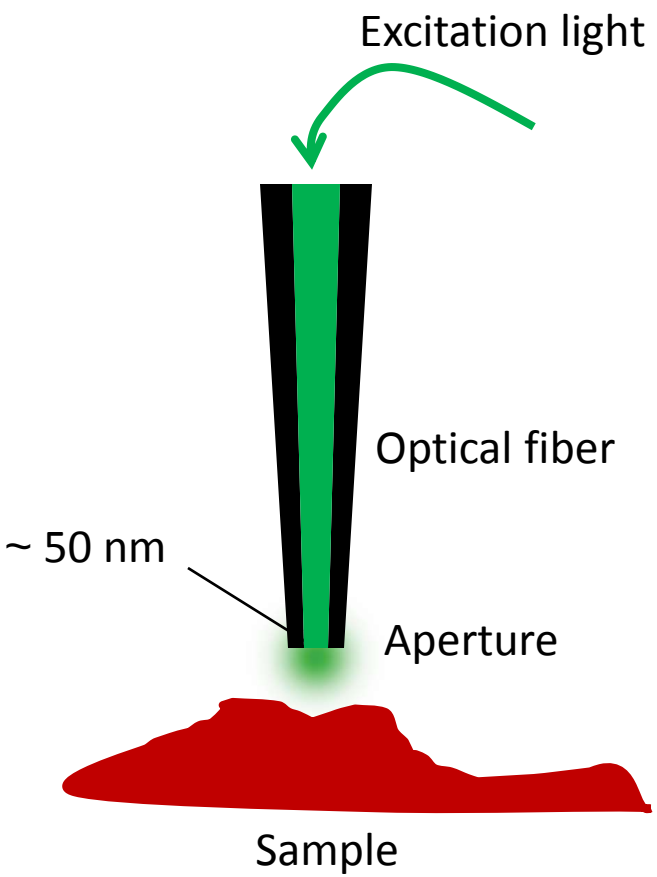
Bo Huang 2013.08.01

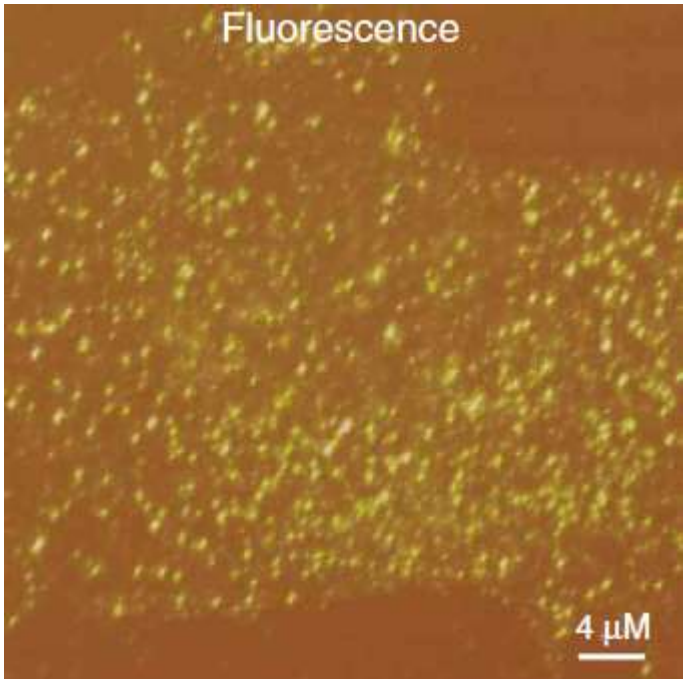
50 years to extend the resolution

- 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



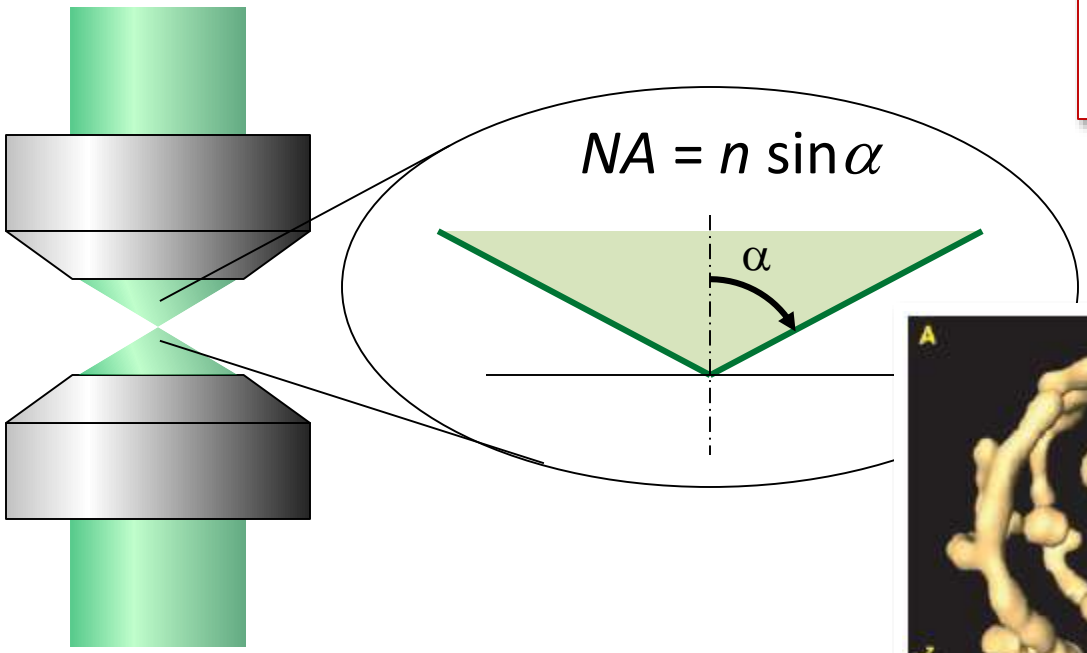
β_2 adrenergic receptor clusters on the plasma membrane

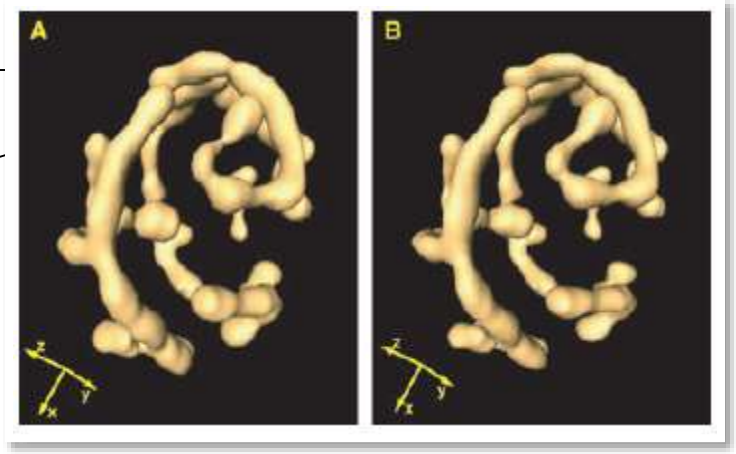


Ianoul et al., 2005

4-Pi / I^{5}M

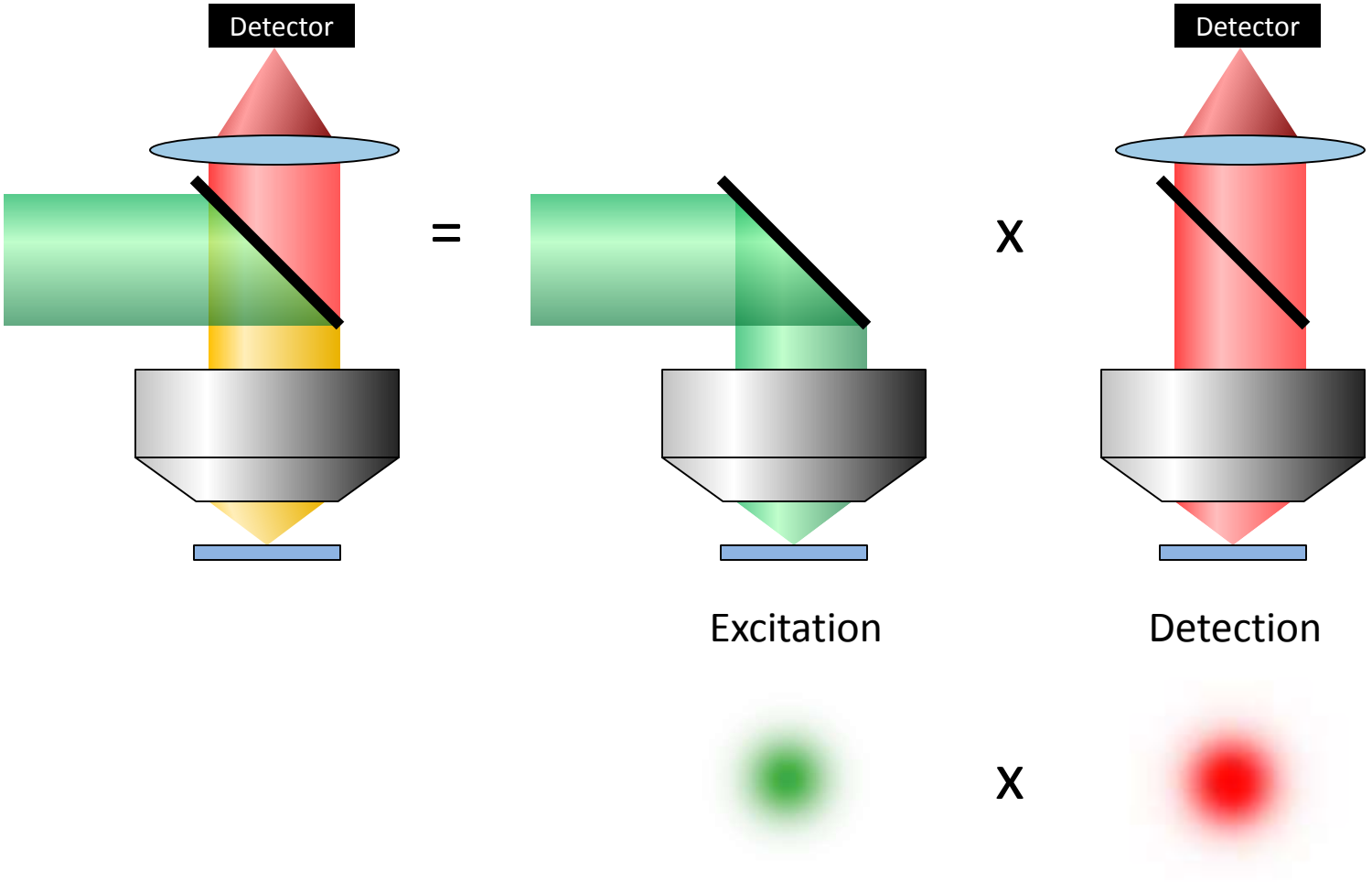
$$d \approx \frac{\lambda}{2\,NA}$$



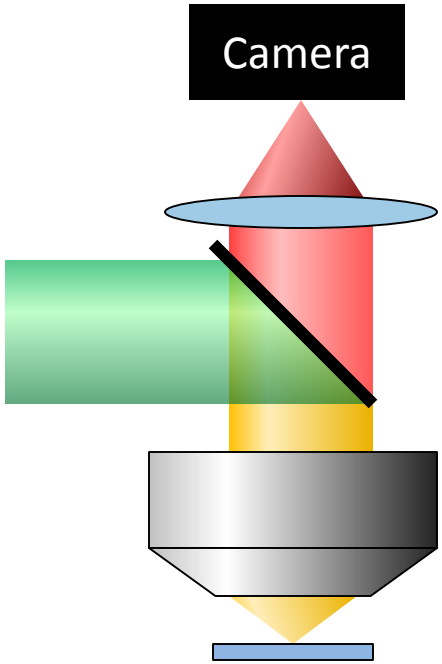


Major advantage:
Similar z resolution as x-y resolution

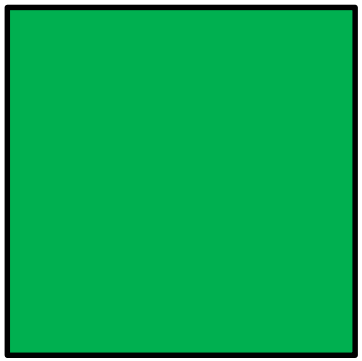
Patterned illumination



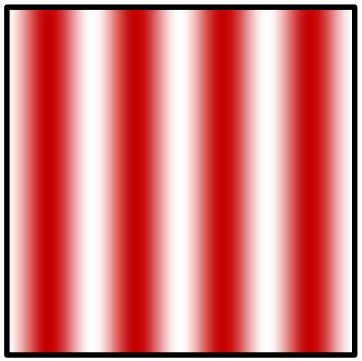
Structured Illumination Microscopy (SIM)



Wide field illumination



Diffraction-limited detection



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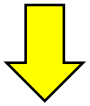
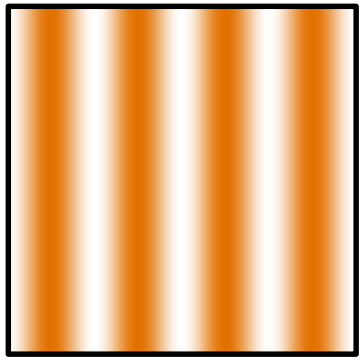
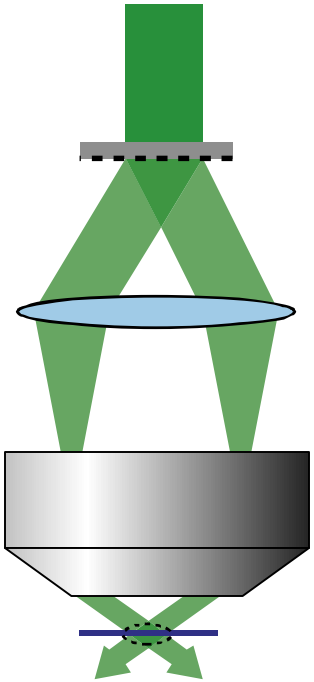


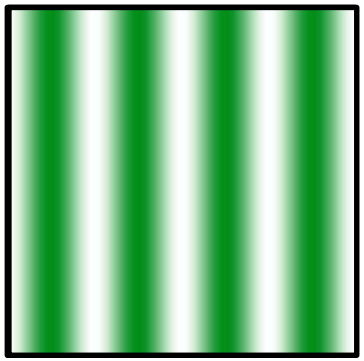
Diffraction-limited image



Structured Illumination Microscopy (SIM)

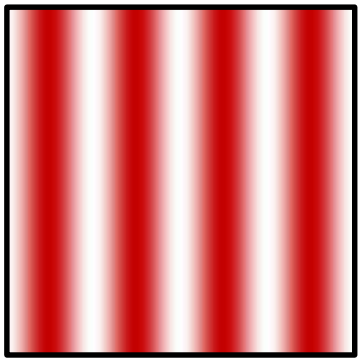


Structured illumination

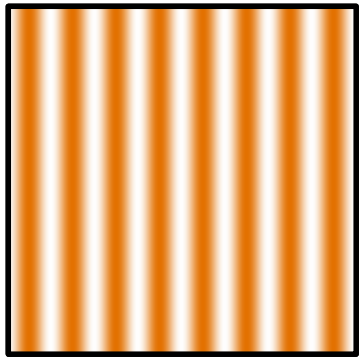


×

Diffraction-limited detection

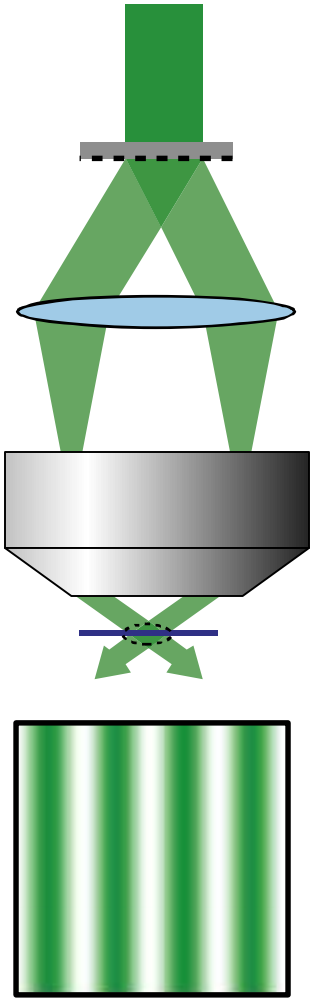


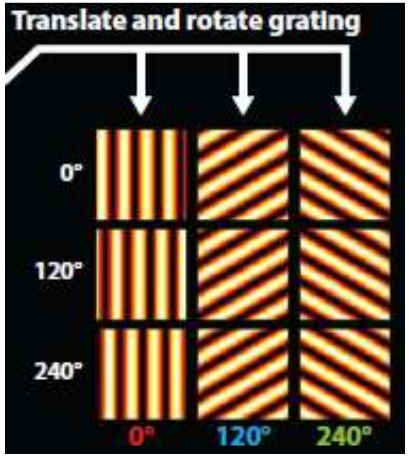
Diffraction-limited image



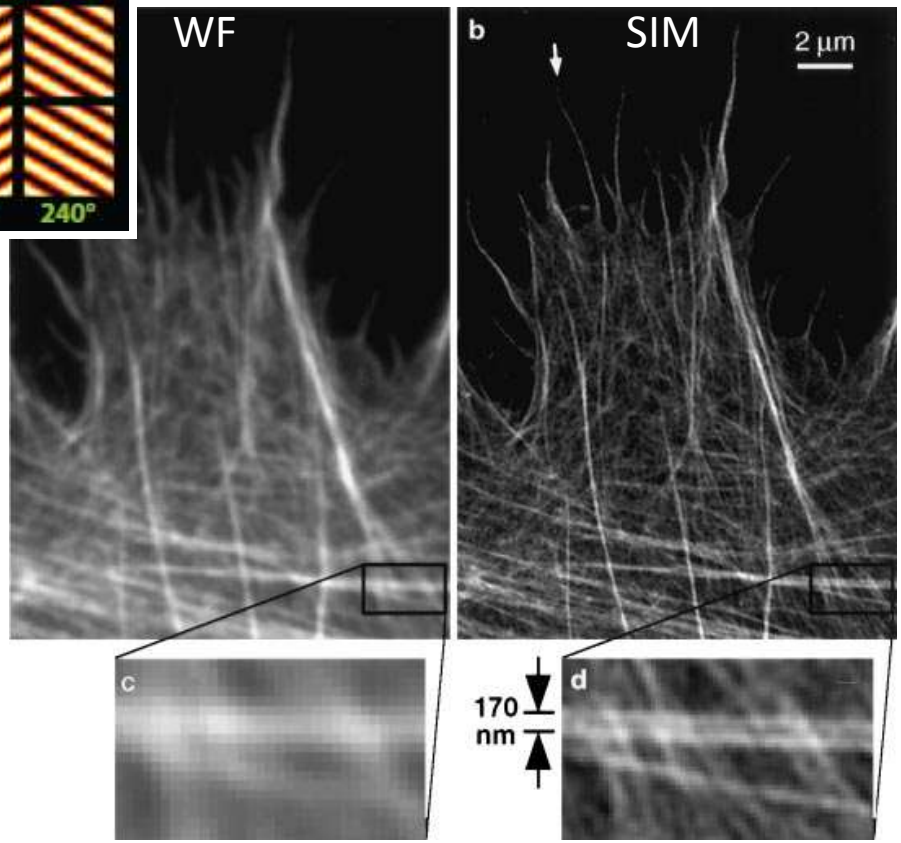
Structured Illumination Microscopy (SIM)

Multiple angles and phases





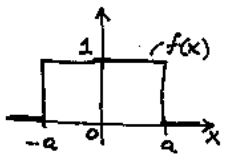
Reconstruction



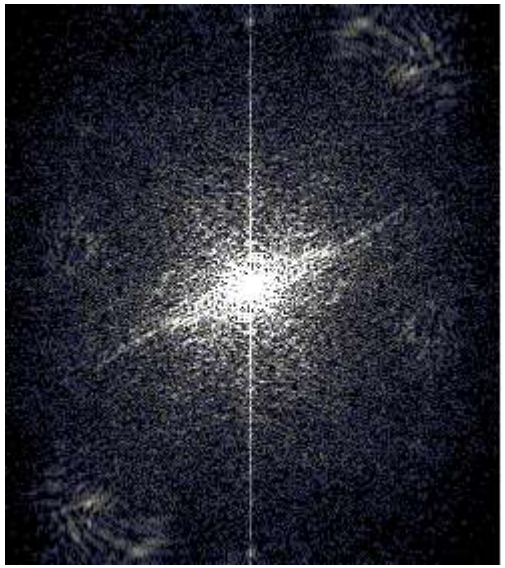
Gustafsson, J Microscopy 2000

Being (slightly) more rigorous about SIM

Fourier transform: Examples

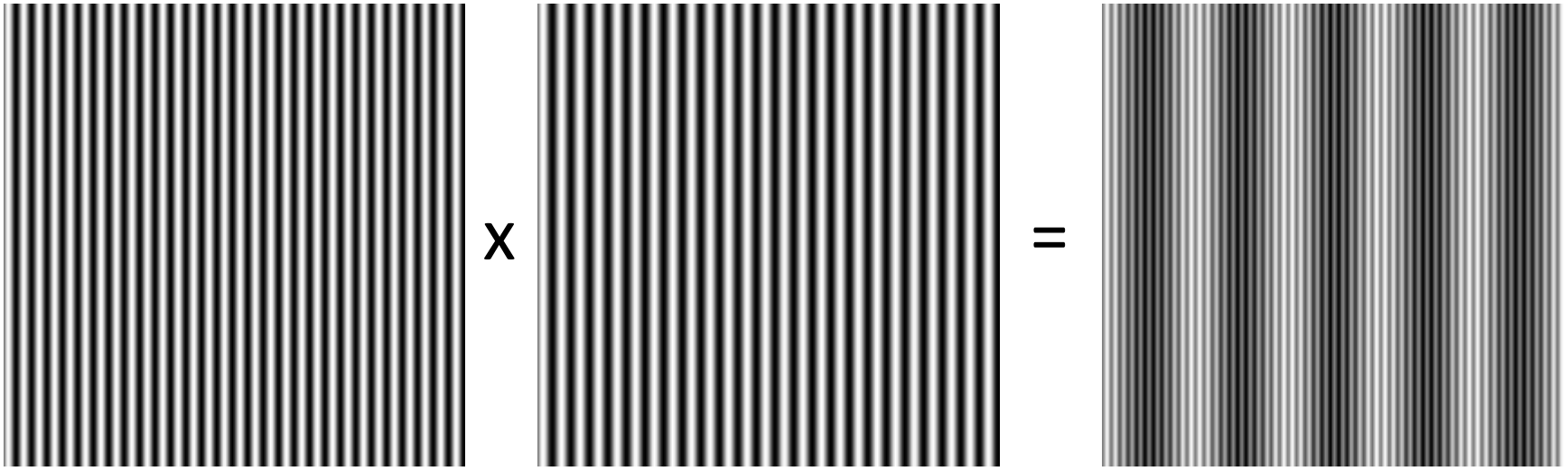
- $f(x) = \delta(x) \;\rightarrow\; \tilde{f}(k) = 1$
- $f(x) = 1 \;\rightarrow\; \tilde{f}(k) = 2\pi\,\delta(k)$
- $f(x) = e^{-a|x|} \;\rightarrow\; \tilde{f}(k) = \frac{2a}{k^2 + a^2}$
- $f(x) = \frac{1}{x^2 + a^2} \;\rightarrow\; \tilde{f}(k) = \frac{\pi}{a} e^{-a|k|}$
- $f(x) = e^{-a\frac{x^2}{2}} \;\rightarrow\; \tilde{f}(k) = \sqrt{\frac{2\pi}{a}}\, e^{-\frac{1}{a}\cdot\frac{k^2}{2}}$
- 

