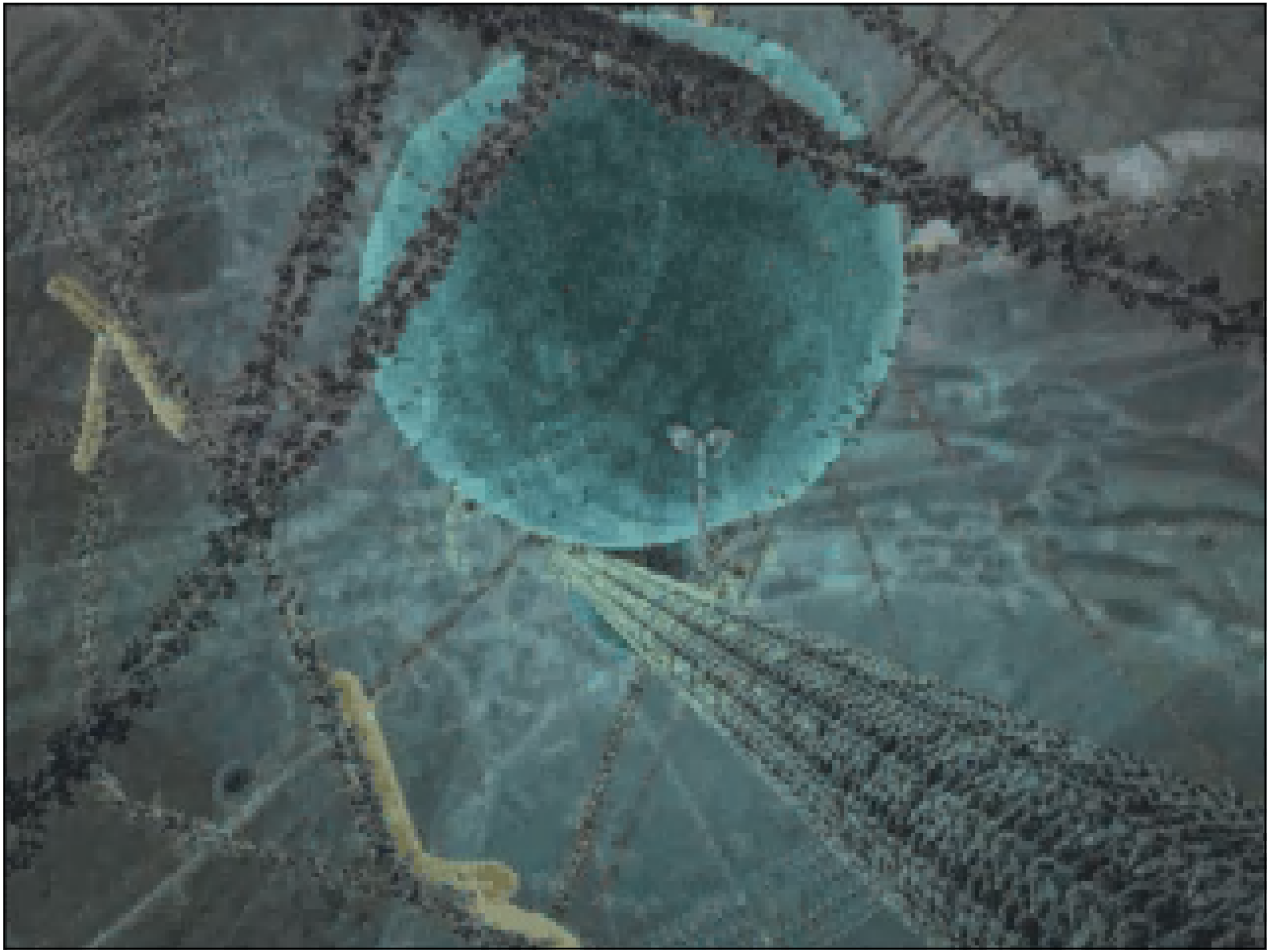
Announcements

I *will* be here on Wed.

Unclear whether we'll have special lecture by Klaus Schulten on magnetic orientation.

Lec. 13 : SHREC, SHRIMP, PALM and STORM

Enhancing Resolution



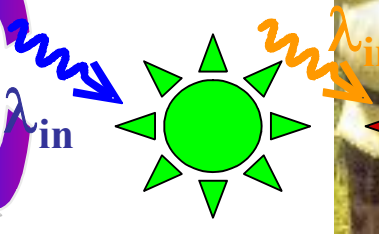
Very good accuracy:
1.5 nm, 1-500 msec

FIONA

Fluorescence **I**maging with
One **N**anometer **A**ccuracy

SHREC

Single molecule **H**igh
REsolution Colocalization

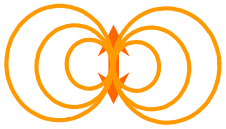


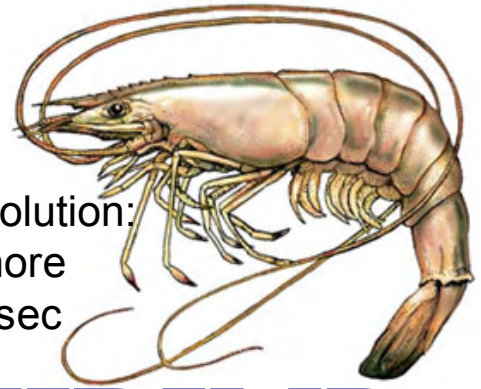
Very good resolution:
8-10 nm, 500 msec

Very good orientation:
10-15°, >500 msec

DOPI

Defocused **O**rientation
Position **I**maging





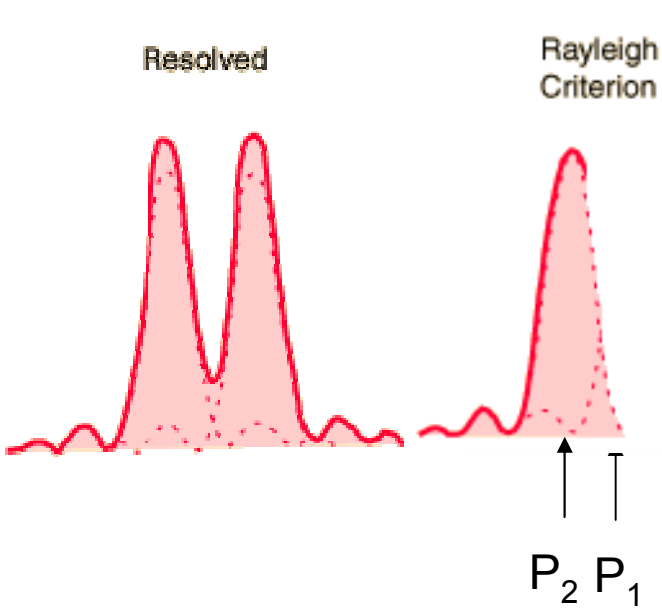
Very good resolution:
same fluorophore
10 nm, 500 msec

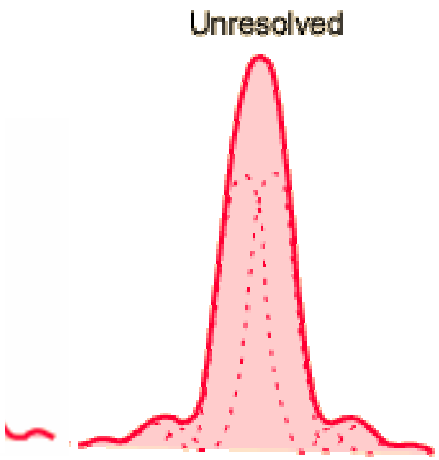
SHRIMP

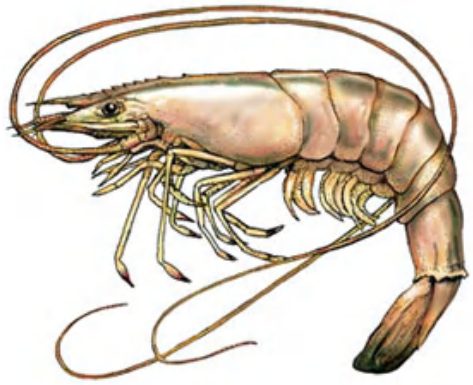
Super **H**igh Resolution
IMaging with **P**hotobleaching

Breaking the Rayleigh criteria: 1st method

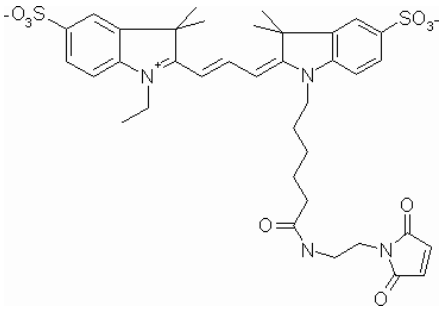
- Can we achieve nanometer *resolution*?
- i.e. resolve two point objects separated by d << λ/2?

