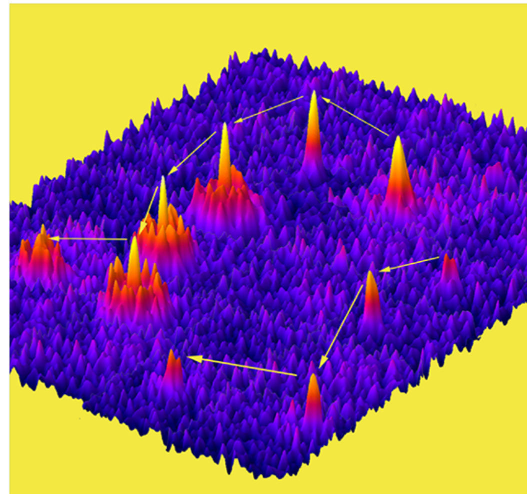
Course „Optics, Forces & Development"



Microscopy Tools

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15. Super resolution microscopy: expansion and STED microscopy
16. Light as tool: fluorescence photobleaching and optical tweezers

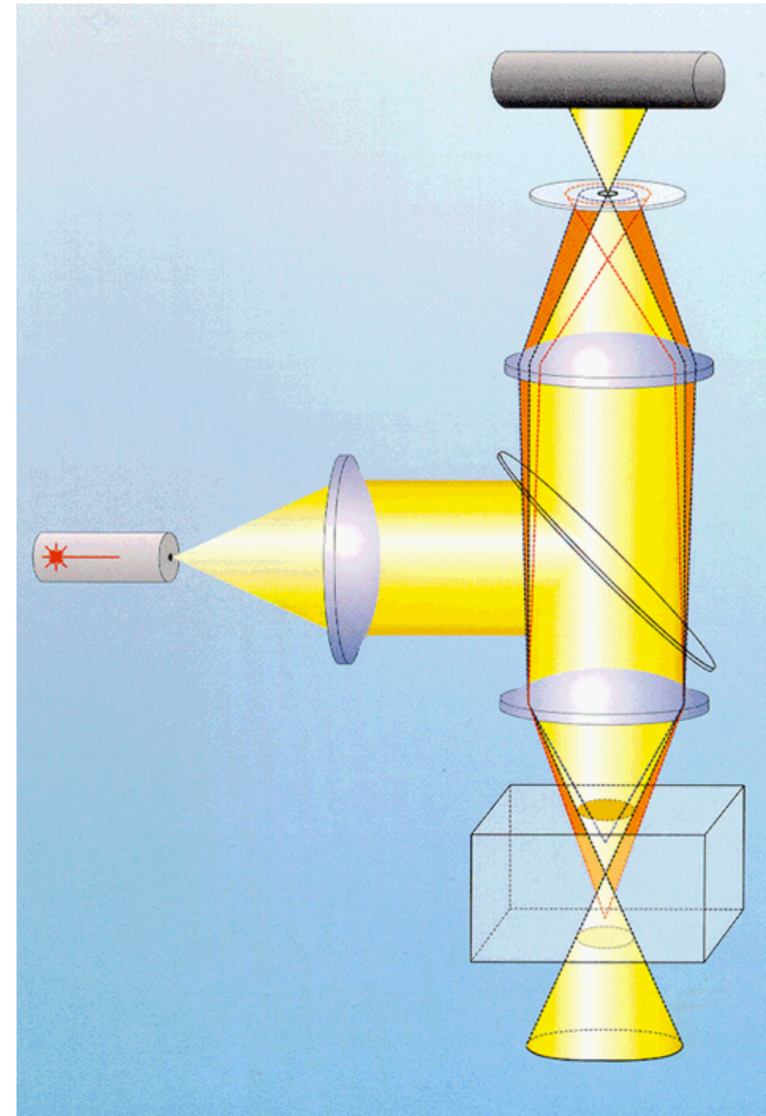
The confocal principle

Combination of focussed laser illumination

and

detection through pinhole placed in a conjugated optical plane

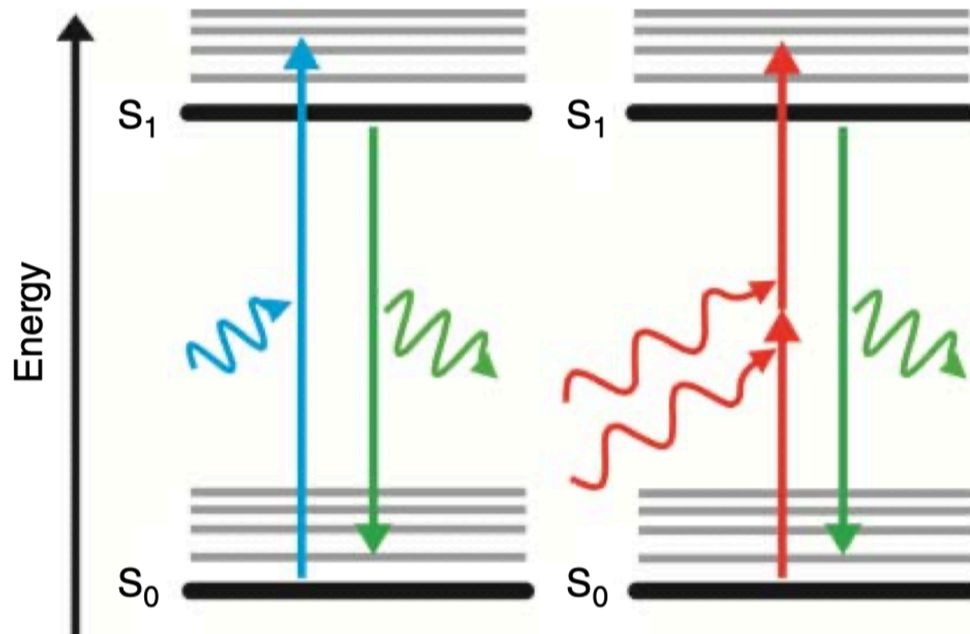
yields efficient background subtraction and axial resolution: „Optical Sectioning“



Source: Carl Zeiss, info on laser scanning microscopy

12. 2-photon microscopy

2-photon fluorescence excitation



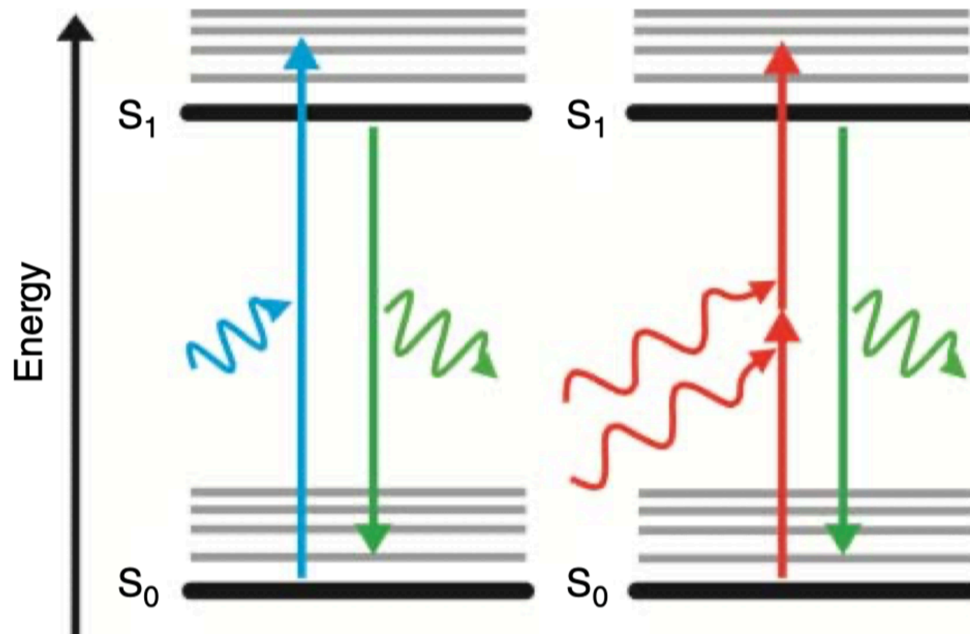
David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

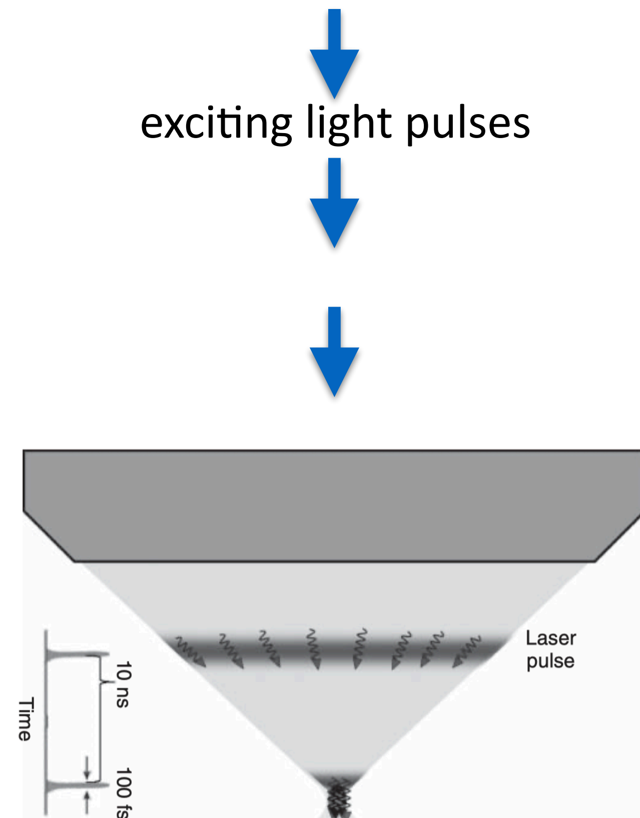
Parameters for 1 and 2 photon excitation microscopy

	One-photon (linear) absorption	Two-photon (non-linear) absorption
1	$N_{ex} = \sigma I$	$N_{ex} = \delta I^2$
2	$\sigma = 10^{-16}$ cm^2	$\delta = 10^{-50}$ cm^4 s/photon
3	$I_{ex} = 1$ MW cm^{-2}	$I_{ex} = 10^6$ MW cm^{-2}
4	CW laser	$t = 10^{-13}$ s
		Freq. = 100 MHz
5	$P_{sat} = 1$ mW	$P_{sat} = 10$ mW

