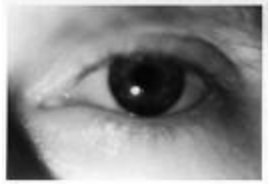
Super-Resolution Optical Microscopy

Bo Huang

Mar 30, 2012



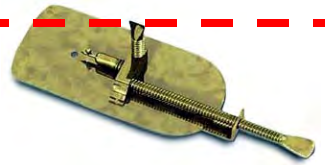
Naked eye: ~ 50-100

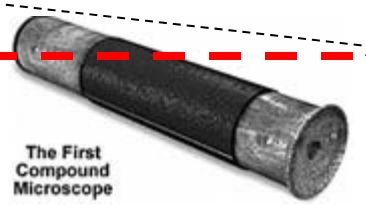
$$d \approx \frac{\lambda}{2\,NA}$$

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

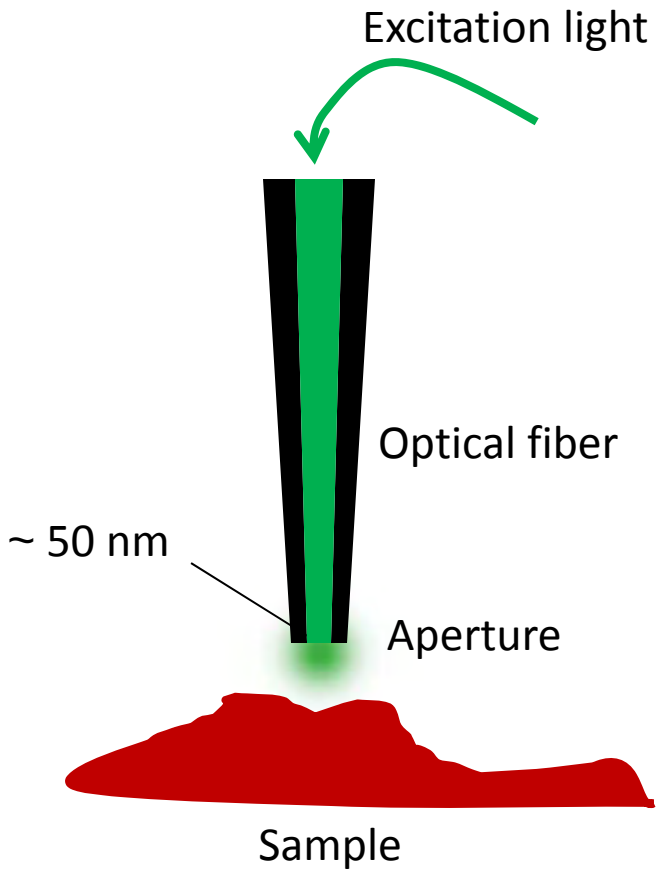
0.1mm
10μm
1μm
100nm
10nm
1nm
1Å

1600 1700 1800 1900 2000

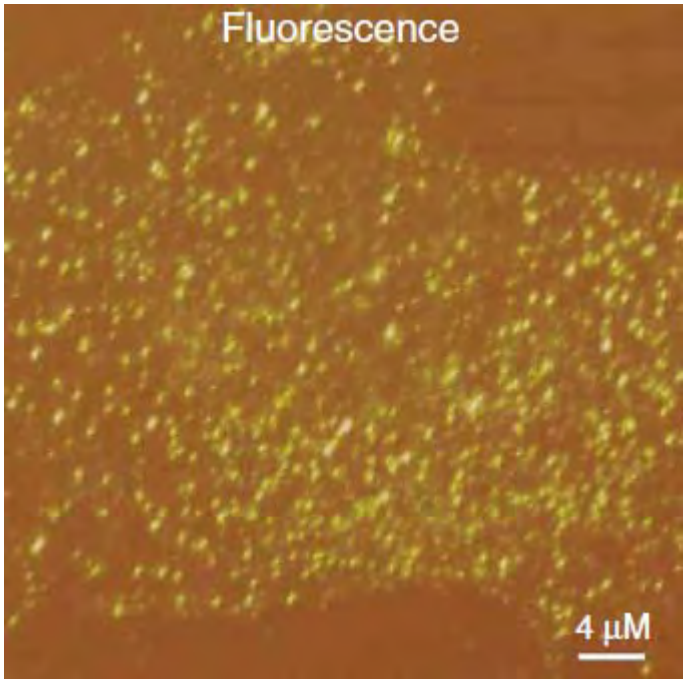
50 years to extend the resolution

- 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



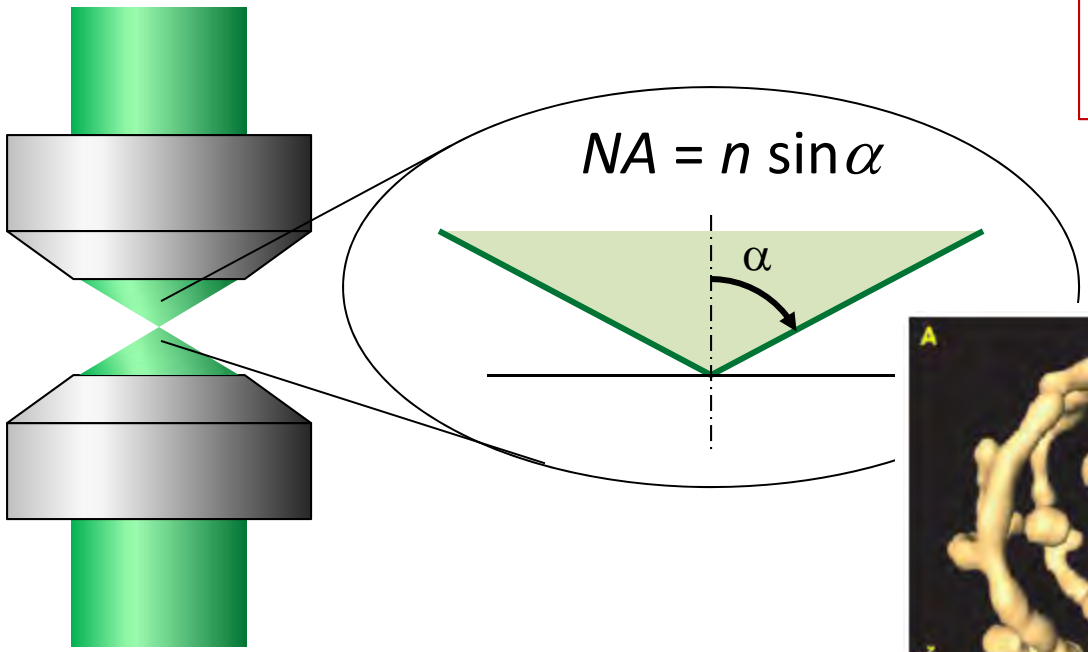
β_2 adrenergic receptor clusters on the plasma membrane

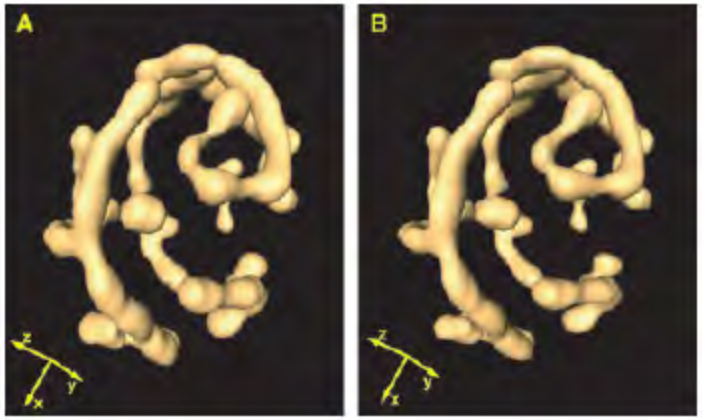


Ianoul et al., 2005

4-Pi / I^{5}M

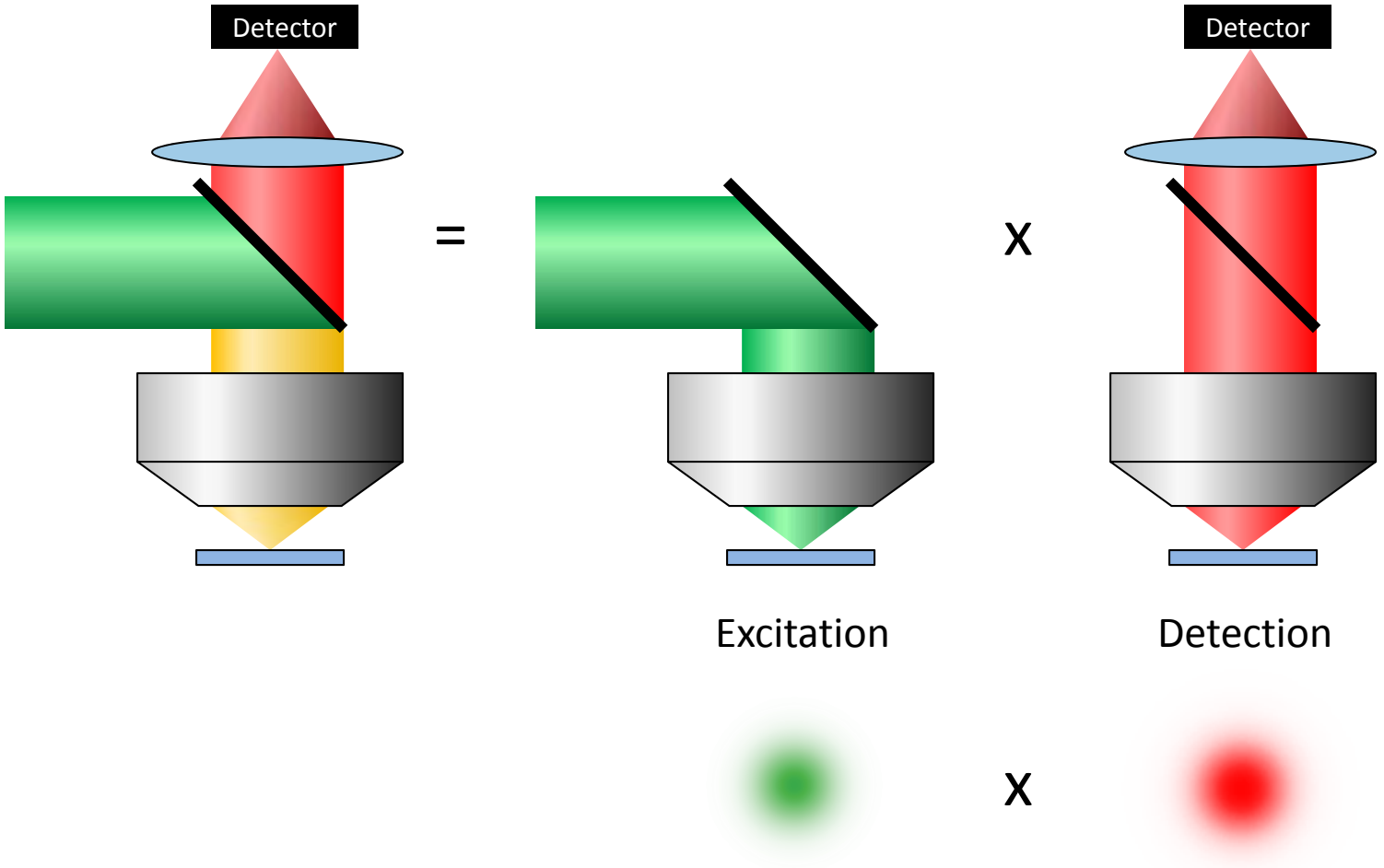
$$d \approx \frac{\lambda}{2\,NA}$$



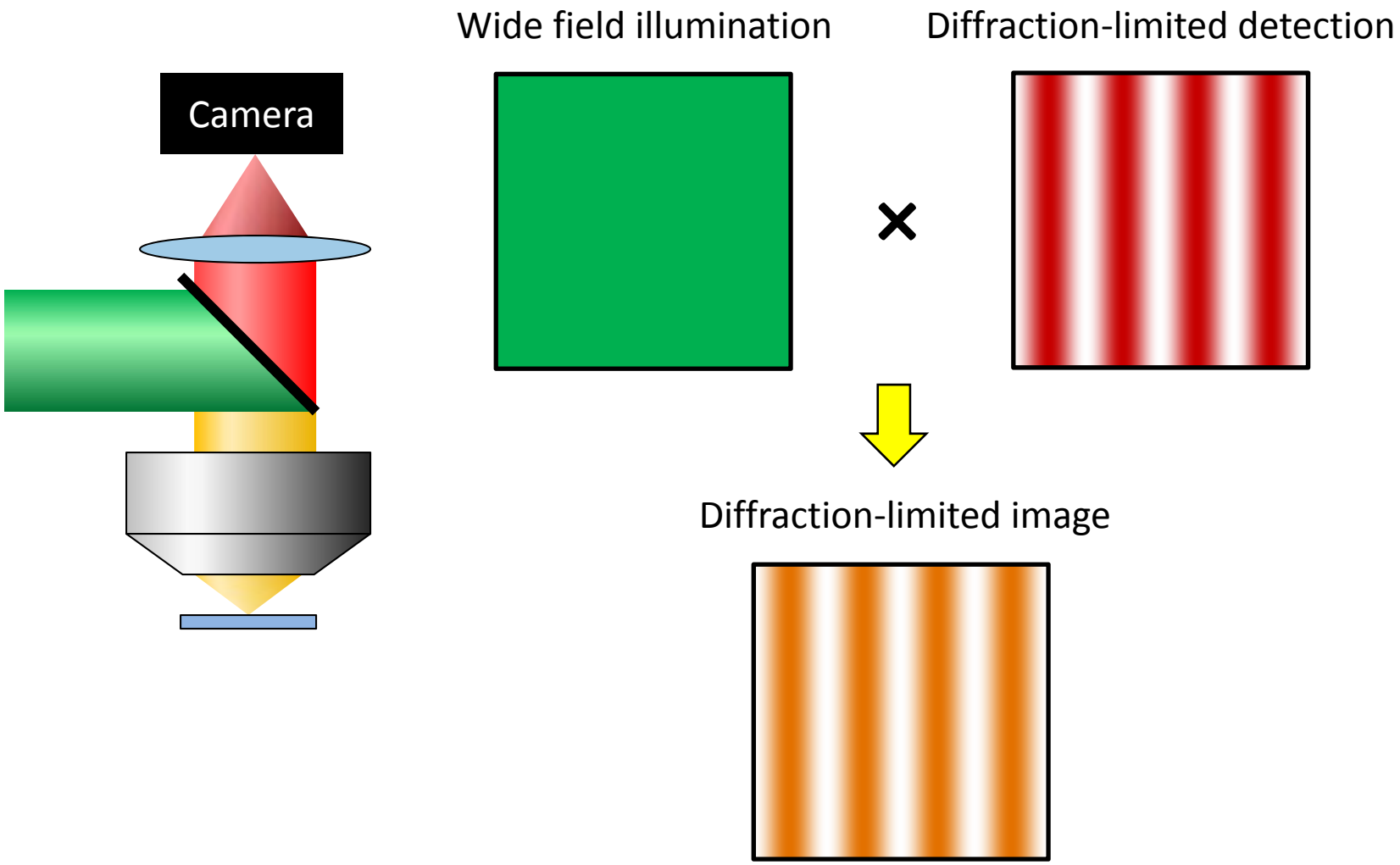


Major advantage:
Similar z resolution as x-y resolution

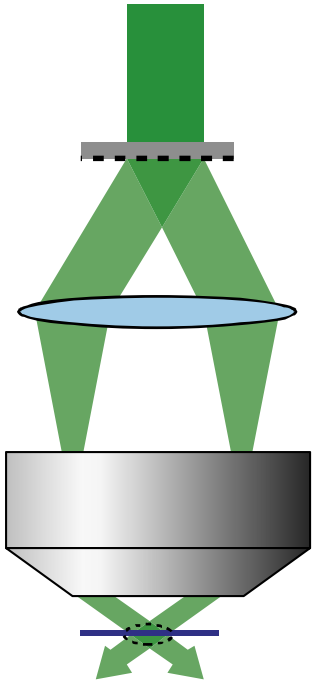
Patterned illumination



Structured Illumination Microscopy (SIM)



Structured Illumination Microscopy (SIM)

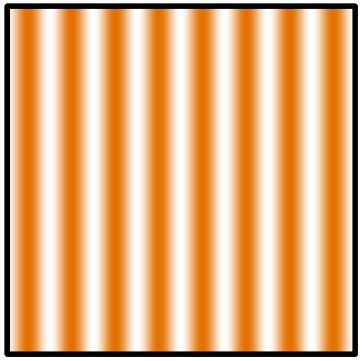


Structured illumination

Diffraction-limited detection

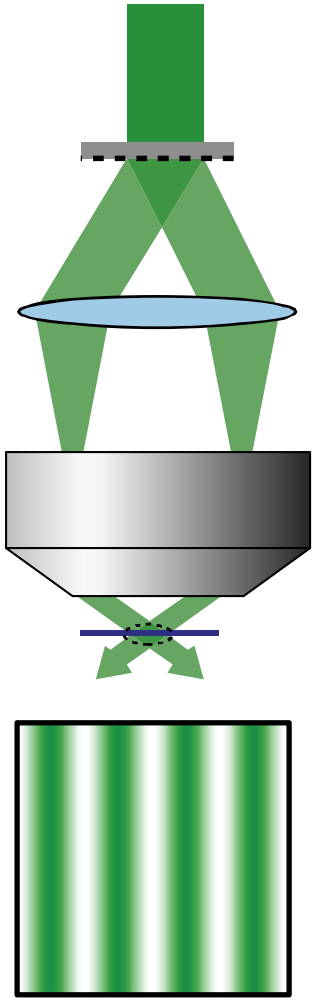
×

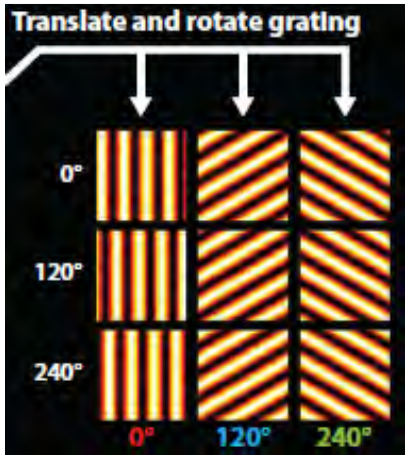
Diffraction-limited image



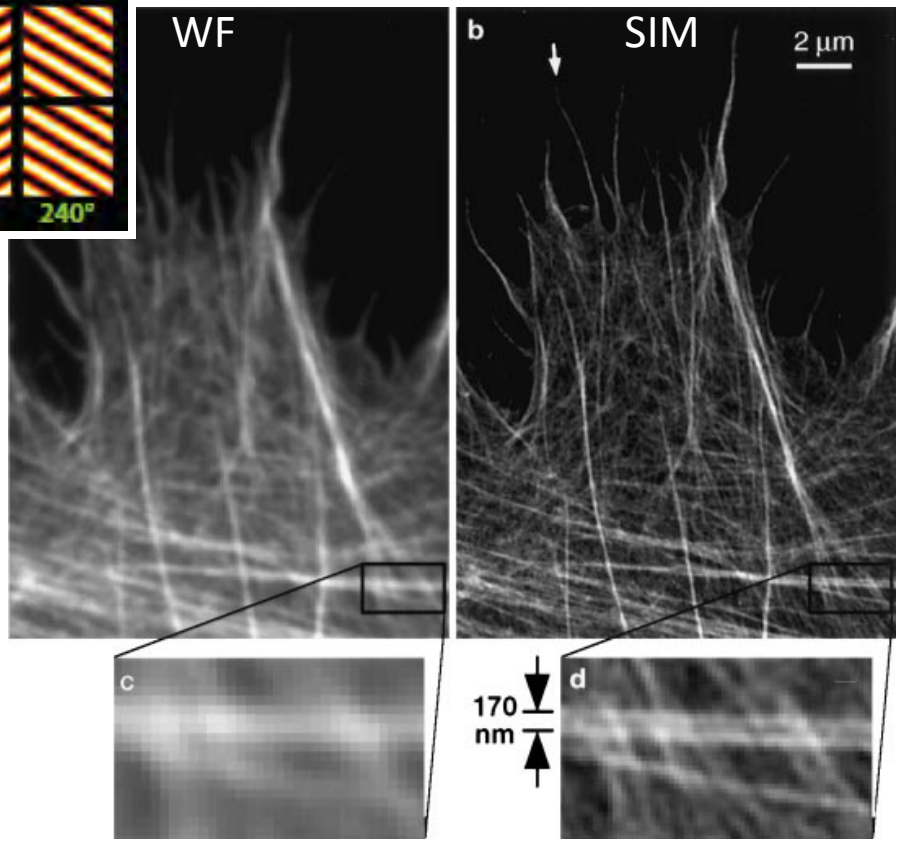
Structured Illumination Microscopy (SIM)

