Laboratory for **SCIAN** Scientific Image Analysis

BIOMEDICAL NEUROSCIENCE INSTITUTE

CIMT CENTRO DE INFORMATICA MEDICA Y TELEMEDICINA

Microscopía y Procesamiento de Imágenes

MiM MAGISTER EN INFORMATICA MEDICA

Microscopía para el Estudio de Biofilms Bacterianos

Instituto de Investigaciones Biológicas Clemente Estable (IIBCE)
Instituto de Neurociencia Biomédica (BNI, ICBM, F-Med, U-Chile)
18 Oct - 1 Nov 2021

URUGUAY   **CHILE**

Prof. Dr. Steffen Härtel: 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)
Anatomy and Developmental Biology Program
Escuela de Postgrado
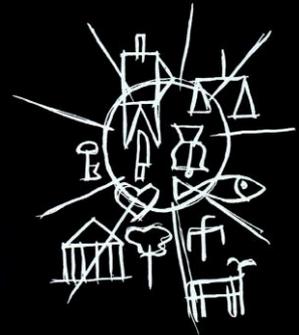Facultad de Medicina, Universidad de Chile

FACULTAD DE **MEDICINA** UNIVERSIDAD DE CHILE

Richard Feynman (1918-1988)

Joaquín Torres García 1874-1949
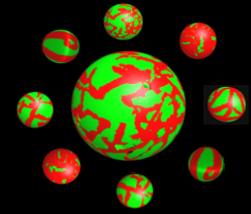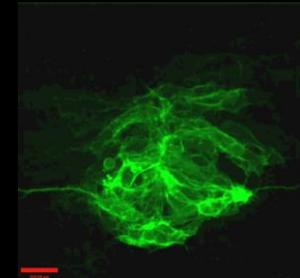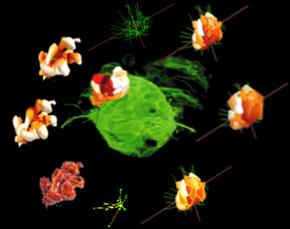
Maria Goeppert-Mayer 1906-1972
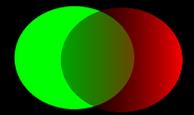
Mats Gustafson 2006 - 2011

René Descartes (1596-1650)

Ernst Abbe 1840- 2005

E Betzig   S Hell   W Moerner

Schermelleh et al 2010 JBC
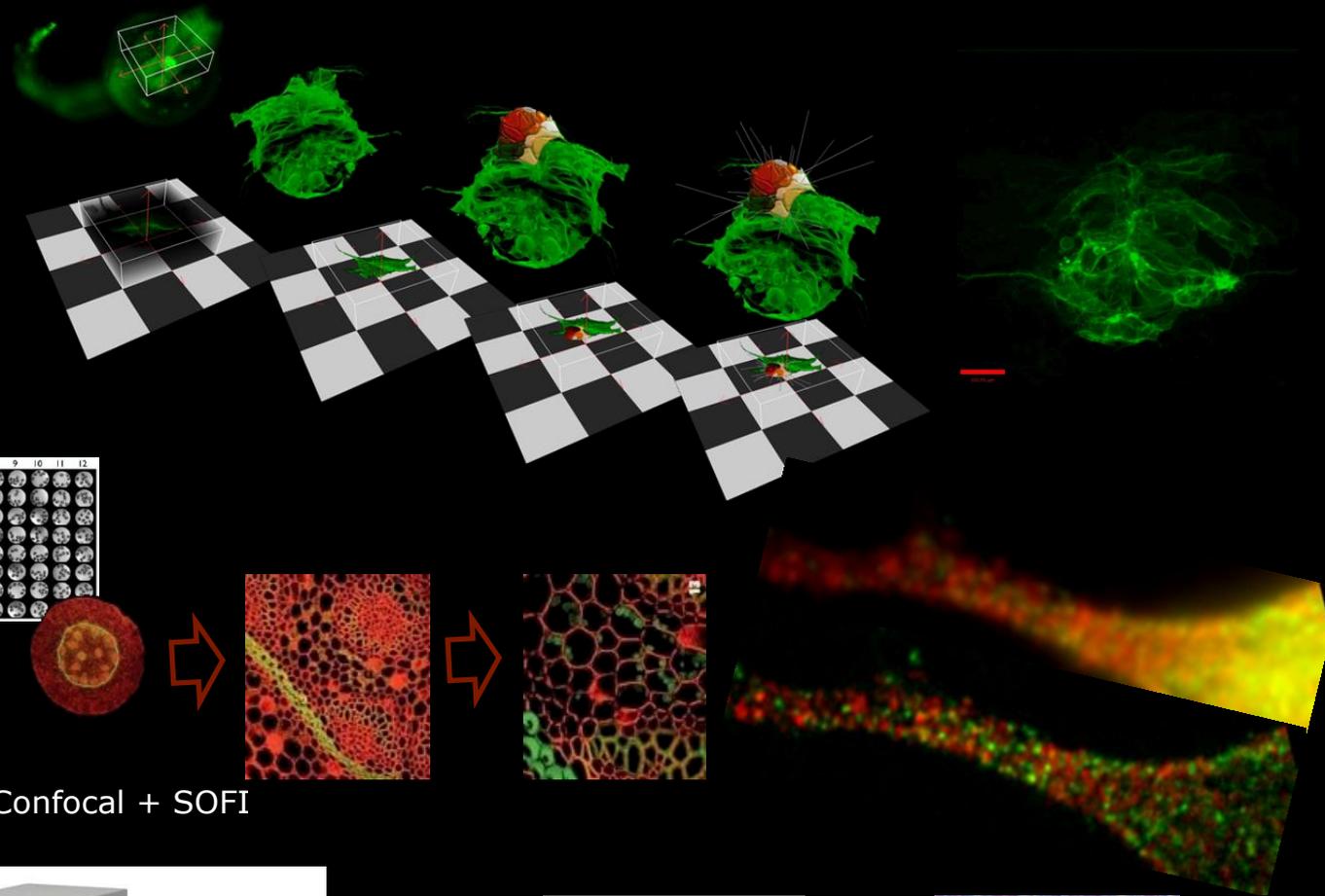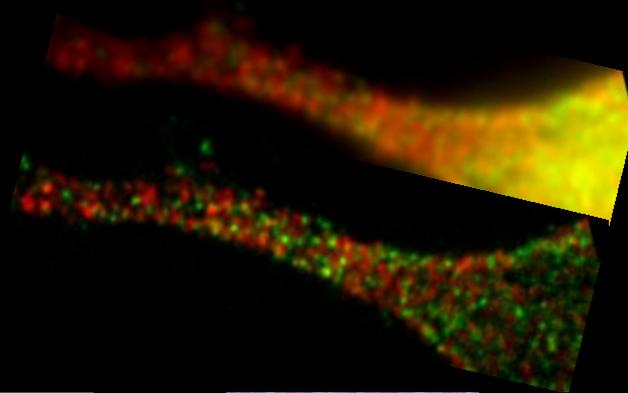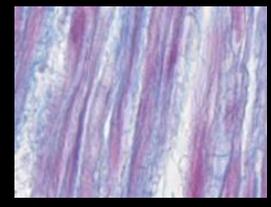
|-> Microscopy @ SCIAN-Lab

