Announcements

Assignment due today.

Pick: 1) an article, 2) a review article,

You stand up there and want to convince people to listen to you.

What is big idea?

Why is it important?

What have they done that makes it a significant advance?

How have they done the significant advance.

What have they found out.

End with give a one sentence summary of the paper.

Homework Assigned Today; due next Wednesday.

March 10th : Mid-term Exam! (Dylan Reid Proctors)

March 12th : Yann Chemla– Optical Traps

March 27th & 28th (evenings) : Your presentations (?)

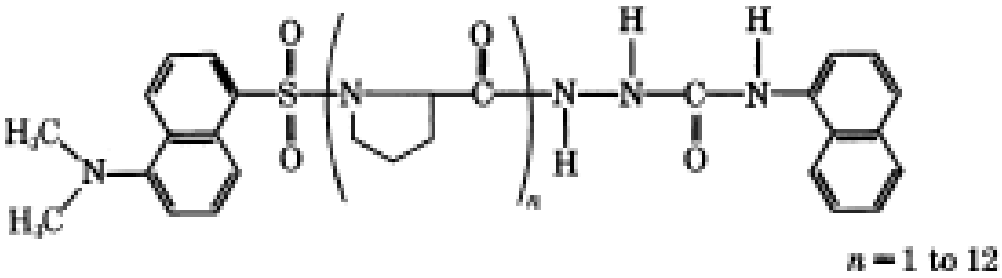
Example

1. Topic today is Science article by Yildiz.
2. They discovered how molecular motors move.
3. Important because virtually everything that needs to move around inside a cell, needs to be carried by molecular motors.
4. Leading choice was between a “hand-over-hand” motion and inchworm fashion.
5. Problem was this involved either a 74nm step, or an 37nm step, too small to see by ordinary diffraction limit.
6. Overcame it with FIONA, which was able to localize a fluorophore to 1.5 nm (in a ½ second).
7. They did this by locating the center of the fluorescence.
8. They applied this to myosin V and found that the stepping was a “hand-over-hand,” or “walking.”

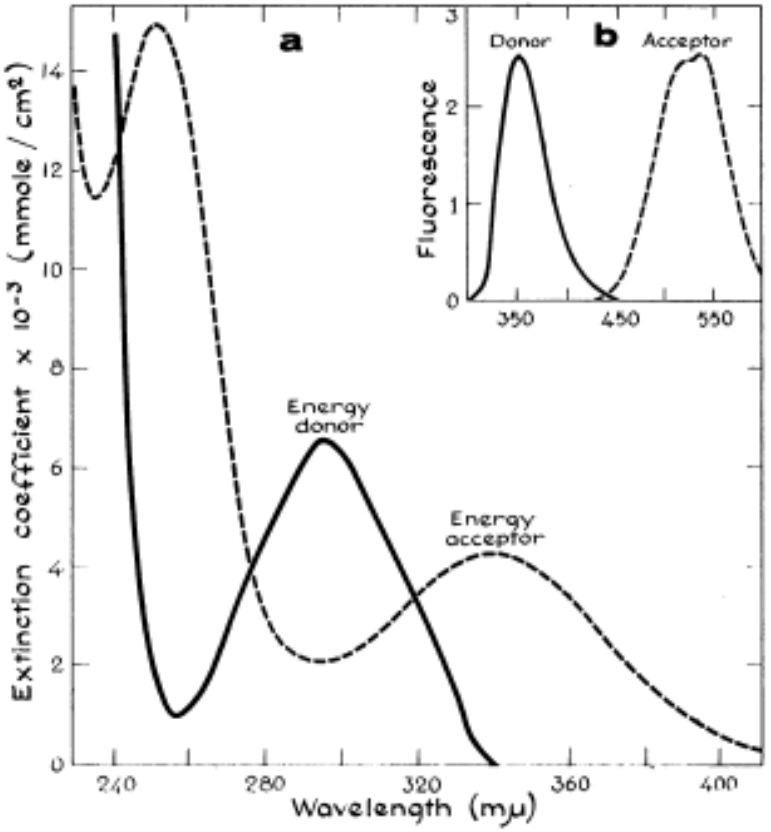
Example of Energy Transfer

Stryer & Haugland, PNAS, 1967

Donor Linker Acceptor



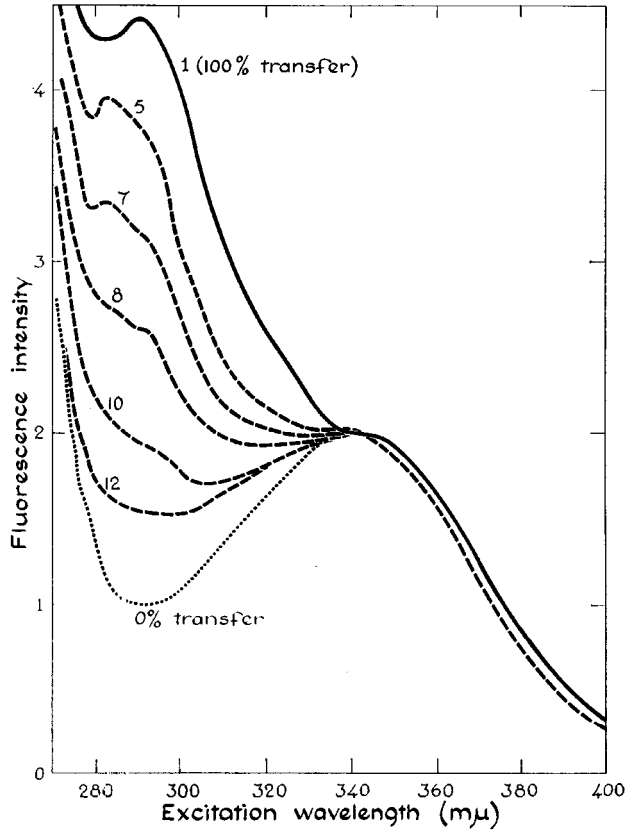
Spectral Overlap

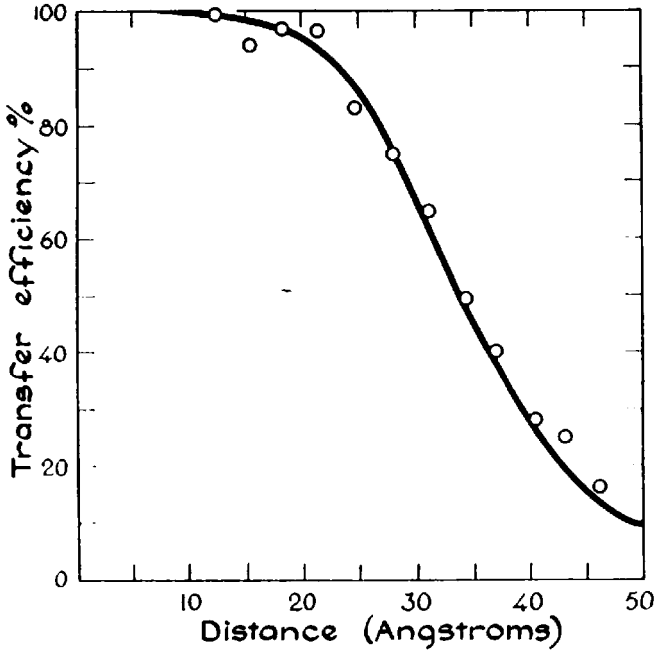


Energy Transfer

Excitation Spectrum of Acceptor = A = $\varepsilon_a + E\varepsilon_D$

(A different way)



