Welcome

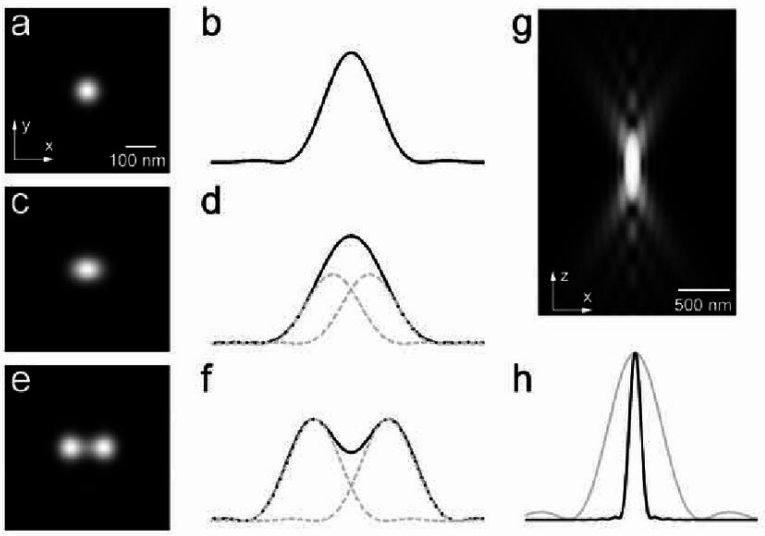
Prof. Dr. Steffen Härtel

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

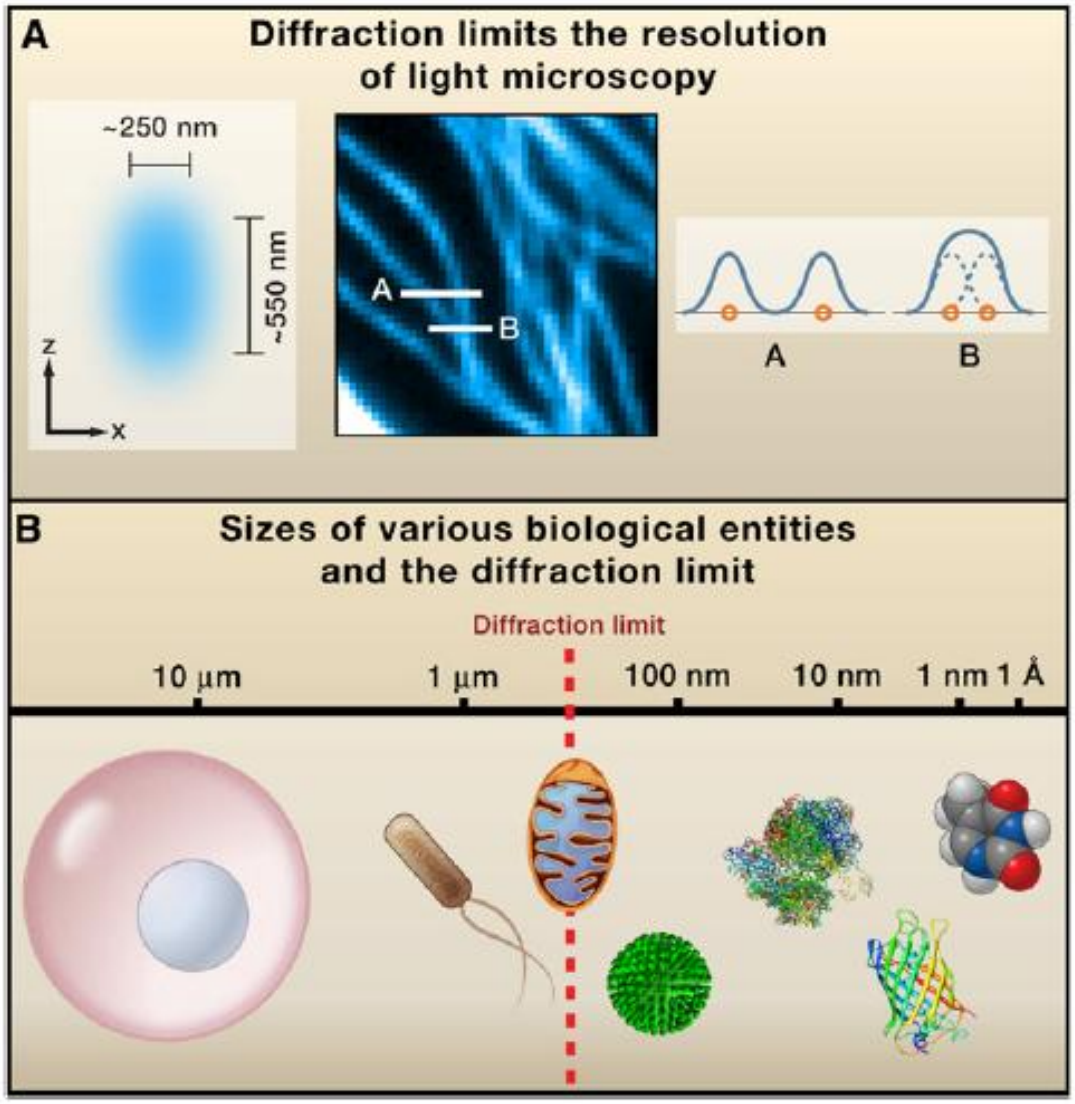
Laboratory for Scientific Image Analysis (SCIAN-Lab)
Centro de Informática Médica y Telemedicina (CIMT)
Centro Nacional en Sistemas de Información en Salud (CENS)
Biomedical Neuroscience Institute (BNI)
Red de Salud Digital de Universidades del Estado (RSDUE)
Institute of Biomedical Sciences (ICBM)
Núcleo Milenio de AutoOrganización y Mecánica de Tejidos (SELFO)

Facultad de Medicina, Universidad de Chile

|-> 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

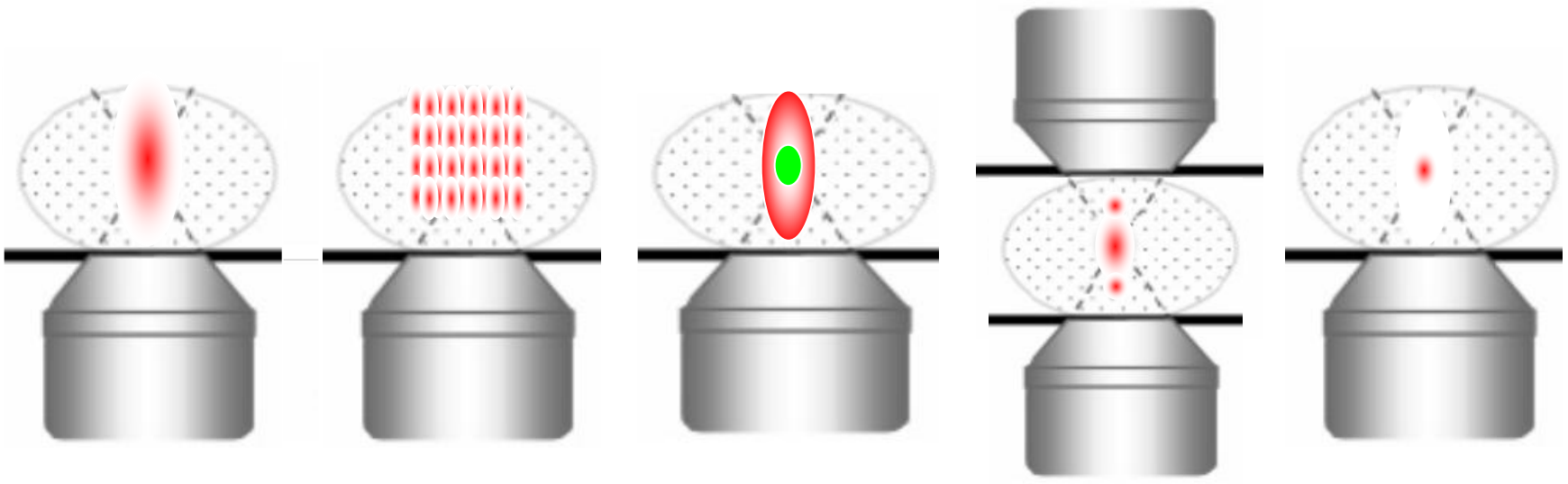
M Goeppert-Mayer
1906-1972

M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

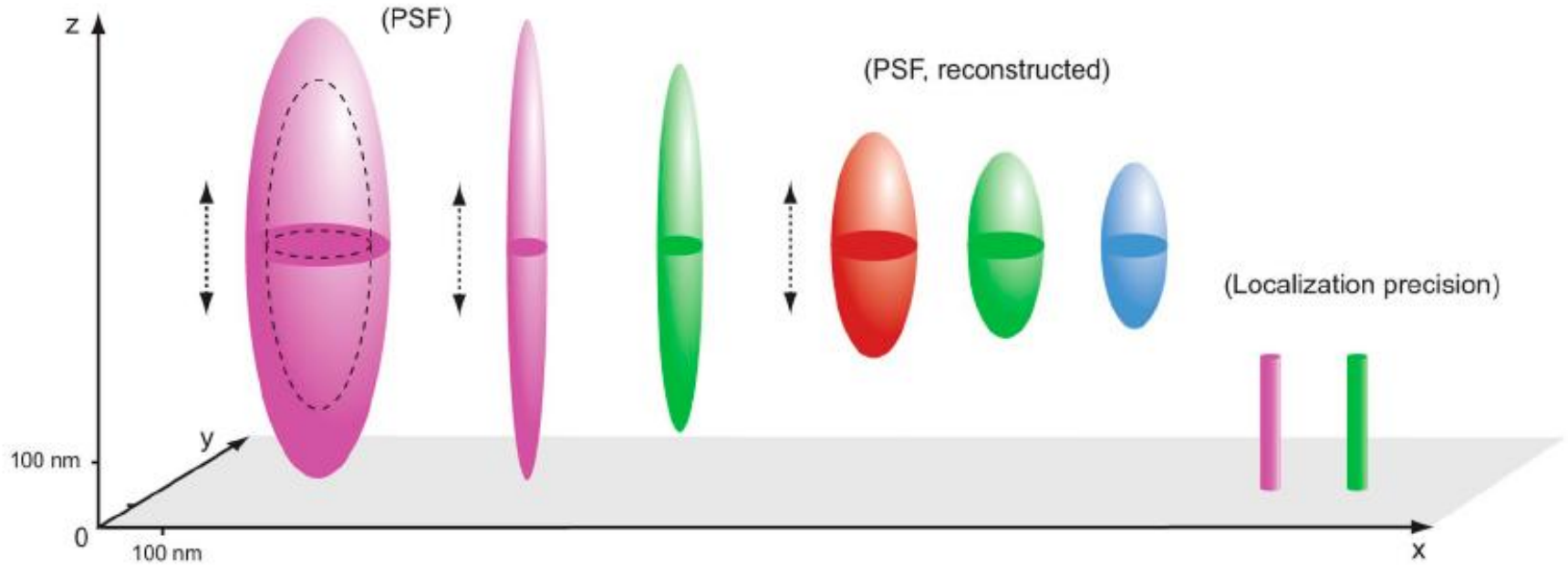
FWHM(xy) ~ λ/2 | ~ λ/4 | ~ λ/∞ | ~ λ/4 | ~ λ/100



