Laboratory for SCIAN
Scientific Image Analysis

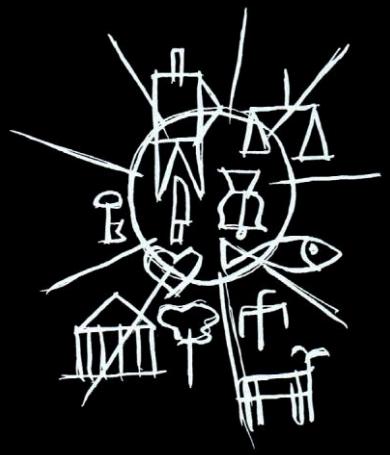
BIOMEDICAL NEUROSCIENCE INSTITUTE

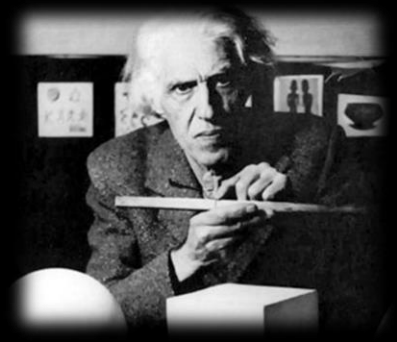
Procesamiento de Imágenes y Bioseñales I & II

Steffen Härtel
www.scian.cl

Laboratory of Scientific Image Analysis (SCIAN-Lab)
Centro de Informatica Medica 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
ICBM, Faculty of Medicine
University of Chile

Santiago de Chile, Septiembre 2018

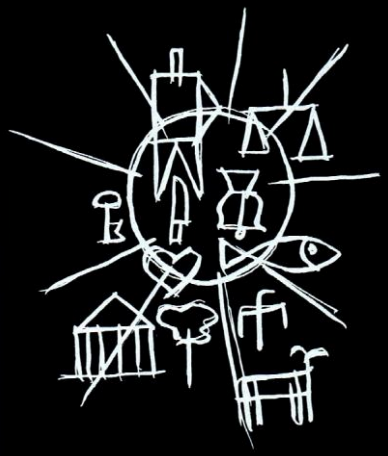


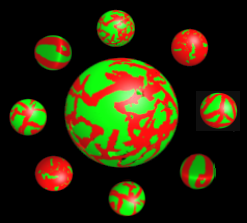


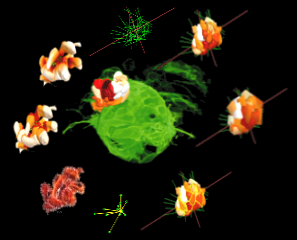
Joaquín Torres García 1874-1949

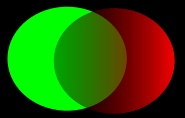
Welcome


FACULTAD DE MEDICINA
UNIVERSIDAD DE CHILE
Cursos de postgrado
Procesamiento de Imágenes y Bioseñales I y II
Inicio: 1 de septiembre de 2018
Contenidos
• Fundamentos de microscopía óptica
• Conceptos de microscopía óptica masiva y subdifracción (súper-resolución)
• Análisis de estructuras en imágenes digitales
• Interpretación de información morfológica, topológica y dinámica en imágenes biológicas y biomédicas
Actividades
Clases presenciales, prácticos de microscopía y procesamiento de imágenes, seminarios dentro del marco de los prácticos y seminario bibliográfico
Profesores participantes
Dr. Steffen Härtel
Dr. Víctor Castañeda
Dr. Mauricio Cerda
Dr.(c) Jorge Jara
Dr. Jorge Toledo
MSc. Susana Vargas
MSc. Jimena López
MSc. Stefan Sigle
Dr. Enzo Brunetti
Dr. Enzo Aguilar
Organización
Dr. Steffen Härtel
Dr. Mauricio Cerda
Lugar
Laboratorio de Procesamiento de Imágenes Científicas SCIAN-Lab
Programa de Anatomía y Biología del Desarrollo
Facultad de Medicina, Universidad de Chile
Av. Independencia 1027, Diente A
SCIAN
Información y consultas: mauriciocerda@med.uchile.cl
CPDAI
REDECA
VISUAL-D
CEDAI
MicroMundo
BNI









Informaciones en:

-> www.scian.cl

-> courses

-> 2018 Curso PIB

Notas:

Prácticos (25%)

Seminarios (25%)

Examen Final (50%)

4 Grupos a 6 Cabezas:

- Seminarios Prácticos

10 min presentación por cabeza !!!

- Seminarios Bibliográficos

10 min presentación +

10 min preguntas y discusión

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-> www.scian.cl

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10 min presentación por cabeza !!!

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VISUAL·D

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REDECA EQUIPOS CIENTÍFICOS AVANZADOS

HHN Hochschule Heilbronn Technik Wirtschaft Informatik

CIMT Centro de Informatica Medica y Telemedicina

Universität Heidelberg Zukunft seit 1386

chip

CENS Centro Nacional en Sistemas de Información de Salud

ICBM Instituto de Ciencias Biomédicas

BNI Chile

Basic Science	Scientific Platforms	Human Capital Formation & Research in Medical Informatics	Institutes
FONDECYT CONICYT	FONDEF CORFO	DAAD / DFG / STIC-AMSUD / NIH	ICM / ICBM

SCIAN-Lab Members

PIs	●●	Biophysics / Computer Science
PostDocs / Young Academics	●●●●●	Biology / Computer Sc / Electric Engineer / Mathematics
PhD - students	●●●●	Computer Sc / Electric Engineer / Biochemistry
Master - students	●●●●●…	Medical Technology / Electric Engineer / Medical Informatics
Undergraduate	●●●●	Computer Science/Biology
Research – Assistants	●●●●●	Medicine / Computer Sc / Electrical Engineer / Biology
Technicians	●●●	Biotechnology / Labtechnician / Administration

Diseño de un modelo de **PRESTADORES DE SALUD** en Chile.
InnovaChile/CORFO Bien Público Estratégico de Alto Impacto.

TERMINOLOGÍA FARMACÉUTICA CHILENA.
InnovaChile/CORFO Bienes Públicos Estratégicos.

EXZELLENZZENTRUM SANTIAGO DE CHILE-HEIDELBERG I y II:
Center of Excellence for Innovative Research and Education
Medical Informatics, DAAD 2009/2014 y 2014/2019

CHIP / PAGEL: CHILEAN HEALTH INFO AND PROCESS CHALLENGE
Partnerships for the Health Sector in Developing Countries. DAAD: 2016 – 2019

2017 Healthier World Challenge Grant, Johns Hopkins University

-> FONDECYTs como PIs / 3 FONDECYTs como CO-Is, Anillos, …
-> Telemedicina HCUCH/CIMT.
-> CENS: Centro Nacional en Sistemas de Información en Salud.

-> 2 Cursos Anuales Pregrado Medicina en Informática Medica y Telemedicina.
-> Cursos e-learning, histopatología virtual U-Chile-UMAG.
-> Escuelas de Verano y Diplomas en Informática Medica.
-> Tercera generación del Magister en Informática Medica 2018:
https://youtu.be/L2rQNpKfuOw

- Image Processing & Microscopy

2D/3D · Morpho-topology, Motion estimation, Tracking

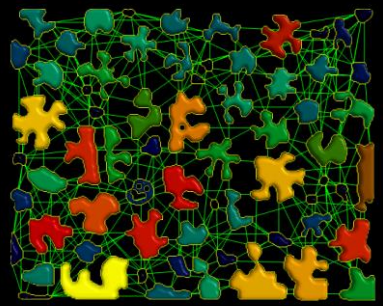
2018 Immunobiology
2017 Brain
2017 Nature Communications
2017 Developmental Dynamics
2016 Journal of Physics: Conference Series
2016 Cell Reports
2016 Pathogens and Disease
2015 Acta Tropica
2015 J of Clinical and Experimental Pathology
2015 The American Journal of Tropical Medicine
2014 Current Molecular Medicine
2014 Medical Image Analysis
2013 Development
2012 European Biophysics Journal
2012 PLosOne
2011 J Microbiol Methods …

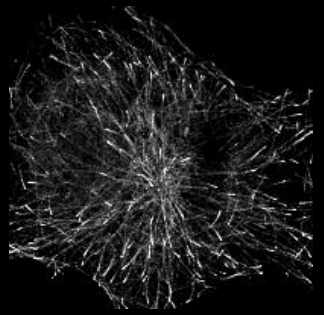
Colocalization

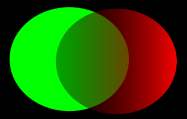
2017 Frontiers Molecular Neuroscience
2015 Gene
2014 Chromatine Research
2014 Frontiers Molecular Neuroscience
2012 PLosOne
2011 JBC
2011 Arthritis & Rheumatism

- (Bio)Medical Informatics

2018 PLOS Medicine
2018 Computer Physics Communications
2017 Computers in Biology and Medicine I y II
2016 Computers in Biology and Medicine I
2015 Science Translational Medicine
2015 IPOL
2013 IEEE Transactions on Pattern Analysis …
2013 Biological Cybernetics
2013 Reproduction, Fertility Development
2012 Machine Vision Applications







IPOL

Image Processing On Line

ImageJ
Image Processing and Analysis in Java

- Image Processing & Microscopy

2D/3D · Morpho-topology, Motion estimation, Tracking

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NASA World Wind screenshot.

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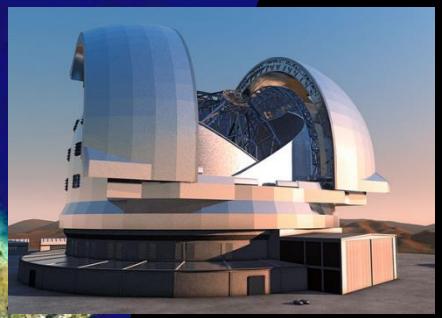
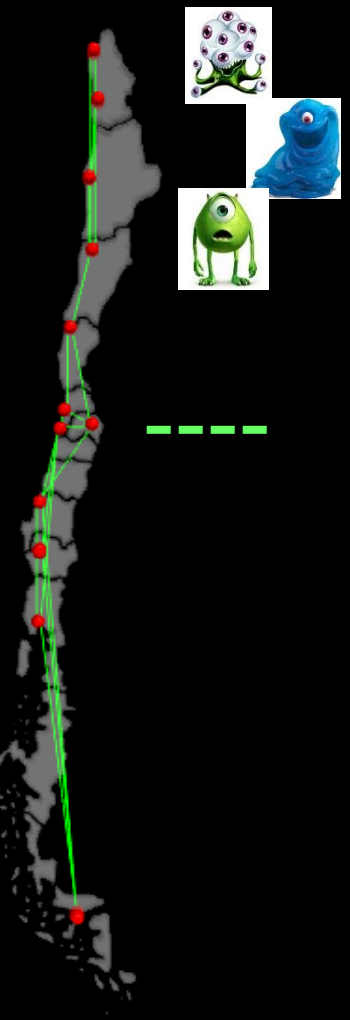
Very Large Telescope (VLT),
4 Telescopes, 8m, 2600 m

Atacama Large Millimeter/submillimeter Array (ALMA), 66 Antenna, 5000 m



E-ELT European Extremely Large Telescope, 39 m, 3000 m

NASA World Wind screenshot



Extraterrestrial Monster Science produces:
TeraB, PetaB, ExaB, ZettaB, YottaB

… Tera is a unit prefix in the metric system denoting multiplication by 10E12 or 1.000.000.000.000 !

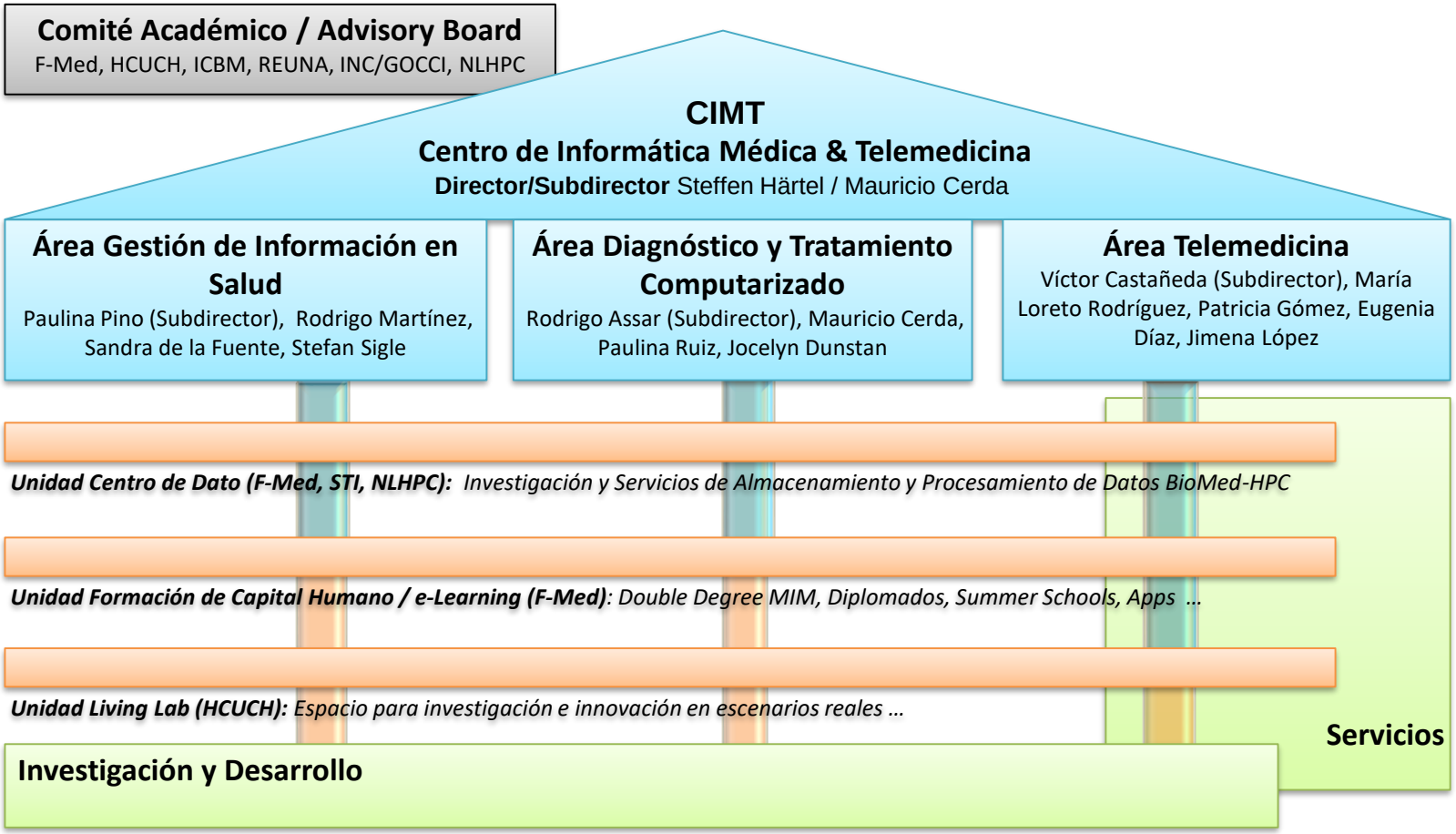
… Tera is derived from Greek τέρας (teras), meaning “monster”.

… Tera was confirmed for use in the SI in 1960…. Wiki !

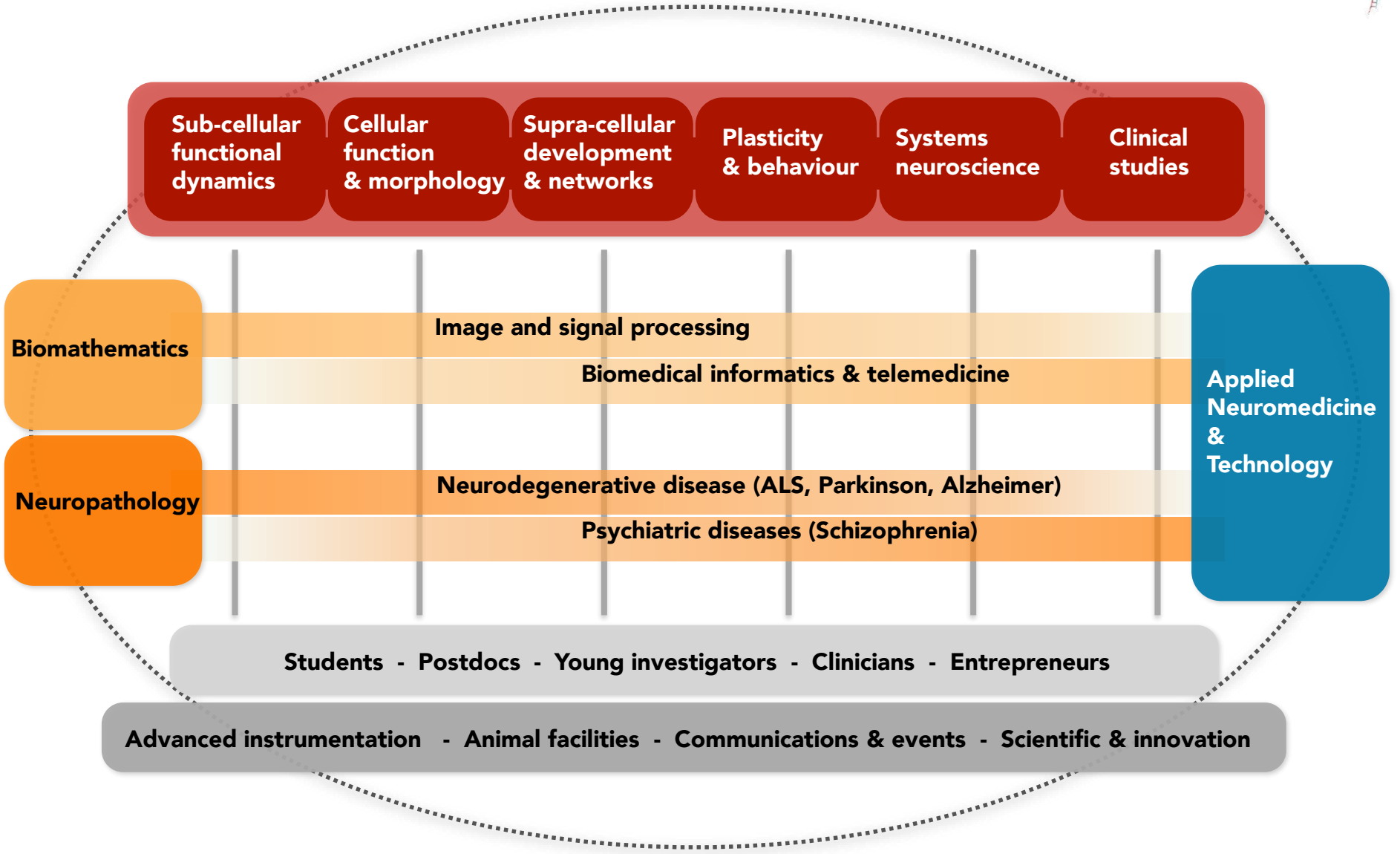
Metric prefixes

Prefix	Symbol	1000^m	10^n	Decimal	English word[n 1]	Since[n 2]
yotta	Y	1000^8	10^{24}	1 000 000 000 000 000 000 000 000	septillion	1991
zetta	Z	1000^7	10^{21}	1 000 000 000 000 000 000 000	sextillion	1991
exa	E	1000^6	10^{18}	1 000 000 000 000 000 000	quintillion	1975
peta	P	1000^5	10^{15}	1 000 000 000 000 000	quadrillion	1975
tera	T	1000^4	10^{12}	1 000 000 000 000	trillion	1960
giga	G	1000^3	10^9	1 000 000 000	billion	1960
mega	M	1000^2	10^6	1 000 000	million	1960
kilo	k	1000^1	10^3	1 000	thousand	1795
hecto	h	$1000^{2/3}$	10^2	100	hundred	1795
deca	da	$1000^{1/3}$	10^1	10	ten	1795
		1000^0	10^0	1	one	–
deci	d	$1000^{-1/3}$	10^{-1}	0.1	tenth	1795
centi	c	$1000^{-2/3}$	10^{-2}	0.01	hundredth	1795
milli	m	1000^{-1}	10^{-3}	0.001	thousandth	1795
micro	μ	1000^{-2}	10^{-6}	0.000 001	millionth	1960
nano	n	1000^{-3}	10^{-9}	0.000 000 001	billionth	1960
pico	p	1000^{-4}	10^{-12}	0.000 000 000 001	trillionth	1960
femto	f	1000^{-5}	10^{-15}	0.000 000 000 000 001	quadrillionth	1964
atto	a	1000^{-6}	10^{-18}	0.000 000 000 000 000 001	quintillionth	1964
zepto	z	1000^{-7}	10^{-21}	0.000 000 000 000 000 000 001	sextillionth	1991
yocto	y	1000^{-8}	10^{-24}	0.000 000 000 000 000 000 000 001	septillionth	1991

El Centro… www.cimt.cl



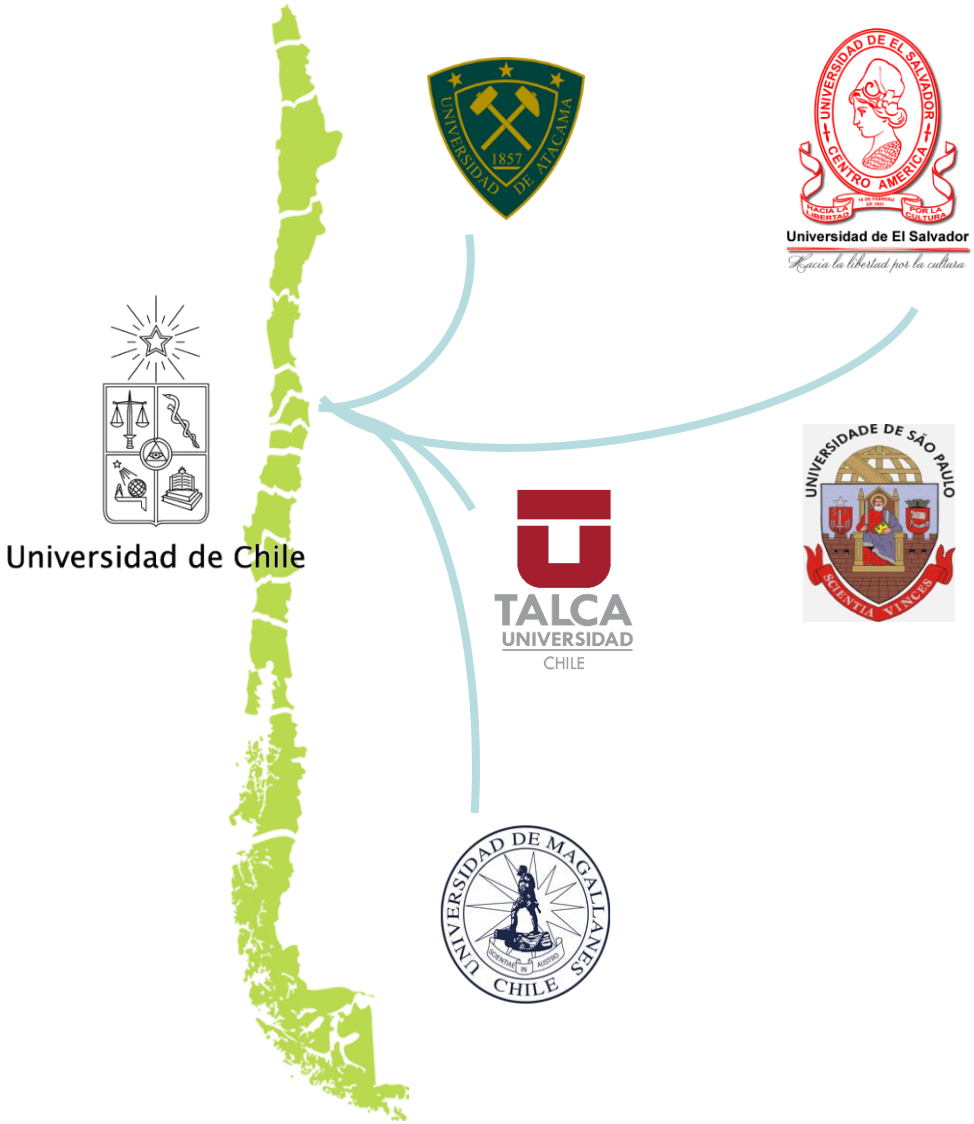
● El Instituto … www.bni.cl



Spin off y Servicios

VirtualMicro: vm.scian.cl

- Primera iniciativa en Chile de plataforma virtual para enseñanza en histopatología.
- Aplicado en tres Universidades Chilenas, el Salvador, Brazil.
- Implementación rápida y concordante con los requerimientos de los usuarios.
- La plataforma de MV apoya en forma efectiva la docencia universitaria.



redeca.med.uchile.cl

Bienvenid@s a REDECA

REDECA provee apoyo tecnológico, acceso y acompañamiento en el uso de servicios y equipos avanzados. Usuarios de la institución y externos pueden solicitar asistencia para la selección de servicios o equipos adecuados a sus desafíos experimentales, el almacenamiento y el análisis de sus datos.

