Superresolution Microscopy by Single Molecule Switching and Localziation

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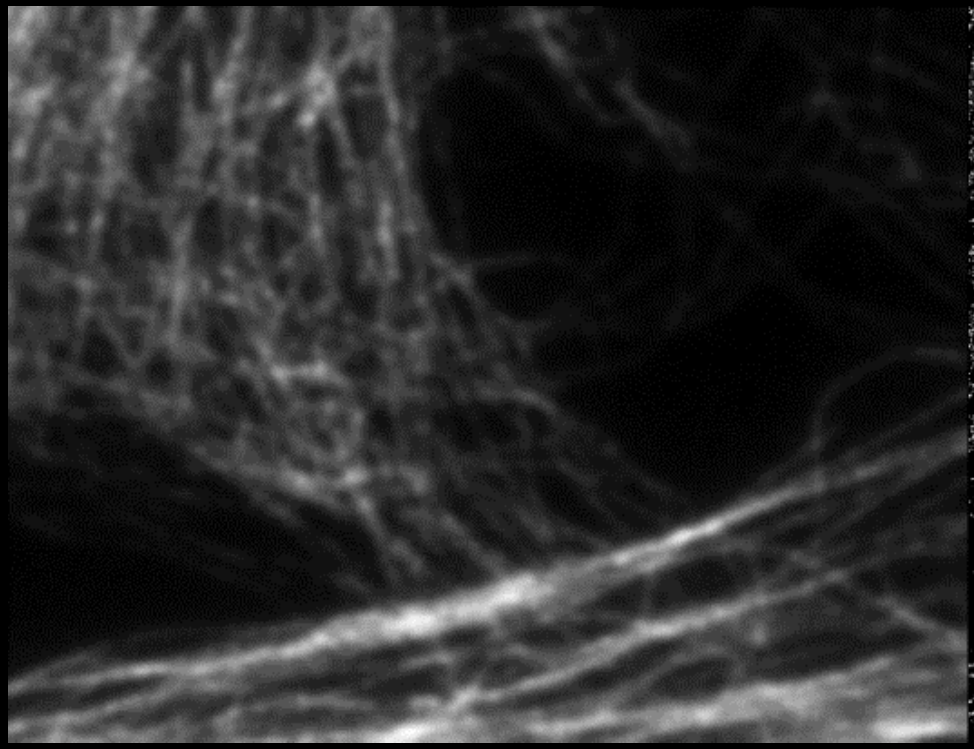
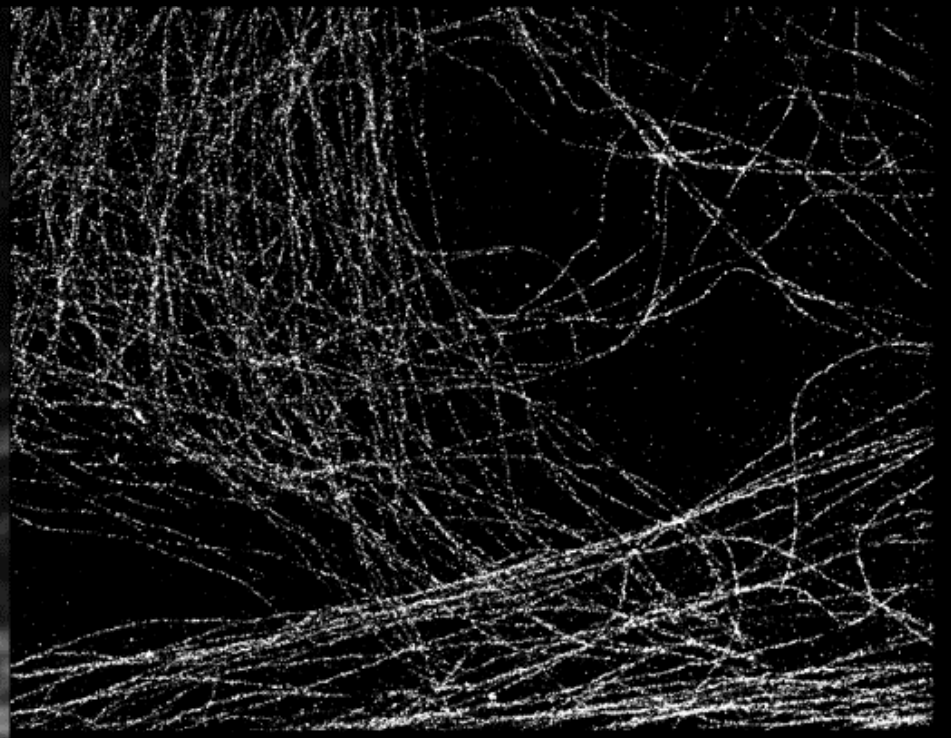
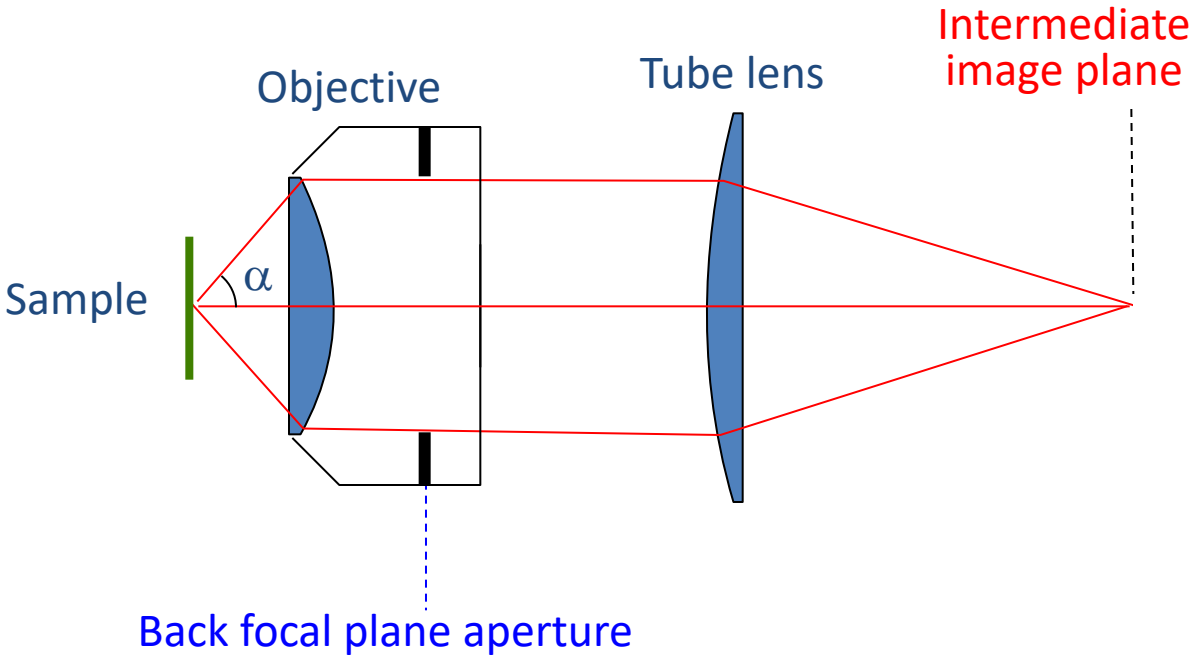
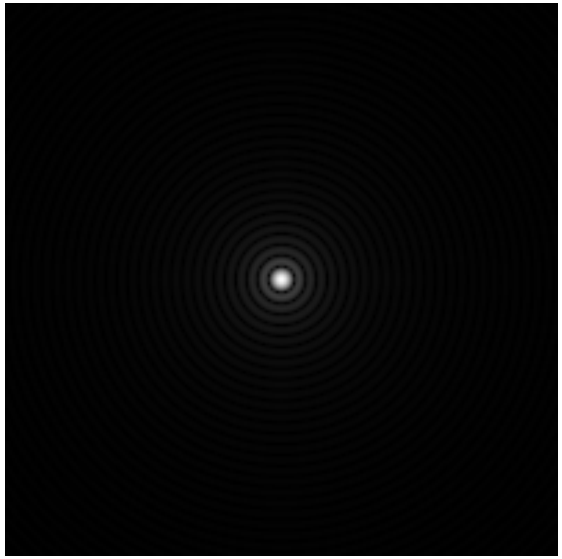




The Resolution Limit in Light Microscopy



Diffraction spot on image plane (resolution)



- Image resolution improves with Numerical Aperture (NA)

$NA = n \sin(\alpha)$

where:

α = light gathering angle
n = refractive index of sample

The Resolution Limit in Light Microscopy

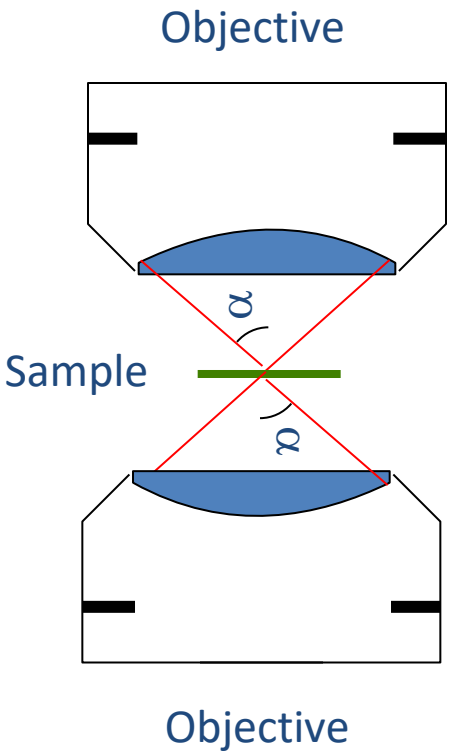
Improving The Resolution Limit in Light Microscopy

- $d = \lambda / 2n \sin \alpha$
- To improve resolution (smaller d):

- Use smaller λ – UV objectives

- Use larger n – Sapphire coverslips

- Use larger α – 4pi / I5M



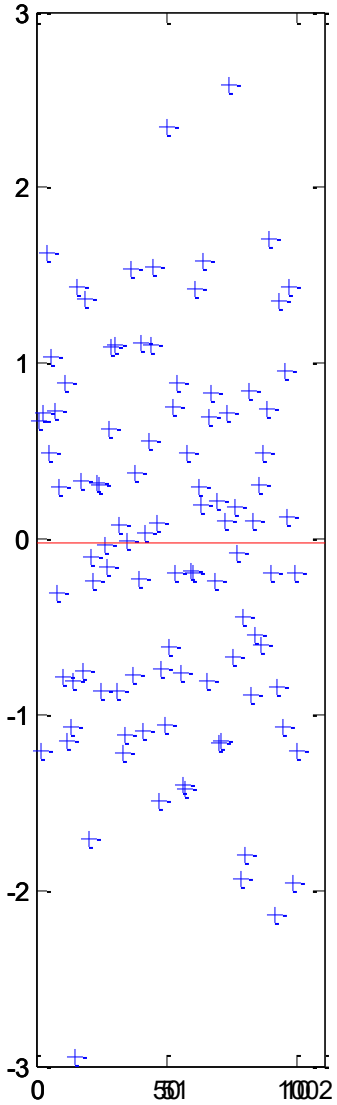
Improving The Resolution Limit in Light Microscopy

- $d = \lambda / 2n \sin \alpha$
- What does this assume?
  - Uniform illumination
  - Continuous sample
- What if we break it?
  - Structured illumination
  - Localize single molecules (PALM/STORM/etc.)

How does imaging single molecules help us?

