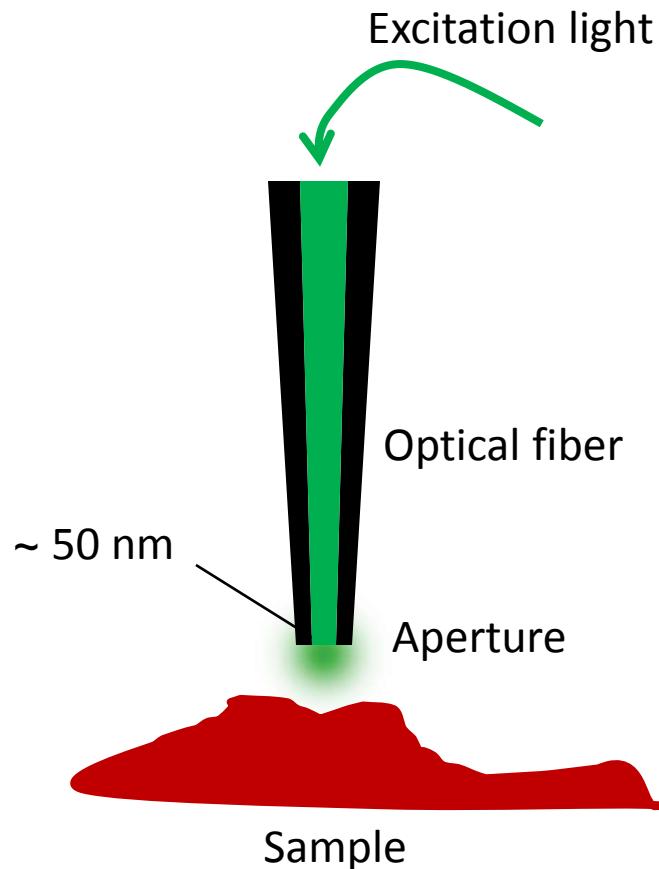


STED nanoscopy **SIM**
GSD **RESOLFT** SSIM
Super-resolution
STORM GSDIM **dSTORM**
PAINT SMACM BBB **PALMIRA** FPALM
SOFI CALM **BALM** **PALM**

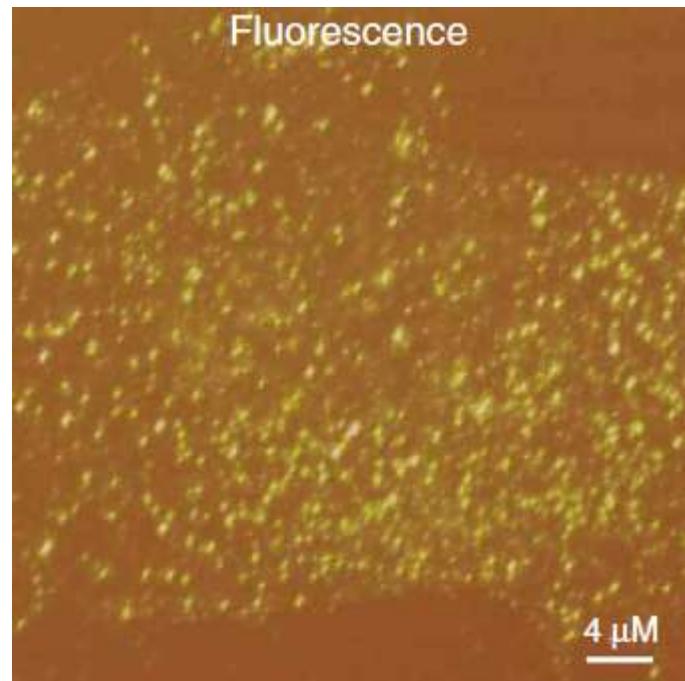
50 years to extend the resolution

- 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

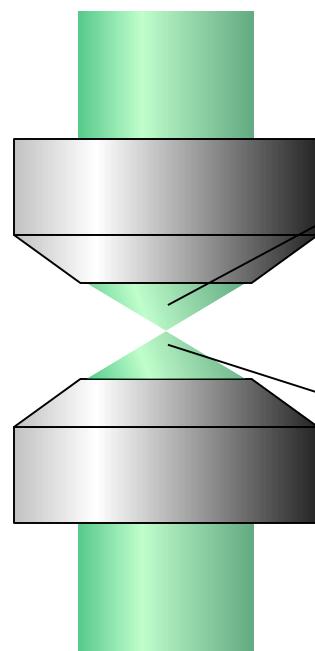


β_2 adrenergic receptor clusters
on the plasma membrane



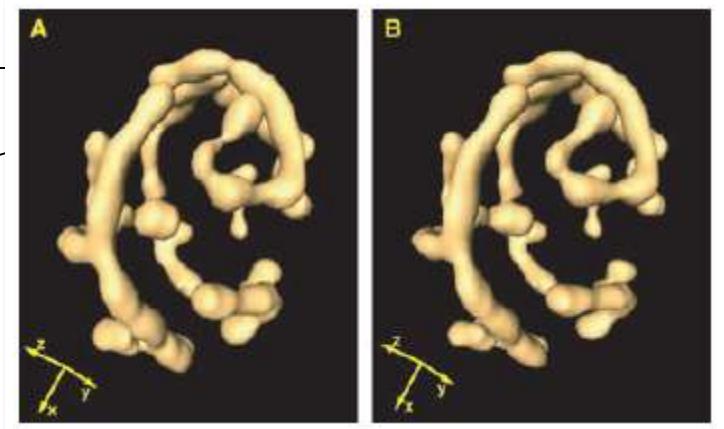
Ianoul et al., 2005

4-Pi / I^5M



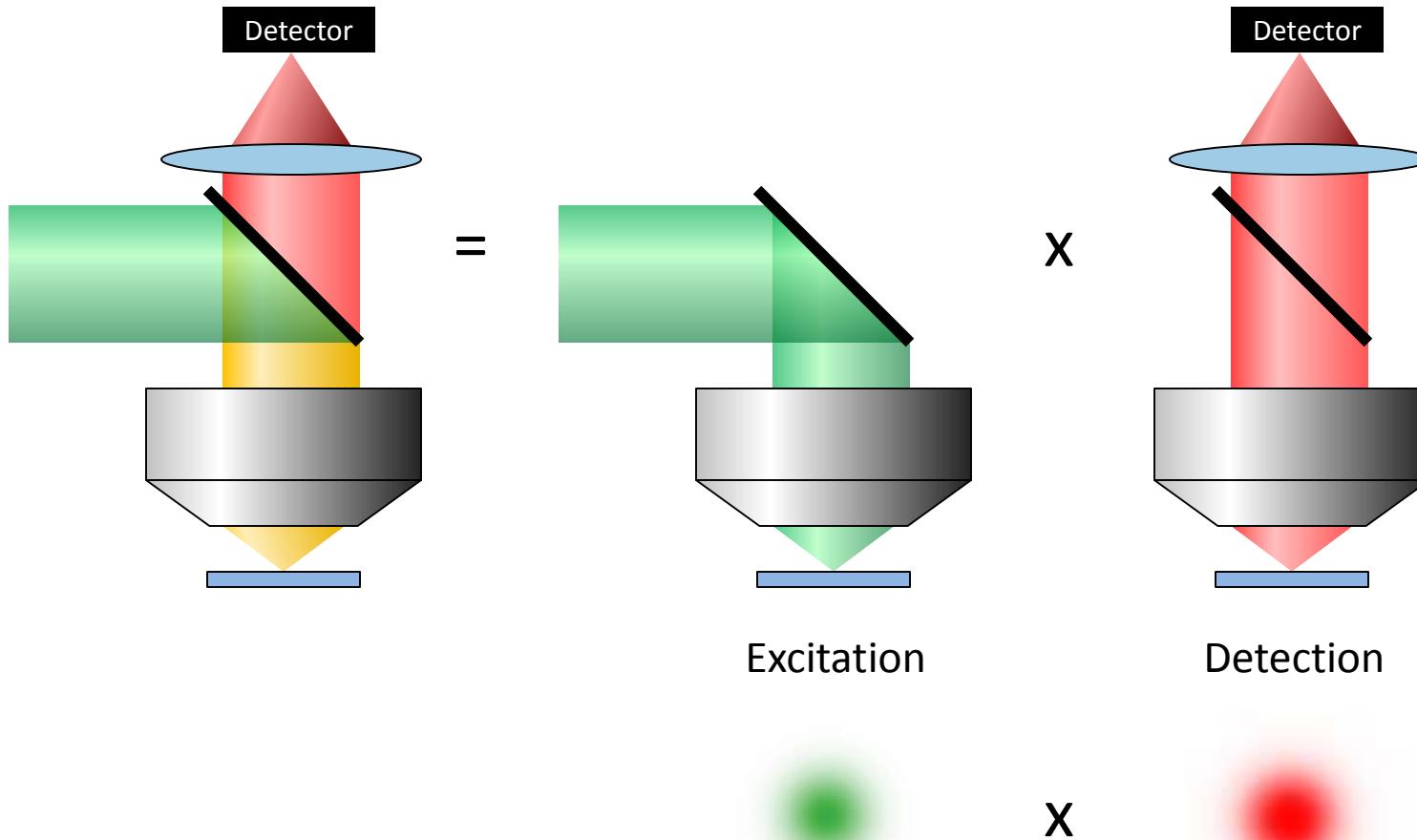
$$NA = n \sin \alpha$$

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

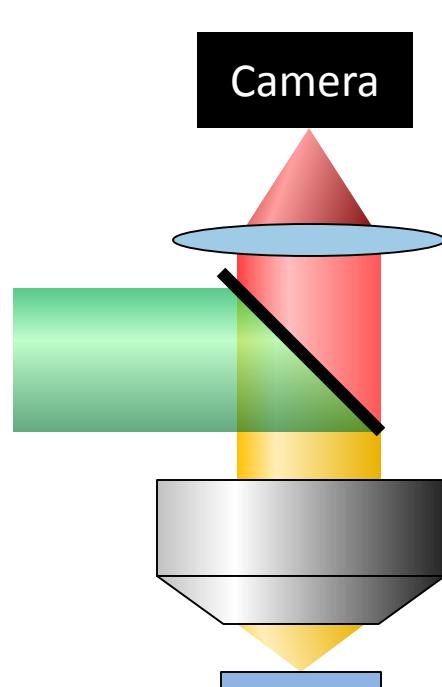


Major advantage:
Similar z resolution as x-y resolution

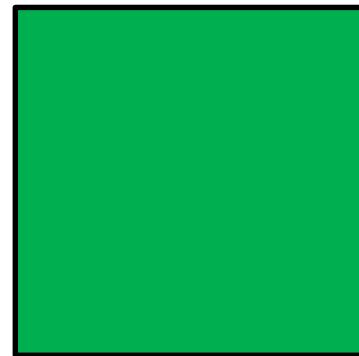
Patterned illumination



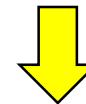
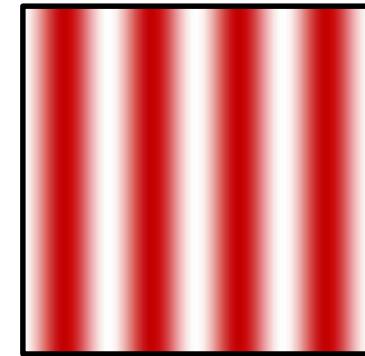
Structured Illumination Microscopy (SIM)



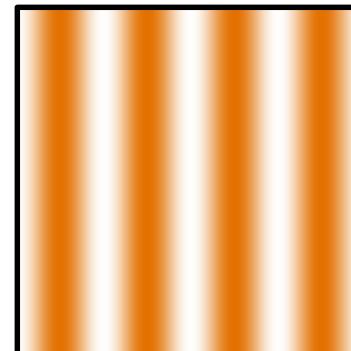
Wide field illumination



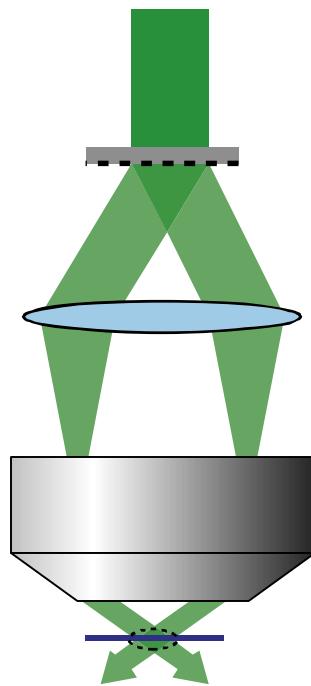
Diffraction-limited detection



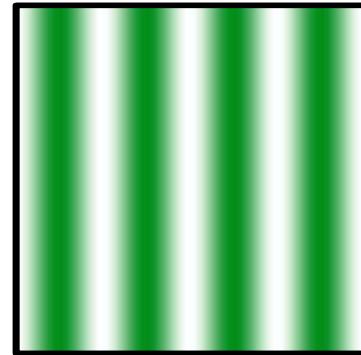
Diffraction-limited image



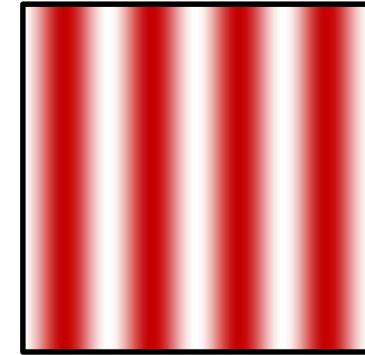
Structured Illumination Microscopy (SIM)



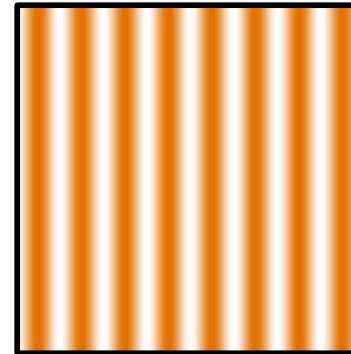
~~Solid field illumination~~



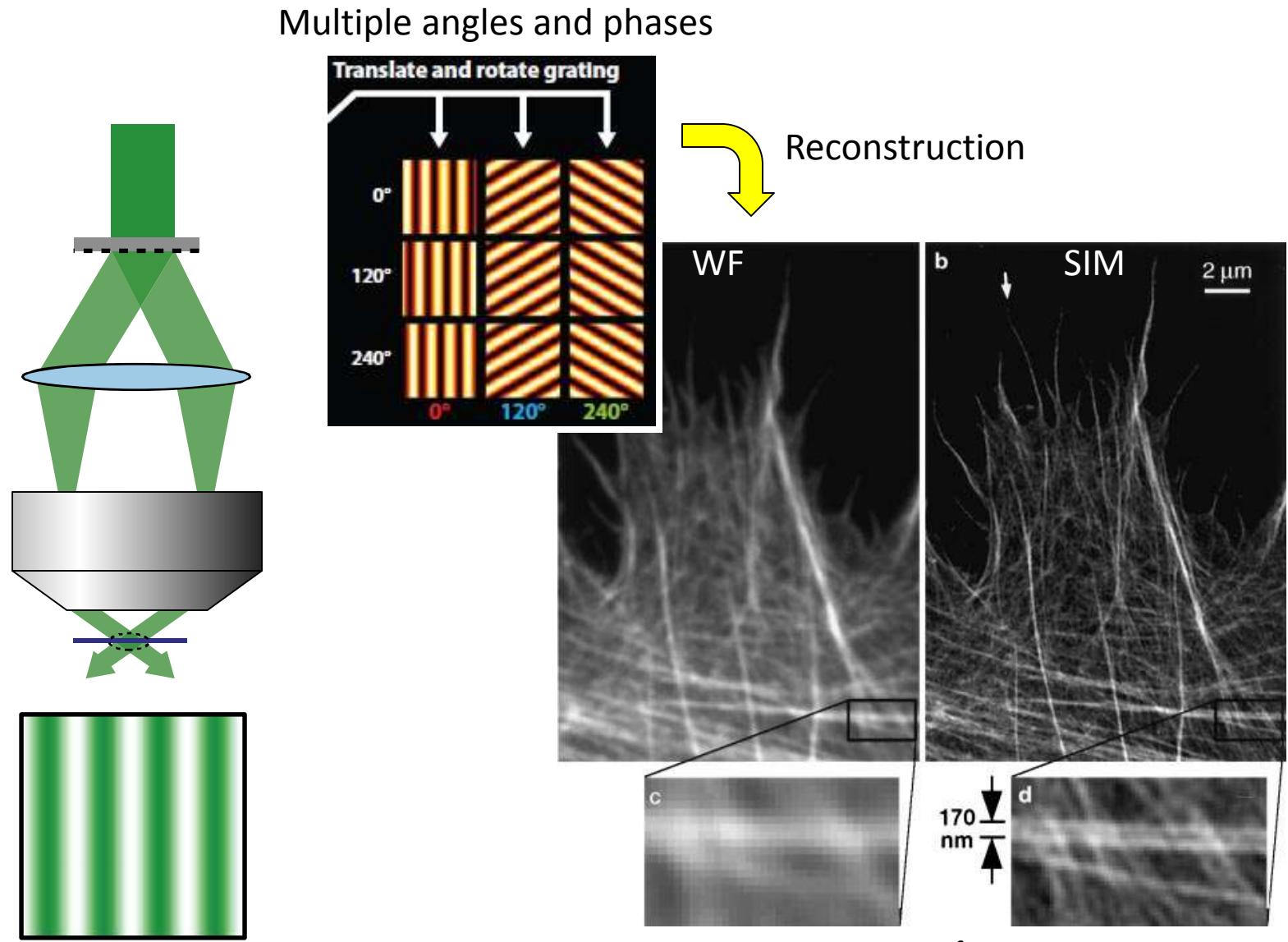
Diffraction-limited detection



Diffraction-limited image



Structured Illumination Microscopy (SIM)



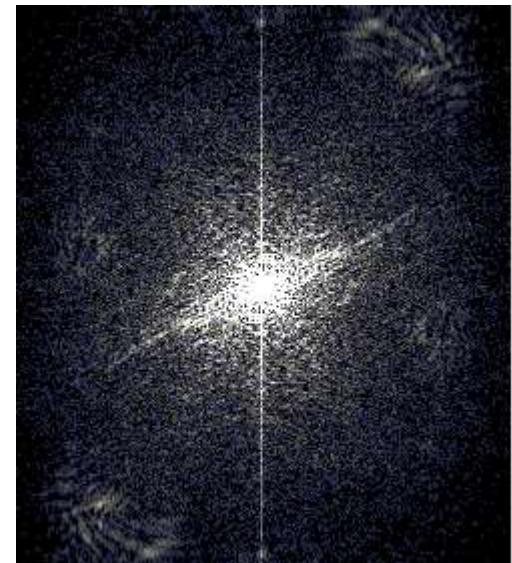
Gustafsson, J Microscopy 2000

Being (slightly) more rigorous about SIM



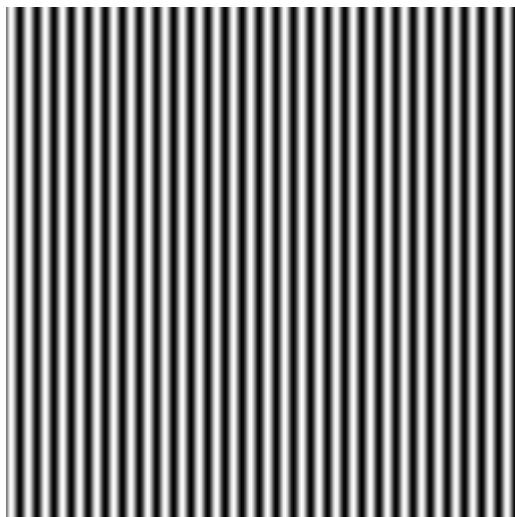
Fourier transform: Examples

- $f(x) = \delta(x) \quad \rightarrow \quad \tilde{f}(k) = 1$
- $f(x) = 1 \quad \rightarrow \quad \tilde{f}(k) = 2\pi \delta(k)$
- $f(x) = e^{-ax|x|} \quad \rightarrow \quad \tilde{f}(k) = \frac{2a}{k^2 + a^2}$
- $f(x) = \frac{1}{x^2 + a^2} \quad \rightarrow \quad \tilde{f}(k) = \frac{\pi}{a} e^{-ak/k}$
- $f(x) = e^{-a\frac{x^2}{2}} \quad \rightarrow \quad \tilde{f}(k) = \sqrt{\frac{2\pi}{a}} e^{-\frac{1}{a} \cdot \frac{k^2}{2}}$
- $$f(x) = \begin{cases} 1 & -a \leq x < 0 \\ 0 & 0 \leq x \leq a \end{cases} \quad \rightarrow \quad \tilde{f}(k) = 2 \frac{\sin(ak)}{k}$$
A graph showing a rectangular pulse function $f(x)$ plotted against x . The function is 1 for x between - a and a , and 0 elsewhere. The x -axis is labeled with $-a$, 0 , and a . The y -axis is labeled $f(x)$ and has a value 1 marked above the pulse.

