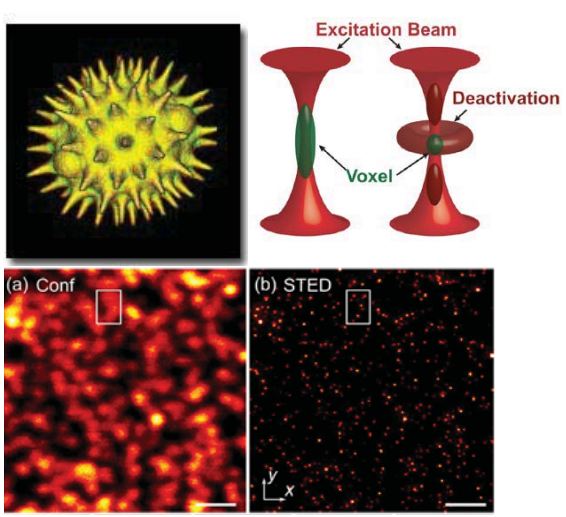
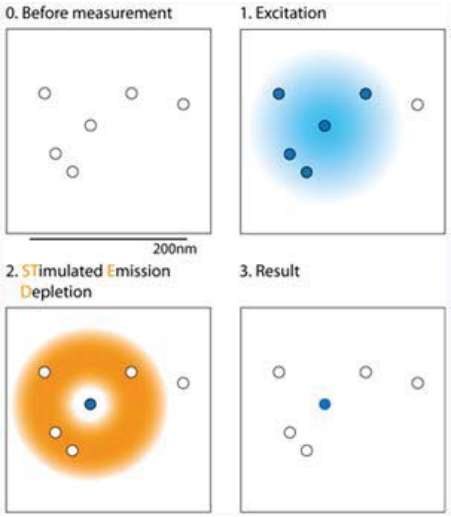
Chapter 5

Advanced Techniques

Confocal Scanning Microscopy, 3D Optical Imaging,
Super-Resolution using PALM, STORM, STED, NSOM





Contents – Chapter 5

Advanced Techniques of Optical Microscopy

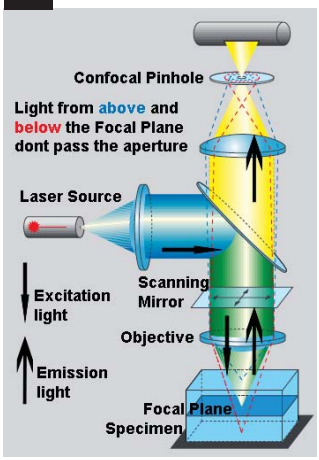
5.1 Scanning Optical Microscopy

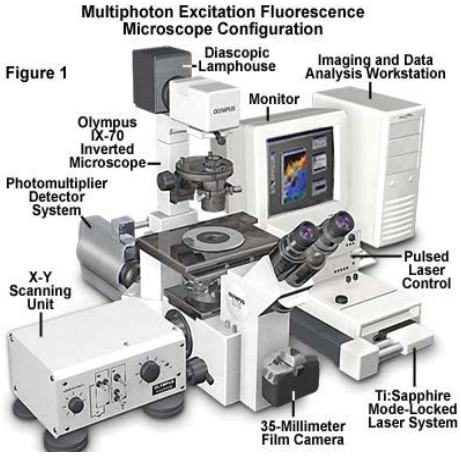
Many advanced optical microscopy methods are based on ***scanning techniques*** in which a focused light spot is ***raster scanned*** across the sample.

Detected is either (1) the reflected or transmitted light or (2) the locally emitted ***luminescence*** or ***fluorescence*** (= scanning fluorescence microscope) from the excited sample volume.

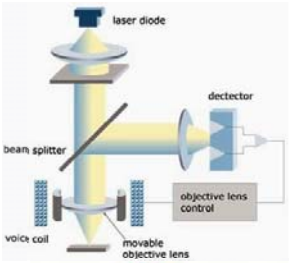
Fluorescence is usually recorded as a function of wavelength using spectral filtering (spectrometer).

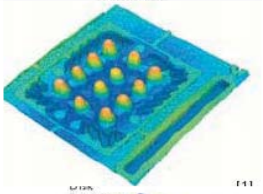
1 Scanning confocal microscope



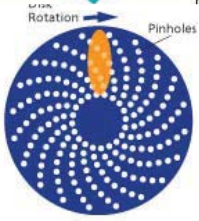


2 Scanning laser profiler



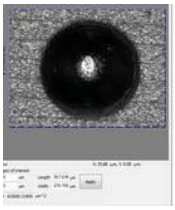


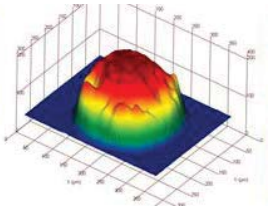
Scanning of the light spot can be achieved by (i) moving the sample underneath a fixed laser focus using a (x,y,z) scan stage, or by (ii) moving the laser spot across the sample by movable mirrors. (iii) Also, spinning Nipkow disks with pinholes and array detectors can be employed to increase the image acquisition speed.



5.1.1 Spatial Resolution in x, y and z Direction (see Chapter 5 for details)

A small spot size is obtained by *focusing* the light beam (laser, etc.) by ***optical elements*** onto the sample.

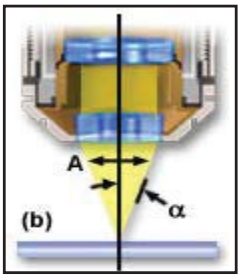




Thus, the probe size d_{probe} is limited by the *same mechanisms* as the resolution in imaging microscopy, i.e., by *diffraction* and *lens aberrations*.

The **lateral probe size** $r_{x,y}$ is given by

$$r_{x,y} = (r^2_{dif} + r^2_{aber})^{1/2}$$ where $$r_{diff} = 0.61\,\lambda / NA$$

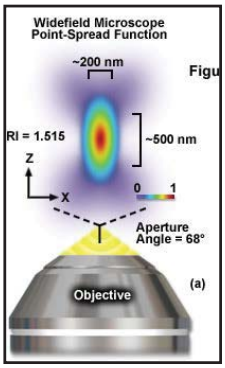


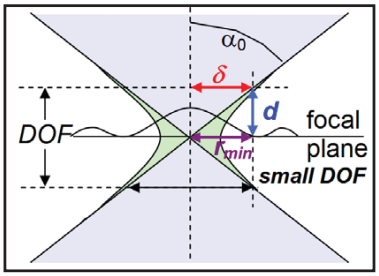
The lateral probe size therefore, decreases with

(i) increasing numerical aperture,
(ii) decreasing wavelength and
(iii) improved lens corrections

This yields at best an optical resolution of:

$$r_{x,y} \sim 0.61\,\lambda = 200 - 400\ nm$$





The **vertical probe size** is given by the intensity distribution along the *z*-axis and is essentially given by the depth-of-focus DOF of the optical system defined by:

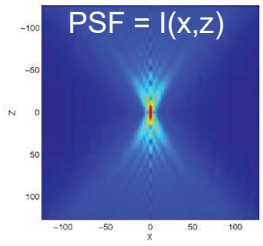
$$r_z \approx 1.22\,\lambda / NA^2$$

$$r_z \sim \lambda = 500 - 1000\ nm.$$

5.1.2 Three-Dimensional Point Spread Function

As described in Chapter 6, in general terms the resolution is given by the ***width*** *of the* microscope's ***point spread function***. For a diffraction limited optical system it is given by the **Airy function**.

Diffraction limited point spread functions of the light cone:



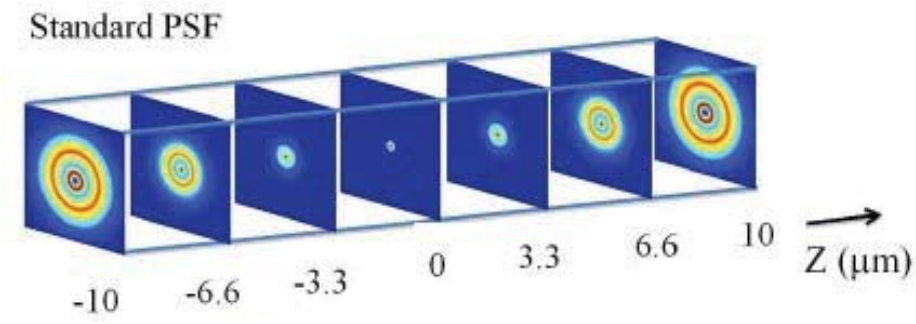
(1) Lateral PSF I(x,y):

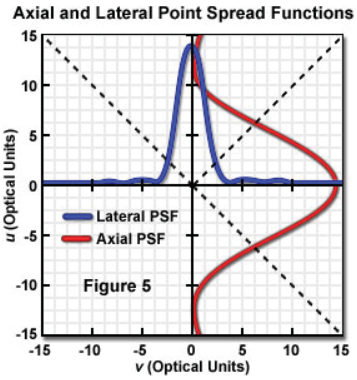
$$I_{Airy}(x, z=0) = I_0 \cdot \left[\frac{2 \cdot J_1(2\pi \cdot n \sin\alpha \cdot x / M\lambda)}{2\pi \cdot n \sin\alpha \cdot x / M\lambda}\right]^2$$

(2) Axial PSF (z, x=y=0)

$$I_{Airy}(x=0, z) = C \cdot \frac{NA^4}{M^2}\left[\frac{\sin(\pi NA^2 \cdot z / 2n * M^2\lambda)}{\pi NA^2 \cdot z / 2n * M^2\lambda}\right]^2$$

where J_1 is the first order Bessel function, α is the aperture angle, n the refractive index of the medium between the object and lens, M the magnification and λ the wavelength of the light.





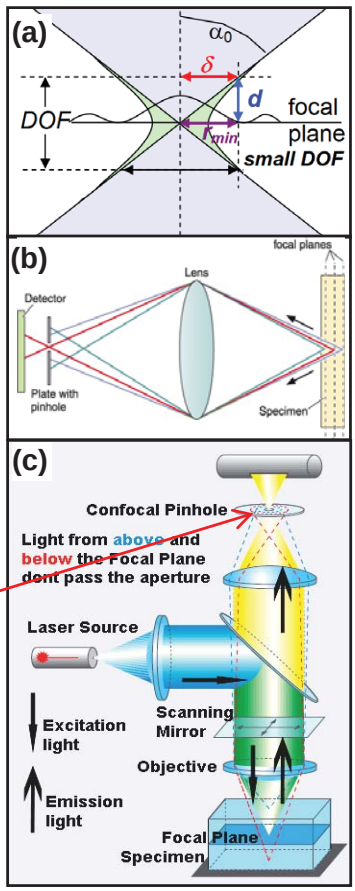
$$\Delta x_{Airy} = 0.61 \cdot M\lambda / n \sin\alpha \qquad \Delta z_{Airy} = 2n^* \cdot M^2\lambda / NA^2$$

5.2 Scanning Confocal Microscopy

Motivation:

In **conventional scanning microscopy** of semi-transparent specimen, all absorption or emission excited within the light cone of the impinging beam falls on the detector. Due to the large divergence of the focused illumination beam required for high lateral resolution, a ***relatively large sample volume is thus excited***.

However, the fluorescence/absorption from *above and below* the focal plane is ***not in focus*** of the microscope objective. Therefore, it produces a broad diffuse background signal on the detector that is superimposed on the high resolution signal collected from the focal plane. As a result, the whole image appears ***significantly blurred*** as shown on the next page.



Solution: Confocal Microscopy

In *confocal microscopy* the blurred signal from out-of-focus sample regions is eliminated such that the detected signal is restricted to the region of the focused excitation spot.

This is achieved by blocking the diffuse light emitted from the out-of-focus regions by inserting a ***pin-hole aperture*** in the back-focal plane of the objective lens, such that only the signal fromt the focal plane within the sample reaches the detector.

This means that *excitation* and *detection* are ***confocal*** to each other and thus, the detected sample region *is well localized* in space not only in the x,y but also in the z-direction.

Enhancement of Vertical Resolution

The **principle** of scanning confocal scanning fluorescence microscopy is shown on the right.

The sample is excited with a narrowly focused laser beam that is scanned over the sample.

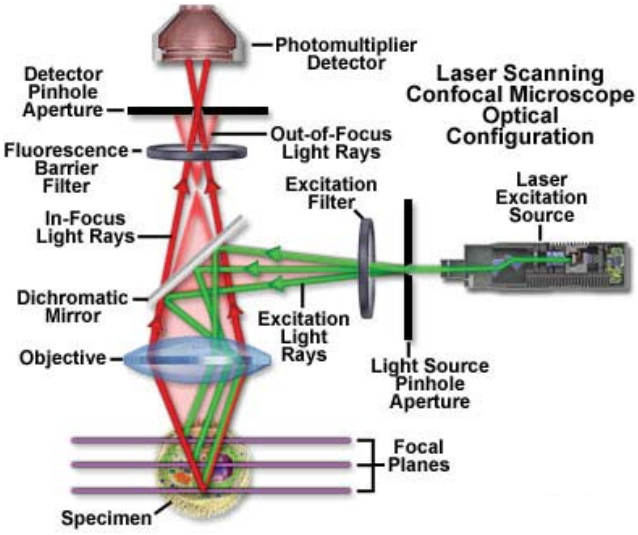
The emitted florescence is collected by a micros-cope objective and detected through a *pinhole* in the back-focal plane, which blocks the defocused diffuse light produced above and below the focal spot from reaching the detector.

