Localization microscopy and single-molecule imaging

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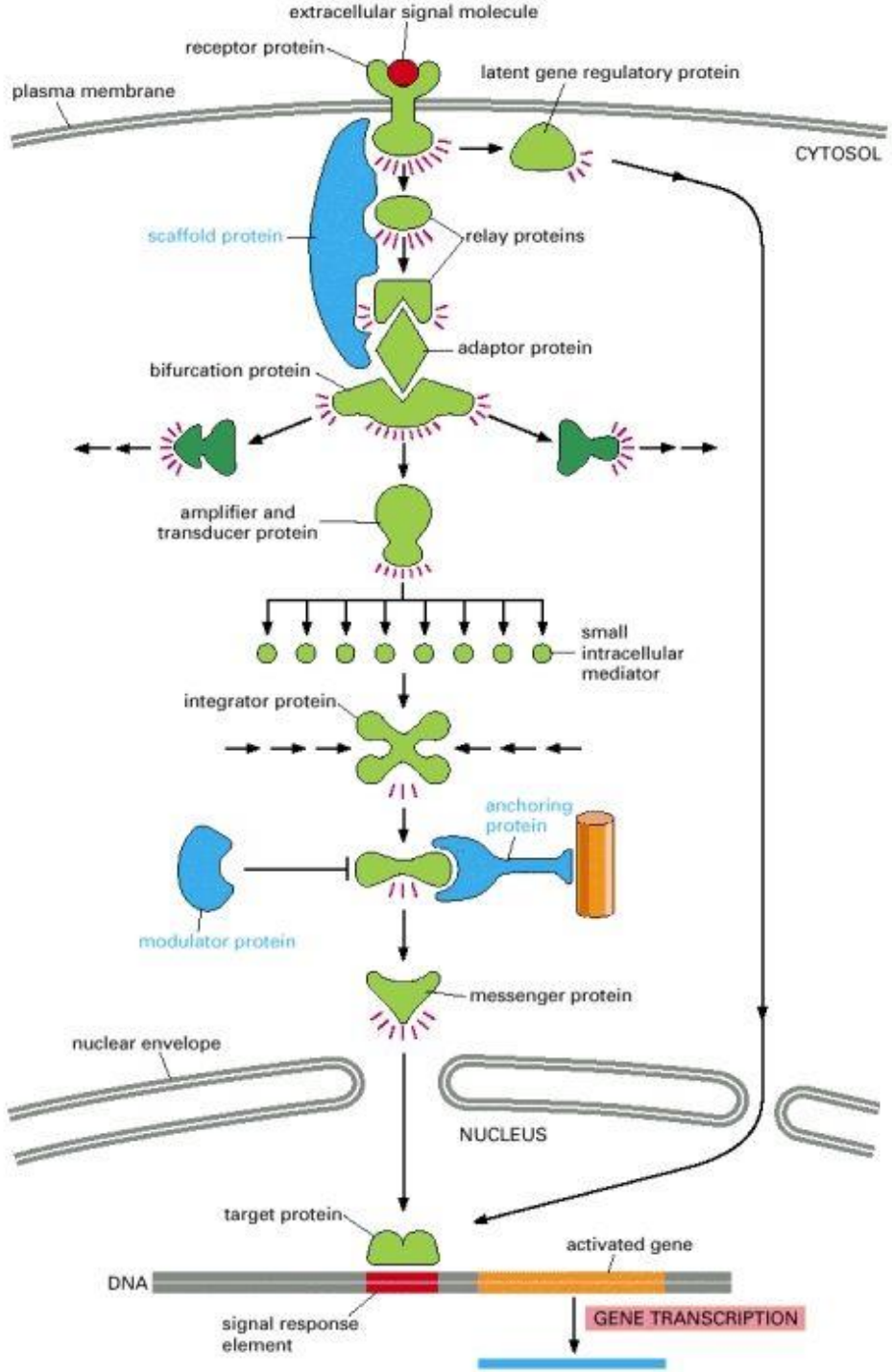
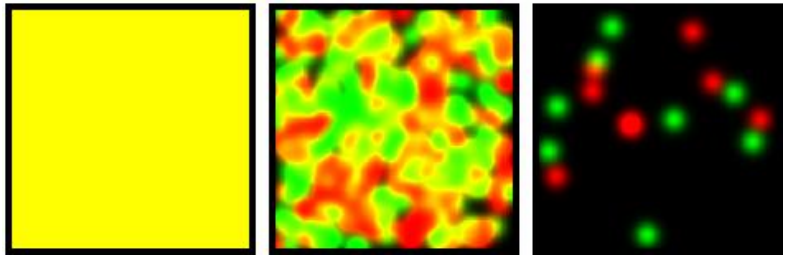

extracellular signal molecule
receptor protein
latent gene regulatory protein
plasma membrane
CYTOSOL
scaffold protein
relay proteins
adaptor protein
bifurcation protein
amplifier and
transducer protein
small
intracellular
mediator
integrator protein
anchoring
protein
modulator protein
messenger protein
nuclear envelope
NUCLEUS
target protein
activated gene
DNA
signal response
element
GENE TRANSCRIPTION

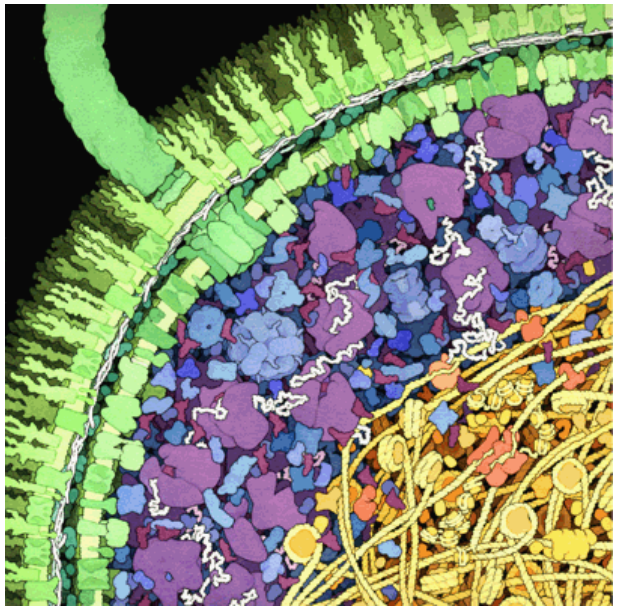
Why single-molecule imaging?

- Single molecules are the units of biological processes.
- There is a lot of heterogeneity in biology.



Single-molecule experiments avoid population-averaging:

- ➢ heterogeneous populations of molecules
- ➢ heterogeneous environments
- ➢ transient states
- ➢ unsynchronised dynamics
- ➢ rare events, low numbers

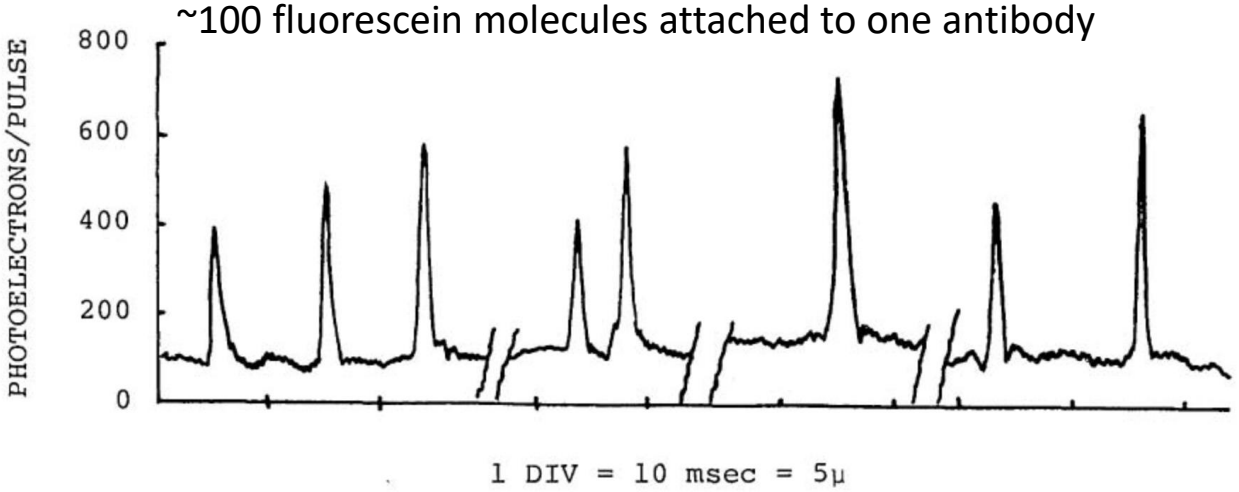


By David Goodsell

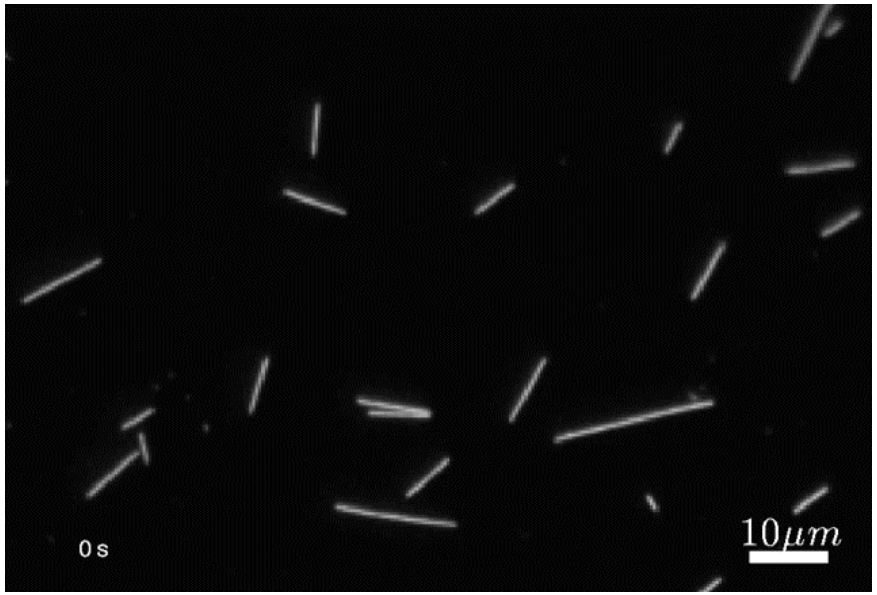
- Seeing is believing.

Single-molecule fluorescence imaging

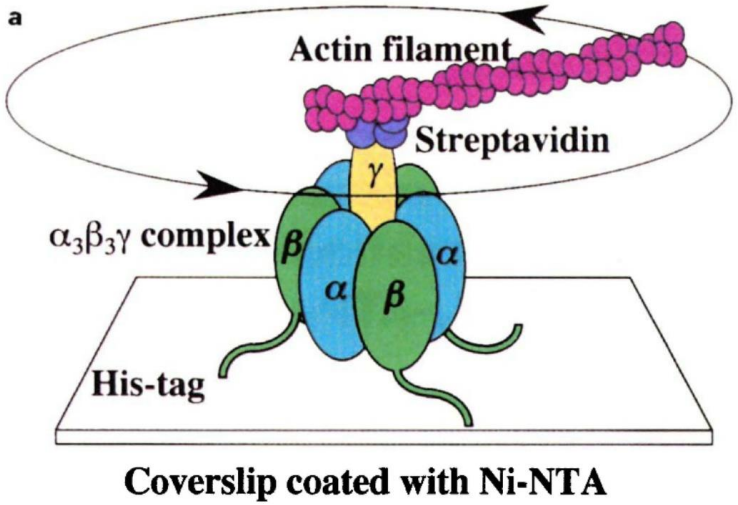
~100 fluorescein molecules attached to one antibody

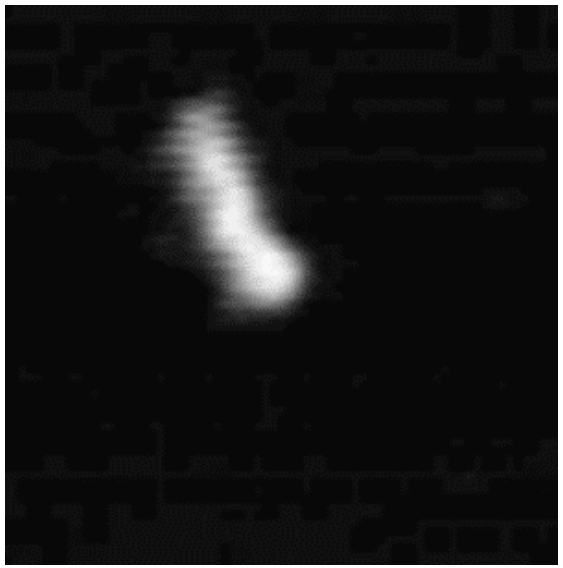


T. Hirschfeld Optical microscopic observation of single small molecules. Appl Optics 1976



Howard et al. Movement of microtubules by single kinesin molecules. Nature 1989
Scharrel et al. Biophys J 2014





Noji et al. Direct observation of the rotation of F1-ATPase. Nature 1997

Achieving single-fluorophore sensitivity

Laser illumination: high energy density, narrow spectrum

Sensitive detector: EMCCD or sCMOS cameras, avalanche photodiode (APD)

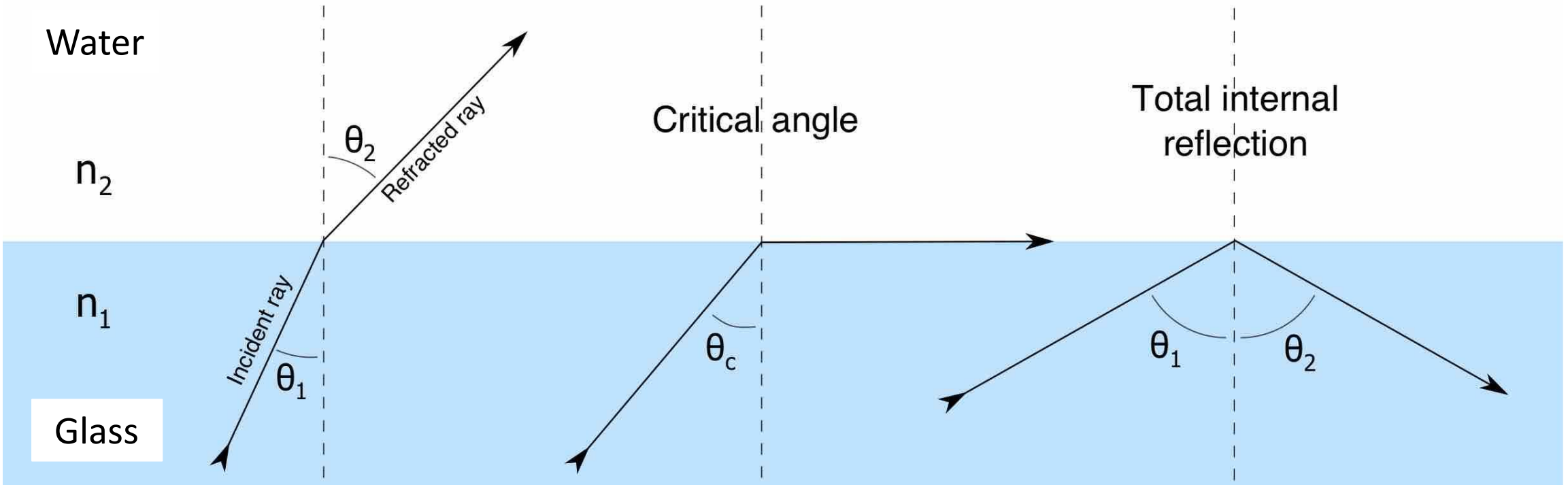
Bright fluorophores: synthetic dyes, quantum dots, bright fluorescent proteins

Low-background samples: cleaned coverslips, clear buffer/growth media

Low vibration & drift: stable instrumentation, optical table, drift correction

Reducing out-of-focus fluorescence: e.g. via Total Internal Reflection (TIRF) illumination

Total internal reflection (TIRF) illumination

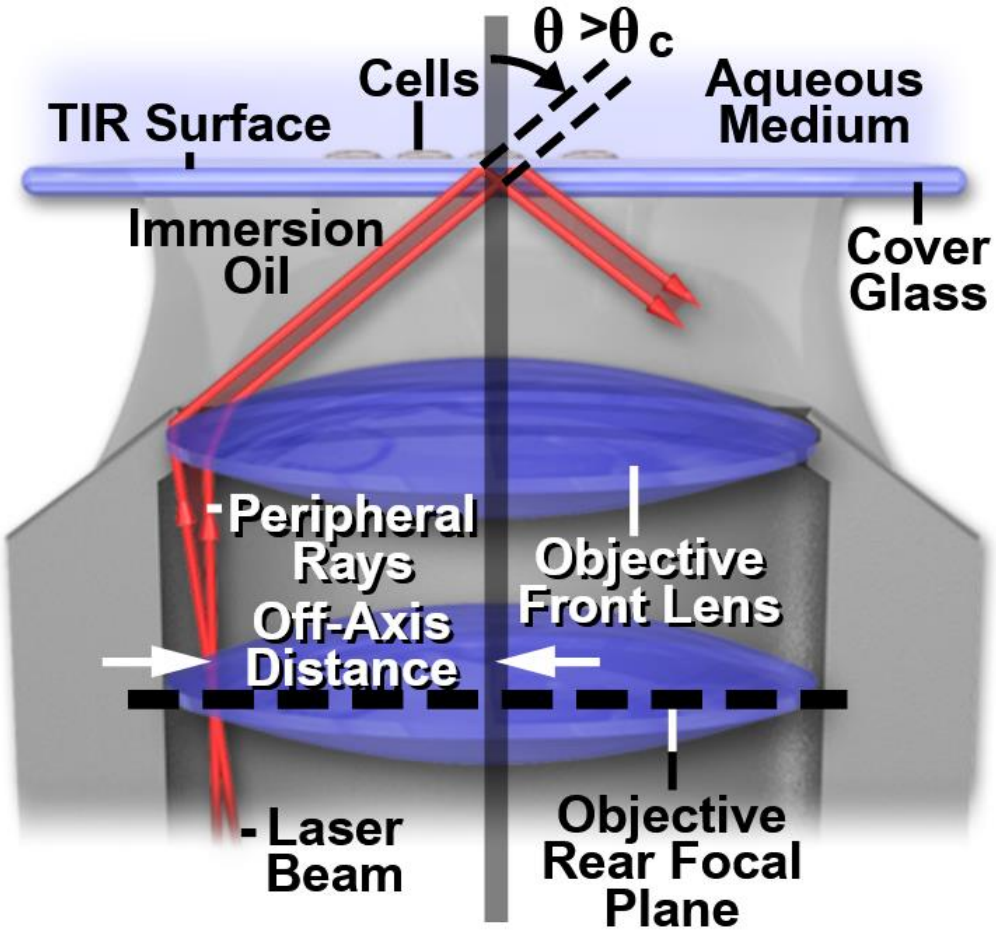


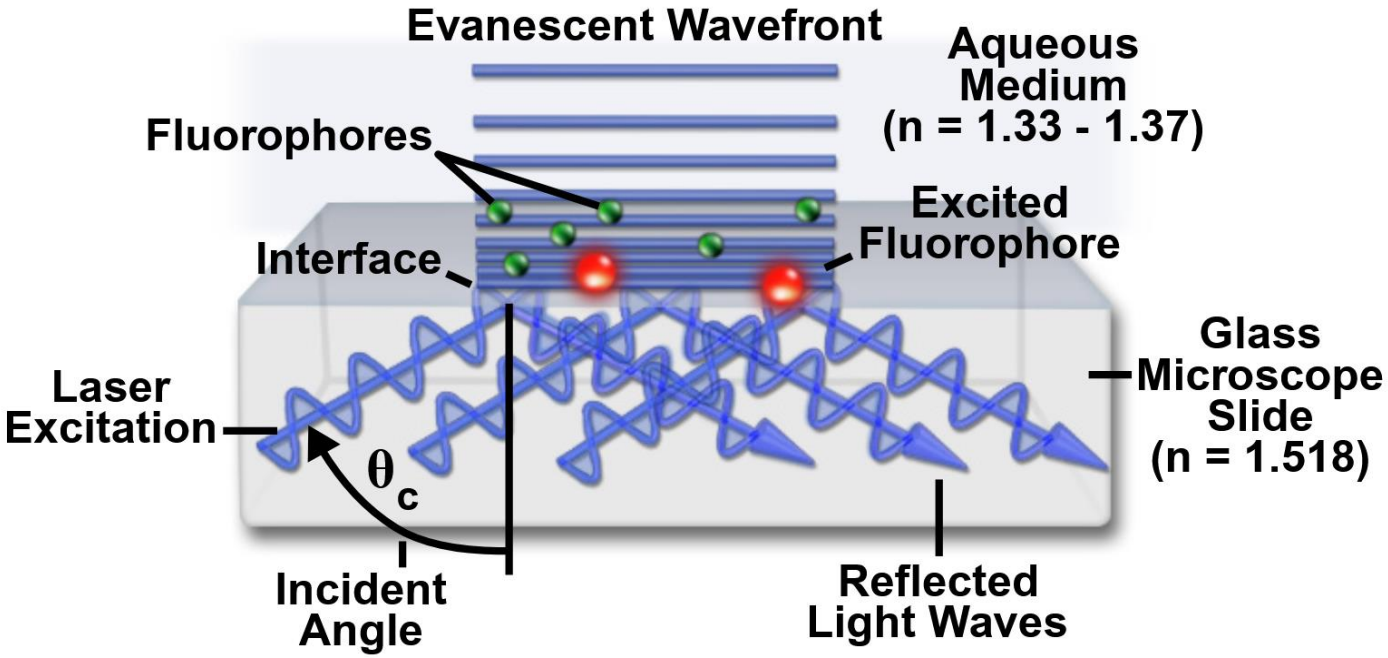
Boundless

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

$$\text{critical angle } \theta_c = \theta_1 = \arcsin\left(\frac{n_2}{n_1}\right)$$

