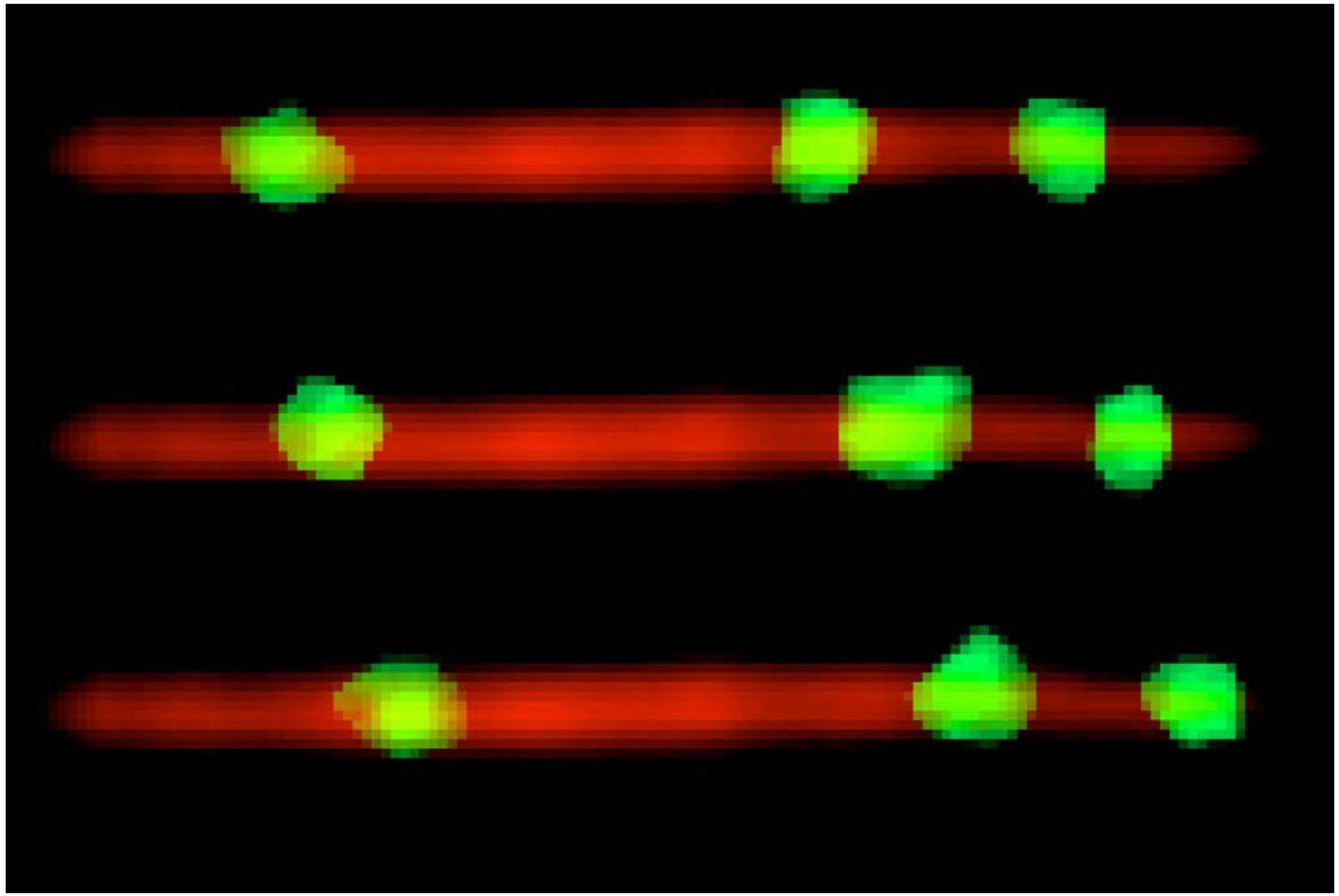
Single molecule imaging, localization

- Last class
  - TIRF
  - Geometrical operators
- This class
  - Single molecule imaging
  - Localization
  - FLIM

Single molecule imaging

Single molecule imaging

- Single kinesin GFP on a microtubule



Single molecule imaging

- Single molecules are neither bright nor photostable
- Have to restrict the size of illumination or emission
- Photons captured with very high efficiency

Nobel prize in 2014 – WE Moerner

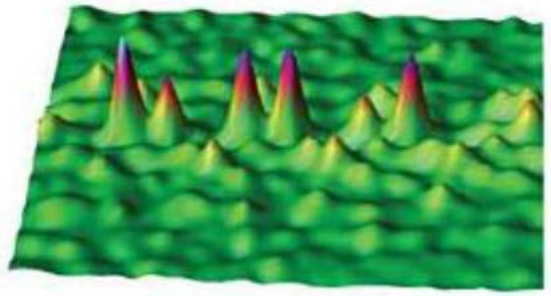


Figure 23.8. Single-molecule image of GFP mutant T203Y. The image was obtained using TIR illumination and a CCD camera. The GFP mutant was immobilized in poly(acrylamide). The image is 2.4 x 2.4 μm. Images courtesy of Dr. W. C. Moerner from Stanford University.

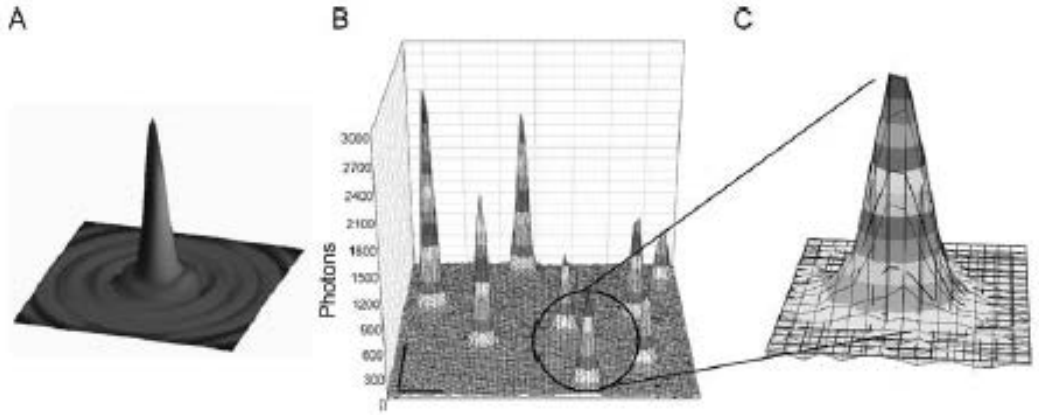


Figure 1.2. A. The Airy pattern of a diffraction-limited spot in two dimensions. **B.** Fluorescence images of several single Cy3-DNA molecules immobilized on a glass surface. The data were taken with a total internal reflection fluorescence microscope in 0.5 sec. **C.** Expanded view of one point spread function (PSF) with two-dimensional elliptical Gaussian curve fit (*solid lines*). The center of this PSF can be located to within 1.5 nm (σ_μ). (From Yildiz et al. [14].)

