Laboratory for SCIAN
Scientific Image Analysis

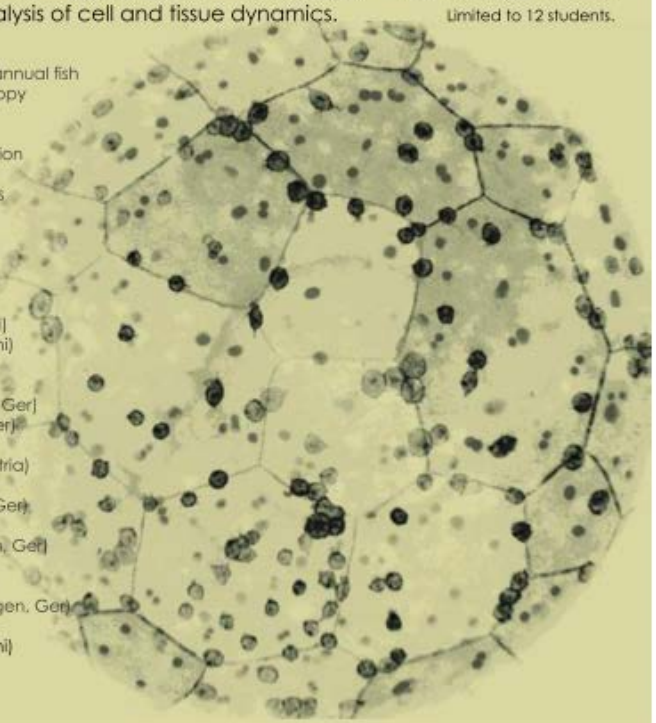
BIOMEDICAL NEUROSCIENCE INSTITUTE

Course: Optics, forces & development

In vivo 3D-microscopy for the analysis of cell behaviour in developing embryos

Santiago – Chile, January 14-29th, 2013

This practical and theoretical course is aimed at graduate students from Latin America interested in the use of optics and microscopic techniques for *in vivo* 3D visualisation and analysis of cell and tissue dynamics. Limited to 12 students.



Topics:

. Development of zebrafish and annual fish
. Confocal and spinning microscopy
. Light-sheet microscopy
. Super-resolution microscopy
. Photoactivation and laser ablation
. In vivo electroporation
. Force estimation in cells & tissues
. Optical tweezers
. Particle tracking
. Image processing and analysis

Teachers:

. Roberto Bernal (U. Santiago, Chi)
. Sebastián Brauchi (U. Austral, Chi)
. Miguel Concha (U. Chile, Chi)
. Mauricio Cerda (U. Chile, Chi)
. Jorg Enderlein (Univ. Gottingen, Ger)
. Nikta Fakhri (Univ. Gottingen, Ger)
. Steffen Härtel (U. Chile, Chi)
. Carl-Philipp Heisenberg (IST. Austria)
. Jorge Jara (U. Chile, Chi)
. Ulrich Kubitscheck (Univ. Bonn, Ger)
. Omar Ramirez (U. Chile, Chi)
. Florian Rehfeldt (Univ. Gottingen, Ger)
. German Reig (U. Chile, Chi)
. Felipe Santibañez (U. Chile, Chi)
. Christoph Schmidt (Univ. Gottingen, Ger)
. Jan Spille (Univ. Bonn, Ger)
. Juan Pablo Staforelli (CEFOP, Chi)

Organisers:

. Miguel Concha (U. Chile)
. Steffen Härtel (U. Chile)

Travel Fellowships will be available. Indicate your interest in the application.

To apply send:
. Curriculum Vitae
. Letter of Intention
. Reference from supervisor/mentor
To: mconcha@med.uchile.cl

Deadline for application - December 26th 2012
Results - December 28th 2012

LEO SCIAN

Universidad de Chile

CONICYT
FONDECYT 1120558, 1120579 & 3130598
FONDEF D11I1096

Iniciativa Científica Milenio ICM

Biomedical Neuroscience Institute
CHILE
ICM P09-015-F

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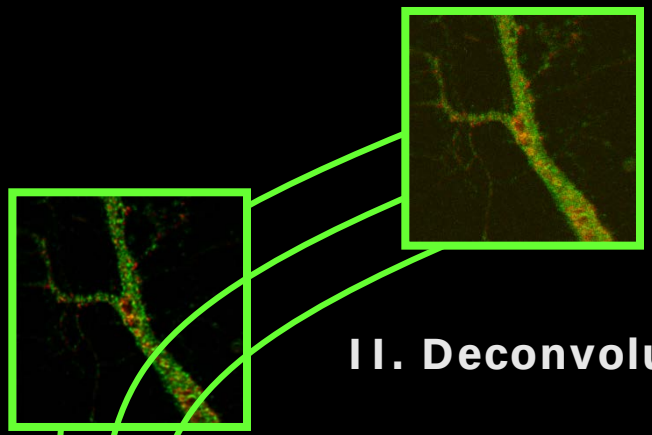


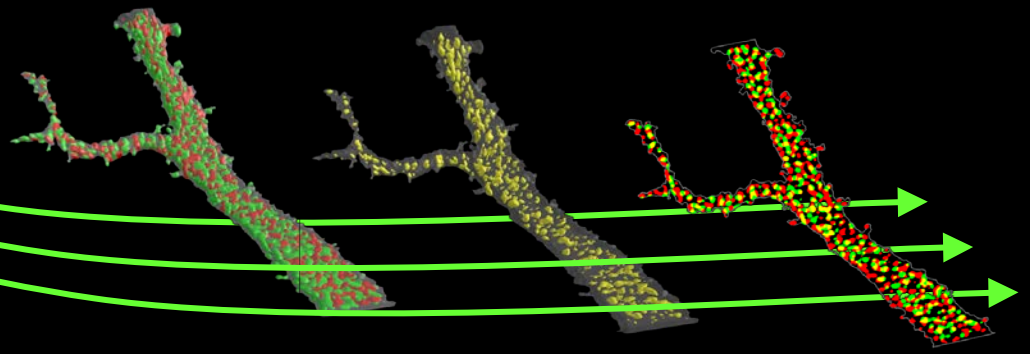
I. Image Adquisition

I.a|-> Motivation
I.b|-> Fundamentos de la microscopía confocal
I.c|-> Fundamentos de la fluorescencia

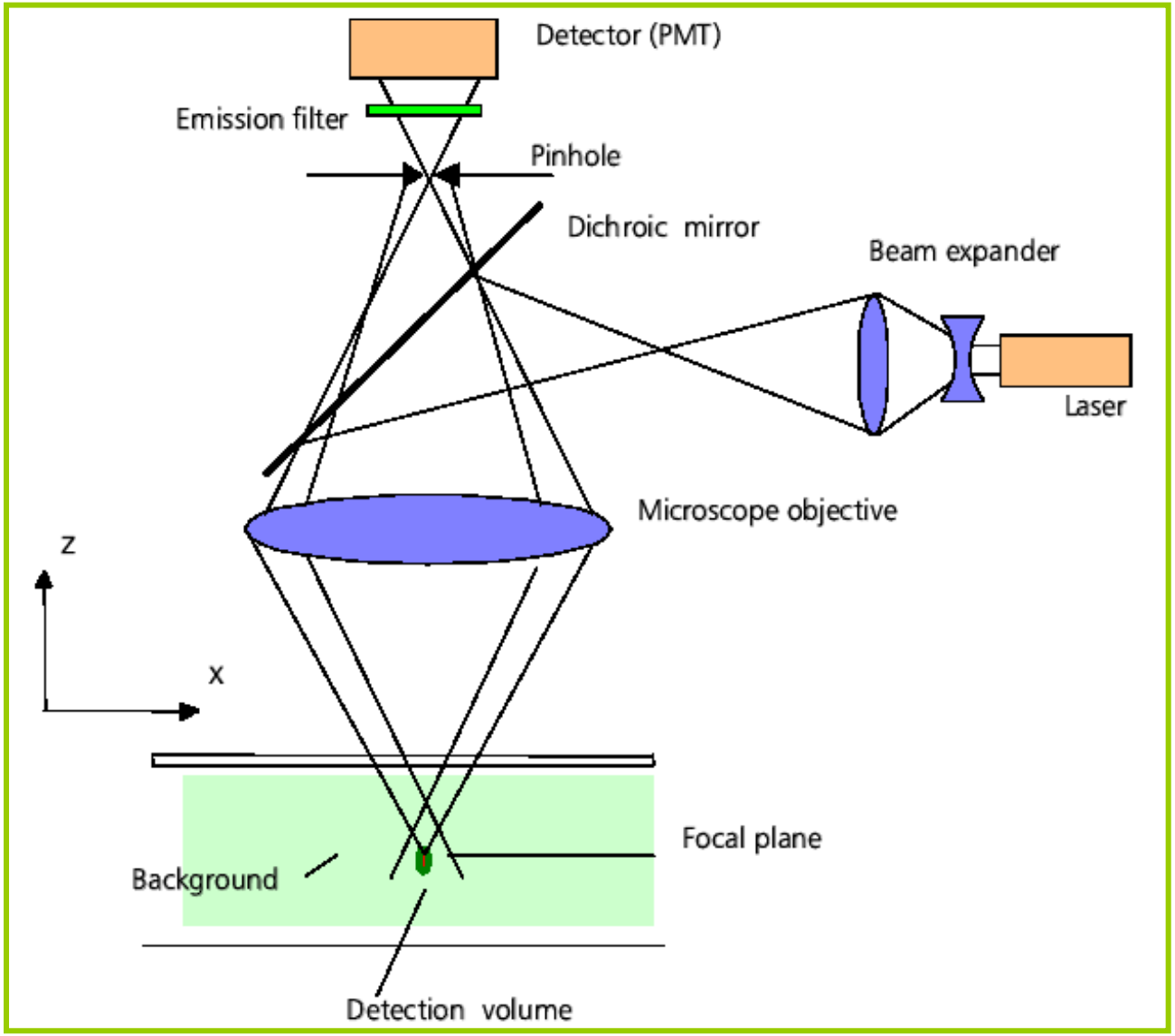
II. Deconvolution

III. Segmentation

IV. Analisys



|-> Diffraction limited 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.

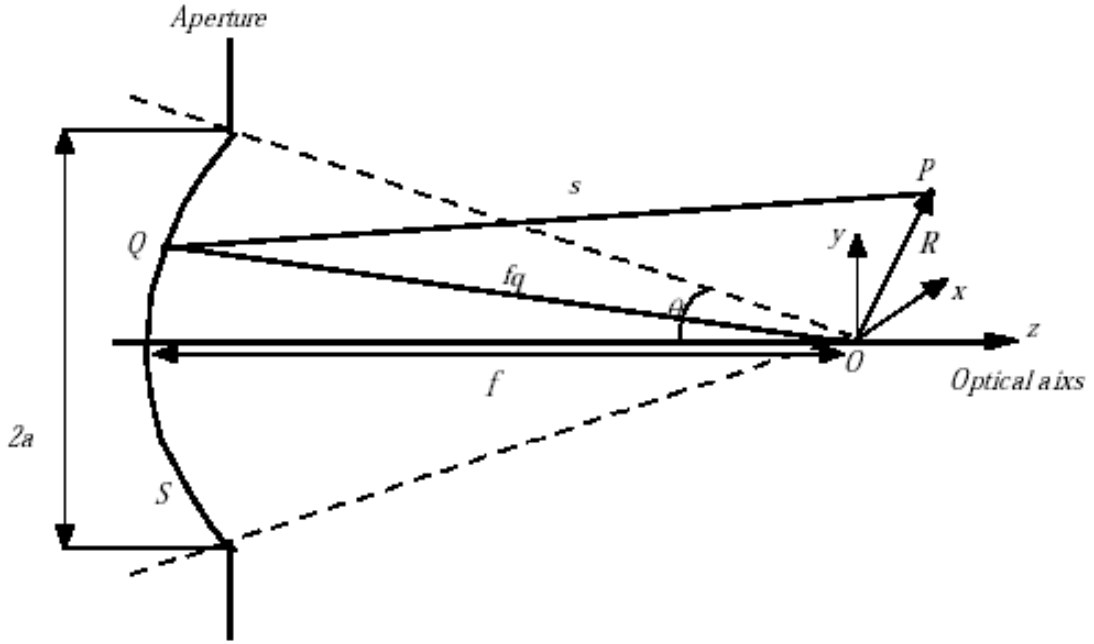


Figure 2.1 Diffraction of a converging spherical wave at a circular aperture

|-> Diffraction limited Microscopy

Óptica no-geométrica /

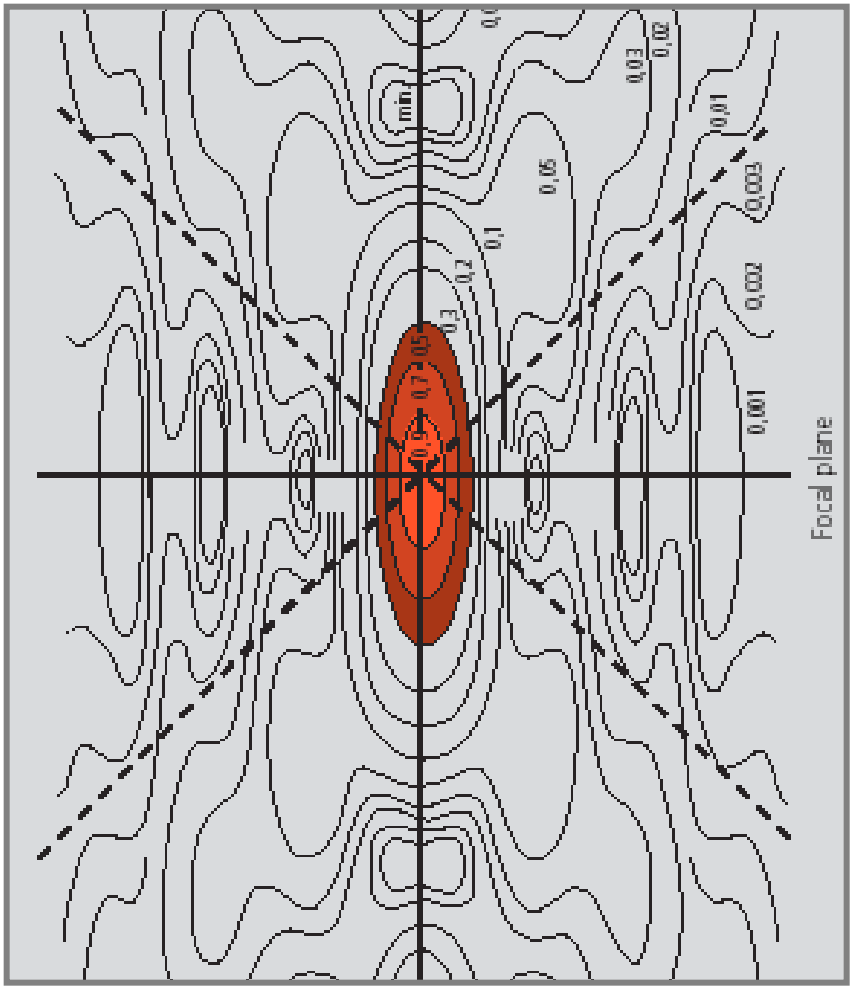
Teoría de difracción

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)