¡ REDECA… haz ciencia con nosotros !

Beneficios …

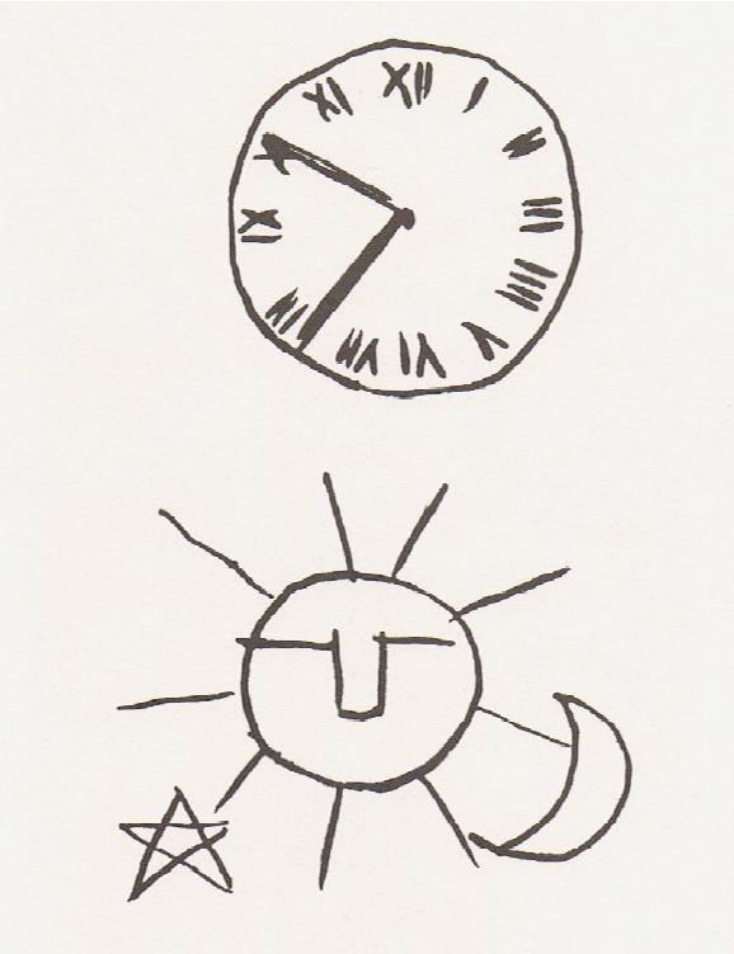
… para el usuario de equipos y servicios

- … fácil acceso a equipos, soporte técnico y diseño experimental.
- … fácil cotización, facturación y traspaso interno.
- … apoyo formulación de proyectos de investigación y equipamiento.

Unidades de servicios

- Unidad de Microscopía
- Unidad de Citometría
- Unidad de Ultracentrifugación
- Unidad de Datos

SCIAN-Lab: BioMedHPC connects via 10 Gbps F-Med, FCFM, STI, REUNA



Joaquín Torres García

Uruguayo, pintor y pensador,

Montevideo, Barcelona, París, NY …

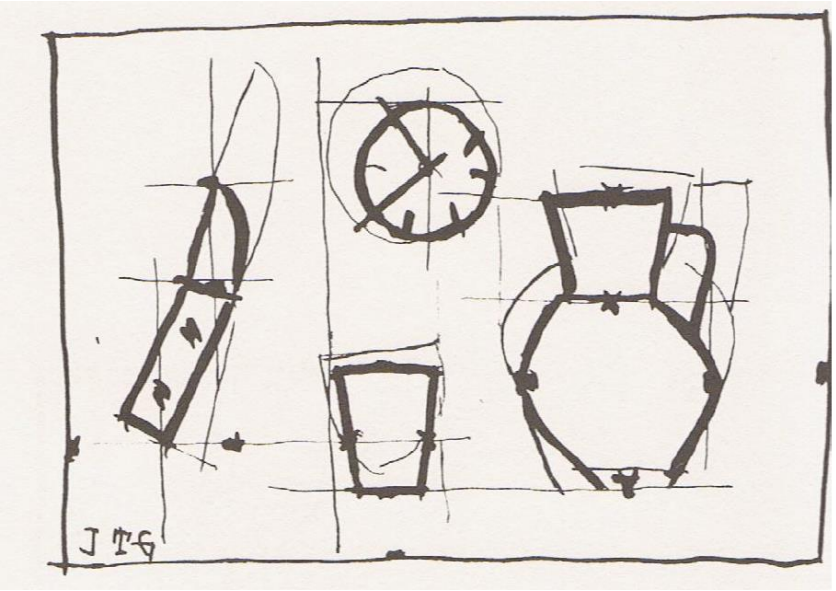
PENSAR ES GEOMETRIZAR

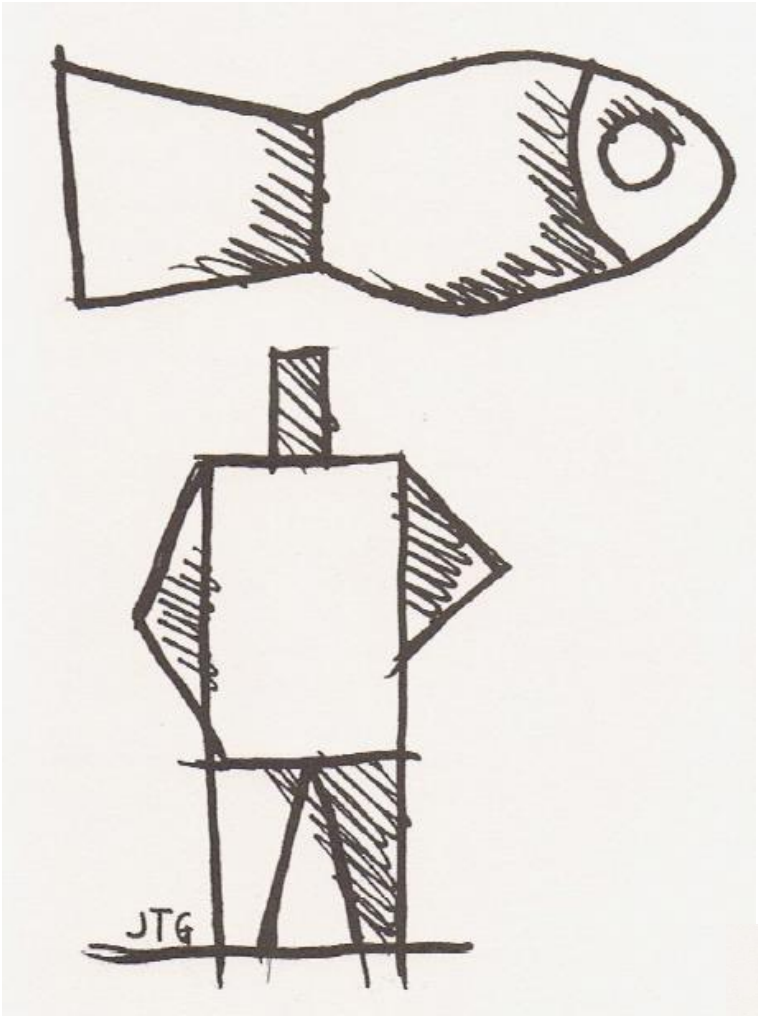
La geometría es como un teclado de lenguajes …

… curvas, regulares o no, rectas, ángulos, circunferencias, arcos …

… son los pocos elementos universales con que se puede expresar todo !!!!

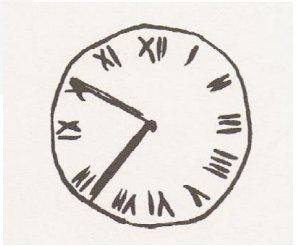
morfología





topología

tiempo



UNIVERSALISMO CONSTRUCTIVO

Laboratory for SCIAN Scientific Image Analysis

... www.scian.cl

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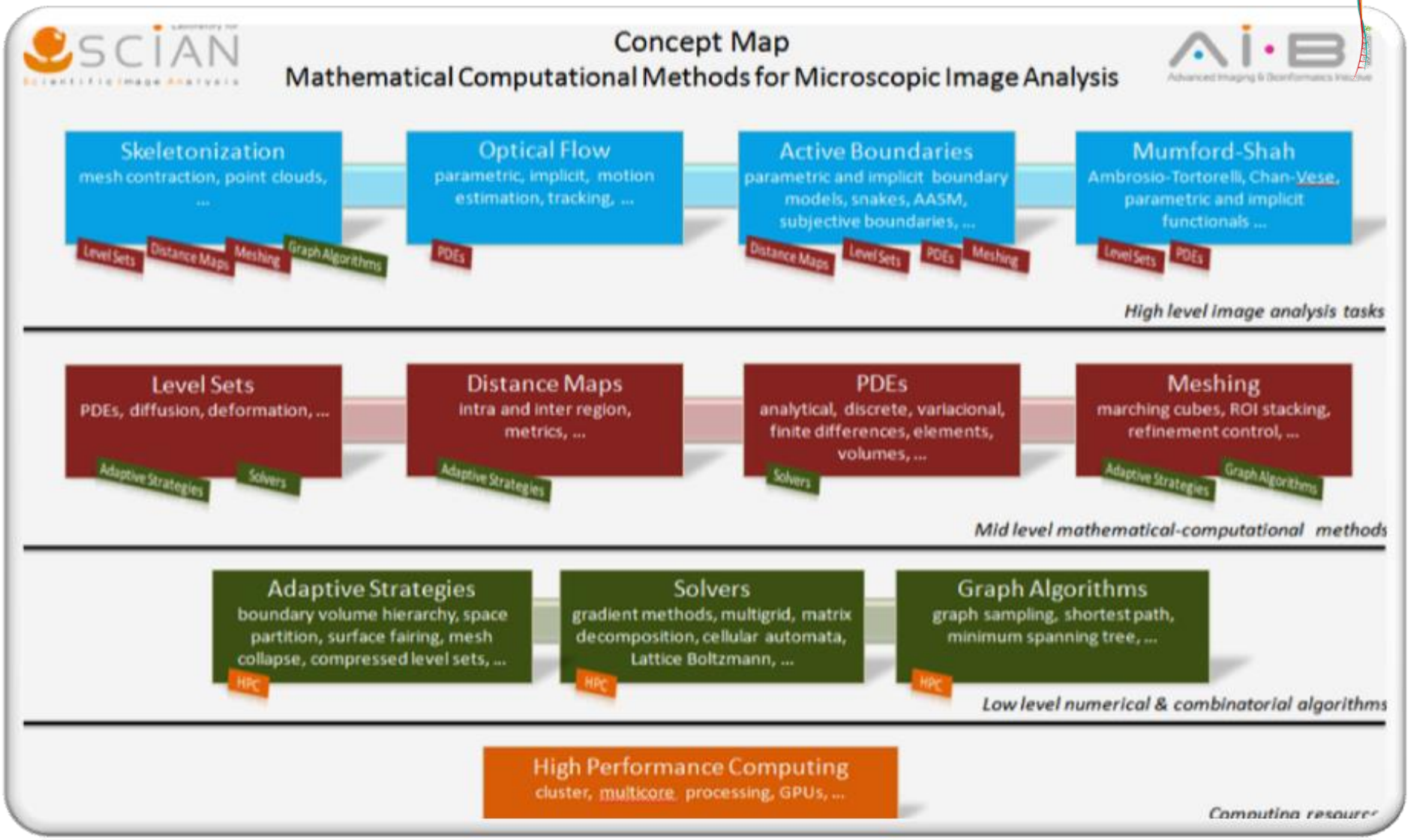
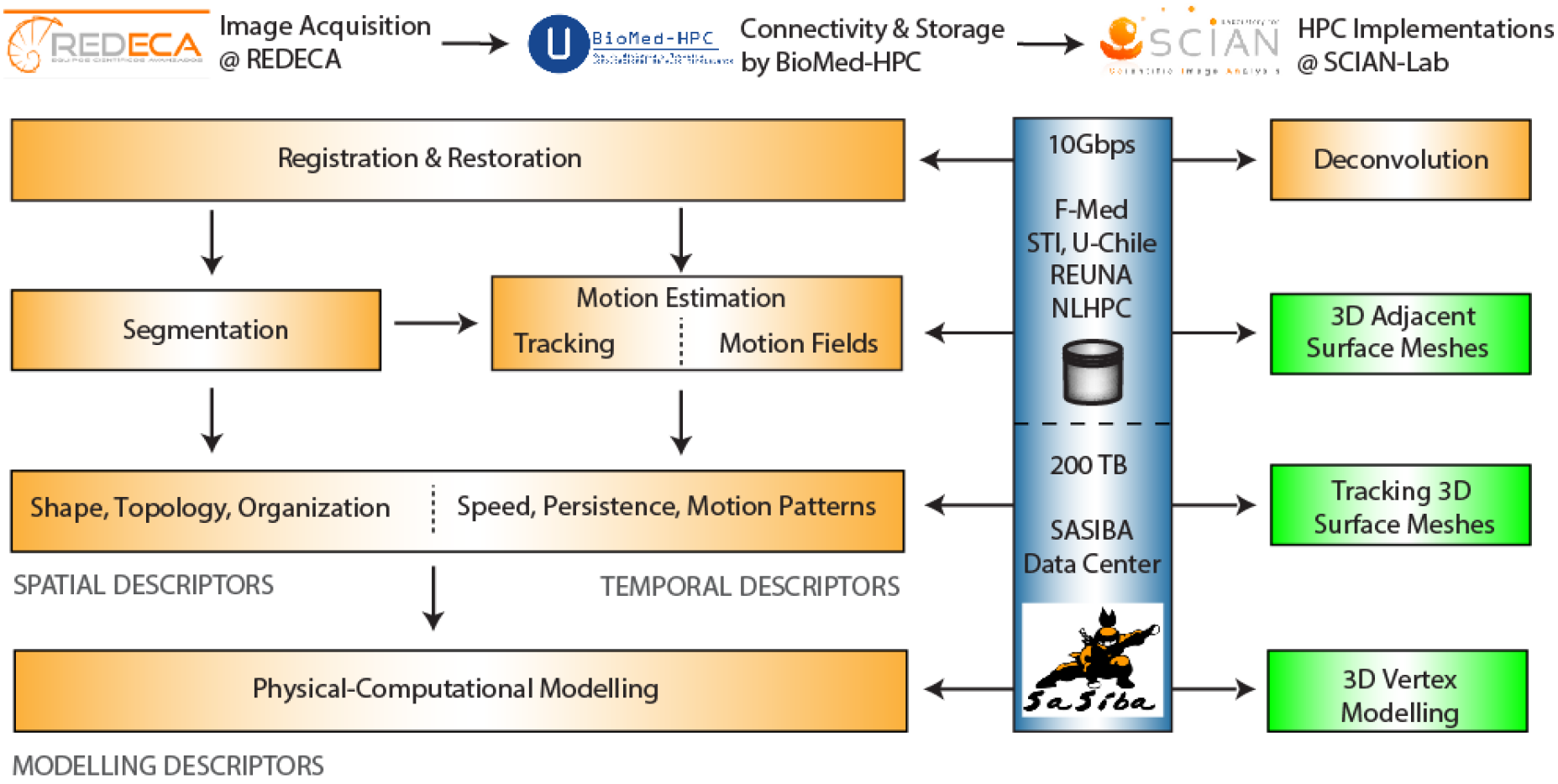
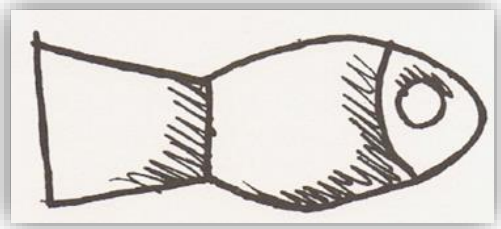



REDECA
Image Acquisition
@ REDECA
BioMed-HPC
Connectivity & Storage
by BioMed-HPC
SCIAN
HPC Implementations
@ SCIAN-Lab
Registration & Restoration
Segmentation
Motion Estimation
Tracking
Motion Fields
Shape, Topology, Organization
Speed, Persistence, Motion Patterns
SPATIAL DESCRIPTORS
TEMPORAL DESCRIPTORS
Physical-Computational Modelling
MODELLING DESCRIPTORS
10Gbps
F-Med
STI, U-Chile
REUNA
NLHPC
200 TB
SASIBA
Data Center
Deconvolution
3D Adjacent
Surface Meshes
Tracking 3D
Surface Meshes
3D Vertex
Modelling

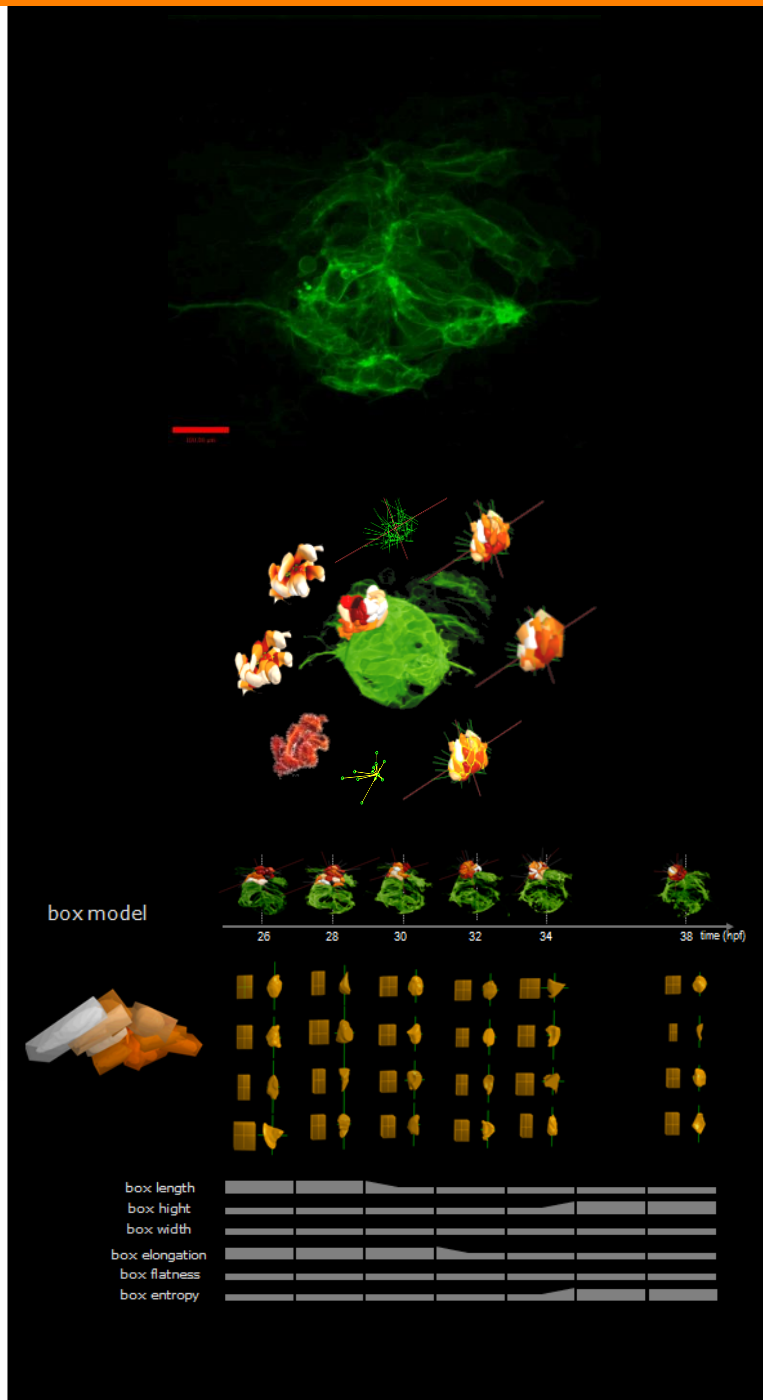
Miguel Concha
Biología del Desarrollo



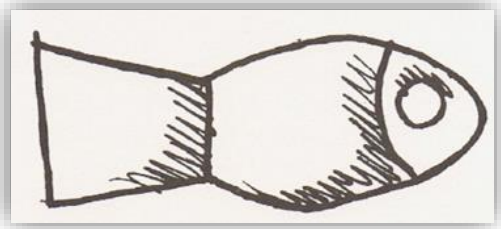
Heidelberg Collaboratory for Image Processing

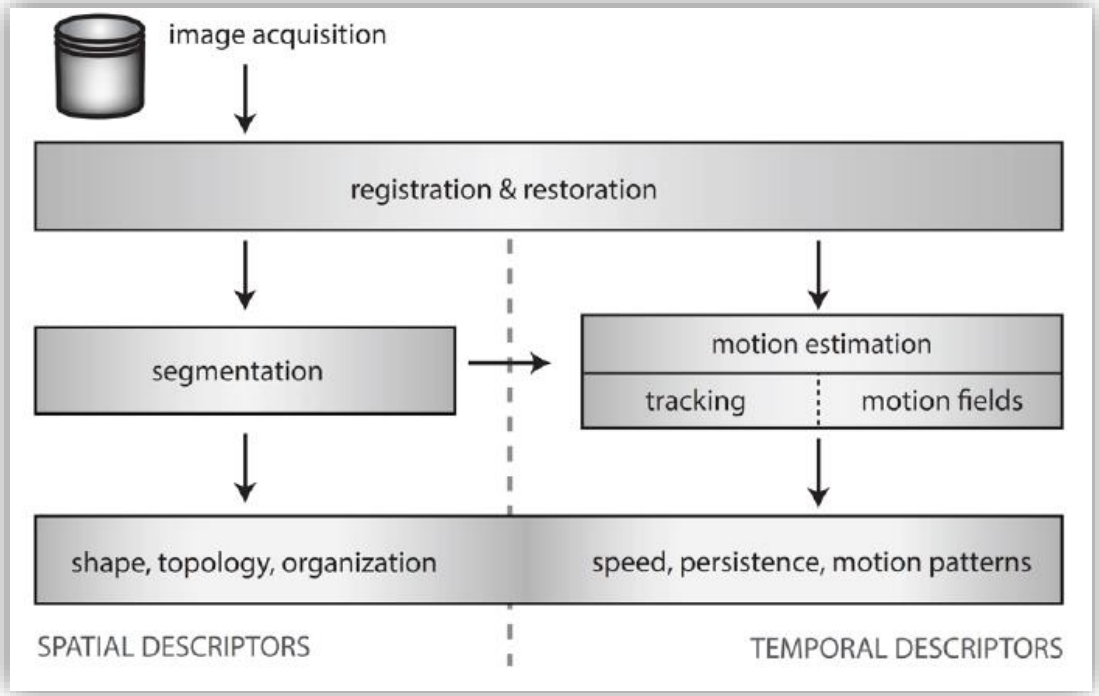
University->Faculty of Mathematics and Computer Science->HCI
University->Faculty of Physics and Astronomy->HCI
Universitv->IWR->HCI

