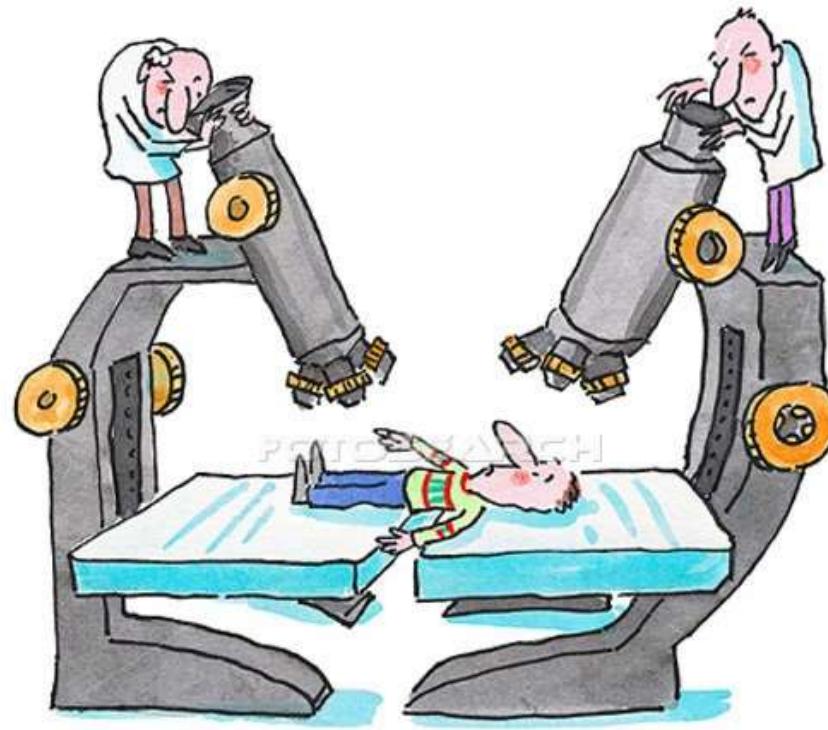


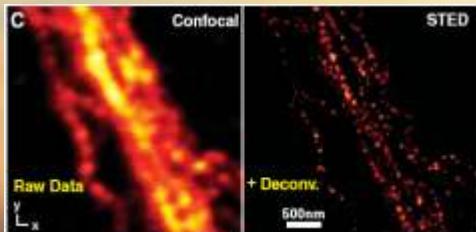
Super-Resolution Microscopy

Single-molecule switching

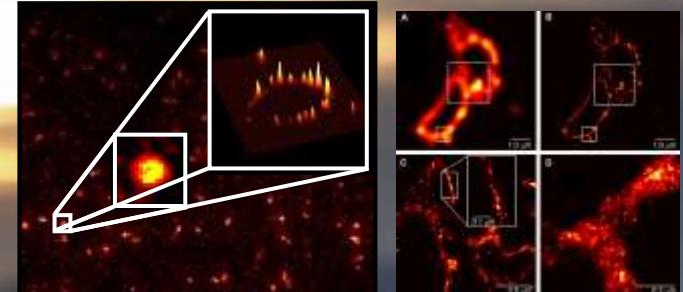


Bo Huang

Super-resolution optical microscopy



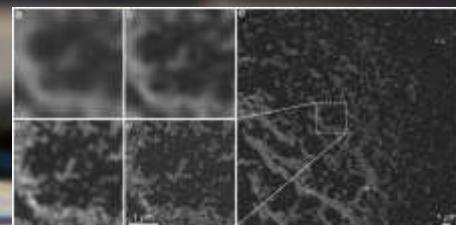
Hell, *Science*, 2007; Hell, *Nat Methods*, 2008



Rust, Bates & Zhuang, *Nat Methods*, 2006

Betzig et al., *Science*, 2006

Hess, Girirajan and Mason, *Biophys. J.*, 2006



Gustafsson, *PNAS*, 2005

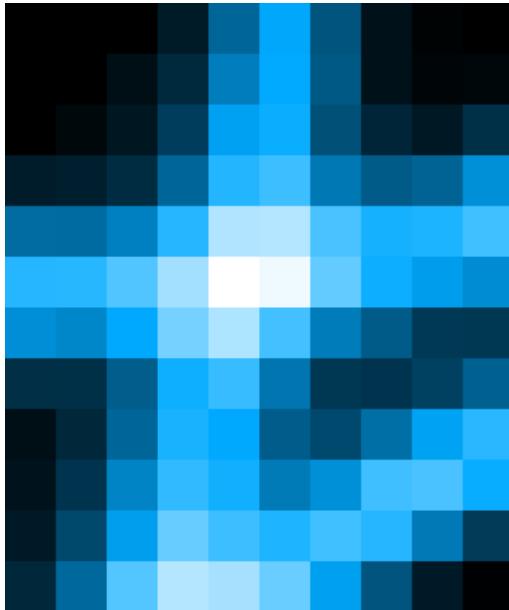
STED

(S)SIM

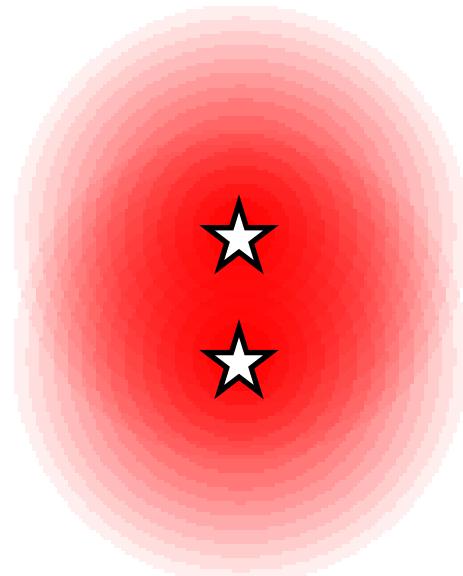
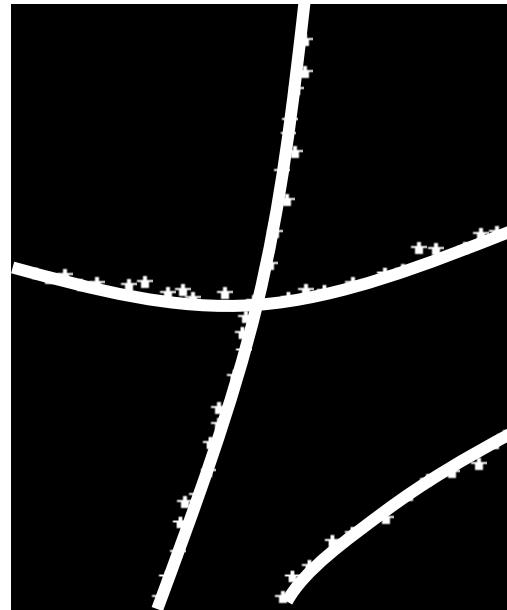
STORM/(F)PALM

Super-resolution by...

Fluorescence image

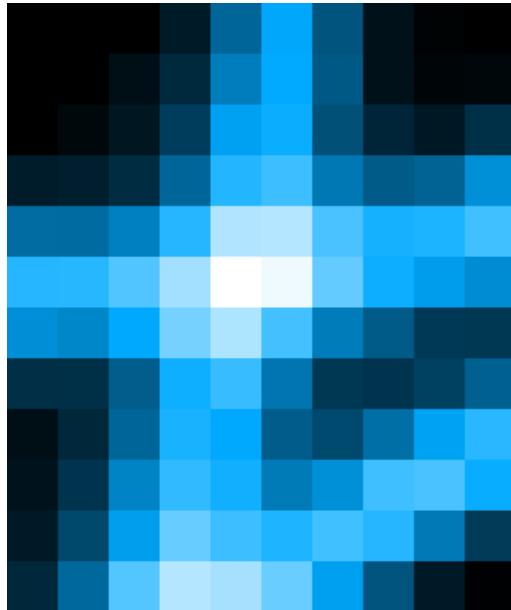


Underlying structure

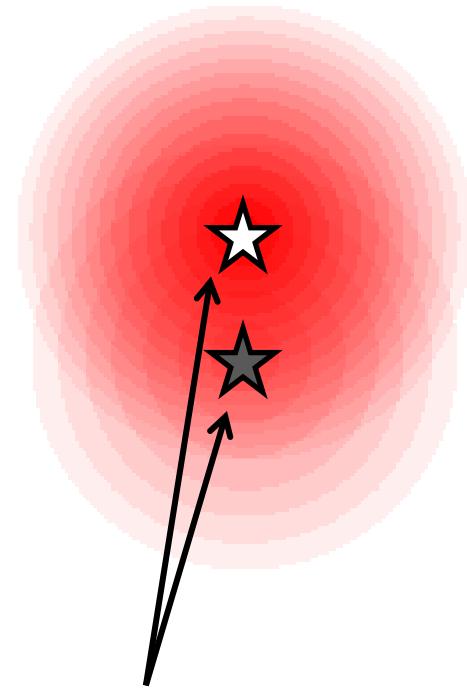
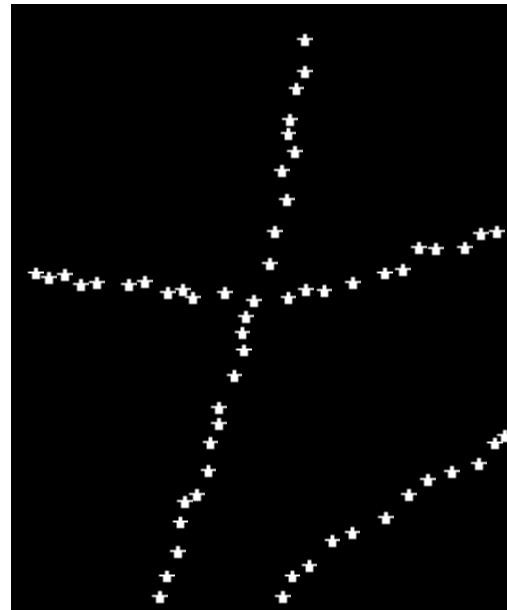


Super-resolution by spatial modulation

Fluorescence image



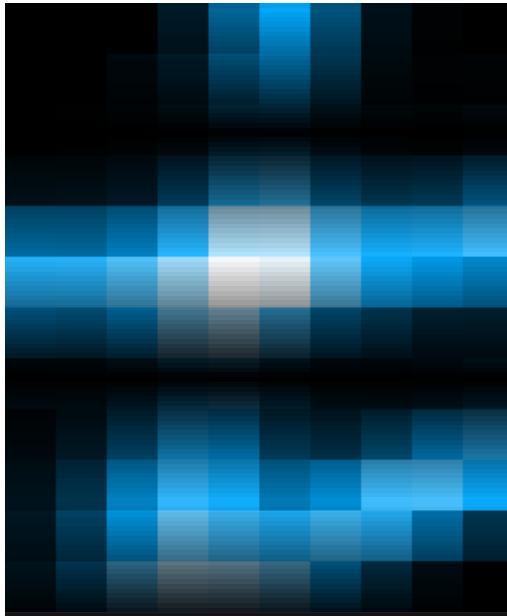
Underlying structure



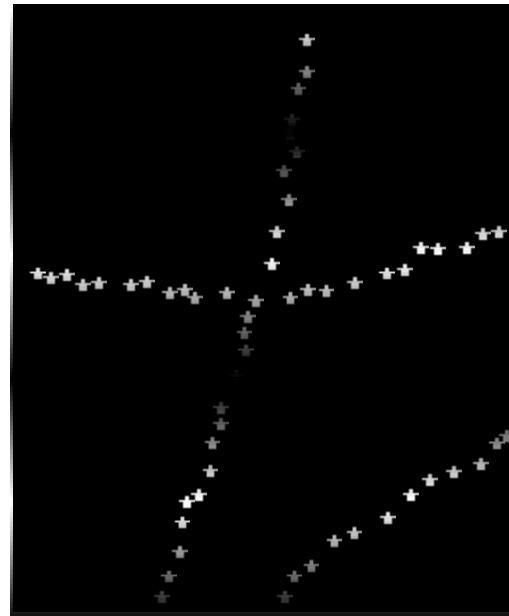
Differential modulation
of the fluorescence response

Super-resolution by differential excitation

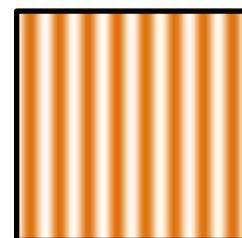
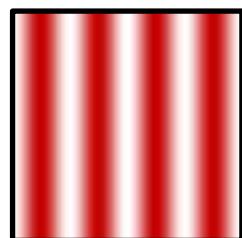
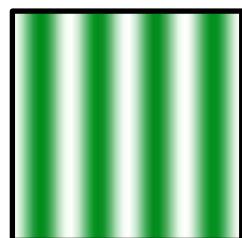
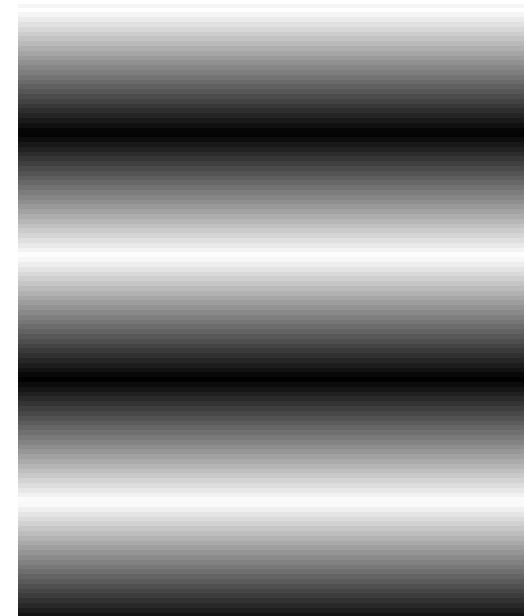
Fluorescence image



Underlying structure



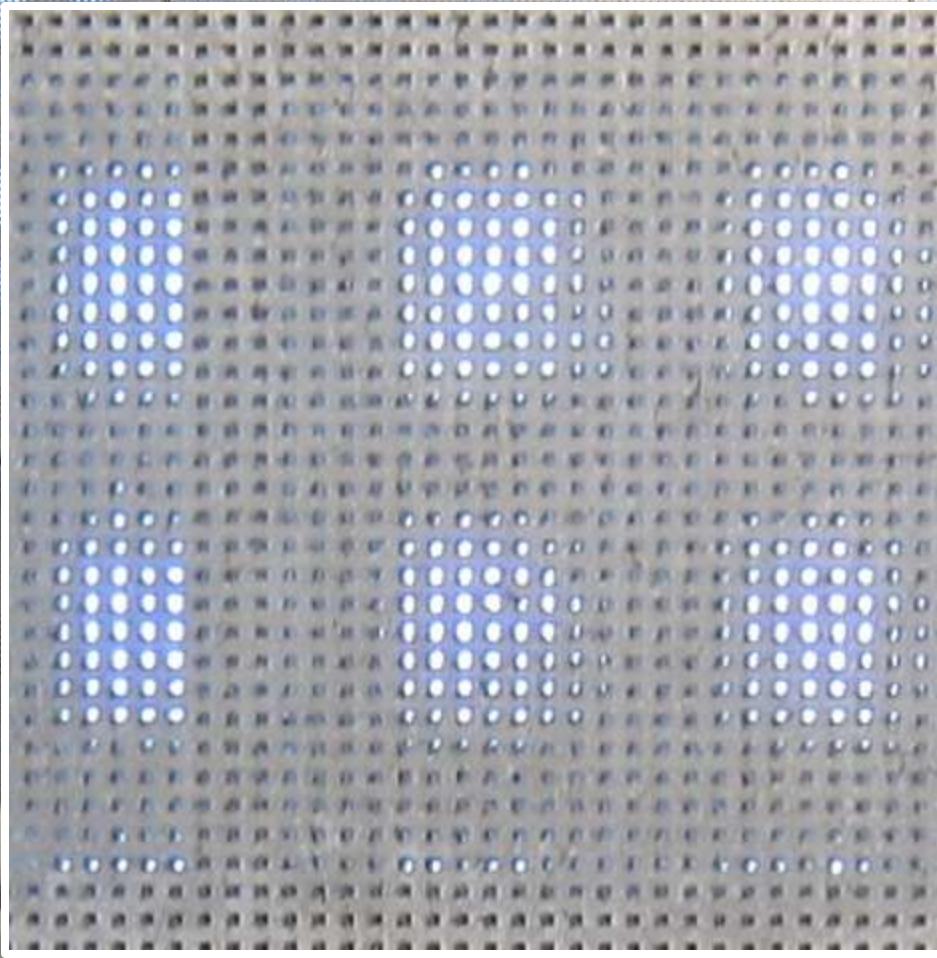
Excitation pattern



SIM (Gustafsson / Heintzmann)
SSIM (Gustafsson 2005)

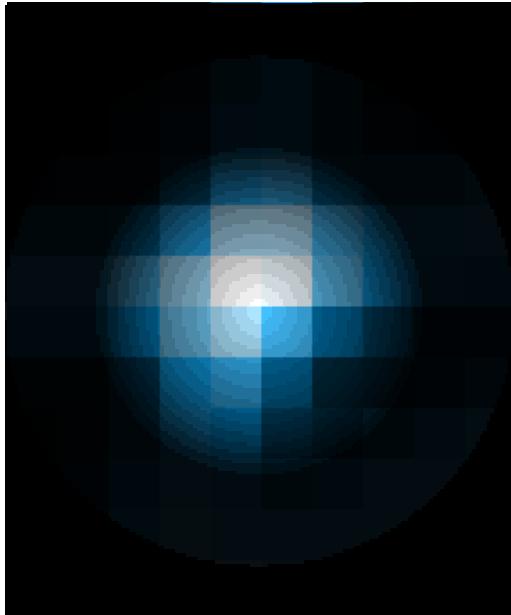
Diffraction limited excitation and emission

Doubled resolution

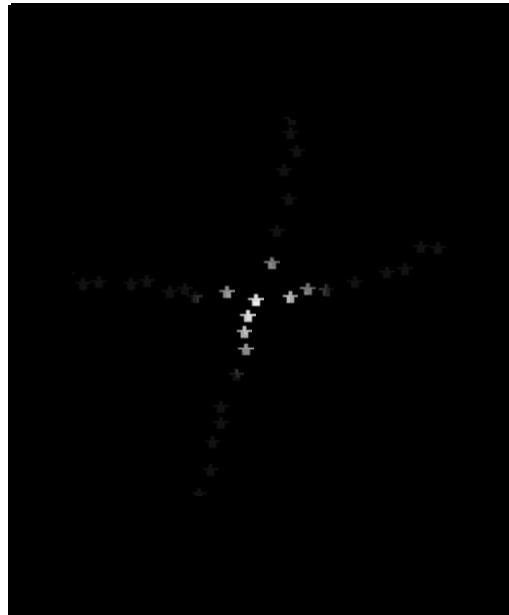


Super-resolution by differential depletion

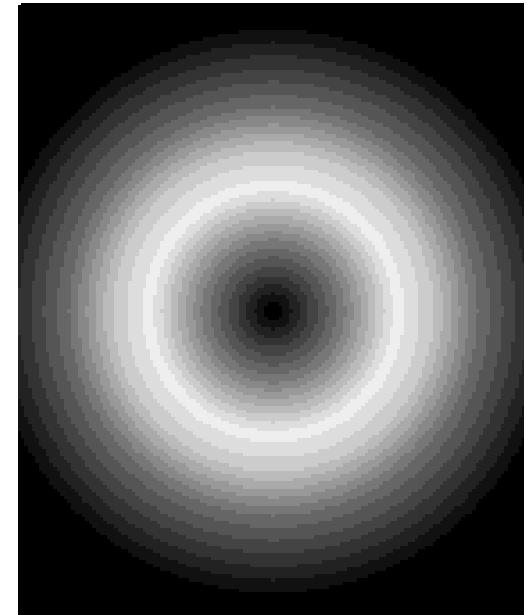
Fluorescence image



Underlying structure



Depletion pattern



Diffraction limited PSF Saturated depletion = Smaller effective PSF

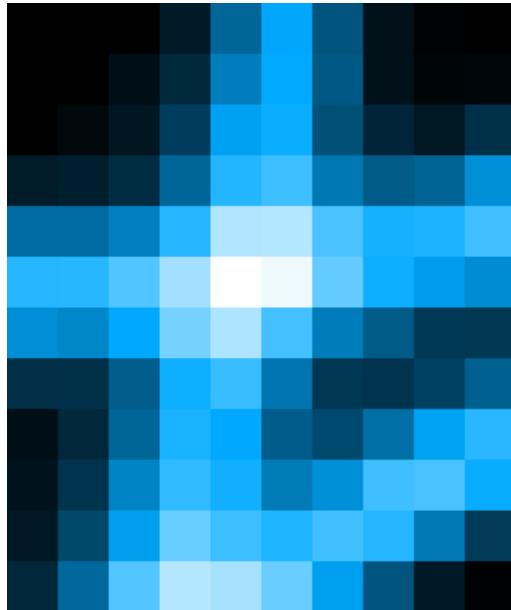
STED (Hell 1994, Hell 1999)

GSD (Hell 1995, Hell 2007)

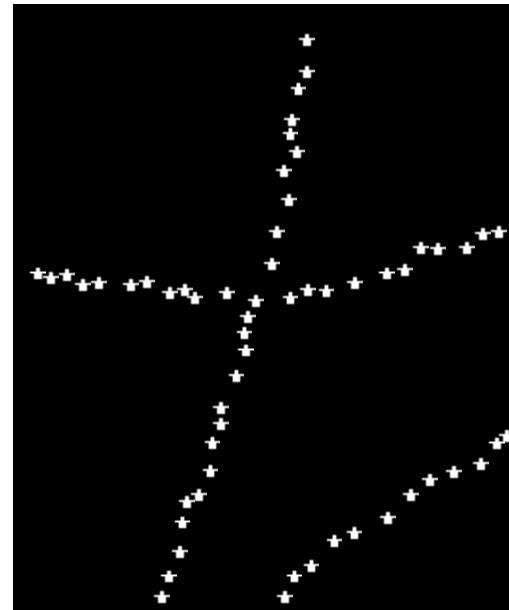
RESOLFT (Hell 2003, Hell 2011)

