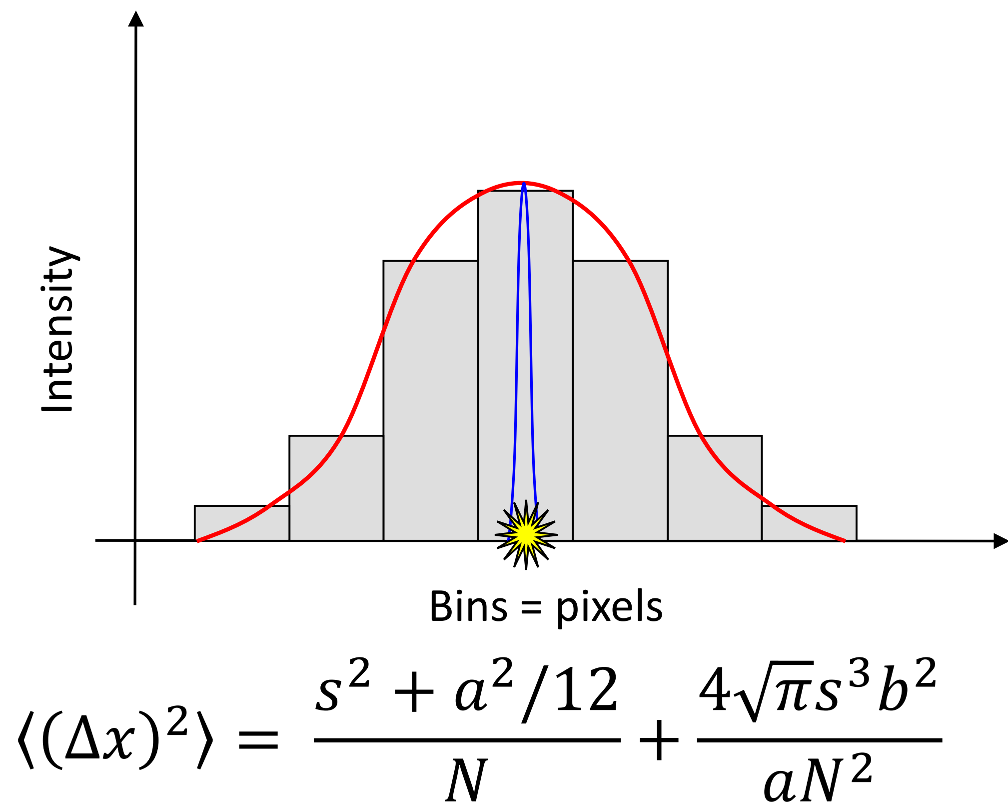
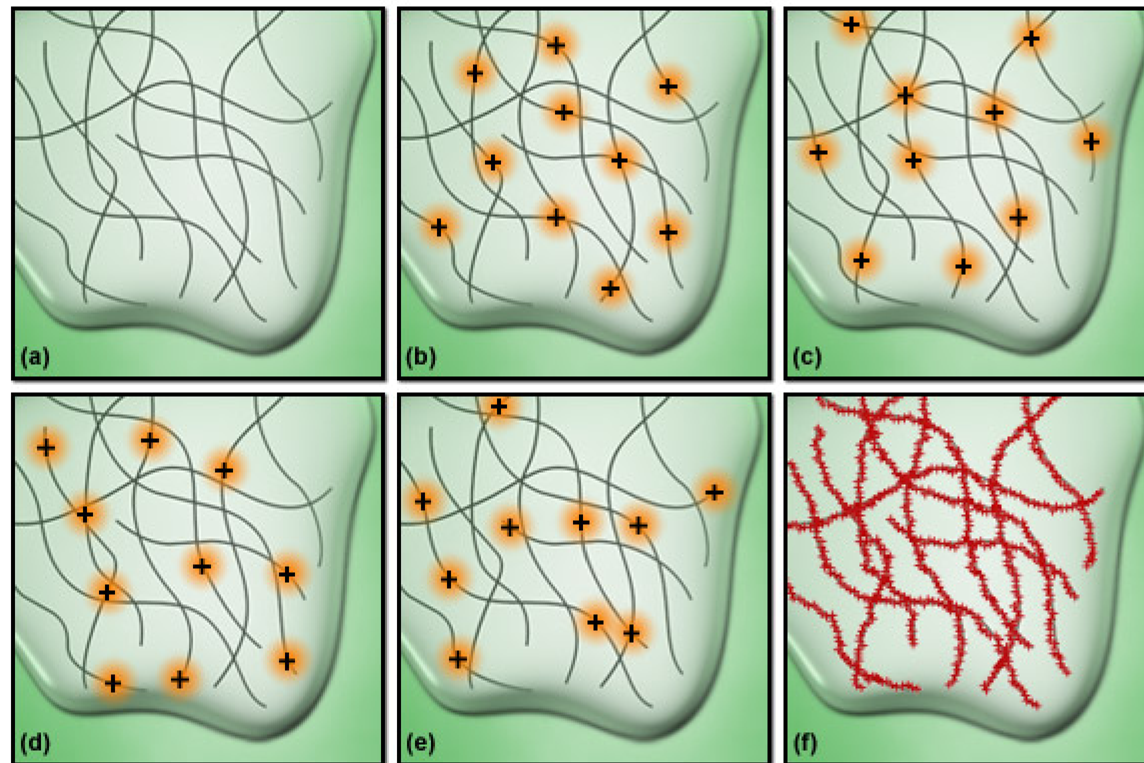
PALM/STORM, BALM, STED

- Last class
  - 2-photon
  - Intro to PALM/STORM
  - Cyanine dyes/DRONPA
- This class
  - Finish localization super-res
  - BALM
  - STED

Localization microscopy



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



Practicalities in STORM

- To resolve small features, labeling has to be VERY dense
- The reconstructed image will represent a pixelated view of the actual sample
- To resolve features, must label AT LEAST at the Nyquist frequency
- With a resolution of 30 nm, must have probes every 15 nm
- Fluorophore size becomes an issue – antibodies are big
- If you're using antibodies, have to ensure high efficiency and high coverage of labeling

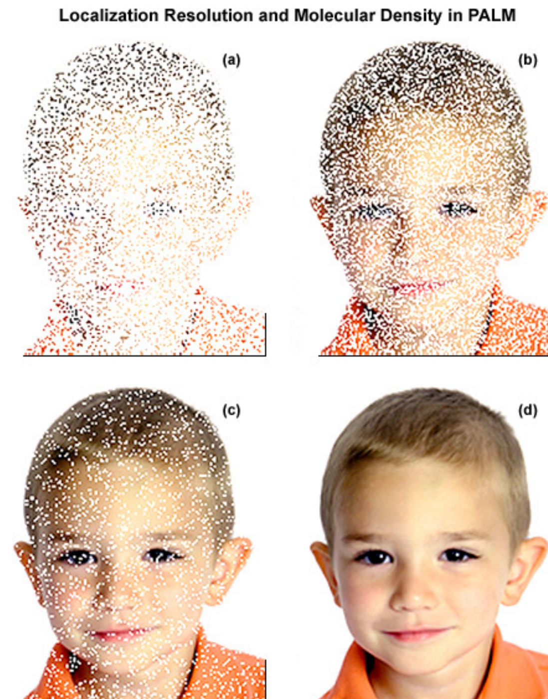


Figure 5

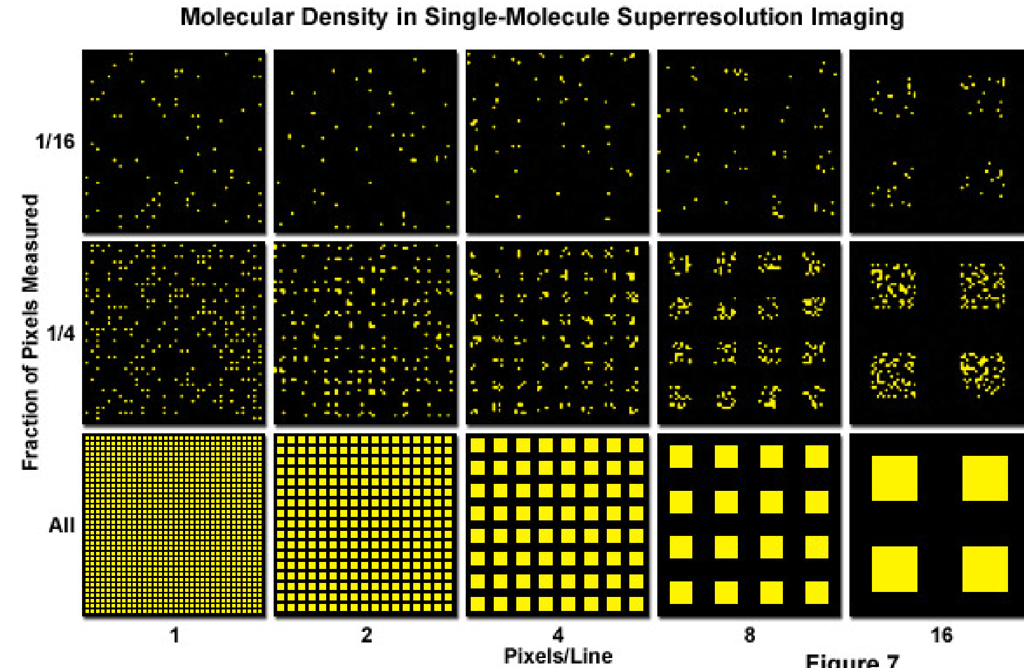


Figure 7

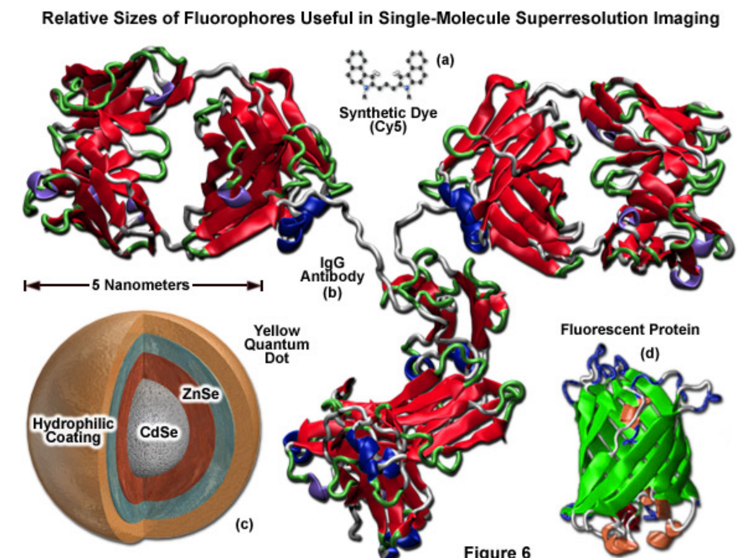


Figure 6

Contrast ratio and spontaneous activation

- In active experiment, #Dark Cy5 >> #Active Cy5
- Noise can creep through spontaneous activation, and dark state fluorescence
- Contrast ratio measures fluorescence in bright state/dark state
- The red laser can drive a little bit of Cy5 photoactivation
- Between 1 and 5 in 10,000 fluorophores turn on spontaneously
- If sample is very densely packed, can override ability