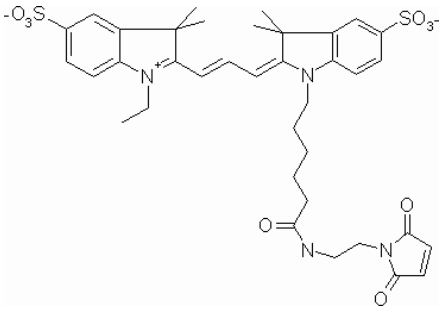






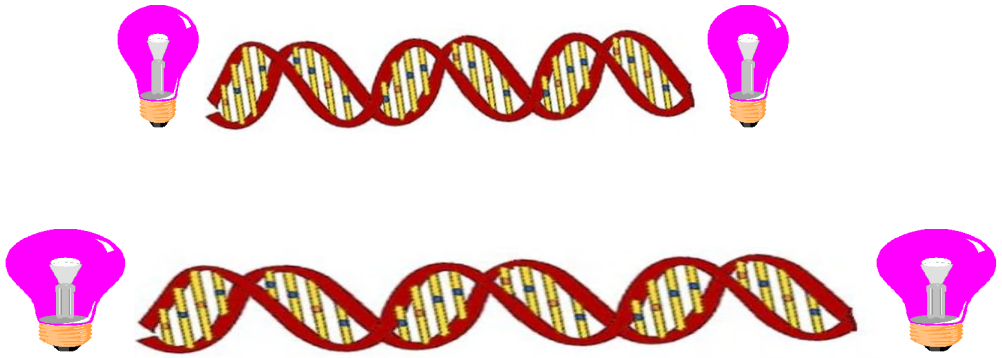
SHRIMP





Super **H**igh Resolution **IM**aging with **P**hotobleaching

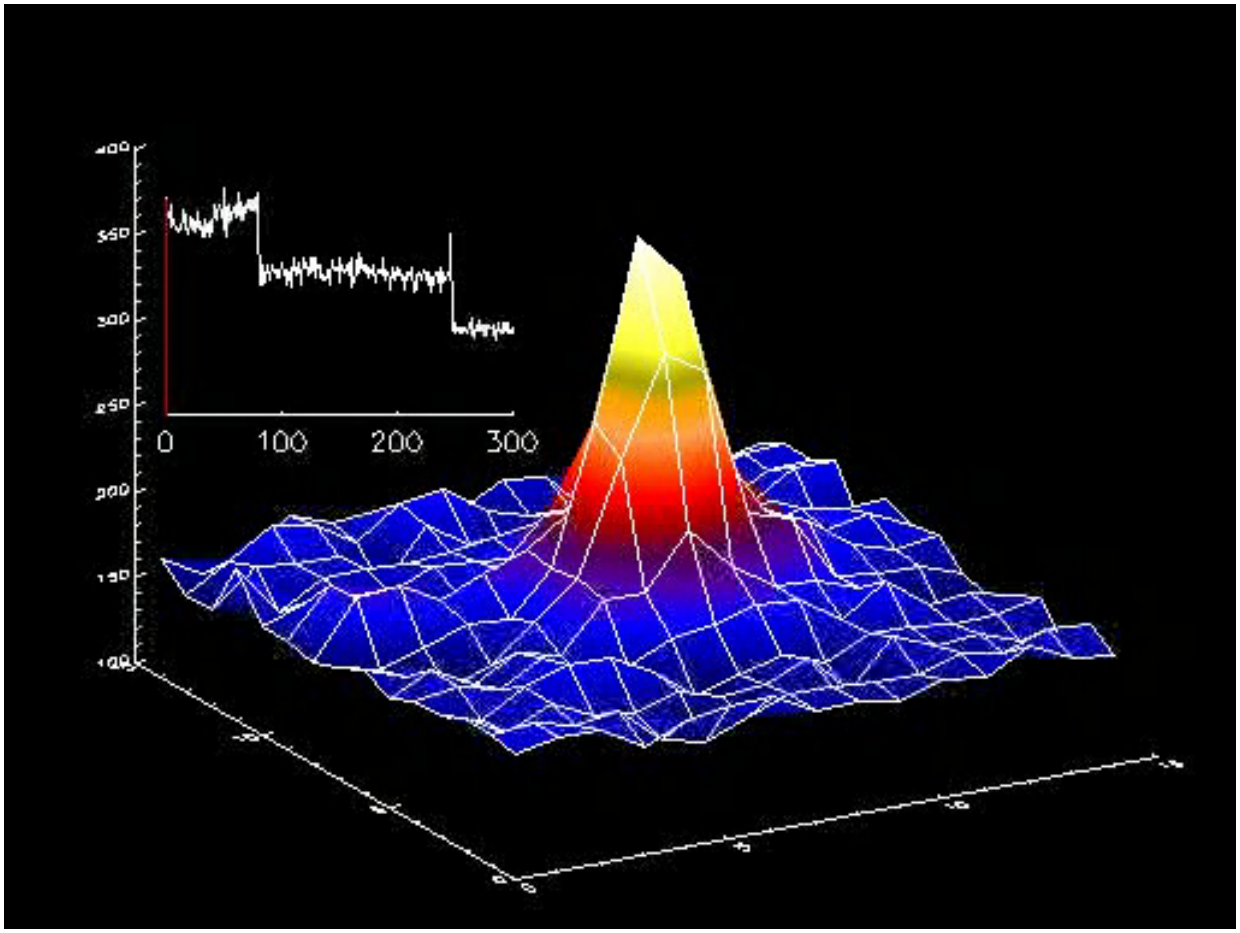
Example of SHRIMP



At what point can you tell that there are two well-resolved fluorophores?

