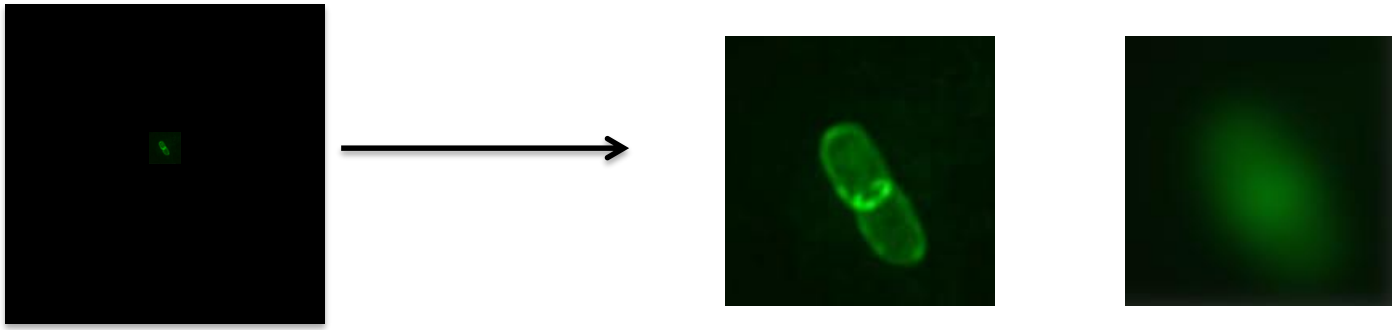
Week 5

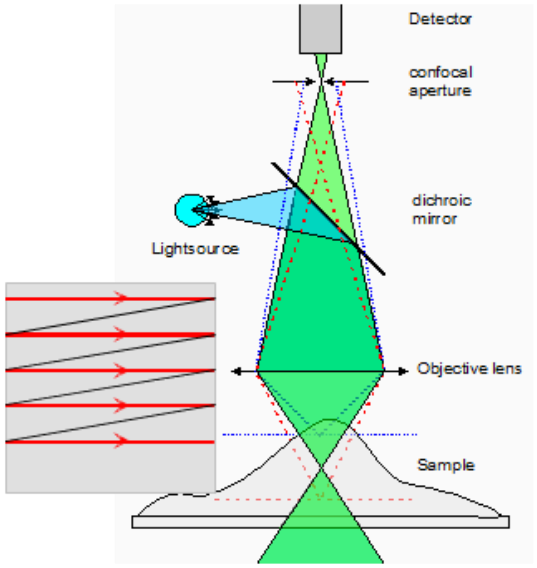
High Resolution Fluorescence Microscopy



Diffraction limited high resolution microscope Methods

1. Laser scanning confocal
2. Spinning disc confocal
3. Multi-photon confocal microscopy

Point Scanning Confocal



Spinning Disk Confocal

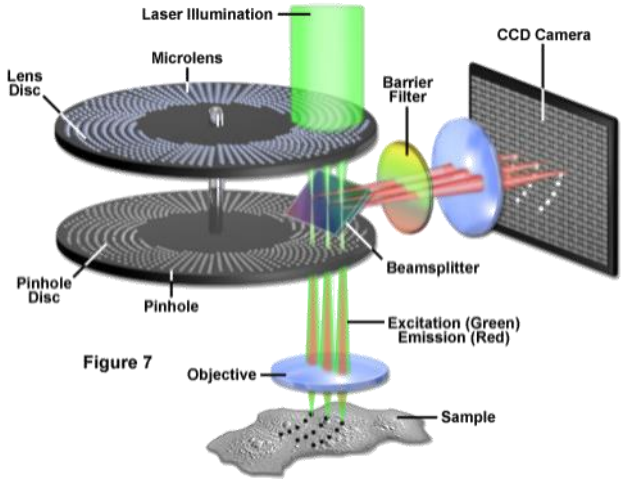
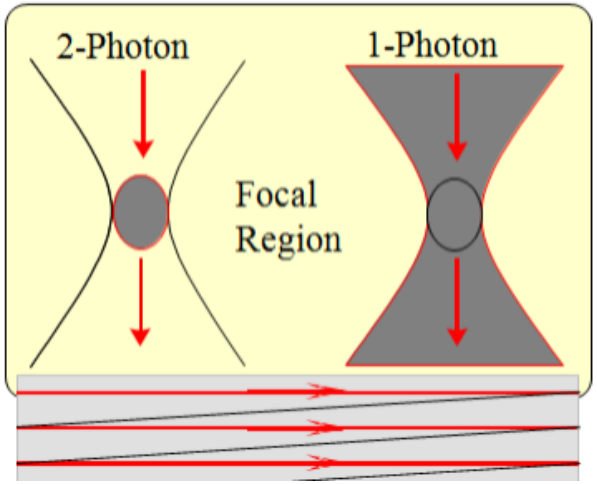


Figure 7

Multiphoton

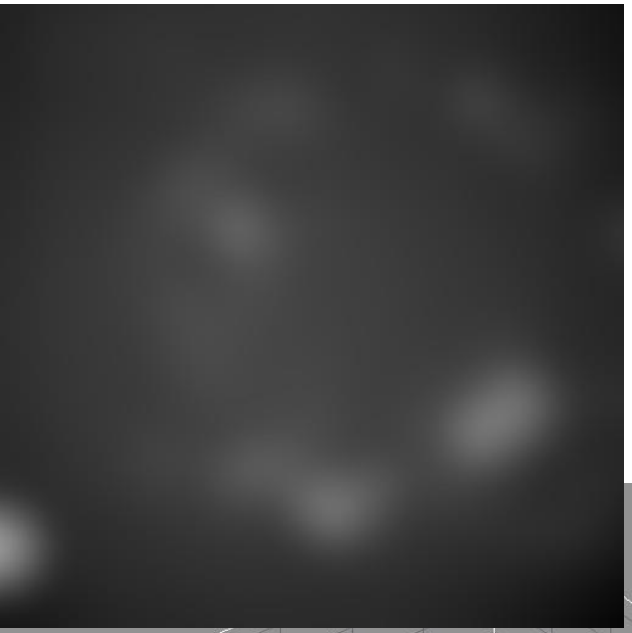


What is the main limitation for all microscope techniques?

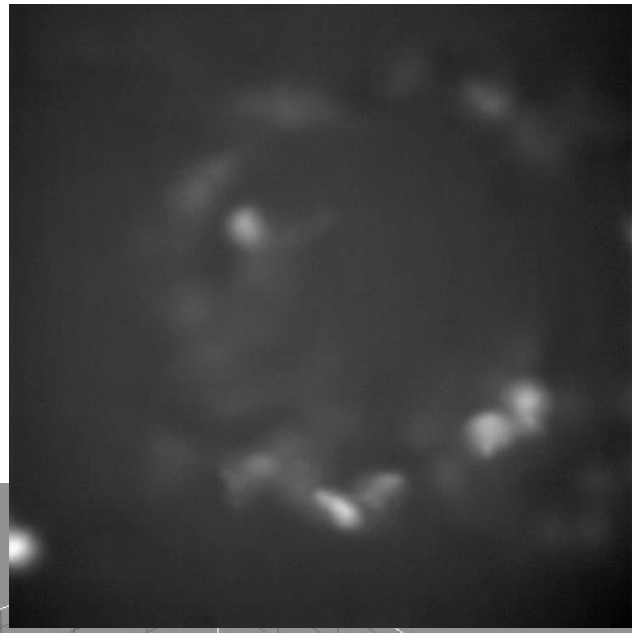
Can you define the problem? Is this an important problem?

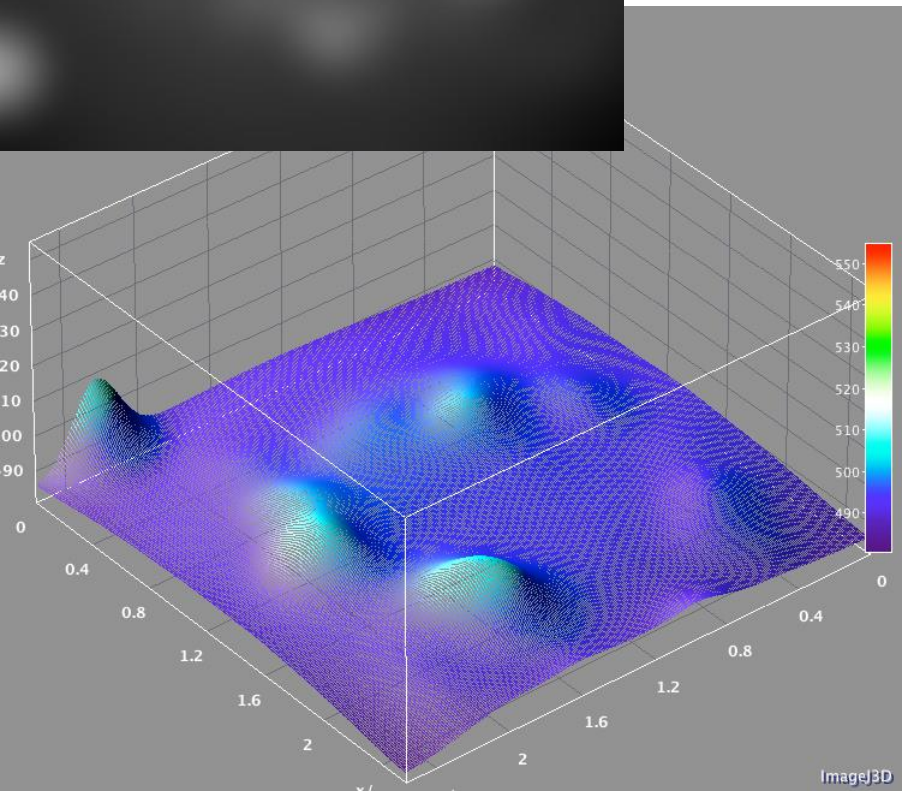
What cause the resolution problem? What is the limiting factor?
What are your solutions?
Is it possible to have a resolution beyond the diffraction limit?