Total internal reflection (TIRF) illumination

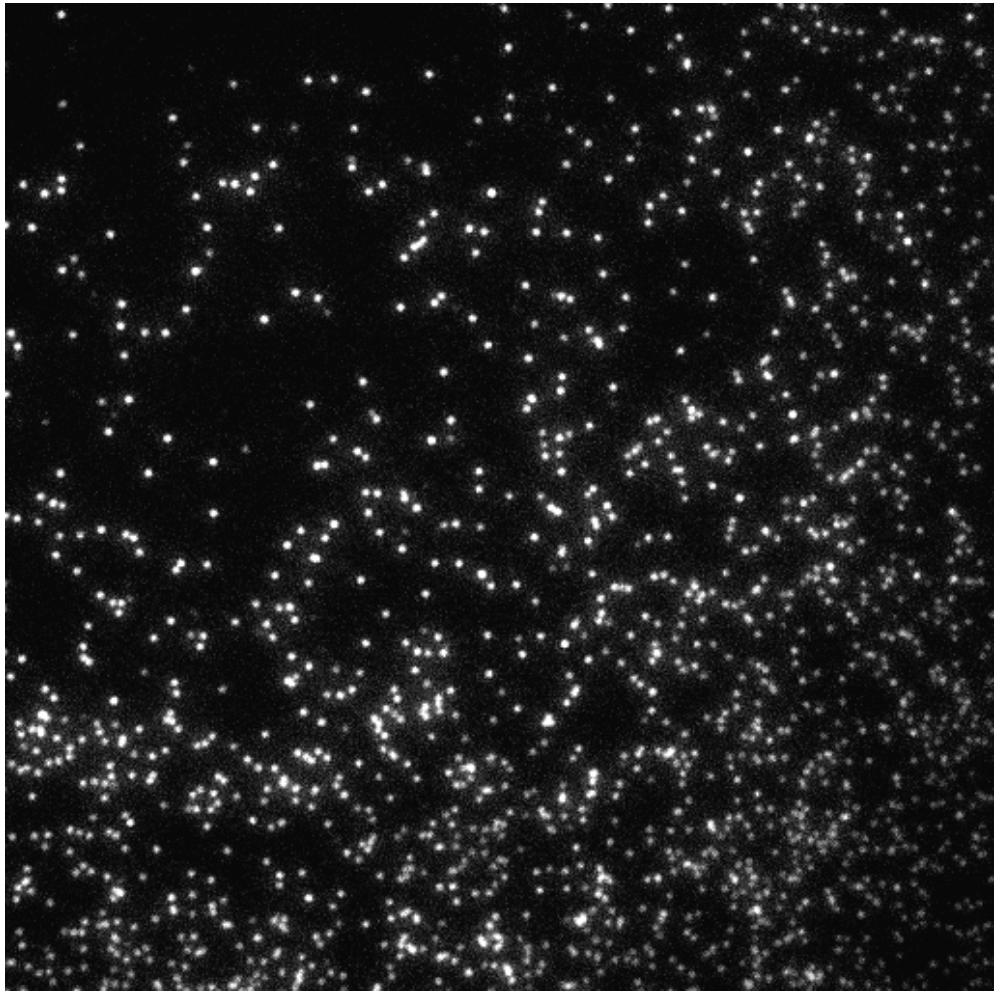




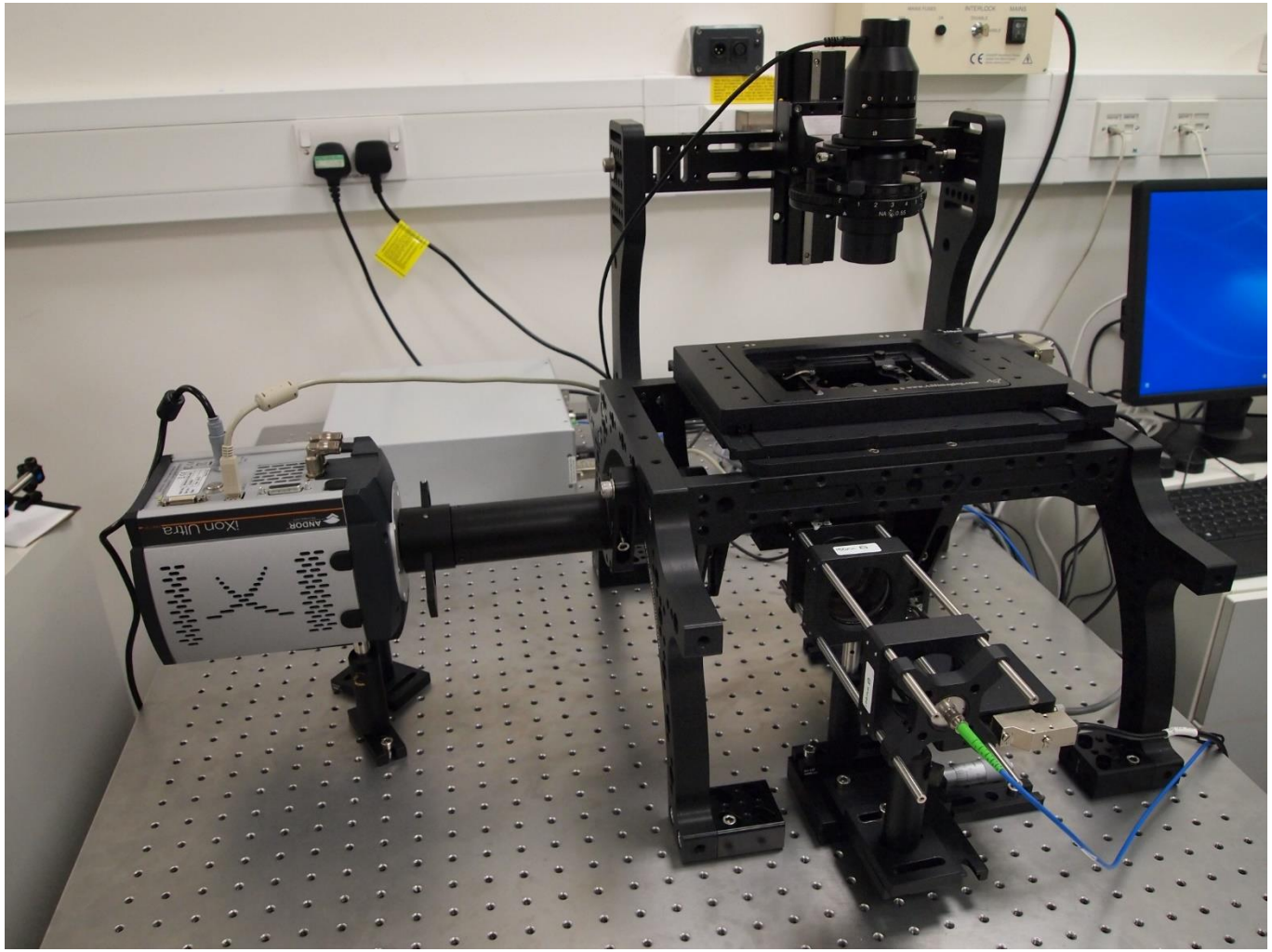
MicroscopyU

Total internal reflection (TIRF) illumination

Single Cy3B fluorophores on a coverslip

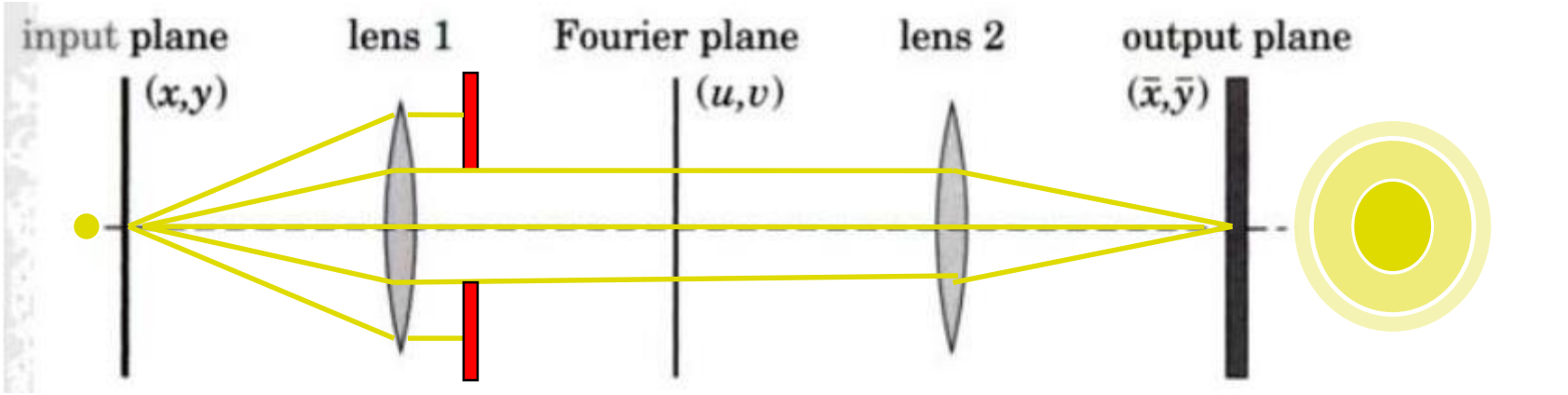


50 µm



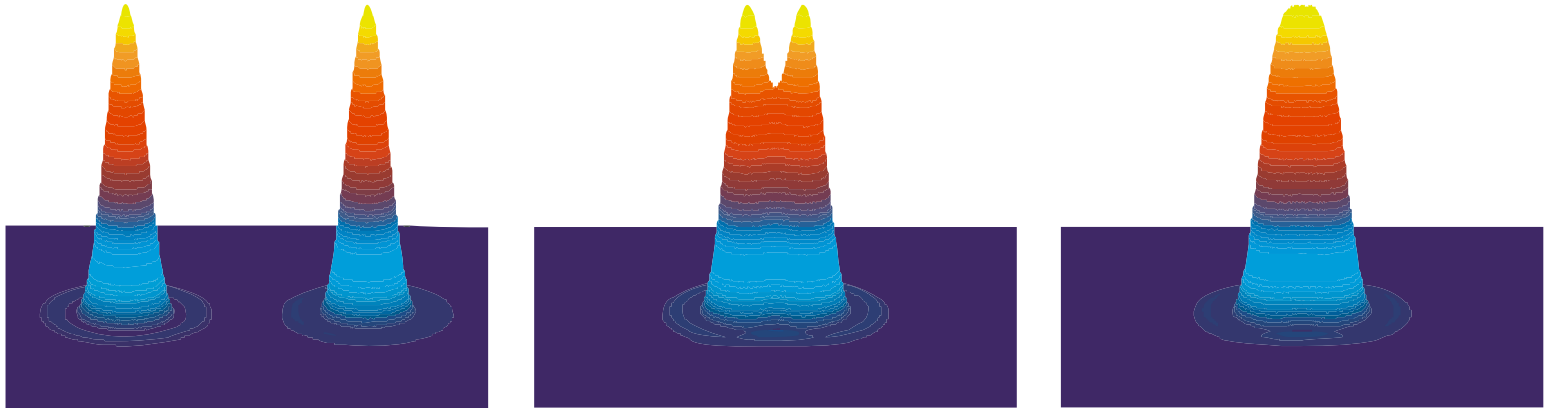
Diffraction Limit of Resolution

Point Spread Function (PSF): Response function of the microscope



Point Source | Objective | Tube Lens | Image: Airy Disk

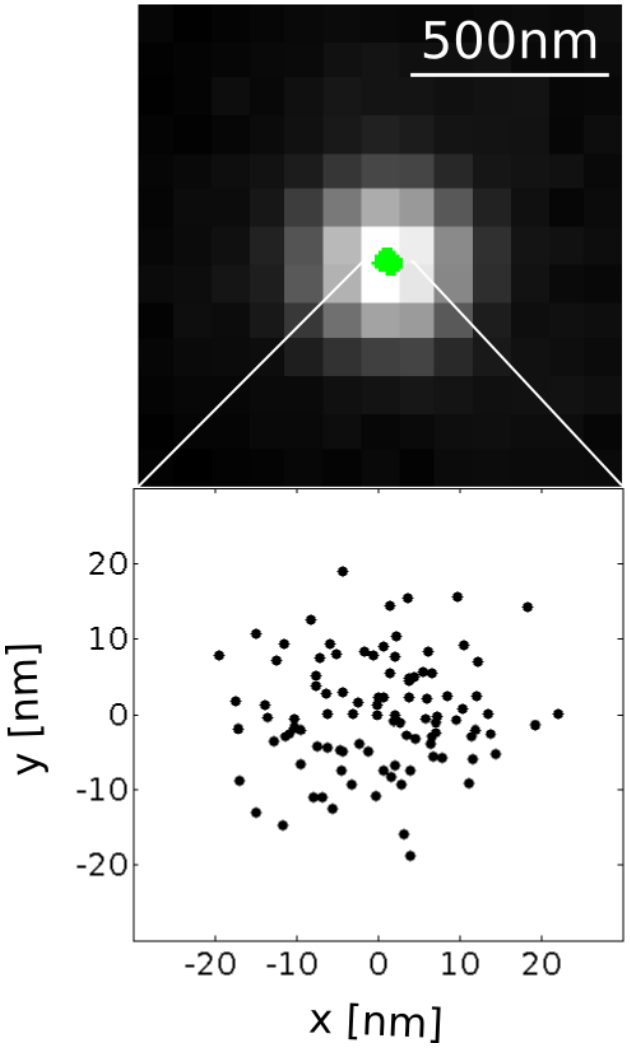
Limited objective aperture: Unwanted lowpass-filter



$$\Delta x = \frac{1.22\lambda}{2\mathrm{NA}} \qquad \mathrm{NA} = n \sin \alpha$$

$$\Delta x \geq \frac{\lambda}{2} \approx 250 \text{ nm}$$

Localization precision of an isolated point source is not limited by diffraction



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

s: Point spread function width

N: number of photons reporting on the location of the fluorophore

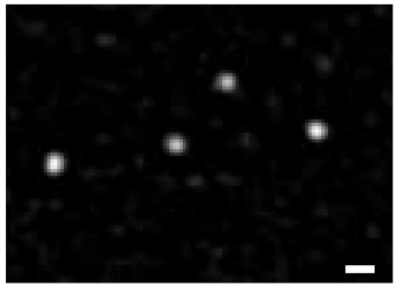
σ: Localization precision

σ is related to the standard error of the mean, limited by photon number

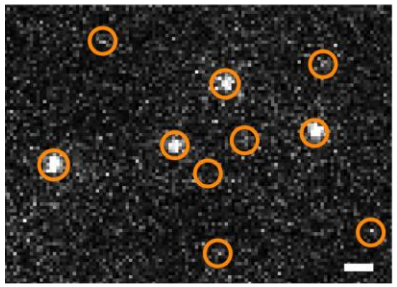
Additional factors: backround noise, pixel size, localization algorithm

Localization analysis

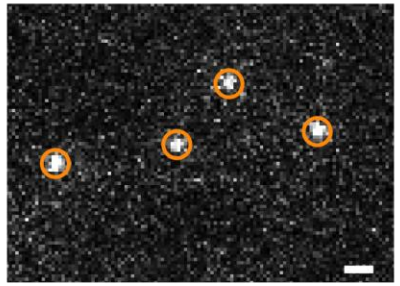
band-pass filter



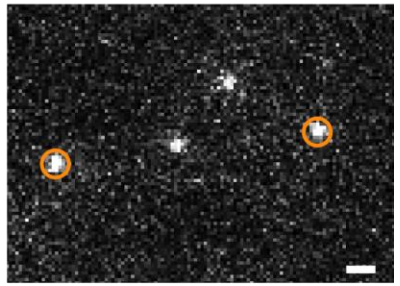
threshold 1.5 SNR



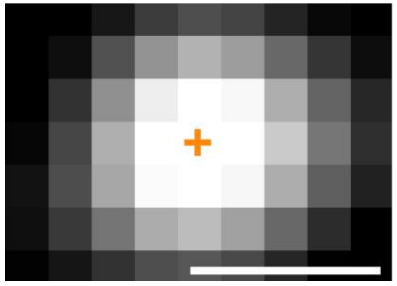
4.5 SNR



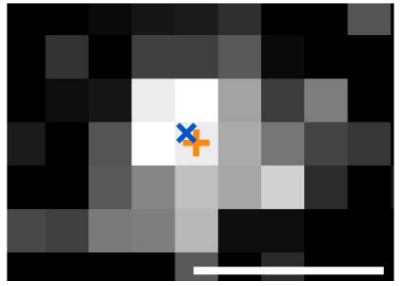
13.5 SNR



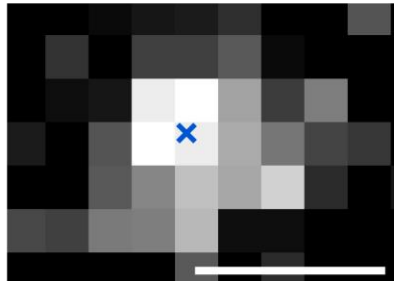
filtered image
intial guess

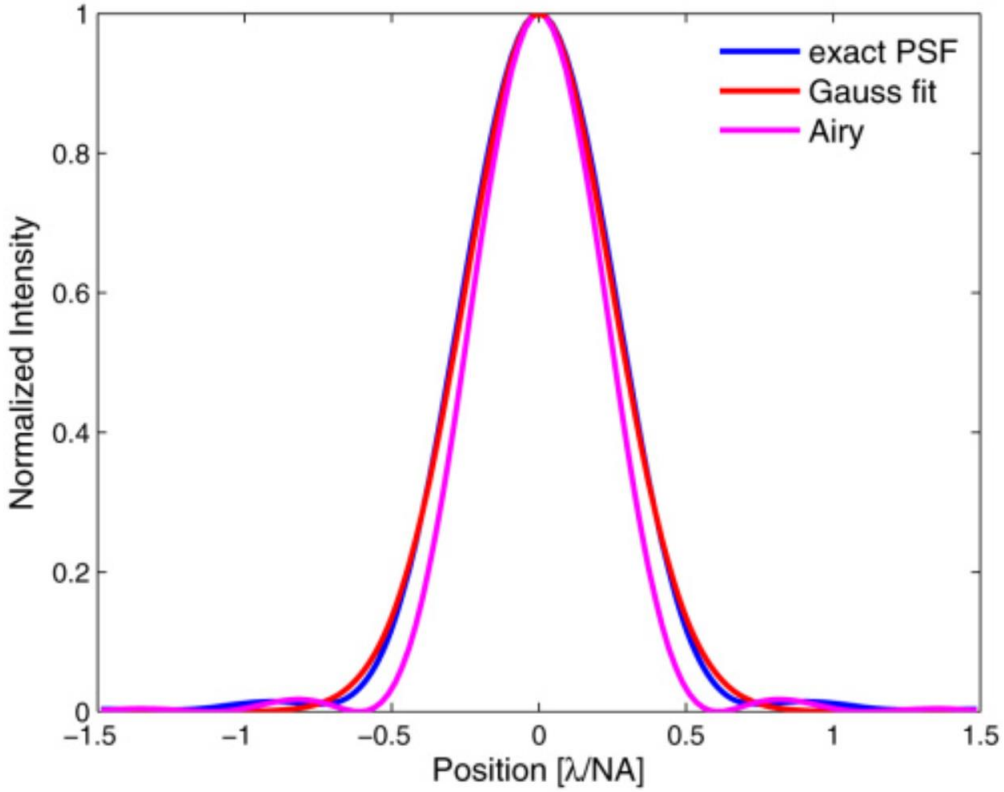


raw image
fitting



raw image
final localization



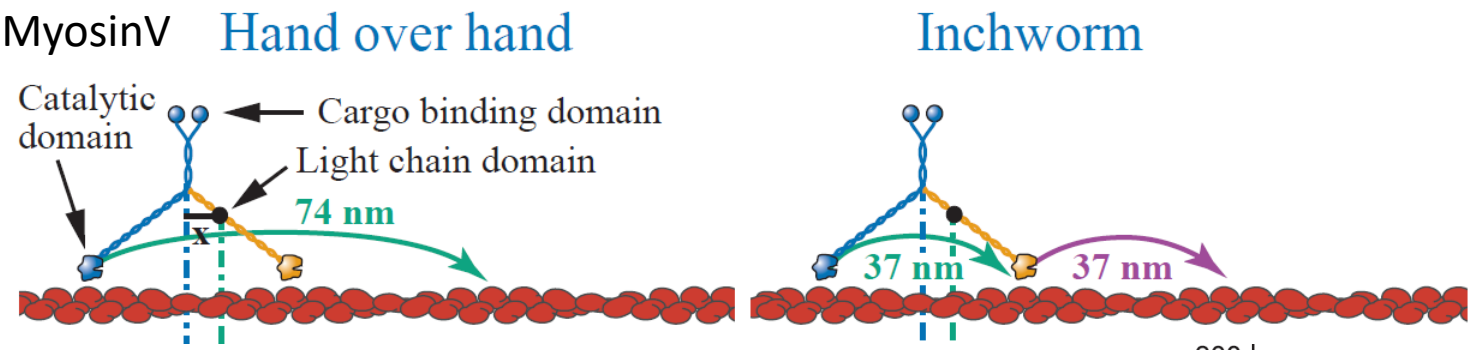


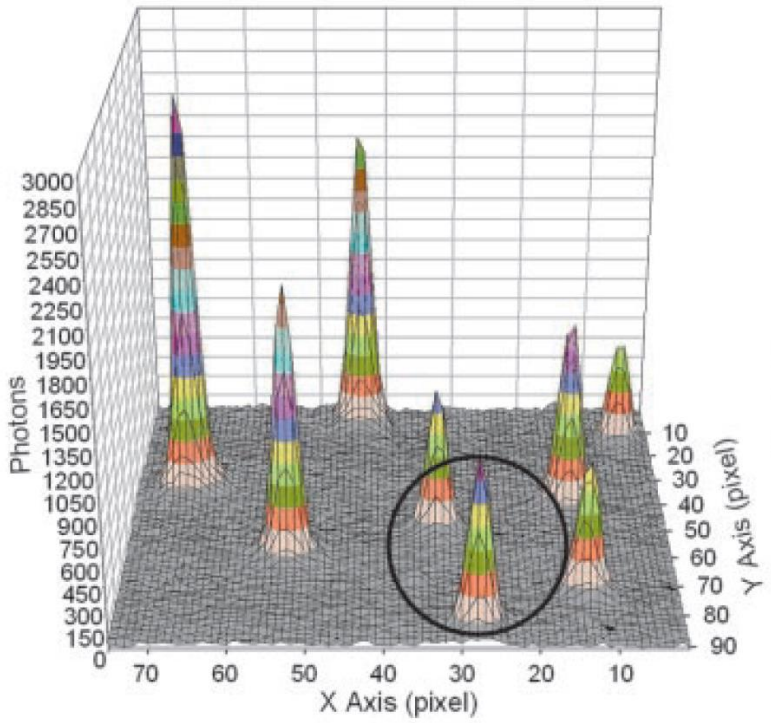
Stallinger et al. Optics Express 2010

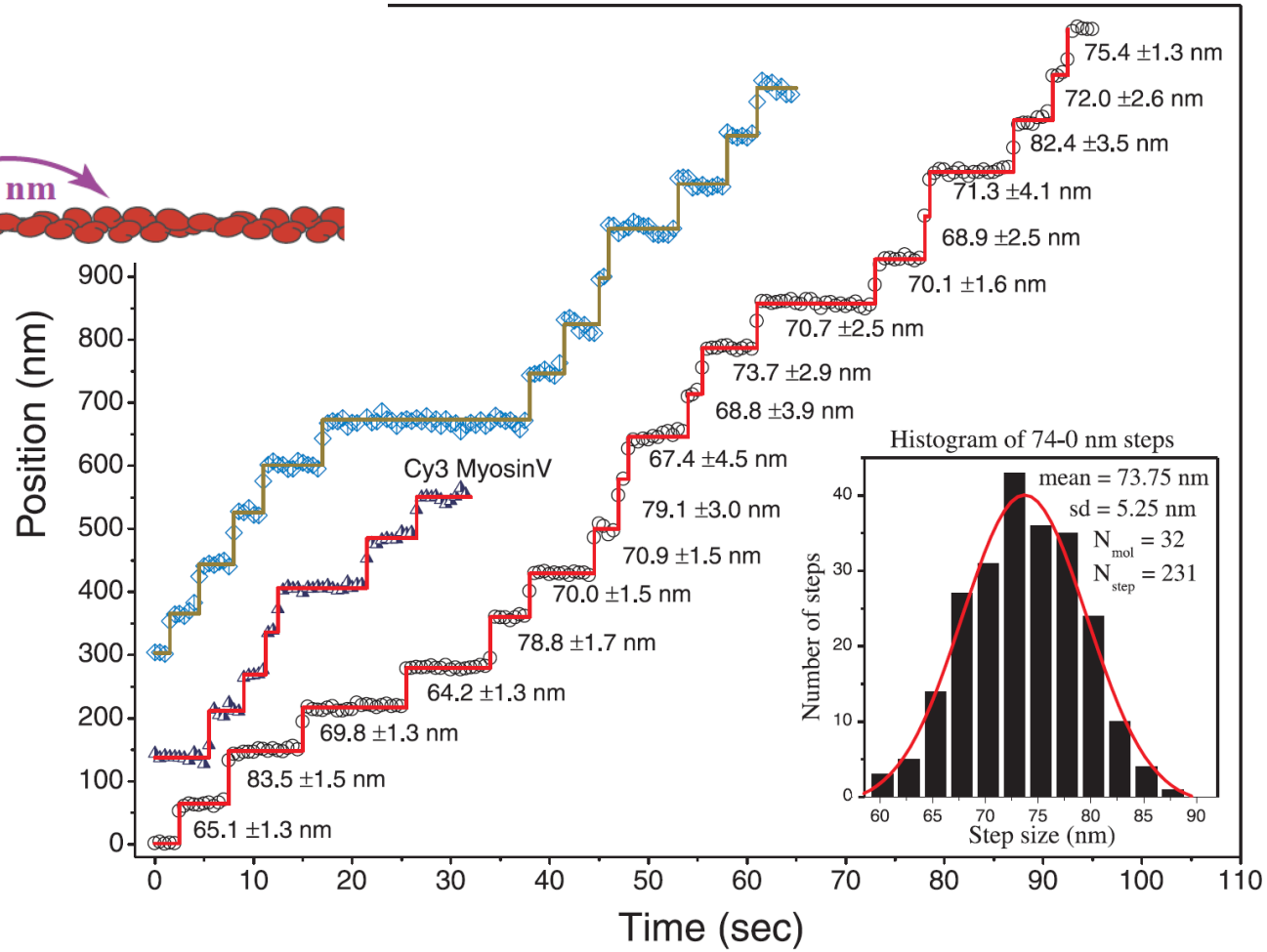
Uphoff Methods Mol Biol 2016

Fluorescence imaging with one nanometre accuracy (FIONA)