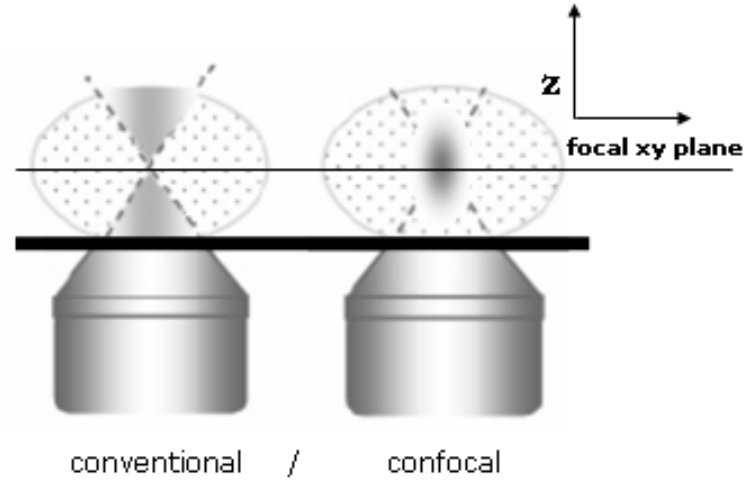


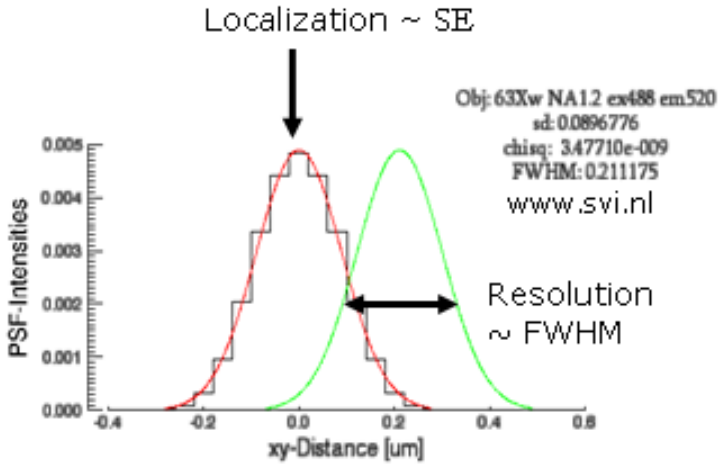
| Diffraction limited microscopy

E. Abbe († 1905)

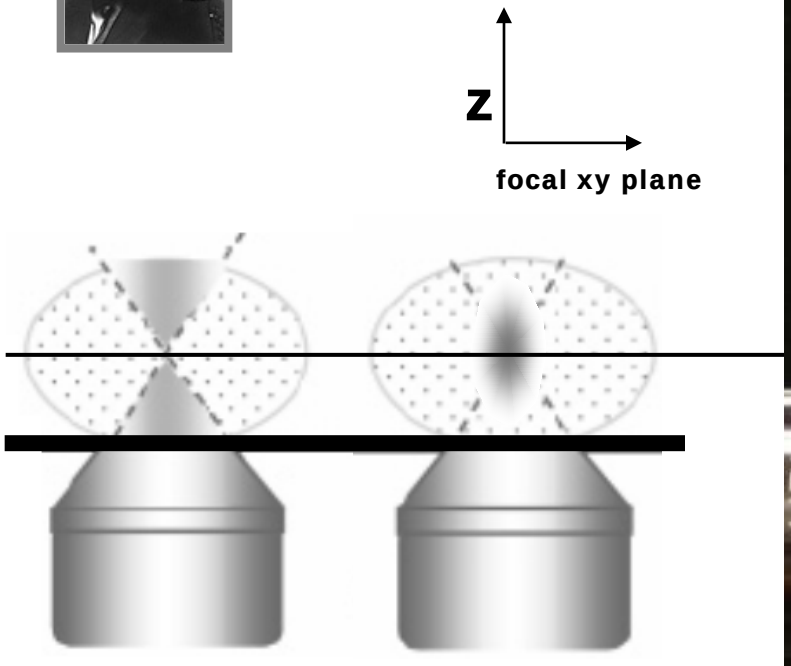
$\lambda / 2 \cdot NA \sim \lambda / 2$ Resolution (Full Width at Halft Maximum, FWHM)

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

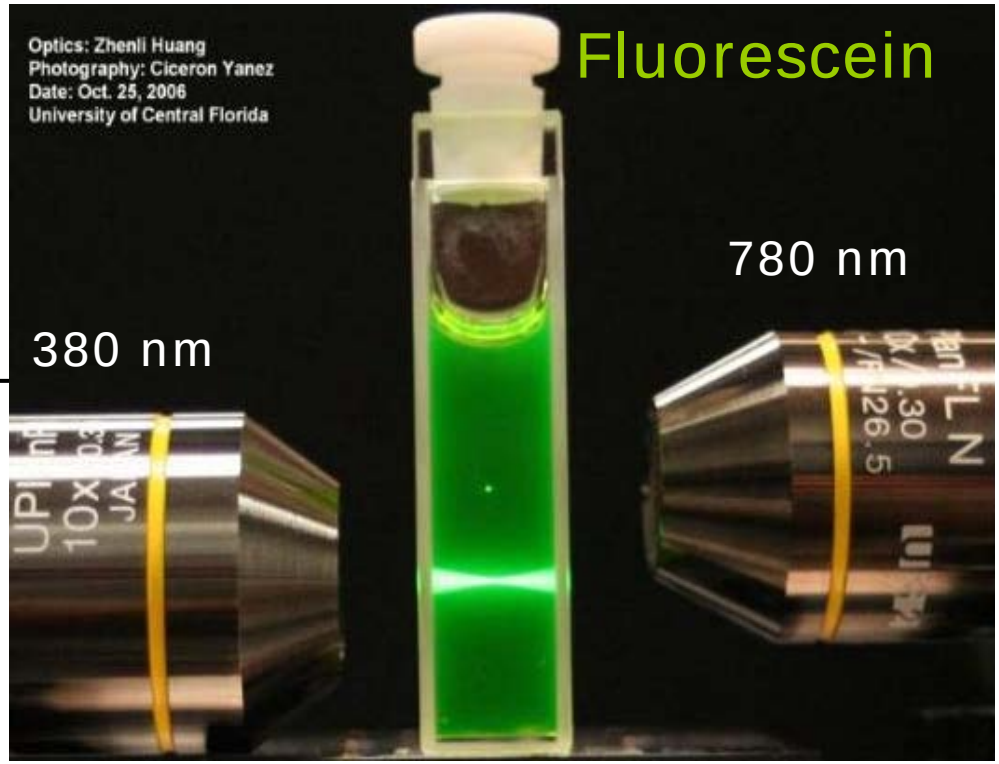




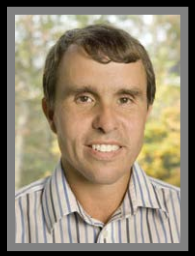
| Best localization: confocal microscopy

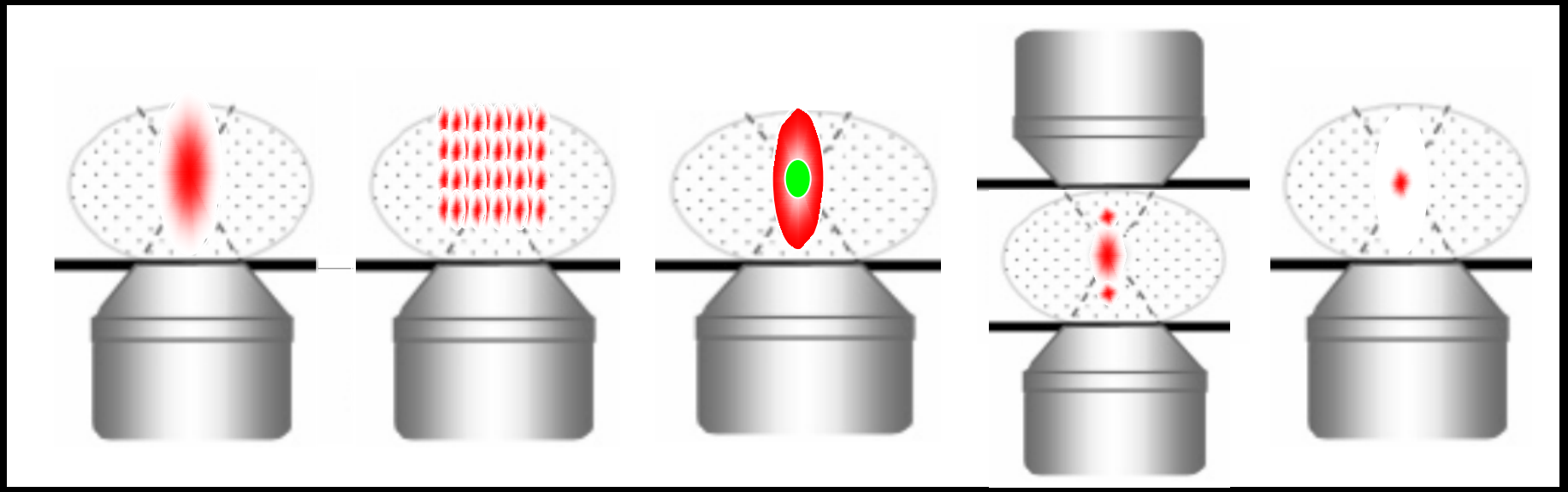


convencional / confocal



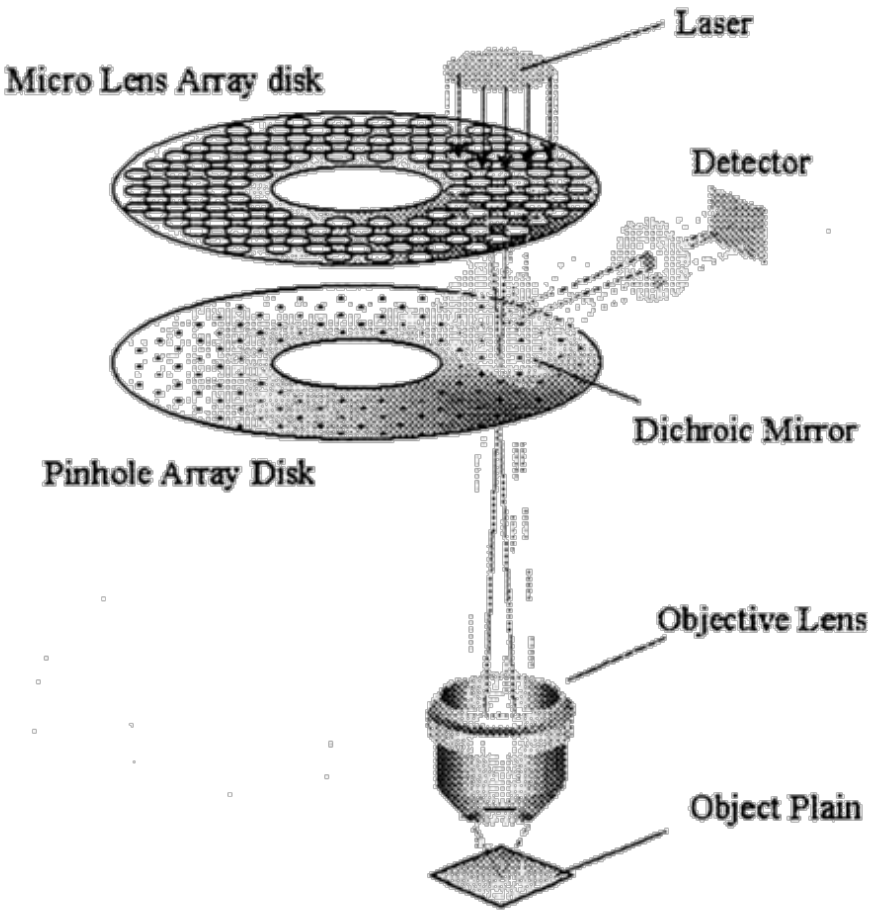
|-> Beyond diffraction

M Goeppert-Mayer	M Gustafson	S Hell		E Betzig
1906-1972	1960-2011	MPI Göttingen BIOQUANT Hdg		Janelia Farm
				
