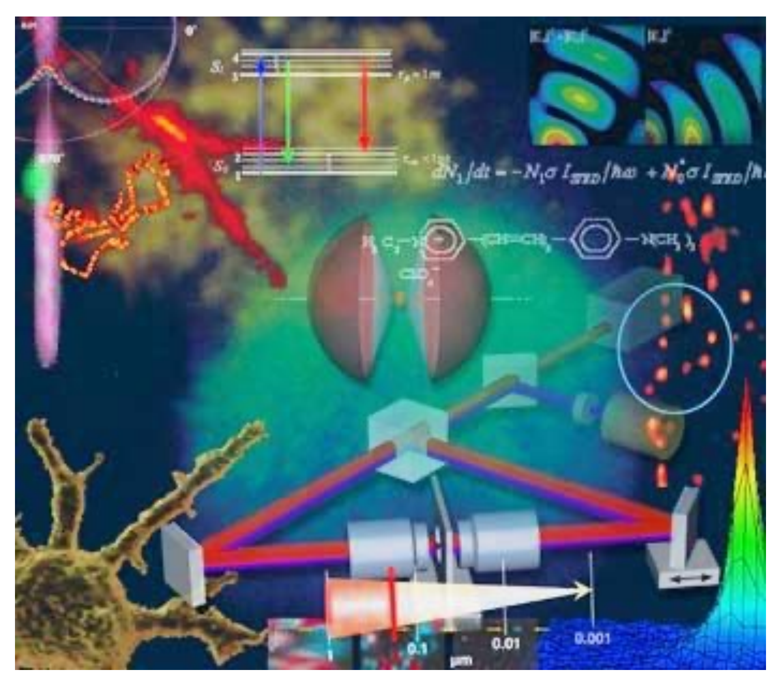
Max Planck Society

Fluorescence Nanoscopy

4Pi / STED / RESOLFT



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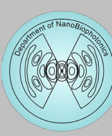


19

Far-Field Optical Nanoscopy

Stefan W. Hell

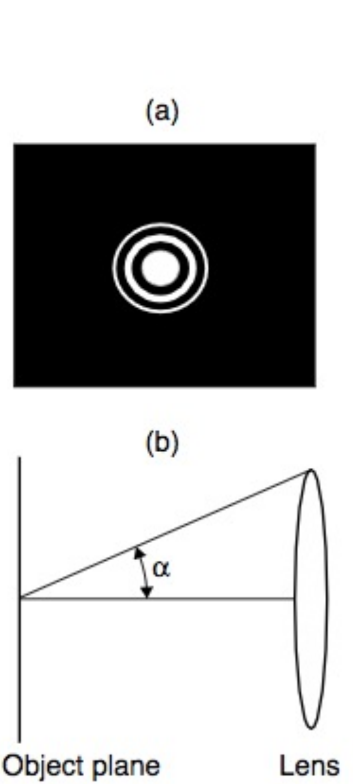
By providing a spatial resolution down to the atomic scale, electron and scanning probe microscopy have revolutionized our understanding of life and matter. Nonetheless, optical microscopy has maintained its key role in many fields, in particular in the life sciences. This stems from a number of rather exclusive advantages, such as the noninvasive access to the interior of (living) cells and the specific and highly sensitive detection of cellular constituents through fluorescence tagging. As a matter of fact, lens-based fluorescence microscopy would be almost ideal for investigating the three-dimensional (3D) cellular interior if it could resolve details far below the wavelength of light. However, until not very long ago, obtaining a spatial resolution on the nanometer scale with an optical microscope that uses lenses and focused visible light was considered unfeasible [1, 2].

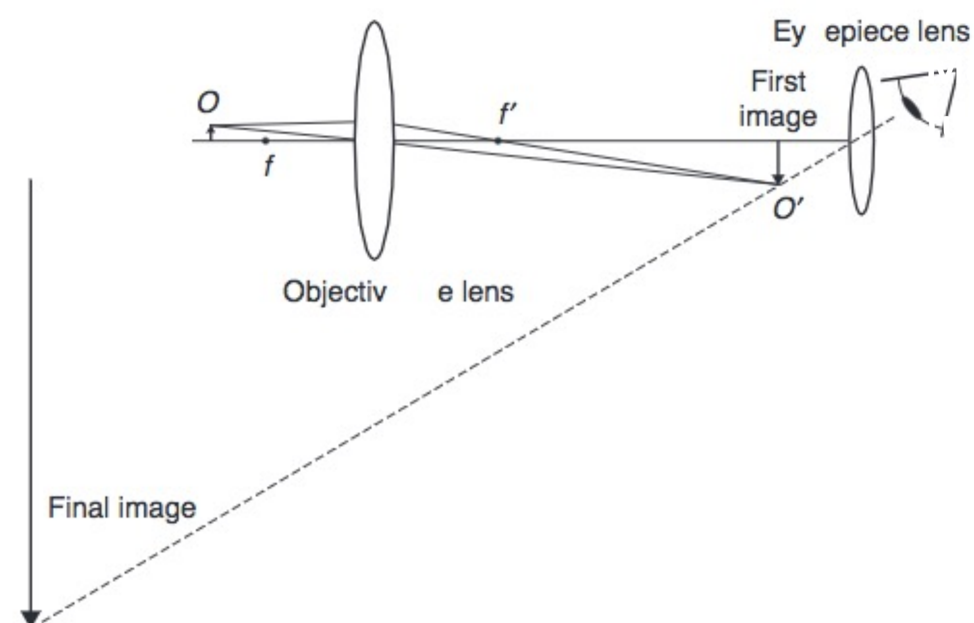
S. Hell, “Far field optical nanoscopy” Springer

PRINCIPLES OF OPTICAL MICROSCOPY

Compound lens microscope

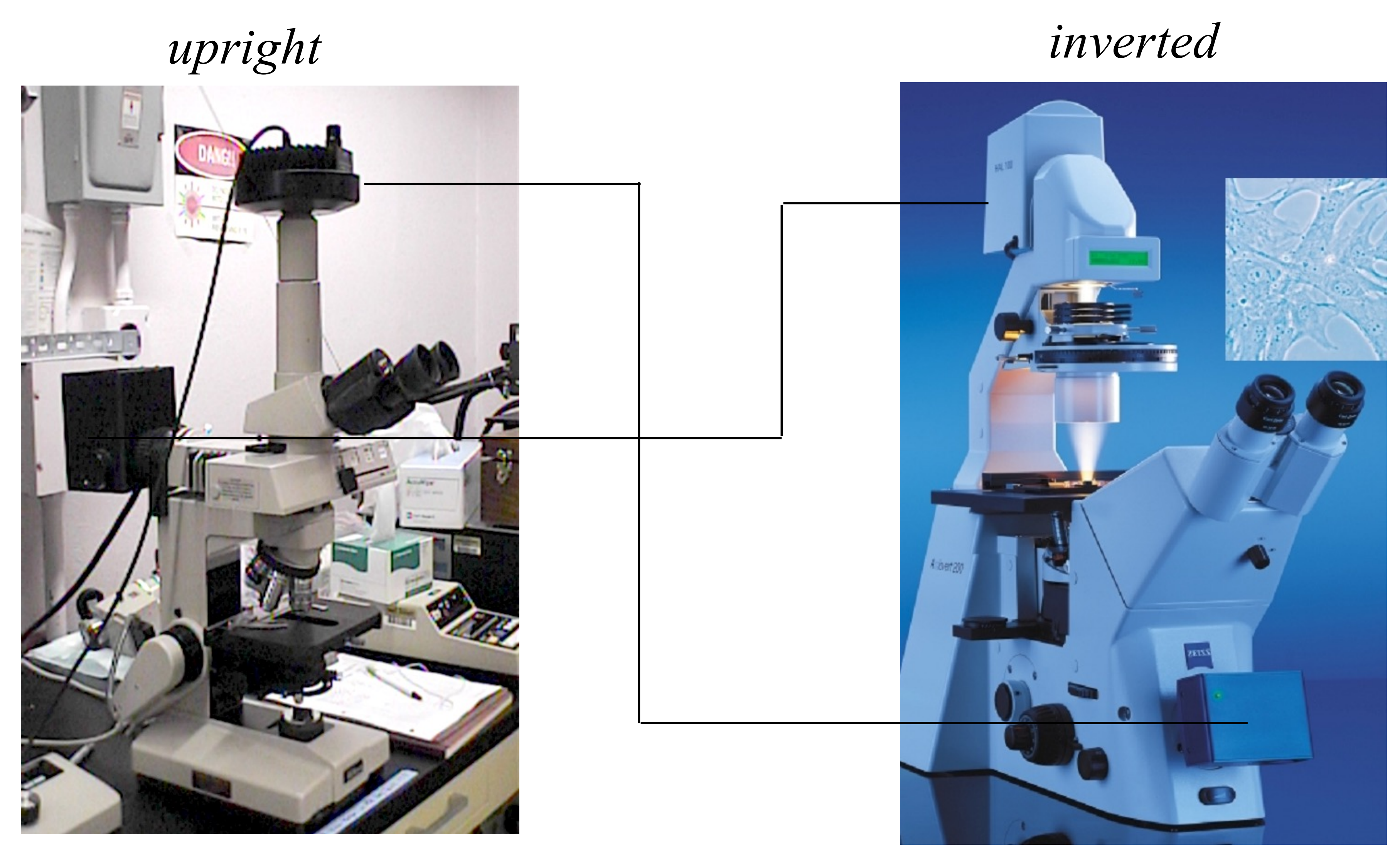
Requires two lenses: an objective plus an eyepiece



