Welcome

Microscopía y Procesamiento de Imágenes

Prof. Dr. Steffen Härtel

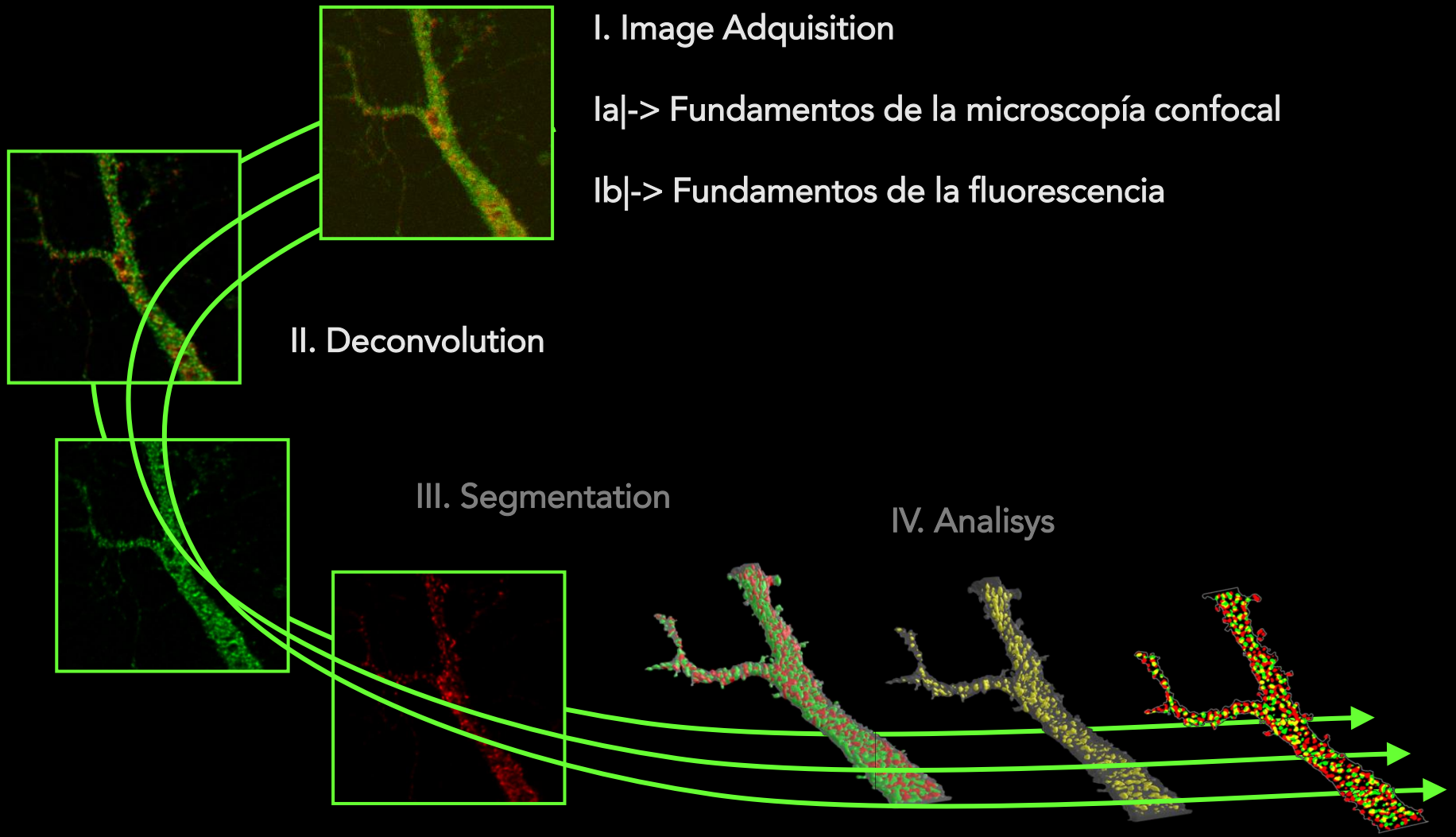
www.scian.cl / www.cimt.cl / www.cens.cl www.bni.cl / www.rsdue.cl

Laboratory for Scientific Image Analysis (SCIAN-Lab)
Centro de Informática Médica y Telemedicina (CIMT)
Centro Nacional en Sistemas de Información en Salud (CENS)
Biomedical Neuroscience Institute (BNI)
Red de Salud Digital de Universidades del Estado (RSDUE)
Institute of Biomedical Sciences (ICBM)
Anatomy and Developmental Biology Program
Escuela de Postgrado
Facultad de Medicina, Universidad de Chile

Laboratory for SCIAN Scientific Image Analysis

Welcome

BIOMEDICAL NEUROSCIENCE INSTITUTE



|-> Joseph R. Lakowicz

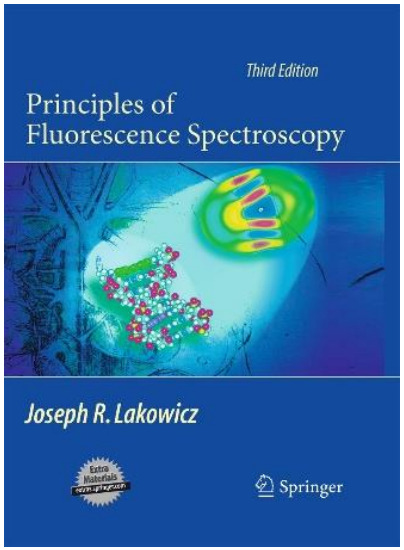
- Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz 4.1 Introduction to Fluorescence

|-> Quenching, Bleaching ...

|-> Polarisation ...

|-> Steady-State and Time-Resolved Fluorescence...

|-> Förster Resonance Energy Transfer ...



- Photomultiplier www.olympus-lifescience.com/en/microscope-resource/primer/digitalimaging/concepts/photomultipliers
- Chrome Spectra Viewer www.chroma.com/spectra-viewer
- Thermo Fisher Spectra www.thermofisher.com/order/spectra-viewer

|-> Fluorescencia I

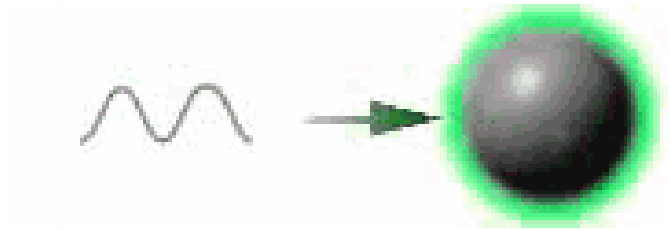
Luminescencia:

- ***Fluorescencia*** $\Delta t \sim 10^{-8}s$
- ***Fosforescencia*** $\Delta t \sim 10^{-3}\text{-}10^{0}s$

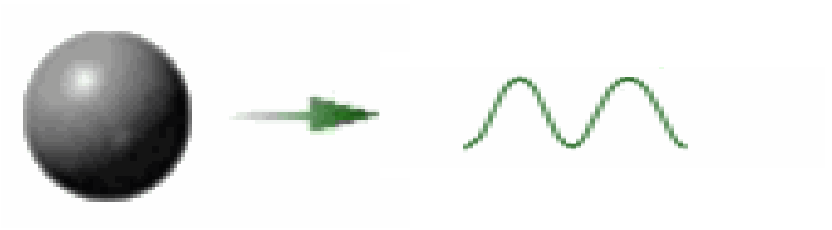
Interacciones ...
- **intra- e inter moleculares ...**

producen cambios ...
- **espectrales**
- **tiempos de vida**
- **polarización**
- **intensidad ...**

← Δt →

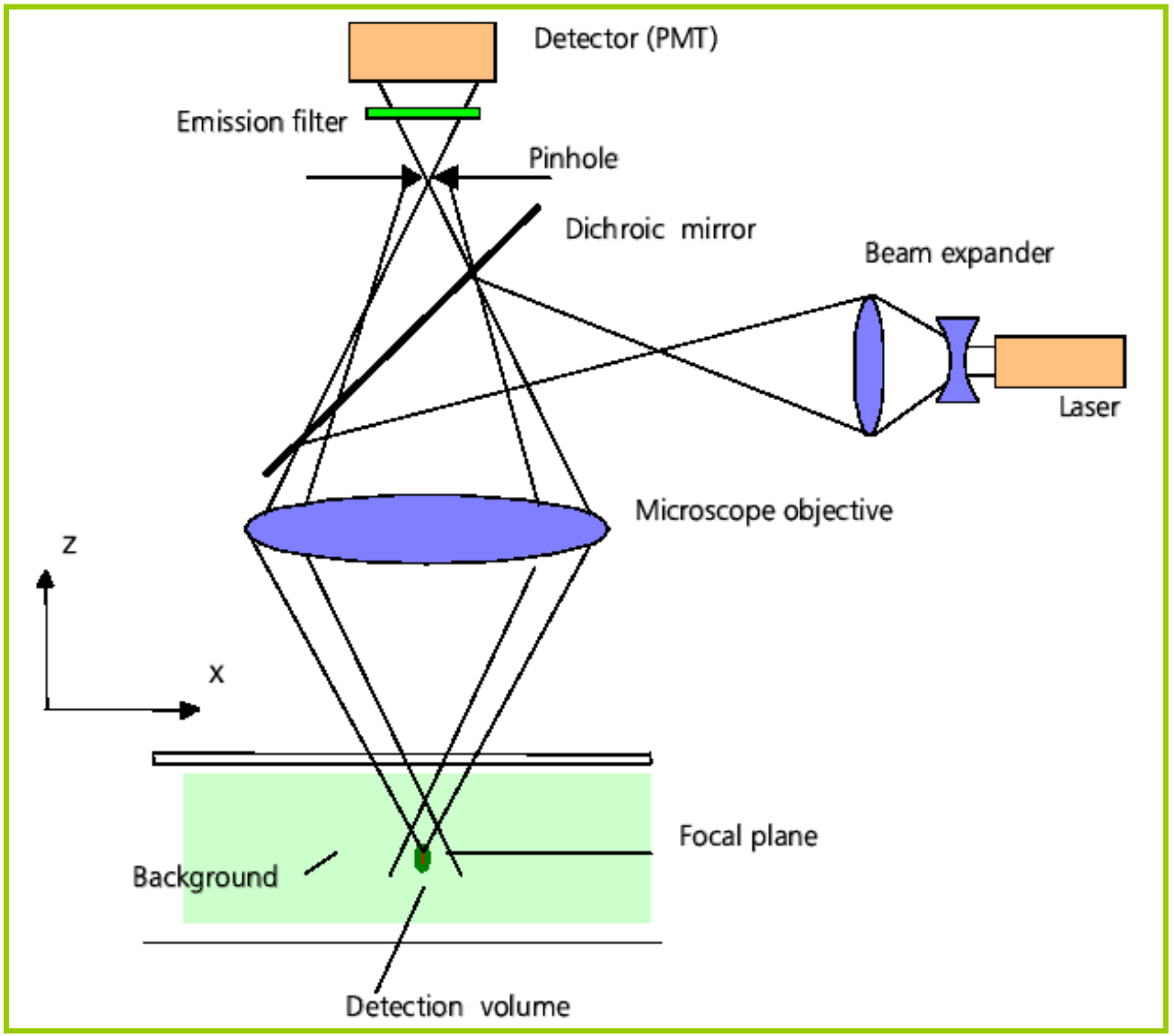


Absorción / Excitación

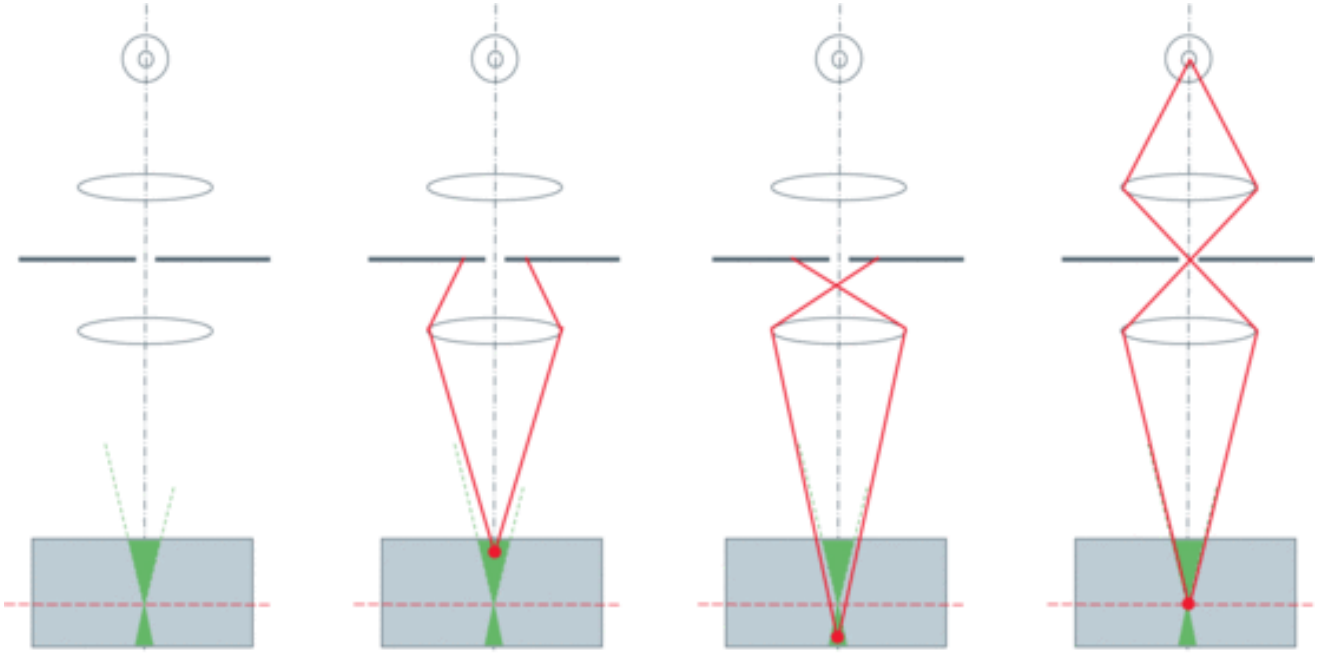


Emissión

|-> Diffraction limited Microscopy



|-> Diffraction limited Microscopy



Rebanada óptica en µm, modificable según Airy units del pinhole

http://www.leica-microsystems.com/science-lab/confocal-microscopy-optical-path/

From Geometric Optics to Diffraction Theory*:*

Diffraction: The deviation of an electromagnetic wavefront from the path predicted by geometric optics when the wavefront interacts with a physical object such as an opening or an edge.