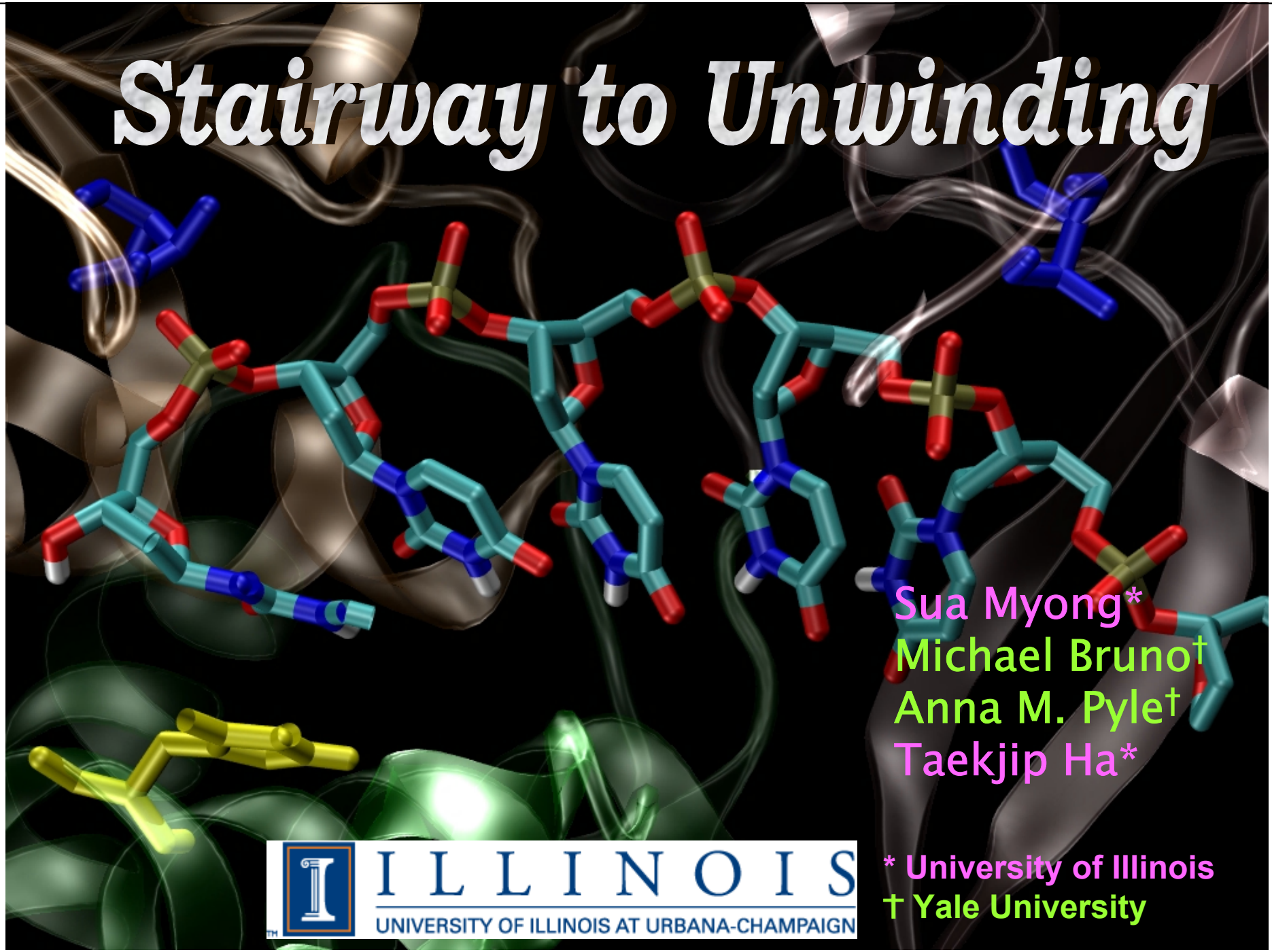


Excitation Spectrum: no E.T., exc. Spectra = absorption spectra of acceptor.

For 100% E.T., Exc. Spectrum = sum of absorption of two chromophores.

Stryer & Haugland, PNAS, 1967

Stairway to Unwinding



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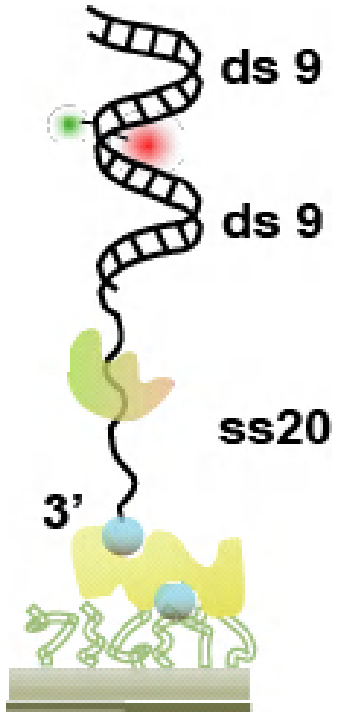
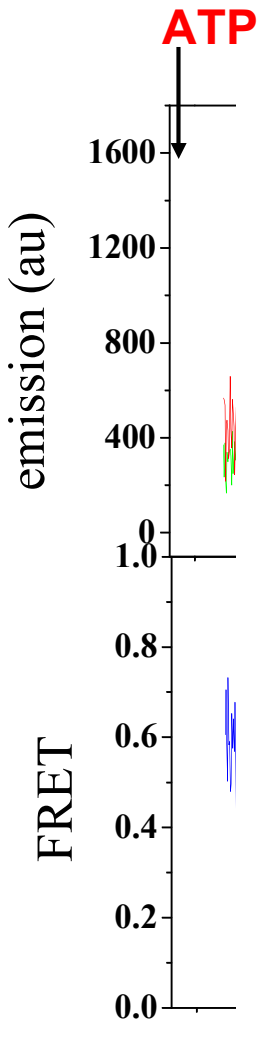
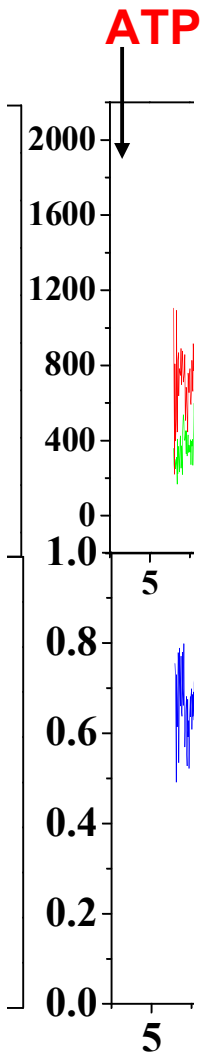
† Yale University

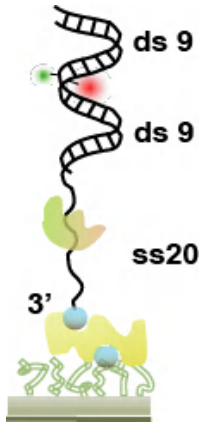
THREE steps in 9 bp unwinding

[NS3]=25nM
[ATP]=4mM
Temp = 32°C

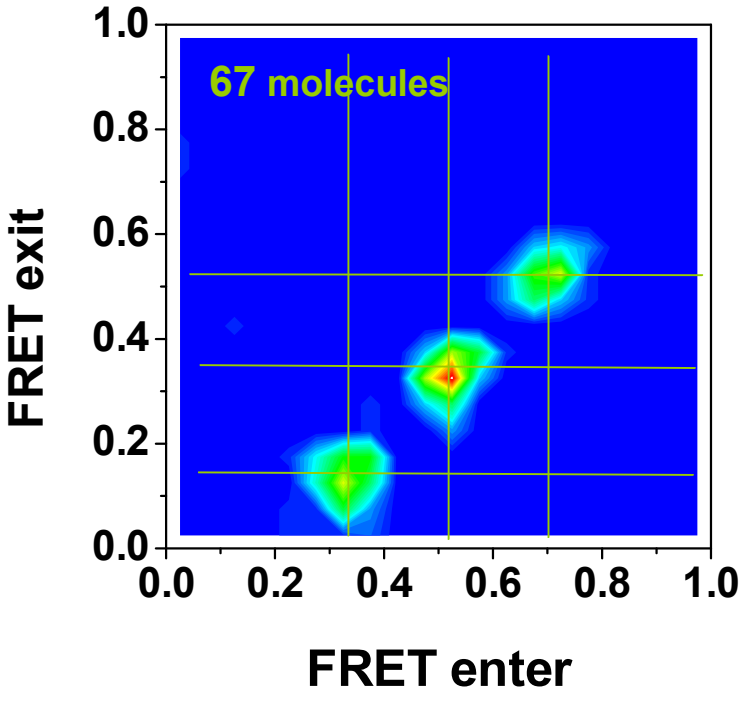


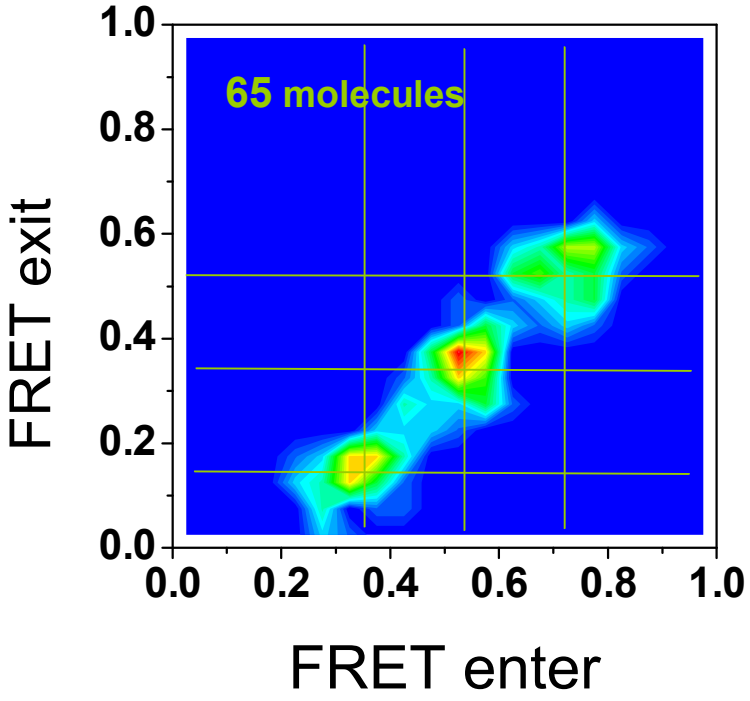






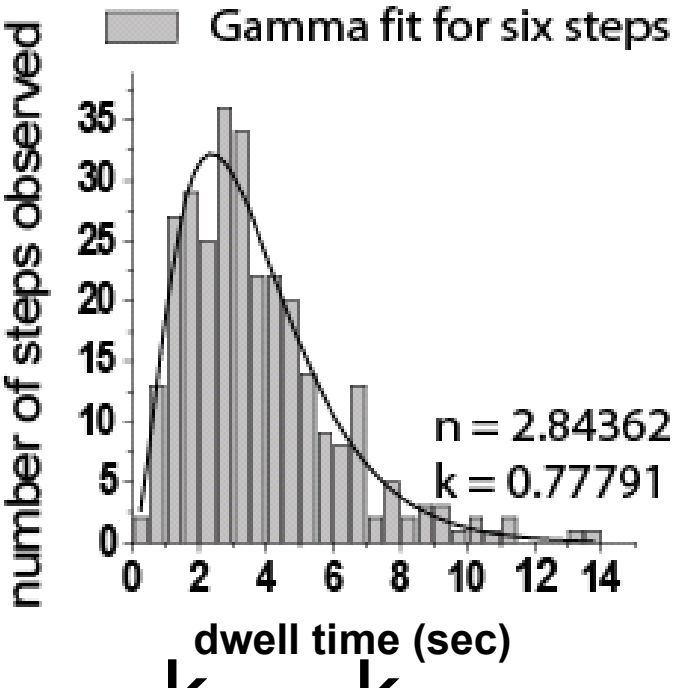
Transition Density Plot (3 steps)