MyosinV



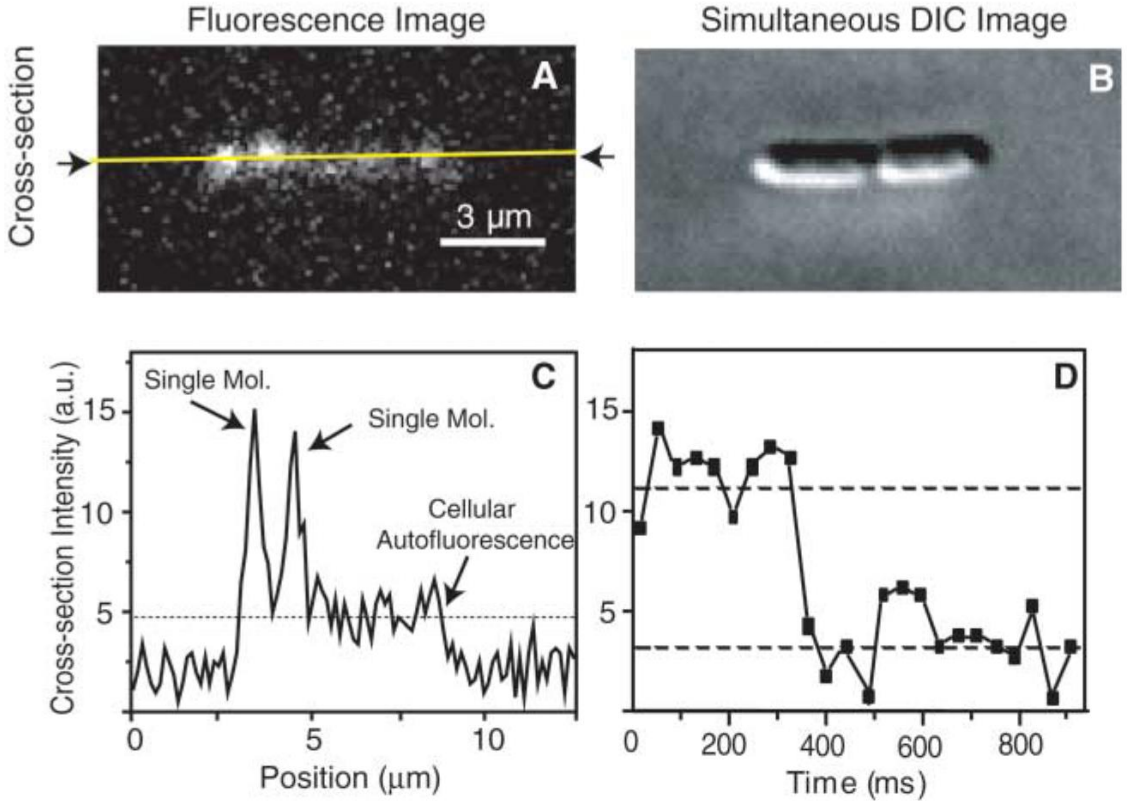


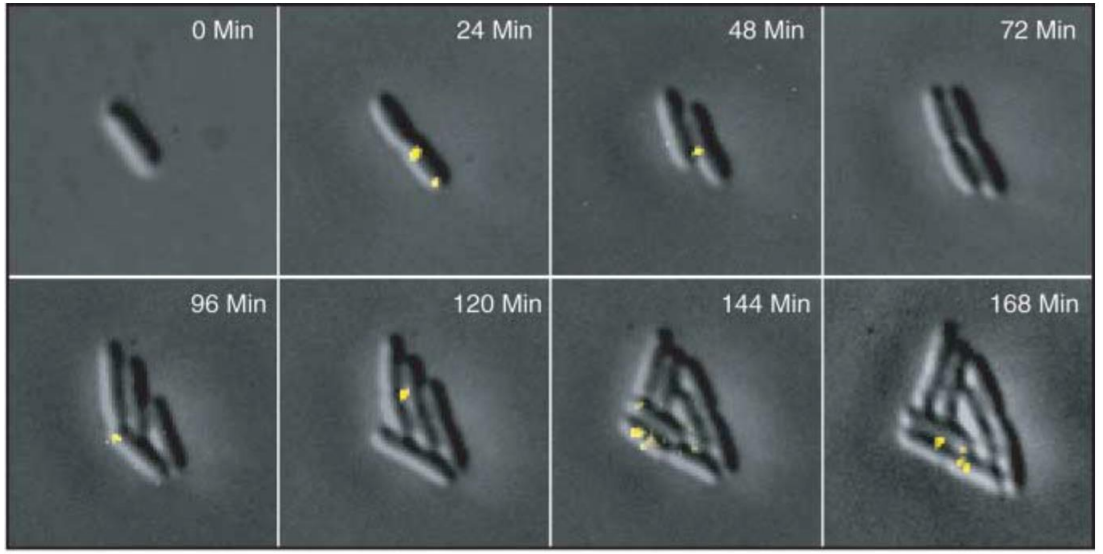


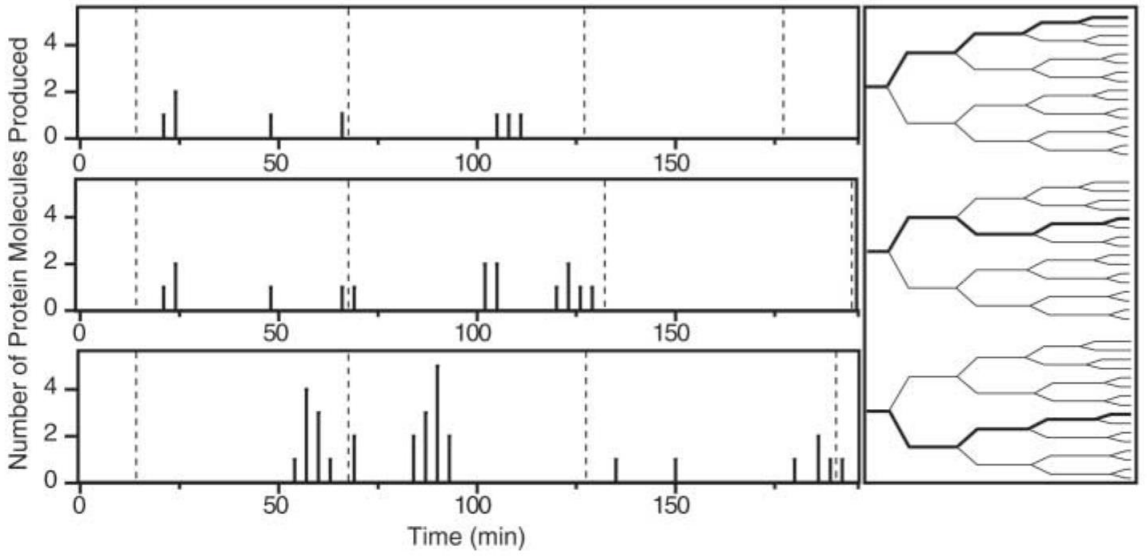
Yildiz et al. Science 2003

Counting single proteins in living cells

Tsr membrane protein fused to Venus fluorescent protein

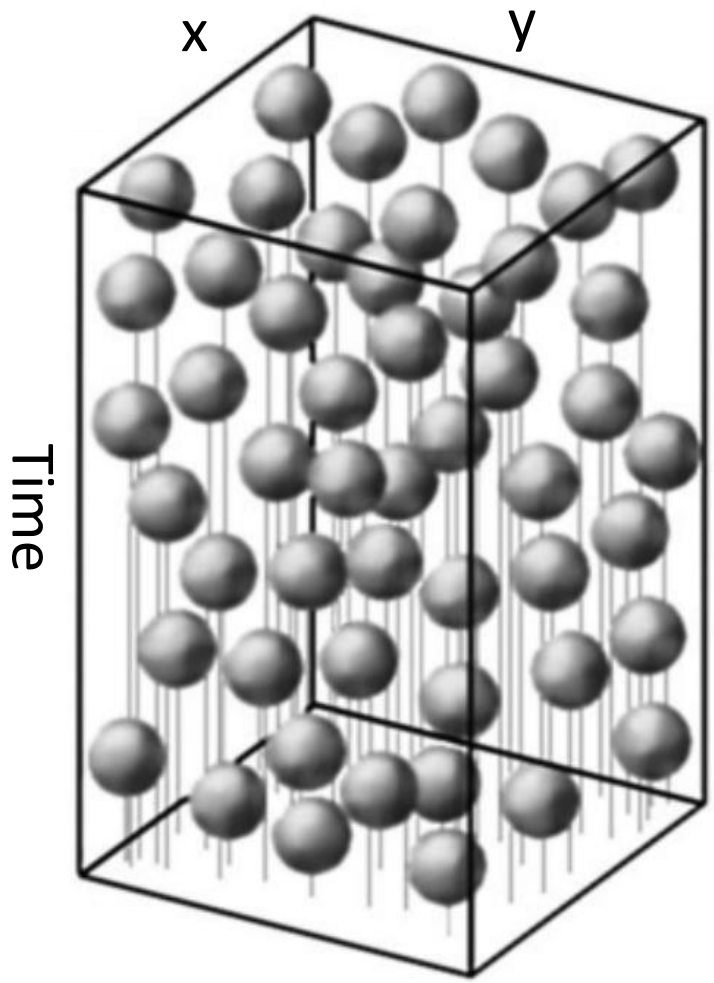


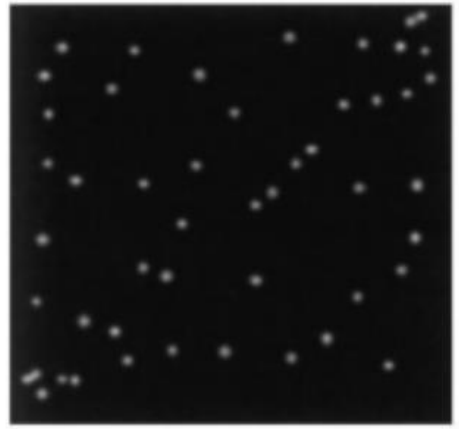




Yu et al. Science 2006

Super-resolution localization microscopy



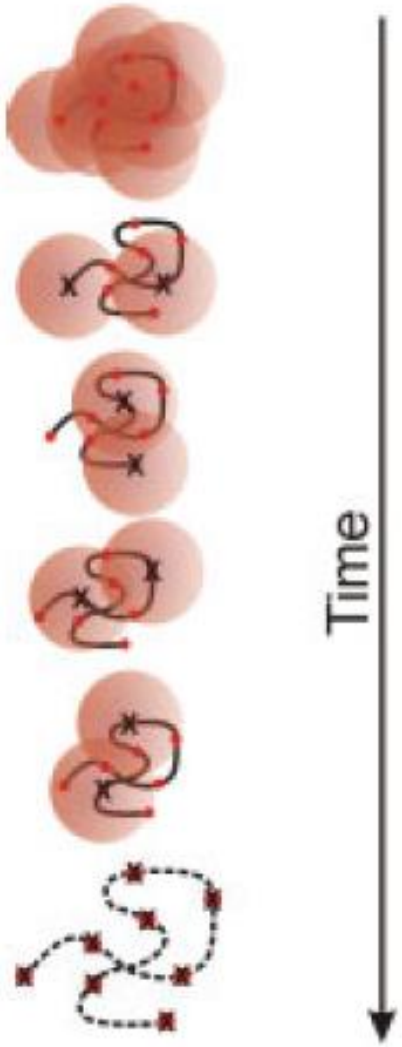


Betzig et al. Optics Lett 1995

Nobel Prize Chemistry 2018

Betzig, Hell, Moerner

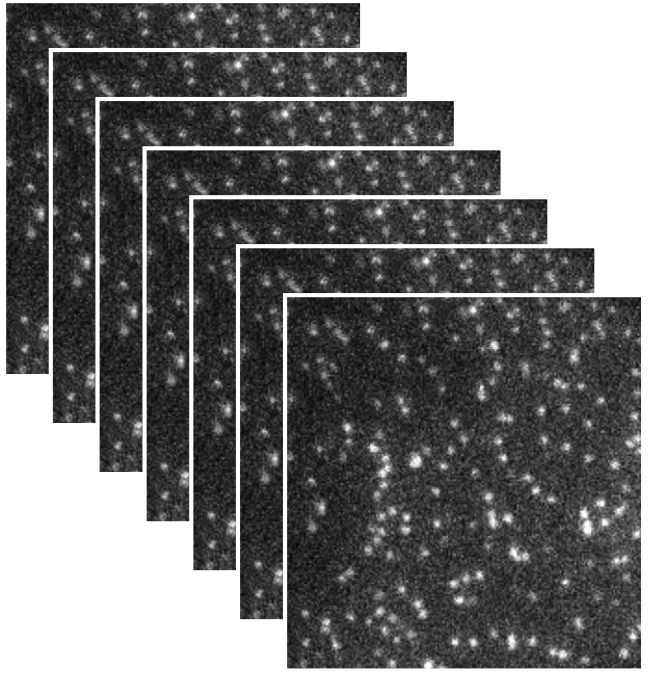
Super-resolution localization microscopy



Isolate a resolvable subset of fluorophores

Localize

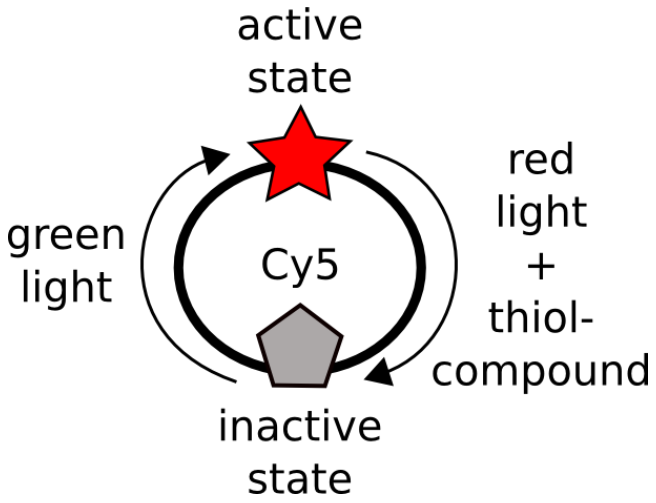
Repeat

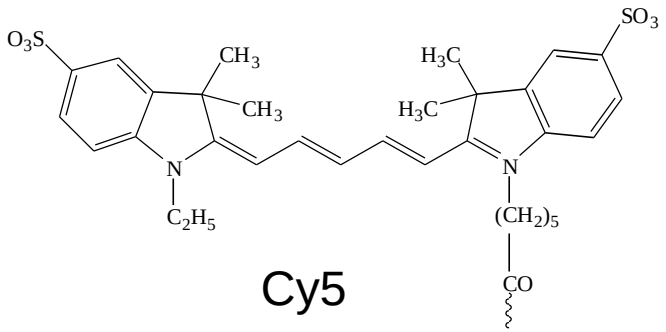


Reconstruct image from localizations

Heilemann et al. Laser & Photon Rev. 2009

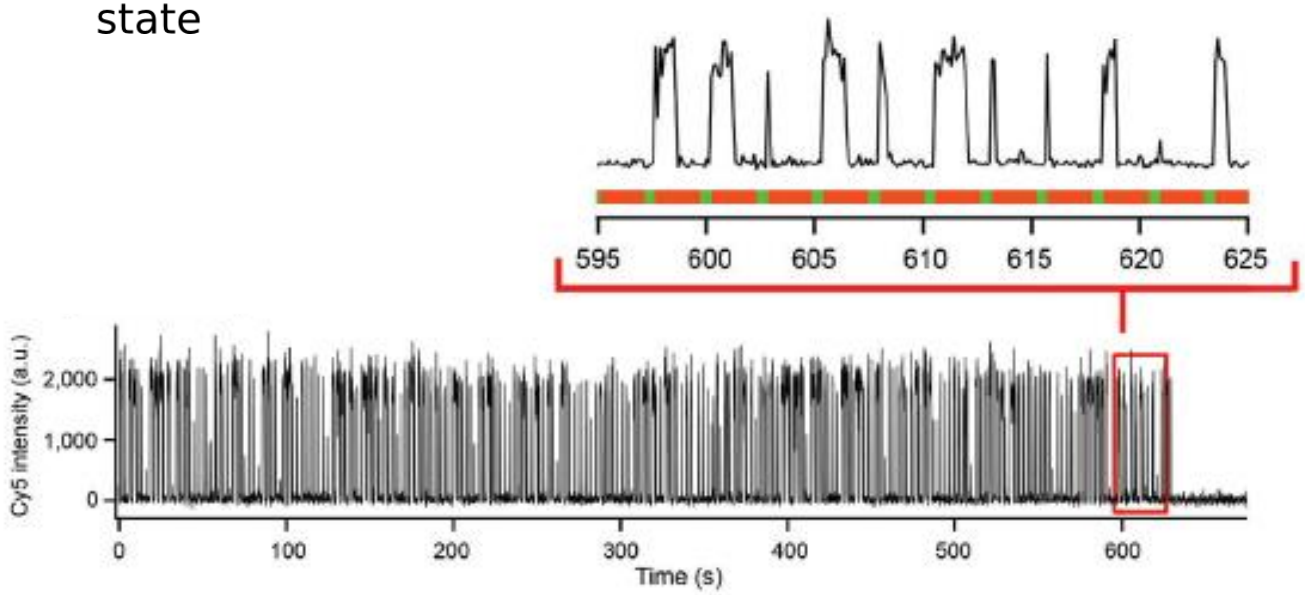
Isolating molecules using photoswitchable fluorophores

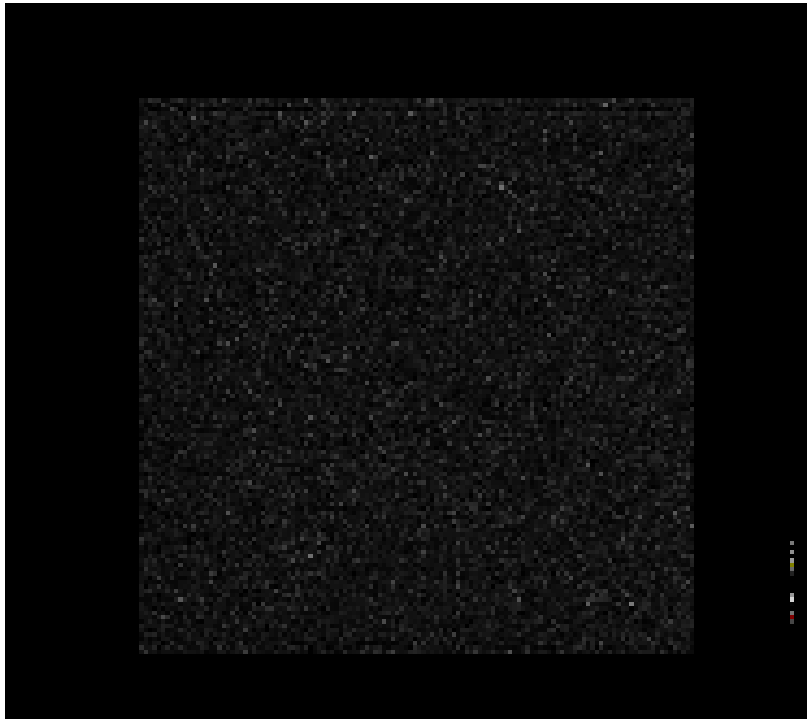




Cy5

Switching buffer:
Oxygen scavenging system
Thiol reducing agent (BME, MEA)