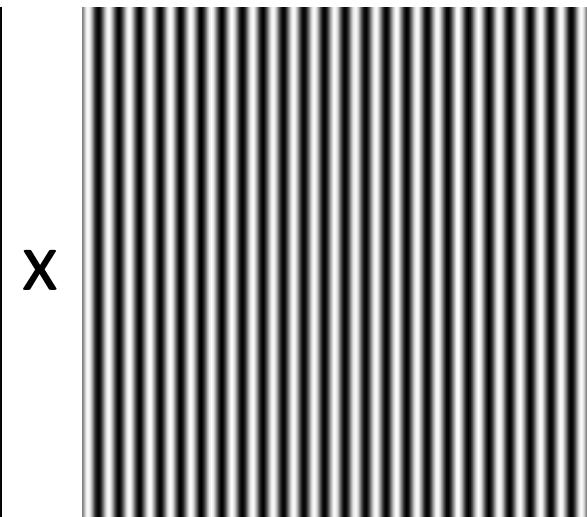


Extending the measurable freq. range

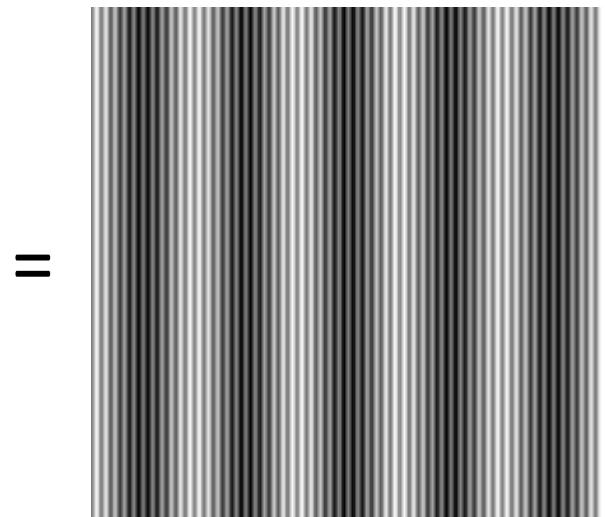
Excitation(x) \times Sample(x) = Observed Signal(x)



Freq = 30



Freq = 25

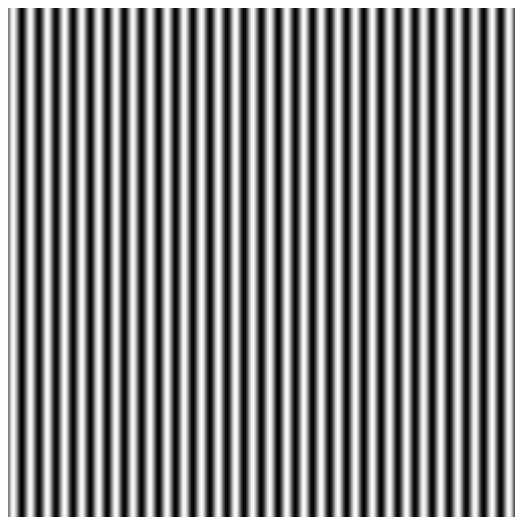


Freq = 55 & 5

$$\sin A \cdot \sin B = (\cos(A - B) - \cos(A + B)) / 2$$

Extending the measurable freq. range

$$\text{Excitation}(x) \times \text{Sample}(x) = \text{Observed Signal}(x)$$

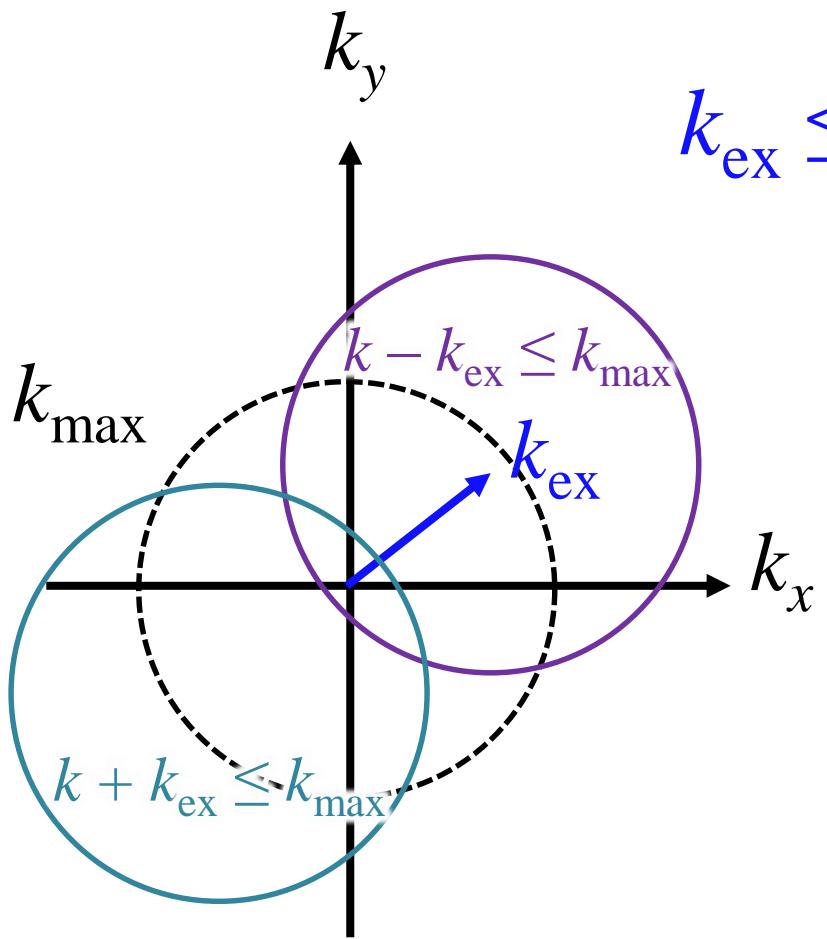


Freq = 30

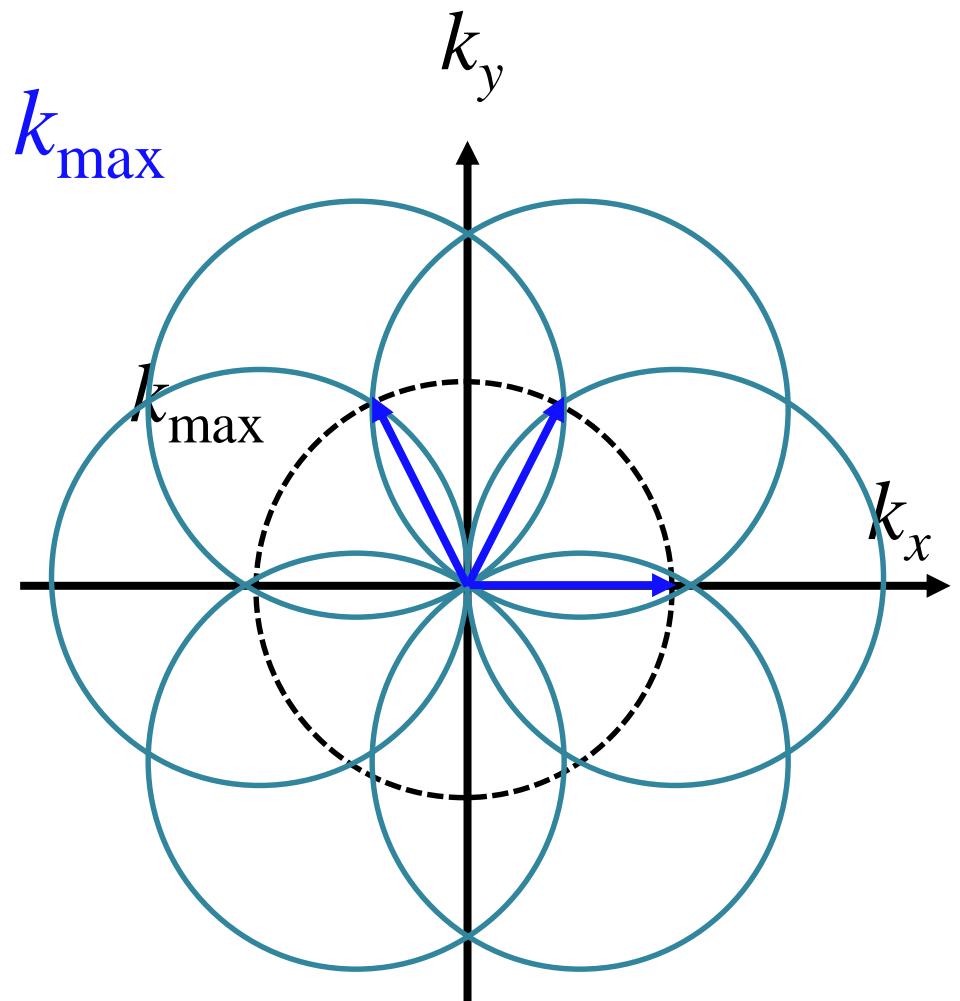
$\sin A \cdot \sin$



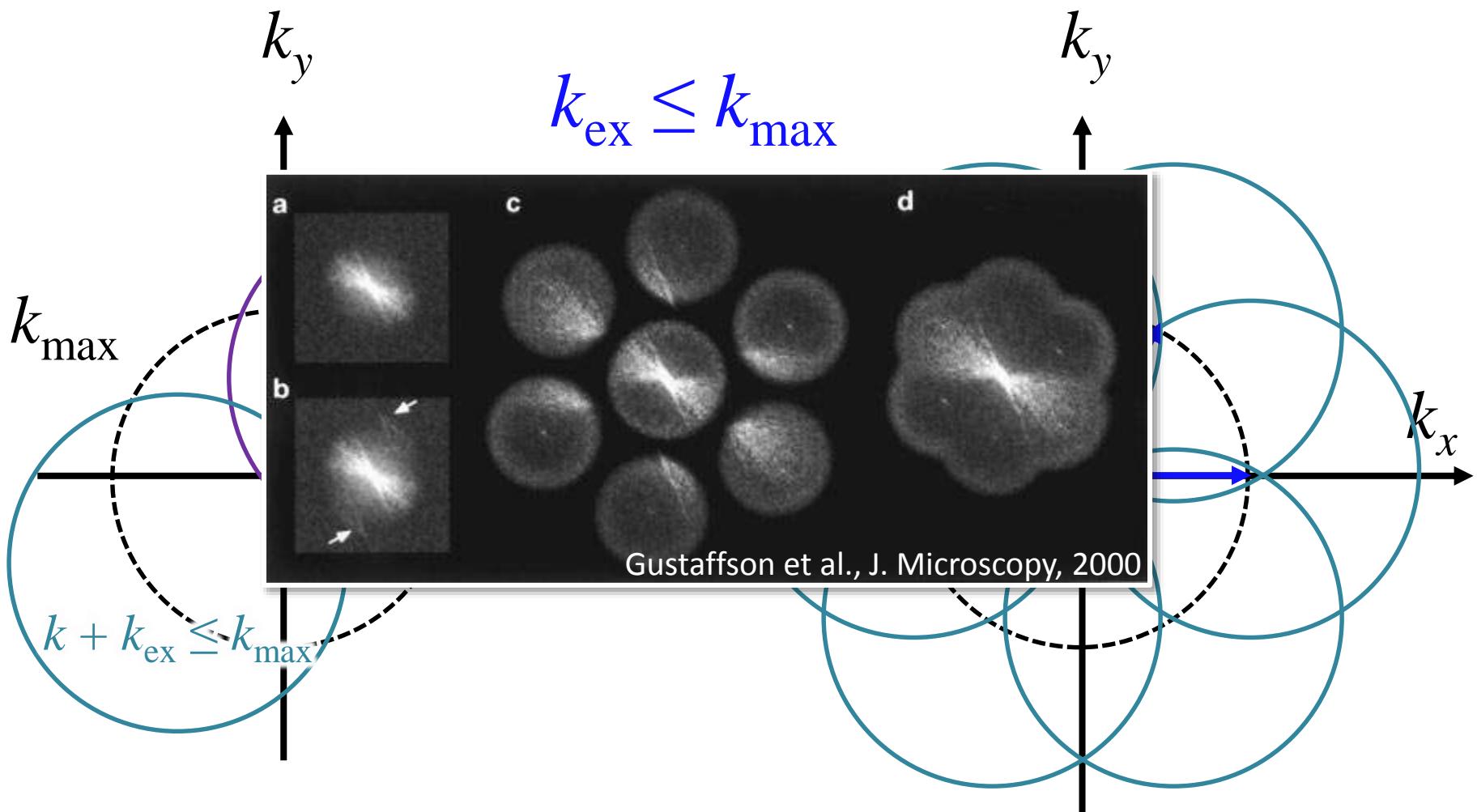
Extending the measurable freq. range



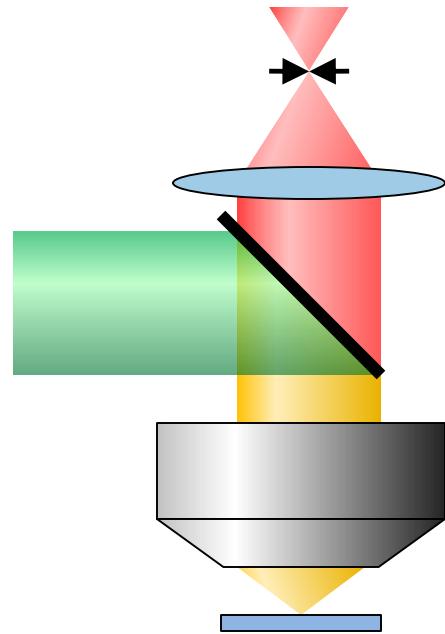
$$k_{\text{ex}} \leq k_{\max}$$



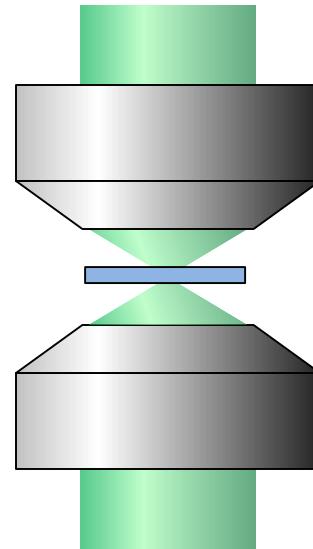
Extending the measurable freq. range



The diffraction limit still exists

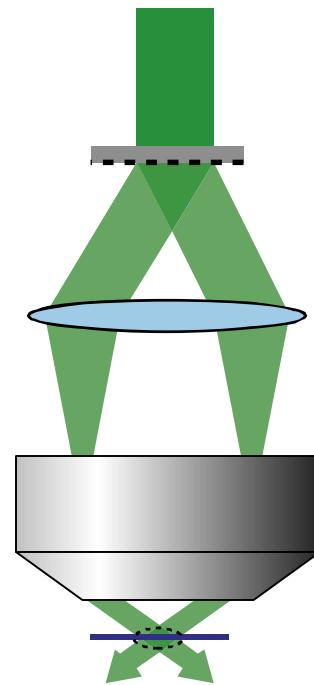


Confocal



$4\text{Pi} / \text{I}^5\text{M}$

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

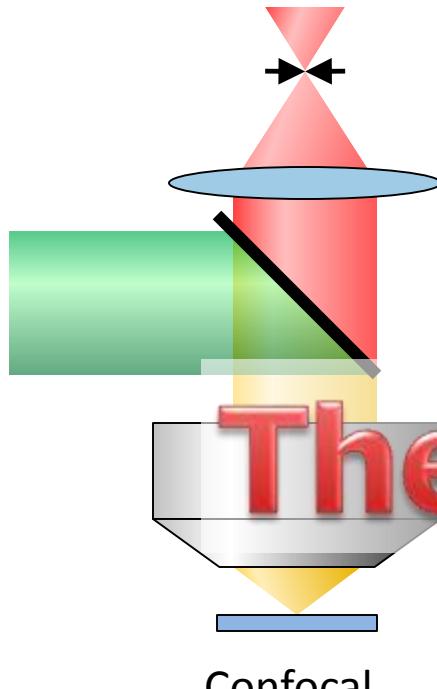


SIM

Breaking the diffraction barrier



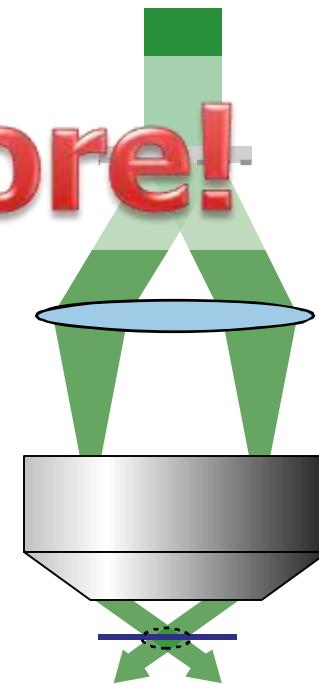
Breaking the diffraction barrier



Confocal



$4\text{Pi} / \text{I}^5\text{M}$

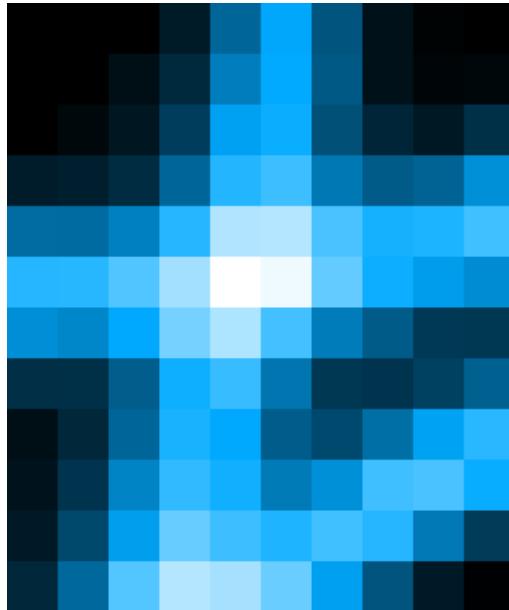


SIM

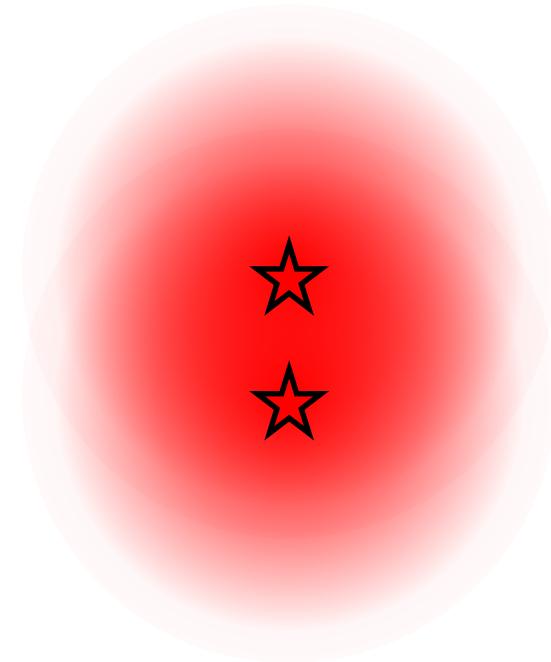
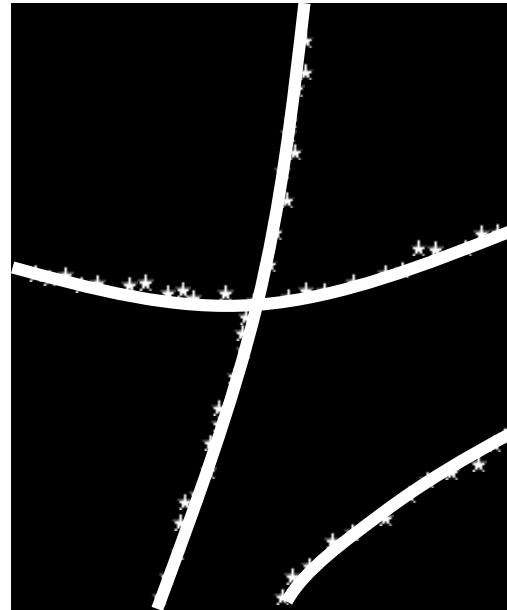
The Fluorophore!

Super-resolution by...

Fluorescence image

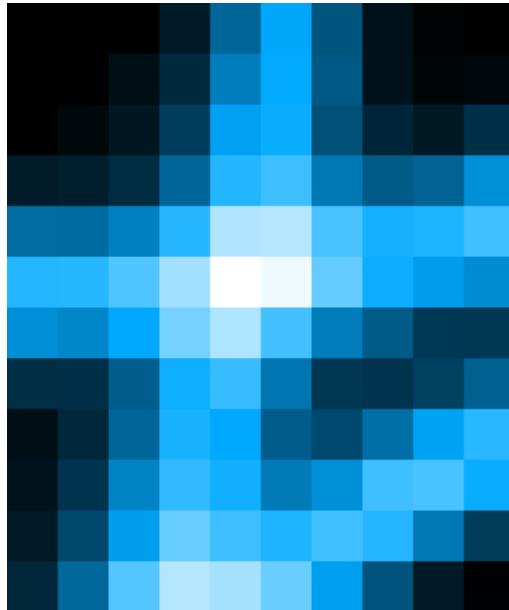


Underlying structure

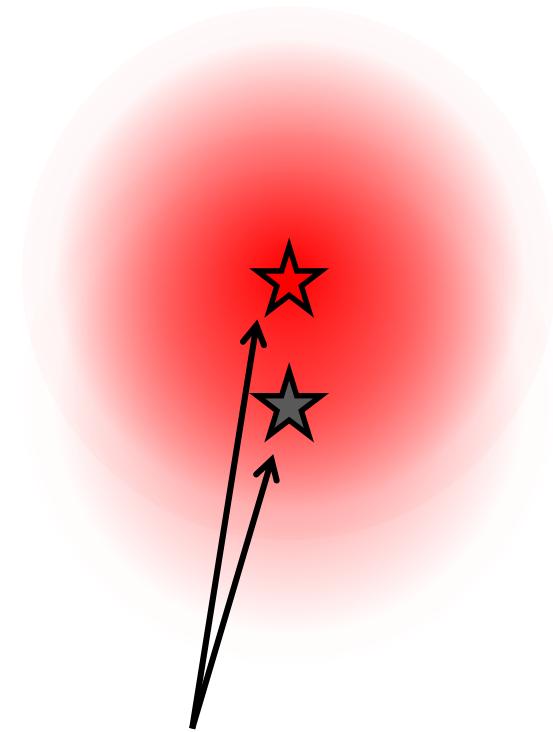
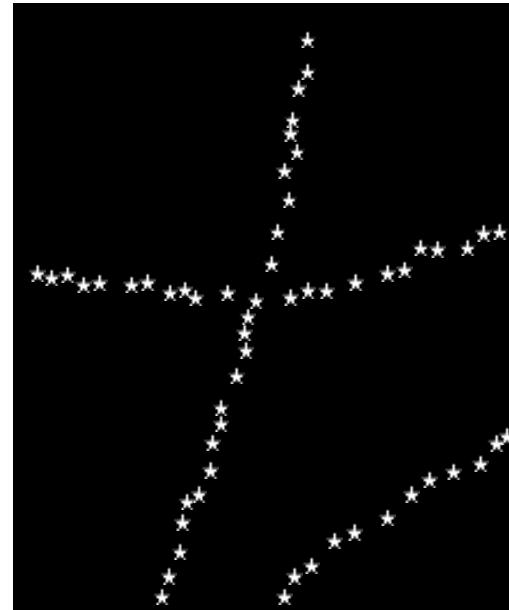


Super-resolution by spatial modulation

Fluorescence image



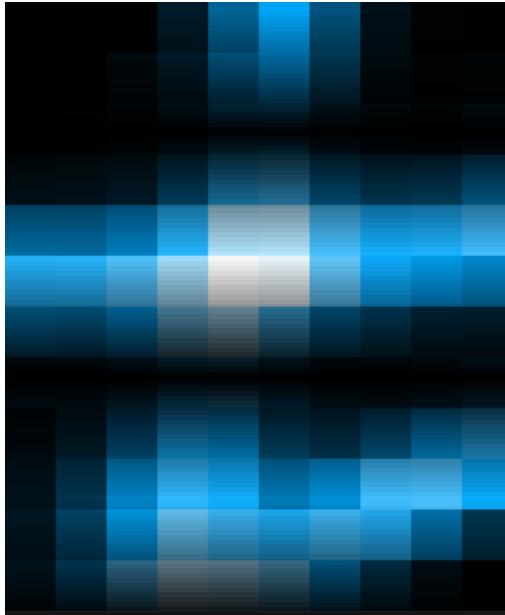
Underlying structure



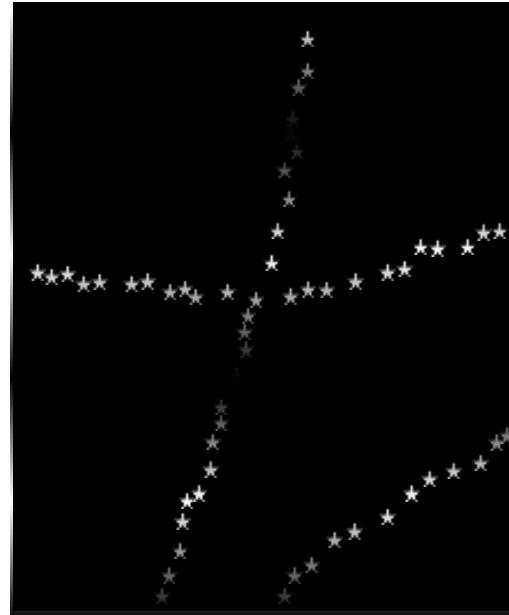
Differential modulation
of the fluorescence response