David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

2-photon fluorescence excitation



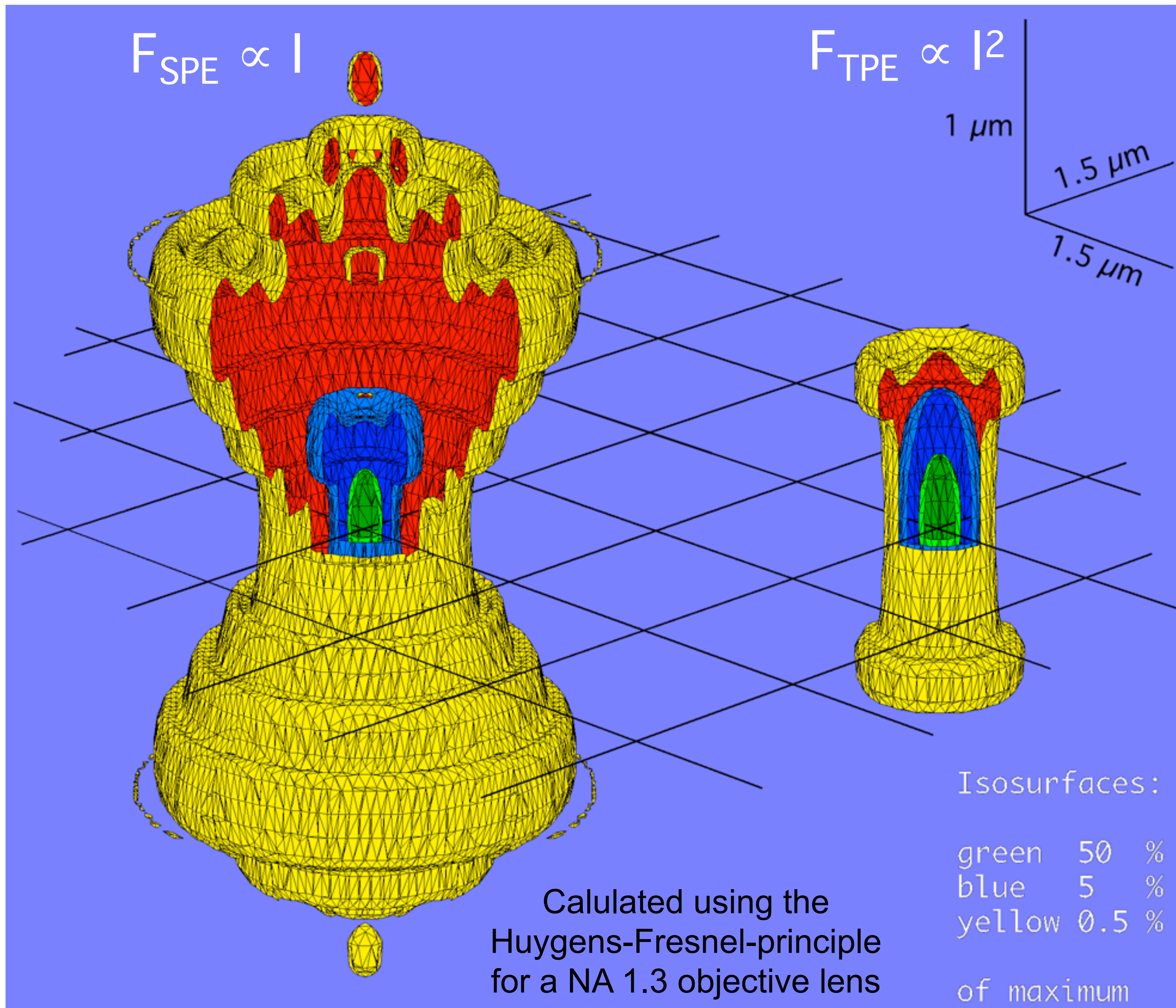


David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

1- and 2-Photon Excitation Profiles

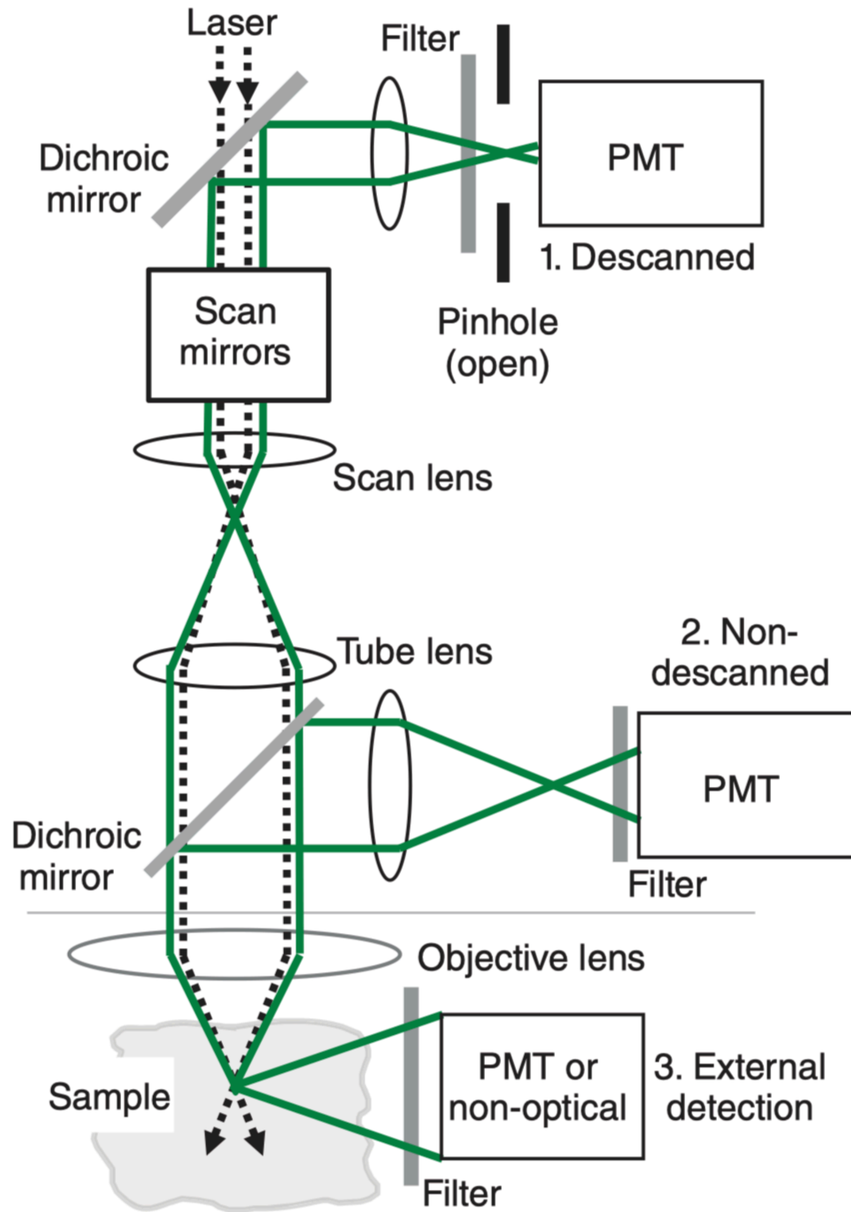
SPE, λ=488 nm

TPE, λ=800 nm



Courtesy of Dr. Christian Verbeek

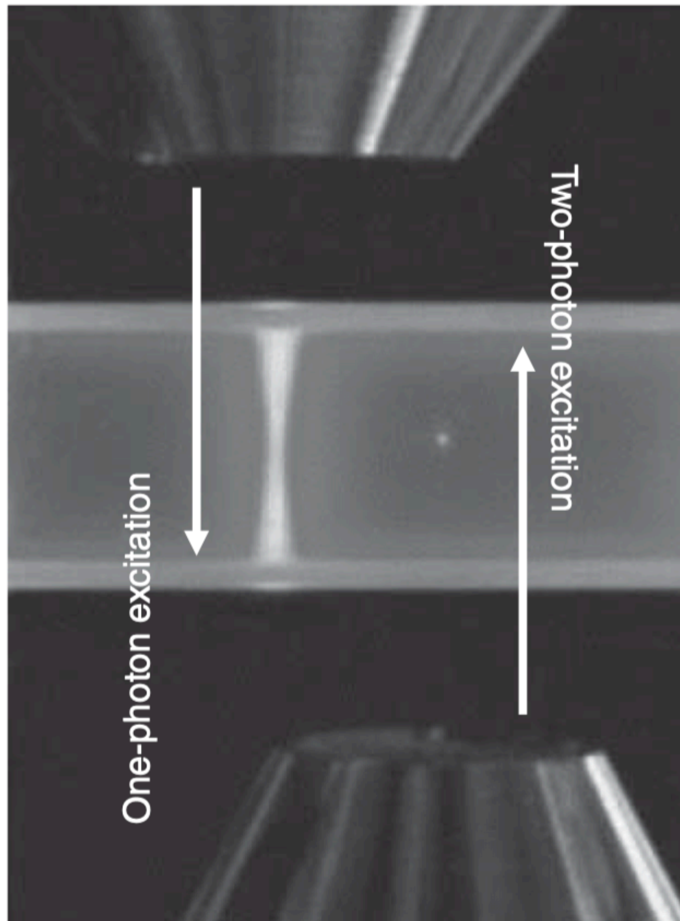
2 photon fluorescence microscope



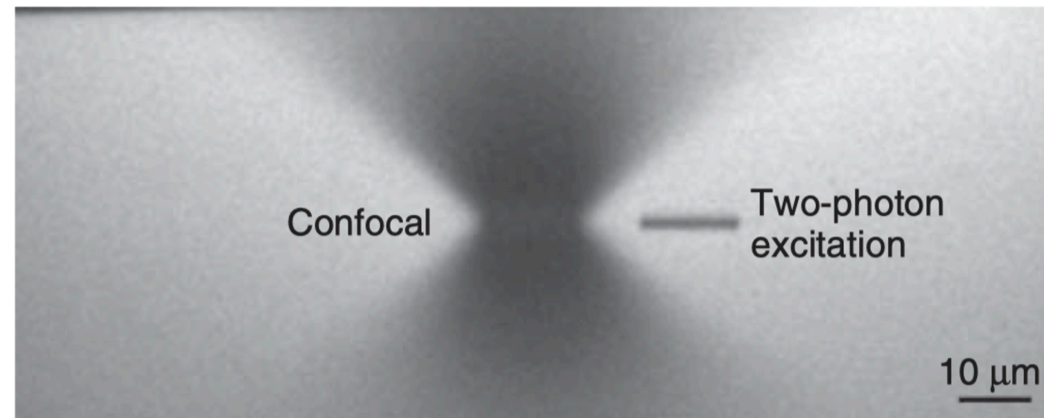
David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Localized excitation and bleaching in 2 photon excitation microscopy

Excitation



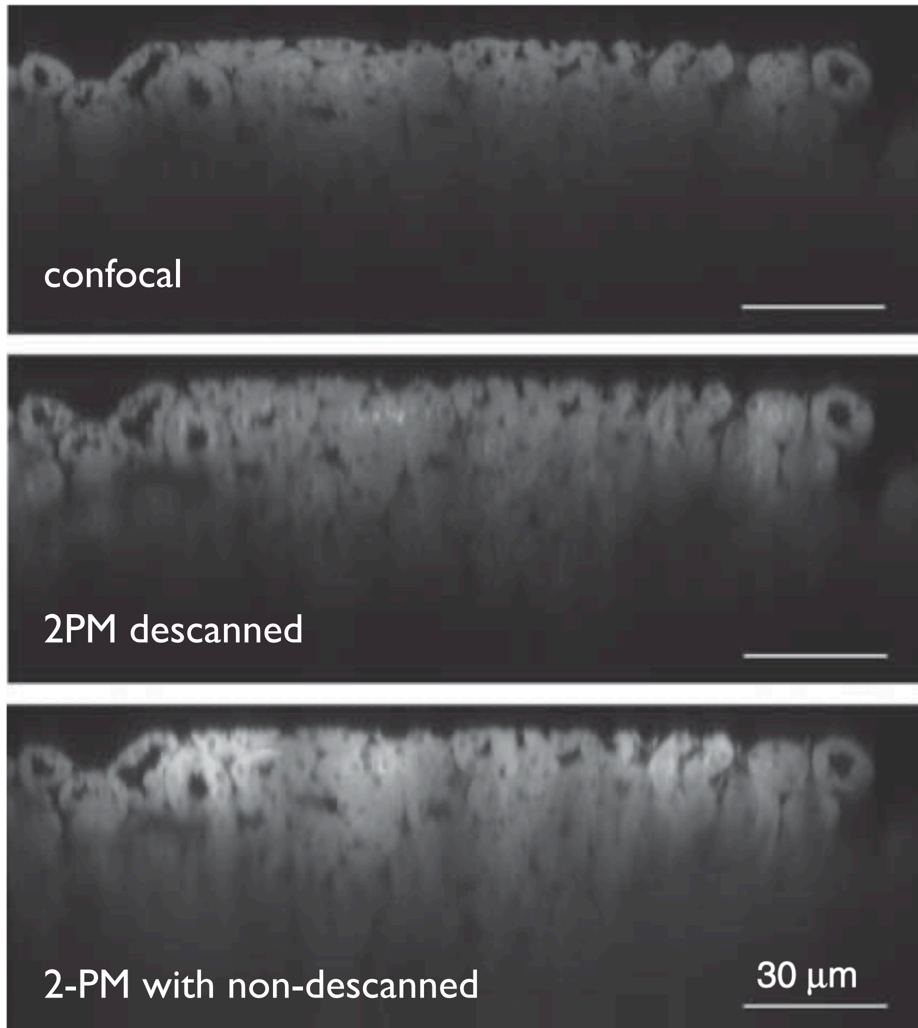
Photobleaching



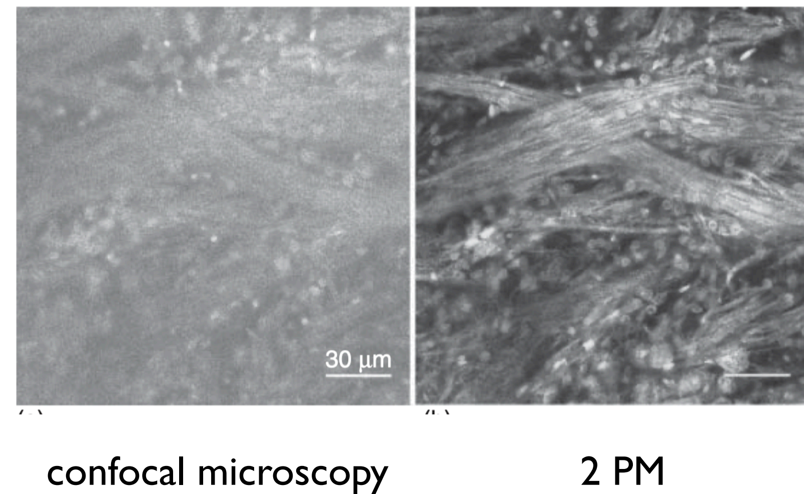
David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Penetration depth and contrast in 1 and 2p microscopy

X–Z profiles through an eosin-stained mouse kidney sample imaged in a depth of 70 μ m with various detection schemes



Same X–Y plane imaged 50 μ m deep in a rhodamine-stained slice of mouse brain

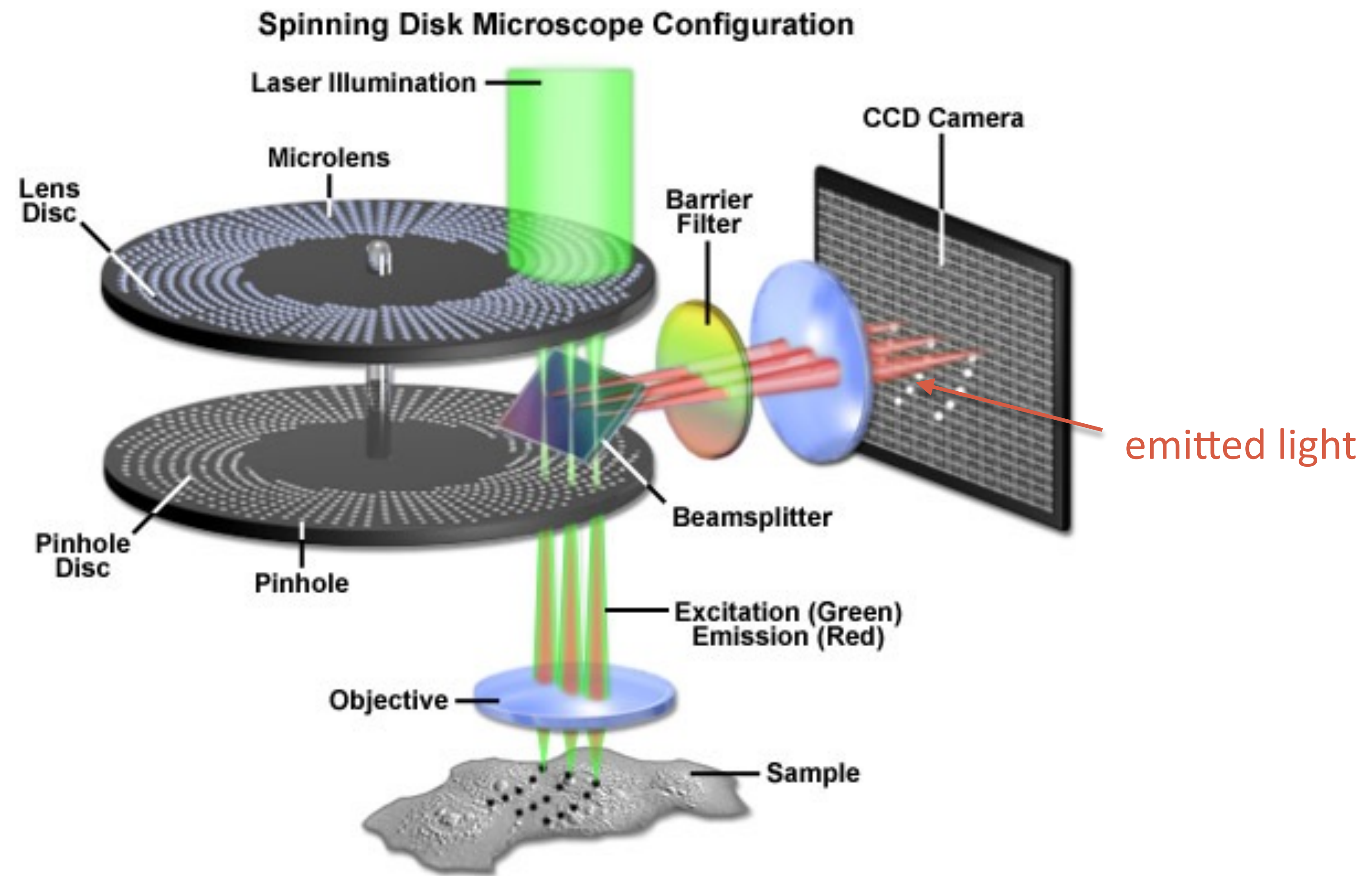


confocal microscopy 2 PM

David W. Piston, Chapter 6 „2-Photon Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

13. Spinning disk confocal microscopy

Spinning disk confocal microscope



Sketch of how the two Nipkow disks are mounted in the microscope.

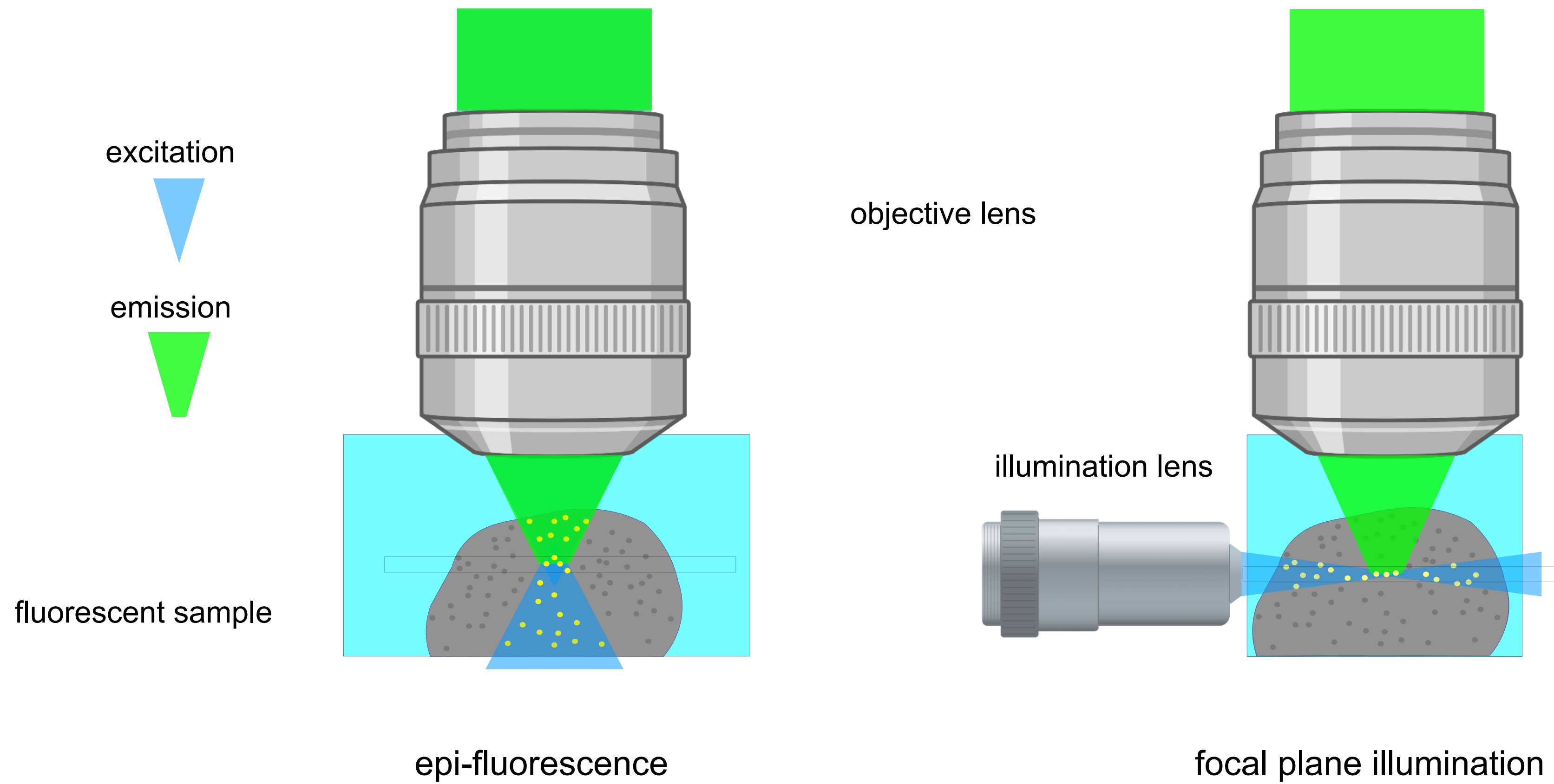
Source: http://zeiss-campus.magnet.fsu.edu/articles/livecellimaging/techniques.html

