

STED

nanoscopy

SIM

GSD

RESOLFT

SSIM

Super-resolution

STORM

GSDIM

dSTORM

PALMIRA

FPALM

SMACM

BBB

PAINT

SPRAINT

SOFI

CALM

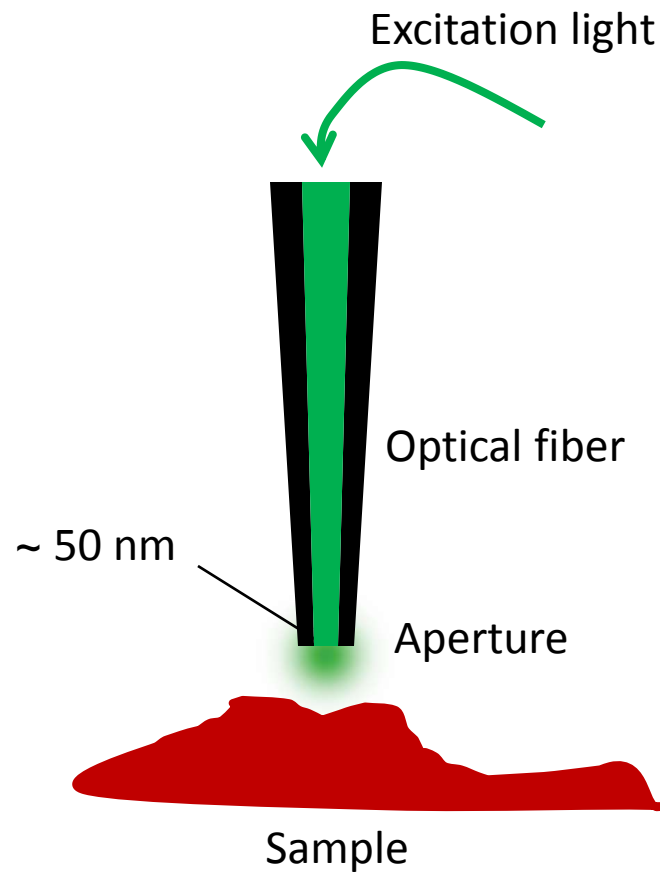
BALM

PALM

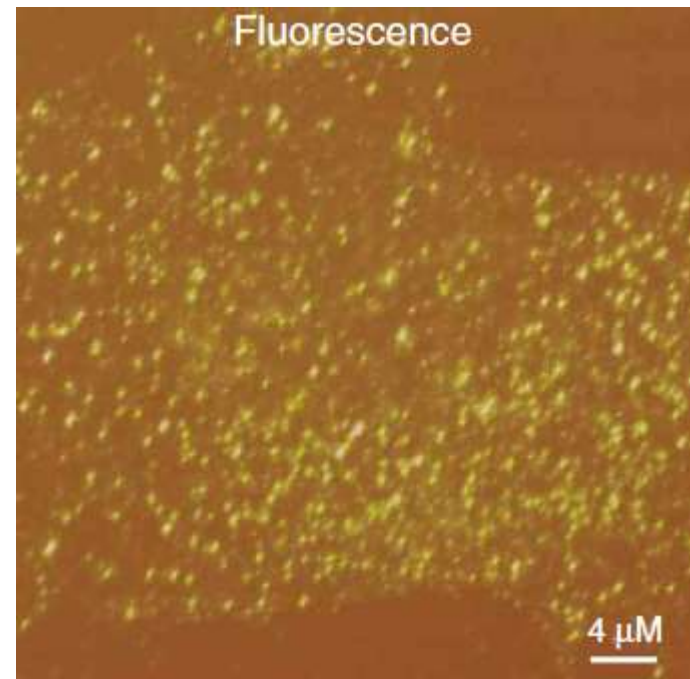
50 years to extend the resolution

- Confocal microscopy (1957)
- Near-field scanning optical microscopy (1972/1984)
- Multiphoton microscopy (1990)
- 4-Pi microscopy / I⁵M (1991-1995)
- Structured illumination microscopy (2000)
- Negative refractive index (2006)

Near-field scanning optical microscopy



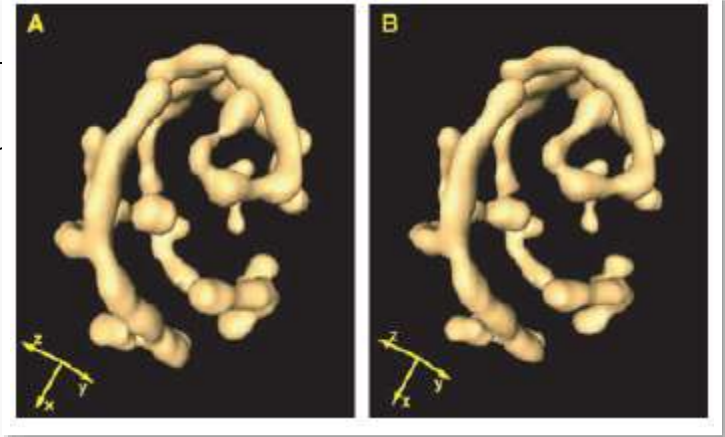
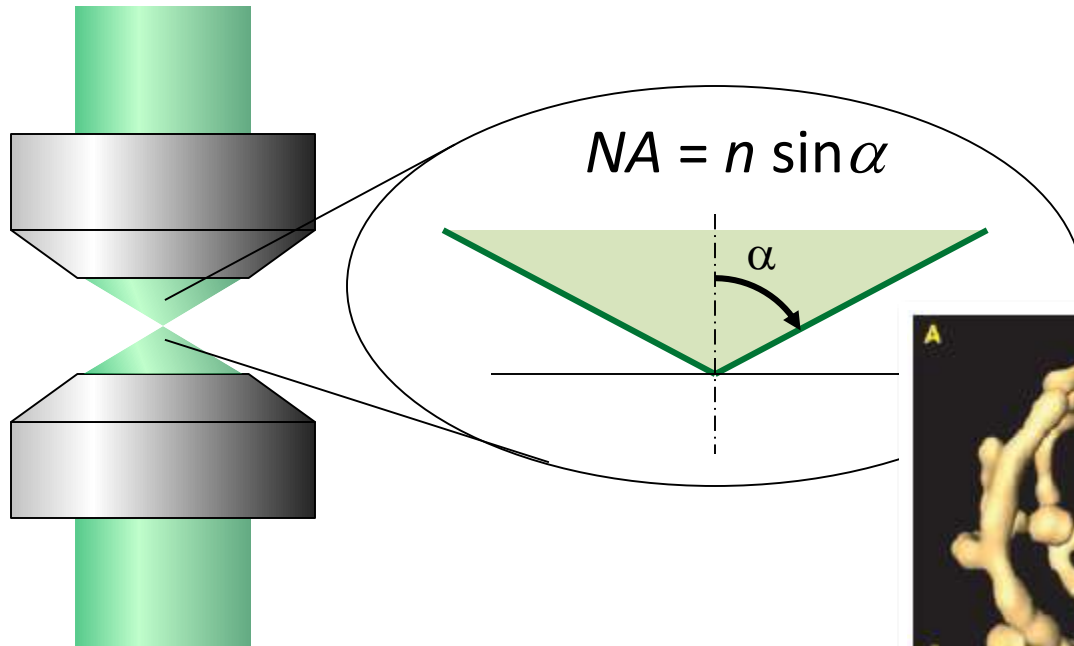
β_2 adrenergic receptor clusters
on the plasma membrane



Ianoul et al., 2005

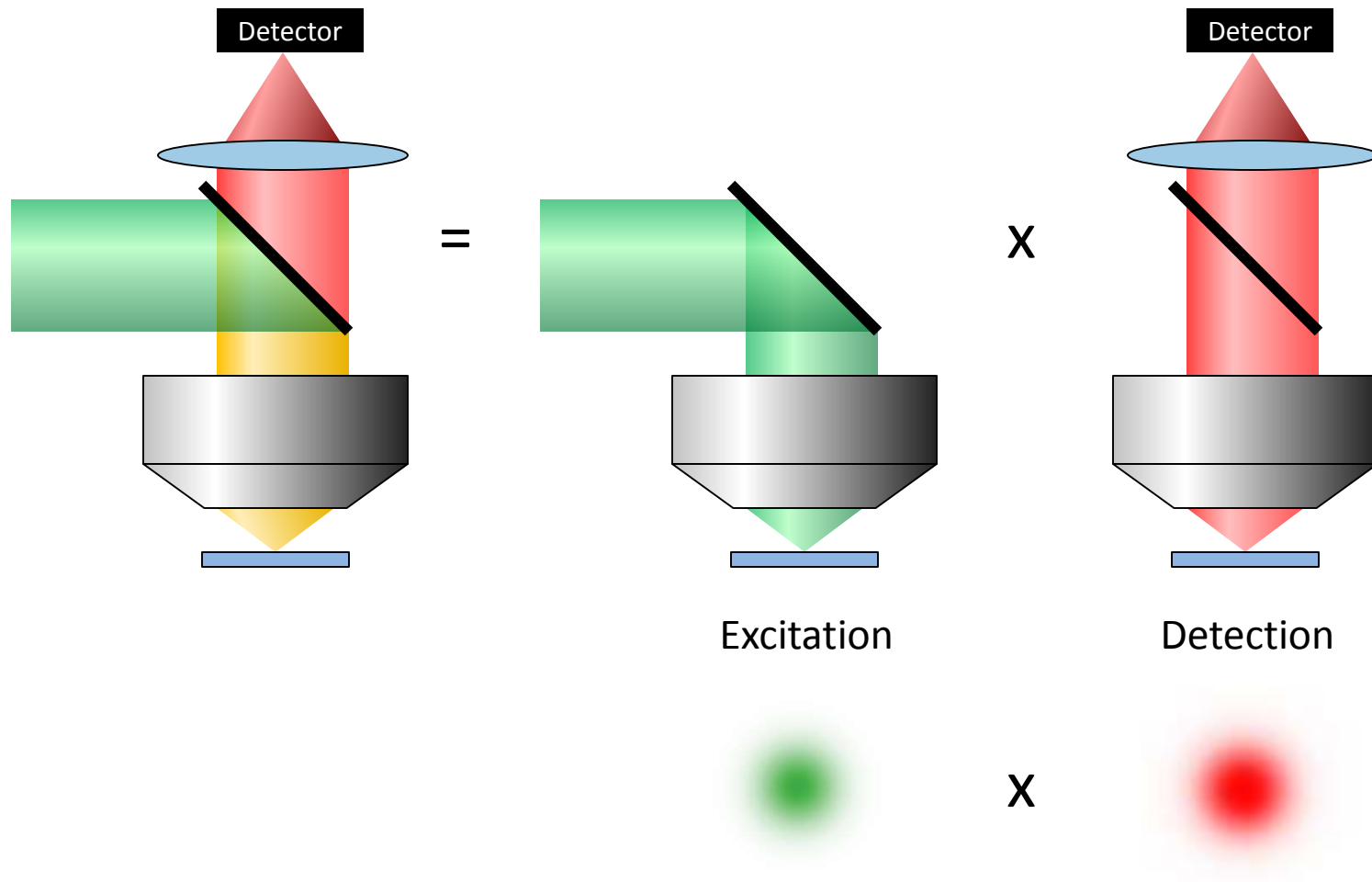
4-Pi / I⁵M

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

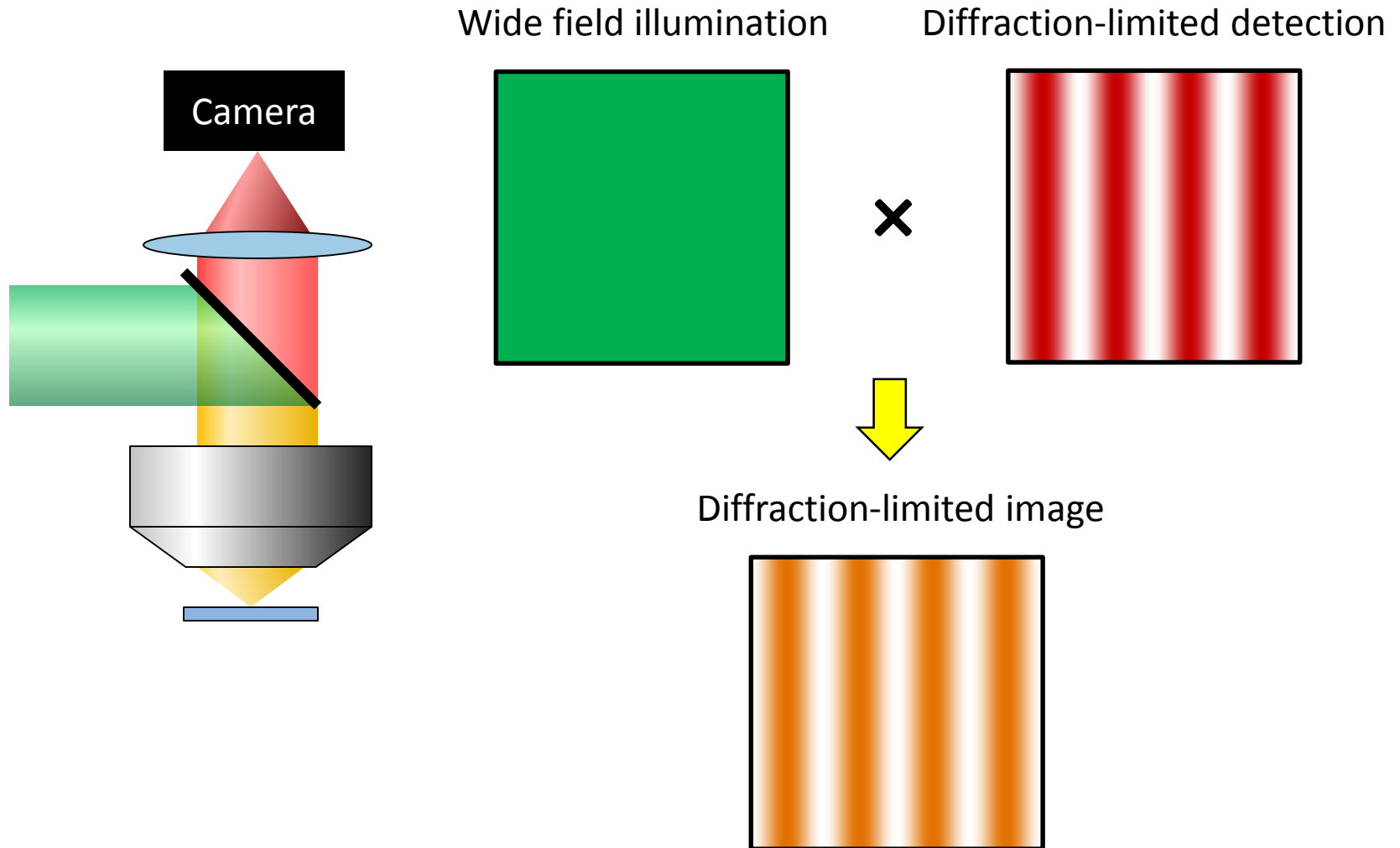


Major advantage:
Similar z resolution as x-y resolution

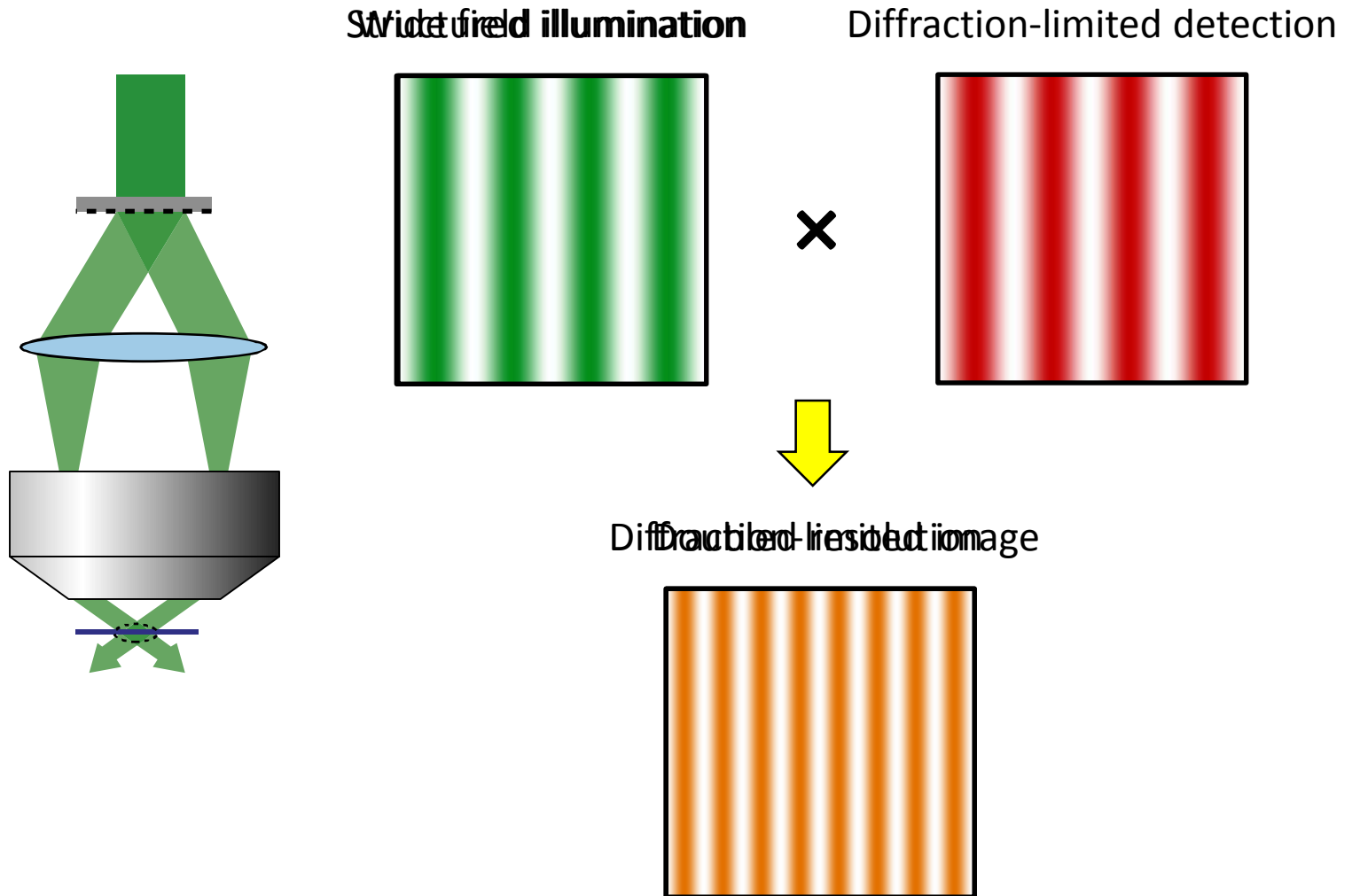
Patterned illumination



Structured Illumination Microscopy (SIM)

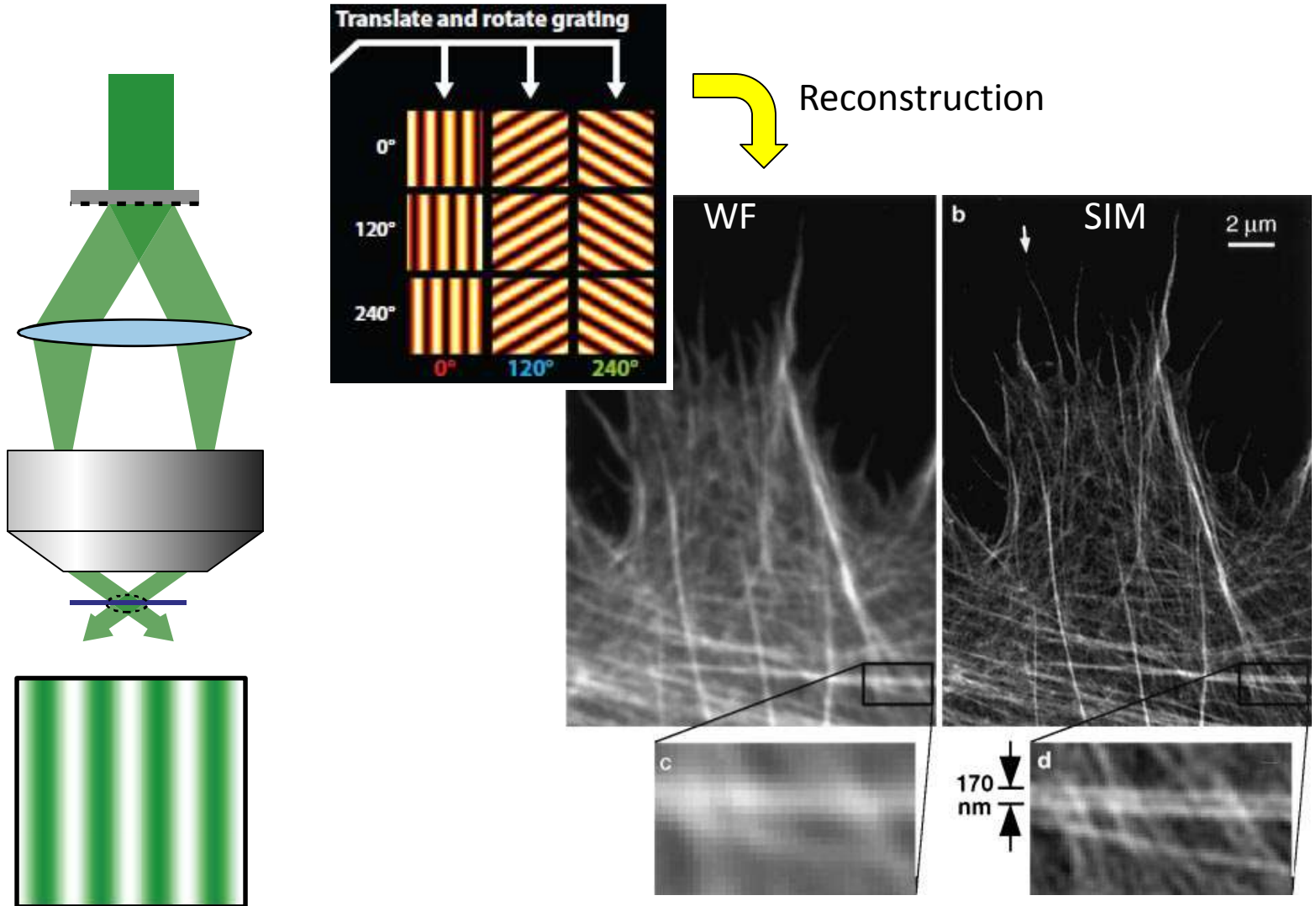


Structured Illumination Microscopy (SIM)