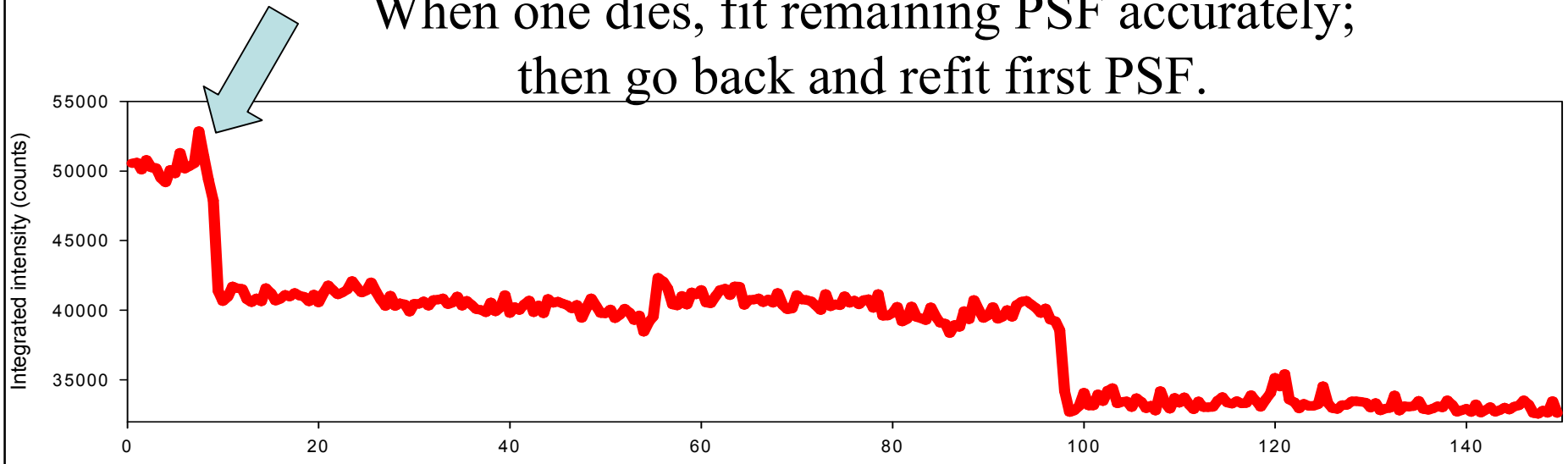
SHRIMP on DNA

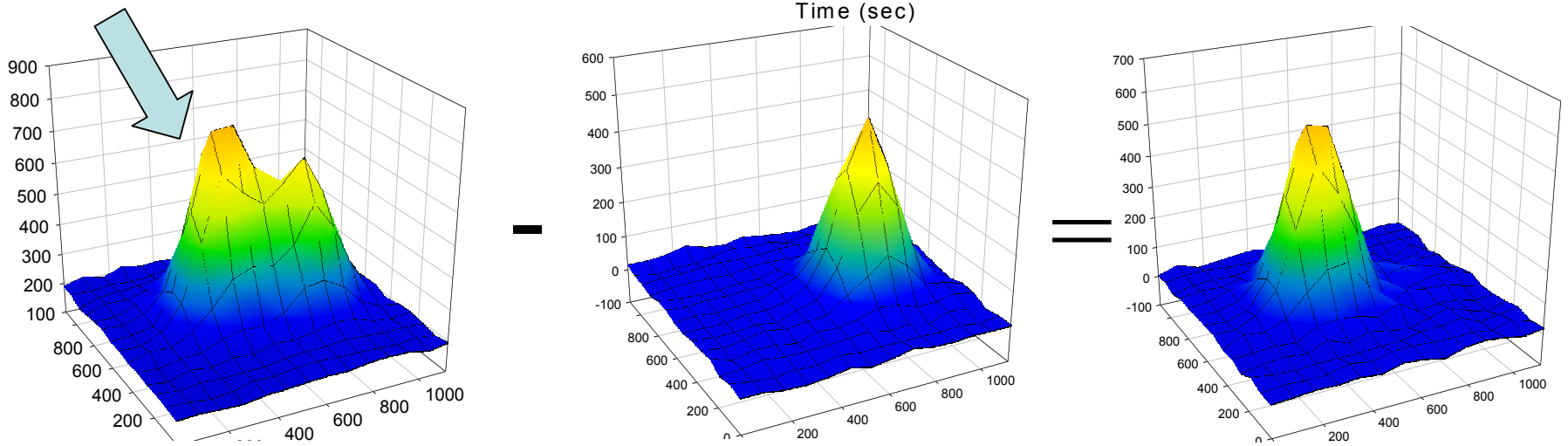
(Super-High Resolution Imaging with Photobleaching)



Utilizing Photobleaching for Colocalization

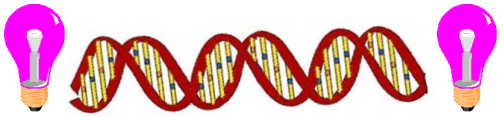
Additional knowledge: 2 single dyes
When one dies, fit remaining PSF accurately;
then go back and refit first PSF.

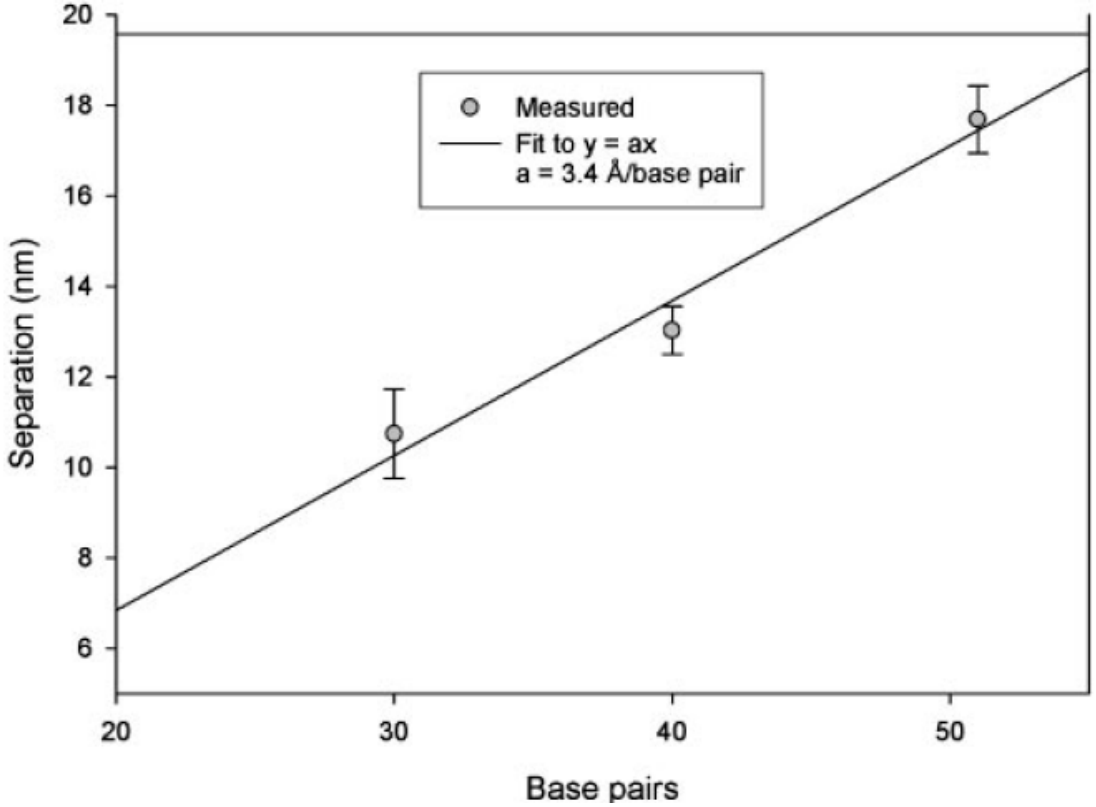




Separation = 329.7 ± 2.2 nm Separation = 324.6 ± 1.6 nm

Control: DNA Molecule End-to-End Separation





Sample	Measured Distance
51-mer	17.7 ± 0.7 nm
40-mer	13.0 ± 0.5 nm
30-mer	10.7 ± 1.0 nm

Conventional resolution: ~300 nm

Unconventional resolution: few nm!

Gordon et al. PNAS, 2003

- DNA labeled Cy3 on 5' end, hydridized.
- Flowed over coverslip coated with nitrocellulose to prevent adverse photophysical interaction of dye with glass
- DNA binds non-specifically to NC surface
- *DNA is stretched by fluid flow to 150% extension (Bensimon, Science, 1994).

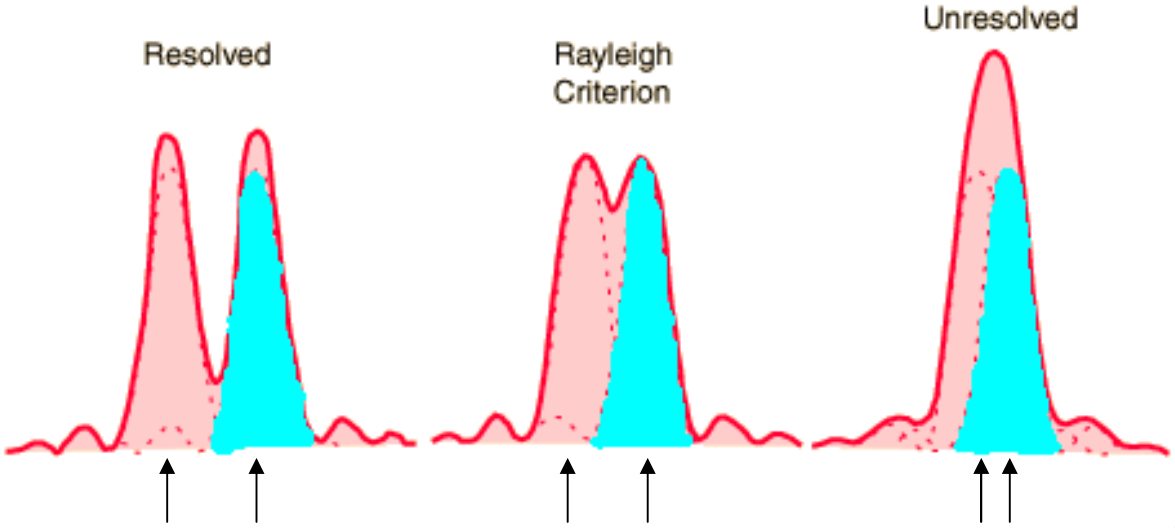
Problem:
SHRIMP only good for one point in time.

SHREC is good at all times.

Breaking the Rayleigh criteria

Can we achieve nanometer *resolution*?

i.e. resolve two point objects separated by d << $\lambda/2$?



1. Make 2 colors– can tell their PSF's

(Point Spread Function),

even if PSF's are a few nm apart.