FWHM(xy) ~ λ/2	~ λ/4	~ λ/∞	~ λ/4	~ λ/100

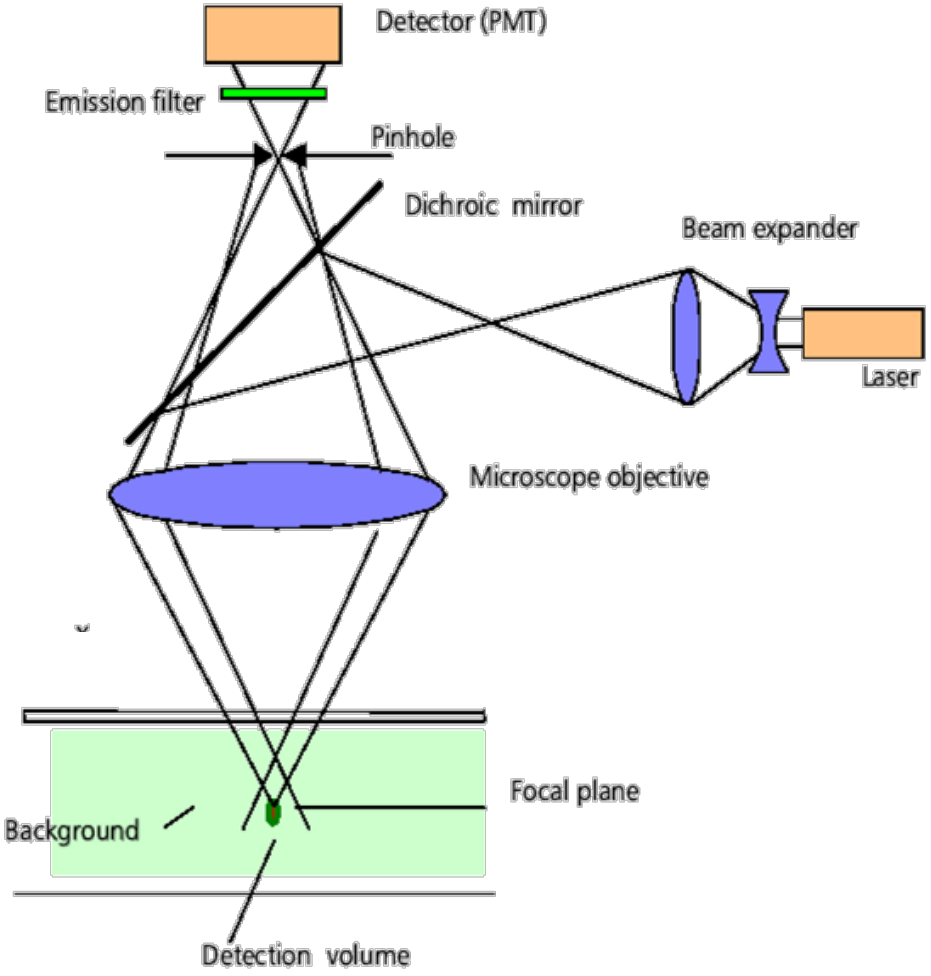


2-photon SIM STED 4-π PALM

|-> Spinning Disk

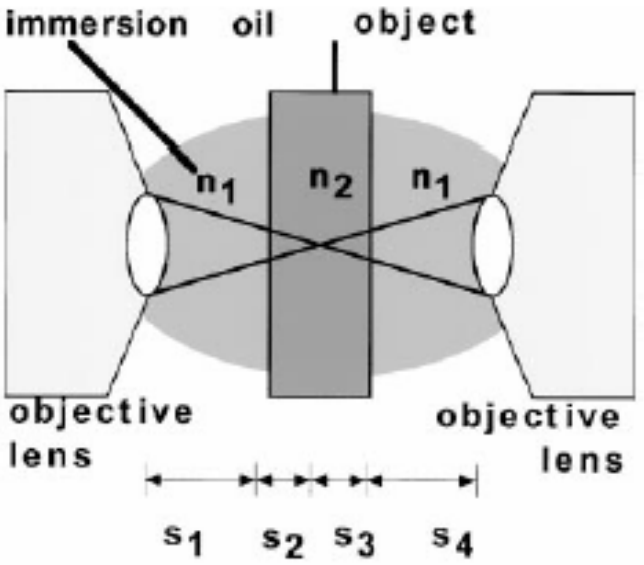


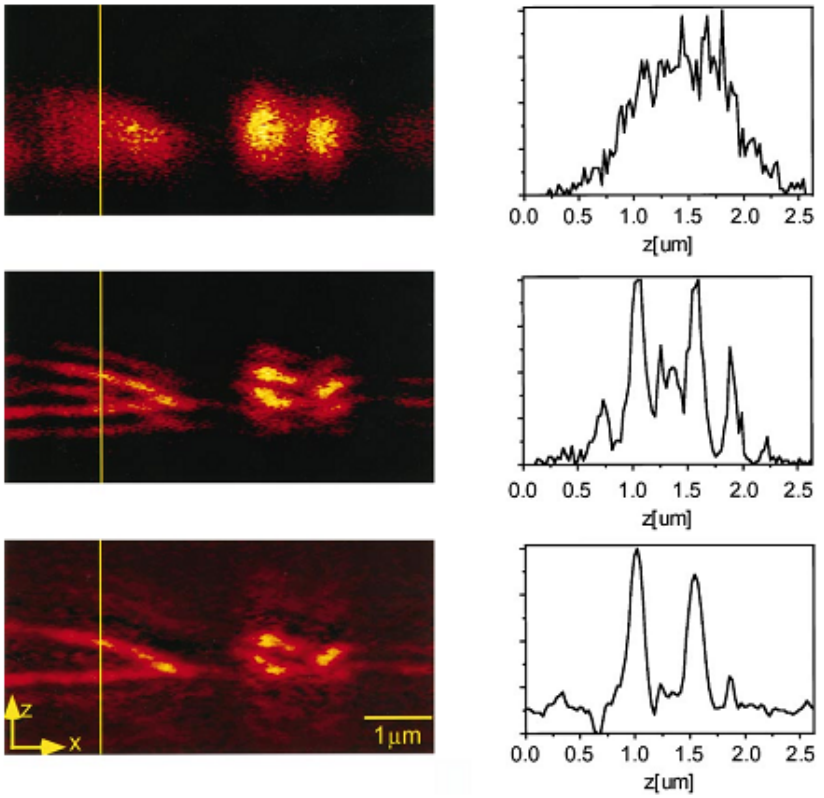
spinning disk



confocal

|-> 4π Microscopy





4Pi-Confocal Imaging in Fixed Biological Specimens

Martin Schrader,* Karsten Bahlmann,* Günter Giese,# and Stefan W. Hell*

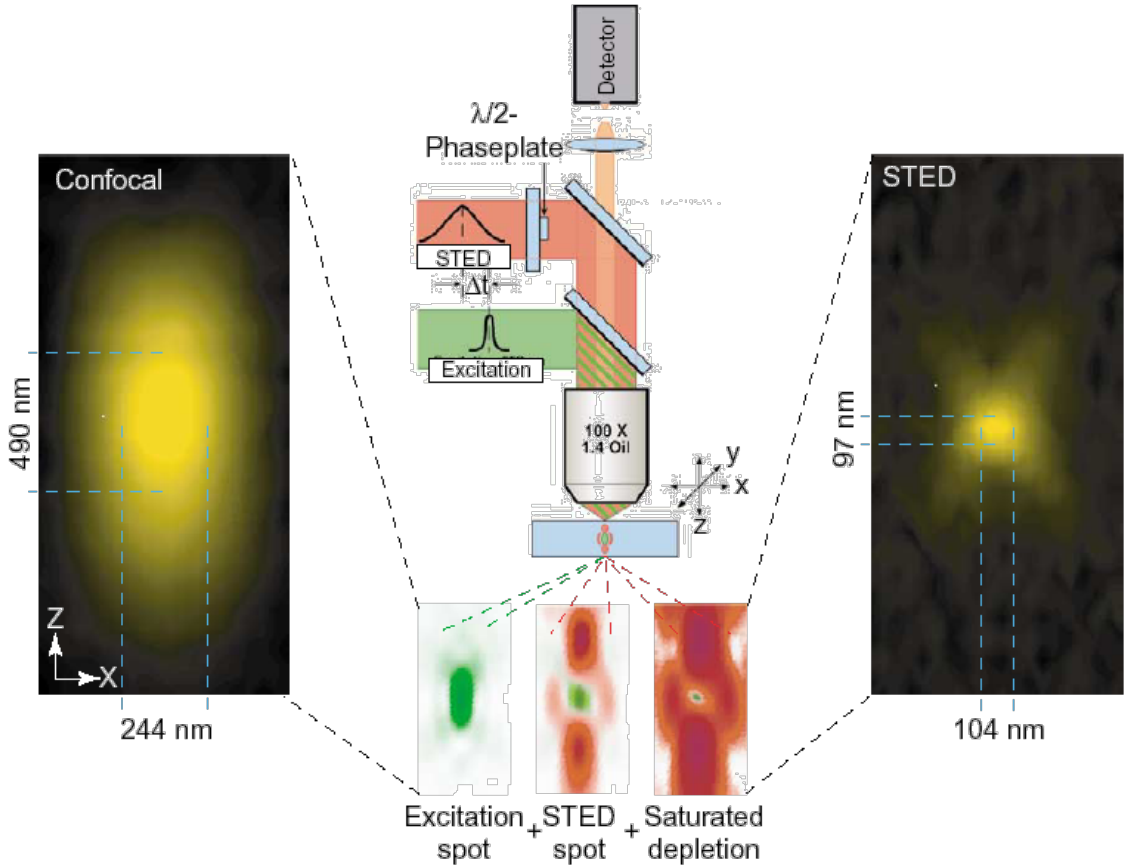
Biophysical Journal Volume 75 October 1998 1659–1668

STED Microscopy

Stimulated

Emission

Depletion



Concepts for nanoscale resolution in fluorescence microscopy

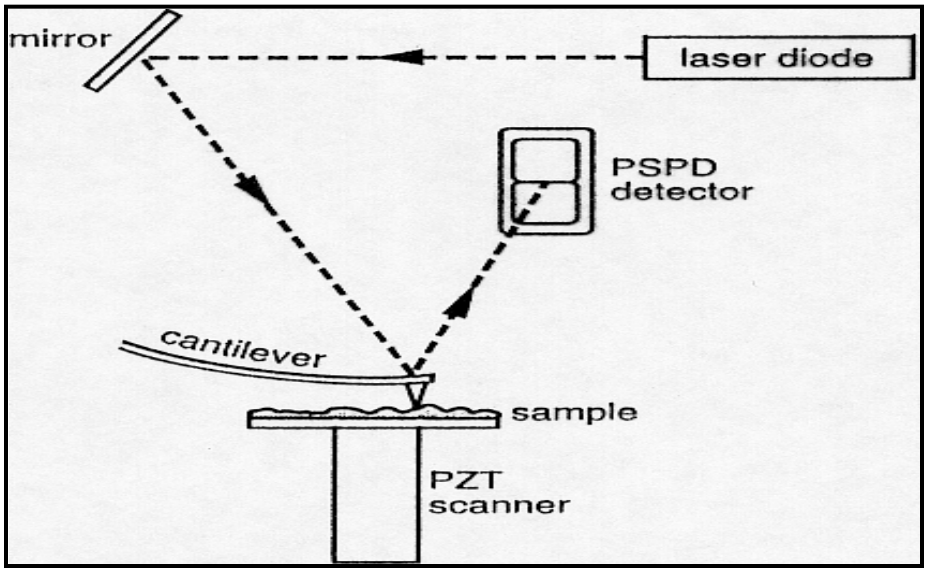
Stefan W Hell*, Marcus Dyba[1] and Stefan Jakobs[2]

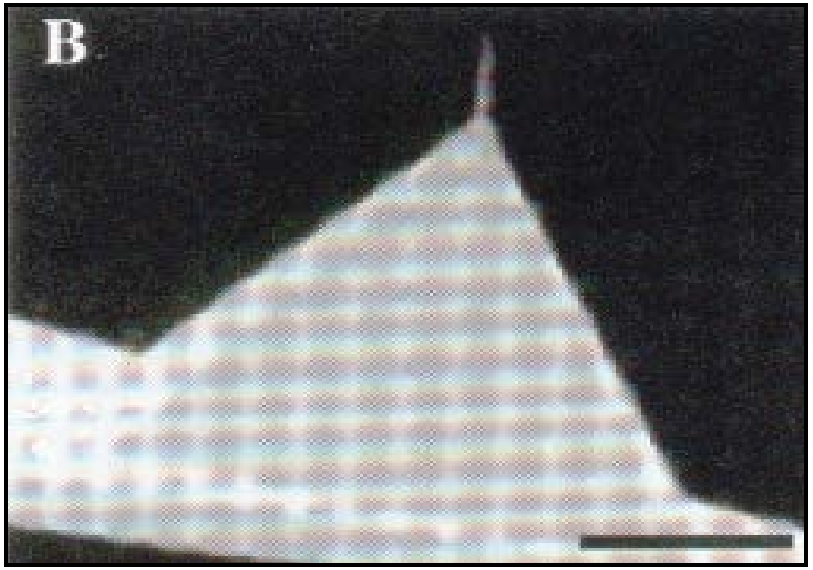
Current Opinion in Neurobiology 2004, **14**:599–609

|-> Atomic Force Microscopy

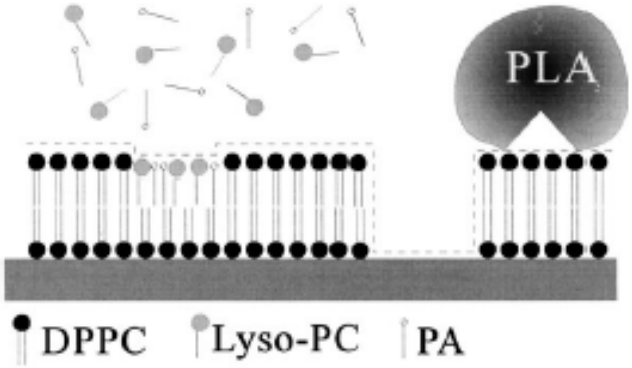
AFM allows the investigation of structural and functional properties of biomolecules in liquid environments, by a unique combination of :