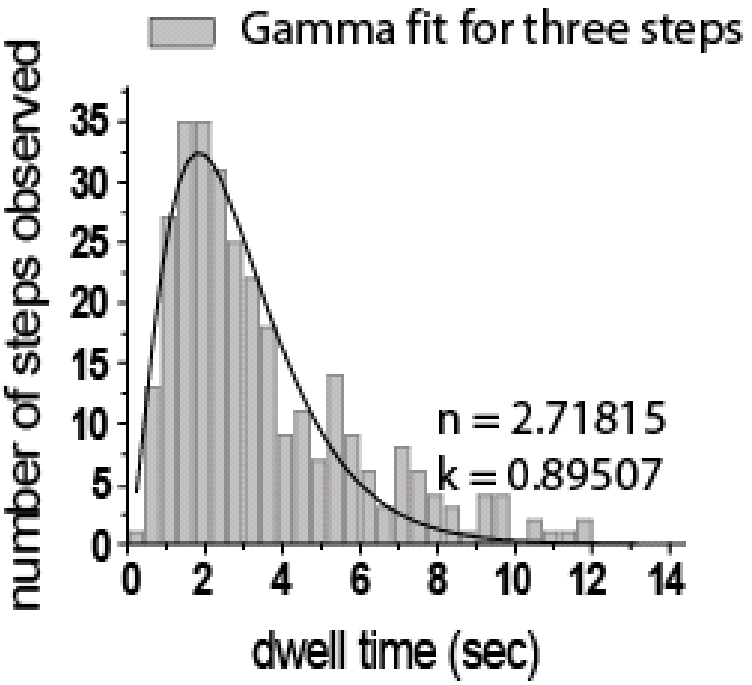


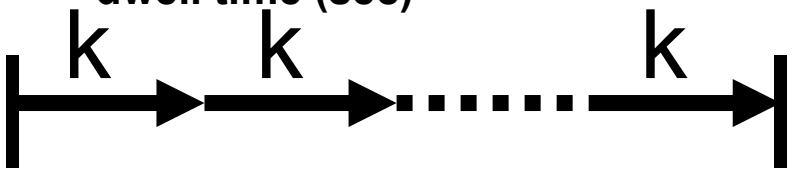


Non-exponential dwell time histograms

Smallest substep = Single basepair !







$$t^{n-1}e^{-kt}$$

n irreversible steps

A model for how NS3 moves

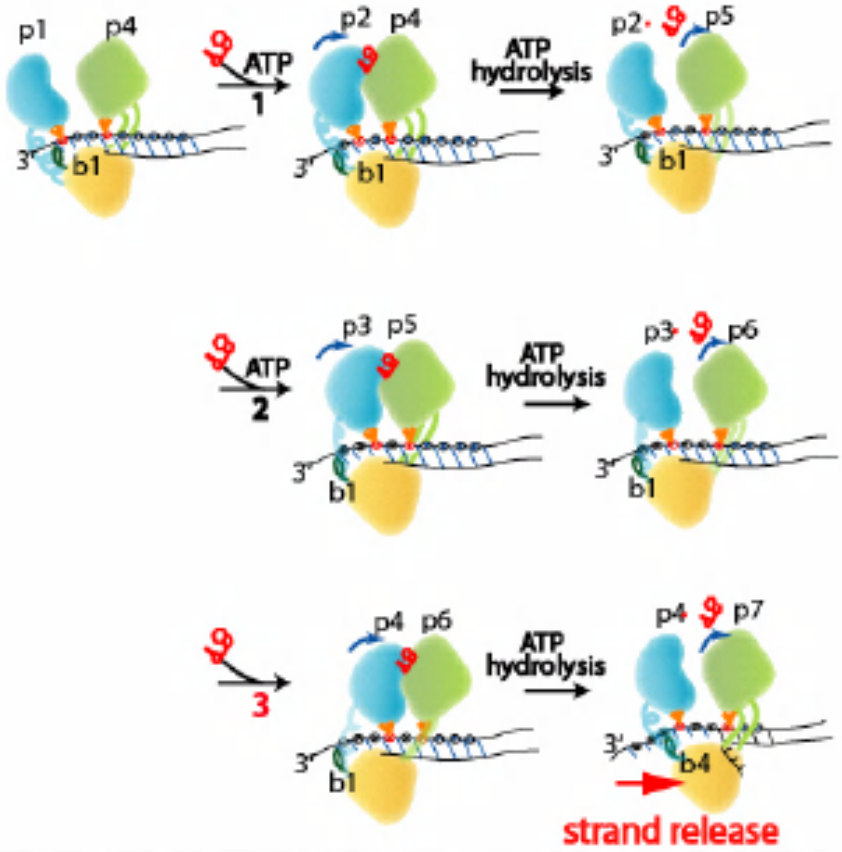


Fig. S9 Based on our data and the previously reported structure of NS3 we propose an unwinding mechanism involving three base pair step composed of three individual steps of one base pair. Domains 1 and 2 colored blue and green respectively, position themselves 3 nt away from each other via two threonine residues interacting with two phosphates reperesnted by two orange protrusions at protein-DNA interface. The two domains move in concerted manner by closing and opening as ATP binds and ADP is released thereby translocating one base toward 5' end (movement denoted by p1 p2 etc). Such single base translocation continues three times while domain 3 remains base stacked through tryptophan 501 (dark green hexagon) generating strain on itself. This spring-loaded domain 3 moves in a burst of 3 nt unzipping three base pairs as a result. The displaced strand gets loaded on domain 3 and maintains contact with the enzyme while it undergoes further unwinding, possibly forming a loop around it.

Why are threonine's involved?

What's the evidence for this?

Read original article!

Myong et al, Science,2007

A movie for how NS3 moves

We made a little movie out of our results and a bit of imagination. The two domains over the DNA move in inchworm manner, one base at a time per ATP while the domain below the DNA stays anchored to the DNA through its interaction, possibly the tryptophan residue. Eventually, enough tension builds and DNA is unzipped in a three base pairs burst.

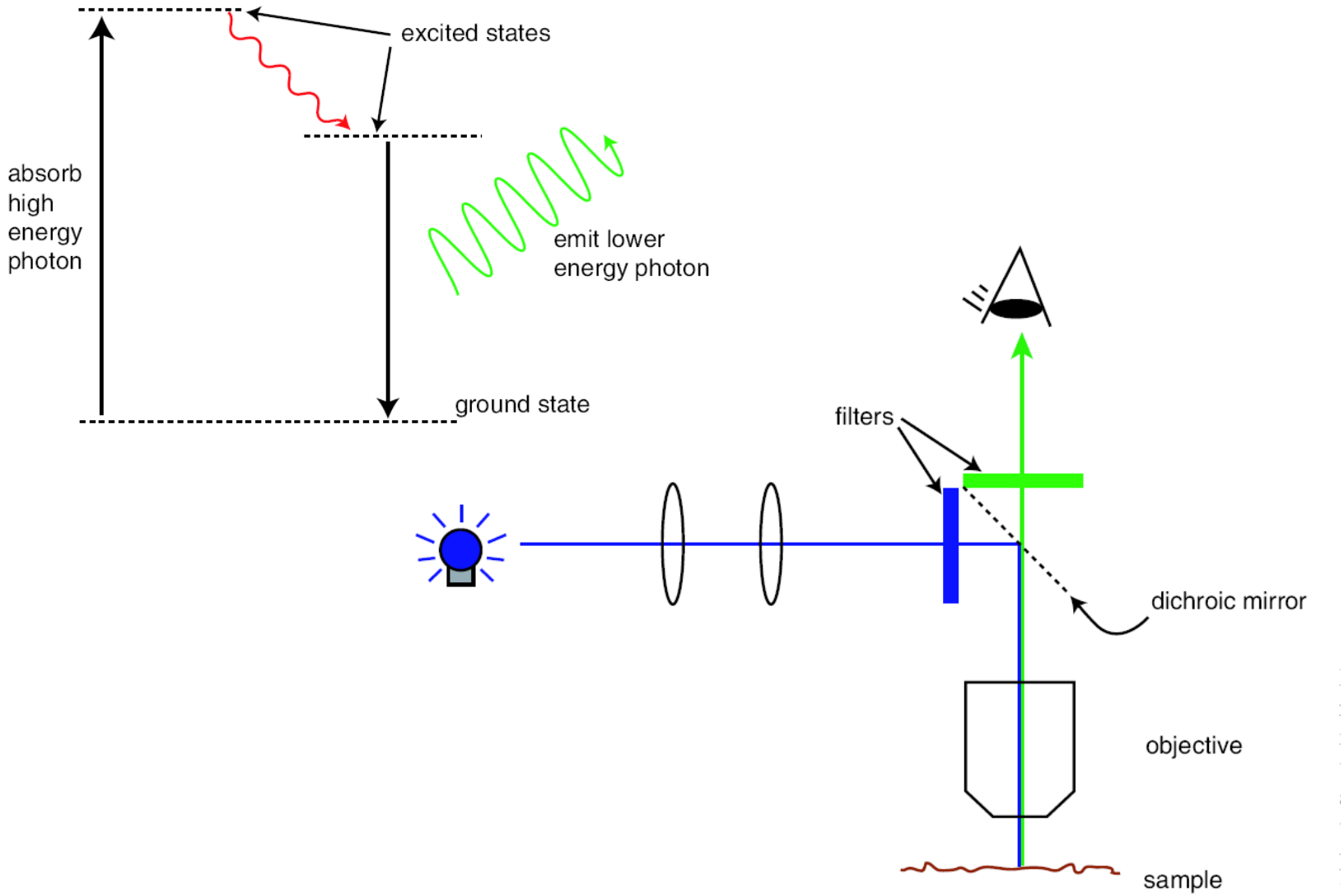
Fluorescence microscopy

How to get z-resolution—confocal

- 1 photon
- 2 photon

How to get super-resolution—STED.