SHREC

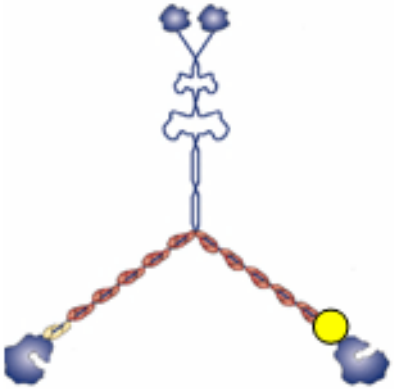
Single molecule **H**igh
REsolution Colocalization

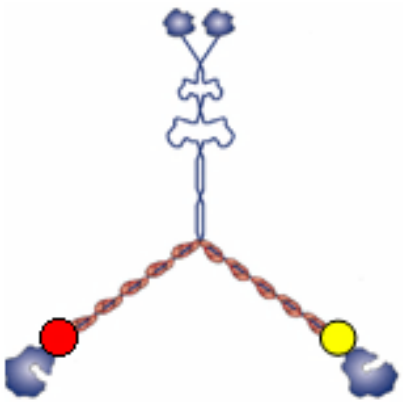
Single Molecule High Resolution Colocalization = SHREC

(S. Churchill, J. Spudich) PNAS, 2004

Two color FIONA = SHREC

1. Seeing every transition
(No 0 nm steps– always 74 nm with Myosin V)
2. Getting distances between points,
Resolving >10 nm

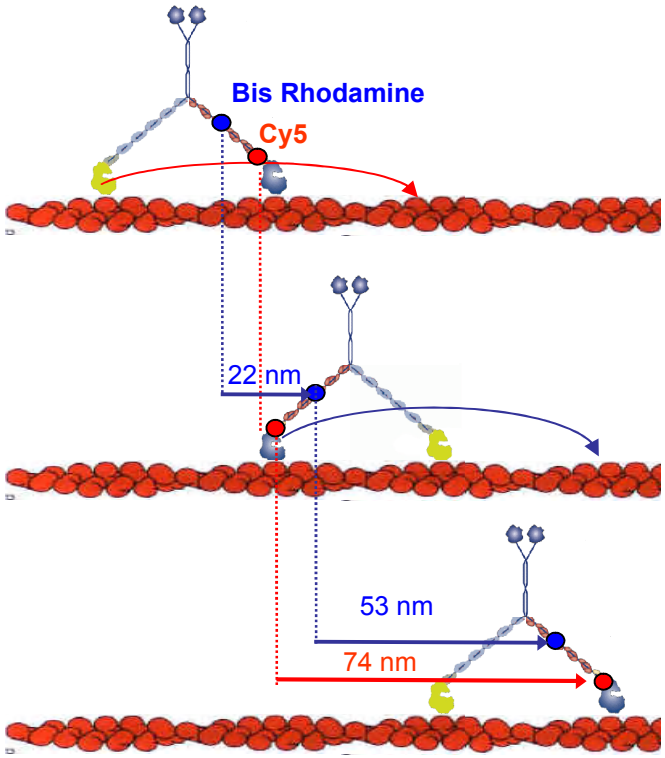


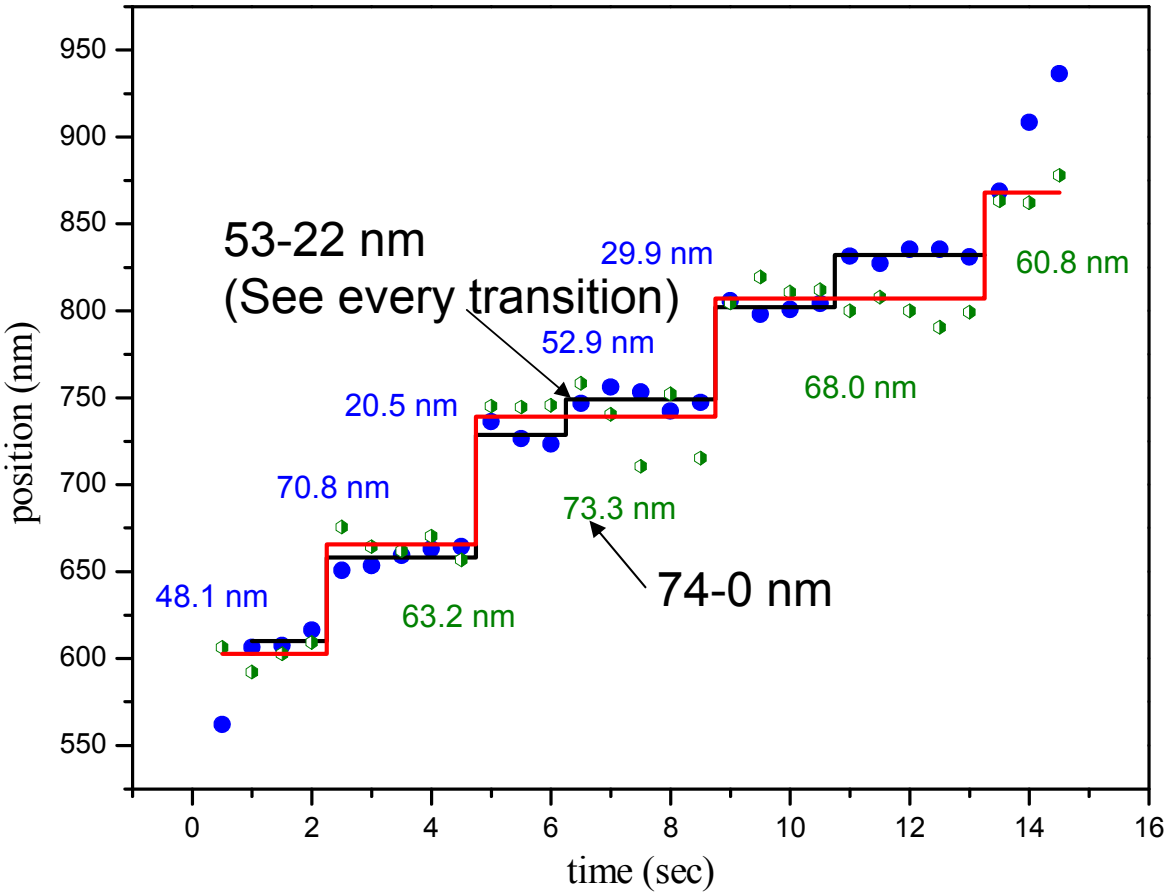


SHREC

Dyes are on the same lever arm: 53-22 nm & 74-0 nm steps:

Resolution = 8 nm





True High-Resolution Imaging

PhotoActivated Localization Microscopy (PALM)

Eric Betzig et al., Imaging intracellular fluorescent proteins at nanometer resolution *Science* **313,** 1642-5 (2006).

Idea:

1. Consider a cell where nothing is moving (no dynamics)– e.g. fixed cell.

2. Label at very low density– less than one per diffraction limited spot. Take image.

3. Using FIONA, get very high accuracy.

(25 nm common– limited by photostability.)

4. Repeat, generating a whole image.

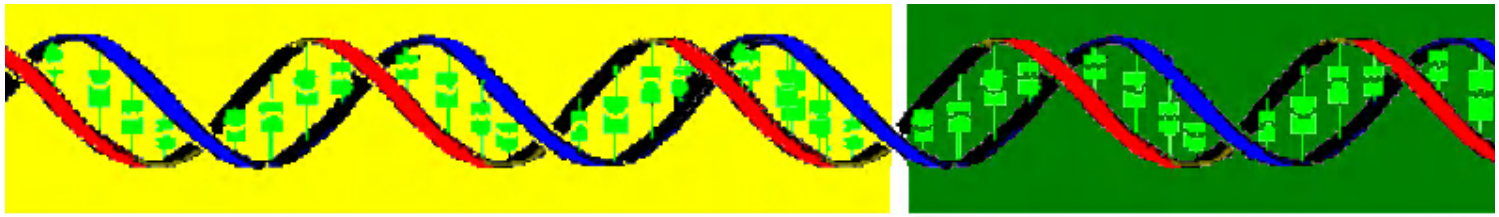
How to repeat? Using photoactivatable dyes. Turn on with 1 wavelength (at short wavelength). Excite fluorescence at 2nd wavelength until it photobleaches. Then repeat.