- ***subnanometer*** spatial resolution
- ***millisecond*** temporal resolution
- ***piconewton*** force sensitivity

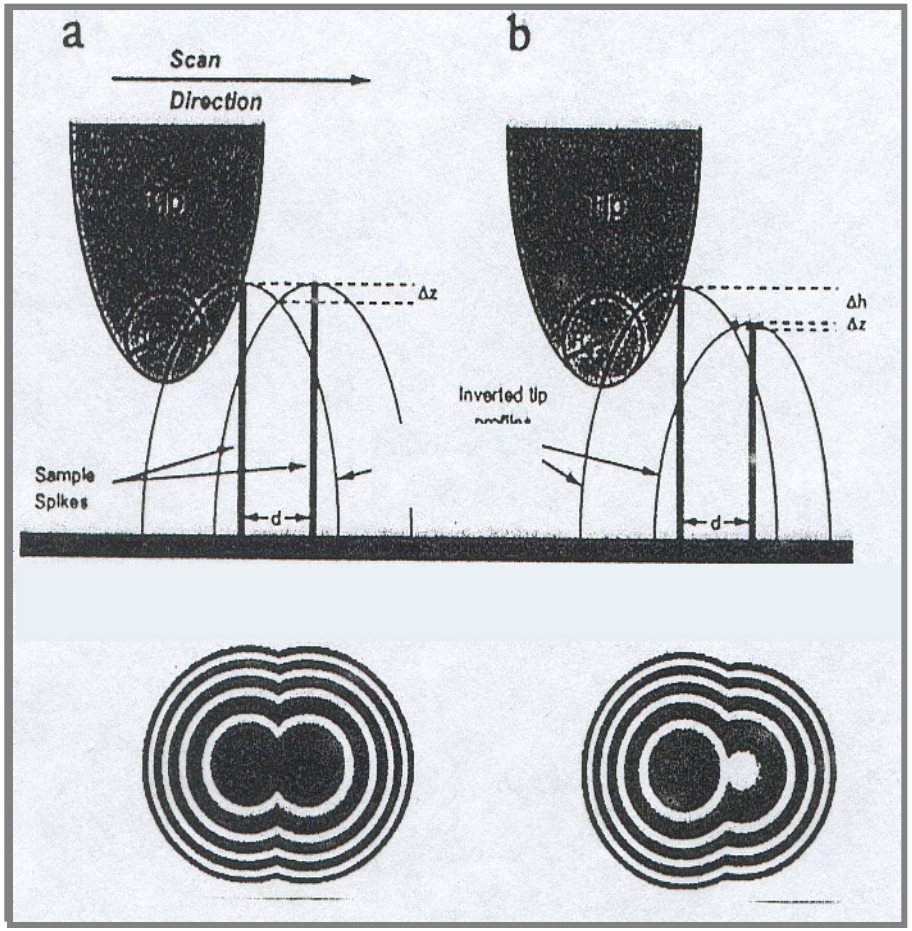




|-> Atomic Force Microscopy



M Grandbois et al. (1998) Biophys J.



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|-> PSF

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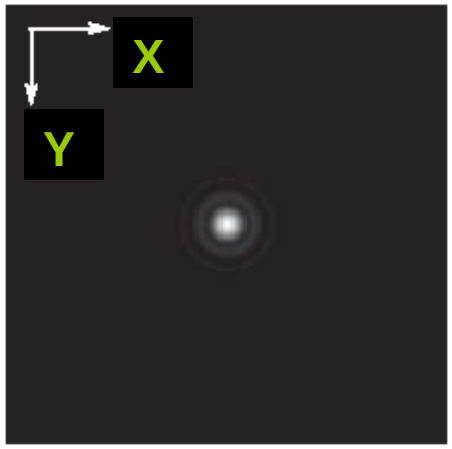
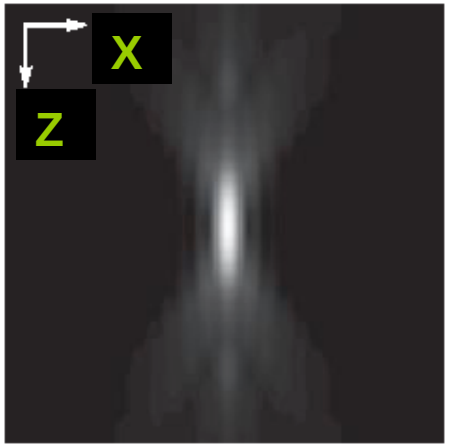
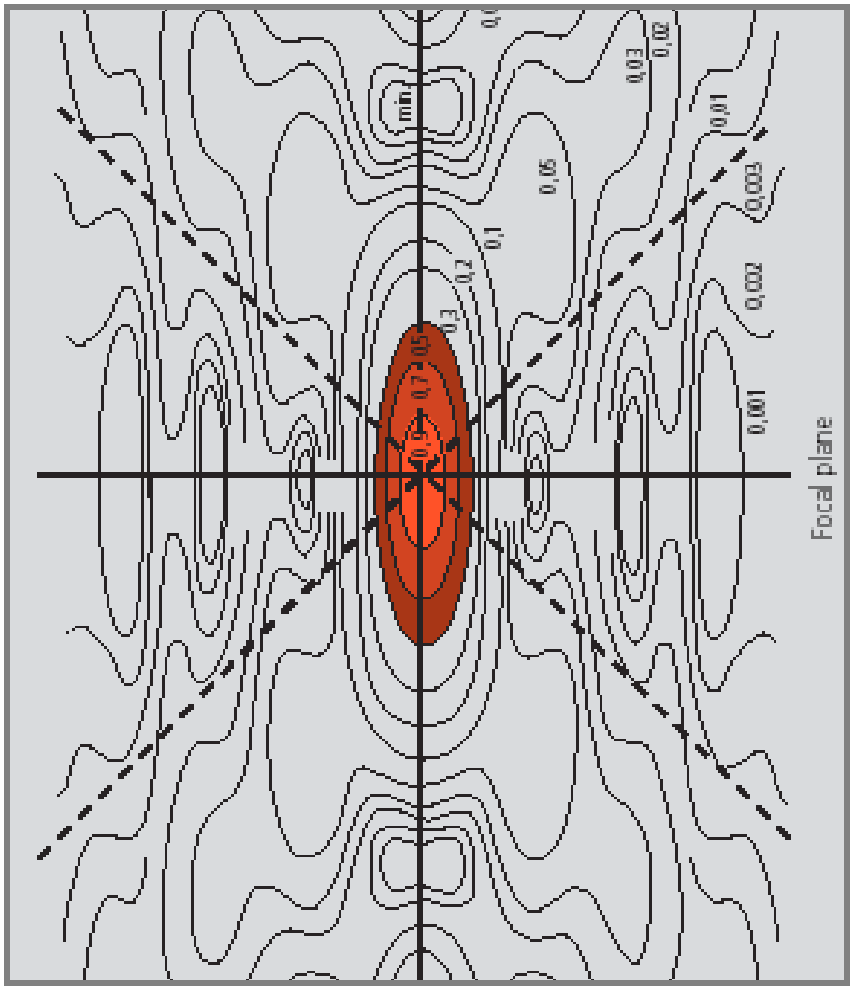
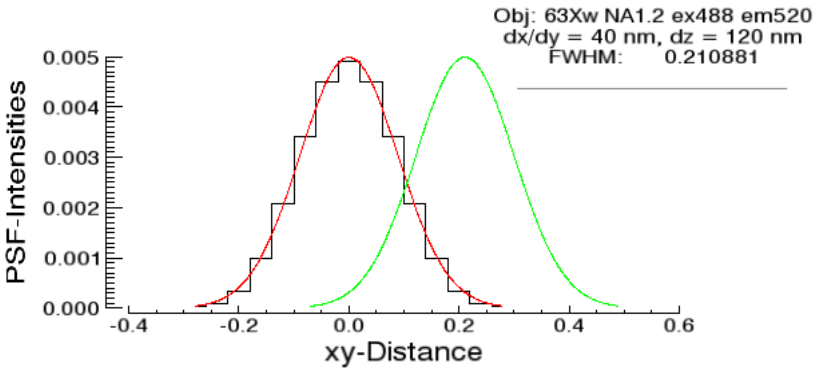
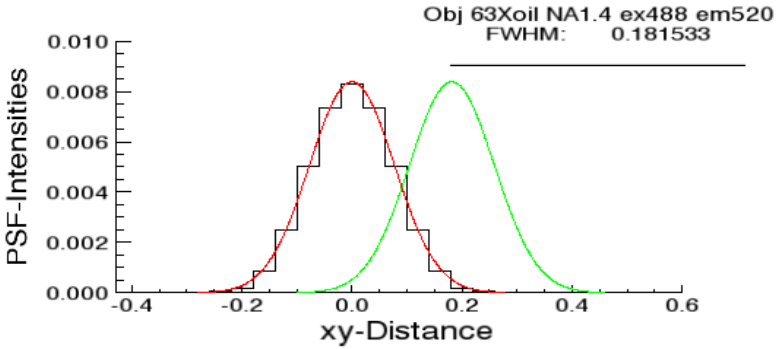


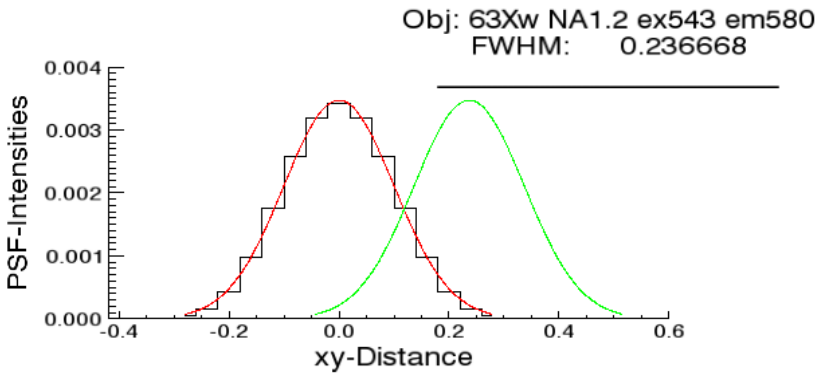


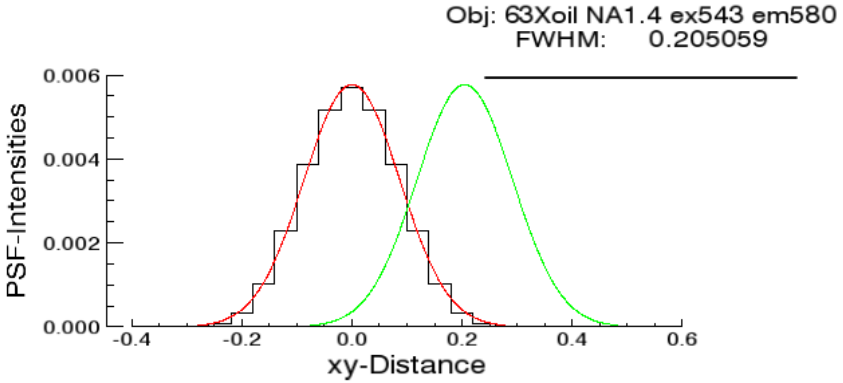


|-> PSF

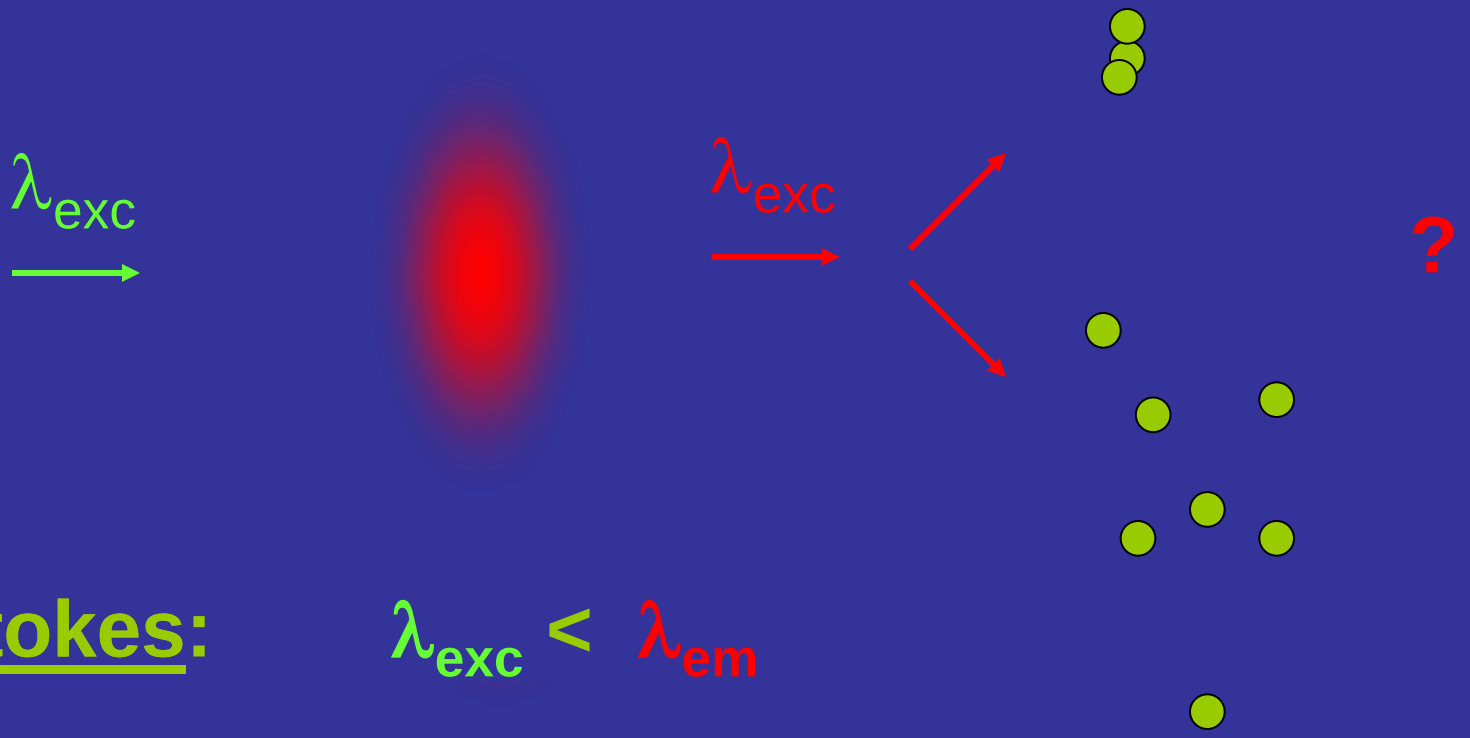

Obj: 63Xw NA1.2 ex488 em520
dx/dy = 40 nm, dz = 120 nm
FWHM: 0.210881
PSF-Intensities
0.005
0.004
0.003
0.002
0.001
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance


