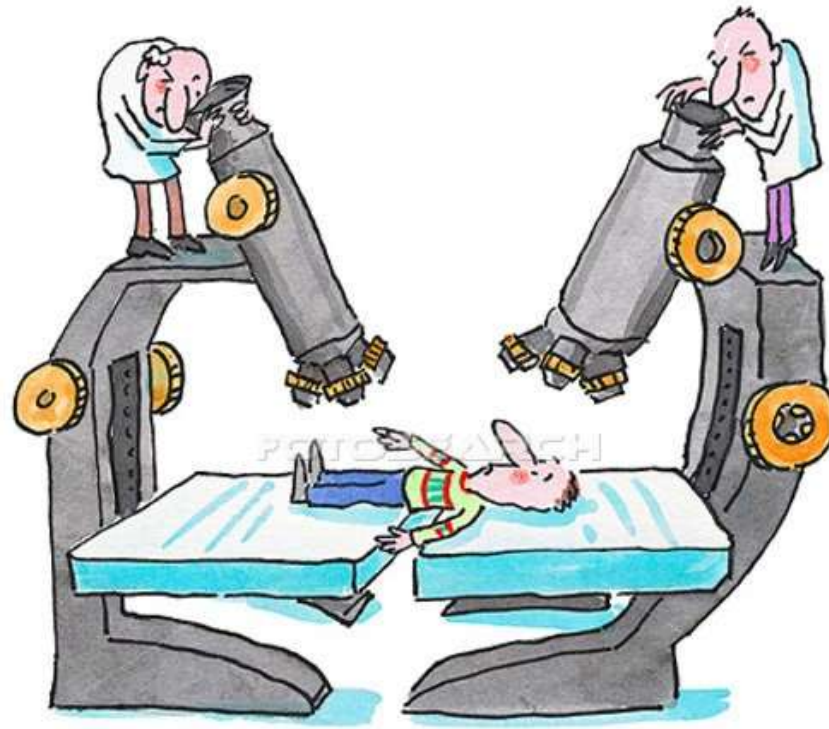


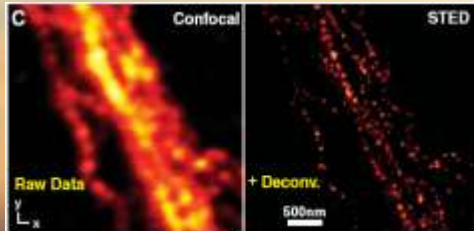
Super-Resolution Microscopy

Single-molecule switching

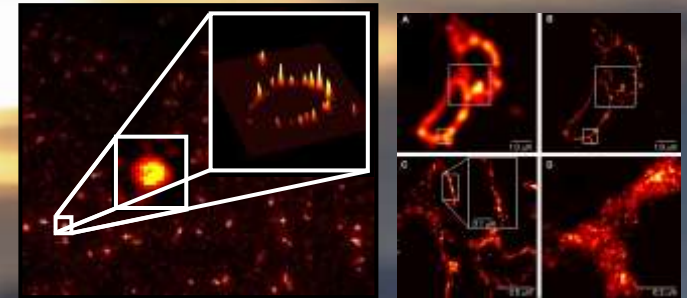


Bo Huang

Super-resolution optical microscopy



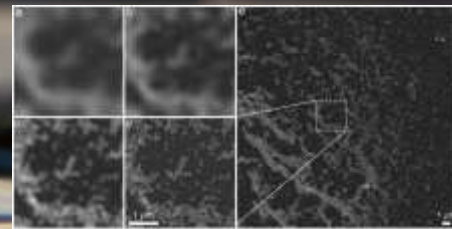
Hell, *Science*, 2007; Hell, *Nat Methods*, 2008



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

Betzig et al., *Science*, 2006

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



Gustafsson, *PNAS.*, 2005

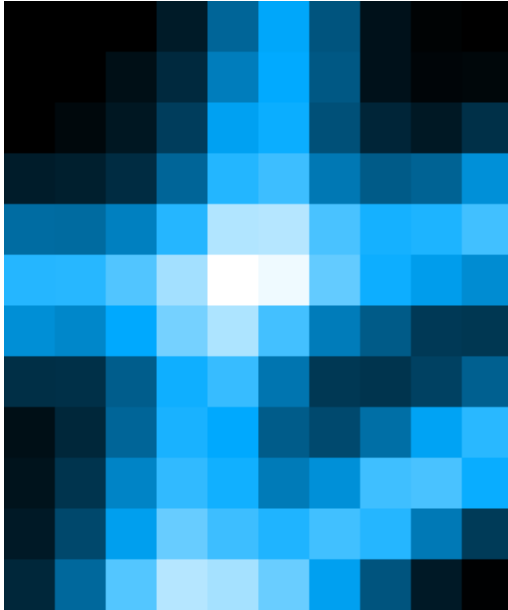
STED

(S)SIM

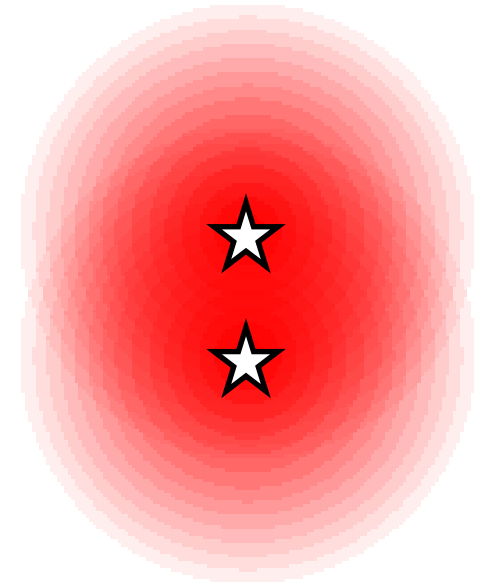
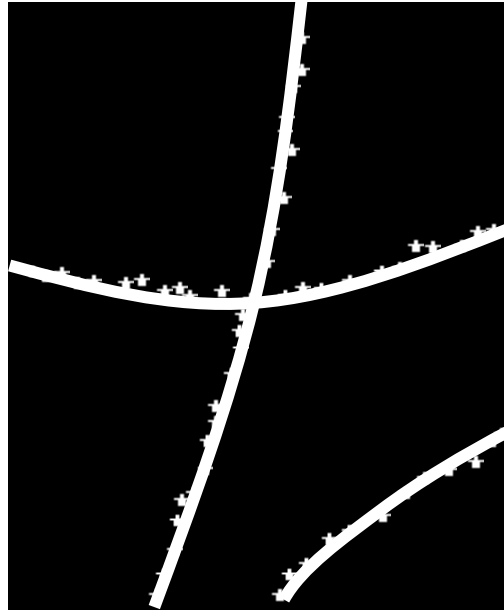
STORM/(F)PALM

Super-resolution by...

Fluorescence image

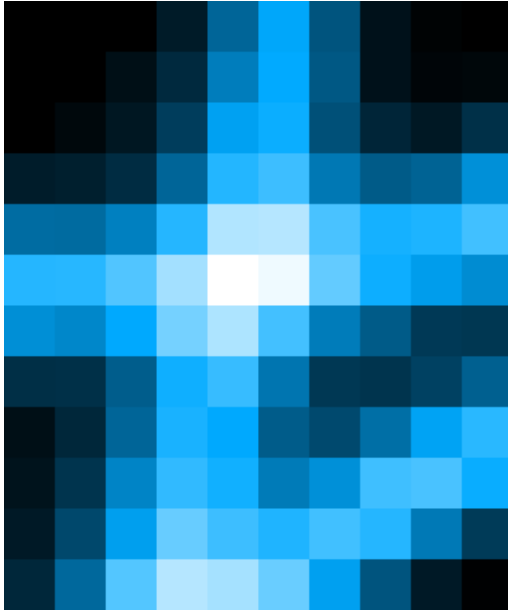


Underlying structure

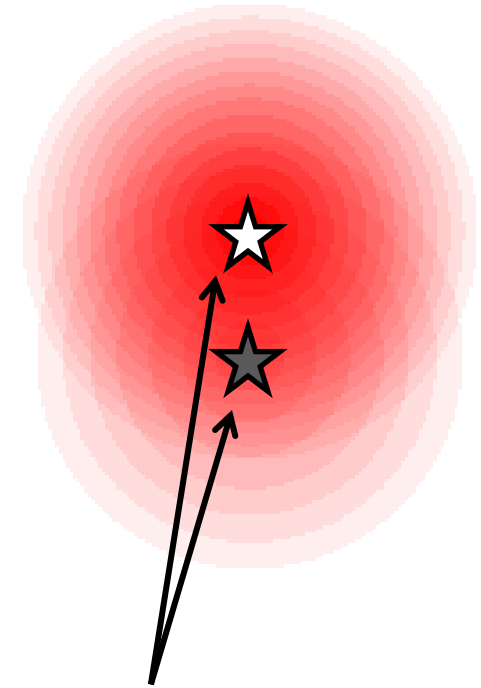
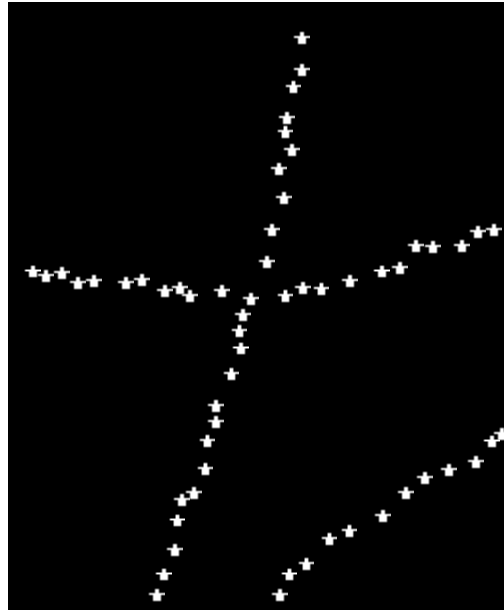


Super-resolution by spatial modulation

Fluorescence image



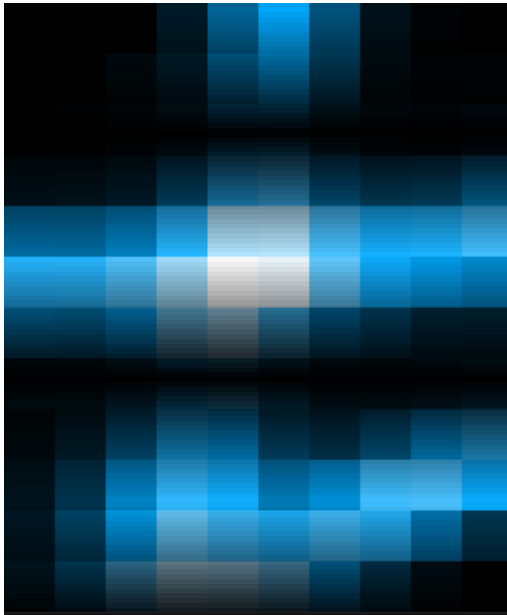
Underlying structure



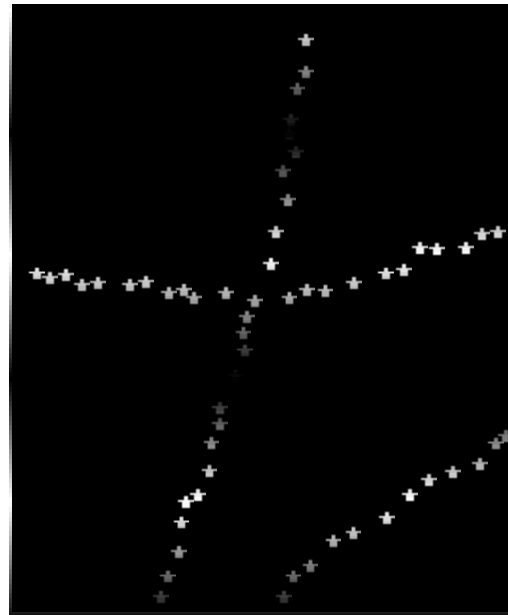
Differential modulation
of the fluorescence response

Super-resolution by differential excitation

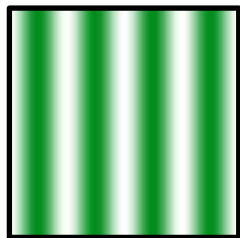
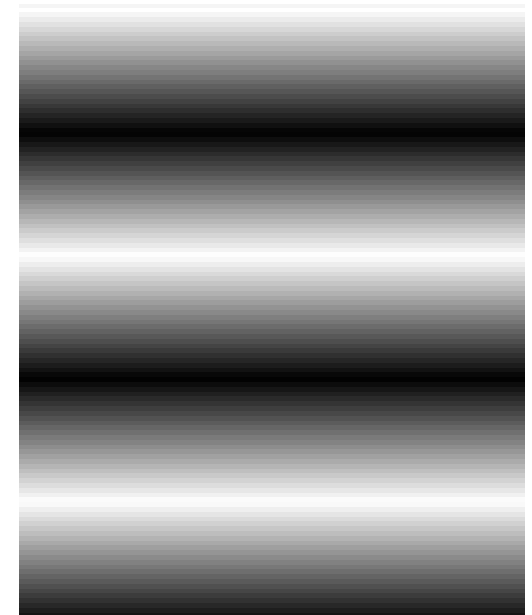
Fluorescence image



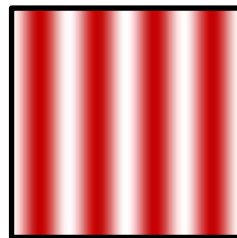
Underlying structure



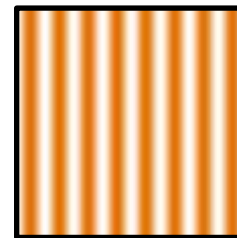
Excitation pattern



×



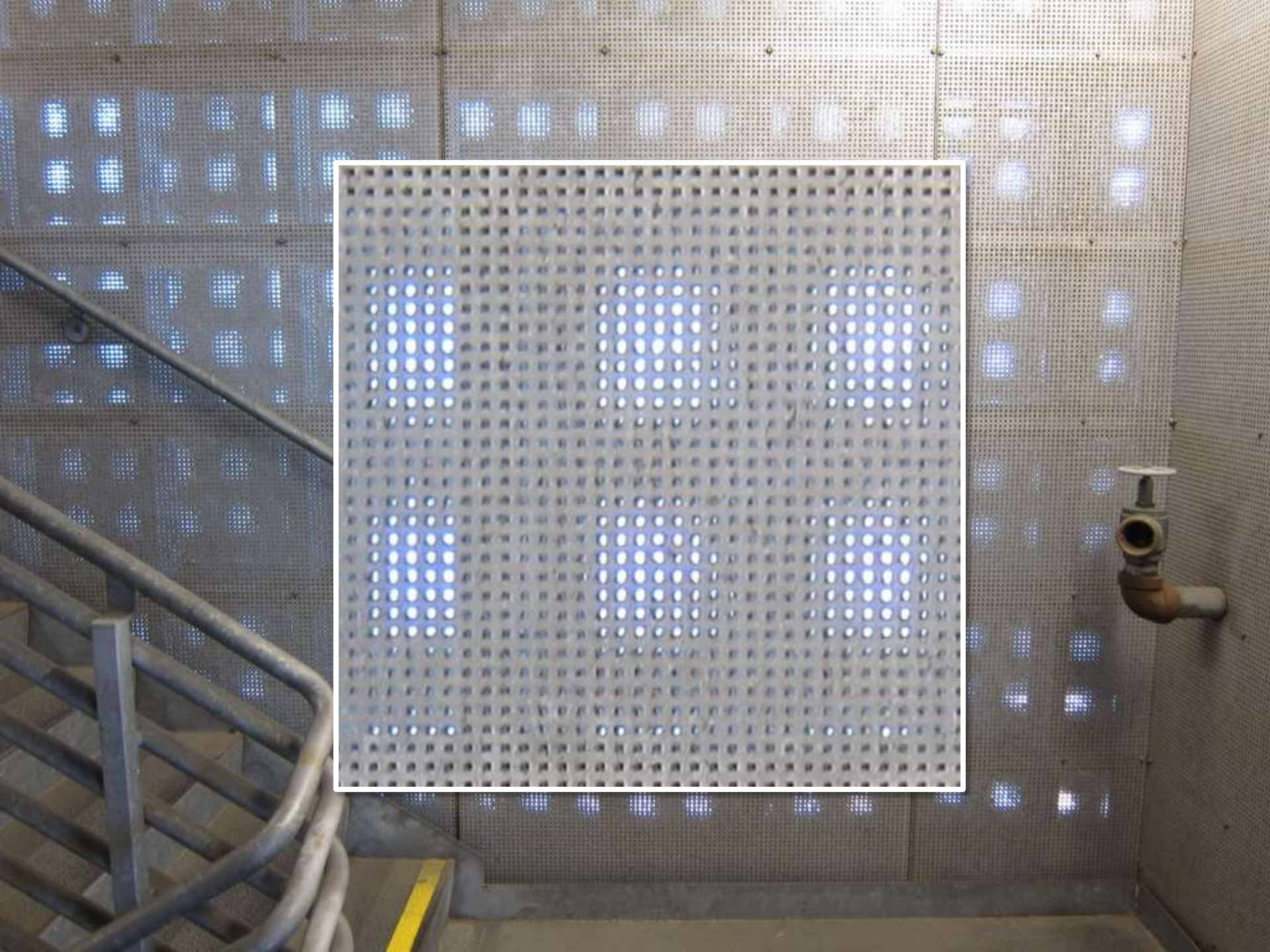
=



SIM (Gustafsson / Heintzmann)
SSIM (Gustafsson 2005)

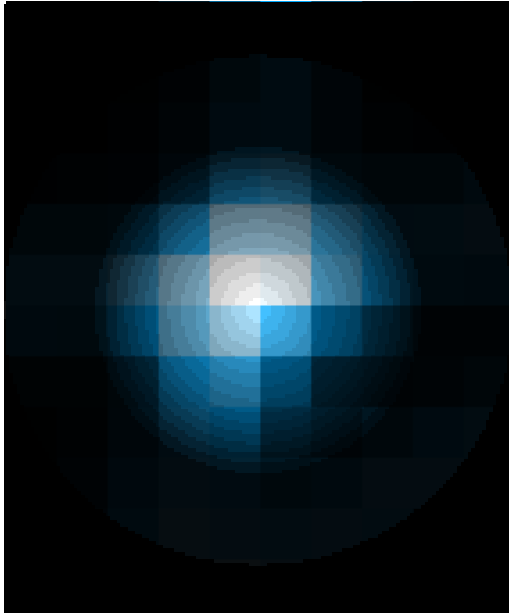
Diffraction limited excitation and emission