Multiple angles and phases





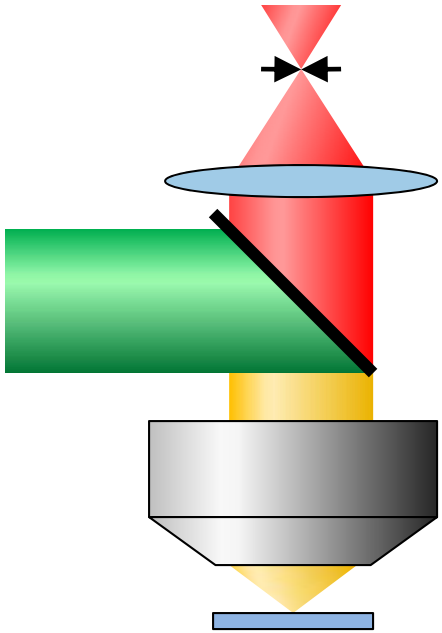
Reconstruction



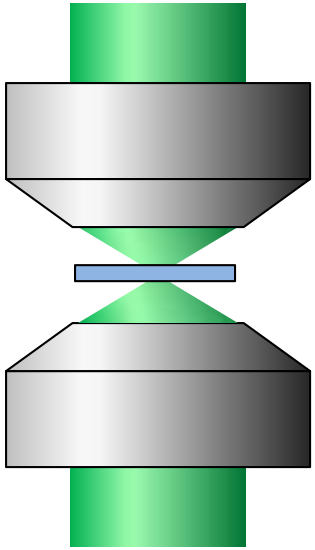
Gustafsson, J Microscopy 2000

The diffraction limit still exists

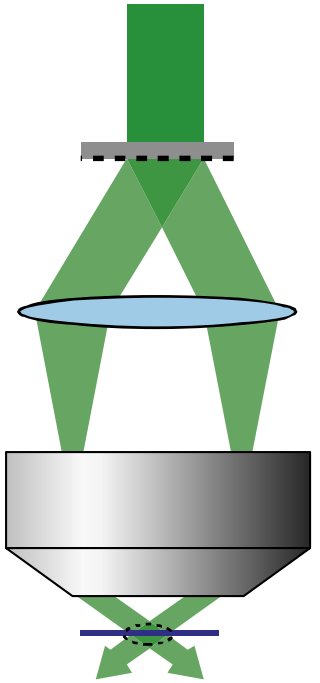
$$d \geq \frac{1}{\mathbf{2}} \cdot \frac{\lambda}{2NA}$$



Confocal



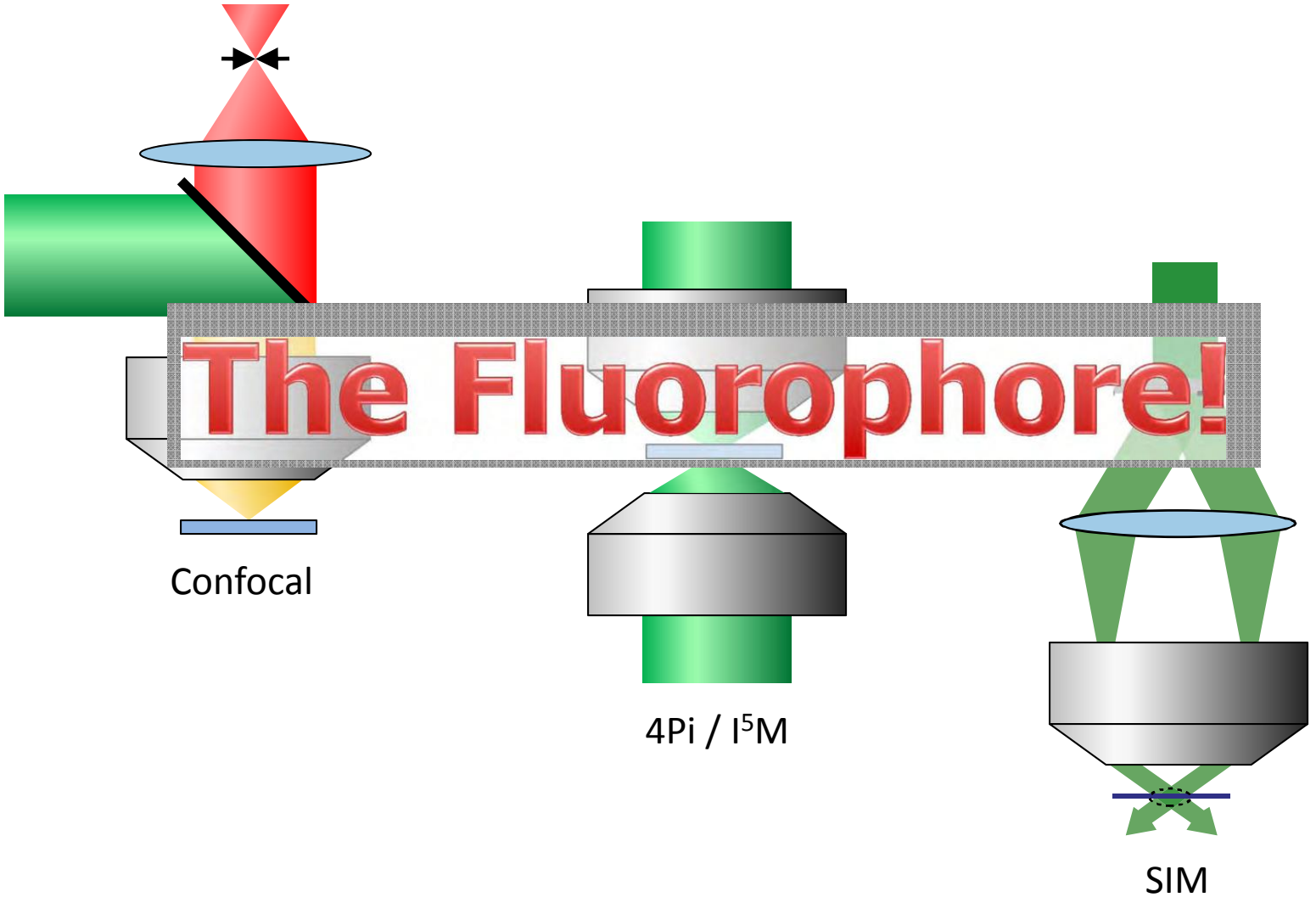
4Pi / I^{5}M



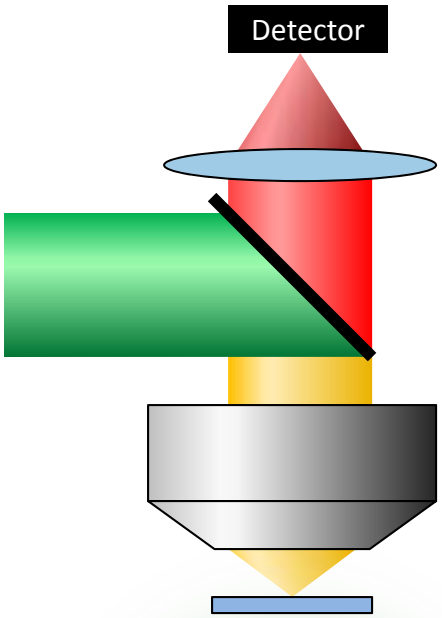
SIM

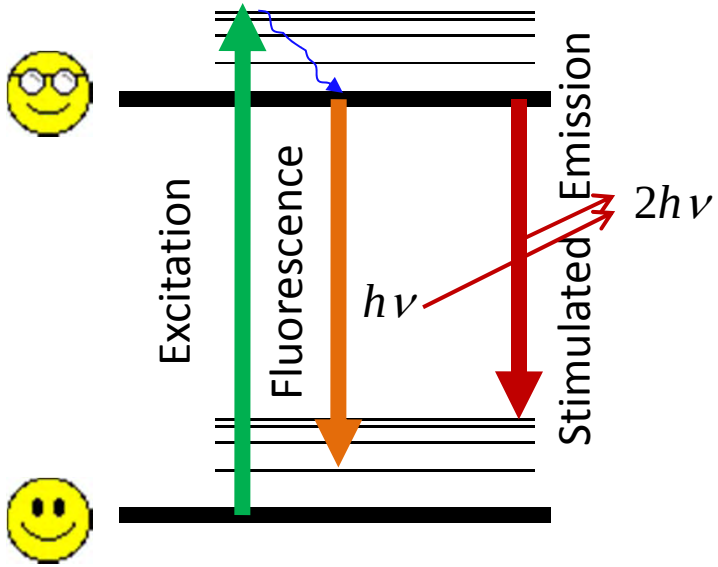
Breaking the diffraction barrier

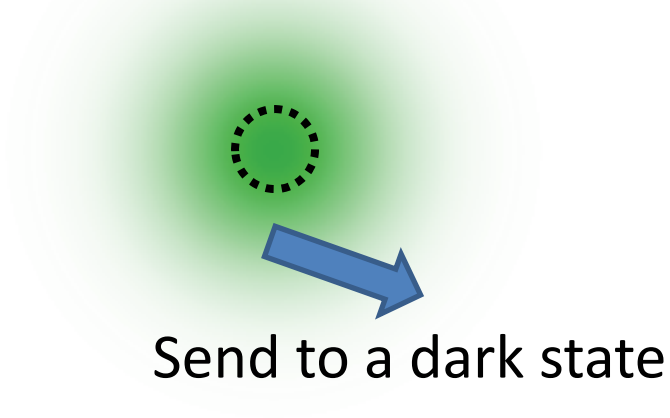
Breaking the diffraction barrier

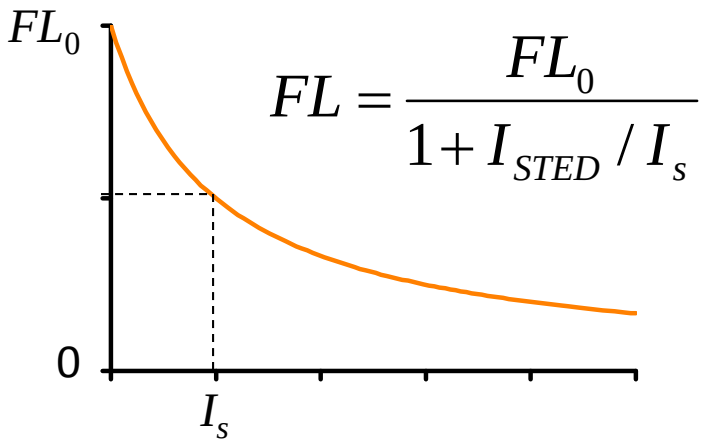


Stimulated Emission Depletion (STED)