Structured Illumination Microscopy (SIM)

Multiple angles and phases

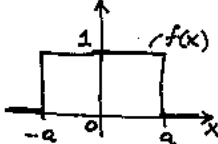


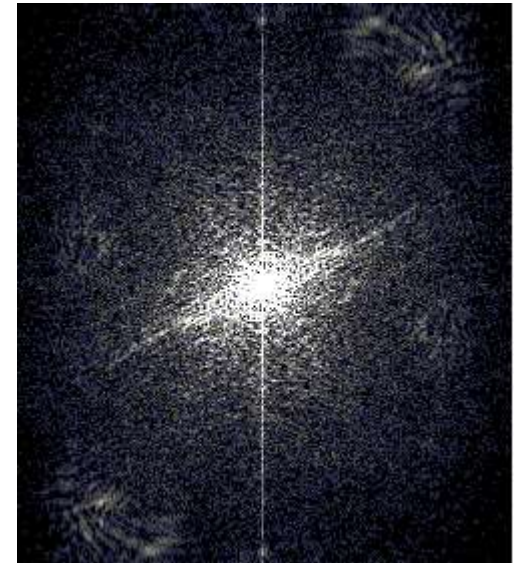
Being (slightly) more rigorous about SIM



Fourier transforms: Examples

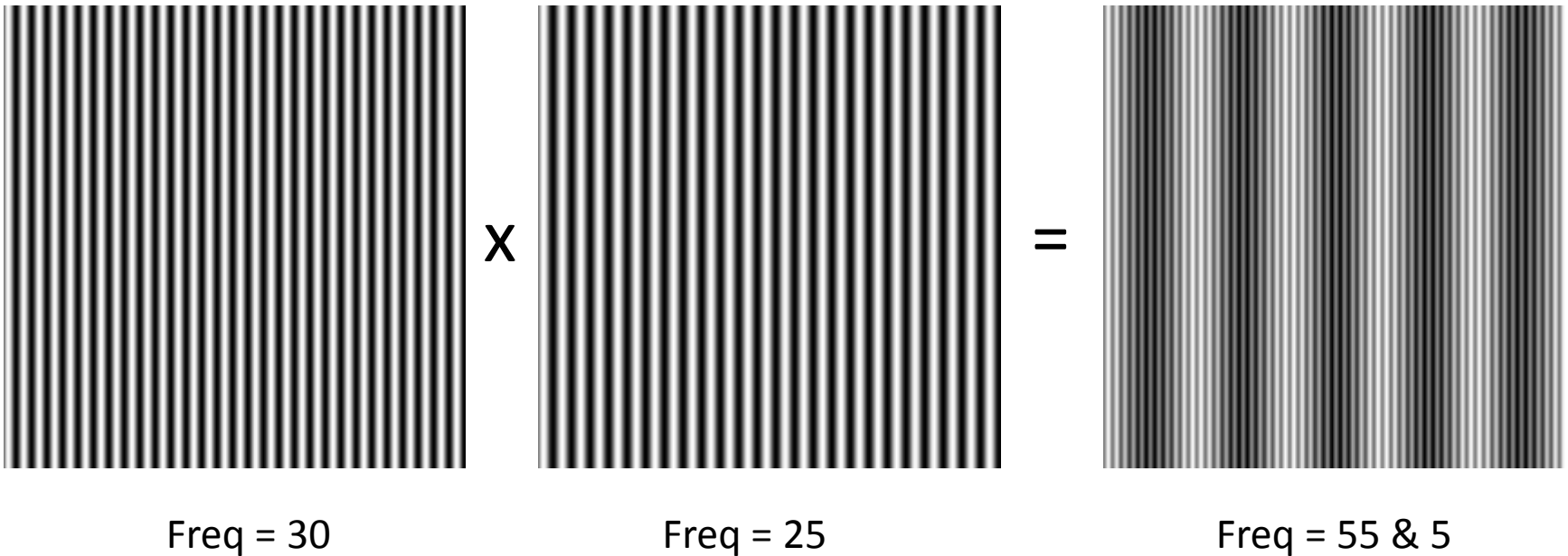
- $f(x) = \delta(x) \quad \rightarrow \quad \tilde{f}(k) = 1$
- $f(x) = 1 \quad \rightarrow \quad \tilde{f}(k) = 2\pi \delta(k)$
- $f(x) = e^{-a|x|} \quad \rightarrow \quad \tilde{f}(k) = \frac{2a}{k^2 + a^2}$
- $f(x) = \frac{1}{x^2 + a^2} \quad \rightarrow \quad \tilde{f}(k) = \frac{\pi}{a} e^{-a|k|}$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

-  $\rightarrow \quad \tilde{f}(k) = 2 \frac{\sin(ak)}{k}$



Extending the measurable freq. range

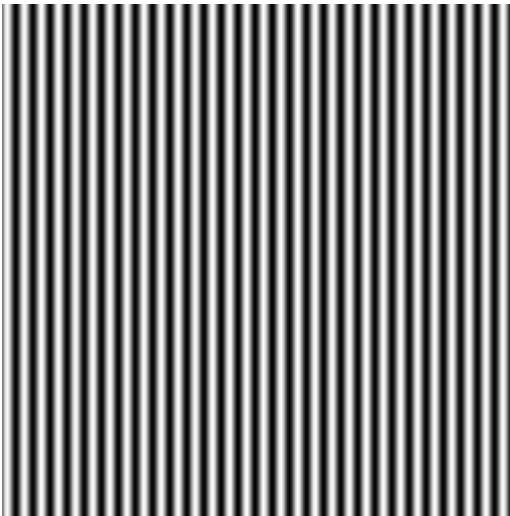
$$\text{Excitation}(x) \times \text{Sample}(x) = \text{Observed Signal}(x)$$



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

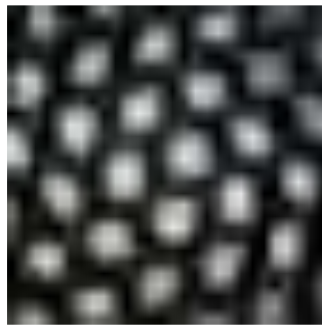
Extending the measurable freq. range

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



Freq = 30

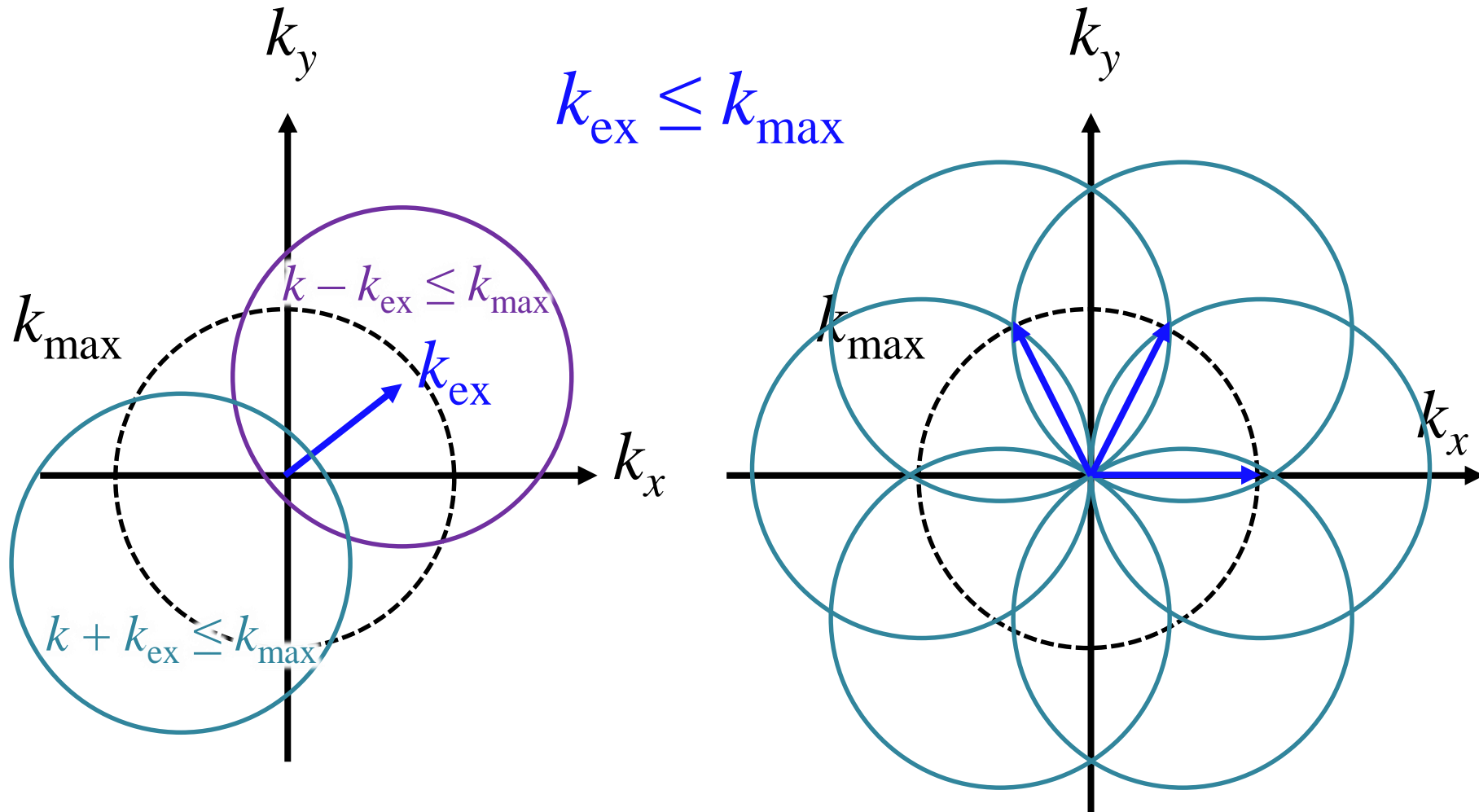
x



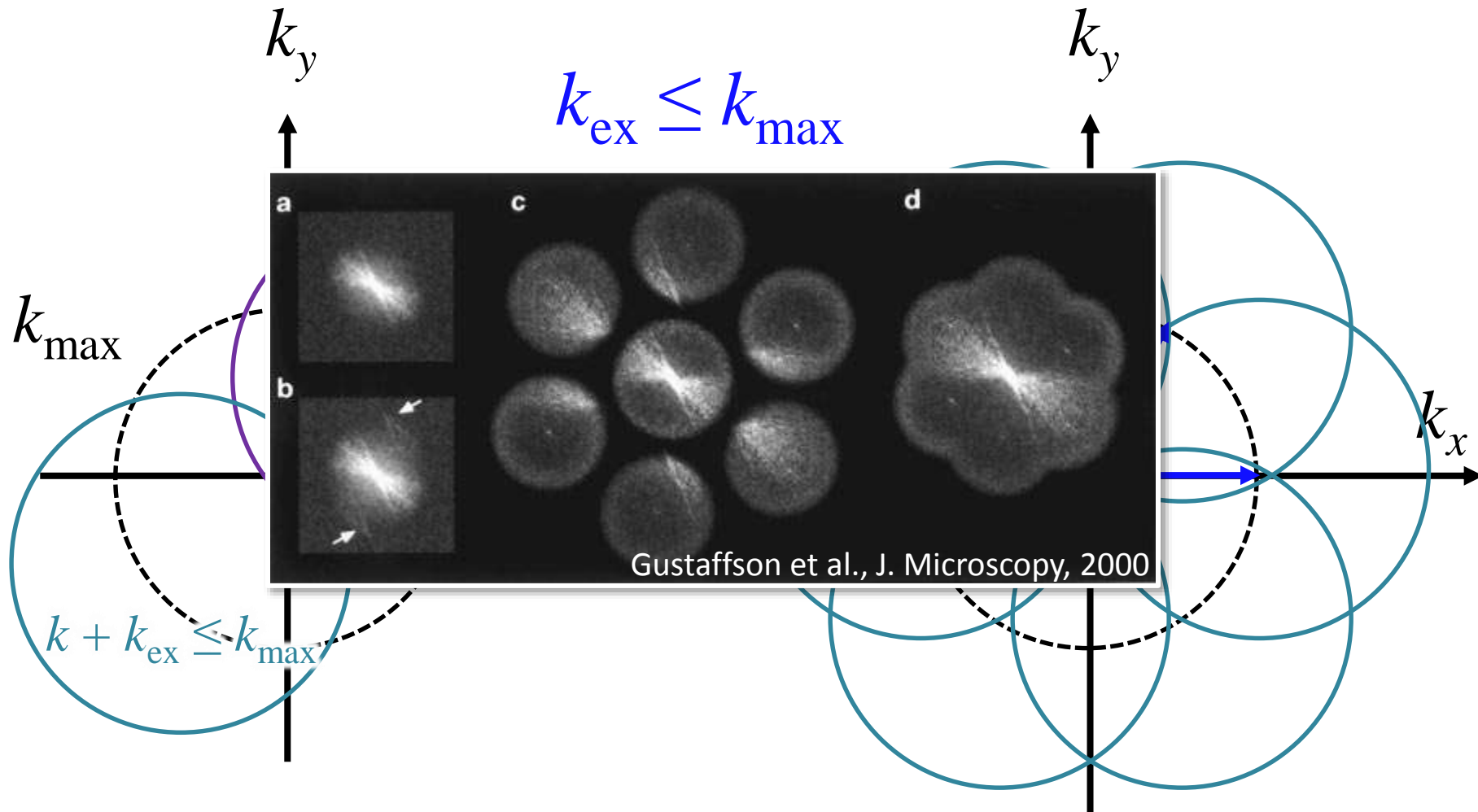
$\sin A \cdot \sin$



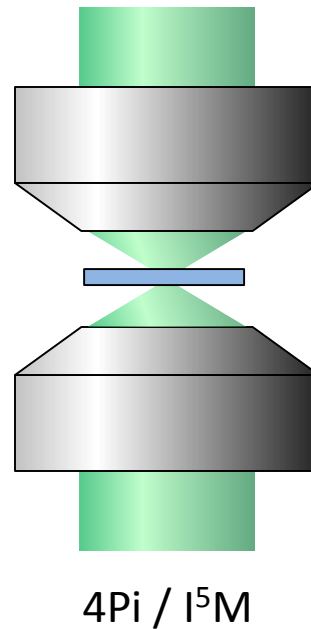
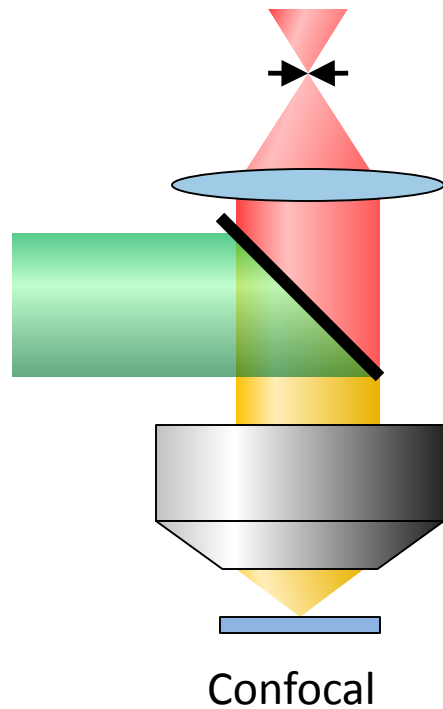
Extending the measurable freq. range



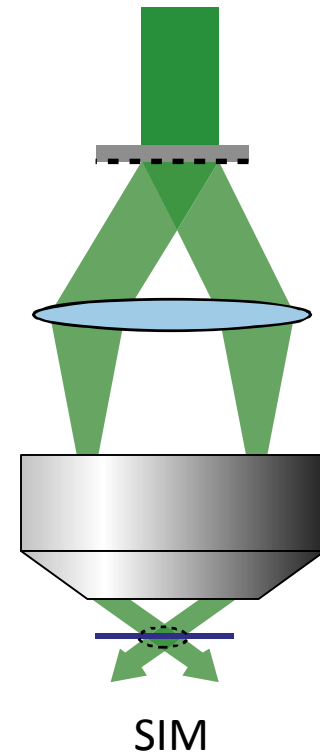
Extending the measurable freq. range



The diffraction limit still exists



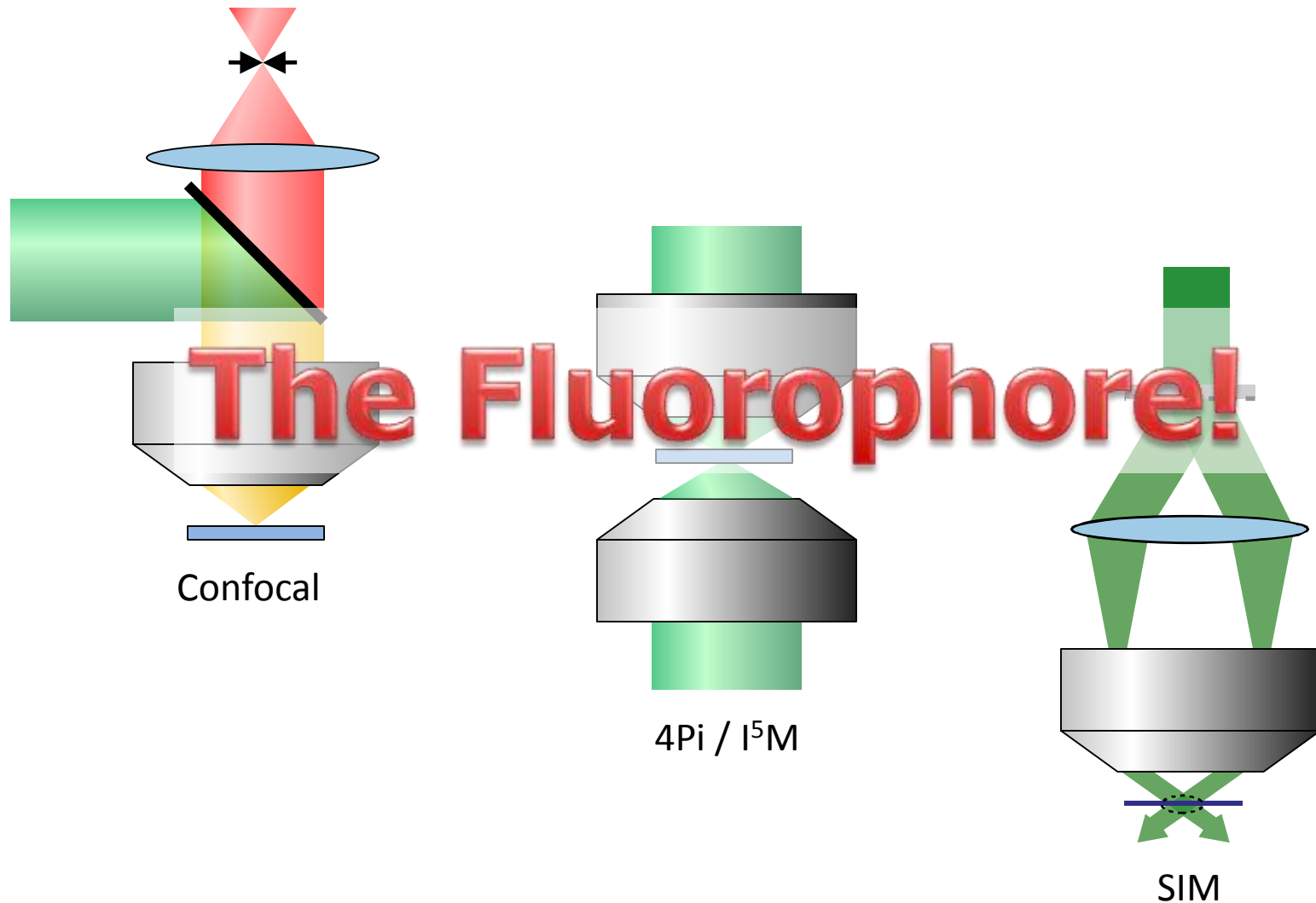
$$d \geq \frac{1}{2} \cdot \frac{\lambda}{2NA}$$



Breaking the diffraction barrier

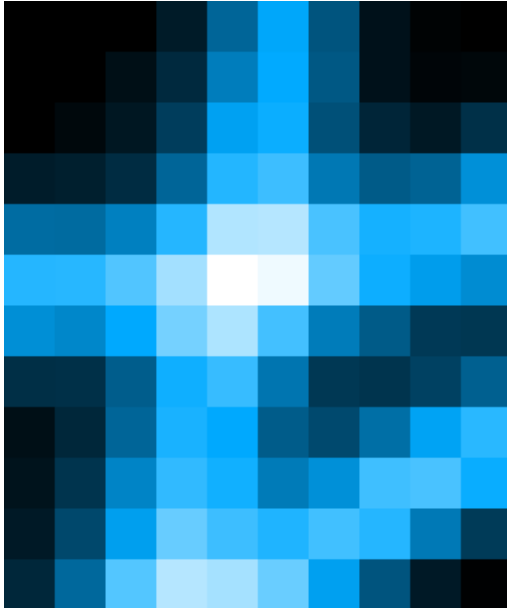


Breaking the diffraction barrier

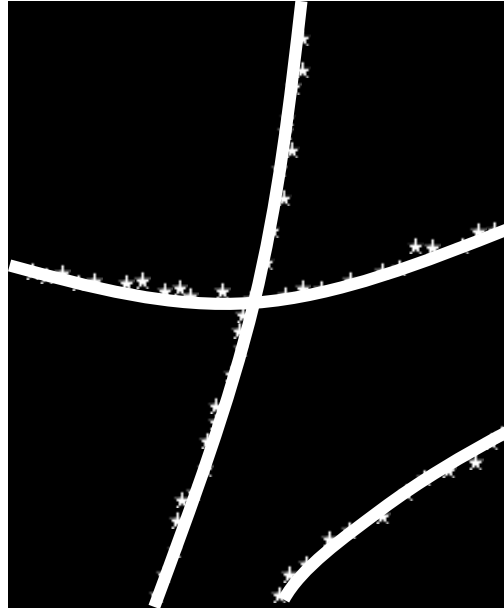


Super-resolution by...