Super-resolution by differential excitation

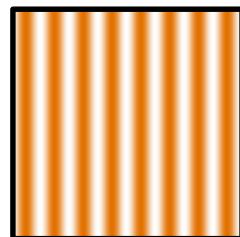
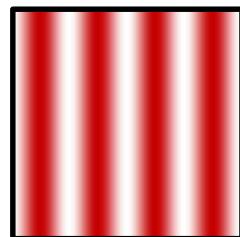
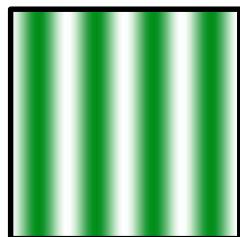
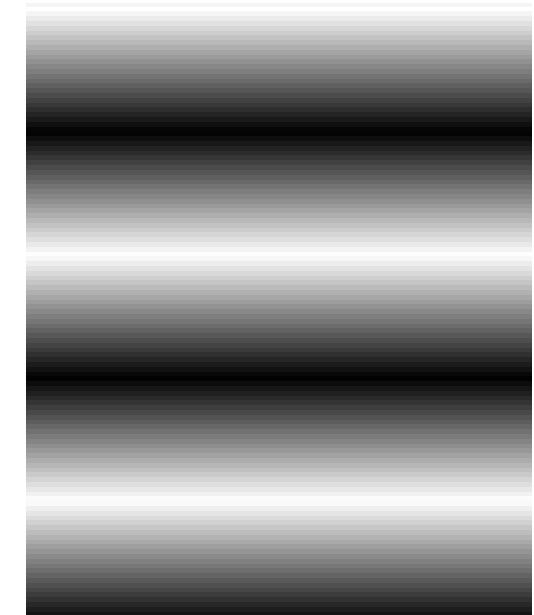
Fluorescence image



Underlying structure



Excitation pattern



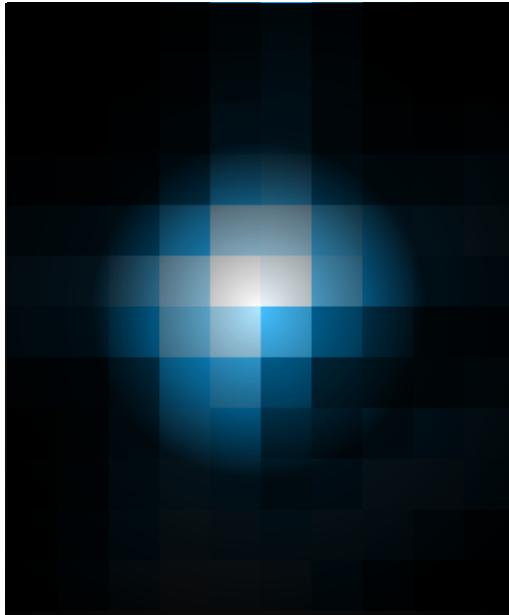
SIM (Gustafsson / Heintzmann)
SSIM (Gustafsson 2005)

Diffraction limited excitation and emission

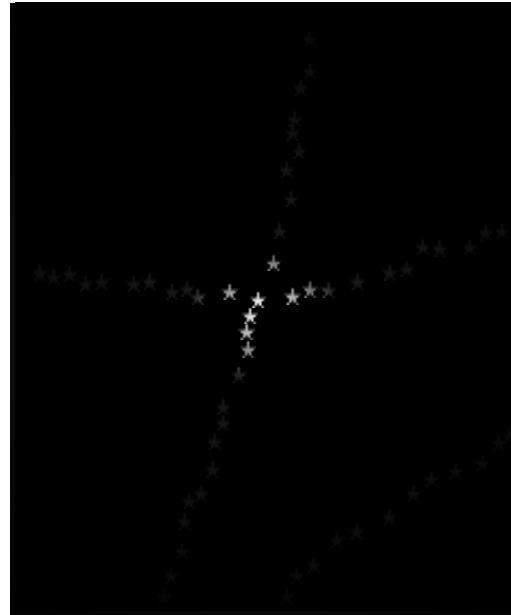
Doubled resolution

Super-resolution by differential depletion

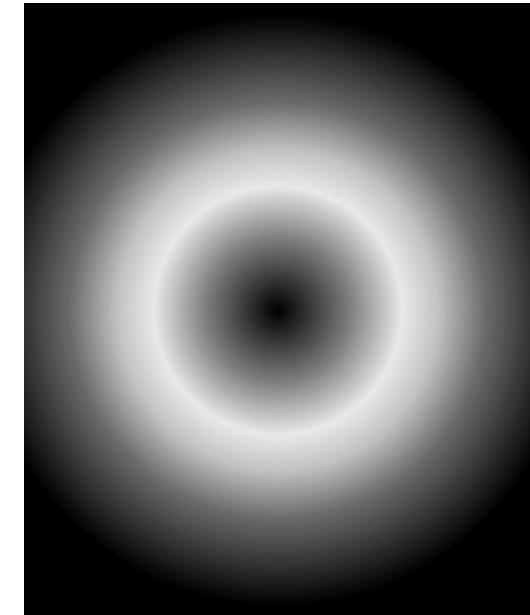
Fluorescence image



Underlying structure



Depletion pattern



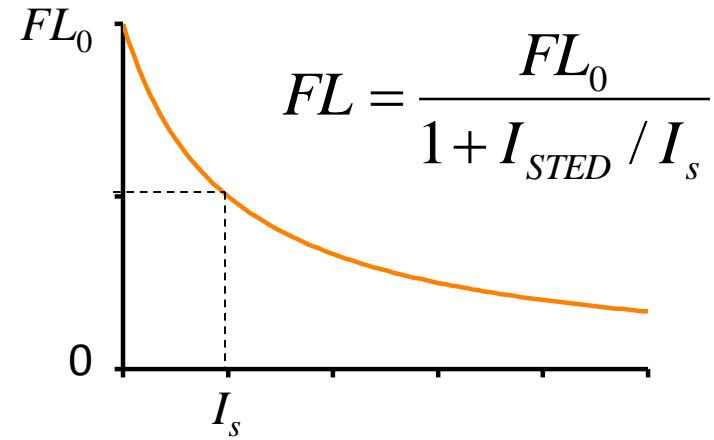
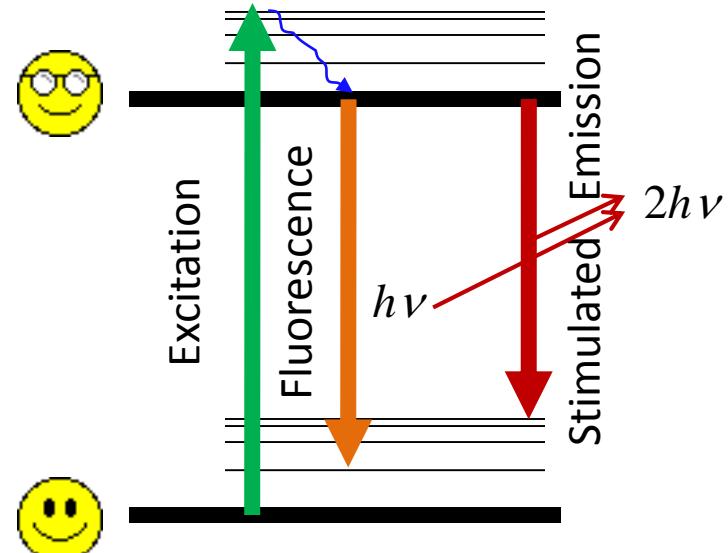
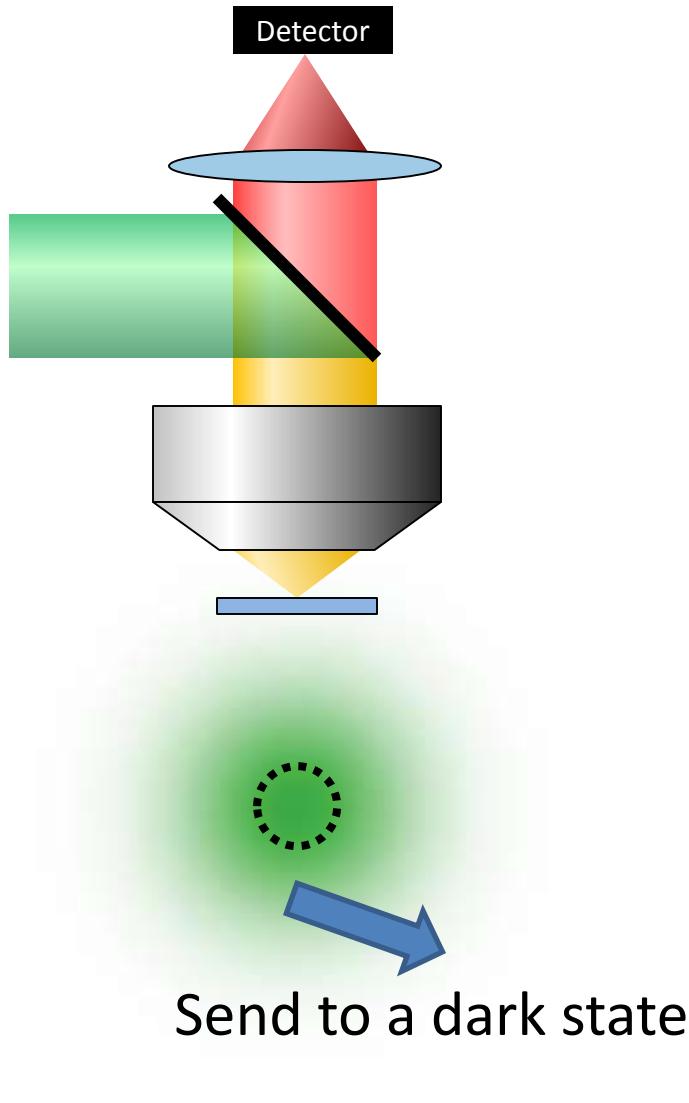
Diffraction limited PSF Saturated depletion = Smaller effective PSF

STED (Hell 1994, Hell 1999)

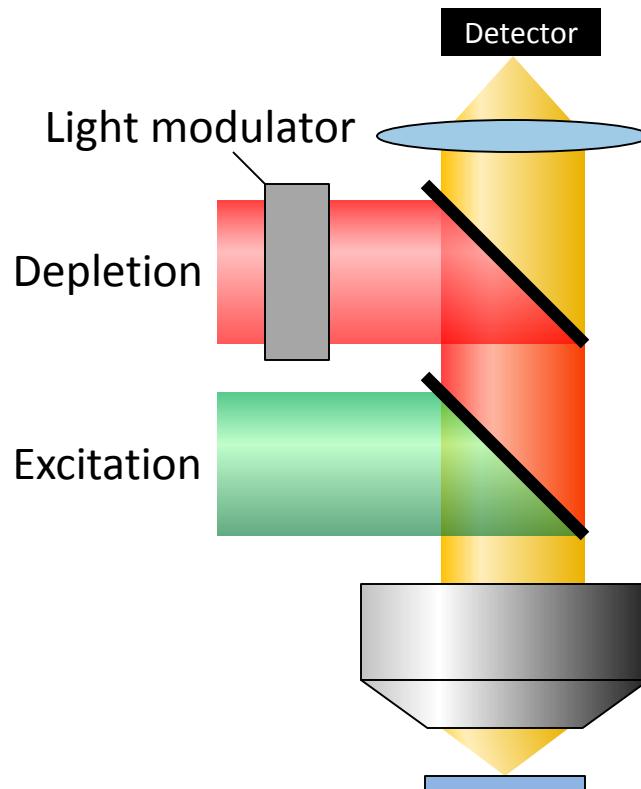
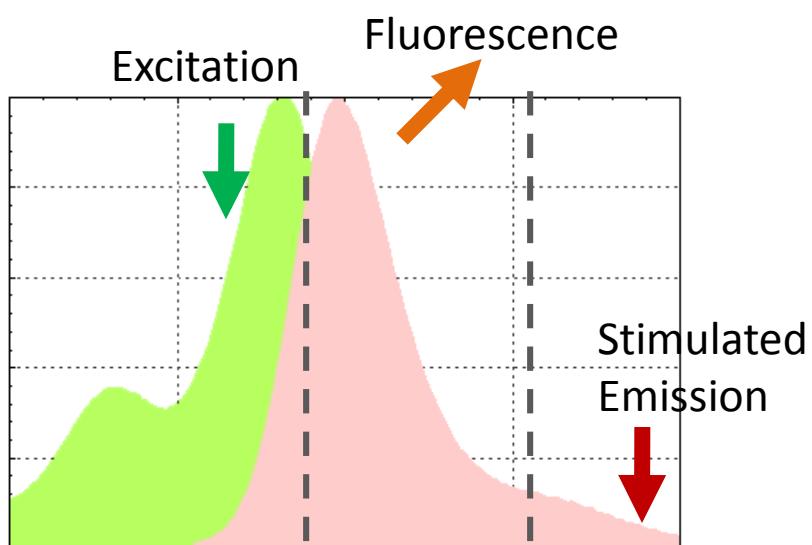
GSD (Hell 1995, Hell 2007)

RESOLFT (Hell 2003, Hell 2011)

Stimulated Emission Depletion (STED)



STED microscopy



Excitation

STED
pattern

Effective
PSF



÷

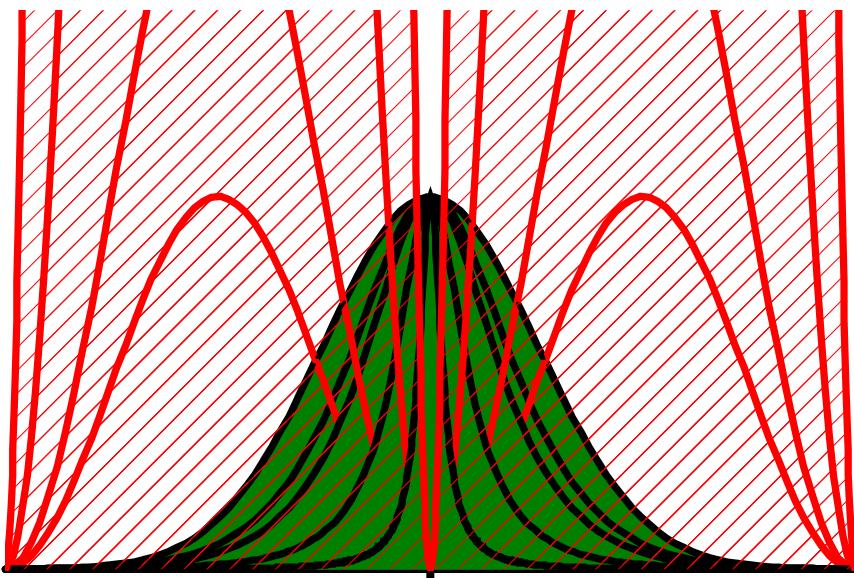


=



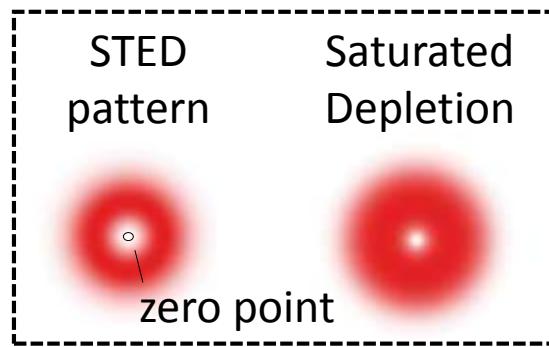
?

Saturated depletion

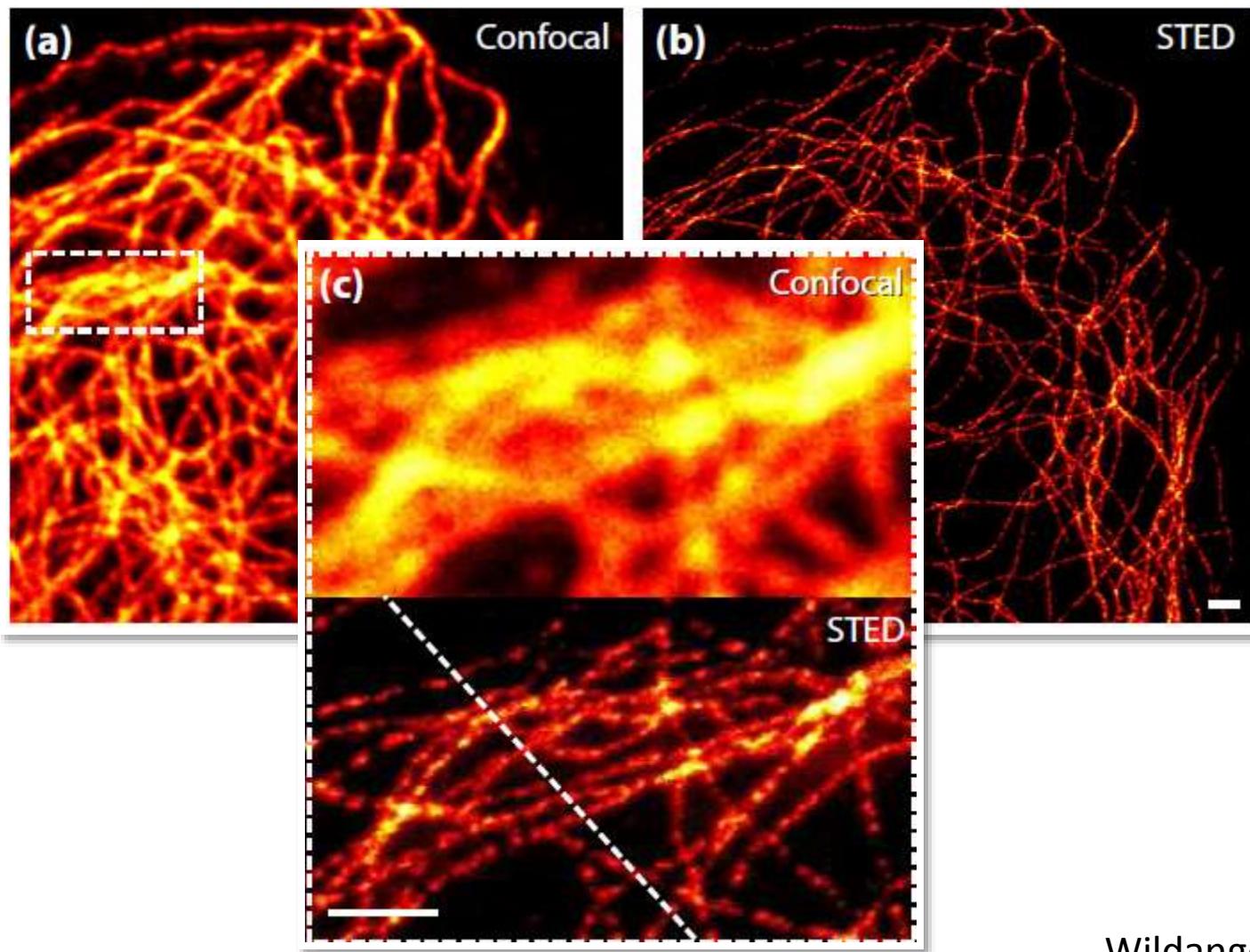


$$I_{\text{STED}} = I_s Q Q_S S_S$$

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

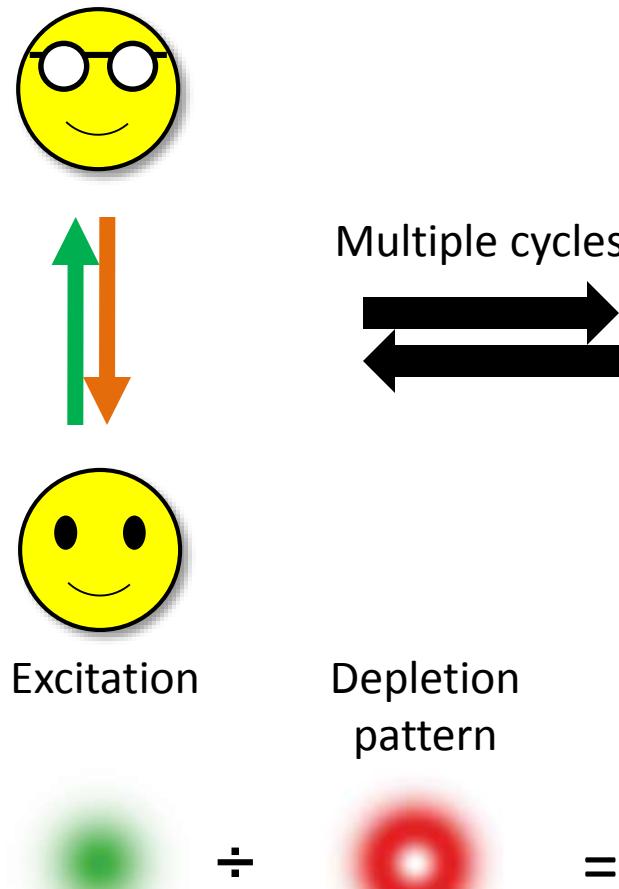


STED images of microtubules

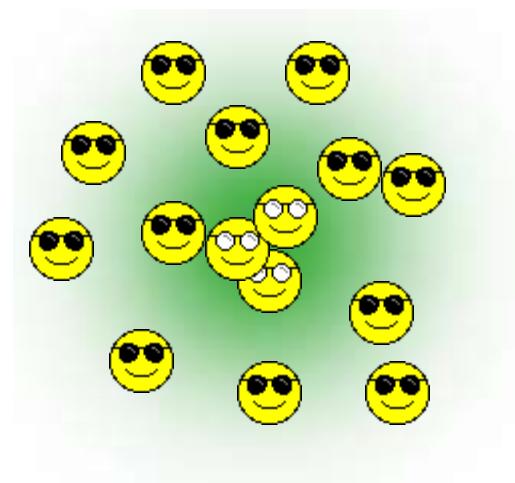


Wildanger et al., 2009

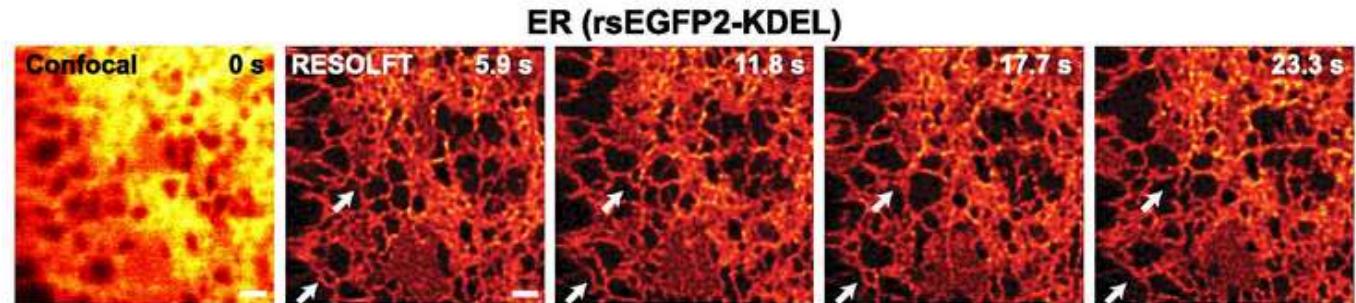
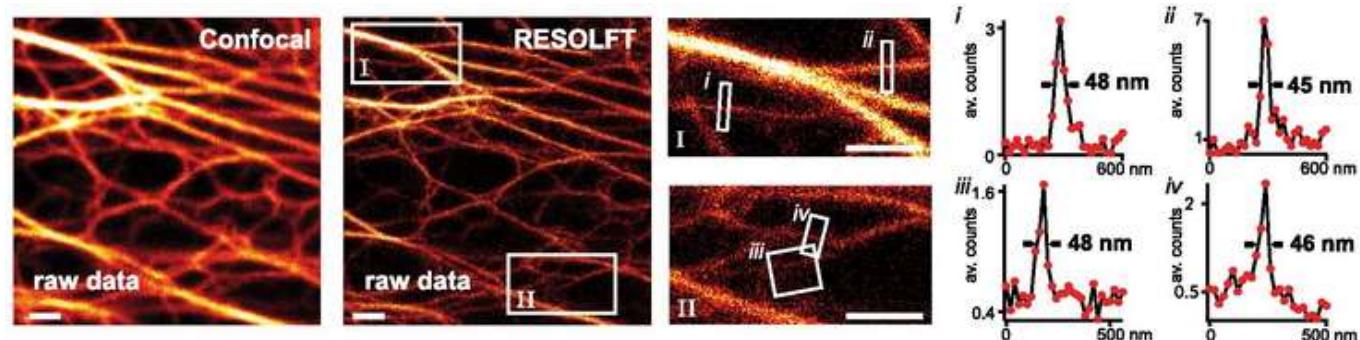
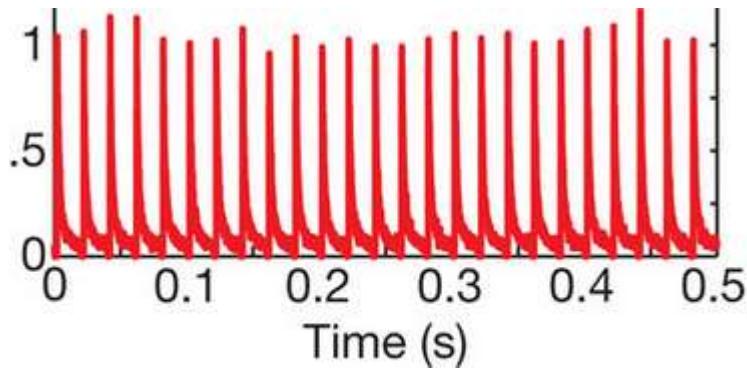
The “patterned illumination” approach



