Debye Summer School 2015

Optical Microscopy of Single Nano Particles

Breaking the Barriers of Light Microscopy

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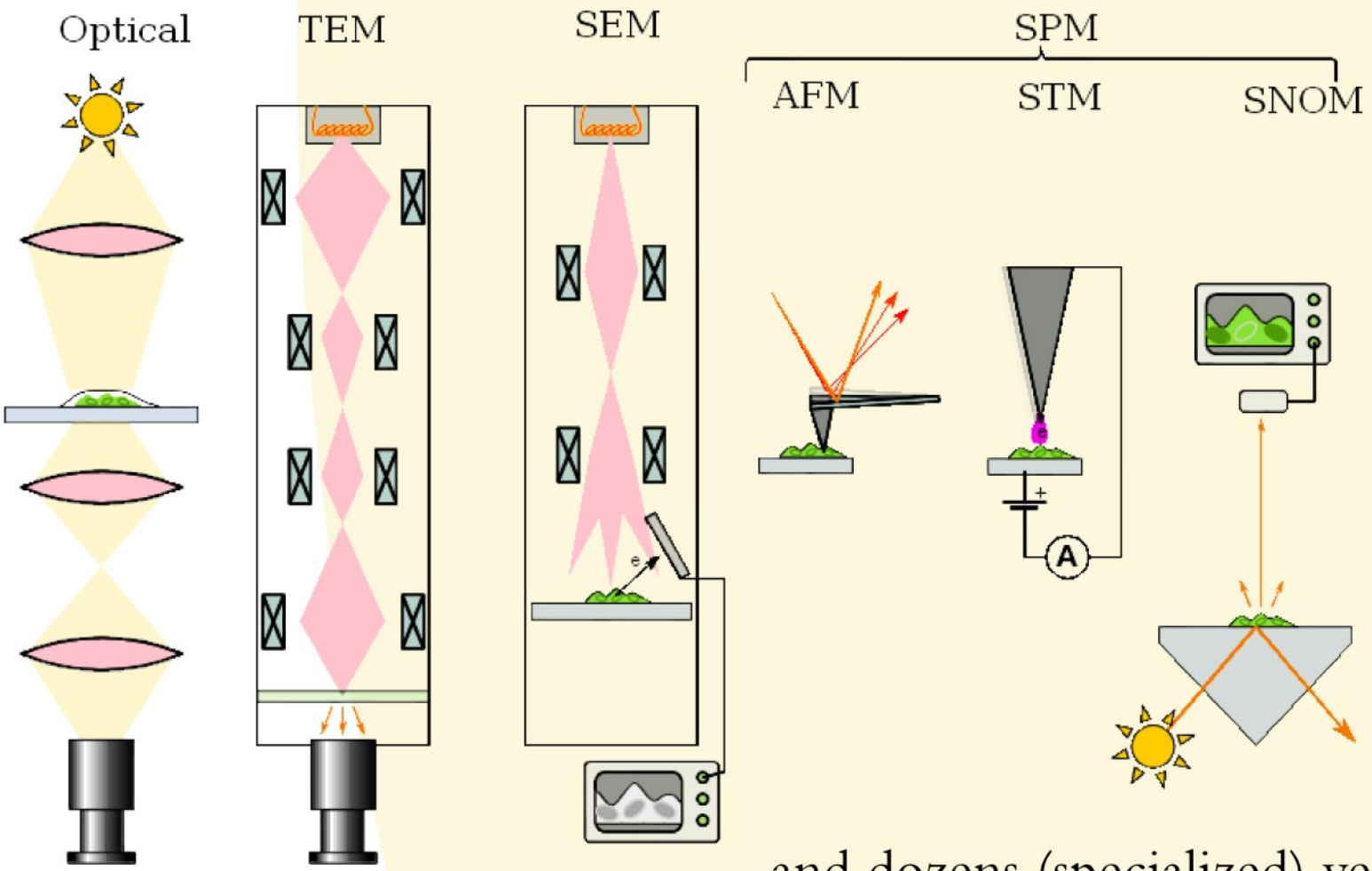
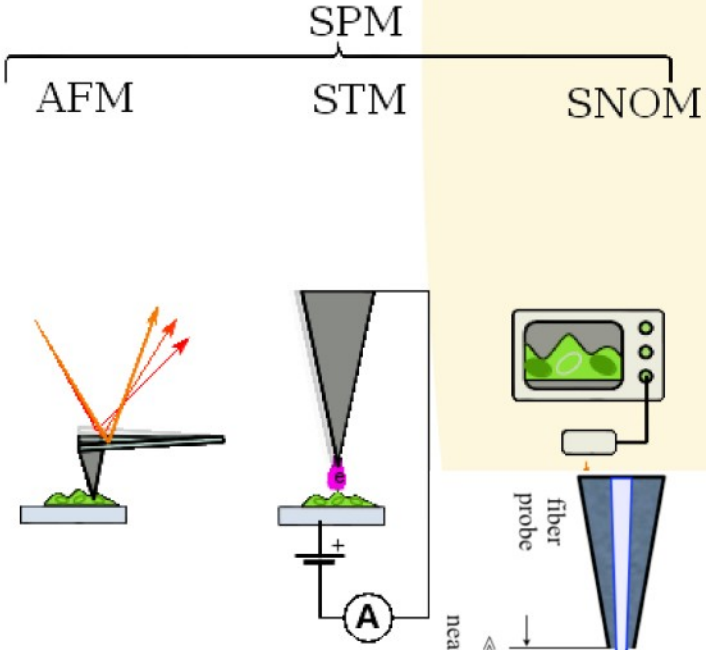
Then What is a Microscope?

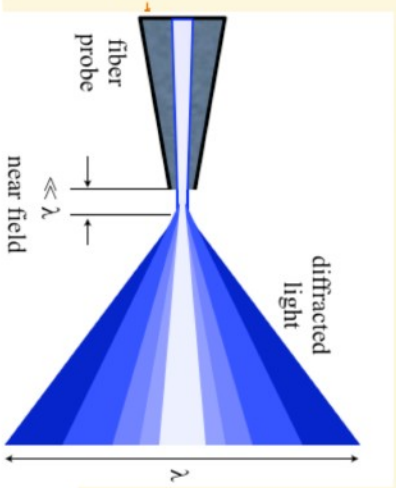


… and dozens (specialized) versions

Source: wikipedia, user FDominec

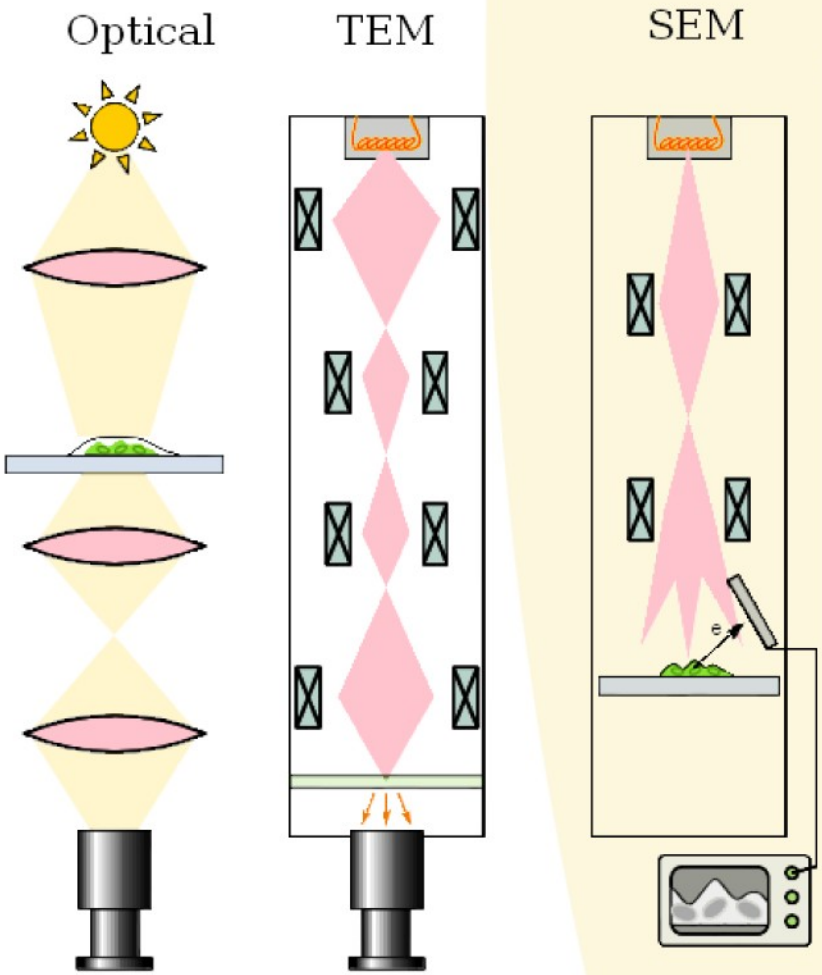
Scanning Probe Microscopy
Near-Field Microscopy





- Local effect
  - Tip (almost) in contact with the sample
- Optics
  - Usually not necessary (or at least not to 'see' the sample
- Detection of the sample by observing the reaction of the probe/tip (amplitude, frequency, current) or light emitted from extremely small region

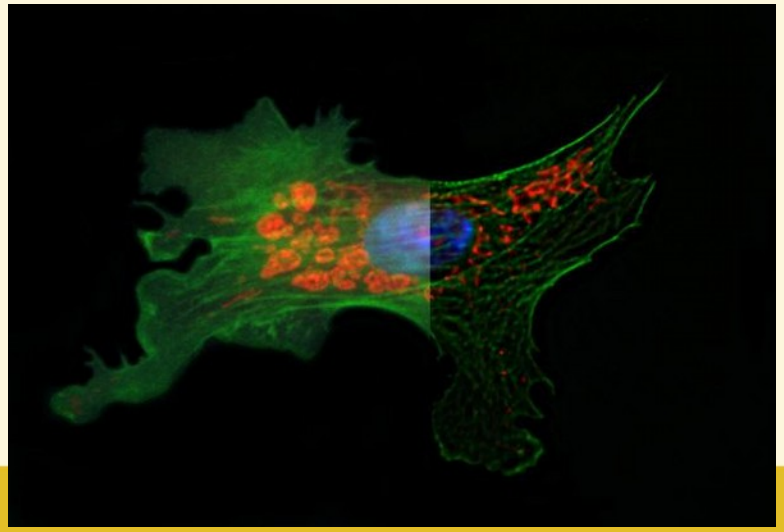
Far-Field ("Regular") Microscopy



- Illumination source
  - Coherent and monochromatic, for preference
- Optics
  - for Magnification
  - to form an Image
- Detector
  - Eye, Camera (full image)
  - Point detector (scanning)
  - (frequency/energy) spectrum ...

Seeing Requires Differences

- In order to detect (a particle, details in a specimen), we require differences: **contrast**.
  - Difference in light absorption or colour
  - Difference in Phase (DIC)
- Alternatively, we might detect emitted light: **Fluorescence**
  - Wavelength (colour)
  - Lifetime
  - Polarization ("Anisotropy")



Seeing Is Changing

- We need to make (interesting) parts of the sample visible:
  - Attachment of Labels
  - (Bio)Chemical Modification
- Interaction of light or matter wave with the sample (or label) transfers energy:
  - 1 μm (IR) → 1.24 eV/photon, ~48 kT, 82 kcal/mol
  - 100 nm (UV) → 12.4 eV/photon
  - for visible light, these values become comparable to typical bond energy!
- Electrons also have a wavelength depending on their energy (de Broglie wavelength, typically << 1 nm)
- **There is a length scale associated with 'seeing'**

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

$$p = \hbar k = \frac{h}{\lambda} = \frac{E}{c}$$

$$\lambda_B = \frac{h}{p}$$

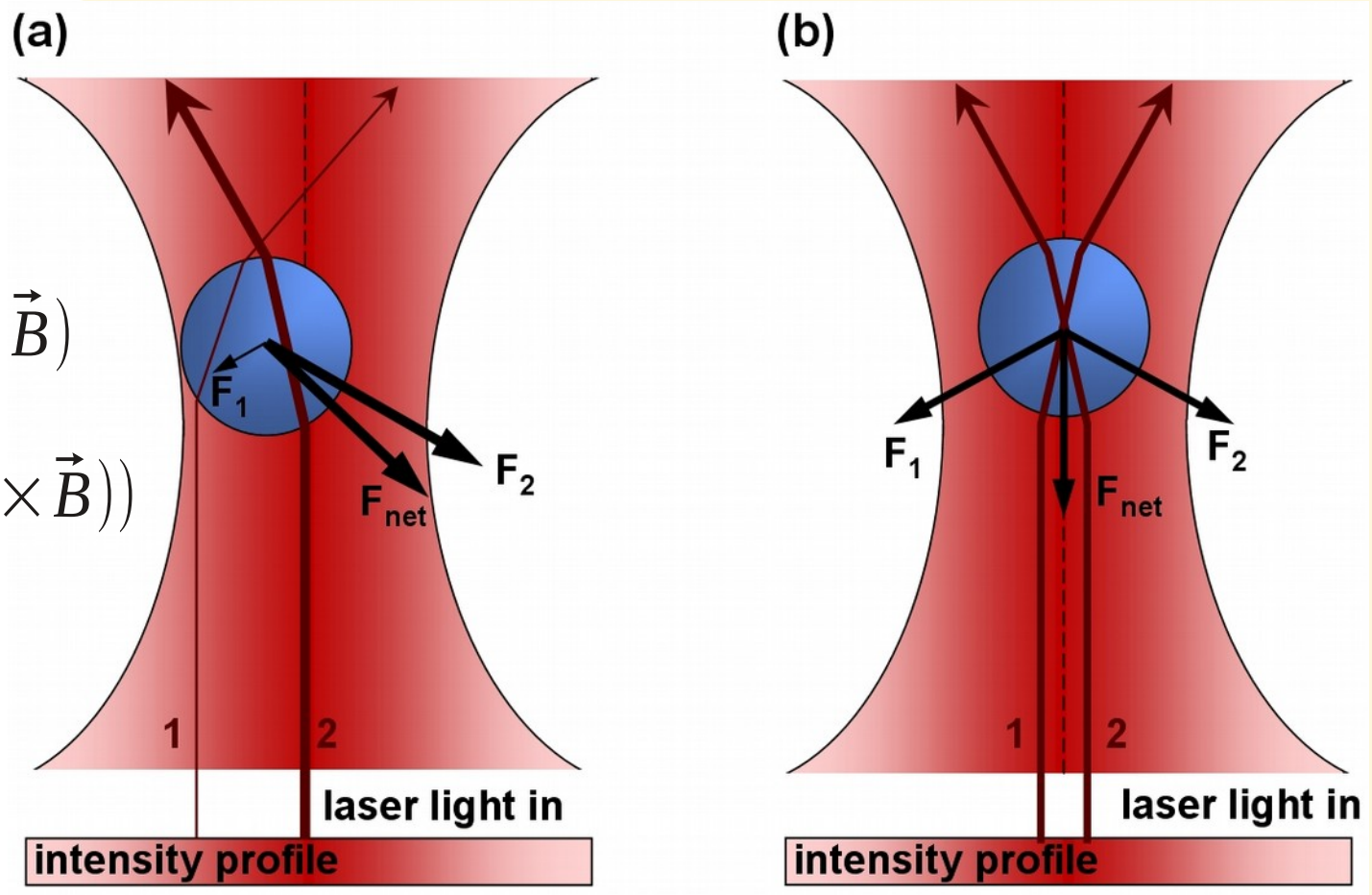
Source: andrewmichaelroberts.blogspot.com (via google image search)