Fluorescence image

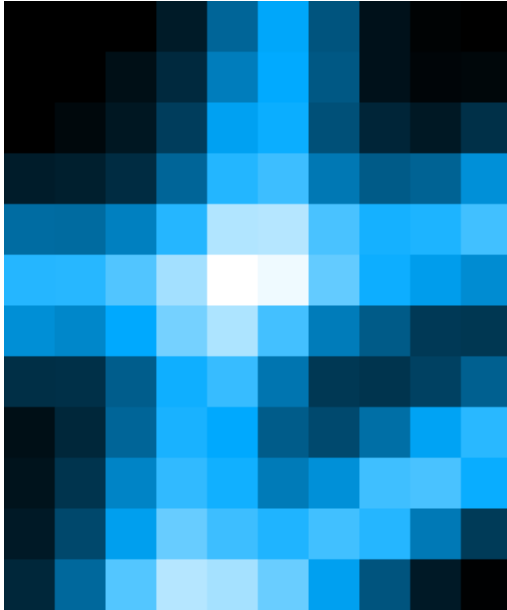


Underlying structure

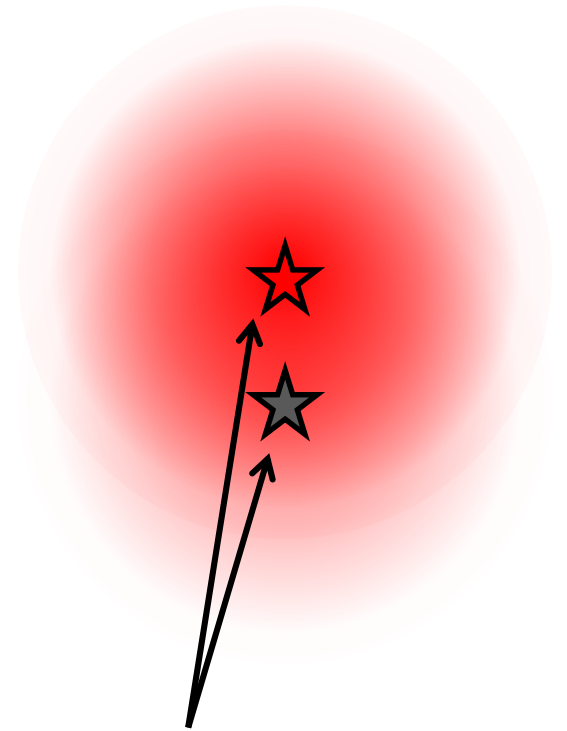
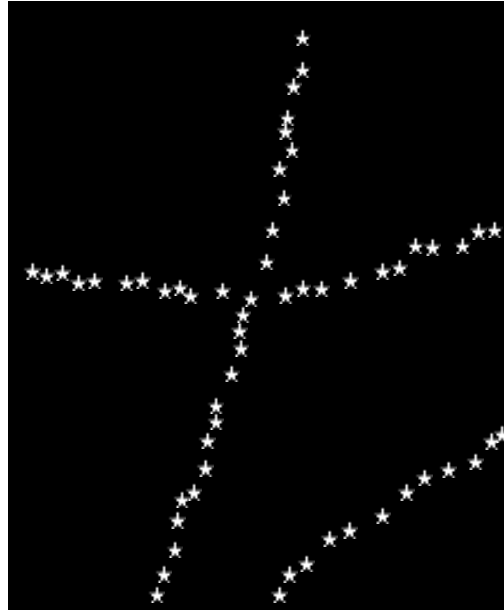


Super-resolution by spatial modulation

Fluorescence image



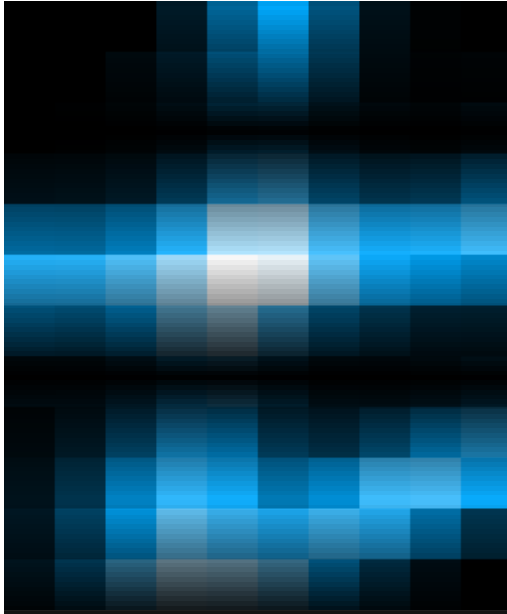
Underlying structure



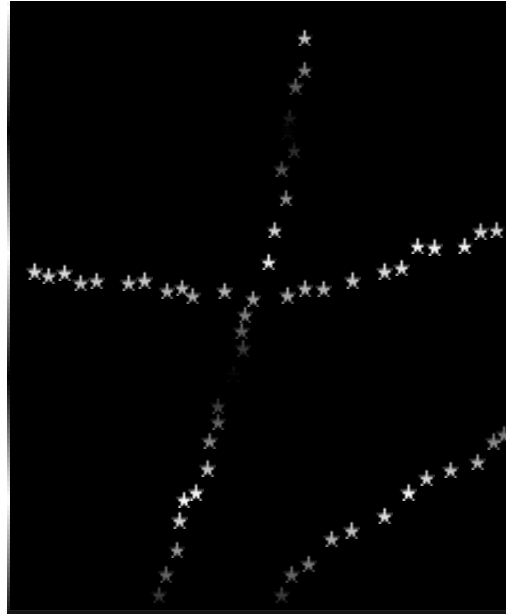
Differential modulation
of the fluorescence response

Super-resolution by differential excitation

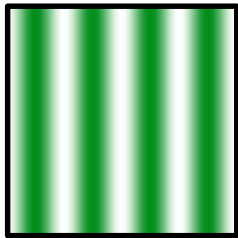
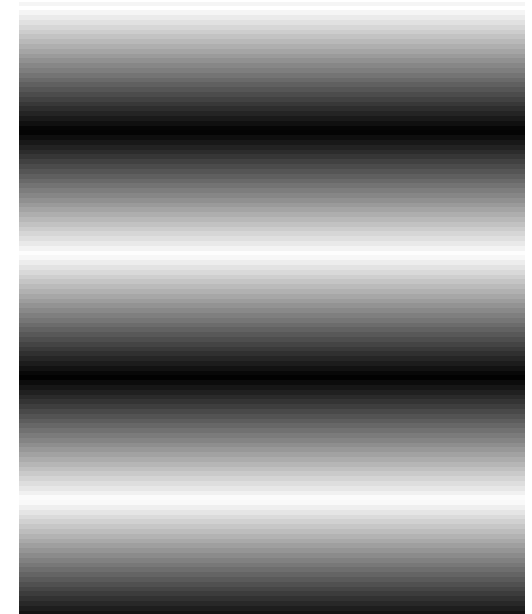
Fluorescence image



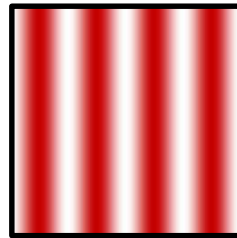
Underlying structure



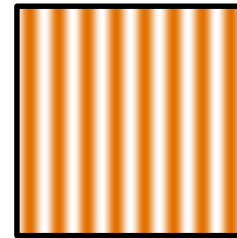
Excitation pattern



×



=



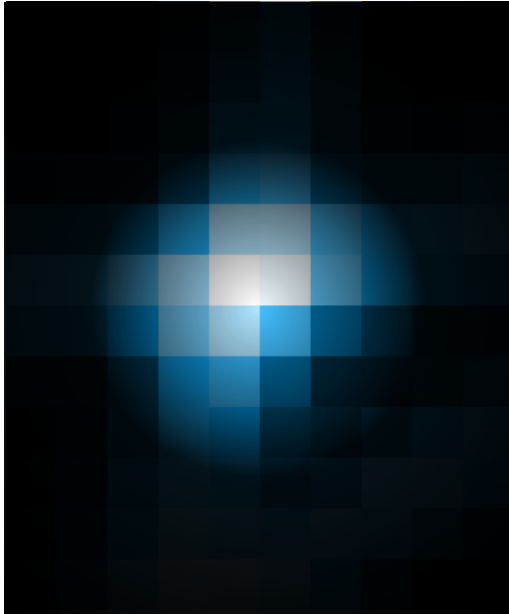
SIM (Gustafsson / Heintzmann)
SSIM (Gustafsson 2005)

Diffraction limited excitation and emission

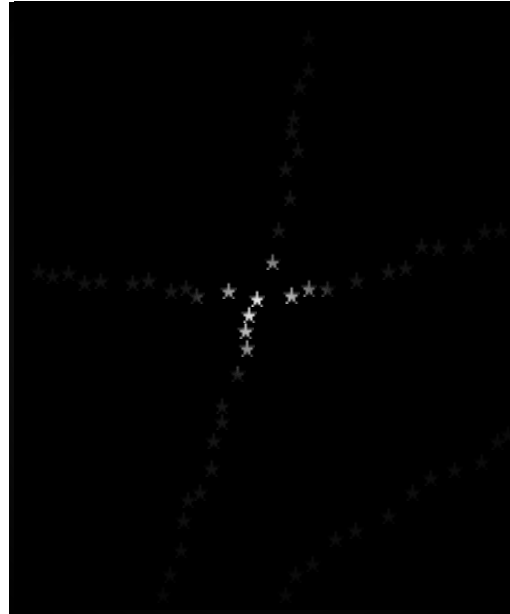
Doubled resolution

Super-resolution by differential depletion

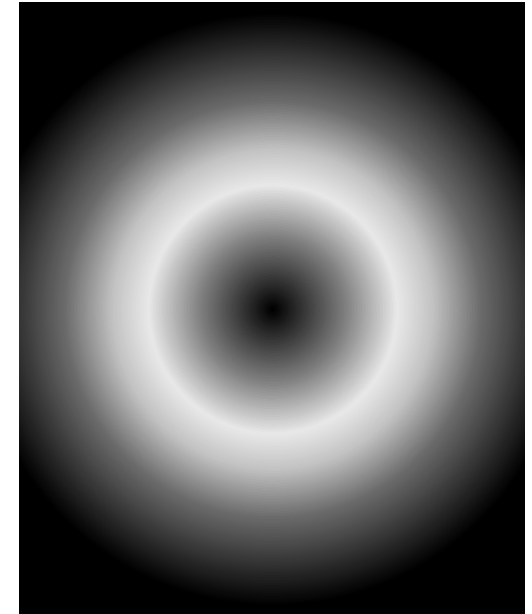
Fluorescence image



Underlying structure



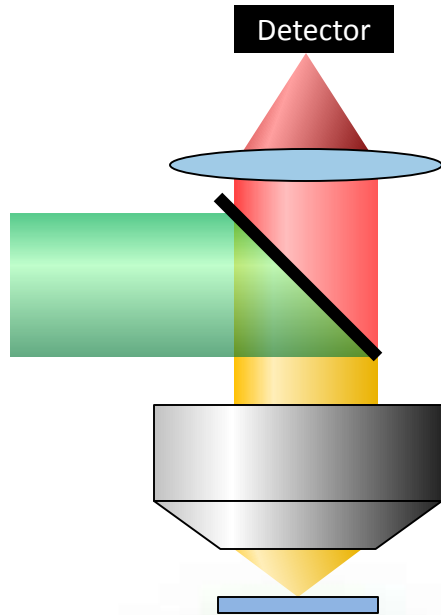
Depletion pattern



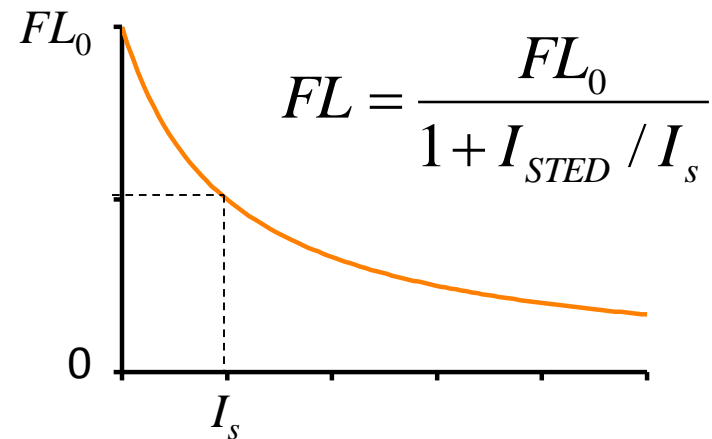
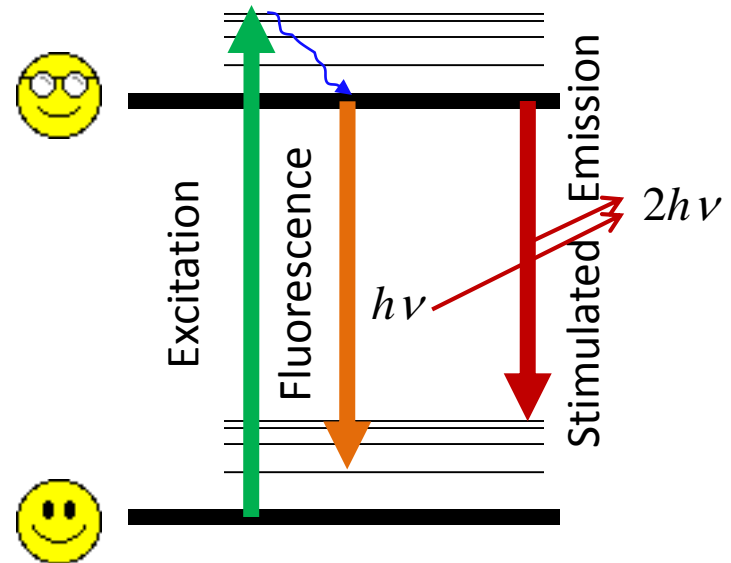
STED (Hell 1994, Hell 1999)
GSD (Hell 1995, Hell 2007)
RESOLFT (Hell 2003, Hell 2011)

Diffraction limited PSF Saturated depletion Smaller effective PSF

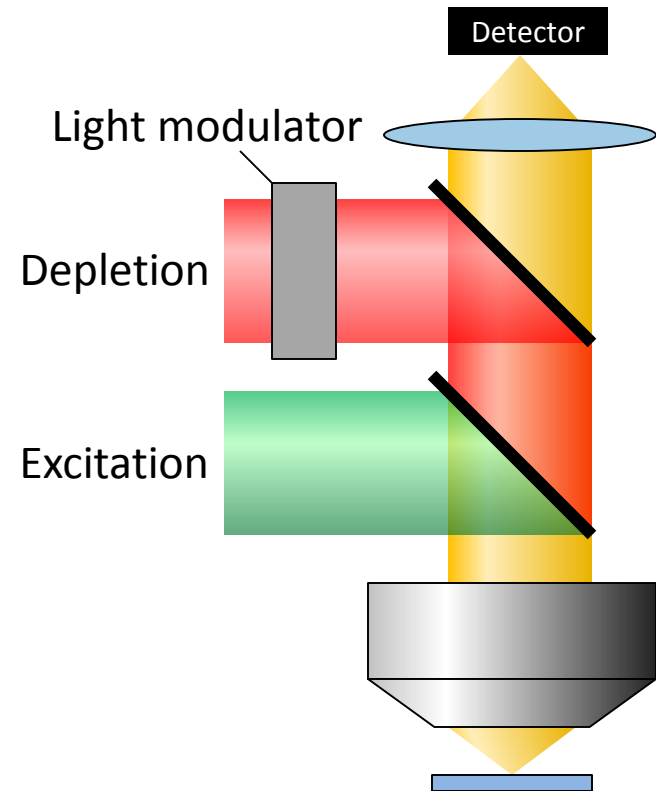
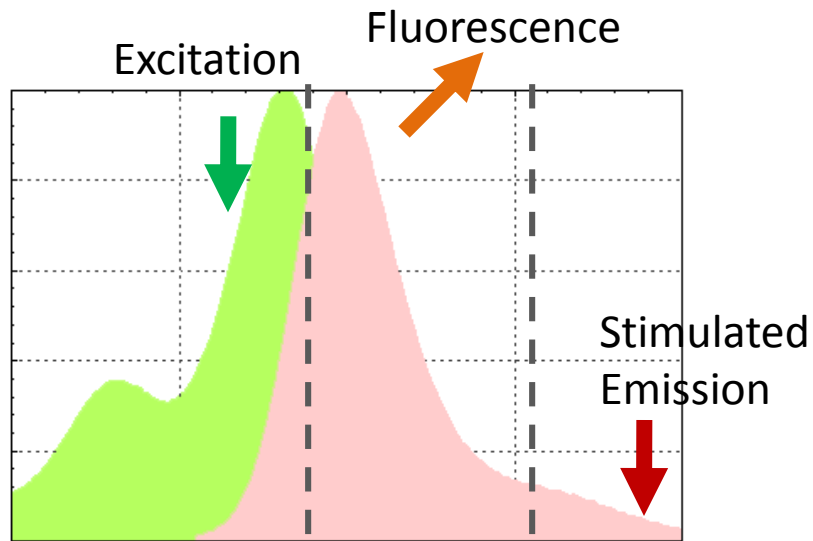
Stimulated Emission Depletion (STED)



Send to a dark state



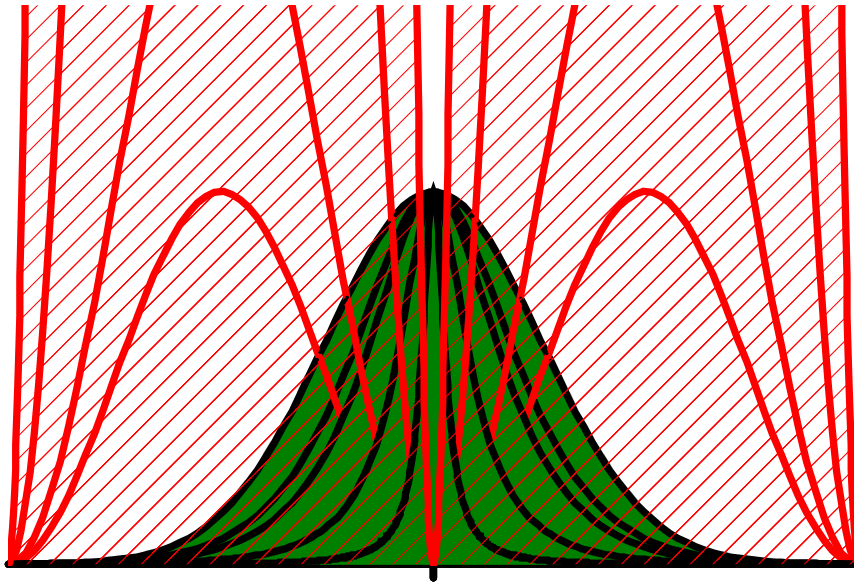
STED microscopy



Excitation STED pattern Effective PSF

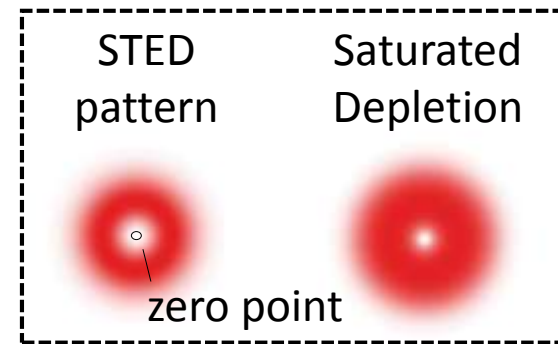
$$\text{Excitation} \div \text{STED pattern} = \text{Effective PSF} \quad ?$$

