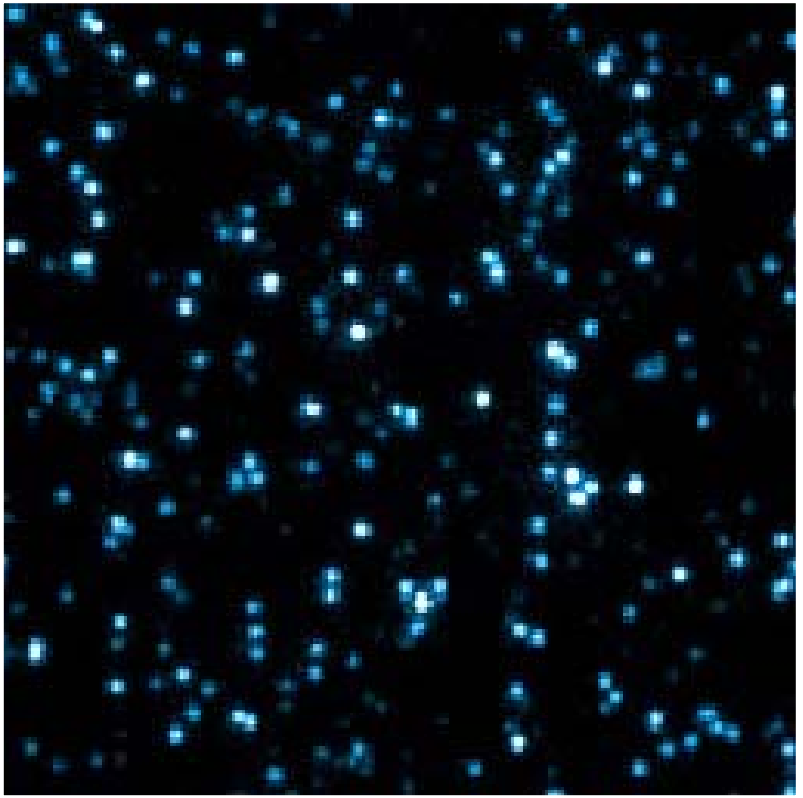
3-color imaging with one excitation wavelength and two detection channels

