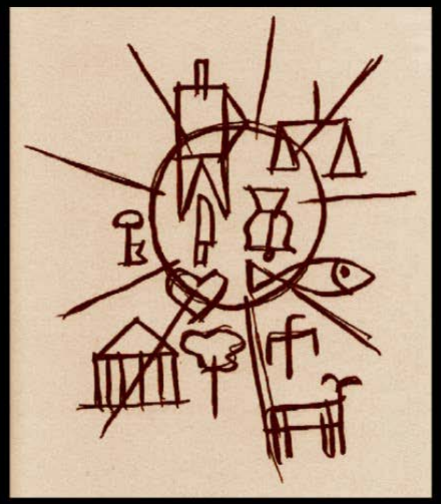
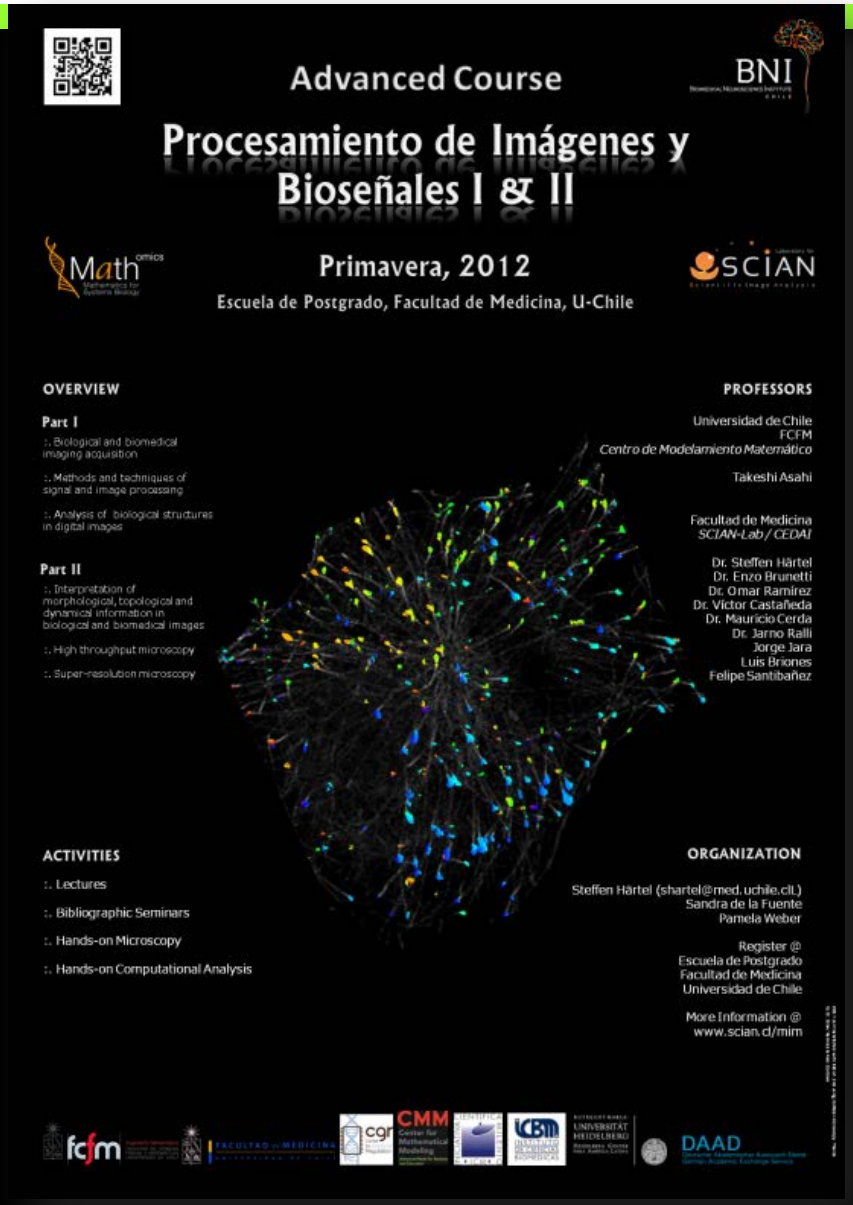
Welcome

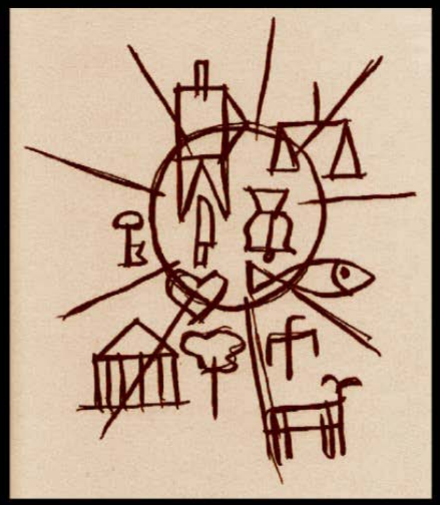


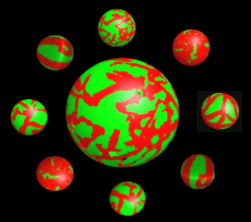
Laboratory for SCIAN Scientific Image Analysis

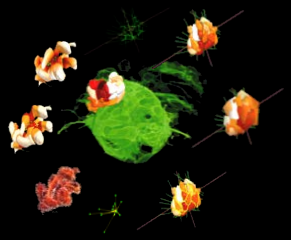
Welcome

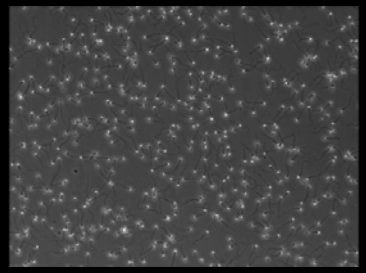
Biomedical Neuroscience Institute

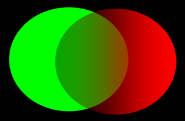


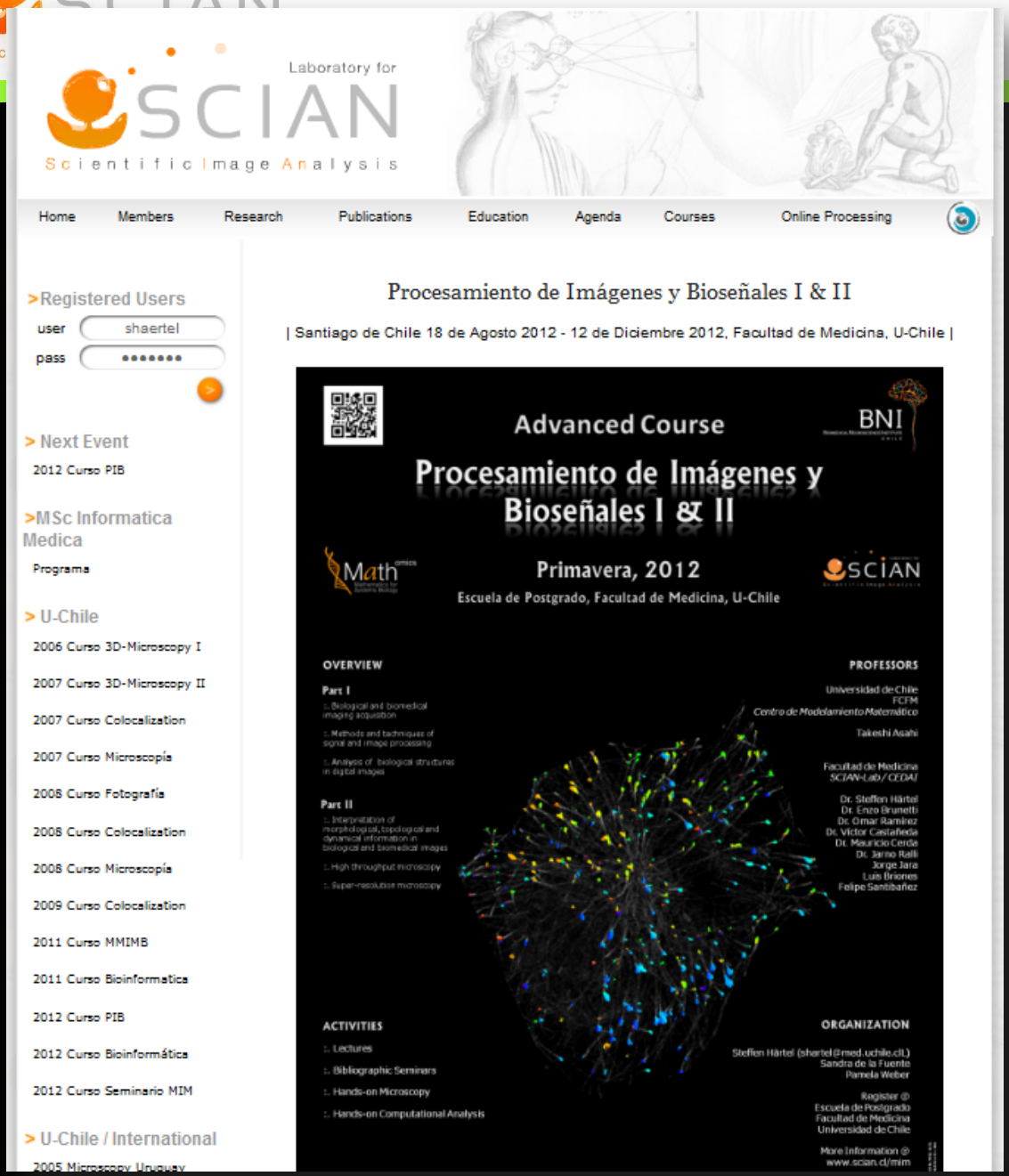












Informaciones en:

-> www.scian.cl

-> www.scian.cl/mim

Notas:

Prácticos (25%)

Seminarios (25%)

Examen Final (50%)

5 grupos a 3:

- Seminarios Prácticos

20 min presentación y pyd

- Seminarios Bibliográficos

20 min presentación +

20 min preguntas y discusión

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www.mascienciaparachile.cl

Basic Science	R&D	Human Capital Formation Medical Informatics	Basic Science
FONDECYT CONICYT	FONDEF CONICYT	DAAD / DFG	ICM
50 M$Ch 100 kUS$ / year	110 M$Ch 220 kUS$ / year	60 M$Ch 120 kUS$ / year	60 M$Ch 120 kUS$ / year

PI	●	Biophysics
PostDocs	●●●	Biology / Computer Sc / Electrical Engineer
PhD - students	●●●●	Computer Sc / Electrical Engineer
Master - students	●●	Medical Technology / Electrical Engineer
Undergraduate	●	Computer Sc
Research – Assistants	●●●●●	Medicine / Computer Sc / Electrical Engineer / Biology
Technicians	●●●	Biotechnology / Labtechnician / Administration

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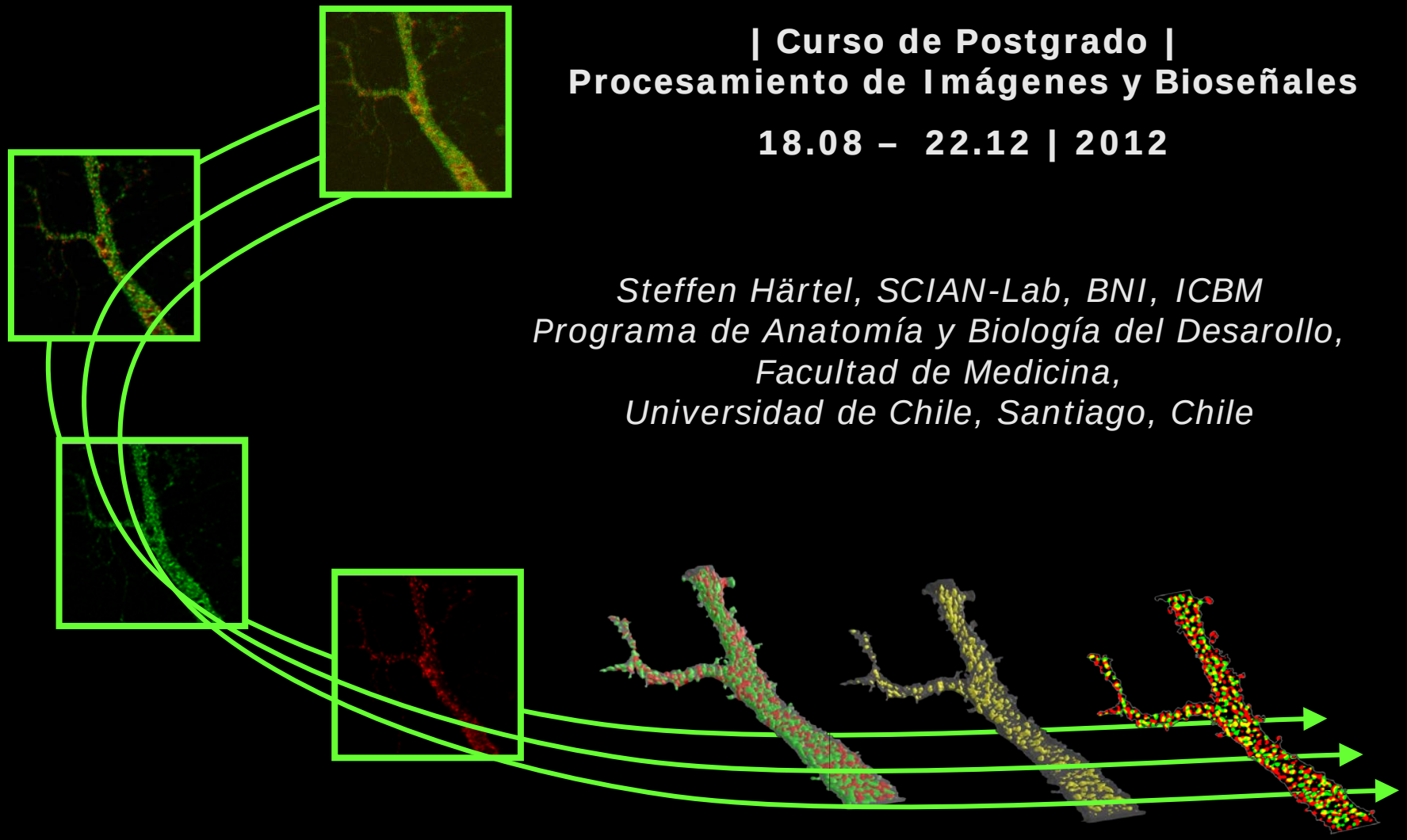
| Curso de Postgrado |
Procesamiento de Imágenes y Bioseñales

18.08 – 22.12 | 2012

Steffen Härtel, SCIAN-Lab, BNI, ICBM
Programa de Anatomía y Biología del Desarollo,
Facultad de Medicina,
Universidad de Chile, Santiago, Chile



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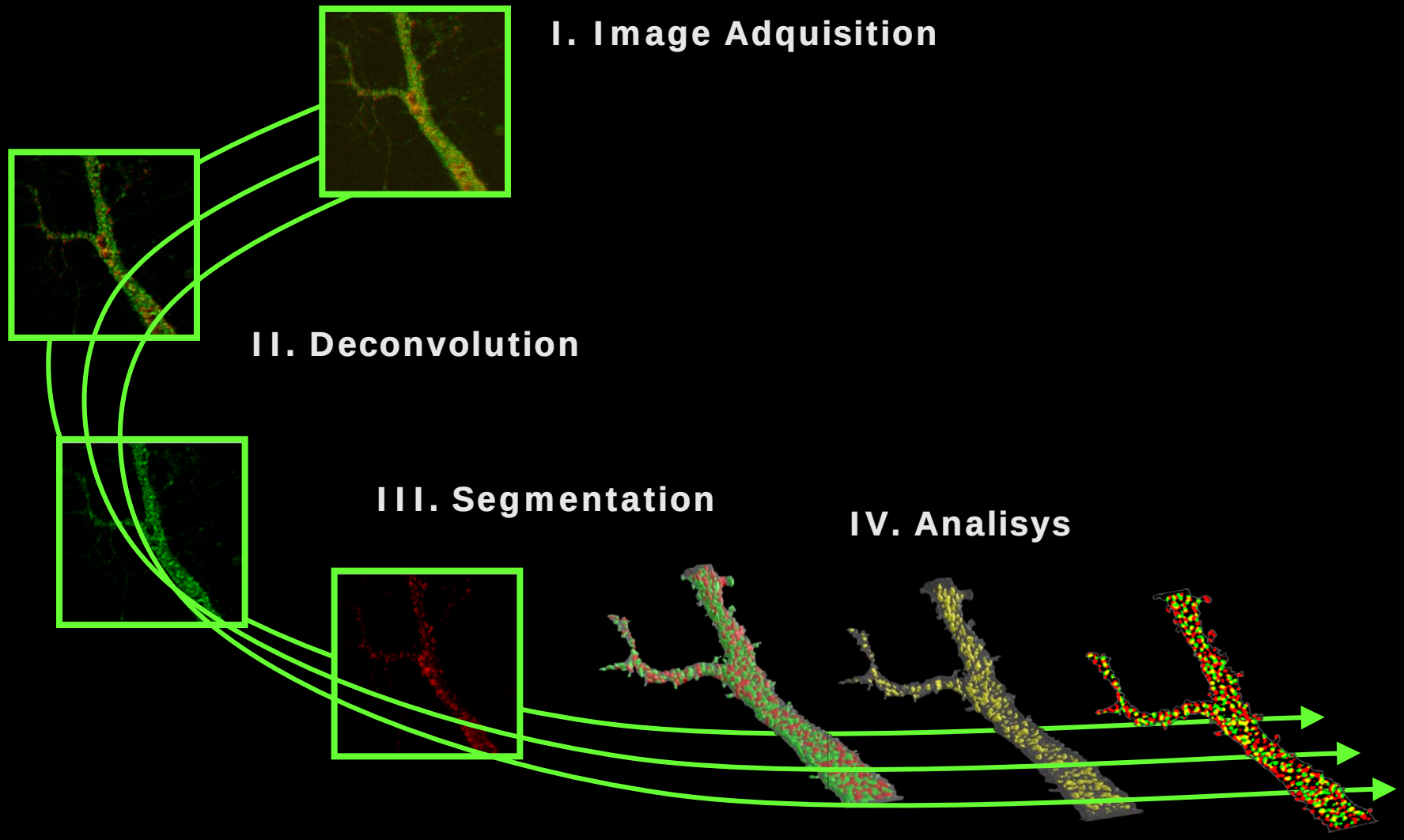

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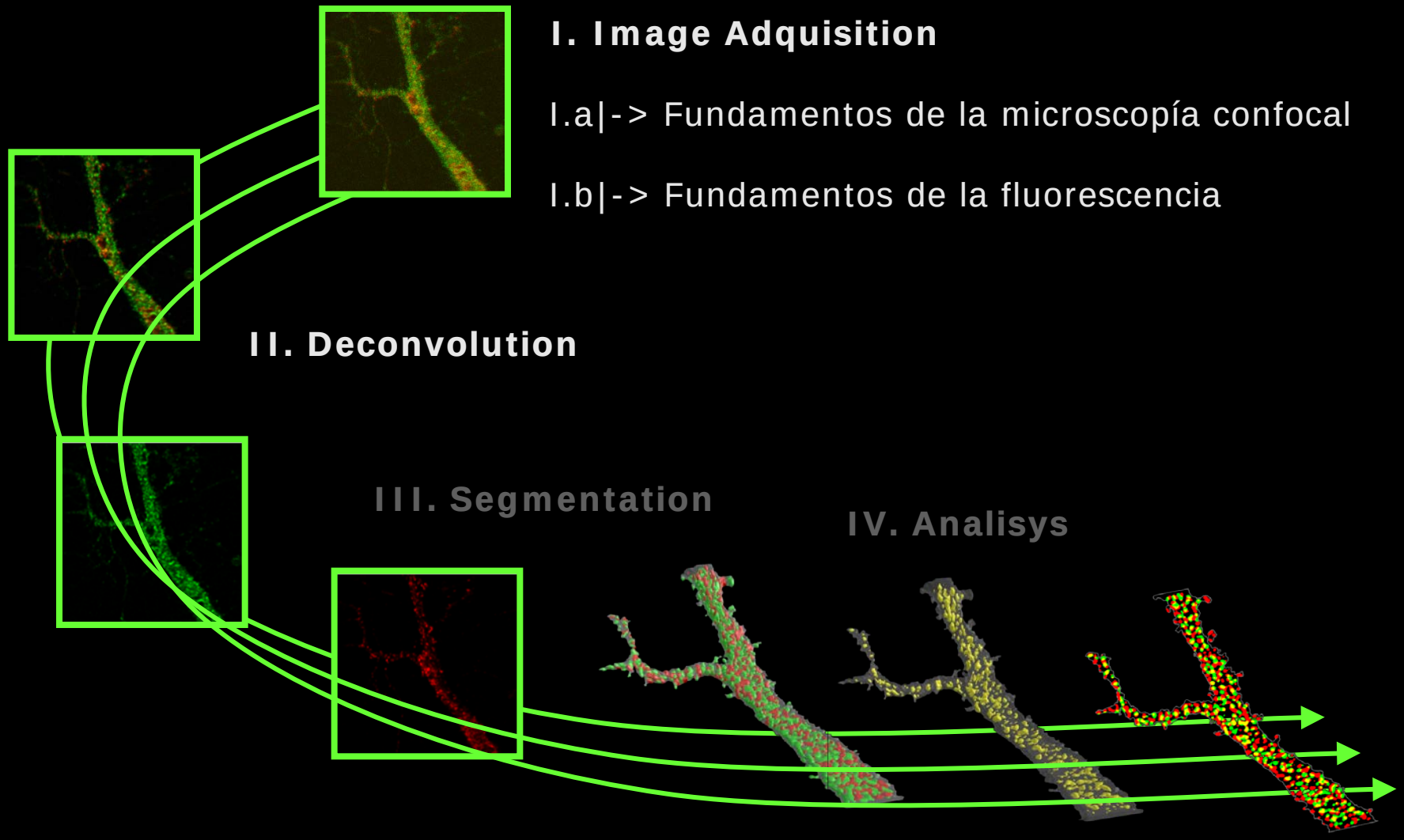
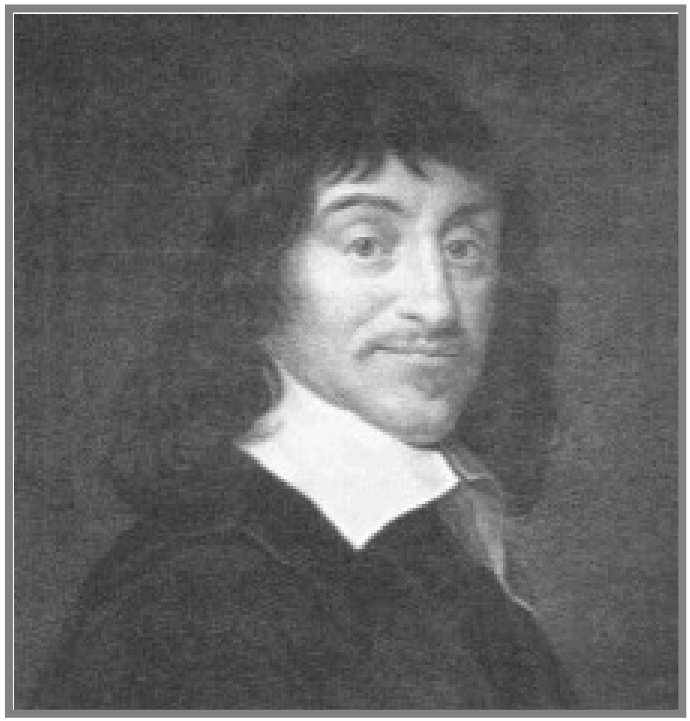


"It is very easy to answer many of these fundamental biological questions. You just look at the thing !

Make microscopes a hundred times more powerful and many problems of biology would be made very much easier."

Richard Feynman (1918-1988)



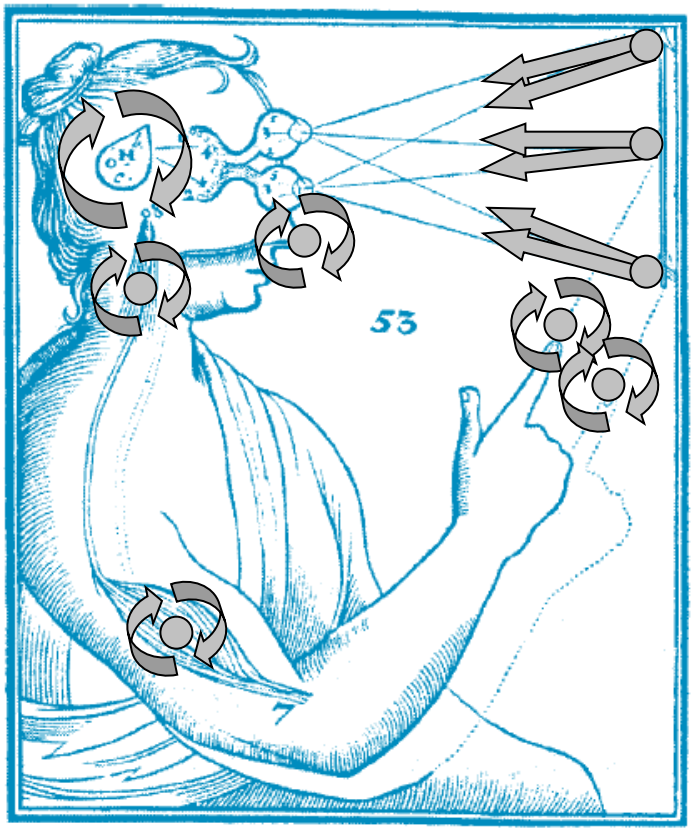
René Descartes (1596-1650)

... just look at the thing ...

