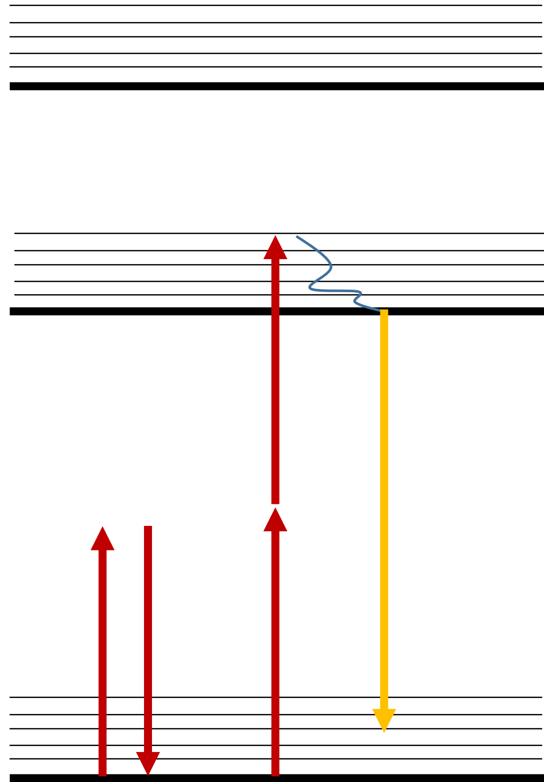
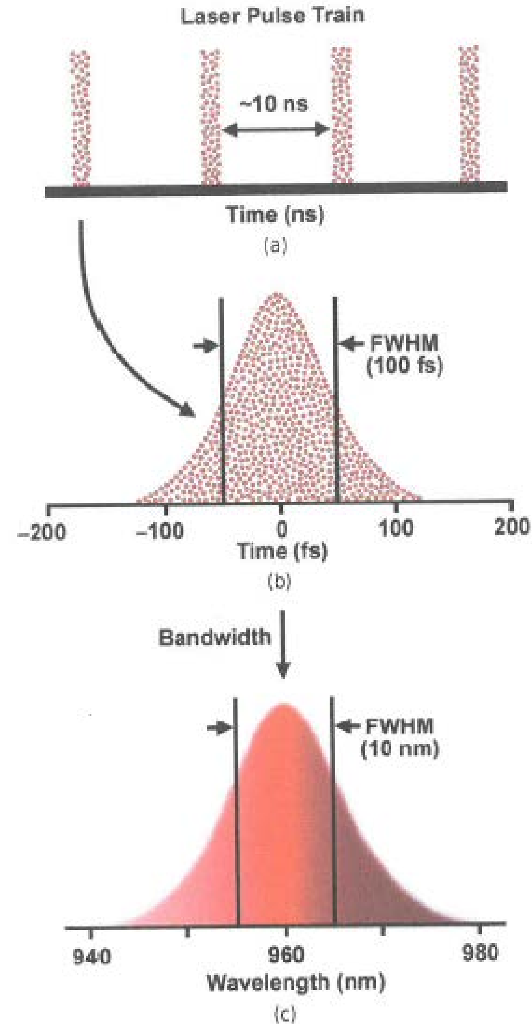
Finish 2 photon, super resolution

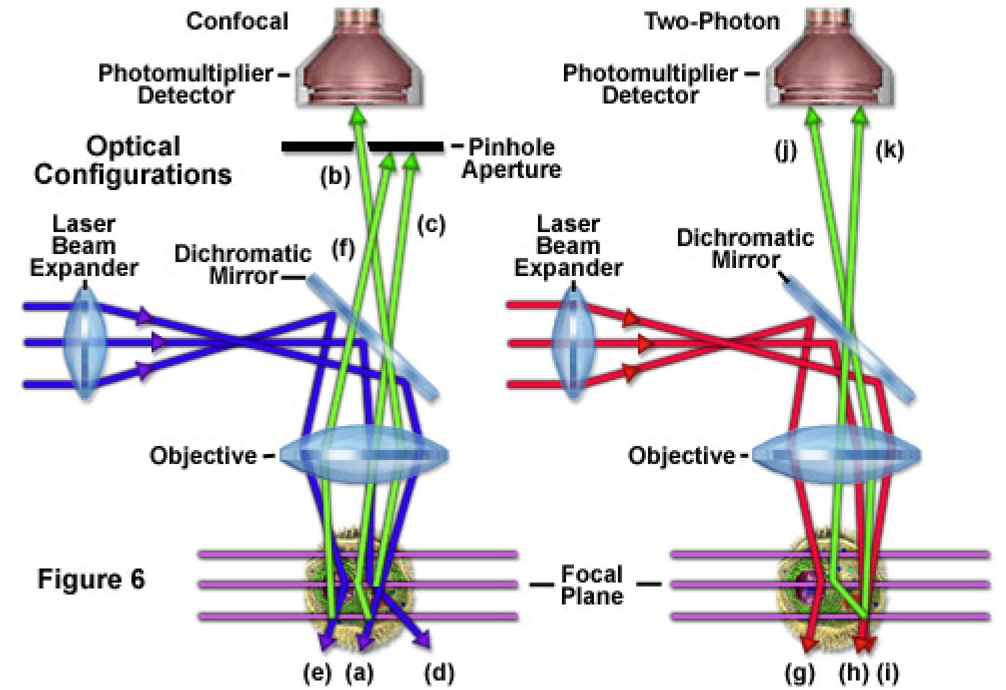
- HW 6 due
- Midterm on Wed, covers everything since 1st midterm
- Guest lecture on Mon – Joe Dragavon

- Last class
  - Spinning disk
  - 2-photon
- This class
  - 2-photon equipment
  - 2-photon examples
  - Super-resolution