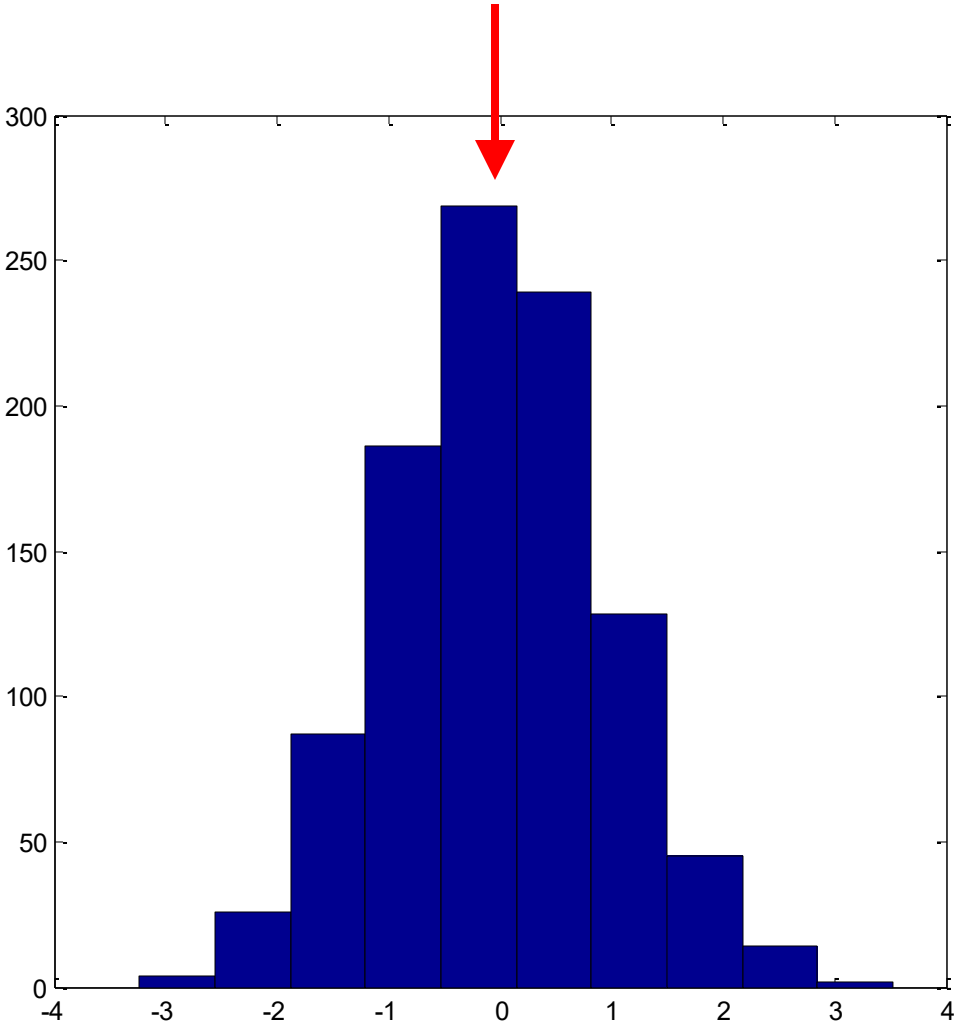
- Imagine taking a measurement
- Multiple times
- Adding measurements improves our ability to estimate the mean

$$SEM = \frac{\sigma}{\sqrt{N}}$$



How does imaging single molecules help us?

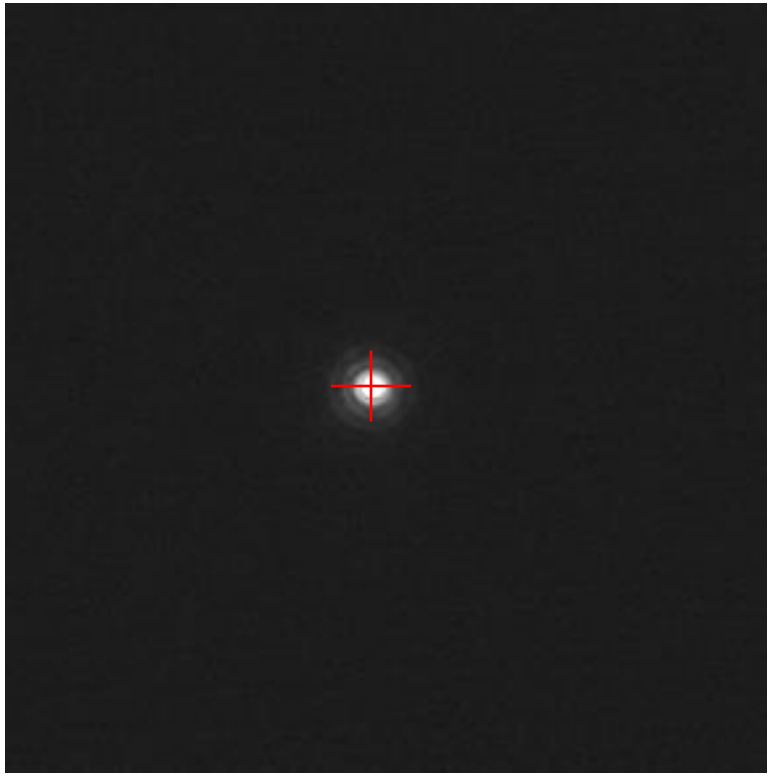
- Can measure the center of a distribution higher accuracy than any single measurement



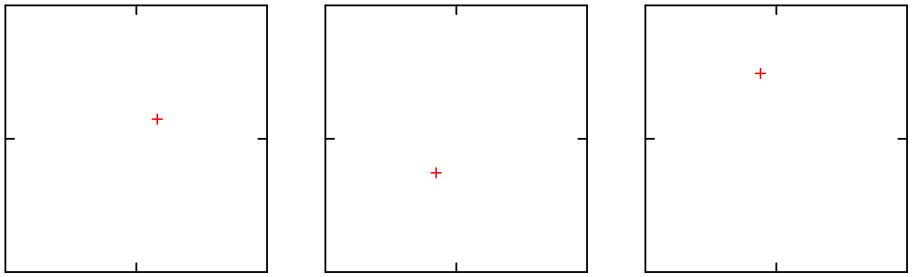
How does imaging single molecules help us?

- Can measure the center of a distribution higher accuracy than it's width.

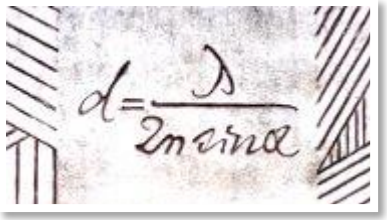
- Can determine the center of a single molecule to much higher accuracy than it's width.

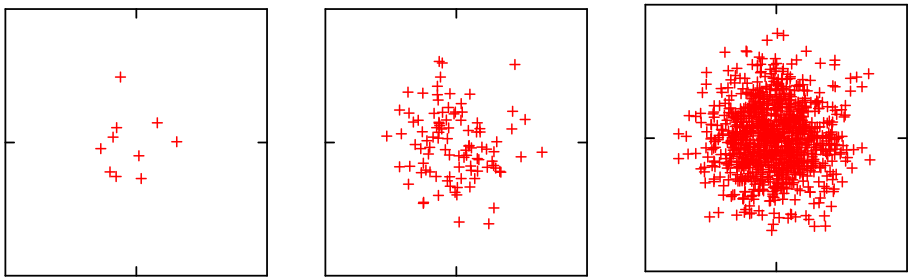


Single-molecule localization precision

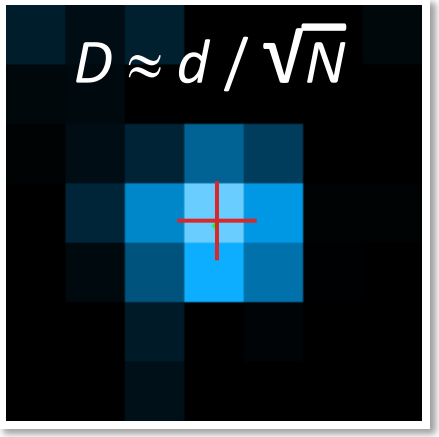


1 photon





10 photons 100 photons 1000 photons



Single molecules can be precisely localized

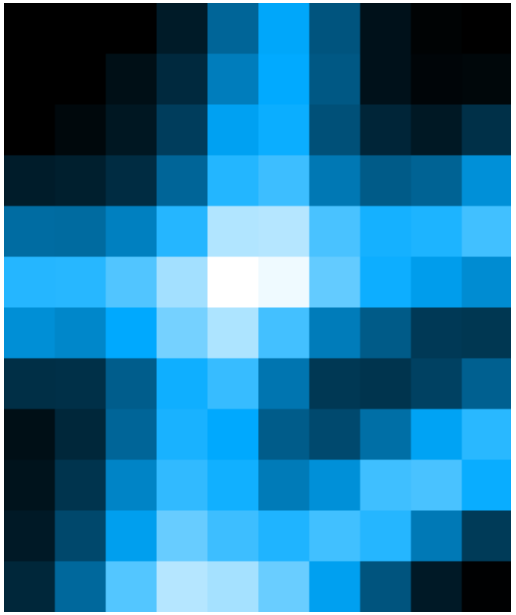
How can we apply this to imaging?

- Problem: in our image, we can't see single molecules – we see lots and lots of molecules and we can resolve them from each other.

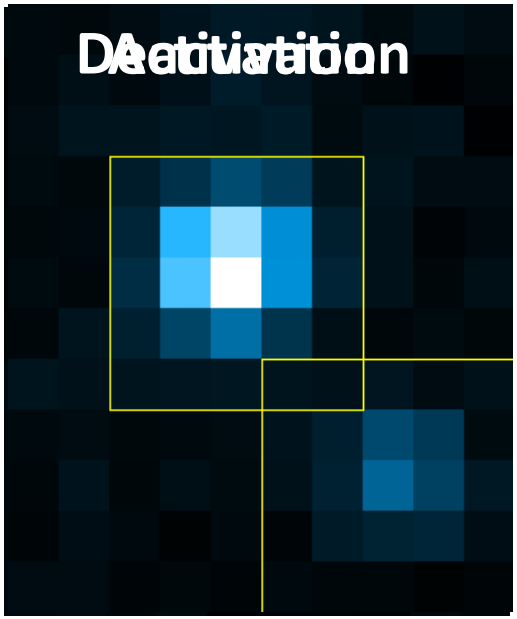
- What if we could turn off all the molecules, and then turn them on a few at a time?

