Welcome

MIM 2022: Procesamiento de Imágenes y Bioseñales I & II

Procesamiento de Imágenes y Bioseñales I

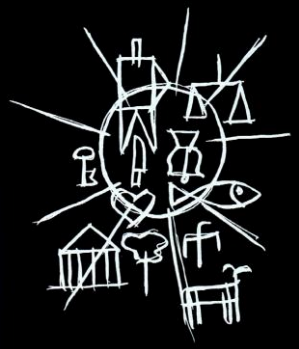
Prof. Dr. Steffen Härtel / Dr. Jorge Jara: www.scian.cl / www.cimt.cl / www.cens.cl

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)
Institute of Biomedical Sciences (ICBM)
Red de Salud Digital de Universidades del Estado (RSDUE)
Program for Integrative Biology
Escuela de Postgrado
Facultad de Medicina, Universidad de Chile

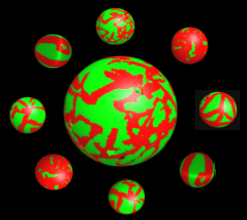
Welcome

Joaquín Torres García 1874-1949

Richard Feynman (1918-1988)

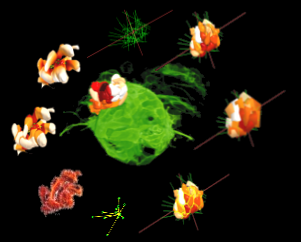


Mats Gustafson 2006 - 2011

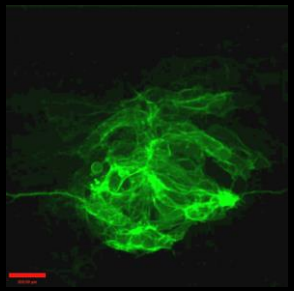


Maria Goeppert-Mayer 1906-1972

René Descartes (1596-1650)



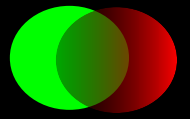
Ernst Abbe 1840- 2005

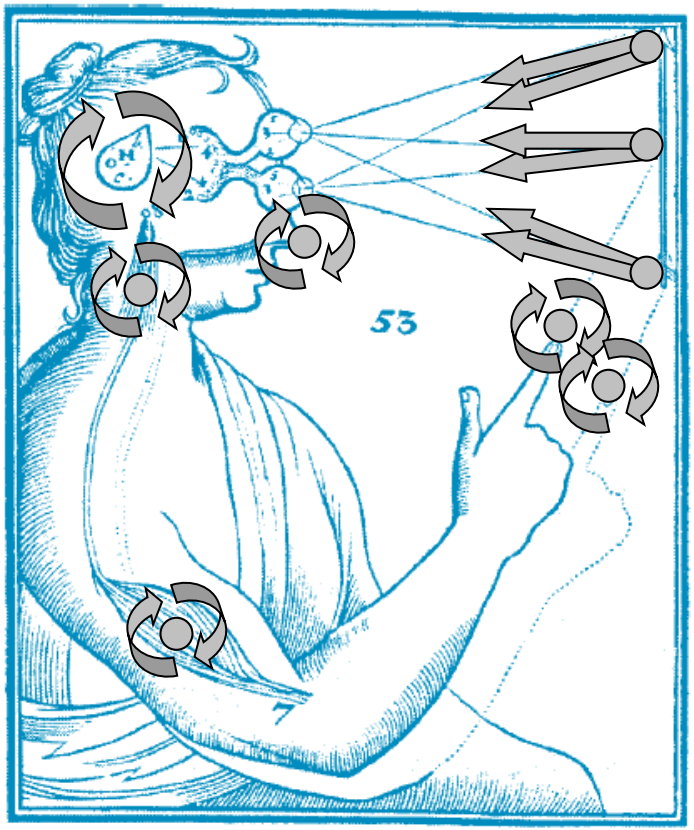


E Betzig

S Hell

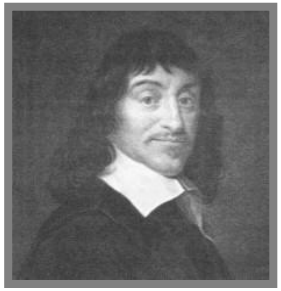
W Moerner





***glandula pinealis* / pineal organ**

A combination of ...



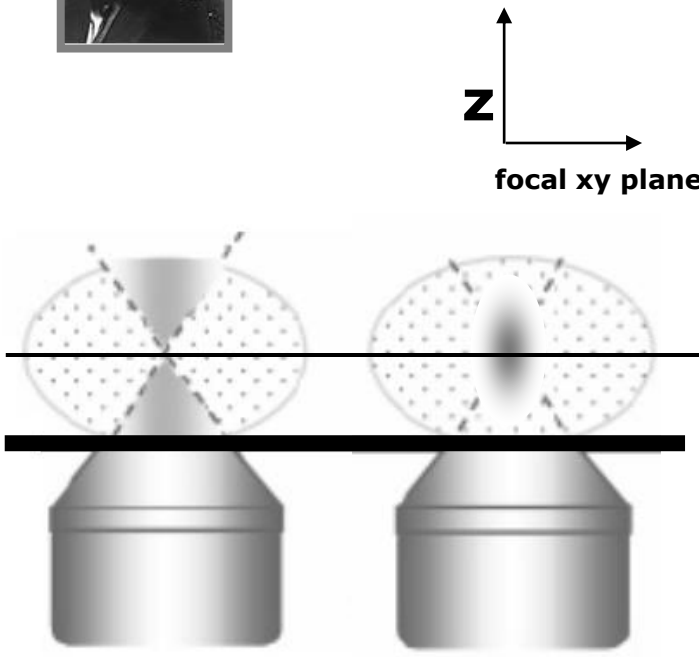
1| direct signals ...

2| signals from other senses ...

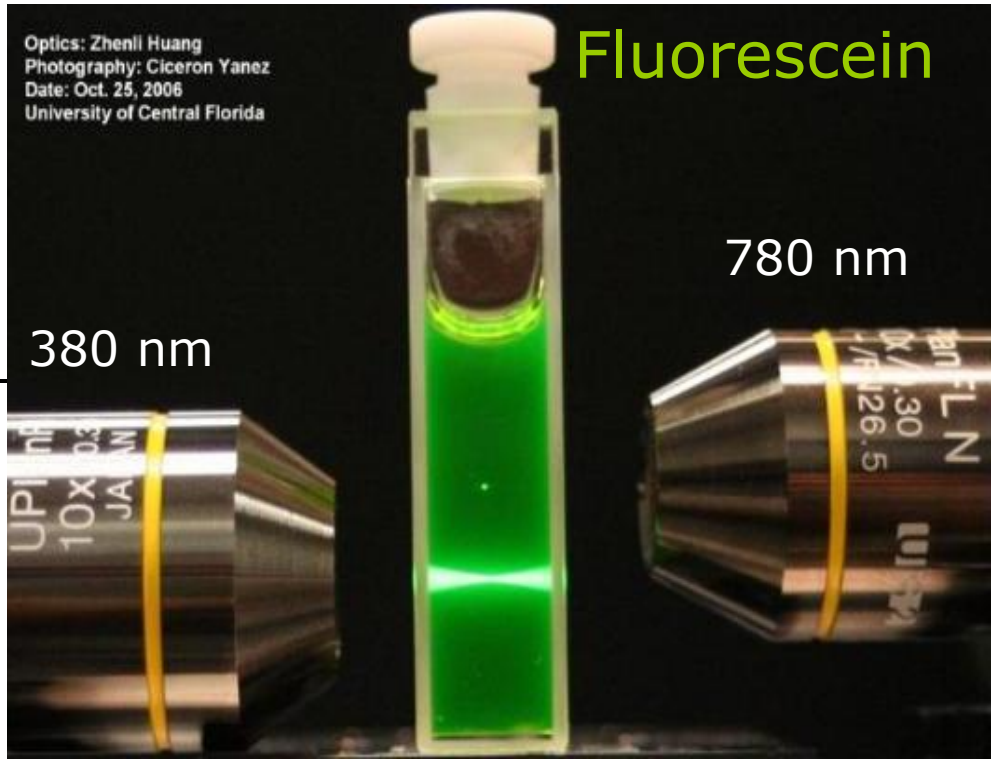
3| feedback loops ...

... produce a symbolic representation of an object.

| Best localization: confocal microscopy



convencional / confocal

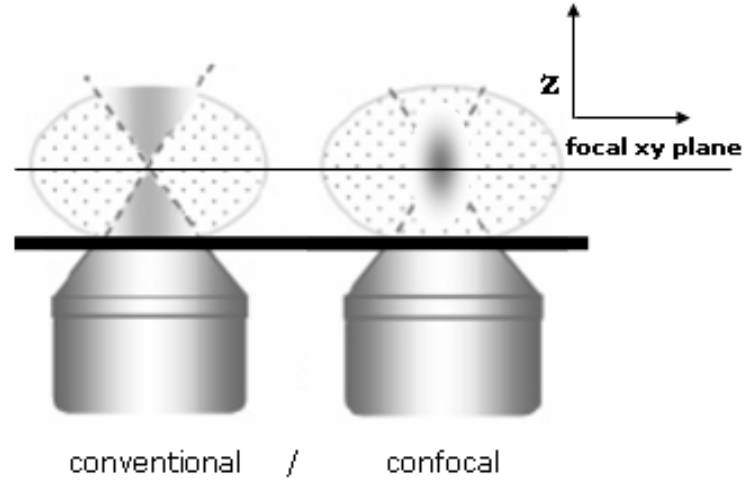


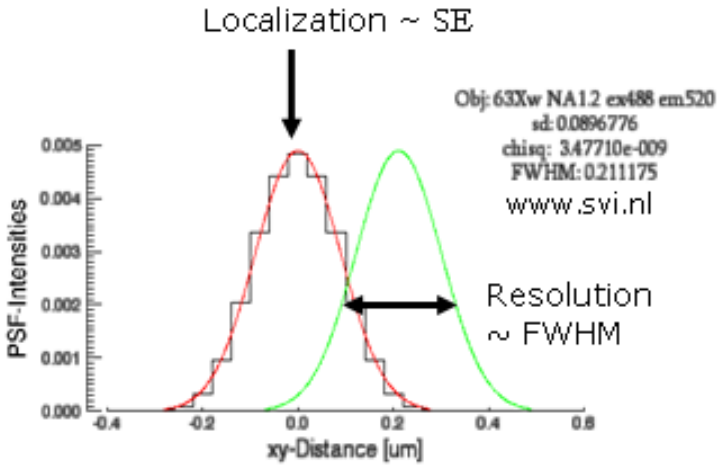
| Diffraction limited microscopy

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





|-> Microscopy

Seeing is believing? Alison J. North, The Journal of Cell Biology, Vol. 172, No. 1, January 2, 2006 9–18

THE JOURNAL OF CELL BIOLOGY

Seeing is believing? A beginners' guide to practical pitfalls in image acquisition

Imaging can be thought of as the most direct of experiments. You see something; you report what you see. If only things were truly this simple. Modern imaging technology has brought about a revolution in the kinds of questions we can approach, but this comes at the price of increasingly complex equipment. Moreover, in an attempt to market competing systems, the microscopes have often been inappropriately described as easy to use and suitable for near-beginners. Insufficient understanding of the experimental manipulations and equipment setup leads to the introduction of errors during image acquisition. In this feature, I review some of the most common practical pitfalls faced by researchers during image acquisition, and how they can affect the interpretation of the experimental data.

This article is targeted neither to the microscopy gurus who push forward the frontiers of imaging technology nor to my imaging specialist colleagues who may wince at the overly simplistic comments and lack of detail. Instead, this is for beginners who gulp with alarm when they hear the word "confocal pinhole" or sigh as they watch their cells fade and die in front of their very eyes time and time again at the microscope. Take heart, beginners, if microscopes were actually so simple then many people (including myself) would suddenly be out of a job!

All data are subject to interpretation

Deliberate scientific fraud exists, but in modern microscopy a far greater number of errors are introduced in complete innocence. As an example of a common problem, take colocalization. Upstairs in the lab, a researcher collects a predominantly yellow merged image on a basic microscope, naturally interpreted as colocalization of green and red signals. Run on the confocal microscope, there is no yellow in the merged images.

"When you employ the microscope, shake off all prejudice, nor harbour any favorite opinions; for, if you do, 'tis not unlikely fancy will betray you into error, and make you see what you wish to see." Henry Baker, chapter 15, "Cautions in viewing objects" of The Microscope Made Easy, 1742.

How can this be? Many factors contribute. Here, I take the reader through the imaging process, from sample preparation to selection of the imaging and image-processing methods. Throughout, we will be on the look-out for problems that can produce misleading results, using colocalization as the most common example. Because one short article cannot be an exhaustive "how to" guide, I have also assembled a bibliography of a few highly recommended textbooks and microscopy web sites, which readers should consult for more extensive treatments of the critical issues introduced here.

Sample preparation

"Garbage in = garbage out" is the universal motto of all microscopists. A worrying tendency today is to assume that deconvolution software or confocal microscopes can somehow override the structural damage or suboptimal immunolabeling induced by poor sample preparation. The importance of appropriate fixation, permeabilization, and labeling methods for preserving cellular morphology or protein localization is well known to electron microscopists (Hayat, 2000), but often underestimated in optical microscopy (Fig. 1).

Many labs use one standardized protocol for labeling with all antibodies, irrespective of whether the targets are membrane- or cytoskeleton-associated, nuclear or cytosolic. However, inappropriate fixation can cause antigen redistribution and/or a reduction in antigenicity. It is therefore important to test each antibody on samples fixed in a variety of ways, ranging from solvents such as methanol to chemical cross-linking agents such as paraformaldehyde and glutaraldehyde (for protocols see Beckerman et al., 1995; Allan, 1999), although glutaraldehyde fixation often reduces antigenicity and increases background autofluorescence. Consult textbooks for notorious pitfalls: phalloidin labeling is incompatible with methanol fixation, while microtubules are inadequately fixed by formaldehyde. Moreover, certain cell types, such as yeast cells, require specialized fixation protocols (Hagan and Ayscough, 1999).

Permeabilization is also critical in achieving a good compromise between antigen accessibility and ultrastructural integrity. Specific detergents will produce different effects (for example, Saponin treatment produces smaller holes in

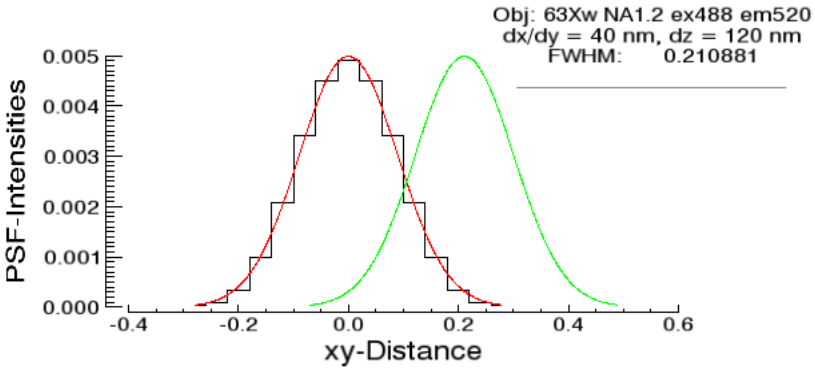
The objective lens is the most critical component of a microscope and yet few researchers grasp the differences between specific objective classes.

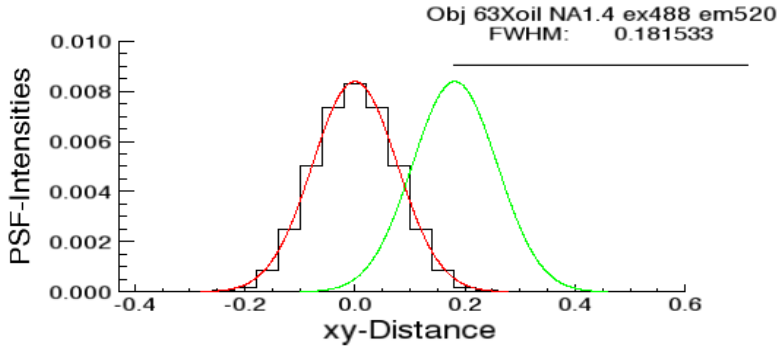
"When you employ the microscope, shake off all prejudice, nor harbour any favorite opinions; for, if you do, 'tis not unlikely fancy will betray you into error, and make you see what you wish to see." Henry Baker, chapter 15, "Cautions in viewing objects" of The Microscope Made Easy, 1742.

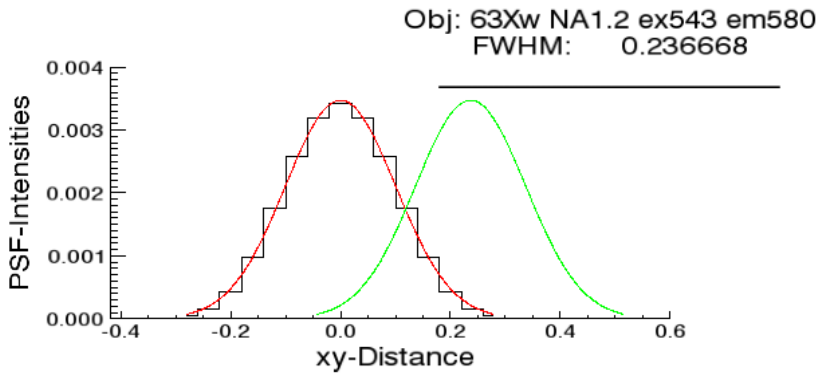
Keep the acquisition settings constant between specimens to be compared quantitatively and particularly between sample and control.