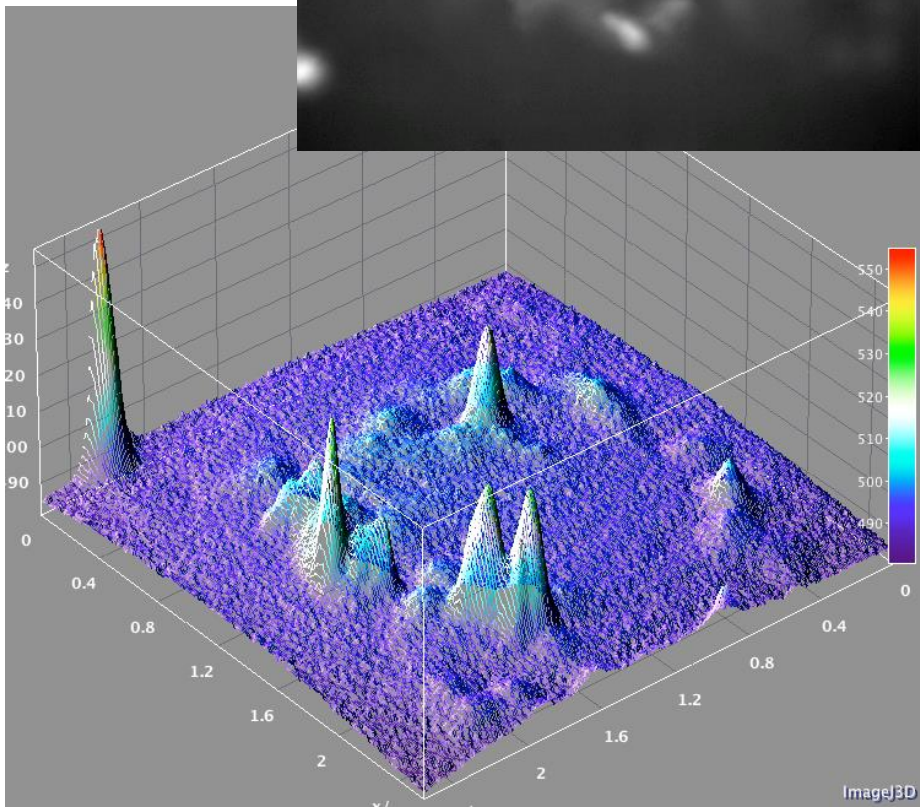
Point Spread Function



GFP expressing cells

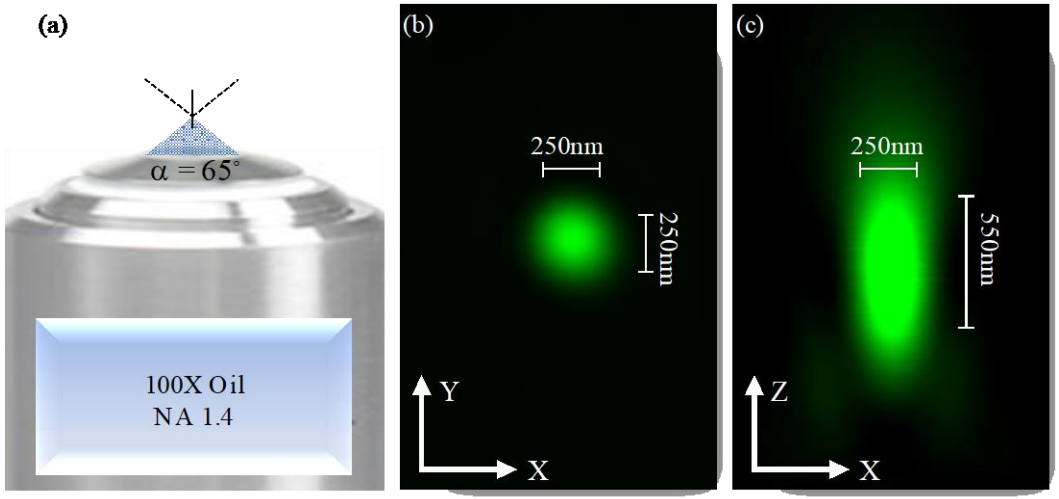






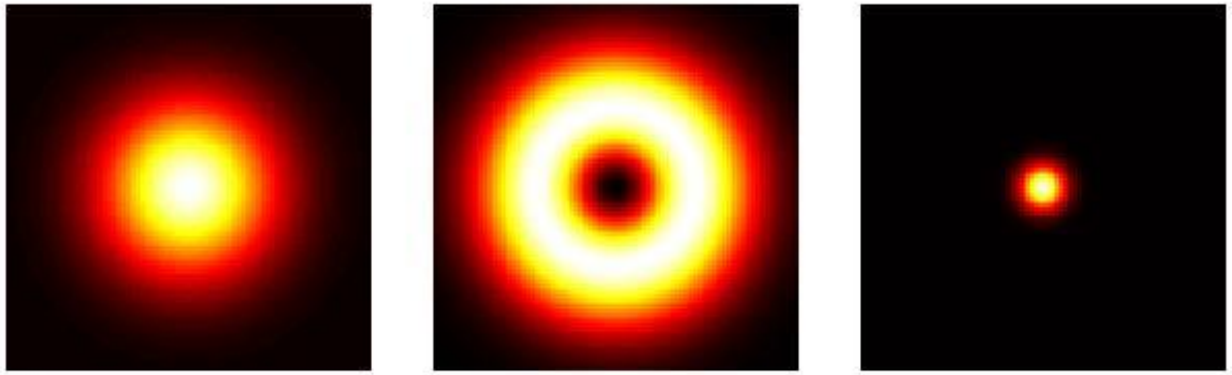
Super Resolution Microscopy

- STORM : Stochastic Optical Reconstruction Microscope
- PALM : Photo activated Localization Microscope
- STED: Stimulated Emission Depletion



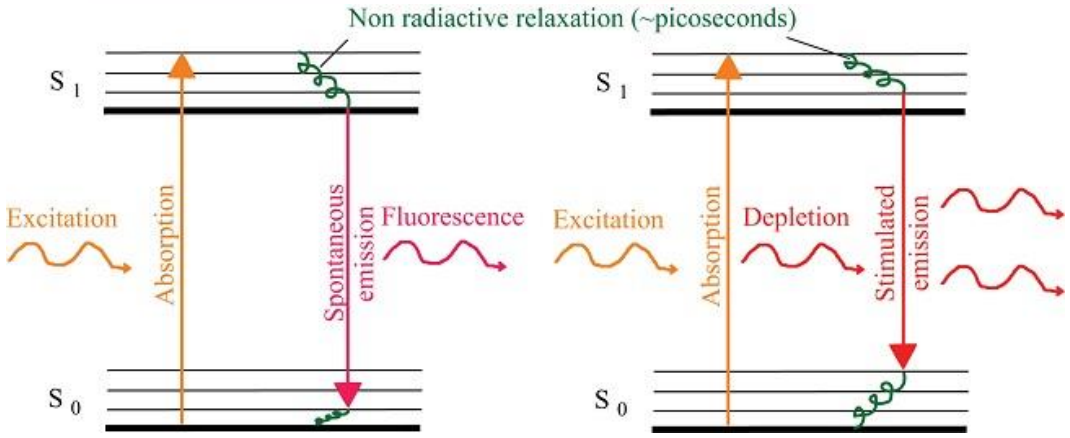
Can we reduce the size of the point spread function?
What other solutions are possible?