Recent technologies have made single molecule imaging easier

- **Ideal Hardware:**
- High NA objectives allow TIRF with low aberrations
- Improved fluorophore brightness and stability
- Sensitive and fast cameras
- Stable illumination sources

Ideal single molecule fluorophore:

High brightness (absorption and QY)

Steady emission (no blinking)

Photostable (under high illumination)

Non-toxic

Single molecule brightness

- Single fluorophore emission
- Process of absorption, vibrational decay, and fluorescence emission are repeated many times
- Rate of fluorescence is dependent on rates of absorption and QY.
- Cross section of GFP is: 6.5×10^{-17} cm^2

Population at concentration c

$$B = QY * \varepsilon$$

$$I_{em} = I_{ex} * QY * (1 - e^{-b\epsilon c})$$

Single fluorophore

$$B_s = QY * \sigma = \mathrm{QY} * \frac{2.3\varepsilon}{N_A}$$

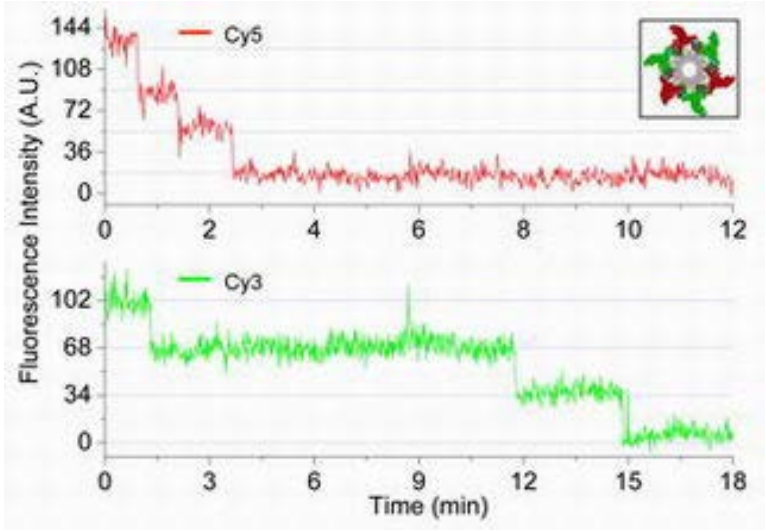
$$I_{em} = I_{ex} * B_s$$

Population of fluorophores
QY = Quantum Yield
ε = absorption coefficient

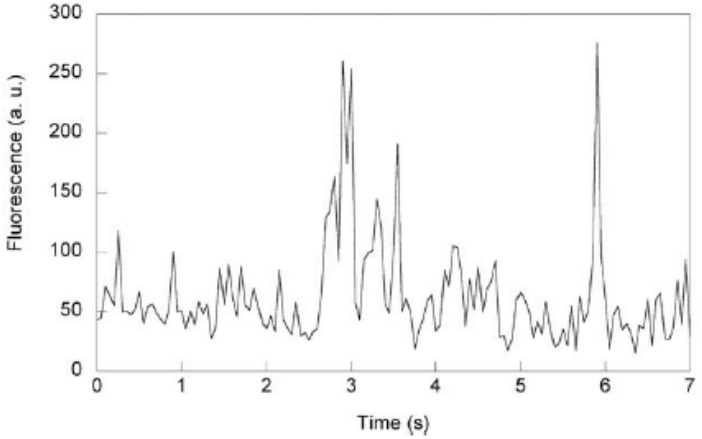
Single fluorophore
QY = Quantum Yield
s = cross section
N_A = Avagadro's #
ε = absorption coefficient

Single molecule detection

- In practice
  - Expect ~10% photon detection efficiency by your scope
  - Do you want to take a movie
  - Brightness of fluorophore (population) > 3 x 10^4 (1.0 for single fluorophore) to detect individual
  - Blue/green excites more autofluorescence, harder to detect
  - TIRF, confocal, or some other background rejection is necessary



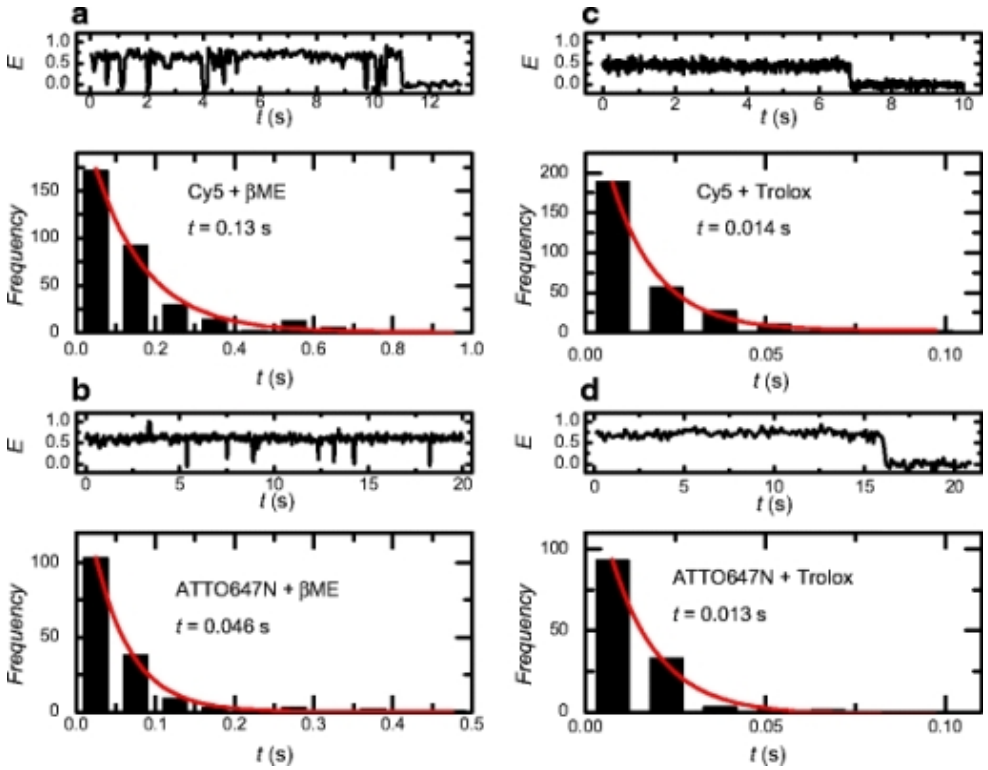
Photobleaching now an irreversible step.
Best way to verify you are looking at single fluorophore