Perkin Elmer Spinning Disk
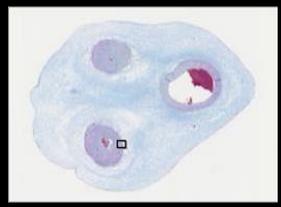
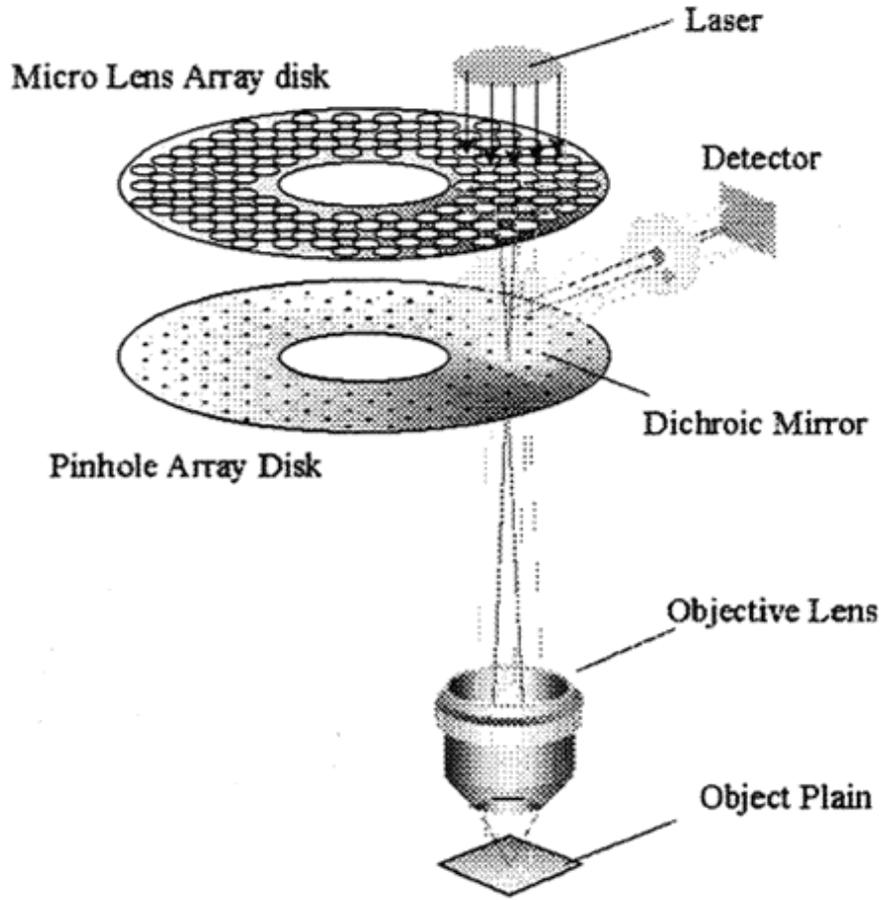Leica TCS LSI: Super Zoom Confocal + SOFI
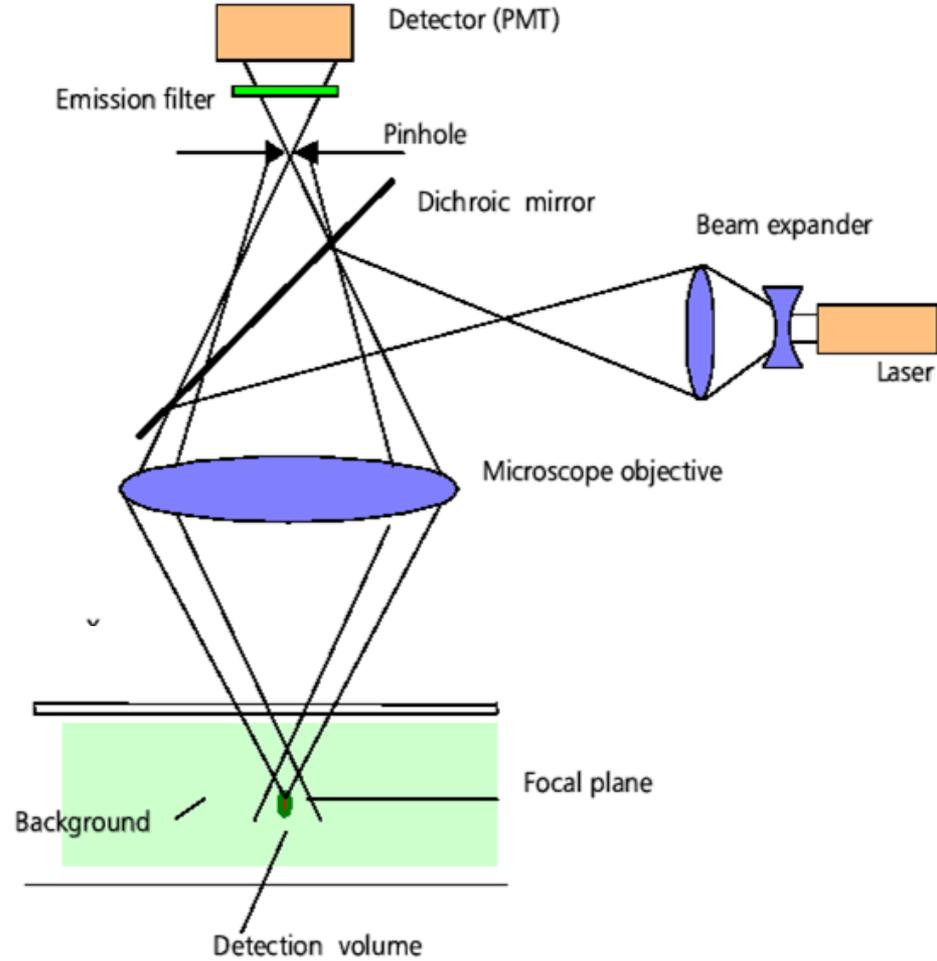
TB Data per Experiment

