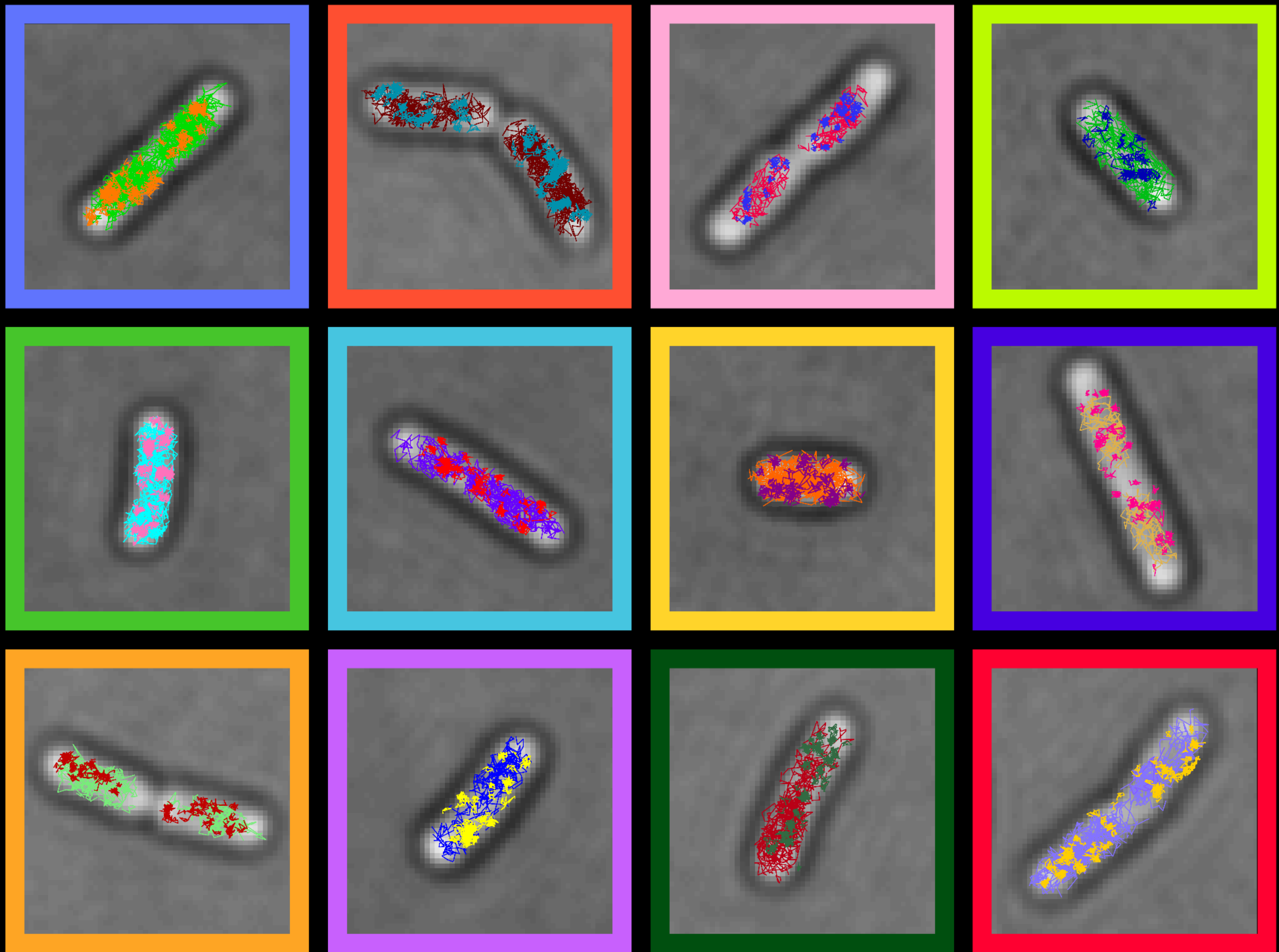
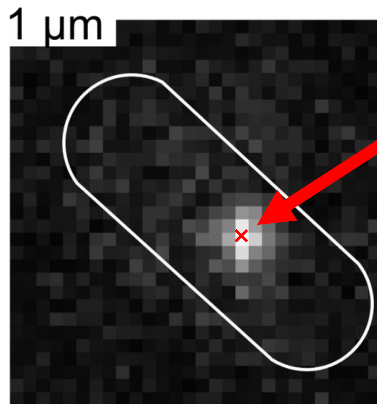
Picture courtesy of Mathew Stracy