Using Light to Change
Optical Tweezers

Movie (does not embed :( )

Gradient Forces

$$F = q\left(\vec{E} + \vec{v} \times \vec{B}\right)$$
$$\vdots$$
$$\alpha\left(\nabla E^2 + \frac{\partial}{\partial t}\left(\vec{E} \times \vec{B}\right)\right)$$

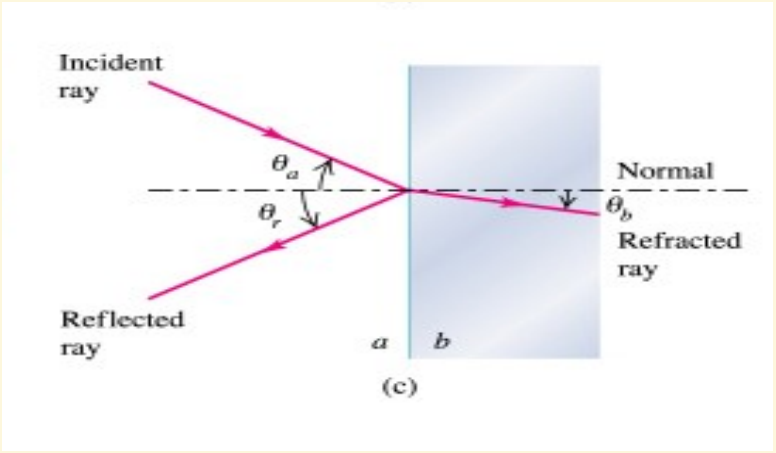


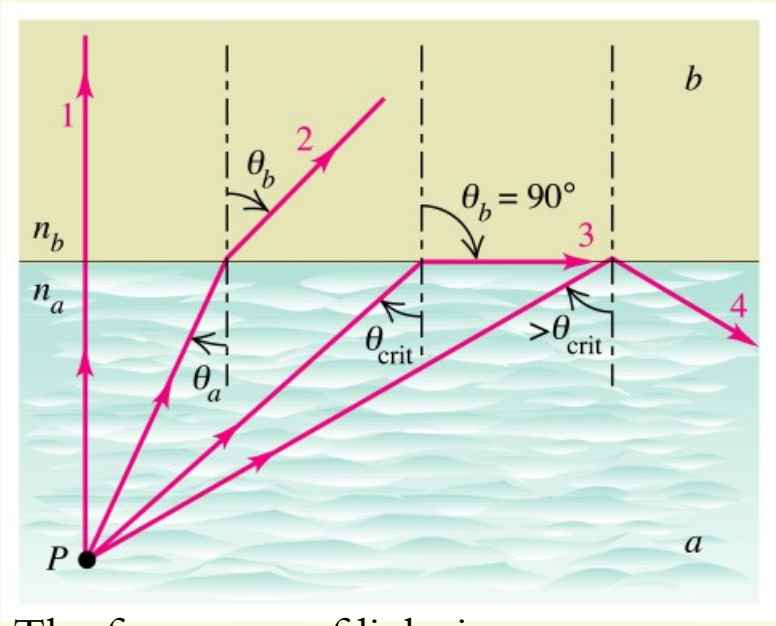
Lenses

- Optical Lenses work on the principle of Refraction (Snell's Law)

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

- The path that light takes is the path of least time (not necessarily the shortest!)
- In addition, there is also Reflection (loss!) at optical interfaces.

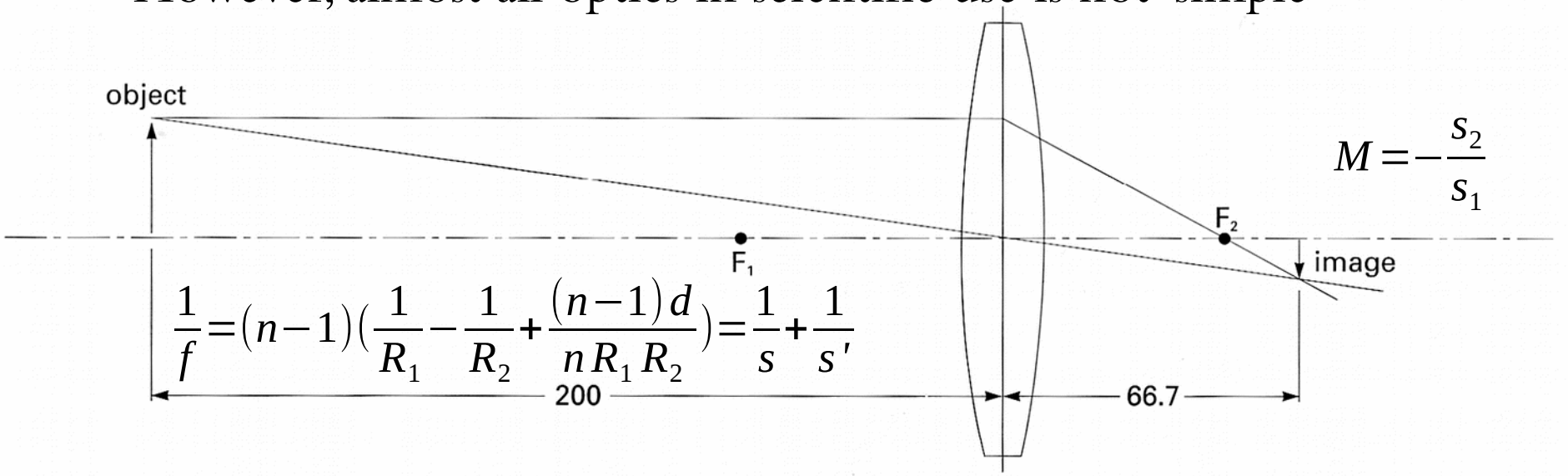




The frequency of light is constant across a boundary, its speed and wavelength are smaller in a medium

Lenses & Geometric Optics

- The simplest description of a lens with optical density *n* is given by the 'lensmaker's equation'
- In good approximation, we can find focal distances and magnification of such a lens.
- However, almost all optics in scientific use is not 'simple'



$$\frac{1}{f} = (n-1)\left(\frac{1}{R_1} - \frac{1}{R_2} + \frac{(n-1)d}{nR_1R_2}\right) = \frac{1}{s} + \frac{1}{s'}$$

$$M = -\frac{s_2}{s_1}$$

"Simple Optics"

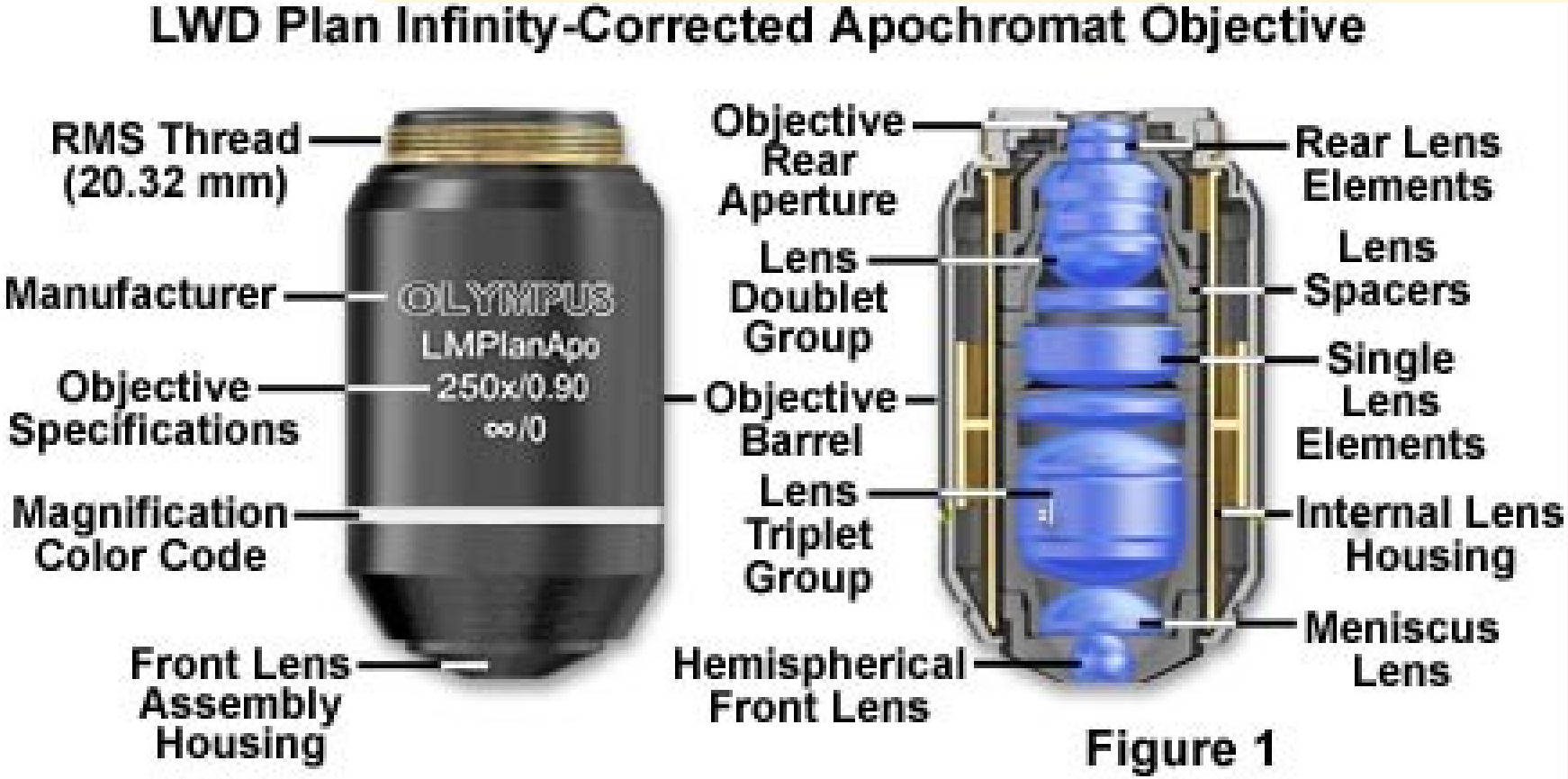
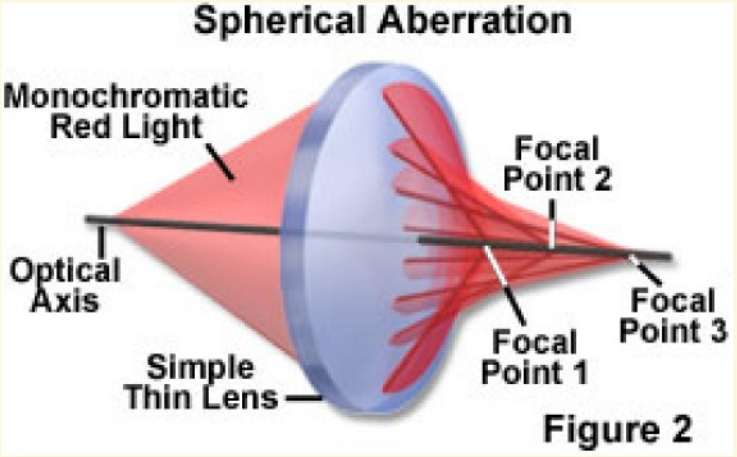


Figure 1

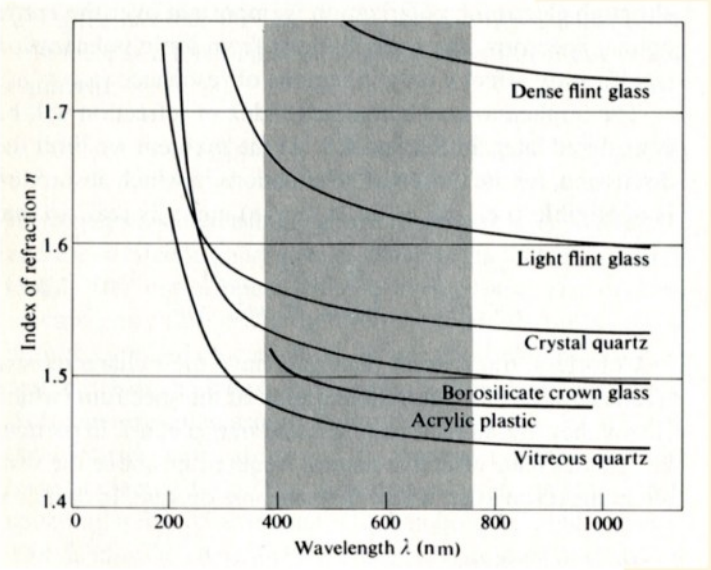
Aberrations

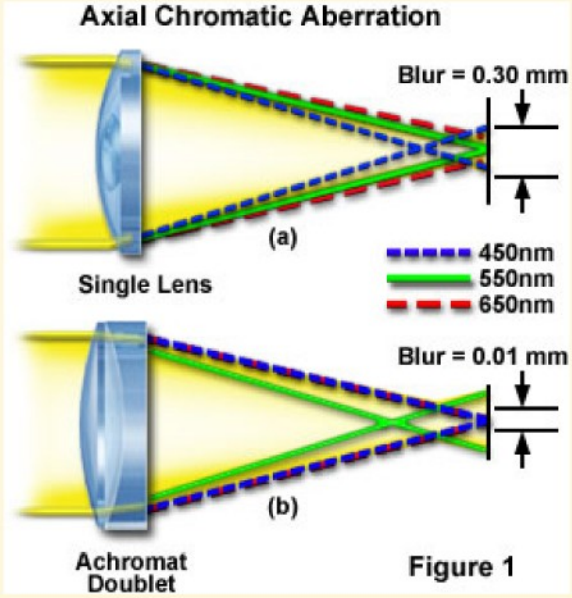
- In addition to monochromatic aberration – results from imperfections of the lens or a break-down of our approximations, there are chromatic aberrations
- Six empirical "Sellmeier coefficients" describe n as a function of wavelength

