Today's Announcements

1. Test given back today. In general you did very well!
2. HW assigned today. Due Wednesday, 4/4/12.
3. Next Monday—1st discussion about Individual Projects.

Today's take-home lessons

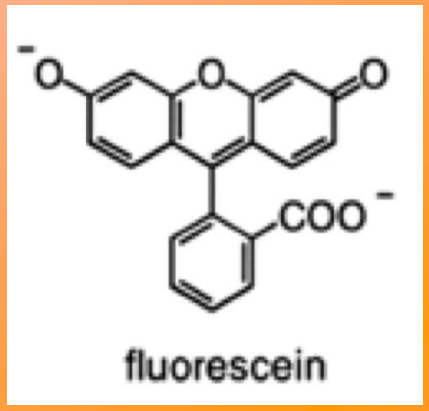
(i.e. what you should be able to answer at end of lecture)

1. How to label things so they fluoresce—ext. labels, GFP.
2. Total Internal Reflection Fluorescence (TIRF)- near surface.
3. Accuracy and Resolution—how fine can you see.
4. Fluorescence. What is it (amplitude, time-scale)?

Fluorophores & Quantum Yield

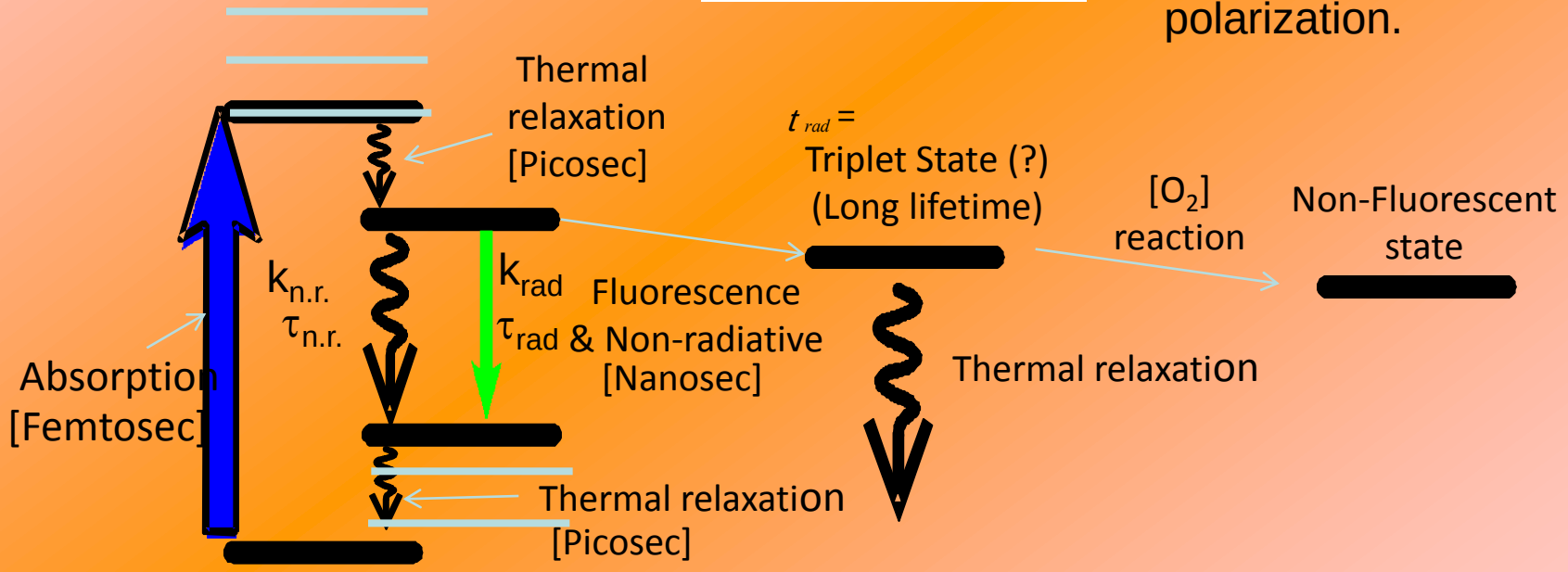
$k = k_{rad} + k_{n.r.}$; $\tau = \tau_{rad} + \tau_{n.r.}$
quantum yield $= k_{rad}/(k_{rad} + k_{n.r})$

Very good dyes have q.y. approaching one ($k_{n.r} \approx 0$)



fluorescein

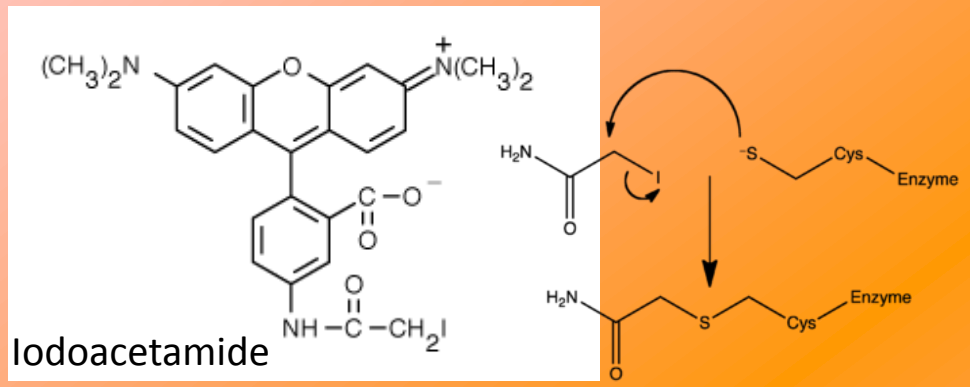
Have ≥ 1 electron that is free to move. Excitation light moves e's around, i.e. a dipole, and it can re-radiate, often with polarization.