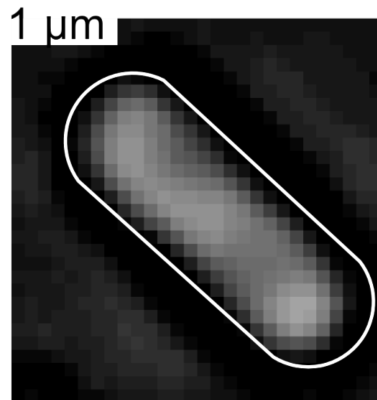


Nanometer resolution by localisation microscopy

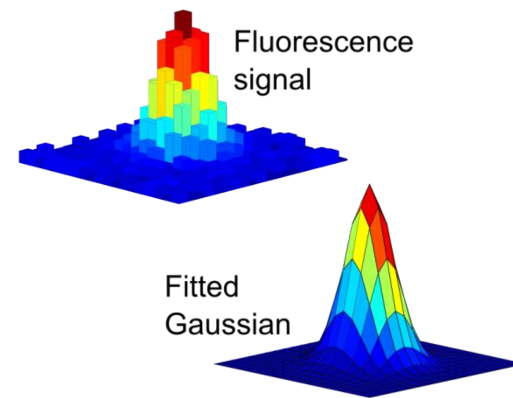
Localising single molecules



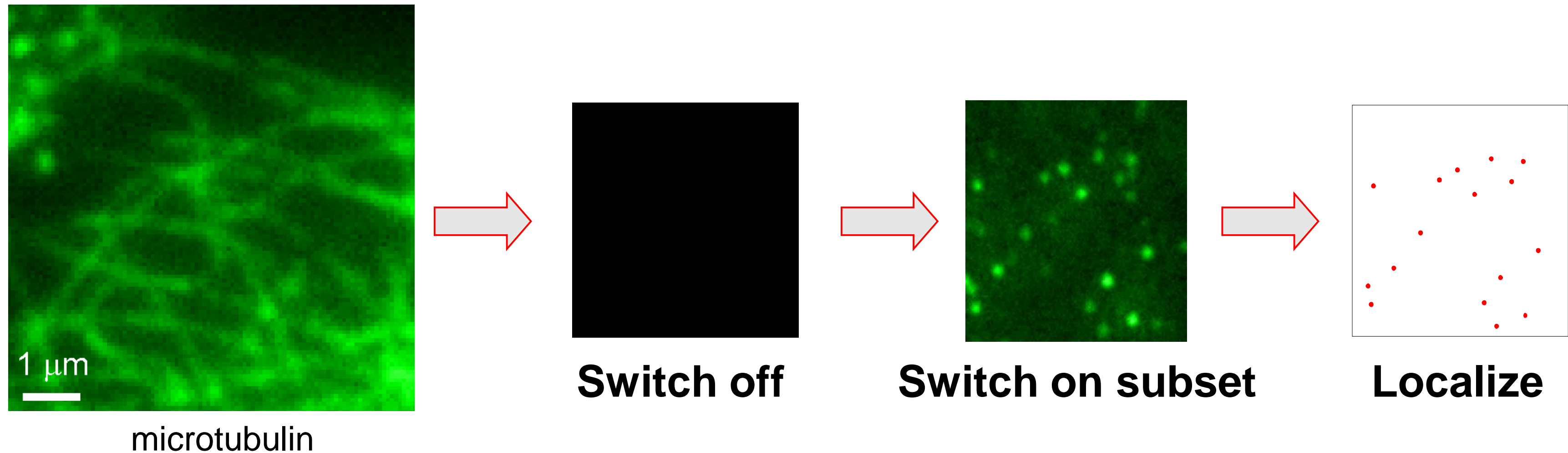
Single labelled protein

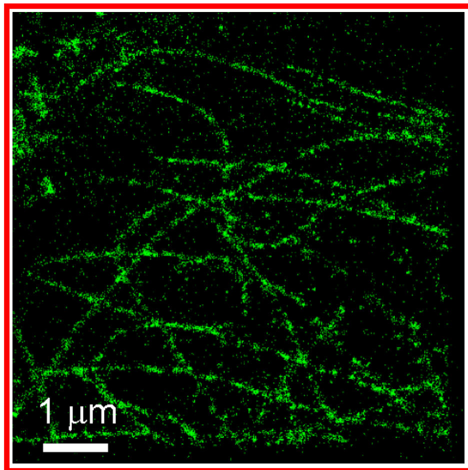


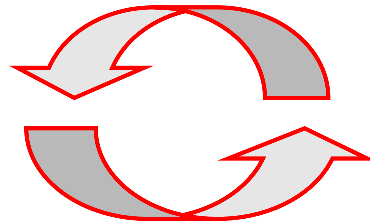
Many labelled proteins

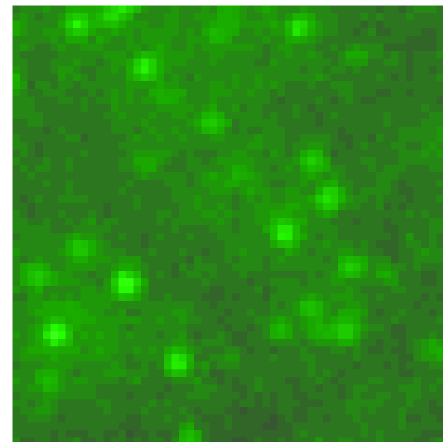


Localisation-based super-resolution imaging









Some history

SCIENCE

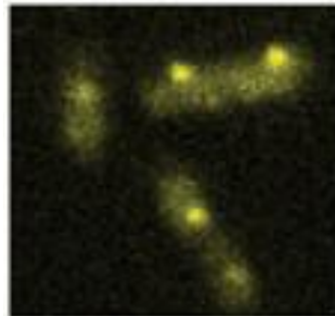
Nobel Laureates Pushed Limits of Microscopes

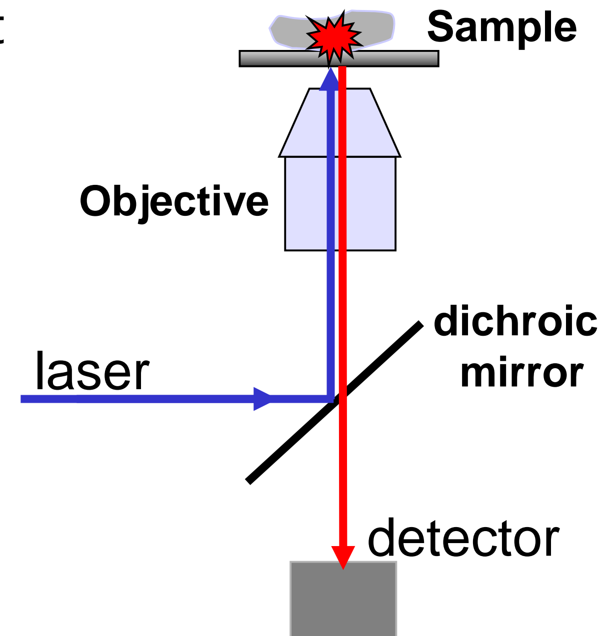
By KENNETH CHANG OCT. 8, 2014

Stefan Hell William Moerner Eric Betzig

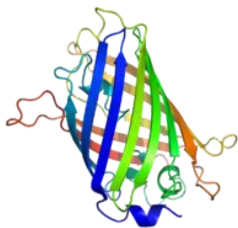
How to image single molecules in 3 simple steps

Step 1: Label your molecule with a fluorescent probe

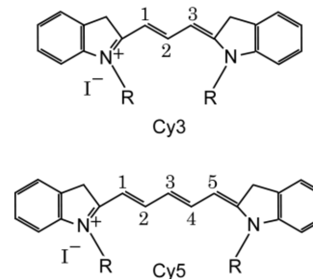




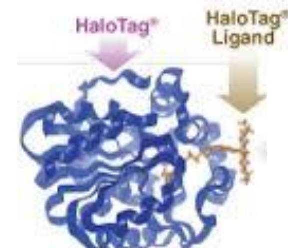
fluorescent proteins



synthetic fluorophores

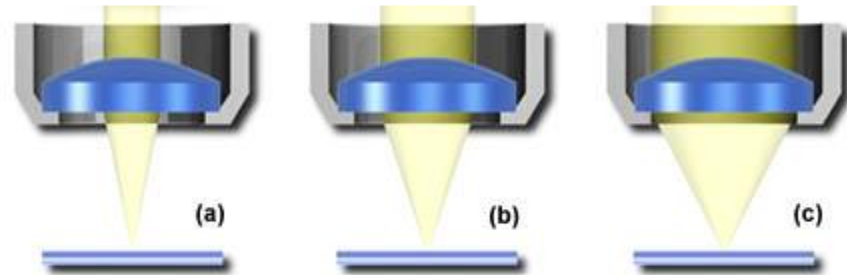


tag labelling



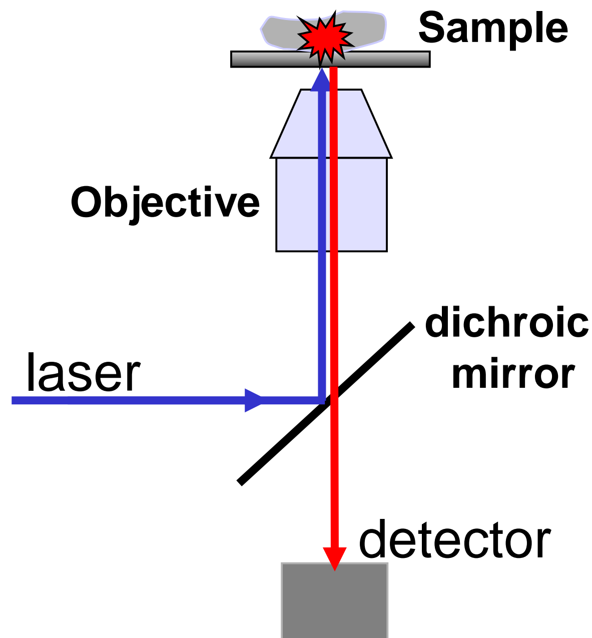
How to image single molecules in 3 simple steps

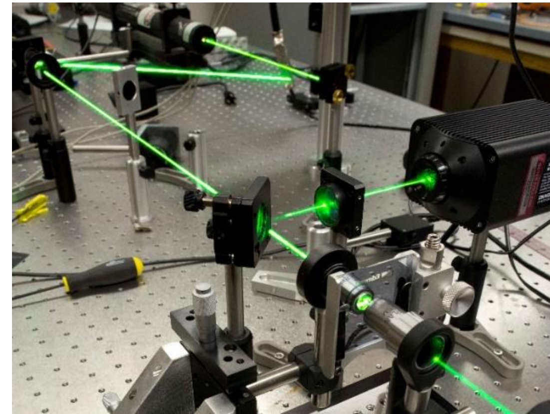
- High numerical aperture objective

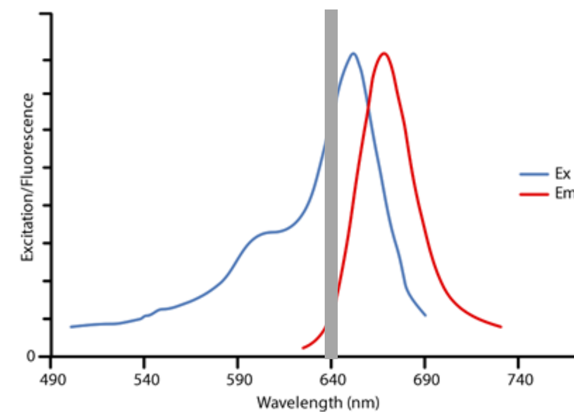


How to image single molecules in 3 simple steps

- High numerical aperture objective
- Laser excitation







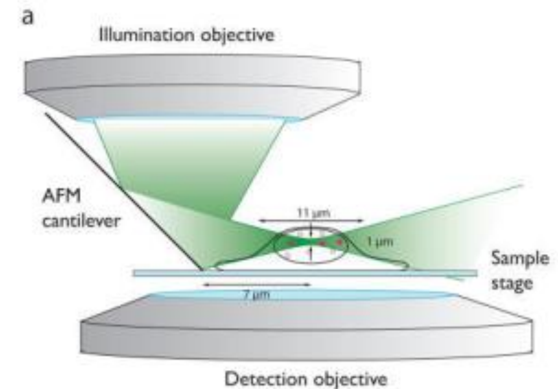
How to image single molecules in 3 simple steps