Super-resolution by localization

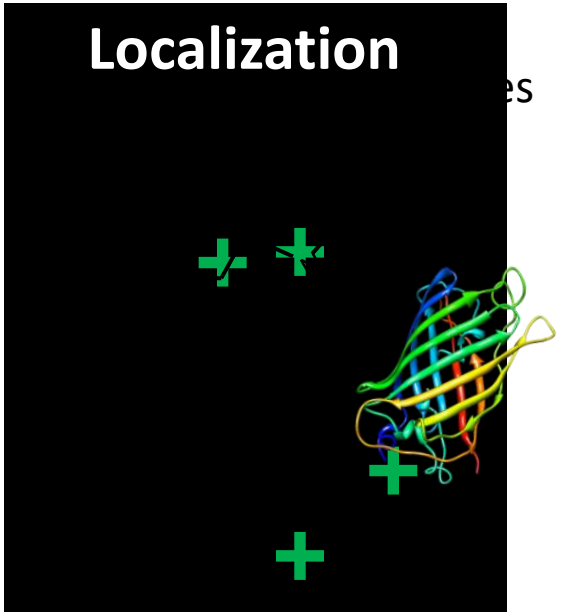
Fluorescence image



Raw images



STORM Image

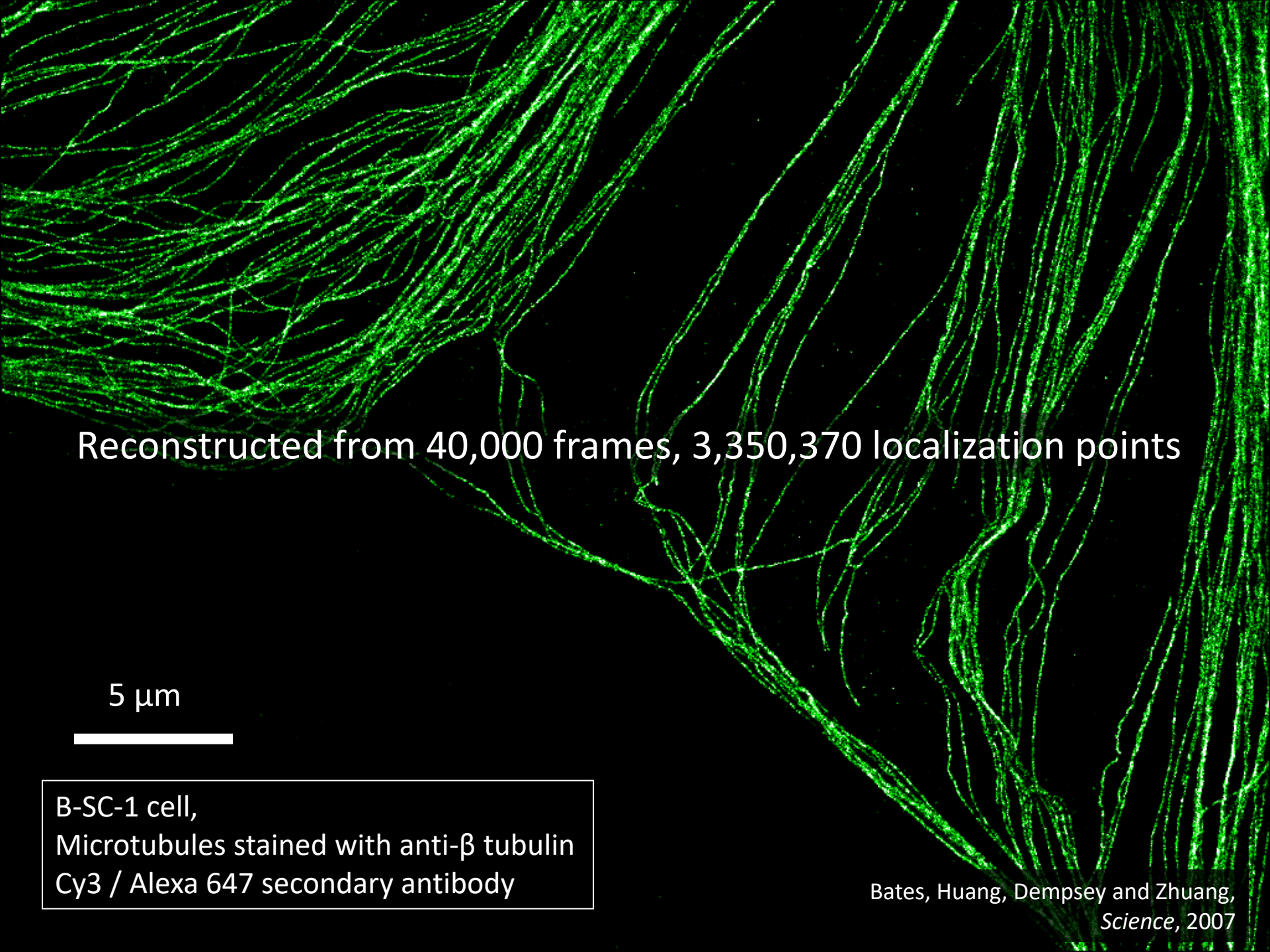


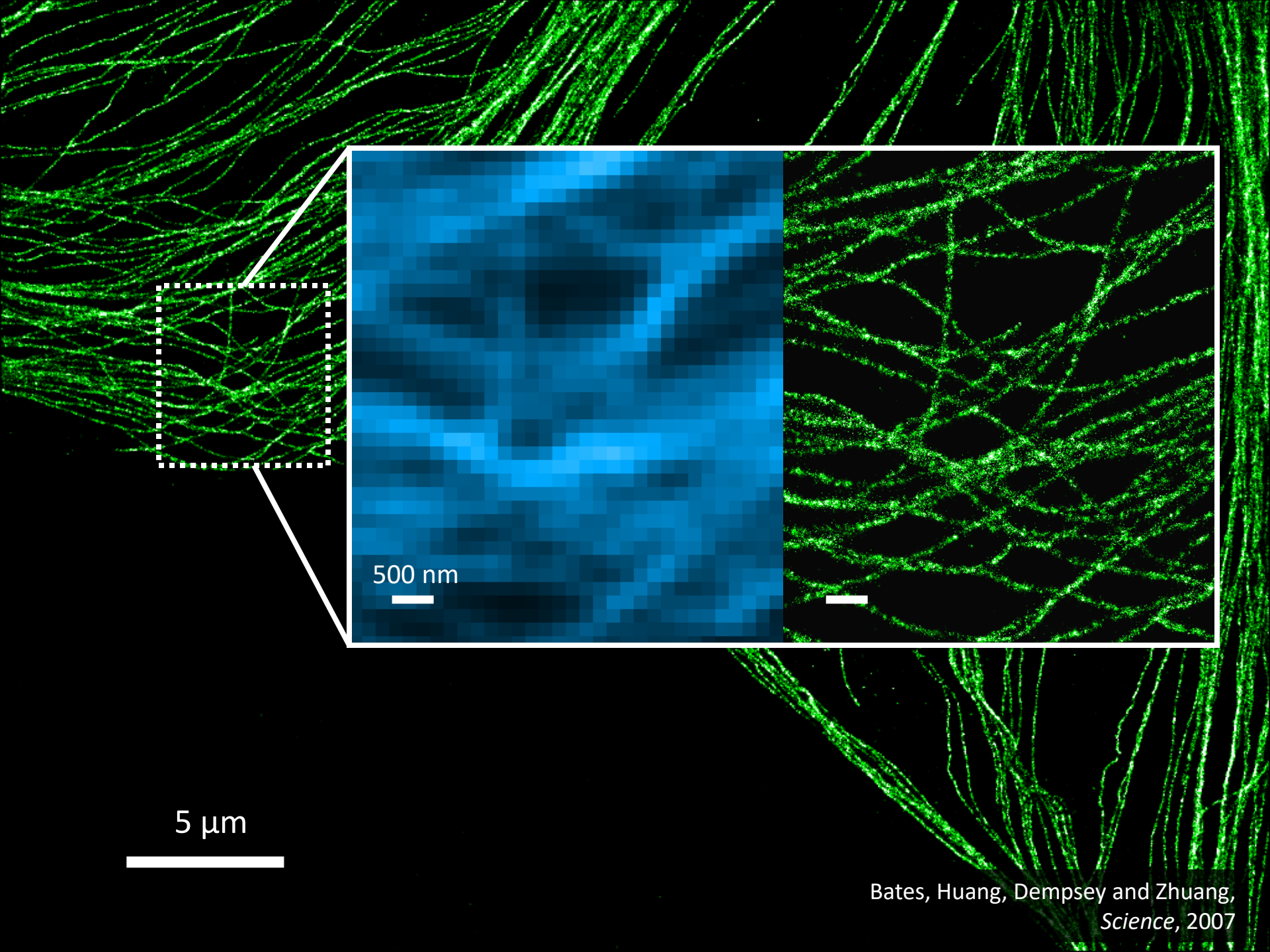
2x real time

Stochastic Optical Reconstruction Microscopy = STORM

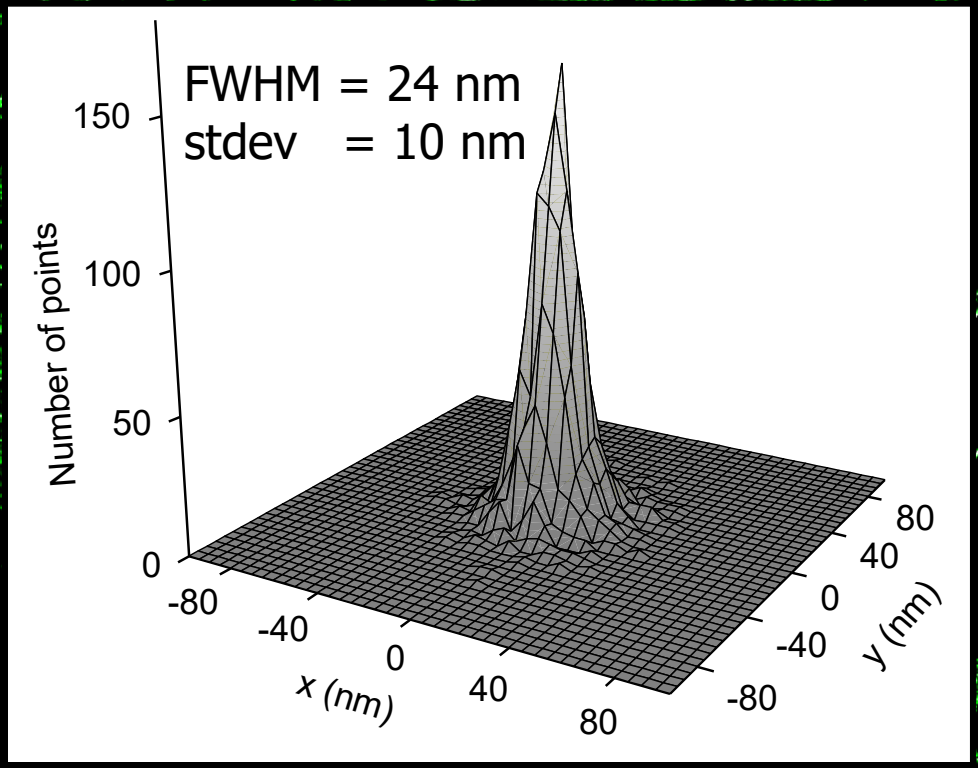
Also named as PALM (Betzig et al., Science, 2006) and FPALM (Hess et al., Biophys. J. 2006)

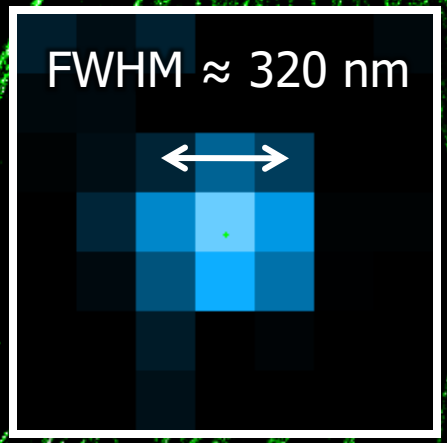
Rust, Bates & Zhuang, Nat. Methods, 2006
Bates, Huang, Dempsey & Zhuang, Science, 2007


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

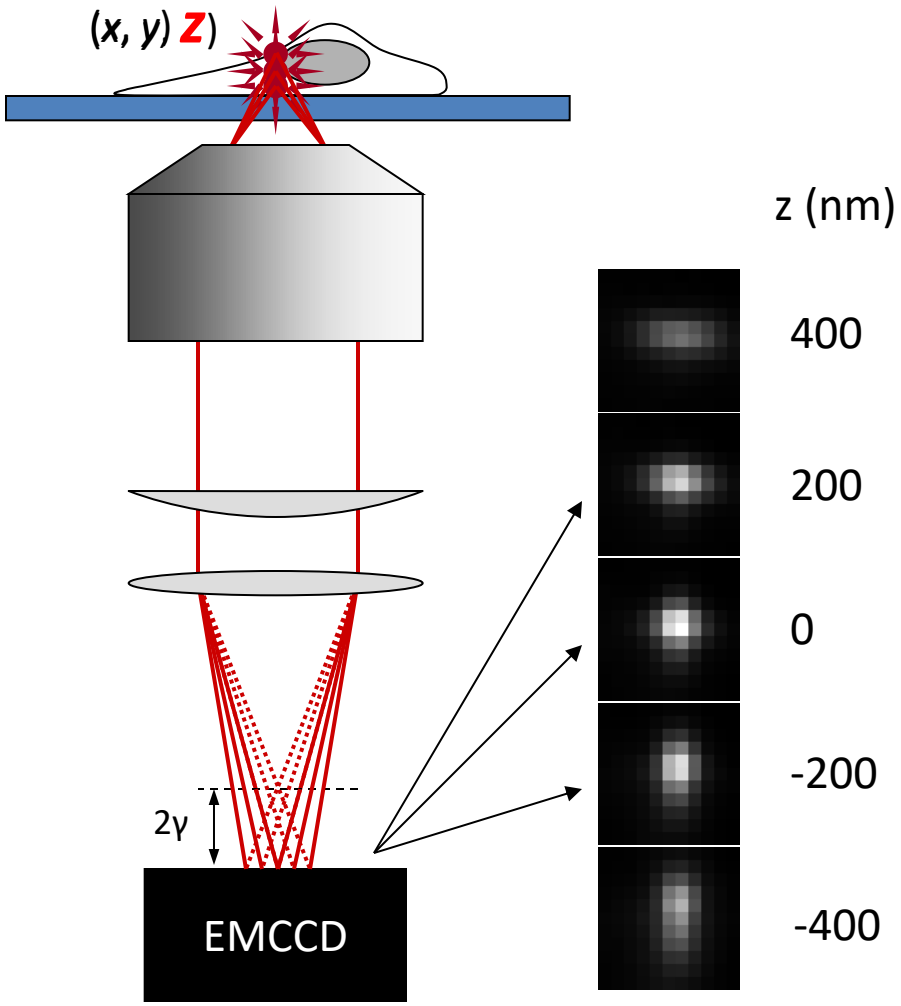




5 µm

Bates, Huang, Dempsey and Zhuang,
Science, 2007

3D Imaging: Localization in the Third Dimension



z (nm)

