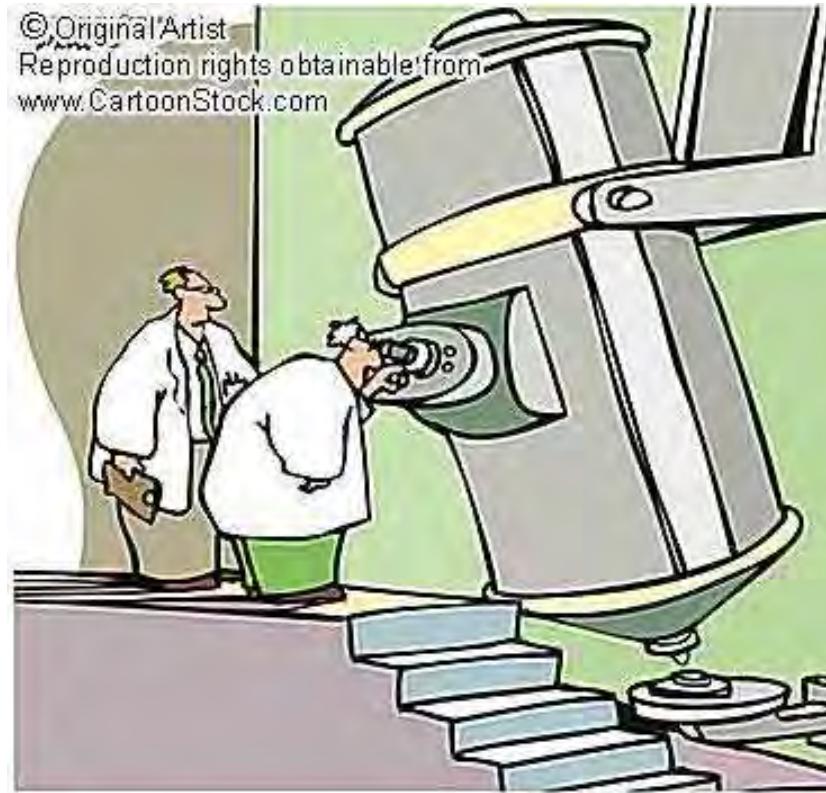
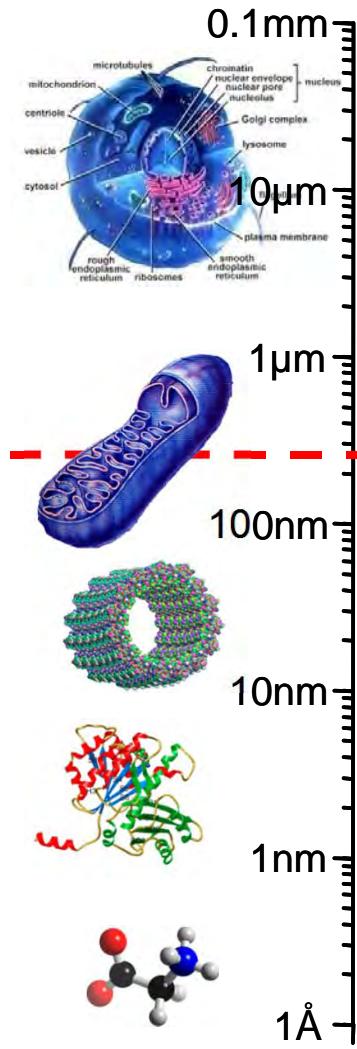


Super-Resolution Optical Microscopy



Bo Huang
Mar 30, 2012



Naked eye: ~ 50-100

PLATE XXIV

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



The First Compound Microscope

Compound microscope
>1000x

1600 1700 1800 1900 2000

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

fig: A

fig: B

fig: E

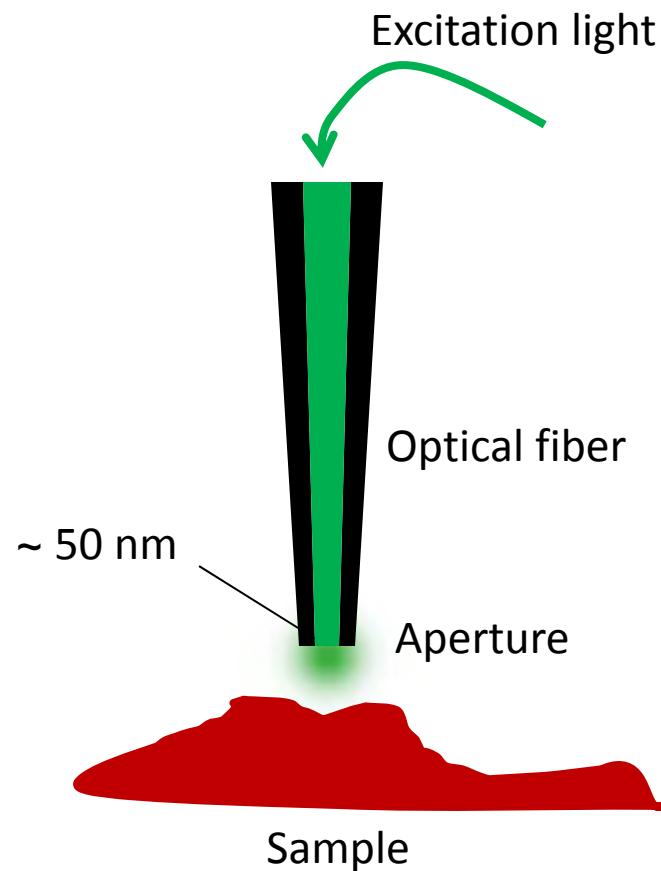
fig: R

Ernst Abbe (1840-1905)
The “physical” diffraction limit

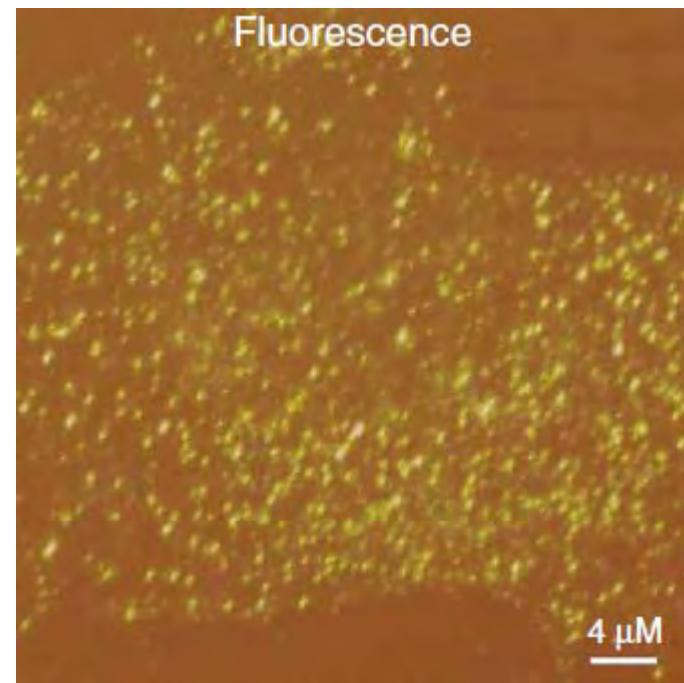
50 years to extend the resolution

- 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

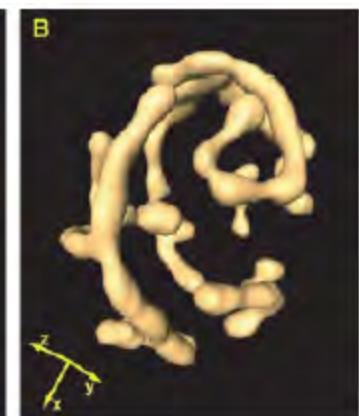
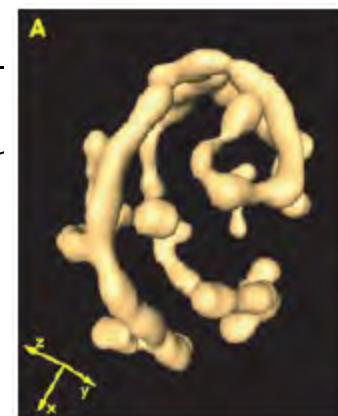
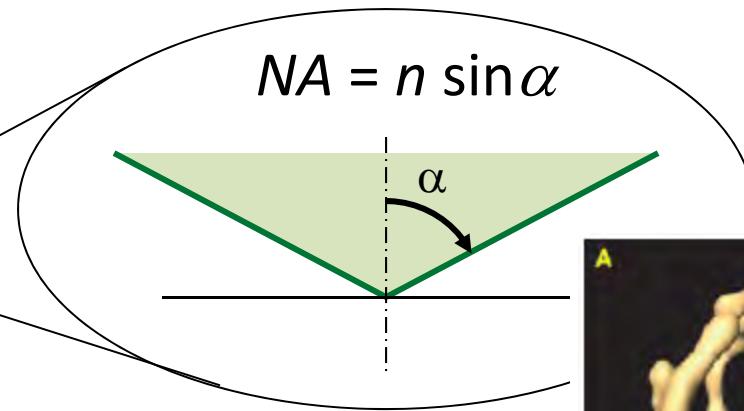
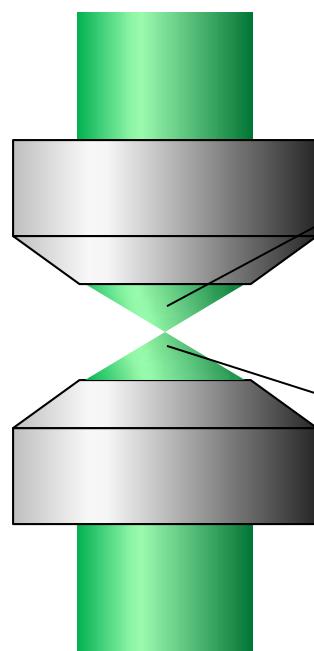