NanoZoomer: Tissue Imaging

spinning disk   **confocal**
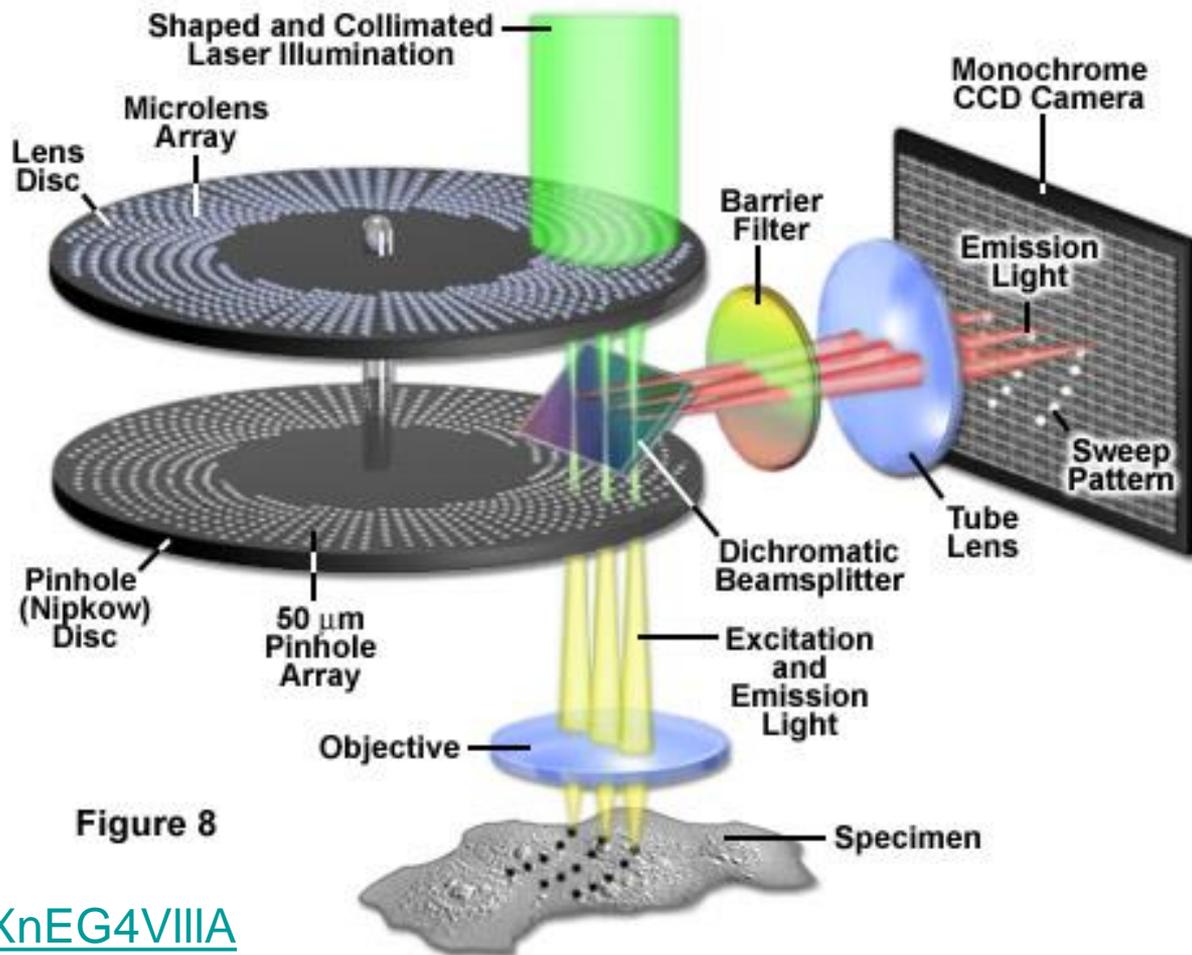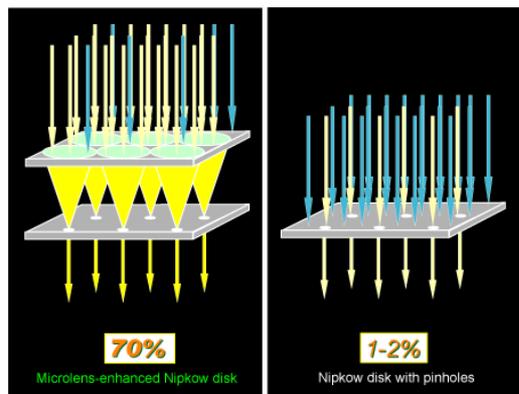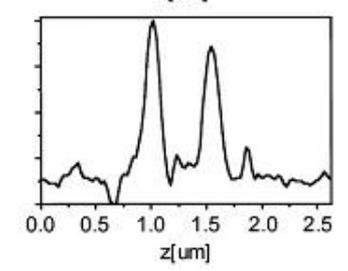
Yokogawa Spinning Disk Unit Optical Configuration