400

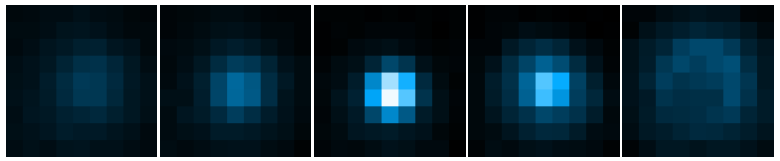
200

0

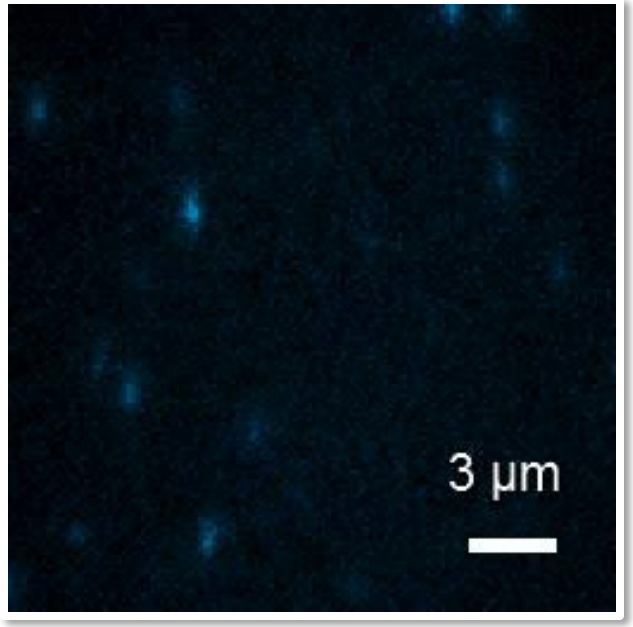
-200

-400

In focus

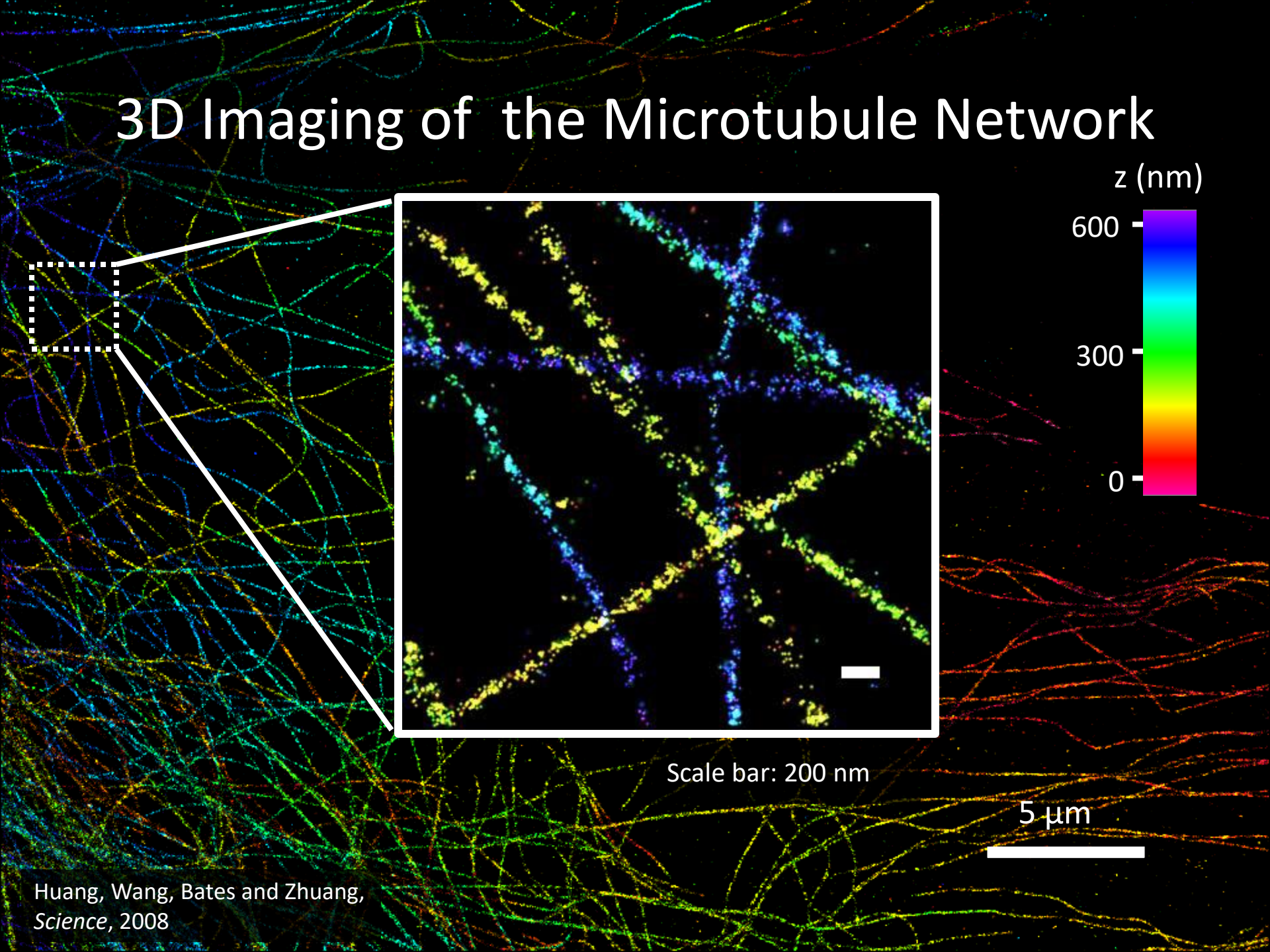


Scan z = -600 to 600 nm



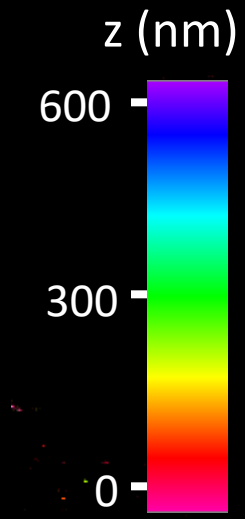
Huang, et al., *Science*, 2008

3D Imaging of the Microtubule Network

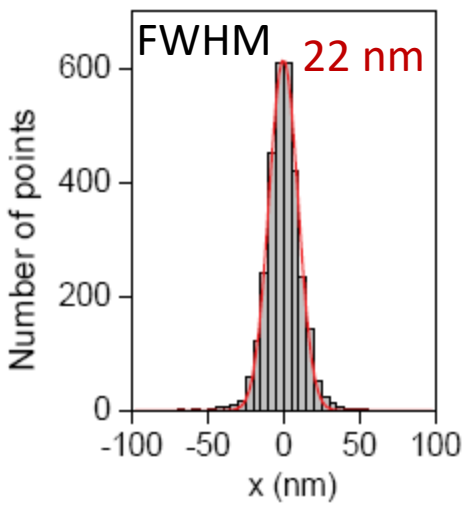


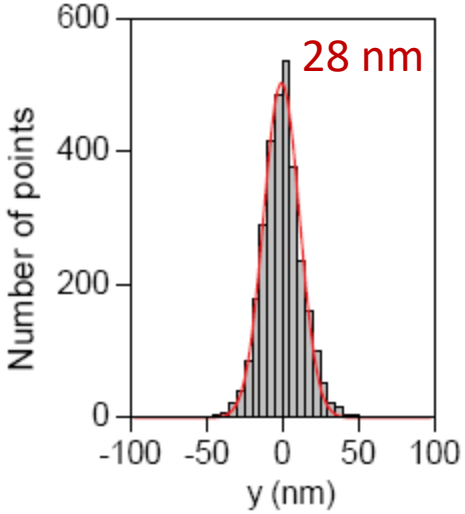
Huang, Wang, Bates and Zhuang,
Science, 2008

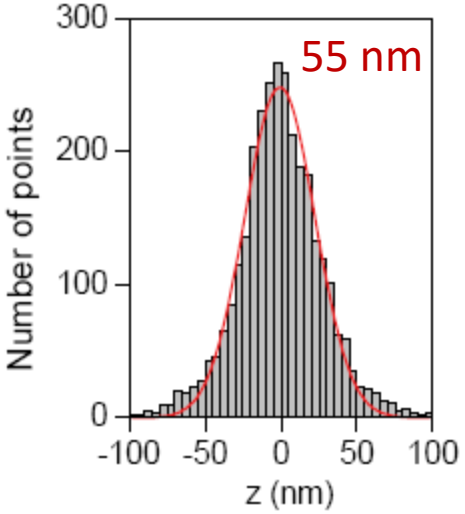
3D Imaging of the Microtubule Network



Small, isolated clusters



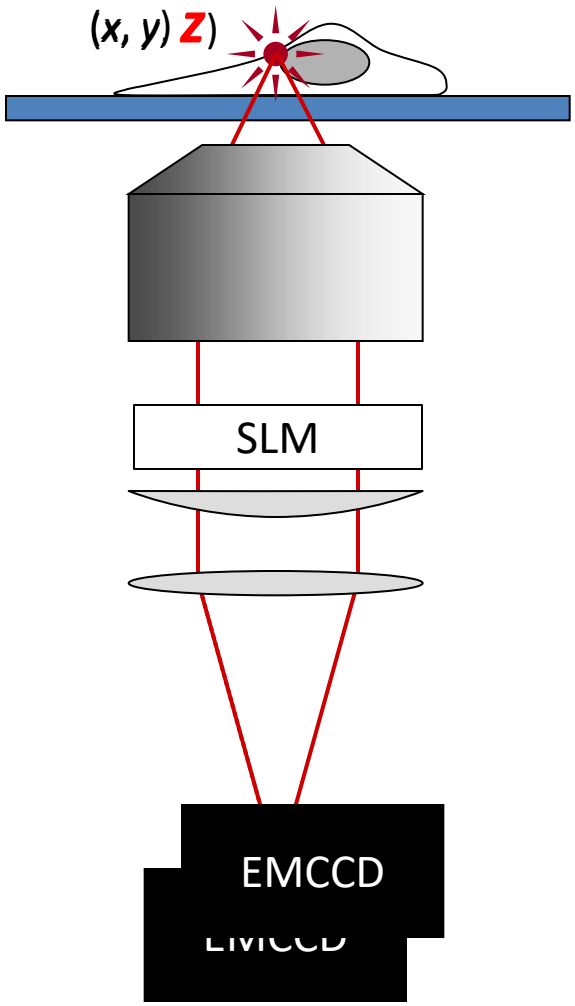




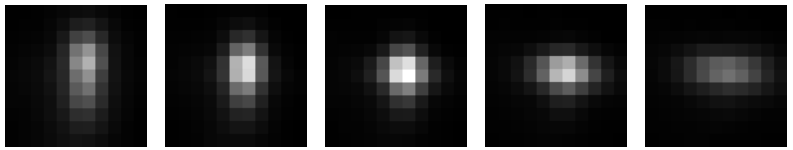
5 µm

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

Other 3D localization method



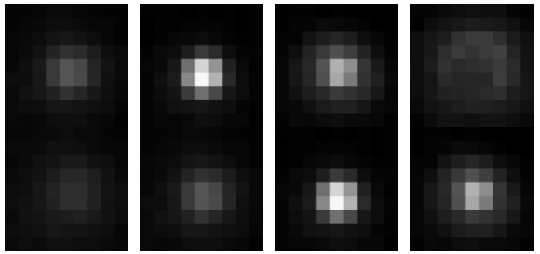
Astigmatic imaging



z (nm) -400 -200 0 200 400

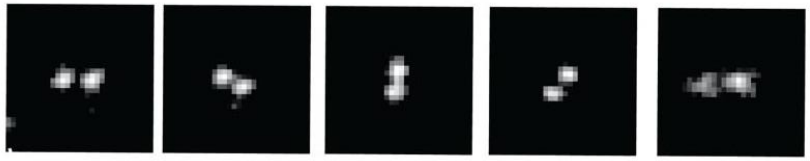
Huang et al., Science 2008

Bi-plane imaging



Juette et al., Nat Methods 2008

Double-helical PSF



z (nm) -900 -500 0 600 1400

Pavani et al., PNAS 2009

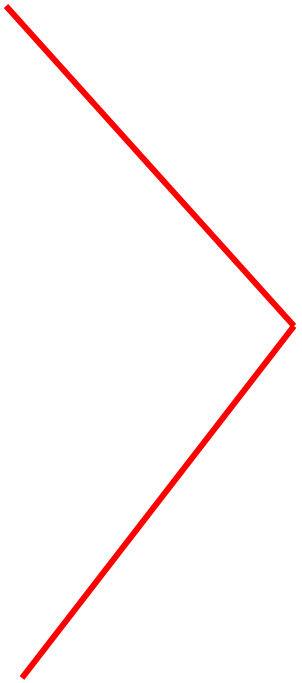
Super-resolution by localization

- Combines two ideas:
  - Localization Microscopy
  - Single Molecule Switching

Single-Molecule Switching Approaches

- Lots of acronyms:

PALM
FPALM
STORM
dSTORM
GSDIM
PALMIRA
SMACM
PAINT
SPRAIPAINT



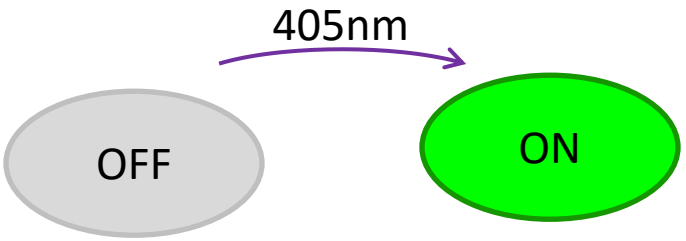
All use single-molecule localization

Main differences are how you switch the molecules on and off

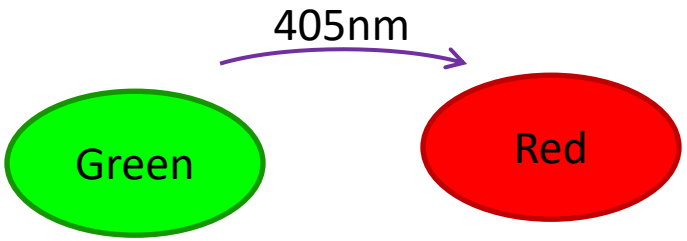
Single Molecule Switching using Fluorescent Proteins

PALM, FPALM

- Photoactivatible molecules: PA-GFP, PA-mCherry, PA-TagRFP



- Photoconvertible molecules: mEos2/3, PS-CFP2, PSmOrange



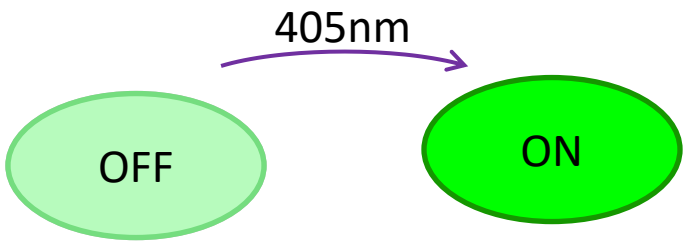
Parameters for evaluating dyes

- Number of photons emitted before bleaching / blinking

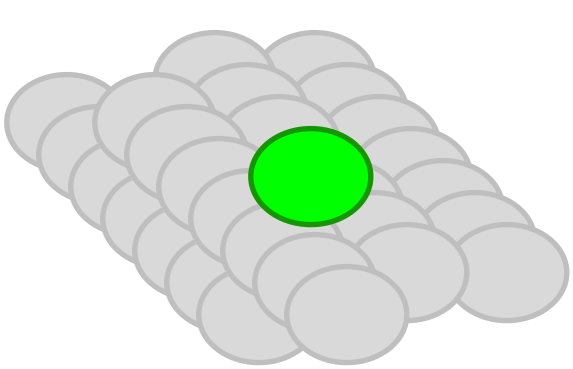
$$SEM = \frac{\sigma}{\sqrt{N}} \quad \Rightarrow \quad D = \frac{D_{diffraction}}{\sqrt{N_{photons}}}$$

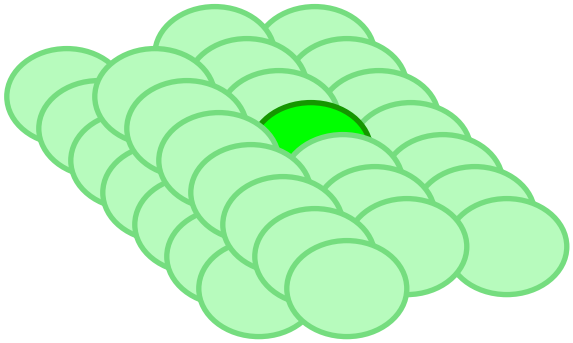
- Contrast ratio – brightness of ON state relative to OFF state

Why Contrast Matters



- What if your OFF state isn't completely off?