¿ Human visual perception ?

***Treatise of man* (~ 1637)**

***Passions of the soul* (~ 1649)**



glandula pinealis / pineal organ

A combination of ...

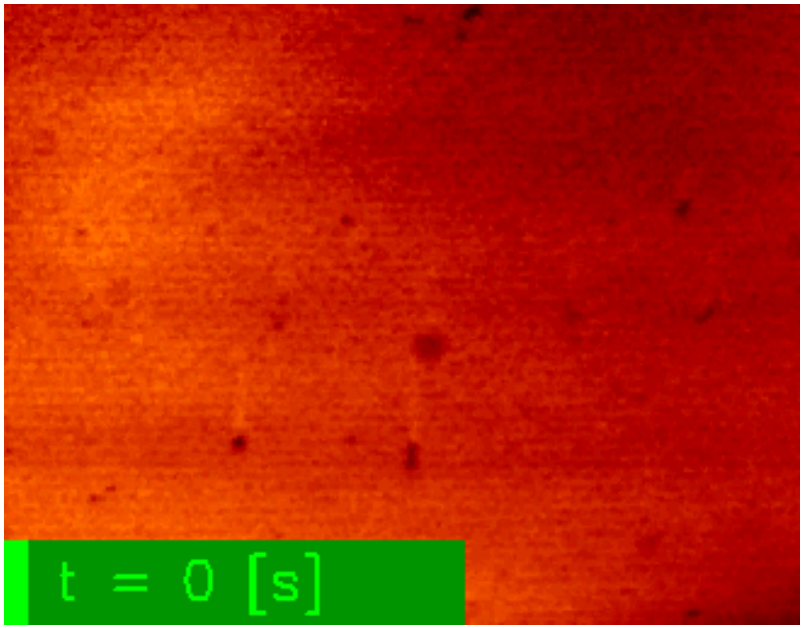
1| direct signals ...

2| signals from other senses ...

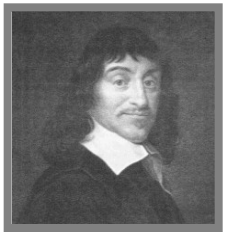
3| feedback loops ...

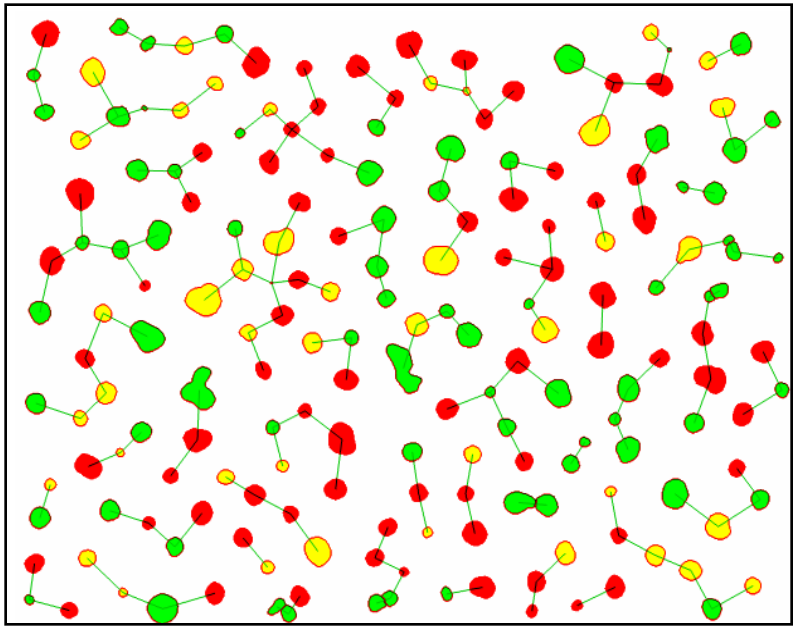
... produce a symbolic representation of an object.

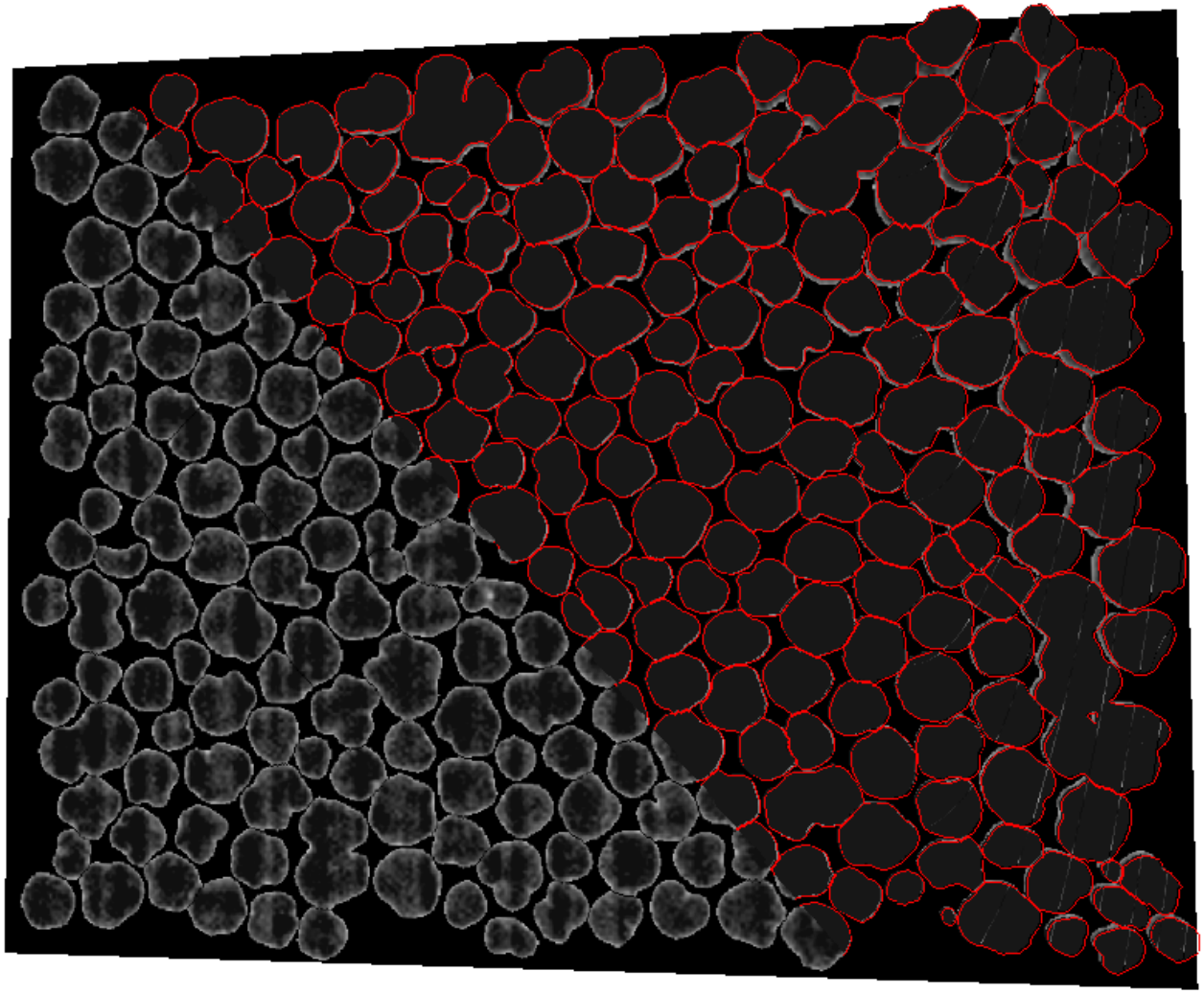
| Best resolution in t & x …



|| Humas vs machine vision







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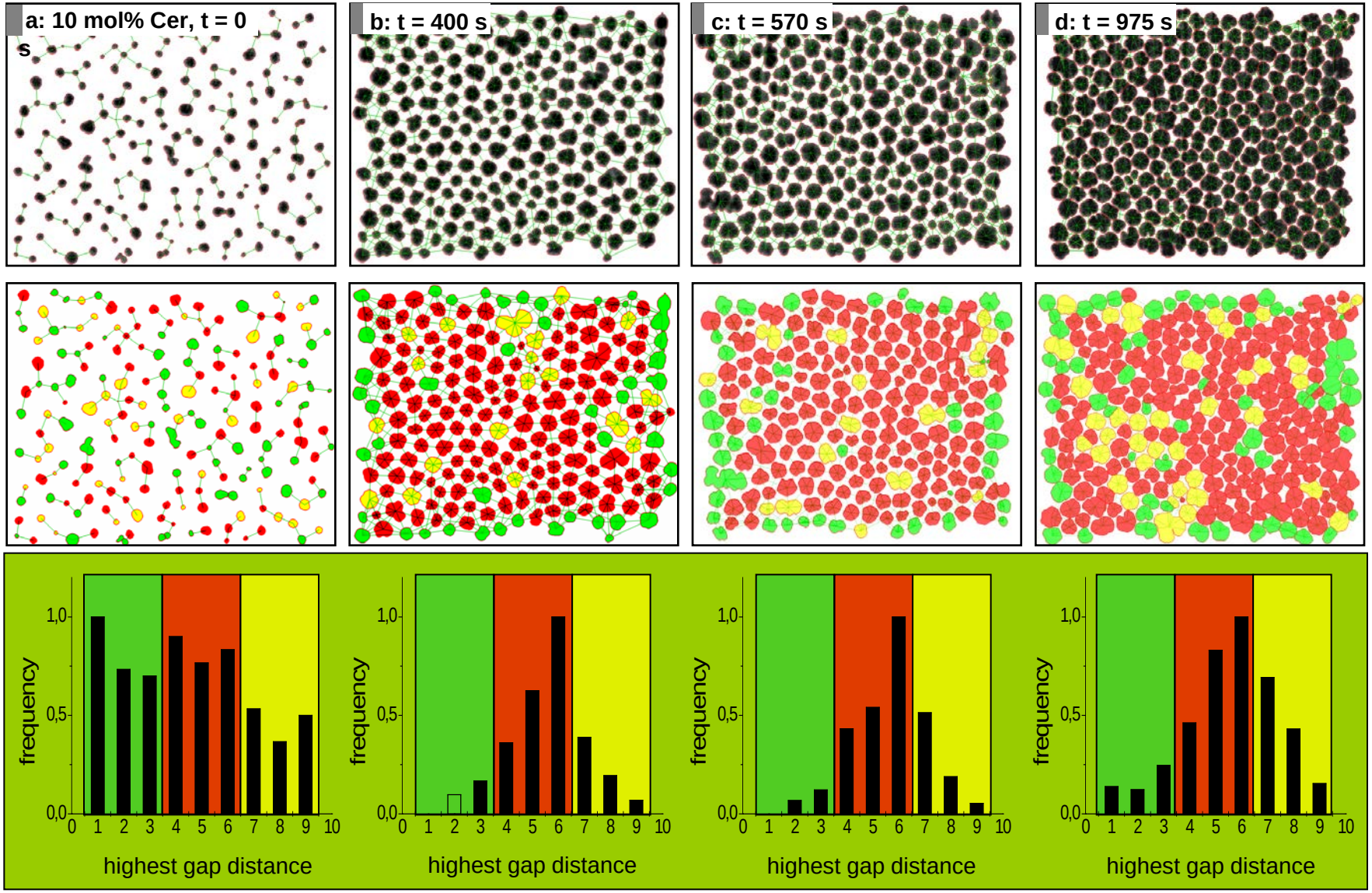
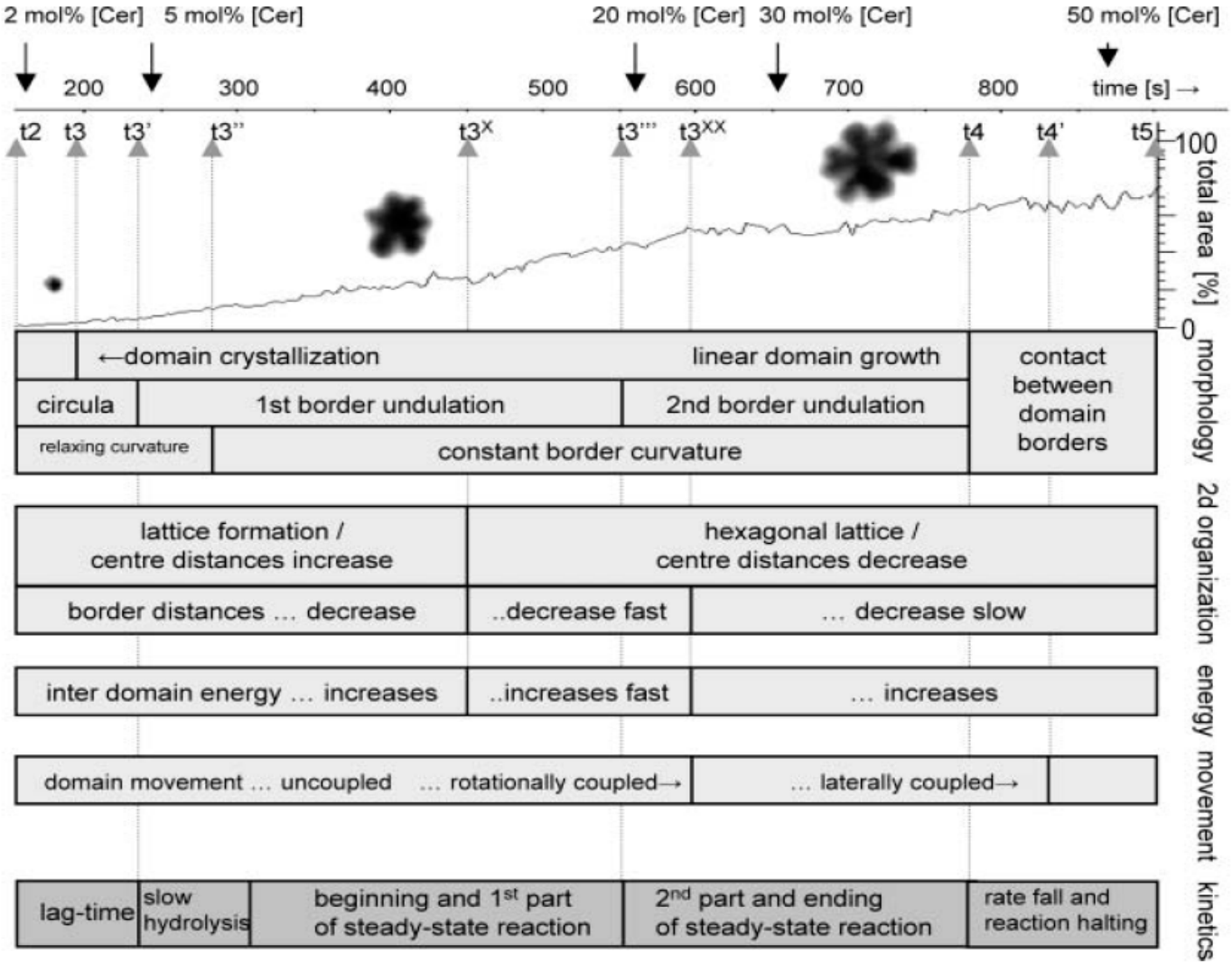
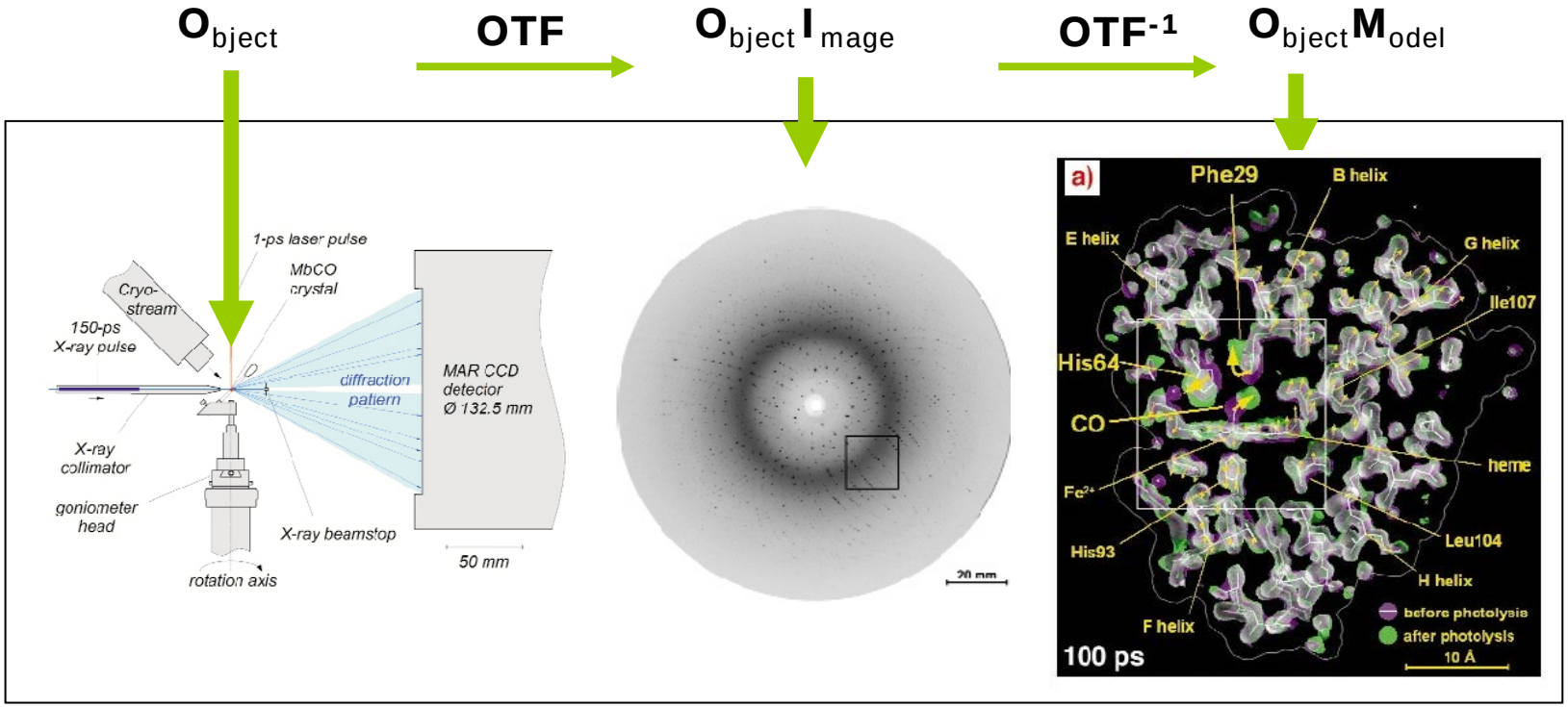



2 mol% [Cer]
5 mol% [Cer]
20 mol% [Cer]
30 mol% [Cer]
50 mol% [Cer]
200
300
400
500
600
700
800
time [s] →
t2
t3
t3'
t3''
t3X
t3'''
t3XX
t4
t4'
t5
100
0
total area [%]
morphology
←domain crystallization
linear domain growth
contact between domain borders
circula
1st border undulation
2nd border undulation
relaxing curvature
constant border curvature
2d organization
lattice formation / centre distances increase
hexagonal lattice / centre distances decrease
border distances … decrease
..decrease fast
… decrease slow
energy
inter domain energy … increases
..increases fast
… increases
movement
domain movement … uncoupled
… rotationally coupled→
… laterally coupled→
kinetics
lag-time
slow hydrolysis
beginning and 1st part of steady-state reaction
2nd part and ending of steady-state reaction
rate fall and reaction halting

OTF: Object/Optical Transfer Function

Myoglobin in Action |Picosecond Laue Crystallography Diffraction Data

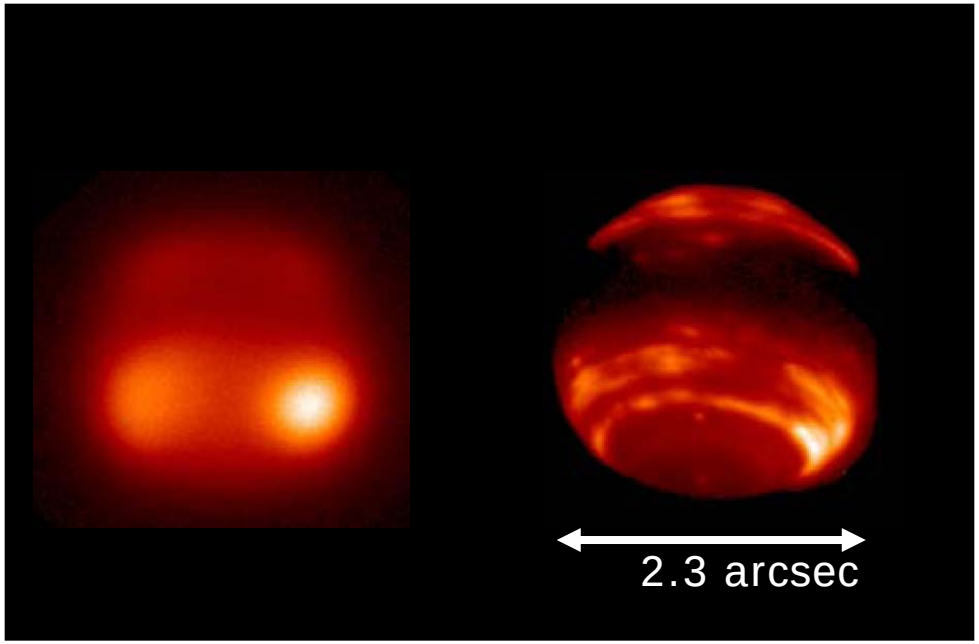
Schotte et al (2003) Science



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.

$$\mathbf{O}_{\text{bject}} \xrightarrow{\mathbf{OTF}} \mathbf{O}_{\text{bject}}\mathbf{I}_{\text{mage}} \xrightarrow{\mathbf{OTF}^{-1}} \mathbf{O}_{\text{bject}}\mathbf{M}_{\text{odel}}$$



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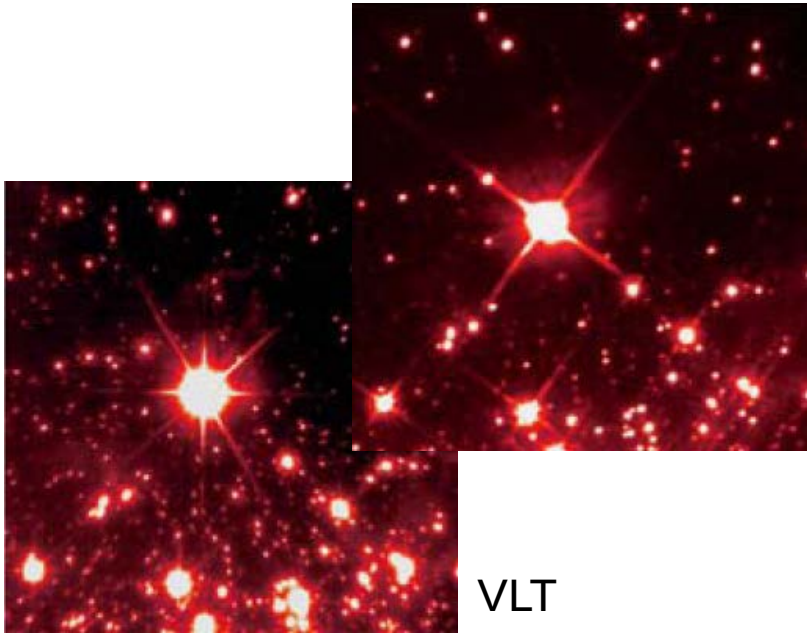


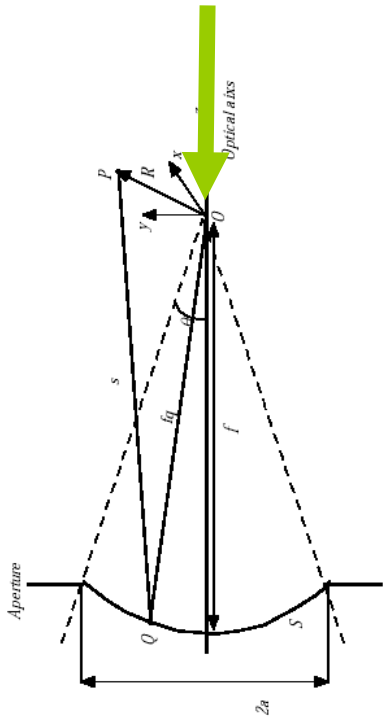
Hubble

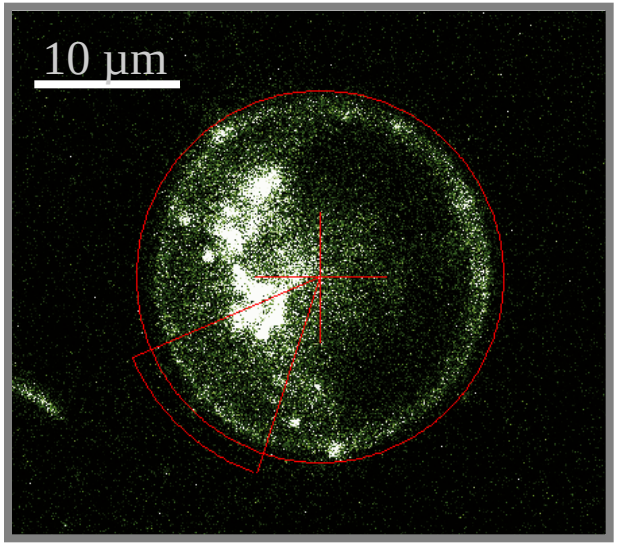
VLT

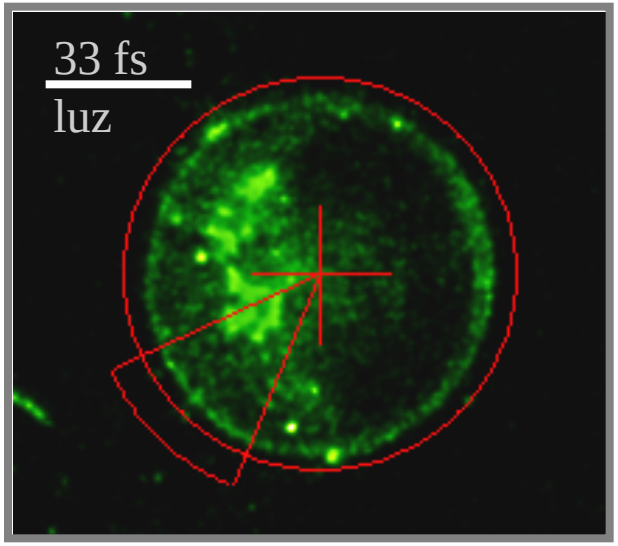
Confocal Microscopy | From Geometric Optics to Diffraction Theory

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







|-> 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

JCB: FEATU

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

Imaging can be thought of as the most honest 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. But on the confocal microscope, there is no yellow in the merged image.