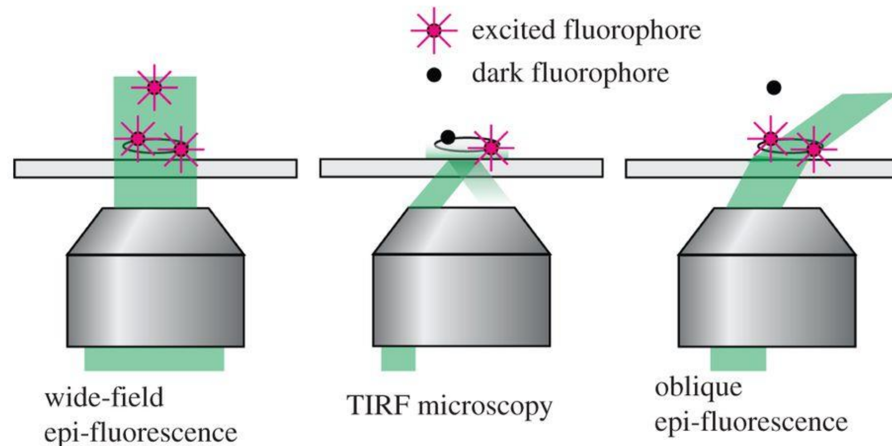
- High numerical aperture objective
- Laser excitation
- Sensitive camera (such as an Electron multiplying CCD)

How to image single molecules in 3 simple steps

Step 3: Get rid of all background fluorescence

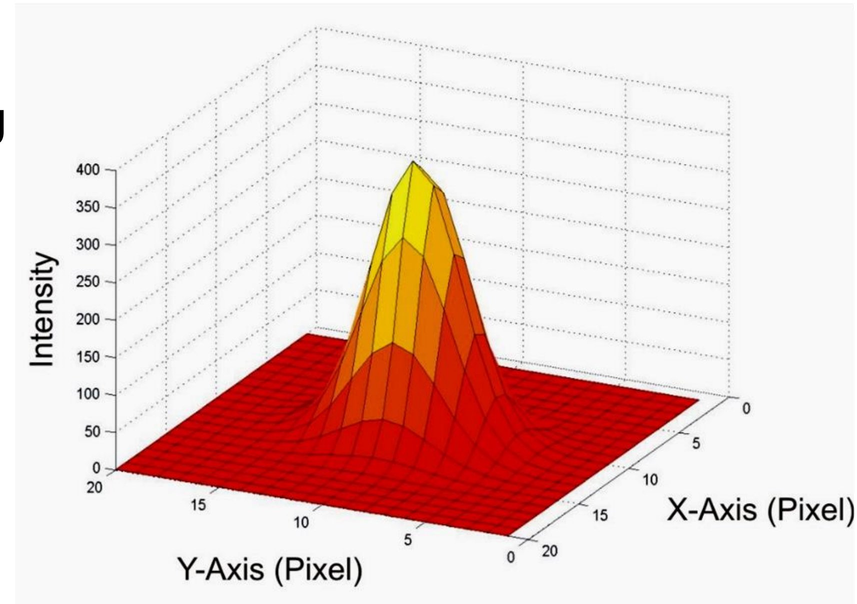
- Clean coverslips
- Low fluorescence growth media
- Narrow illumination area





How to get nm precision

Determine center of emission pattern by fitting a 2D Gaussian to the PSF



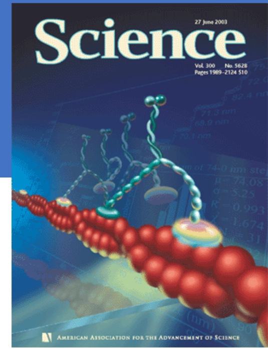
$$I(x, y) = I_0 + A \cdot e^{-\frac{(x-x_c)^2}{2s_x^{\,2}}} \cdot e^{-\frac{(y-y_c)^2}{2s_y^{\,2}}}$$

Standard deviation of Gaussian fit along x or y

$$\sigma_\mu \sim \frac{s_i}{\sqrt{N}}$$

for 10^4 photons
precision of 125nm/100
~ 1.25 nm (!)

How does myosin V "walk"?



Myosin V: cargo-carrying processive motor

Two actin binding, ATPase "heads" joined by a stalk

cargo binding domain

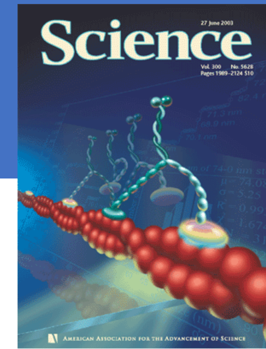
head

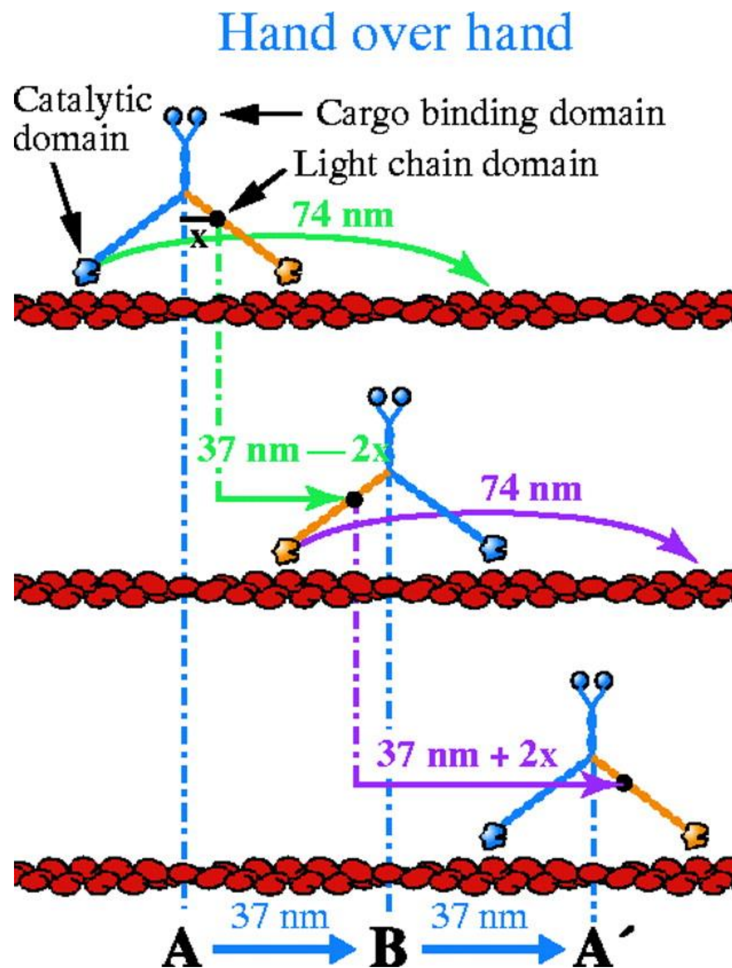
head

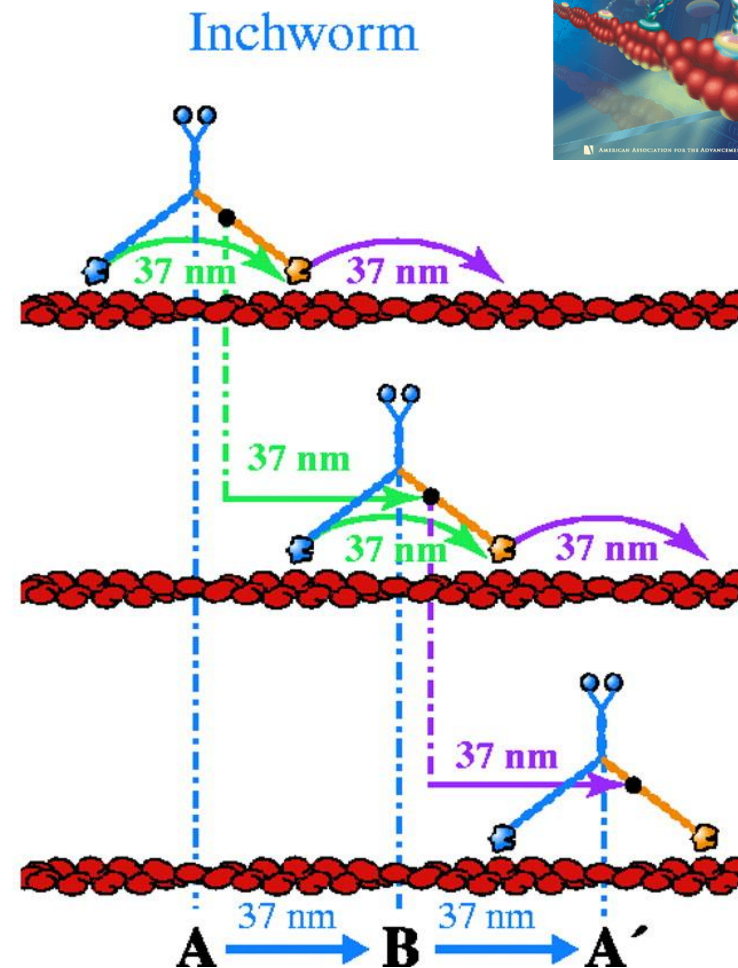
actin

37nm

How does myosin V "walk"?