Stimulated Emission Depletion (STED) Fluorescence Microscope



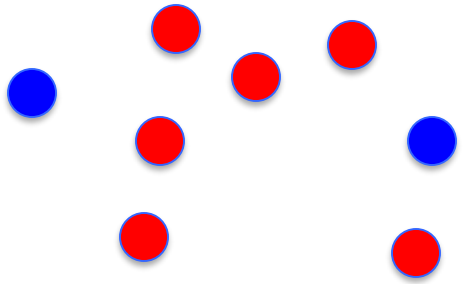
Stefan Hell

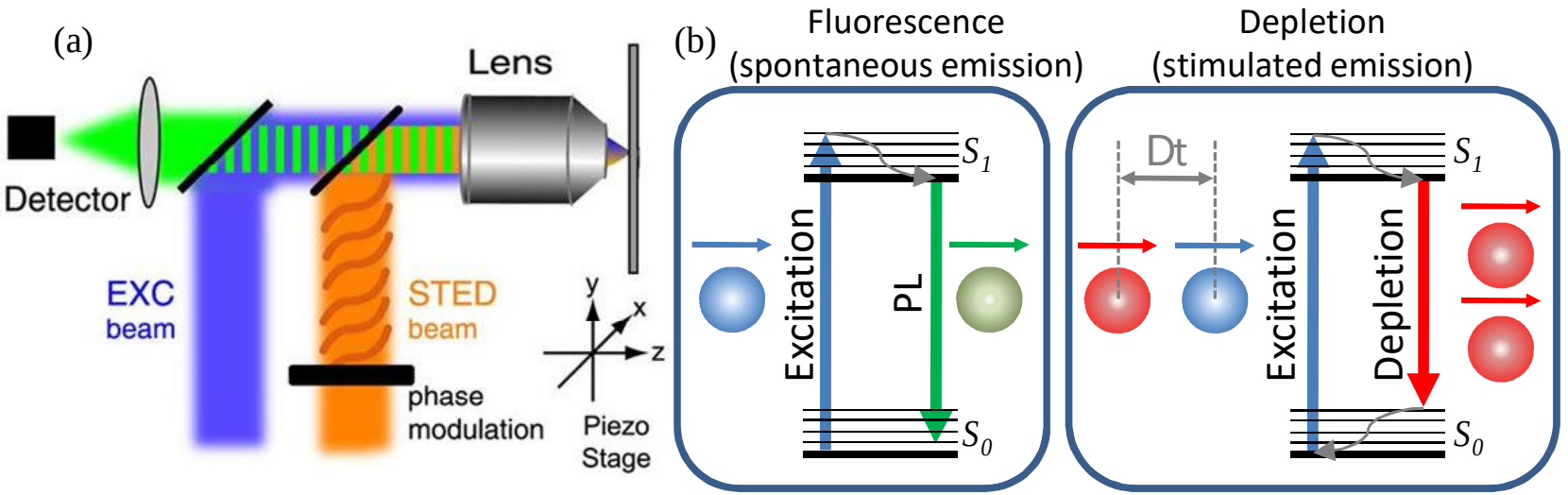
Max Planck Institute

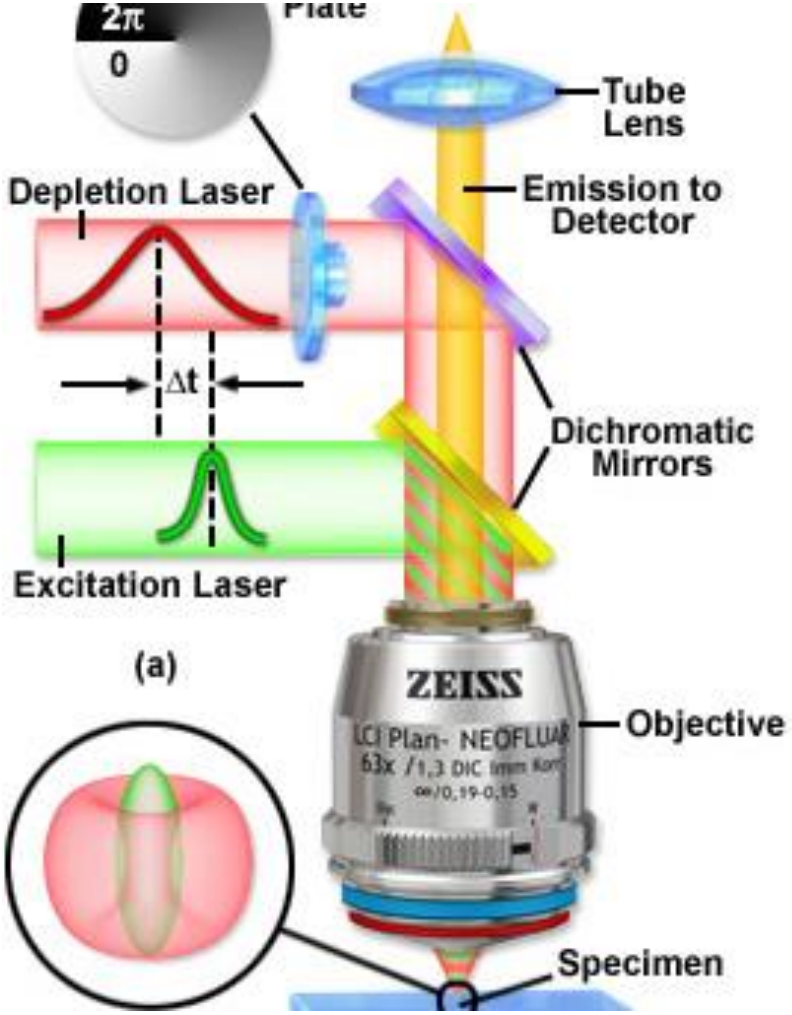


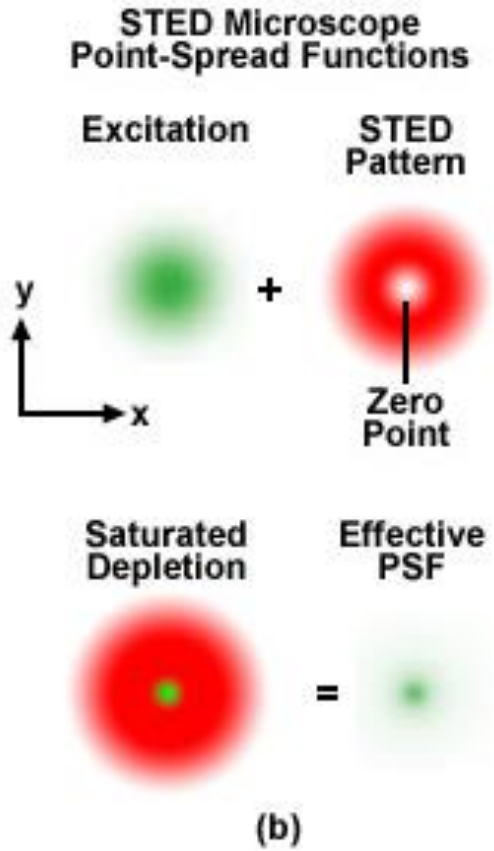
About **30-80nm lateral resolution** has been reported in various biological samples

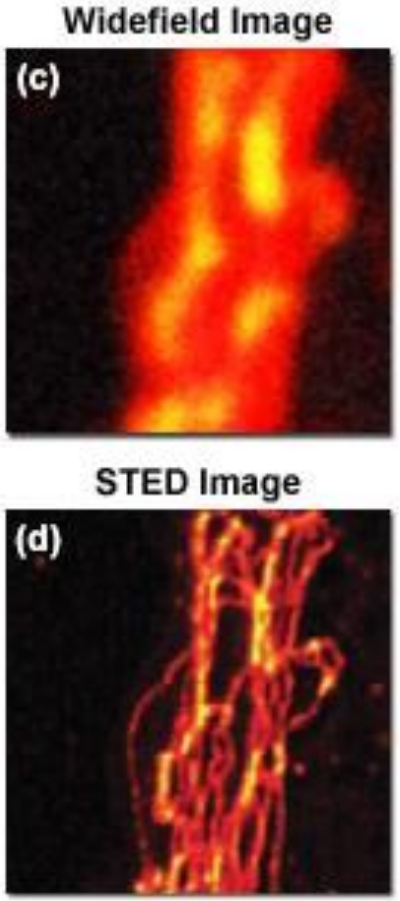
Many dye molecules are excited that reduce any good resolution. As the light of the different dye molecules can not be distinguished.



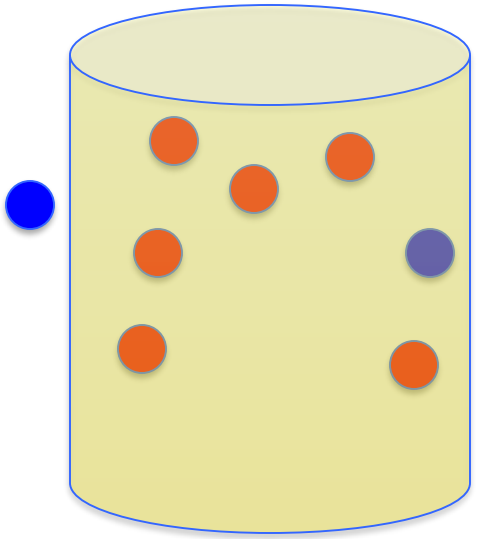



2π
0
Plate
Tube Lens
Depletion Laser
Emission to Detector
Δt
Dichromatic Mirrors
Excitation Laser
(a)
ZEISS
Objective
Specimen


STED Microscope
Point-Spread Functions
Excitation
STED Pattern
y
x
+
Zero Point
Saturated Depletion
Effective PSF
=
(b)

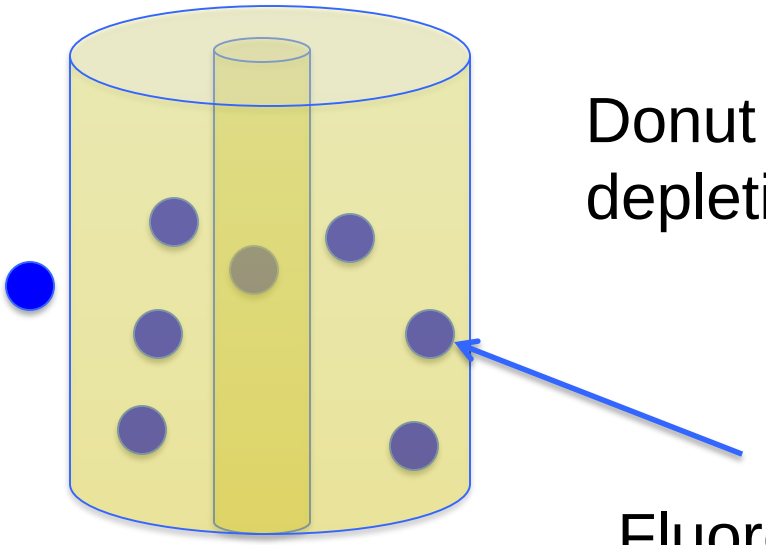

Widefield Image
(c)
STED Image
(d)

Many dye molecules are excited that reduce any good resolution. As the light of the different dye molecules can not be distinguished.



Many molecules are excited by a laser spot.

Laser beam deexcite the molecules by stimulated emission.

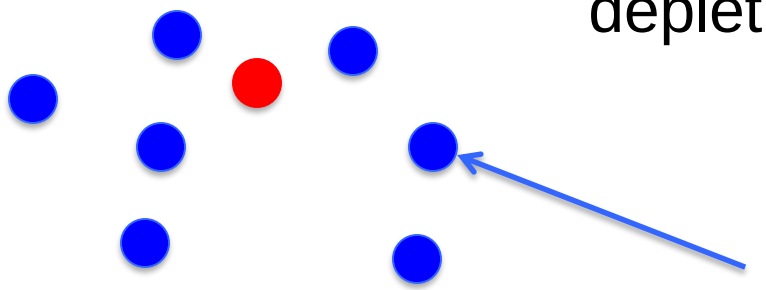


Donut shape depletion beam

Fluorescence of these molecules are shifted

Only molecule at the center of donut is excited and emit a photon. The others are depleted.

Laser beam deexcite the molecules by stimulated emission.



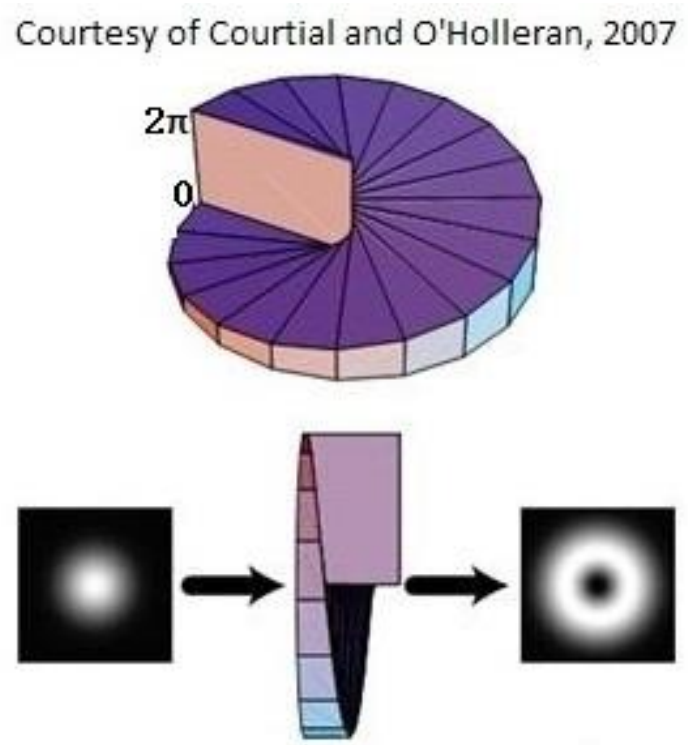
Donut shape depletion beam

Fluorescence of these molecules are shifted

Only molecule at the center of donut is excited and emit a photon. The others are depleted.

