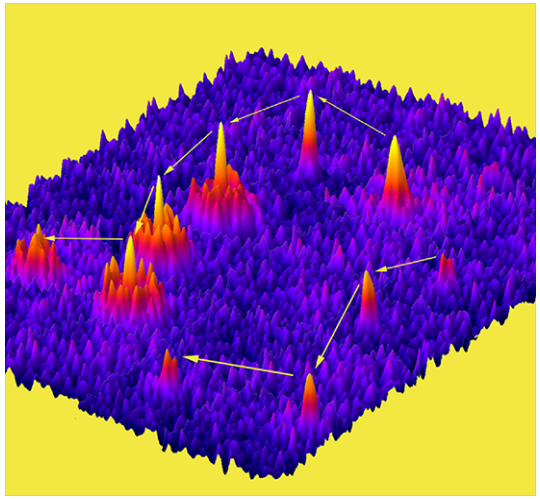
Course „Optics, Forces & Development“

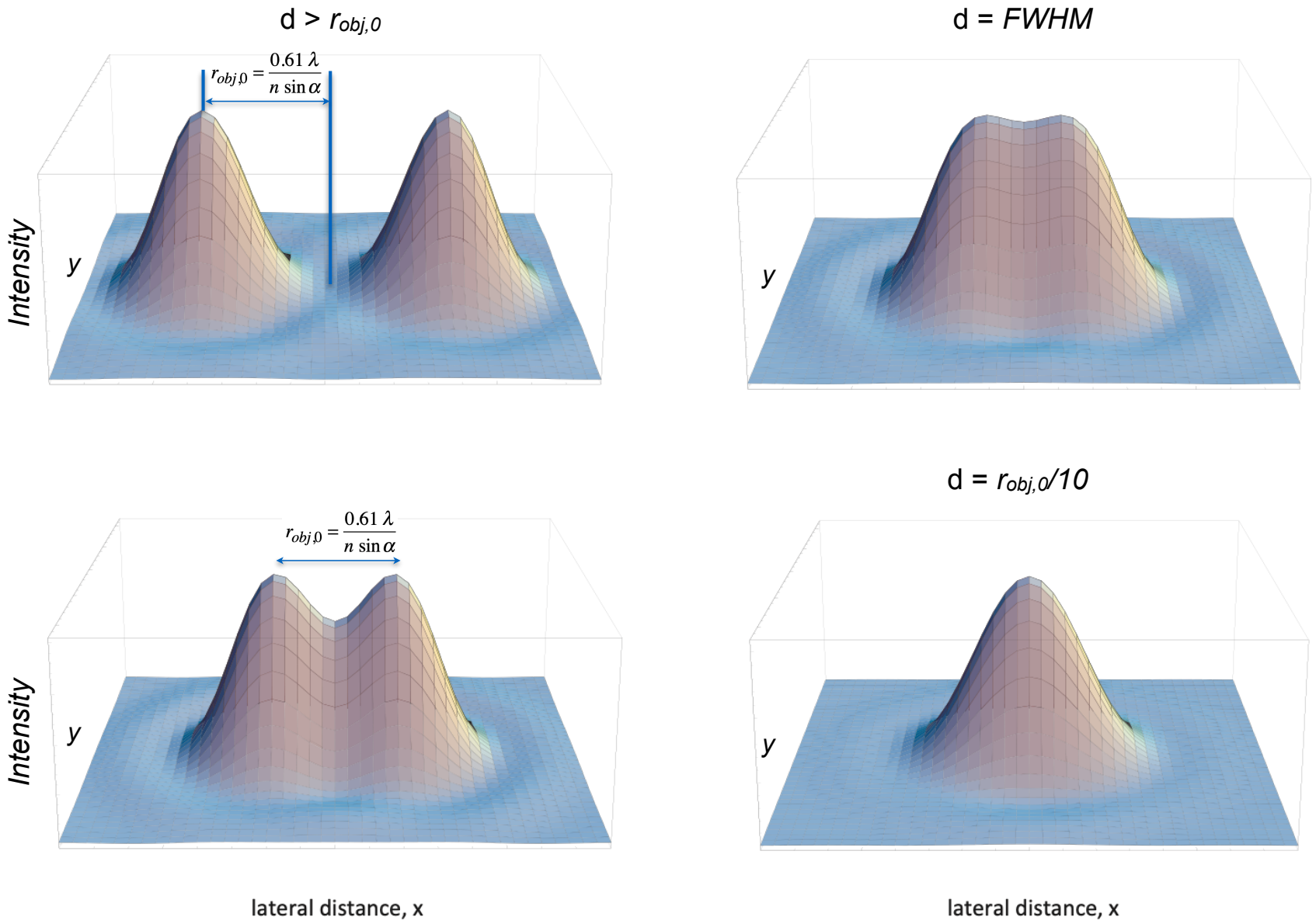


Principles of Optics II

Ulrich Kubitscheck

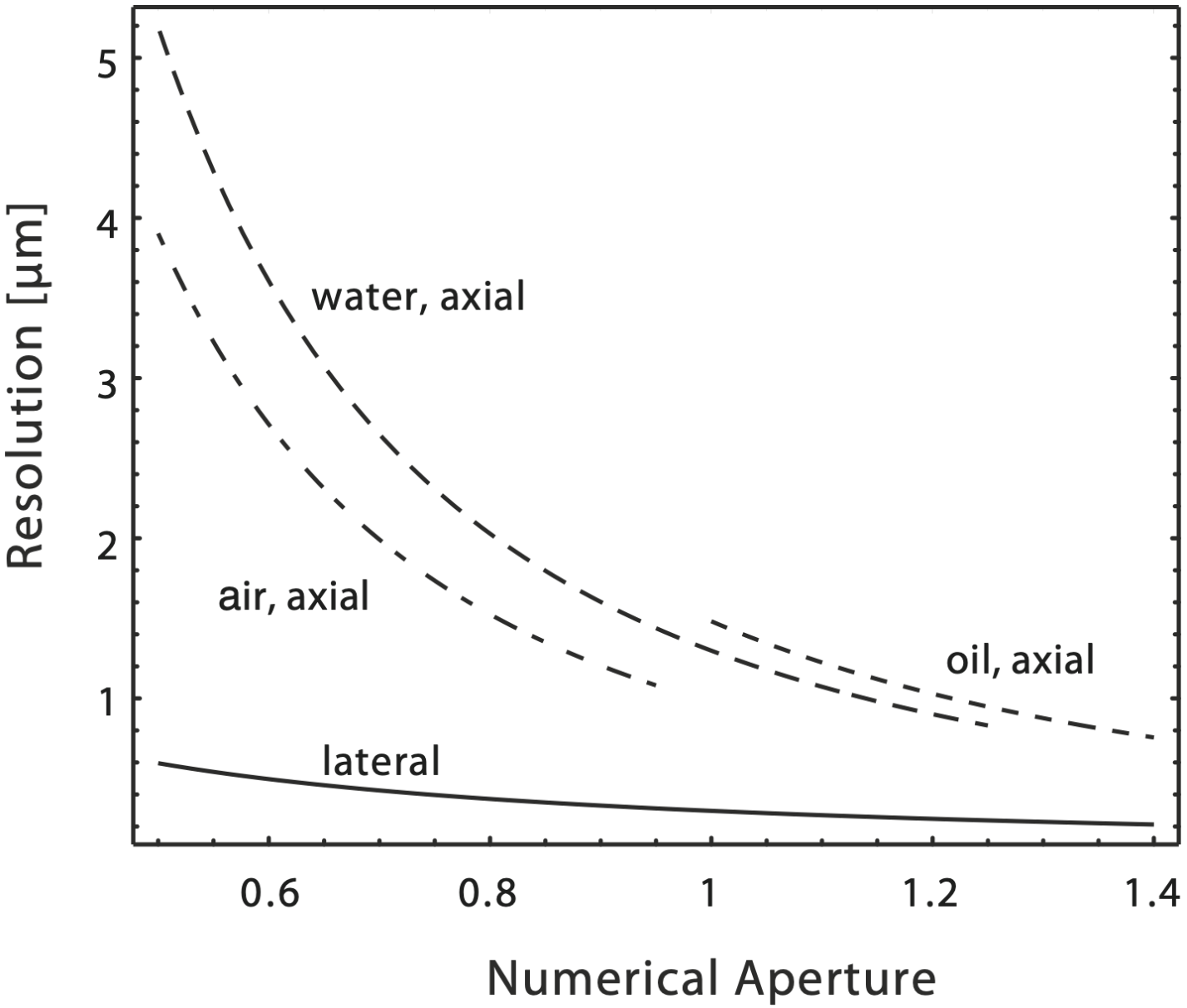
Clausius-Institute of Physical and Theoretical Chemistry
Rheinische Friedrich-Wilhelms-Universität Bonn

Sum of Point Spread Functions for Incoherent Point Objects



From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition

Radial and axial resolution as function of the NA



From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition, Fig. 2.13

The resolution limit in the biological context

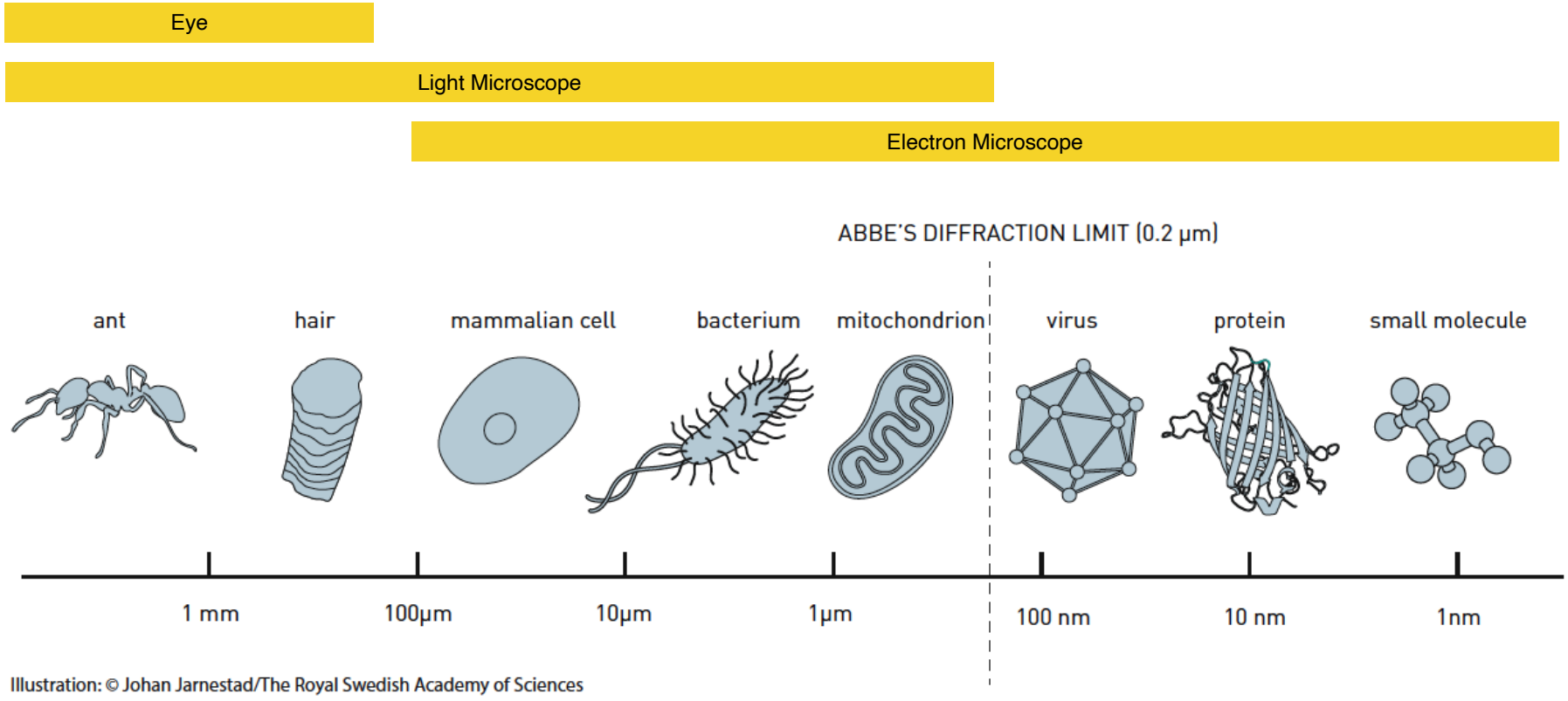
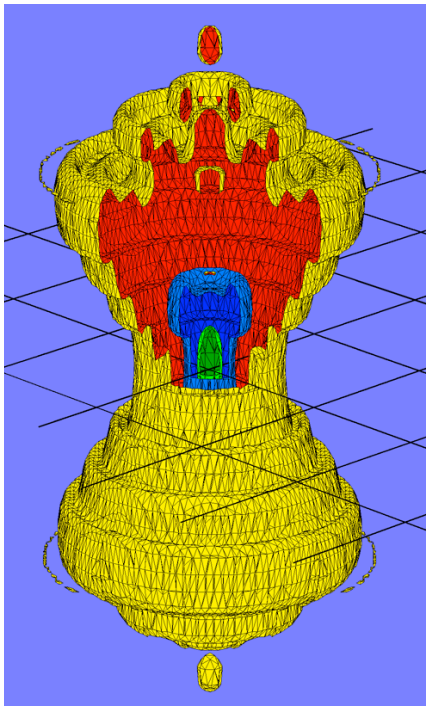
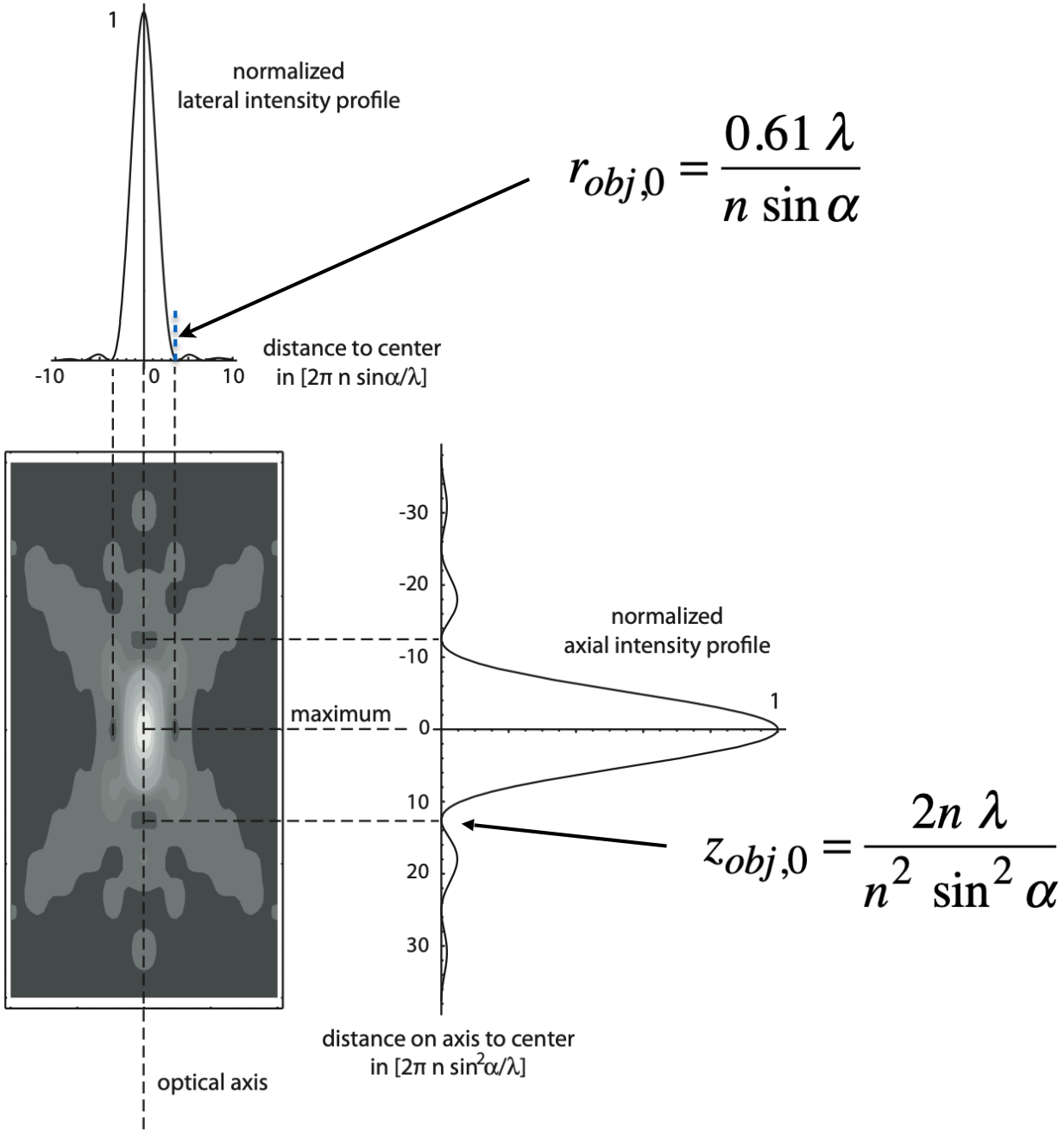


Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences

3D Point spread function (PSF)



isosurfaces at
50, 5, 0.5% of max

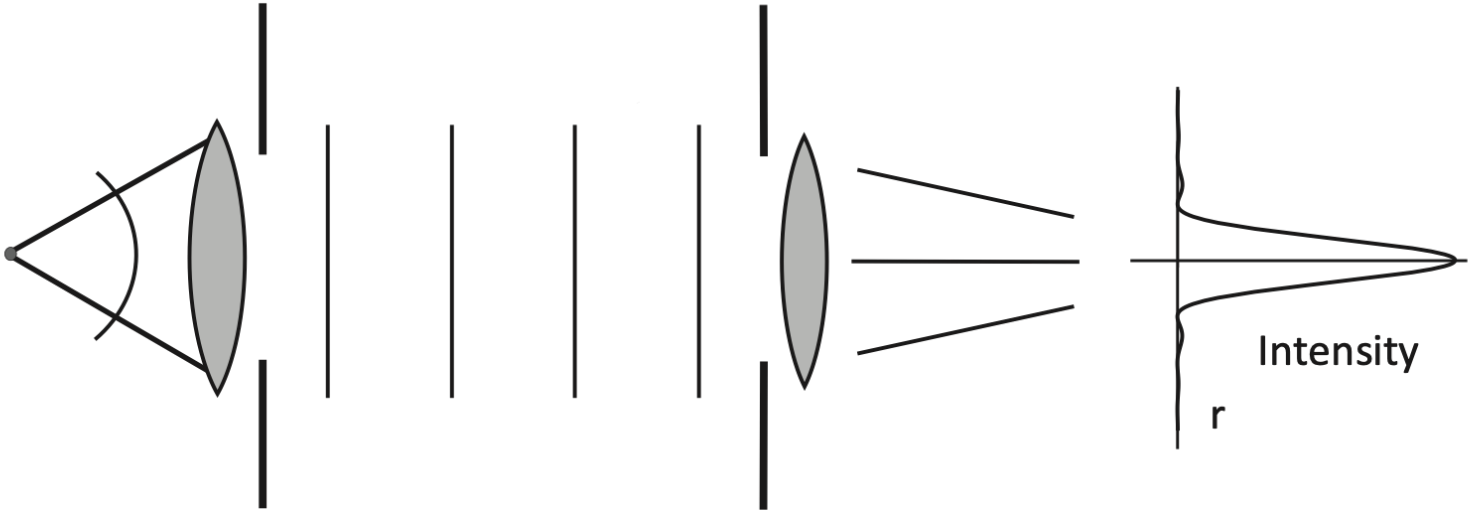


$$r_{obj,0} = \frac{0.61\,\lambda}{n \sin\alpha}$$

$$z_{obj,0} = \frac{2n\,\lambda}{n^2 \sin^2\alpha}$$

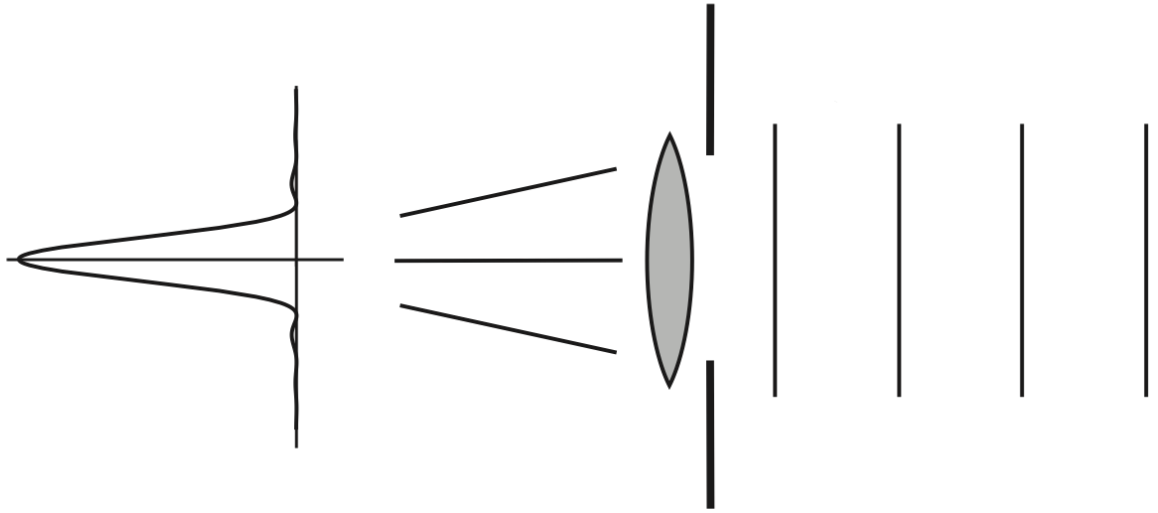
Radial and axial intensity profile of the light distribution in the focus of a lens

Imaging of a point



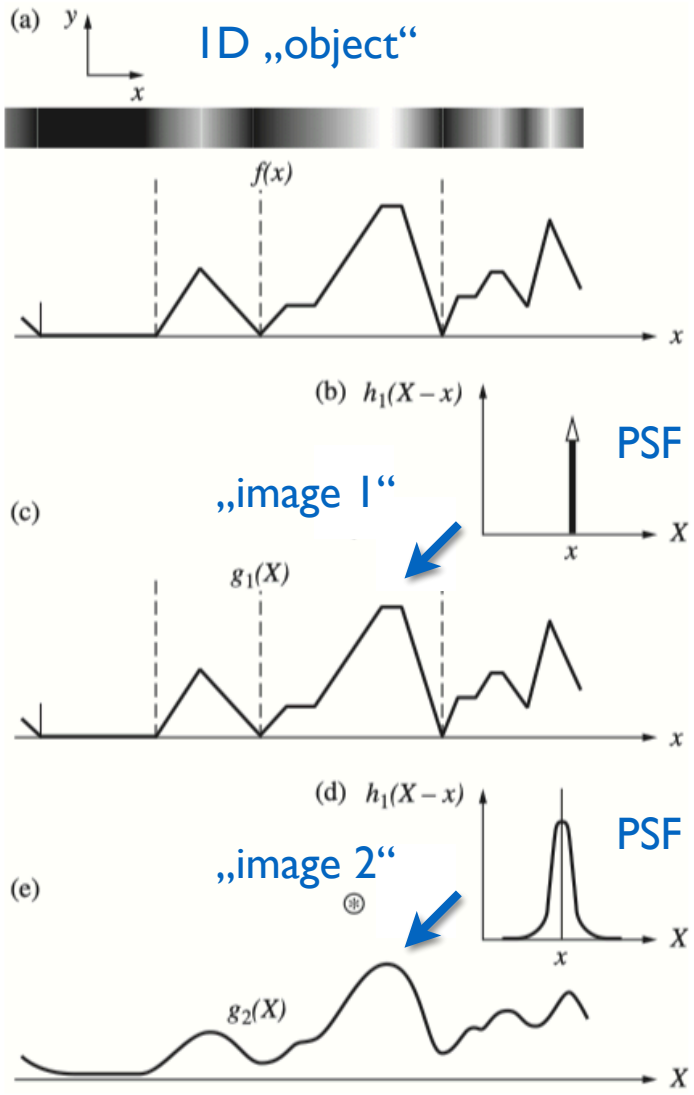
object space

Illumination of a „point“

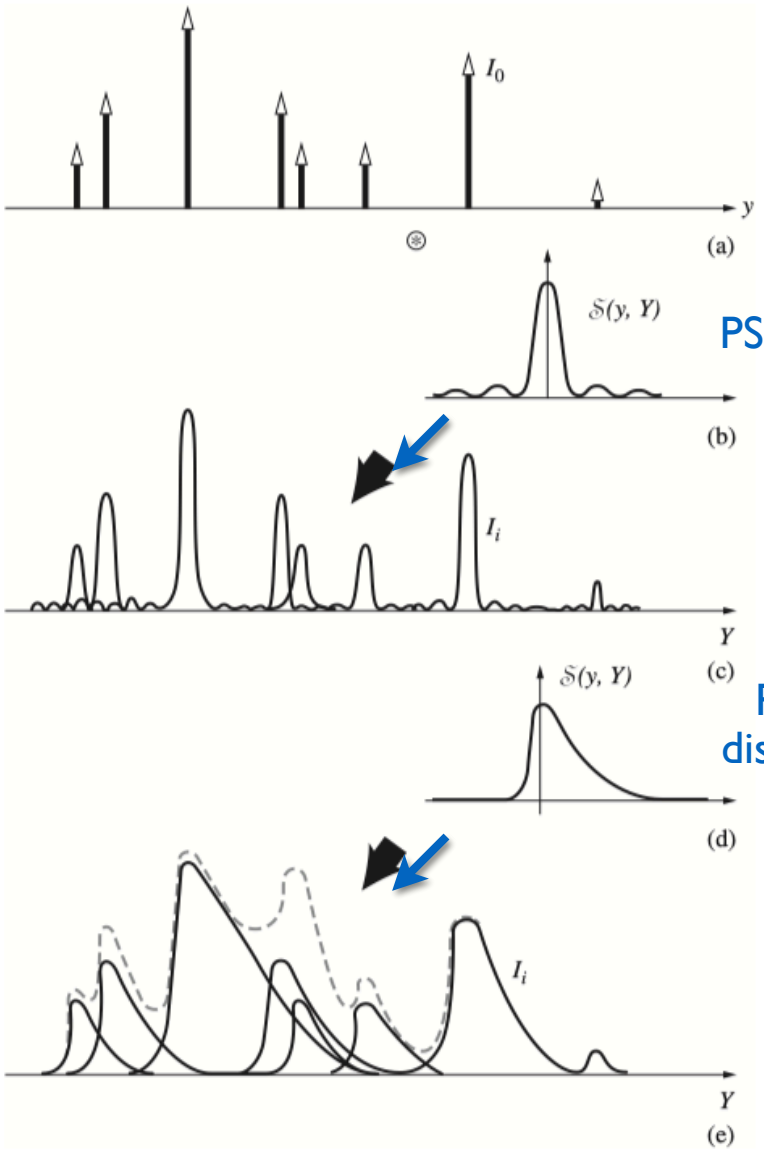


6. More PSF: convolution and deconvolution

More on the PSF: convolution in microscopy



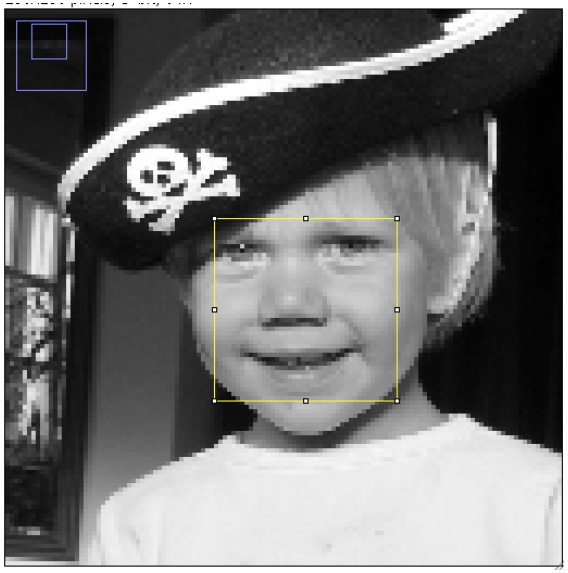
The irradiance distribution is converted to a function $f(x)$ shown in (*a*). This is convolved with δ-function (*b*) to yield a duplicate of $f(x)$. By contrast, convolving $f(x)$ with the spread function h_2 in (*d*) yields a smoothed-out curve represented by $g_2(x)$ in (*e*).

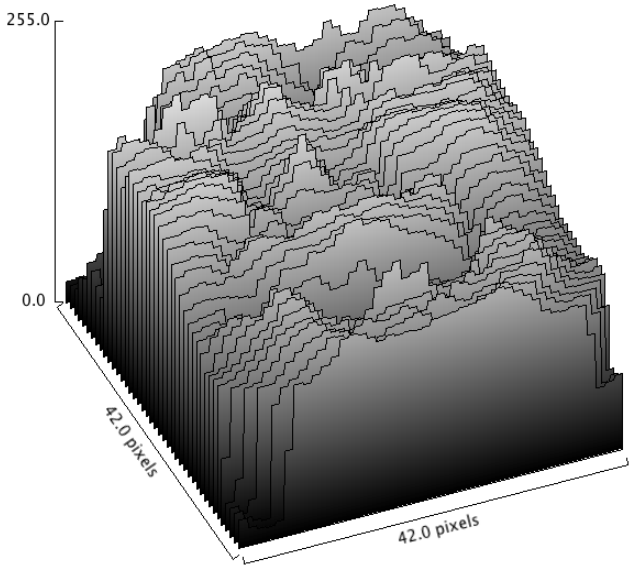


Here (*a*) is convolved first with (*b*) to produce (*c*) and then with (*d*) to produce (*e*). The resulting pattern is the sum of all the spread-out contributions, as indicated by the dashed curve in (*e*).

Adapted from Eugene Hecht, Optics 2017

Images are arrays of integer values h(i,j)

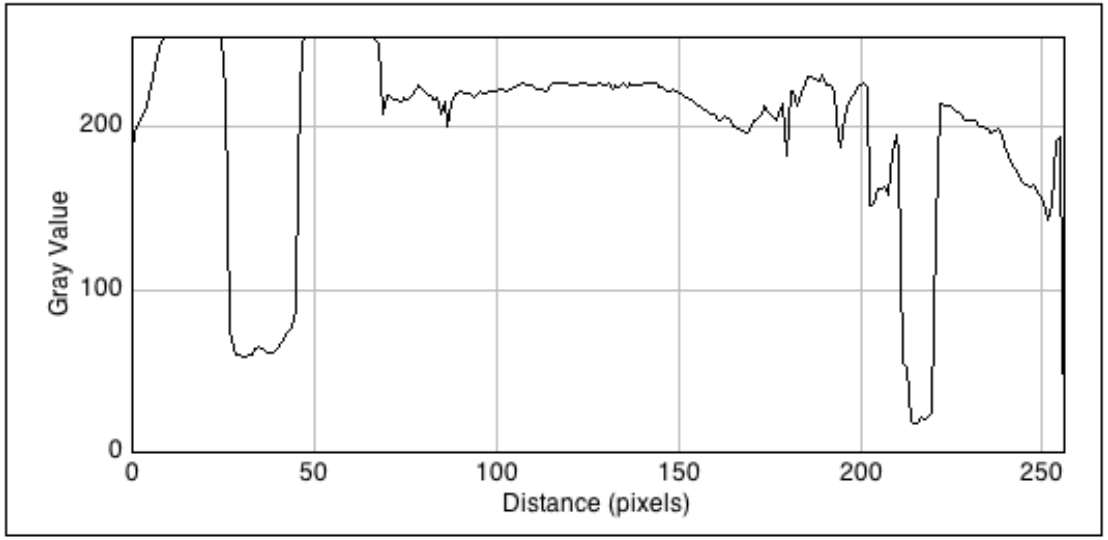




Surface profile

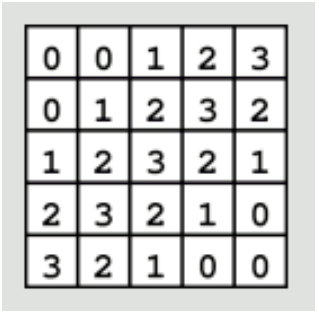
i, j-values correspond to position

Line profile



Gauss filter for image smoothing… and convolution

$$h'(i,j) = \{h(i-1,j-1) + 2\,h(i-1,j) + h(i-1,j+1)$$
$$+ 2\,h(i,j-1) + 4\,h(i,j) + 2\,h(i,j+1)$$
$$+ h(i+1,j-1) + 2\,h(i+1,j) + h(i+1,j+1)\} / 16$$



0	0	1	2	3
0	1	2	3	2
1	2	3	2	1
2	3	2	1	0
3	2	1	0	0

or

$$h'(i,j) = \sum_{u=-1}^{1}\sum_{v=-1}^{1} h(i+u,j+v)\cdot\, g(u+1,v+1)$$

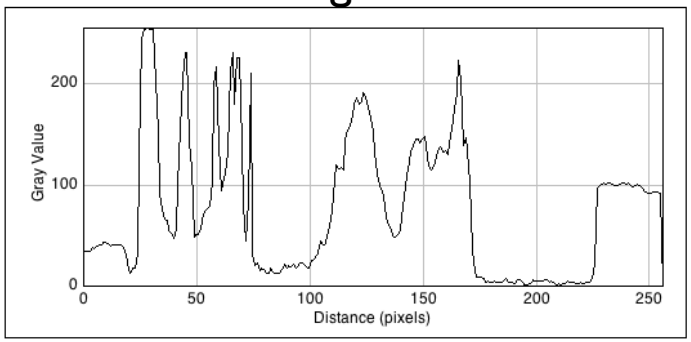
$$g(u,v) = \frac{1}{16}\begin{pmatrix} 1 & 2 & 1 \\ 2 & 4 & 2 \\ 1 & 2 & 1 \end{pmatrix}$$

or

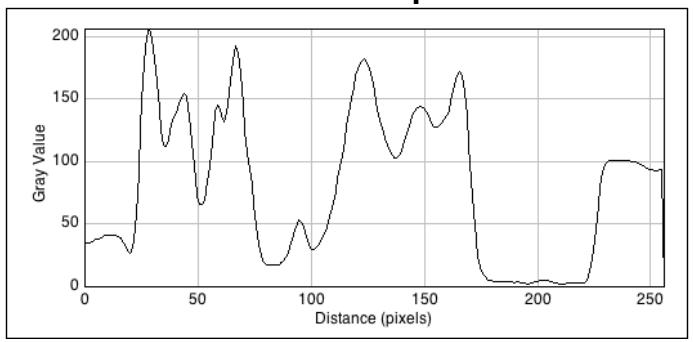
$h' = h \otimes g$ h' is the result of the „convolution“ of h with g

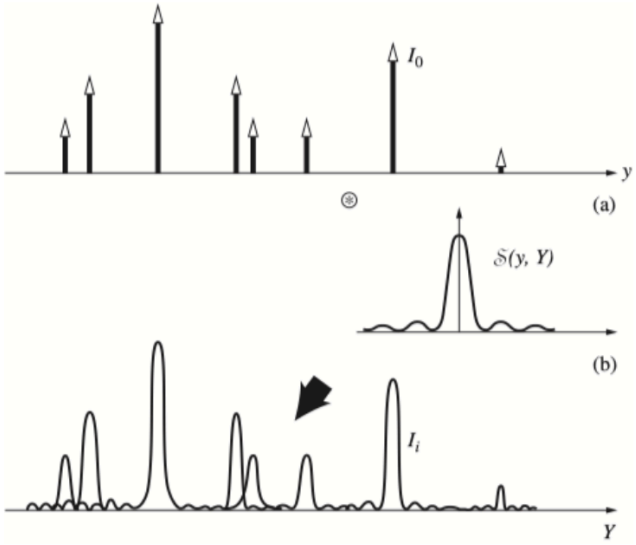
Image smoothing = low pass filtering

original



smoothed or low pass filtered





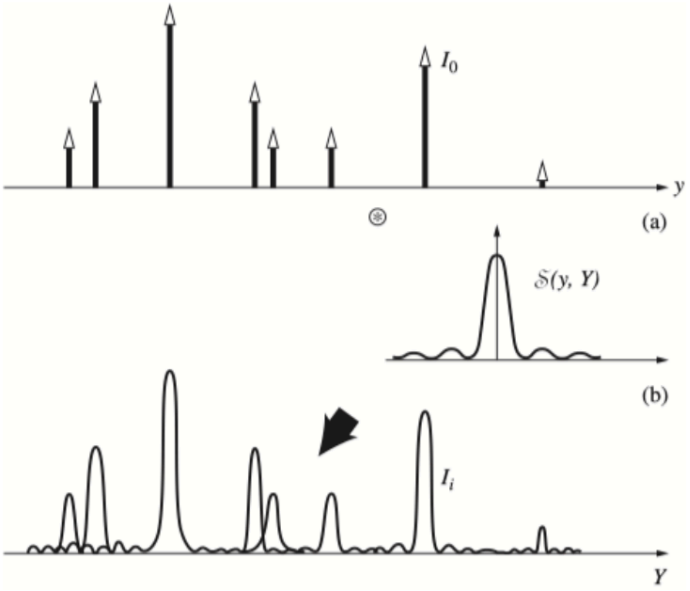
„original“

„lens“

„image“

„lenses“ are nothing else than „optical low pass filters“

Restoration of original object function by deconvolution



object function $O(x,y,z) \longleftrightarrow \mathbb{O}(\omega_x,\omega_y,\omega_z)$

lens function $PSF(x,y,z) \longleftrightarrow \mathbb{OTF}(\omega_x,\omega_y,\omega_z)$

image function $I(x,y,z) \longleftrightarrow \mathbb{I}(\omega_x,\omega_y,\omega_z)$