Doubled resolution

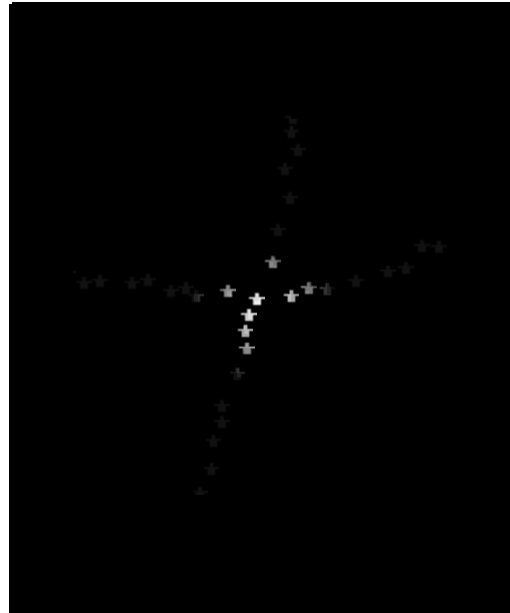


Super-resolution by differential depletion

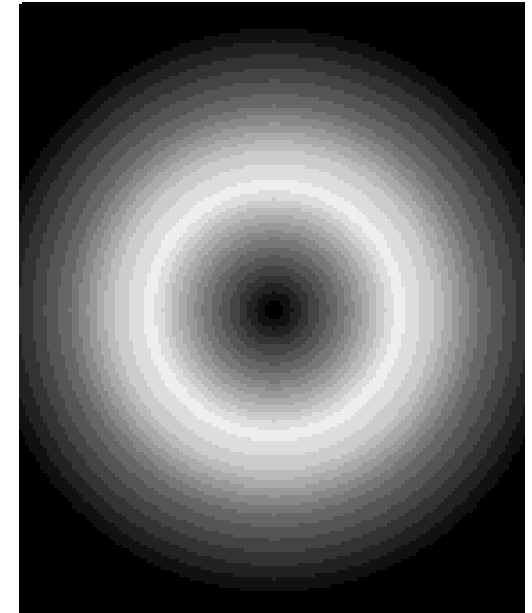
Fluorescence image



Underlying structure



Depletion pattern

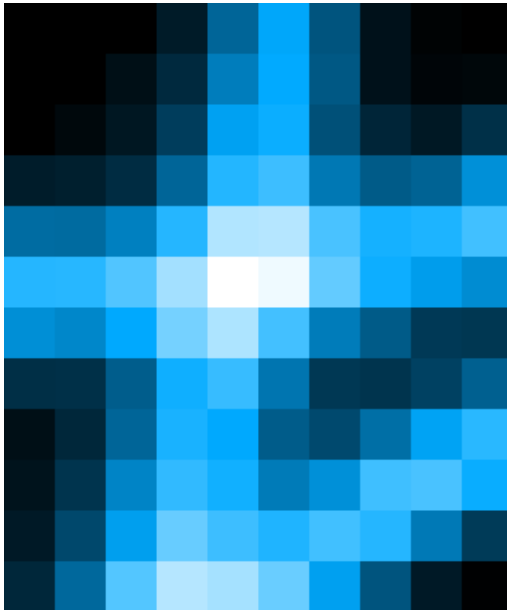


- STED** (Hell 1994, Hell 1999)
- GSD** (Hell 1995, Hell 2007)
- RESOLFT** (Hell 2003, Hell 2011)

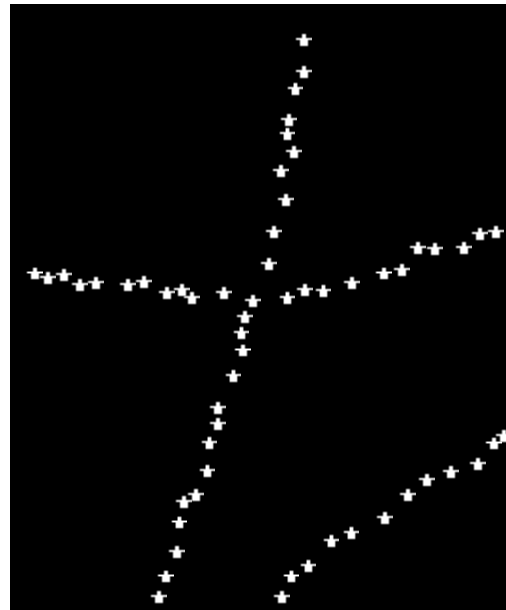
Diffraction limited PSF Saturated depletion Smaller effective PSF

Super-resolution by single-molecule switching

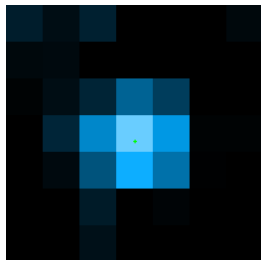
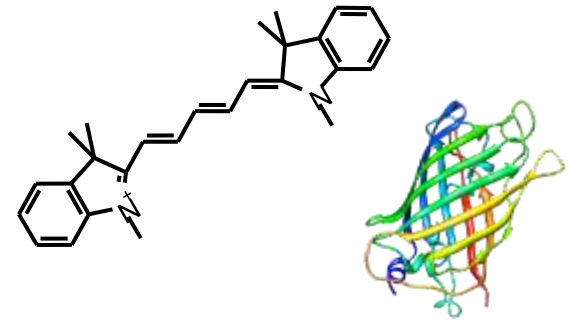
Fluorescence image



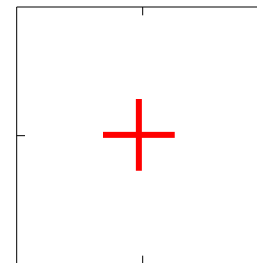
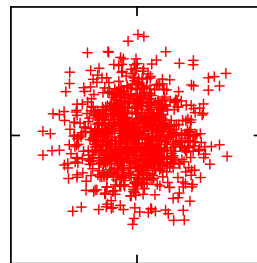
Underlying structure



Photoswitchable molecules



=



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

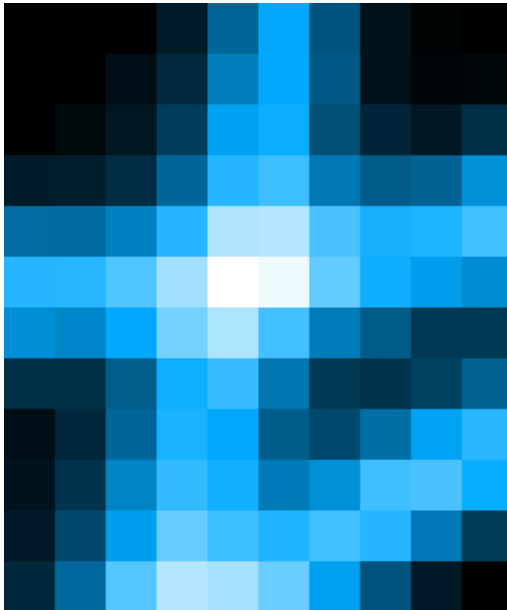
Single molecule image

N photons

Single-molecule localization

Super-resolution by single-molecule switching

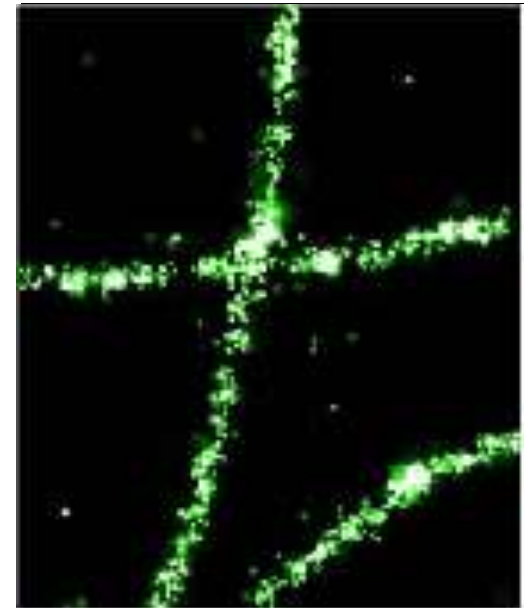
Fluorescence image



Raw images



STORM Image



2x real time

STORM = Stochastic Optical Reconstruction Microscopy (Zhuang 2006)

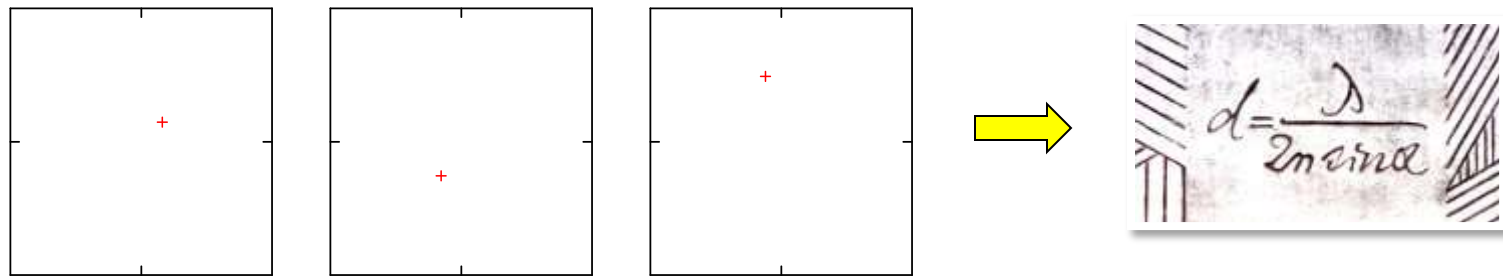
PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)

FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)

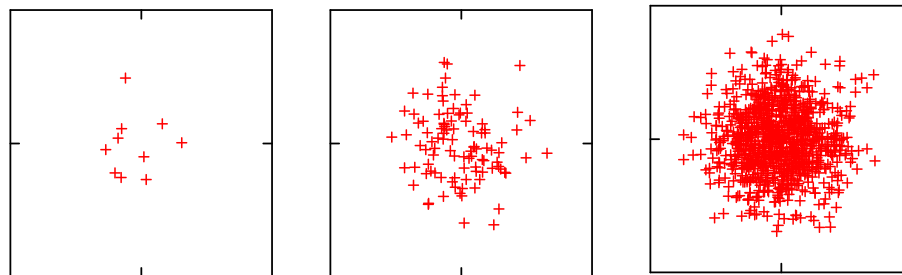
PALMIRA (Hell 2007), **GSDIM** (Hell 2008), **dSTORM** (Sauer 2008), **SMACM** (Moerner 2008)

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

Single-molecule localization precision



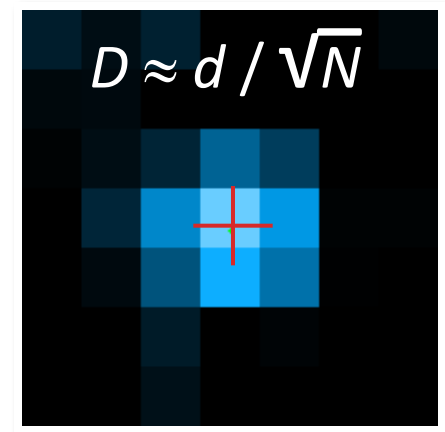
1 photon



10 photons

100 photons

1000 photons



A 3D reconstruction of a microtubule network in a B-SC-1 cell. The microtubules are shown as a dense, interconnected network of green filaments. The network is highly organized, with many filaments running parallel to each other, particularly in the lower right quadrant. The overall structure is complex and shows a clear degree of order and connectivity.

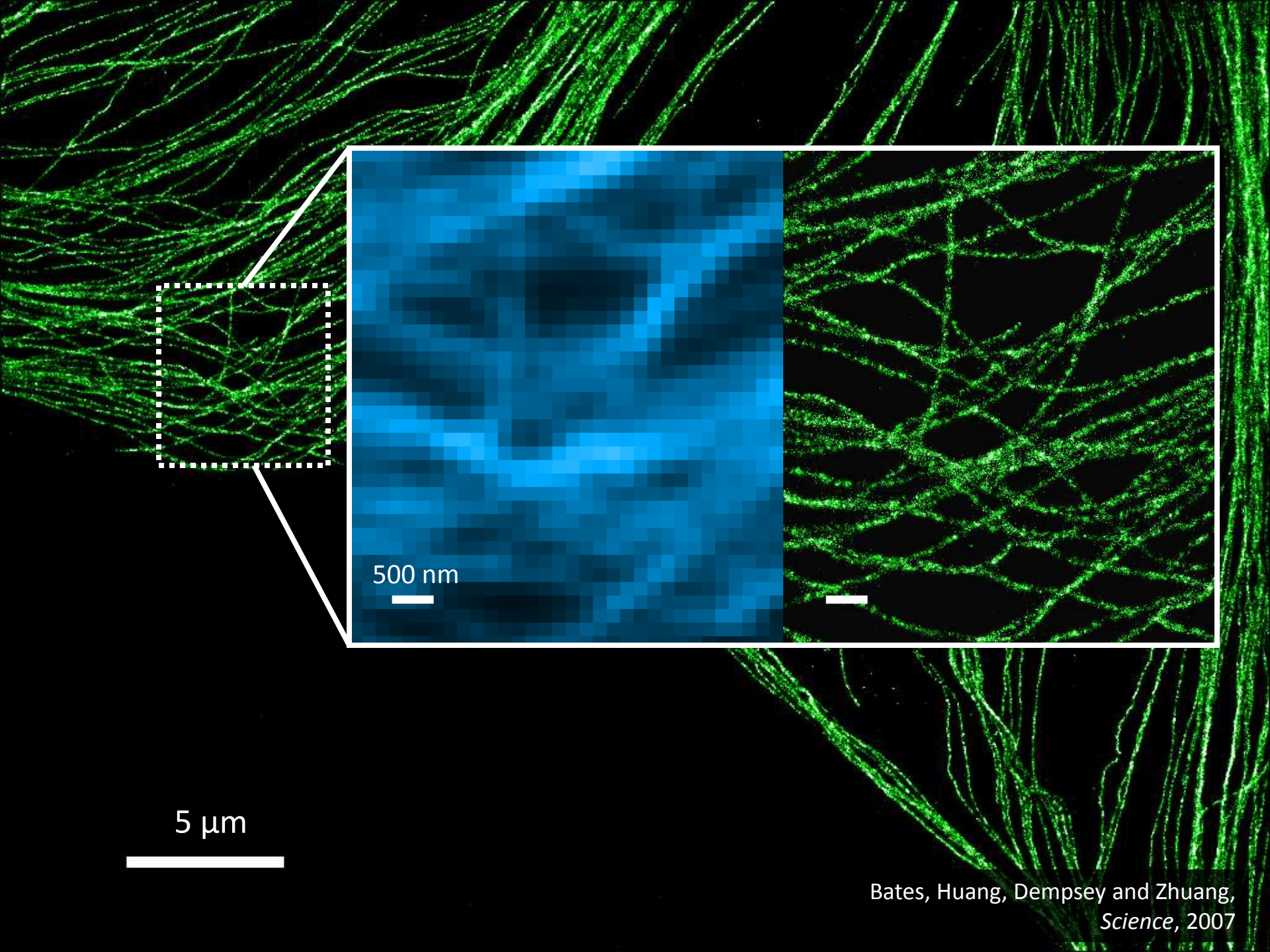
Reconstructed from 40,000 frames, 3,350,370 localization points

5 μm

A white horizontal scale bar representing 5 micrometers.

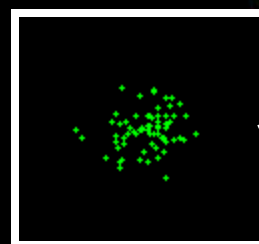
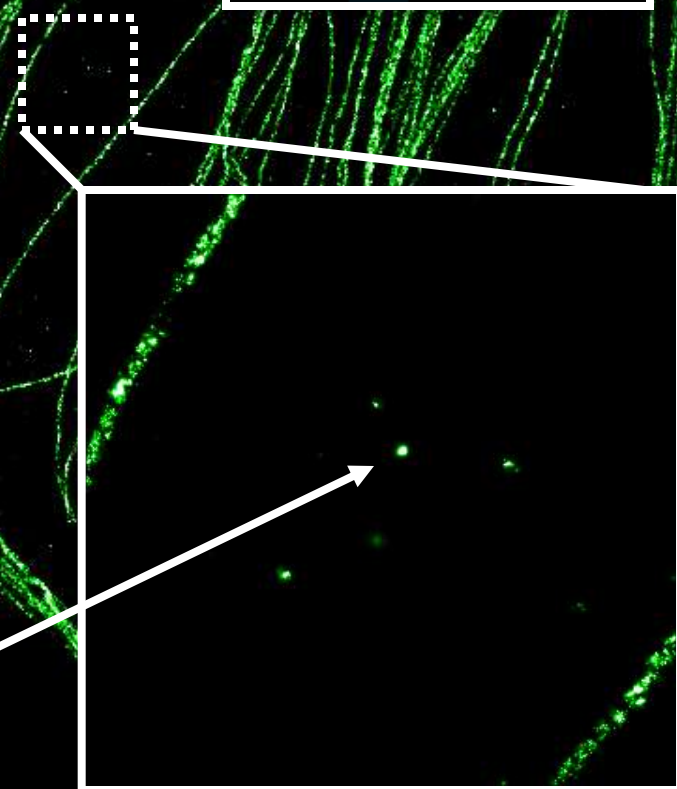
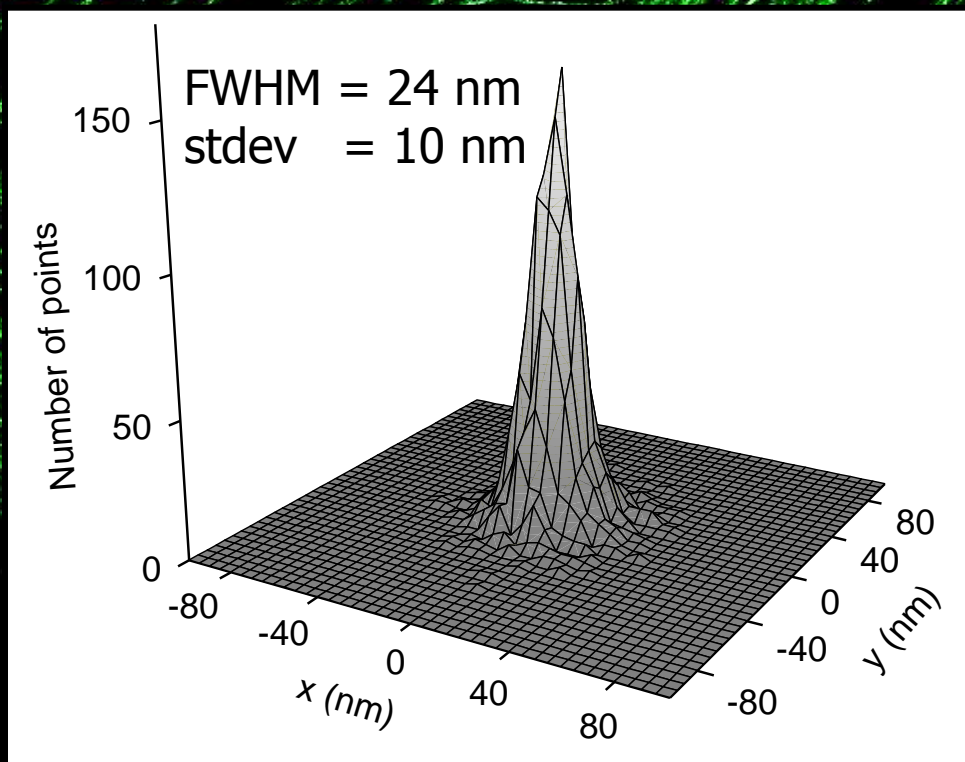
B-SC-1 cell,
Microtubules stained with anti- β tubulin
Cy3 / Alexa 647 secondary antibody