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

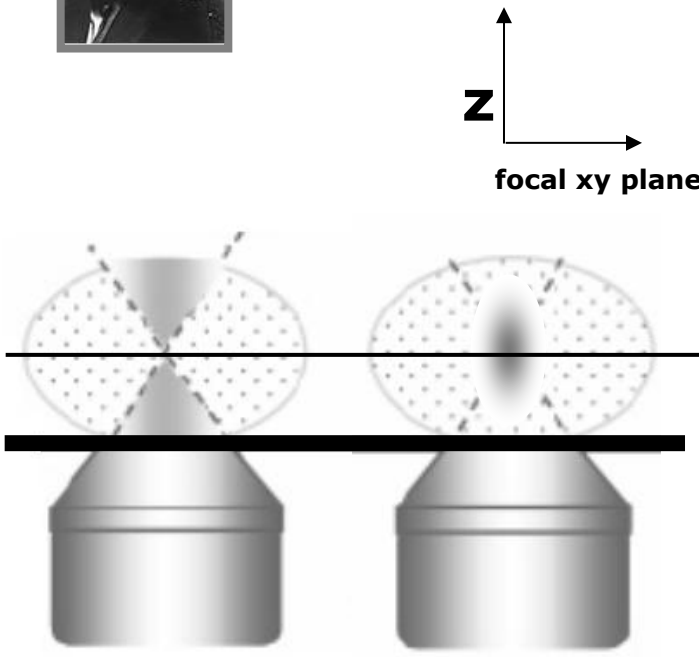


~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

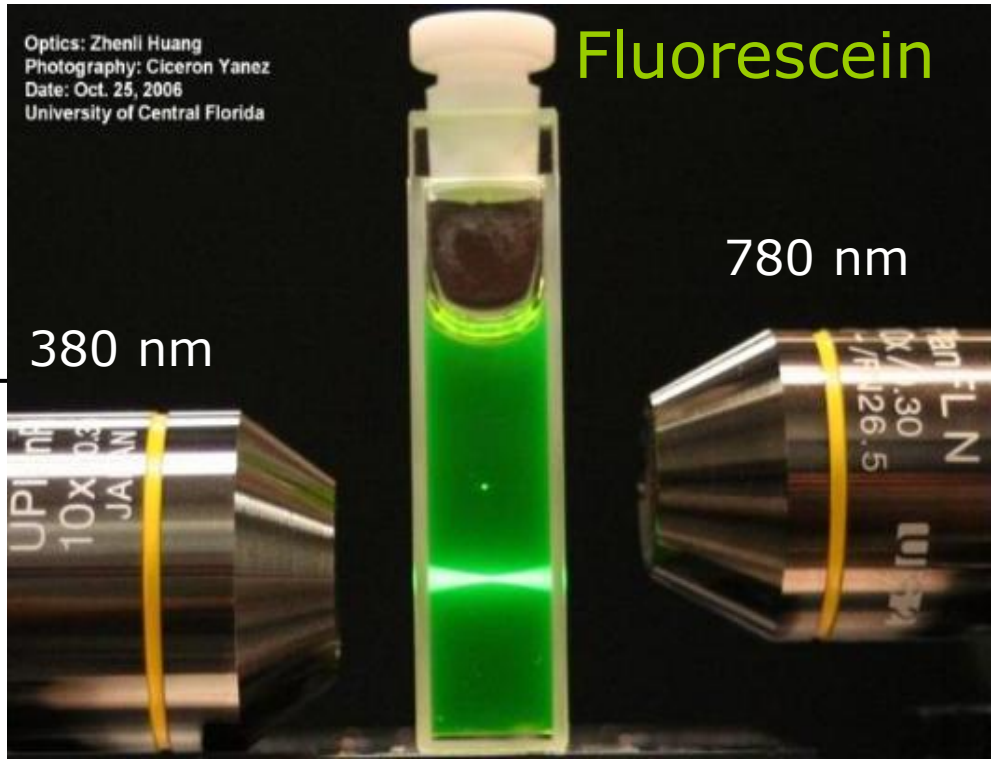
Schermelleh et al 2010 JBC

|-> Diffraction limited Microscopy

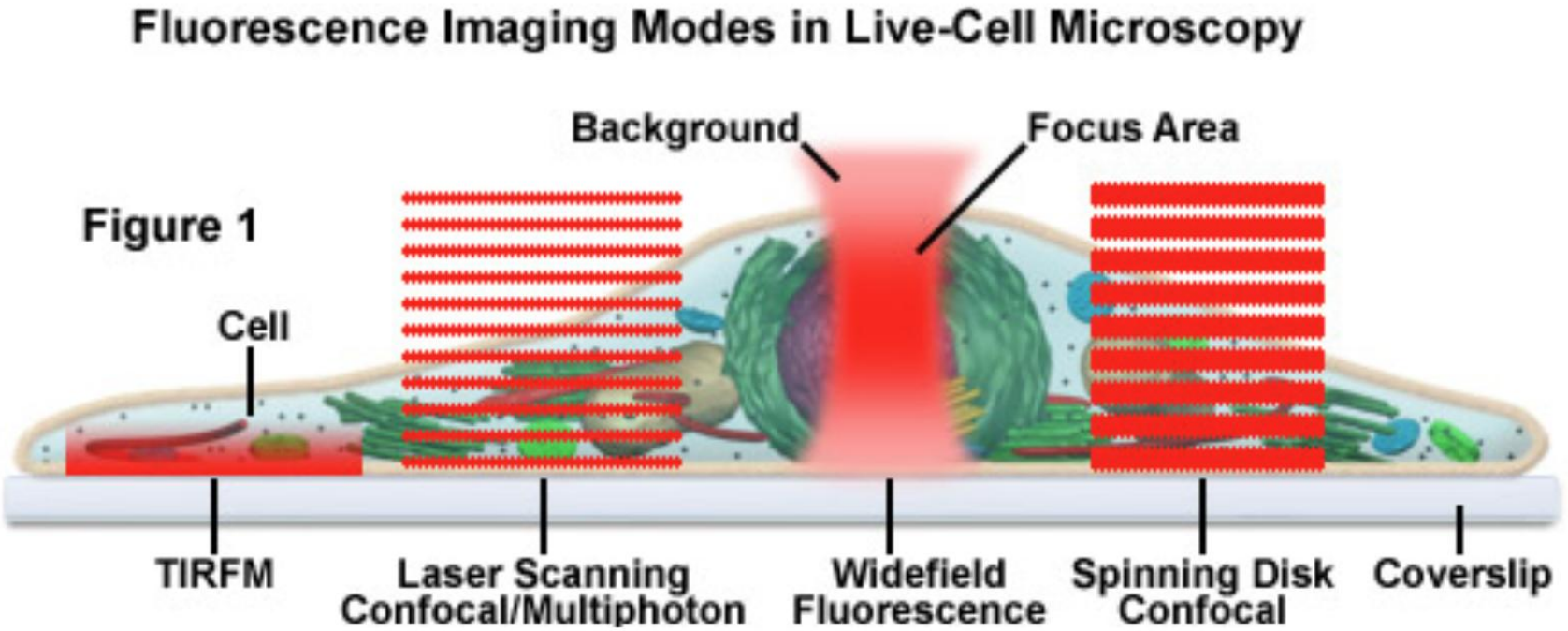
| Best localization: confocal microscopy



convencional / confocal



|-> Diffraction limited Microscopy



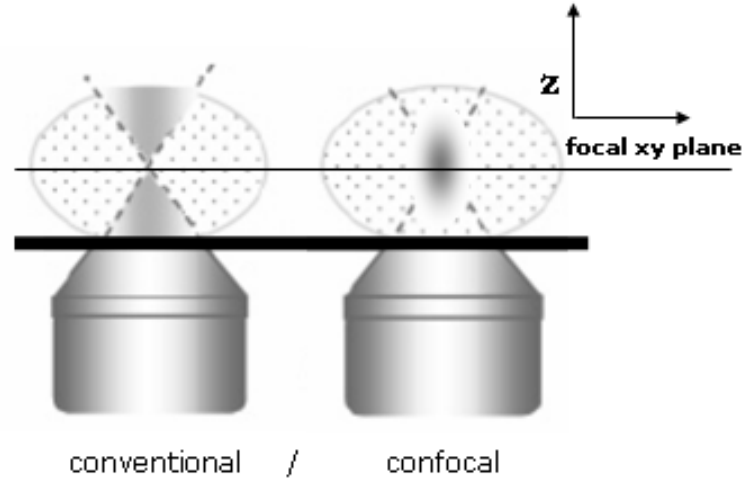
https://zeiss-campus.magnet.fsu.edu/print/livecellimaging/techniques-print.html

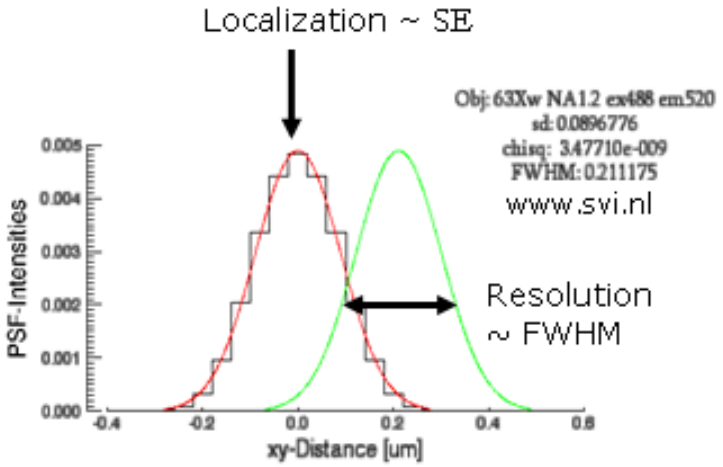
| Diffraction limited microscopy

E. Abbe († 1905)

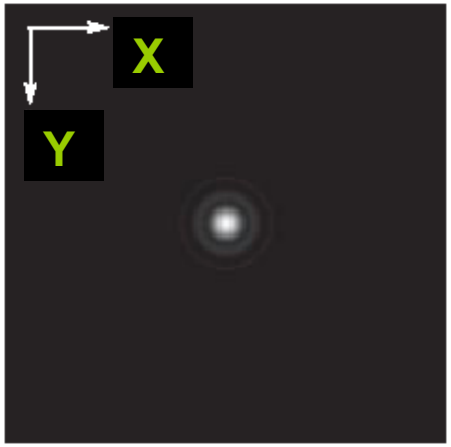
$\lambda / 2 \cdot NA \sim \lambda / 2$ Resolution (Full Width at Halft Maximum, FWHM)

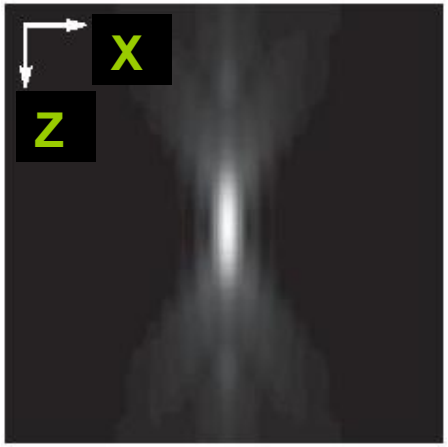
$FWHM / N^{1/2}$ Localization, N number of photons