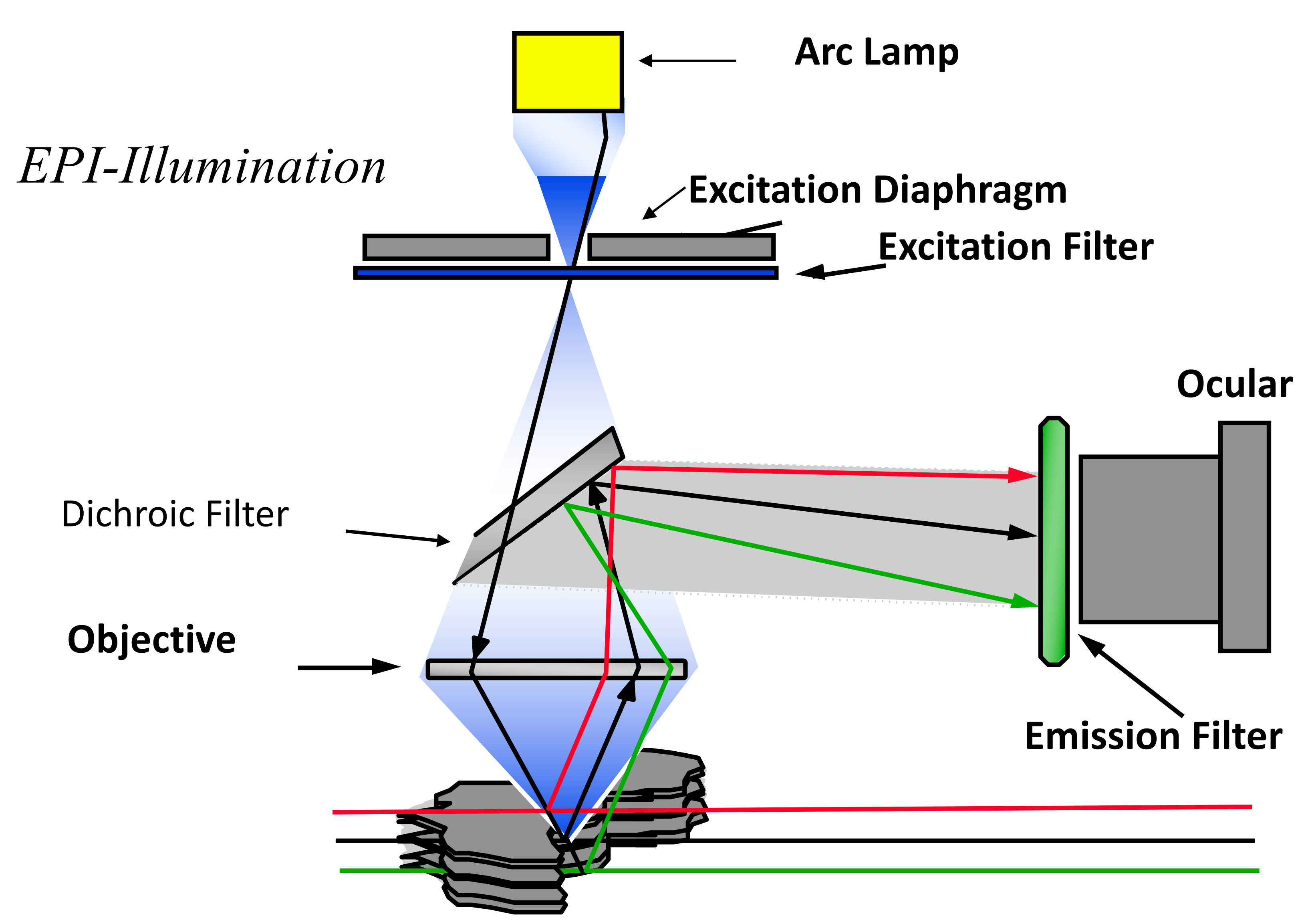


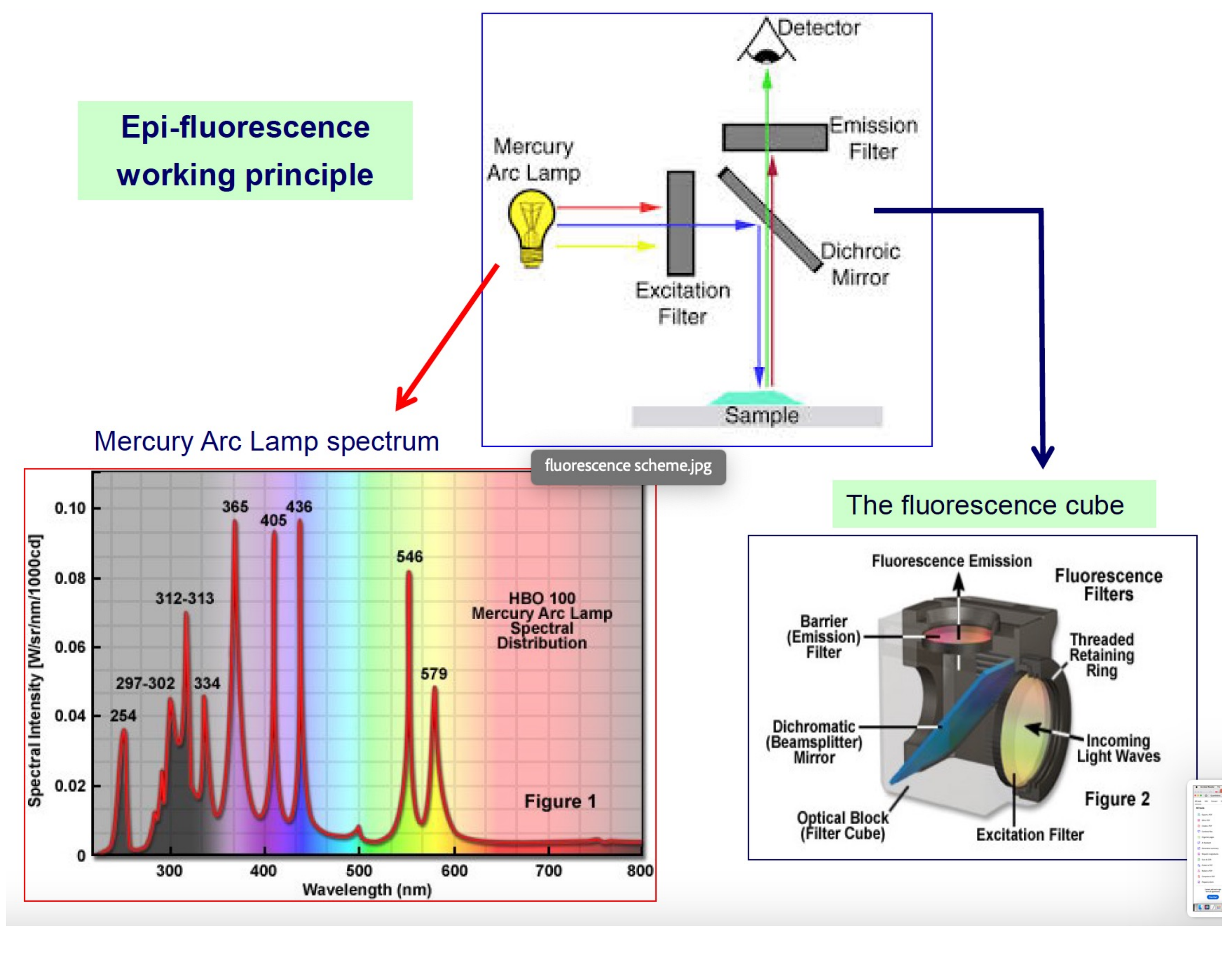
The object is positioned beyond the objective. The object-magnified image is further magnified by the eyepiece and focused onto the retina of the eye

Fluorescence Microscope



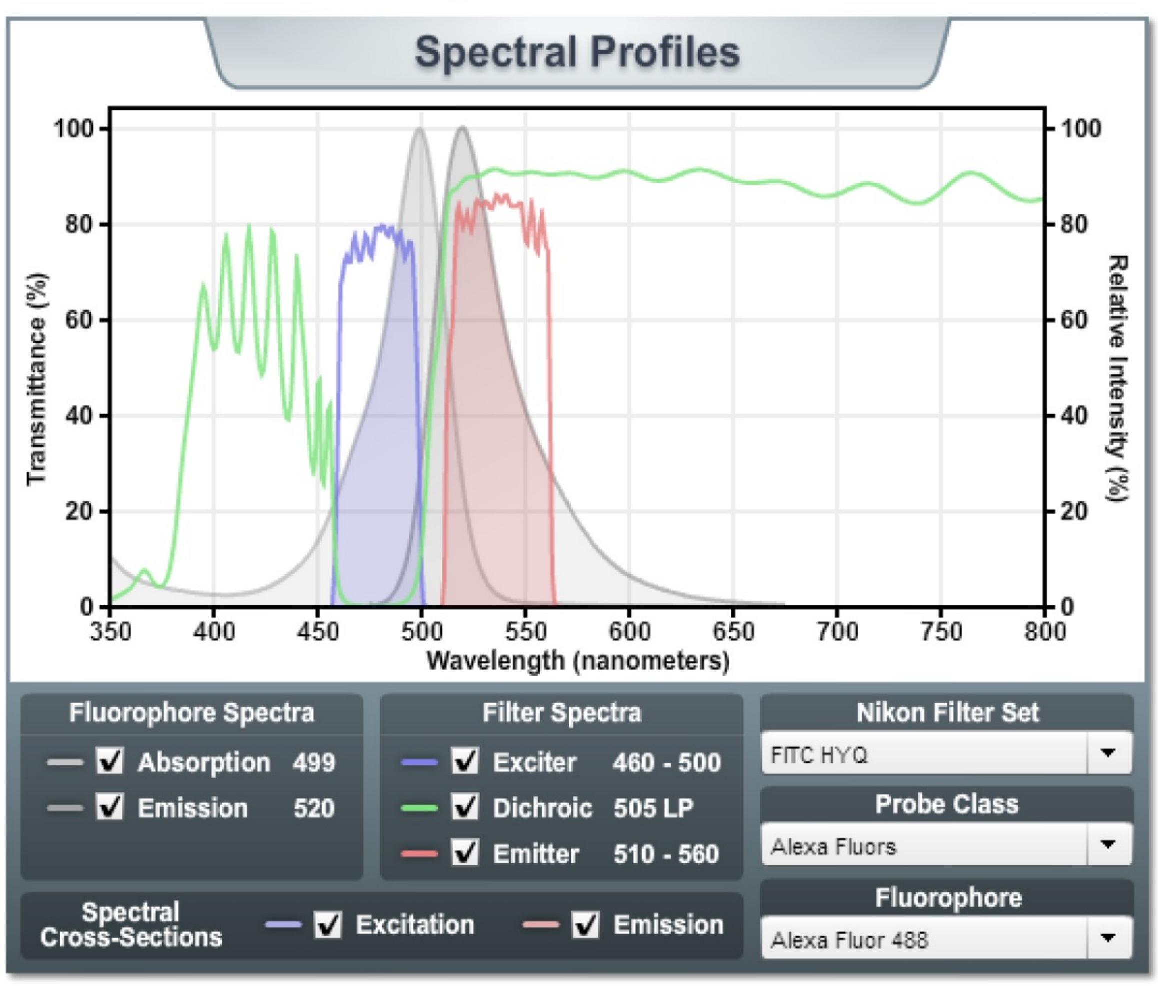
Fluorescent Microscope





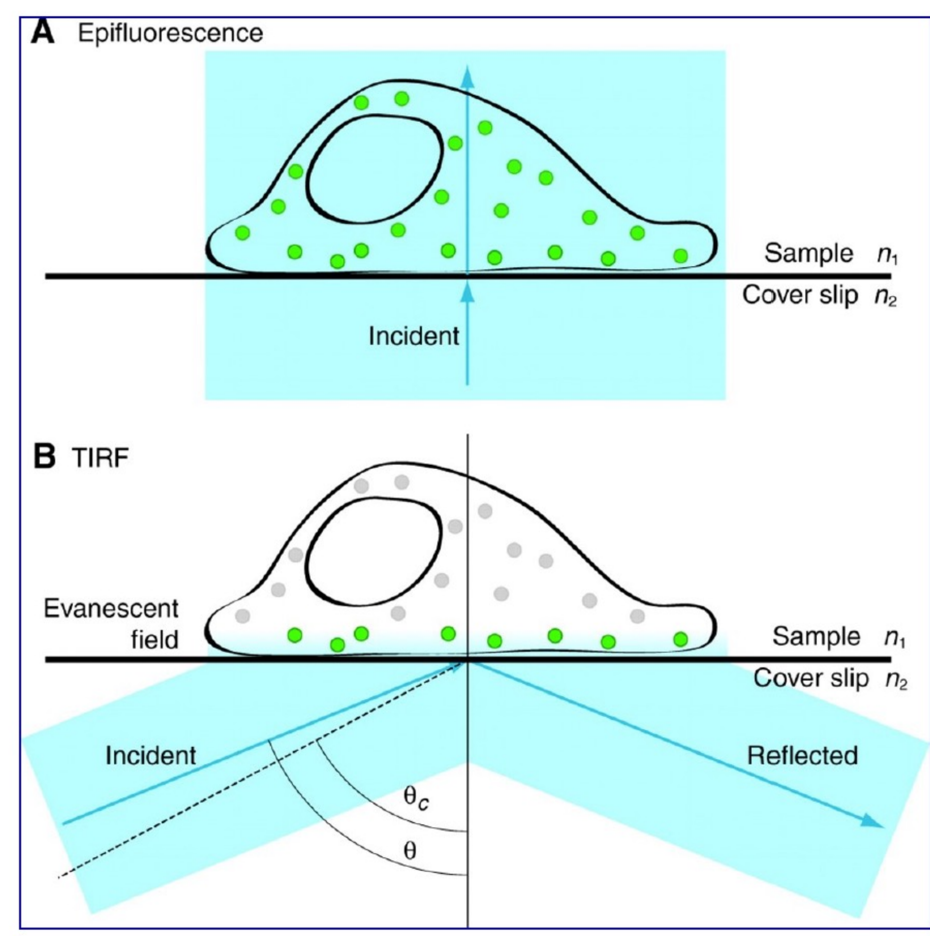
Epi-fluorescence working principle
Detector
Emission Filter
Mercury Arc Lamp
Dichroic Mirror
Excitation Filter
Sample
fluorescence scheme.jpg
Mercury Arc Lamp spectrum
0.10
0.08
0.06
0.04
0.02
0
Spectral Intensity [W/sr/nm/1000cd]
365
405
436
546
312-313
297-302
334
254
579
HBO 100 Mercury Arc Lamp Spectral Distribution
Figure 1
300
400
500
600
700
800
Wavelength (nm)
The fluorescence cube
Fluorescence Emission
Fluorescence Filters
Barrier (Emission) Filter
Threaded Retaining Ring
Dichromatic (Beamsplitter) Mirror
Incoming Light Waves
Figure 2
Optical Block (Filter Cube)
Excitation Filter

Excitation Filter ; Dichroic ; Emission Filter



http://www.microscopyu.com/

Total Internal Reflection Fluorescence (TIRF) Microscopy



TIRF microscopy provides a means to selectively excite fluorophores near the adherent cell surface while minimizing fluorescence from intracellular regions.

This serves to reduce cellular photodamage and increase the signal-to-noise S/N ratio.