2-photon imaging

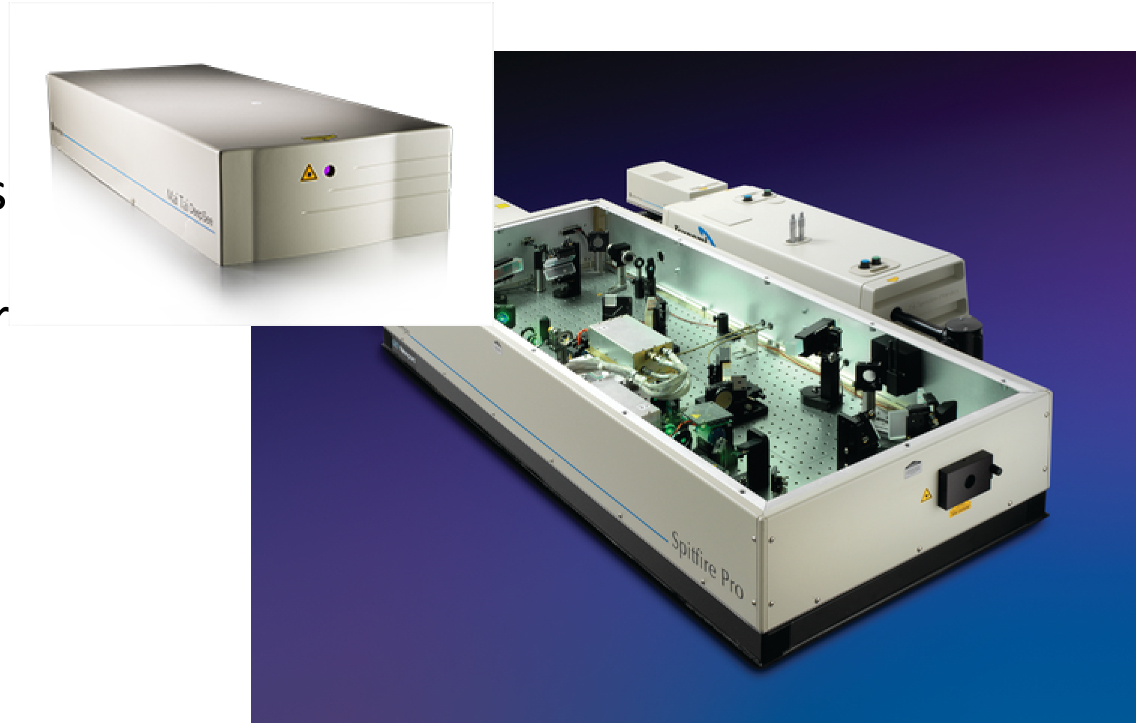


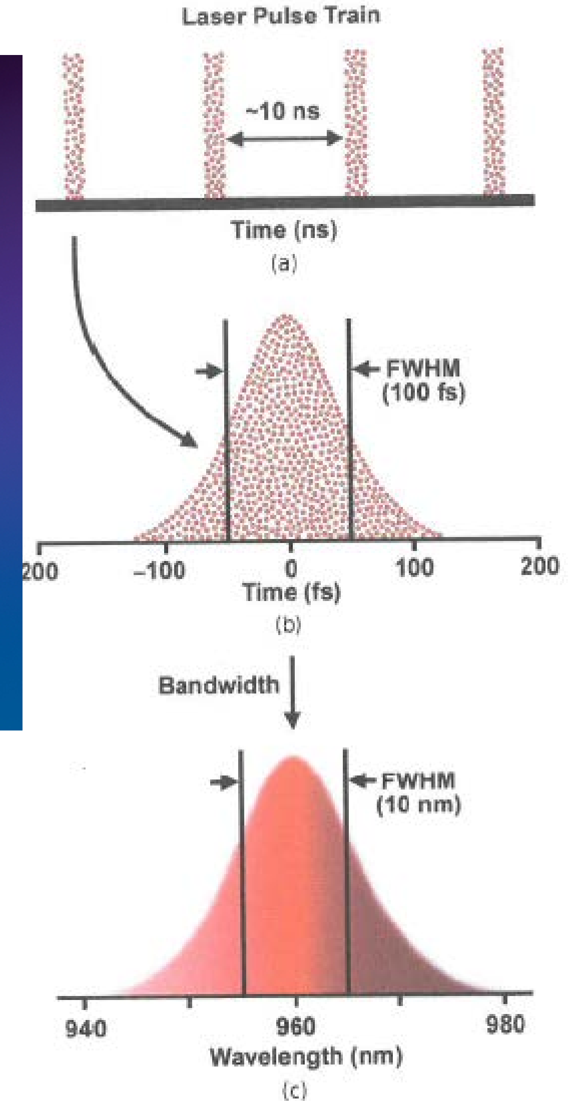




2 Photon Equipment - Laser

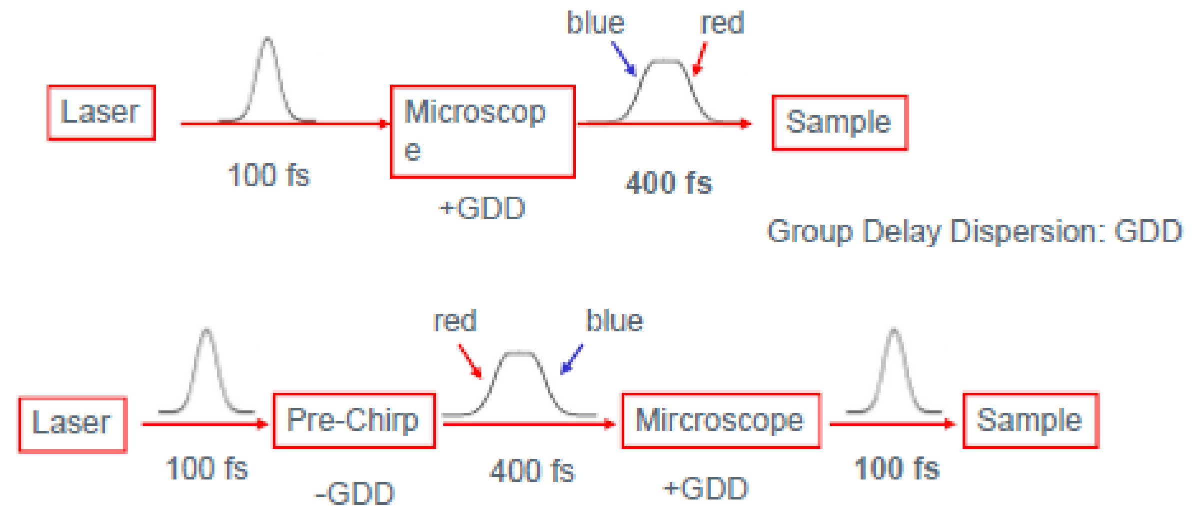
- Ultrafast laser – Titanium sapphire oscillator produces pulses at 800 nm
- Optical parametric oscillator (OPO) enables tuning from ~680-1200 nm
- Pulse traveling through a lens (objective) increases dispersion, broadens pulse in time
- Possible to compensate by applying negative dispersion early on
- EXPENSIVE!!

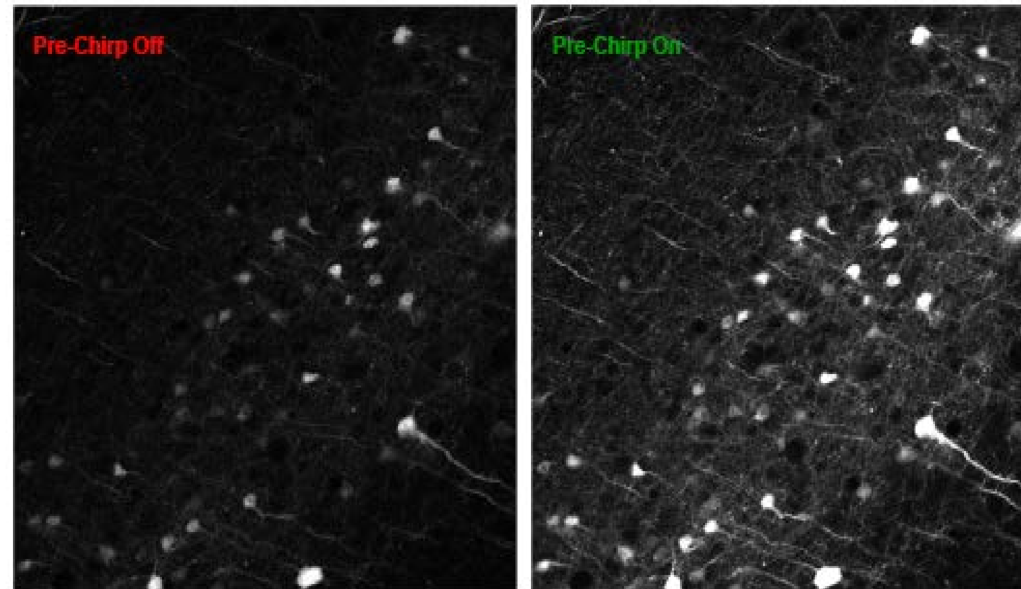




Dispersion

- Requires ~ 10^{14} W/m^2 required for 2 photon excitation (Surface of the sun is ~10^8 W/m^2)
- Dispersion affects intensity
- Intensity affects fluorescence^2
- A 4 fold increase in time duration of pulse -> a 16x reduction in fluorescence





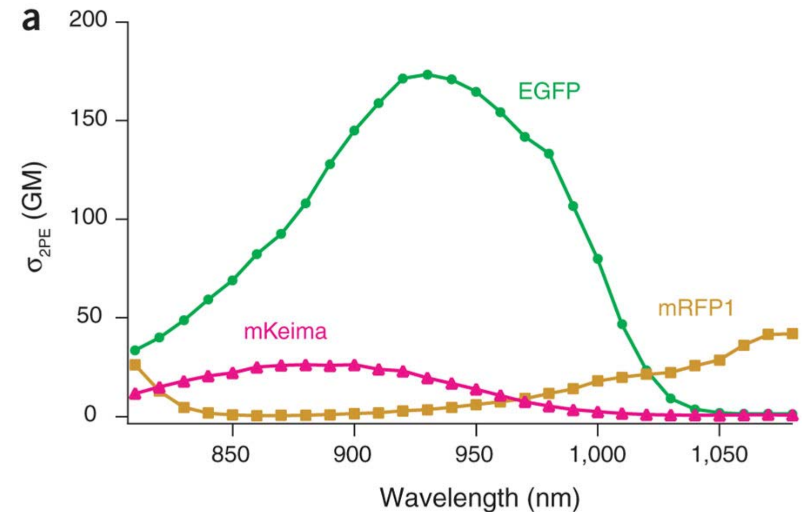
2 Photon equipment – objectives, detectors

- Ideal to have high NA
- Must transmit in the IR
- Water immersion to dip into samples (or brains)
- Long working distance
- High field number
- EXPENSIVE

- Still use PMT, but due to low signals, choose the most sensitive ones available.
- OK to have a large area, as it will collect more scattered fluorescent photons

Downsides of 2 photon

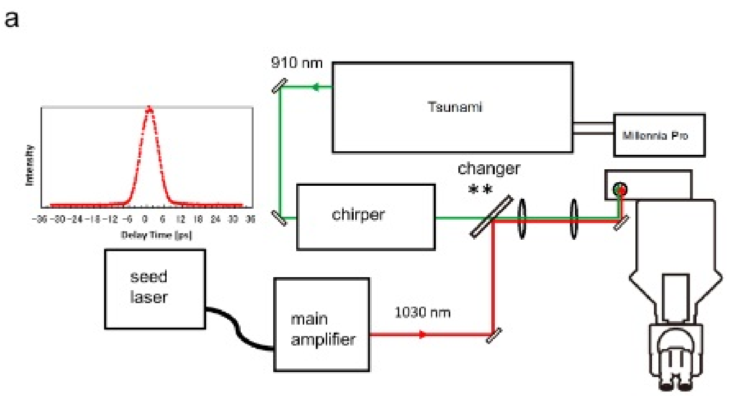
- Very expensive
- Slow scan rates (unless you use holographic dispersion)
- Typically limited to imaging one color (takes fair amount of time to switch laser color)
- Low 2 photon cross sections typically make for dim samples
- Equipment often requires regular upkeep to maintain image quality

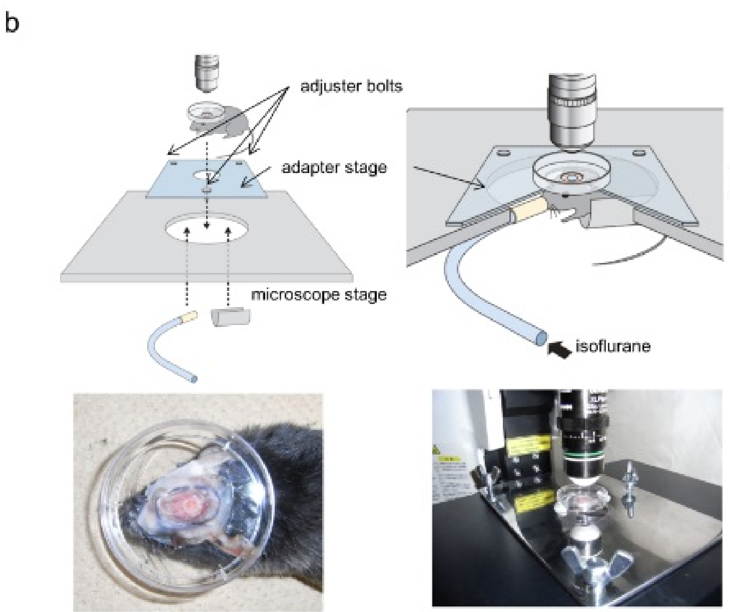


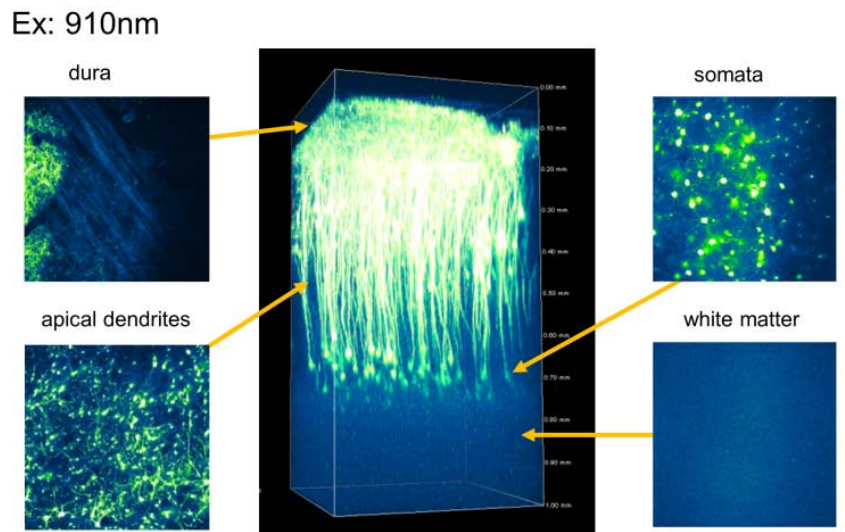
Can use mKeima and GFP to image 2 colors at the same time