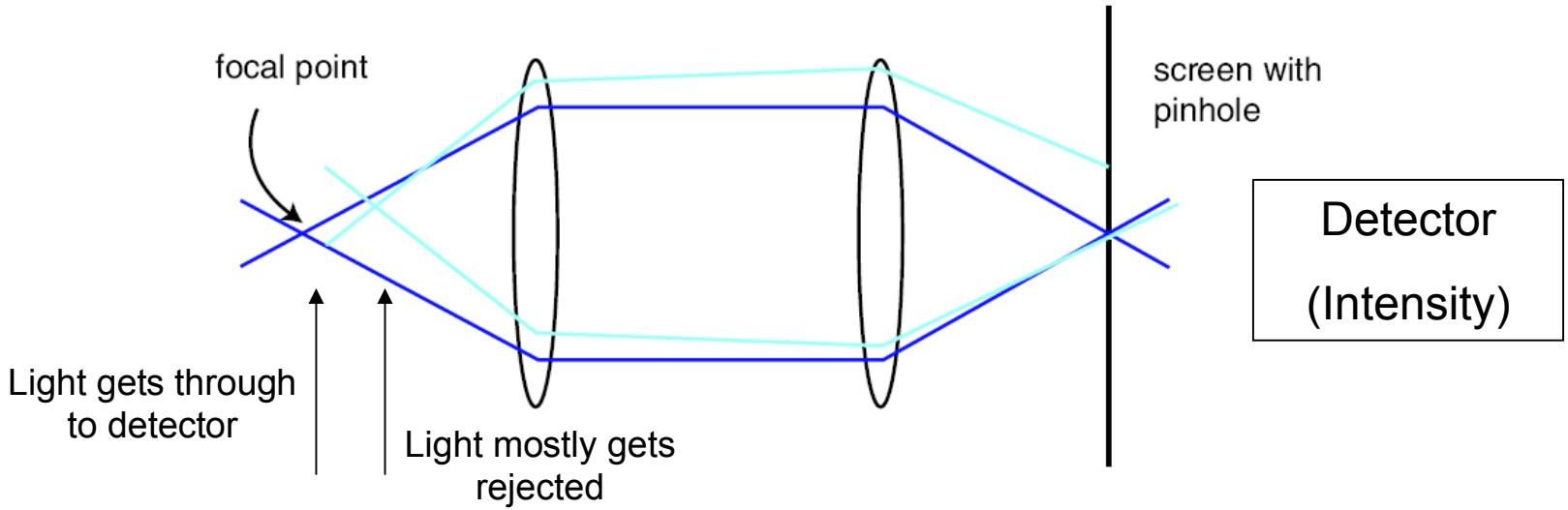
Basic Set-up of Fluorescence Microscope



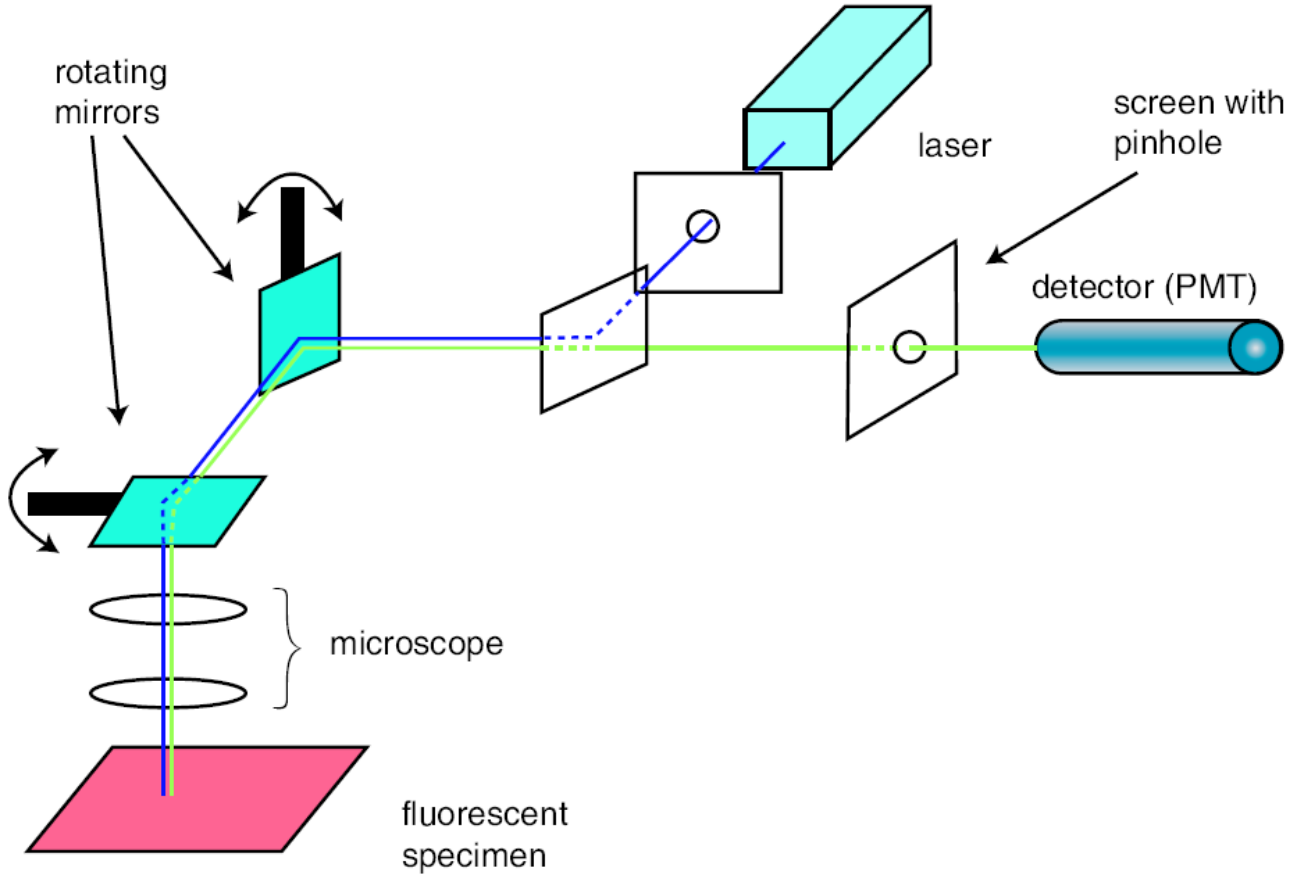
Semwogerere & Weeks, Encyclopedia of Biomaterials and Biomedical Engineering, 2005

Confocal Detection

A pinhole allows only in-focus light through



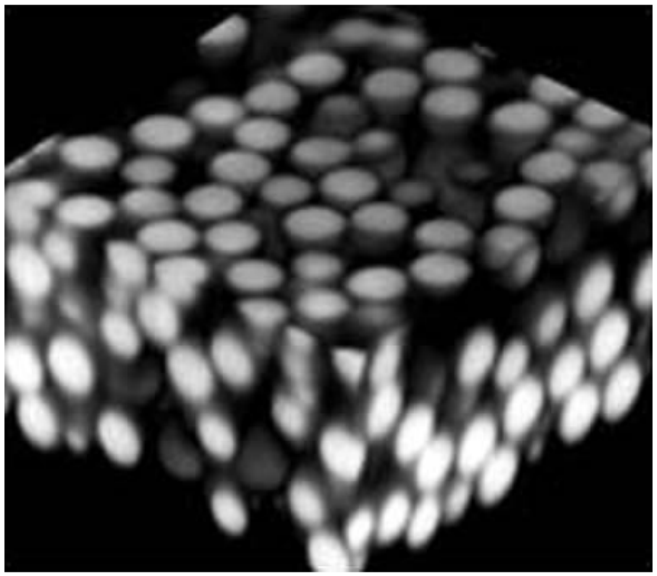
Confocal Microscopy



Light from the laser is scanned across the sample (in x-y plane) and then detected.

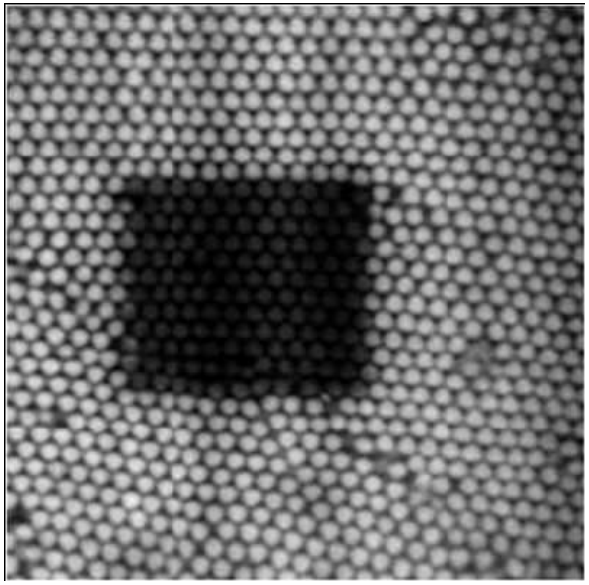
For 3-D, move sample in z-direction.