Super-resolution by single-molecule switching

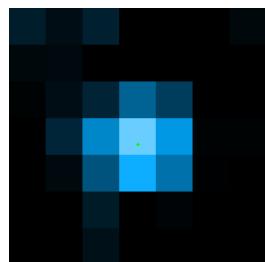
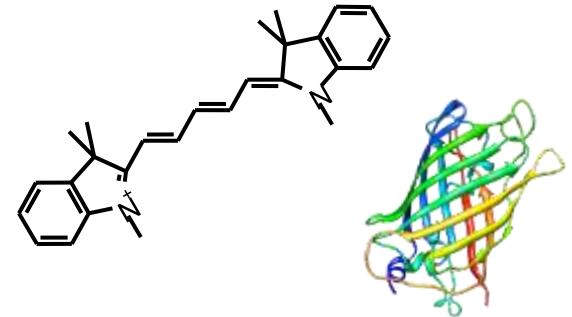
Fluorescence image



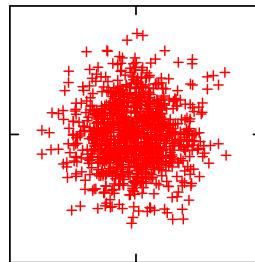
Underlying structure



Photoswitchable molecules



=



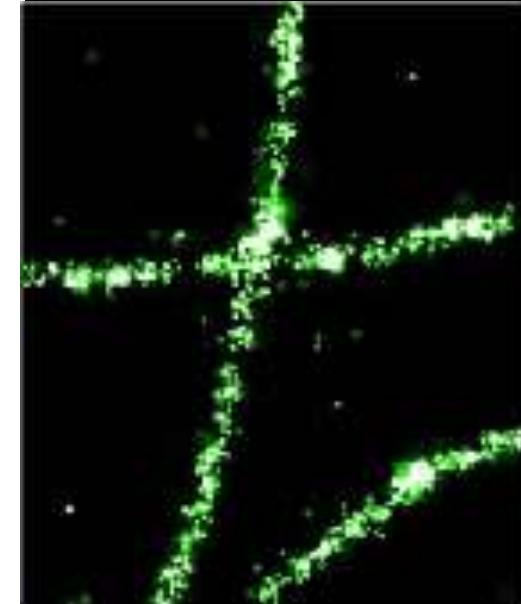
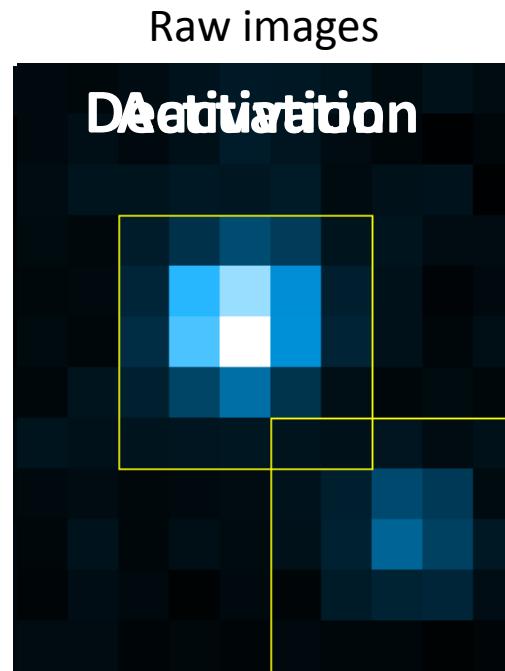
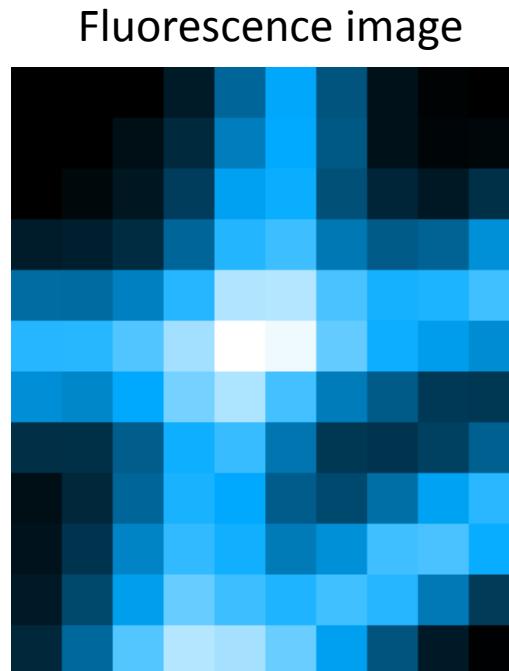
$$D \approx d / \sqrt{N}$$

Single molecule image

N photons

Single-molecule localization

Super-resolution by single-molecule switching



2x real time

STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)

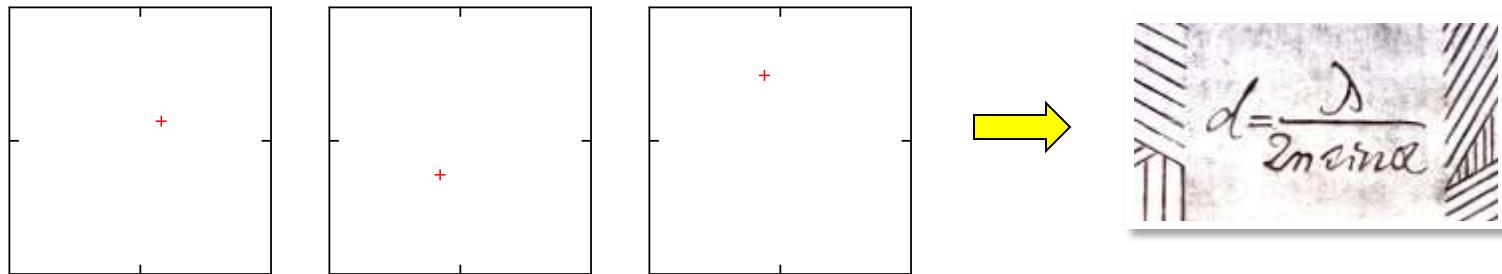
PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)

FPALM = Fluorescence Photoactivated Localization Microscopy (Hess 2006)

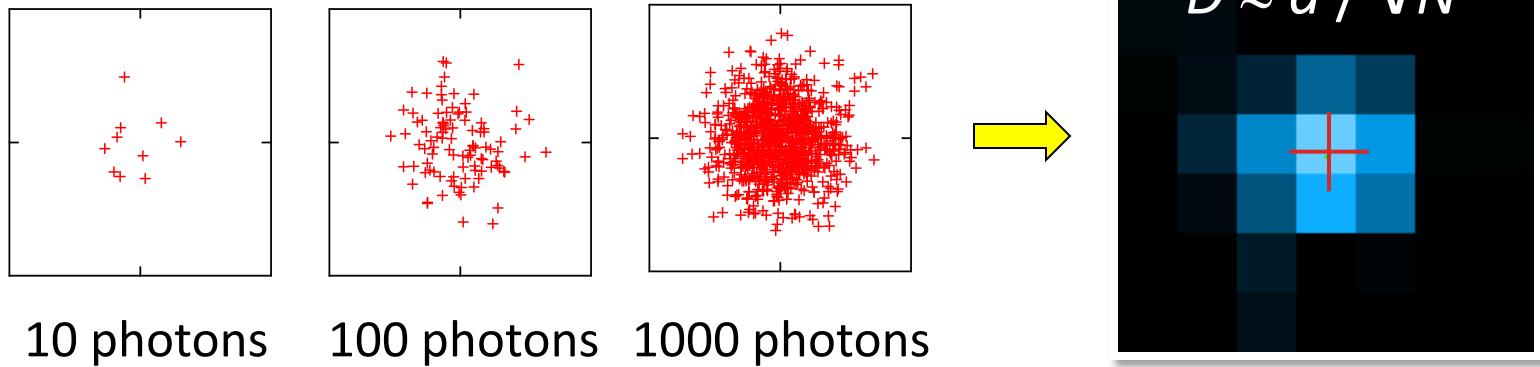
PALMIRA (Hell 2007), **GSDIM** (Hell 2008), **dSTORM** (Sauer 2008), **SMACM** (Moerner 2008)

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

Single-molecule localization precision



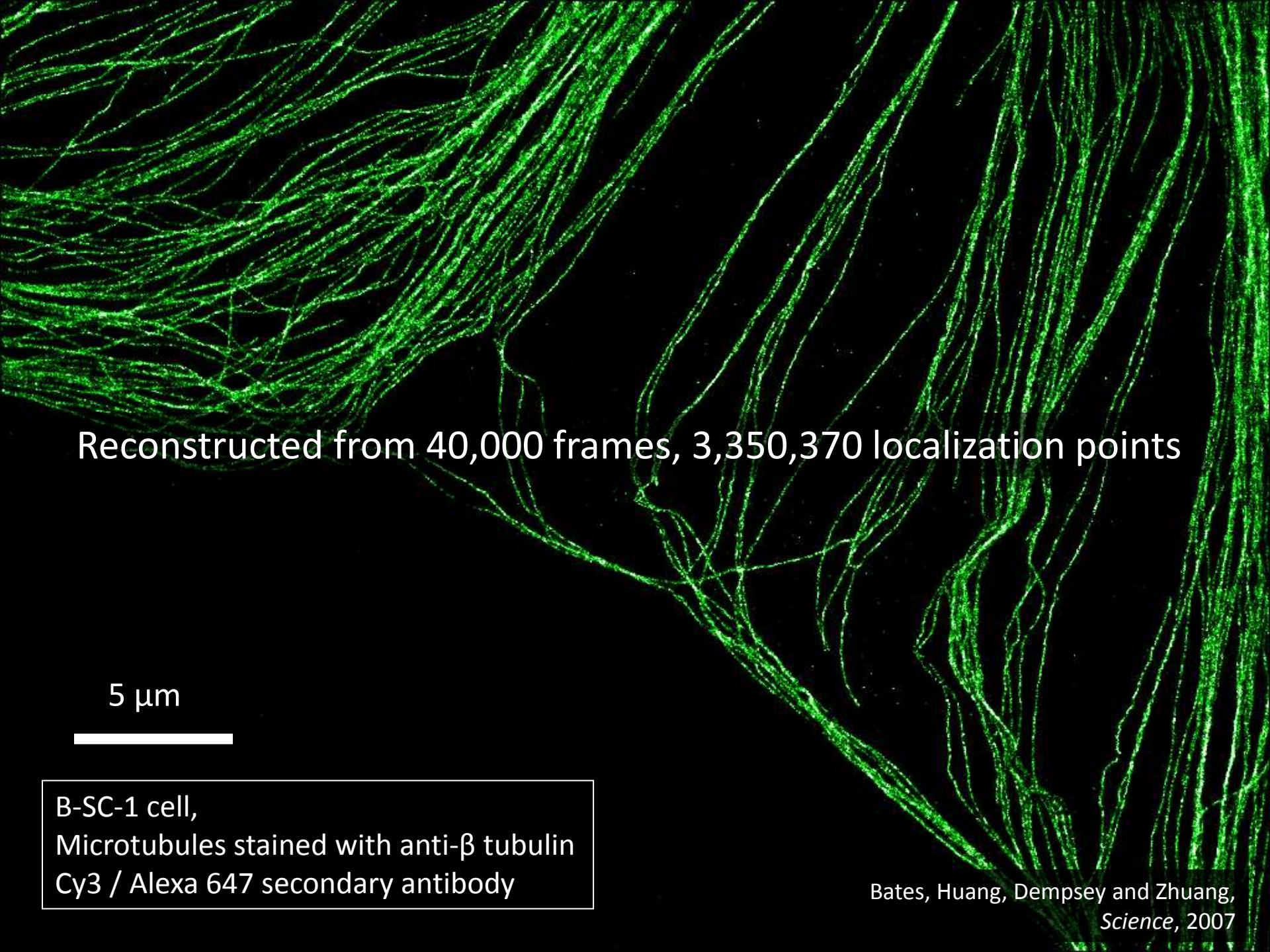
1 photon



10 photons

100 photons

1000 photons

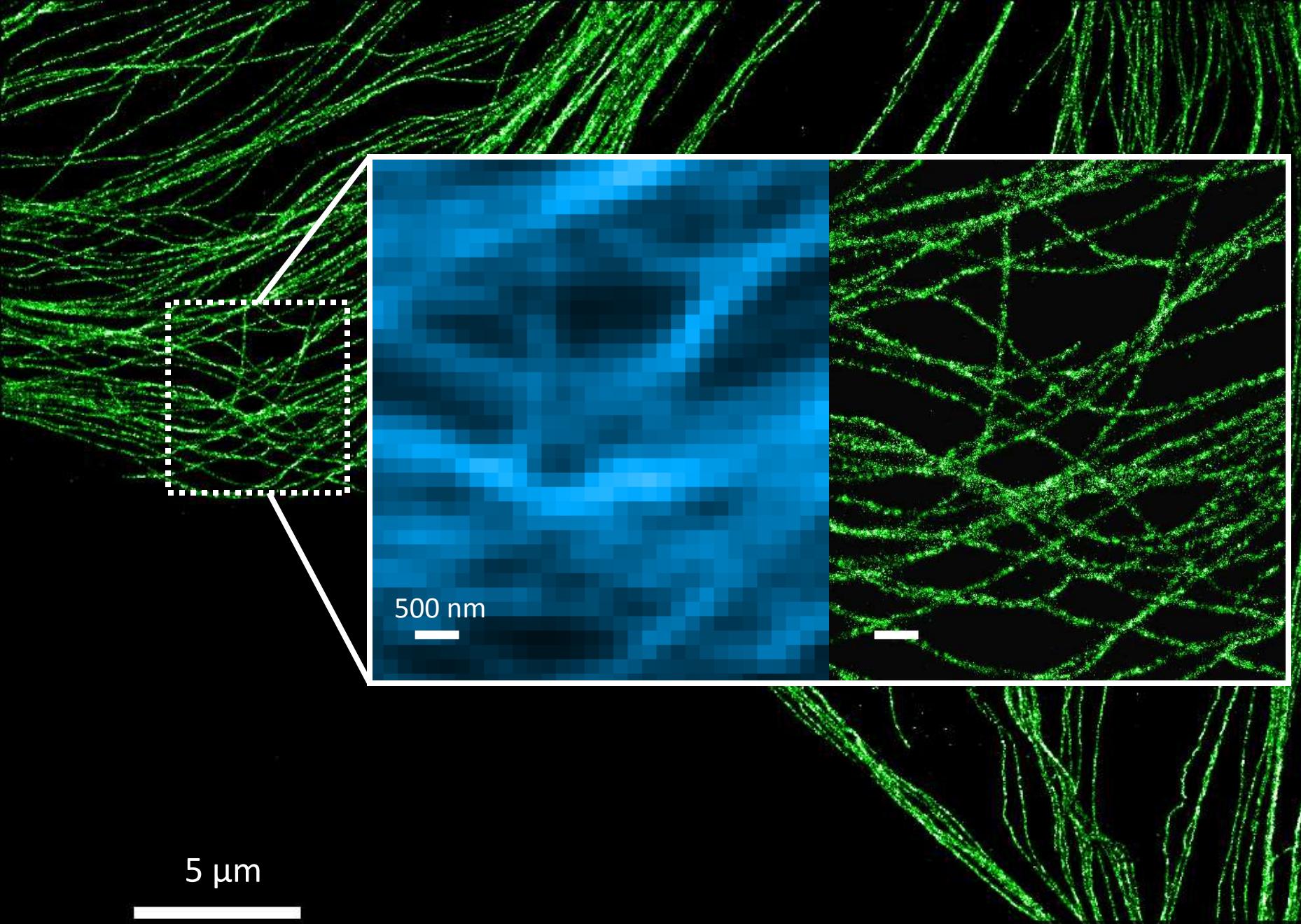
A fluorescence microscopy image showing a dense network of green-stained microtubules against a black background. The microtubules are represented by numerous thin, curved lines of green fluorescence, forming a complex web-like structure.

Reconstructed from 40,000 frames, 3,350,370 localization points

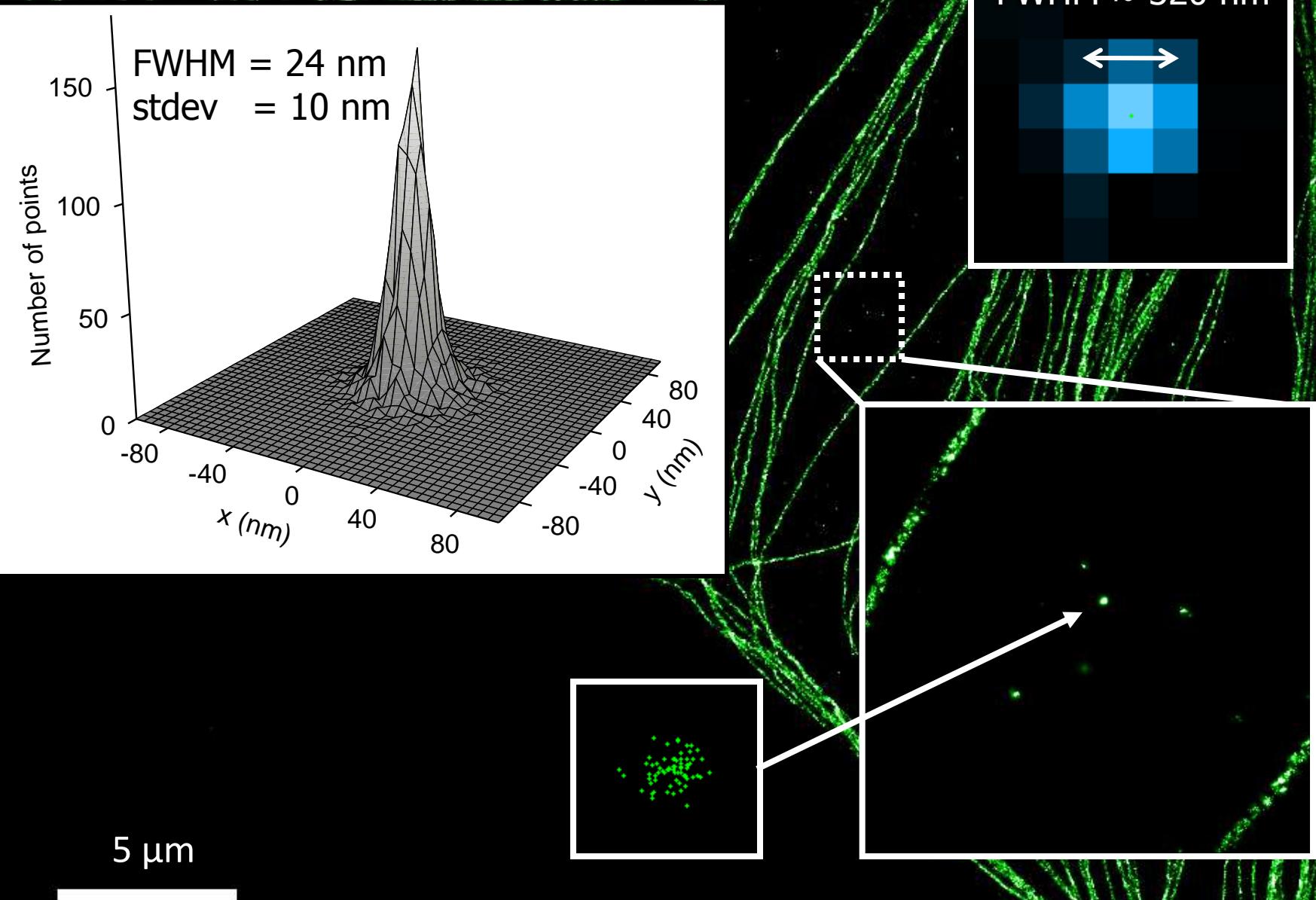
5 μ m

B-SC-1 cell,
Microtubules stained with anti- β tubulin
Cy3 / Alexa 647 secondary antibody

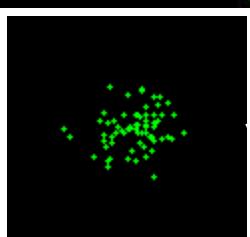
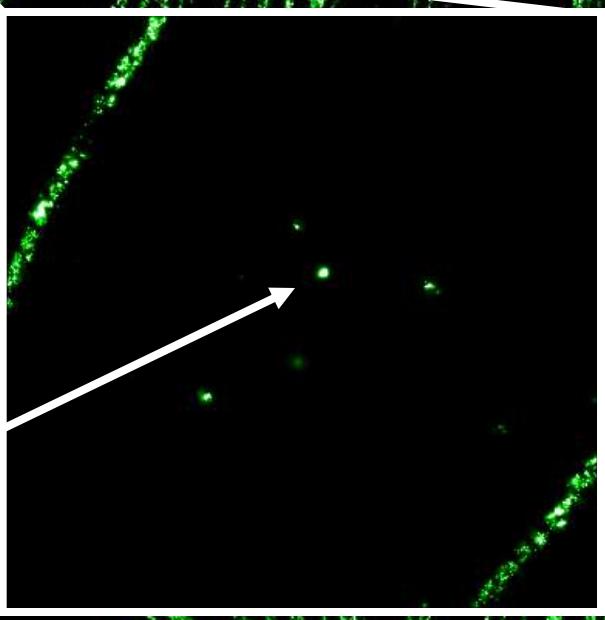
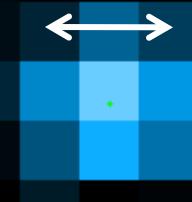
Bates, Huang, Dempsey and Zhuang,
Science, 2007