Sidepoint

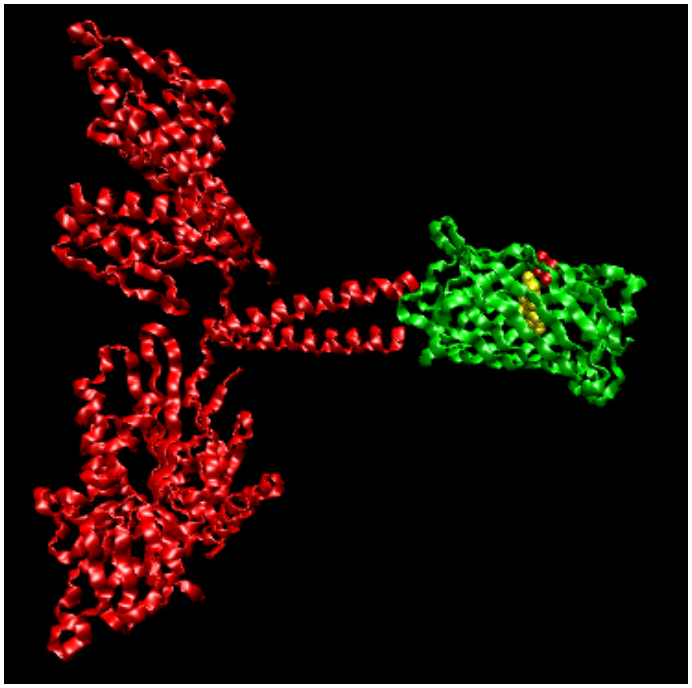
Use GFP's. Get perfect specificity --label only the objects that you want to

Green Fluorescent Protein

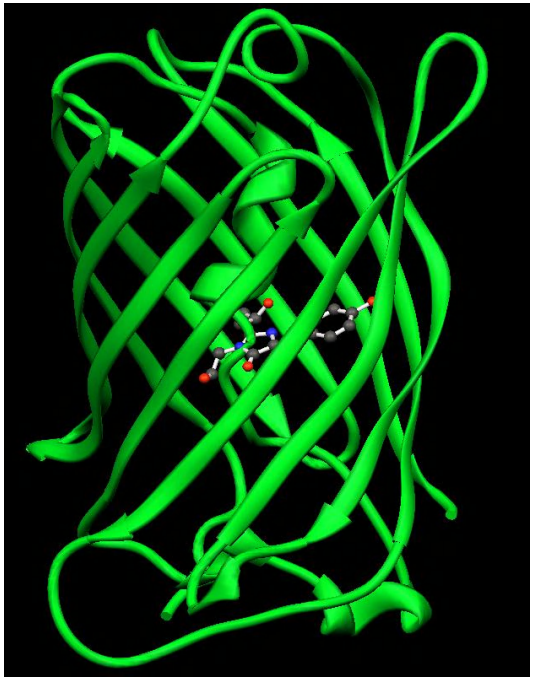
GFP – genetically encoded dye (fluorescent protein)

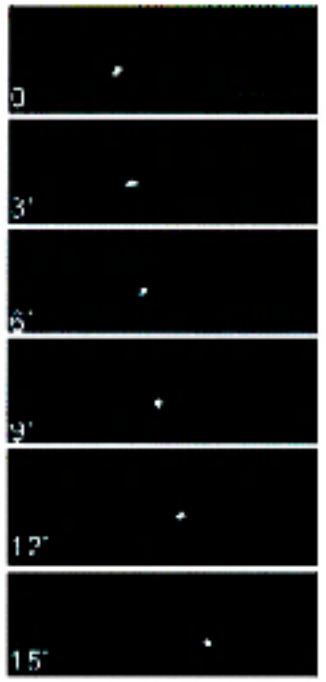


(Motor) protein GFP



Kinesin – GFP fusion



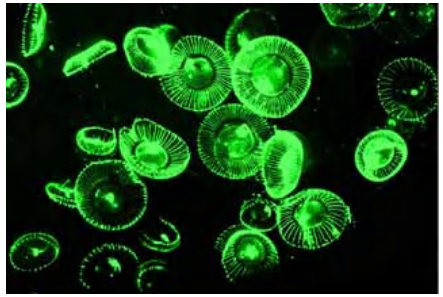


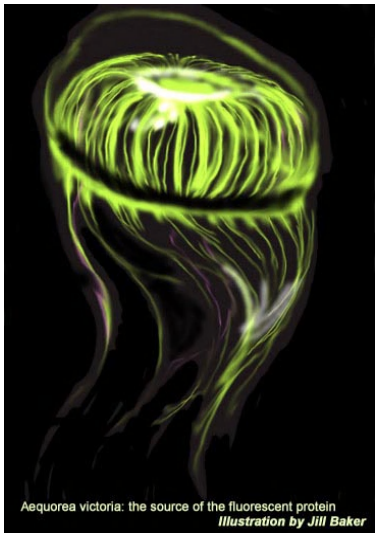
Wong RM et al. PNAS, 2002

Genetically encoded → perfect specificity.

Green Fluorescent Protein: Genetically-encoded dye

Fluorescent protein
from jelly fish

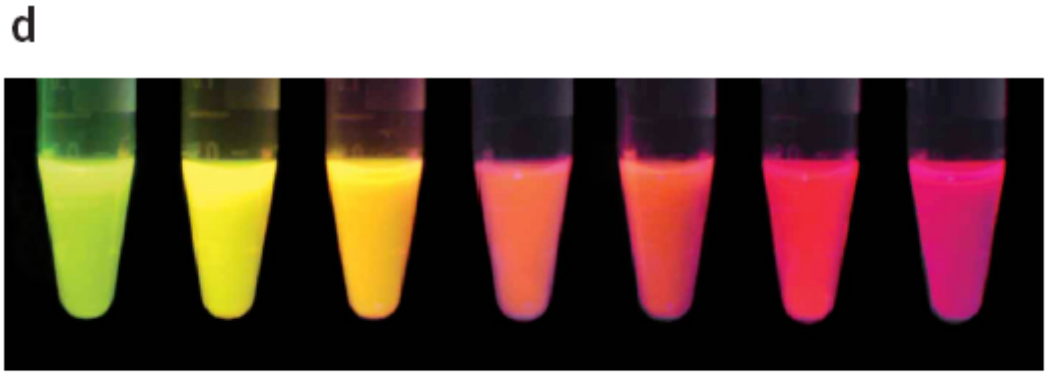




Different “GFPs”