"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 Bacallao et al., 1995; Allen, 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 Aysough, 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

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The Journal of Cell Biology, Vol. 172, No. 1, January 2, 2006 9–18
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Supplemental Material can be found at:
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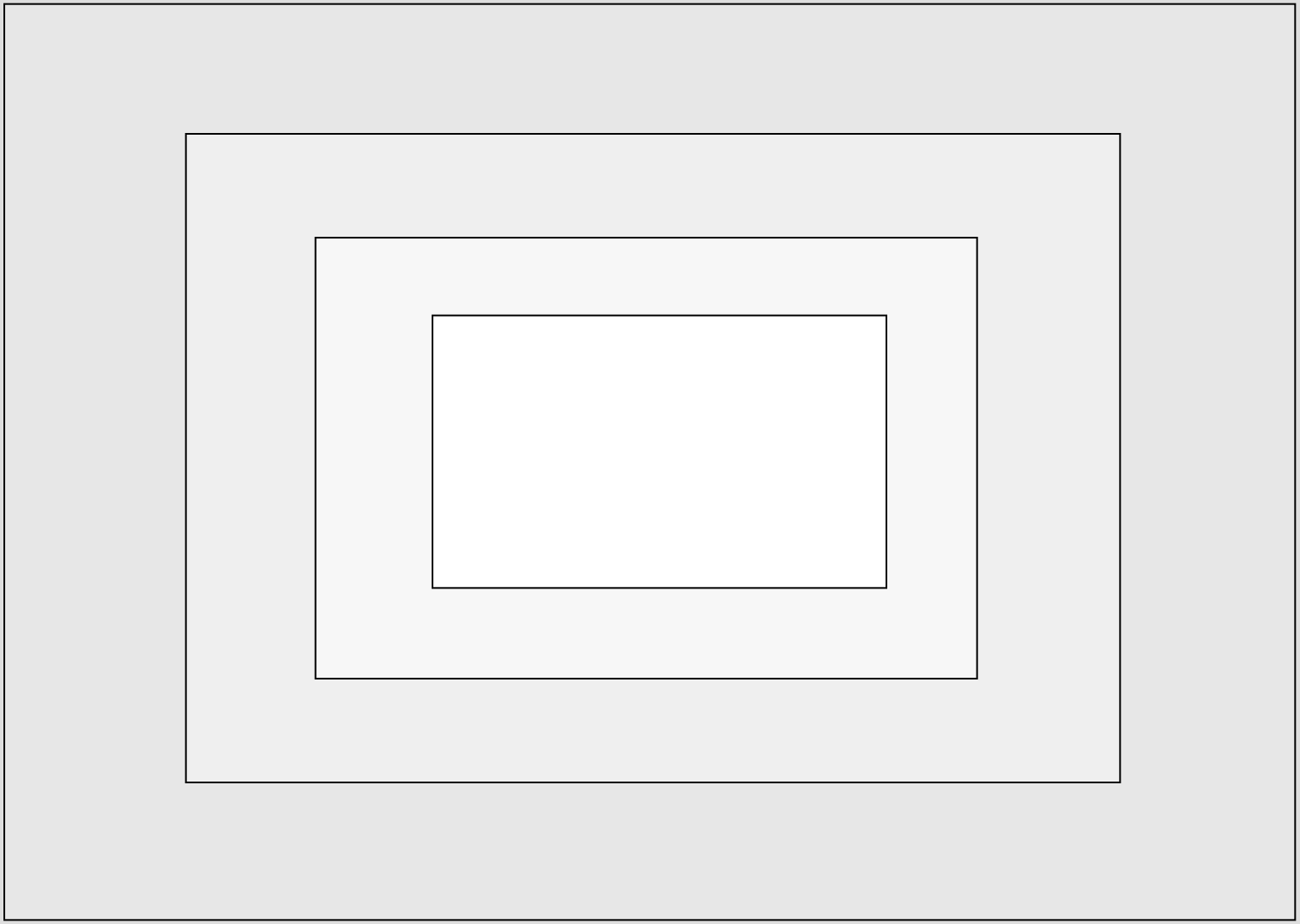
JCB ■

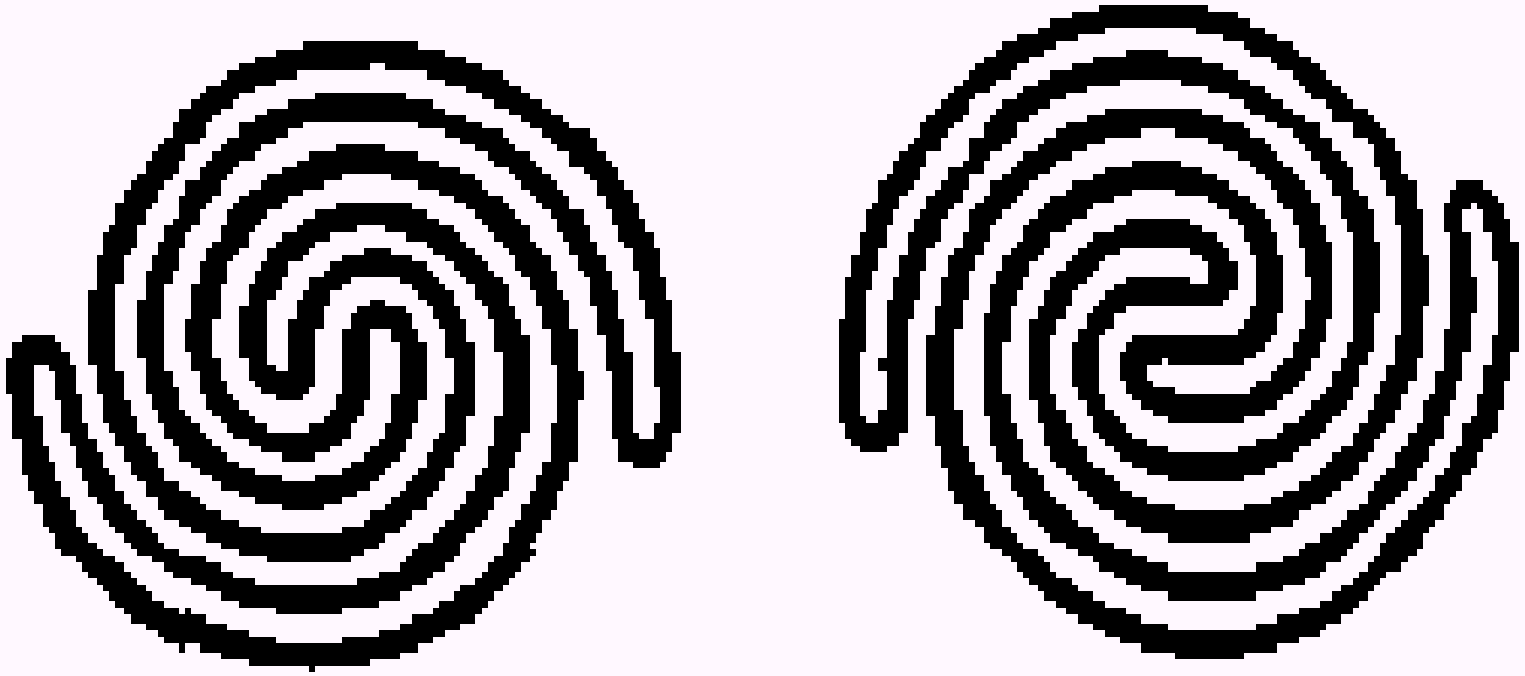
The objective lens is the most critical component of a microscope and yet few researchers grasp the differences between specific objective classes.

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

"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.

"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.

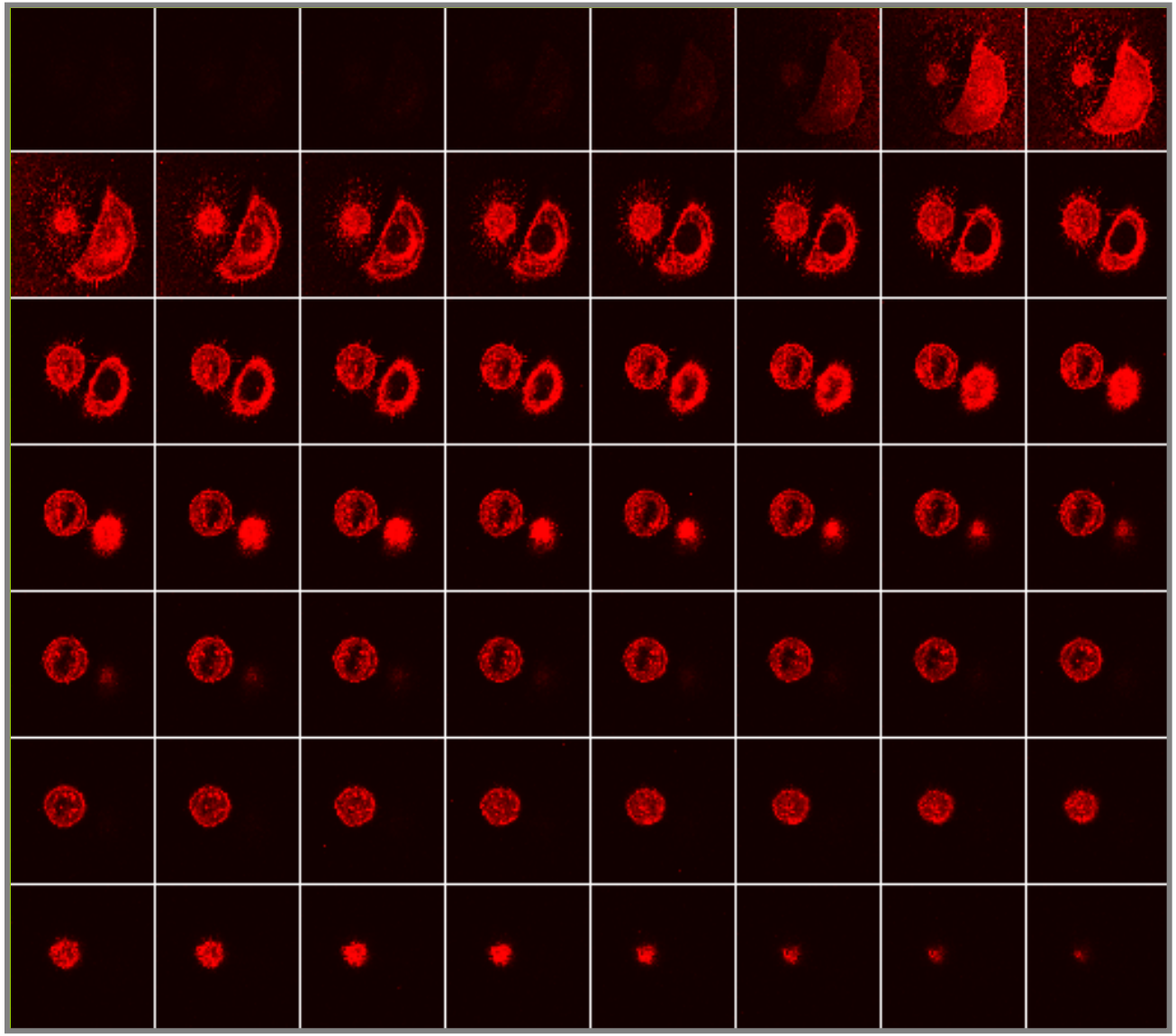




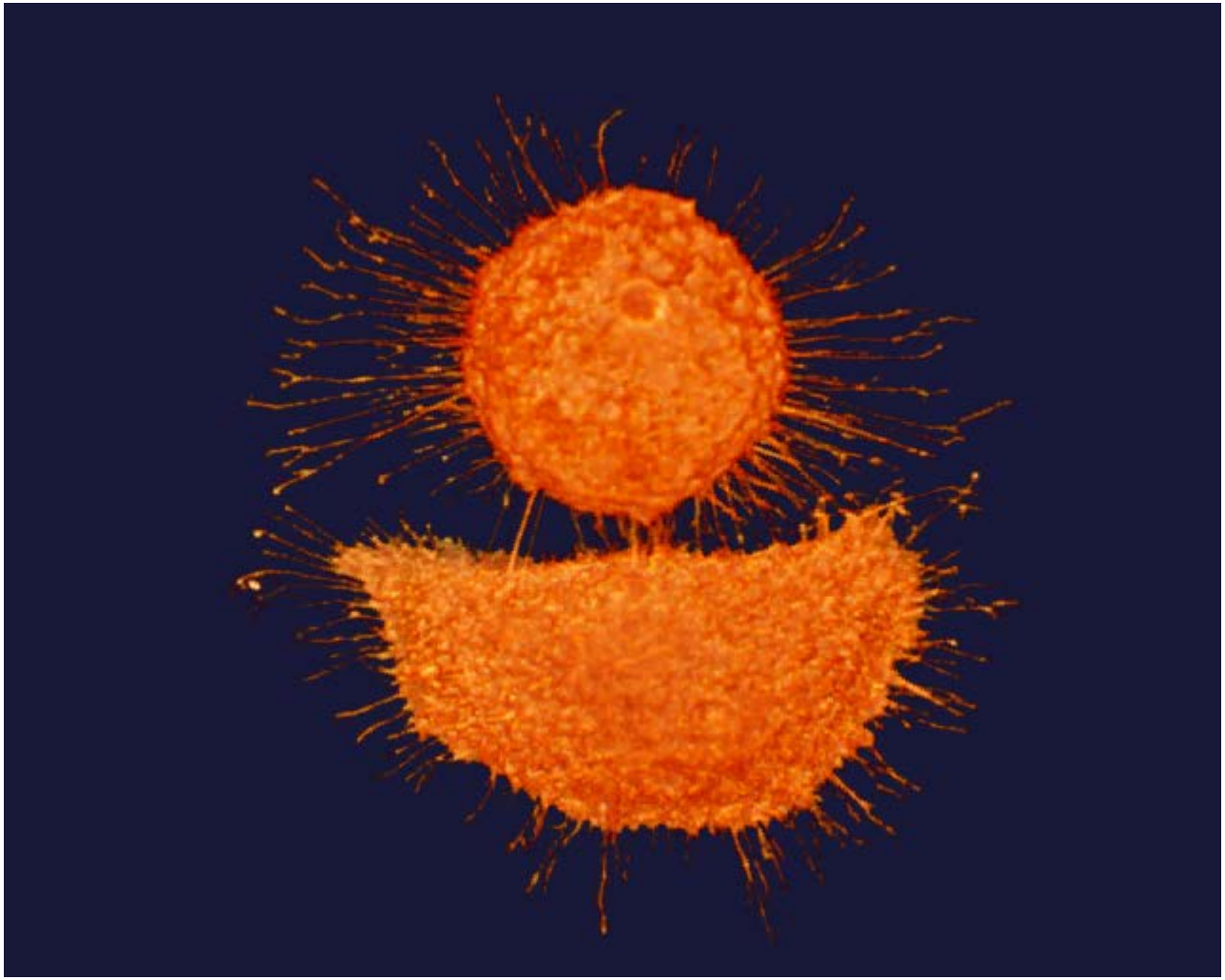
|-> Microscopy

Hans Janssen (1595), ...
Galileo Galilei (1610)

|-> Microscopy



|-> Microscopy



|-> Fluorescencia I

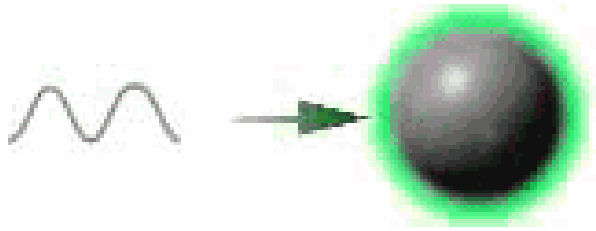
Luminescencia:

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

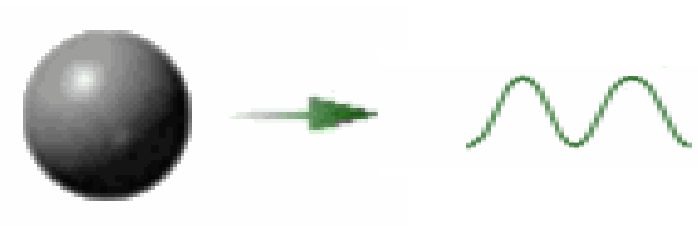
Interacciones ...
- **intra- e inter moleculares ...**

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

← Δt →

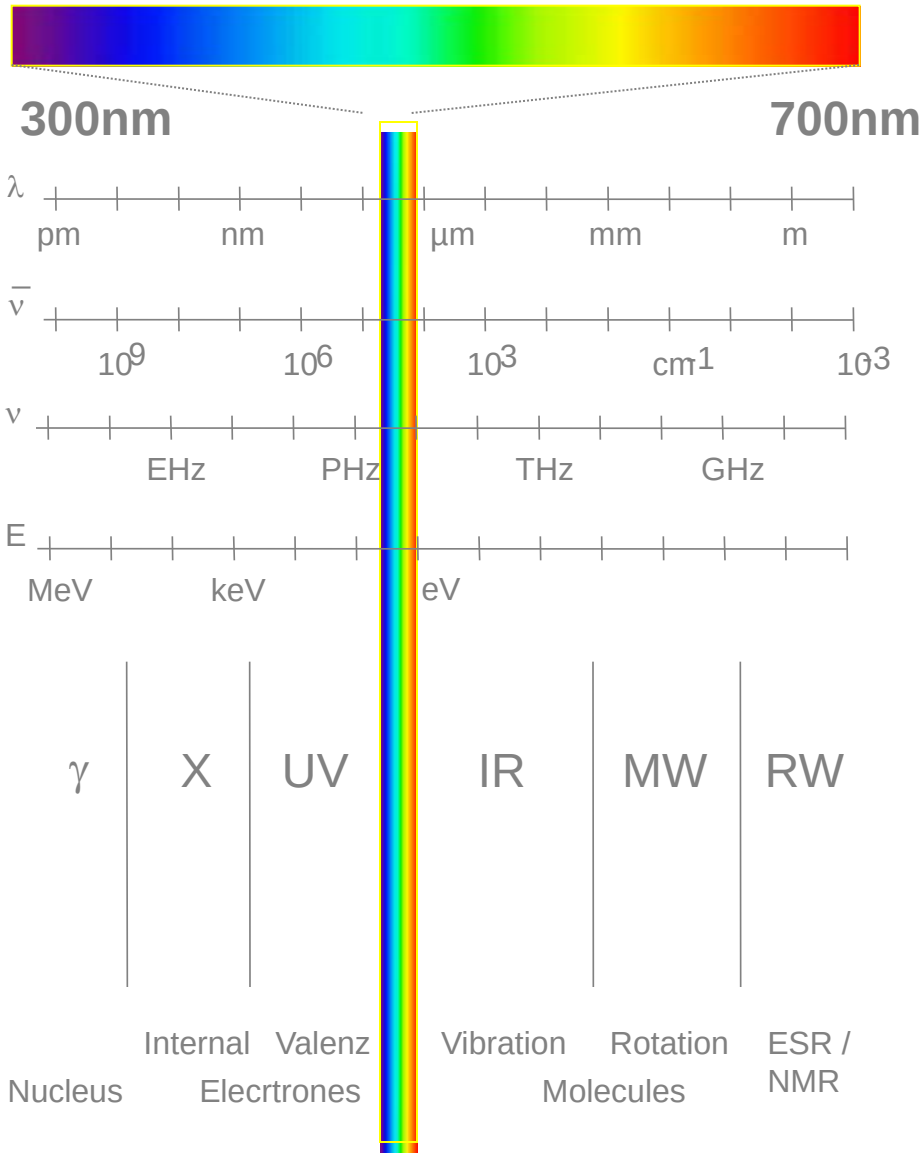


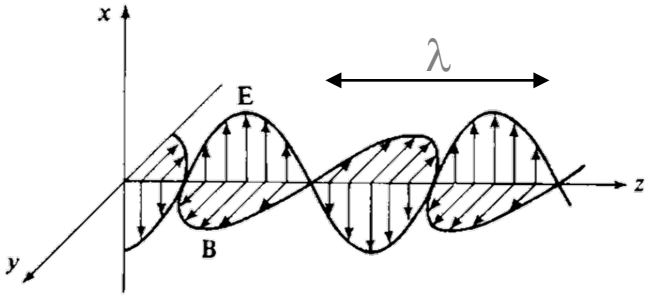
Absorción / Excitación



Emissión

|-> Fluorescencia II





Energía de un fotón: (~1-5eV)

$$E = h\nu = hc\lambda^{-1} \quad | \quad c = \nu\lambda$$

ν, frecuencia [s^{-1}]
h, constante de Planck [$6.626\ 10^{-34}\ Js^{-1}$]
λ, longitud de onda [m]
c, velocidad de luz [$\sim 3\ 10^{8}\ ms^{-1}$]