The strong **improvement of the image resolution** is illustrated in the figure below.

Optical Sectioning:

Since the detected fluorescence signal now comes almost exclusively from the focal plane, the image recorded by scanning the laser beam in (x,y) direction over the samples actually corresponds to an ***optical slice*** of the sample at the focal plane.

When the focal plane is move in the vertical direction by moving the sample in *z*-direction, optical slices are recorded in different sample depths, which can be put together into a ***full 3D tomographic image*** of the sample.



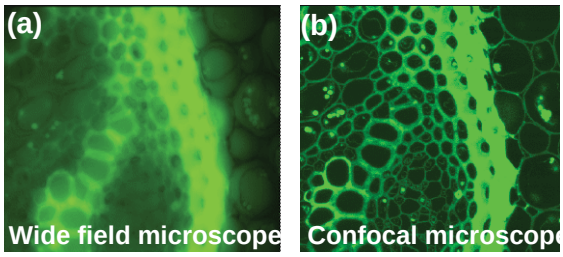
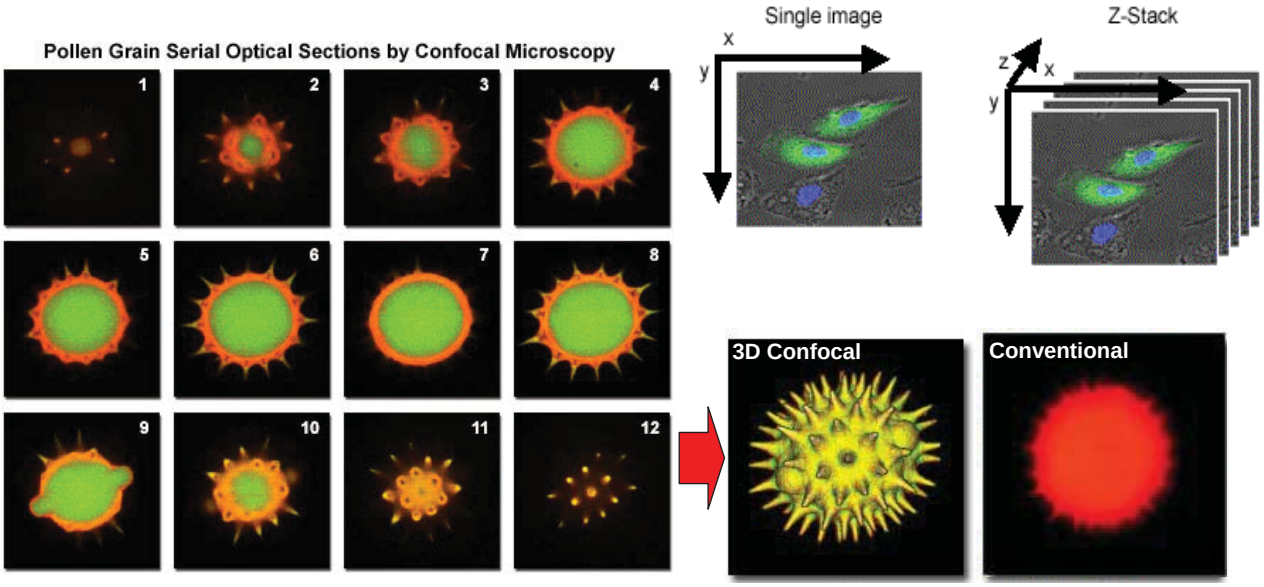


Figure: Comparison of the (a) wide field and (b) confocal microscope image of a specimen.

5.2.1 Optical Sectioning & 3D Imaging in Scanning Confocal Microscopy

The high vertical and lateral resolution of scanning confocal microscopy allows to obtain thin, i.e., 0.3 to 1 µm thick ***optical sections*** of fluorescent specimens with lateral resolution down to 200nm and vertical resolution of ~400nm. Recording a whole series of 2D images (= **z-stack**) by incremental movements of the sample (or optics) in the vertical direction, a ***complete 3D map*** of the sample is obtained. The image quality is also improved due to the reduced background fluorescence and enhanced signal-to-noise ratio.



Stack of optical sections through a sunflower pollen grain revealing internal variations in autofluorescence emission wavelength gathered in 0.5-micrometer steps perpendicular to the z-axis using a dual argon-ion (488 nm; green fluorescence) and helium/neon (543 nm; red fluorescence) laser system. Pollen grains of from this species range between 20 and 40 micrometers in diameter and yield blurred images in wide field fluorescence microscopy (see Figure on the right hand side), which lack information about internal structural details.

Examples for Optical Sectioning using Confocal Microscopy: Pollen Grain

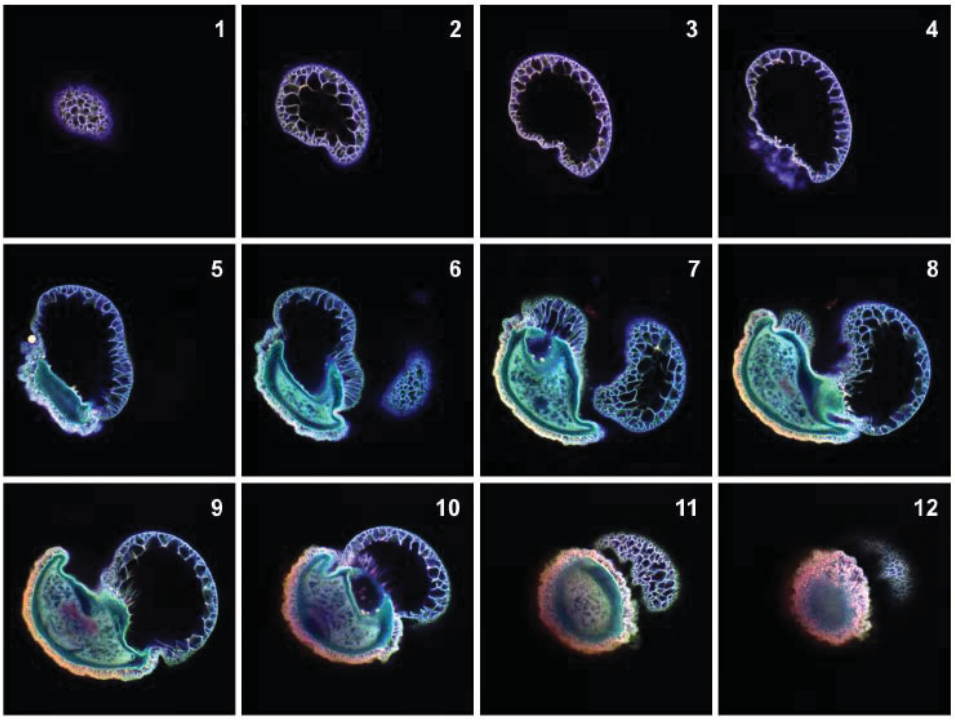
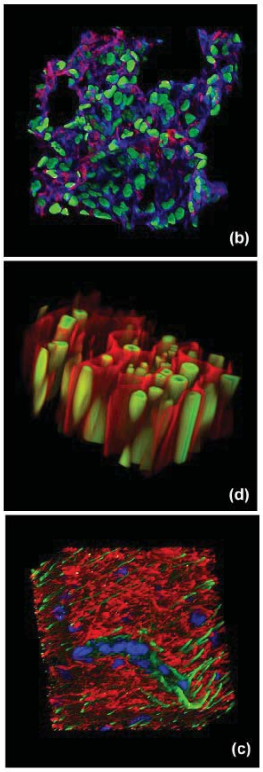


Figure 6. Lodgepole pine (*Pinus contorta*) pollen grain optical sections. Bulk pollen was mounted in CytoSeal 60 and imaged with a 100x oil immersion objective (no zoom) in 1 micrometer axial steps. Each image in the sequence (1-12) represents the view obtained from steps of 3 micrometers.

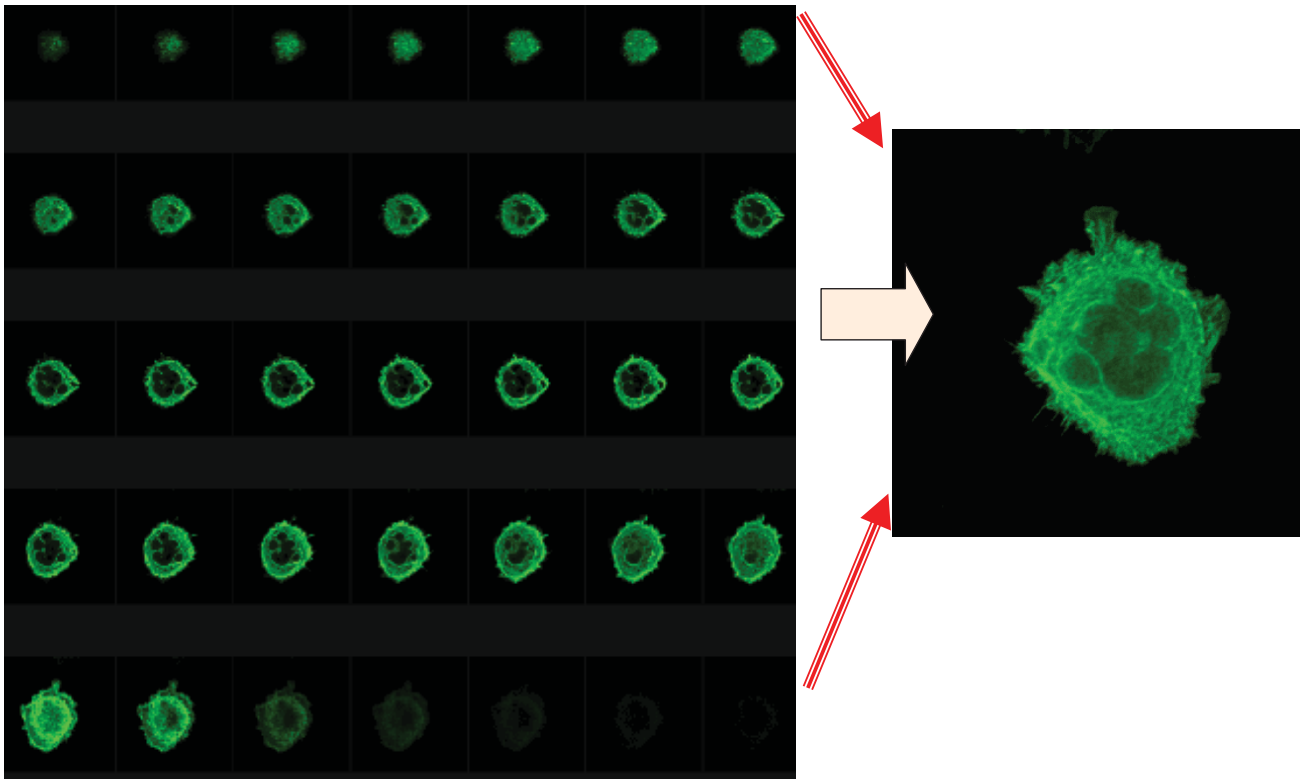


Examples for 3D representation of confocal microscopy images

Examples for Optical Sectioning and 3D Confocal Microscopy Images:

Single confocal microscopy fluorescent slices:

3D tomogram:



5.2.2 Vertical and Lateral Resolution

As described above, the resolution is generally defined by the ***width*** *of the* microscope's ***point spread function***. For a diffraction limited optical system it is determined by the **Airy function**.

For a **confocal microscope**, due to single-point illumination and confocal single-point detection only the fluorophores in the ***shared volume*** of illumination and imaging are detected.

Due to the incoherent superposition, the overall point spread function is thus the ***product*** of the ***independent illumination and detection point spread functions***. As a result, the lateral and vertical (axial) width of the total point spread function is reduced by about 30% compared to that of conventional scanning microscopy (see figure below). This yields a corresponding **factor of ~1.4** ***increase*** **in spatial resolution** provided that the pin-hole aperture is sufficiently small.

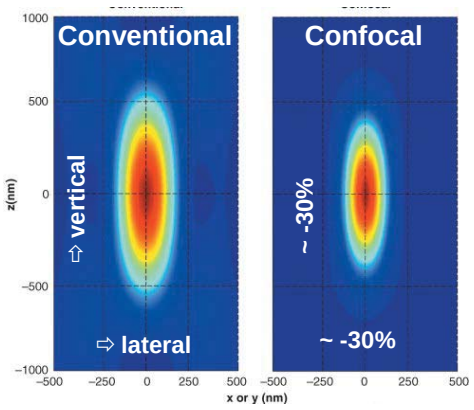
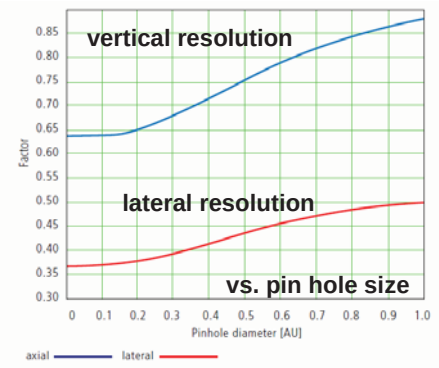


Figure: Comparison of the point spread functions PSF(x,z) for a (a) conventional and (b) confocal fluorescence microscope.