Saturated depletion

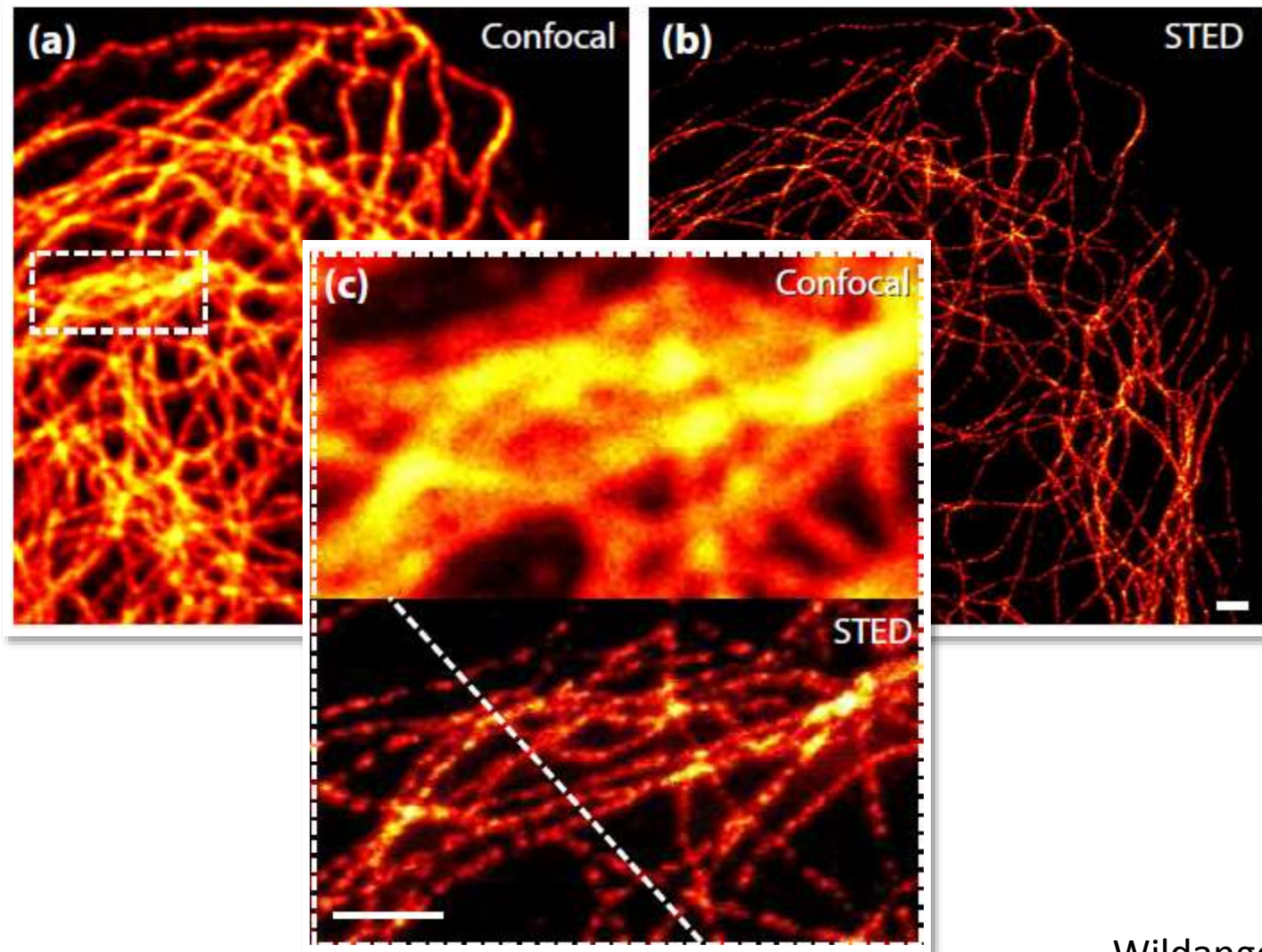


$$I_{\text{STED}} = 200 I_s$$

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

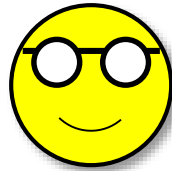


STED images of microtubules



Wildanger et al., 2009

The “patterned illumination” approach



Multiple cycles

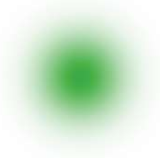


- Ground state
- Triplet state
- Isomerization etc.



Excitation

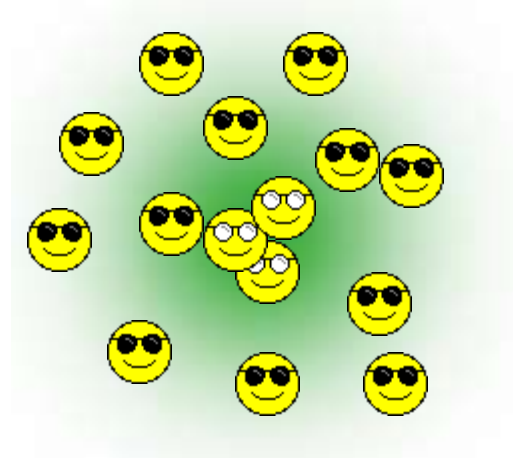
Depletion
pattern



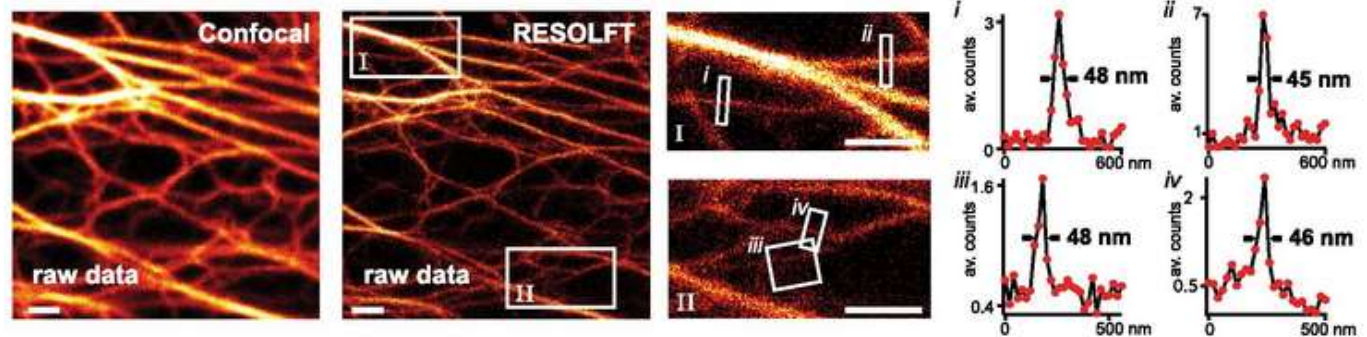
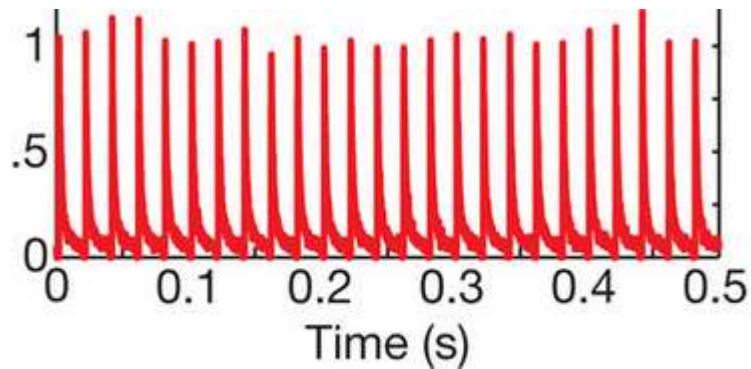
÷



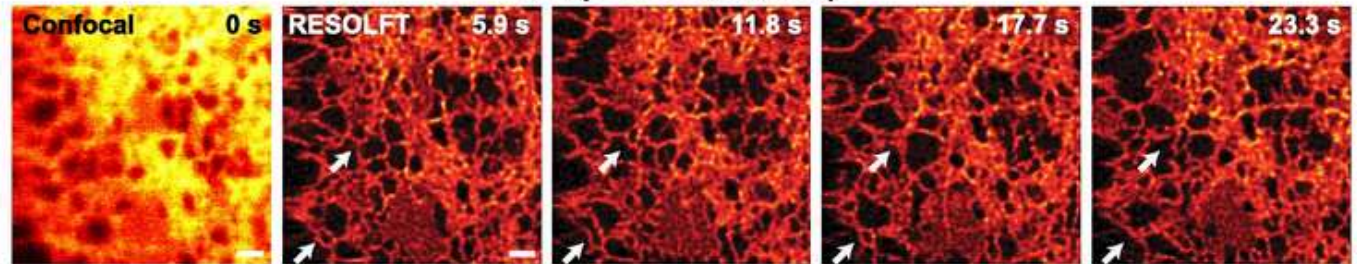
=



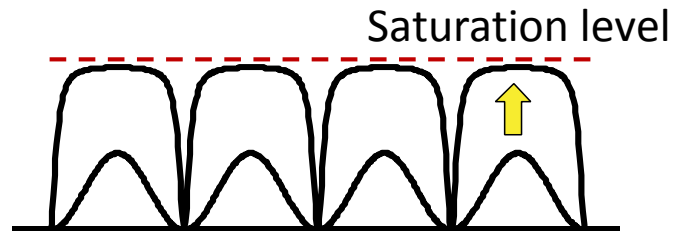
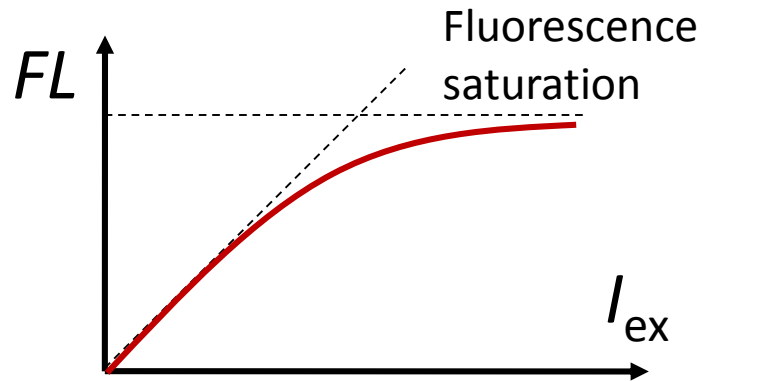
RESOLFT by rsEGFP and rsEGFP2



ER (rsEGFP2-KDEL)



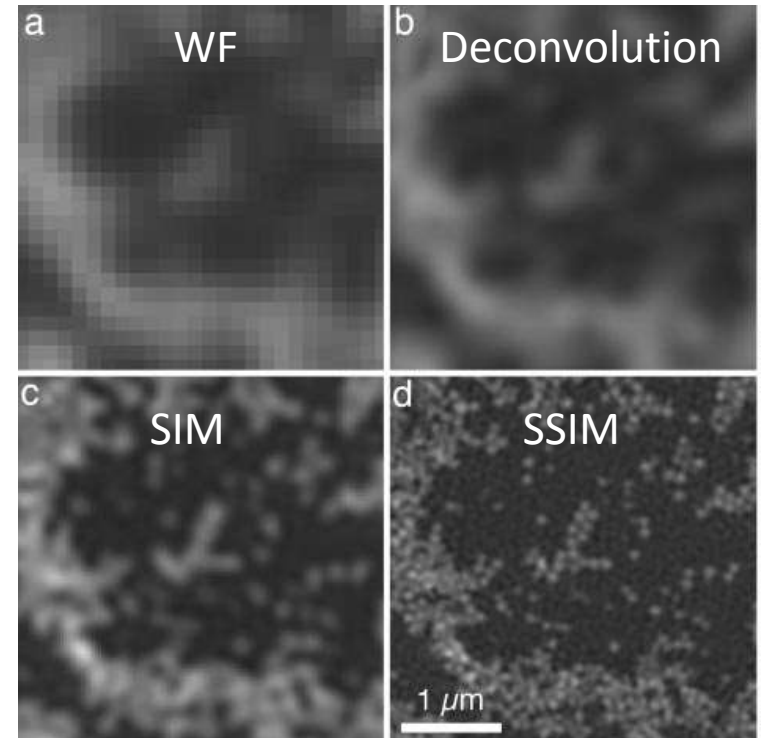
Saturated SIM



Saturated illumination pattern



Sharp zero lines



50 nm resolution

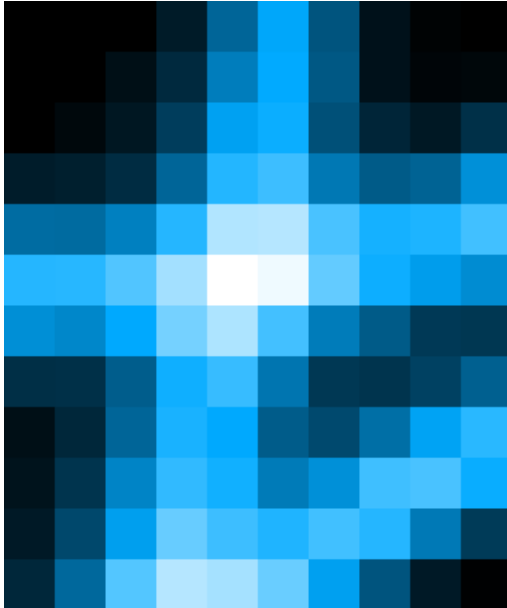
Suffers from fast photobleaching
under saturated excitation condition

Super-resolution by single-molecule switching

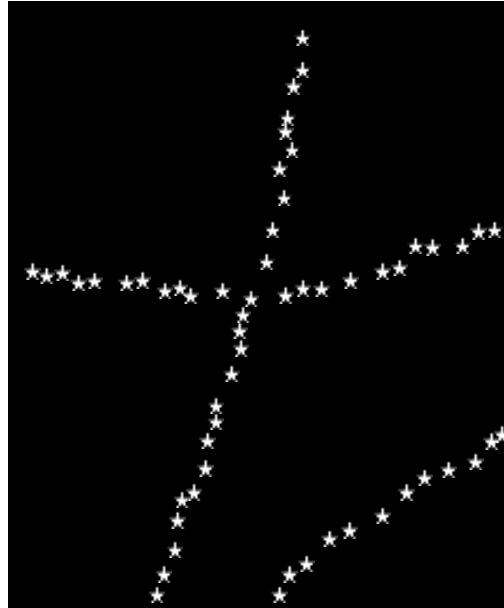


Super-resolution by single-molecule switching

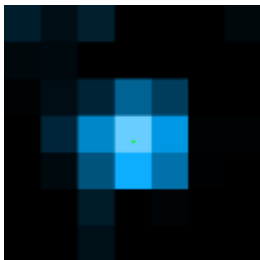
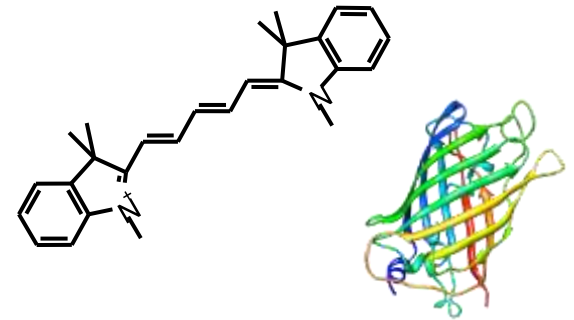
Fluorescence image



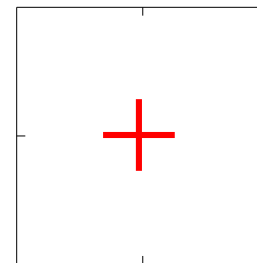
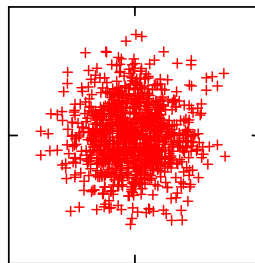
Underlying structure



Photoswitchable molecules



=



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

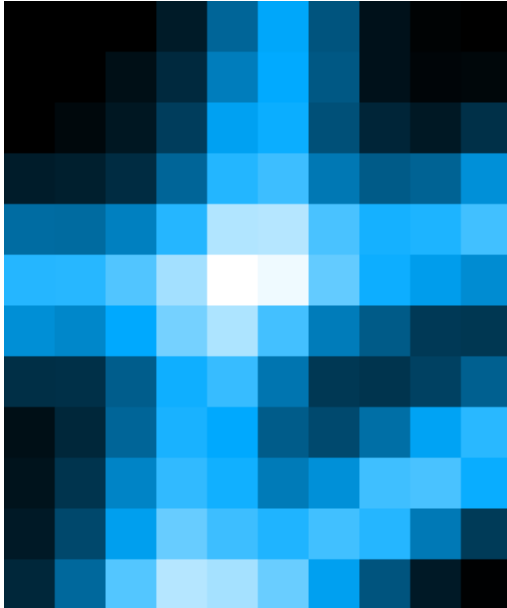
Single molecule image

N photons

Single-molecule localization

Super-resolution by single-molecule switching

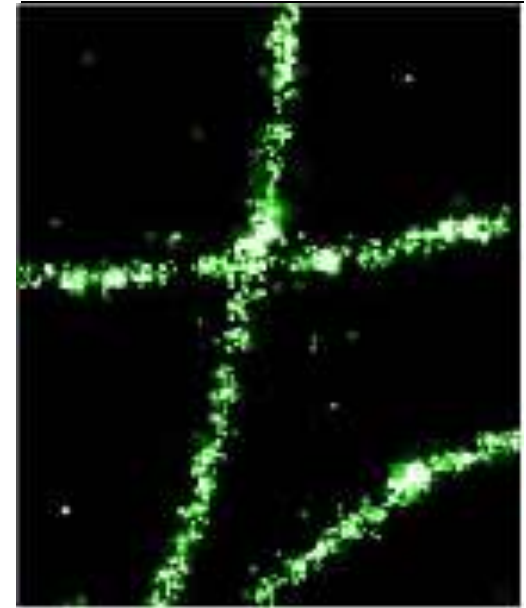
Fluorescence image



Raw images



STORM Image



2x real time

STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)

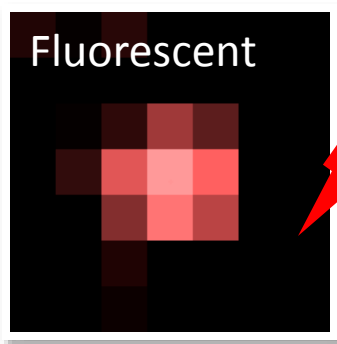
PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)

FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)

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

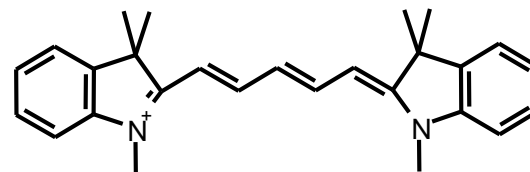
PAINT (Hochstrasser 2006), **SPRAYPAINT** (Moerner 2011), **SOFI** (Weiss 2009)

Photoswitching of red cyanine dyes



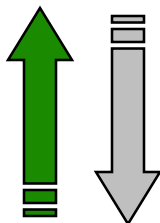
650 nm

+ thiol



Cy5 / Alexa 647

photoactivation

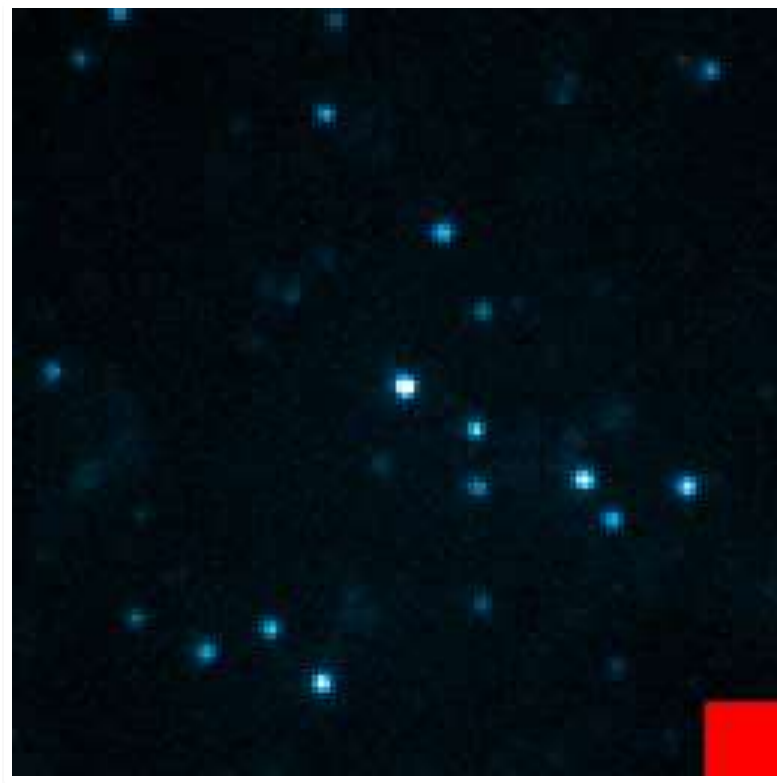


Deactivation

360 nm

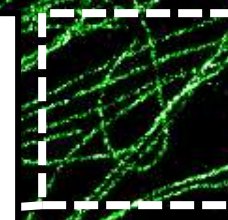
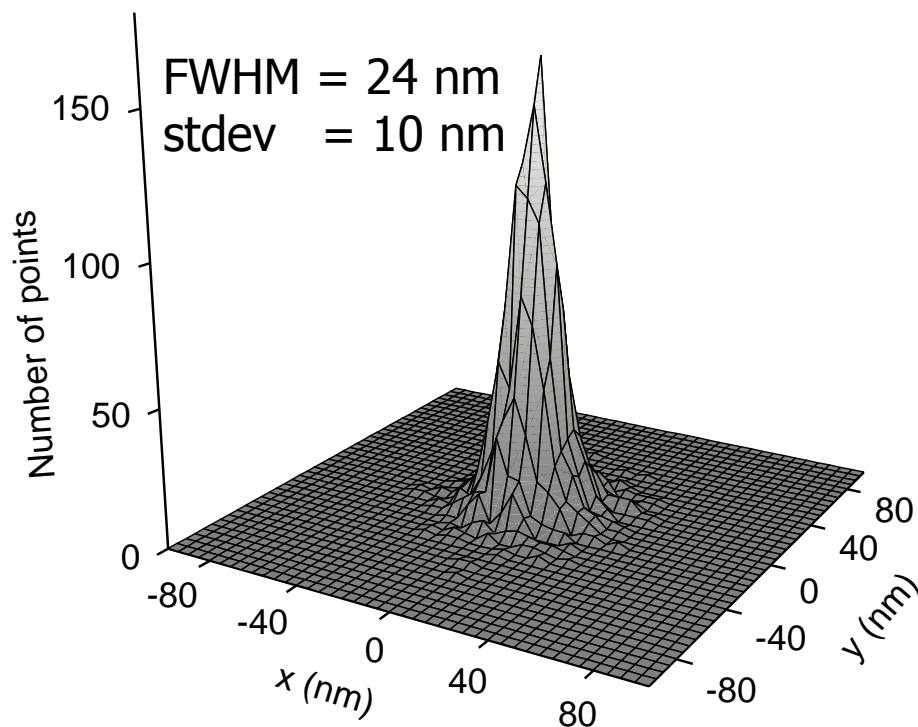
650 nm