3-D sectioning is possible with Confocal



Three-dimensional reconstruction of a series of
2D images of PMMA spheres

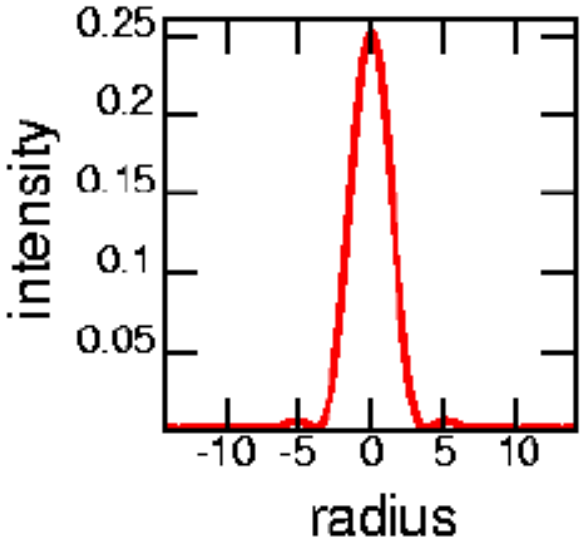
Photobleaching limits confocal

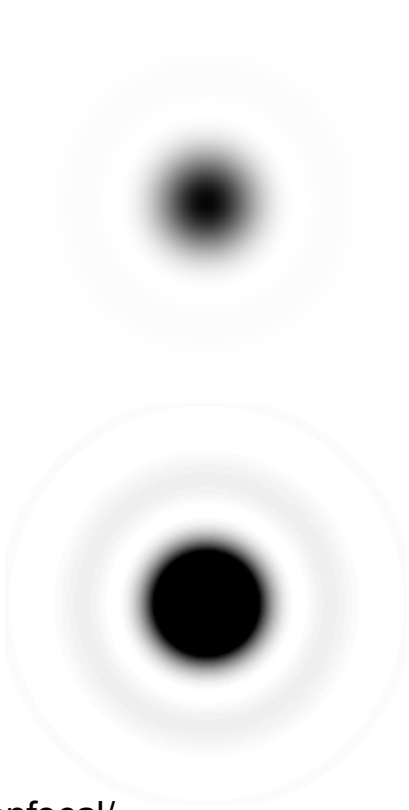


Dyed suspension of densely packed polymethylmethacrylate beads with significant photobleaching. The rectangular region near the center faded after about 30 sec of exposure to excitation light.

Most confocal microscopes generate a single image in 0.1–1 sec.

Size of pinhole
Too small



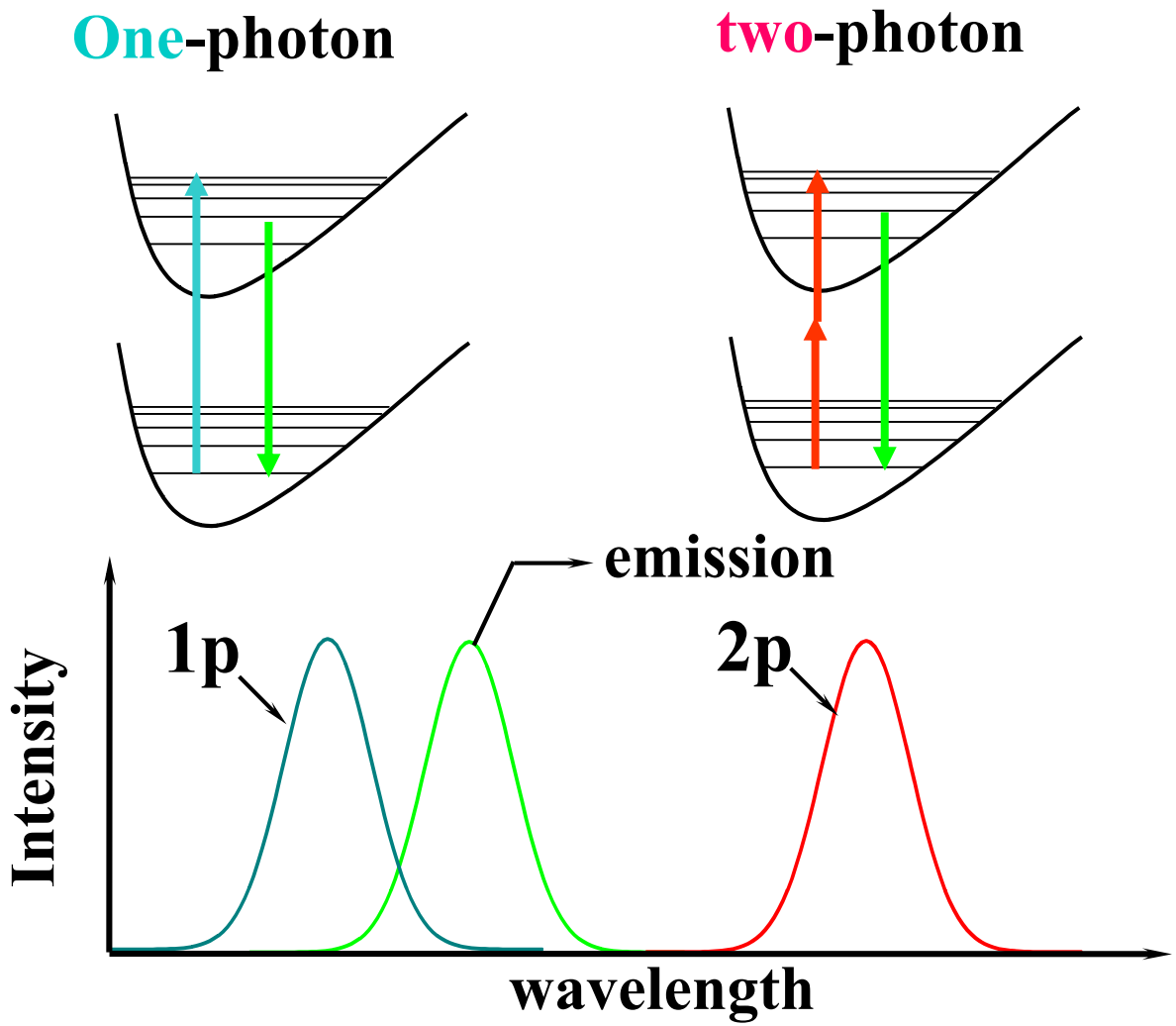


http://www.physics.emory.edu/~weeks/confocal/

Even faster Confocal: Nipkow Disk

An even faster technique is the so-called Nipkow disk microscope. Instead of scanning a single point across the specimen the Nipkow disk microscope builds an image by passing light through a spinning mask of pinholes, thereby simultaneously illuminating many discrete points.