Absorption

Fluorescence

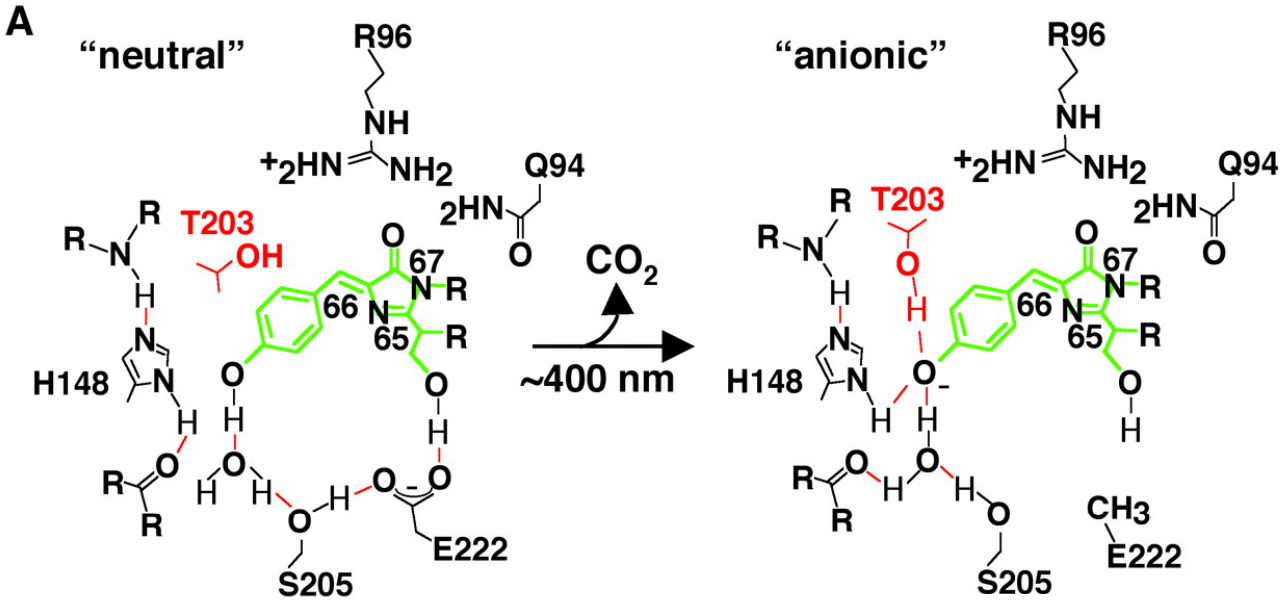


mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry

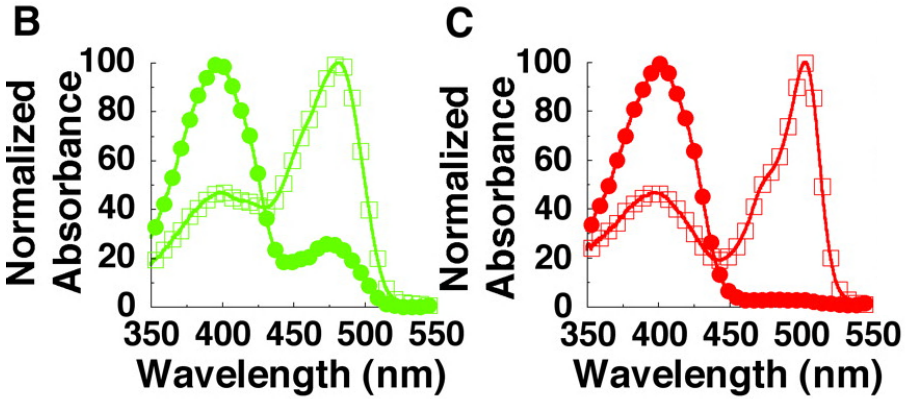
Shaner, Tsien,Nat. Bio., 2004

Photo-active GFP

G. H. Patterson et al., Science 297, 1873 -1877 (2002)



Photoactivatable variant of GFP that, after intense irradiation with 413-nanometer light, increases fluorescence 100 times when excited by 488-nanometer light and remains stable for days under aerobic conditions