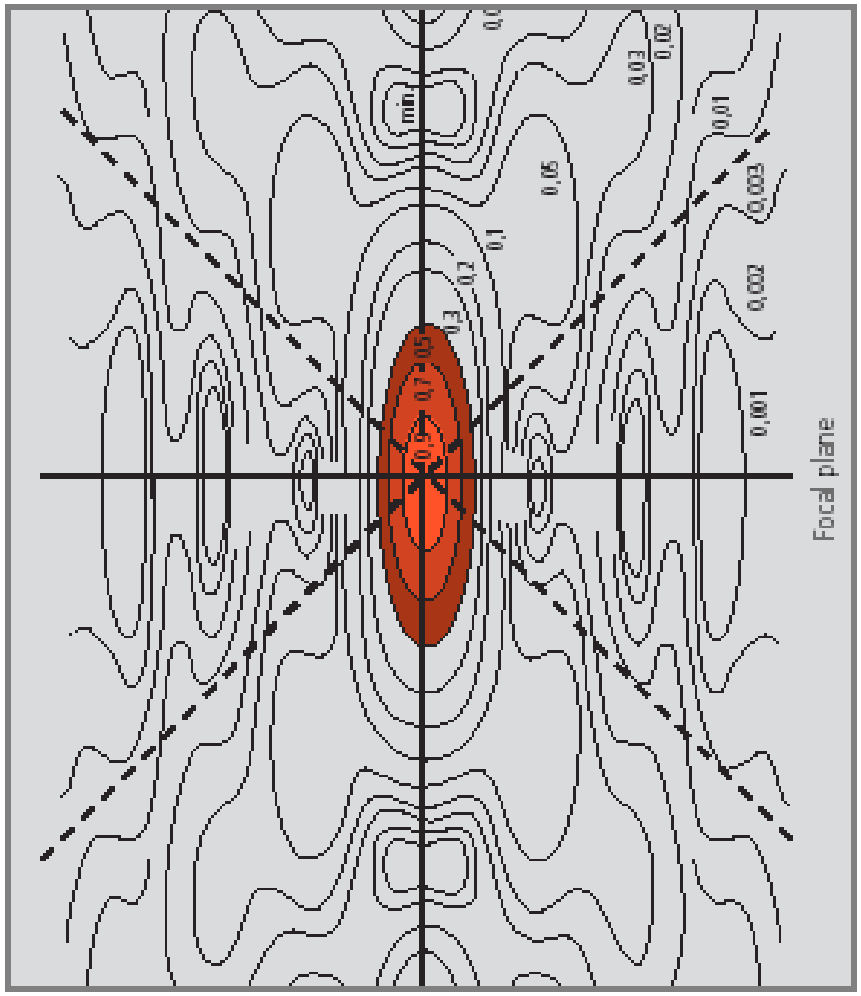




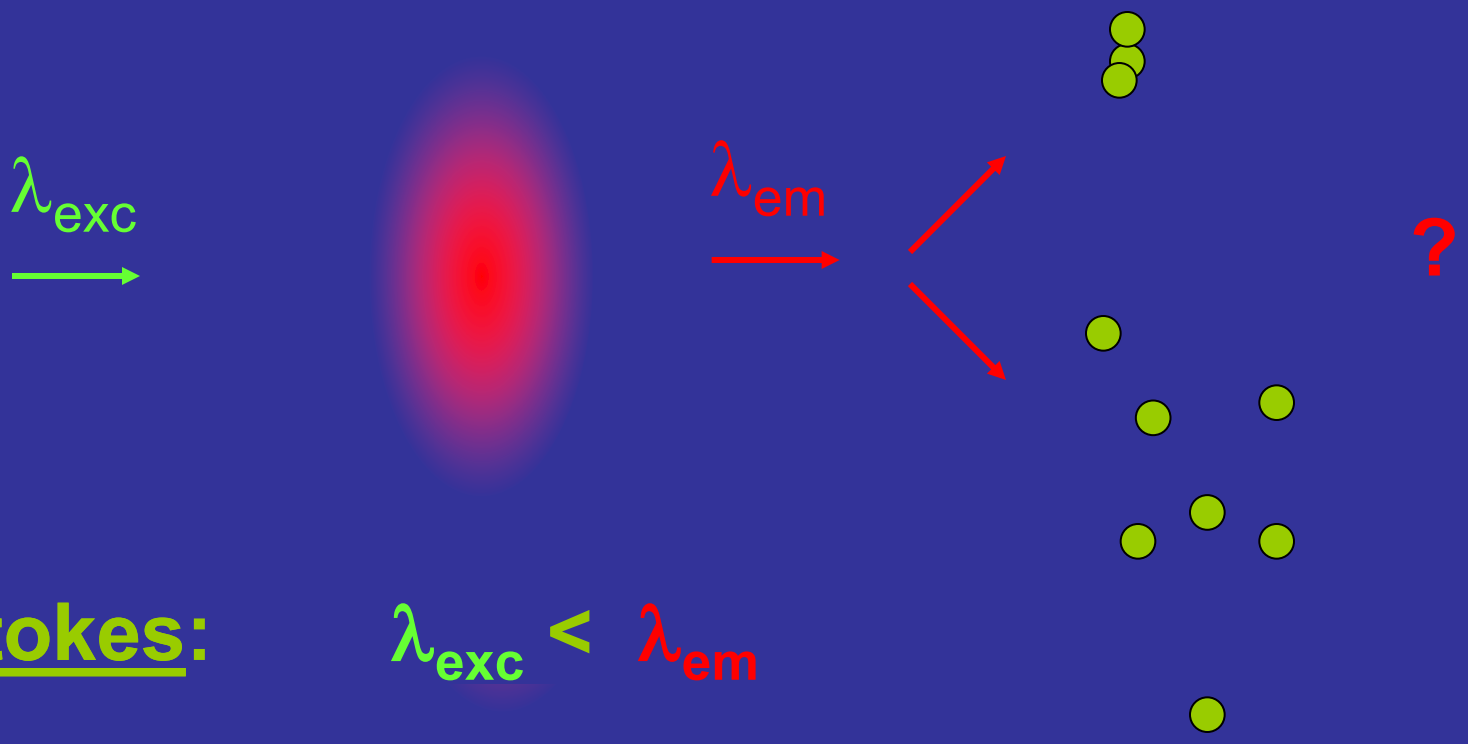
|-> PSF





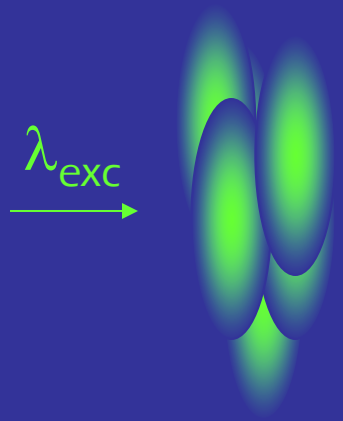


|-> Convolution



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

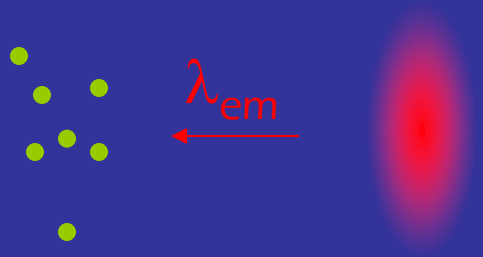
|-> Convolution



λ_{exc}

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

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



λ_{em}

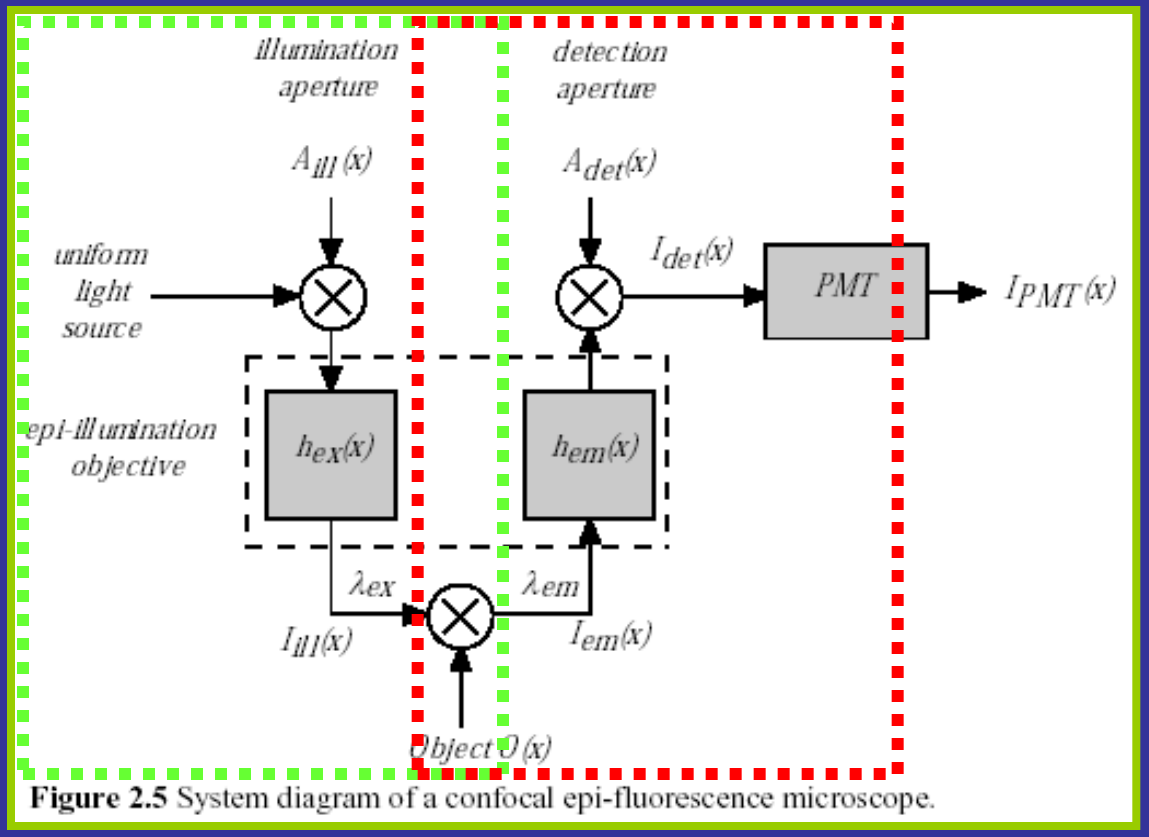
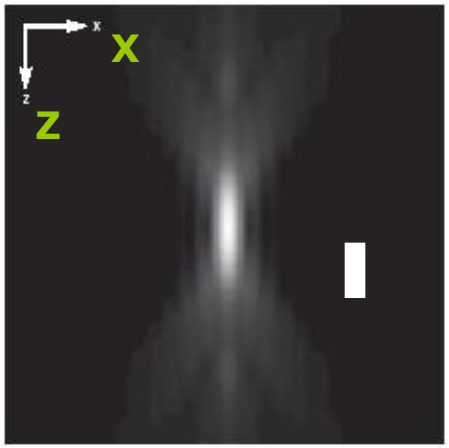
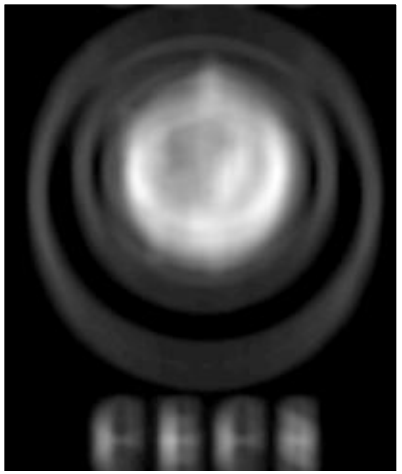