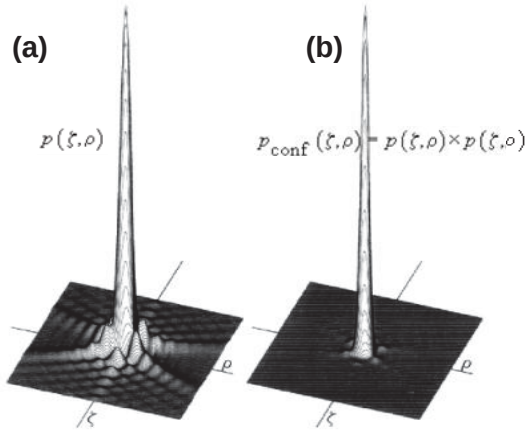
Top : *z*-resolution for conventional and confocal microscopy

Right: Dependence of the axial vertical resolution (blue) and the lateral resolution (red) of a confocal microscope as a function of the pinhole diameter.



Confocal point-spread function

of a confocal microscope: It is roughly given by the ***square*** of the usual Airy spread function of a wide field microscope (see figure below).



Left: Conventional PSF, **right:** Confocal PSF.

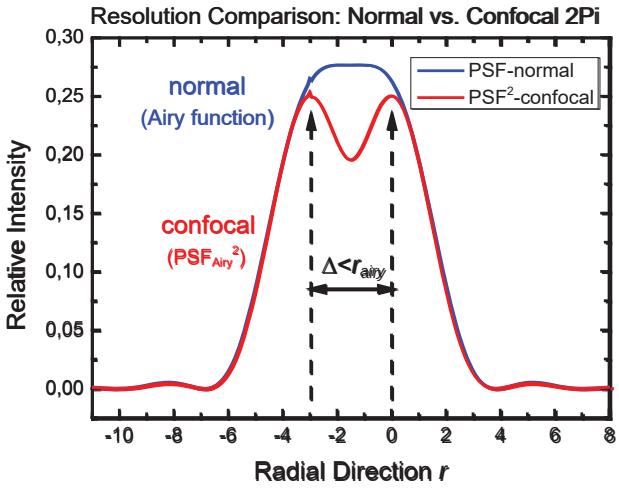


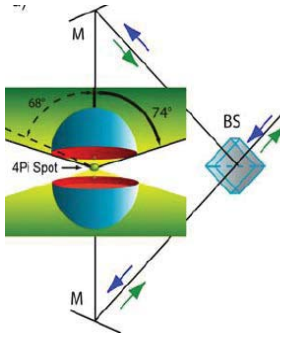
Figure: Superposition of the point spread function (PSF) of two point sources for (i) a **normal microscope (blue)** where the PSF is given by the Airy (Ai) function and $I_{tot} = Ai_1 + Ai_2$, and (ii) for a **confocal microscopy (red)** where the PSF is the square of the Airy function and $I_{tot} = Ai^2_1 + Ai^2_2$. In this case the two points are still resolved !

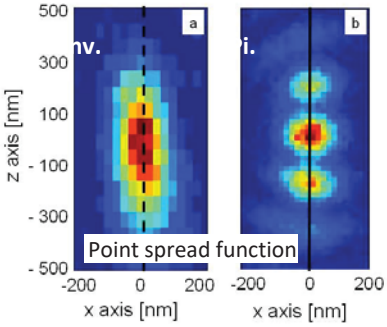
Using the **Rayleigh criterion** for the resolution, for confocal microscopy an ***increase in resolution*** as compared to a conventional optical microscopy is obtained (see right figure on top) according to:

$$r_{conf} = 0.44\frac{\lambda}{n\sin\theta} = 0.88\frac{\lambda'}{D}F \quad \text{versus:} \quad r_{resel} = 0.61\frac{\lambda}{n\sin\theta} = 1.22\frac{\lambda'}{D}F \qquad \lambda' = \lambda/n$$

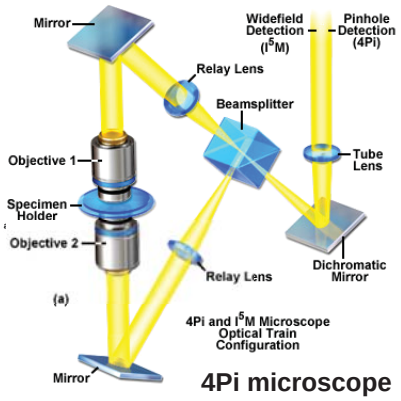
5.2.3 4Pi Confocal Microscopy

In 4Pi microscopy, the sample is illuminated simultaneously from above and below with precisely aligned high NA lenses such that a **single common spot** is formed in the focal plane. The emitted fluorescence is collected with these lenses from above *and* below. When the illumination and detection spot are precisely aligned, the illumination and collection angles each span over nearly 360° (=2Pi). The sum corresponds to **4Pi**, which is the name of this type of configuration.

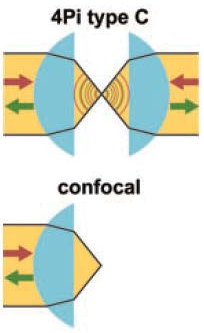


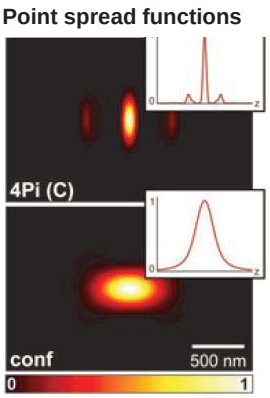


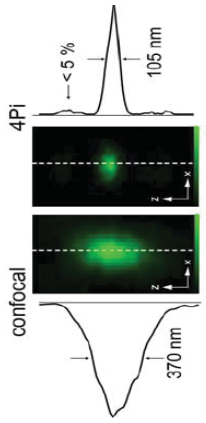
If the illumination from above and below is coherent, a sharp interference pattern is formed in the vertical direction as shown by the point spread function depicted in the figures above and below. This drastically ***improves the vertical resolution*** by as much as a ***factor of 2-3*** compared to 2Pi microscopy.
The lateral resolution, however, is unchanged.



4Pi microscope







5.2.4 Further Improvement: Nonlinear Microscopy

Nonlinear or **multiple photon processes** can be utilized to further narrow down the excitation volume to be ***smaller*** than the actual beam spot size.

In a ***nonlinear process***, the optical response *S*, i.e., absorption, excitation density, emission, optical polarization, etc. depends ***non-linearly*** on the excitation power I_{pump} according to:

$$S_{exc} = \alpha \cdot I_{pump} + \beta \cdot I_{pump}^2 + \gamma \cdot I_{pump}^3 + ...$$

⇨ At *low pump power*, the excitation S_{exc} is usually linear to the pump intensity $S \sim I_{pump}$ because the higher order coefficients $\alpha >> \beta >> \gamma >>$..... are usually very small.

⇨ At *high pump power*, the higher order terms start to dominate, *i.e.*, $S_{exc} \sim I^n_{pump}$. Thus, the excitation density is high only in the sample regions where the pump intensity is *very* high.

In the *usual linear excitation*, the detected signal *S* is: $S_{lin} \sim I_{pump} \times I_{det}$

whereas for a ***nonlinear excitation***, the signal is $\sim I^n$, i.e.,: $S_{nonlin} \sim I^n_{pump} \times I_{det}$

If we assume a Gaussian profile of the detection and excitation beam, i.e, $I_{pump} = I_{det} = I_{Gauss}(x)$, then the effective full width of half maximum of the excitation density is given by:

$$I^n_{Gauss}(x) = I_0 e^{-nx^2/2\delta^2} \qquad FWHM(n) = 1.135 \cdot \delta / \sqrt{n}$$

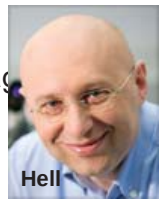
⇨ Thus, the width of the excitation volume *S*(*x*) shrinks as $\sim 1/\sqrt{n}$.

Restriction: For a multiple photon excitation process where two (or more) photons instead of one photon is absorbed at the same time to induce an electronic transition, for a given transition energy ΔE, the wavelength of the two photons must be two times larger than that of the single photon. Thus, the narrowing of the excitation volume is offset by the increase in the wavelength.

5.3 Super-Resolution in Fluorescence Microscopy

Modern ***fluorescence microscopy*** and near-field scanning optical microscopy (see Sec. 5.7) can overcome the classical Abbe diffraction limit of microscopy resolution.

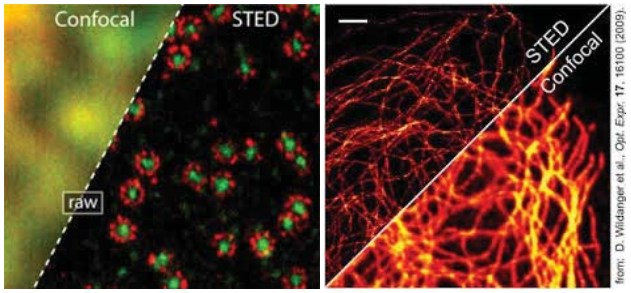
Superresolution in fluorescence microscopy is based on ***two principles***, namely, by

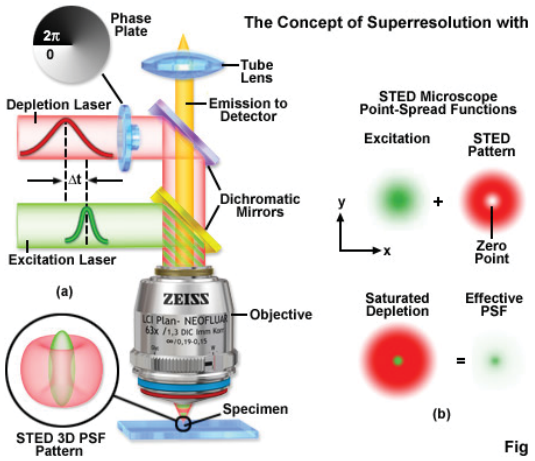


(i) ***Point spread function engineering*** = artificially *narrowing down the excitation volume* to a size smaller than the diffraction limit. This is achieved by taking advantag of the ***non-linear nature*** of fluorescence emission of molecules and its control by multiple wavelength illumination and excitation/depletion.

Example: STED = Stimulated emission depletion fluorescence microscopy, invented 1999 by Stefan Hell, MPI Göttingen, Nobel Prize 2014

Example STED:





GSD = Ground state depletion nonlinear microscopy.
RESOFLT = Reversible saturable (switchable) optical fluorescence transitions.

⇨ Because ***high power excitation*** can be achieved only with a tightly focused laser beam, this method is applied only for *scanning* confocal microscopy.

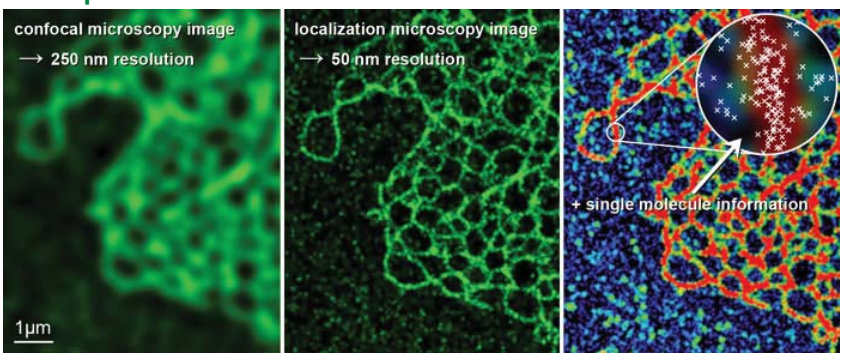
(ii) ***Single molecule localization*** = spatial localization of point-like emitters in the sample using an image processing software.

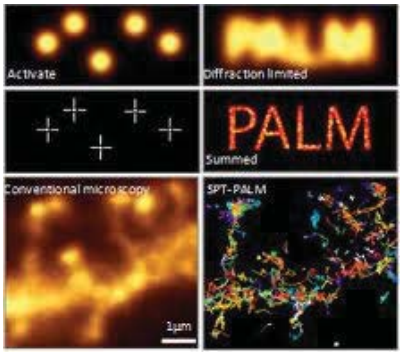
In each image only a small subset of molecules are in an emitting *emitting bright state* whereas most other molecules are in a *non-emitting dark state* => "***Localization microscopy***"

Complete images are then constructed by superposition of many images recorded sequentially.

Fluorescence localization microscopy methods (PALM, STORM, RESOLFT) were invented in 2006 by Eric Betzig at Harvard and William Moerner at Stanford (Nobel prize 2014).

Example PALM:





Background: Fluorescence Excitation and Emission

In fluorescence microscopy, *fluorophoric molecules* (or *fluorescence markers*) are introduced into the sample and these are excited with short wavelength photons to *emit light* at a characteristic fluorescence wavelength that is determined by the optical transitions within the molecule.