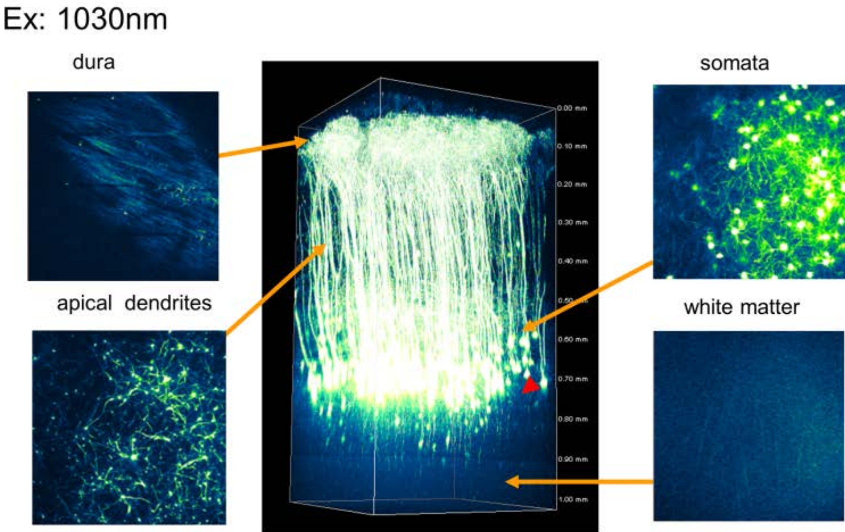
CHARACTERISTIC	dKeima-Red	mKeima-Red
Oligomerization	Dimer	Monomer
Number of amino acid	222	222
Excit./Emiss. maxima (nm)	440/616	440/620
Molar extinction coefficient ($M^{-1}cm^{-1}$)	24,600 (440 nm)	14,400 (440 nm)
Fluorescence quantum yield	0.31	0.24
Brightness*[1]	7.6	3.5

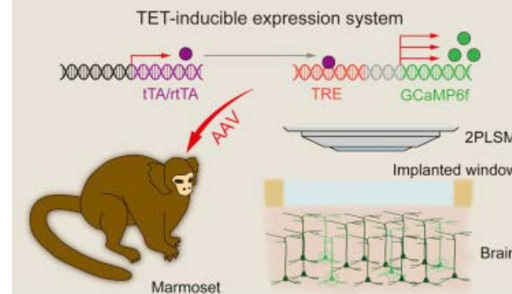
Using 2 photon

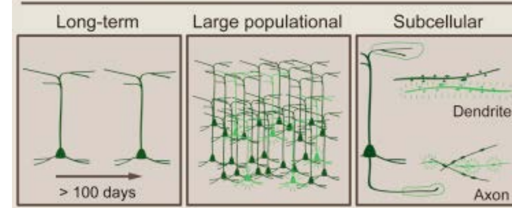


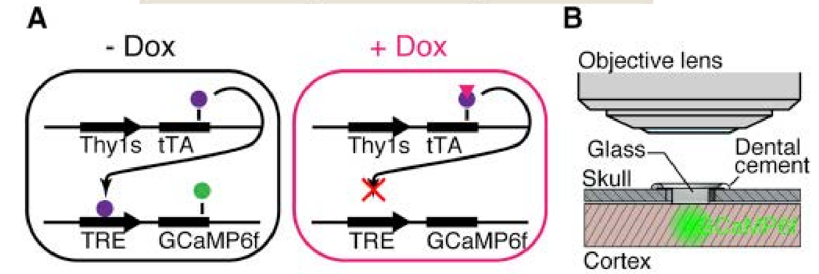


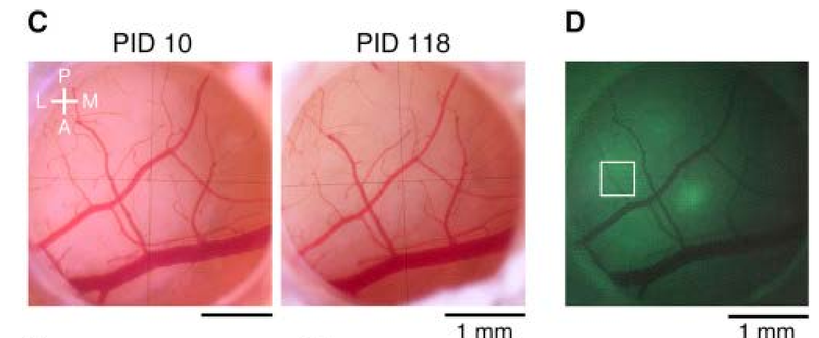


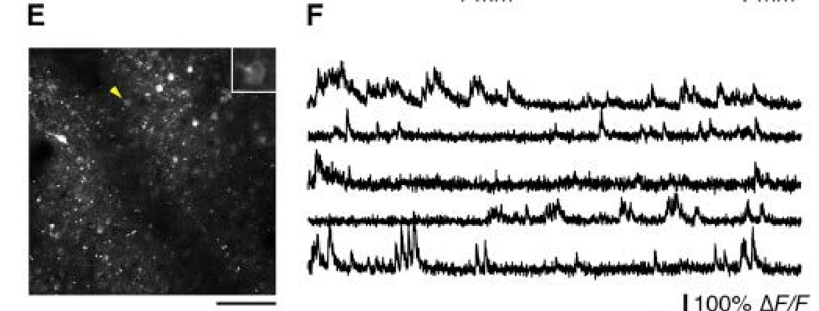






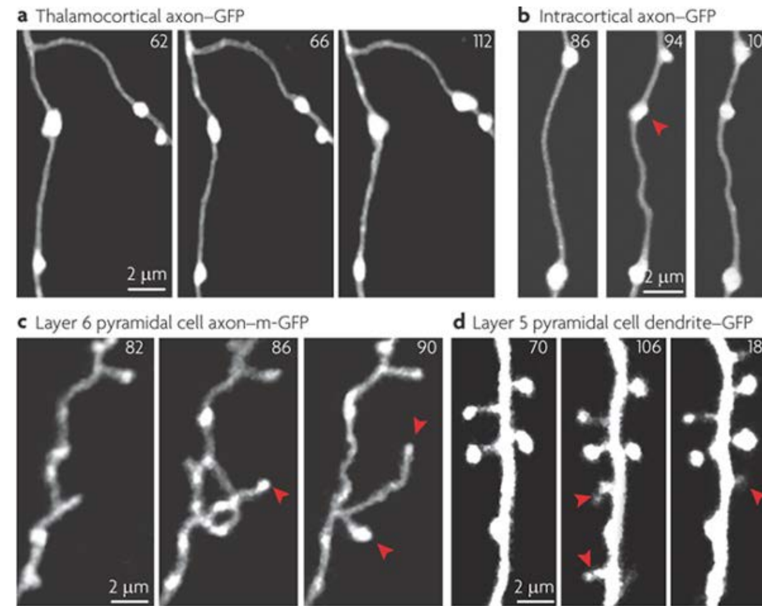


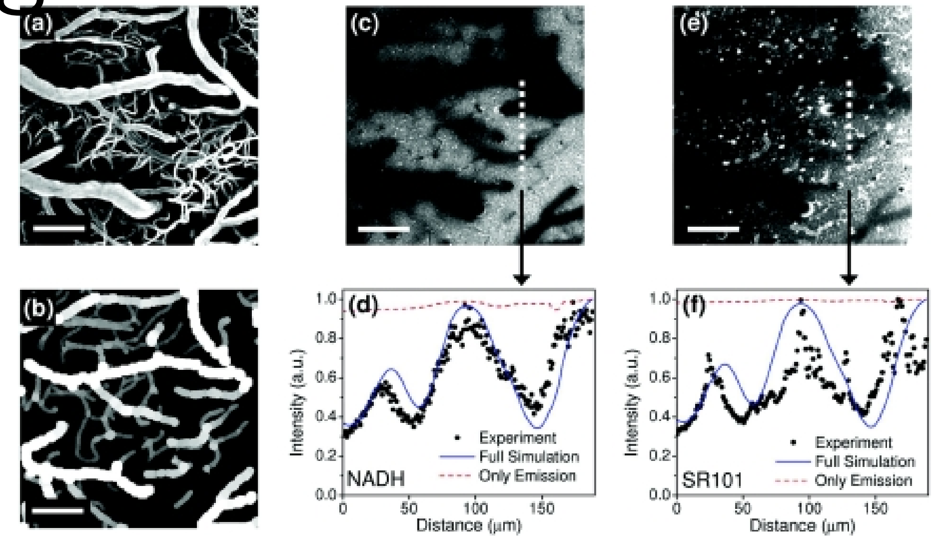


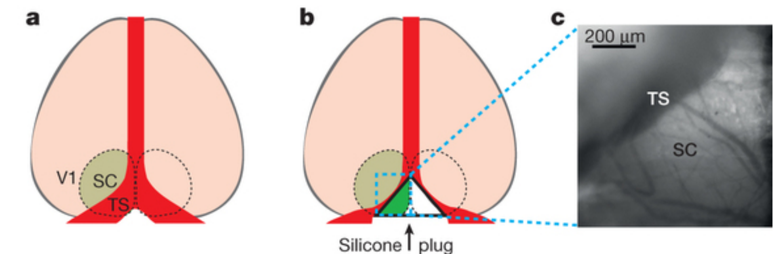


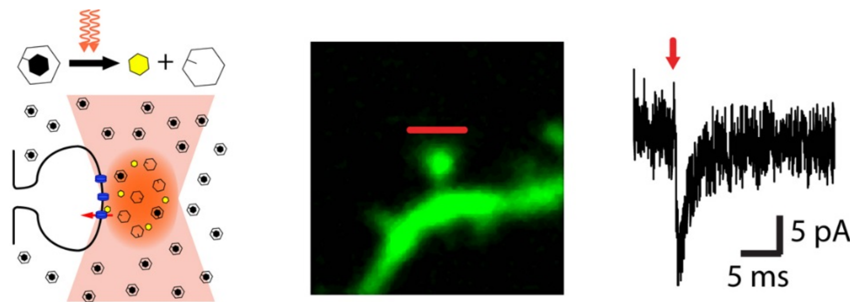
Applications of 2 photon imaging

- 2 Photon can image 300-600 µm into tissue
- Long term, deep brain imaging
- 2 photon NADH imaging, brain metabolism
- 2 photon uncaging