$\rightarrow\; \tilde{f}(k) = 2\,\frac{\sin(ak)}{k}$



Extending the measurable freq. range

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

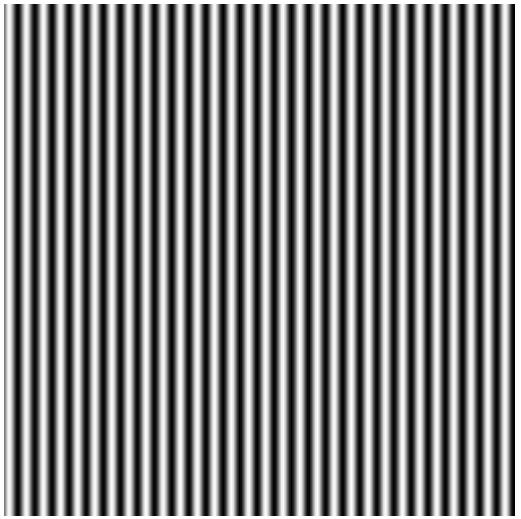


Freq = 30 Freq = 25 Freq = 55 & 5

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

Extending the measurable freq. range

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

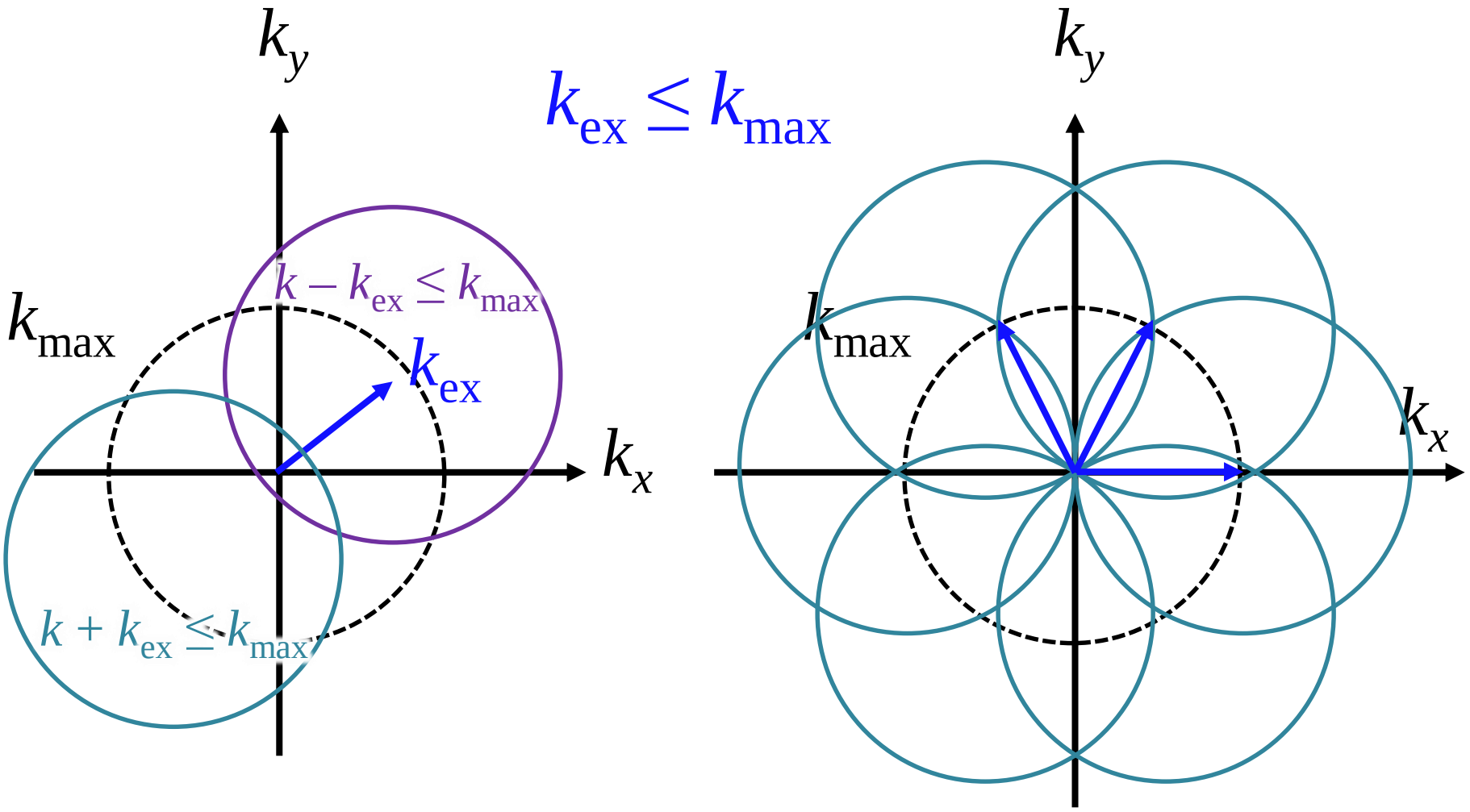


Freq = 30

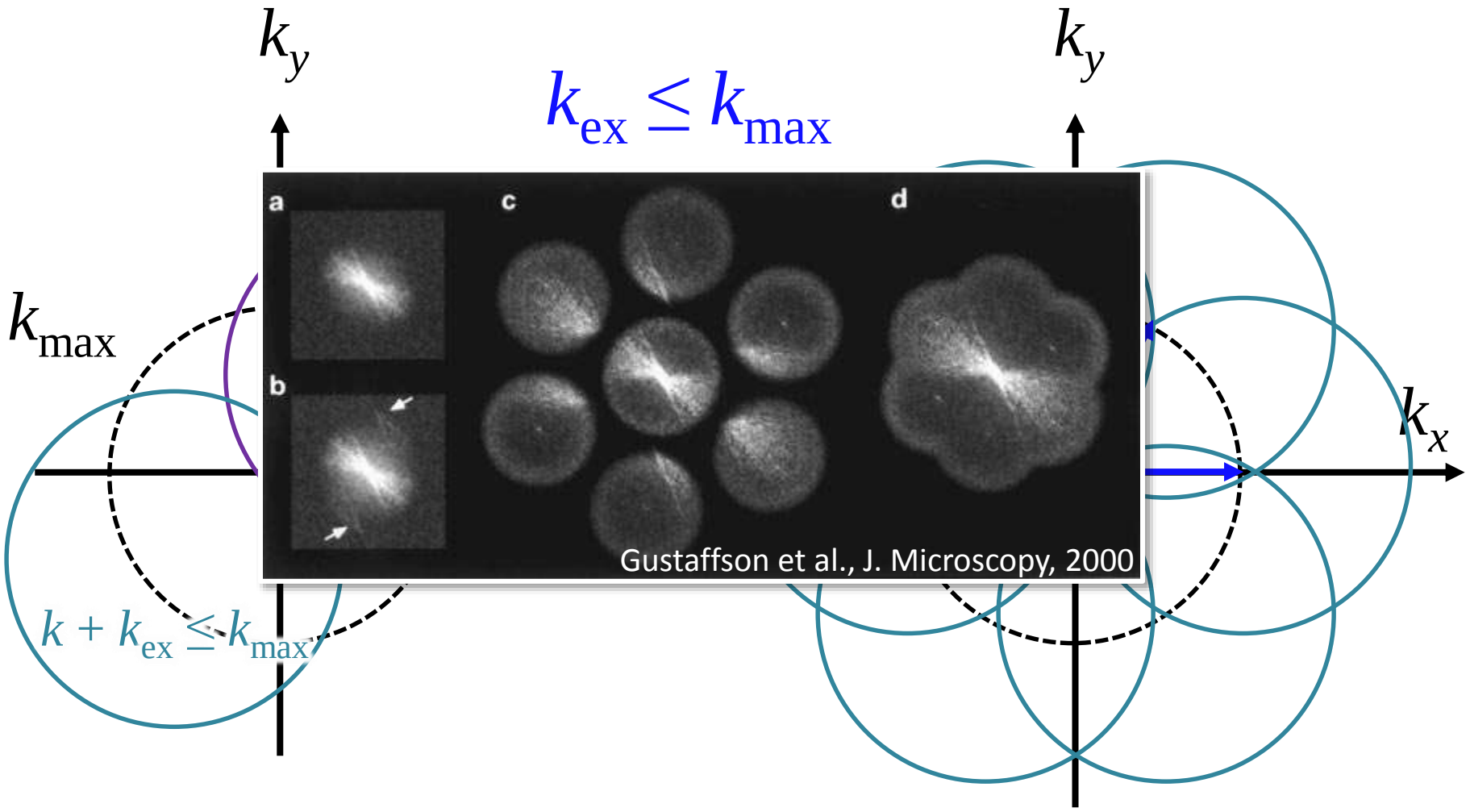
x

$\sin A \cdot \sin$

Extending the measurable freq. range

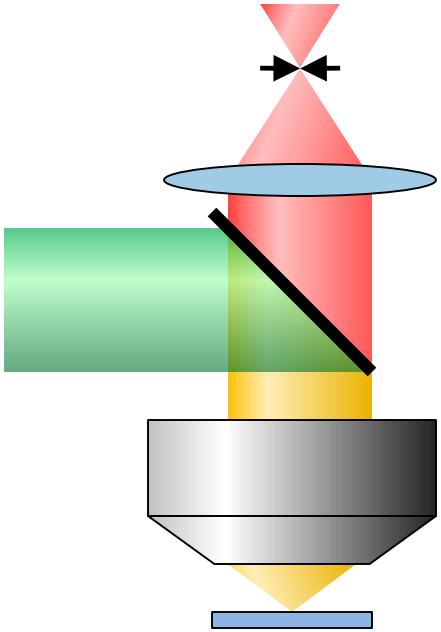


Extending the measurable freq. range

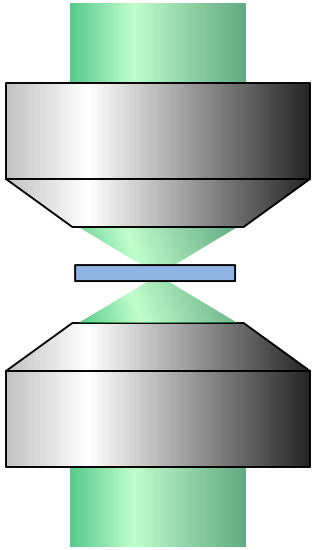


The diffraction limit still exists

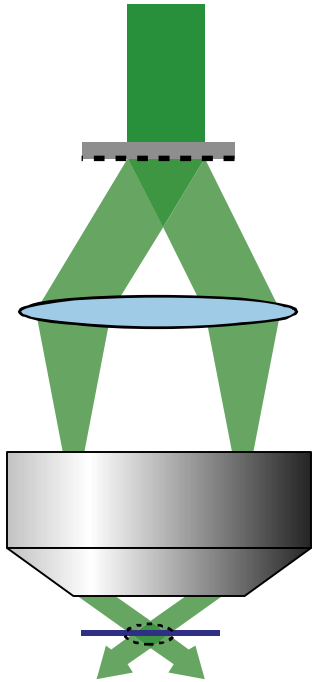
$$d \geq \frac{1}{2} \cdot \frac{\lambda}{2NA}$$



Confocal



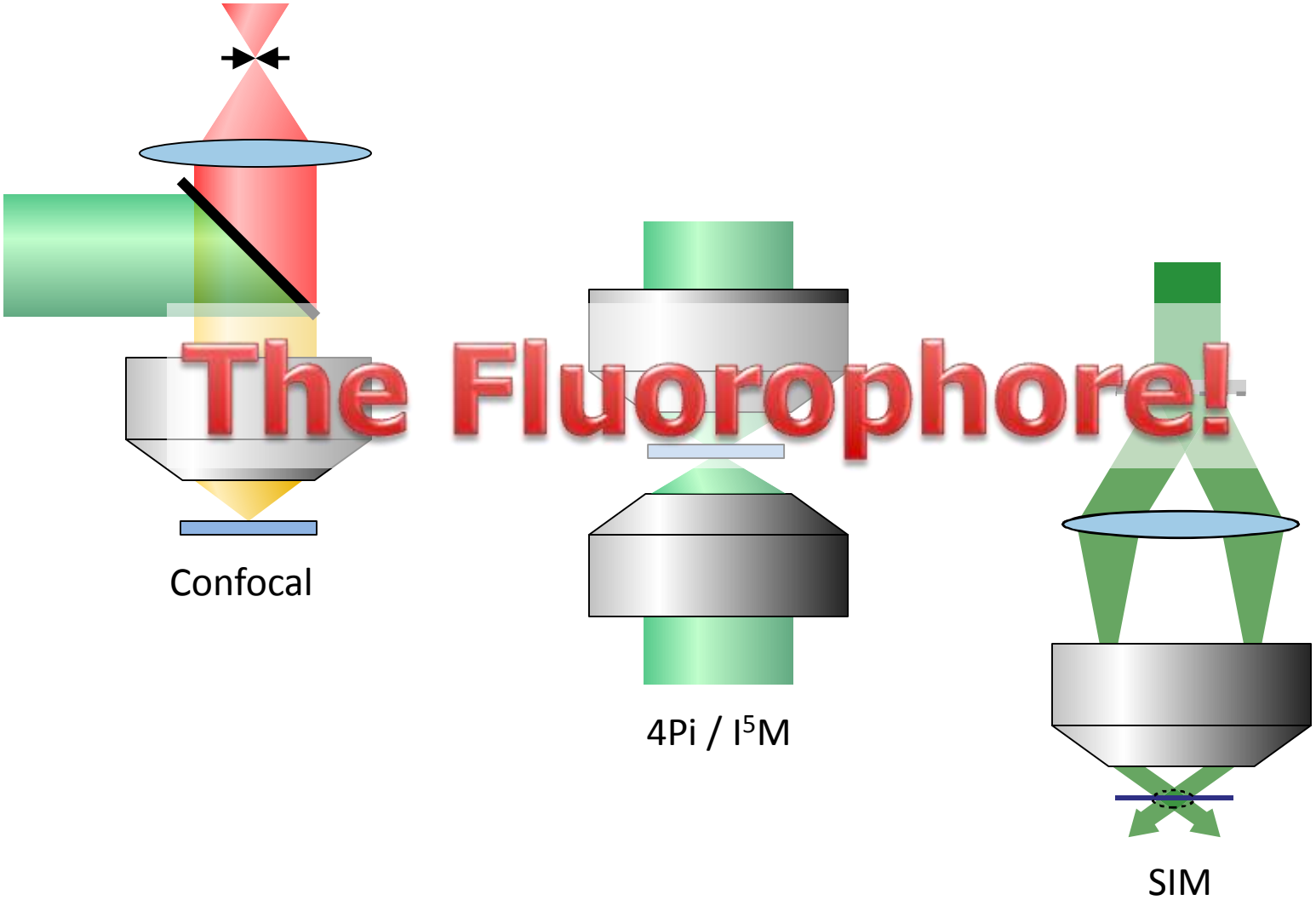
4Pi / I^{5}M



SIM

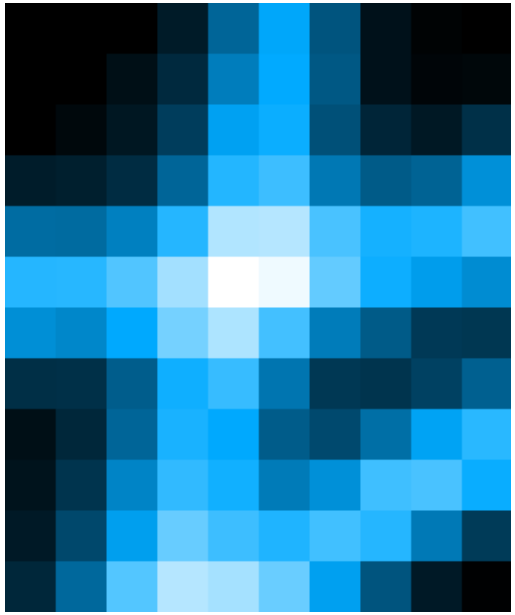
Breaking the diffraction barrier

Breaking the diffraction barrier

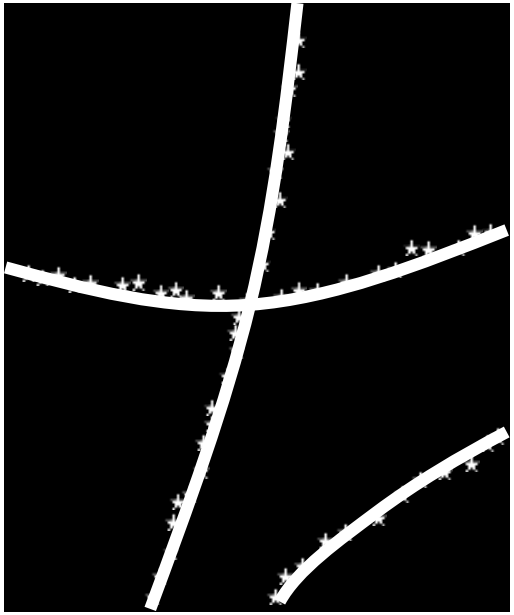


Super-resolution by…

Fluorescence image

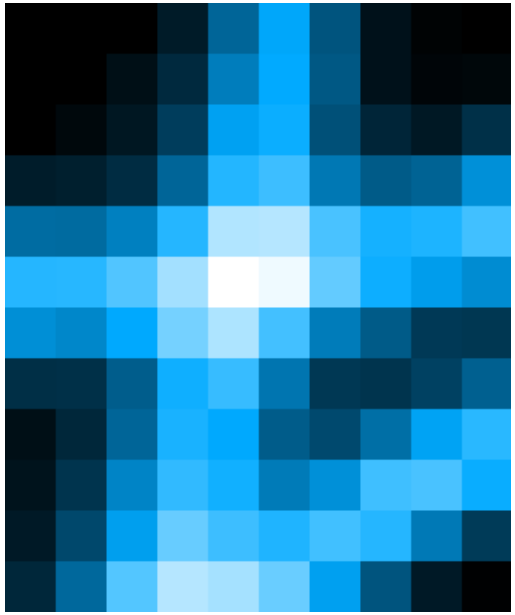


Underlying structure

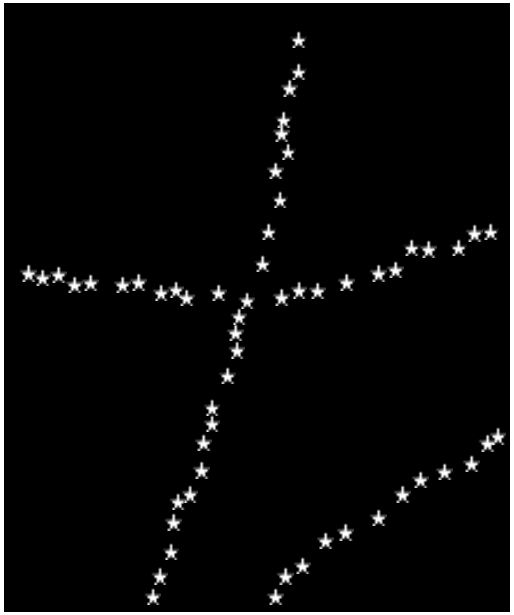


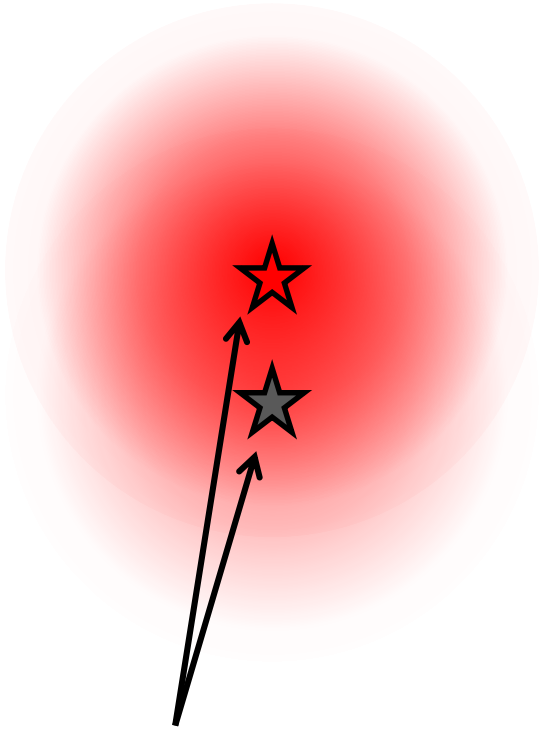
Super-resolution by spatial modulation

Fluorescence image



Underlying structure

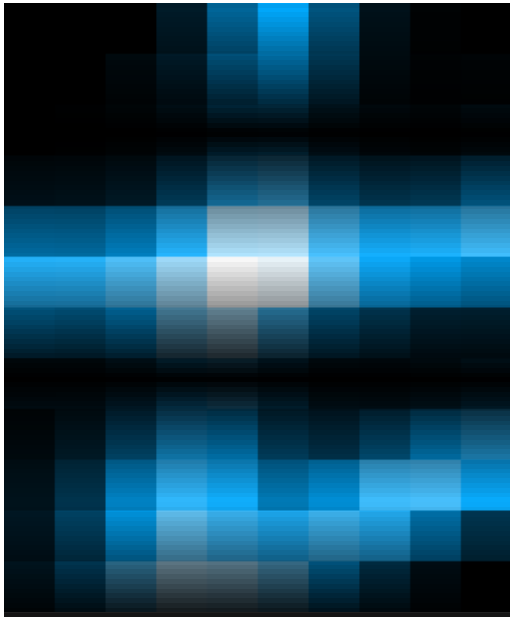




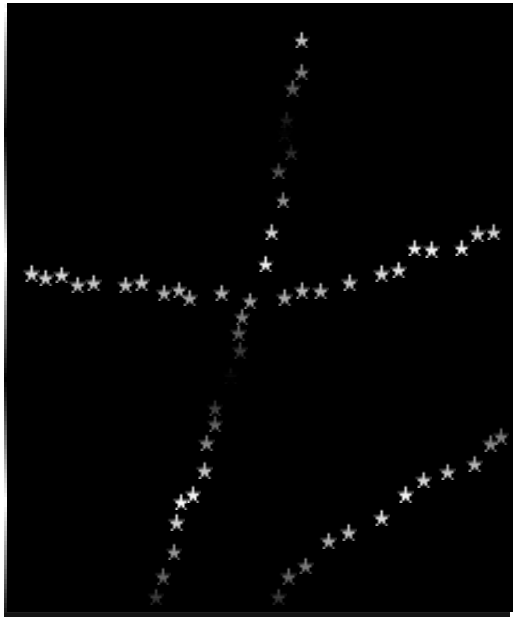
Differential modulation
of the fluorescence response