Bates, Huang, Dempsey and Zhuang,
Science, 2007



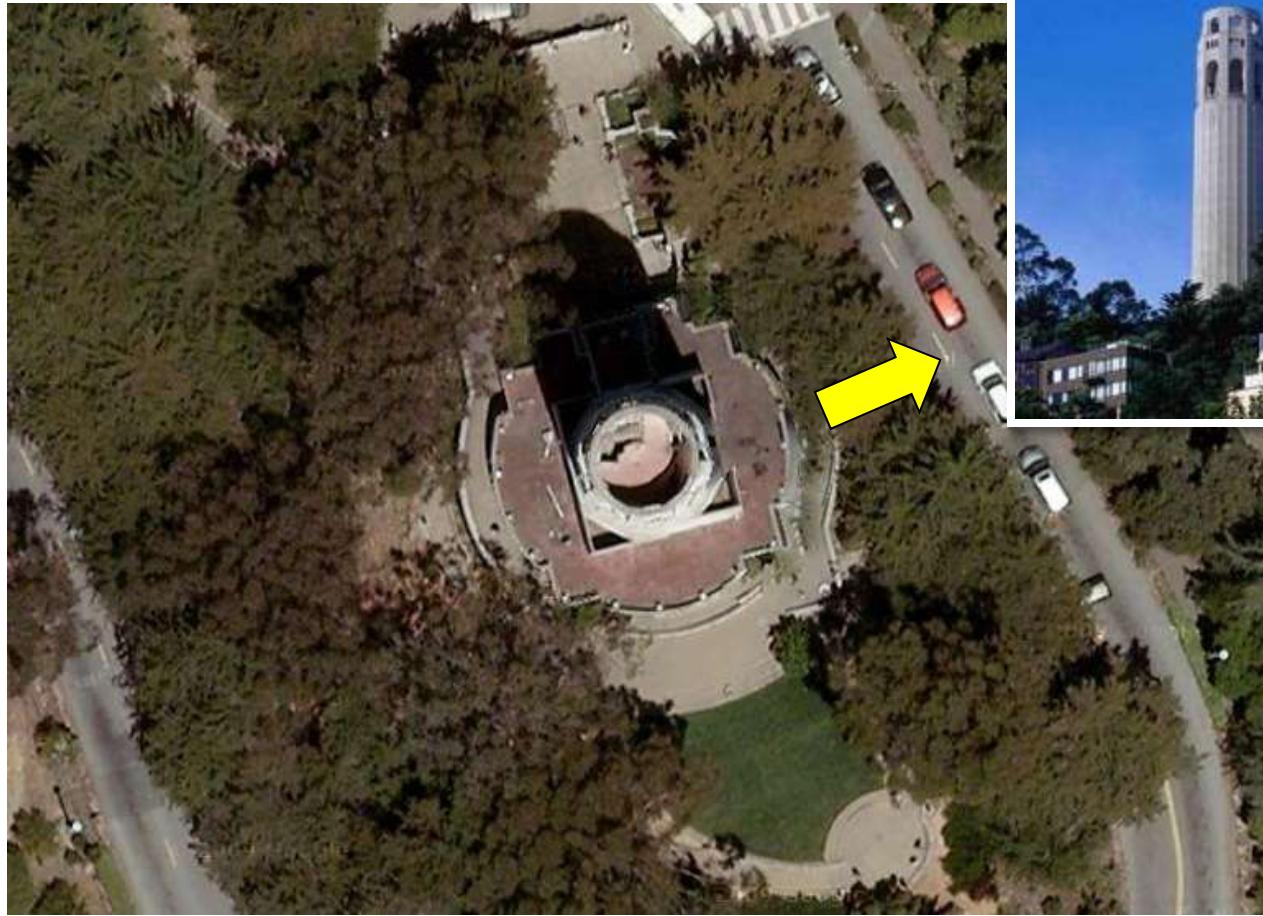
FWHM \approx 320 nm



3D Imaging

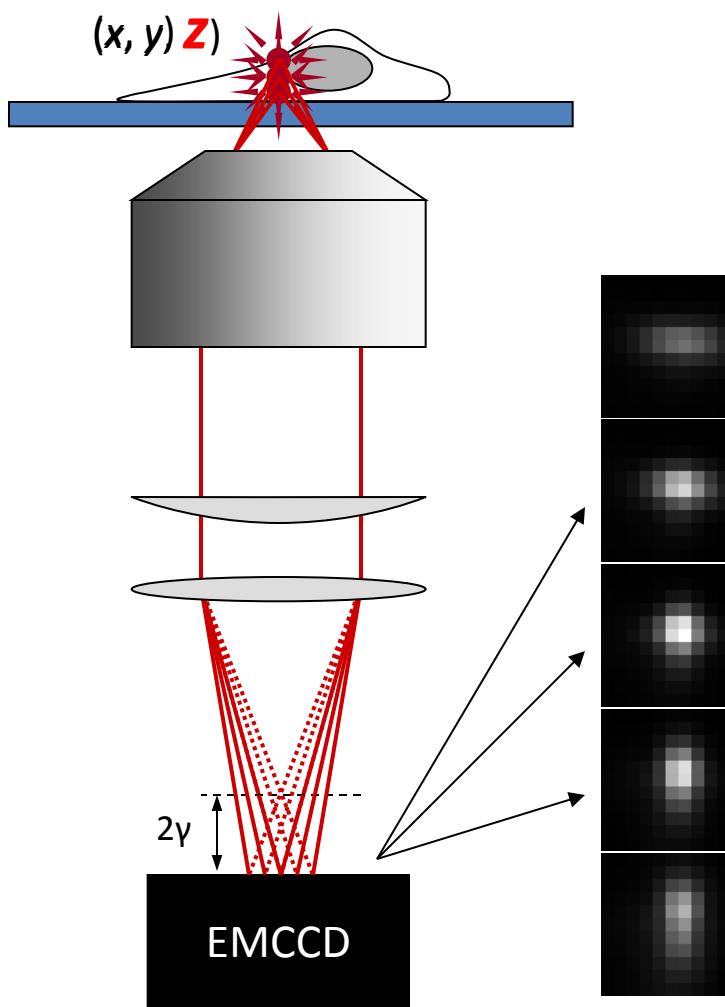
In a 2D world...

Satellite image of ???



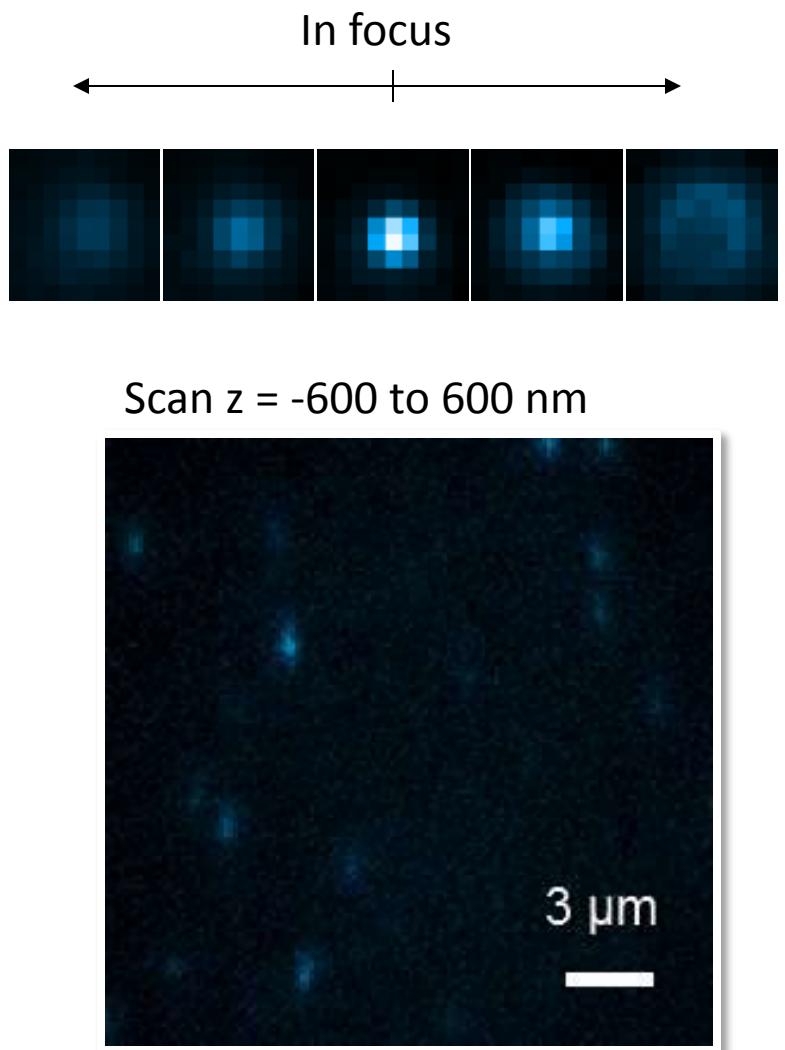
Google maps

3D Imaging: Localization in the Third Dimension

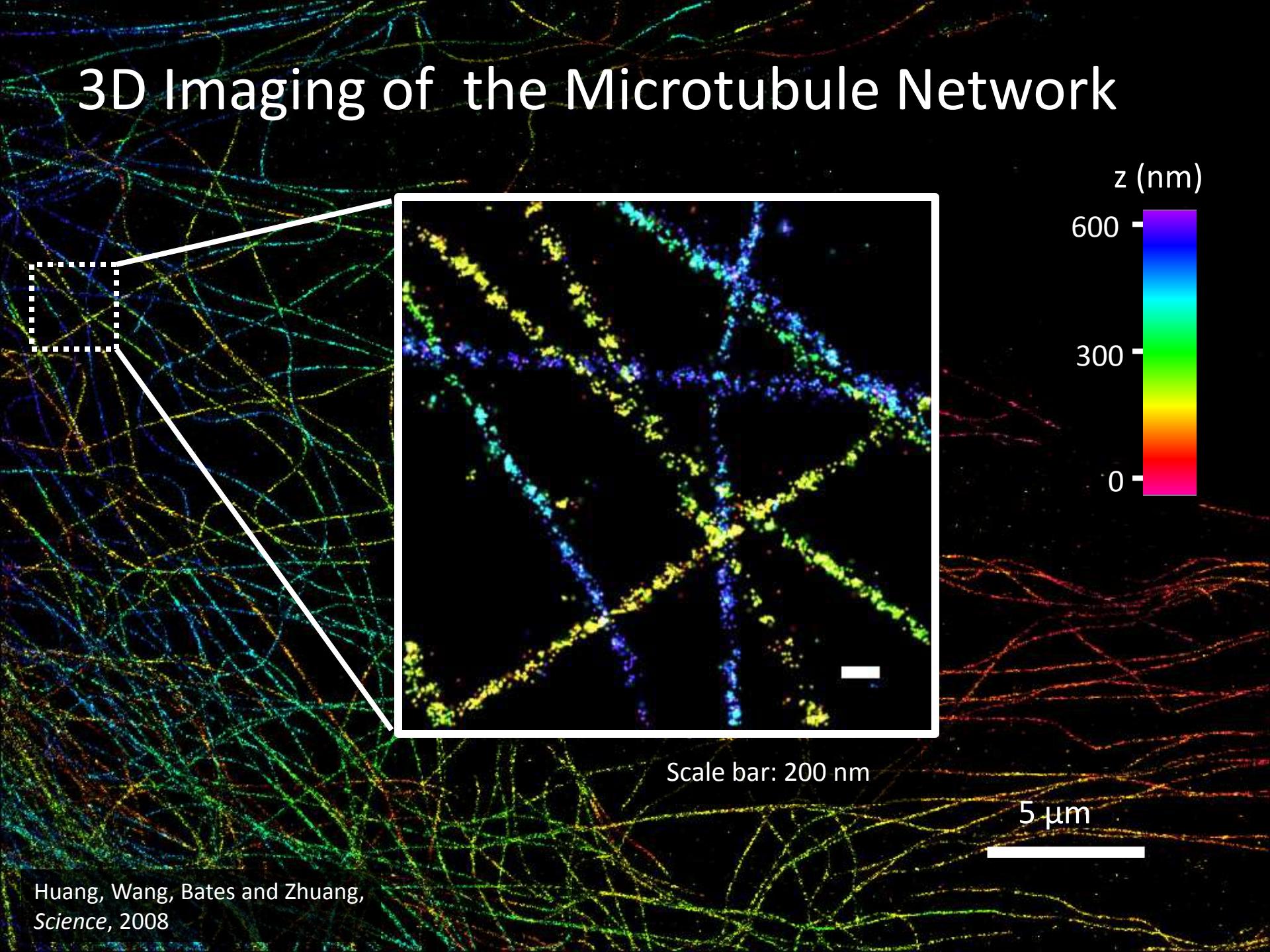


z (nm)

400
200
0
-200
-400

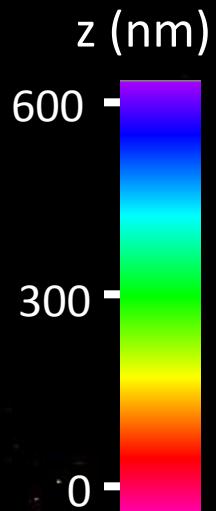
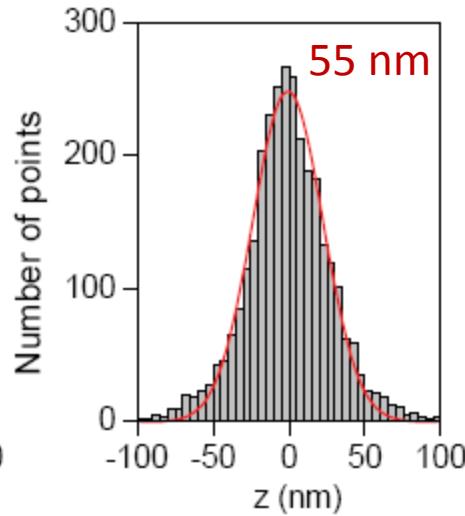
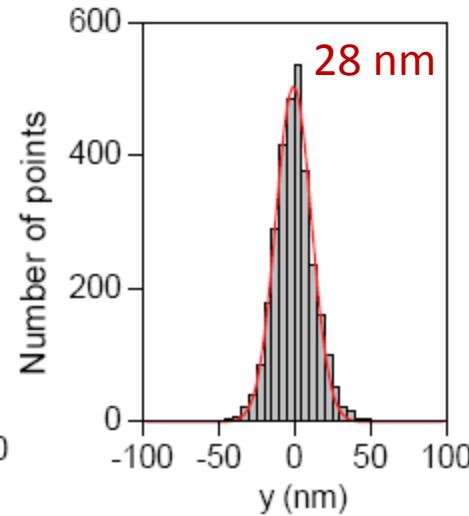
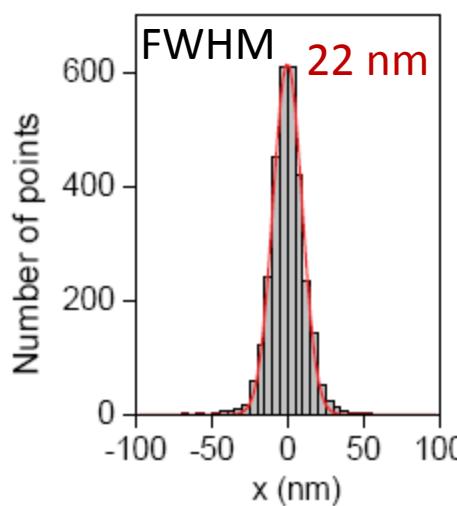


3D Imaging of the Microtubule Network



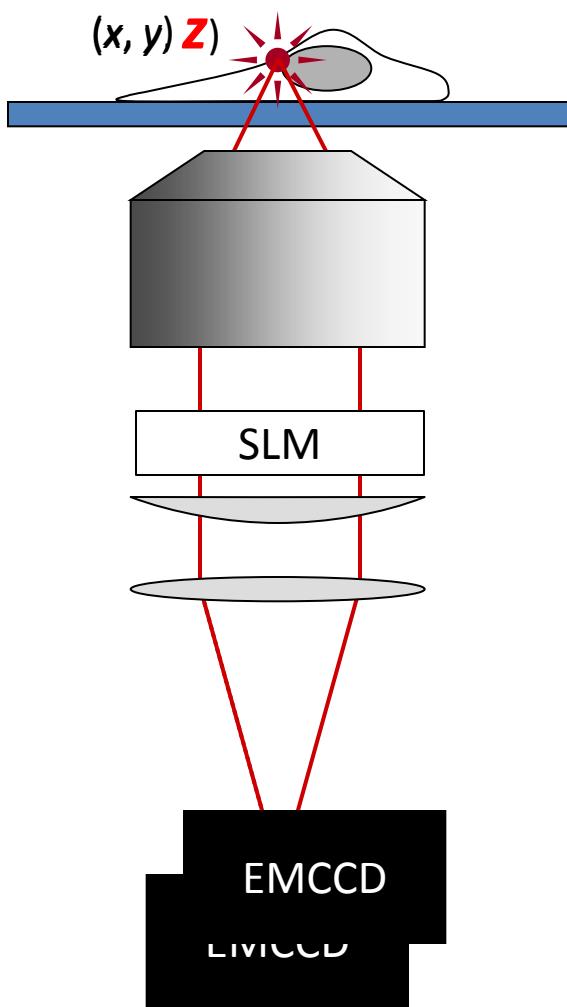
3D Imaging of the Microtubule Network

Small, isolated clusters

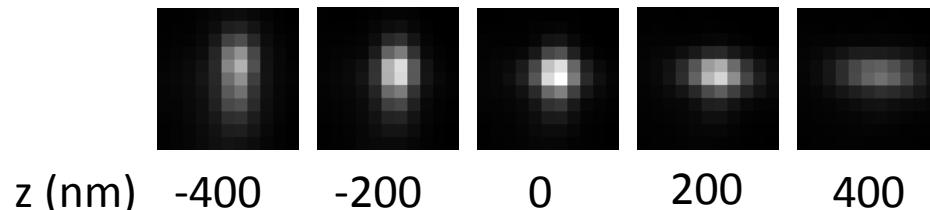


5 μ m

Other 3D localization method

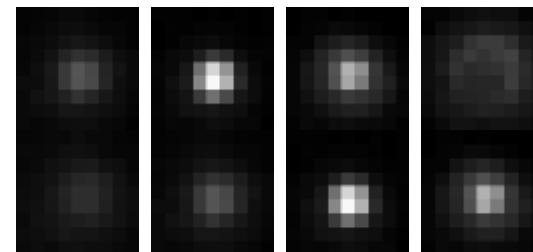


Astigmatic imaging



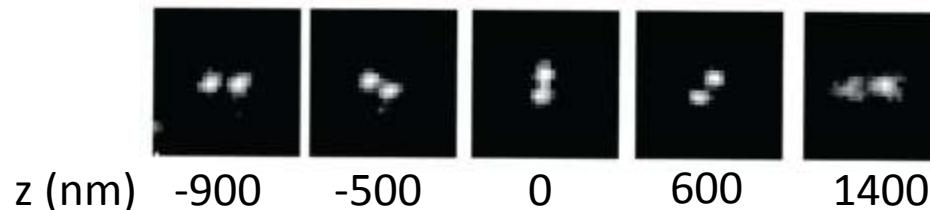
Huang et al., Science 2008

Bi-plane imaging



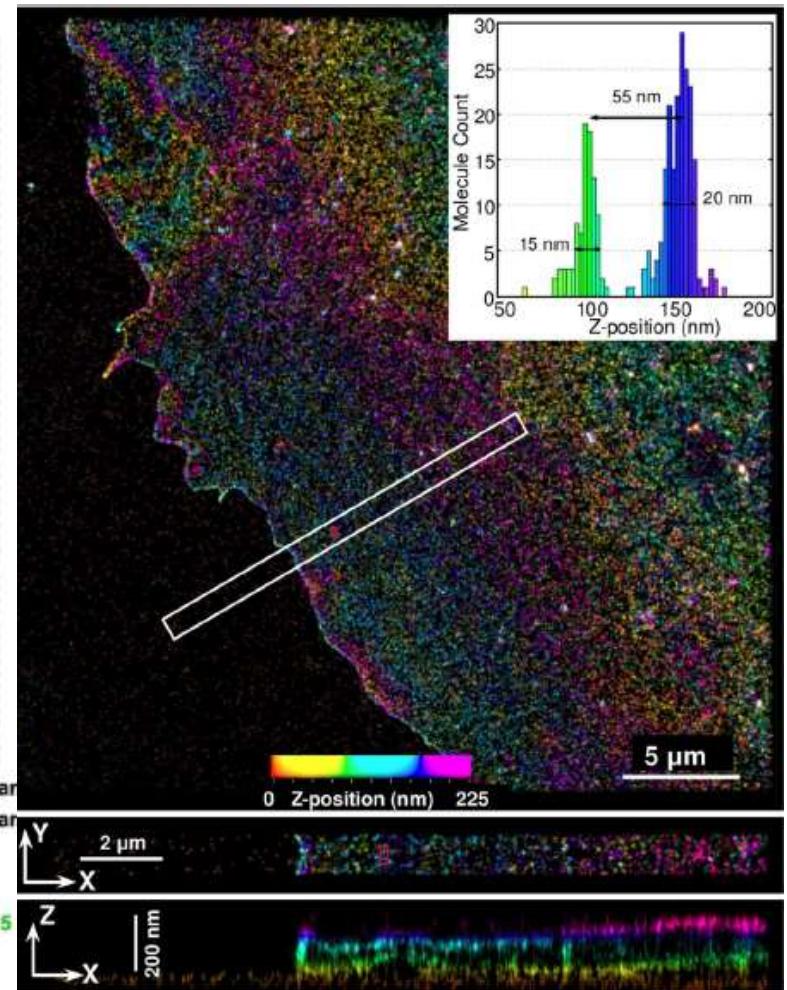
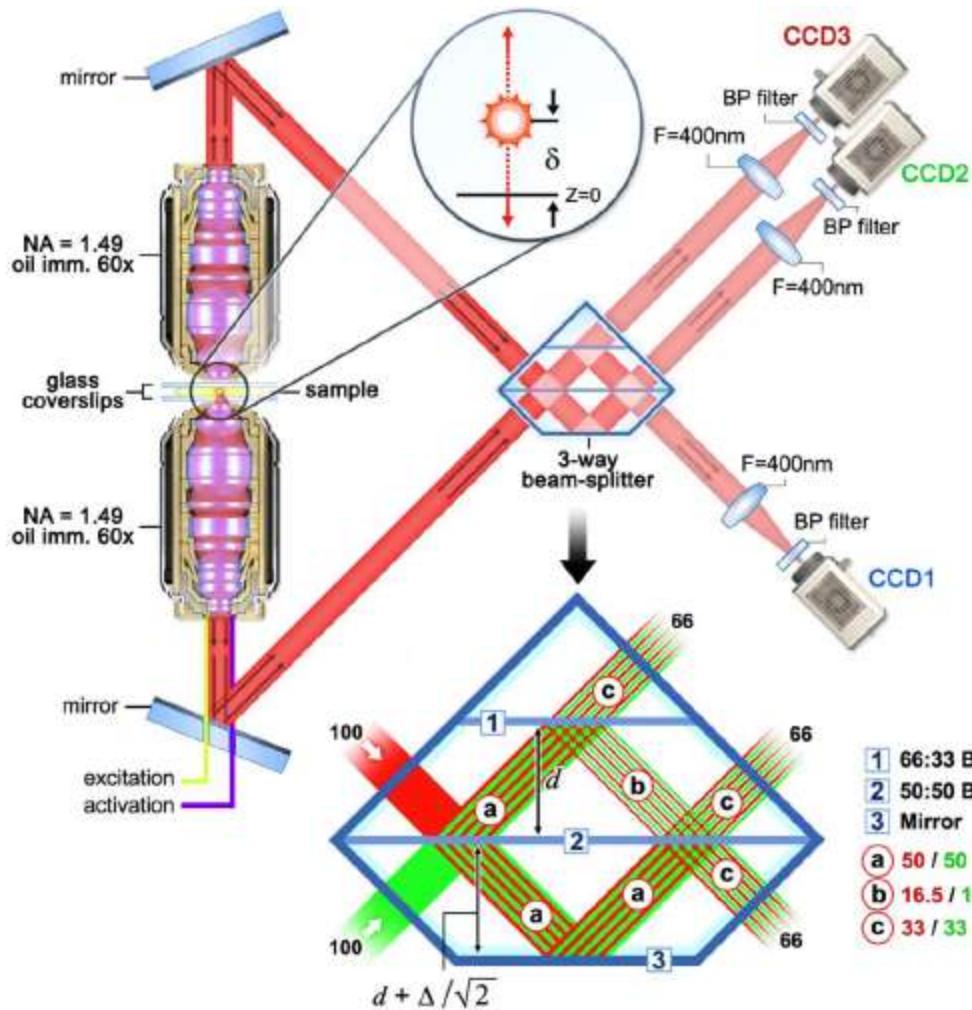
Juette et al., Nat Methods 2008