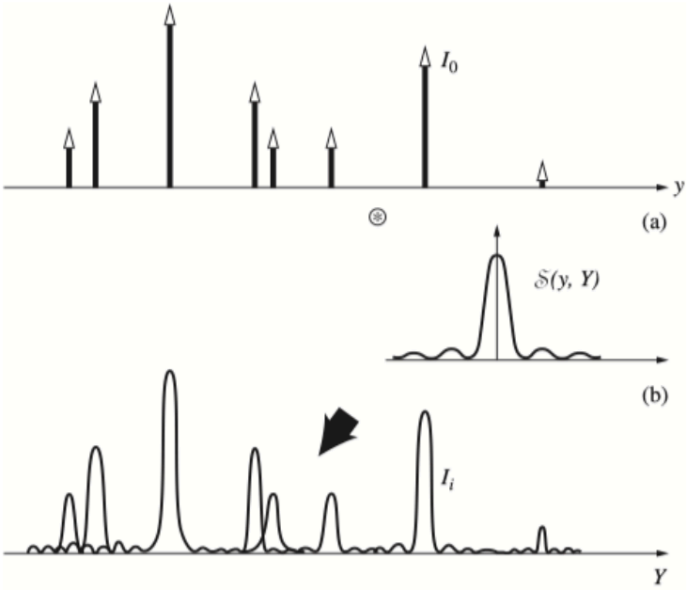
$$I(x,y,z) = O(x,y,z) \otimes PSF(x,y,z) \longleftrightarrow \mathbb{I}(\omega_x,\omega_y,\omega_z) = \mathbb{O}(\omega_x,\omega_y,\omega_z) \cdot \mathbb{OTF}(\omega_x,\omega_y,\omega_z)$$

$$\Rightarrow \quad \mathbb{O}(\omega_x,\omega_y,\omega_z) = \mathbb{I}(\omega_x,\omega_y,\omega_z) \,/\, \mathbb{OTF}(\omega_x,\omega_y,\omega_z)$$

$$\Rightarrow \quad \mathbb{O}(\omega_x,\omega_y,\omega_z) \longleftrightarrow O(x,y,z)$$

Theoretically, we can invert the effect of the PSF by „deconvolution“

Restoration of original object function by deconvolution



object function $O(x,y,z) \longleftrightarrow \mathbb{O}(\omega_x,\omega_y,\omega_z)$

lens function $PSF(x,y,z) \longleftrightarrow \mathbb{OTF}(\omega_x,\omega_y,\omega_z)$

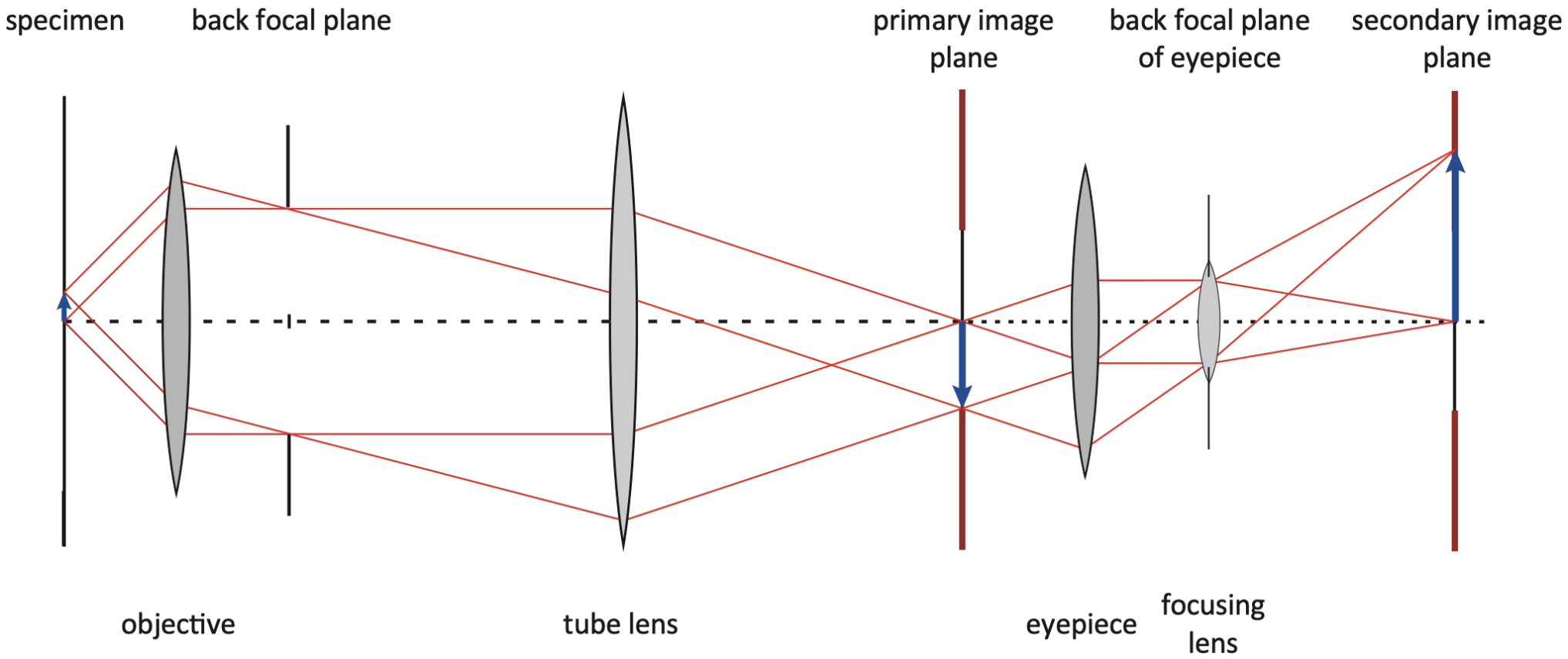
image function $I(x,y,z) \longleftrightarrow \mathbb{I}(\omega_x,\omega_y,\omega_z)$

$$I'(x,y,z) = O(x,y,z) \otimes PSF(x,y,z) + Noise(x,y,z)$$

Theoretically, we can invert the effect of the PSF by „deconvolution“.
Practically, this is far from easy!

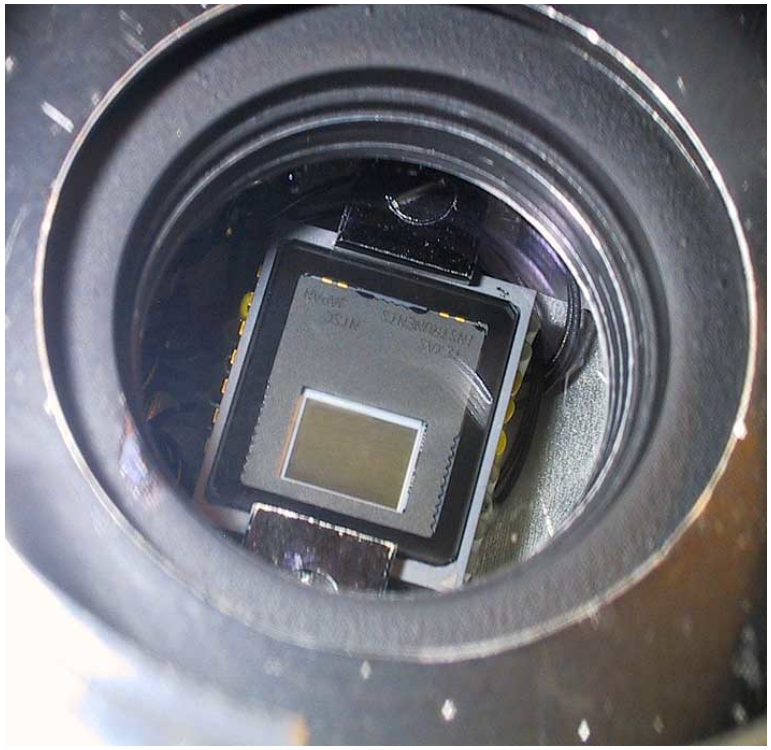
7. Image detection by cameras and pixel size

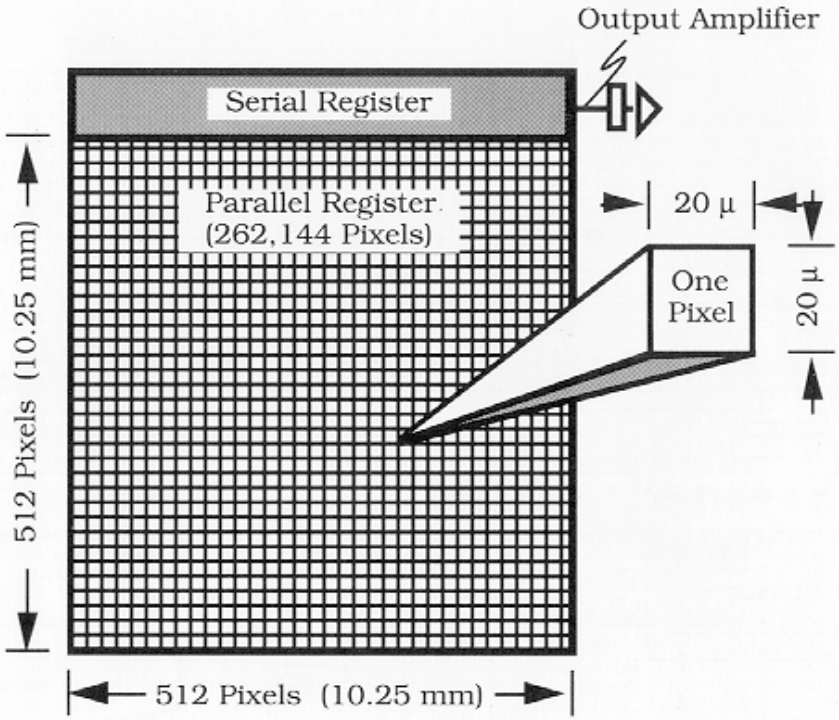
Construction of a microscope
by combination of two magnification stages

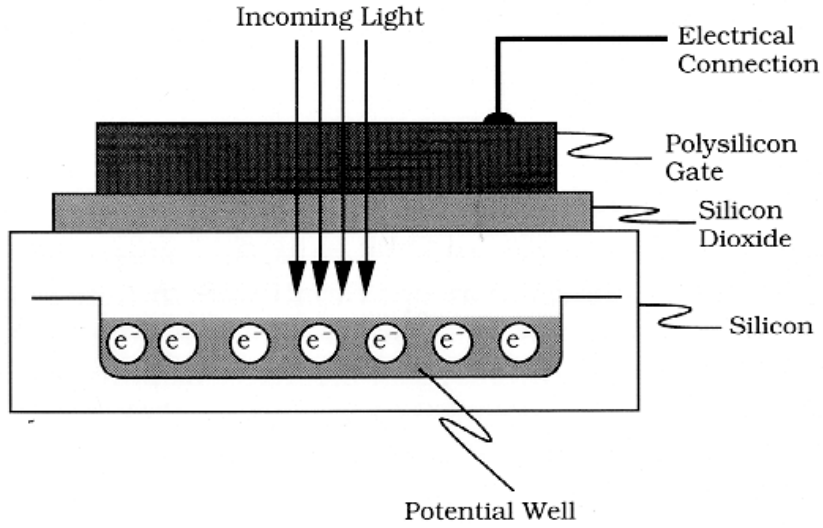


From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition

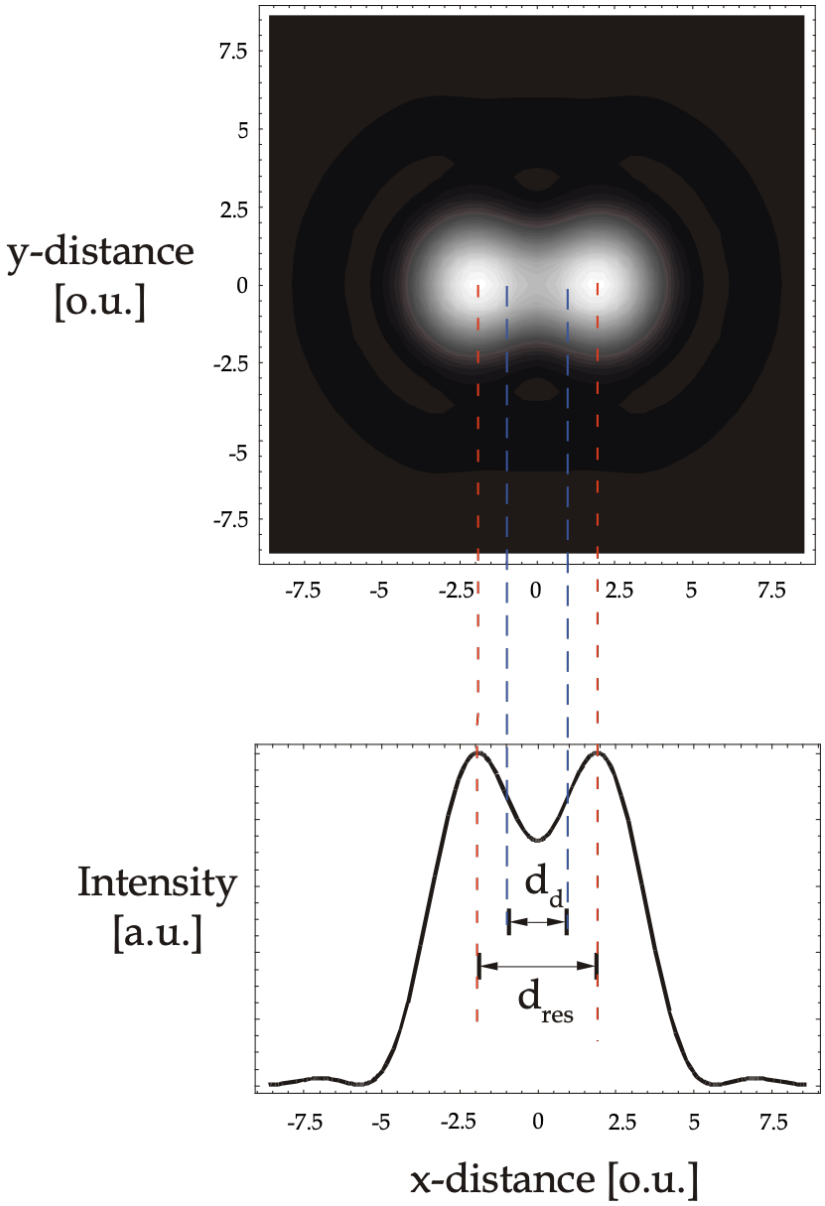
CCD-Chips

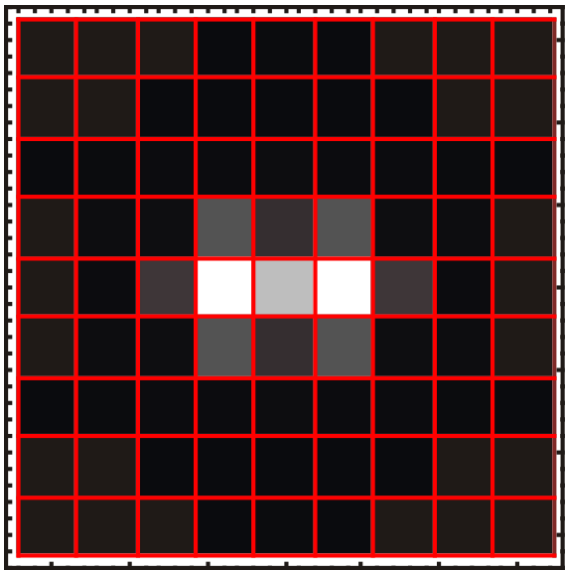



Output Amplifier
Serial Register
Parallel Register
(262,144 Pixels)
20 μ
One
Pixel
20 μ
512 Pixels (10.25 mm)
512 Pixels (10.25 mm)


Incoming Light
Electrical
Connection
Polysilicon
Gate
Silicon
Dioxide
Silicon
e⁻
Potential Well

Optical Resolution and Choice of Detection Pixel Size





$$d_d = 0.5\, d_{res}$$

$$d_d \leq \frac{d_{res}}{2}$$

Nyquist-Theorem

From “Fluorescence Microscopy: From Principles to Biological Applications“

Minimal Magnification Required to Avoid Resolution Losses

$$d_d \leq M \frac{d_{res}}{2}$$

$$M_{opt} \geq 2 \frac{d_d}{d_{res}}$$

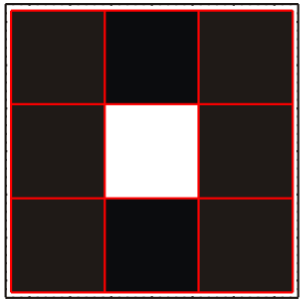
CCD camera:

$d_{res} = 0.25\,\mu m$

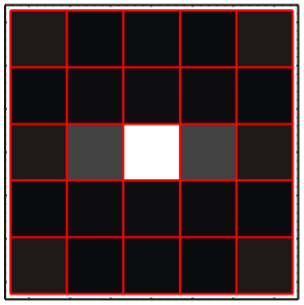
$d_d = 10\,\mu m$

$\rightarrow \quad M_{opt} \geq 80$

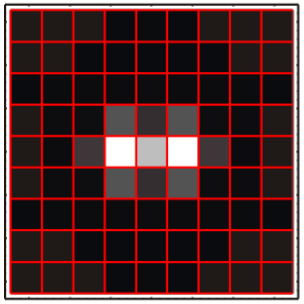
under-sampling



$d_d = 1.5\, d_{res}$

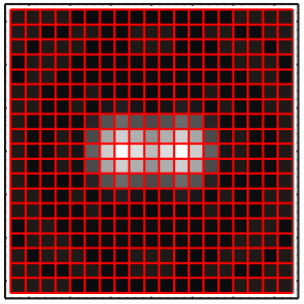


$d_d = 0.9\, d_{res}$



$d_d = 0.5\, d_{res}$

over-sampling

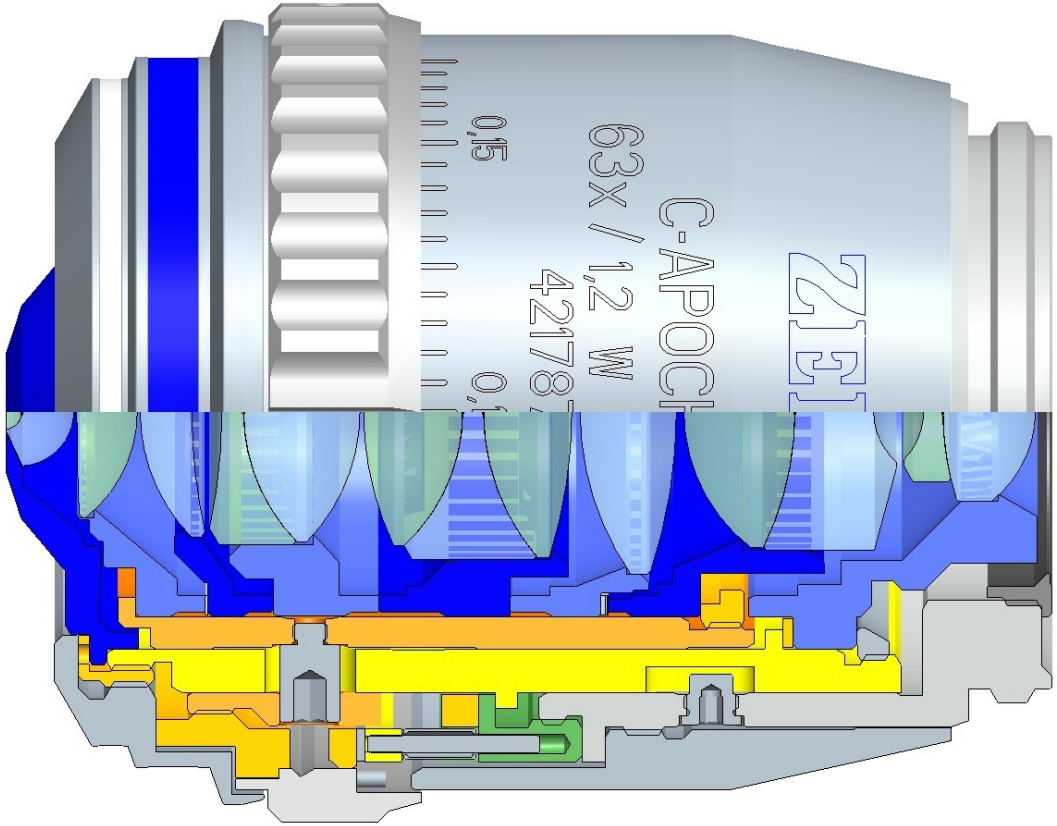


$d_d = 0.24\, d_{res}$

From “Fluorescence Microscopy: From Principles to Biological Applications“

8. Objective lenses

Objectives



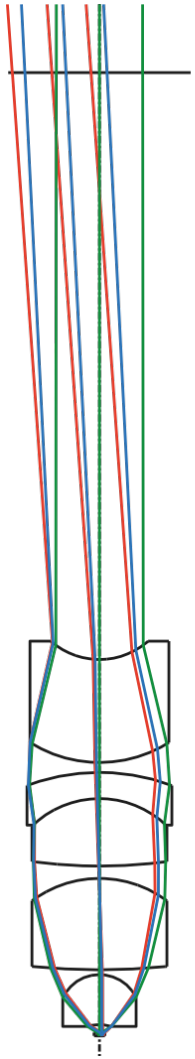
Color code	Magnification
white	100 - 160x
dark blue	60 - 63x
light blue	40 - 50x
green	16 - 32x
yellow	10x
orange	6.3x
red	4 - 5x
brown	2.5x
grey	1.6x
black	1 - 1.25x

Color code	Immersion
white	water
orange	glycerol
red	Multi-immersion
black	oil

With kind permission by Carl Zeiss Microscopy GmbH

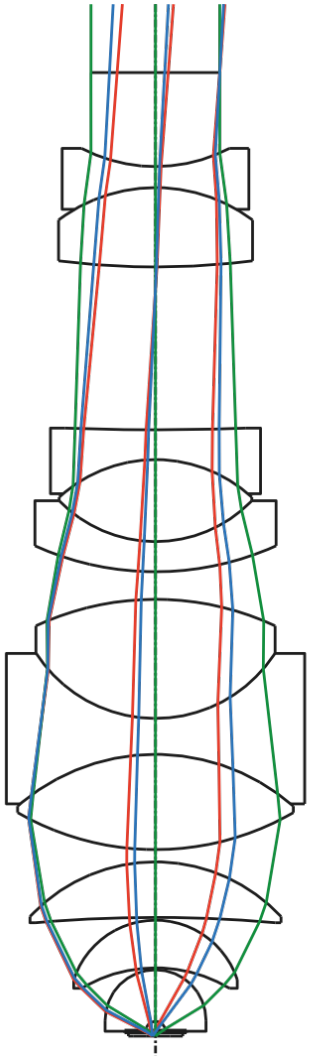
From “Fluorescence Microscopy: From Principles to Biological Applications“

Objective Lens Classes



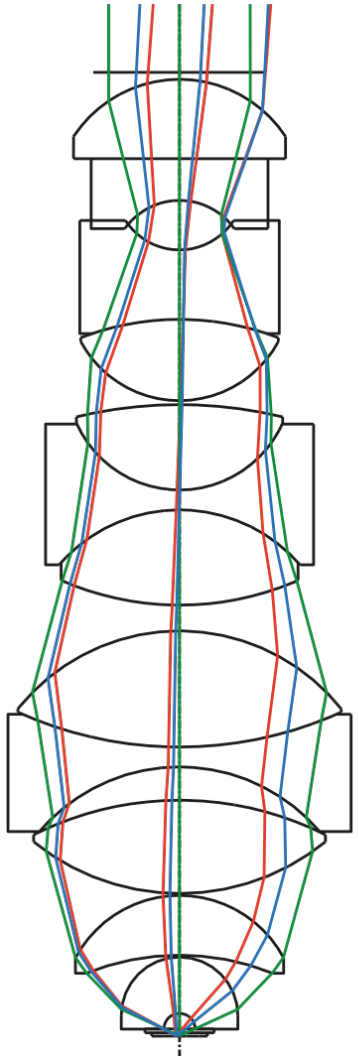
N-Achroplan NA 0.85

air immersion;



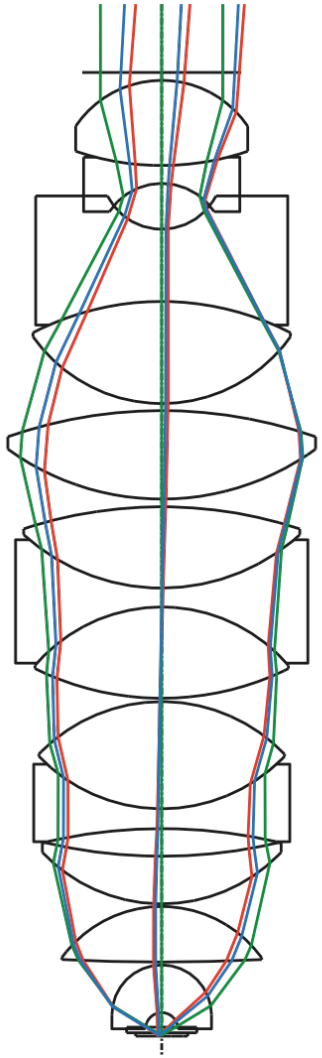
Plan-Neofluar NA 1.25

oil immersion



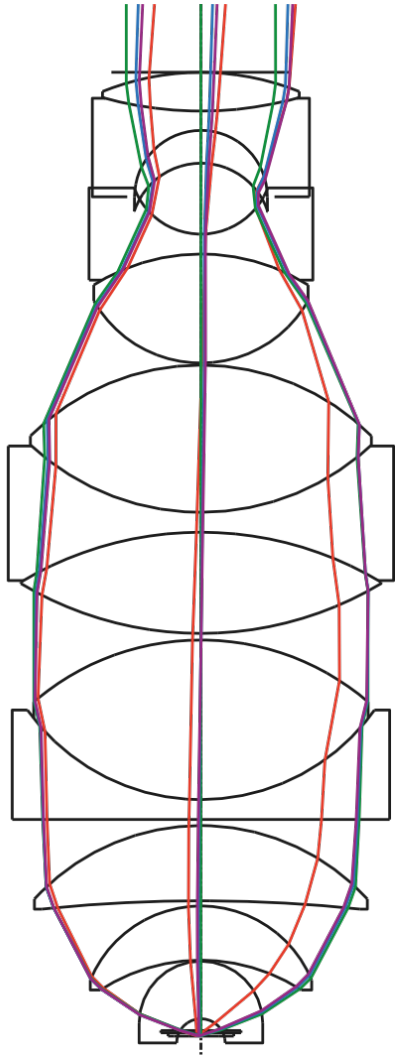
Plan-Apochromat NA 1.4

oil immersion



C-Apochromat NA 1.2

water immersion



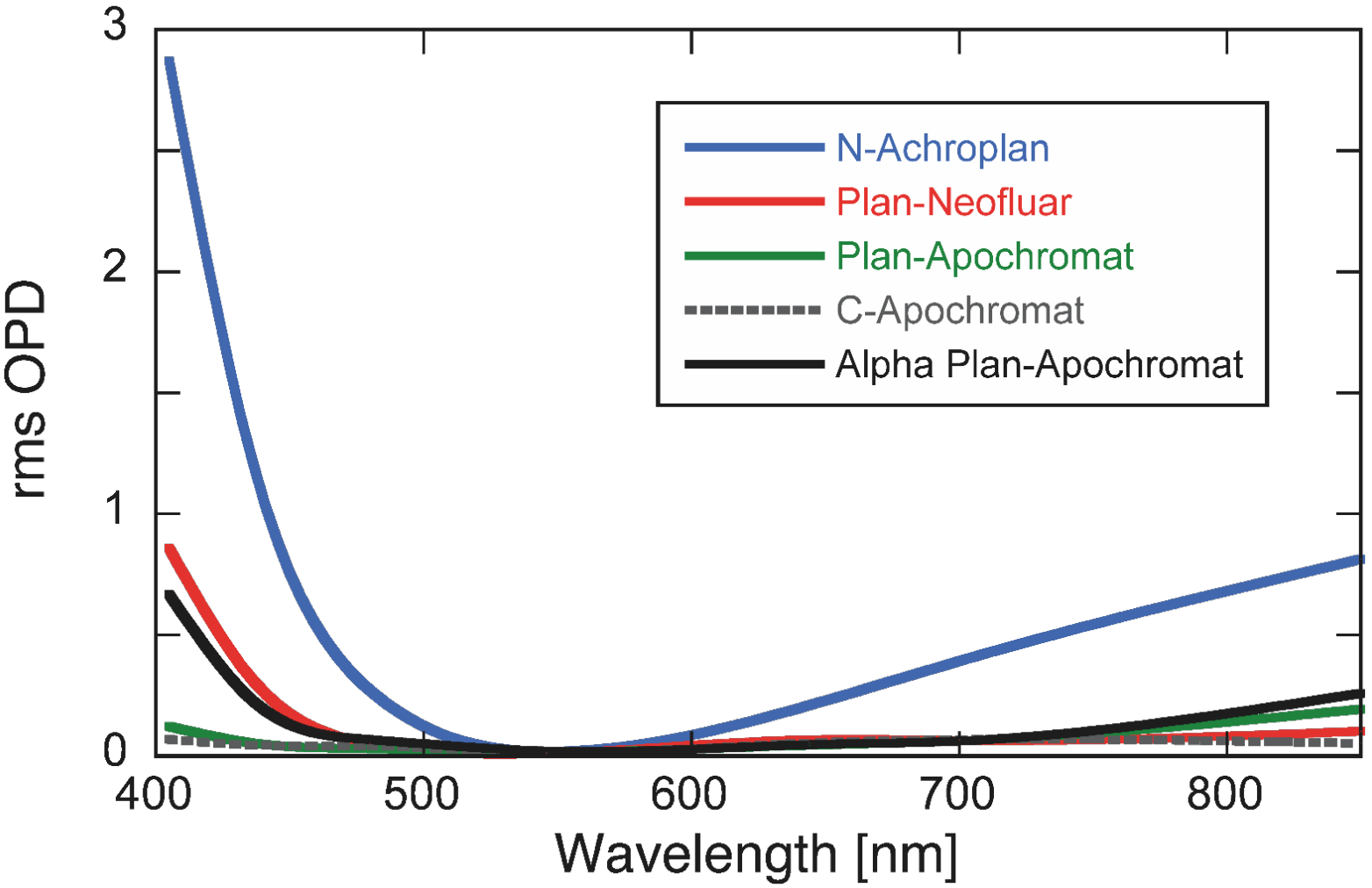
Alpha Plan-Apochromat NA 1.4

oil immersion

From “Fluorescence Microscopy: From Principles to Biological Applications“

63x objectives from Zeiss. With kind permission by Carl Zeiss Microscopy GmbH

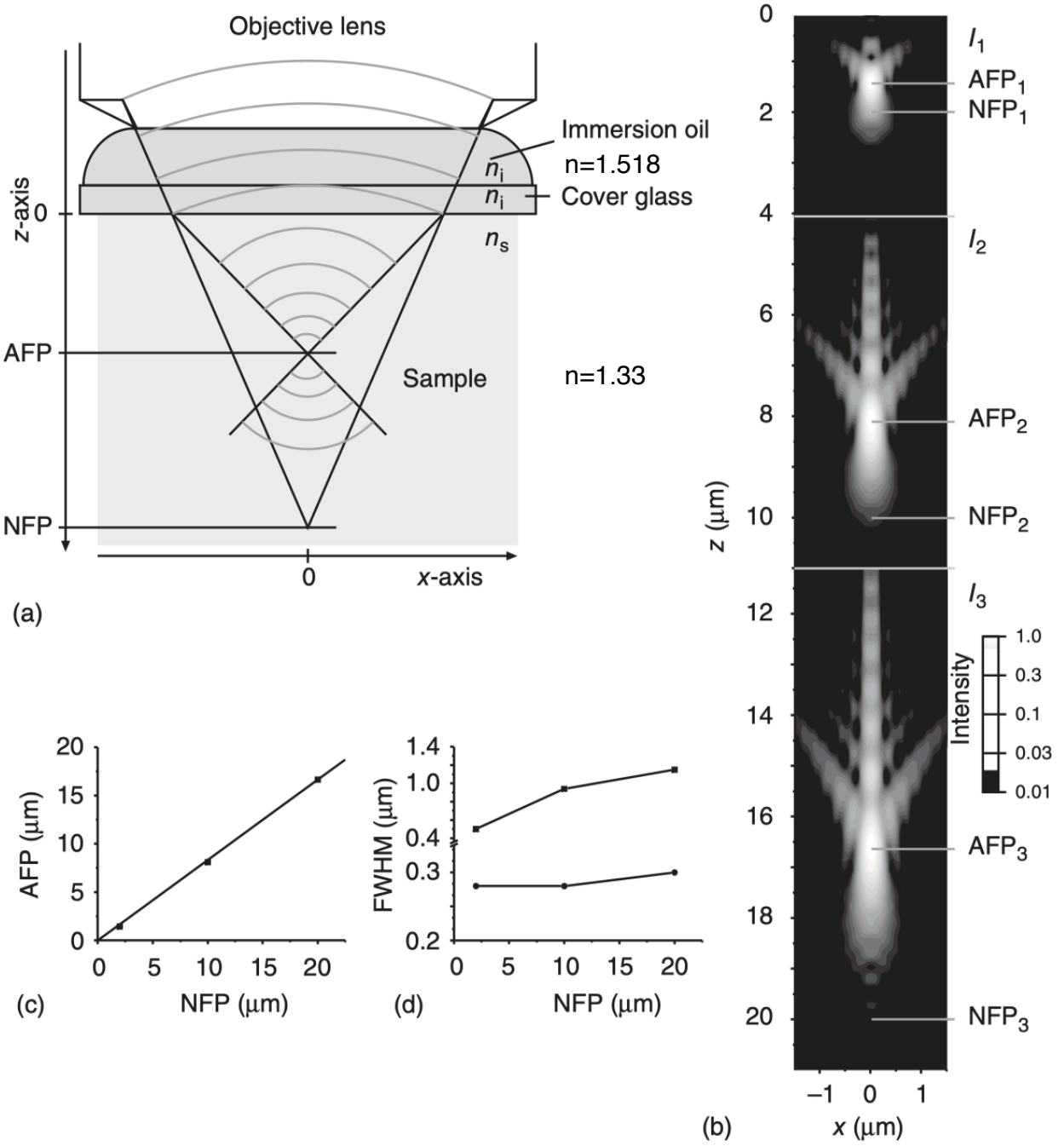
Optical Correction of Objectives



Comparison of the degree of correction for the objectives shown above in terms of the root-mean-square deviations of the optical path length difference (rms-OPD) in the exit pupil of the objective plotted as a function of the wavelength

From “Fluorescence Microscopy: From Principles to Biological Applications“
With kind permission by Carl Zeiss Microscopy GmbH

Using an oil immersion objective for imaging in water



objective NA 1.3

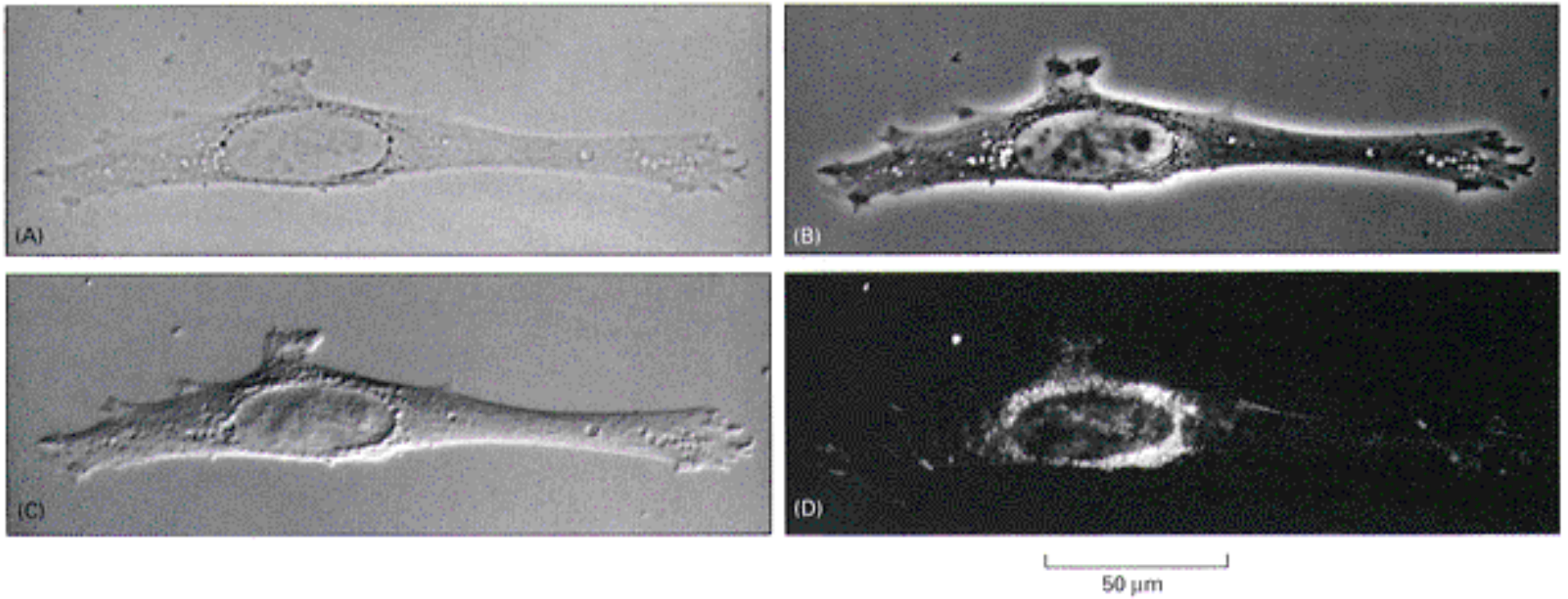
note
logarithmic scale

From “Fluorescence Microscopy: From Principles to Biological Applications“

The contrast problem: physical contrast techniques

bright field

phase contrast



Differential interference contrast (DIC)

dark field

⇒ Image contrast is no inherent object property!