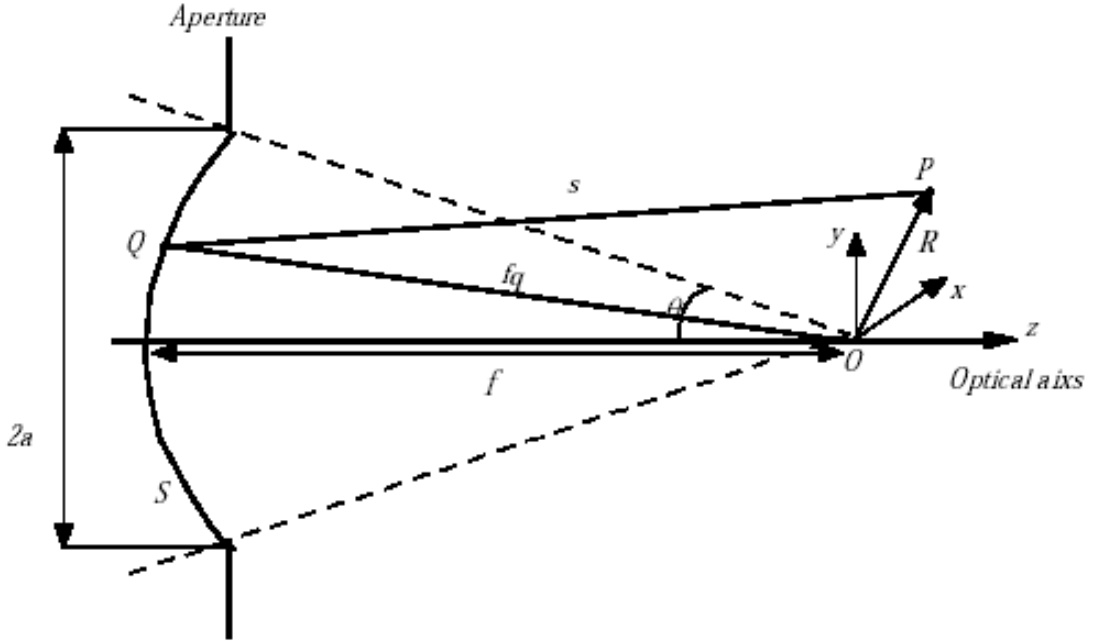


Figure 2.1 Diffraction of a converging spherical wave at a circular aperture

|-> Diffraction limited Microscopy

Óptica no-geométrica /

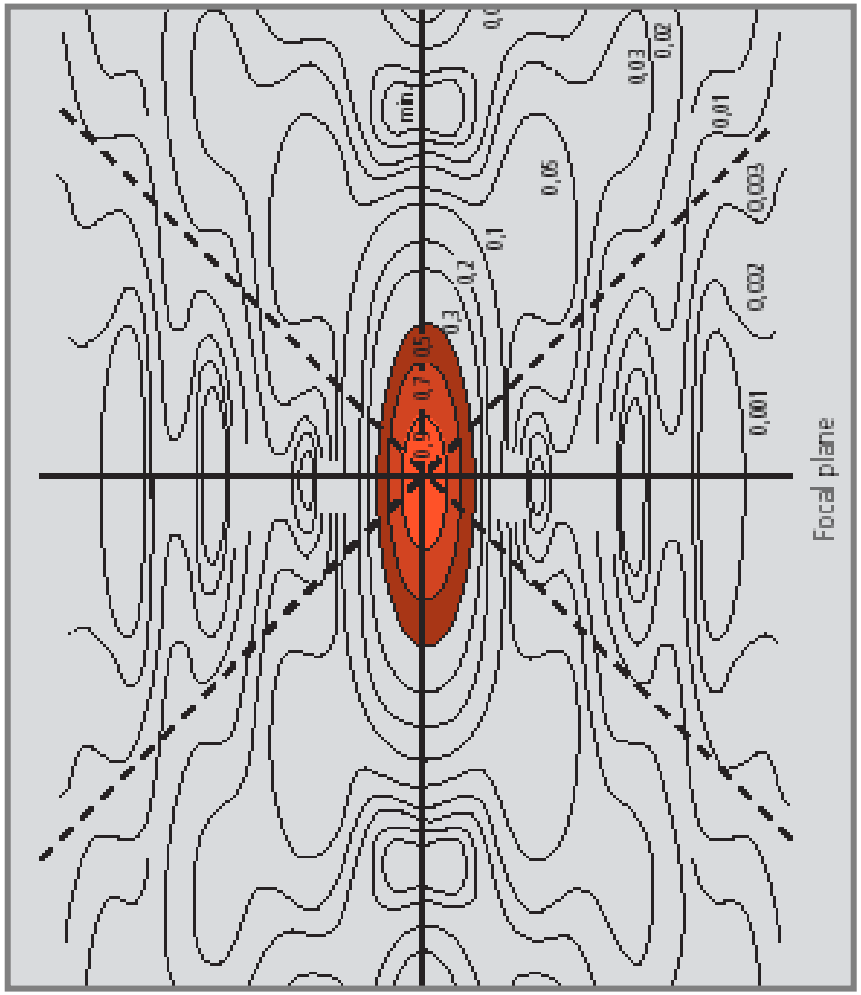
Teoría de difracción

PSF = $|U|^2 = f(J_0)$

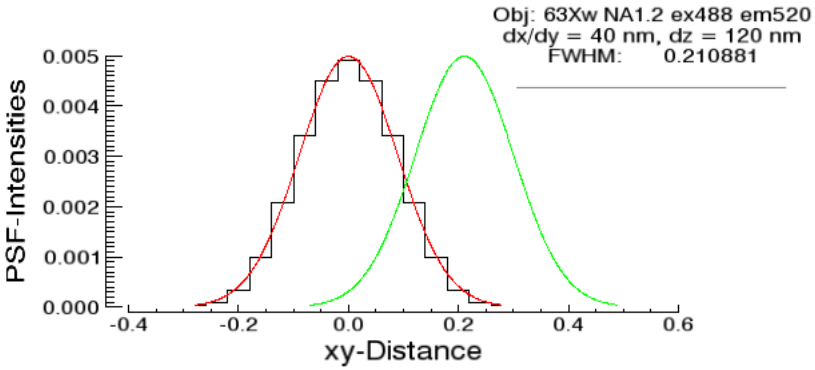
U, Integral de Difracción de Kirhoff

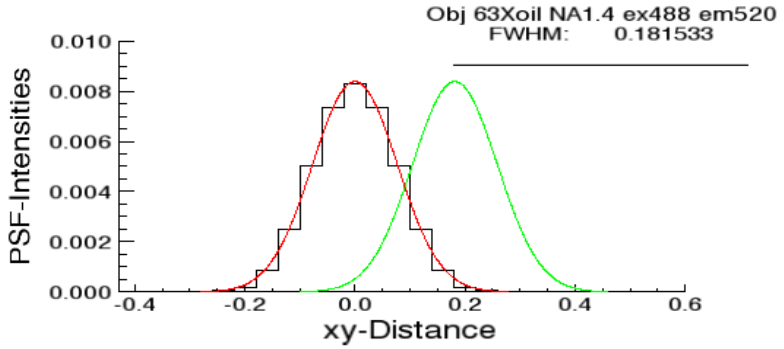
J_0, Serie de funciones de Bessel

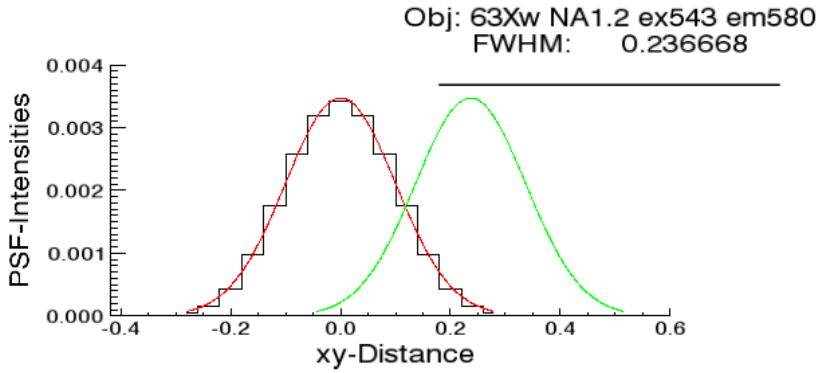
(Born & Wolf, Priniples of Optics, 6th edition 1988, Pergamon Press)