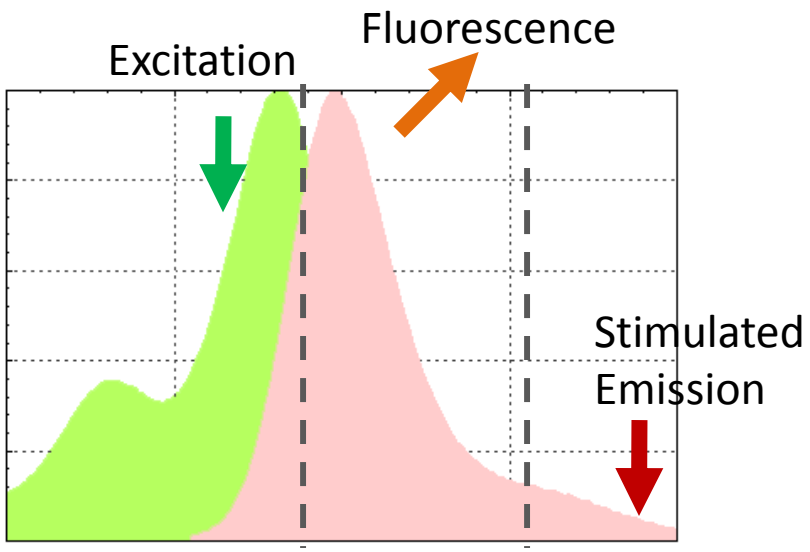


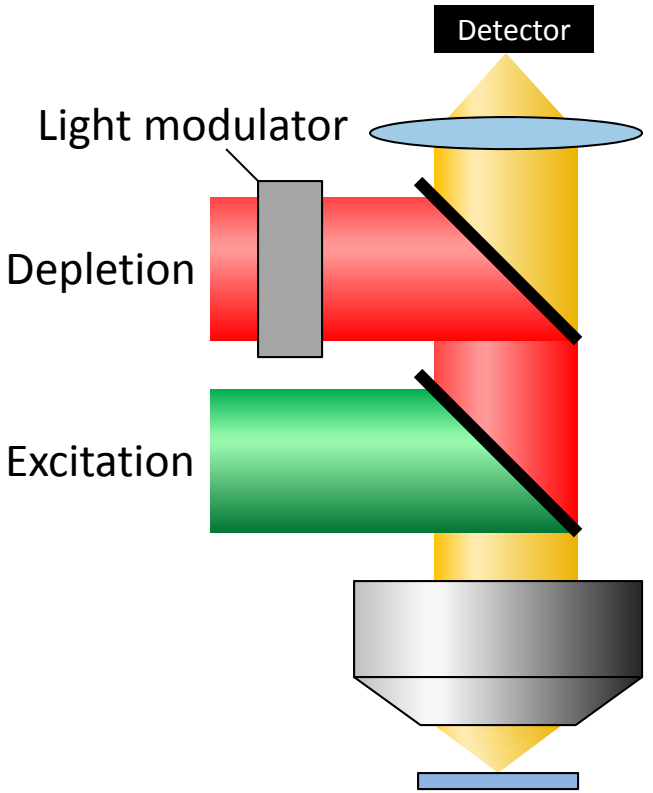


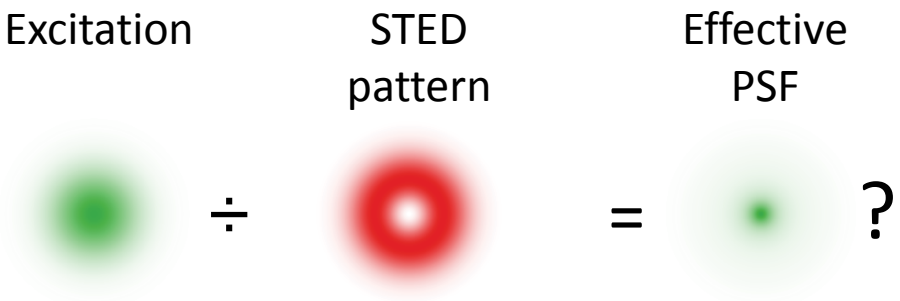




STED microscopy

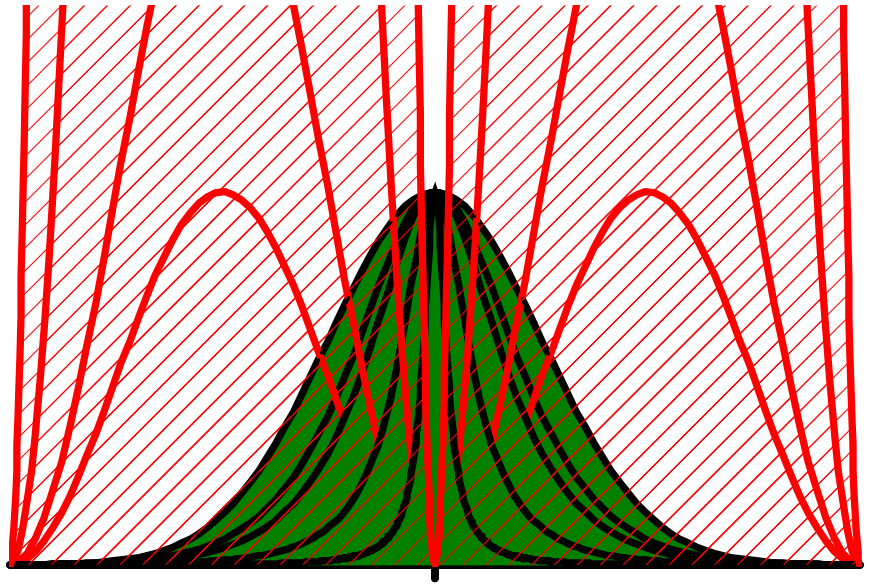






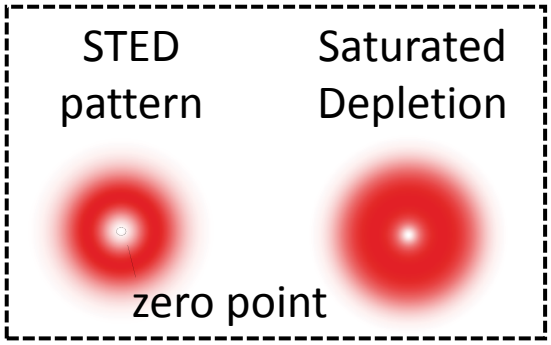
Hell 1994, Hell 2000

Saturated depletion

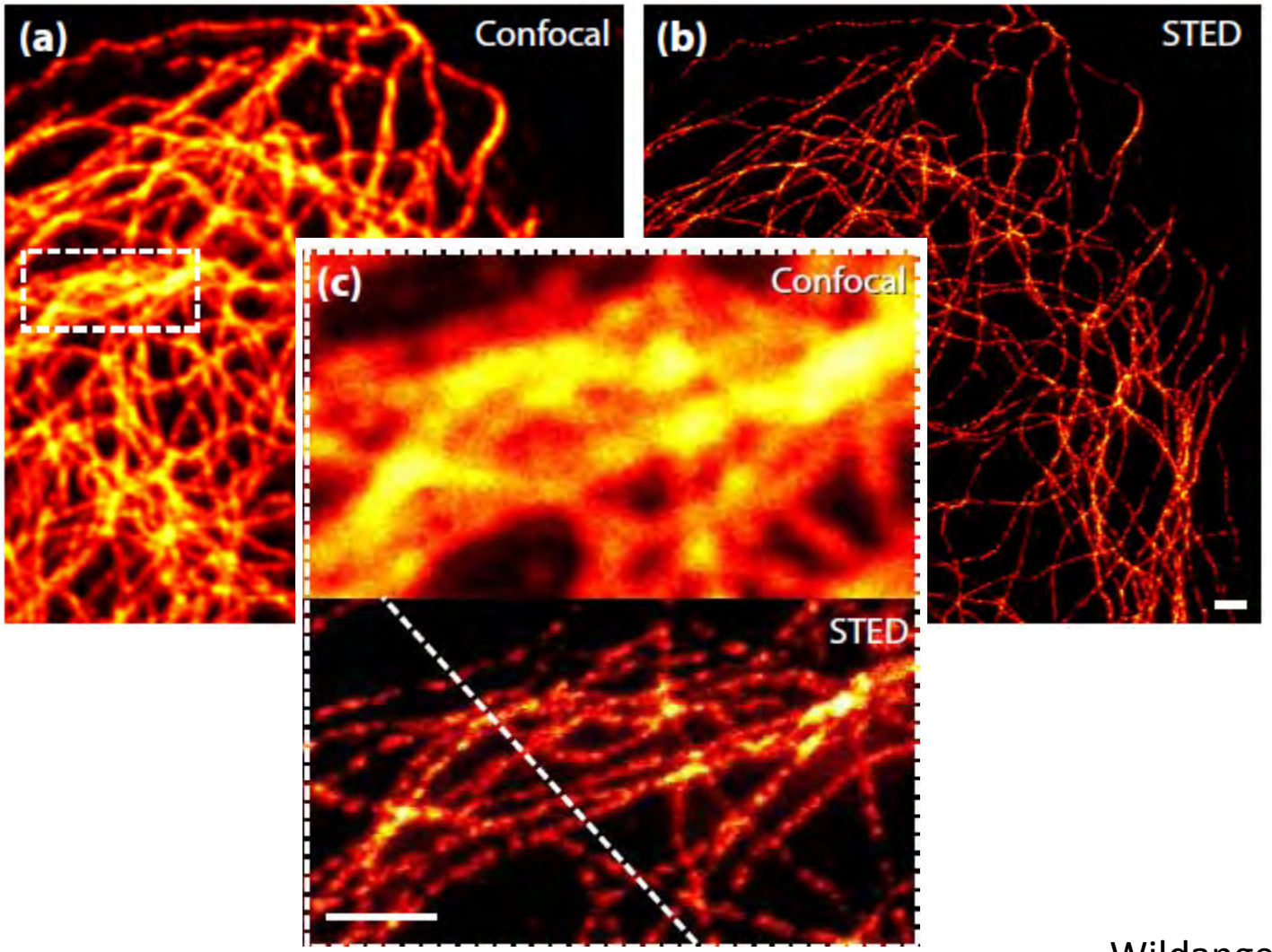


$$I_{\mathrm{STED}} = 100 I_S$$

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

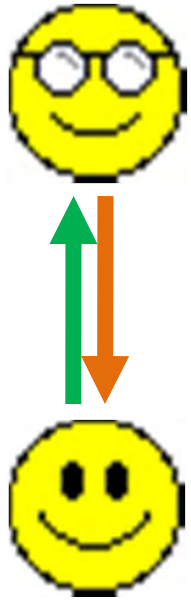


STED images of microtubules



Wildanger et al., 2009

The “patterned illumination” approach



Multiple cycles

- Ground state
- Triplet state
- Isomerization

etc.

Excitation

Depletion pattern

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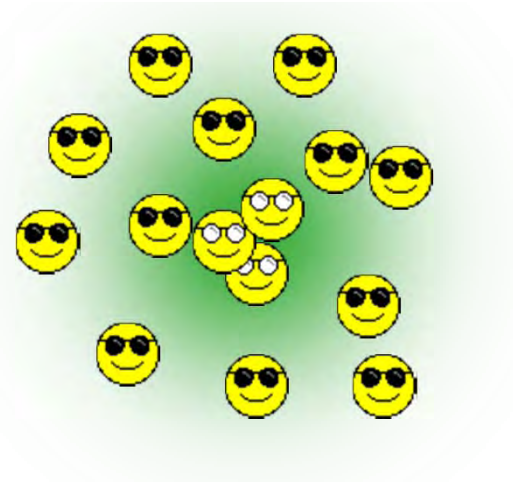
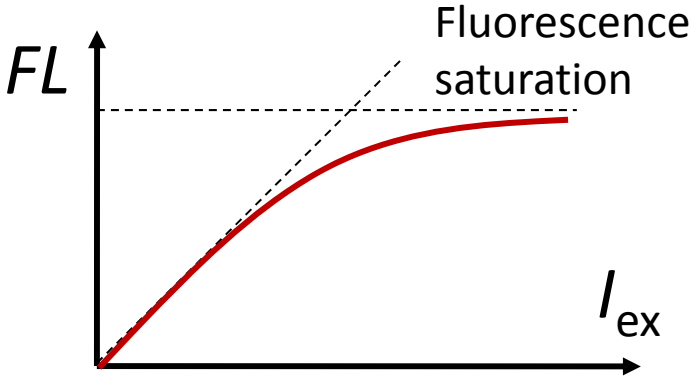
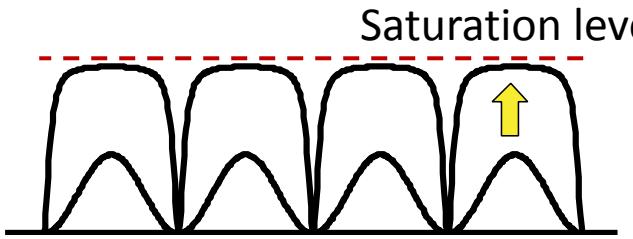
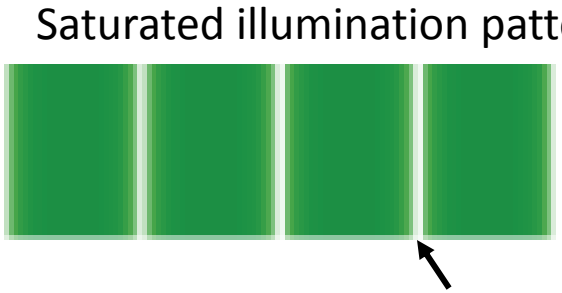
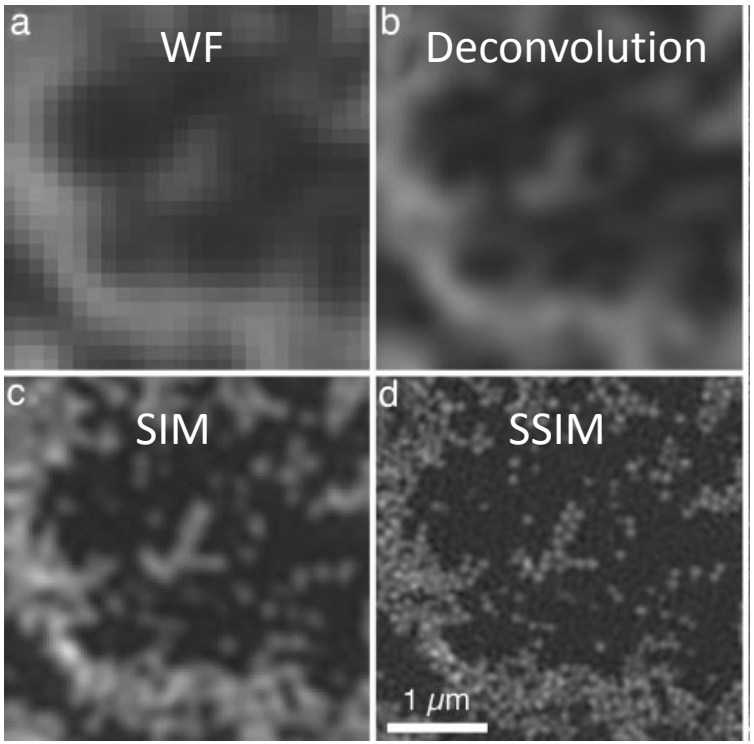


Saturated SIM









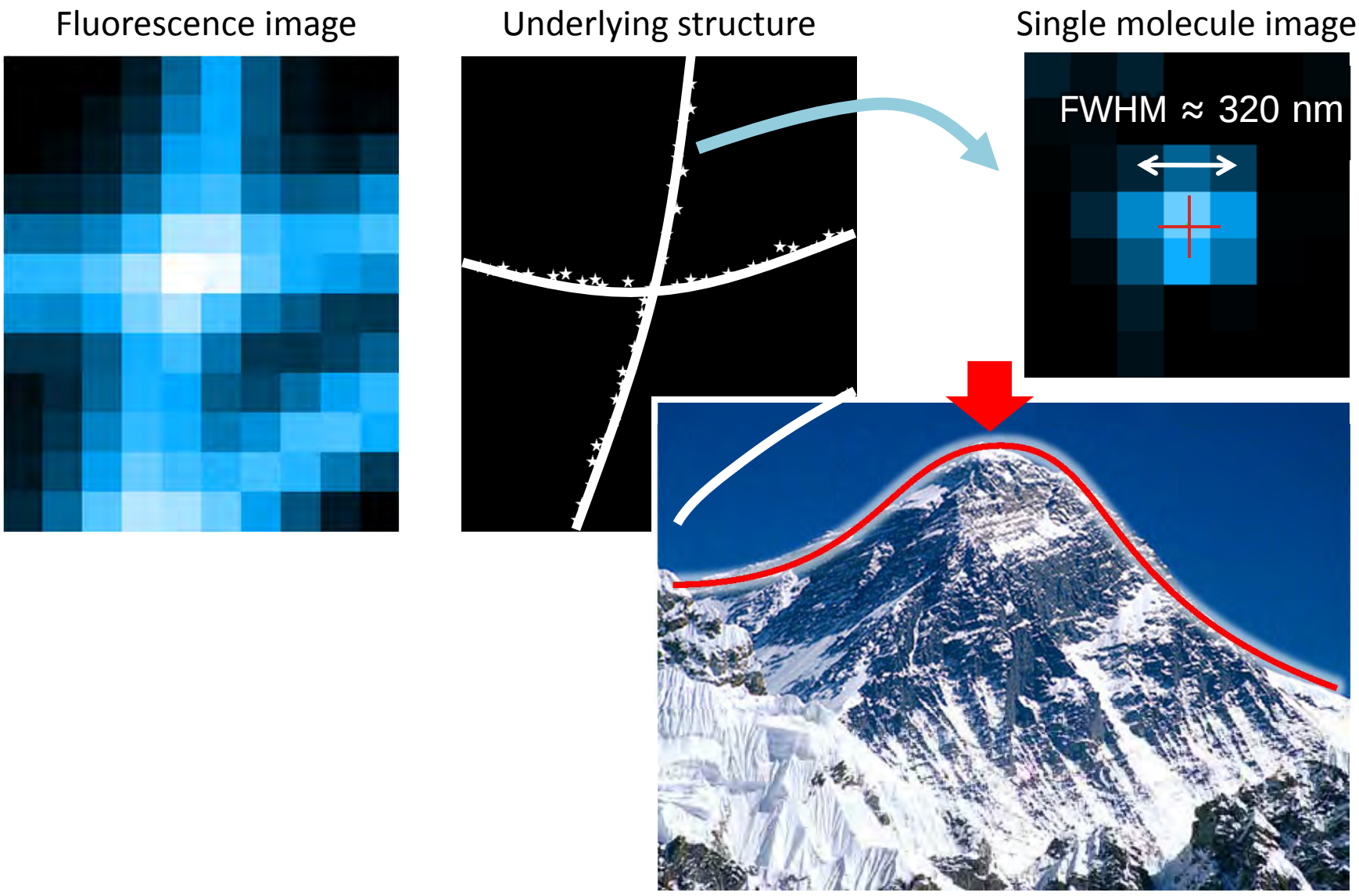
50 nm resolution

Suffers from fast photobleaching under saturated excitation condition

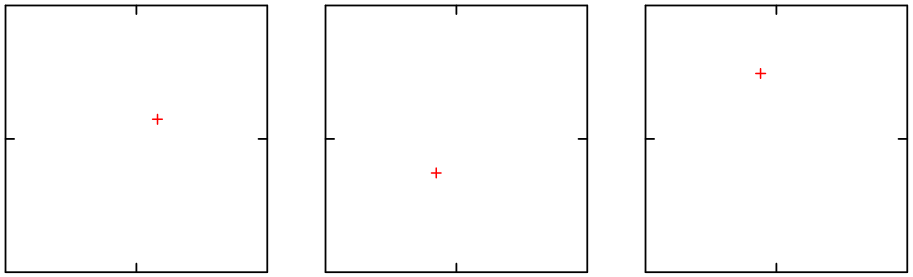
Gustaffson, PNAS 2005

The single-molecule switching approach

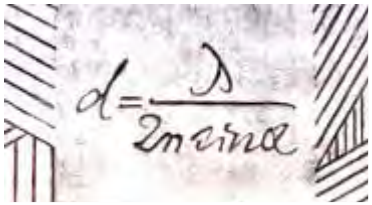
STORM/PALM: Single molecule localization