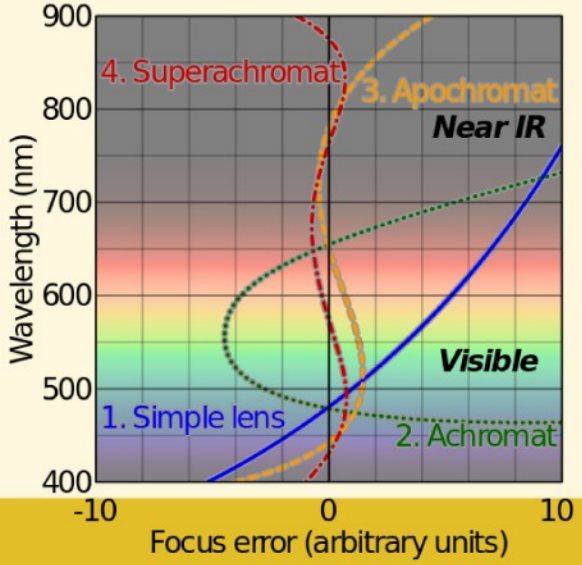


$$n^2(\lambda) = 1 + \sum_i \frac{B_i \lambda^2}{\lambda^2 - C_i}$$

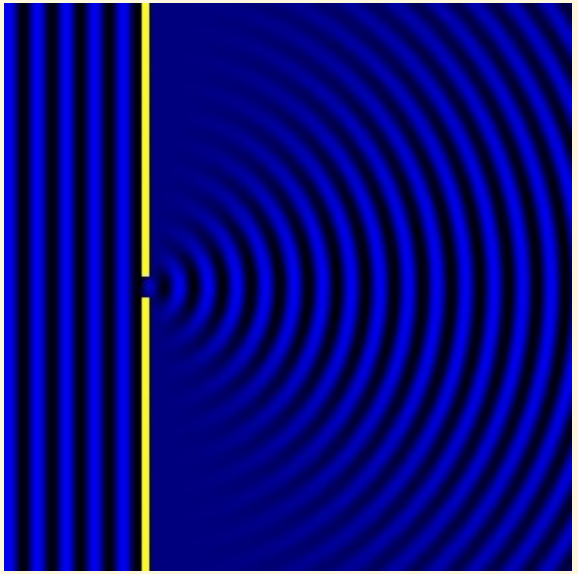
"Sellmeier equation"

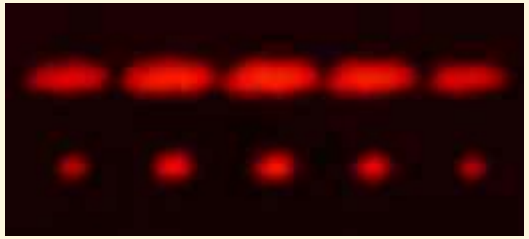






Diffraction





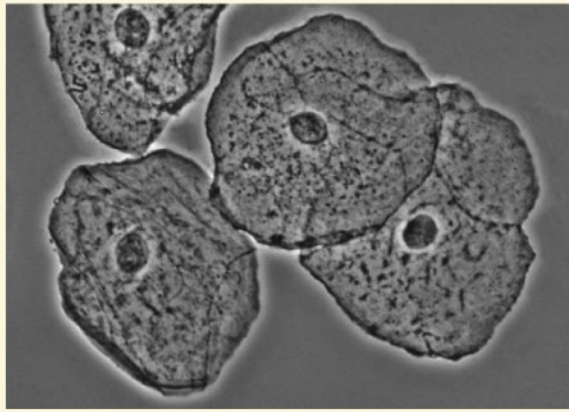
- Refraction alone is not enough to describe optics
- Light has not only particle properties (photons), it also behaves like a wave with amplitude ***and*** phase.
- Any finite aperture will cause diffraction!
- The math is complex[1], but we have two approaches:
  - Kirchhoff-Fresnel diffraction (near field)
  - Fraunhofer diffraction (far field or lens focus)
- The diffraction pattern of a circular aperture is called an "Airy Disk"

> No-one has ever been able to define the difference between interference and diffraction satisfactorily. It is just a question of usage, and there is no specific, important physical difference between them.
>
> RP Feynman

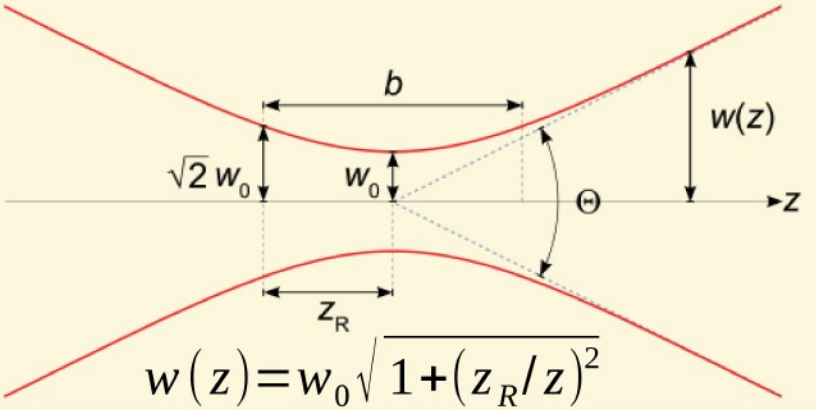
[1] no pun intended!

Consequences of Diffraction

- Diffraction can be used to focus instead of refraction (e.g. if no suitable lens material is available): ***Fresnel Zone Plates***
- Interference can be used to enhance contrast of samples: ***Phase Contrast Microscopy***
- ***Dark-Field Imaging*** uses diffracted or scattered light to form images, while rejecting the (much stronger) directly transmitted light.



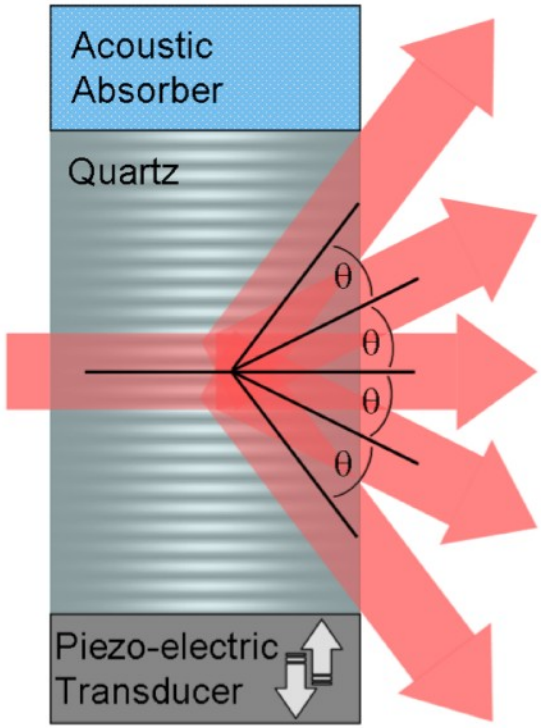
- On the other hand, even a perfect lens – even in paraxial approximation – is ***diffraction limited*** → there is a limit to the resolution of a microscope



$$w(z) = w_0 \sqrt{1 + (z_R / z)^2}$$

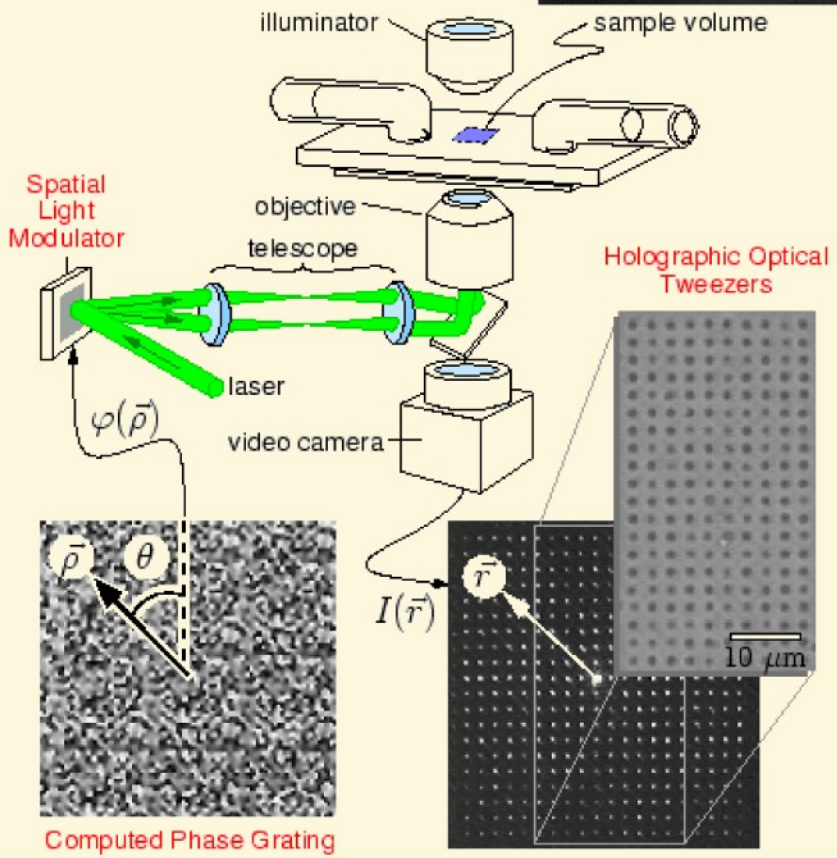
$$\Theta \simeq \frac{\lambda}{2\pi w_0}$$

And yet other uses of Diffraction



← Move a Beam (AOM)

Shape a beam → (SLM)



Source: Grier Lab, NYU

And finally…

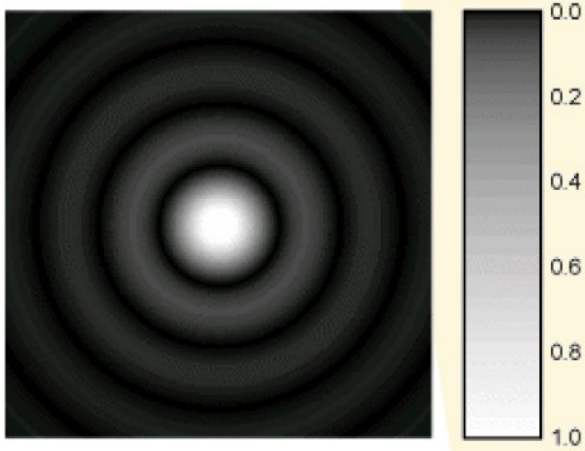
- Microscopes come in all sizes, shapes, and price ranges.
- A (near-field) tip, light (or for that matter, matter-) waves interact with the sample to generate an image. Make sure that the interactions do not alter that in which you are interested!
- **Refractive** elements ("lenses") allow us to focus waves into small spaces, and in principle so do Diffractive elements ("zone plates")
- However, diffraction also puts a limit to how tightly one can focus, and how small a detail we can resolve.

Part Two

- **Limits to Resolution**
  - Confocal "Gold Standard"
- **Improving Data Analysis**
  - Deconvolution
- **Improving Detection**
- **Improving Illumination**
- **Redefine "Resolution"**

Airy Disc and Resolving Power

British Astronomer George Airy discovered and described the diffraction pattern of a circular aperture.



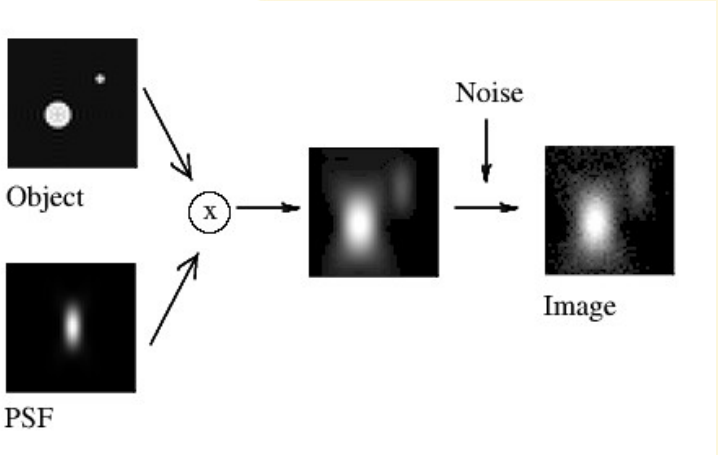
$$r_{min} = 0.61 \frac{\lambda}{NA}$$

λ ..... wavelength of light (~ 532 nm)
NA .... numerical aperture (~ 1.3)
r_{min} ..... resolution (~ 250 nm)

Sir George Airy
(1801-1892)

Defining the Starting Point

Resolution of “standard”