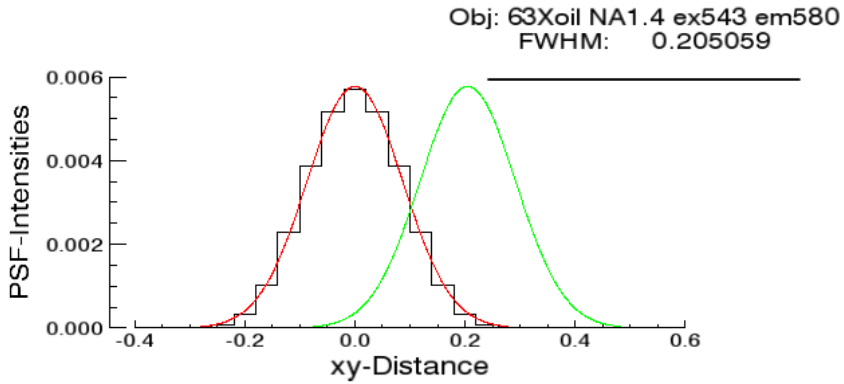


|-> Diffraction limited Microscopy

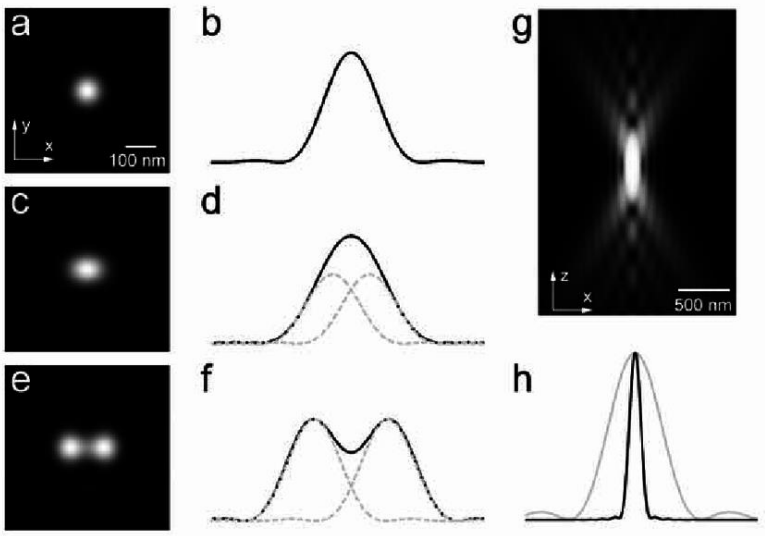




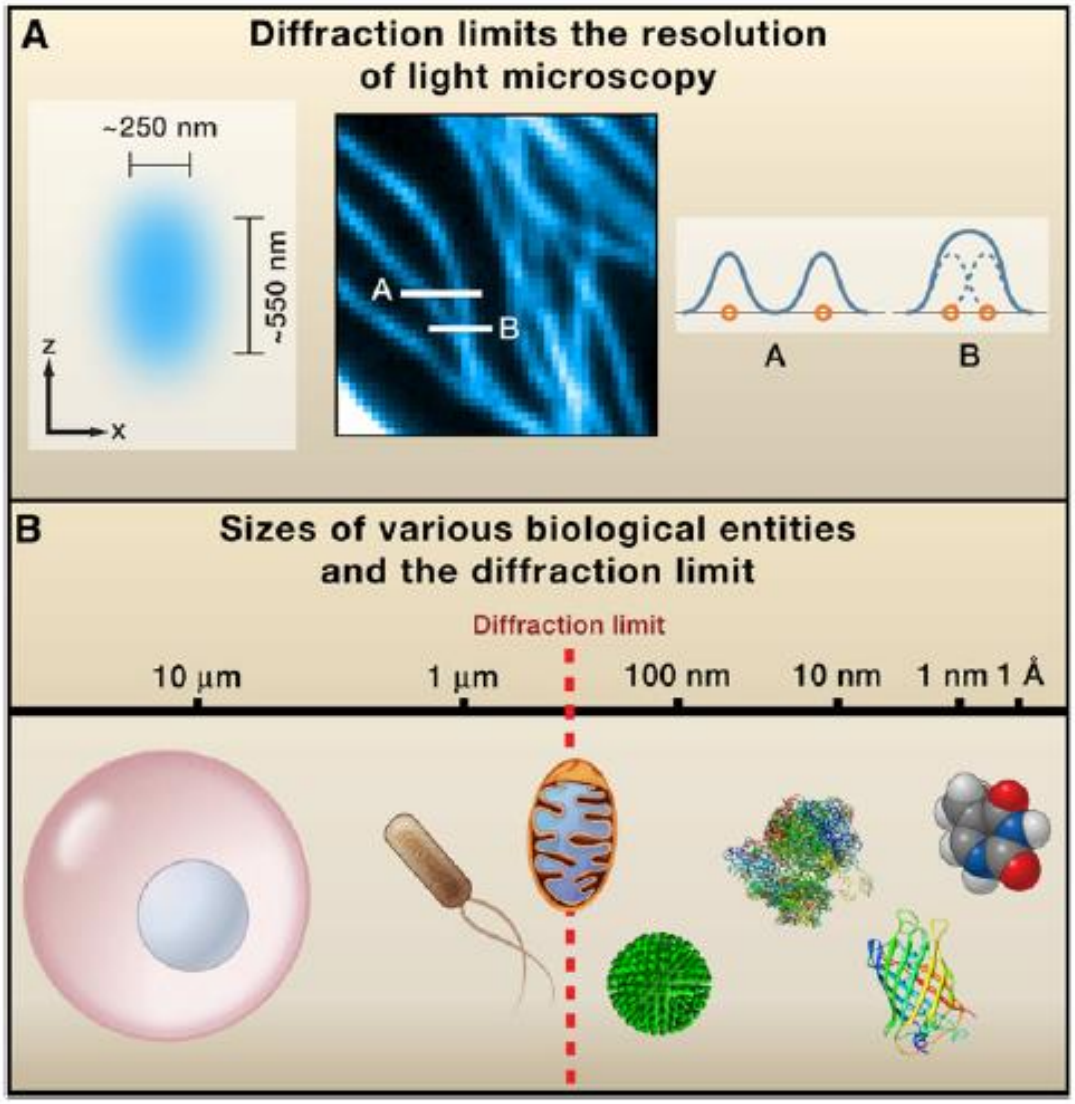




|-> Diffraction limited Microscopy



Bertocchi, Cristina & Goh, Wah & Zhang, Zhen & Kanchanawong, Pakorn. (2013). Nanoscale Imaging by Superresolution Fluorescence Microscopy and Its Emerging Applications in Biomedical Research. Critical reviews in biomedical engineering. 41. 281-308. 10.1615/CritRevBiomedEng.2014010448.



Bo Huang, Hazen Babcock, Xiaowei Zhuang, Breaking the Diffraction Barrier: Super-Resolution Imaging of Cells, Open Archive Published:December 17, 2010 DOI:https://doi.org/10.1016/j.cell.2010.12.002

|-> Beyond diffraction

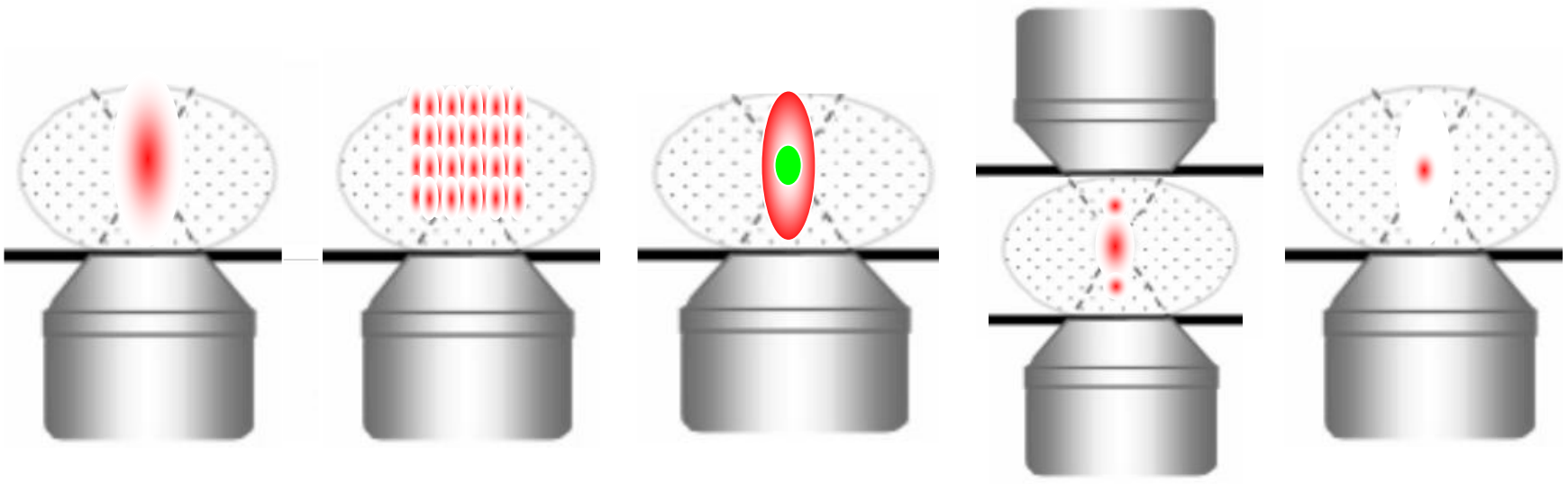
M Goeppert-Mayer
1906-1972

M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

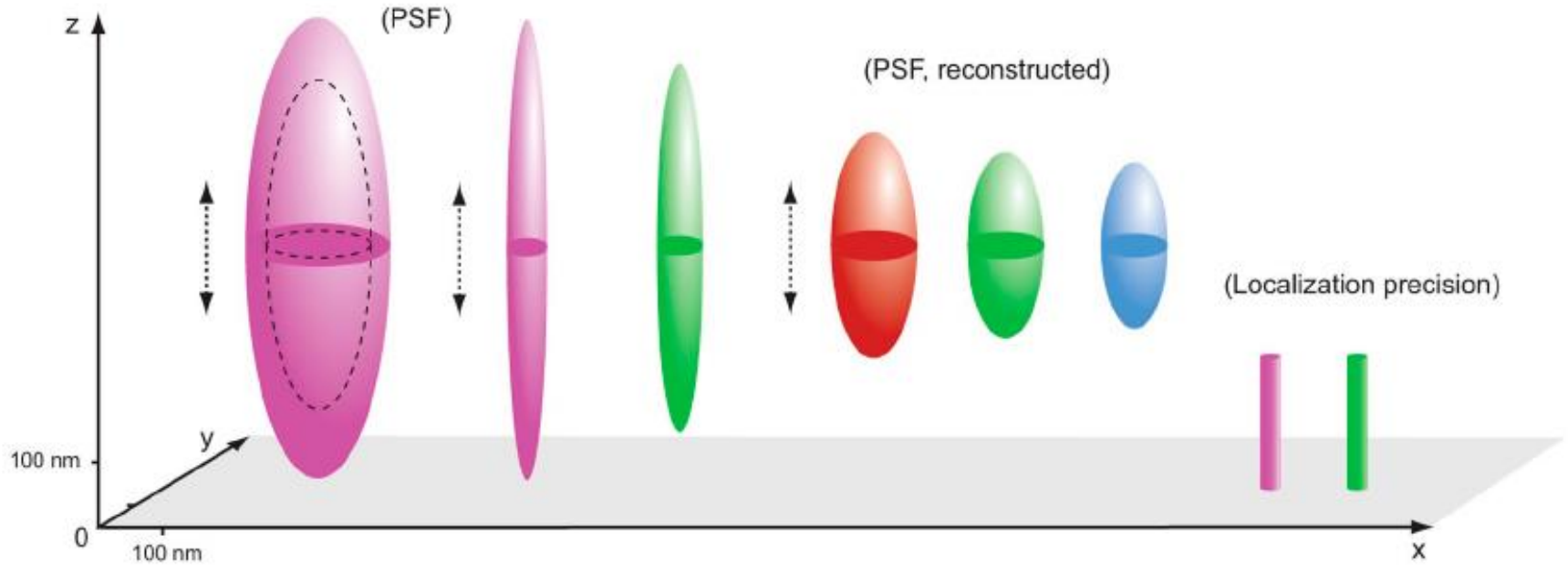
FWHM(xy) ~ λ/2 | ~ λ/4 | ~ λ/∞ | ~ λ/4 | ~ λ/100



2-photon | SIM | STED | 4-π | PALM

|-> PSF overview

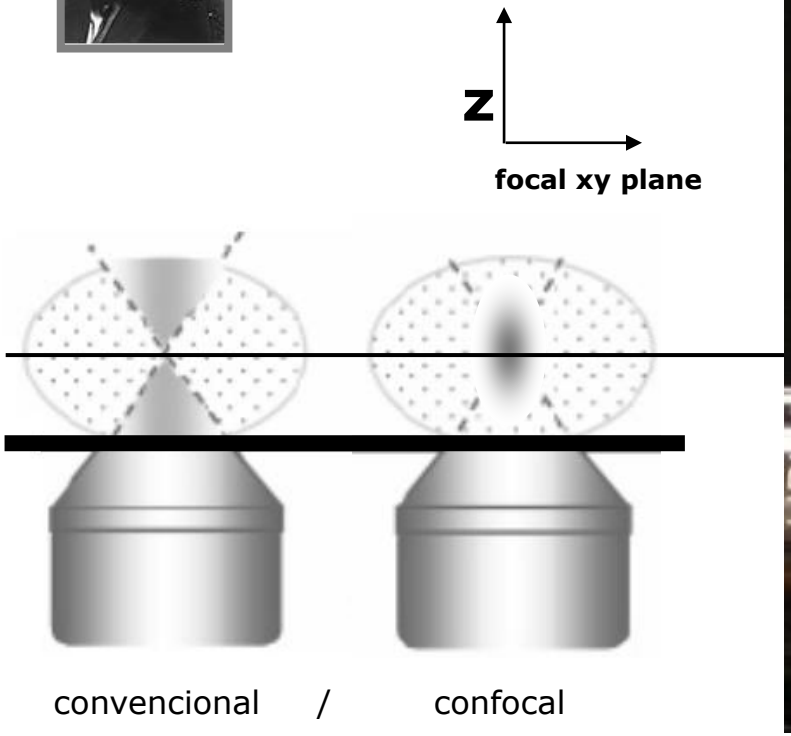
	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520