β_2 adrenergic receptor clusters
on the plasma membrane



Ianoul et al., 2005

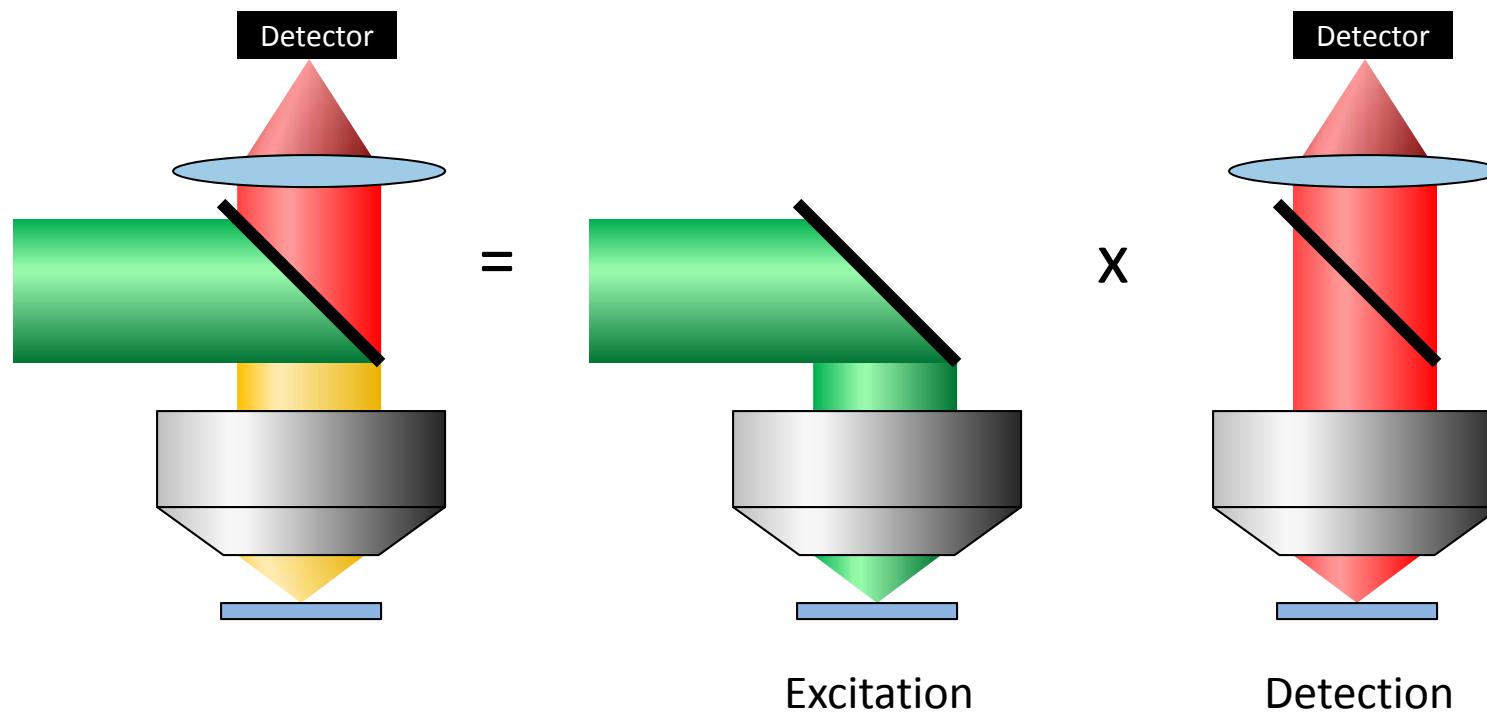
4-Pi / $I^5 M$



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

Major advantage:
Similar z resolution as x-y resolution

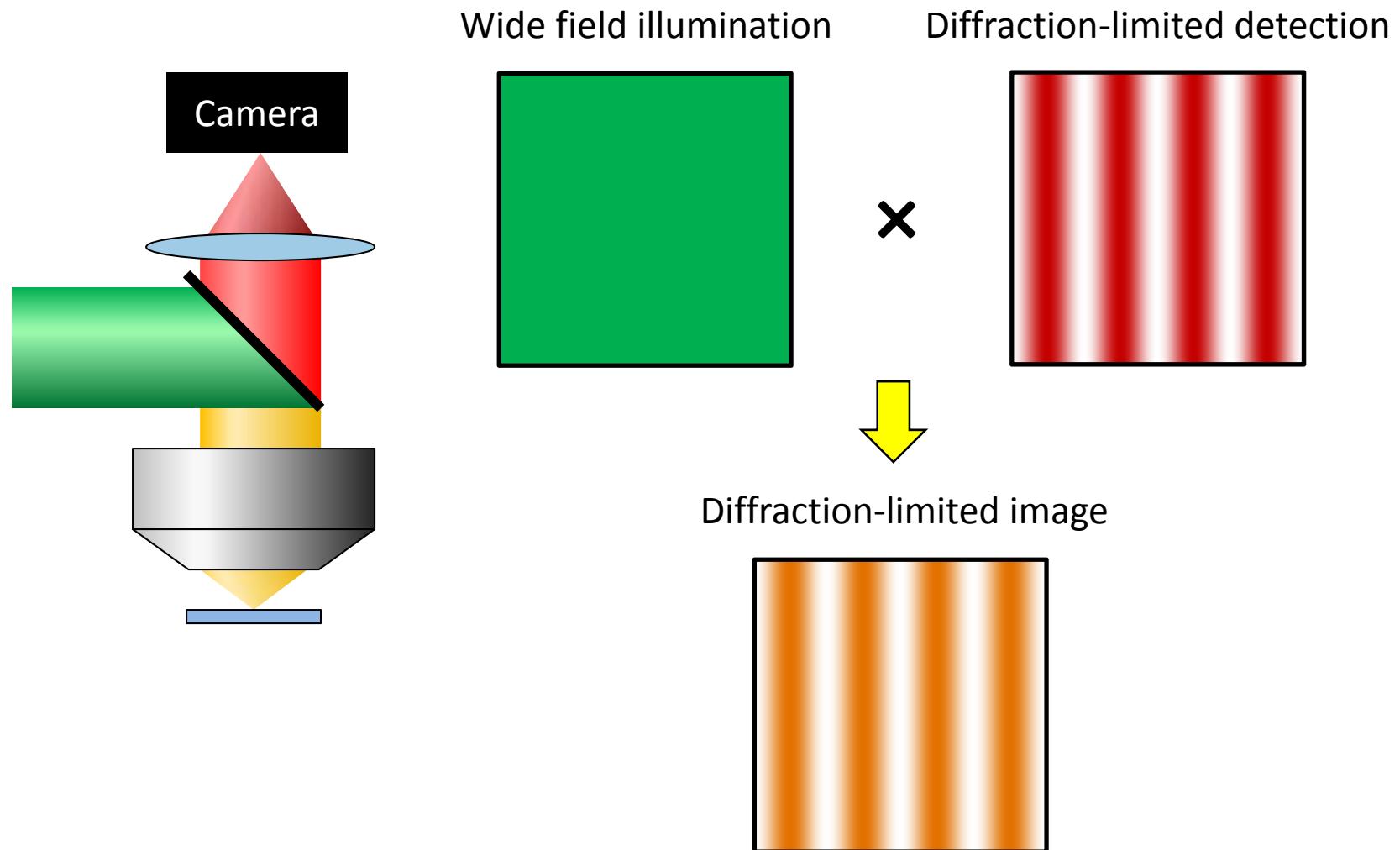
Patterned illumination



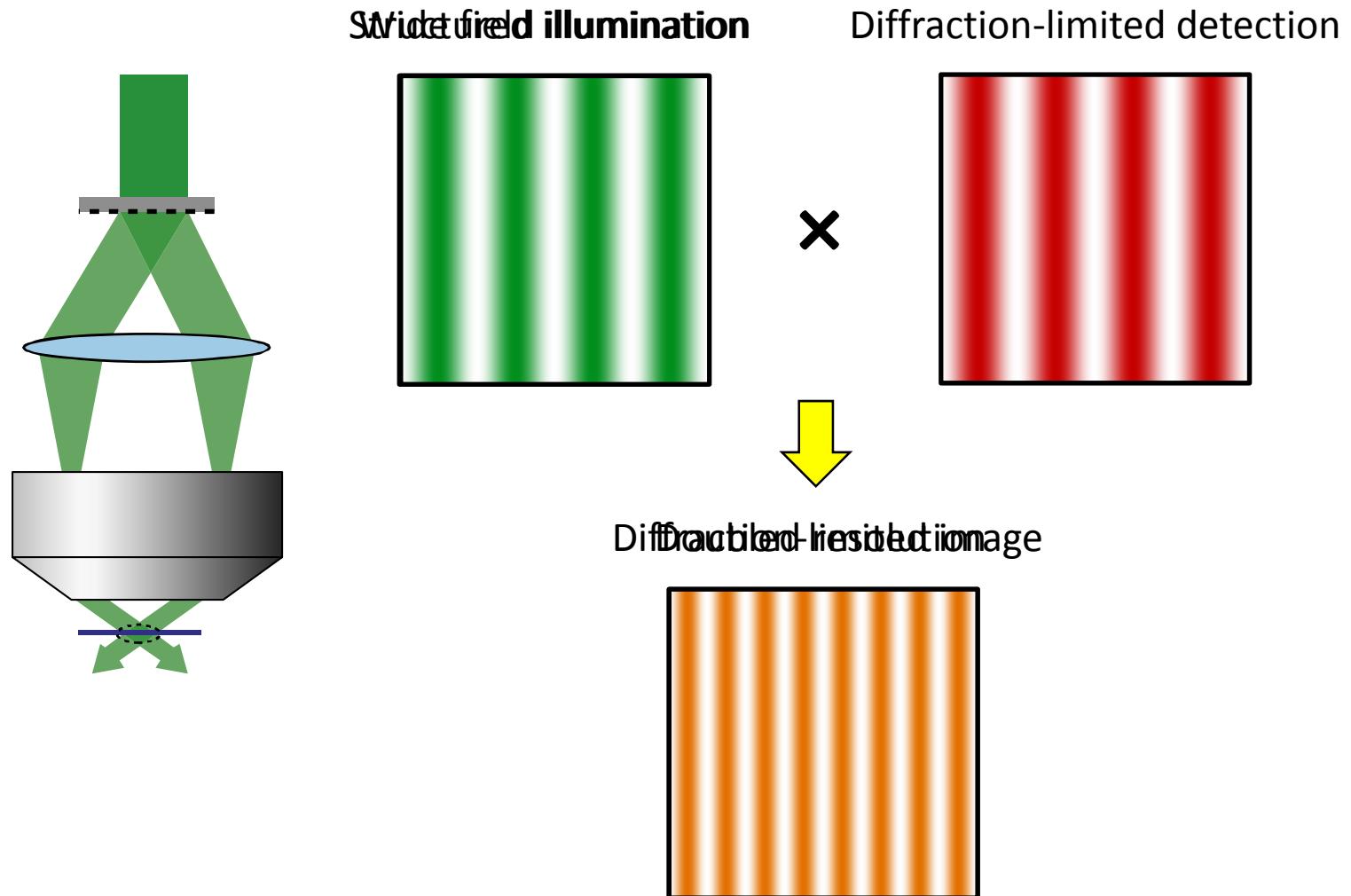
x



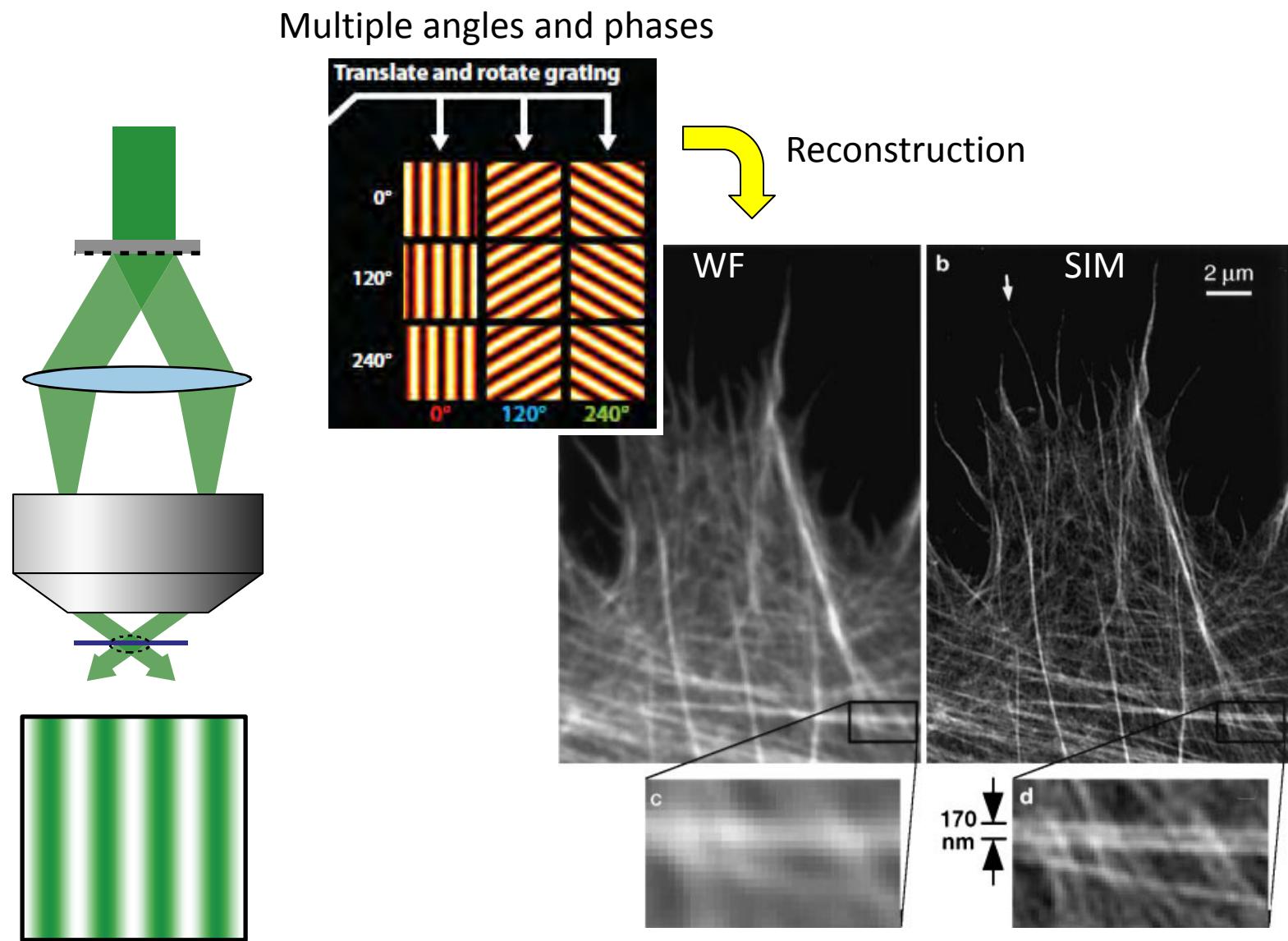
Structured Illumination Microscopy (SIM)



Structured Illumination Microscopy (SIM)

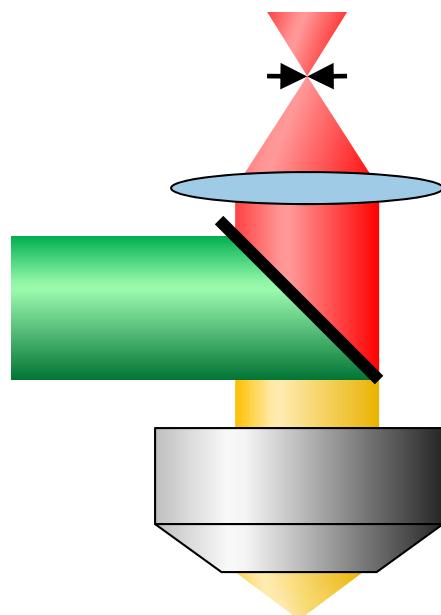


Structured Illumination Microscopy (SIM)

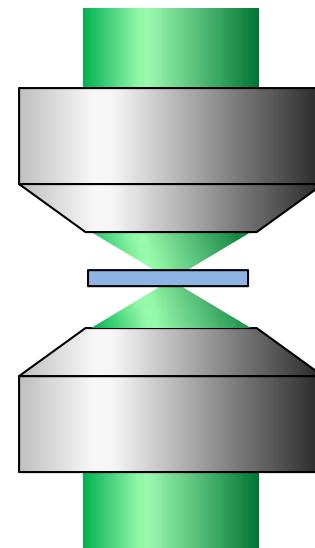


Gustafsson, J Microscopy 2000

The diffraction limit still exists

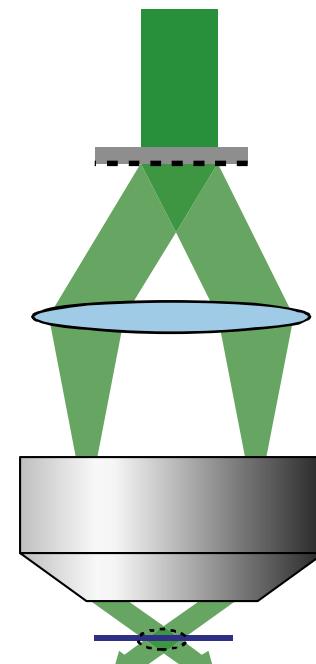


Confocal



$4\pi / I^5M$

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

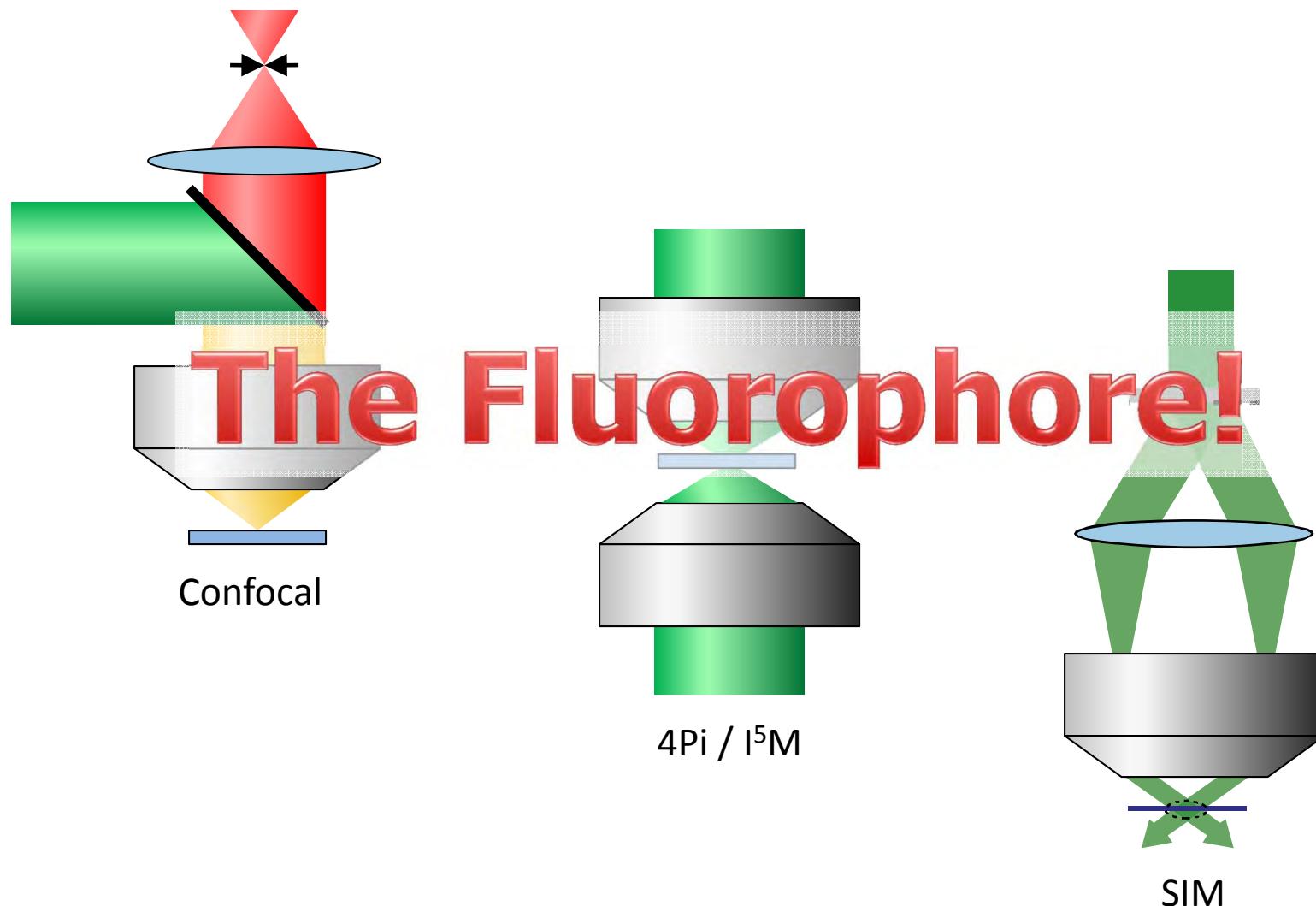


SIM

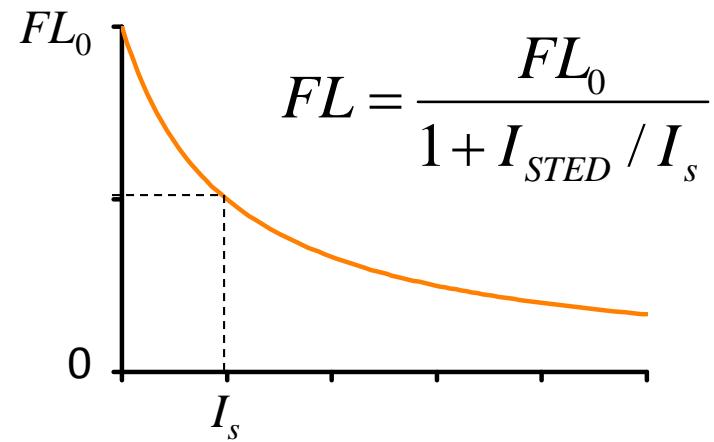
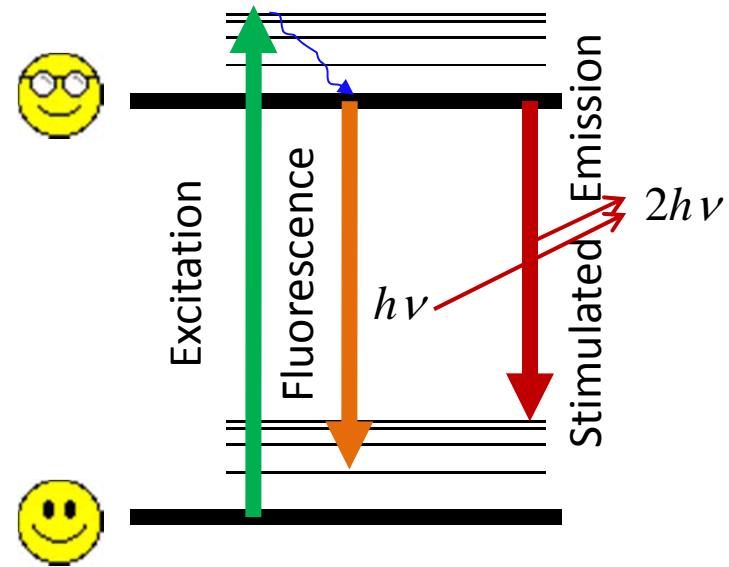
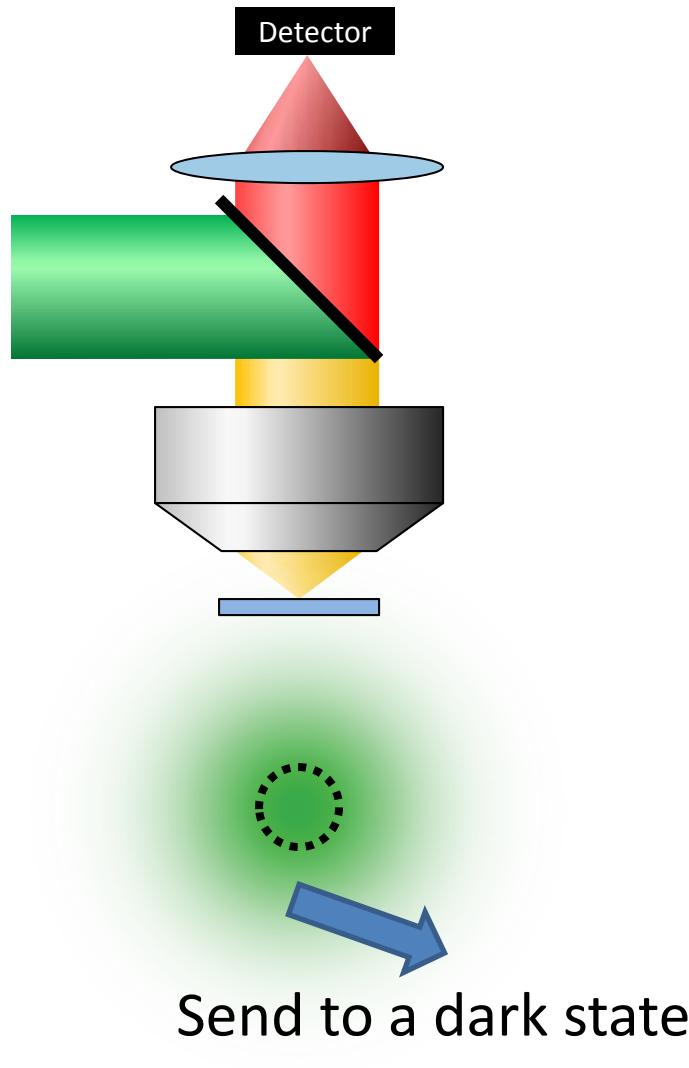
Breaking the diffraction barrier



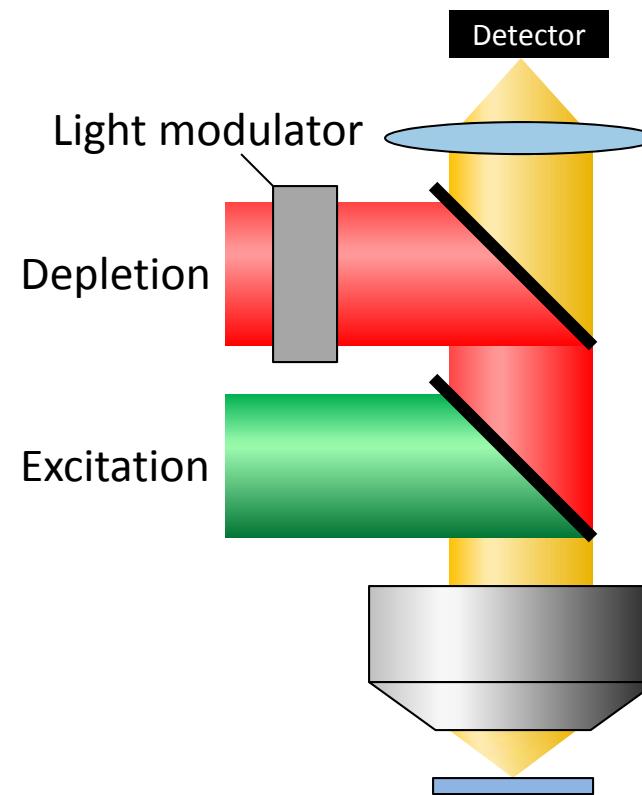
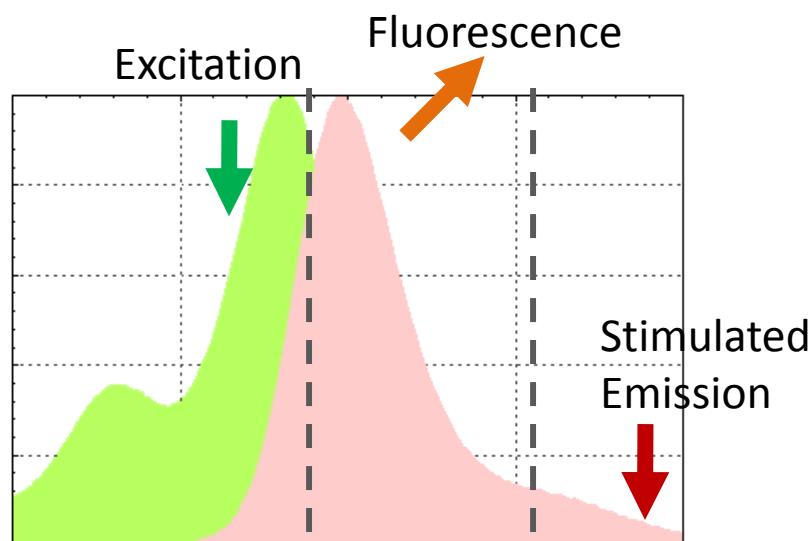
Breaking the diffraction barrier



Stimulated Emission Depletion (STED)



STED microscopy



Excitation

STED
pattern

Effective
PSF



÷



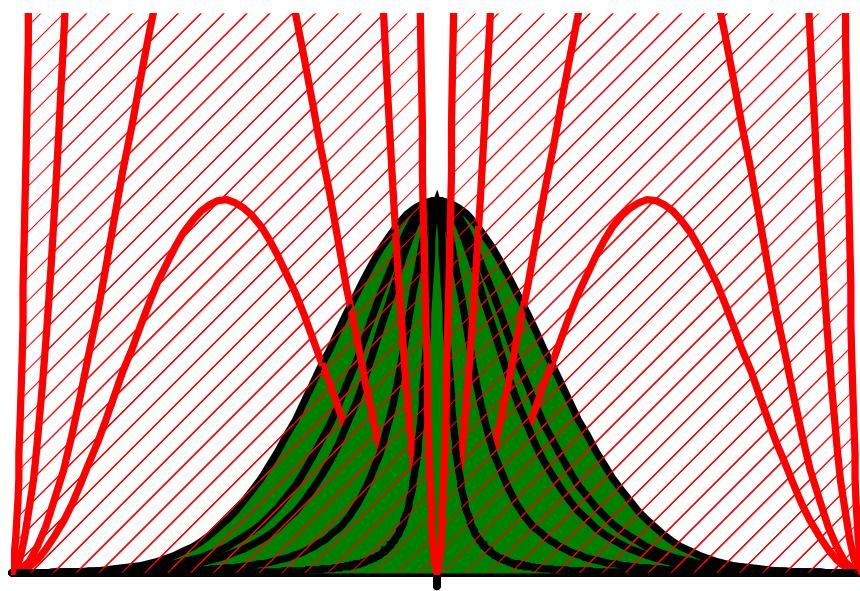
=



?