Figure 8

www.youtube.com/watch?v=fXnEG4VIIIA

|-> 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

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

M Grandbois et al. (1998) Biophys J.

|-> PSF

λ_{exc}

λ_{em}

?

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.

PSF:
Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: *Point Spread Function*

f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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

Objeto (f) PSF Objeto borroso PSF ⊗ f + b Imagen con ruido (I)

Deconvolución

Resultado ~ f
Mejor representación
de la realidad

|-> 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

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

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

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

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

Informa... Theo... atistical ...ysics

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

PSF: *Point Spread Function*

f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

- *Black Body Irrdiation (Poisson)*

- *Detector Noise (Gauss)*

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

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

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

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

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

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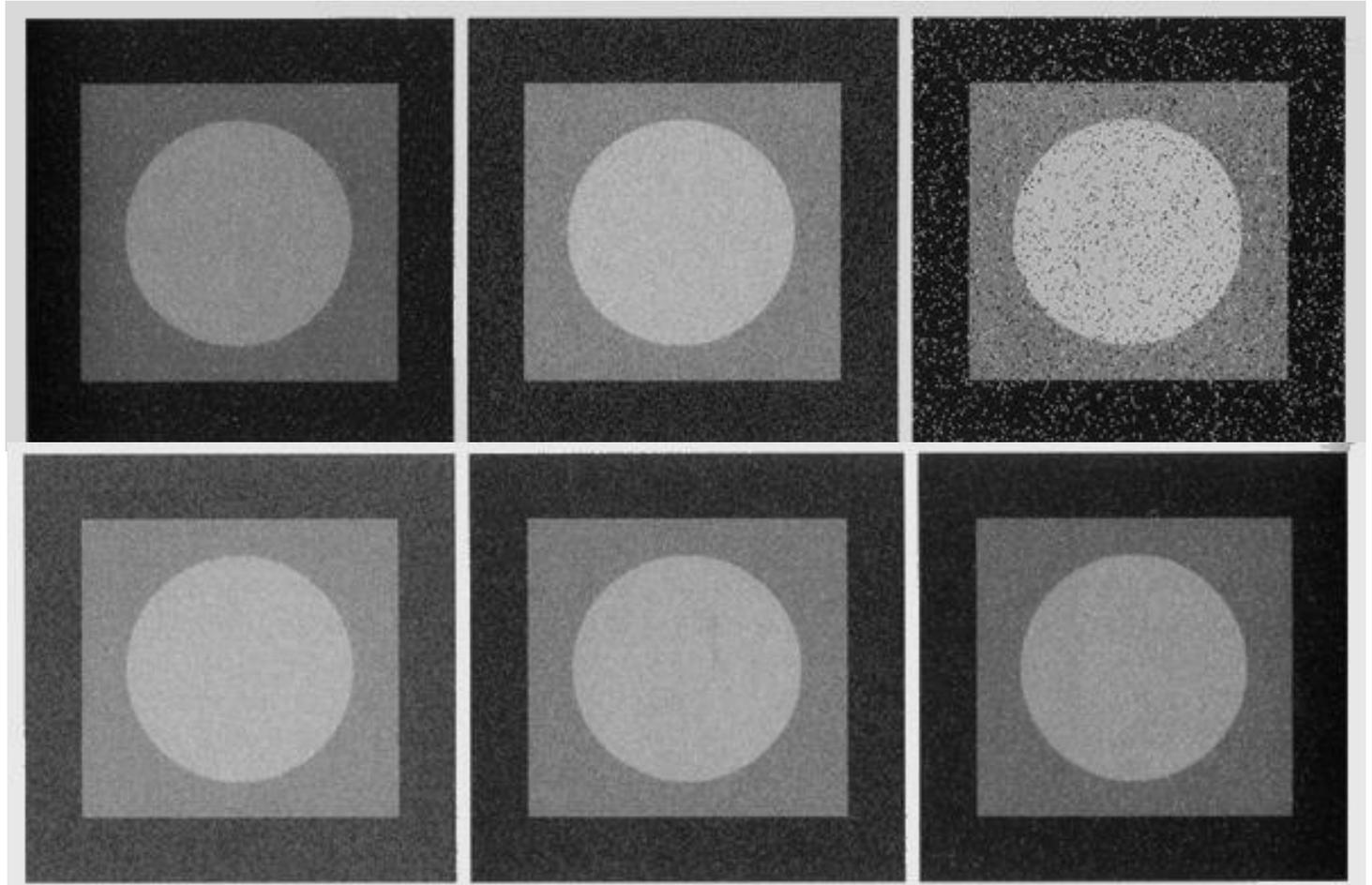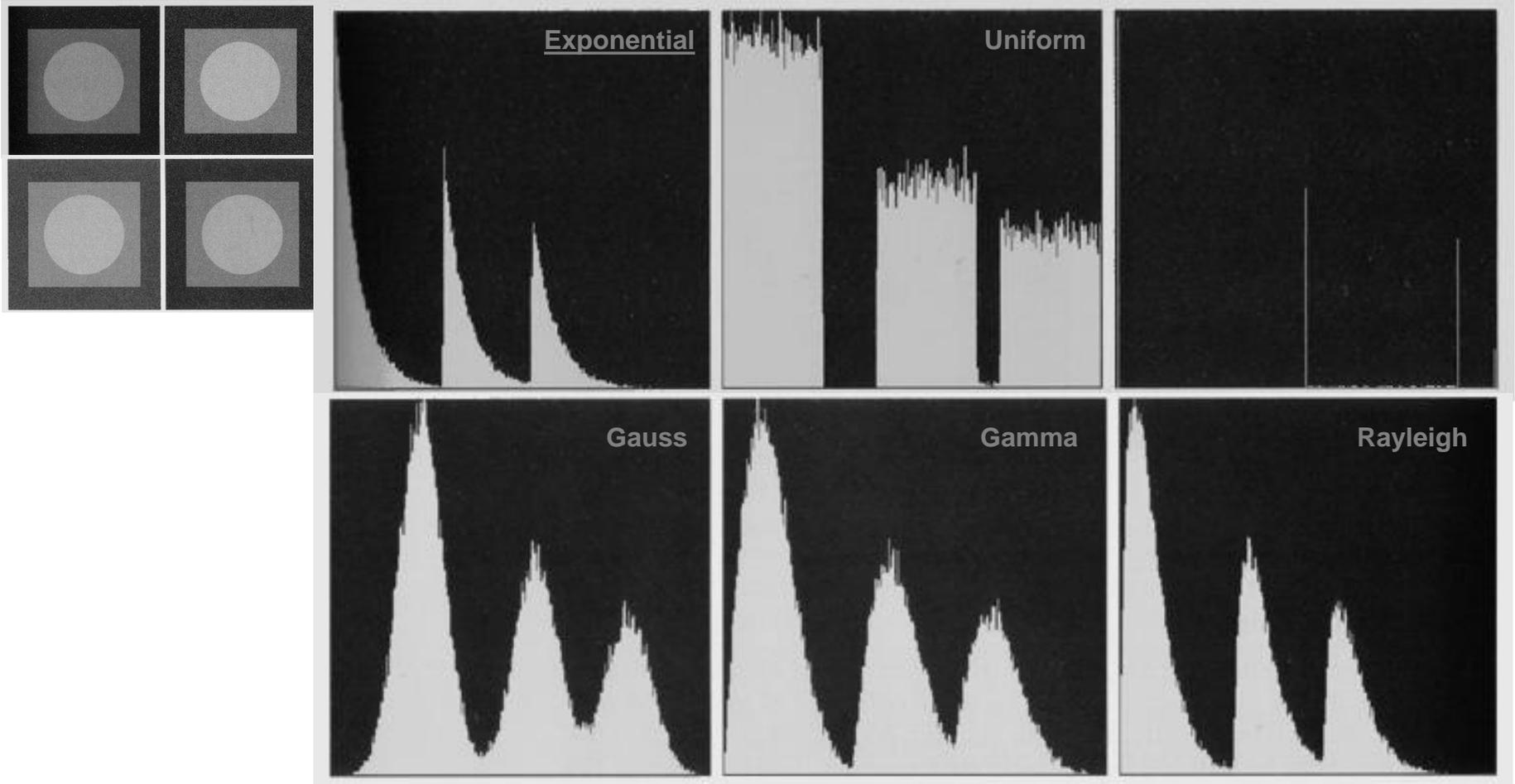 bright areas in your object image. In the figure at left you see examples of such textures obtained from originally the same object image to which various levels of poisson noise were added.