Fluorescence: After excitation, the electrons loose some vibrational energy within picoseconds and relax to the lowest excited singlet state from where they relax to the ground state through fluorescent emission.

Thus, the fluorescence has a ***longer wavelength*** than the excitation light (= ***Stokes shift***), as is illustrated in the fuorescence and absorption spectrum below and described by ***Jablonski diagrams.***

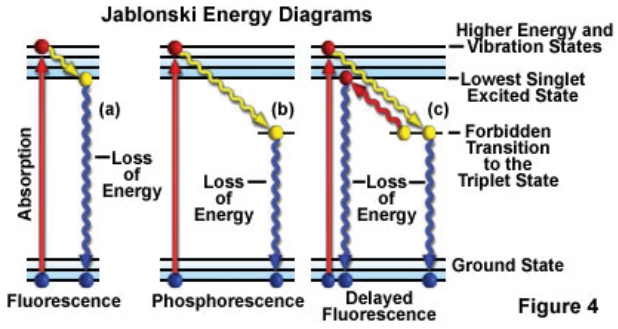
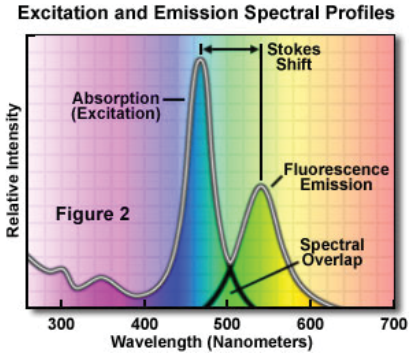


Figure 4



The Stokes shift allows to ***separate*** the emission from the excitation by spectral filters.

Phosphorescence: In some cases, excited electrons make a forbidden transition to a metastable long lived triplet state, from where relaxation to the ground by fluorescence is delayed up to several seconds.

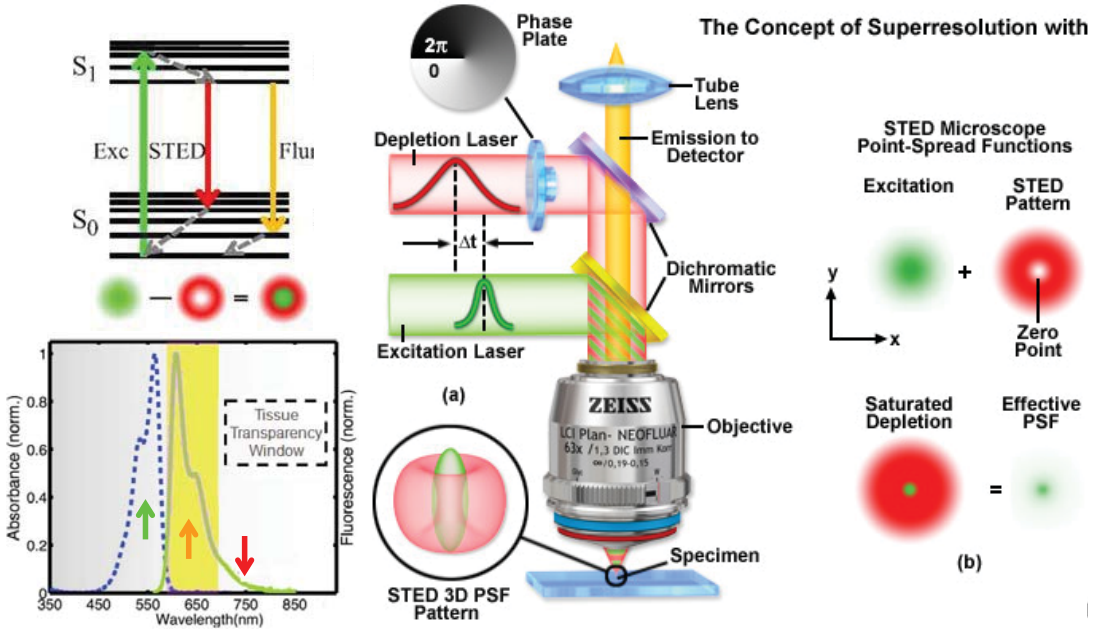
Photobleaching: Under continuous excitation many fluorescence molecules show bleaching, i.e., irreversible diminishing of fluorescence due to photo-induced reactions with oxygen or surrounding atoms.

5.4 Stimulated Emission-Depletion Fluorescence Microscopy (STED)

Working Principle: A very small excitation volume is created by illumination of the samples with ***two*** **collinear and confocal laser beams** with different wavelength *and* different beam profiles which are then simultaneously raster scanned over the sample. The two laser beams consist of a:

(i) **Gaussian-shaped *excitation laser*** at short wavelength that excites fluorescence emission at intermediate longer wavelengths (e.g. in yellow) that is detected through a band pass filter.

(ii) **Donut shaped red depletion laser** beam (=STED laser) at longer wavelength that locally ***depletes*** i.e., quenches the excited state by inducing stimulated emission from the molecules at a red (long) wavelength.

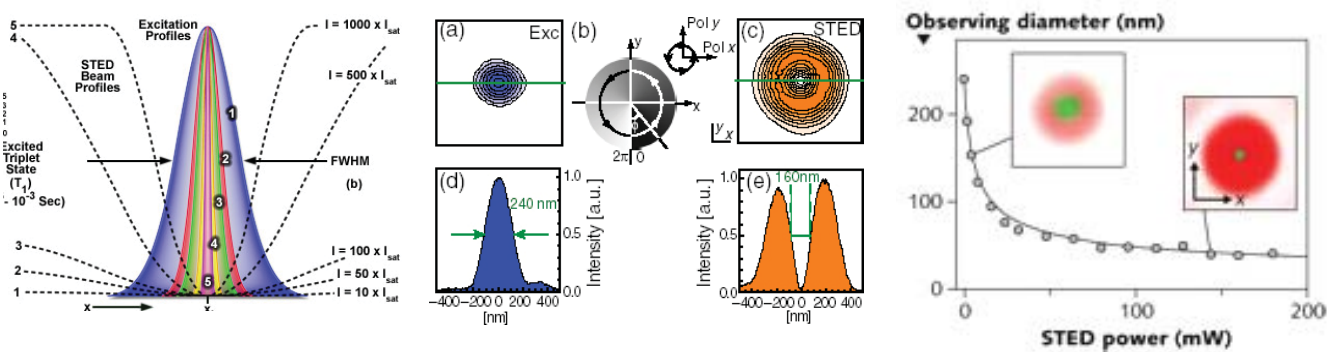


5.4.1 Shrinking the Excitation Volume and Increasing the Resolution

The high power ***depletion laser*** depletes the spontaneous fluorescence by inducing stimulated emission.

Stimulated emission is ***highly nonlinear*** and requires a certain ***threshold intensity I_{sat}*** of the STED laser (often exceeding 250 MW/cm^2). At $I >> I_{Sat}$, the fluorescence is quenched everywhere ***except at the central node*** of the donut-shaped STED beam, where its intensity of the depletion laser is nearly zero.

Thus, fluorescence emission occurs ***only*** from the center of the excitation spot with FWHM < r_{diff} .



By increasing the depletion laser intensity, the diameter of the central node where fluorescence is not quenched shrinks to a size much smaller than the Abbe diffraction limit.
Thereby, the effective excitation volume is reduced.

The ***spatial resolution Δr*** of STED is given by:

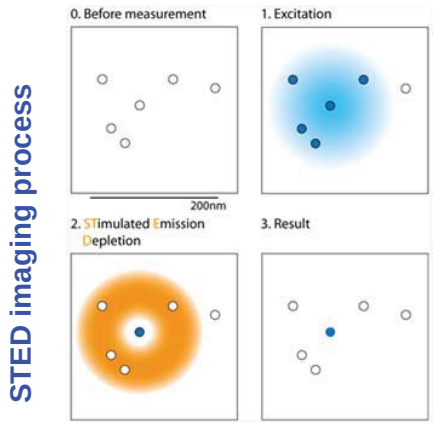
Thus, it ***improves*** with increasing STED intensity.

$$\Delta r = \frac{\sqrt{8}}{\pi\sqrt{1 + I_{\mathrm{STED}}^{\max}/I_{\mathrm{sat}}}} \frac{\lambda}{2n\sin\alpha}$$

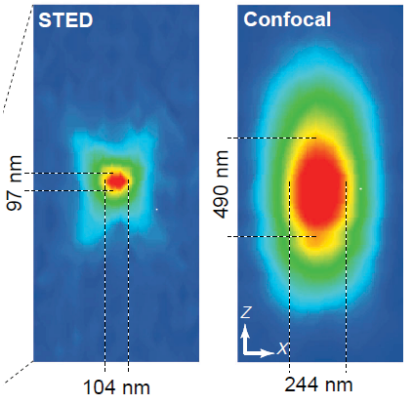
Experimentally, ***a factor of 10 improvement*** in ***spatial resolutions*** down to 10 nm have been demonstrated. Thus, high resolution images are obtained *without any image processing*, contrary to PALM/STORM.

Alternative to stimulated depletion, a transition to a non-emitting state can be also induced by driving the fluorophores into a metastable triplet state, by formation of charge-transfer states, or by photoswitching.

5.4.2 STED Imaging Process and Spatial Resolution



Point spread function



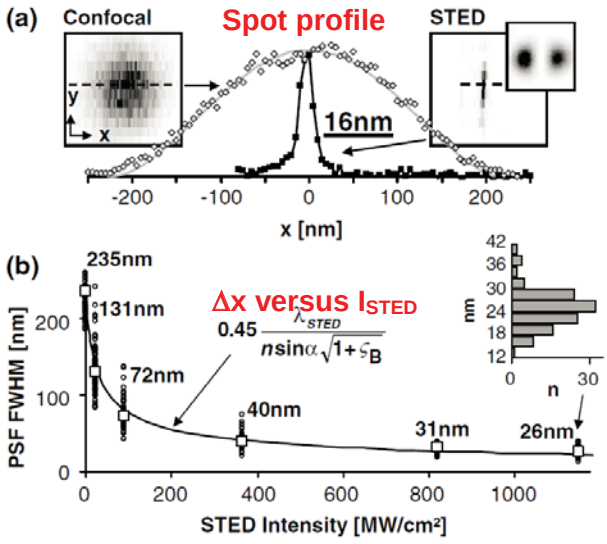


FIG. 2. Reducing the fluorescence focal spot size to far below

Figure: Reducing the fluorescence focal spot size far below the diffraction limit: (a) spot of a confocal microscope (left) compared with that in a STED microscope (right) squeezing the spot in the *x* direction to 16 nm width. (b) The average focal spot size (squares) decreases with the STED intensity following a square-root law, in agreement with the equation shown as insert. The right insert shows the histogram of the measured spot sizes with 26 nm average FWHM.