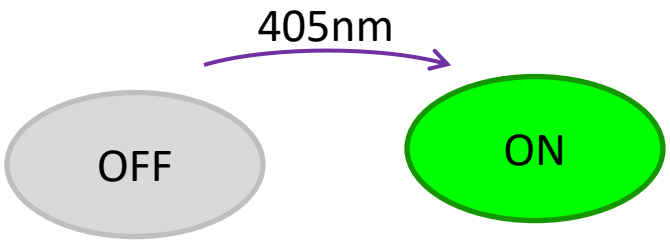
- Can no longer distinguish single molecules above background

Fluorescent Proteins for Single Molecule Switching

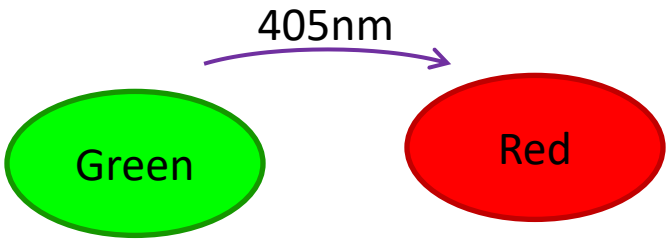
Protein	λex	λem	Contrast Ratio	Nphotons (median)	Notes
PS-CFP2	490	511	2000	260	Cyan-to-green
mGeos-M	503	514		387	Reversibly photoactivatible
PATagRFP	562	596	540	500?	Photoactivatible
PAmCherry	564	595	4000	348	Photoactivatible
tdEos	569	581		499	Green-to-red
mEos2	573	584		379	Green-to-red
mEos3.2	572	580		482	Green-to-red
PSmOrange	631	662	10700	337	Orange-to-far red

Full table at
http://nic.ucsf.edu/dokuwiki/doku.php?id=storm:fps

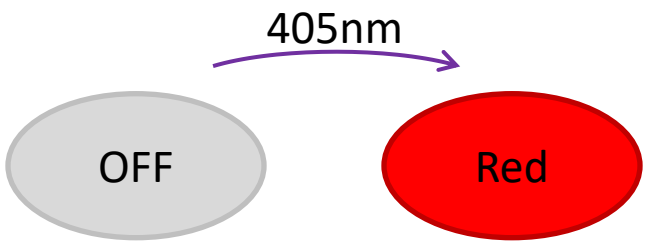
Multi-color and FPs



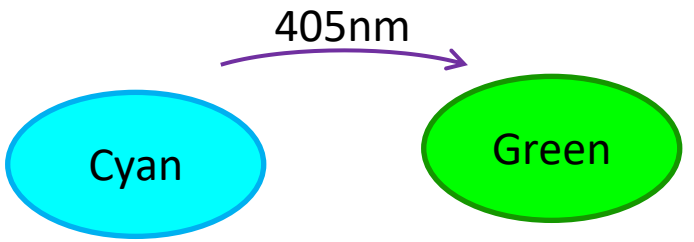
e.g. PA-GFP, mGeos-M



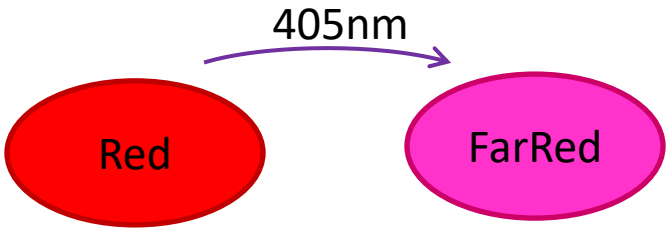
e.g. mEos2/3



e.g. PA-TagRFP, PA-mCherry



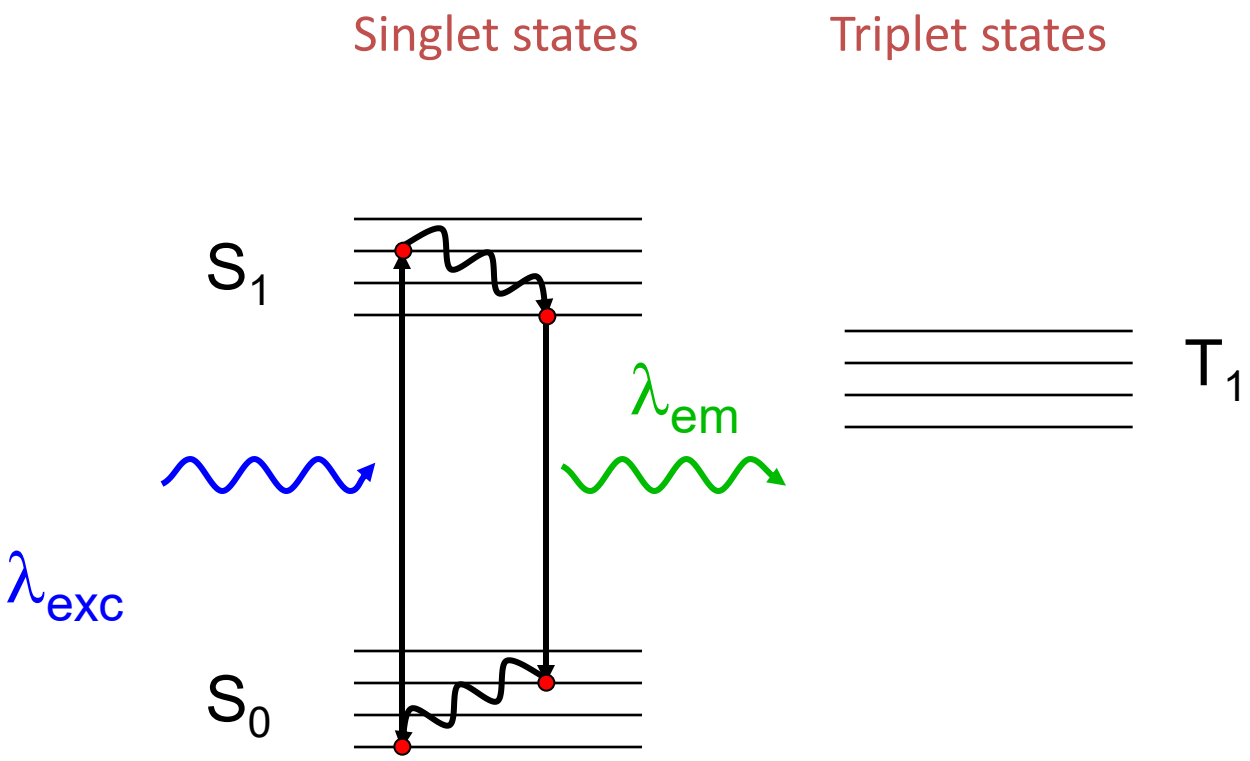
e.g. PS-CFP



e.g. PSmOrange

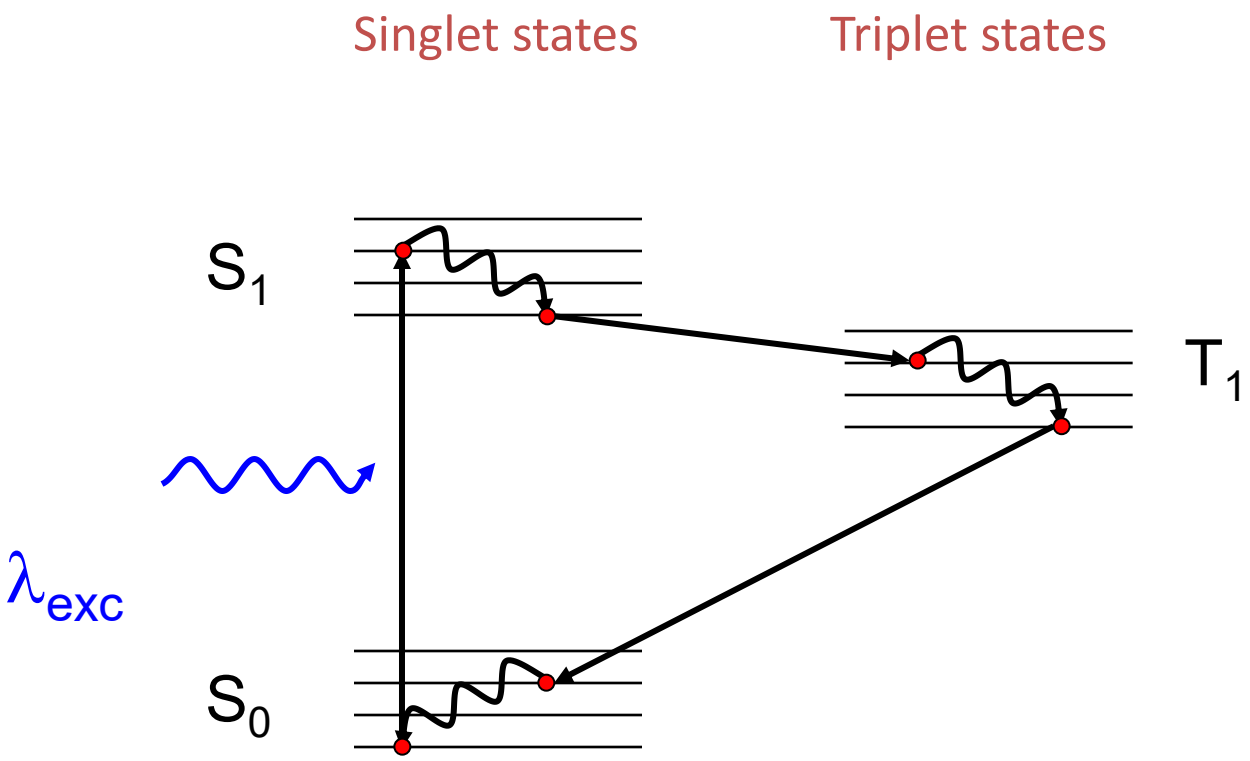
Single Molecule Switching Using Small Molecule Dyes