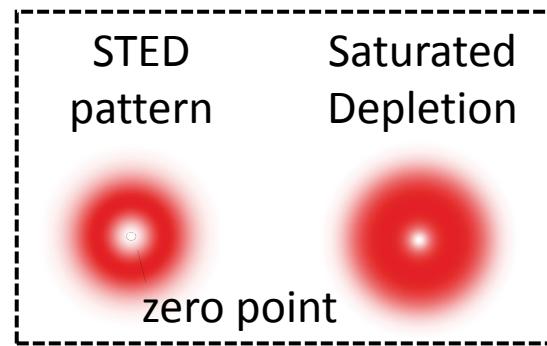
Hell 1994, Hell 2000

Saturated depletion

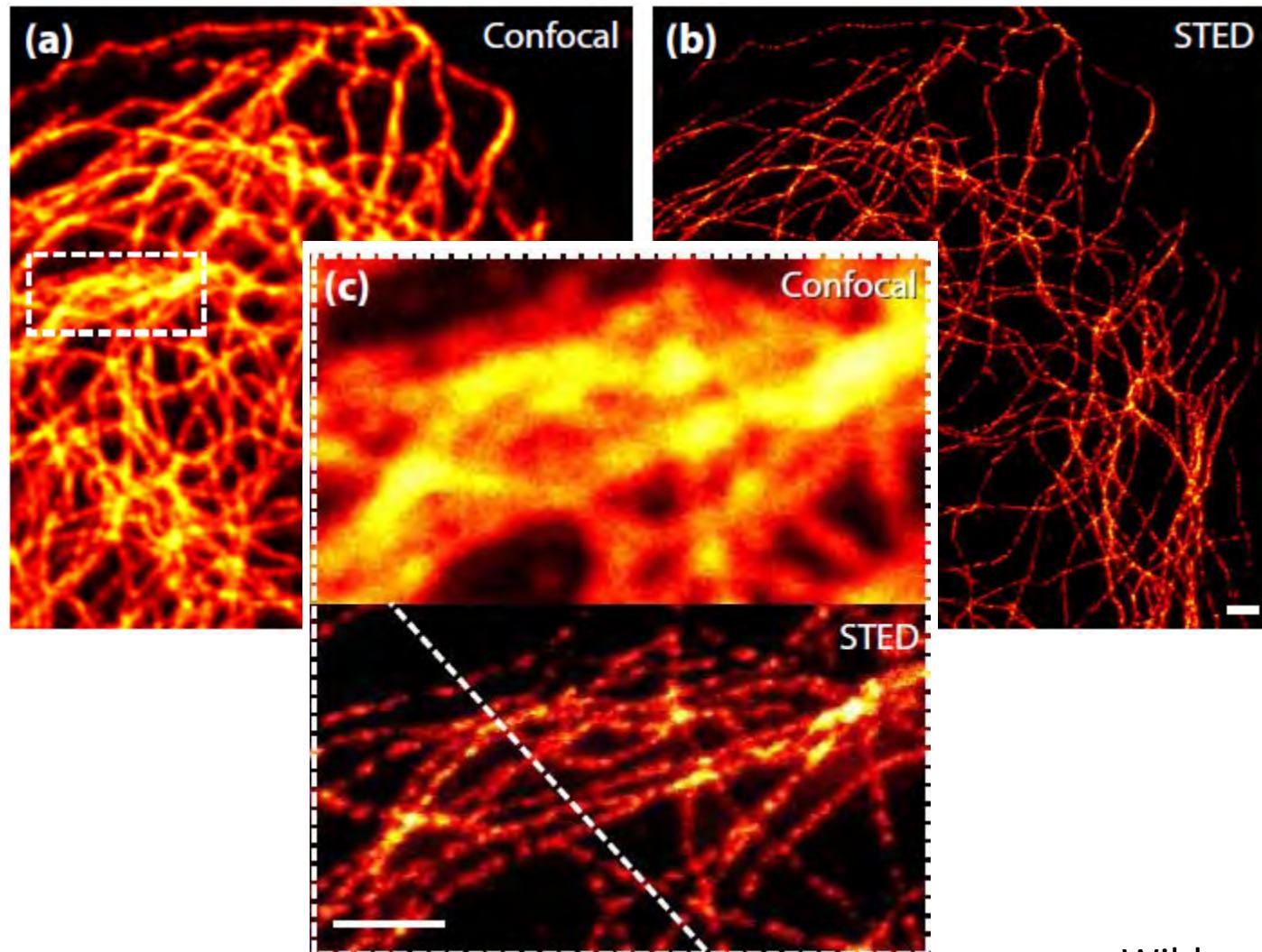


$$I_{\text{STED}} = 20 Q_S I_S$$

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

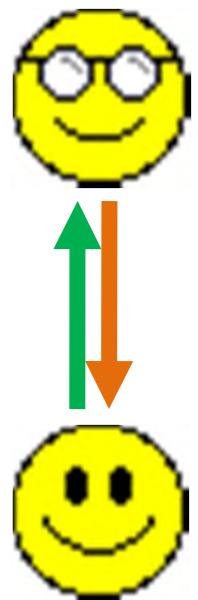


STED images of microtubules



Wildanger et al., 2009

The “patterned illumination” approach



Excitation

Multiple cycles



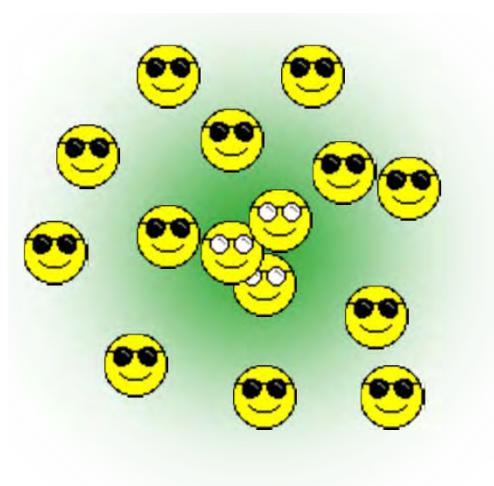
- Ground state
- Triplet state
- Isomerization
- etc.



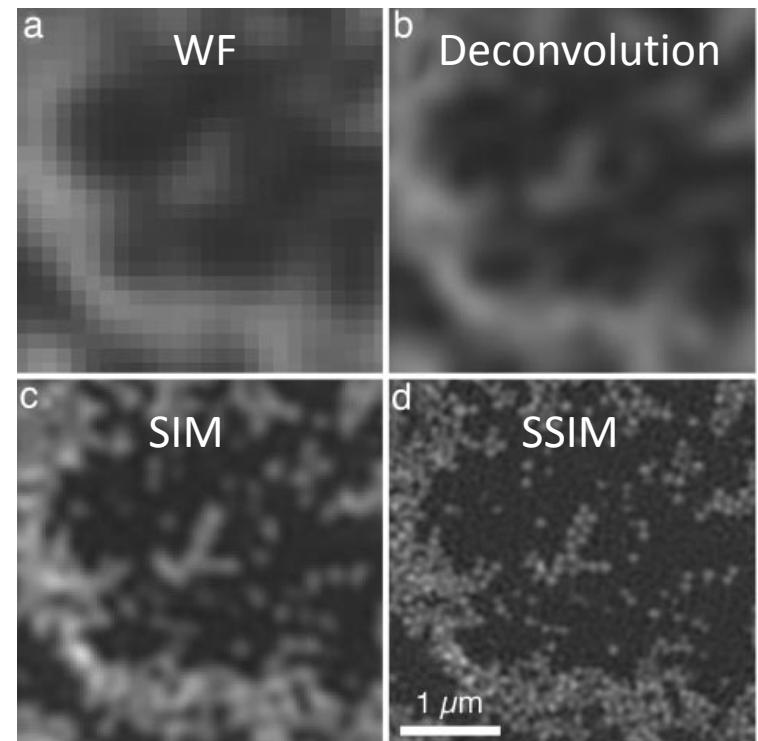
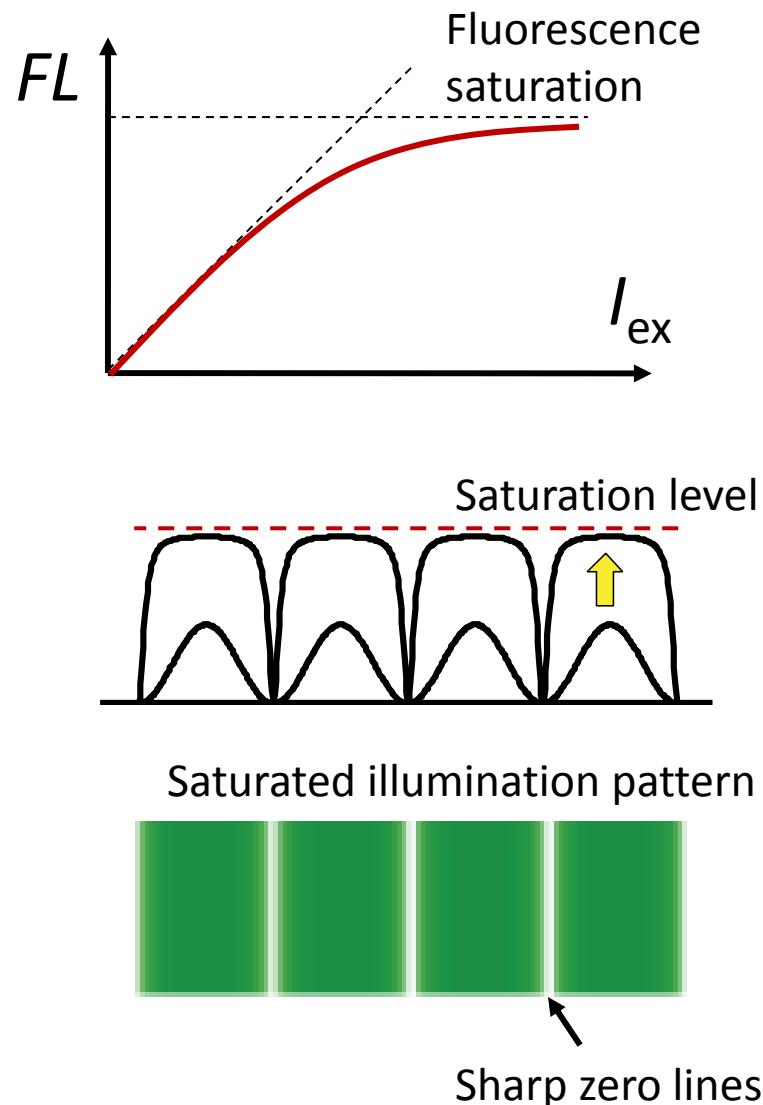
÷

Depletion
pattern

=



Saturated SIM

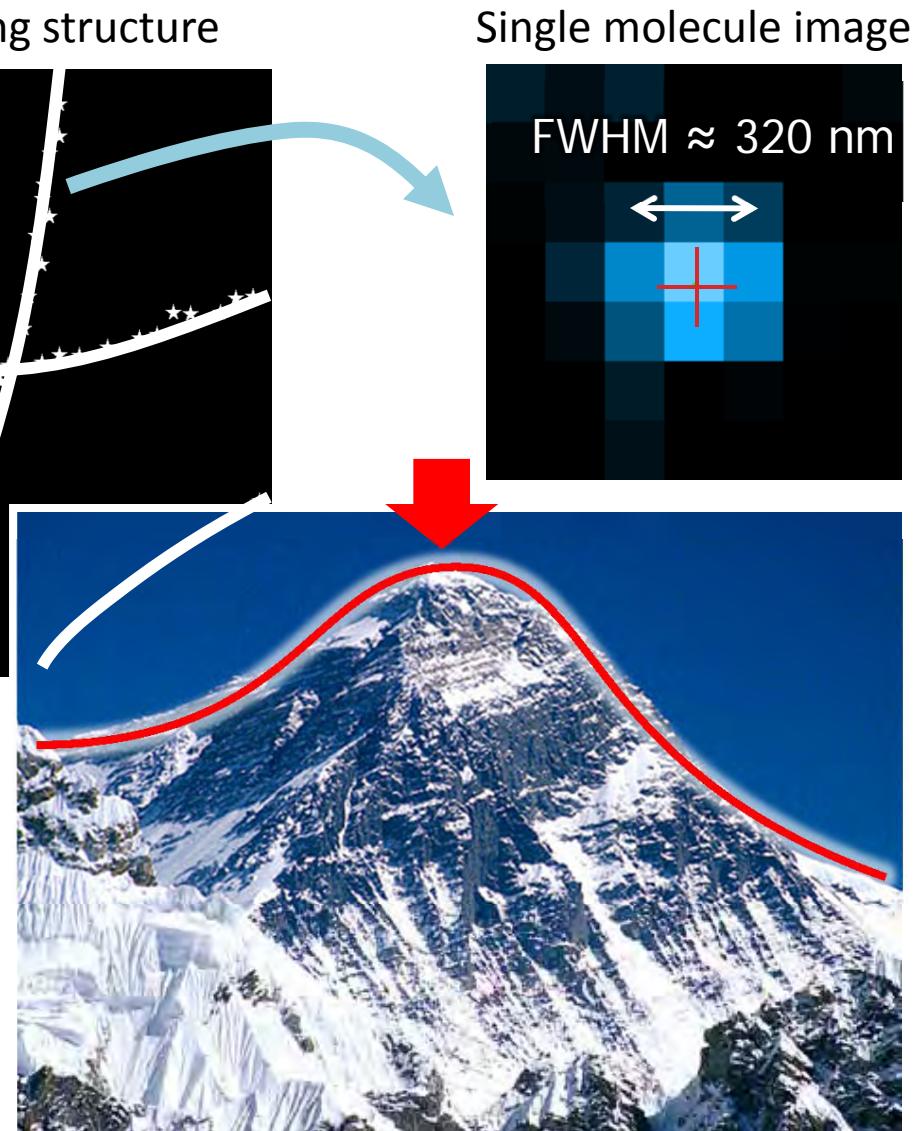
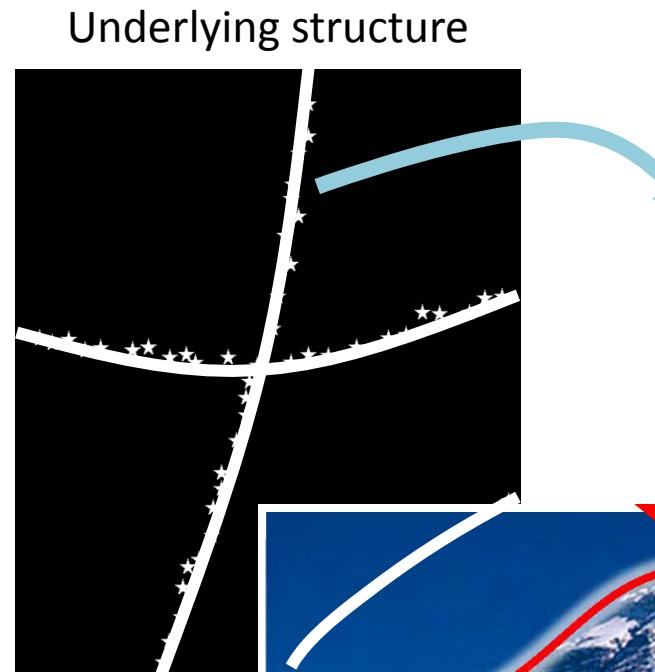
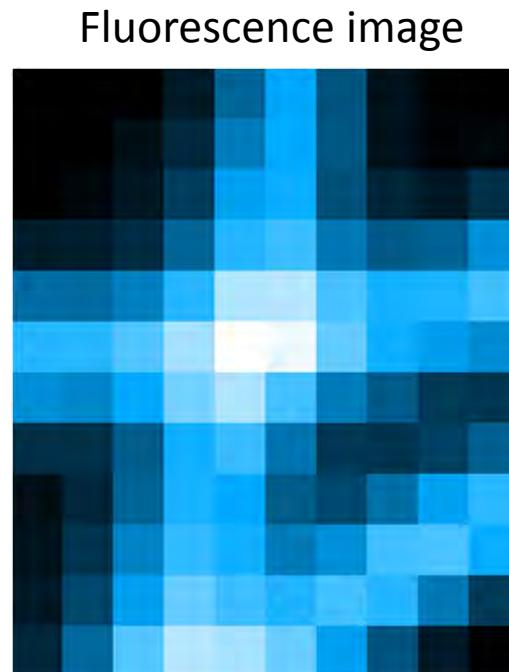


Suffers from fast photobleaching
under saturated excitation condition

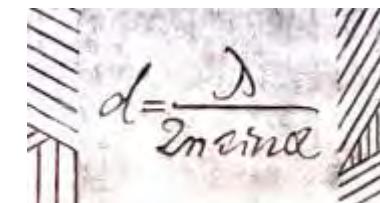
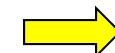
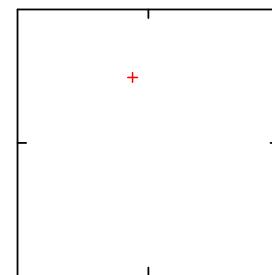
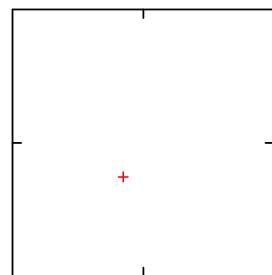
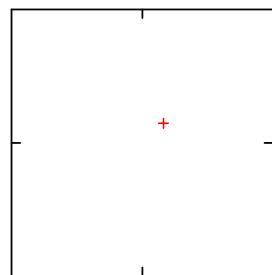
The single-molecule switching approach