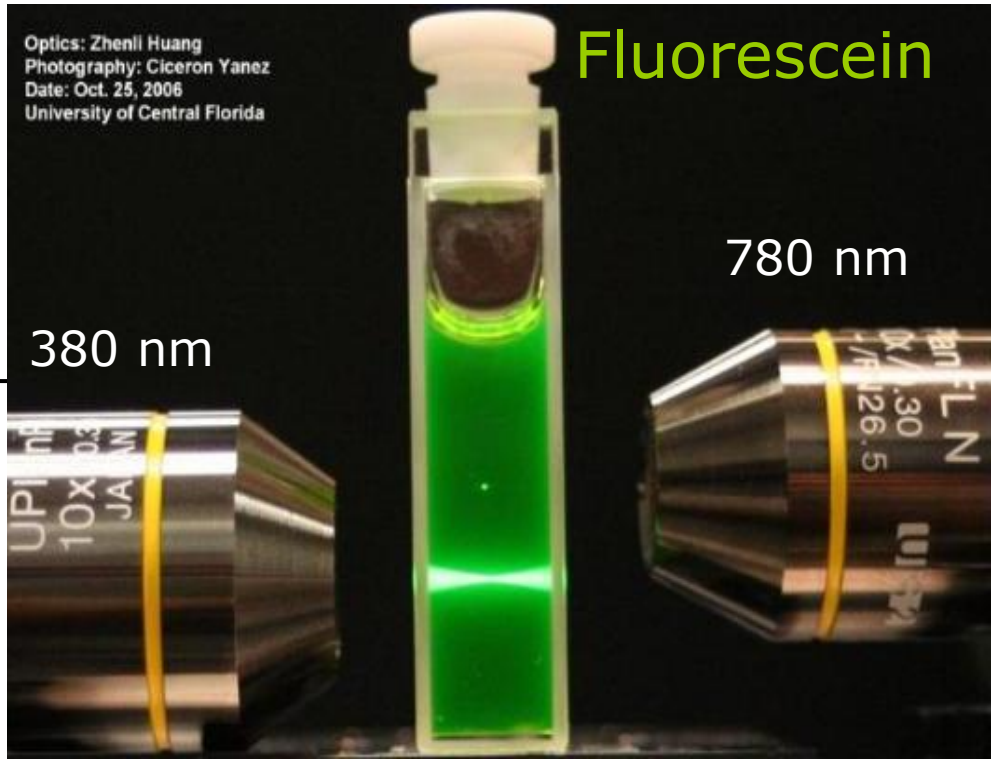


~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Schermelleh et al 2010 JBC

| Best localization: confocal microscopy



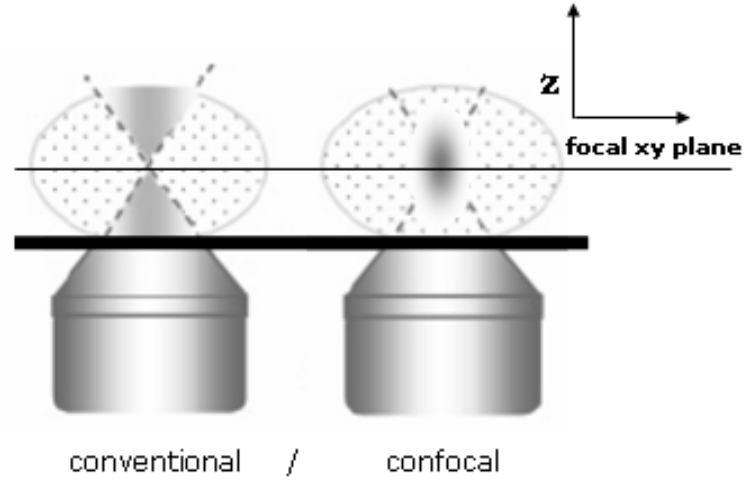


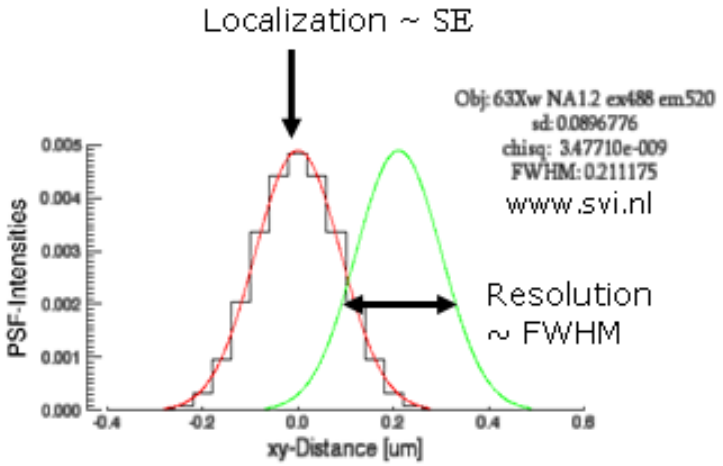
| Diffraction limited microscopy

E. Abbe († 1905)

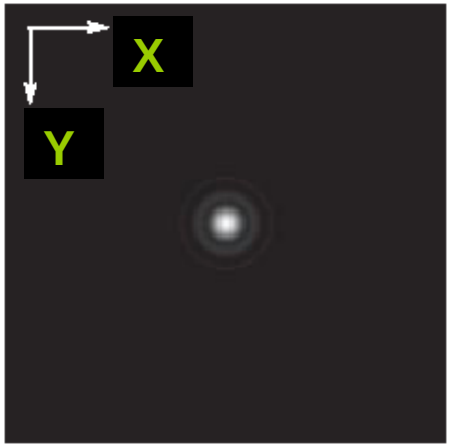
$\lambda / 2 \cdot NA \sim \lambda / 2$ Resolution (Full Width at Halft Maximum, FWHM)

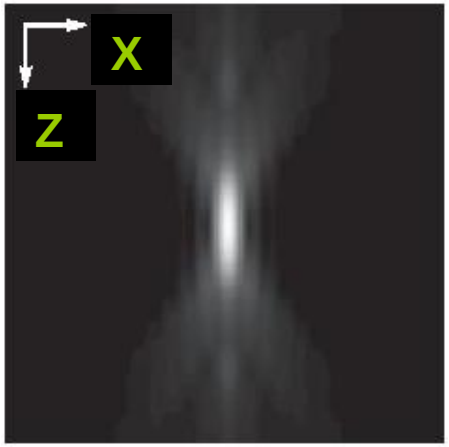
$FWHM / N^{1/2}$ Localization, N number of photons