Dark

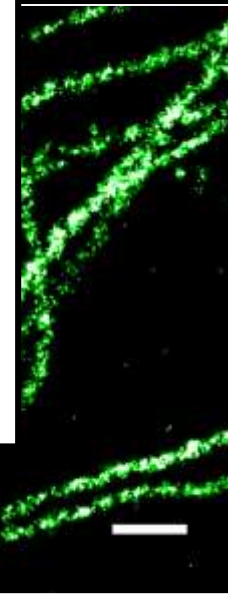


B-SC-1 cell, anti- β tubulin

Commercial **Alexa 647** secondary antibody



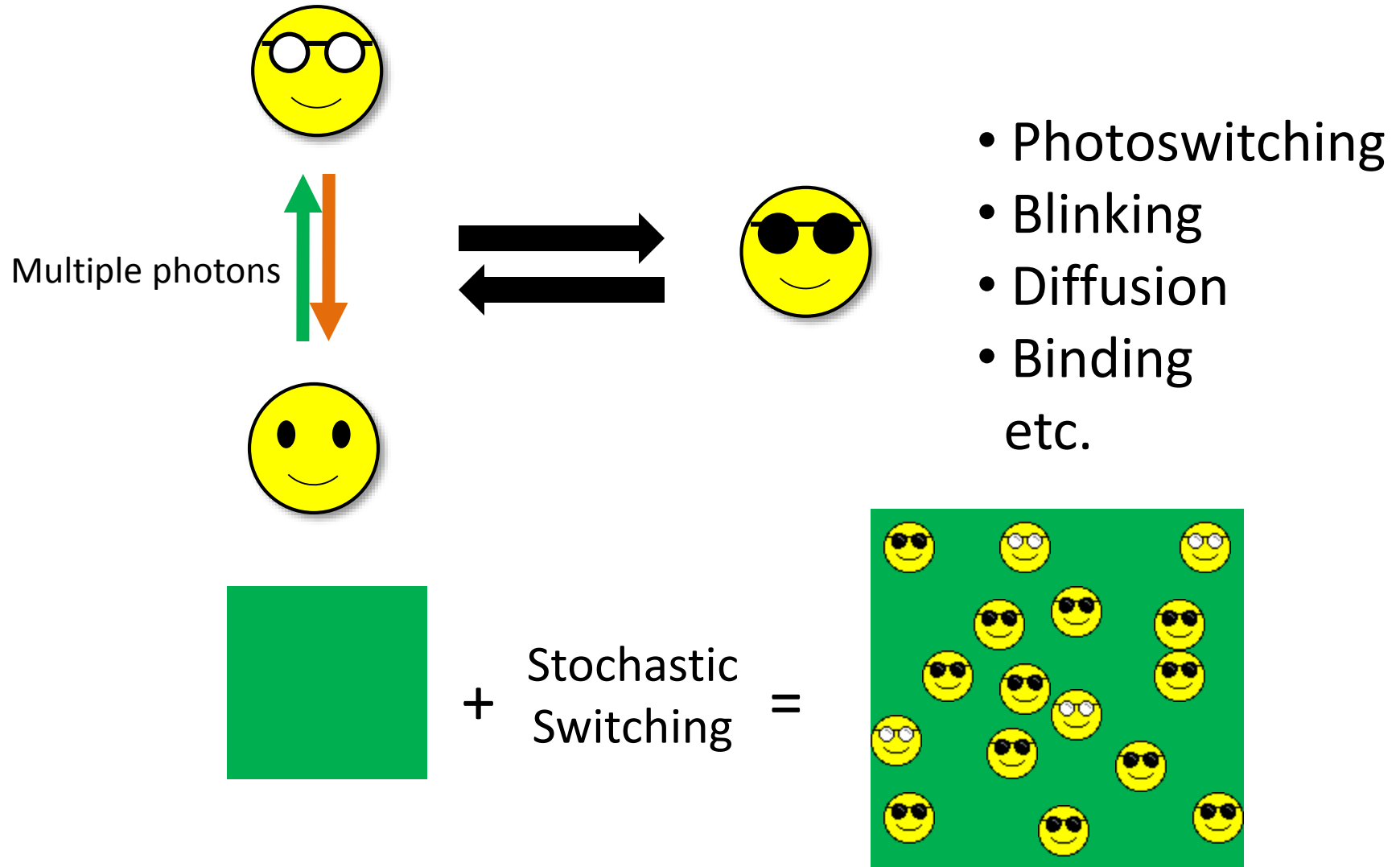
alization points



5 μ m

500 nm

The “single-molecule switching” approach



Photoswitchable probes readily available

400

500

600

700 nm

Simple dyes (+ thiole / redox system)



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

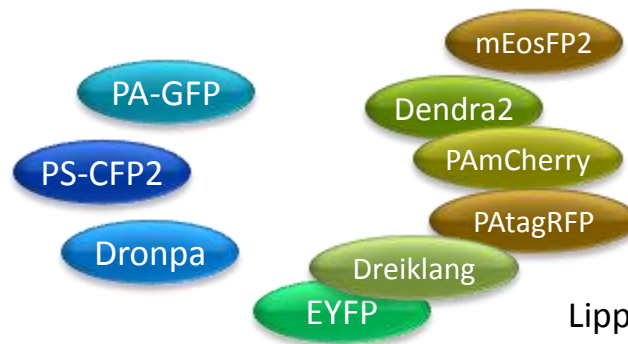
Heilemann et al., 2009

Functional dyes



Shim 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

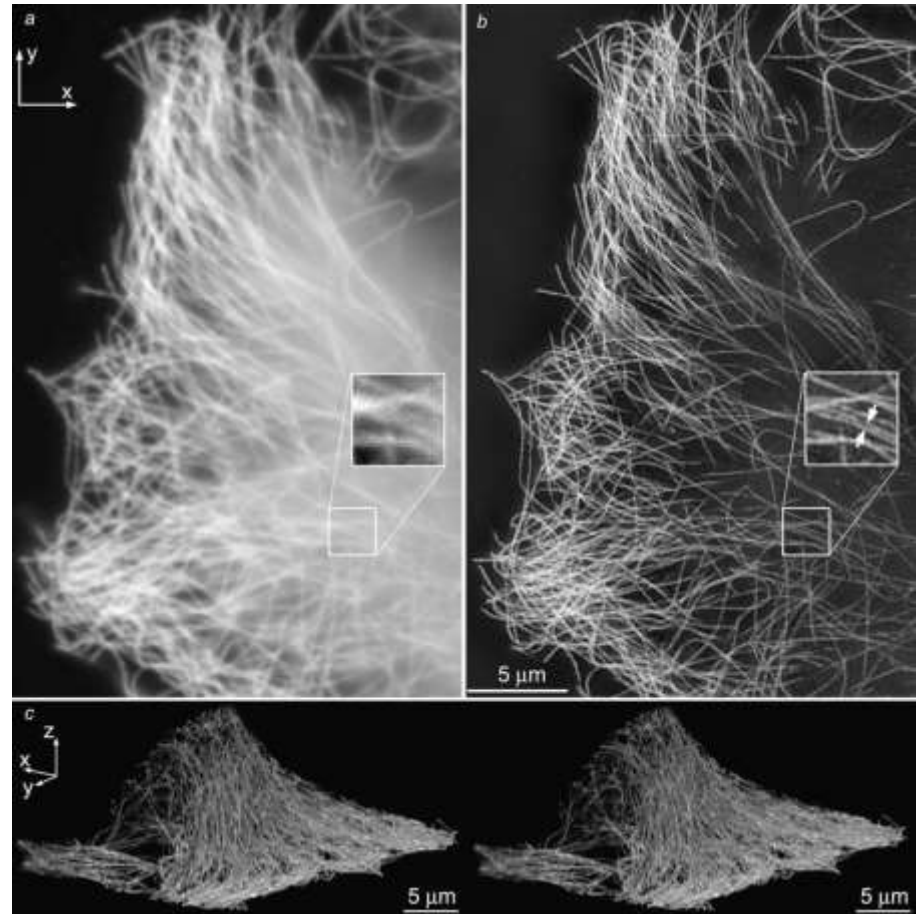
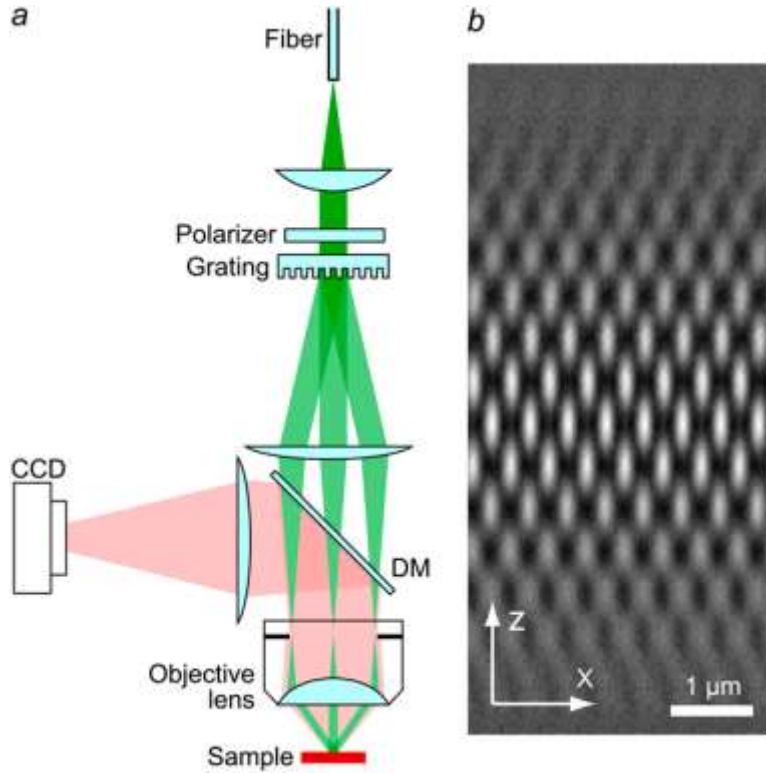
In a 2D world...

Satellite image of ???

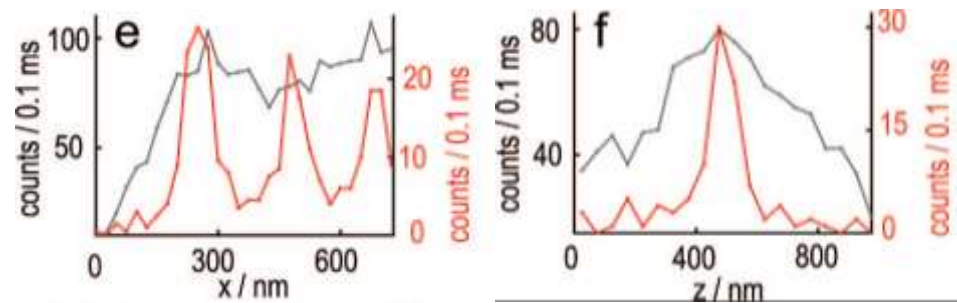
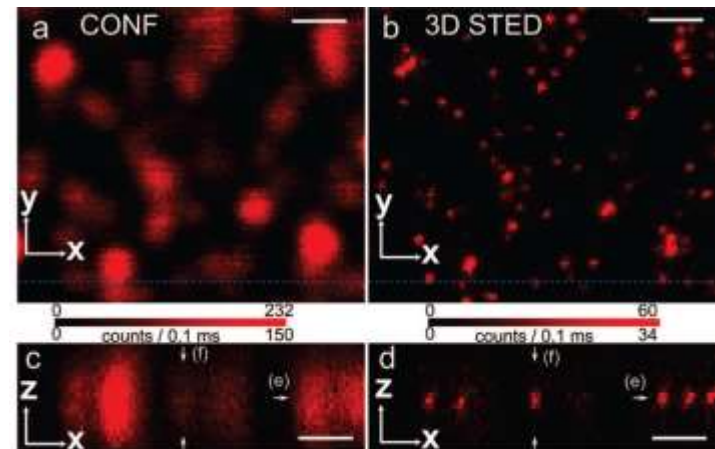
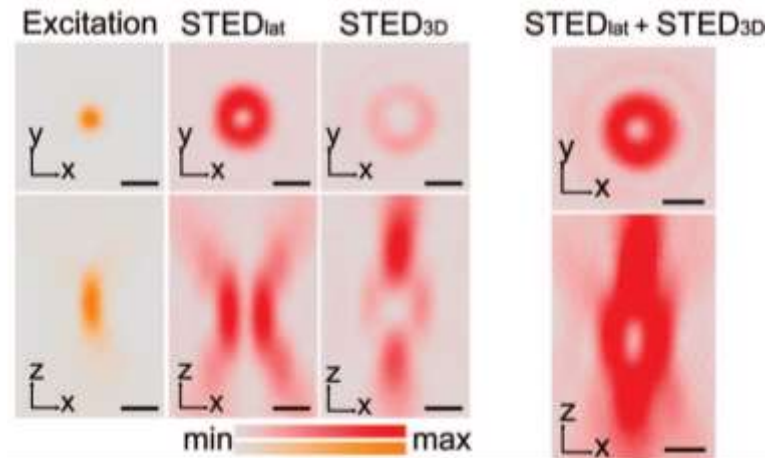