Summary: confocal microscopy

- out-of-focus light is reduced by confocal pinhole
- lateral resolution is improved by a factor of up to 1.4
- optical sectioning is possible
- measured intensity is proportional to dye concentration
- 3D-data sets describe spatial structure of objects
- rendering and ray-tracing allow 3D-visualization
- high hard- and software demands
- low light efficiency: about 1‰ - 1% (video microscope 3-10 %
- 2 photon excitation microscopy yields specific features
- Nipkow disk microscopy allows to speed up imaging

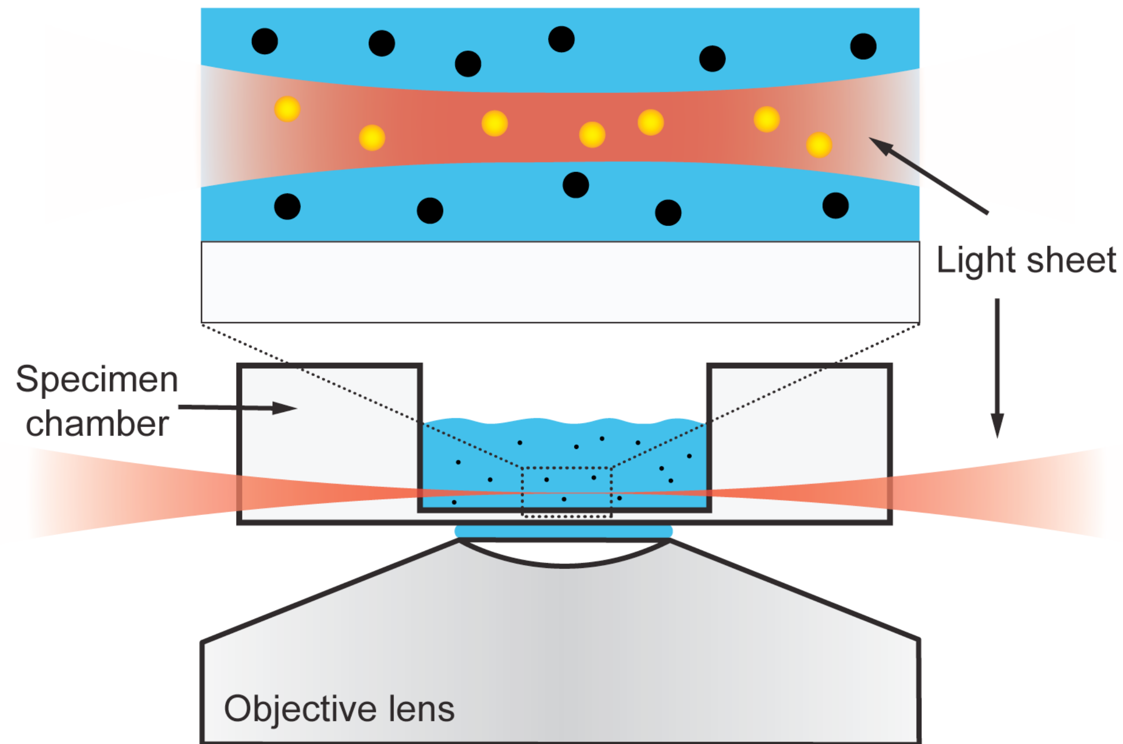
14. Light sheet fluorescence microscopy

High contrast by light-sheet based microscopy



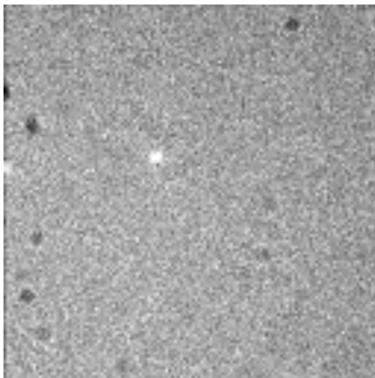
Zsigmondy, 1903
Voie et al, 1993
Huisken et al., 2004
Dodt et al., 2007
.....

Single molecule imaging in solution



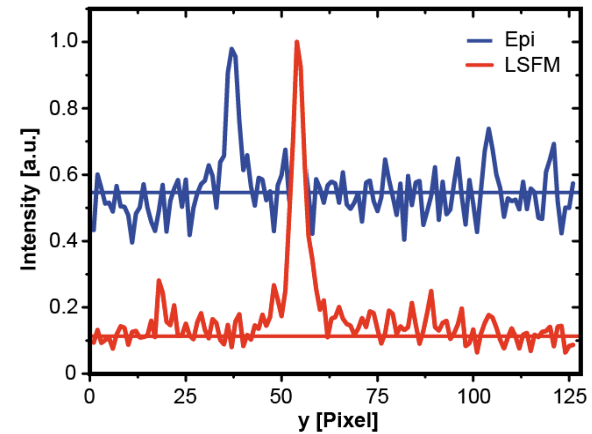
Comparison between epi- and light sheet illumination

500 kDa dextran-Atto633 in buffer
40X, NA 1.2W objective lens
Image field 19.2 µm
Image acquisition 100 Hz, display 33 Hz



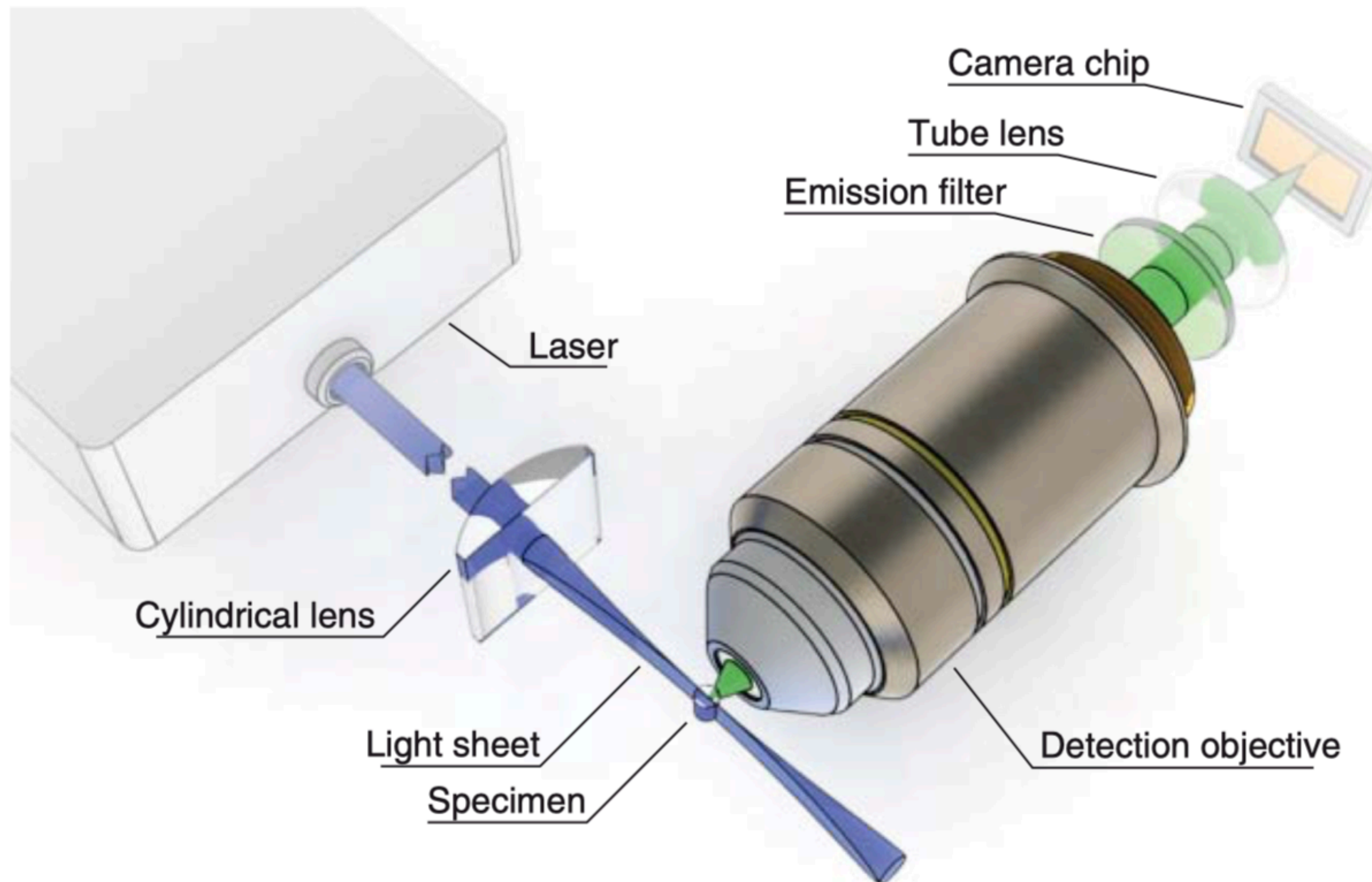
Epi-illumination
Contrast 0.37

Sheet illumination
Contrast 0.97



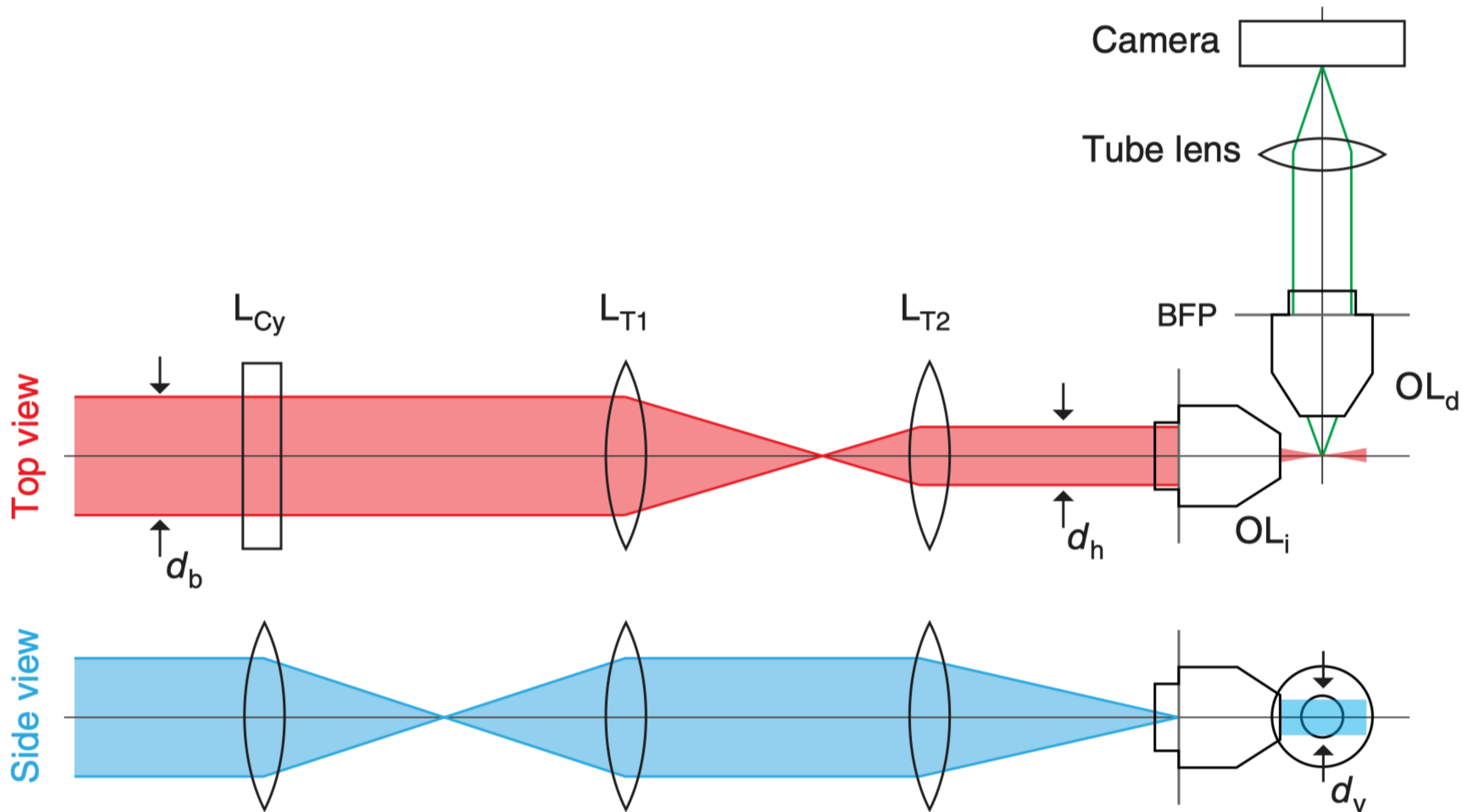
D= 7.1 ± 0.2 $\mu m^2/s$
as theoretically expected