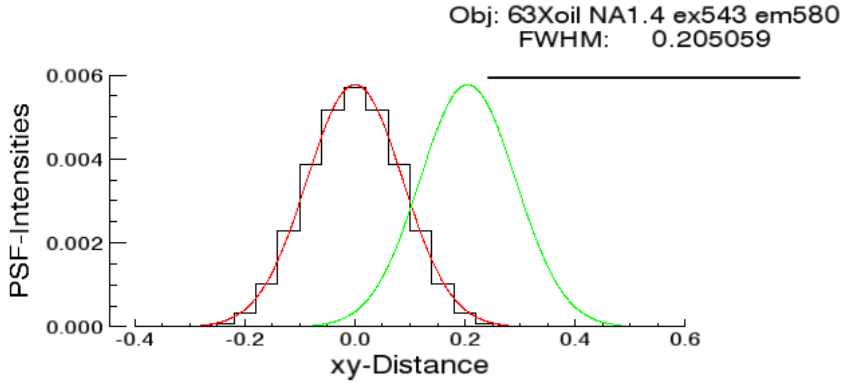
"Remember that truth alone is the matter that you are in search after; and if you have been mistaken, let not vanity seduce you to persist in your mistake." Henry Baker, The Microscope Made Easy, 1742.

|-> Diffraction limited Microscopy

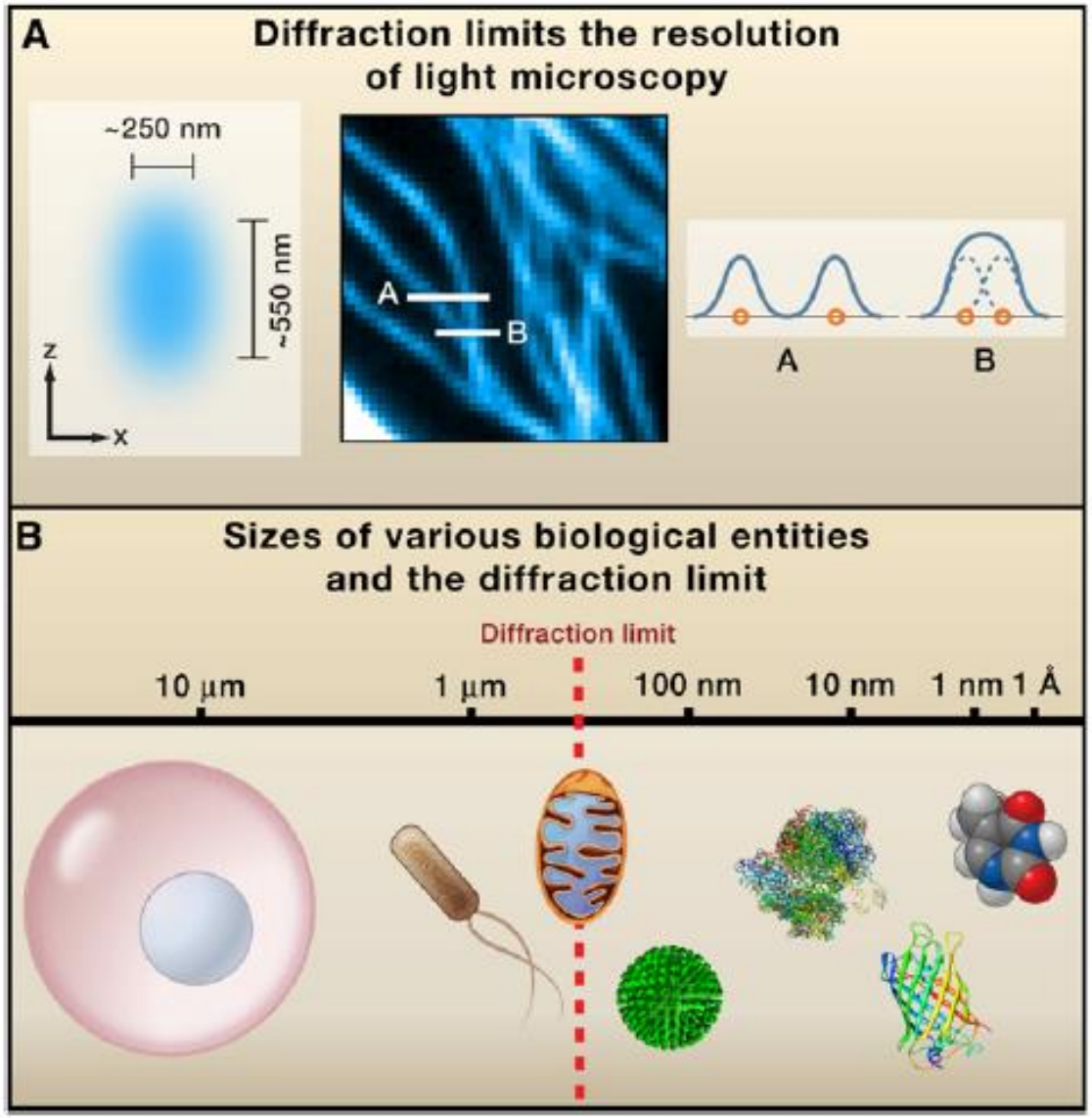








|-> Diffraction limited Microscopy



|-> Beyond diffraction

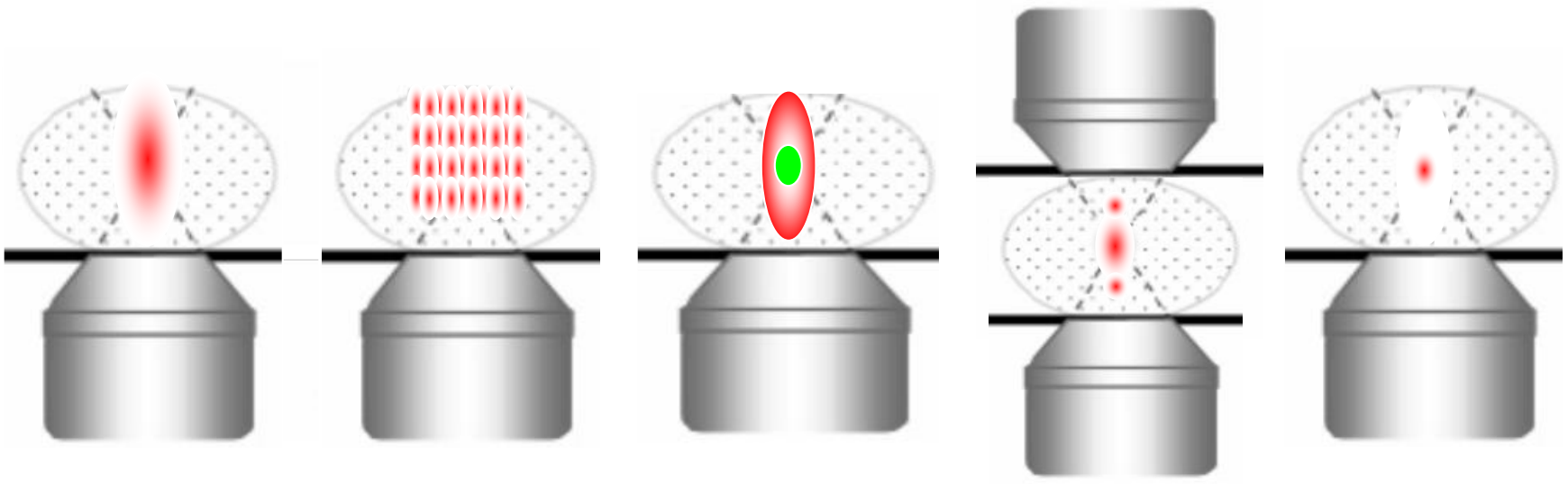
M Goeppert-Mayer
1906-1972

M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

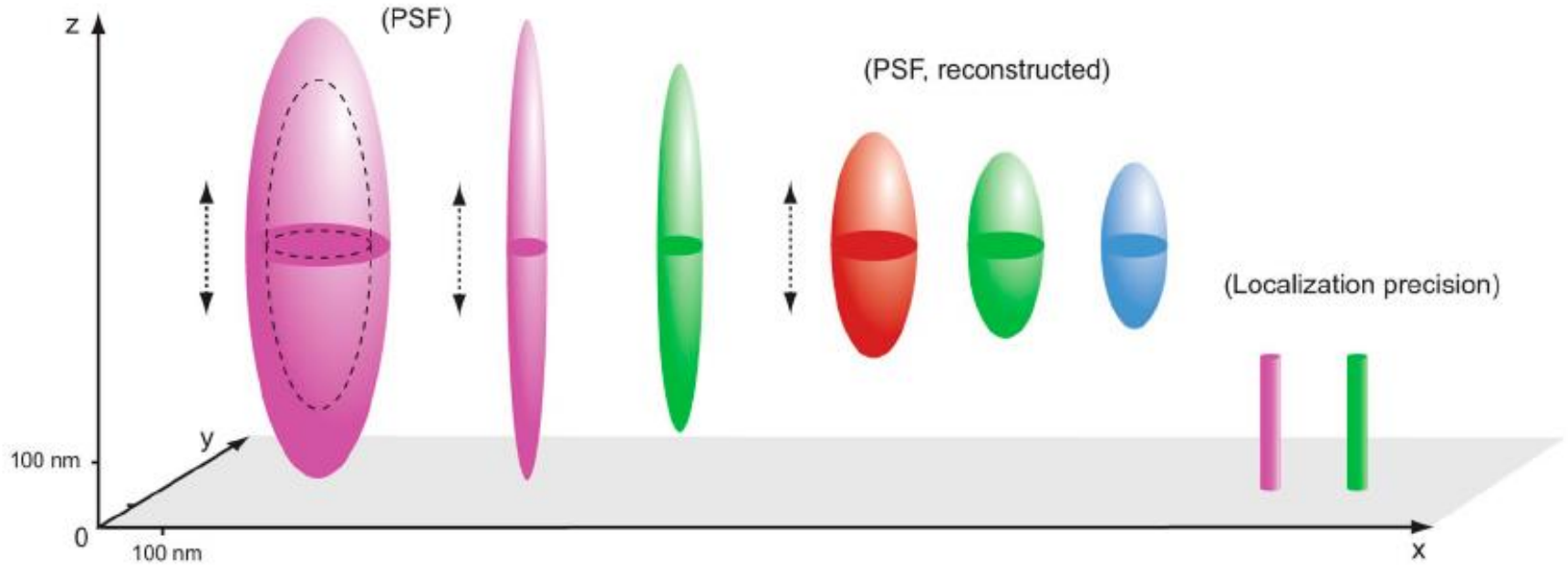
FWHM(xy) ~ λ/2 | ~ λ/4 | ~ λ/∞ | ~ λ/4 | ~ λ/100



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

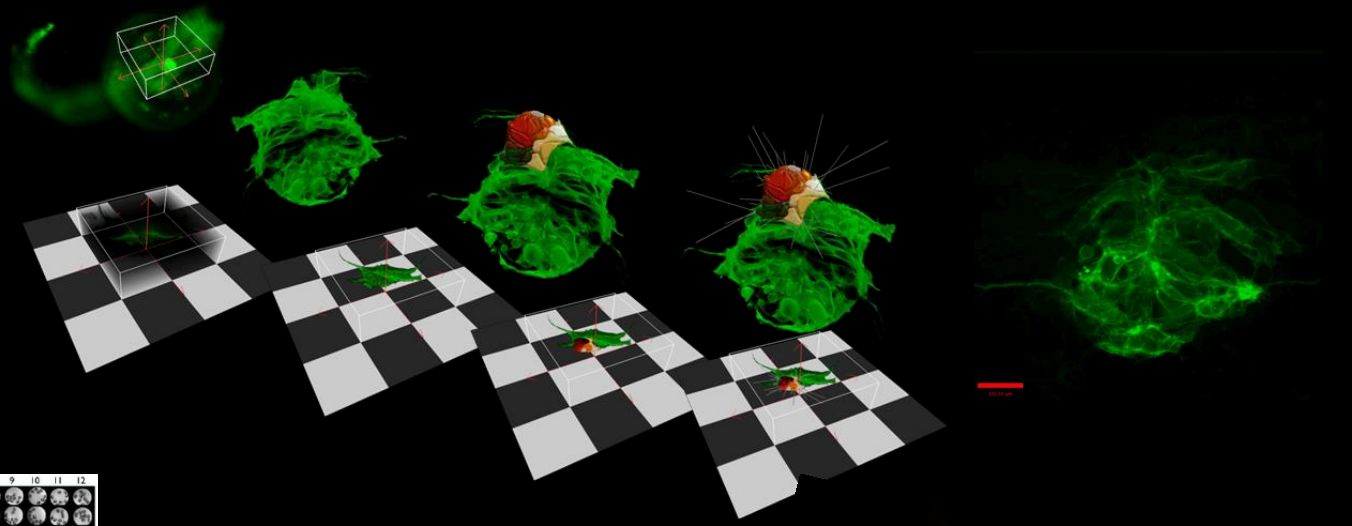


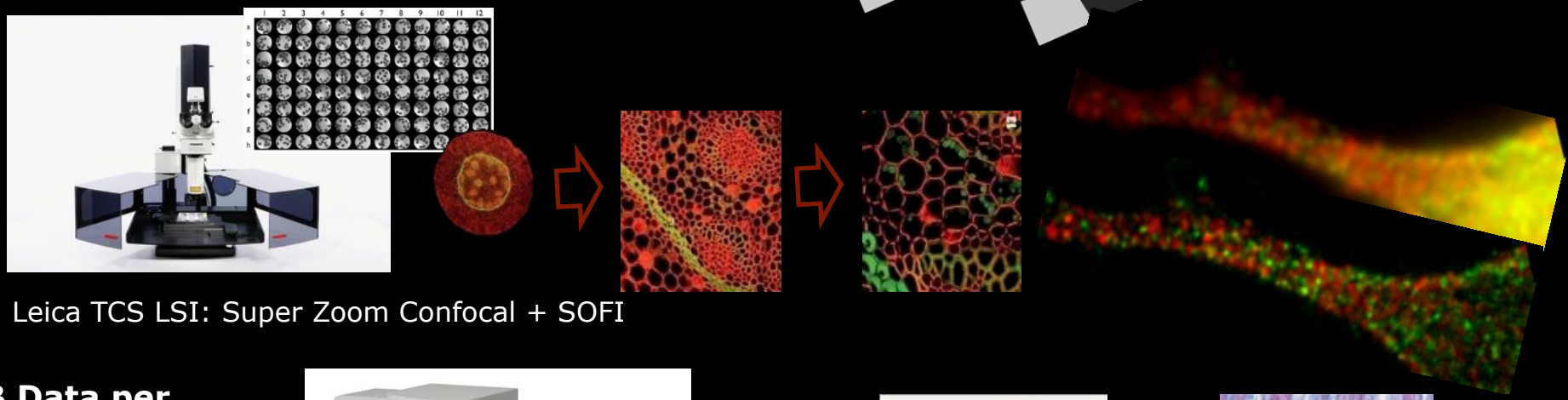
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Schermelleh et al 2010 JBC

|-> Microscopy @ SCIAN-Lab

Perkin Elmer Spinning Disk



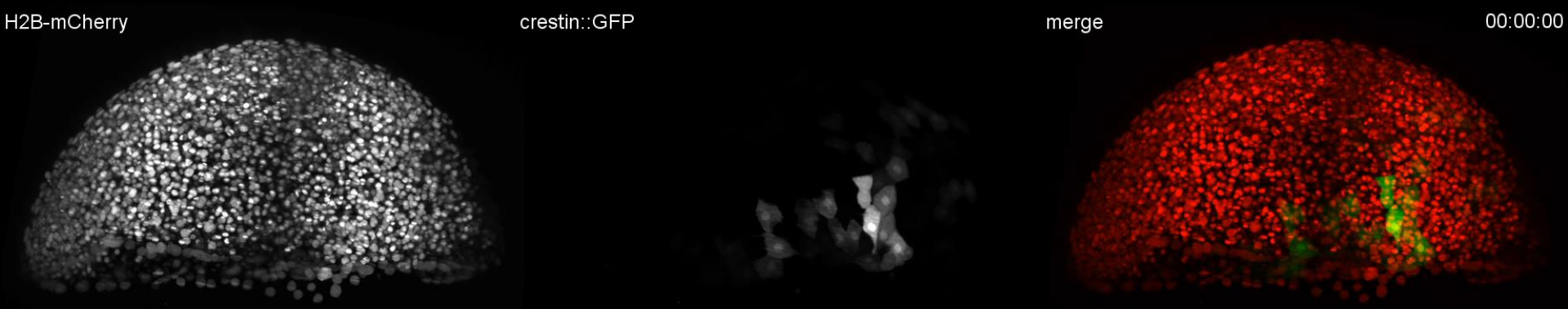


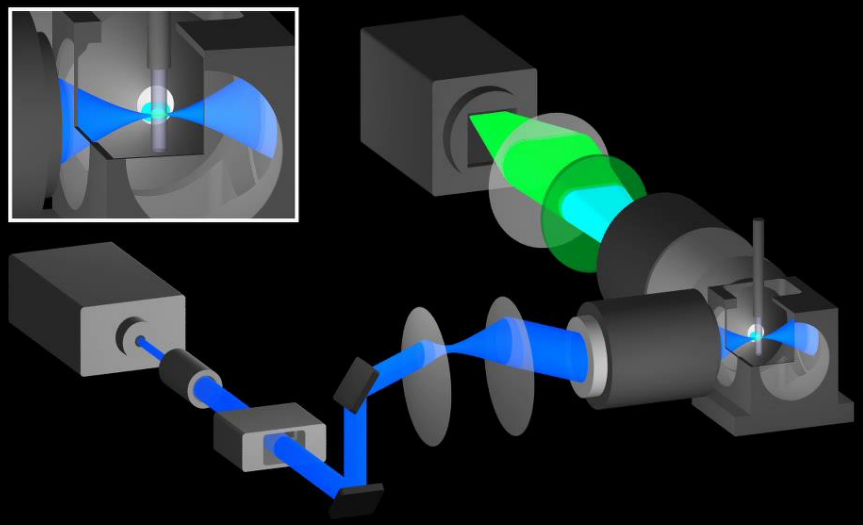
Leica TCS LSI: Super Zoom Confocal + SOFI