Bates, Huang, Dempsey and Zhuang,
Science, 2007



500 nm

5 μm



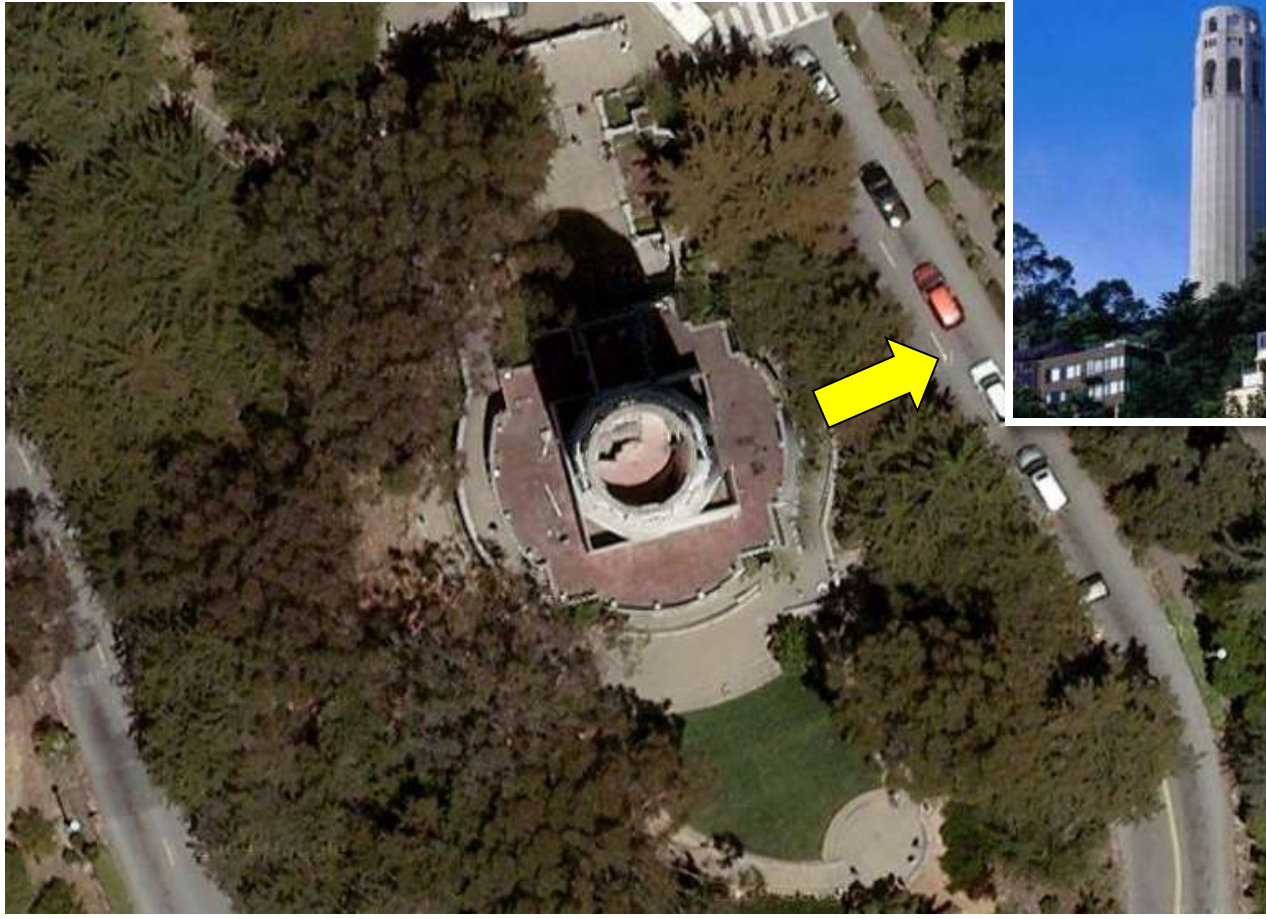
5 μ m



3D Imaging

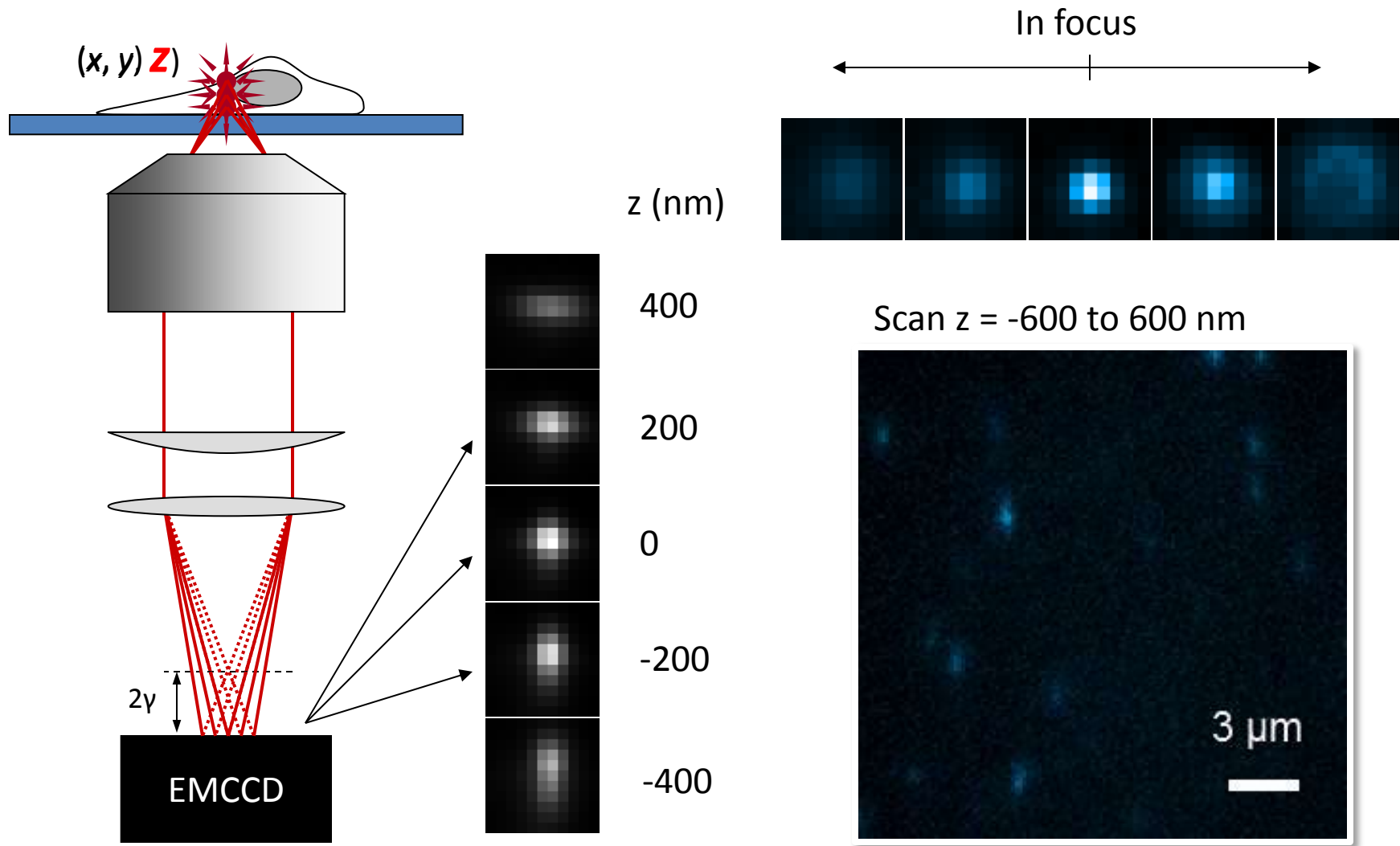
In a 2D world...

Satellite image of ???

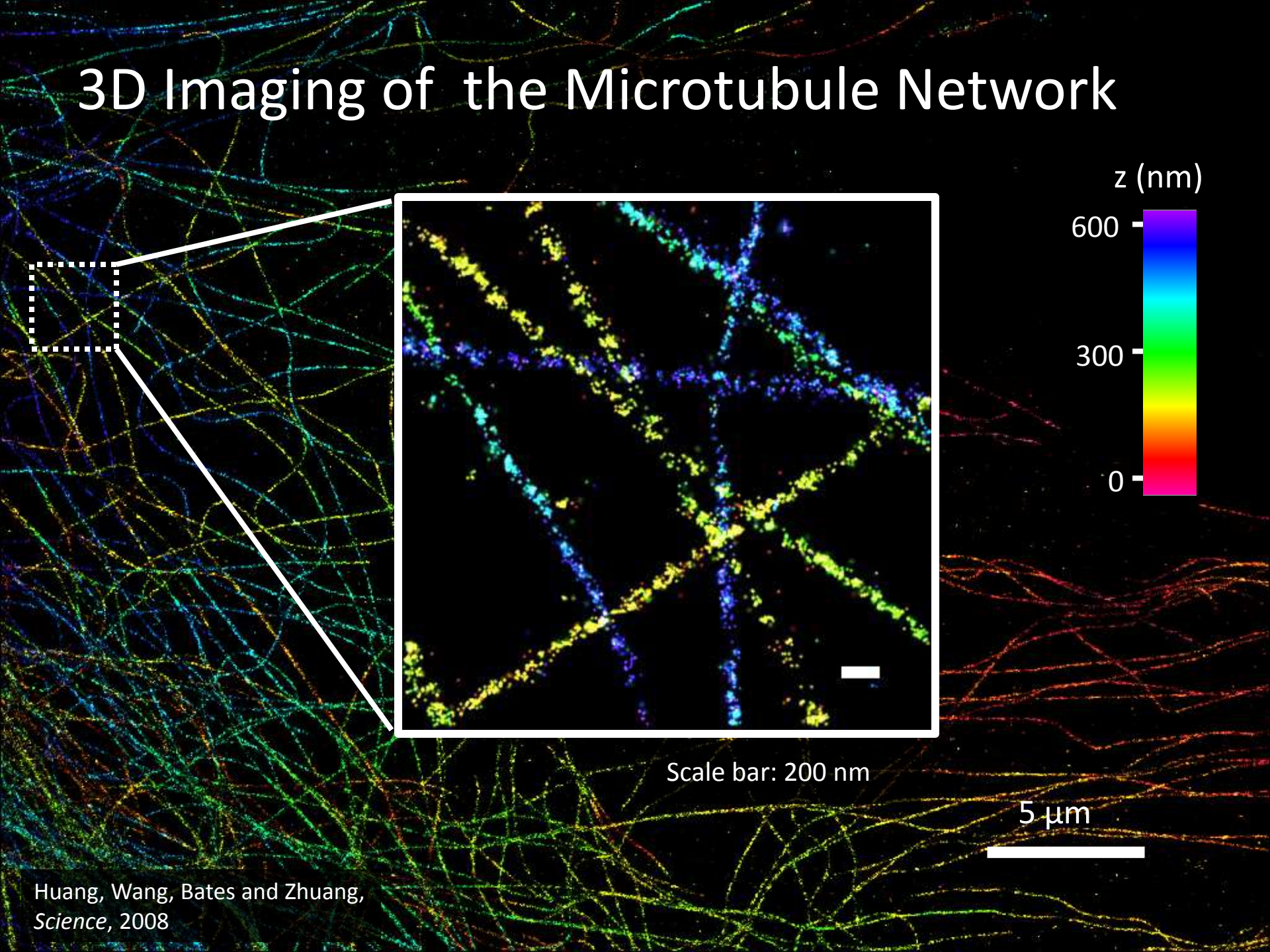


Google maps

3D Imaging: Localization in the Third Dimension



3D Imaging of the Microtubule Network



z (nm)

600

300

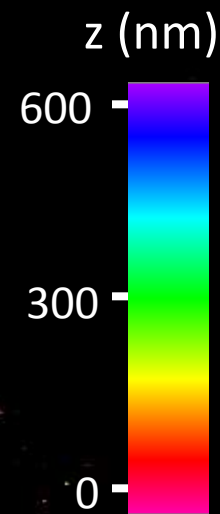
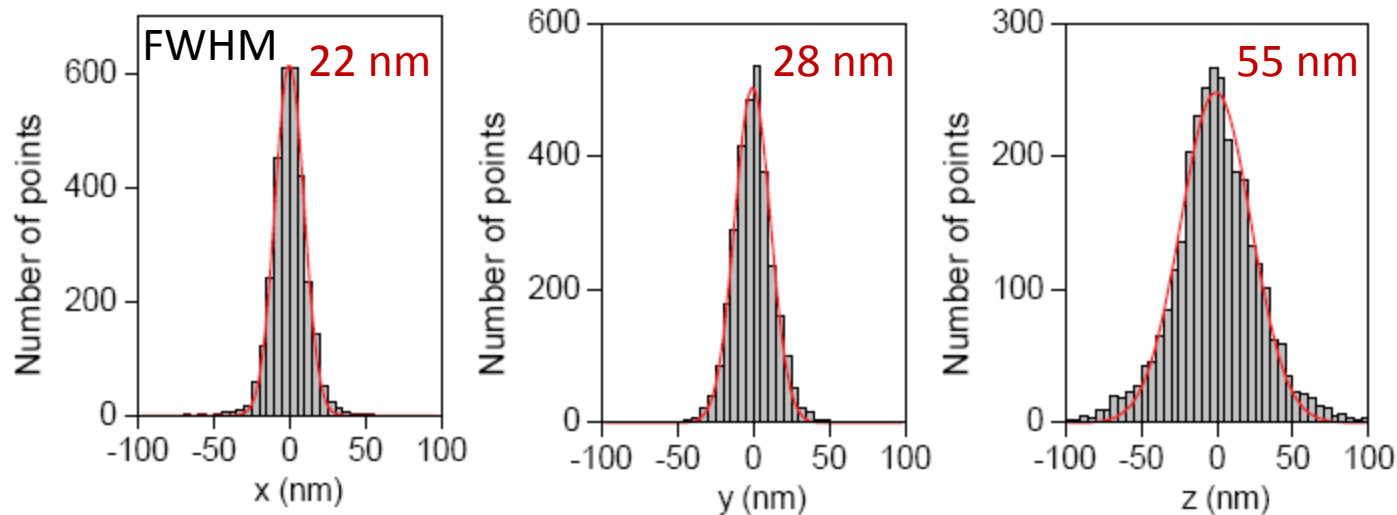
0