Obj 63Xoil NA1.4 ex488 em520
FWHM: 0.181533
PSF-Intensities
0.010
0.008
0.006
0.004
0.002
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance


Obj: 63Xw NA1.2 ex543 em580
FWHM: 0.236668
PSF-Intensities
0.004
0.003
0.002
0.001
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance

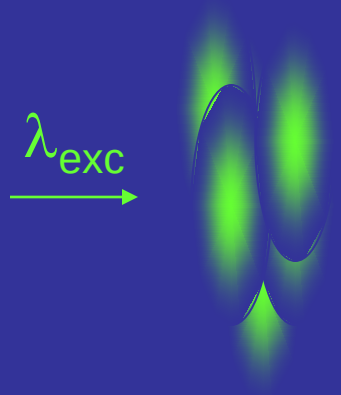

Obj: 63Xoil NA1.4 ex543 em580
FWHM: 0.205059
PSF-Intensities
0.006
0.004
0.002
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance

|-> Convolution



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

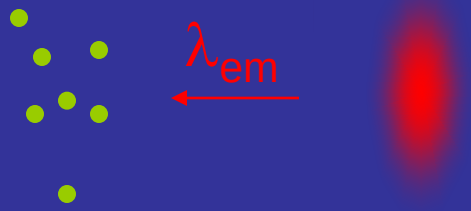
|-> Convolution



λ_{exc}

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

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



λ_{em}

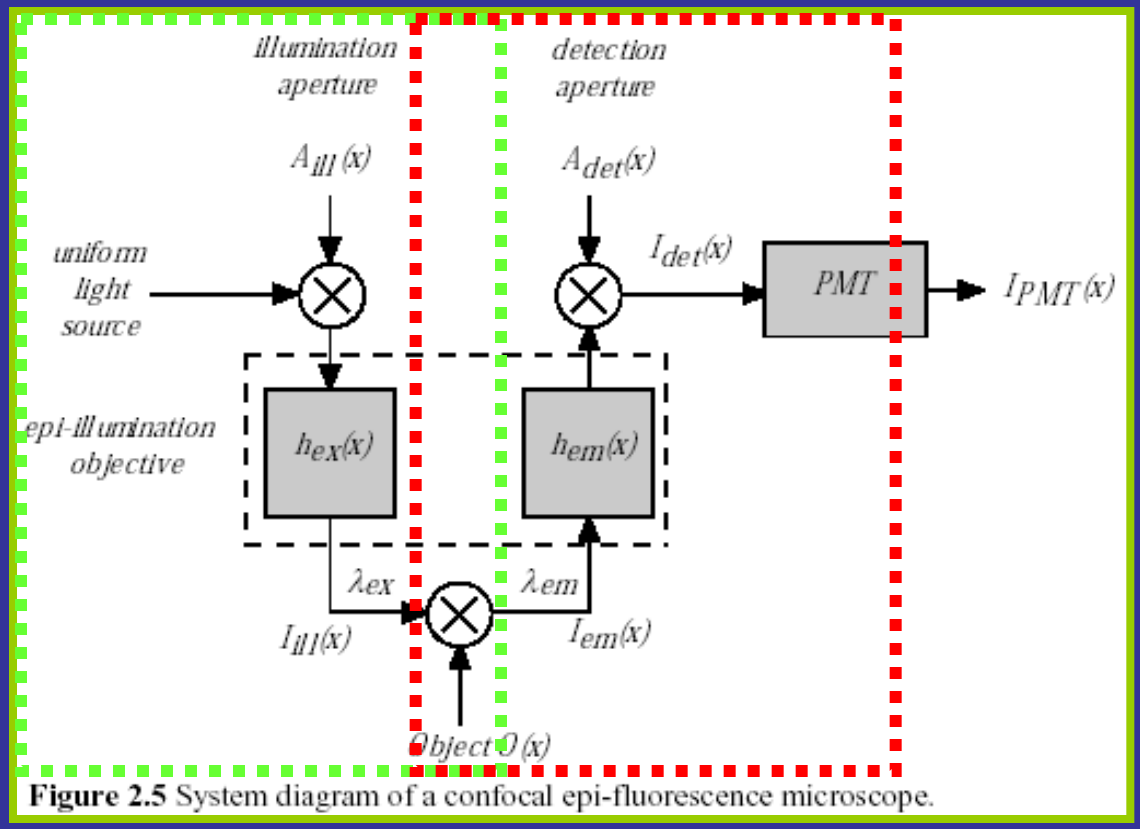
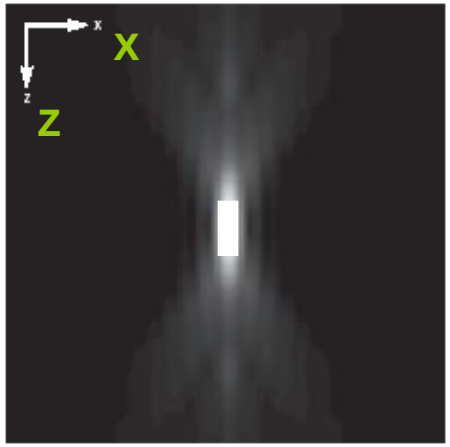


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

|-> Convolution



PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: *Point Spread Function*

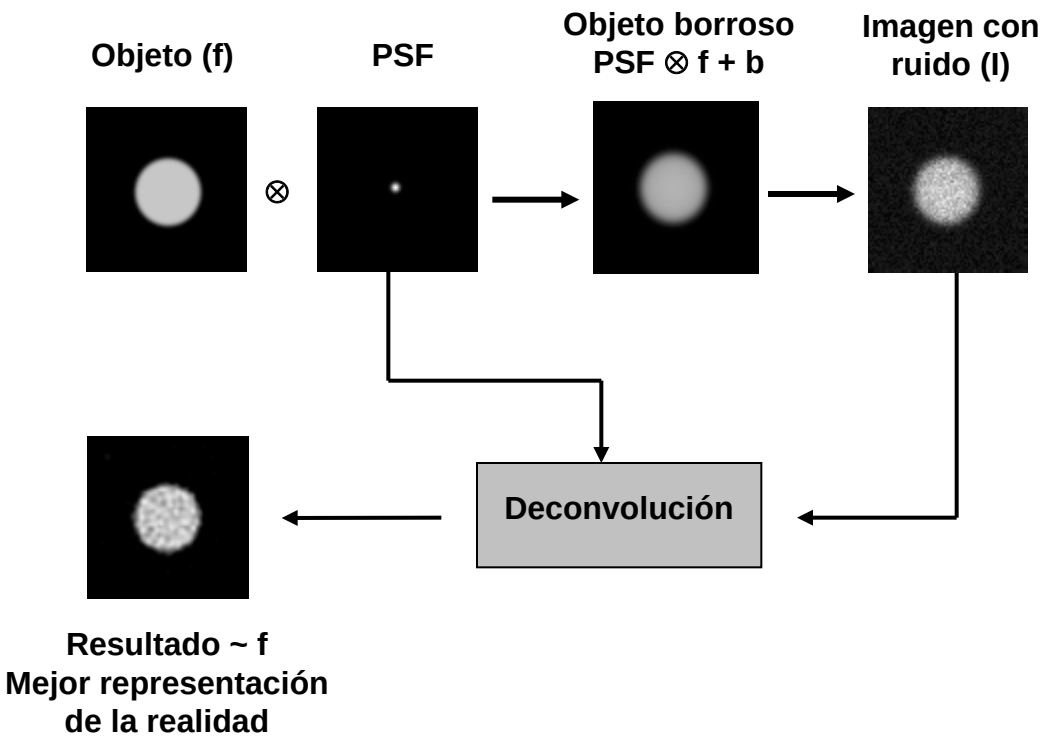
f: *Object Function*

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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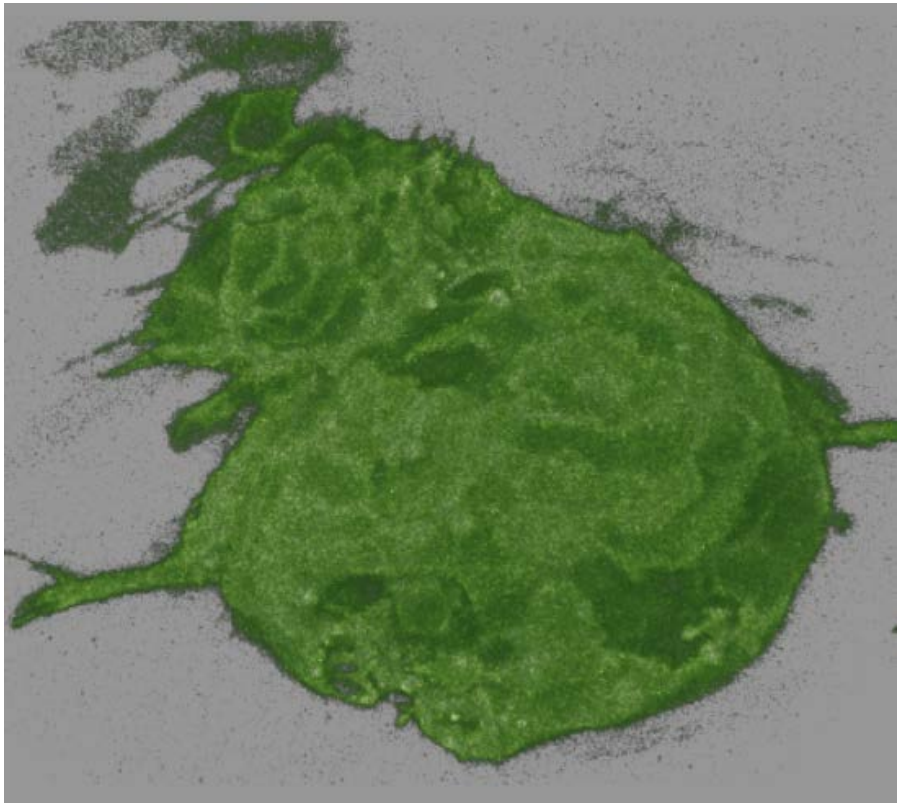
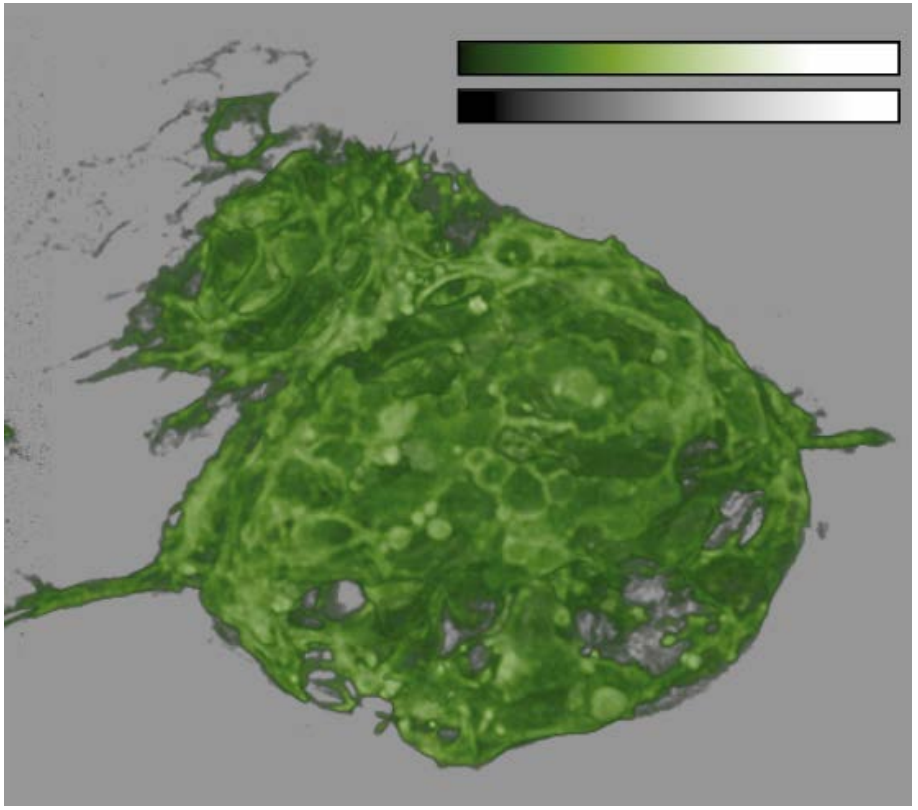