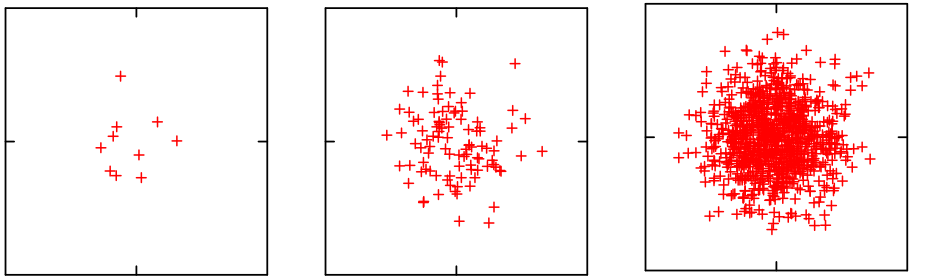


Single-molecule localization precision

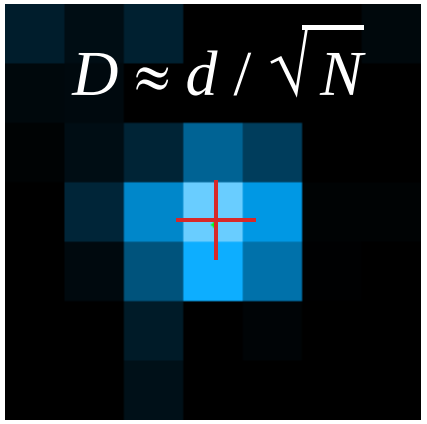


1 photon



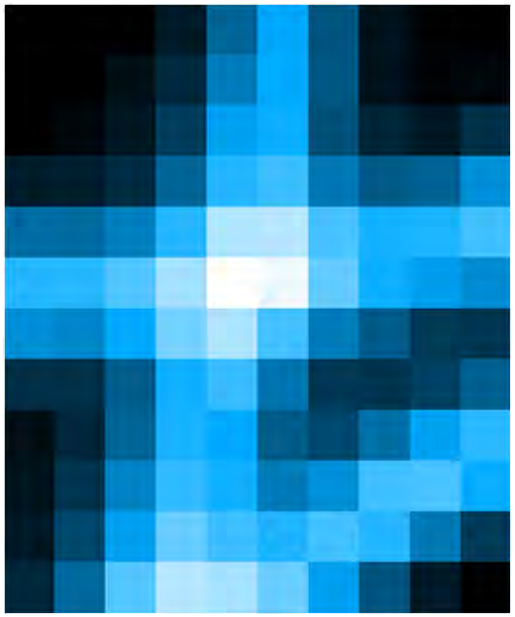


10 photons 100 photons 1000 photons

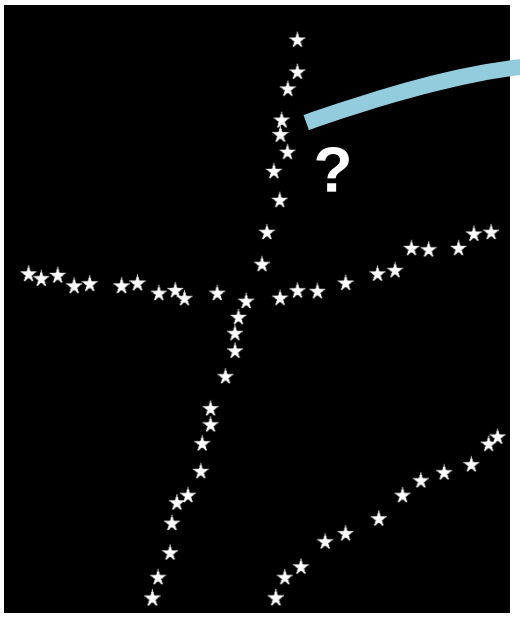


STORM/PALM: Single molecule localization

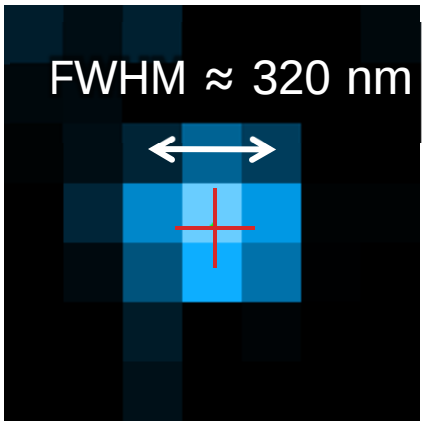
Fluorescence image



Underlying structure

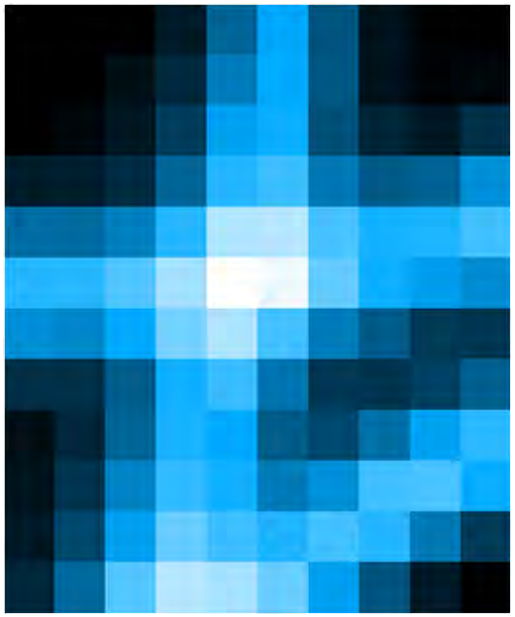


Single molecule image

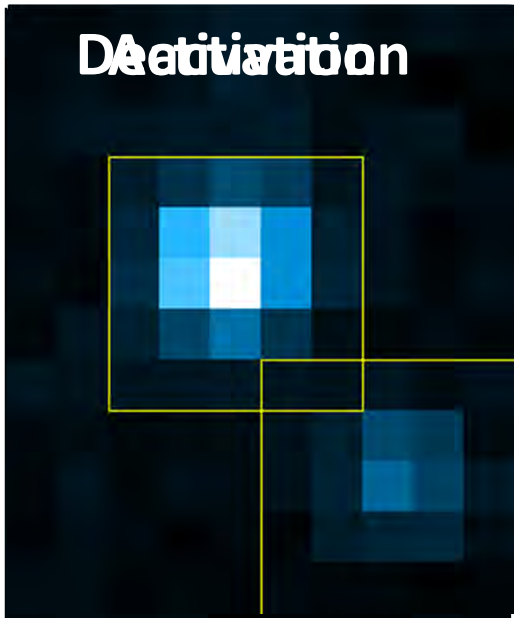


STORM/PALM: Single molecule switching

Fluorescence image



Raw images



STORM Image

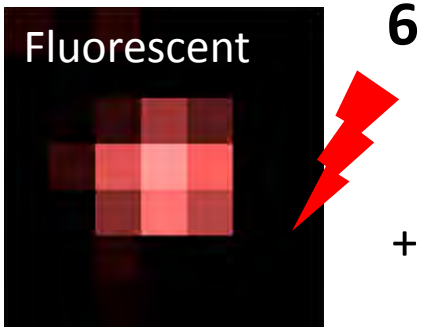
2x real time

Stochastic Optical Reconstruction Microscopy = STORM

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

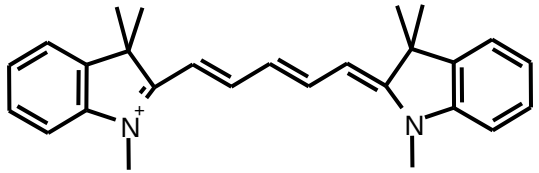
Rust, Bates & Zhuang, Nat. Methods, 2006
Bates, Huang, Dempsey & Zhuang, Science, 2007

Photoswitching of red cyanine dyes



650 nm

+ thiol



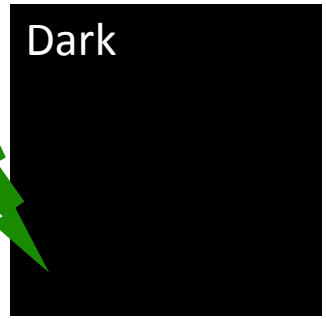
Cy5 / Alexa 647

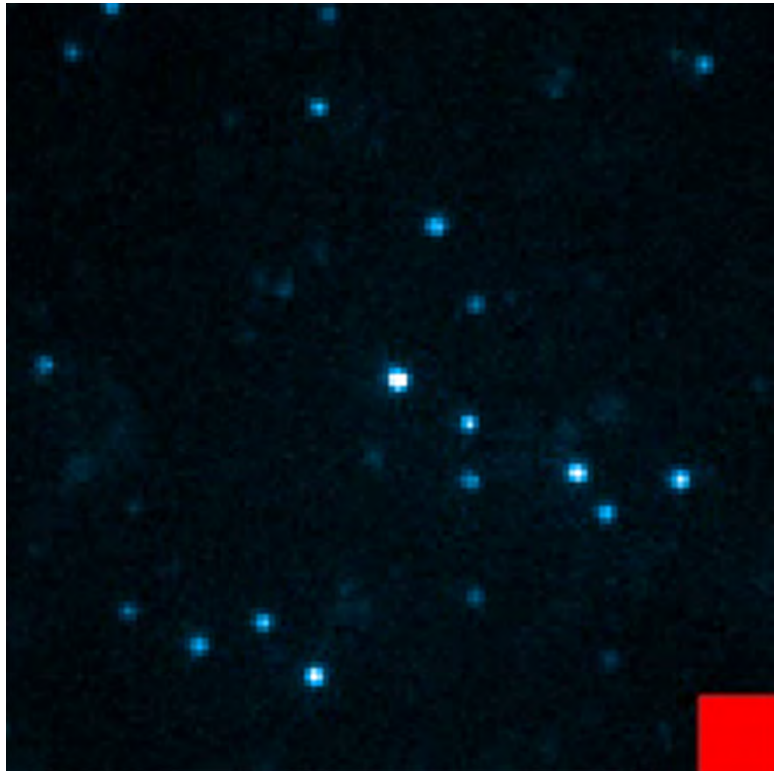
photoactivation

Deactivation

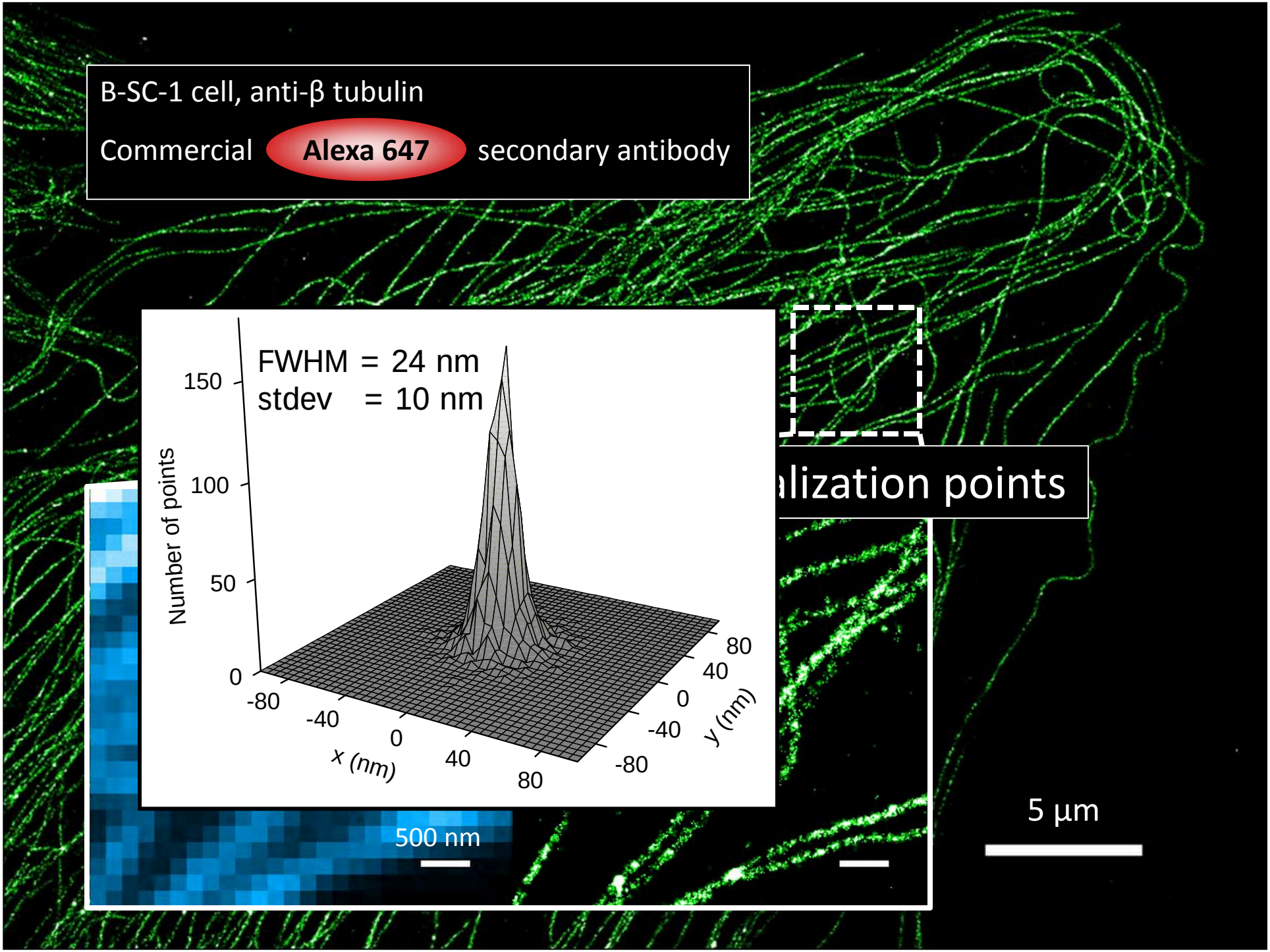
360 nm

650 nm

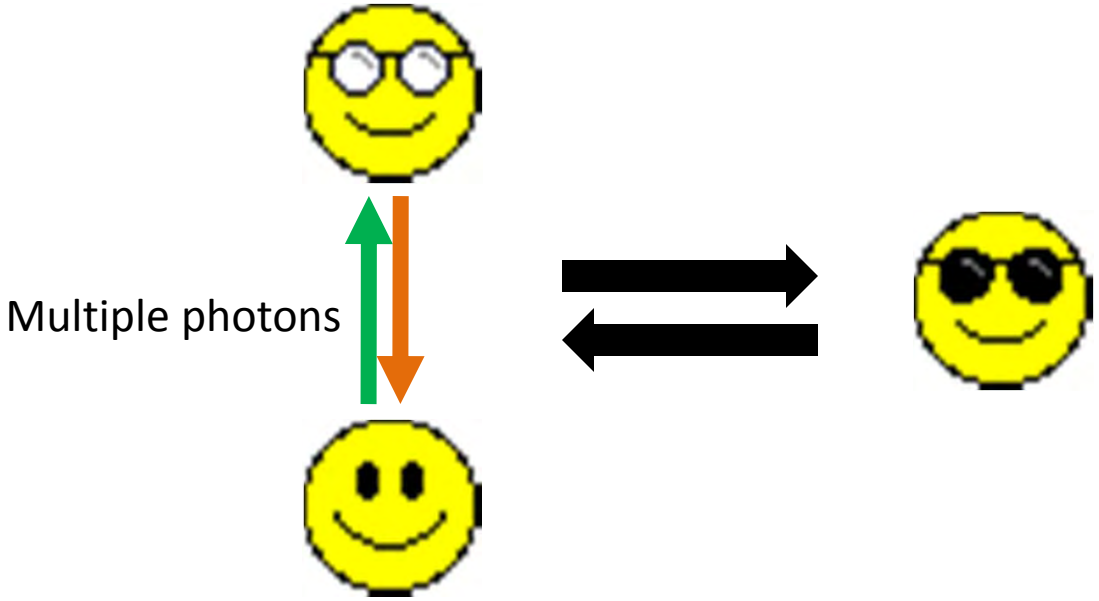




Bates eta l., *PRL* 2005, Bates et al., *Science* 2007, Dempsey et al., *JACS* 2009


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

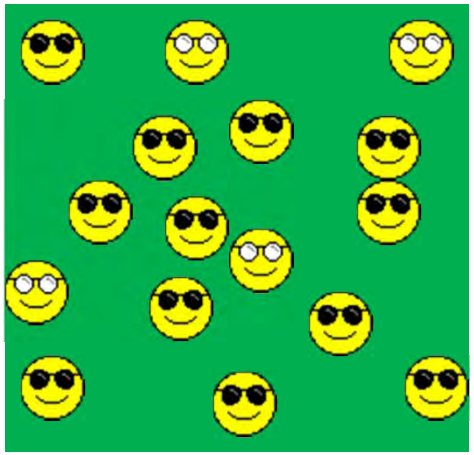
The “single-molecule switching” approach



- Photoswitching
- Blinking
- Diffusion
- Binding

etc.

\+ Stochastic Switching =



Photoswitchable probes readily available

Cyanine dye + thiol system

Alexa647
Cy5.5
Cy7
Cy5

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

Rhodamine dye + redox system

Atto565
Alexa488
Alexa532
Atto590
Atto655
Atto700
Atto520
Alexa568

Heilemann et al., 2009, Dempsey et al., 2012

Photoactivatible fluorescent proteins

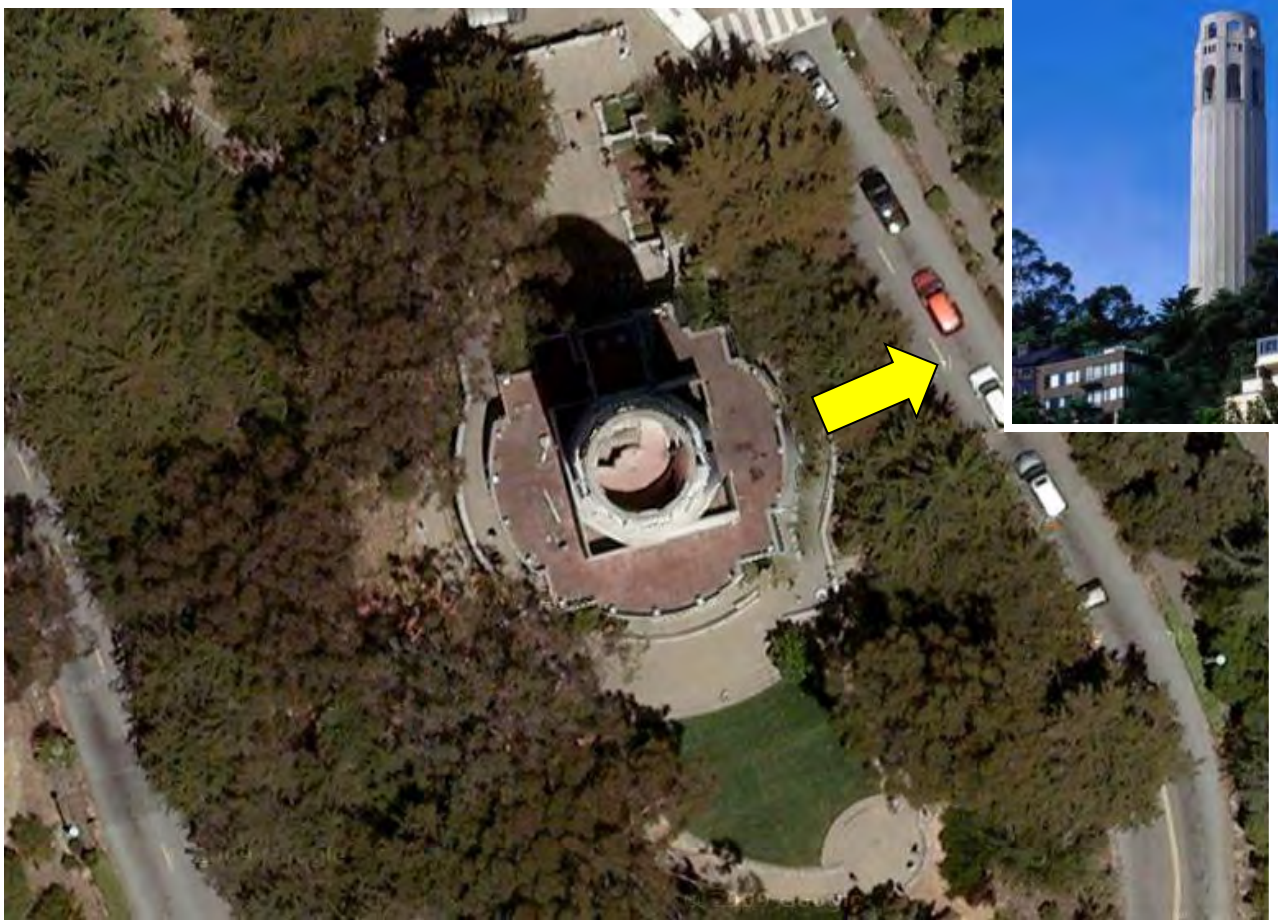
mEosFP2
PA-GFP
Dendra2
PS-CFP2
PAmCherry
PAtagRFP
Dronpa
Dreiklang
EYFP

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

3D Imaging

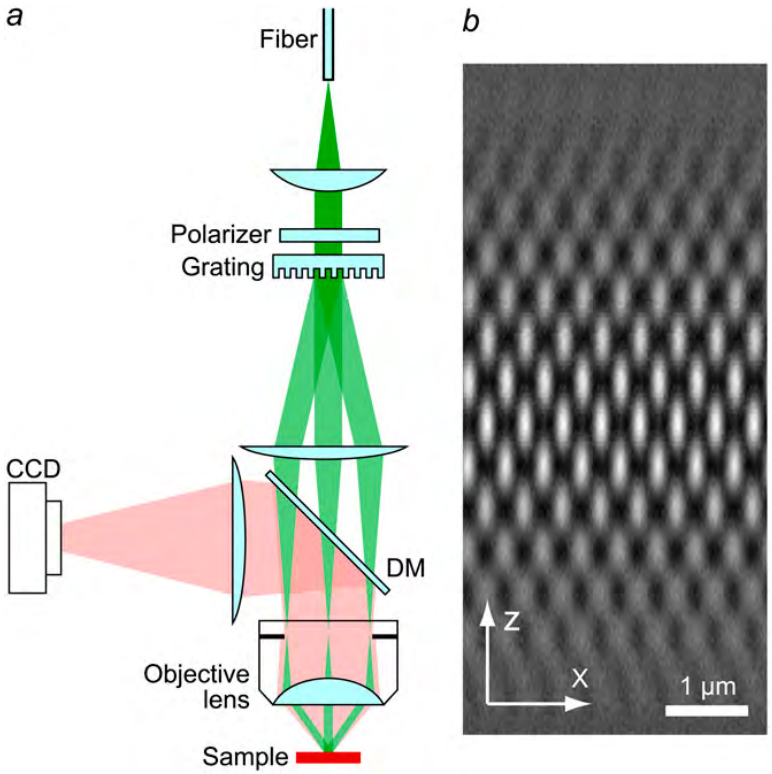
In a 2D world…

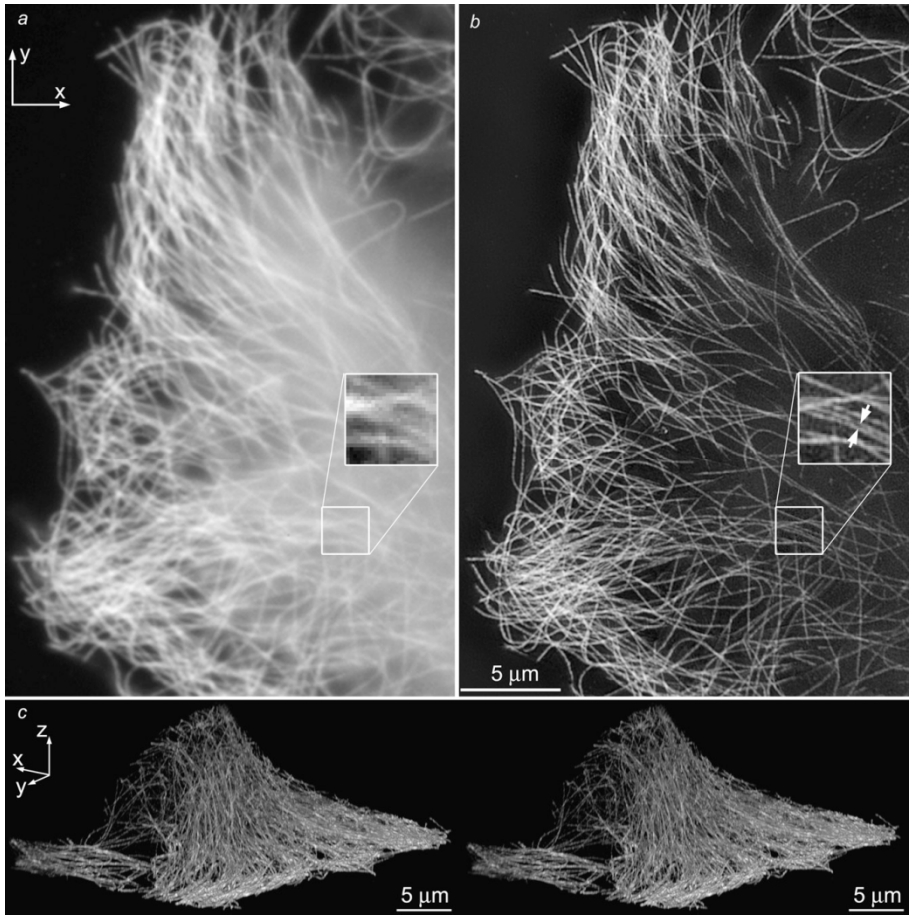
Satellite image of ???