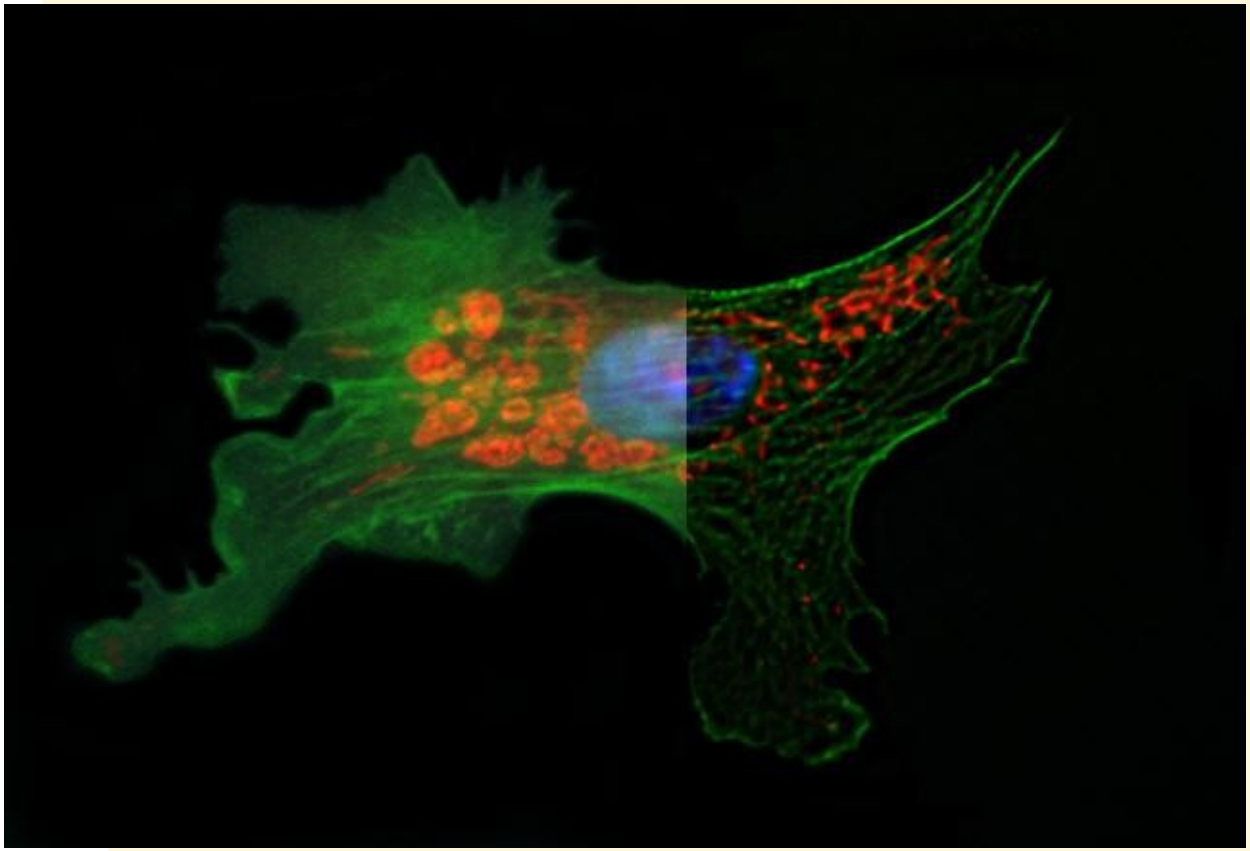


Standard **wide field** microscope: $d_{min} = 0.61\ \lambda/NA \sim 250$ nm

Confocal microscope: $d_{min,lat} = 0.45\ \lambda/NA \sim 175$ nm

$d_{min,ax} = 1.4\ \lambda / (n\ .\ \sin^2(\alpha)) \sim 600$ nm

1.) Cleaning Up the Data: De-convolution

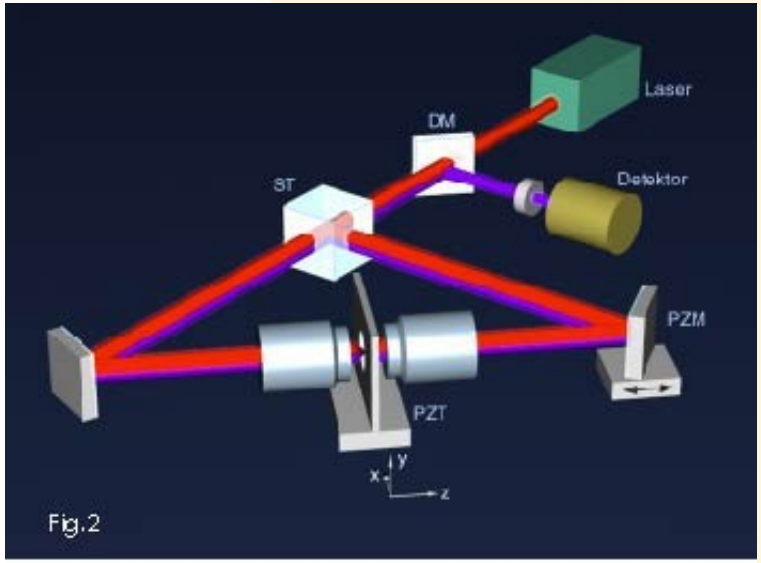


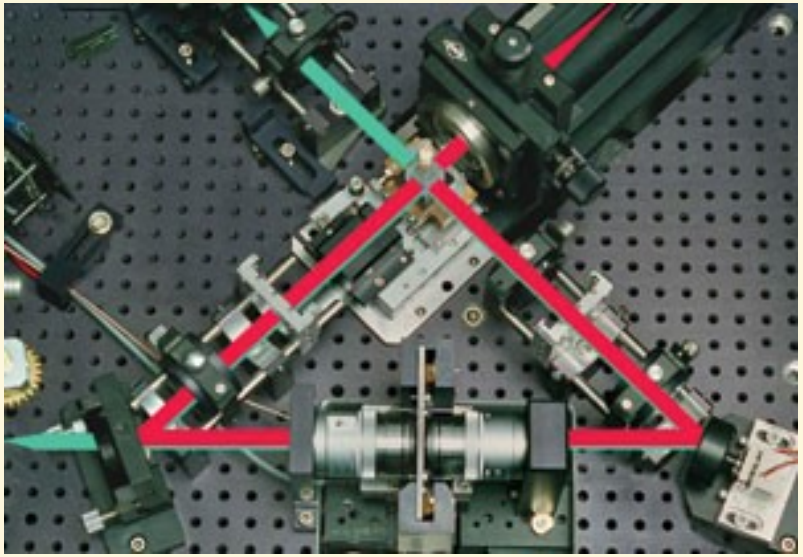
http://www.svi.nl/ImageGallery

Image created by Dr. Jeff Tucker and Dr. Holly Rutledge from NIEHS, NIH, USA

The Power of Deconvolution. Experimental point spread functions were generated for the red, green, and blue channels on an epifluorescence microscope and then used to deconvolve a standard Invitrogen Floucells #1 prepared slide, containing bovine pulmonary artery endothelial cells stained for mitochondria (red), F-actin (green), and nuclei (blue). Before (left) and after (right) deconvolution images were merged side by side to display the

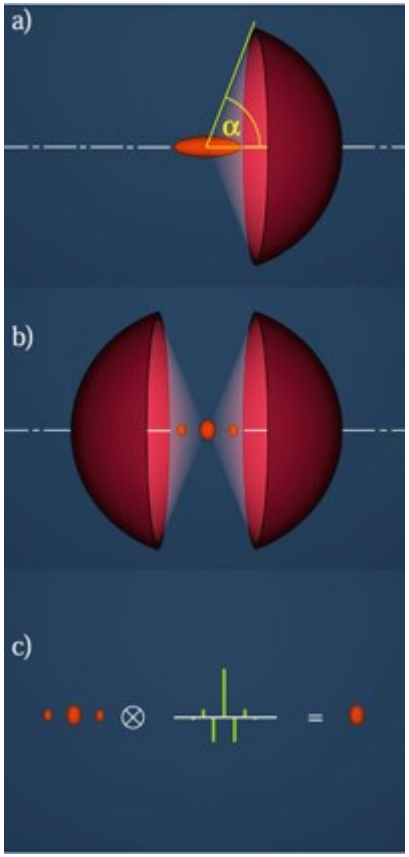
2.) Increasing NA: 4Pi microscopy

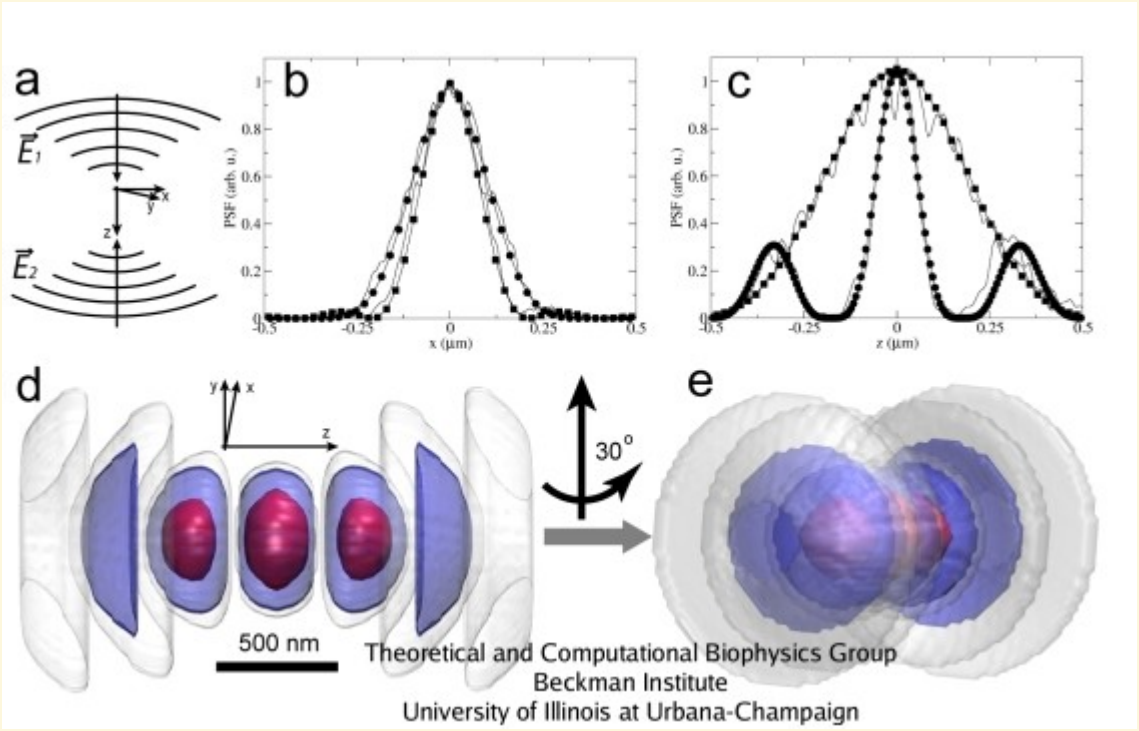




Increase the NA
by using 2 objectives

4Pi microscopy – Side Lobes

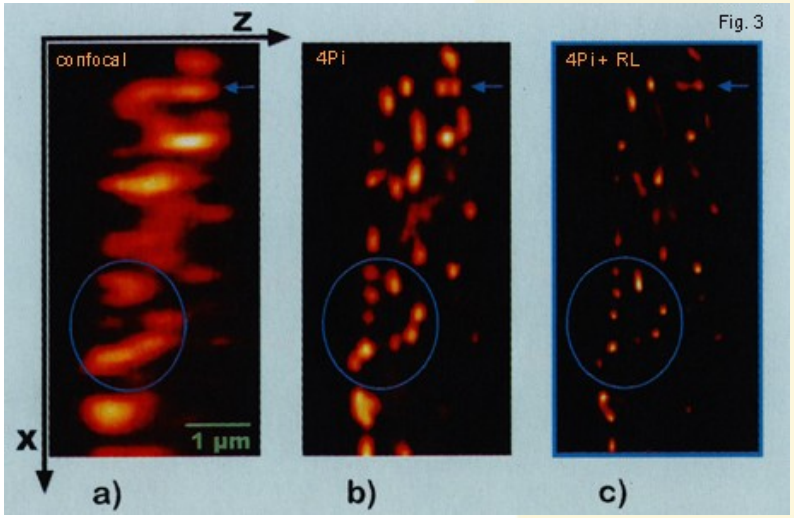




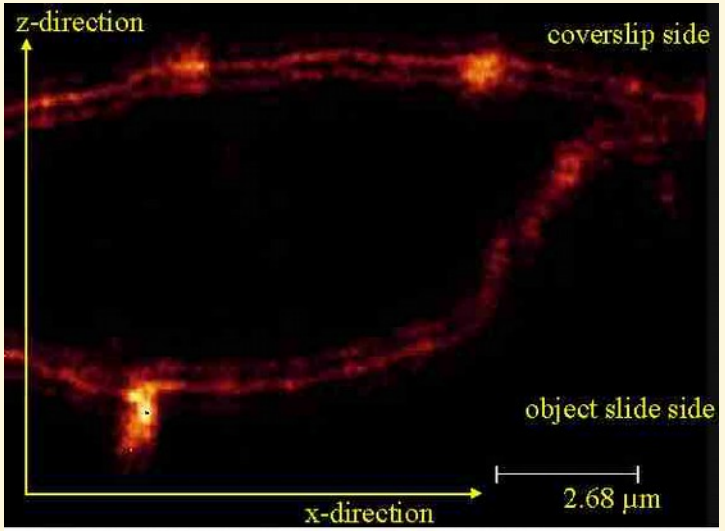
Apply filter (image processing) to get rid of side lobes

Resolution approximately uniform in all directions: ~ 100 nm

4Pi microscopy



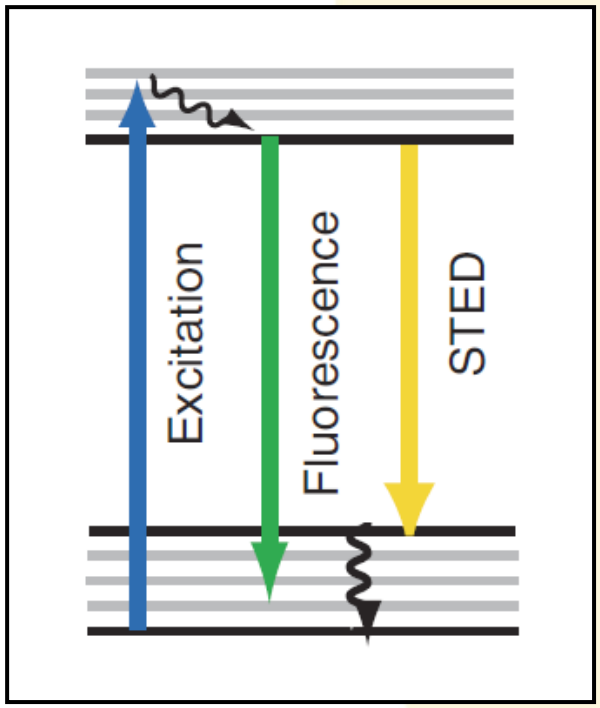
Test specimen fluorescent beads



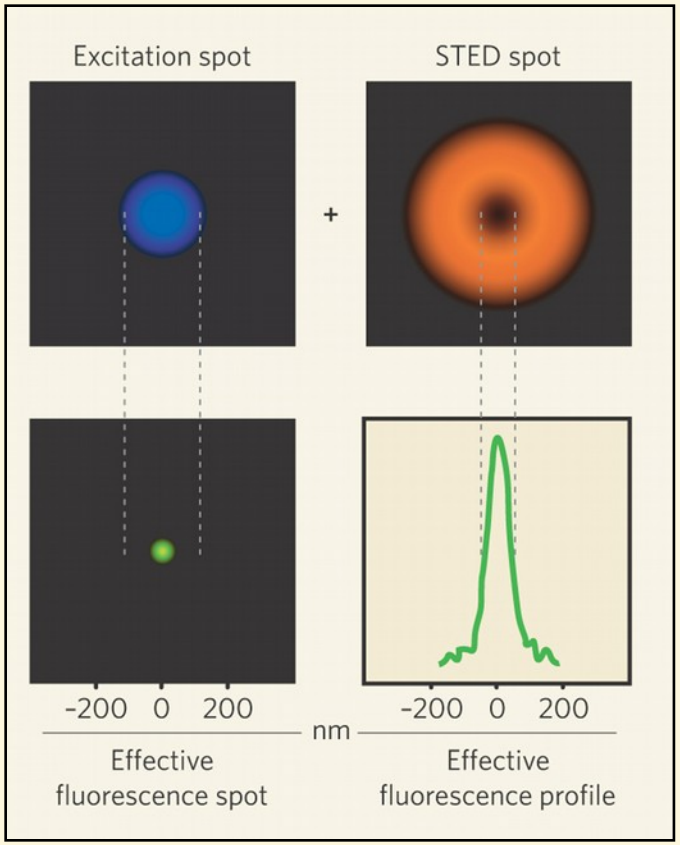
Kir2.1-GFP (potassium channel) in HEK 291 cells

Leica stopped producing 4Pi microscopes
Stability issues, hard to use, expensive, ...