9. Fluorescence

Advantages of Fluorescence Microscopy

- specific labeling

- excellent contrast

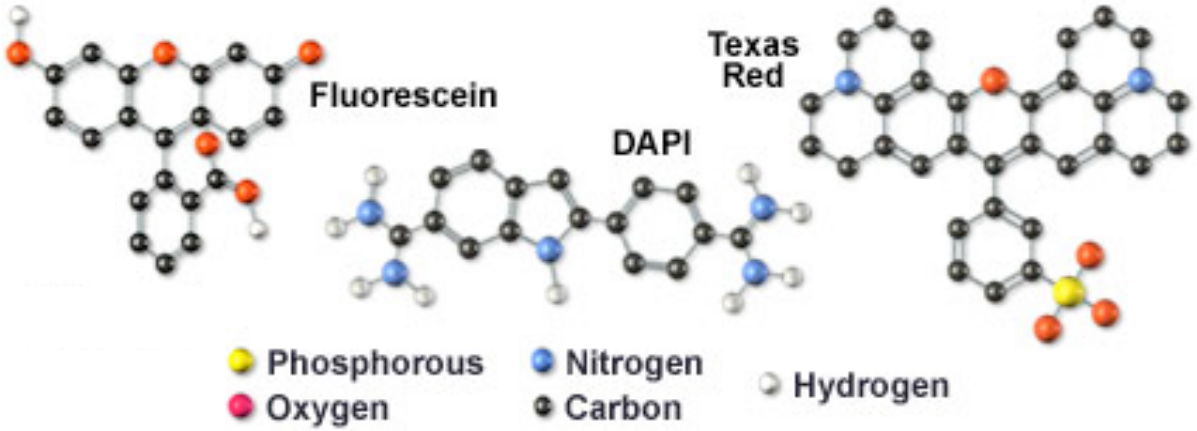
- in vivo observation

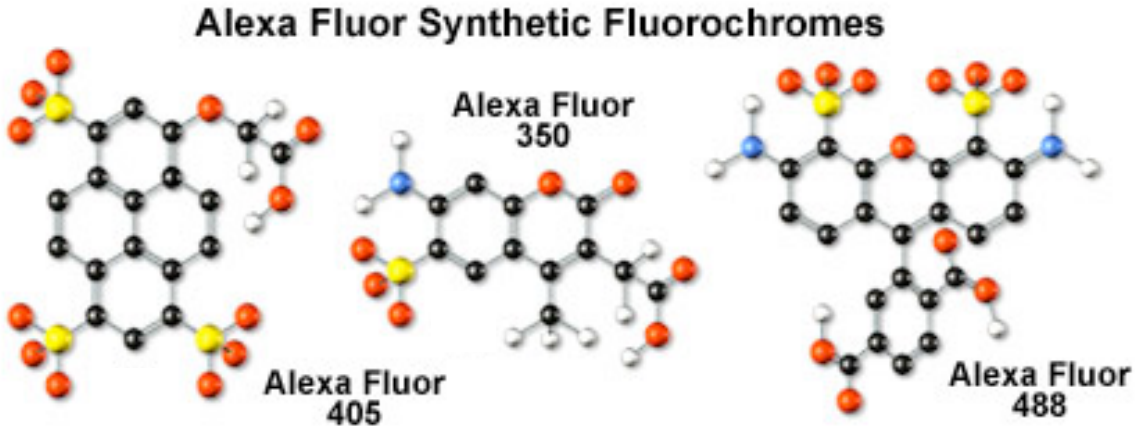
- observation of dynamics

green: nuclear envelope labeled by NTF2-GFP
red: large ribosomal subunits labeled by eIF6-Halo

Fluorescent dyes

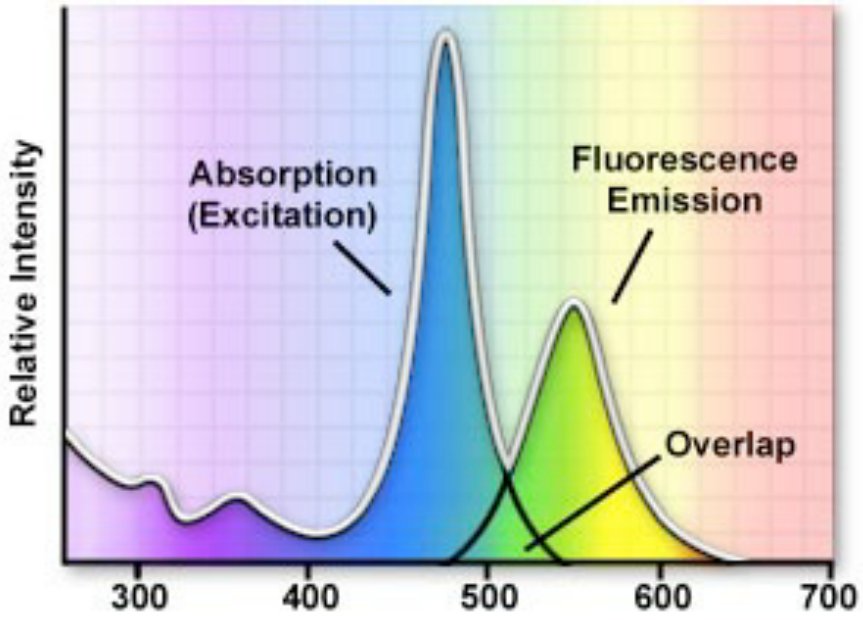
⟝length ≈ 1.3 nm⟞



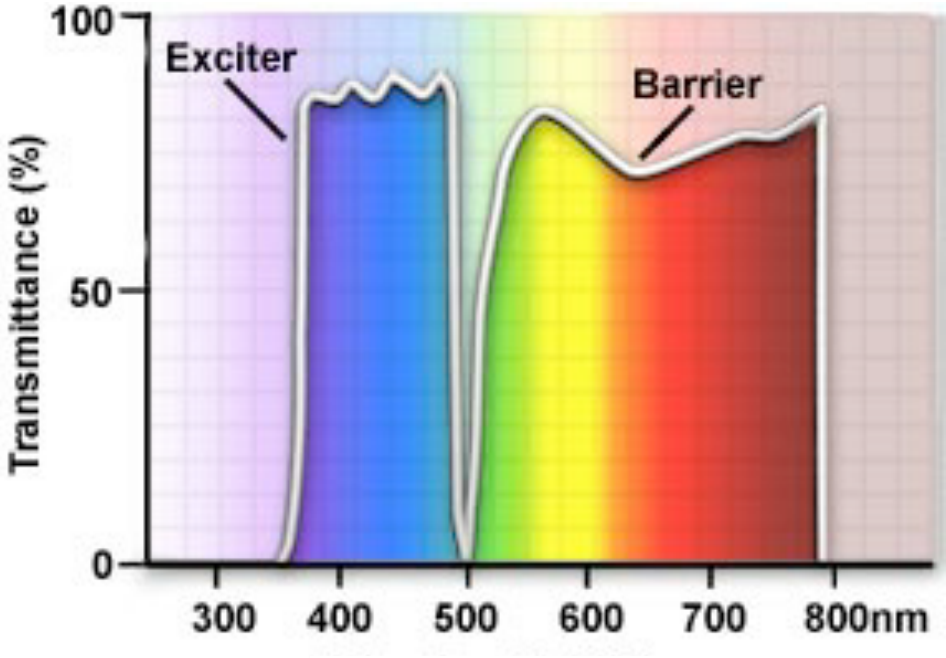


Source: microscopy primer, https://micro.magnet.fsu.edu/primer/

Absorption and emission spectra & corresponding band pass filters



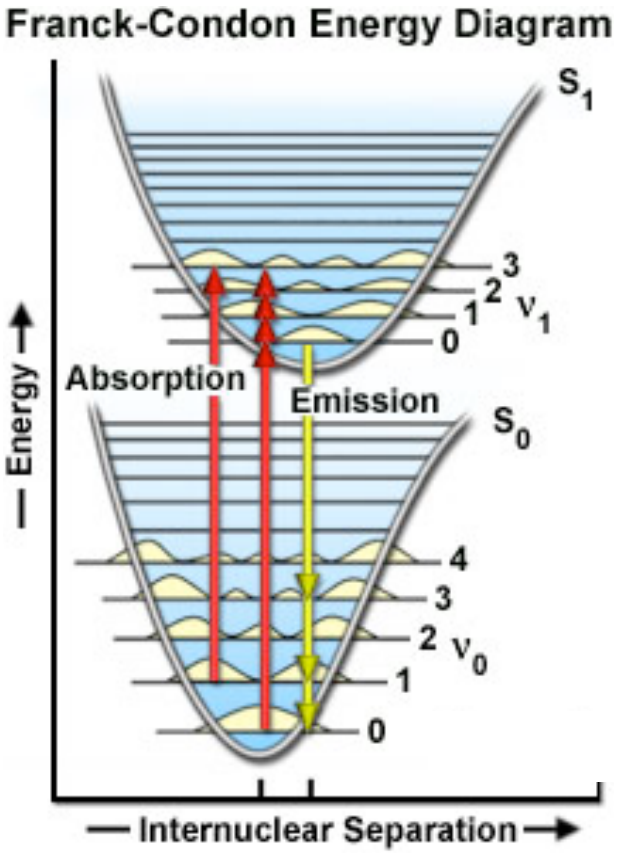
Wellenlänge (nm)

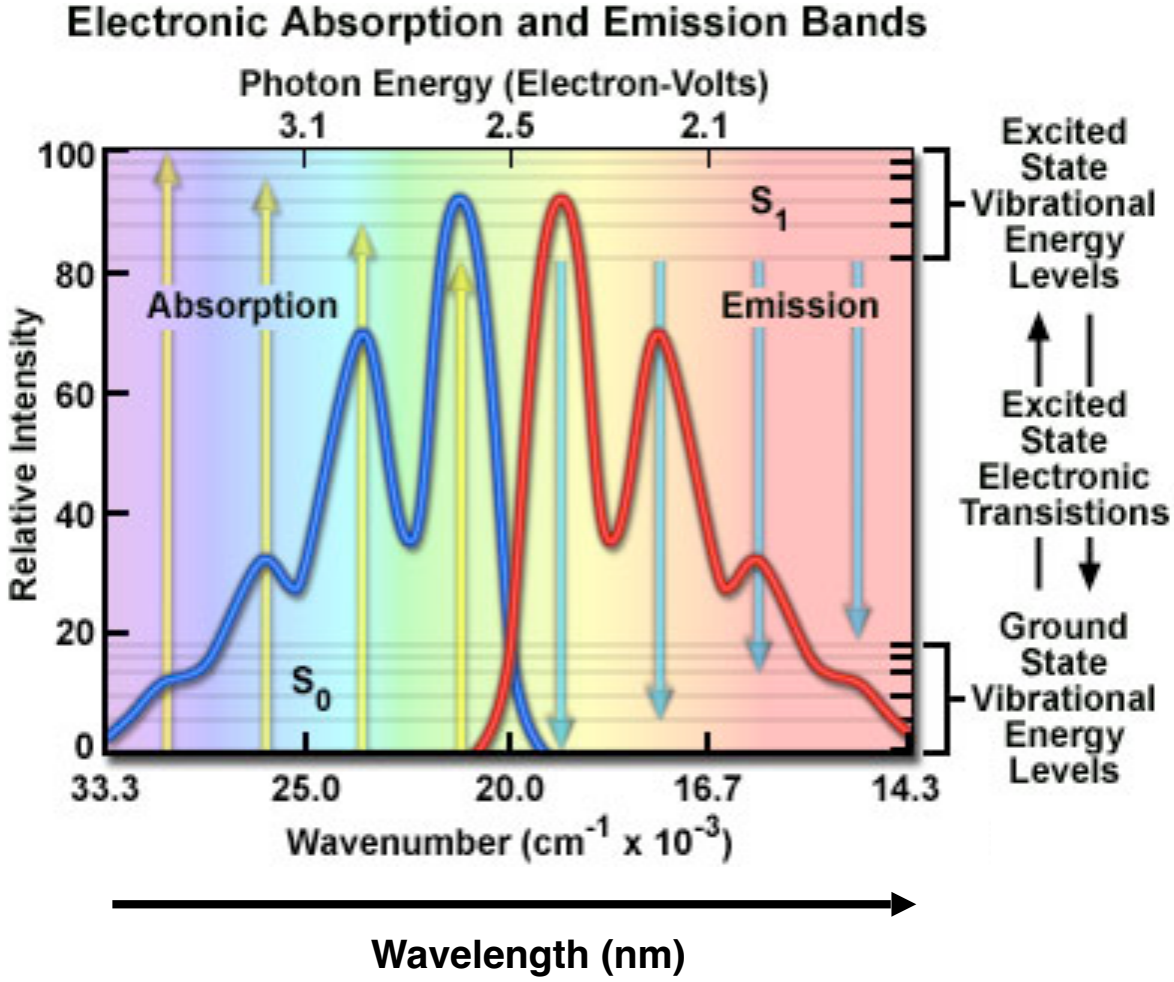


Wellenlänge (nm)