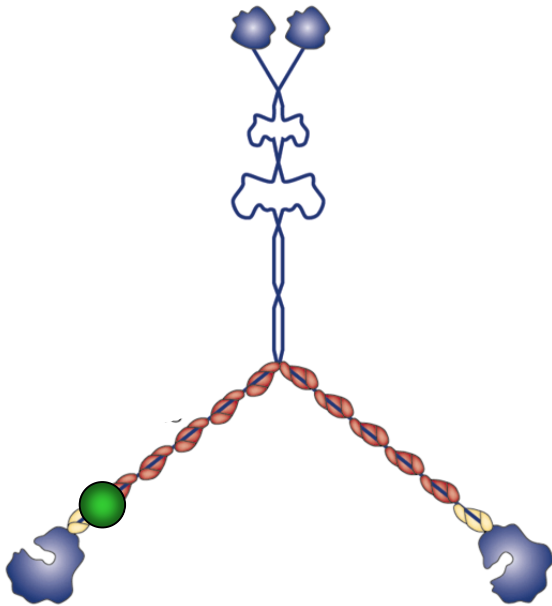
Probe step sizes:
74, 0, 74, 0, 74

Probe step sizes:
37, 37, 37, 37

Yildiz *et al* (2003) *Science* 300, 2061

Measuring myosin V steps using FIONA
(Fluorescence Imaging with One-Nanometer Accuracy)

1) labeled myosin V



2) Add labeled myosin V to actin; located probe

3) Add ATP: myosin moves

4) Measure step size

B

Photons: 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200

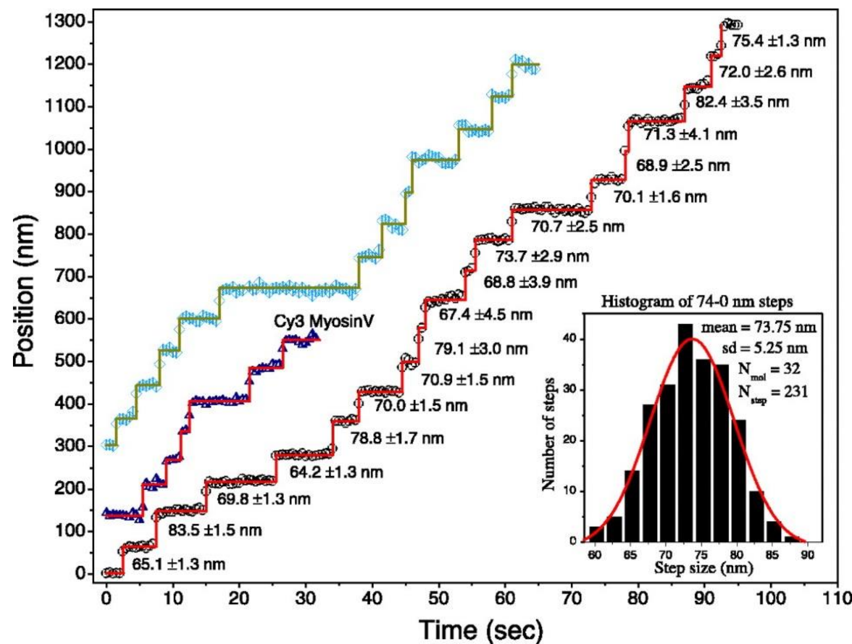
Y Axis (pixel): 12, 8, 4

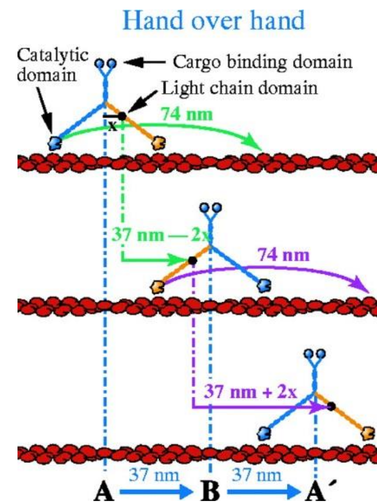
X Axis (pixel): 4, 8, 12, 16

Yildiz *et al* (2003) *Science* 300, 2061

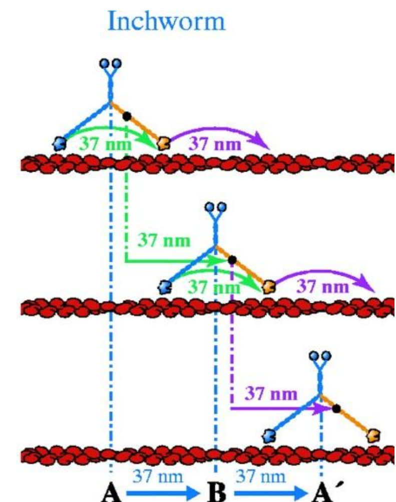
Measuring myosin V steps using FIONA

(Fluorescence Imaging with One-Nanometer Accuracy)





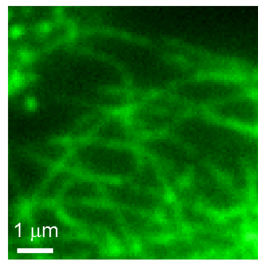
Probe step sizes:
74, 0, 74, 0, 74

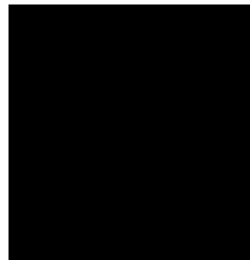


Probe step sizes:
37, 37, 37, 37

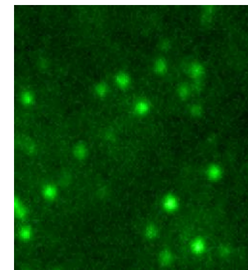
Yildiz *et al* (2003) *Science* 300, 2061

Photoactivation and photo-switching





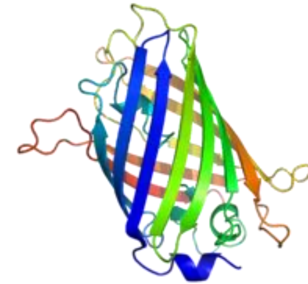
Switch off



Switch on subset

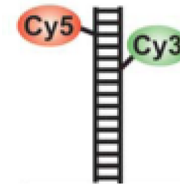
Photoactivation and photo-switching

PALM: Photoactivated Localization Microscopy

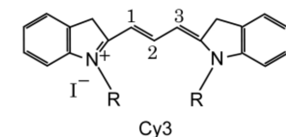


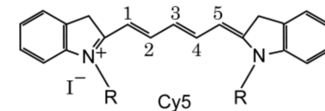
- Photoactivatable fluorescent proteins (PA-GFP, PAmCherry etc)
- Irreversibly convert from an initial non-fluorescent state to a fluorescent state upon irradiation with 405 nm (UV) light.
- Photoactivation levels depend on intensity of 405 nm