Double-helical PSF

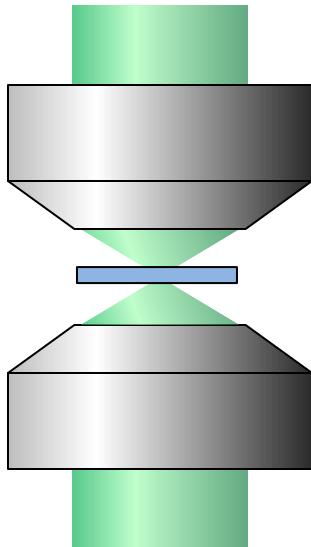


Pavani et al., PNAS 2009

Better 3D by two-objective interference



The use of two opposing objectives

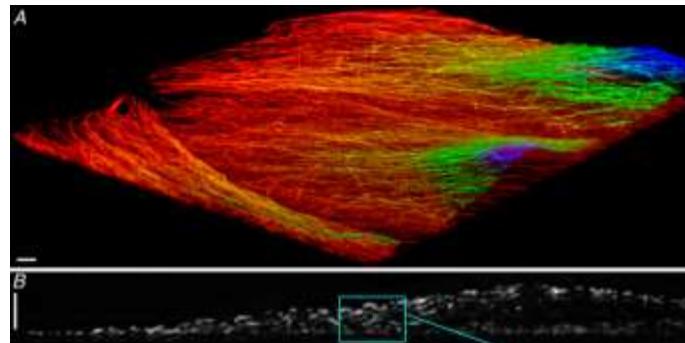


4Pi scheme



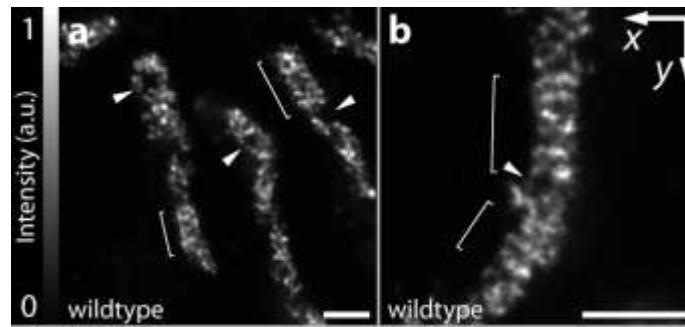
Near isotropic
3D resolution

$|^5S$



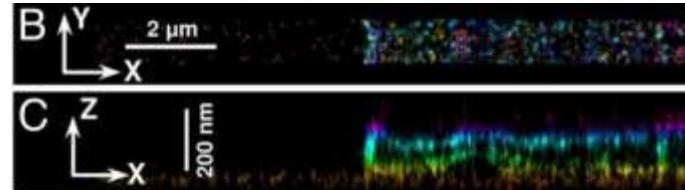
Shal et al., Biophys J 2008

isoSTED



Schmidt et al., Nano Lett 2009

iPALM



Shtengel et al., PNAS 2009

Photoswitchable fluorophores



Photoswitchable probes readily available

400

500

600

700 nm

Simple dyes (+ thiole / redox system)

Alexa488

Alexa532

Atto520

Atto565

Alexa568

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

Dil

DiD

LysoTracker Red

ER Tracker Red

Shim et al., 2012

Photoactivatable fluorescent proteins

PA-GFP

PS-CFP2

Dronpa

mEosFP2

Dendra2

PAmCherry

PAtagRFP

Dreiklang

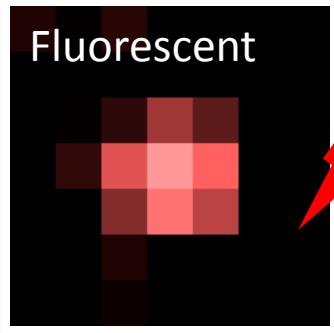
EYFP

Reviews:

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

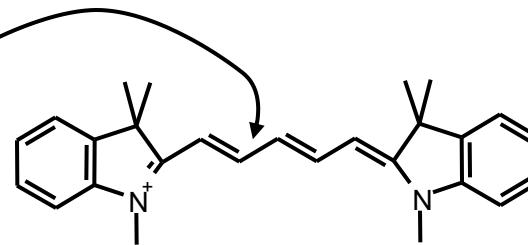
Lippincott-Schwartz et al., Trends Cell Biol., 2009

Photoswitching of red cyanine dyes

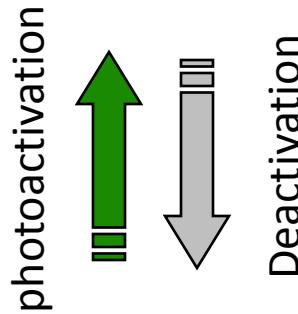


650 nm

+ thiol

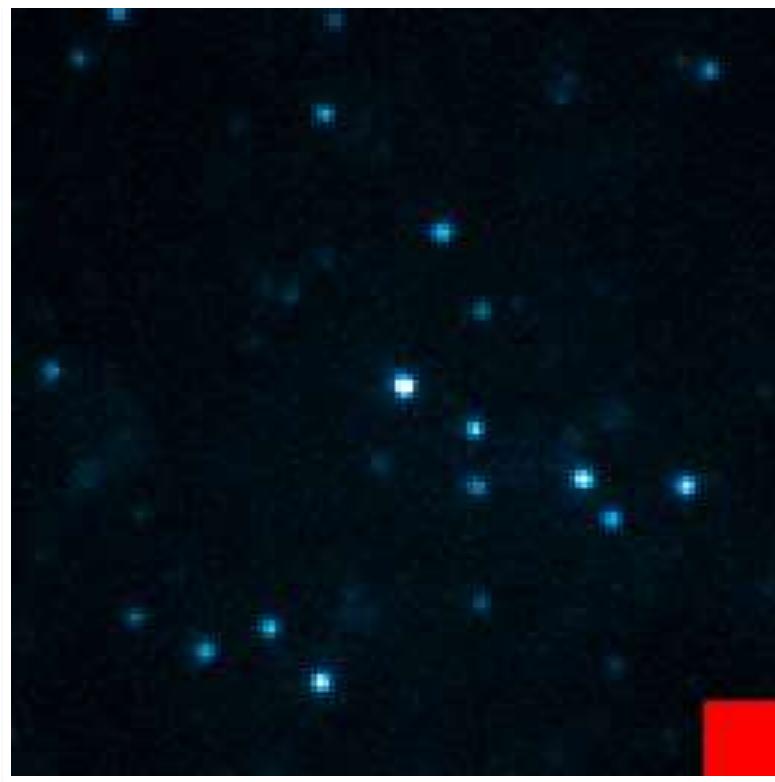


Cy5 / Alexa 647



360 nm

650 nm

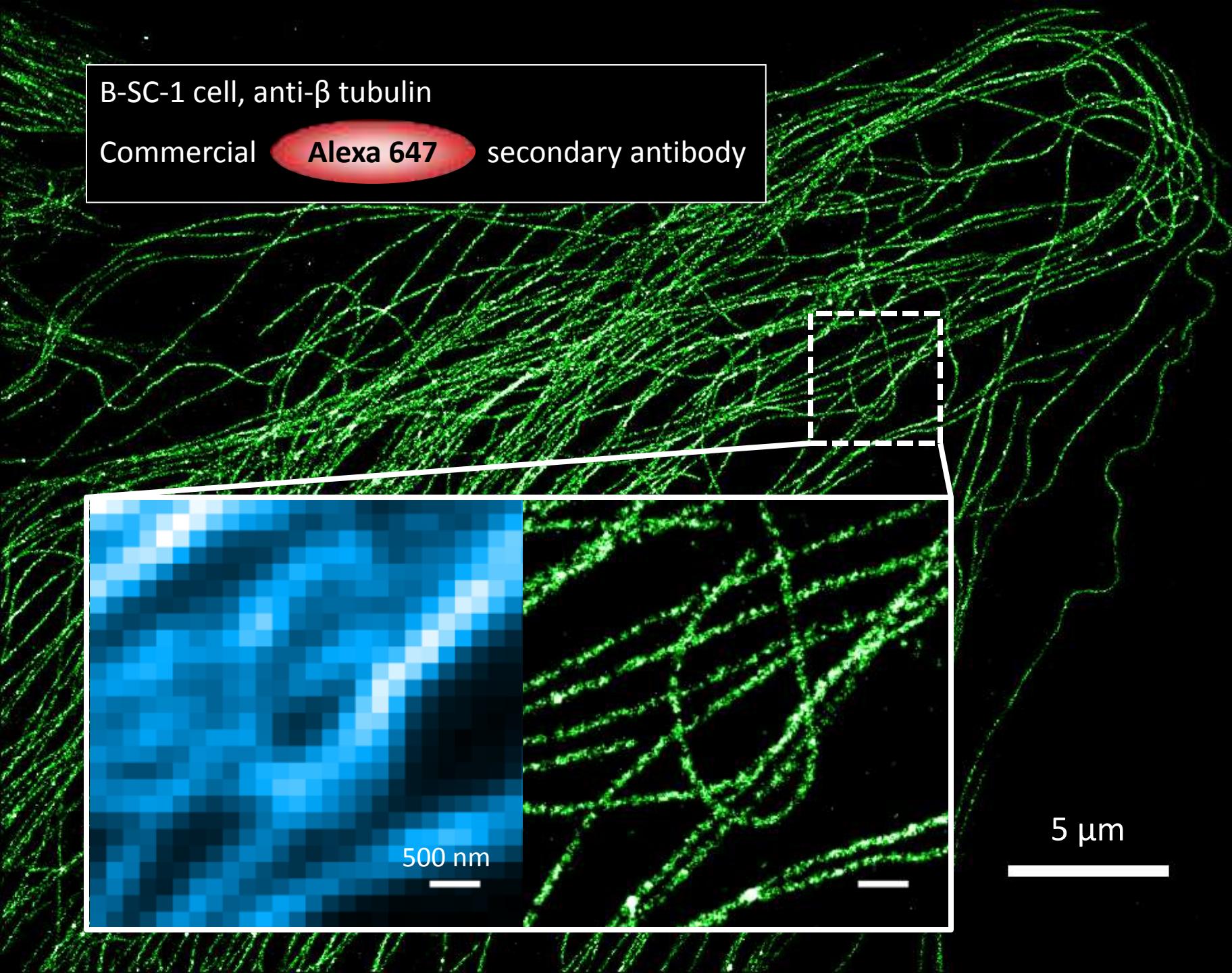
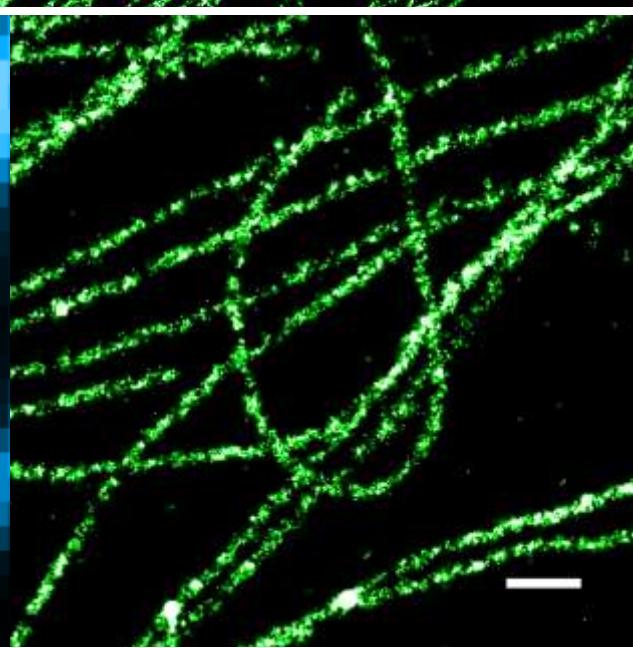
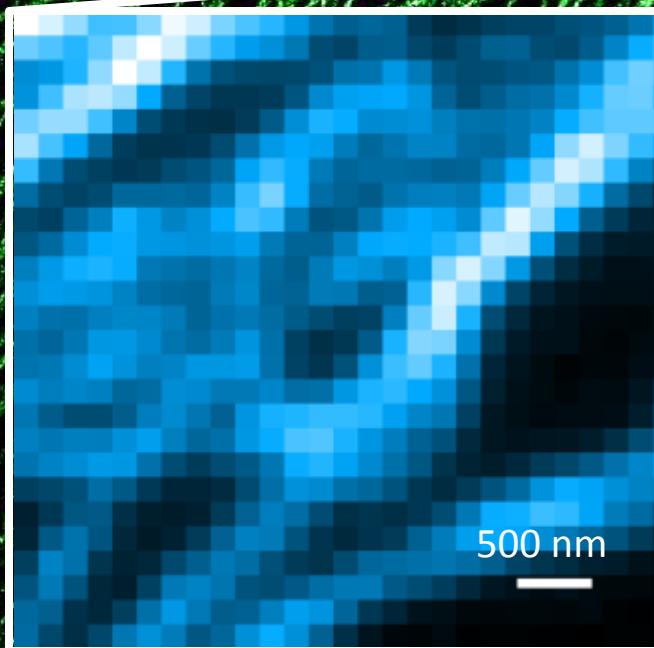