Principle of light sheet microscopy



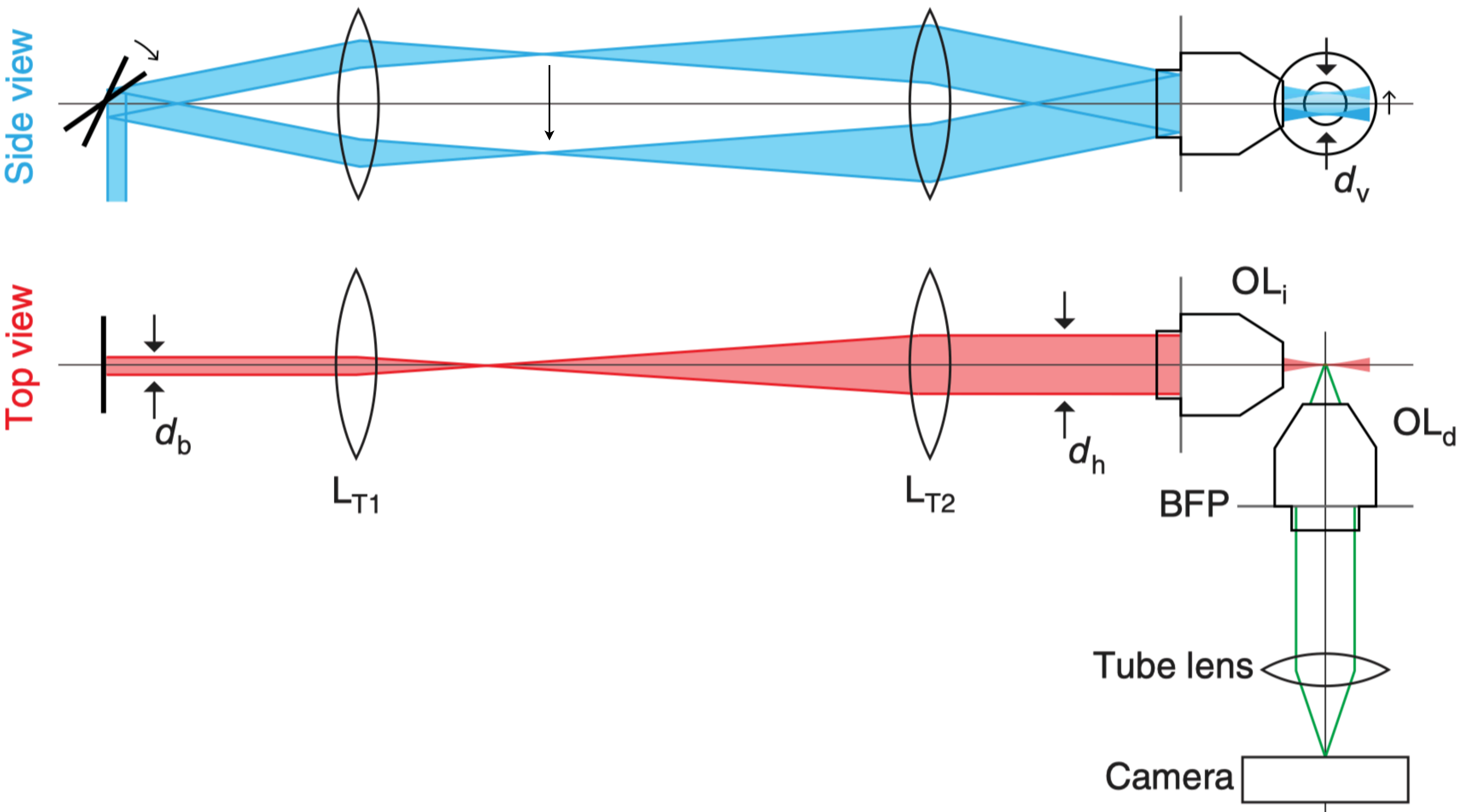
Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Light Sheet Microscopy using a cylindrical lens



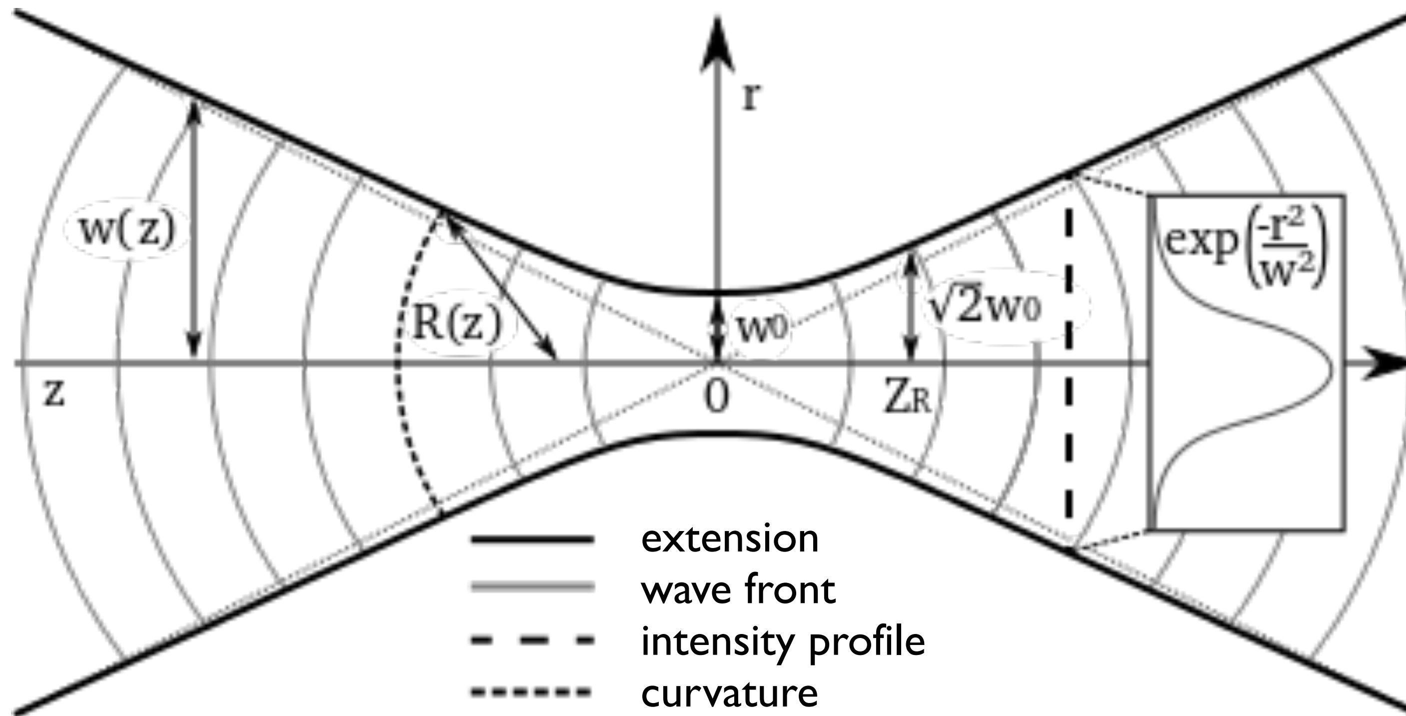
Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy" in „Fluorescence Microscopy", ed. U. Kubitscheck, 2017 Wiley VCH

Scanned Light Sheet Microscopy



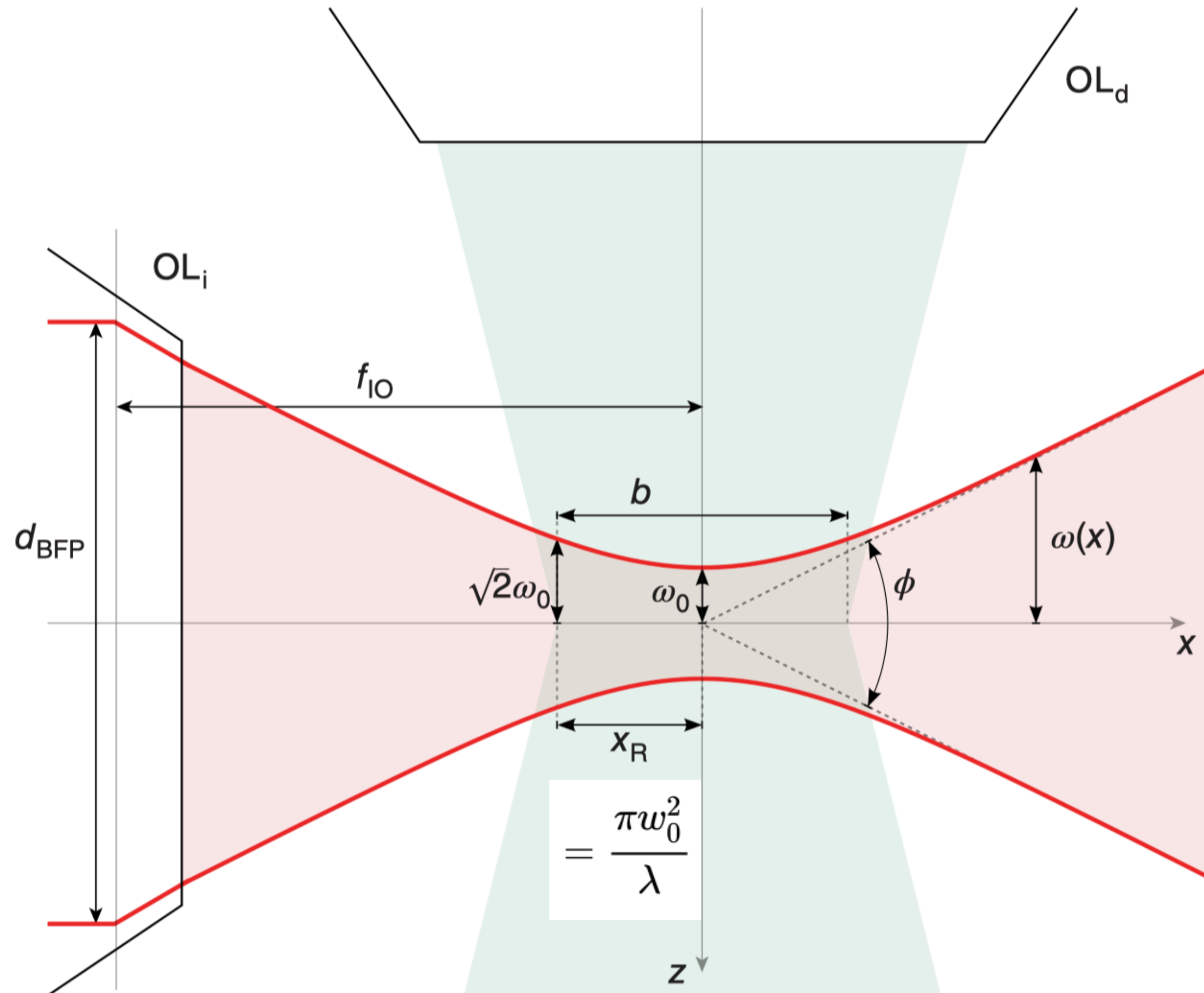
Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy" in „Fluorescence Microscopy", ed. U. Kubitscheck, 2017 Wiley VCH

Gaussian beam



Source: א (Aleph) - Eigenes Werk, CC BY-SA 2.5, https://commons.wikimedia.org/w/index.php?curid=3807143

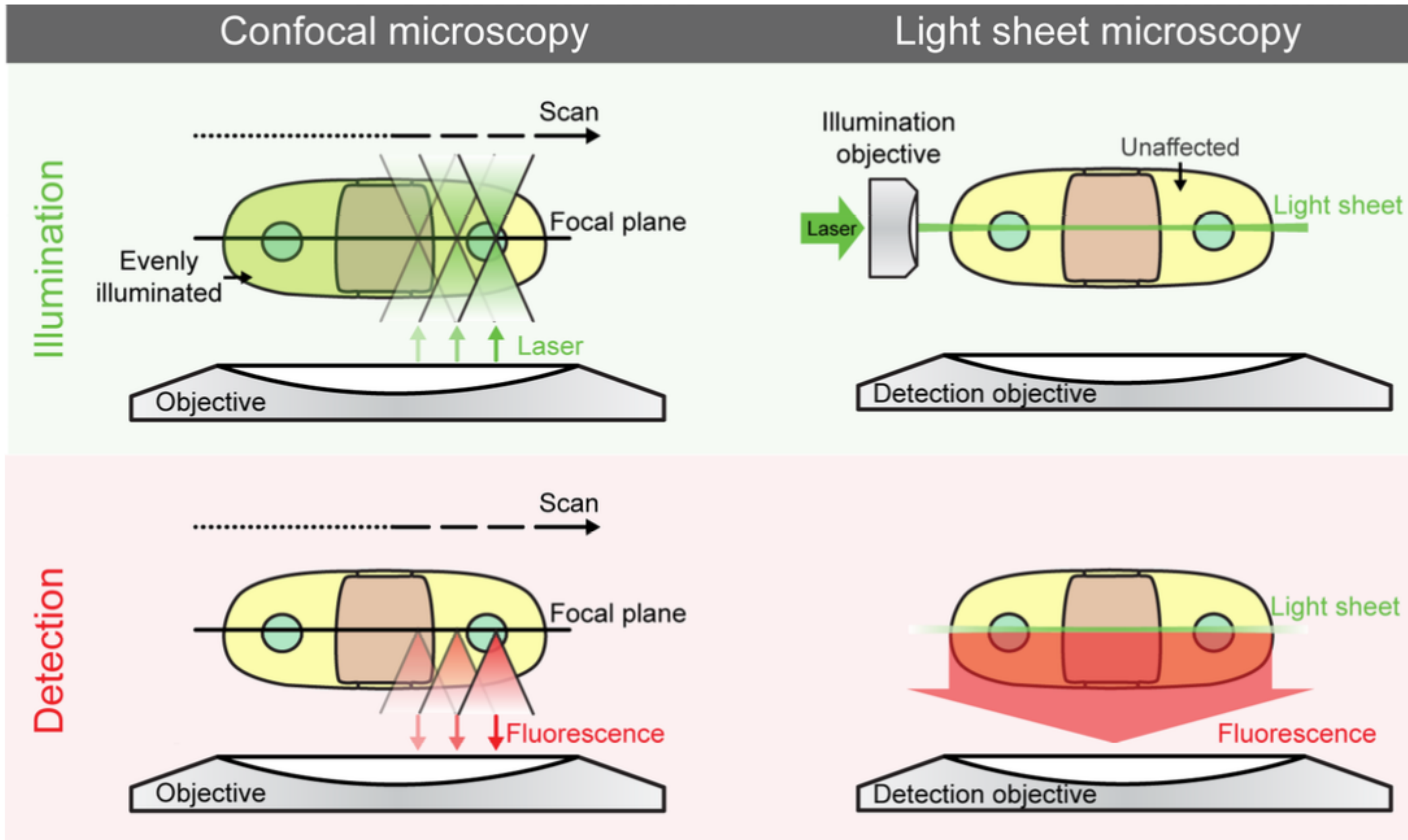
Relationship between light sheet dimensions and field of view



Gaussian beam illumination:
limited observation field size
Width and length are closely related to each other

Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Image generation in confocal and light sheet microscopy

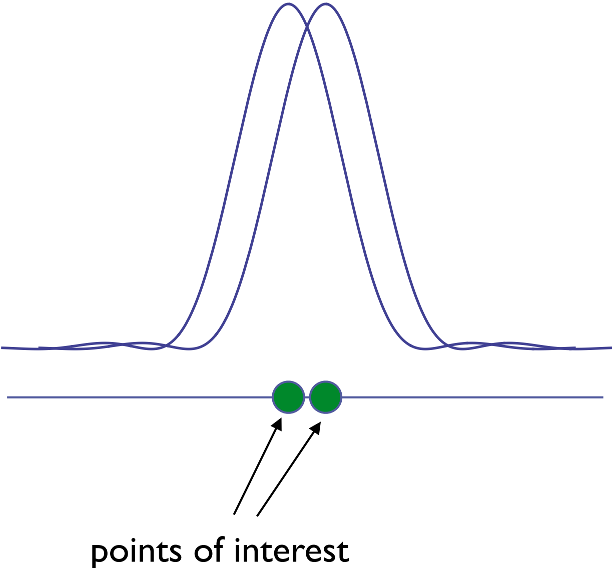


Jörg Ritter, Bonn 2011

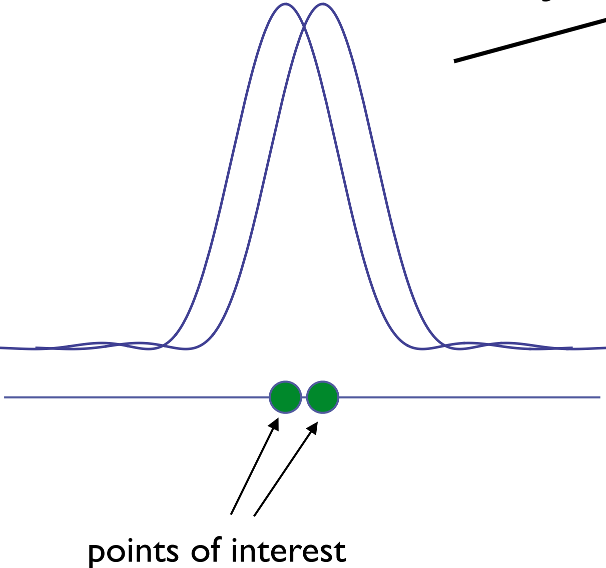
15. Super resolution microscopy

(a) Expansion microscopy
(b) STED microscopy

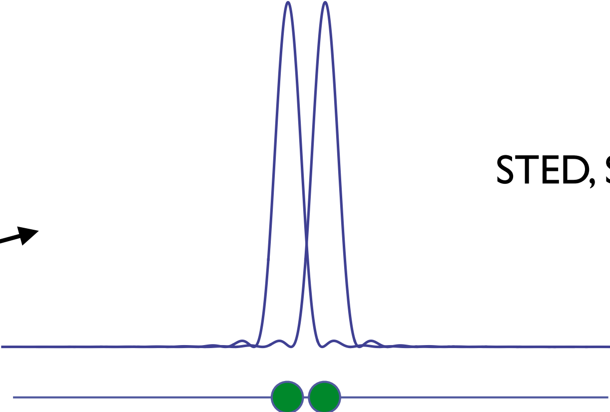
How to image a sample with sub-resolution features?