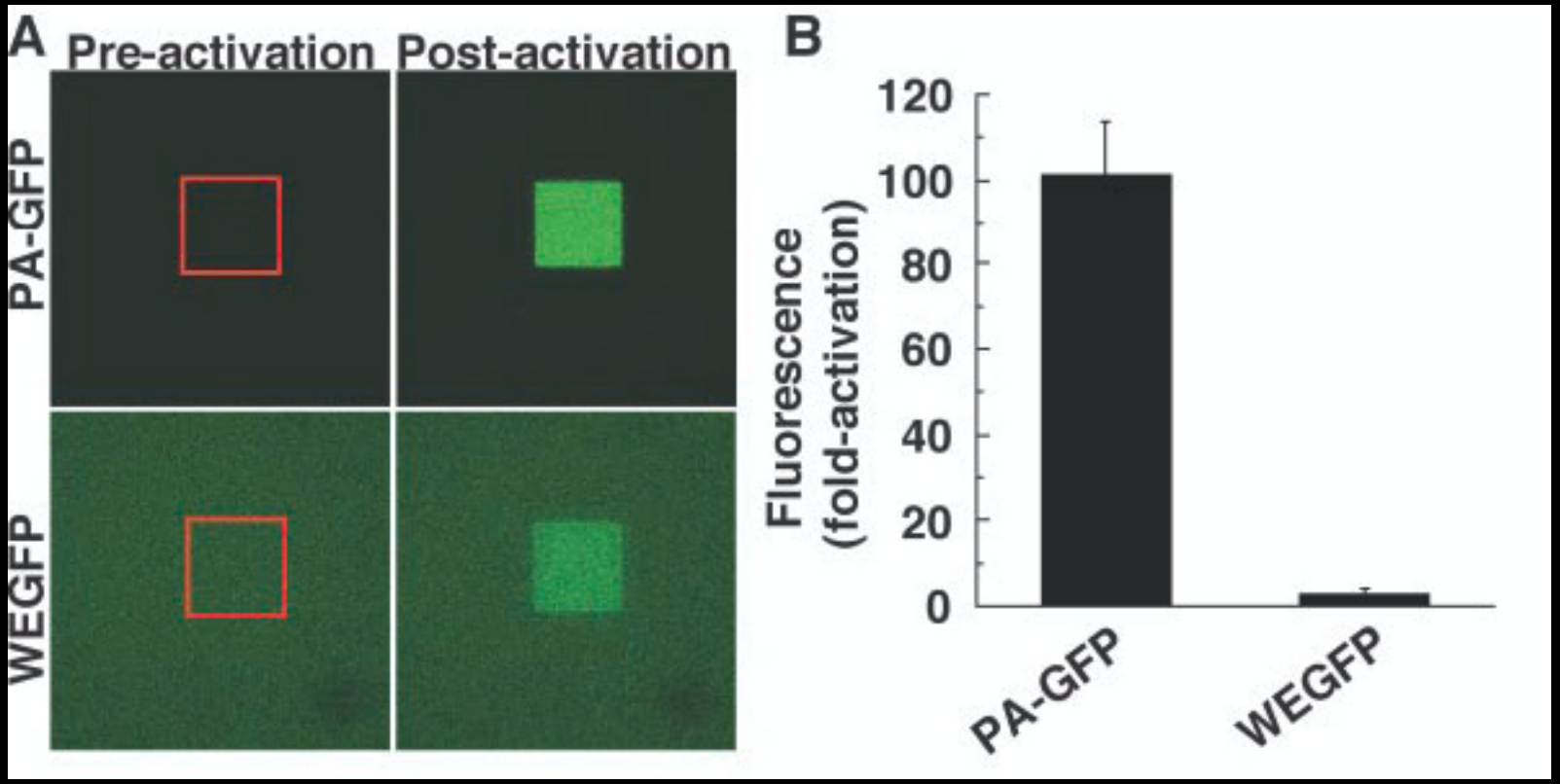
Wild-type GFP

T203H GFP: PA-GFP

Native= filled circle

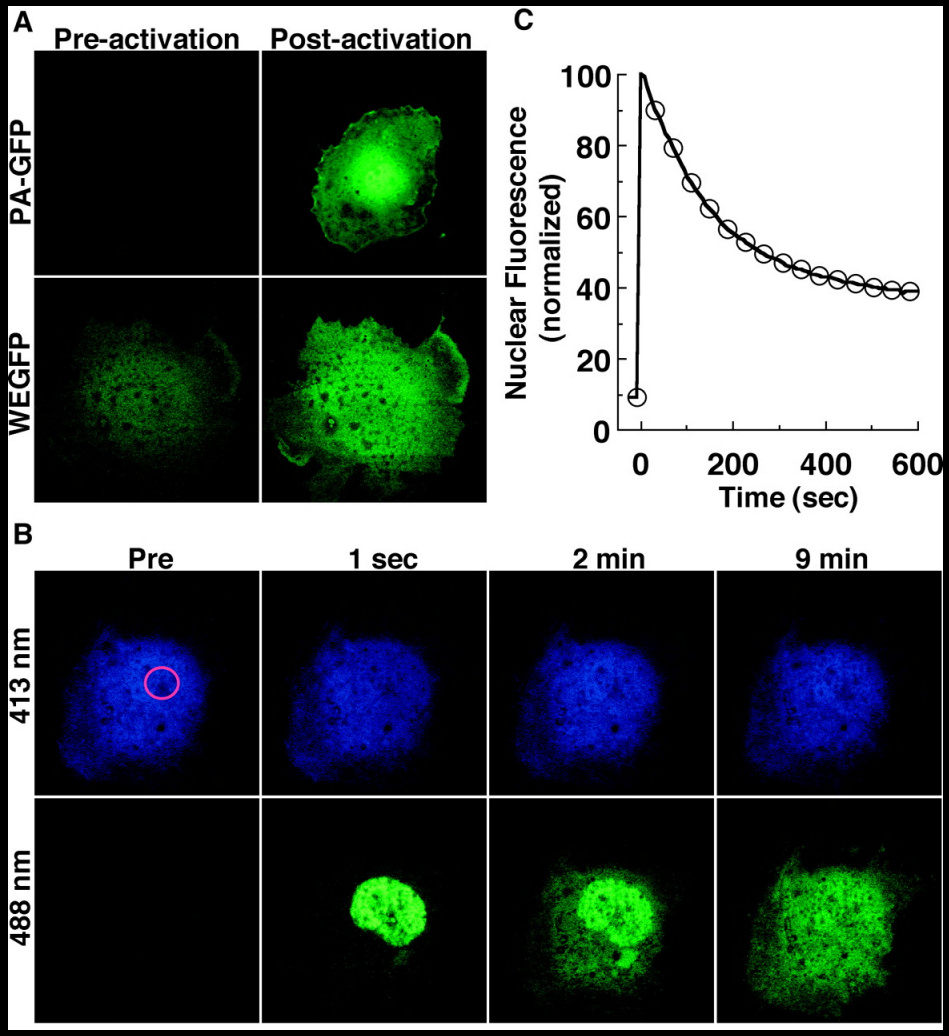
Photoactivated= Open squares

Photoactivation and imaging in vitro



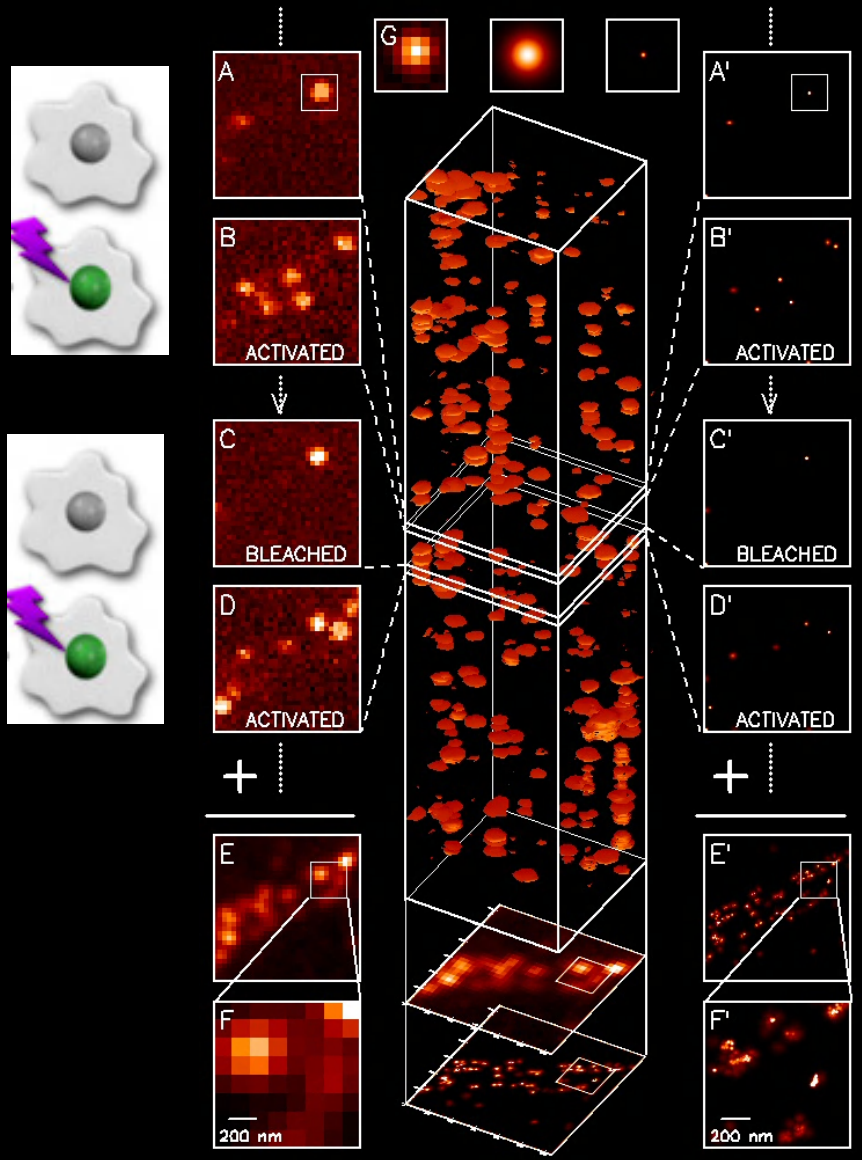
G. H. Patterson et al., Science 297, 1873 (2002)

Photoactivation and imaging in vivo



Science
AAAS

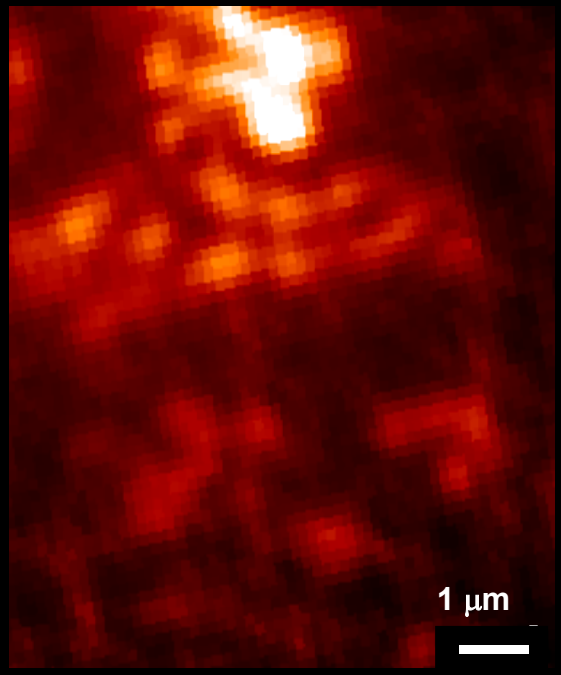
PALM: Photoactivated localization microscopy

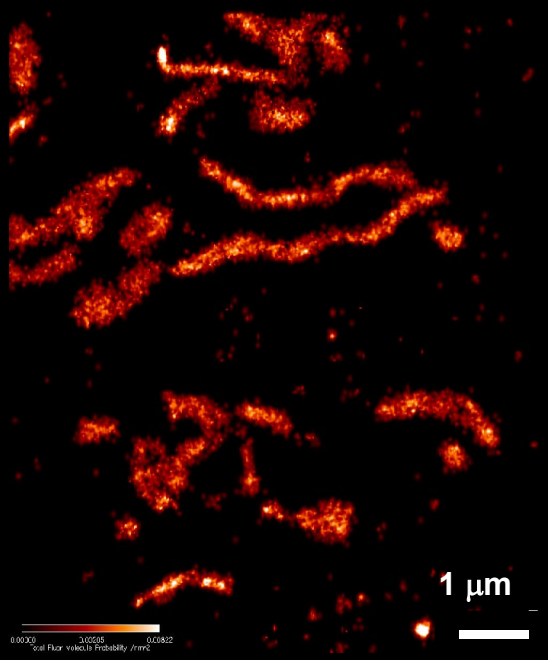


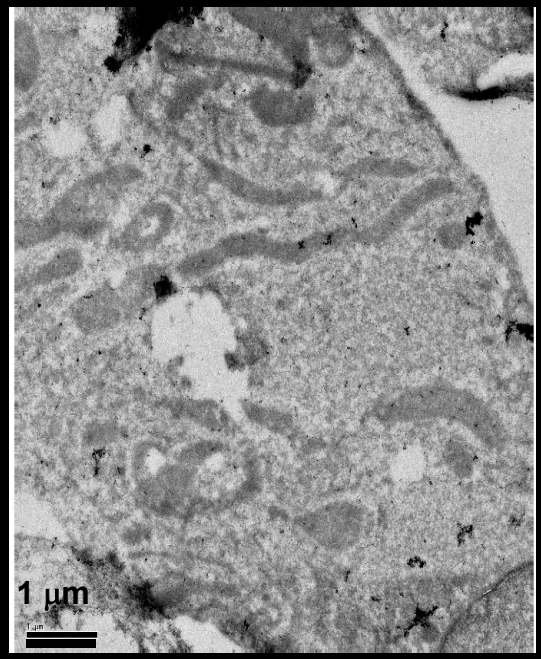
Numerous sparse subsets of photoactivatable fluorescent protein molecules were activated, localized (to ~2 to 25 nanometers), and then bleached. The aggregate position information from all subsets was then assembled into a super-resolution image.

Fig. 1. A sparse subset of PA-FP molecules are attached to proteins of interest and then fixed within a cell are activated (**A** and **B**) with a brief laser pulse at λ_{act} = 405 nm and then imaged at λ_{exc} = 561 nm until most are bleached (C). This process is repeated many times (**C** and **D**) until the population of inactivated, unbleached molecules is depleted. Summing the molecular images across all frames results in a diffraction-limited image (**E** and **F**). However, if the location of each molecule is first determined by fitting the expected molecular image given by the PSF of the microscope [(**G**), center] to the actual molecular image [(G), left], the molecule can be plotted [(G), right] as a Gaussian that has a standard deviation equal to the uncertainty s *x*,*y* in the fitted position. Repeating with all molecules across all frames (**A'** through **D'**) and summing the results yields a super-resolution image (**E'** and **F'**). Scale: 1 x 1 μm in (F) and (F'), 4 x 4 μm elsewhere.