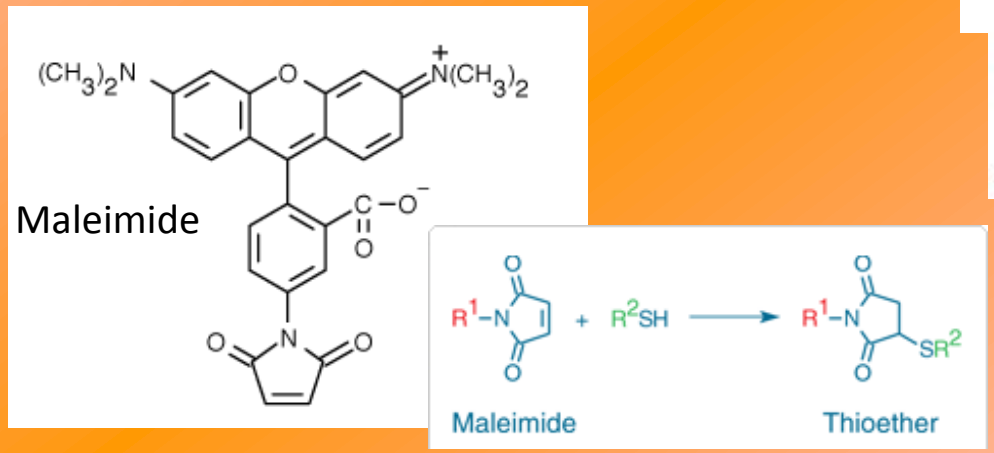


Basics of Labeling: In vitro

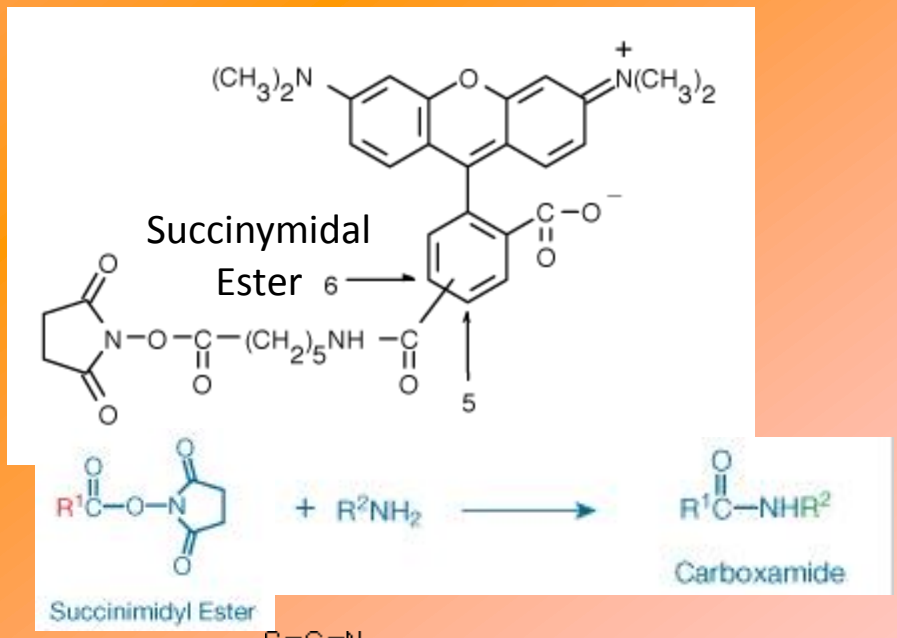
Bind to: free cysteines (R-SH) (often only one or a few in proteins)
: free lysines (R-NH_2) (many per protein)

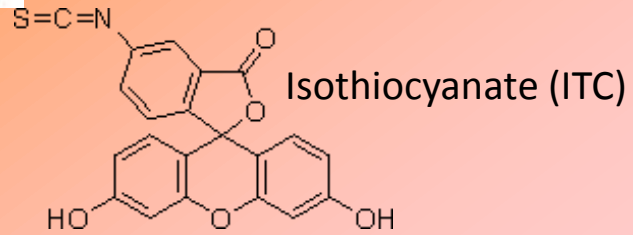
Cysteine Reactive

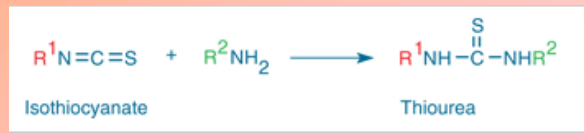




Amine Reactive





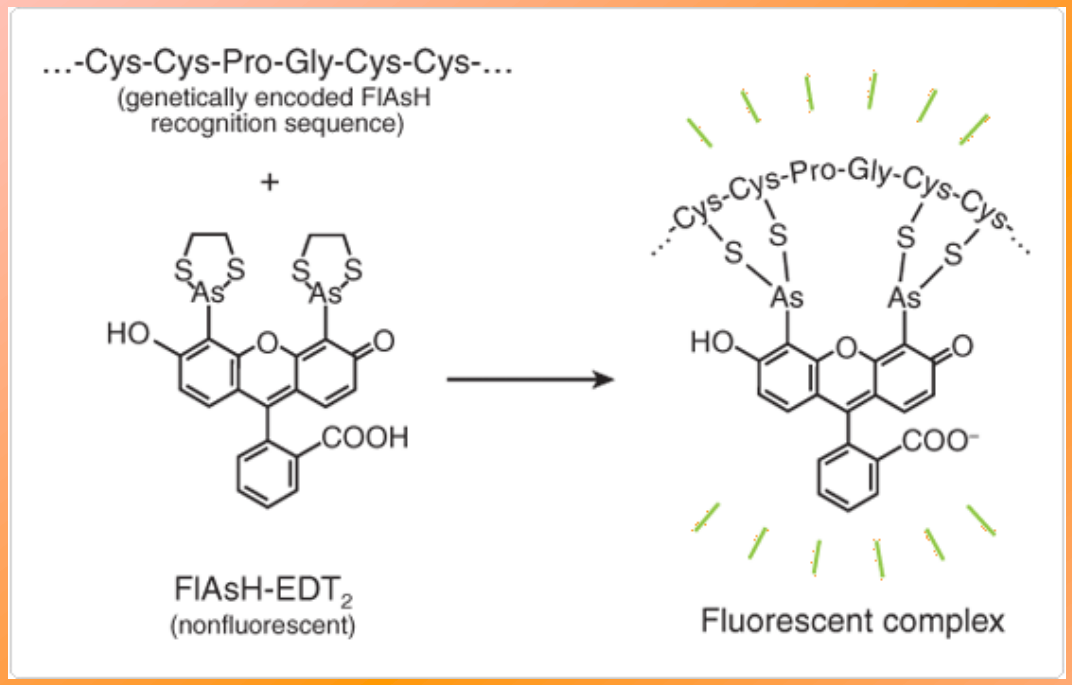


Basics of Labeling In vivo (inside cell)