B-SC-1 cell, anti- β tubulin

Commercial

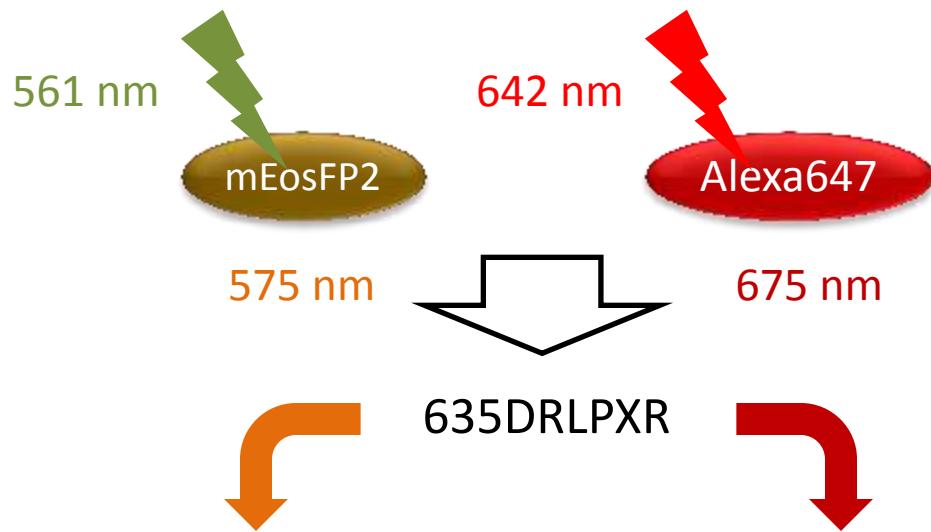
Alexa 647

secondary antibody



Multi-color Imaging

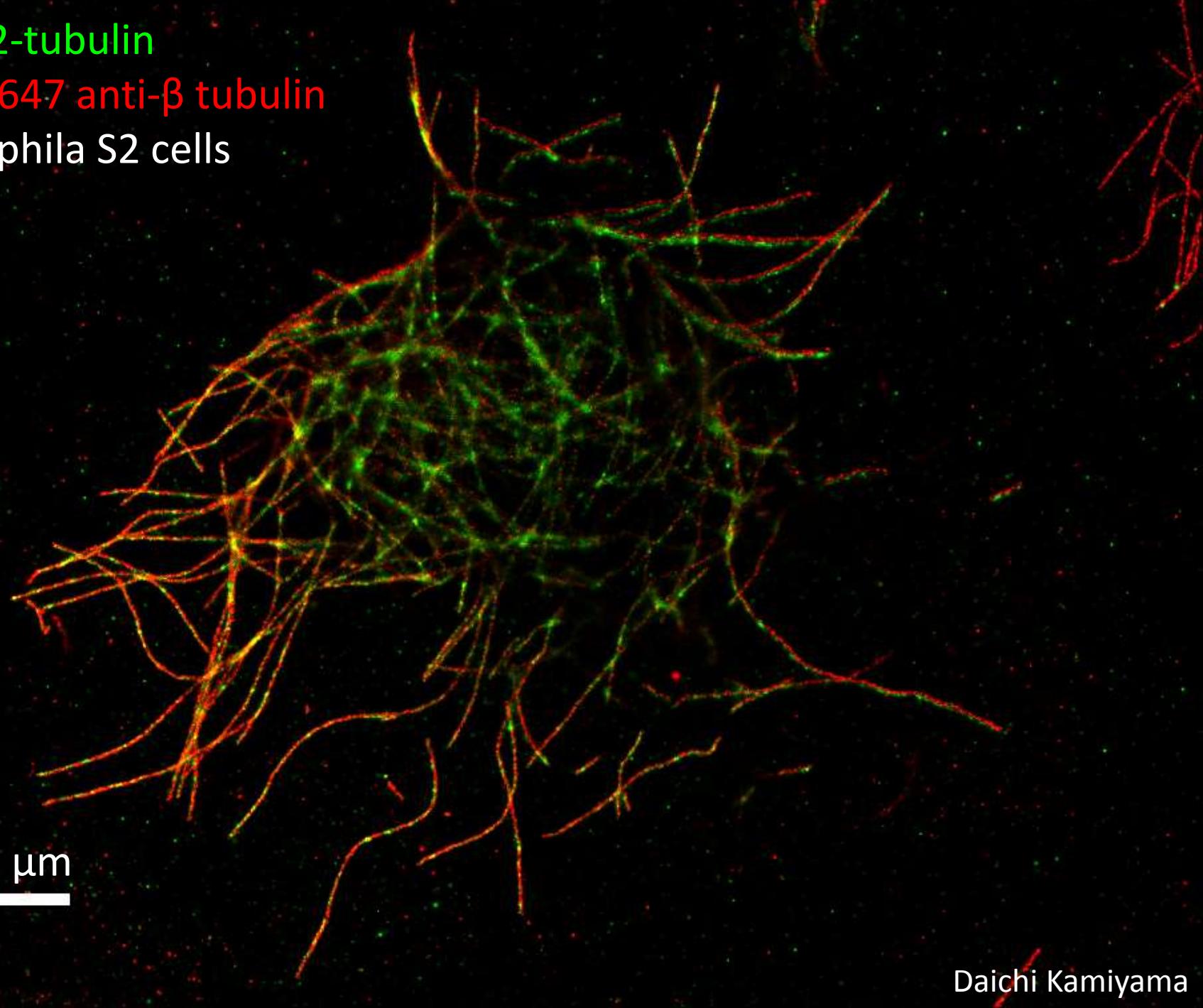
Multicolor STORM/PALM



mEos2-tubulin

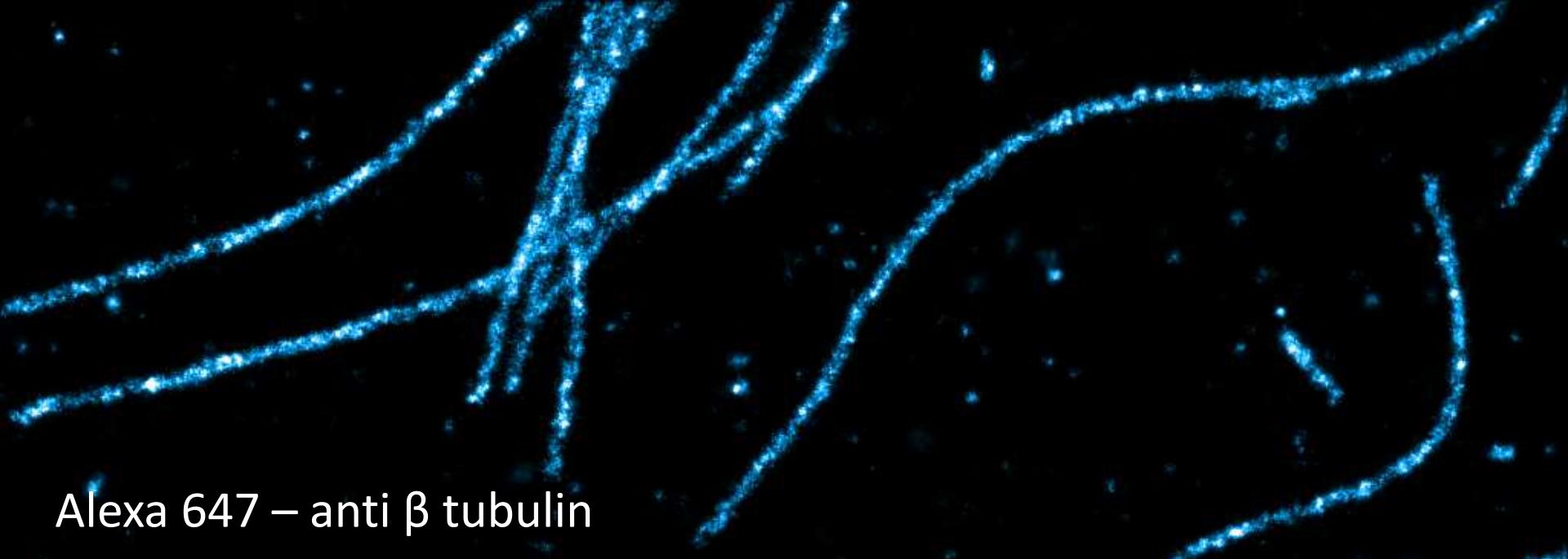
Alexa 647 anti- β tubulin

Drosophila S2 cells

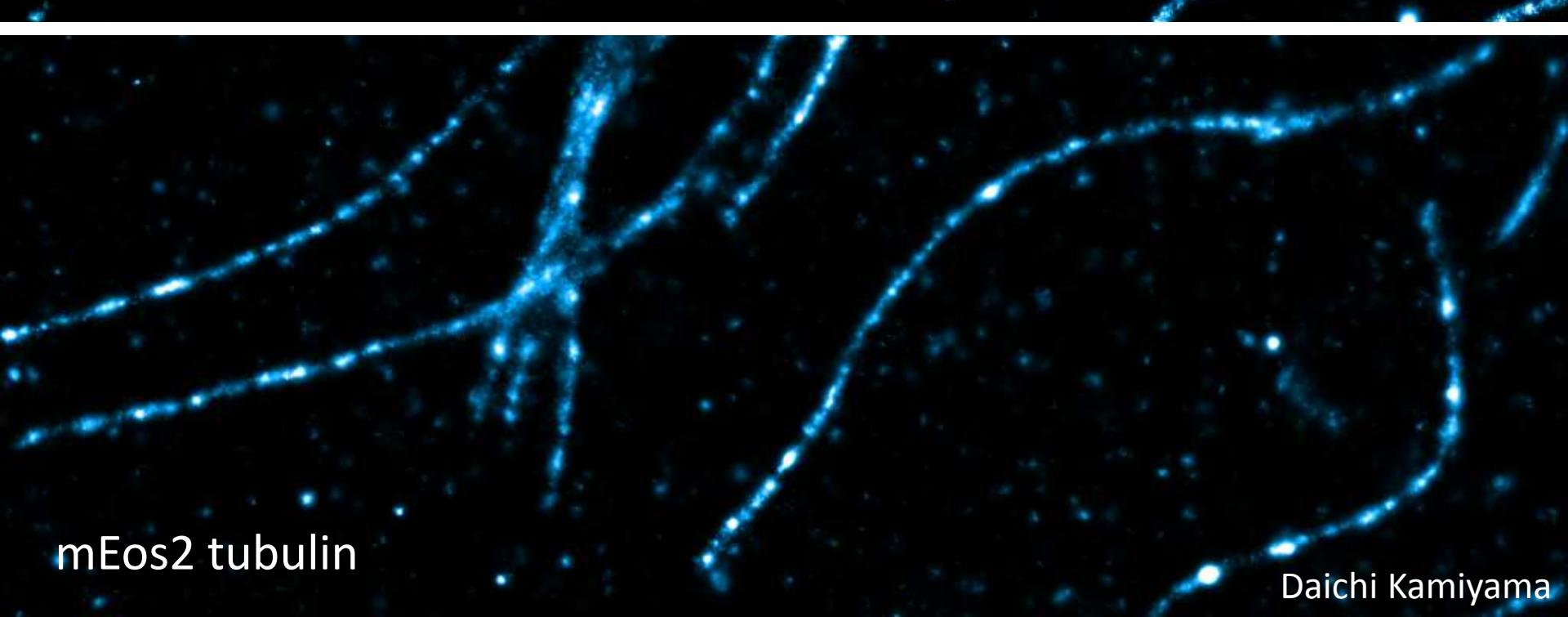


2 μ m

Daichi Kamiyama



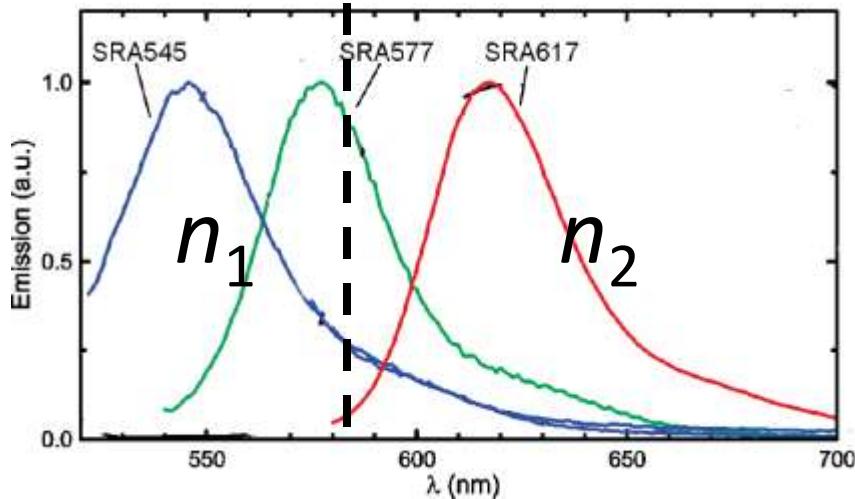
Alexa 647 – anti β tubulin



mEos2 tubulin

Daichi Kamiyama

Multicolor STORM/PALM: Emission

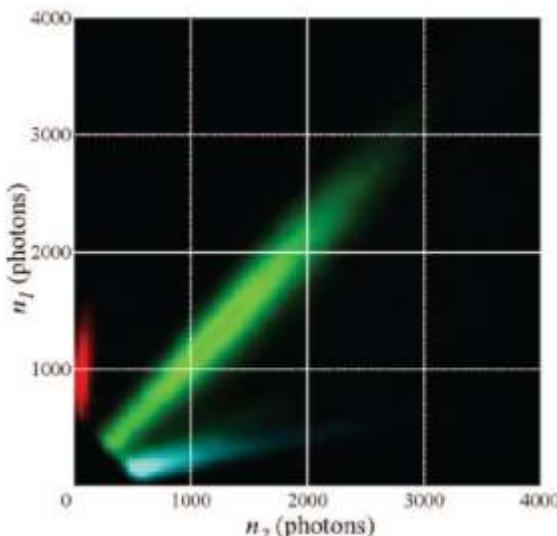


$$n_1 = n_2$$

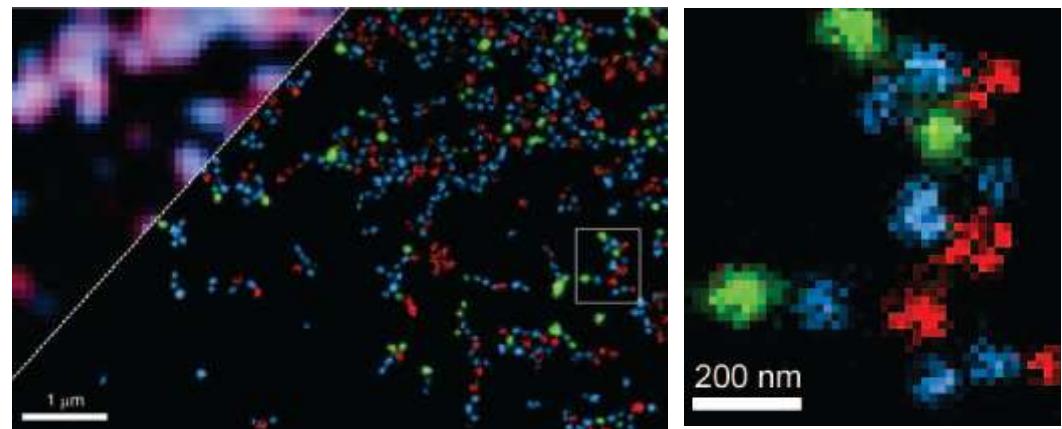
→ 50% SRA545 + 50% SRA617?

→ 100% SRA577?

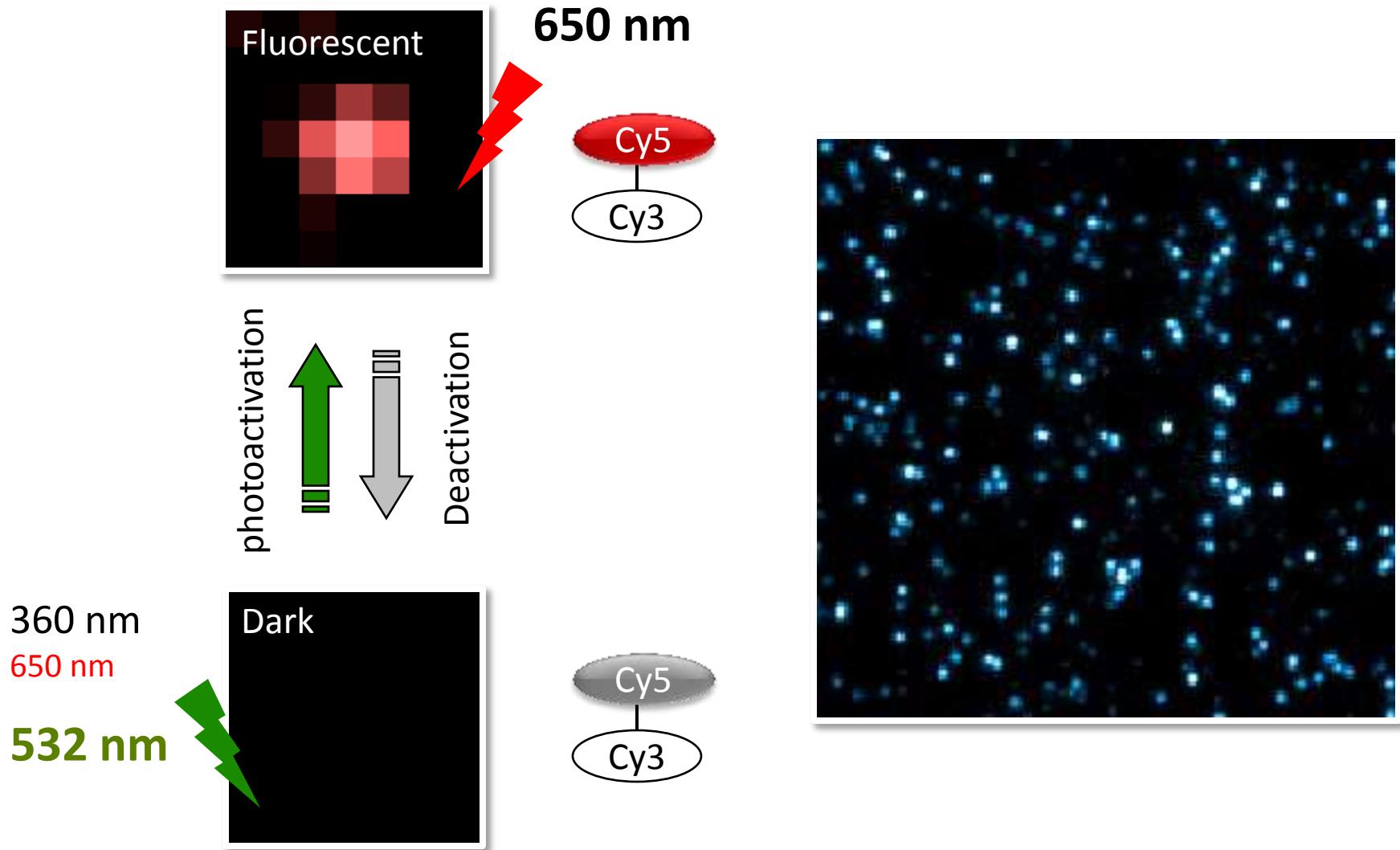
Single-molecule detection!



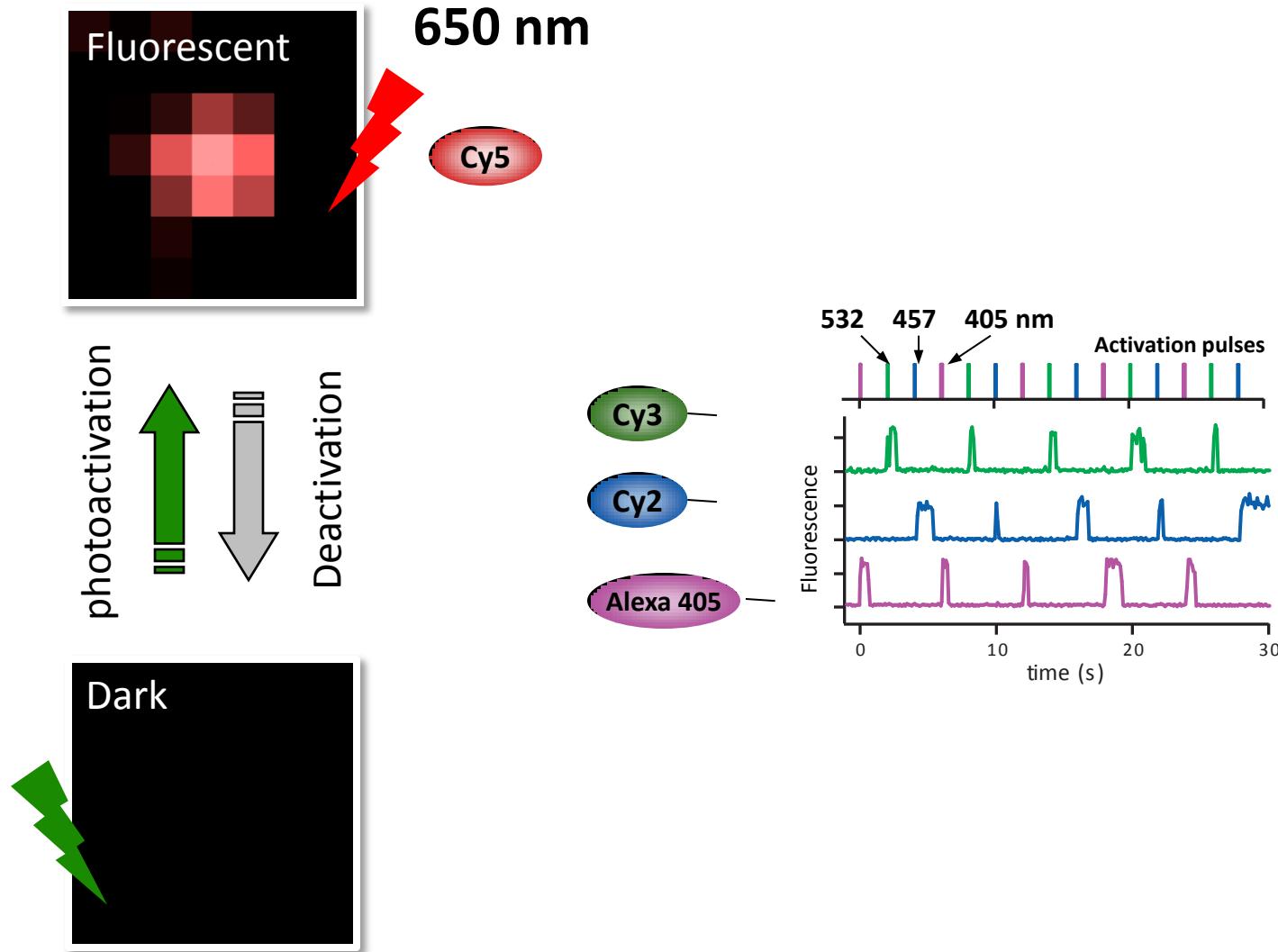
3-color imaging with one excitation wavelength
and two detection channels

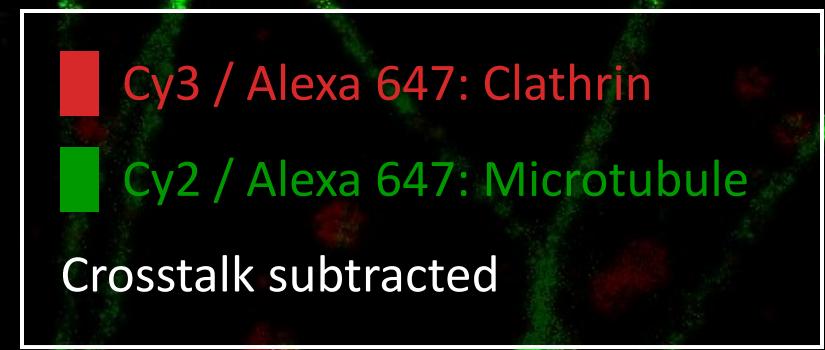
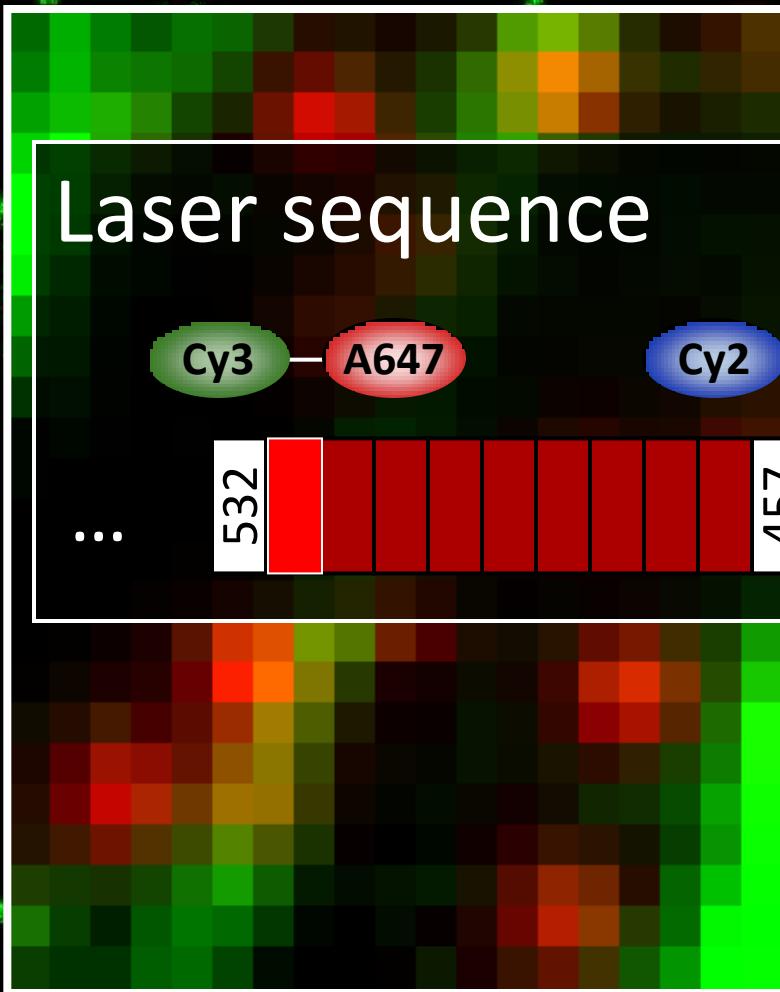


Multicolor STORM/PALM: activation



Controlling the activation of Cy5





1 μm

Bates, Huang, Dempsey and Zhuang,
Science, 2007

Multicolor imaging approaches

By emission wavelengths

- Simple fluorophores
- Low crosstalk
- Continuous imaging
- Multi-channel detection optics
- Needs nanometer scale image alignment

By activation wavelengths

- Dye-pairs
- Crosstalk from nonspecific activation
- Laser sequences
- Single channel detection
- Images naturally aligned



Phone



Palm Pre

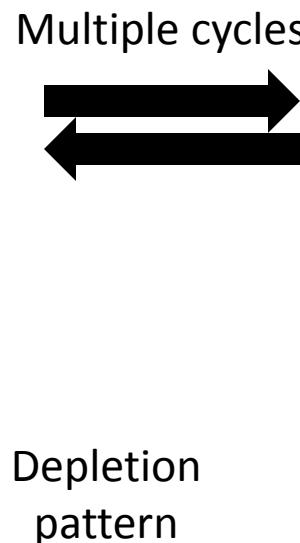
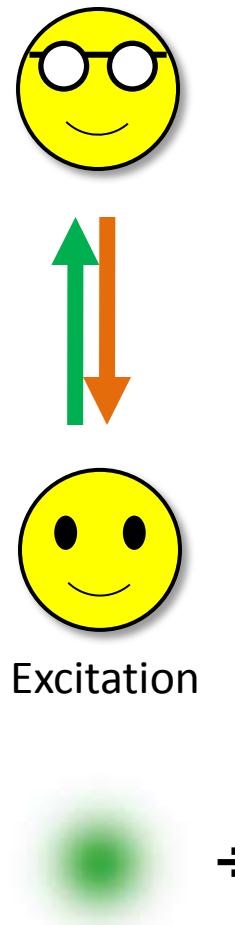
BlackBerry Storm 2

Manufacturer	Palm Inc.	Research In Motion
Platform	WebOS	Proprietary
Availability	6 Jun 2009	September 2009
Carrier	Sprint	Verizon
Price	\$199 (after rebate)	Unknown
Data Plan	From \$69/month - 2	Unknown
Size and weight	100.5 x 59.5 x 10 mm, 130g	Lighter, lighter than BlackBerry Storm
Display	WebOS, Webkit based	Full HTML
Keyboard	TFT capacitive touchscreen, 320x480 pixels, 3.1 inches	TruePress technology
Accelerometer	Physical, Full QWERTY	Software, Full QWERTY
Headphone Jack	Yes	Yes
Bluetooth	Yes, 3.5mm	Yes, 3.5mm
Voice	GSM QuadBand	GSM QuadBand
Data	HSDPA TriBand (US)	HSDPA TriBand (US)
Wi-Fi	Yes, Wi-Fi 802.11 b/g	Yes, Wi-Fi 802.11 b/g
Memory	8GB	1 GB storage, 128 MB RAM