- Computer Vision(Ommer lab)
- Digital Image Processing (Jähne lab)
- Image and Pattern Analysis (Schnörr lab)
- Image Processing and Modelling (Garbe lab)
- Multidimensional Image Processing (Hamprecht lab)

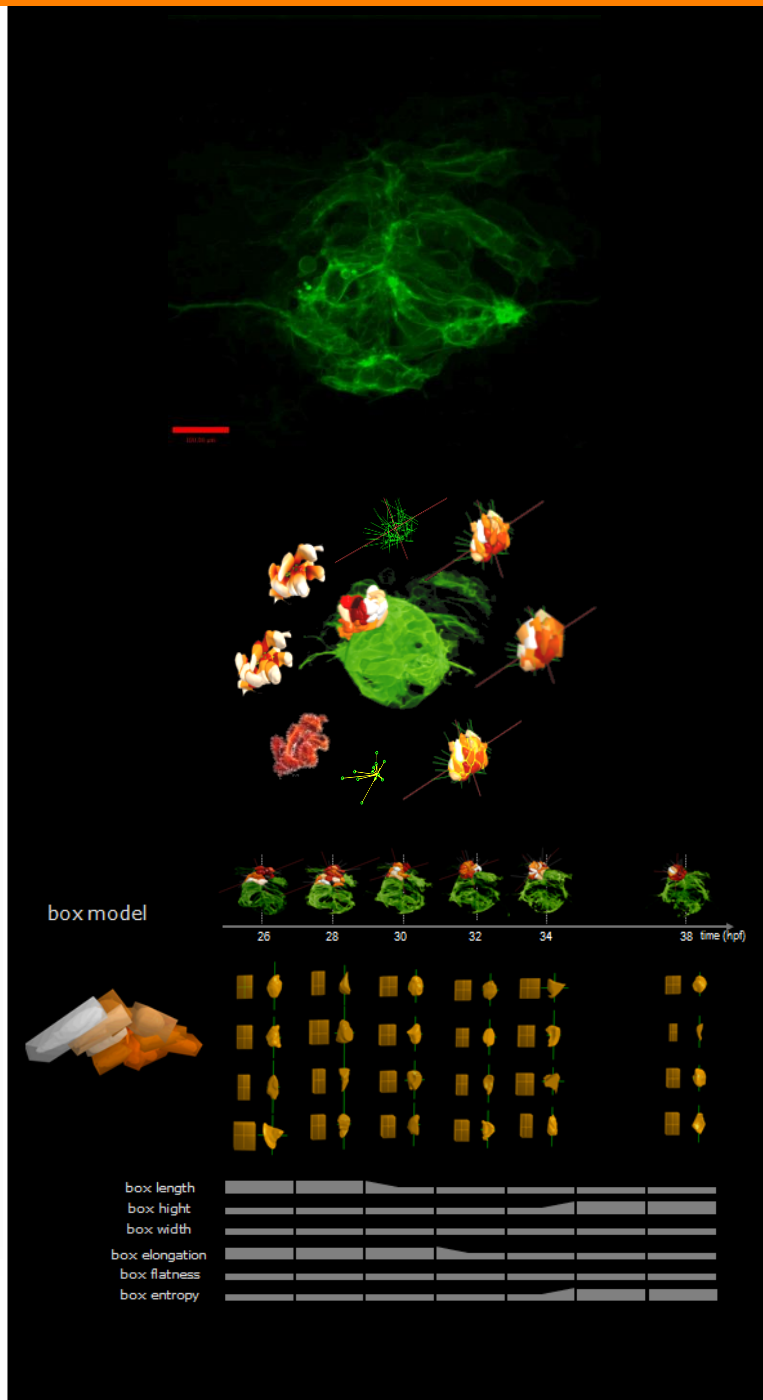


Miguel Concha
Biología del Desarrollo





Castañeda et al 2014



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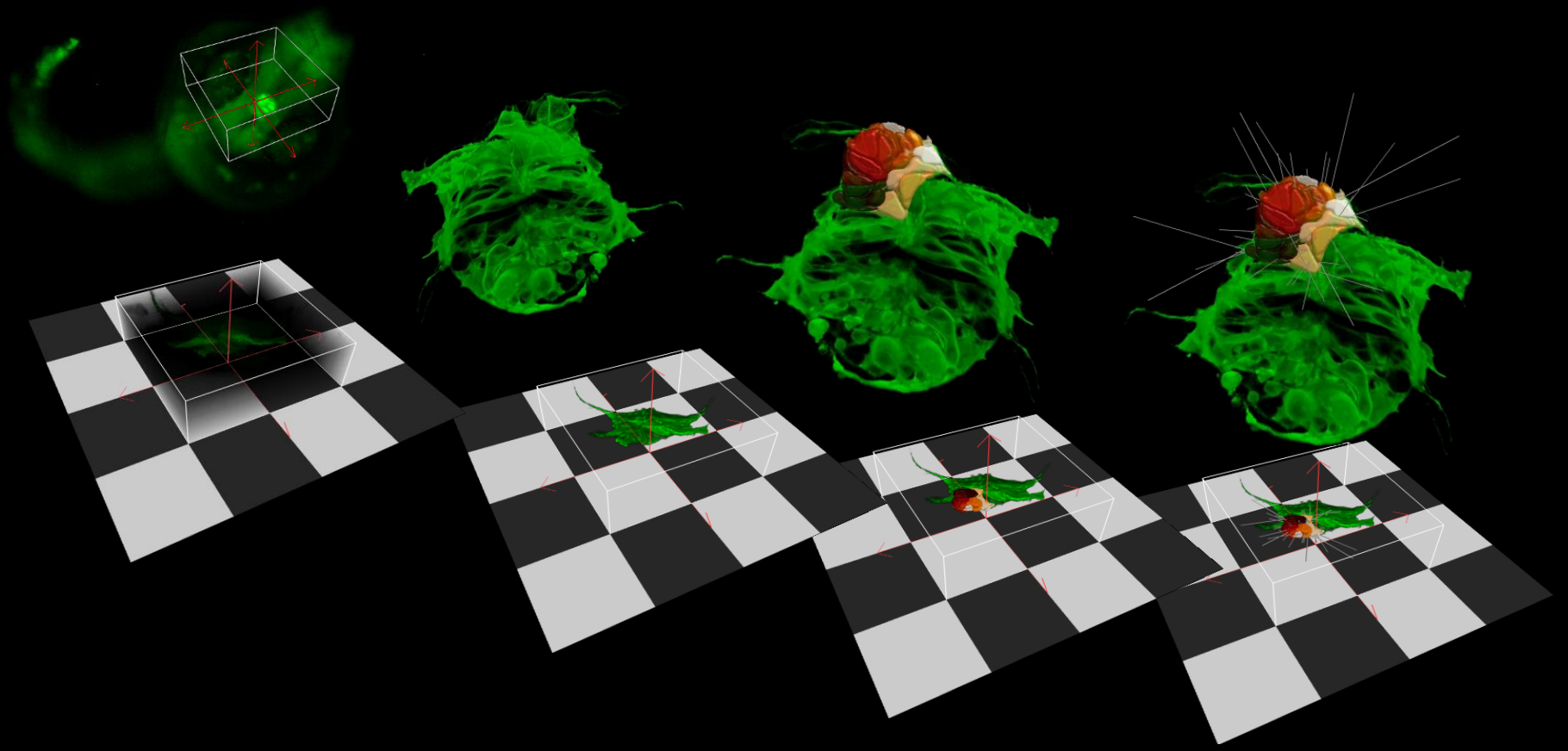
Biomedical Neuroscience Institute

Morpho-genetic mechanisms, form, function in developmental biology

Transgenic ***flh*::GFP**



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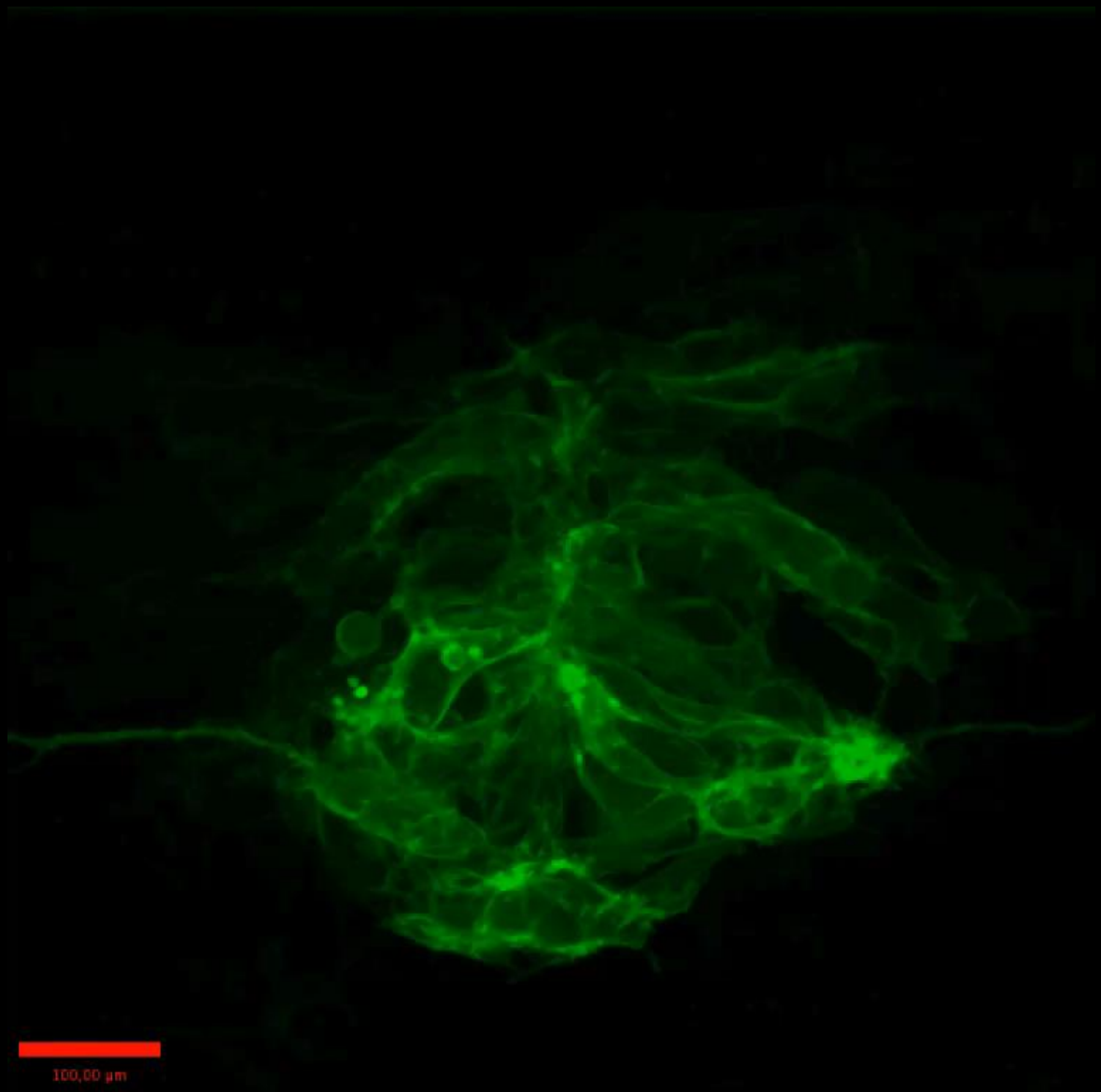

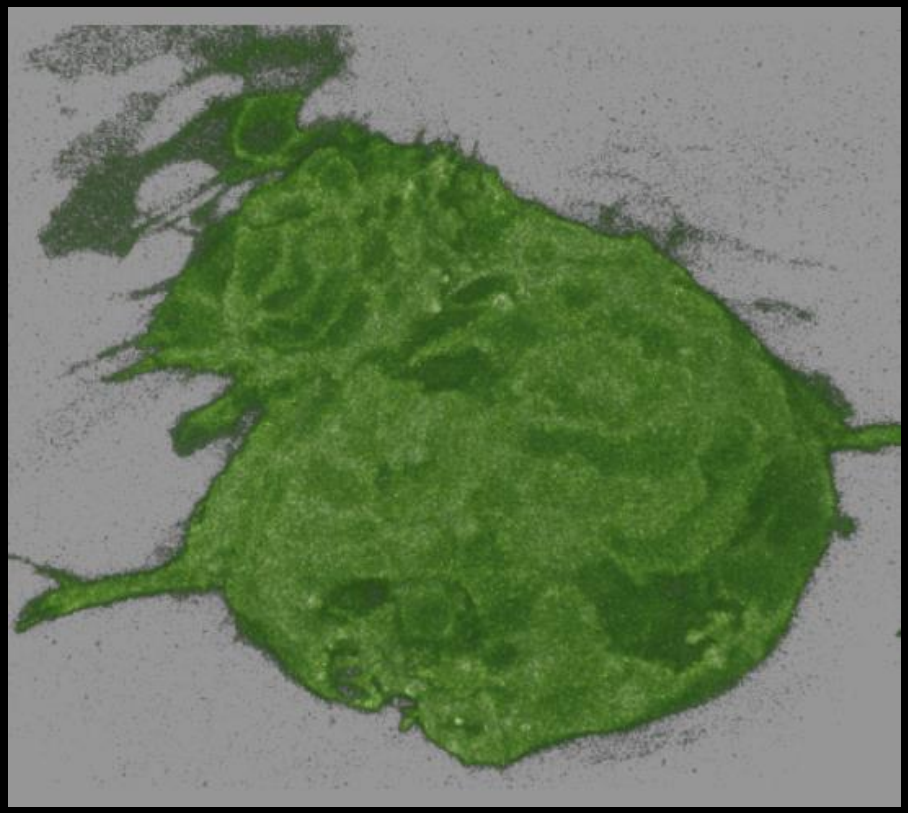

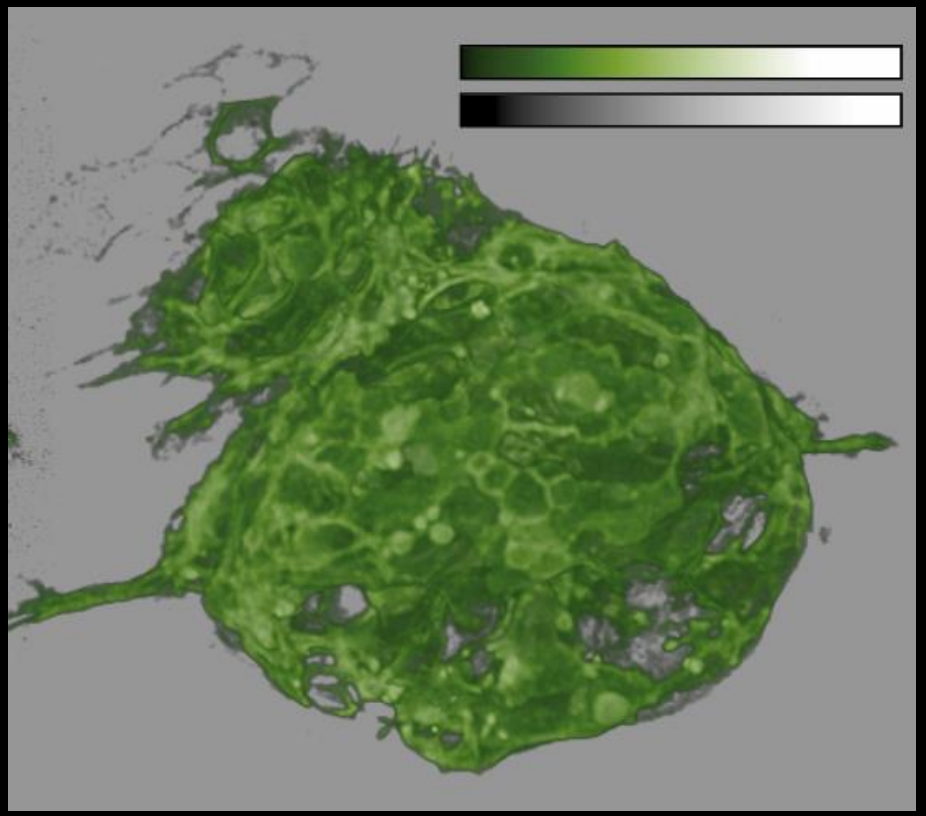

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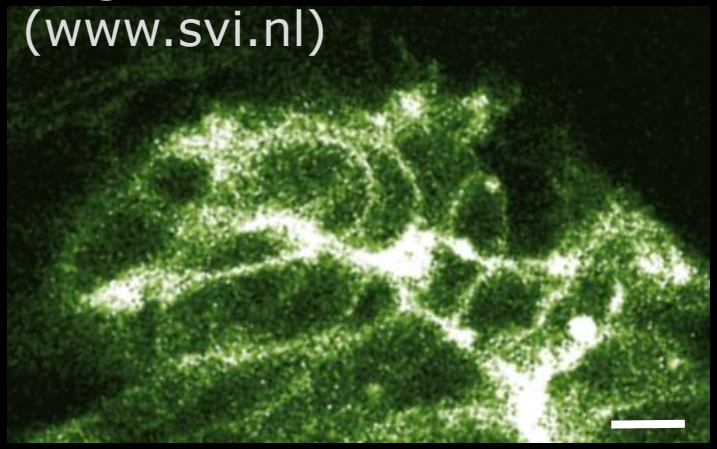
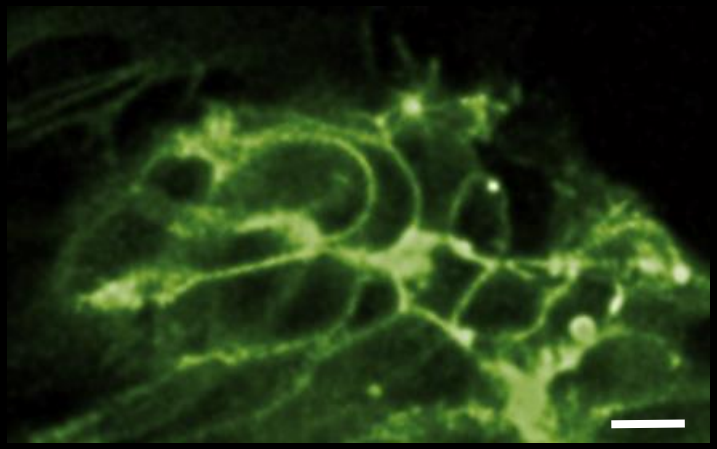
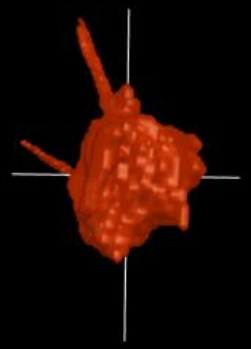
Biomedical Neuroscience Institute

Original Data (www.svi.nl)

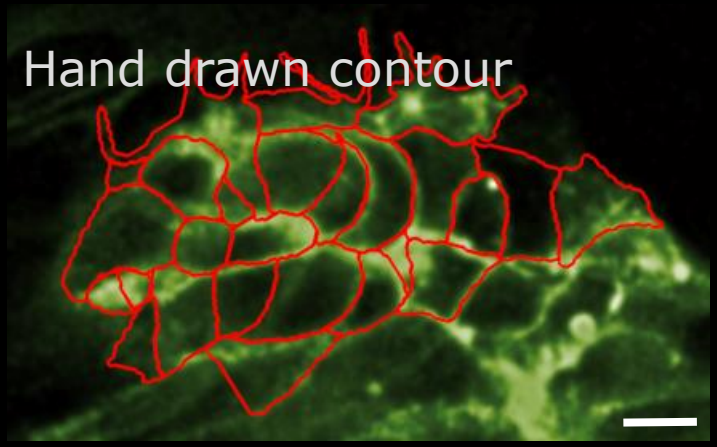


Deconvolved Data

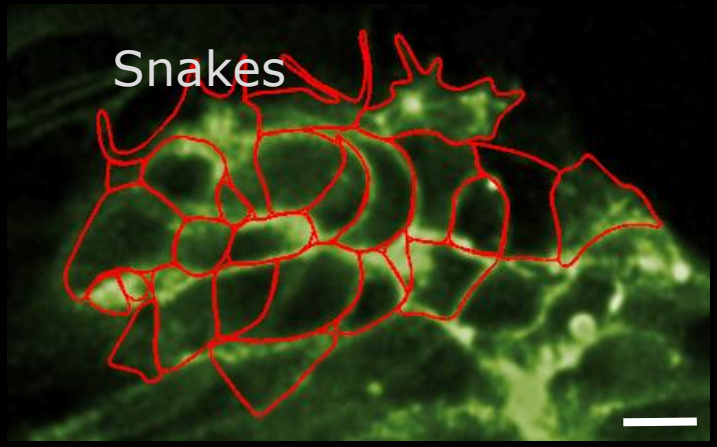


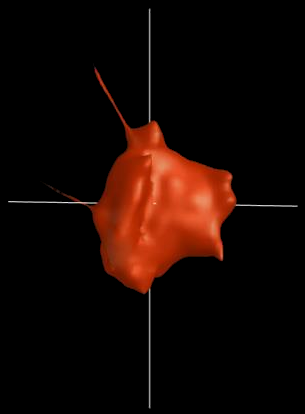


Hand drawn contour



Snakes





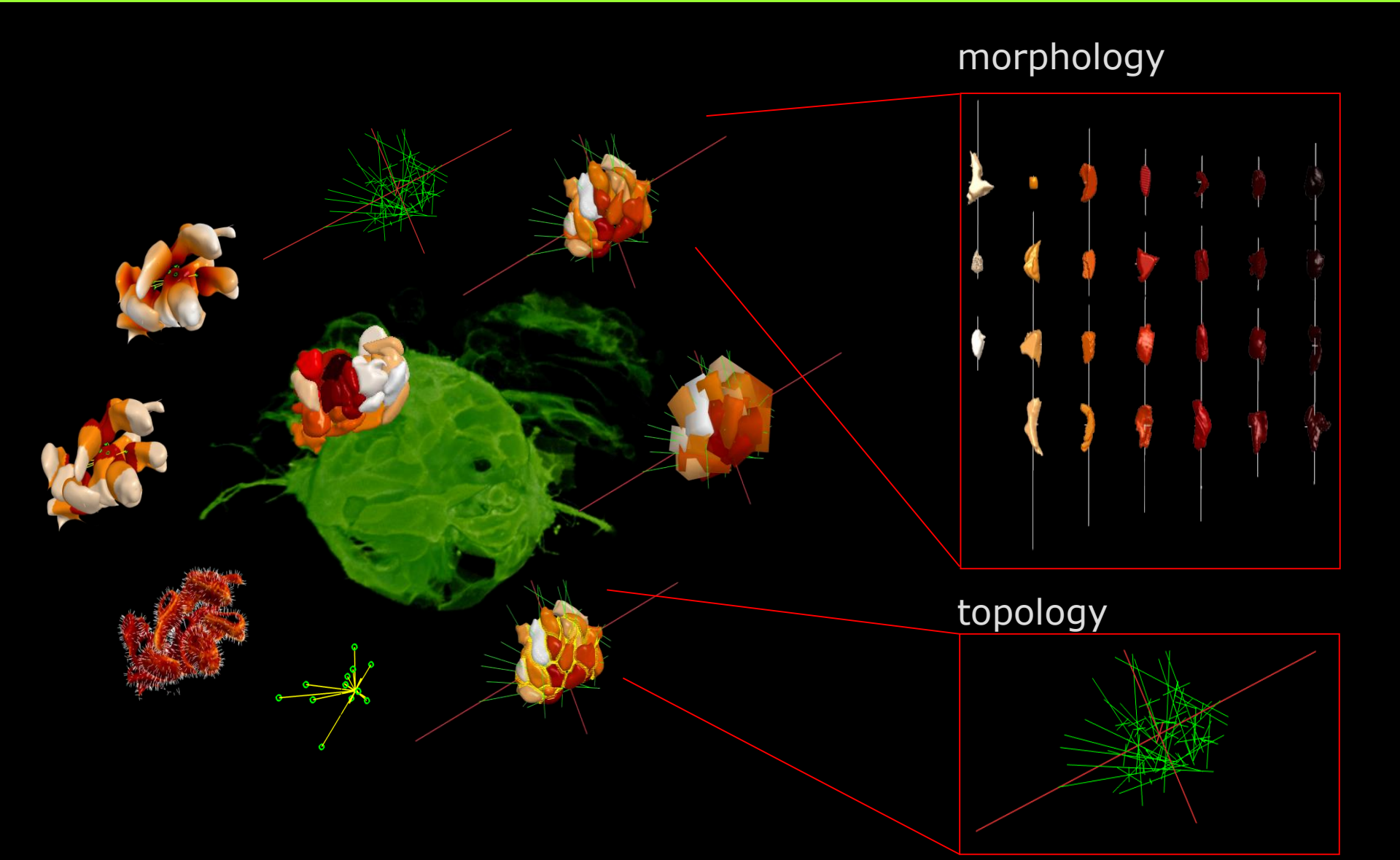
Kass M. et al (1988) Int. J. Comp. Vis. 1:321-331.
Xu & Prince (1998) Signal Processing 71: 131-139.