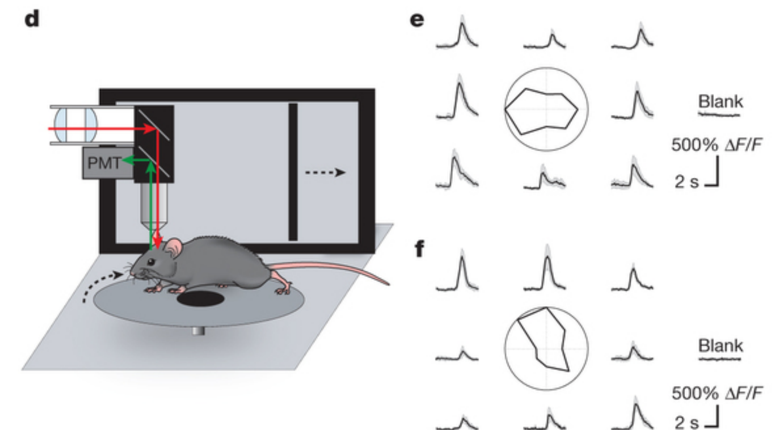




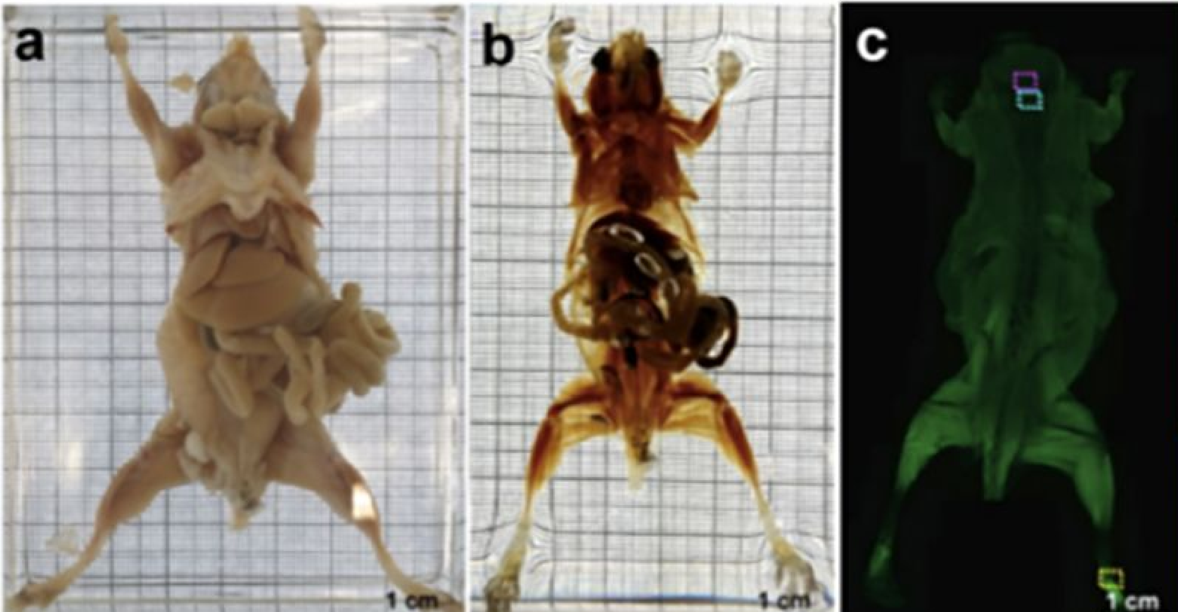


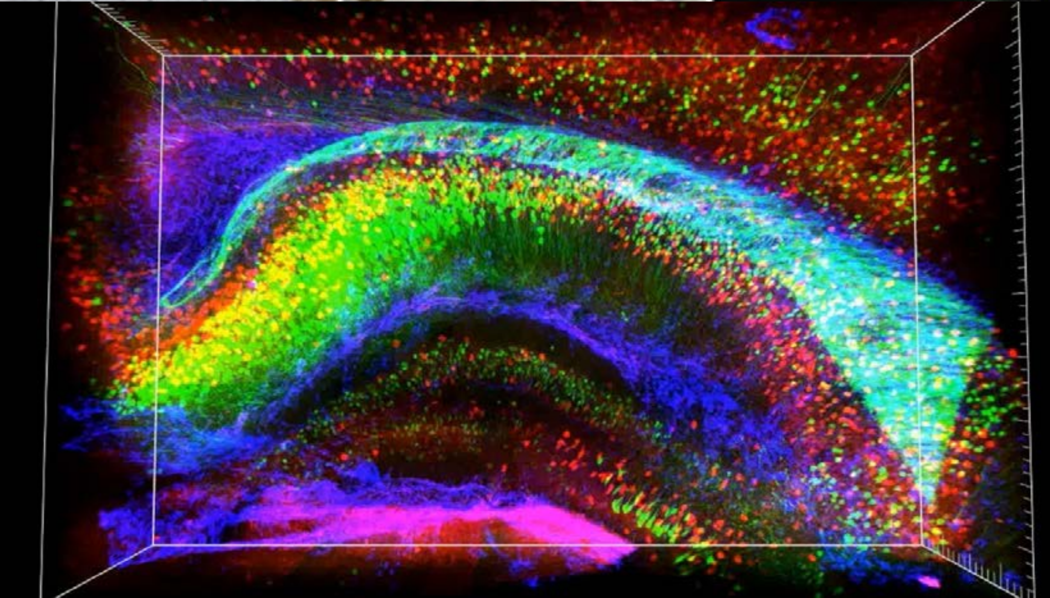


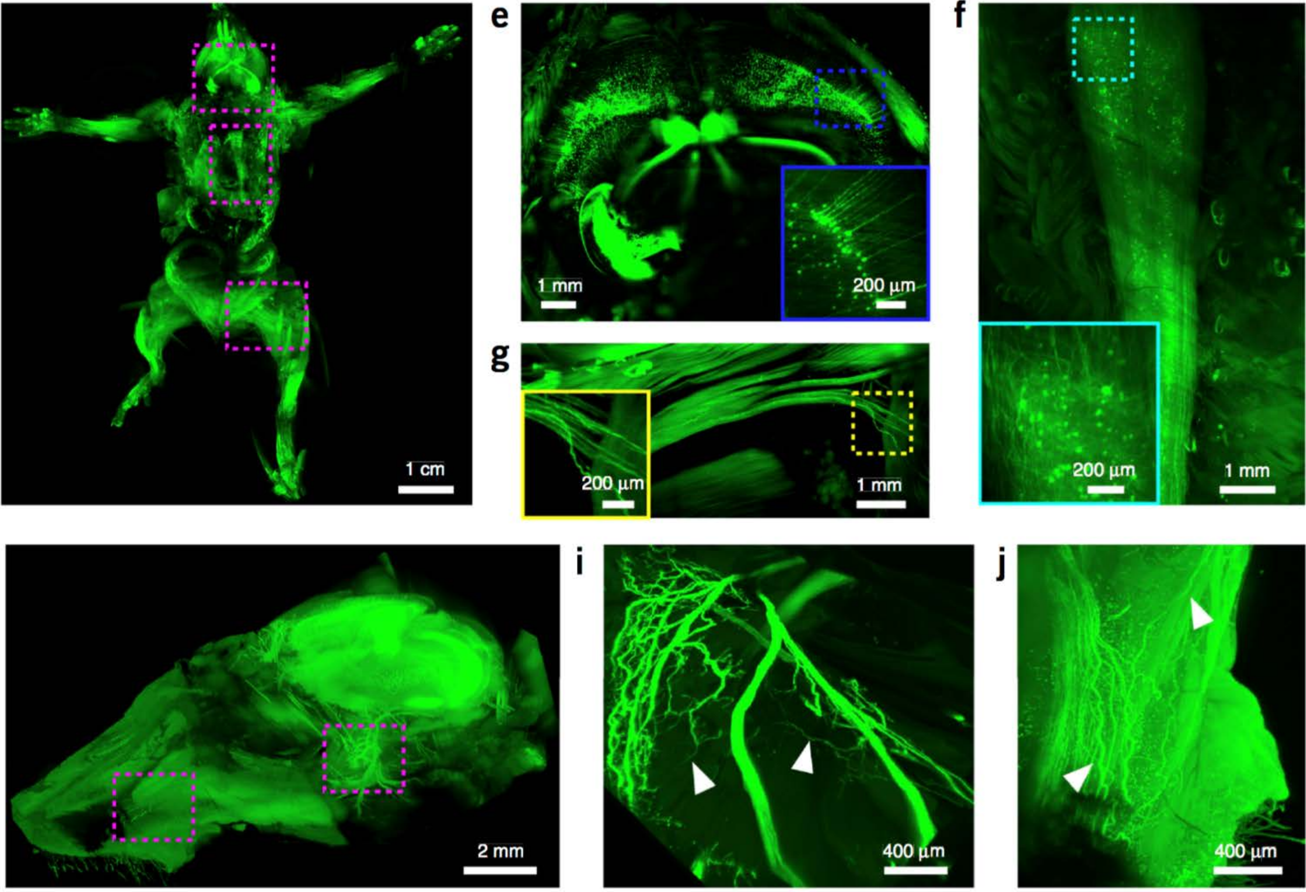
Activation of synaptic glutamate receptors by 2-photon glutamate uncaging.