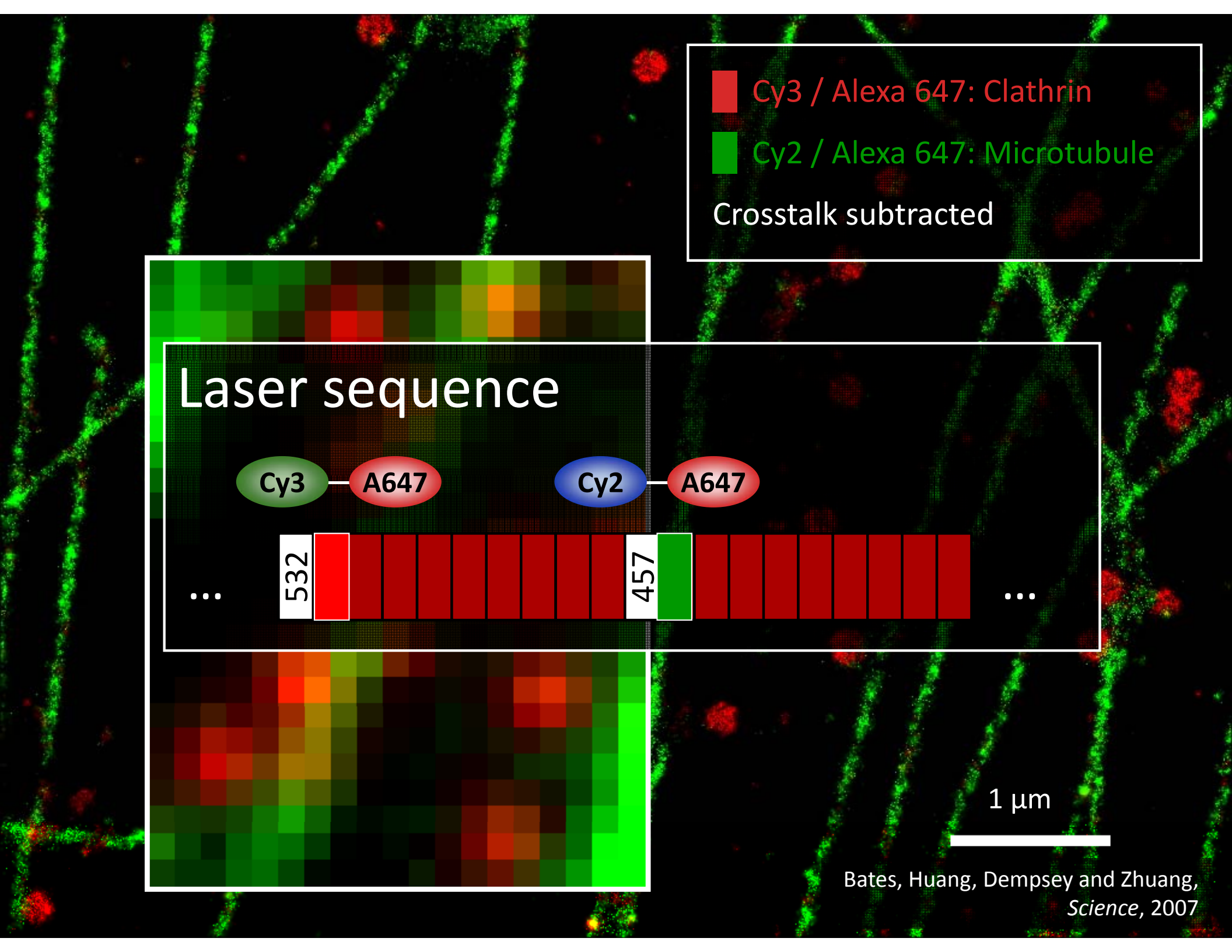


Bossi et al., Nano Lett 2008

Multicolor STORM/PALM: activation







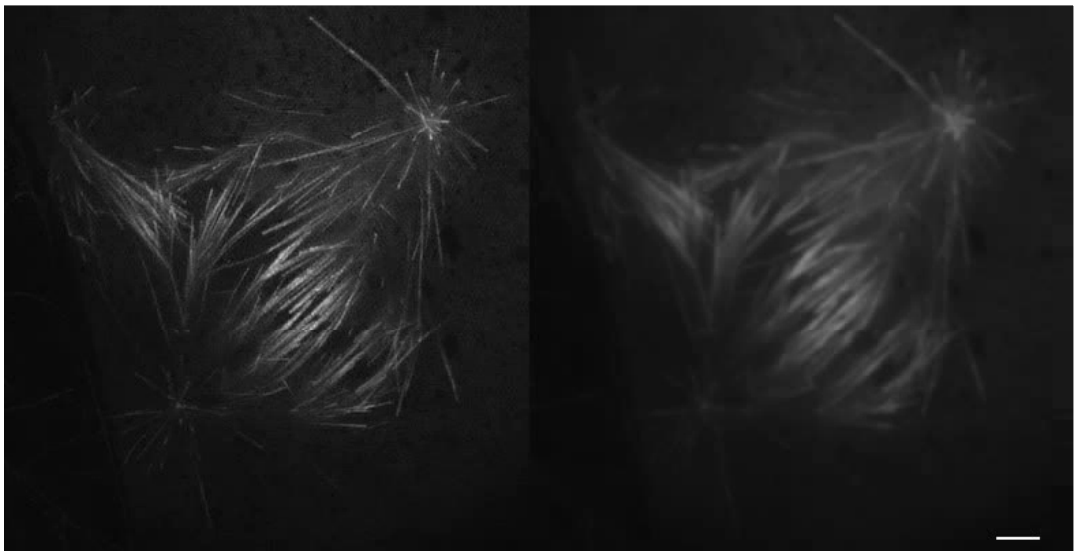
Bates, Huang, Dempsey and Zhuang,
Science, 2007