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

|-> Convolution





PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: Point Spread Function

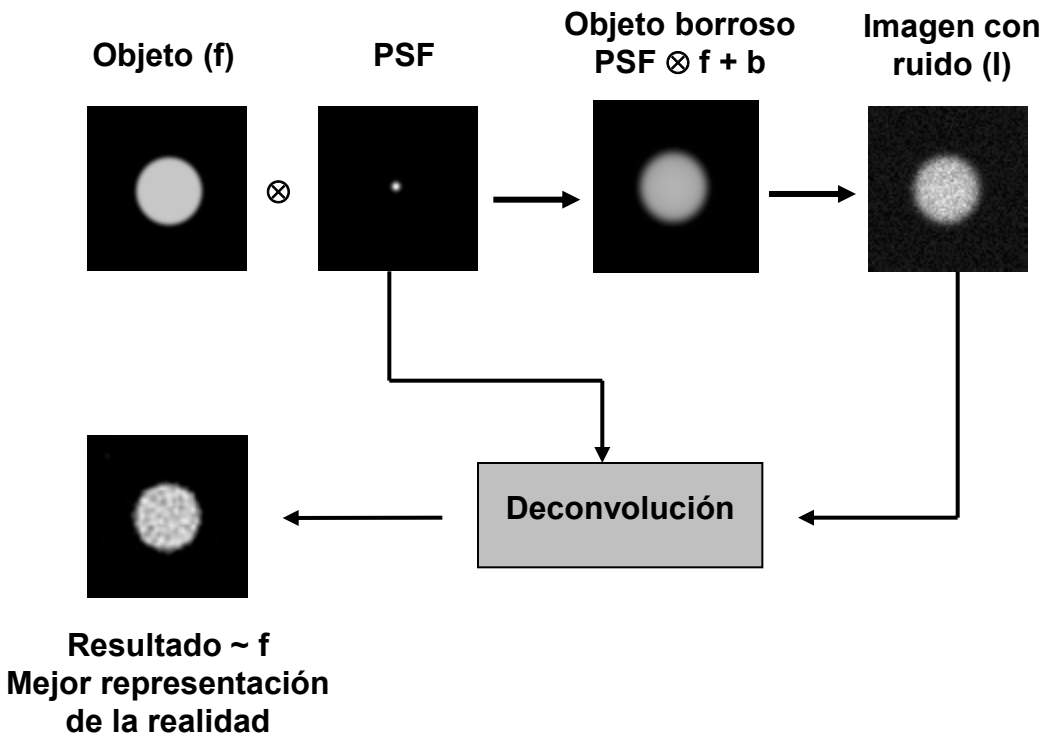
f: Object Function

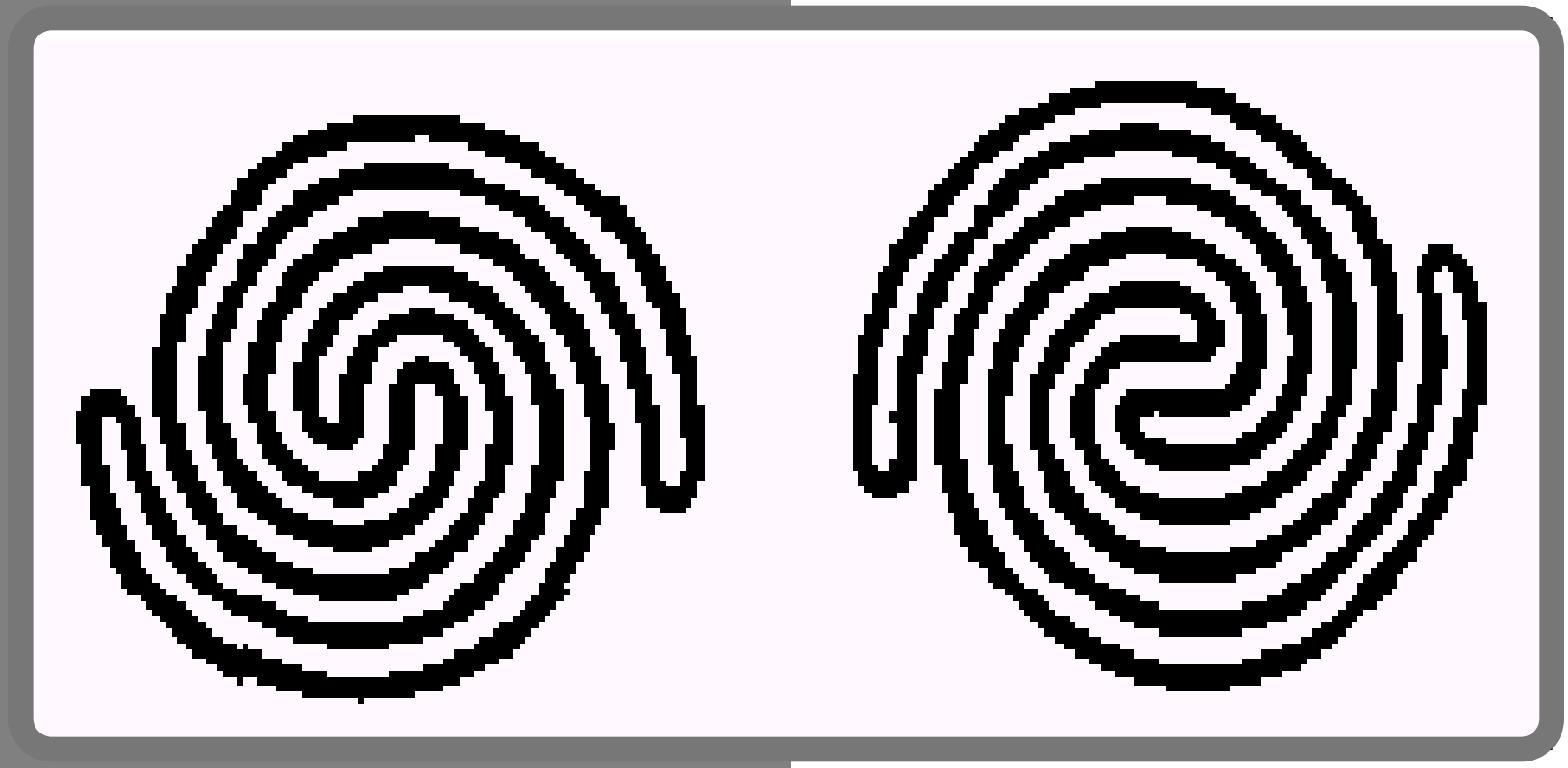
b: Offset Function

I: Image Matrix

N: Noise Function

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



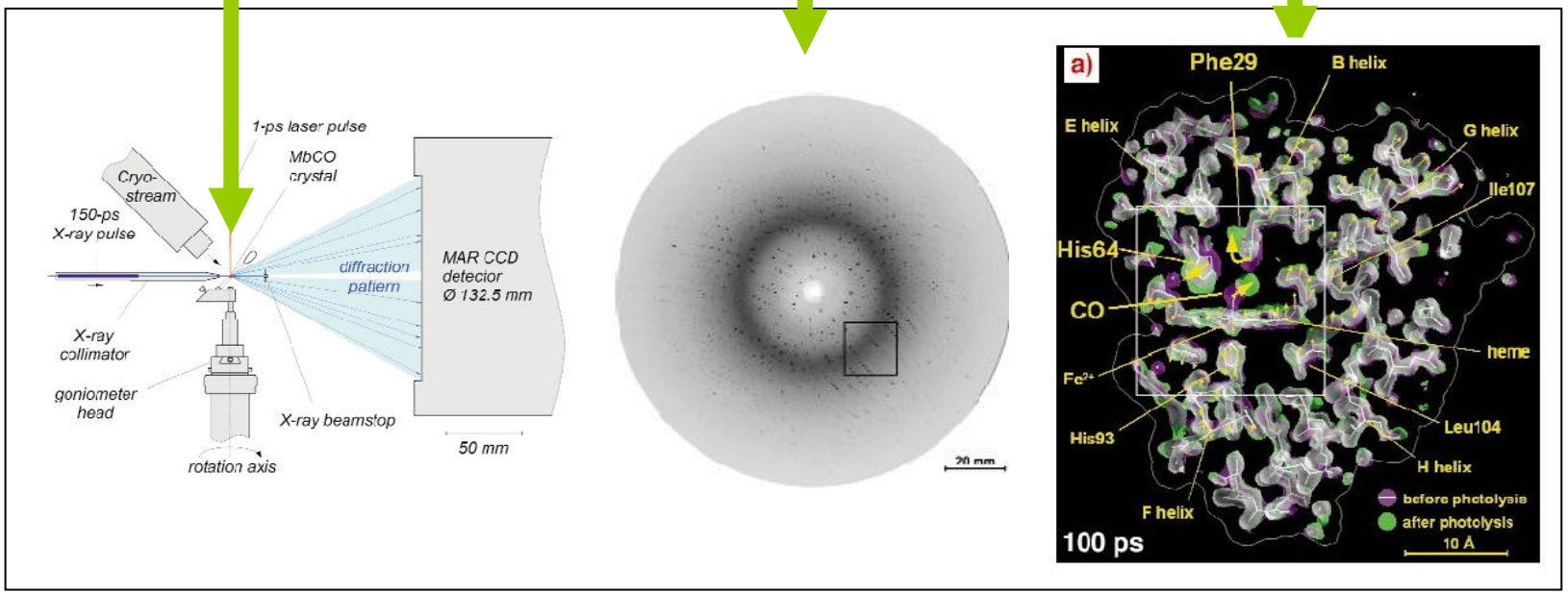


OTF: Object/Optical Transfer Function

Myoglobin in Action |Picosecond Laue Crystallography Diffraction Data

Schotte et al (2003) Science

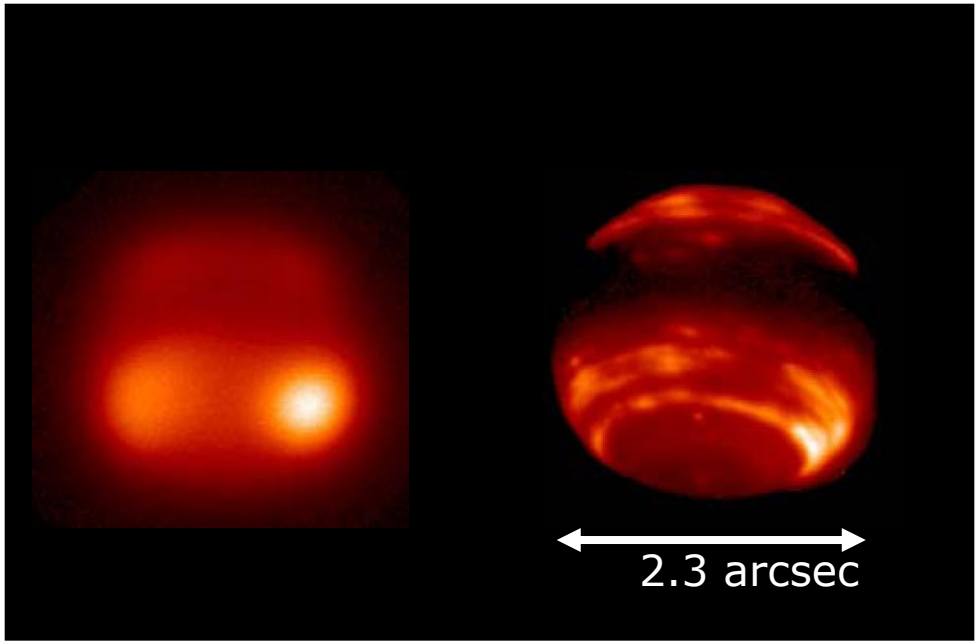
Object → **OTF** → **O**bject**I**mage → **OTF^{-1}** → **O**bject**M**odel



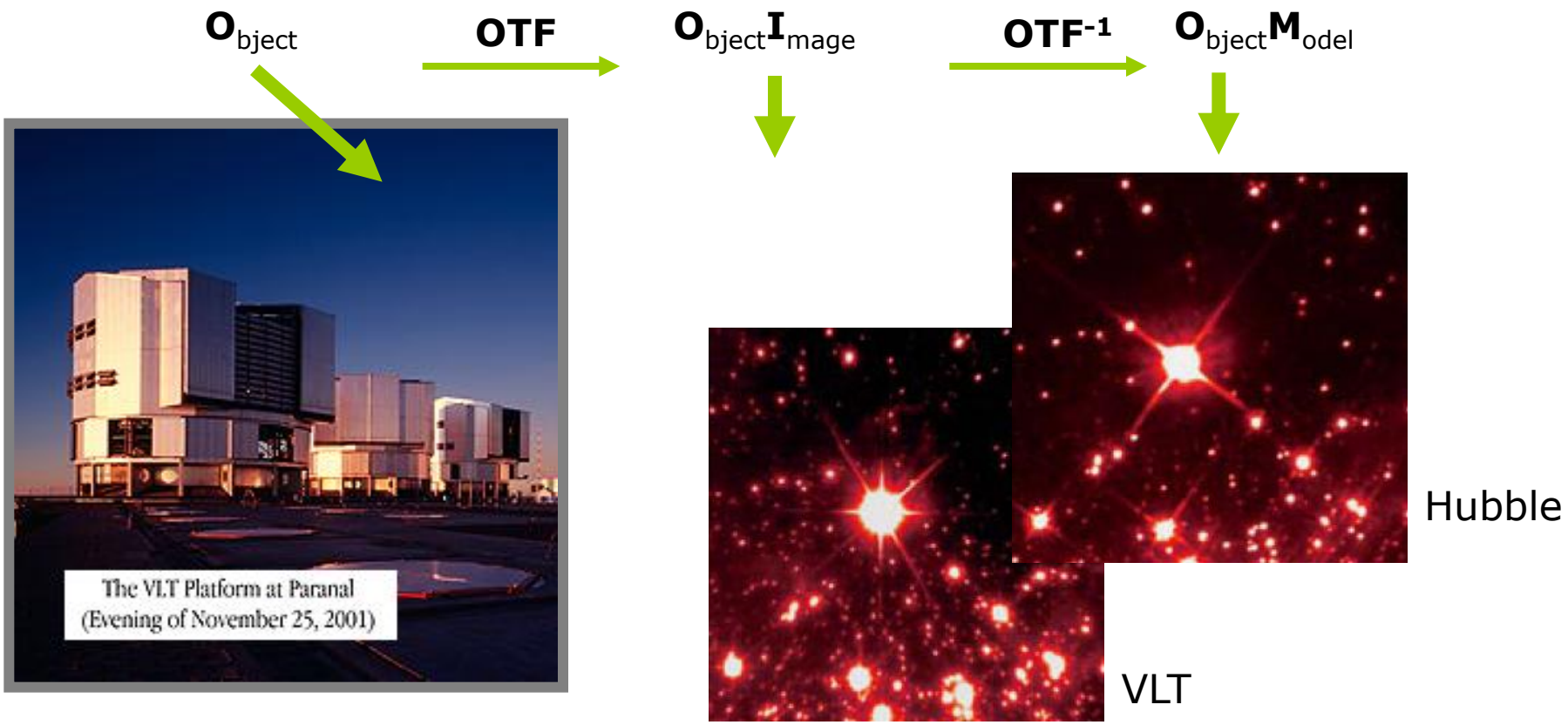
http://www.youtube.com/watch?v=lnKIBZYarzM

Diffraction Limited Resulution for a 10m telescope ~ λ/D ~ 0.01 arcsec
is limited to ~ 0.5 arcsec by the turbulent atmosphere.
NAOS creates an artificial star at 90 km altitude in the Earth´s mesosphere.
The Laser Guide Star is used to correct atmospheric effects

O_{bject} → **OTF** → $O_{bject}I_{mage}$ → OTF^{-1} → $O_{bject}M_{odel}$



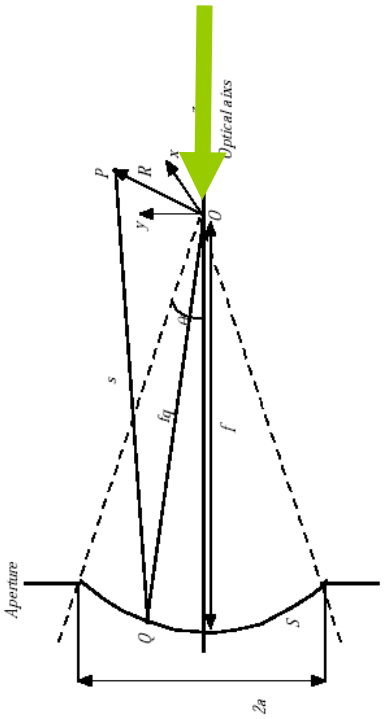
Diffraction Limited Resulution for a 10m telescope ~ λ/D ~ 0.01 arcsec is limited to ~ 0.5 arcsec by the turbulent atmosphere.

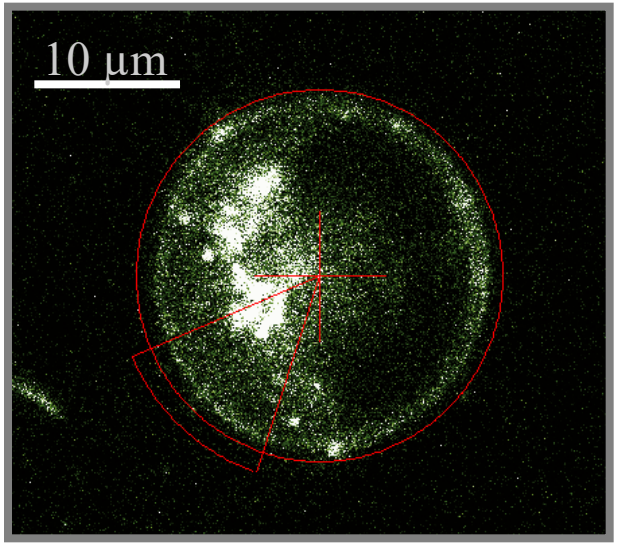


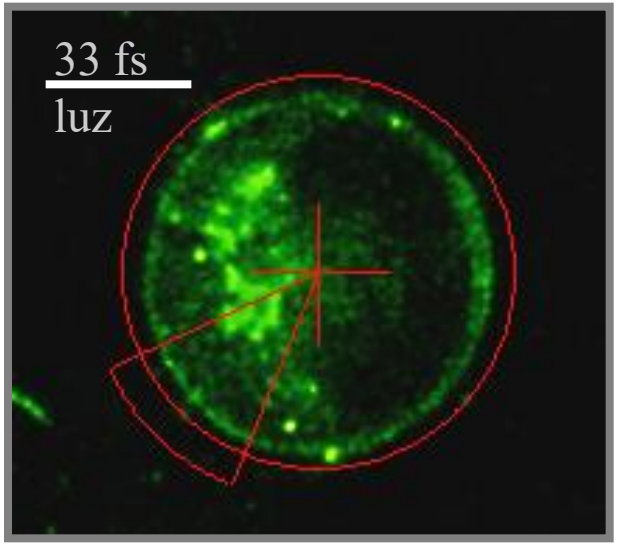
Confocal Microscopy | From Geometric Optics to Diffraction Theory

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.