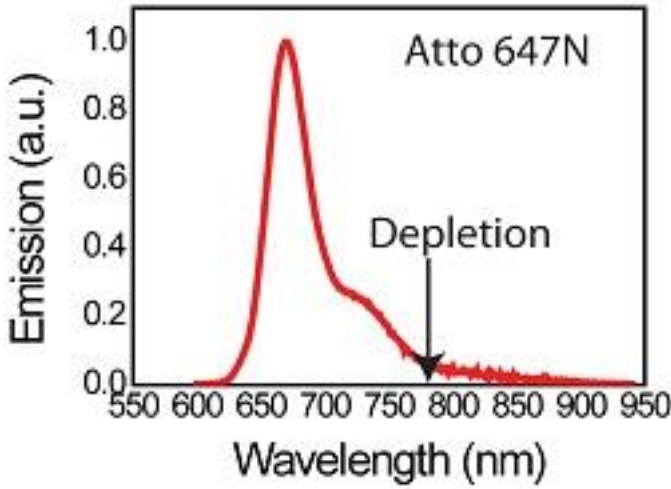
How to make a hole at the center of the beam?

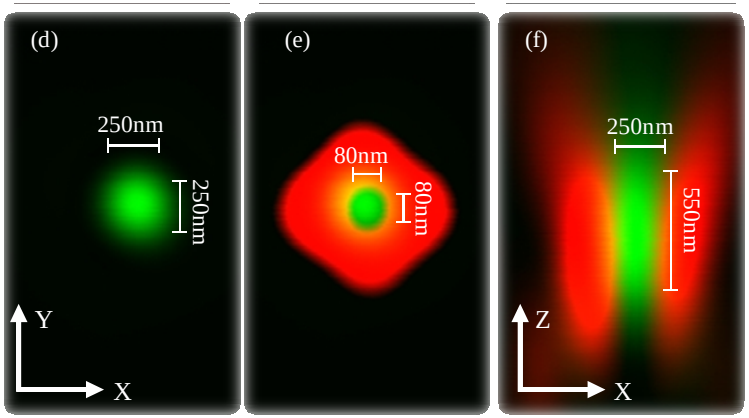
- Optical vortex method can be used to make a twisted light with a center in the hole. Phase plate can be used to make optical vortex.
- Optical vortex has a zero of an optical field.

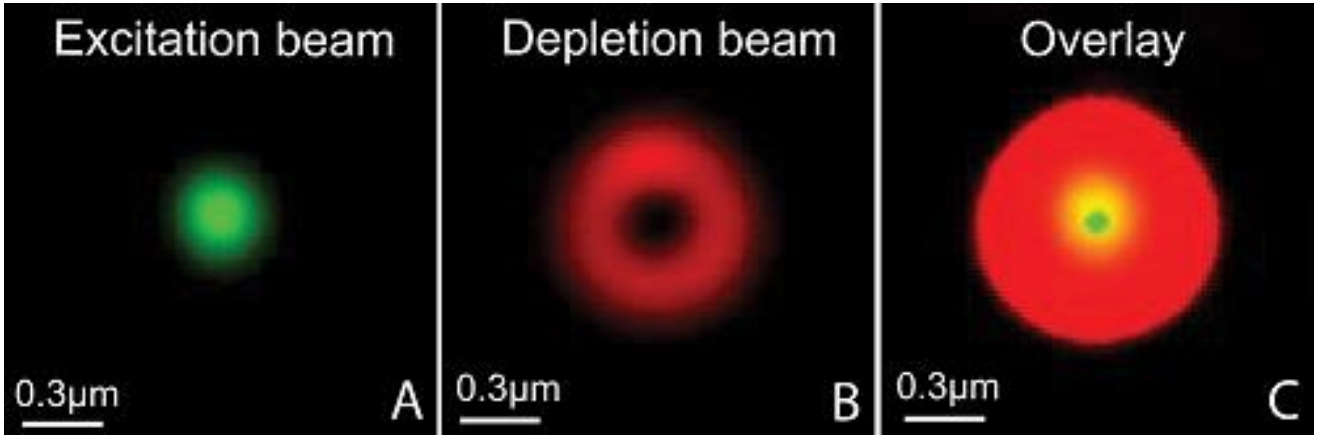


Wavelength of emission can be controlled by stimulated emission

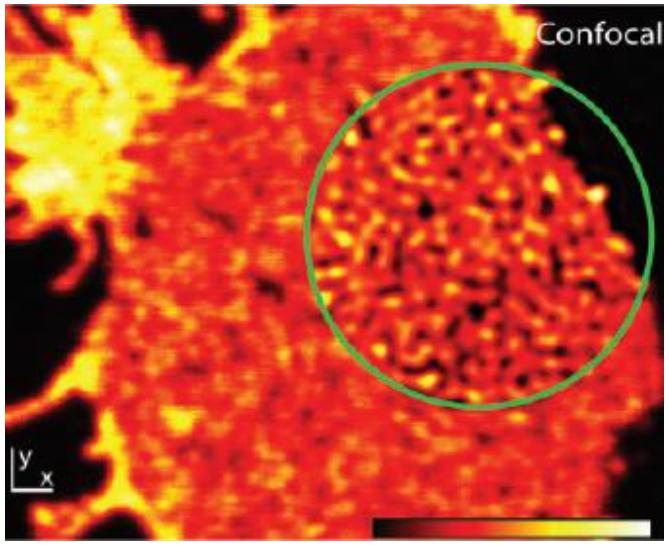
Stimulated emission wavelength is different than the wavelength of fluorescence.

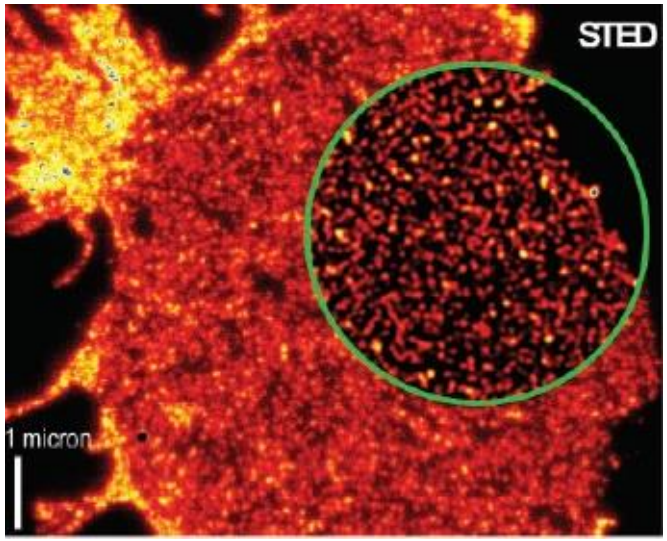






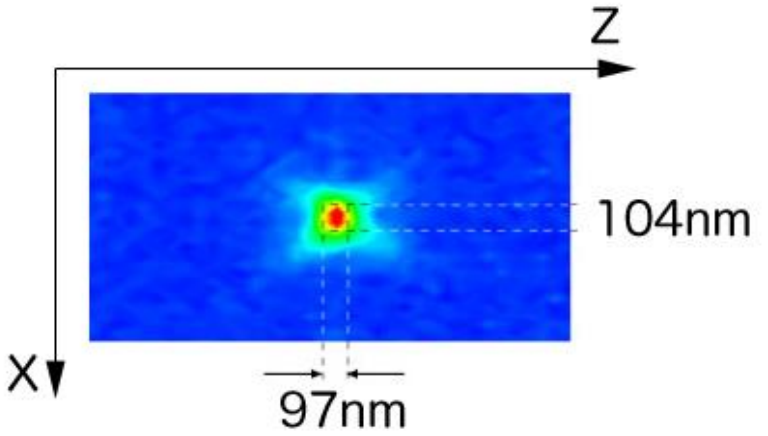
STED vs Confocal Microscope

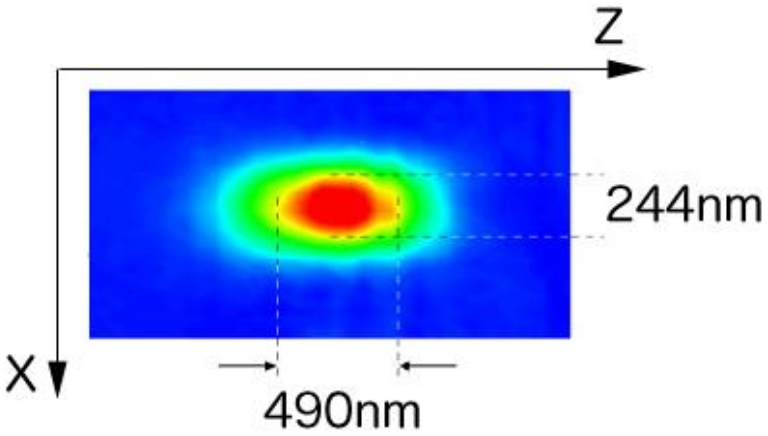




STED Resolution

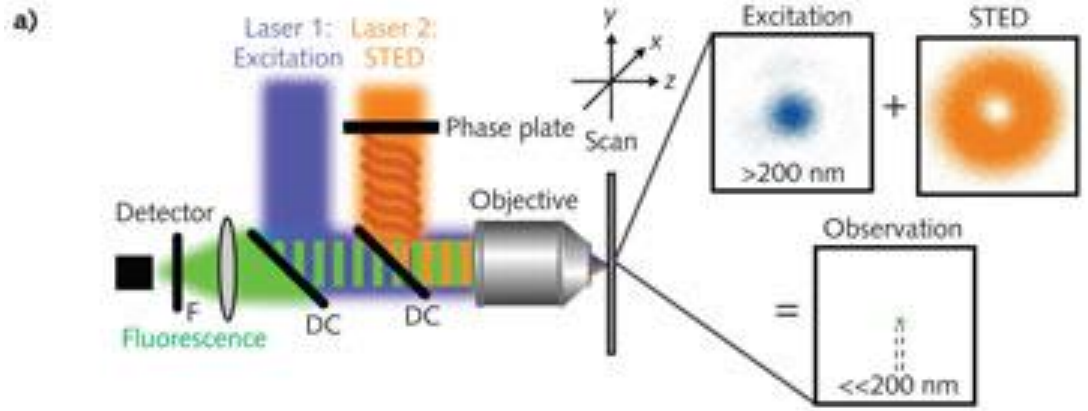
- STED PSF : 97nm resolution in Z and 104nm in XY
- Confocal PSF : 490nm resolution in Z and 244nm in XY