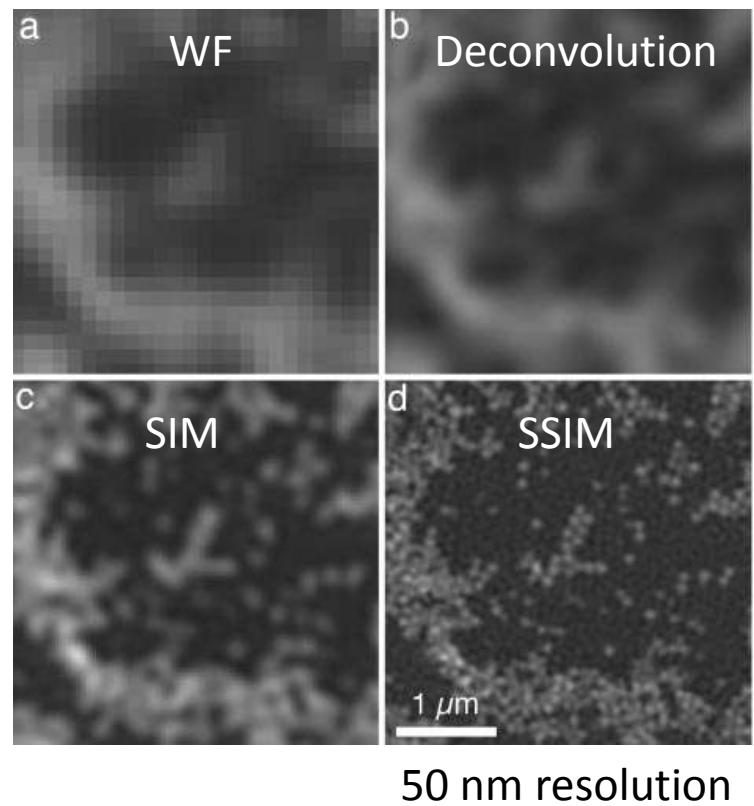
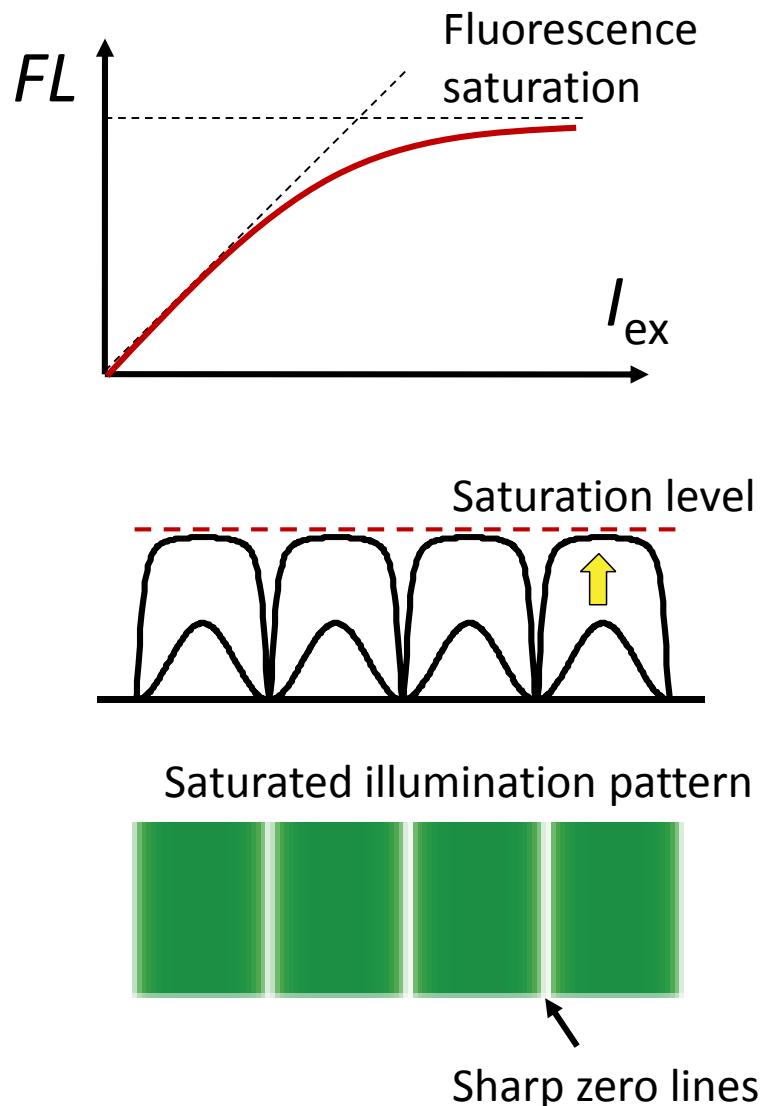
- Ground state
- Triplet state
- Isomerization
- etc.



RESOLFT by rsEGFP and rsEGFP2



Saturated SIM



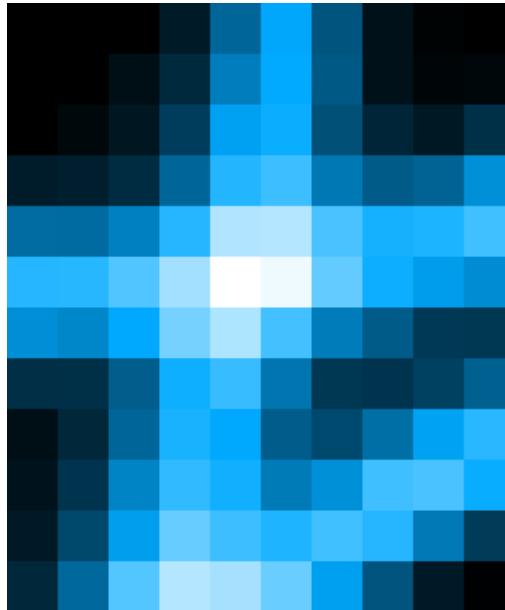
Suffers from fast photobleaching
under saturated excitation condition

Super-resolution by single-molecule switching

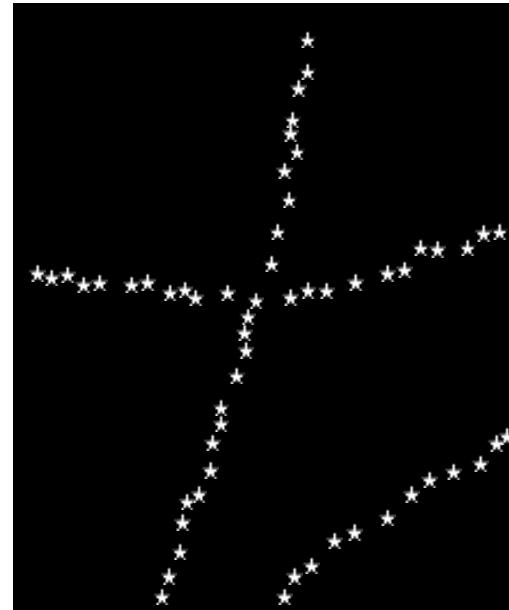


Super-resolution by single-molecule switching

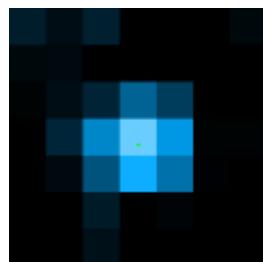
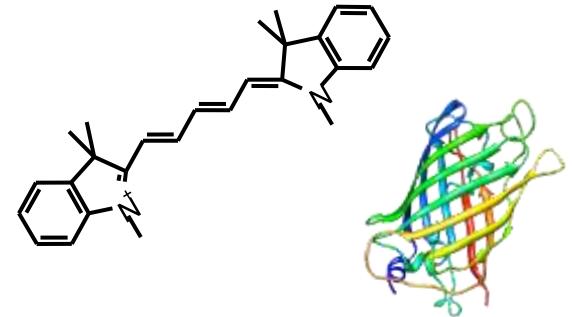
Fluorescence image



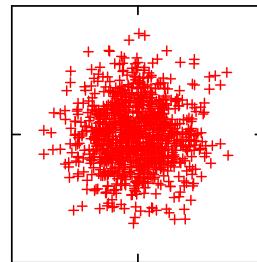
Underlying structure



Photoswitchable molecules



=



$$D \approx d / \sqrt{N}$$

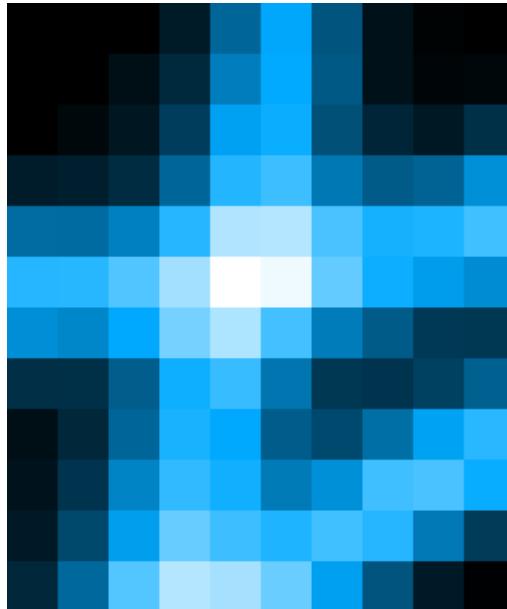
Single molecule image

N photons

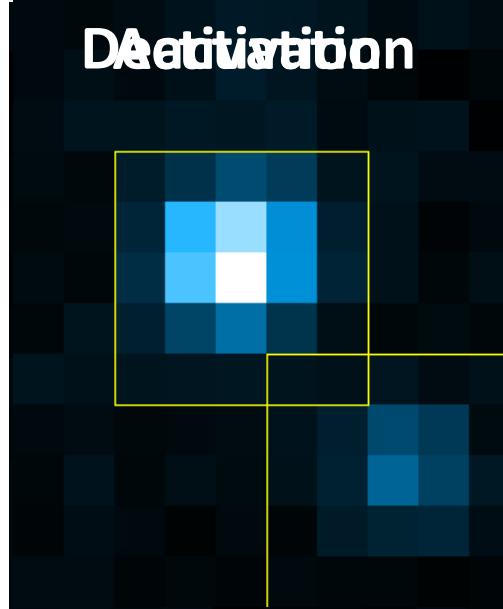
Single-molecule localization

Super-resolution by single-molecule switching

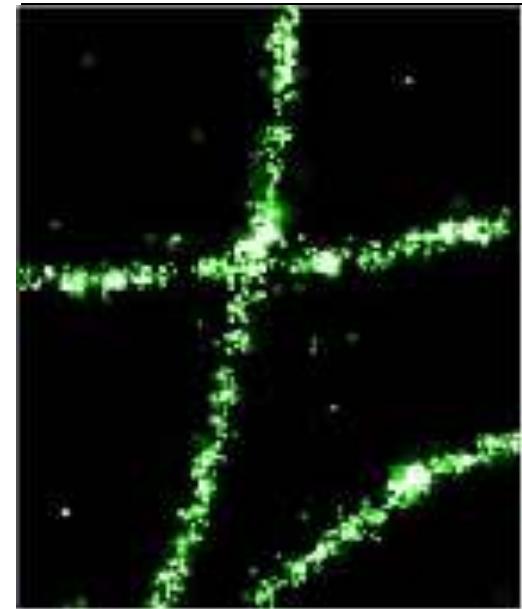
Fluorescence image



Raw images



STORM Image



2x real time

STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)

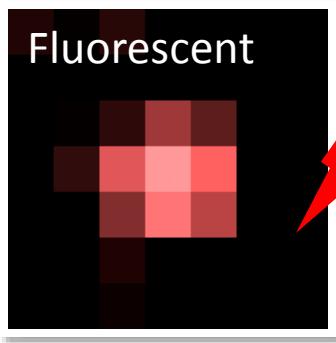
PALM = Photoactivation Localization Microscopy (Betzig & Hess 2006)

FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)

PALMIRA (Hell 2007), **GSDIM** (Hell 2008), **dSTORM** (Sauer 2008), **SMACM** (Moerner 2008)

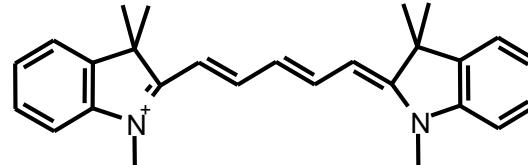
PAINT (Hochstrasser 2006), **SPRAYPAINT** (Moerner 2011), **SOFI** (Weiss 2009)

Photoswitching of red cyanine dyes

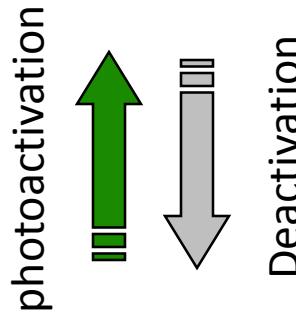


650 nm

+ thiol



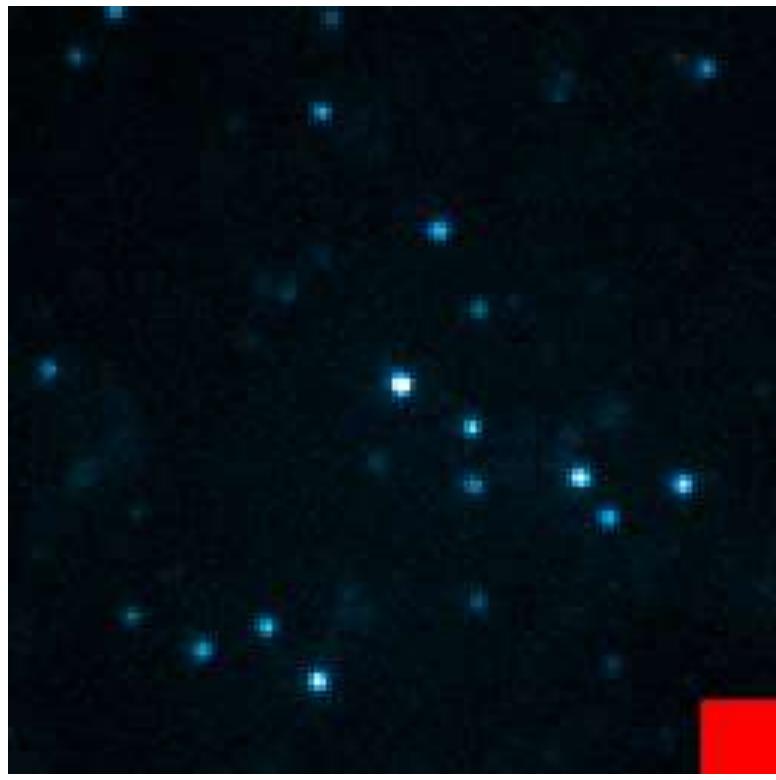
Cy5 / Alexa 647



360 nm



650 nm

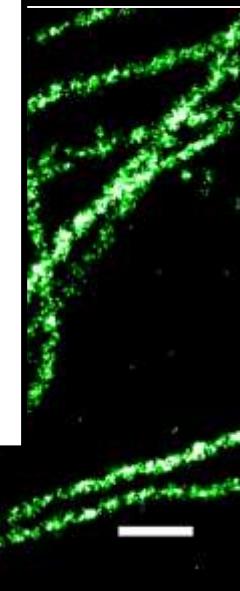
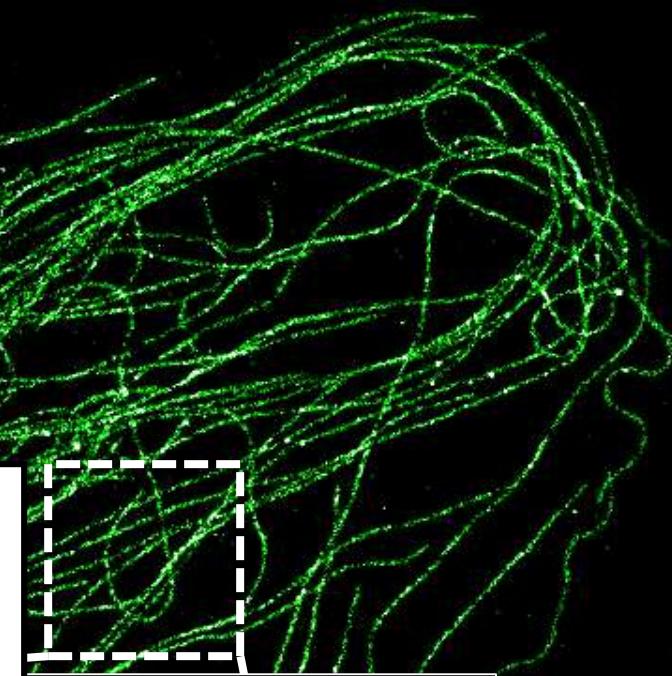
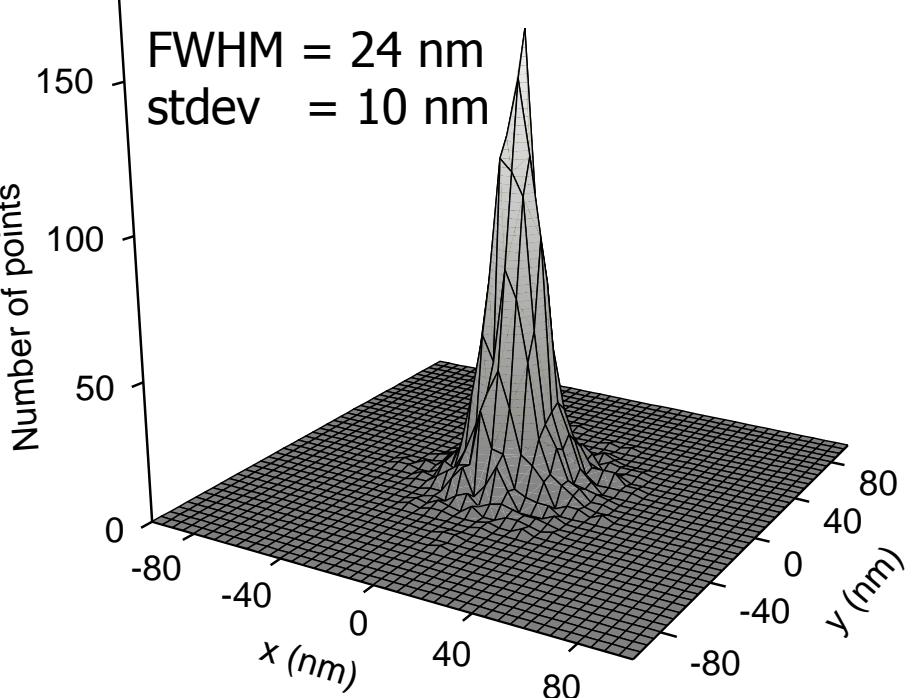


B-SC-1 cell, anti- β tubulin

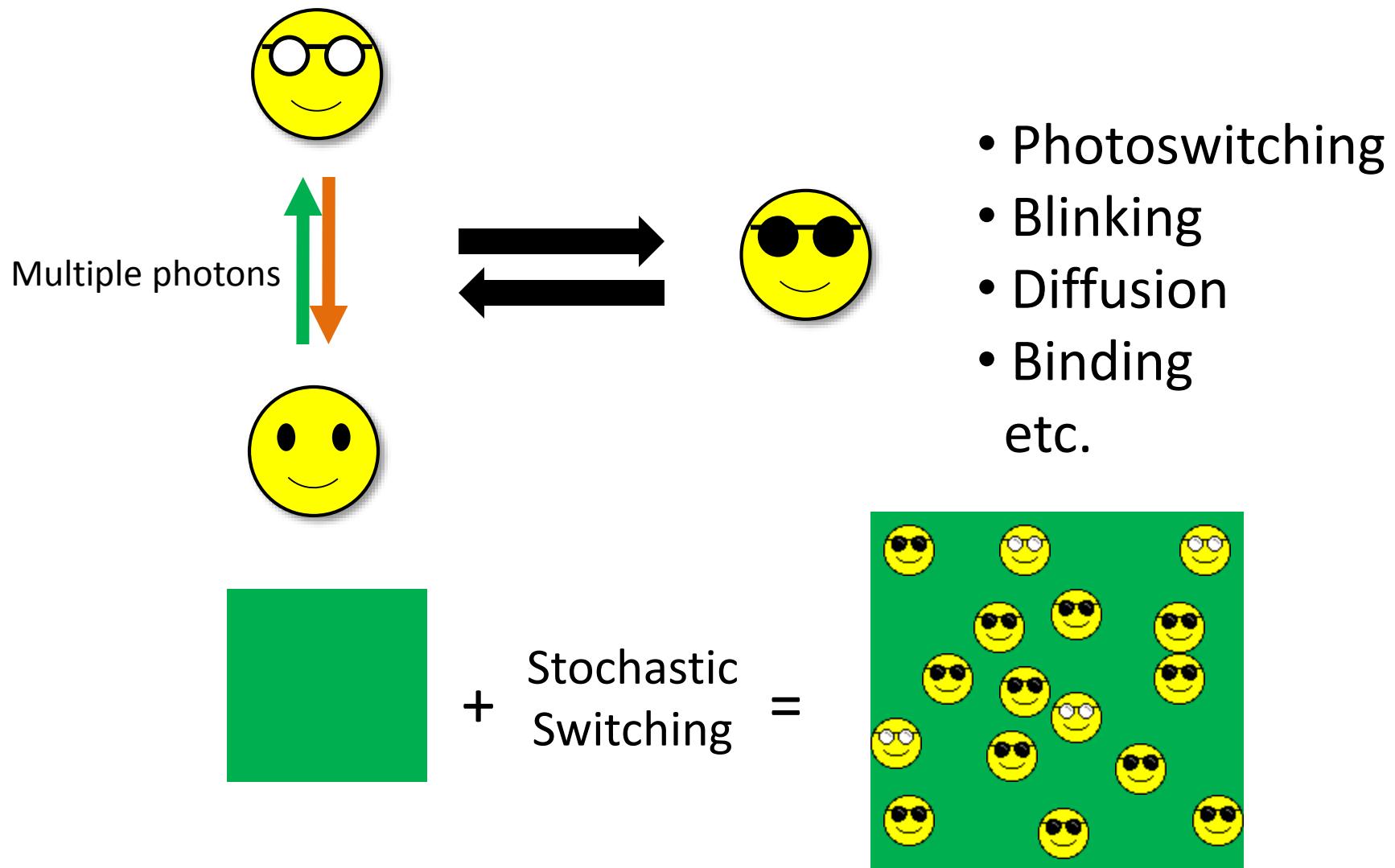
Commercial

Alexa 647

secondary antibody



The “single-molecule switching” approach



Photoswitchable probes readily available

400

500

600

700 nm

Simple dyes (+ thiole / redox system)