V. Westphahl & S. Hell, PRL 2005

5.4.3 Examples for High-Resolution STED Microscopy

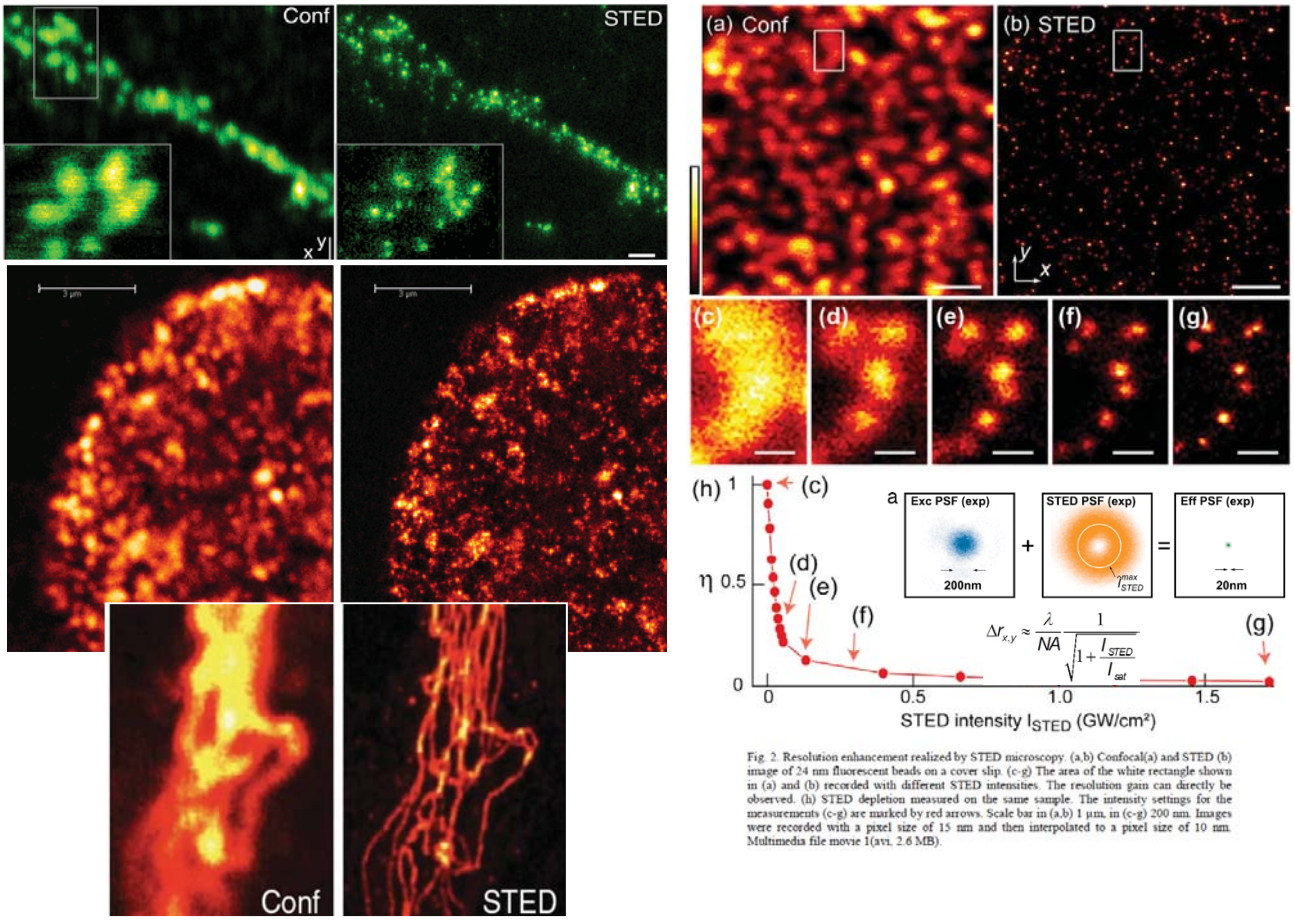


Fig. 2. Resolution enhancement realized by STED microscopy. (a,b) Confocal(a) and STED (b) image of 24 nm fluorescent beads on a cover slip. (c-g) The area of the white rectangle shown in (a) and (b) recorded with different STED intensities. The resolution gain can directly be observed. (h) STED depletion measured on the same sample. The intensity settings for the measurements (c-g) are marked by red arrows. Scale bar in (a,b) 1 µm, in (c-g) 200 nm. Images were recorded with a pixel size of 15 nm and then interpolated to a pixel size of 10 nm. Multimedia file movie 1(avi, 2.6 MB).

Example for a 2Pi STED set-up

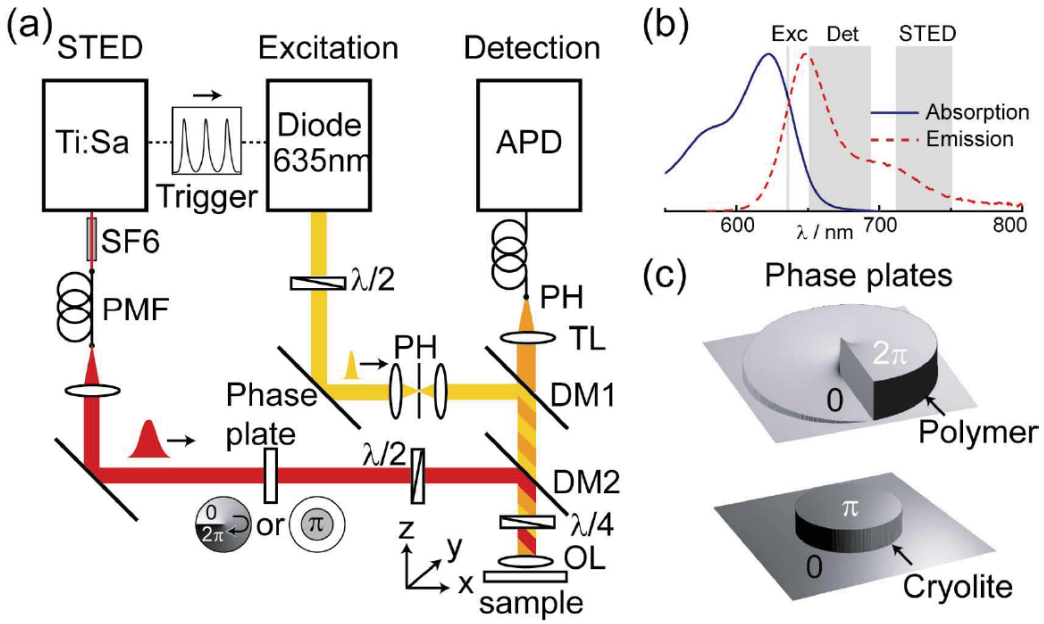
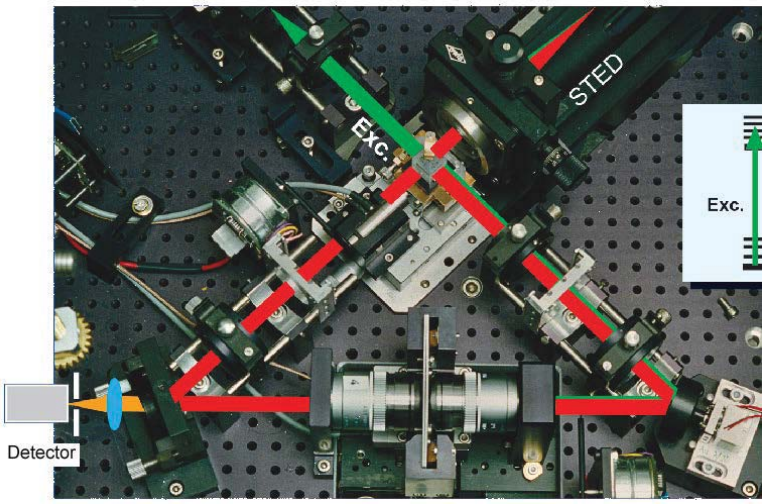
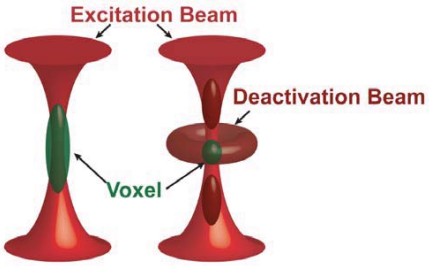
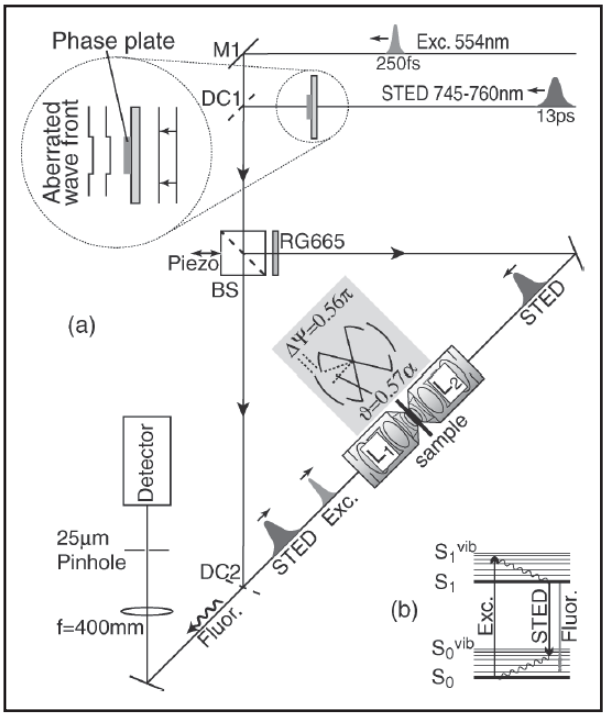


Fig. 1. Experimental platform; (a) Setup. PMF: polarisation maintaining fibre; APD: avalanche photo diode; PH: pinhole; TL: tube lens; DM: dichroic mirror; OL: objective lens; SF6: glass rods. (b) Absorption and emission spectrum of a solution of 24 nm fluorescent beads used in the presented measurements. (c) 3D view of the phase masks used for the measurements.

By applying a 4Pi excitation geometry, also axial resolutions down to 50 nm have been demonstrated. Thus, fluorescence microscopy has developed into a powerful nanocharacterization technique, which however is applicable only to self-luminous samples containing appropriate fluorescing molecules.





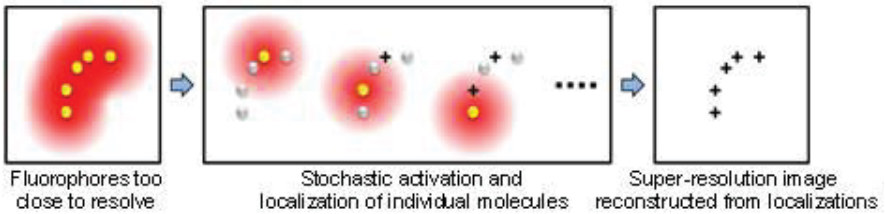


5.5 PALM/STORM Single Molecule Localization Microscopy

PALM = Photo-Activated Localization Microscopy, **STORM** = Stochastic Optical Reconstr. Microscopy

Working principle: PALM and STORM determine the precise location of individual single-molecule emitters incorporated in the sample by switching the fluorescence emission "on" only for small subsets of the molecules at a time such that their average distance is large enough to precisely determine their positions.

From a whole series of images, in which each time different molecules light up, a high resolution image can be constructed.



Resolution: Although the emission of each molecule creates a resolution limited broad signal on the detector array, by fitting the intensity distribution for each "blob" by a Gaussian, the *position* of each *individual molecule* can be determined with an accuracy of down to *few nanometers*.

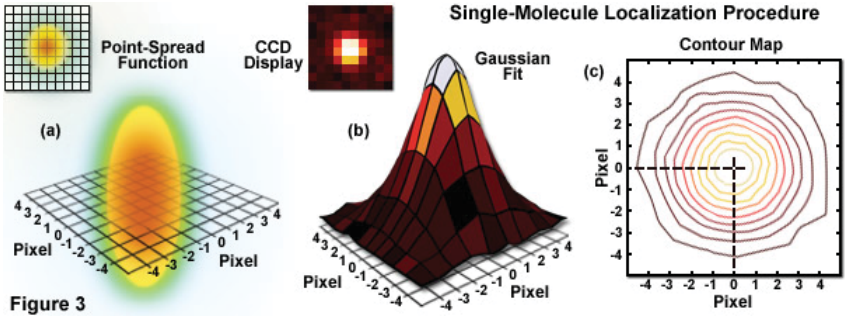


Figure 3

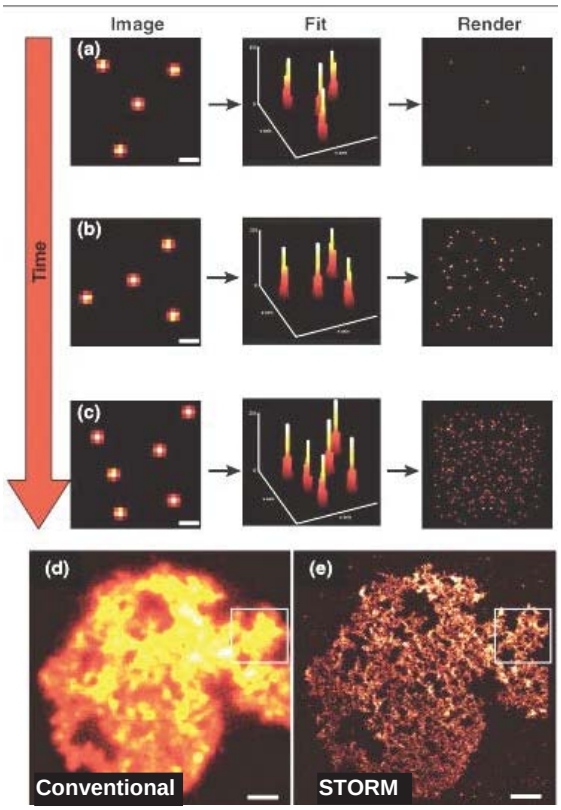
This requires that enough photons are gathered from each molecule and that their separation is larger than about 200 nm.

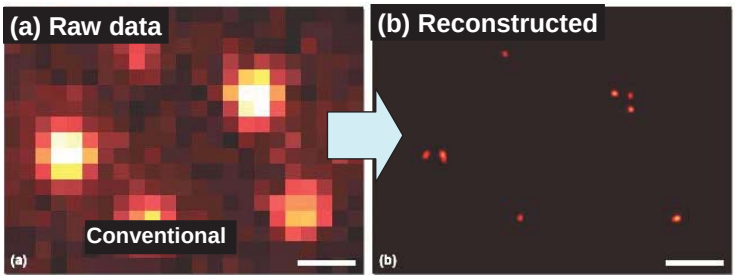
5.5.1 Data Acquisition and Image Reconstruction

Data acquisition: A whole ***series of images*** is recorded sequentially at different times or under different excitation (or detection) conditions.

In each image, only a ***sparse subset*** *of individual fluorescence molecules appears* due to (i) the non-simultaneous stochastic fluorescence nature (blinking) of single molecules, (ii) *spectral filters* in front of the detector or (iii) due to the use of *different excitation* wavelengths. In the sparse images, the separation of the emitting molecules is larger than the Abbe resolution limit such that the individual molecules are resolved.

***Image* reconstruction:** In each image, the position of the individual molecules is determined by fitting and the superposition of these positions yields a final map of the molecular emission centers with few nm resolution.