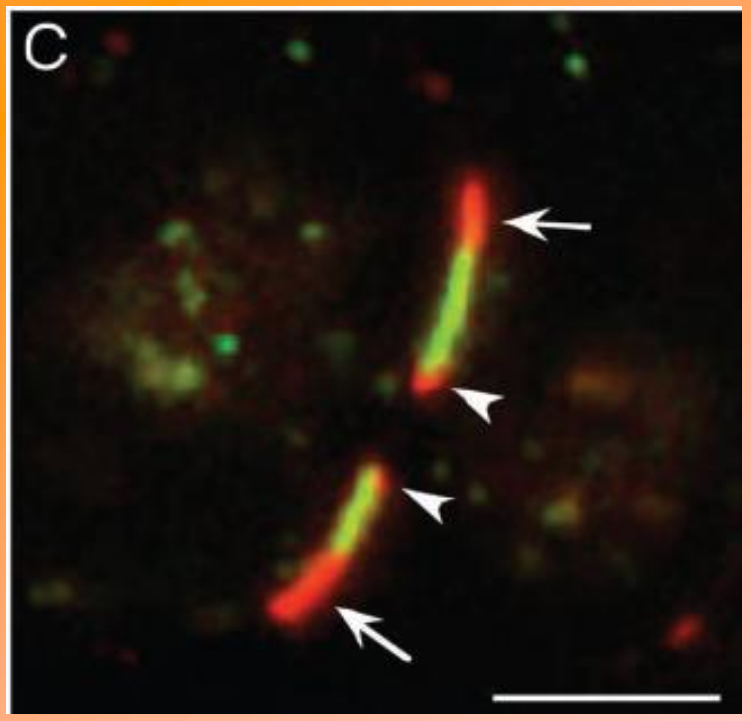
Cell has a membrane, which is, in general, impermeant to dyes!

Bi-Arsenic FLASH, Fluorescent Proteins, SNAP-tag, Halo-tag

Bi-Arsenic FLASH, ReASH…



Tsien, Science, 1998

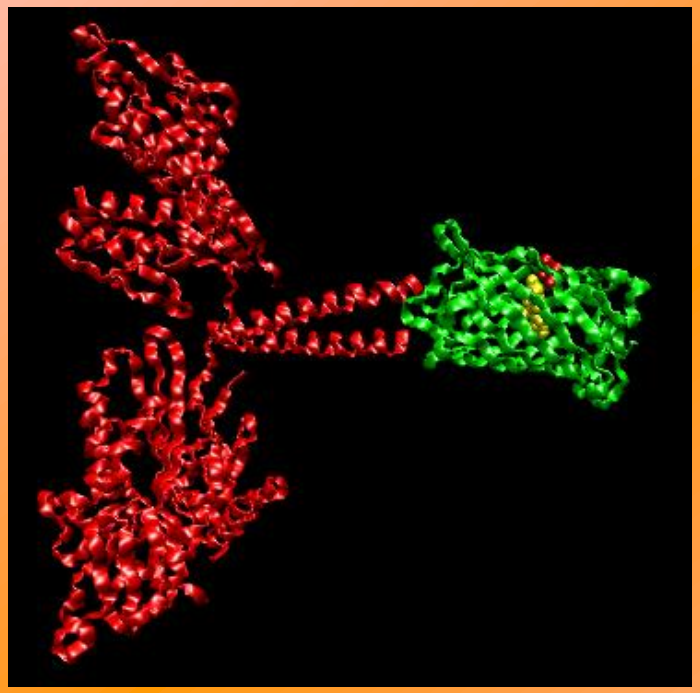


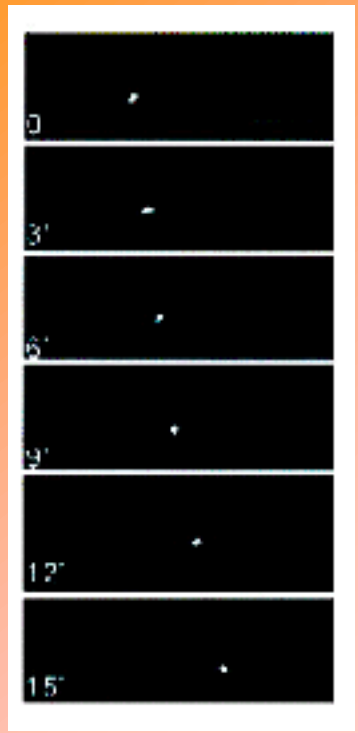
Tsien, Science, 2002

Green Fluorescent Protein (Nobel, 2009)

Genetically encoded dye (fluorescent protein)