Alexa488

Atto520

Alexa532

Alexa568

Atto565

Atto590

Alexa647

Cy5

Cy5.5

Cy7

Atto655

Atto700

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

Heilemann et al., 2009

Functional dyes

MitoTracker Red

DiD

Dil

LysoTracker Red

ER Tracker Red

Shim et al., 2012

Photoactivatable fluorescent proteins

PA-GFP

PS-CFP2

Dronpa

mEosFP2

Dendra2

PAmCherry

PAtagRFP

Dreiklang

EYFP

Reviews:

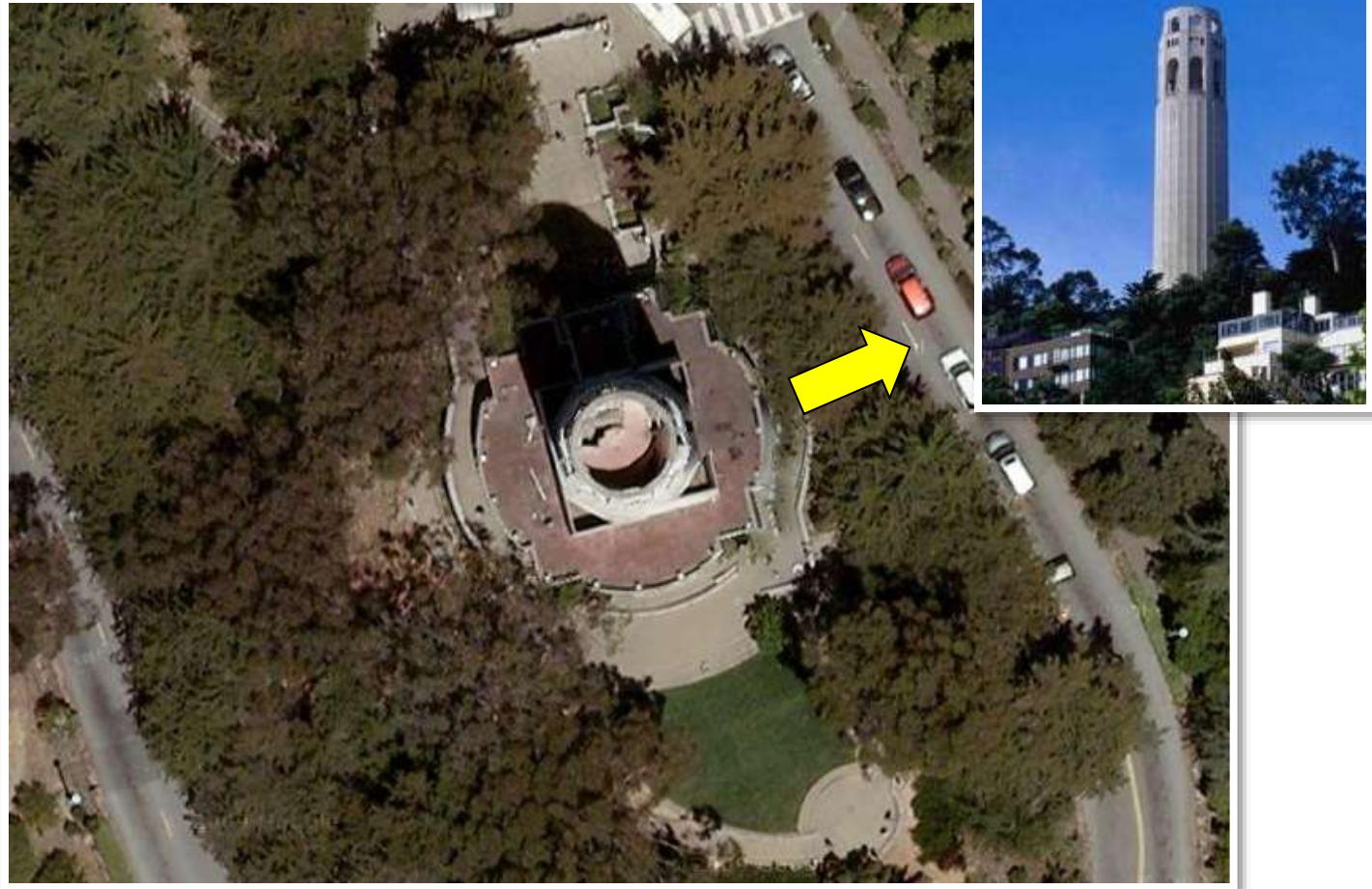
Lukyanov et al., Nat. Rev. Cell Biol., 2005

Lippincott-Schwartz et al., Trends Cell Biol., 2009

3D Imaging

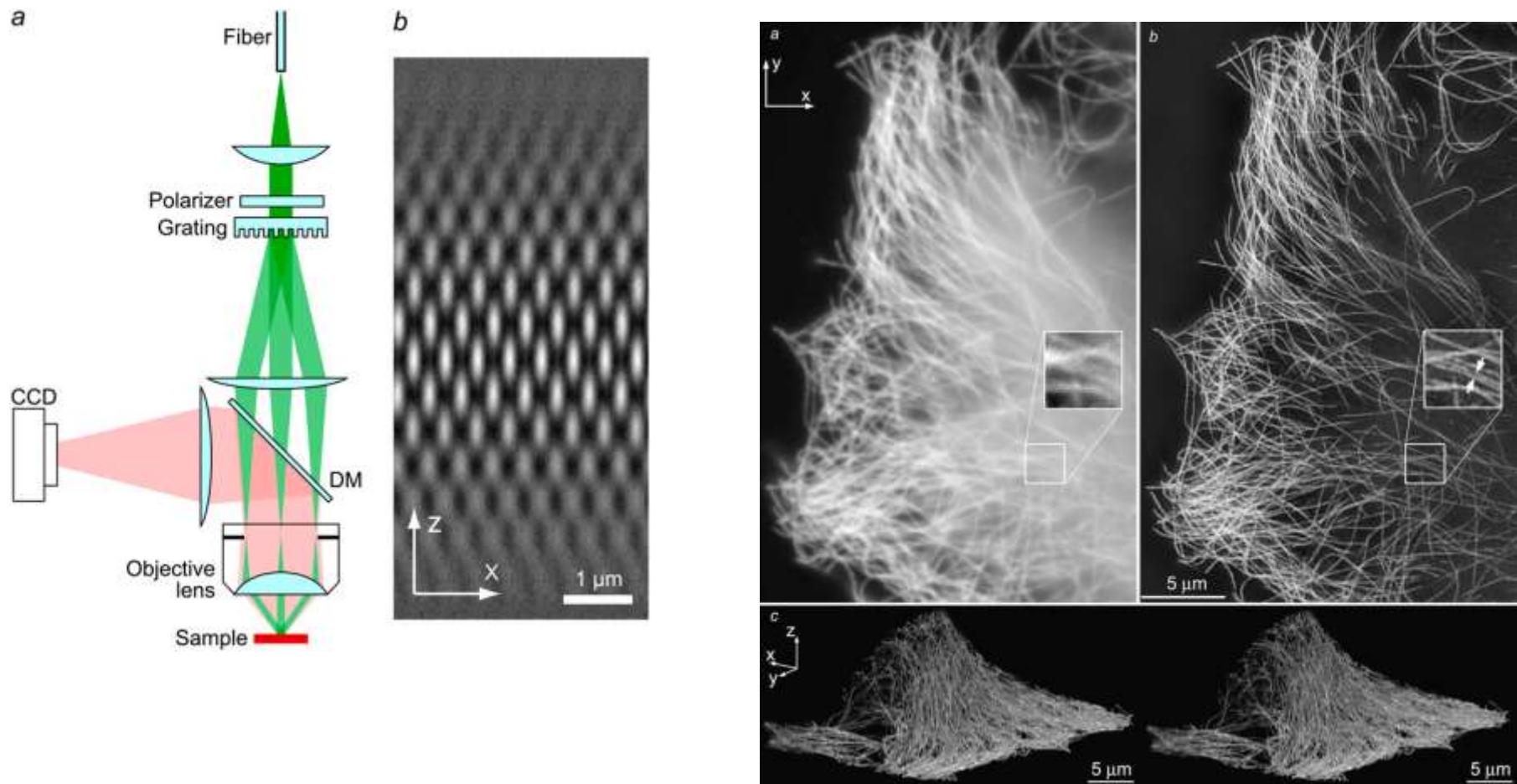
In a 2D world...

Satellite image of ???

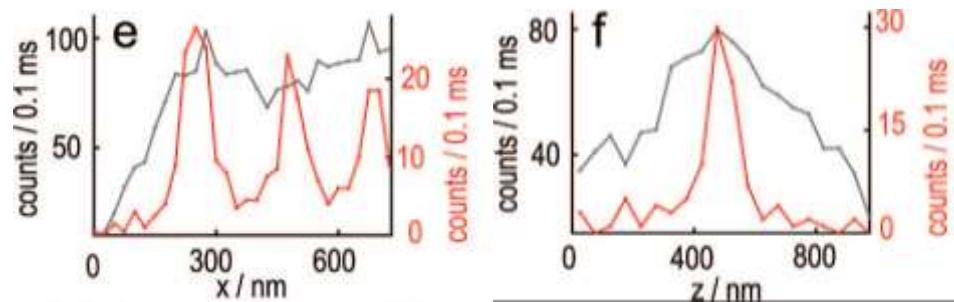
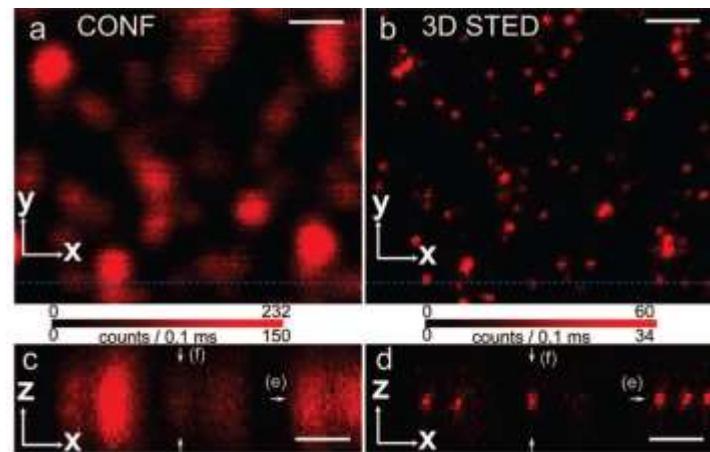
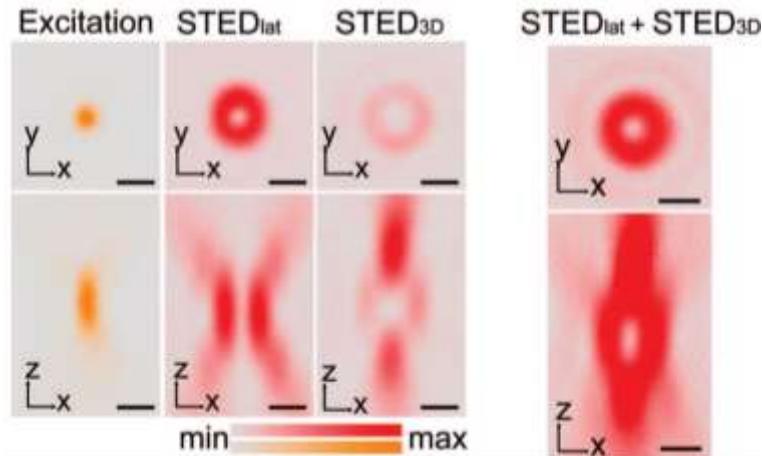


Google maps

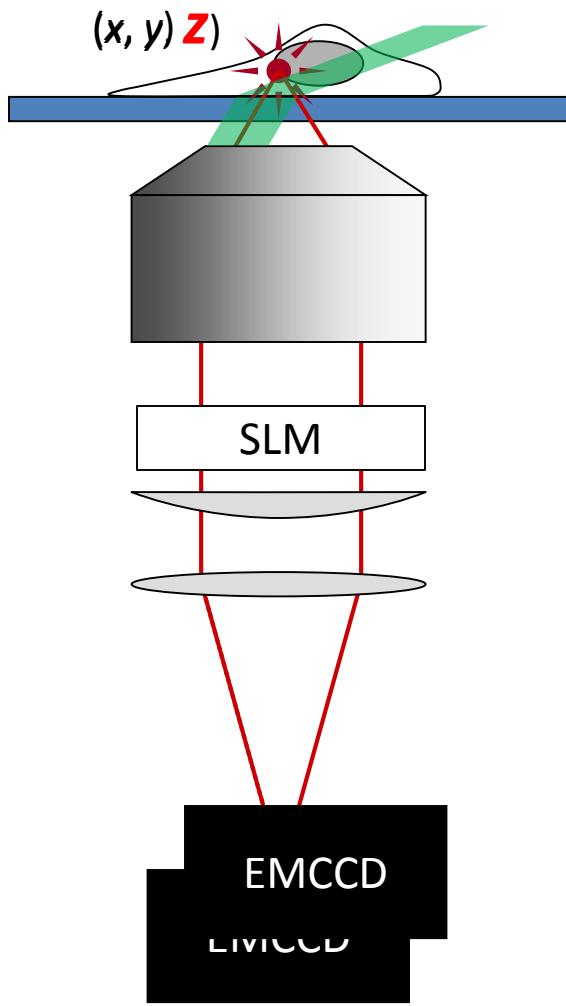
3D SIM



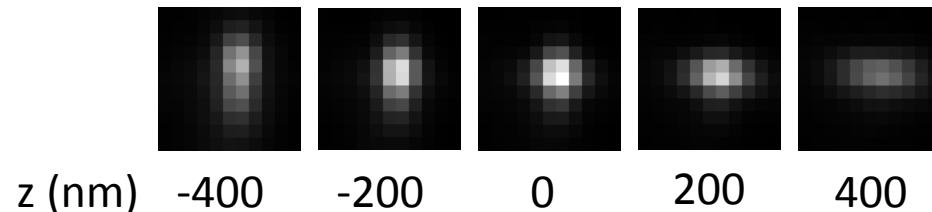
3D STED



3D STORM/PALM

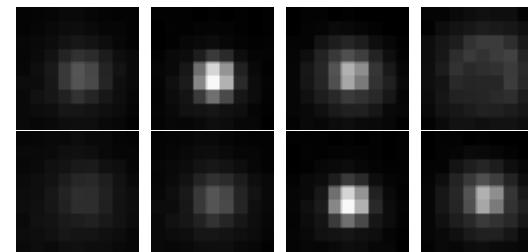


Astigmatic imaging



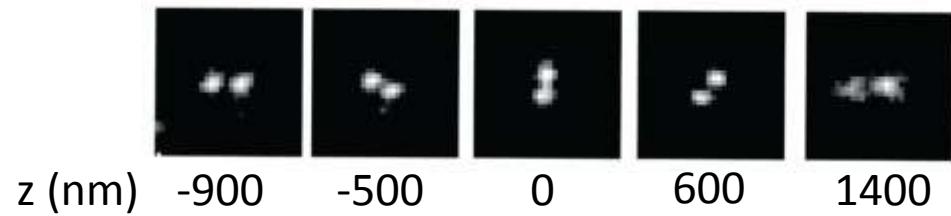
Huang et al., Science 2008

Bi-plane imaging



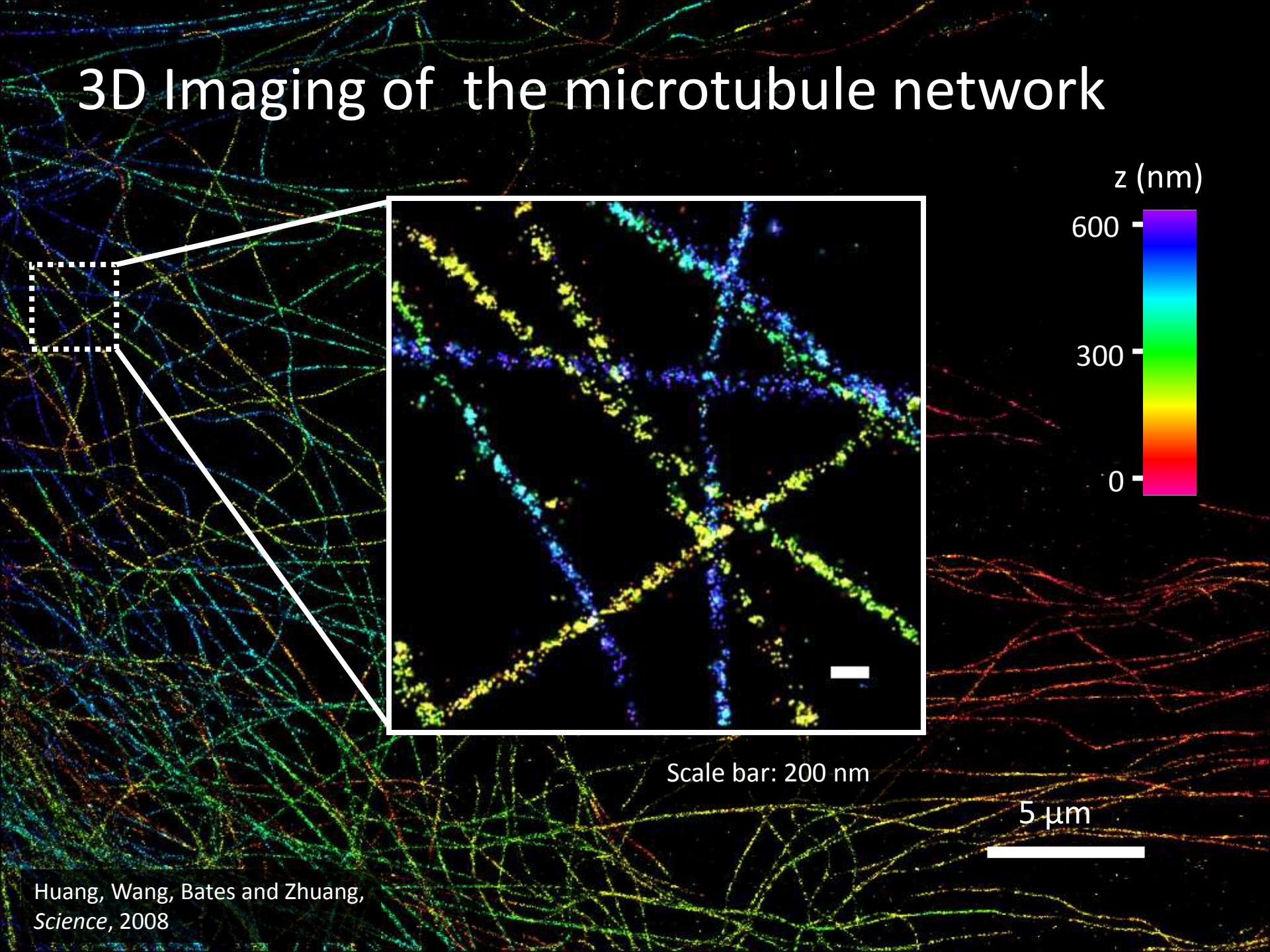
Juette et al., Science 2008

Double-helical PSF

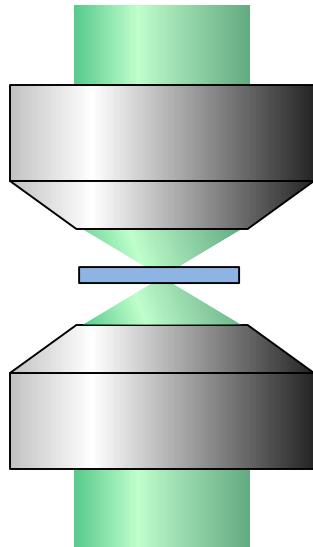


Pavani et al., PNAS 2009

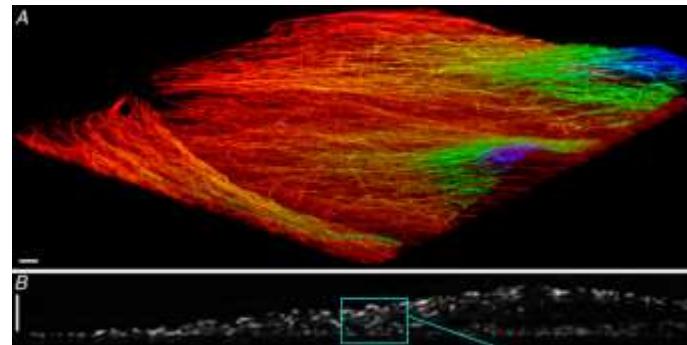
3D Imaging of the microtubule network



The use of two opposing objectives

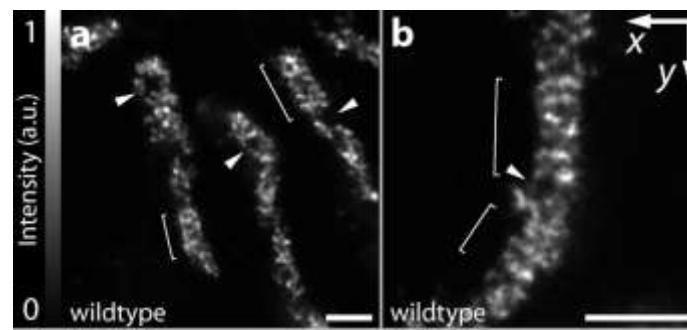


$|^5S$



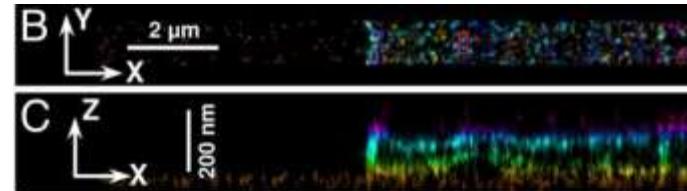
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