Super-resolution by differential excitation

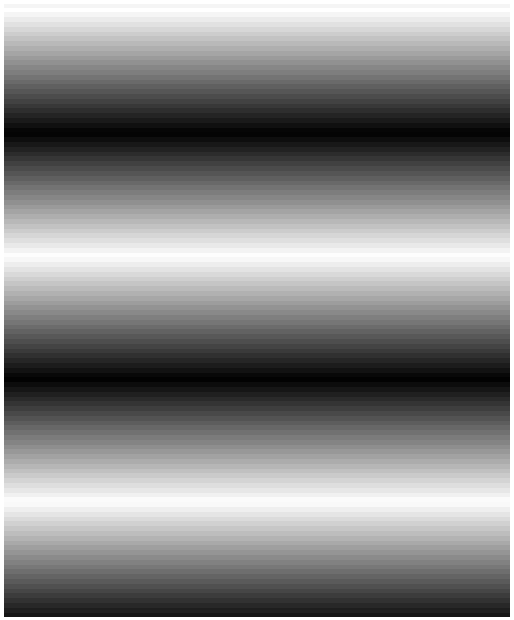
Fluorescence image

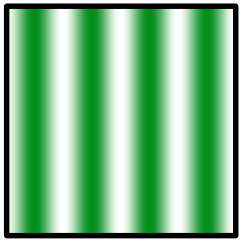


Underlying structure



Excitation pattern





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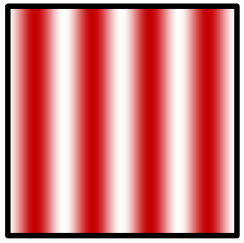

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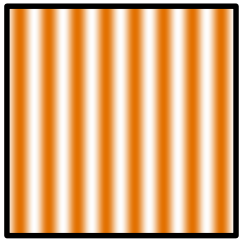


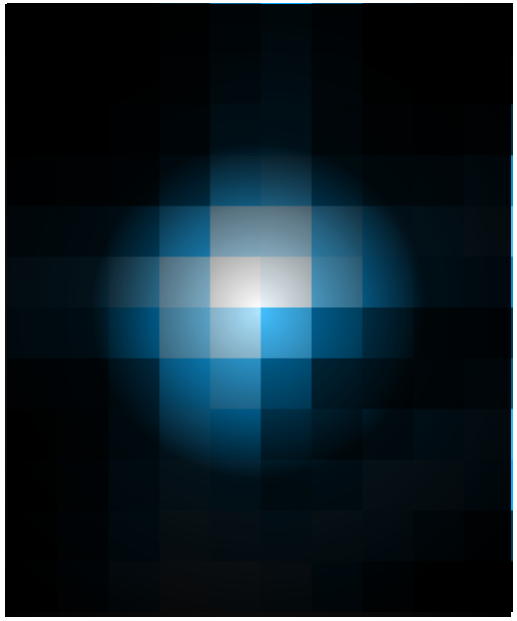
SIM (Gustafsson / Heintzmann)
SSIM (Gustafsson 2005)

Diffraction limited excitation and emission

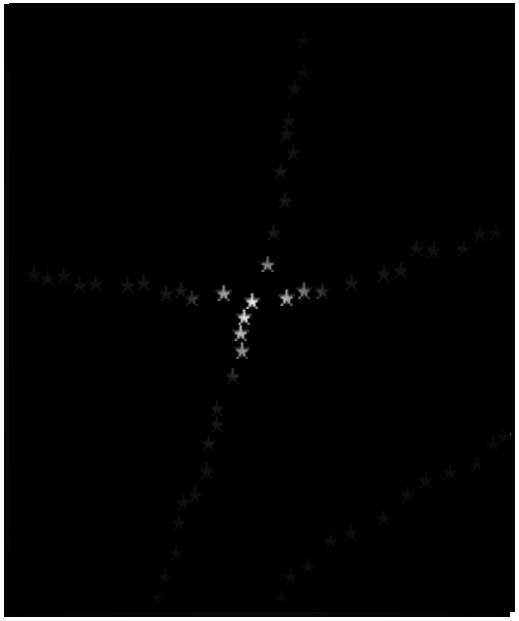
Doubled resolution

Super-resolution by differential depletion

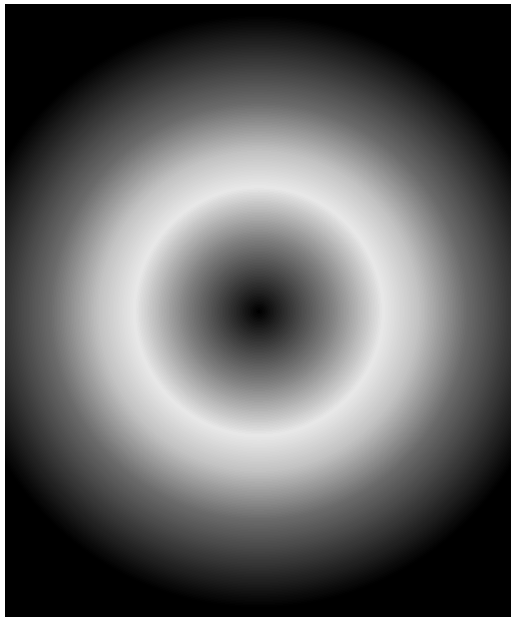
Fluorescence image



Underlying structure



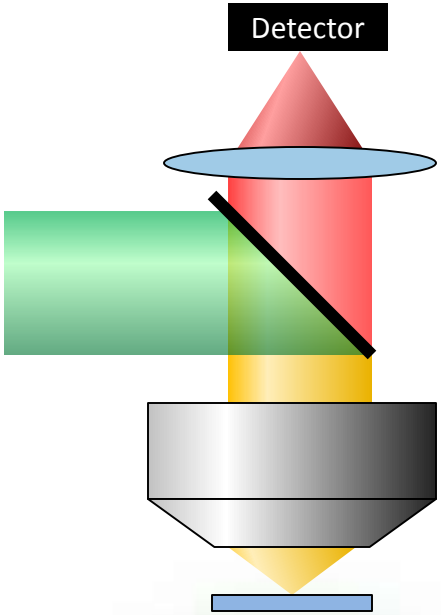
Depletion pattern

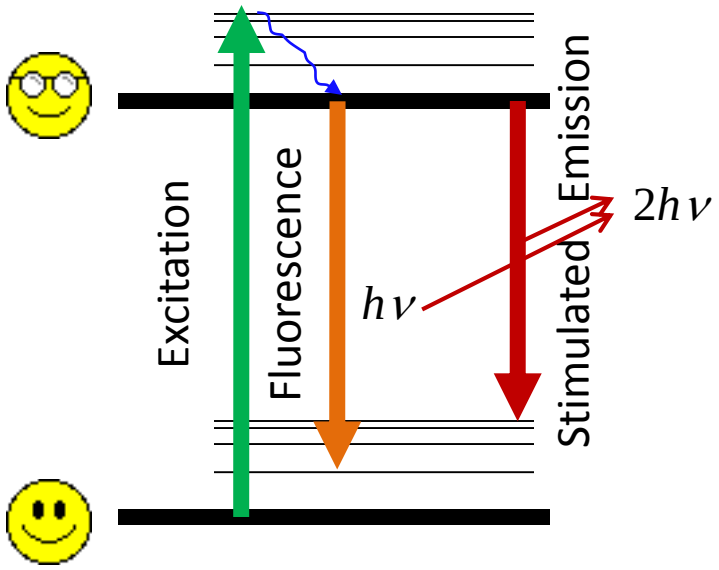


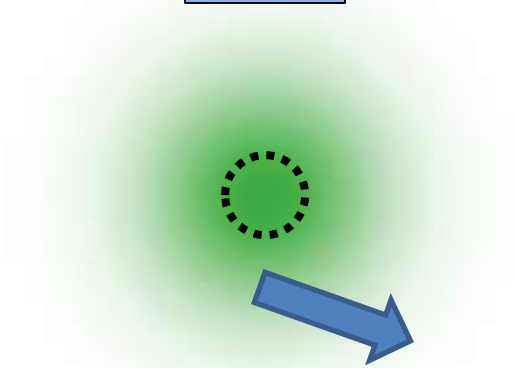
Diffraction limited PSF Saturated depletion Smaller effective PSF

STED (Hell 1994, Hell 1999)
GSD (Hell 1995, Hell 2007)
RESOLFT (Hell 2003, Hell 2011)

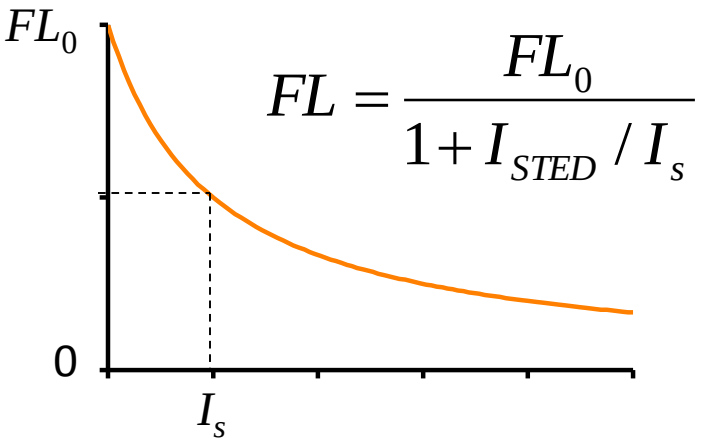
Stimulated Emission Depletion (STED)