Scale bar: 200 nm

5 μm

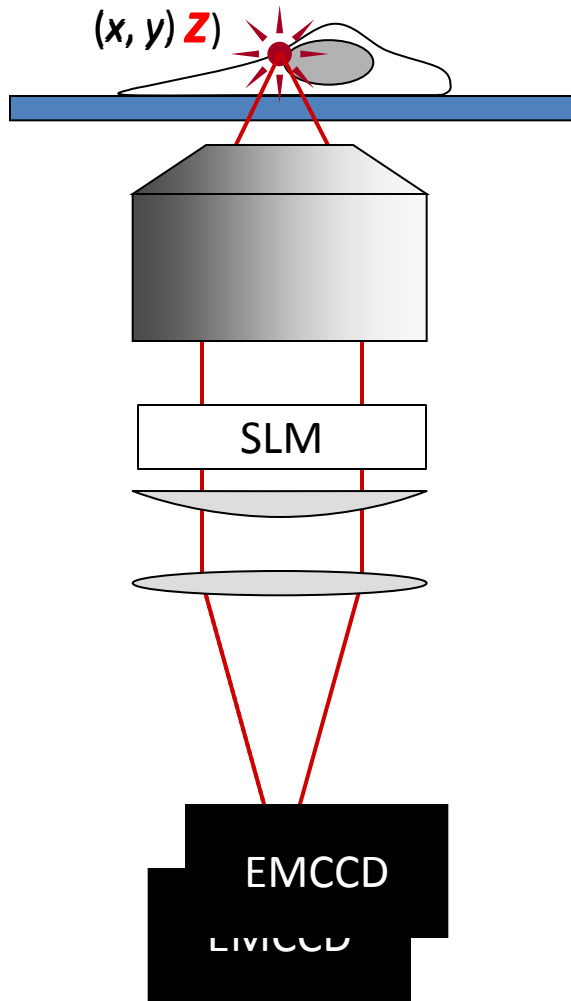
3D Imaging of the Microtubule Network

Small, isolated clusters

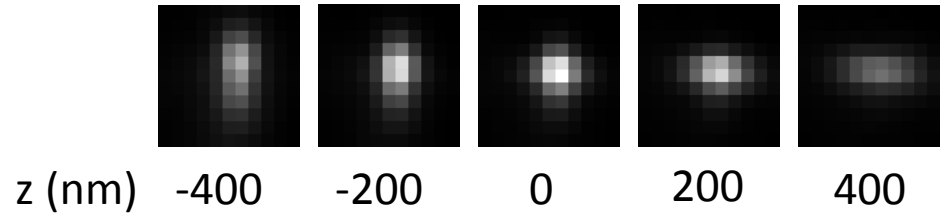


5 μm

Other 3D localization method

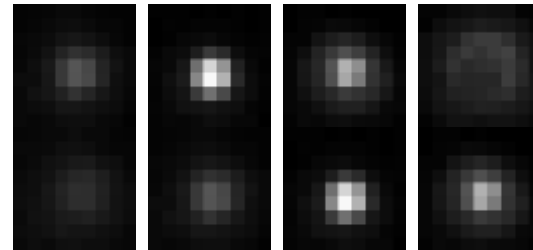


Astigmatic imaging



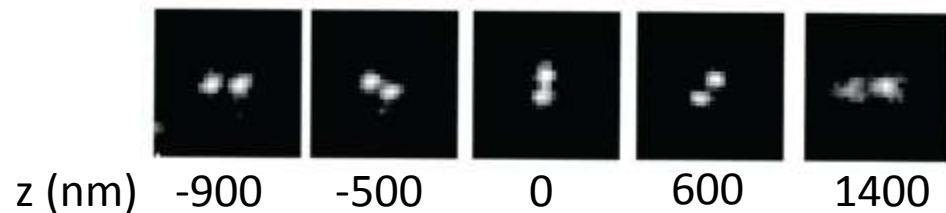
Huang et al., Science 2008

Bi-plane imaging



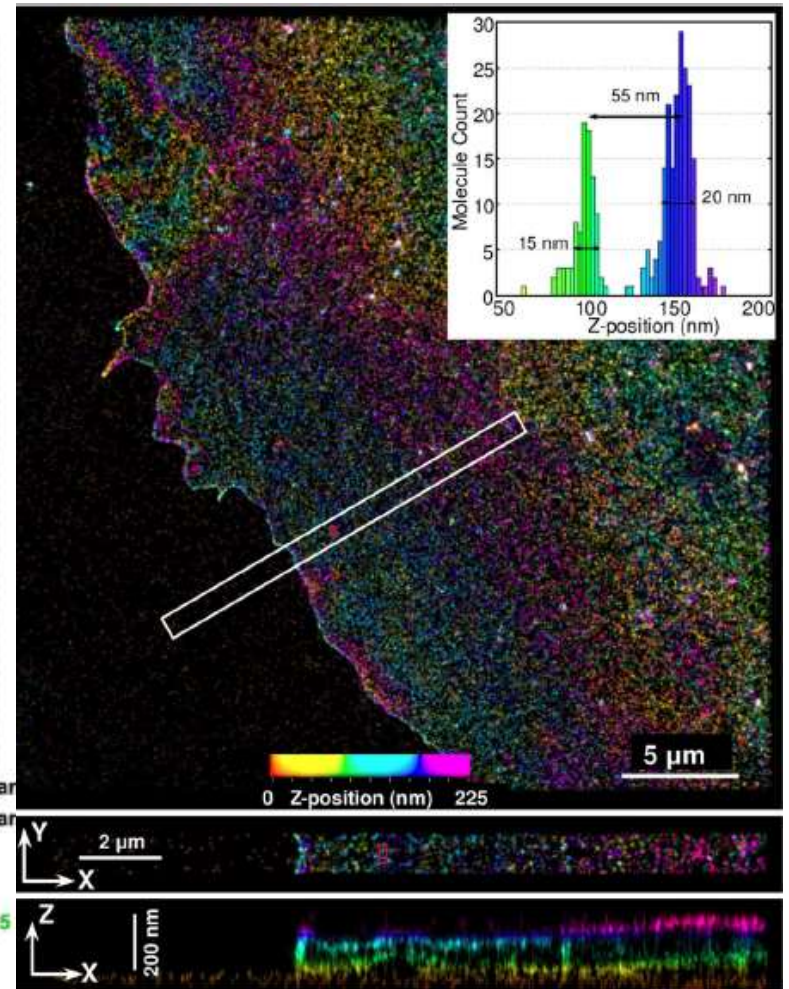
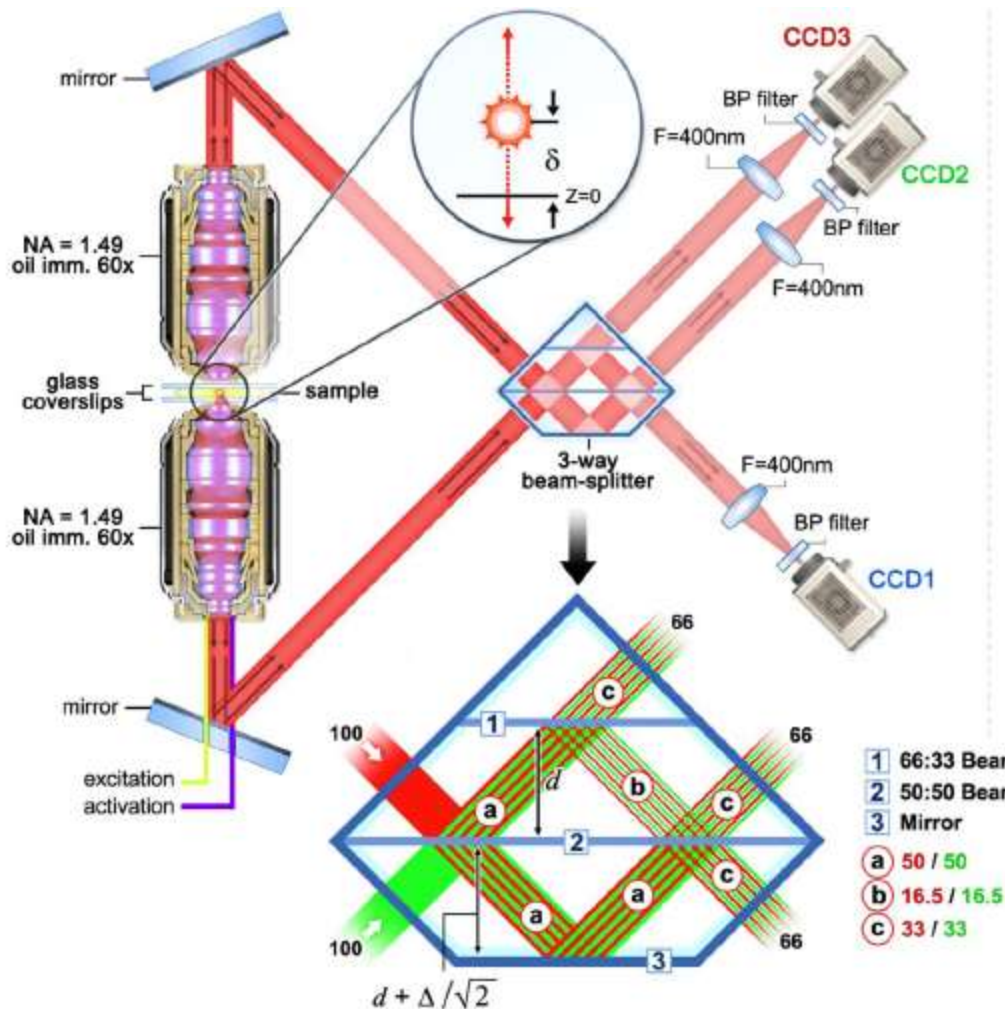
Juette et al., Nat Methods 2008

Double-helical PSF

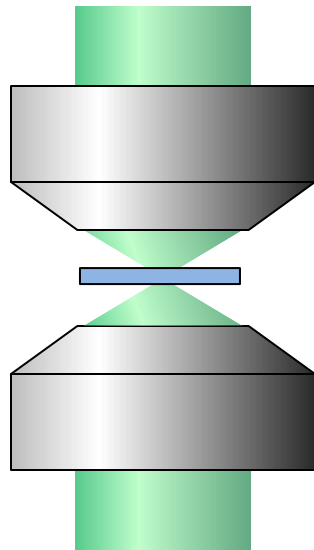


Pavani et al., PNAS 2009

Better 3D by two-objective interference



The use of two opposing objectives

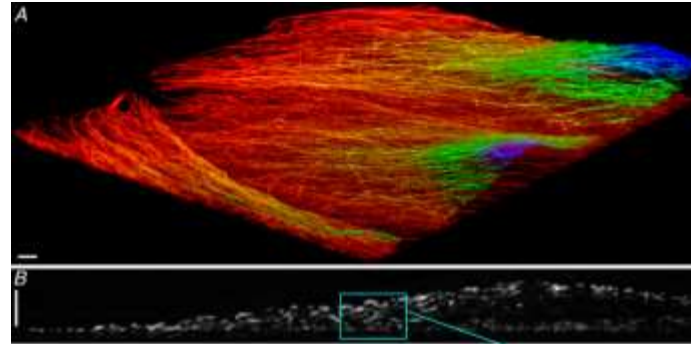


4Pi scheme



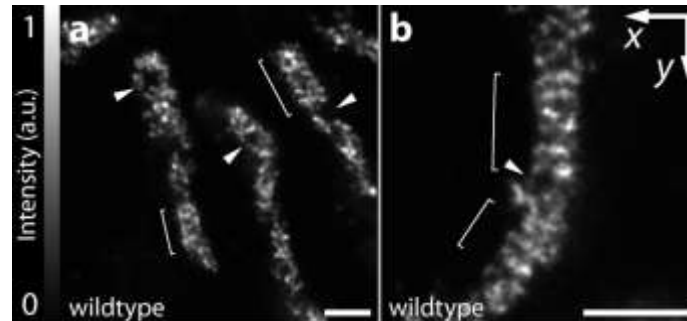
Near isotropic
3D resolution

I⁵S



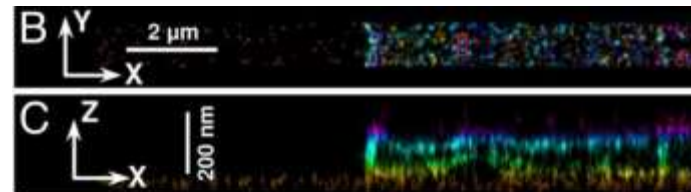
Shal et al., Biophys J 2008

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)



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

Heilemann et al., 2009

Functional dyes



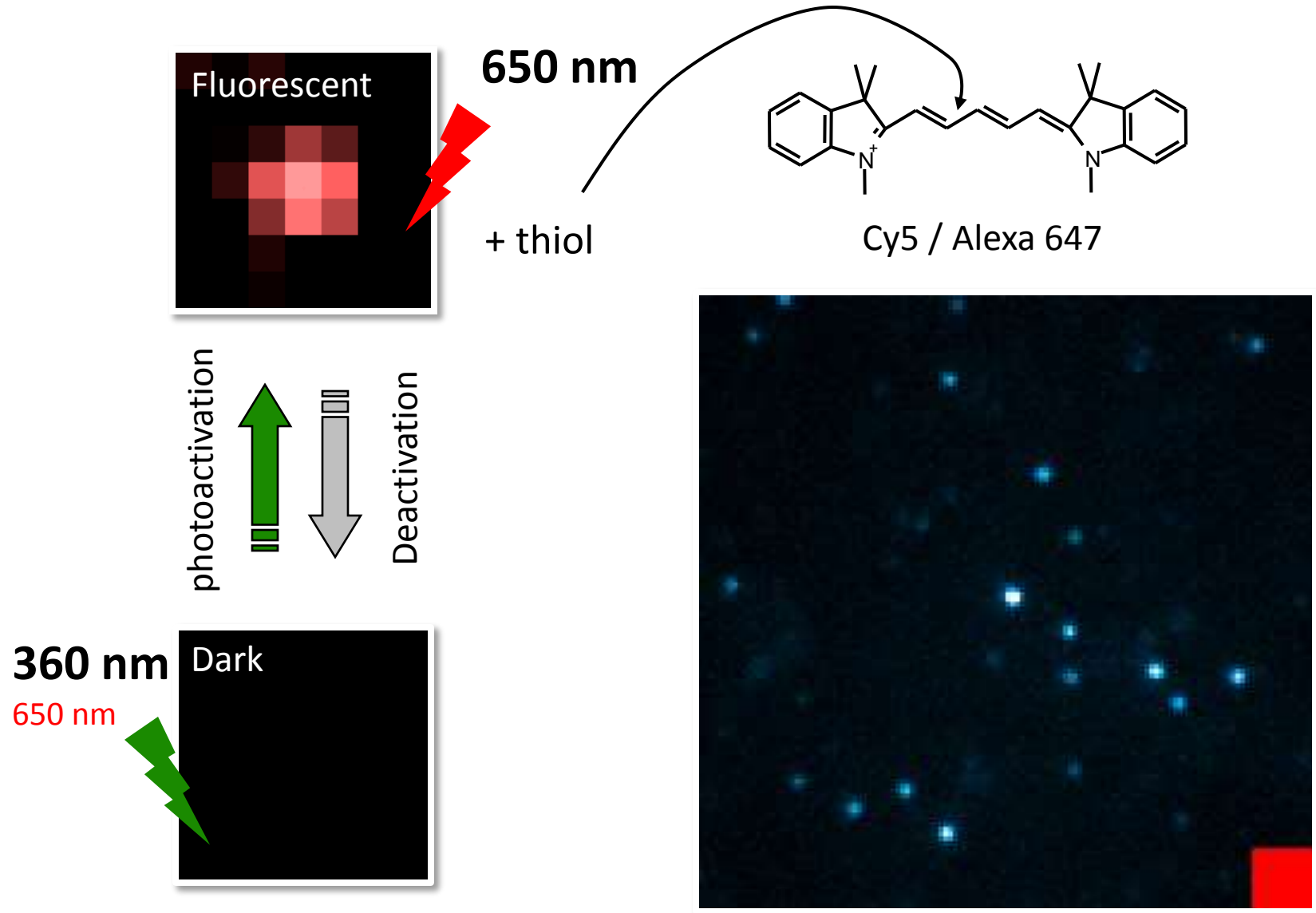
Shim et al., 2012

Photoactivatable fluorescent proteins



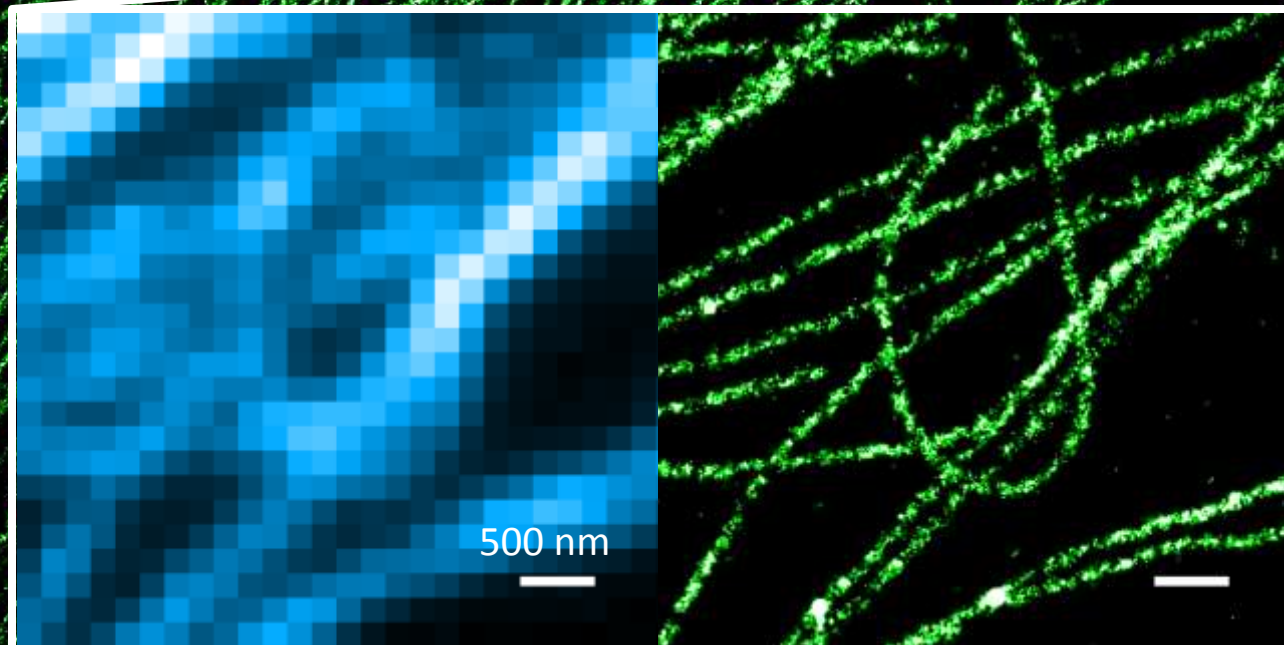
Reviews:
Lukyanov et al., Nat. Rev. Cell Biol., 2005
Lippincott-Schwartz et al., Trends Cell Biol., 2009

Photoswitching of red cyanine dyes



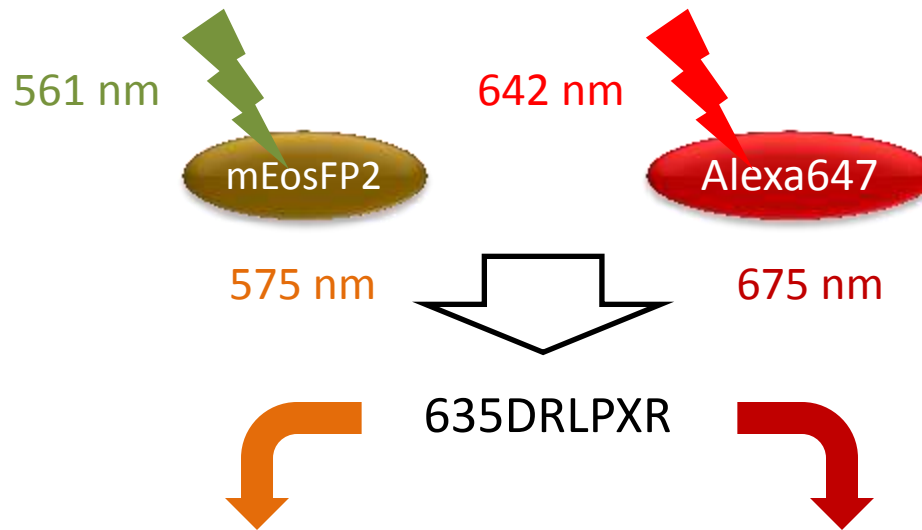
B-SC-1 cell, anti- β tubulin

Commercial **Alexa 647** secondary antibody



Multi-color Imaging

Multicolor STORM/PALM



mEos2-tubulin

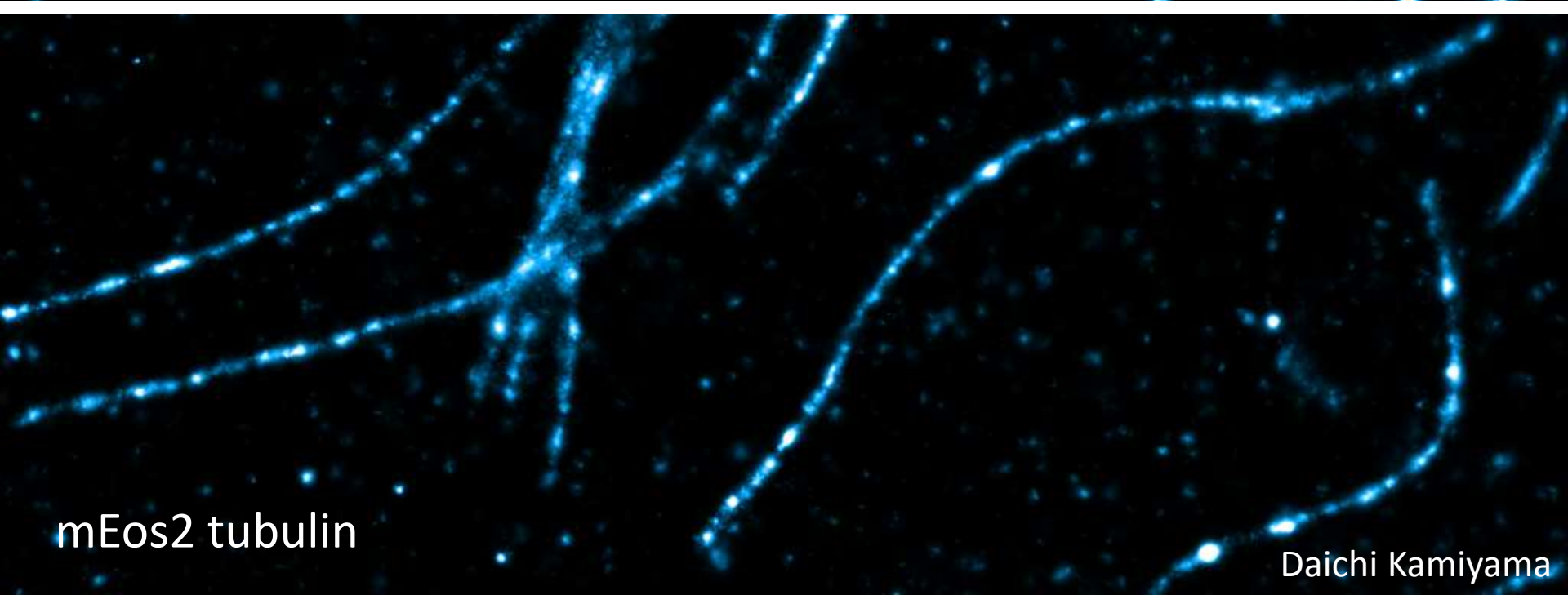
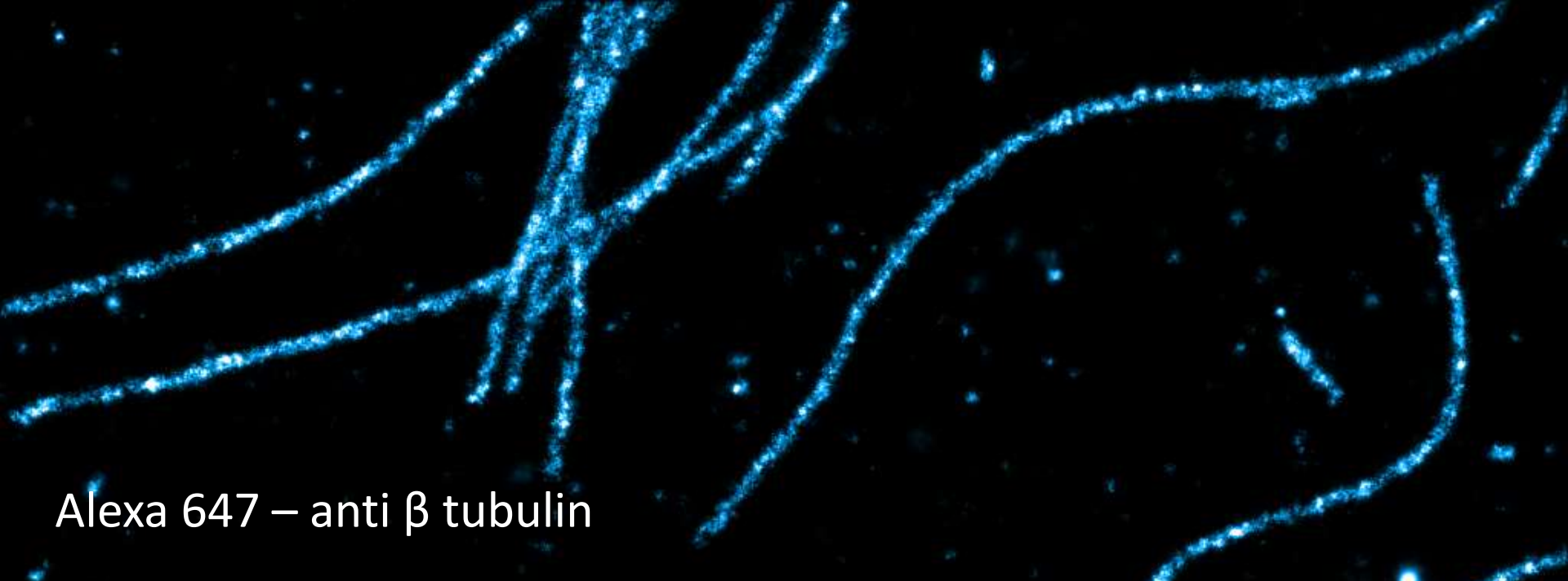
Alexa 647 anti- β tubulin