SN = 30   SN = 15   SN = 5

Figure 9. Images with different generated noise levels

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

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

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

- **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 $\geq 2\nu$?

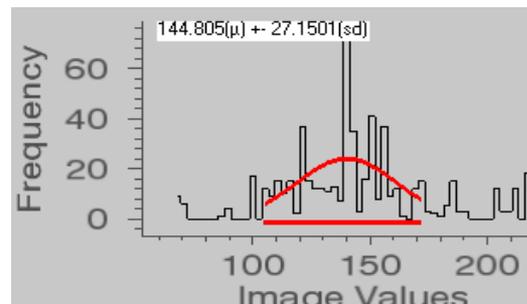**! Diffraction theory calculates lateral NR ~ 20 pixel/μm(~50 nm/pixel) !**
... laxial NR ~ (~150 nm/pixel)

|-> 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*

f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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

Nyquist sampling (x,y,z in nm): **46, 46, 165**

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

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:

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

$n = n(\lambda)$!
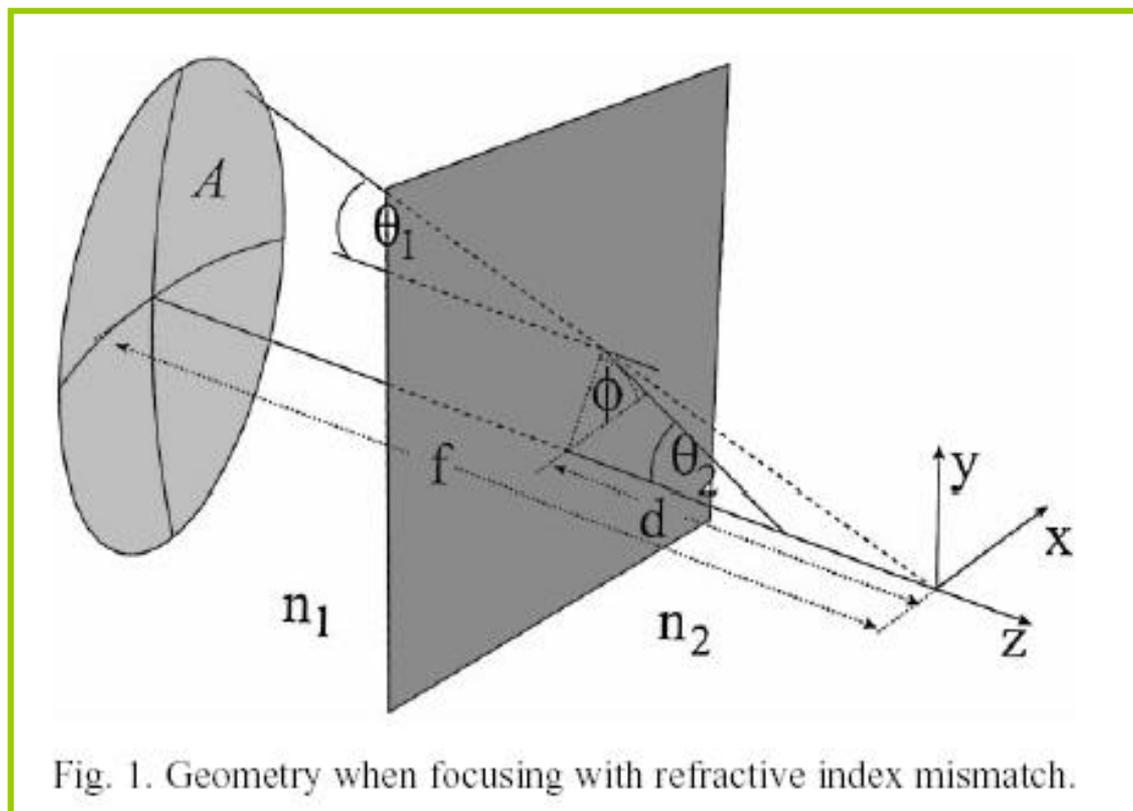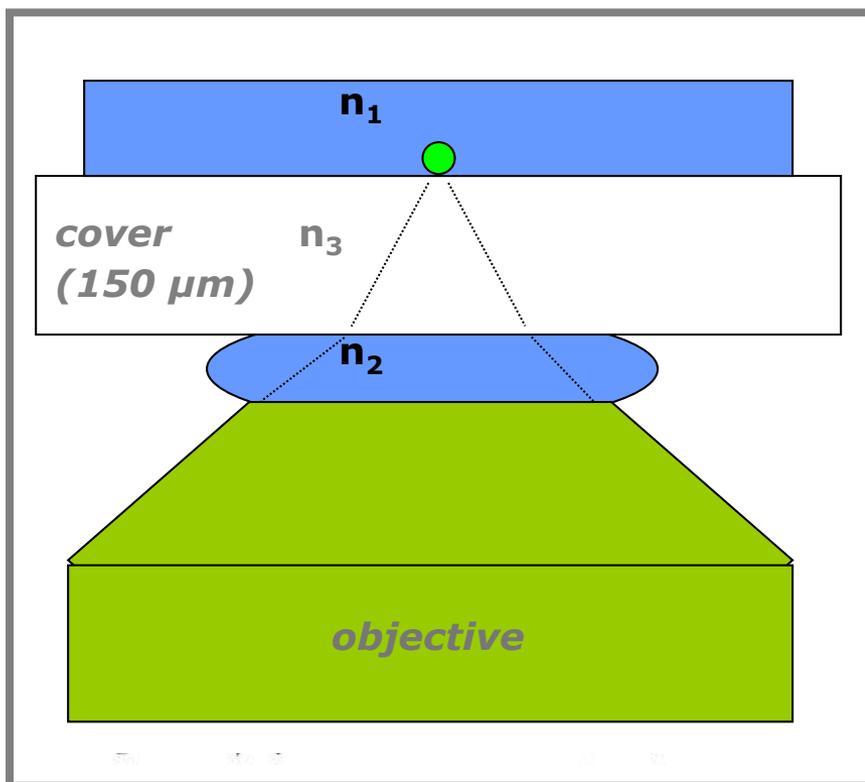
- **1.518 [Zeiss]**
- **1.33 [Water]**
- **1.0008 [Air]**