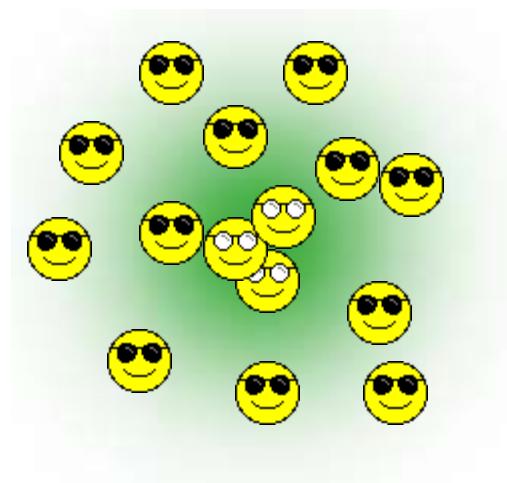
Super resolution microscopy spec sheets



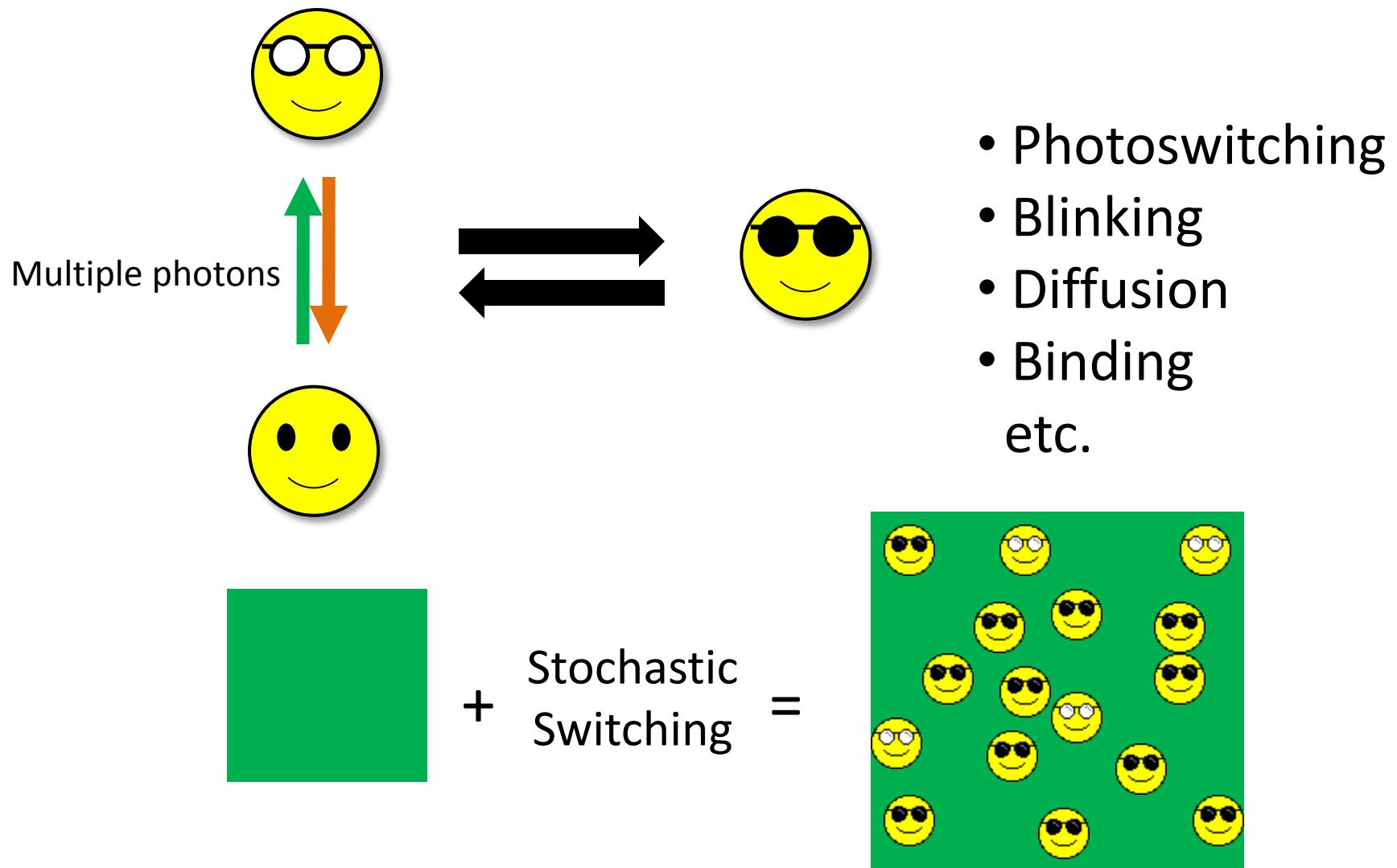
The “patterned illumination” approach



- Ground state
- Triplet state
- Isomerization
- etc.



The “single-molecule switching” approach



3D spatial resolution

	x-y (nm)	z (nm)	Opposing objectives (nm)	Two-photon
Conventional	250	600	4Pi: 120	500 µm deep
SIM	100	250	I ⁵ S: 120 xyz	
STED	~30	~100	isoSTED: 30 xyz	100 µm deep
STORM/PALM	20-30	50-60	iPALM: 20 xy, 10 z	10 µm

Multicolor imaging

Multicolor capability	
Conventional SIM	4 colors in the visible range
STED	2 colors so far
STORM/PALM	3 activation x 3 emission

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 (6 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 (6 sec) – no scan!

Practical issues

	SIM	STED	STORM/PALM
Fluorophore limitation	-	x	x
Instrument complexity	xx	xxx	x
Data analysis	xxx	-	xx
Cost (rapidly changing)	xx	xxx	x



The limit of “Super-Resolution”

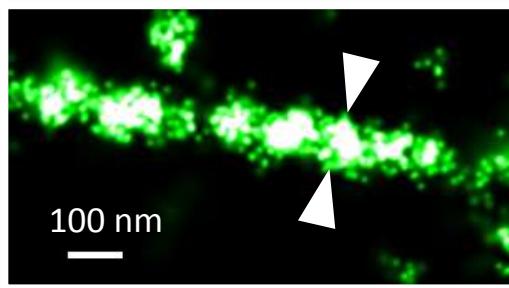
Unbound theoretical resolution

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

- STORM/PALM
 - $S \approx \sqrt{N}$
 - 6,000 photons \rightarrow 5 nm
 - 100,000 photos during Cy5 life time \rightarrow < 1 nm

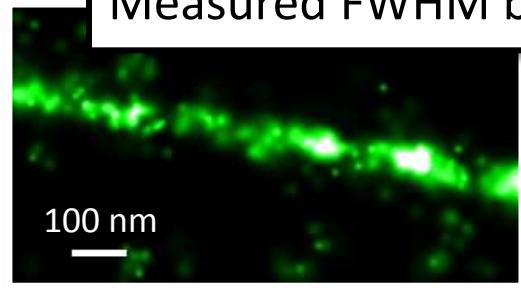
Effective resolution: Probe matters

Antibodies:
~ 10 nm



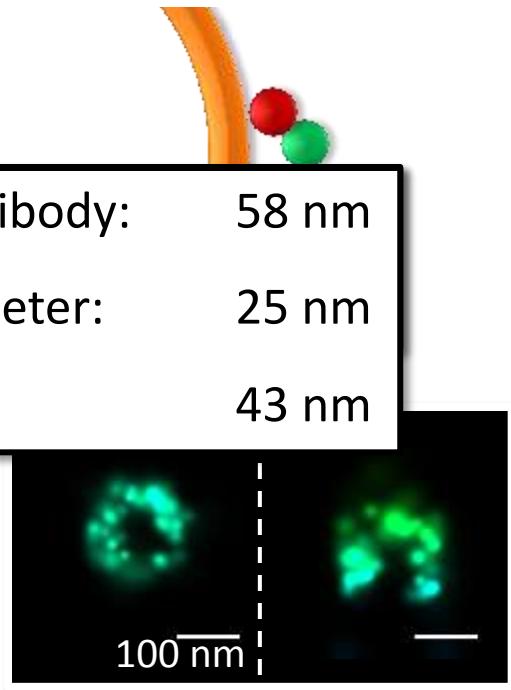
~ 6000 photons

Fluorescent Proteins:
~ 3 nm



< 1000 photons

Small fluorophores:
~ 1 nm



~ 6000 photons

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

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

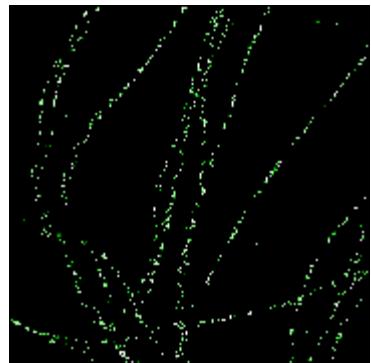
- Multicolor imaging so far challenging

- More versatile for multicolor imaging

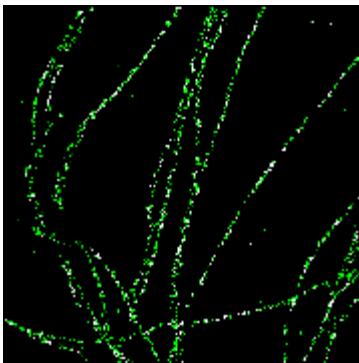
Effective resolution: Density matters

Frames for image reconstruction:

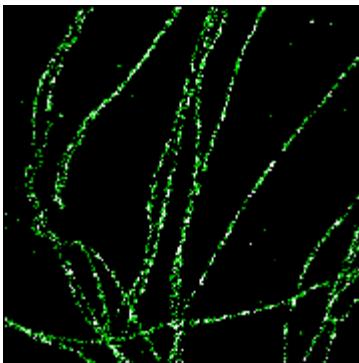
200



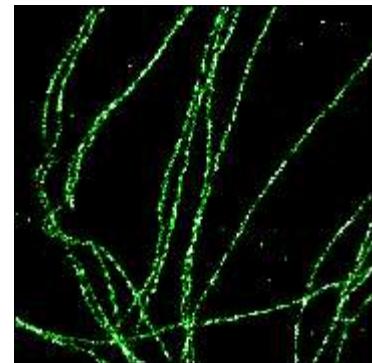
500



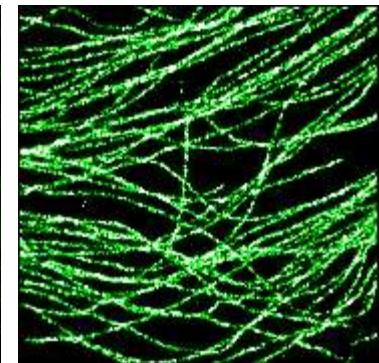
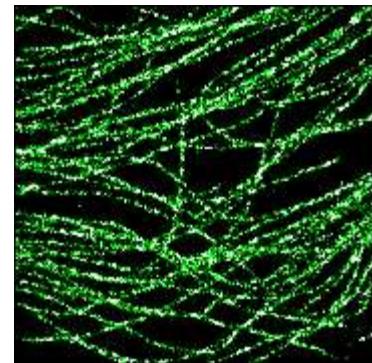
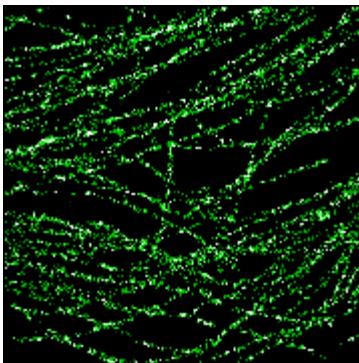
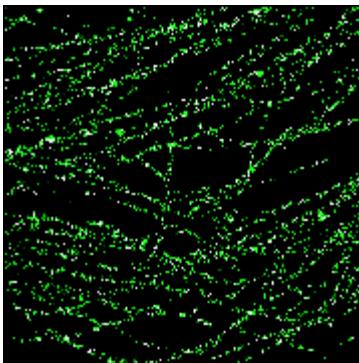
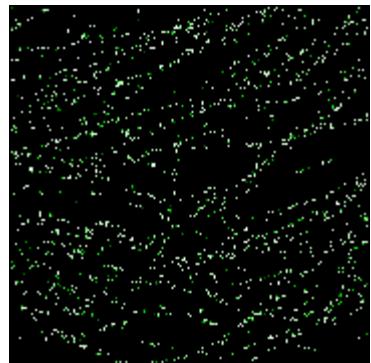
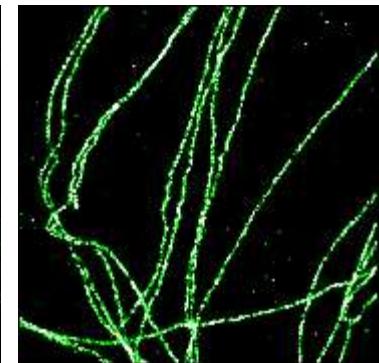
1,000



5,000



40,000



Effective resolution: Density matters

Frames for image reconstruction:

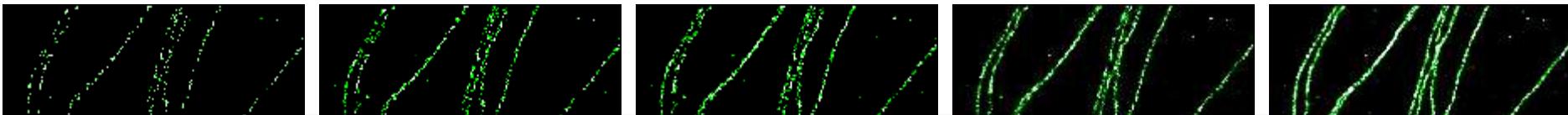
200

500

1,000

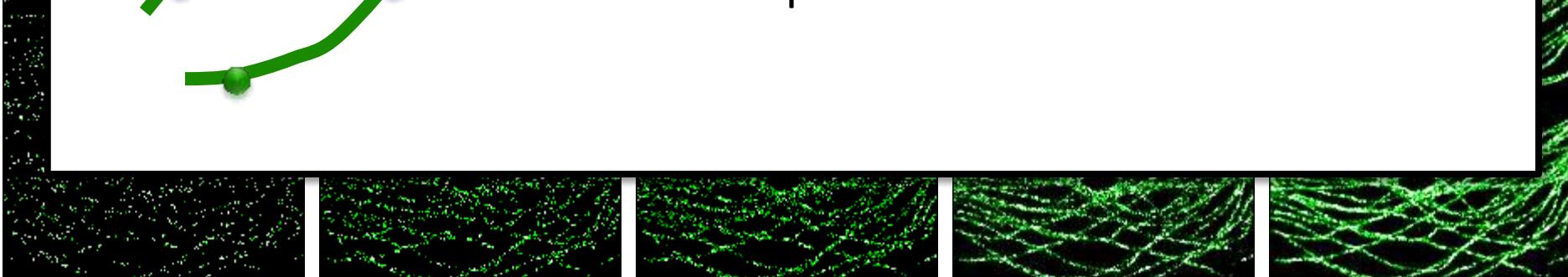
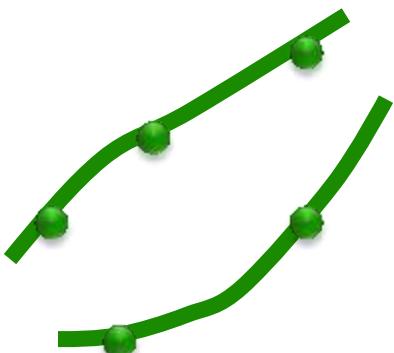
5,000

40,000



Nyquist criteria 

Point to point distance \approx Feature size



Effective resolution: Density matters

Frames for image reconstruction:

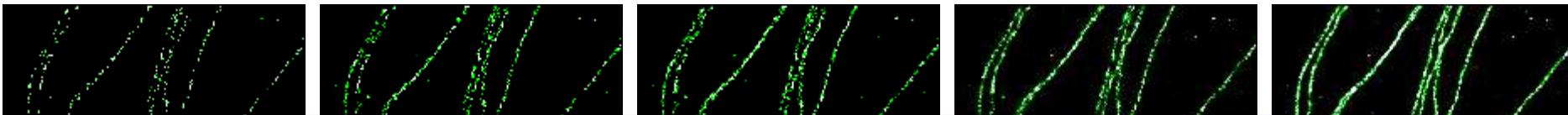
200

500

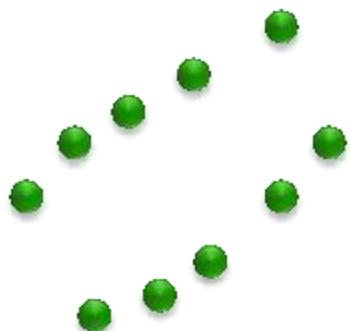
1,000

5,000

40,000



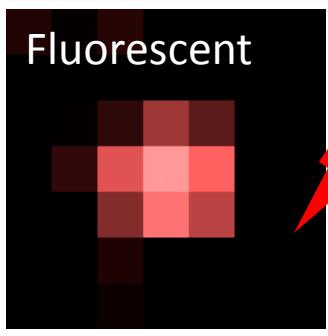
Nyquist criteria



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

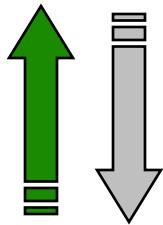


650 nm

e.g. 1%

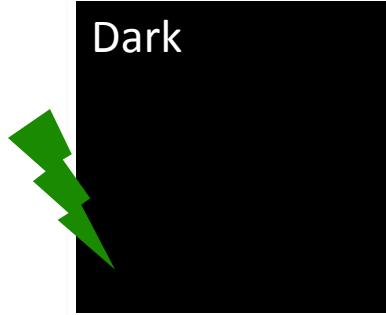


photoactivation



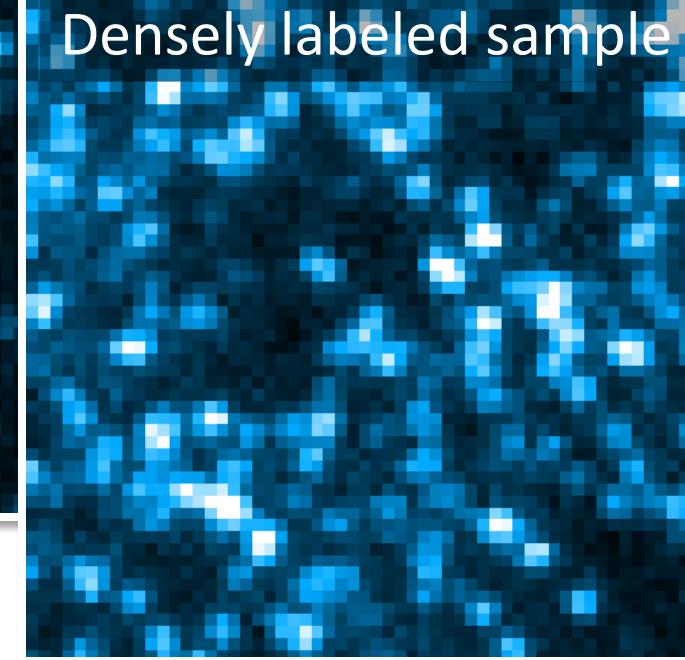
Deactivation

650 nm

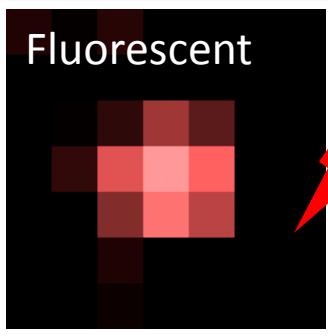


e.g. 99%

1% means...

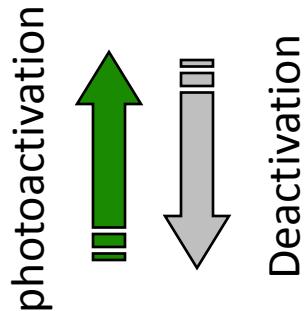


Effective resolution: Contrast matters

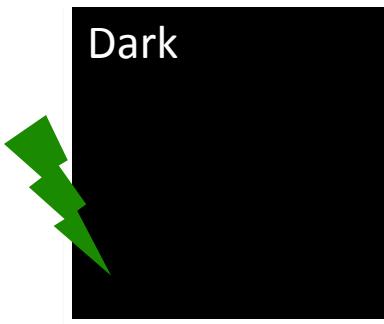


650 nm

e.g. 1%



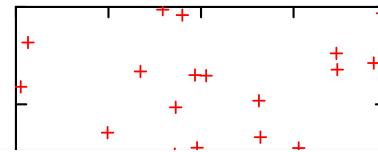
650 nm



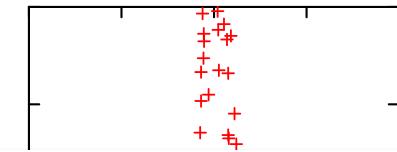
e.g. 99%

1% means...