Drosophila S2 cells

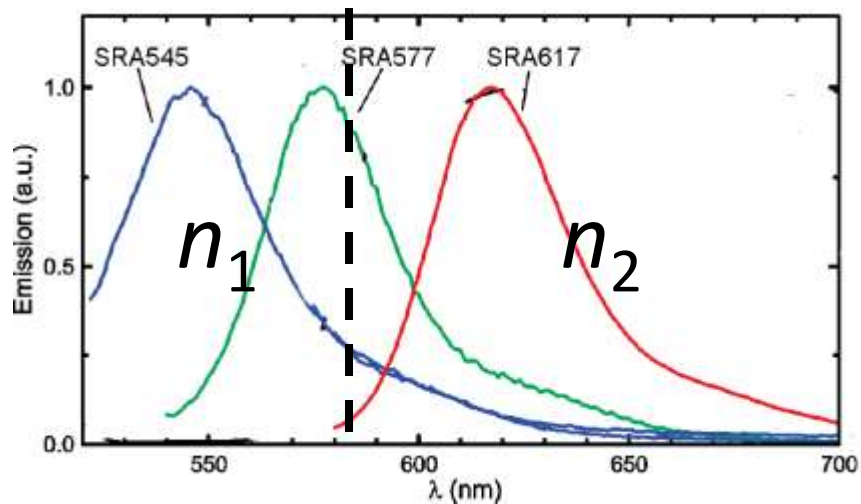
2 μ m



Daichi Kamiyama



Multicolor STORM/PALM: Emission



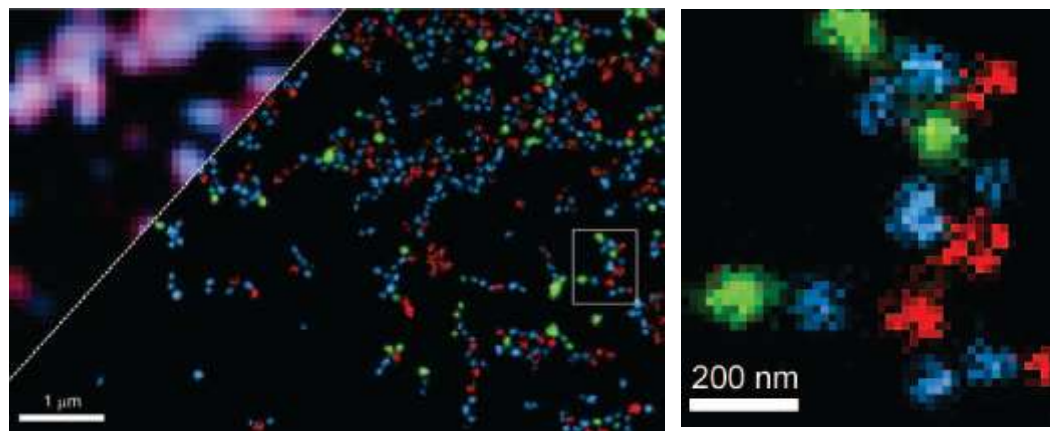
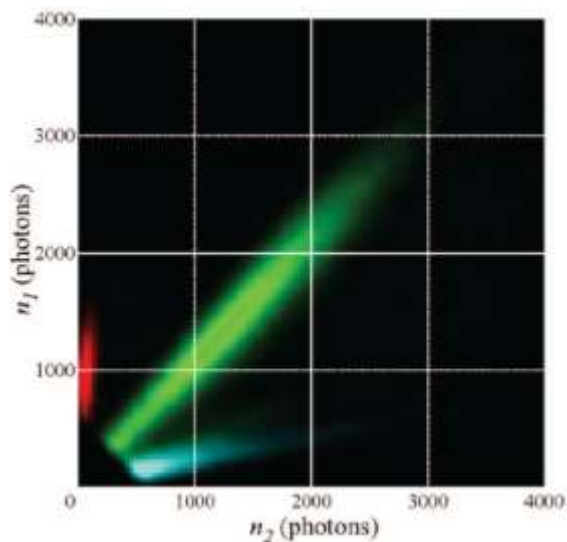
$$n_1 = n_2$$

→ 50% SRA545 + 50% SRA617?

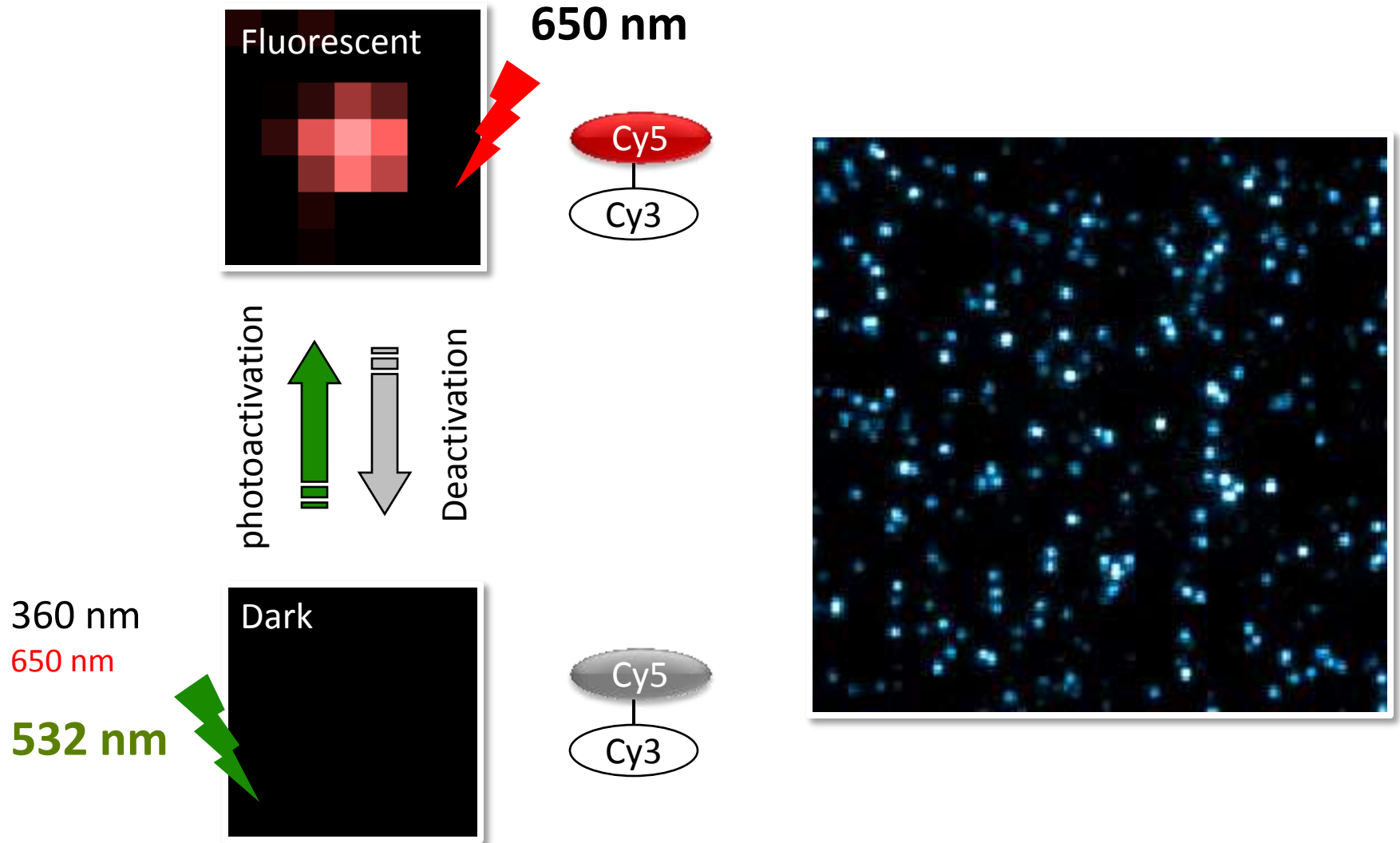
→ 100% SRA577?

Single-molecule detection!

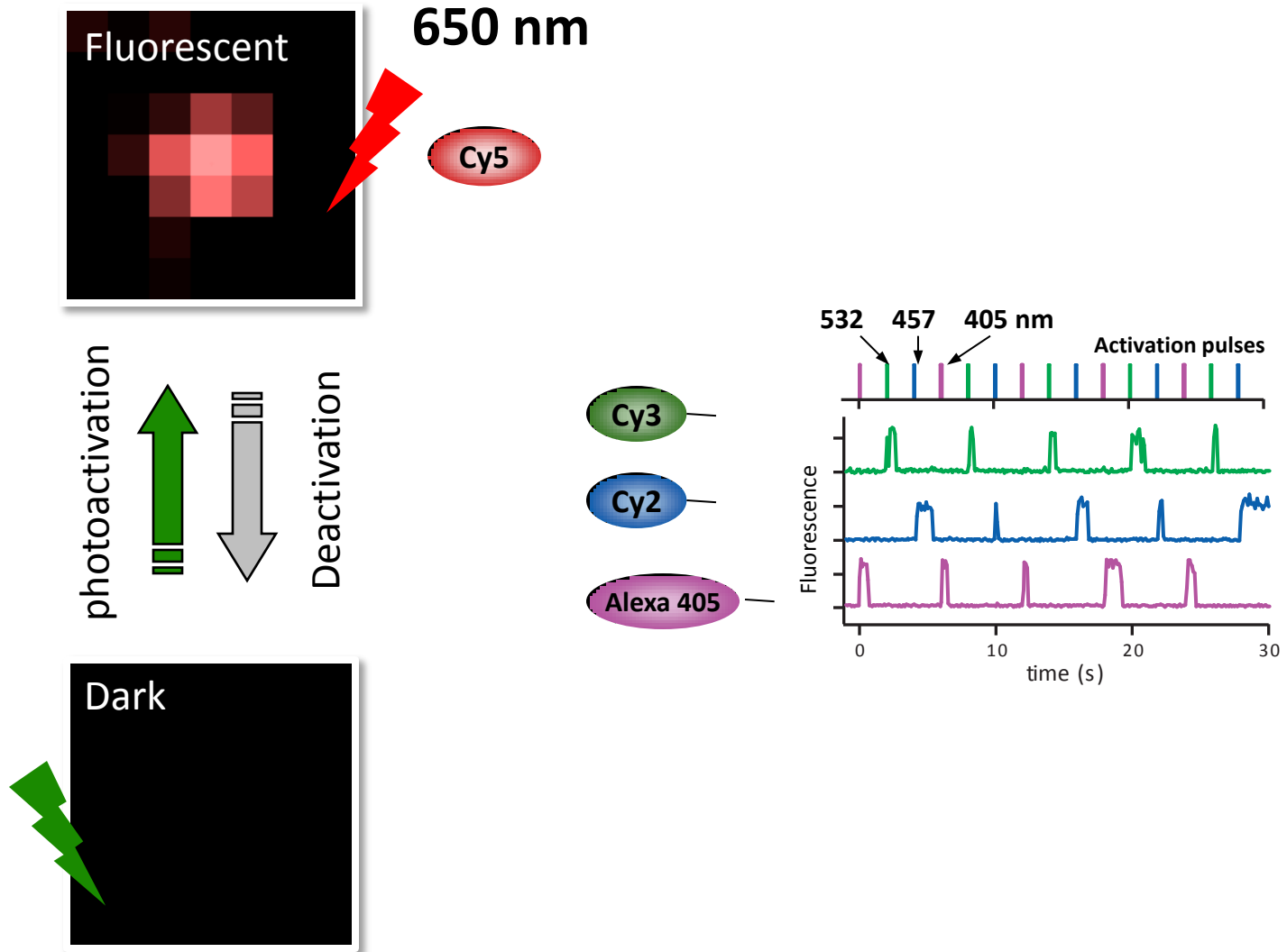
3-color imaging with one excitation wavelength and two detection channels



Multicolor STORM/PALM: activation



Controlling the activation of Cy5



Cy3 / Alexa 647: Clathrin

Cy2 / Alexa 647: Microtubule

Crosstalk subtracted

Laser sequence



1 μ m

Multicolor imaging approaches

By emission wavelengths



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

By activation wavelengths

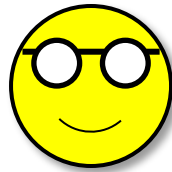
- Dye-pairs
- Crosstalk from nonspecific activation
- Laser sequences
- Single channel detection
- Images naturally aligned



Super resolution microscopy spec sheets

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 16.5 mm, 130g	Slimmer, lighter than BlackBerry Storm
OS	WebOS, Webkit based	Full HTML
Display	TFT capacitive touchscreen, 320x480 pixels, 3.1 inches	TruePress technology
Keyboard	Physical, Full QWERTY	Software, Full QWERTY
Accelerometer	Yes	Yes
Headphone Jack	Yes, 3.5mm	Yes, 3.5mm
Bluetooth	Yes, v2.1 with A2DP	Yes
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

The “patterned illumination” approach



Excitation

Multiple cycles



- Ground state
- Triplet state
- Isomerization etc.

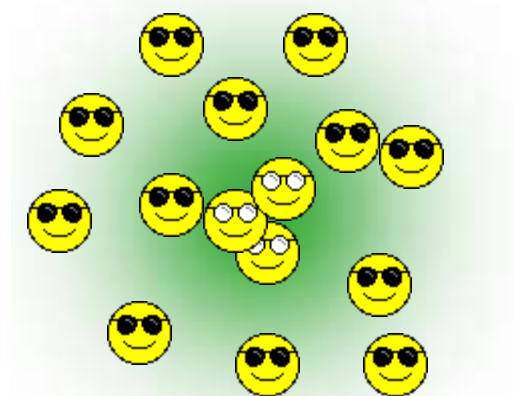


÷

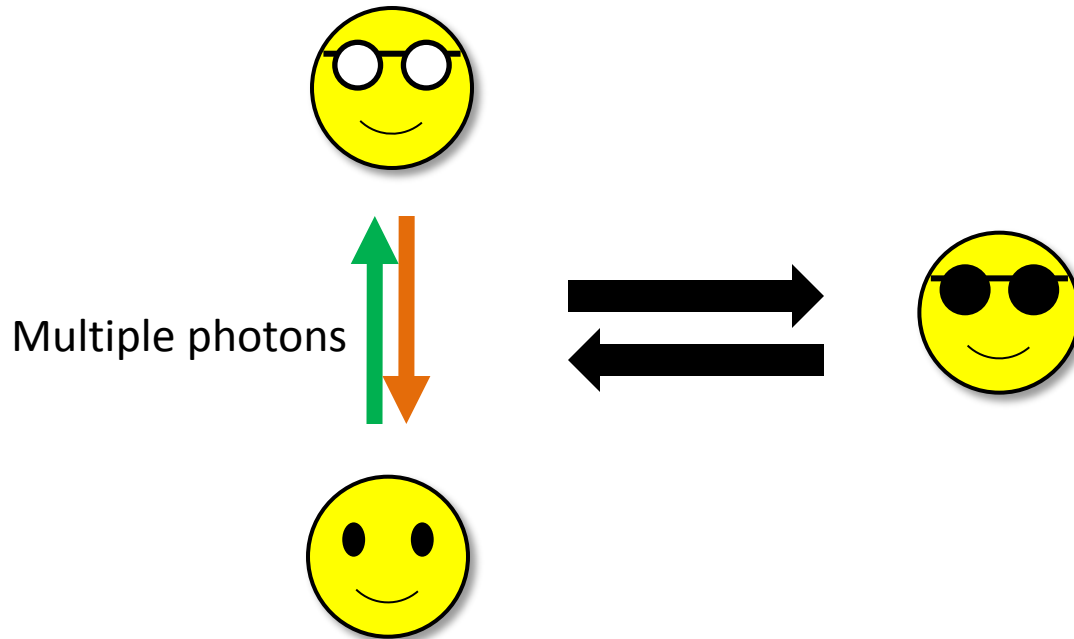
Depletion
pattern



=



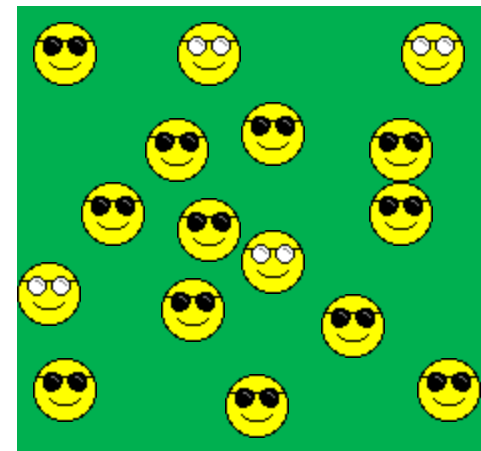
The “single-molecule switching” approach



- Photoswitching
- Blinking
- Diffusion
- Binding etc.