Clarity/uDisco

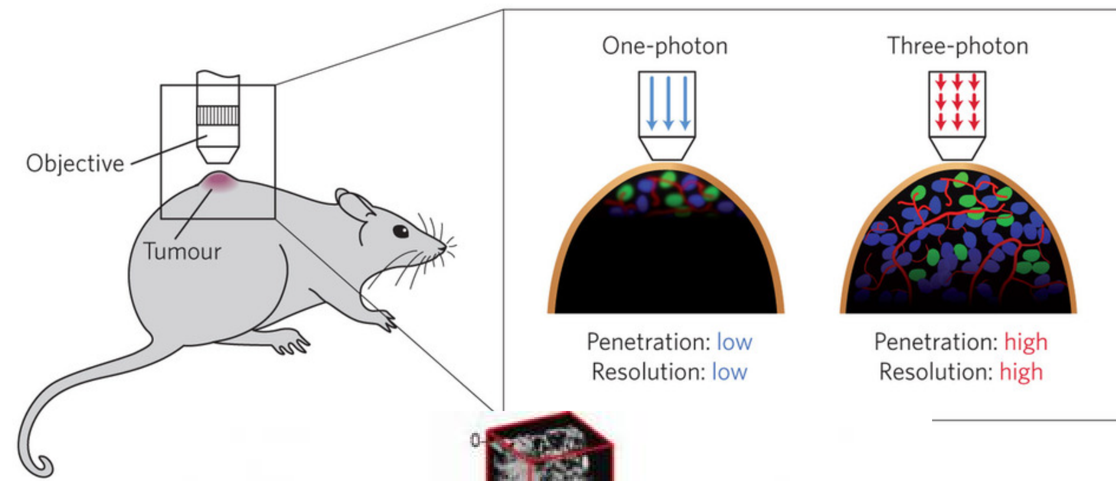


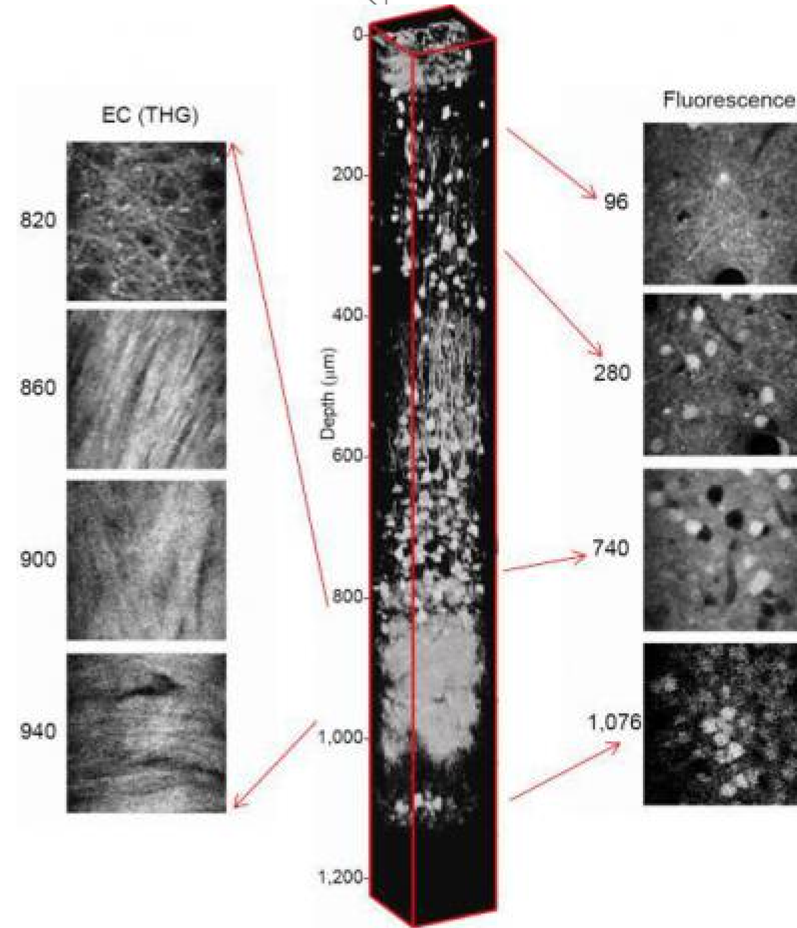




3 photon imaging

- Sure, why not…
- Absorption increases as Intensity^3
- 10x greater photon density needed for 3 photon as compared to 2
- Same laser can be used to excite 3 photon UV, and 2 photon green
- Eventually you run into absorption of IR light by water
- Technically possible to use 4 photon, but nobody does





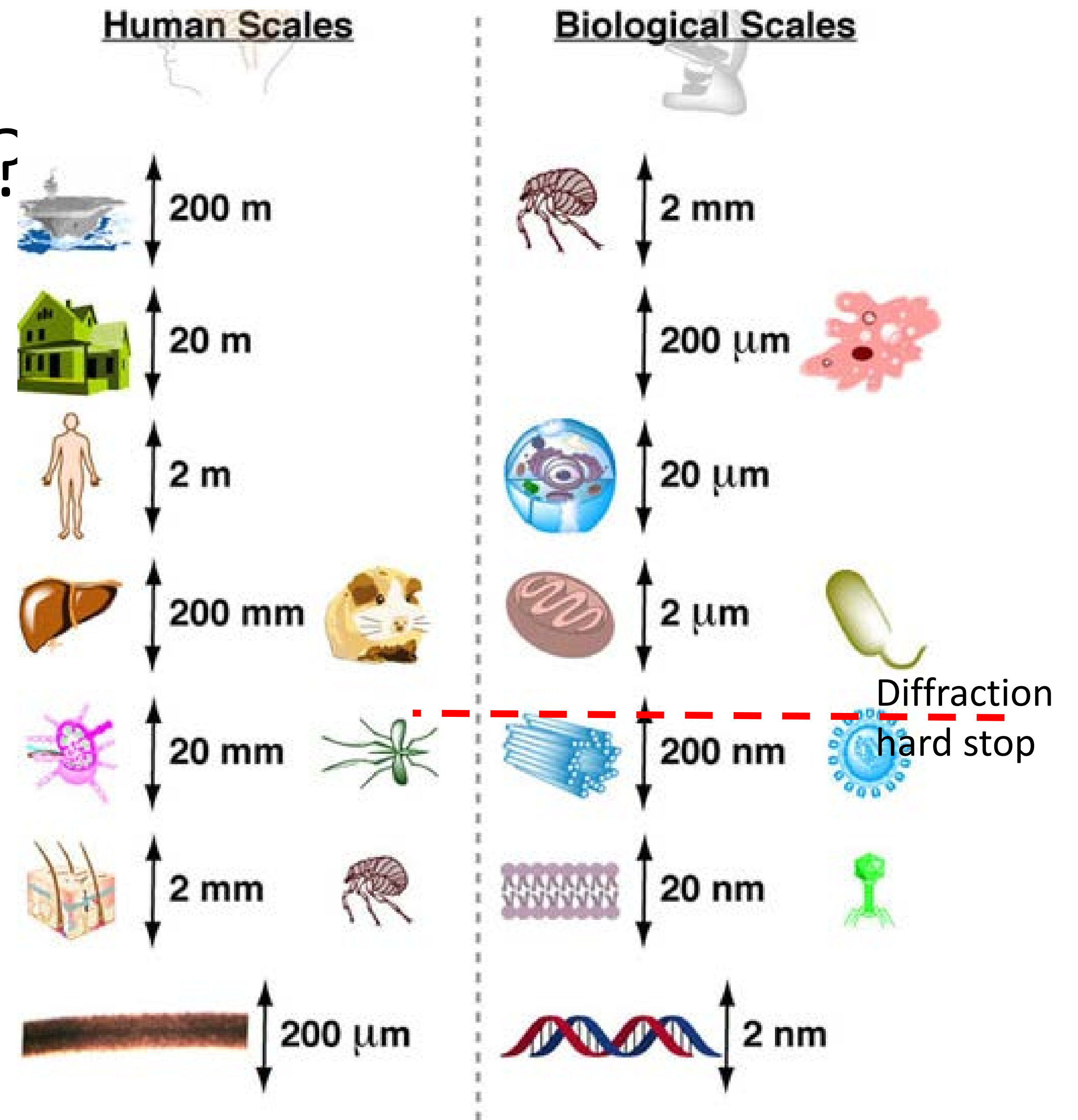
In vivo imaging of RFP neurons beyond the white matter

Super resolution

- When 200 nm just isn't enough
- Superresolution is defined as resolving features better than Abbe's limit
- 2014 Nobel prize – Eric Betzig/Stefan Hell

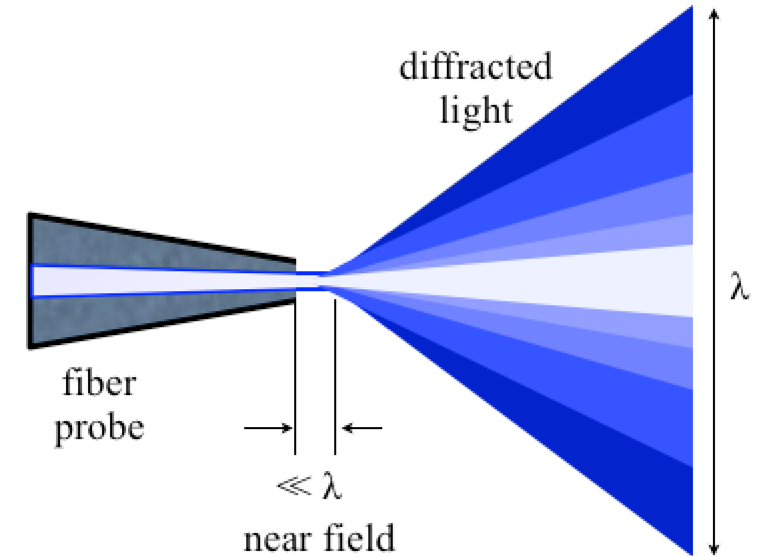
Why superresolution?

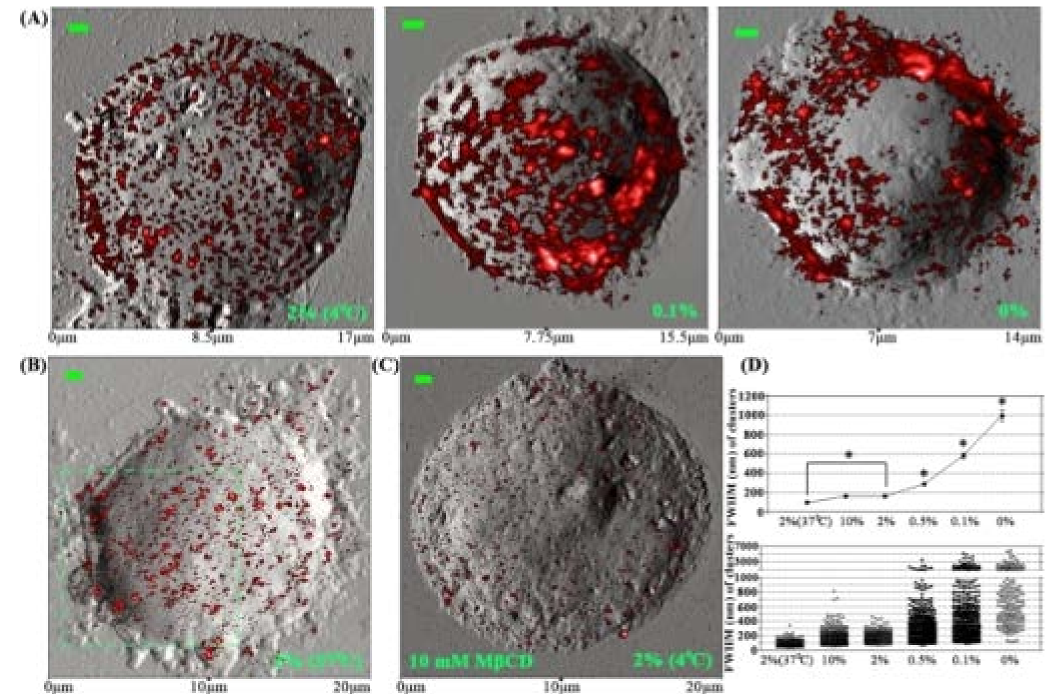
- There are things in biology smaller than 250 nm that you might be interested in looking at
- EM has VERY high resolution (0.5 nm), but your samples sure aren't moving
- It can also be difficult to label electron dense regions of your sample