|-> Deconvolution

PSF: *Point Spread Function*

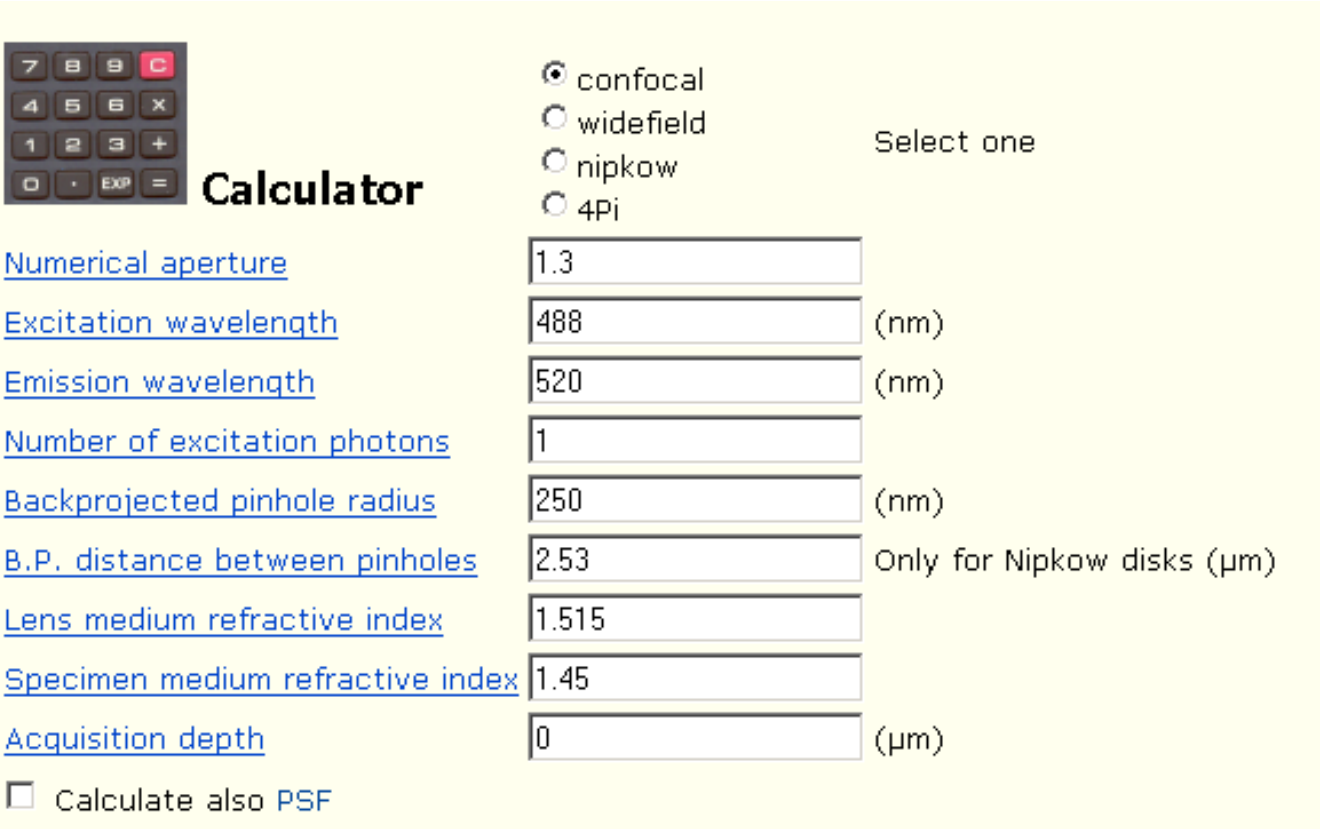
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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



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

|-> Pinhole

PSF: *Point Spread Function*

f: *Object Function*

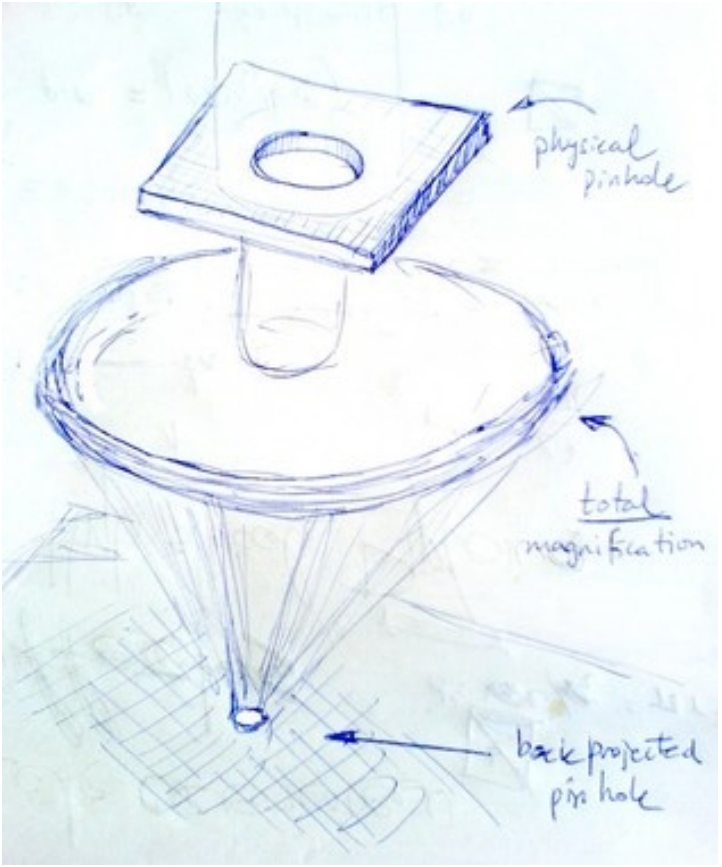
b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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

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*

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

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

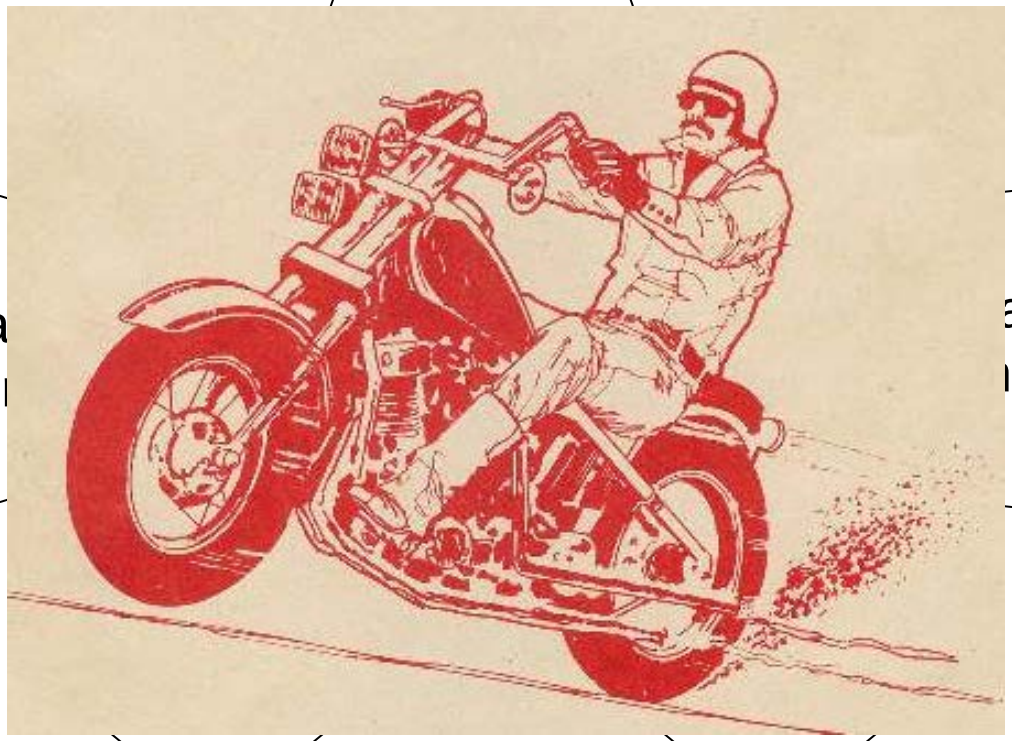
- Olympus FV300
- Olympus FV500
- Olympus FV1000

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)*

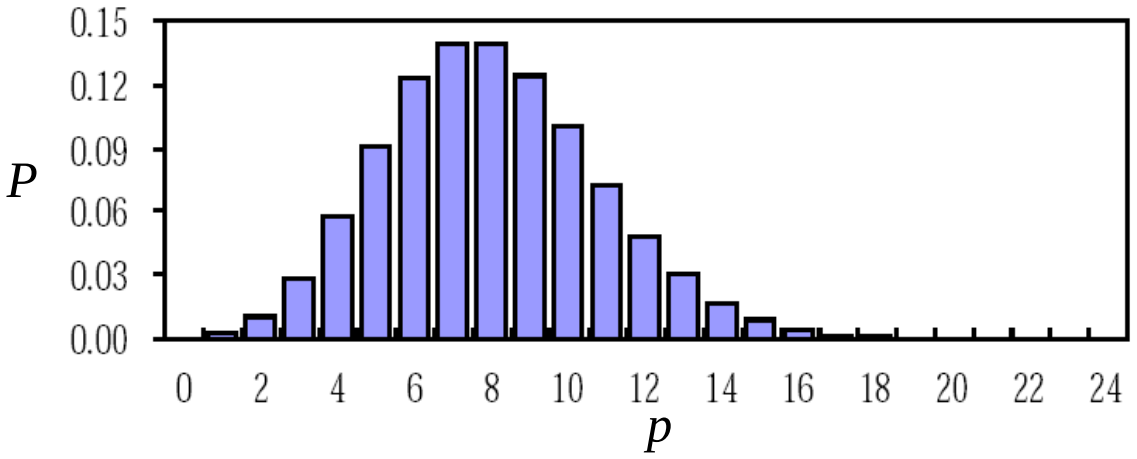
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

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

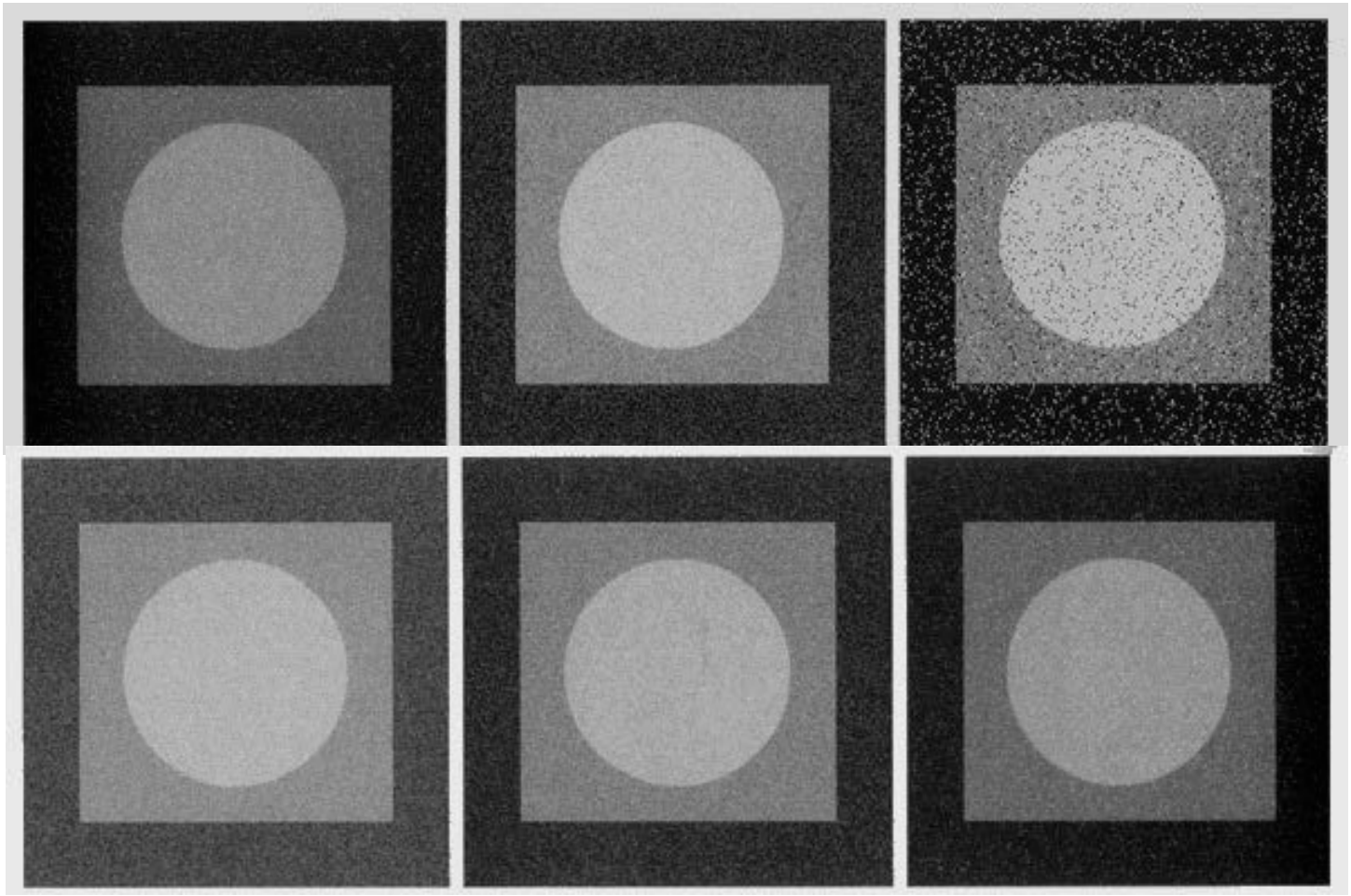
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