+ Stochastic Switching =



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

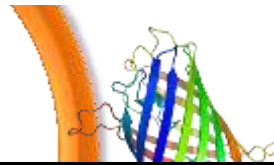
- 100,000 photos during Cy5 life time \rightarrow < 1 nm

Effective resolution: Probe matters

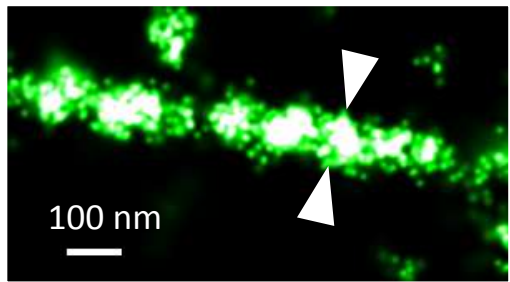
Antibodies:
~ 10 nm

Fluorescent Proteins:
~ 3 nm

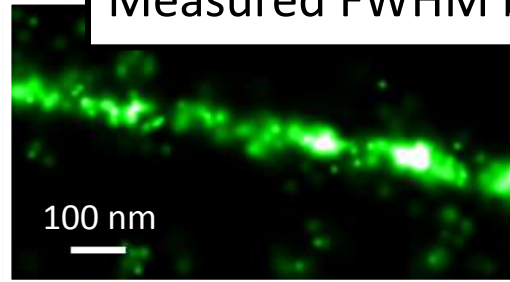
Small fluorophores:
~ 1 nm



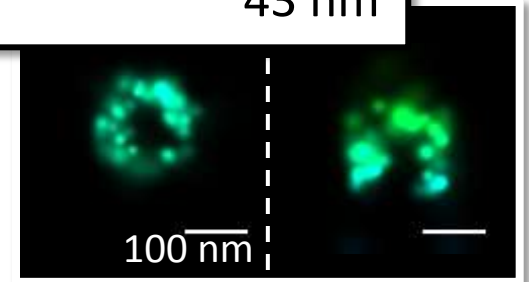
Measured FWHM by antibody:	58 nm
Actual microtubule diameter:	25 nm
Measured FWHM by FP:	43 nm



~ 6000 photons



< 1000 photons



~ 6000 photons

Fluorescent protein vs. Antibody

Fluorescent protein fusion

- Live sample labeling
- High specificity
- High labeling efficiency
- Genetically encoded
- Lower S/N
- Multicolor imaging so far challenging

Antibody immunofluorescence

- Fixed sample
- High specificity labeling
- High labeling efficiency
- Labeling of endogenous proteins
- High localization precision
- 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:

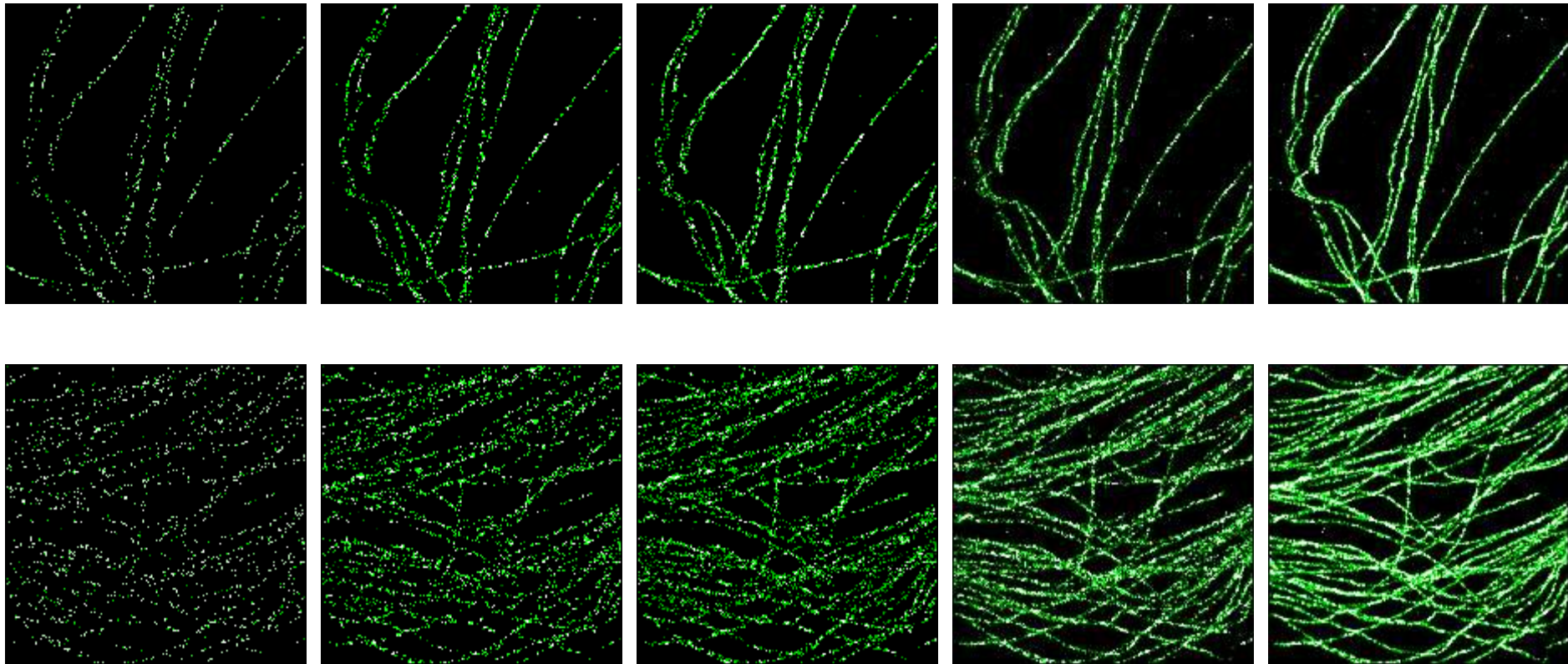
200

500

1,000

5,000

40,000



Effective resolution: Density matters

Frames for image reconstruction:

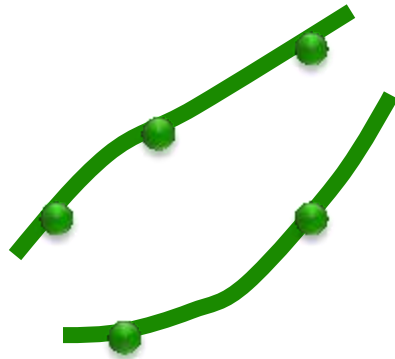
200

500

1,000

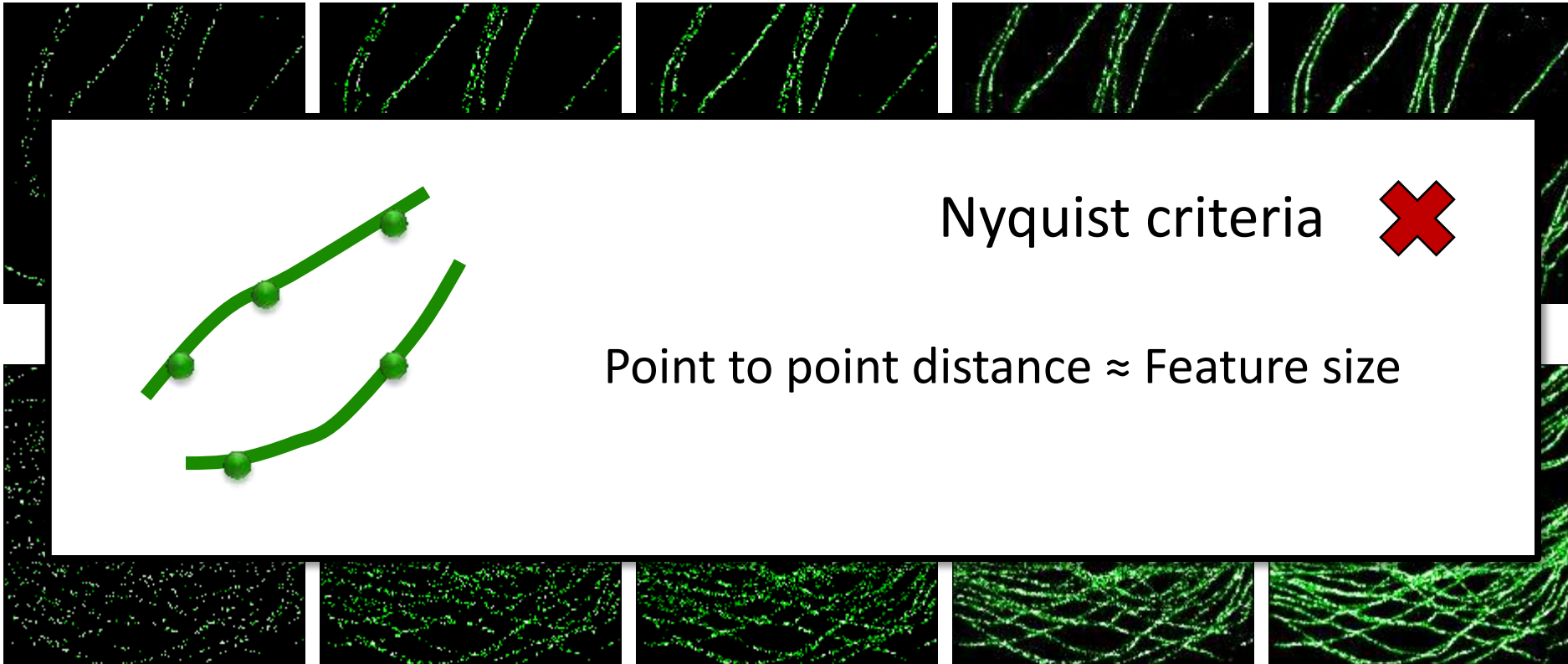
5,000

40,000



Nyquist criteria 

Point to point distance \approx Feature size



Effective resolution: Density matters

Frames for image reconstruction:

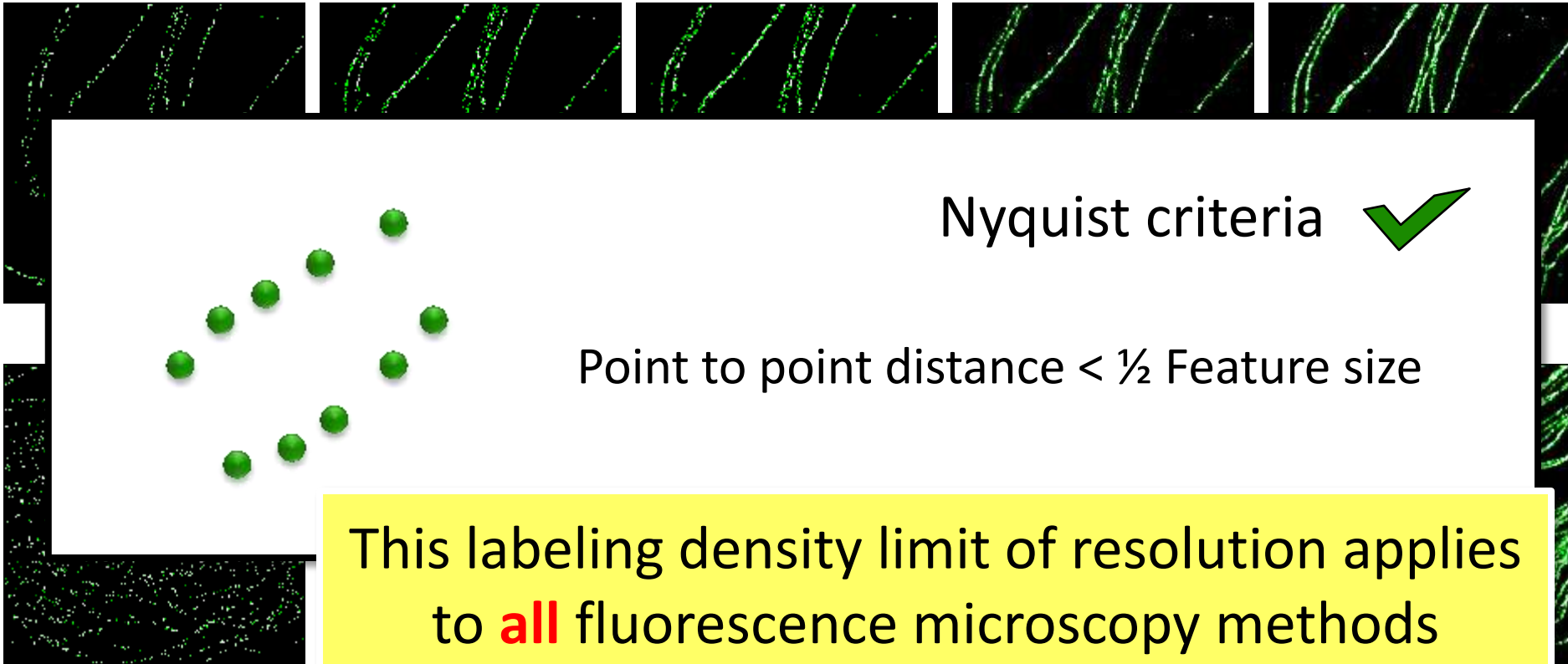
200

500

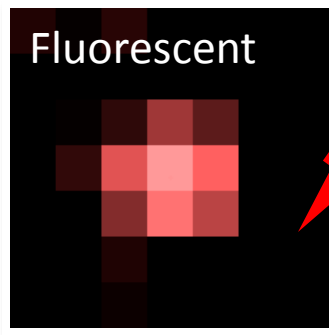
1,000

5,000

40,000



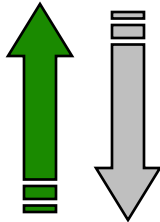
Effective resolution: Contrast matters



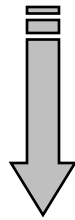
650 nm

e.g. **1%**

photoactivation



Deactivation



650 nm



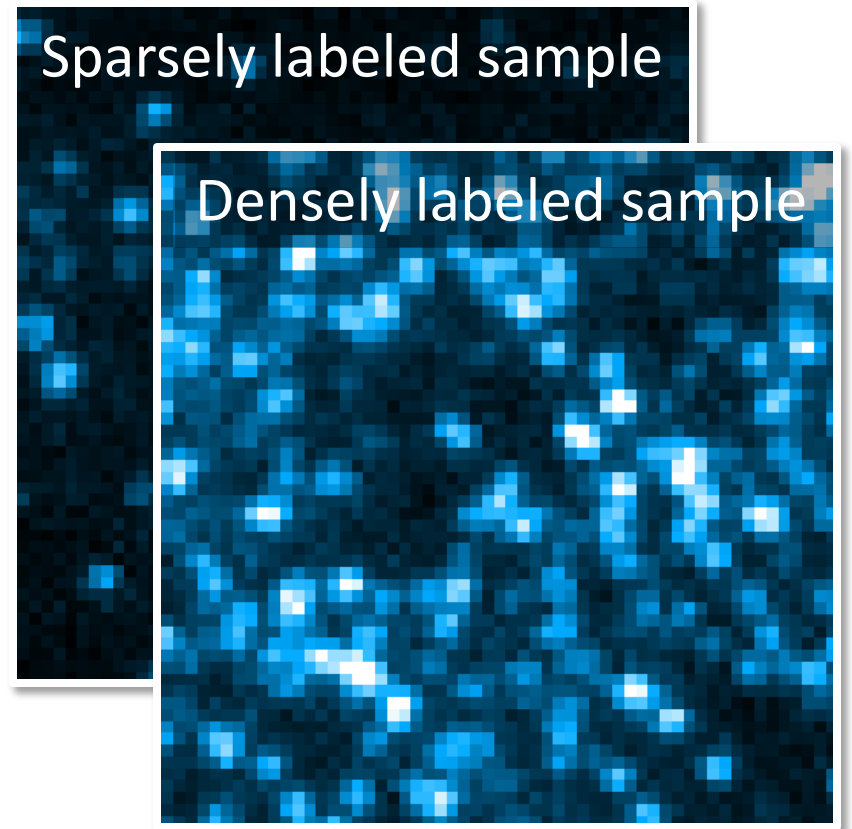
Dark

e.g. **99%**

1% means...

Sparsely labeled sample

Densely labeled sample



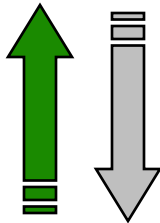
Effective resolution: Contrast matters



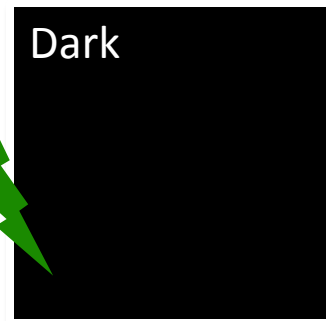
650 nm

e.g. **1%**

photoactivation



Deactivation

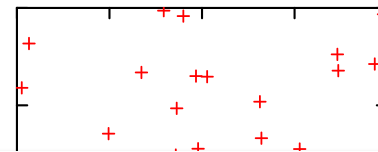


650 nm

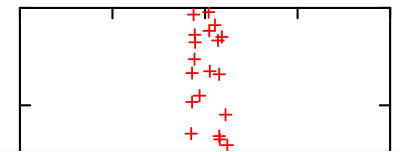
e.g. **99%**

1% means...