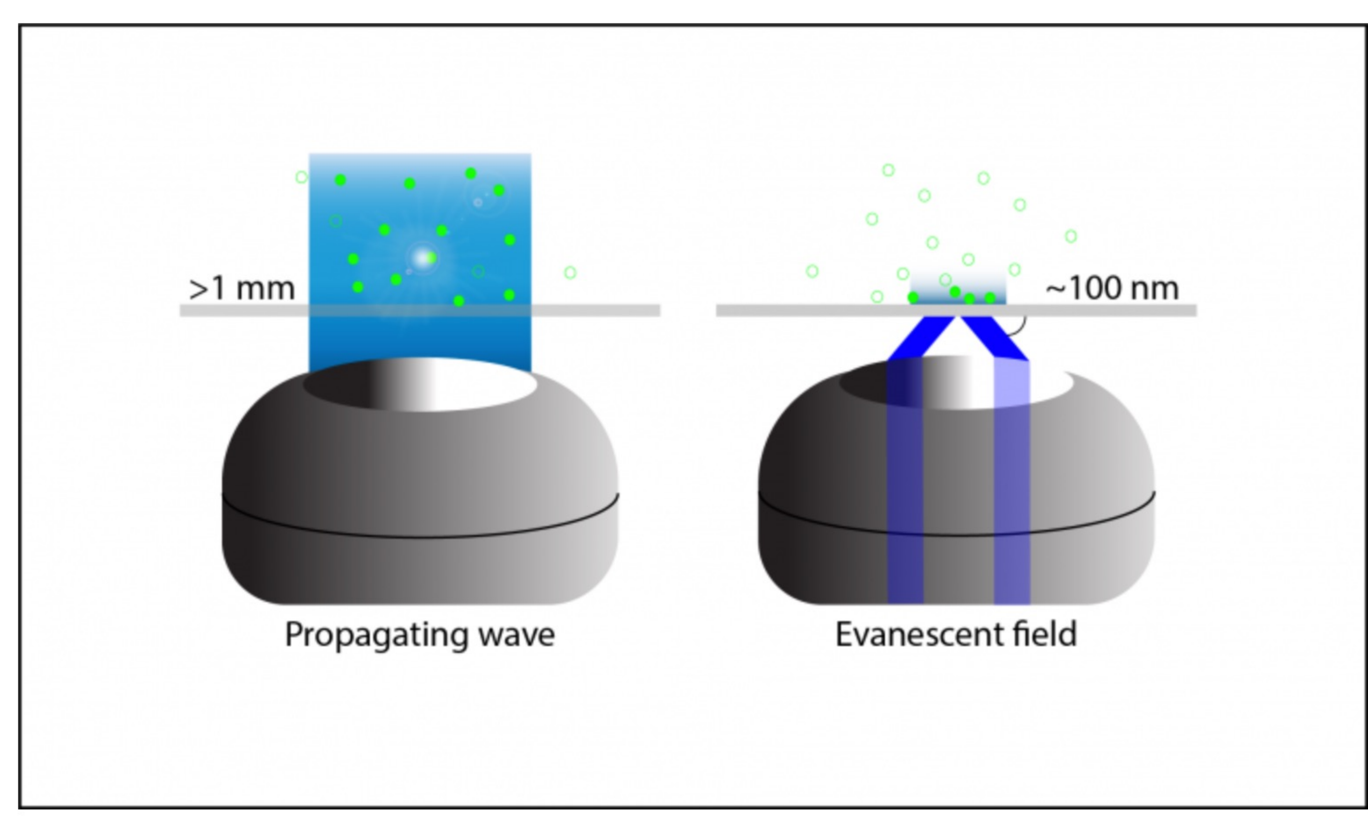
Note:

TIRF is particularly well suited to analysis of the localization and dynamics of molecules and events near the plasma membrane.

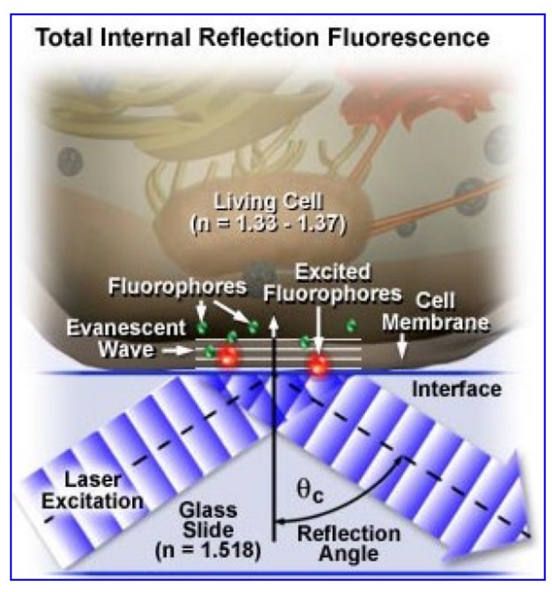
TIRF provides a better axial resolution but does not increase lateral resolution.

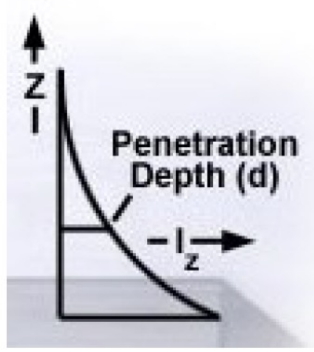
Imaging with total internal reflection fluorescence microscopy for the cell biologist
Alexa L. Mattheyses, Sanford M. Simon, Joshua Z. Rappoport
Journal of Cell Science 2010 123: 3621-3628; doi: 10.1242/jcs.056218

$$\Theta_C = \sin^{-1}(n1/n2)$$

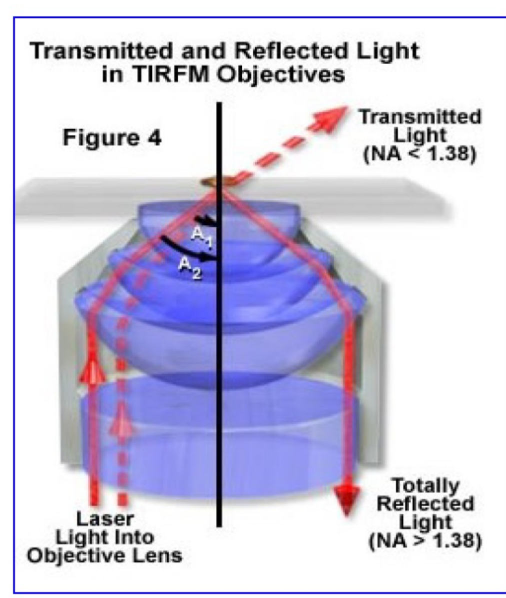


TIRF microscopy implementation





$$I(z) = I(o)e^{-z/d}$$



I(z) - the **intensity** at distance **z** from the interface

I(o) - the intensity at the interface

d - the characteristic **penetration depth**.

(d_max (for which I = 0) < wavelength)

www.olympusmicro.com/

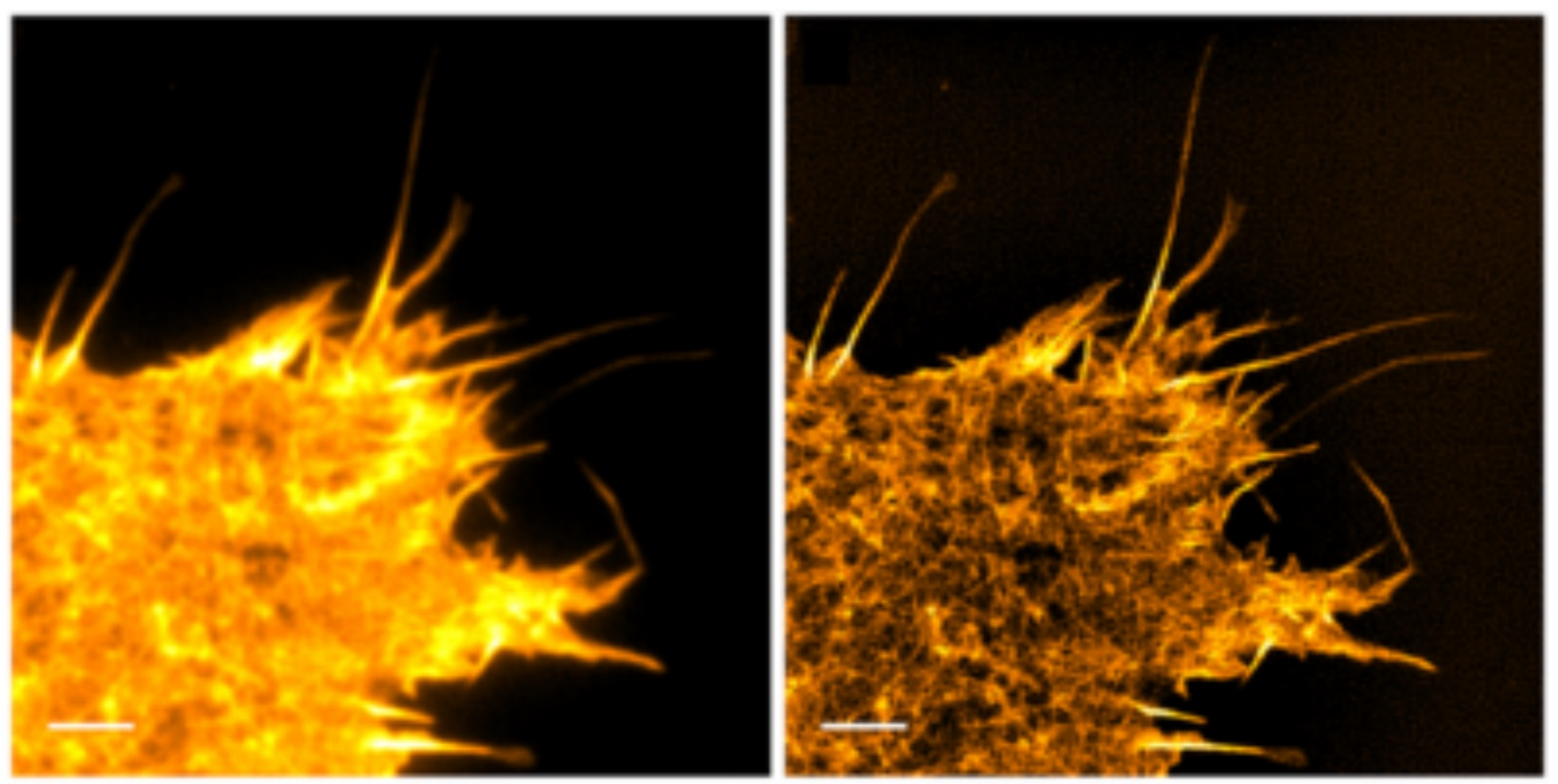
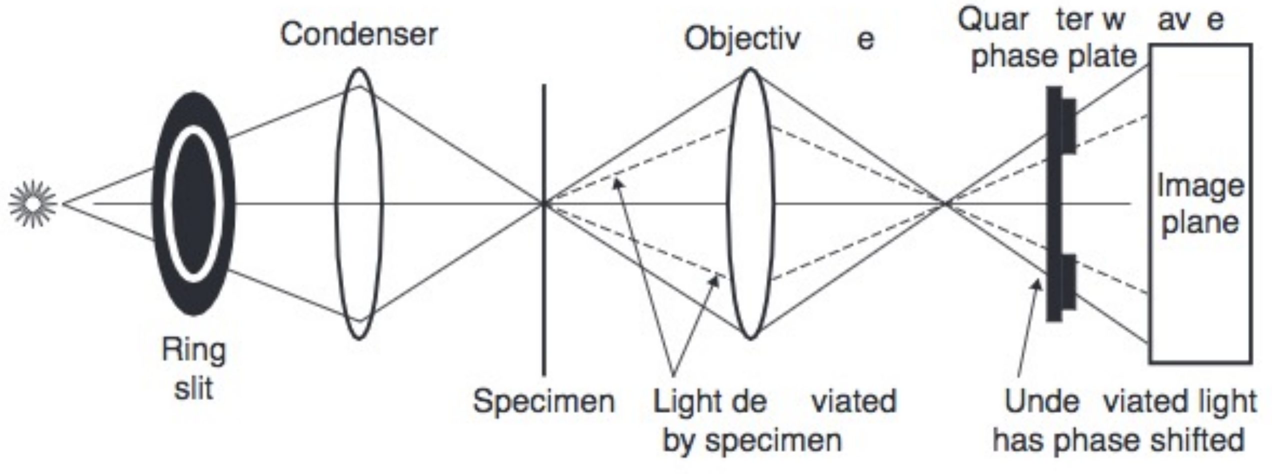