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

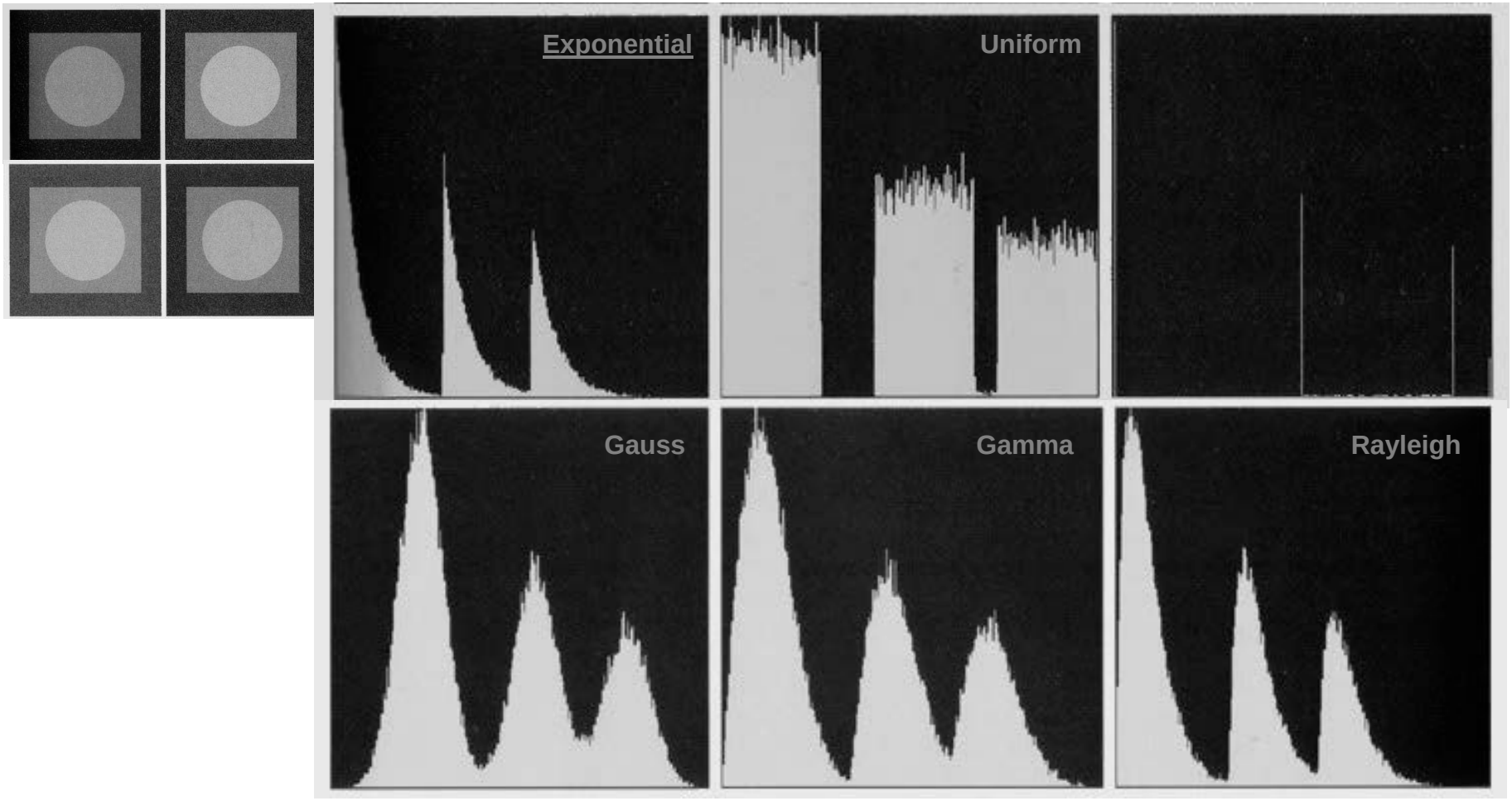
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> Noise



|-> Noise



|-> 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.

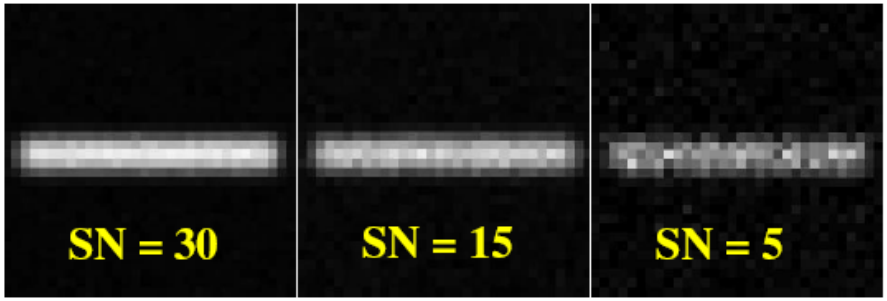
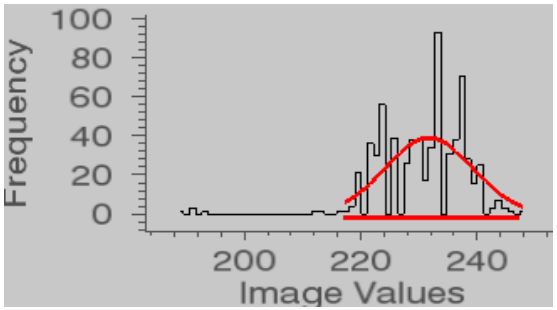
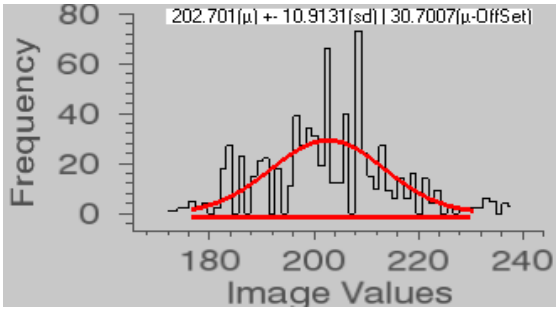
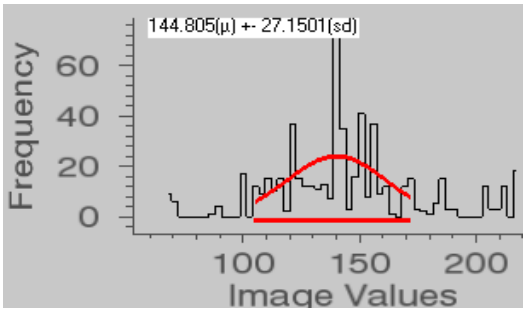


Figure 9. Images with different generated noise levels





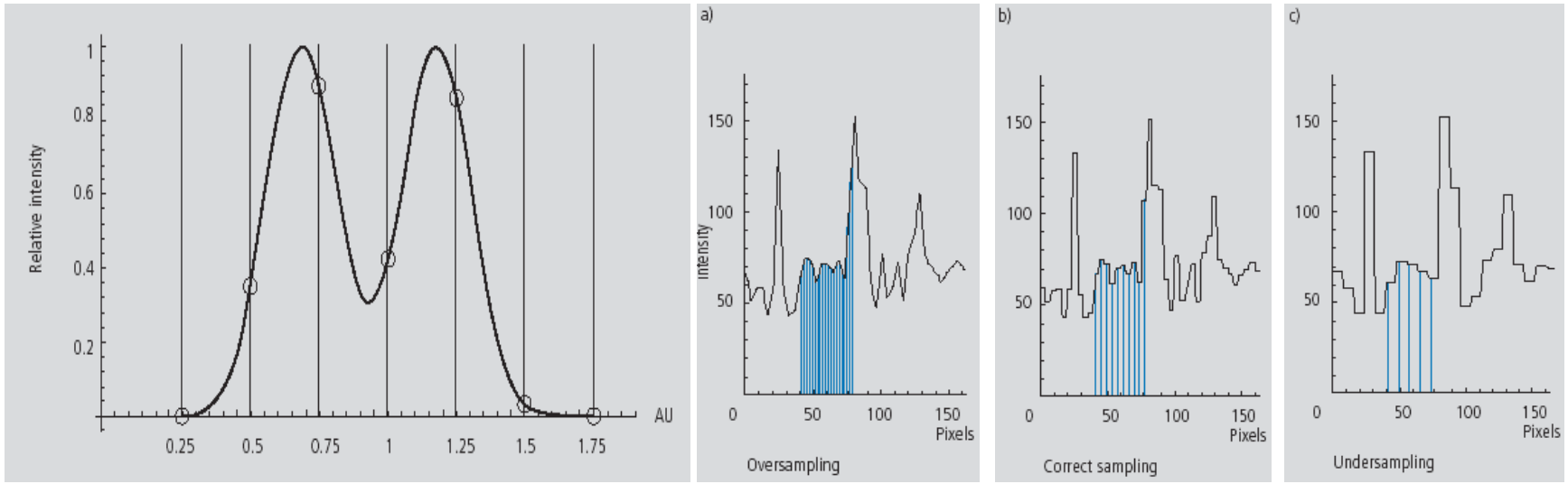


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

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

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

|-> 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

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

|-> PSF calculator

PSF: *Point Spread Function*

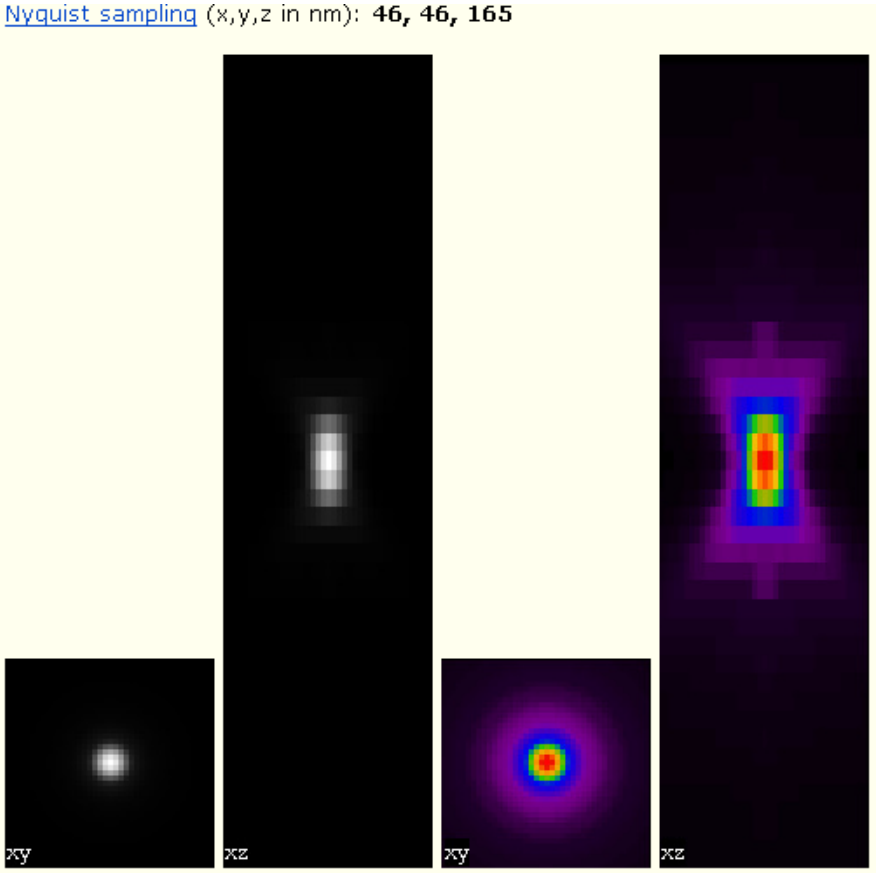
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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



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

|-> Snell‘s Law

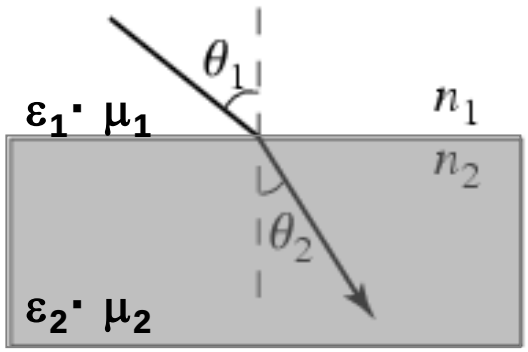
Index of refraction: $n = (\varepsilon \cdot \mu)^{1/2} = c/v$,

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:

$RI = n_1 / n_2 = v_2 / v_1$

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]