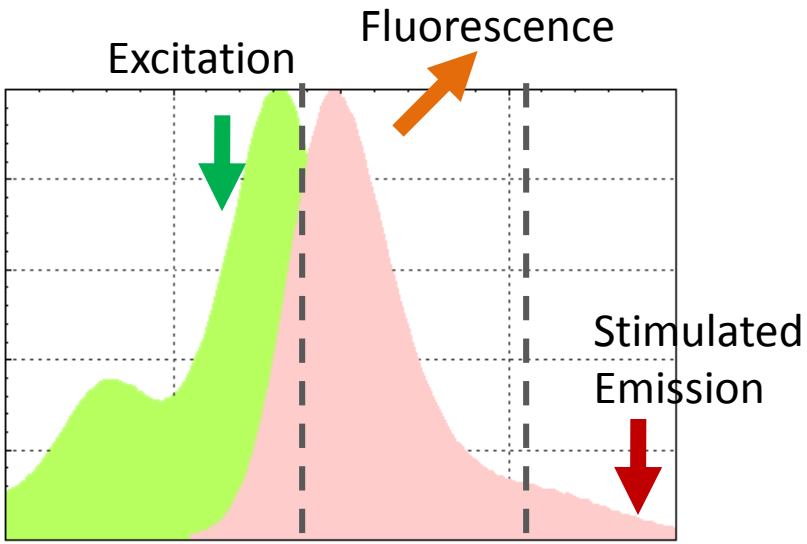


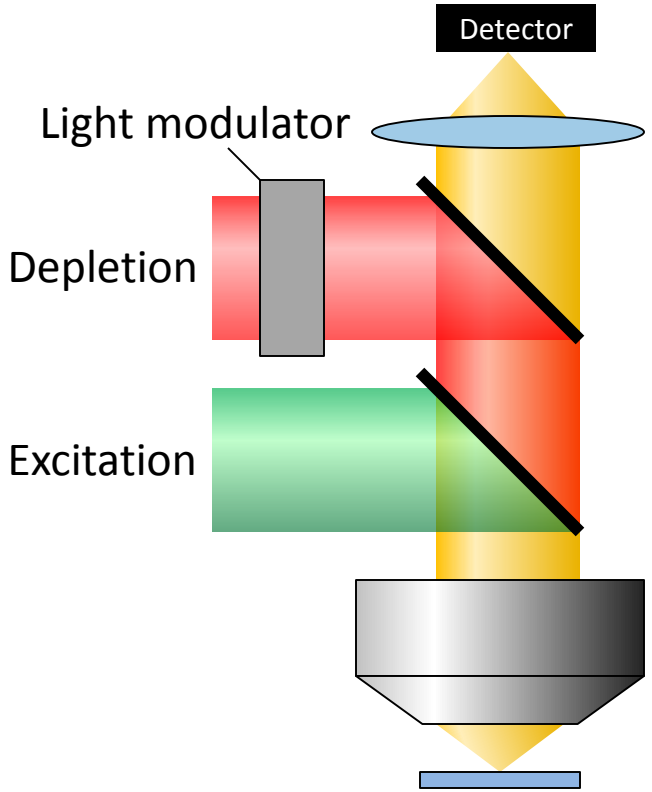
Send to a dark state

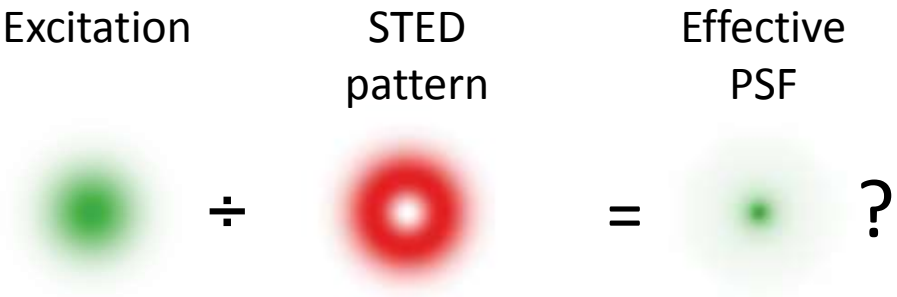


$$FL = \frac{FL_0}{1 + I_{STED} / I_s}$$

STED microscopy

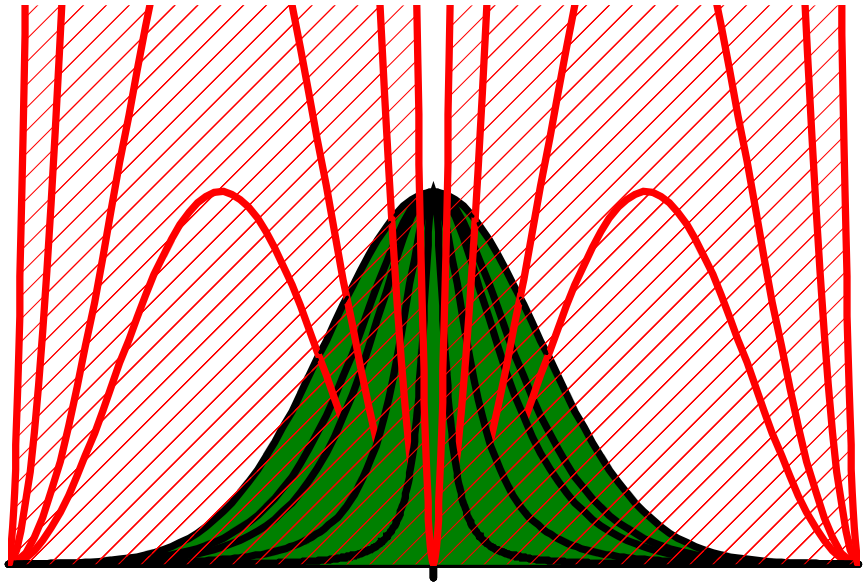






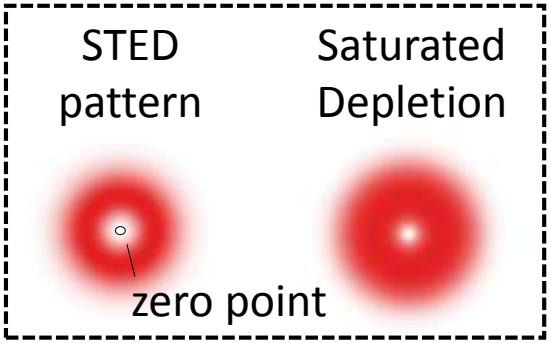
Hell 1994, Hell 2000

Saturated depletion

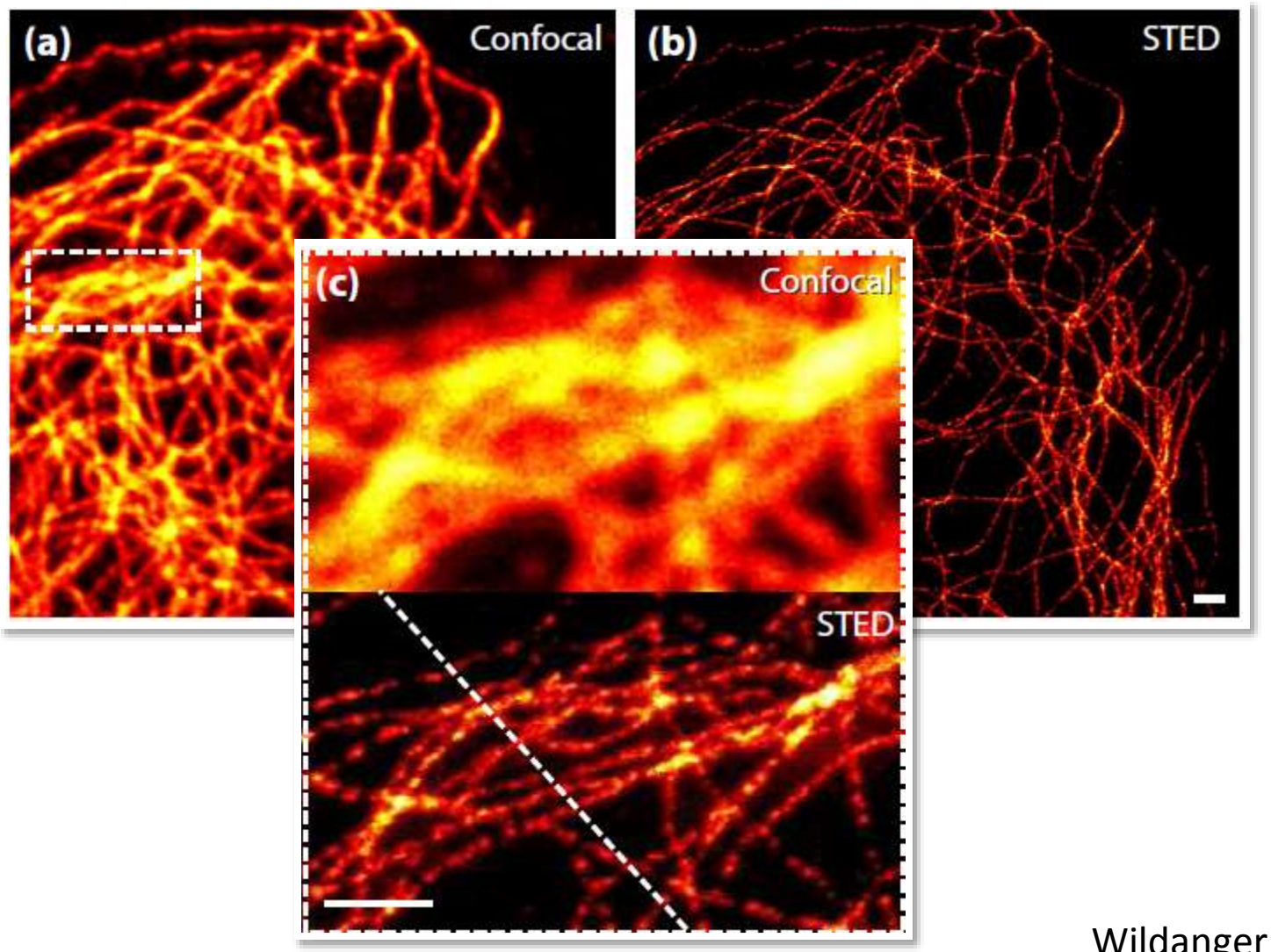


$$I_{\mathrm{STED}} = 200\, I_S$$

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

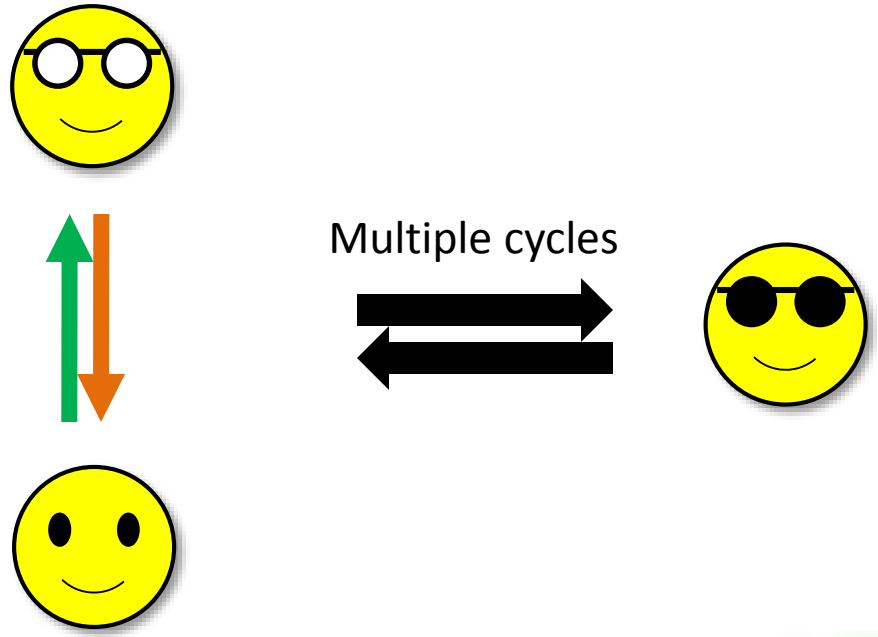


STED images of microtubules



Wildanger et al., 2009

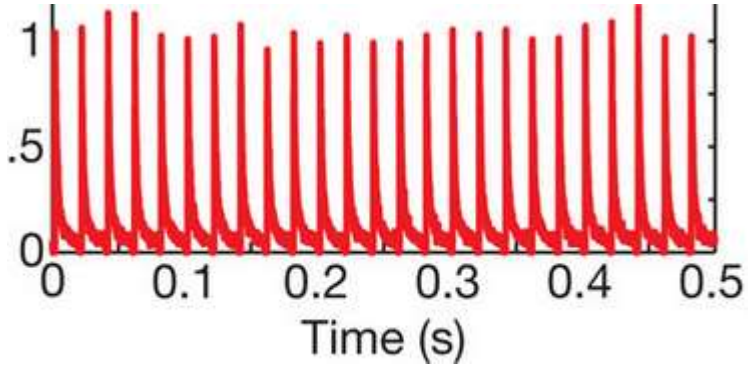
The “patterned illumination” approach

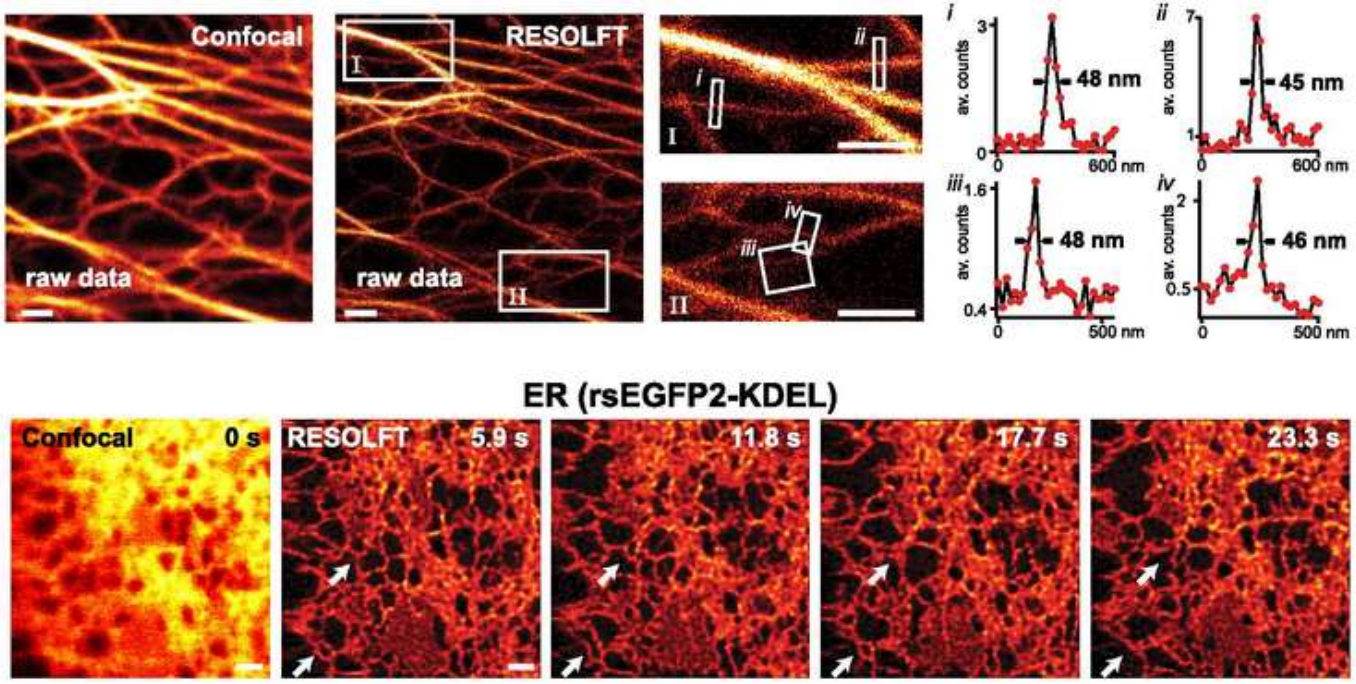


- Ground state
- Triplet state
- Isomerization

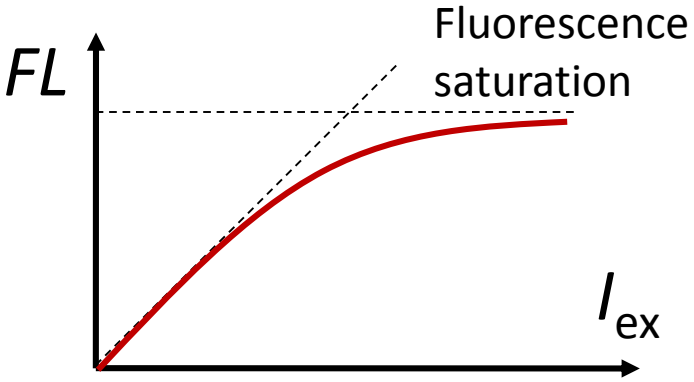
etc.

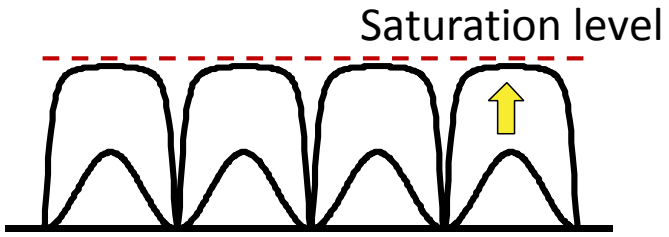
RESOLFT by rsEGFP and rsEGFP2



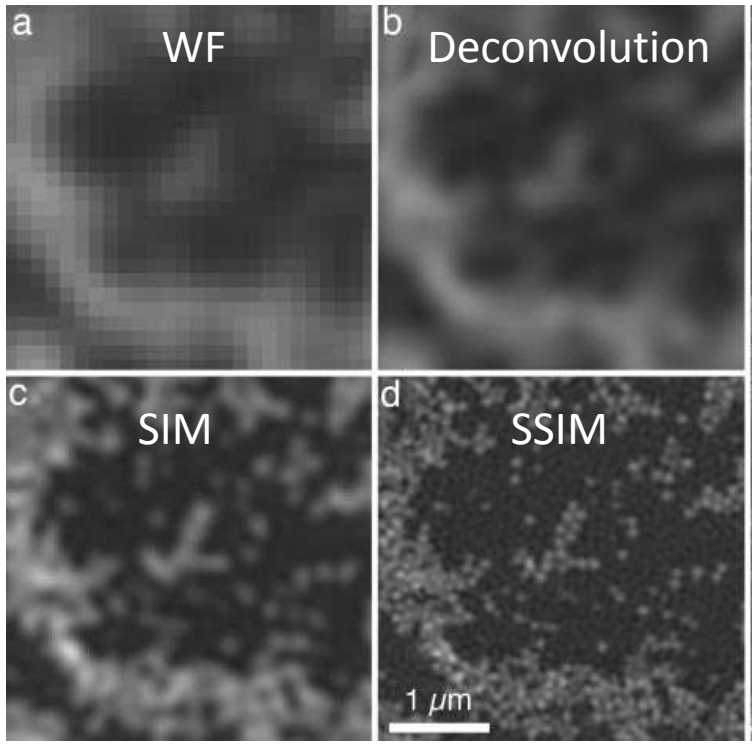


Saturated SIM





Saturated illumination pattern



50 nm resolution

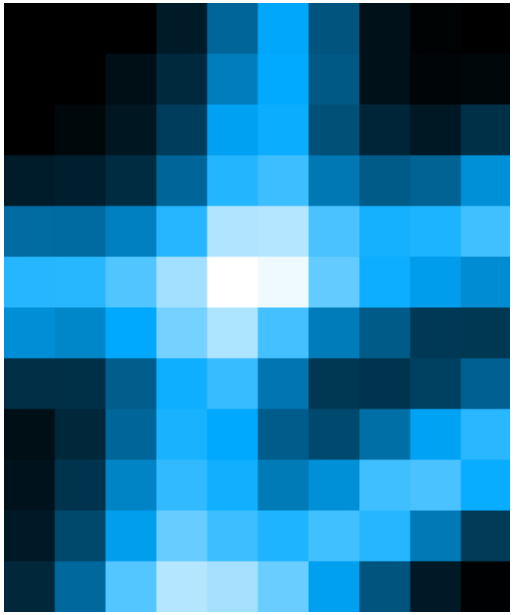
Suffers from fast photobleaching under saturated excitation condition

Gustaffson, PNAS 2005

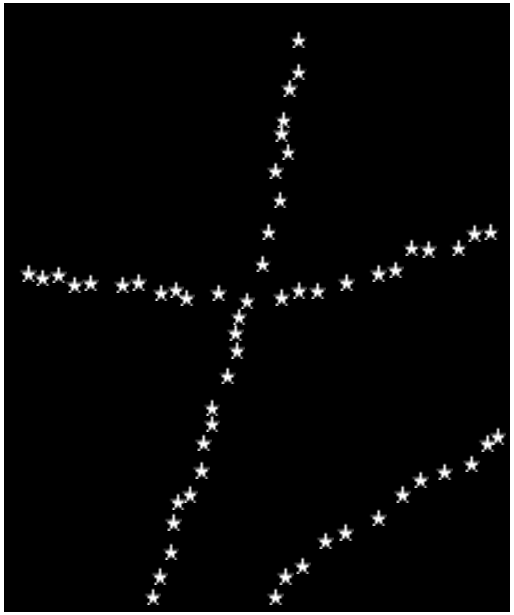
Super-resolution by single-molecule switching

Super-resolution by single-molecule switching

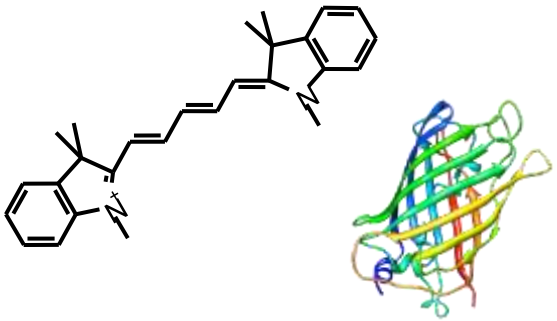
Fluorescence image

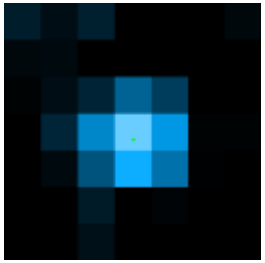


Underlying structure



Photoswitchable molecules





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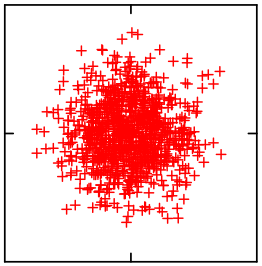
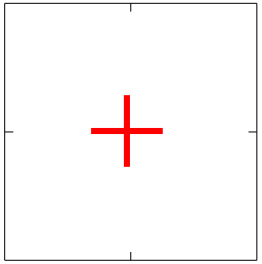




$$D \approx d / \sqrt{N}$$

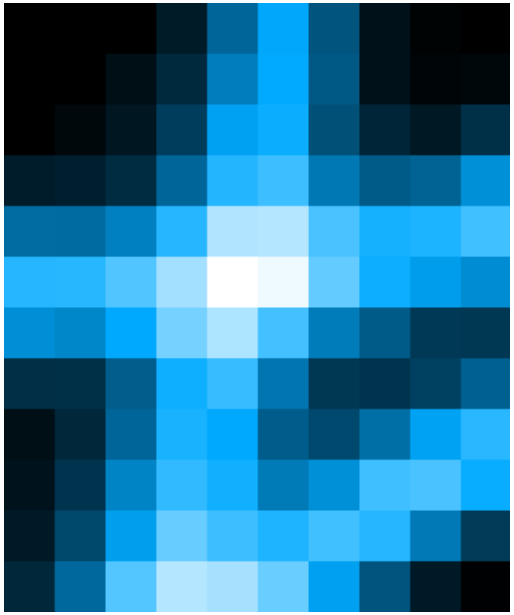
Single molecule image

N photons

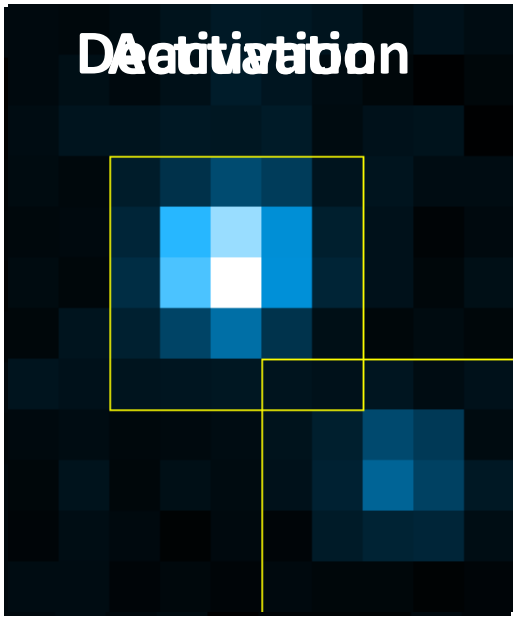
Single-molecule localization

Super-resolution by single-molecule switching

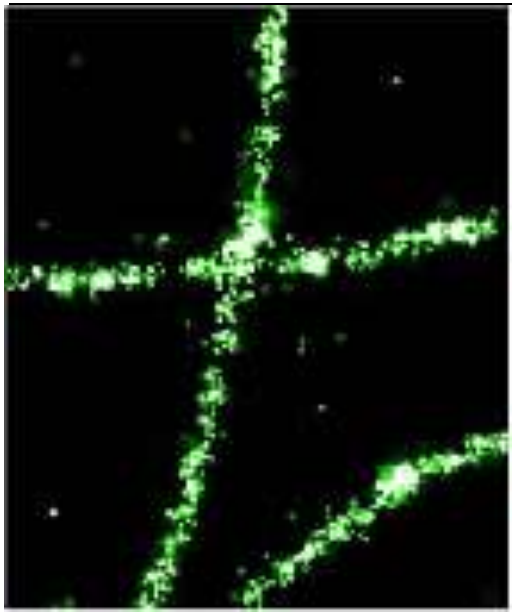
Fluorescence image



Raw images



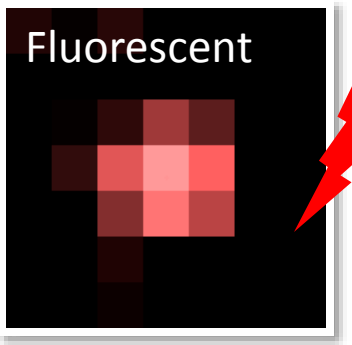
STORM Image



2x real time

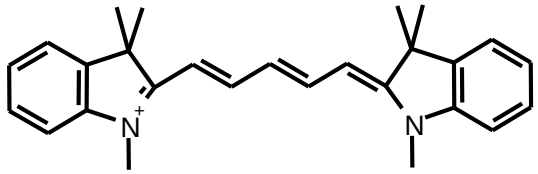
STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)
PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)
FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)
PALMIRA (Hell 2007), GSDIM (Hell 2008), dSTORM (Sauer 2008), SMACM (Moerner 2008)
PAINT (Hochstrasser 2006), SPRAYPAINT (Moerner 2011), SOFI (Weiss 2009)

Photoswitching of red cyanine dyes



650 nm

+ thiol



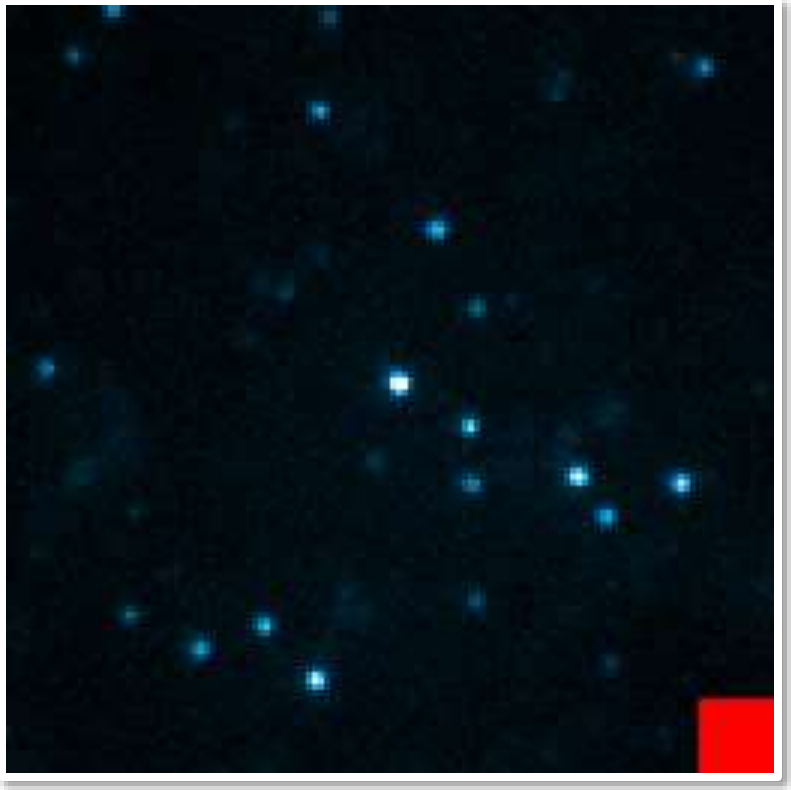
Cy5 / Alexa 647

photoactivation

Deactivation

360 nm

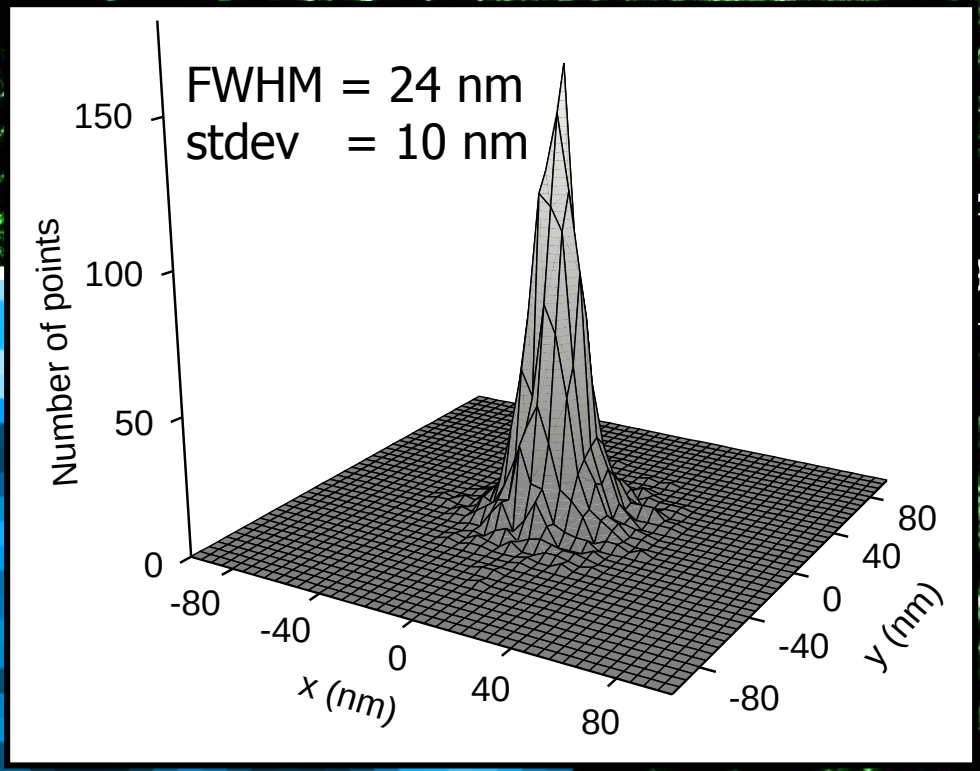
650 nm



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

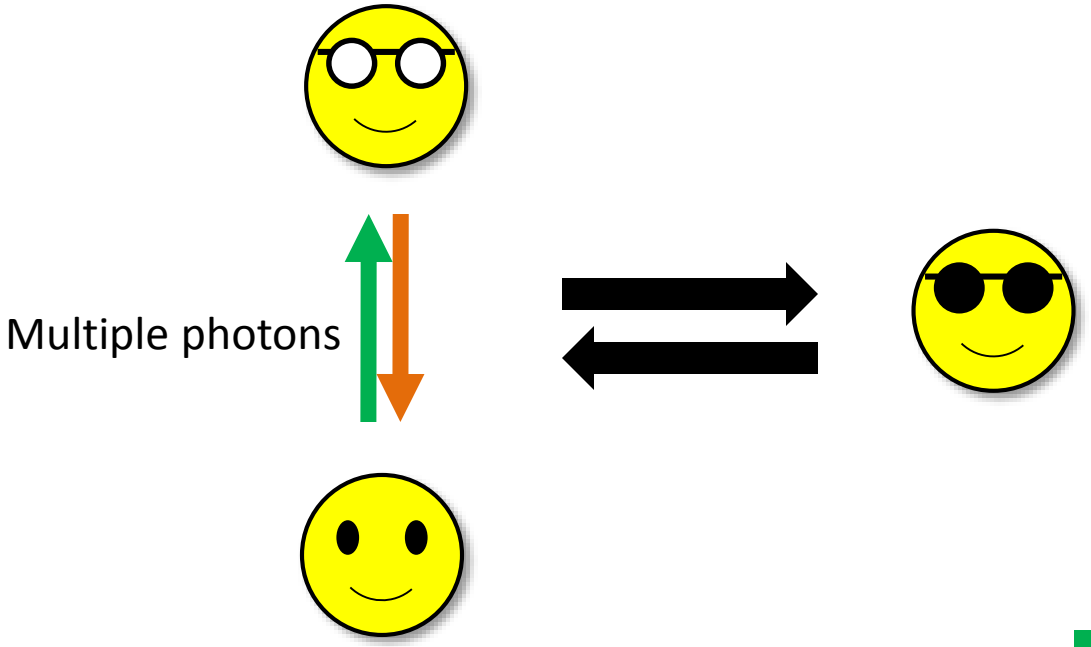


lization points

500 nm

5 µm

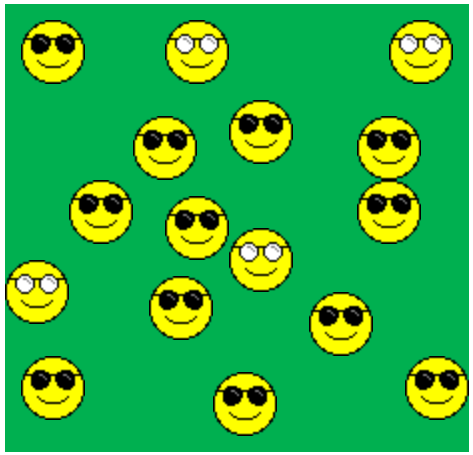
The “single-molecule switching” approach



- Photoswitching
- Blinking
- Diffusion
- Binding

  etc.