Homogeneous sample



Microtubule



Common blinking dyes: >3%

Cy5 + mercaptoethylamine: 0.1-0.2%

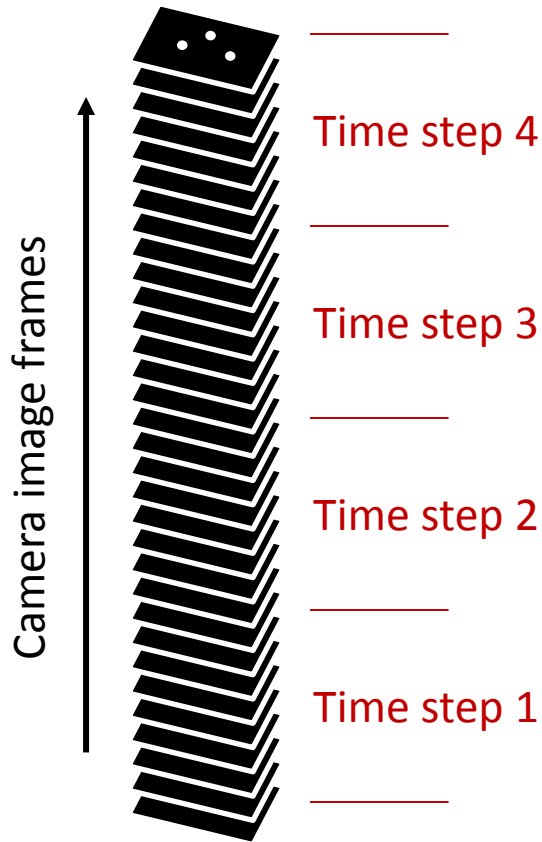
mEosFP: 0.001%

40 nm

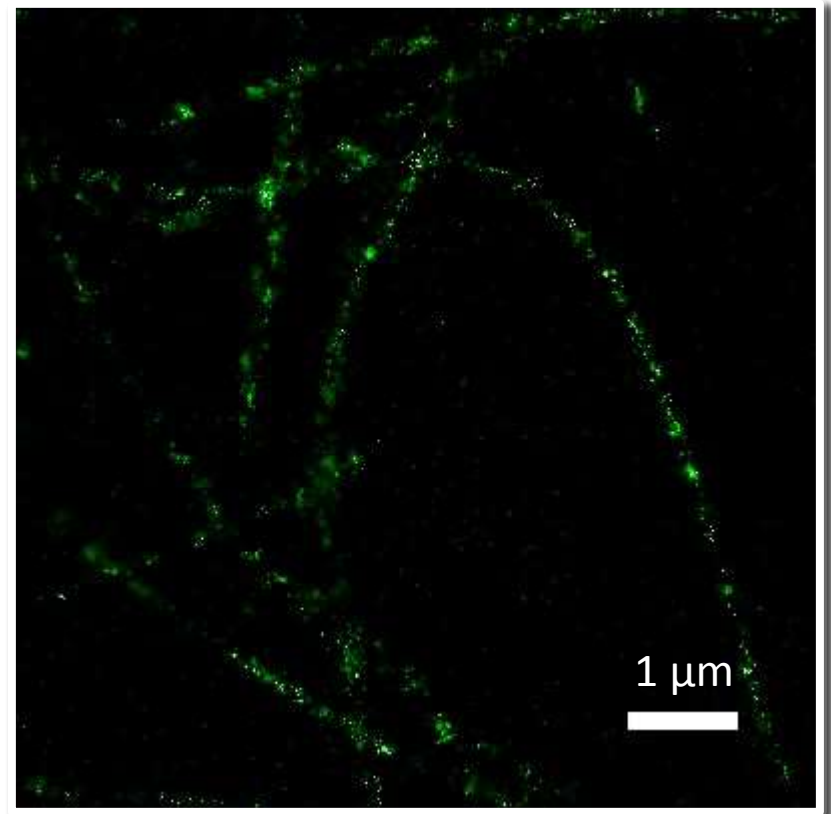
14 nm

Live Cell Imaging

Live cell STORM

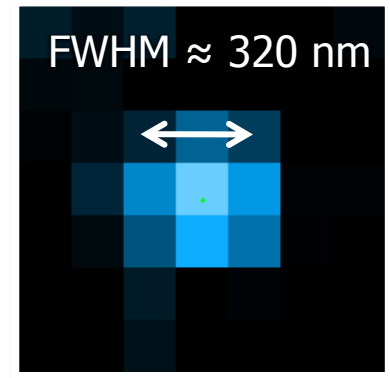
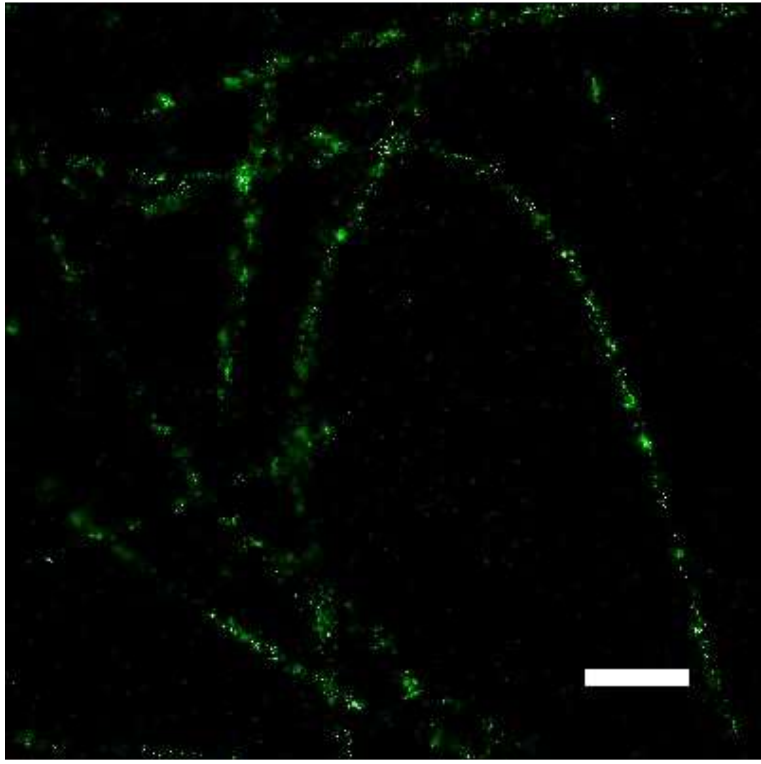


mEos2 labeled microtubule in live S2 cells



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

Spatial-temporal resolution trade-off



Assuming:

1 molecule occupies 500×500 nm



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



70 nm resolution \equiv 2000 frames

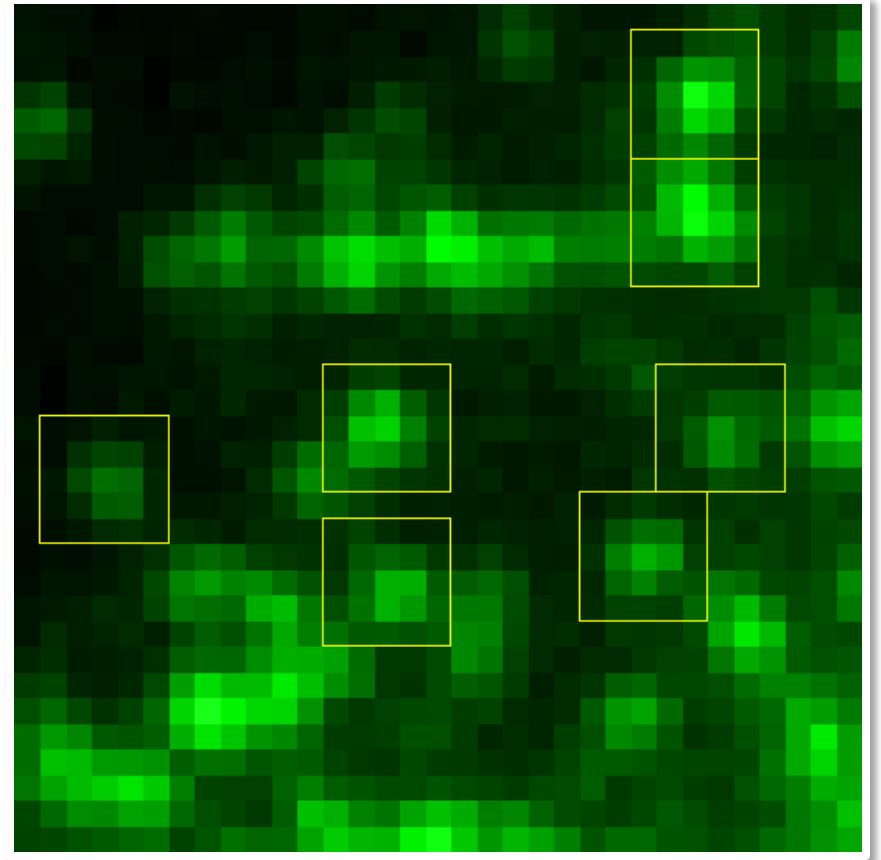
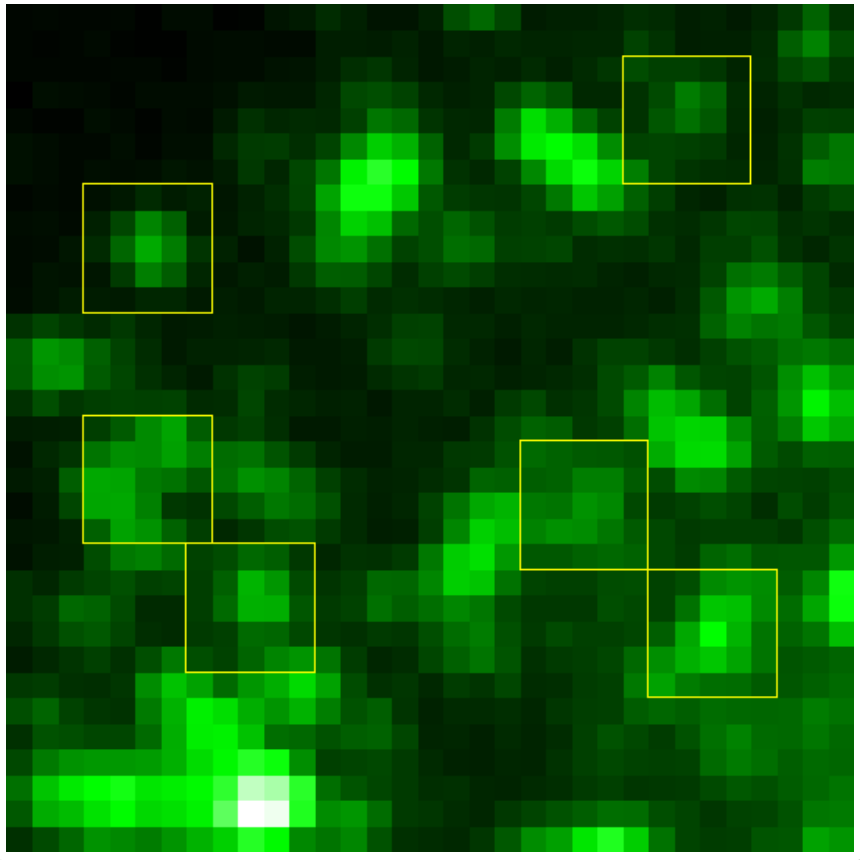


100 fps = 20 sec time resolution

1000 fps

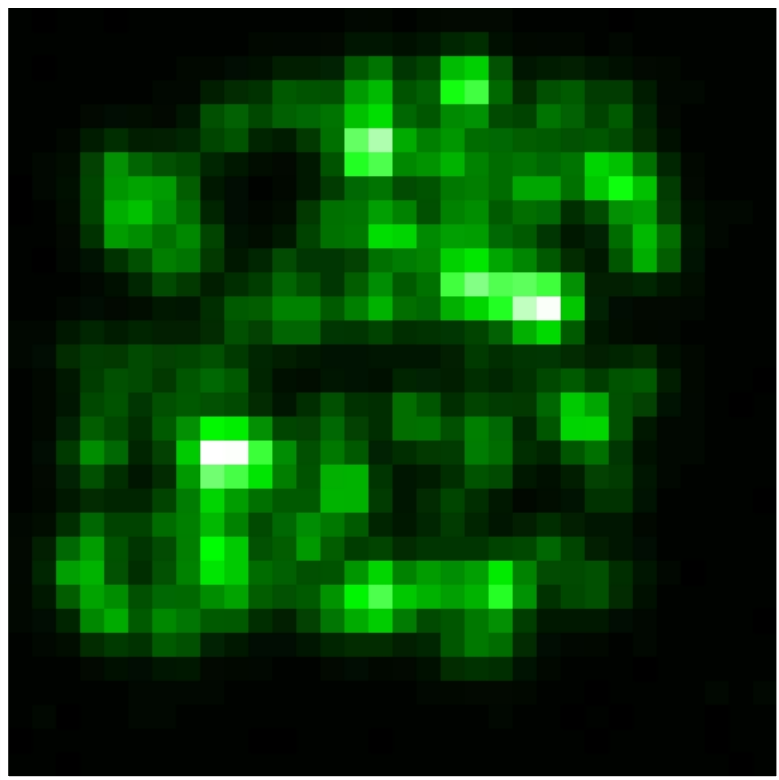


More molecules per camera image?