TB Data per Experiment

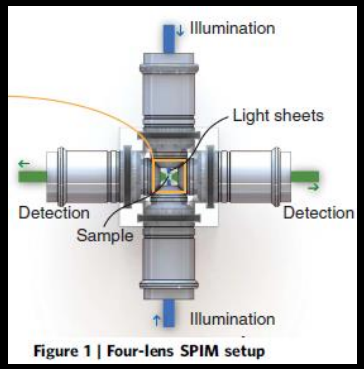
NanoZoomer: Tissue Imaging

|-> Light Sheet Microscopy



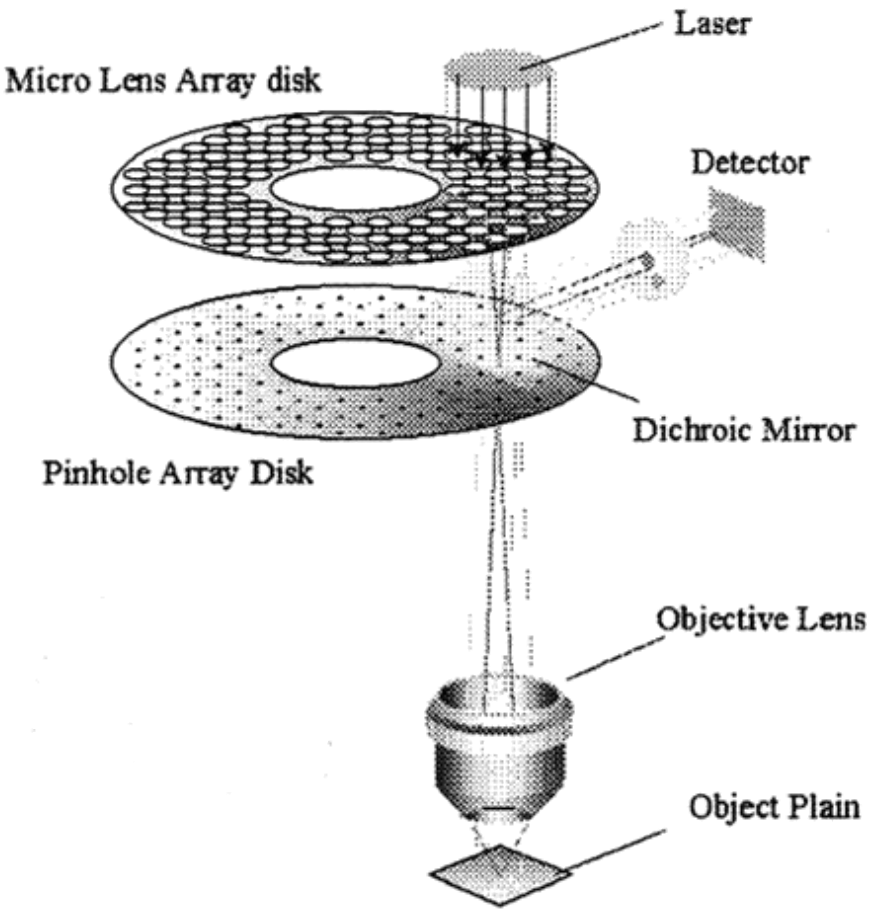


Ulrich Kubitscheck
Bonn

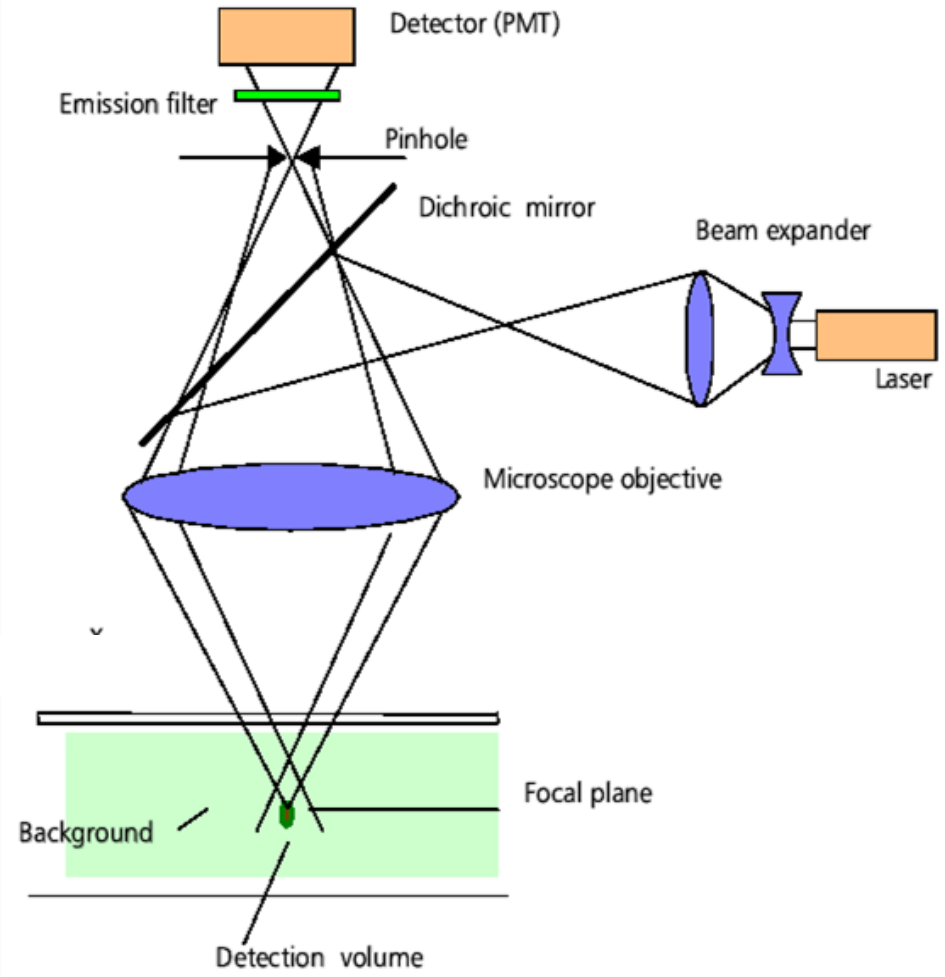


Jan Huisken
Dresden

|-> Spinning Disk

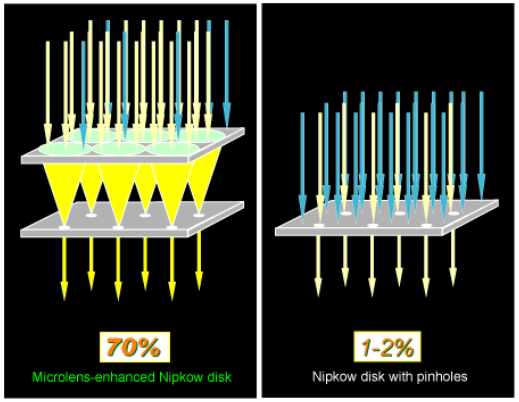


spinning disk



confocal

|-> Diffraction limited microscopy



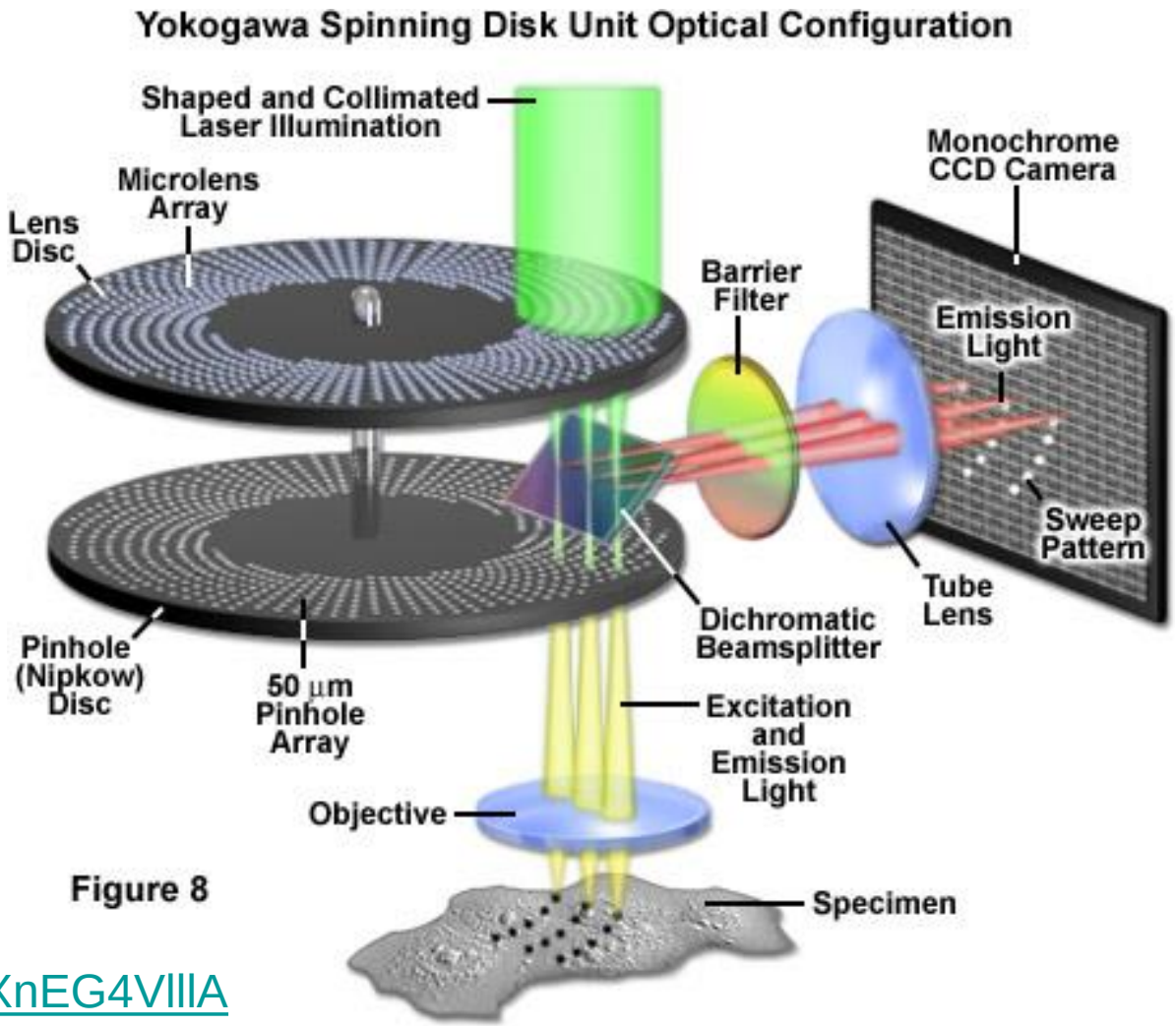
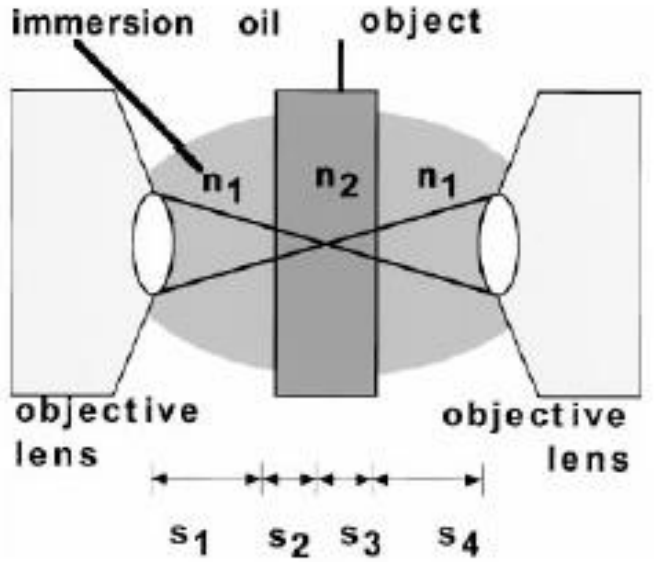


Figure 8

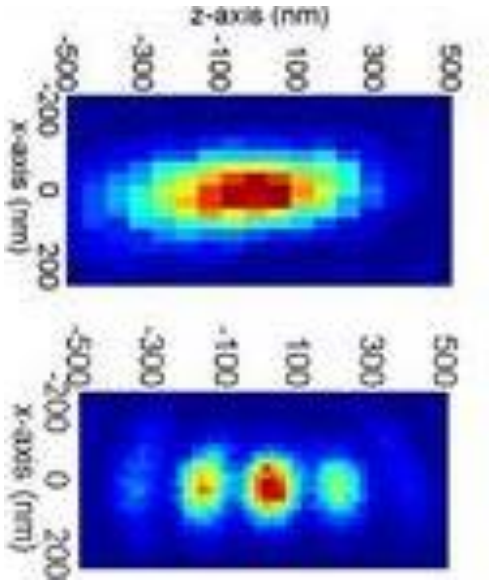
www.youtube.com/watch?v=fXnEG4VIlIA

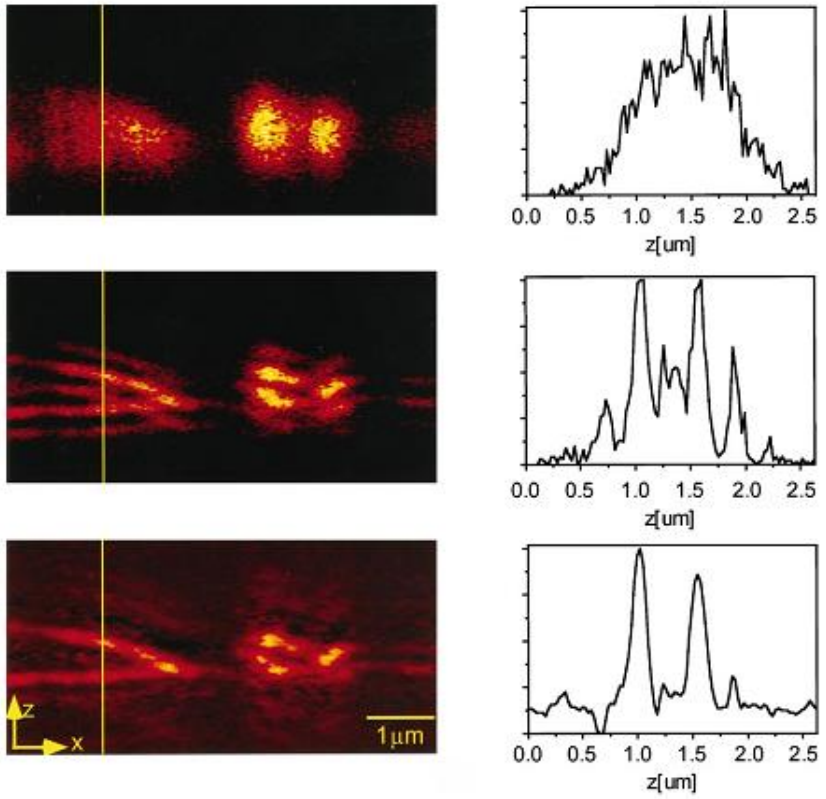
|-> 4π Microscopy



|-> confocal

|-> 4π





4Pi-Confocal Imaging in Fixed Biological Specimens

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

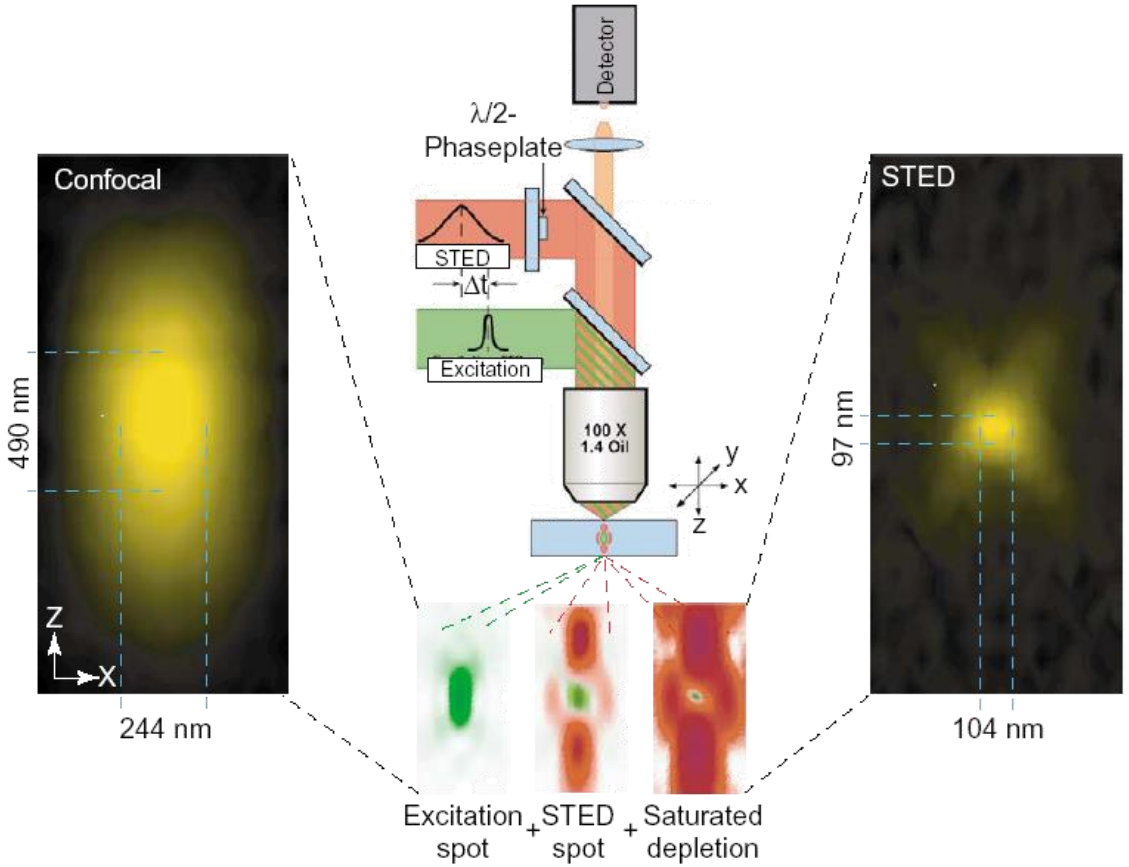
Biophysical Journal Volume 75 October 1998 1659–1668