STORM, dSTORM, GSDIM



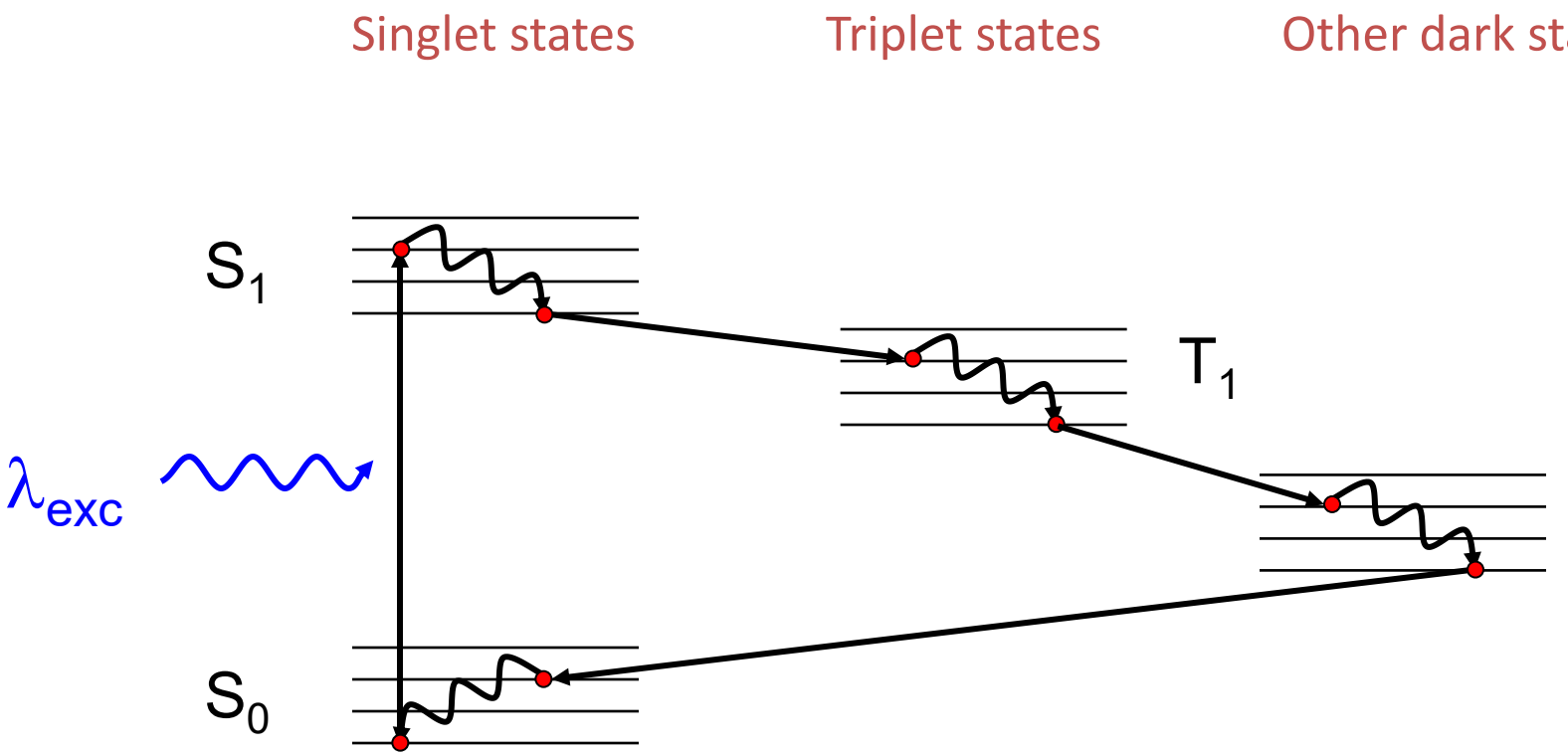
Single Molecule Switching Using Small Molecule Dyes

STORM, dSTORM, GSDIM



Single Molecule Switching Using Small Molecule Dyes

STORM, dSTORM, GSDIM



Dark states can be long-lived

Single Molecule Switching Using Small Molecule Dyes

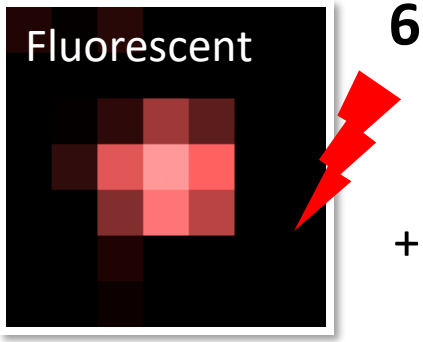
STORM, dSTORM, GSDIM

- The idea:

- Drive the majority of molecules into a dark state
  - Use high laser power
  - Special buffers

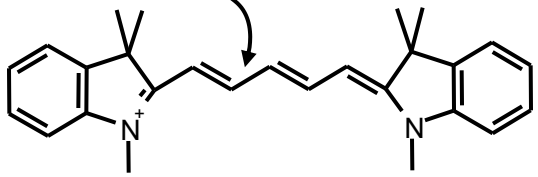
- Wait for molecules to spontaneously return to ground state
  - Image them until the bleach or return to dark state

Photoswitching of red cyanine dyes



650 nm

+ thiol



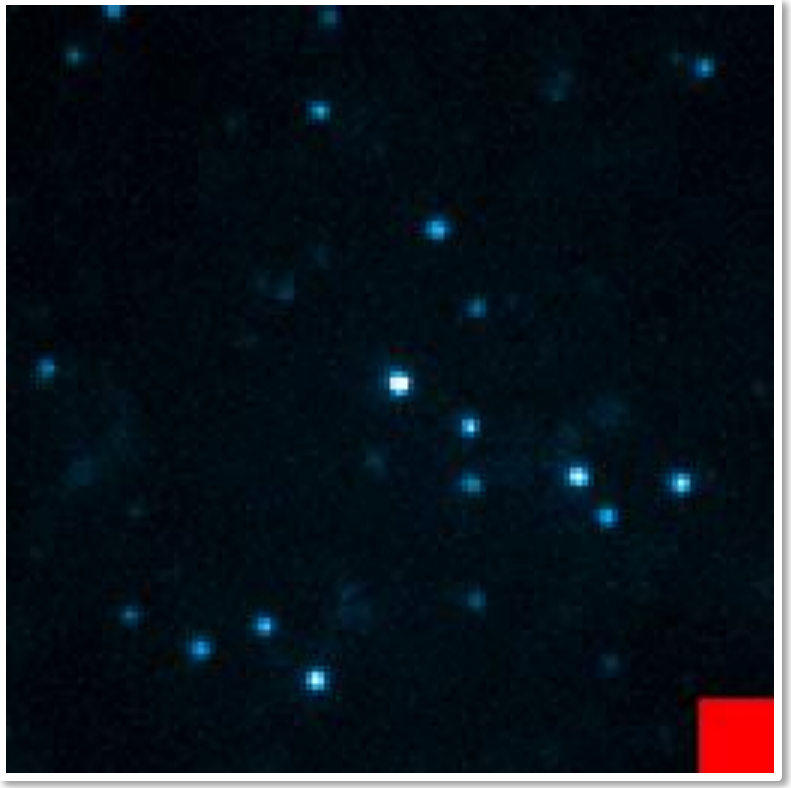
Cy5 / Alexa 647

photoactivation

Deactivation

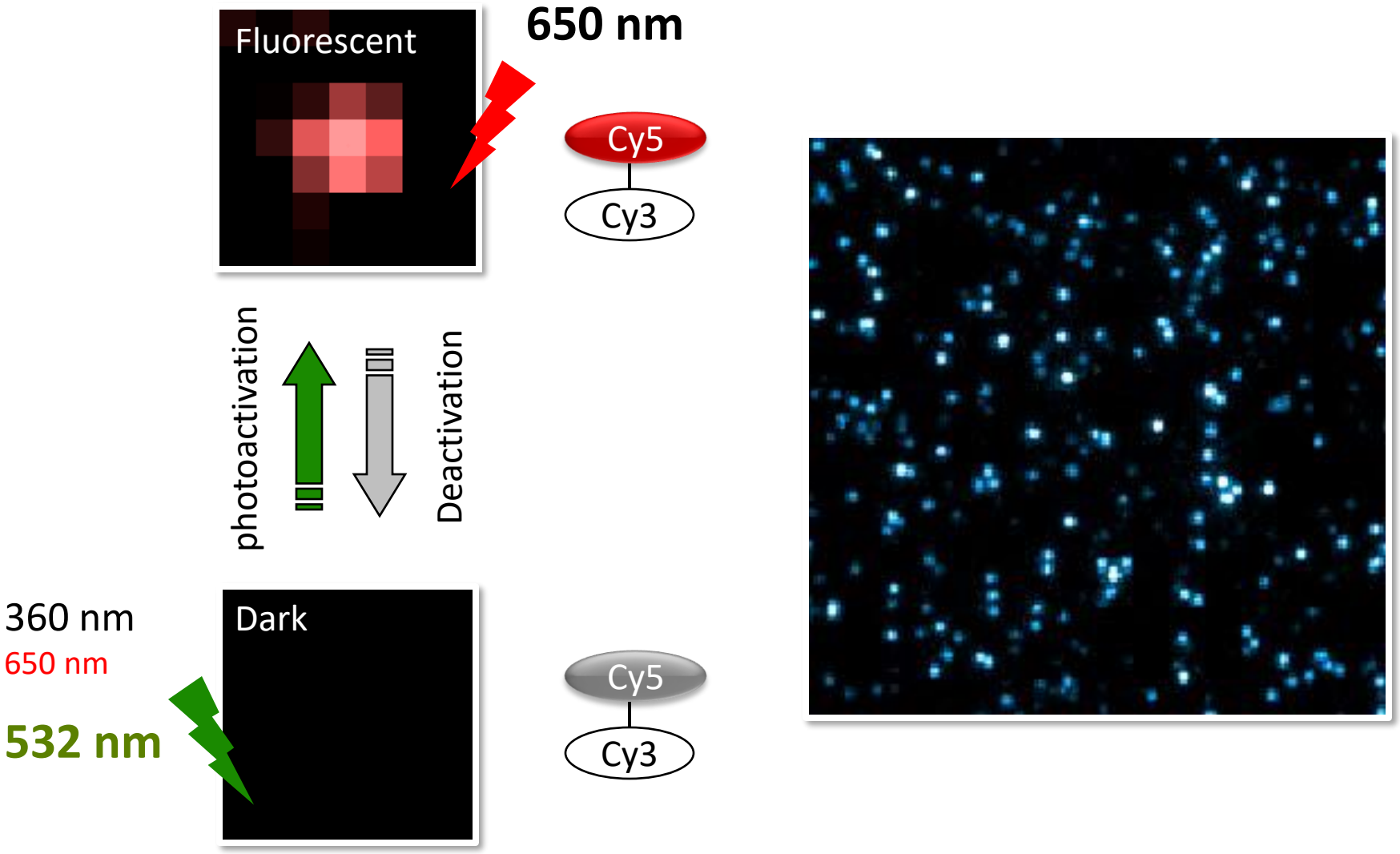
360 nm

650 nm

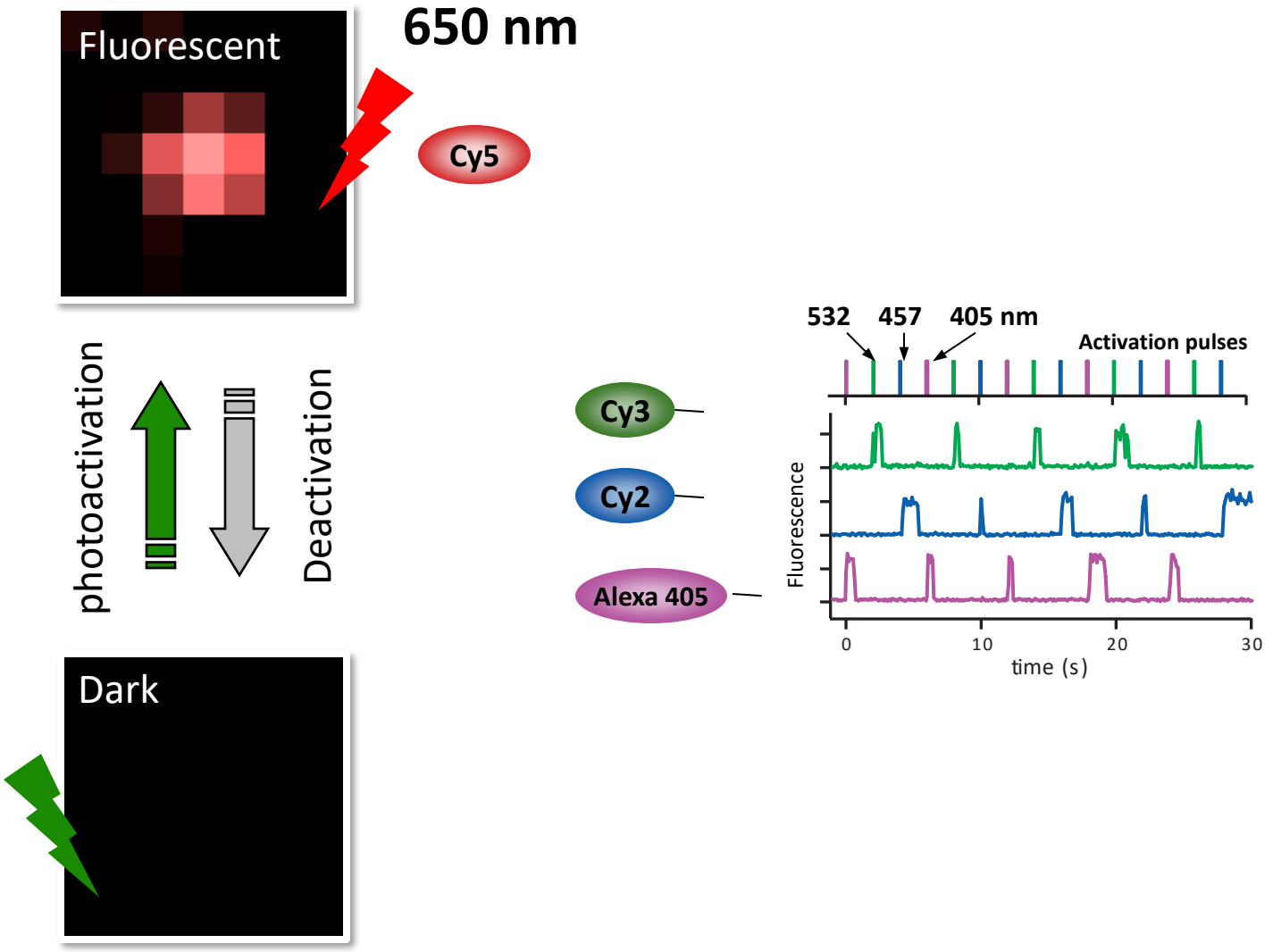


Bates eta l., *PRL* 2005, Bates et al., *Science* 2007, Dempsey et al., *JACS* 2009