2D

3D

Laboratory for SCIAN Scientific Image Analysis

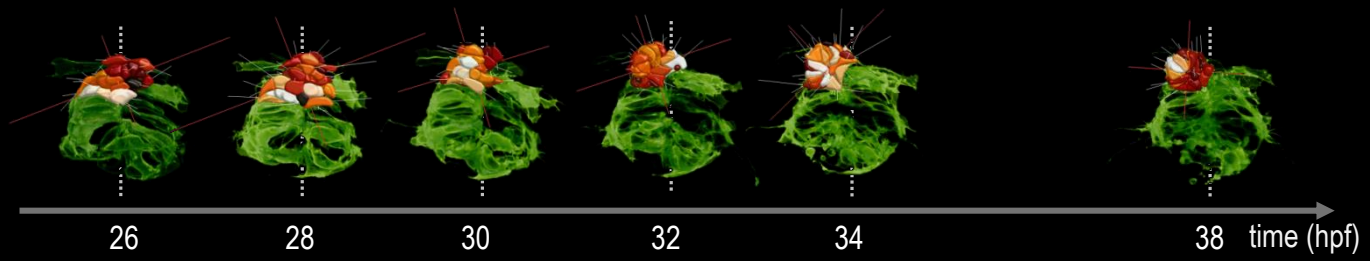
Biomedical Neuroscience Institute

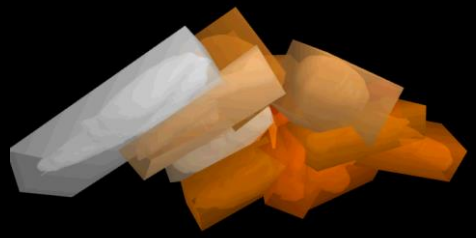


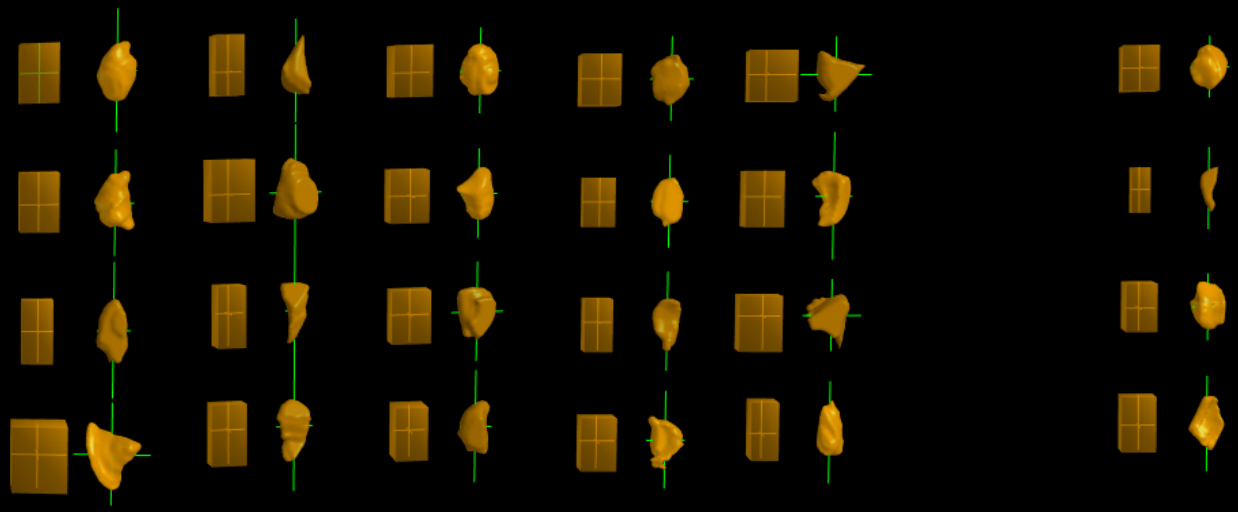
Laboratory for SCIAN Scientific Image Analysis

BIOMEDICAL NEUROSCIENCE INSTITUTE

box model







box length

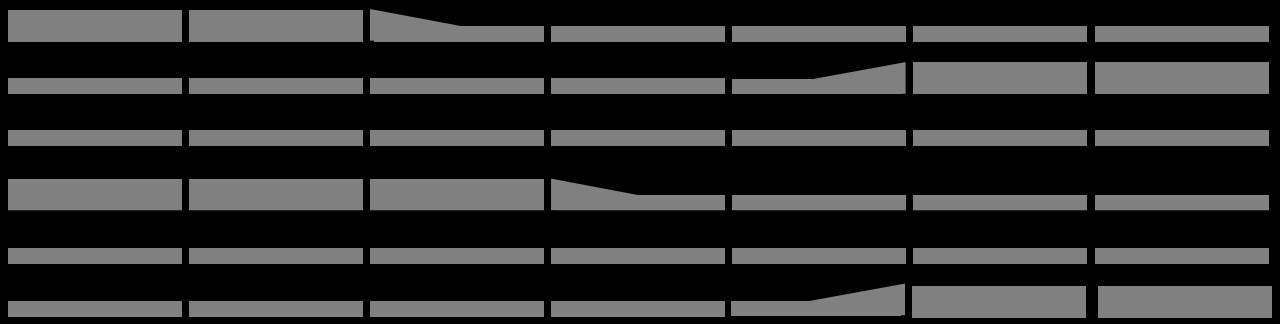
box hight

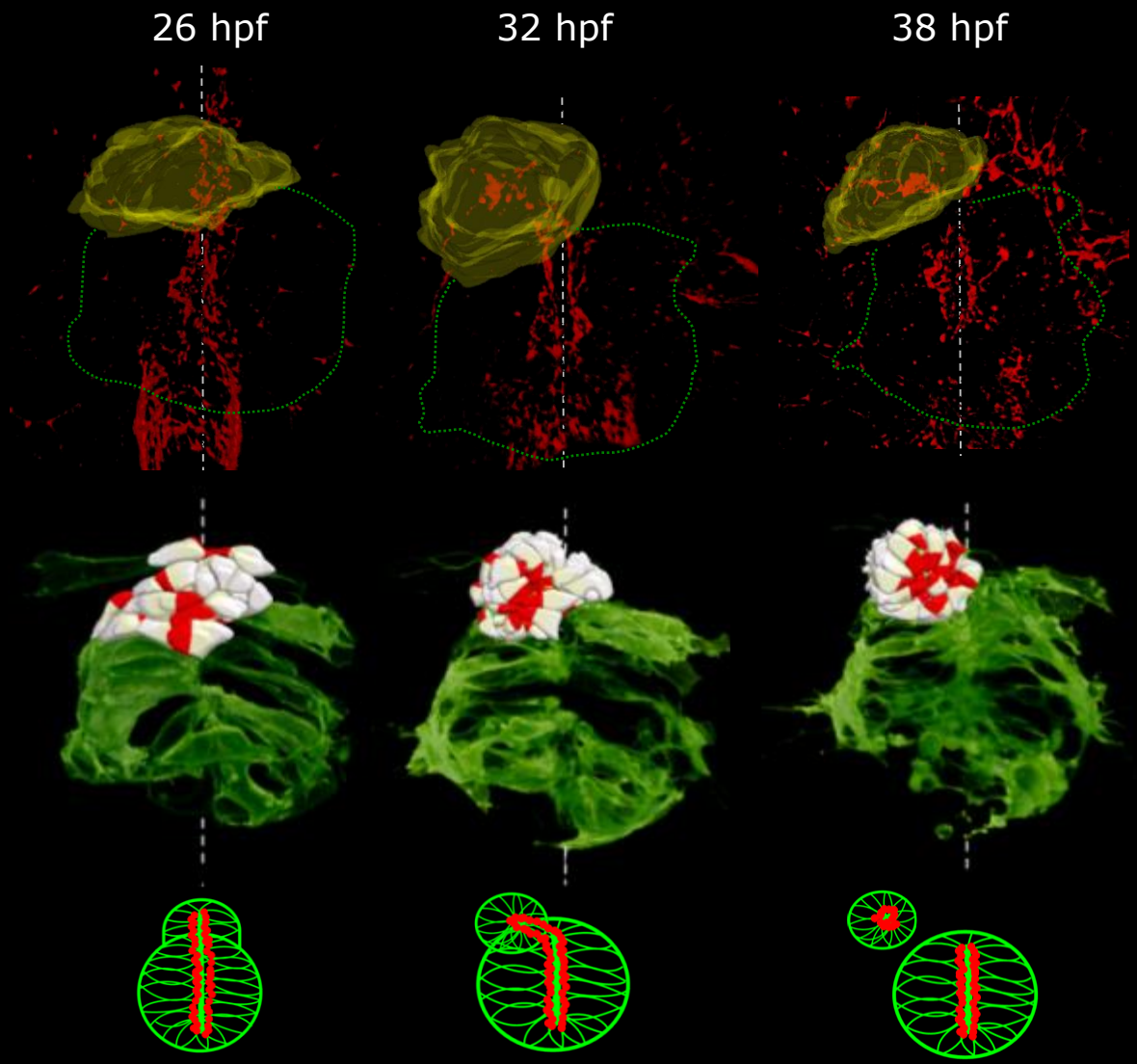
box width

box elongation

box flatness

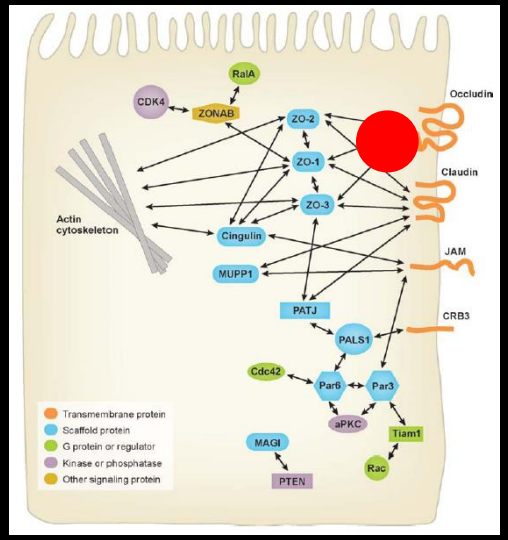
box entropy





ZO-1

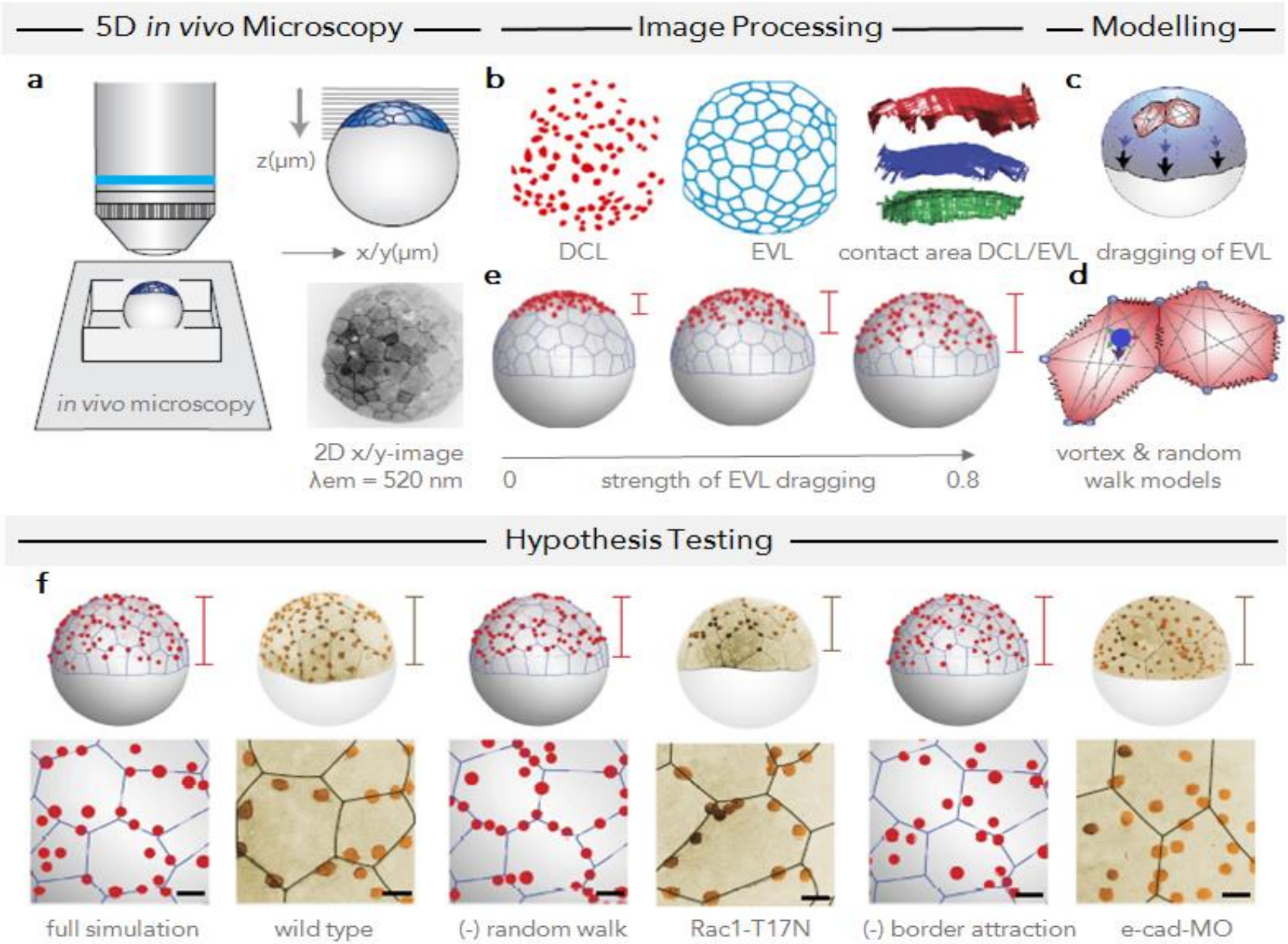
apical



basolateral

Similar for α-tubulin,
γ-tubulin,
Phalloidin,

nature COMMUNICATIONS



Scientific Background on the Nobel Prize in Chemistry 2014

SUPER-RESOLVED FLUORESCENCE MICROSCOPY

Photo: Matt Staley/HHMI

Eric Betzig

Prize share: 1/3

© Bernd Schuller, Max-Planck-Institut

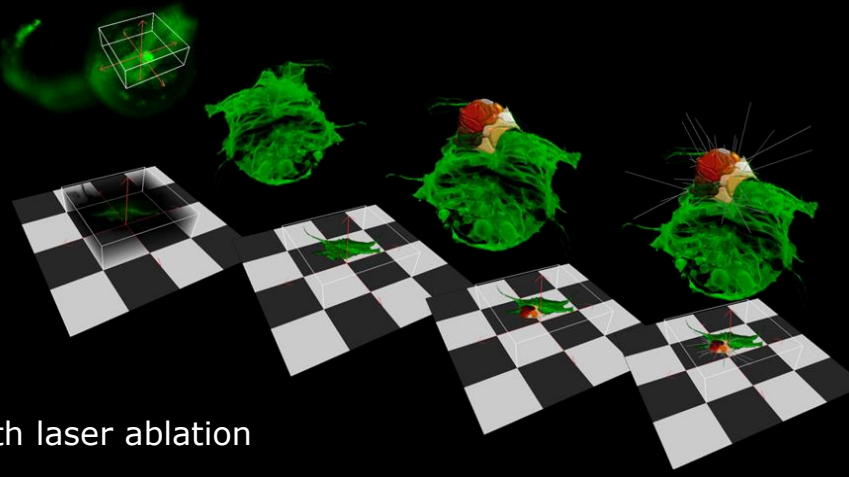
Stefan W. Hell

Prize share: 1/3

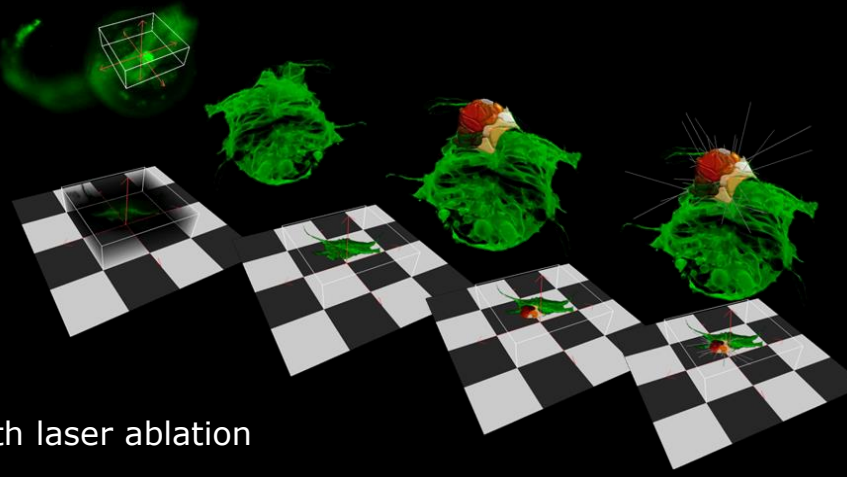
Photo: K. Lowder via Wikimedia Commons, CC-BY-SA-3.0

William E. Moerner

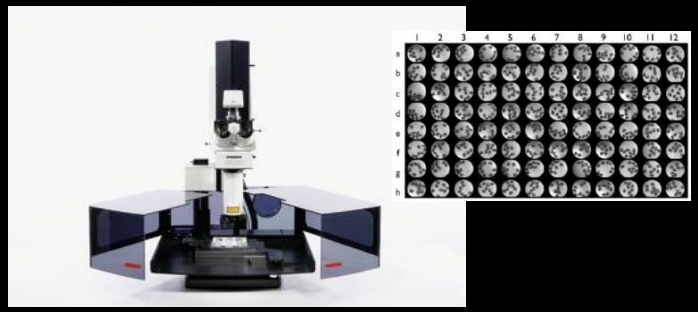
Prize share: 1/3

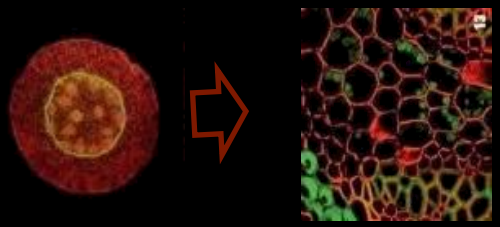


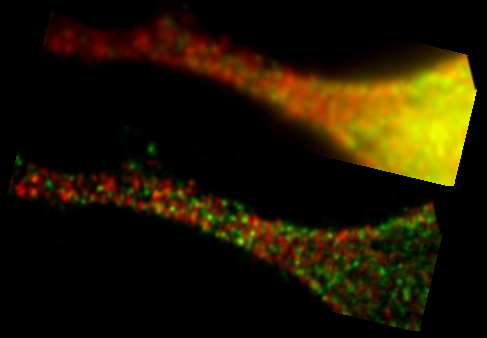
Scian/Leo-Lab: Perkin Elmer Spinning Disk with laser ablation



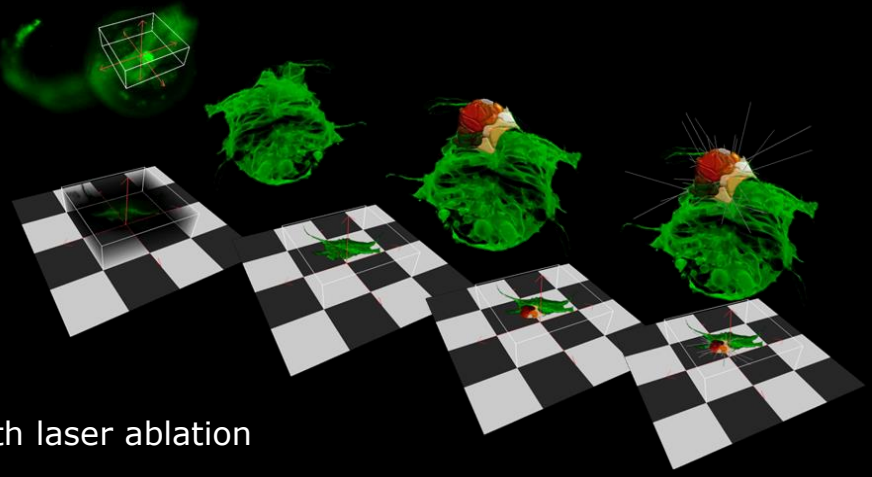
Scian/Leo-Lab: Perkin Elmer Spinning Disk with laser ablation



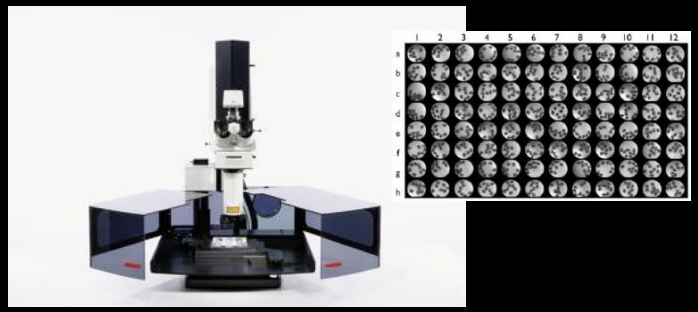


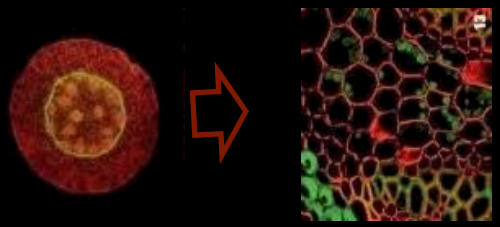


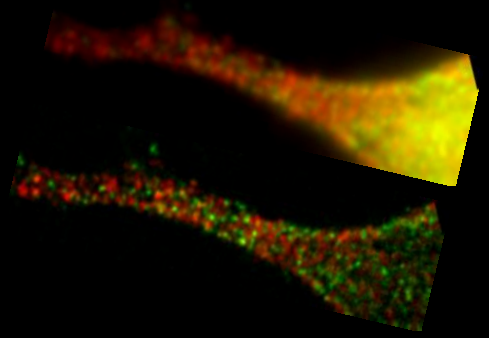
SCIAN-Lab Leica TCS LSI Super Zoom Spectral Confocal + Superresolution Optical Fluctuation Imaging SOFI



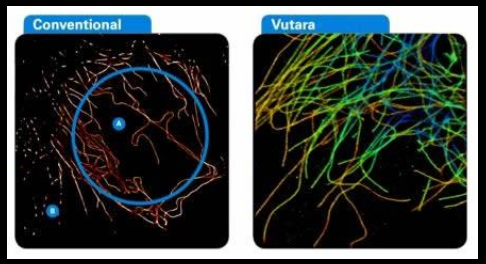
Scian/Leo-Lab: Perkin Elmer Spinning Disk with laser ablation