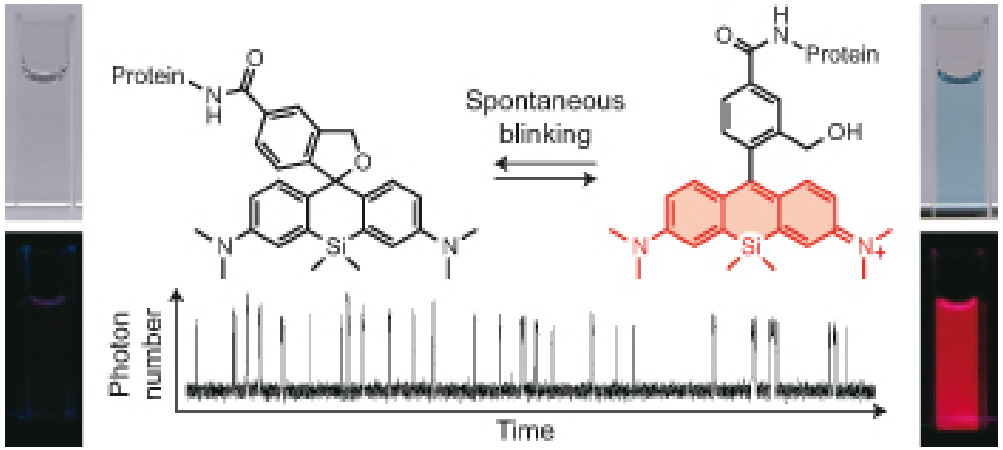


Blinking is a reversible dark state. Dependent on illumination intensity

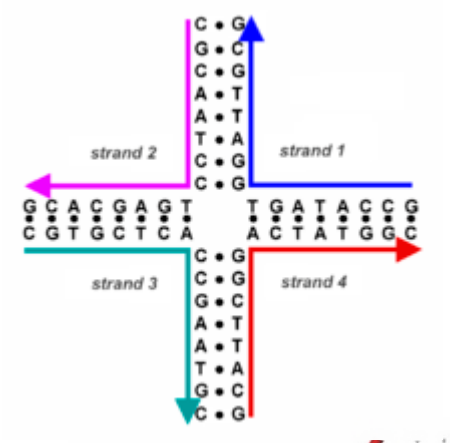
Fluorophore blinking

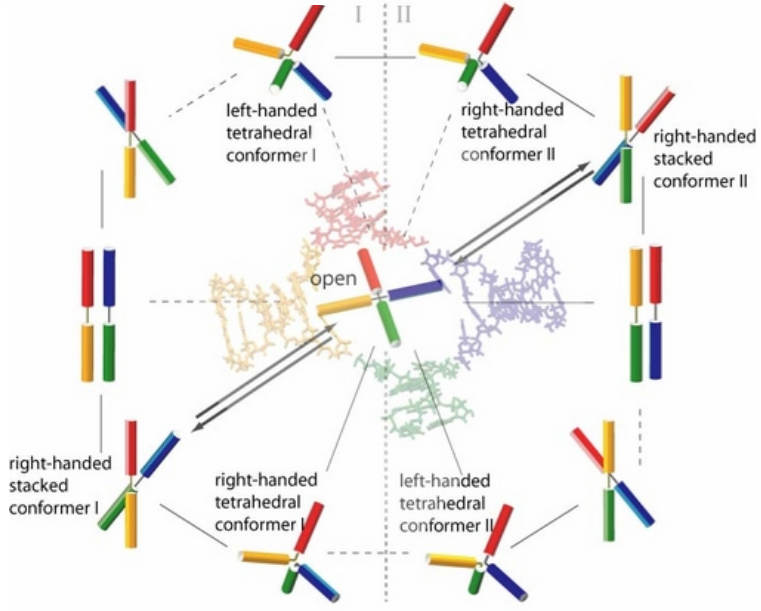
- Part of every fluorophore
- Often due to reversible isomerization around pi bond
- Organic dyes will enter triplet state
- Quantum dots blink due to ejection of electrons from small cores
- Can be reduced using triplet state quenchers
- Can be used for good – see super resolution imaging in a few lectures…

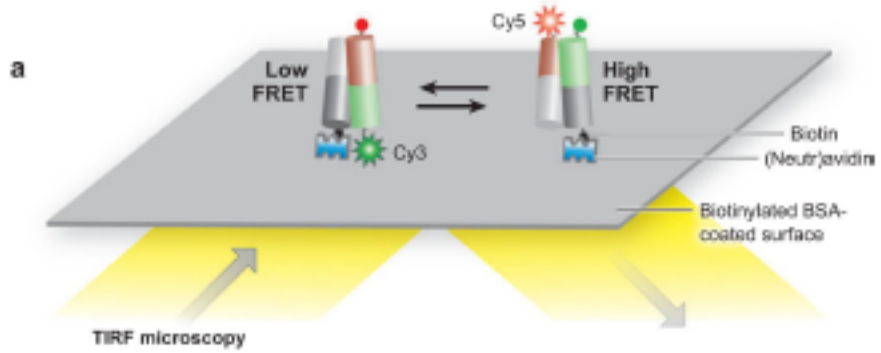


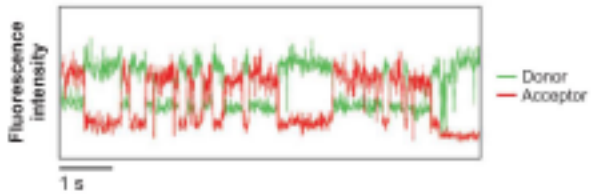


Single molecule applications
Holliday Junctions





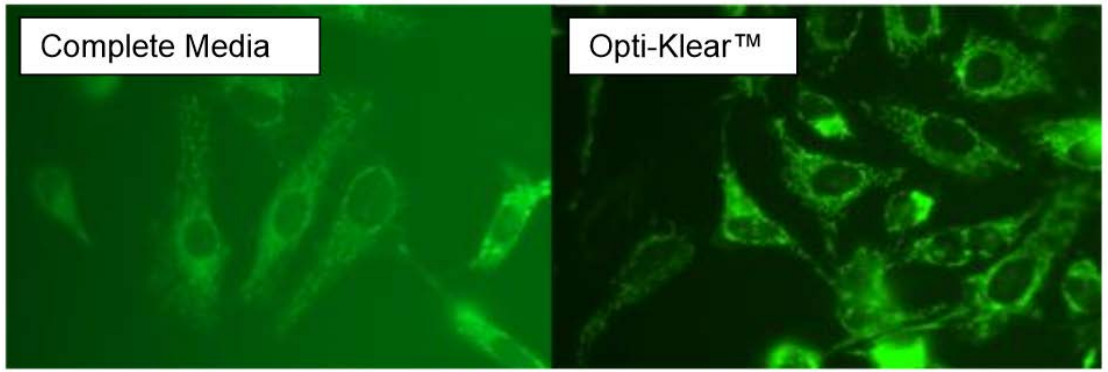


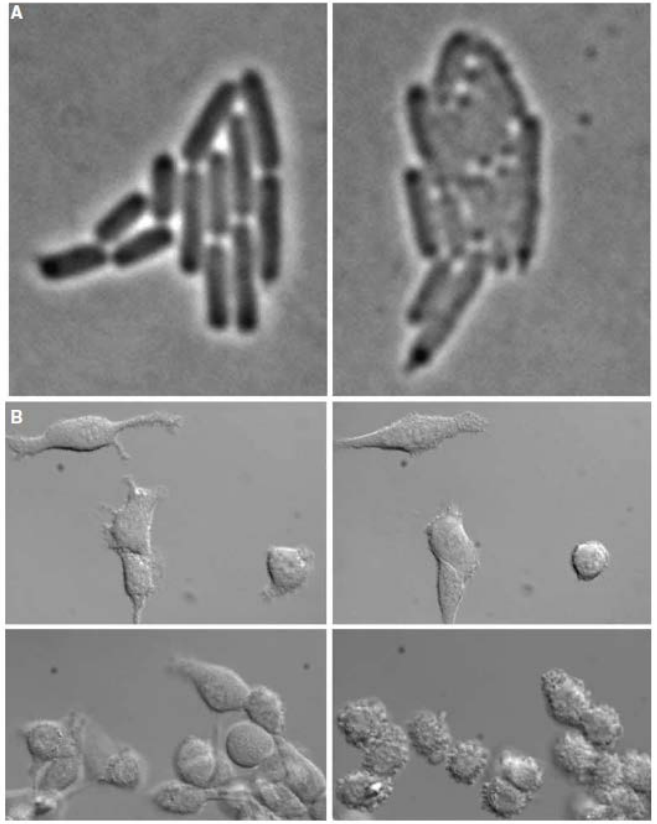


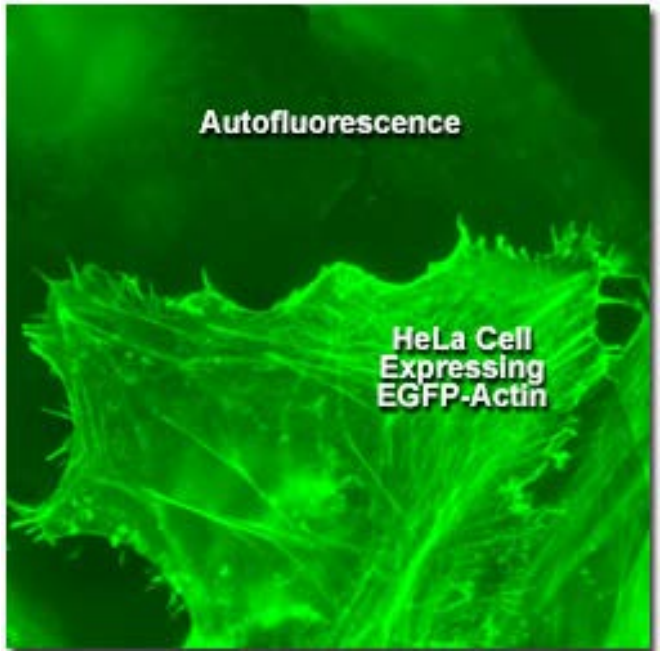
Holliday junctions are tethered via biotin-neutravidin conjugation on the bovine serum albumin (BSA)-coated surface. The conformational dynamics of Holliday junction is shown in a fluorescence time trace