Multicolor Single Molecule Switching: Activation



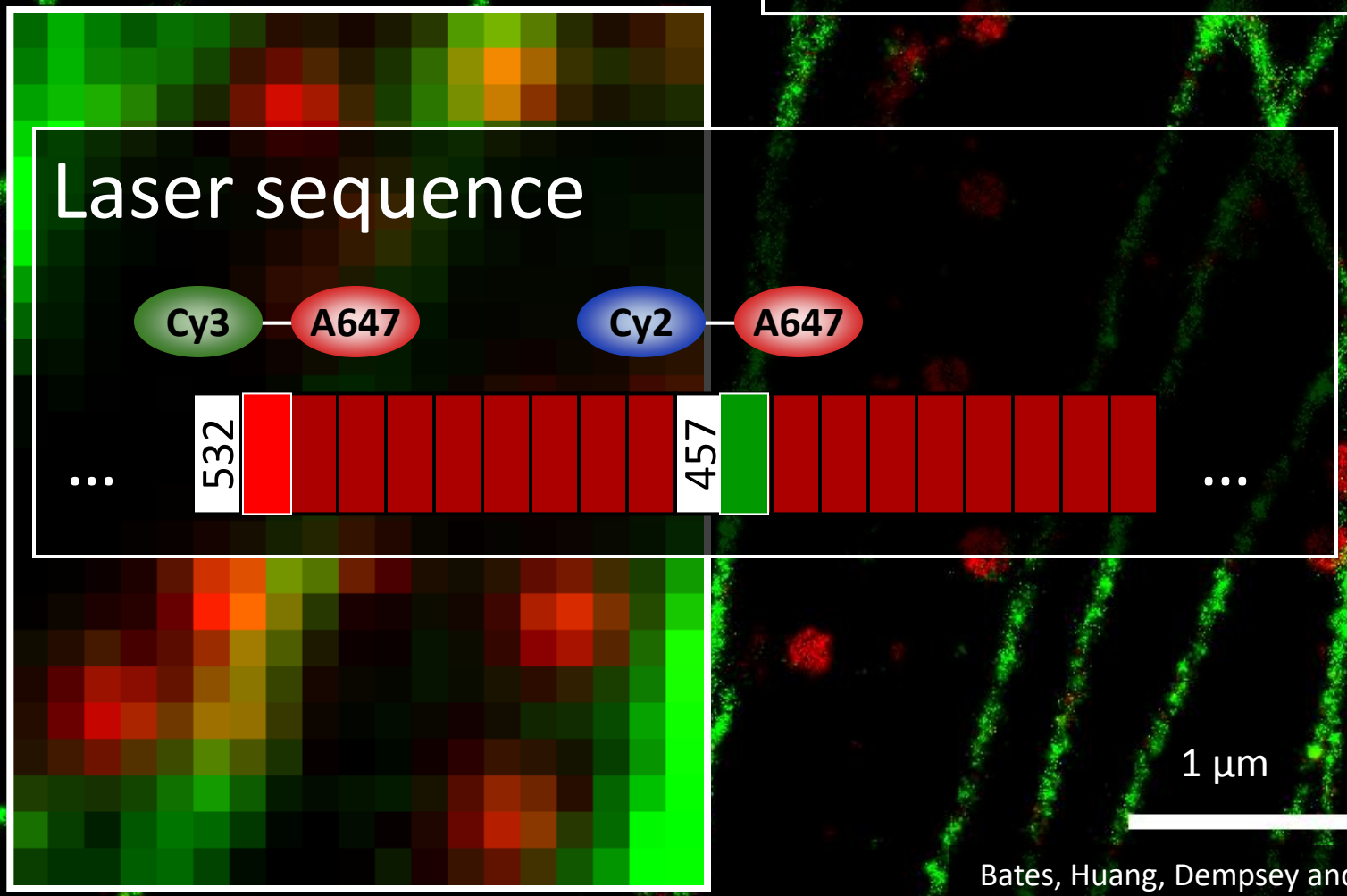
Controlling the activation of Cy5



Cy3 / Alexa 647: Clathrin

Cy2 / Alexa 647: Microtubule

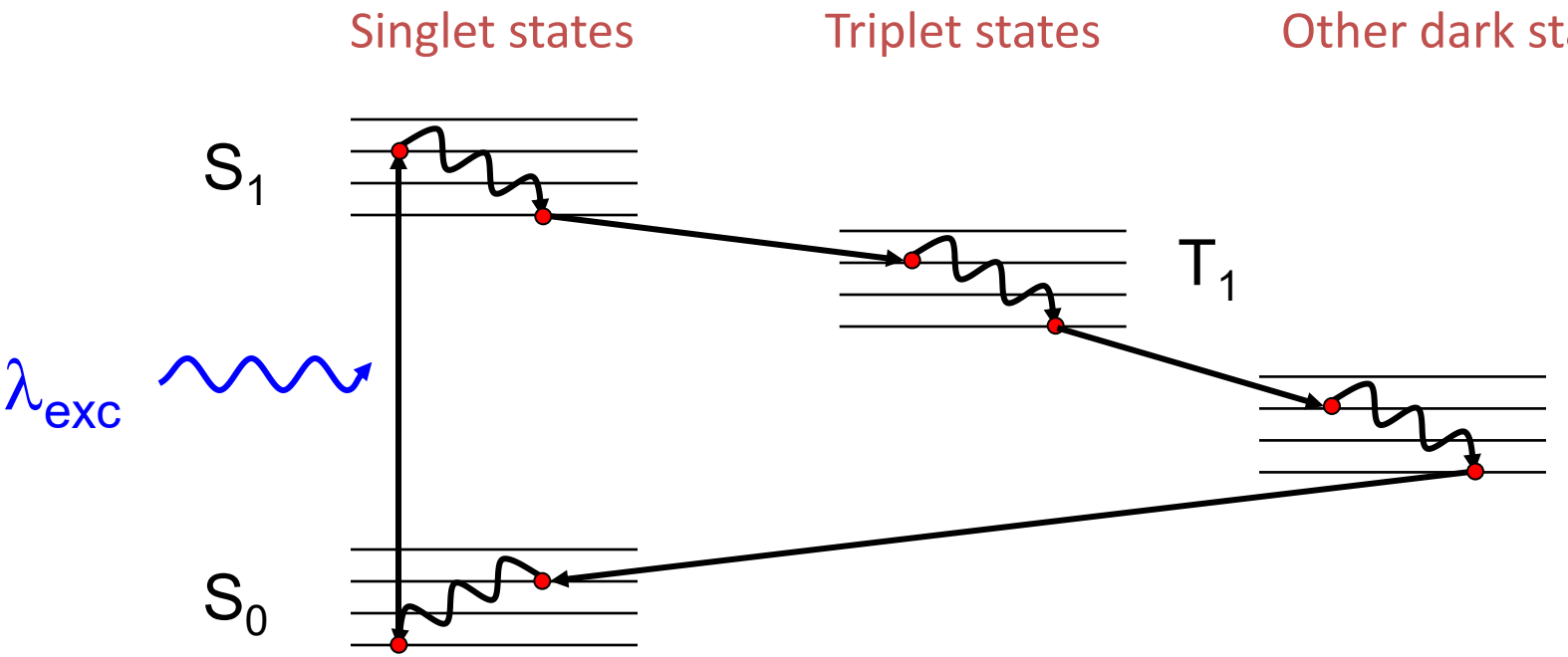
Crosstalk subtracted



1 µm

Bates, Huang, Dempsey and Zhuang,
Science, 2007

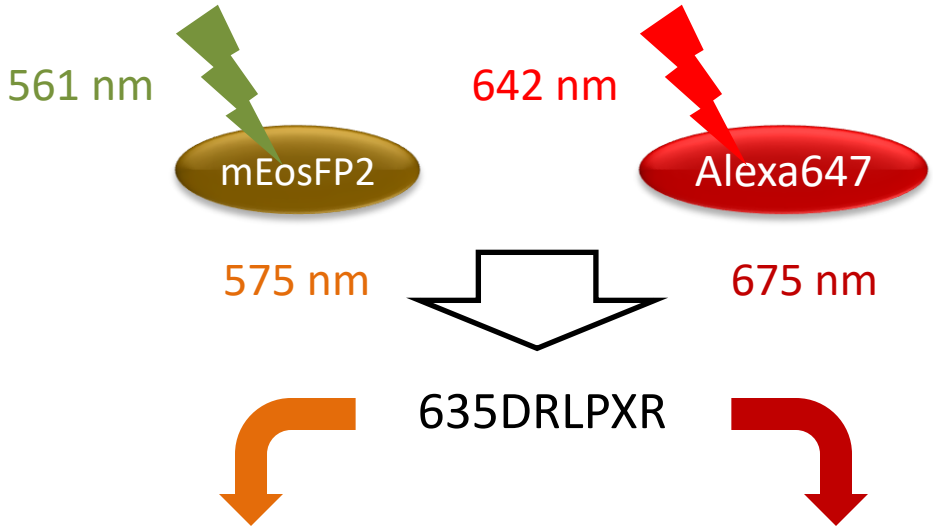
Multicolor Single Molecule Switching: Emission