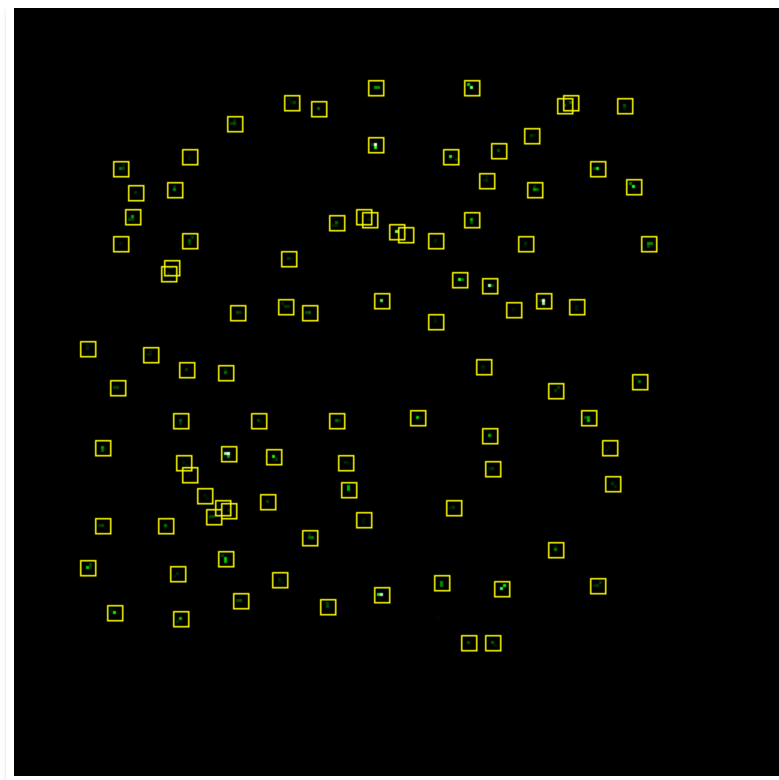


Molecule identification by compressed sensing

Simulated camera image



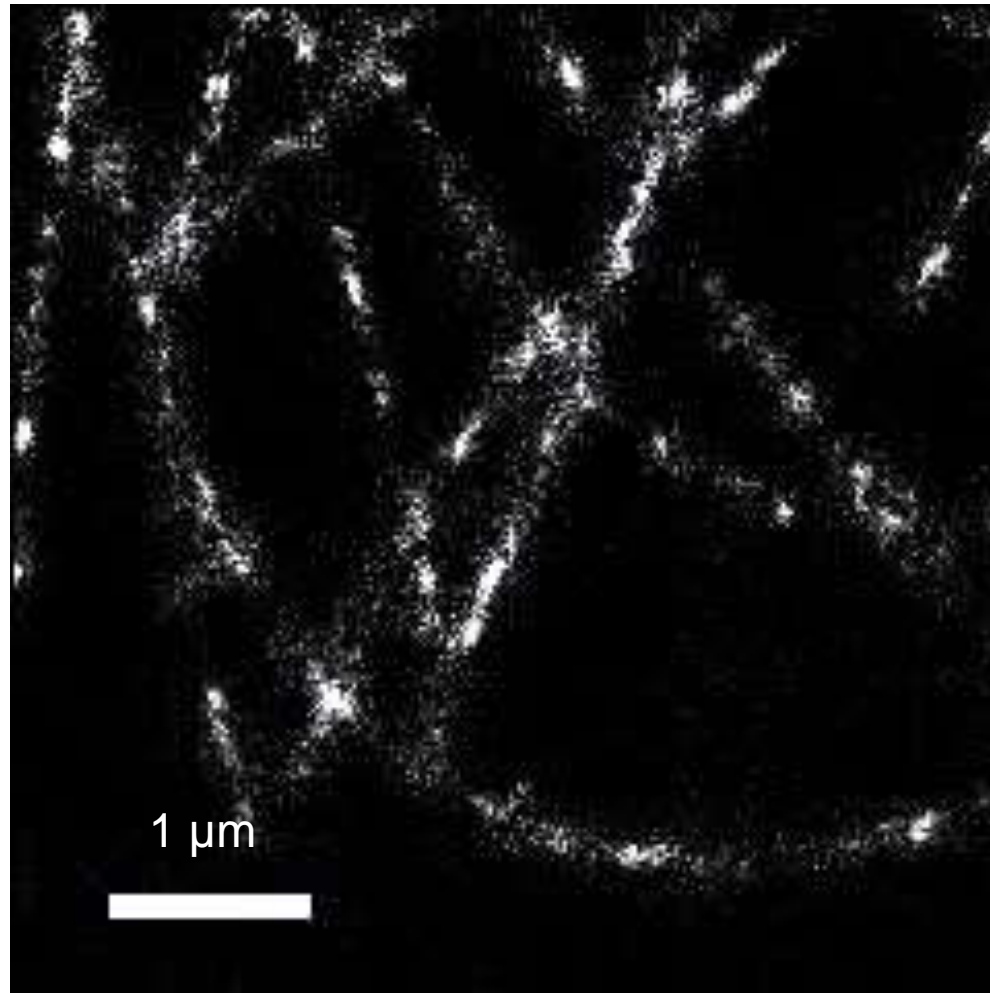
Compressed sensing



94 recovered

100 molecules

Fast live cell imaging by compressed sensing

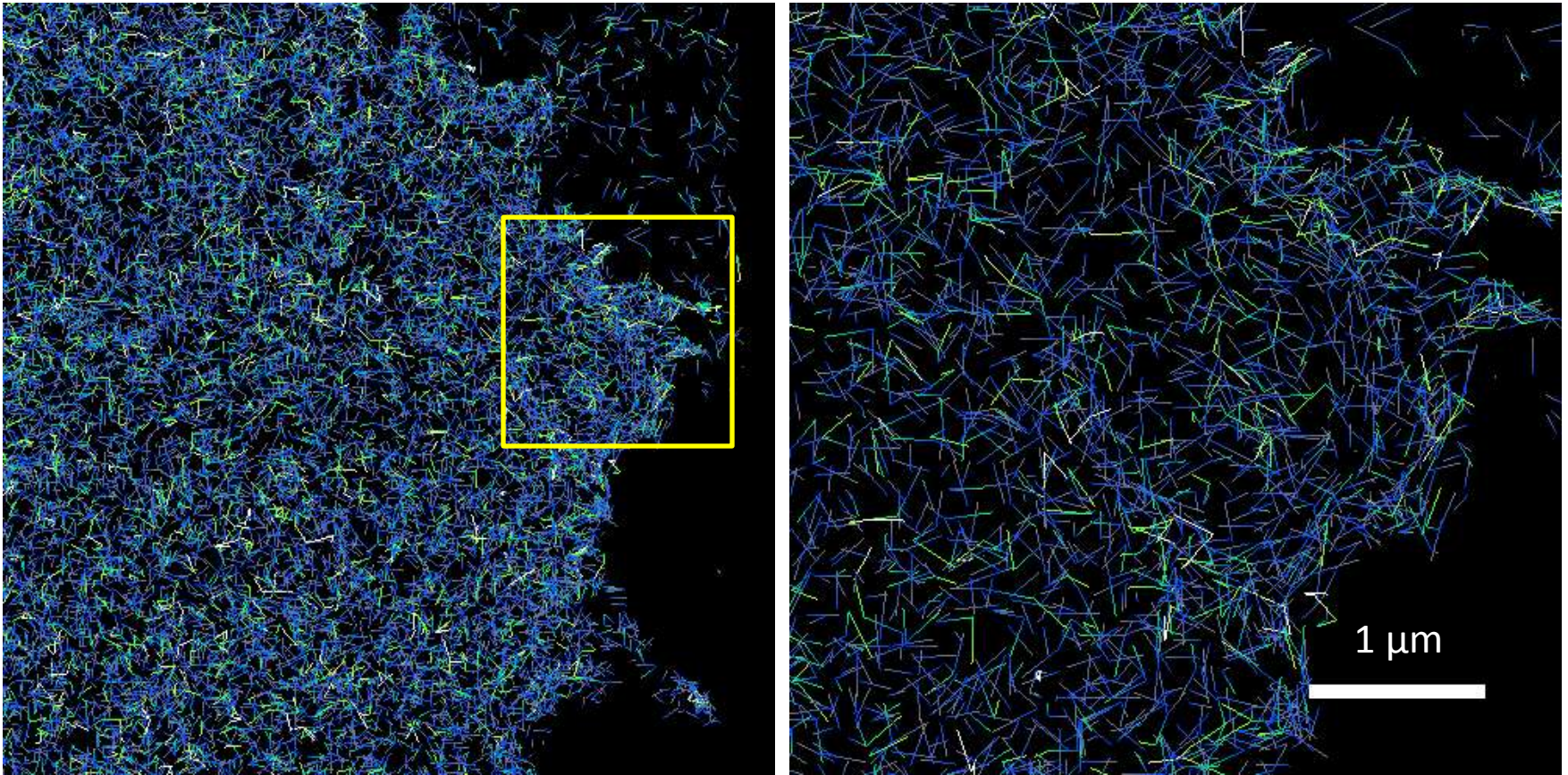


mEos2-tubulin in S2 cell, 3 sec time resolution, 11.8x real time

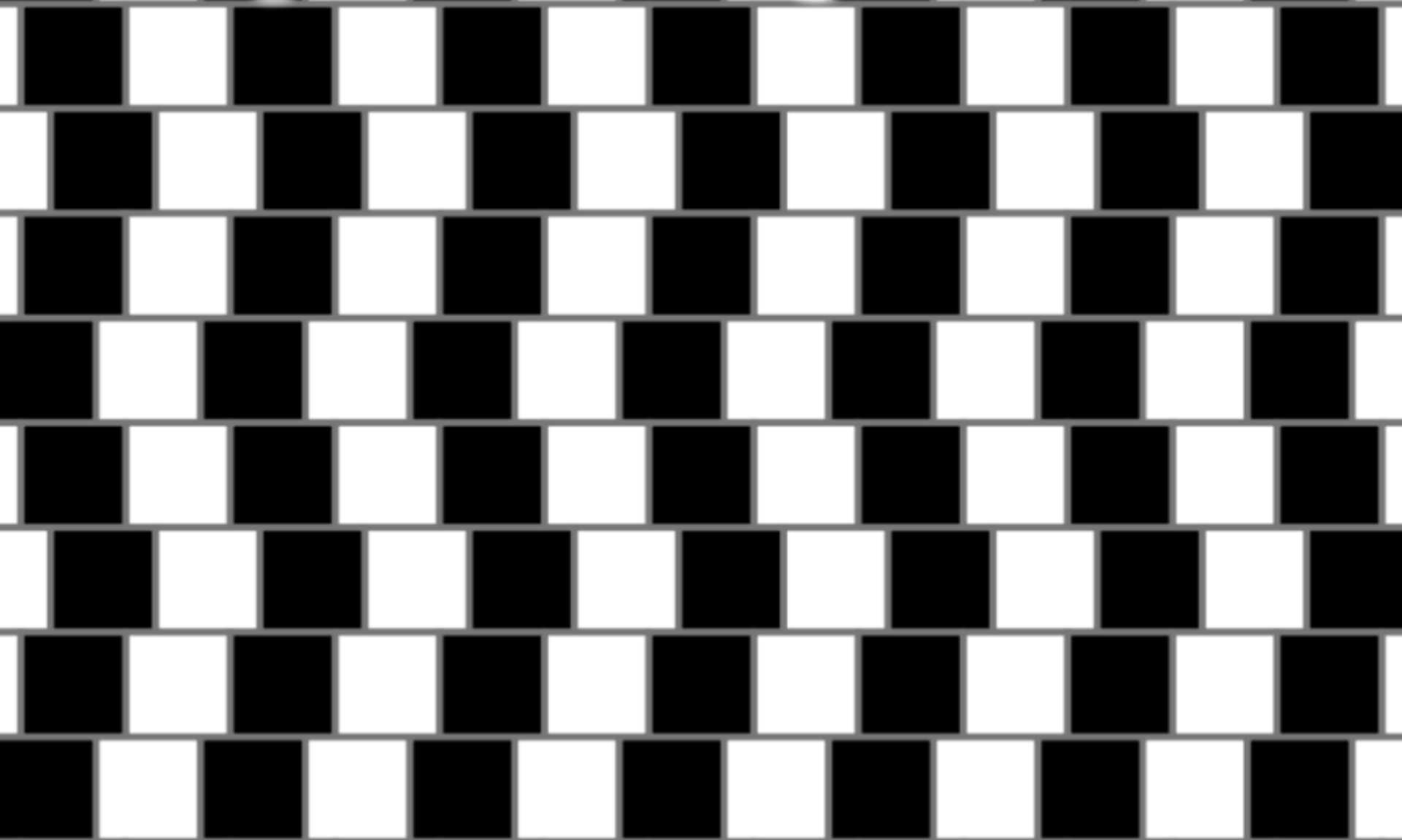
Fast... and even faster?



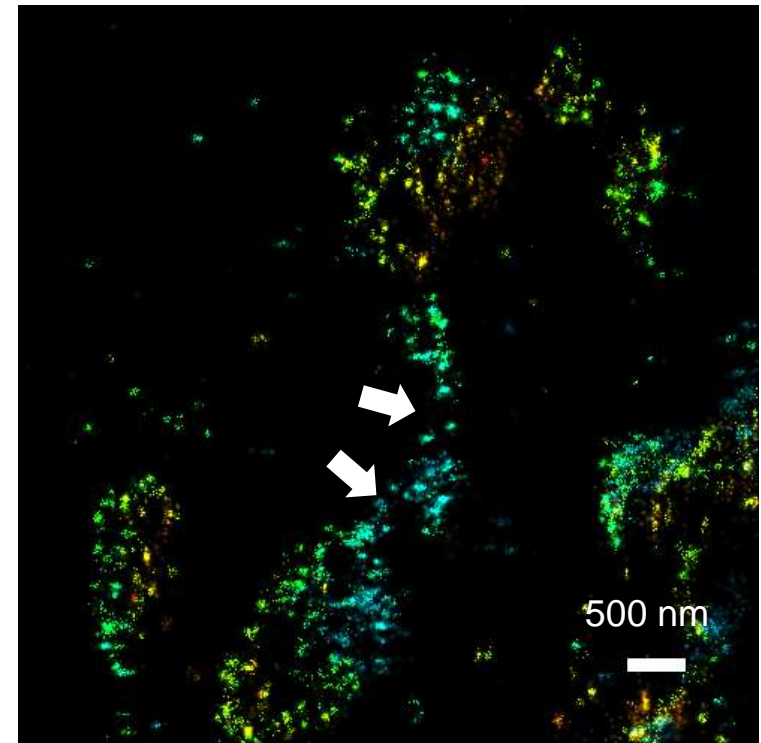
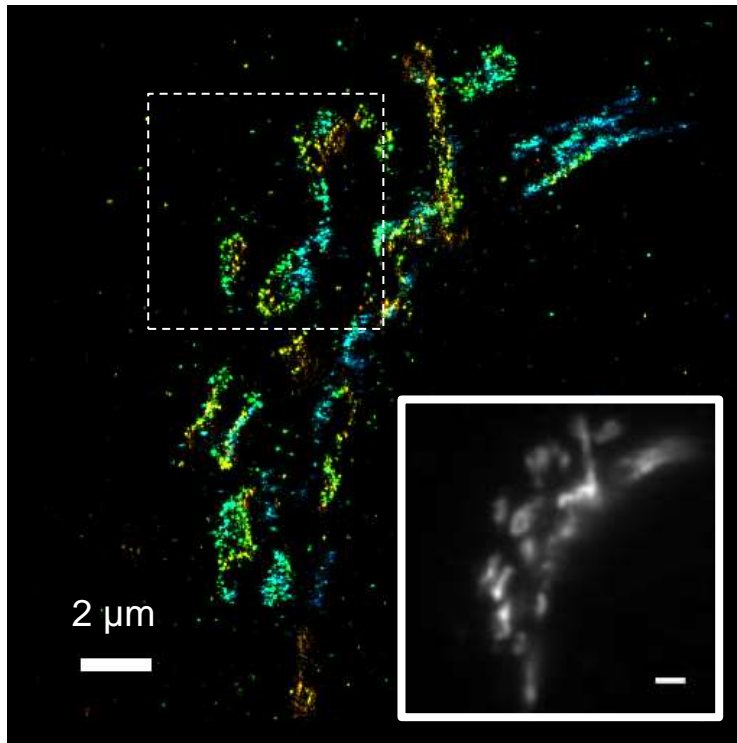
Tracking DiD in the membrane



Seeing can be deceiving...

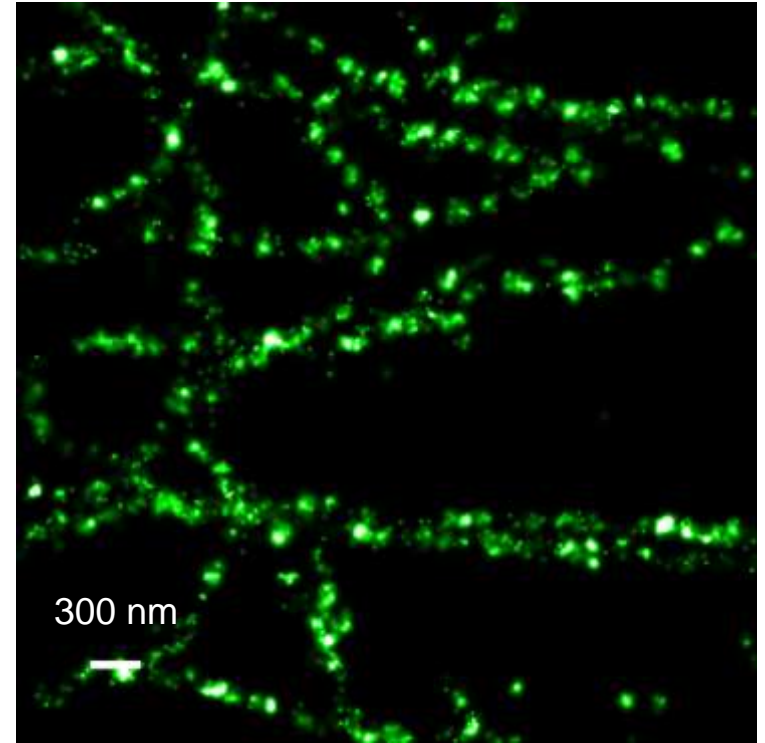
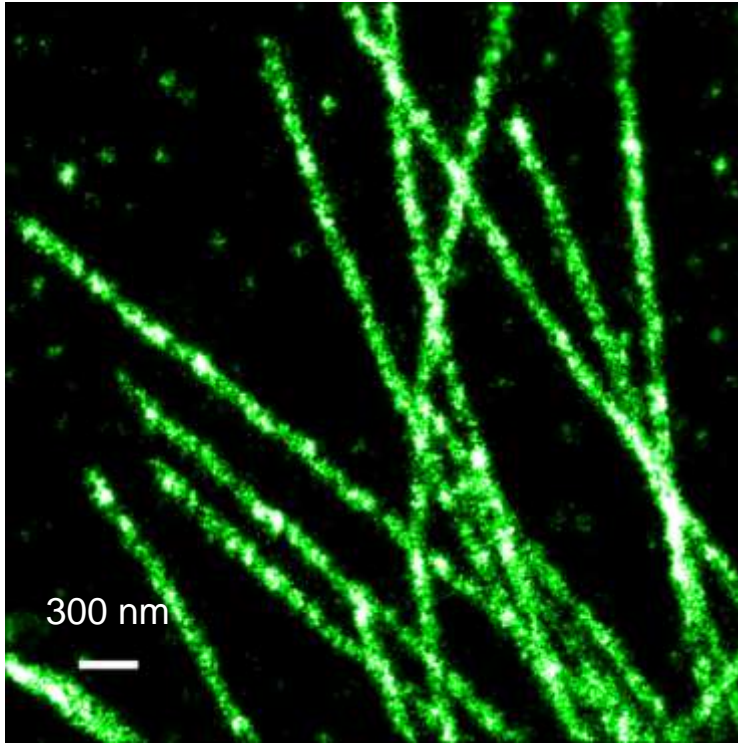


Super-resolved artifacts: sparse labeling



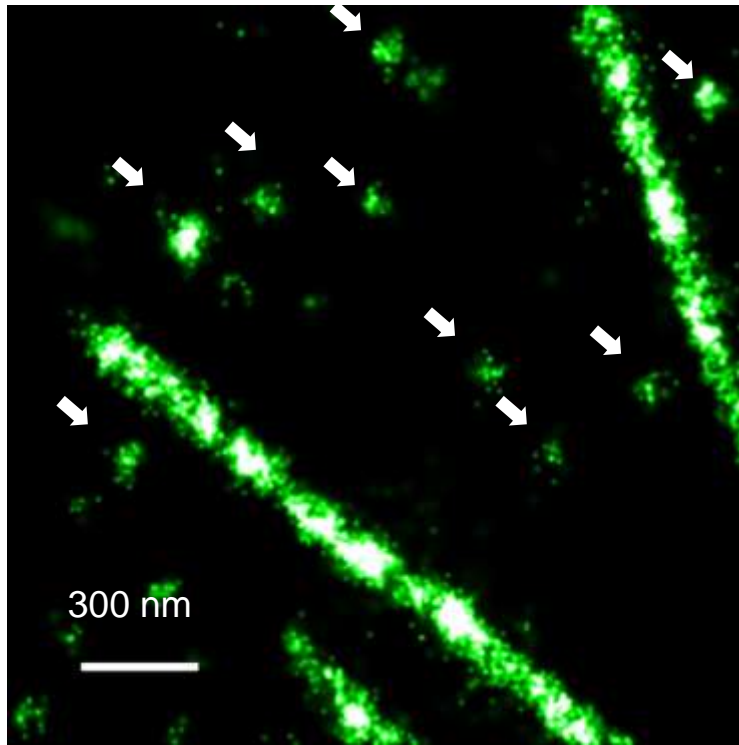
Golgi: Giantin immunofluorescence

Super-resolved artifacts: poor fixation

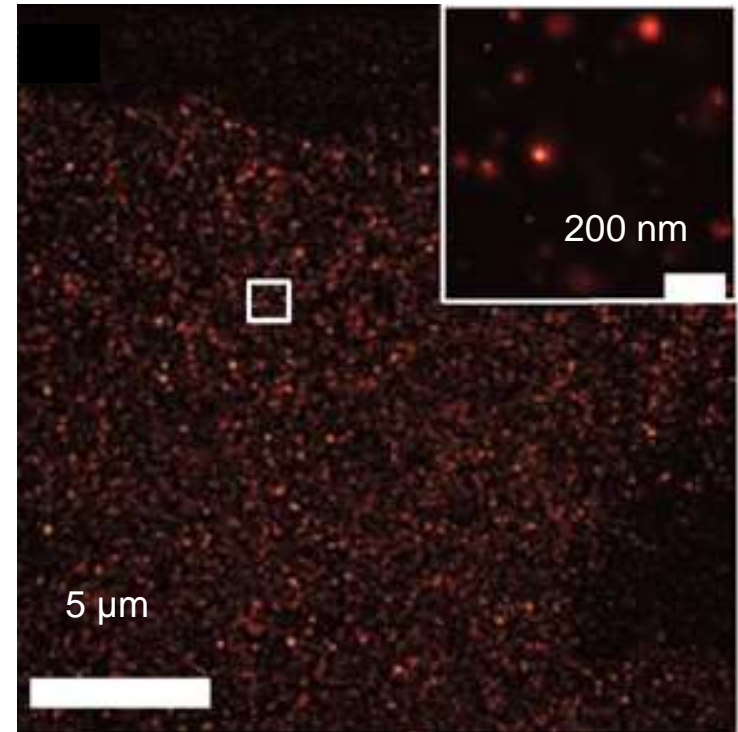


Microtubules: good and bad fixation

Super-resolved artifacts: clustering

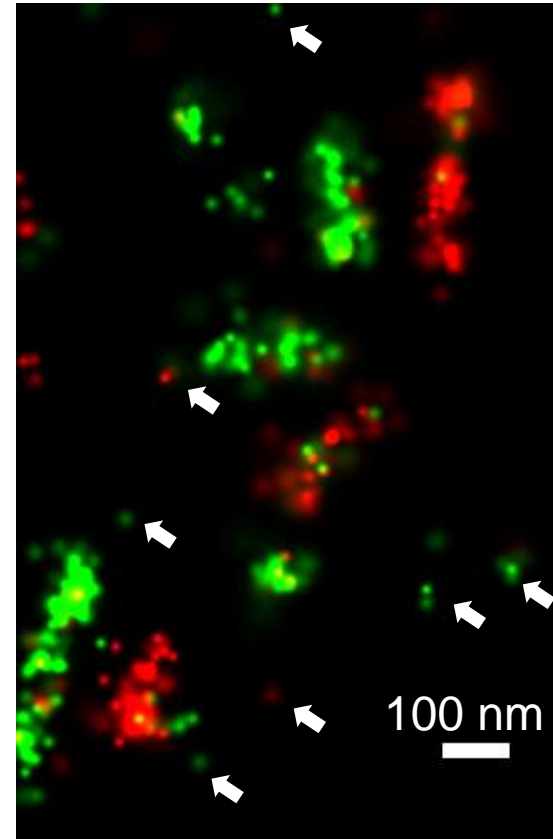
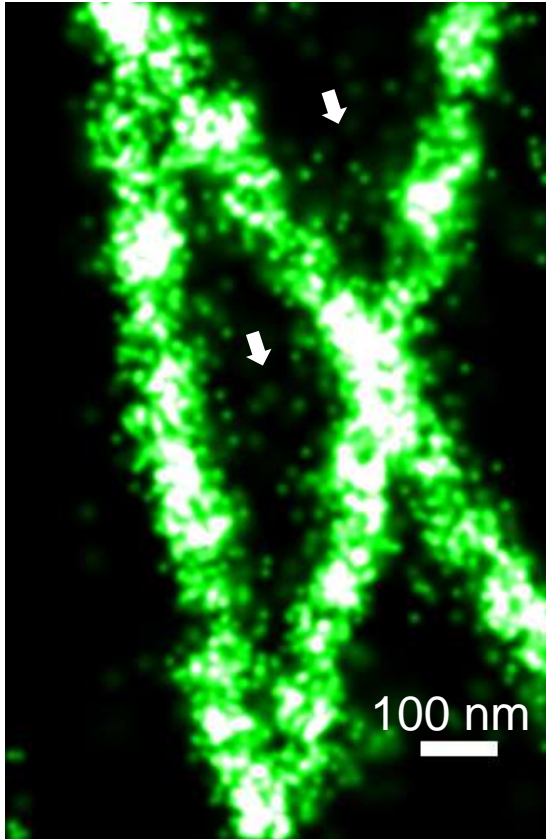


Clusters from single antibodies



Clusters from blinking FPs

Super-resolved artifacts: “noise” points



Noise from misidentified molecules, crosstalk and background

With the creation of new tools...