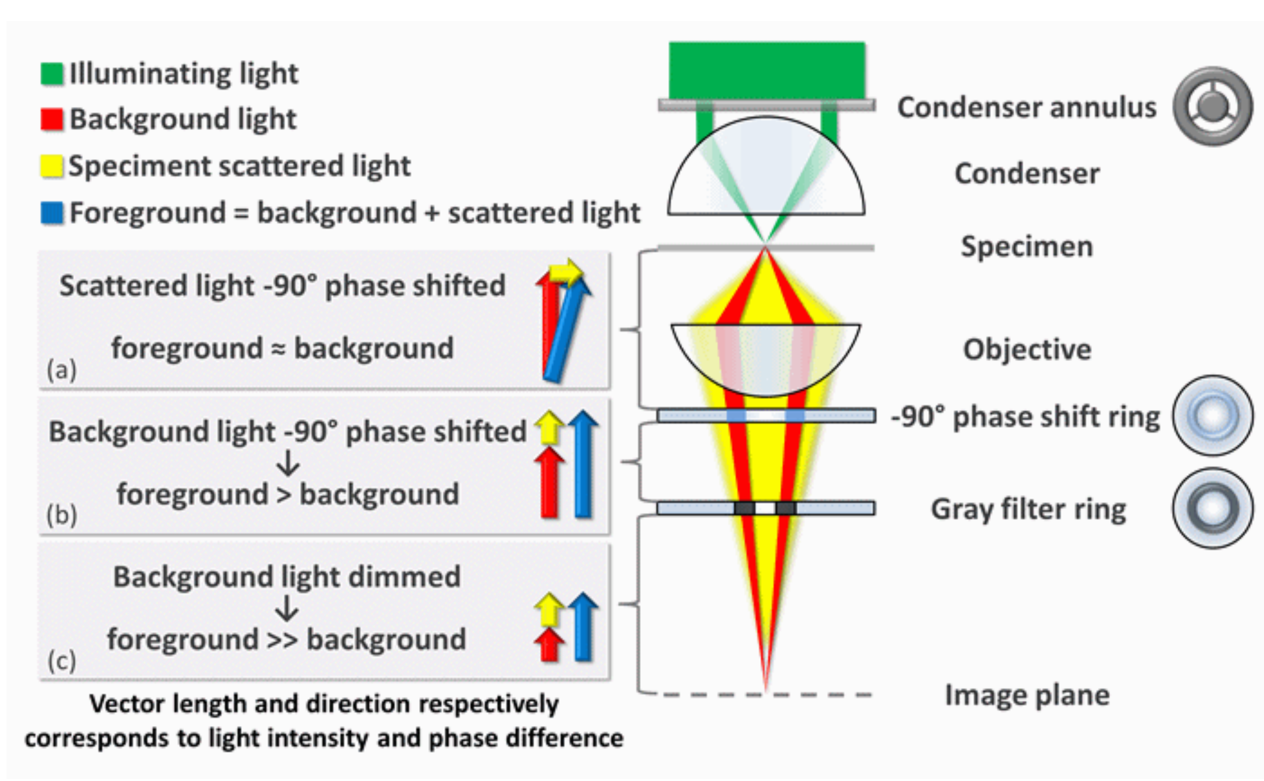
Figure 4: *TIRF Live cell imaging by compared to SIM TIRF in cos 7 cells expressing LifeAct GFP. Left Expressing the fluorescent actin LifeAct GFP. Right SIM TIRF of the same field of view, providing an increase in lateral resolution and contrast by computing spatial information from phase and frequency data. Scale bar: 3 µm. Adapted from Young et al. (2016).*

Key ideas in optical microscopy

- The light microscope is an instrument for producing enlarged images of objects that are too small to be seen unaided; such images may be viewed directly with a viewing screen or photographic apparatus or special electronic device.
- Because biological macromolecules are generally transparent to light numerous contrast-enhancing techniques have been developed including dark-field, phase-contrast, polarization and interference microscopy.
- The diffraction limit of resolution power of a microscope is about half of the wavelength of the illuminating light; an increase of the resolving power of the microscope is possible only by increasing the numerical aperture of the optical system or by using a shorter wavelength.
- In contrast to the ordinary light microscope, the confocal microscope employs a point-like illumination and detection arrangement; by restricting the observed volume, the technique keeps nearby scatterers from contributing to the detected signal.

Phase contrast microscopy



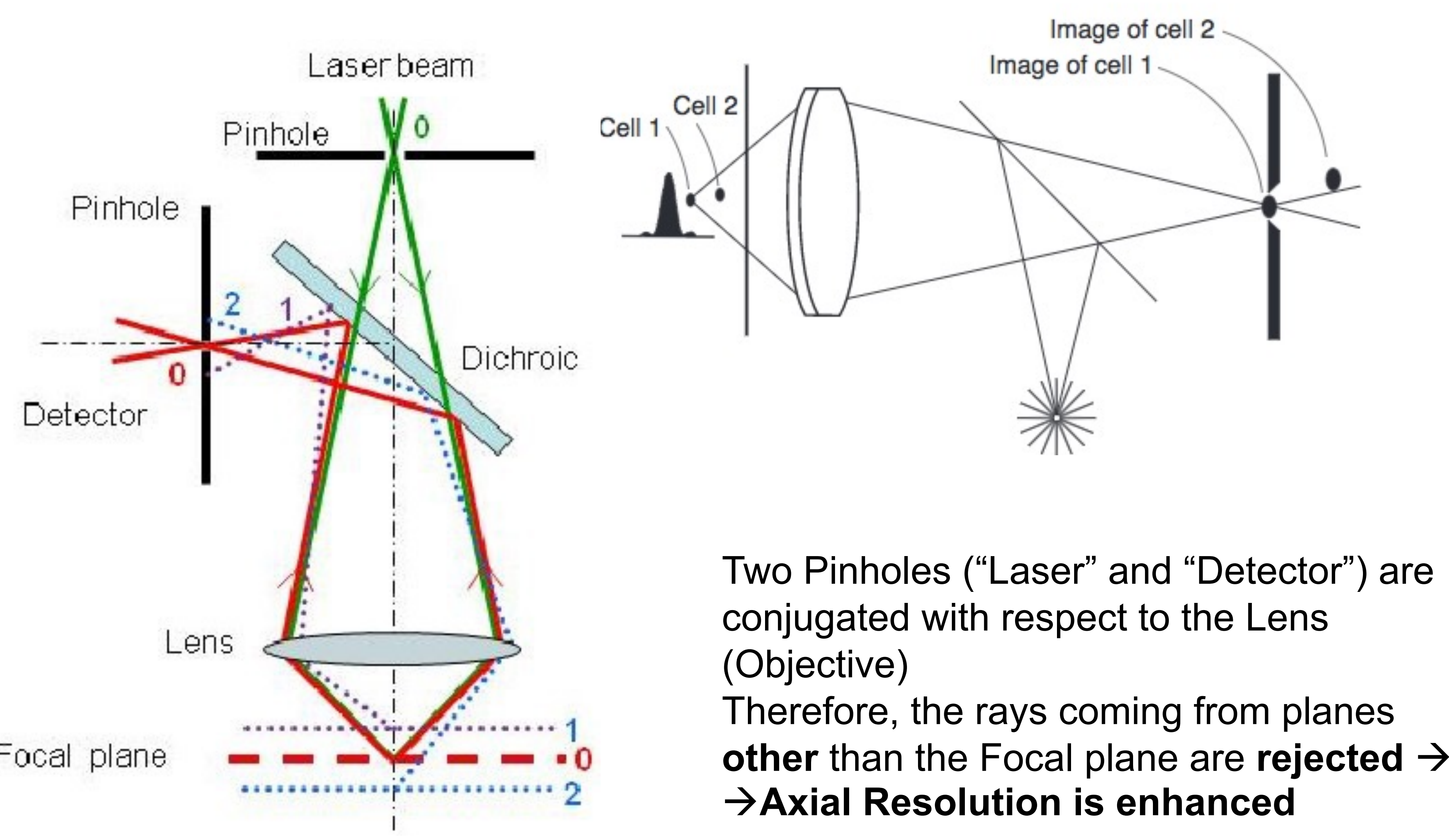


Confocal optical microscopy

Confocal fluorescence microscopy has also been connected with resolution improvement (Fig. 19.1a) [15–17]. Illuminating with a diffraction limited focused spot and detecting with a symmetrically arranged point detector, the effective focal spot, i.e., the effective PSF of this microscope is described by the product of the diffraction pattern for illumination and for detection: $I(x,y,z)\,I_{\mathrm{em}}(x,y,z) \approx I^2(x,y,z)$. The multiplication of intensities and the nearly quadratic dependence on the intensity in this formula reduces the FWHM of its central spot by $\sim \sqrt{2}$. In the Fourier domain, the multiplication expands the optical bandwidth of spatial frequencies even by a factor of two. In practice, however, $I(x,y,z)$ and $I_{\mathrm{em}}(x,y,z)$ are not really identical and the detector is not point-like [16, 17], meaning that the process is not really quadratic and the limited bandwidth expansion by a factor of two not practically realized. However, even if it were, the newly gained higher frequencies are heavily damped, which is why confocal microscopy did not really provide a higher resolution. Its actual benefit was the improved 3D-imaging and superb background rejection [16, 17].

S. Hell, “Far field optical nanoscopy” Springer

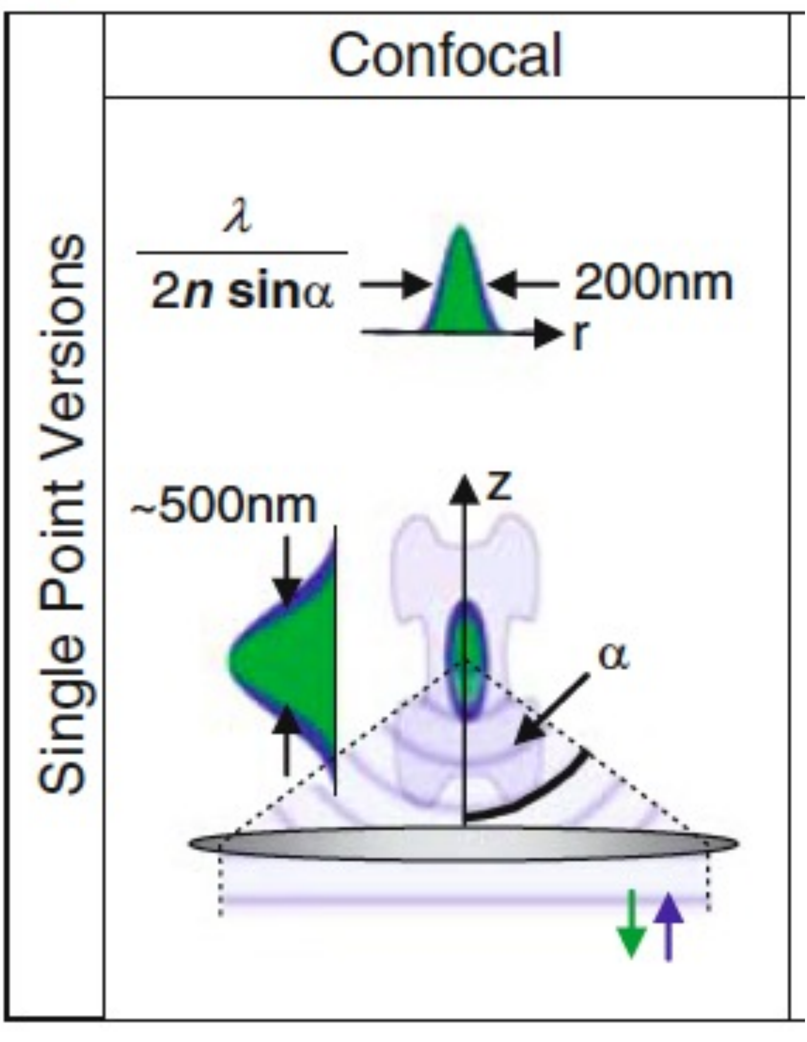
Confocal microscopy



Two Pinholes (“Laser” and “Detector”) are conjugated with respect to the Lens (Objective)
Therefore, the rays coming from planes **other** than the Focal plane are **rejected** →
→**Axial Resolution is enhanced**

Diffraction limit

Focusing a propagating light wave means causing it to interfere constructively at a certain point in space, called the geometrical focal point (0,0,0). Due to diffraction a focal intensity pattern $I(x, y, z)$ emerges around (0,0,0), which is also referred to as the intensity point-spread-function (PSF) of the lens. $I(x, y, z)$ features a central maximum called the focal spot (Fig. 19.1a) whose full-width-half-maximum (FWHM) is $\Delta r \approx \lambda/(2n \sin \alpha)$ in the focal plane and $\Delta z \approx \lambda/(n \sin^2 \alpha)$ along the optic axis [3]. λ is the wavelength of light, α denotes the semi-aperture angle of the lens, and n is the refractive index of the object medium (Fig. 19.1a). Discerning similar objects lying within this spot is usually precluded because they are illuminated in parallel and hence give off (fluorescence) photons in parallel. Likewise, the propagation of the emitted (fluorescence) light that is collected by a lens and focused to an image plane is governed by a similar function $I_{\mathrm{em}}(x, y, z)$, describing the blur of the coordinate from where the photons originated.



the gain in resolution (given by the spot size) is obtained **mechanically limiting** the size of the excitation and detection spots (by means of pinholes).