Energía molecular:

$$E = E_{rot} + E_{vib} + E_{el}$$

$$1 : 10^3 : 50 \cdot 10^3$$

Energía térmica:

$$E = k\,T \quad (\sim 2.5\ 10^{-2}\ eV,\ T \sim 20°C)$$

k = Constante de Bolzmann ($0.86\ 10^{-4}$ eV/K)

|-> Jablonski Diagram

Transiciones: → con radiación

sin radiación |

Niveles: —— vibracionales

▬ electrónicos

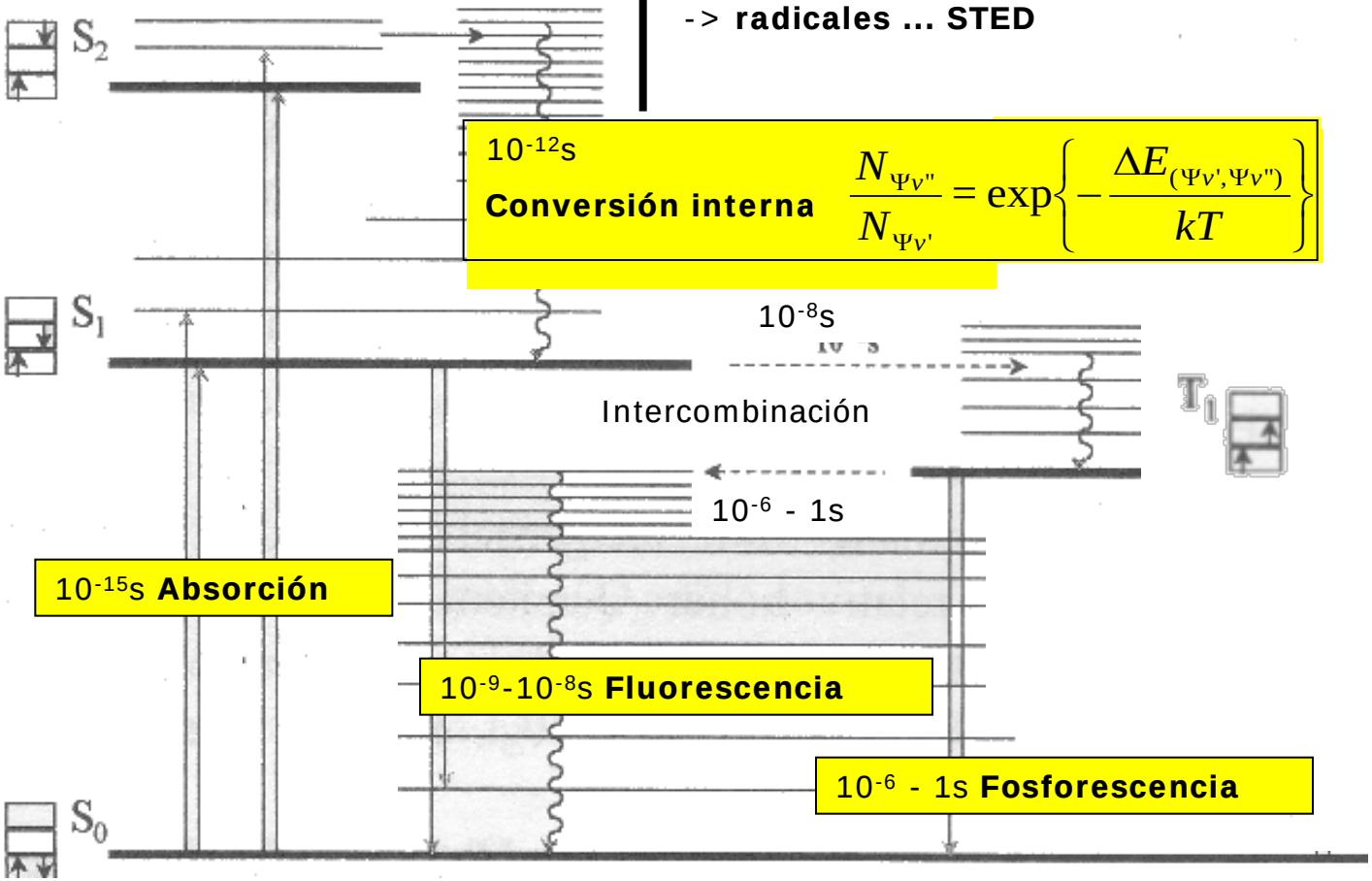
-> **calor**

-> **transferencia de energía (FRET)**

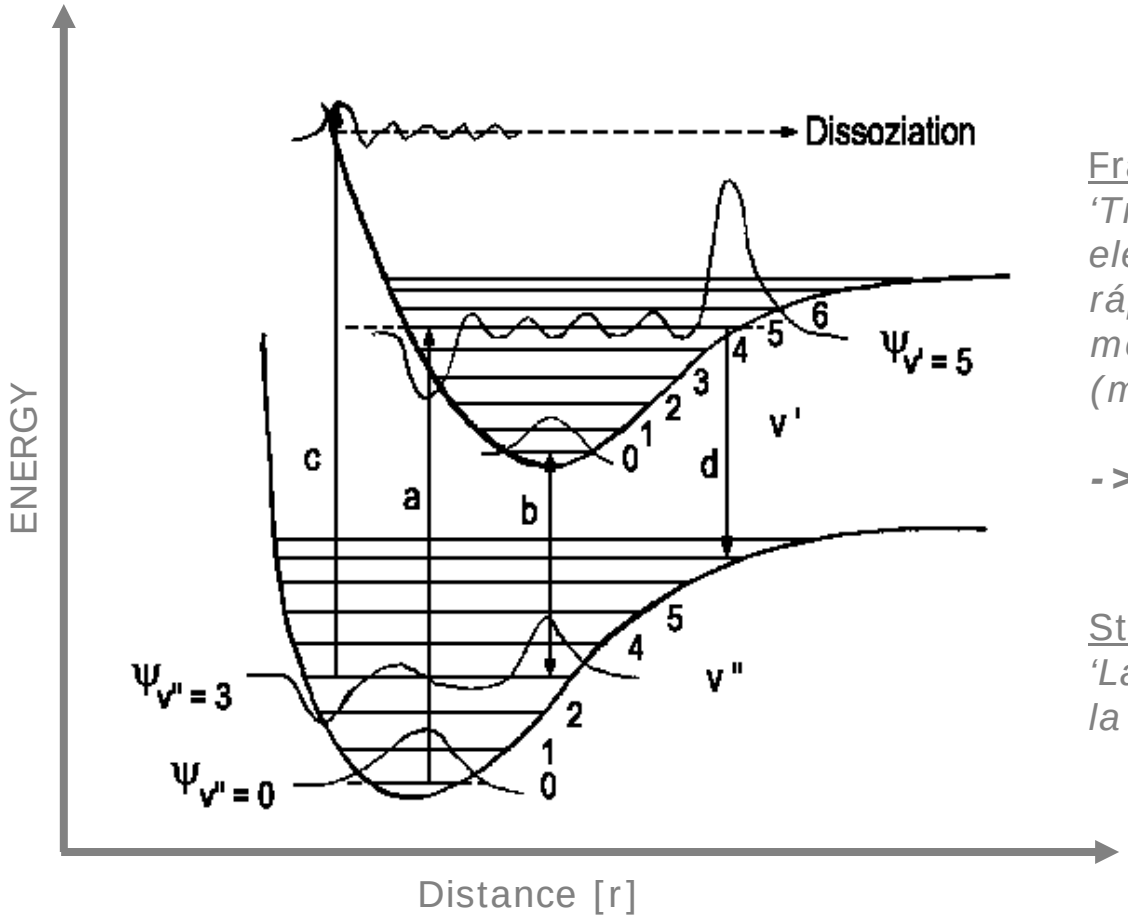
-> **radicales ... STED**

z

x / y



|-> Franck Condon



Frank Condon **:**

'Transiciones entre niveles electrónicos ocurren mucho mas rápido que movimientos de núcleos moleculares.'

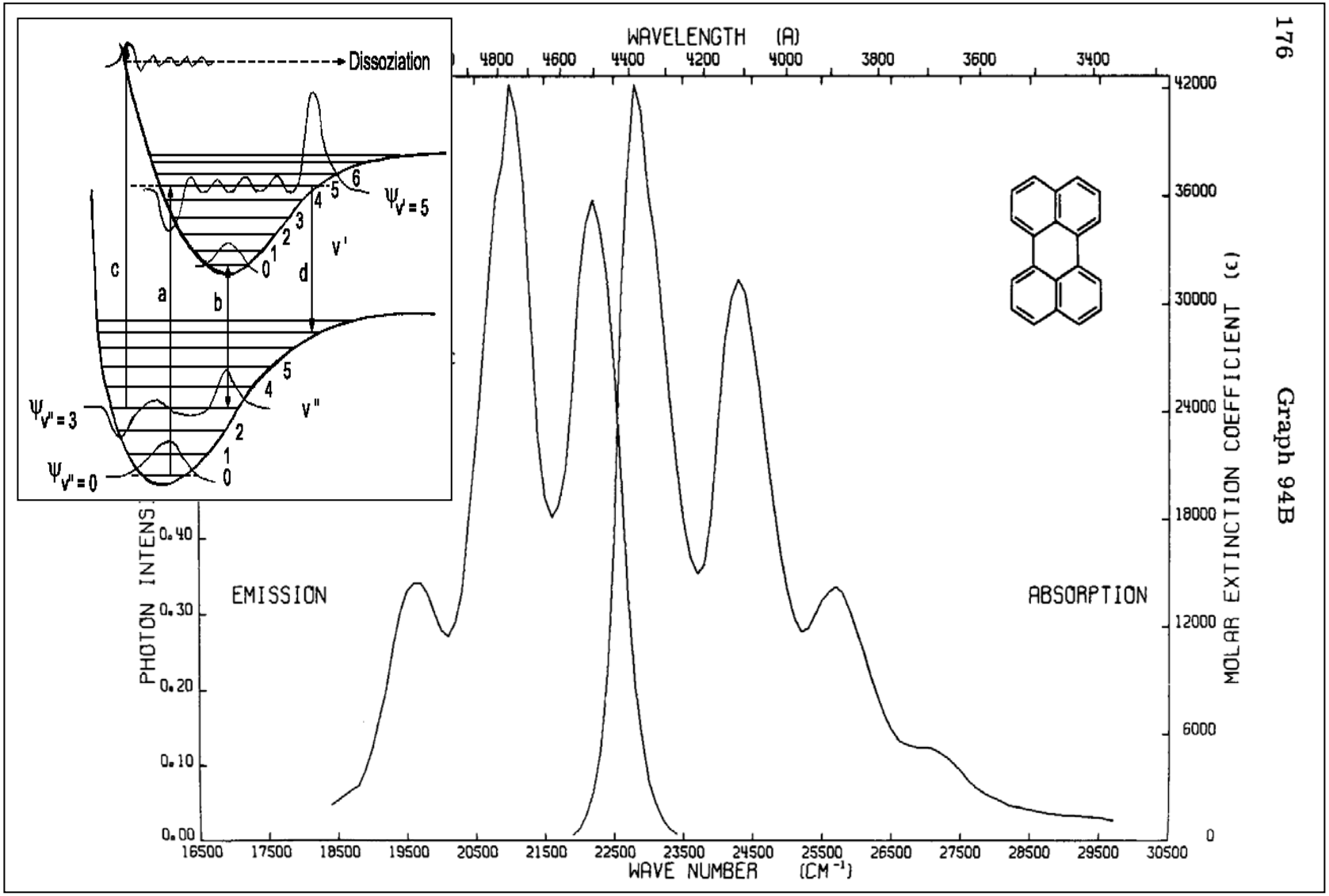
(masa$_{electron}$ / masa$_{atom}$: 1 : 2000)

-> Mirror Image Rule

Stokes (Shift) **:**

'La energía de emisión es menor a la energía de excitación.'

|-> Mirror Image Rule

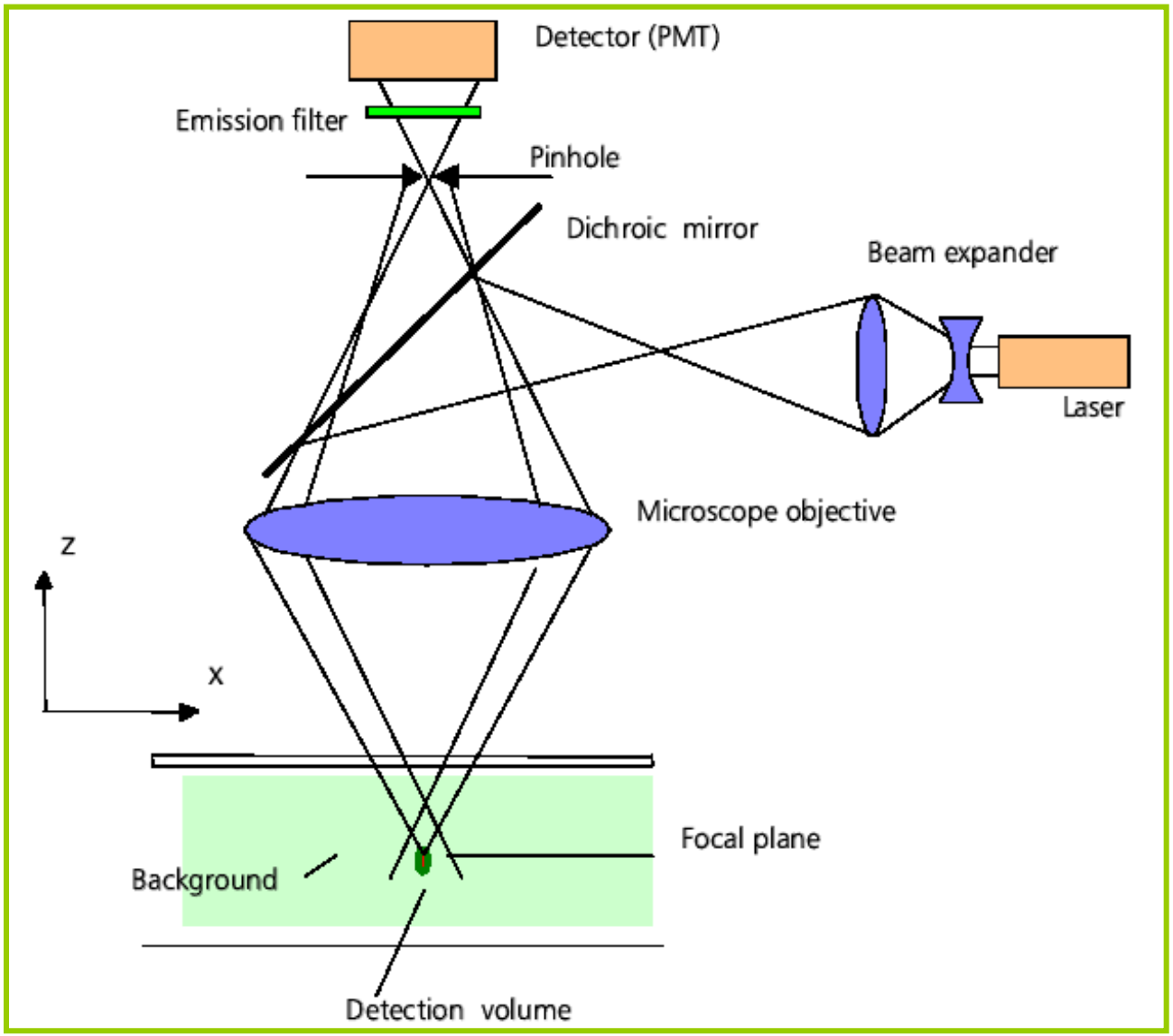


|-> Joseph R. Lakowicz

"Principles of Fluorescence Spectroscopy" Chapter 1

|-> Polarisation ...
|-> Steady-State and Time-Resolved Fluorescence...
|-> Resonance Energy Transfer ...
|-> Quenching, Bleaching ...

|-> Diffraction limited Microscopy



|-> 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.

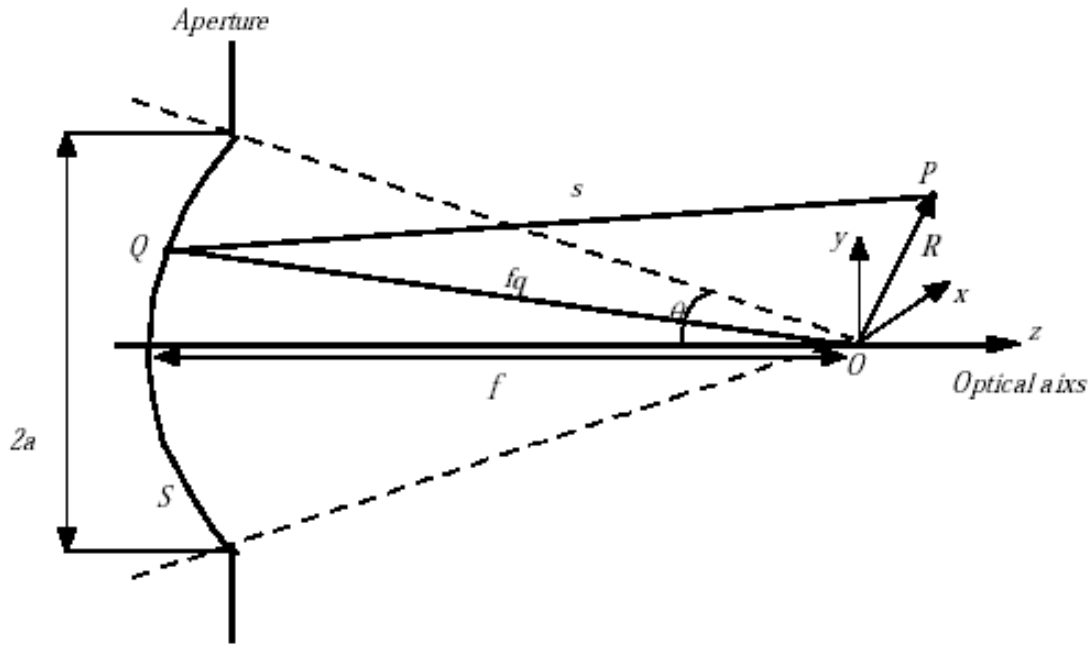


Figure 2.1 Diffraction of a converging spherical wave at a circular aperture

|-> Diffraction limited Microscopy

Óptica no-geométrica /

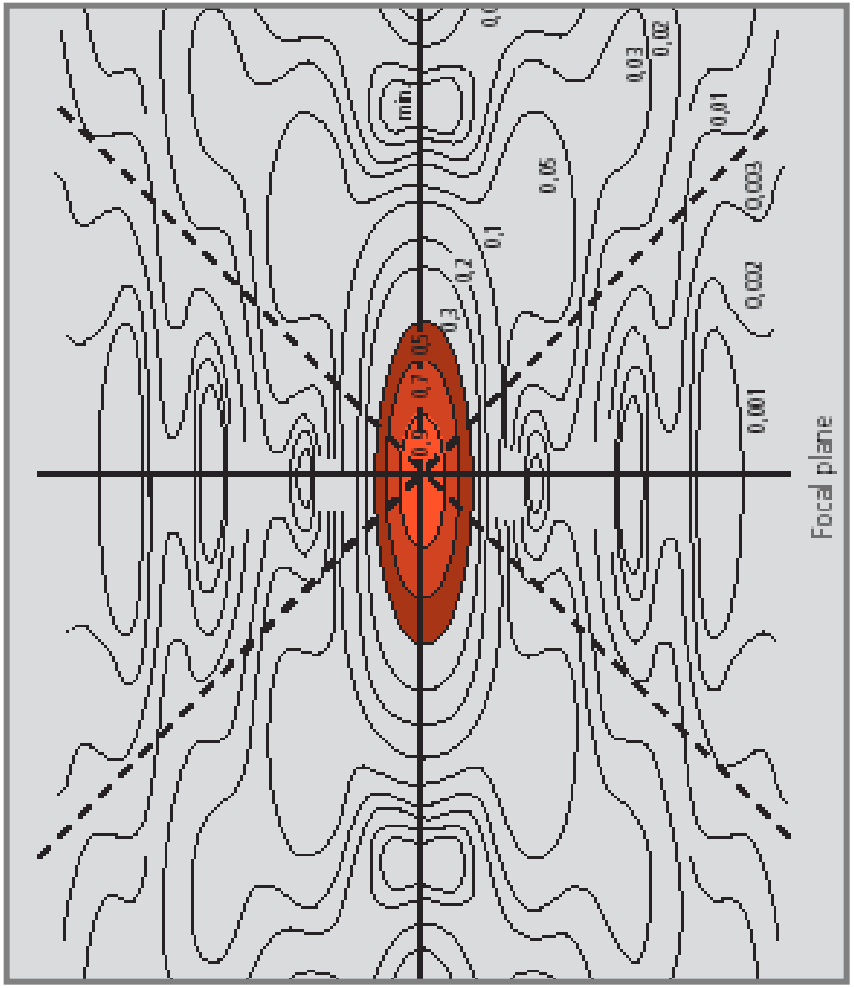
Teoría de difracción

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

U, Integral de Difracción de Kirhoff

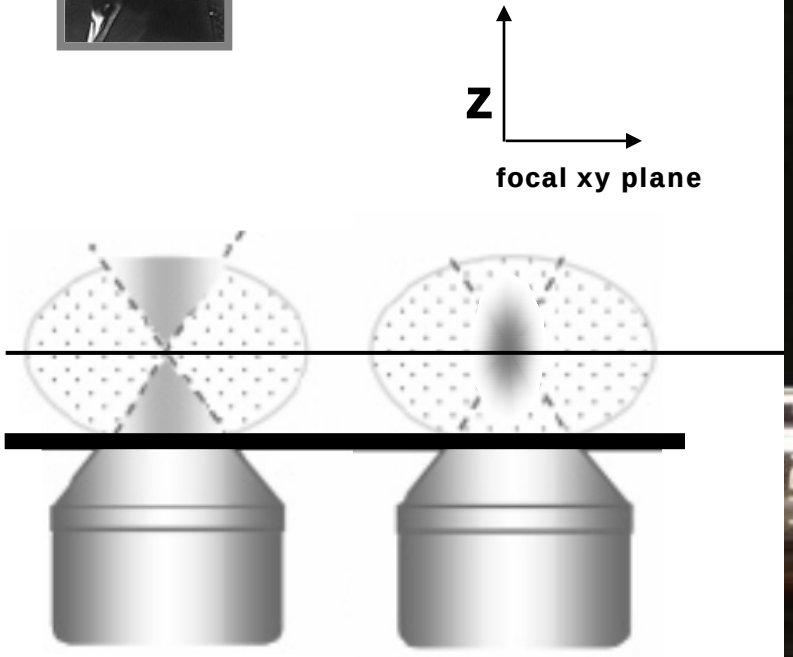
J_0, Serie de funciones de Bessel

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

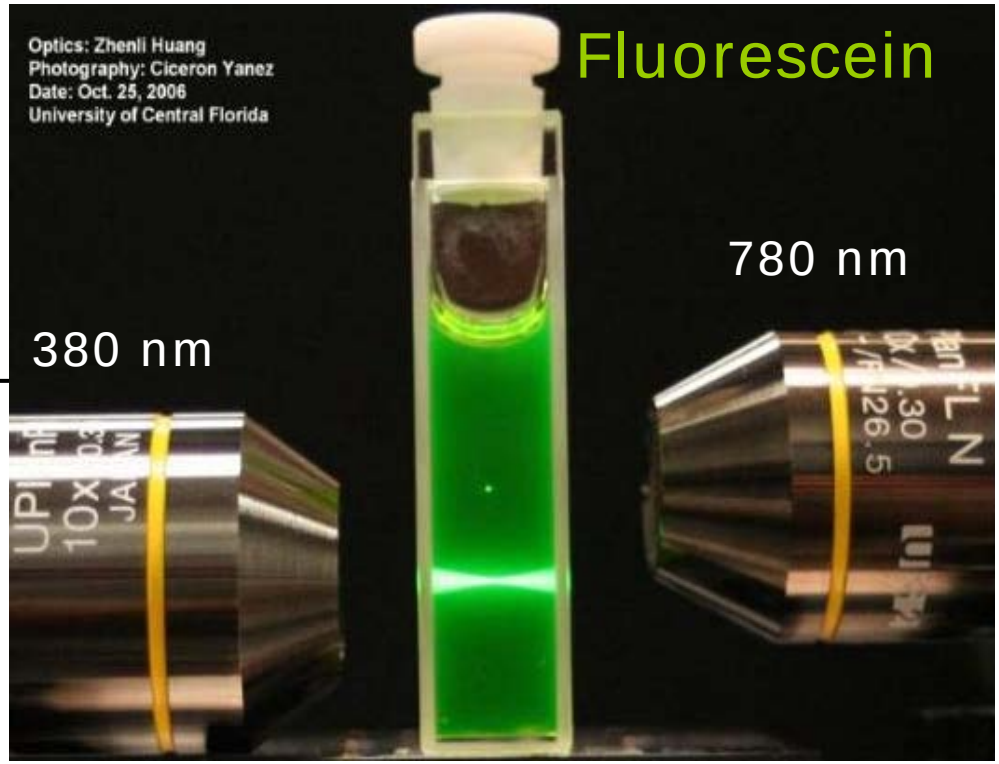


|-> Diffraction limited Microscopy

| Best localization: confocal microscopy

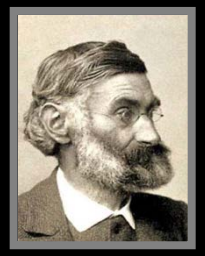


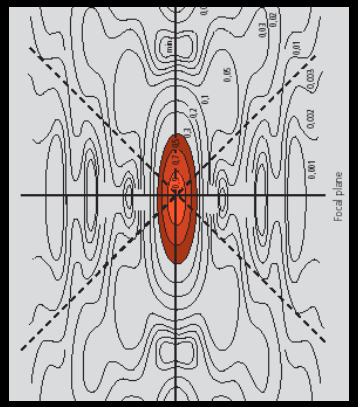
convencional / confocal

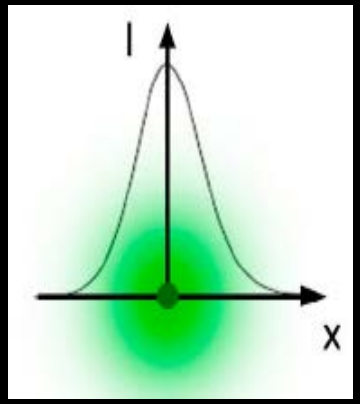


|-> Diffraction limited Microscopy

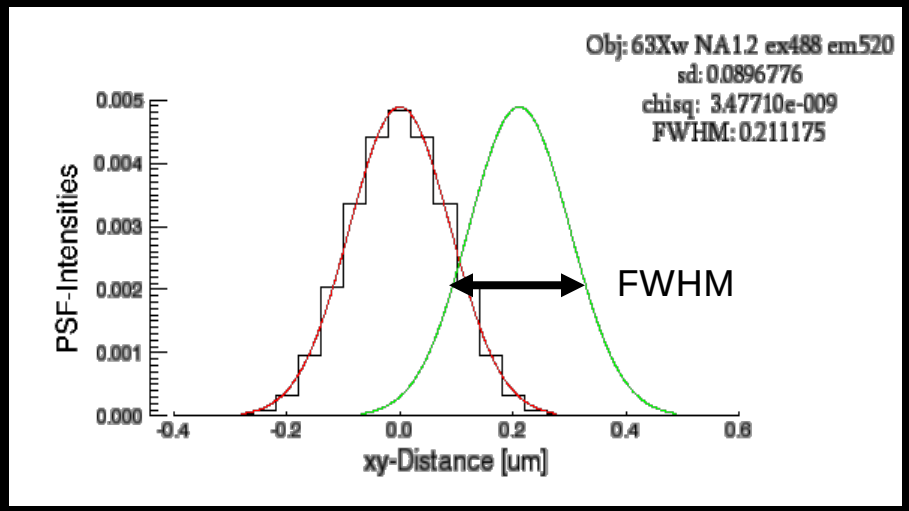
Ernst Abbe
1840 - 1905







Resolution ~ λ / 2 ~ Full Width at Halft Maximum (FWHM)