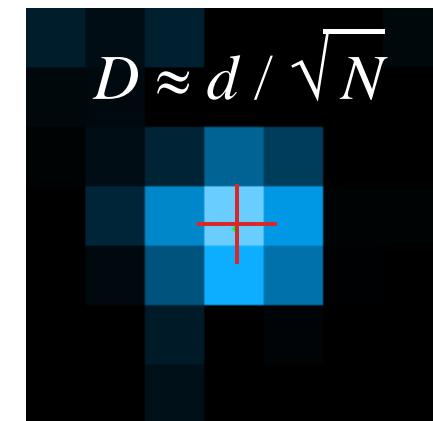
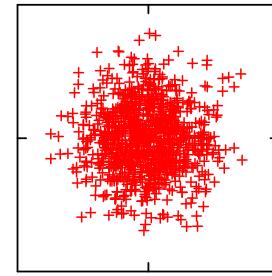
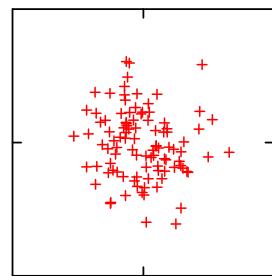
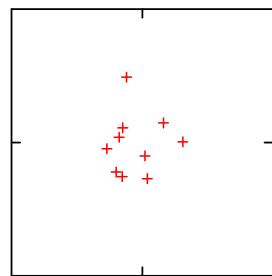
STORM/PALM: Single molecule localization



Single-molecule localization precision



1 photon



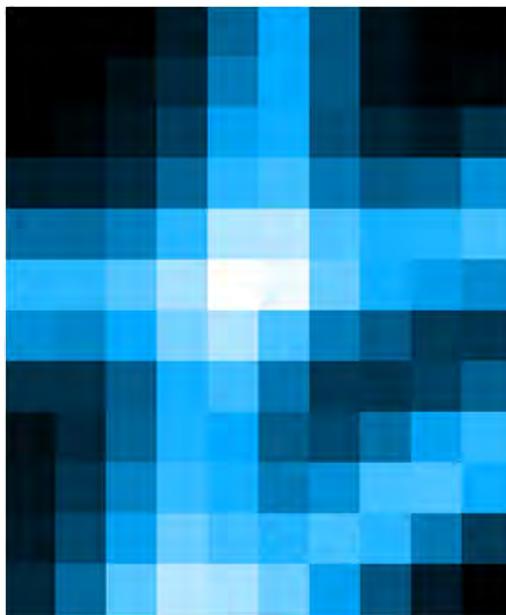
10 photons

100 photons

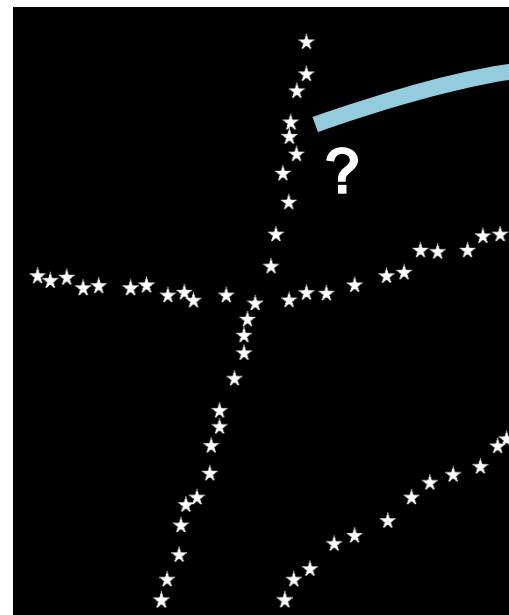
1000 photons

STORM/PALM: Single molecule localization

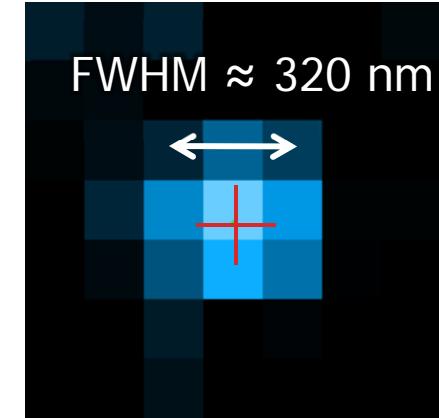
Fluorescence image



Underlying structure

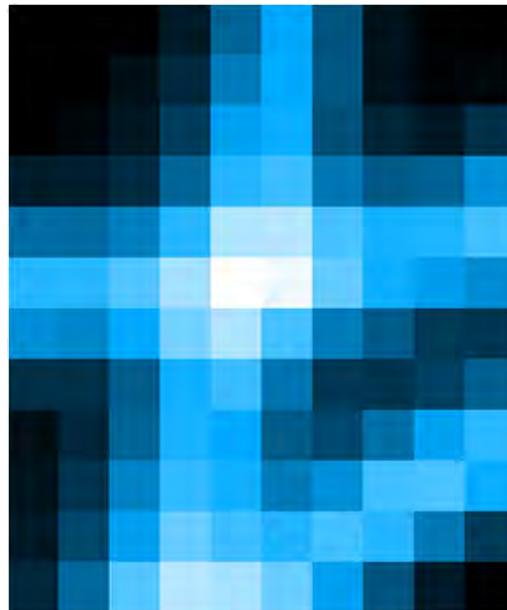


Single molecule image

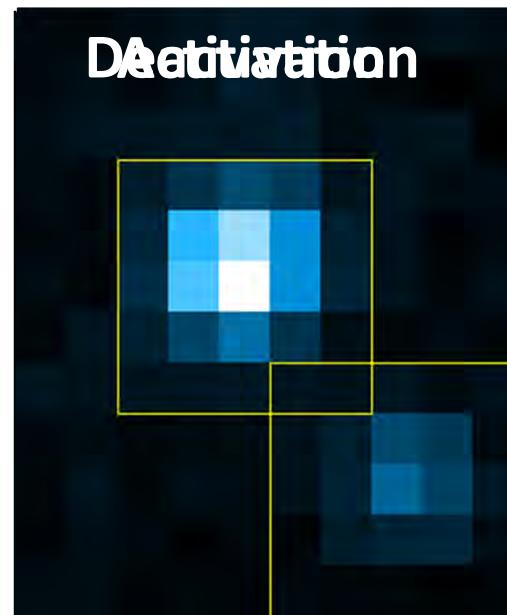


STORM/PALM: Single molecule switching

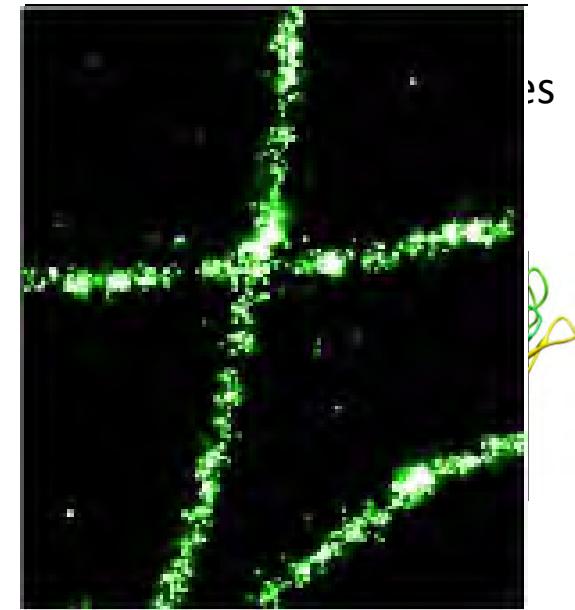
Fluorescence image



Raw images



STORM Image



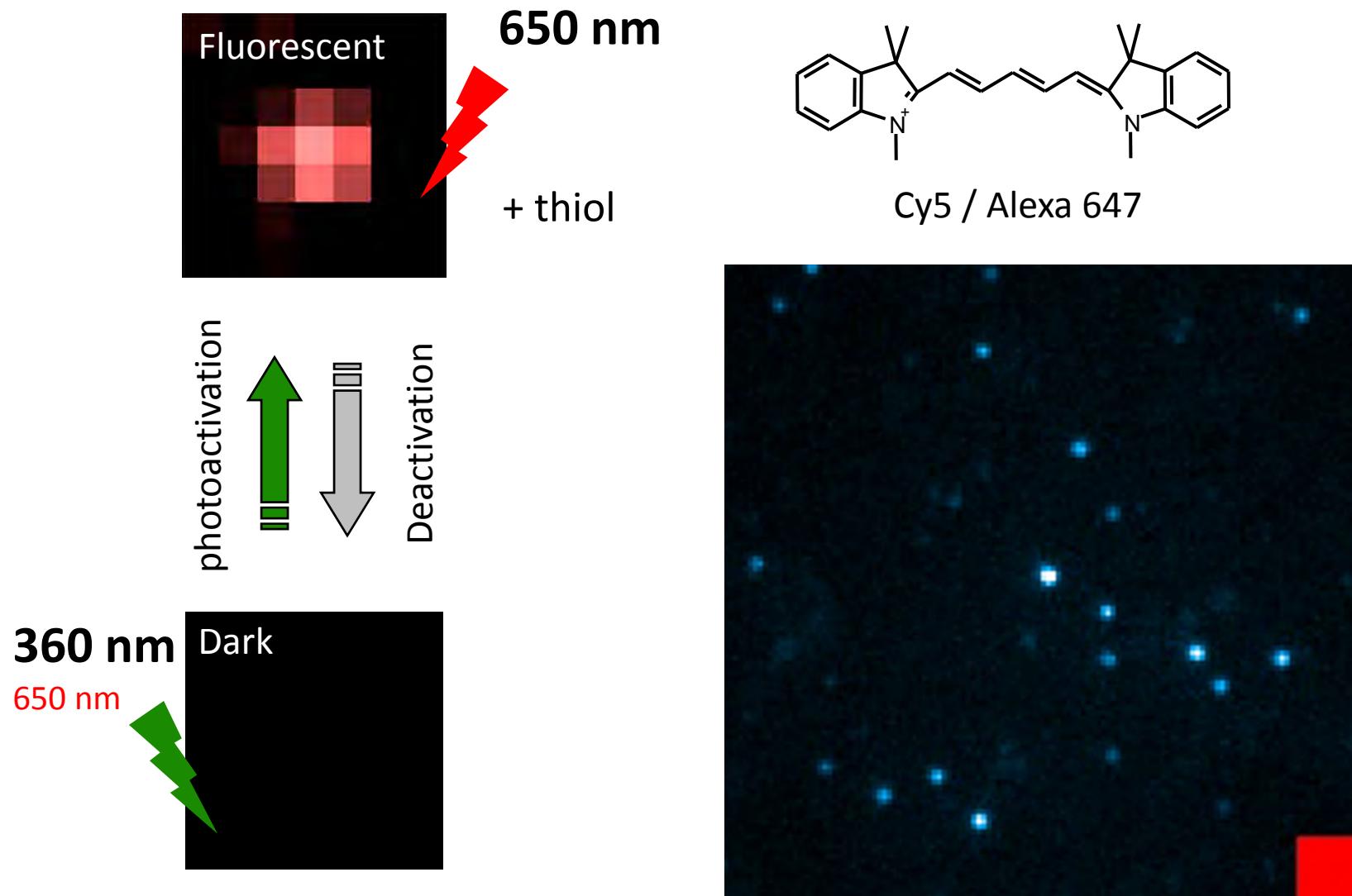
2x real time

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



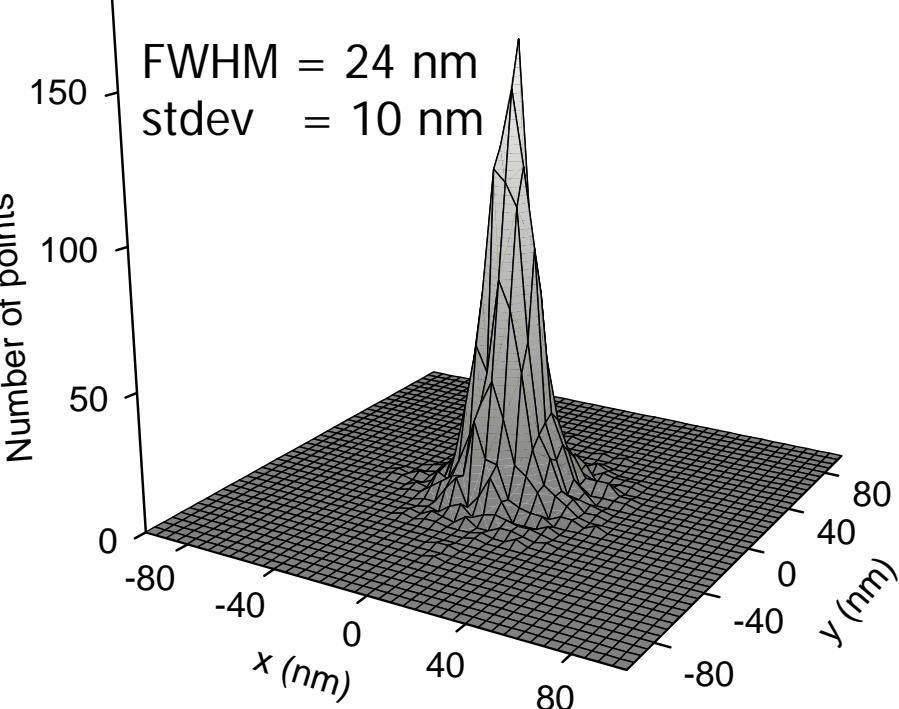
Bates et al., PRL 2005, Bates et al., Science 2007, Dempsey et al., JACS 2009

B-SC-1 cell, anti- β tubulin

Commercial

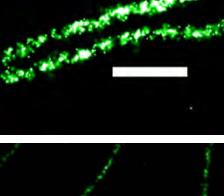
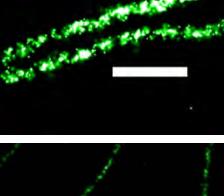
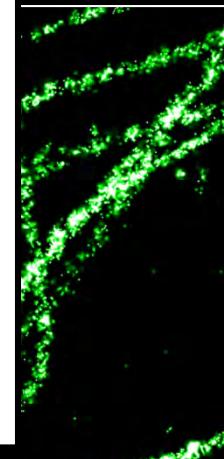
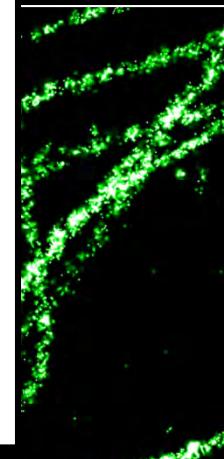
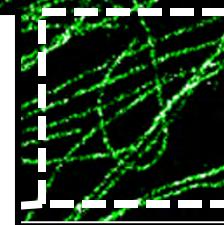
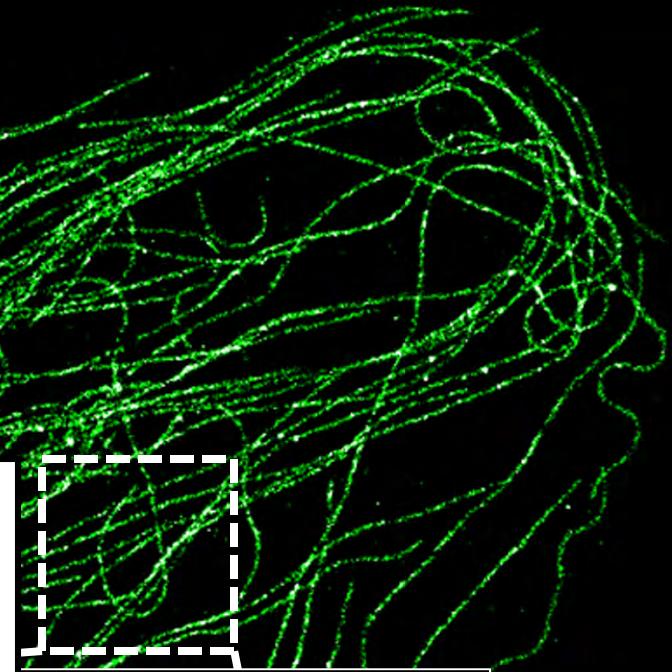
Alexa 647

secondary antibody



500 nm

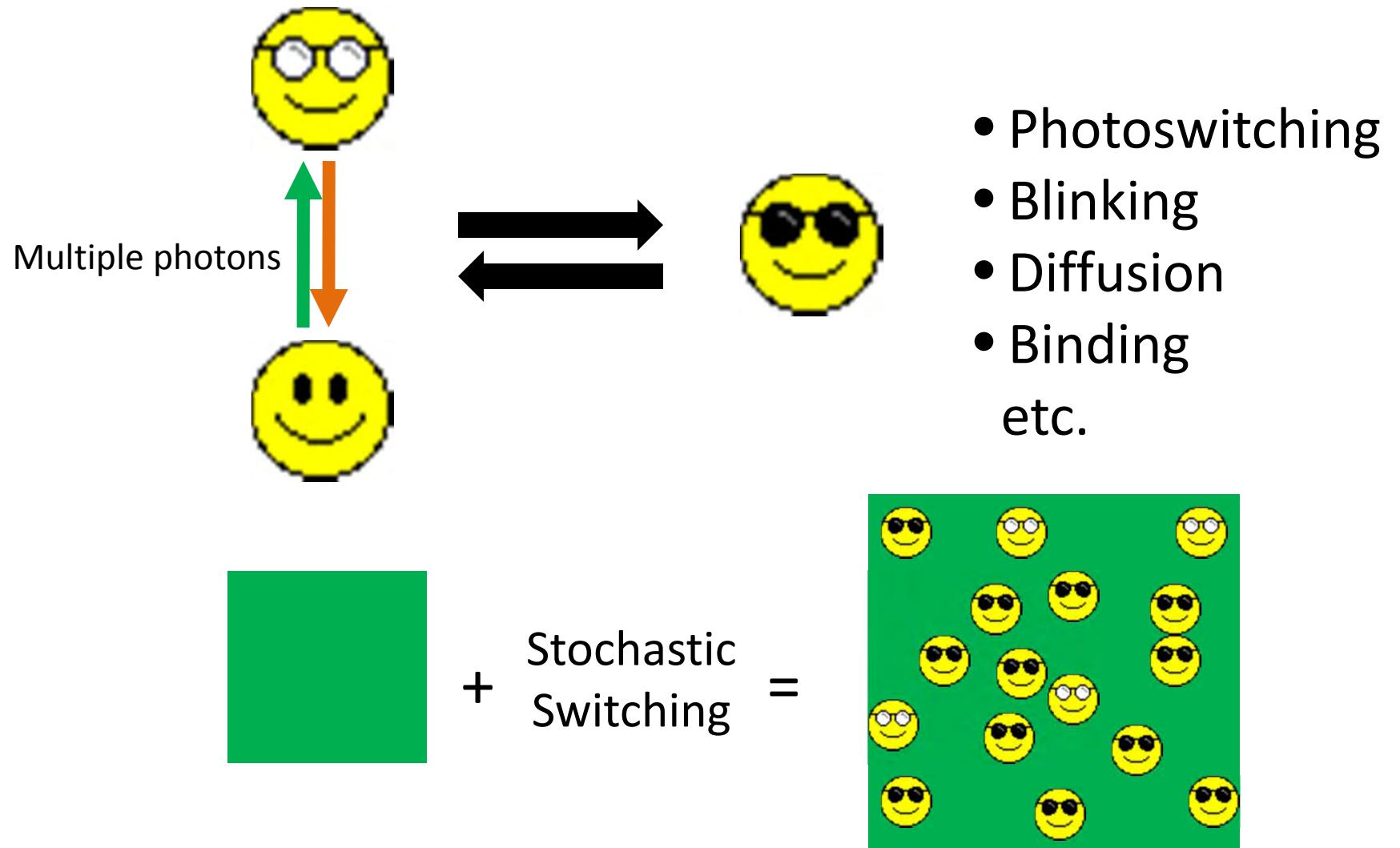
500 nm



localization points

5 μ m

The “single-molecule switching” approach



Photoswitchable probes readily available



Cyanine dye + thiol system



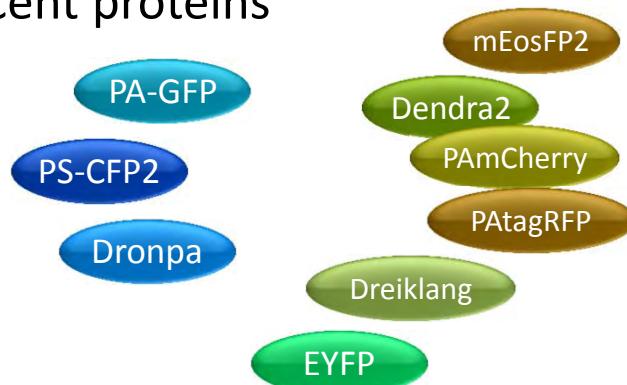
Bates et al., 2005, Bates et al., 2007, Huang et al., 2008