STED Microscopy

Stimulated

Emission

Depletion



Concepts for nanoscale resolution in fluorescence microscopy

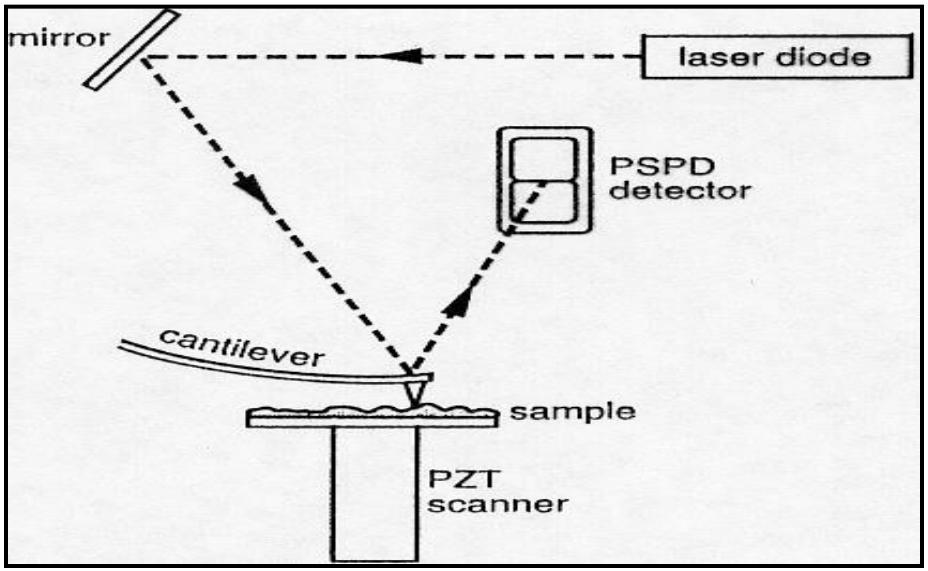
Stefan W Hell*, Marcus Dyba[1] and Stefan Jakobs[2]

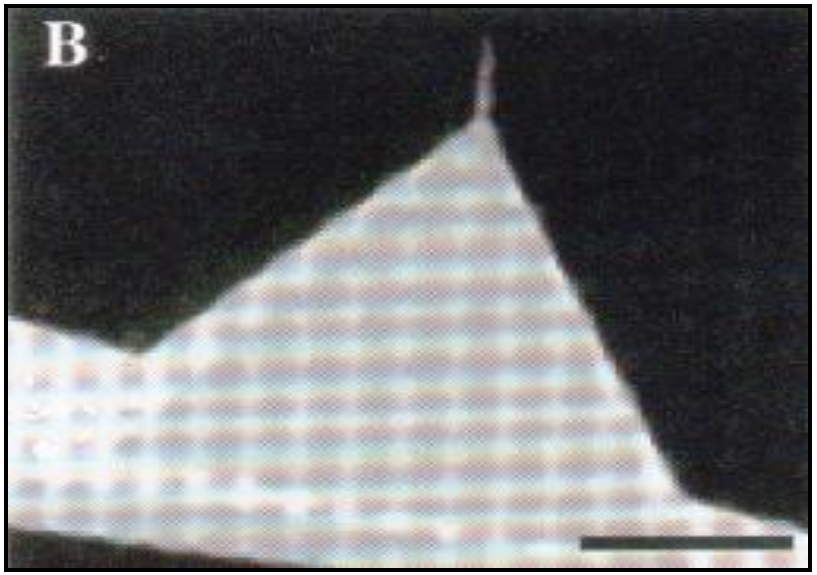
Current Opinion in Neurobiology 2004, **14**:599–609

|-> Atomic Force Microscopy

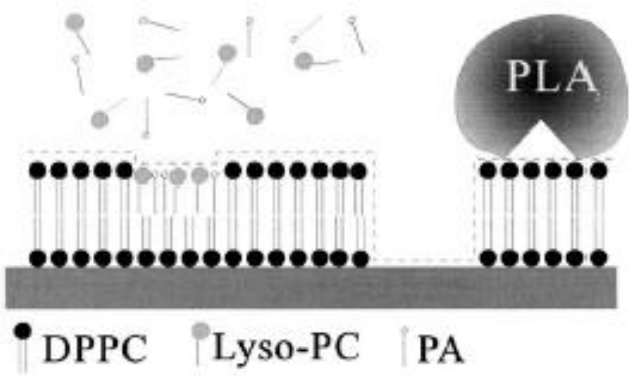
AFM allows the investigation of structural and functional properties of biomolecules in liquid environments, by a unique combination of :

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

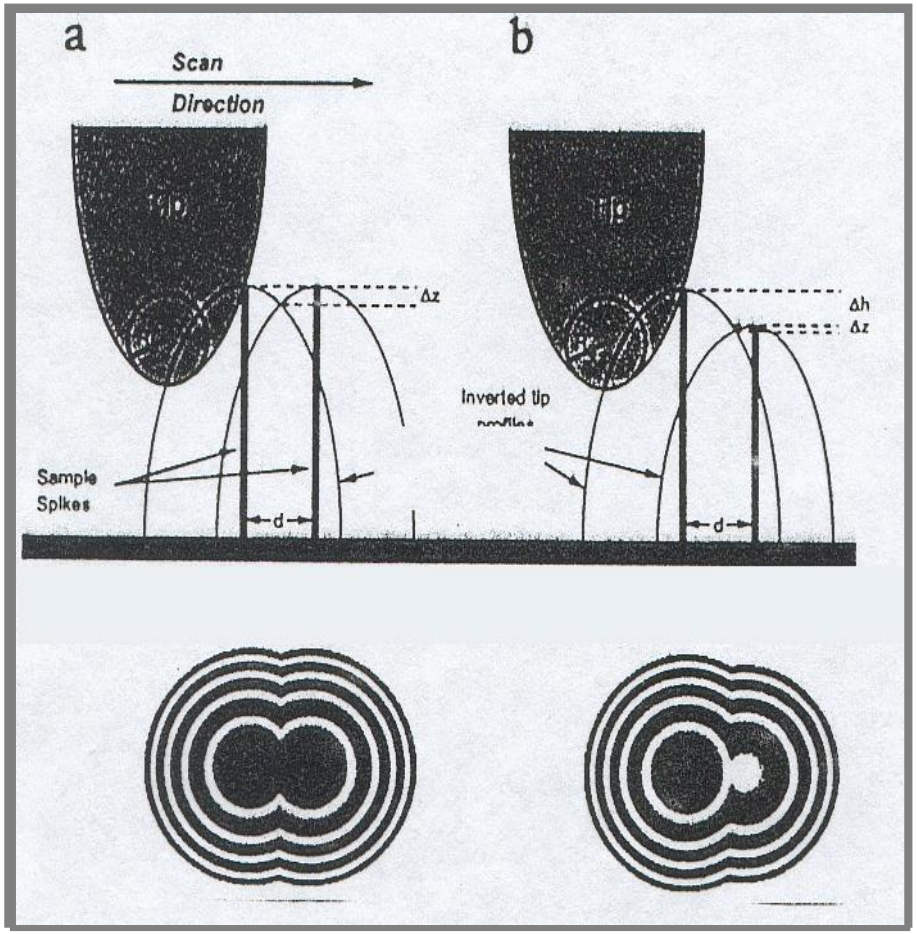




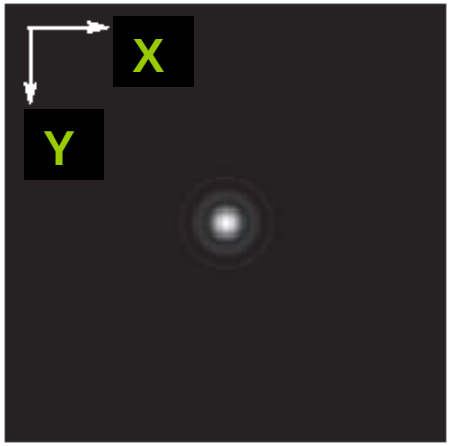
|-> Atomic Force Microscopy

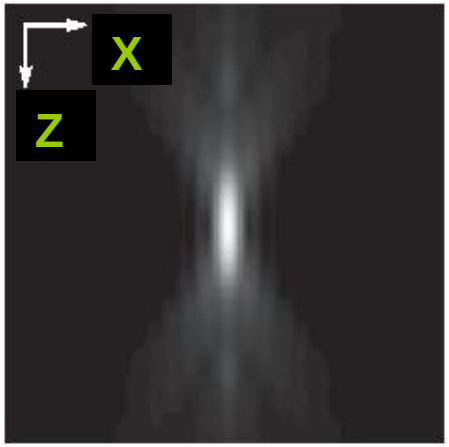


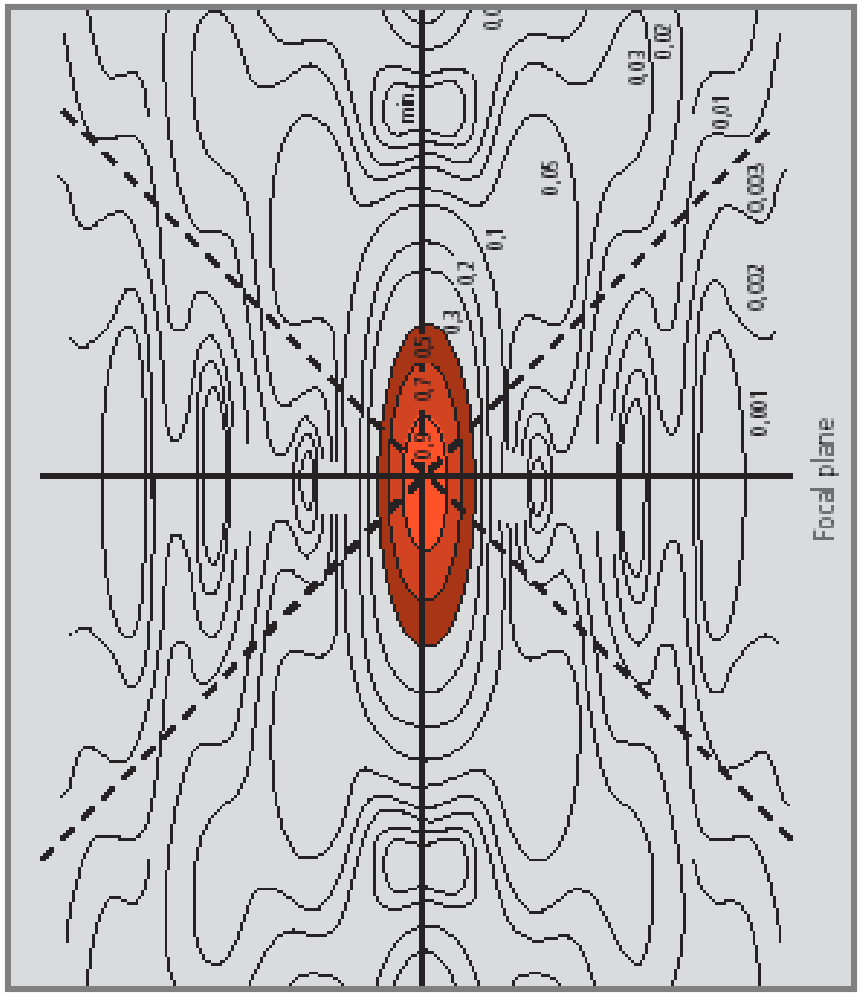
M Grandbois et al. (1998) Biophys J.