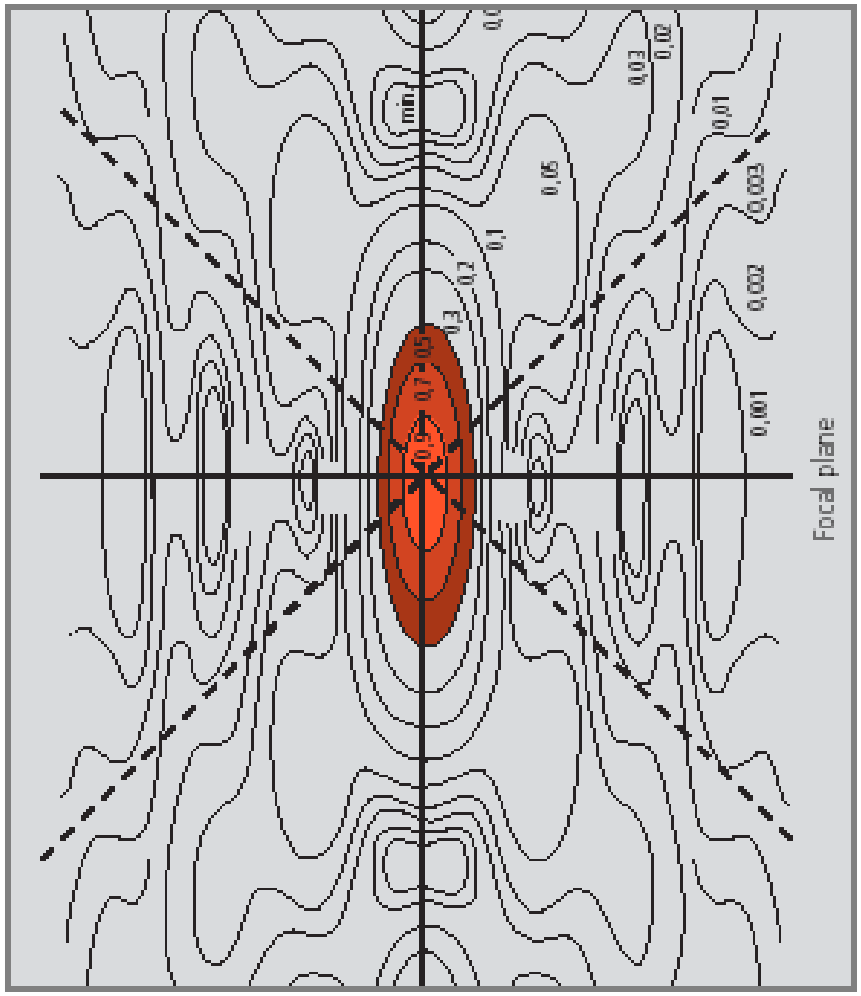




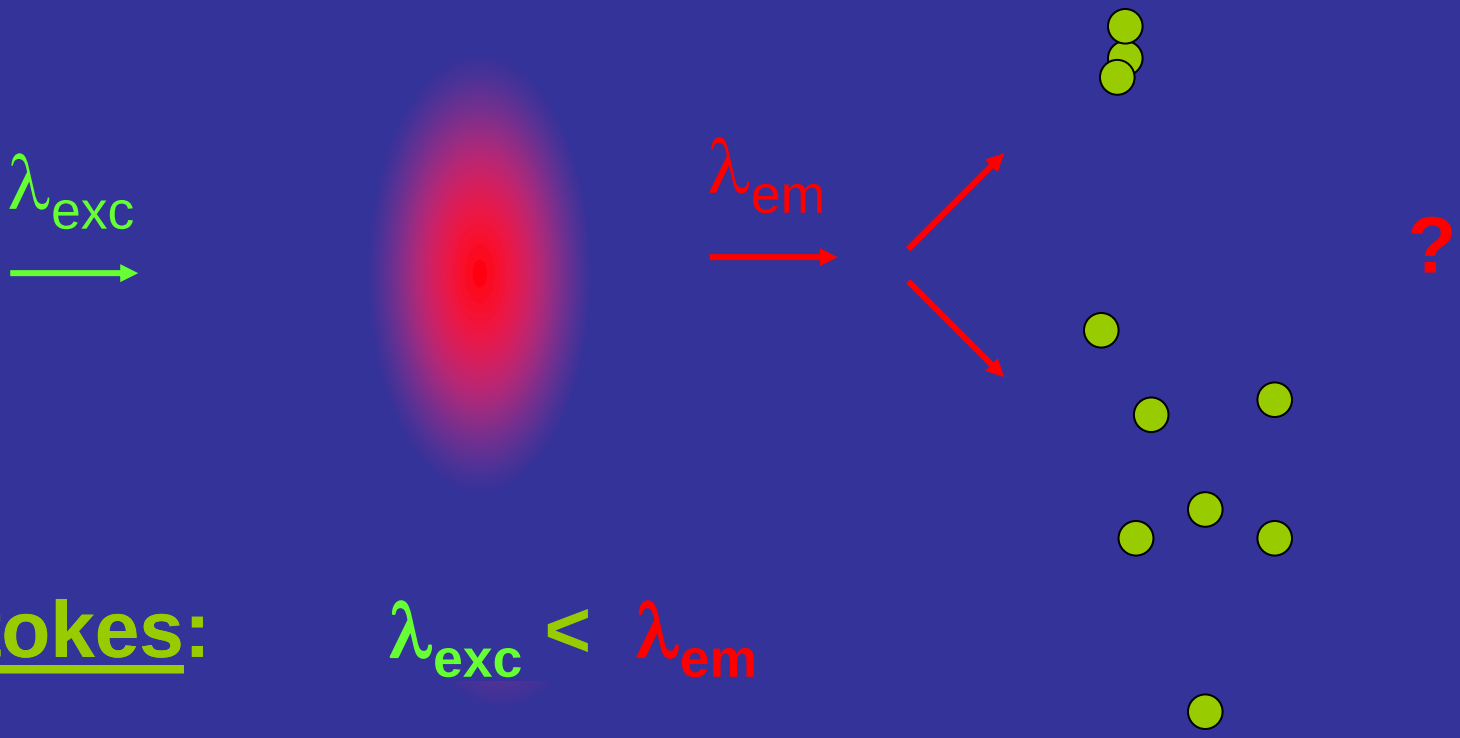
|-> PSF





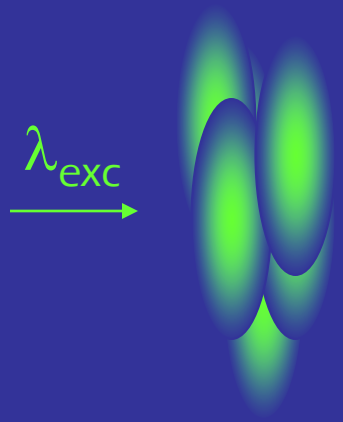


|-> Convolution



Stokes: $\lambda_{exc} < \lambda_{em}$

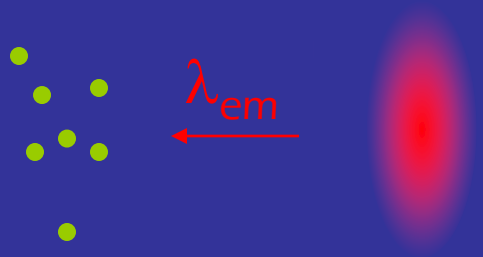
|-> Convolution



λ_{exc}

Stokes: $\lambda_{exc} < \lambda_{em}$

$n(\lambda_{exc}) > n(\lambda_{em})$



λ_{em}

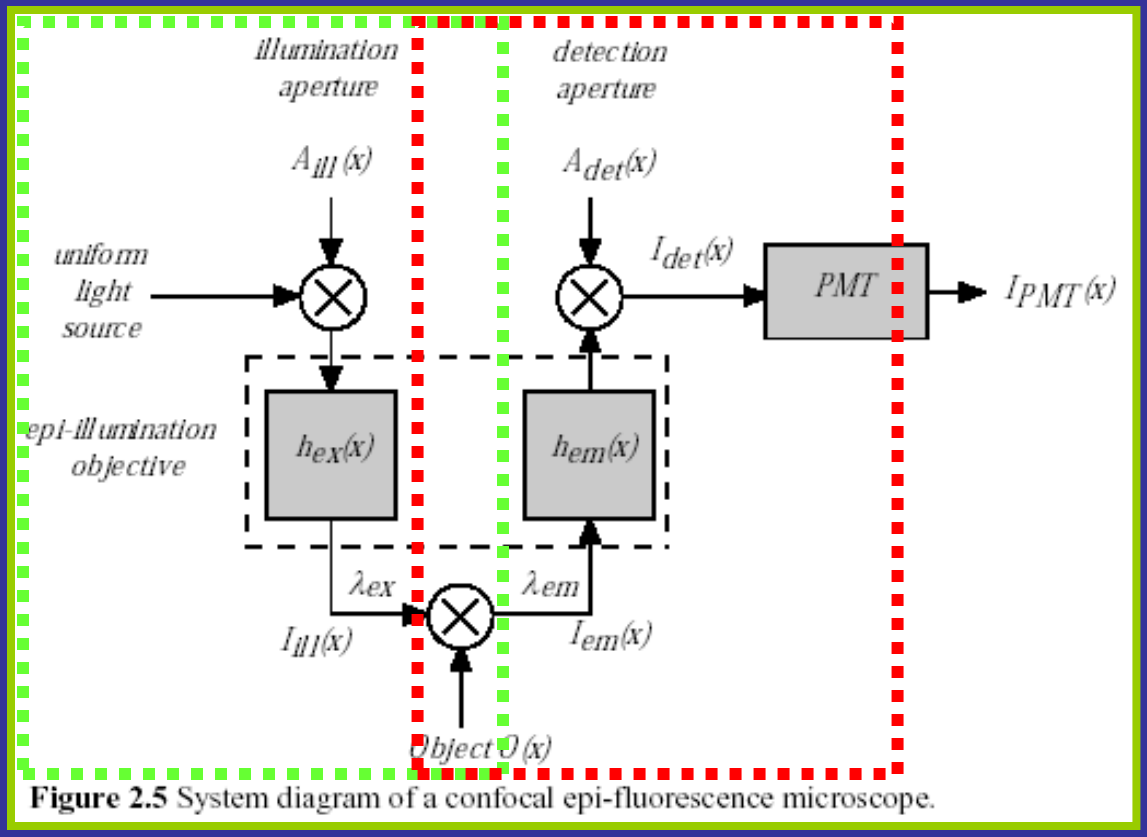
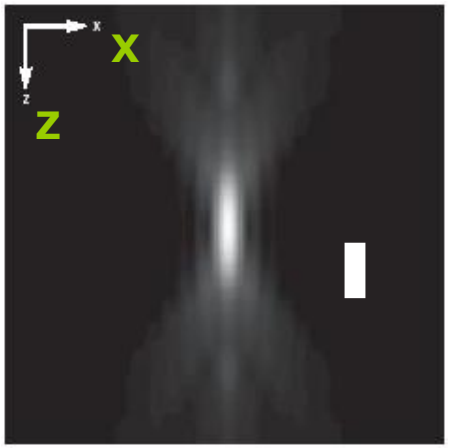


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

|-> Convolution



PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: Point Spread Function

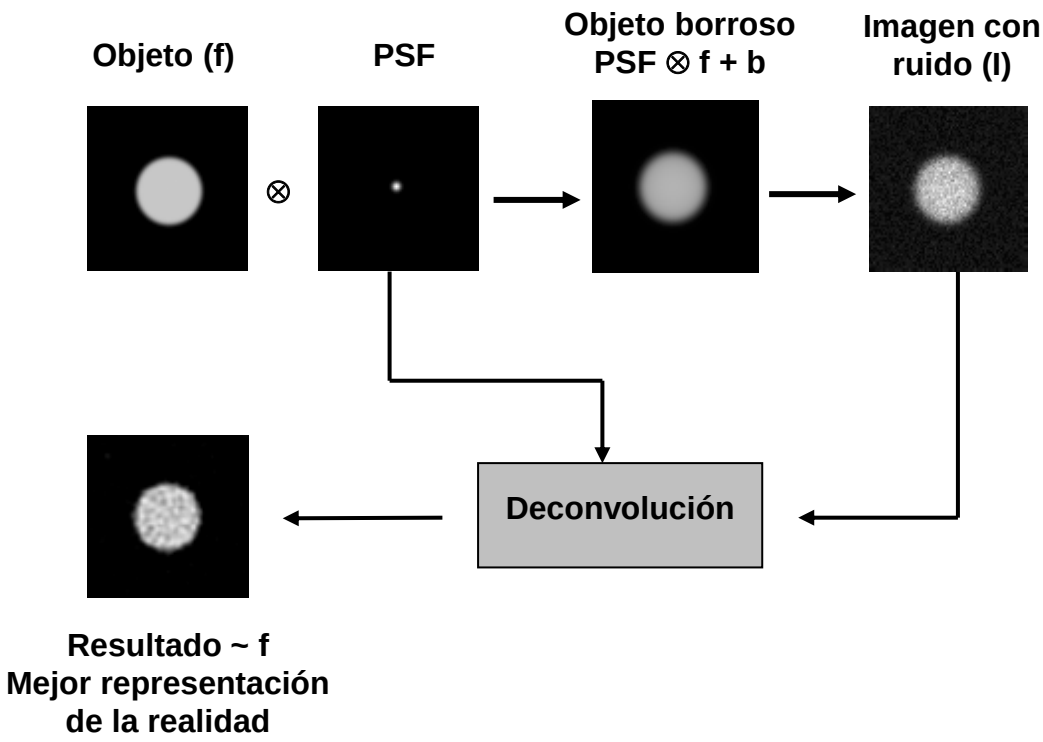
f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$



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|-> Deconvolution

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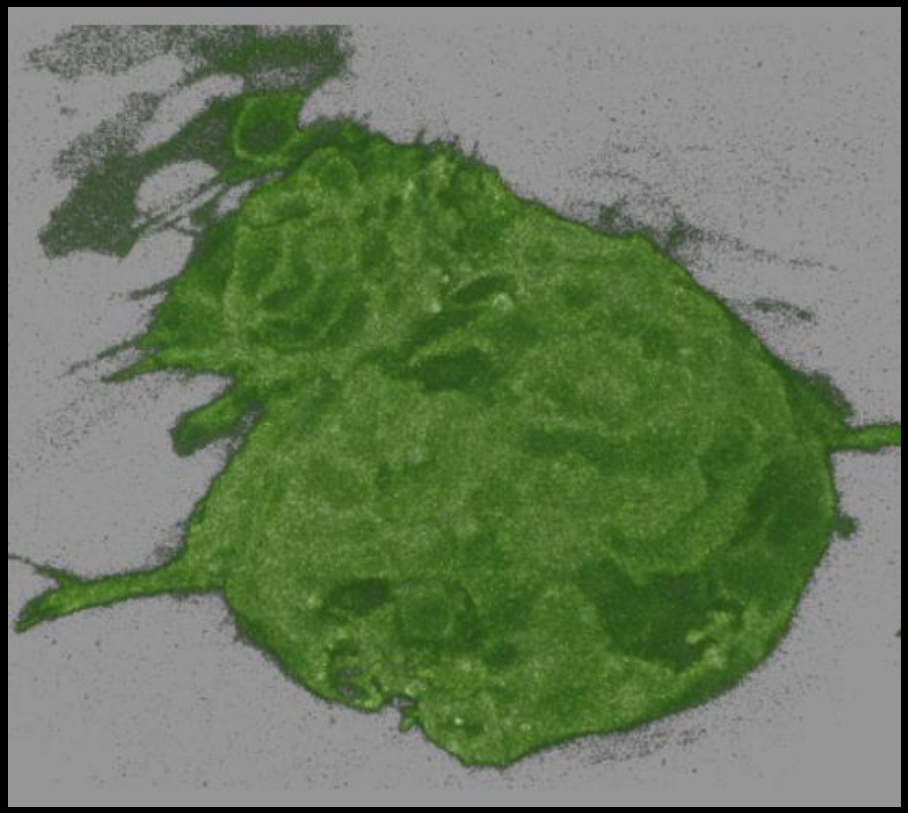
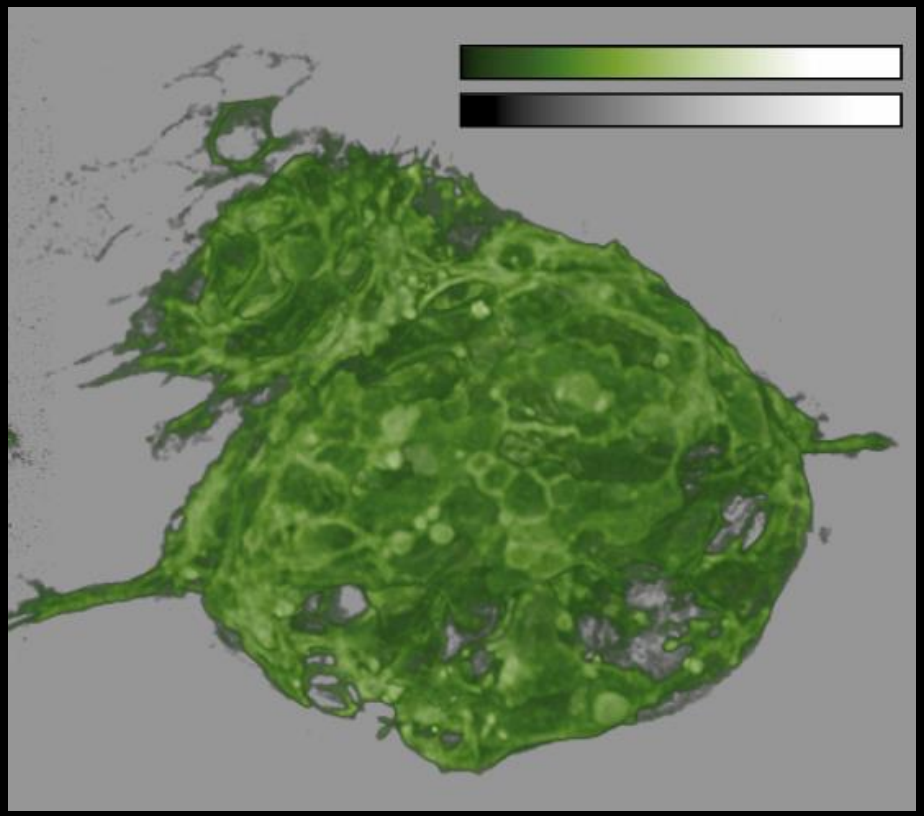
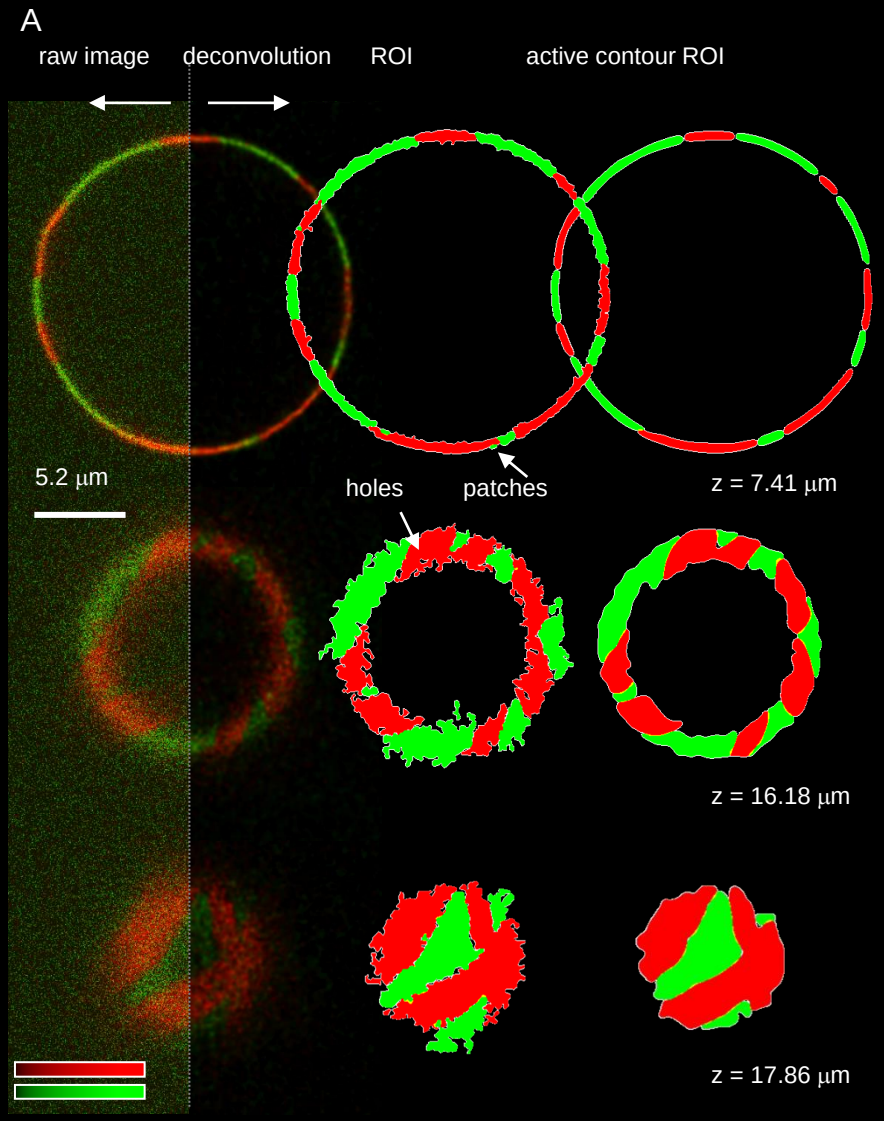
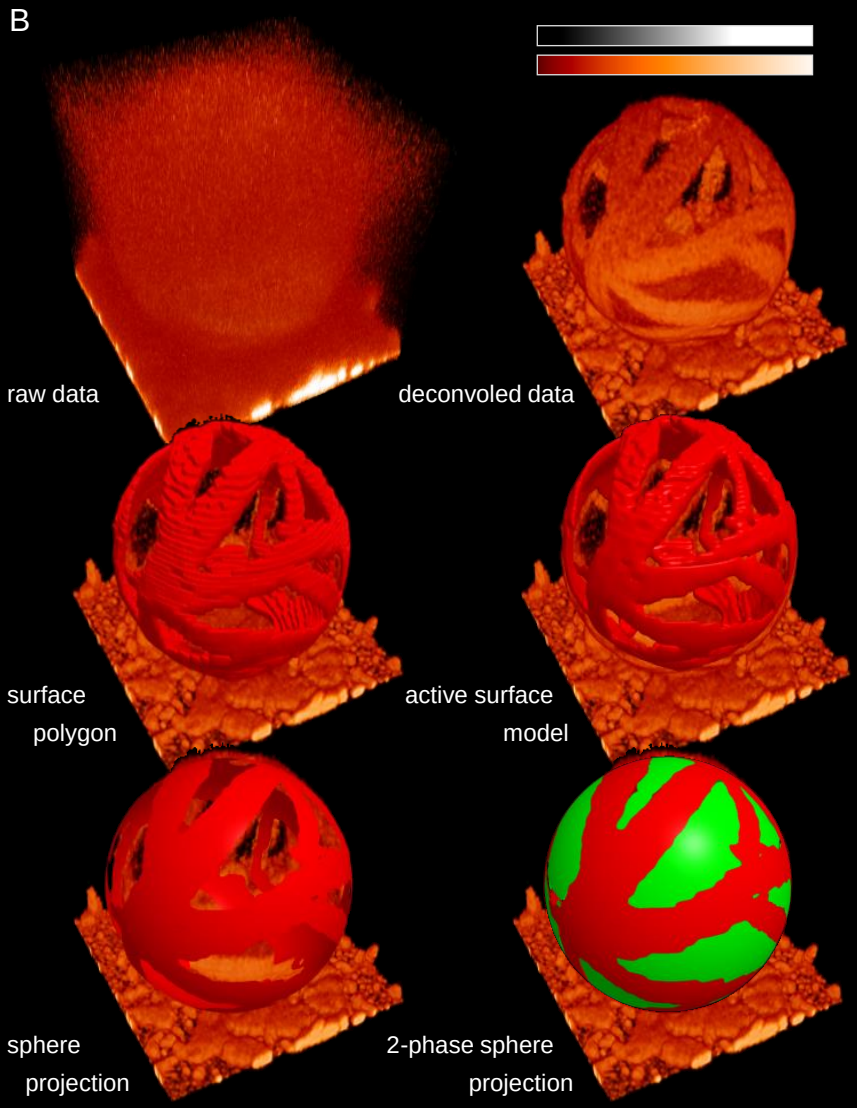