3.) Shaping the Laser Focus: STED
(Stimulated Emission Depletion)



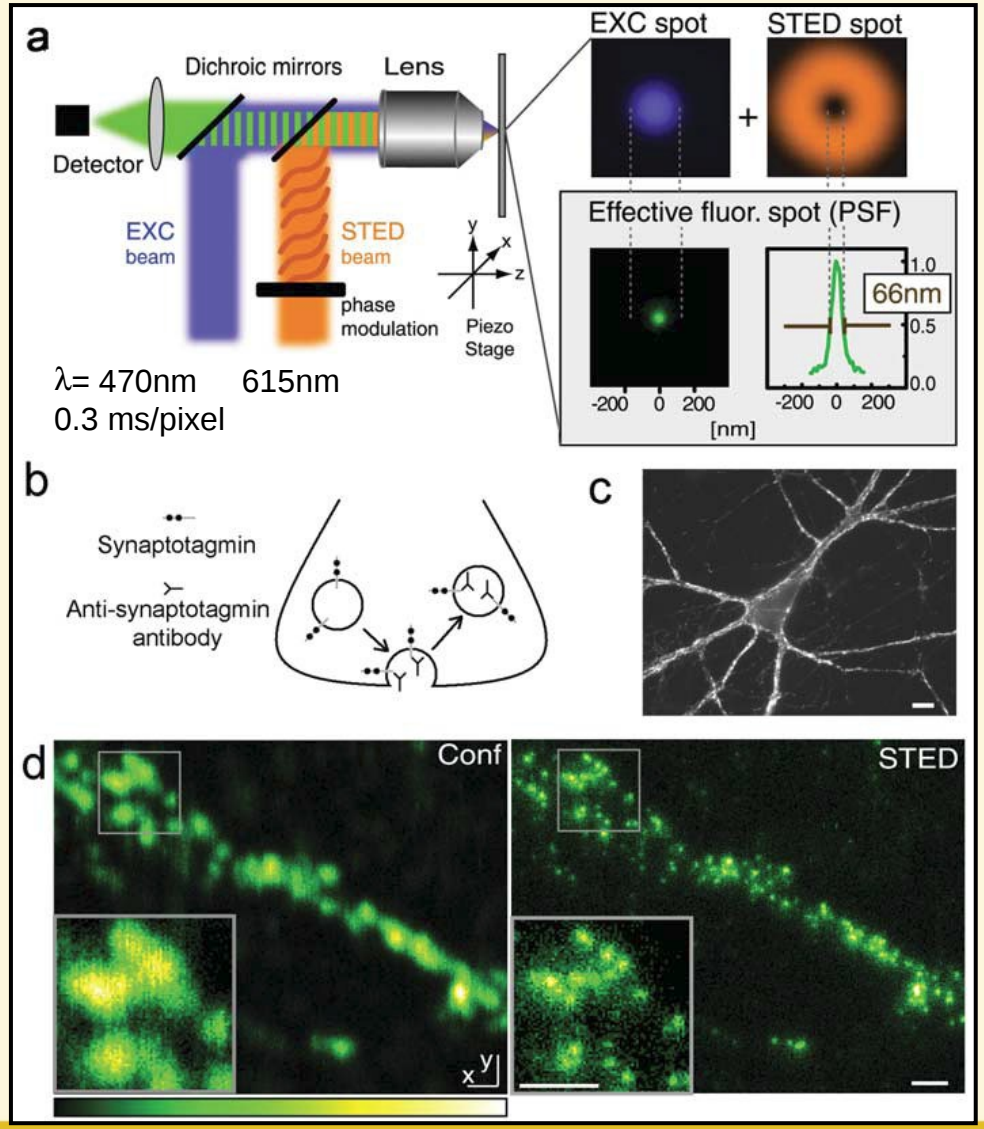
STED can be used to improve resolution



Stimulated Emission Depletion (STED) Microscopy

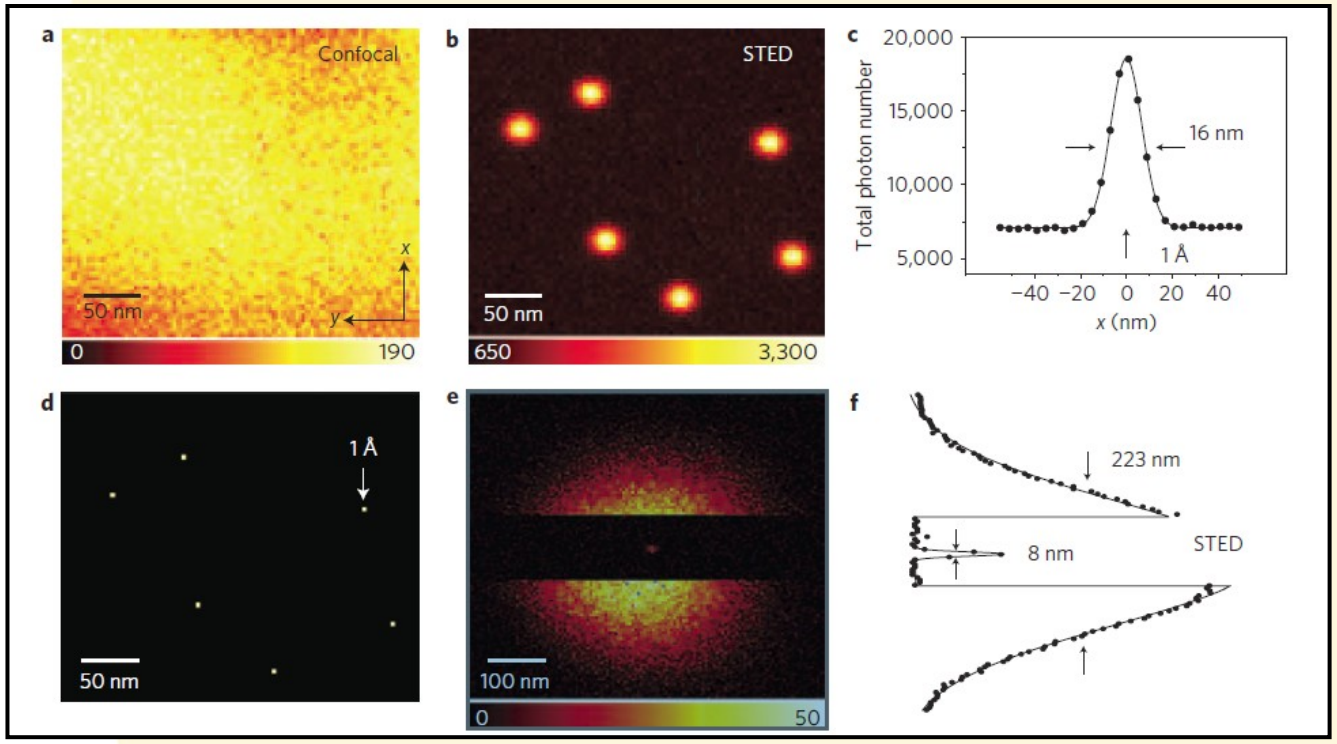
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1 + I_{max}/I_s}}$$

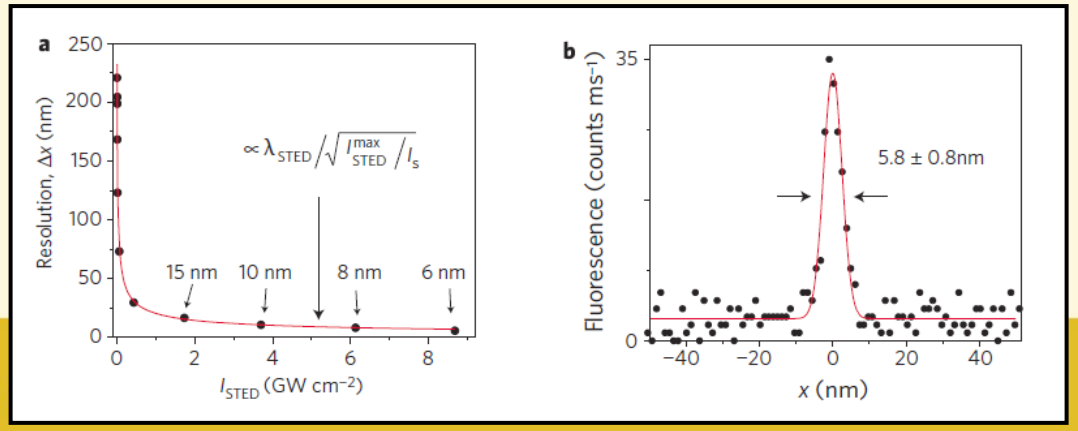
STED spot generated using a helical phase pattern (SLM,Vortex phase plate)



K.I. Willig et al. NATURE, Vol 440,13 April 2006

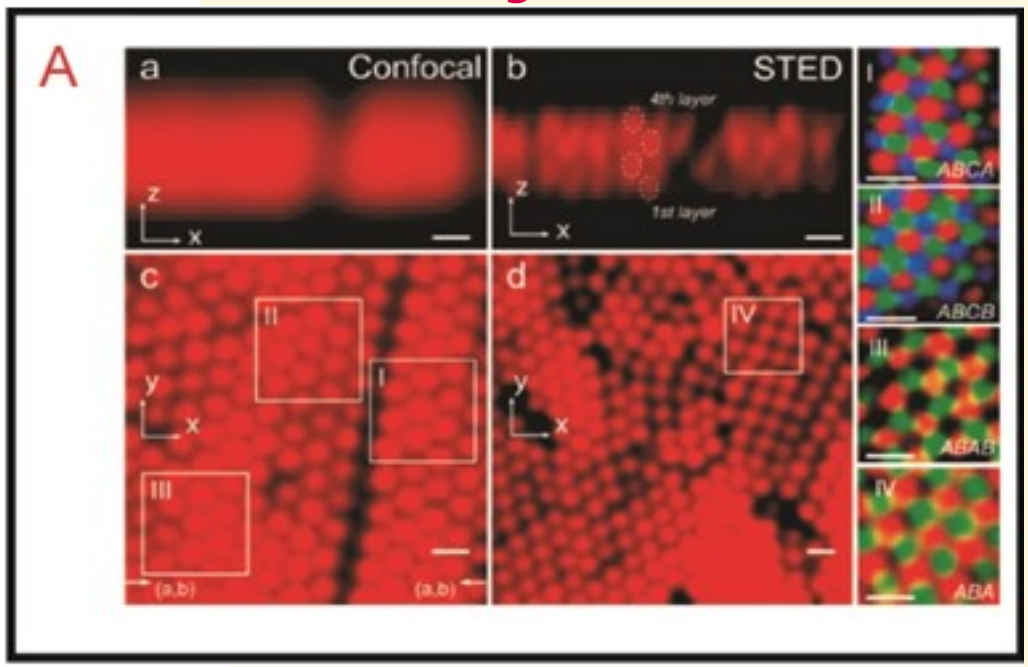
STED on Nitrogen Vacancies in diamond





E. Rittweger et al. NATURE PHOTONICS, VOL 3, MARCH 2009

STED on Colloid Crystals



Colloidal crystals, linearly deconvolved data (scale bars = 250 nm).

(a) Confocal

(b) STED images of a four-layers crystal

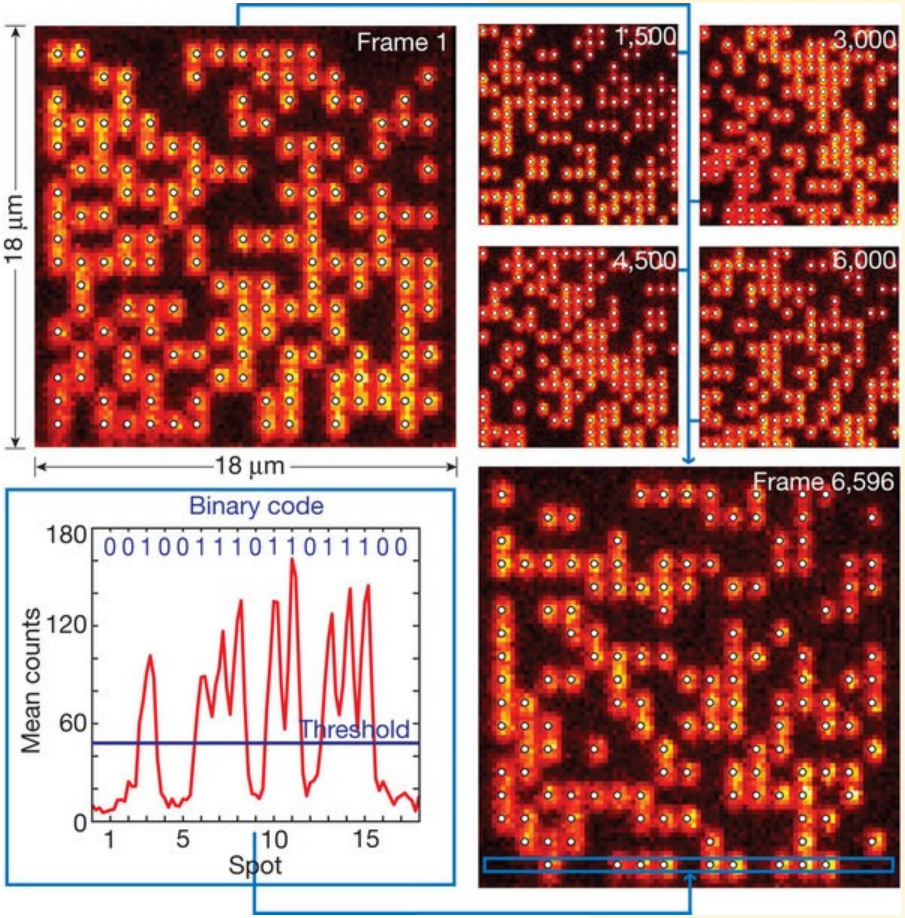
(c) First layer of the previous crystal.