Near isotropic
3D resolution



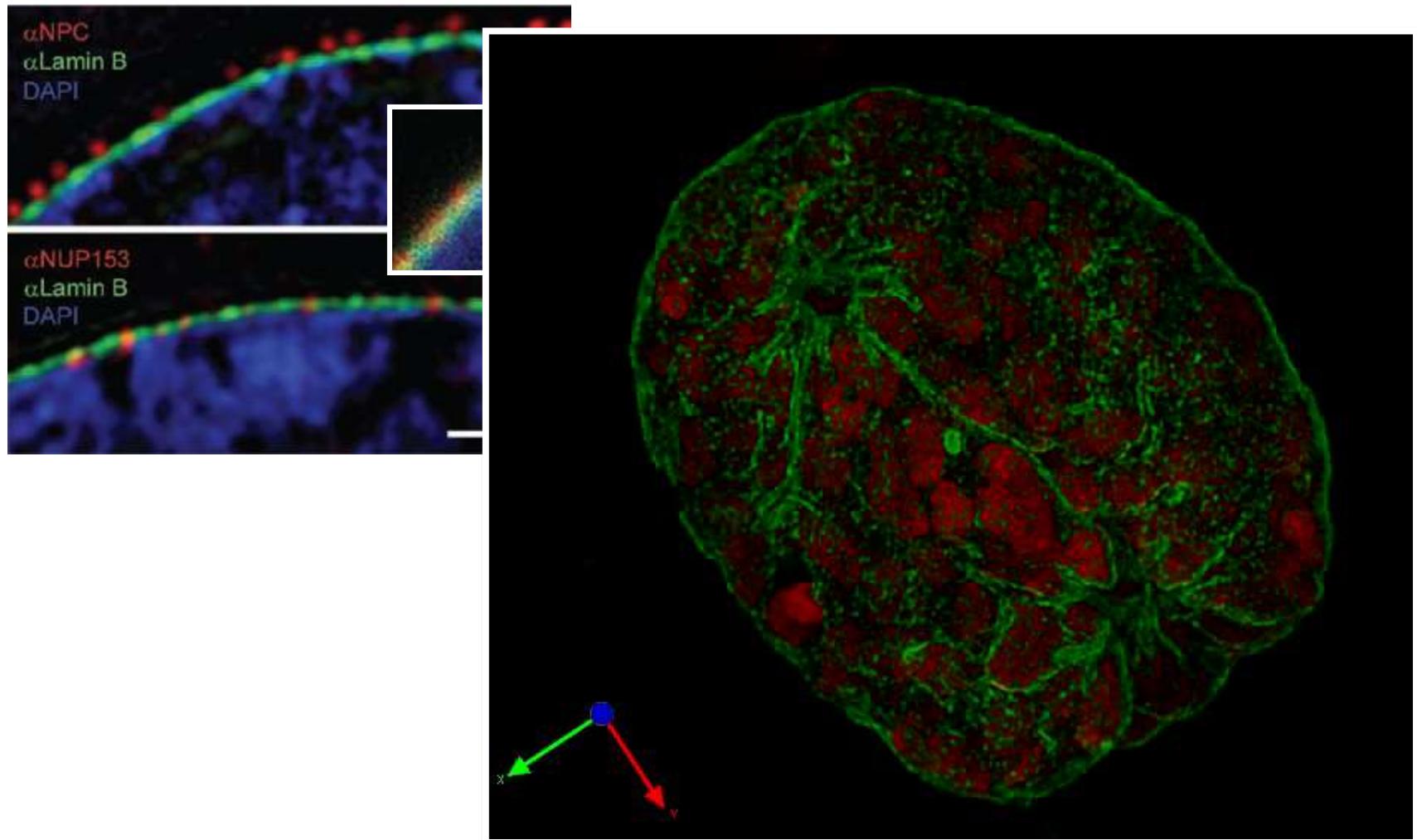
3D resolution of super-resolution methods

	x-y (nm)	z (nm)	Opposing objectives (nm)	Deep tissue
Conventional	250	600	4Pi: 120	
SIM	100	250	I ⁵ S: 120 xyz	
STED	~30	~100	isoSTED: 30 xyz	2 photon
STORM/PALM	20-30	50-60	iPALM: 20 xy, 10 z	2 photon, SPIM

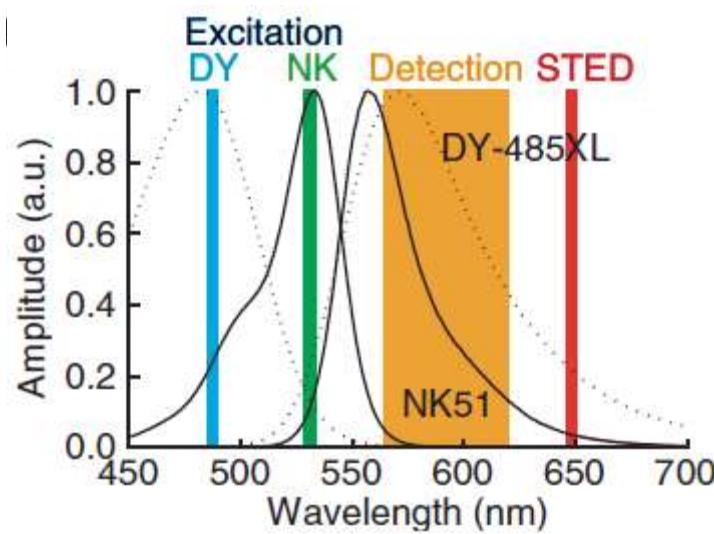
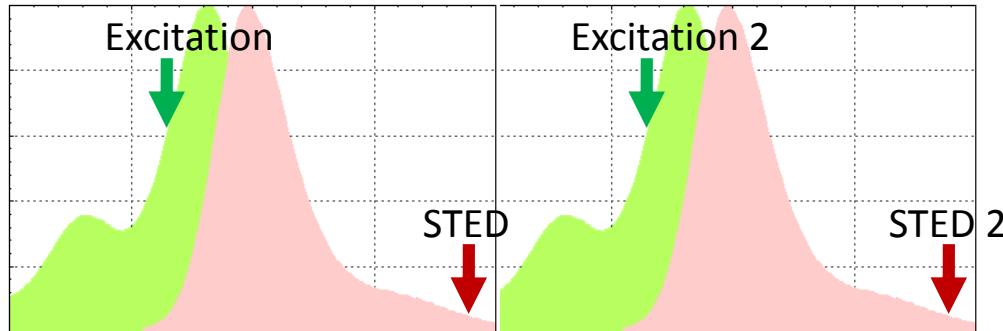
Multi-color Imaging

Multicolor SIM

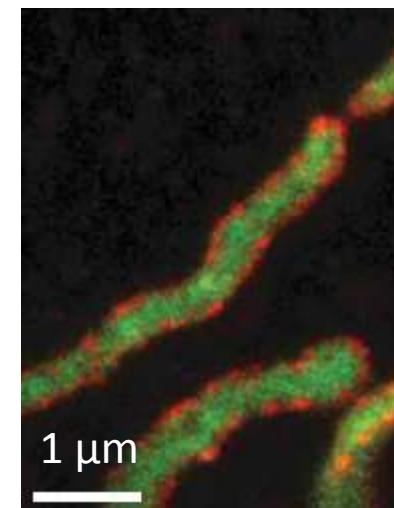
Same as conventional fluorescence microscopy!



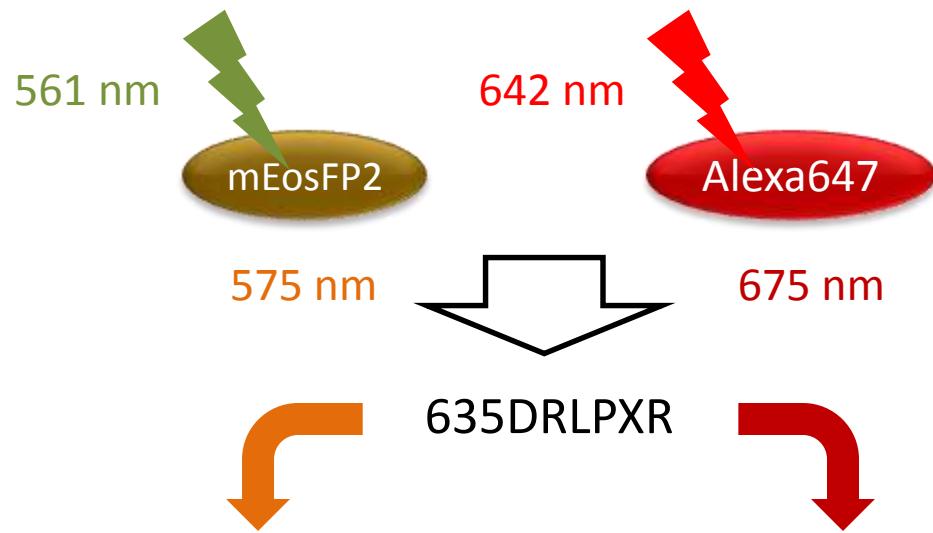
Muticolor STED



2 color isoSTED resolving
the inner and outer membrane
of mitochondria



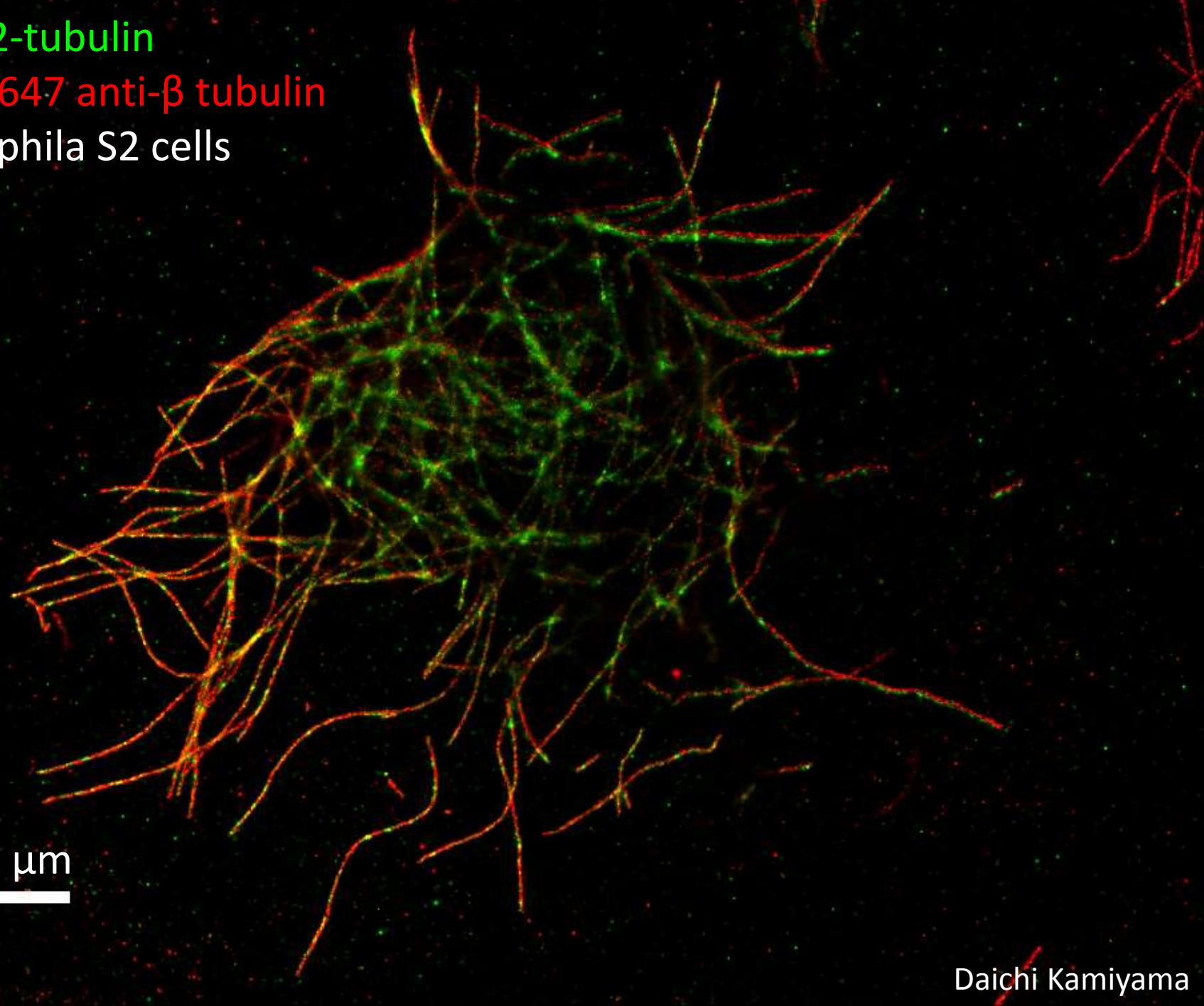
Multicolor STORM/PALM



mEos2-tubulin

Alexa 647 anti- β tubulin

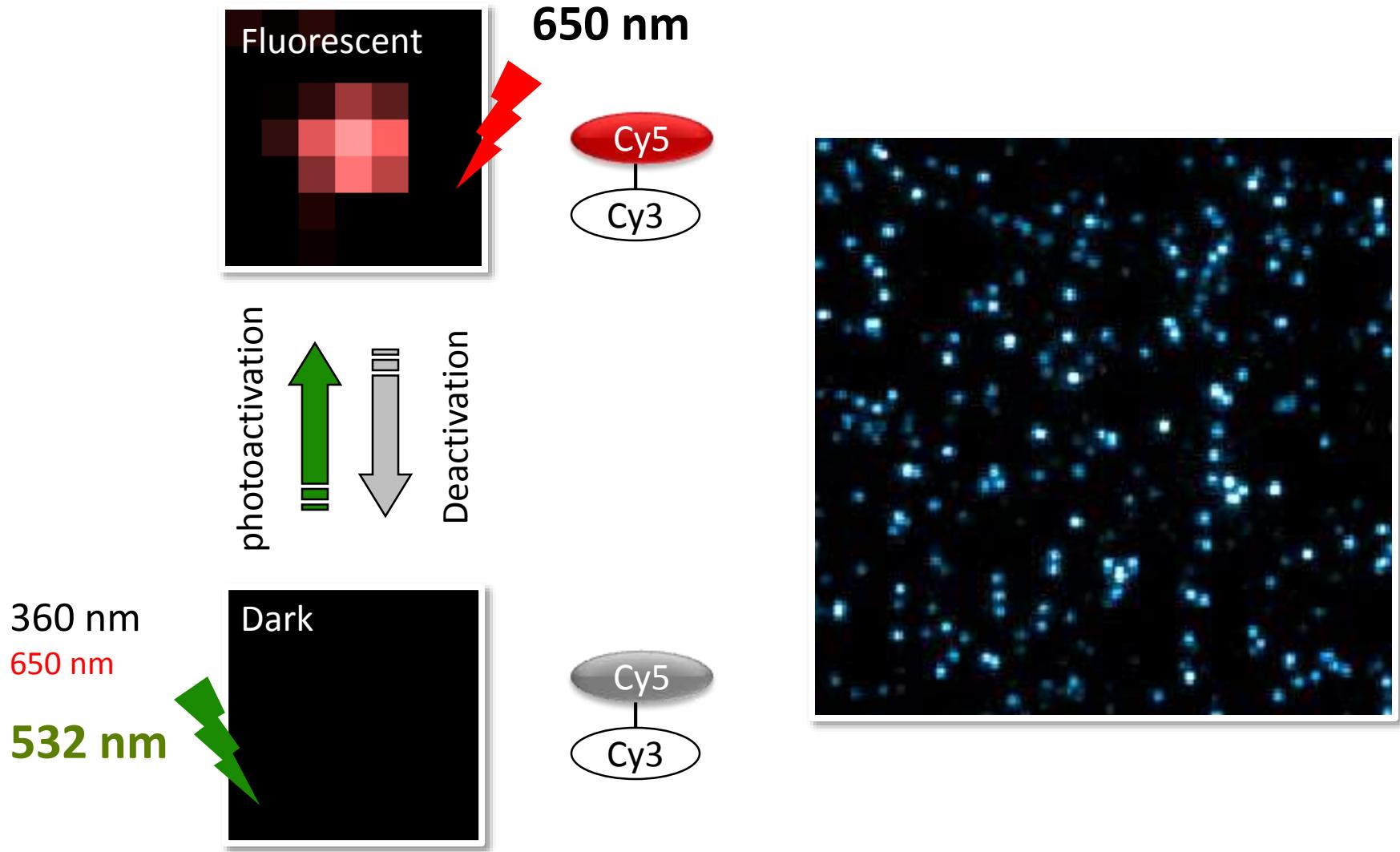
Drosophila S2 cells

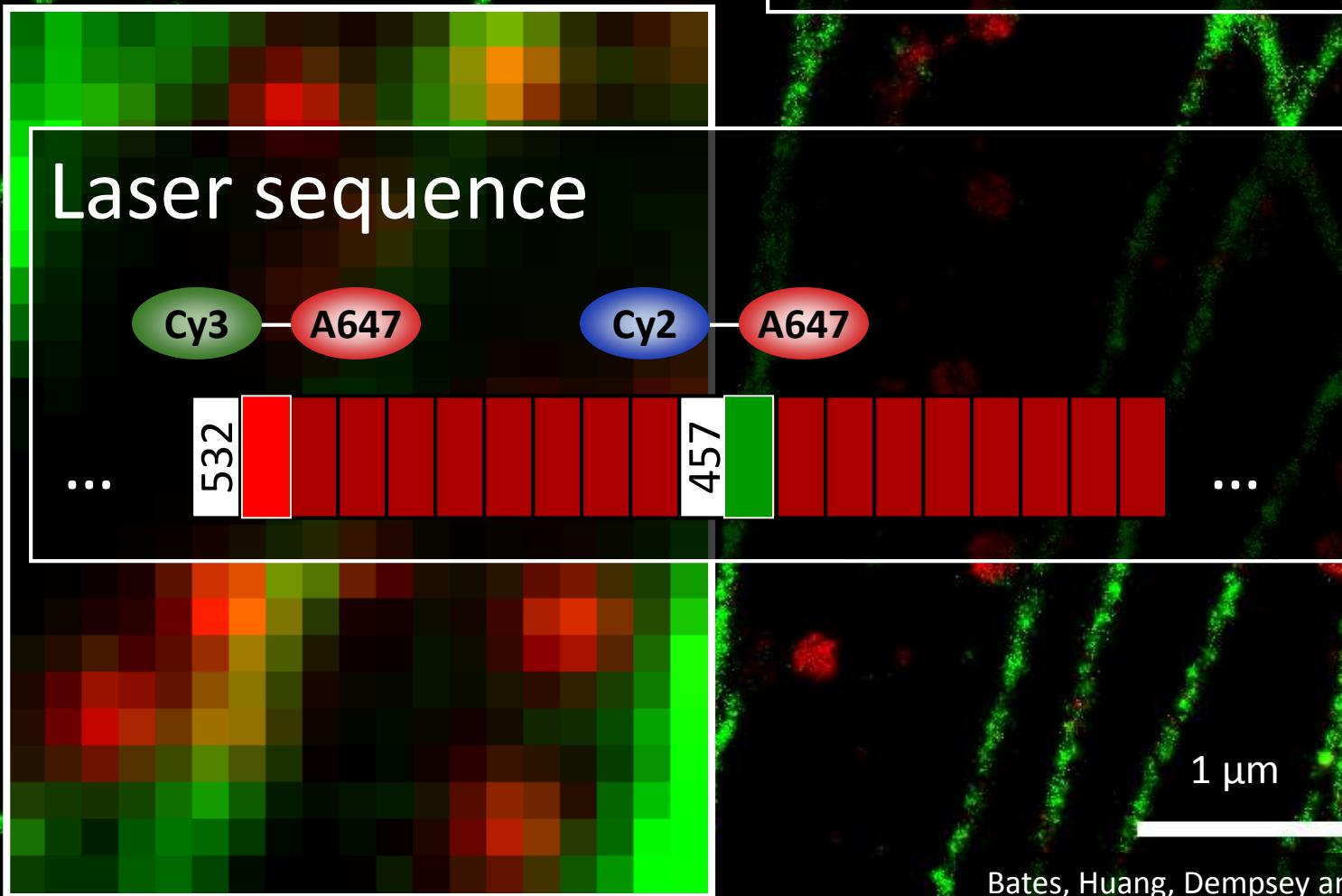


2 μ m

Daichi Kamiyama

Multicolor STORM/PALM: activation

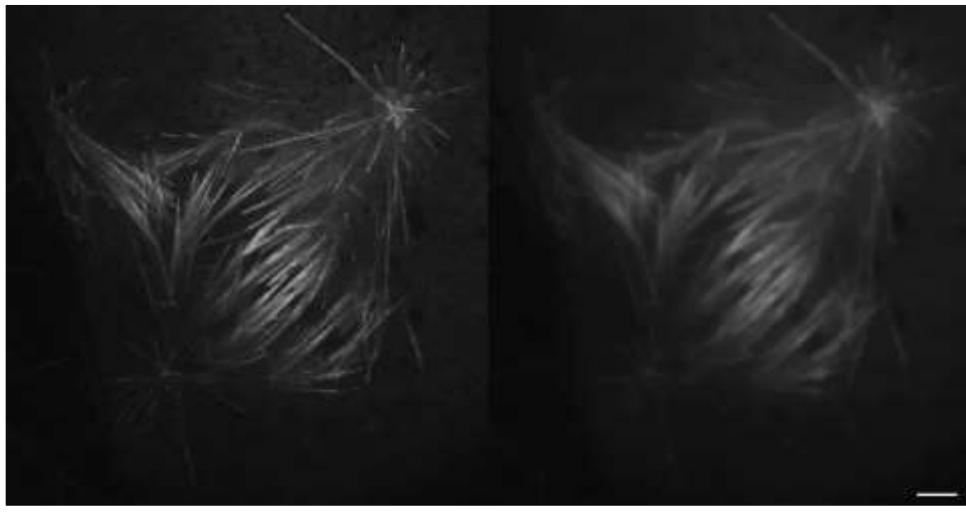




Multicolor imaging

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

Kner, Chhun et al., Nat Methods, 2009

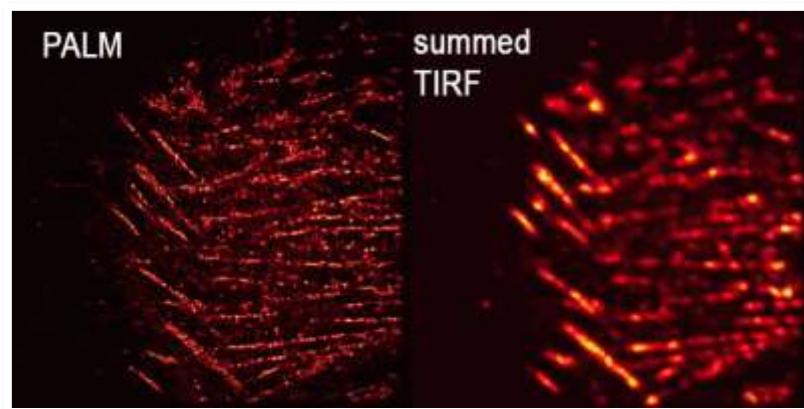
2 μm

STORM/PALM

STED



Nagerl et al., PNAS, 2008



Schroff et al., Nat Methods, 2008



The limit of “Super-Resolution”