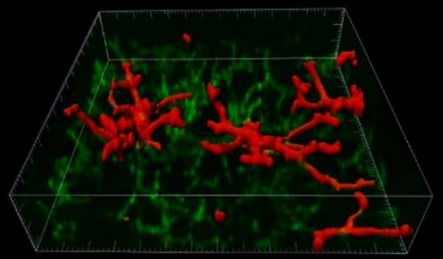




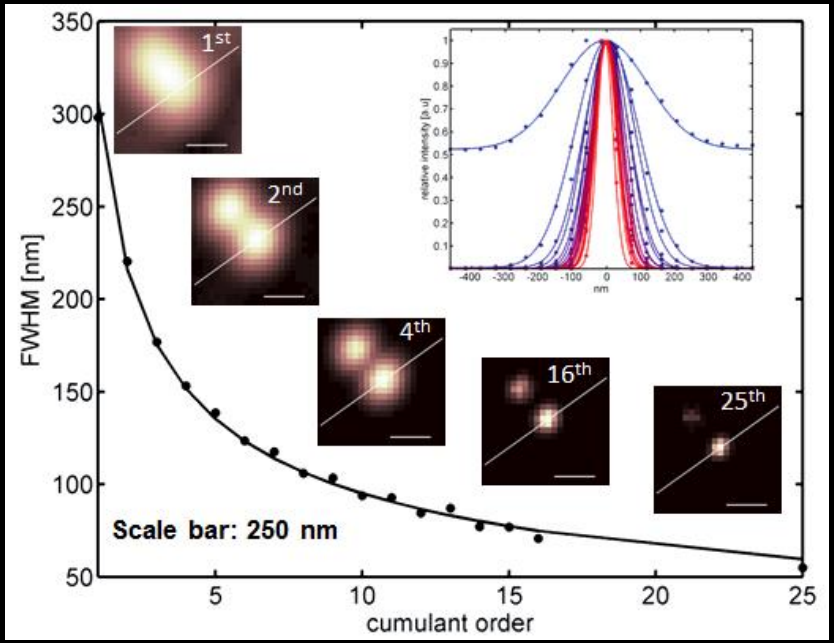


SCIAN-Lab Leica TCS LSI Super Zoom Spectral Confocal + Superresolution Optical Fluctuation Imaging SOFI

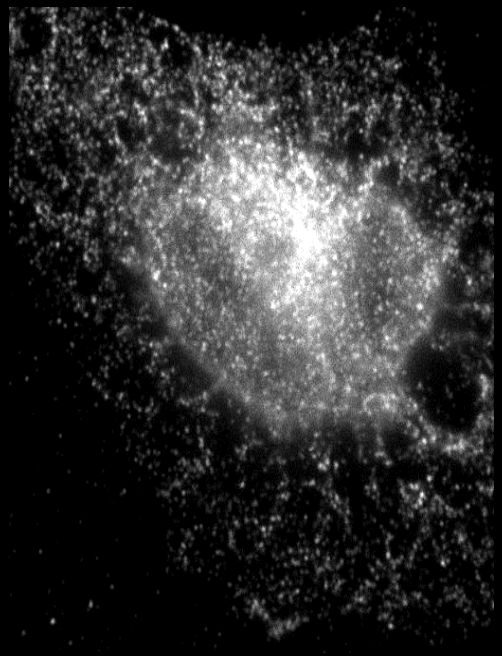


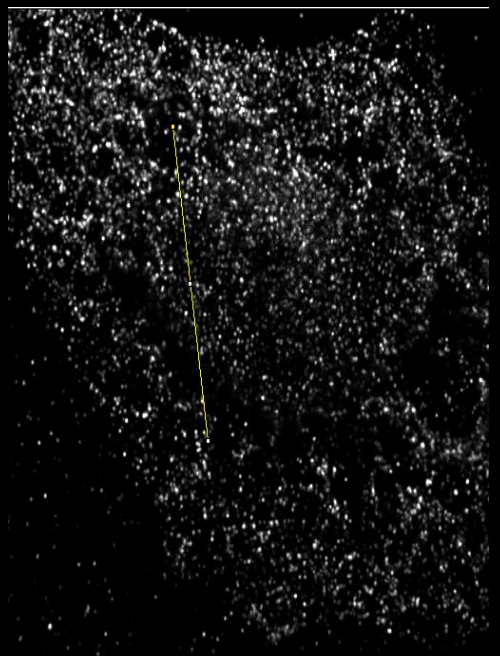


Couve-Lab PALM Vtarar 200, Bliplane 3D



Dertinger et al 2009 PNAS



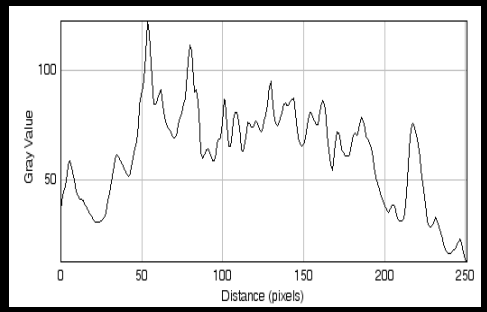


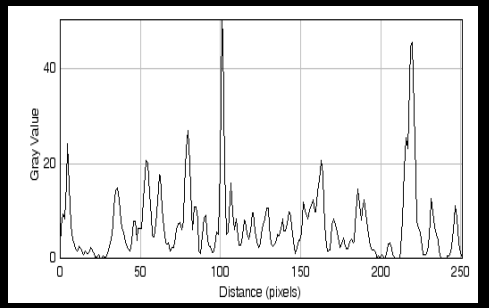
COS7 myc-GABABR1 conjugados con QDots 525 nm

Jörg Enderlein
Gôttingen

Mayor Challenges:

- vibrations
- computational backbone
- sustainability



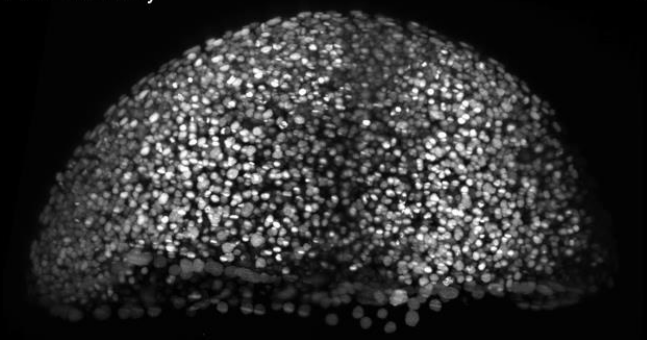


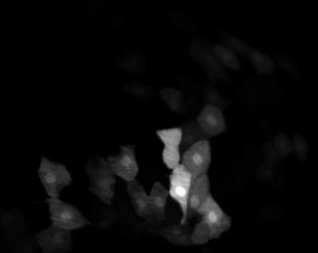
H2B-mCherry

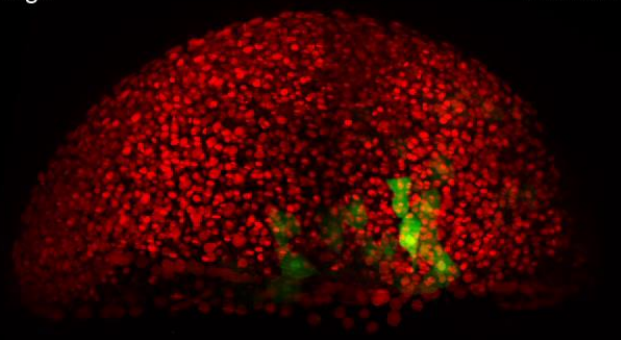
crestin::GFP

merge

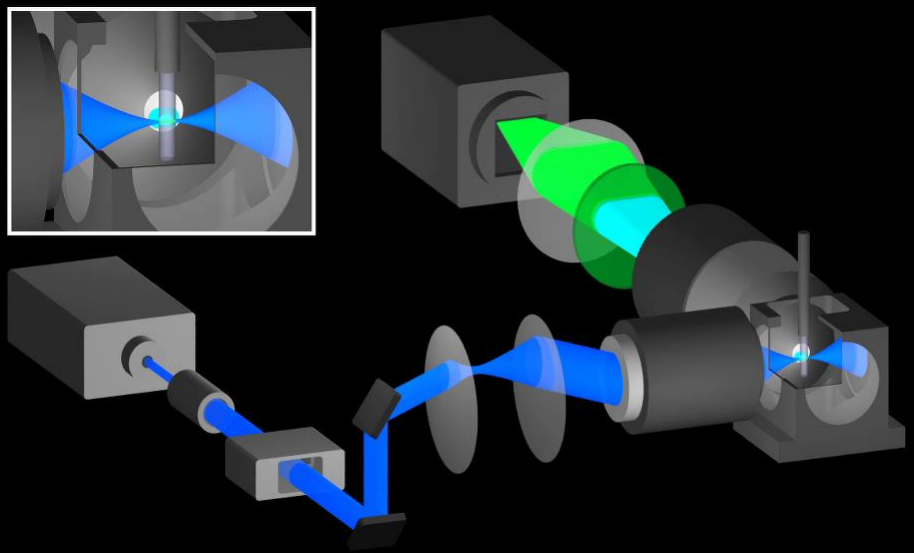
00:00:00







Pulgar, Keller, Concha, unpublished



Ulrich Kubitscheck
Bonn

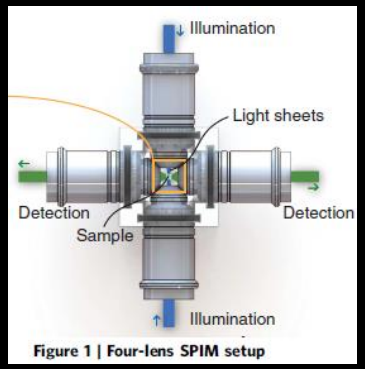
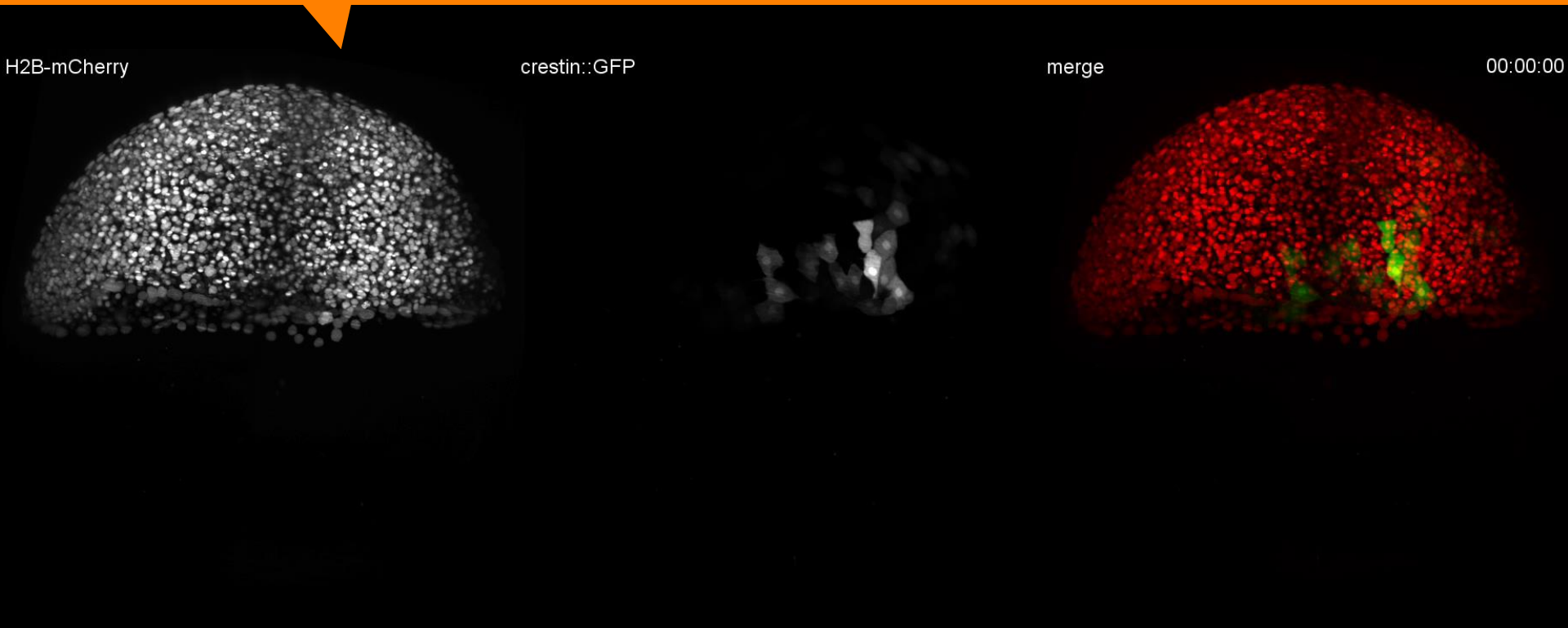
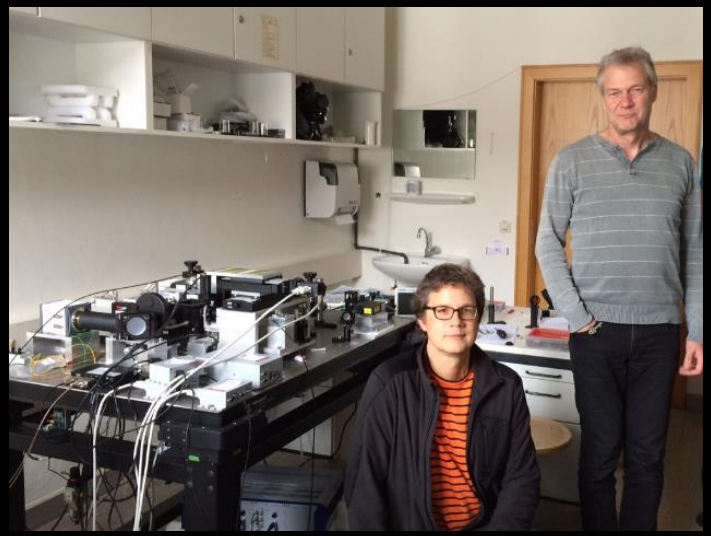


Figure 1 | Four-lens SPIM setup

Jan Huisken
Dresden



Pulgar, Keller, Concha, unpublished



Hetz-Lab: Drug Discovery

Natural Chilean compounds

Preclinical models

SCIAN-Lab: Digital Pathology

High Speed Whole Slide Imaging

Digital Patholoy

Quantitative Microscopy

Medical Education & Outreach

BNI
BIOMEDICAL NEUROSCIENCE INSTITUTE
CHILE

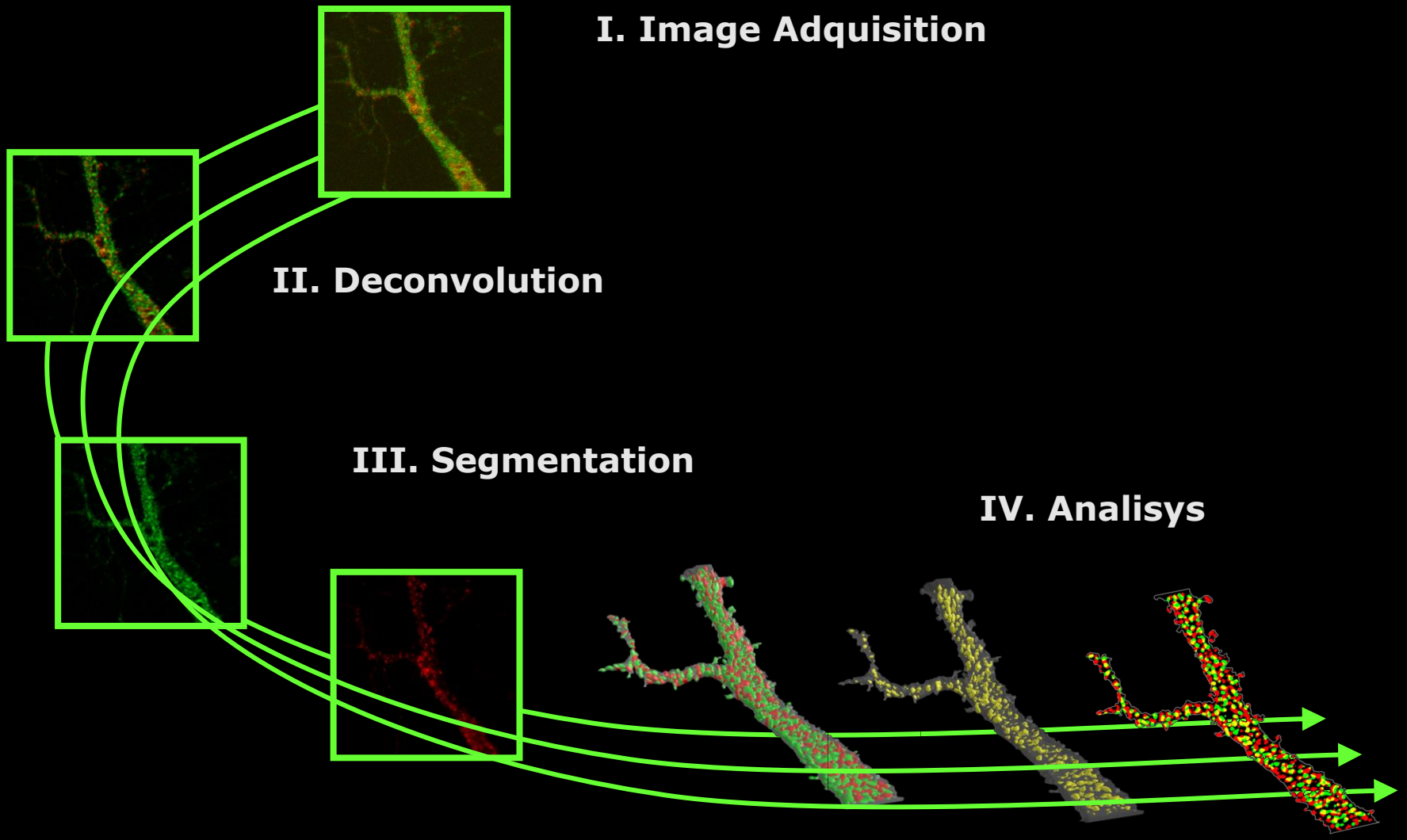
R&D: Applied Science

Impact on Health System

Impact on Public Opinion

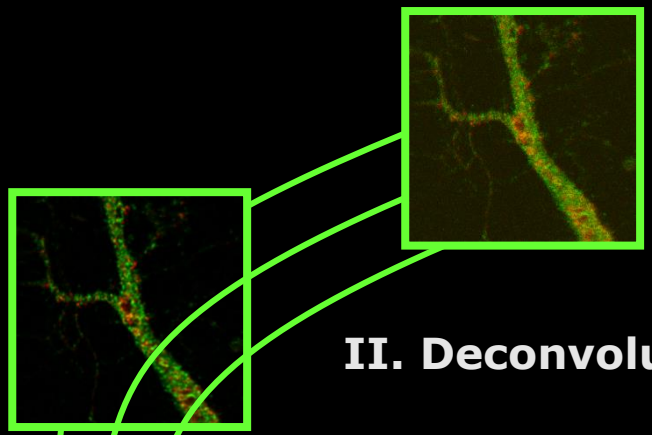
Laboratory for SCIAN Scientific Image Analysis

BIOMEDICAL NEUROSCIENCE INSTITUTE



Laboratory for SCIAN Scientific Image Analysis

BIOMEDICAL NEUROSCIENCE INSTITUTE



I. Image Adquisition

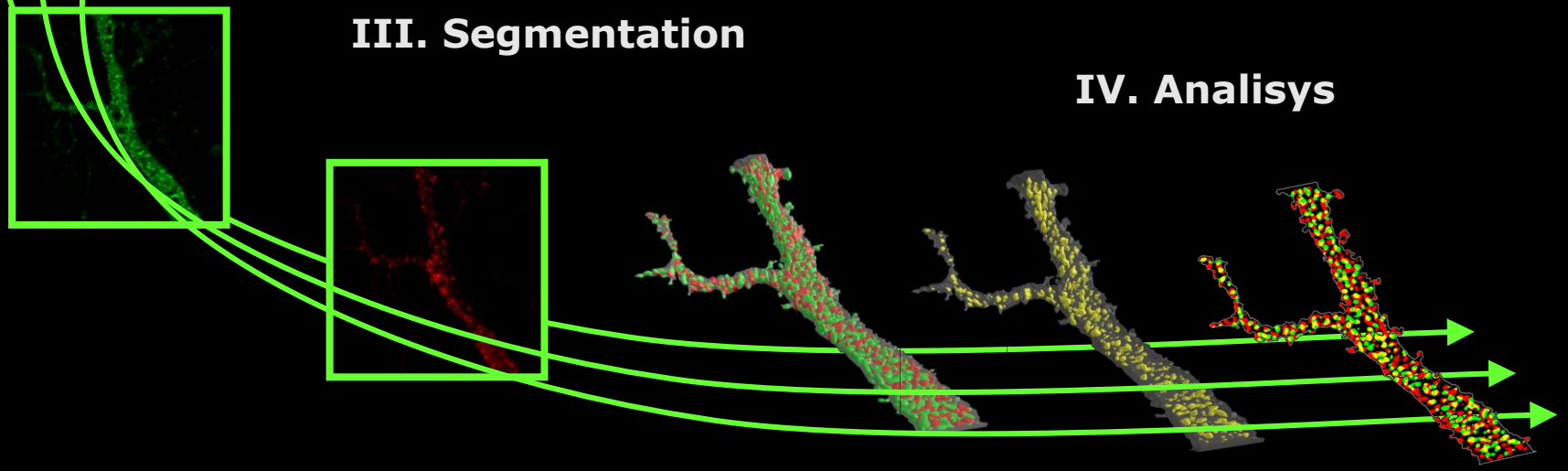
I.a|-> Fundamentos de la microscopía confocal

I.b|-> Fundamentos de la fluorescencia

II. Deconvolution

III. Segmentation

IV. Analisys



"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."

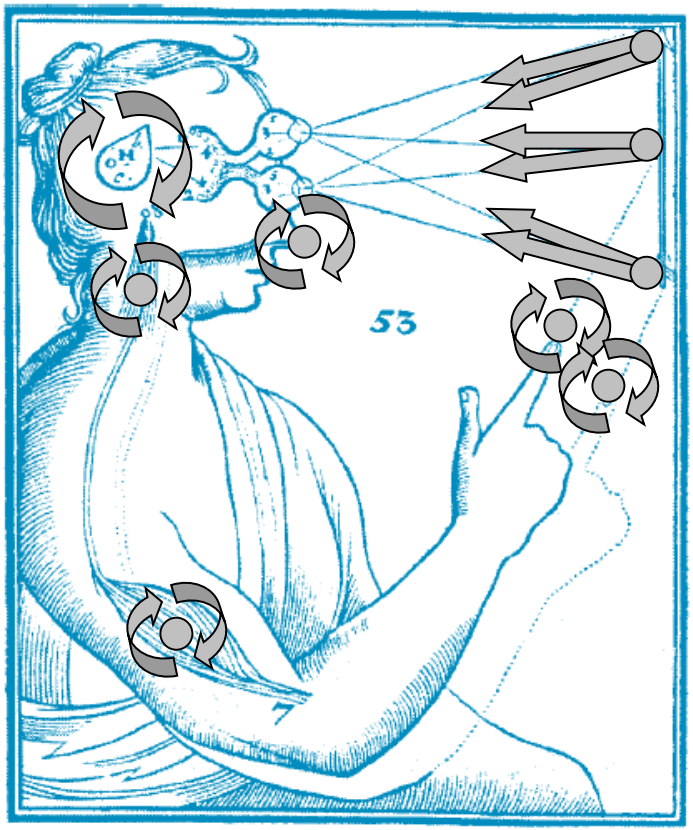
Richard Feynman (1918-1988)

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 ...