$$Contrast = \frac{F_{br\ state}}{F_{dk\ state}} \sim 1000$$

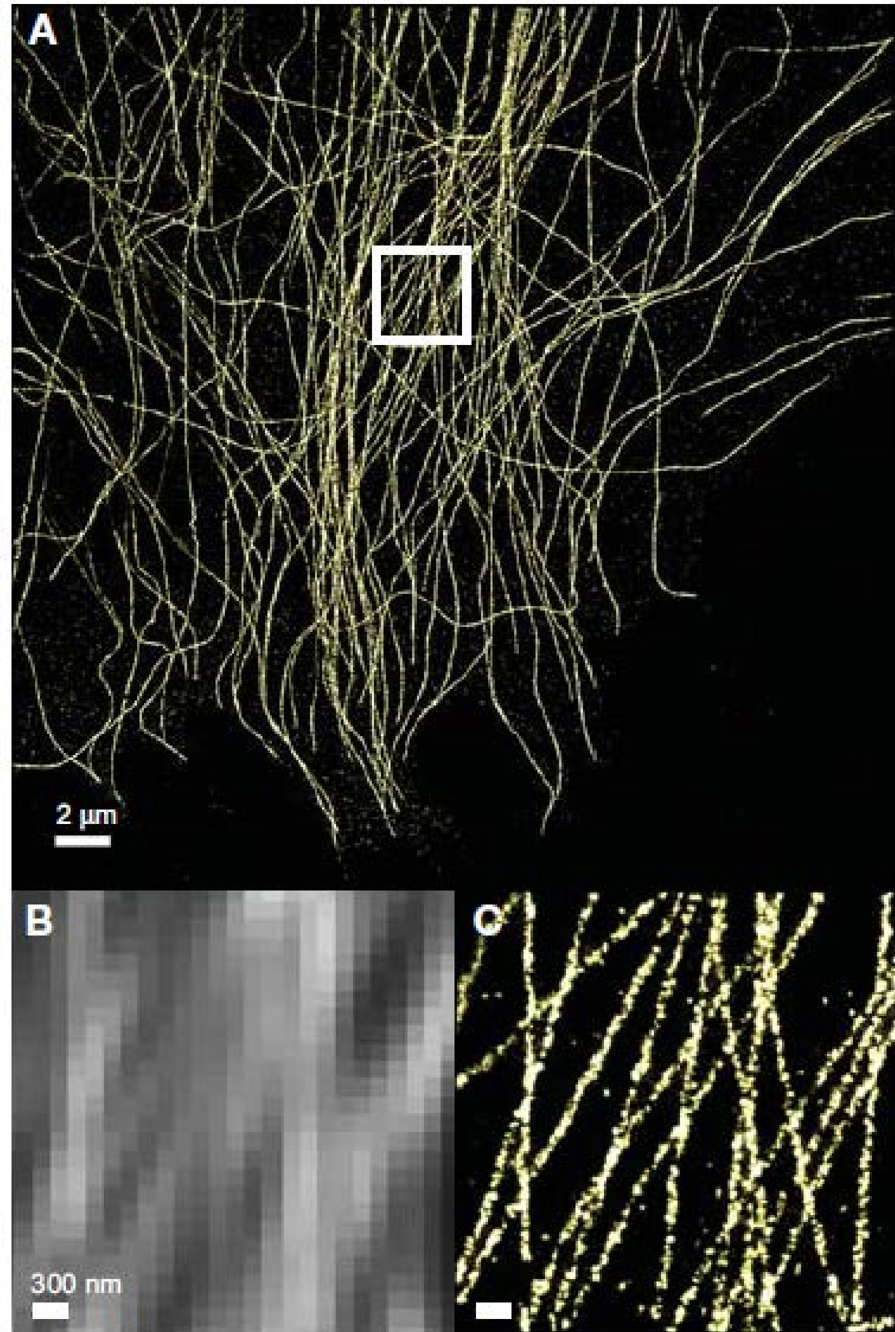
1000 dark fluorophores = 1 bright
Can erode ability to detect and fit peaks

$$\%Active = \frac{k_{blink}}{k_{blink} + k_{off}} \sim .0005$$

k_{off} = rate of switching off with red light
k_{blink} = rate of reactivation by red light

STORM image display

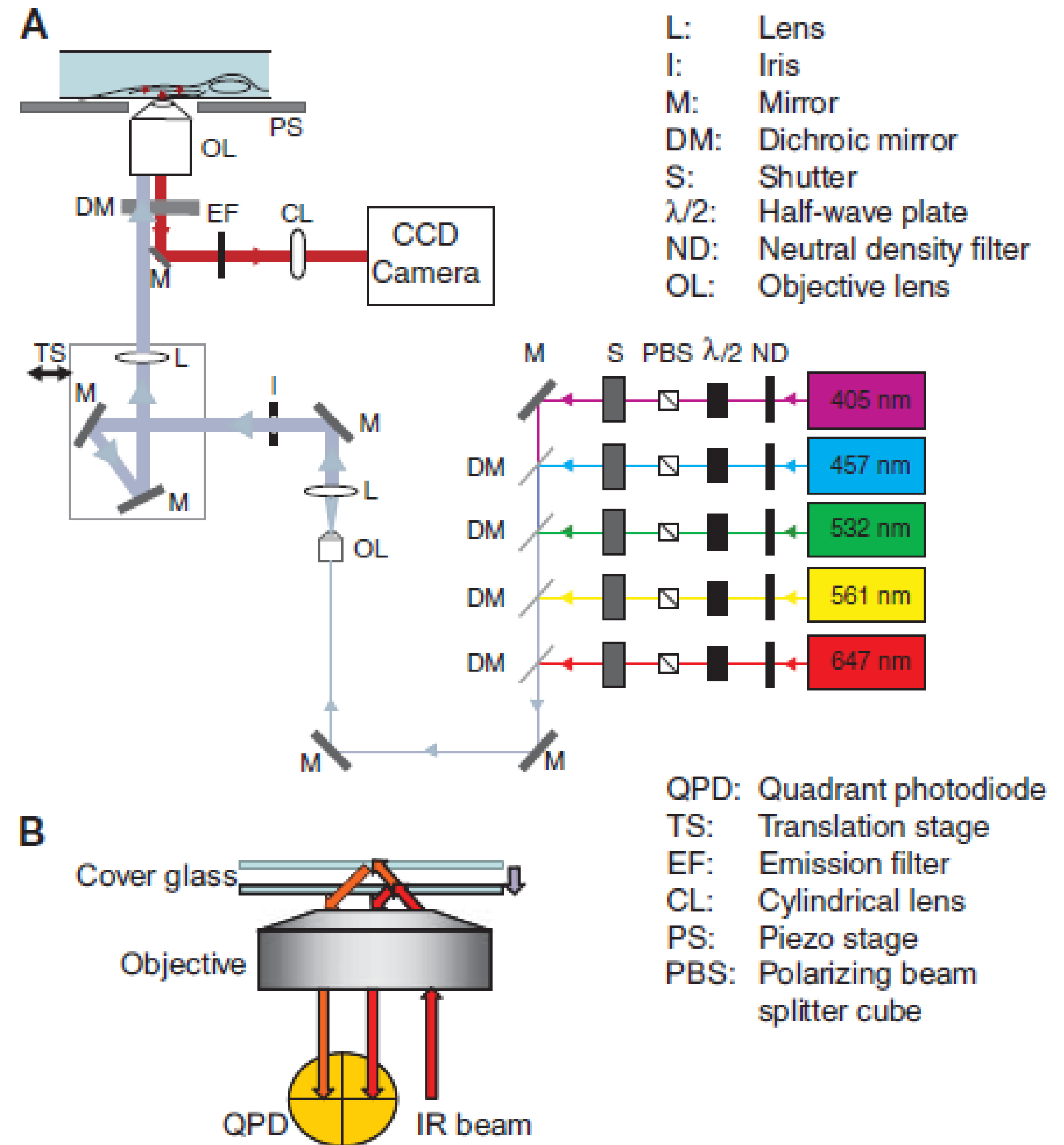
- Concatenate all fitted points from collected images and display them on single picture
- Often use a Gaussian of calculated uncertainty width
- A 40μm x 40μm image displayed at 1 nm resolution would take ~4.5Gb of memory



Microtubules with Cy3-Alexa647 2.4×10^6 localizations

STORM Equipment

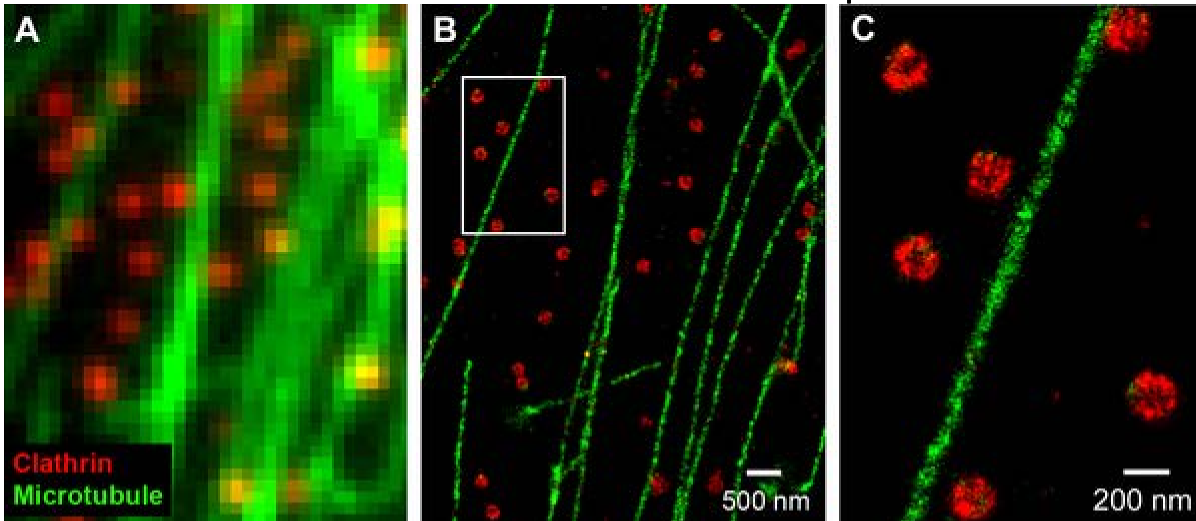
- High NA objective (probably TIRF)
- Multicolor laser excitation (at least 2)
- High sensitivity camera
- Drift correction (if your sample moves by 20 nm, that’s now a lot in your image)
- Images take a long time to collect
- Autofocus (Quadrant photodiode with IR laser)

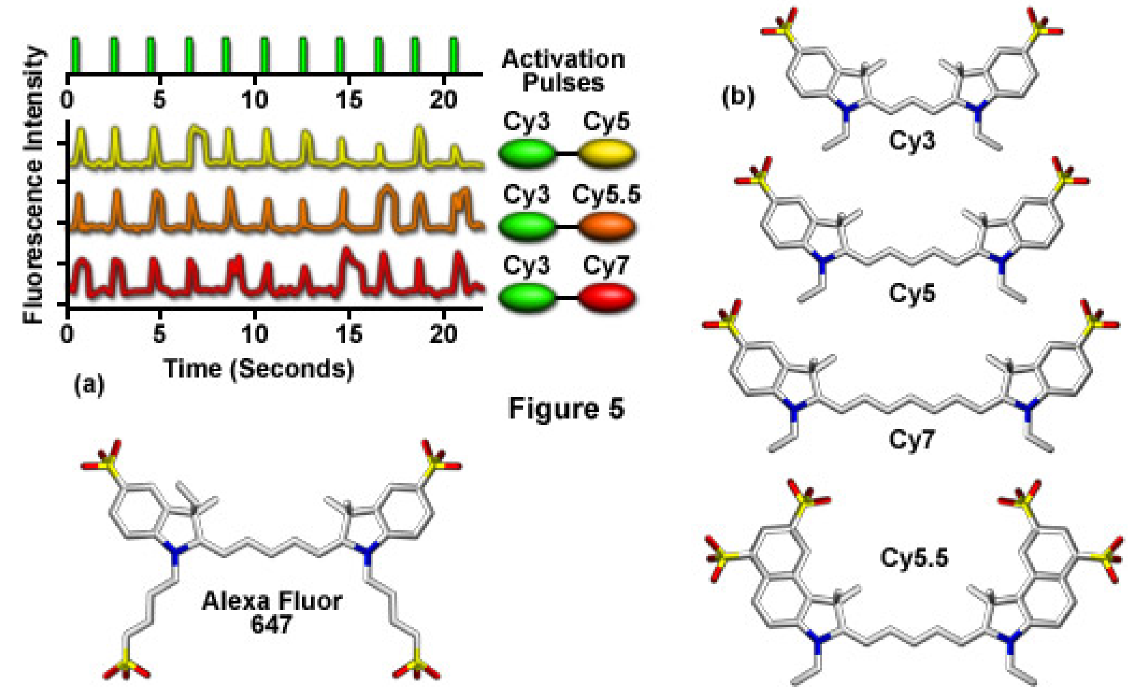


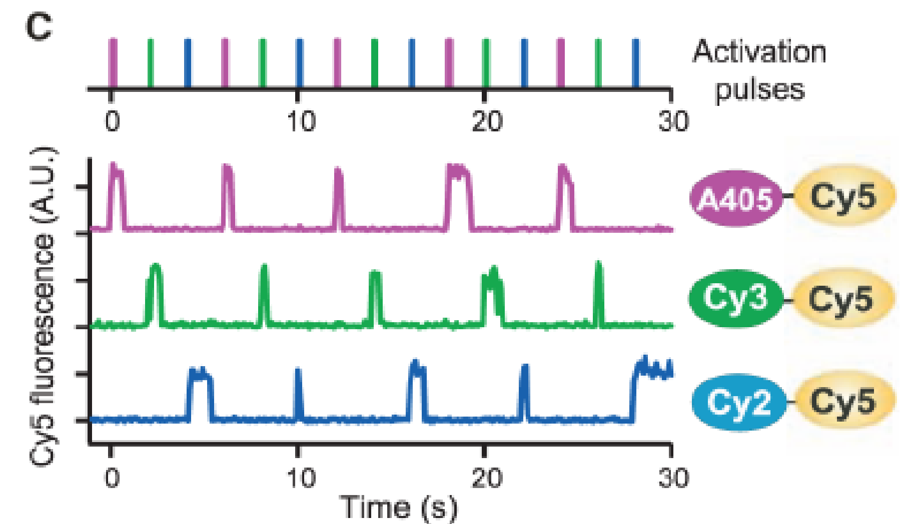
Multicolor STORM

- Two potential options
  - Single donor, multicolor acceptors
  - Many donors, single acceptor