\+ Stochastic Switching =



Photoswitchable probes readily available

Simple dyes (+ thiole / redox system)

Alexa647
Cy5
Cy5.5
Cy7
Atto565
Alexa532
Alexa488
Atto520
Alexa568
Atto590
Atto655
Atto700

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

Heilemann et al., 2009

Functional dyes

MitoTracker Red
DiD
DiI
LysoTracker Red
ER Tracker Red

Shim 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

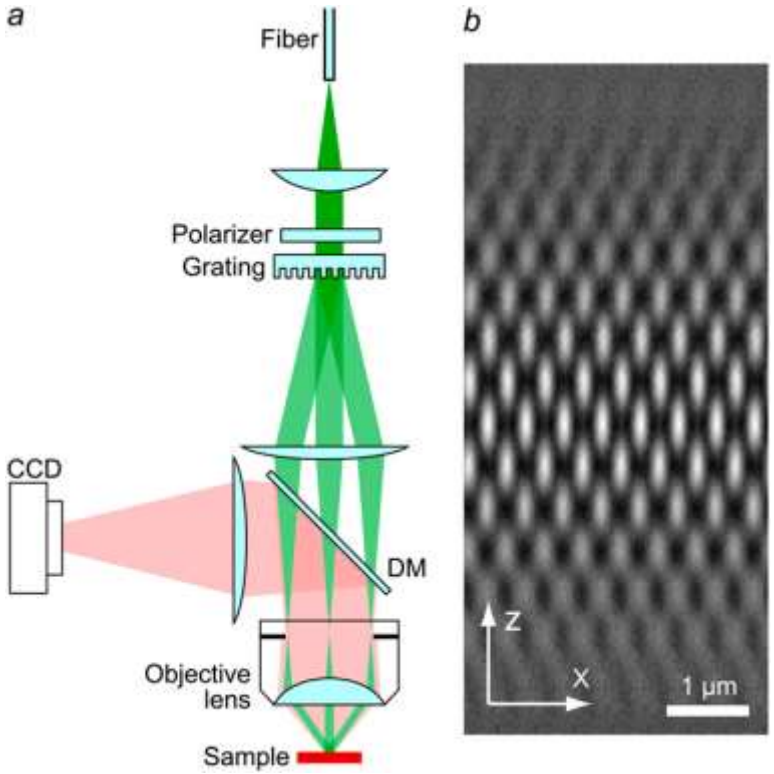
3D Imaging

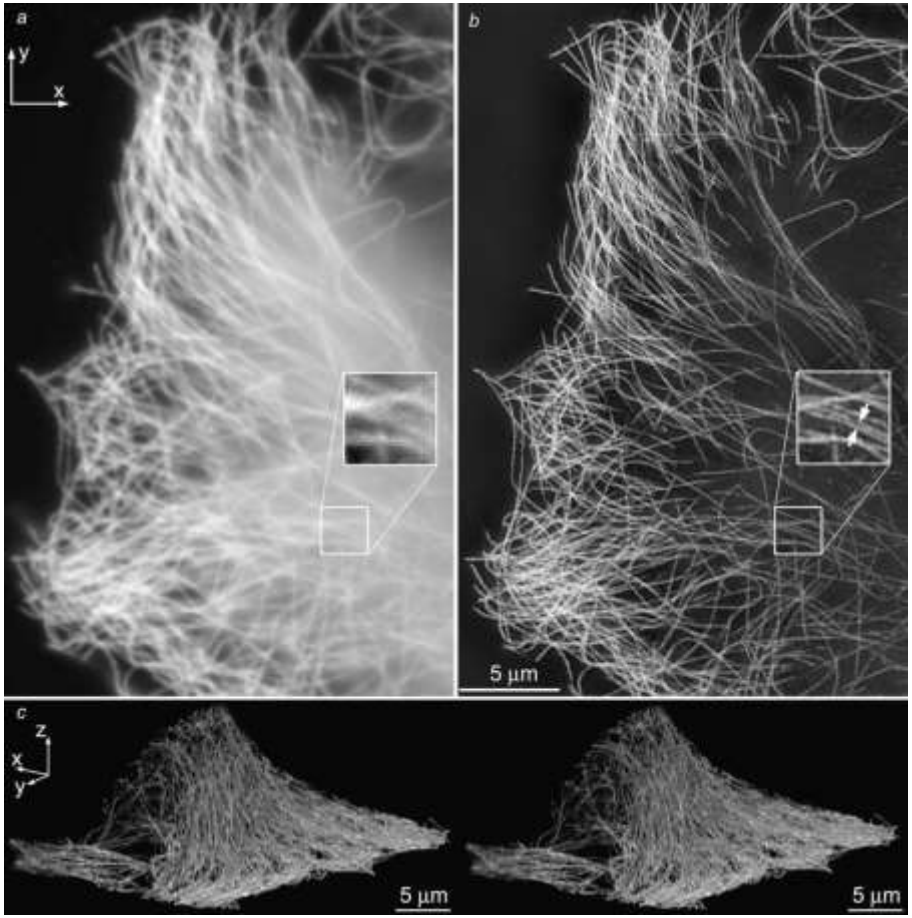
In a 2D world…

Satellite image of ???

Google maps

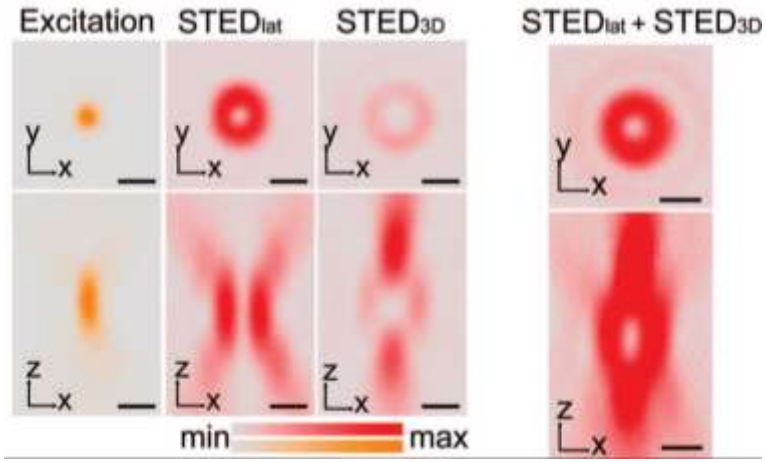
3D SIM

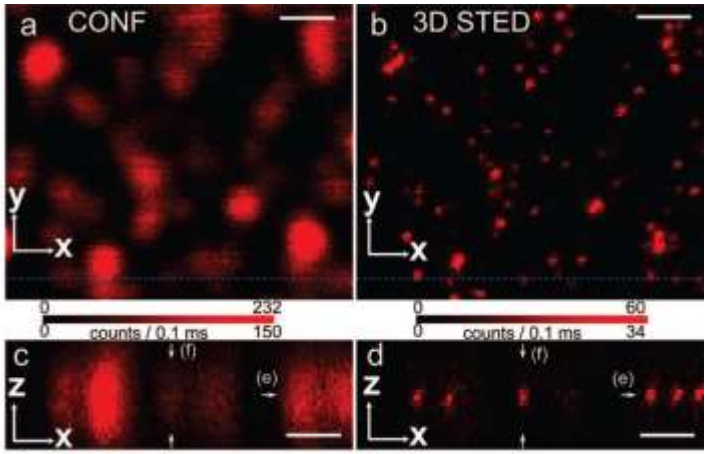


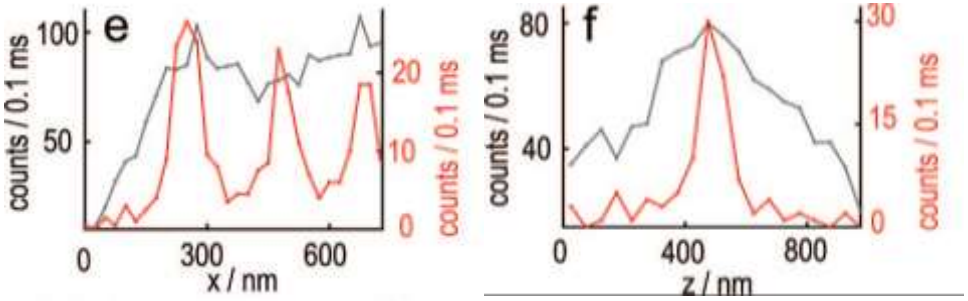


Schermellech et al., Science 2008, Gustafsson et al., Biophys J. 2008

3D STED

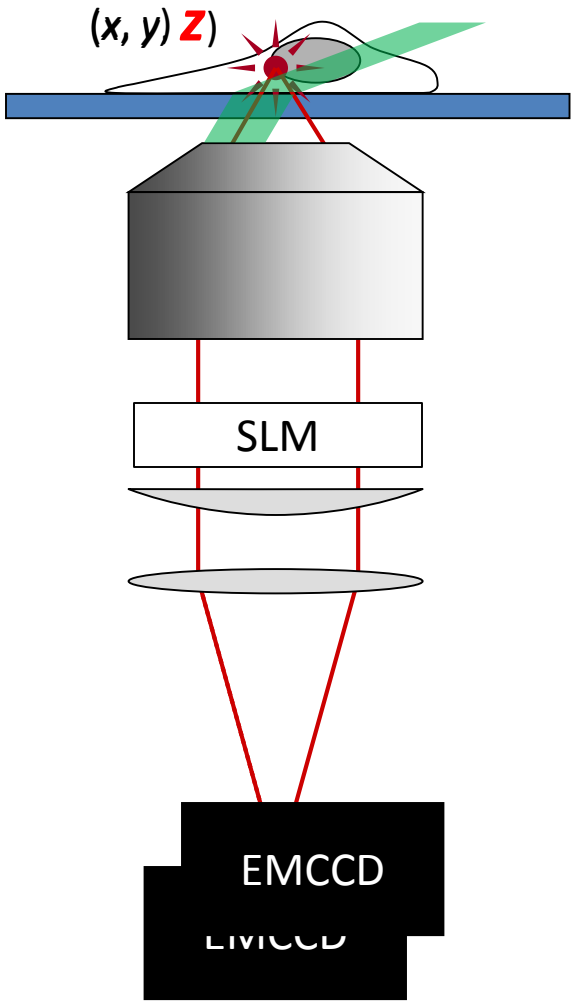




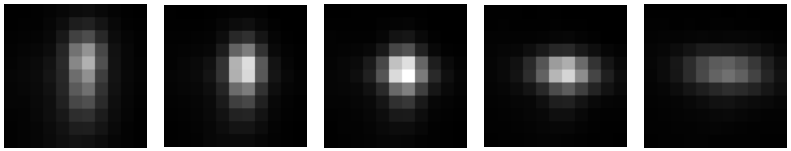


Harke et al., Nano Lett, 2008

3D STORM/PALM

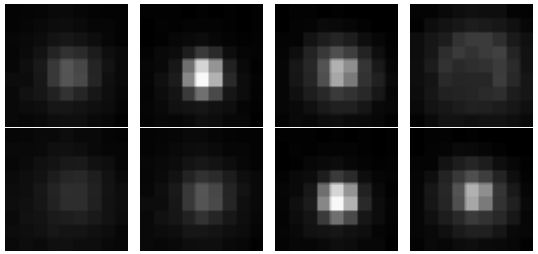


Astigmatic imaging



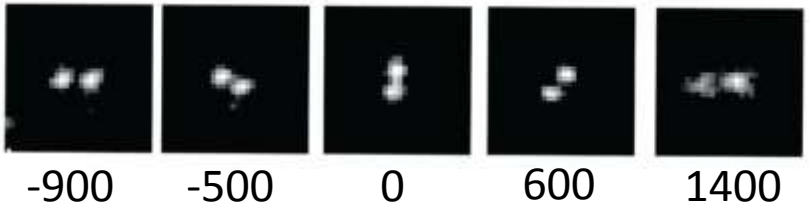
Huang et al., Science 2008

Bi-plane imaging



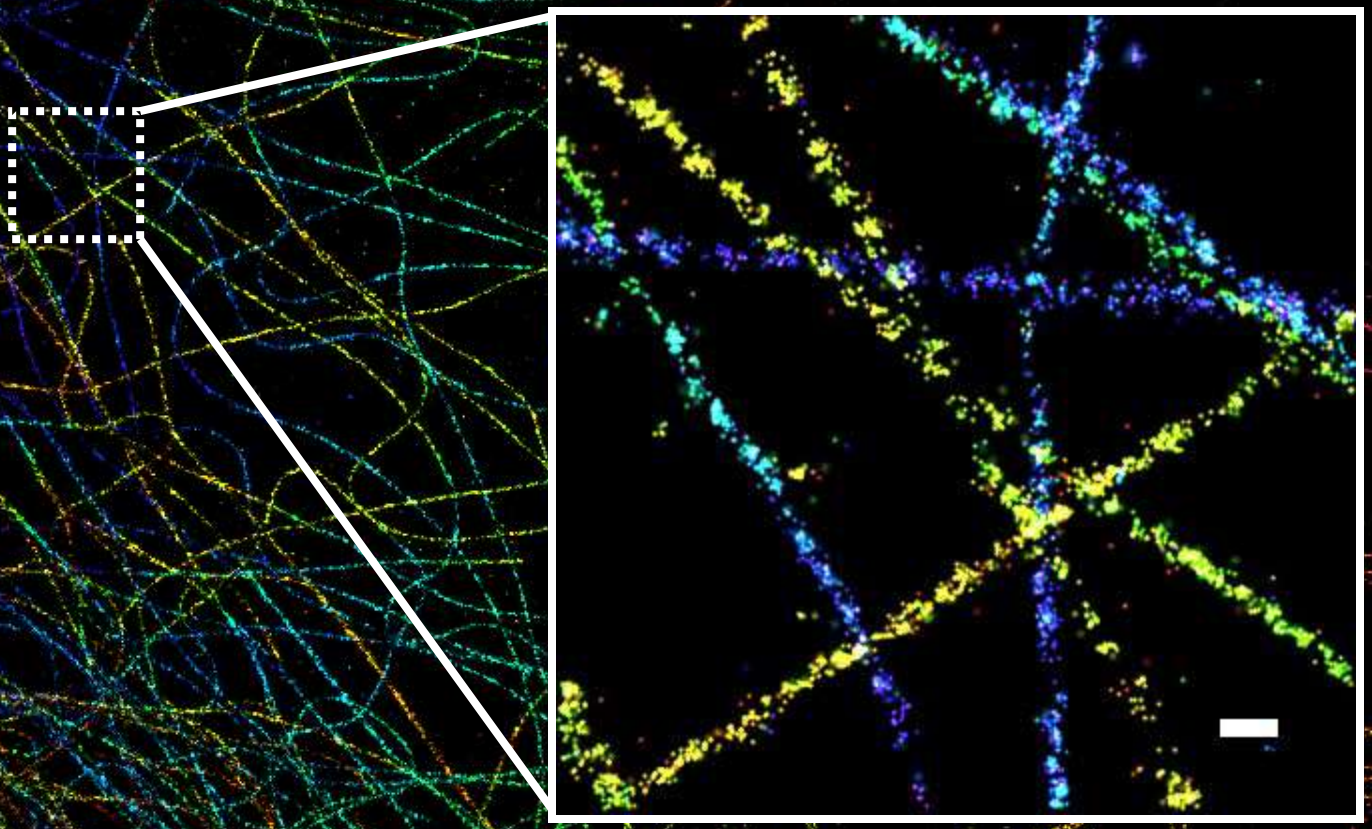
Juette et al., Science 2008

Double-helical PSF



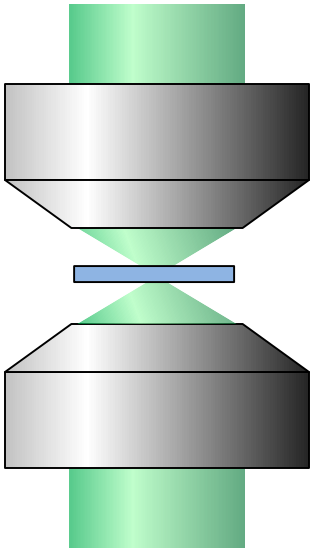
Pavani et al., PNAS 2009

3D Imaging of the microtubule network



Huang, Wang, Bates and Zhuang, *Science*, 2008

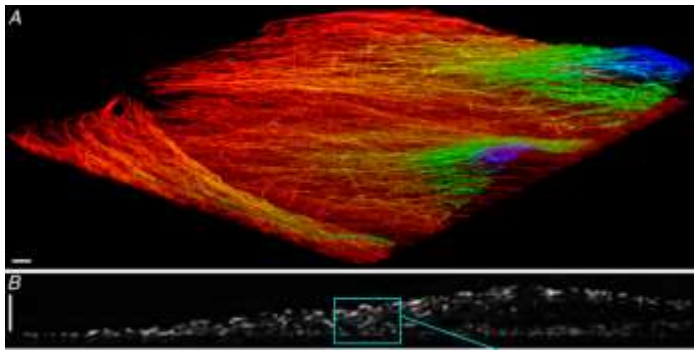
The use of two opposing objectives



4Pi scheme

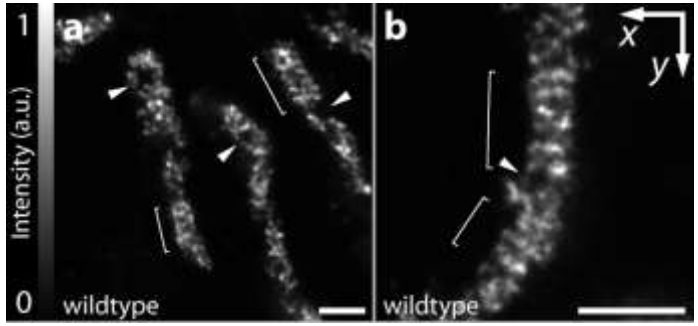
Near isotropic
3D resolution

I^{5}S



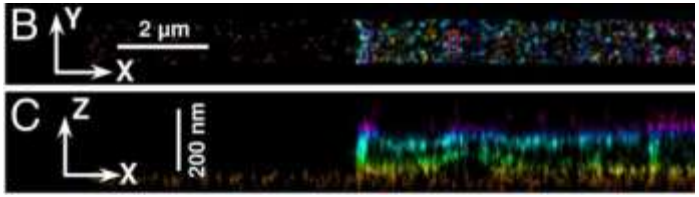
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

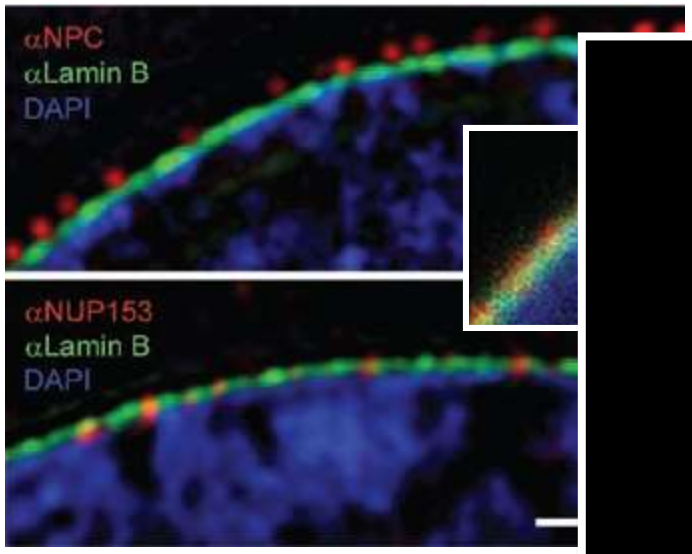
3D resolution of super-resolution methods

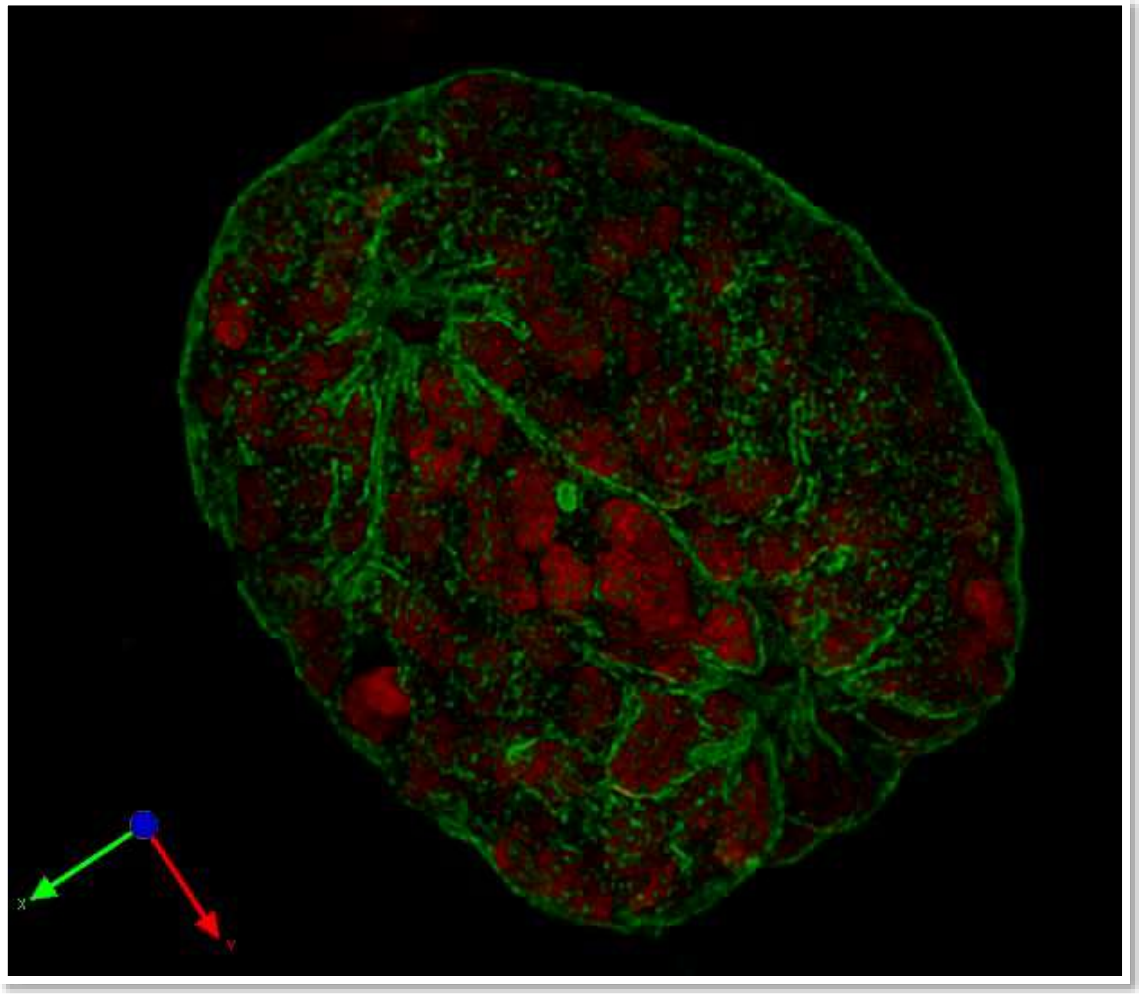
	x-y (nm)	z (nm)	Opposing objectives (nm)	Deep tissue
Conventional	250	600	4Pi: 120	
SIM	100	250	I^5S: 120 xyz	
STED	~30	~100	isoSTED: 30 xyz	2 photon
STORM/PALM	20-30	50-60	iPALM: 20 xy, 10 z	2 photon, SPIM

Multi-color Imaging

Multicolor SIM

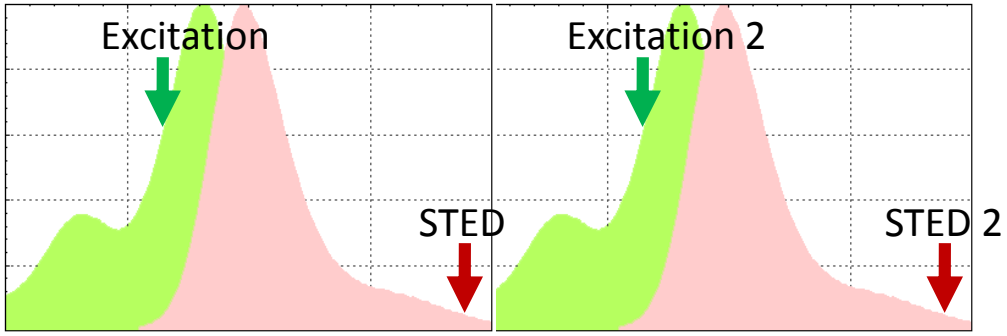
Same as conventional fluorescence microscopy!