500 nm

$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$

α

200 nm

λ

Wavelength

Lens

E. Abbe (1873), *Arch. Mikroskop. Anat.* 9, 413.

Risoluzione: criterio di Reyleigh

schermo

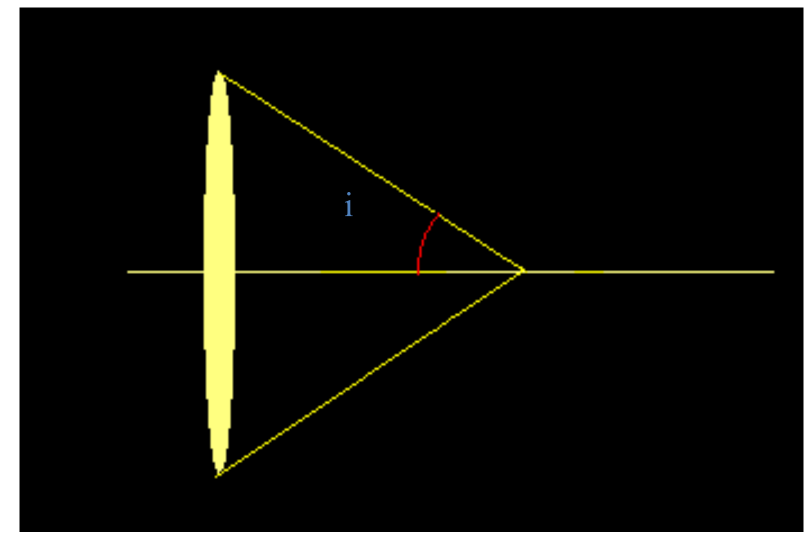
Intensita'

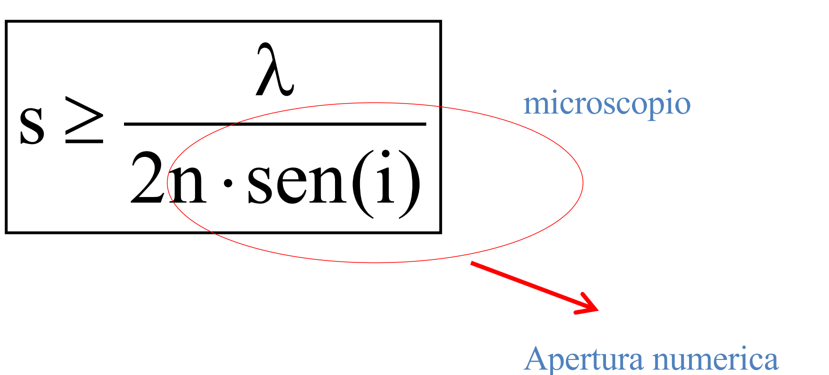
S_1 S_2 α

$\alpha = 2\theta_1$ $\alpha = \theta_1$ $\alpha = \theta_1/2$

$$\alpha \geq \theta_1 = 1.22 \frac{\lambda}{D}$$

telescopio





$$s \geq \frac{\lambda}{2n \cdot \mathrm{sen}(i)}$$

microscopio

Apertura numerica

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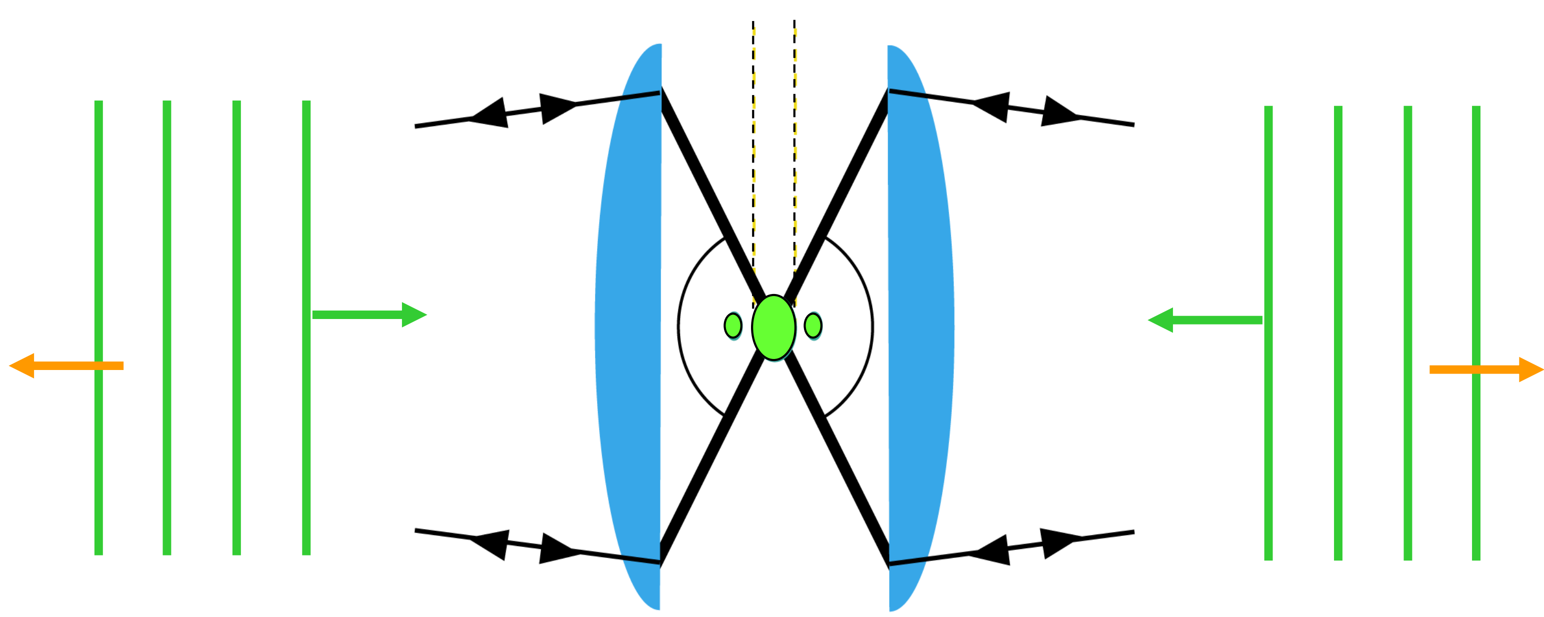
4Pi- Microscopy: resolution improvement in **z**

4Pi- Microscopy:

70 - 140 nm



$$\vec{E}^{4Pi}(r,z,\varphi)=\vec{E}_1(r,z,\varphi)+\vec{E}_2(r,-z,\varphi)$$

Coherent illumination and/or fluorescence detection

S.W. Hell (1990), *Europ. Patent* OS 0491289.
S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

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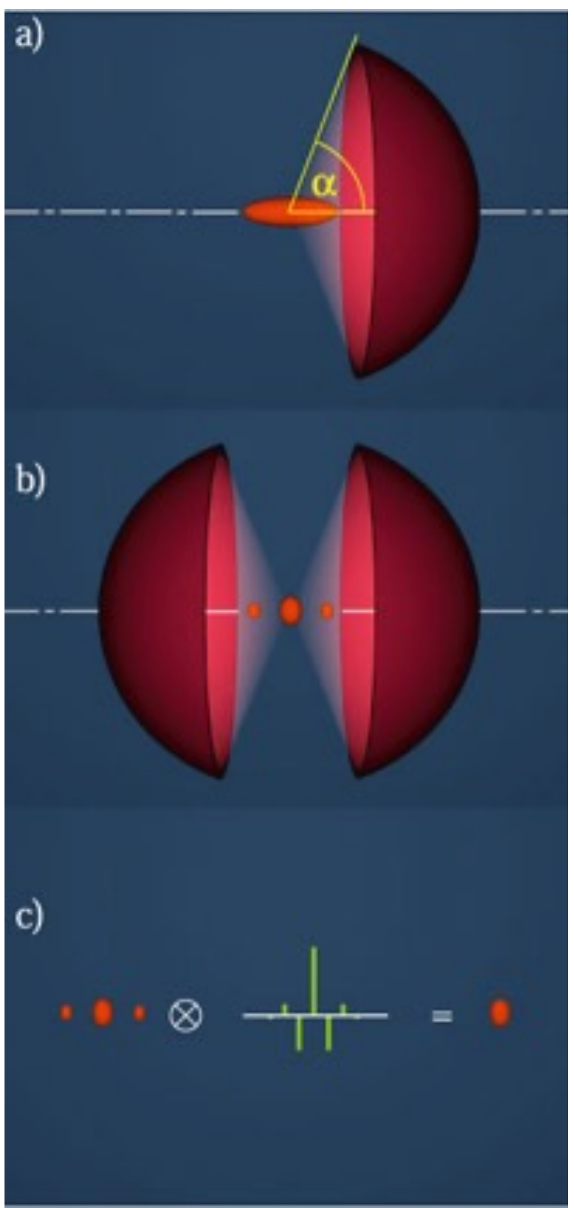
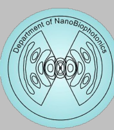


Ernst Abbe discovered that the focal spot size decreases with the microscope's aperture angle i.e. with the size of the spherical wavefront that is produced by the objective lens. But a regular objective lens, even of the largest aperture, produces just a segment of a spherical wavefront coming from a single direction. As a result the focal spot is longer (z) than wide (x,y) [Fig. 1a]. By contrast, a full spherical wavefront of a solid angle of 4π would lead to a spherical spot and hence to an improvement of spatial resolution in the z-direction.



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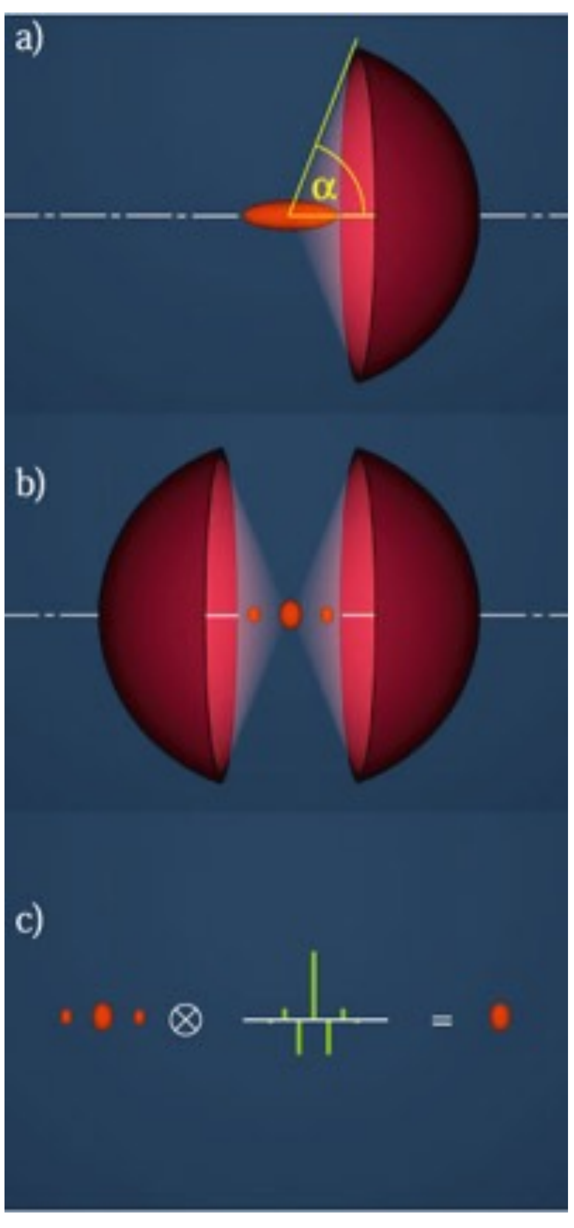

The idea: Since there are no lenses or mirrors that could provide such a wavefront across a significantly large field of view, the idea behind our 4Pi-microscope is to mimic the 'close to ideal' situation by **using two opposing objective lenses coherently**, so that the two wavefronts add up and join forces.

Allowing the illumination wavefronts to constructively interfere in the sample produces a main focal spot that is sharper in the z-direction by about 3-4 times (4Pi of type A). A similar improvement is obtained if the lenses add their collected fluorescence wavefronts in a common point detector (4Pi of type B). Doing both together is best, of course, and leads to a 5-7-fold improvement of resolution along z (4Pi of type C).