Microtubules and clathrin coated pits

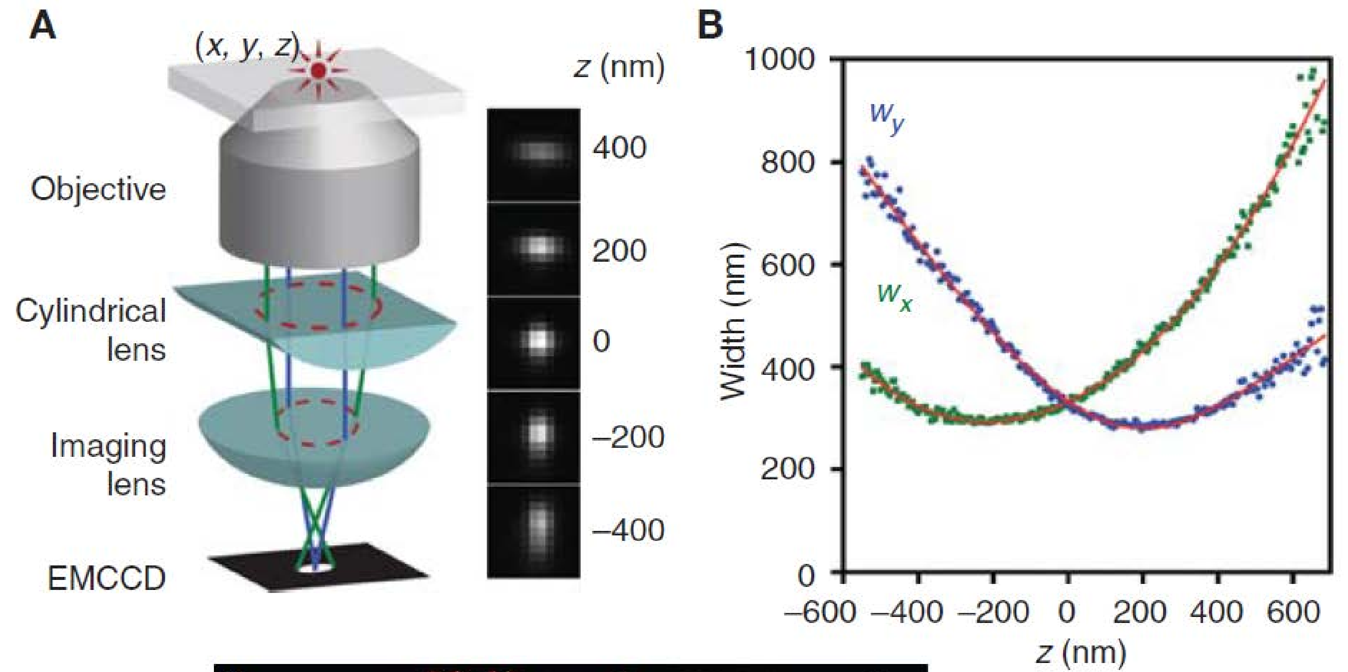


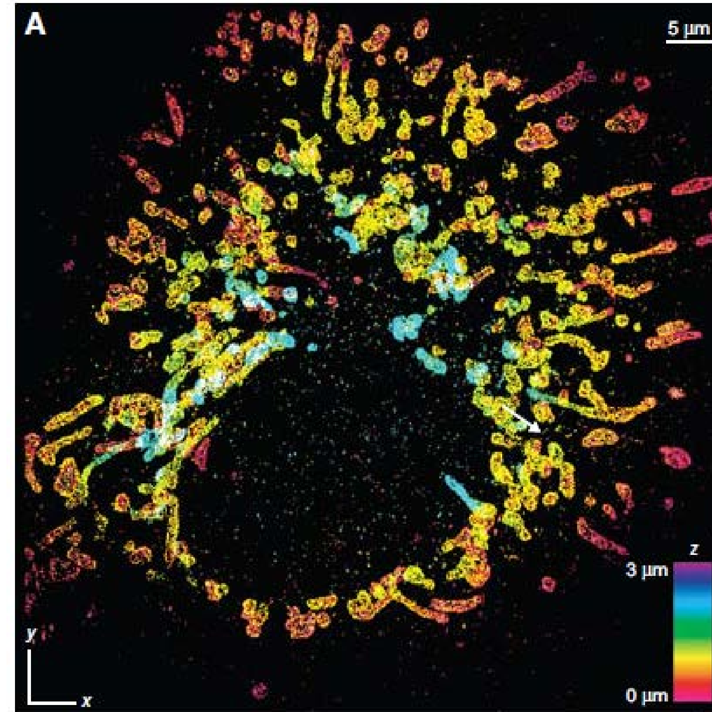




3D STORM

- Use an cylindrical lens to purposely introduce astigmatism
- PSF changes as a function of depth
- Keep measurements with high ellipticity, and then assign a z-depth according to a/b ratio