(d) First layer of a three-layers crystal.

(e) Projection images of the insets

I (face centered cubic), II (randomly stacked hexagonal close packed), III (hexagonal close packed), IV (body centered cubic).

STED Fairy Tales and Photo-Switchable GFP

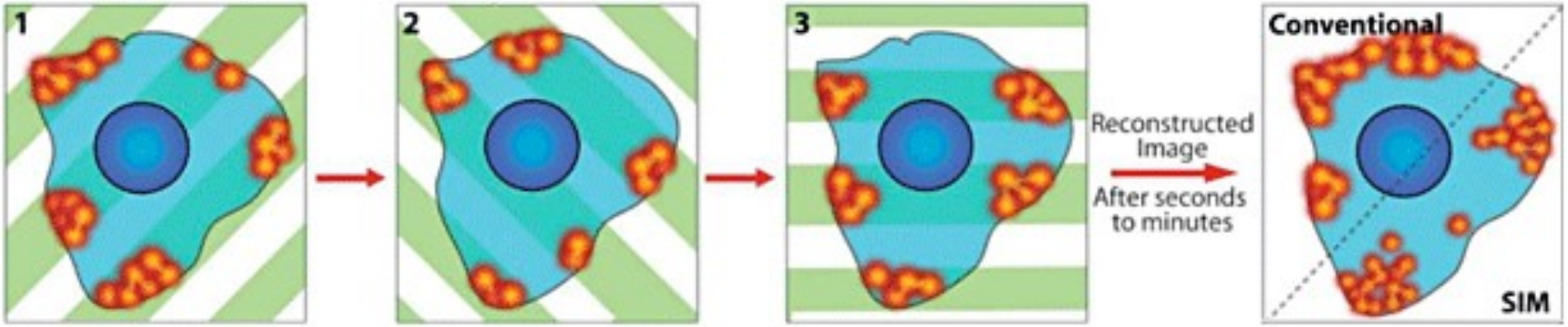


Rewritable data storage. The text of 25 Grimm's fairy stories (1.9Mbits) consecutively written and read on a 17x17 mm area of a PAA layer containing rsEGFP, with bits written as spots (representative frames shown).

The white dots mark spots that were recognized as set bits ('1's).
The graph shows an intensity profile along the indicated area, averaged over three pixels along the y-axis. The blue line indicates the threshold used to assign read spots to '0's or '1's.

200 nm bit distance

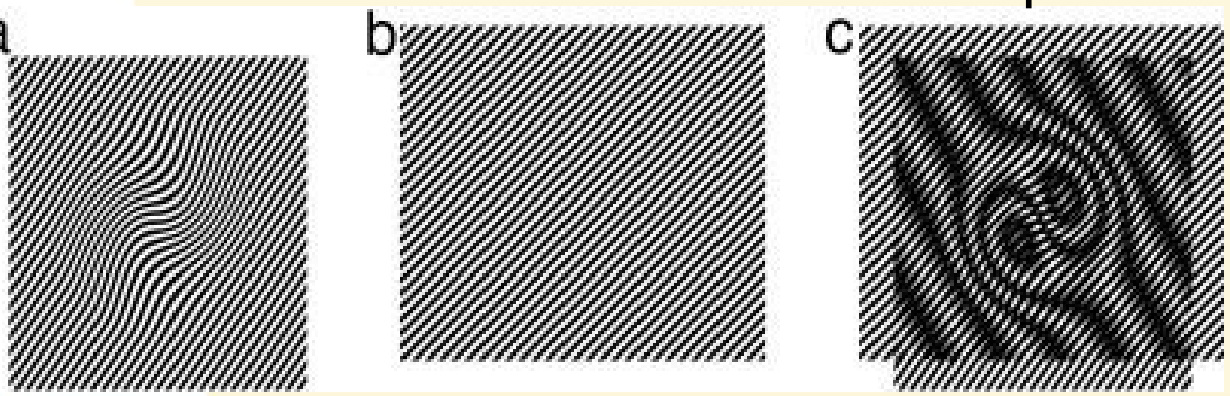
3a.) Structured Illumination



http://www.api.com/super-res-tech.asp

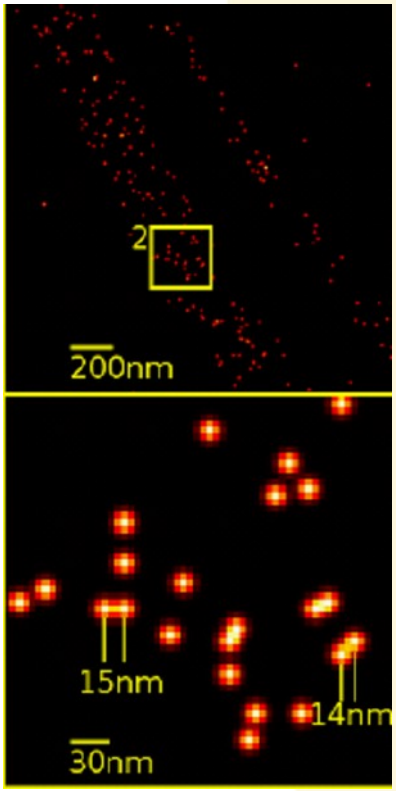
(S)SIM

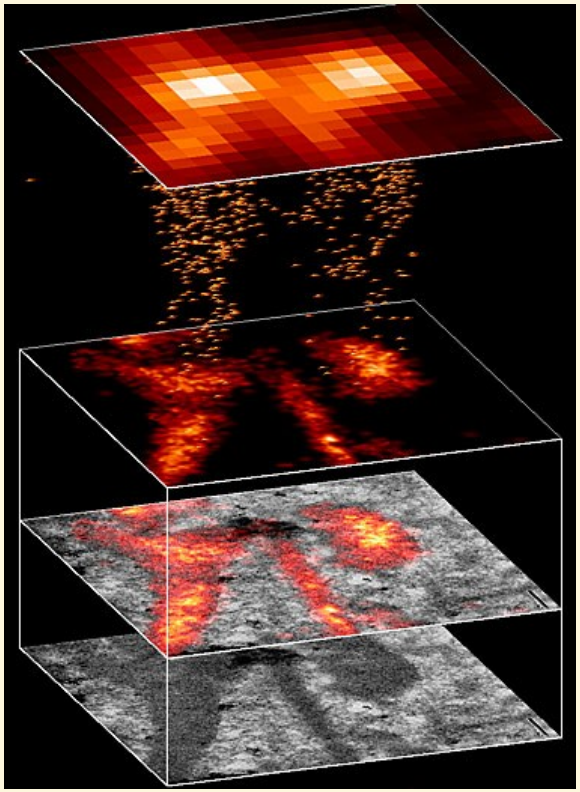
Image reconstructed from many pictures with different illumination – subresolution features show up as moire pattern



Gustafsson, M.G.: Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Micr 198:82-87 (2000)

4.) Redefining Resolution: Localization Microscopy





Record signal from single (isolated) fluorescent molecules. This can be realized by using e.g. photoactivatable fluorescent molecules

Determine *position* of the molecules by fitting. Error in position and thus the resolution depends on S/N.

Combine positions of many recordings and construct image

It Always Starts with One

Signal transduction

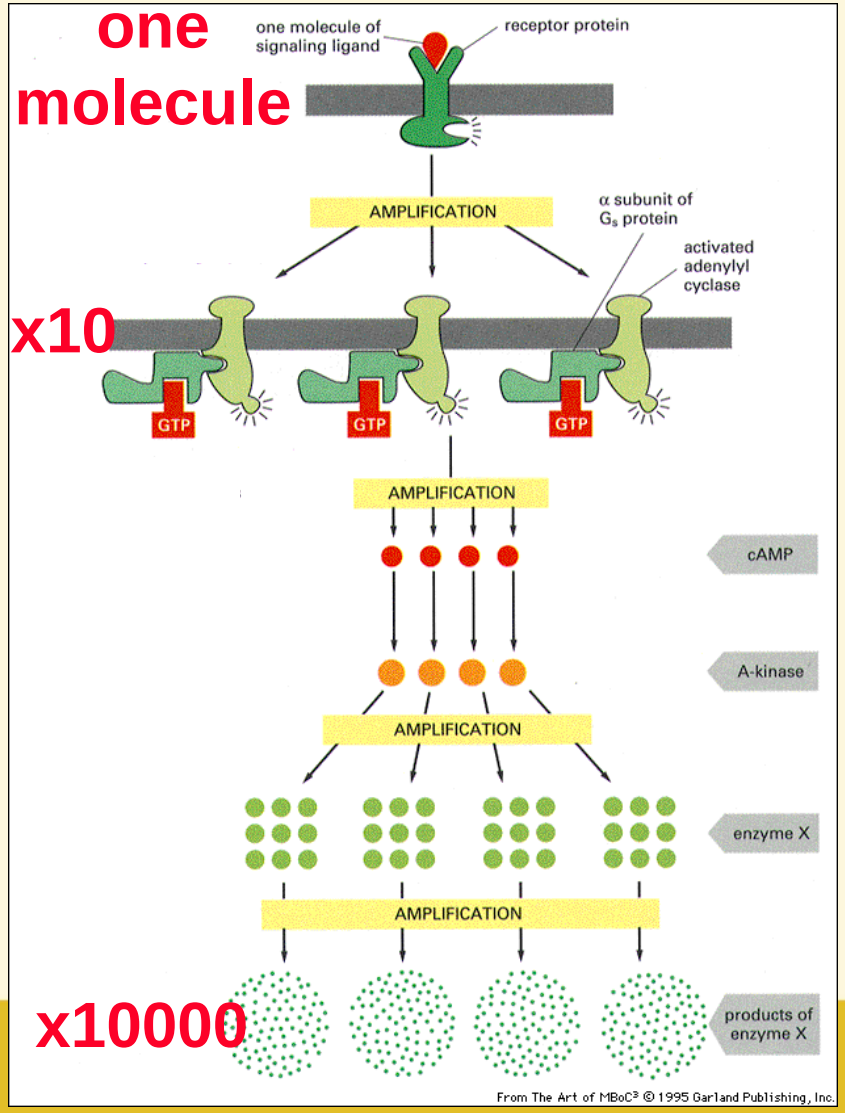
started by one molecule

Amplification steps

The "Snowball-Effect"

Final result

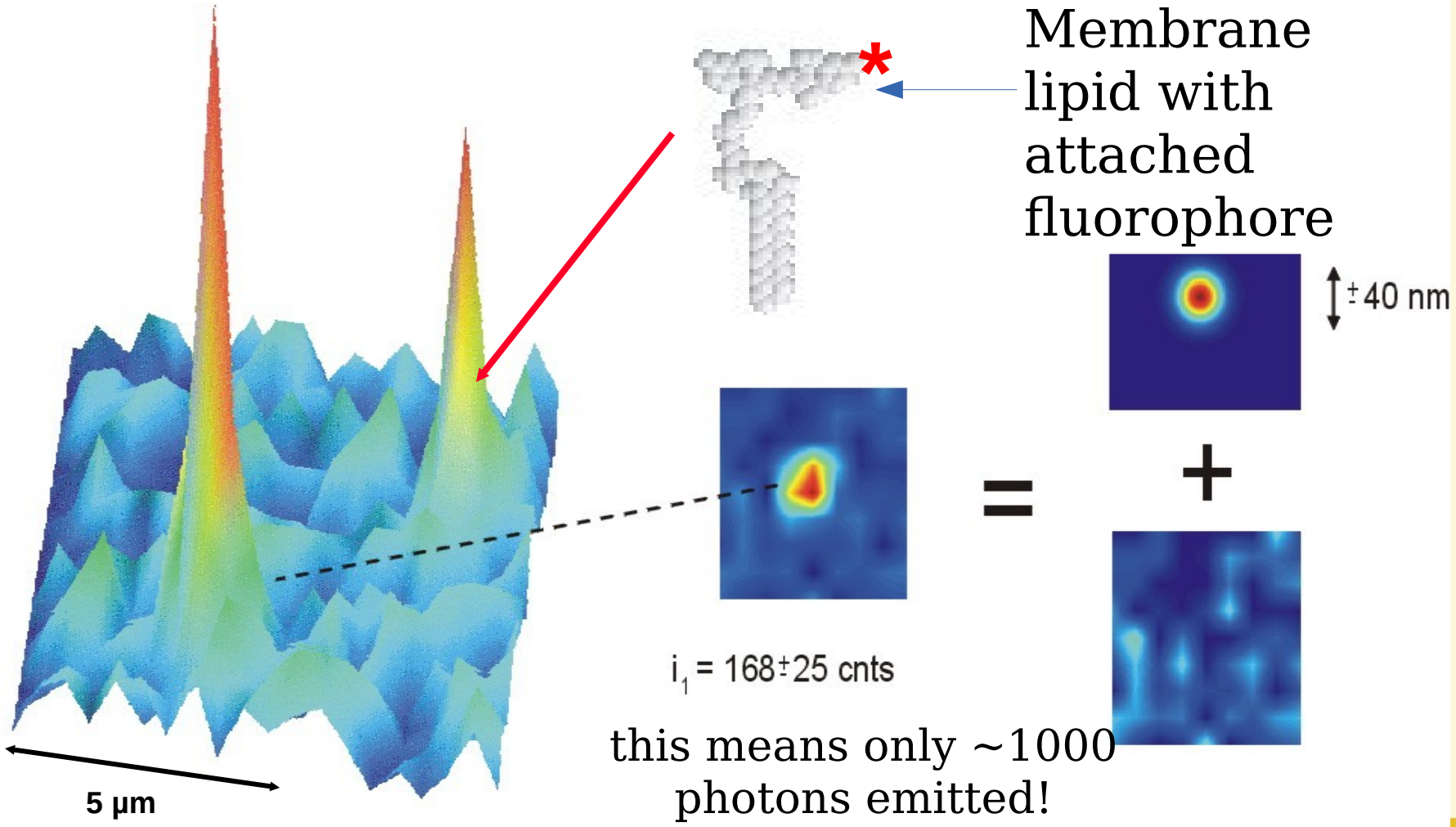
10000+ molecules



Only Use One Molecule at a Time

- The resolution of a microscope is limited by the wavelength of light
- This is not a problem if one is only interested in the position of one molecule
- To see only one molecule at a time one needs to strongly dilute my sample or to use labels on a small number of molecules of interest.
- Such labels can be metallic or fluorescent.
- There are many different fluorophores with different colors and for different applications