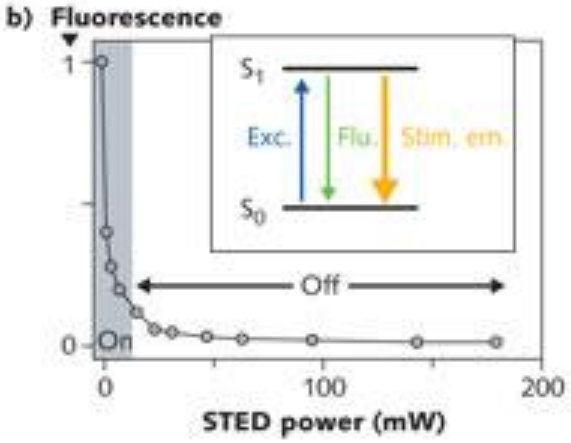


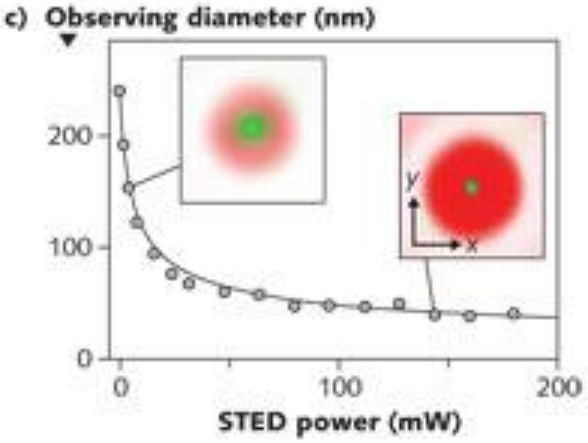


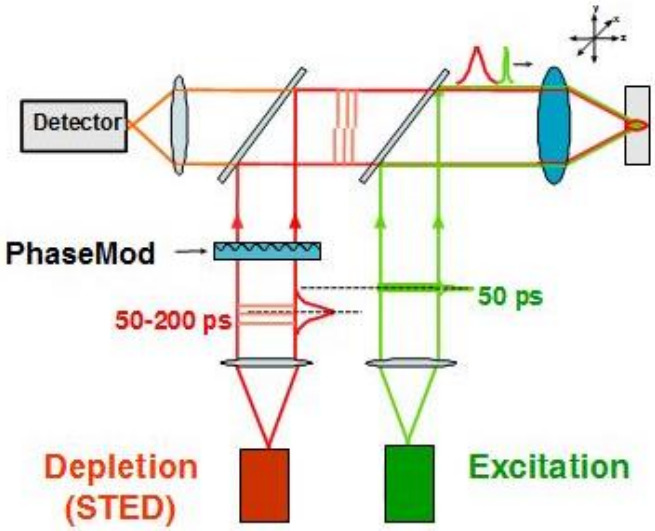
$$\Delta x = \frac{\lambda}{2 \cdot NA \cdot \sqrt{1 + \frac{I_{STED}}{I_{Sat}}}}$$

STED Setup









The power dependence delivers subdiffraction size observation volumes on the spot. The volume in which fluorescence emission is allowed (green, insets) but decreases with increasing STED laser power.

The resolution can be increased to 80nm by using fluorescence depletion to illuminate small spot to.

Advantage of STEM

Z resolution can be as low as 30 nm
No computation is required to construct image
High resolution (80nm) in 3D can be obtained

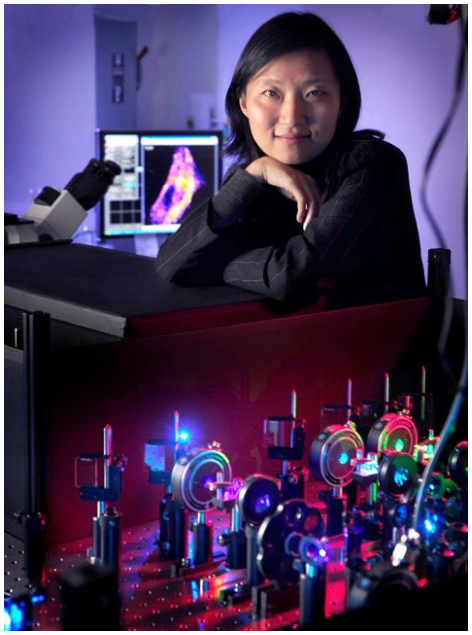
Disadvantage of STEM

Image with multiple dye is difficult
The system is very expensive
It takes a long time to capture image. A little slow for live imaging. But fast scanning resonant mirrors have been applied to STED microscopy to perform live imaging experiments.

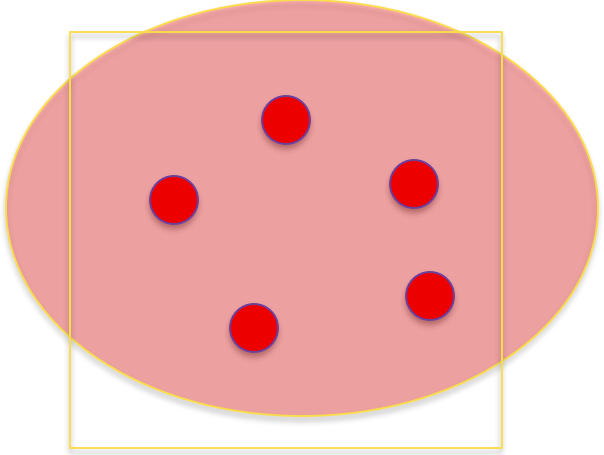
STORM MICROSCOPY

- The main principle of this imaging method is to figure out the position of the each fluorophore. Then, each spot is deconvoluted.

- The molecules are activated and imaged stochastically in the image plane.