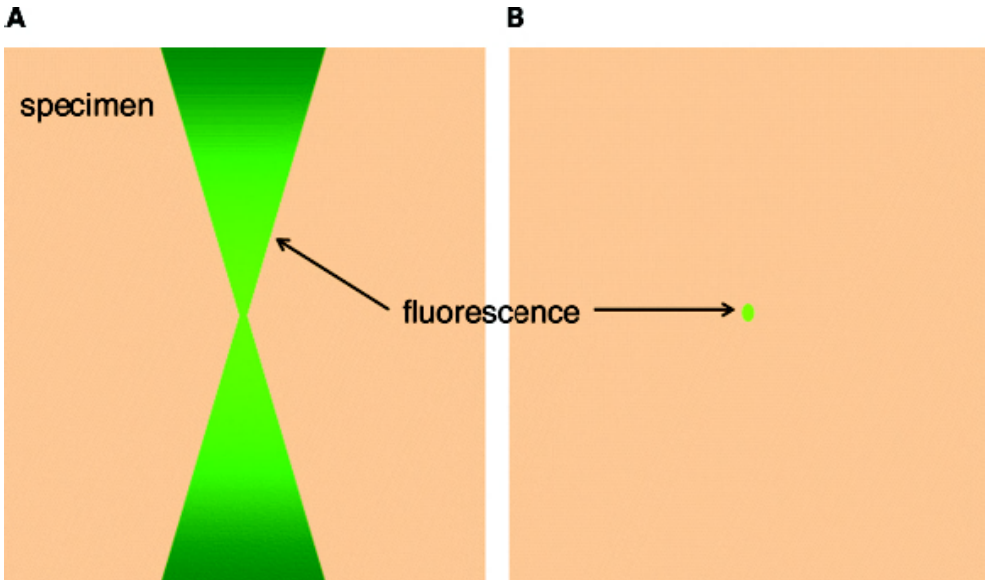
Two photon principle



$$E = \frac{hc}{\lambda}$$

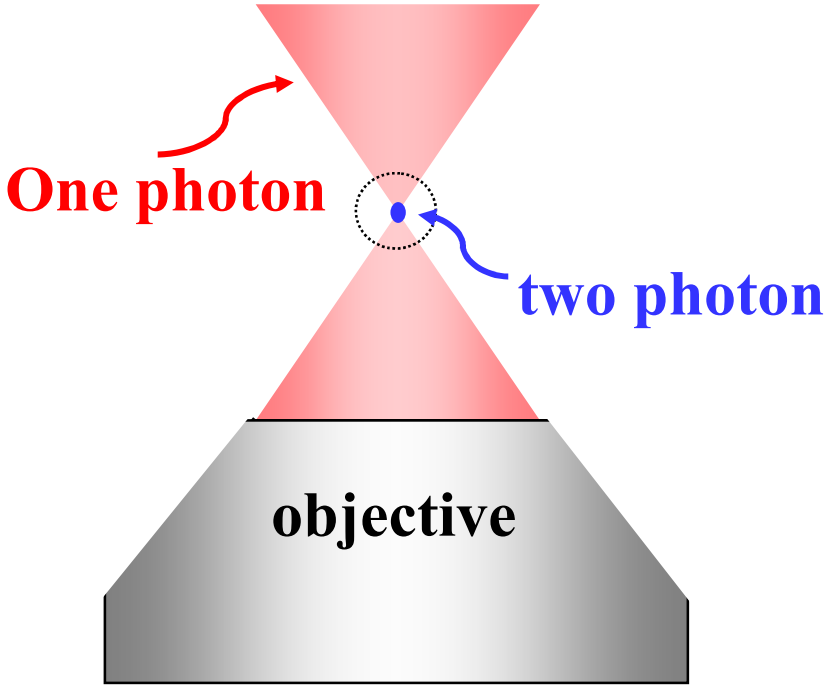
Simultaneous absorption of two photons

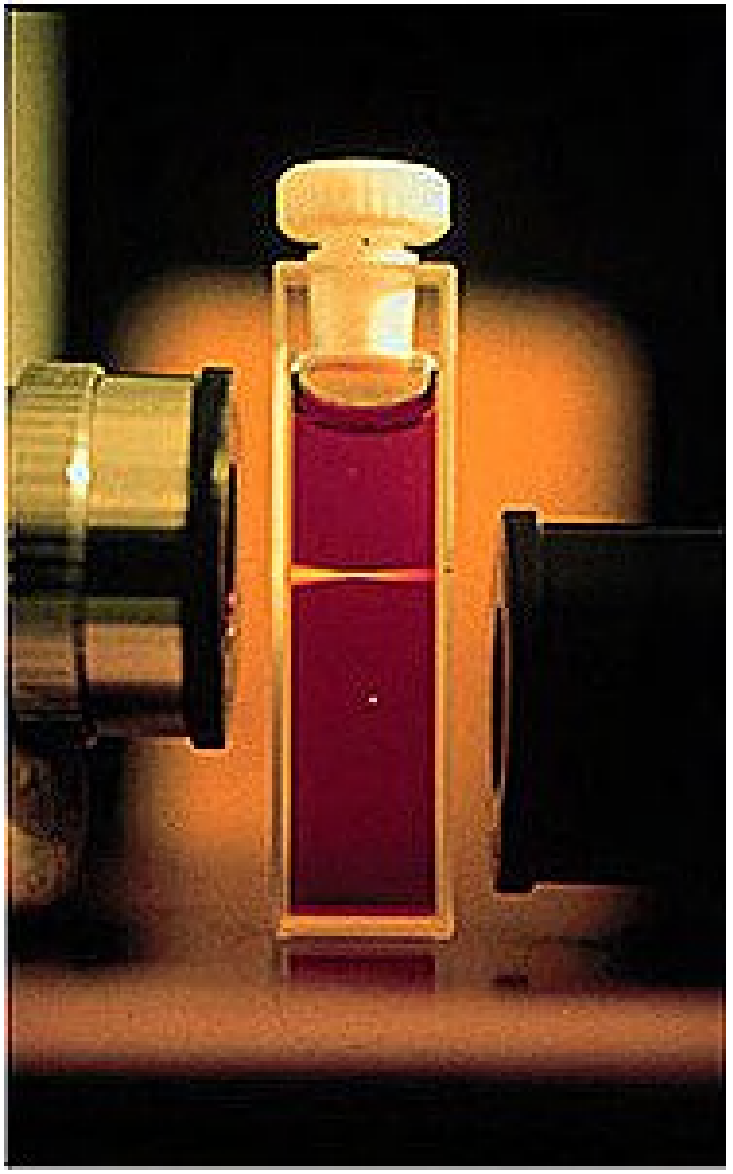
Two-photon (automatically Confocal)



Greatly reduces out-of-focus photobleaching

2-photon optical sectioning



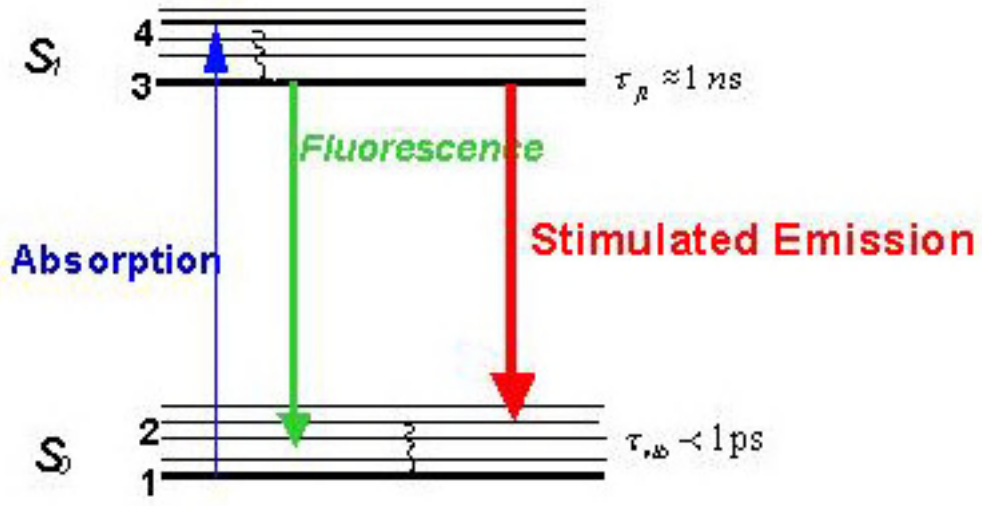


- **inherent spatial resolution**

Stimulated Emission Depletion (STED-) microscope

The perhaps most straightforward way to sharpen the fluorescence focal spot is to selectively inhibit the fluorescence at its outer part.

Do this by causing a loss of fluorescence by stimulated emission with a second pulse that is in the shape of a doughnut.

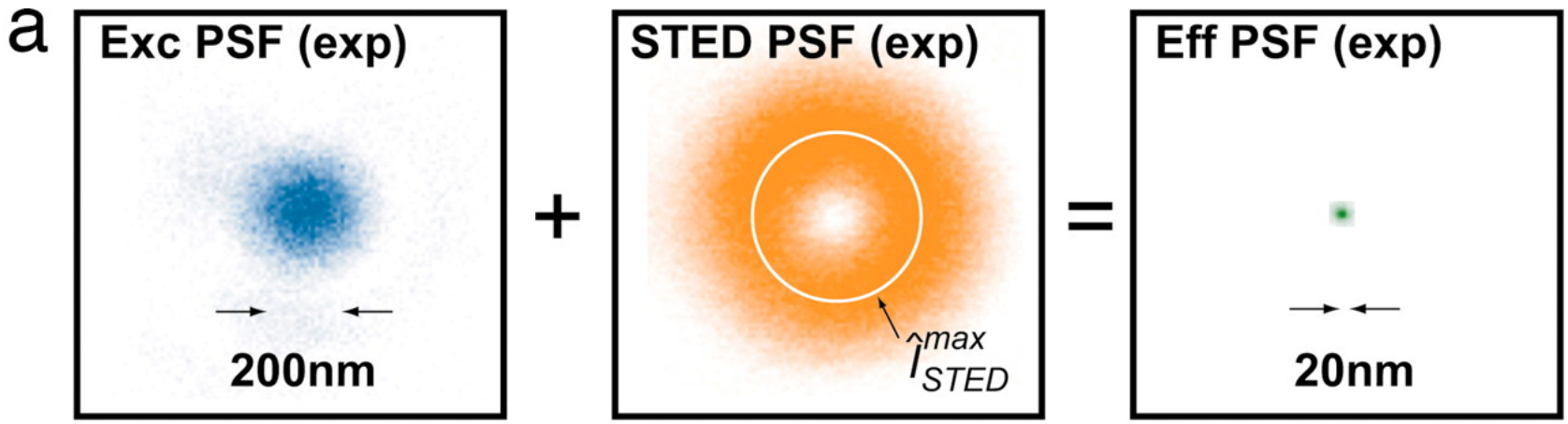


Excite fluorescence with blue laser. A little bit later, excite with doughnut-shaped red beam, which causes stimulated emission from the doughnut shape, i.e. fluorescence dies away *very fast,* and doesn't affect remaining fluorescence.