Rhodamine dye + redox system



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

Photoactivatable fluorescent proteins



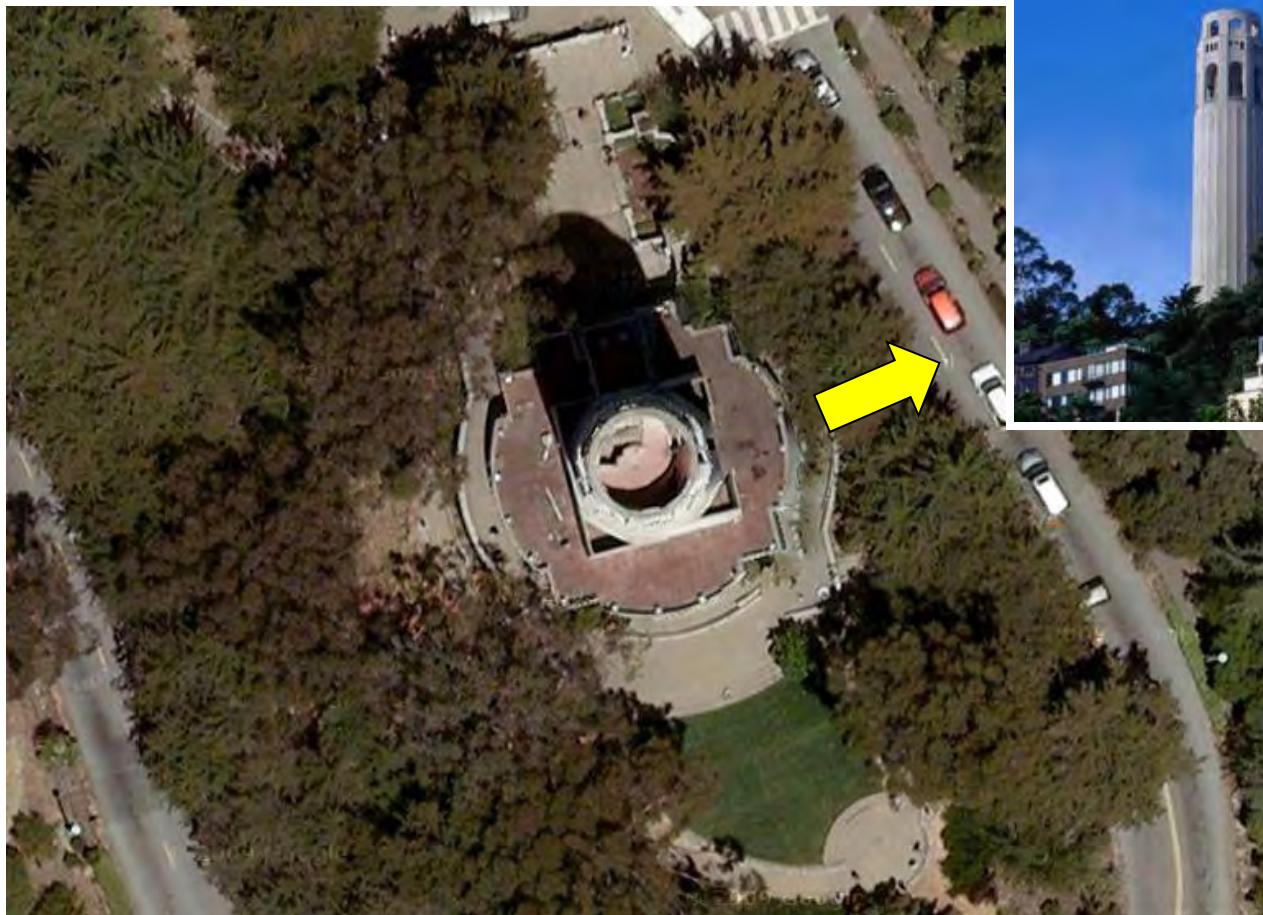
Reviews:
Lukyanov et al., Nat. Rev. Cell Biol., 2005
Lippincott-Schwartz et al., Trends Cell Biol., 2009

3D Imaging

2D Imaging

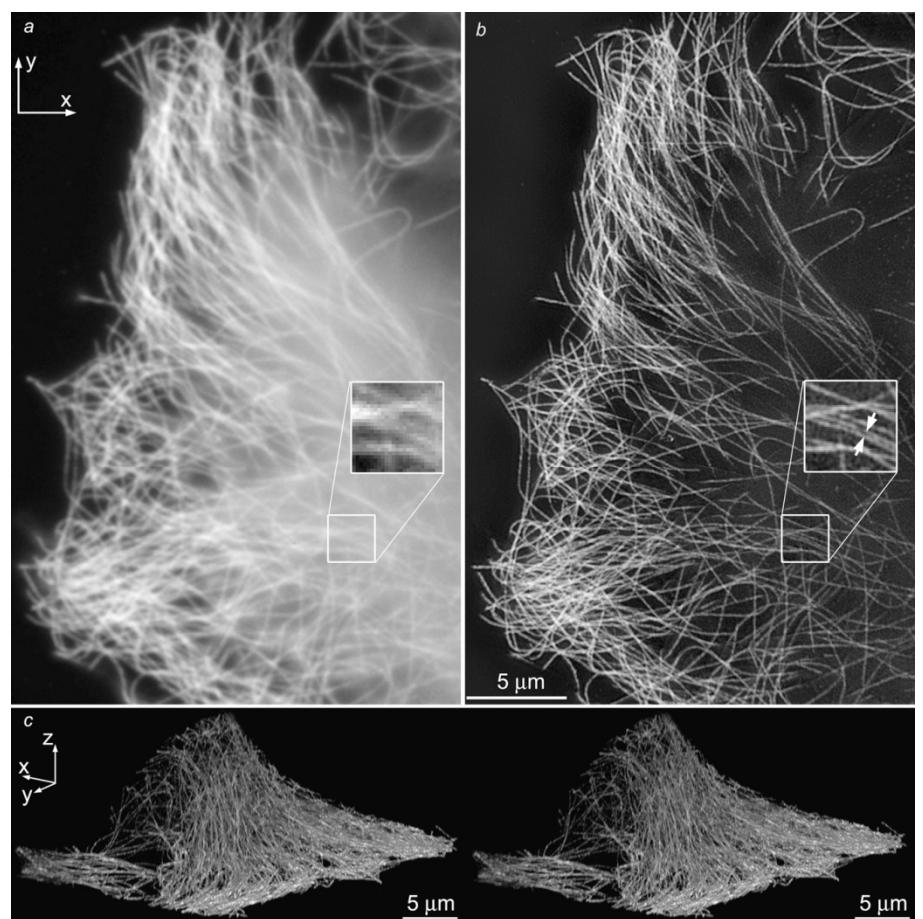
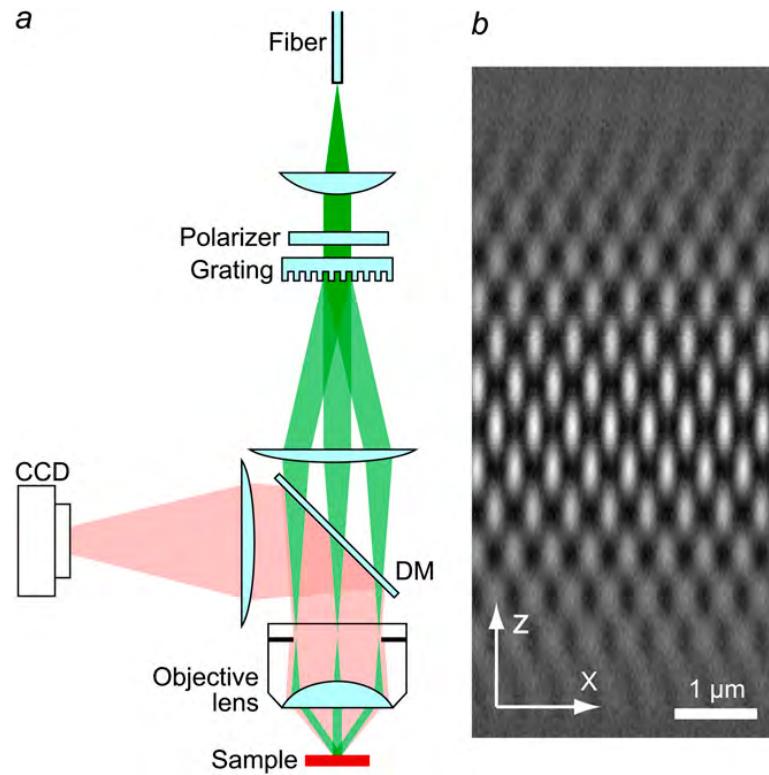
In a 2D world...

Satellite image of ???