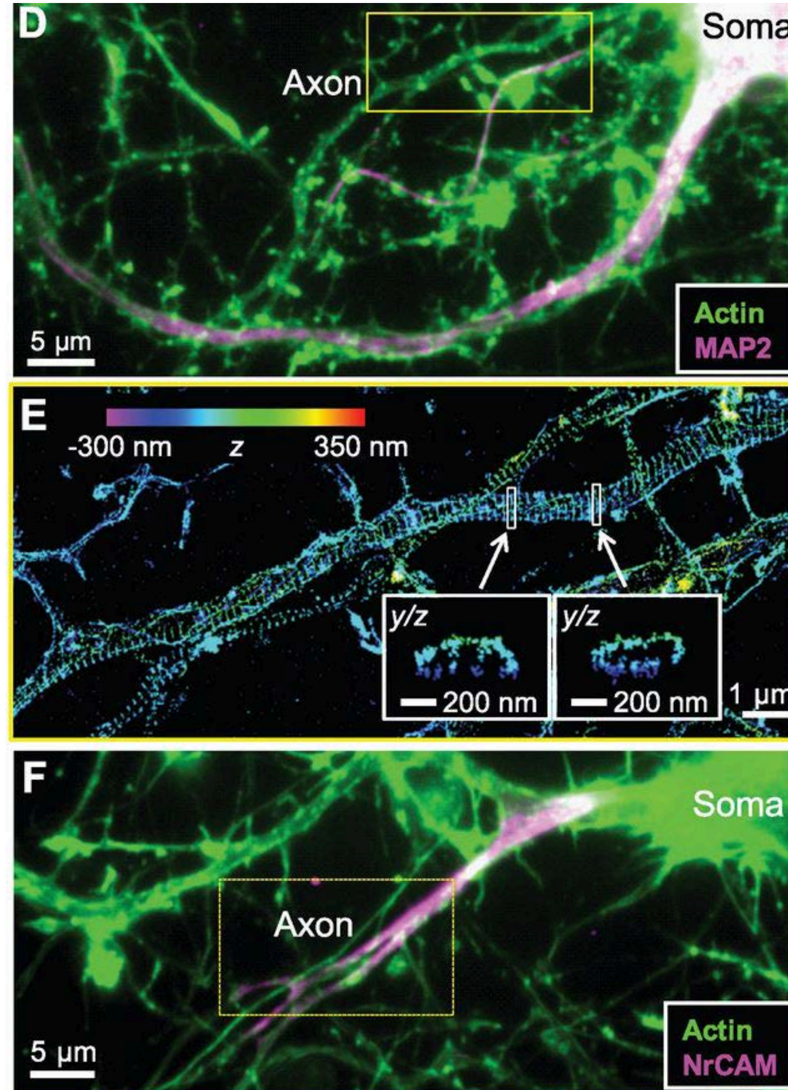


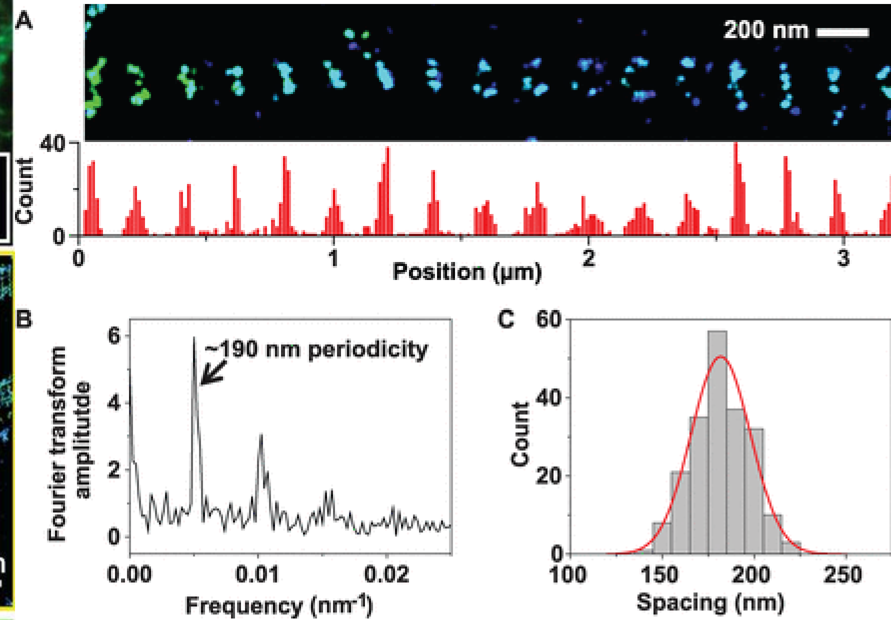


Mitochondrial network with color encoding depth

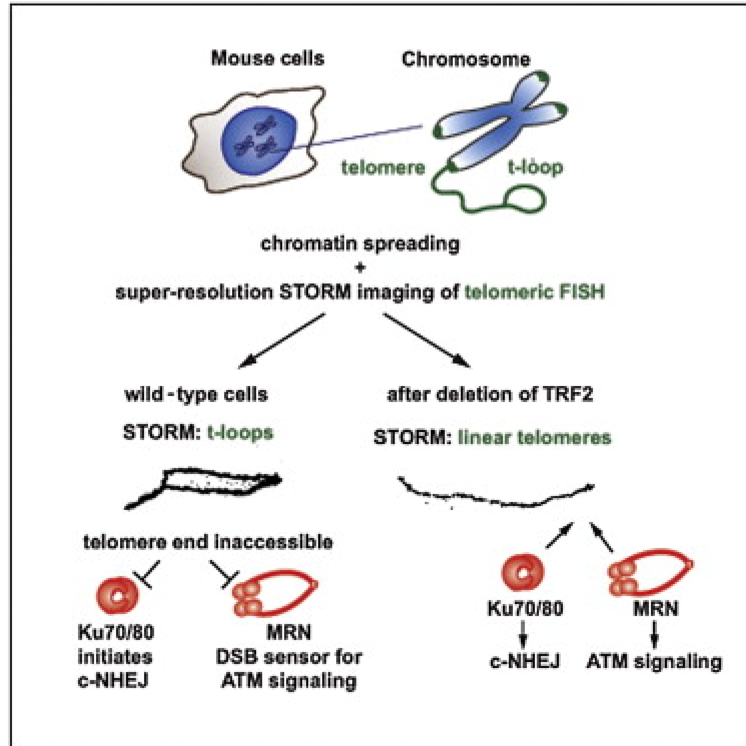
STORM applications

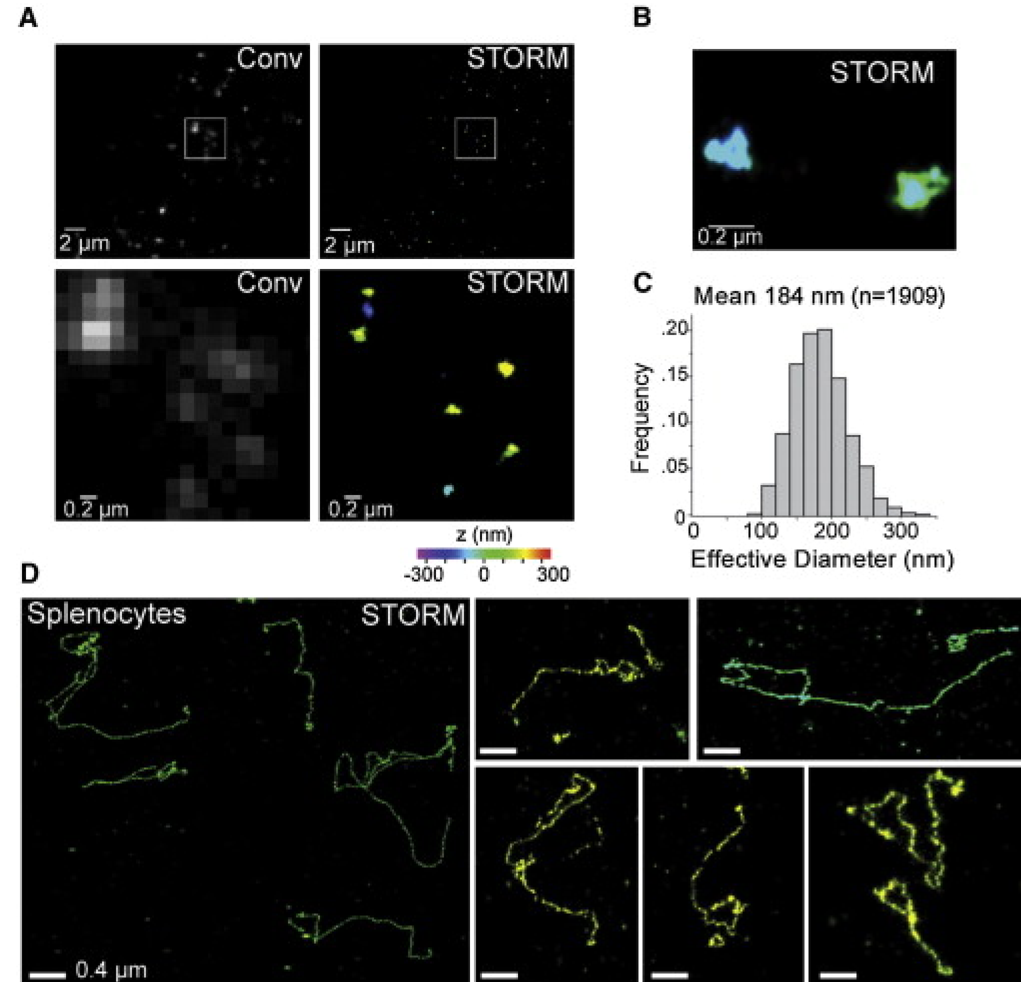
- Cytoskeletal arrangement too small to be seen by widefield
- Axon specific rings, dendrites don't show this pattern
- One of my favorite examples of new biology using super-resolution





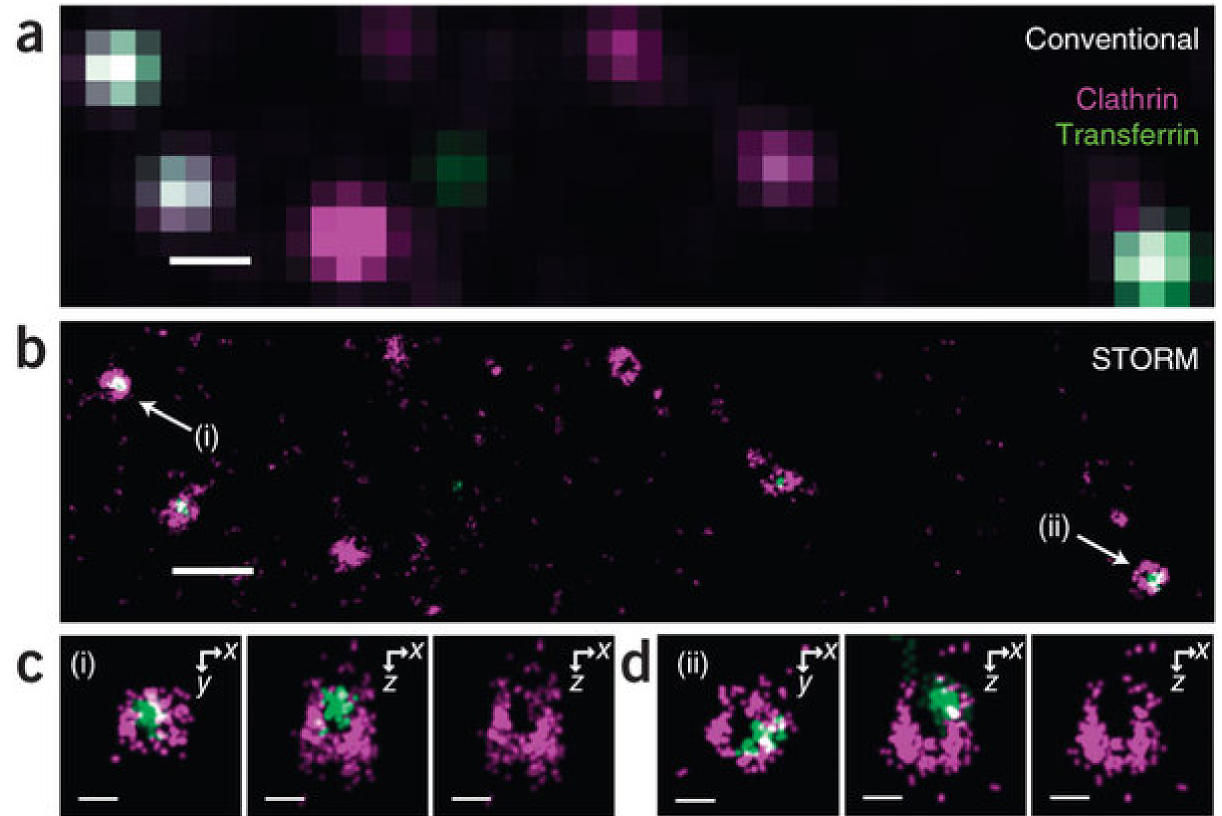
STORM applications – telomere FISH





Live cell STORM

- Possible, but slow and hard
- Have to keep a thiol reagent (toxic) and blast cells with light
- Labels can obviously move, now, makes it harder to localize
- Time resolution on the order of seconds

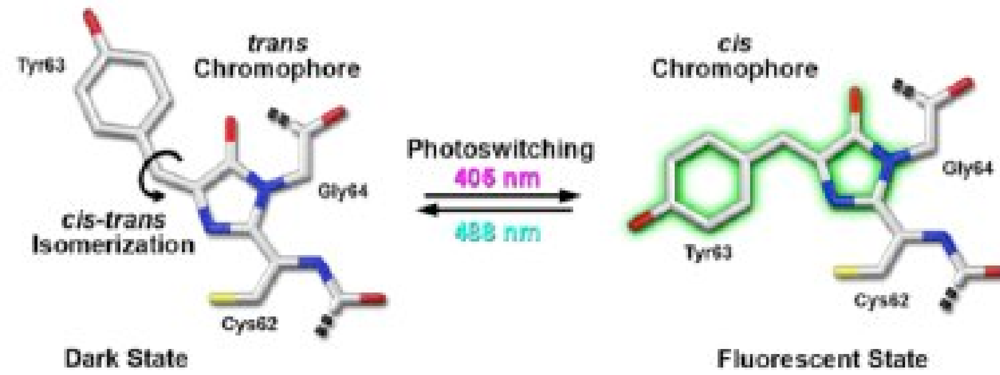


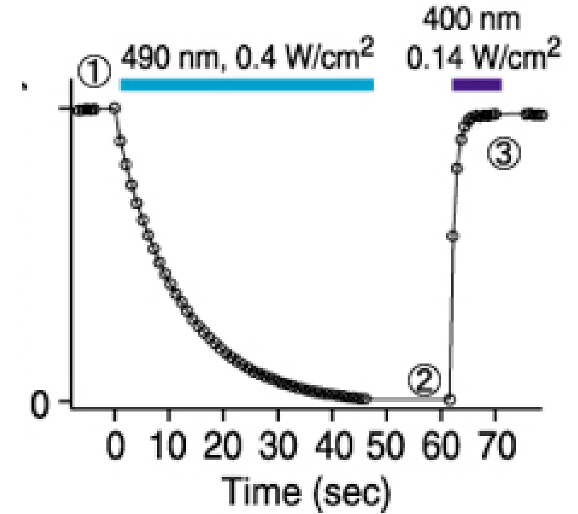
Two color, 3D, live cell STORM
Clathrin and transferrin

PALM

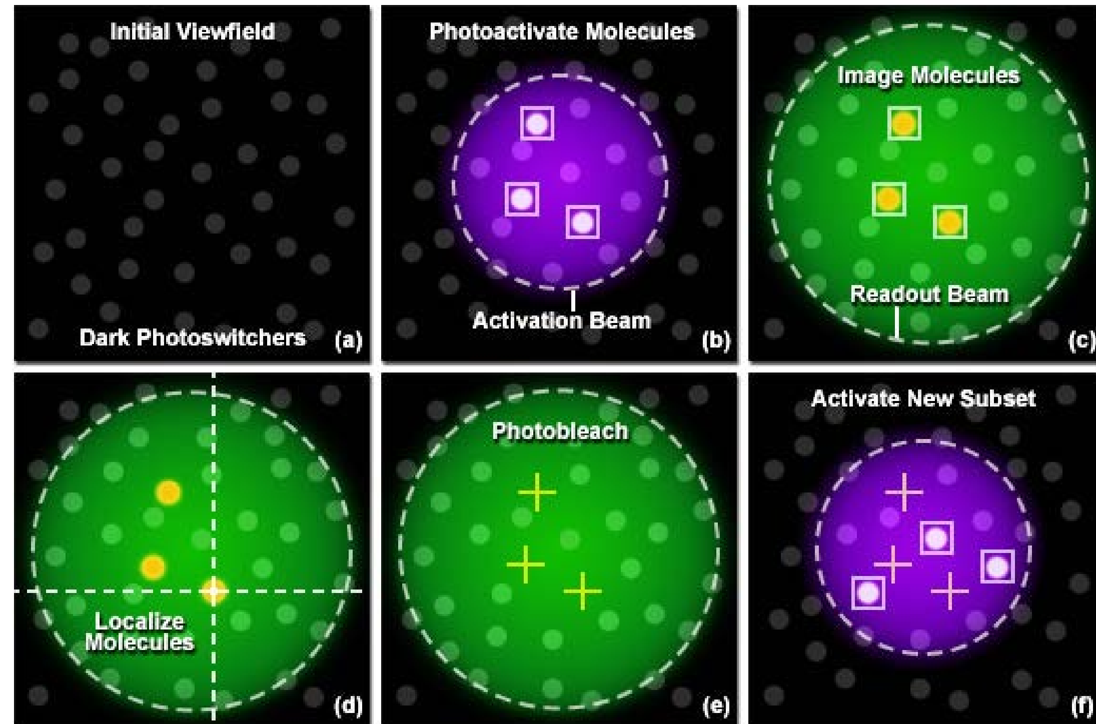
- Photoswitchable proteins instead of Cy3-Cy5 pairs
- Dronpa (photoswitchable) and mEos (photoconvertible) are two most popular
- FPs are bigger and less bright
- Gain all the advantages of genetic labeling

DRONPA – photoswitchable protein



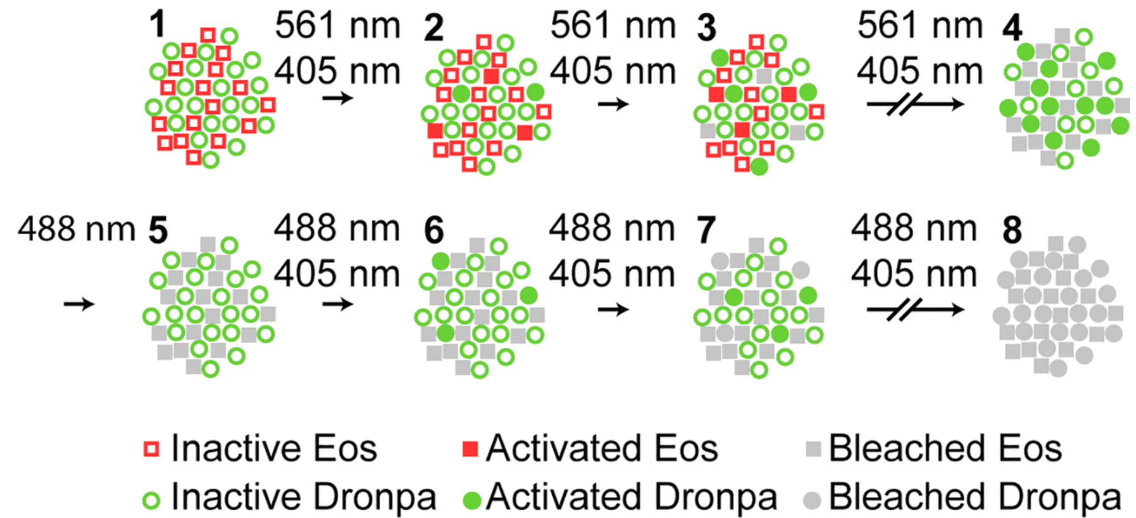


Principle of Single-Molecule Localization Microscopy



Two color PALM

- Can use Dronpa and tdEos
- Dronpa significantly overlaps tdEOS pre-conversion
- First photoactivate (405 nm) and localize tdEos (561 nm)
- Photobleach all tdEos
- Then use 488 nm to localize and bleach Dronpa
- There also exists a photoswitchable mCherry