Object → **OTF** → **O**bject**I**mage → **OTF**$^{-1}$ → **O**bject**M**odel



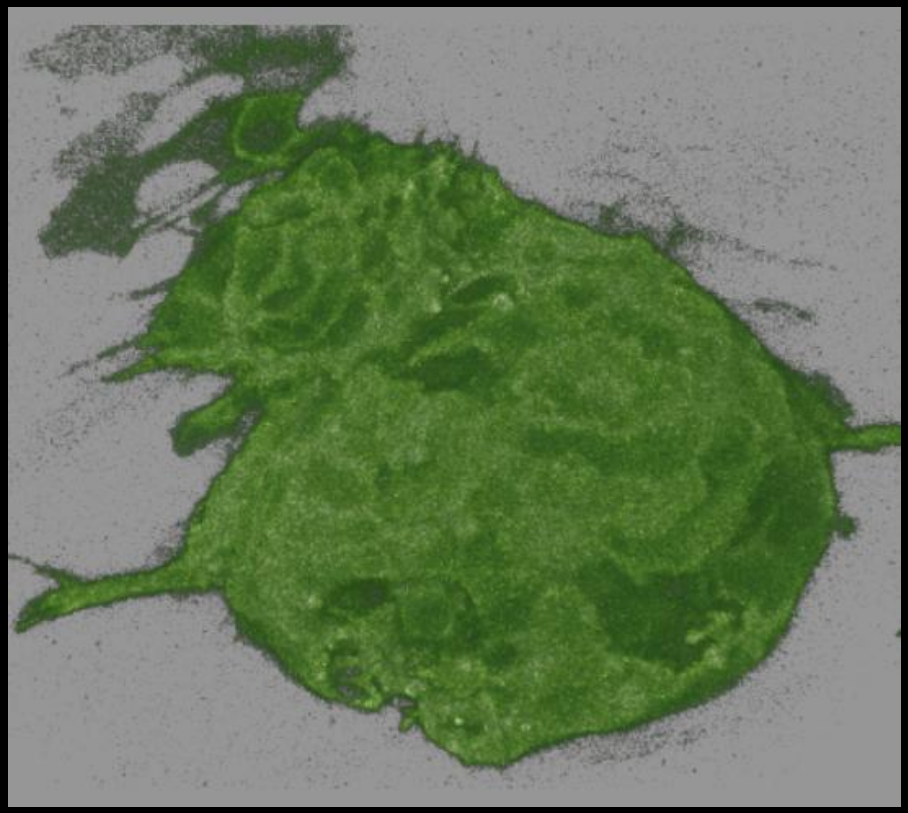


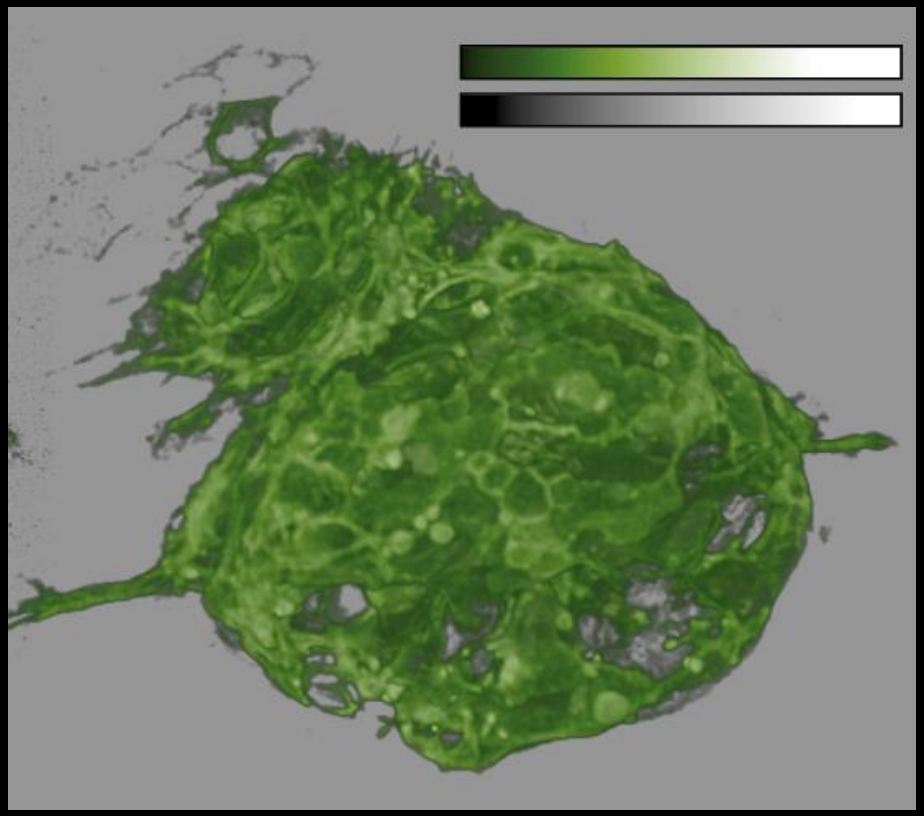


Laboratory for SCIAN Scientific Image Analysis

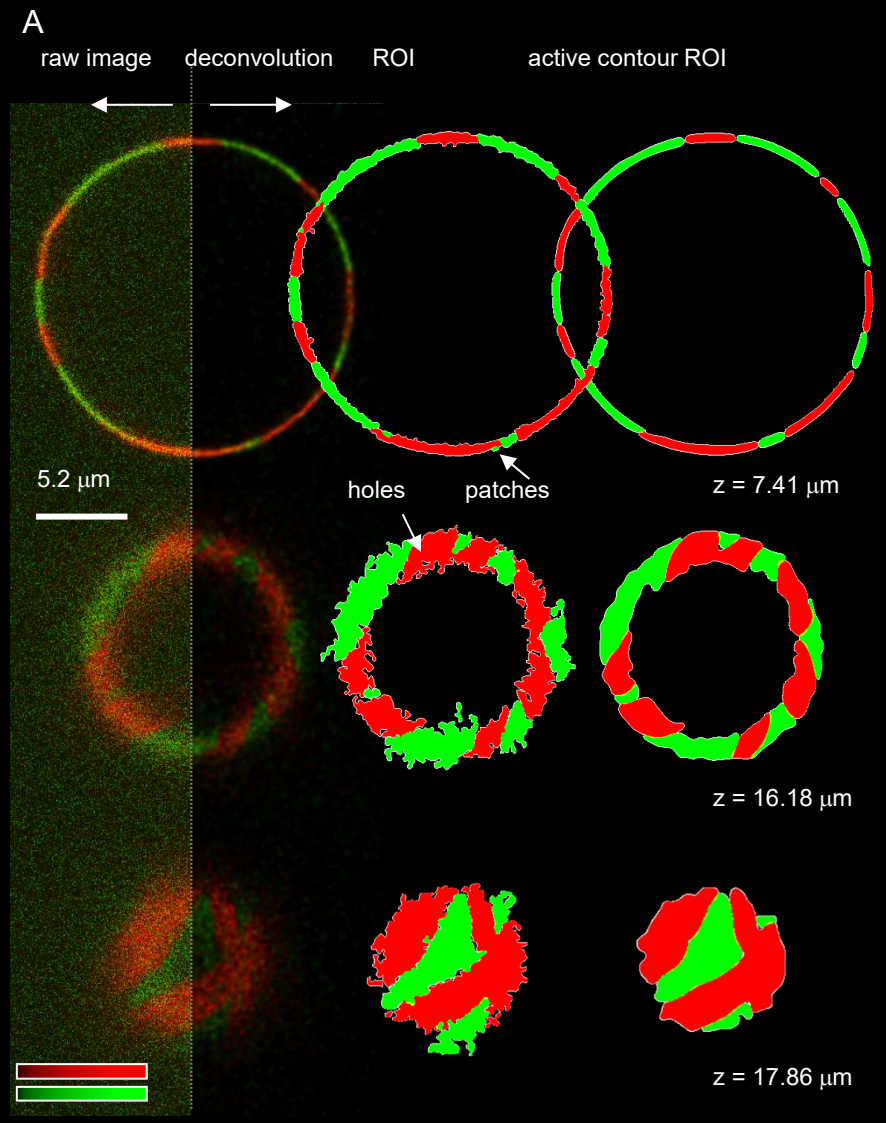
|-> Deconvolution

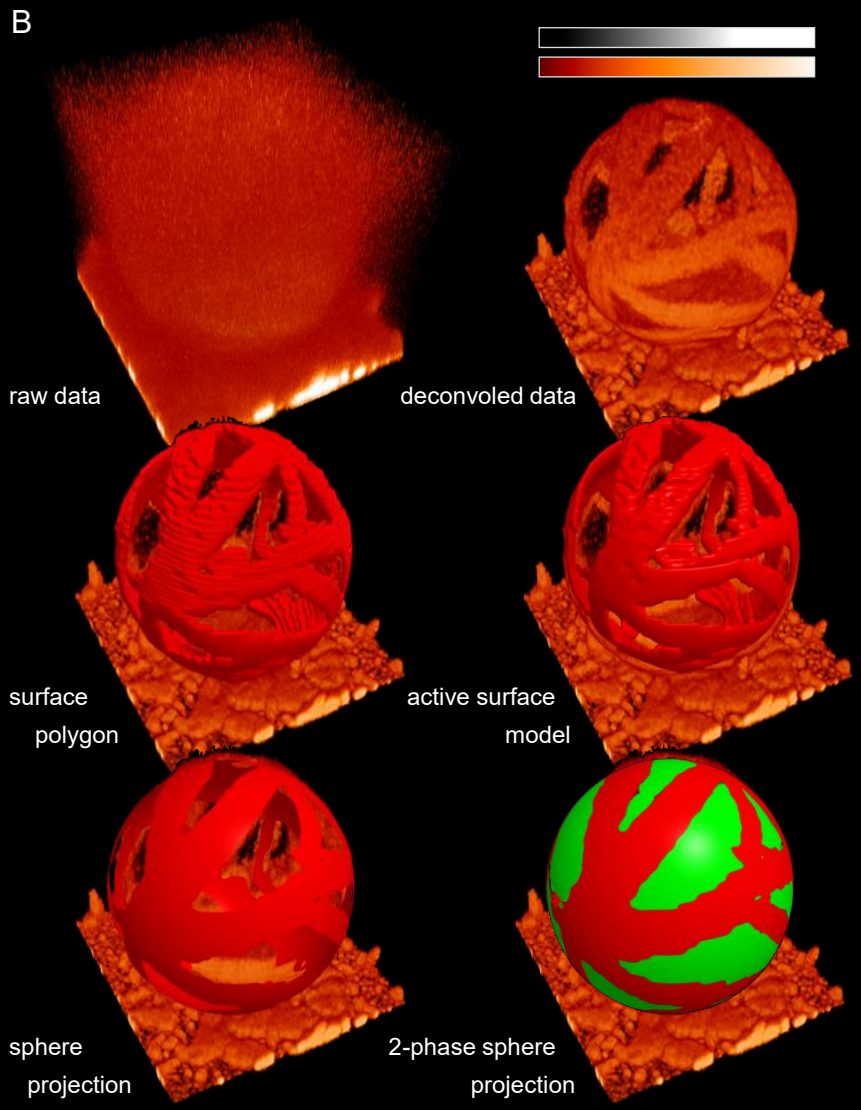
BIOMEDICAL NEUROSCIENCE INSTITUTE





|-> Deconvolution





|-> Deconvolution

PSF: Point Spread Function

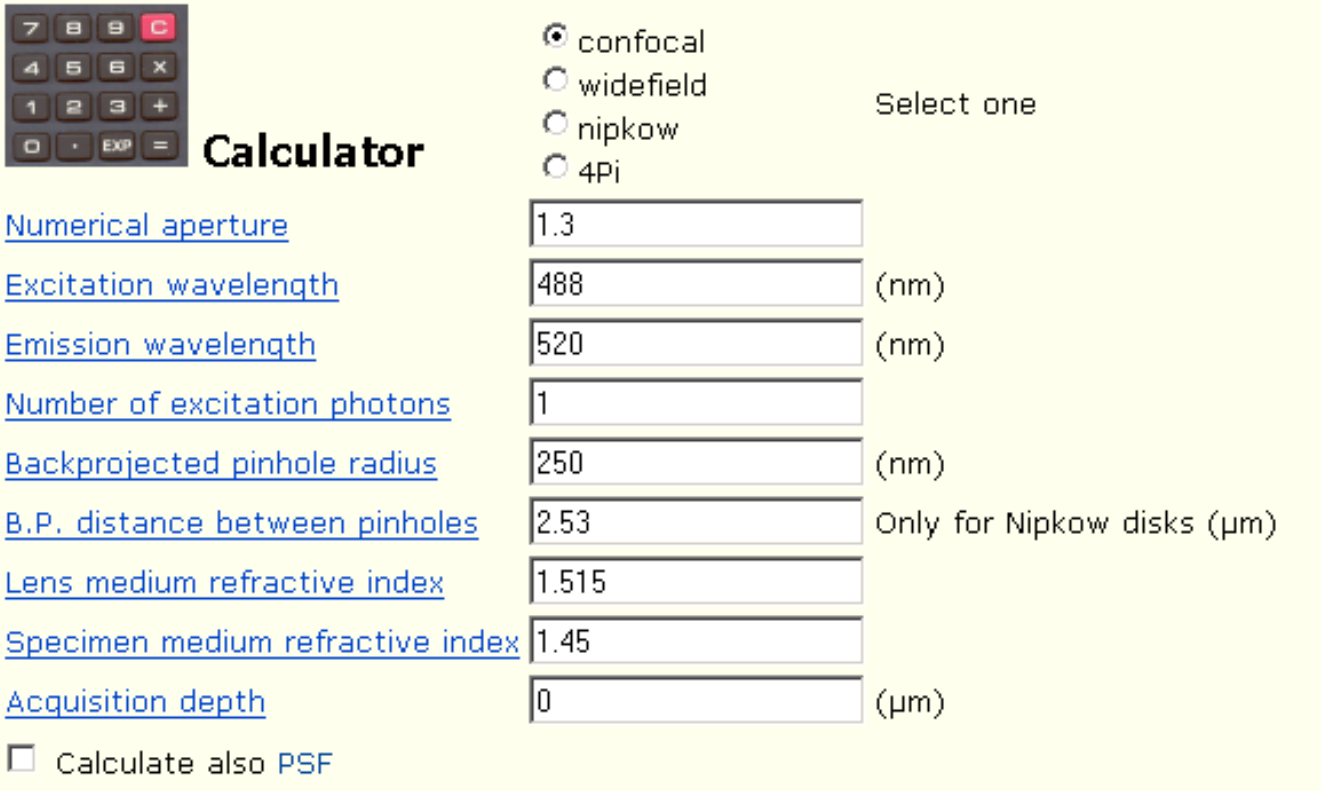
f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