Increase optical resolution by „sharpening“ the point spread function

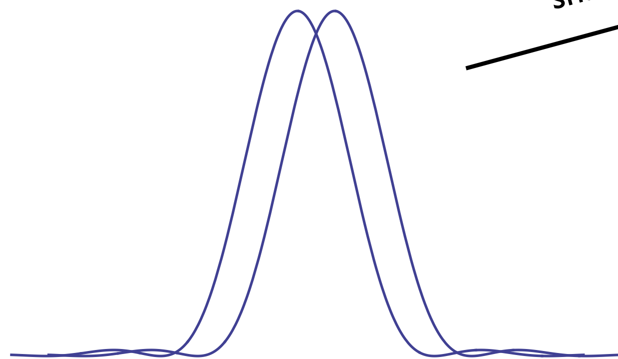


sharpen PSF

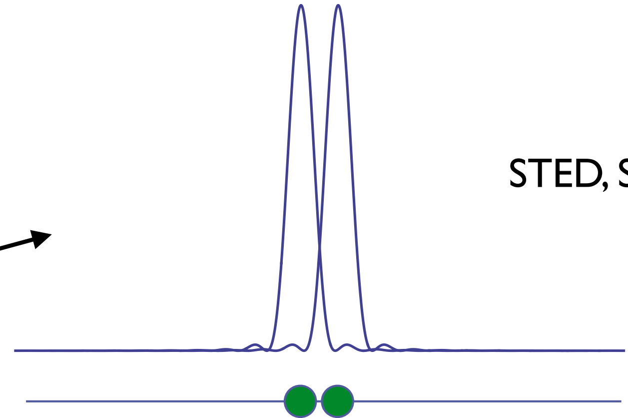


… or use expansion microscopy

classical microscopy

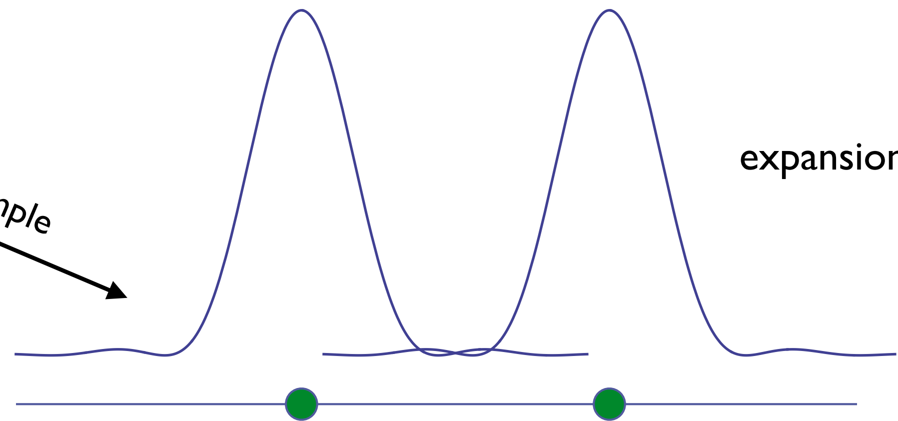


sharpen PSF



STED, SIM, STORM

expand sample



expansion microscopy

15a. Expansion microscopy

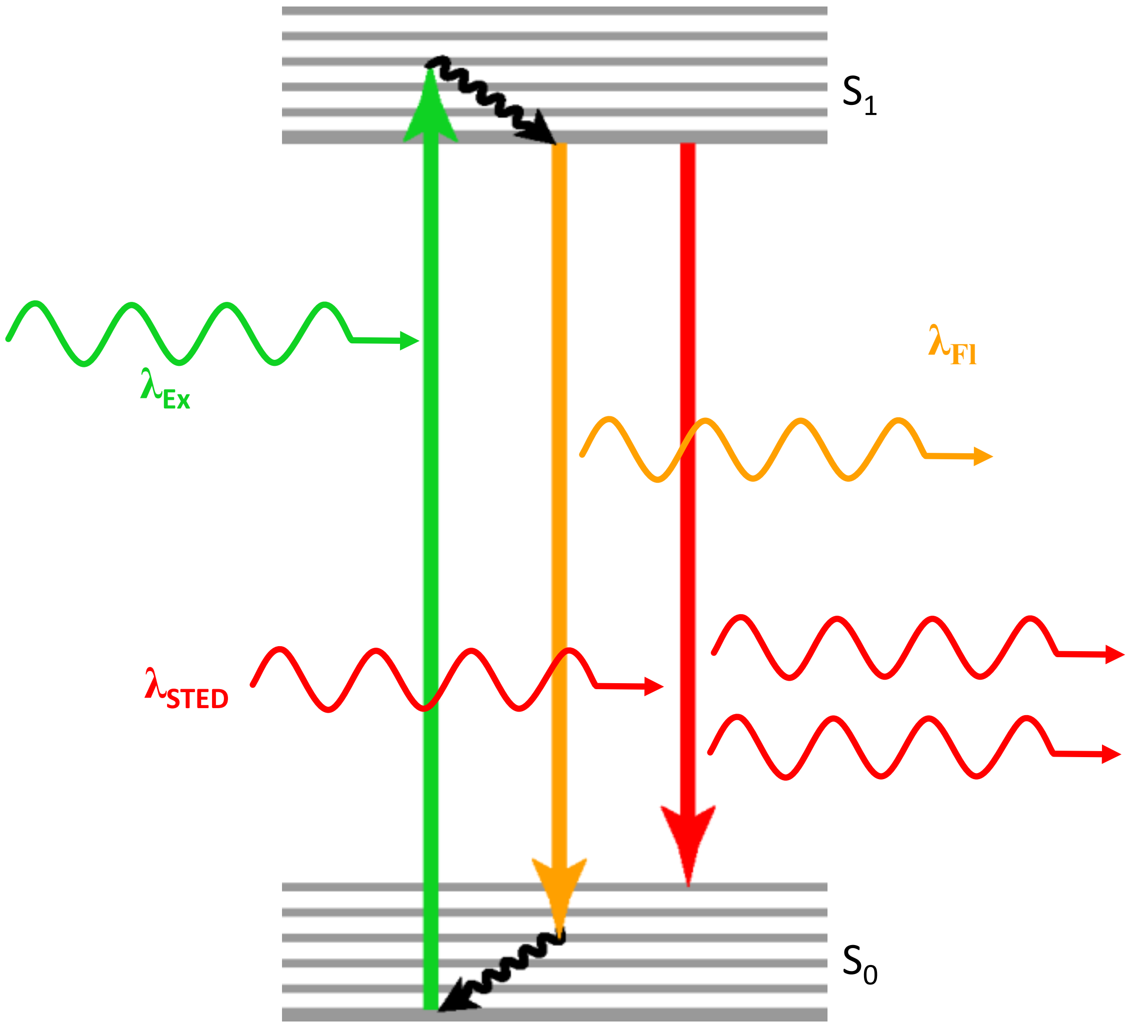
Light sheet fluorescence expansion microscopy

→ PhD defense Dr. Juan Eduardo Rodríguez-Gatica

February 22nd, 2024

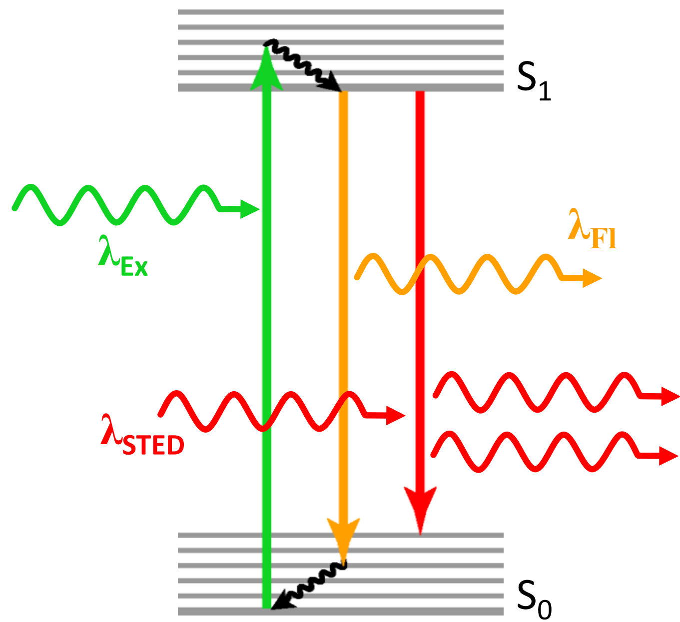
15b. Stimulated emission depletion microscopy STED

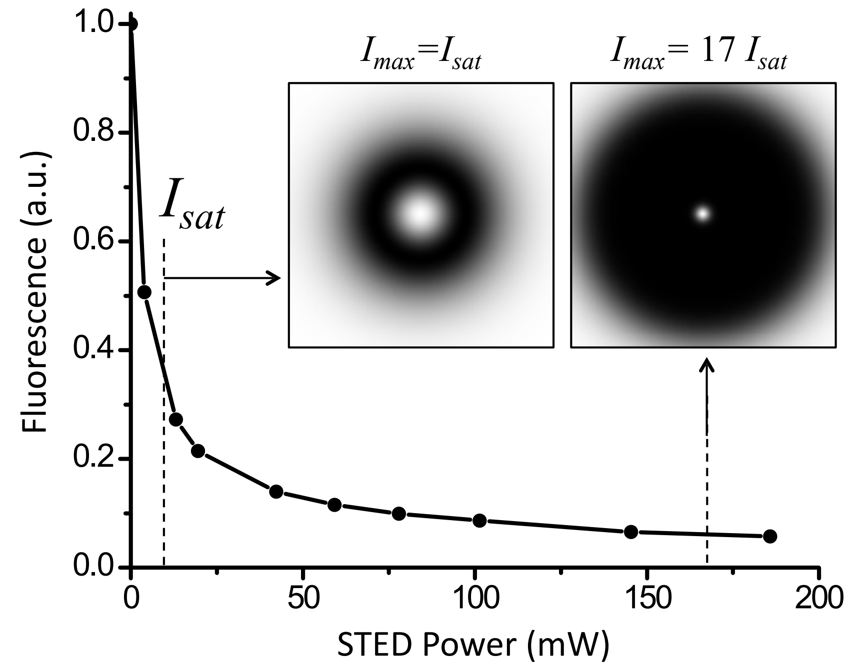
Stimulated emission



Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy" in „Fluorescence Microscopy", ed. U. Kubitscheck, 2017 Wiley VCH