(Motor) protein GFP





Kinesin – GFP fusion

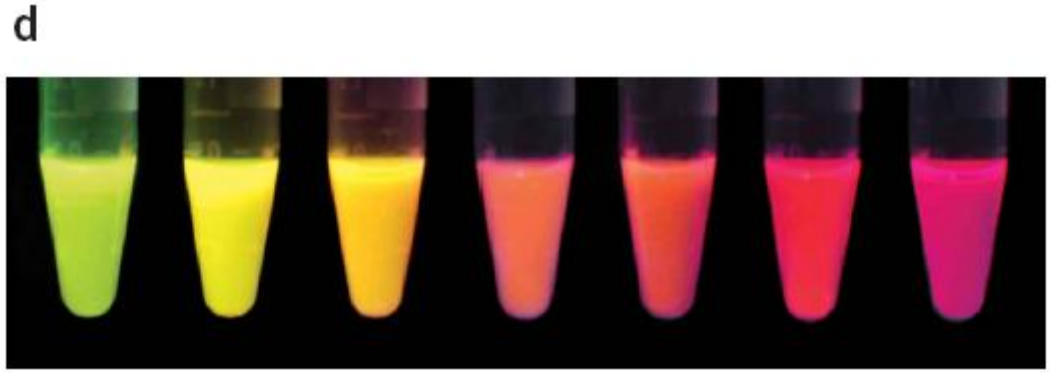
Wong RM et al. PNAS, 2002

Genetically encoded → perfect specificity

Different Fluorescent Proteins

Absorption

Fluorescence

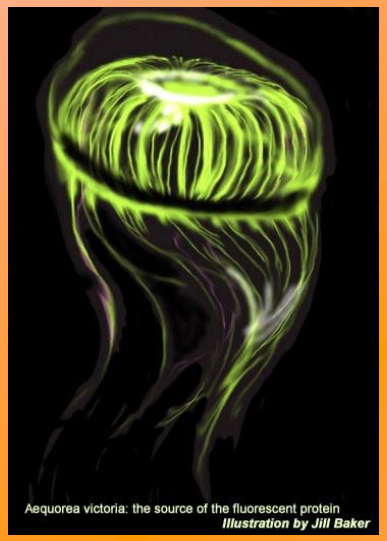


mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry

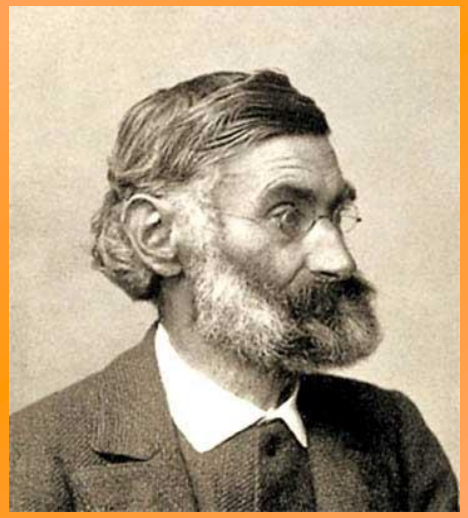
Shaner, Tsien, Nat. Bio., 2004

Green Fluorescent Protein: Genetically-encoded dye

Fluorescent protein from jelly fish



How fine can you see?
The Limits of Microscopy



Ernst Abbe

For visible microscopy,

<u>Resolution is limited to ~250 nm</u>

Ernst Abbe & Lord Rayleigh

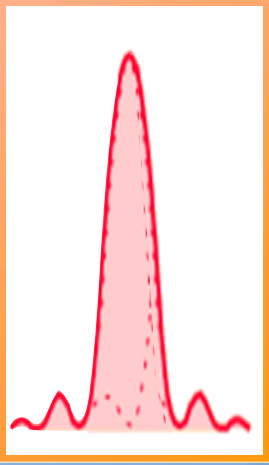
Recent microscopy: 1-100 nm,

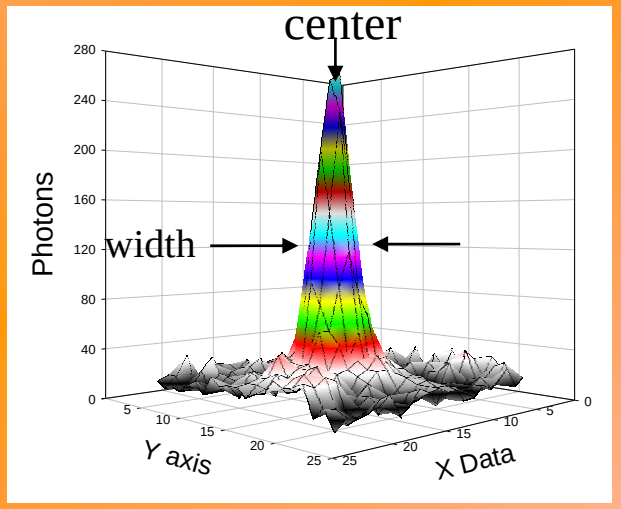
Here we present techniques which are able to get super-accuracy (1.5 nm) and/or super-resolution (<10 nm, 35 nm)

Super-Accuracy
(Accuracy << 250 nm: 1.5 nm, 1-500 ms)

FIONA

Fluorescence **I**maging with **O**ne **N**anometer **A**ccuracy





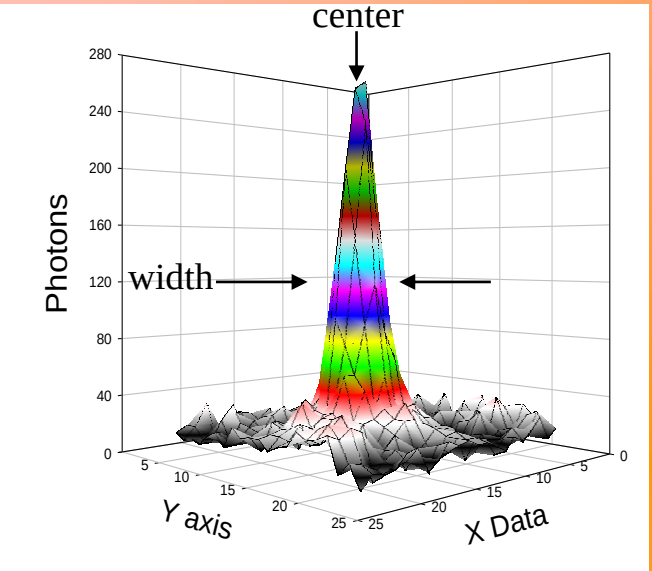
Center can be found much more accurately than width

S/N ≈ width /√N
≈ 250/√10^4 ≈ 1.3 nm

When light gets dim,
what happens to your ability to find the center?

How well can you localize?

Depend on 3 things



1. # of Photons Detected (N)