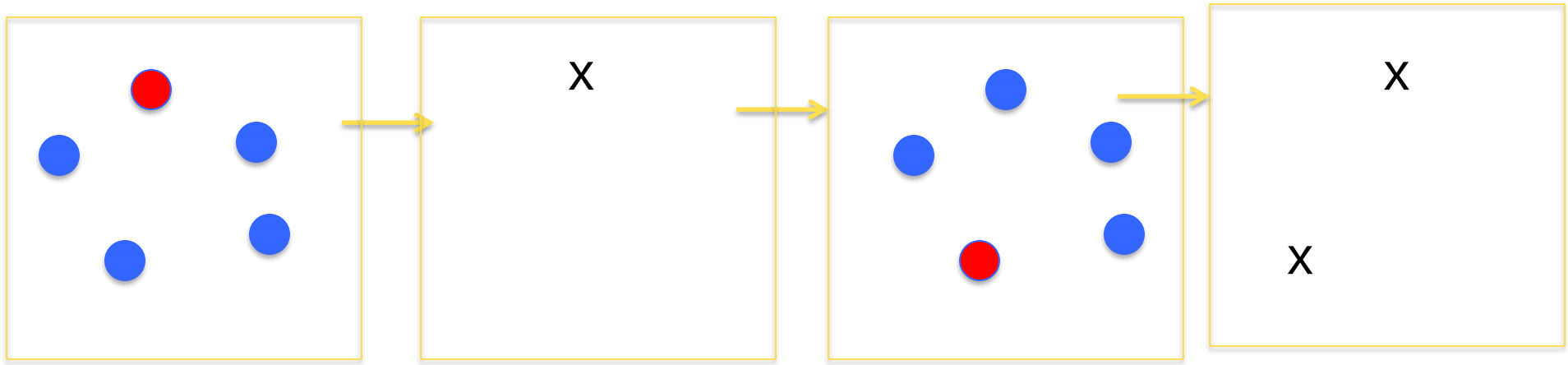


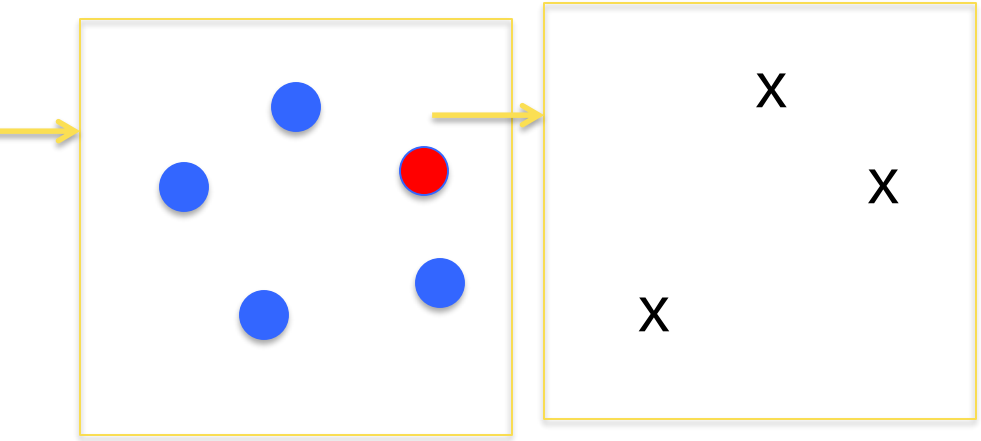
Harvard – Chemistry Department



Confocal Spot illuminates many fluorophore distance less than diffraction limit

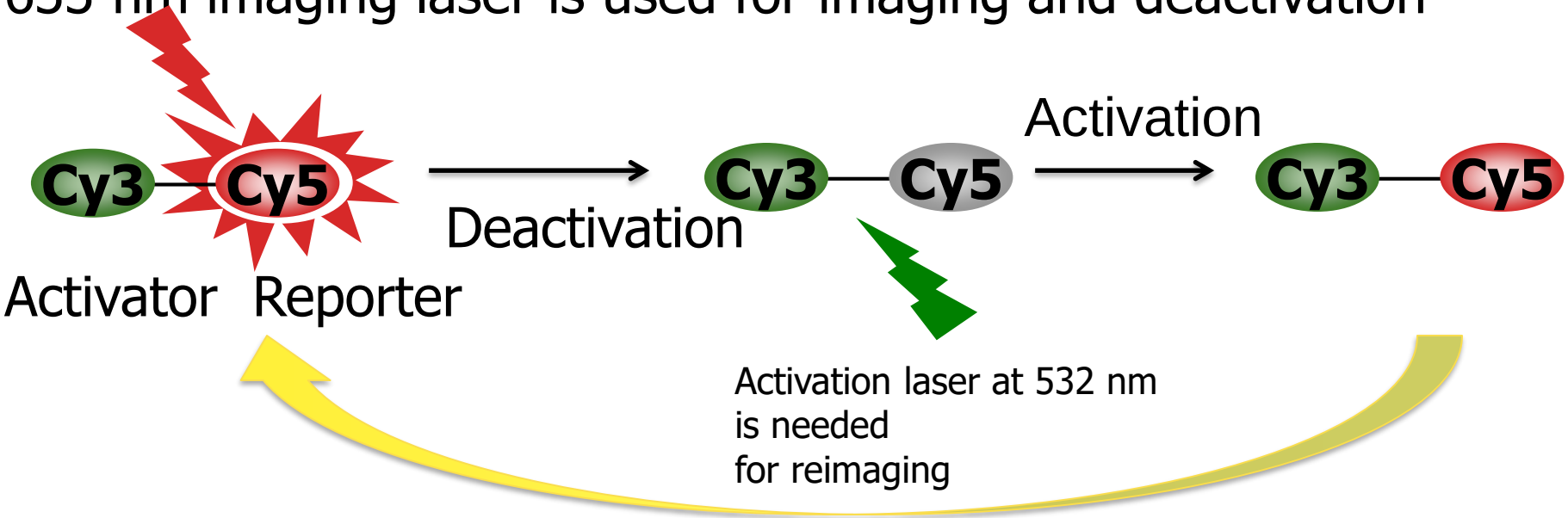
STORM IMAGING



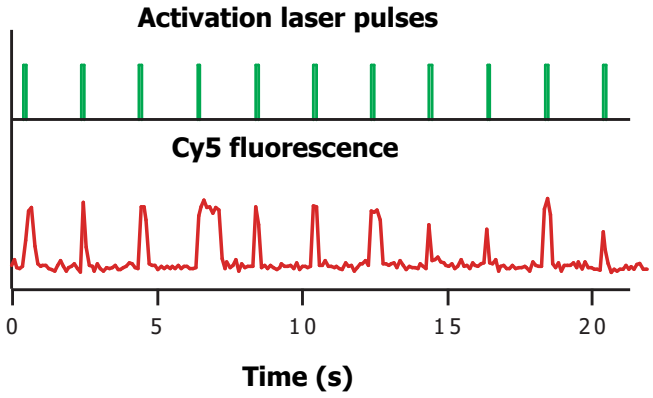


Fluorophore are imaged stochastically. Then, positions of fluorophore are determined

633 nm imaging laser is used for imaging and deactivation



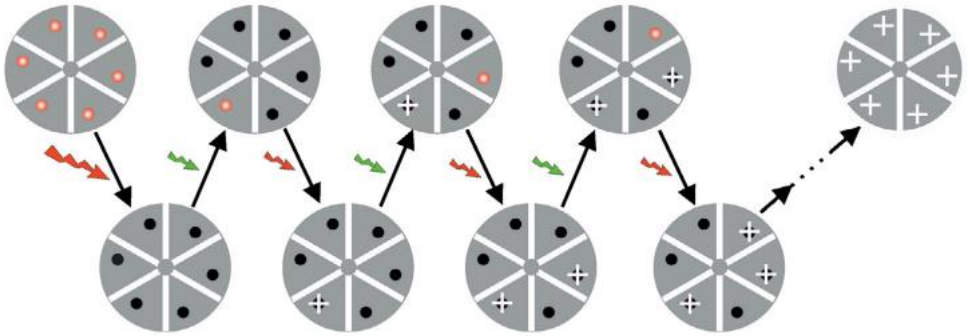
Cycled – many photons is needed



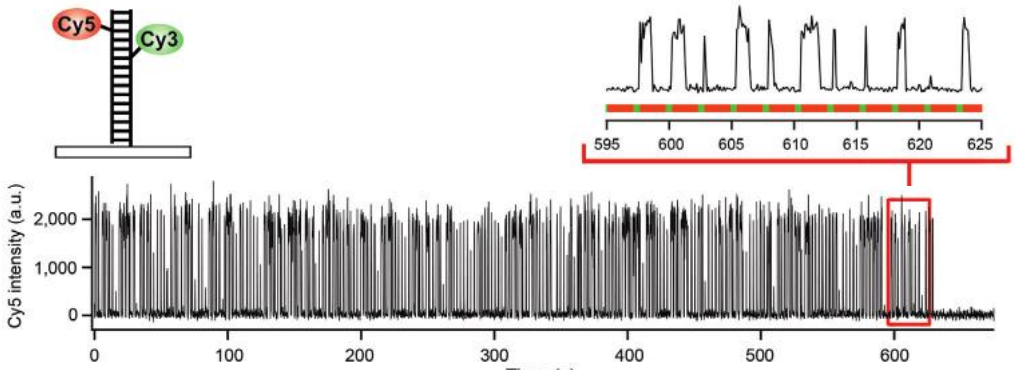
Green laser pulse is used to switch on only a fraction of the fluorophores to give an optically resolvable set of active fluorophores. A single Cy5 fluorescent dye can be turned on and off for more than 100 cycles before its permanently Photobleached.