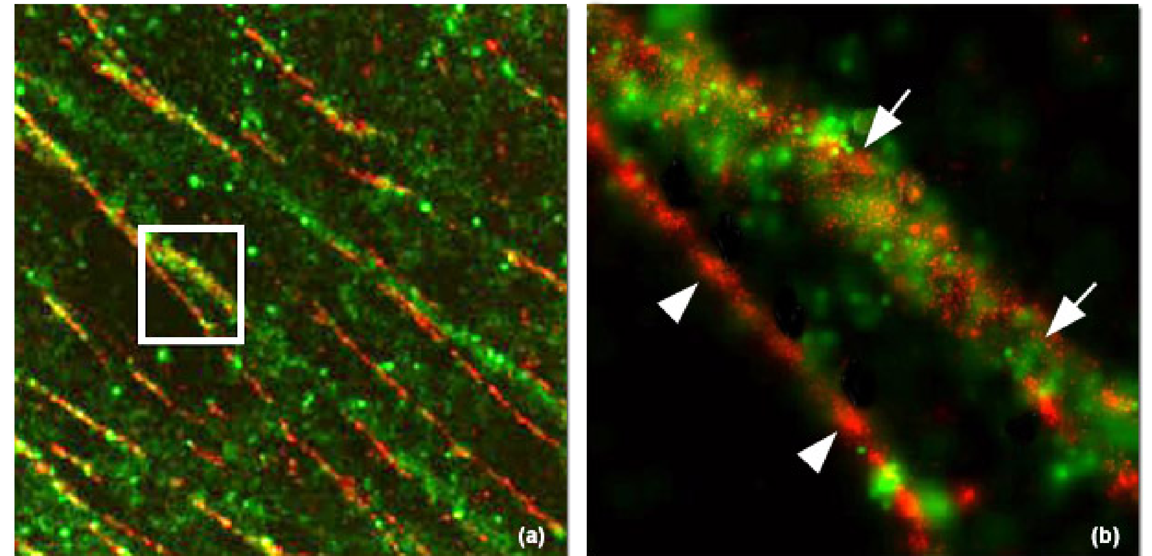
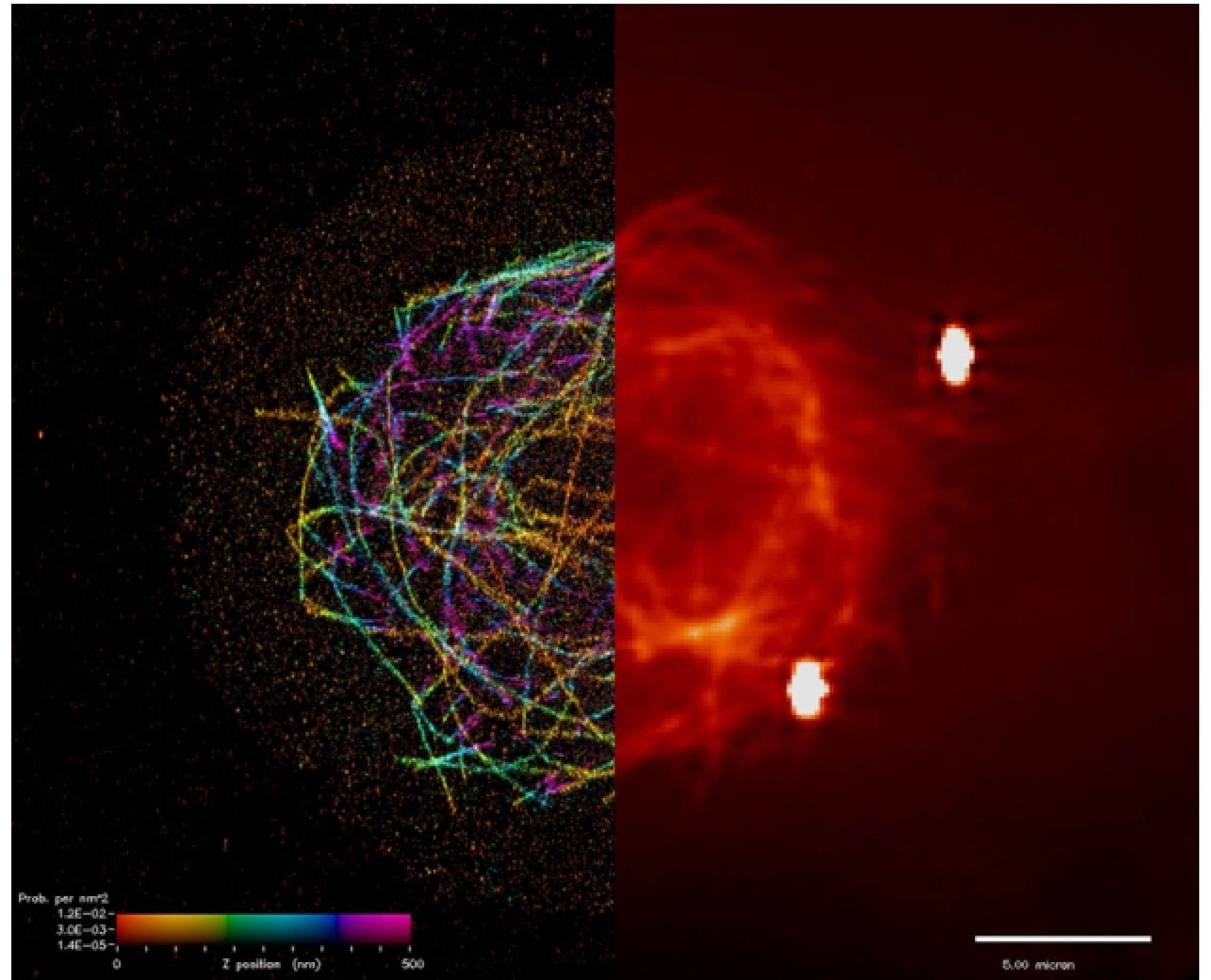


Figure 8

PALM vs STORM

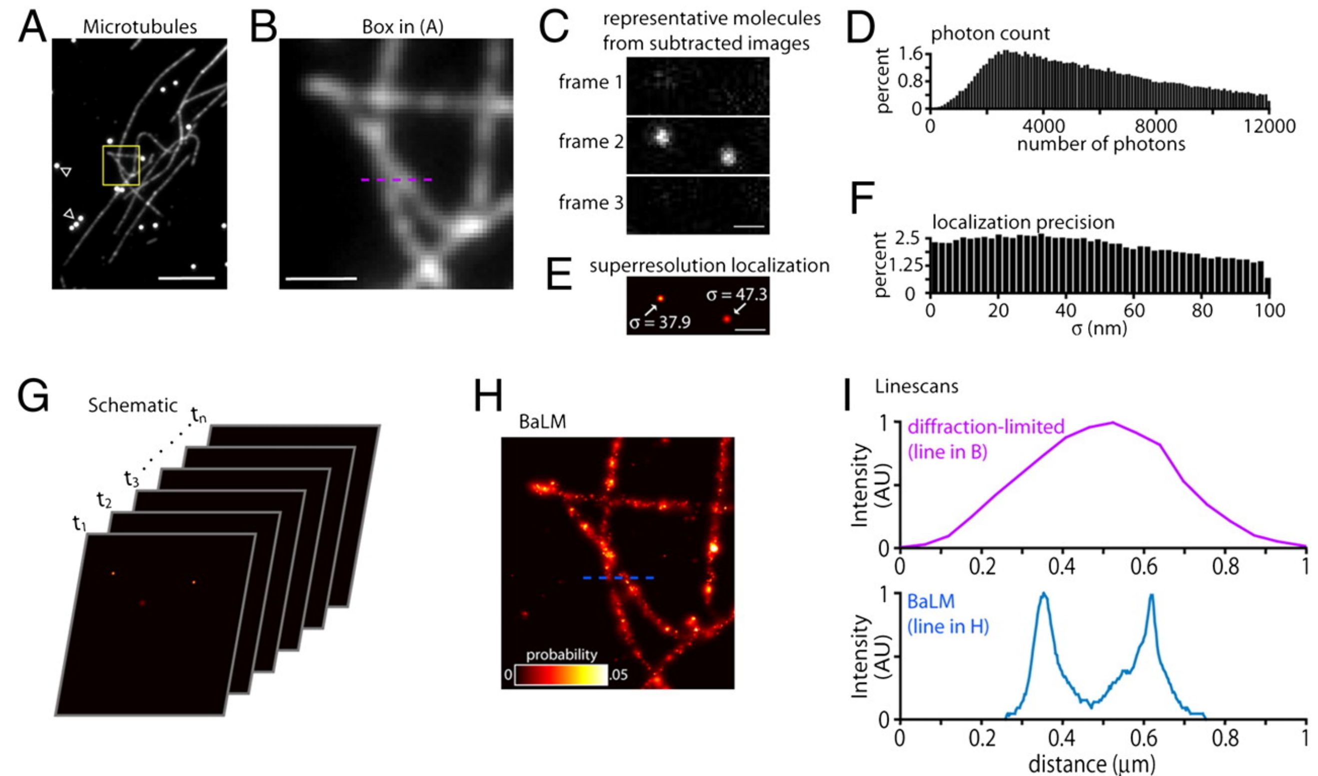
- Same exact fitting protocol
- PALM uses FPs – easier to genetically attach, worse as fluorophores
- STORM uses photoswitching dyes, requires toxic thiol buffers
- Easier to do 3 colors in STORM
- Really, not much difference



Couldn’t find an image to represent differences, went with this cool picture

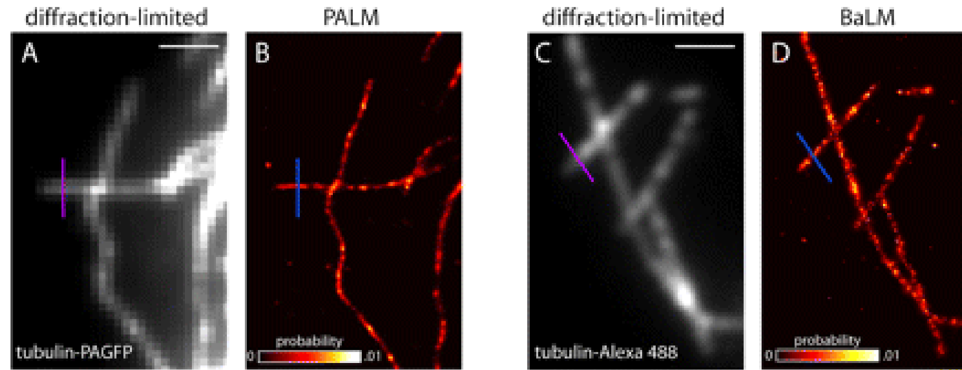
BALM - blinking assisted localization microscopy

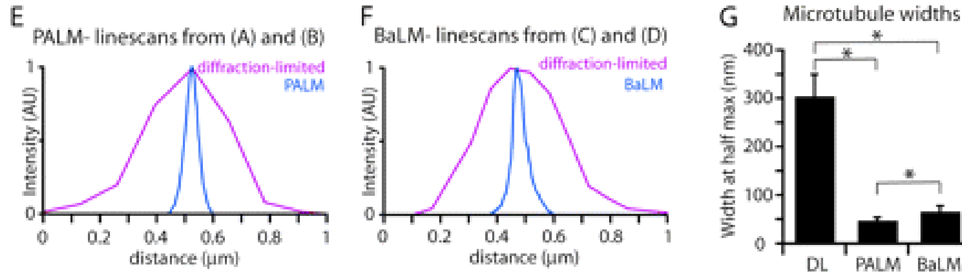
- Rather than photoswitch, rely on endogenous blinking to isolate molecules
- Let laser and camera run continuously
- Take difference images between frames
- If dye blinks on, it will appear like a negative spot
- If dye blinks off, it will appear as positive spot
- Average together those frames to get single molecule spot