|-> Deconvolution





|-> Deconvolution

PSF: Point Spread Function

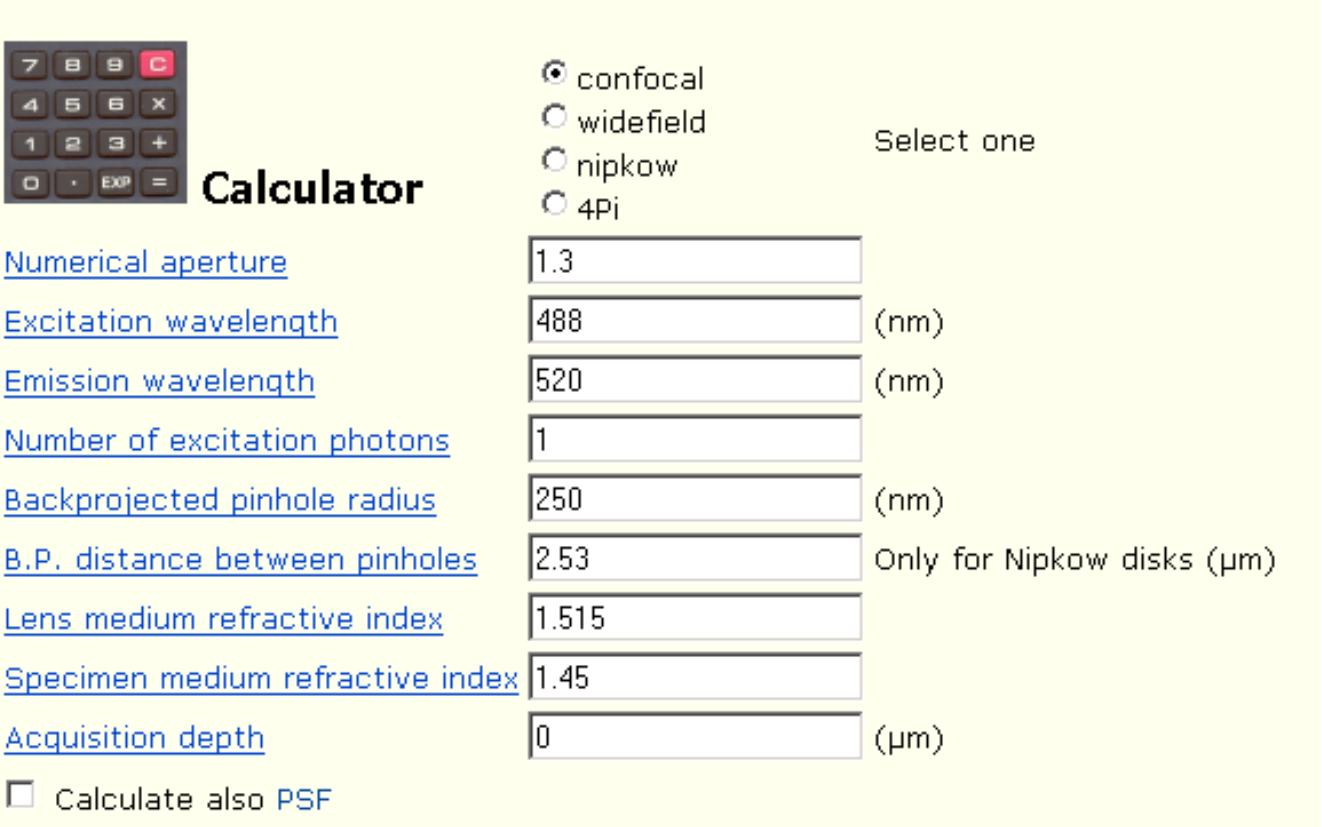
f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$



http://support.svi.nl/wiki/NyquistCalculator

|-> Pinhole

PSF: Point Spread Function

f: Object Function

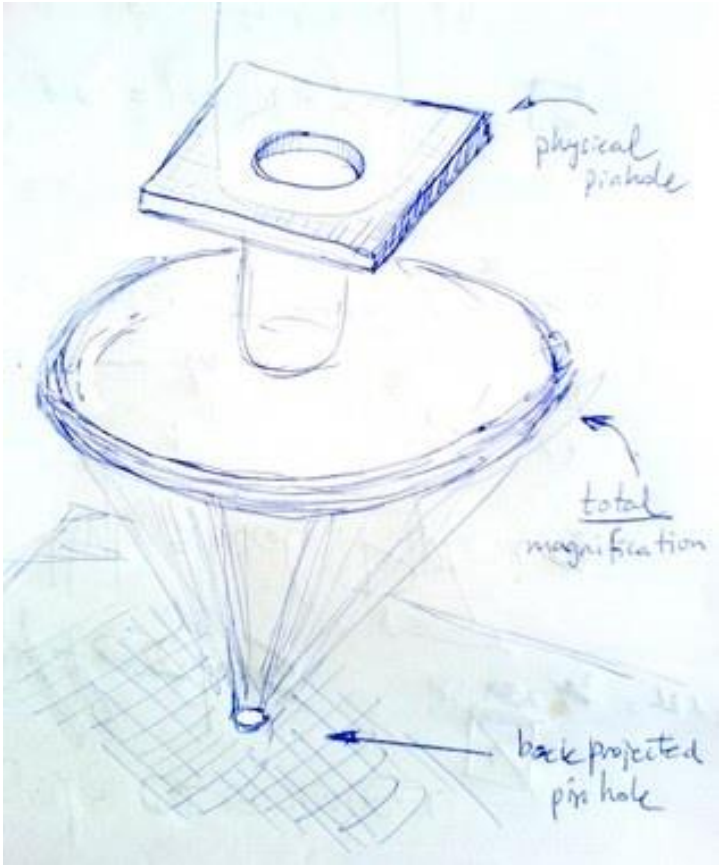
b: Offset Function

I: Image Matrix

N: Noise Function

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

Backprojected confocal pinhole



http://support.svi.nl/wiki/NyquistCalculator

|-> Pinhole

PSF: Point Spread Function

f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

Biorad

- Biorad MRC 500, 600 and 1024
- Biorad Radiance

Leica

- Leica confocals TCS 4d, SP1 and NT
- Leica confocal SP2
- Leica confocal SP5

Nikon

- TE2000-E with the C1 scanning head

Olympus

- Olympus FV300
- Olympus FV500
- Olympus FV1000

Zeiss

- Zeiss LSM410 inverted
- Zeiss LSM510

http://support.svi.nl/wiki/NyquistCalculator

|-> Noise

Literature: eg. Noise Theory and Application to Physics: Philippe Réfrégier, Springer

|-> Noise

PSF: Point Spread Function

f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

- *Black Body Irrdiation (Poisson)*
- *Detector Noise (Gauss)*

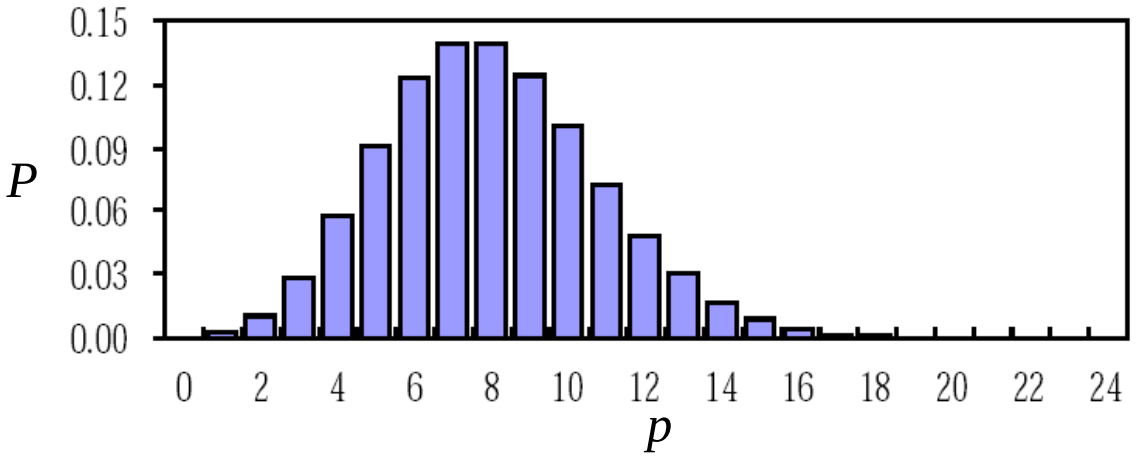
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

$$P(p, \mu) = \frac{\mu^p}{p!} \cdot e^{-\mu}$$

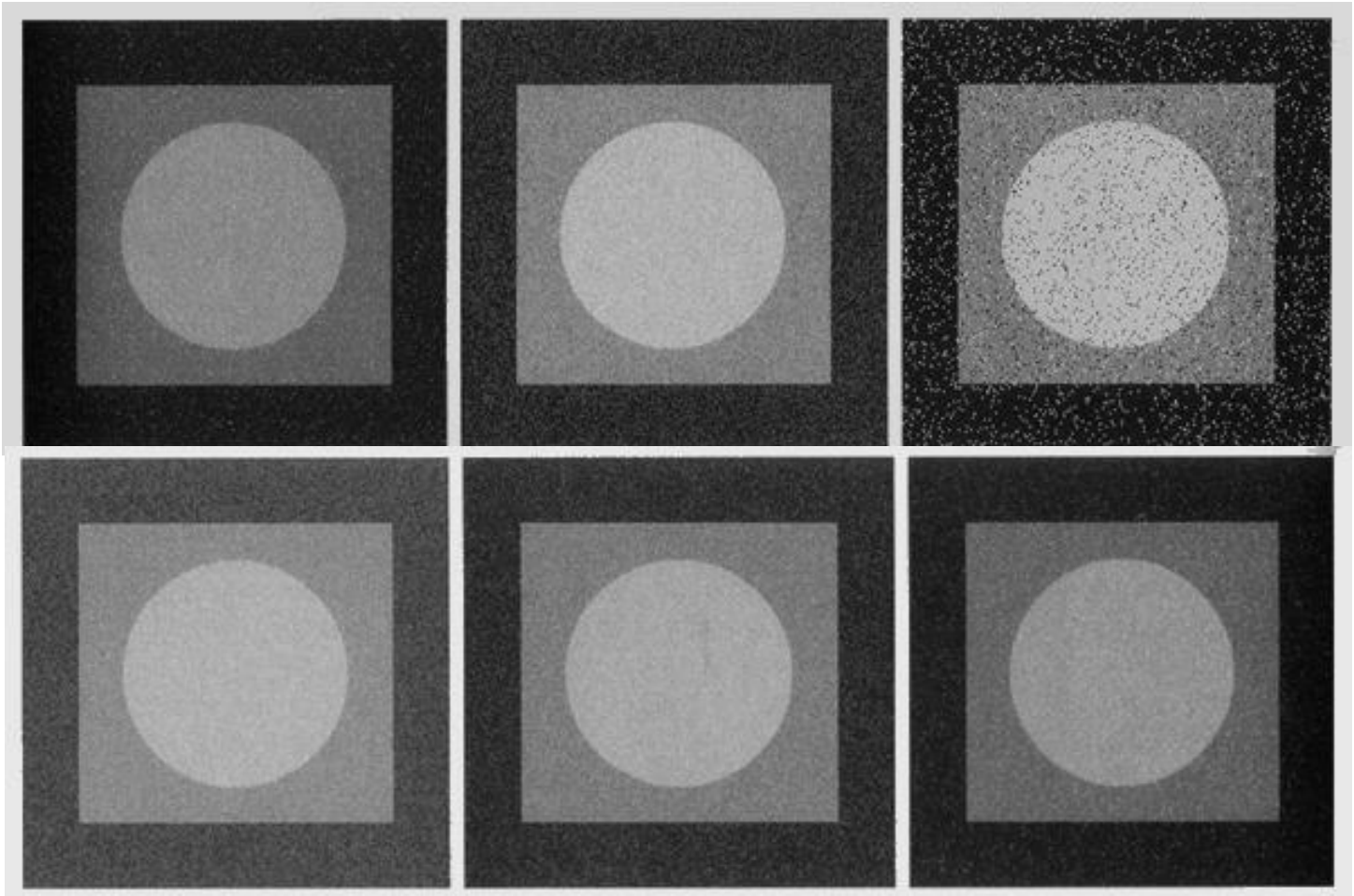
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