Single molecules in cells

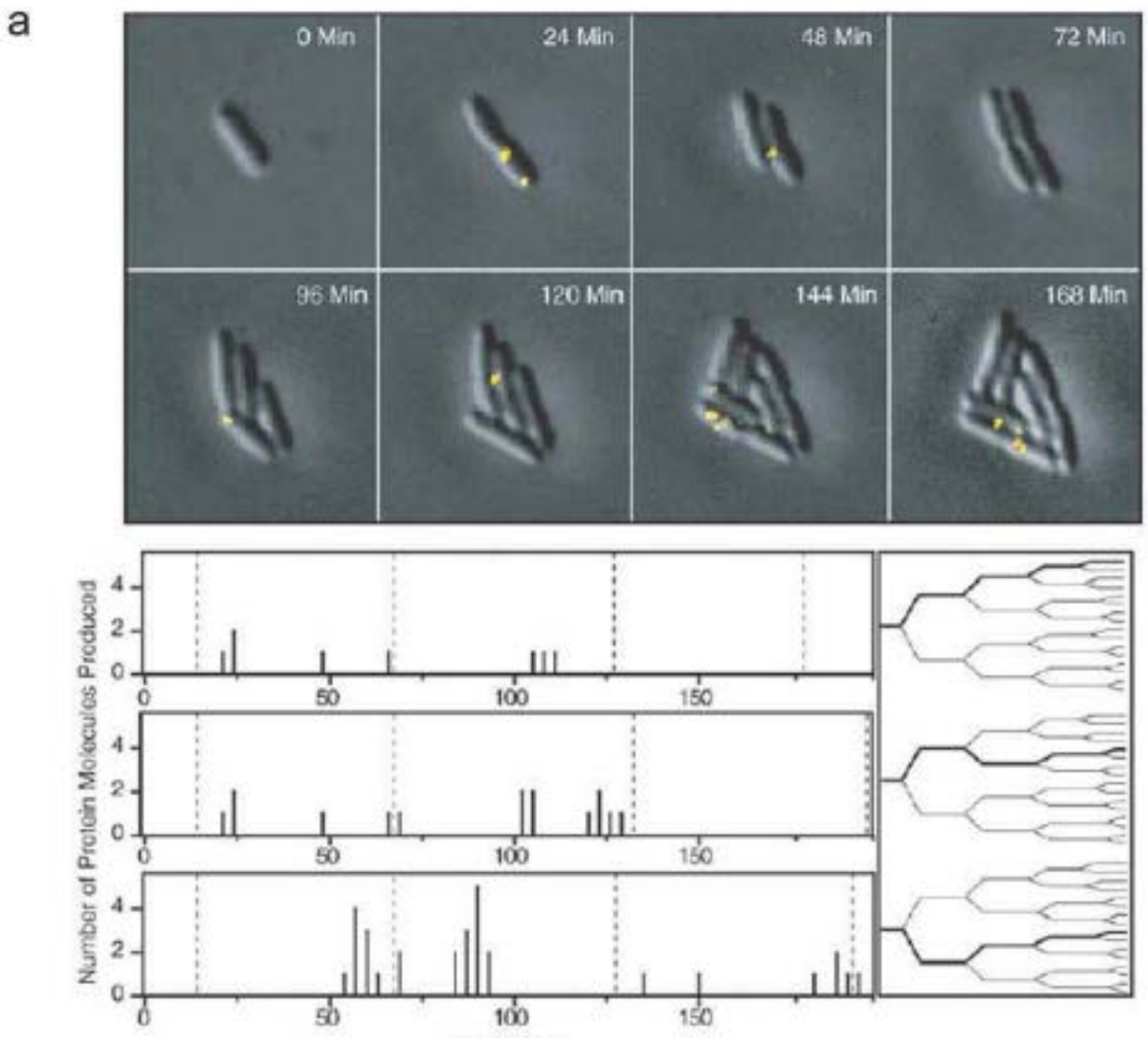
- Autofluorescence – both in cells and medium, try to use red light. YFP and reds are preferred FPs
- Laser induced photodamage – very high intensities, can kill cells, try to separate exposures in time, lowest possible intensity
- Protein diffusion – harder to detect across a large area
- Environmental controls – do you need heat and CO_2?







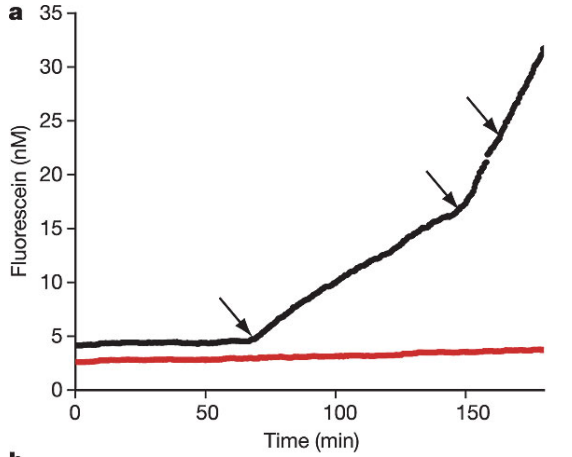
Stochastic gene expression in bacteria

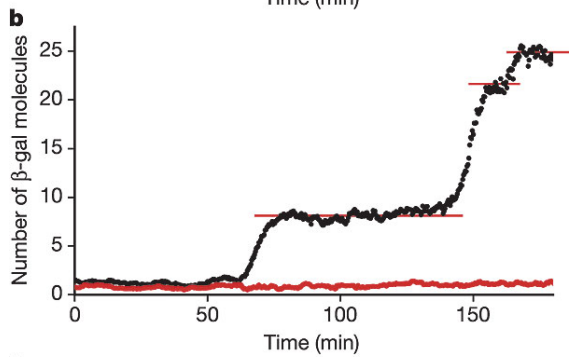


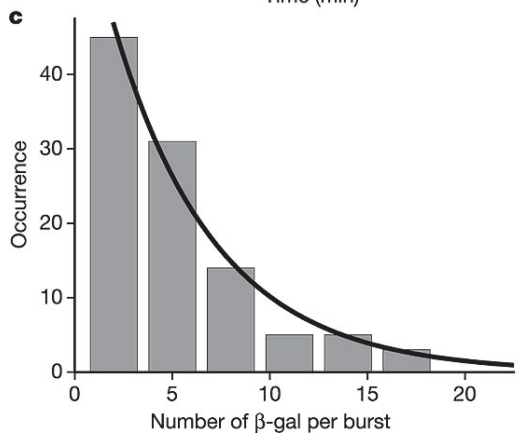
Proteins are generated in bursts in time with Poission statistics.

Each cell, though they came from a single progenitor, has varying components.