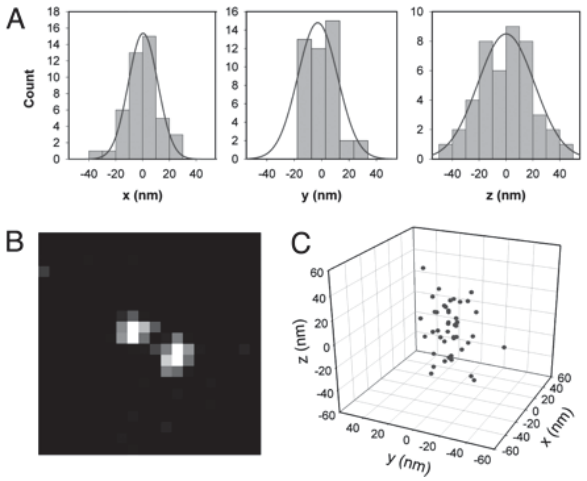
5.5.2 Resolution due to Localization Accuracy

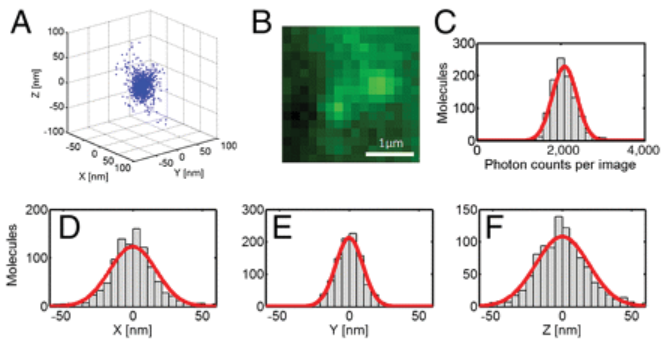
The number of photons that can be recorded from the emission from a single molecule is limited due to photo bleaching. This means that the signal, i.e., intensity distribution recorded is “noisy” and not perfectly smooth.

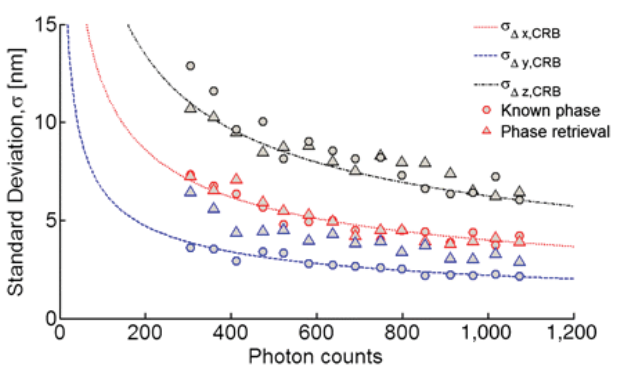
As a result there is always a statistical error of the fit of the data, i.e., the precision of the localization is limited.

The ***localization accuracy Δx*** for the individual molecules depends on the **signal-to-noise ratio**, i.e., the ***number of photons m*** detected.

The localization accuracy is: $$\Delta x \approx \frac{\lambda}{2NA\sqrt{m}}$$

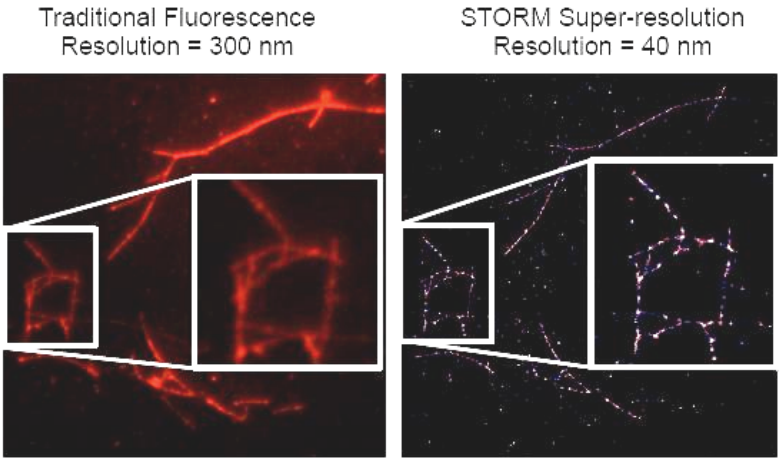






STED Resolution Limit

Because the localization accuracy increases infinitely with increasing count statistics, PALM and STORM can *theoretically provide unlimited localization accuracy*, i.e., unlimited spatial resolution of a fraction of the optical wavelength much below the Abbe limit.



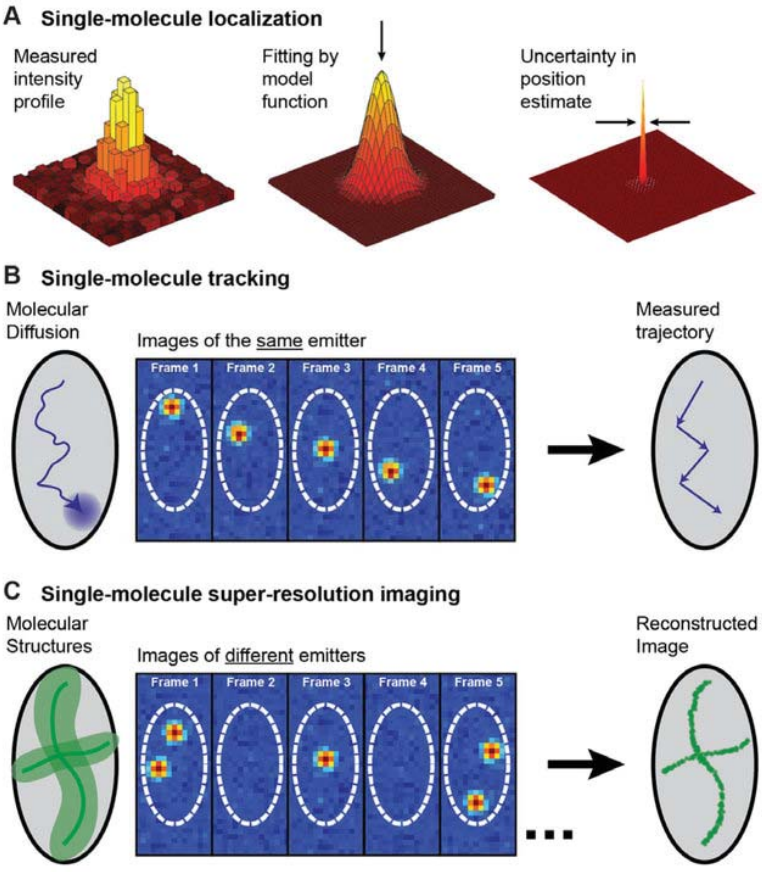
Microtubule biopolymers labeled with Alexa-647

When two single-molecule emissions overlap with a separation distance shorter than the diffraction limit, the centroid for each fluorophore may still be individually localized by subtracting the point-spread function of one fluorophore from the other.

⇨ A very favourable feature of PALM, STORM and related wide-field methods is that they **do not require modification of the optical set-up** of standard optical microscopes. Thus, these methods can be implemented in any instrument that is capable of single-molecule imaging.

Other Resolution Limitation: Diffusion of Position

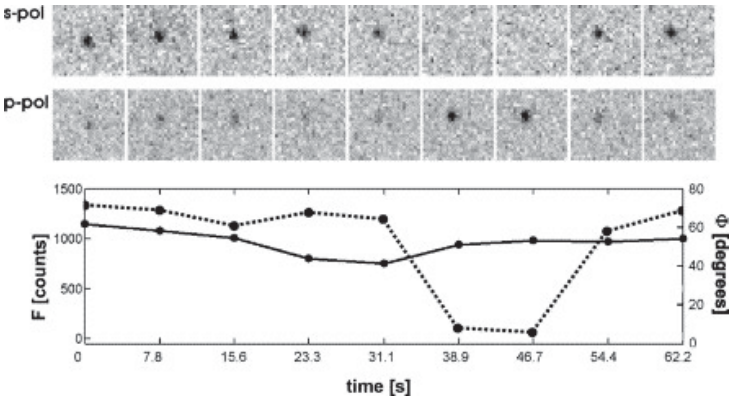
A second effect is that the position of the molecule may slightly change during time due to their diffusion within the sample, which leads to an additional smearing of the position.

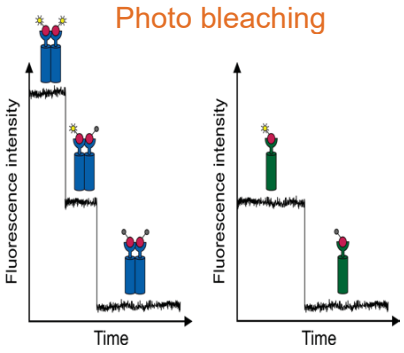


5.5.3 Non-Simultaneous Emission of the Molecules

To achieve the situation that only a small subset of molecules emit light at the same time, special molecules are required that live in two different states, namely, a ***bright state*** in which they efficiently emit light, and a ***dark state*** in which the do not emit light. These two states are often caused by conformational changes of the molecule

Switching between a dark and bright state is a ***stochastic process*** that occurs naturally due to thermal switching between two metastable states of the molecules (STORM). This effect is called **blinking**, but it is preferable to control it by an activation laser as in PALM microscopy.





Photobleaching is an additional effect in which the emission of activated molecules dies out after some time. Emission and absorption, as well as photo-activation or deactivation of molecules usually occurs at slightly ***different wavelengths*** . Therefore, different subsets of molecules can be also detected when the image is recorded at different wavelengths selected by a spectrometer.

5.5.4 Photo-Activated Localization Microscopy: PALM

PALM relies on photo-activated switching of the molecules between a dark and bright state. Upon excitation with an *activation laser* a small percentage of the molecules are turned on, then imaged and localized. During imaging the molecules photobleach and cease to emit after some time. Repeating this process for multiple cycles enables the reconstruction of superresolution images.

Sequence of data acquisition:

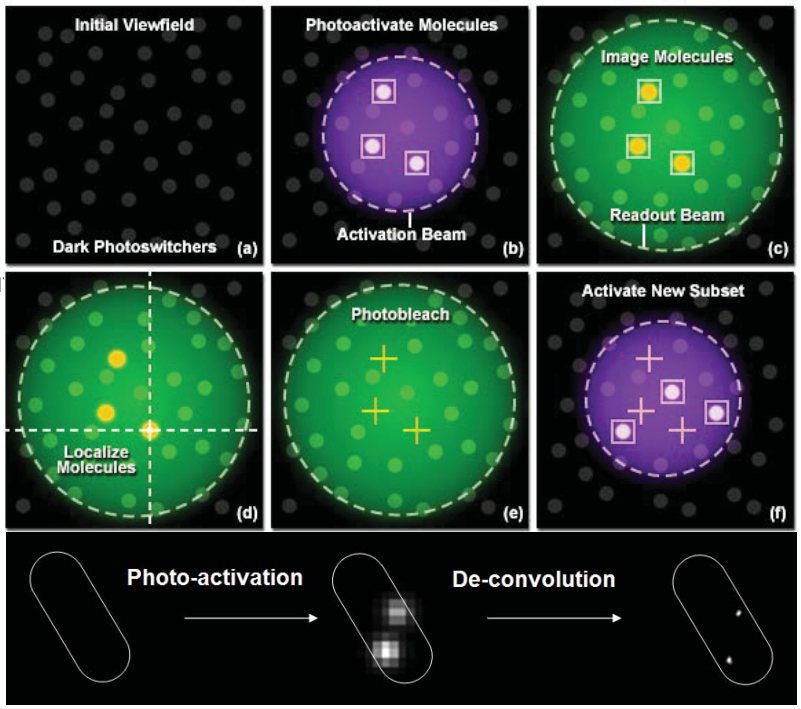
1) A **violet laser** is used to ***photo-activate*** a sparse subset of molecules (boxes).

2) A **green laser** is then used for ***readout*** of the resulting fluorescent singal, keeping the number of emitting molecules low by using a weak readou intensity.

3) The image of activated molecules is analyzed their position **localized**.

4) After some time, the photoactivated molecules eventually photobleach and become inactive.

5) Then, a ***new subset*** of molecules is photoactivated, and the whole sequence is repeated until all molecules in the specimen have been exhausted. The final image is obtained by superposition.



5.5.5 Examples for STORM and PALM Images

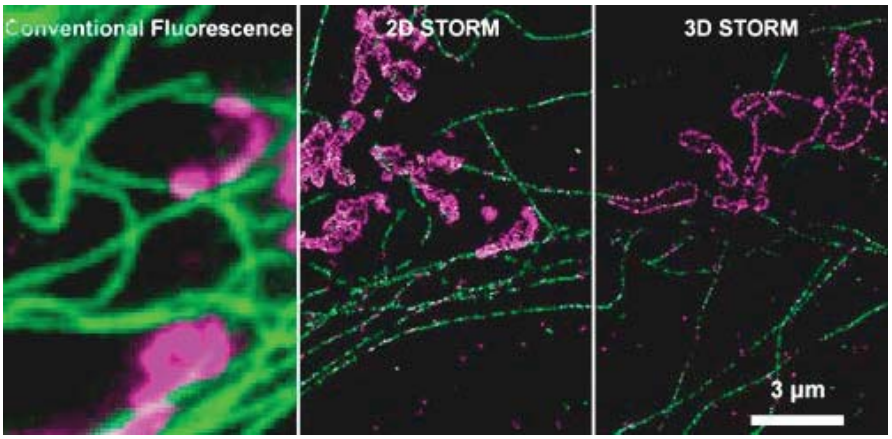


Figure: Conventional wide-field fluorescence image (left), corresponding 2D STORM image (middle) and 50 nm thick z section from the 3D STORM image (right) of microtubules (green color) and mitochondria (magenta color) in BS-C-1 cells.