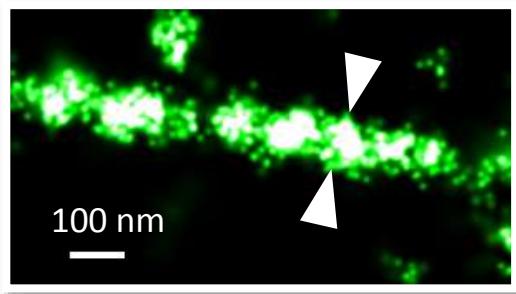
Unbound theoretical resolution

$$D = \frac{d_{\text{Diffraction}}}{S}$$

- $S \approx \sqrt{N}$
- 6,000 photons for Cy5 \rightarrow 5 nm
- 1,000,000 photos for Hydro-Cy5 \rightarrow < 1 nm
(Vaughn et al., 2012)

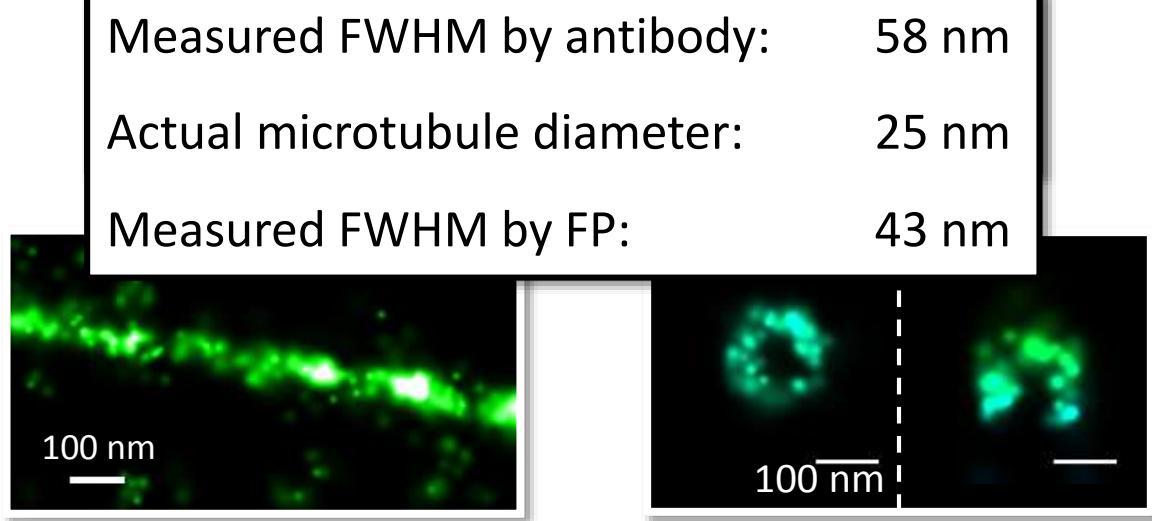
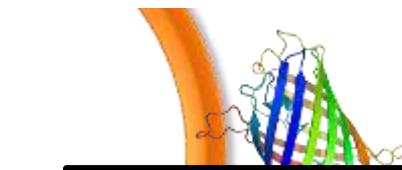
Effective resolution: Probe matters

Antibodies:
~ 10 nm



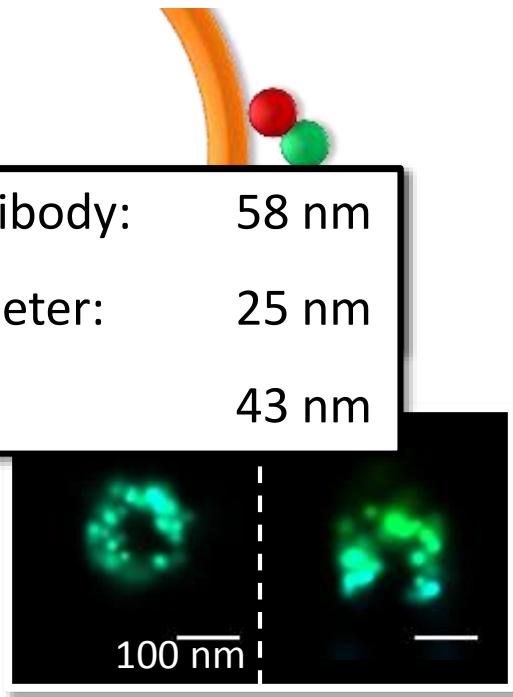
~ 6000 photons

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

Antibody immunofluorescence

- Fixed sample

Newer labeling methods

- Enzymatic tags
SNAP-tag, HALO-tag, TMP-tag, etc.
- Nanobodies
- RNA aptamers

precision

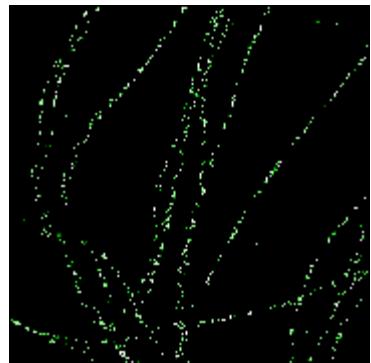
- Multicolor imaging so far challenging

- More versatile for multicolor imaging

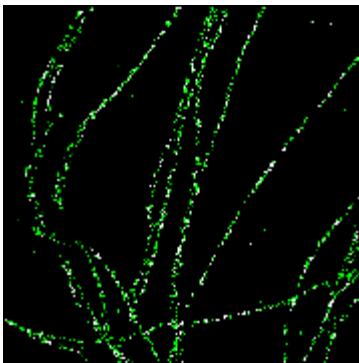
Effective resolution: Density matters

Frames for image reconstruction:

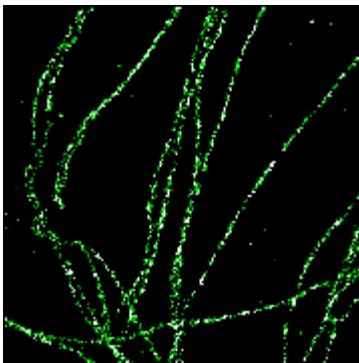
200



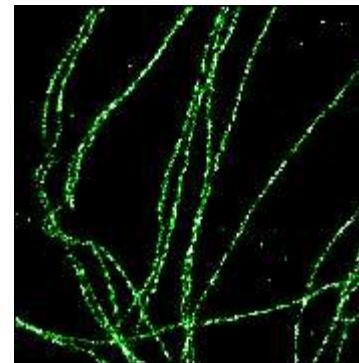
500



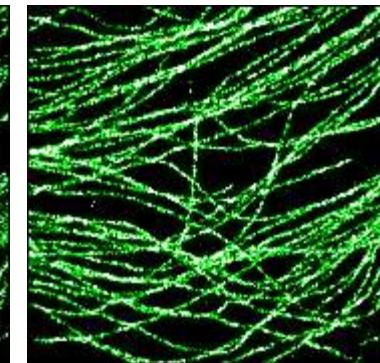
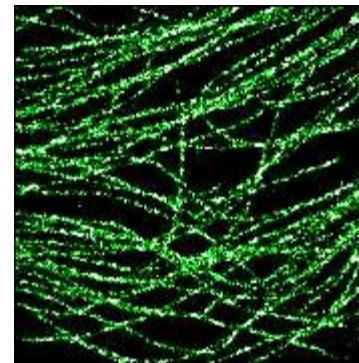
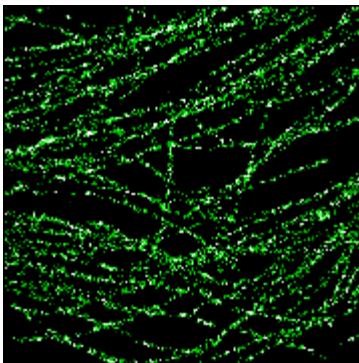
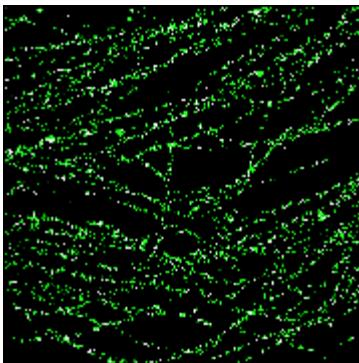
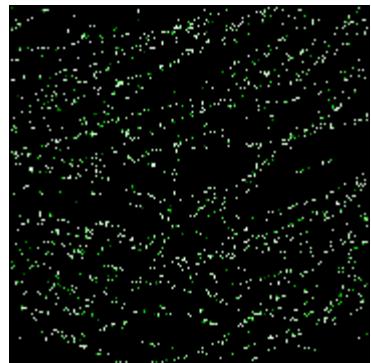
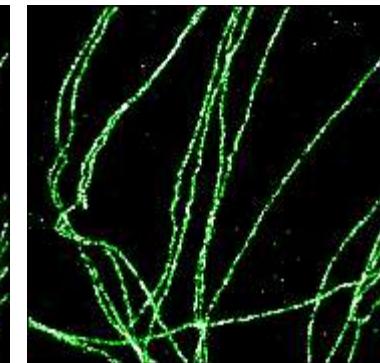
1,000



5,000



40,000



Effective resolution: Density matters

Frames for image reconstruction:

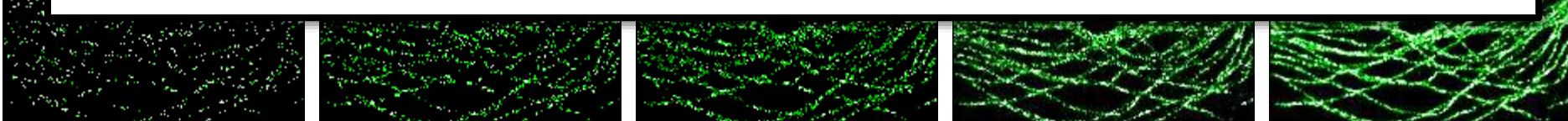
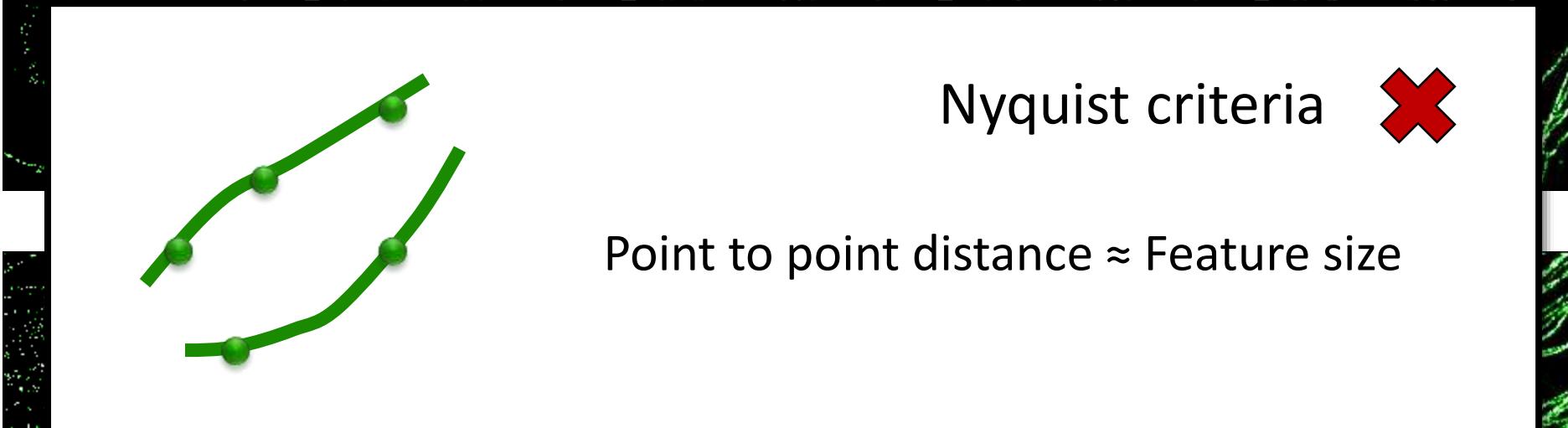
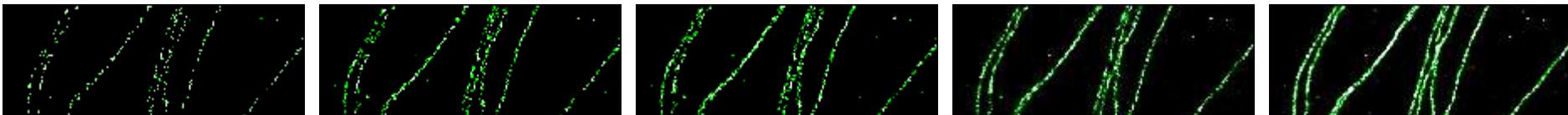
200

500

1,000

5,000

40,000



Effective resolution: Density matters

Frames for image reconstruction:

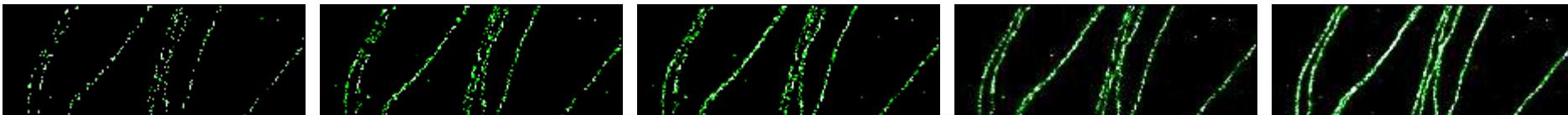
200

500

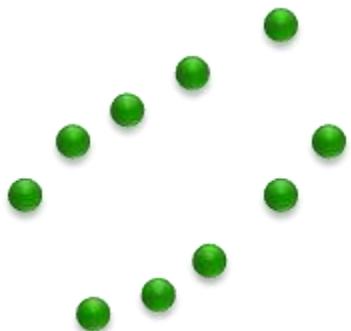
1,000

5,000

40,000



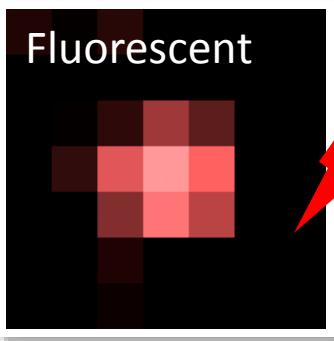
Nyquist criteria



Point to point distance < $\frac{1}{2}$ Feature size

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

Effective resolution: Contrast matters

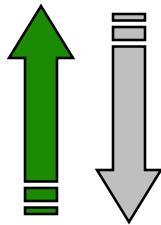


650 nm

e.g. 1%

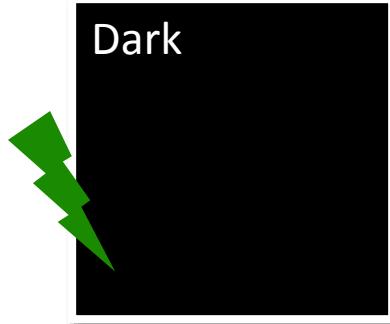


photoactivation



Deactivation

650 nm



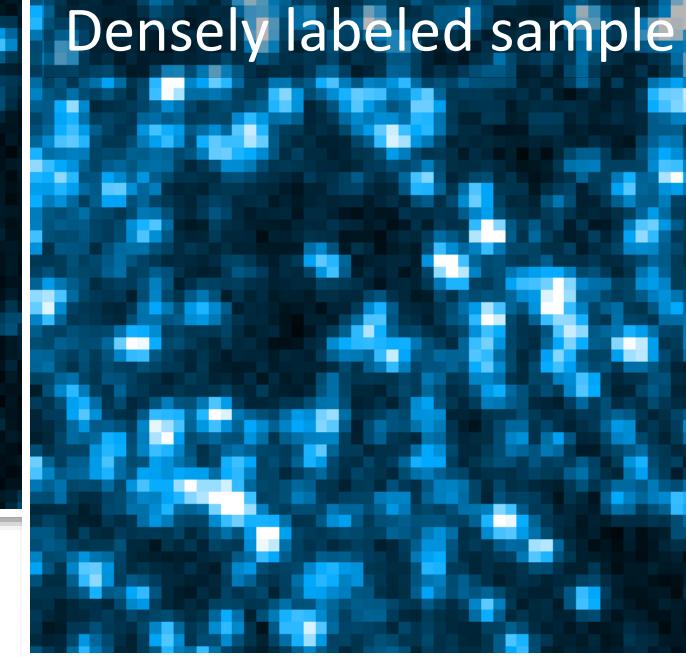
e.g. 99%



1% means...

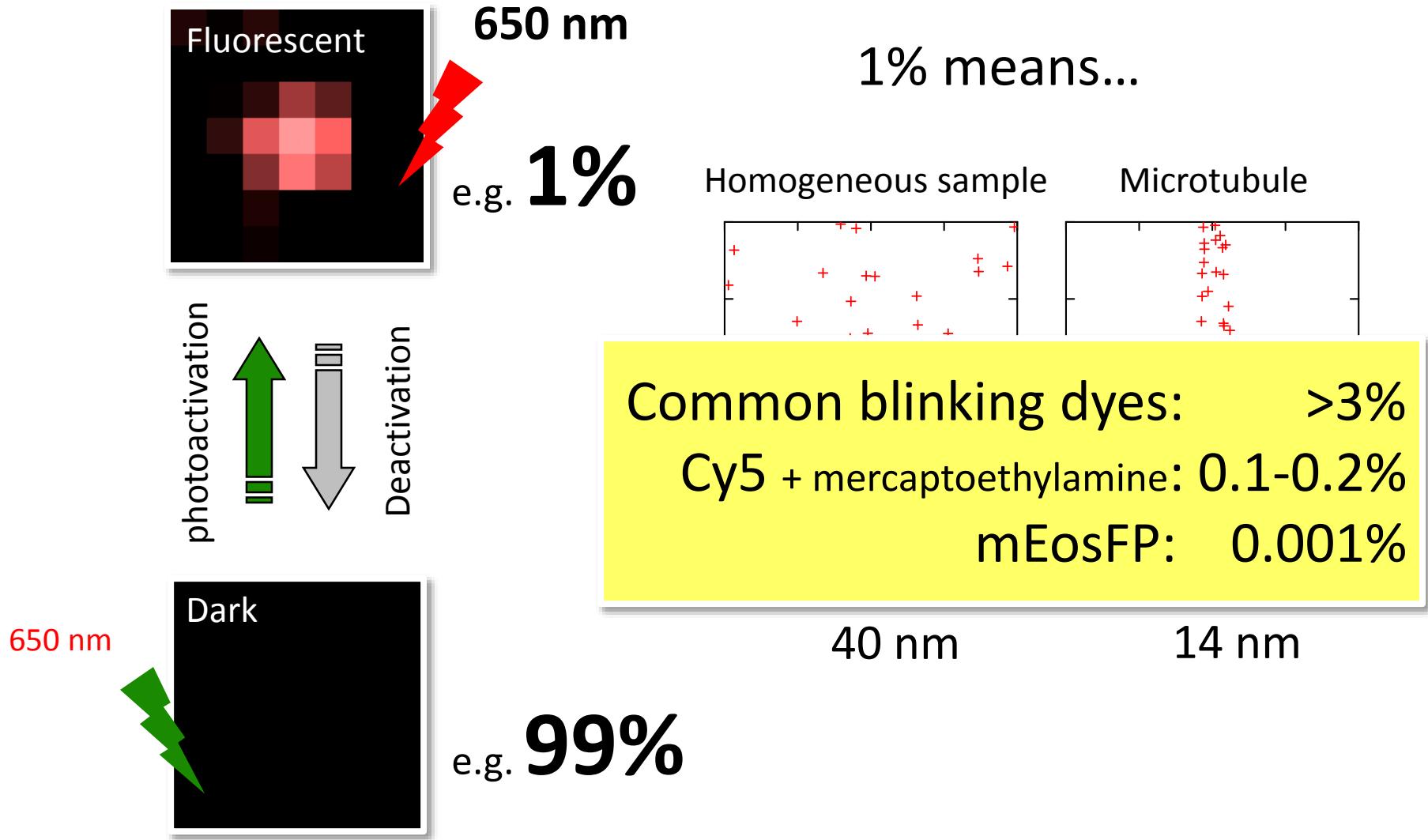


Sparsely labeled sample



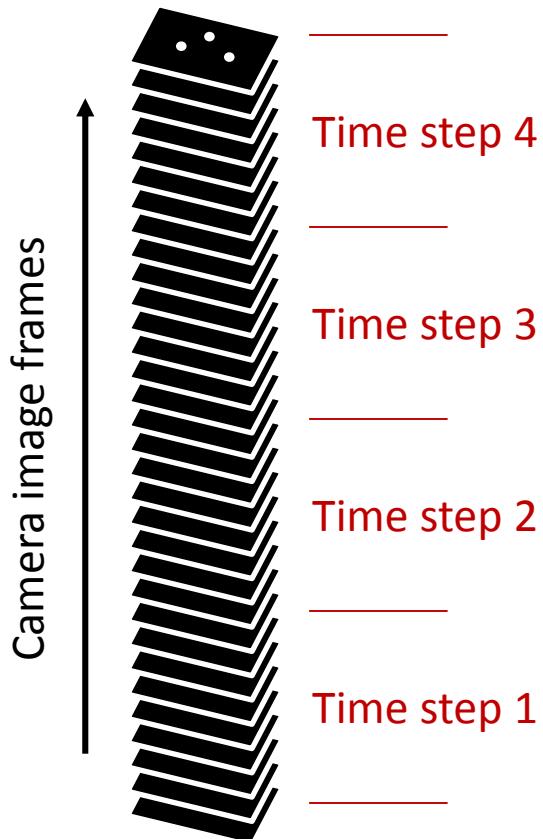
Densely labeled sample

Effective resolution: Contrast matters

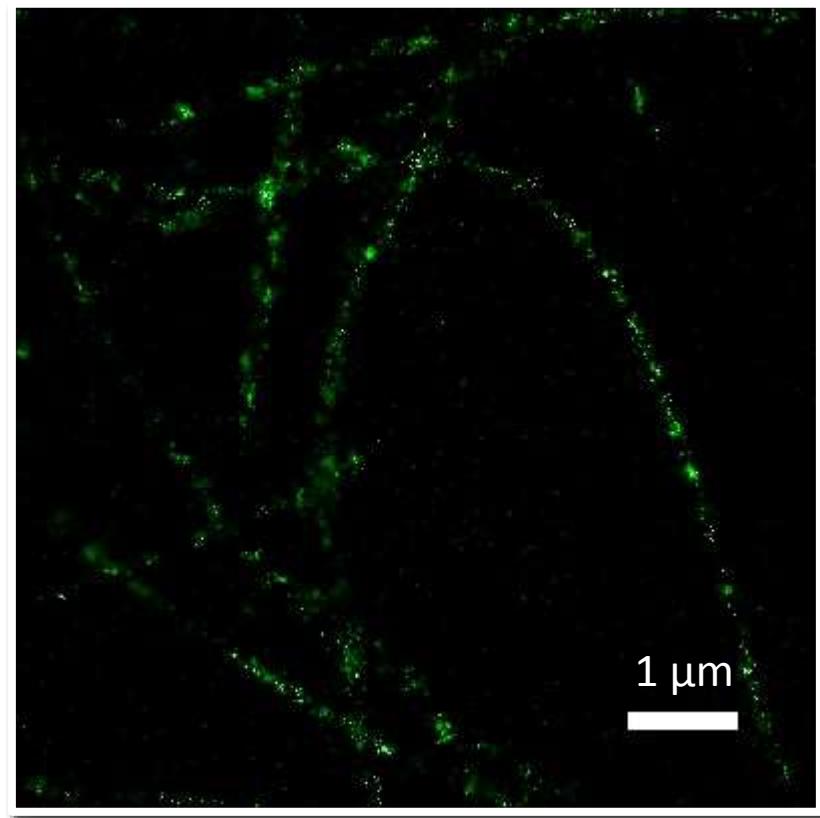


Live Cell STORM/PALM

Live cell STORM/PALM



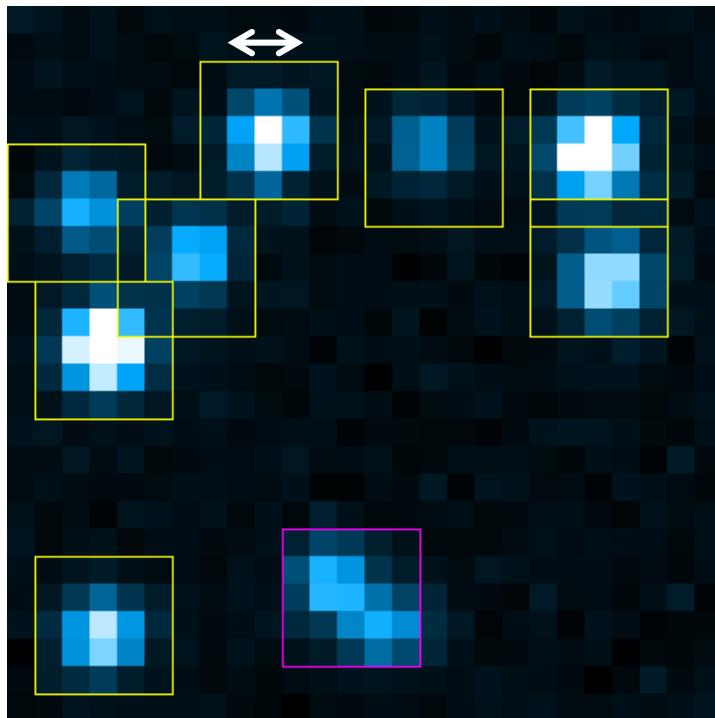
mEos2 labeled microtubule in live S2 cells