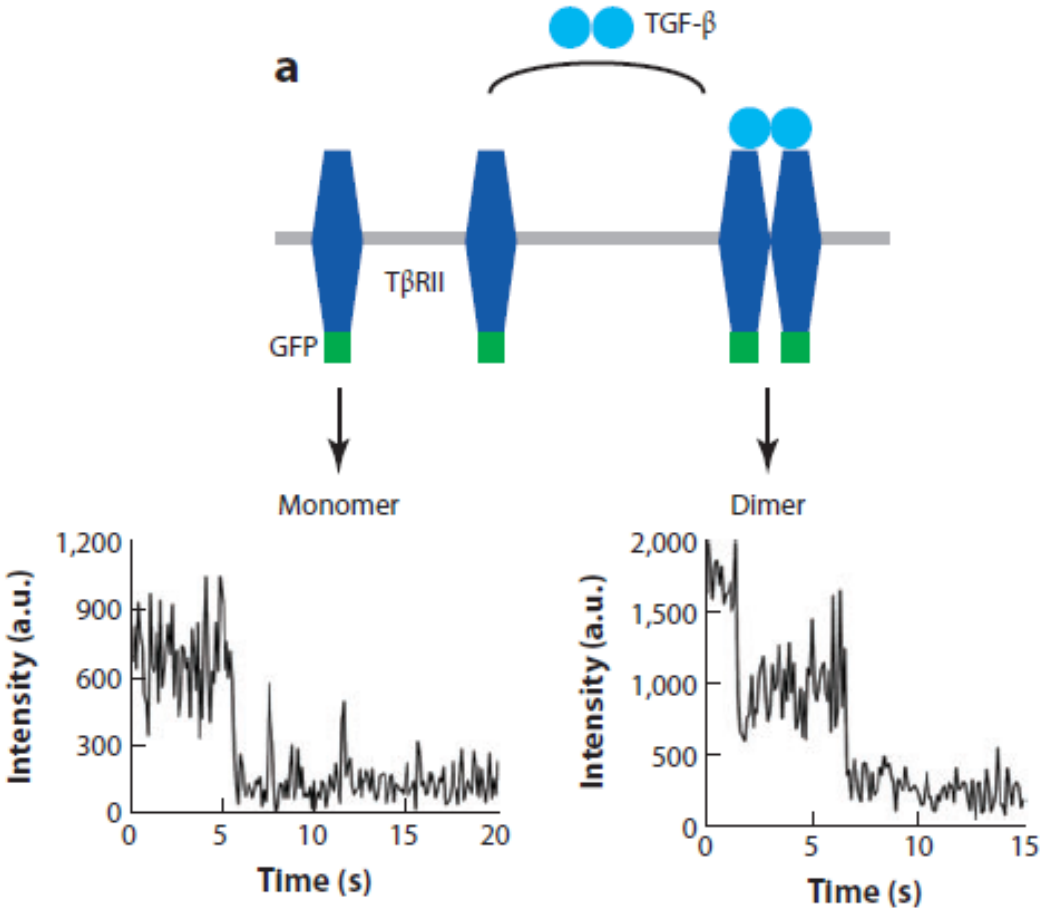
Non-genetic heterogeneity

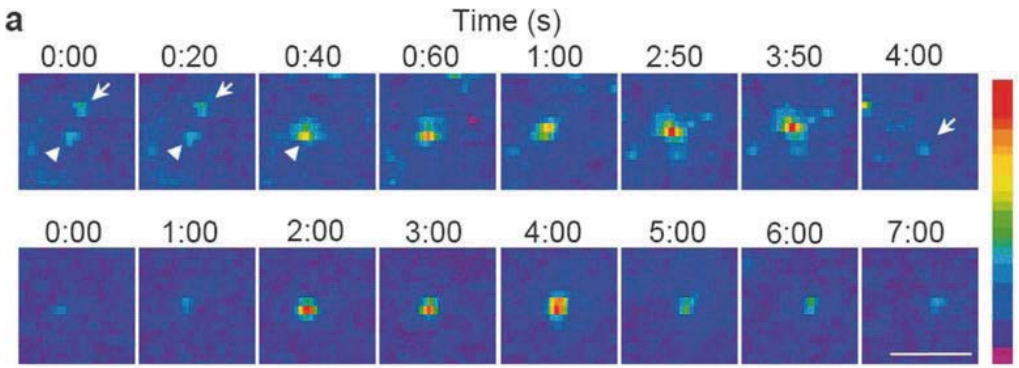


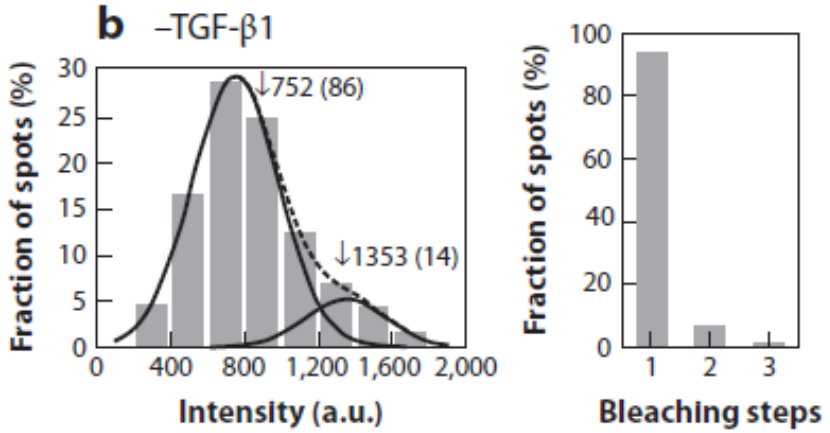


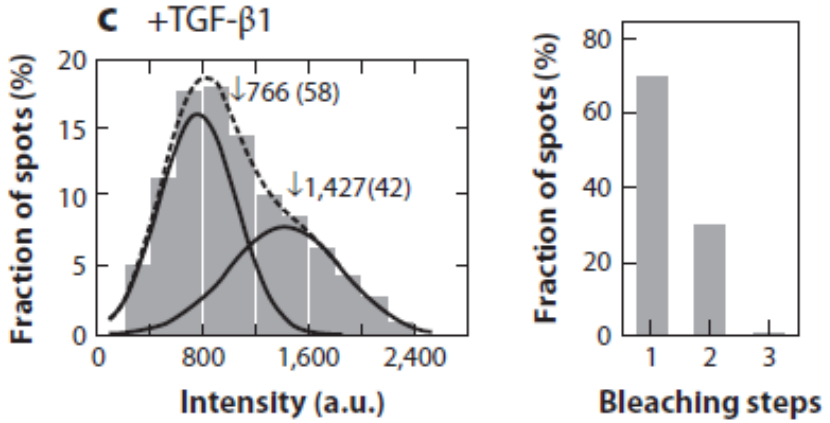


Single molecule applications
Stoichiometry



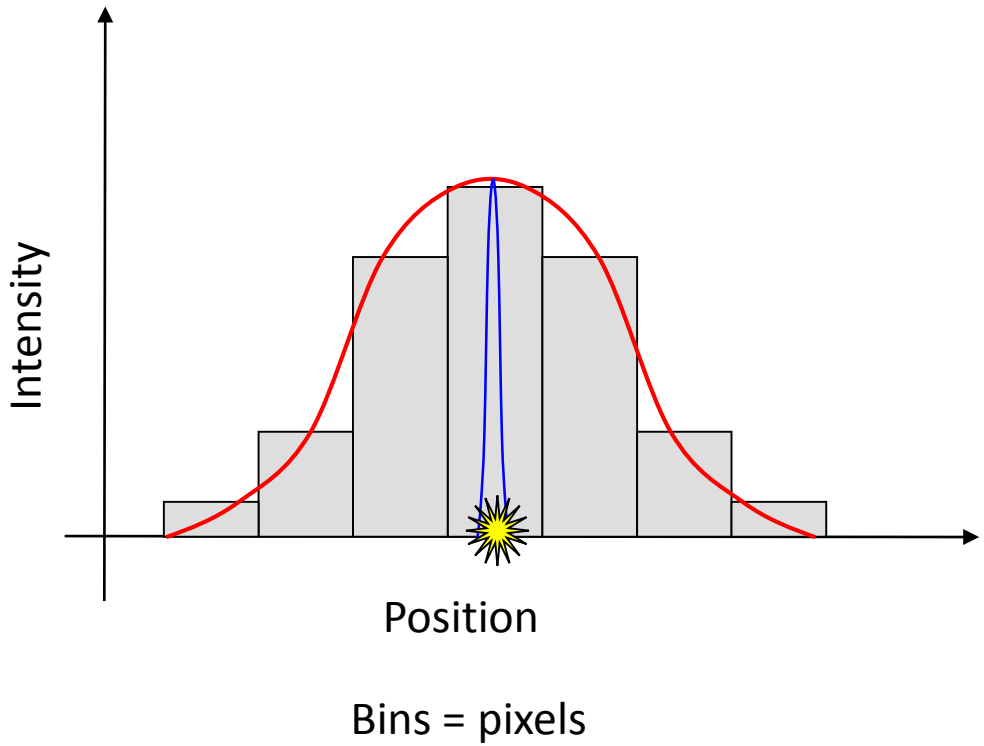






Single molecule localization

- We can localize individual fluorophores to better than the diffraction limit of our optics, IF they are single fluorophores
- We have to know the point spread function of the scope
- Best is FIONA – fluorescent imaging with one nanometer accuracy
- N ~ 10,000 photons for FIONA