Multicolor imaging

	Multicolor capability
Conventional SIM	4 colors in the visible range
STED	2 colors so far
STORM/PALM	3 activation x 3 emission

Live Cell Imaging

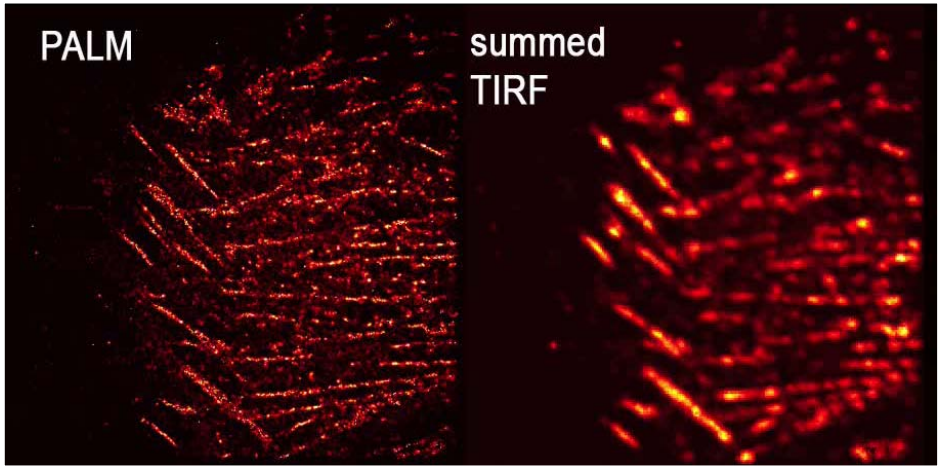
SIM



2 µm

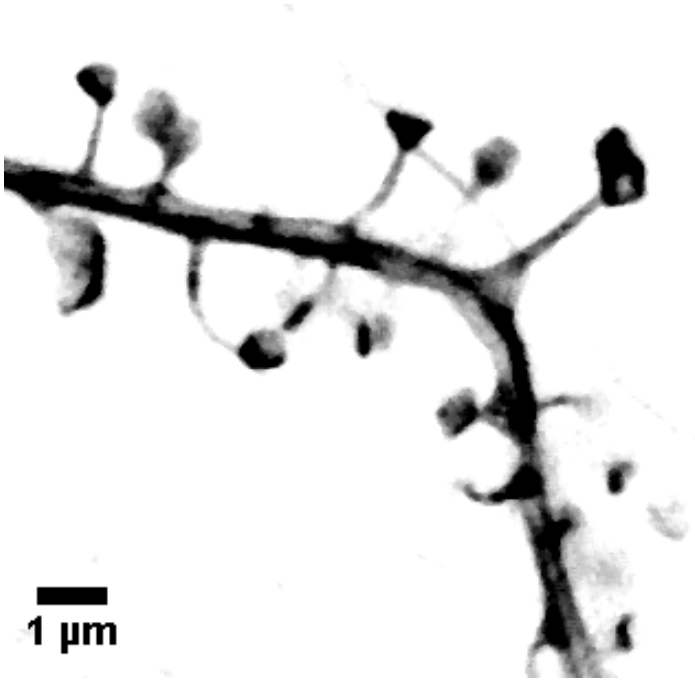
Kner, Chhun et al., Nat Methods, 2009

STORM/PALM



Schroff et al., Nat Methods, 2008

STED



Nagerl et al., PNAS, 2008

The limit of “Super-Resolution”