$$2.\ counting:\ \bar{p} \pm \sqrt{\bar{p}}$$

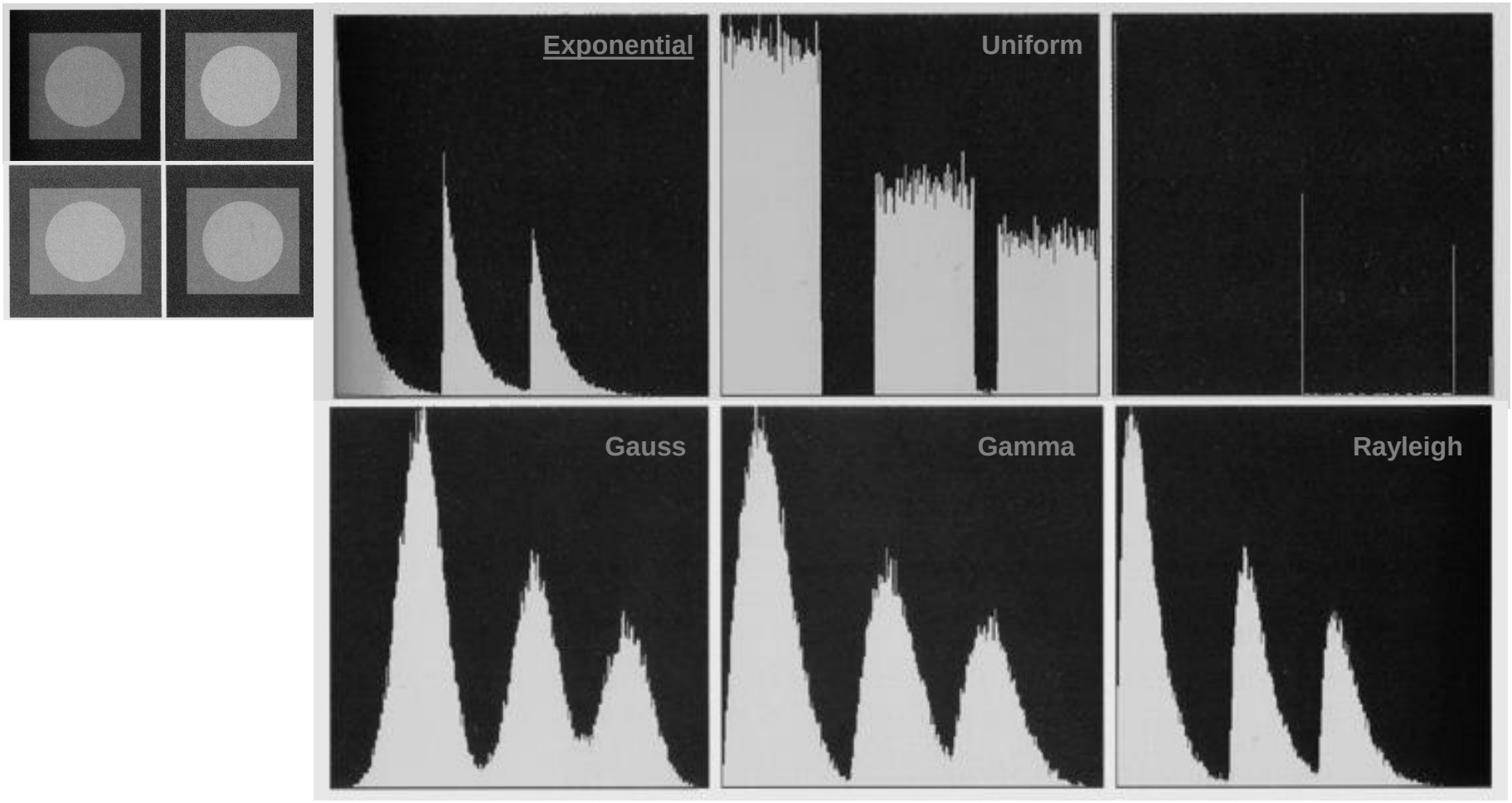
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> Noise



|-> Noise



The Signal to Noise ratio (SN) is a number not always easy to estimate. The easiest way to obtain some fi gureis to look at the textures of bright areas in your object image. In the fi gure at left you see examples of such textures obtained from originally the same object image to which various levels of poisson noise were added.

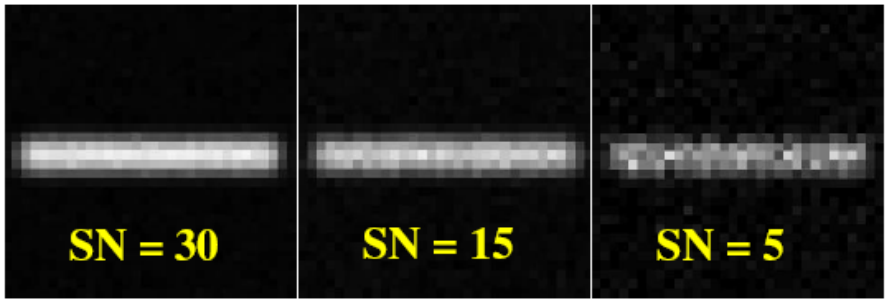
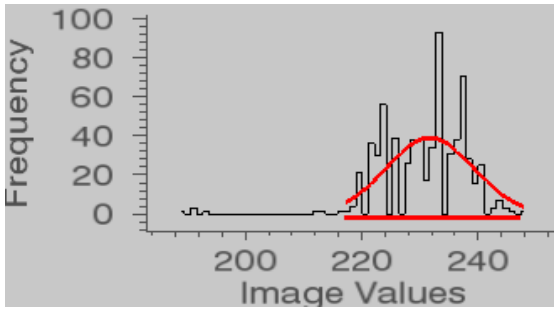
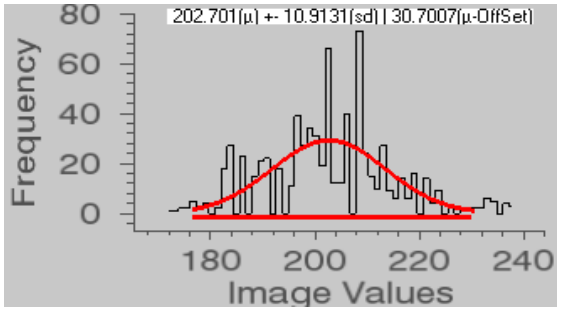
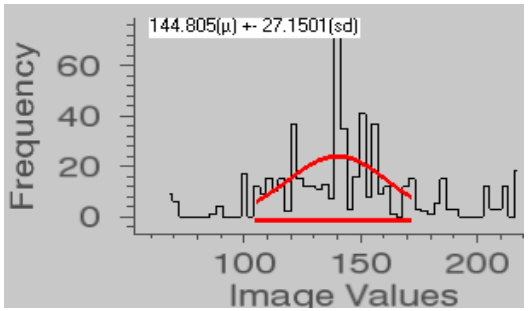


Figure 9. Images with different generated noise levels





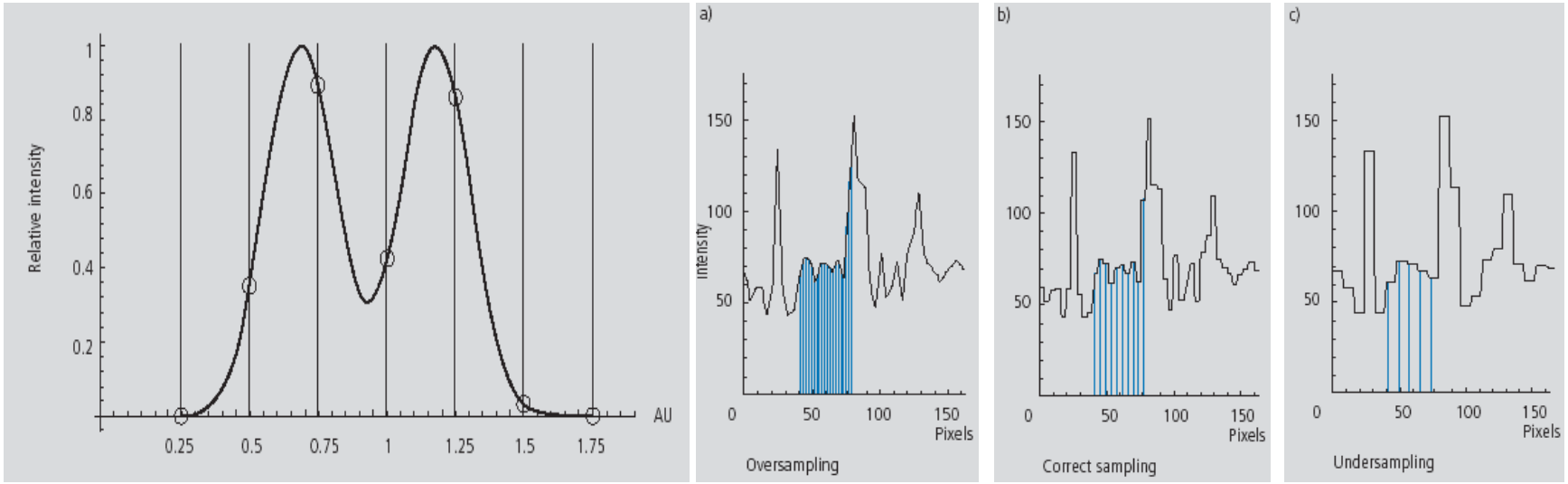


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{200}{10}$$

$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{139}{27}$$

|-> Nyquist /Shannon Theorem



- Undersampling looses structures.
- Oversampling waists memory/computation time.

The 'Nyquist /Shannon Theorem' or 'Sampling Theorem' for the digital sampling of analogue signals suggests a Nyquist rate NR $\geq 2\nu$?