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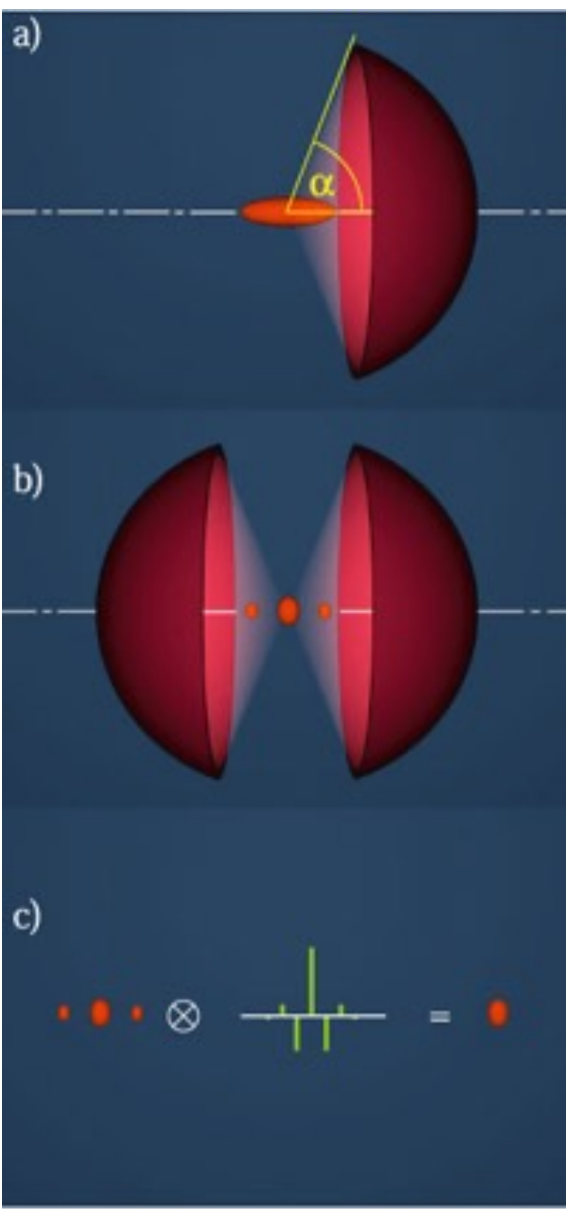
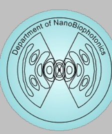


The sidelobe challenge: If the two segments were full spherical halves, the focal spot would be a (nearly) spherical spot, too. But since a considerable solid angle is not provided by the lenses, interference typically spawns off 2 axial side-lobes which, if not taken into account, lead to artefactual images. We deal with this challenge by an appropriate mathematical filter. This filter does not require any information about the object, apart from the height and location of the lobes. Linear filtering is possible if the lobes are significantly less than 50% of the main sharp maximum. This can be reliably fulfilled if multiphoton excitation of the dye is applied. Linear mathematical filtering is fast and a single effective spot is readily achieved.



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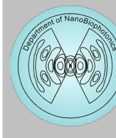
Laser
DM
Detektor
ST
PZM
PZT
y
x
z

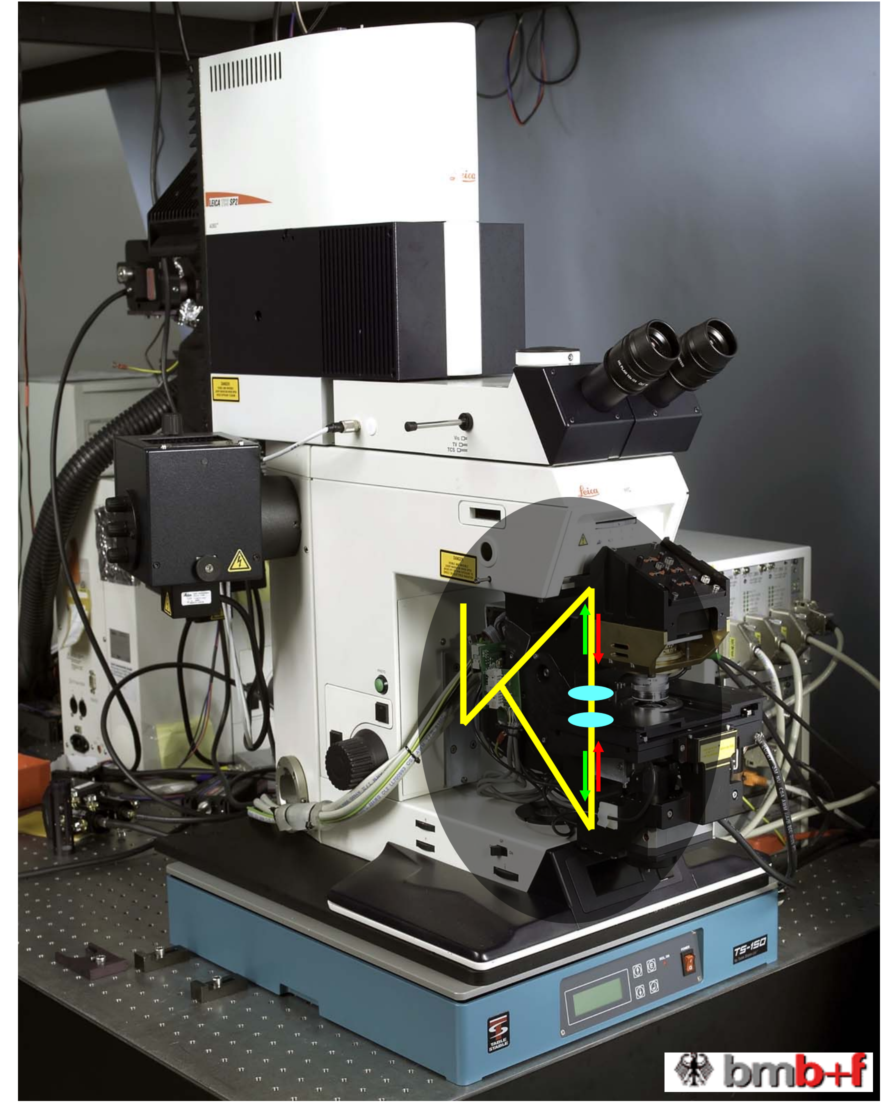
Fig.2

Microtubules, mouse fibroblast
Immunofluor, Oregon Green

S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

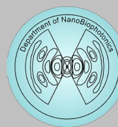


Commercial 4Pi-microscope



Z- resol < 90 nm (Live cells /aqueous cond.)

H. Gugel, et al. (2004), *Biophys J* **87**, 4146.



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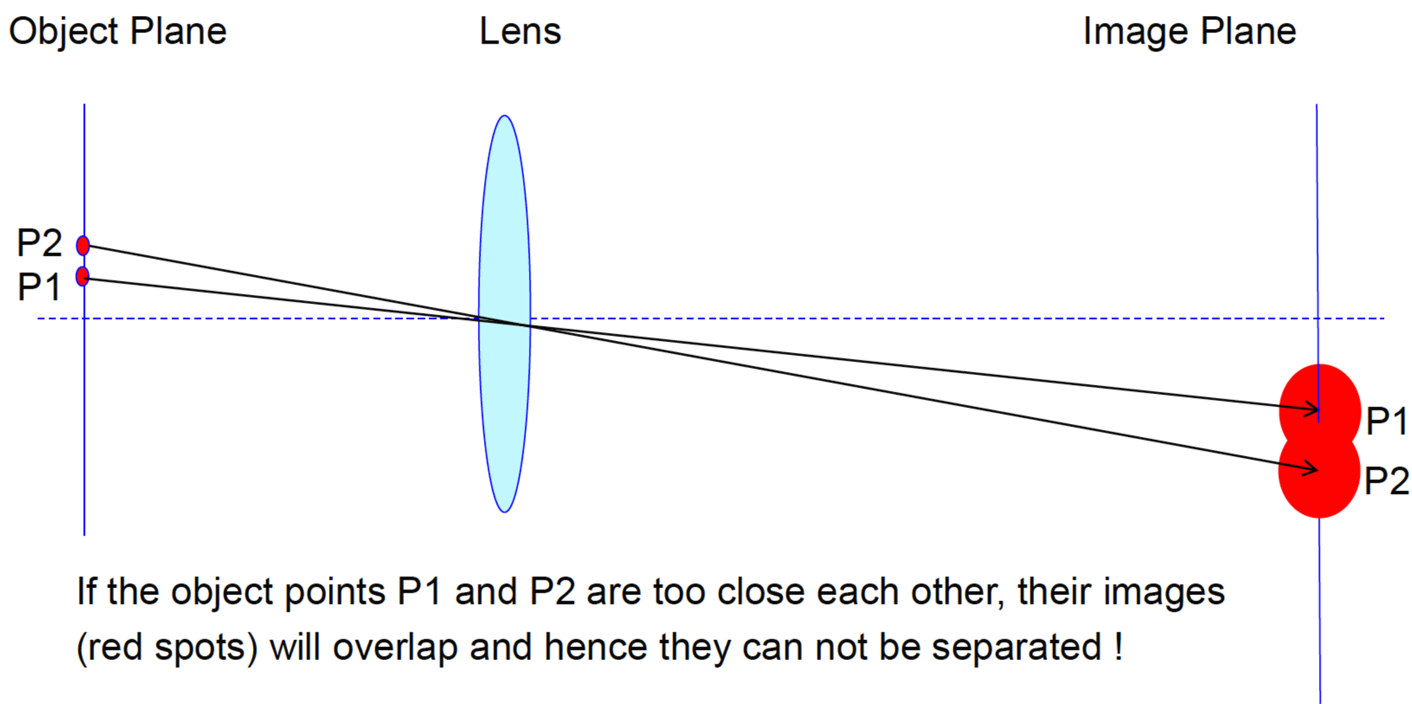
STED microscopy

1st physical concept to break the
diffraction barrier in
far-field
fluorescence microscopy

S.W. Hell & J. Wichmann (1994), *Opt. Lett.* **19**, 780.

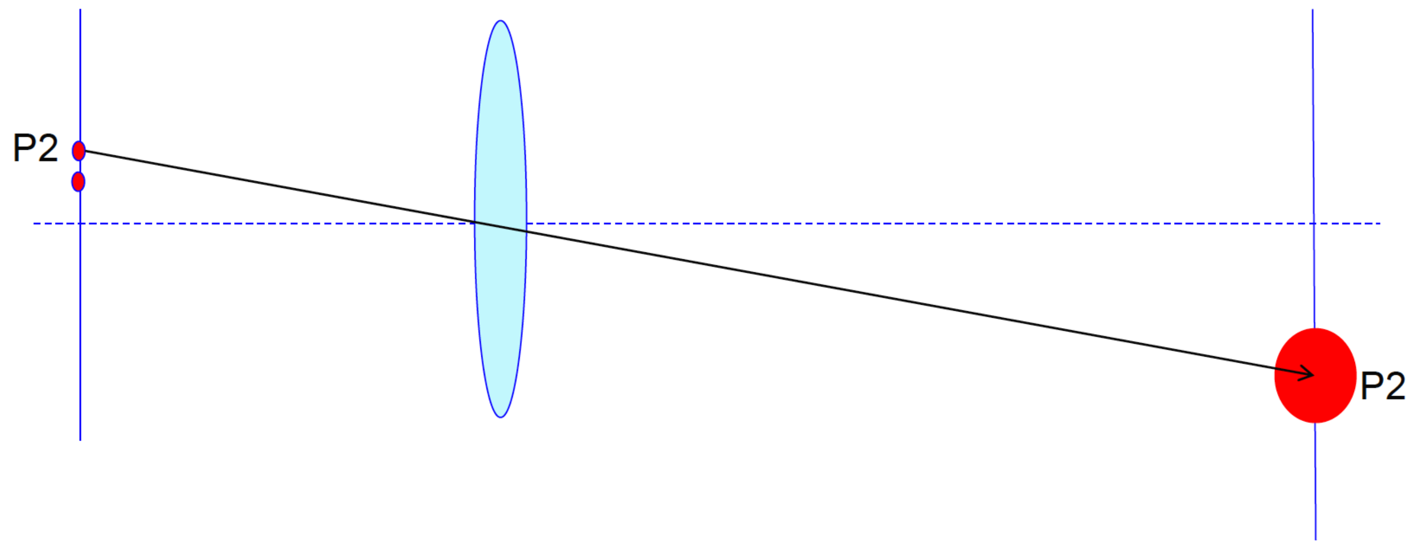
Imaging TWO fluorescent points through a lens



This limits the imaging resolution.

We say that he imaging is diffraction limited.

But what if we can **switch ON/OFF the fluorescence** of the two points ?



We can RESOLVE / IMAGE the two points below the diffraction limit !

Do not separate just by focusing the light !