Each point are imaged stochastically

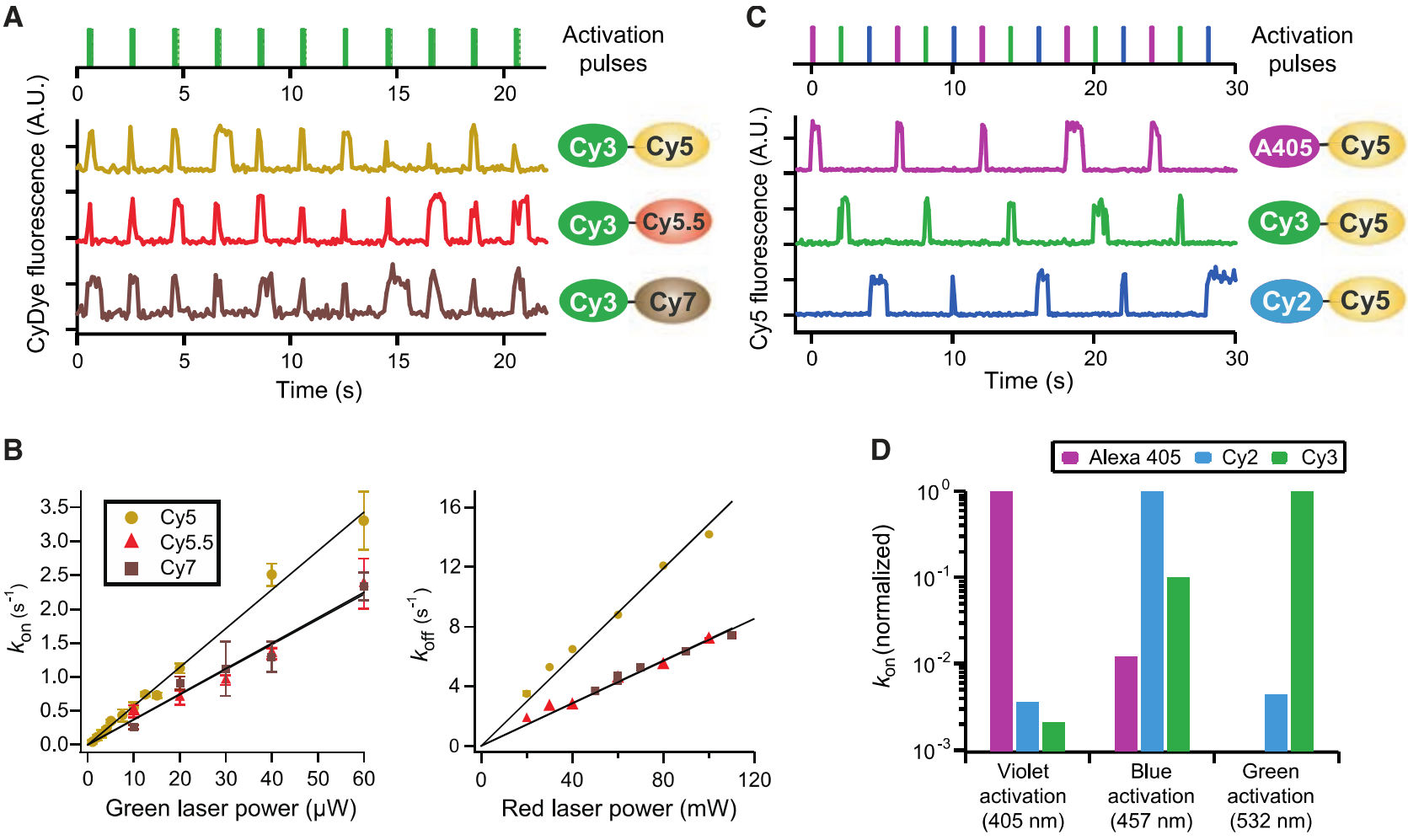
a



b



Multi color storm



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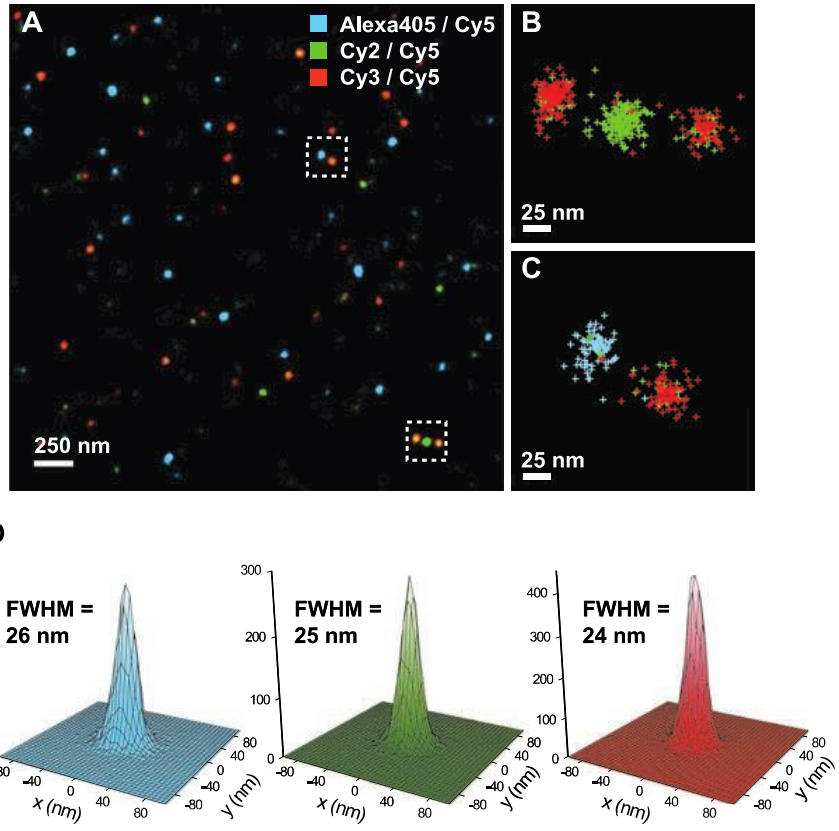
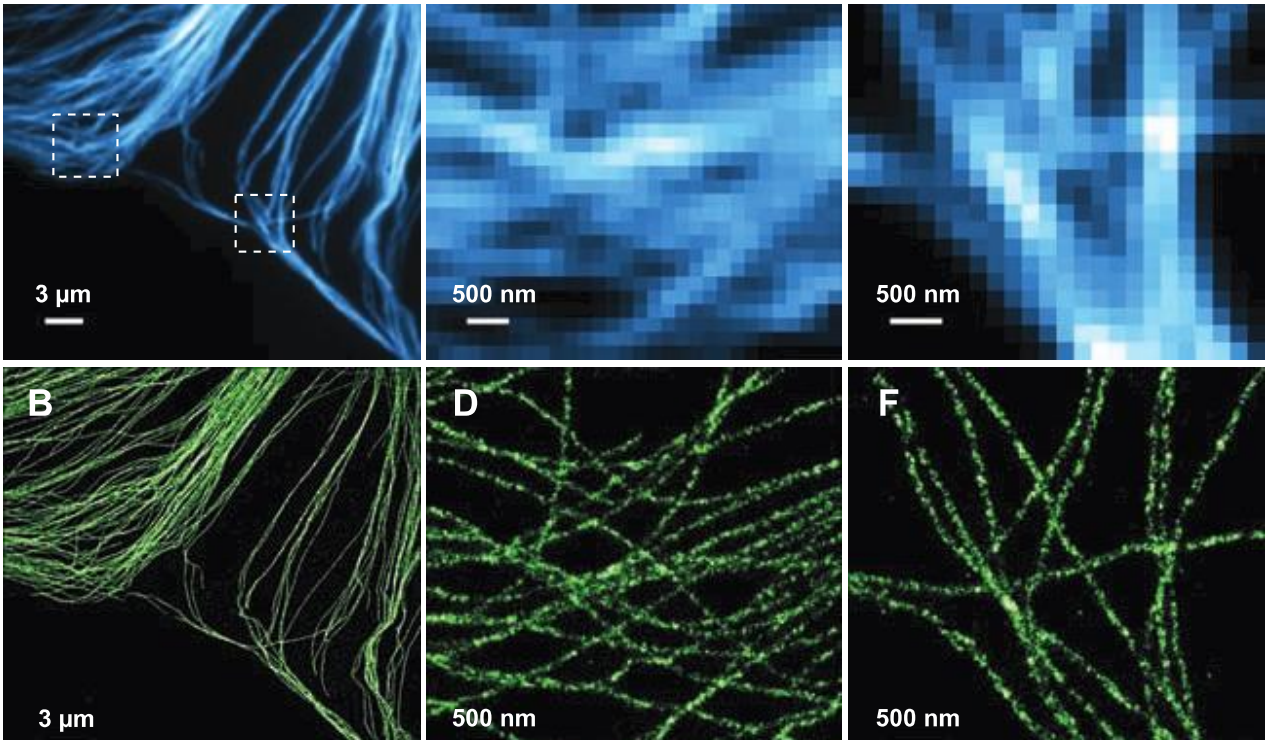
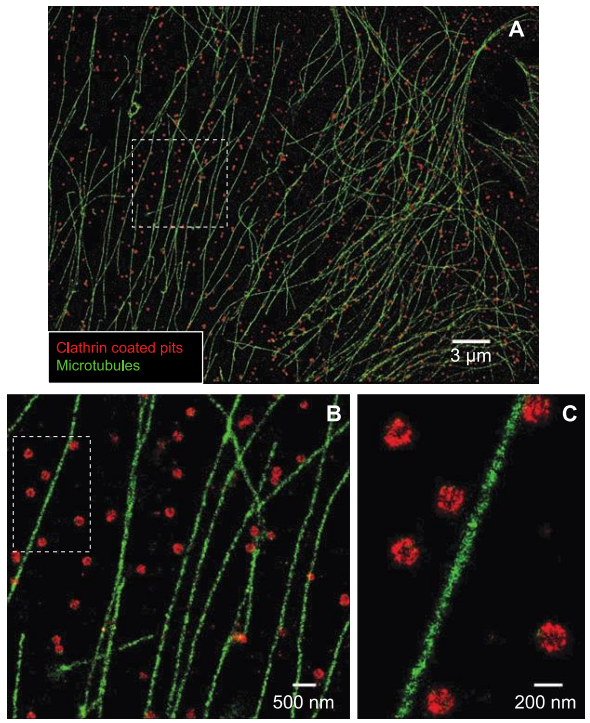
Multi color storm imaging



microtubules in a mammalian cell

Two-color STORM imaging of microtubules and CCPs in a mammalian cell.





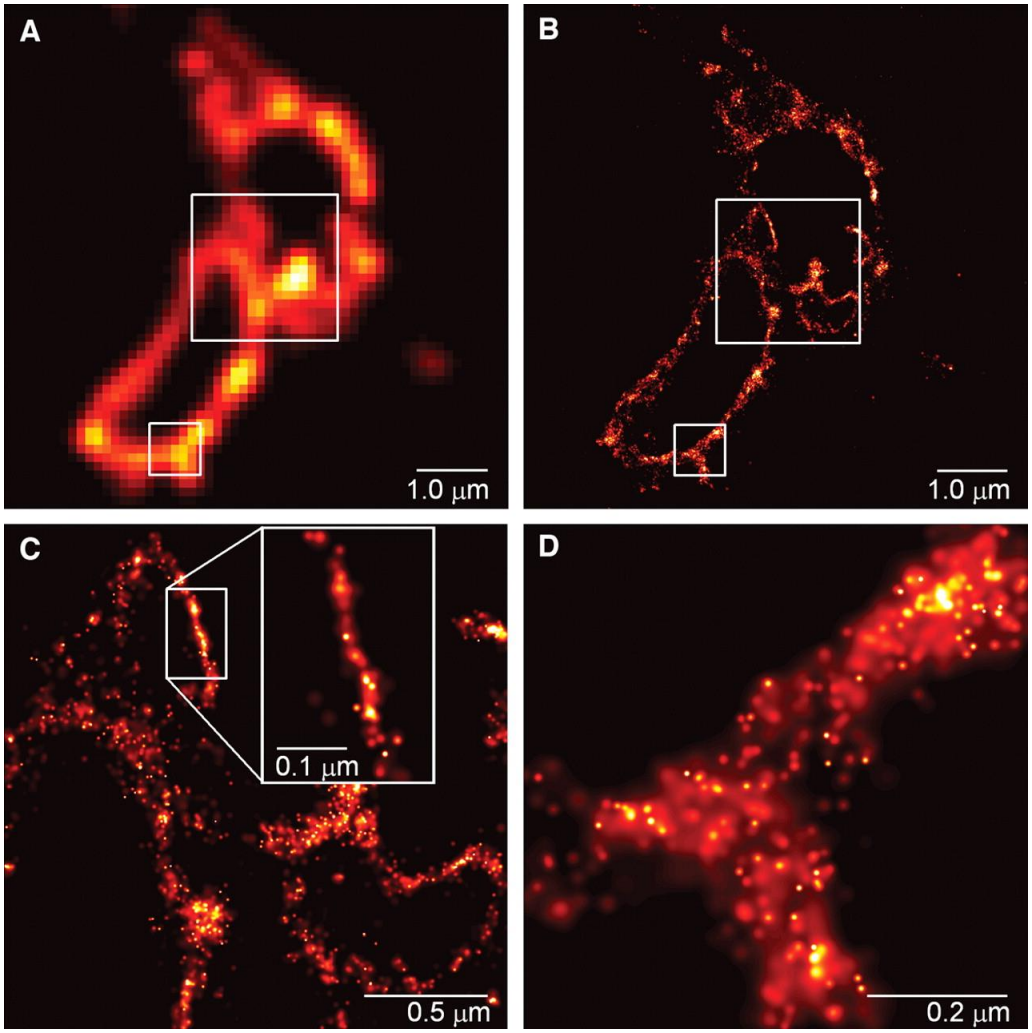
PALM

- one dye at a time can be activated, Then dye position is measured by PSF, you can separate two dyes which distance is less than optical resolution.

Jenalia Farm Research Center

Eriz Betzig, Jenalia FARM, Science **313,** 1642-5 (2006).