Google maps

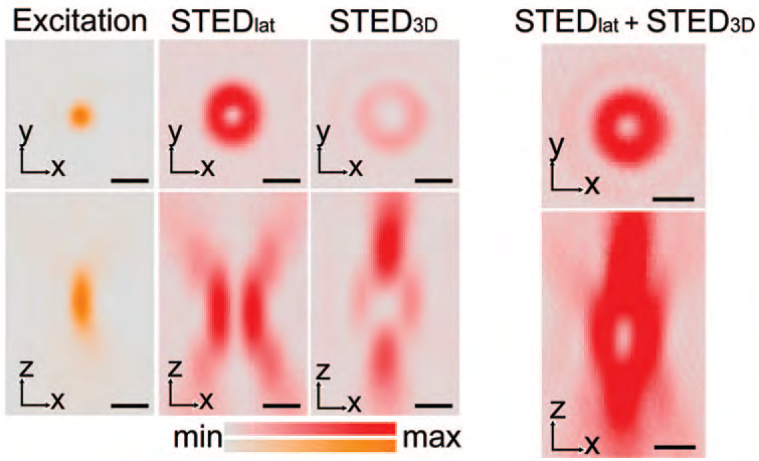
3D SIM

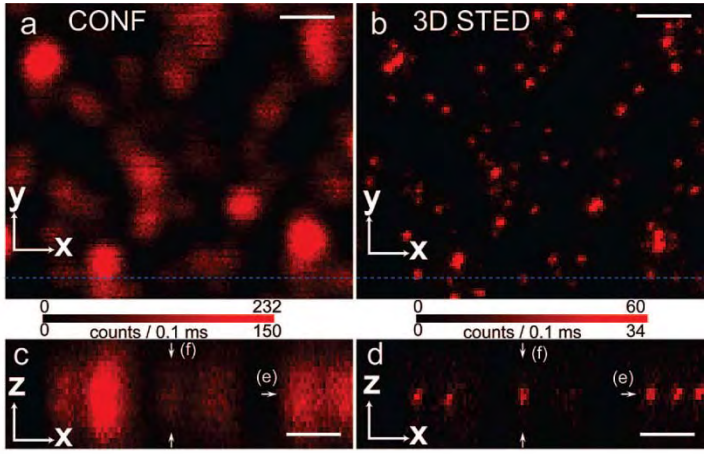


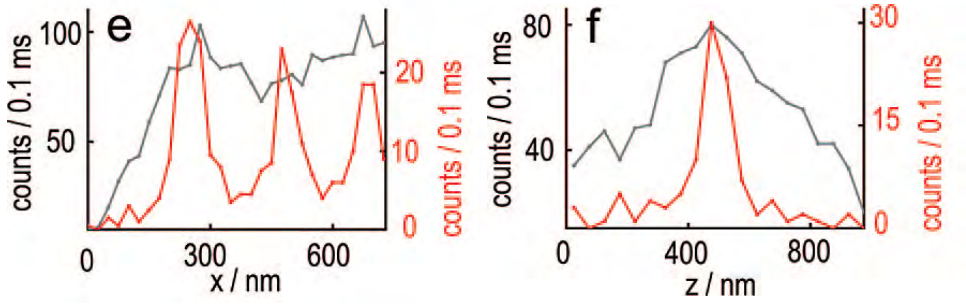


Schermellech et al., Science 2008, Gustafsson et al., Biophys J. 2008

3D STED

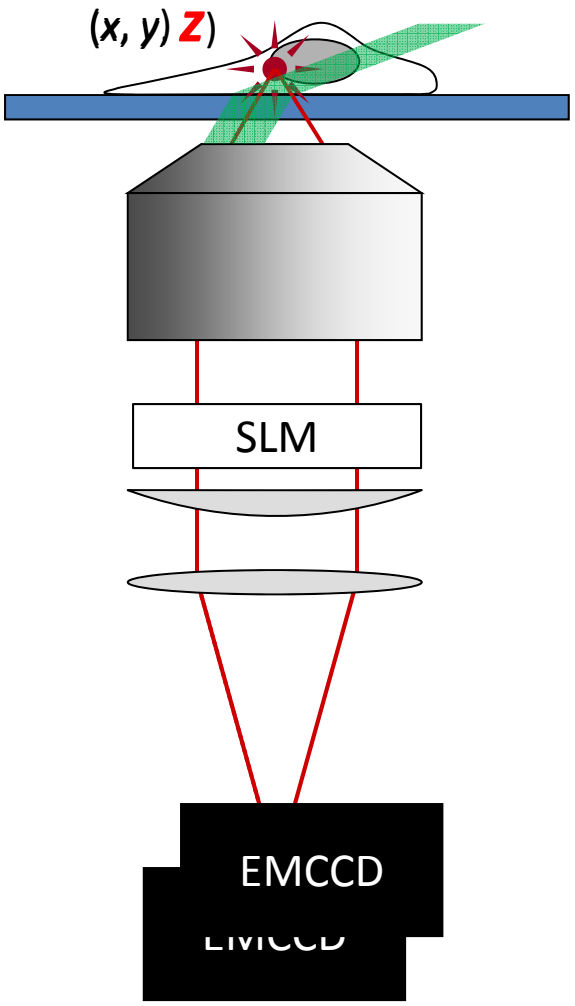




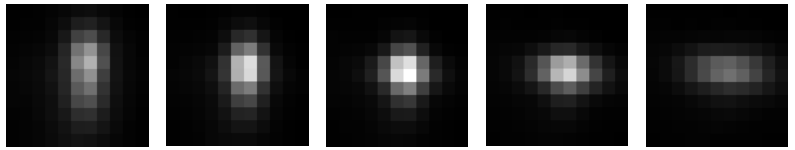


Harke et al., Nano Lett, 2008

3D STORM/PALM



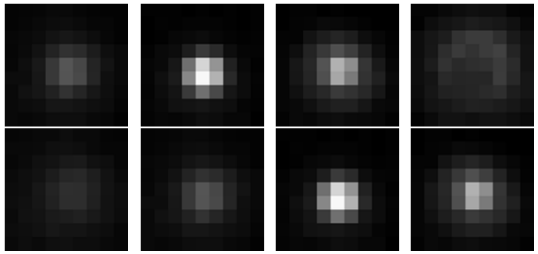
Astigmatic imaging



z (nm) -400 -200 0 200 400

Huang et al., Science 2008

Bi-plane imaging



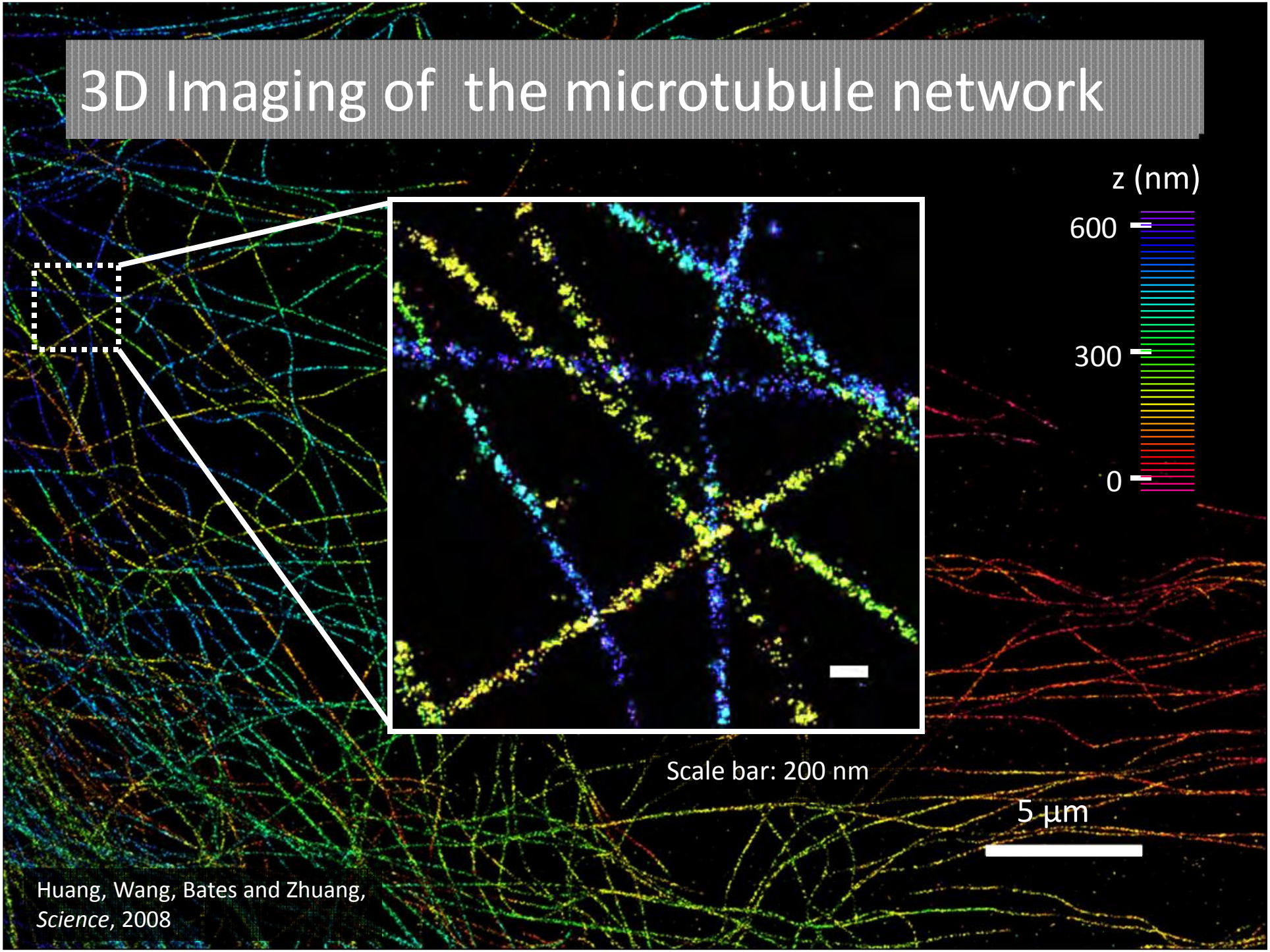
Juette et al., Science 2008

Double-helical PSF

z (nm) -900 -500 0 600 1400

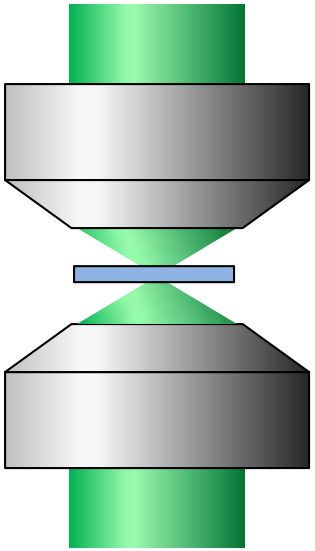
Pavani et al., PNAS 2009

3D Imaging of the microtubule network



Huang, Wang, Bates and Zhuang,
Science, 2008

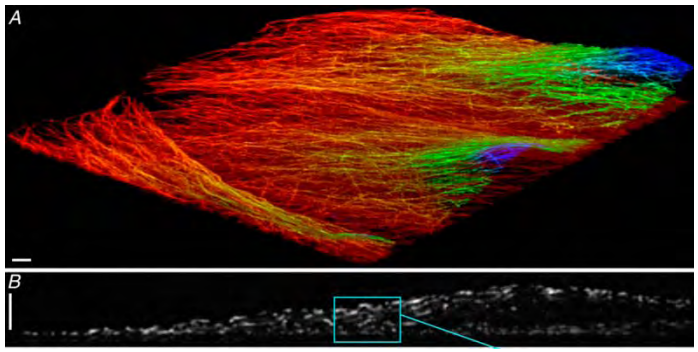
The use of two opposing objectives



4Pi scheme

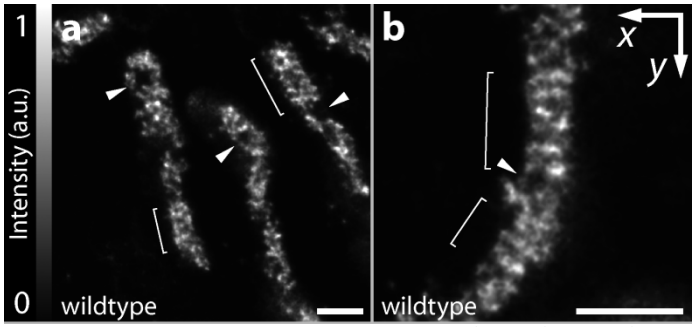
Near isotropic
3D resolution

I^{5}S



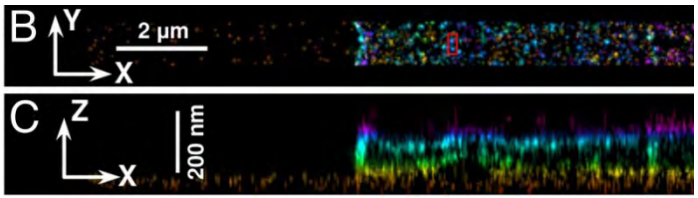
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

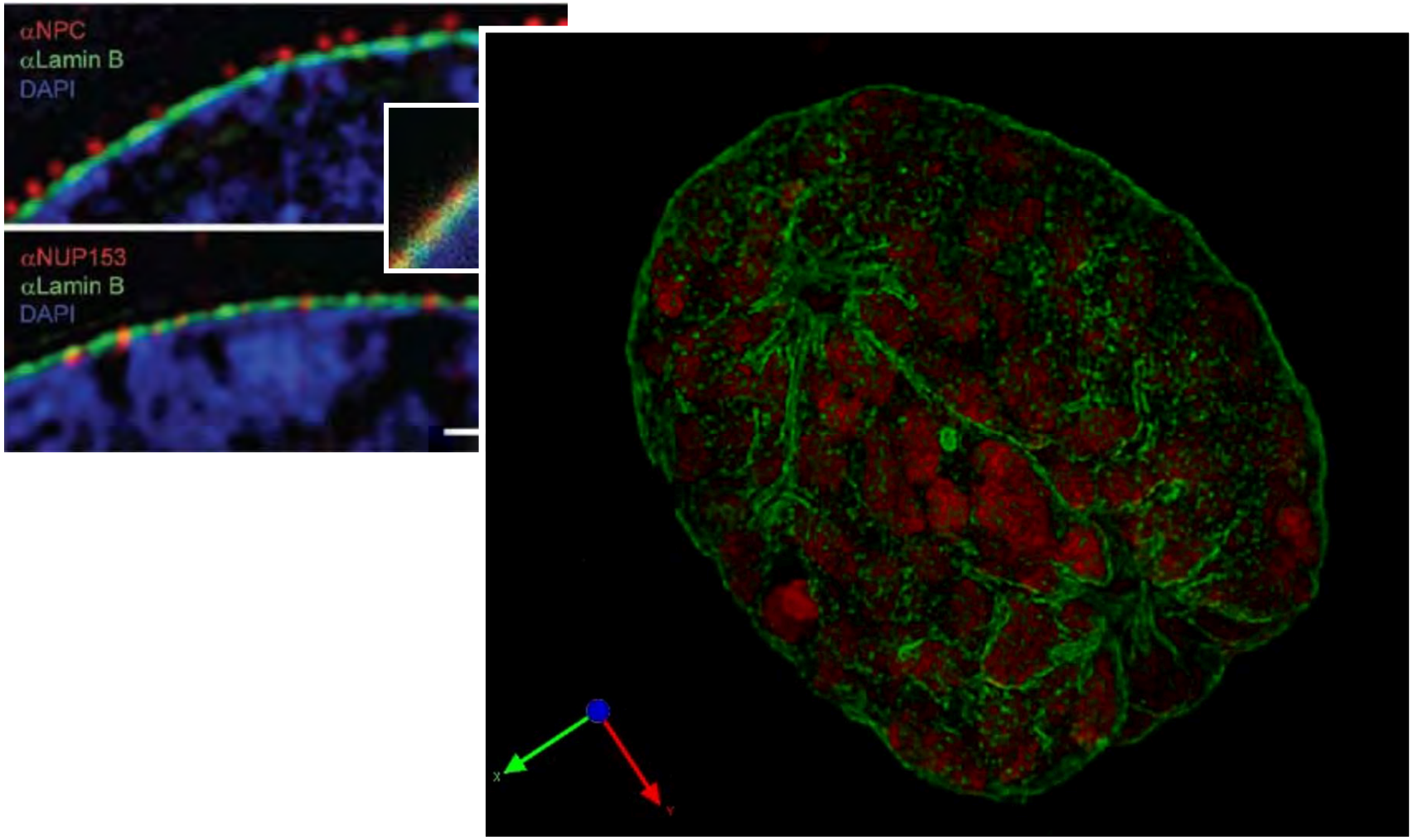
3D resolution of super-resolution methods

	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

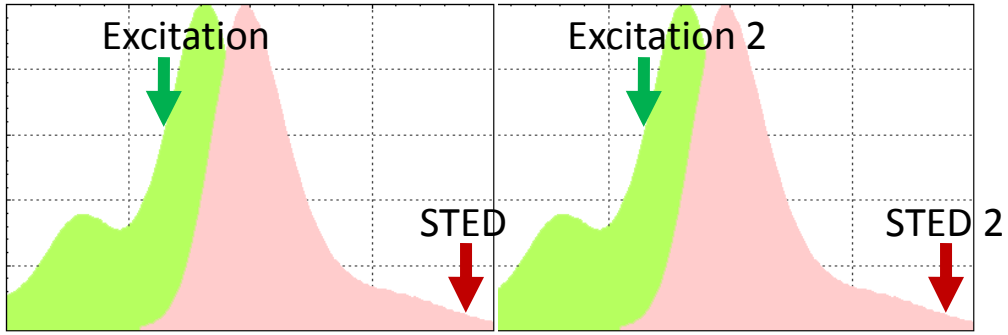
Multicolor SIM

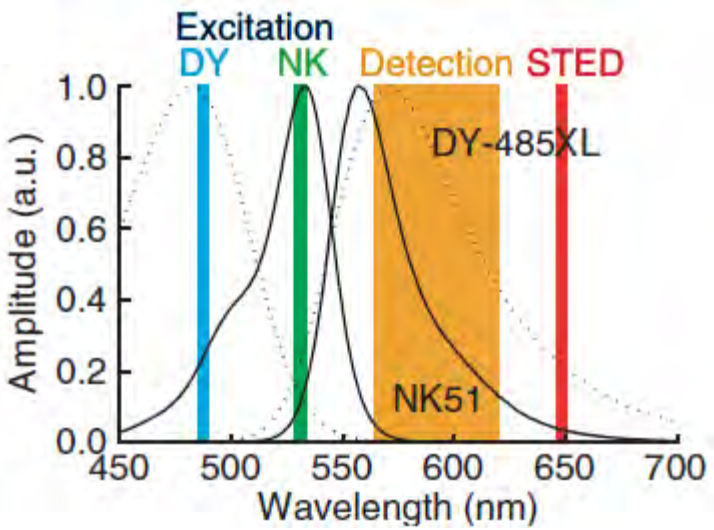
Same as conventional fluorescence microscopy!