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



https://svi.nl/Nyquist-Calculator

|-> Pinhole

PSF: Point Spread Function

f: Object Function

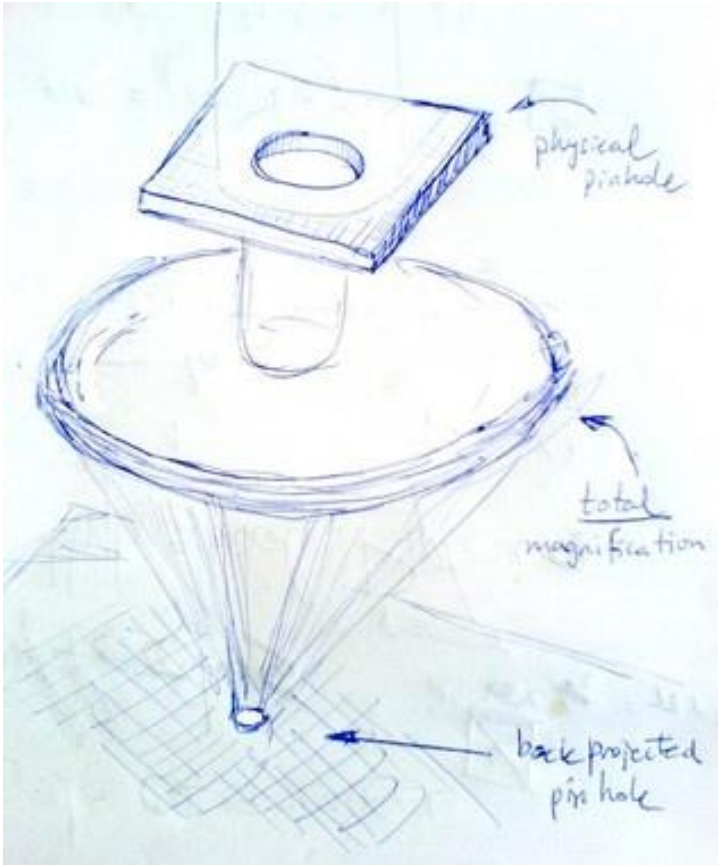
b: Offset Function

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



https://svi.nl/Nyquist-Calculator

|-> Pinhole

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

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

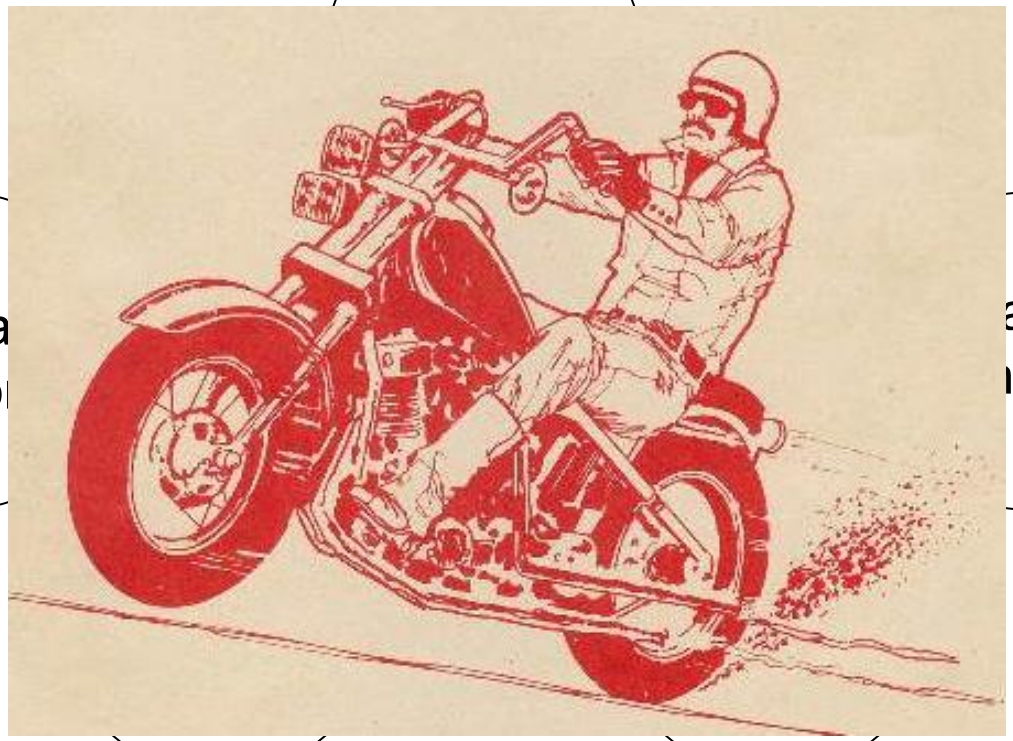
- Olympus FV300
- Olympus FV500
- Olympus FV1000

Zeiss

- Zeiss LSM410 inverted
- Zeiss LSM510

https://svi.nl/Nyquist-Calculator

|-> 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)*

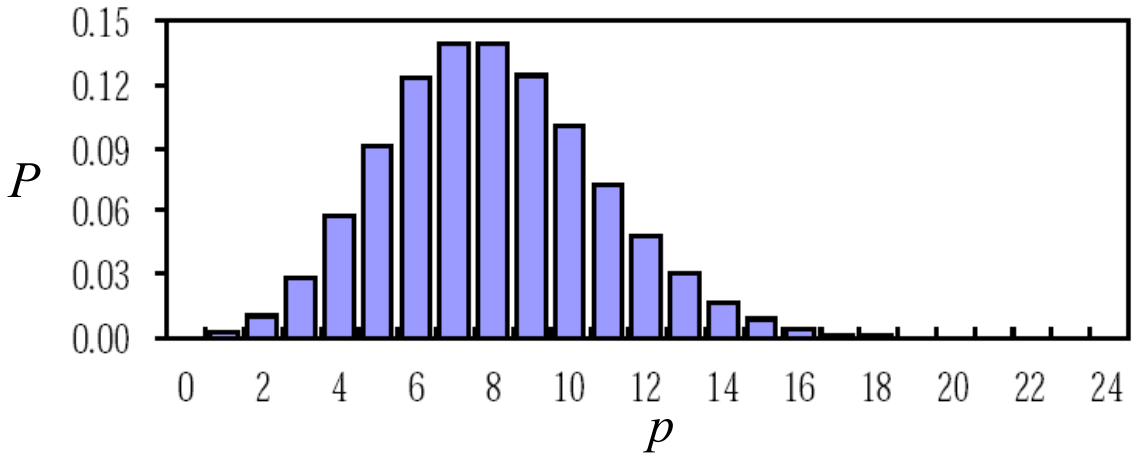
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

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

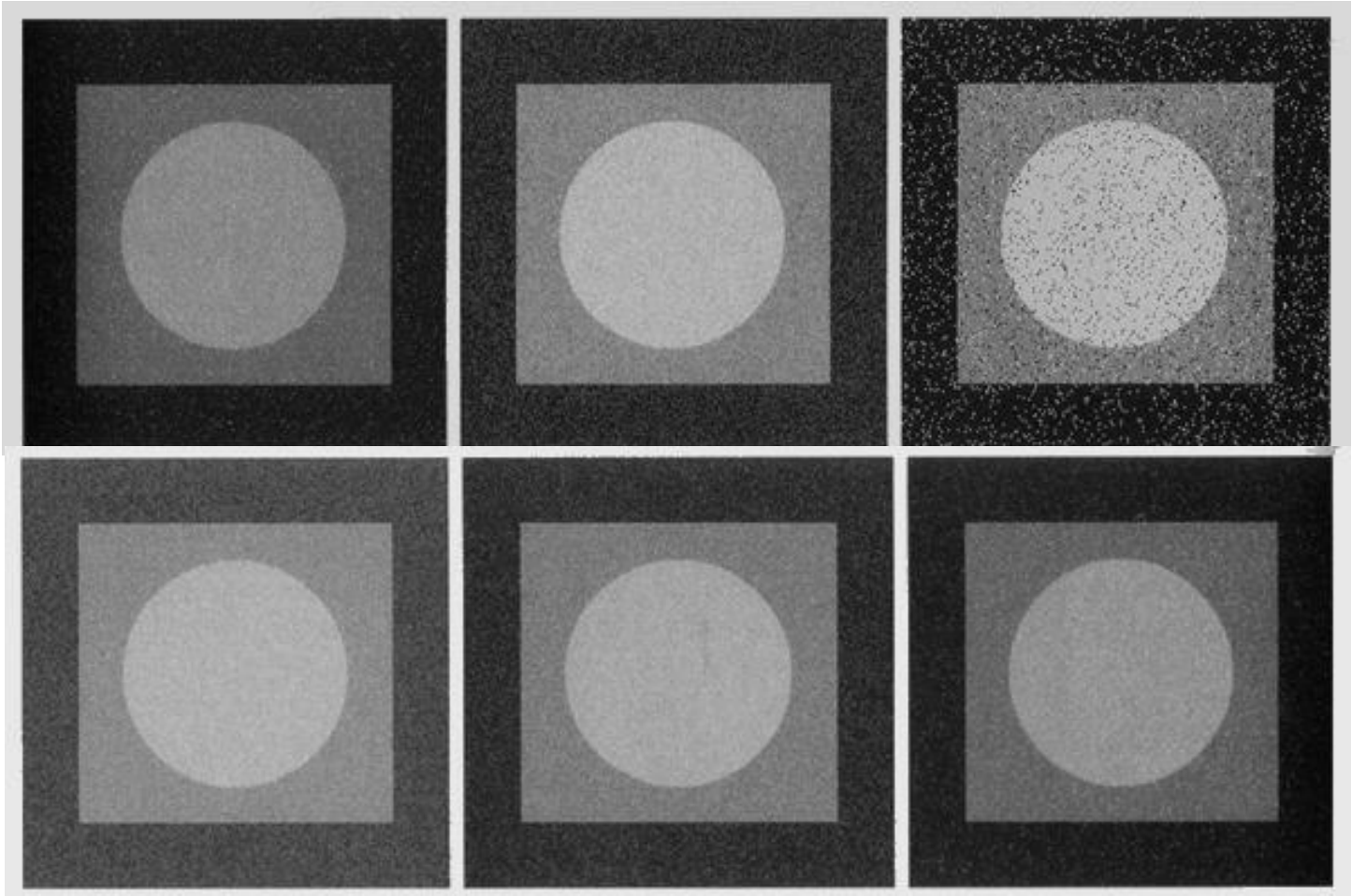
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