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

(Egner et al 1998)

🟢 **Micro-esfera*:*** $\varnothing$ = 6 µm

n$_1$

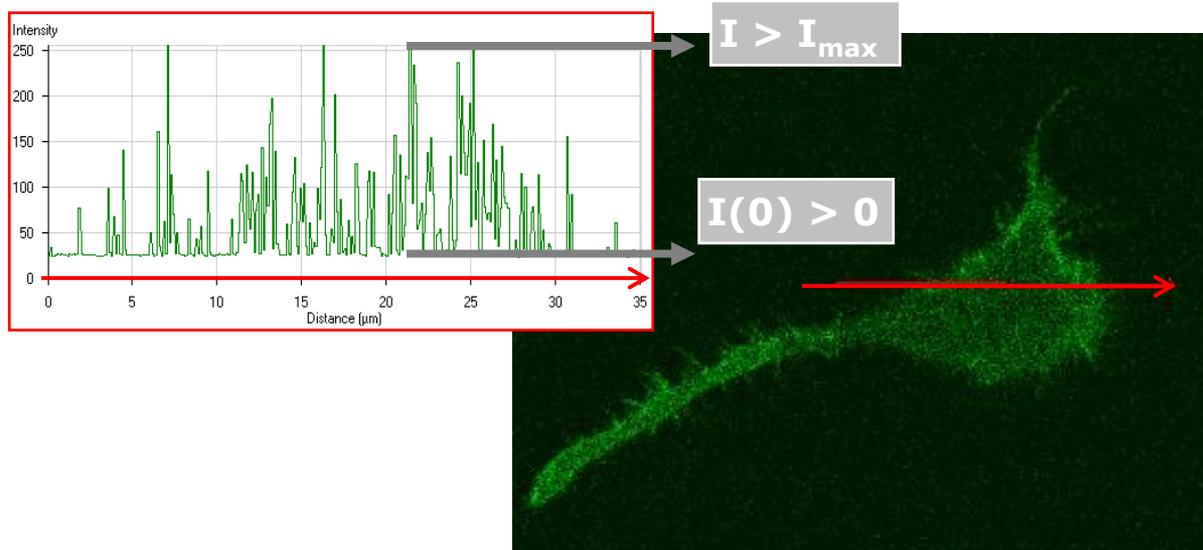*cover*   **n$_3$**
(150 µm)