Fig. 2. 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

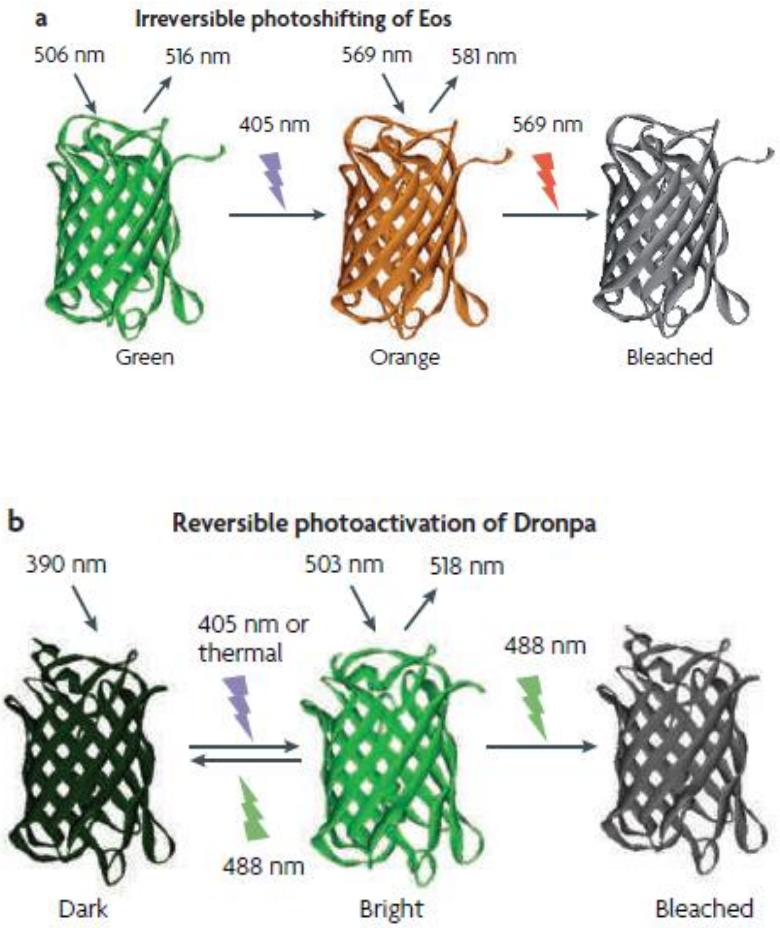


COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede.

Lysosomes can not be observed on TIRF check image A, compare with D.

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

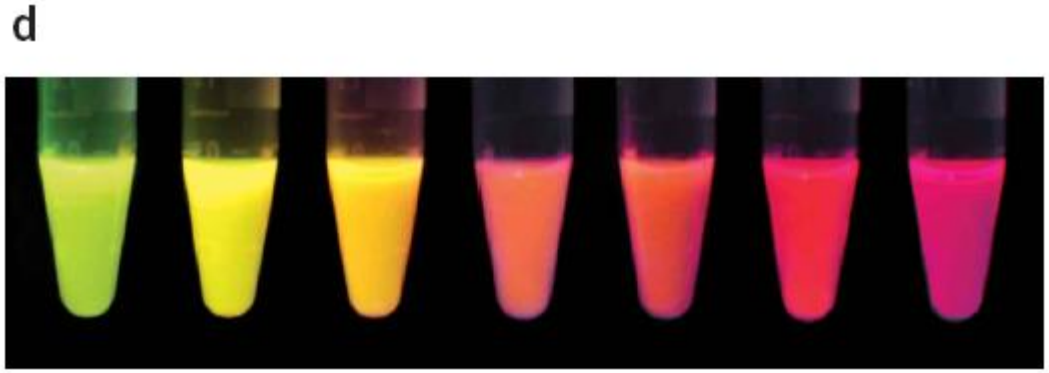
Activation in PALM



Different “GFPs”

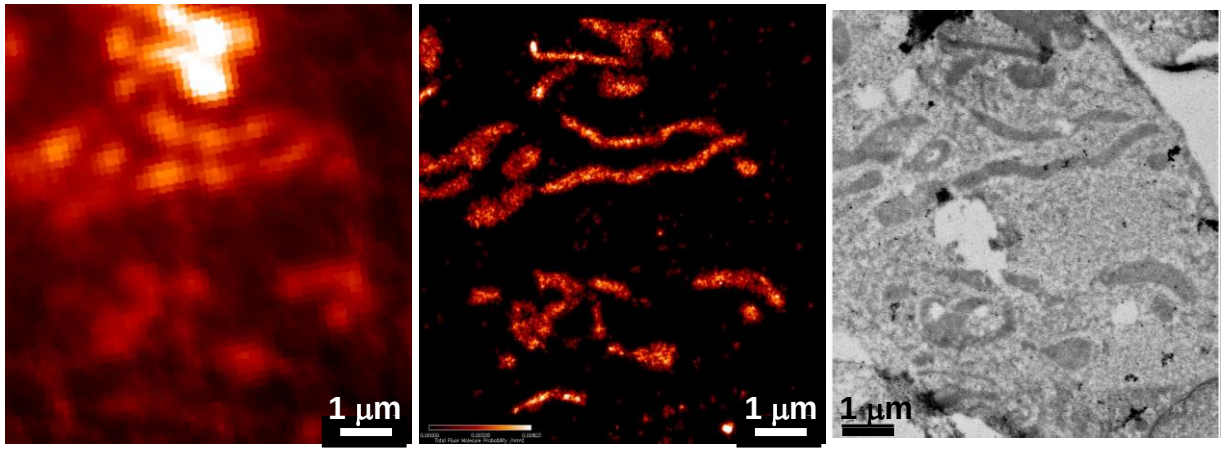
Absorption

Fluorescence



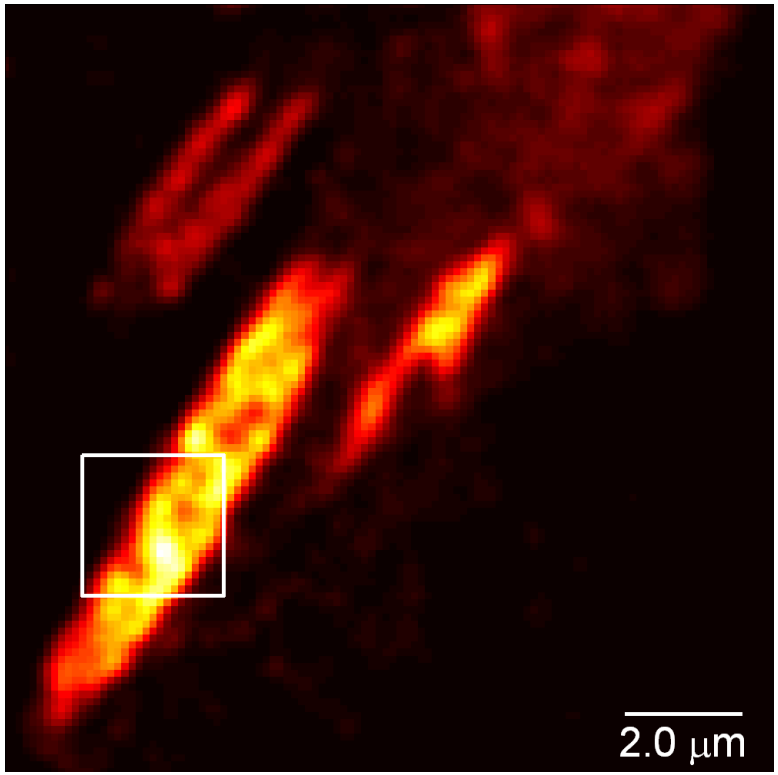
mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry

Shaner, Tsien,Nat. Bio., 2004

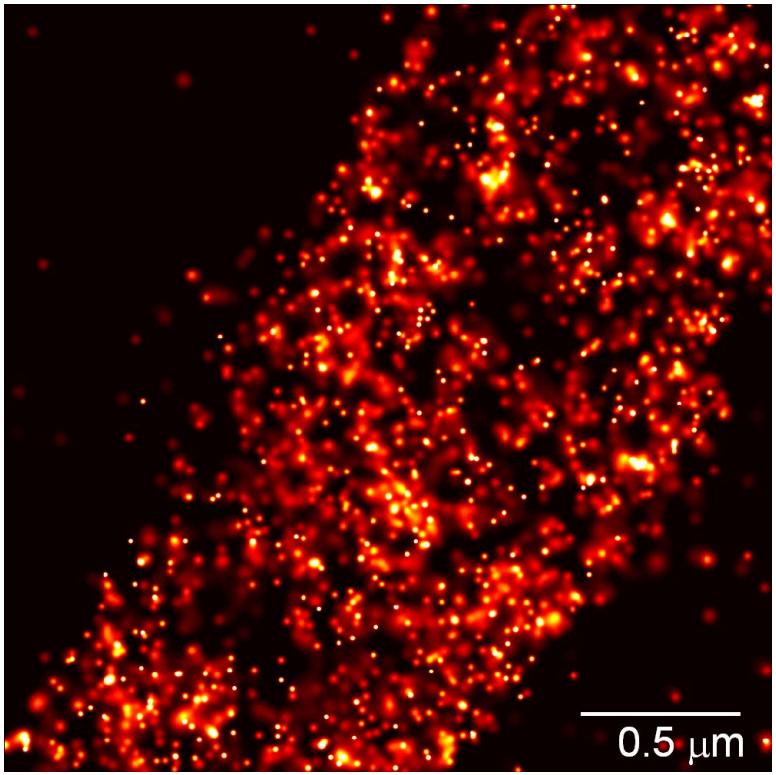


TIRF PALM TEM

Mitochondrial targeting sequence tagged with mEOS



TIRF image



Magnified PALM image

Vinculin-tagged dEosFP

Palm and Storm microscopy

Spatial resolution of both technique can be as small as 20 nm along the x-y plane. Z plane resolution can be as low as 60 nm.

Some disadvantages of STORM and PALM

- Long exposure is needed.
- The direct imaging is not possible. Image reconstruction and processing are required.
  - Different Analysis software can output different result.
  - Need to evaluate system performance.
- Image with multiple dyes as compare to confocal microscope is difficult.

  - Two Color PALM microscopy was demonstrated with EosFP and Dronpa fluorescent proteins.
  - STORM can be used easily as a multi color imaging compare to PALM.

Take home message

- There is no more resolution limits theoretically for far field microscopy.
- As you see that Abbes Law of limit for microscopy can be reformulated.
- STORM and PALM use a stochastic imaging method and intensive computing is required.
- STED method engineers the point spread function using depletion beam.