Google maps

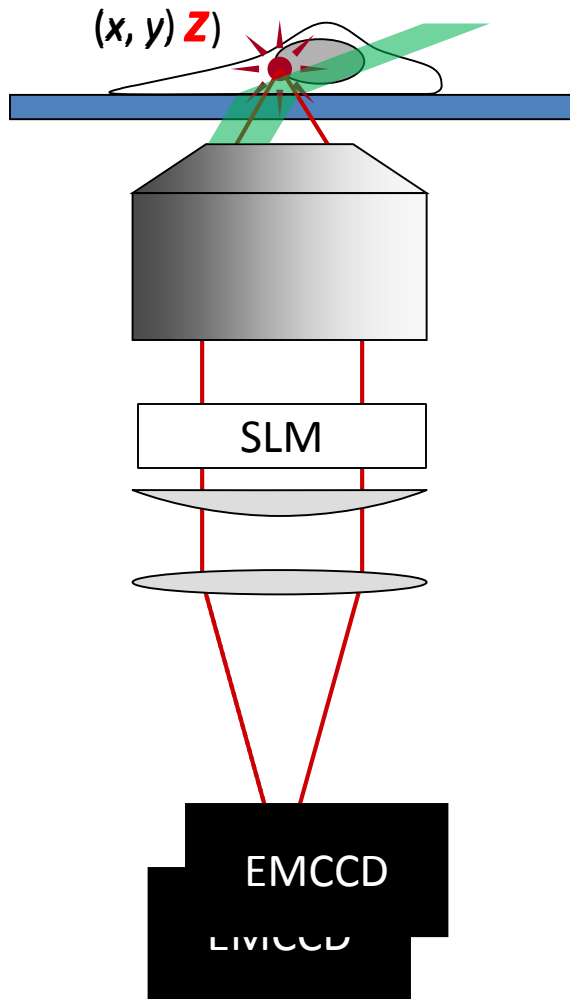
3D SIM



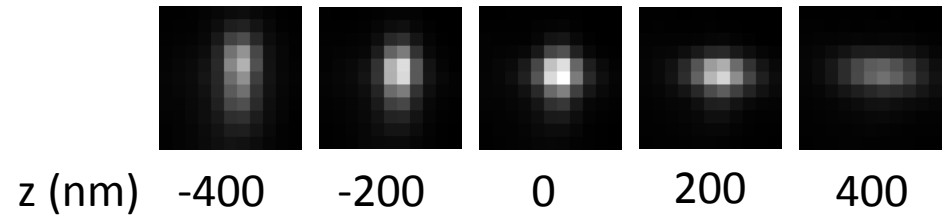
3D STED



3D STORM/PALM

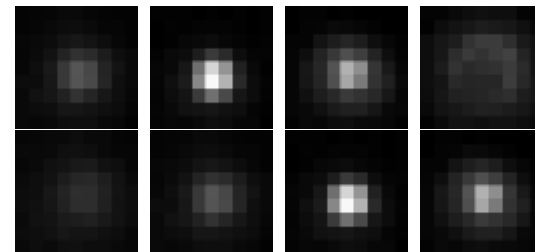


Astigmatic imaging



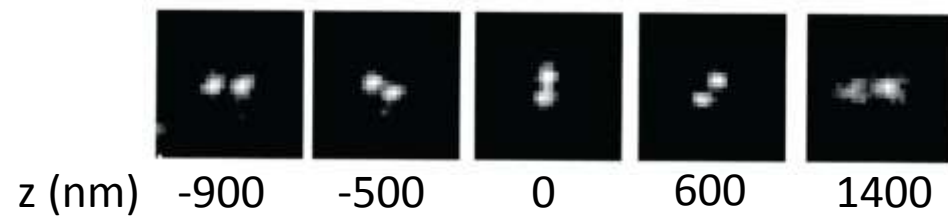
Huang et al., Science 2008

Bi-plane imaging



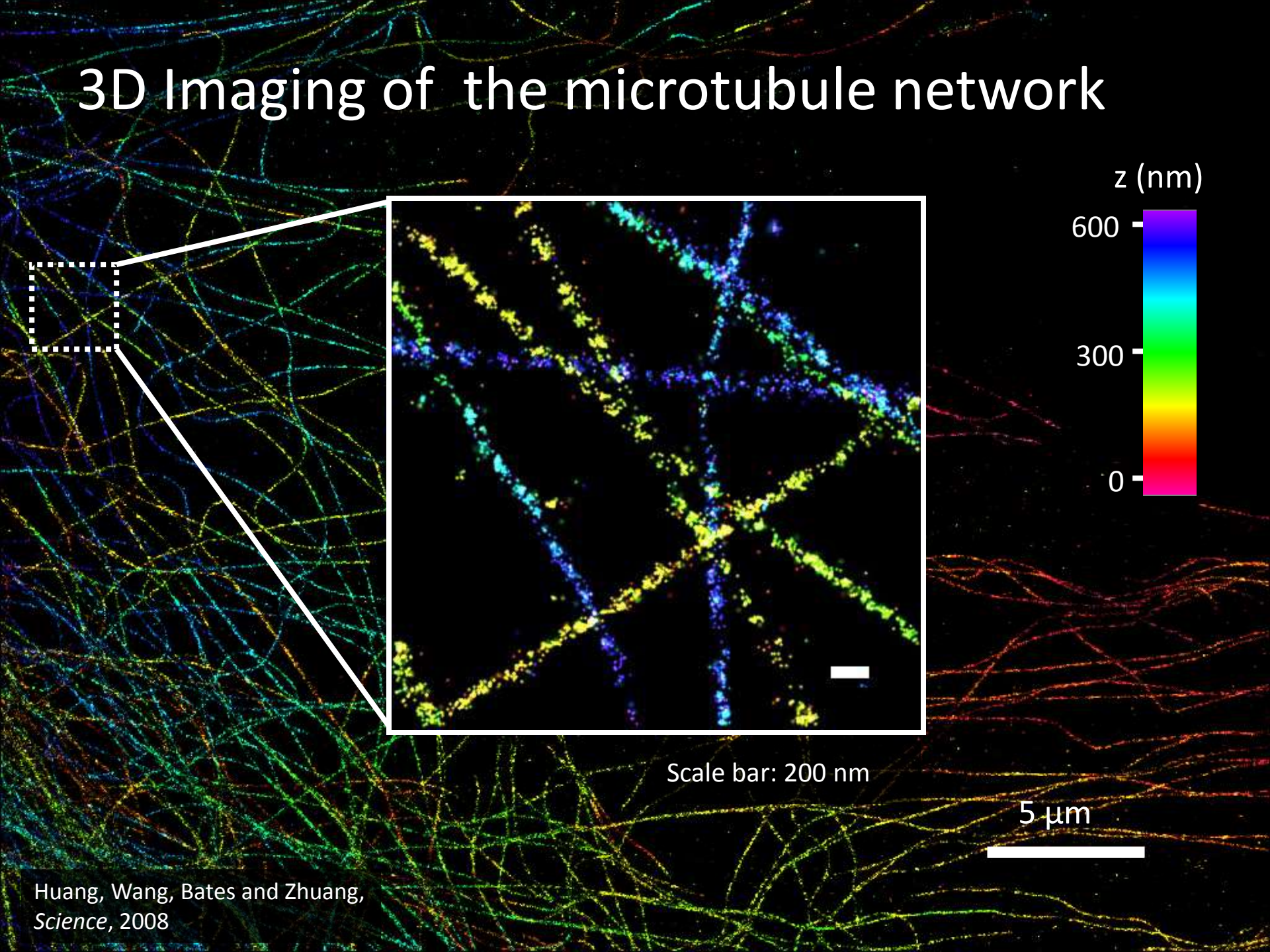
Juette et al., Science 2008

Double-helical PSF

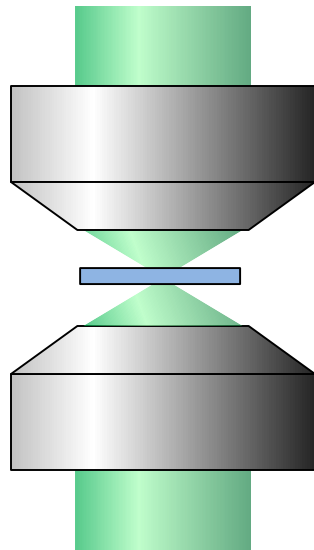


Pavani et al., PNAS 2009

3D Imaging of the microtubule network



The use of two opposing objectives

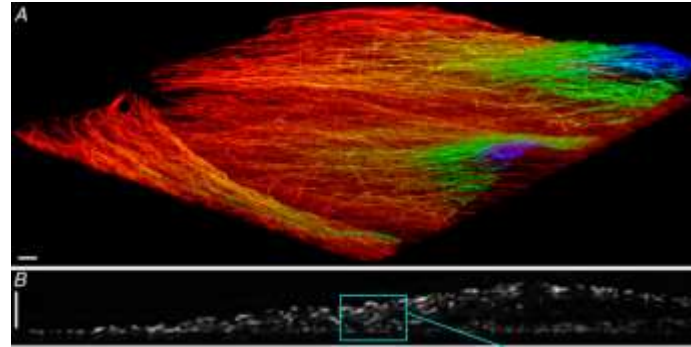


4Pi scheme



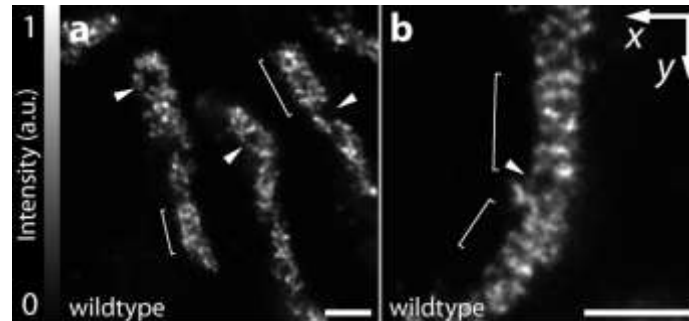
Near isotropic
3D resolution

I⁵S



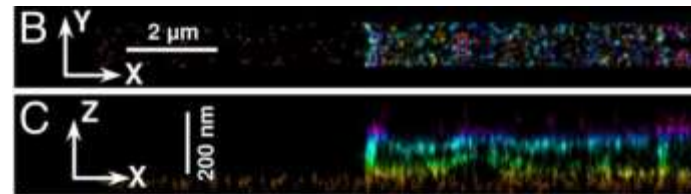
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

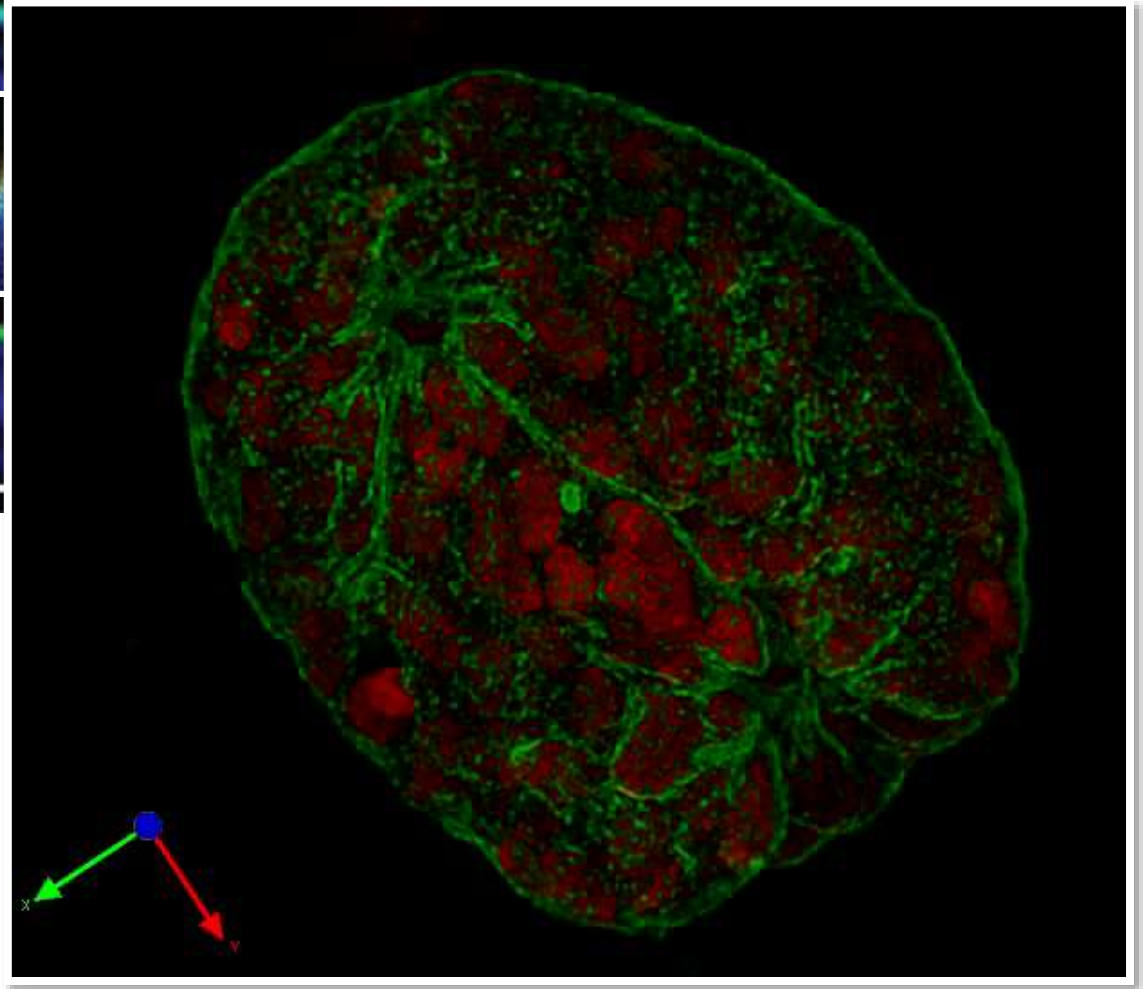
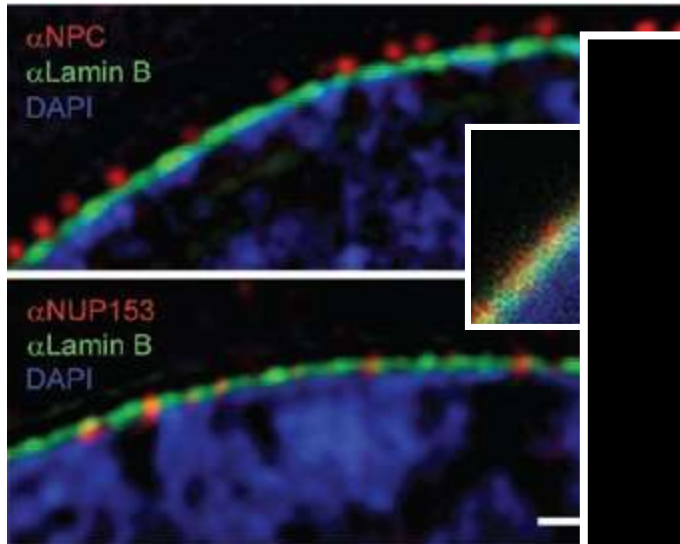
3D resolution of super-resolution methods

	x-y (nm)	z (nm)	Opposing objectives (nm)	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

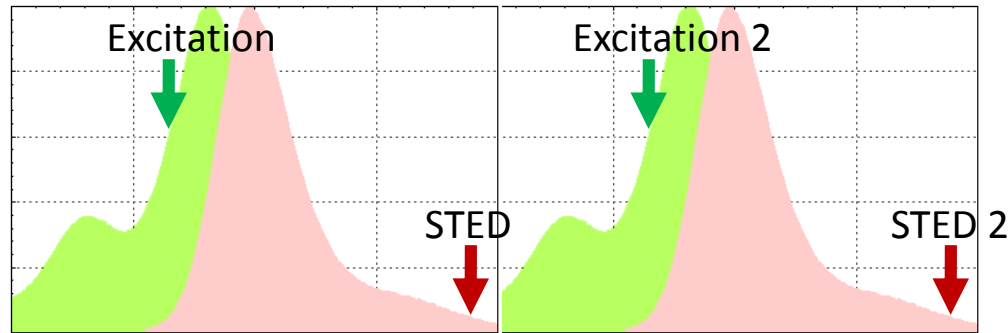
Multi-color Imaging

Multicolor SIM

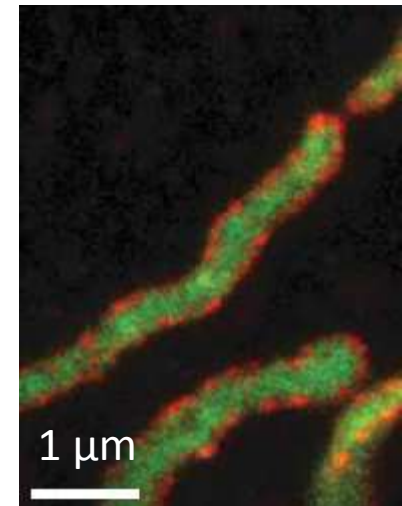
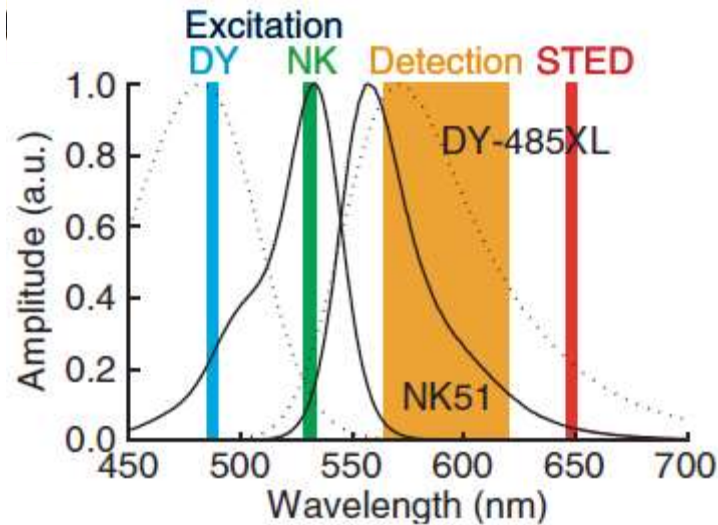
Same as conventional fluorescence microscopy!



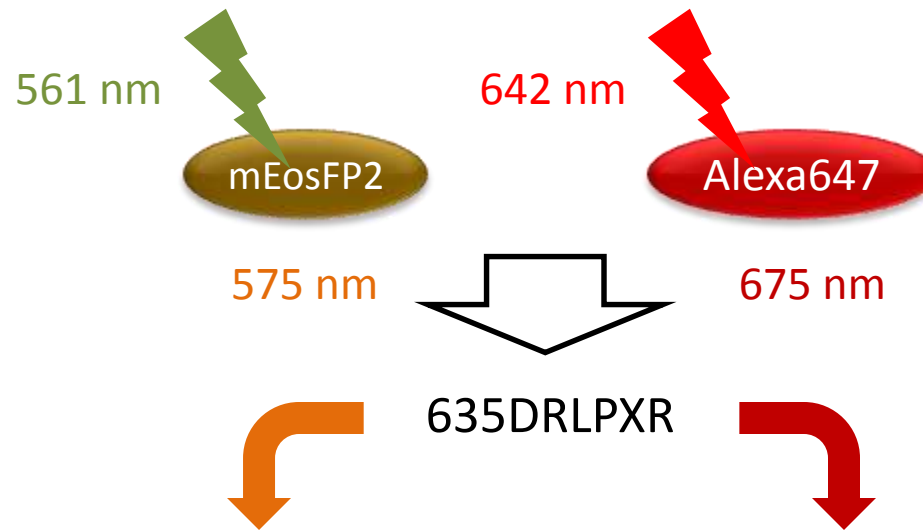
Multicolor STED



2 color isoSTED resolving
the inner and outer membrane
of mitochondria



Multicolor STORM/PALM



mEos2-tubulin

Alexa 647 anti- β tubulin

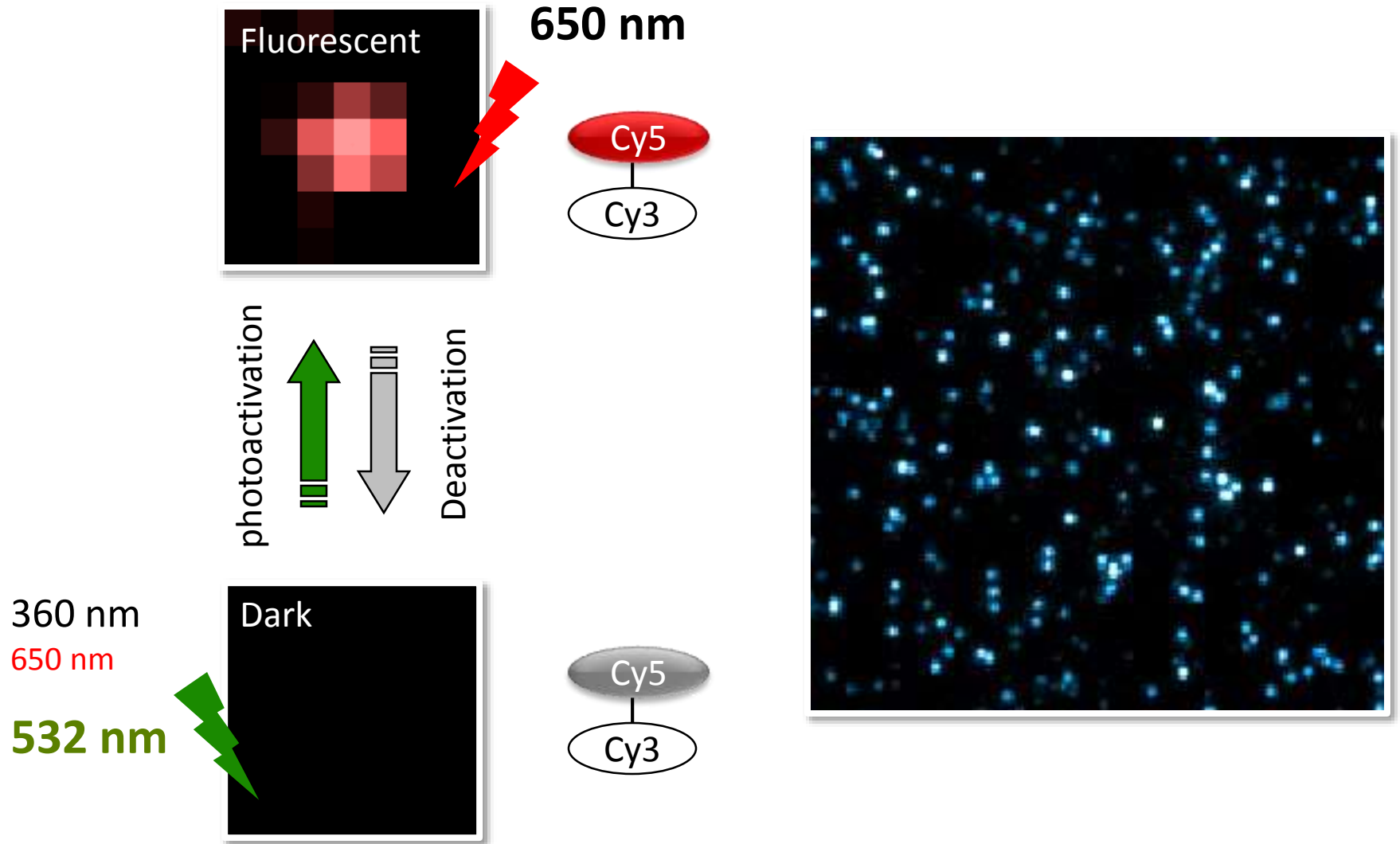
Drosophila S2 cells

2 μ m



Daichi Kamiyama

Multicolor STORM/PALM: activation



■ Cy3 / Alexa 647: Clathrin
■ Cy2 / Alexa 647: Microtubule
Crosstalk subtracted

Laser sequence

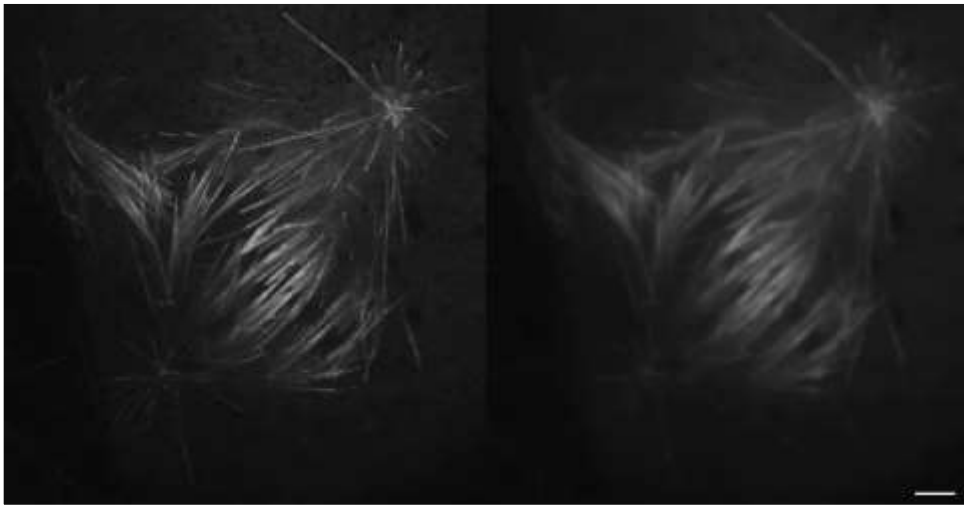


1 μ m

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

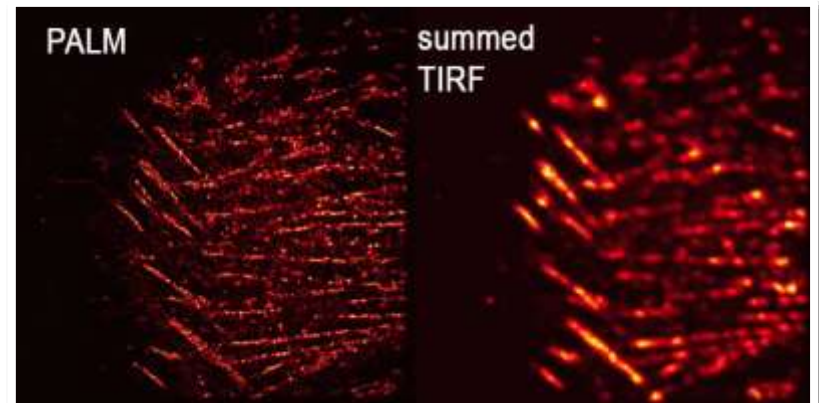


SIM

2 μm

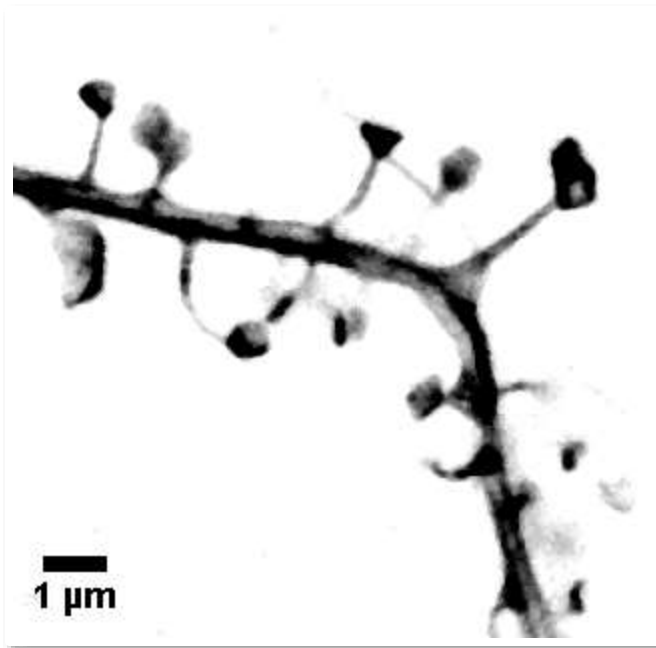
Kner, Chhun et al., Nat Methods, 2009

STORM/PALM



Schroff et al., Nat Methods, 2008

STED



Nagerl et al., PNAS, 2008



The limit of “Super-Resolution”