Homogeneous sample



Microtubule



Common blinking dyes: >3%

Cy5 + mercaptoethylamine: 0.1-0.2%

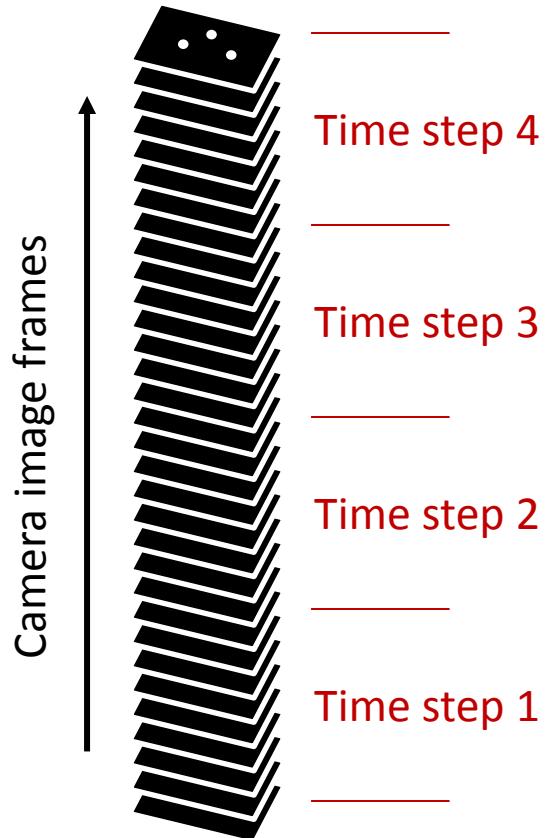
mEosFP: 0.001%

40 nm

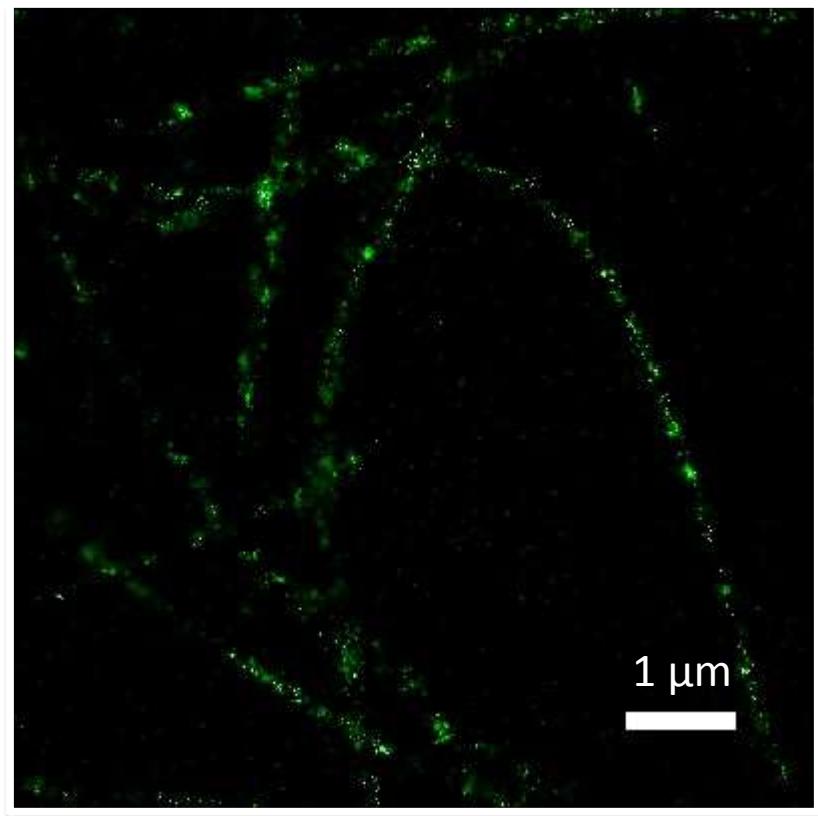
14 nm

Live Cell Imaging

Live cell STORM

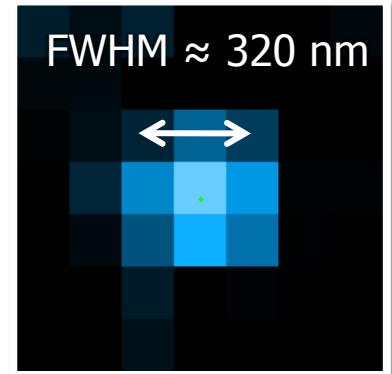
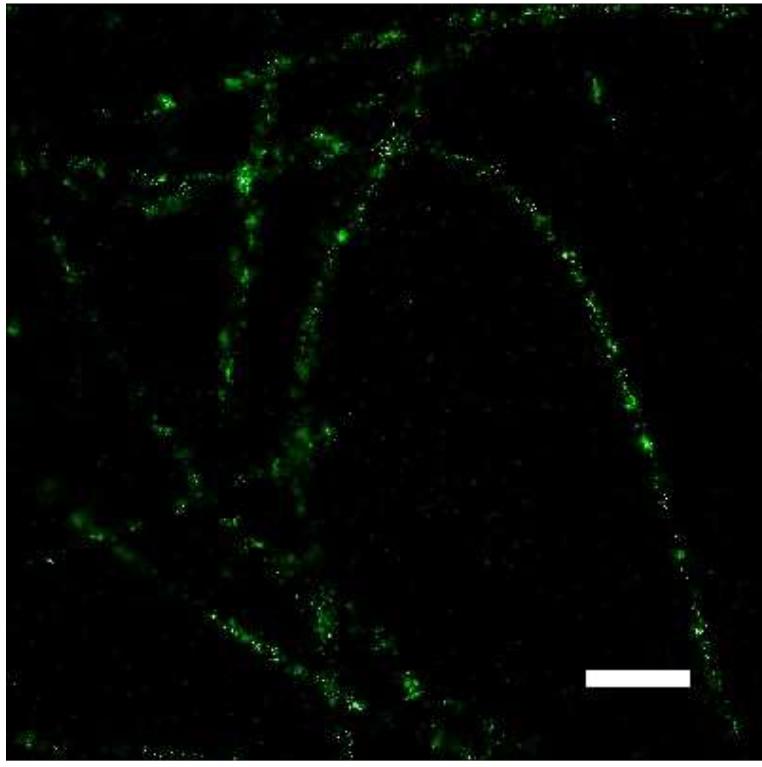


mEos2 labeled microtubule in live S2 cells



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

Spatial-temporal resolution trade-off



Assuming:

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

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

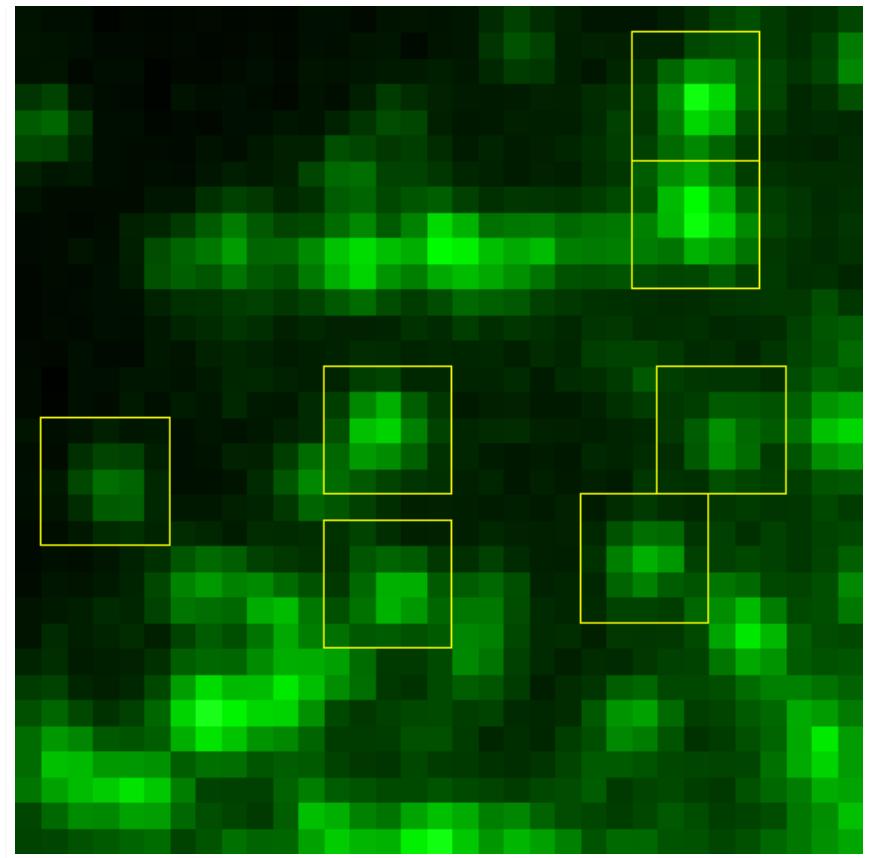
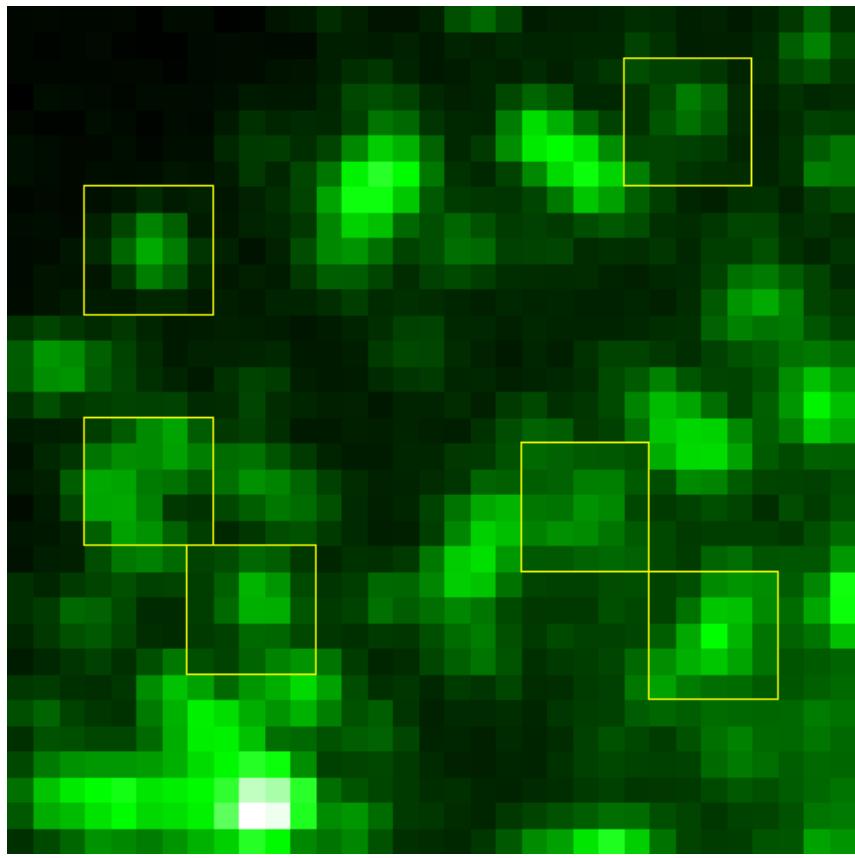
\downarrow
70 nm resolution \equiv 2000 frames

\downarrow
100 fps = 20 sec time resolution

1000 fps

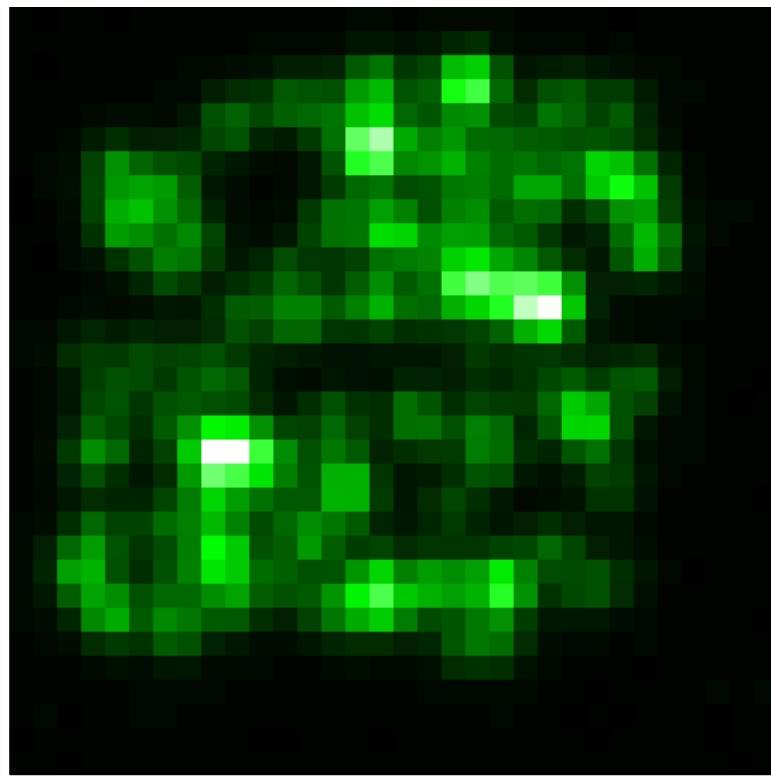


More molecules per camera image?

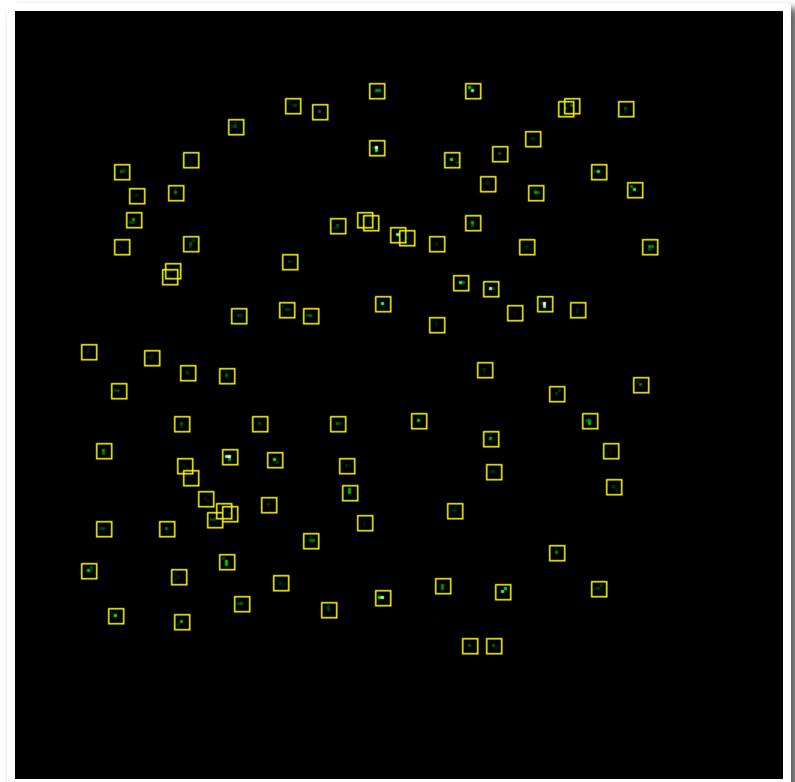


Molecule identification by compresses sensing

Simulated camera image



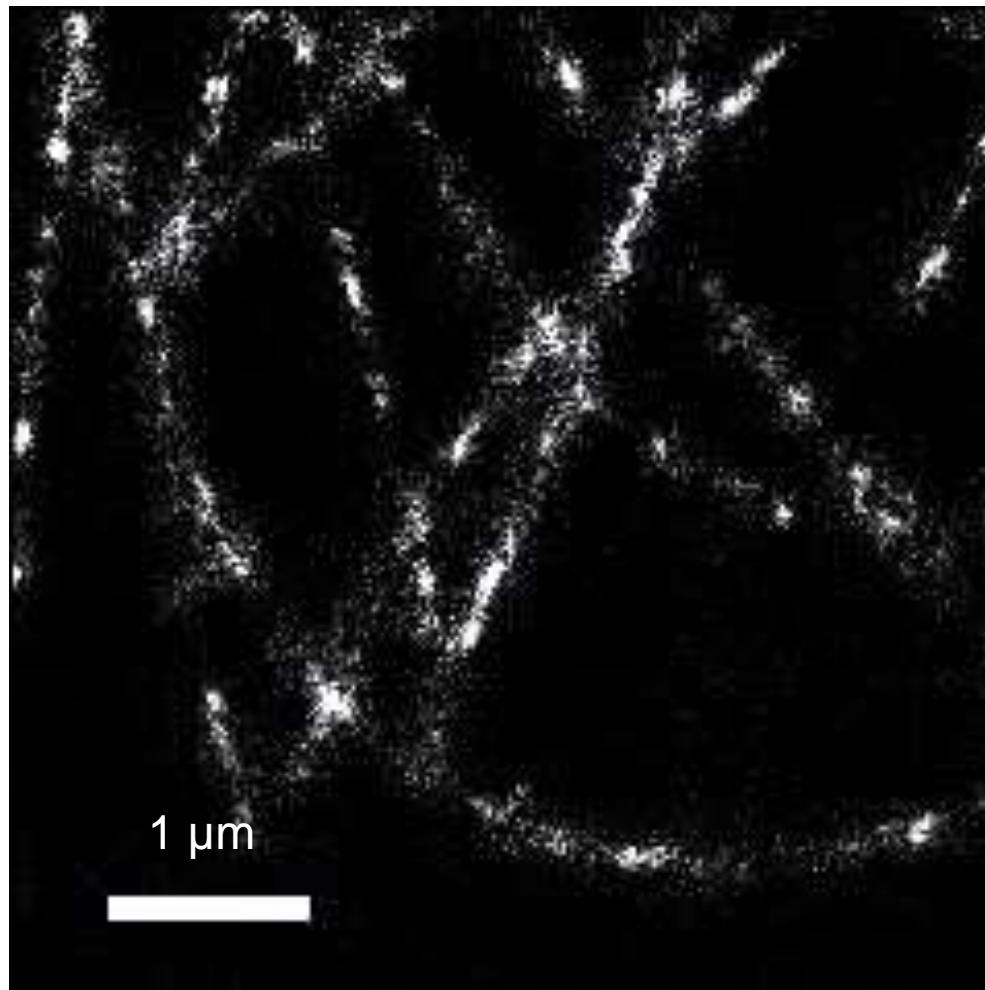
Compressed sensing



94 recovered

100 molecules

Fast live cell imaging by compressed sensing

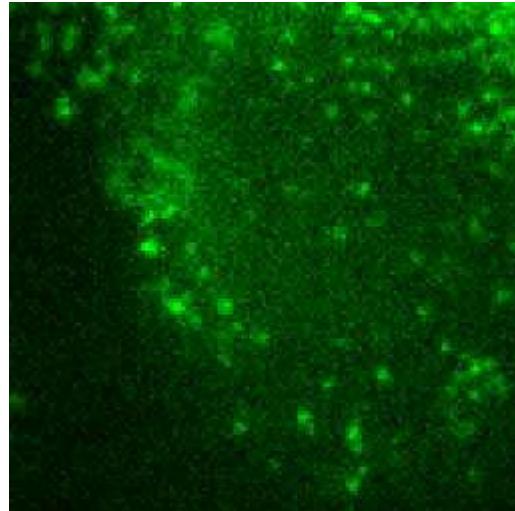
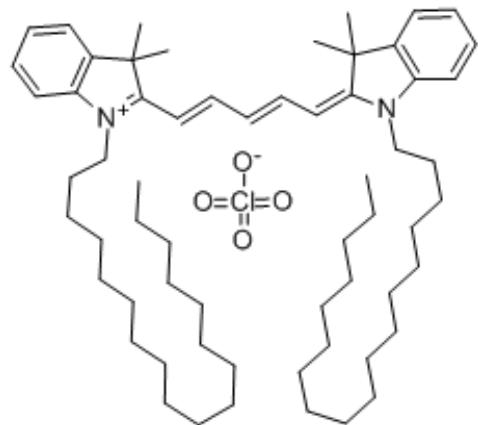


mEos2-tubulin in S2 cell, 3 sec time resolution, 11.8x real time

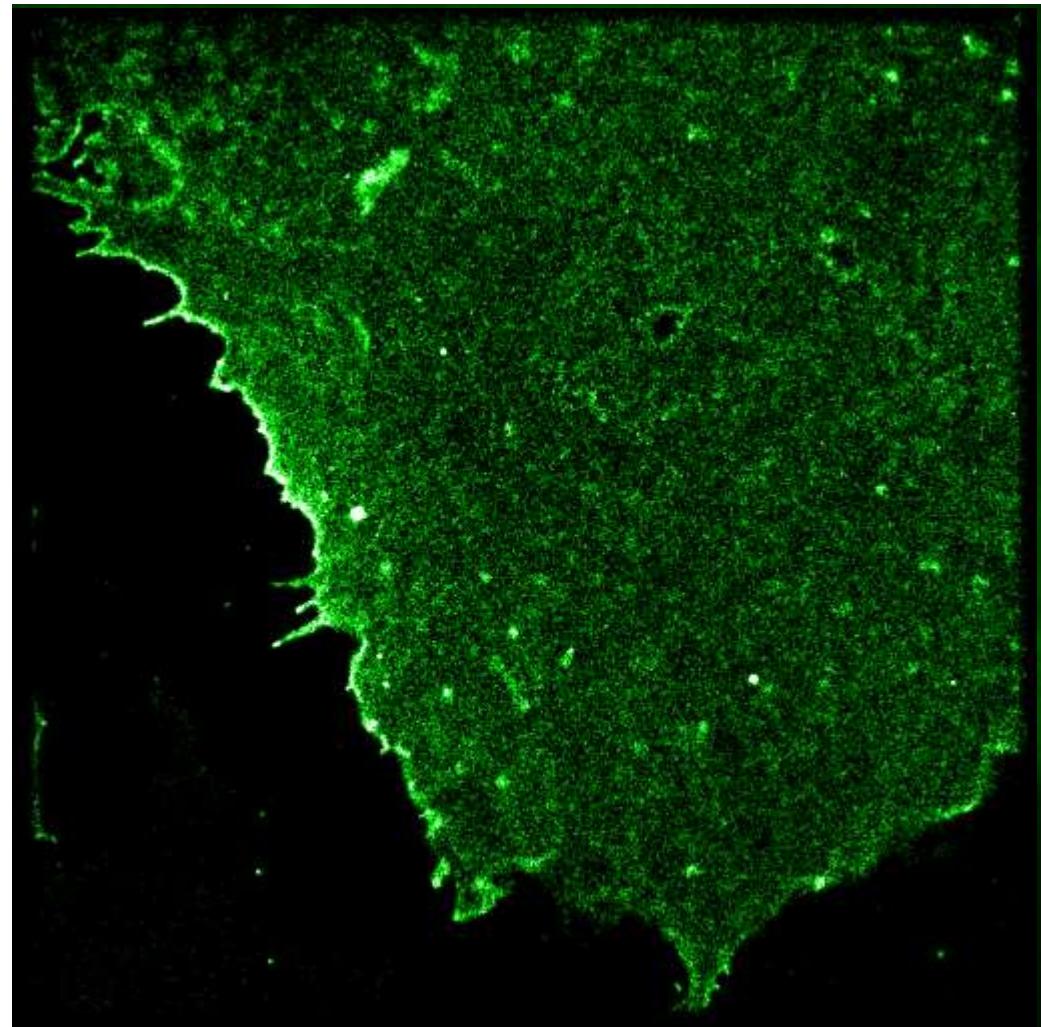
Fast... and even faster?