Source: microscopy primer, https://micro.magnet.fsu.edu/primer/

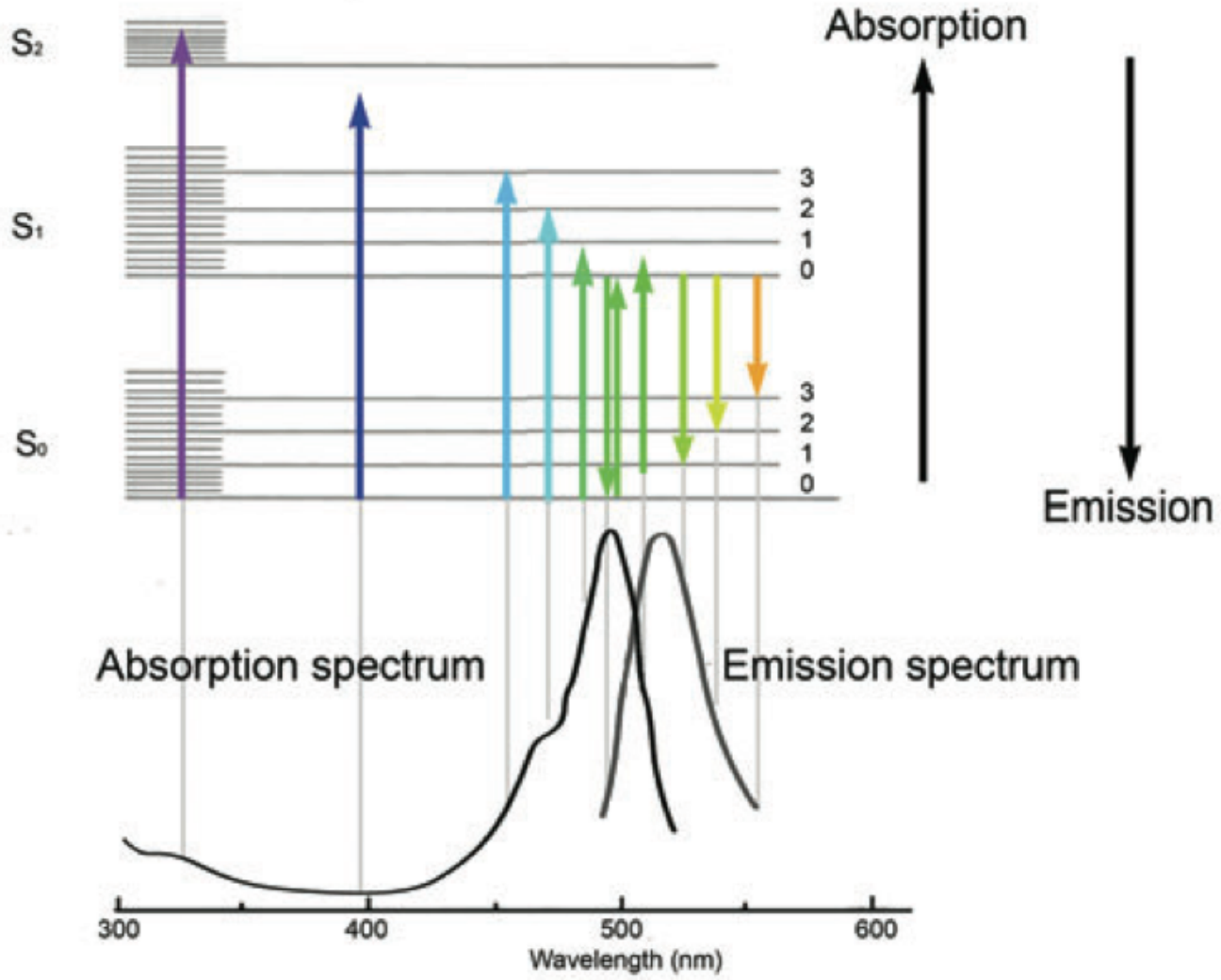
Stokes shift and mirror image rule



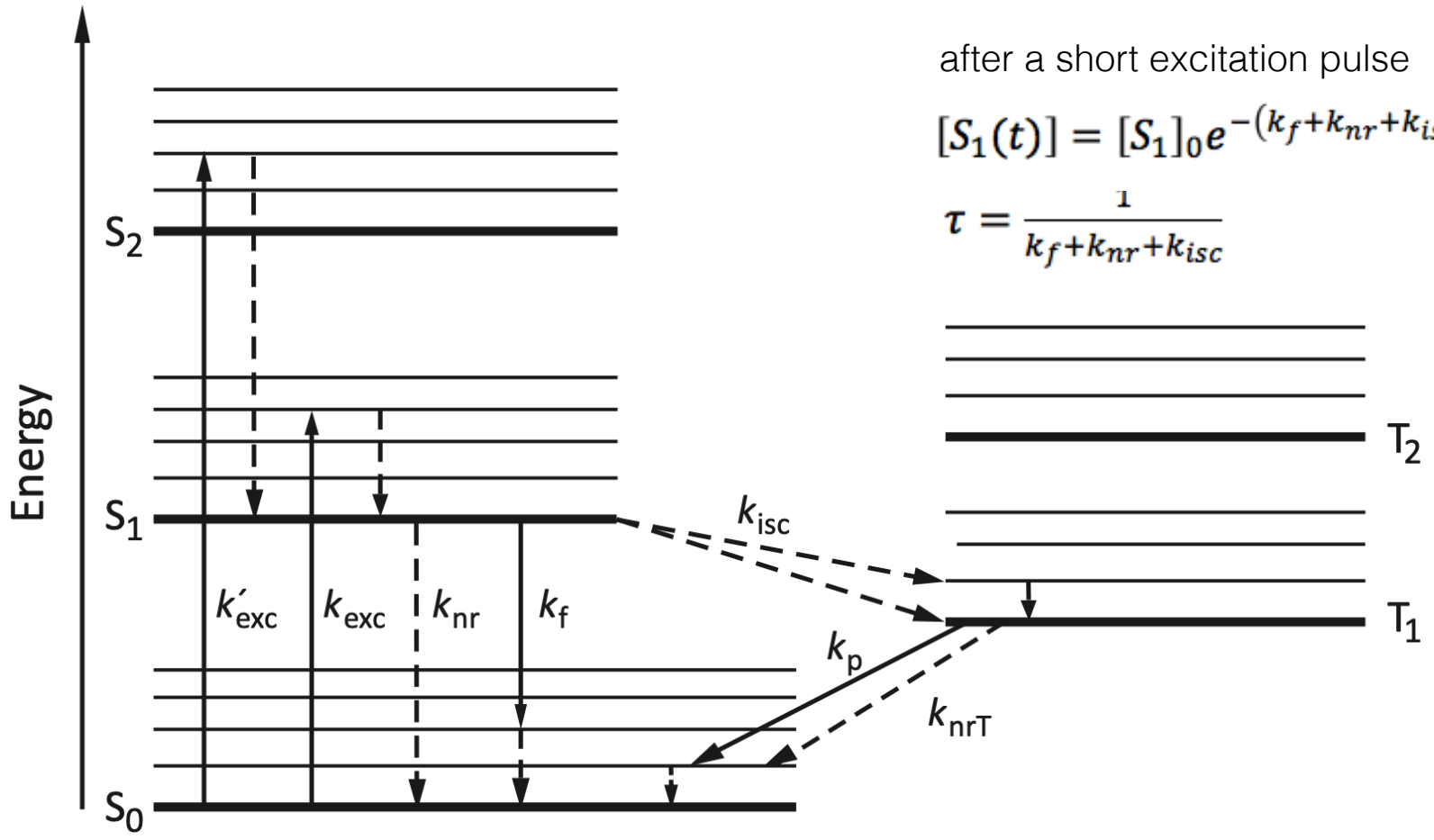


Source: microscopy primer, https://micro.magnet.fsu.edu/primer/

Jablonski diagram



Jablonski diagram



after a short excitation pulse

$$[S_1(t)] = [S_1]_0 e^{-(k_f + k_{nr} + k_{isc})\, t}$$

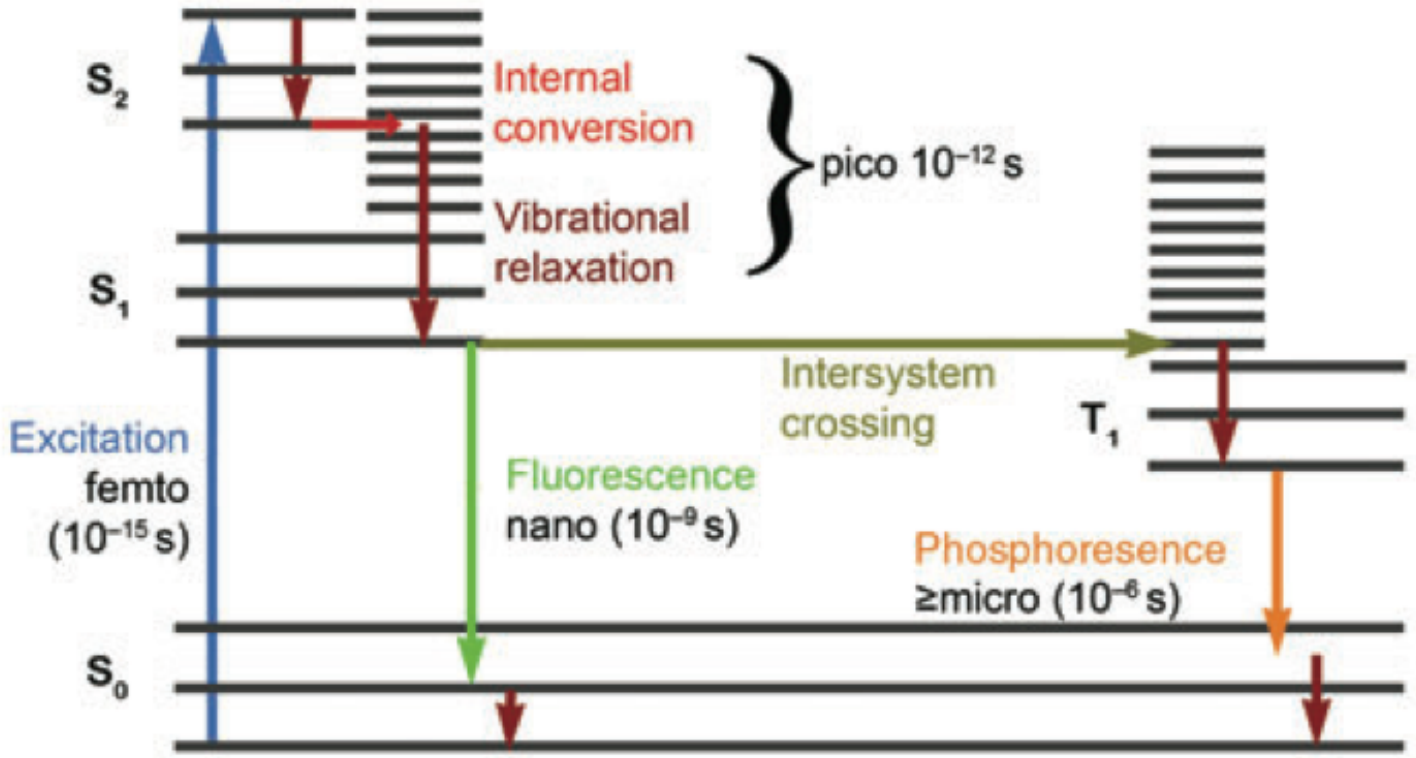
$$\tau = \frac{1}{k_f + k_{nr} + k_{isc}}$$

„quantum efficiency“

$$\Phi = \frac{k_f}{k_f + k_{nr} + k_{isc}}$$

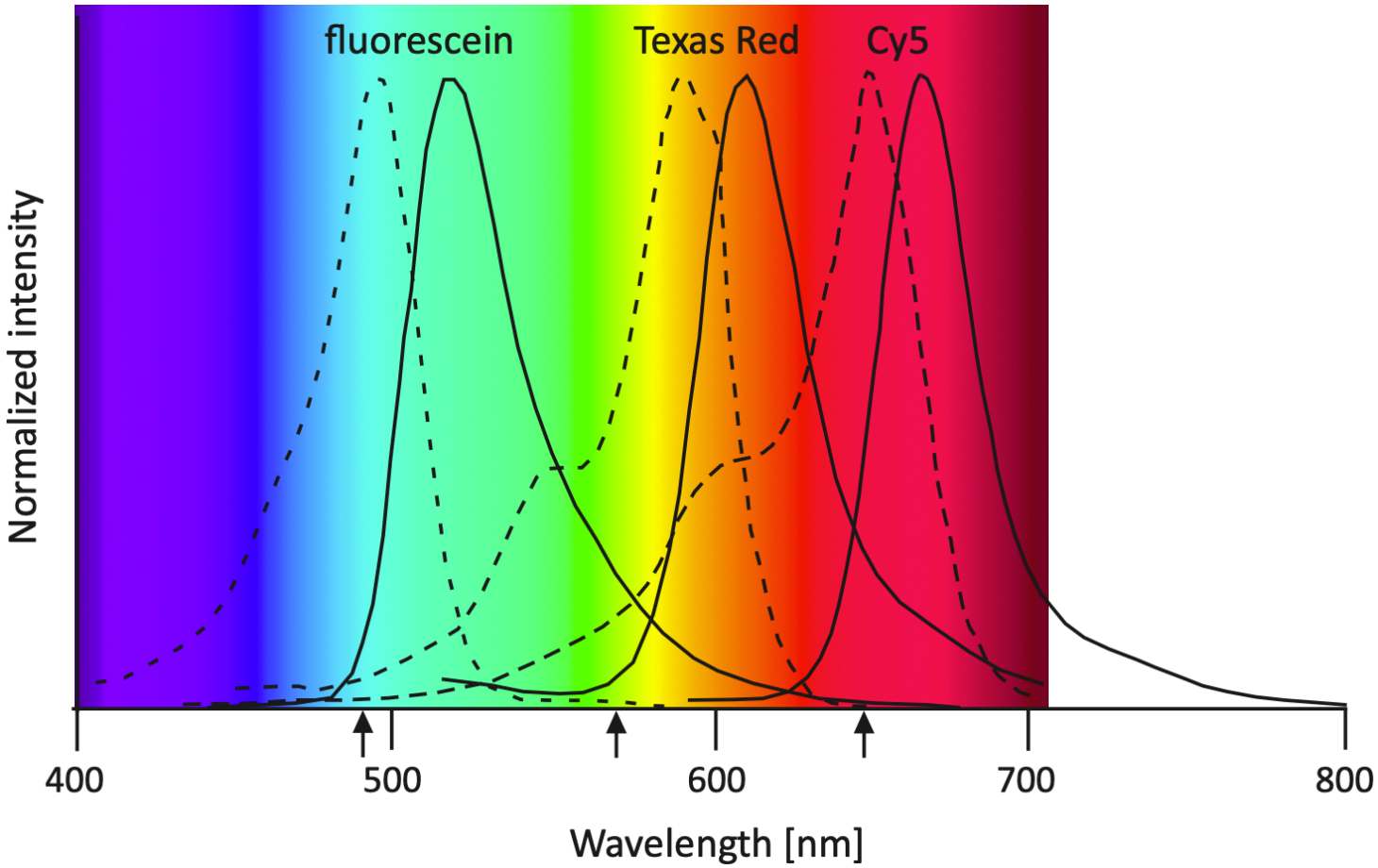
From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition, Fig. 3.1

Time scales of relaxation processe



Source: microscopy primer, https://micro.magnet.fsu.edu/primer/

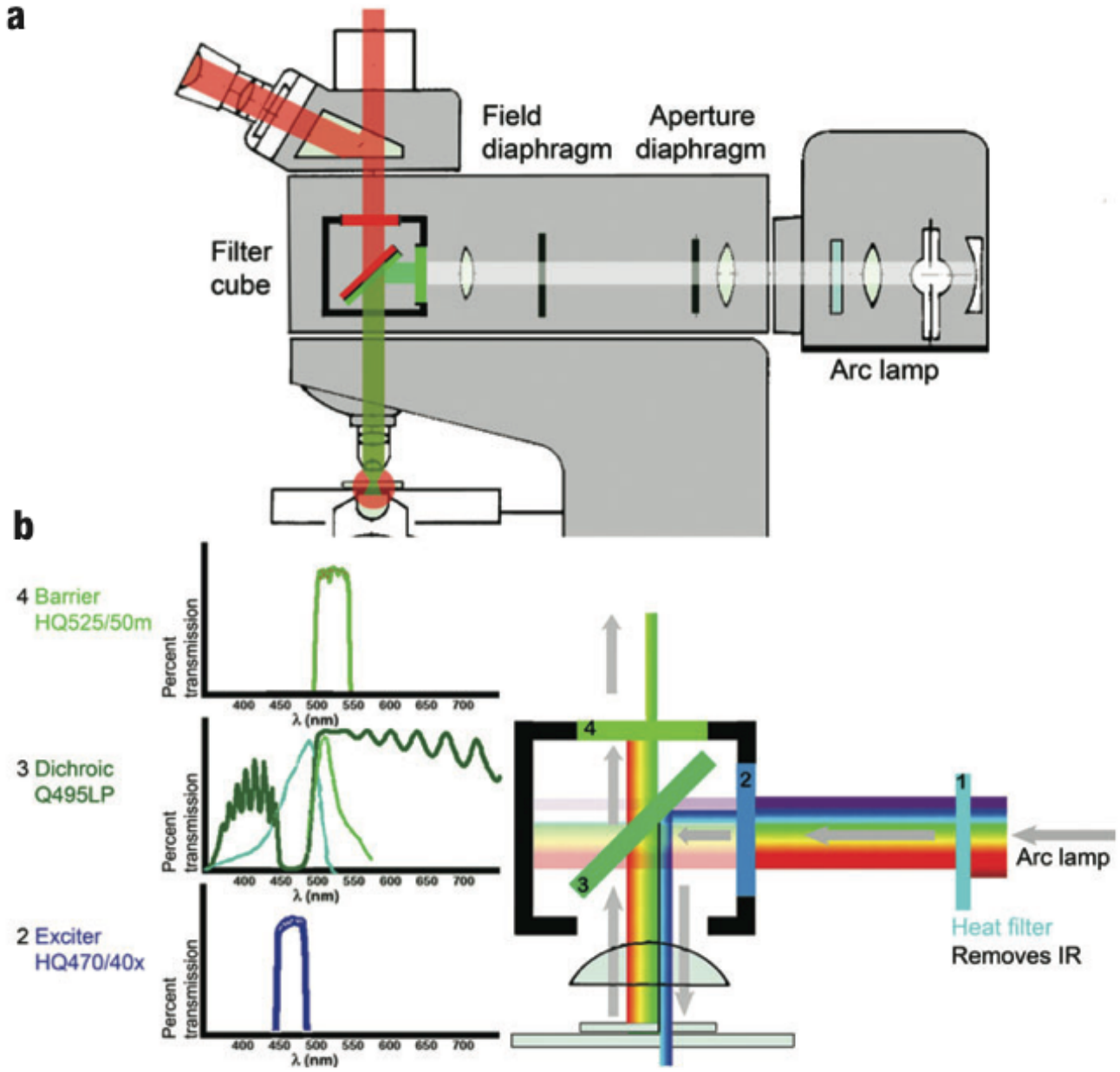
Fluorescence spectra



From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition

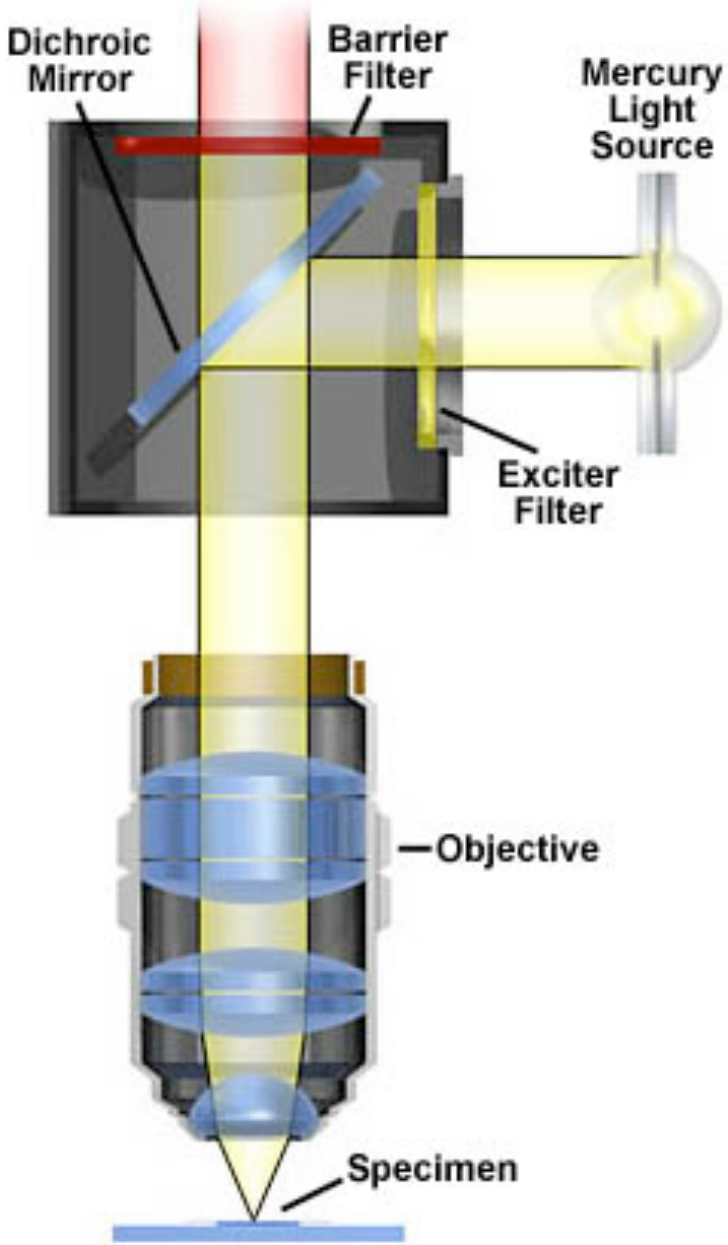
10. Fluorescence microscopy

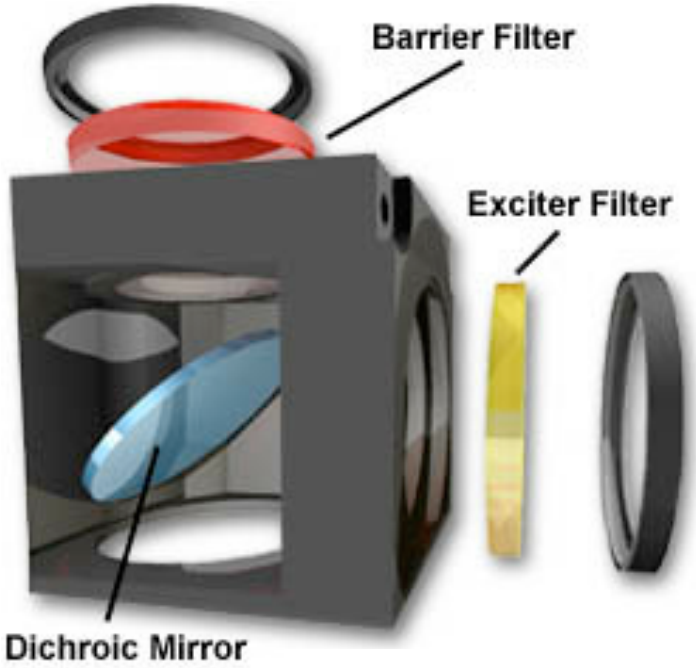
Upright fluorescence microscope



Conchello & Lichtman, 2005. Optical sectioning microscopy. *Nature Methods* 2(12), p. 920