Unbound theoretical resolution

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

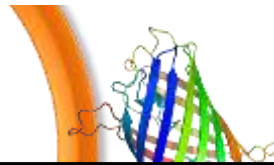
- $S \approx \sqrt{N}$
 - 6,000 photons for Cy5 \rightarrow 5 nm
 - 1,000,000 photos for Hydro-Cy5 \rightarrow < 1 nm
- (Vaughn et al., 2012)

Effective resolution: Probe matters

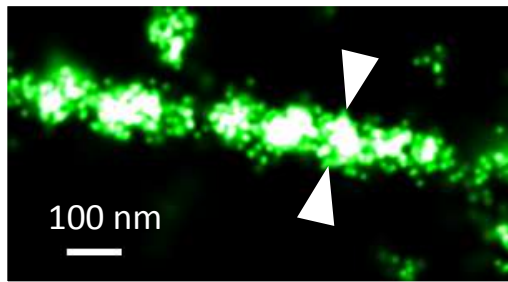
Antibodies:
~ 10 nm

Fluorescent Proteins:
~ 3 nm

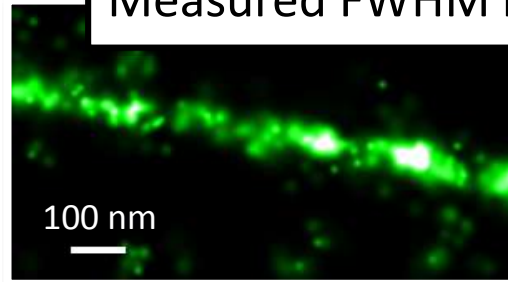
Small fluorophores:
~ 1 nm



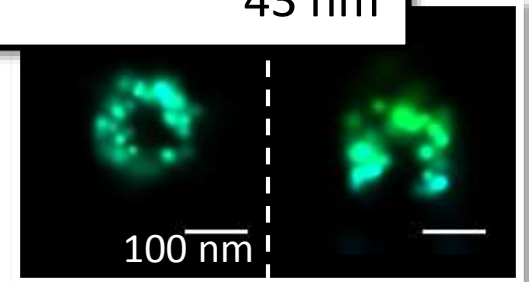
Measured FWHM by antibody:	58 nm
Actual microtubule diameter:	25 nm
Measured FWHM by FP:	43 nm



~ 6000 photons



< 1000 photons



~ 6000 photons

Fluorescent protein vs. Antibody

Fluorescent protein fusion

- Live sample labeling

- High specificity

- High labeling efficiency

- Genetically

- Lower S/N

- Multicolor imaging so far challenging

Antibody immunofluorescence

- Fixed sample

specific labeling

efficiency

ogenous proteins

high 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:

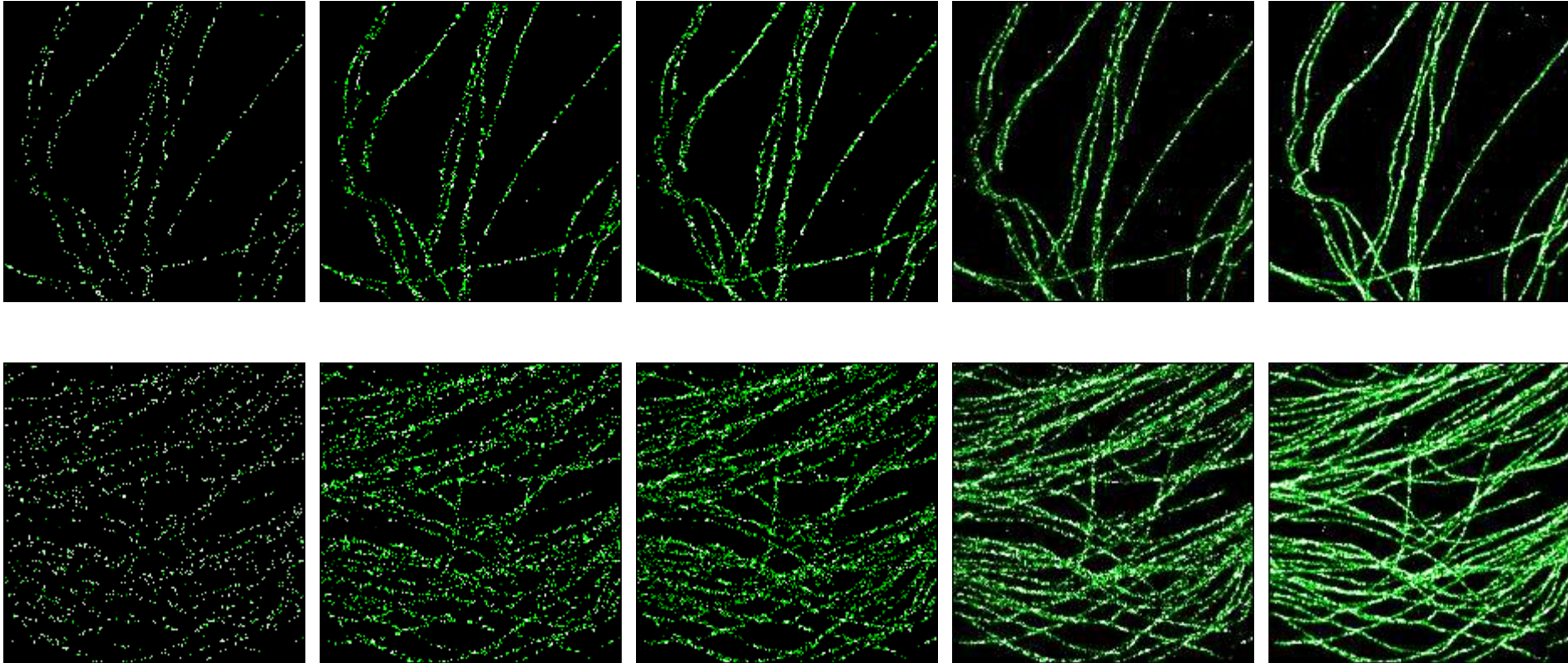
200

500

1,000

5,000

40,000



Effective resolution: Density matters

Frames for image reconstruction:

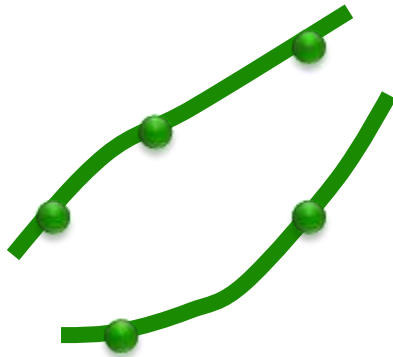
200

500

1,000

5,000

40,000



Nyquist criteria



Point to point distance \approx Feature size

Effective resolution: Density matters

Frames for image reconstruction:

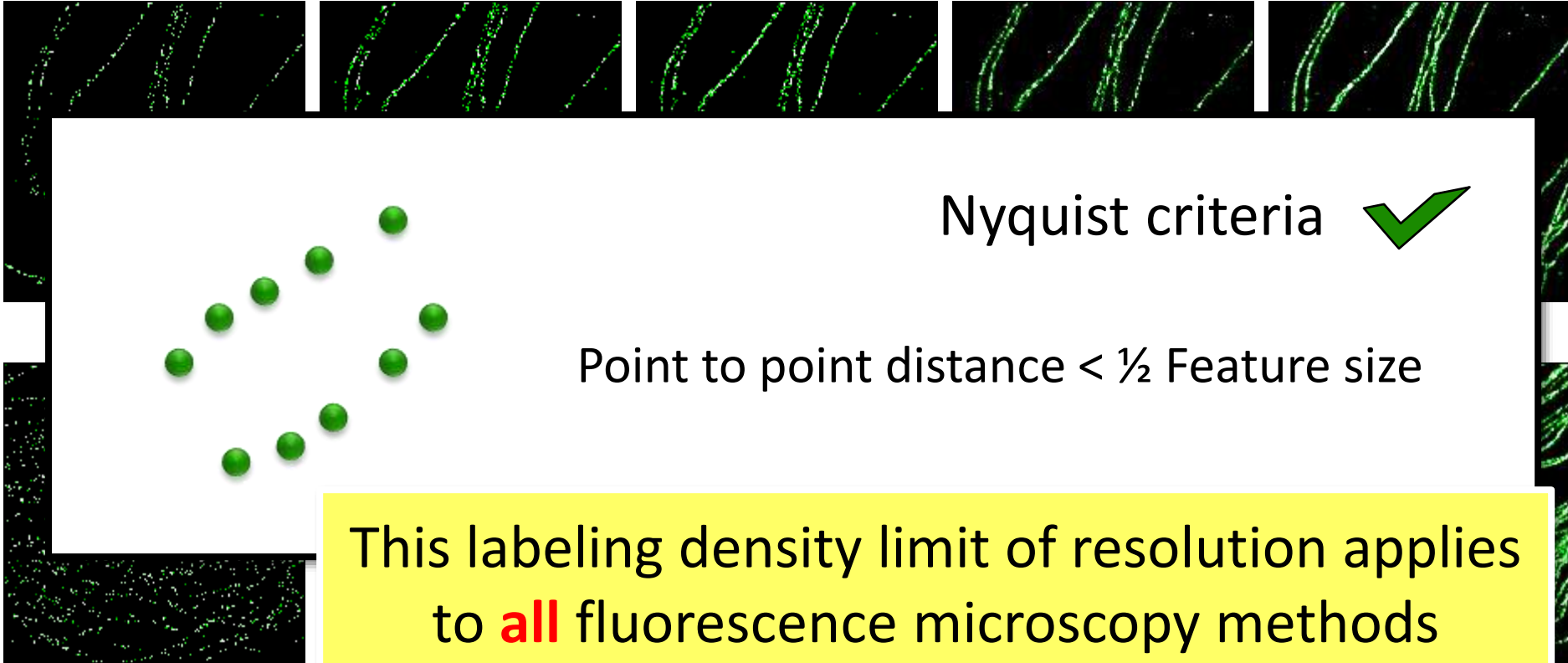
200

500

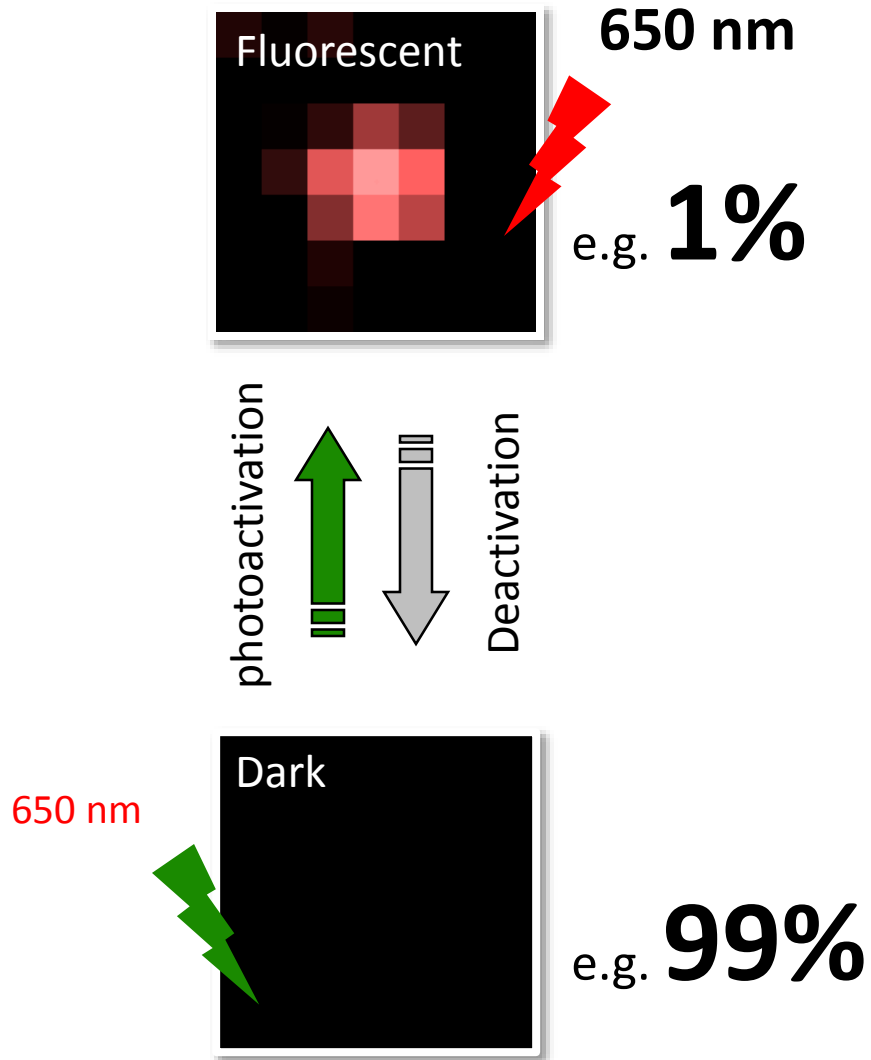
1,000

5,000

40,000



Effective resolution: Contrast matters

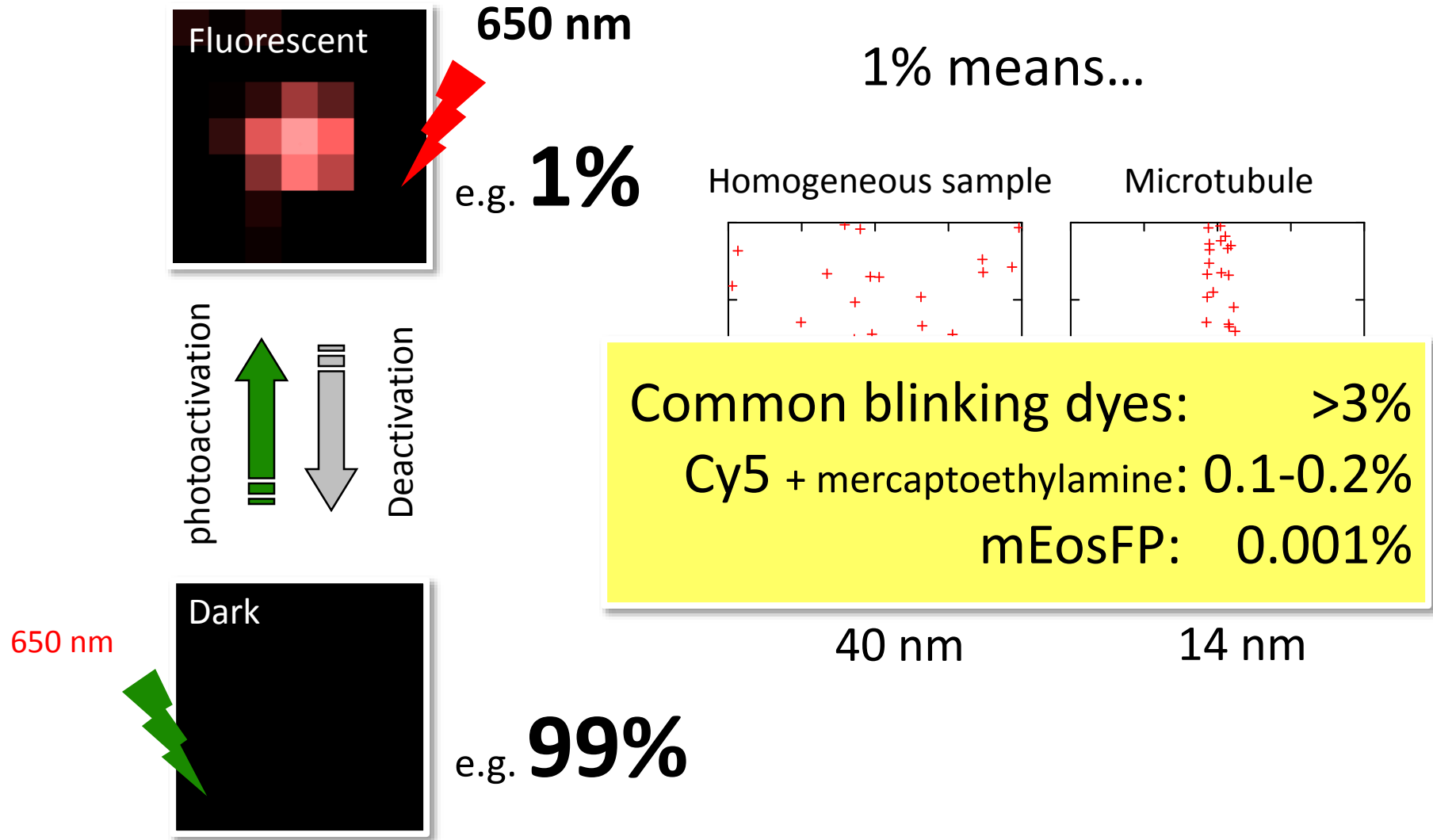


1% means...

Sparsely labeled sample

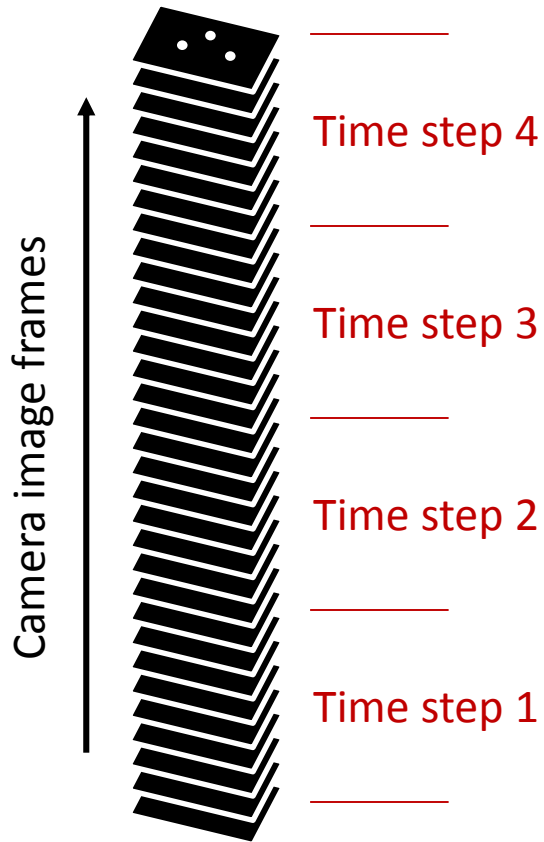
Densely labeled sample

Effective resolution: Contrast matters

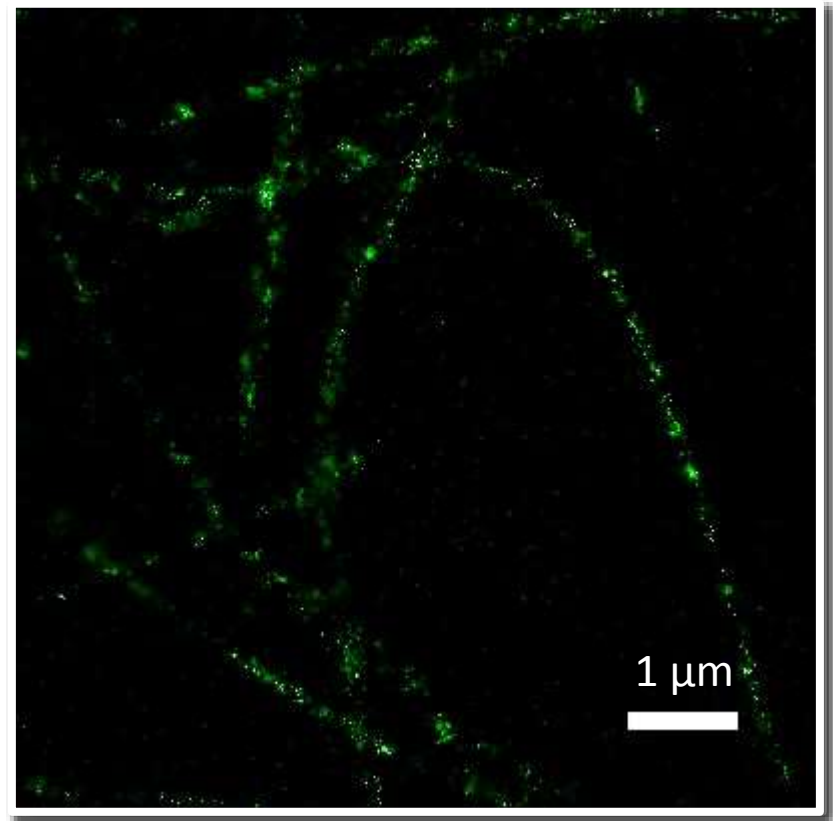


Live Cell STORM/PALM

Live cell STORM/PALM



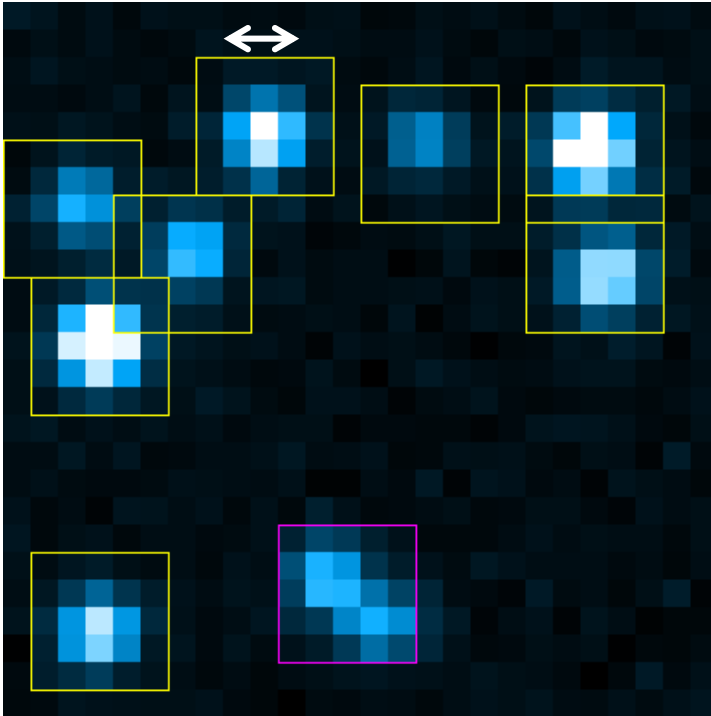
mEos2 labeled microtubule in live S2 cells