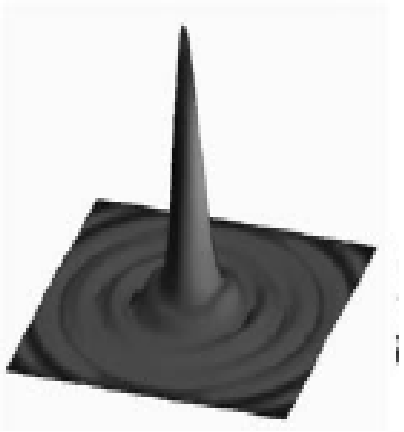


Calculating uncertainty in localization

- Have to know your point spread function very accurately
- Measure with beads smaller than diffraction limit
- Accuracy is a function of fluorophore, microscope PSF, background noise

$$I(\theta) = I(0)\left[\frac{2J_1(ka\sin(\theta)}{ka\sin(\theta)}\right]^2$$

PSF fits to Bessel function if your scope is well aligned (or Gaussian)

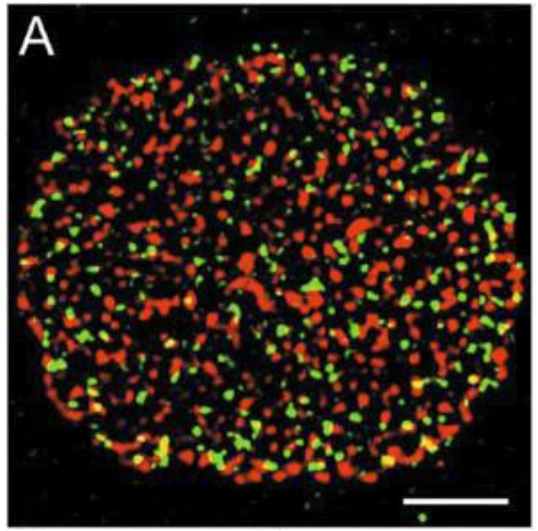


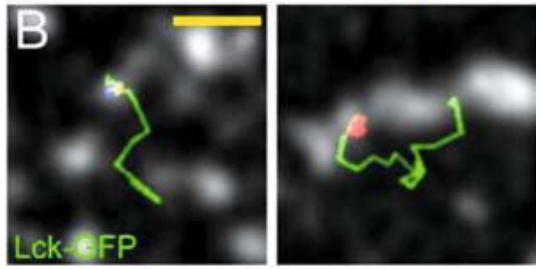
$$\sigma_{\mu_i} = s_i/\sqrt{N}$$

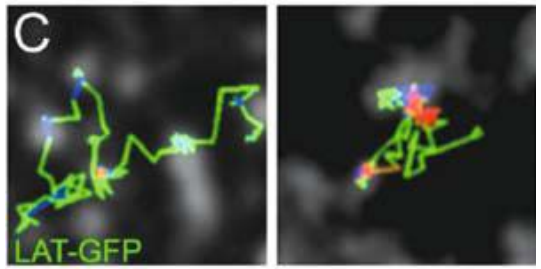
In a perfect world, localization only depends on # of photons

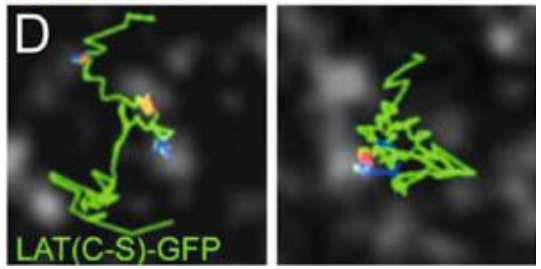
$$\langle(\Delta x)^2\rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi}\,s^3b^2}{aN^2},$$

N = number of photons
a = pixel size
b = background signal
s = standard deviation of PSF