2. Pixel size of Detector (a)

3. Noise (Background) of Detector (b)

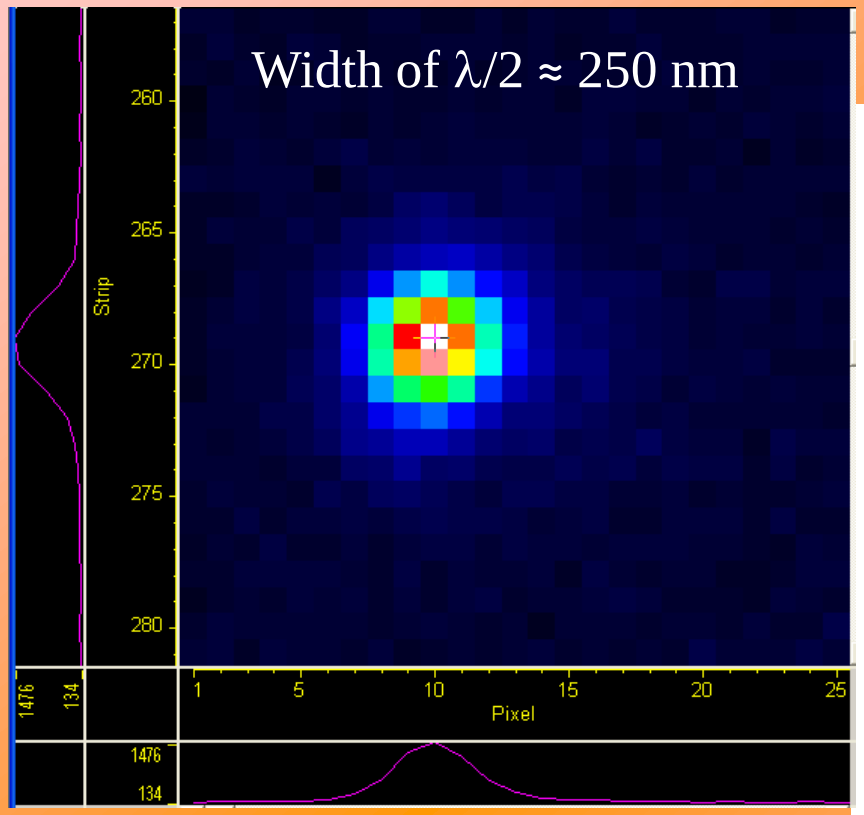
(includes background fluorescence and detector noise)

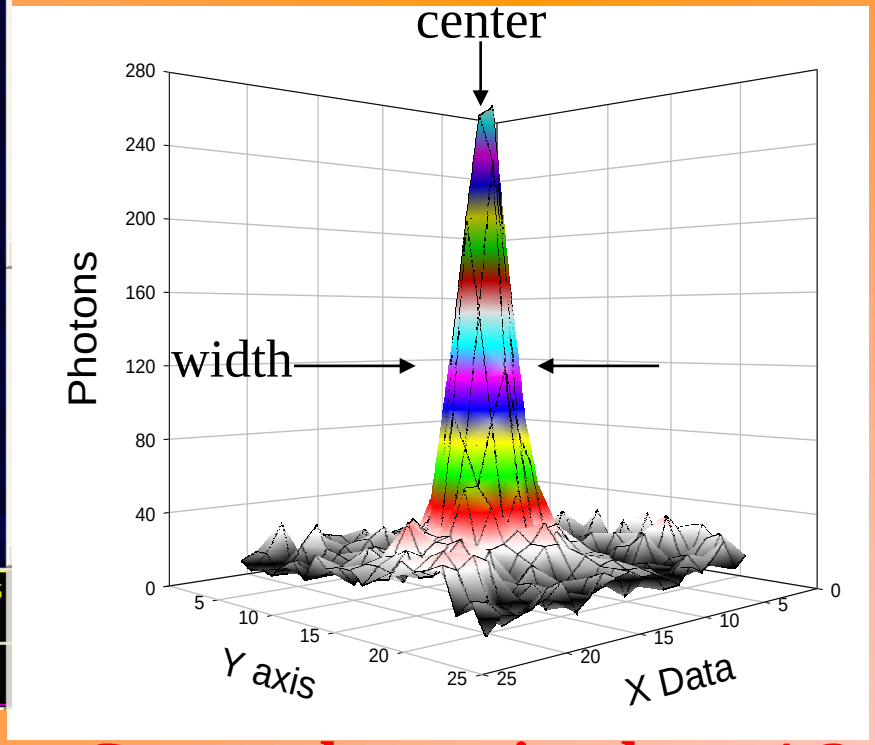
$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)}$$

derived by Thompson et al. (Biophys. J.).

Diffraction limited spot: Single Molecule Sensitivity

Accuracy of Center = width/ S-N
= 250 nm / $\sqrt{10^4}$ = 2.5 nm = ± 1.25nm





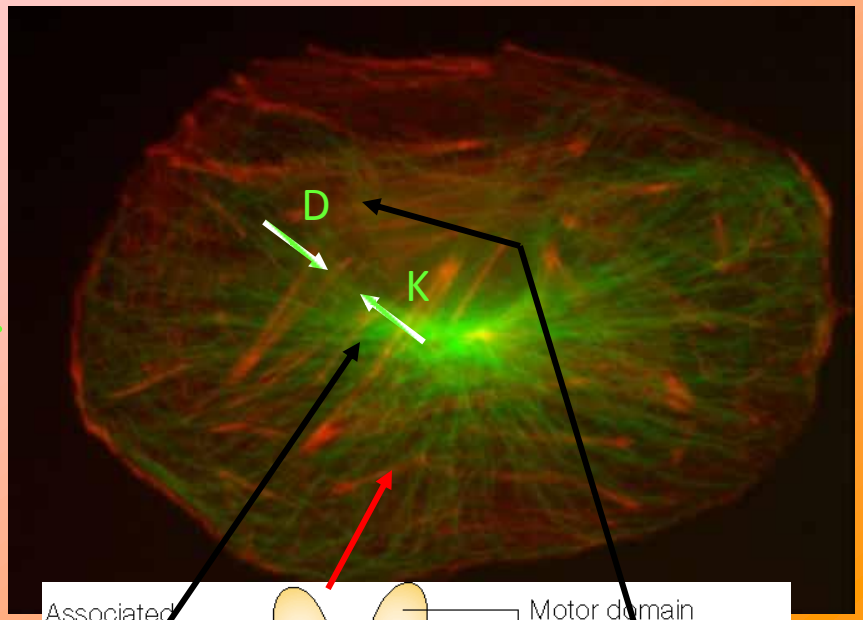
Enough photons (signal to noise)…**Center determined to ~1.3 nm**

Dye lasts 5-10x longer -- typically ~30 sec- 1 min. (up to 4 min)