Unbound theoretical resolution

$$d = \frac{1}{\boldsymbol{S}} \cdot \frac{\lambda}{2NA}$$

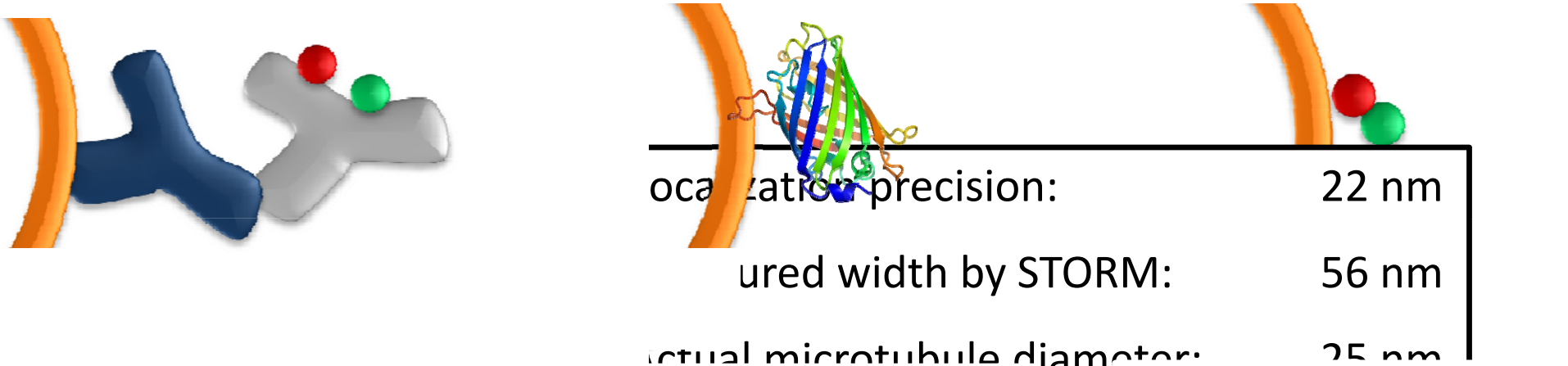
- STORM/PALM $S = \sqrt{N}$
  - 6,000 photons → 5 nm
  - 100,000 photos during Cy5 life time → < 1 nm
- STED $S = \sqrt{1 + I/I_s}$
  - 1:100 contrast of the donut → 20 nm
  - Diamond defects: 8 nm

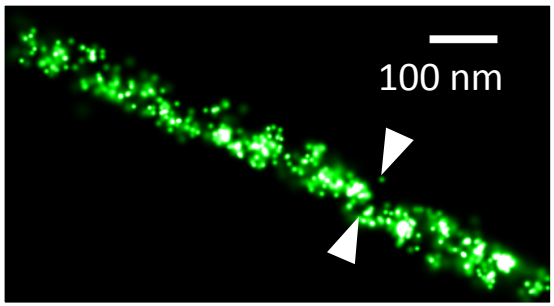
Effective resolution: Probe size matters