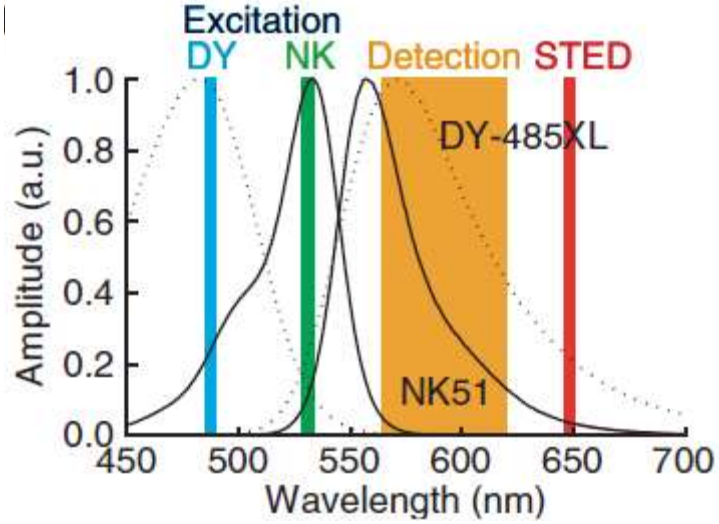


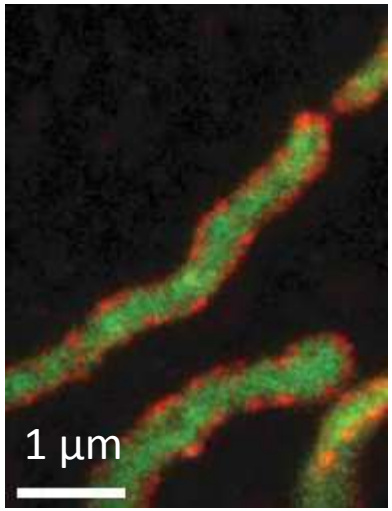
Schermelleh et al., Science 2008

Muticolor STED



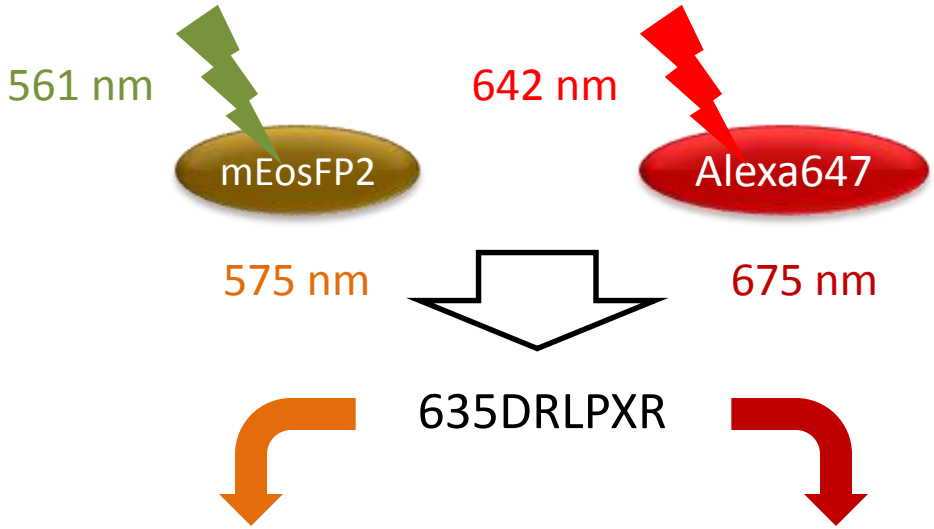
2 color isoSTED resolving
the inner and outer membrane
of mitochondria

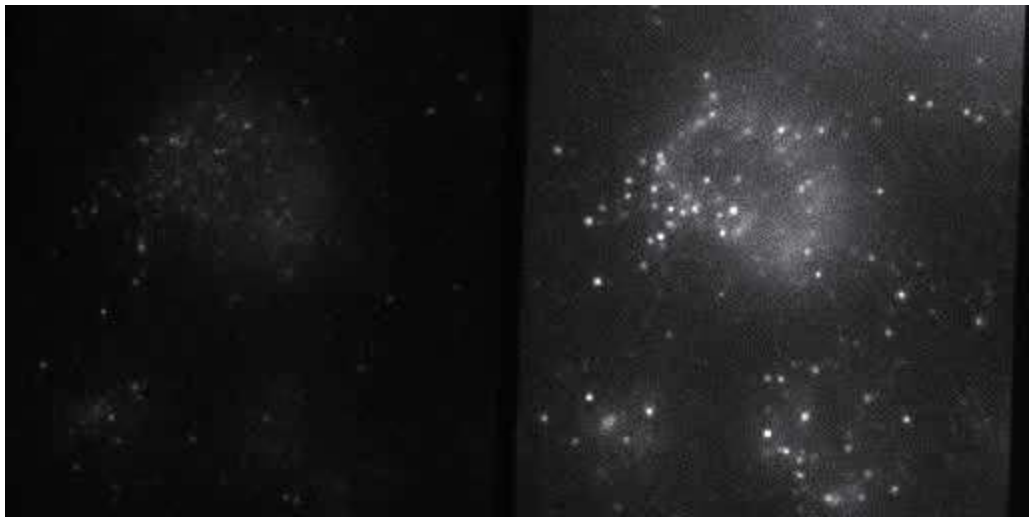


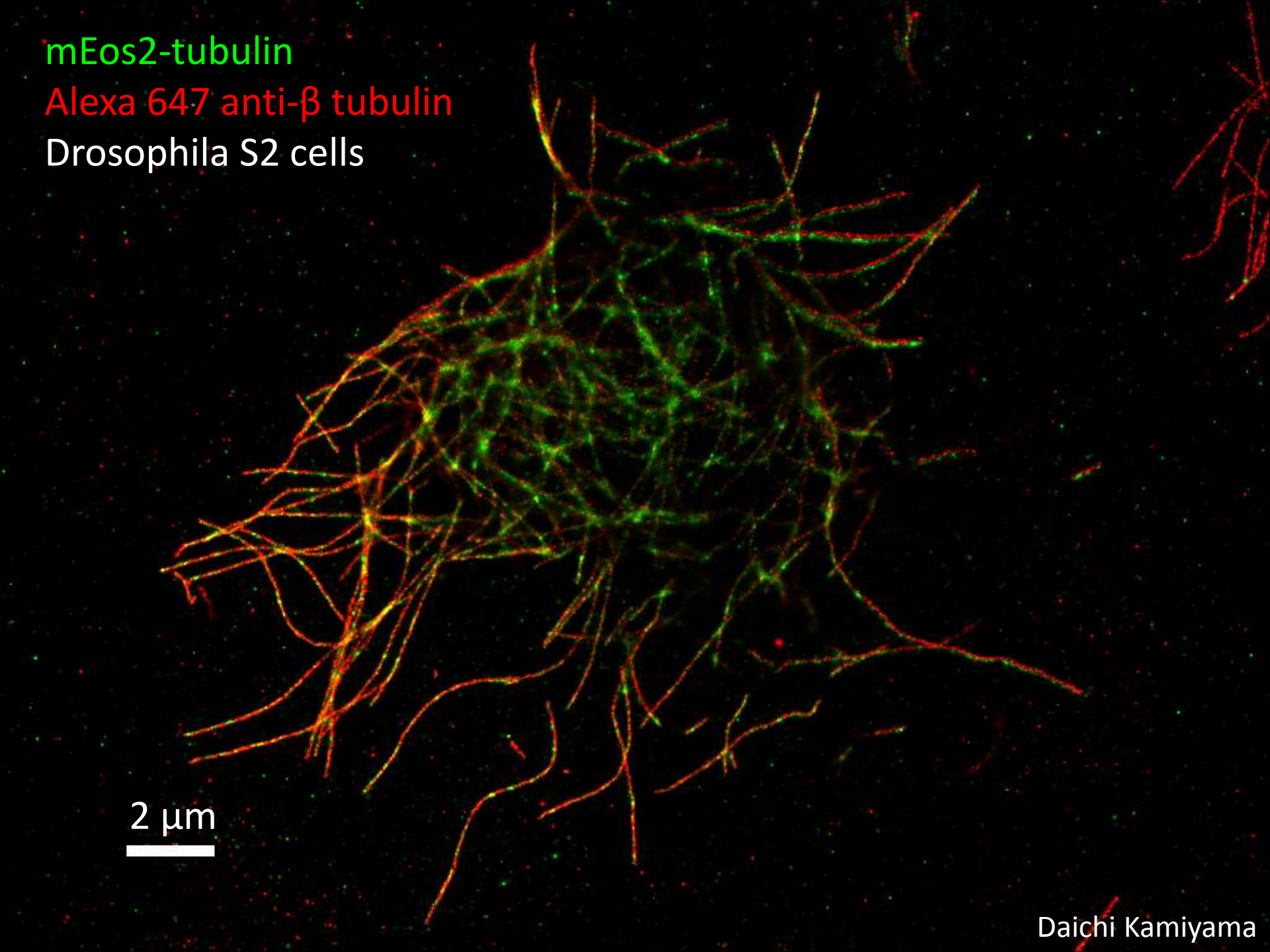


Schmidt et al., Nat Methods 2008

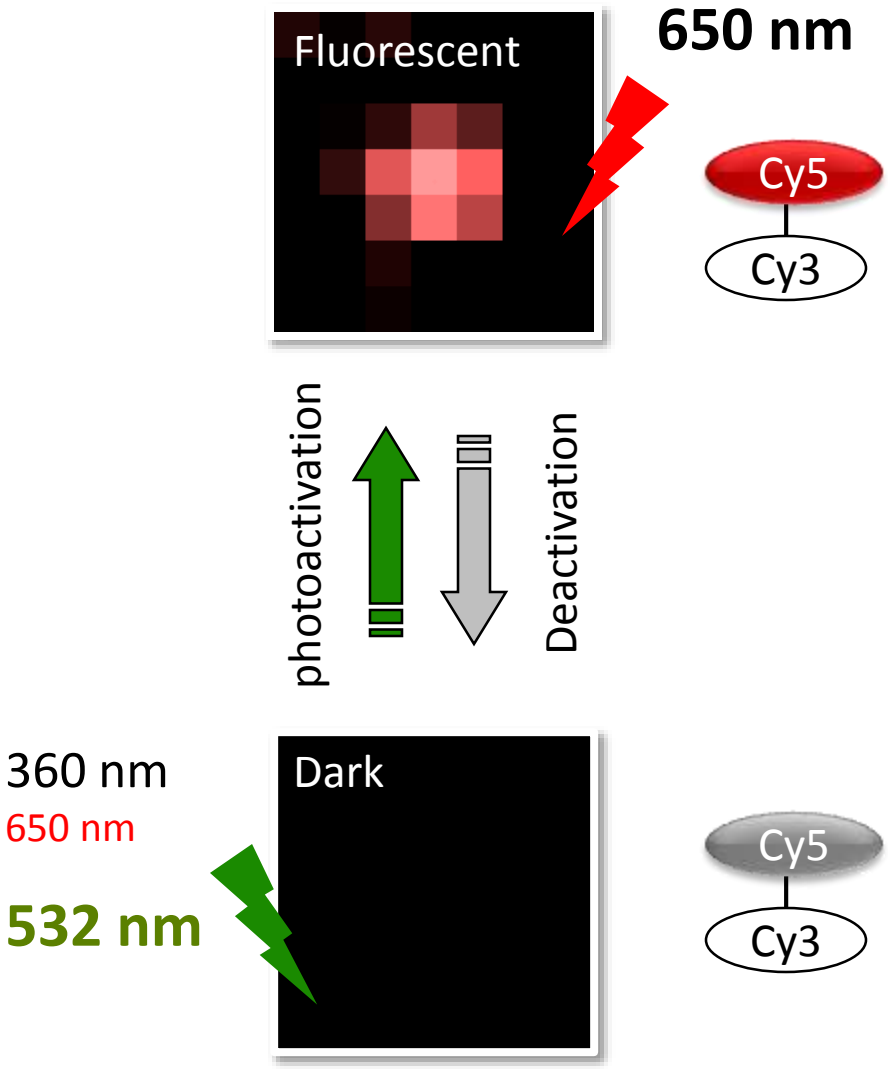
Multicolor STORM/PALM

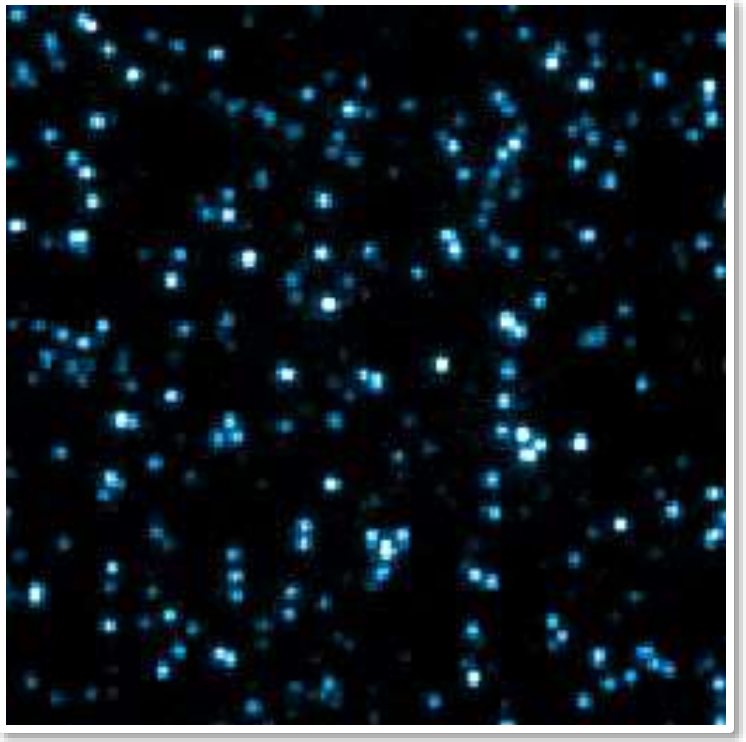





mEos2-tubulin
Alexa 647 anti-β tubulin
Drosophila S2 cells
2 µm
Daichi Kamiyama

Multicolor STORM/PALM: activation

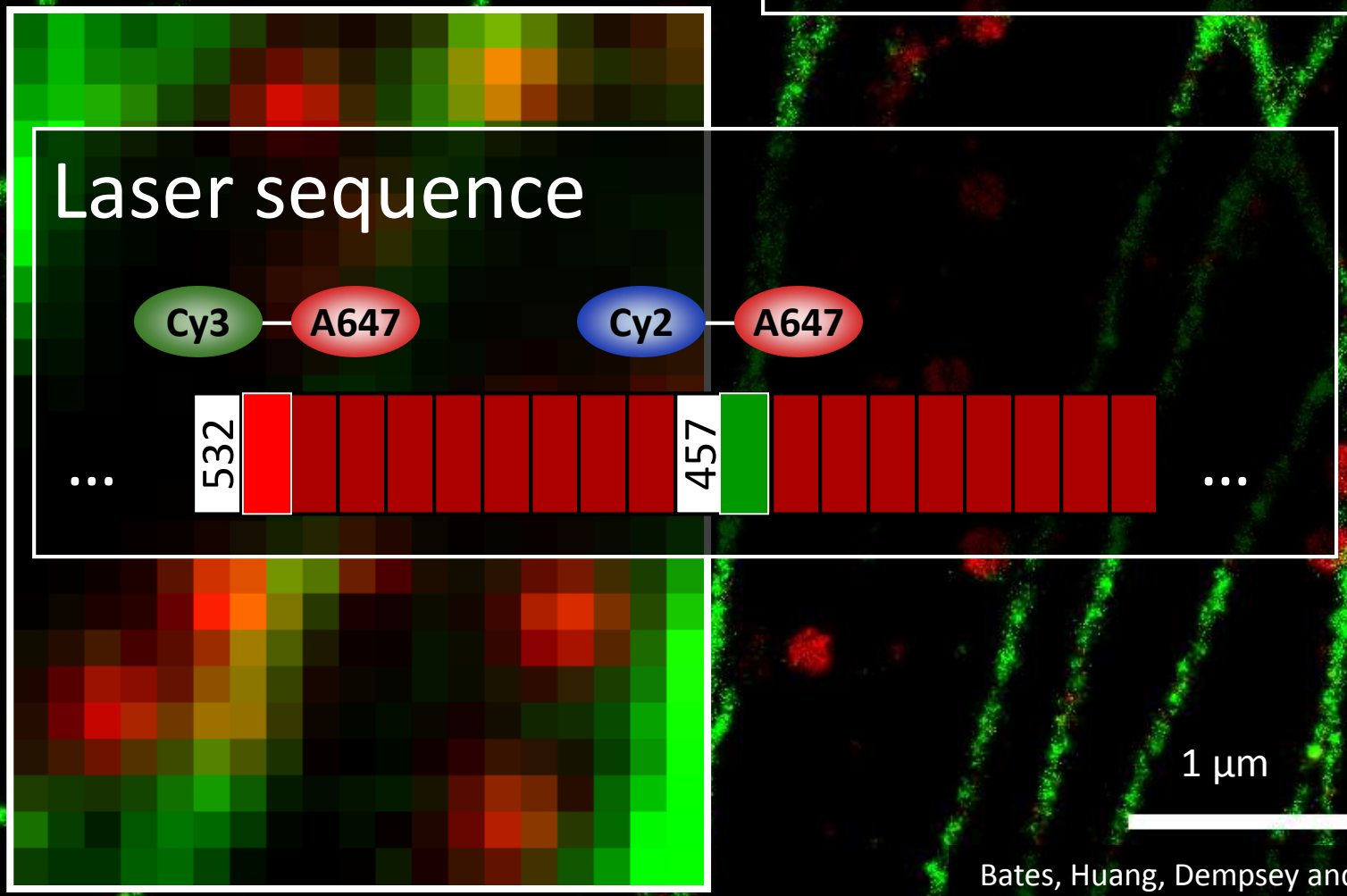




Cy3 / Alexa 647: Clathrin

Cy2 / Alexa 647: Microtubule

Crosstalk subtracted



1 µm

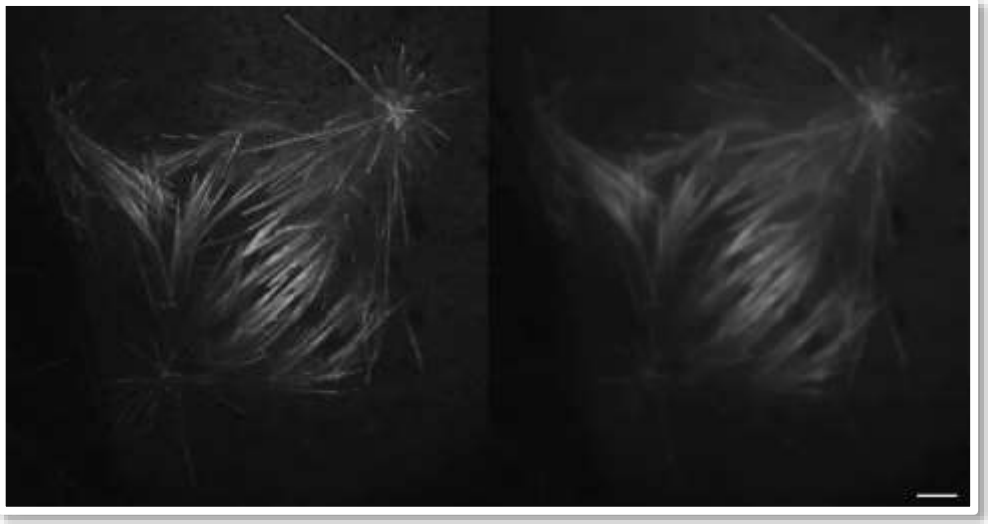
Bates, Huang, Dempsey and Zhuang,
Science, 2007

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

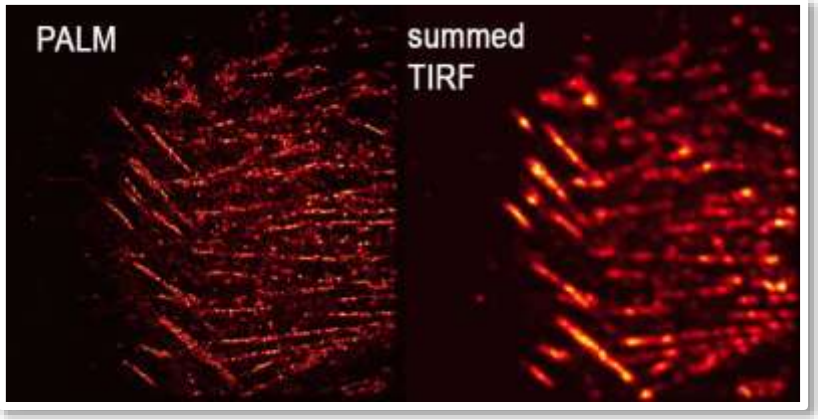
SIM



2 µm

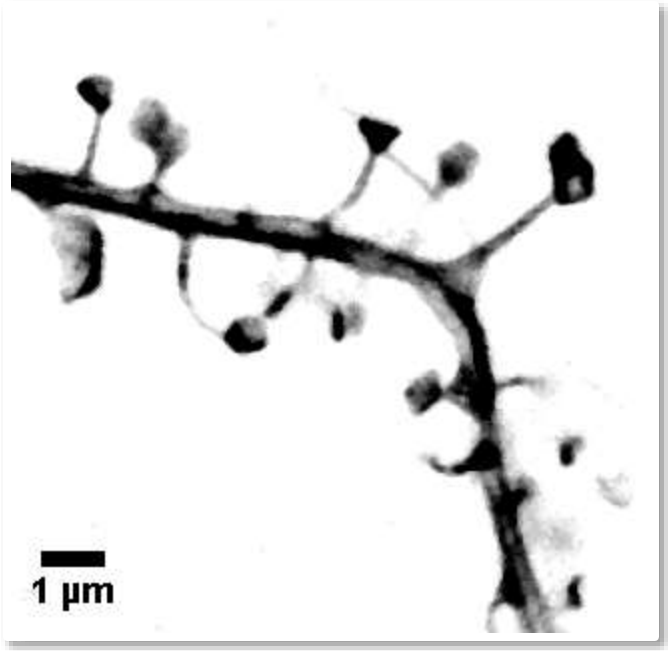
Kner, Chhun et al., Nat Methods, 2009

STORM/PALM



Schroff et al., Nat Methods, 2008

STED



Nagerl et al., PNAS, 2008

The limit of “Super-Resolution”