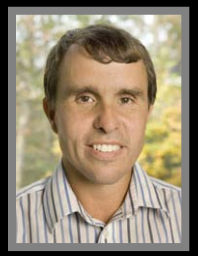
|-> Beyond diffraction

M Goeppert-Mayer
1906-1972

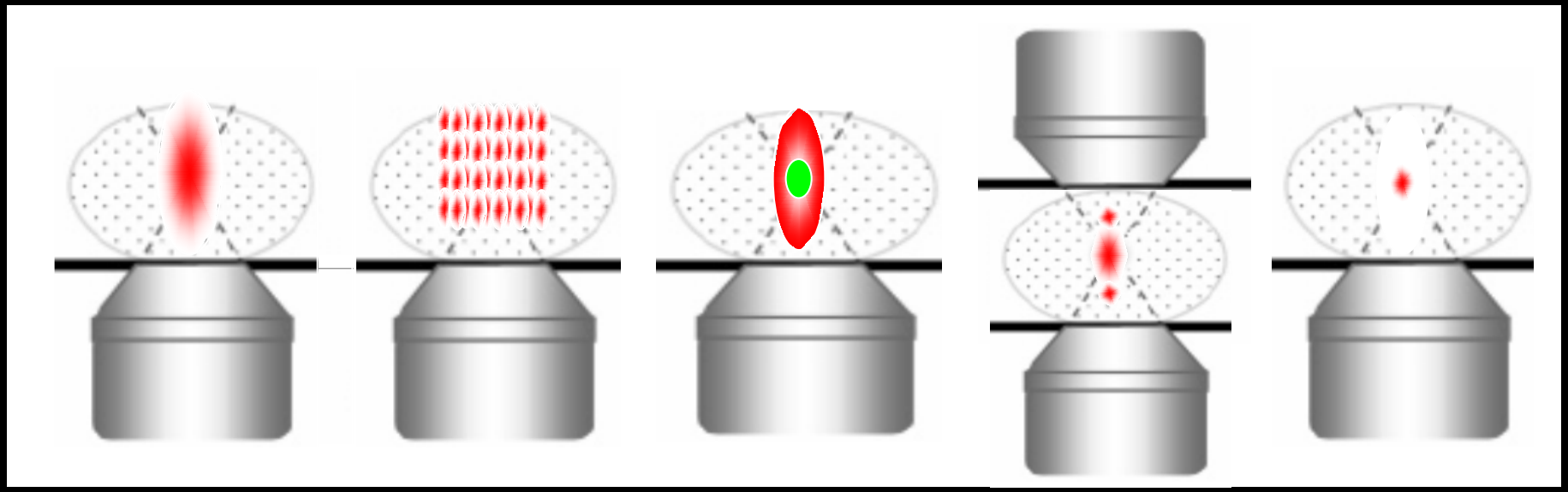
M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

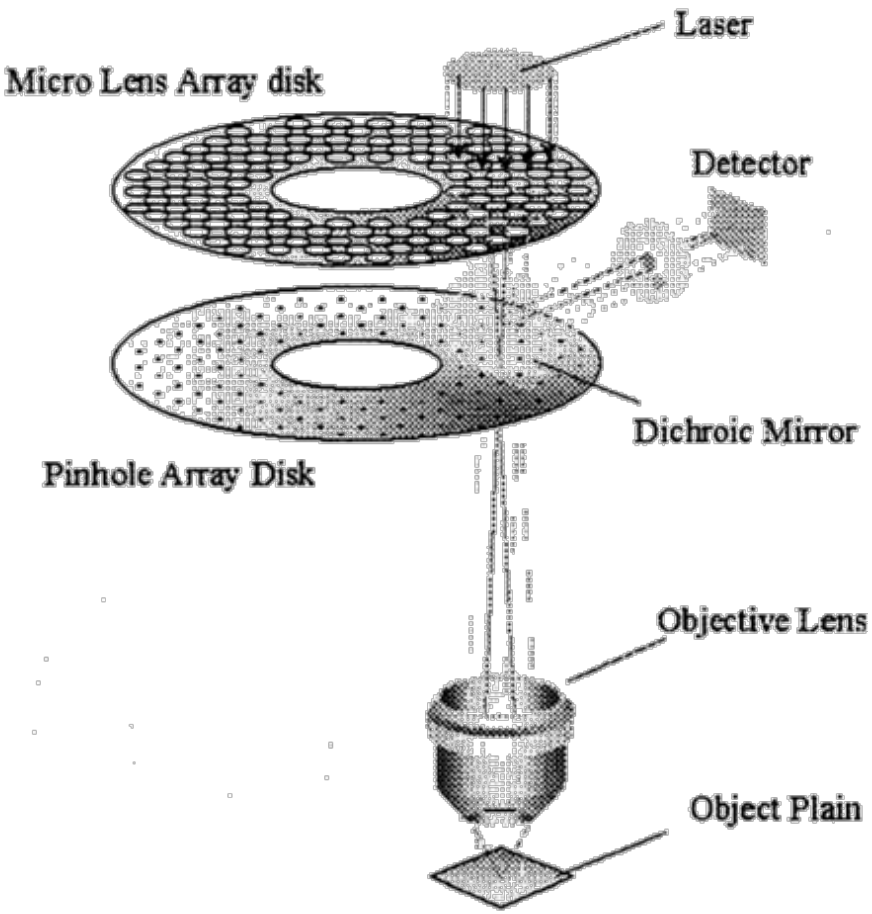


FWHM(xy) ~ $\lambda/2$ | ~ $\lambda/4$ | ~ λ/∞ | ~ $\lambda/4$ | ~ $\lambda/100$

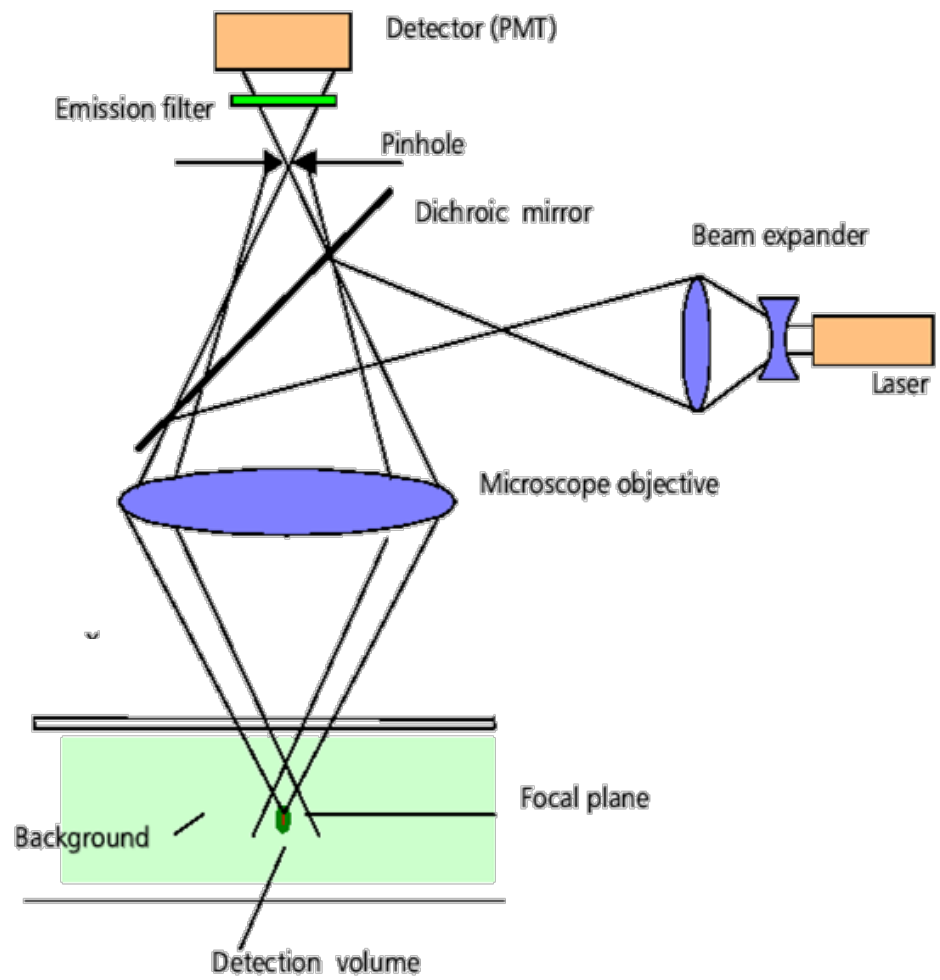


2-photon | SIM | STED | 4-π | PALM

|-> Spinning Disk

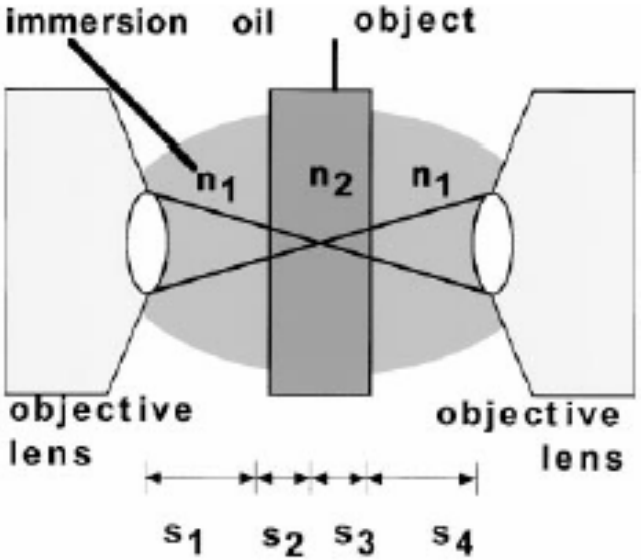


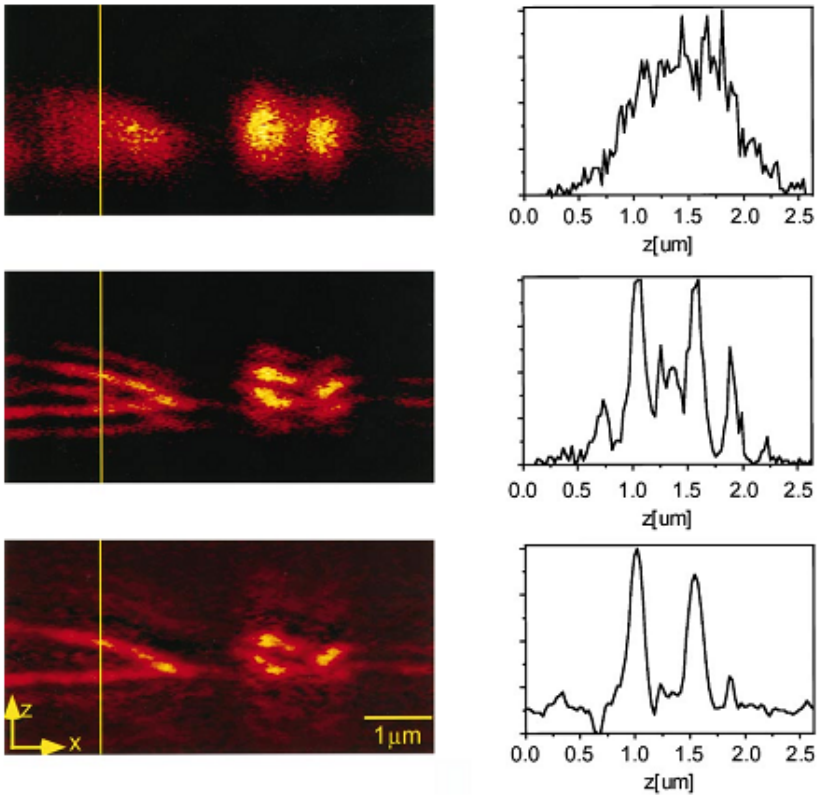
spinning disk



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

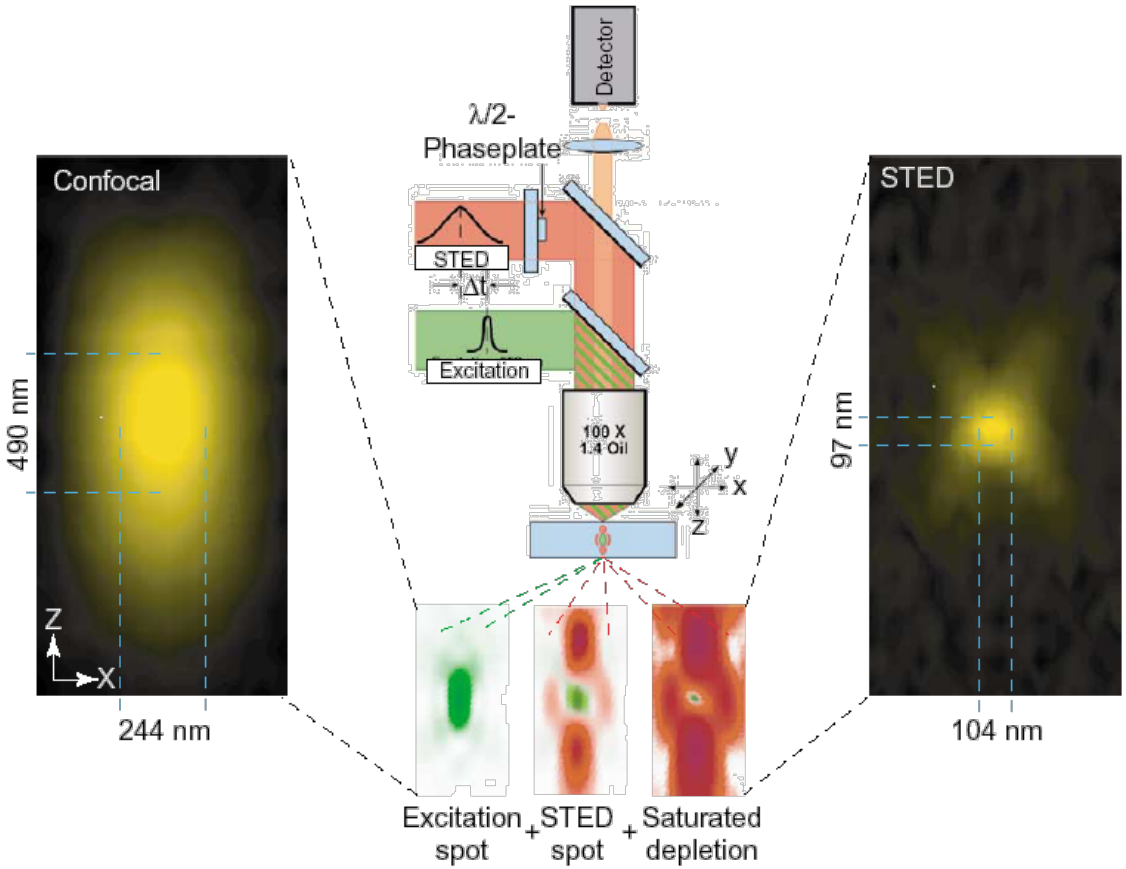
|-> STED

STED Microscopy

Stimulated

Emission

Depletion



Concepts for nanoscale resolution in fluorescence microscopy

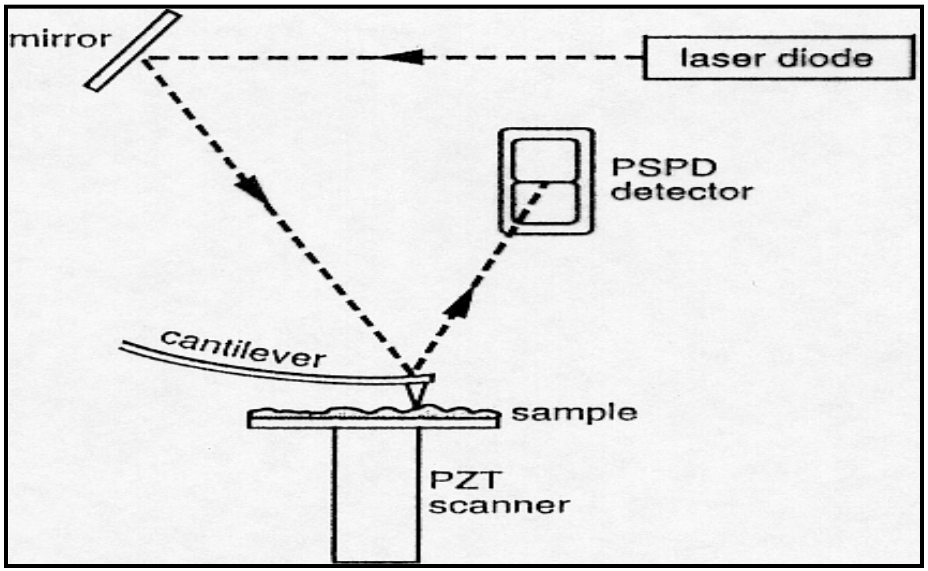
Stefan W Hell*, Marcus Dyba[1] and Stefan Jakobs[2]

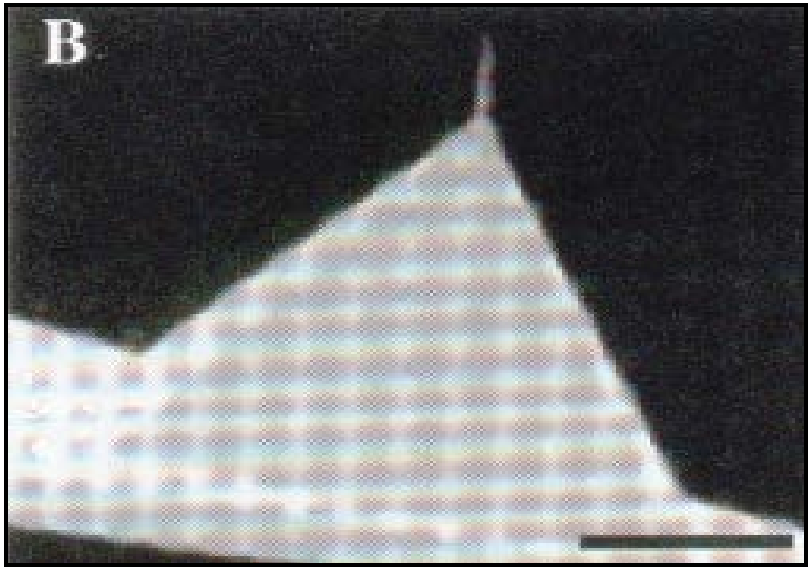
Current Opinion in Neurobiology 2004, **14**:599–609

|-> Atomic Force Microscopy

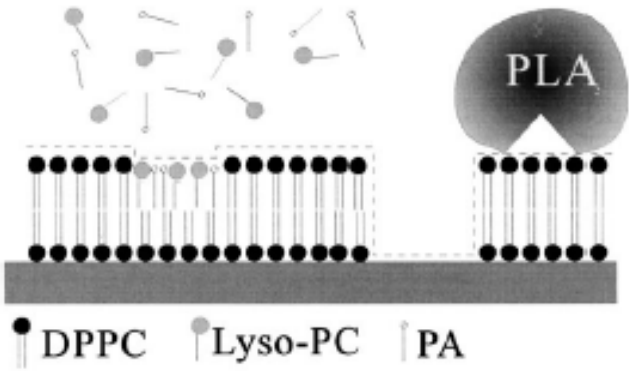
AFM allows the investigation of structural and functional properties of biomolecules in liquid environments, by a unique combination of :

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

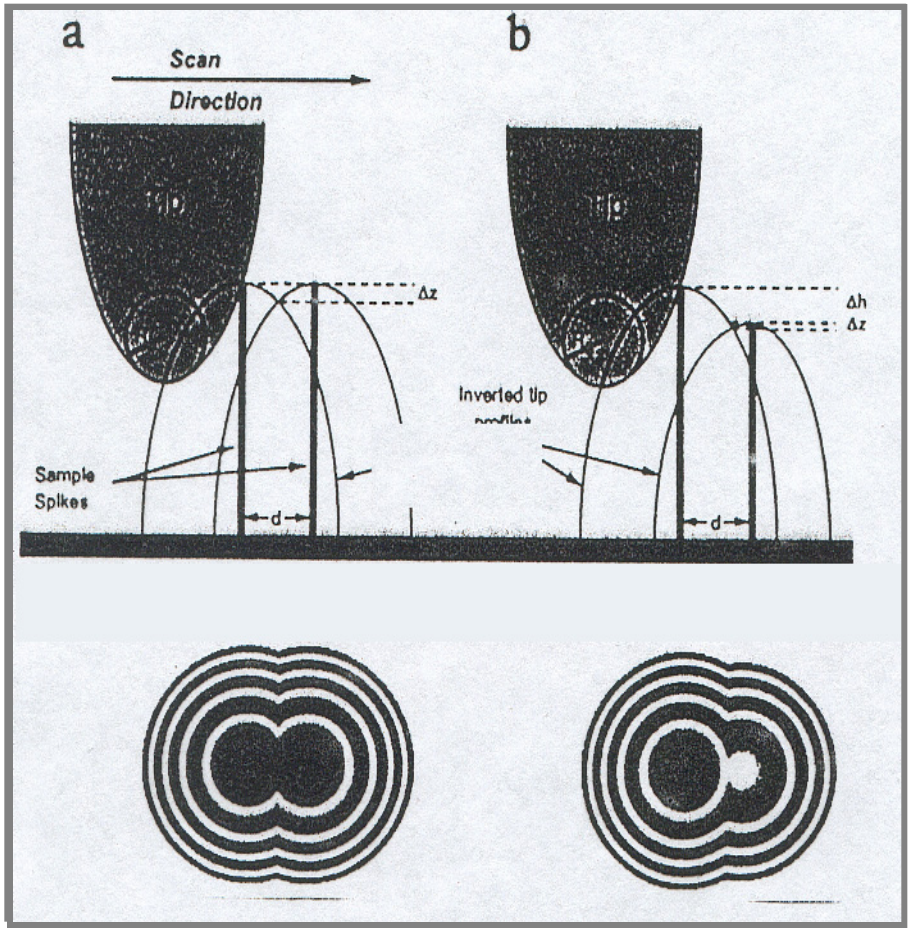




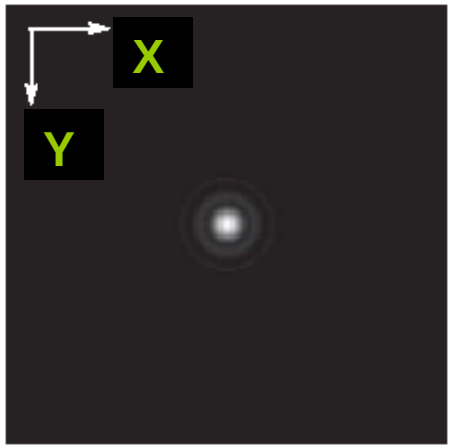
|-> Atomic Force Microscopy

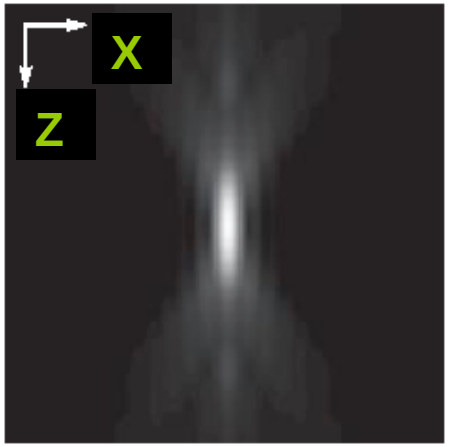


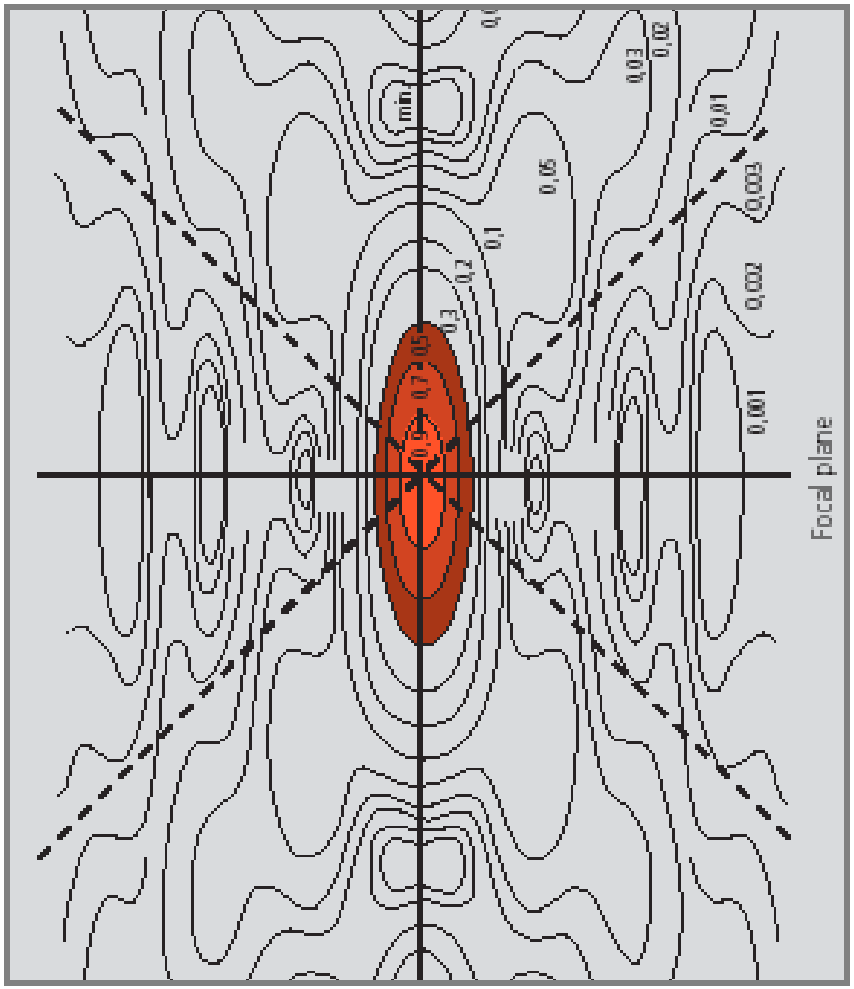
M Grandbois et al. (1998) Biophys J.