Start of high-accuracy single molecule microscopy

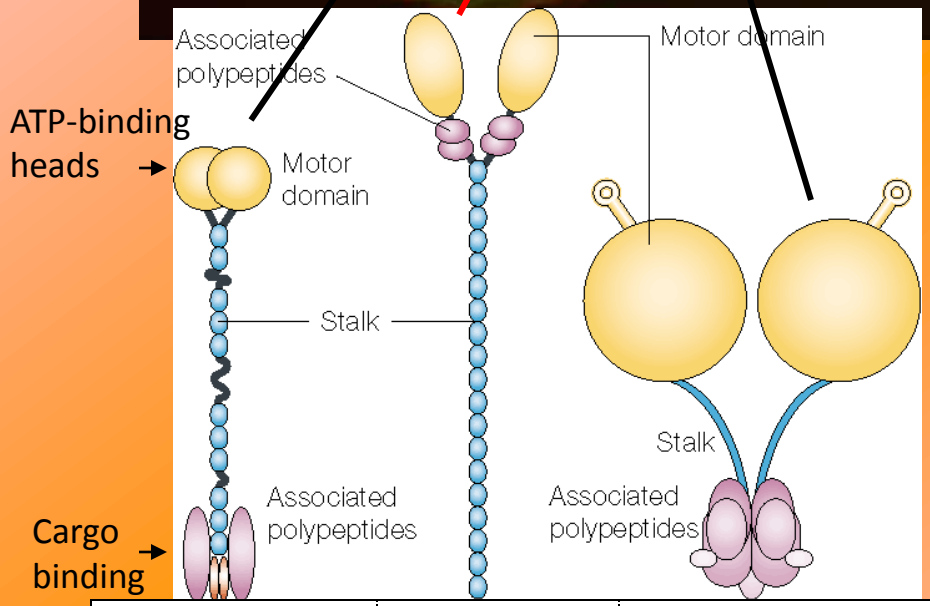
Thompson, BJ, 2002; Yildiz, Science, 2003

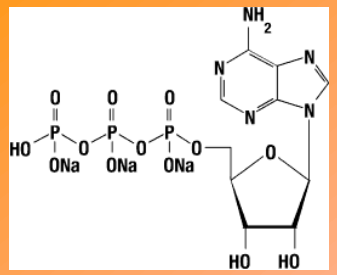
Biomolecular Motors: Intra- & Extra-Cellular Motion



Characteristics

- nm scale
- Move along tracks
- intracellular directional movement
- cell shape changes & extracellular movement
- Use ATP as energy source