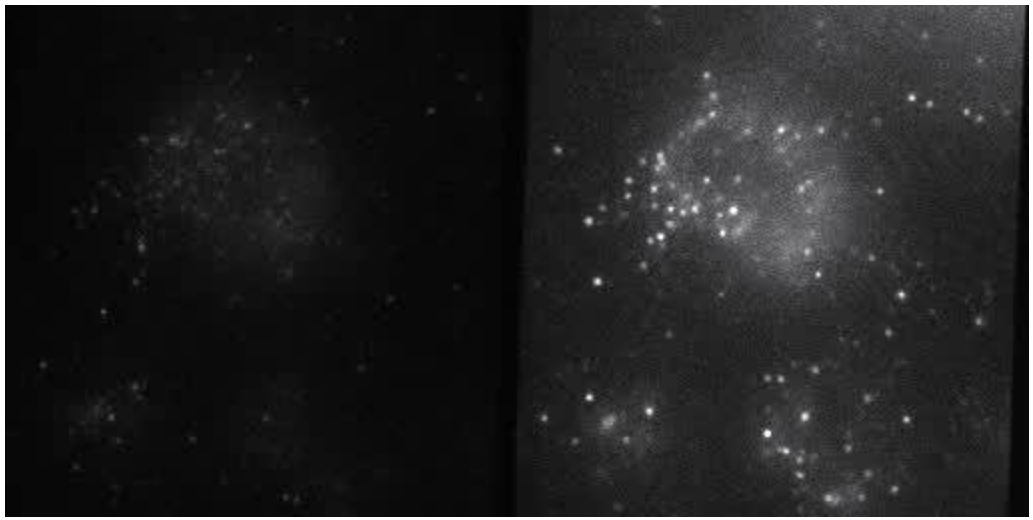


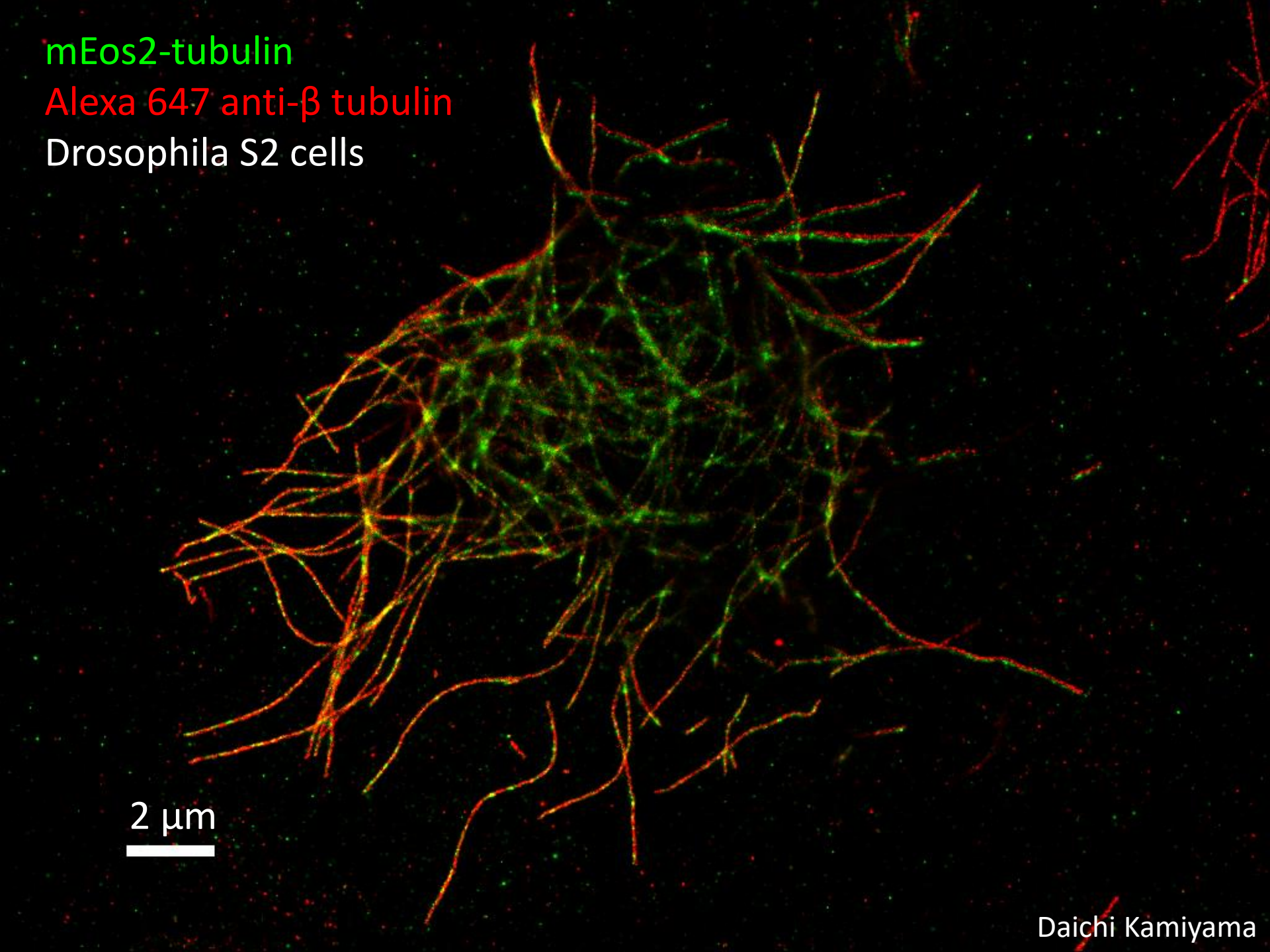
- If you use high enough laser power, many molecules can be driven to the dark state
- Cy3B, Atto488, …

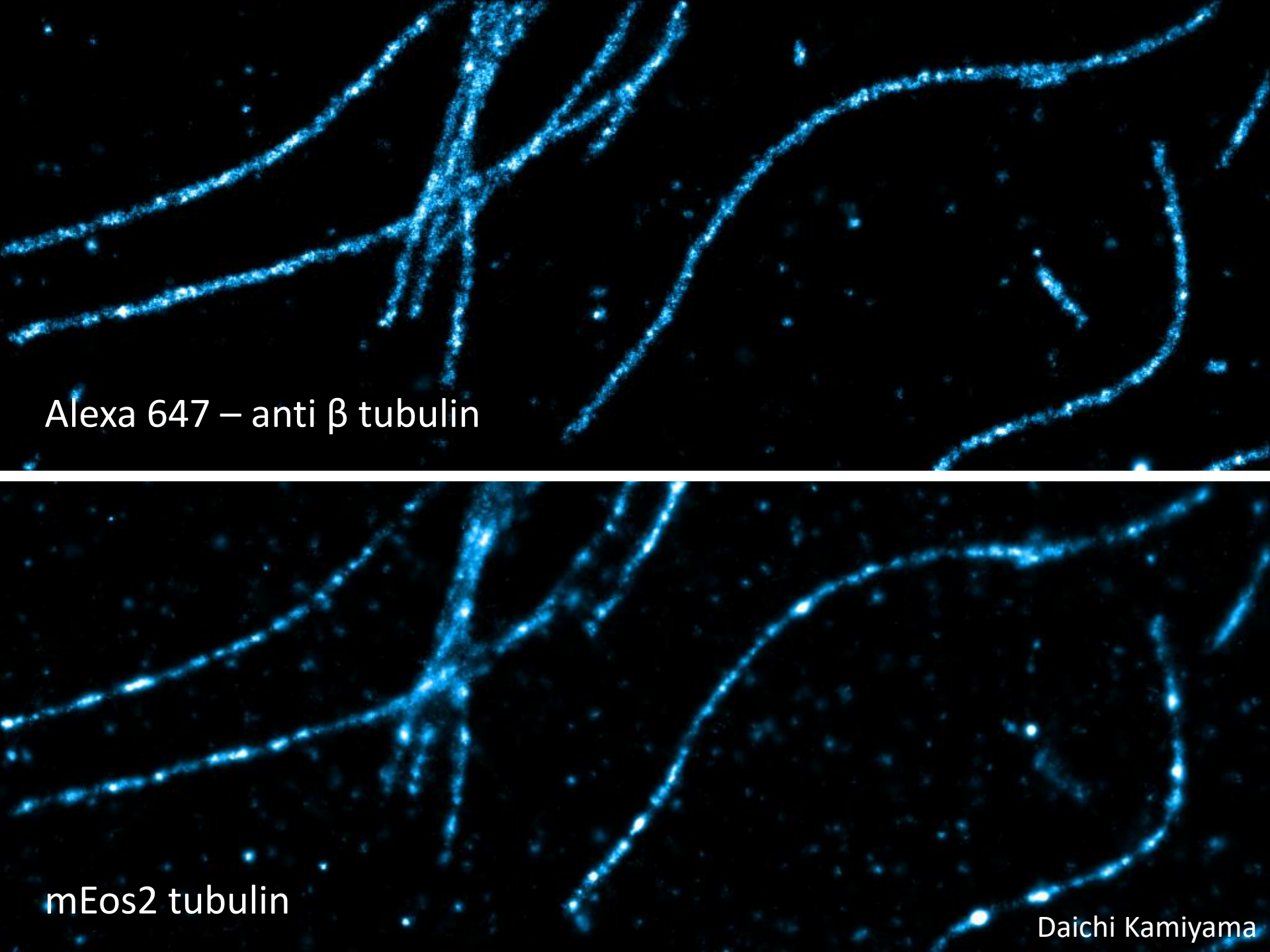
See Dempsey et al. Nat. Meth. 2011, p. 1027

Multicolor STORM/PALM



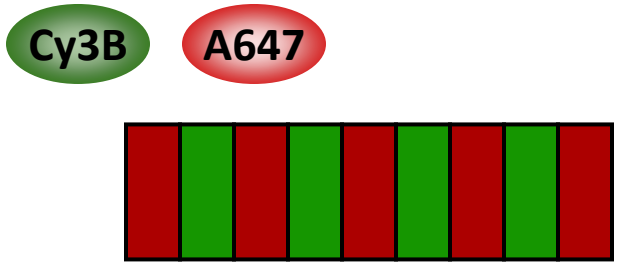



mEos2-tubulin
Alexa 647 anti-β tubulin
Drosophila S2 cells
2 µm
Daichi Kamiyama


Alexa 647 – anti β tubulin
mEos2 tubulin
Daichi Kamiyama

An alternative strategy: time slicing

- Use a multi-pass emission filter
- Alternate laser excitation so that every other frame is a different dye.



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

Summary: Labeling Strategies

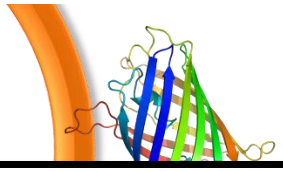
- Fluorescent proteins
  - mEos is a good starting point
  - Multicolor may be challenging
- Cy5 / A647 plus activator dyes
  - Need to label your own antibodies
  - Crosstalk a problem
- Dyes driven to dark state
  - Need high laser power
- Photoswitchable / photoactivatible dyes
  - Not many options yet (Aberrior)

Effective resolution: Probe matters

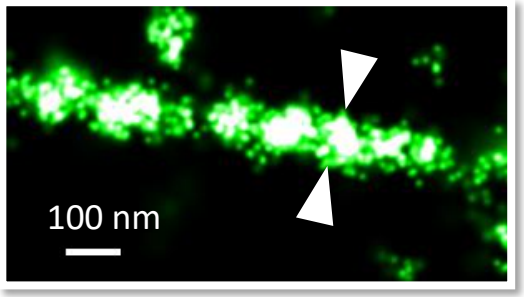
Antibodies:
~ 10 nm

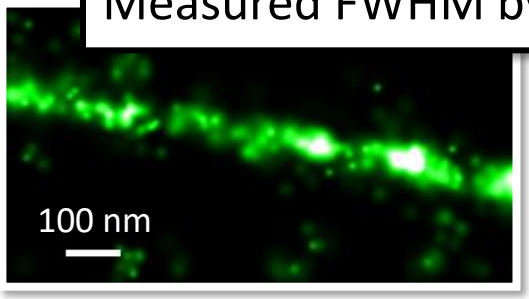
Fluorescent Proteins:
~ 3 nm

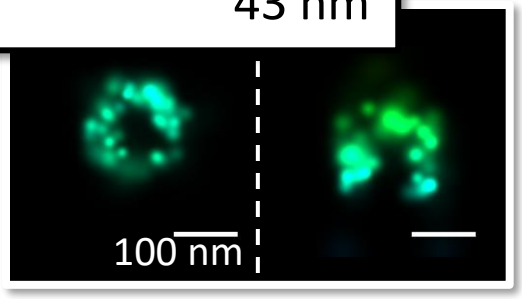
Small fluorophores:
~ 1 nm



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

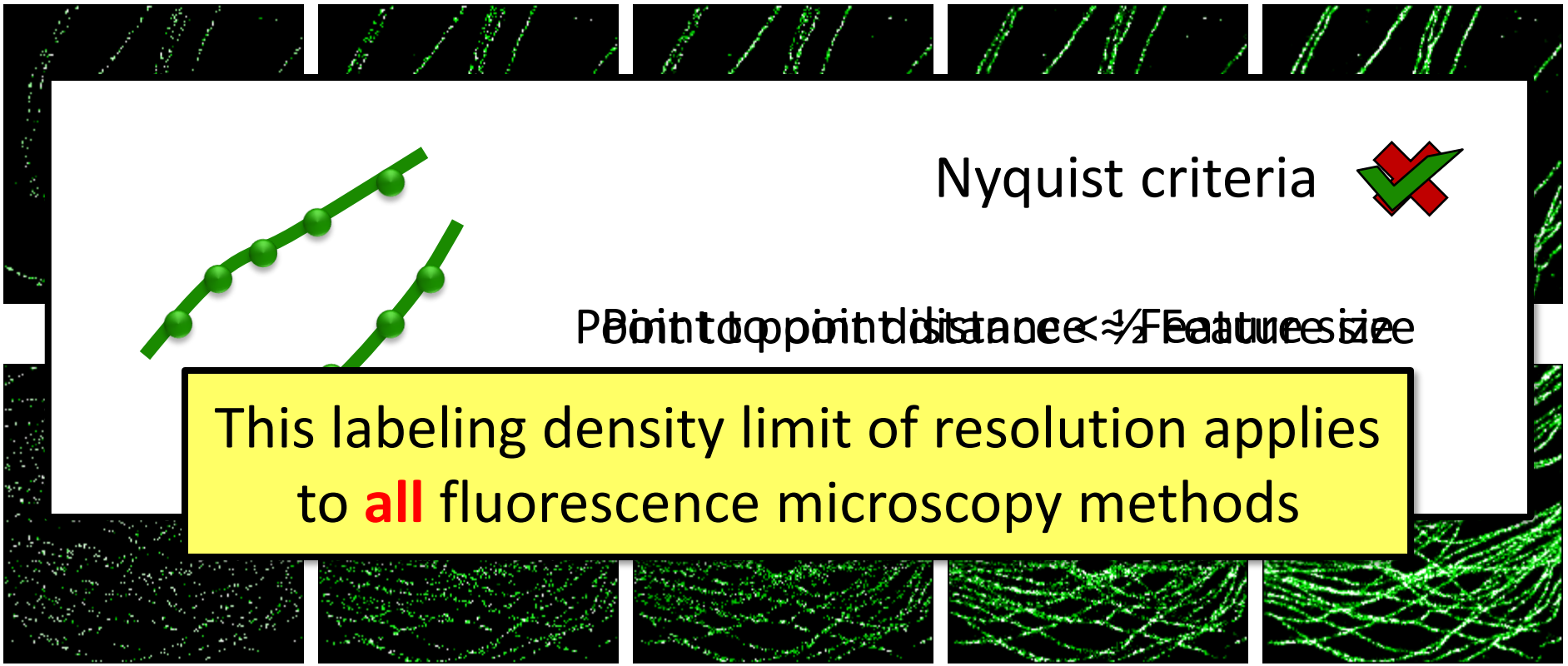
Antibody immunofluorescence

- Fixed sample
- Potential nonspecific labeling
- Lower labeling efficiency
- Labeling endogenous proteins
- High signal = high localization precision
- More versatile for multicolor imaging

Effective resolution: Density matters

Frames for image reconstruction:

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



Nyquist criteria

Point to point distance ≤ ½ Feature size

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

Other issues

- Sample drift
  - Dedrifting by cross-correlation
  - Fiducial markers (gold particles, beads)

- Fixing
  - Background fluorescence
  - Sample preservation

Acknowledgements / Further Reading

- Bo Huang
- http://nic.ucsf.edu/dokuwiki/doku.php?id=storm
- Lots of papers