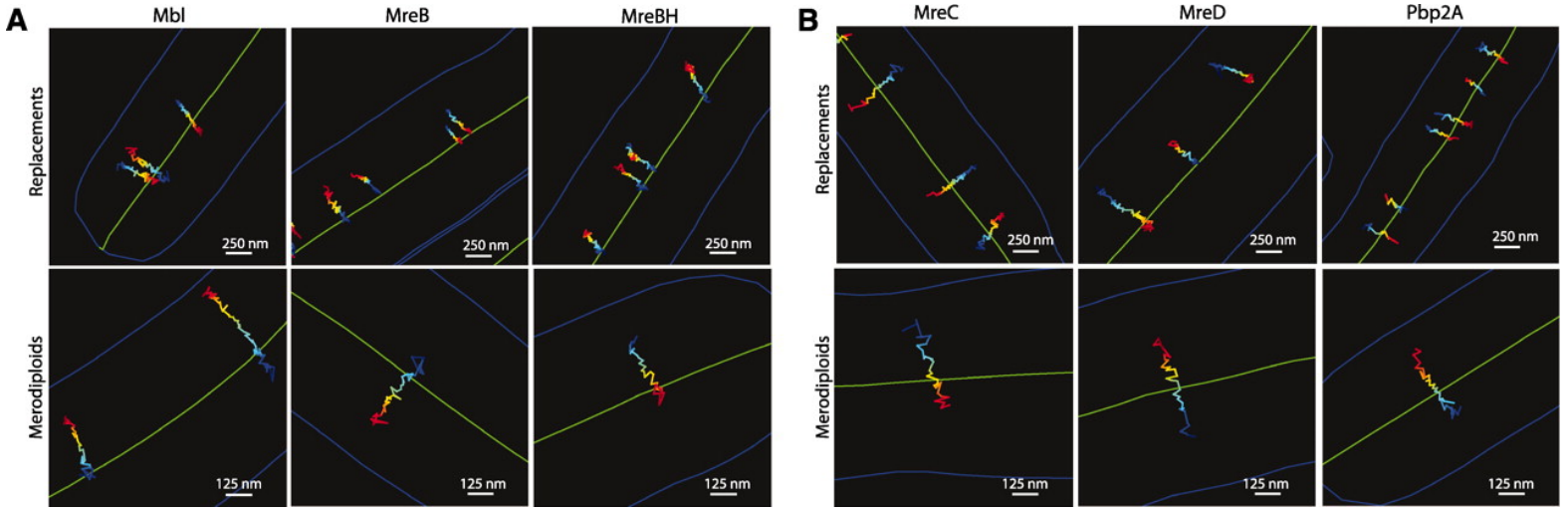


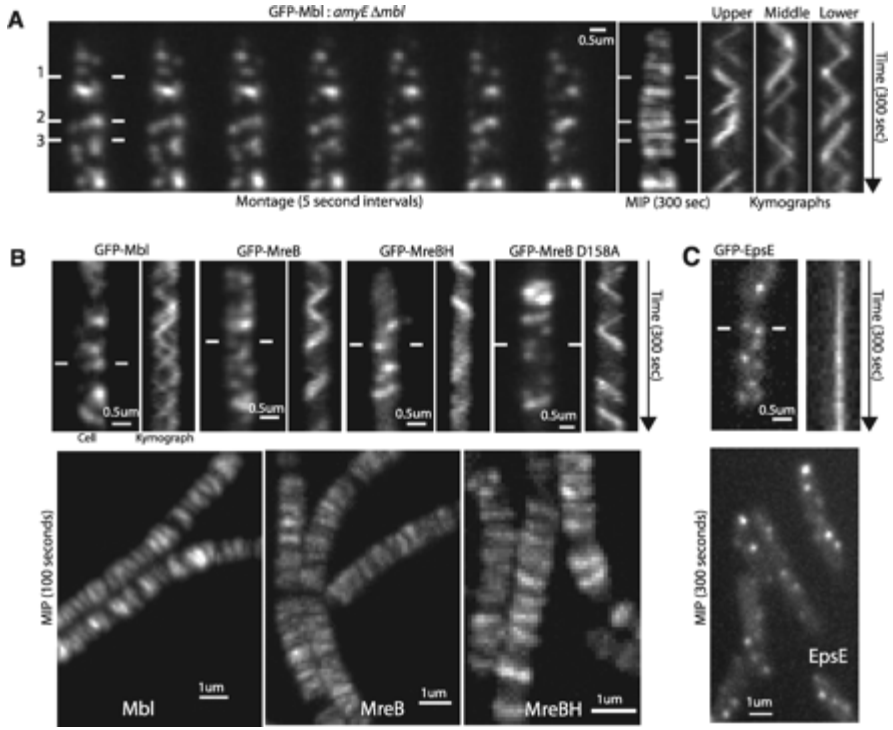




Movement of individual receptors in T cells

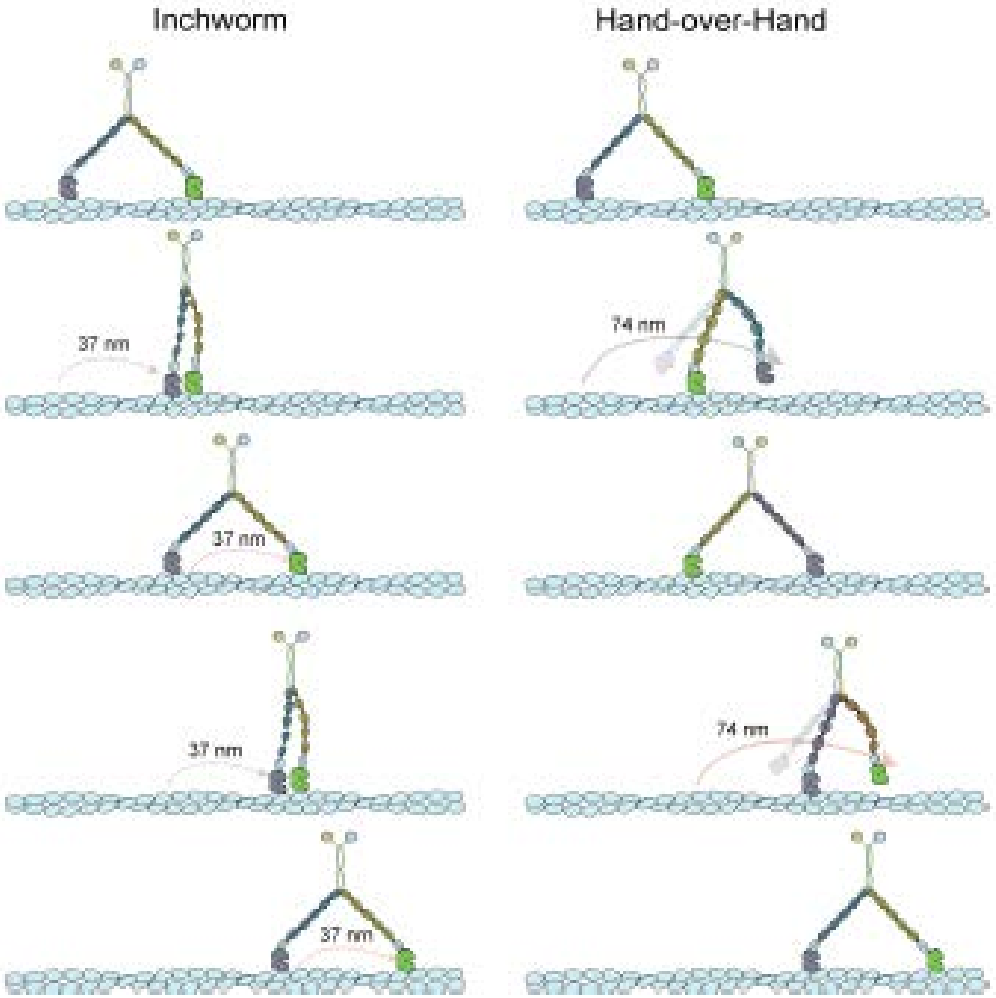
Showed clustering of proteins independent of lipid rafts

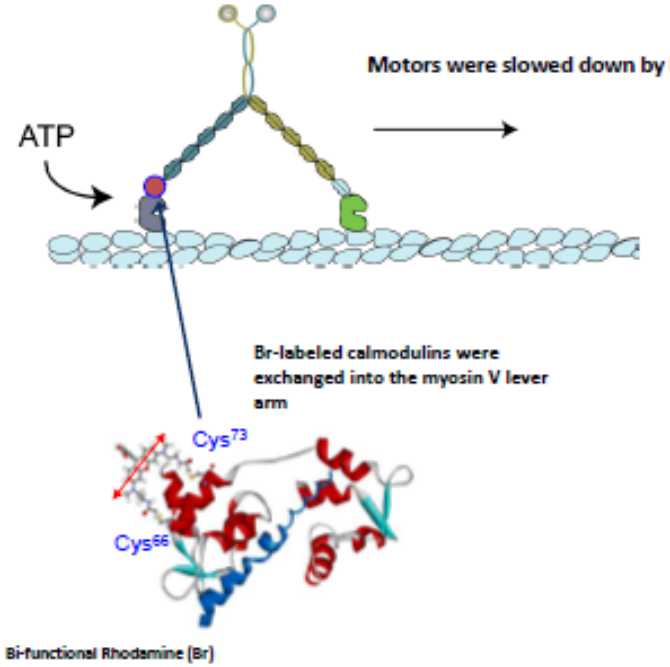


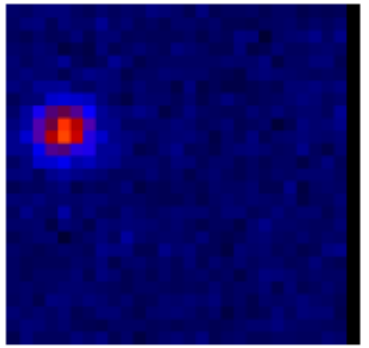


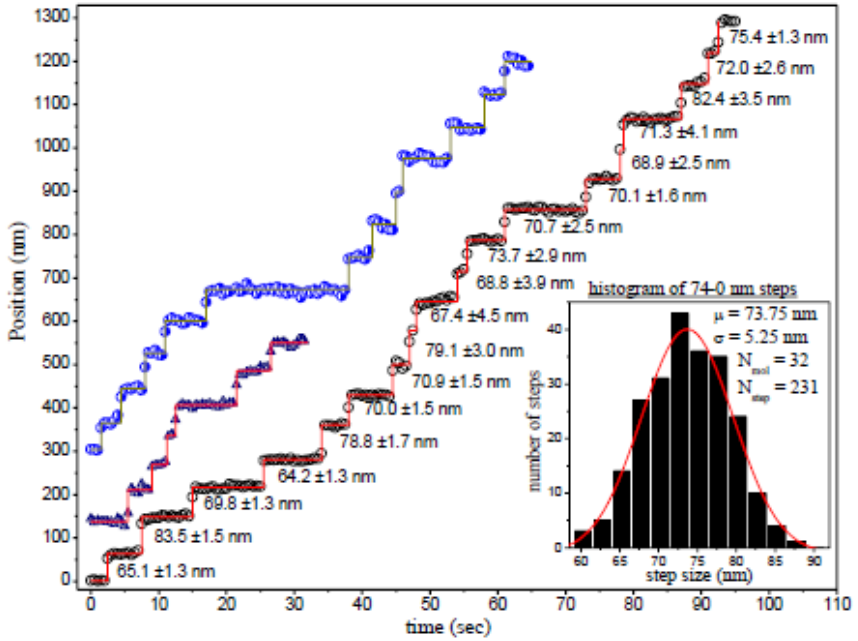
Lipid incorporation to outer membrane of *B. subtilis*