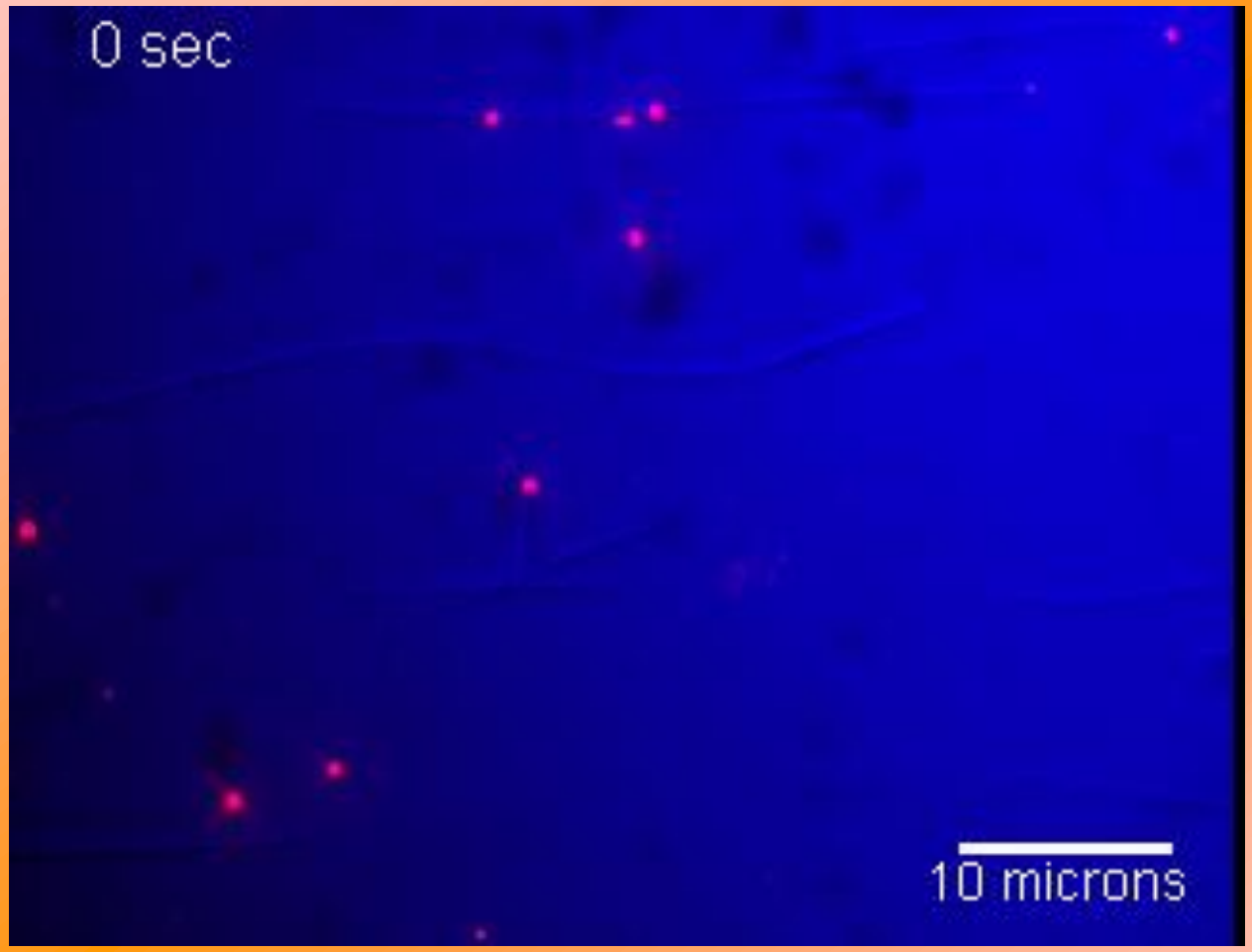




ATP → mechanical work

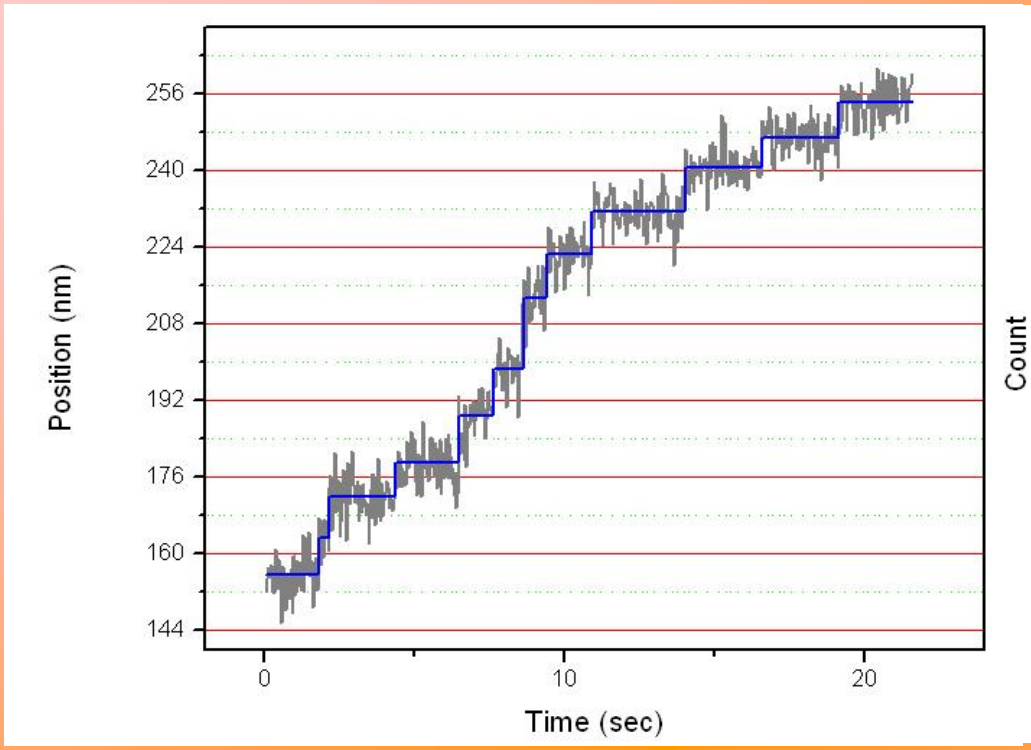
Kinesin	Myosin	Dynein	← Motor
Microtubule	actin	Microtubule	← polymer

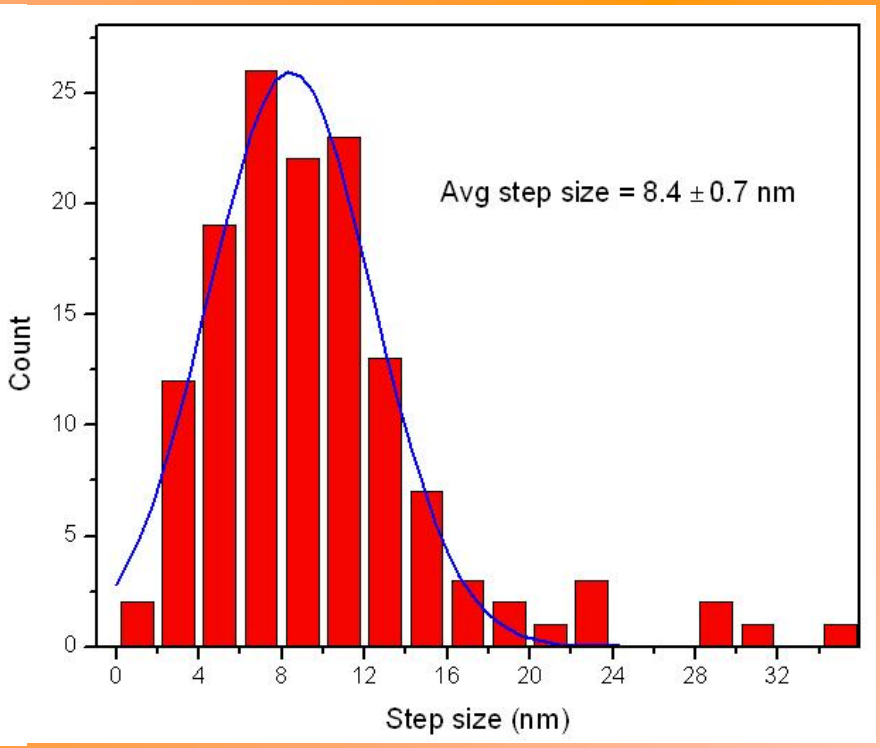
Motility of quantum-dot labeled Kinesin (CENP-E)



8.3 nm/step from optical trap

Kinesin (Center-of-Mass) Moving

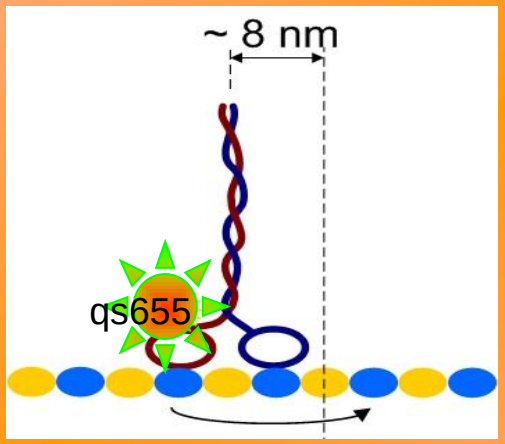




Kinesin moves with 8.4 nm /ATP step size.

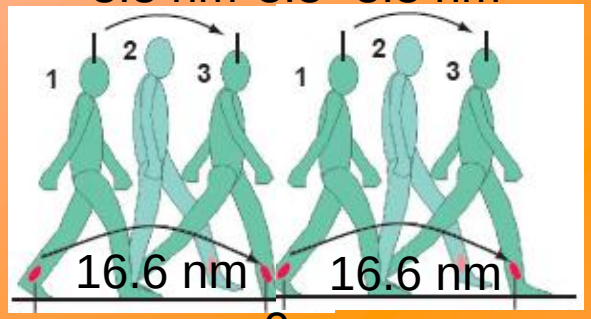
Kinesin: Hand-over-hand or Inchworm?