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

Spatial-temporal resolution trade-off

FWHM ≈ 320 nm



0.68 molecules per μm^2

Assuming:

1 molecule occupies 500×500 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



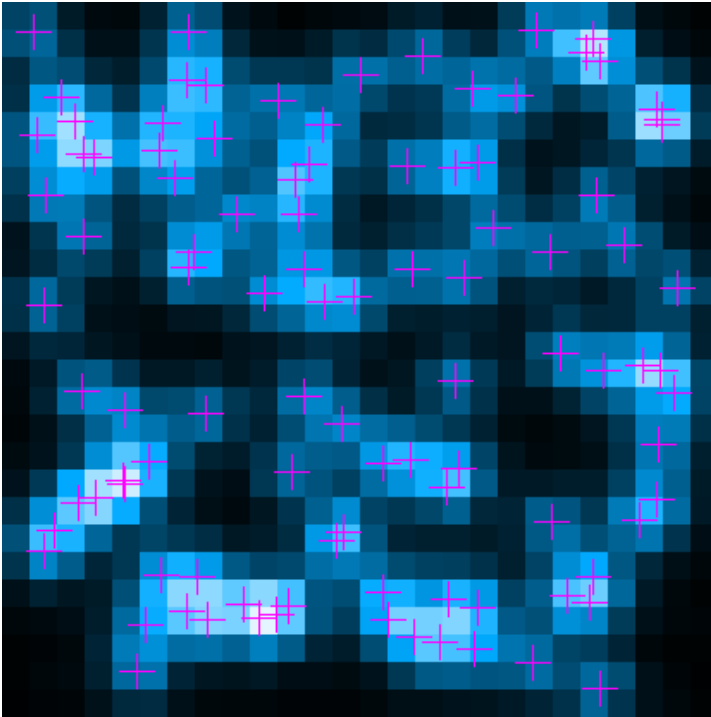
100 fps = 20 sec time resolution



1000 ~ 3000 fps

Spatial-temporal resolution trade-off

FWHM ≈ 320 nm



6.8 molecules per μm^2

Assuming:

1 molecule occupies 500×500 nm

8 per μm^2

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

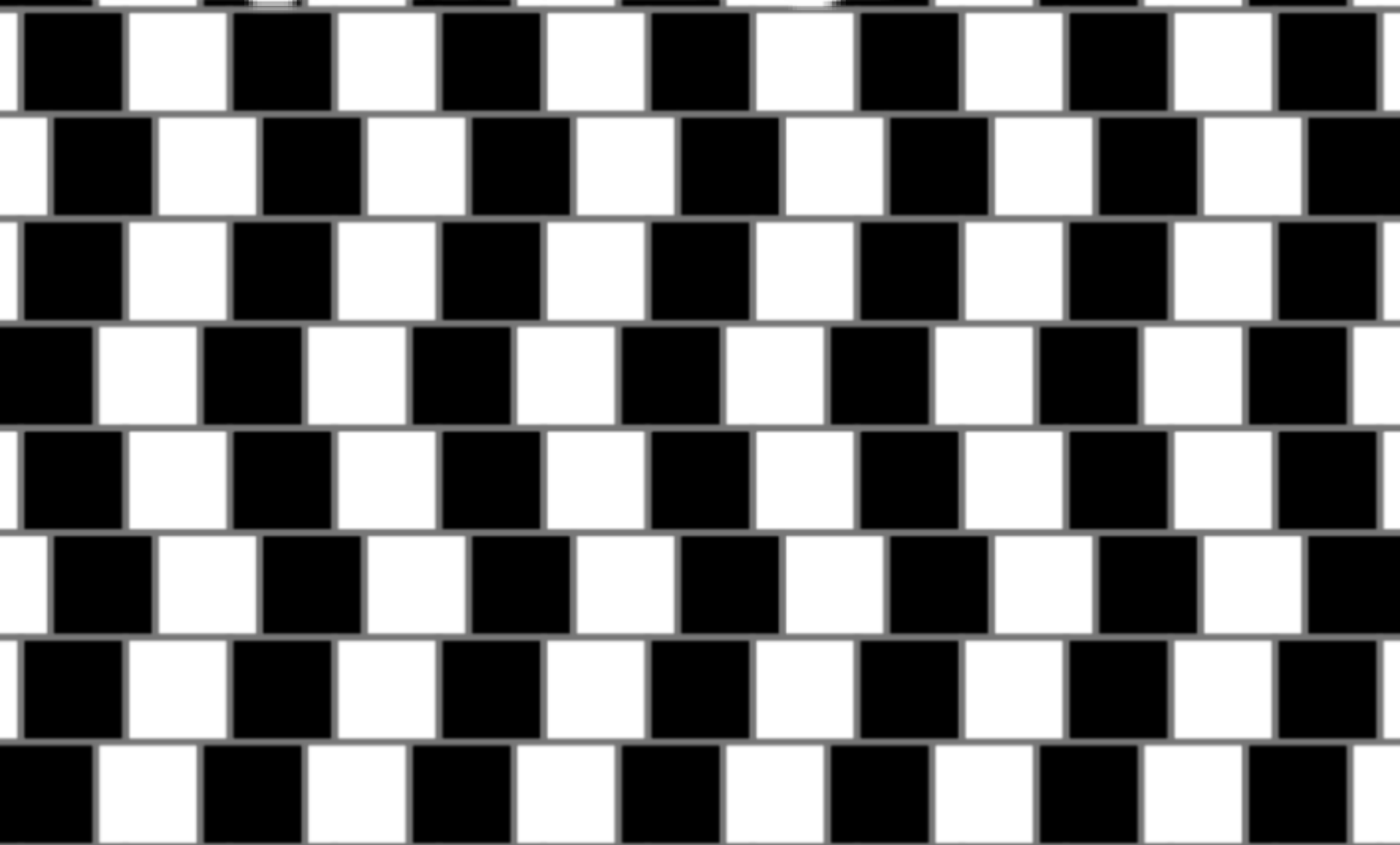
Comparison of time resolution

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)

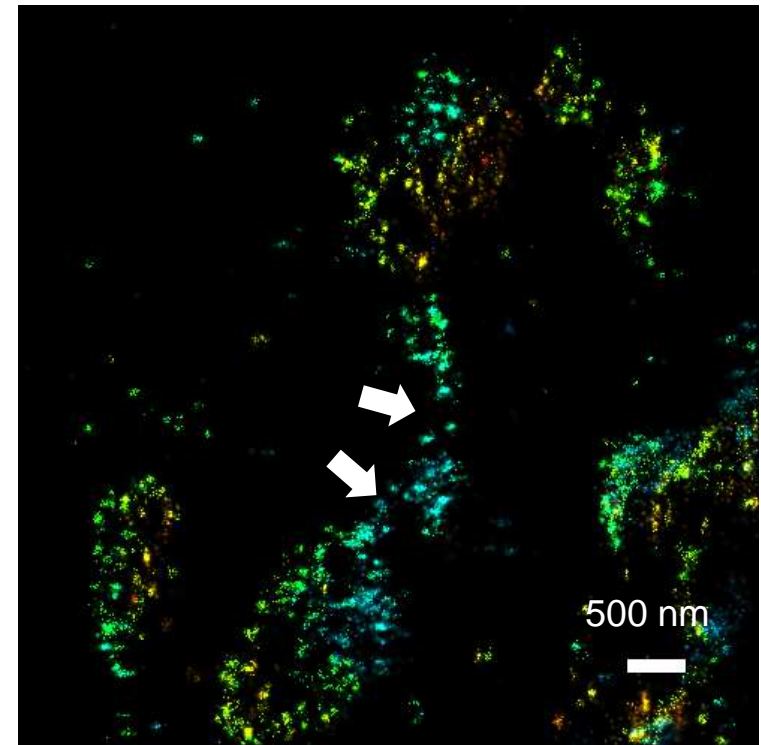
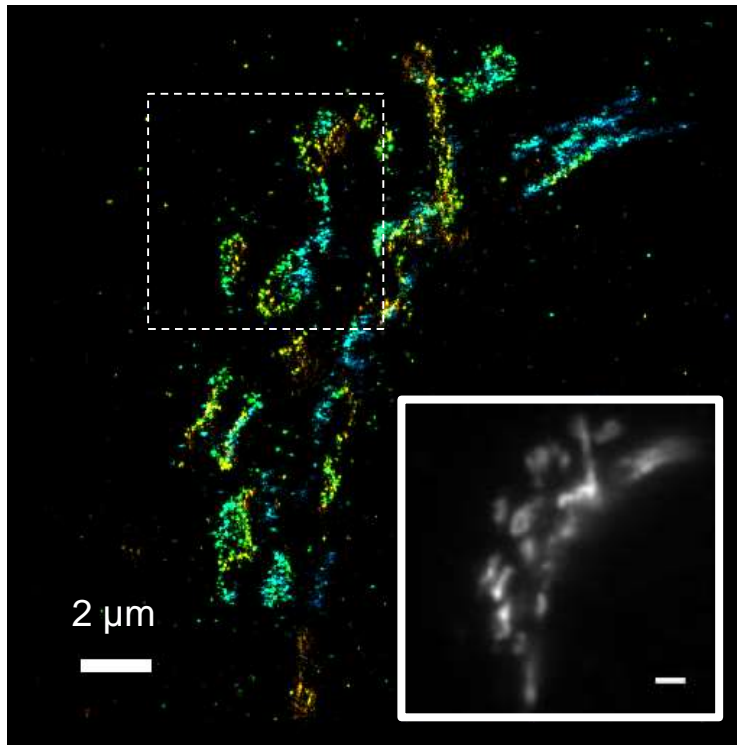
3D		Spatial resolution	Time resolution
SIM	Wide-field	120 nm	15 frames x 10 (1.5 sec)
STED	Scanning	60 nm	1 x 2 x 0.6 μm : 0.6 sec 10 x 20 x 0.6 μm : 60 sec
STORM/PALM	Wide-field	60 nm	3000 frames (3 sec) – no scan!

Multipoint scanning RESOLFT...

Seeing can be deceiving...

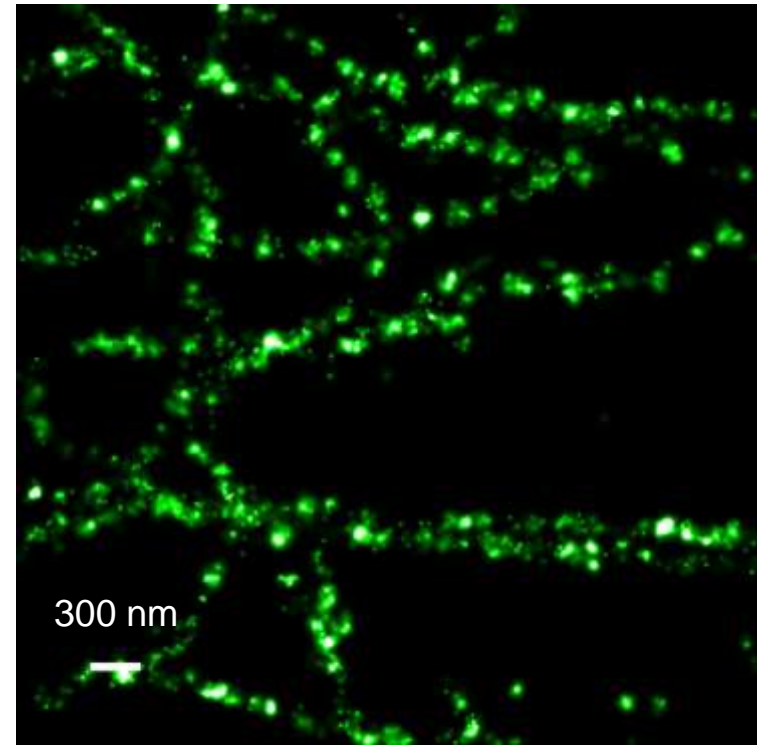
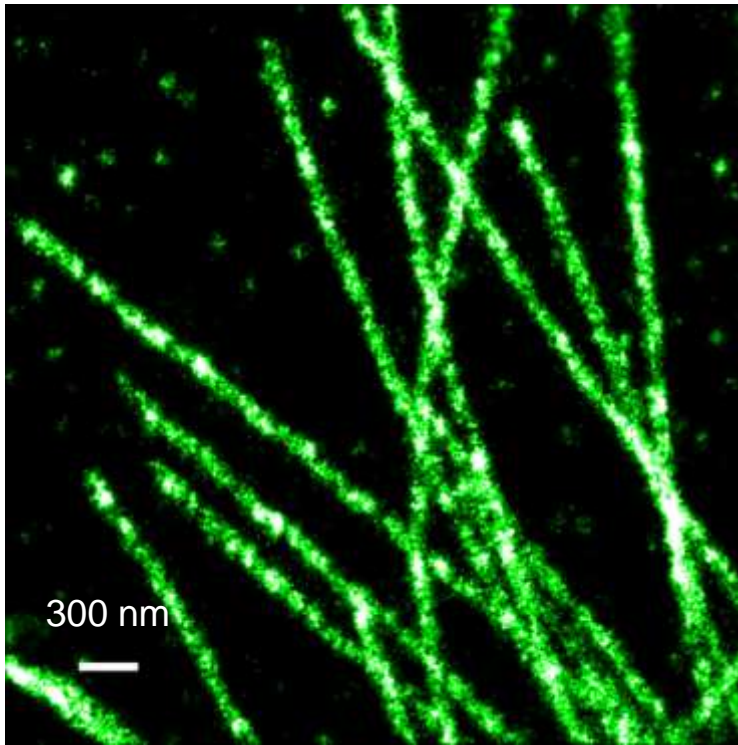


Super-resolved artifacts: sparse labeling



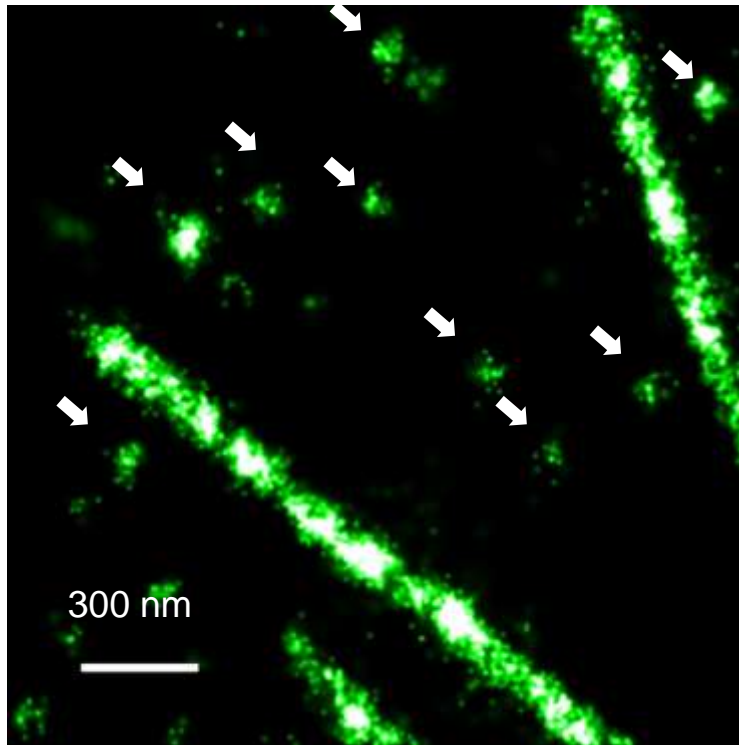
Golgi: Giantin immunofluorescence

Super-resolved artifacts: poor fixation

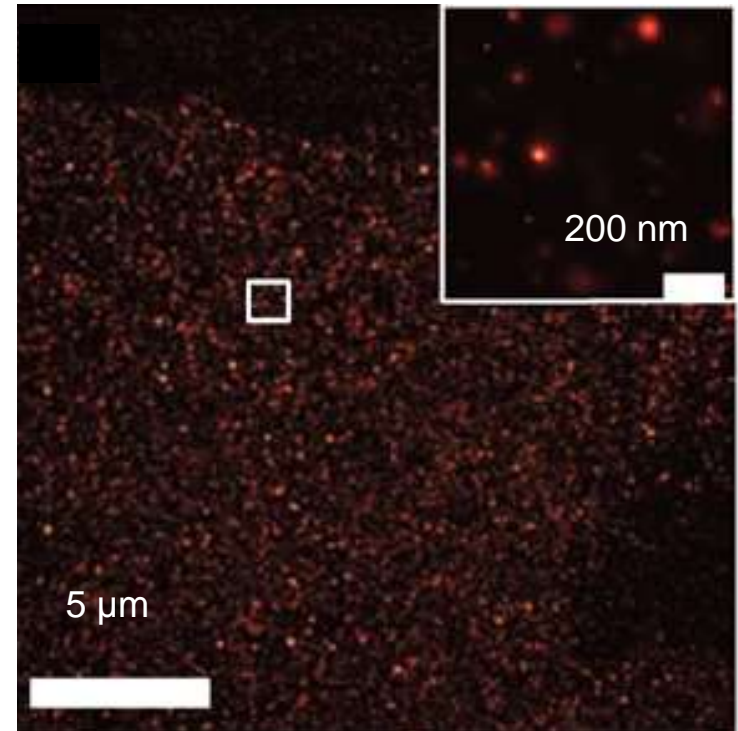


Microtubules: good and bad fixation

Super-resolved artifacts: clustering

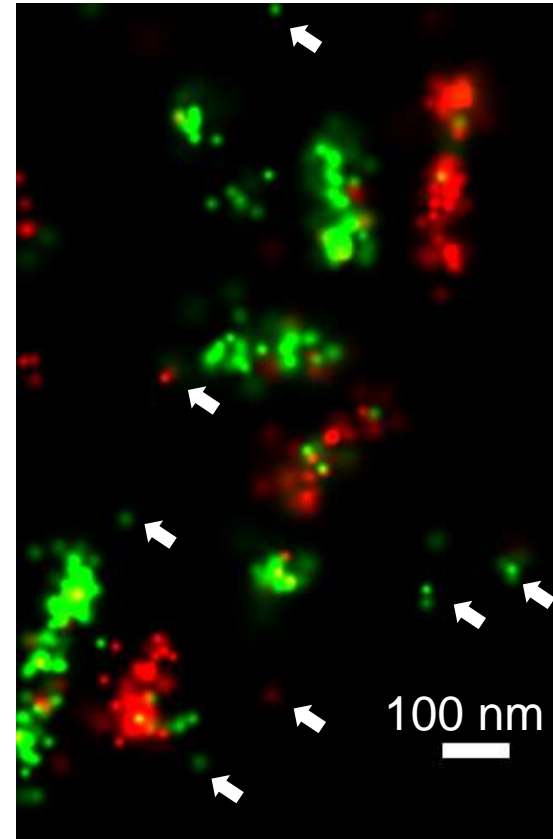
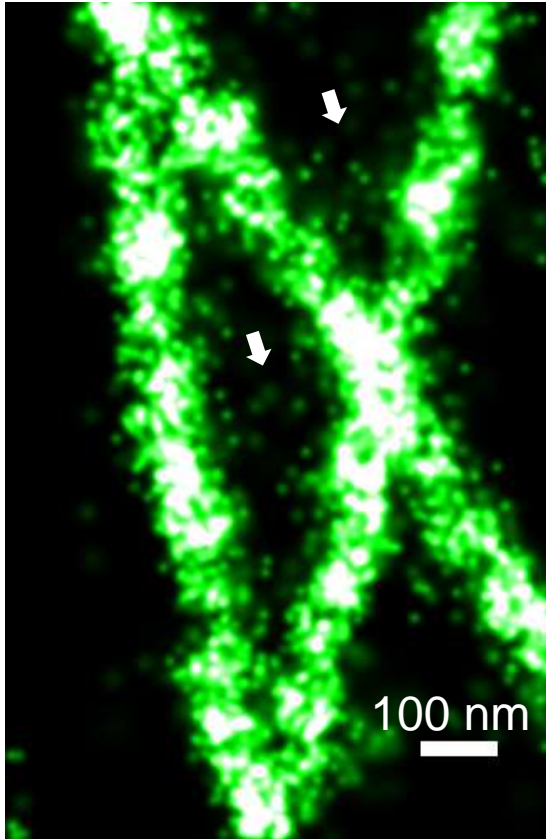


Clusters from single antibodies



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).