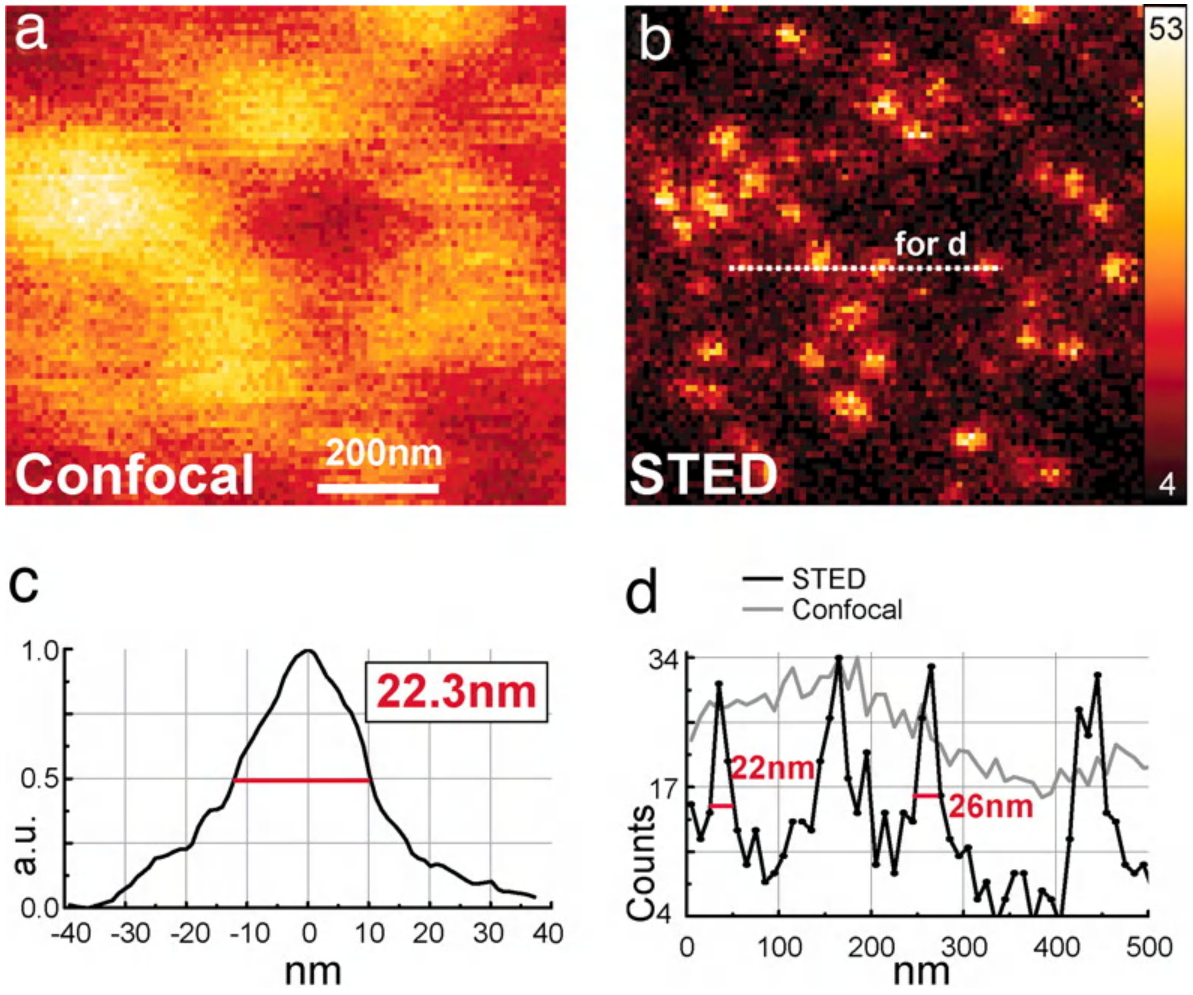
Net result is a smaller Point Spread Function.

http://www.mpibpc.gwdg.de/groups/hell/

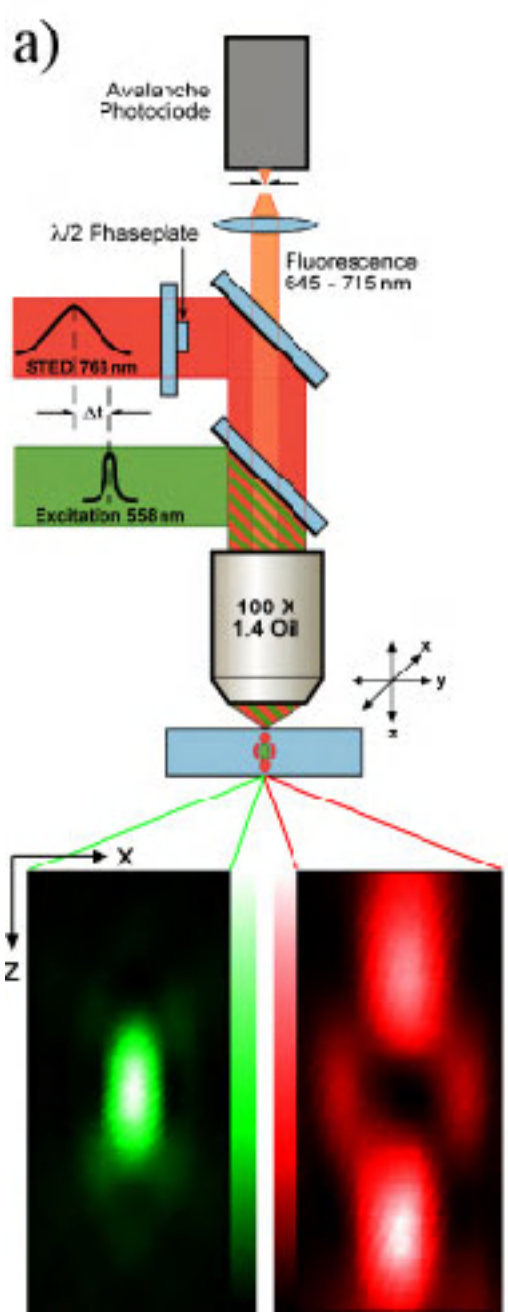
Fiddling with PSF via STED beam



Increased Resolution



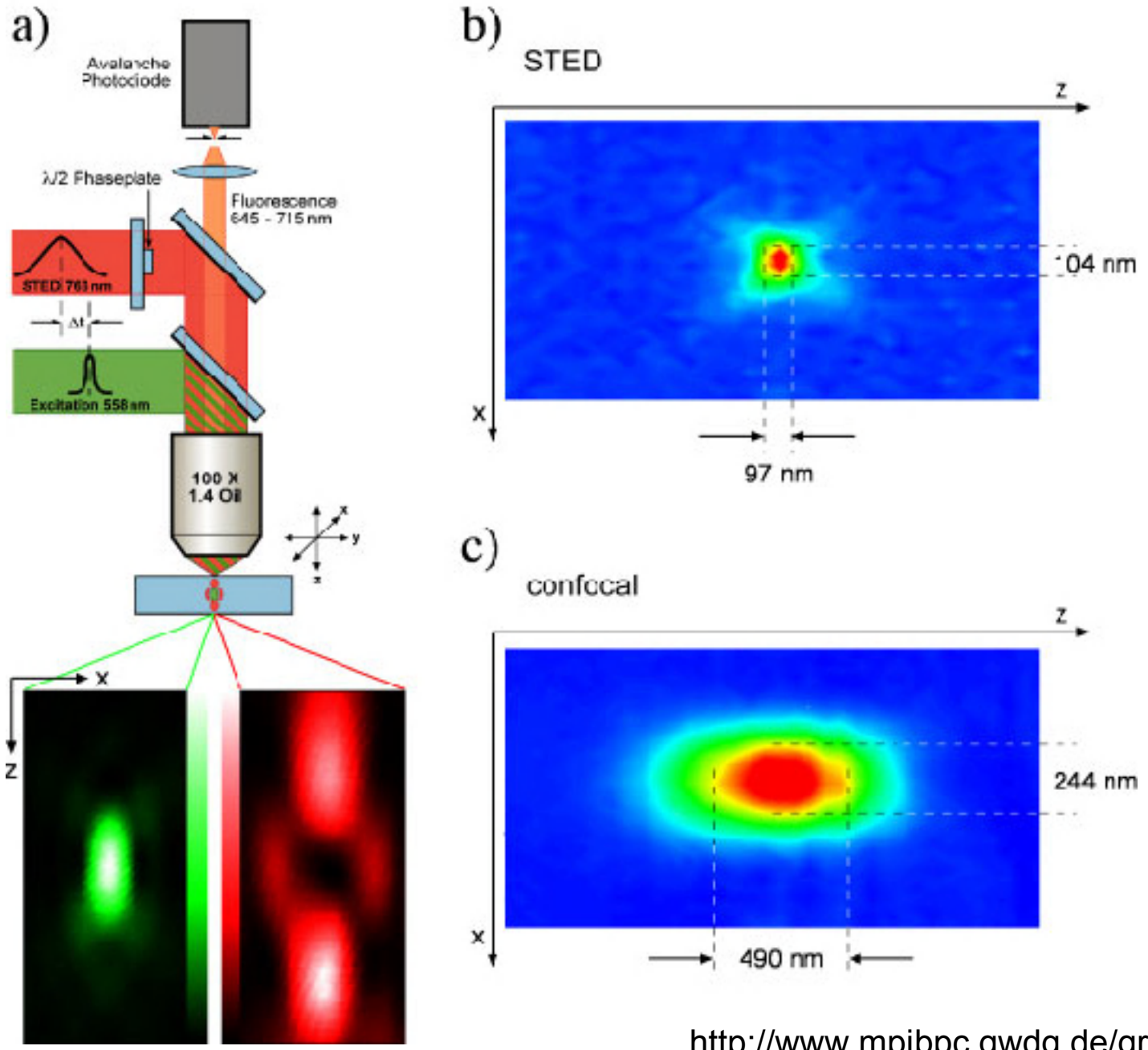
Donnert, Gerald et al. (2006) Proc. Natl. Acad. Sci. USA 103, 11440-11445