8.3 nm, 8.3 nm

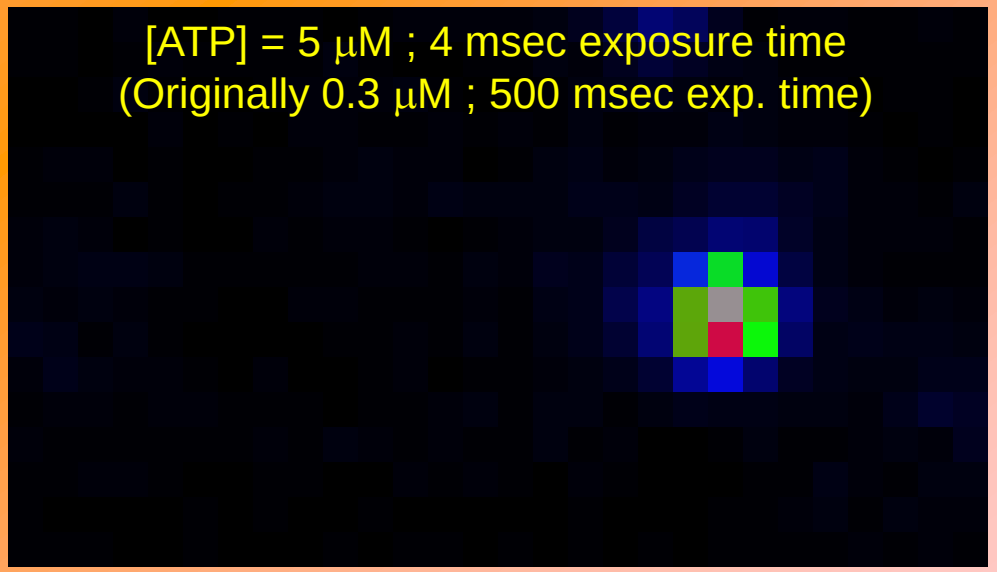


16 nm

8.3 nm 8.3 8.3 nm

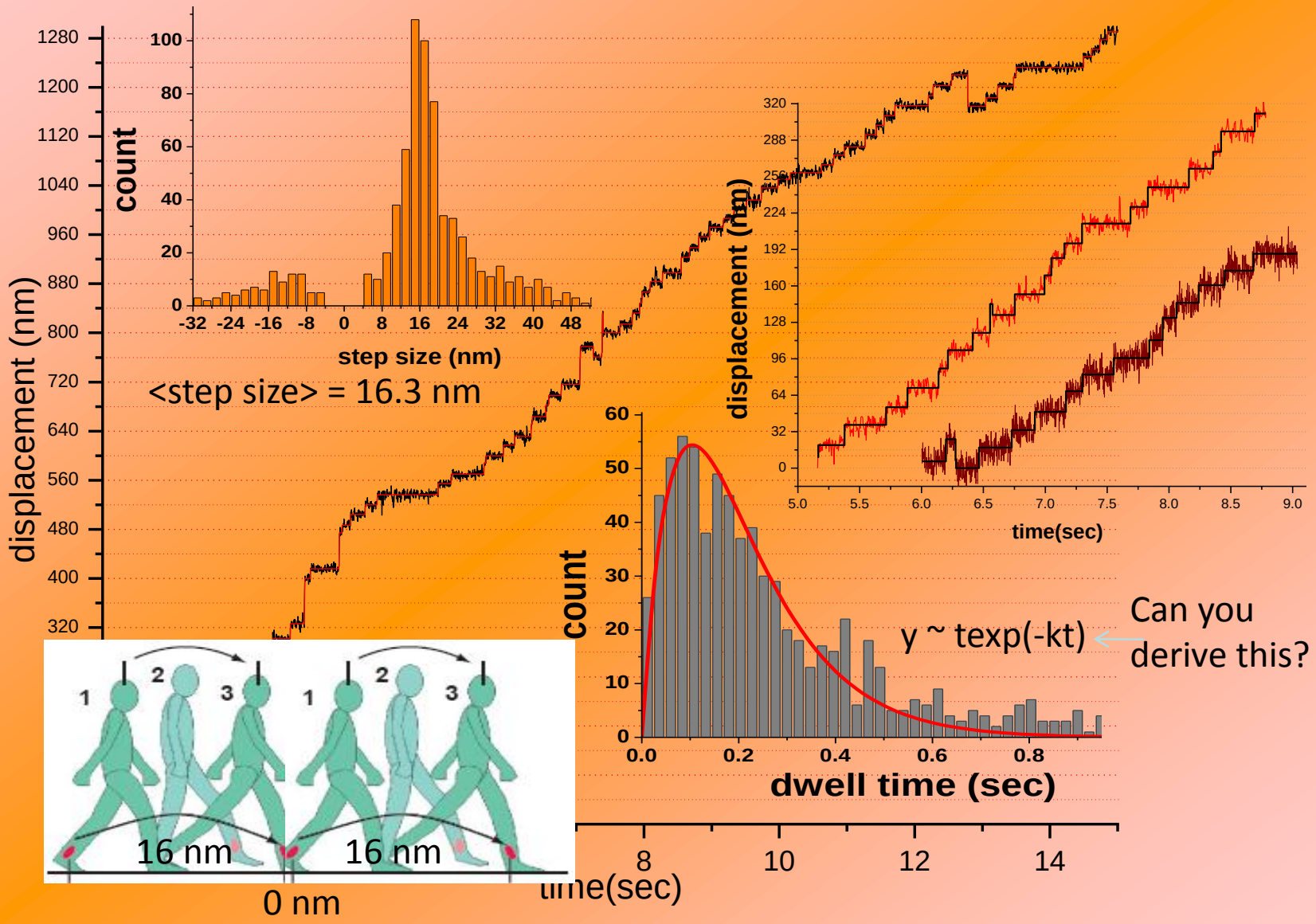


16.6, 0, 16.6 nm, 0…



pixel size is 160nm
2 x real time

Kinesin



Takes 16 nm hand-over-hand steps (even at 5μM)

Class evaluation

1. What was the most interesting thing you learned in class today?

2. What are you confused about?

3. Related to today's subject, what would you like to know more about?

4. Any helpful comments.

Answer, and turn in at the end of class.