Separate also by molecular ON/OFF states !

Stefan W. Hell, Nobel Prize Lecture 2014

Switching characteristics

To switch a fluorescent molecule ON or OFF requires **two states**:

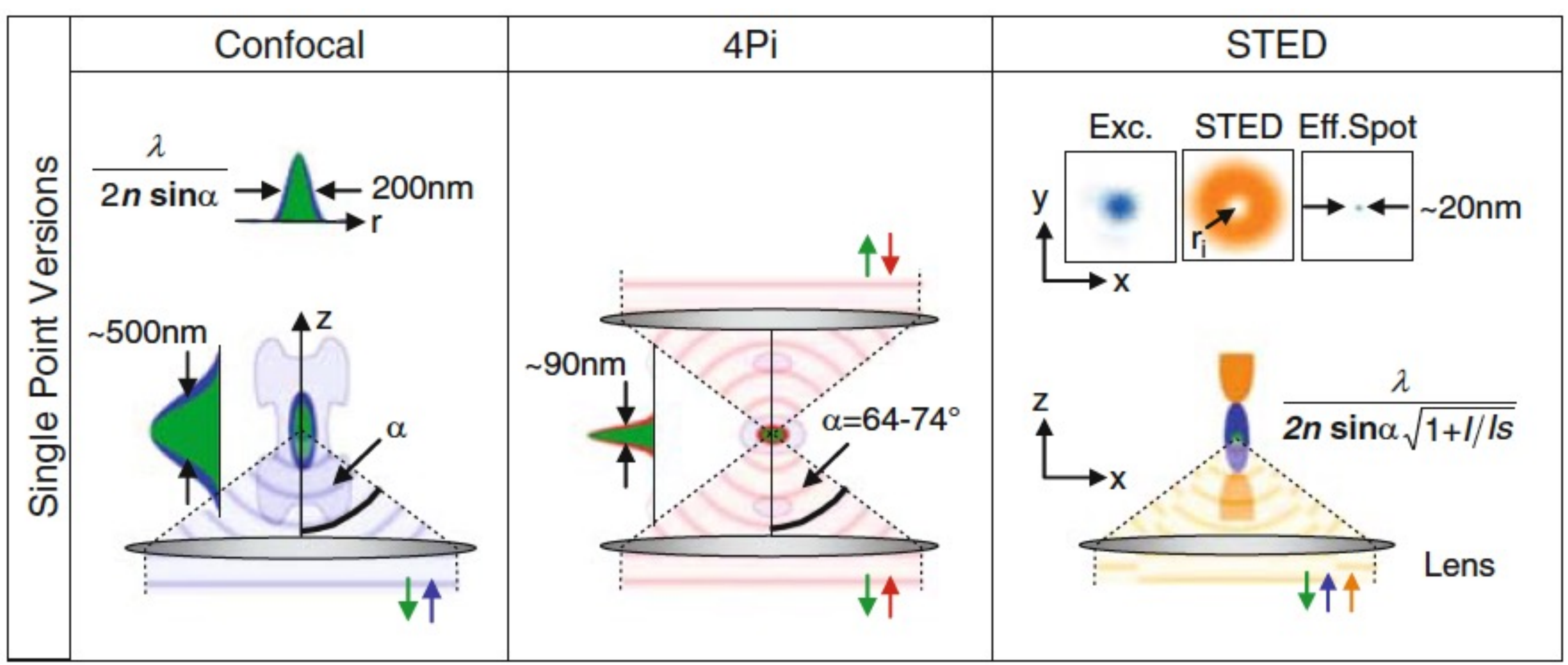
a **fluorescent (ON)** state and a **dark (OFF)** state,

connected by a transition representing the actual switch.

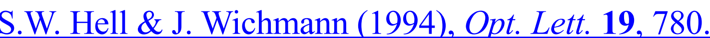
Several states in a fluorophore are suitable for such transitions:

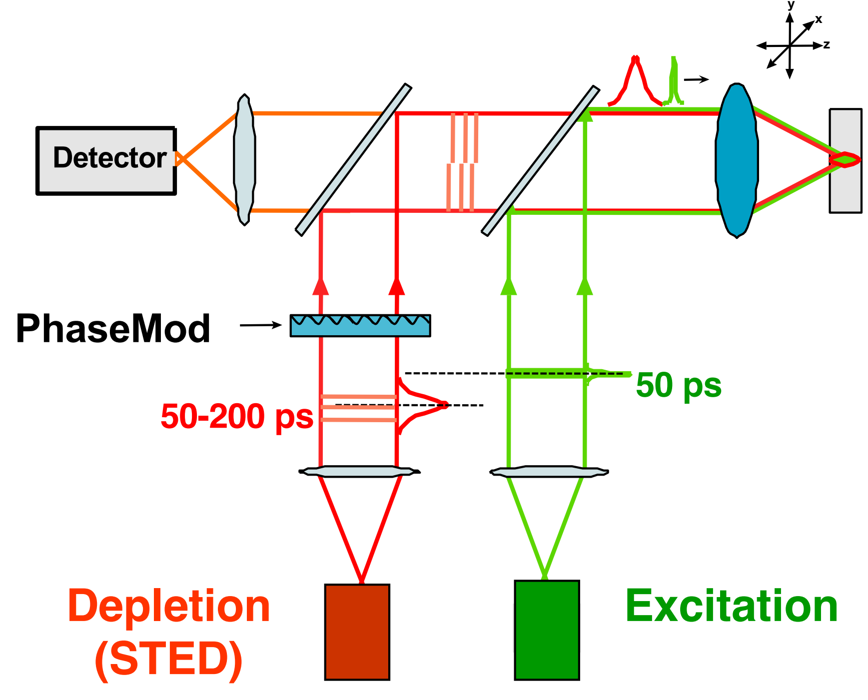
- S0 (ground state) and S1 (singlet state) represents the basic and obvious pair of ON/OFF states. It is used in **Stimulated Emission-Depletion (STED),** Saturated Pattern Excitation M/Saturated Structured Microscopy (SPEM/SSIM), Ground State Depletion (GSD).

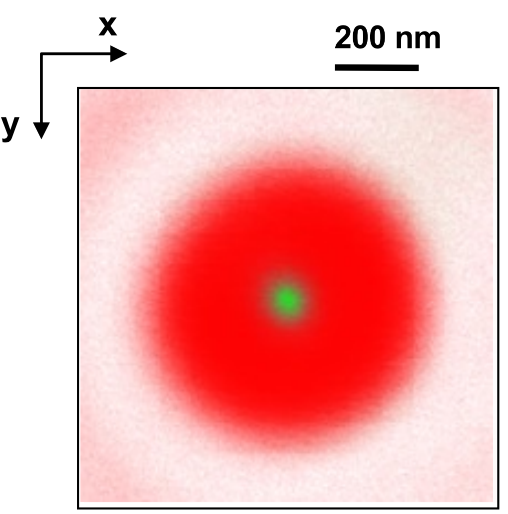
- The concept referred to as **Reversible Saturable Optical Fluorescent Transition** (**RESOLFT**) includes switching isomerization (*cis–trans*) states and other optically bistable transitions in fluorophores, as do the landmark concepts **PhotoActivation Localization Microscopy (PALM)** and **Stochastic Optical Reconstruction Microscopy (STORM)**


Single Point Versions
Confocal
4Pi
STED
$\frac{\lambda}{2n \sin\alpha}$
200nm
r
~500nm
z
α
~90nm
α=64-74°
Exc.
STED
Eff.Spot
y
x
~20nm
r_i
z
x
$\frac{\lambda}{2n \sin\alpha \sqrt{1+I/Is}}$
Lens

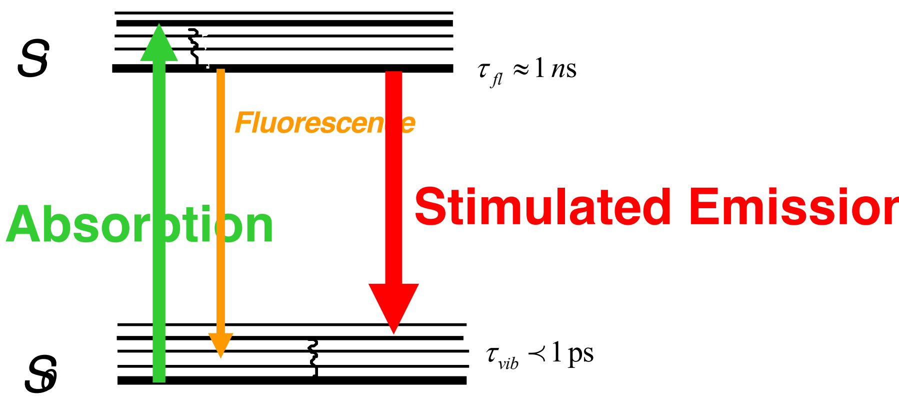
STED Microscopy

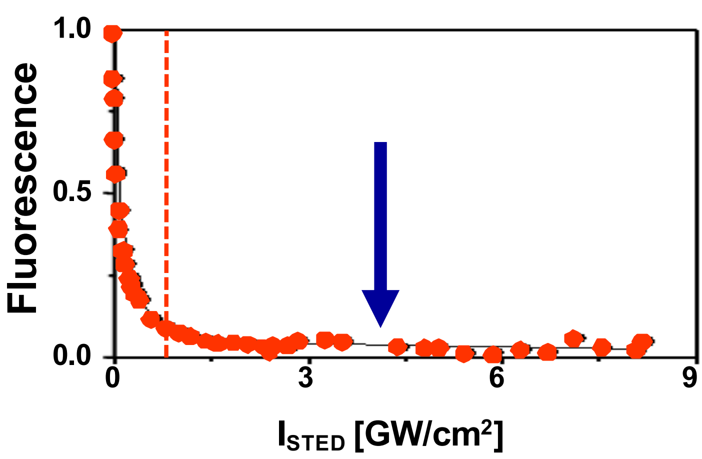






The stronger the STED beam the narrower the fluorescent spot!





Focal spot *... probed with 1 molecule*

Fluorescence
1.0
0.5
0.0
0
3
6
9
I_{STED}
$I/I_{sat} \cong 20$

λ_{STED}= 770 nm

STED
200nm

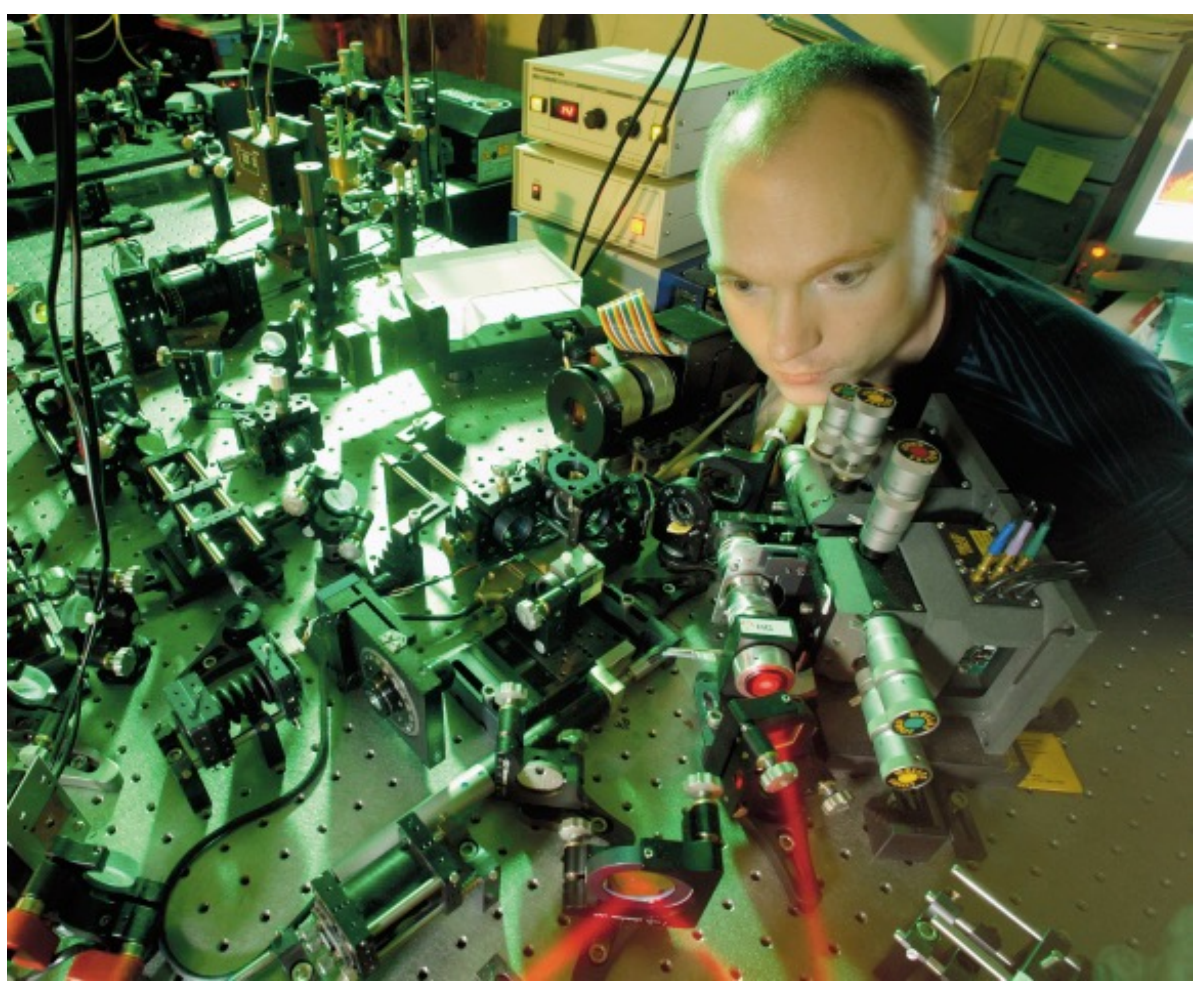
48 nm

254 nm

Confocal

-250
-150
-50
50
150
250
x [nm]

V. Westphal & S.W. Hell (2005), Phys. Rev. L



Imaging 40 nm fluorescence beads:

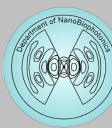
Confocal

10 counts/0,3ms 204

STED

... just physics !