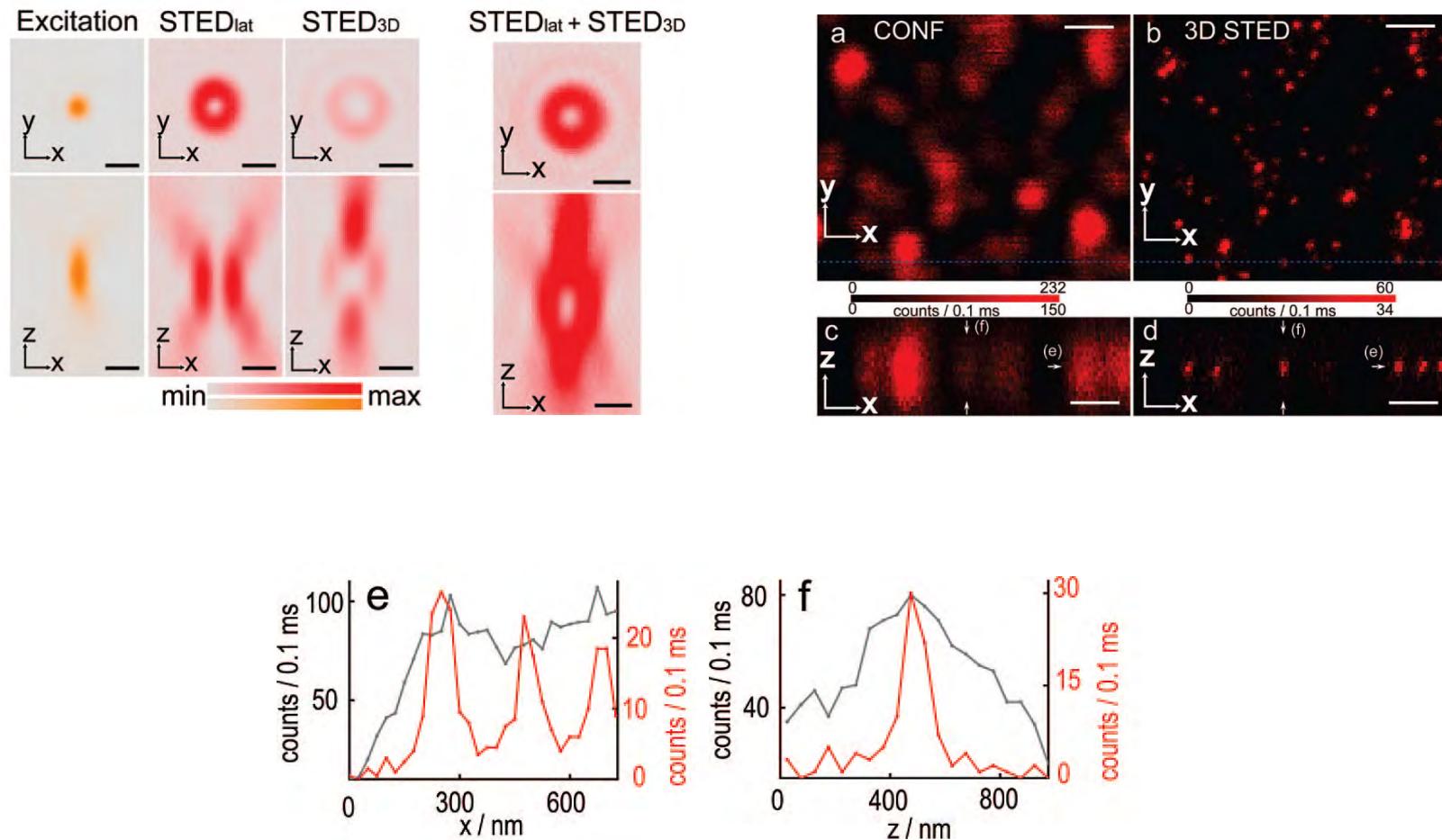
Google maps

3D SIM

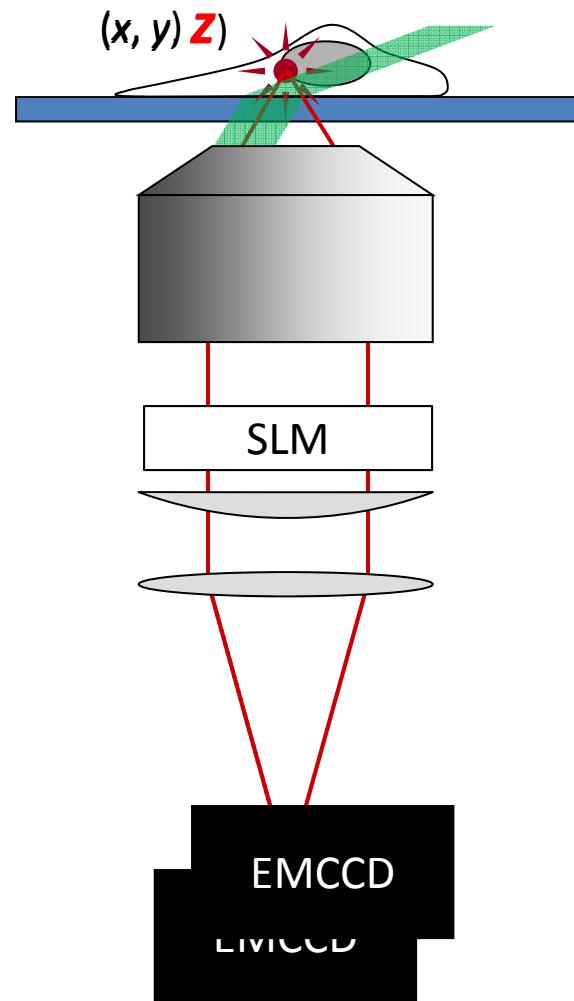


Schermellech et al., Science 2008, Gustafsson et al., Biophys J. 2008

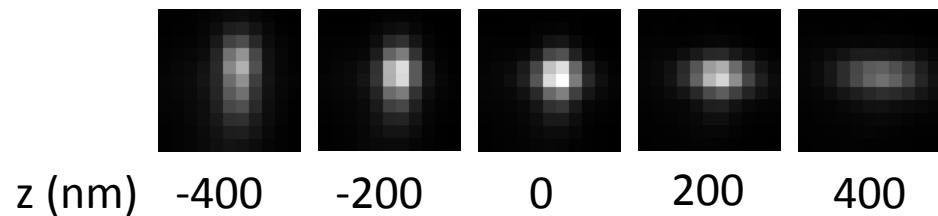
3D STED



3D STORM/PALM

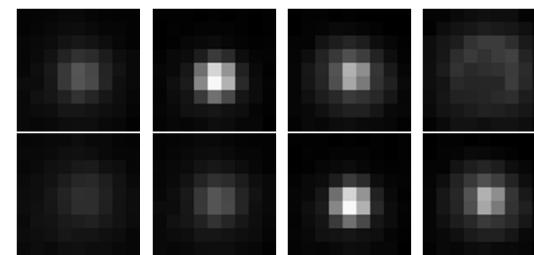


Astigmatic imaging



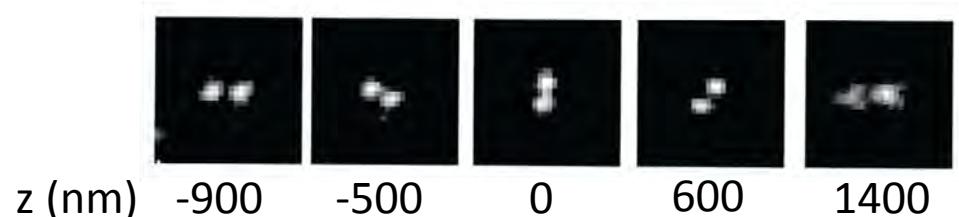
Huang et al., Science 2008

Bi-plane imaging



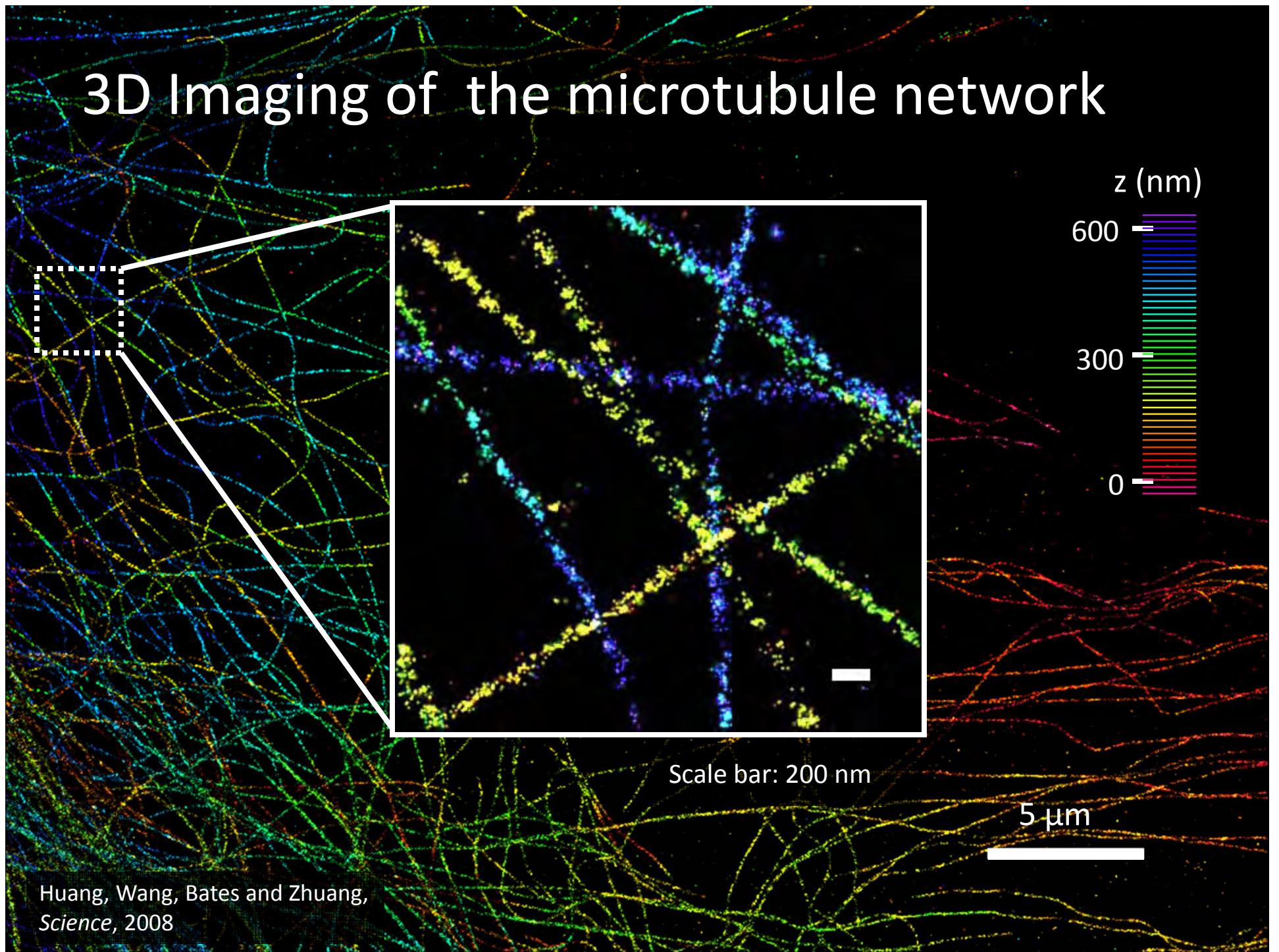
Juette et al., Science 2008

Double-helical PSF

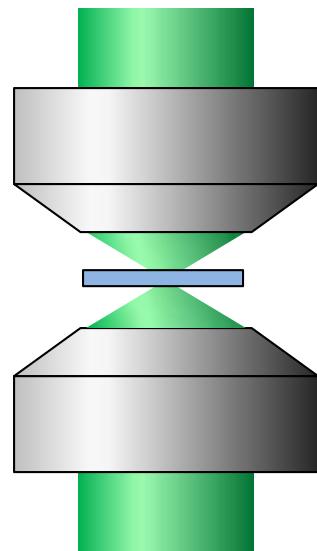


Pavani et al., PNAS 2009

3D Imaging of the microtubule network



The use of two opposing objectives

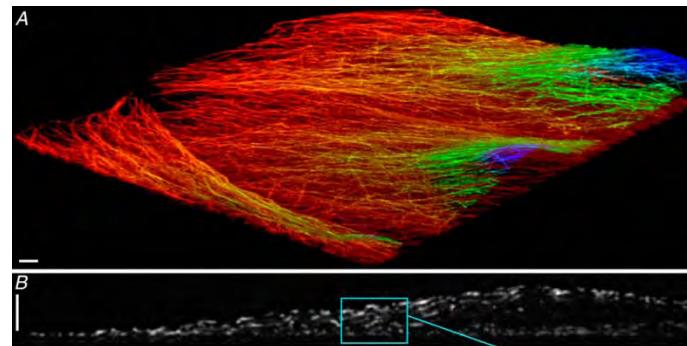


4Pi scheme



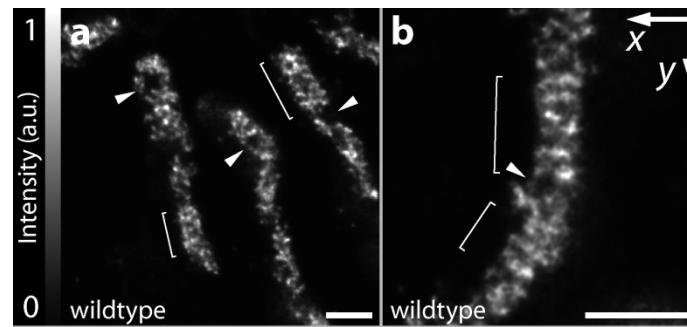
Near isotropic
3D resolution

I⁵S



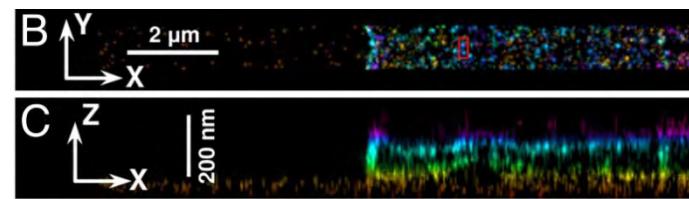
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

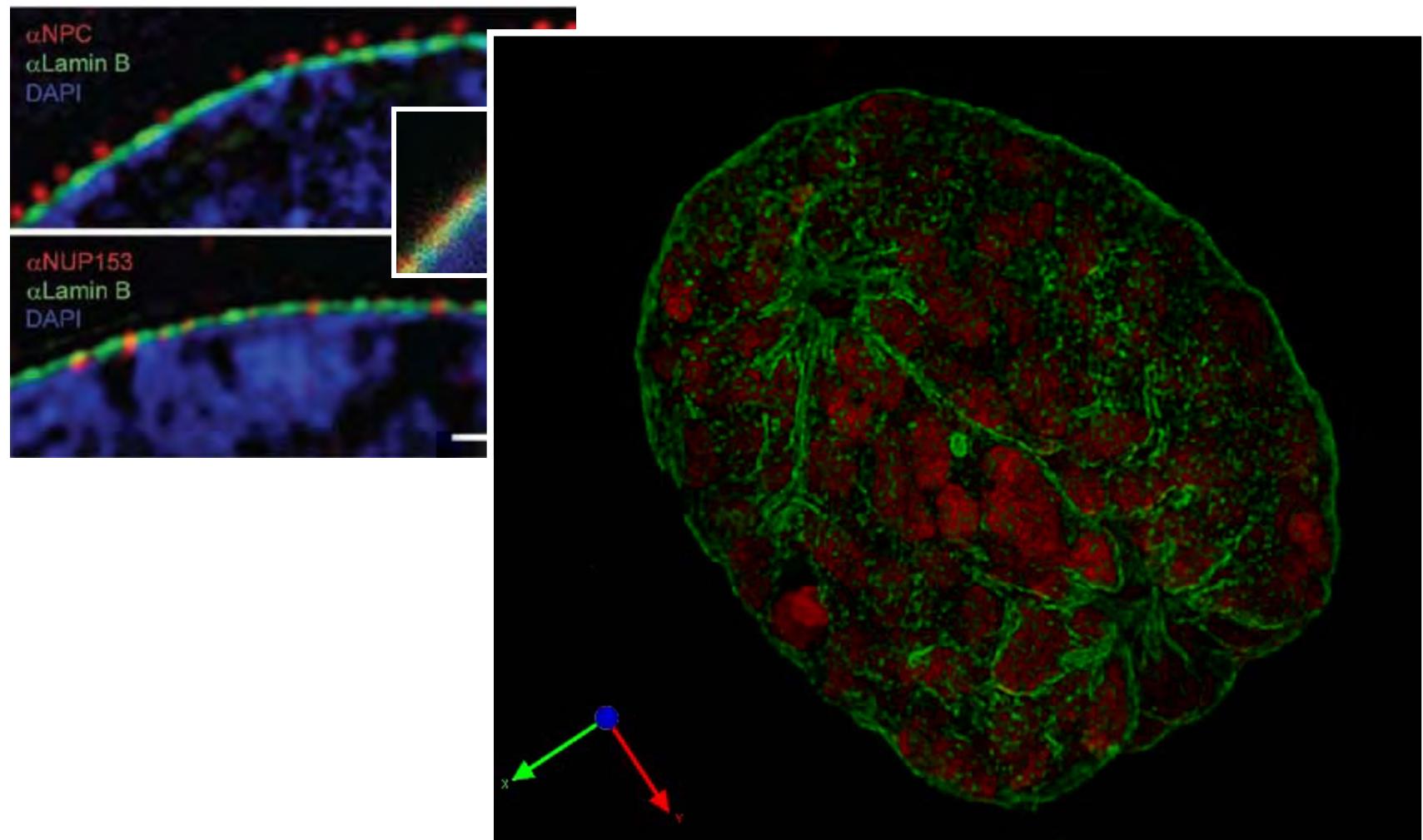
3D resolution of super-resolution methods

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

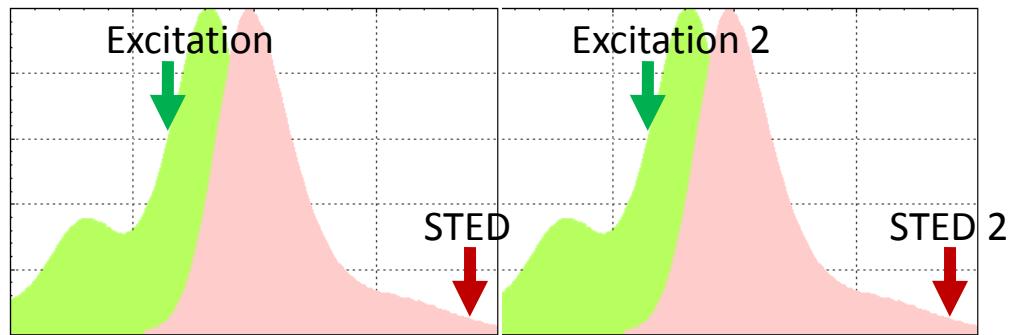
Multi-color Imaging

Multicolor SIM

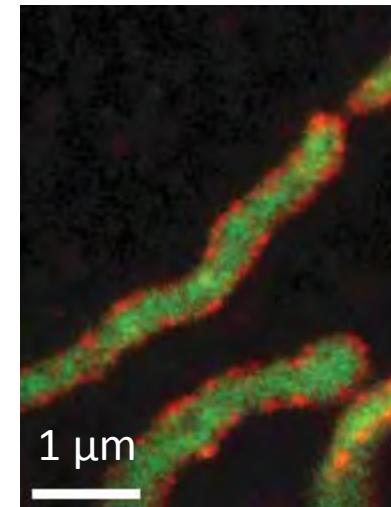
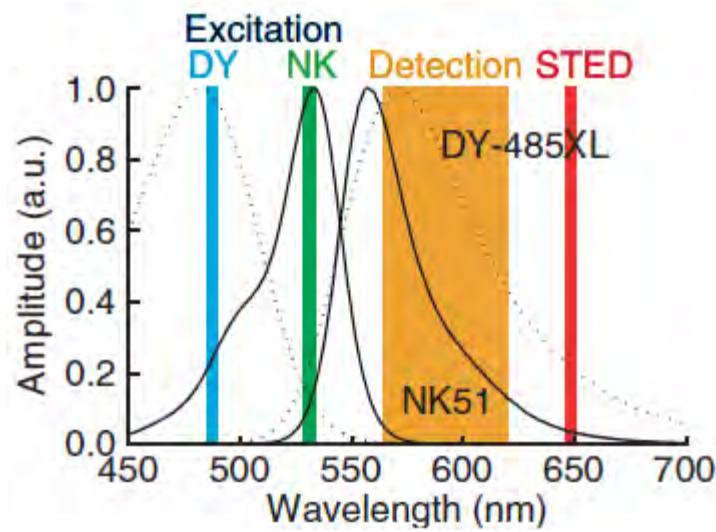
Same as conventional fluorescence microscopy!



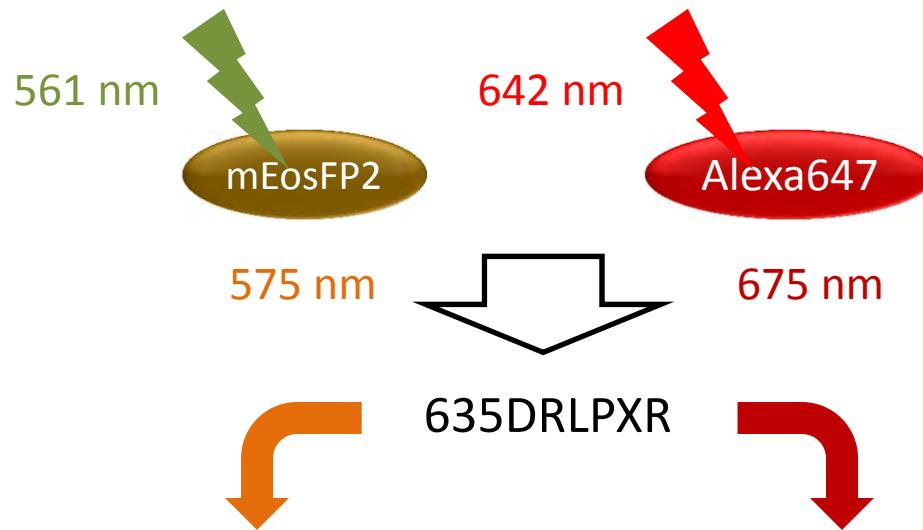
Muticolor STED



2 color isoSTED resolving
the inner and outer membrane
of mitochondria



Multicolor STORM/PALM



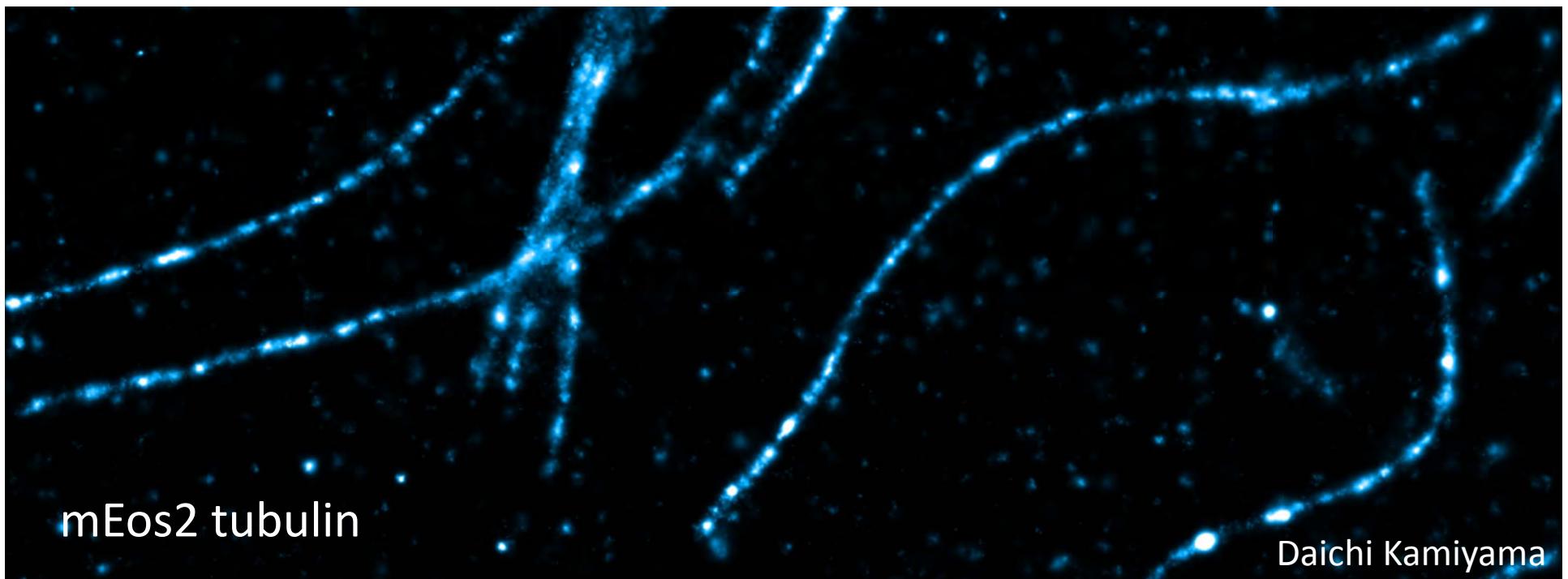
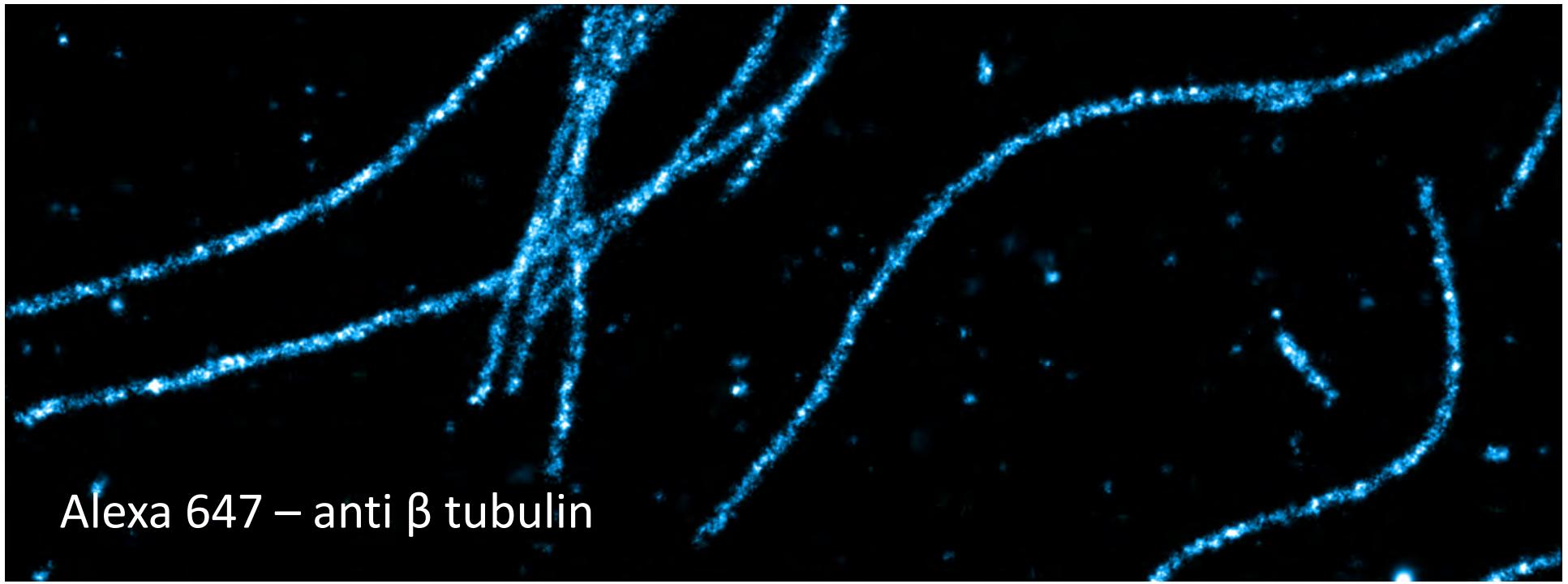
mEos2-tubulin

Alexa 647 anti- β tubulin

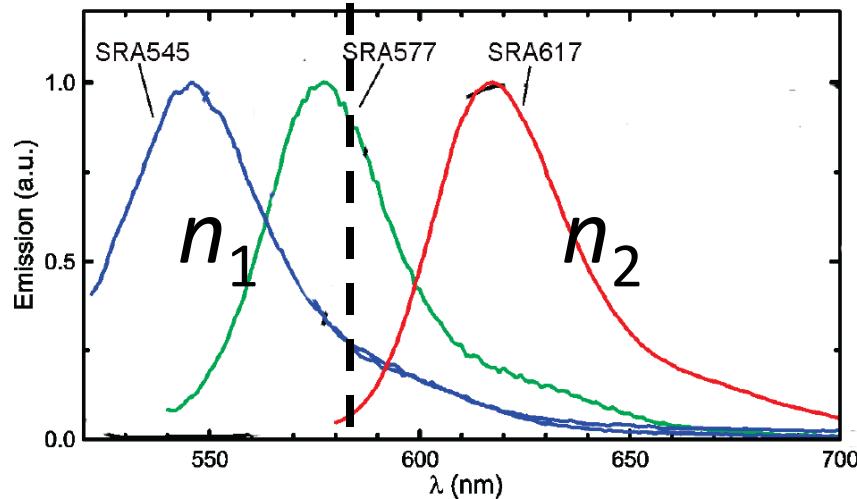
Drosophila S2 cells

2 μ m

Daichi Kamiyama



Multicolor STORM/PALM: Emission

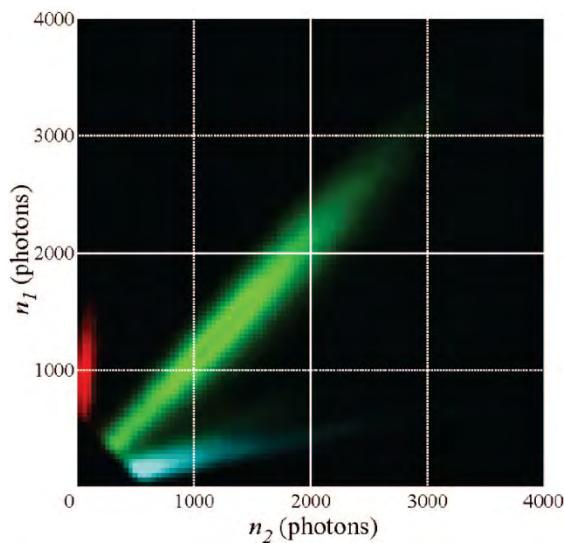


$$n_1 = n_2$$

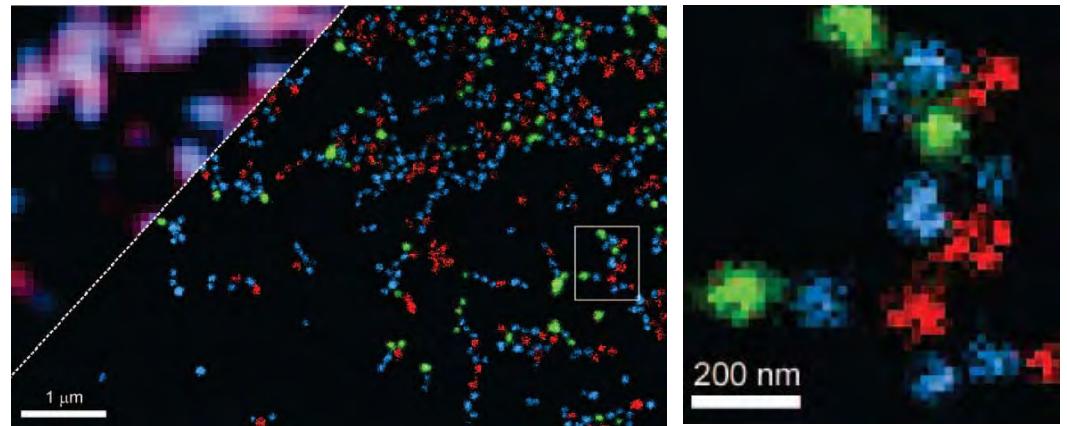
→ 50% SRA545 + 50% SRA617?