Older techniques you won't use NSOM

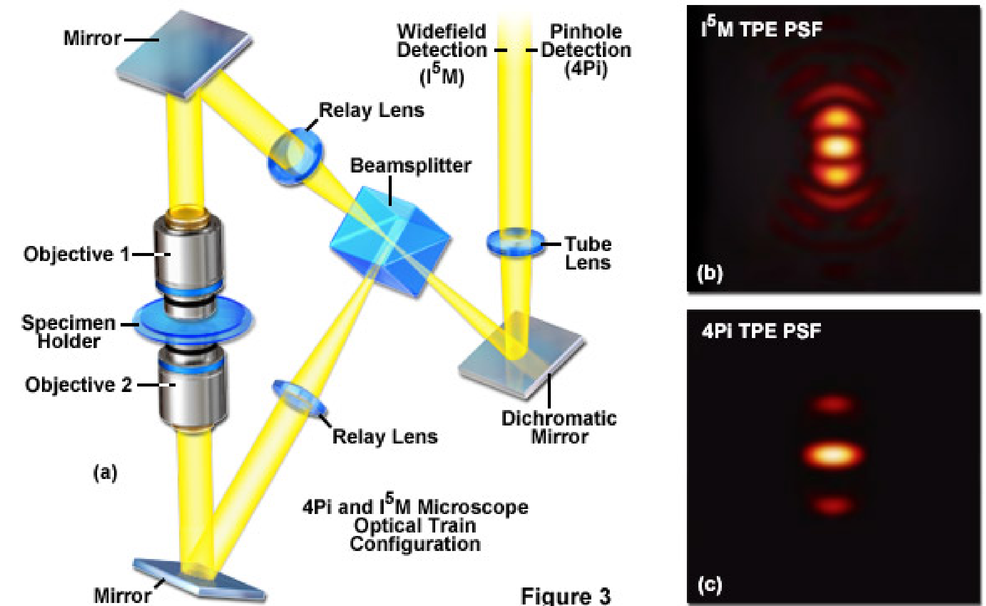
- NSOM – Near-field scanning optical microscopy
- Bring a probe VERY close to the surface
- Similar effects to TIRF, if you get the probe close enough (< 100 nm), the diffraction doesn't have space to develop
- Can get lateral resolutions of 20-50 nm
- First instrument in 1972
- VERY challenging
- Only useful for investigating right at cell membrane

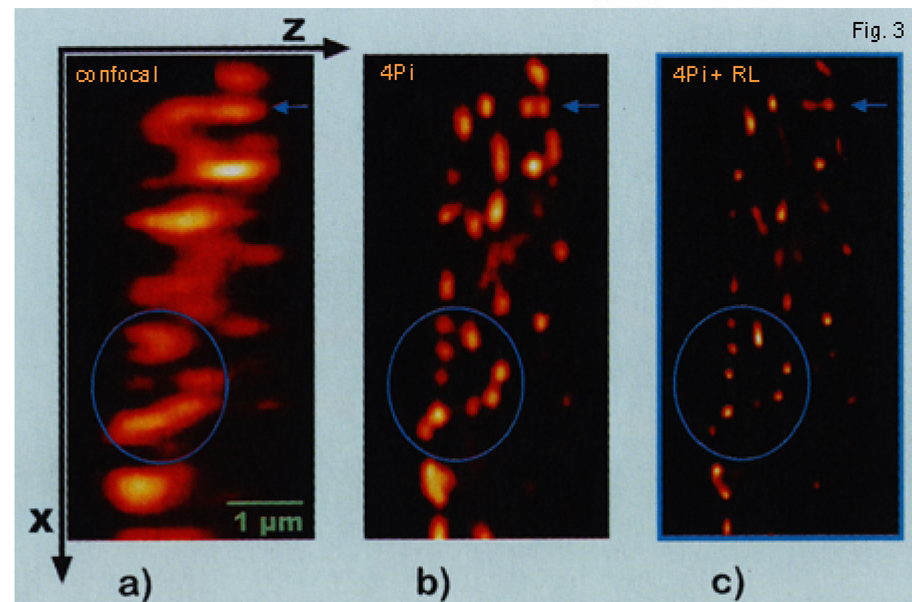


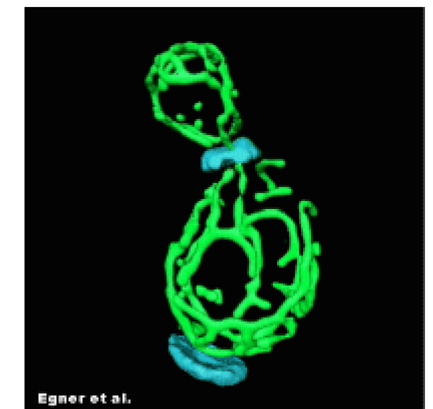


Older techniques you won't use
4Pi microscopy

- 4 Pi involves not one, but two high NA objectives
- Allows collection of 2x the number of photons
- Lateral resolution is equivalent, but axial resolution (z) can be reduced to 100 nm
- Challenging setup, no improvement in resolution
- First experiment in 1994 by Stefan Hell
- Slow, but some groups still use 4pi

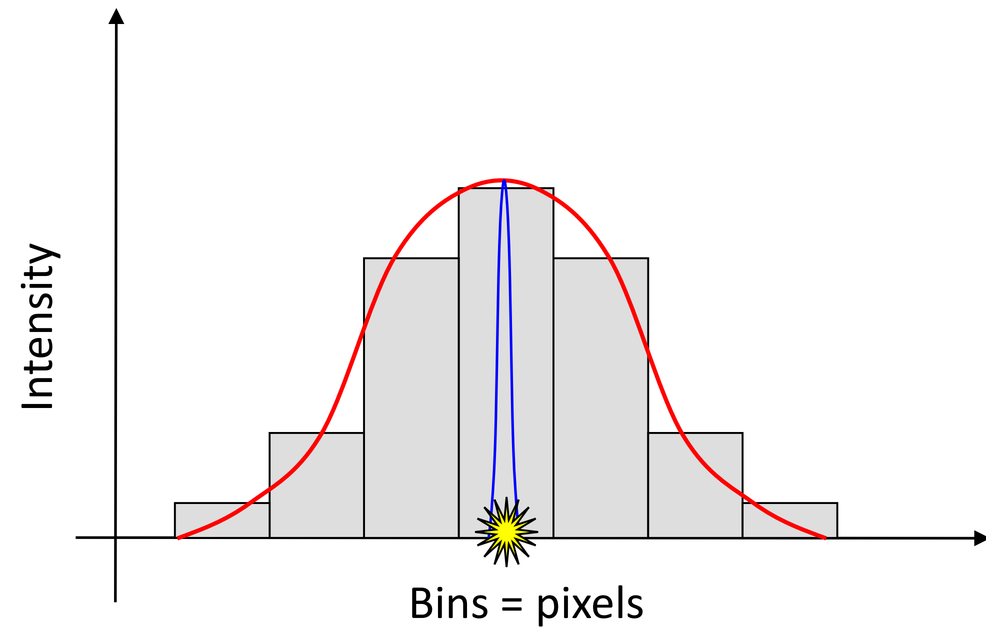






STORM/PALM microscopy

- STORM = Stochastic Optical Reconstruction Microscopy
- PALM = PhotoActivated Localization microscopy
- The problem is that when you have many fluorophores in a sample, they bleed together due to diffraction, and you can't tell where one starts and the next ends
- Solution proposed in 2003 by Eric Betzig – relies on imaging individual fluorophores



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

Single-Molecule Localization Microscopy for Superresolution

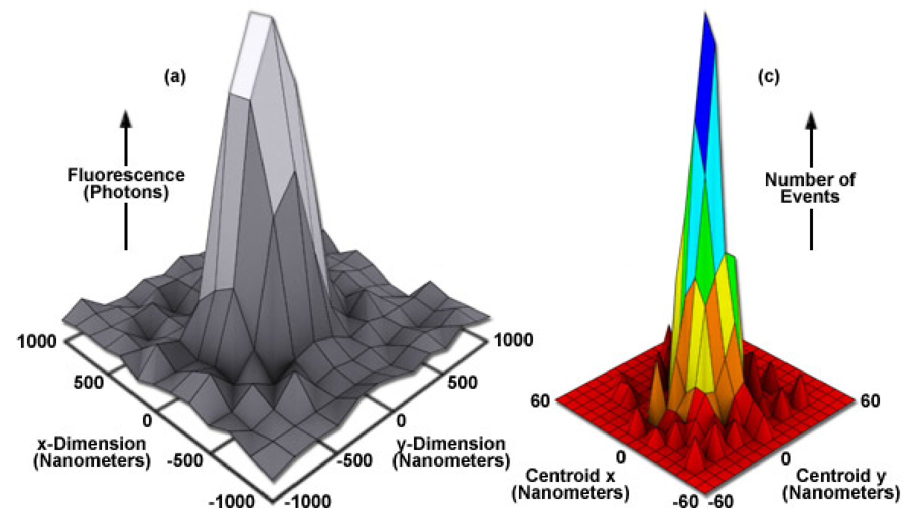
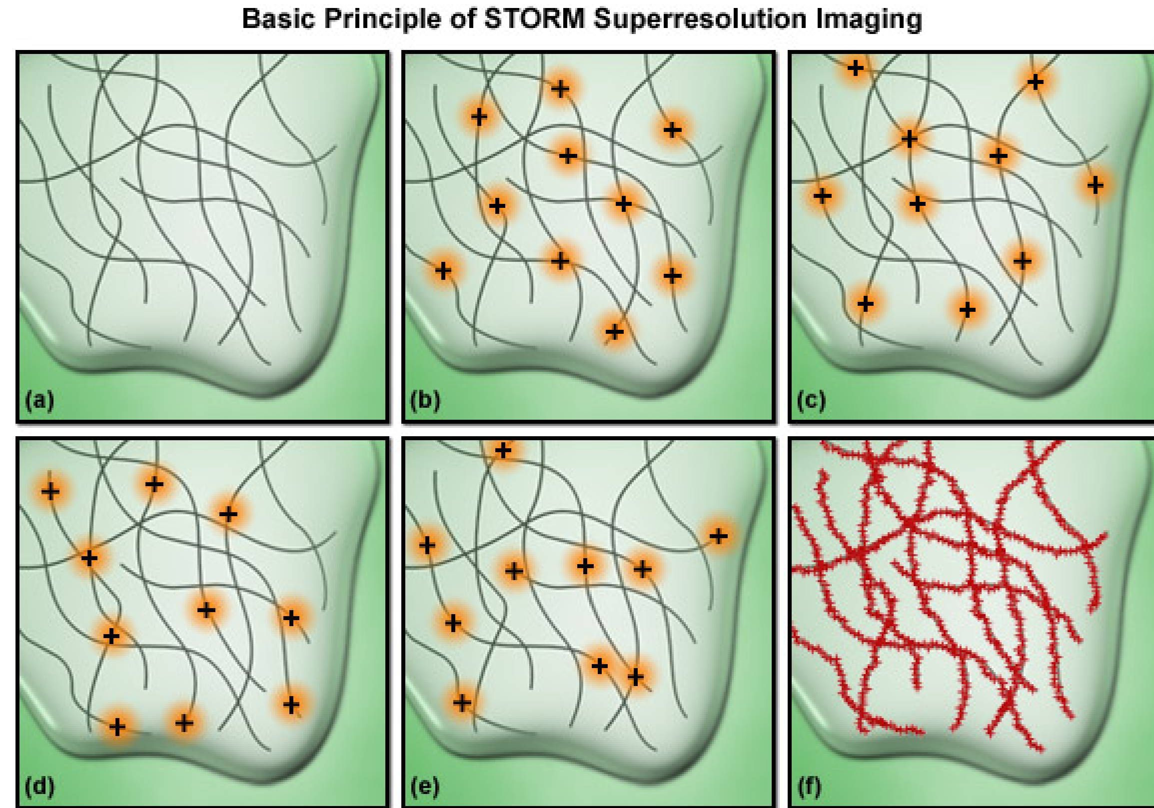