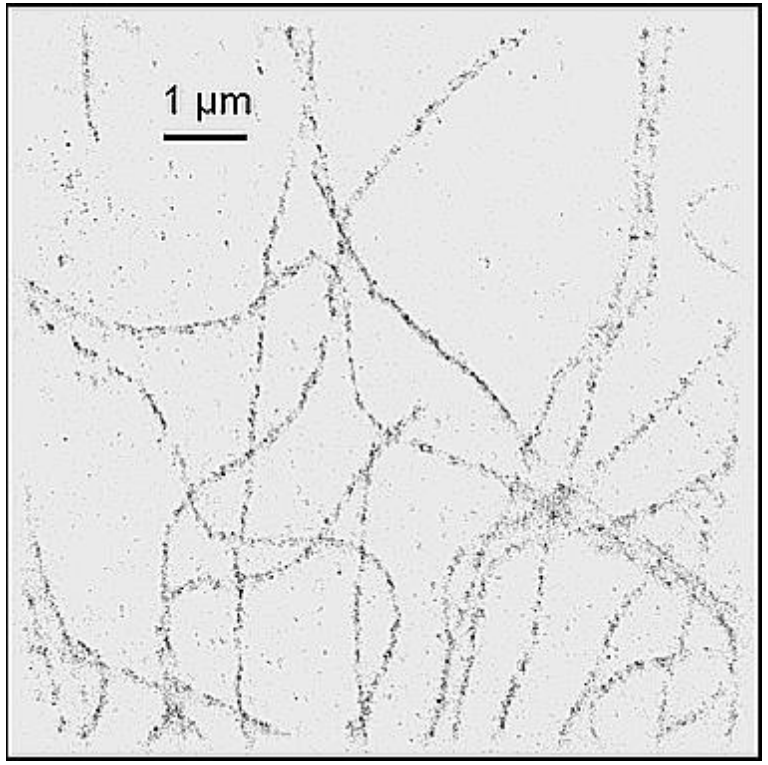




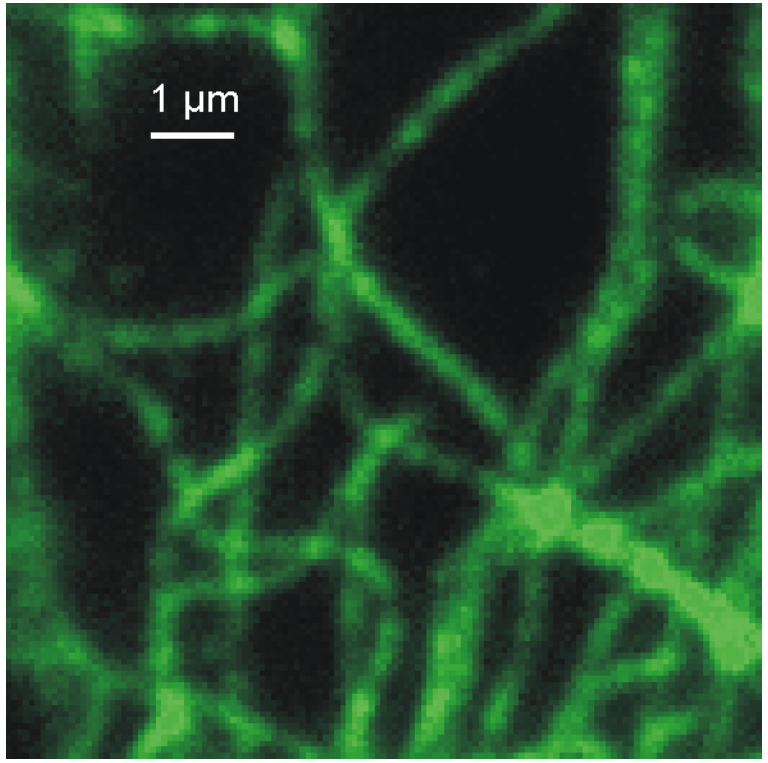
Heilemann et al., JACS 2005; Bates et al., PRL 2005

Stochastic optical reconstruction microscopy (STORM)

STORM



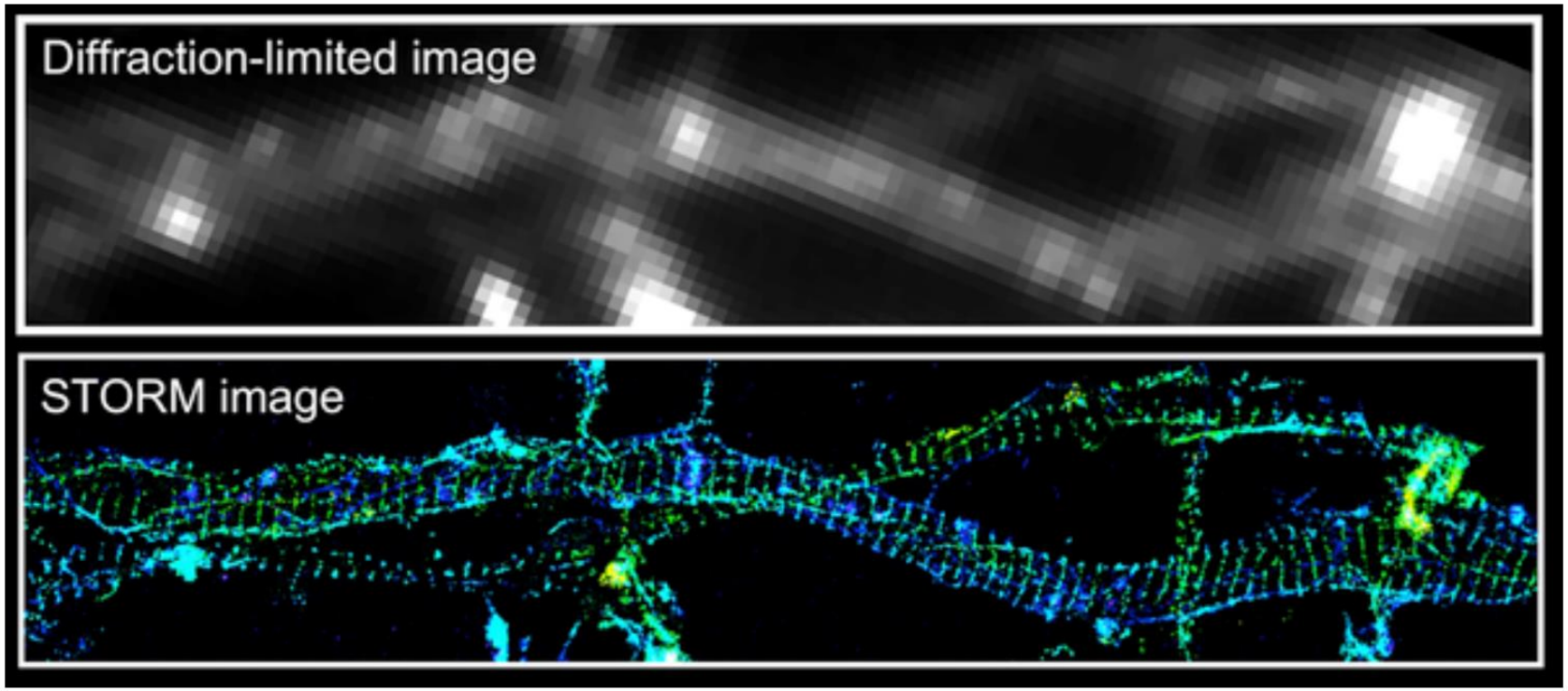
TIRF



Microtubules immuno-labelled with Cy5 dye

By Mike Heilemann

STORM

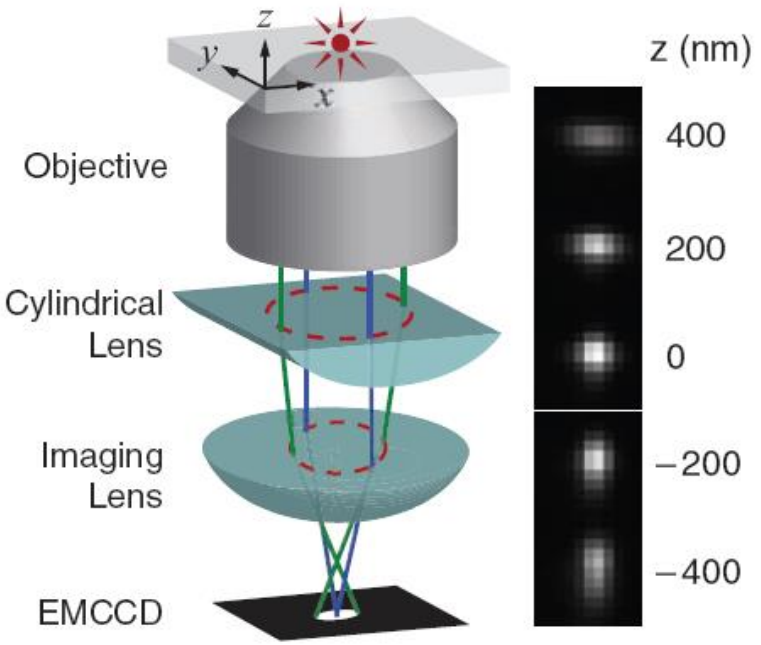


Comparison of conventional (upper) and 3D STORM (lower) images of actin in the axons of neurons. Actin is labeled with phalloidin conjugated to photoswitchable dyes. STORM image revealed a novel periodic, actin-spectrin-based membrane skeleton in axons.

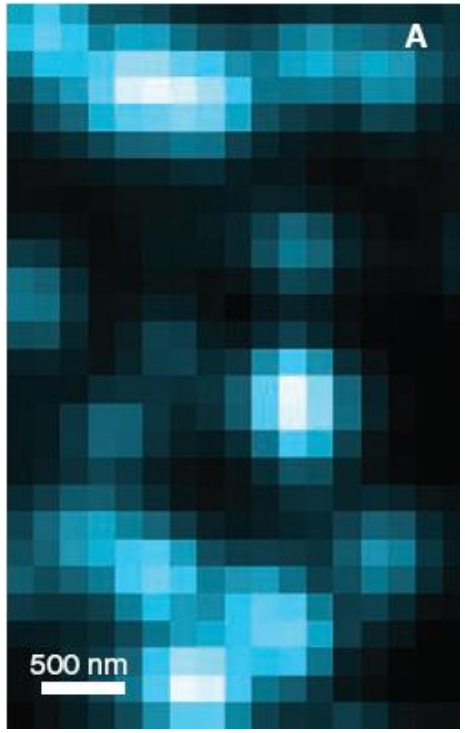
Xu et al. Science 2013

3D STORM

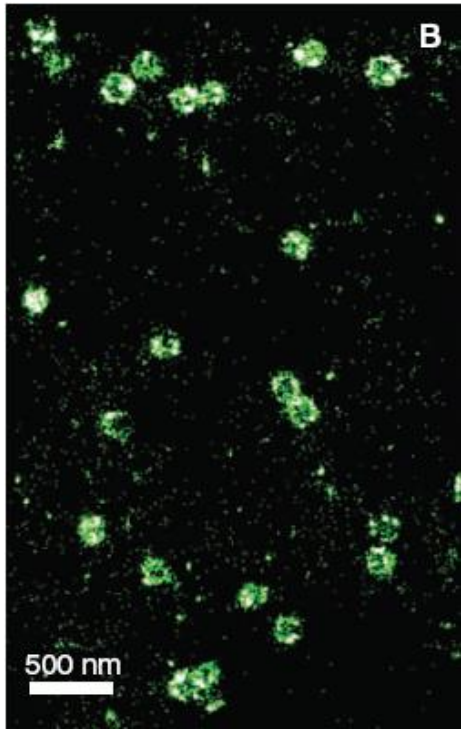
clathrin-coated pits



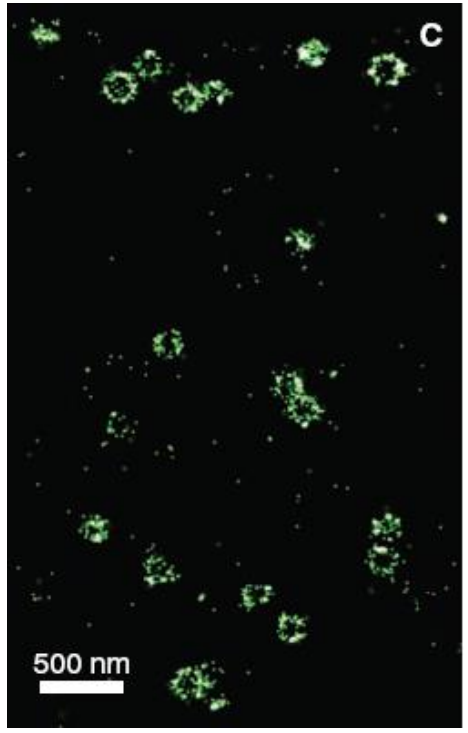
Astigmatism



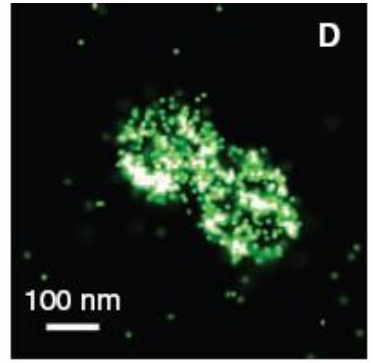
Widefield

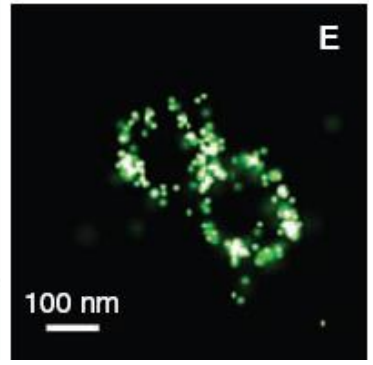


STORM projection



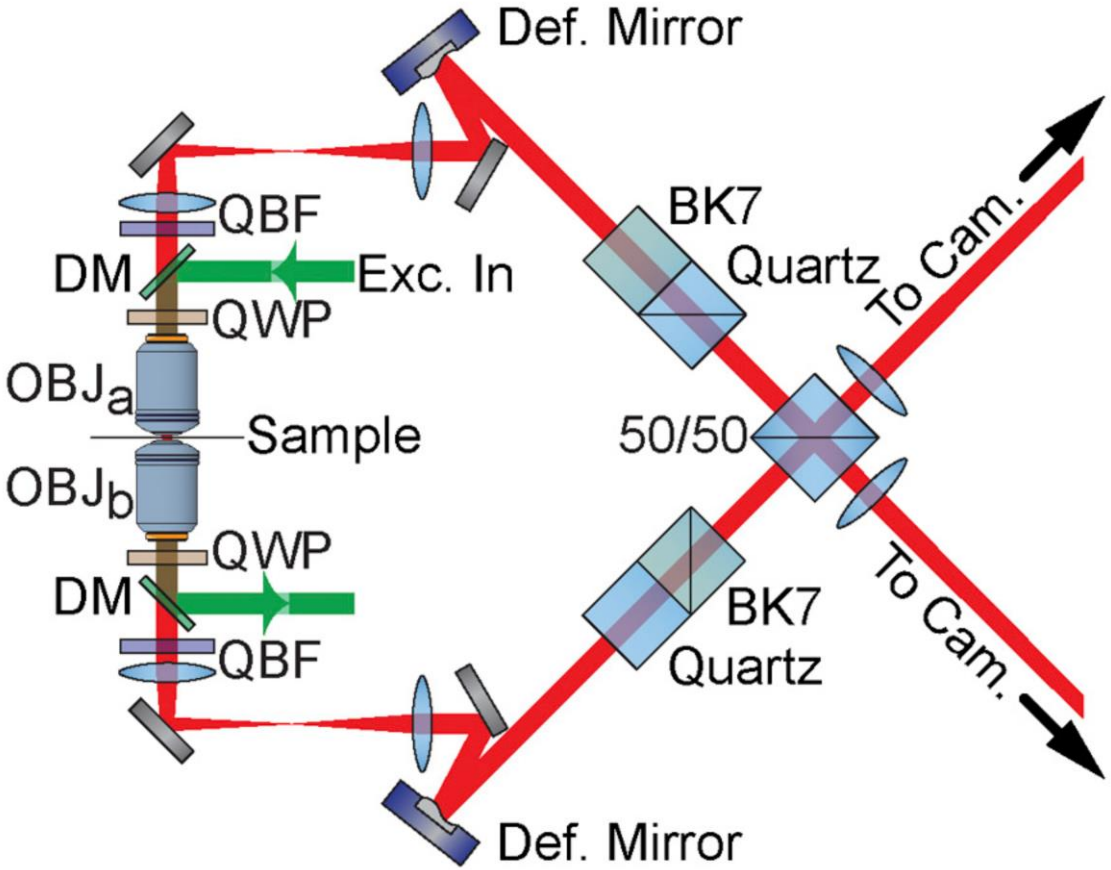
2D cross-section





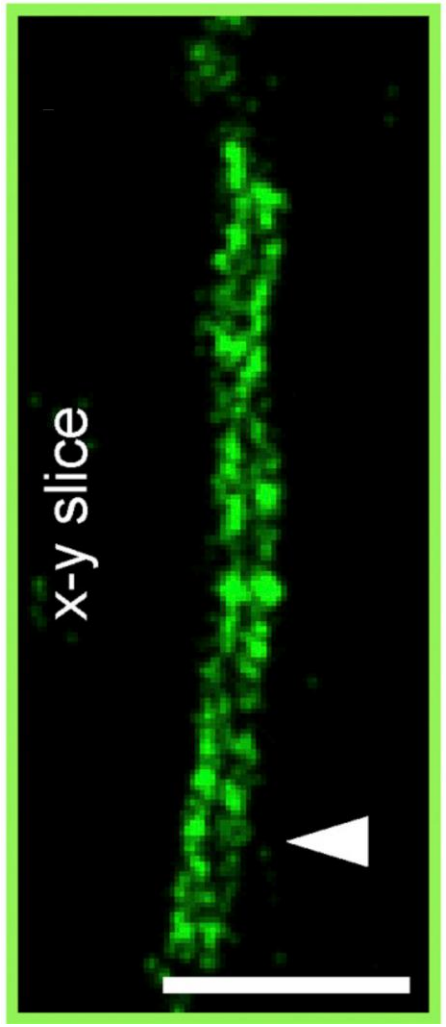
Huang et al. Science 2008

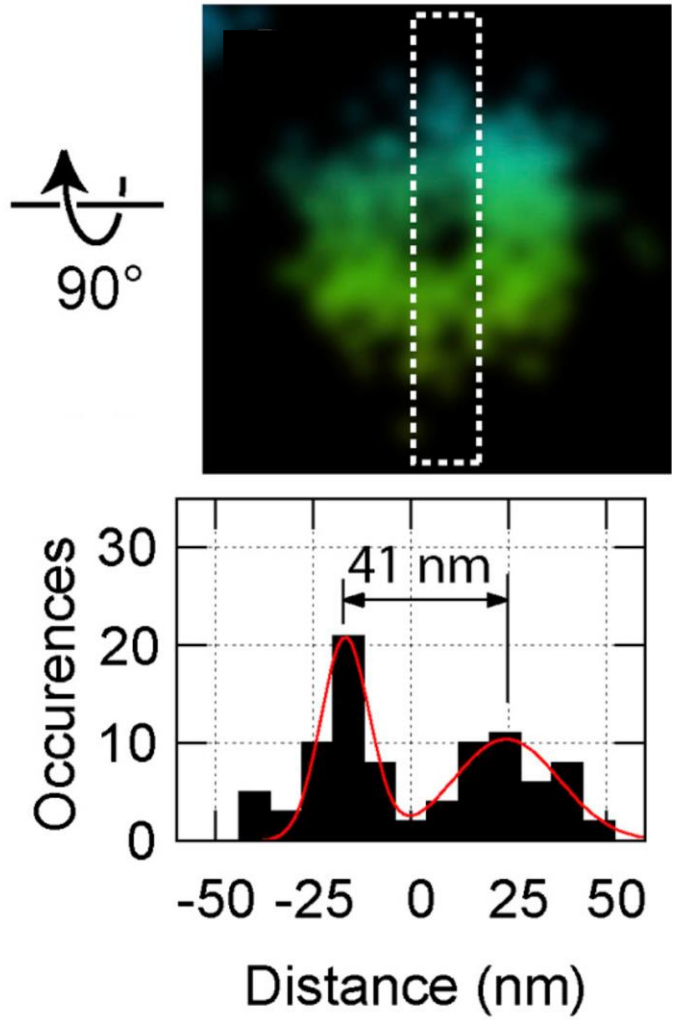
Using interference to enhance 3D resolution



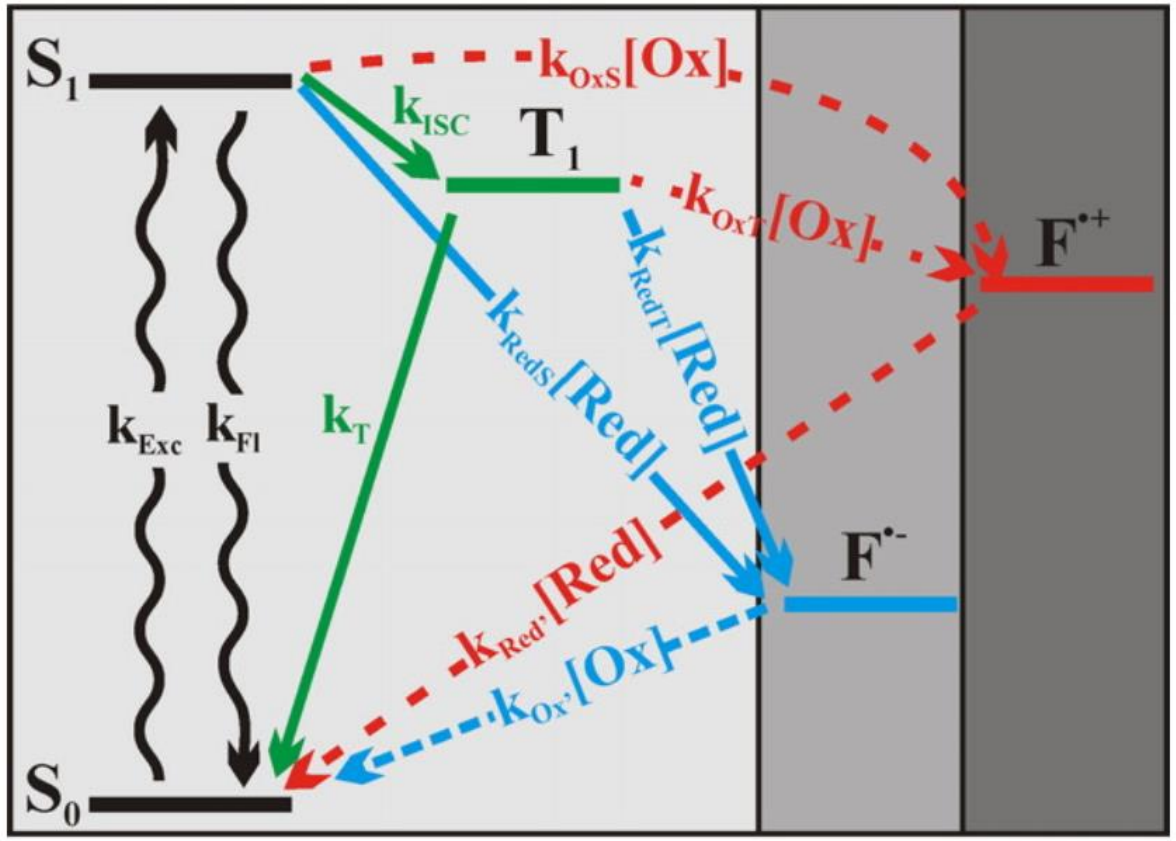
Huang et al. Cell 2016

Microtubule





Why do fluorophores blink?