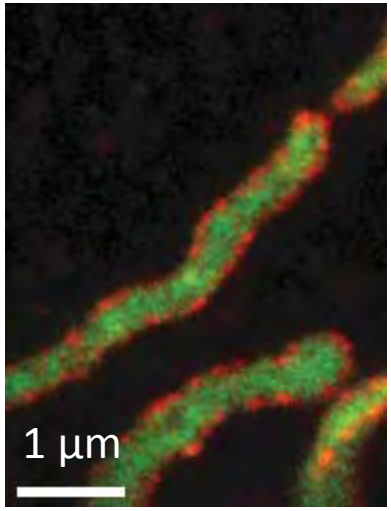
Schermelleh et al., Science 2008

Muticolor STED



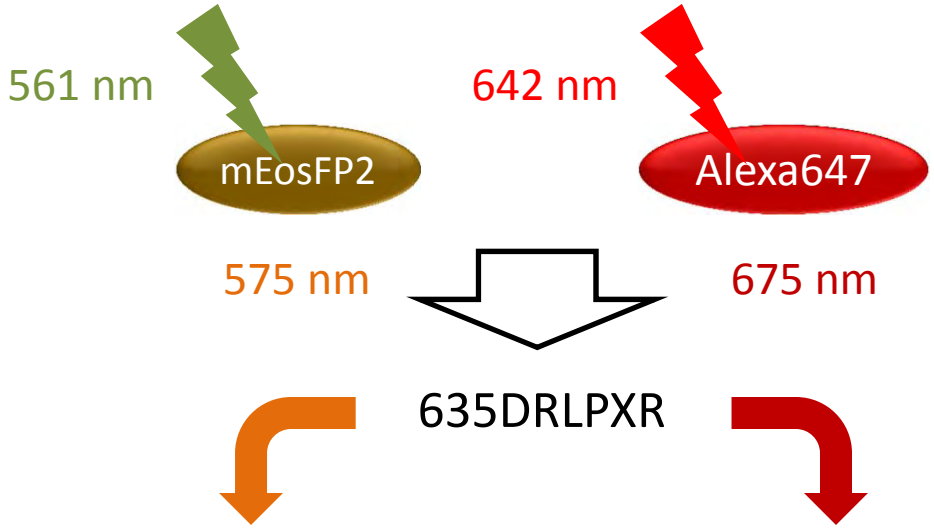


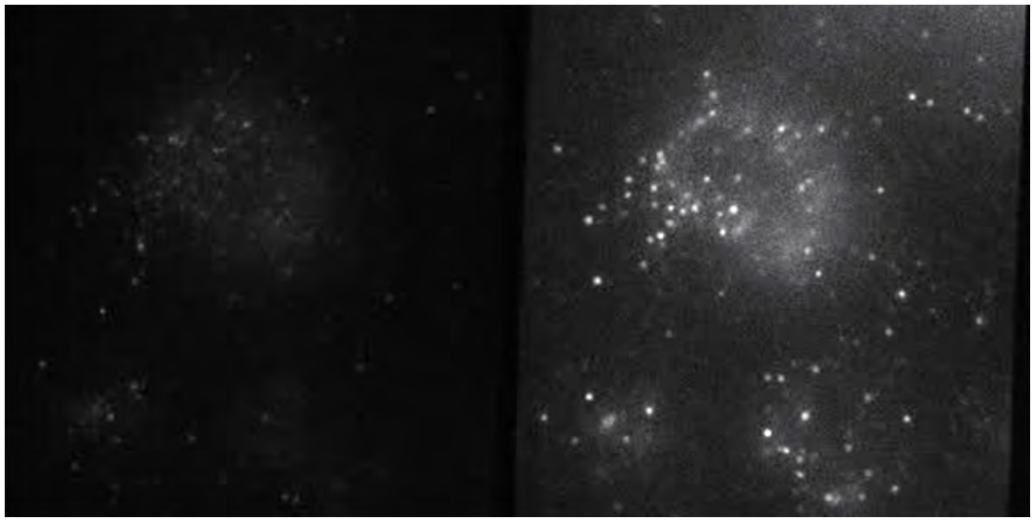
2 color isoSTED resolving
the inner and outer membrane
of mitochondria

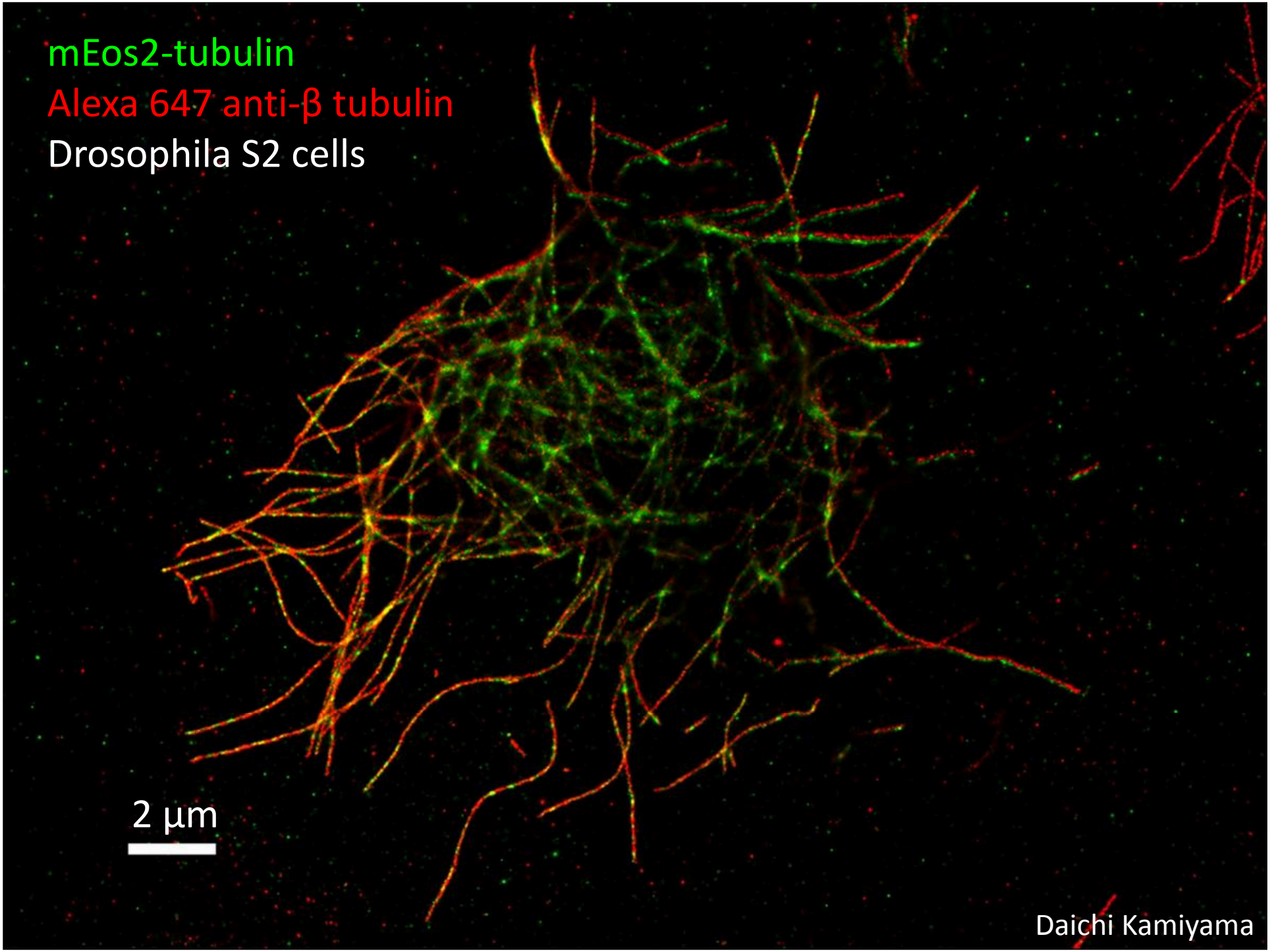


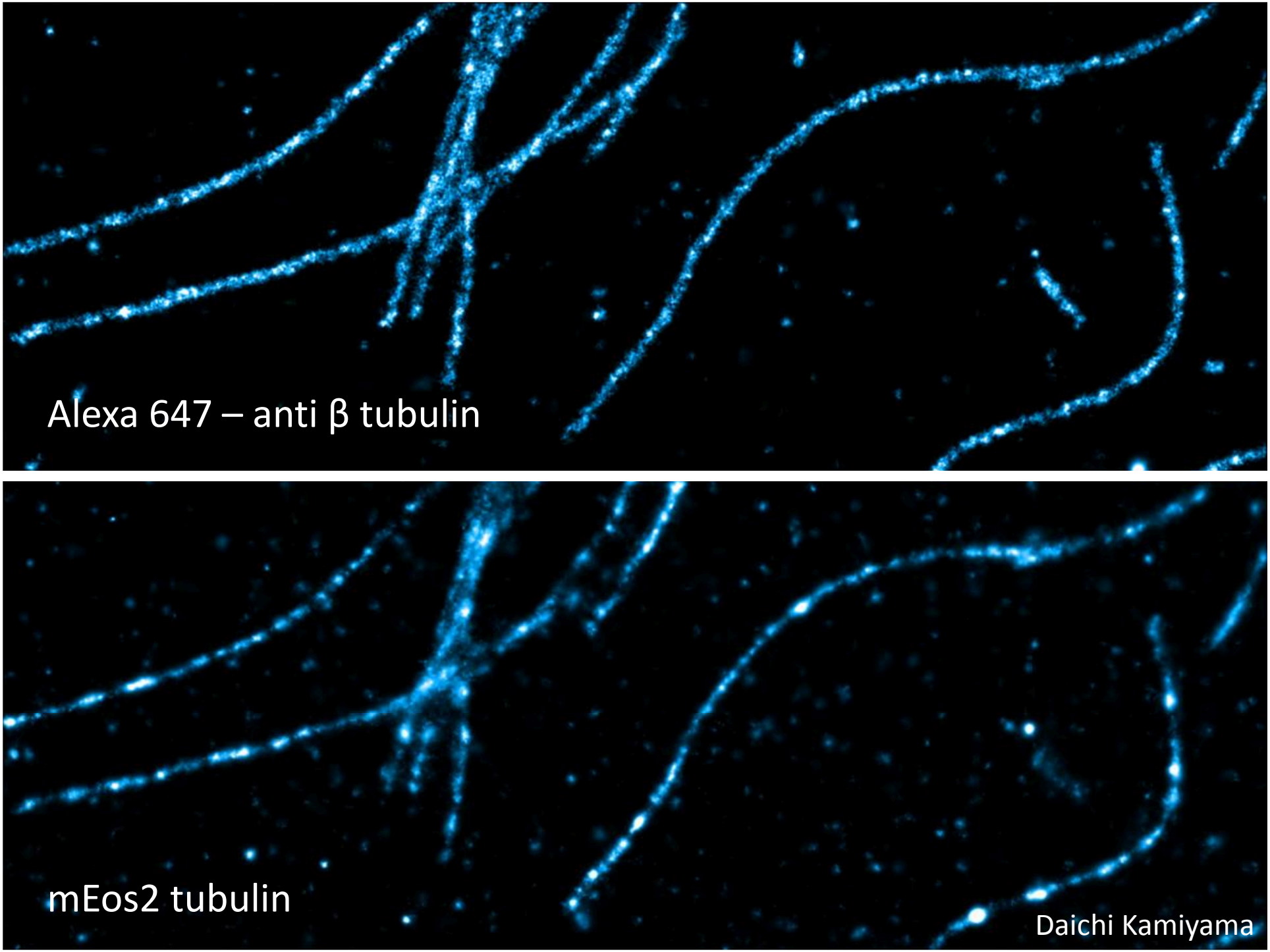
Schmidt et al., Nat Methods 2008

Multicolor STORM/PALM

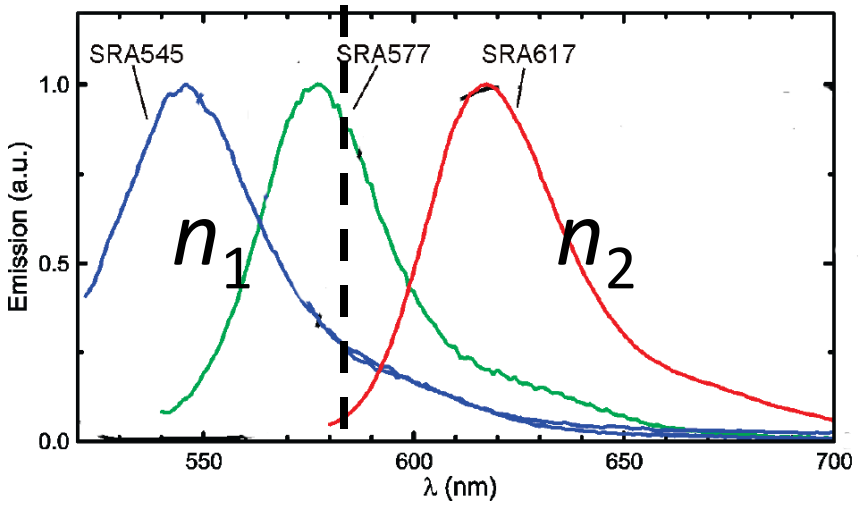





mEos2-tubulin
Alexa 647 anti-β tubulin
Drosophila S2 cells
2 µm
Daichi Kamiyama


Alexa 647 – anti β tubulin
mEos2 tubulin
Daichi Kamiyama

Multicolor STORM/PALM: Emission

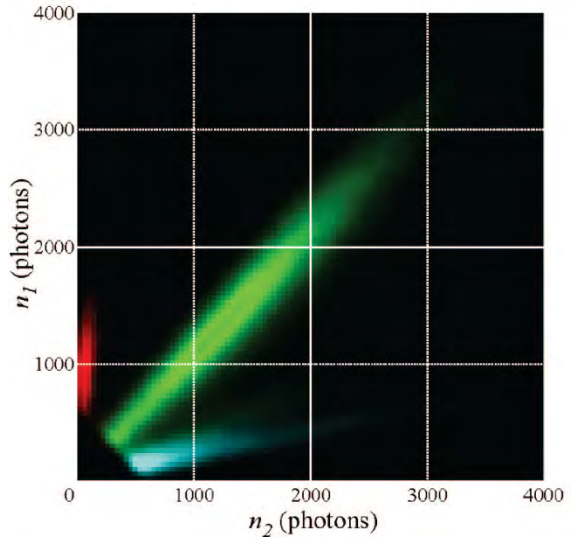


$n_1 = n_2$

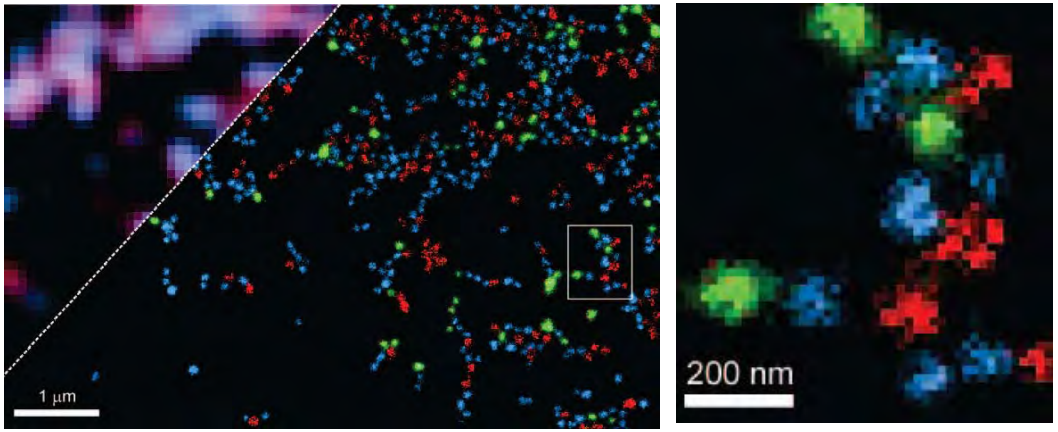
➔ 50% SRA545 + 50% SRA617?

➔ 100% SRA577?

Single-molecule detection!