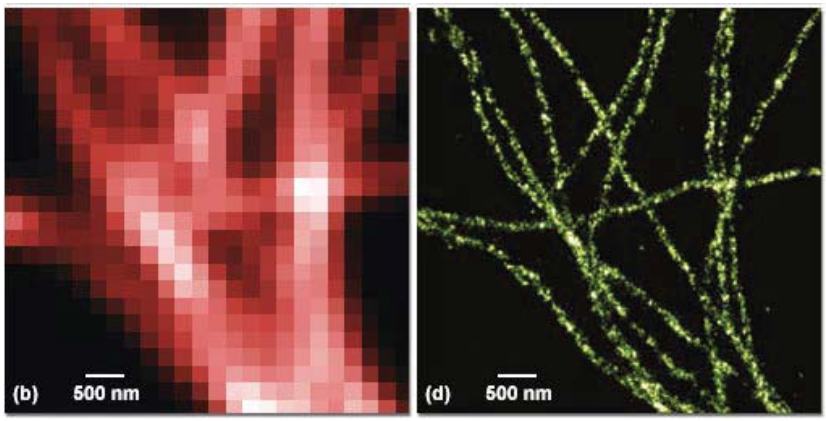
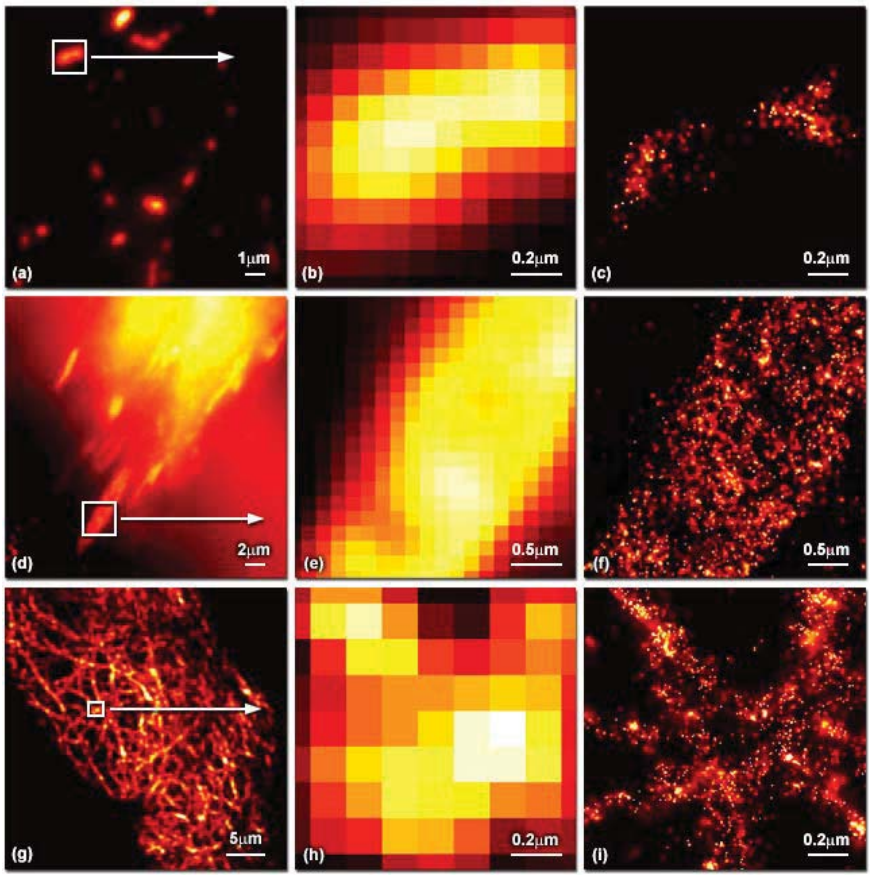


Figure: Pseudocolored images of microtubule network in a mammalian fibroblast kidney cell imaged with conventional confocal microscopy (left) and STORM (right) with superresolution.

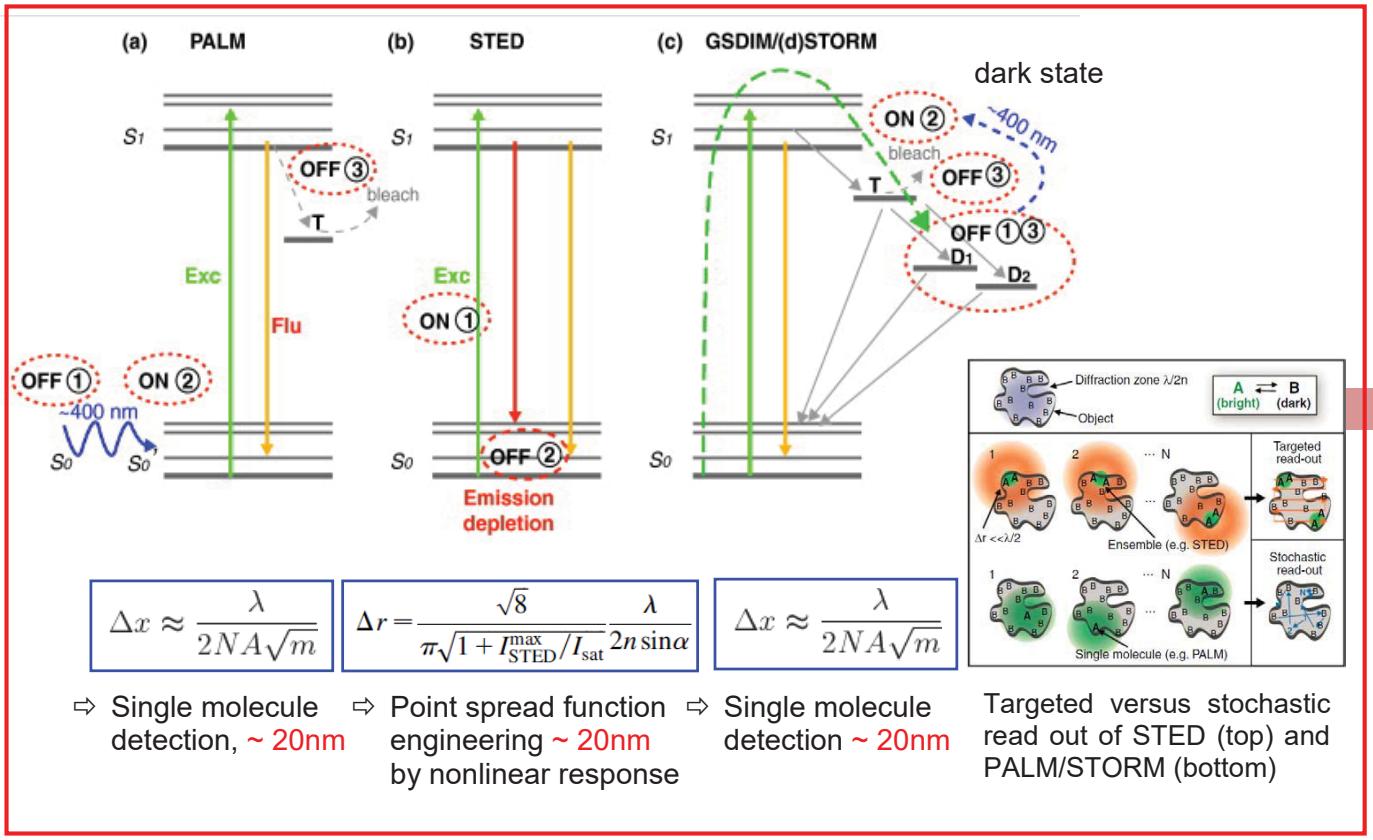
STORM images were generated using a green activation laser (λ =532 nanometers) and a far-red imaging laser ((λ =657 nanometers).



Examples for PALM

Figure: Examples of super-resolution images generated using PALM coupled with the fluorescent proteins, tandem dimer Eos and monomeric Eos. Figures (a) through (c) show tdEos fused to a short mitochondrial targeting signal in wide field total internal reflection (a) as well as the boxed region expanded to the size of the PALM image (b), and the corresponding superresolution PALM image (c). Likewise, images of tdEos fused to vinculin are shown in (d) to (f). A fusion of mEos2 to human cytokeratin is presented in figures (g) to i)) to illustrate the dramatic increase in resolution afforded by PALM imaging, where features ranging in size from 30 to 50 nanometers are resolved with high localization precision.

Comparison of the Optical Transitions used in PALM, STED, GSD and STORM



$$\Delta x \approx \frac{\lambda}{2NA\sqrt{m}}$$

$$\Delta r = \frac{\sqrt{8}}{\pi\sqrt{1 + I_{\mathrm{STED}}^{\max}/I_{\mathrm{sat}}}} \frac{\lambda}{2n\sin\alpha}$$

$$\Delta x \approx \frac{\lambda}{2NA\sqrt{m}}$$

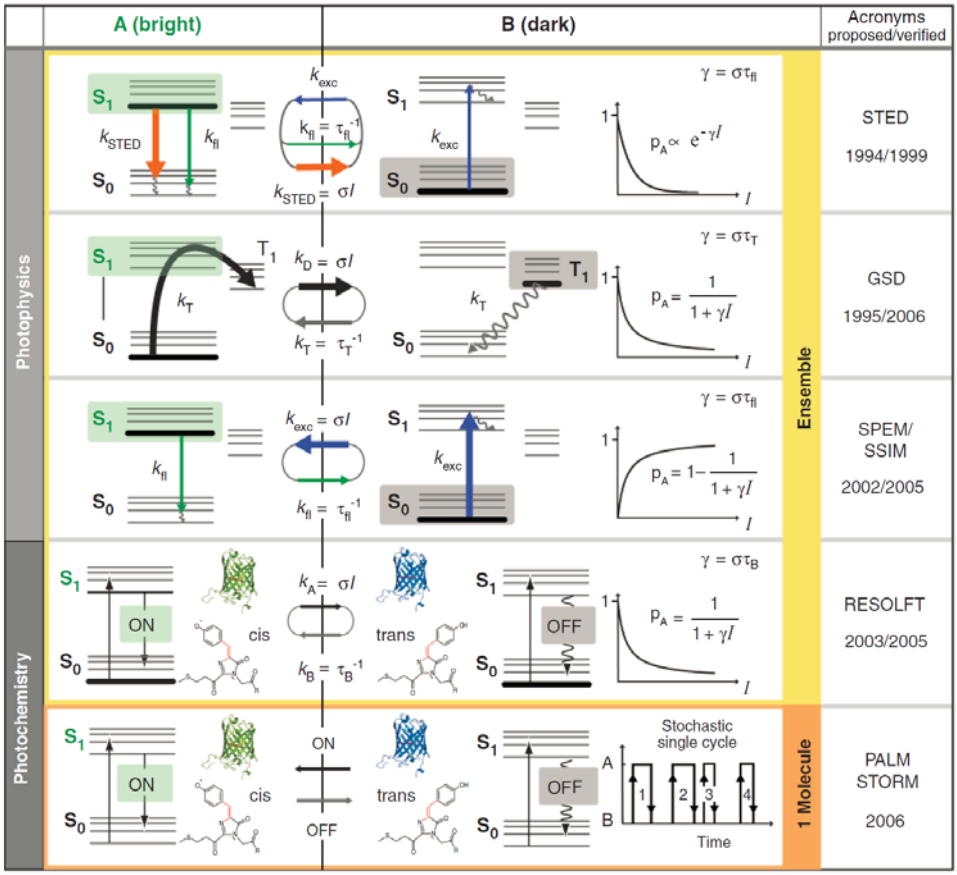
⇨ Single molecule detection, ~ 20nm

⇨ Point spread function engineering ~ 20nm by nonlinear response

⇨ Single molecule detection ~ 20nm

Targeted versus stochastic read out of STED (top) and PALM/STORM (bottom)

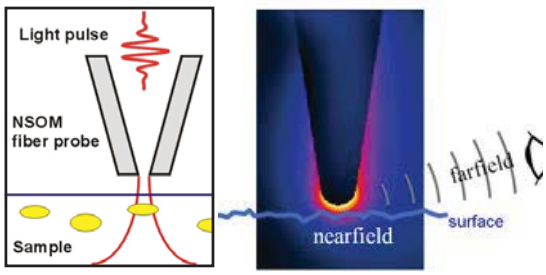
See: Reviews by S. Hell, Science 316 2007, Optics Express 2008, Nature Biology 2003



5.6 Near-field Scanning Optical Microscopy - NSOM

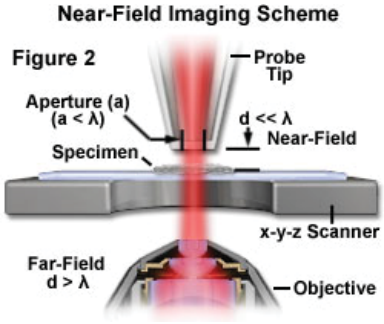
In **NSOM**, a small ***fiber tip*** with *subwavelength diameter aperture* is scanned in very close proximity (near-field) over the sample such that the surface is within the highly localized evanescent electromagnetic waves emitted or collected through the aperture.