Correlative PALM-EM imaging



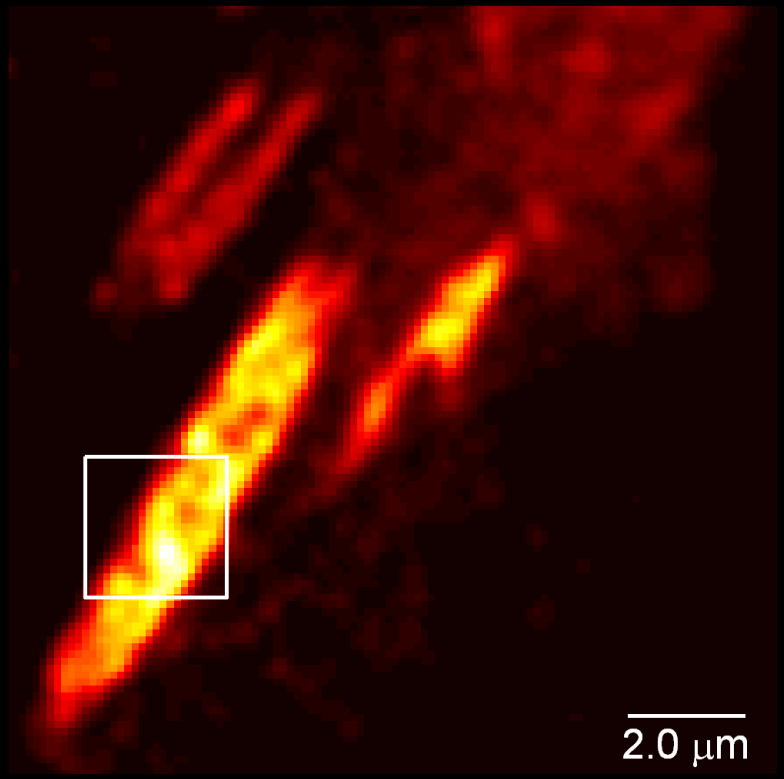




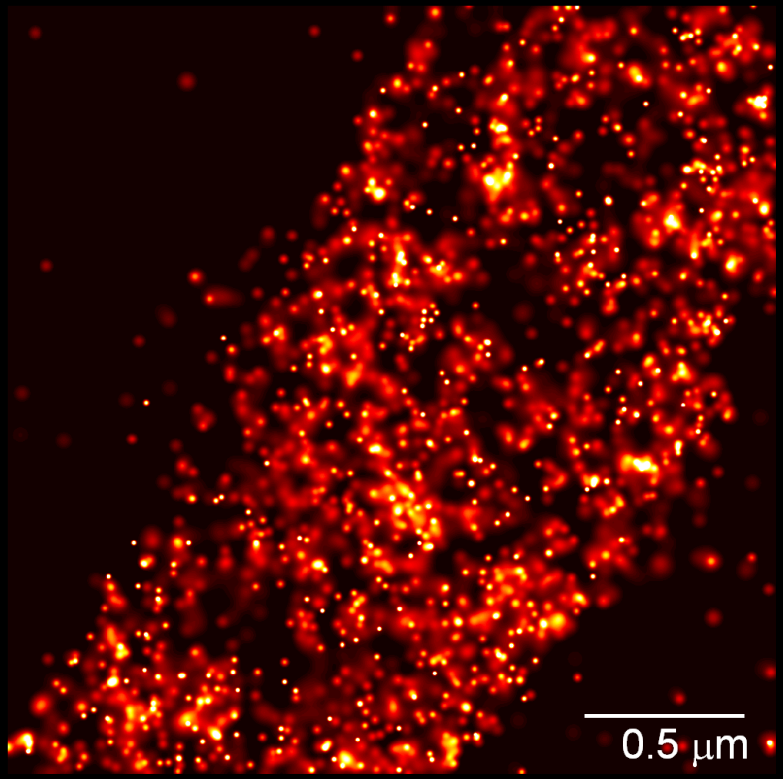
TIRF PALM TEM

Mitochondrial targeting
sequence tagged with mEOS

Comparative TIRF and PALM images



TIRF image

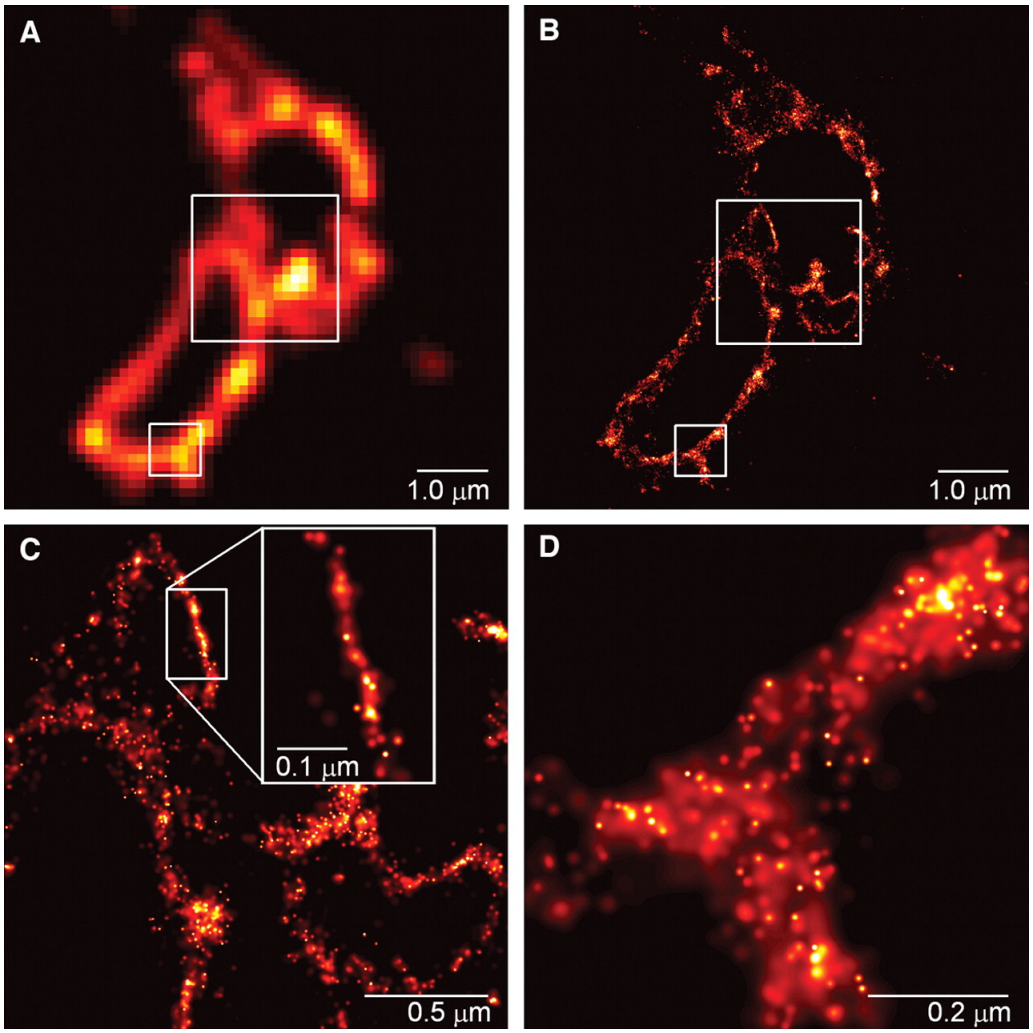


Magnified PALM image

Vinculin-tagged dEosFP

Fig. 2. Comparative summed-molecule TIRF (A) and PALM (B) images of the same region within a cryo-prepared thin section from a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede

Fig. 2. Comparative summed-molecule TIRF (**A**) and PALM (**B**) images of the same region within a cryo-prepared thin section from a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede. The larger boxed region in (B), when viewed at higher magnification (**C**) reveals smaller associated membranes that may represent interacting lysosomes or late endosomes that are not resolvable by TIRF. In a region where the section is nearly orthogonal to the lysosomal membrane, the most highly localized molecules fall on a line of width 10 nm (inset). In an obliquely cut region [(**D**), from the smaller boxed region in (B)], the distribution of CD63 within the membrane plane can be discerned.



E. Betzig et al., Science 313, 1642 -1645 (2006)