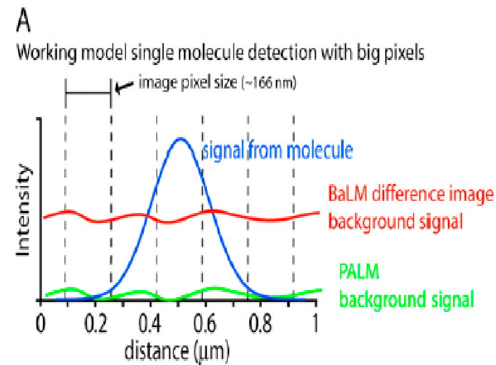


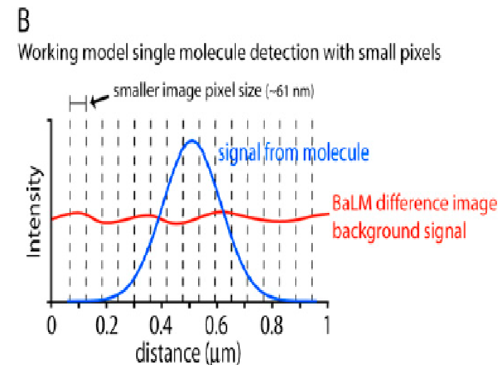
BALM

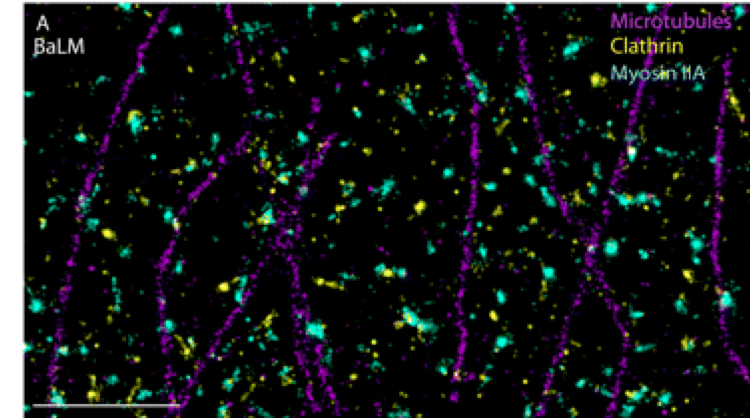
- Due to higher background, localization is not as good as PALM/STORM
- Still significant improvement over diffraction limited systems
- Easy to do multi-color BALM

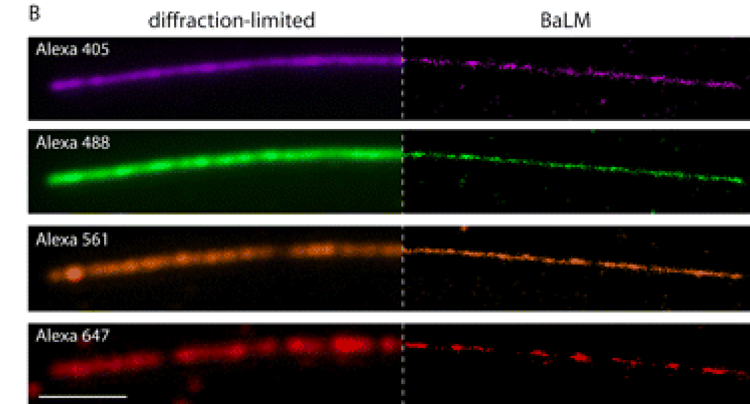






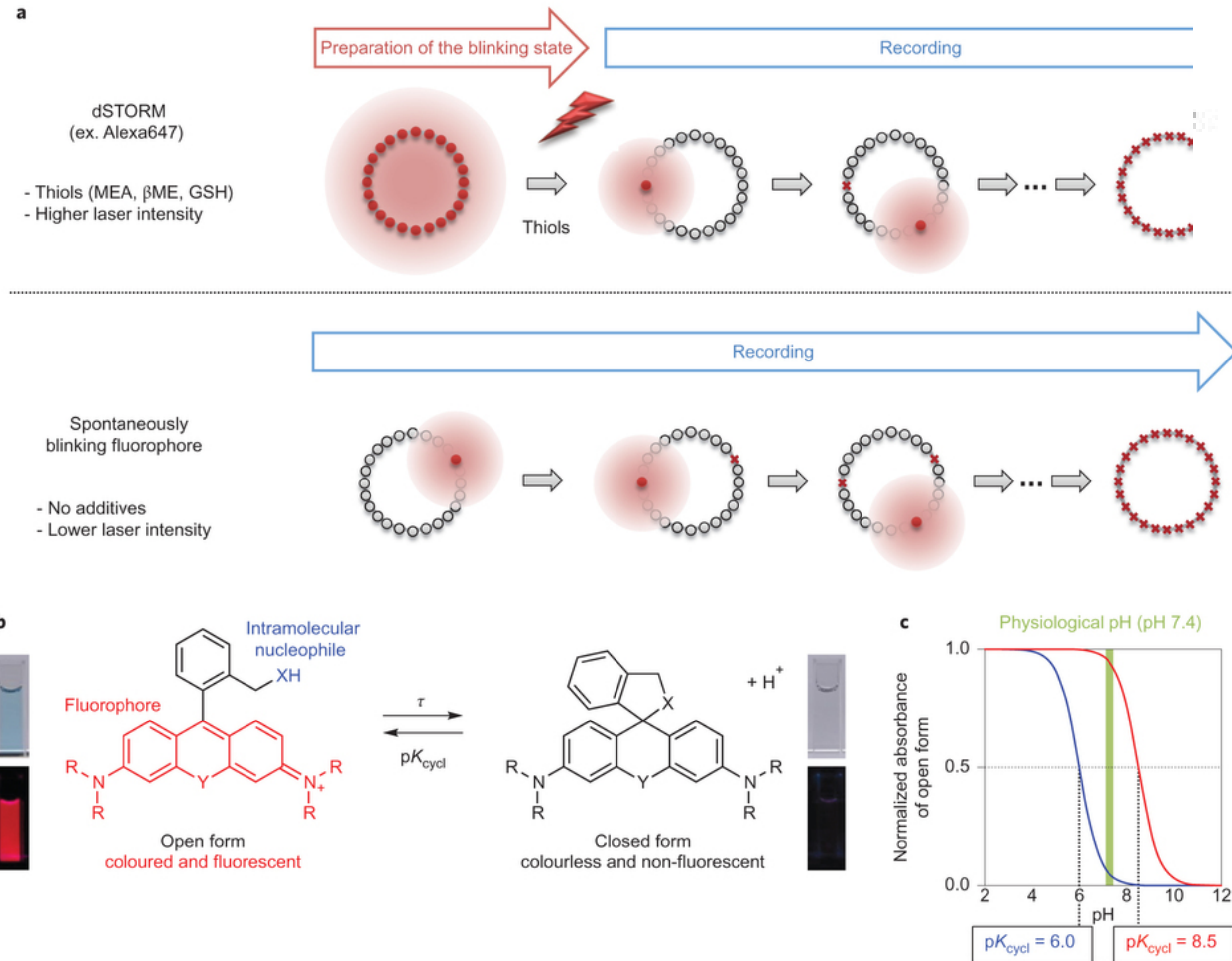


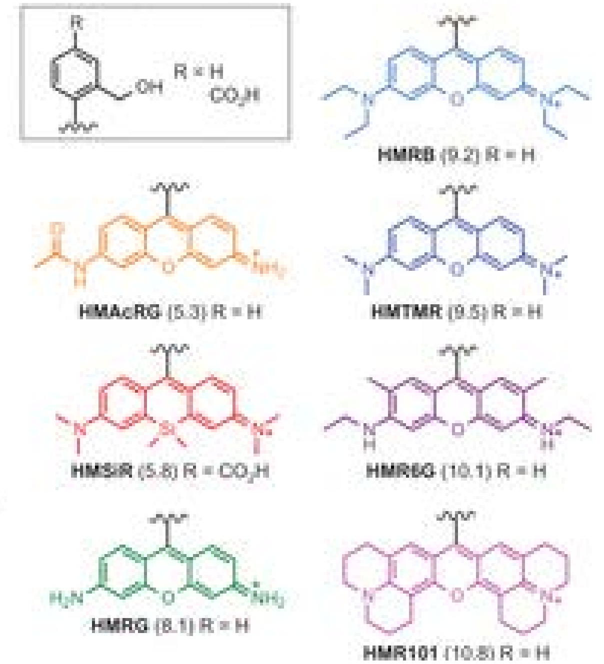


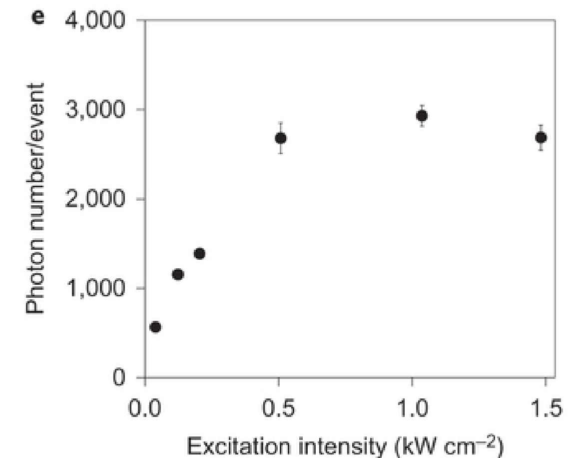


Increasing fluorophore blinking

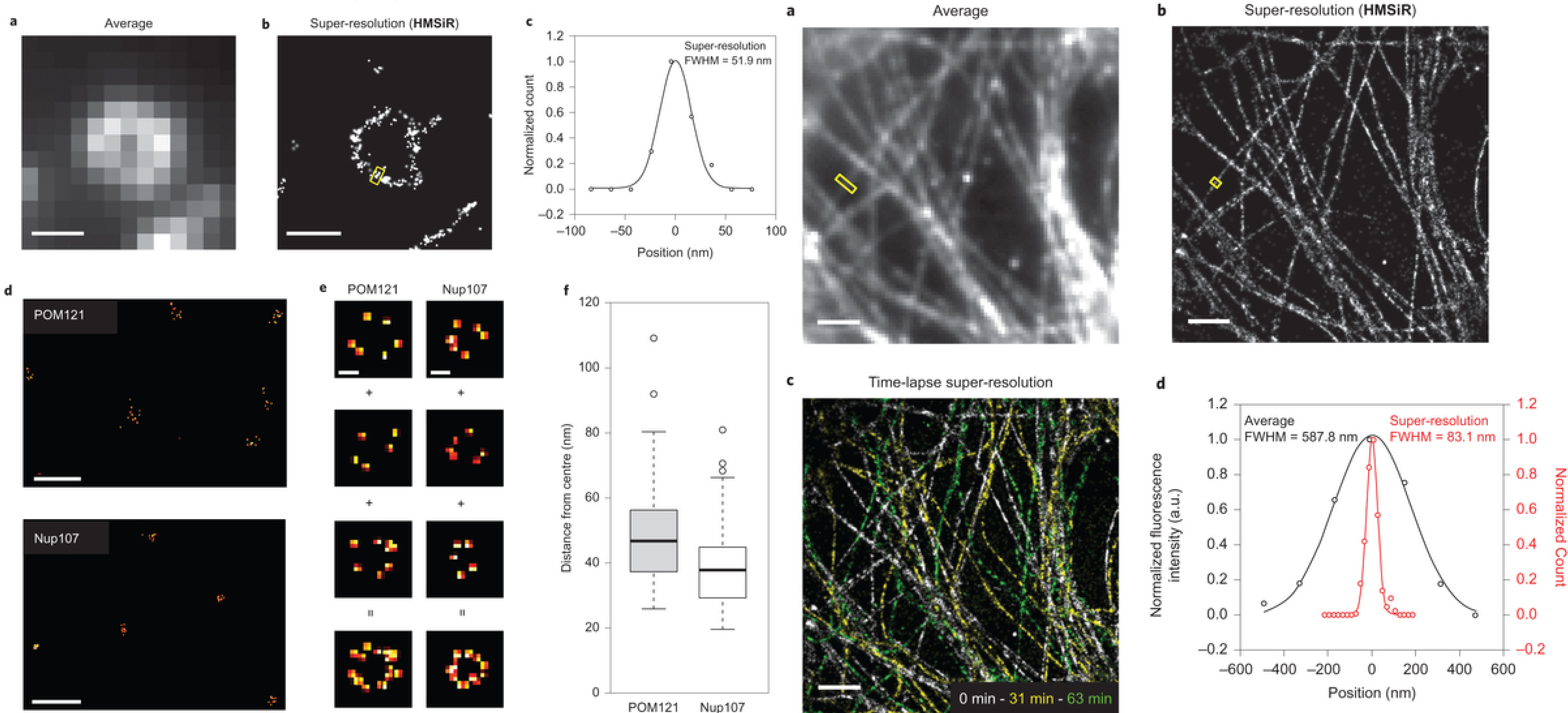
- Engineering fluorophores to undergo blinking with appropriate kinetics
- Too much blinking -> not enough signal for each fluorophore, too many on at one time
- Too little blinking -> Takes forever to build image