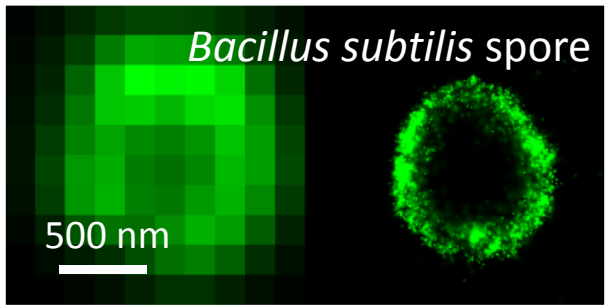
Antibodies:
~ 10 nm

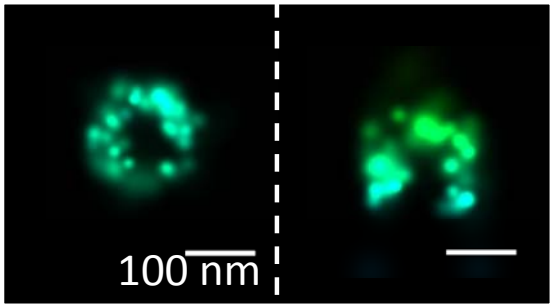
Fluorescent Proteins:
~ 3 nm

Small fluorophores:
~ 1 nm







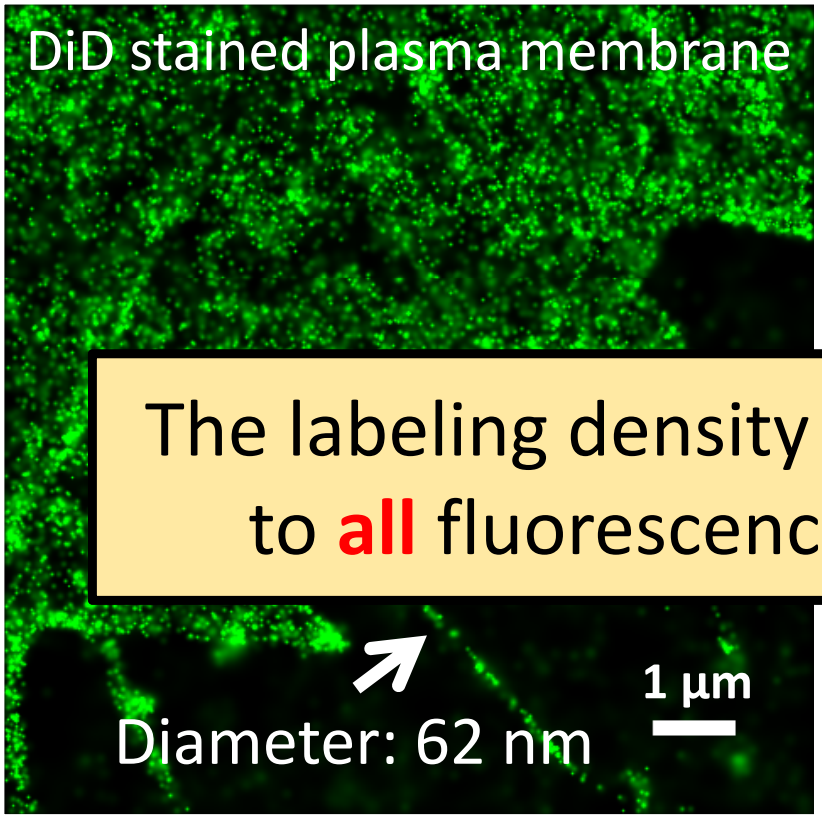


~ 6000 photons

< 1000 photons

~ 6000 photons

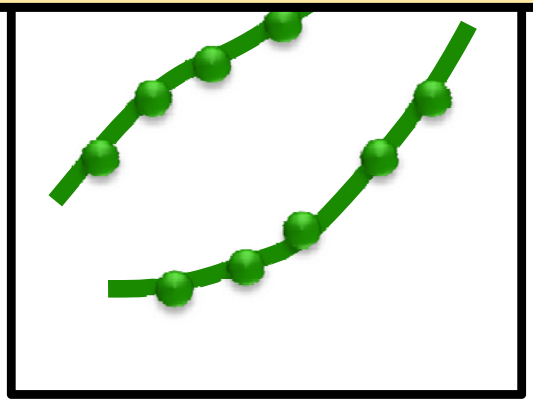
Effective resolution: Density matters



1000 frames, 10 sec total time

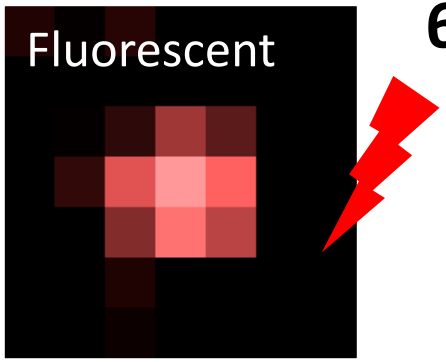
- Localization precision
  - 1000 photons -> 20 nm
- Localization density

The labeling density limit of resolution applies to **all** fluorescence microscopy methods