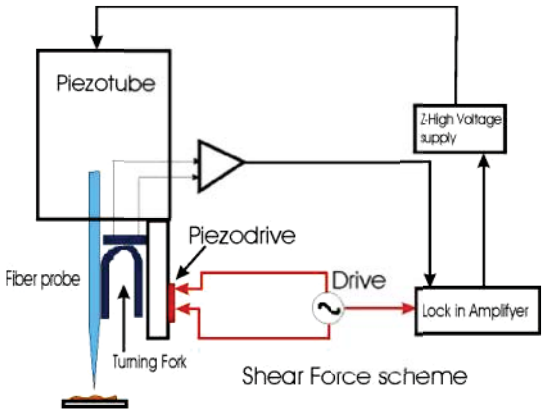
Detected can be the transmitted, reflected or excited light (fluorescence). Detection can be also done in the far field using a wide field objective. In this case, only the sample excitation is done with the near field probe.



Scanning is usually performed using an x,y,z piezo scanner similar as used for atomic force microscopy. Since the signal and resolution critically depends on the tip sample-distance.

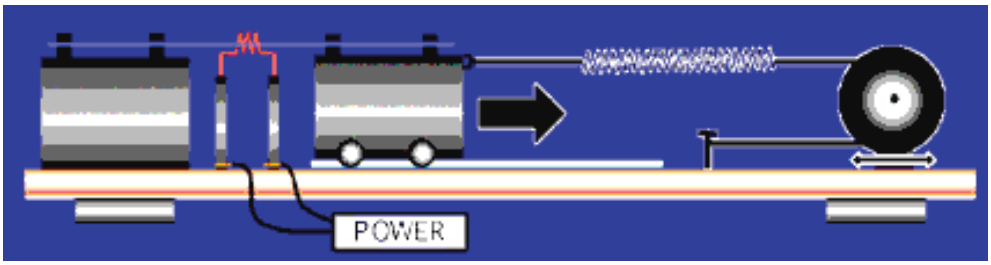
To ***control the tip-sample distance*** the probe is attached to an oscillating quartz tuning fork and the damping due to the sample detected. Using a feed-back loop, in this way the tip-sample distance can be held constant.

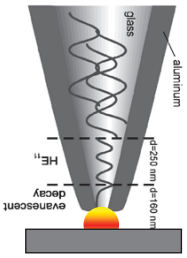




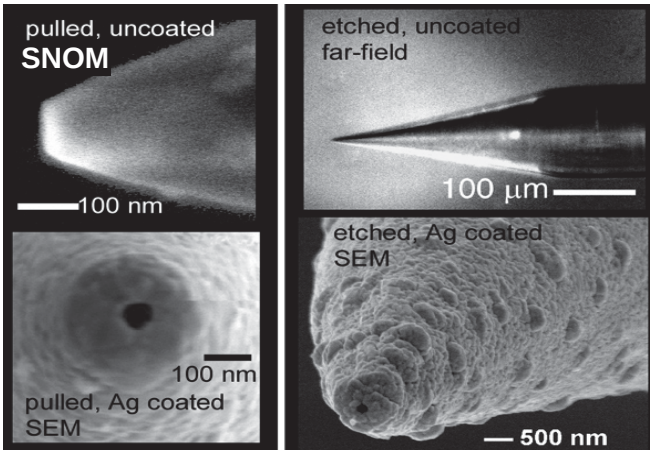
Realization of Tapered Fiber Probes

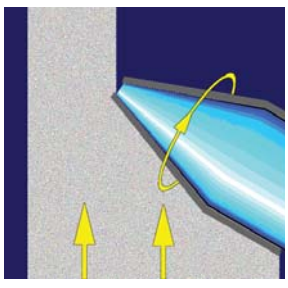
Fabrication: Heated and pulled to finally ruptured glass fiber results in a tapered end with diameters down to only 50 – 200 nm, which is smaller than the used optical wavelength.

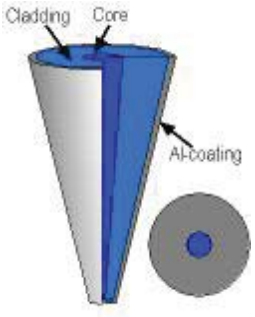




To reduce the leakage of the light out of the fiber side wall and thus lower intensity losses and a higher transmission, the side wall of the fibers tips are coated with a reflecting metal layer







Metal evaporation from the side for reflective coating of the fiber surface for better confinement of the light within the fiber core.

Resolution of NSOM

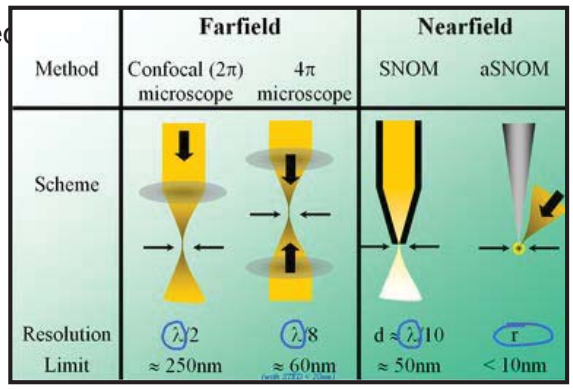
Determined by the ***aperture size d*** and the ***distance*** between the probe and the sample:

$$r_{min} \sim \sqrt{(\lambda \cdot z)^2 + d^2}$$

⇨ ***High subwavelength resolution*** can be achieved because the resolution **not limited by diffraction** but by the probe diameter.

d_{probe} **= 50 - 300 nm** (tapered optical fiber)

⇨ However, with decreasing aperture size, the ***optical transmission drastically decreases***. Therefore, the practical usable aperture size is limited.



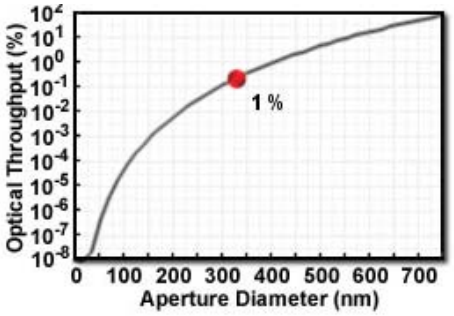
Limitation of NSOM:

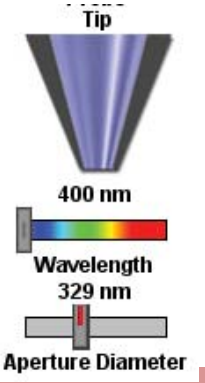
The transmission of small apertures and fibers strongly decreases with decreasing tip diameter:

Optical Transmission T ~ (d/λ)^4

Thus, the higher the resolution the lower the signal intensity and signal/noise ratio.

Aperture Diameter (nm)	Transmission
50	~10^{-5}
100	~10^{-4}
200	~10^{-3}
500	~10^{-1}





Dependence of the resolution on the tip-sample separation:

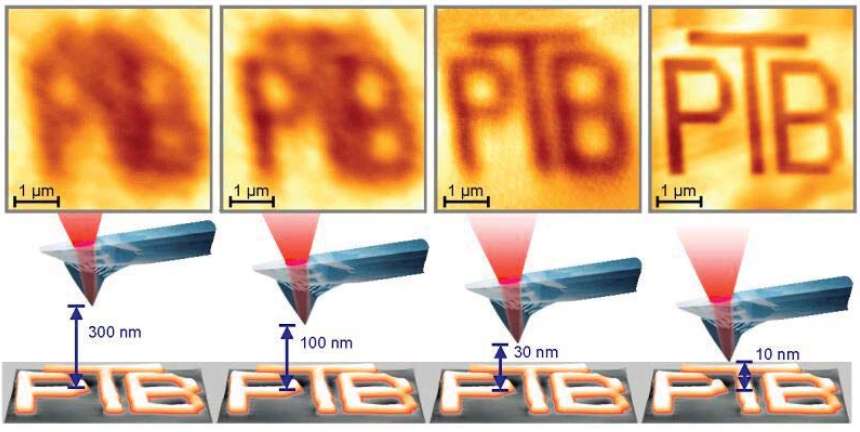
For any proximal probe, the interaction spreads out laterally when the probe-sample distance is increased, i.e., the strength of the near-field interactions decreases.

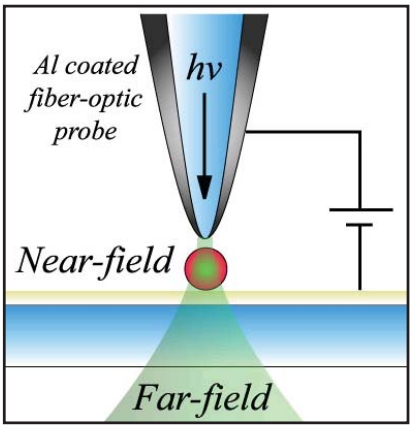
$$r_{min} \sim \sqrt{\lambda \cdot z}$$

⇨ As a result, the *sensitivity* as well as the *lateral resolution* rapidly *decreases* with *increasing* tip-sample distance.

⇨ For highest resolution the tip-sample distance should be kept as small as possible !

Example: Distance dependence of the ***NSOM resolution*** illustrated by a series of scanning transmission NSOM images of gold/palladium test structures on silicon recorded at different scan heights *z*. For the smallest tip-sample distance *z*=10nm, a lateral resolution of ~ 80 nm is achieved for λ =1064 nm.





⇨ **The same effect occurs for STM, AFM, MFM and all other proximal probe techniques.**

5.7 Summary

- **Confocal scanning microscopy** allows optical sectioning of transparent samples. Thus, ***3D tomographic images*** of the samples can be obtained.
- **Advanced fluorescence microscopy** allows to break the fundamental resolution limit of diffraction achieving super sup-wavelength resolution down to the < 20nm limit.

  (a) Single molecule detection and localization (= PALM, STORM), where the individual position of isolated nanometer size luminescence centers are localized by fitting the image spots by Gaussians. Full sample images are then obtained by Superposition of many of such images. The ***resolution limit*** is given by the size of the individual emission centers as well as the localization precision (signal-noise ratio).

  (b) Point spread function engineering by utilizing nonlinear optical processes and saturable or switchable optical transitions (= STED, GSD). The excitation volume is artificially reduced below the diffraction limit by non-linear (stimulated) depletion of the outer part of the excitation spot by a second donut shaped depletion laser. The resolution limit is determined by the power of the depletion laser.
- **Scanning near-field optical microscopy** (NSOM) using tapered optical fibers with sub-wavelength apertures scanning in close proximity to the sample surface (=near-field limit) also provides a spatial resolution below the diffraction limit.
  However, with decreasing aperture size the *signal intensity strongly decreases* due to the reduction of the optical fiber transmission.

Summary: Resolution of Optical Microscopy Methods

Method	Common abbreviations	Abbe applies	Remarks	Best resolution in nm	Ref.
Widefield	WF	Yes	The resolution depends on the numerical aperture, NA (not the magnification!). High NA is achieved by immersion objectives. The sample has to be immersed in the same refractive index as immersion medium.	~230 (XY) ~1000 (Z)	[46]
Confocal	CLSM, LSM	Yes	In theory: Twice the resolution of widefield microscopy. Local dose of light is very high for a short time. Potentially more photo-damage. Photons are blocked by the pinhole, wasting useful information. Fine with living cells.	~180 (XY) ~500 (Z)	[46]
4Pi	4Pi (Type A,B,C)	Yes	A second lens is used and adjusted to coherently participate in the imaging. Demonstrated with living cells, multiple foci preferred.	~200 (XY) ~90 (Z)	[27, 46]
Localization	Pointillism PALM, STORM	Yes	Optics within the Abbe limit, but the task (localizing single particles) is not restricted to it. PALM and STORM managed (for the first time) to reconstitute full images from many thousand localized molecules. Particle discrimination by colour, fluorescence lifetime, blinking, bleaching, photo-activation followed by bleaching. Not demonstrated in 3D, no living cells yet.	~20 (XY)	[6–9, 12–23]
Stimulated emission depletion	STED	No	The saturation of the stimulated emission circumvents the limit. Combination with 4Pi possible. Not yet demonstrated in 3D. No living cells demonstrated yet.	~16 (X) ~20 (XY) ~50 (Z)	[35–40, 46]
Evanescent Wave	TIRFM	No	Resolution improvement only along Z. Can be combined with structured illumination. Contact to surface of different refractive index required. Fine with living cells. 3D stacks not demonstrated.	~230 (XY) ~100 (Z)	[30, 44, 46]
Near Field	SNOM, NSOM	No	Only applicable if scanning tip is in close proximity (<10 nm) to the sample. Living cells possible. 3D stacks not yet demonstrated.	~30 (XY) ~10 (Z)	[42, 43]