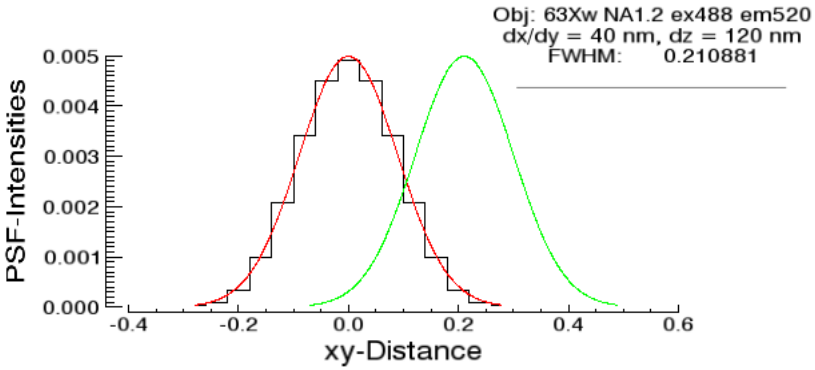
|-> PSF

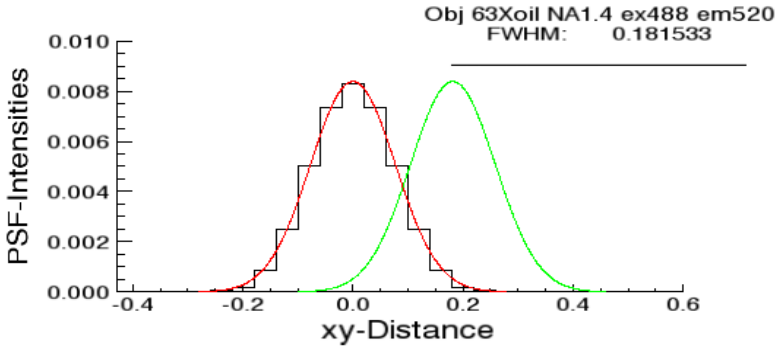


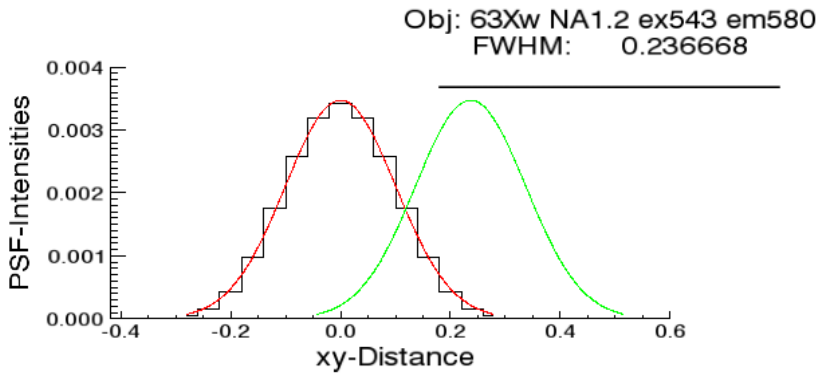




|-> PSF


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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0.003
0.002
0.001
0.000
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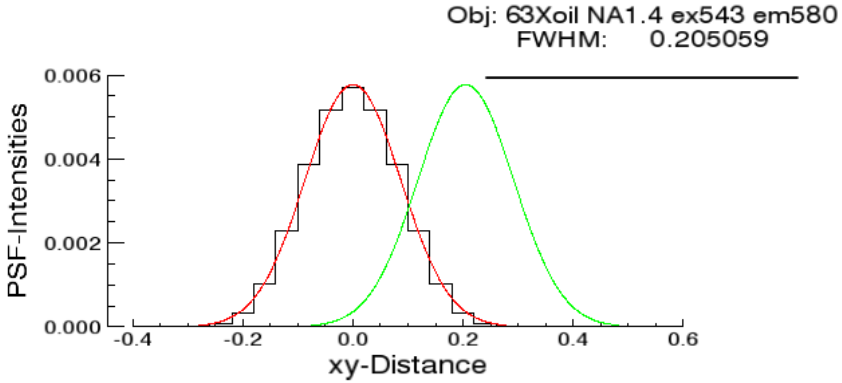
-0.2
0.0
0.2
0.4
0.6
xy-Distance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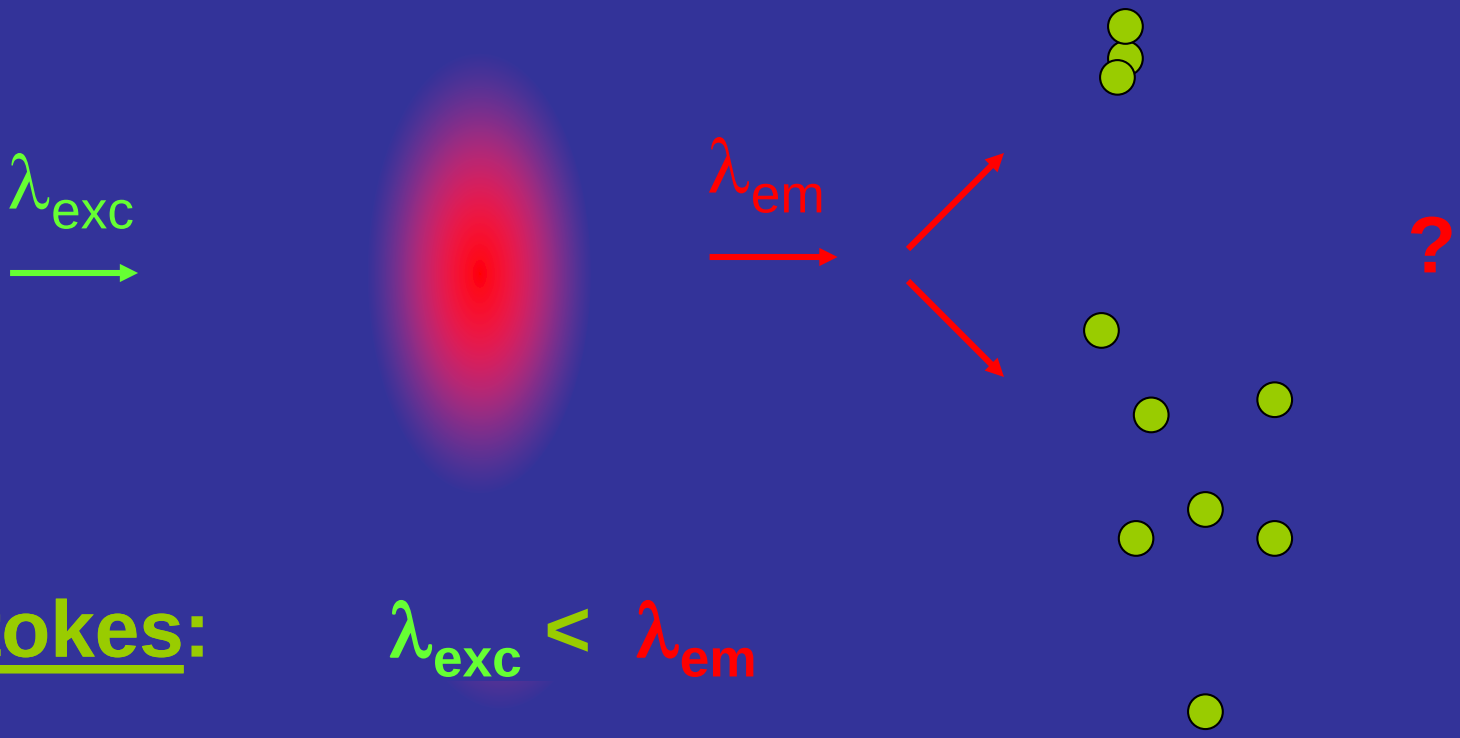
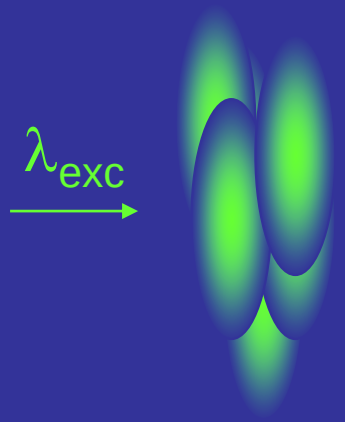
FWHM: 0.205059
PSF-Intensities
0.006
0.004
0.002
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance

|-> Convolution



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

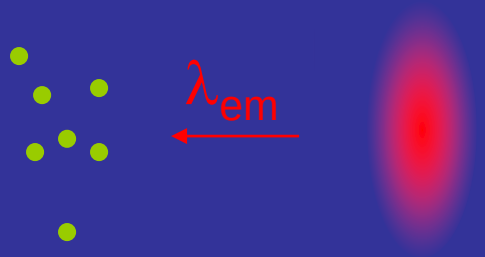
|-> Convolution



λ_{exc}

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

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



λ_{em}

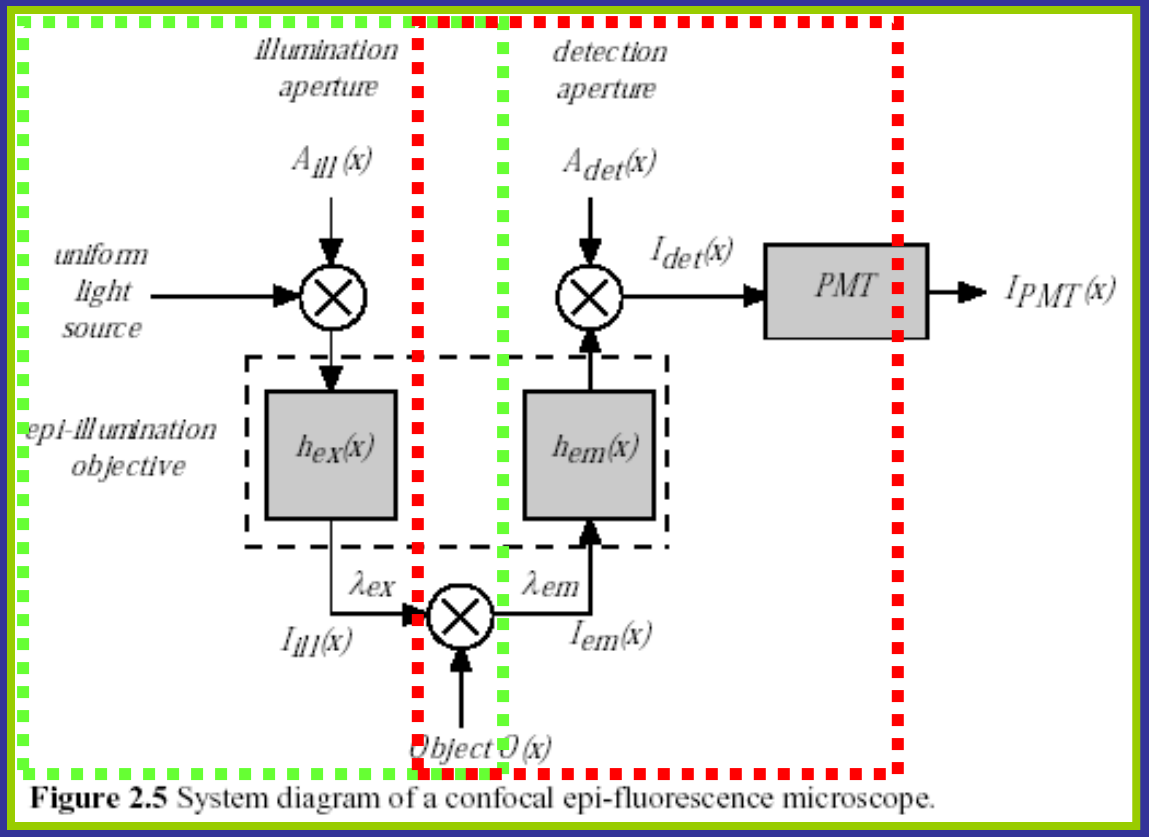
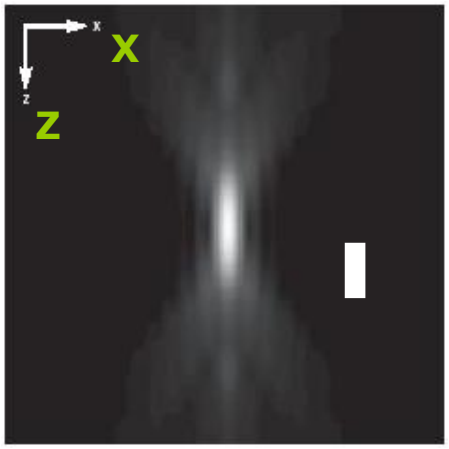


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

Laboratory for SCIAN Scientific Image Analysis

|-> Convolution

BIOMEDICAL NEUROSCIENCE INSTITUTE



PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: *Point Spread Function*

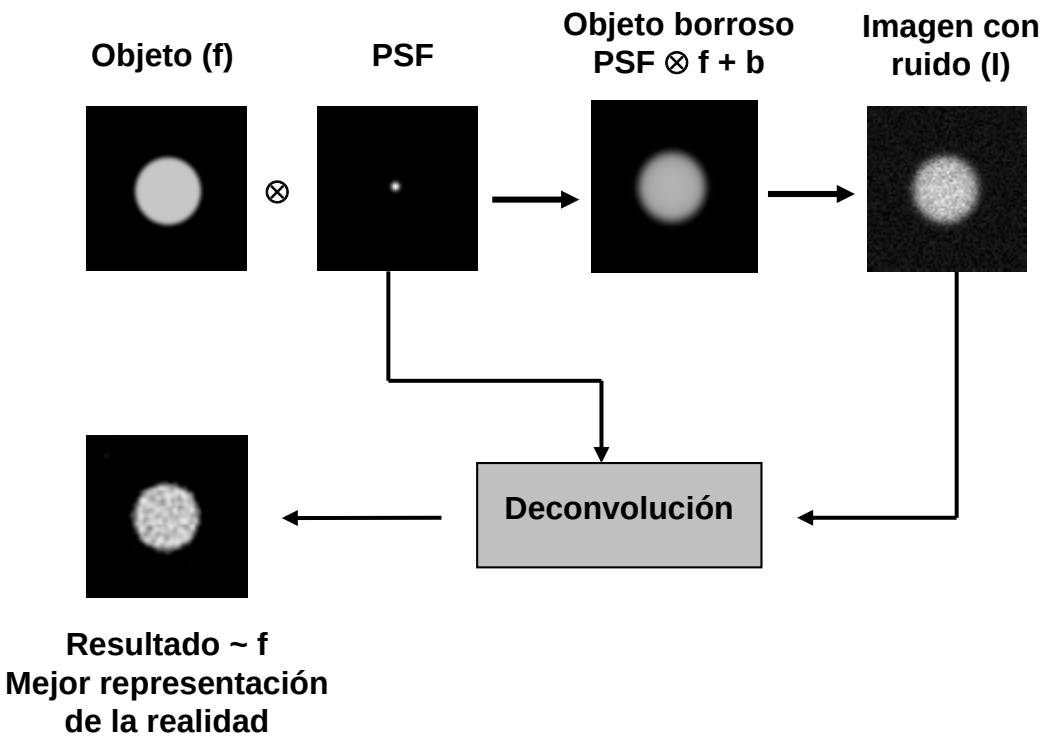
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

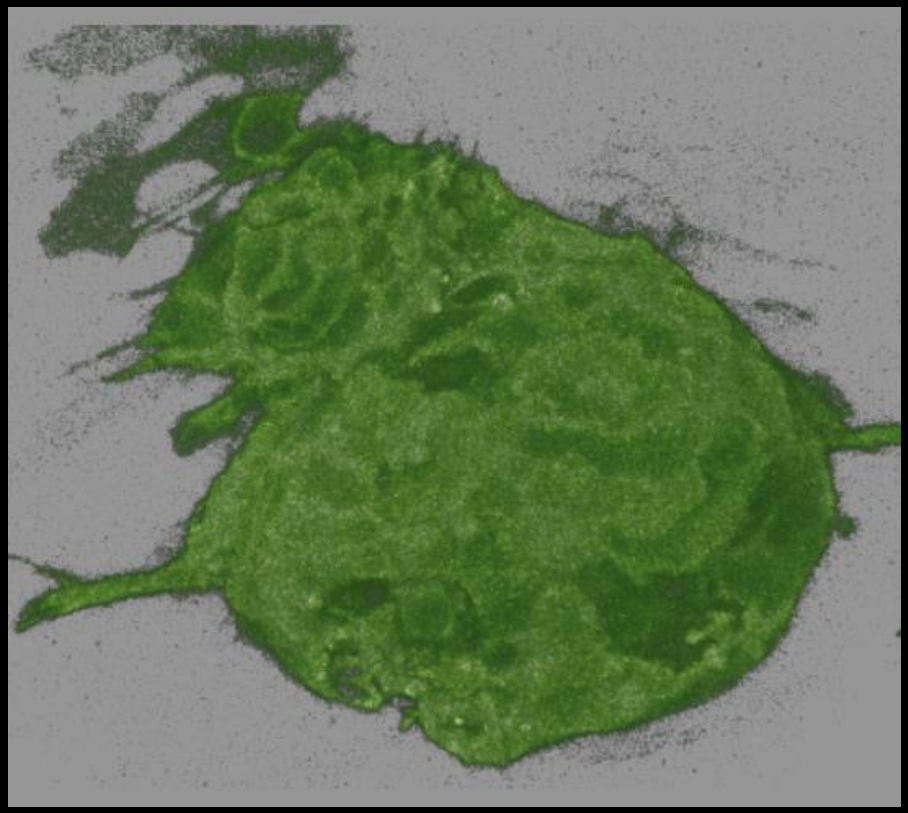
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