Blinking buffer:

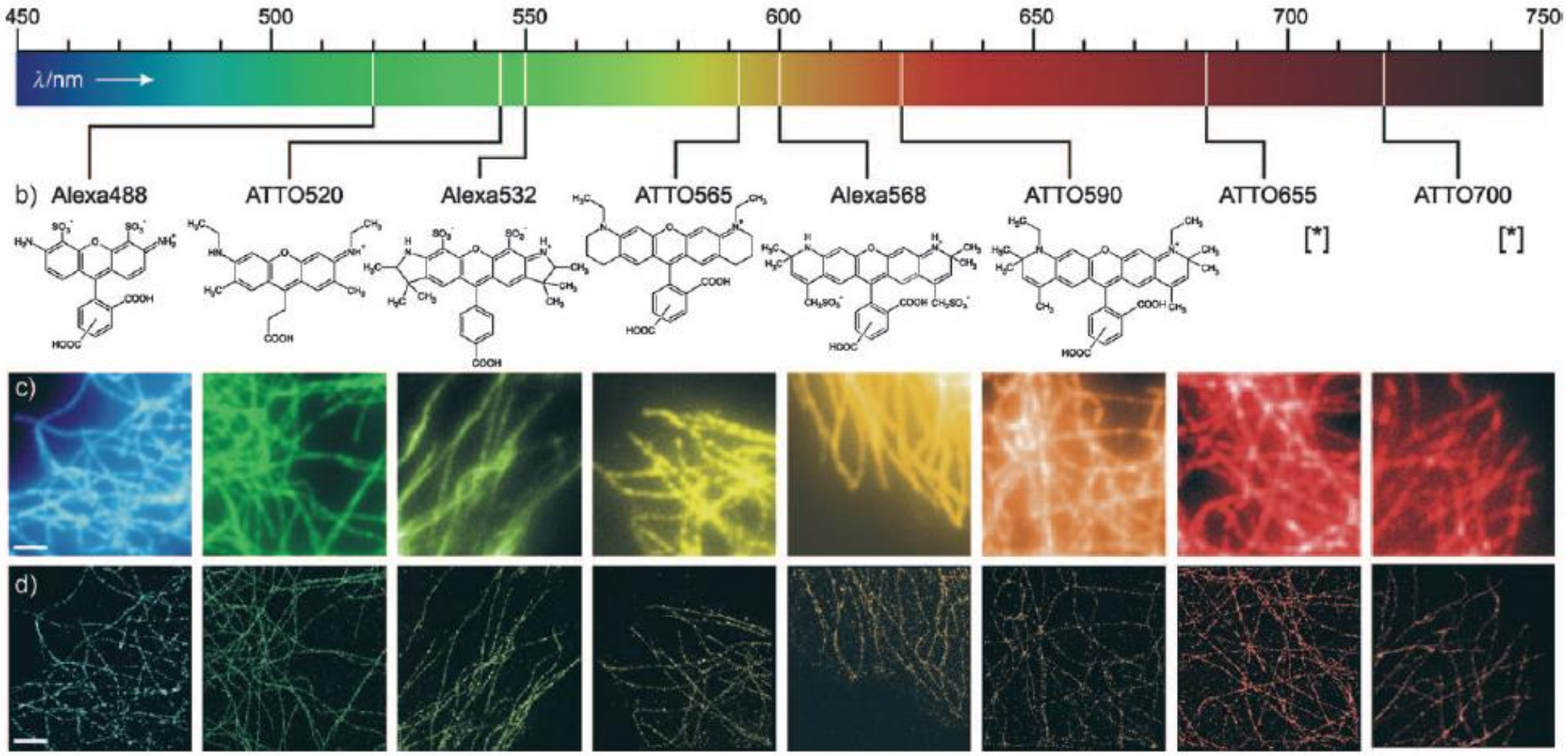
Oxygen scavenging system

Reducing agent (ascorbic acid, BME)

Oxidising agent (methylviologen)

Vogelsang et al. PNAS 2009

Multi-colour STORM

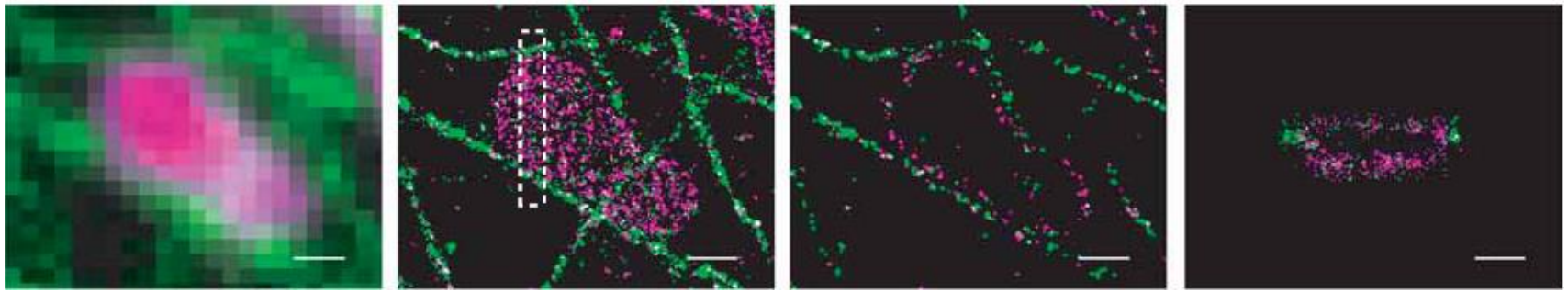


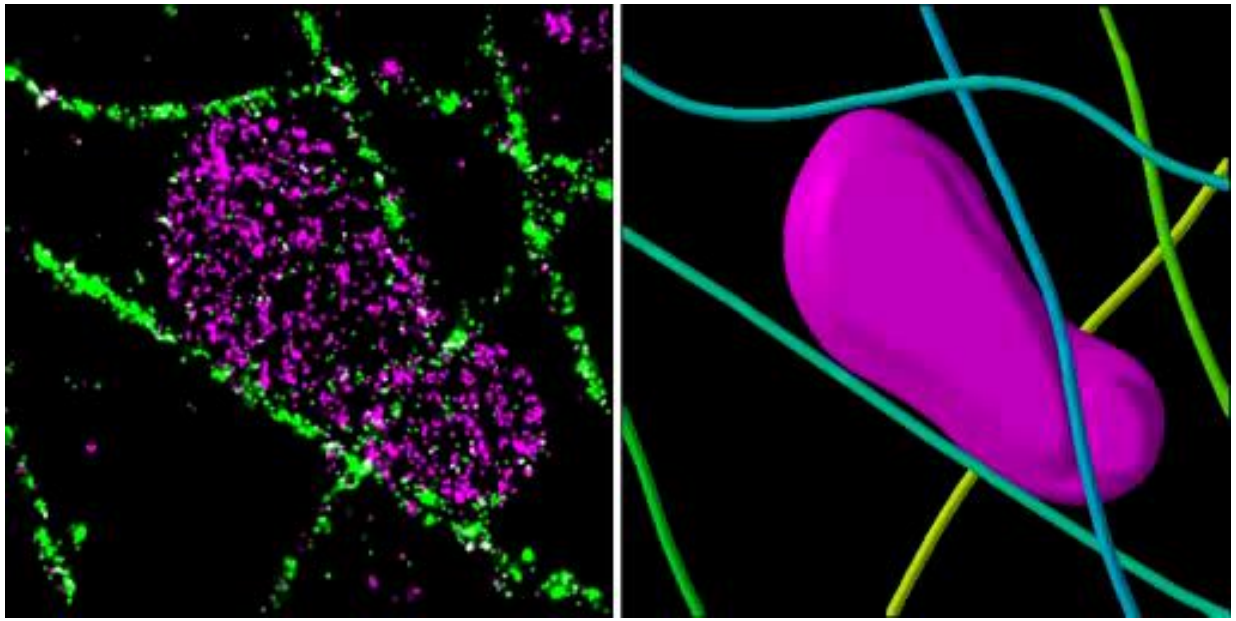
Photoswitchable fluorophores spanning the visible spectrum (Cyanines, Oxazines, Rhodamines, etc)

Heilemann et al. Angewandte Chem. 2009

Multi-colour 3D STORM

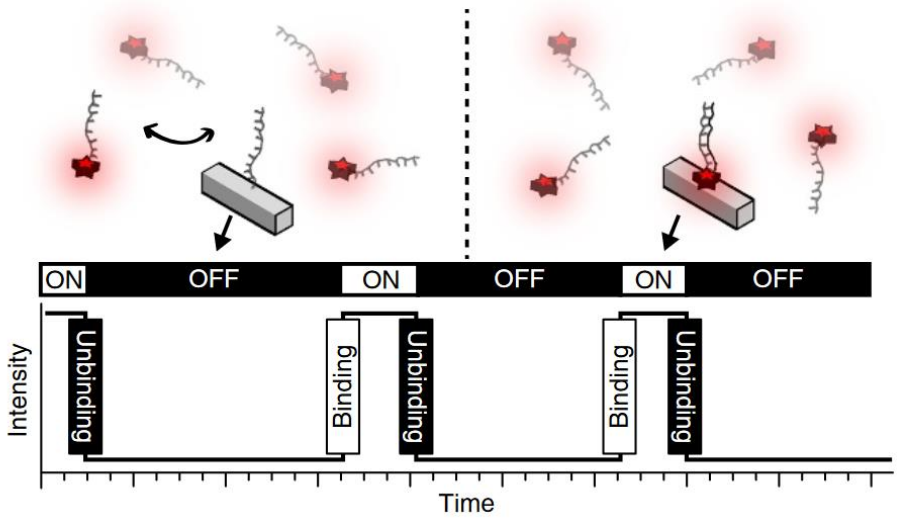
Mitochondria and Microtubule

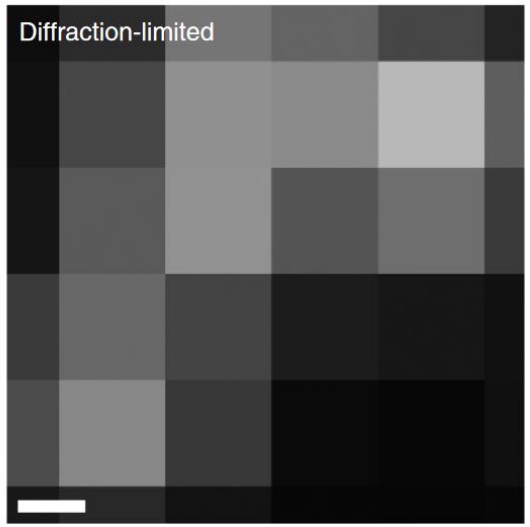


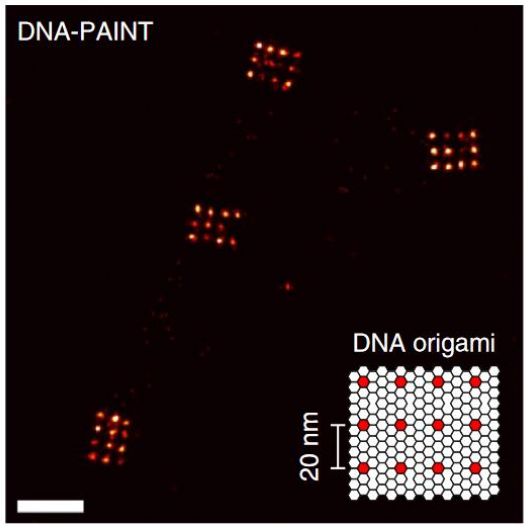


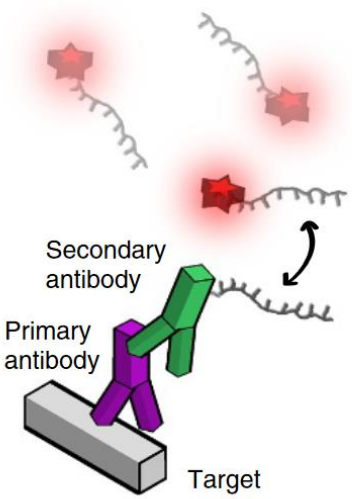
Huang et al. Nature 2008

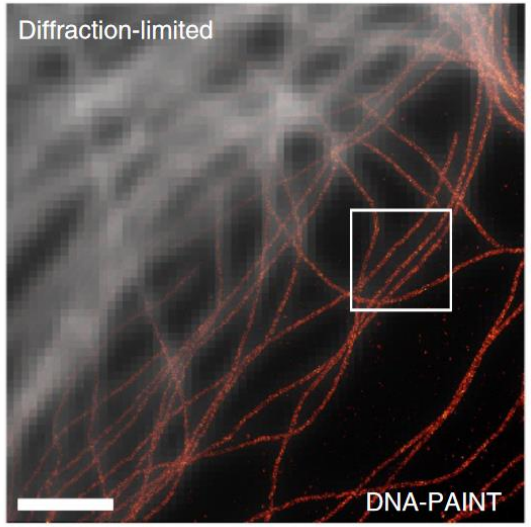
PAINT microscopy

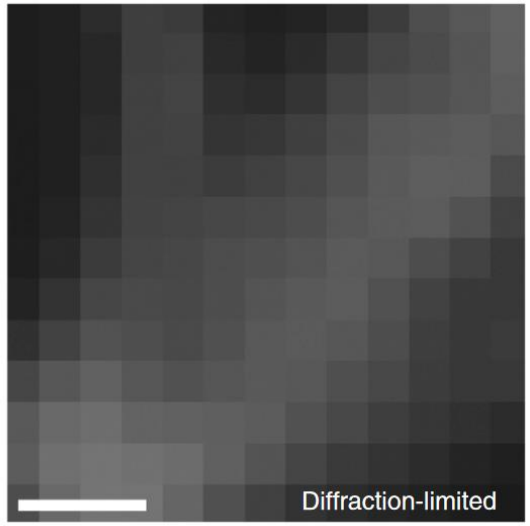


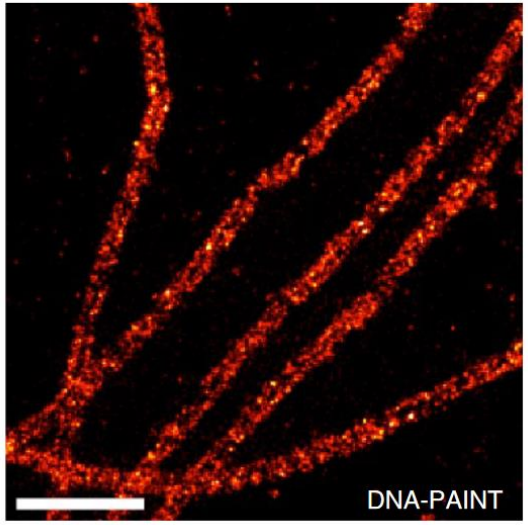






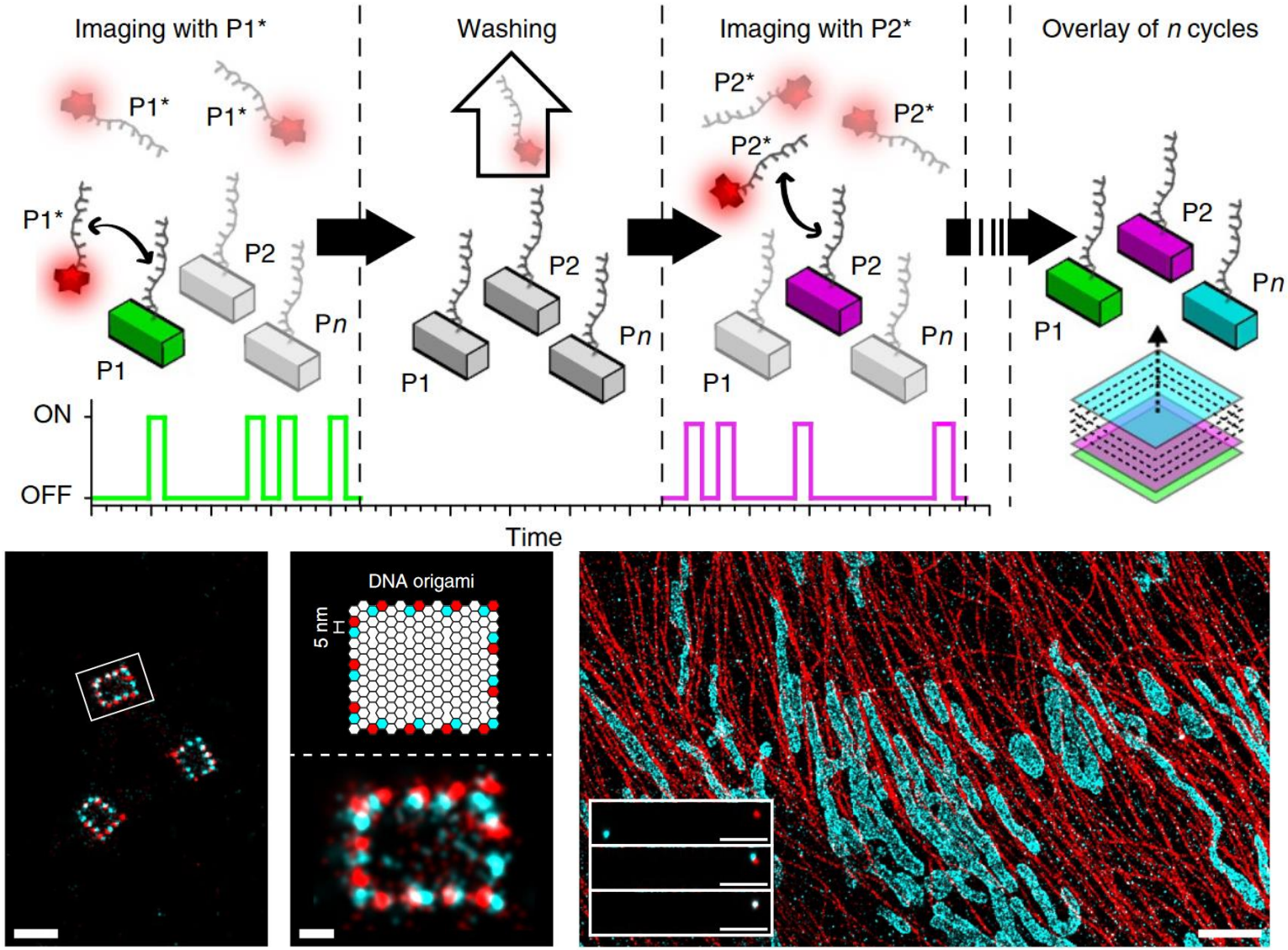






Schnitzbauer et al. Nature Protocols 2017

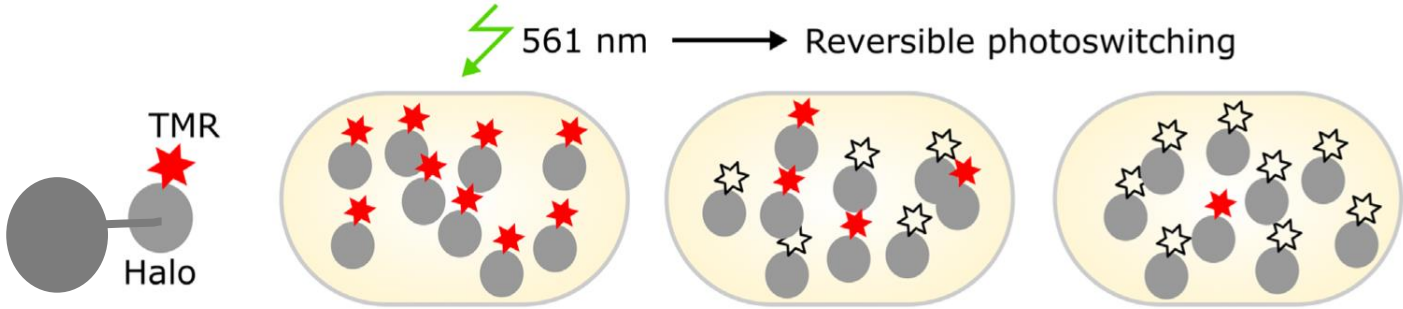
Multiplexed localization microscopy using PAINT



Schnitzbauer et al. Nature Protocols 2017

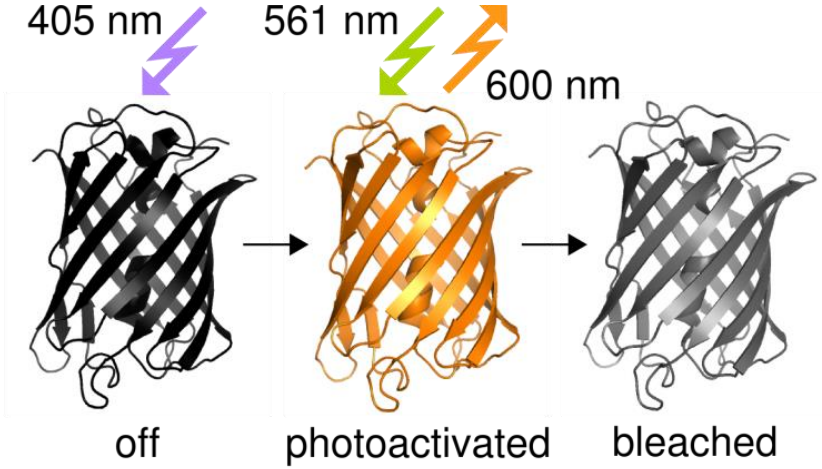
Live-cell localization microscopy using genetically-encoded labels

STORM using protein tags (e.g. Halo, SNAP) labelled with cell-permeable dyes (e.g. TMR, Janelia fluors)

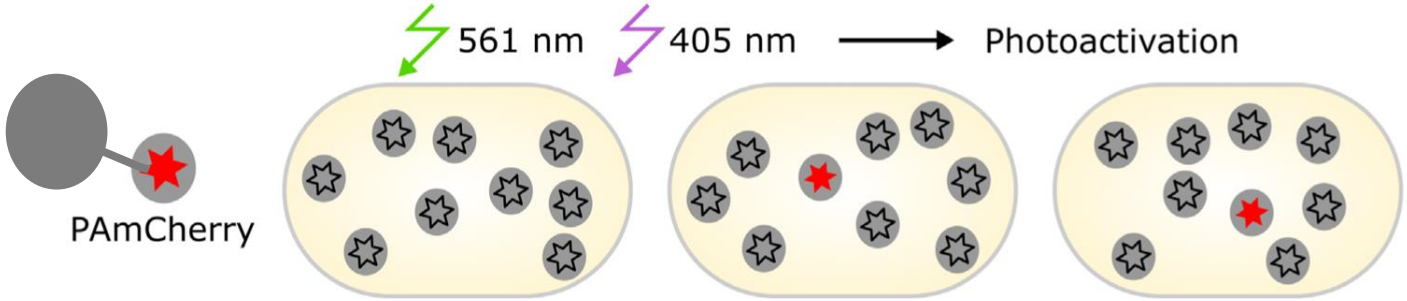


Banaz et al. JPhysD 2019

Photoactivated localization microscopy (PALM) using fluorescent proteins, e.g. PA-GFP, mEOS, PAmCherry