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

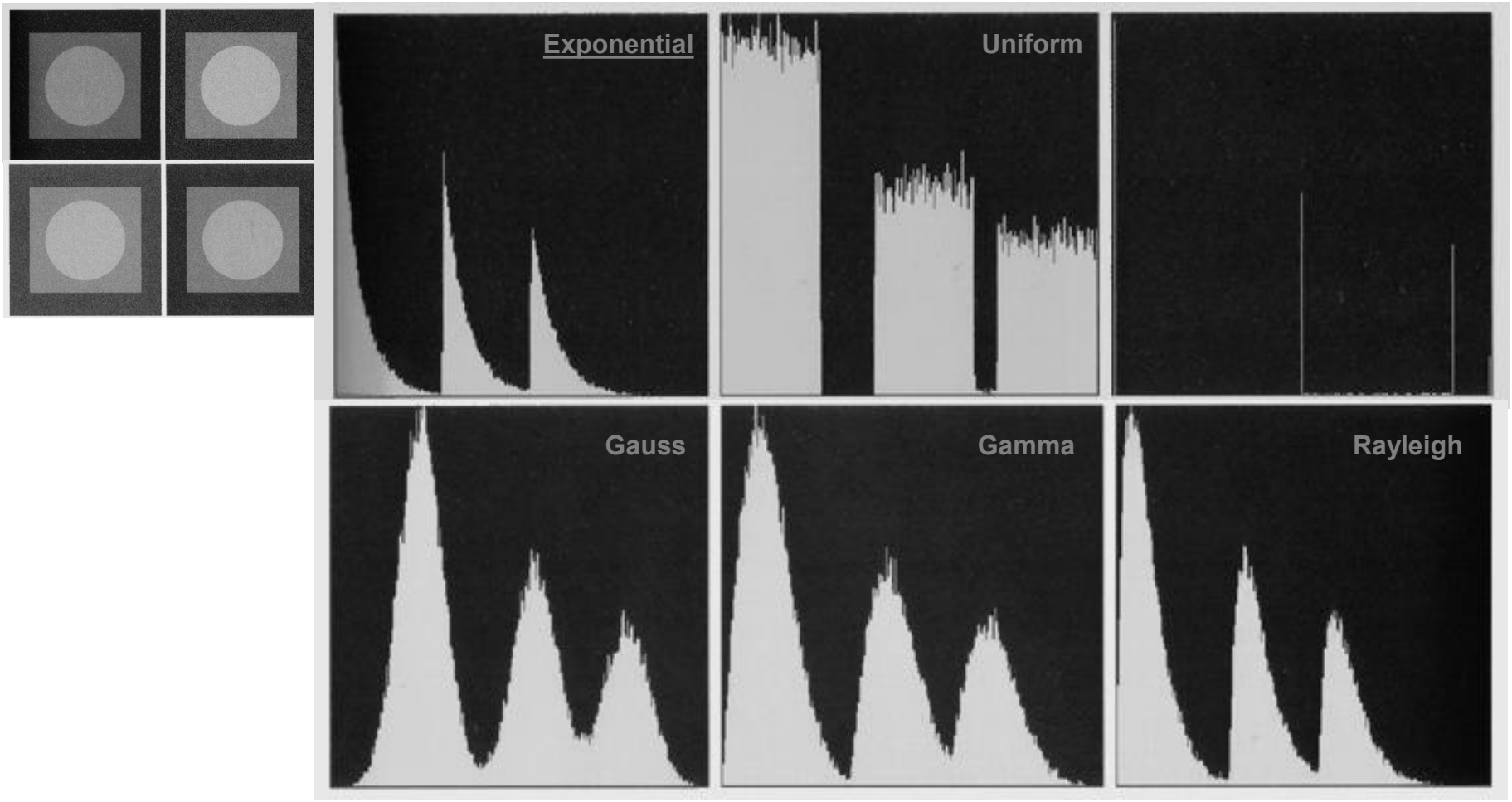
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> Noise



|-> Noise



|-> 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.

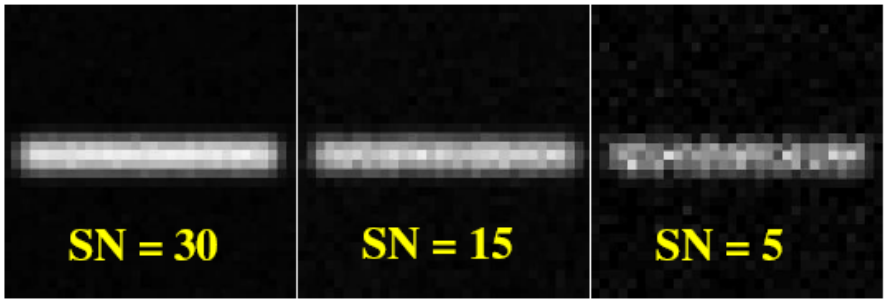
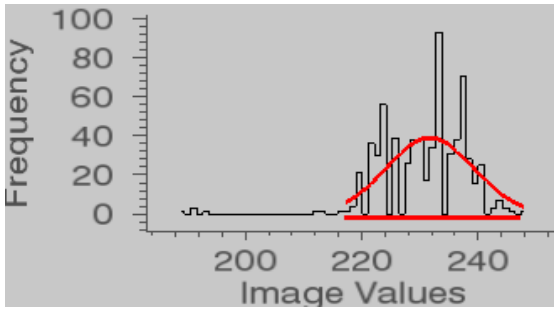
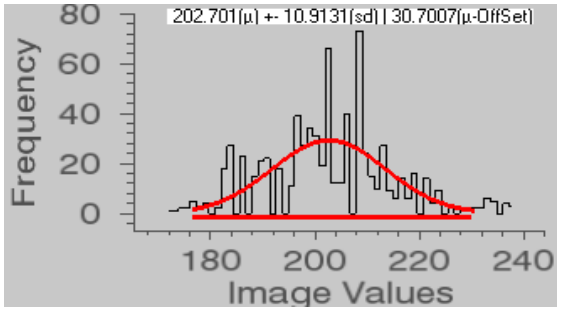
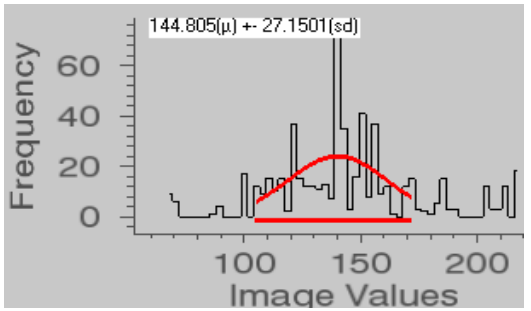


Figure 9. Images with different generated noise levels





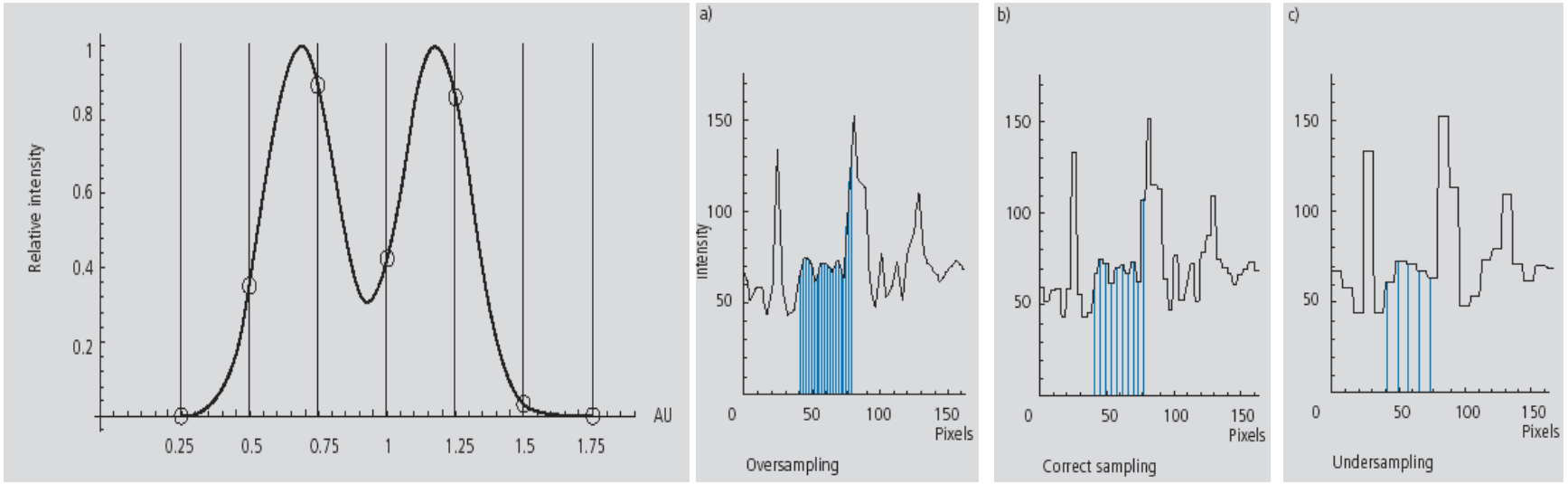


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

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

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

|-> 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

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

Calculator

- (•) confocal
- ( ) widefield
- ( ) nipkow
- ( ) 4Pi

Select one

Numerical aperture	1.3	
Excitation wavelength	488	(nm)
Emission wavelength	520	(nm)
Number of excitation photons	1	
Backprojected pinhole radius	250	(nm)
B.P. distance between pinholes	2.53	Only for Nipkow disks (µm)
Lens medium refractive index	1.515	
Specimen medium refractive index	1.45	
Acquisition depth	0	(µm)

☐ Calculate also PSF

https://svi.nl/Nyquist-Calculator

|-> PSF calculator

PSF: Point Spread Function

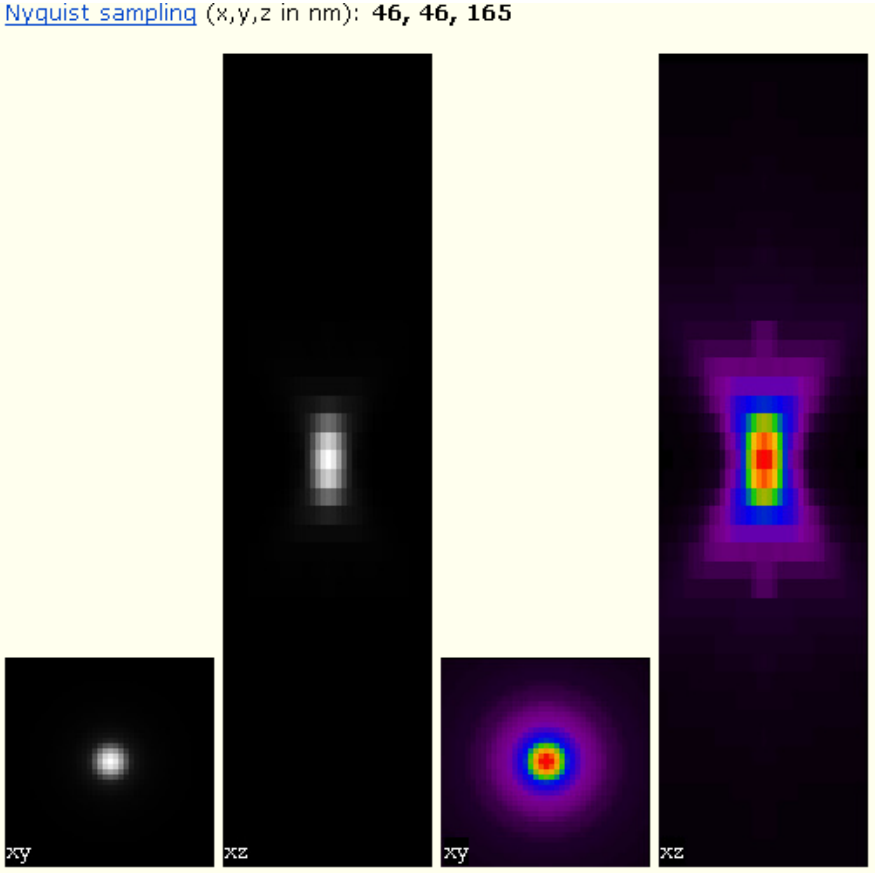
f: Object Function

b: Offset Function

I: Image Matrix

N: Noise Function

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



https://svi.nl/Nyquist-Calculator

|-> Snell's Law

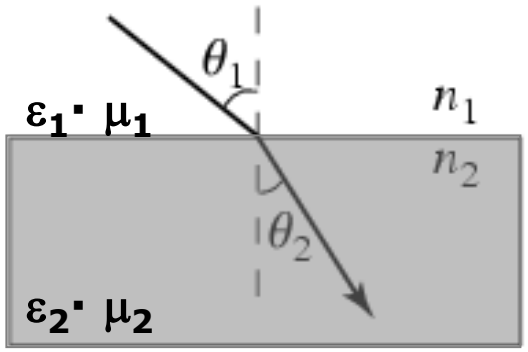
Index of refraction: $n = (\varepsilon \cdot \mu)^{1/2} = c/v$,

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]