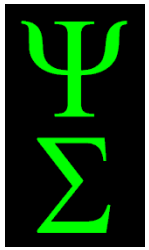
1| direct signals ...

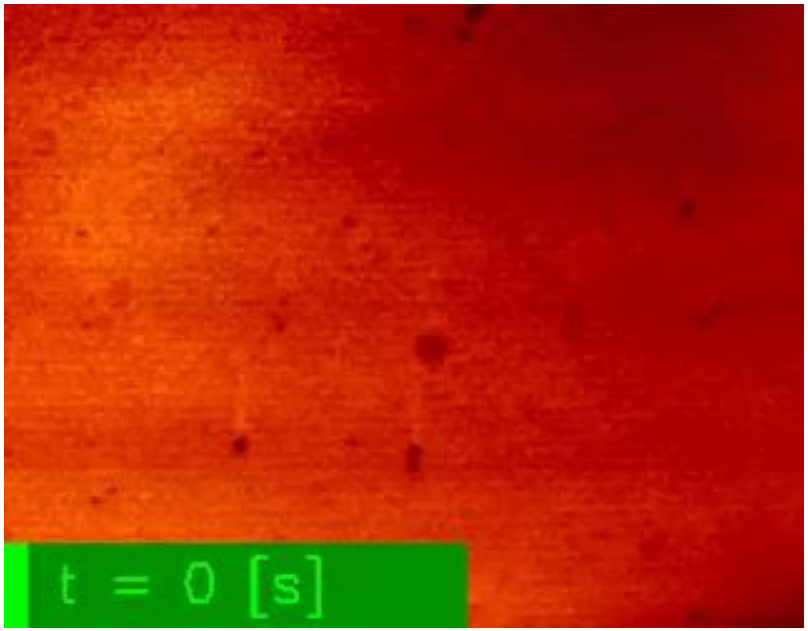
2| signals from other senses ...

3| feedback loops ...

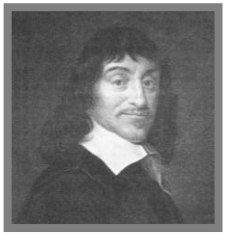
... produce a symbolic representation of an object.

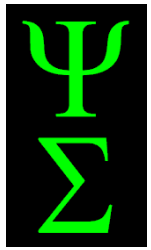
| Best resolution in t & x …

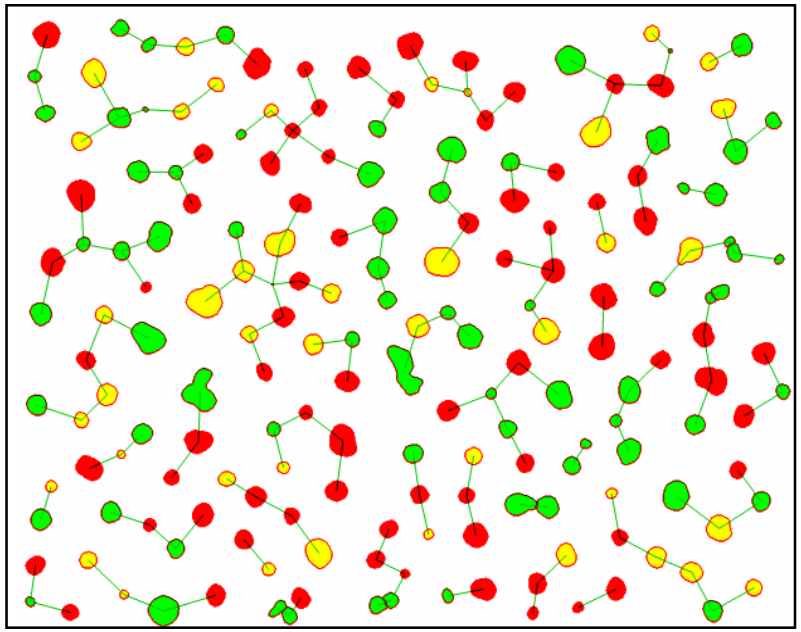


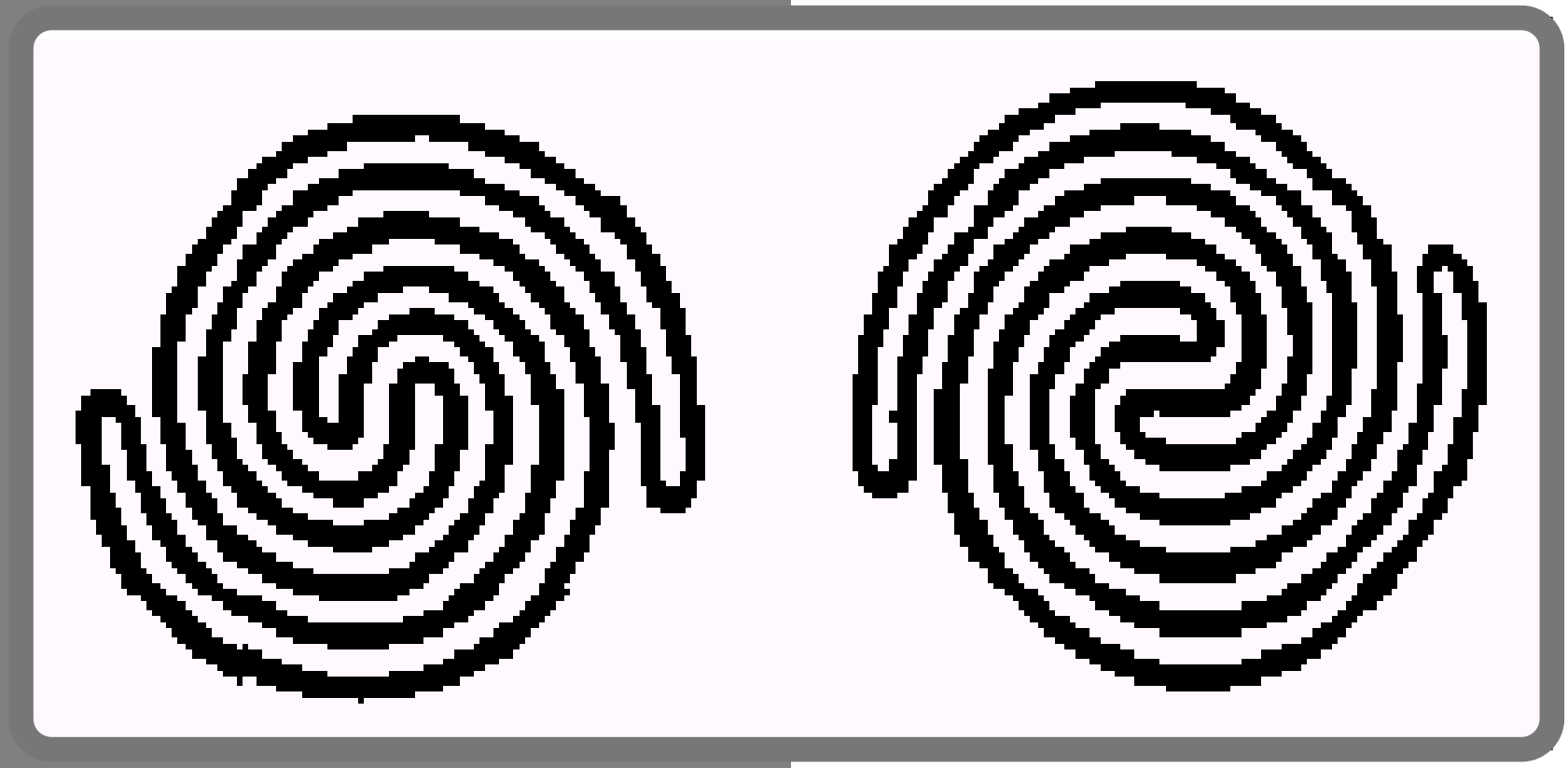


|| Humas vs machine vision





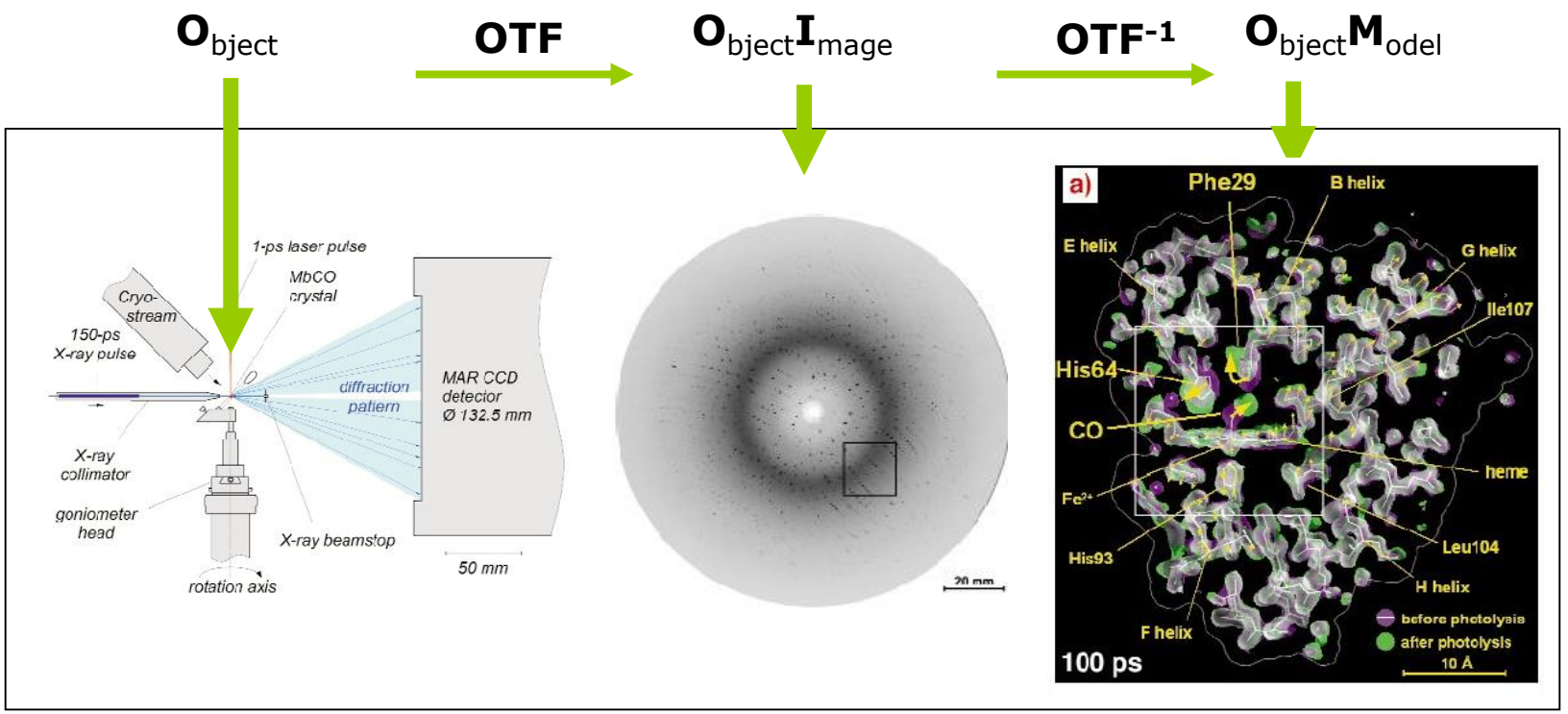




OTF: Object/Optical Transfer Function

Myoglobin in Action |Picosecond Laue Crystallography Diffraction Data

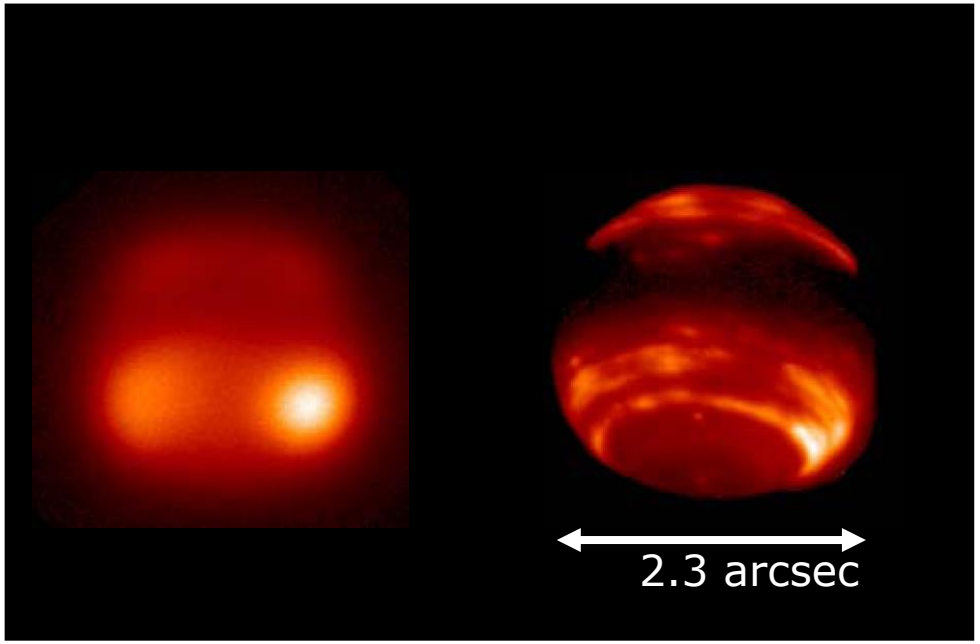
Schotte et al (2003) Science



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

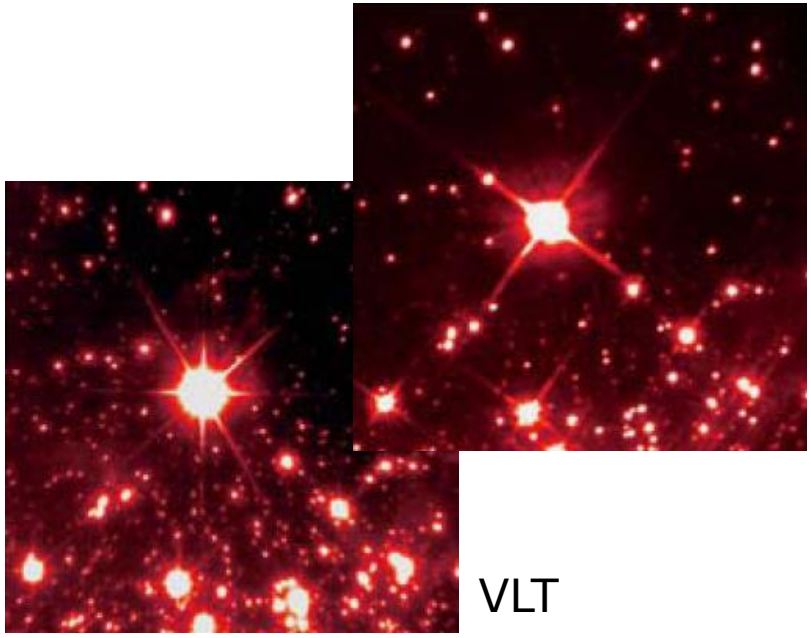
Diffraction Limited Resulution for a 10m telescope ~ λ/D ~ 0.01 arcsec
is limited to ~ 0.5 arcsec by the turbulent atmosphere.
NAOS creates an artificial star at 90 km altitude in the Earth´s mesosphere.
The Laser Guide Star is used to correct atmospheric effects

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



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

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



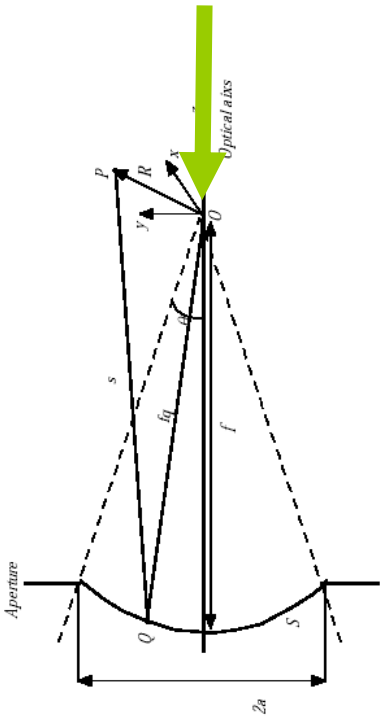
Hubble

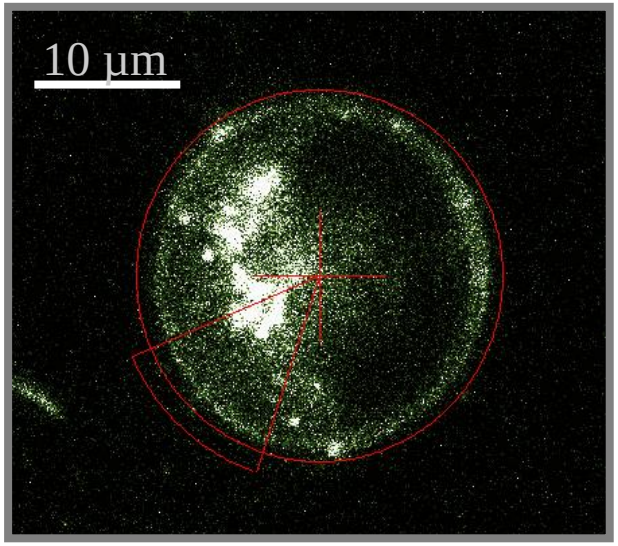
VLT

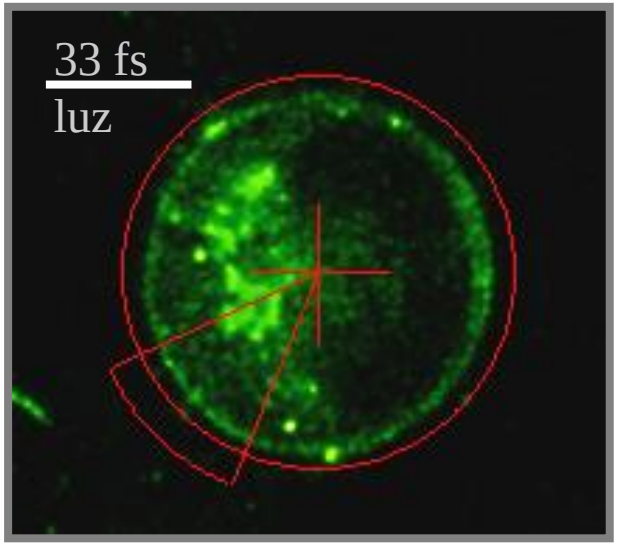
Confocal Microscopy | From Geometric Optics to Diffraction Theory

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

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

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The Journal of Cell Biology, Vol. 172, No. 1, January 2, 2006 9–18
http://www.jcb.org/cgi/doi/10.1083/jcb.200507102

Supplemental Material can be found at:
http://jcb.rupress.org/cgi/content/full/jcb.200507102/DC1

JCB ■

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.

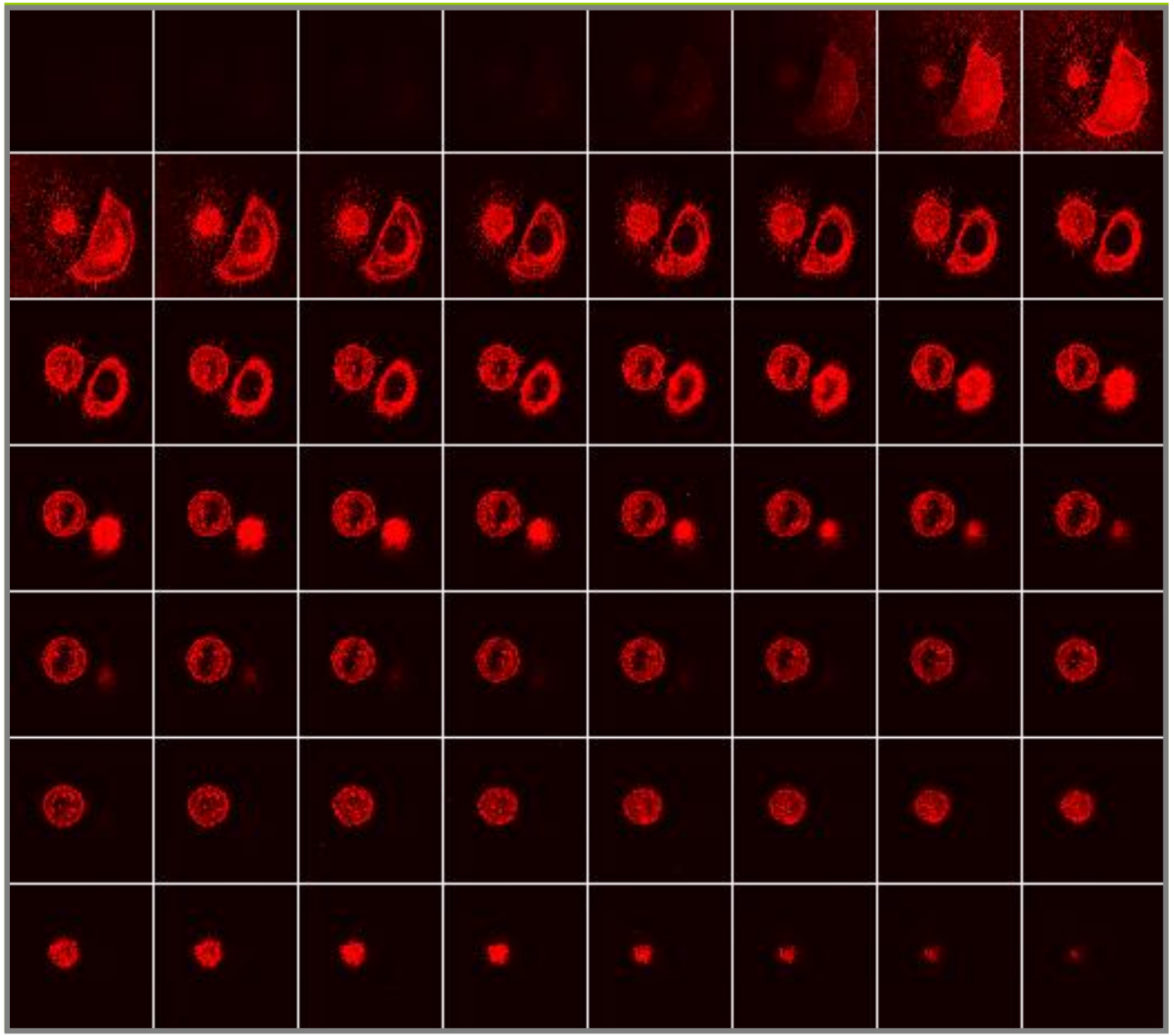
|-> Microscopy

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

Laboratory for SCIAN Scientific Image Analysis

|-> Microscopy

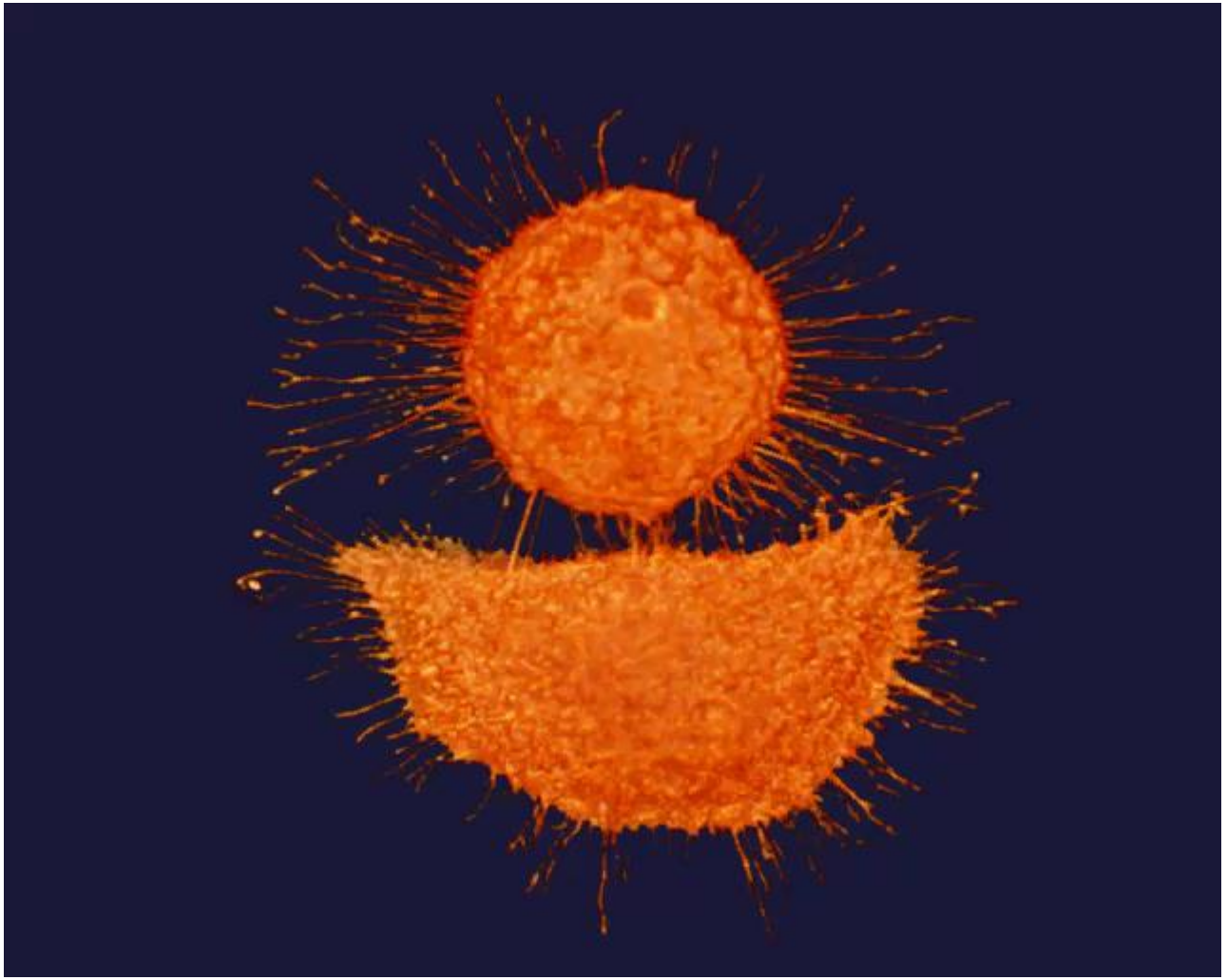
BIOMEDICAL NEUROSCIENCE INSTITUTE



Laboratory for SCIAN Scientific Image Analysis

|-> Microscopy

BIOMEDICAL NEUROSCIENCE INSTITUTE



|-> Fluorescencia I

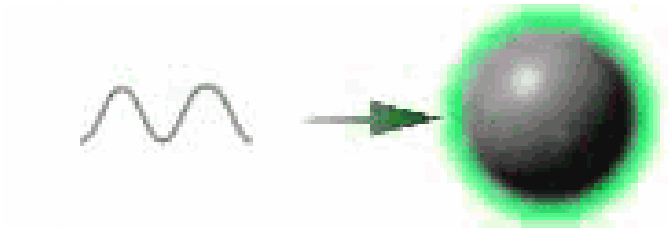
Luminescencia:

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

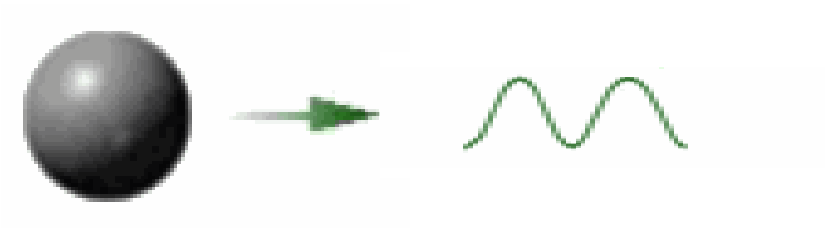
Interacciones ...
- **intra- e inter moleculares ...**

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

← Δt →

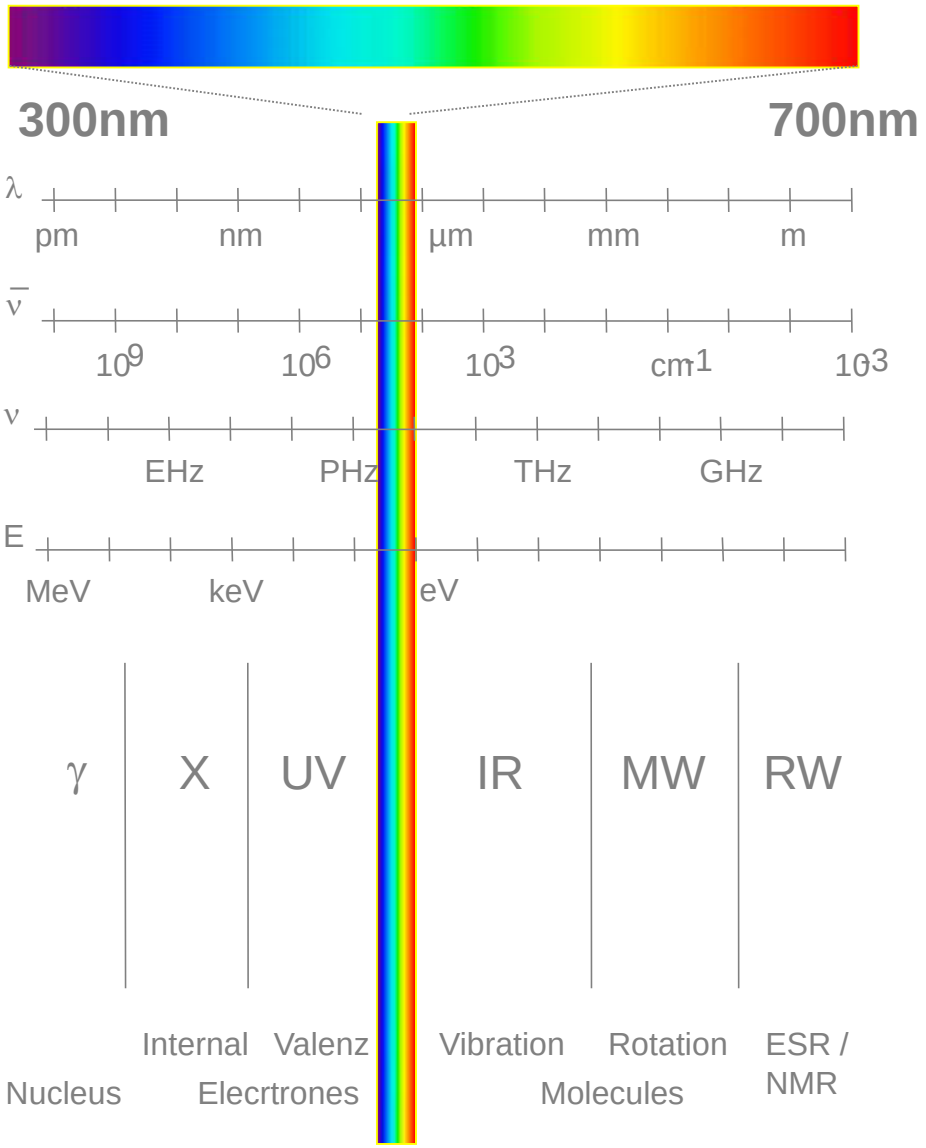


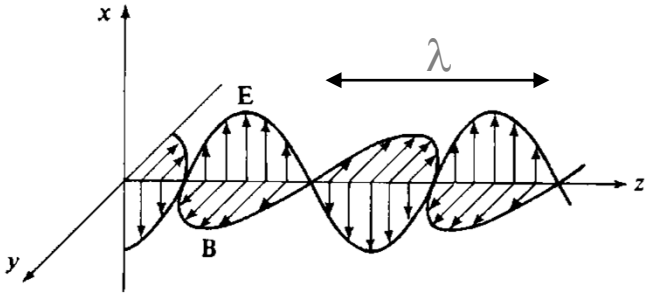
Absorción / Excitación



Emissión

|-> Fluorescencia II





Energía de un fotón: (~1-5eV)

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

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:

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

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

|-> Jablonski Diagram

Transiciones: → con radiación

~ sin radiación |

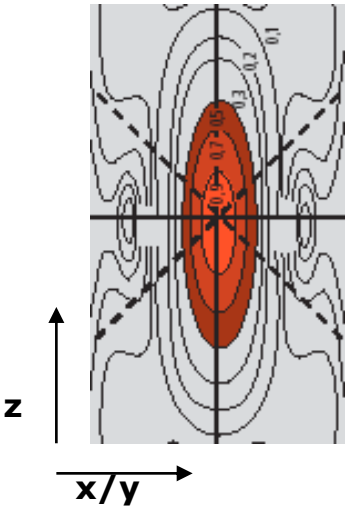
Niveles: —— vibracionales

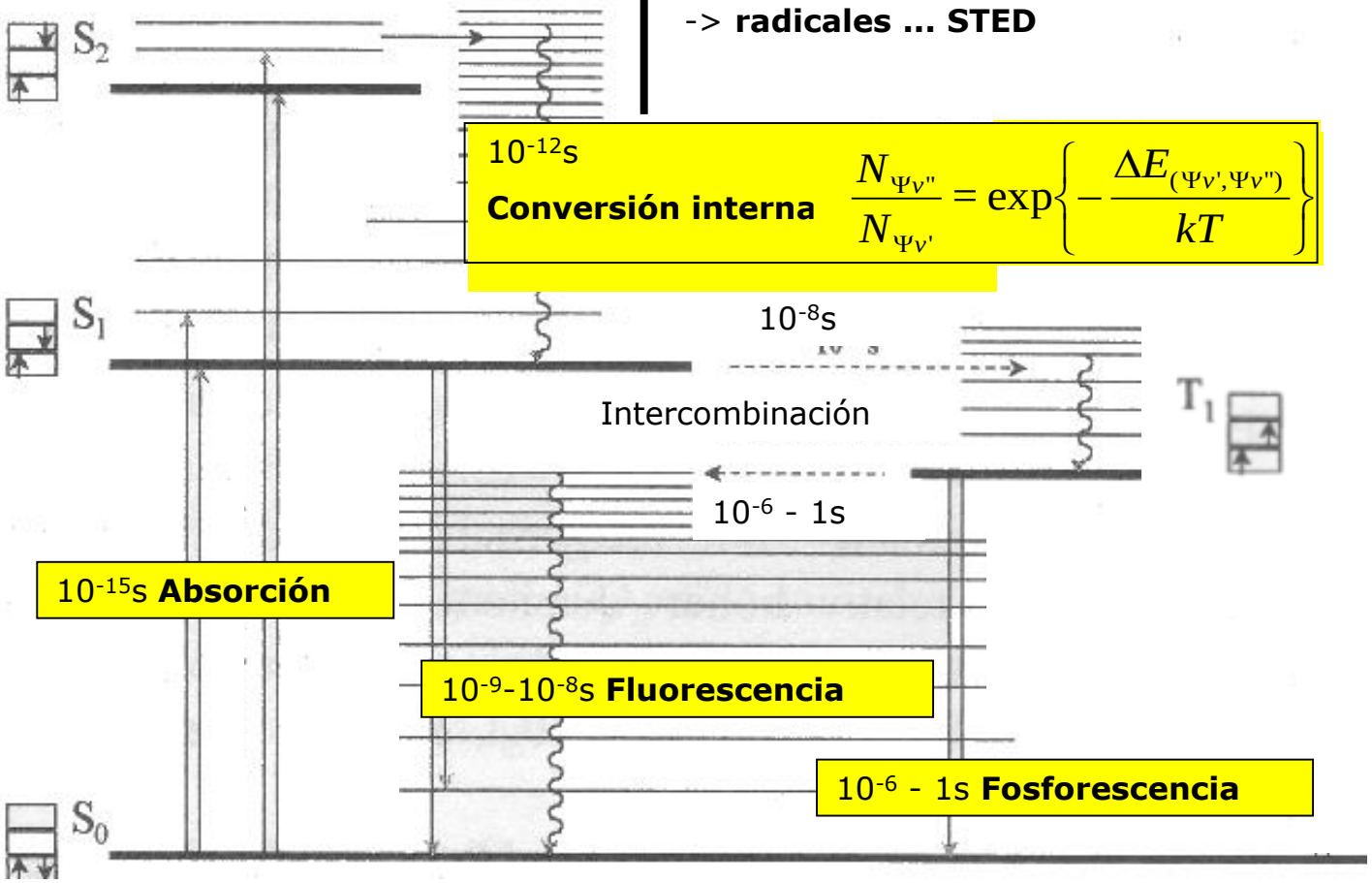
▬ electrónicos

-> **calor**

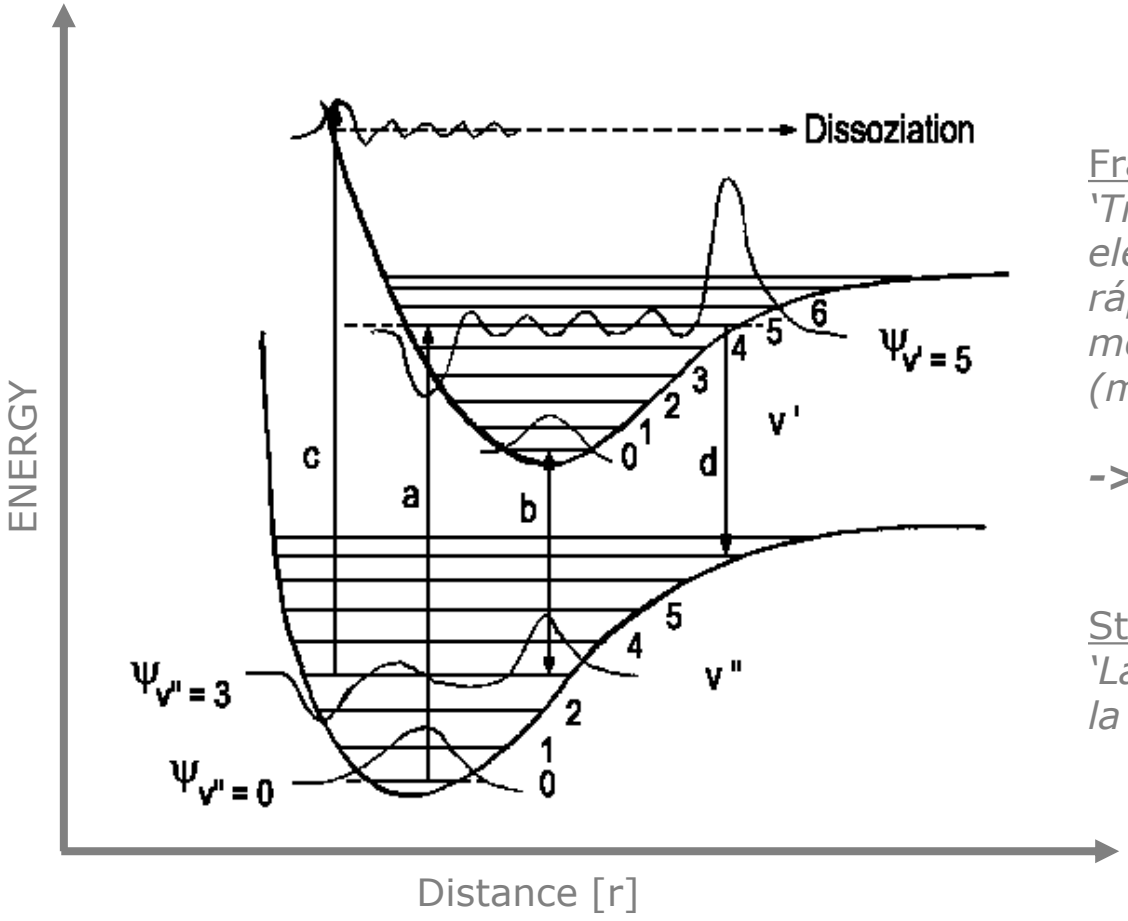
-> **transferencia de energía (FRET)**

-> **radicales ... STED**





|-> Franck Condon



Frank Condon **:**

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

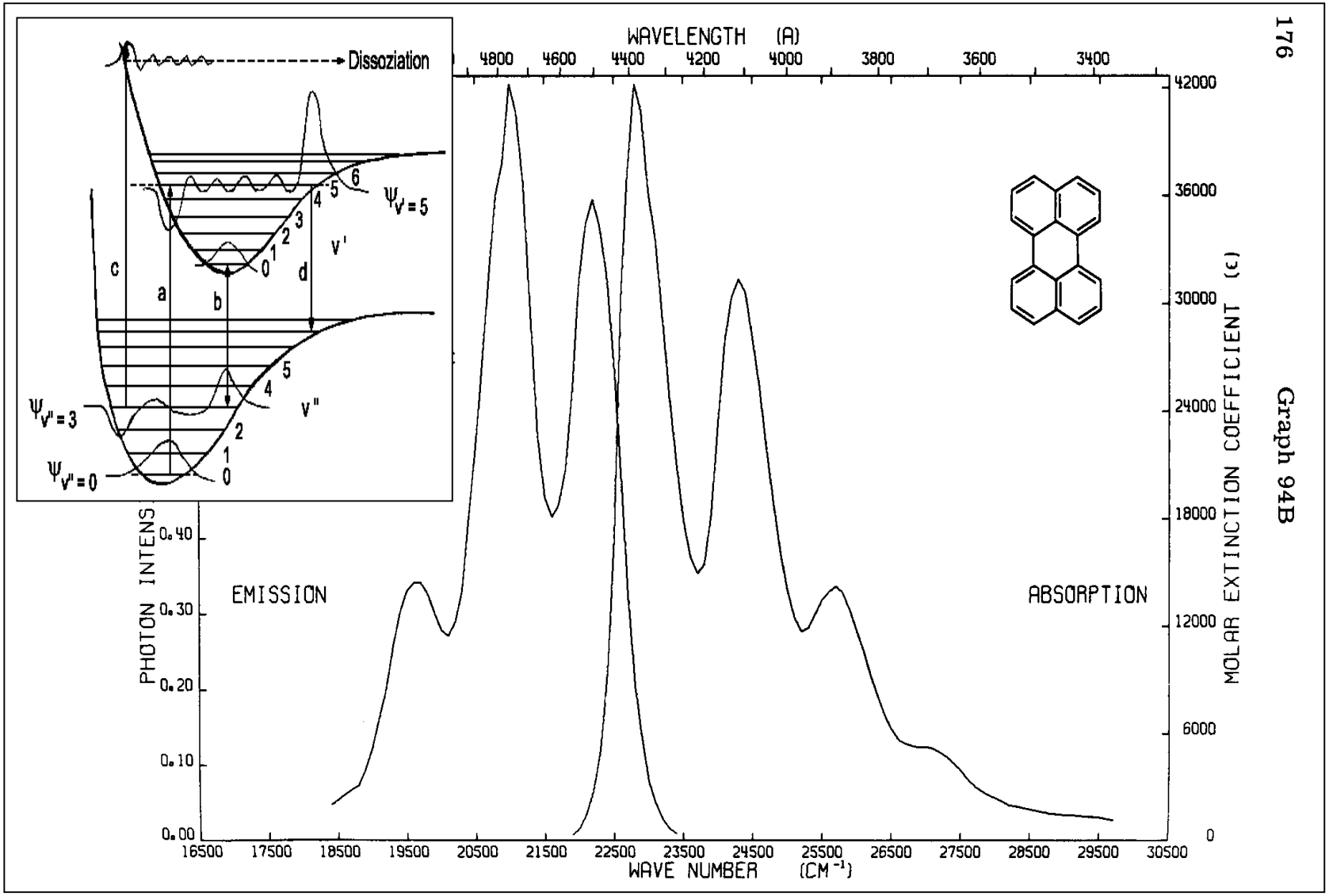
($masa_{electron}$ / $masa_{atom}$: 1 : 2000)

-> Mirror Image Rule

Stokes (Shift) **:**

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

|-> Mirror Image Rule



”Principles of Fluorescence Spectroscopy” Chapter 1

www.scian.cl -> Courses -> Optics, forces & development | Santiago de Chile 14-29 de Enero 2013, Facultad de Medicina, U-Chile |

|-> Quenching, Bleaching ...

|-> Polarisation ...

|-> Steady-State and Time-Resolved Fluorescence...

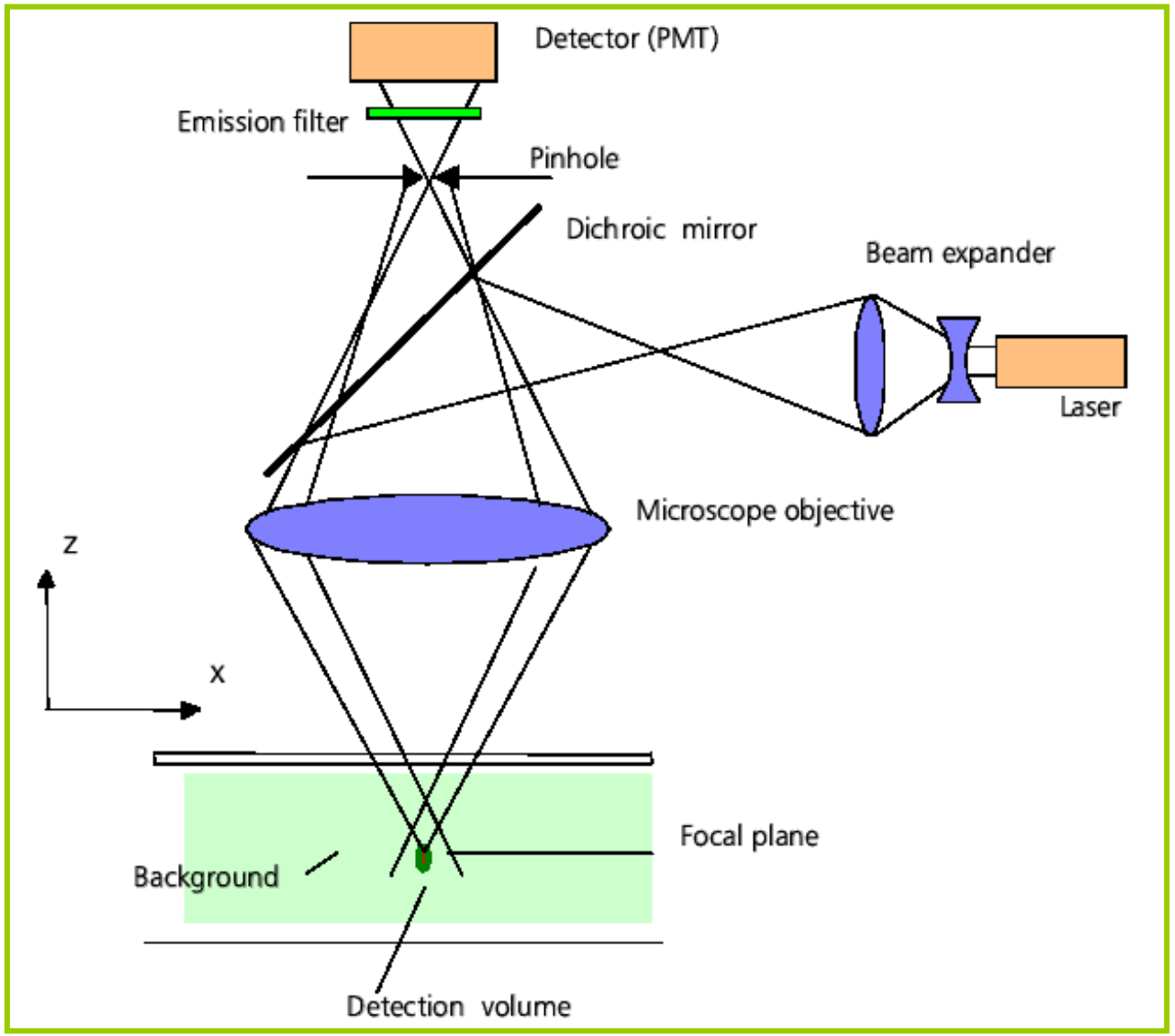
|-> Förster Resonance Energy Transfer ...