BALM applications

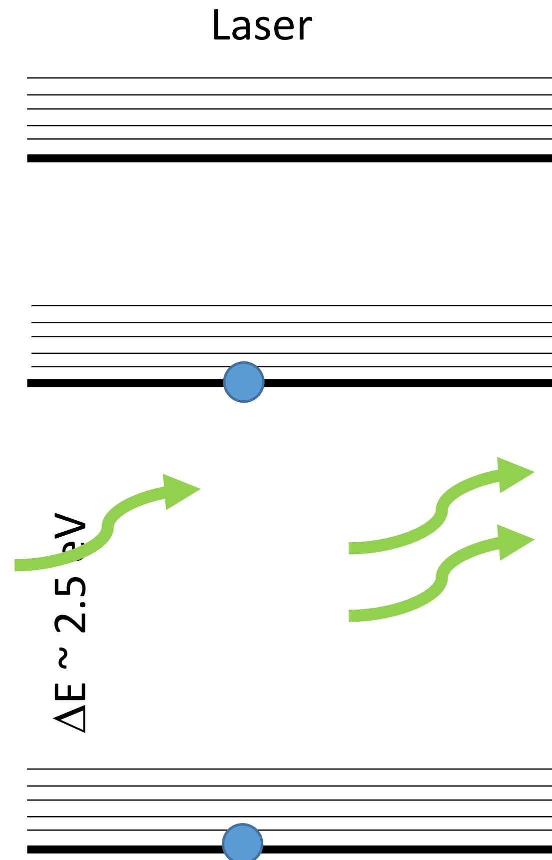


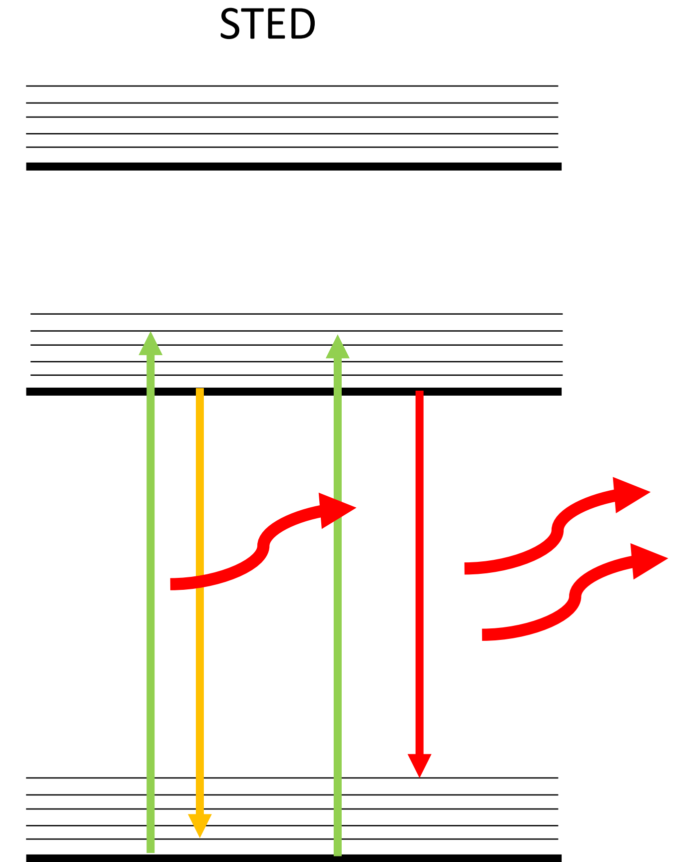
STED – Stimulated Emission Depletion

- Another way to generate superresolution images
- 2014 Nobel prize – Stefan Hell

STED theory

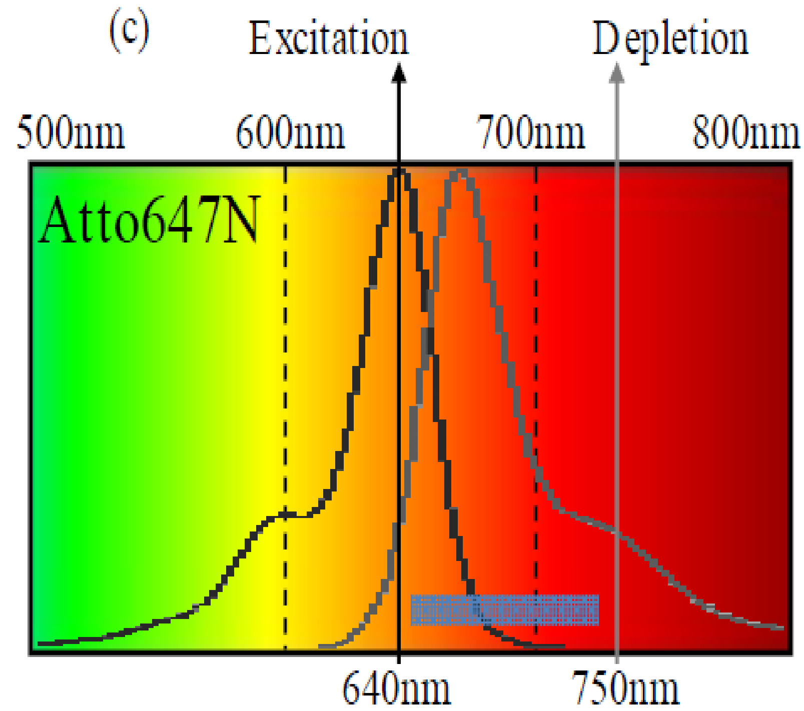
- Consider a laser
- Electron is living in an excited state, doesn't matter how it got there
- If a photon arrives with the energy equivalent to band gap, the electron de-excites, and 2 photons appear

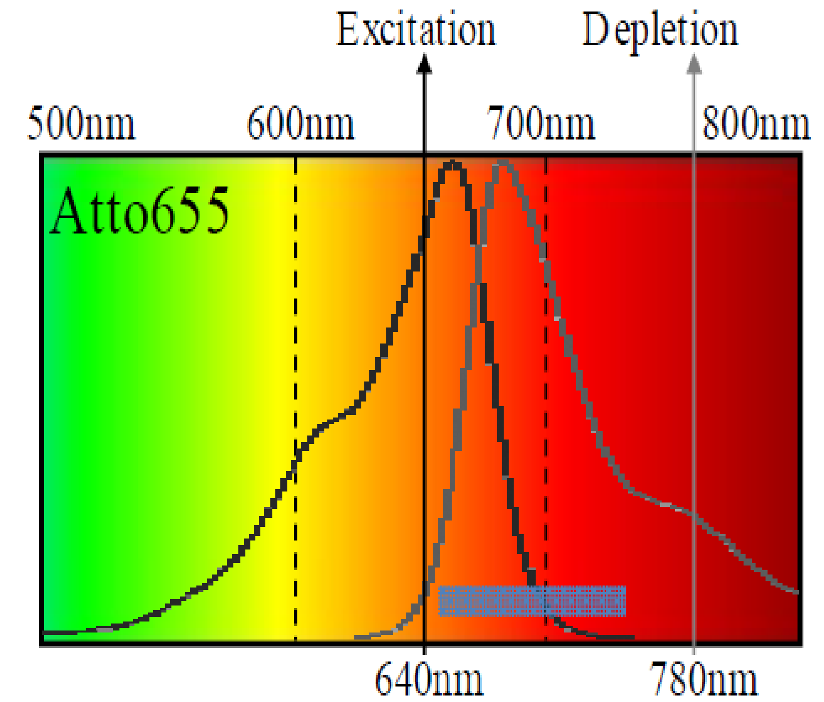




STED theory

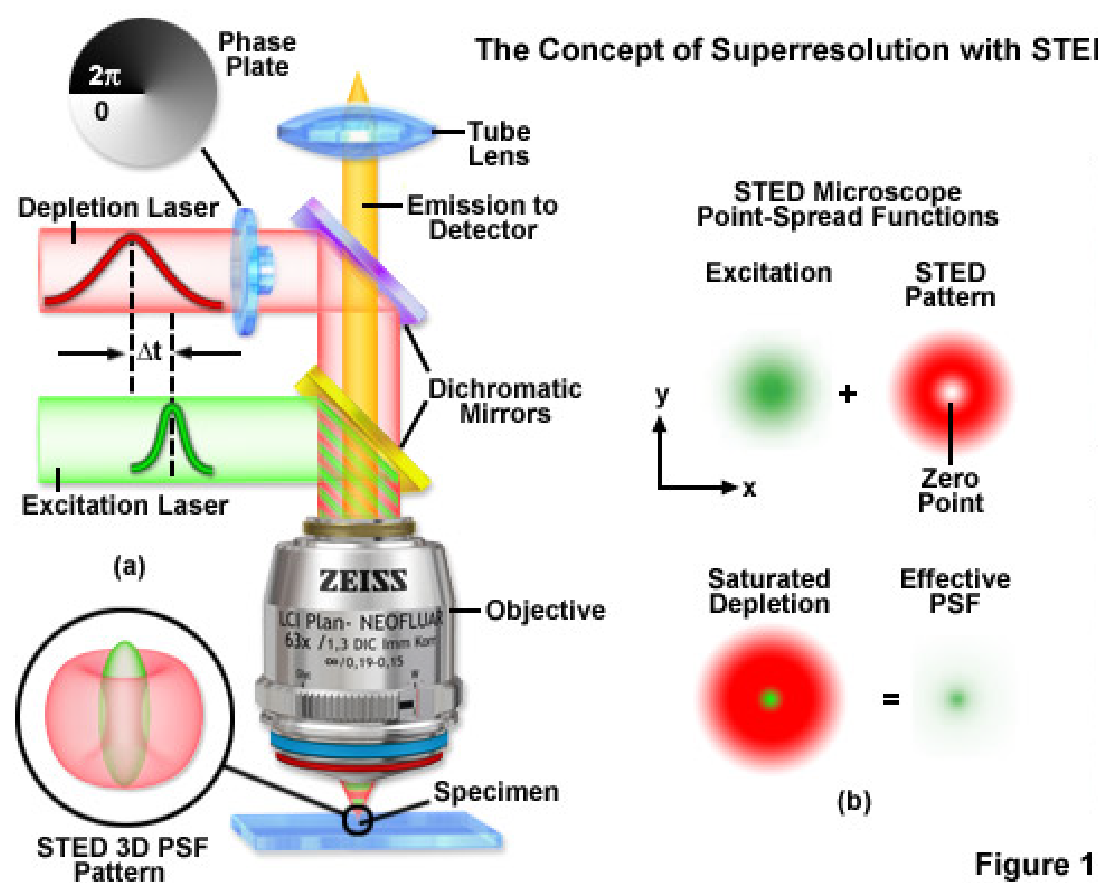
- By sending in light at a given wavelength, we can control the color of the stimulated emission
- Possible to design filters to let normal fluorescent light through, while excluding the stimulated photons



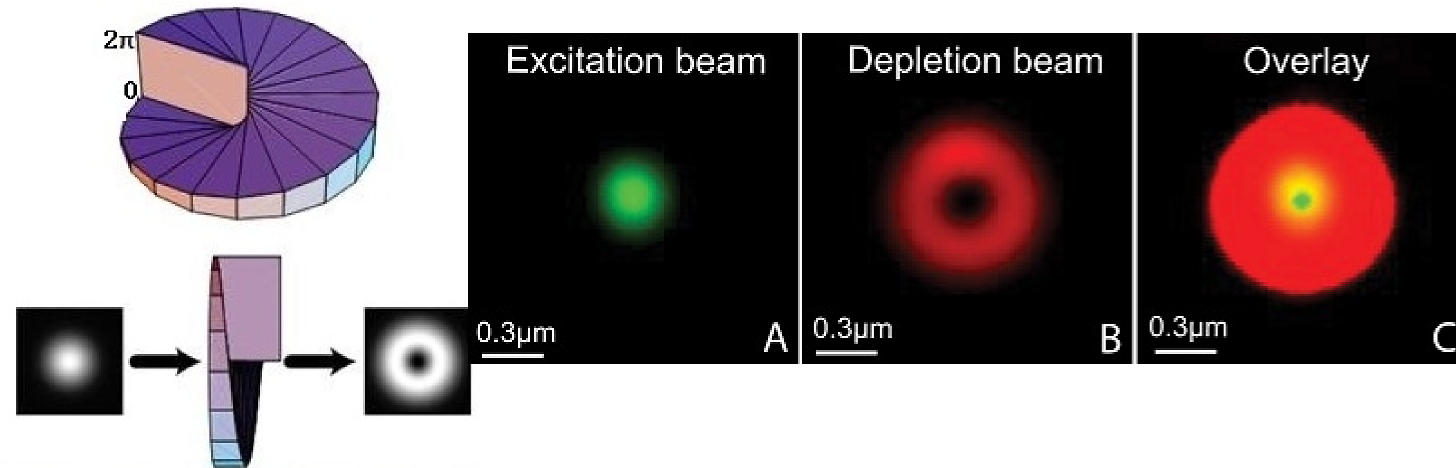


STED theory

- The smallest spot we can excite is a diffraction limited spot (100s of nanometers)
- If we can selectively deplete some chunk of that region, we can limit the region of fluorophores that emit
- STED ideal is to make a donut around the excitation spot, limiting emission from a small region in the center. If you see fluorescent photons, you know where they came from to a much higher precision