Point to point distance ≈ ½ Feature size

Effective resolution: contrast matters



650 nm

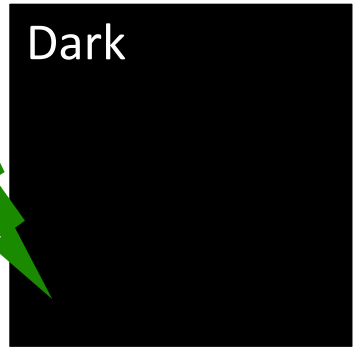
1%

photoactivation

Deactivation

360 nm

650 nm



99%

1% means…

Homogeneous sample Microtubule

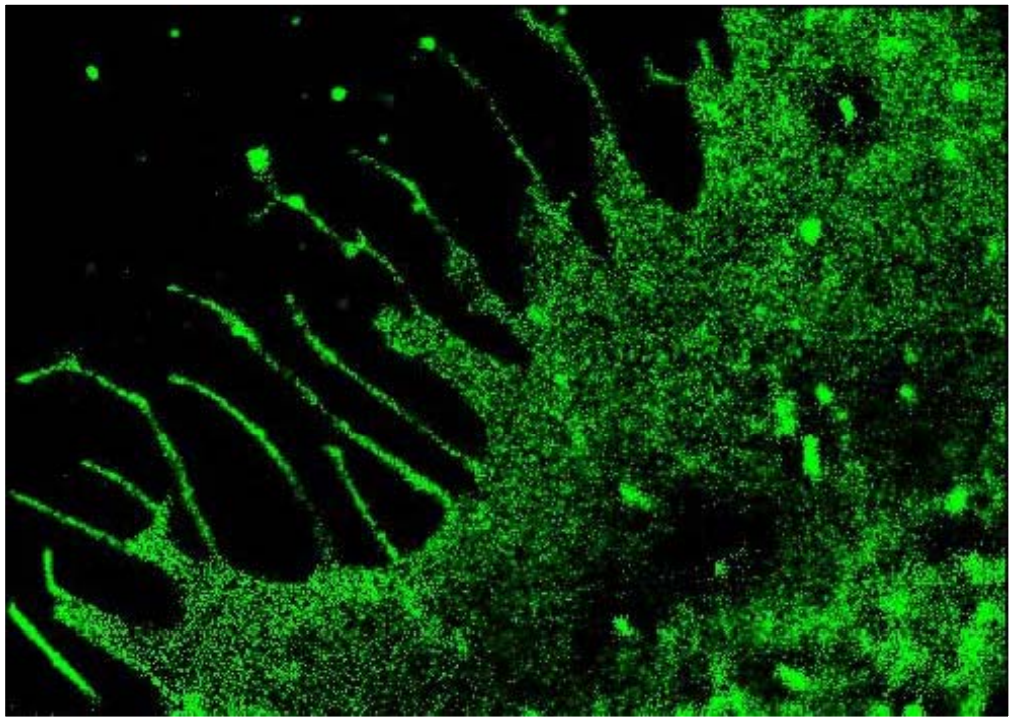
Common dye blinking: >3%

Cy5-MEA: 0.1-0.2%

mEos2: 0.001%

No such limit for non-single methods of SIM and STED

Time resolution: density matters



25 sec time resolution, 100x real time
3 mM mercaptoethylamine
1 µm

Typical Localization accumulation:
28 points / $\mu m^2 \cdot s$

Effective resolution:
70 nm at 25 sec integration time

Now as fast as 2 sec time resolution
with 1000 frames / sec camera

Comparison of time resolution

2D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	9 frames (0.09 sec)
STED	Scanning	60 nm	1 x 2 µm: 0.03 sec 10 x 20 µm: 3 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec)

3D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	15 frames x 10 (1.5 sec)
STED	Scanning	60 nm	1 x 2 x 0.6 µm: 0.6 sec 10 x 20 x 0.6 µm: 60 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec) – no scan!

With the creation of new tools…