“Seeing” Fluorescent Molecules

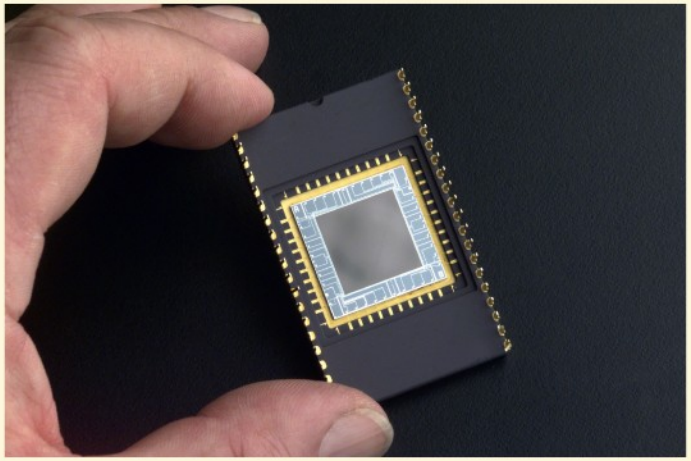


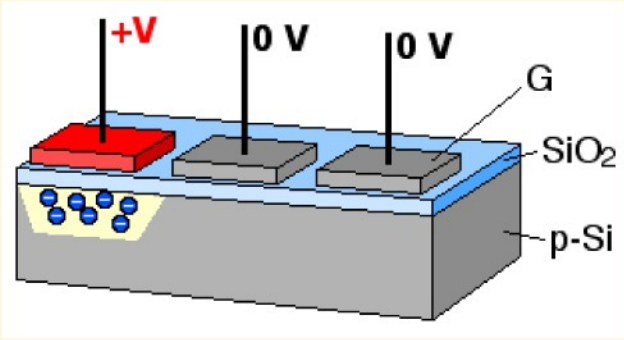
Sensitive Cameras

- Convert photons into electrons

$$\eta = \frac{N_{photons}}{N_{electrons}}$$

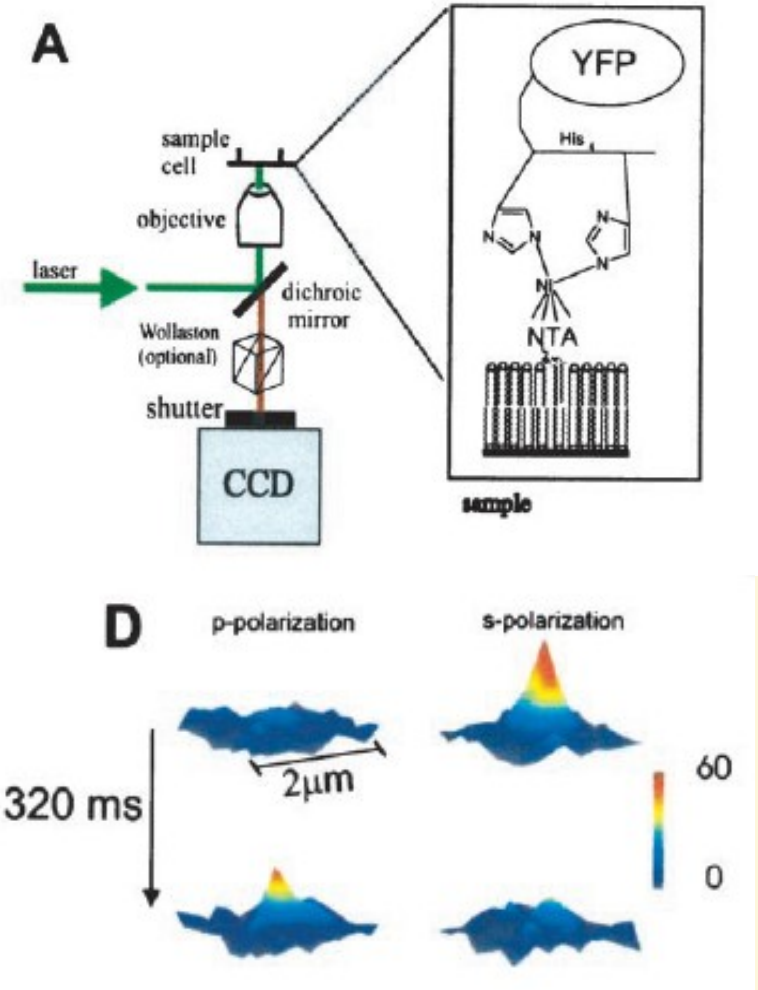
- Convert electrons into signal (amplification)
  - 'dark count'
  - Conversion noise
- Active Pixel (CMOS) – each pixel contains detector and amplifier
- Charge Coupled Device (CCD) – charge is 'shifted' into special region of chip to be read out





time scale: microseconds

Single Molecules – Really only One?



Sample Preparation and Labelli

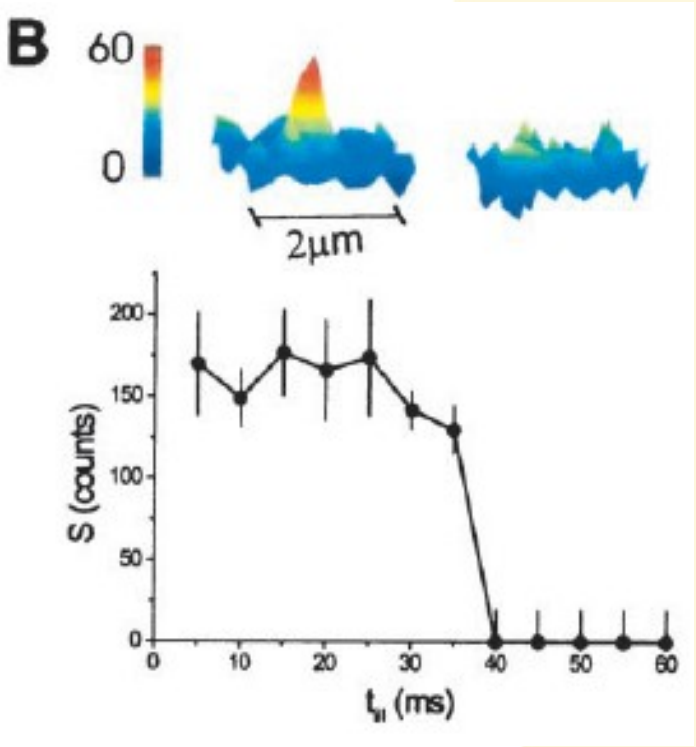
- One label per molecule
- Low Density

Fluorescence Anisotropy

- Not useful for fast rotation or slow image frequency

Image from Harms et al., BiophysJ 80 2396-2408 (2001)

Photobleaching



Single Step Photobleaching

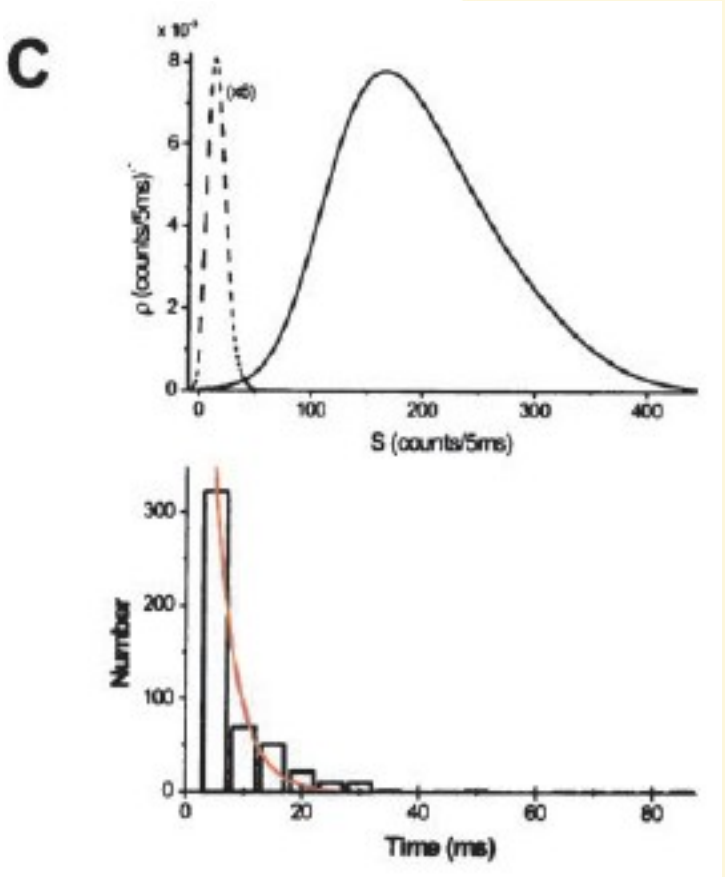
Fluorescent specimens will eventually bleach – single molecules must bleach in a single step!

$$\tau_{bl}(I) = \tau_{bl}^{\infty}(1 + I_S / I)$$

τ_{bl} ... bleaching time

I_S ... saturation intensity

Signal and Saturation



The signal obtained from a single molecule is not a fixed value, but is distributed (Poisson distribution)

$$S_{det}(I,t) = \frac{\eta_{det} k_{\infty} t}{1 + I_S / I}$$

S_{det} … signal

η_{det} ... detection efficiency

k_{∞} ... maximal photon emission rate

I_S … saturation intensity

Exercise 1: Life on a Budget

$$S_{det}(I,t) = \frac{\eta_{det} k_{\infty} t}{1 + I_S/I}$$

$$\tau_{bl}(I) = \tau_{bl}^{\infty}(1 + I_S/I)$$

- Assume a single molecule has an livetime $\tau_{bl}(I)$ after which it bleaches.
- Assume a single molecule has an average (constant) intensity given by S_{det} during this lifetime

- What is the total number of photons emitted by this single molecule during its lifetime
  - Try $I=1/2\ I_S$ and $I=2I_S$; $\eta=0.05$; $k_{\infty}=2\ 10^5$ photons/s; $\tau^{\infty}= 25$ ms
  - Find a general solution
  - What does this general solution mean?

Exercise 1: Life on a Budget

$$S_{det}(I,t) = \frac{\eta_{det} k_{\infty} t}{1 + I_S / I}$$

$$\tau_{bl}(I) = \tau_{bl}^{\infty}(1 + I_S / I)$$

- We are interested in $S_{det}(I,\tau_{bl}(I))$:
  - If you calculate this quantity according to the formula, you will find that $S(\frac{1}{2}I_S) = S(2I_S)=250$ photons
  - If you calculate the general formula, you will find that Sdet no longer depends on the intensity (the term $(1+I_S/I)$ cancels out).
  - This means that we have a fixed number of photons that we can detect, regardless of whether we measure at high power (fast bleaching, short observation time, 'snapshot') or at low power (slow bleaching, long observation time, 'dynamics').

From Budget to Accuracy

- From Excercise 1 we have seen that when dealing with single molecule fluorescence, we have a 'photon budget':
  - We can either measure a high intensity for a short time, or
  - We can measure for long, but at low intensity.

- Low intensity can cause a problem with detection!

- If I want to track / locate my molecule, I need to optimize my experimental parameters!

Exercise 2: Signal / Noise

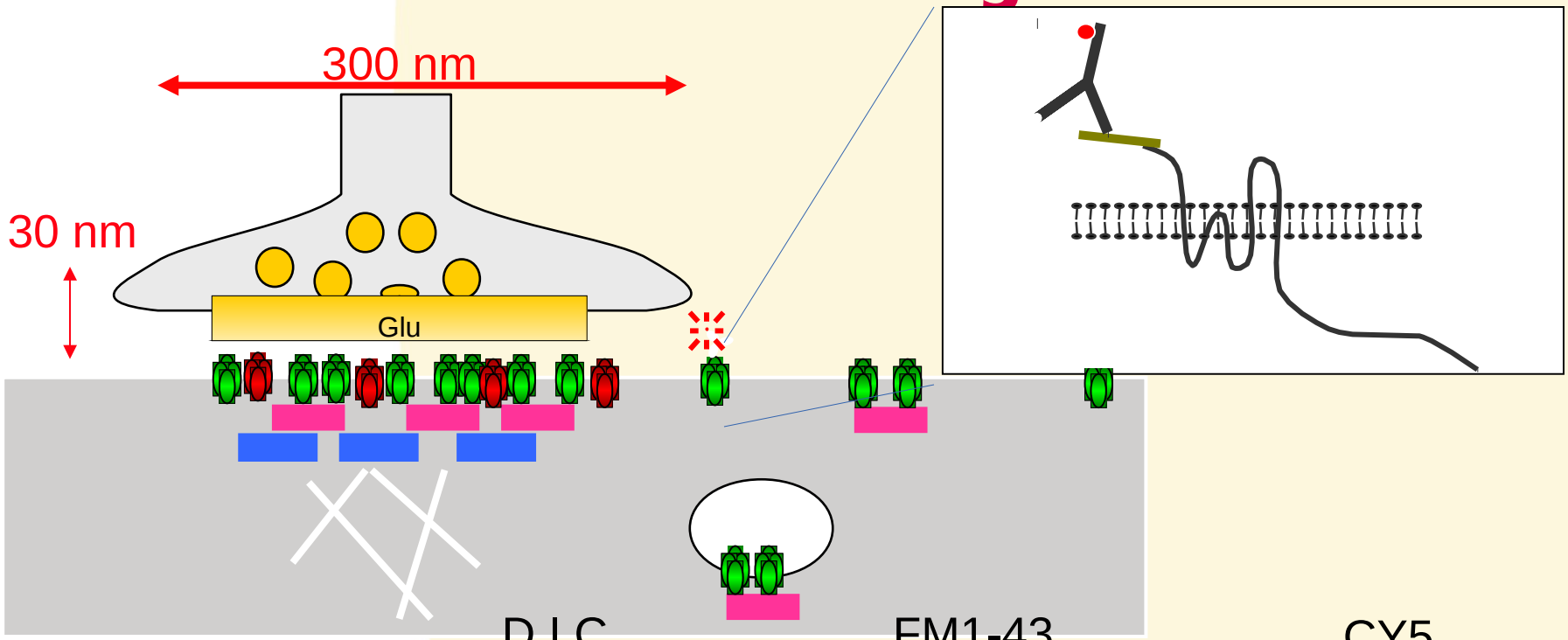
- We want to accurately localize a single molecule, thus we wish to minimise the variance $<\Delta x^2>$