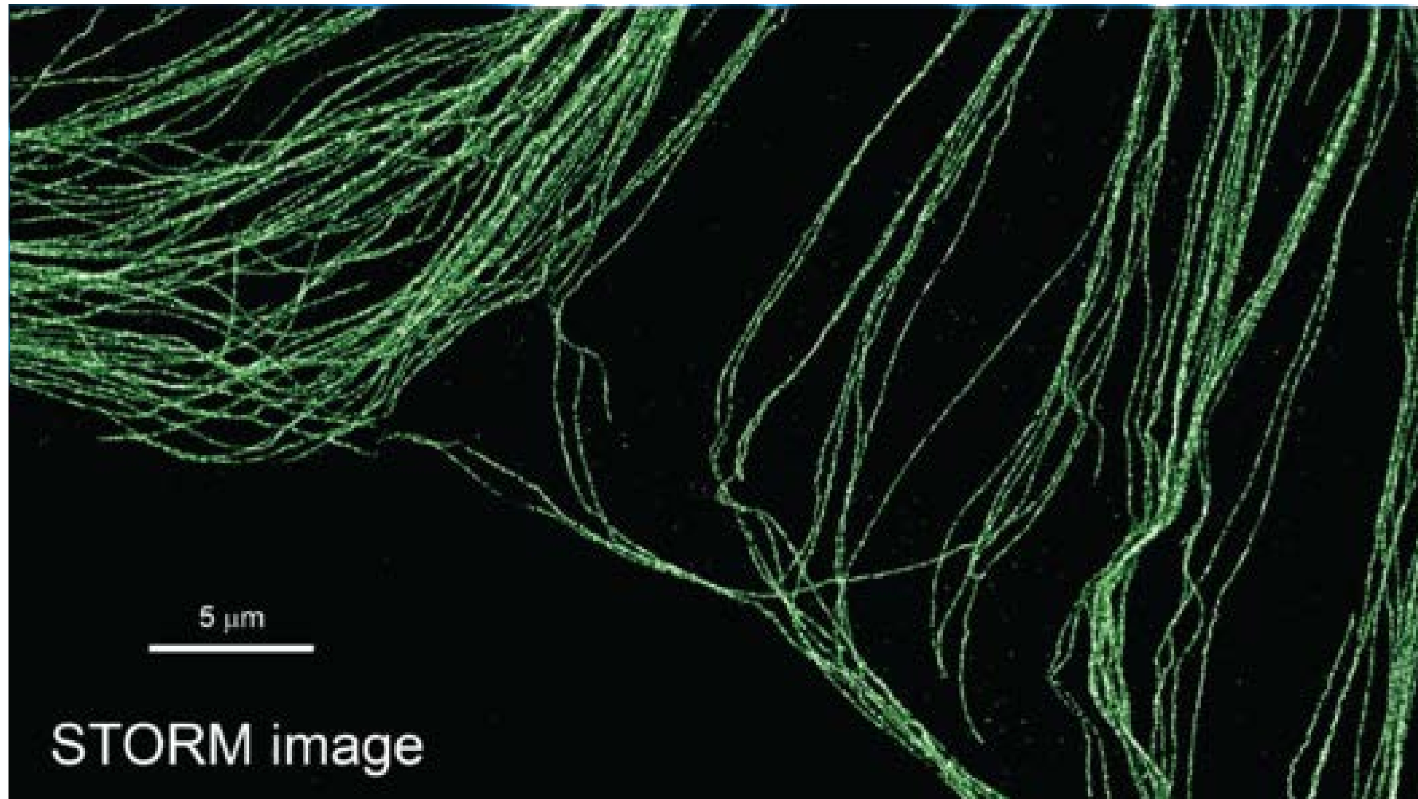
Figure 3

STORM/PALM involves optically turning on small subset of fluorophores in an image

- Imagine a sample densely labeled with dark fluorophores
- Algorithm:
  - Turn on some random subset of fluorophores such that they are individually resolvable
  - Image those fluorophores until they all photobleach
  - Fit those peaks, and put a small dot
  - Repeat steps 1-3
  - Display all fit dots to reconstruct your image
- ONLY works if you turn on small number in FOV

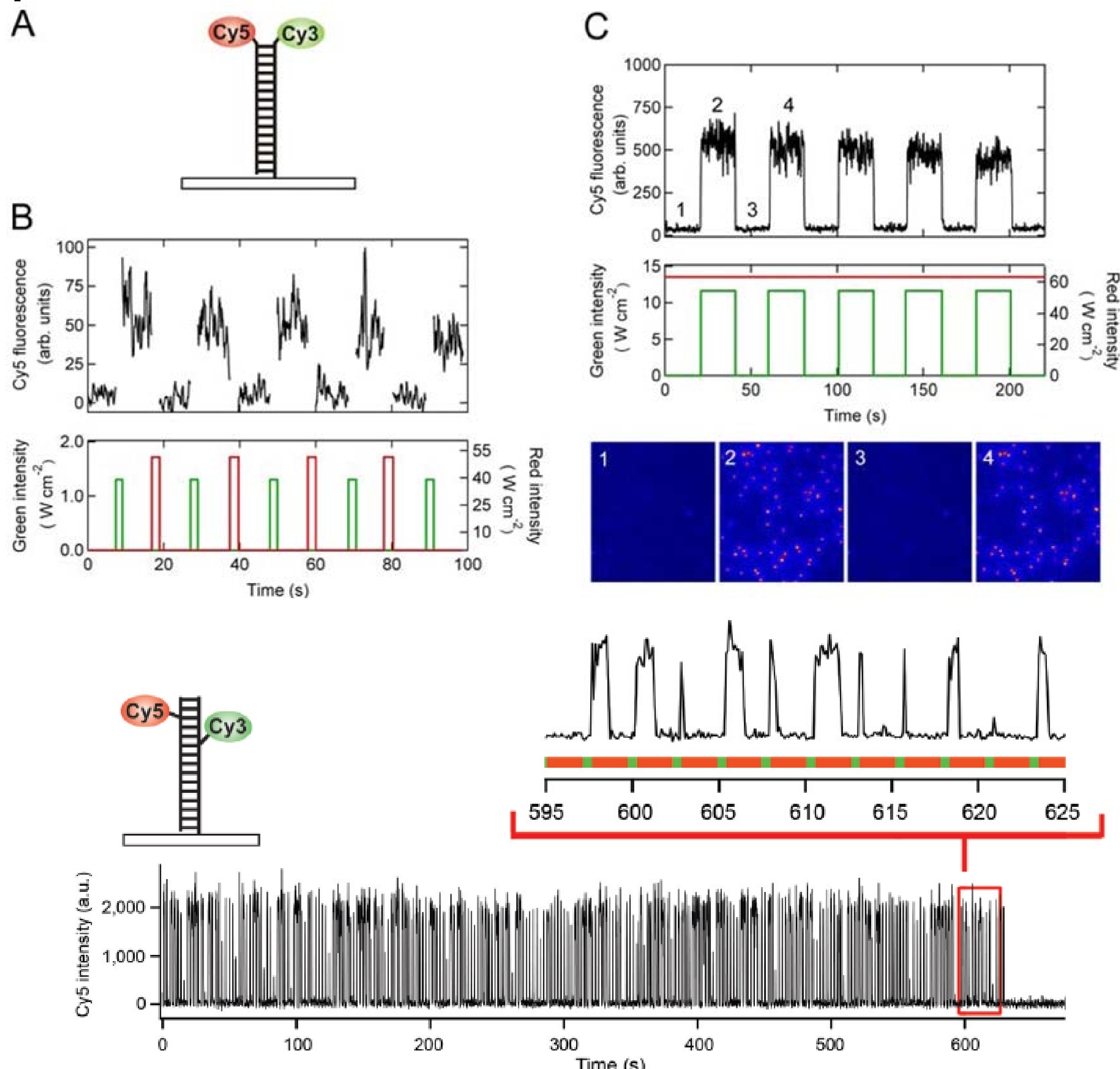


Ooh, aah…



Cy3-Cy5 tandem act as photoswtich

- Cy3+Cy5+triplet quencher and no oxygen
- Red light causes Cy5 fluorescence and photobleaching
- Dark state has lifetime of hours
- Cy3 excitation causes
- Photoswitch can occur ~100x
- Eventually permanently photobleaches