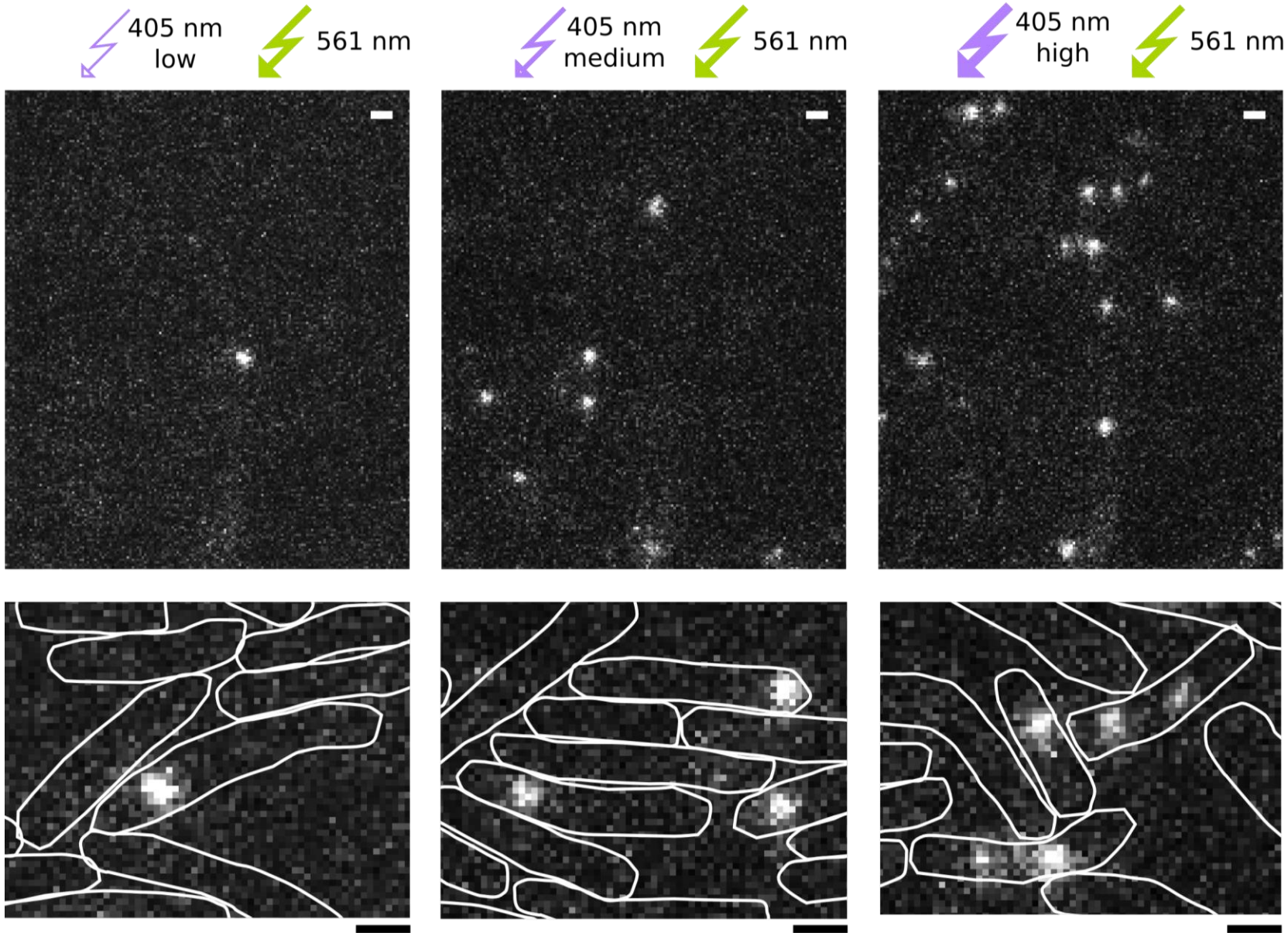


Betzig et al. Science 2006

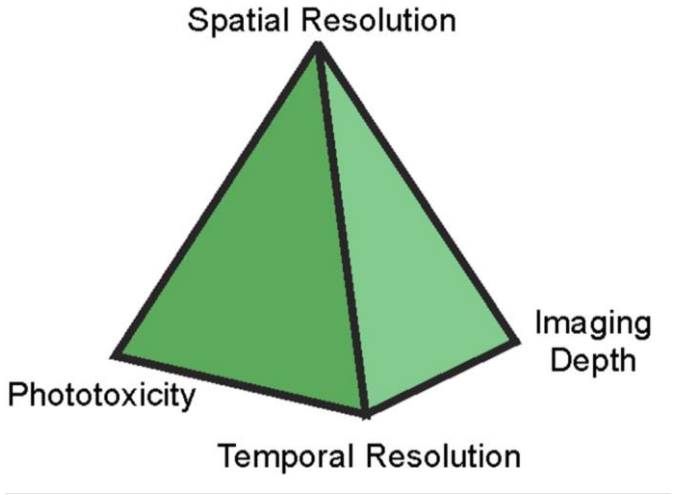


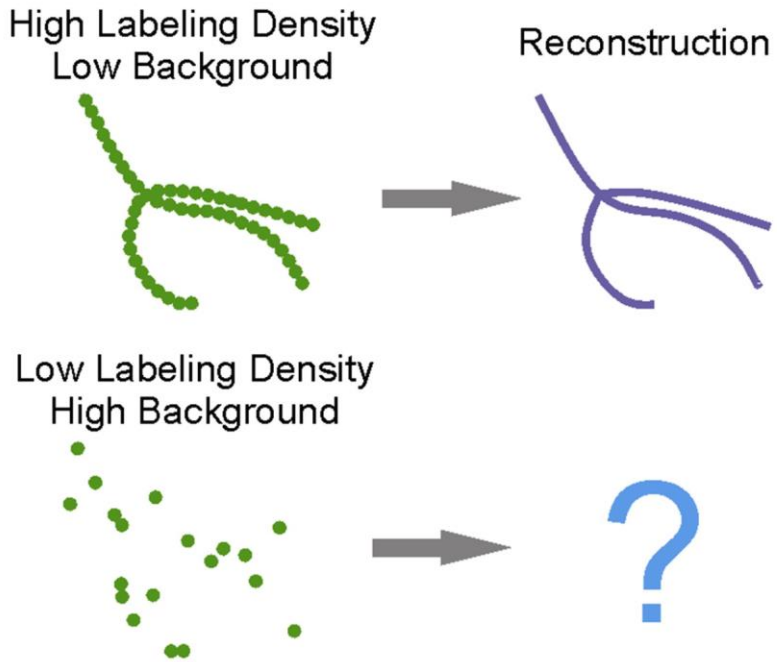
Photoactivated localization microscopy (PALM)

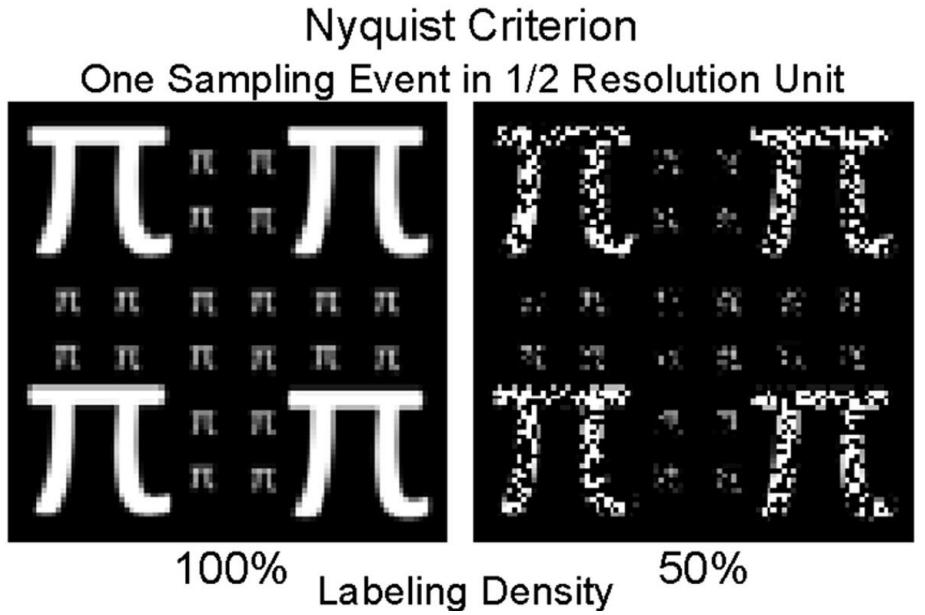
PAmCherry

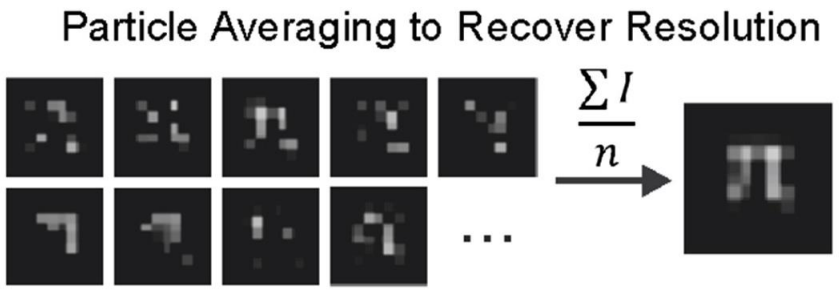


Trade-offs in localization microscopy





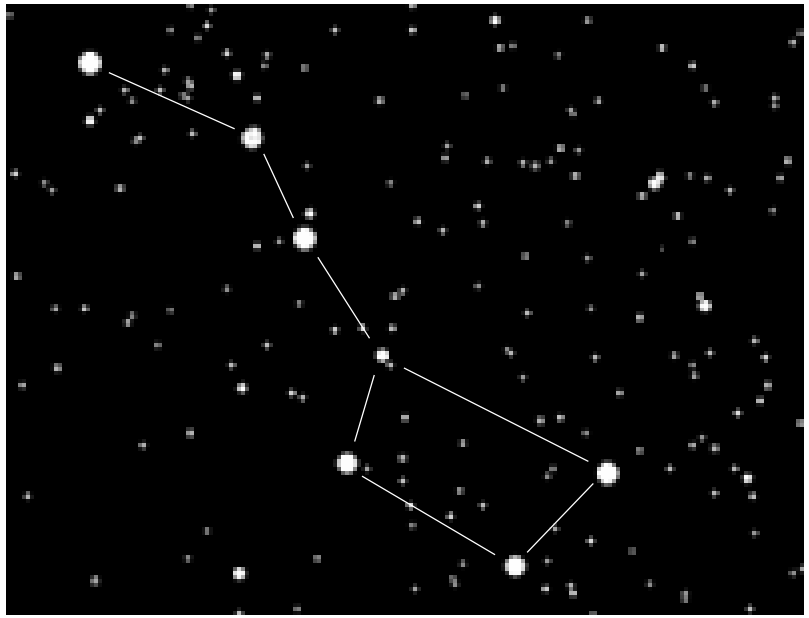


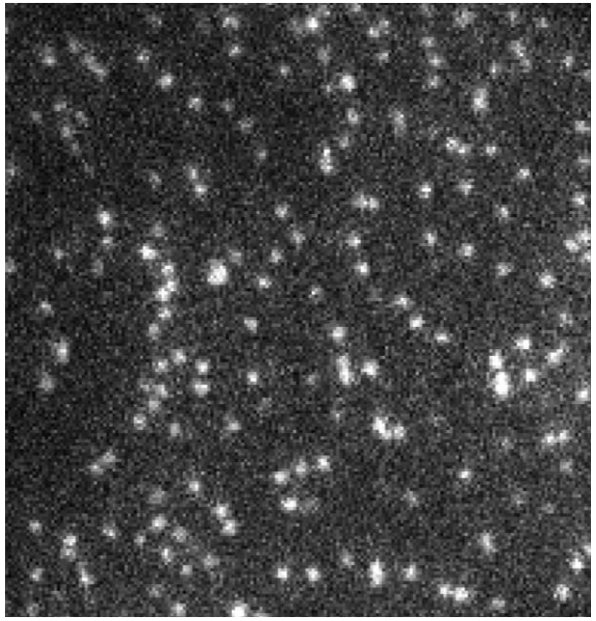


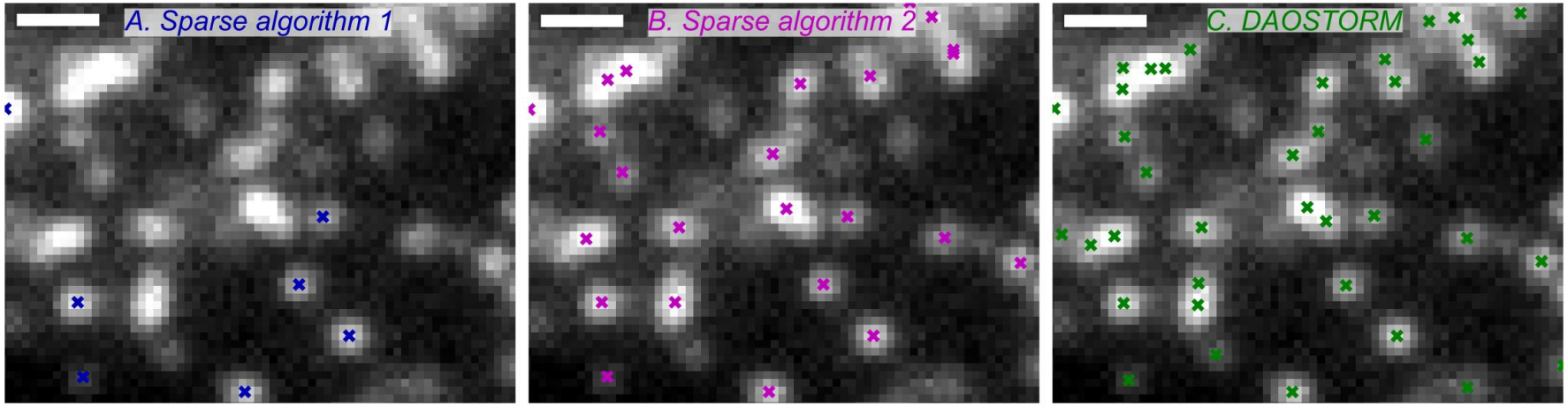
High labelling density requires fluorophores with a long off state!