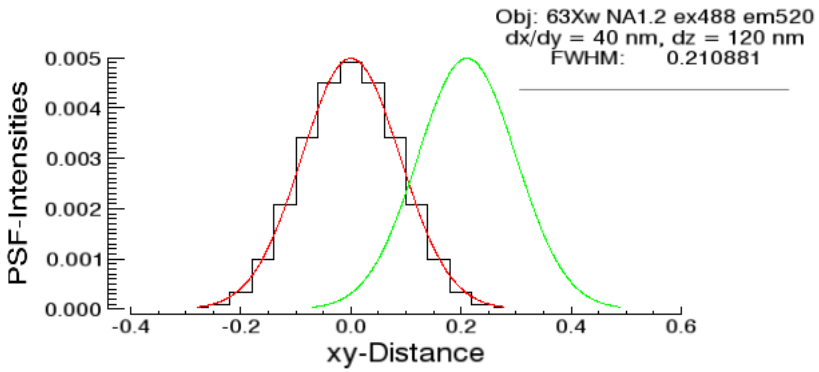
|-> PSF

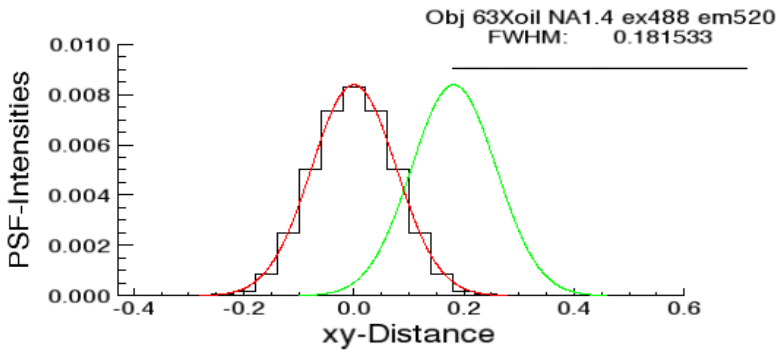

X
Y

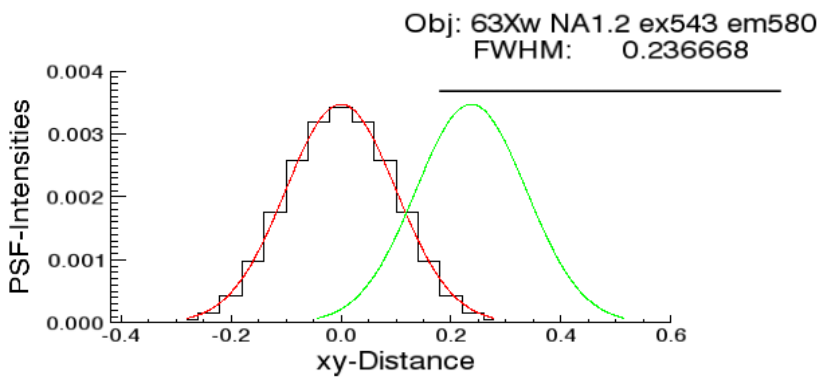

X
Z

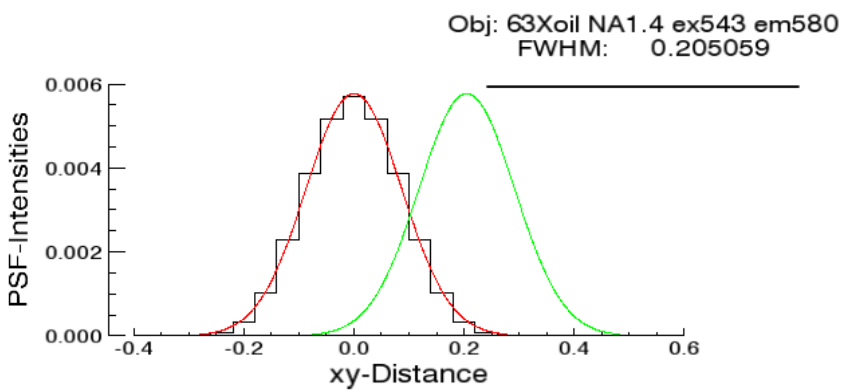

Focal plane
0,001
0,002
0,003
0,01
0,02
0,03
0,05
0,1
0,2
0,3
0,5
0,7
min

|-> PSF

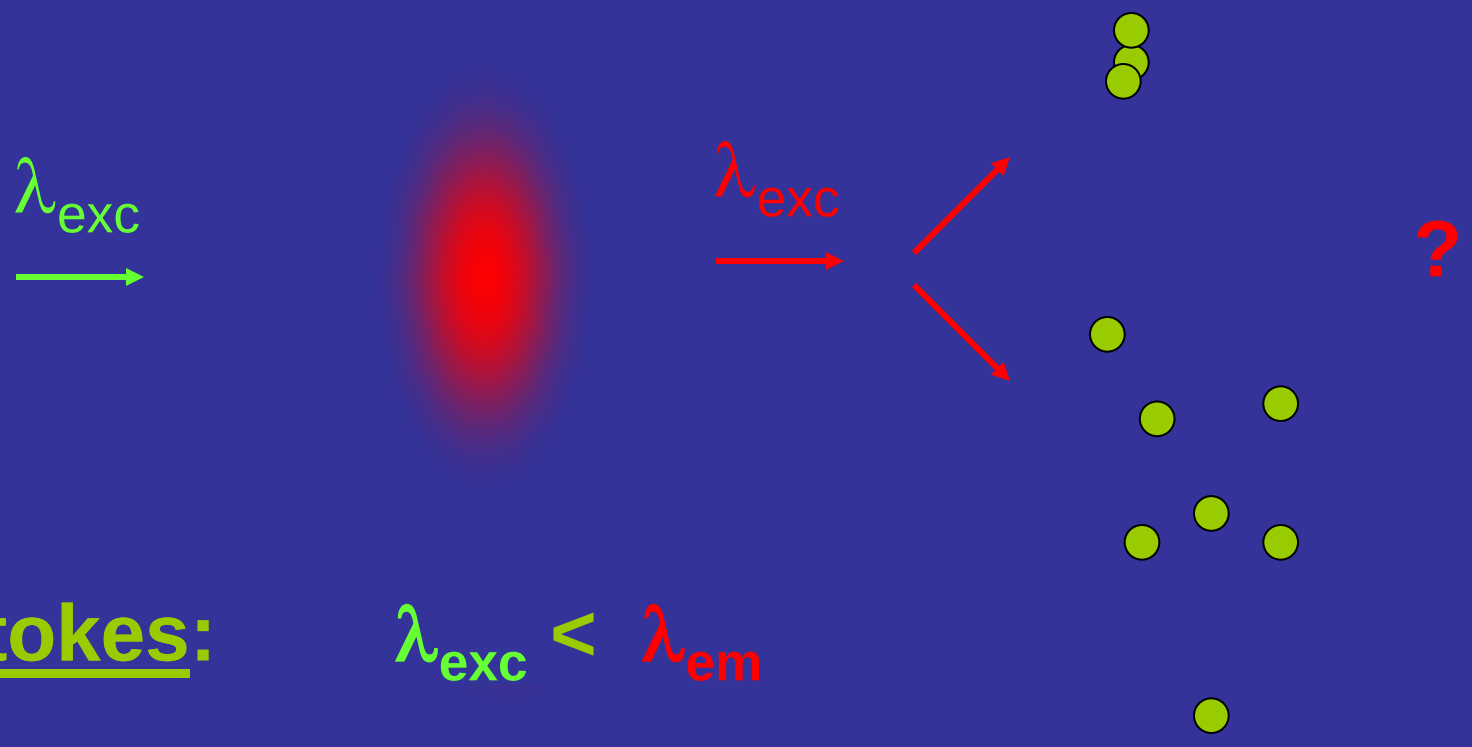

Obj: 63Xw NA1.2 ex488 em520
dx/dy = 40 nm, dz = 120 nm
FWHM: 0.210881
PSF-Intensities
0.005
0.004
0.003
0.002
0.001
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance


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

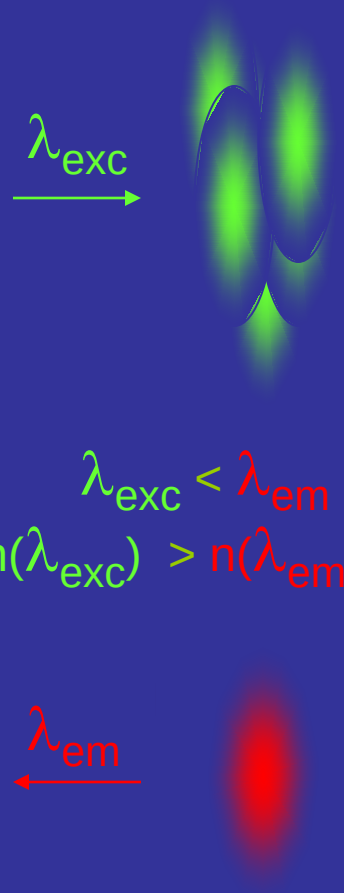

Obj: 63Xoil NA1.4 ex543 em580
FWHM: 0.205059
PSF-Intensities
0.006
0.004
0.002
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance

|-> Convolution



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

|-> Convolution



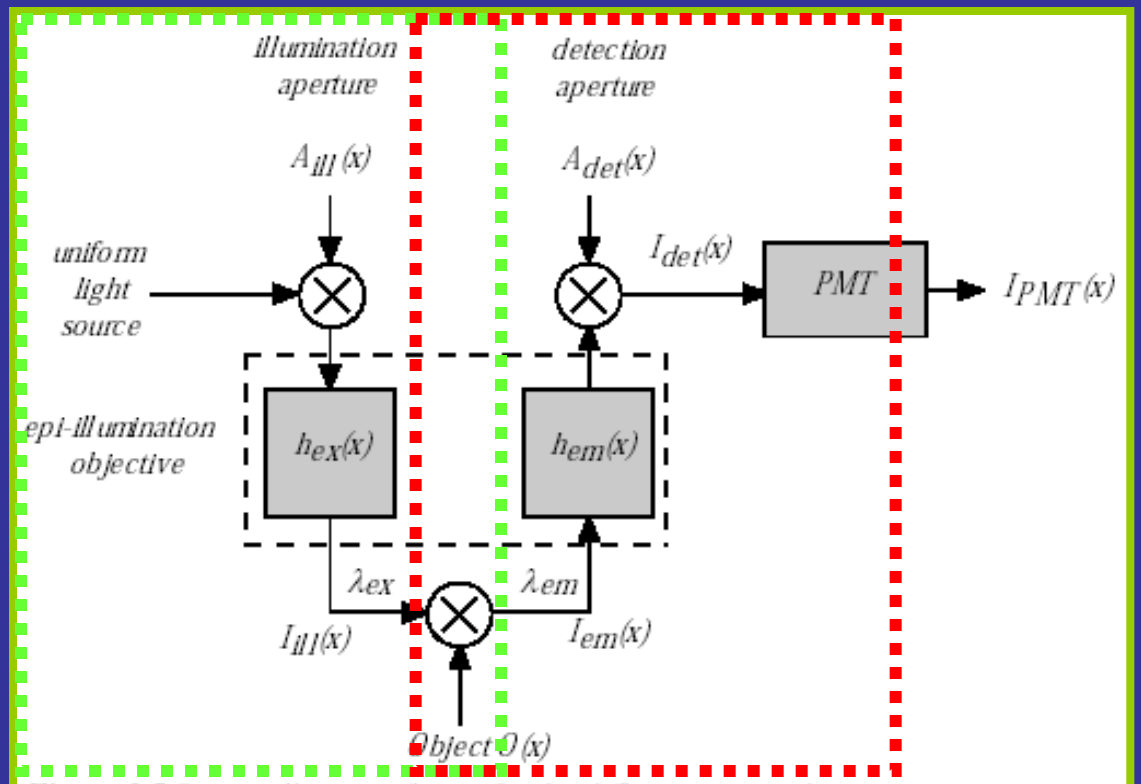
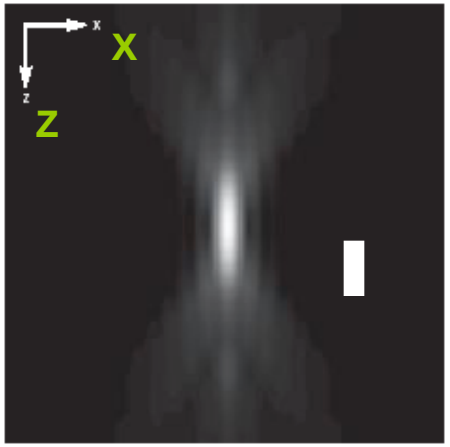
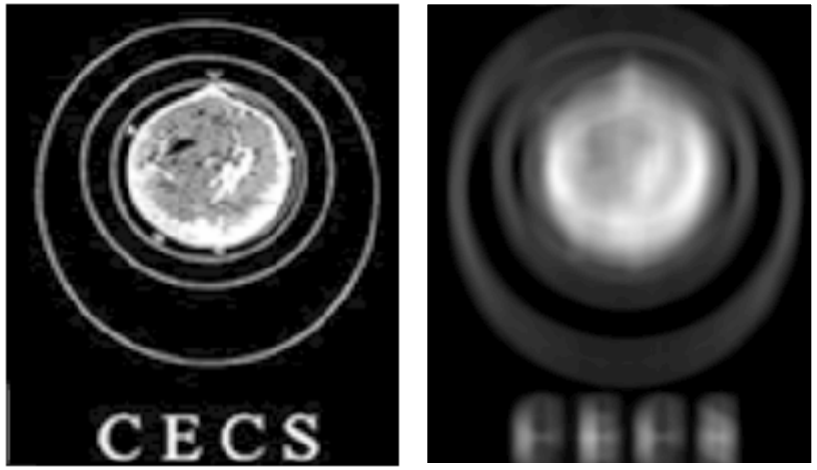