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

Spatial-temporal resolution trade-off

FWHM \approx 320 nm



0.68 molecules per μm^2

Assuming:

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



$1/10$ occupancy $\equiv 0.4$ point per $\mu\text{m}^2 \cdot \text{frame}$



70 nm resolution $\equiv 800$ per $\mu\text{m}^2 \equiv 2000$ frames



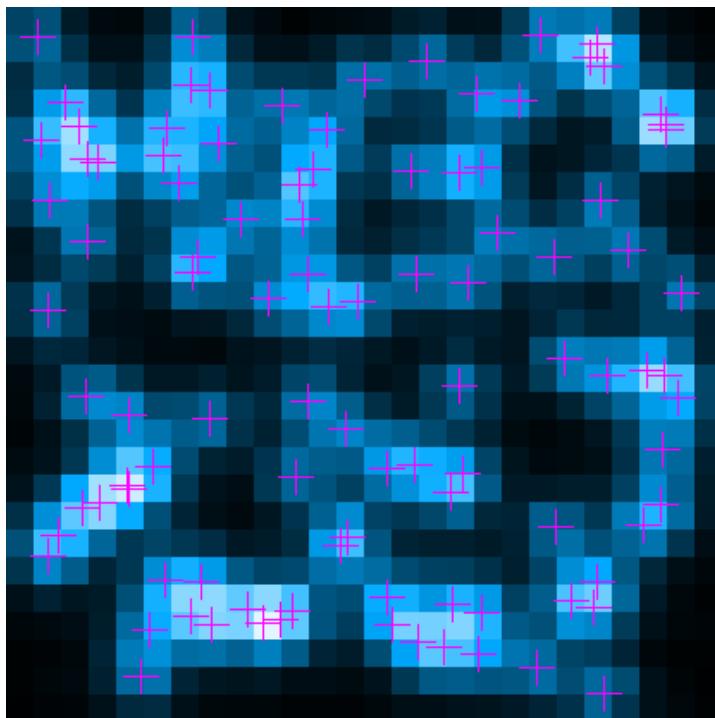
100 fps = 20 sec time resolution



1000 ~ 3000 fps

Spatial-temporal resolution trade-off

FWHM \approx 320 nm



6.8 molecules per μm^2

Assuming:

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

8 per μm^2

$1/10$ occupancy \equiv 0.4 point per $\mu\text{m}^2 \cdot \text{frame}$

$70 \text{ nm resolution} \equiv 800 \text{ per } \mu\text{m}^2 \equiv 2000 \text{ frames}$

\downarrow
100 fps = 20 sec time resolution

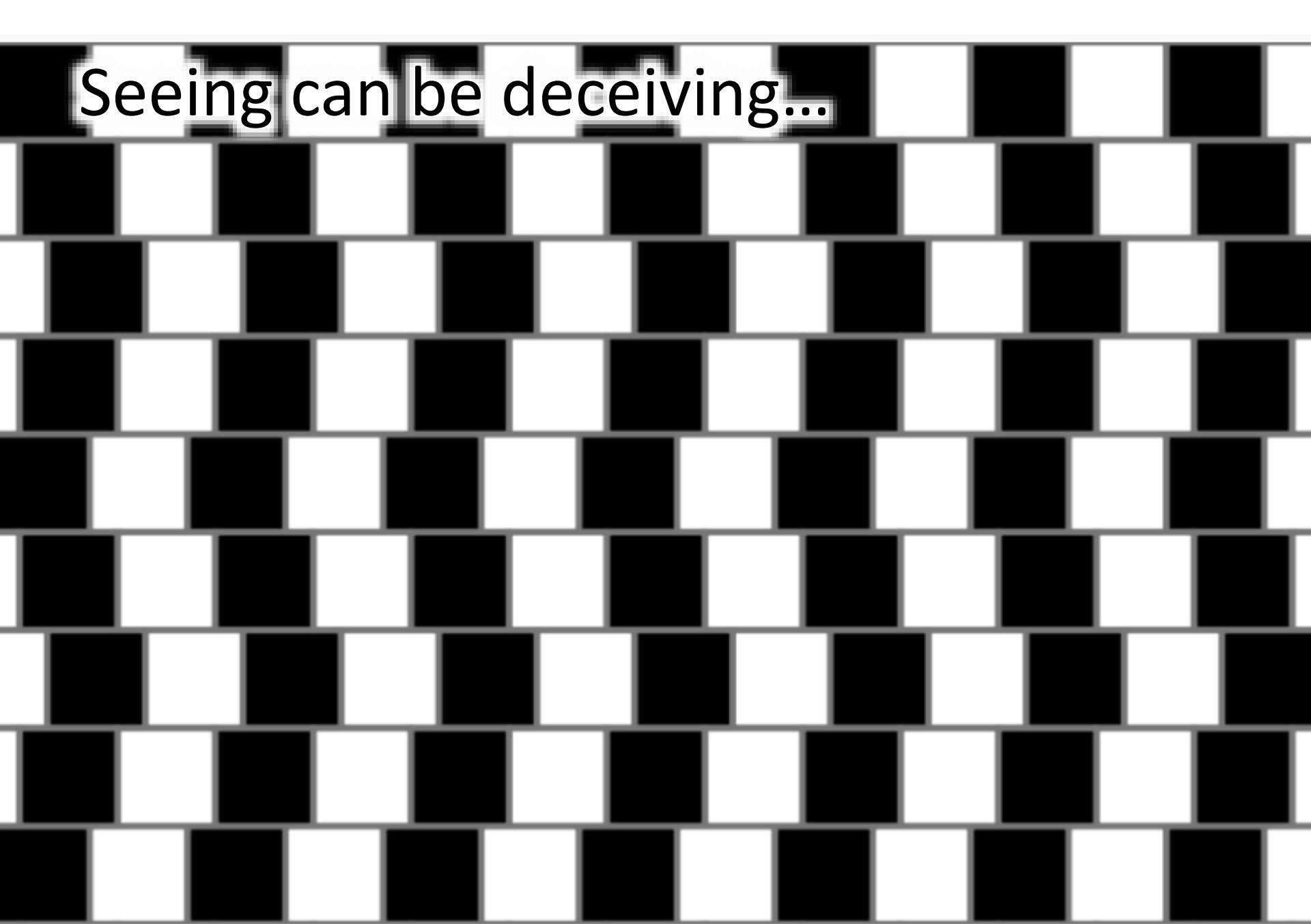
Comparison of time resolution

2D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	9 frames (0.09 sec)
STED	Scanning	60 nm	$1 \times 2 \mu\text{m}$: 0.03 sec $10 \times 20 \mu\text{m}$: 3 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec)

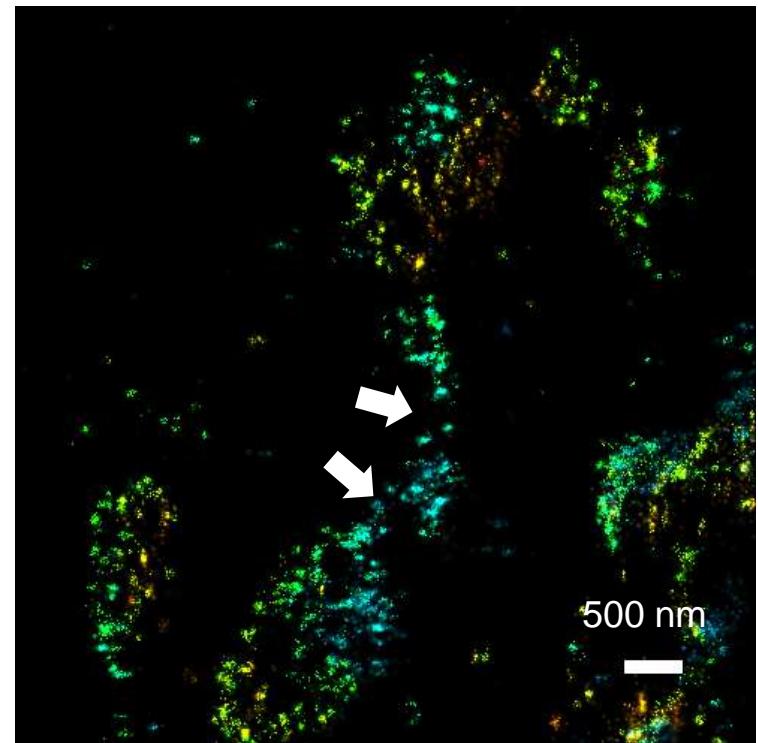
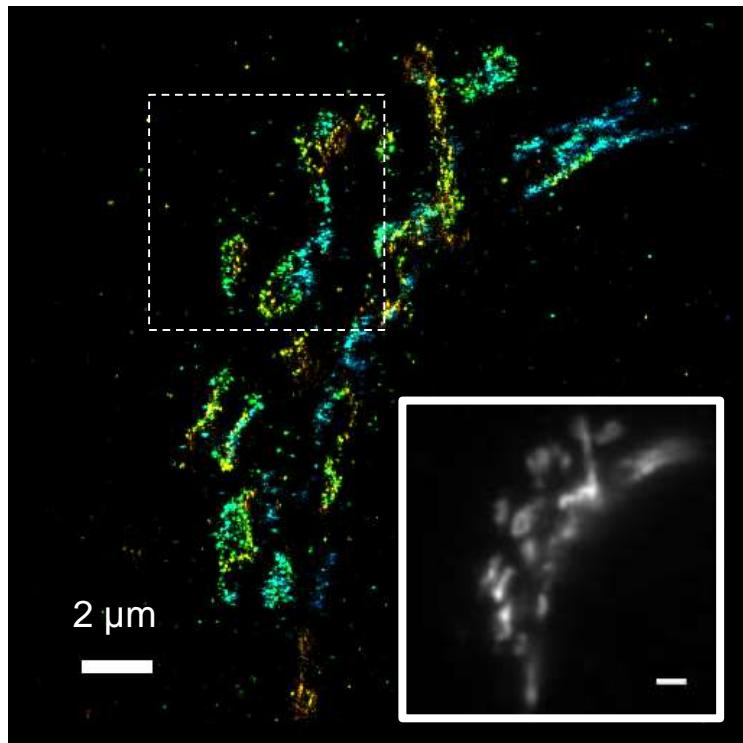
3D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	15 frames x 10 (1.5 sec)
STED	Scanning	60 nm	$1 \times 2 \times 0.6 \mu\text{m}$: 0.6 sec $10 \times 20 \times 0.6 \mu\text{m}$: 60 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec) – no scan!

Multipoint scanning RESOLFT...

Seeing can be deceiving...

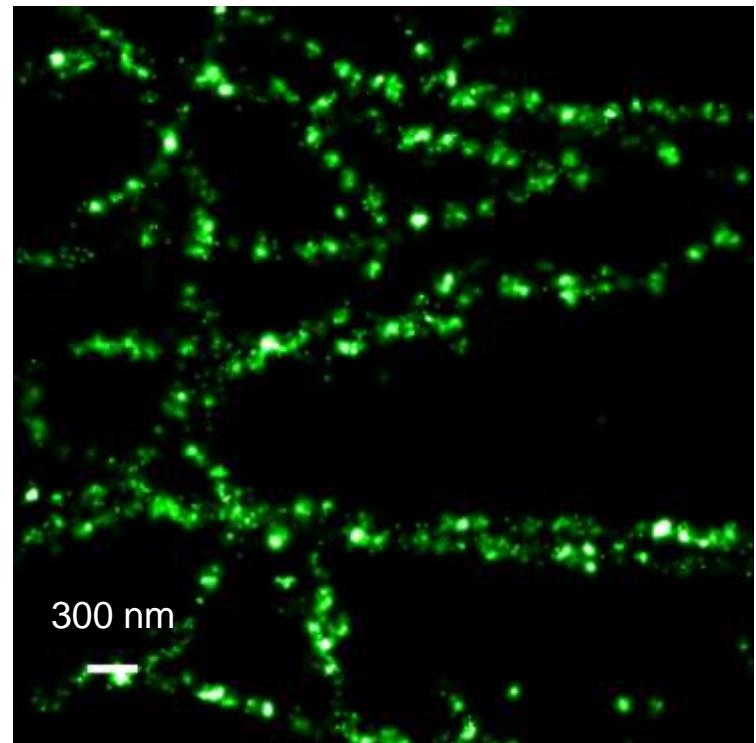
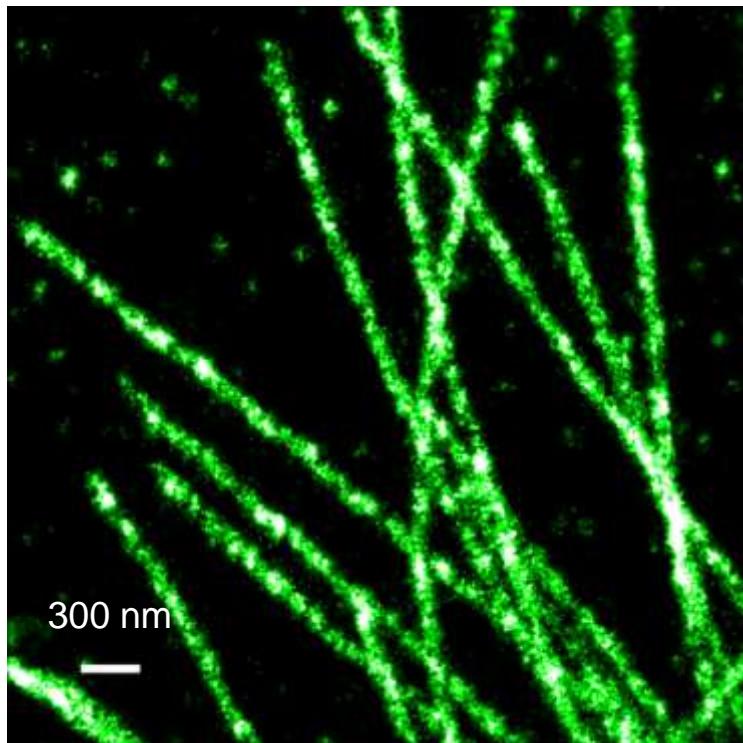


Super-resolved artifacts: sparse labeling



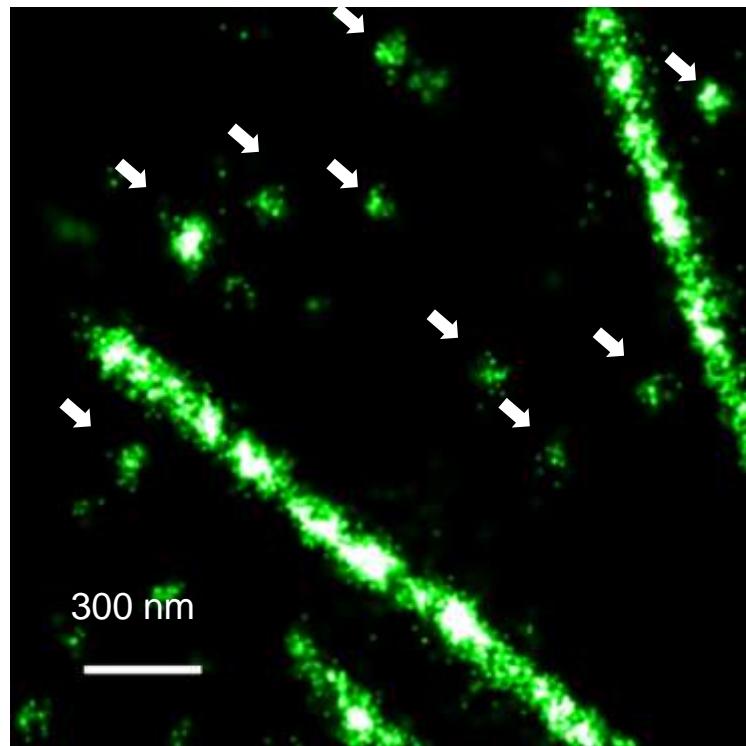
Golgi: Giantin immunofluorescence

Super-resolved artifacts: poor fixation

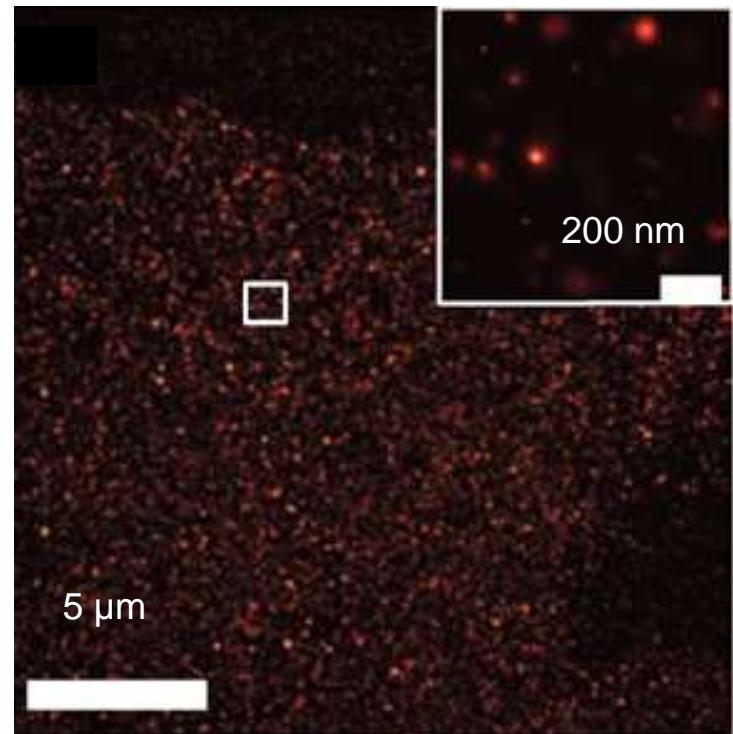


Microtubules: good and bad fixation

Super-resolved artifacts: clustering

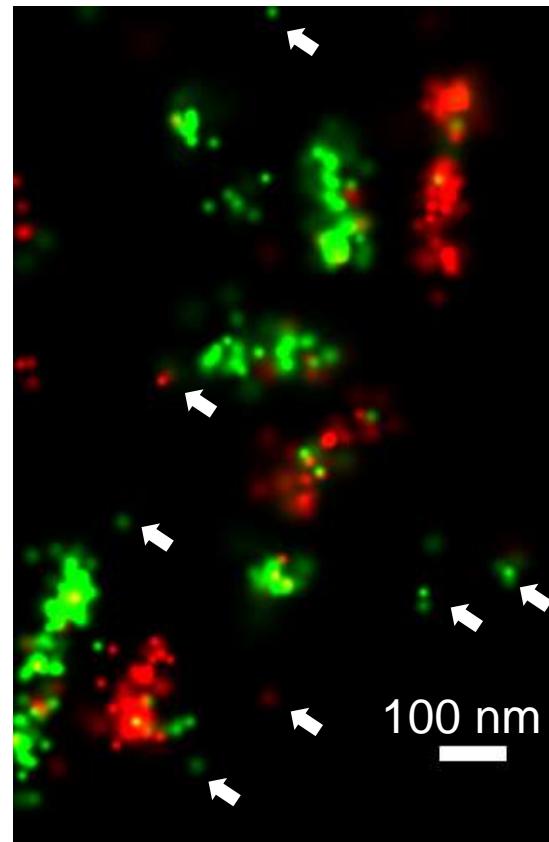
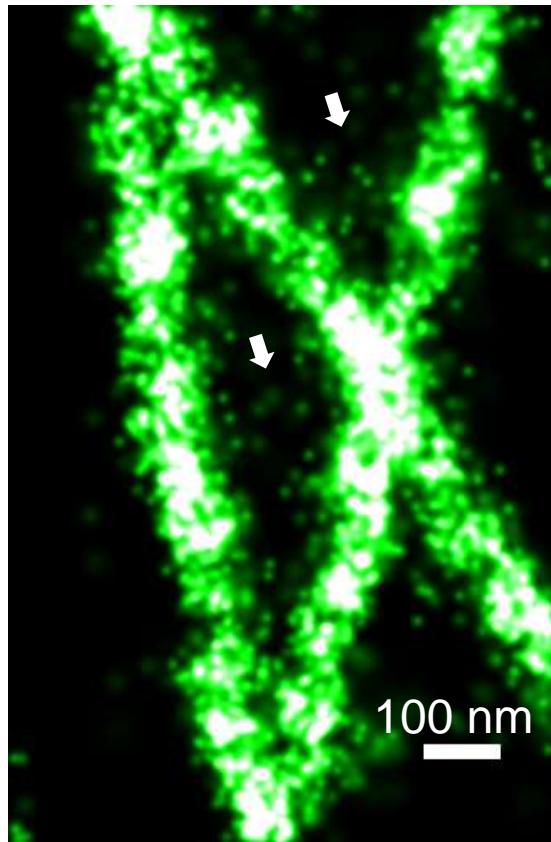


Clusters from single antibodies



Clusters from blinking FPs

Super-resolved artifacts: “noise” points



Noise from misidentified molecules, crosstalk and background

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).
- 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).
- D. Kamiyama, B. Huang, "Development in the STORM", *Developmental Cell*, 23, 1013 (2012).
- 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).