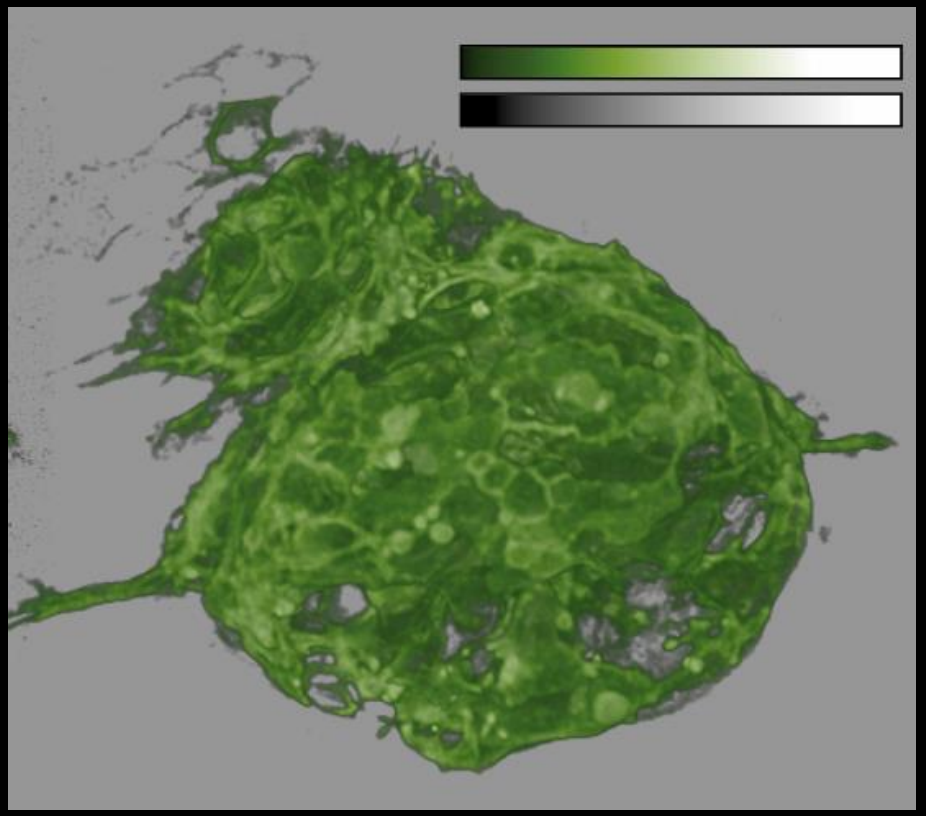


Laboratory for SCIAN Scientific Image Analysis

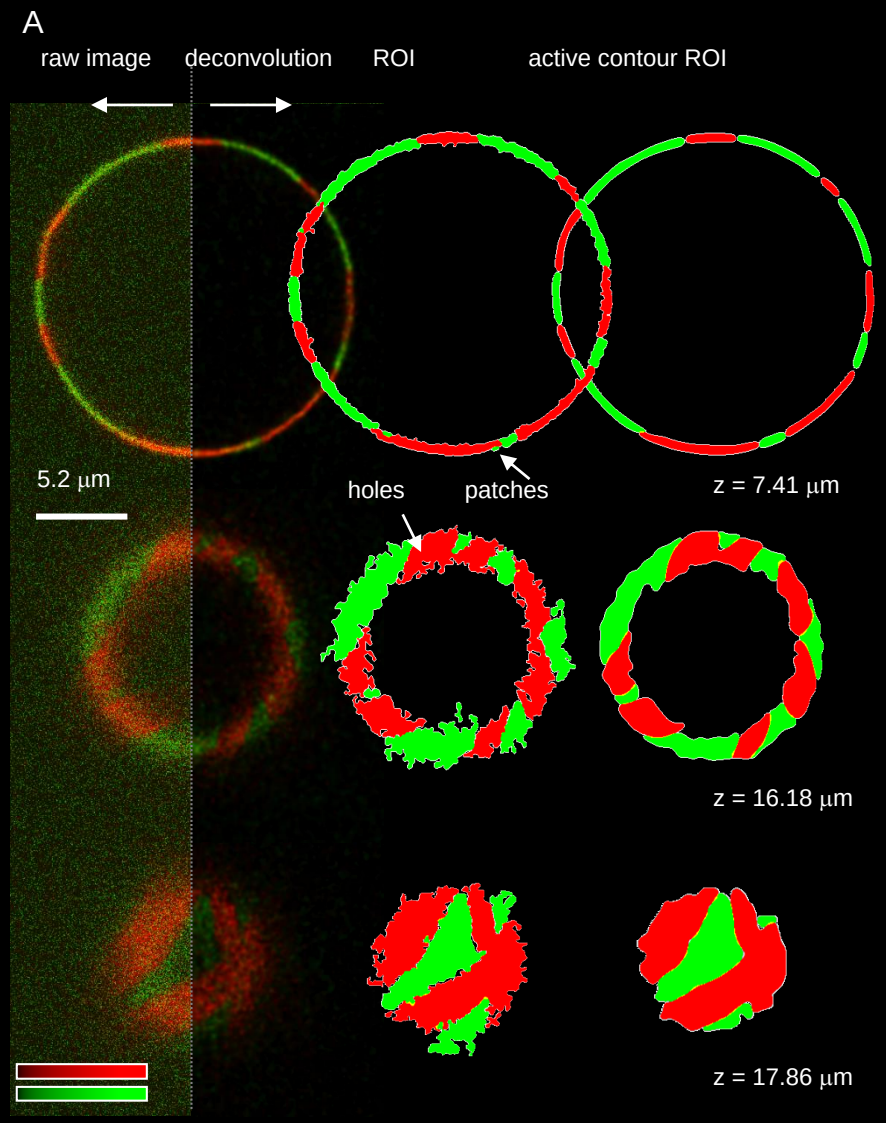
|-> Deconvolution

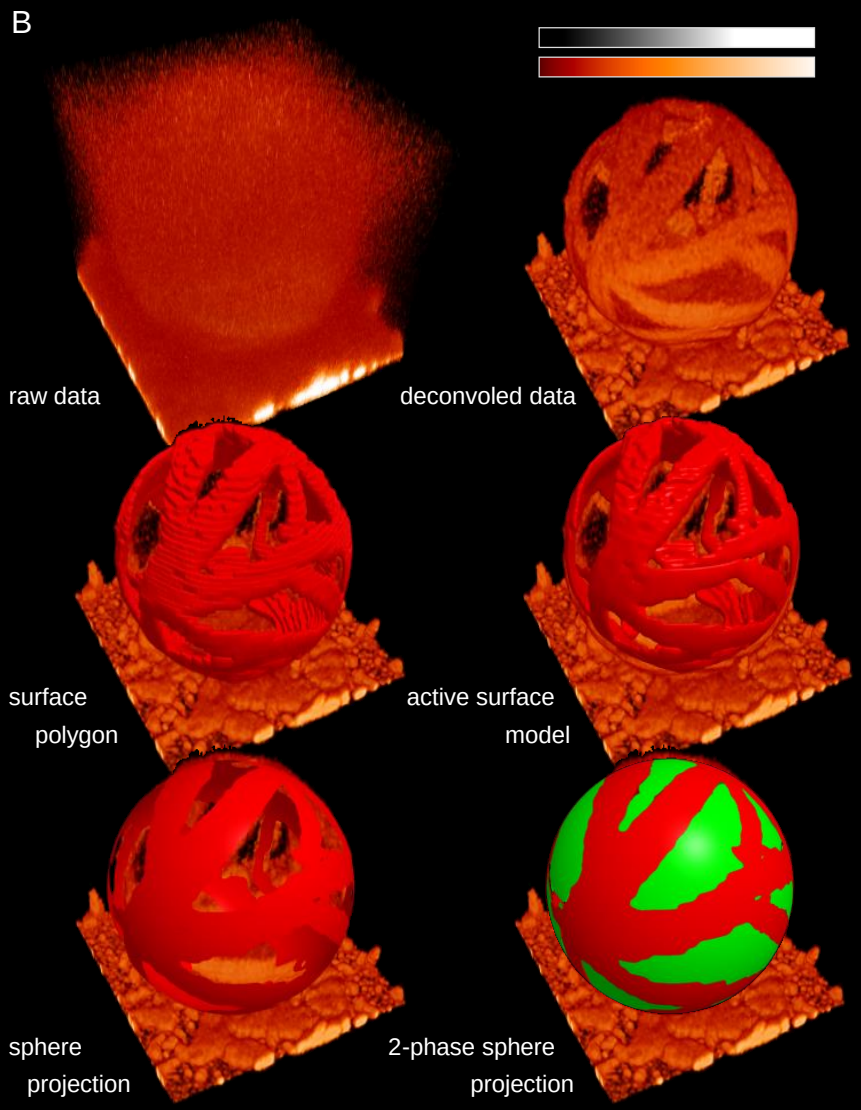
BIOMEDICAL NEUROSCIENCE INSTITUTE





|-> Deconvolution





|-> Deconvolution

PSF: *Point Spread Function*

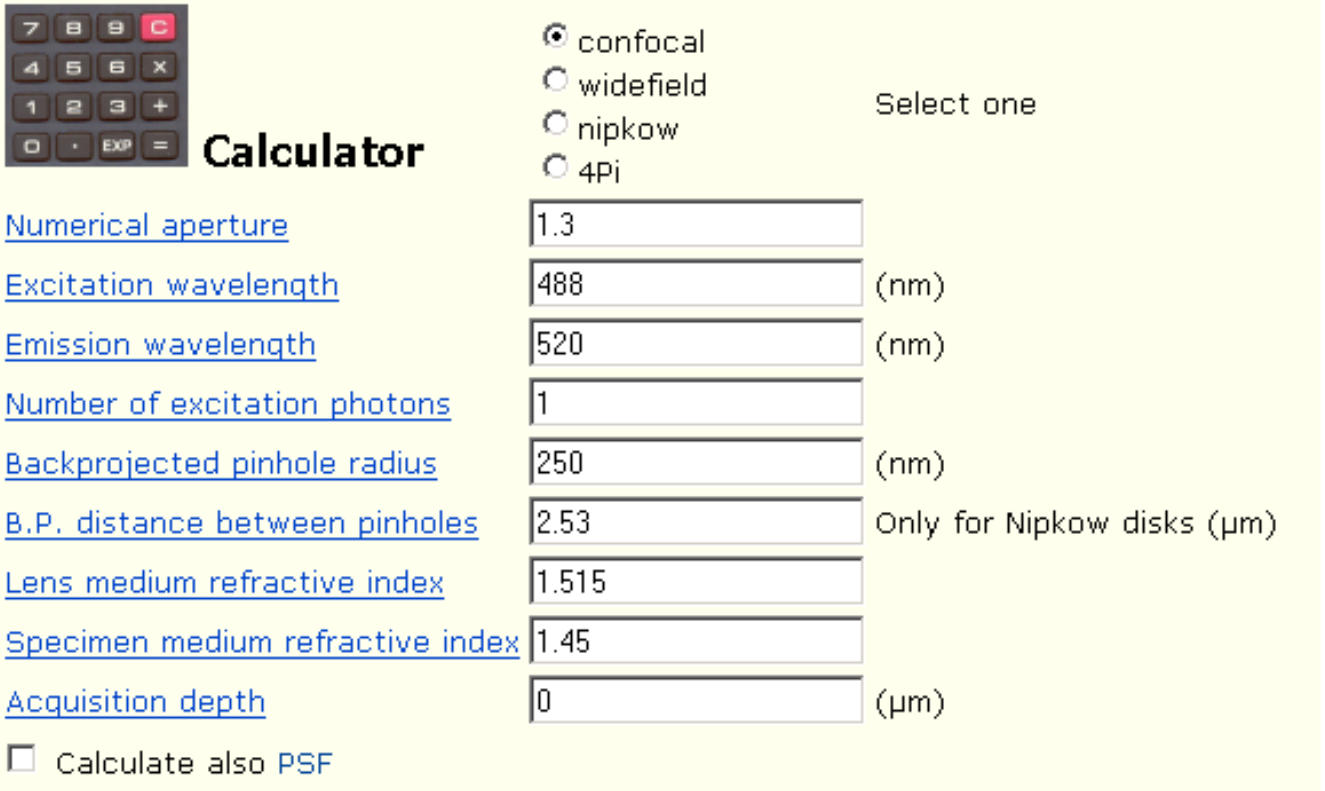
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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



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

|-> Pinhole

PSF: *Point Spread Function*

f: *Object Function*

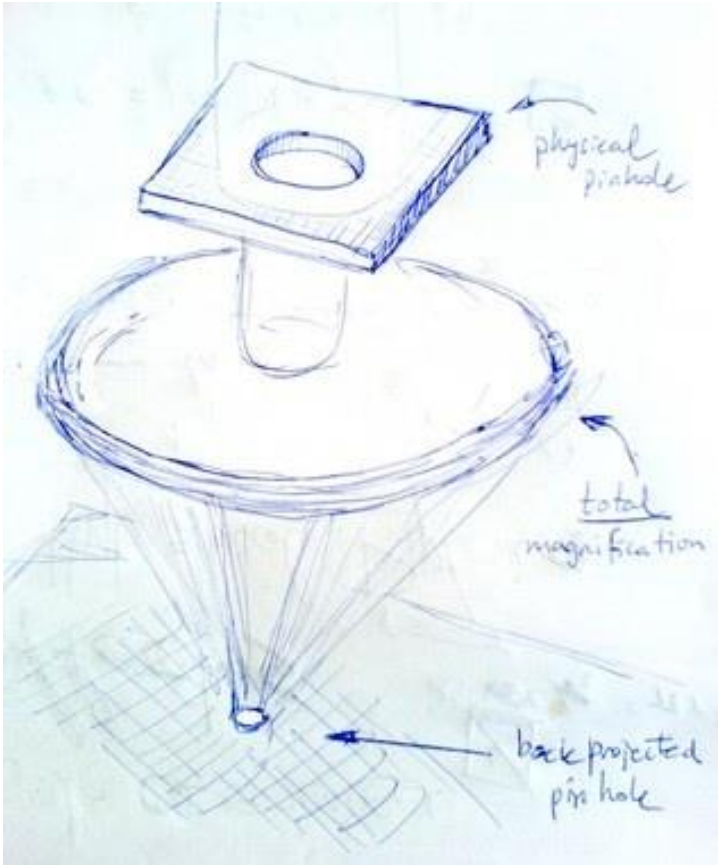
b: *Offset Function*

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

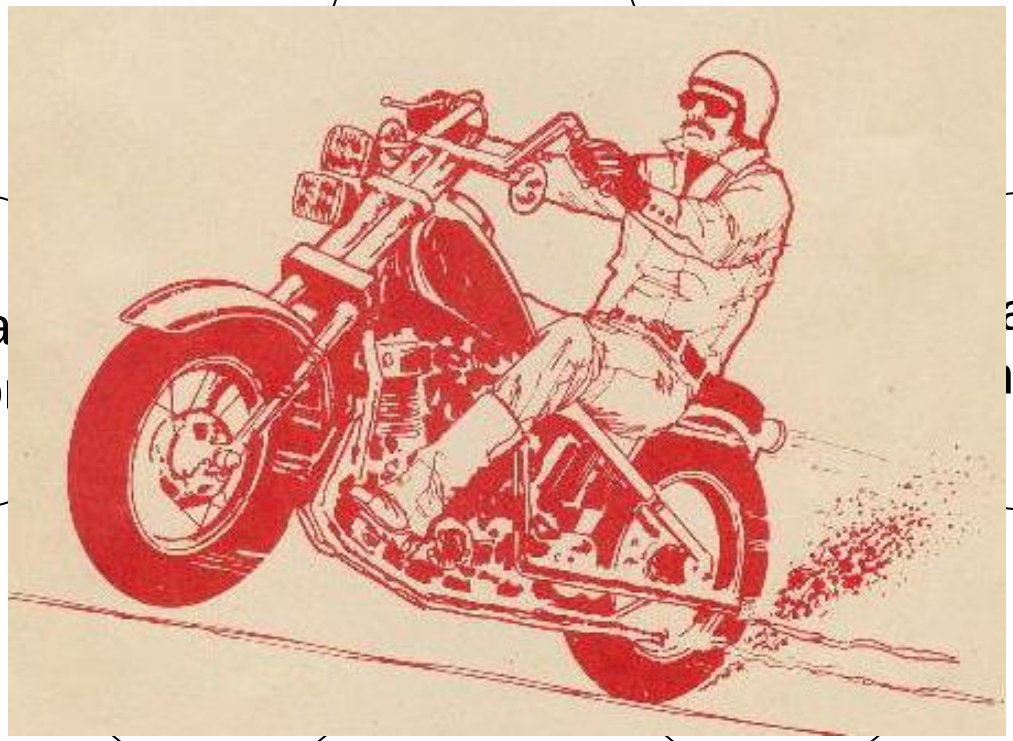
- Olympus FV300
- Olympus FV500
- Olympus FV1000

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)*

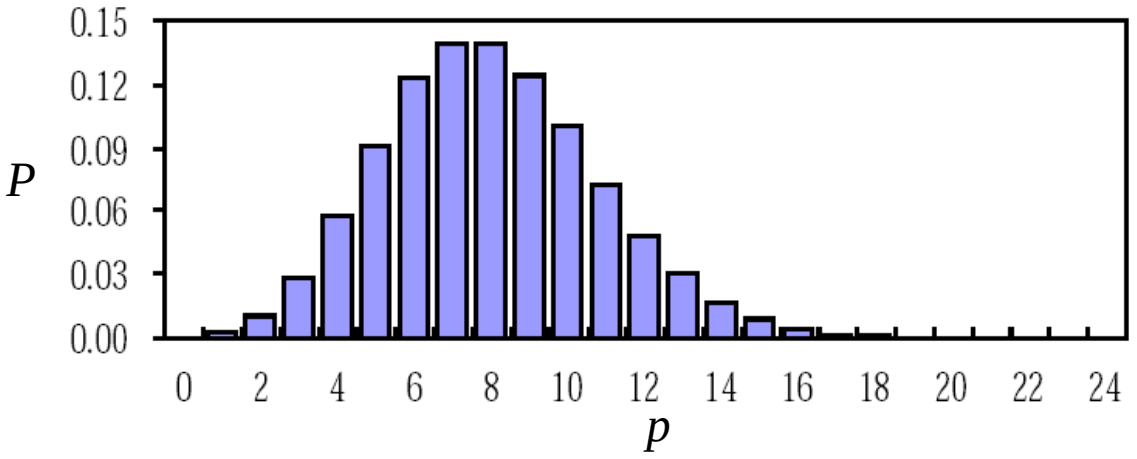
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

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

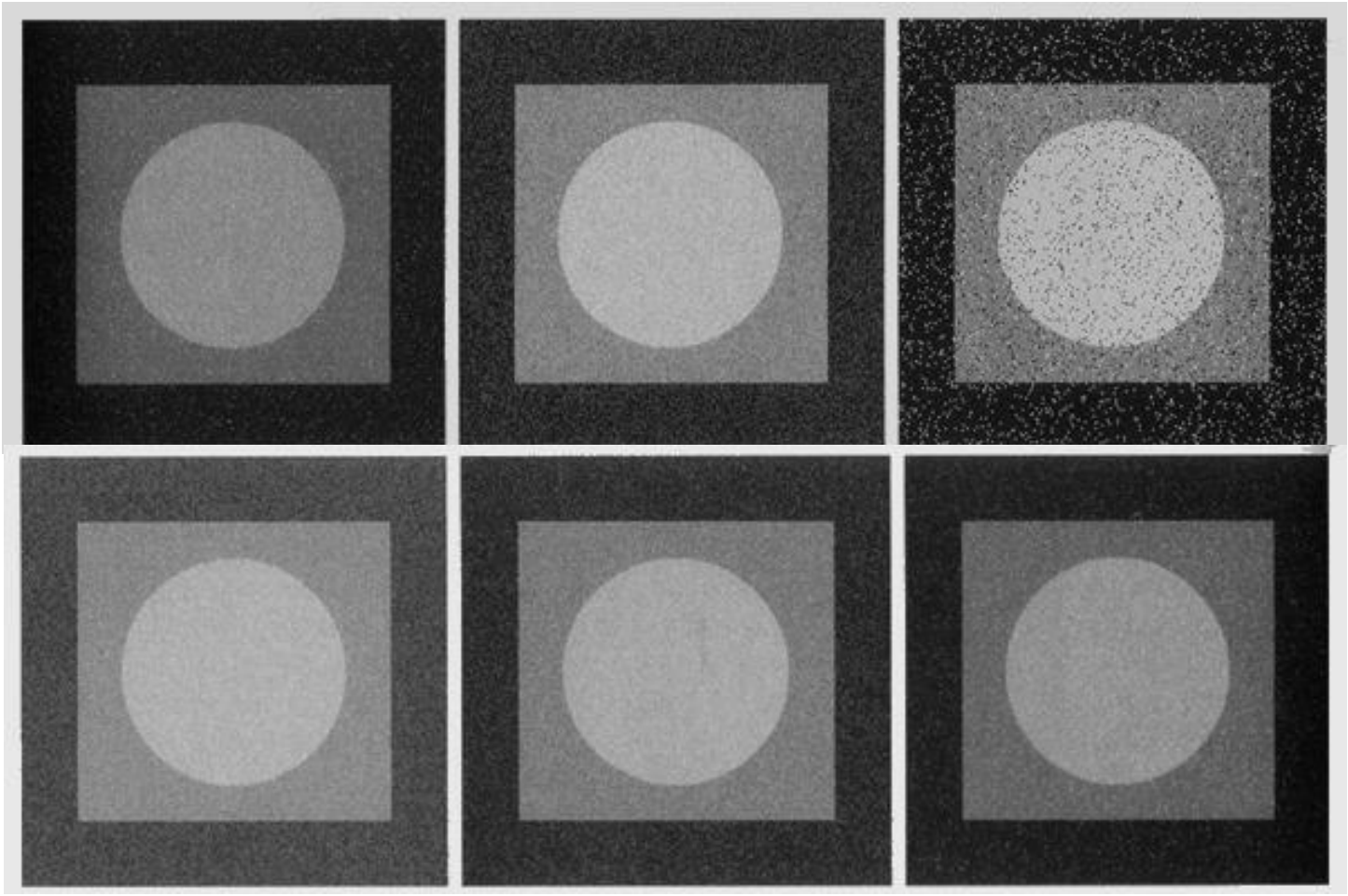
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