STORM: Stochastic Optical Reconstruction Microscopy

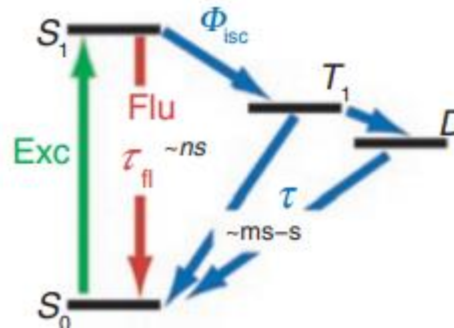


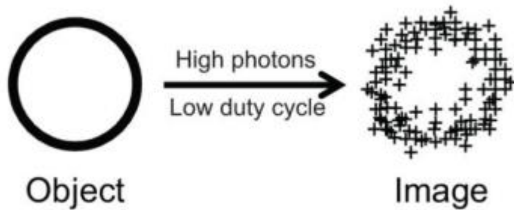
- Also dSTORM, GSDIM depending on fluorophores used
- Originally Cy3-Cy5 pair, photo-switchable synthetic fluorophores, conventional organic dyes
- Frequently fluorophores are conjugated to antibodies for immunostaining
- Transitions to a long lived dark state, and excitation with UV to green illumination recovers the fluorescent state.
- Requires a 'switching buffer' with an oxygen scavenger (glucose oxidase), and a reducing agent (thiol such as β-mercaptoethylamine (BME))

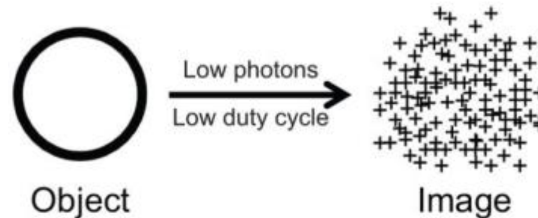


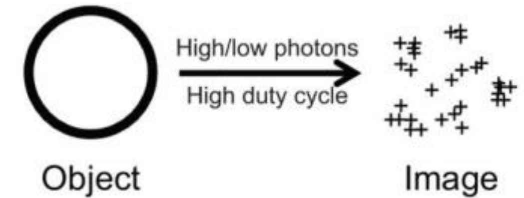


Photoactivation and photo-switching





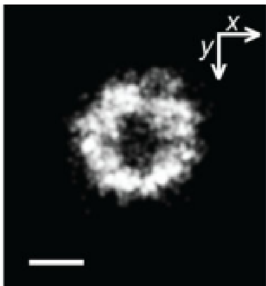


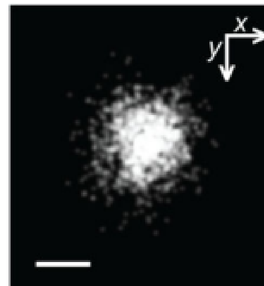


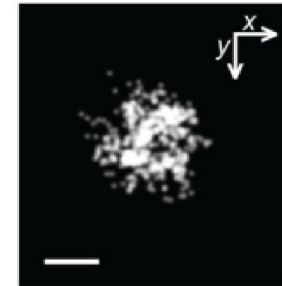
Alexa 647

Atto 655

Cy5.5



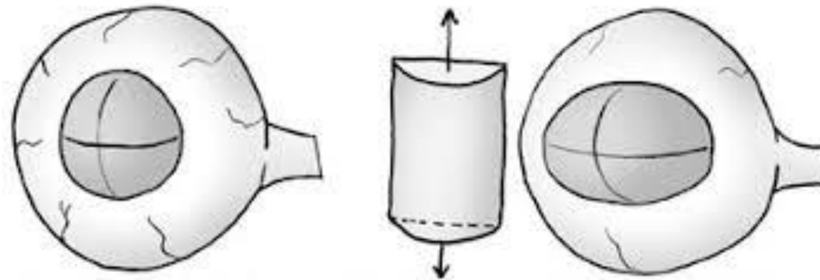




Dempsey *et al* (2012) *Nat Methods*, 8(12): 1027–1036

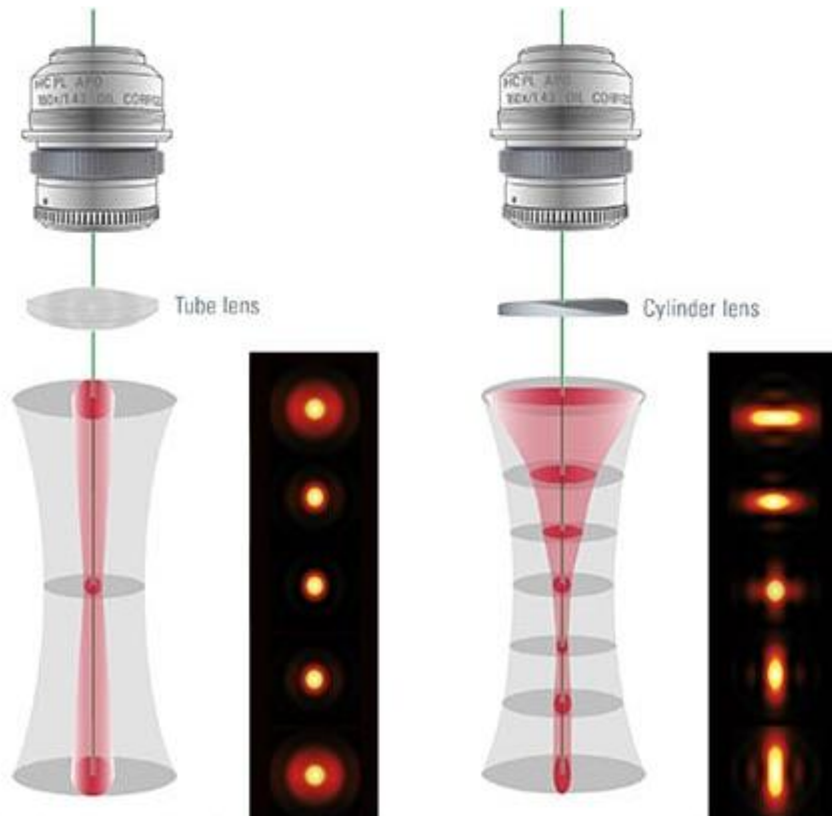
3D localisation microscopy

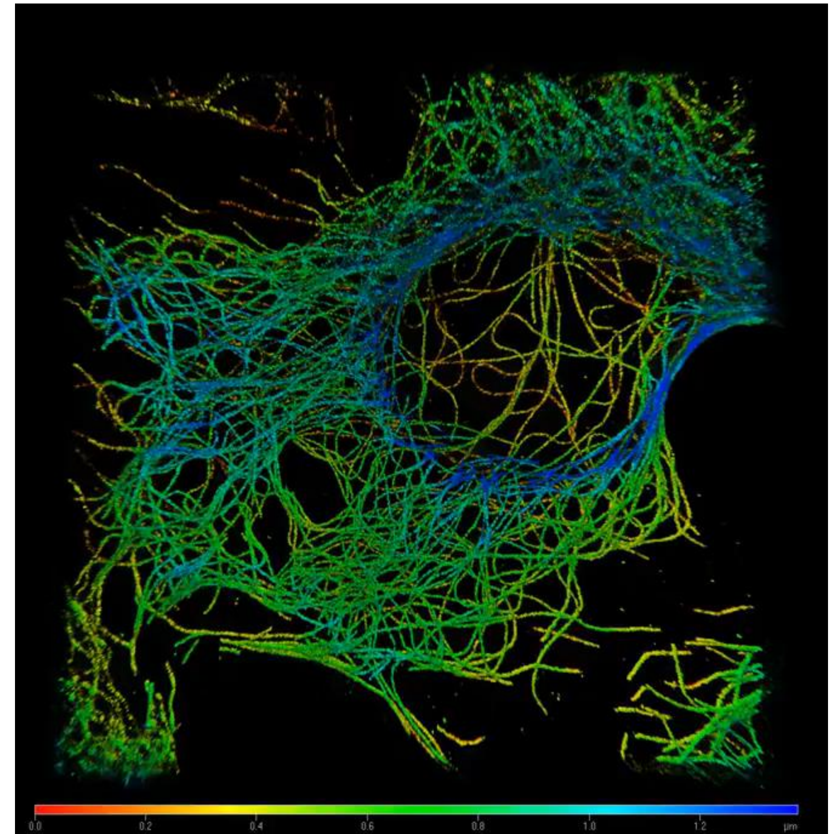
Gaining z position information with astigmatism



3D localisation microscopy

Gaining z position information with astigmatism



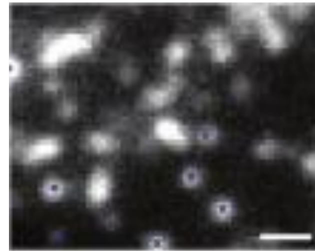


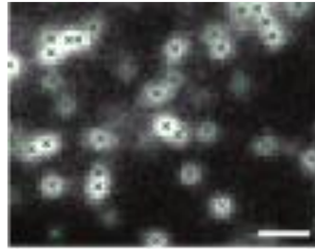
alpha-tubulin labelled with Alexa 647

Pros and cons of localisation microscopy

CONS:

- Slow (tens of thousands of frames needed)
- Not the best for live cell imaging*





Crowded field localisation algorithms allow for faster imaging

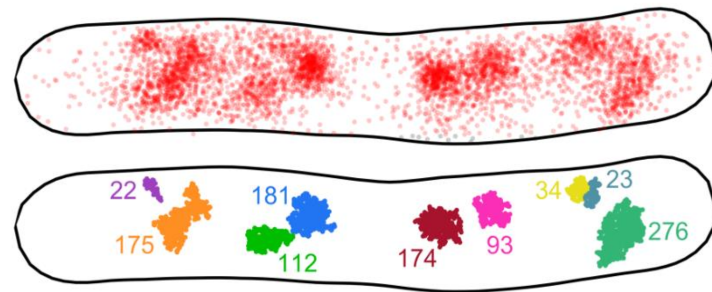
Pros and cons of localisation microscopy

CONS:

- Slow (tens of thousands of frames needed)
- Not the best for live cell imaging*
- Requires using particular fluorophores and/or buffers

PROS:

- Comparatively simple microscope design and analysis
- Can easily extract quantitative information



Mathew Stracy

Pros and cons of localisation microscopy

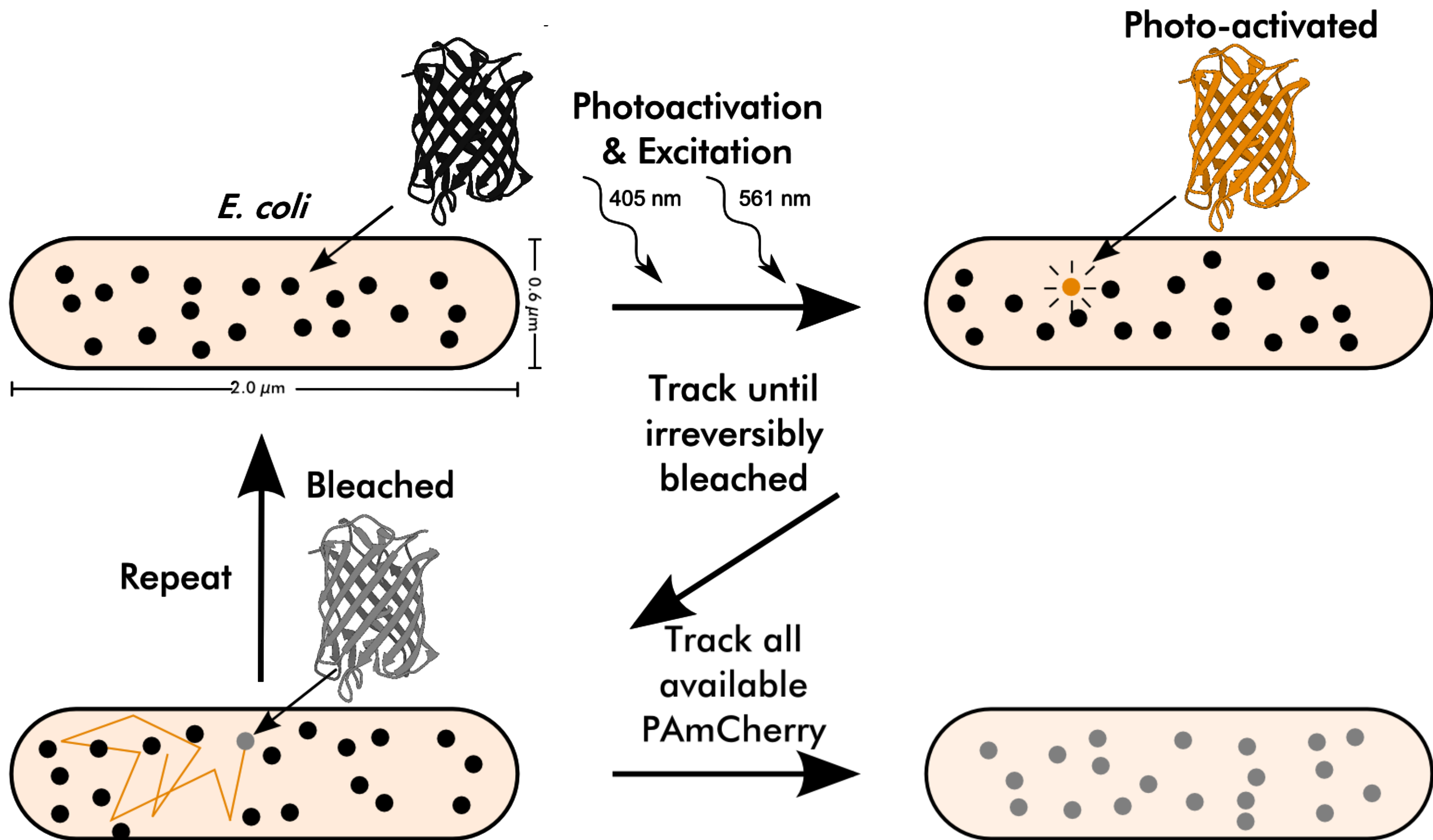
CONS:

- Slow (tens of thousands of frames needed)
- Not the best for live cell imaging*
- Requires using particular fluorophores and/or buffers

PROS:

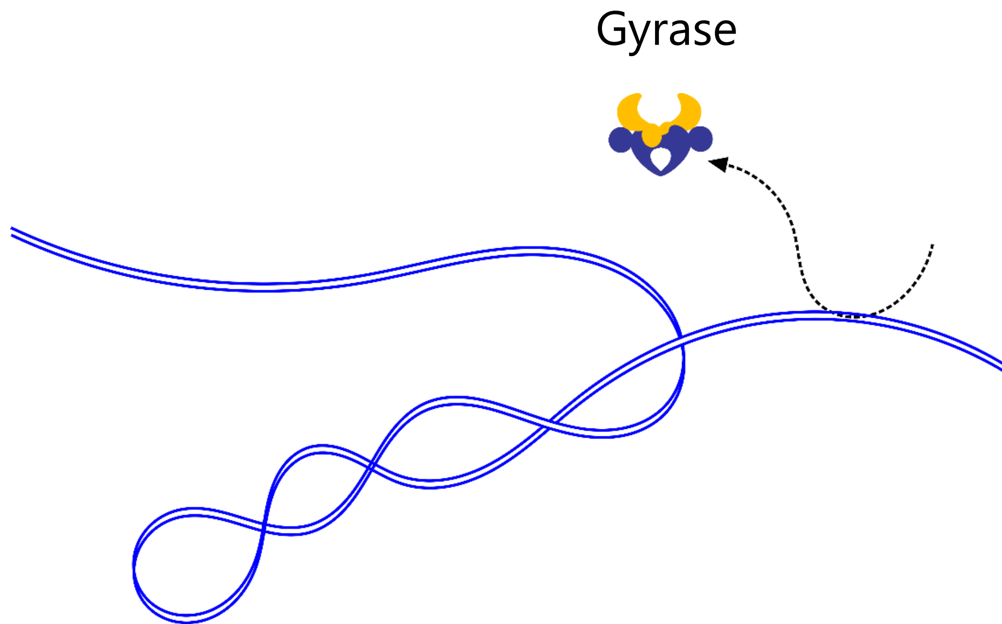
- Comparatively simple microscope design and analysis
- Can easily extract quantitative information
- Can be combined with SINGLE PARTICLE TRACKING!!