Unbound theoretical resolution

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

- $S \approx \sqrt{N}$
- 6,000 photons for Cy5 → 5 nm
- 1,000,000 photos for Hydro-Cy5 → < 1 nm

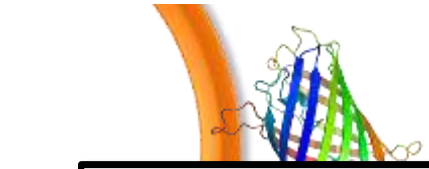
(Vaughn et al., 2012)

Effective resolution: Probe matters

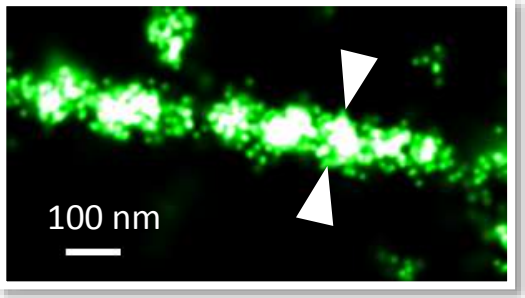
Antibodies:
~ 10 nm

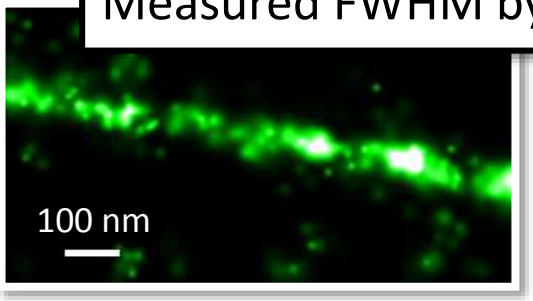
Fluorescent Proteins:
~ 3 nm

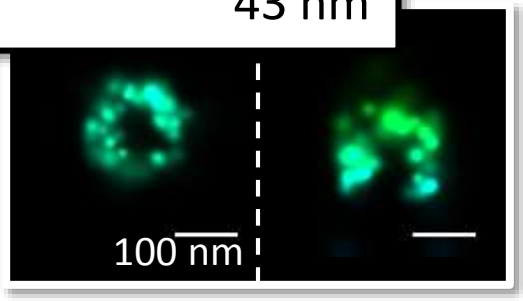
Small fluorophores:
~ 1 nm



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 specific
- High labelin
- Genetically
- Lower S/N
- Multicolor imaging so far challenging

Antibody immunofluorescence

- Fixed sample
- pecific labeling
- efficiency
- genous proteins
- igh localization
- More versatile for multicolor imaging

Newer labeling methods

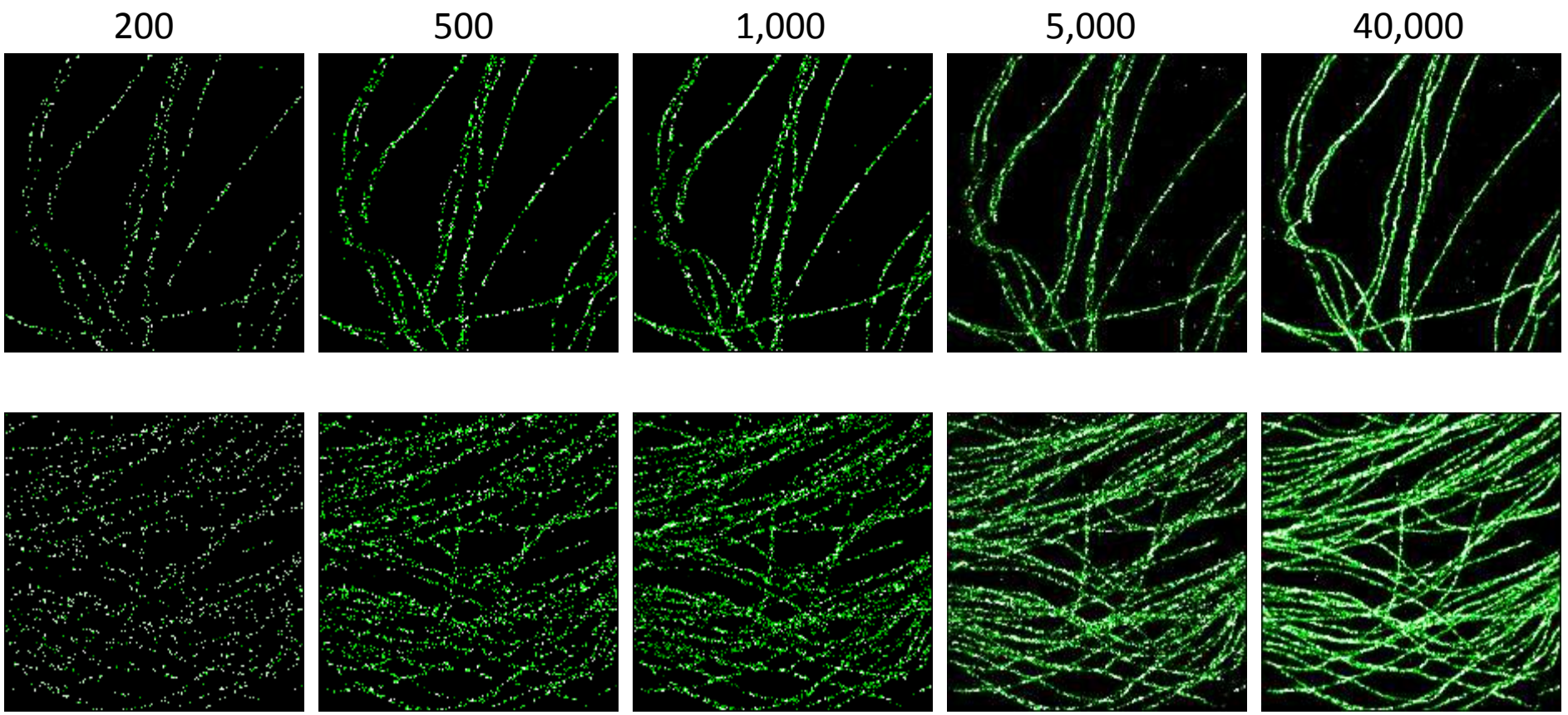
- Enzymatic tags

  SNAP-tag, HALO-tag, TMP-tag, etc.

- Nanobodies
- RNA aptamers

Effective resolution: Density matters

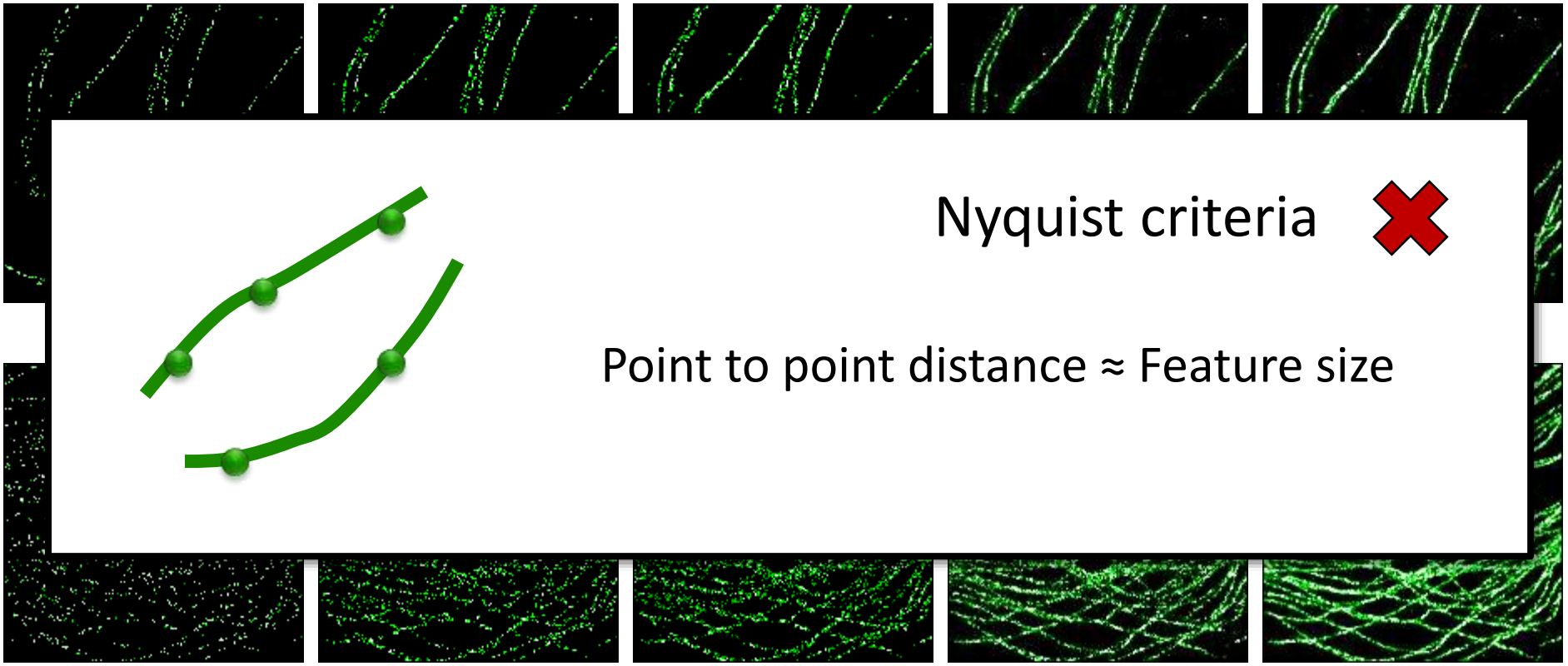
Frames for image reconstruction:



Effective resolution: Density matters

Frames for image reconstruction:

200 | 500 | 1,000 | 5,000 | 40,000



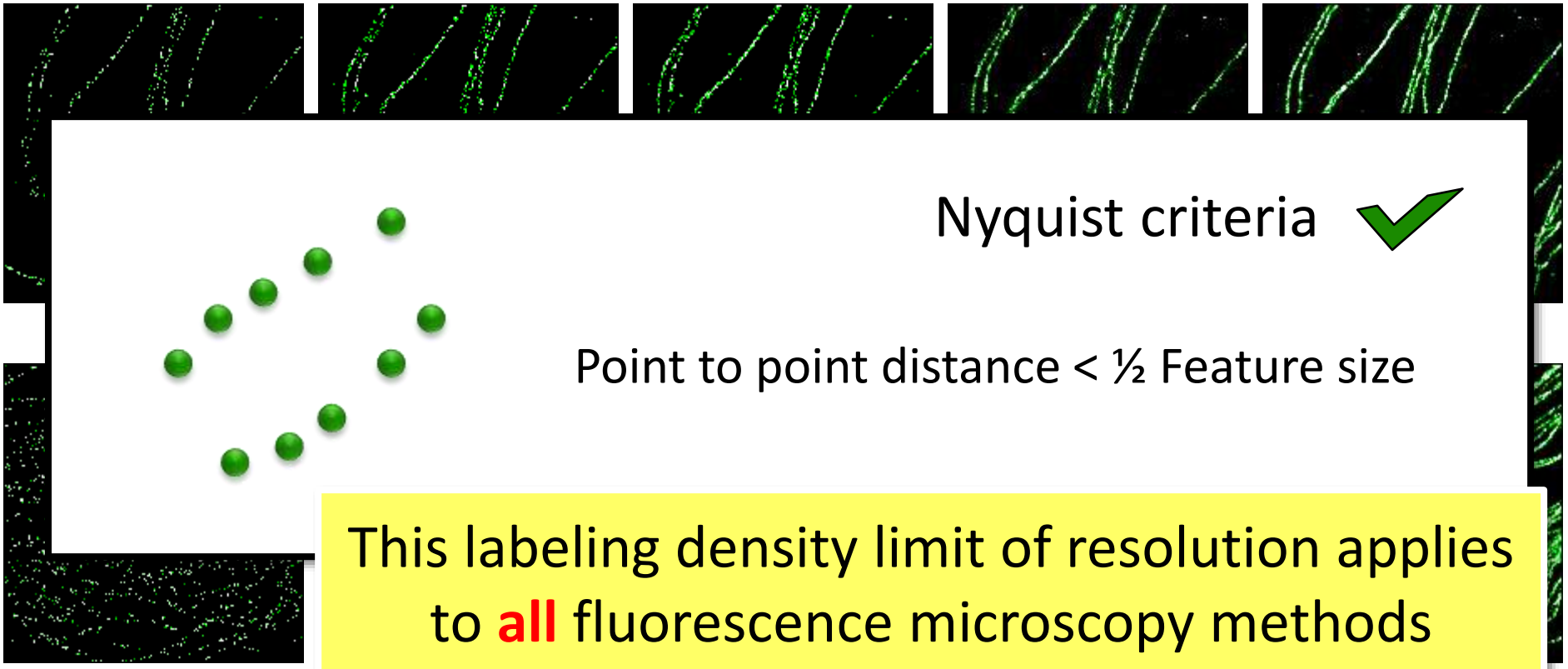
Nyquist criteria ✖

Point to point distance ≈ Feature size

Effective resolution: Density matters

Frames for image reconstruction:

200 | 500 | 1,000 | 5,000 | 40,000

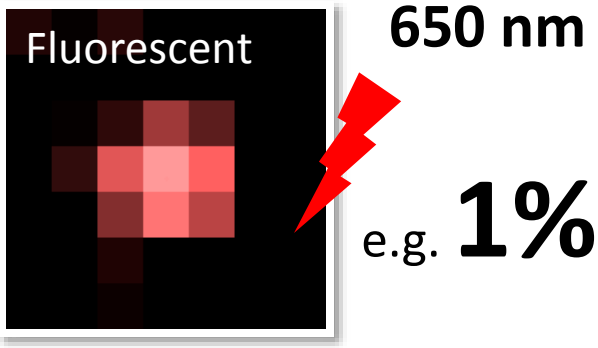


Nyquist criteria ✓

Point to point distance < ½ Feature size

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

Effective resolution: Contrast matters



650 nm

e.g. **1%**

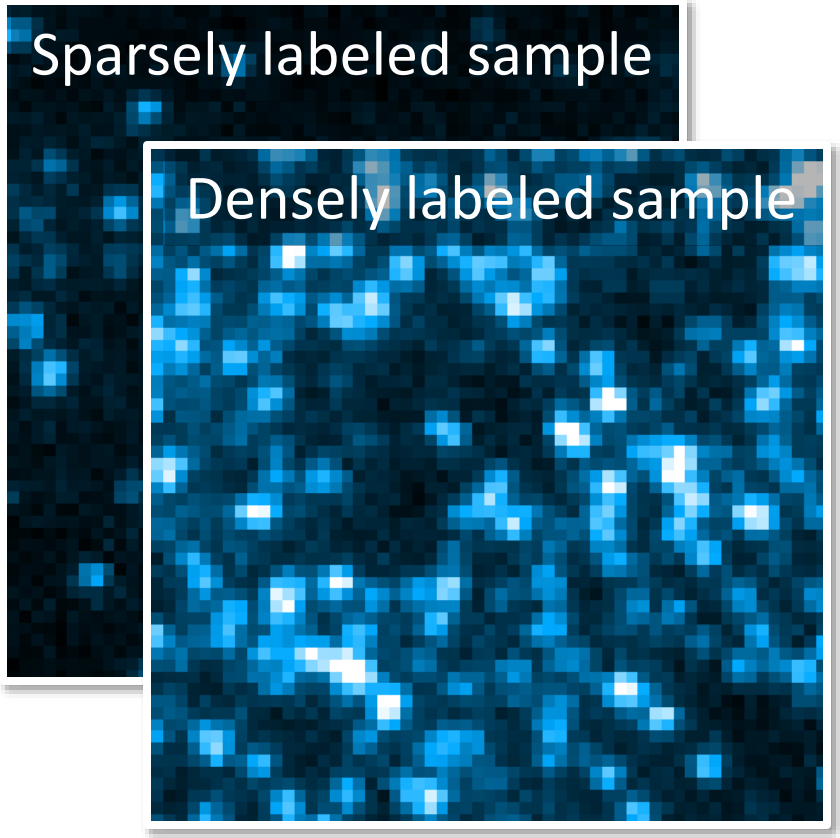
photoactivation

Deactivation

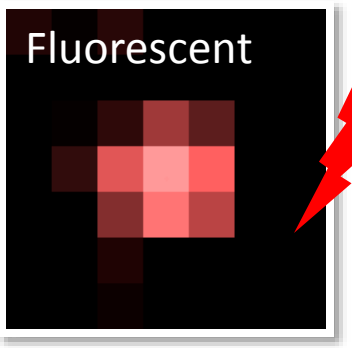
650 nm

e.g. **99%**

1% means…



Effective resolution: Contrast matters

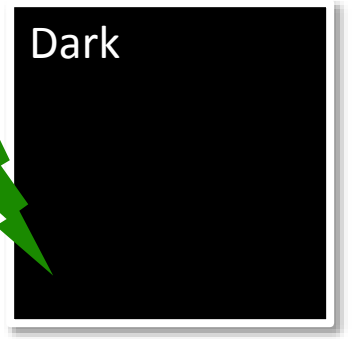


650 nm

e.g. **1%**

photoactivation

Deactivation



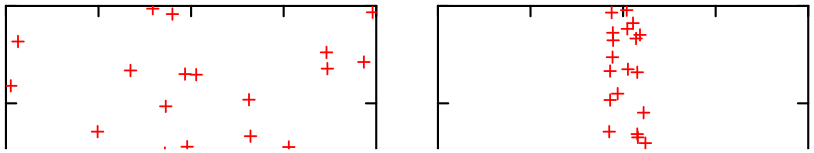
650 nm

e.g. **99%**

1% means…

Homogeneous sample

Microtubule



Common blinking dyes: >3%

Cy5 + mercaptoethylamine: 0.1-0.2%

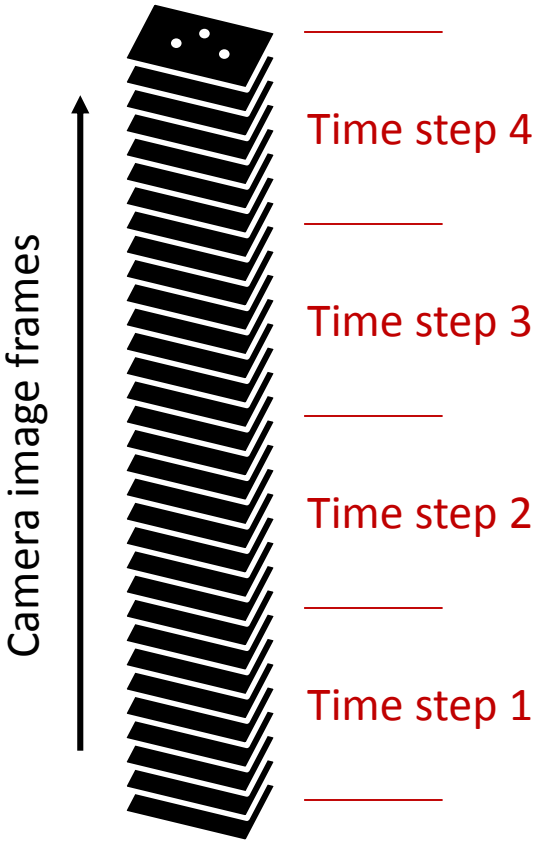
mEosFP: 0.001%

40 nm

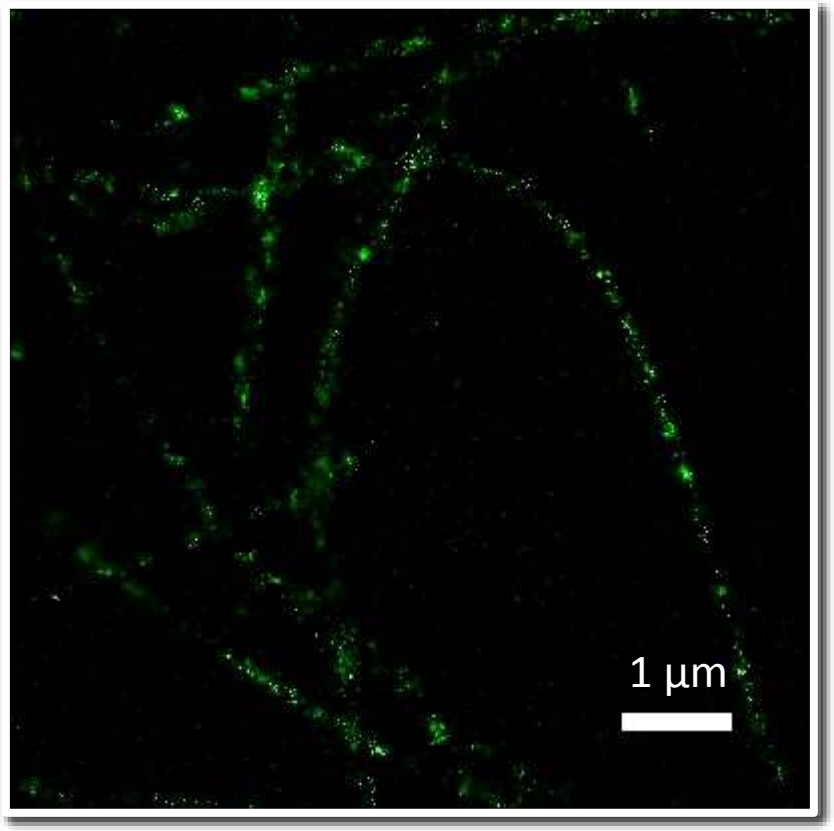
14 nm

Live Cell STORM/PALM

Live cell STORM/PALM



mEos2 labeled microtubule in live S2 cells

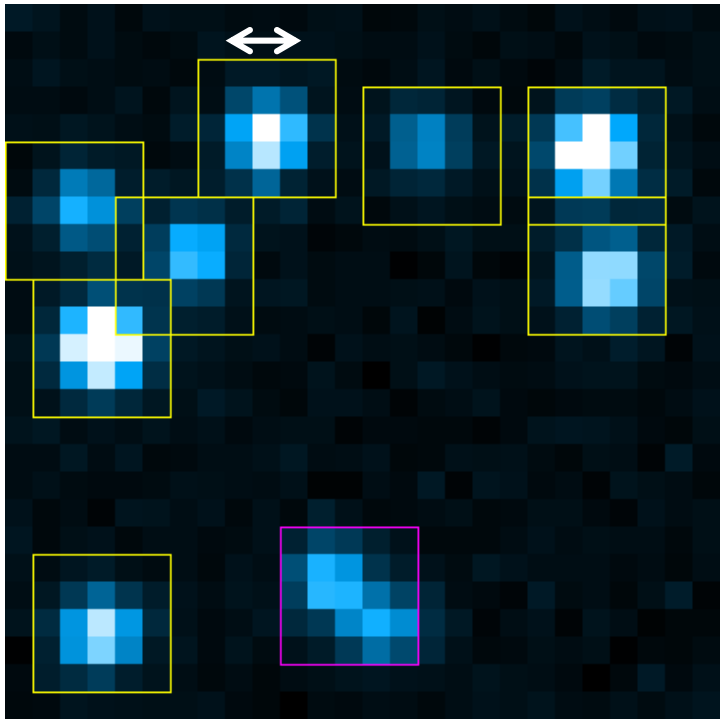


60 frames/sec
1200 frames/step (20 sec time resolution)
50x real time

Wei Zhang, Daniel Elnatan

Spatial-temporal resolution trade-off

FWHM ≈ 320 nm



0.68 molecules per μm²

Assuming:

1 molecule occupies 500 × 500 nm

↓

1/10 occupancy ≡ 0.4 point per μm²·frame

↓

70 nm resolution ≡ 800 per μm² ≡ 2000 frames

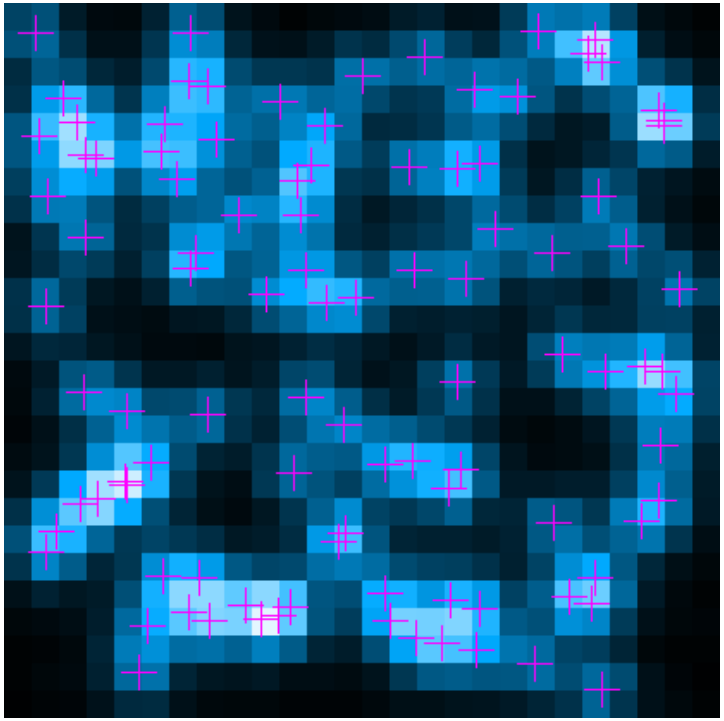
↓

100 fps = 20 sec time resolution

1000 ~ 3000 fps

Spatial-temporal resolution trade-off

FWHM ≈ 320 nm



6.8 molecules per μm²

Assuming:

8 per μm²

1 molecule occupies 500 × 500 nm

↓

1/10 occupancy ≡ 0.4 point per μm²·frame

↓

70 nm resolution ≡ 800 per μm² ≡ 2000 frames

↓

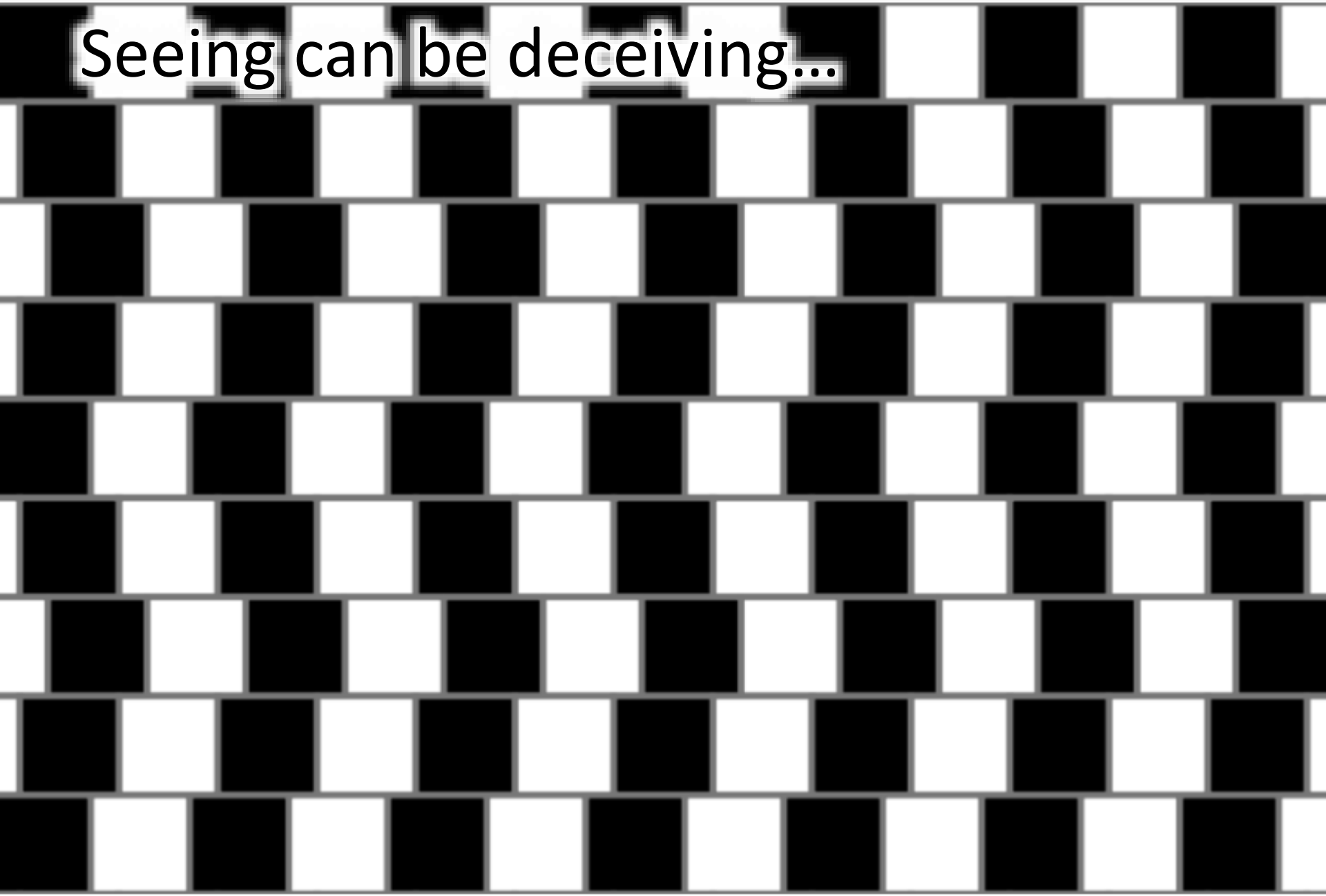
100 fps = 20 sec time resolution

Comparison of time resolution

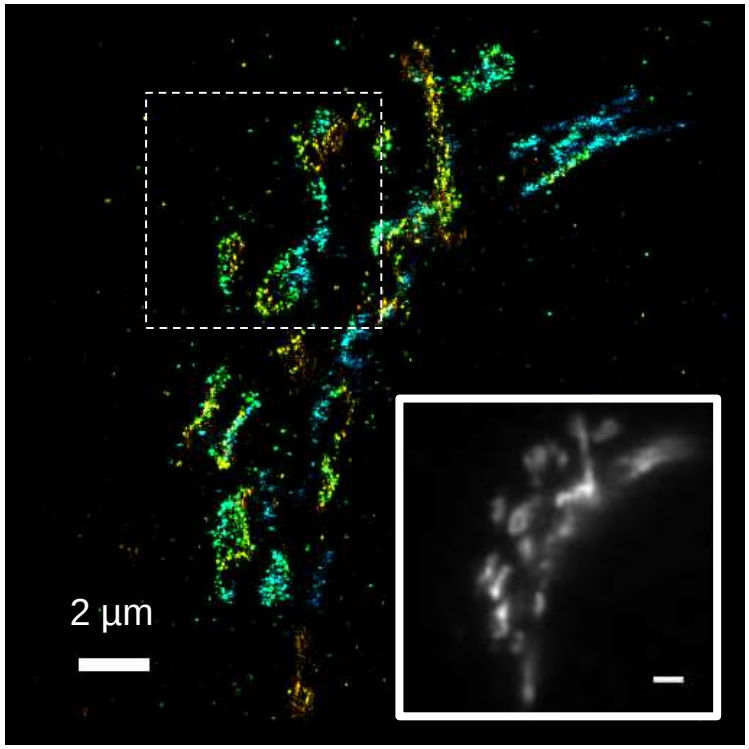
2D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	9 frames (0.09 sec)
STED	Scanning	60 nm	1 x 2 µm: 0.03 sec 10 x 20 µm: 3 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec)

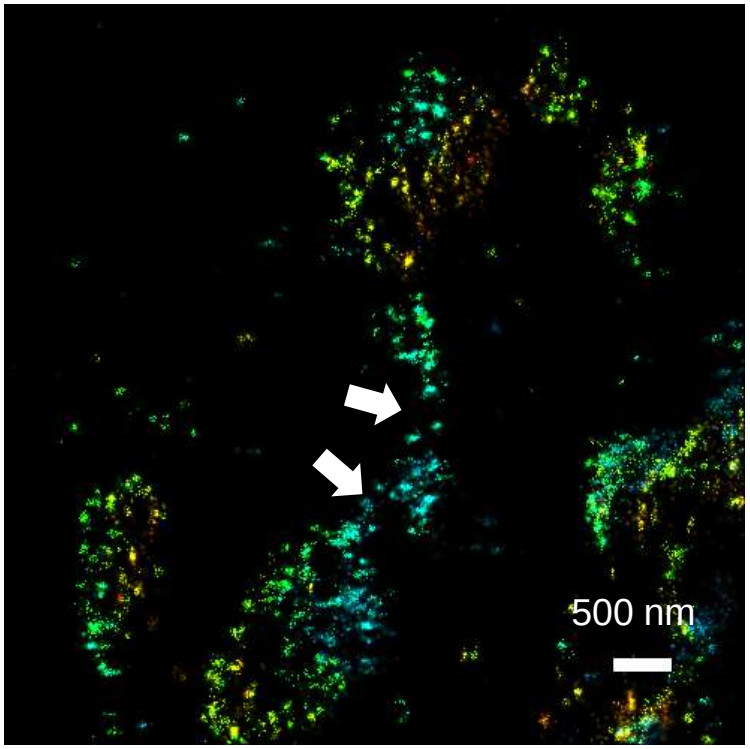
3D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	15 frames x 10 (1.5 sec)
STED	Scanning	60 nm	1 x 2 x 0.6 µm: 0.6 sec 10 x 20 x 0.6 µm: 60 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec) – no scan!

Multipoint scanning RESOLFT…


Seeing can be deceiving…

Super-resolved artifacts: sparse labeling

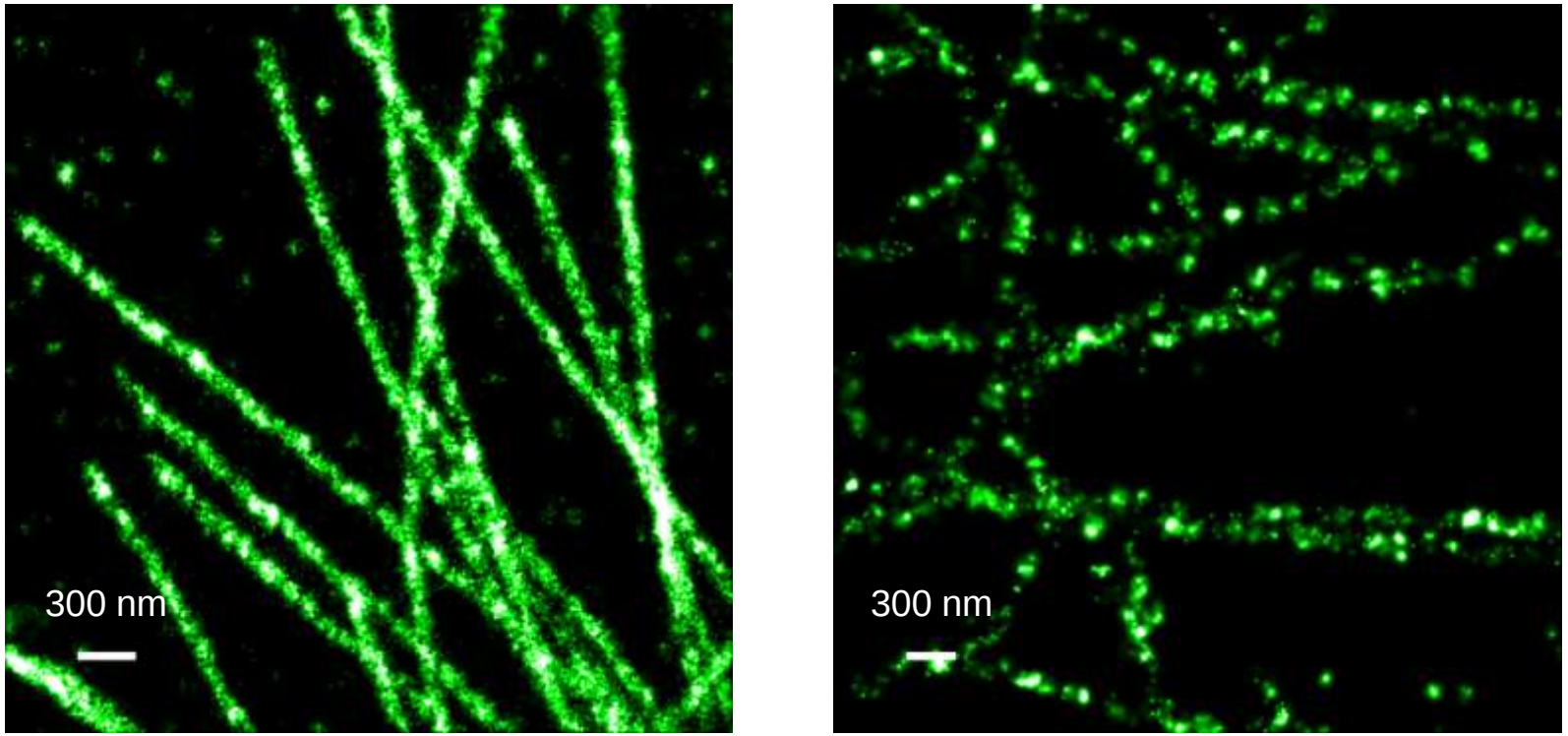




Golgi: Giantin immunofluorescence

Kamiyama and Huang, *Development Cell*, 2012

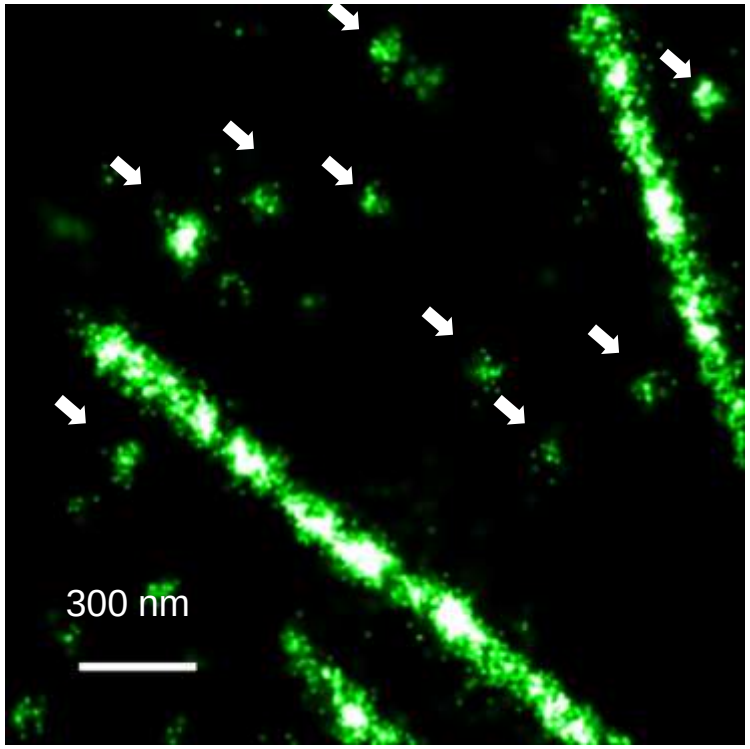
Super-resolved artifacts: poor fixation



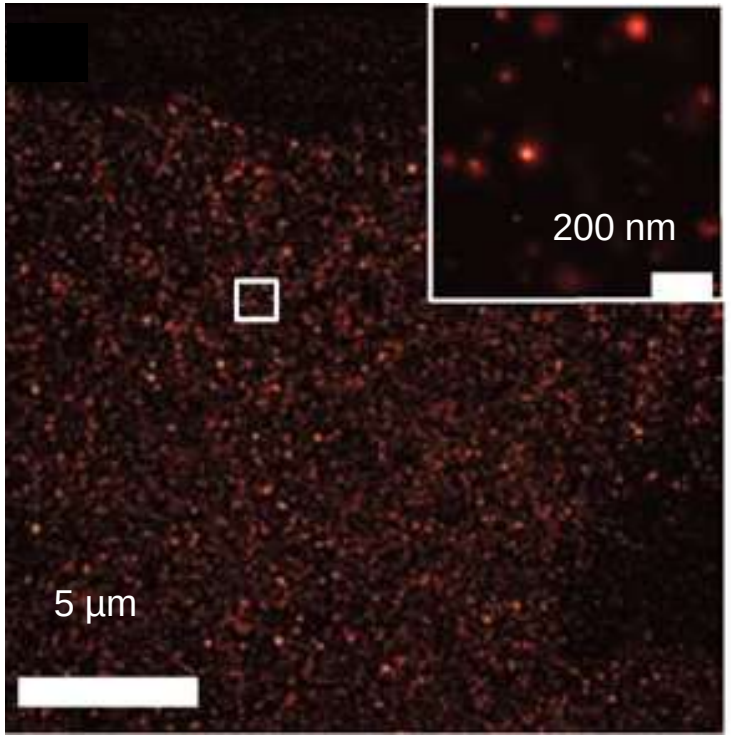
Microtubules: good and bad fixation

Kamiyama and Huang, *Development Cell*, 2012

Super-resolved artifacts: clustering



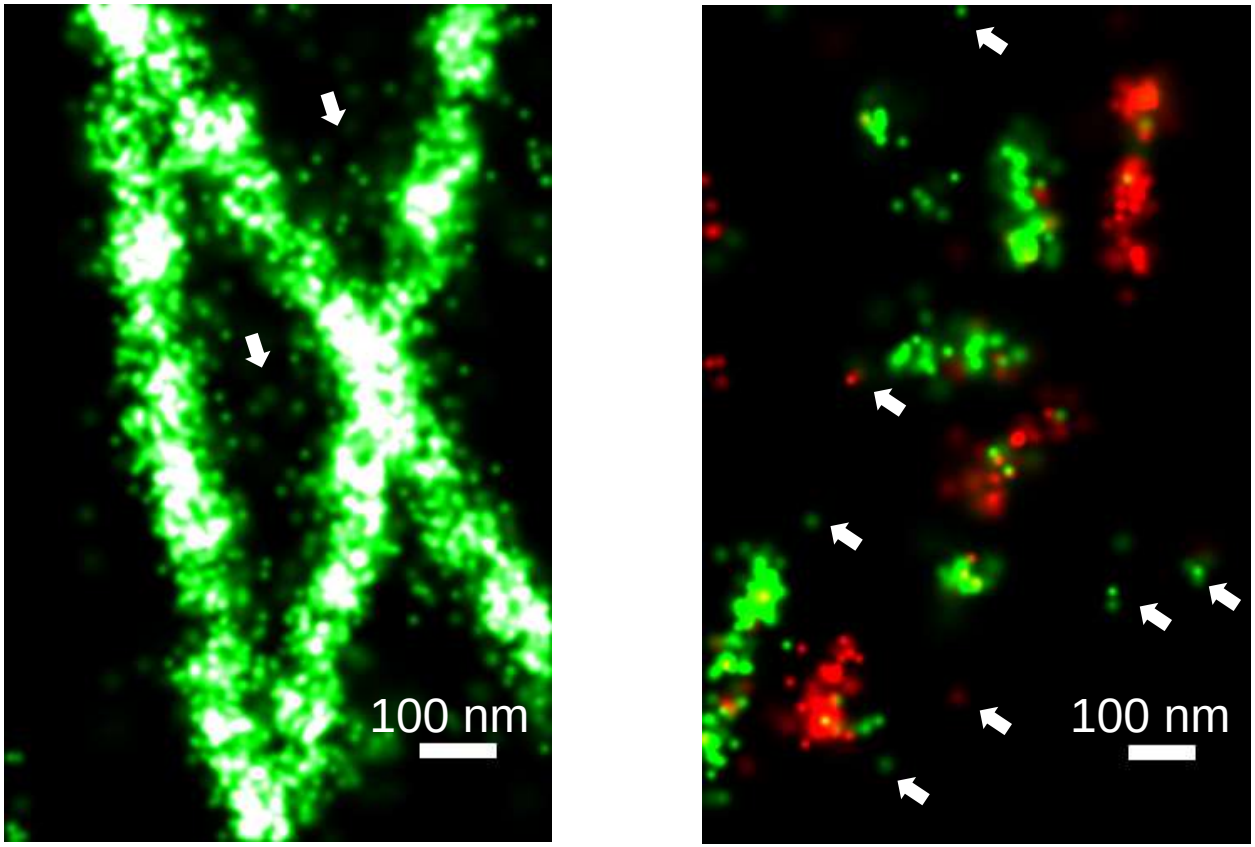
Clusters from single antibodies



Clusters from blinking FPs

Kamiyama and Huang, *Development Cell*, 2012

Super-resolved artifacts: “noise” points



Noise from misidentified molecules, crosstalk and background

Kamiyama and Huang, *Development Cell*, 2012

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