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

|-> Convolution





PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: *Point Spread Function*

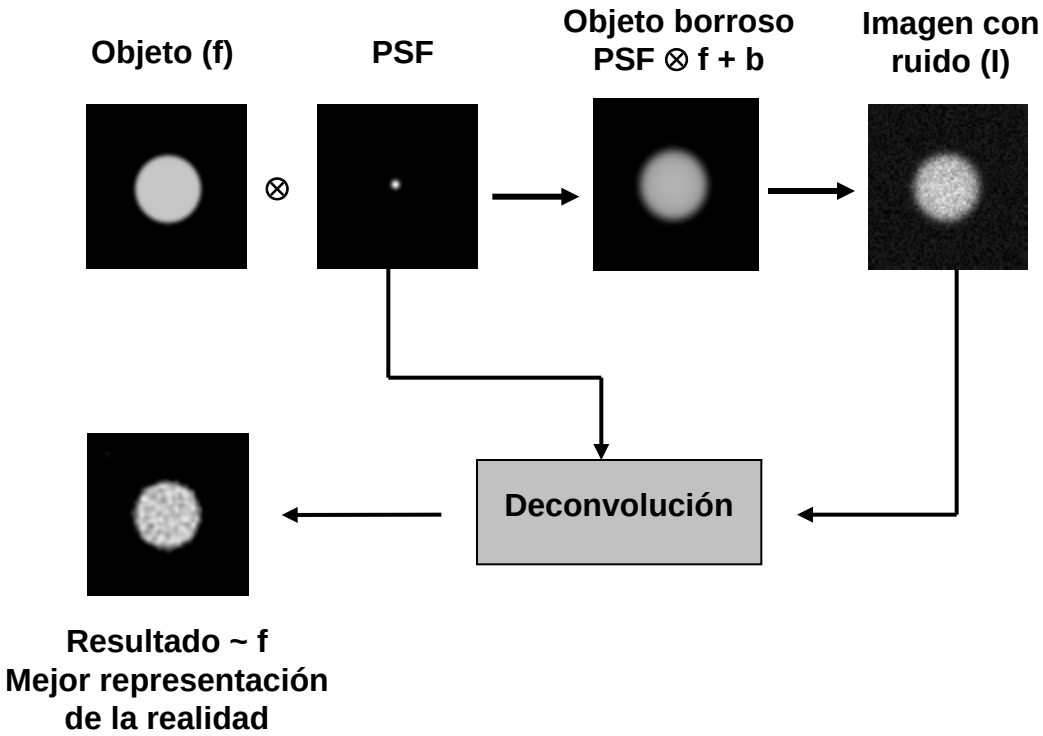
f: *Object Function*

b: *Offset Function*

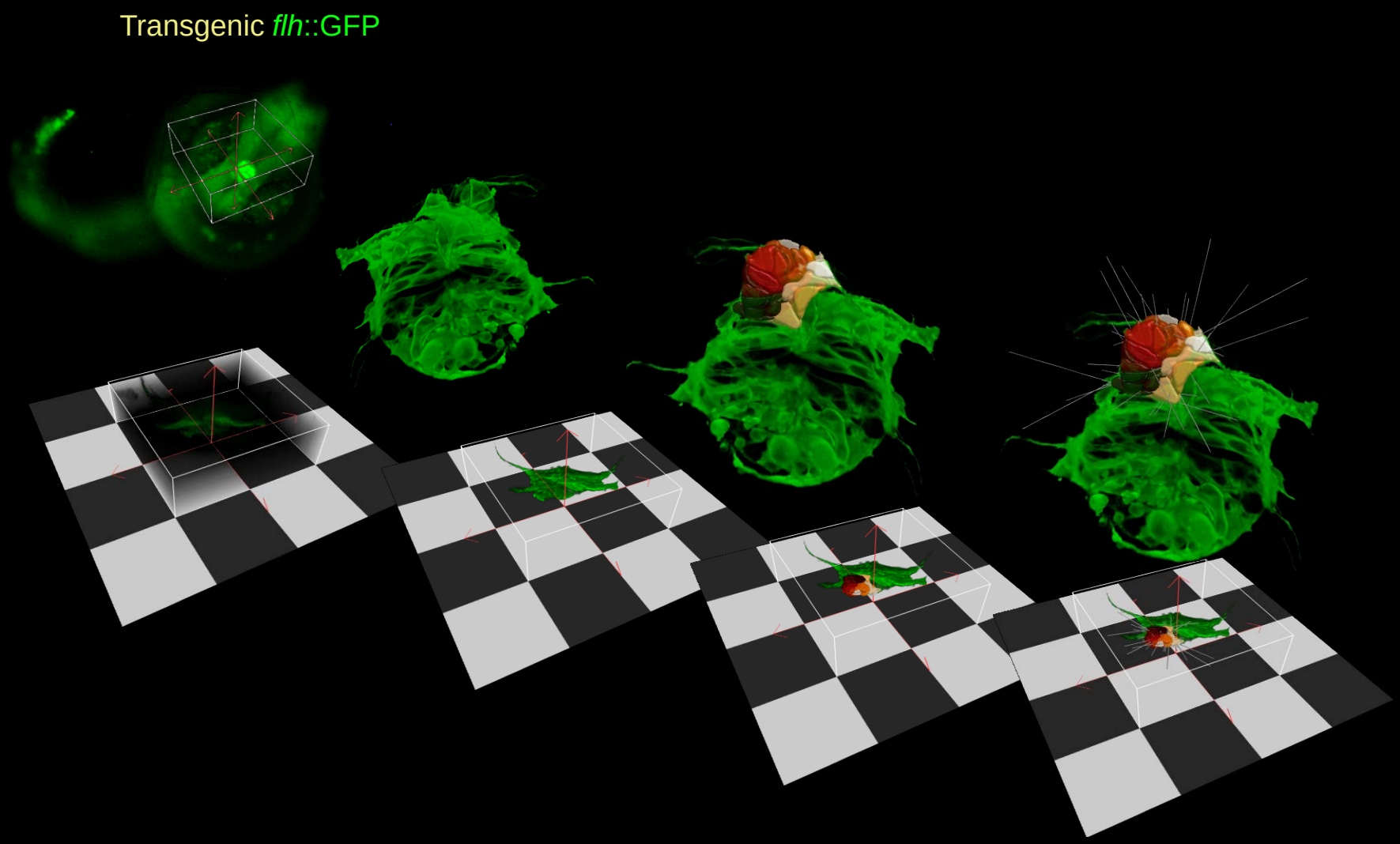
I: *Image Matrix*

N: *Noise Function*

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

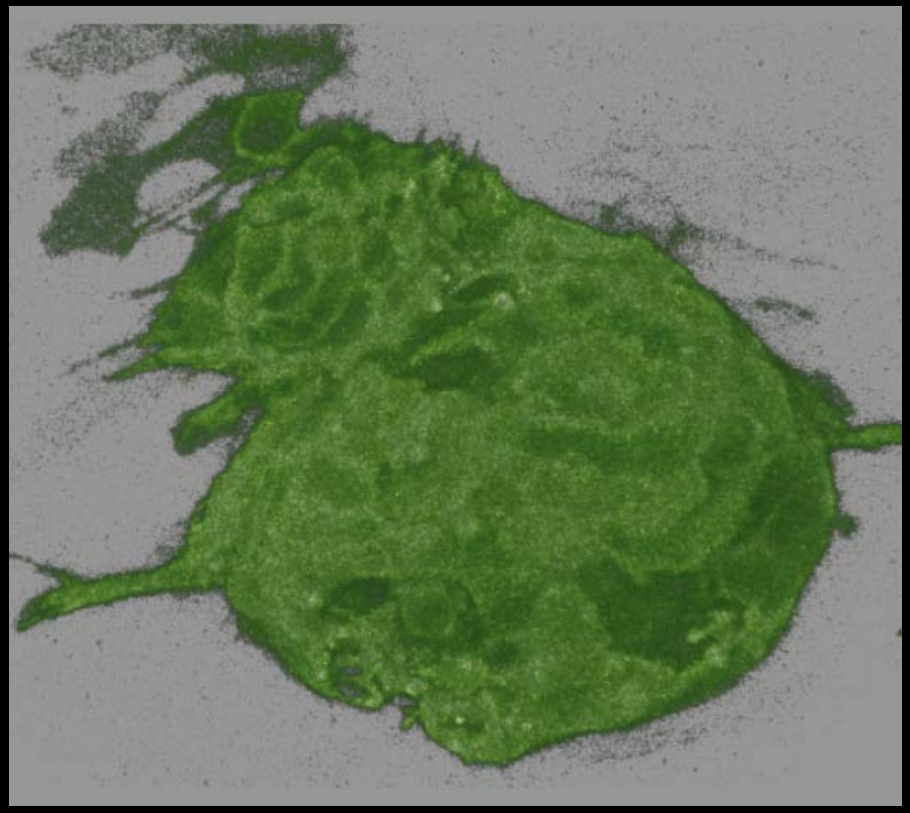


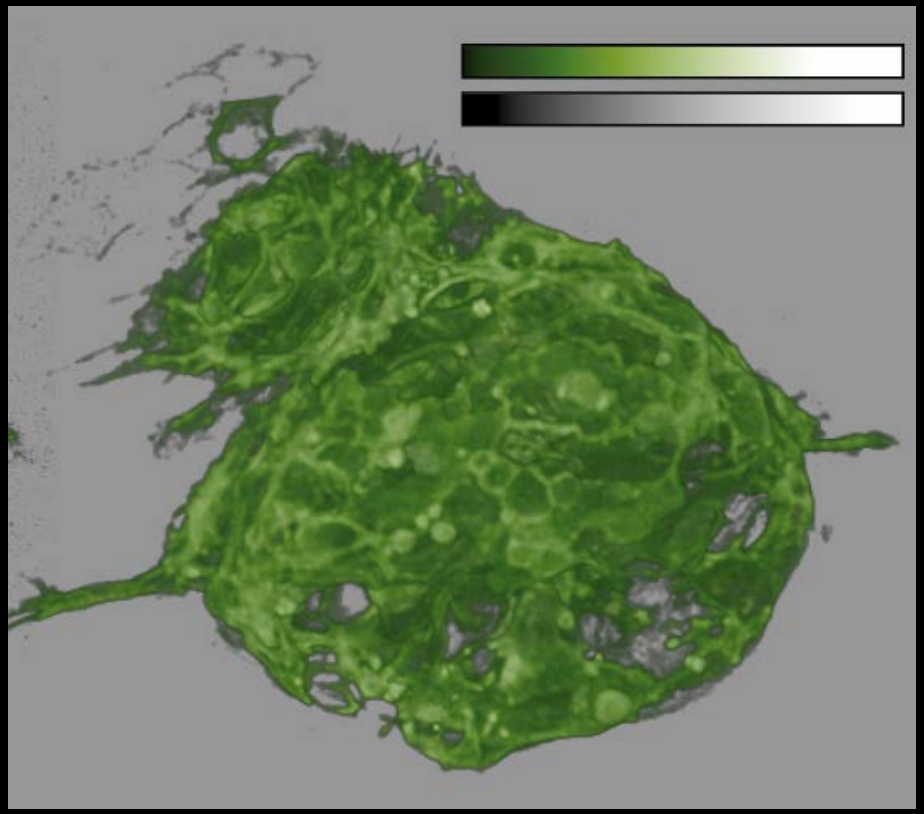
|-> Deconvolution



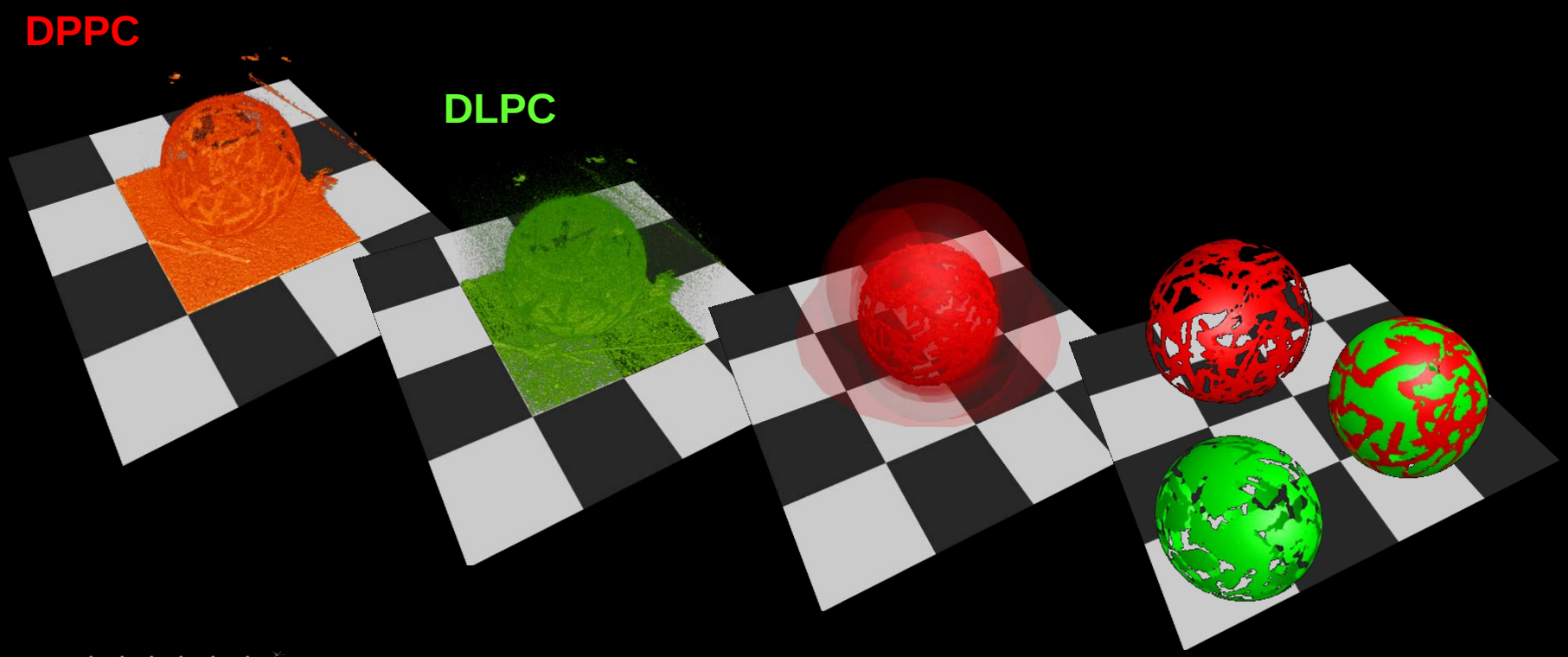
LEO-Lab/SCIAN-Lab … (Härtel et al. 2007 Computational Modelling of Objects Represented in Images. Fundamentals, Methods and Applications)
Morpho-genetic mechanisms – interplay of form and function. .

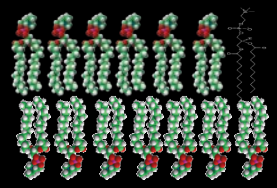
|-> Deconvolution



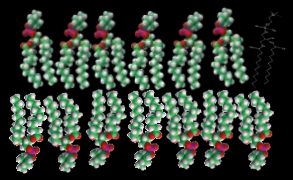


|-> Deconvolution



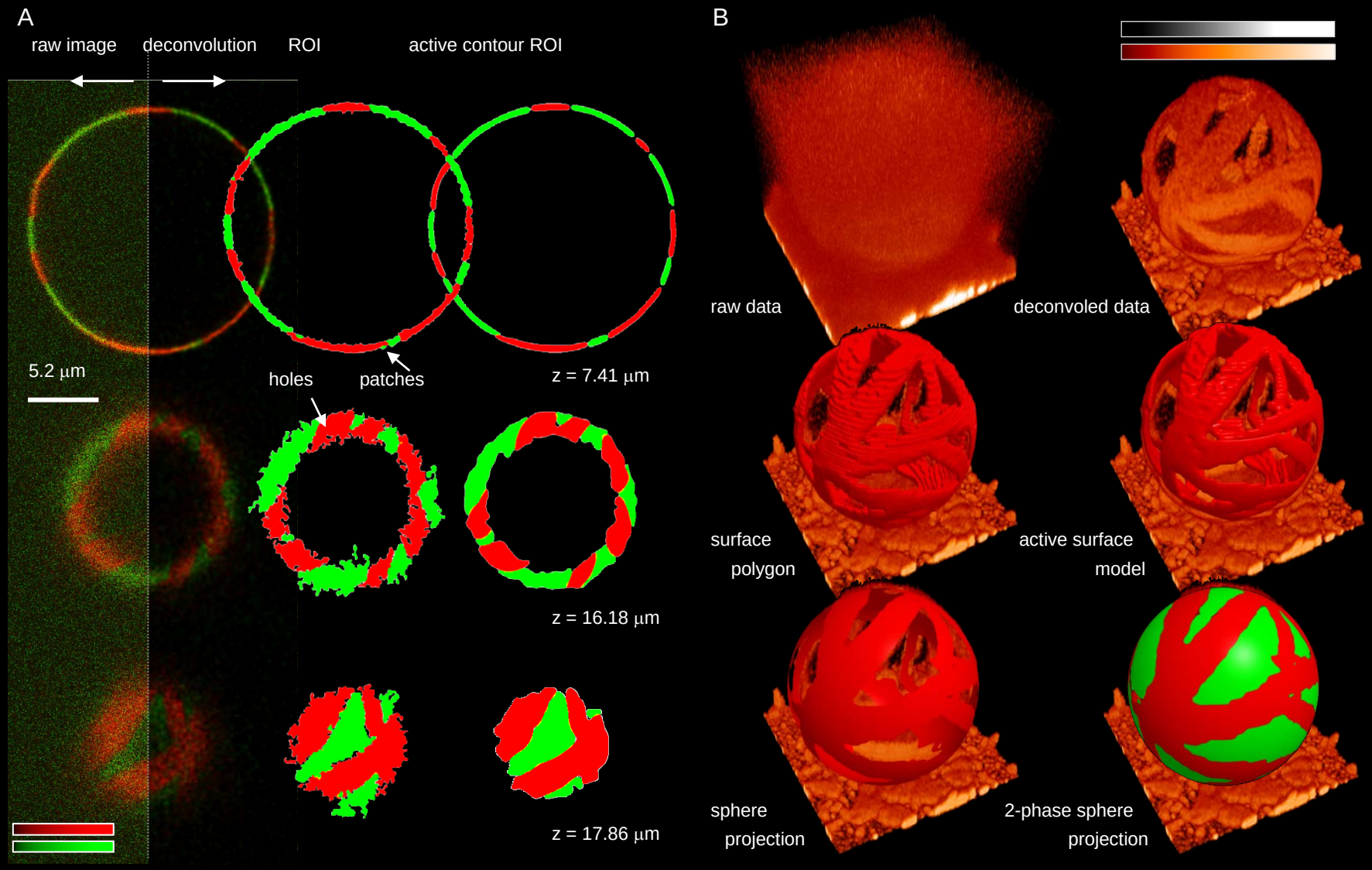


At T = 20 ºC: **Solid Gel or S_0 phase is formed by DPPC** 16:0 (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine) + 0.5 mol% $DiIC_{18}$.

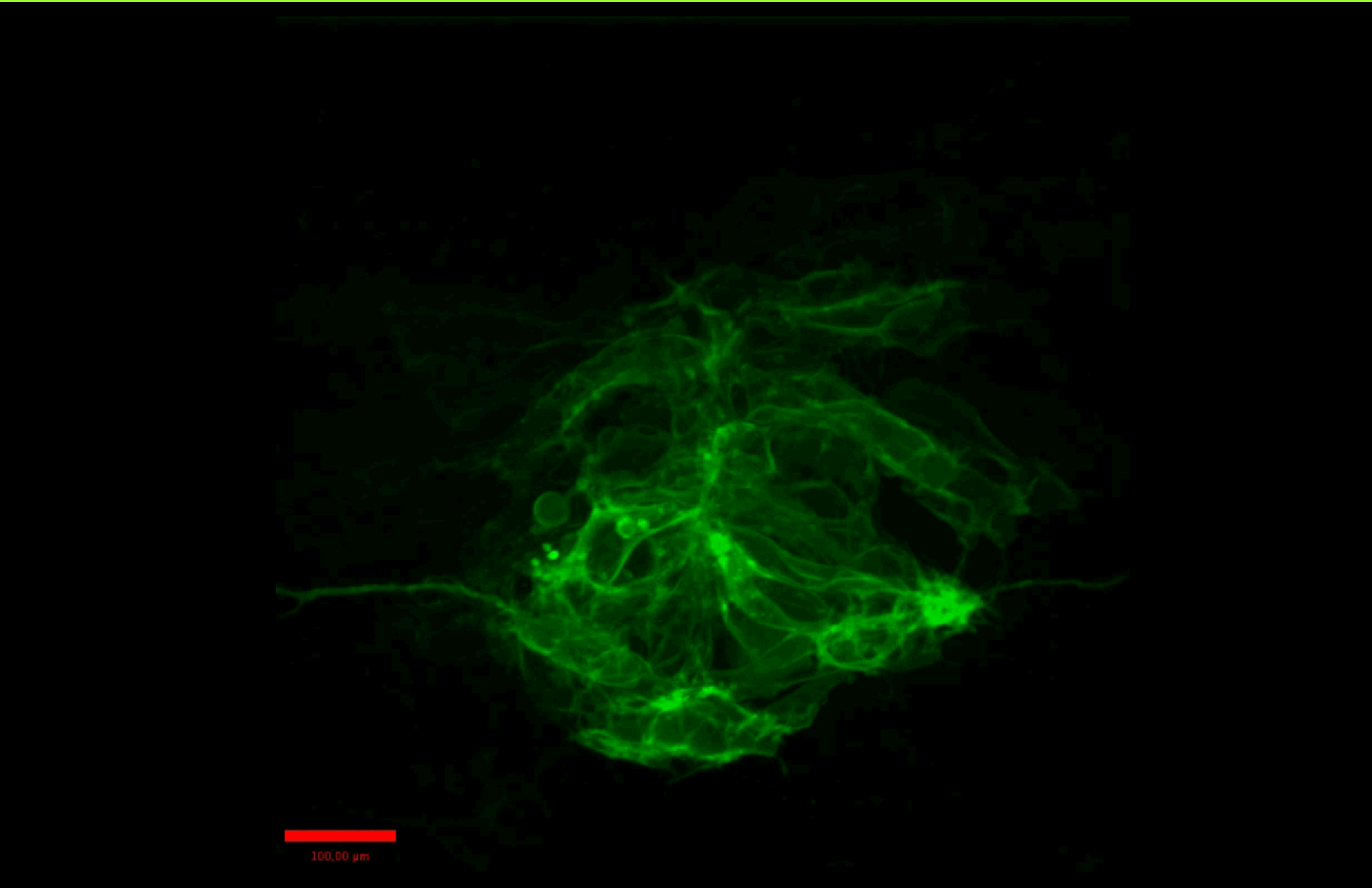


At T = 20 ºC: **Fluid phase is formed by DLPC** 12:0 PC (1,2-Dilauroyl-*sn*-Glycero-3-Phosphocholine) + 0.5 mol% BODIPY-PC.

|-> Deconvolution



|-> Deconvolution



|-> Deconvolution

PSF: *Point Spread Function*

f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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

Calculator

Select one: confocal / widefield / nipkow / 4Pi

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

|-> Pinhole

PSF: *Point Spread Function*

f: *Object Function*

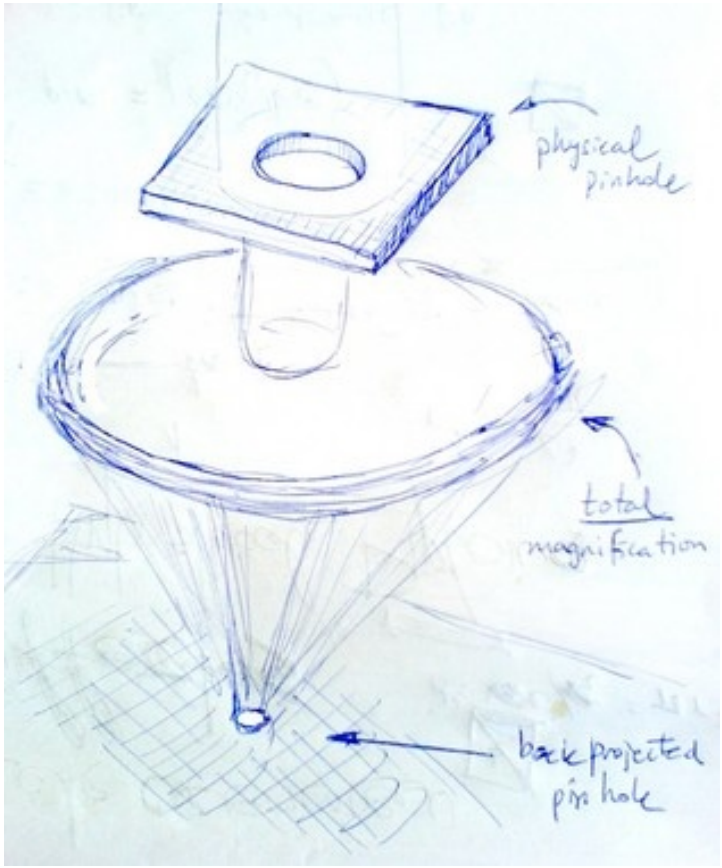
b: *Offset Function*

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)*

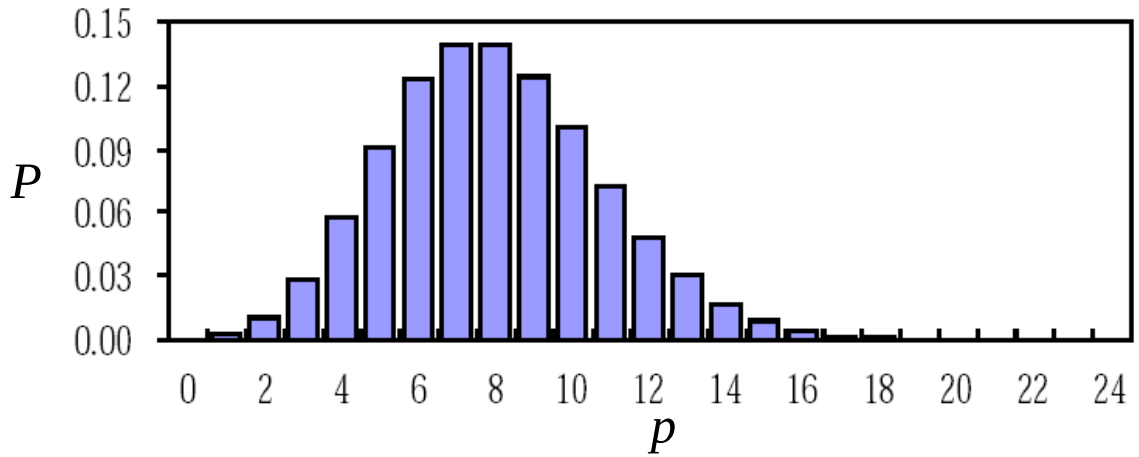
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

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

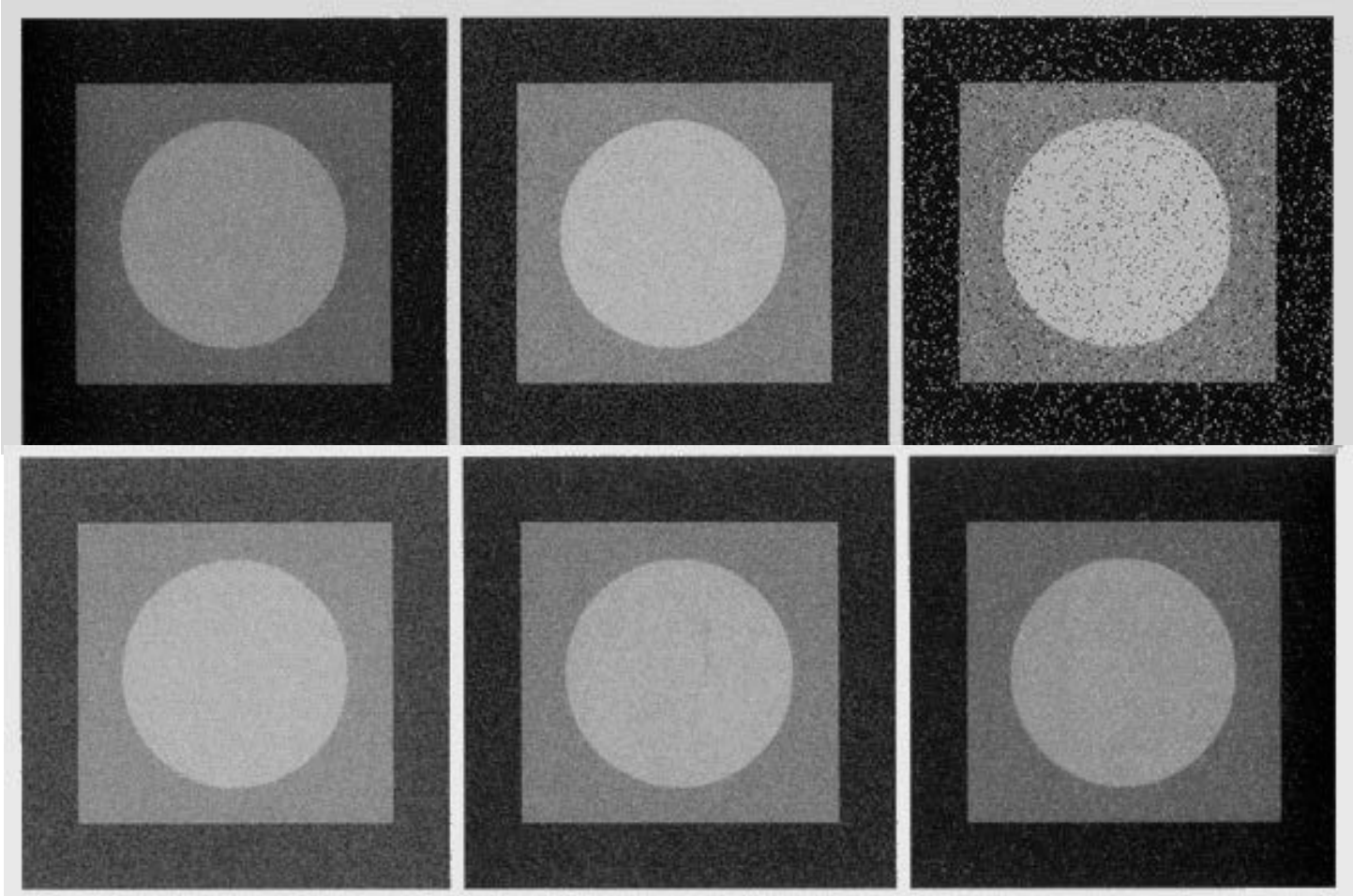
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

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

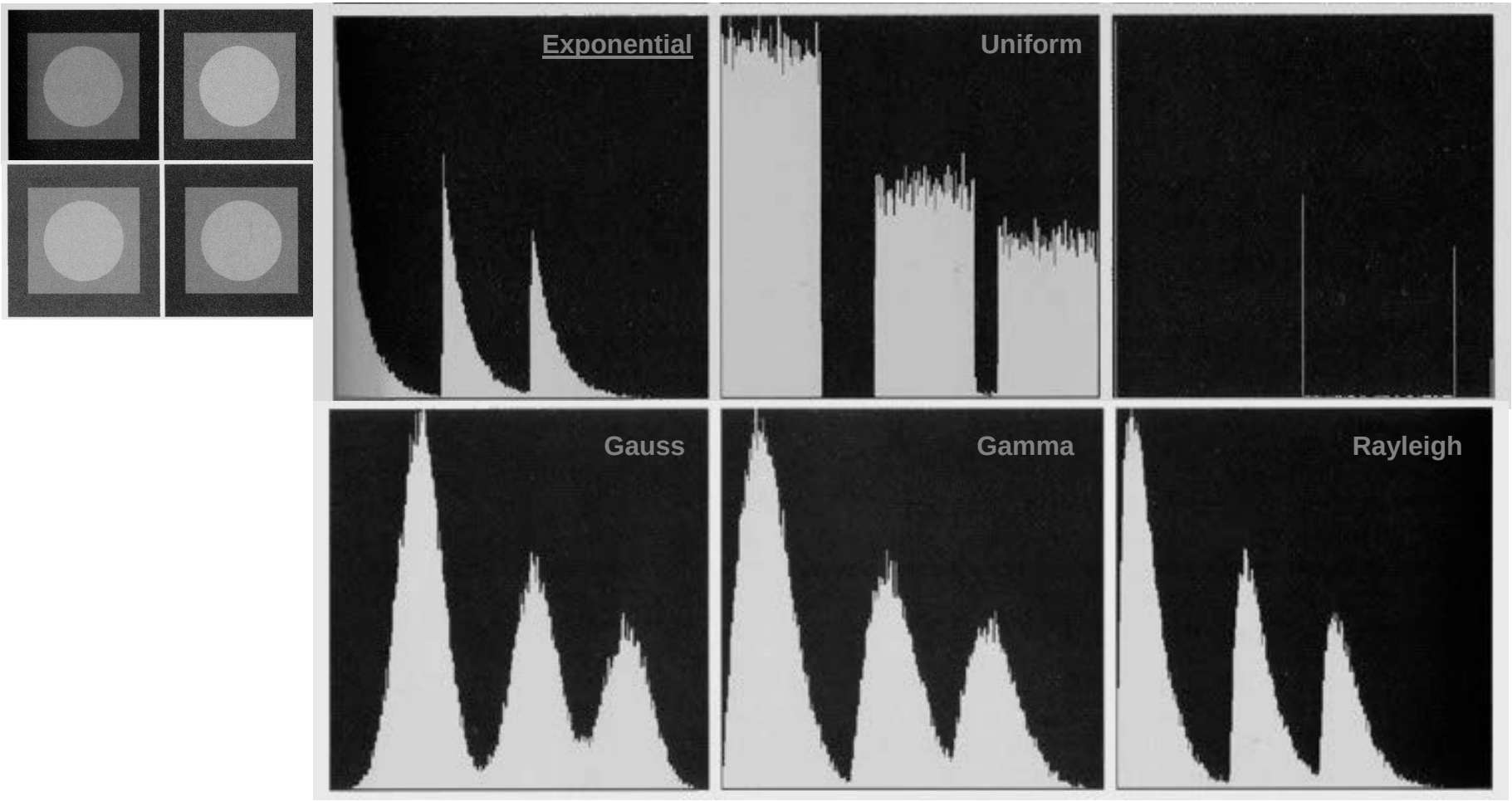
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> Noise



|-> Noise



|-> Noise

The Signal to Noise ratio (SN) is a number not always easy to estimate. The easiest way to obtain some figures 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.