5 counts/0,3ms 89

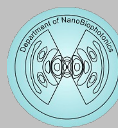


Imaging protein distribution on cell membrane: SNAP 25

...just physics !

Confocal:

STED:



Heavy subunit of neurofilaments in neuroblastoma

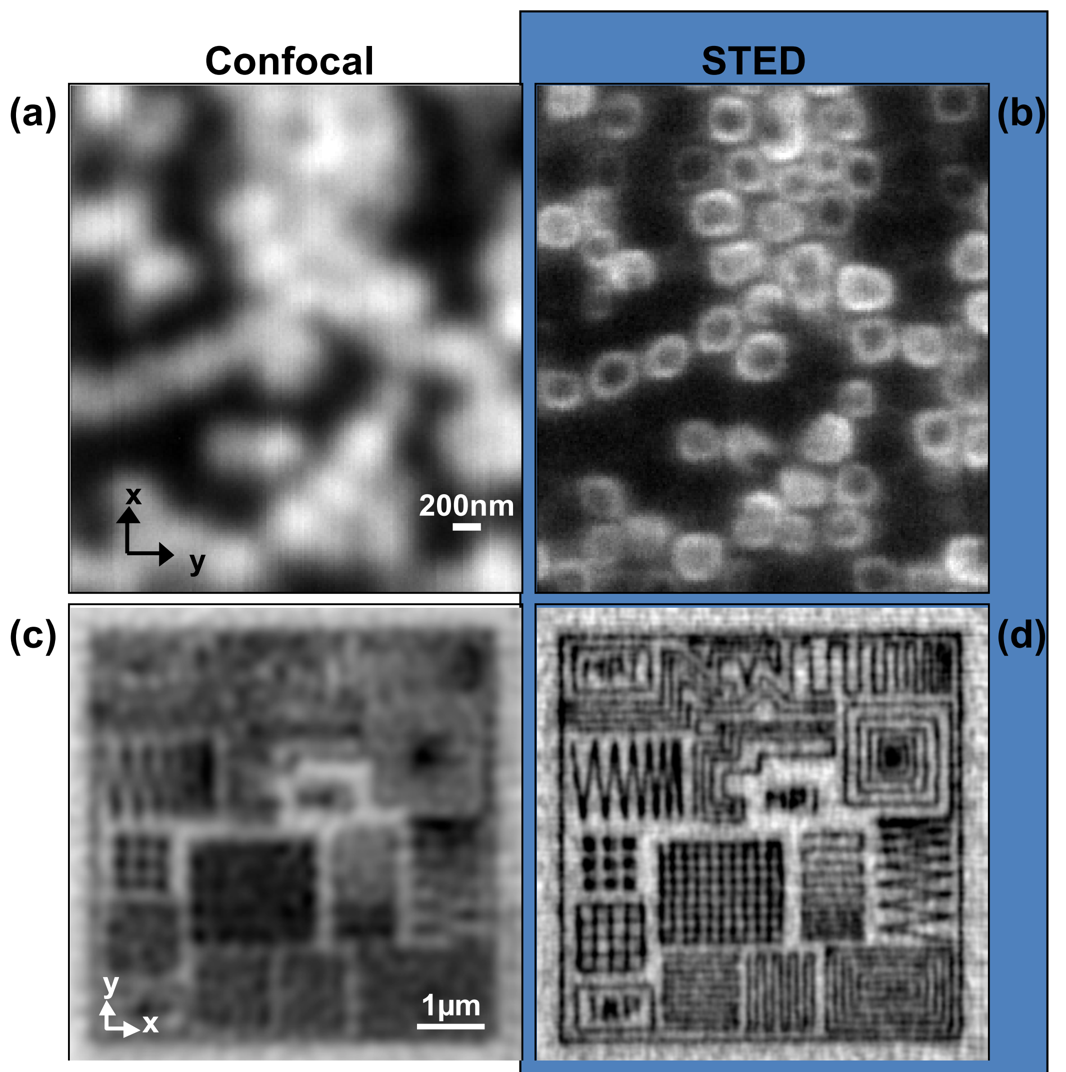
Confocal

500 nm

STED

[G. Donnert, et al. (2006), *PNAS* **103**, 11440.]

STED Microscopy: Sometimes only *resolution*...

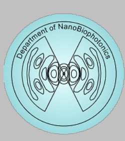


Pores in a porous membrane marked with a fluorescent dye

Fluorescence dye marked nanostructures produced by electron beam lithography in a polymer

...makes subdiffraction images !

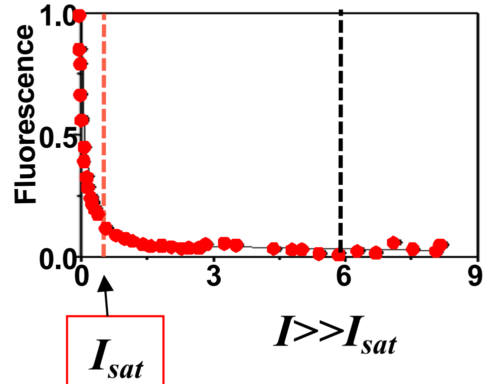
V. Westphal, S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.
V. Westphal, J. Seeger, T. Salditt, S. W. Hell (2005) , *J. Phys. B* **38**, S695.



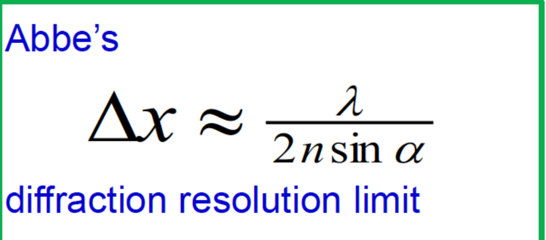
STED microscopy

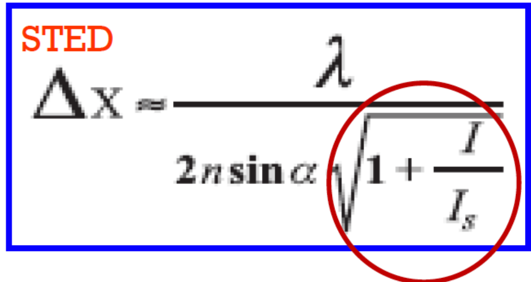
- Resolution is not limited by the wavelength of light!
- Resolution just depends on the level of fluorescence depletion.
- Resolution at the molecular scale is possible with visible light and regular lenses!
- Resolution follows a new law; a modification of Abbe's law:

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_{sat}}}$$



S.W. Hell (2003), *Nature Biotech.* **21**, 1347.
S.W. Hell (2004), *Phys. Lett. A* **326**, 140.
V. Westphal & S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.





- ***I/Is***, is called **"saturation factor"** and determines the increase in resolution

- ***I***, is the Intensity of the STED Laser;

- ***Is,*** is the **saturation intensity**, i.e. the intensity of the STED laser at which the fluorescence drops to 1/e of its initial value. ***Is ~ 1/T*** **!** *T* – transition time

- ***Is*** is a characteristic of the materia (dye used).
  Typical values:10–100 MW/cm^2 for organic dyes

- For a given dye,
  the resoultion is increased, increasing the intensity of the STED laser.

- Another possibility is to find dyes with longer transition time *T* between the two states and hence smaller saturation intensity *Is* → RESOLFT

In the targeted read-out mode, the bright state A is established at coordinate r_i by driving an optical transition $A \rightarrow B$ with a light intensity distribution $I = I(r)$ featuring an intensity minimum, ideally a zero, at coordinate r_i (Figs. 19.1d and 19.2a). Applying $I(r)$ transfers the markers virtually everywhere to B, except at the zero-intensity point r_i where the molecules can still remain in A. The rate of the transition $A \rightarrow B$ is given by $k_{AB} = \sigma I$, with σ denoting the optical cross-section for $A \rightarrow B$. In order to effectively switch the molecule to B, the optically induced rate k_{AB} must outperform any competing spontaneous transitions between A and B. Since these spontaneous rates are given by the inverse lifetimes $\tau_{A,B}$ of the states A and B, we obtain: $k_{AB} = \sigma I \gg (\tau_{A,B})^{-1}$. Therefore, applying an intensity I that is much larger than the "saturation intensity" $I_\mathrm{s} = (\sigma\,\tau_{A,B})^{-1}$ shifts the molecule everywhere to B except in the proximity of the zero-intensity point r_i of $I(r)$. Thus, we obtain a narrowly confined region $r_i \pm \Delta r/2$ in which the molecule can still be in A. The width Δr of this region or spot is readily calculated as

$$\Delta r \approx \frac{\lambda}{2\,n\,\sin\alpha\,\sqrt{1 + aI_{\max}/I_s}}. \tag{19.1}$$

Sharpest focal spot

(a) Confocal
STED
16nm
x [nm]

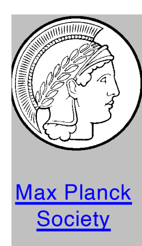
(b)
235nm
131nm
72nm
40nm
31nm
26nm

$$0.45 \frac{\lambda_{STED}}{n \sin\alpha \sqrt{1+\varsigma_B}}$$

PSF FWHM [nm]
STED Intensity [MW/cm²]
nm
n

Validation of square-root resolution law

V. Westphal, S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.



Max Planck
Society

4Pi- STED Microscopy

Axial (z) resolution 30-50 nm and beyond …

The combination: STED-4Pi-Microscopy

↓ Monolayer ↓ Monolayer

confocal

STED-4Pi

53 nm

1600
1200
Counts
800
400
0
0 1 2 3 4
Z / µm
Monolayer Microtubules

x
z

M. Dyba, S. W. Hell

FWHM
53nm
1 2 3
Z/ µm
0 1 2 3 4
Monolayer Microtubules

Fluorescently tagged microtubuli
with an axial resolution of 50-70 nm

M. Dyba, S. Jakobs, S.W. Hell (2003), *Nature Biotechnol.* **21**, 1303.

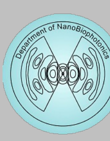
Max Planck Society

RESOLFT

Reversible Saturable (Switchable) Linear Fluorescence Transitions

is

the generalized principle of STED microscopy



RESOLFT: Reversible Saturable Optical (Fluorescent) Transition

S.W. Hell (2003), *Nature Biotechnol.* **21**, 1347.
S.W. Hell, M. Dyba, S. Jakobs (2004), *Curr. Opin. Neurobiol.* **14**, 599.

Acknowledgements / References:

Physics:

G. Donnert et al — *PNAS, 103 (2006)*

K. Willig, J. Keller, M. Bossi, S.W. Hell — *New J Phys, 8 (2006)*

V. Westphal, S.W. Hell — *Phys Rev Lett, 94 (2005)*

L. Kastrup, H. Blom, C. Eggeling, S.W. Hell — *Phys Rev Lett, 94 (2005)*

M. Hofmann, C. Eggeling, S. Jakobs, S.W. Hell — *PNAS, 102 (2005)*

Applications:

K. Willig, S. Rizzoli, R. Jahn, S.W. Hell — *Nature, April 13, (2006)*

R. Kittel, et al — *Science, May 19, (2006)*

Pictures/Movies

www.nanoscopy.de