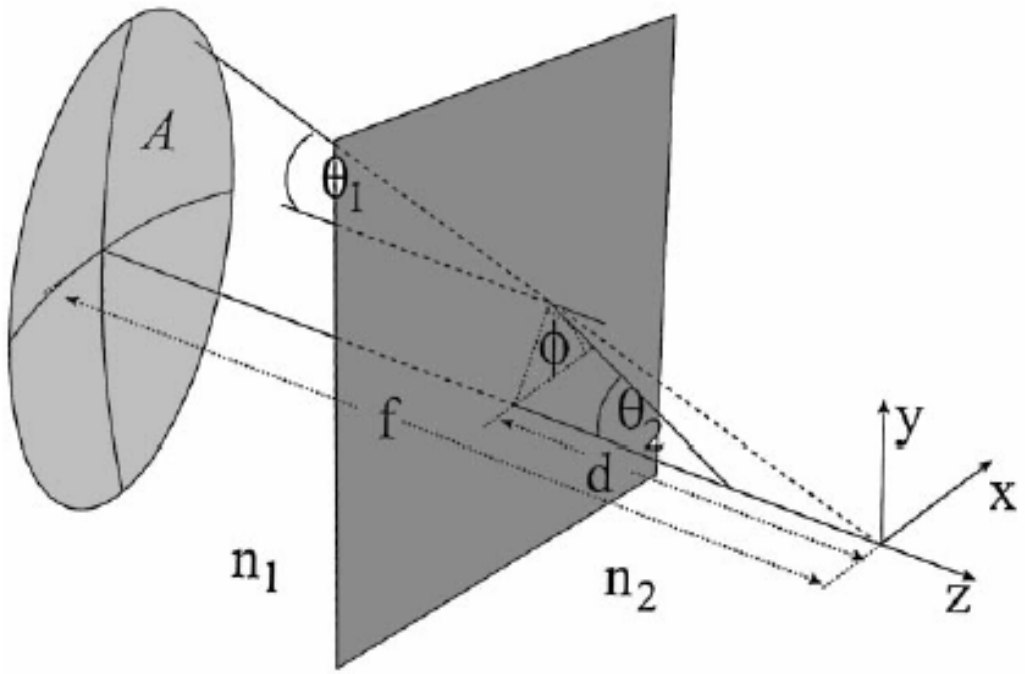
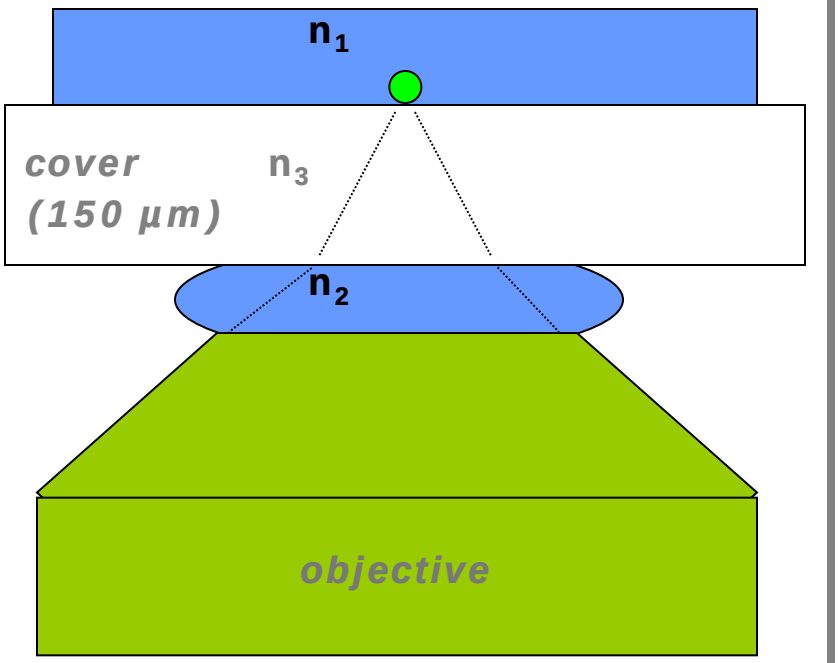


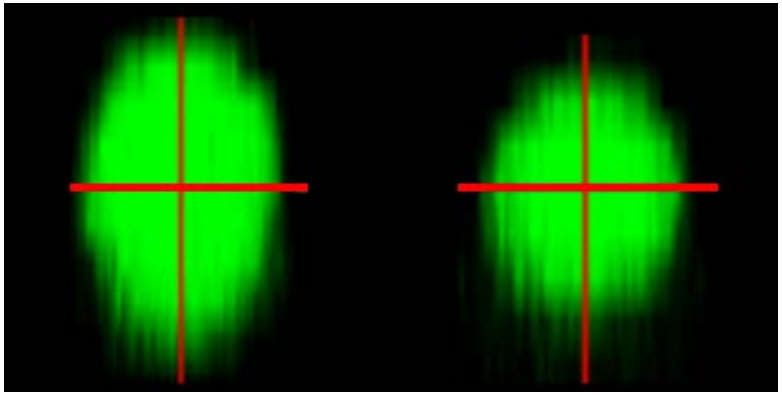
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera: ∅ = 6 µm**





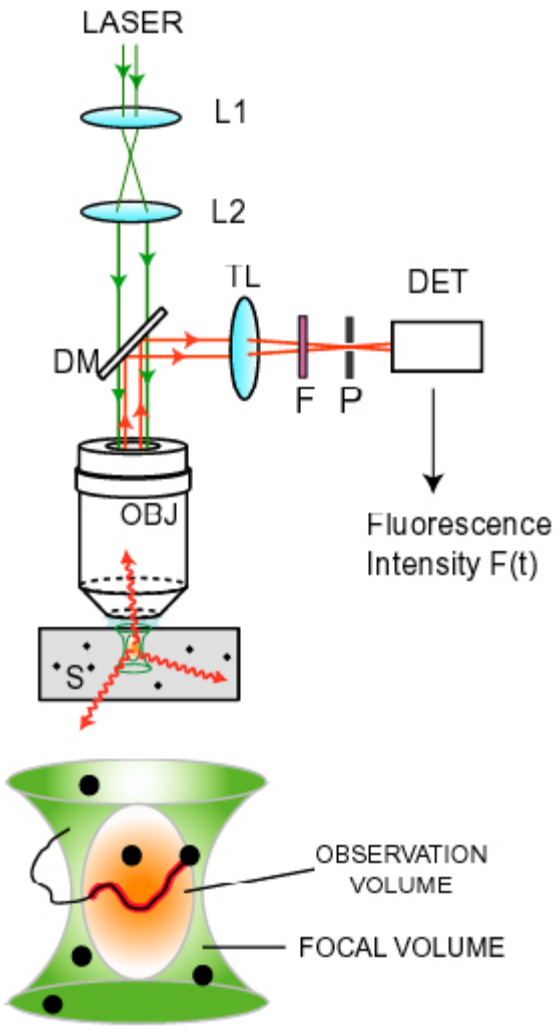
agua / aceite -- *aceite / aceite*

$n_1 \neq n_2$ $n_1 = n_2$

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

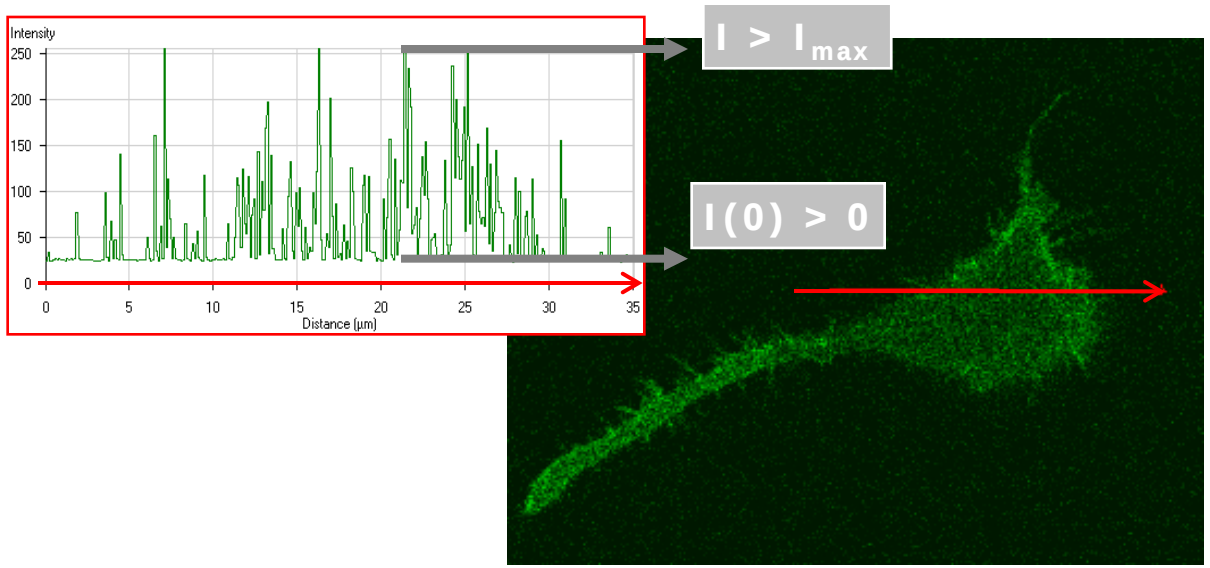
$n = n(\lambda)$!

|-> Resumen I

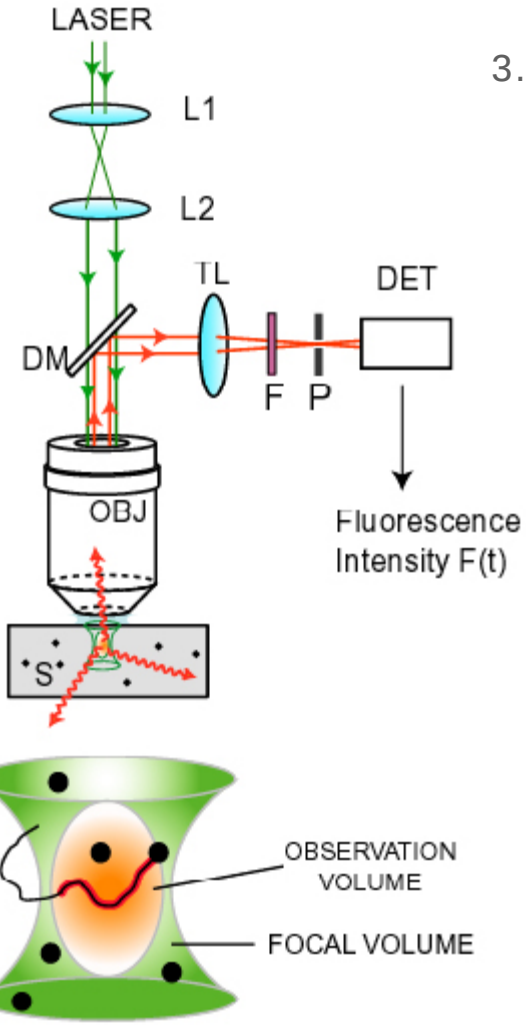


The observation volume (femtoliter) defined by the Point Spread Function must be considered as a mini-sprectrofluorimeter.

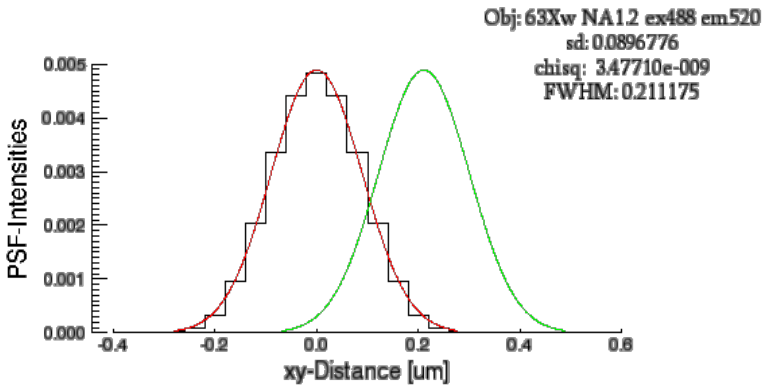
1. You need to consider the Offset I(0) in order to calibrate your signal $I(0) \geq 0$!
2. Never saturate the signal: $I \leq I_{max}$ (255 for 8 bit) !



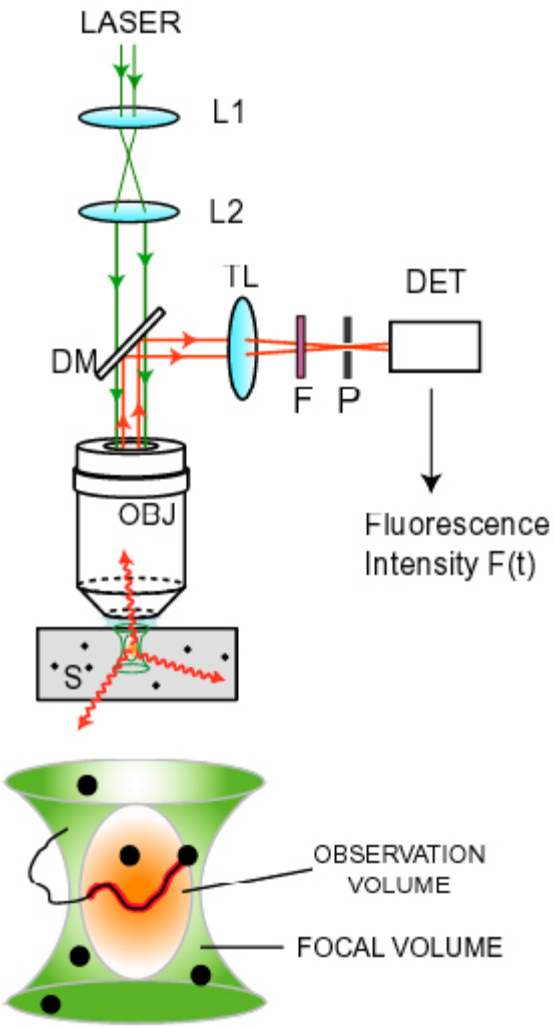
|-> Resumen II



3. You need to consider sampling distances in Δx and $\Delta y \approx 50$ nm and $\Delta z \approx 150\text{-}300$ 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 !