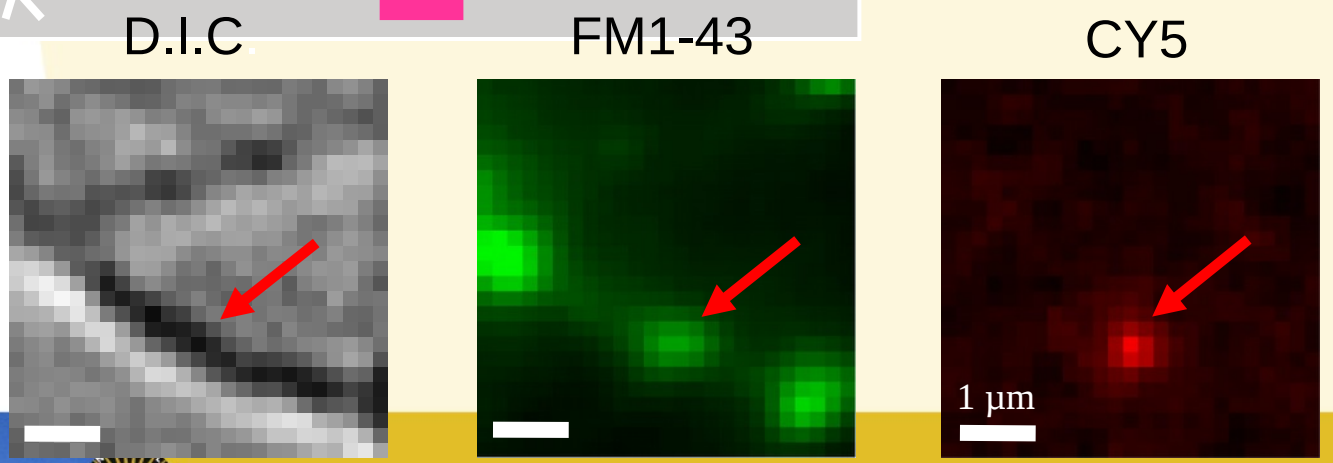
$$\langle \Delta x^2 \rangle = \frac{s^2}{N} + \frac{a^2/12}{N} + \frac{4\sqrt{\pi}\, s^3 b^2}{a N^2}$$

- s is the half width of the PSF, N is the total number of photons, a is the size of the pixel in the image, and b is the background noise per pixel.
- Discuss what the different parts of the variance represent
- Approximately what kind of accuracy Δx can I expect for single molecules (s=250 nm, N=200 cnt, a = 100 nm, b=6 cnt)
- Is there an optimal pixel size? What is the approximate accuracy there?

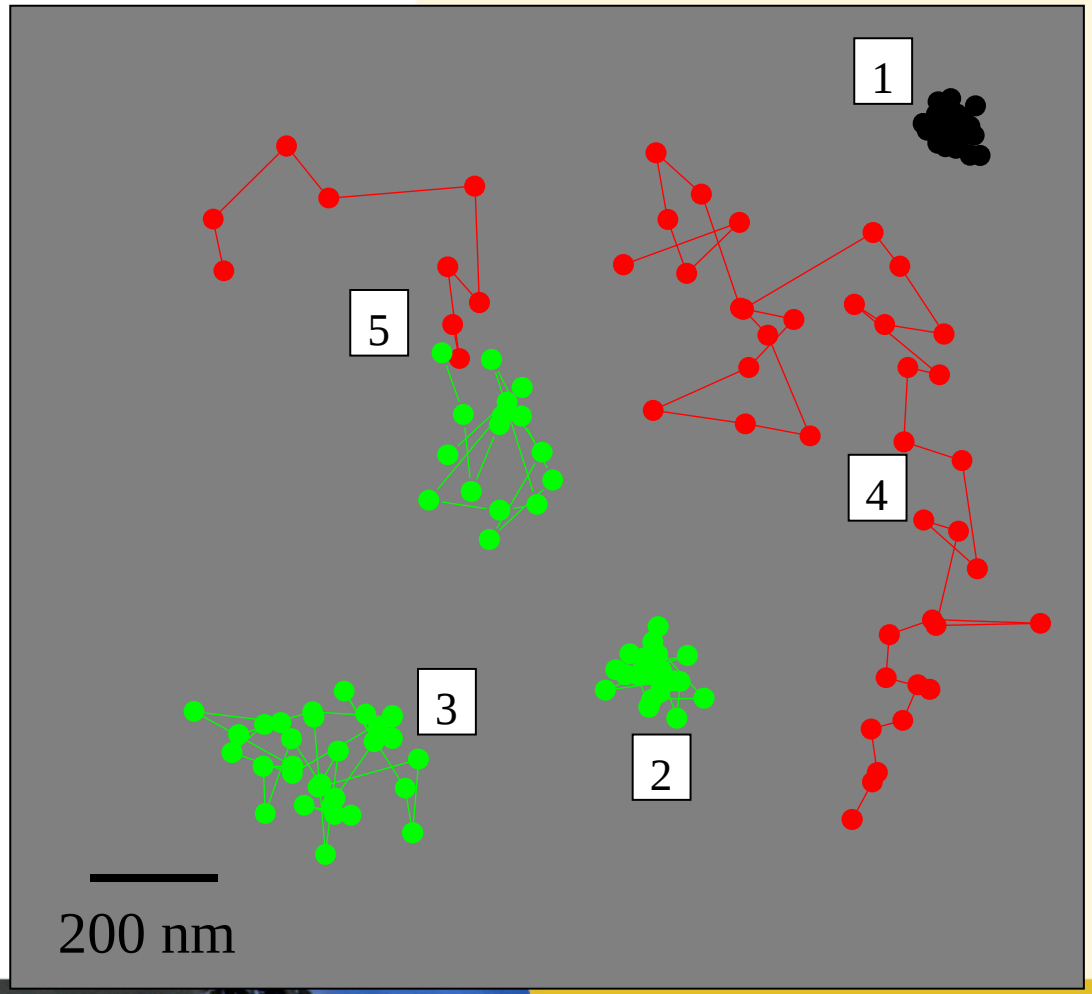
Formula from: Thomason, Larson, and Webb, BiophysJ 80:2775-2783 (2002)

The Need for Sub-Wavelength Localization





Receptors are Mobile



Fixed molecule (1)

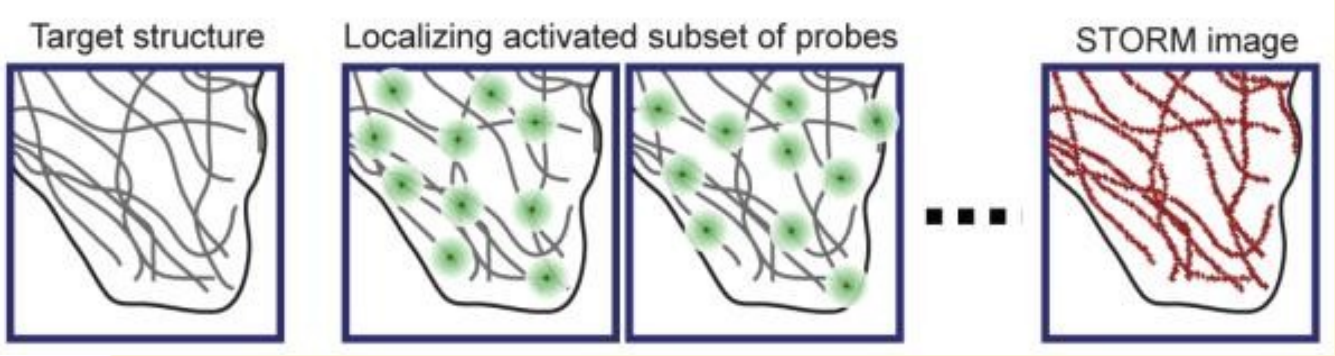
Molecules in synapses immobile (2) and mobile (3)

Molecule outside synapse (4)

Molecule entering the synapse (5)

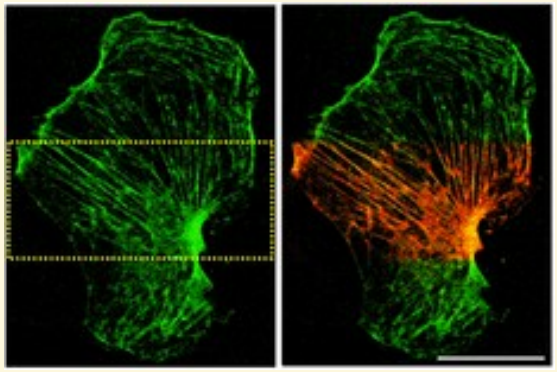
4a.) STORM, PALM

STochastic Optical Reconstruction Microscopy
PhotoActivated Localization Microscopy



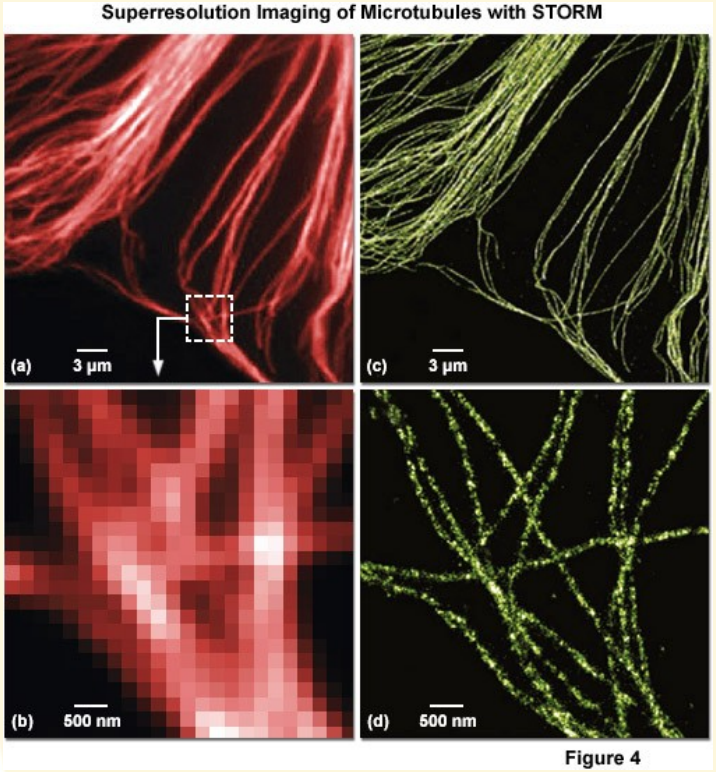
doi: 10.1038/nphoton.2009.101

- Fluorescent probes (Cy5, FP, ...) need to be activatable (STORM) or switchable (PALM)
- The Position of each fluorophore is determined
- The image is reconstructed from the measured positions



(B) Rat primary astrocyte expressing mKikGR-βactin. Images show before (left) and after (right) local photoswitching by irradiating 405 nm laser over the yellow-boxed region. Cells are cultured at 37°C.

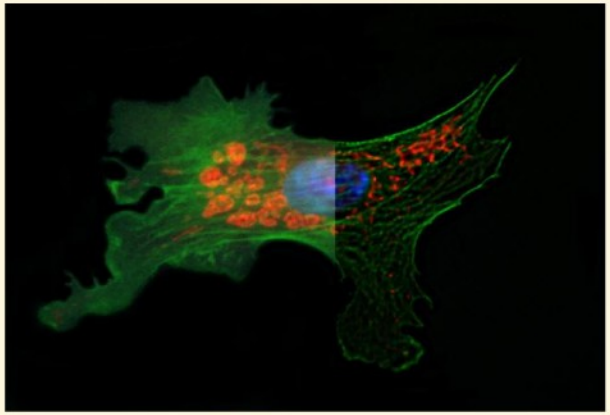
4a.) STORM, PALM



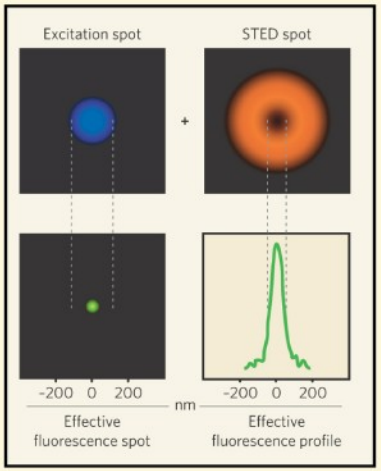
Advantage: high resolutions of 20-30 nm can be realized
not too expensive

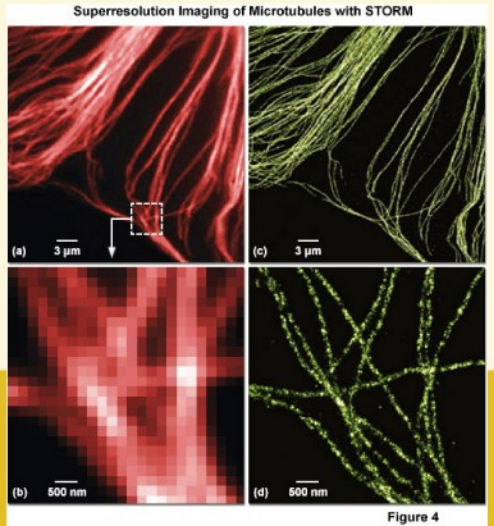
Disadvantage: slow (multiple images), requires extensive post processing

Questions?



- **Improving Data Analysis**
  - Deconvolution
- **Improving Detection**
  - 4PI
- **Improving Illumination**
  - STED, (S)SIM, GSD
    deterministic
- **Redefine "Resolution": Localization Microscopy**
  - Single molecule tracking
    PALM, STORM
    stoichiastic





http://www.BlabLab.nl