n$_2$

objective

agua/aceite   --   aceite/aceite

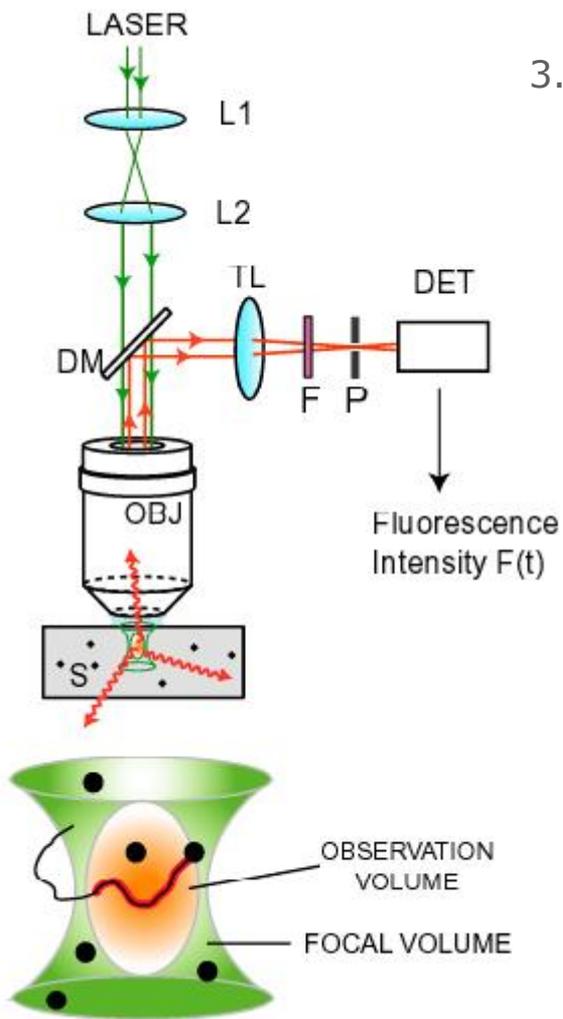**n$_1$ ≠ n$_2$ n$_1$ = n$_2$**

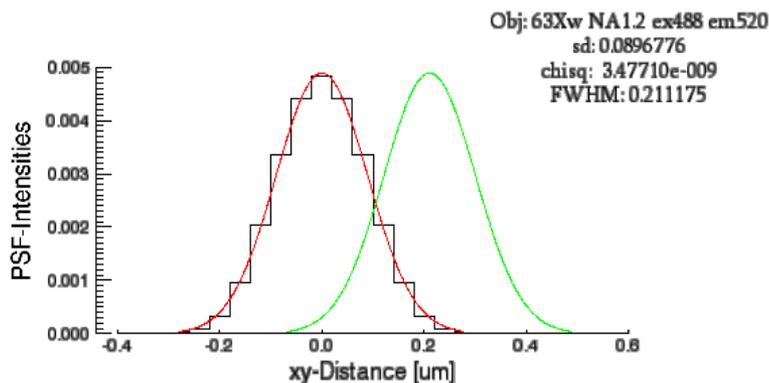**Ley de Snell: n$_i$ · sinΘ_i = n$_k$ · sinΘ_k**
n = n(λ) !

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) !
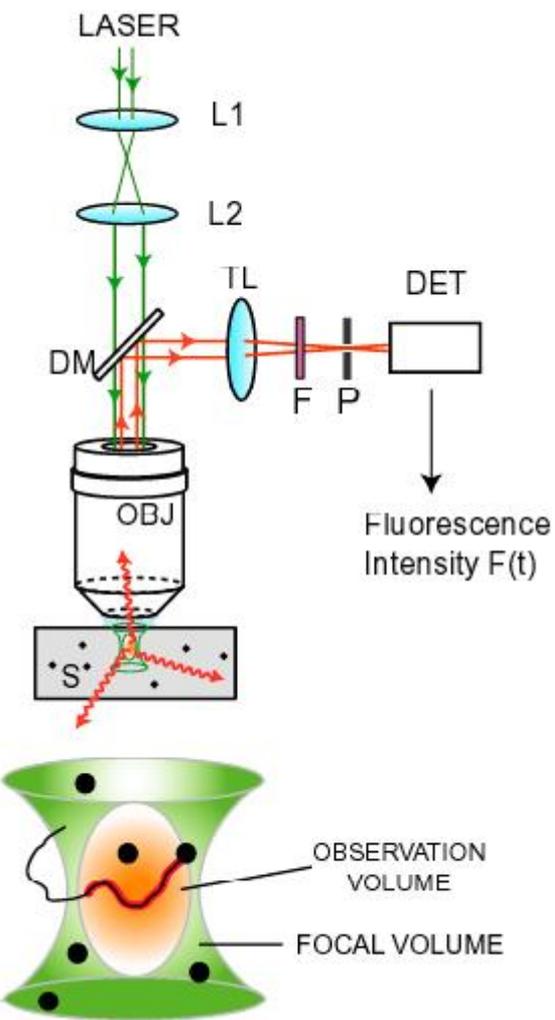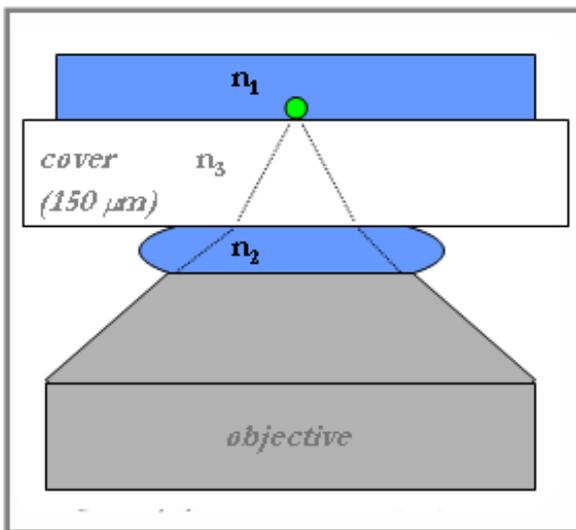
$I > I_{max}$

$I(0) > 0$

3. You need to consider sampling distances in Δx and Δy ≈ 50 nm and Δz ≈ 150-300 nm for later deconvolution, or calculate the explicit sample distances @ http://support.svi.nl/wiki/NyquistCalculator

LASER

L1

L2

TL   DET

DM

F P

OBJ

Fluorescence
Intensity F(t)

'S'

OBSERVATION
VOLUME

FOCAL VOLUME

4. Use the right inmersion setup !

n1 = n2 !

Keep refractive index / index of refraction constant !

● Micro-esfera: $\varnothing = 6 \ \mu m$

n_1

cover n_3
(150 μm)

n_2

objective

agua/aceite   --   aceite/aceite

$n_1 \neq n_2$ $\qquad$ $n_1 = n_2$

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

$n = n(\lambda)$!