Liu et al. Mol Cell 2017

High-density localization algorithms

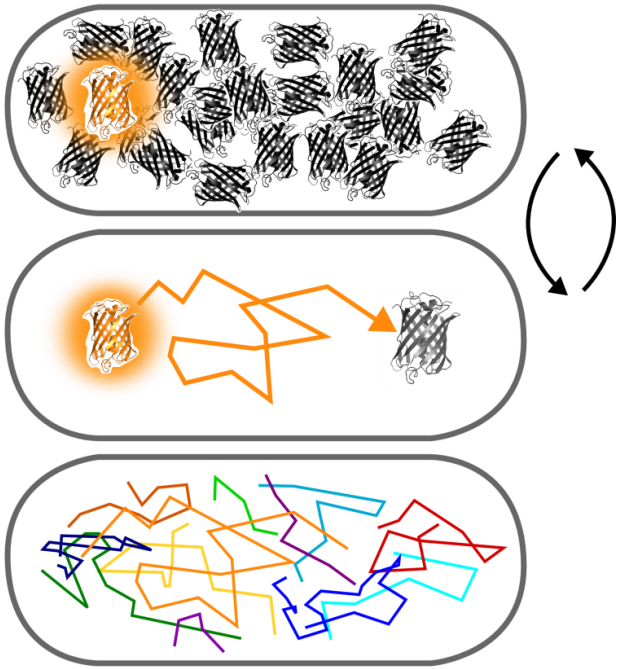


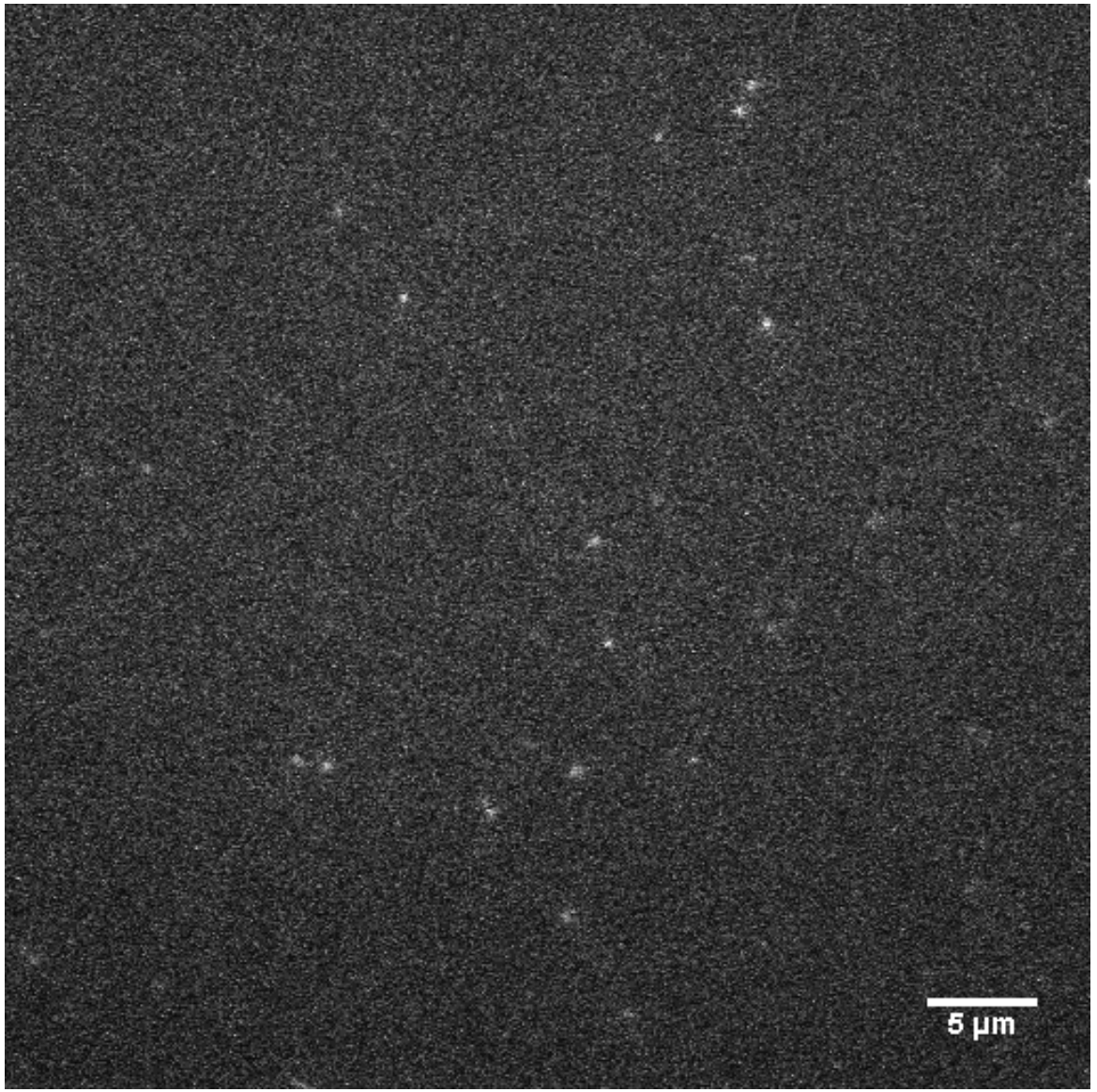




Holden et al. Nature Methods 2011

Photoactivated single-molecule tracking

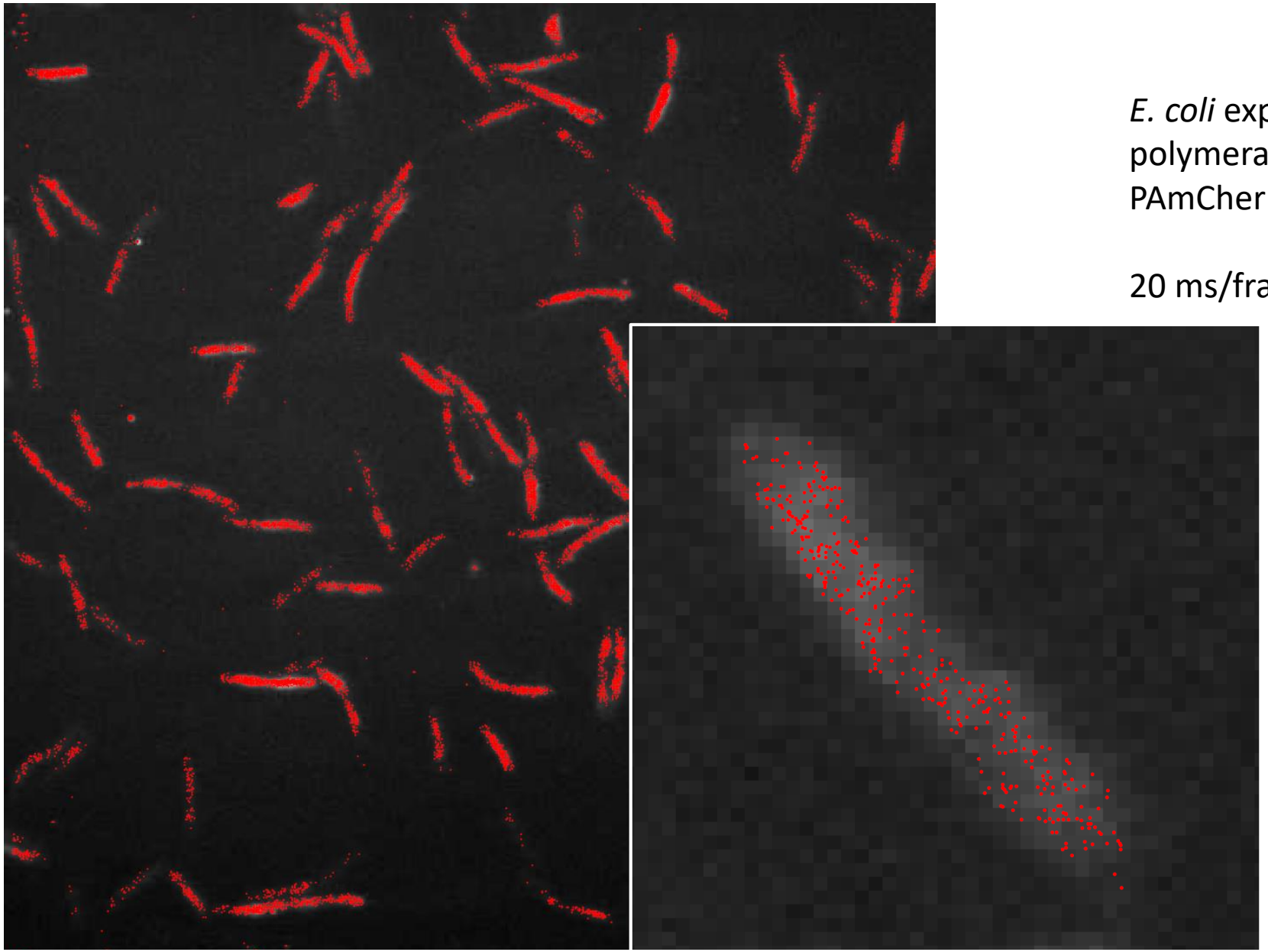




E. coli expressing DNA polymerase fused to PAmCherry

20 ms/frame

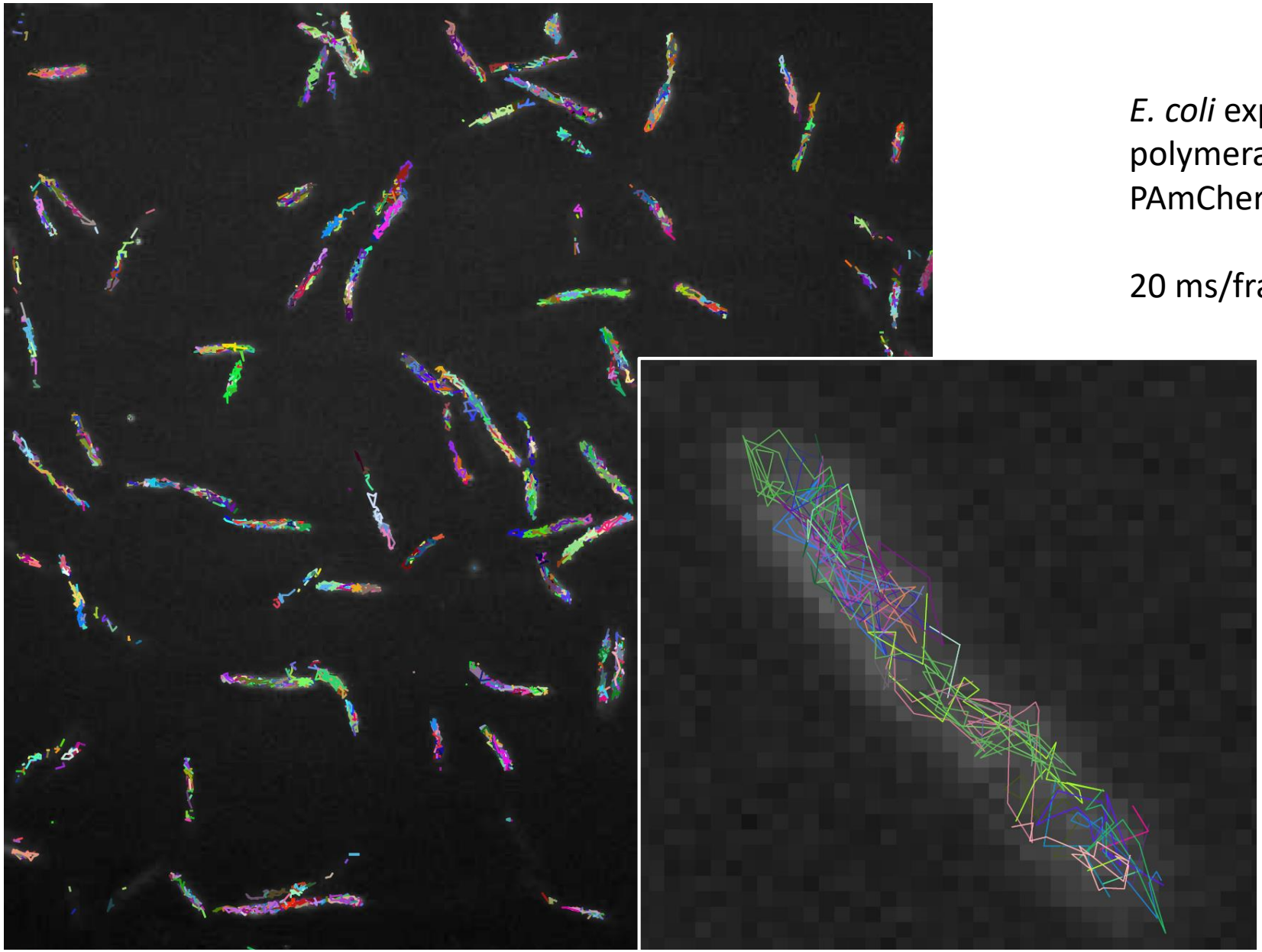
Localization analysis



E. coli expressing DNA polymerase fused to PAmCherry

20 ms/frame

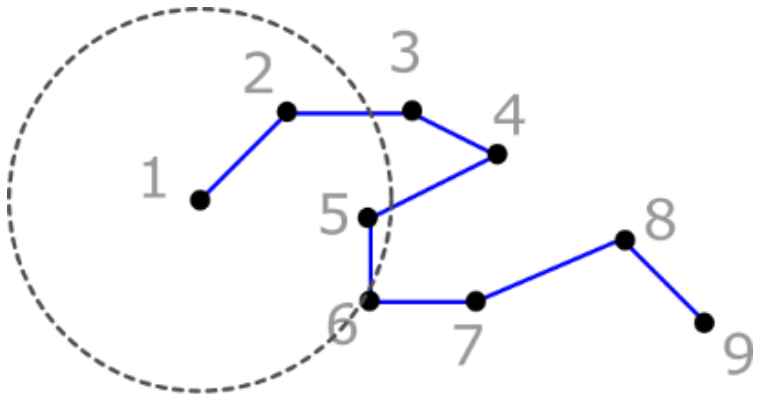
Tracking analysis

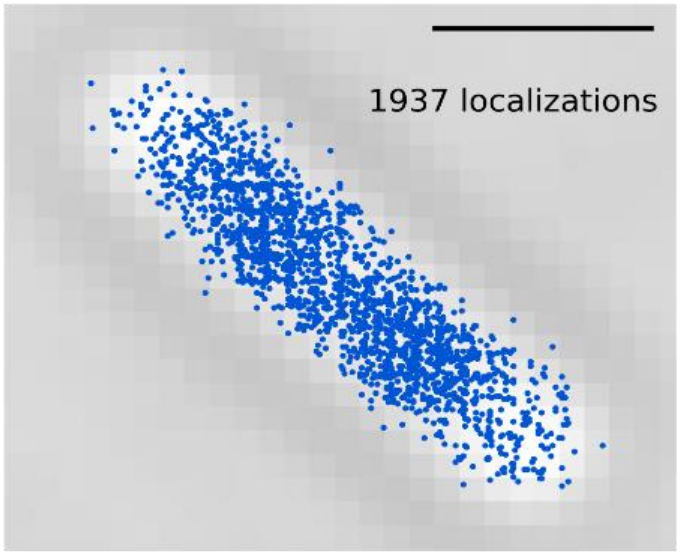


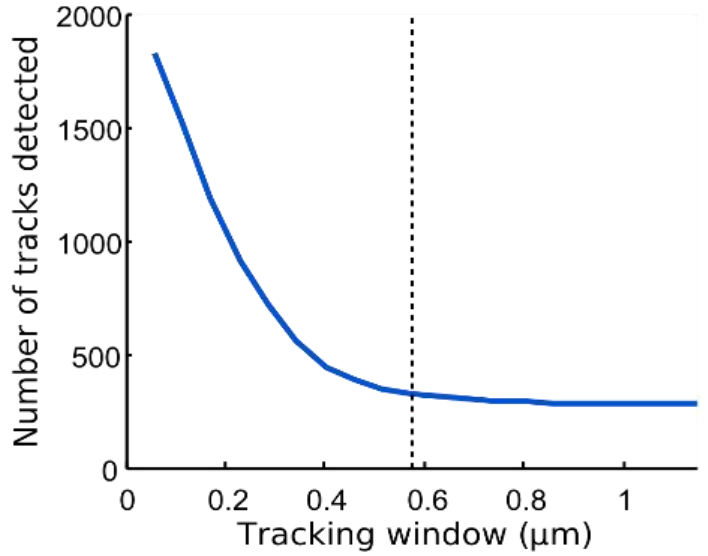
E. coli expressing DNA polymerase fused to PAmCherry

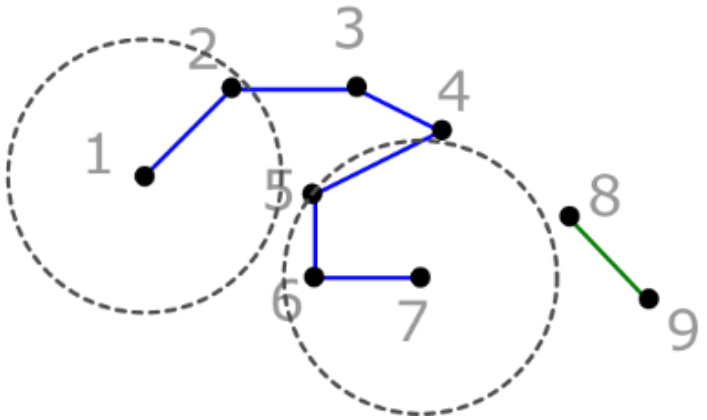
20 ms/frame

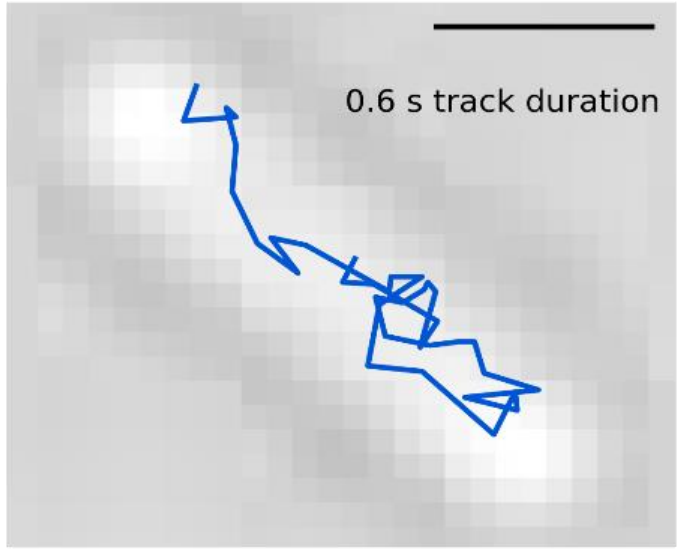
From localizations to tracks

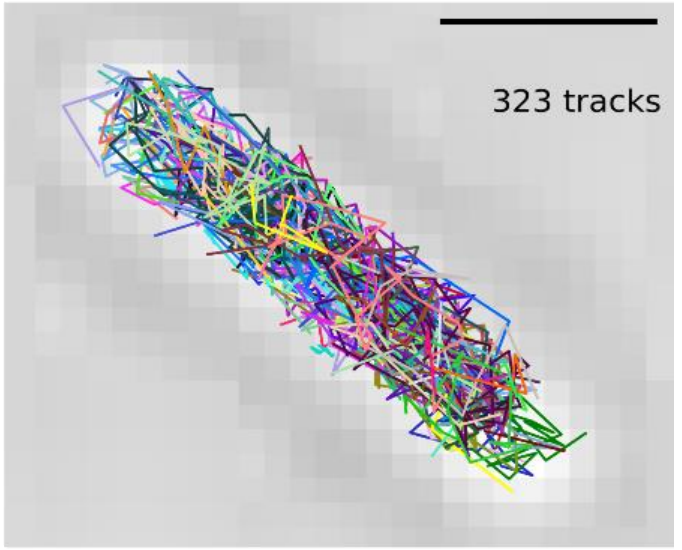




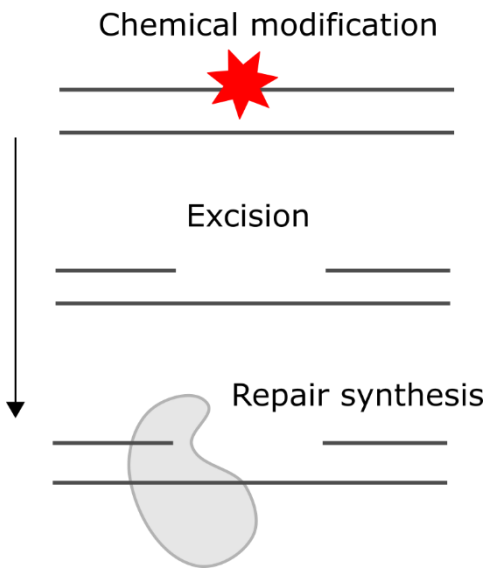


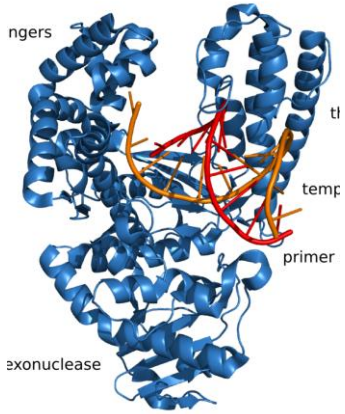




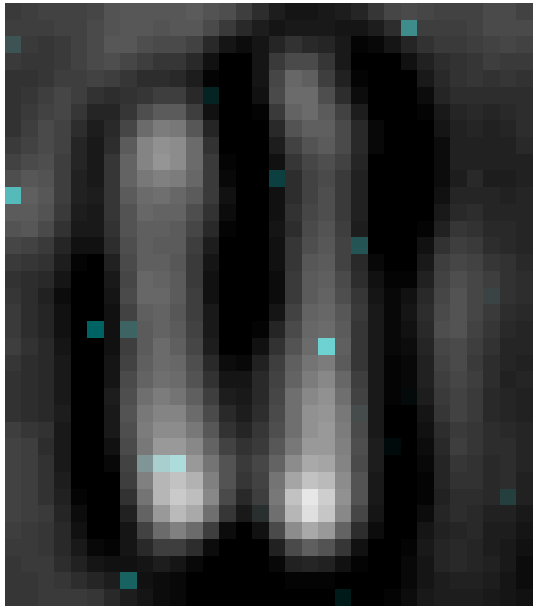


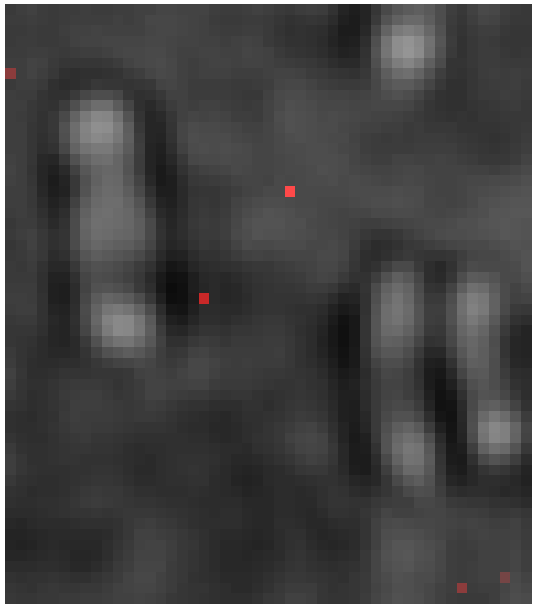
Using tracking to visualise protein function in living cells

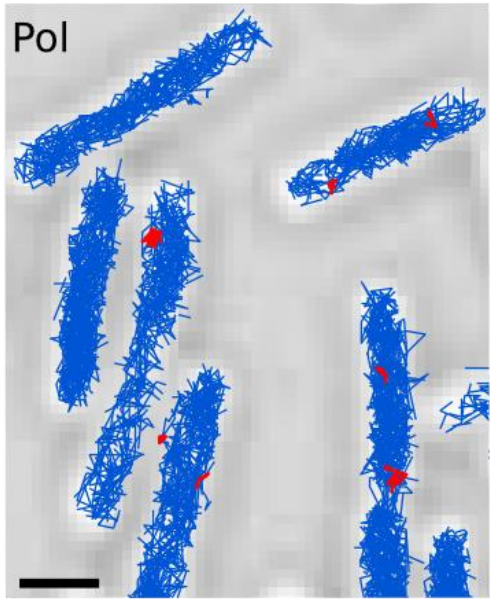


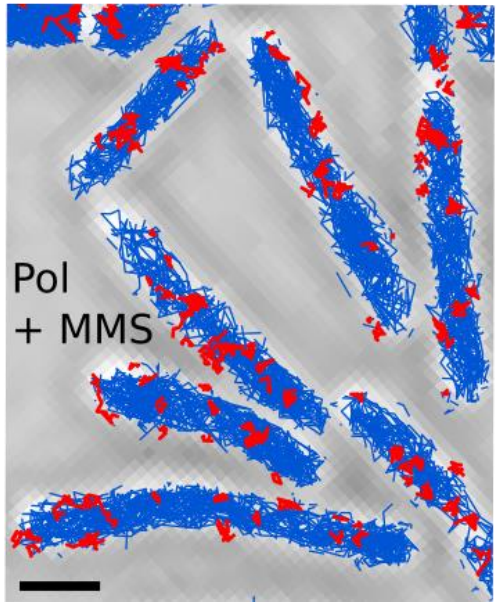


DNA polymerase I





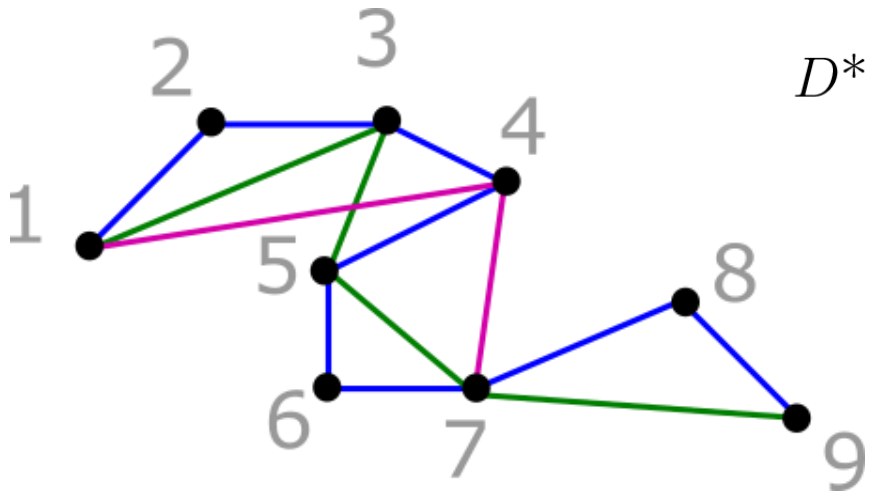




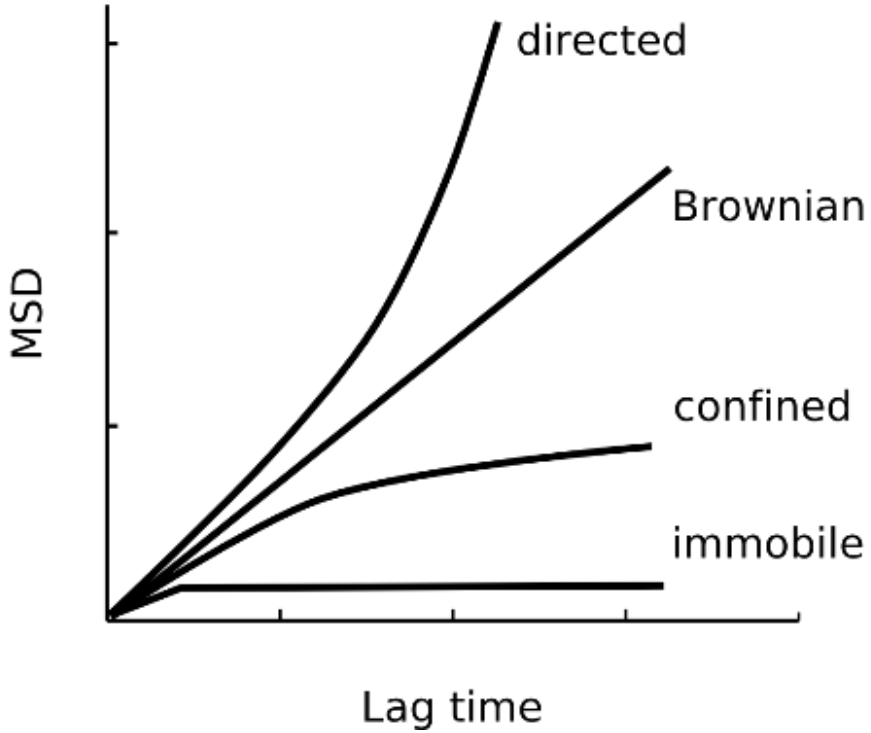
Uphoff et al. PNAS 2013

From tracks to diffusion coefficients

$$\mathrm{MSD}\,(n\Delta \mathrm{t}) = \frac{1}{N-n}\sum_{i=1}^{N-n}\left(x_{i+n}-x_i\right)^2 + \left(y_{i+n}-y_i\right)^2$$

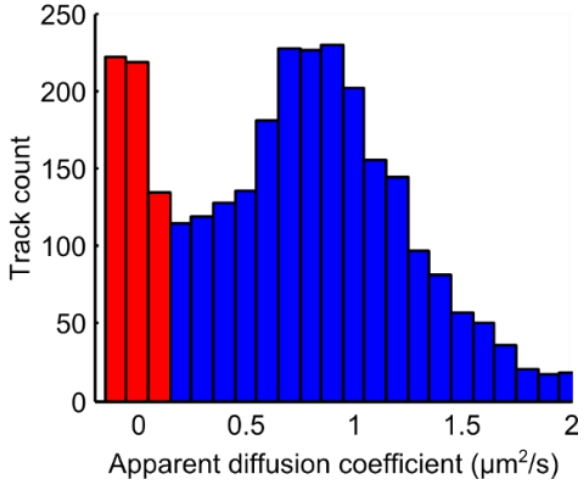


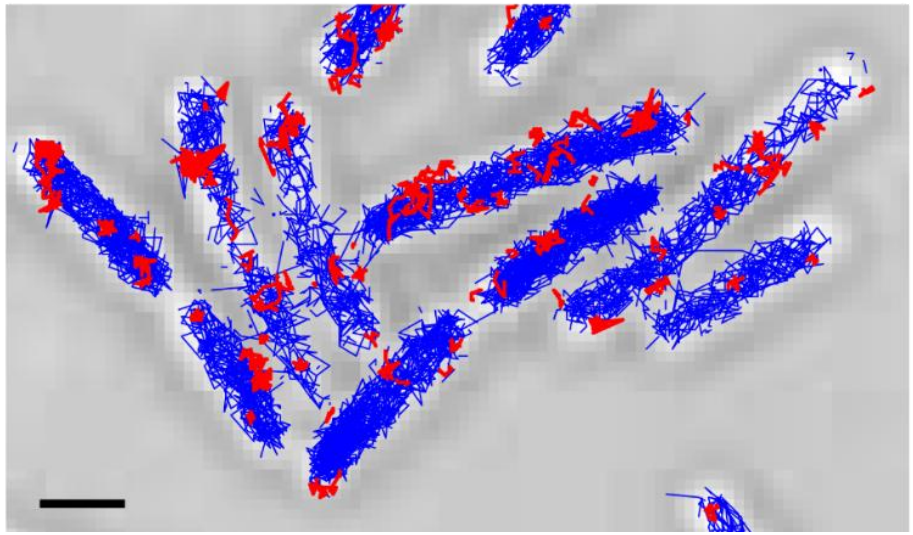
$$D^* = \frac{\mathrm{MSD}}{4\cdot\Delta \mathrm{t}}$$