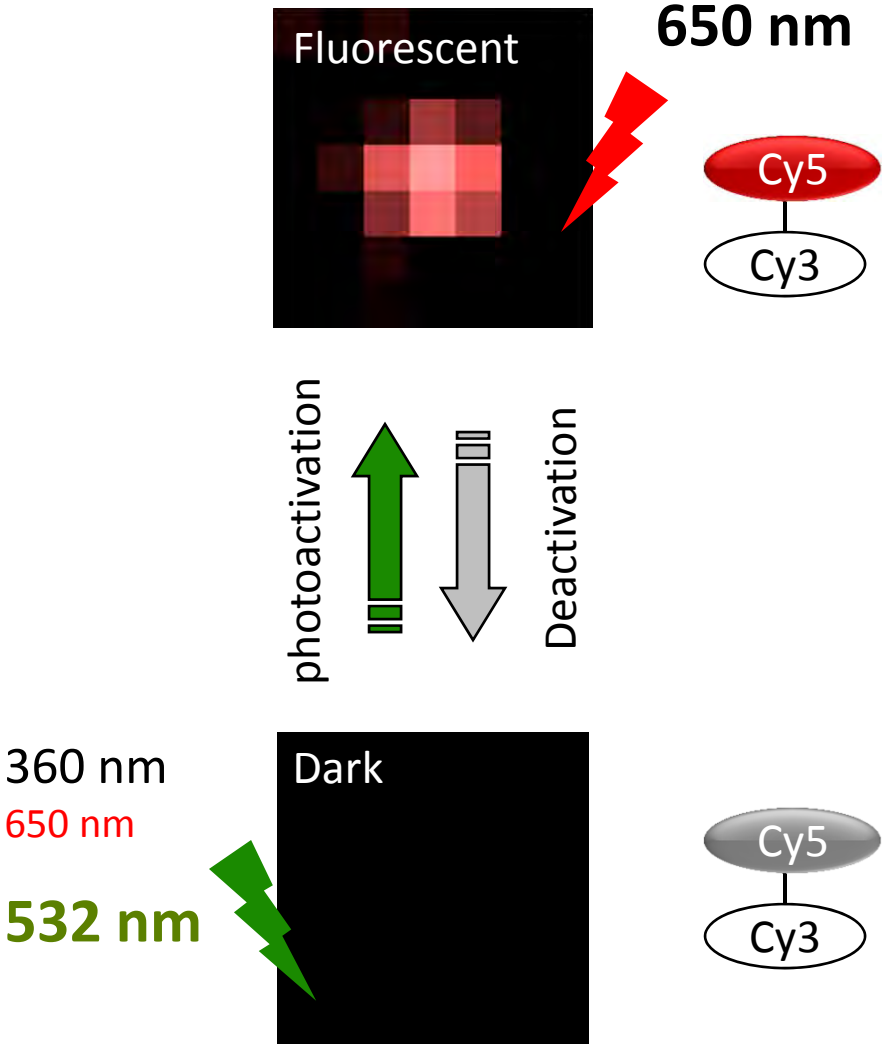


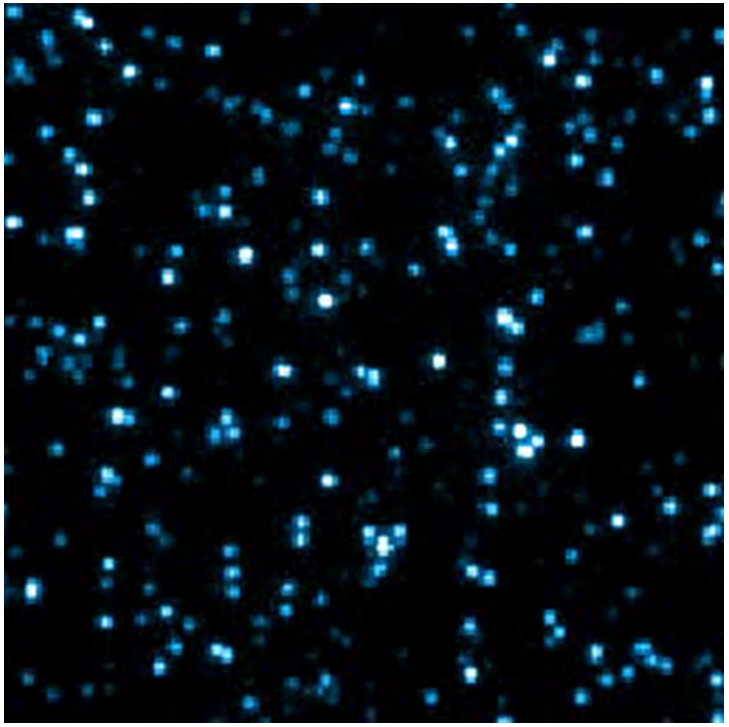
3-color imaging with one excitation wavelength and two detection channels

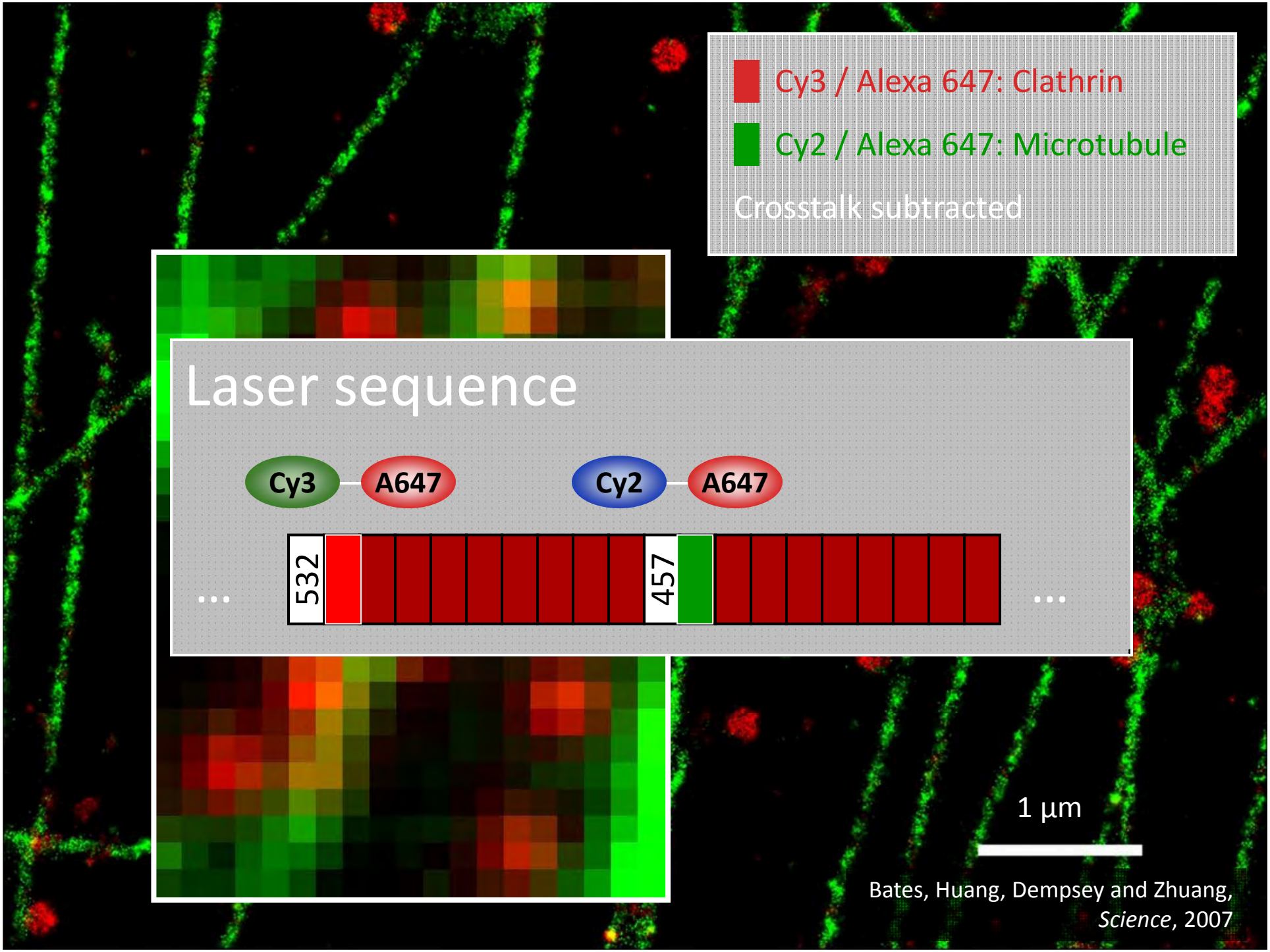


Bossi et al., Nano Lett 2008

Multicolor STORM/PALM: activation





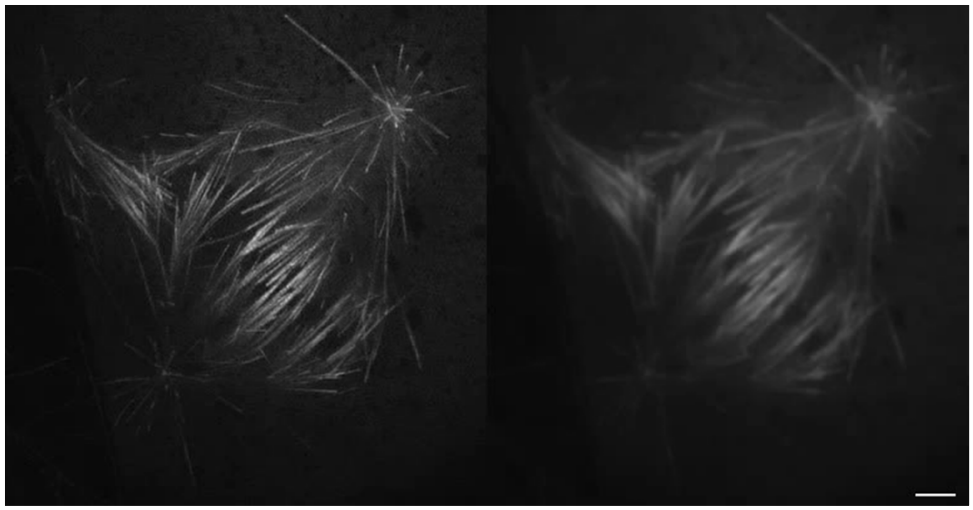

Cy3 / Alexa 647: Clathrin
Cy2 / Alexa 647: Microtubule
Crosstalk subtracted
Laser sequence
Cy3
A647
Cy2
A647
532
457
...
...
1 µm
Bates, Huang, Dempsey and Zhuang,
Science, 2007

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

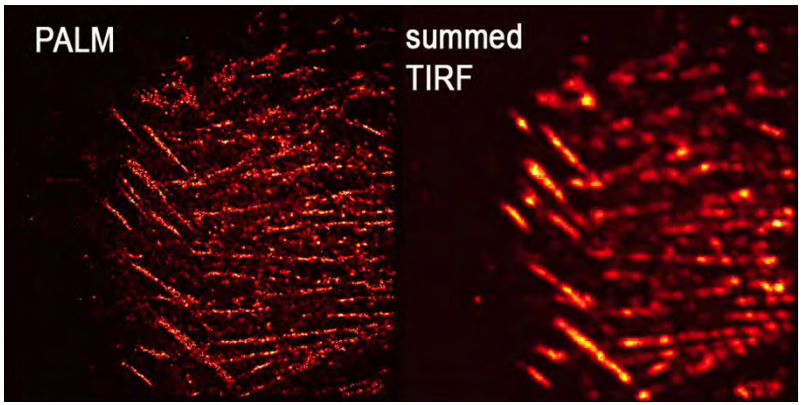
SIM



2 µm

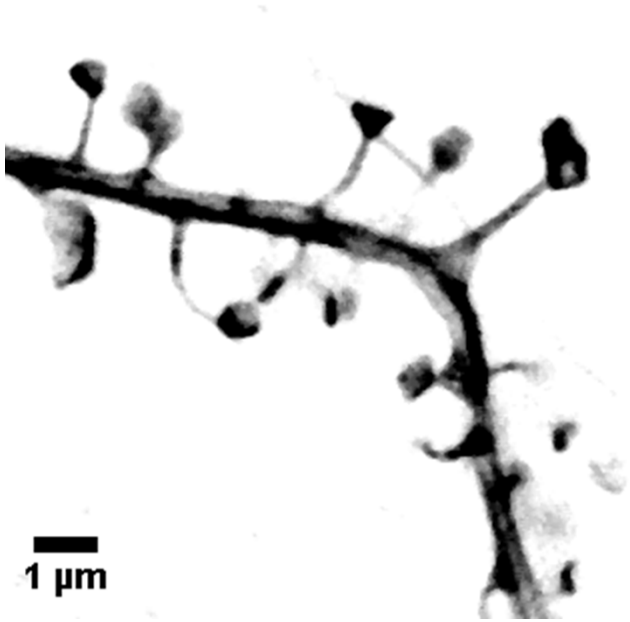
Kner, Chhun et al., Nat Methods, 2009

STORM/PALM



Schroff et al., Nat Methods, 2008

STED



Nagerl et al., PNAS, 2008

The limit of “Super-Resolution”

Unbound theoretical resolution

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

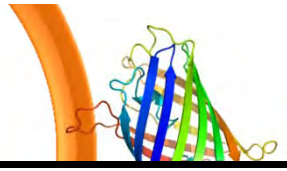
- STORM/PALM $S = \sqrt{N}$
  - 6,000 photons → 5 nm
  - 100,000 photos during Cy5 life time → < 1 nm
- STED $S = \sqrt{1 + I/I_s}$
  - 1:100 contrast of the donut → 20 nm
  - Diamond defects: 8 nm

Effective resolution: Probe matters

Antibodies:
~ 10 nm

Fluorescent Proteins:
~ 3 nm

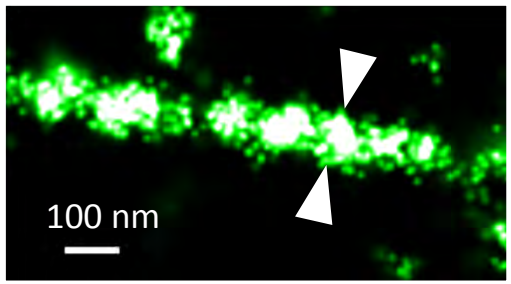
Small fluorophores:
~ 1 nm

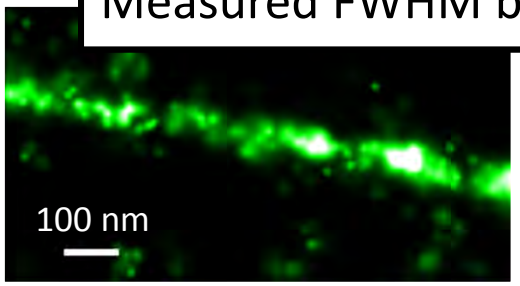


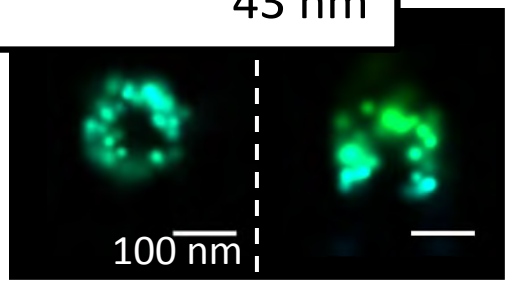
Measured FWHM by antibody: 58 nm

Actual microtubule diameter: 25 nm

Measured FWHM by FP: 43 nm







~ 6000 photons

< 1000 photons

~ 6000 photons

Fluorescent protein vs. Antibody

Fluorescent protein fusion

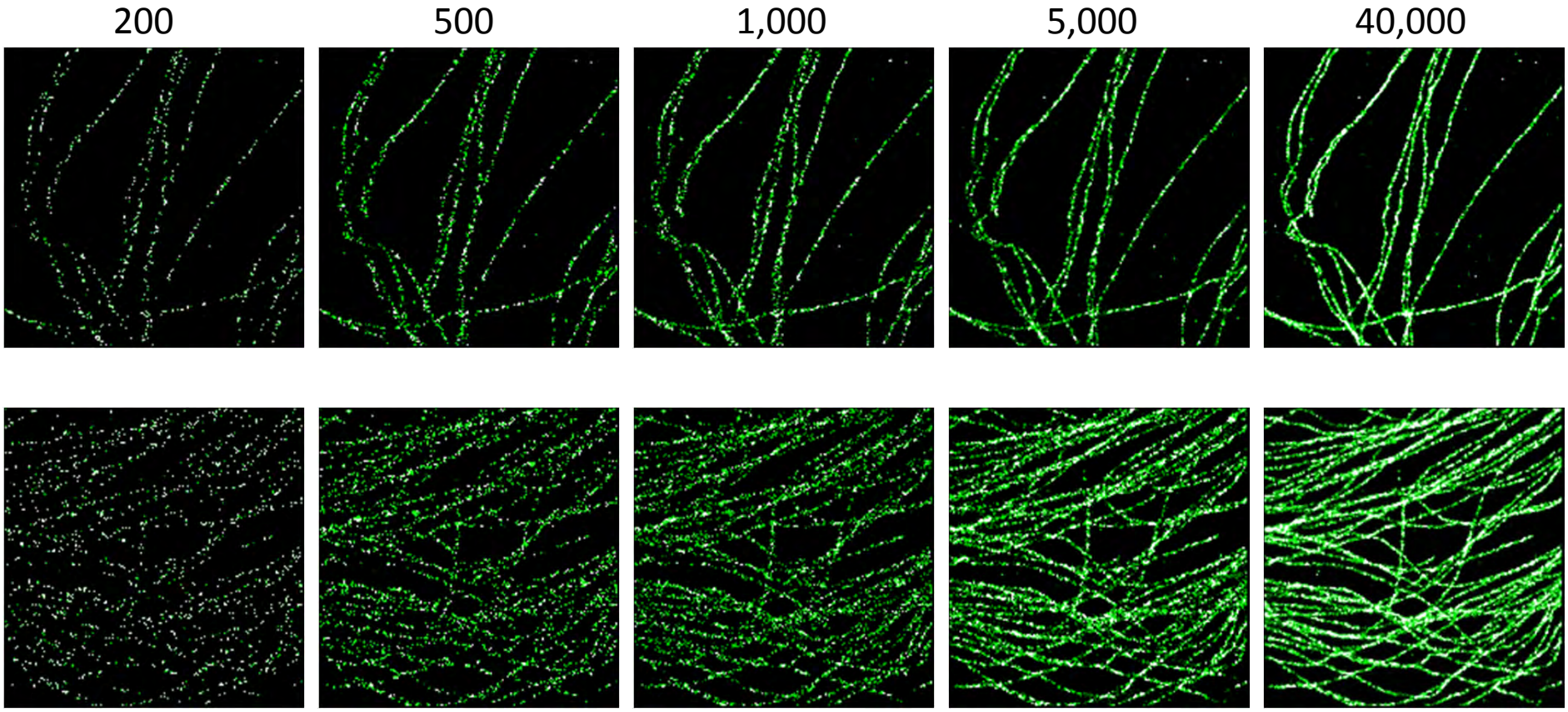
- Live sample labeling
- High specificity
- High labeling efficiency
- Genetically encoded
- Lower S/N
- Multicolor imaging so far challenging