! Diffraction theory calculates lateral NR ~ 20 pixel/µm(~50 nm/pixel) !
... laxial NR ~ (~150 nm/pixel)

|-> PSF calculator

PSF: Point Spread Function

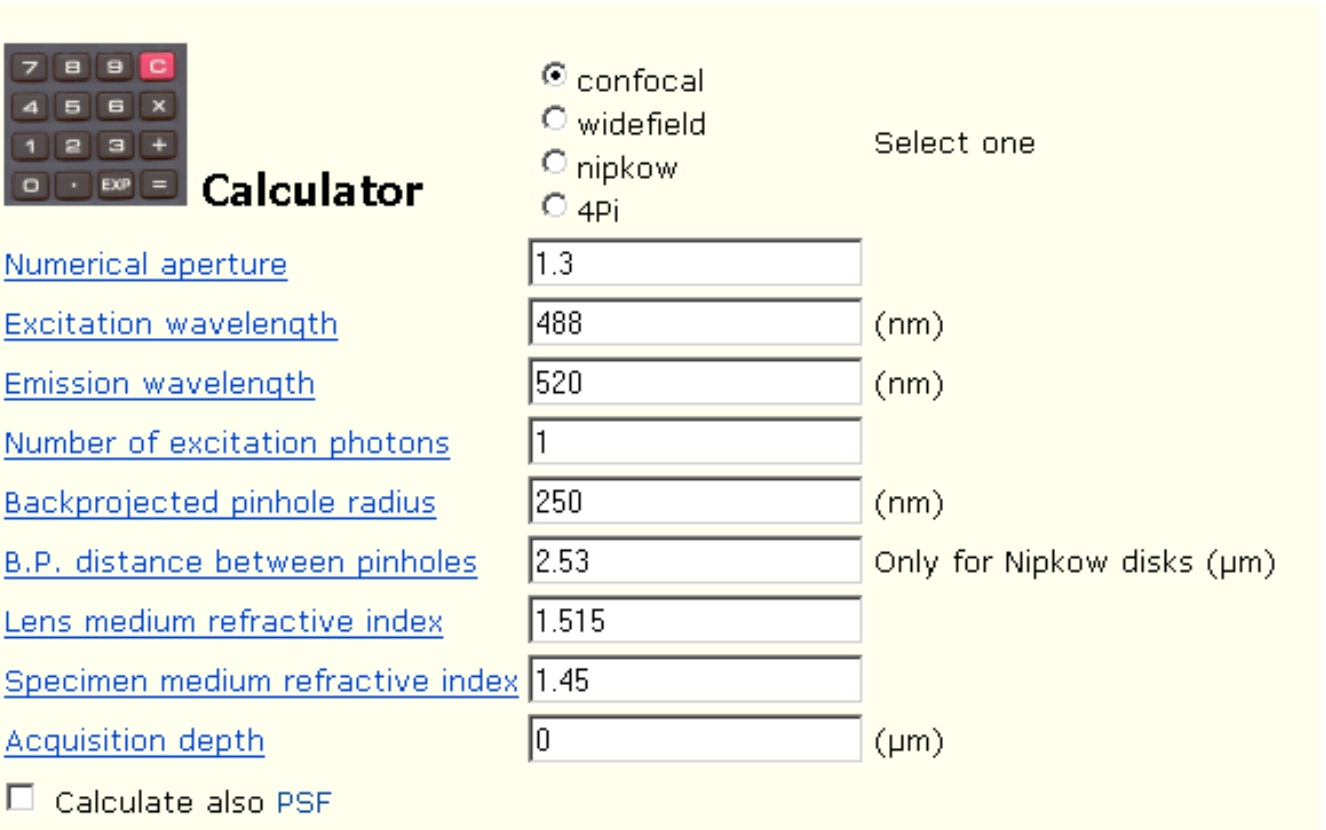
f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$



http://support.svi.nl/wiki/NyquistCalculator

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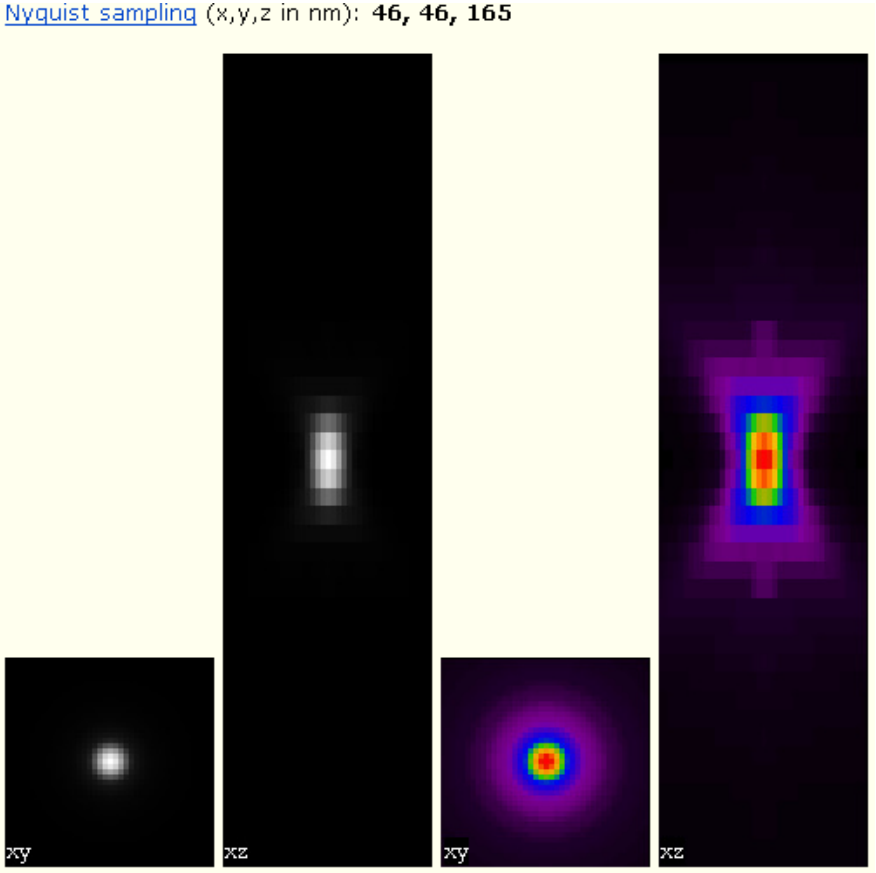


http://support.svi.nl/wiki/NyquistCalculator

|-> Snell's Law

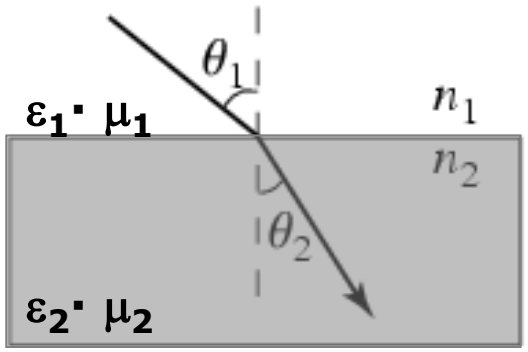
Index of refraction: $n = (\varepsilon \cdot \mu)^{1/2} = c/v$,

ε electric permittivity and μ magnetic permeability.

Snell's Law:

$\sin\Theta_1 \, n_1 = \sin \Theta_2 \, n_2$

- **1.518 [Zeiss Oil]**
- **1.33 [Water]**
- **1.0008 [Air]**



|-> Refractive index

Refractive Index:

$RI = n_1/n_2 = v_2/v_1$

Snell's Law:

$\sin\Theta_1 \, n_1 = \sin \Theta_2 \, n_2$

$n = n(\lambda)$!

- 1.518 [Zeiss]
- 1.33 [Water]
- 1.0008 [Air]

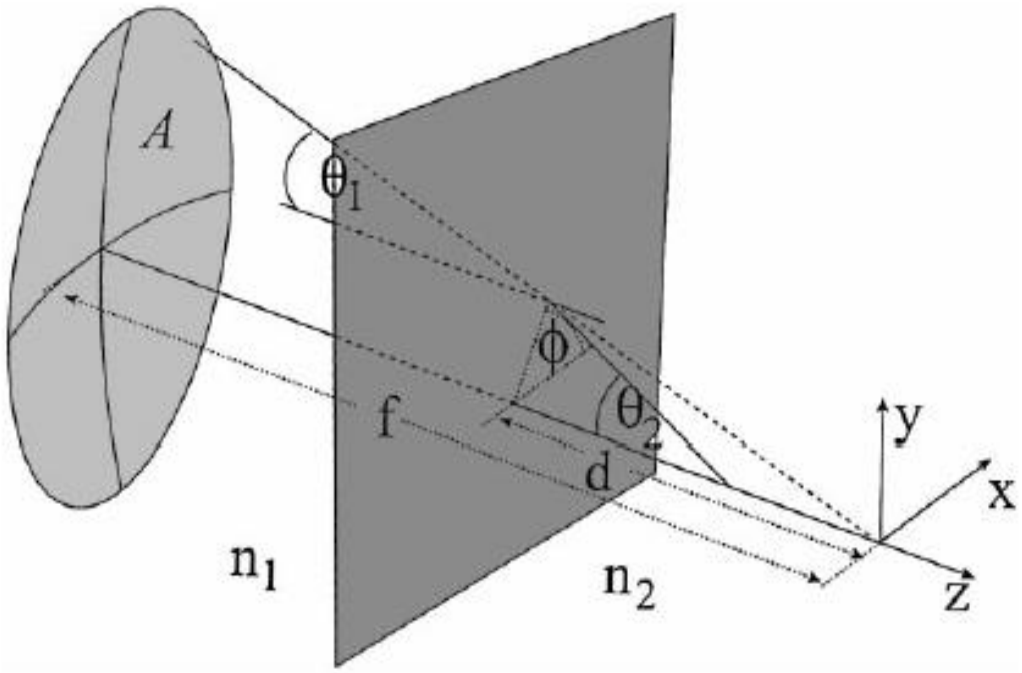
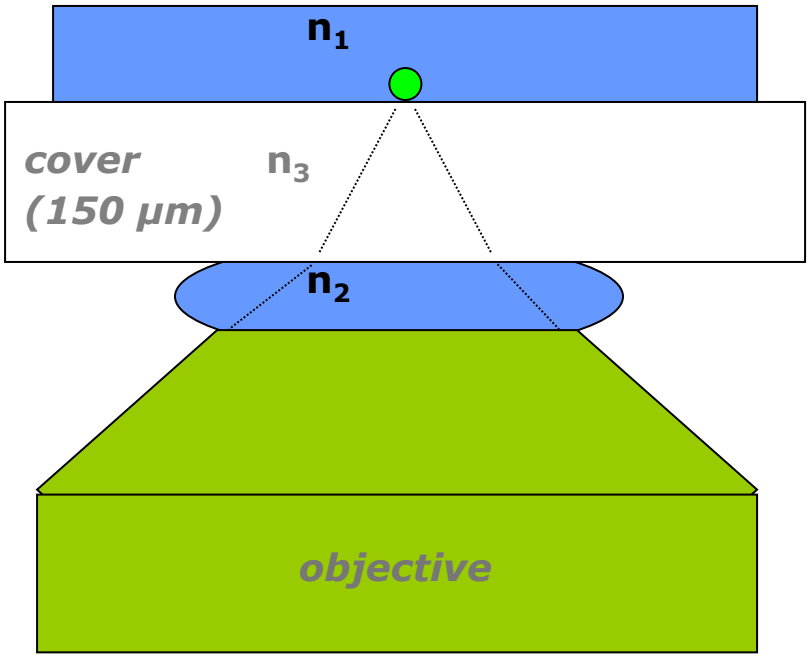


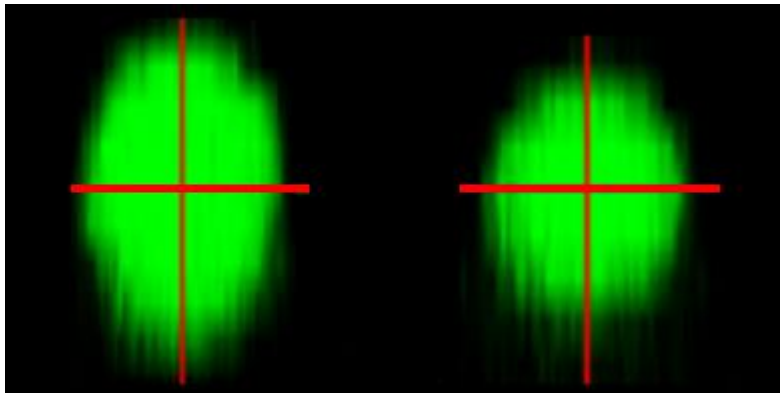
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera:** ∅ = 6 µm





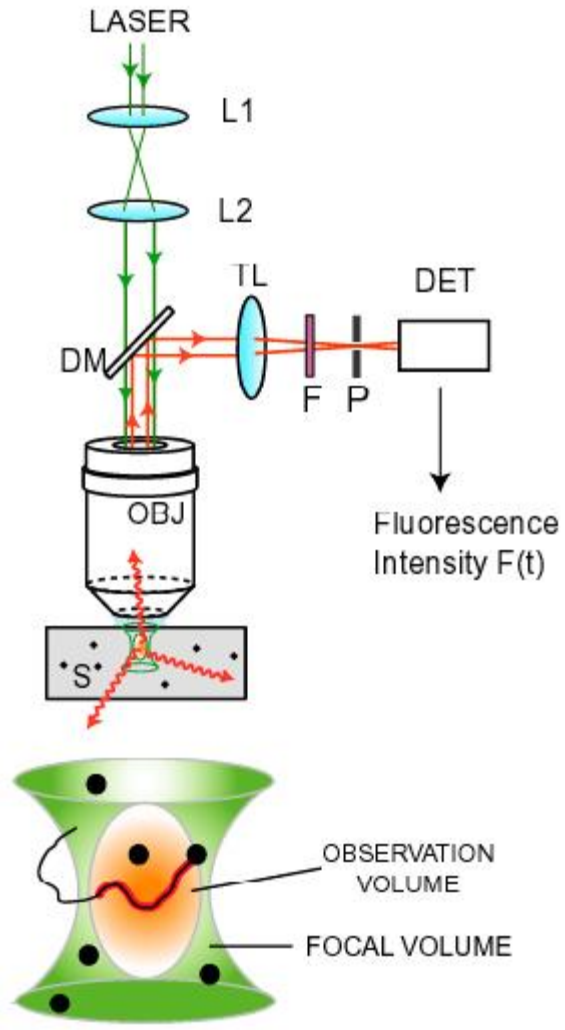
agua/aceite -- *aceite/aceite*

$n_1 \neq n_2$ $n_1 = n_2$

Ley de Snell: $n_i \cdot \sin\Theta_i = n_k \cdot \sin\Theta_k$

$n = n(\lambda)$!

|-> Resumen I



The observation volume (femtoliter) defined by the Point Spread Function must be considered as a mini-sprectrofluorimeter.

1. You need to consider the Offset I(0) in order to calibrate your signal $I(0) \geq 0$!
2. Never saturate the signal: $I \leq I_{max}$ (255 for 8 bit) !