The distribution of diffusion coefficients reveals molecular subpopulations

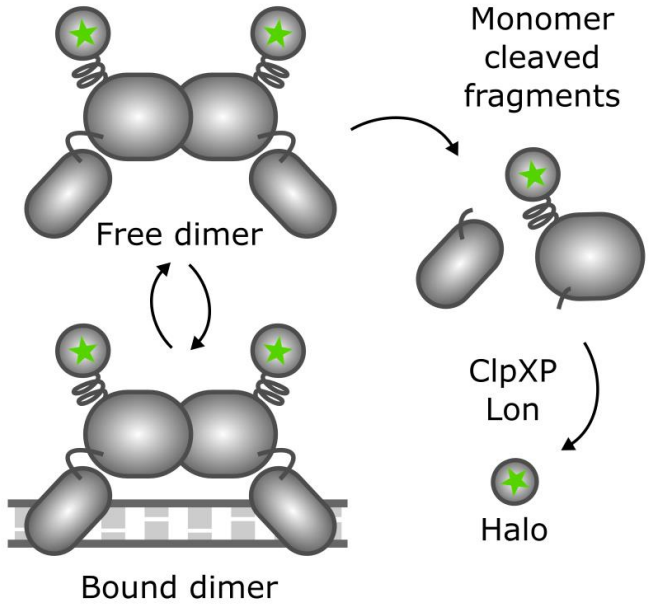
DNA polymerase I

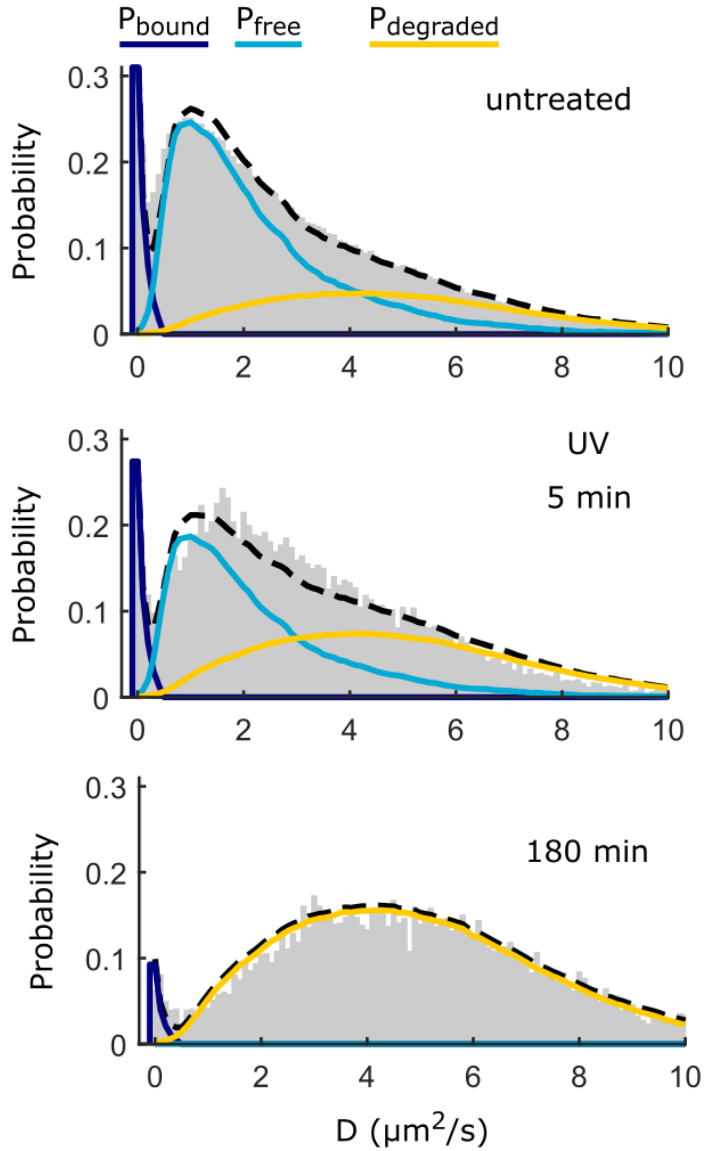




Uphoff Methods Mol Biol 2016

LexA

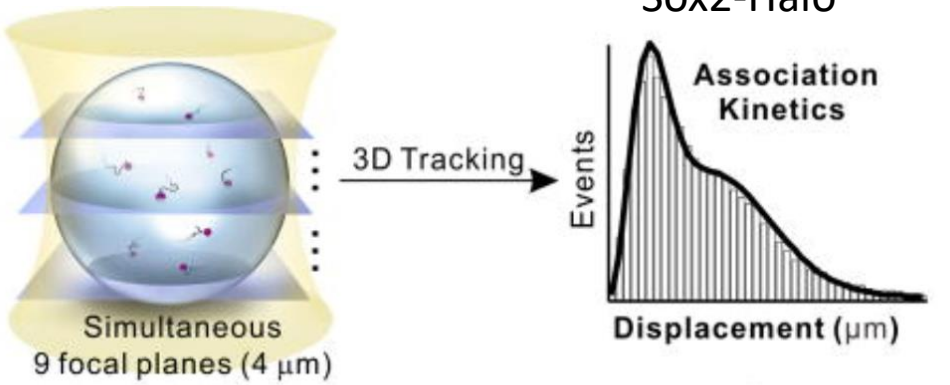


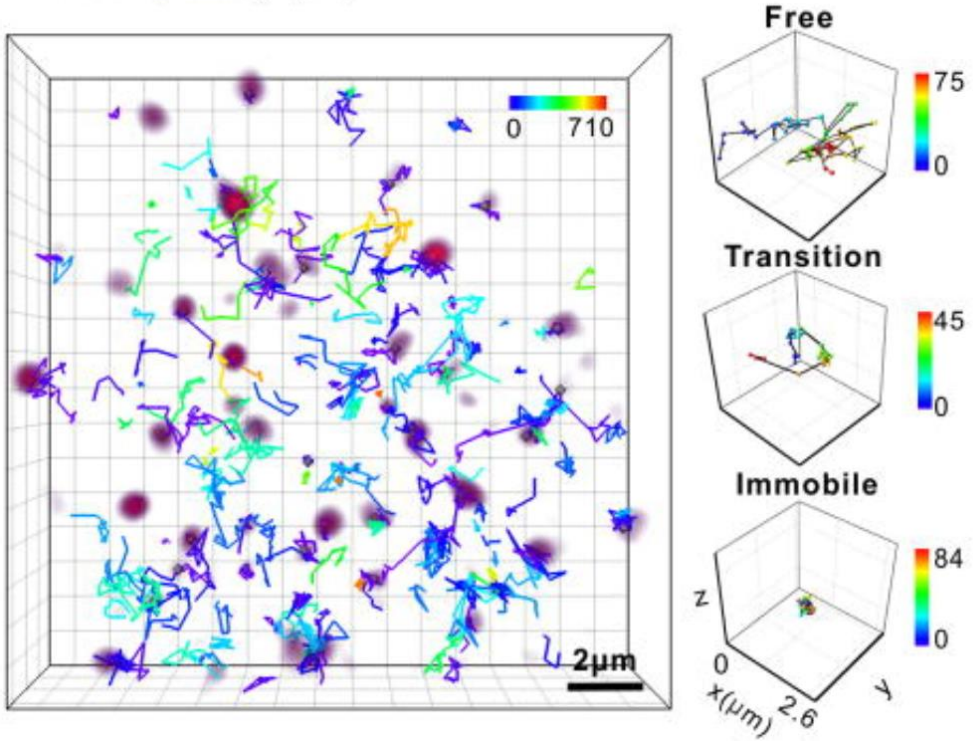


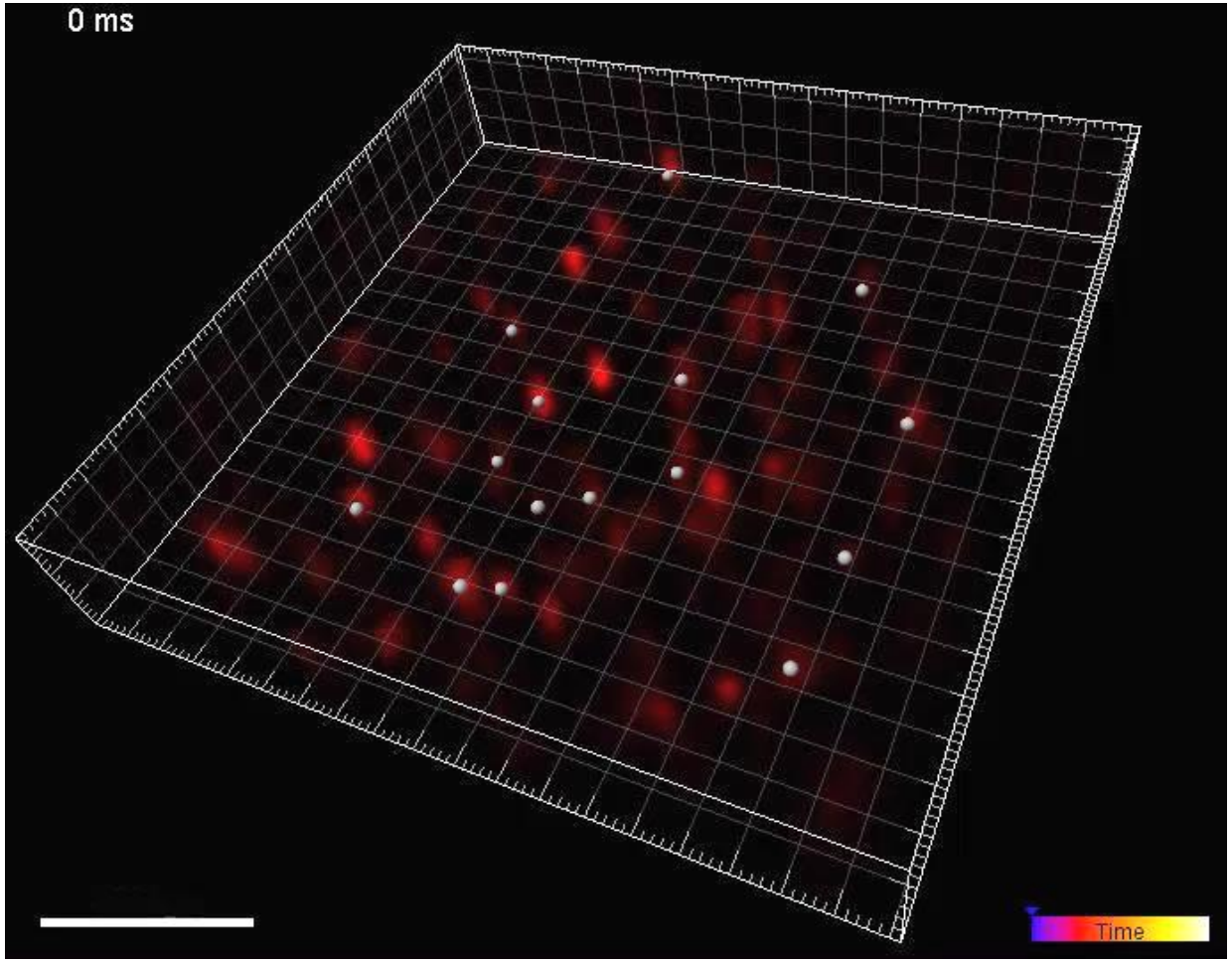
Jones et al. bioRxiv 2020

3D tracking in mammalian nuclei

Sox2-Halo

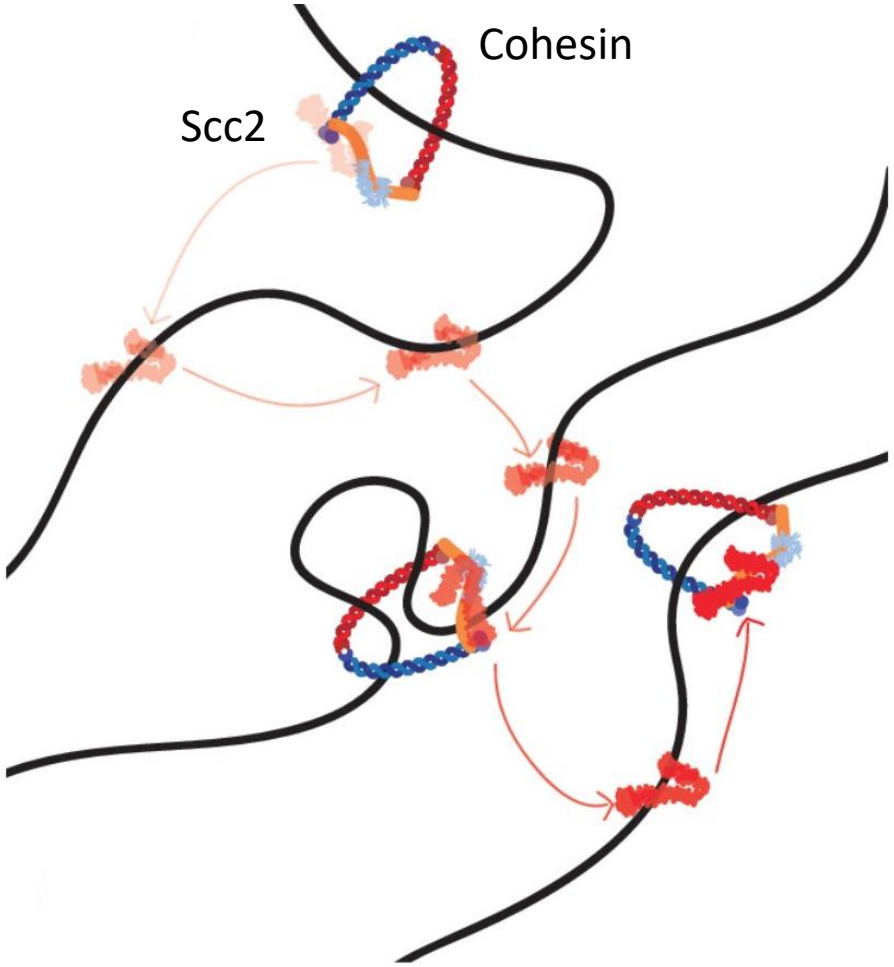


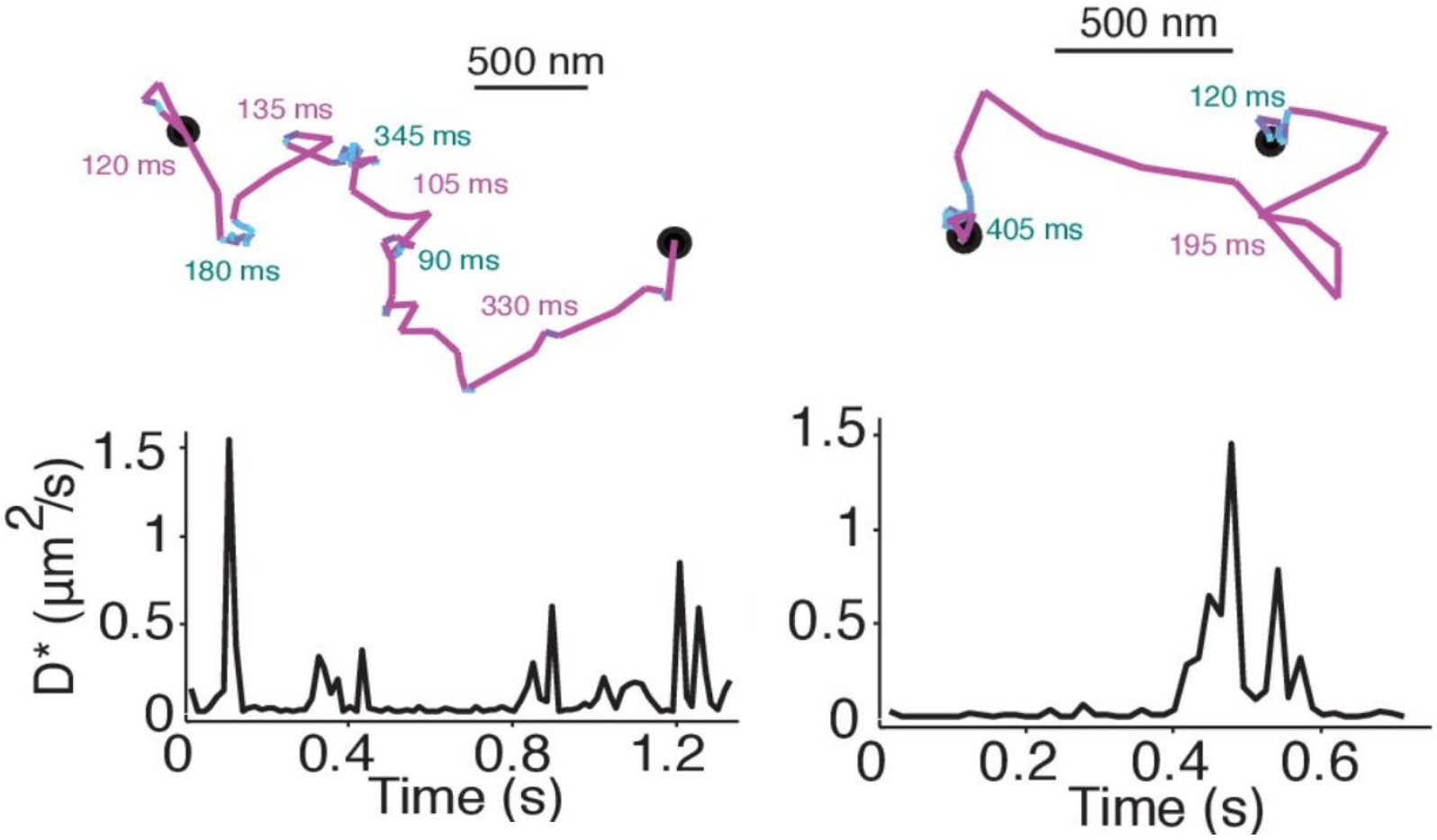




Chen et al. Cell 2014

Measuring transient binding events





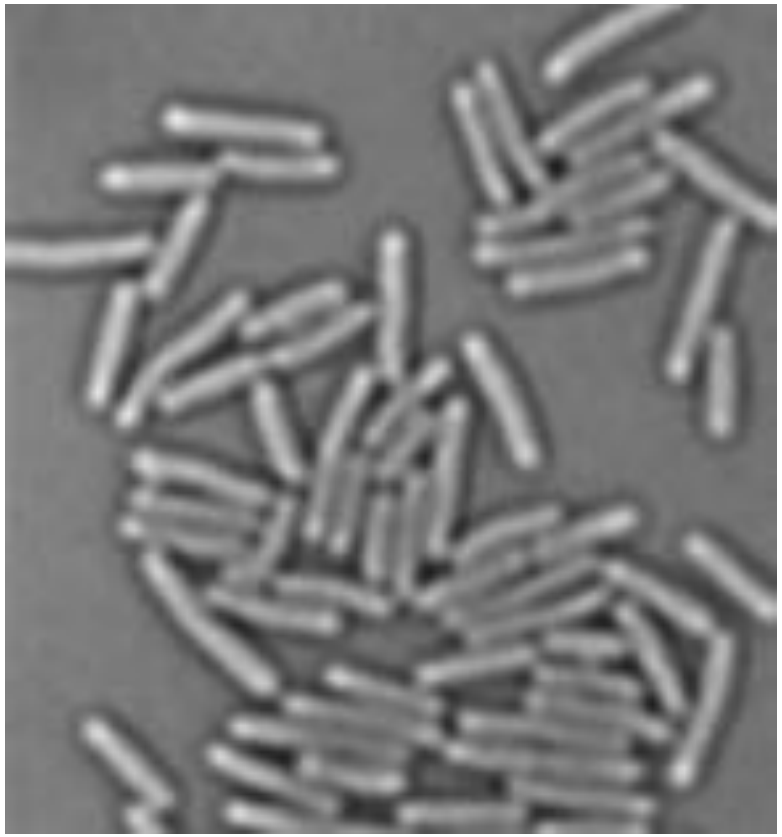
Rhodes et al. eLife 2017

Measuring long-lived binding events

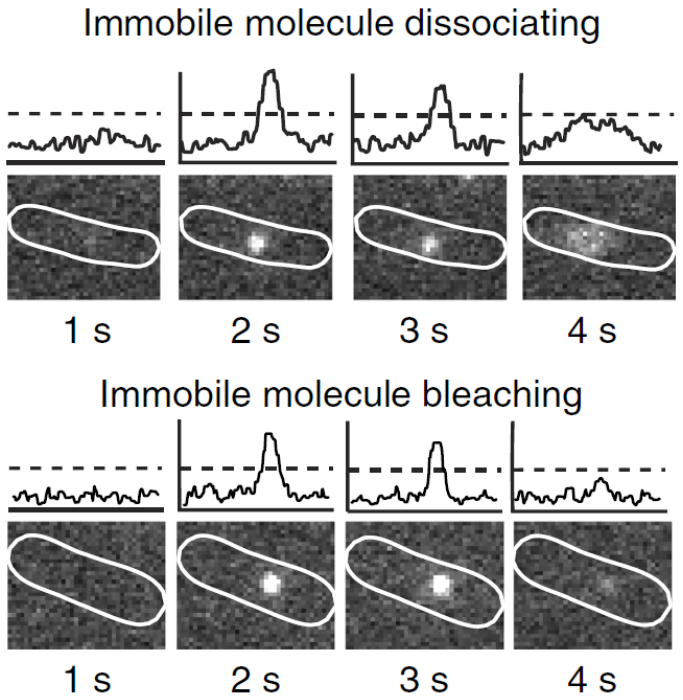
Photobleaching limits the observation time per molecule

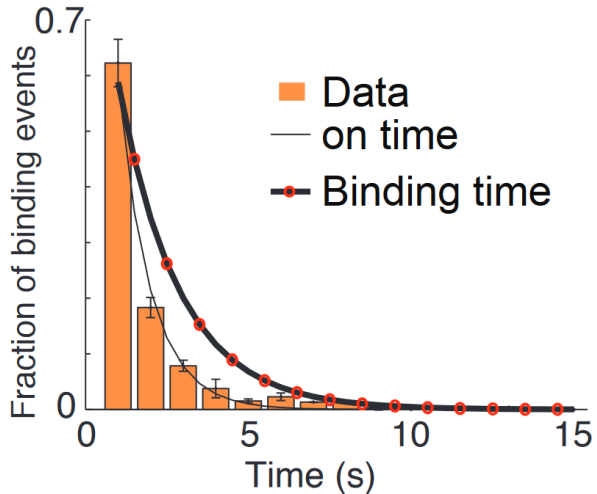
How can we extend the duration of the tracks to measure binding kinetics?

-> Reduce excitation intensity & increase exposure time



1 s/frame





Stracy et al. Nature Comm 2016

Summary

- Very high spatial resolution
- Single-molecule observation
- Localization microscopy offers more than pretty images
  - Molecular trajectories
  - Diffusion coefficients
  - Molecule counting
  - Spatial distributions, clustering
  - Colocalization
  - Interaction kinetics
- Many flavours of the method enable diverse applications
- Comparatively modest cost

Open challenges:

- Phototoxicity in live cells
- Special dyes (+ buffer) required
- Have to record a movie to get an image
- Photophysics, blinking
- Limited observation time per molecule
- Imaging deep / high background
- Data visualisation & interpretation
- One molecule visible at a time means many invisible molecules!
- Difficulty: Advanced