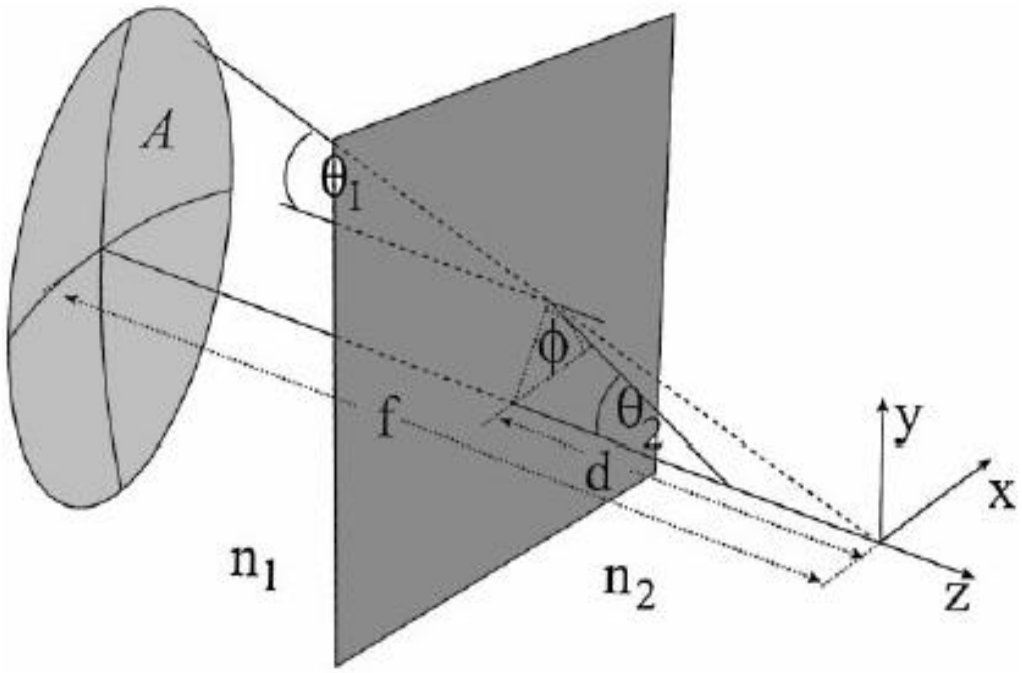
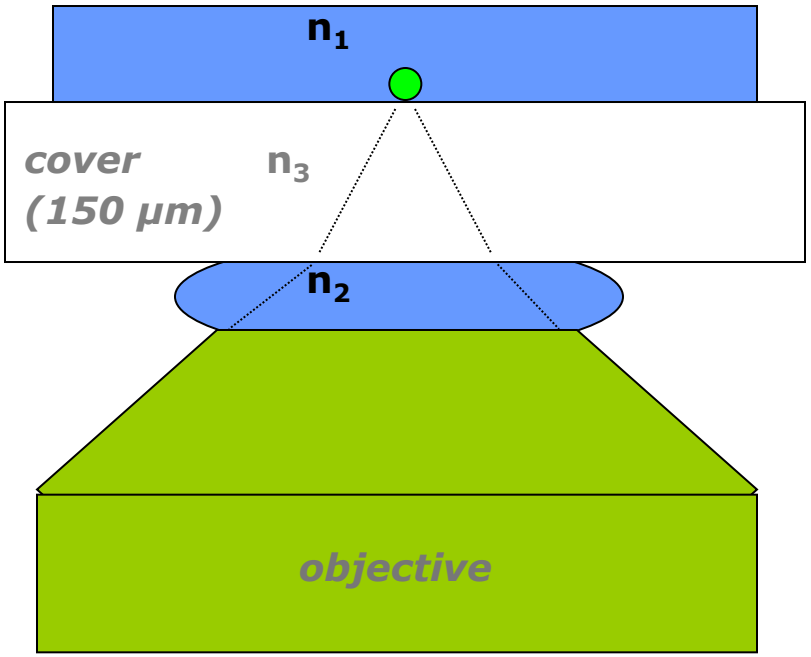


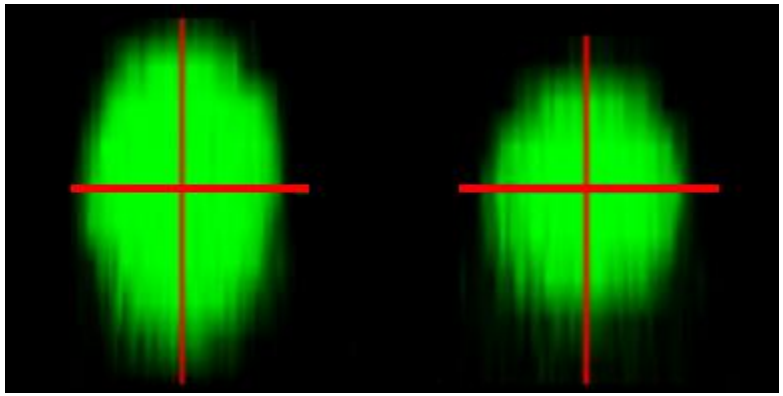
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera:** ∅ = 6 µm





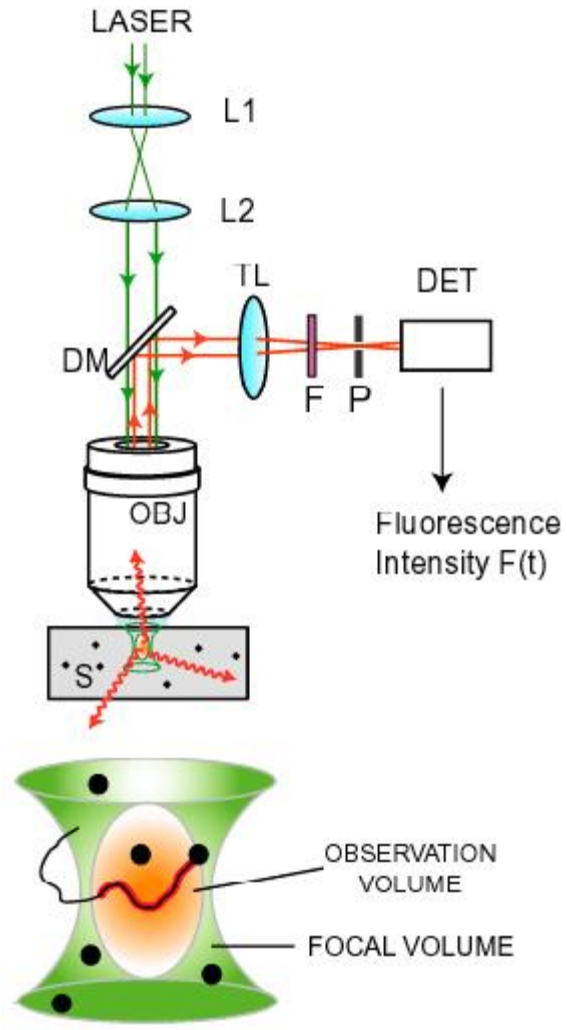
agua/aceite -- *aceite/aceite*

$n_1 \neq n_2$ $n_1 = n_2$

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

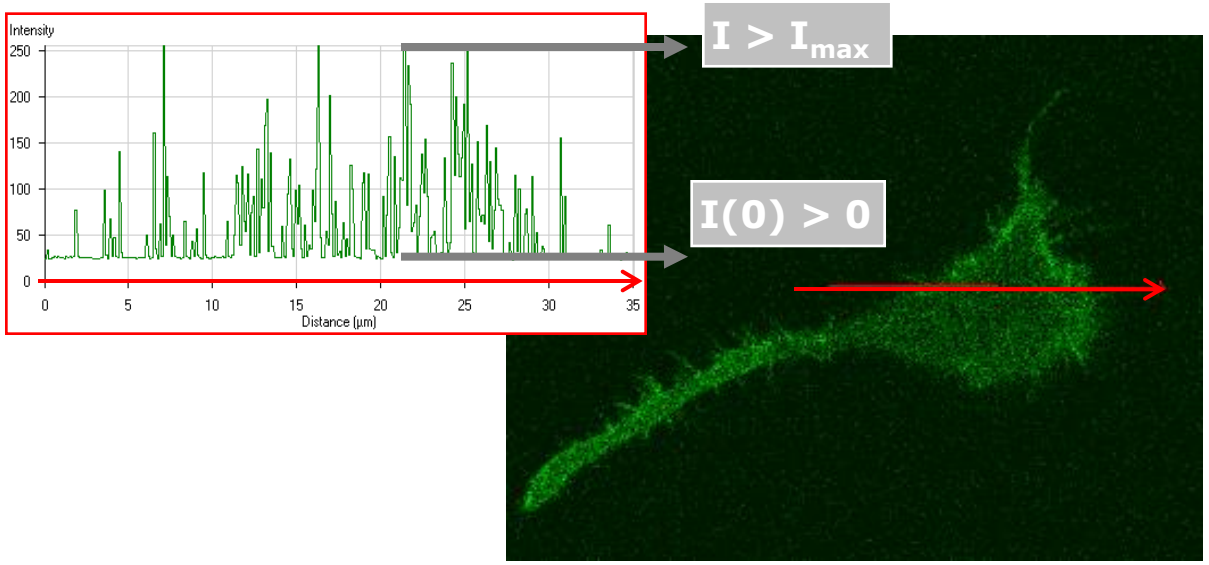
$n = n(\lambda)$!

|-> Resumen I

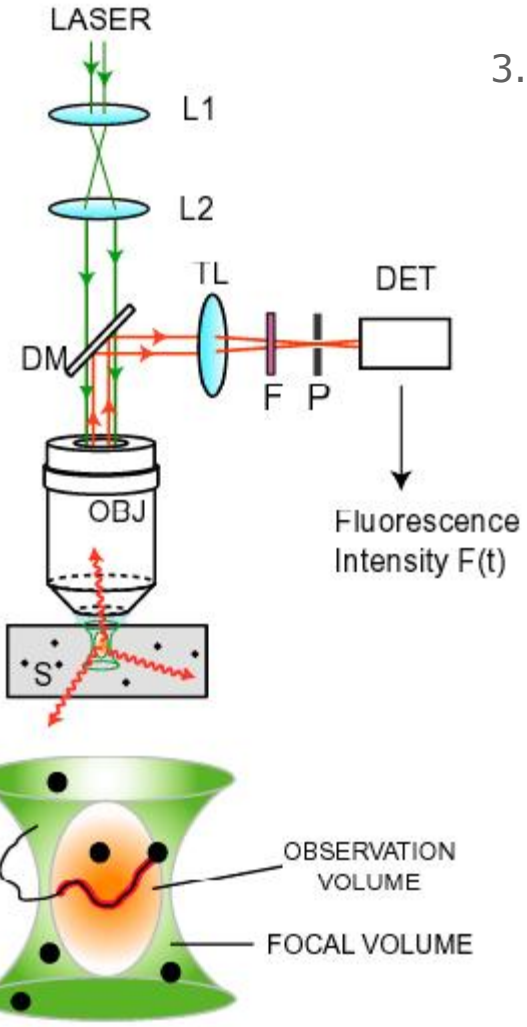


The observation volume (femtoliter) defined by the Point Spread Function must be considered as a mini-sprectrofluorimeter.

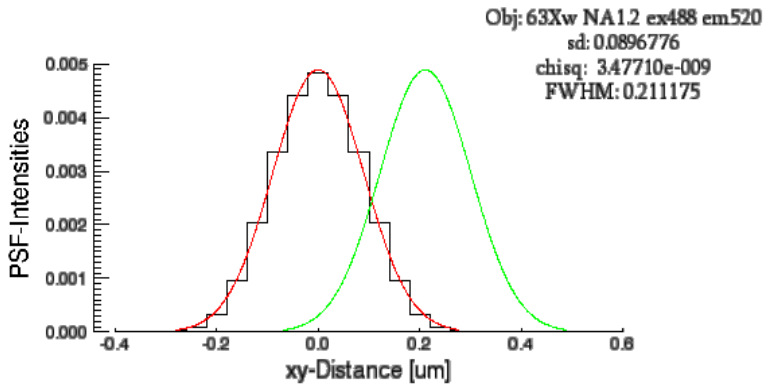
1. You need to consider the Offset I(0) in order to calibrate your signal $I(0) \geq 0$!
2. Never saturate the signal: $I \leq I_{max}$ (255 for 8 bit) !



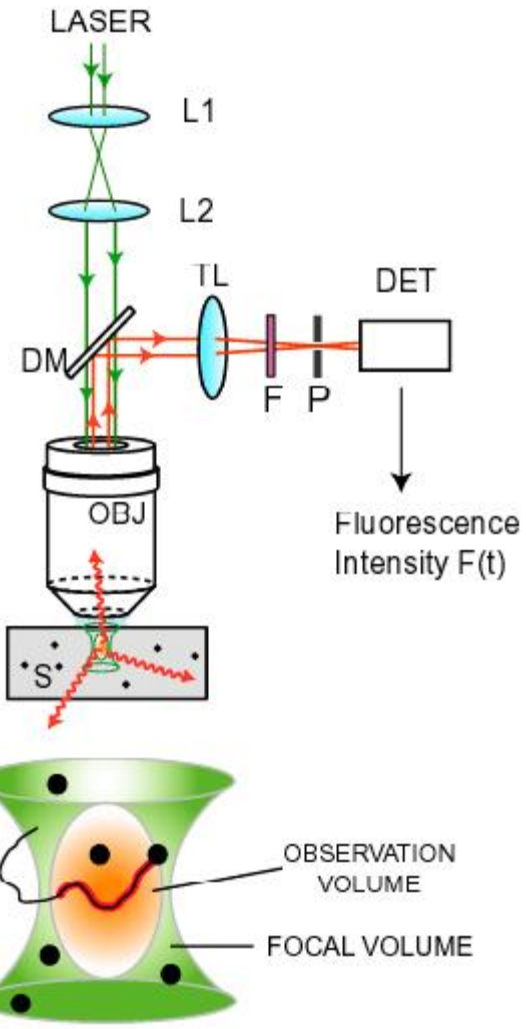
|-> Resumen II



3. You need to consider sampling distances in Δx and $\Delta y \approx 50$ nm and $\Delta z \approx$ 150-300 nm for later deconvolution, or calculate the explicit sample distances @ http://support.svi.nl/wiki/NyquistCalculator



|-> Resumen III



4. Use the right inmersion setup !

   n1 = n2 !

   Keep refractive index / index of refraction constant !