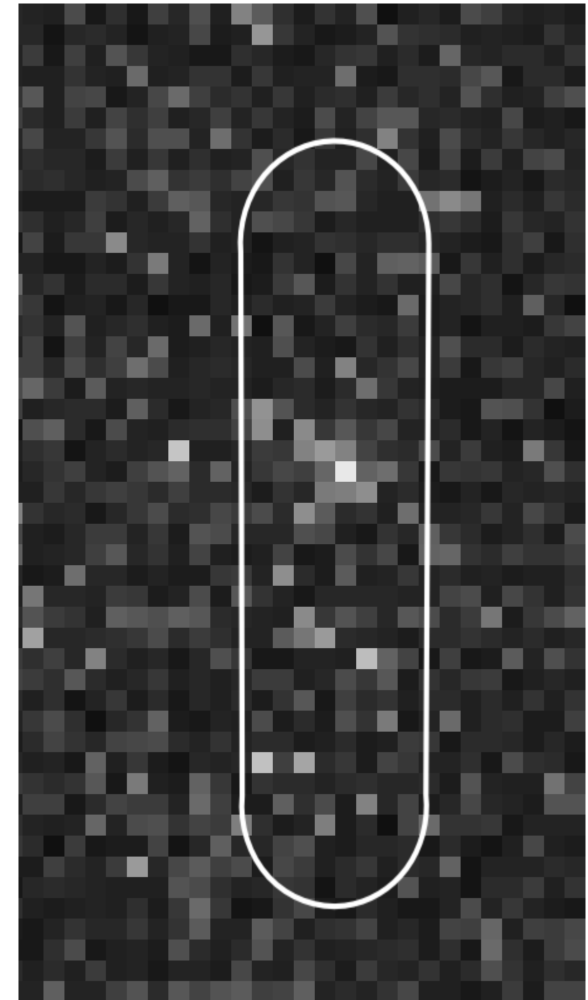
Single-molecule tracking PALM



Manley et al. Nature Methods (2008)