Antibody immunofluorescence

- Fixed sample
- Potential nonspecific labeling
- Lower labeling efficiency
- Labeling endogenous proteins
- High signal = high localization precision
- More versatile for multicolor imaging

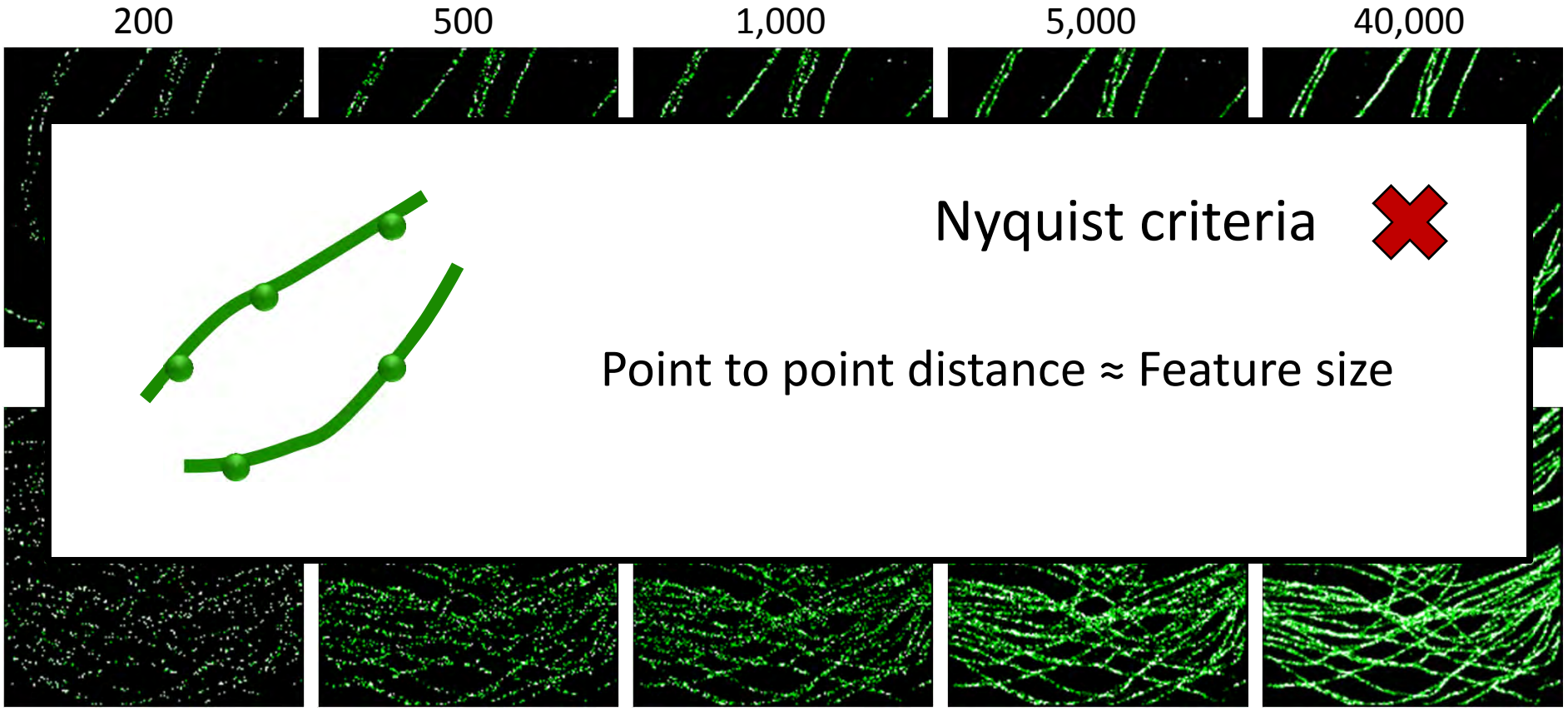
Effective resolution: Density matters

Frames for image reconstruction:



Effective resolution: Density matters

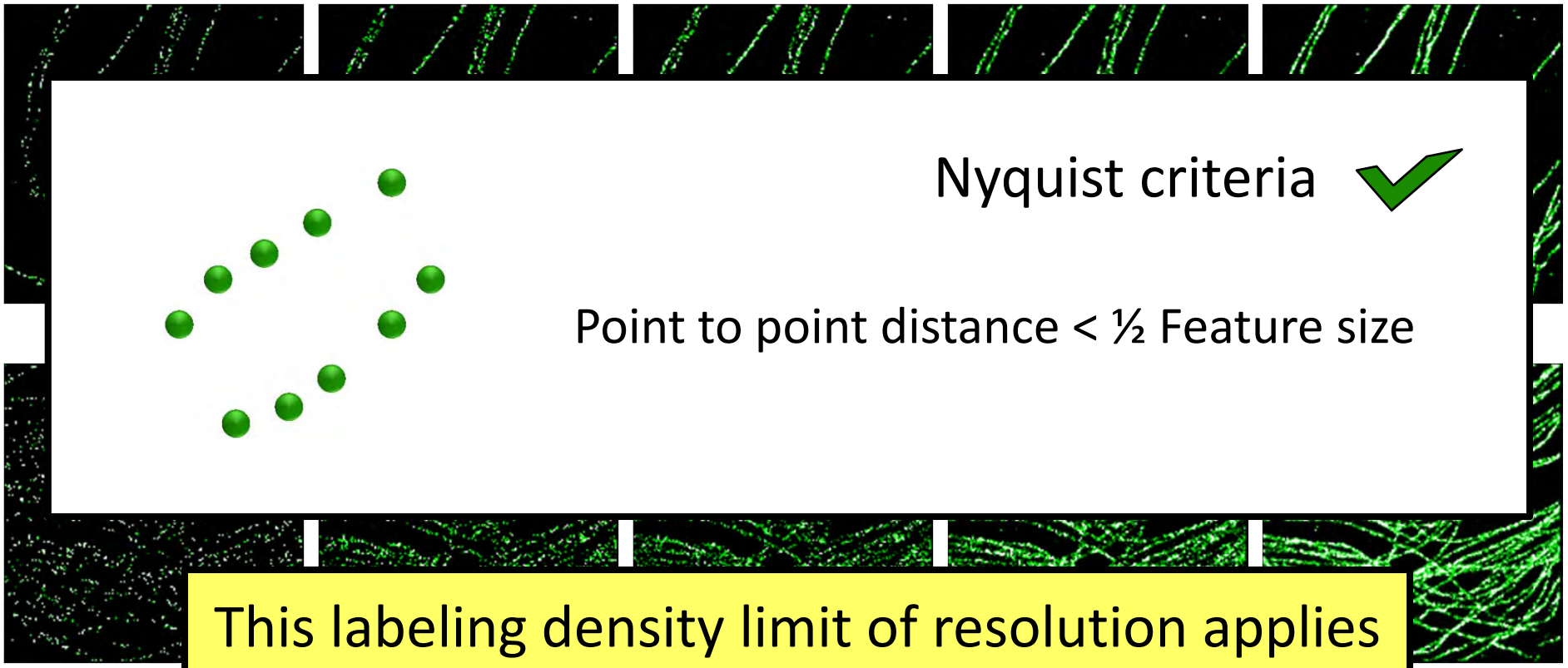
Frames for image reconstruction:



Effective resolution: Density matters

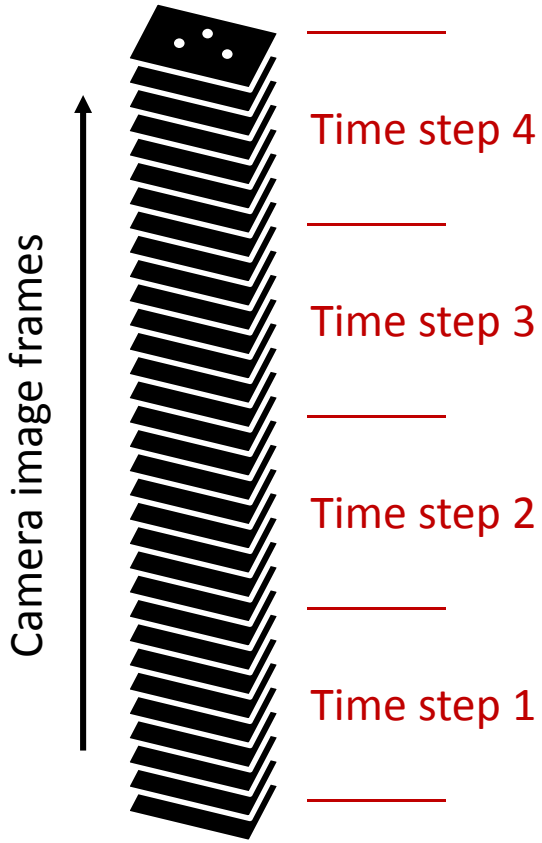
Frames for image reconstruction:

200 500 1,000 5,000 40,000

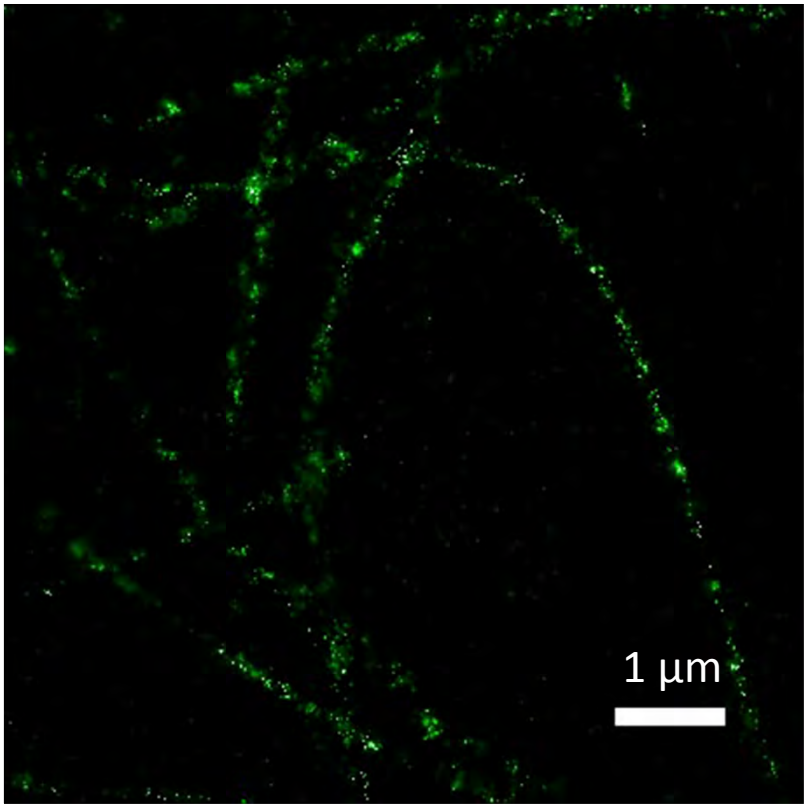


This labeling density limit of resolution applies to **all** fluorescence microscopy methods

Live cell STORM/PALM



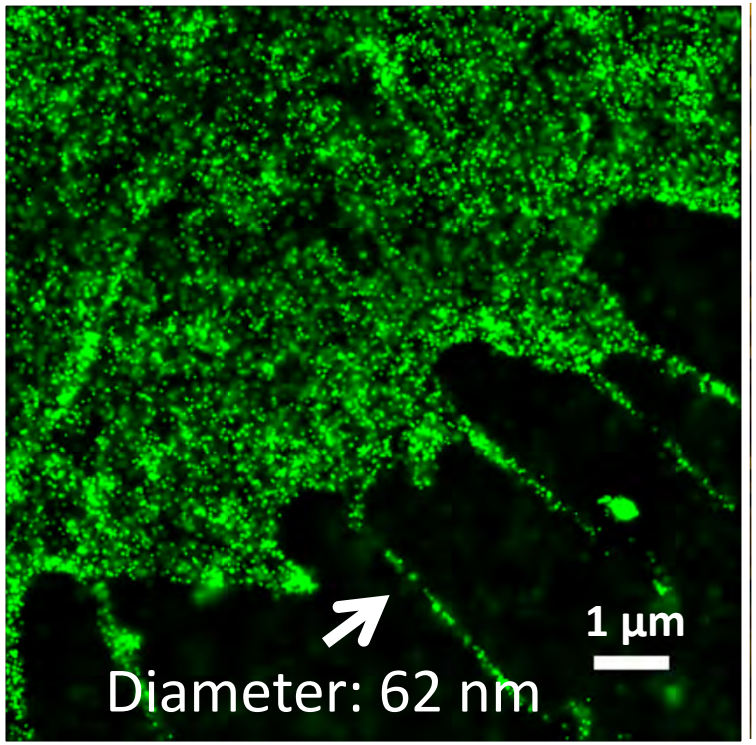
mEos2 labeled microtubule in live S2 cells



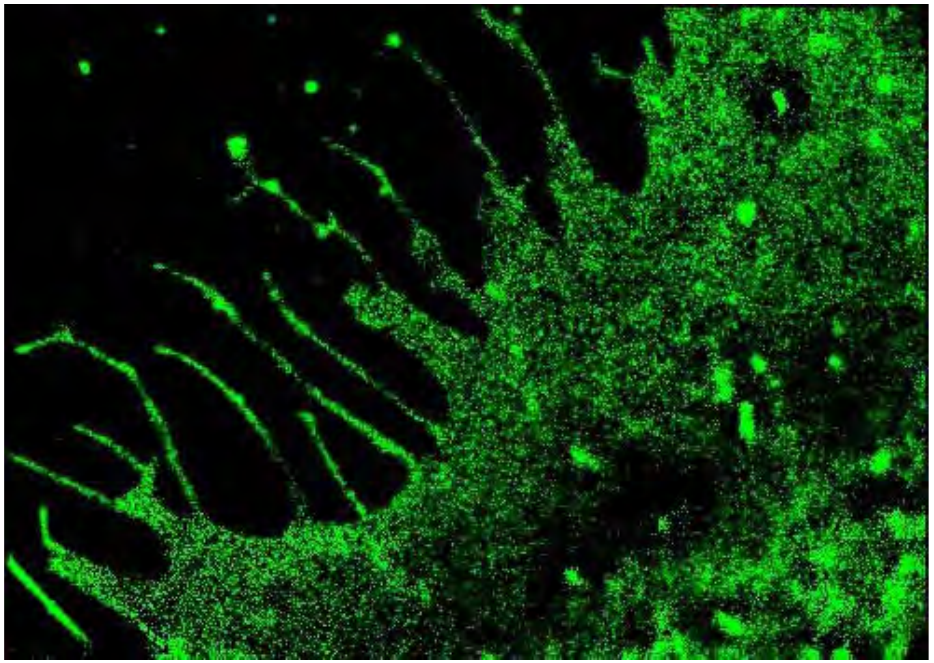
60 frames/sec
1200 frames/step (20 sec time resolution)
50x real time

Live cell imaging of plasma membrane

DiD stained plasma membrane

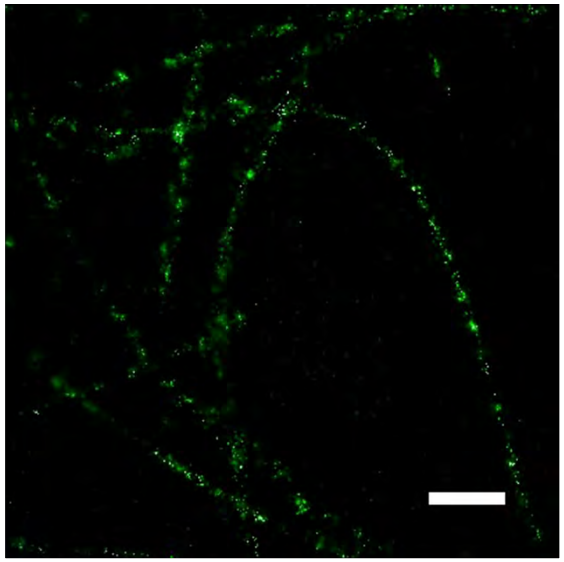


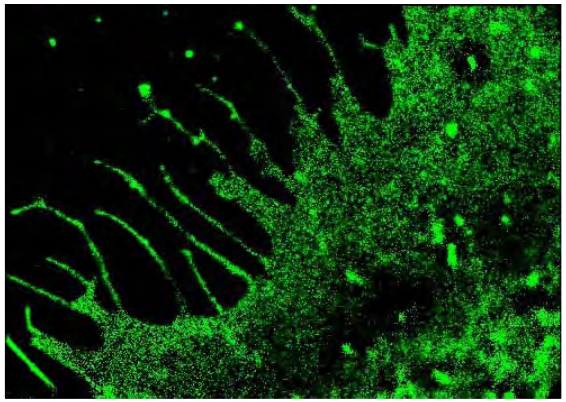
1000 frames, 10 sec total time

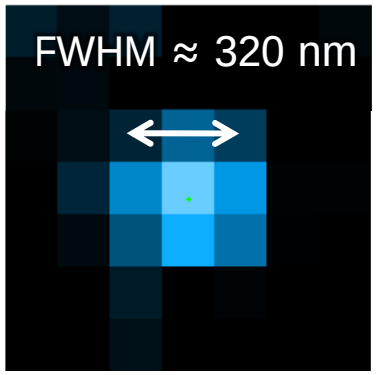


120 frames / sec, 3000 frames (25 sec)
100x real time
3 mM mercaptoethylamine
1 µm

Spatial-temporal resolution trade-off







Assuming:

1 molecule occupies 500 × 500 nm

↓

On average 0.1 point / 0.25 μm²·frame → 2 points

↓

70 nm resolution ≡ 2000 frames

↓

100 fps = 20 sec time resolution → 1000 fps

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!

Useful review articles

- B. Huang, H. Babcock, X. Zhuang, “Breaking the diffraction barrier: super-resolution imaging of cells”, *Cell*, 143, 1047-1058 (2010).
- S. Hell, "Microscopy and its focal switch", *Nat. Methods*, 6, 24-32 (2009).
- B. Huang, M. Bates, X. Zhuang, “Super resolution fluorescence microscopy”, *Ann. Rev. Biochem.*, 17, 993-1016 (2009).
- S. Hell, "Far-field optical nanoscopy", *Science*, 316, 1153-1158 (2007).
- R. Heintzmann, M. G. L. Gustafsson, "Subdiffraction resolution in continuous samples", *Nat. Photonics*, 3**,** 362-364 (2009).
- B. Huang, “Super resolution optical microscopy: multiple choices”, *Curr. Opin. Chem. Biol.*,14, 10-14 (2010).
- M. Fernandez-Suarez, A. Y. Ting, "Fluorescent probes for super-resolution imaging in living cells. *Nat. Rev. Mol. Cell Biol.,* 9, 929-943 (2008).
- J. Lippincott-Schwartz, G.H. Patterson, "Photoactivatible fluorescent proteins for diffraction-limited and super-resolution imaging", *Trends in Cell Biology*, 19, 555-565 (2009).