Motor proteins

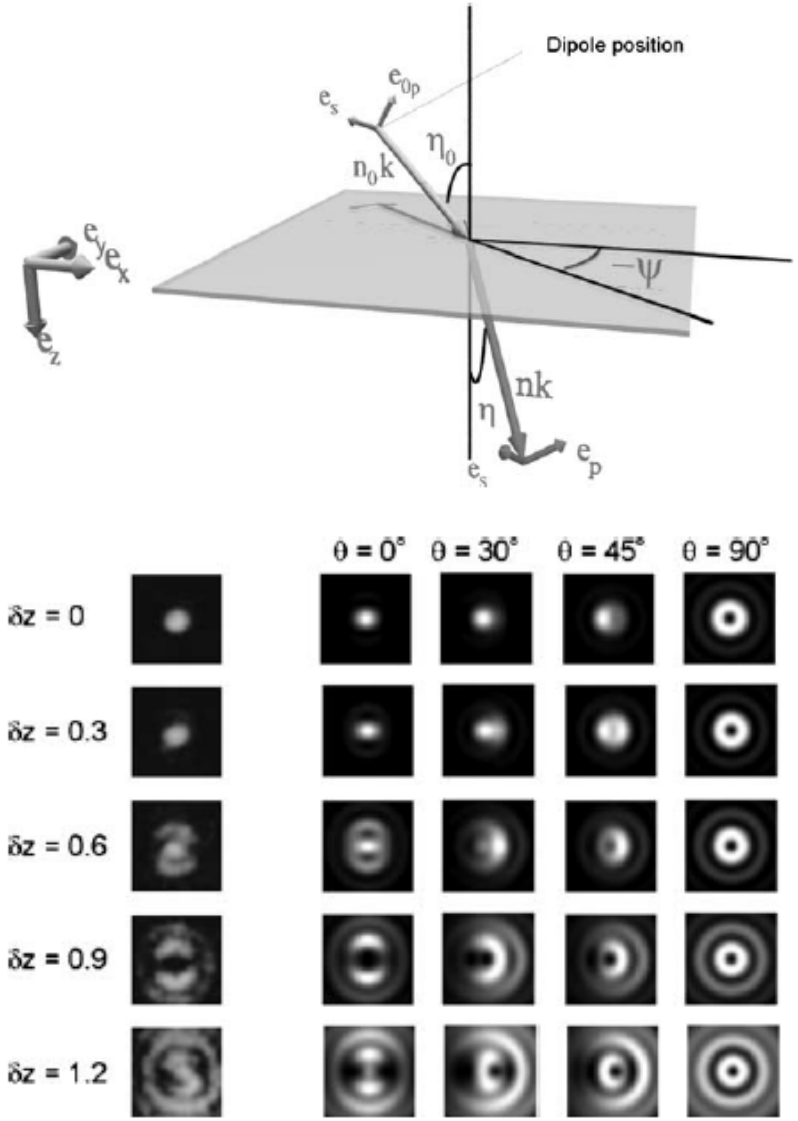


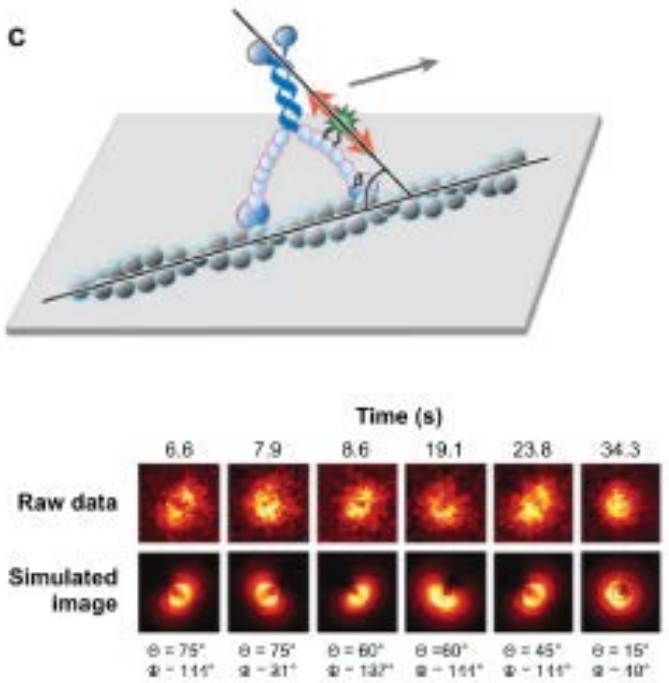


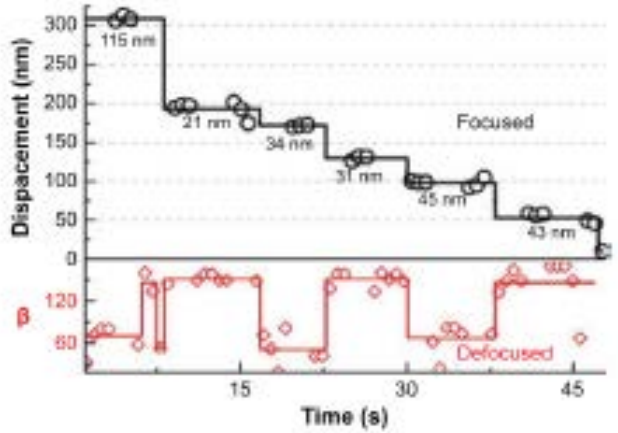




Defocusing fluorescence microscopy







Future of single molecule imaging…

- Brighter fluorophores
  - Better FPs or organic dyes
  - Less intrusive quantum dots
  - Diamond nitrogen vacancy centers
- Better hardware
  - Higher NA objectives with sapphire coverslips
  - More sensitive cameras with lower background noise
  - Ultrastable lasers

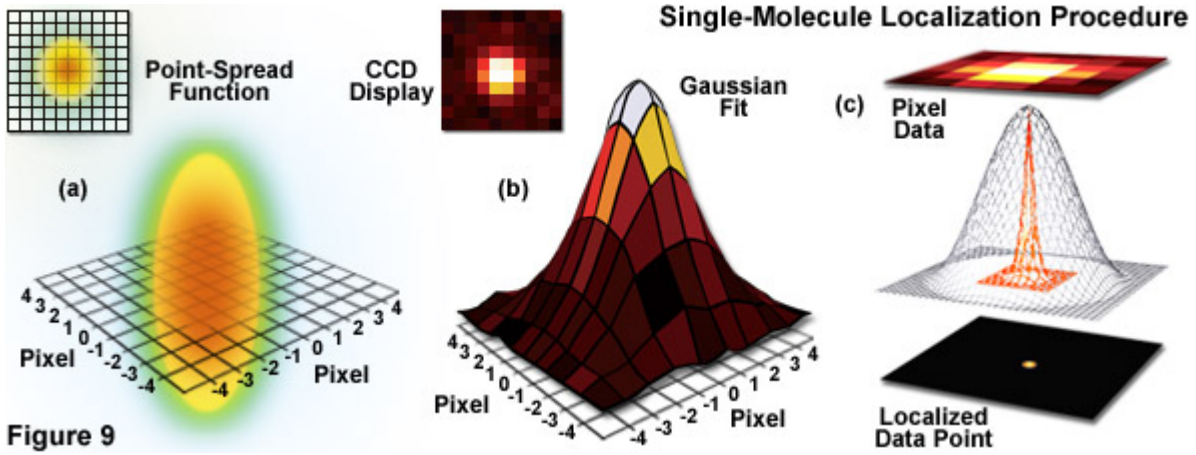


Figure 9

And on to Matlab…