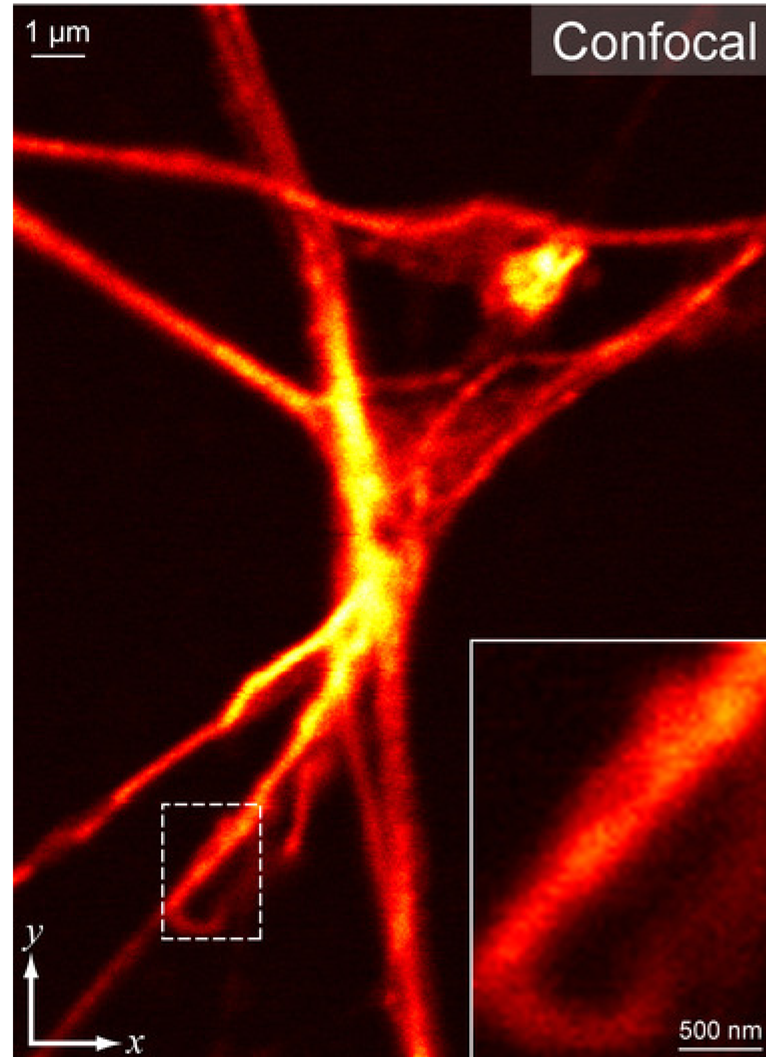


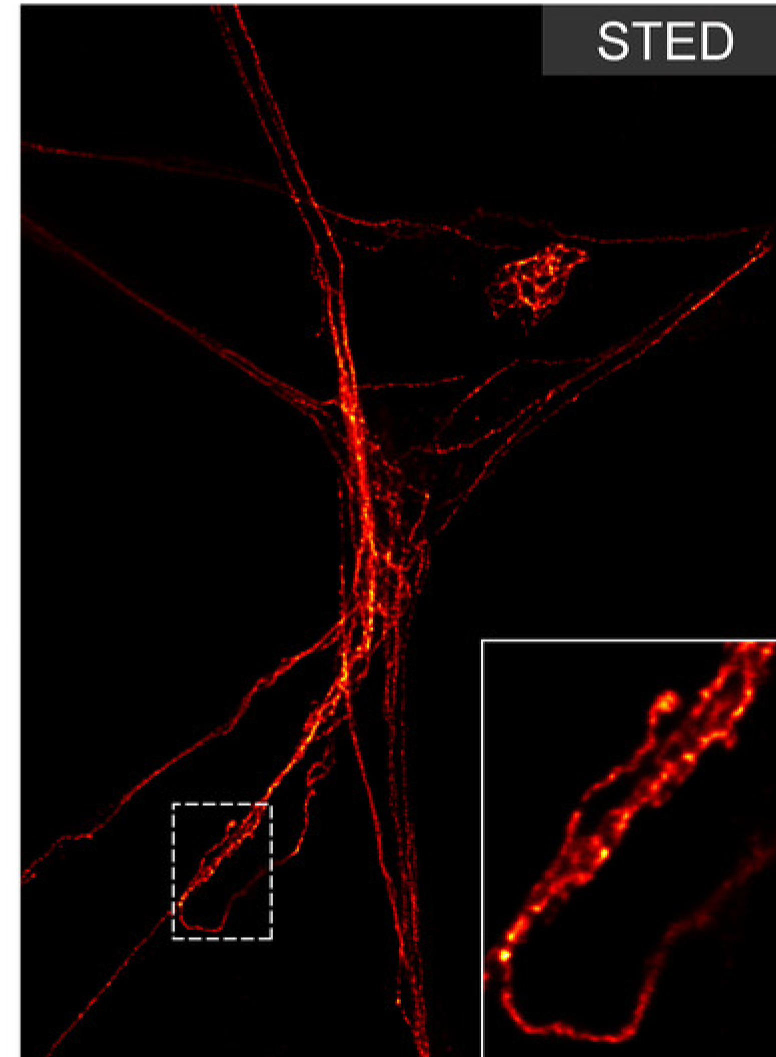
Courtesy of Courtial and O'Holleran, 2007



STED imaging

- Develop image exactly like confocal
- Scan both excitation and STED laser around sample
- But you know below resolution limit where the fluorescence originated from
- Rebuild super-resolution image
- No need to post process images, unlike STORM/PALM





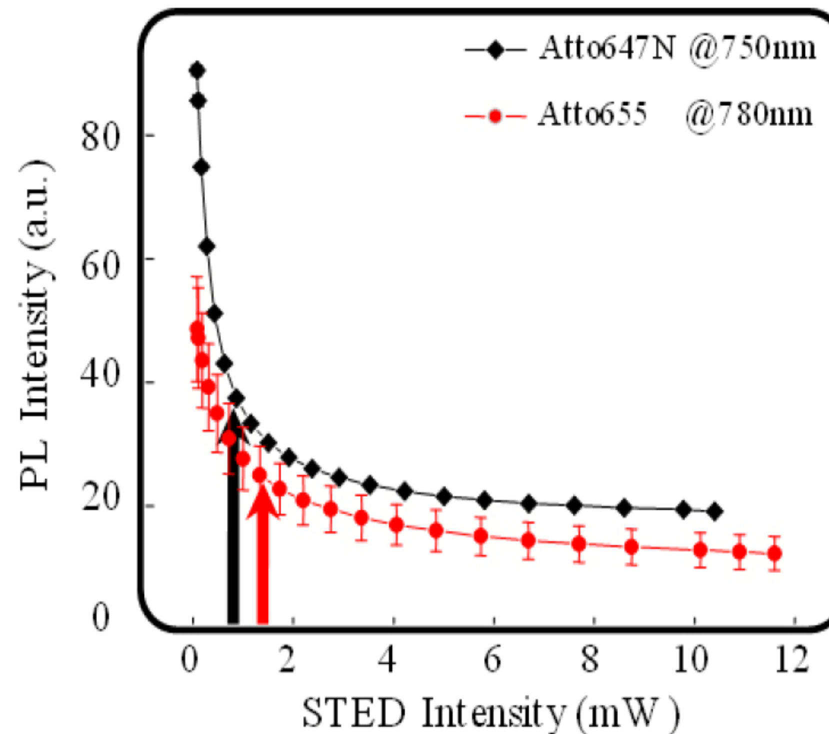
STED resolution

- Unlike STORM/PALM, resolution limit is dependent on intensity of depletion laser
- Intensity must be above I_s, the threshold at which 50% fluorophores undergo stimulated emission (ie. Depleted fluorescence)
- STED pulse must be delayed in time to allow vibrational relaxing. Want to catch electrons in ground state of excited state, but before they decay

$$d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}}$$

d = diffraction limit

I_s = Saturation intensity

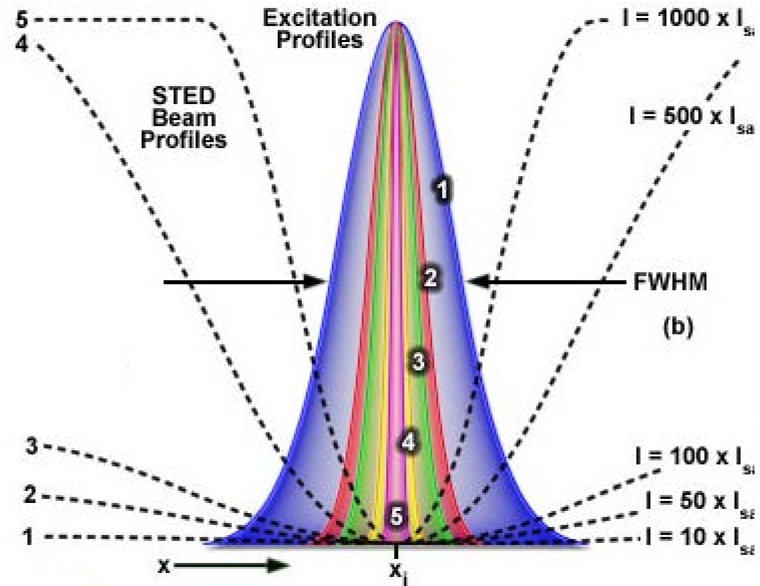


I_s fluorophore and wavelength dependent.

STED PSF is intensity and wavelength dependent

- More power = higher resolution
- Higher photobleaching and toxicity as well
- Diminishing returns in resolution, but increasing returns in cell death with increasing lasers

$$d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}}$$



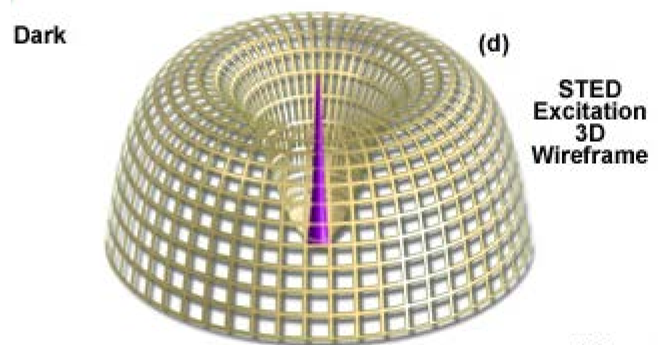


Figure 4

Dye Name	Manufacturer	Exc. λ (nm)	STED λ (nm)
Atto 425	ATTO-TEC GmbH	440	532
Atto 532	ATTO-TEC GmbH	488	615
DY-485XL	Dyomics GmbH	488	647
Alexa Fluor 488	Invitrogen Corp.	488	592
Chromeo 488	Active Motif	488	602
Oregon Green 488	Invitrogen Corp.	488	592
FITC	Cappel Corp.	488	592
DY-495	Dyomics GmbH	488	592
GFP		488	575
Citrine		488	592
YFP		488	598
Atto 565	ATTO-TEC GmbH	532	640-660
MR 121 SE	Roche Diagnostics	532	793
NK51	ATTO-TEC GmbH	532	647
RH 414	Biotium Inc.	554	745
Atto 590	ATTO-TEC GmbH	570	690-710
Alexa 594	Invitrogen Corp.	570	690-710
DyLight 594	Thermo Scientific	570	690-710
Atto 633	ATTO-TEC GmbH	635	750
Atto 647N	ATTO-TEC GmbH	635	750
Atto 655	ATTO-TEC GmbH	635	780
Malachite Green		635	730-750
E2-Crimson		635	750

Full list at: http://nanobiophotonics.mpibpc.mpg.de/old/dyes/

STED Practicalities

- STED depletion pulse is susceptible to changes in index of refraction
- Must use oil that exactly matches glass (type F)
- Must use coverslips that are 170 μm (+/- 3%). Normal coverslips can vary by as much as 20%. Have to order special
- Ultrafast pulsed lasers can be temporally separated to optimize depletion, but then they take longer to scan and cost more monies.

On to Matlab…