Beam splitting cube



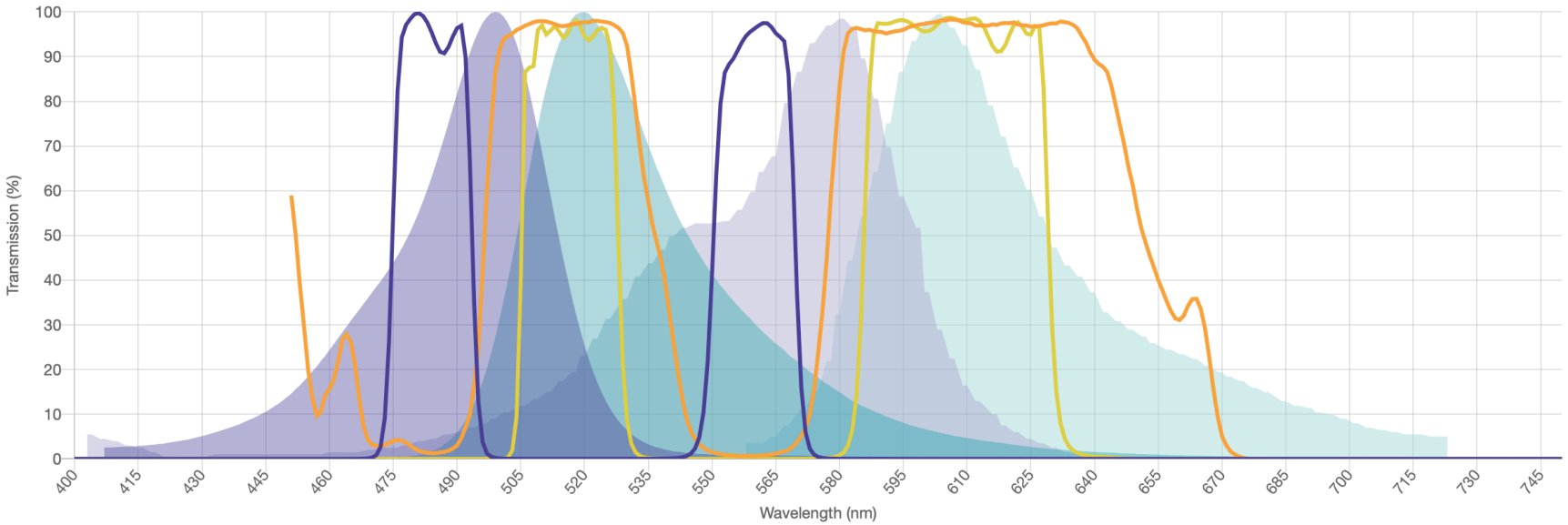


From “Fluorescence Microscopy: From Principles to Biological Applications“

How to find the correct filters for your fluorophore

https://www.omegafilters.com/curvomatic/

Curvomatic Fluorescence Spectral Viewer



Filters and Curves

Wavelength 400 to 750 nm

(Click and drag for horizontal scroll)

Fluorescence Sets

XF452 FITC/ALEXA FLUOR 568 DUAL BAND PREMIUM FLUORESCENCE FILTER SET

485-560DBEX: --- %T at --- nm
485-560DBDR: --- %T at --- nm
520-610DBEM: --- %T at --- nm

Fluorophores

ALEXA FLUOR® 488

Excitation: --- % at --- nm
Emission: --- % at --- nm

ALEXA FLUOR® 568

Excitation: --- % at --- nm
Emission: --- % at --- nm

Alternative:

Spectra Viewer by the MPI for Brain Research, Frankfurt, Germany

11. Confocal microscopy

The confocal principle

Combination of focussed laser illumination

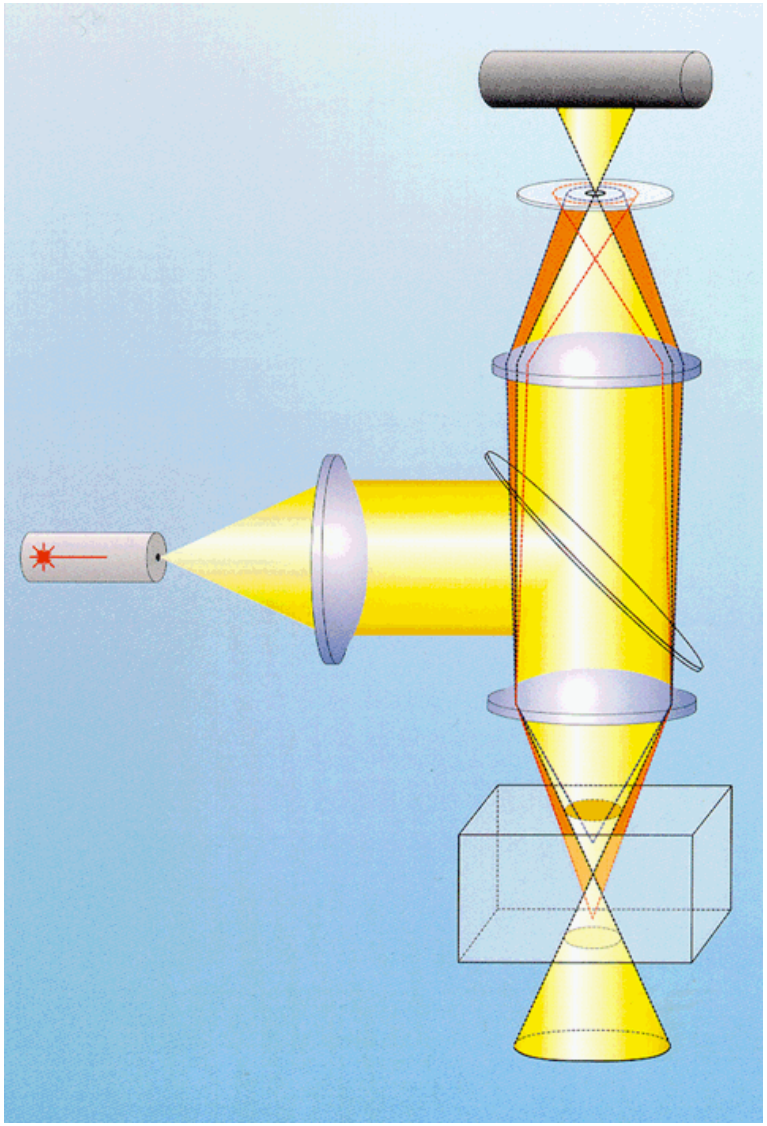
and

detection through in pinhole placed in a conjugated optical plane

yields efficient background subtraction

and axial resolution:

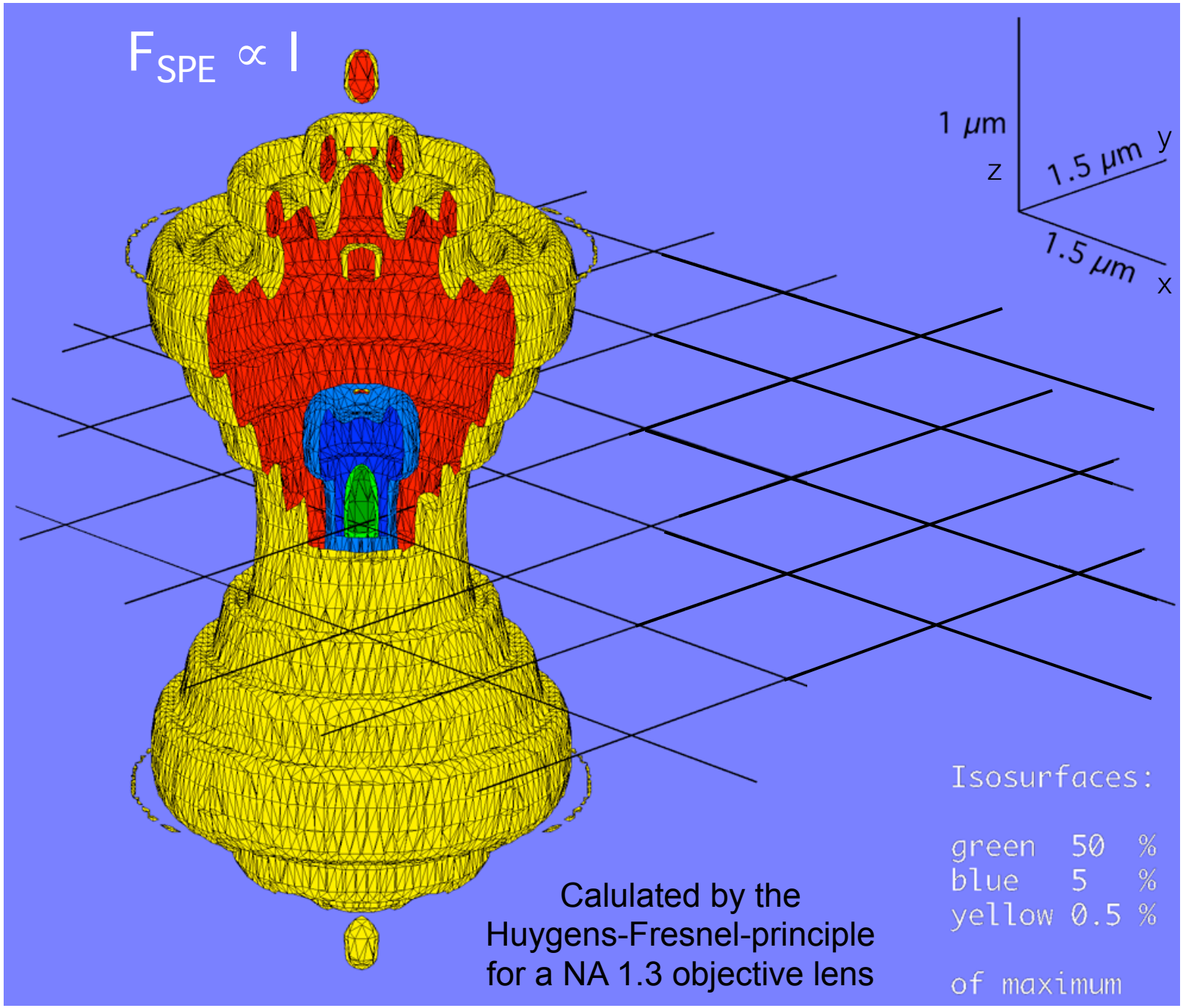
„Optical Sectioning“



Source: Carl Zeiss, info on laser scanning microscopy

1-Photon Excitation Profile

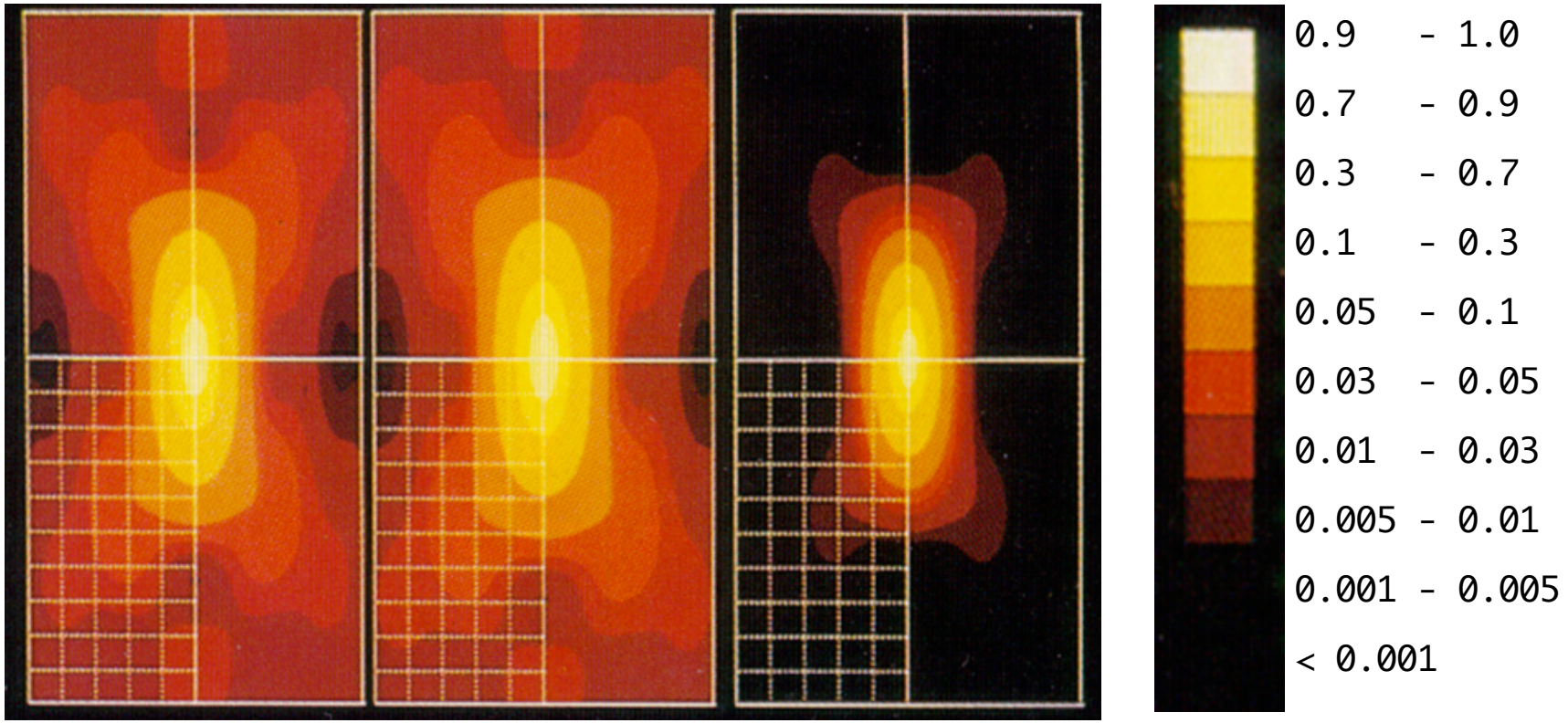
SPE, λ=488 nm



Courtesy of Dr. Christian Verbeek

Confocal point spread function

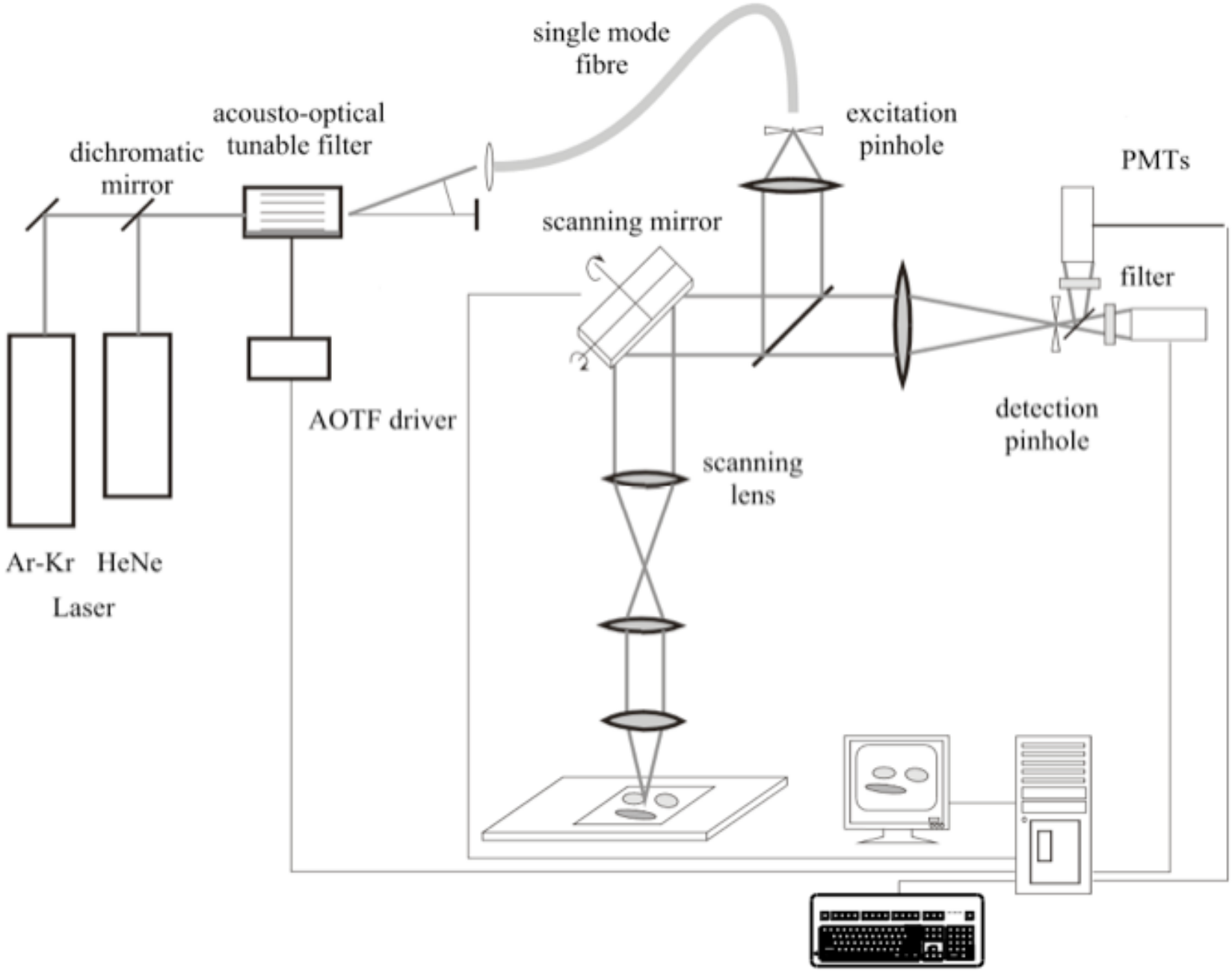
Excitation 488 nm Fluorescence 525 nm



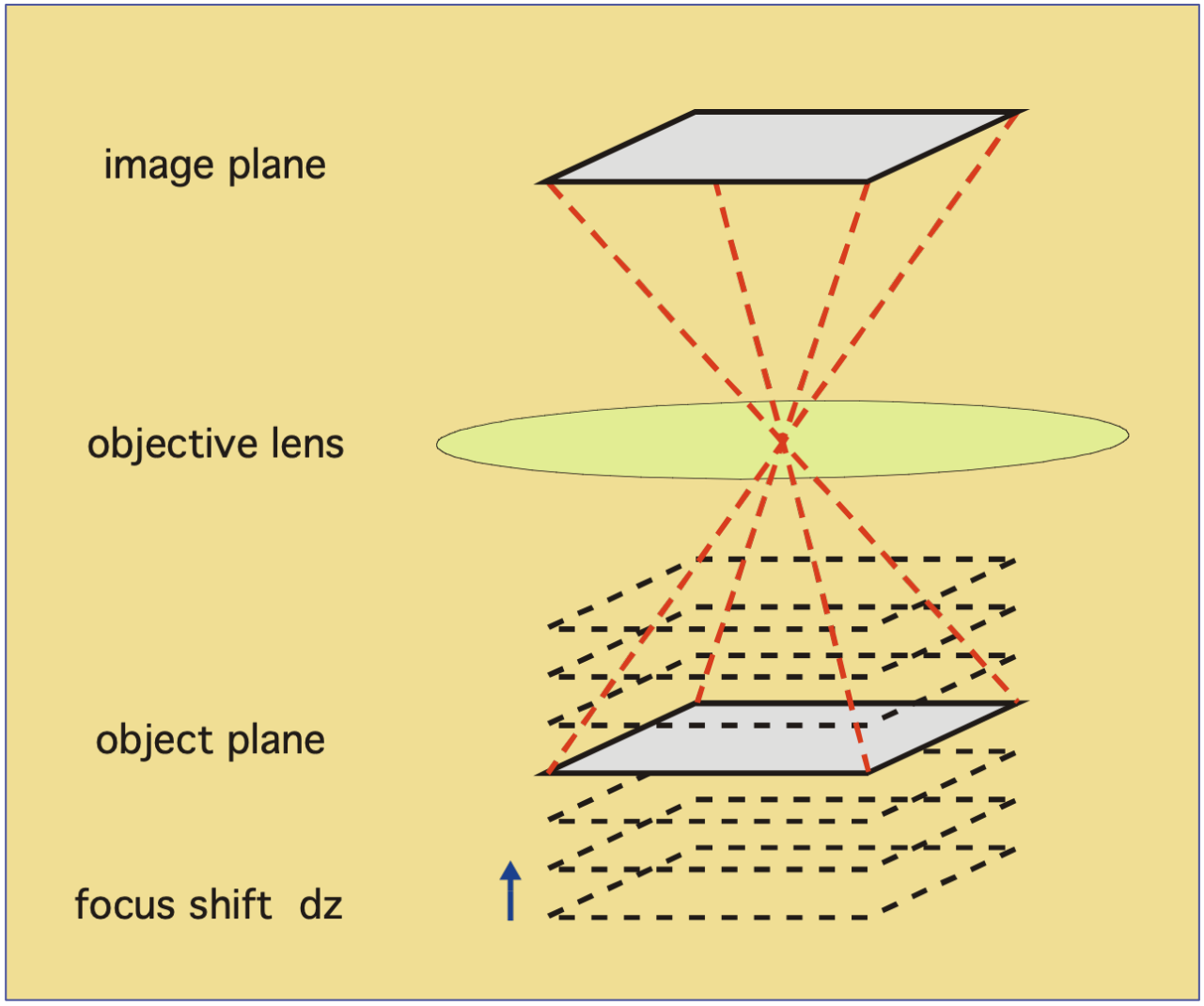
Objective 100X NA 1.32
100 nm/division

Source: publication by Stefan Hell and colleagues, 1990s

Scheme of a point scanning confocal microscope

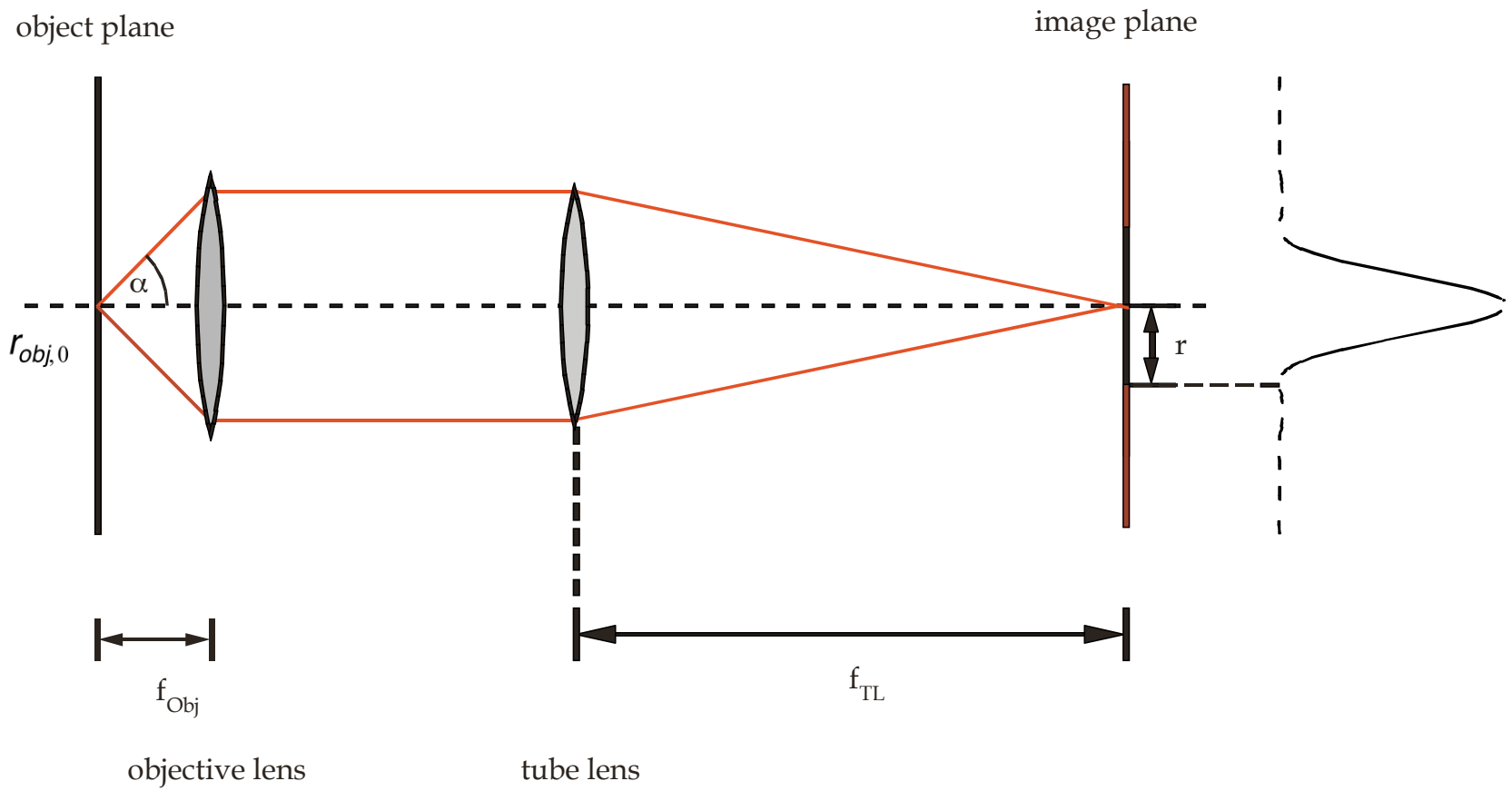


Acquisition of optical slices



Alternatives: shift of object or shift of objective

Role of the detection pinhole



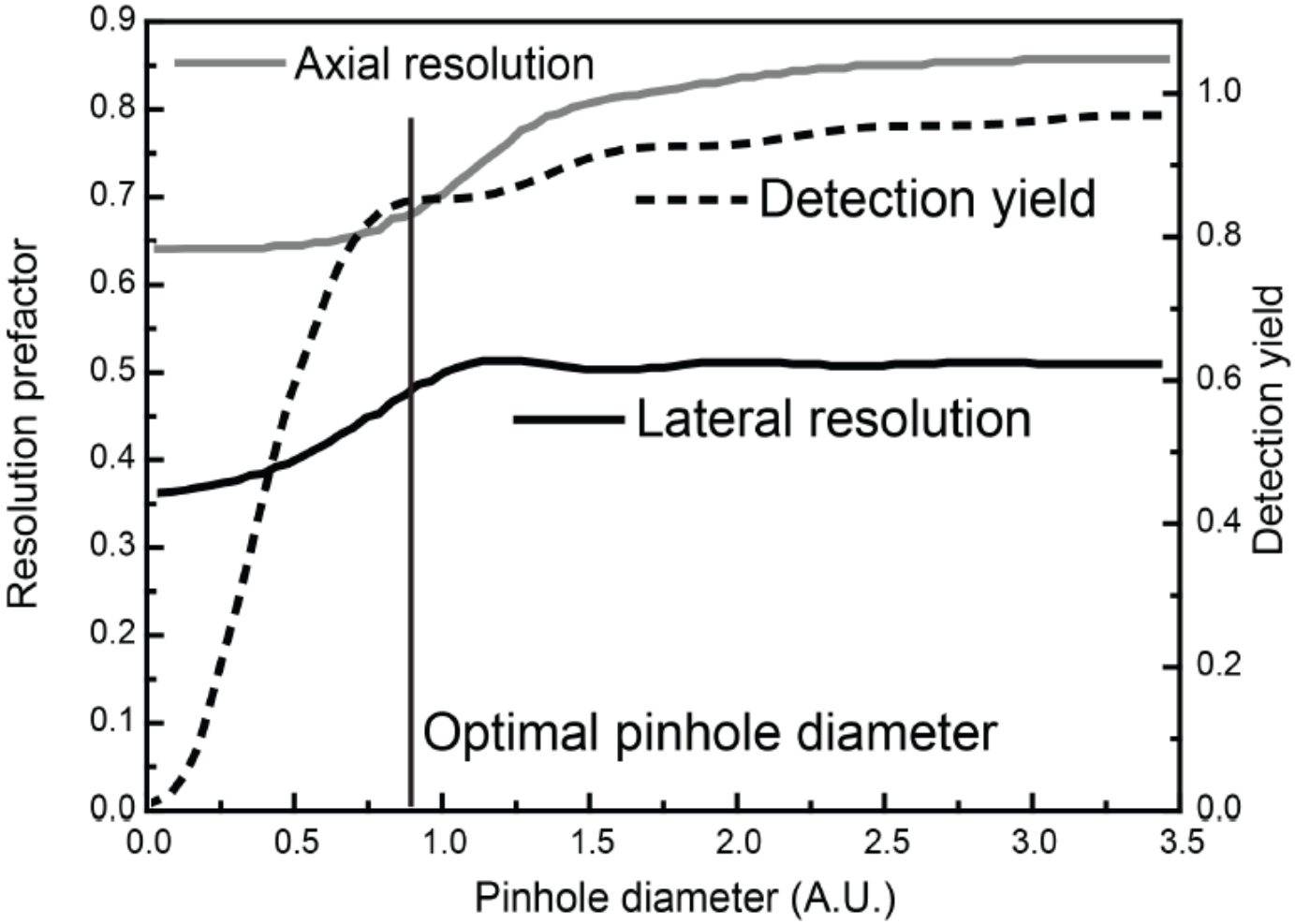
$$r_{obj,0} = \frac{0.61\,\lambda}{n\,\sin\alpha}$$

α: opening angle of the objective lens divided by 2

n the refractive index of the medium in front of the objective lens.

$$NA_{Obj} = n\,\sin\alpha$$

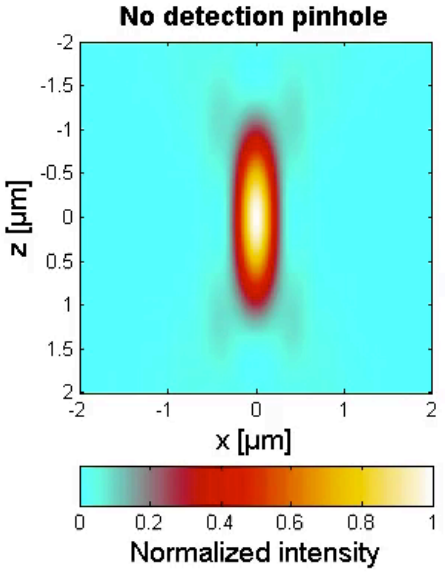
Detection yield, axial and lateral resolution as function of detection pinhole diameter

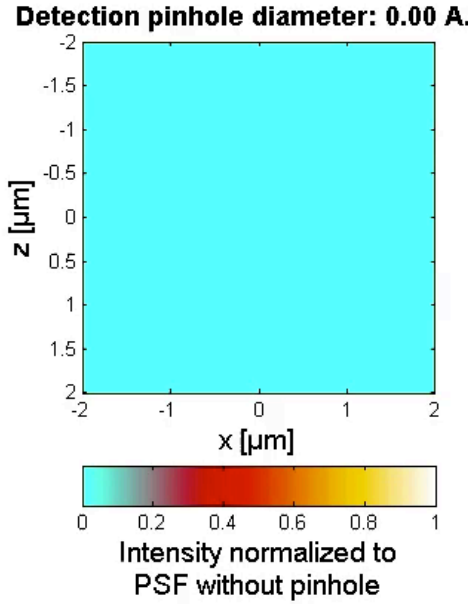


N. Naredi-Rainer, J. Prescher, A. Hartschuh, D.C. Lamb 2013, chapter „Confocal Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck

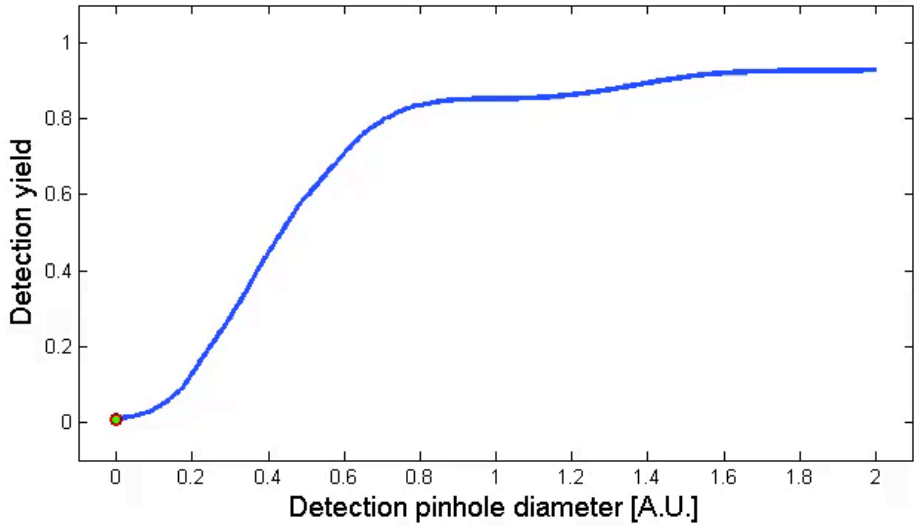
Confocal Point Spread Function (PSF) as Function of Pinhole size

Excitation PSF
without pinhole





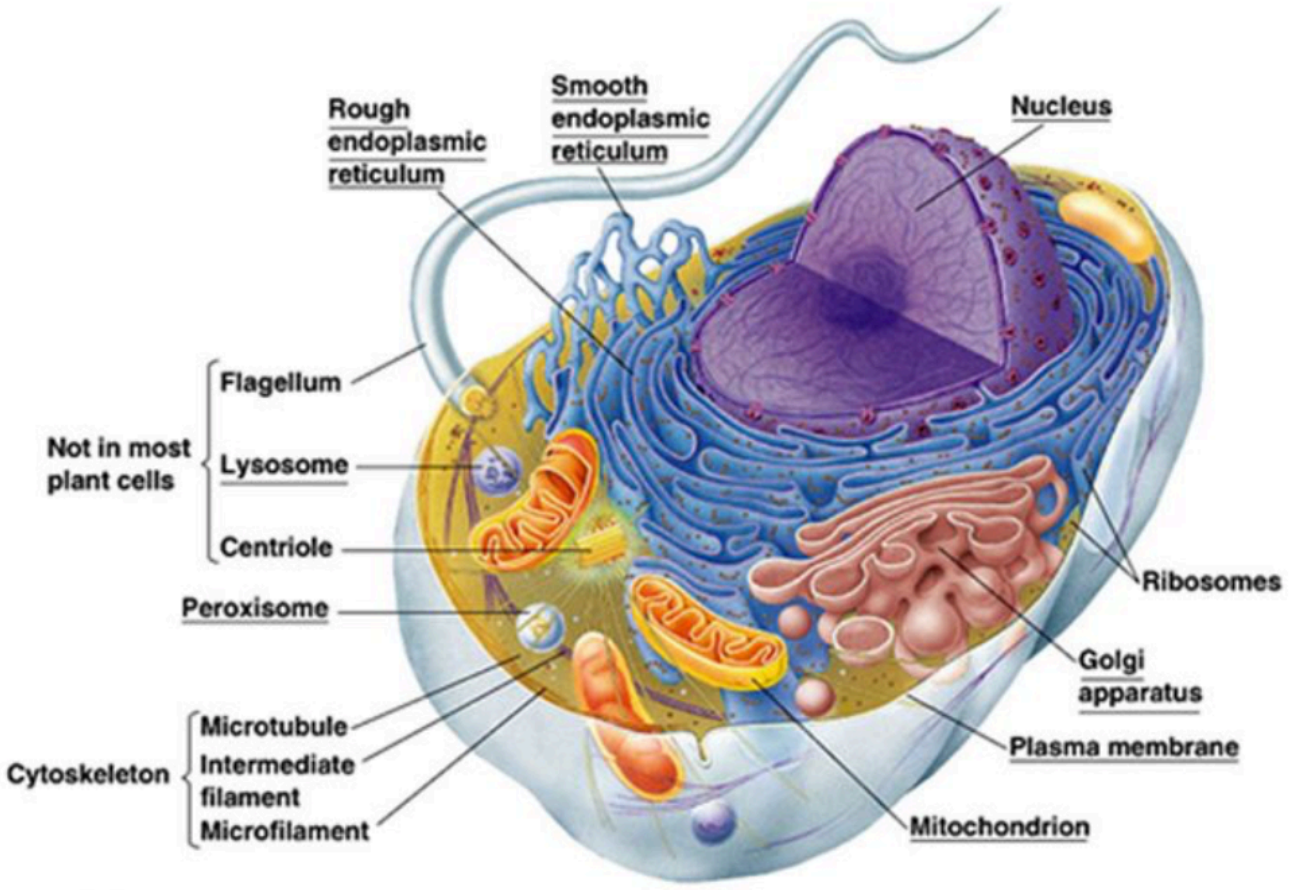
total PSF



Fraction of light that is transmitted through the pinhole is shown with the green cursor marking the current pinhole size for the calculated PSF.
A smaller pinhole size yields a higher overall resolution, but at a significant loss in detection yield.

N. Naredi-Rainer, J. Prescher, A. Hartschuh, D.C. Lamb 2013, Chapter 5 „Confocal Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

Example: nuclear pore complexes in the nuclear envelope



Source: https://slideplayer.com/slide/10634029/

Example: nuclear pore complexes in the nuclear envelope