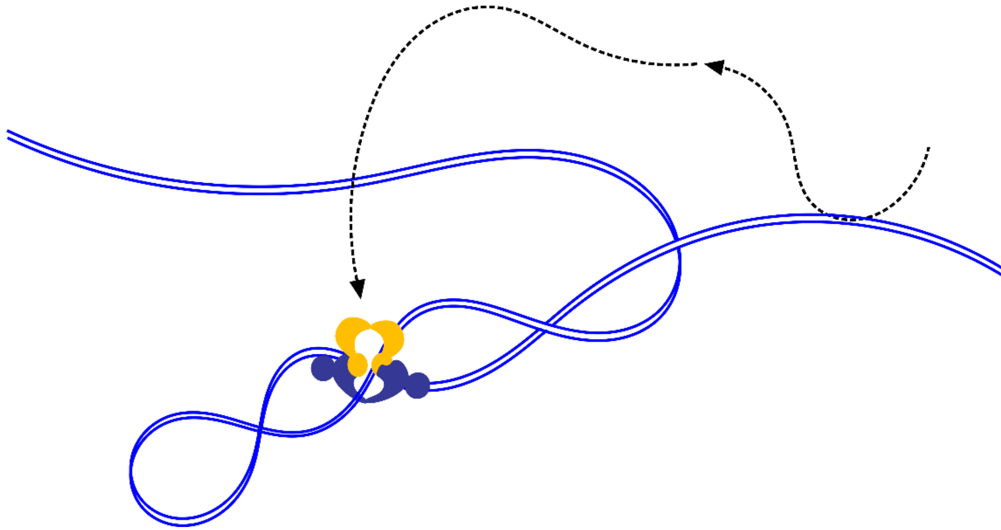
Single-particle tracking PALM

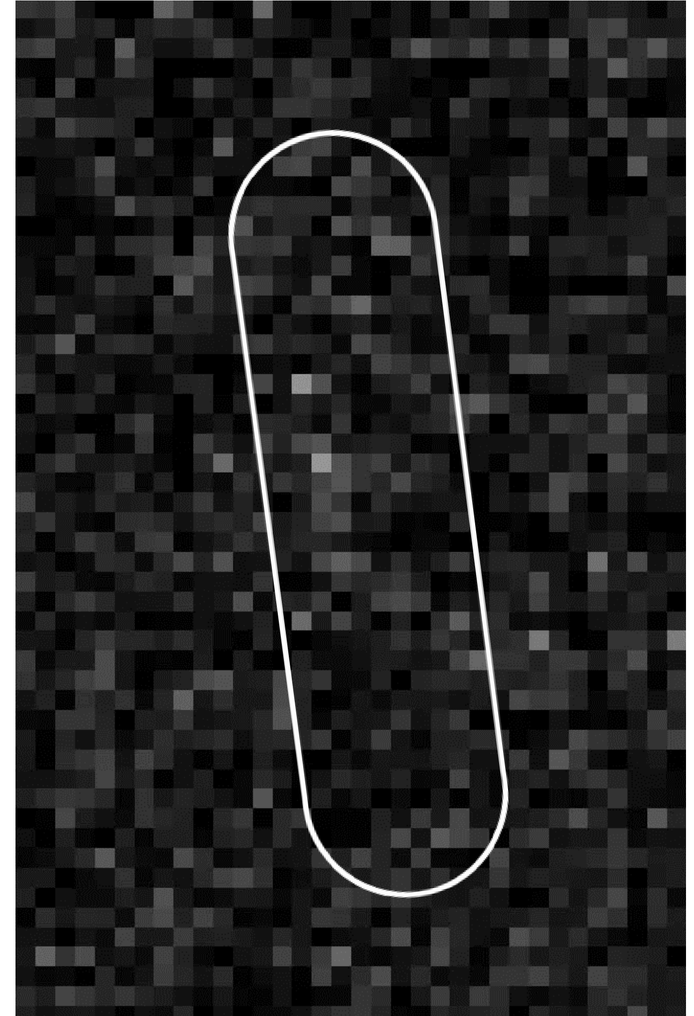




Mathew Stracy

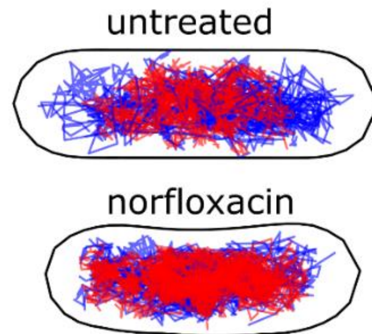
Single-particle tracking PALM

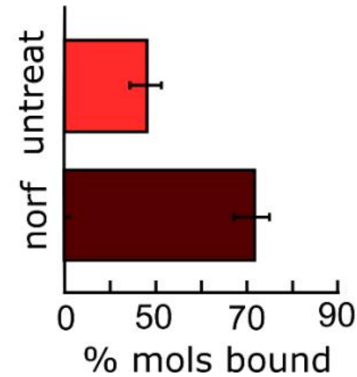


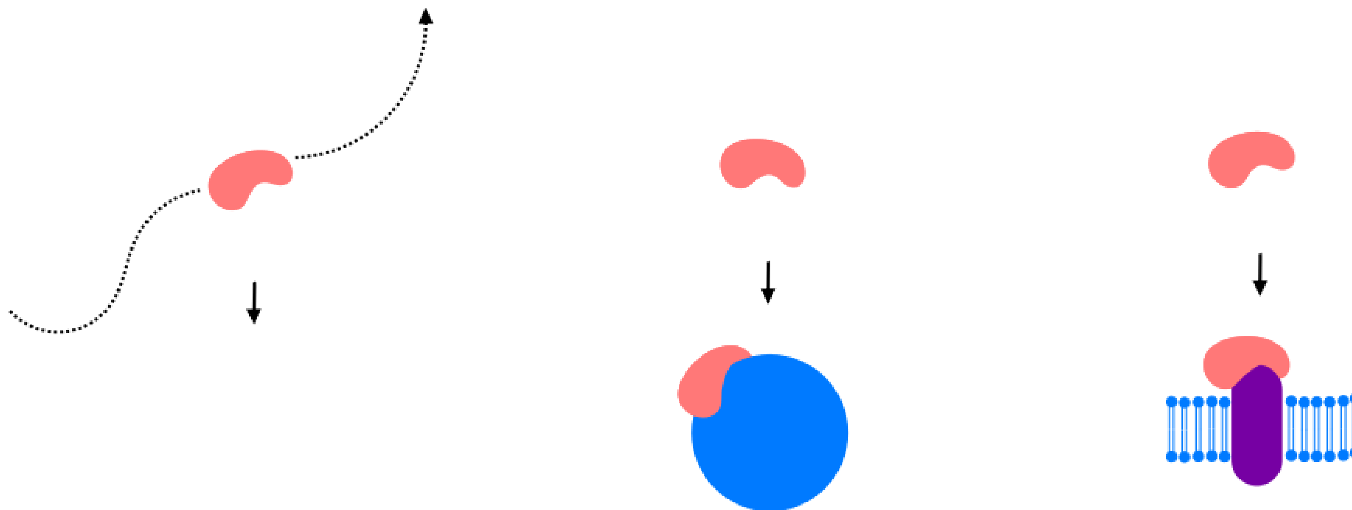


Mathew Stracy

Single-particle tracking PALM





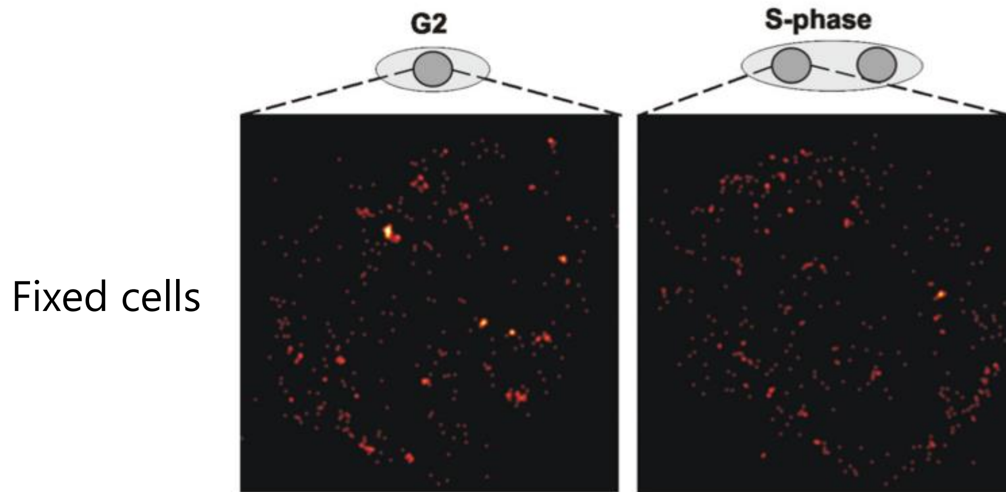


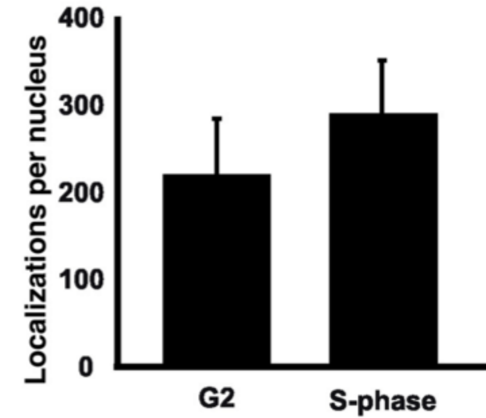
Mathew Stracy

Long exposures

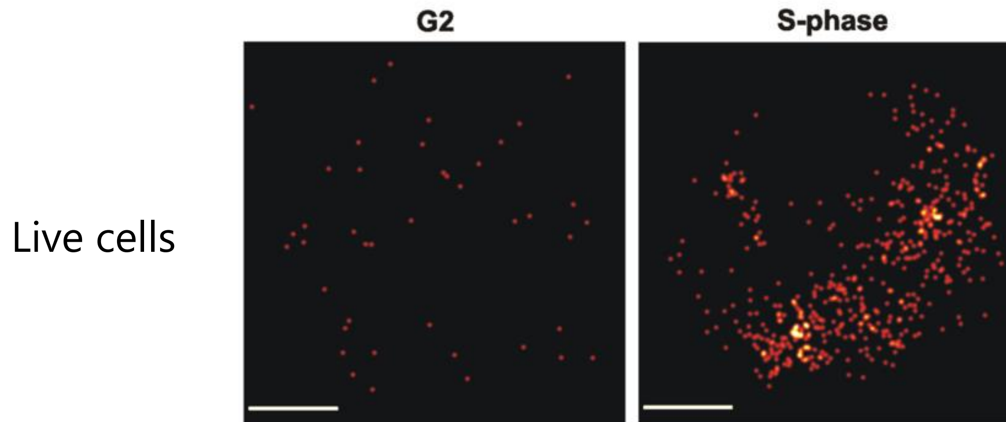
Long exposures

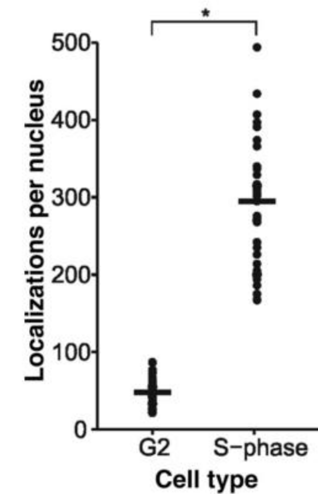
Fixed cells





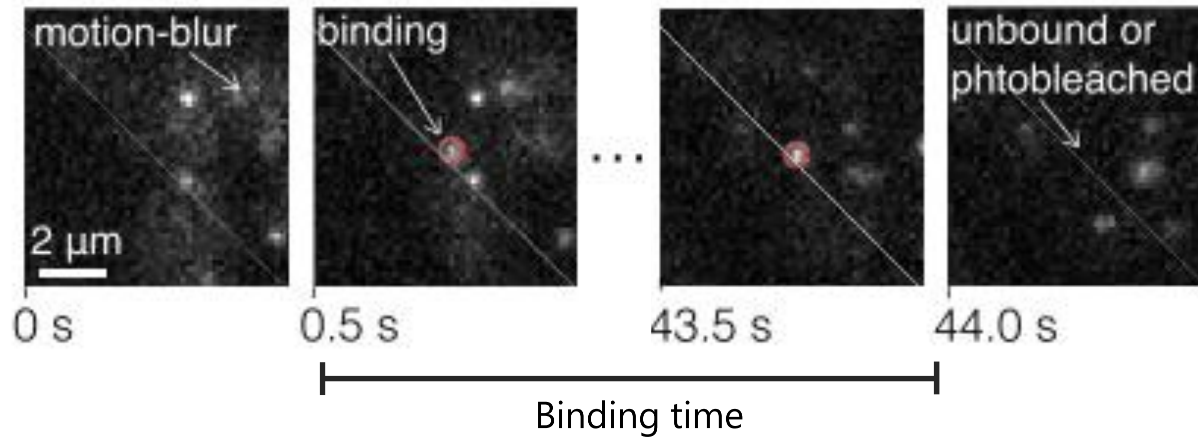
Live cells

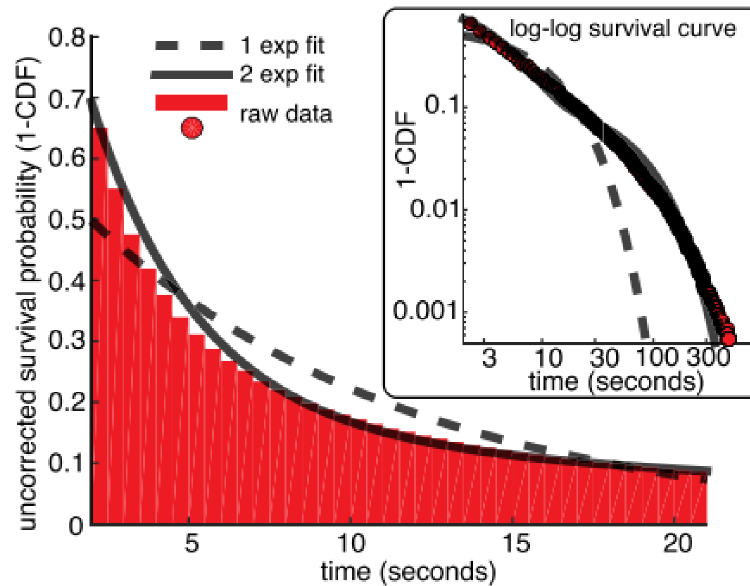


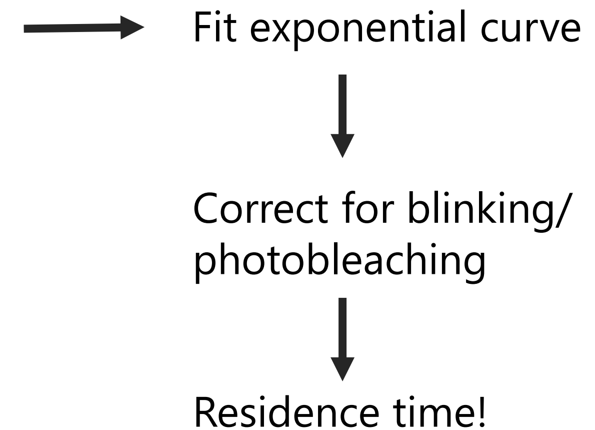


Etheridge et al. *NAR* 2014

Long exposures







Hansen et al. *eLife* 2017

Any questions?