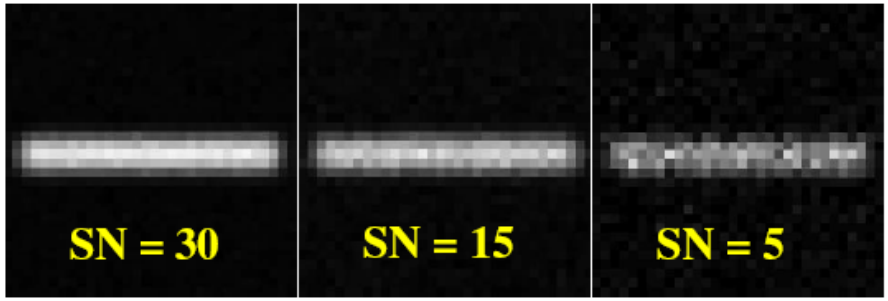
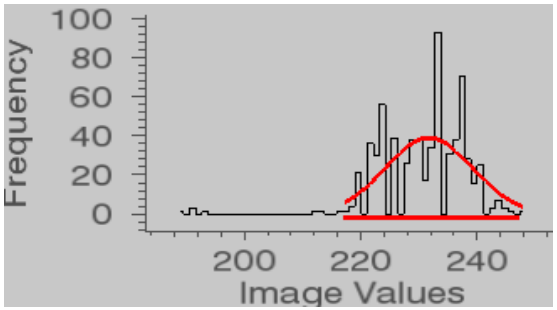
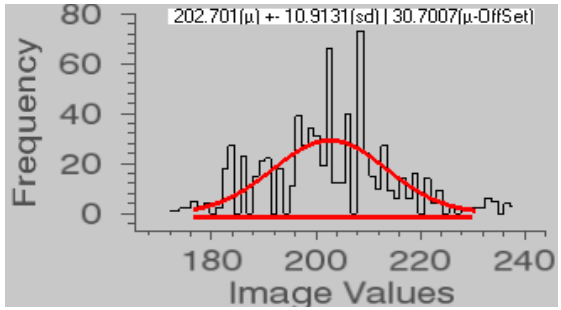
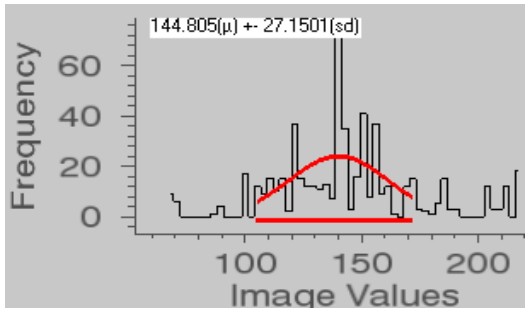


Figure 9. Images with different generated noise levels





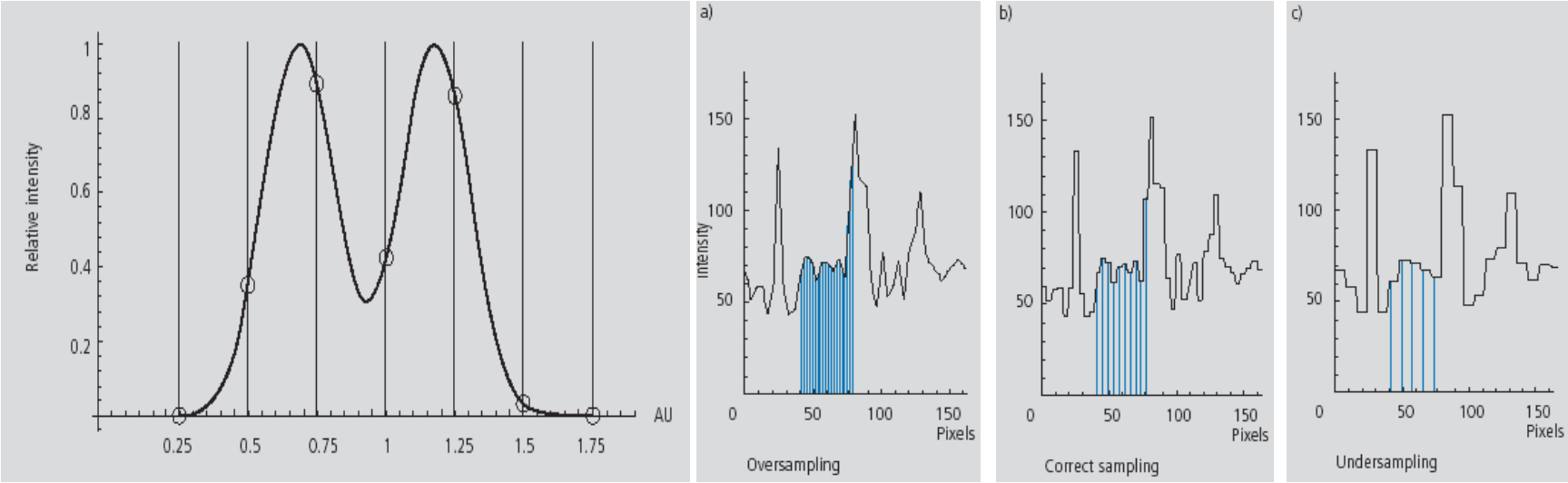


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

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

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

|-> 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*

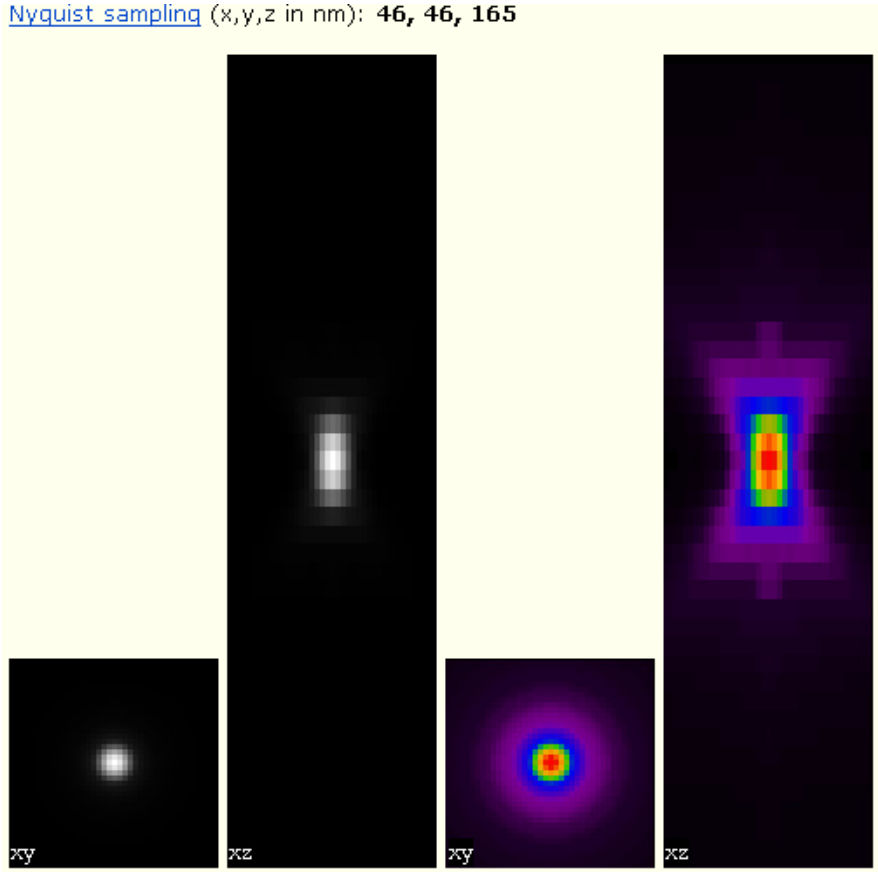
f: *Object Function*

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

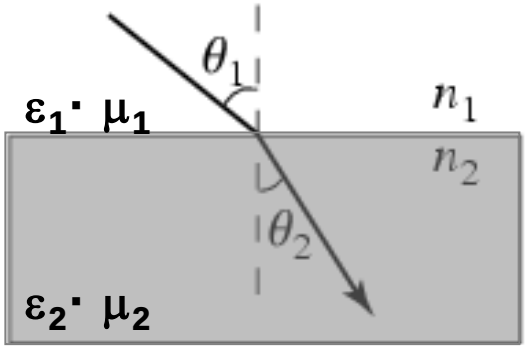
|-> Snell's Law

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

ε electric permittivity and μ magnetic permeability.

<u>Snell's Law:</u>

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



- 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]

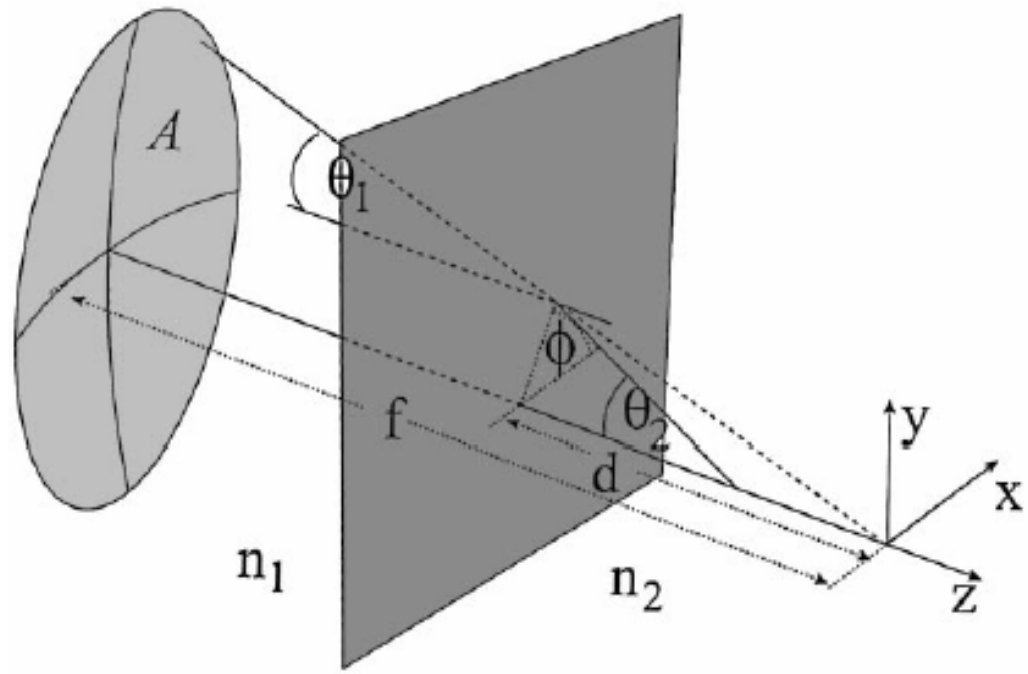
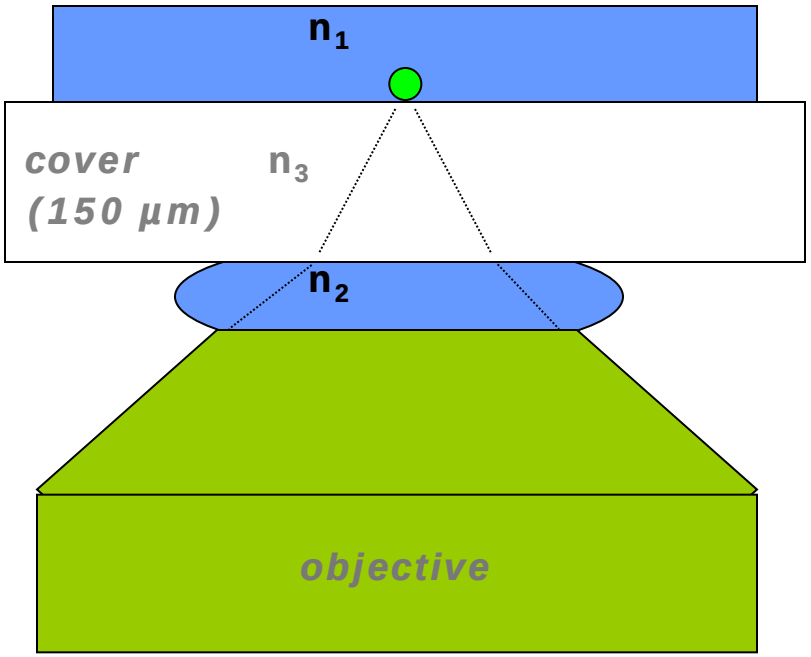


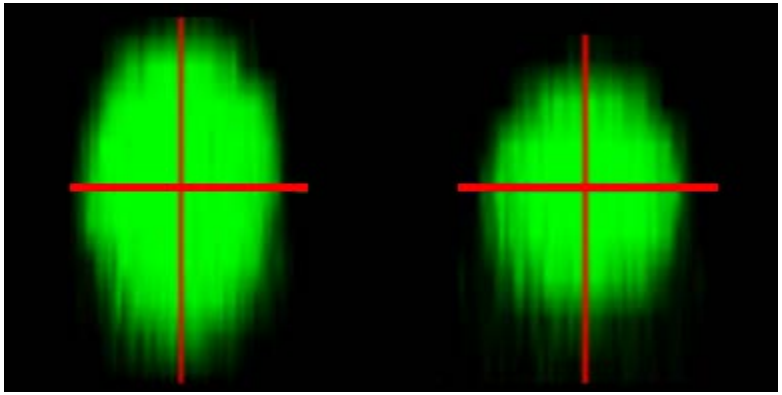
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera:** ∅ = 6 µm





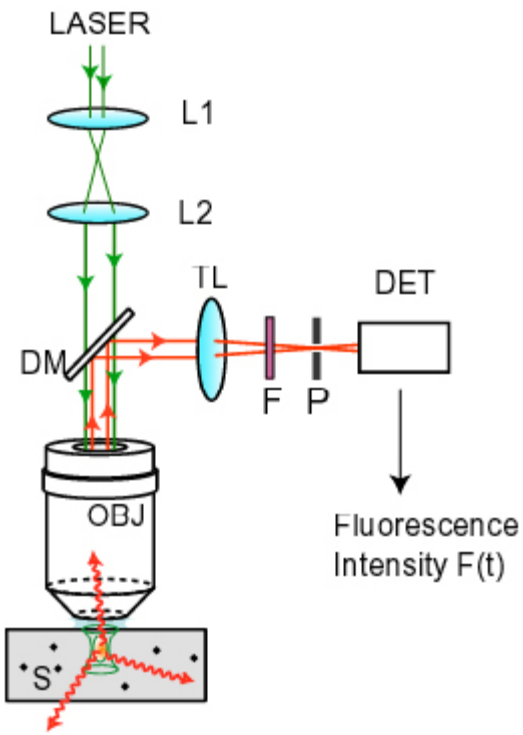
agua / aceite -- *aceite / aceite*

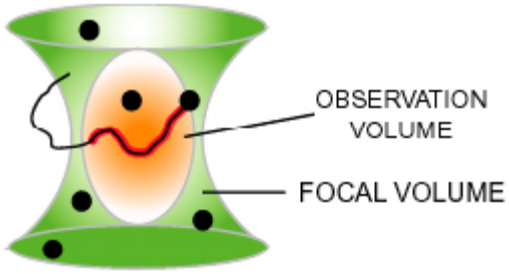
$n_1 \neq n_2$ $n_1 = n_2$

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

$n = n(\lambda)$!

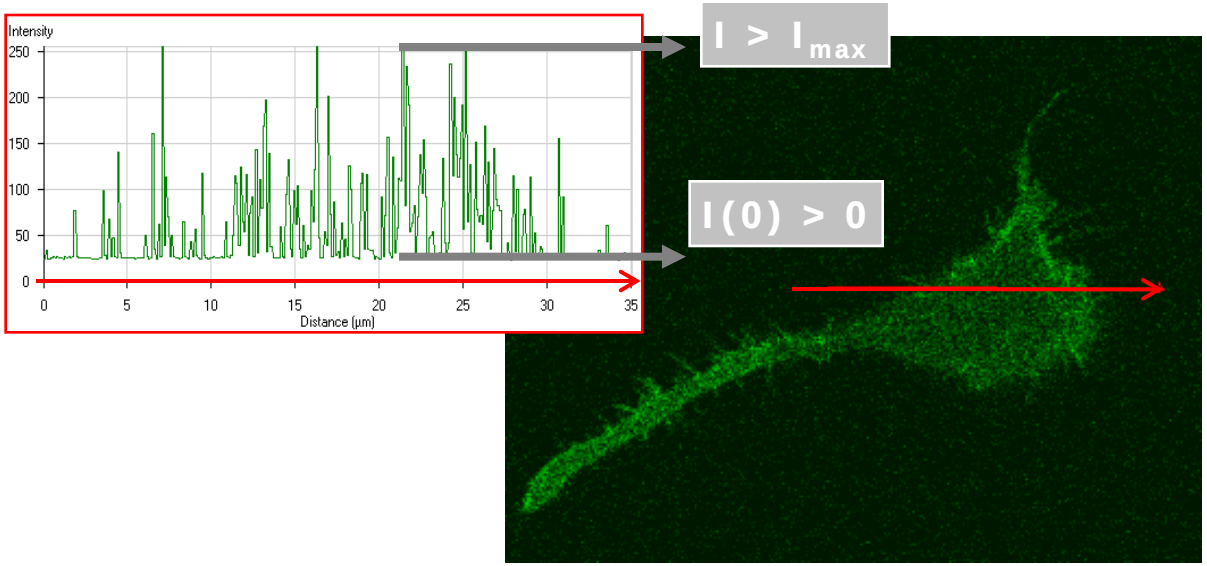
|-> Resumen I



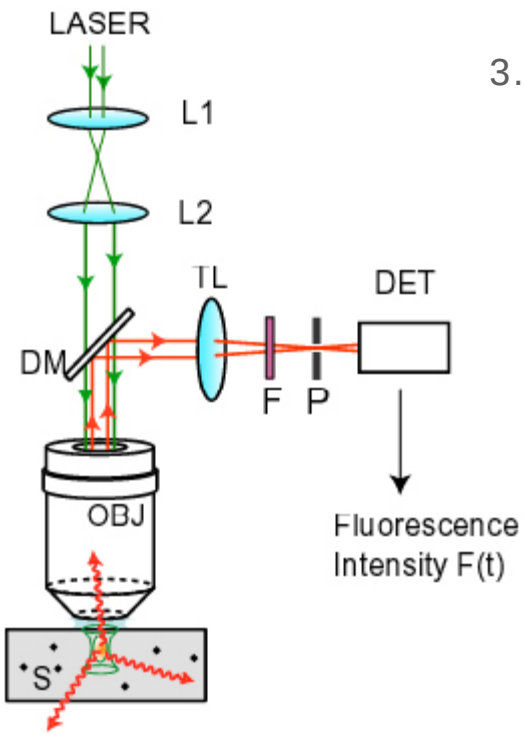


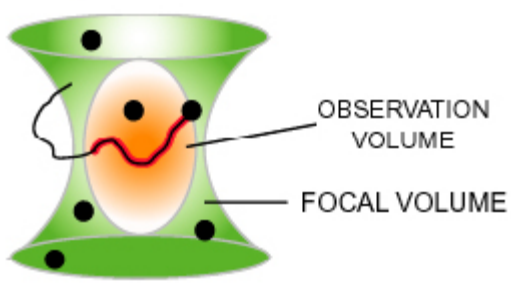
The observation volume (femtoliter) defined by the Point Spread Function must be considered as a <u>mini-sprectrofluorimeter</u>.

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) !

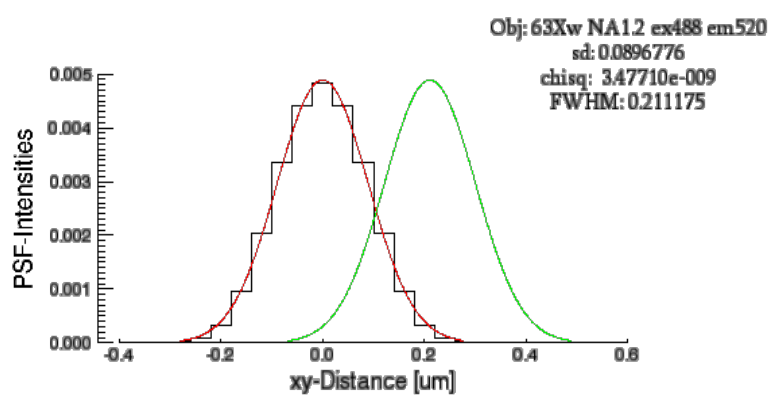


|-> Resumen II

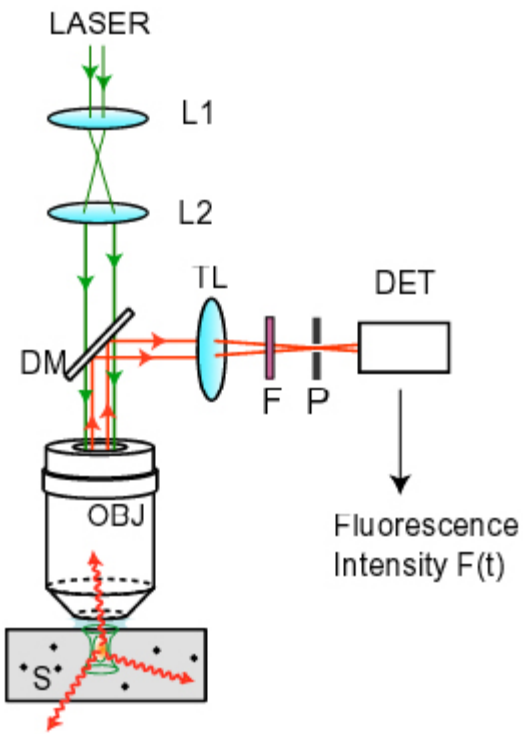


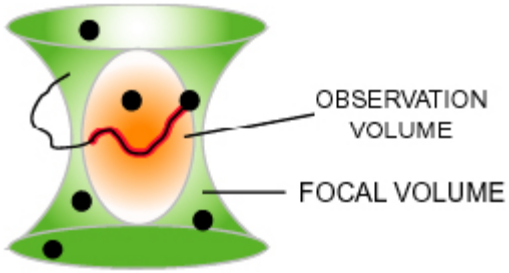


3. You need to consider sampling distances in Δx and $\Delta y \approx 50$ nm and $\Delta z \approx 150$-300 nm for later deconvolution, or calculate the explicit sample distances @ http://support.svi.nl/wiki/NyquistCalculator



|-> Resumen III

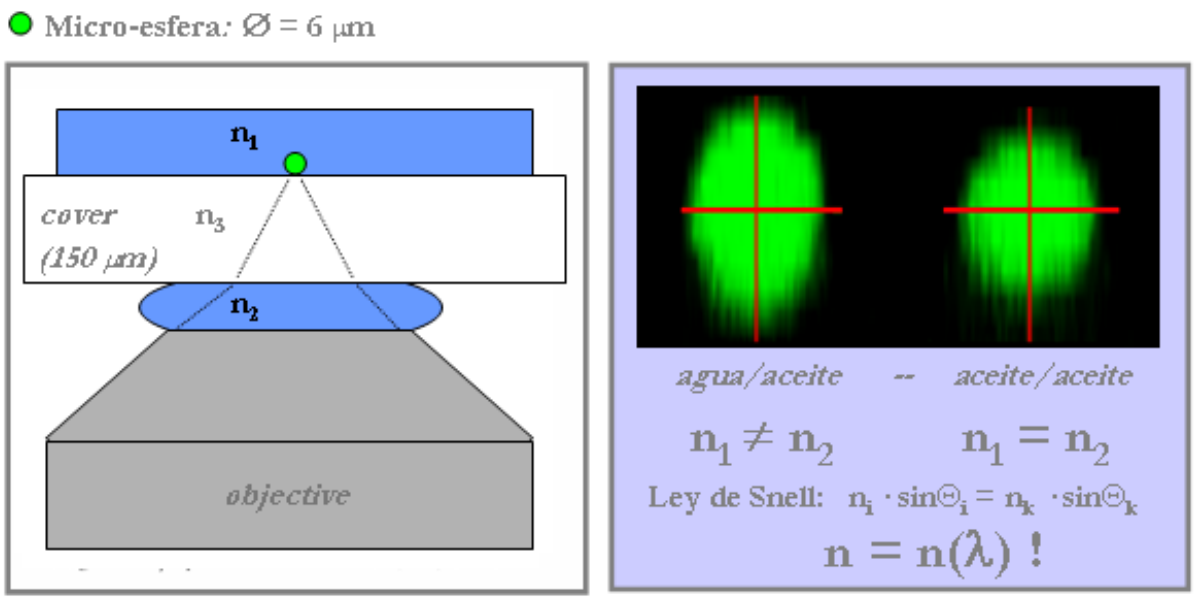




4. Use the right inmersion setup !

   n1 = n2 !

   Keep refractive index / index of refraction constant !



Laboratory for SCIAN Scientific Image Analysis

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