STED-Microscopy

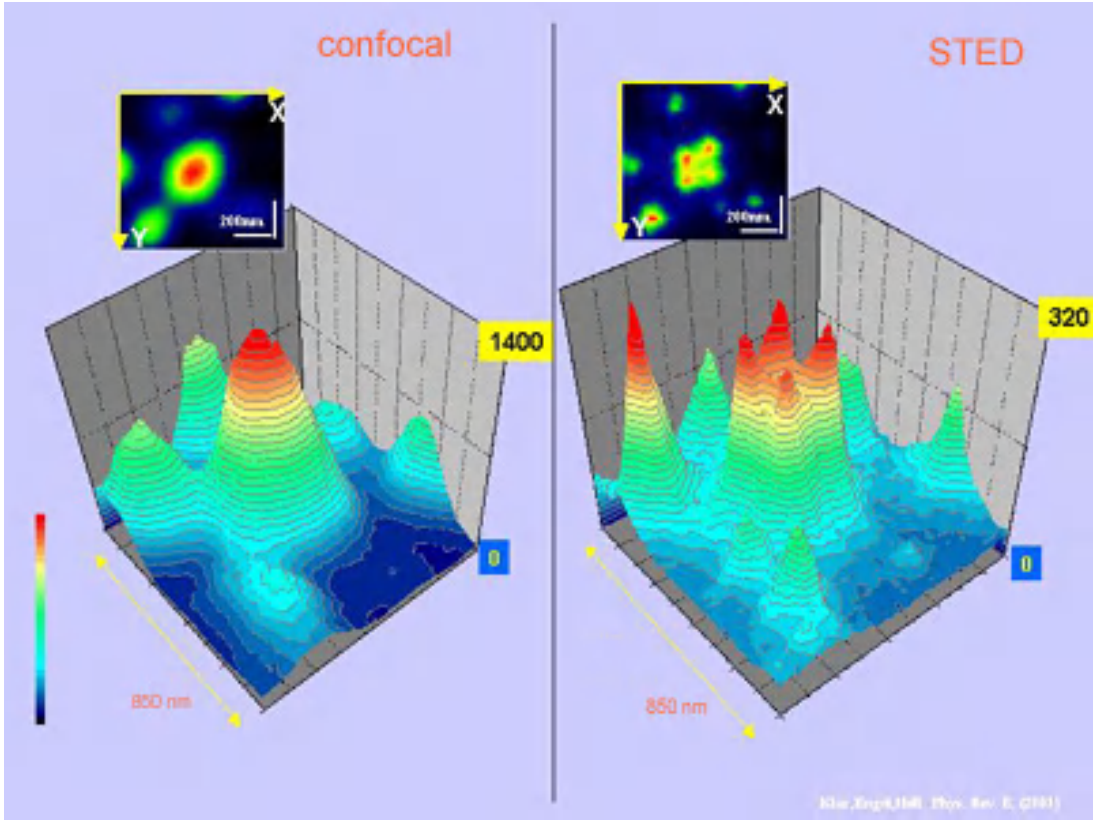
The setup: Synchronized laser pulses. Excitation is performed by a subpicosecond laser pulse that is tuned to the absorption spectrum of the dye, produces ordinary diffraction limited spot of excited molecules. The excitation pulse is immediately followed by a depletion pulse, dubbed 'STED-pulse'. The STED pulse is red-shifted in frequency to the emission spectrum of the dye, so that its lower energy photons act ideally only on the excited dye molecules, quenching them to the ground state by stimulated emission. The net effect of the STED pulse is that the affected excited molecules cannot fluoresce because their energy is dumped and lost in the STED pulse. By spatially arranging the STED pulse in a doughnut mode, only the molecules at the periphery of the spot are ideally quenched. In the center of the doughnut, where the STED pulse is vanishing, fluorescence ideally remains unaffected.

http://www.mpibpc.gwdg.de/groups/hell/



http://www.mpibpc.gwdg.de/groups/hell/

STED has improved Resolution



To date an improvement beyond the diffraction barrier of 3 in the transverse direction and up to 6 along the optical axis has been experimentally demonstrated.

http://www.mpibpc.gwdg.de/groups/hell/

No inherent resolution to STED Microscopy

No resolution limit: By increasing the STED pulse intensity, the depletion becomes complete at the spot's periphery and increasingly more effective towards the middle. At the doughnut hole, however, the fluorescence is ideally not affected at all. Therefore, by increasing the intensity of the doughnut-shaped STED-pulse, the fluorescent spot can be progressively narrowed down, in theory, even to the size of a molecule. This concept signifies a fundamental breaking of the diffraction barrier. The essential ingredient is the saturated reduction of the fluorescence (= depletion) at any coordinate but the focal point.

Comparison with confocal fluorescence microscopy

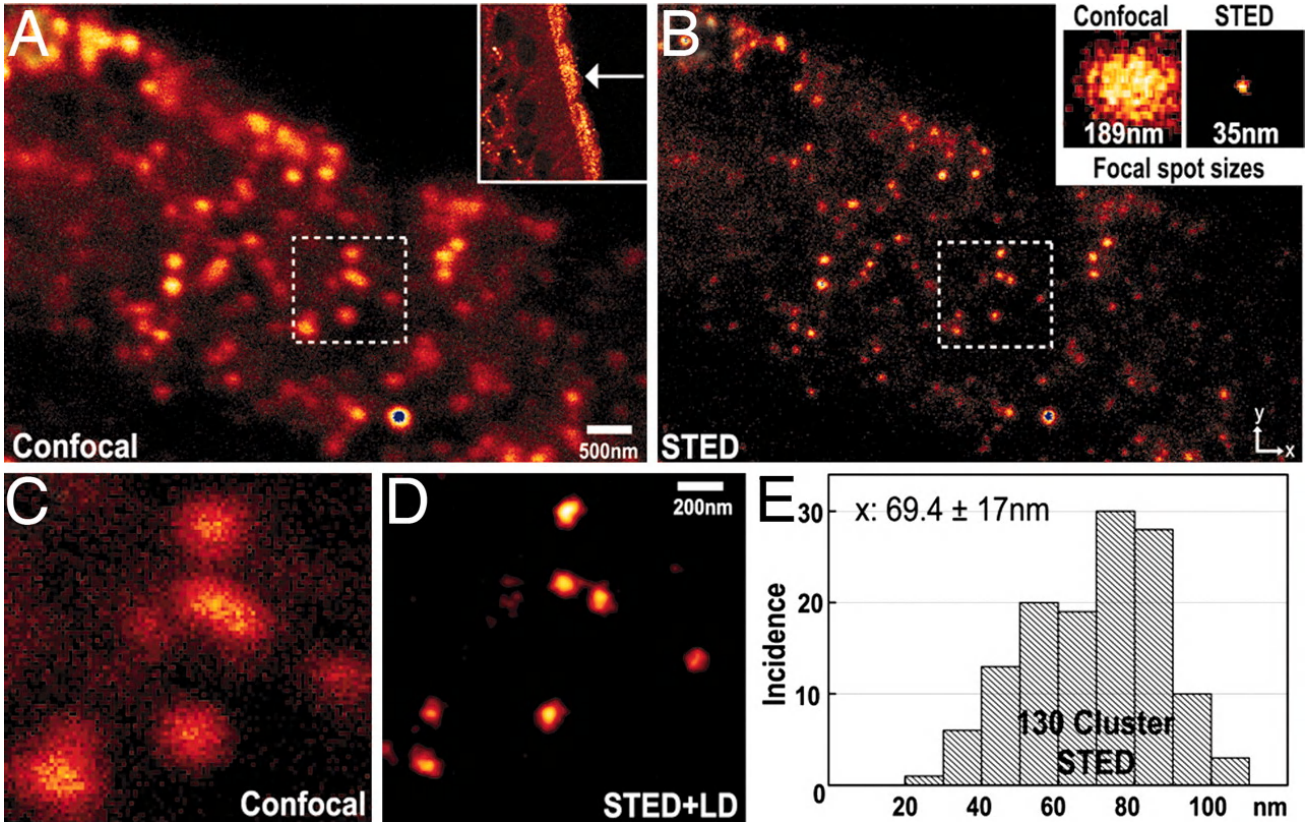
STED is super-resolution microscopy. Confocal is not.

So far, experiments show that the level of saturation will be determined by the bleaching that is inflicted on the dye.

http://www.mpibpc.gwdg.de/groups/hell/

Biological Example of STED

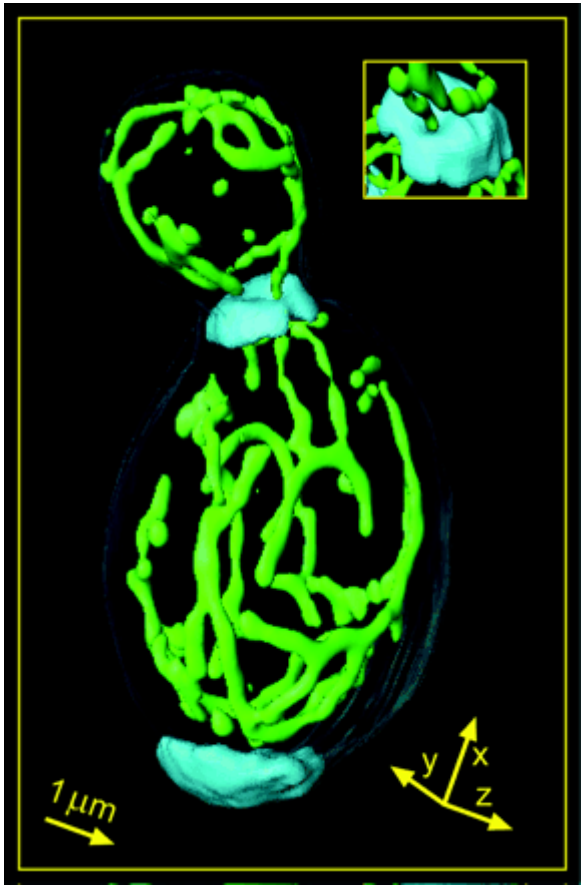
The transient receptor potential channel M5



Analysis of spot size for Confocal (*A*) and STED (*B*) images of TRPM5 immunofluorescence layer of the olfactory epithelium. (*A, C Inset*) Confocal image at a lower (higher; box) magnification taken with a confocal microscope. (*B*) STED image. Effective point-spread function in the confocal (189-nm) and STED (35-nm) imaging modes.

Hell, PNAS, 2007

YFP Mitochondria



Egner, Alexander et al. (2002) Proc. Natl. Acad. Sci. USA 99, 3370-3375

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.