Magic Part 1 – Photoswitchable fluorophores: Dyes

- Some spectral property must be converted by light
- The current preferred method is photoactivation (light turns on fluorescence)
- Photoswitchable FPs - PALM
- Cyanine dyes + thiol molecule – STORM

Properties of Selected Synthetic Probes for STORM Imaging

Name (Acronym)	Ex(nm)	Em(nm)	EC(x 10-3)	QY	N Photons
Cy3B	559	570	130.0	0.67	1400
Cy3.5	581	596	150.0	0.67	5000
Cy5	649	664	250.0	0.28	4000
Cy5.5	675	694	190.0	0.23	6000
Cy7	747	767	200.0	0.28	1000
Alexa Fluor 488	495	519	71.0	0.92	1200
Alexa Fluor 568	578	603	91.3	0.69	2800
Alexa Fluor 647	650	665	240.0	0.33	6000
Alexa Fluor 750	749	775	240.0	0.12	450
ATTO 488	501	523	90.0	0.80	1300
ATTO 520	516	538	110.0	0.90	1200
ATTO 565	563	592	120.0	0.90	20000
ATTO 647	645	669	120.0	0.20	1500
ATTO 647N	644	669	150.0	0.65	3000
ATTO 680	680	700	125.0	0.3	1500
ATTO 740	740	764	120.0	0.1	1000

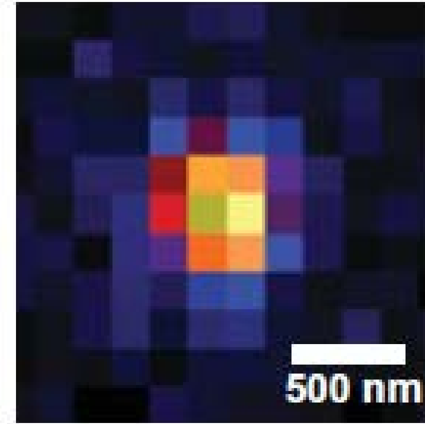
Localizing fluorophores

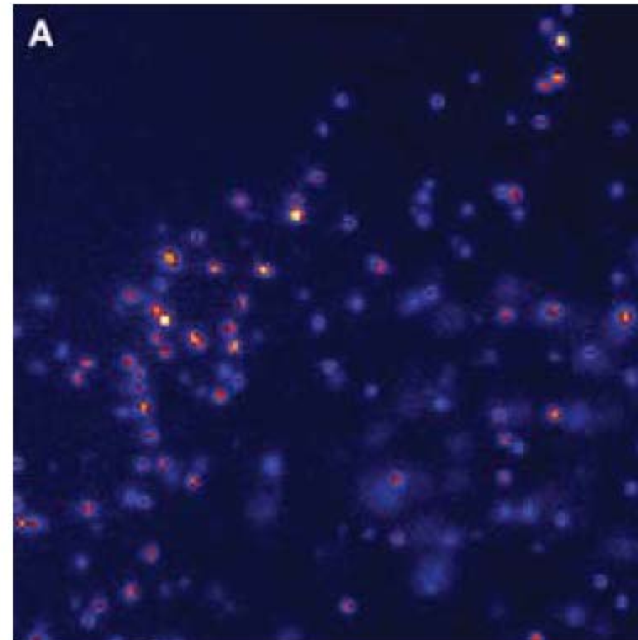
Based on star analysis in astronomy

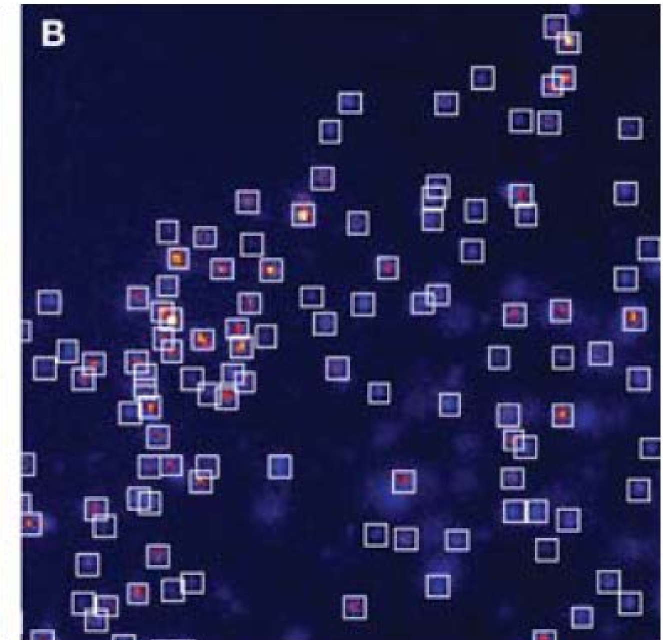
Identify individual fluorophore – convolve with a Gaussian filter with the same width as known PSF

Reduces high frequency noise and low frequency background

Threshold to identify individual fluorophores







STORM peak fitting

- Square region of 7x7 pixels centered on peak
- Fit to ellipsoidal Gaussian (max(a,b)/min(a,b))
- Determine if it's likely to be 1 fluorophore
- Fit to symmetric Gaussian

1st Elliptical Fit

$$I(x,y) = A_0 + I_0 \exp\left\{-\frac{1}{2}\left[\left(\frac{x'}{a}\right)^2 + \left(\frac{y'}{b}\right)^2\right]\right\}$$

$$x' = (x - x_0)\cos\theta - (y - y_0)\sin\theta$$

$$y' = (x - x_0)\sin\theta + (y - y_0)\cos\theta$$

A_0 = background fluorescence
I_0 = Peak amplitude
a,b = widths of Gaussian distributions
x_0,y_0 = Center coordinates
θ = rotation relative to camera

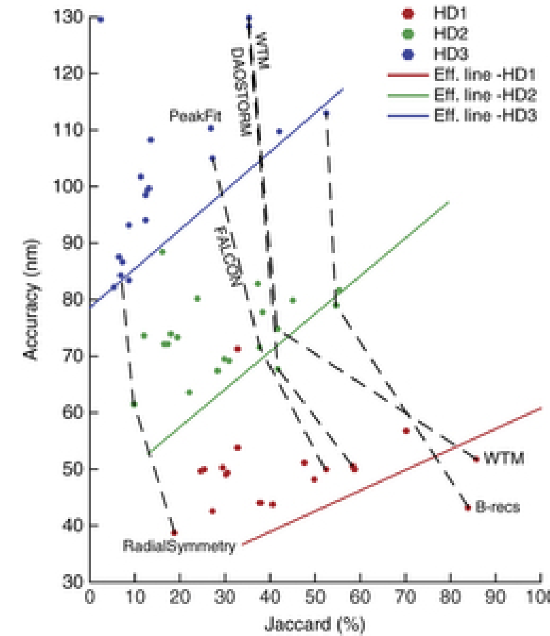
2nd Gaussian Fit

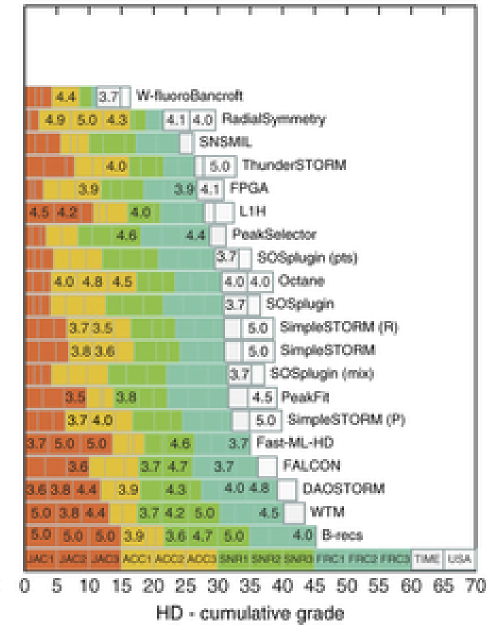
$$I(x,y) = A_0 + I_0 \exp\left\{-\frac{1}{2}\left[\left(\frac{x - x_0}{\sigma}\right)^2 + \left(\frac{y - y_0}{\sigma}\right)^2\right]\right\}$$

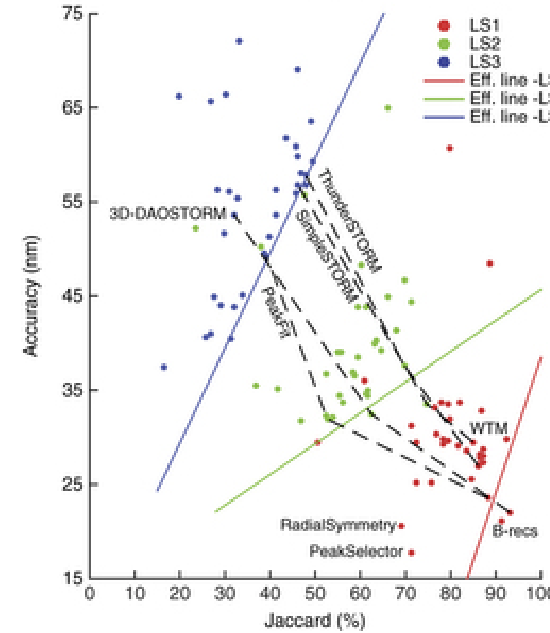
$$\#PhotoElectrons = 4\pi\sigma I_0$$

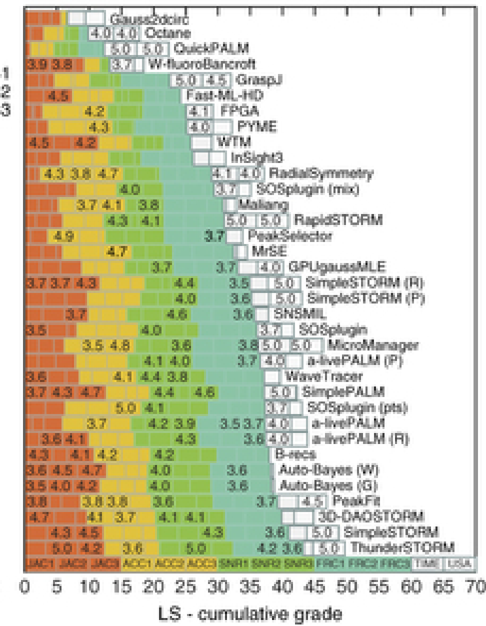
Analyzing images

- Great computational task
- Cottage industry around different fitting algorithms
- Improved software borrows from astronomy, allows for much higher density of turned on fluorophores
- So much so that a group recently tested many of the packages for accuracy and fidelity
- Simple Gaussian fit works well until active fluorophores get really dense









On to Matlab…