→ 100% SRA577?

Single-molecule detection!

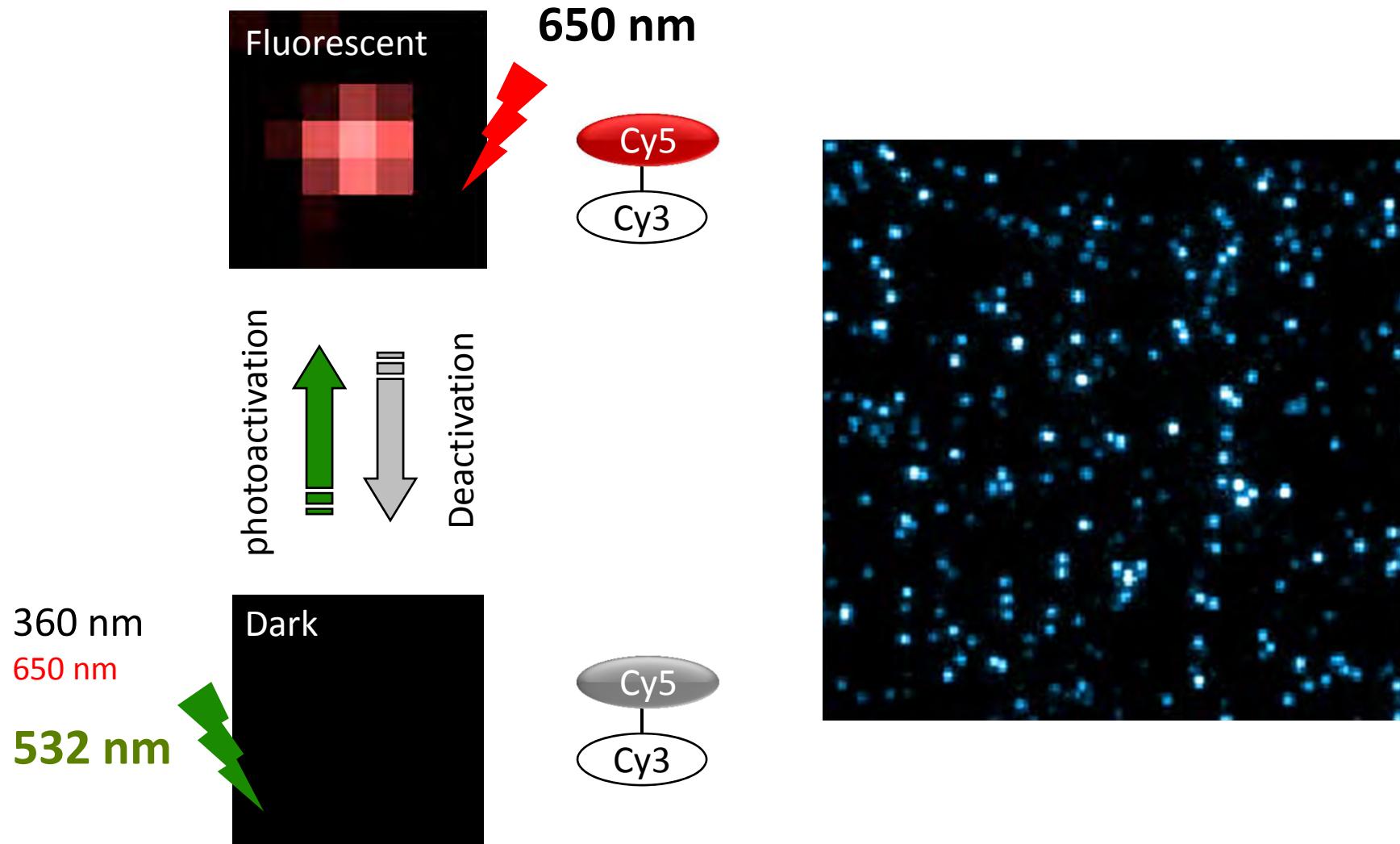


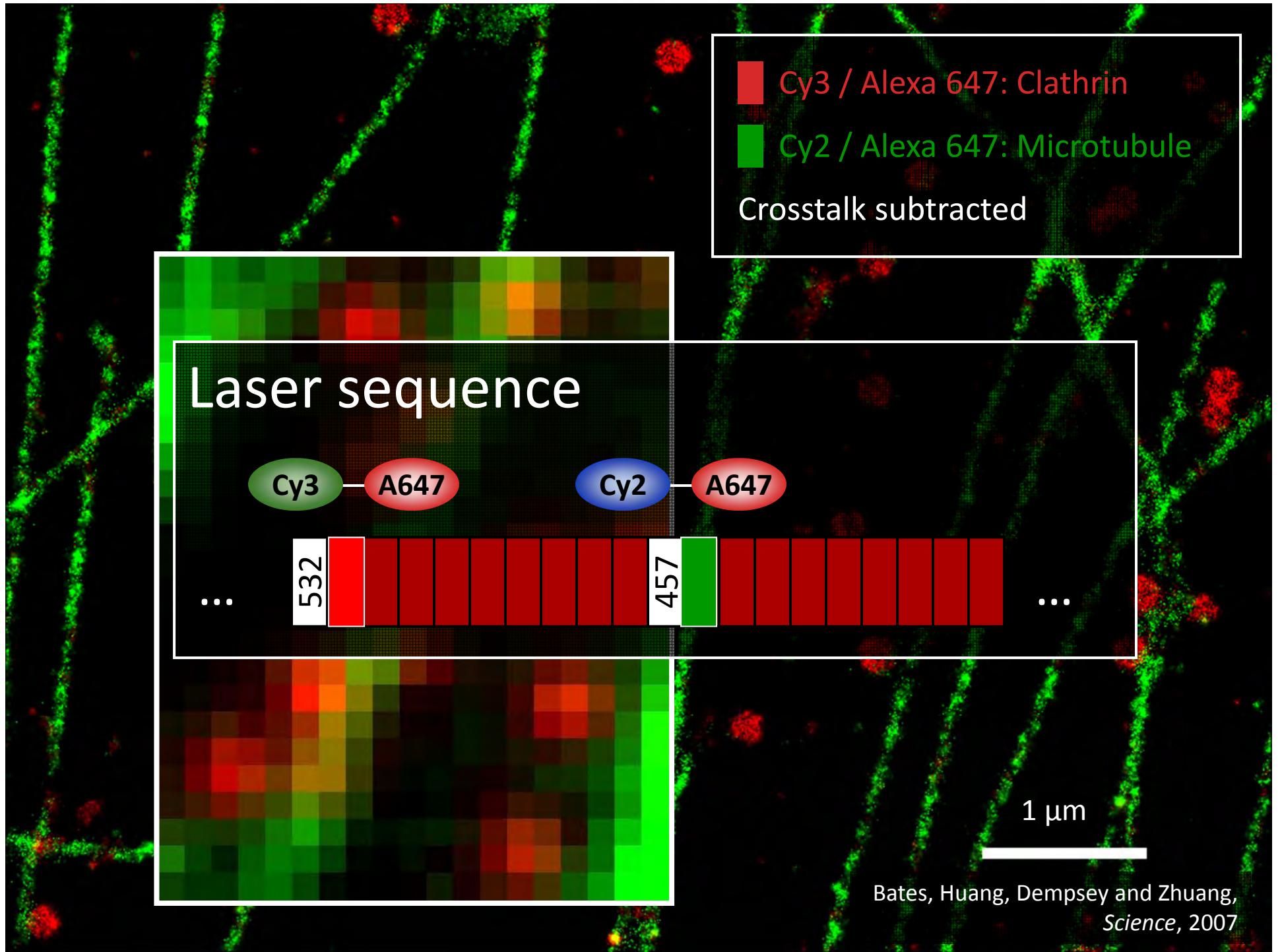
3-color imaging with one excitation wavelength
and two detection channels



Bossi et al., Nano Lett 2008

Multicolor STORM/PALM: activation

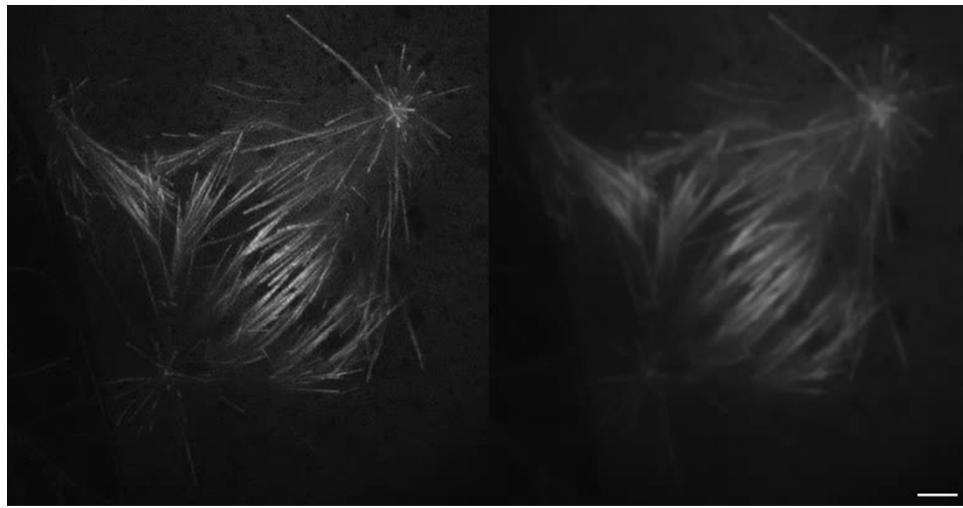




Multicolor imaging

	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



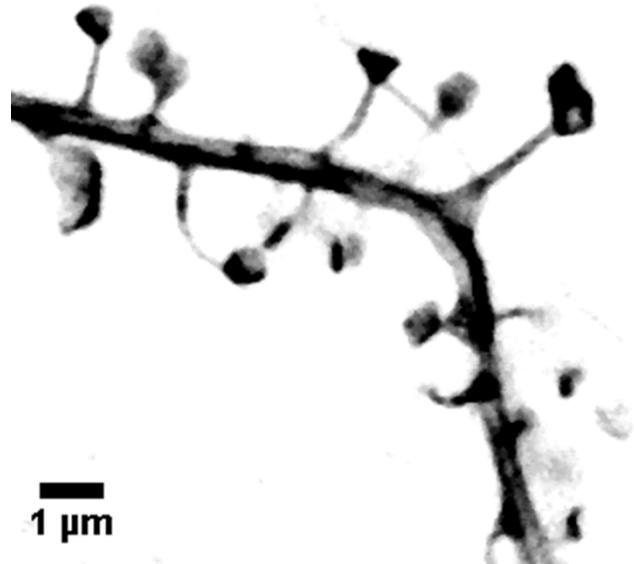
SIM

—
2 μm

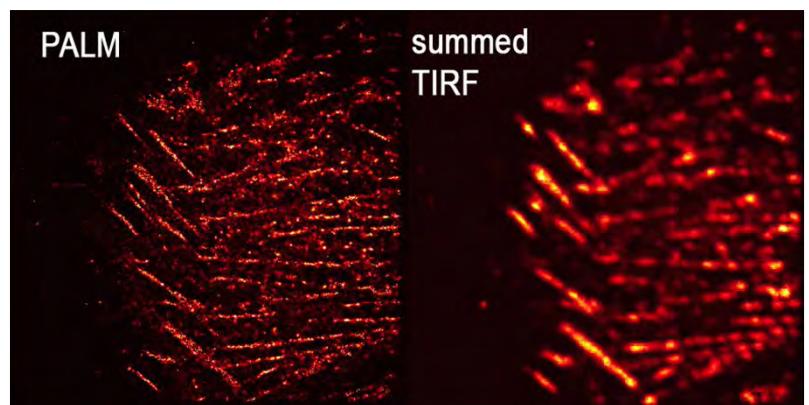
STORM/PALM

Kner, Chhun et al., Nat Methods, 2009

STED



Nagerl et al., PNAS, 2008



Schroff et al., Nat Methods, 2008

A photograph of a paved road curving away from the viewer. In the distance, four police cars are stacked vertically. The top car is positioned on the right side of the road, while the bottom car is on the left. Each car is black with white stripes and features a star-shaped badge on its front grille. The background shows a grassy area with trees and utility poles under a cloudy sky.

The limit of “Super-Resolution”

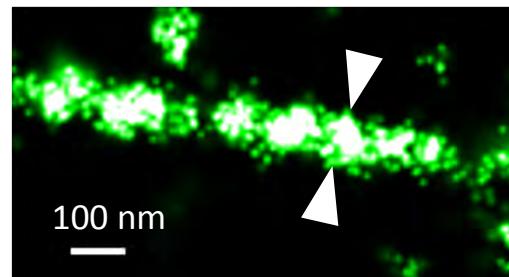
Unbound theoretical resolution

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

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

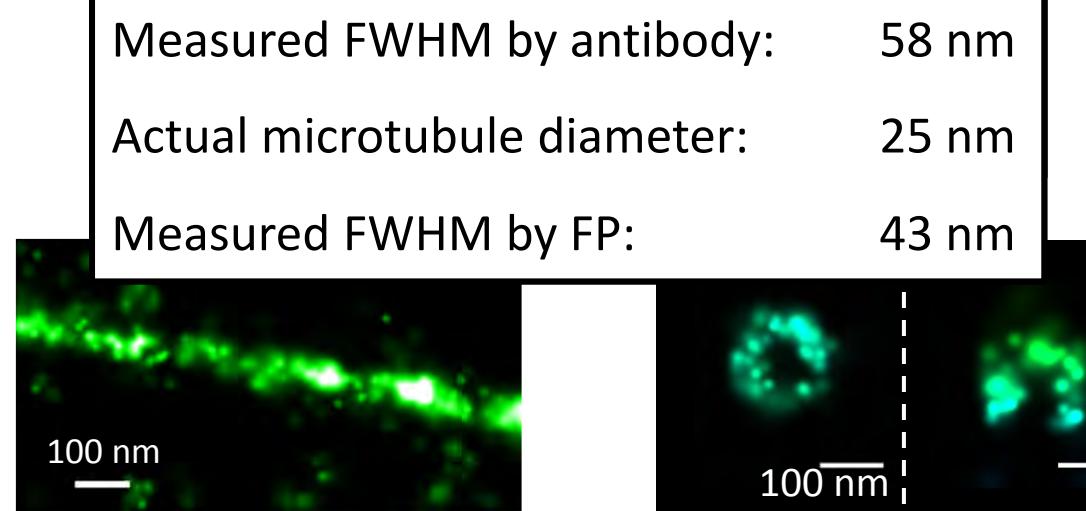
Effective resolution: Probe matters

Antibodies:
~ 10 nm



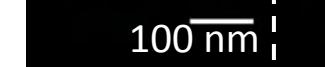
~ 6000 photons

Fluorescent Proteins:
~ 3 nm



< 1000 photons

Small fluorophores:
~ 1 nm



~ 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

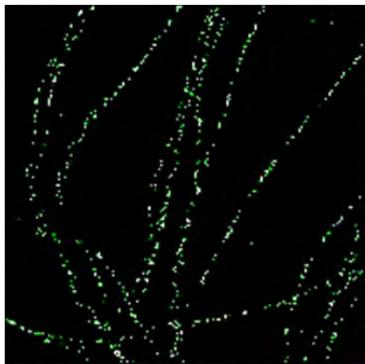
Antibody immunofluorescence

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

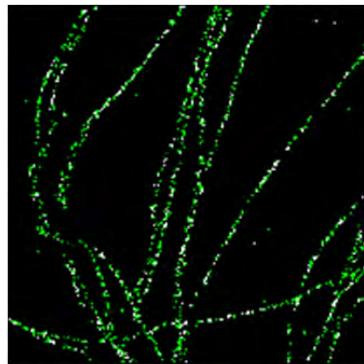
Effective resolution: Density matters

Frames for image reconstruction:

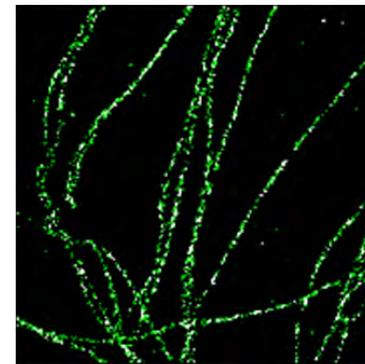
200



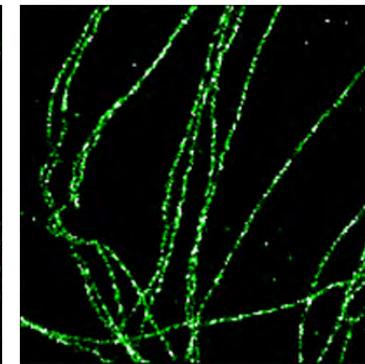
500



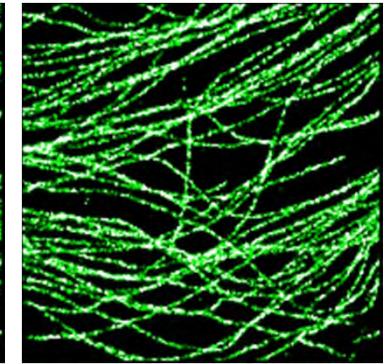
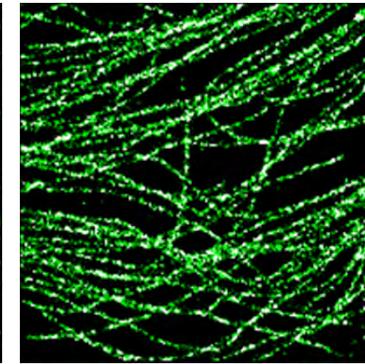
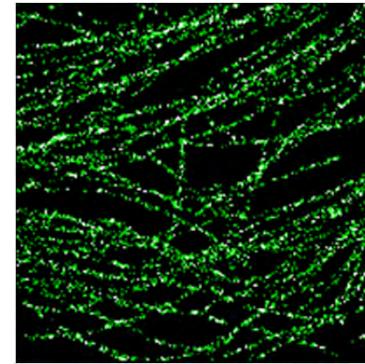
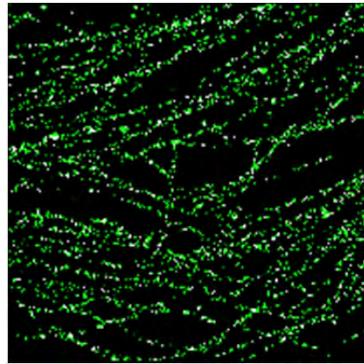
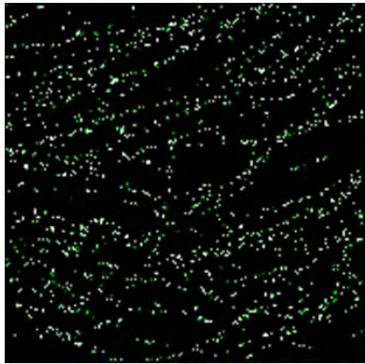
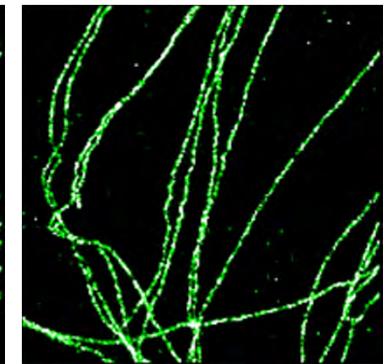
1,000



5,000

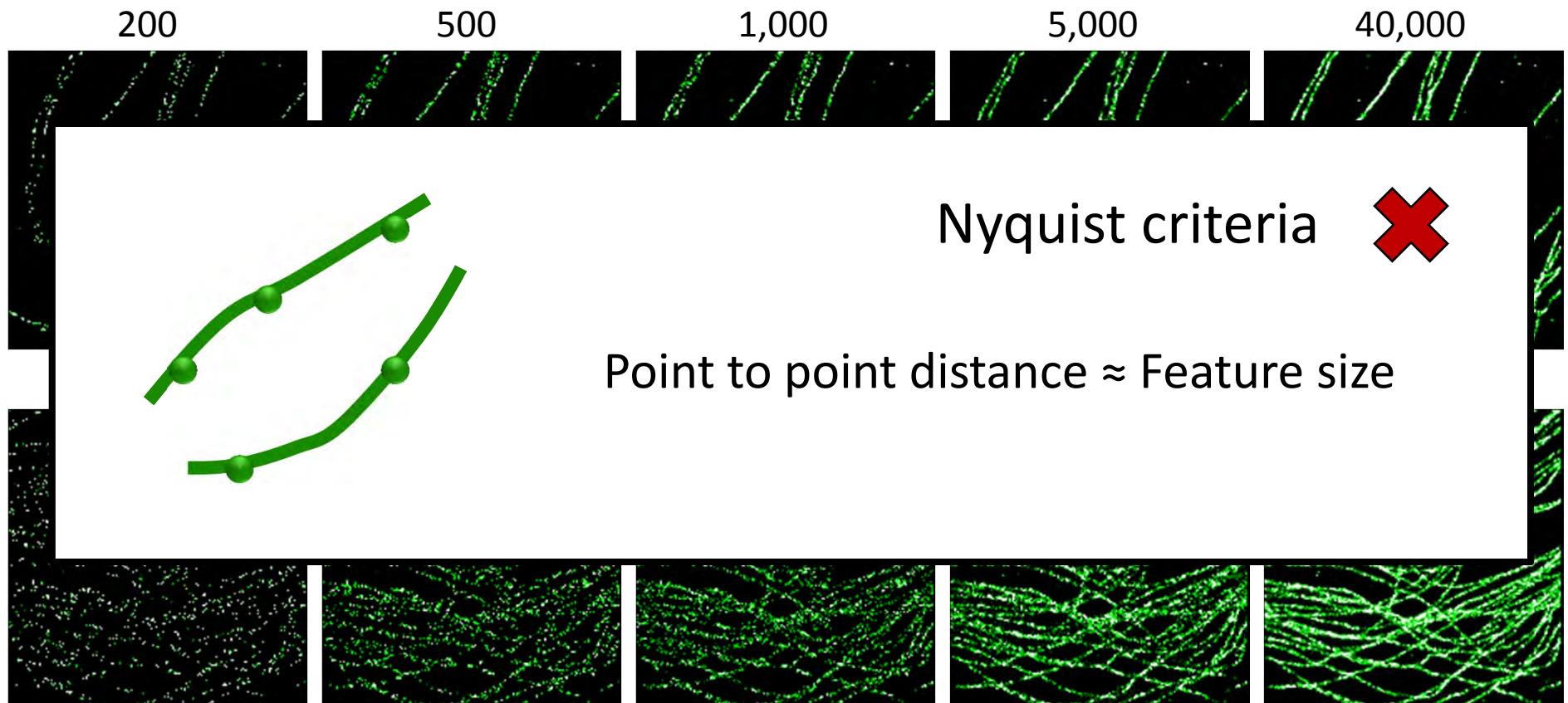


40,000



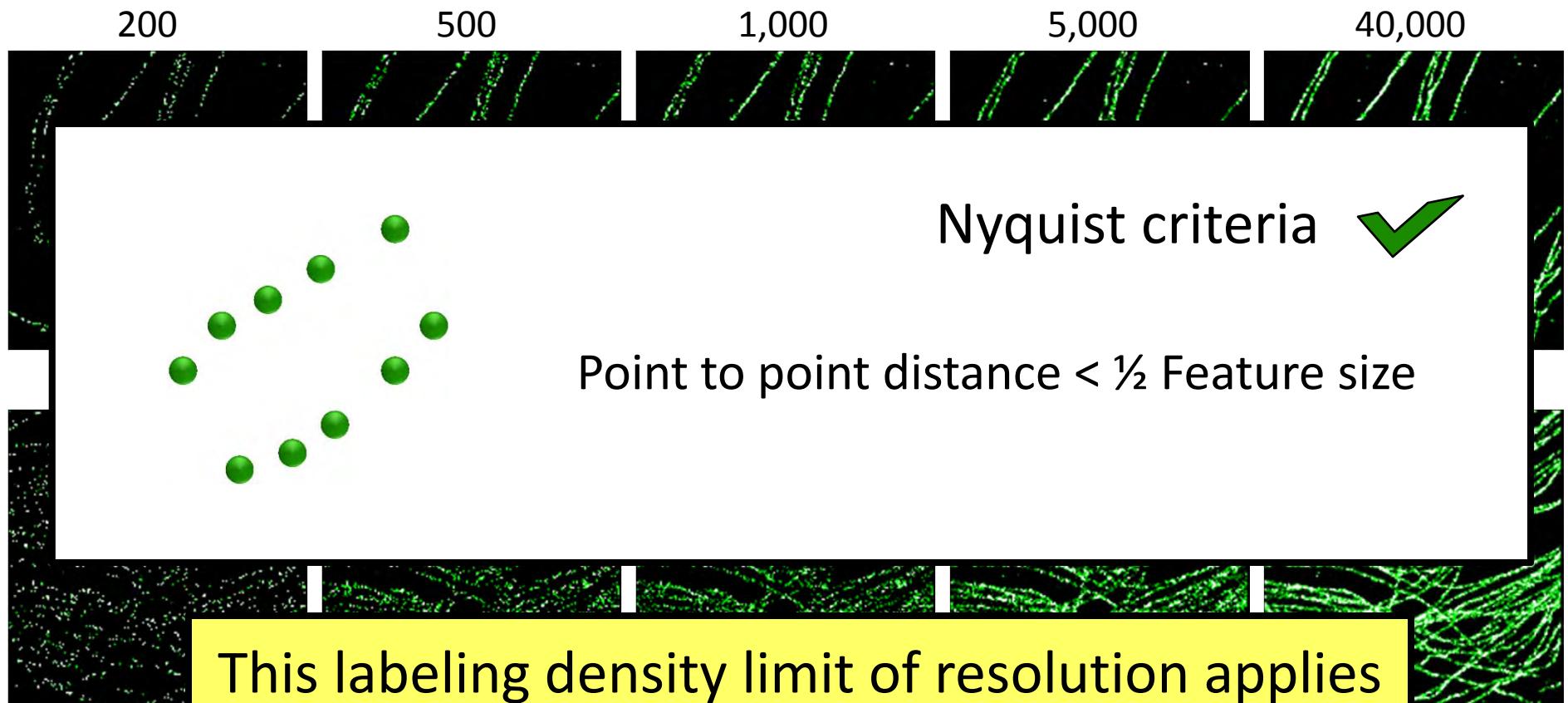
Effective resolution: Density matters

Frames for image reconstruction:



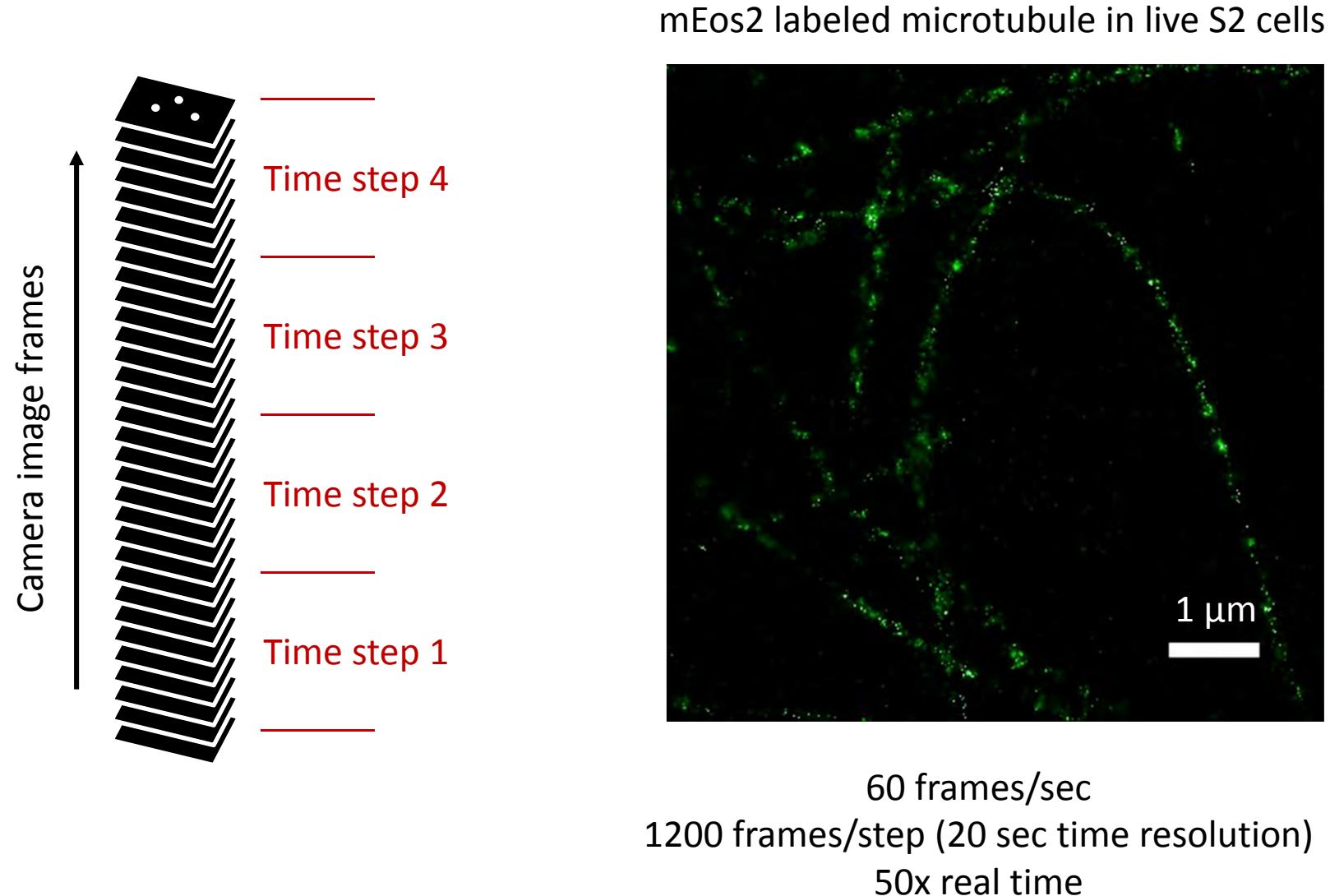
Effective resolution: Density matters

Frames for image reconstruction:



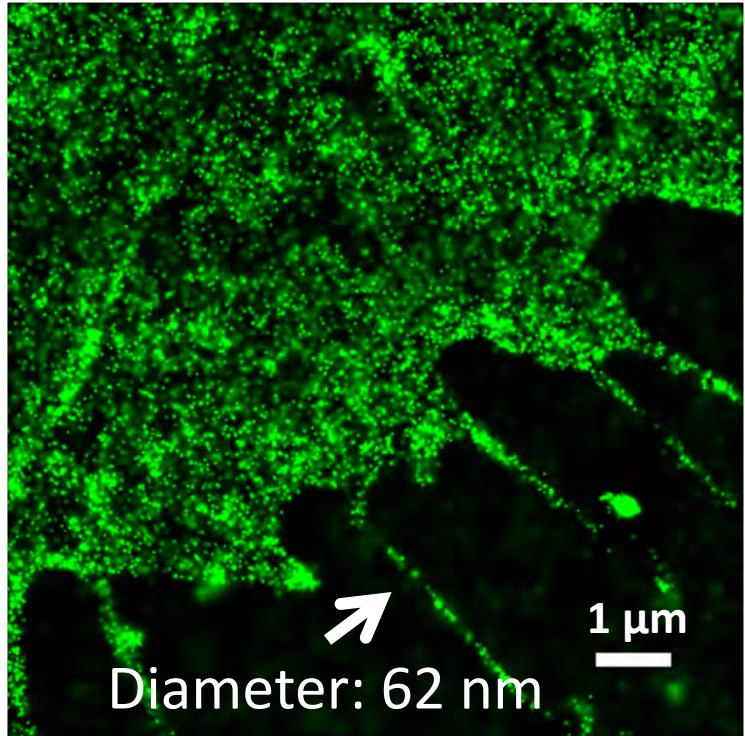
This labeling density limit of resolution applies to **all** fluorescence microscopy methods

Live cell STORM/PALM

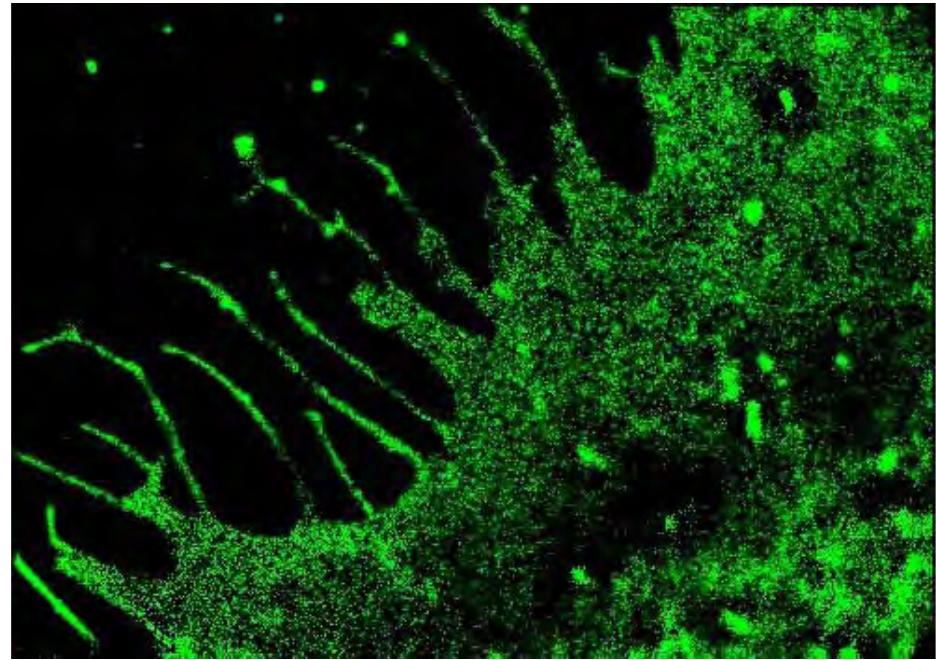


Live cell imaging of plasma membrane

DiD stained plasma membrane



1000 frames, 10 sec total time



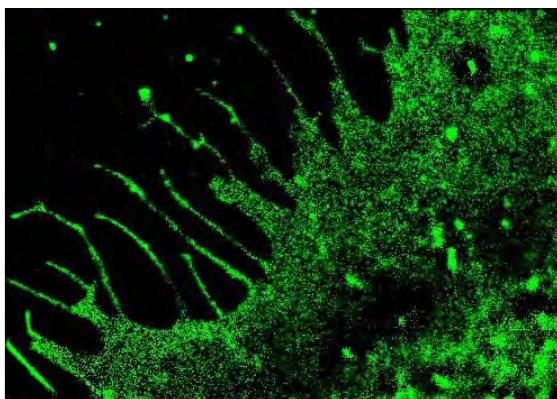
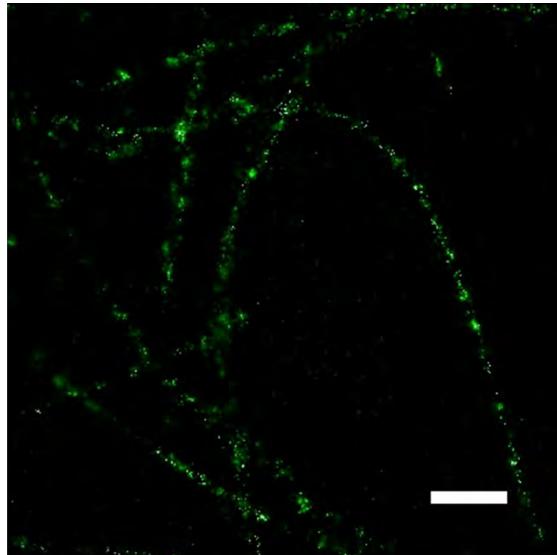
120 frames / sec, 3000 frames (25 sec)

100x real time

3 mM mercaptoethylamine

— 1 μm

Spatial-temporal resolution trade-off



Assuming:

1 molecule occupies $500 \times 500 \text{ nm}$

On average 0.1 point / $0.25 \mu\text{m}^2 \cdot \text{frame}$

70 nm resolution \equiv 2000 frames

100 fps = 20 sec time resolution

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, "Photoactivatable fluorescent proteins for diffraction-limited and super-resolution imaging", *Trends in Cell Biology*, 19, 555-565 (2009).