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

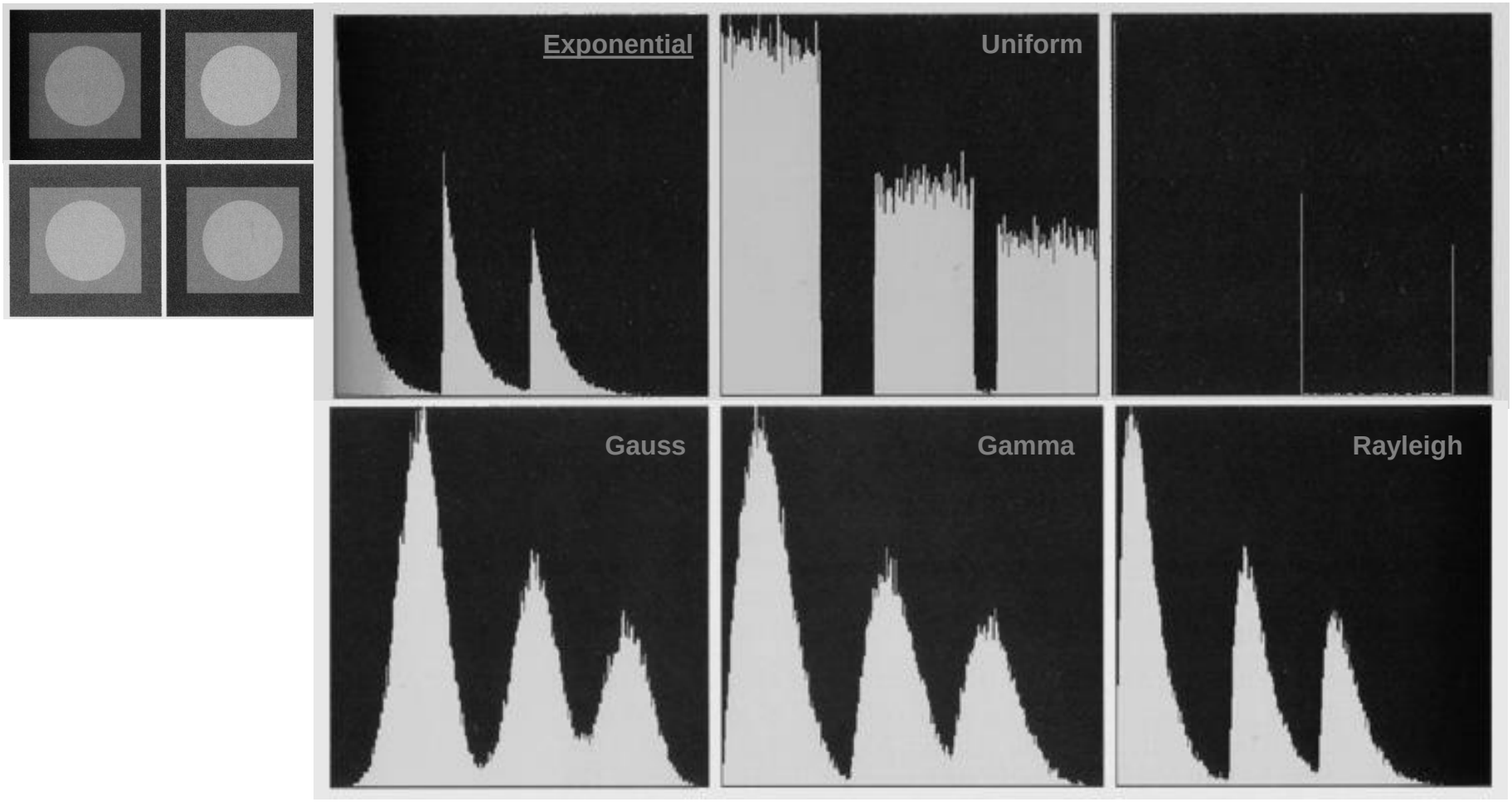
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> 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.

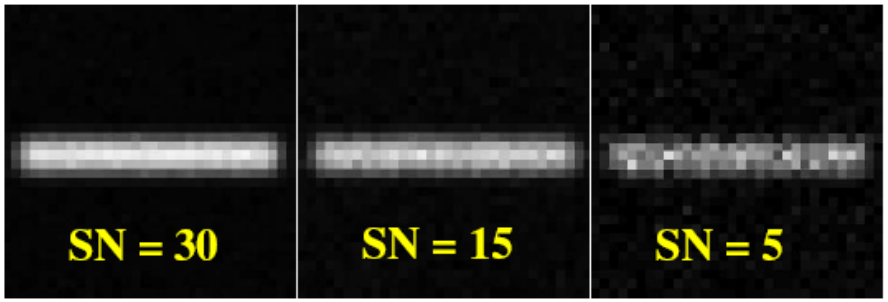
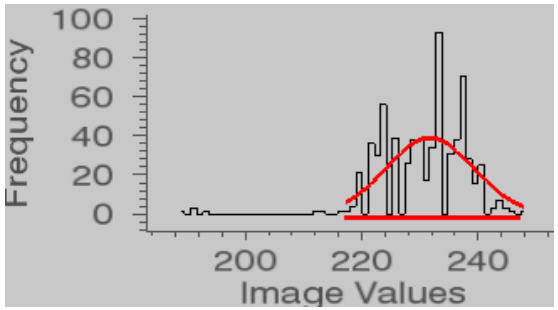
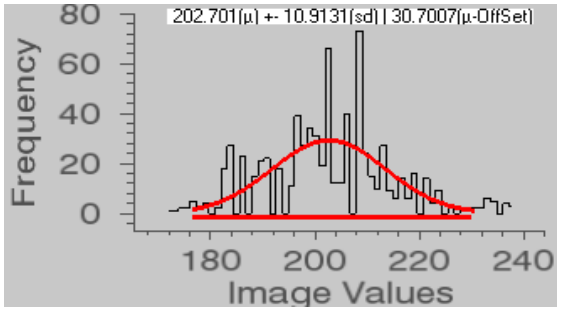
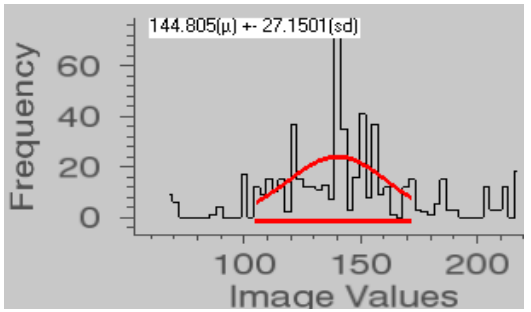


Figure 9. Images with different generated noise levels





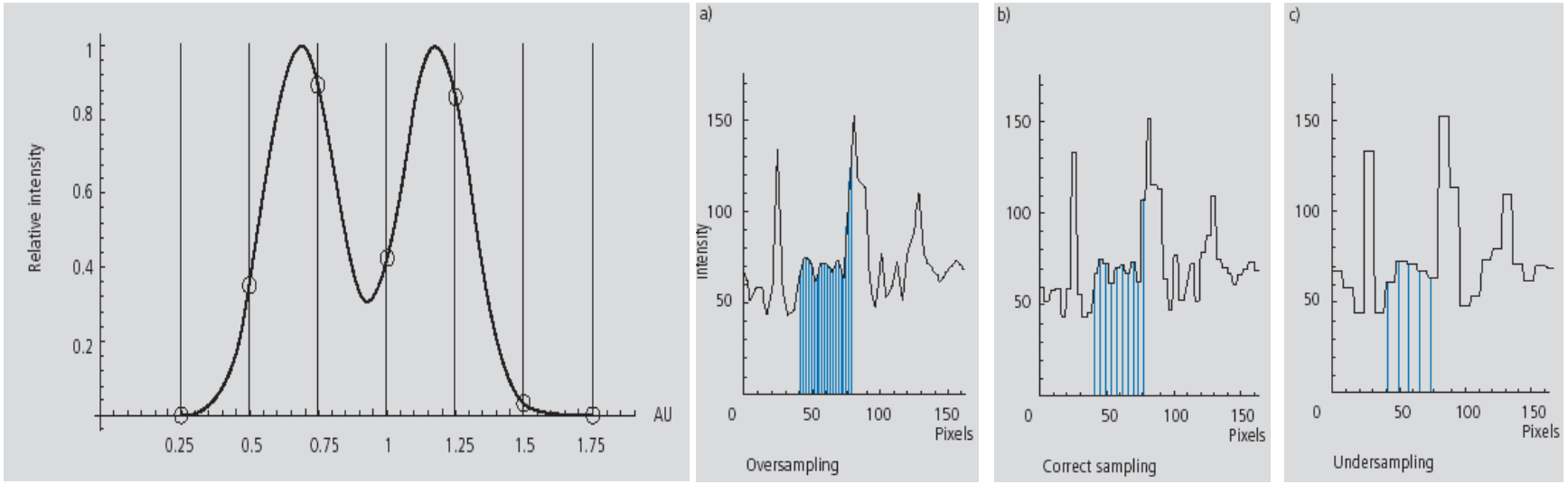


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

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

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

|-> 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*

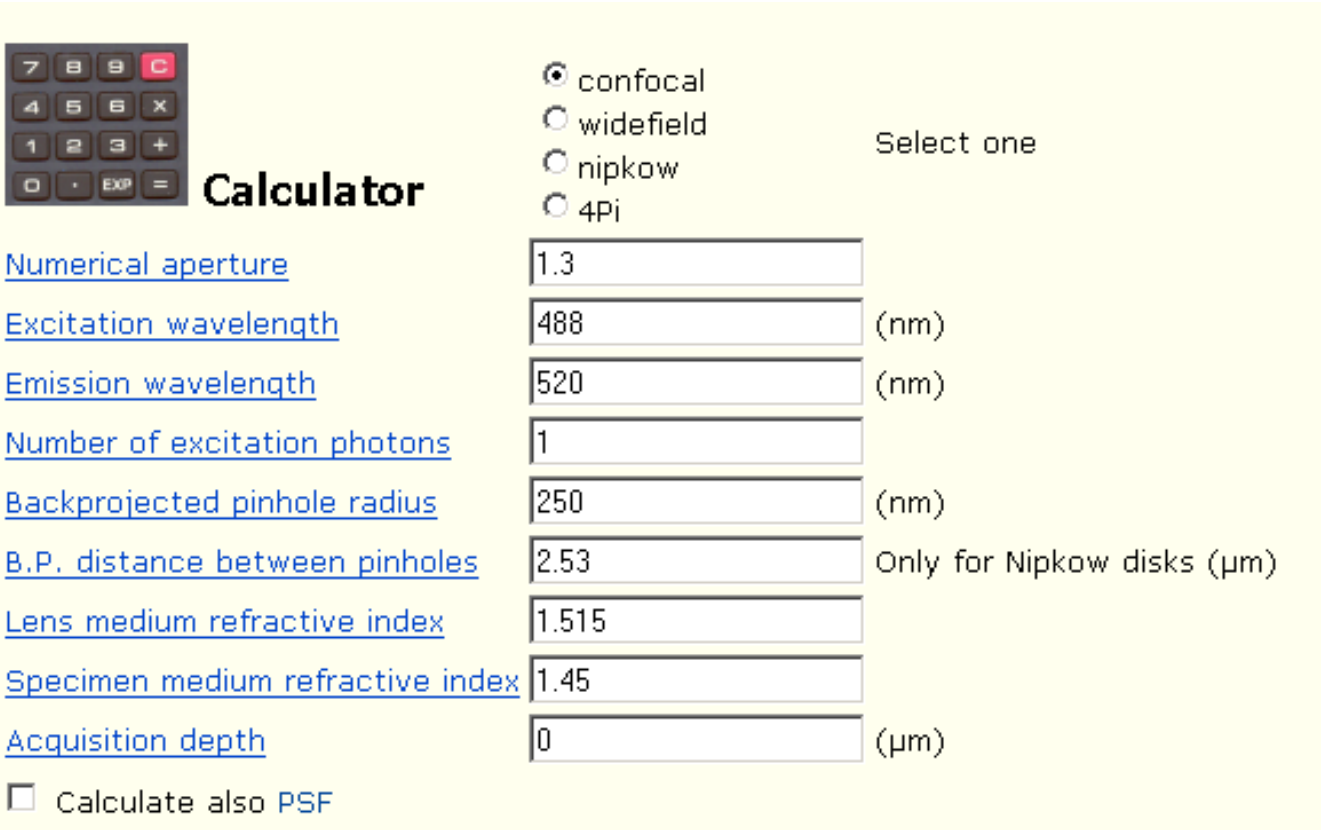
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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



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

|-> PSF calculator

PSF: *Point Spread Function*

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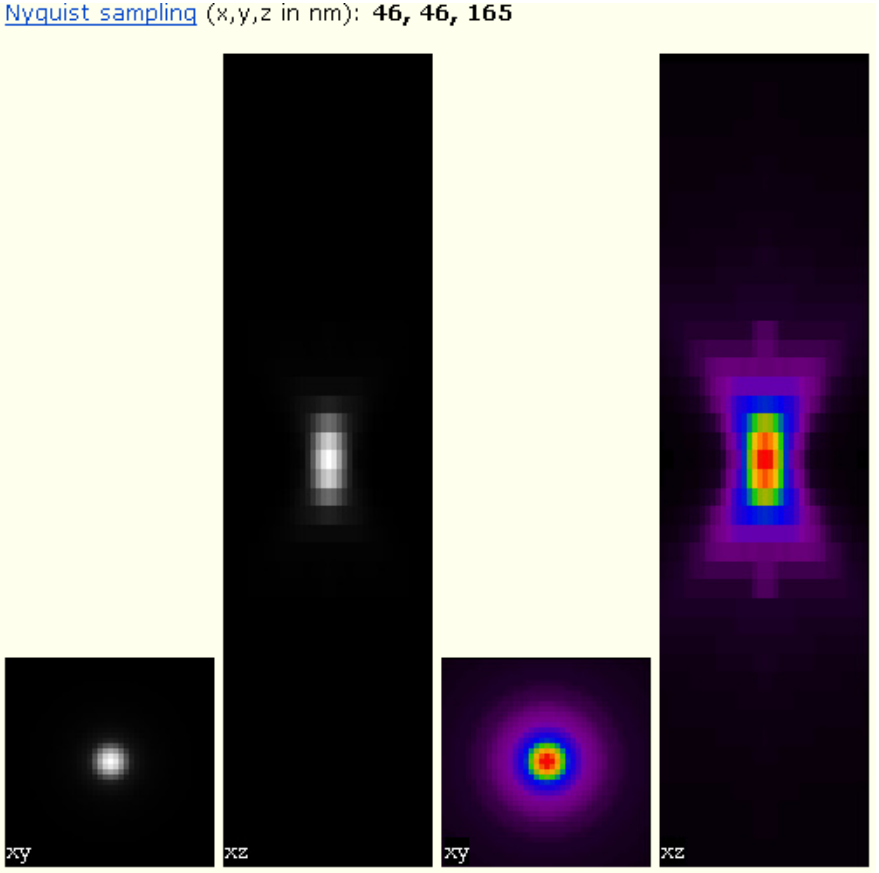
$$N(\mathbf{PSF}(x, y, z) \otimes \mathbf{f}(x, y, z) + \mathbf{b}(x, y, z)) = \mathbf{I}(x, y, z)$$