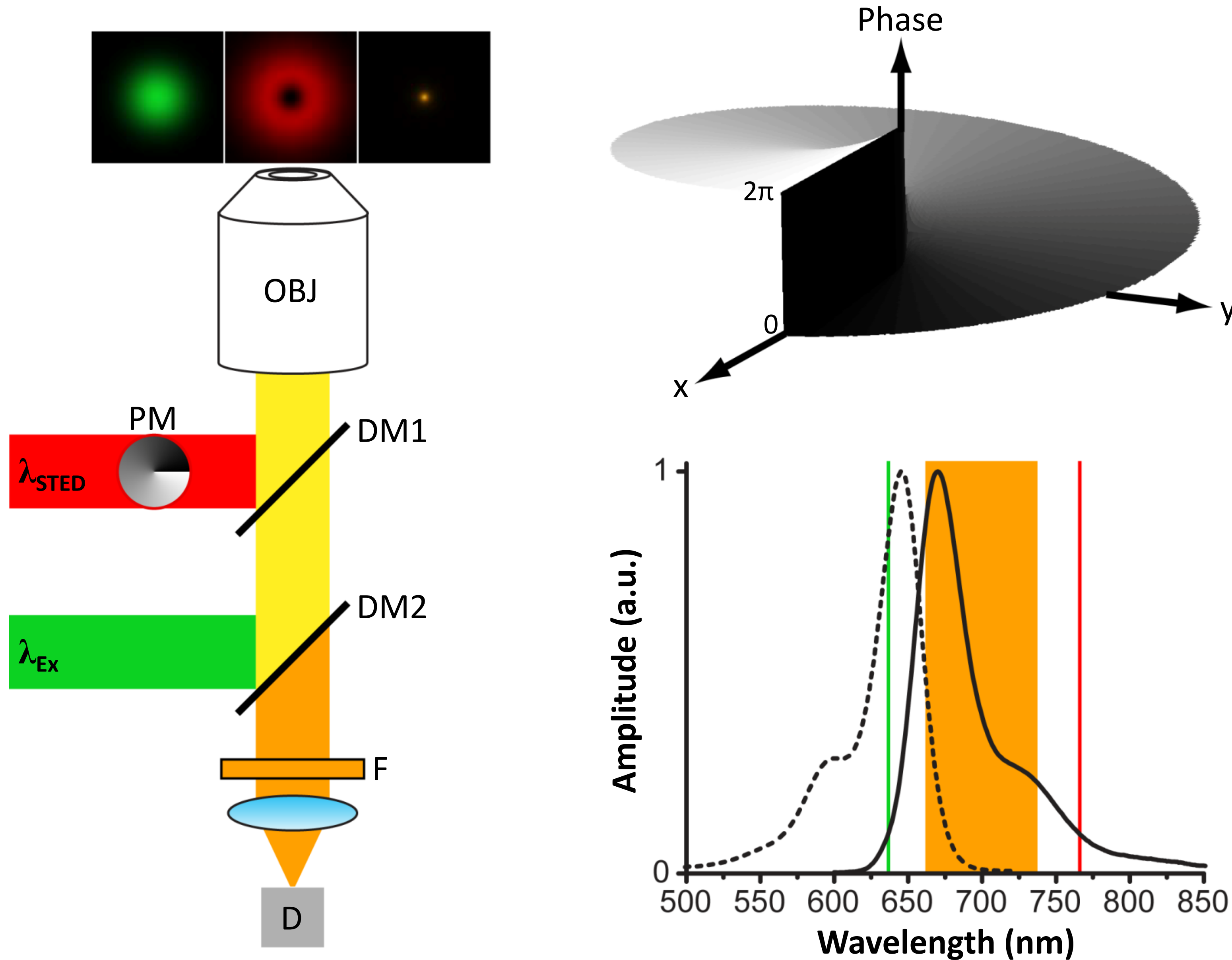
Stimulated emission





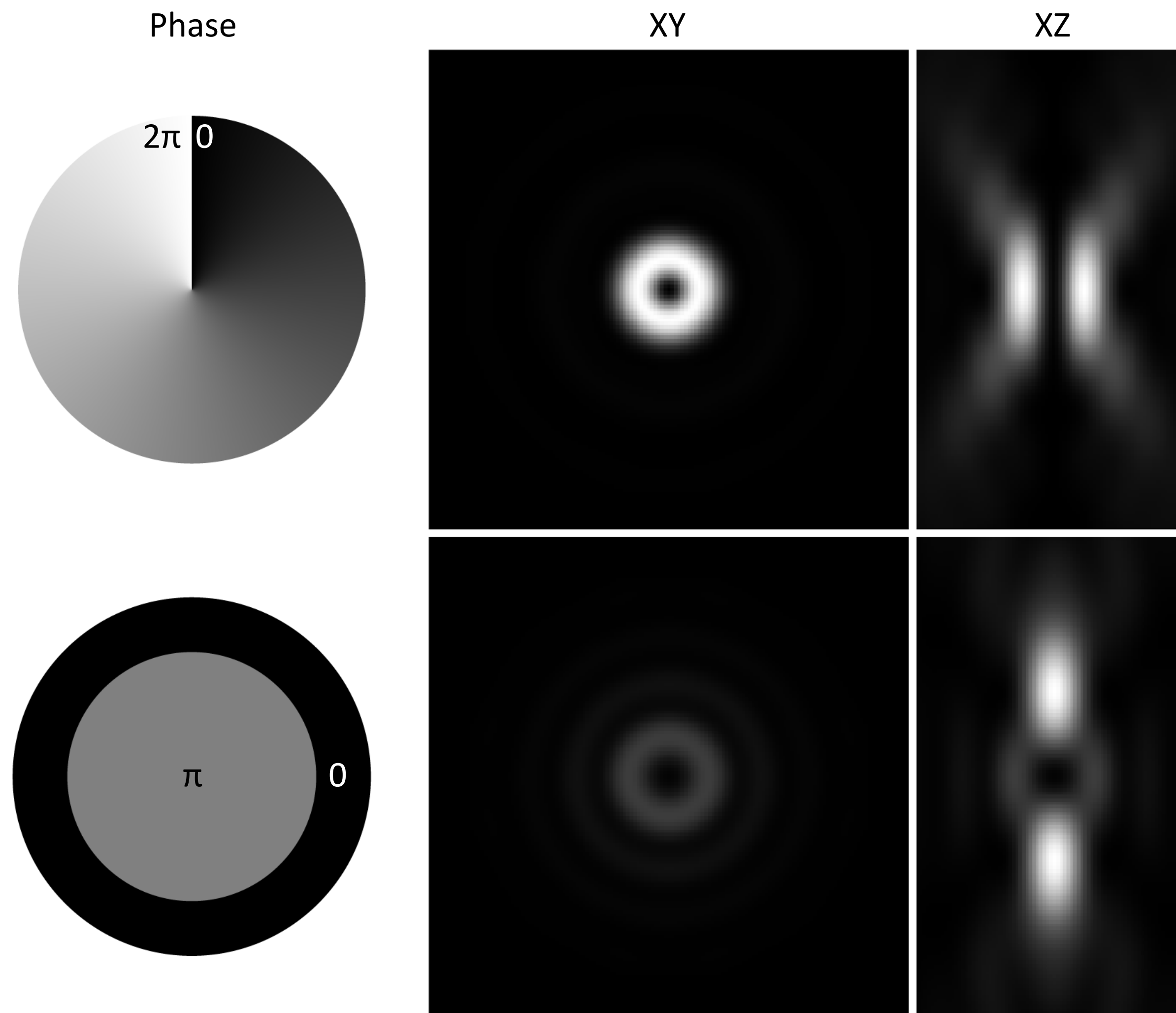
Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Realisation of STED microscopy



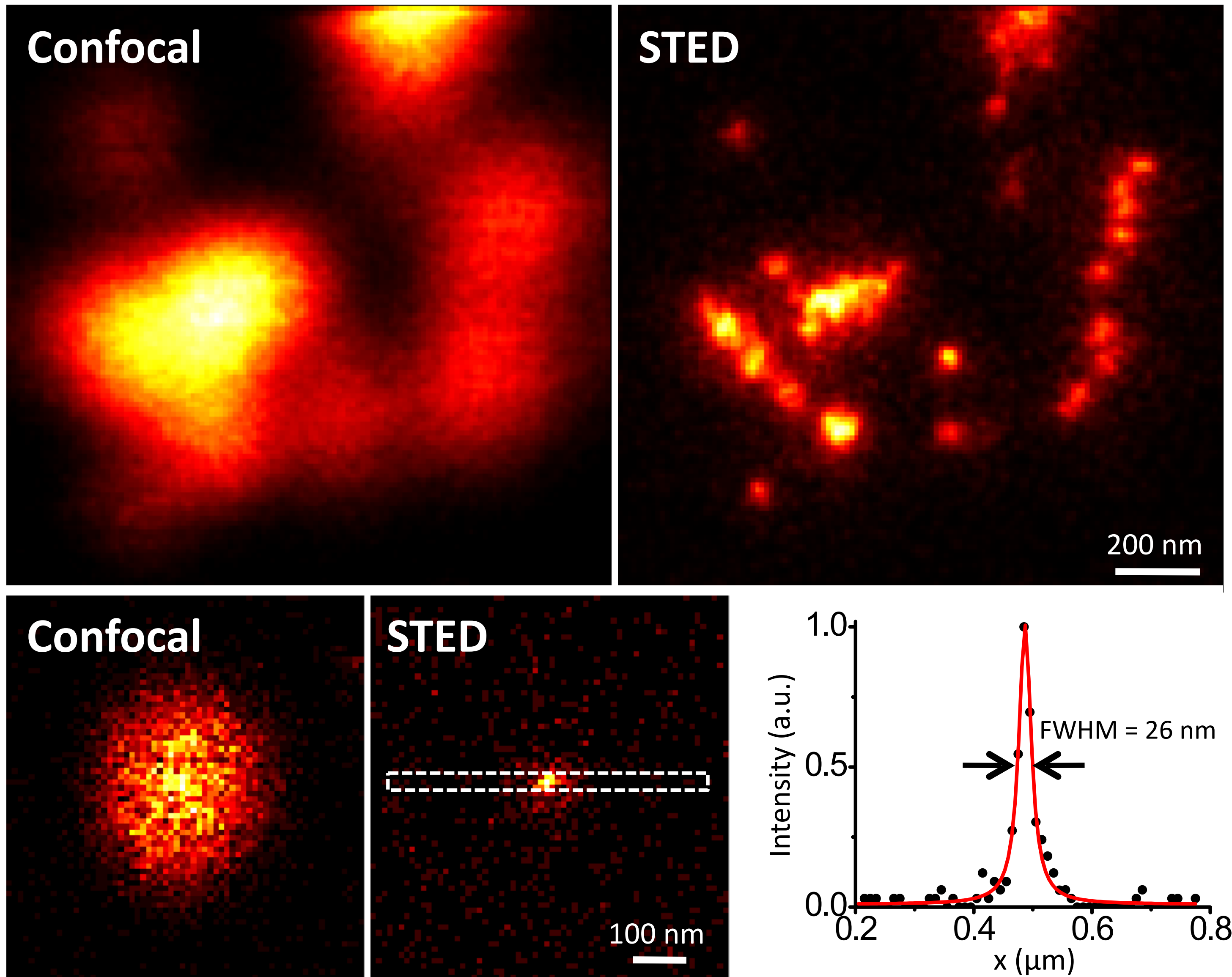
Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy" in „Fluorescence Microscopy", ed. U. Kubitscheck, 2017 Wiley VCH

Phase masks used in the back focal plane in STED microscopy



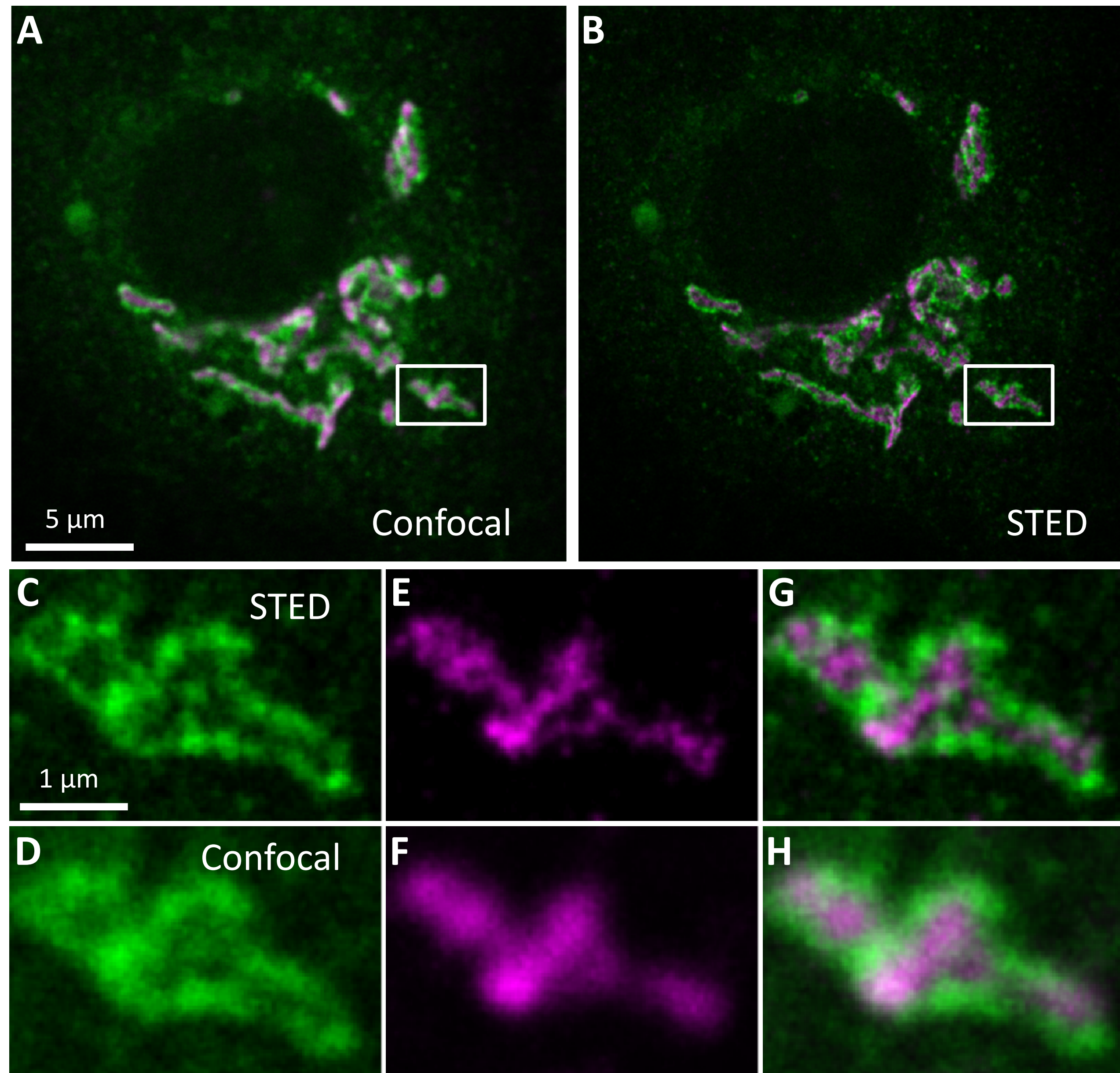
Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Imaging 20 nm fluorescent beads



Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

STED imaging



Dual-color STED image of the endoplasmic reticulum (ER) and mitochondria. (a) Overlay of images of ER labeled with Atto594 (green) and mitochondria labeled with ATTO647N (magenta). (b) Corresponding STED image. (c–h) Zoom-in of boxed areas in (a) and (b). Images were obtained using a custom-built STED instrument and smoothed by Gaussian filters.

Gould, Schroeder, Pellett and Bewersdorf, Chapter 10 „STED Microscopy" in „Fluorescence Microscopy", ed. U. Kubitscheck, 2017 Wiley VCH

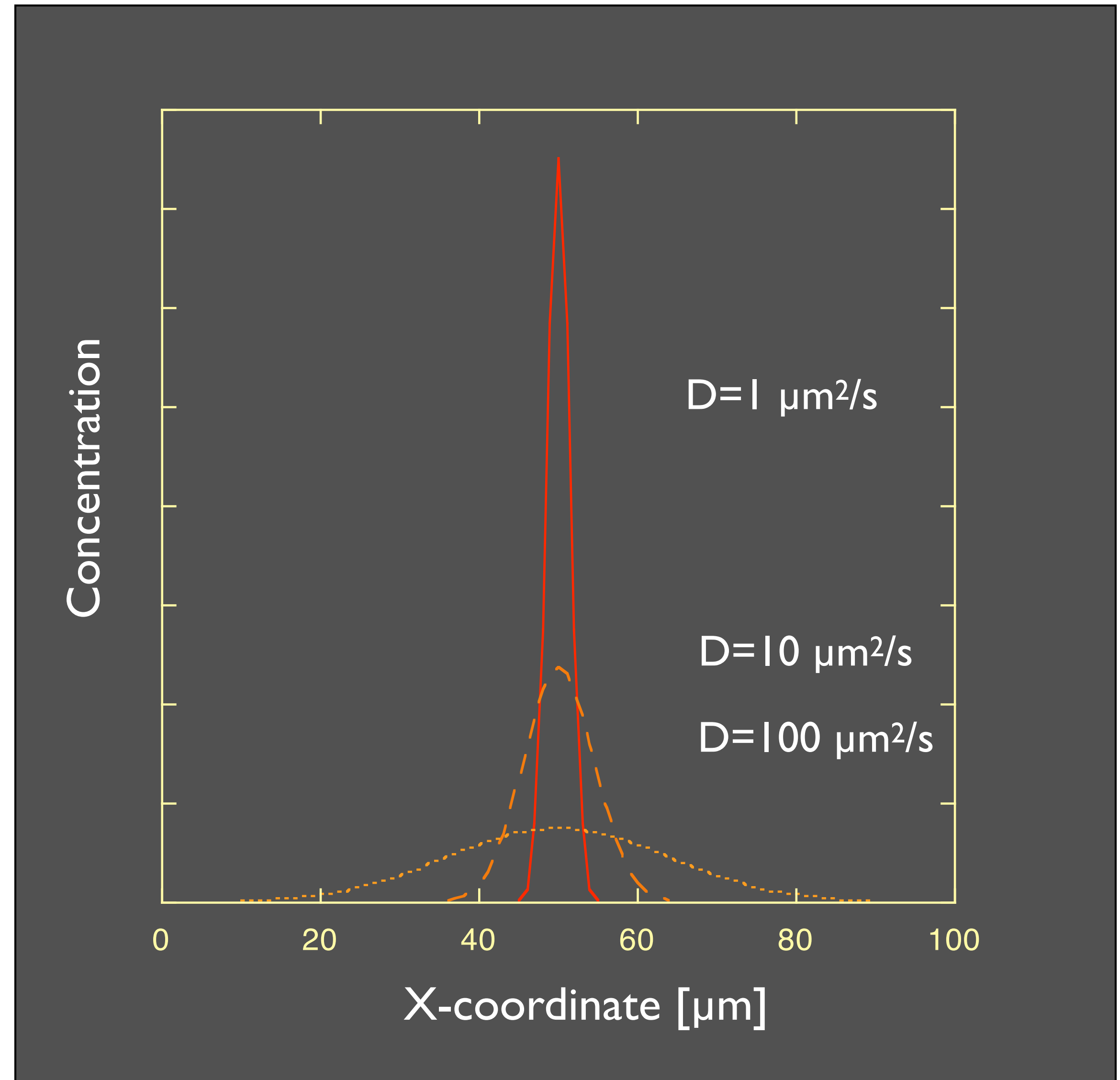
16. Molecular Mobility
Fluorescence recovery after photobleaching
FRAP

Particle transport by diffusion

$$c(x,t) = \frac{1}{2\sqrt{\pi D t}} e^{-\frac{x^2}{4Dt}}$$

Start position: x=50

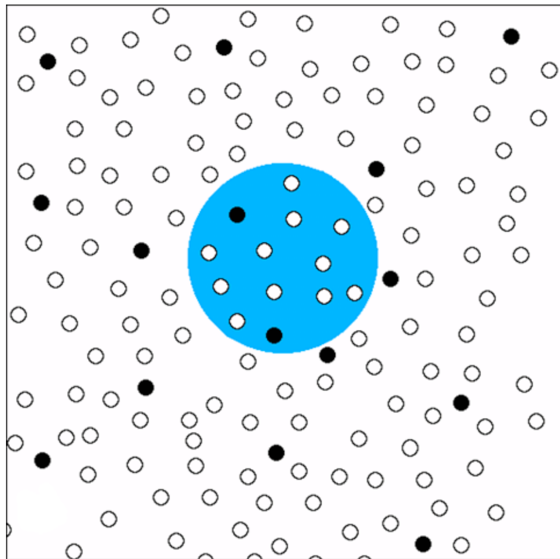
Distribution after 1 second:



Transport measurements by photobleaching:

„Fluorescence Recovery After Photobleaching“

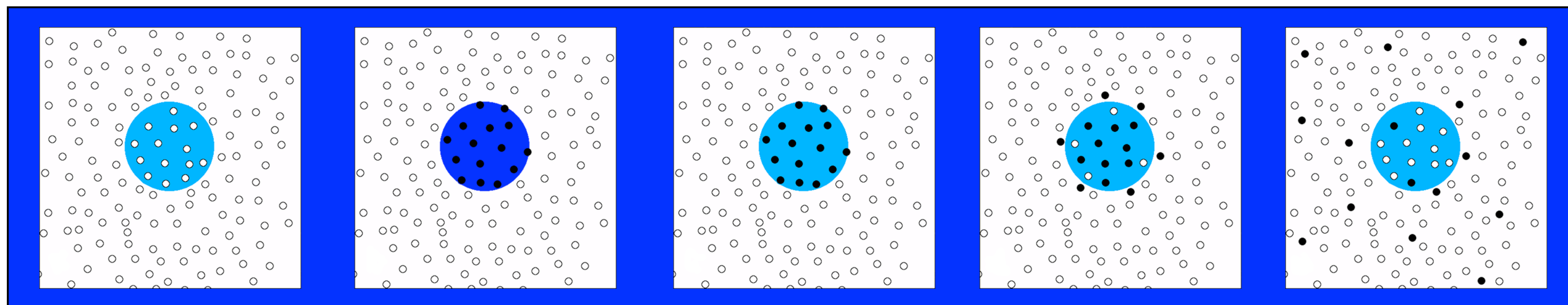
FRAP or FPR



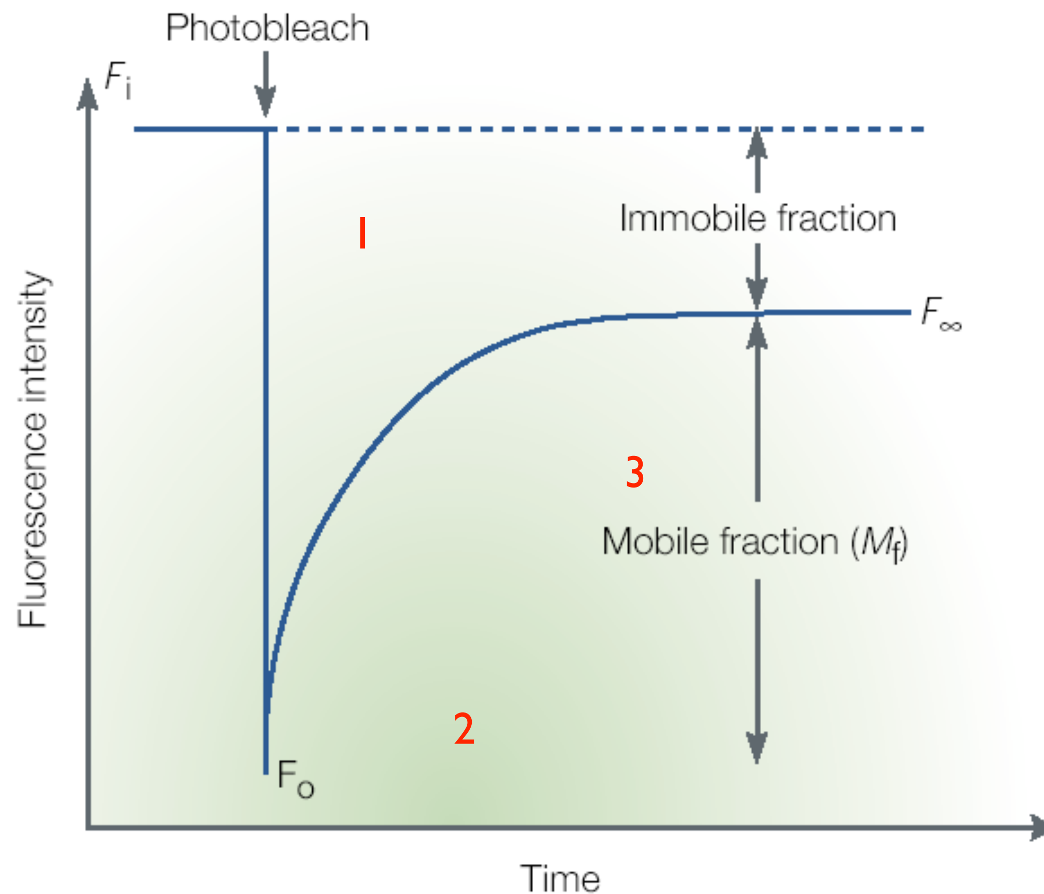
(1) Low intensity: prebleach measurement

(2) High intensity: bleaching (microphotolysis)

(3) Low intensity: monitoring measurement



Time dependence of fluorescence in a FRAP experiment



(1) Low intensity: prebleach measurement

(2) High intensity: bleaching (microphotolysis)

(3) Low intensity: monitoring measurement

Preconditions:
Instantaneous bleaching
No bleaching during monitoring

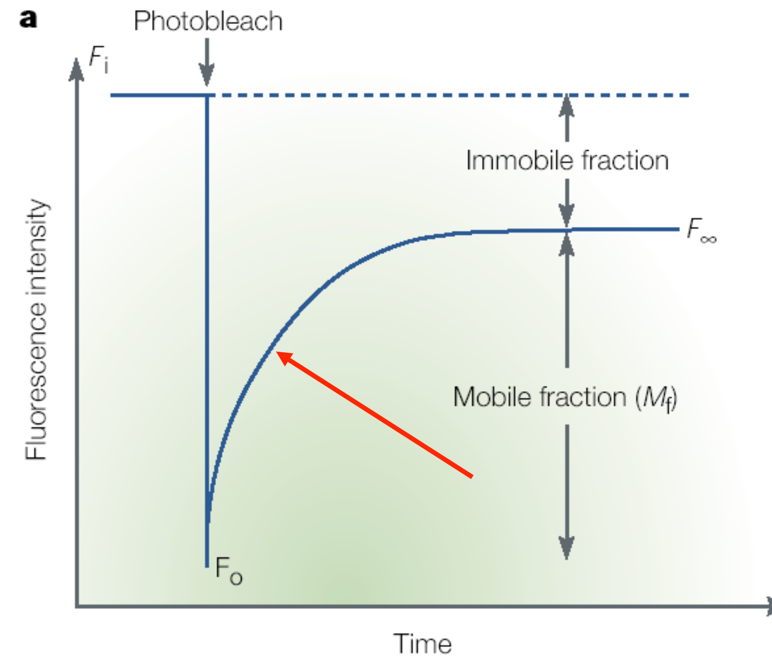
Lippincott-Schwartz et al., 2001

Diffusion time, τ_D

Diffusion time:

$$\tau_D = \frac{w^2}{4D}$$

w, radius of the focussed laser beam
D, diffusion constant

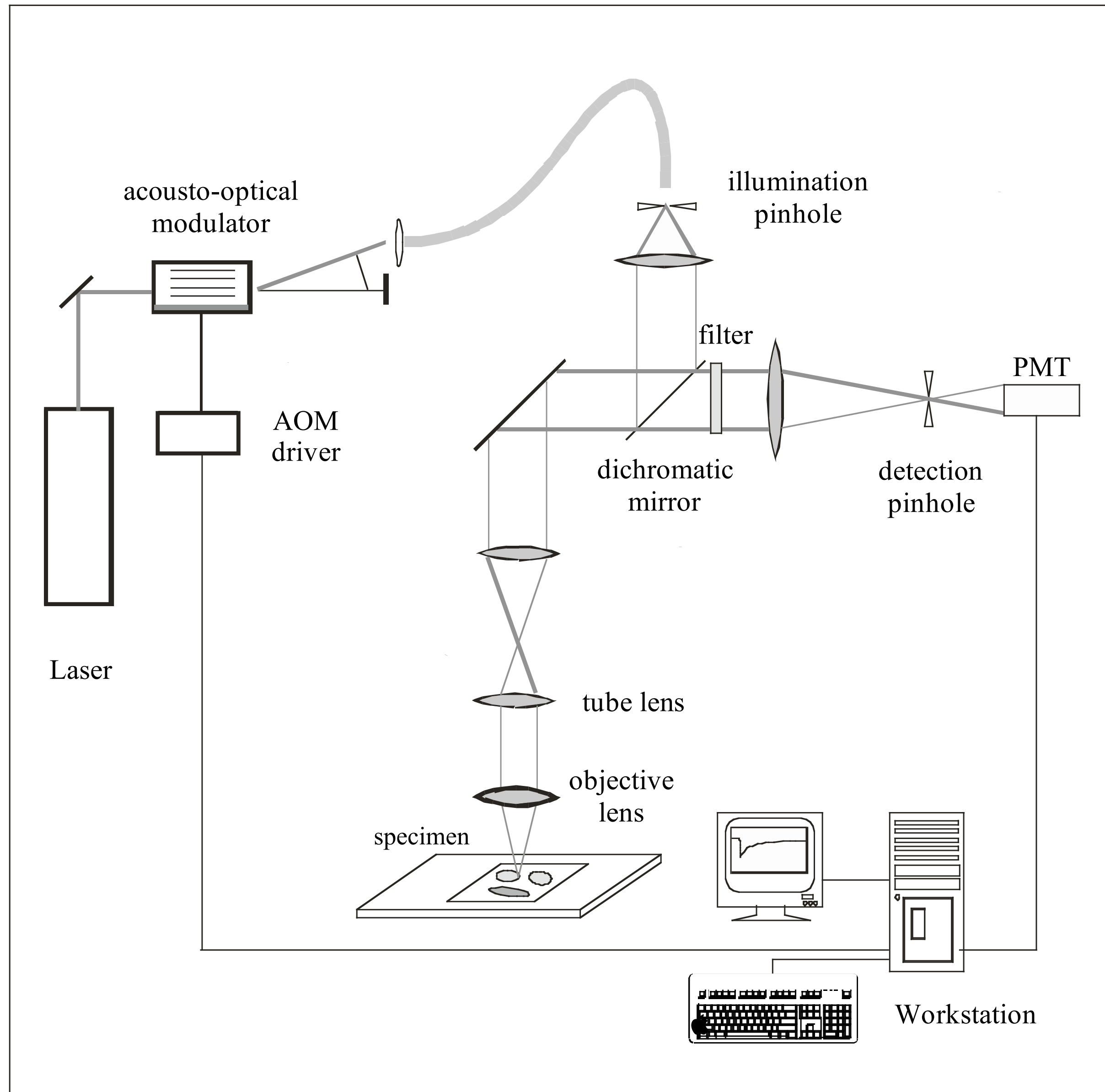


τ_D is obtained plotting the recovery of the relative fluorescence intensity within a bleached area as a function of time, and by fitting this curve by a specific mathematical model

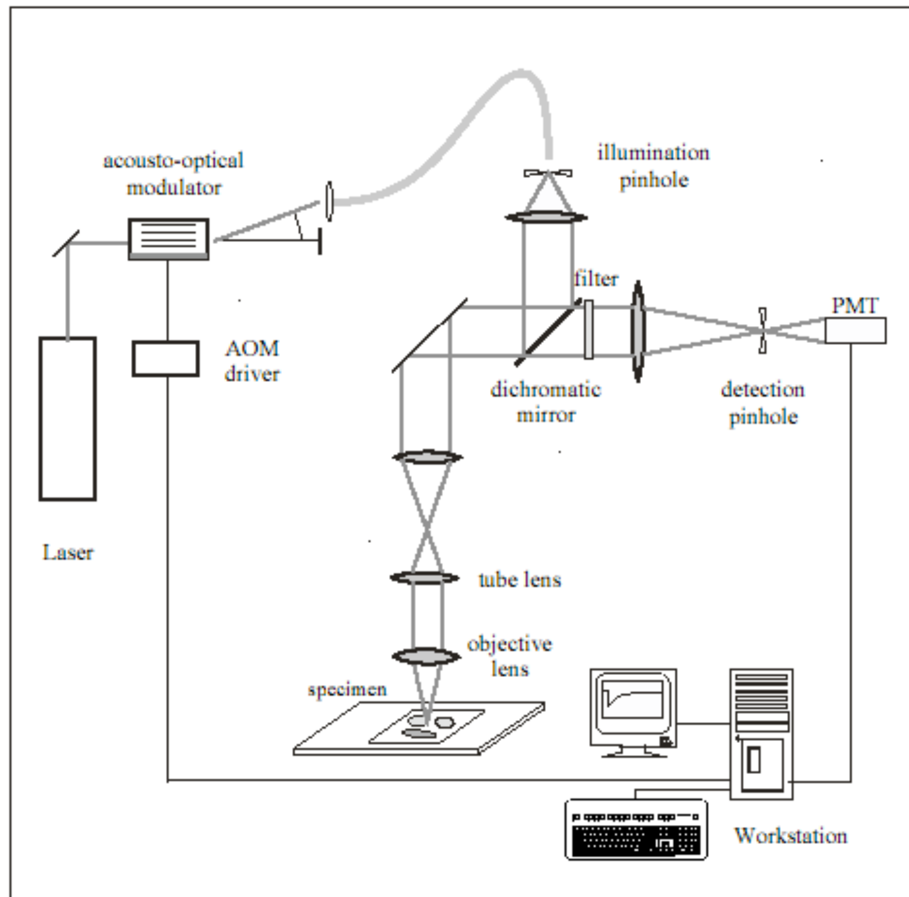
(w is measured)