Lipid in the plasma membrane with DiD

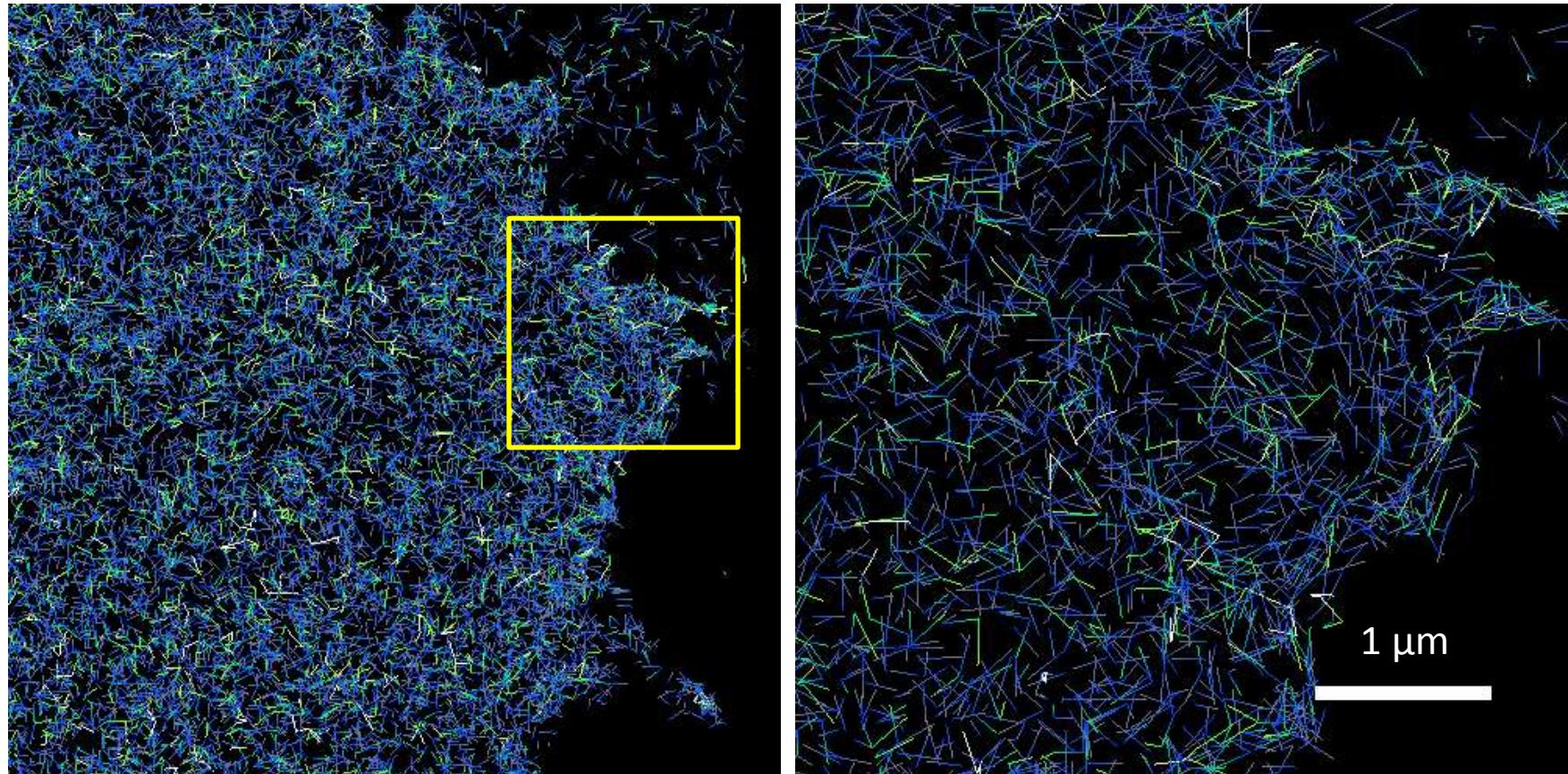


1/3 real time

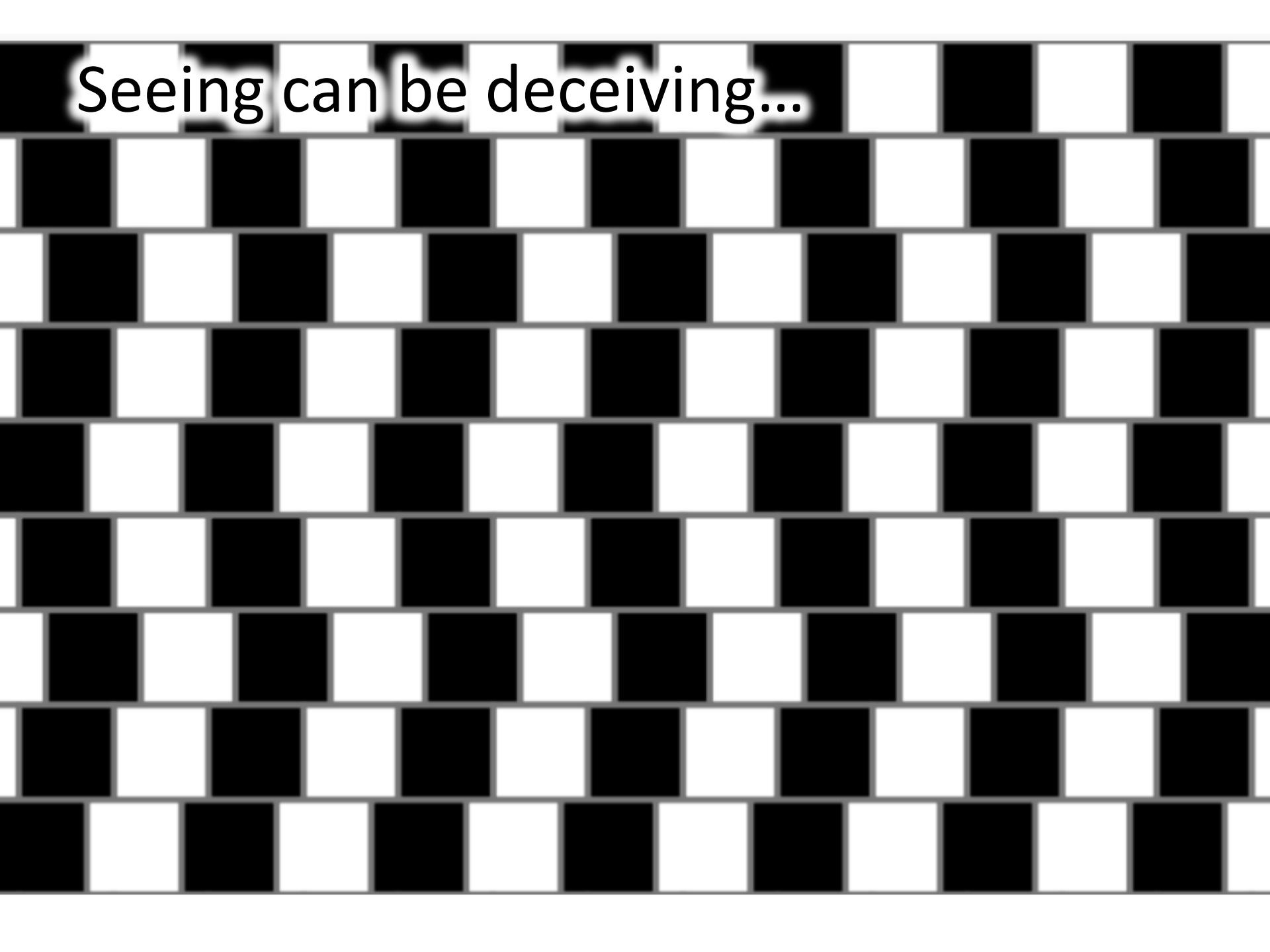


Matthew Bakalar

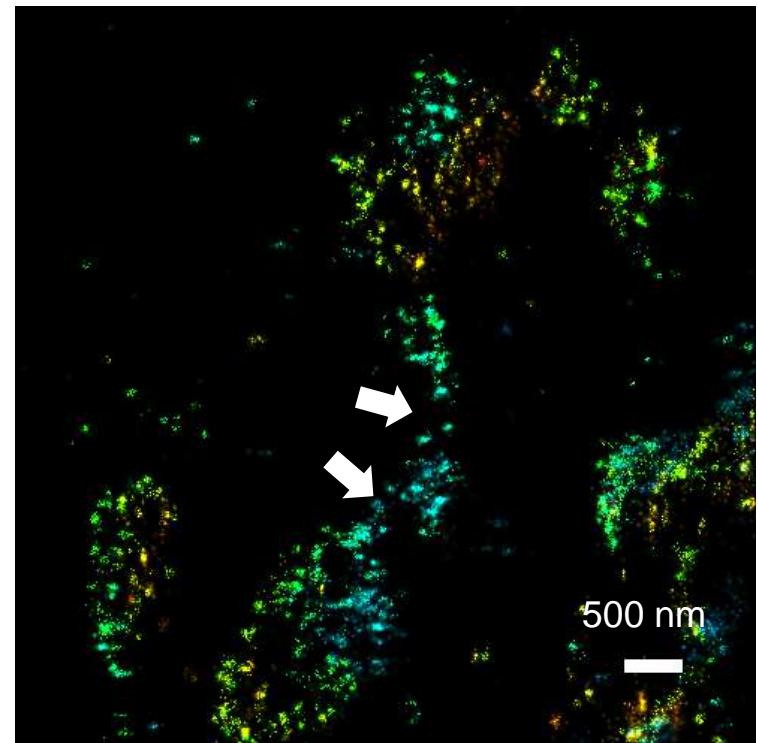
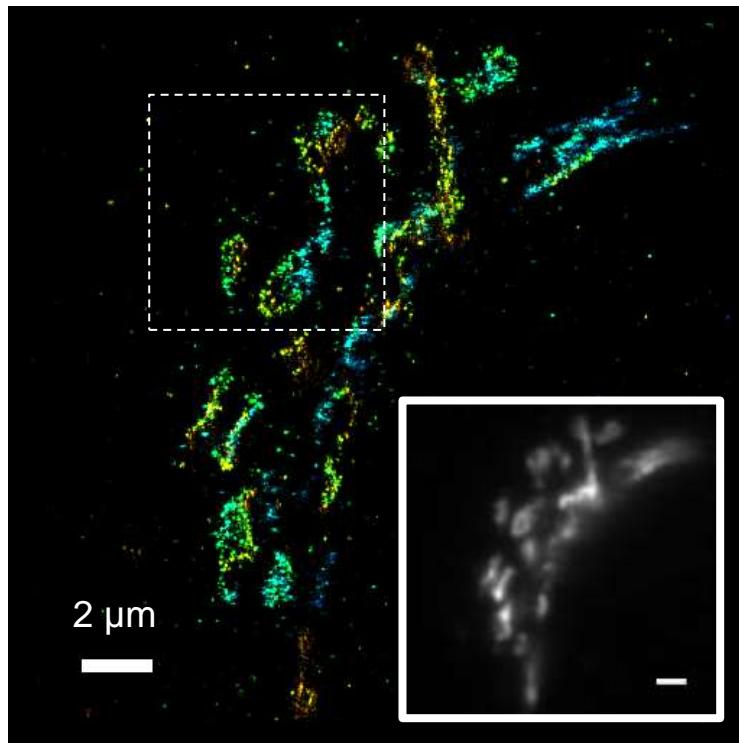
Tracking DiD in the membrane



Seeing can be deceiving...

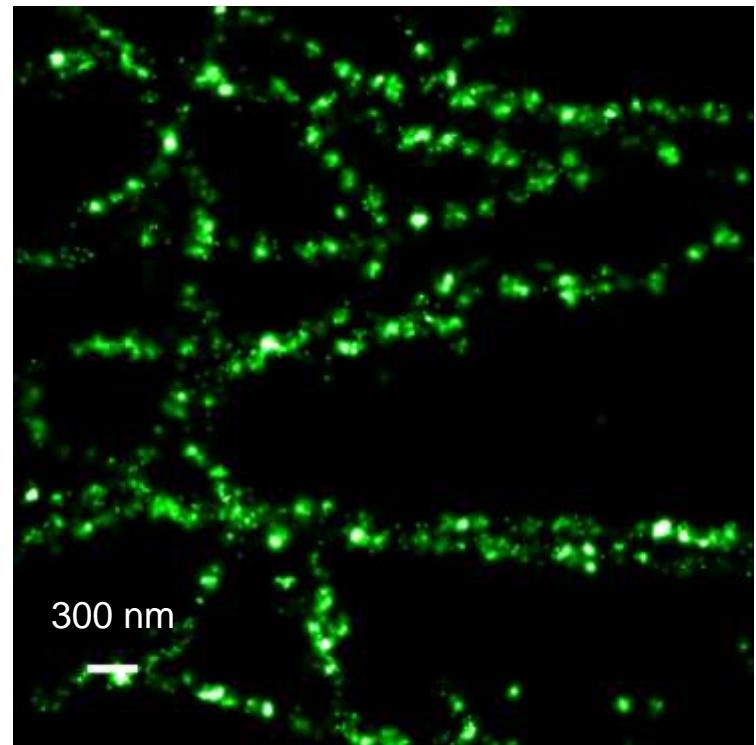
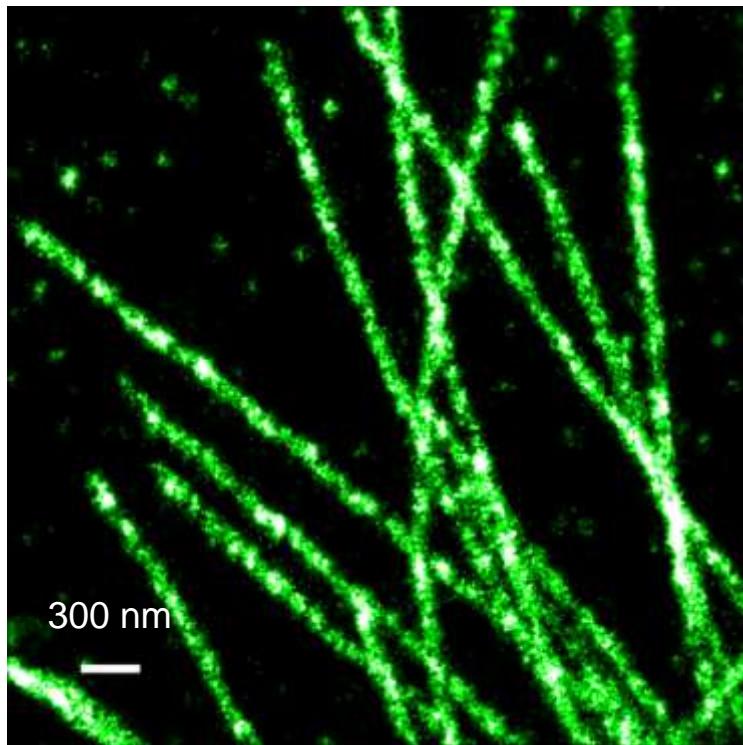


Super-resolved artifacts: sparse labeling



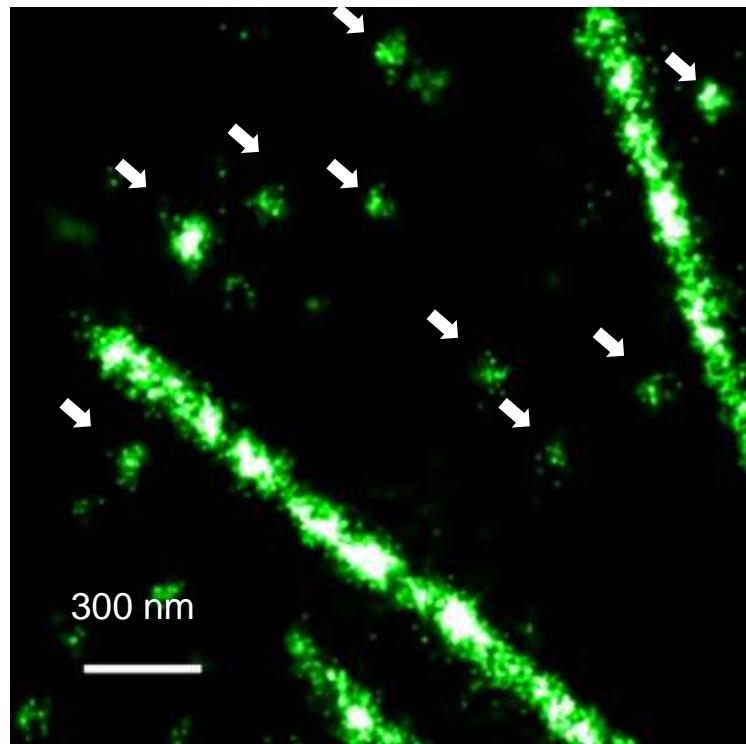
Golgi: Giantin immunofluorescence

Super-resolved artifacts: poor fixation

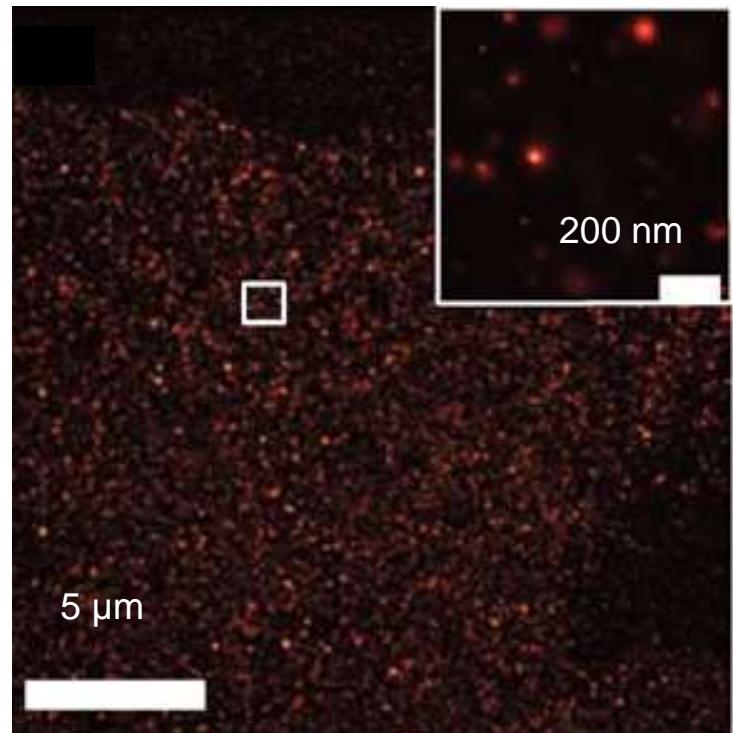


Microtubules: good and bad fixation

Super-resolved artifacts: clustering

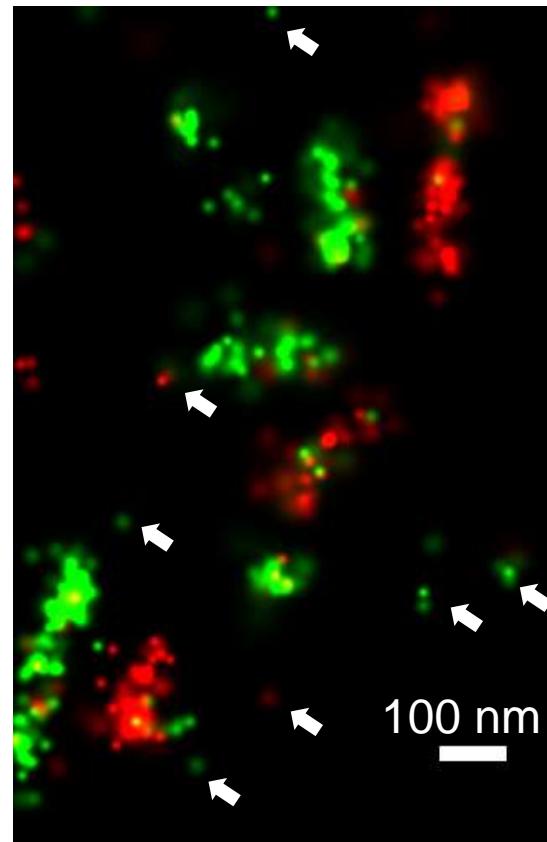
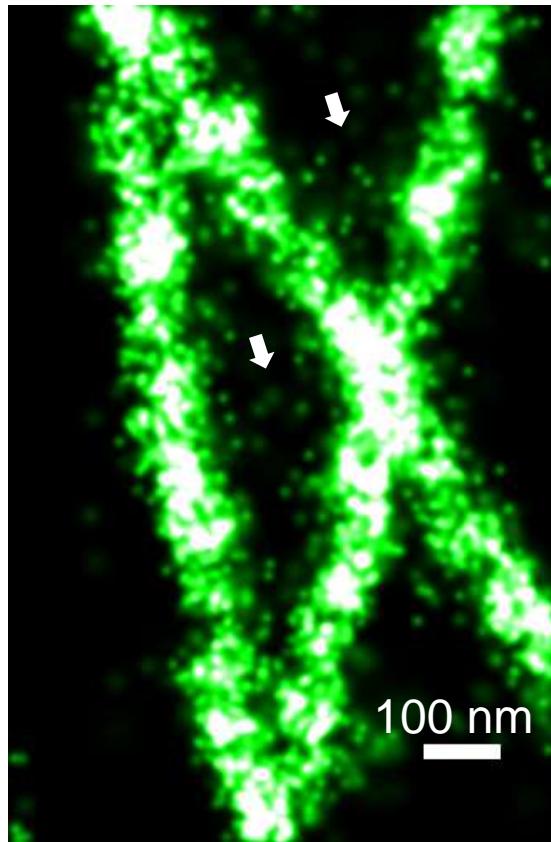


Clusters from single antibodies



Clusters from blinking FPs

Super-resolved artifacts: “noise” points



Noise from misidentified molecules, crosstalk and background

With the creation of new tools...