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

|-> Snell's Law

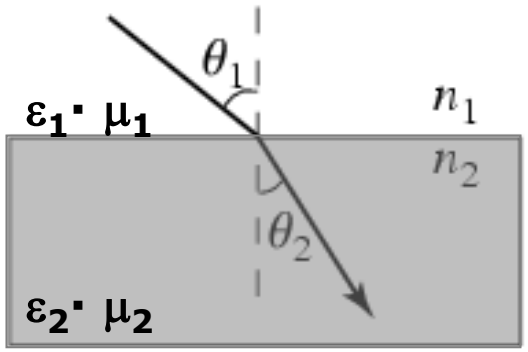
Index of refraction: $n = (\varepsilon \cdot \mu)^{1/2} = c/v$,

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]

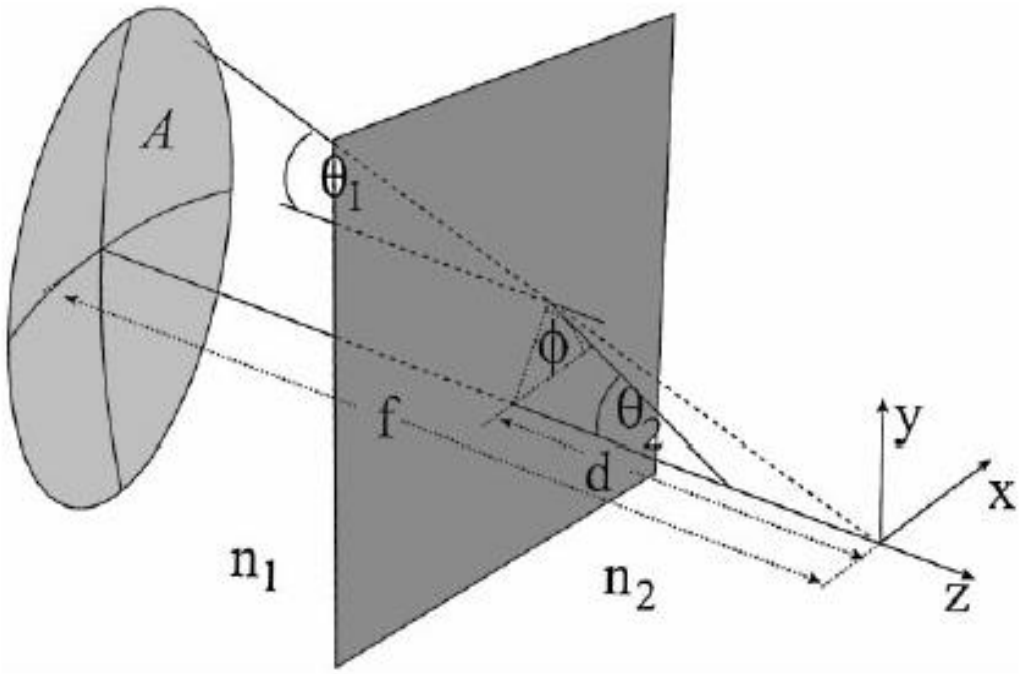
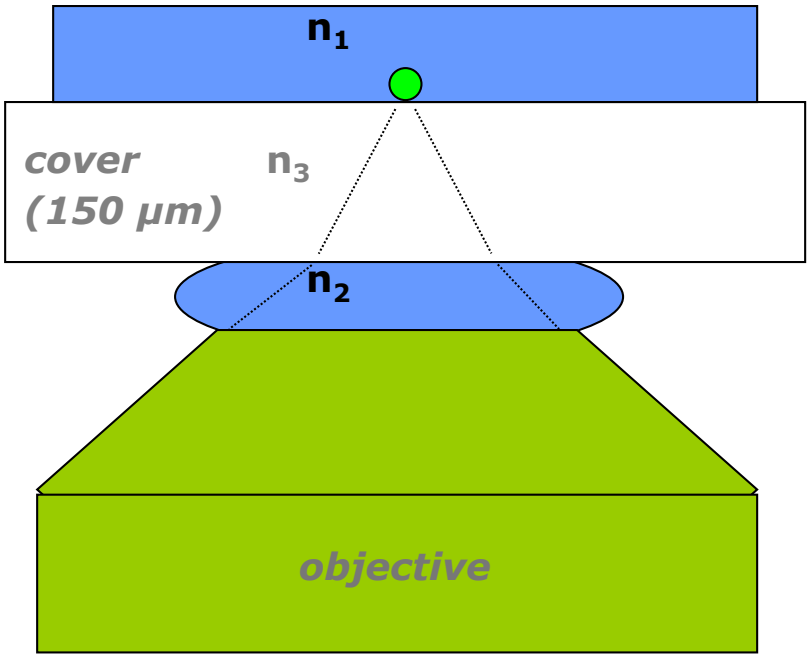


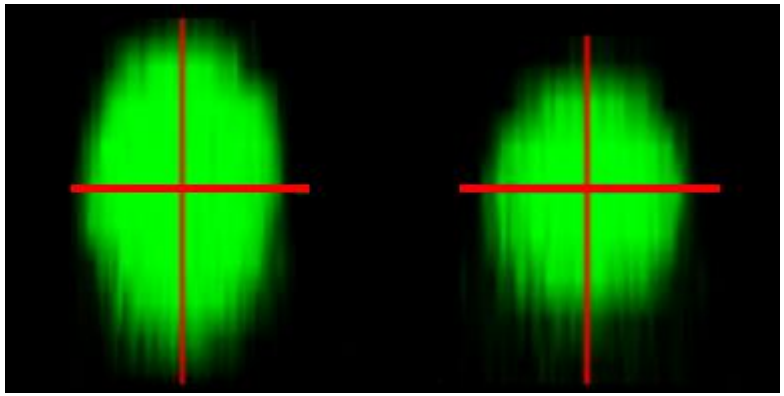
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera:** ∅ = 6 µm





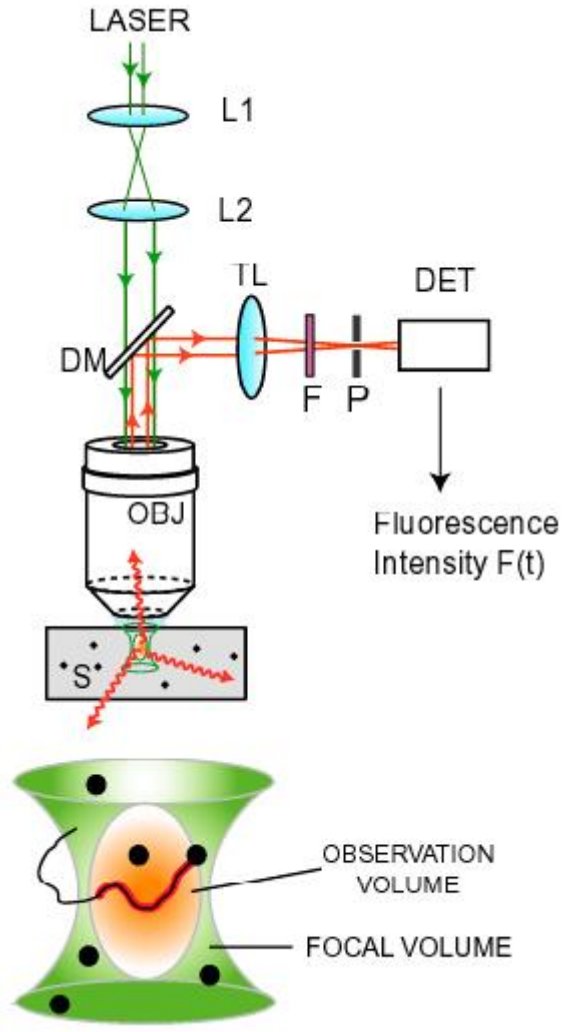
agua/aceite -- *aceite/aceite*

$n_1 \neq n_2$ $n_1 = n_2$

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

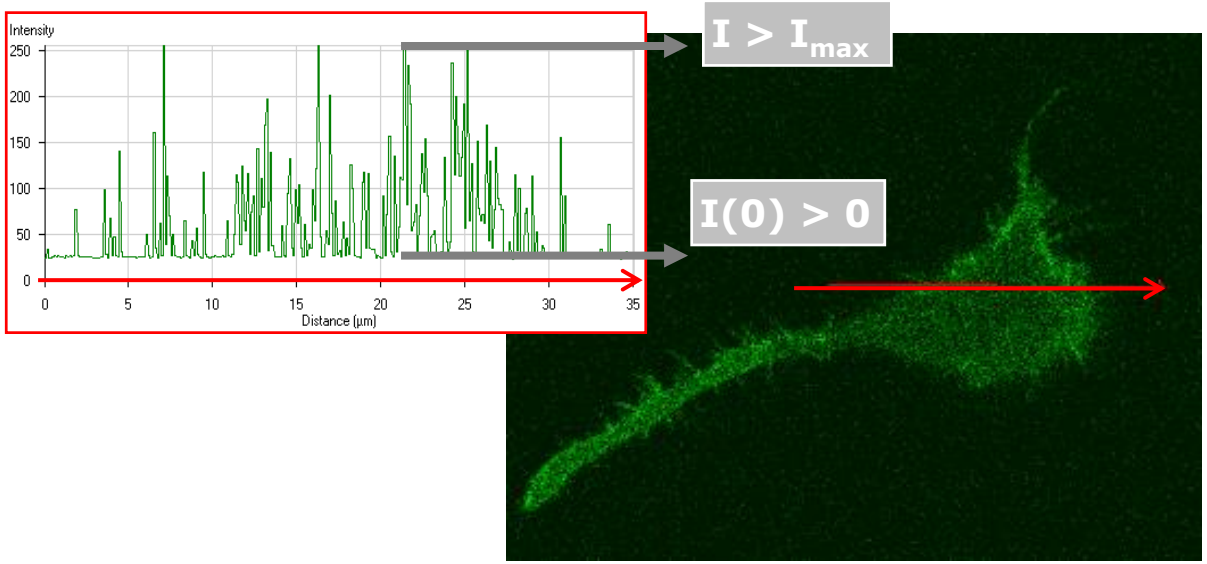
$n = n(\lambda)$!

|-> Resumen I

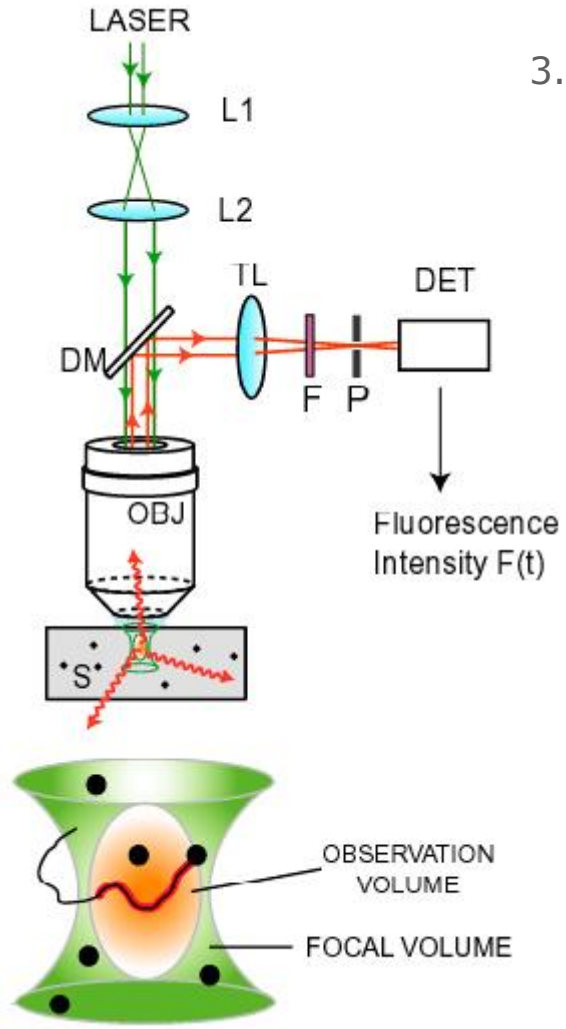


The observation volume (femtoliter) defined by the Point Spread Function must be considered as a mini-sprectrofluorimeter.

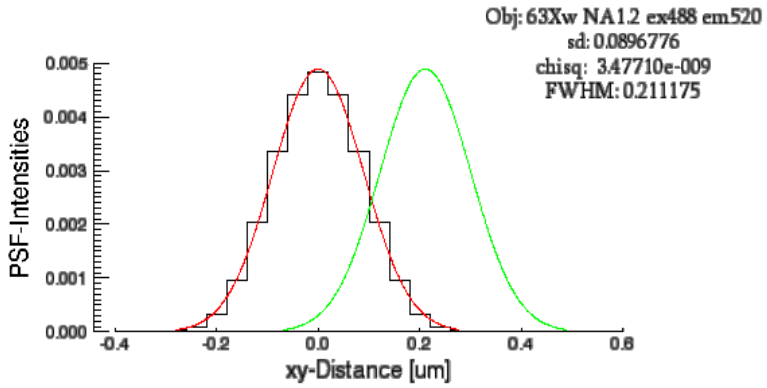
1. You need to consider the Offset I(0) in order to calibrate your signal $I(0) \geq 0$!
2. Never saturate the signal: $I \leq I_{max}$ (255 for 8 bit) !



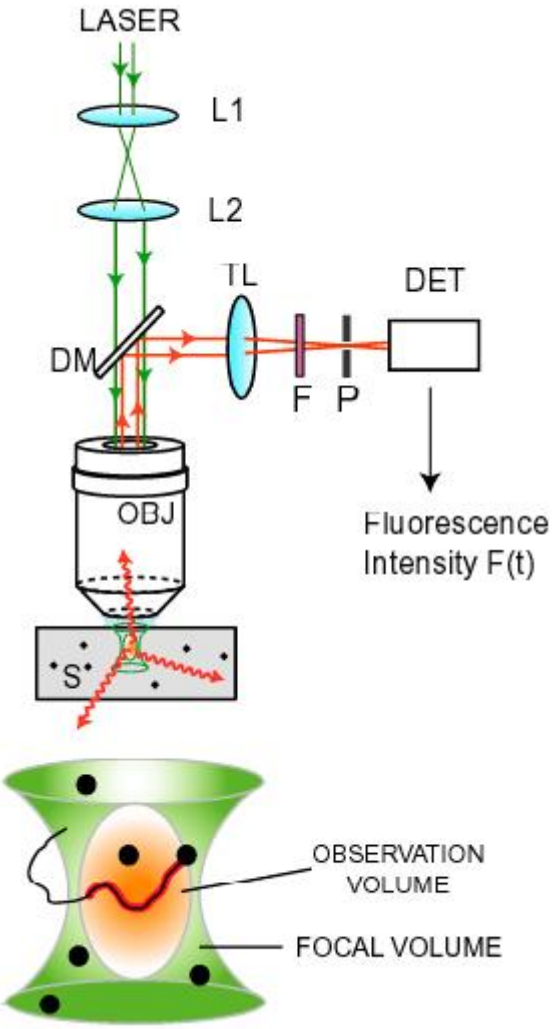
|-> Resumen II



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



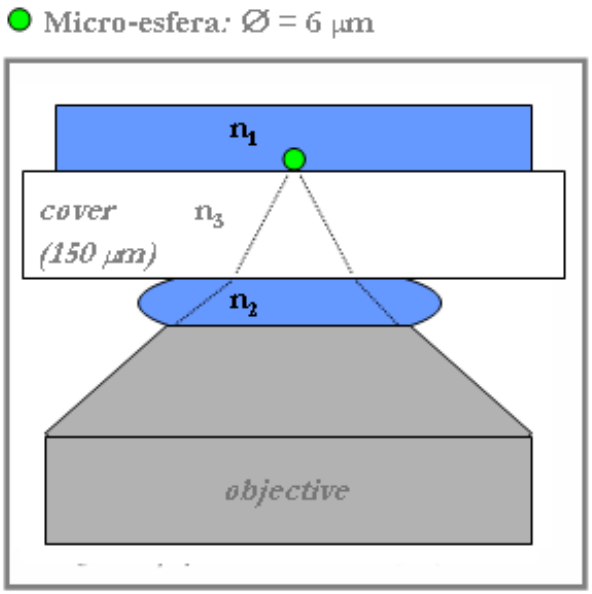
|-> Resumen III

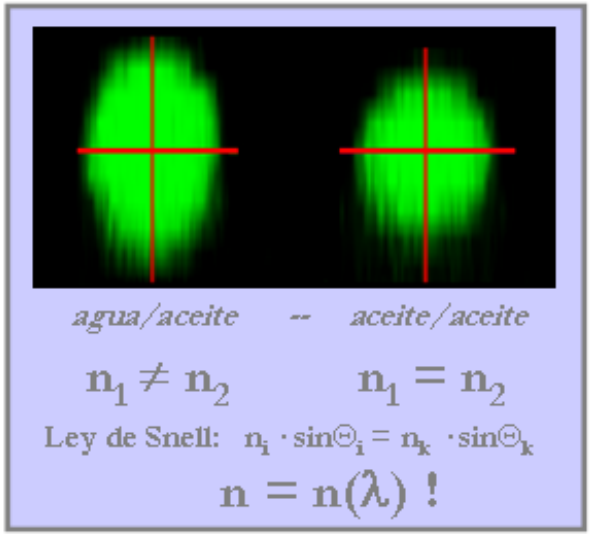


4. Use the right inmersion setup !

   n1 = n2 !

   Keep refractive index / index of refraction constant !





|-> Resumen

I. Image Adquisition

I.a|-> Fundamentos de la microscopía confocal

I.b|-> Fundamentos de la fluorescencia

II. Fundamentos de la Deconvolution

Para prufundizar: ver literatura en la página, presasos y practicos !