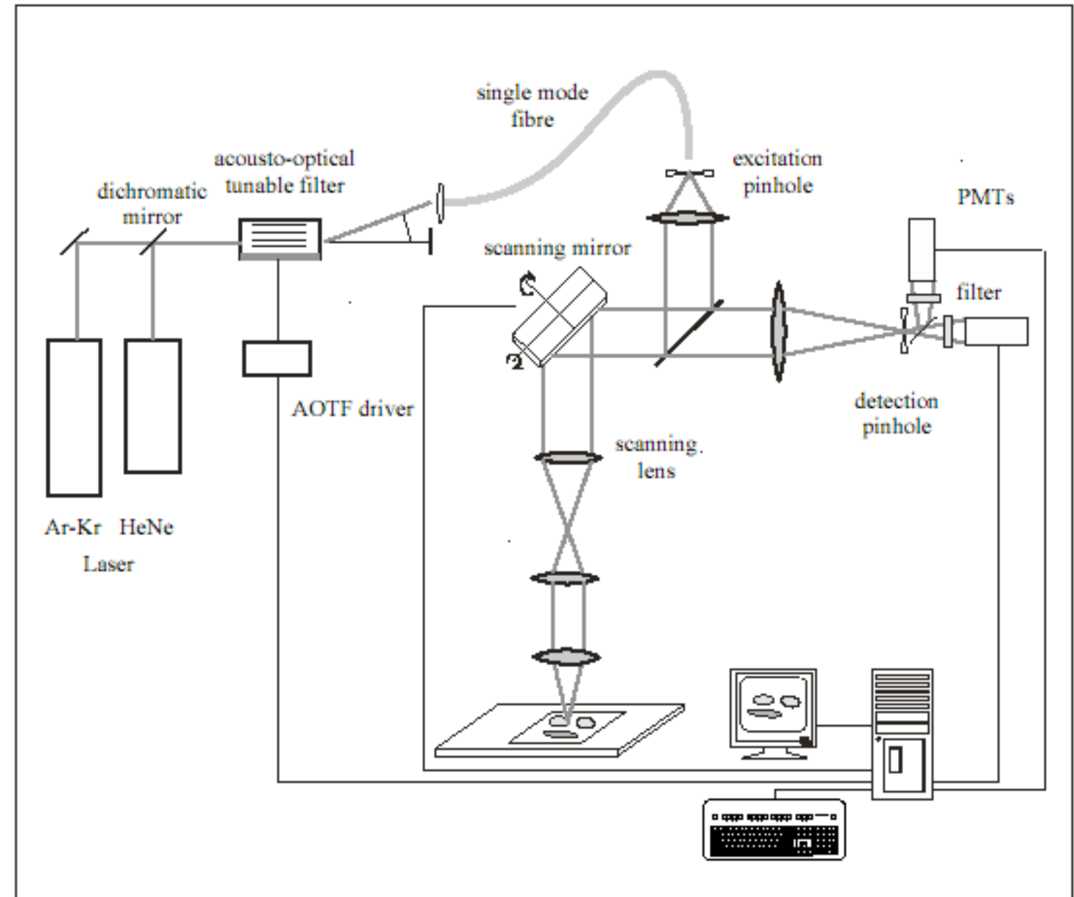
→ determination of D

Photobleaching Instrument

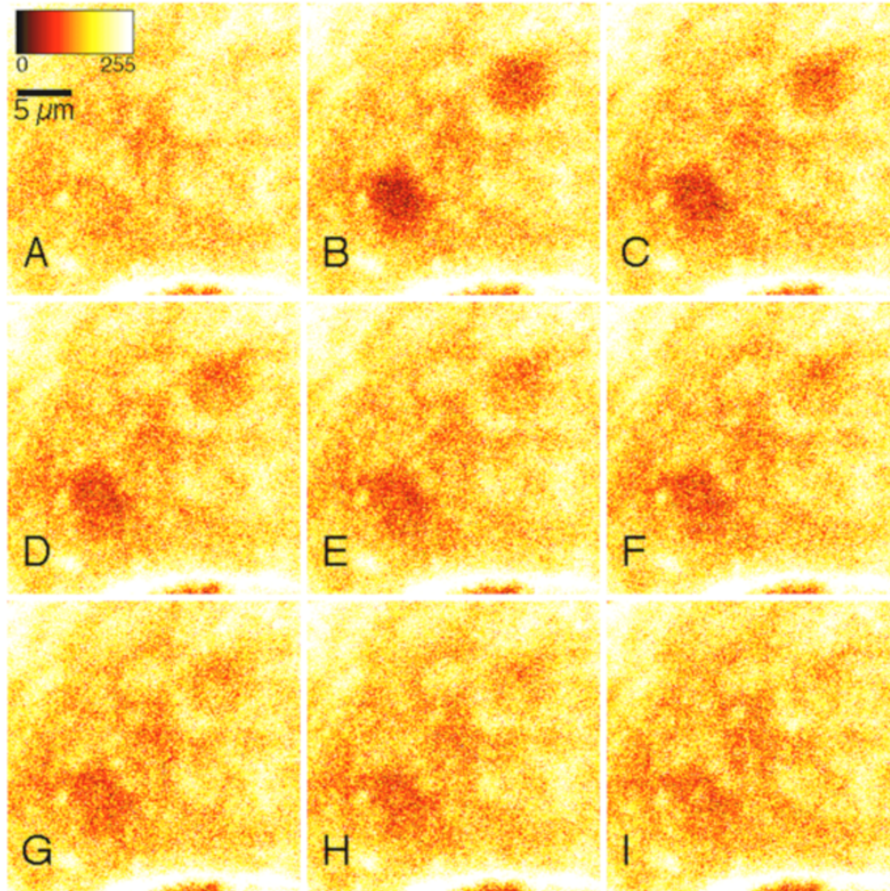


Photobleaching instrument & CLSM





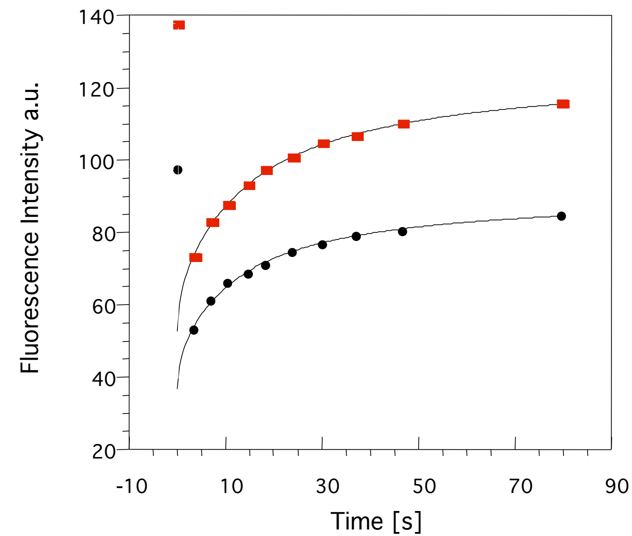
Lateral membrane transport by CLSM-FRAP



Sample:

Membranes of 3T3 cells labeled
by fluorescent lipid analoga

$D = 0.2\ \mu m^2/s$
Fraction of immobile molecules ≈ 5%



Kubitscheck et al., Biophys. J. 67 (1994)

16. Optical tweezers

Photon momentum

$p = m \cdot v$

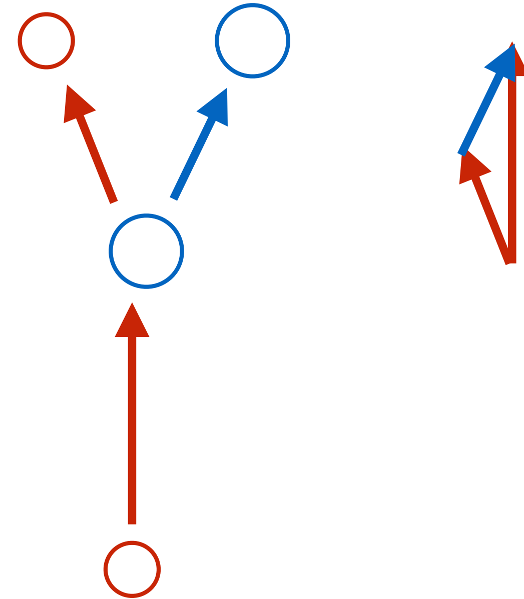
$E = m \cdot c^2 \Rightarrow m = E/c^2$

$E = h\nu \quad \nu\lambda = c$

$p = E/c = h\nu/c = h/\lambda$

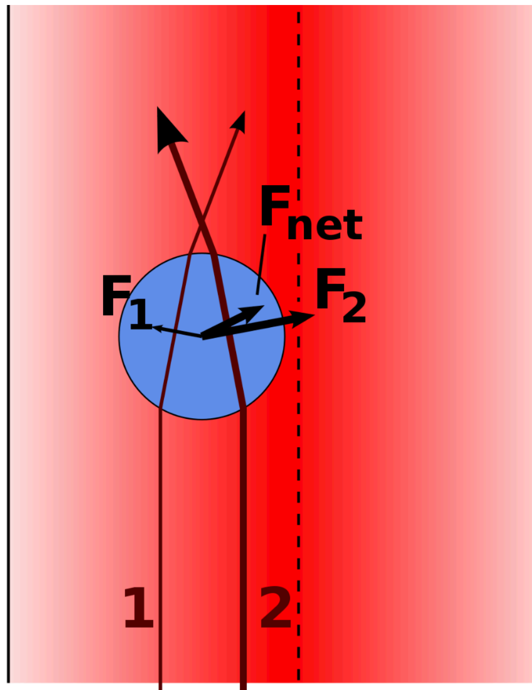
$dp/dt = F$

Change of light momentum causes a force
Principle of conservation of momentum applies

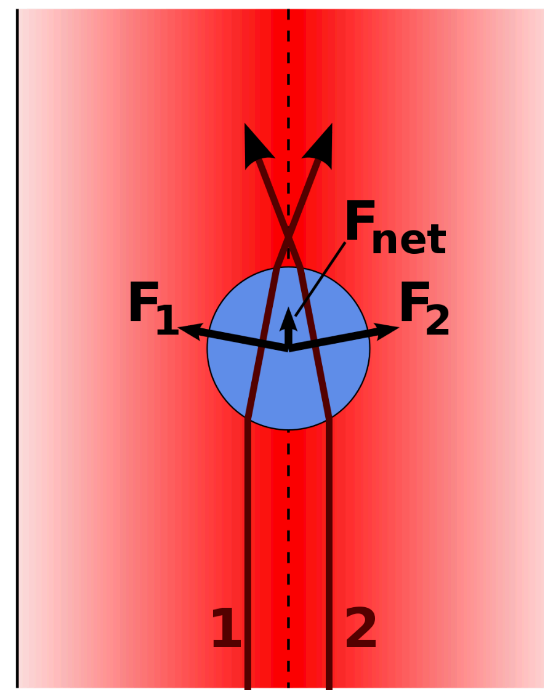


Force on a particle in an unfocused light beam

If diameter d of a trapped particle is significantly greater than λ,
the trapping phenomenon can be explained using ray optics

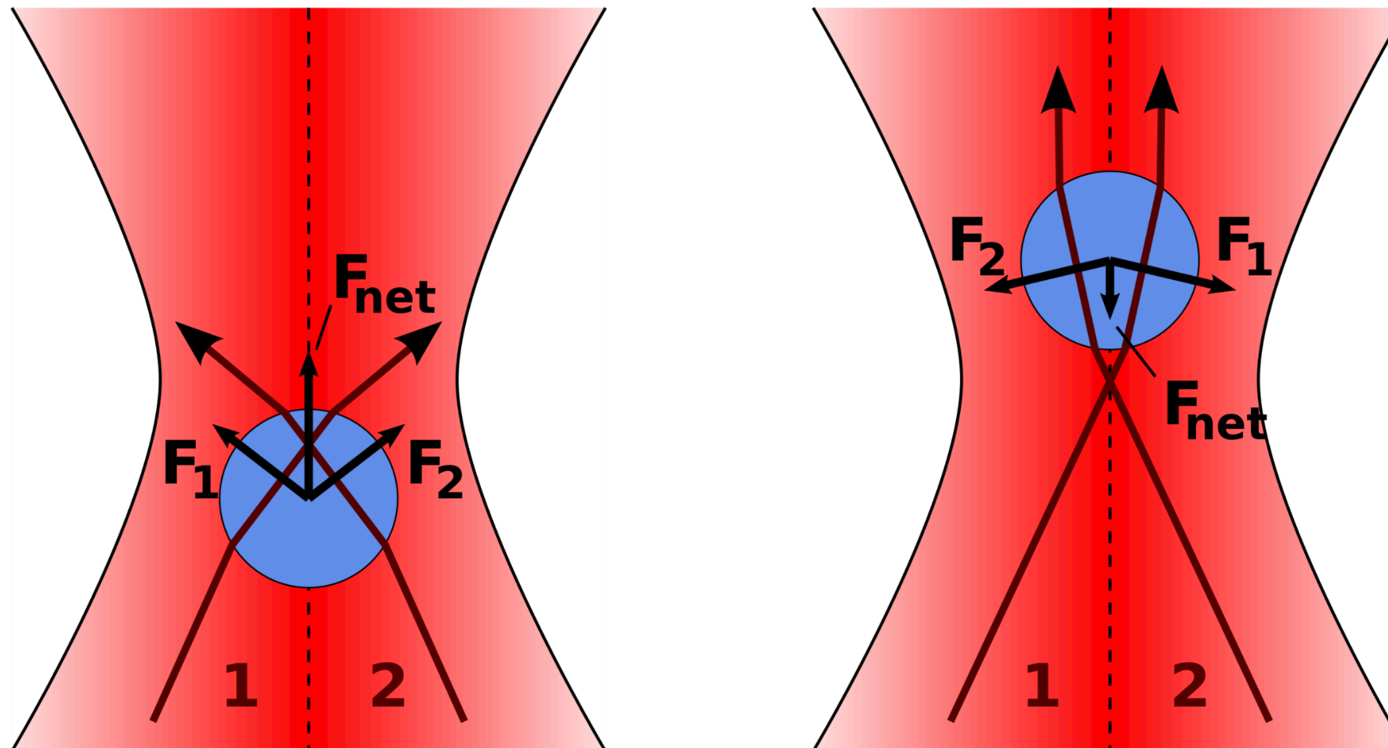


When the bead is displaced from the beam center, the larger momentum change of the more intense rays cause a net force to be applied back toward the center of the laser.



When the bead is laterally centered on the beam (left), the resulting lateral force is zero. But an unfocused laser still causes a force pointing away from the laser

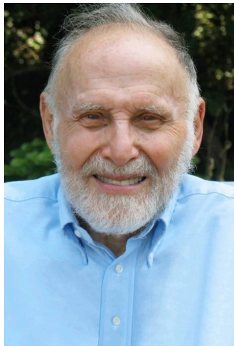
Force on a particle in a focused light beam



In addition to keeping the bead in the center of the laser, a **focused** laser also keeps the bead in a fixed axial position:
The momentum change of the focused rays causes a force towards the laser focus, both when the bead is in front (left) or behind (right) the laser focus.
So, the bead will stay slightly behind the focus, where this force compensates the scattering force.

Example: multiple optical foci

The Nobel Prize in Physics 2018



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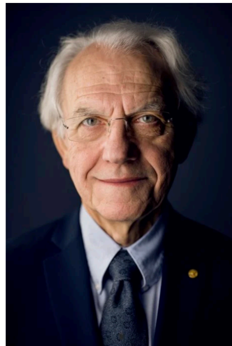
Arthur Ashkin
Prize share: 1/2



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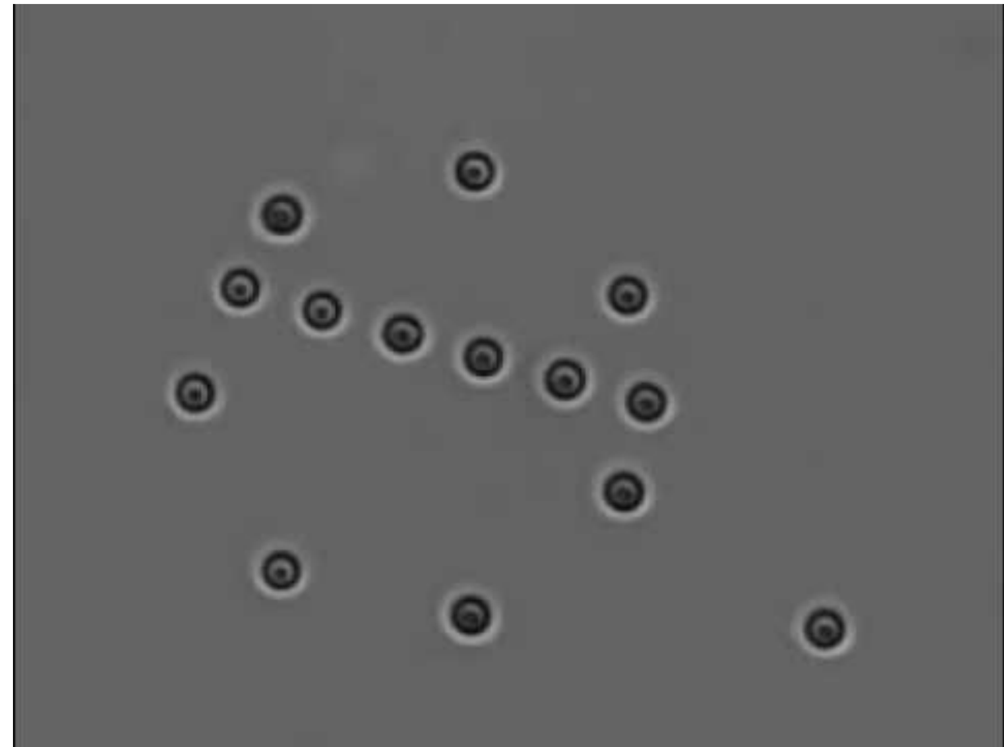

Nobel prize 2018 to Arthur Ashkin for "for the optical tweezers and their application to biological systems"

https://www.nobelprize.org/prizes/physics/2018/summary/

Example: multiple optical foci

Force on the particle due to the field gradient:

$$\mathbf{F} = \frac{1}{2}\alpha\nabla E^2 = \frac{2\pi n_0 a^3}{c}\left(\frac{m^2-1}{m^2+2}\right)\nabla I(\mathbf{r})$$

where
a is the particle radius,
n_0 is the index of refraction of the particle and
$m=n_n/n_1$
is the relative refractive index between the particle and the medium

+ scattering force pushing the particle forward

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Thank you very much for your attention!