Sekar and Periasamy (2003) FRET microscopy imaging, JBC, Volume 160, Number 5, 629–633

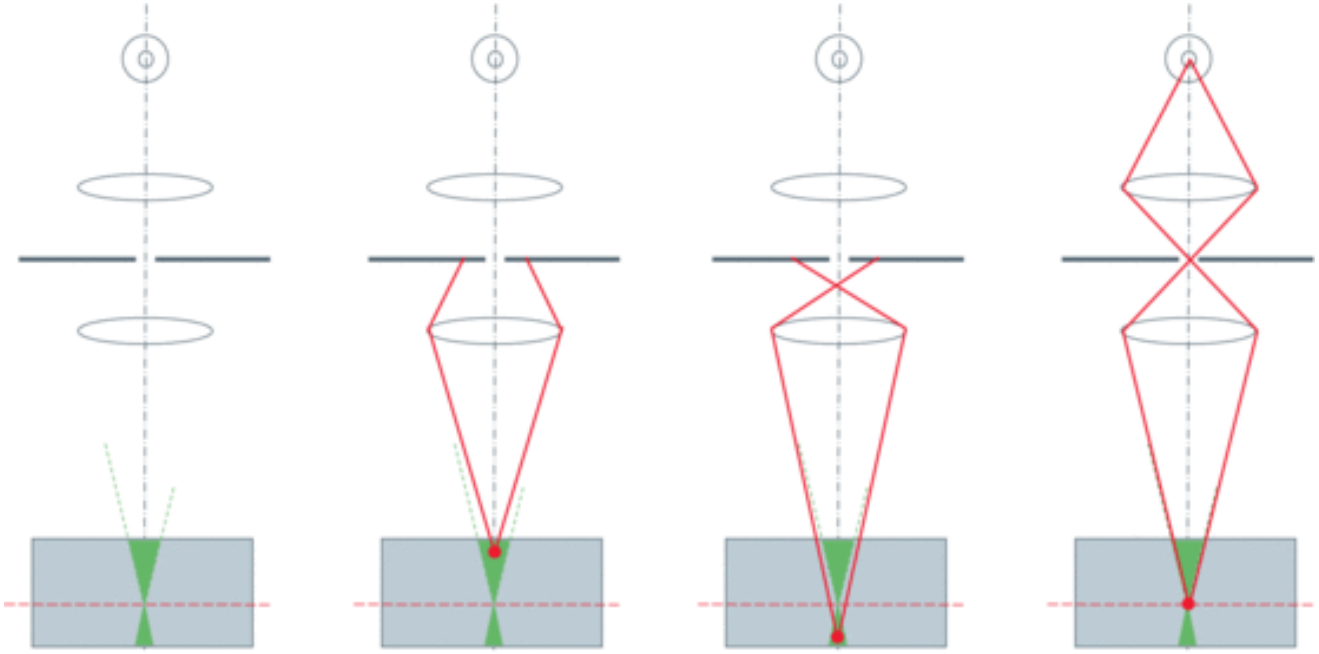
Feige et al (2005) PixFRET, an ImageJ plug-in for FRET calculation which can accommodate variations in spectral bleed-throughs MICROSCOPY RESEARCH AND TECHNIQUE 68, 51–58

Vogel et al (2006) Fanciful FRET. Cell Signaling Technology

|-> Diffraction limited Microscopy



|-> Diffraction limited Microscopy



Rebanada óptica en µm, modificable según Airy units del pinhole

http://www.leica-microsystems.com/science-lab/confocal-microscopy-optical-path/

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.

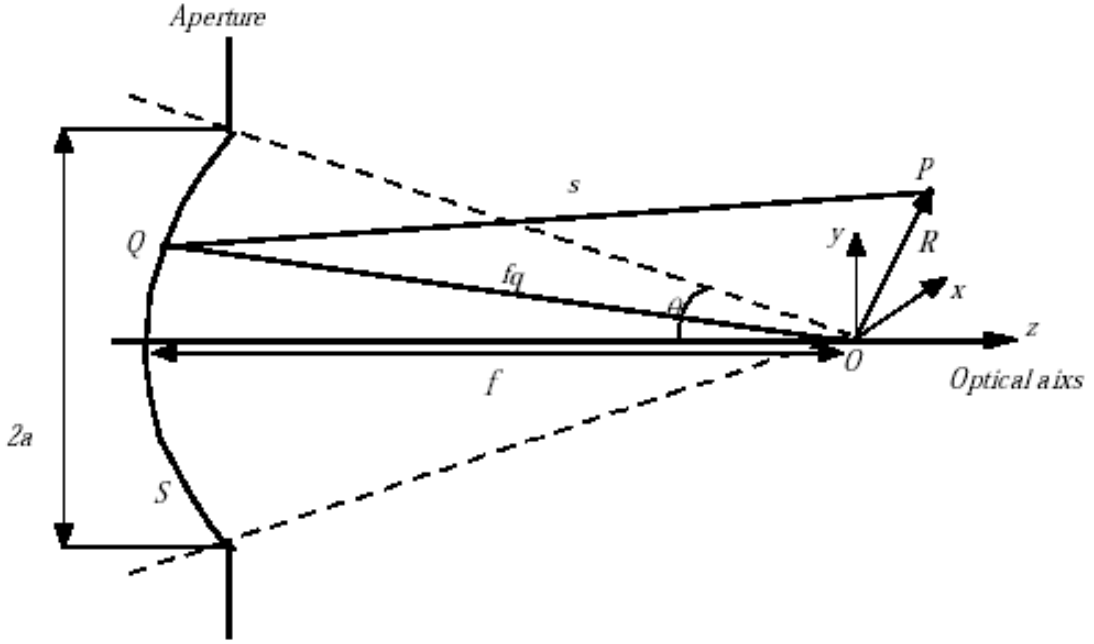


Figure 2.1 Diffraction of a converging spherical wave at a circular aperture

|-> Diffraction limited Microscopy

Óptica no-geométrica /

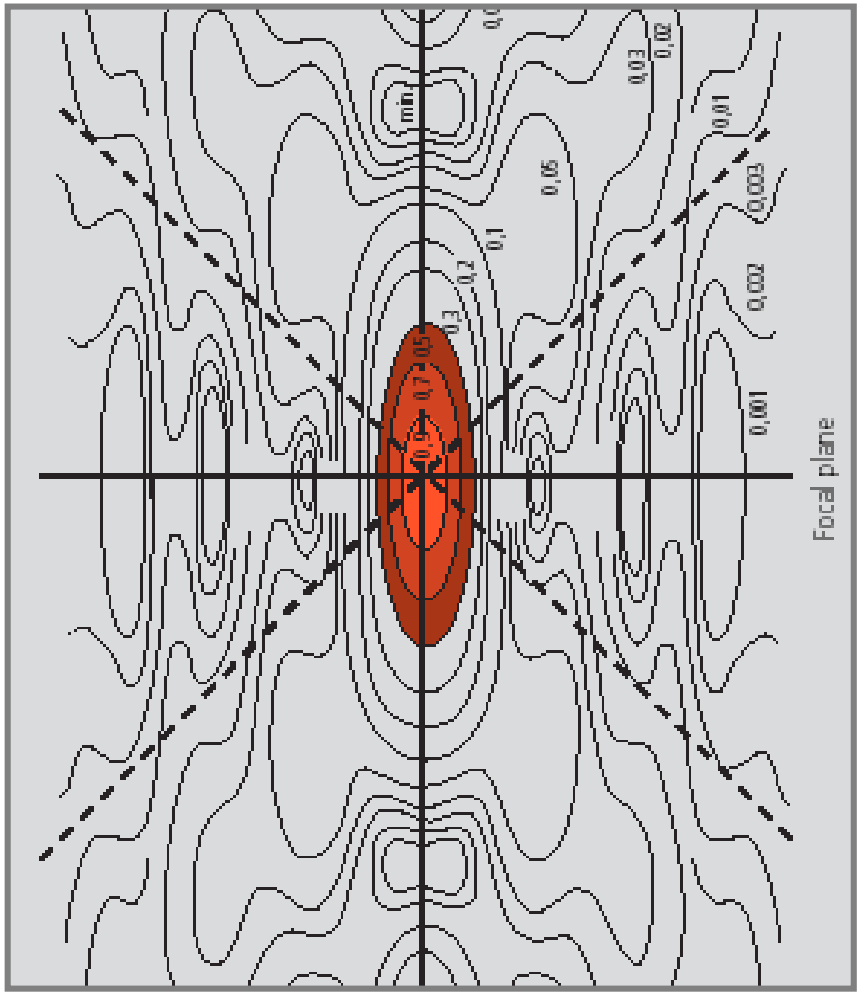
Teoría de difracción

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

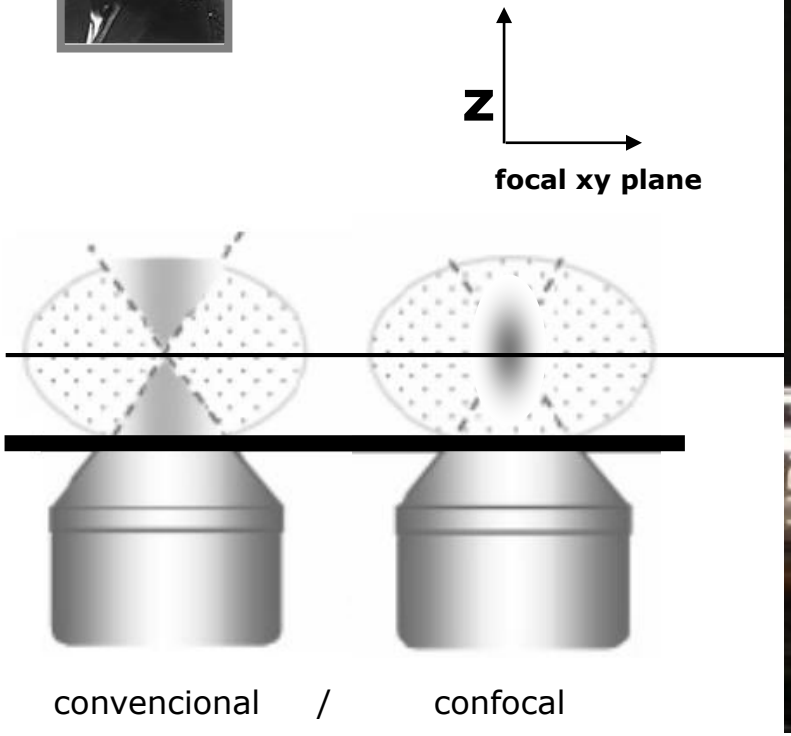
U, Integral de Difracción de Kirhoff

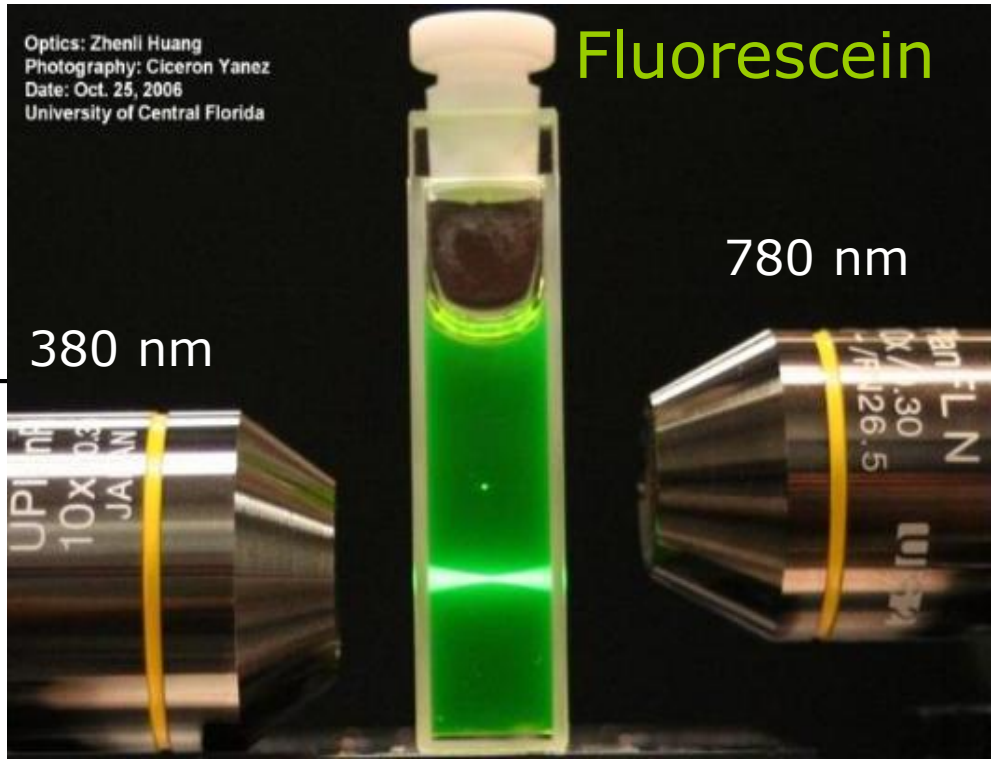
J_0, Serie de funciones de Bessel

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



| Best localization: confocal microscopy



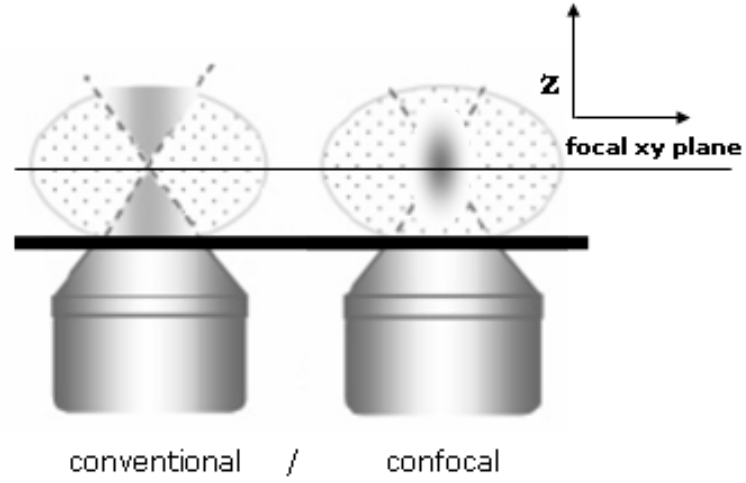


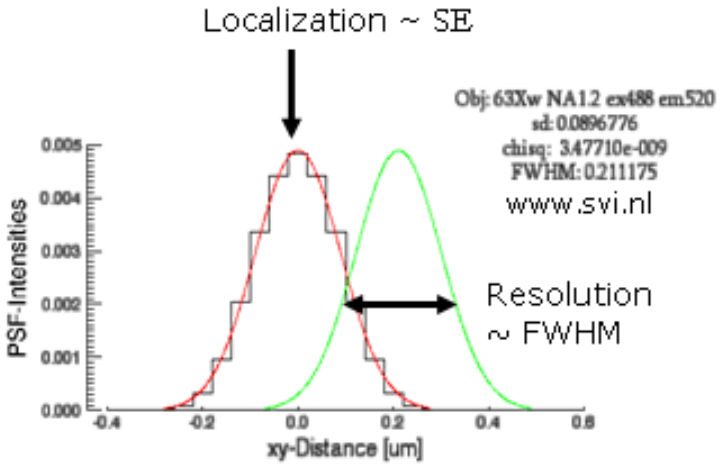
| Diffraction limited microscopy

E. Abbe († 1905)

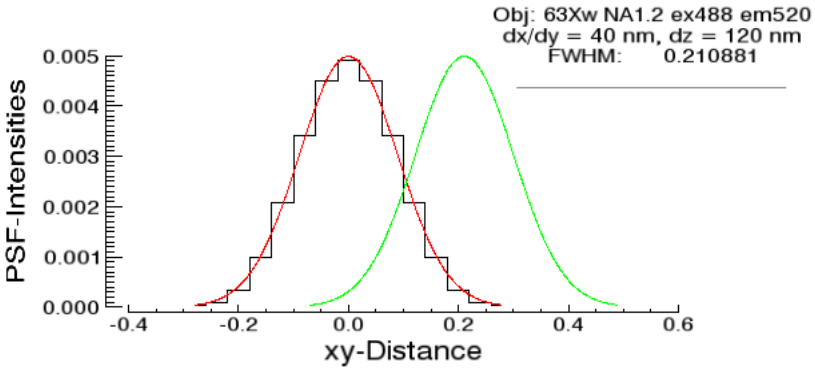
$\lambda / 2 \cdot NA \sim \lambda / 2$ Resolution (Full Width at Halft Maximum, FWHM)

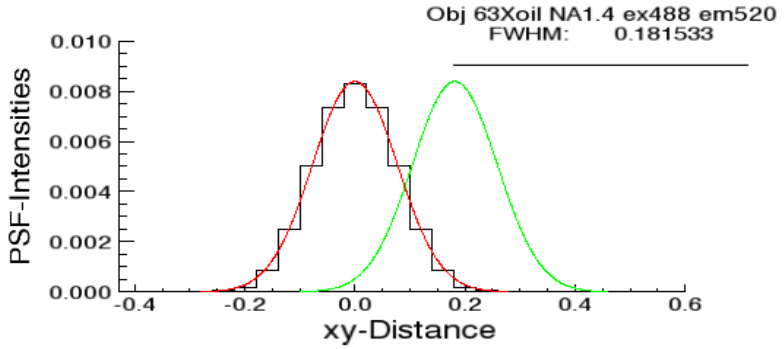
$FWHM / N^{1/2}$ Localization, N number of photons

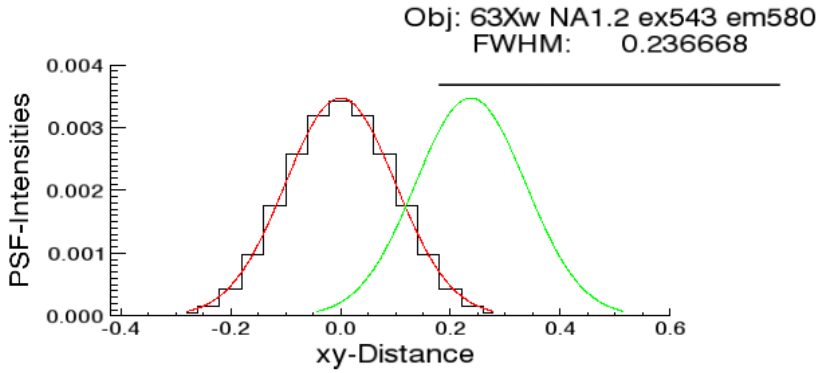


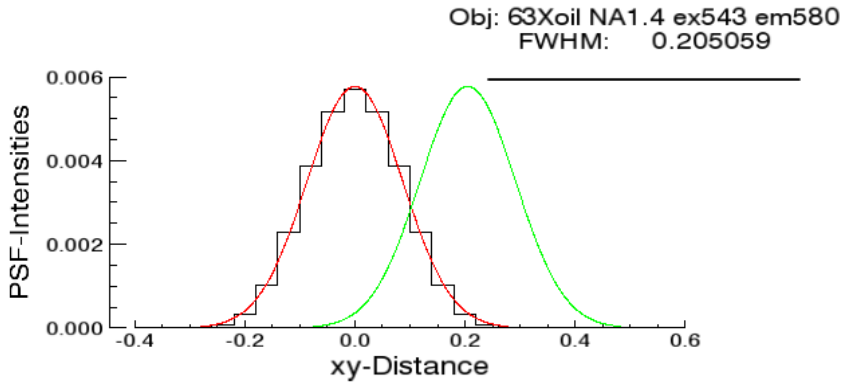


|-> Diffraction limited Microscopy

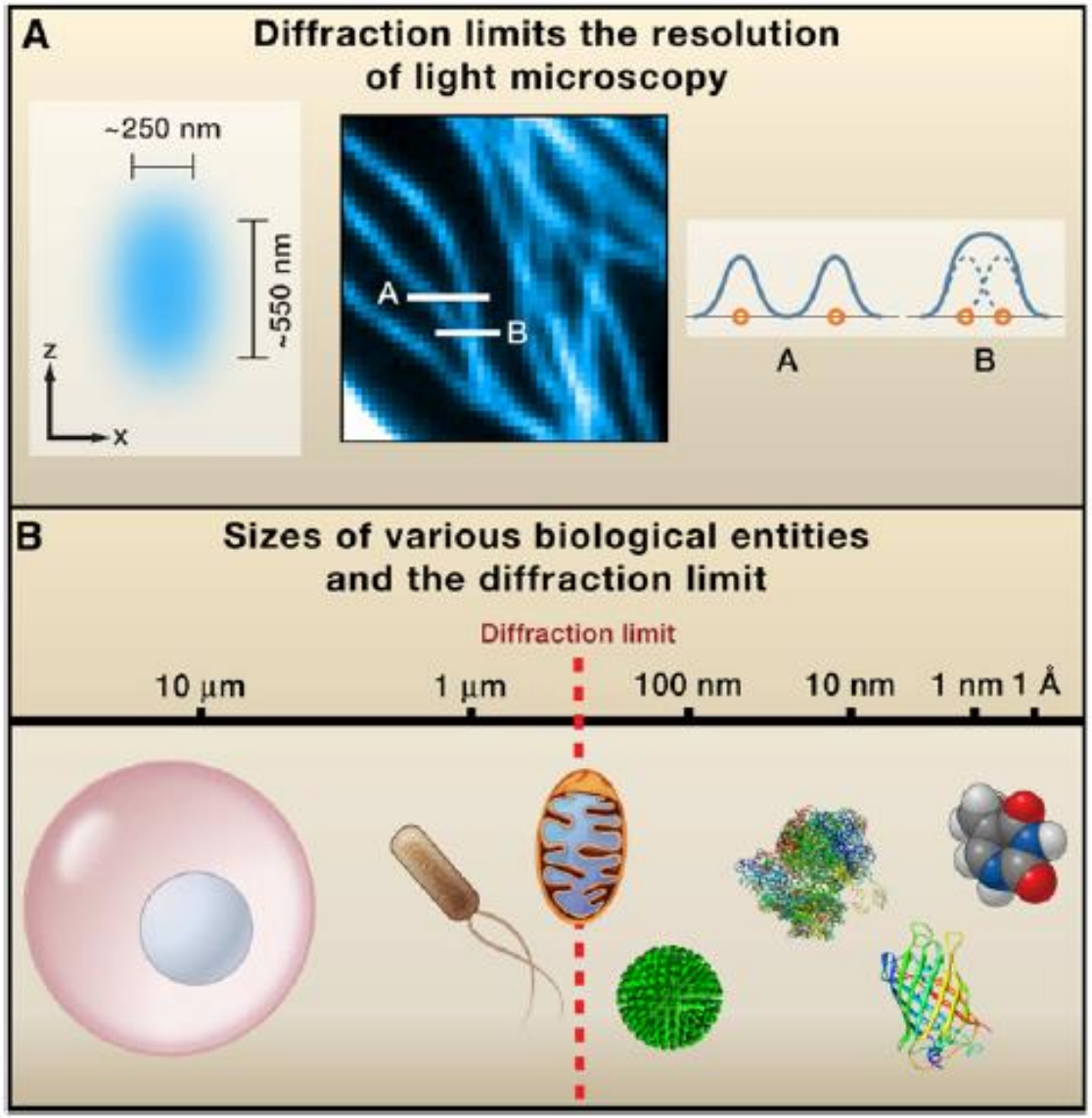








|-> Diffraction limited Microscopy



|-> Beyond diffraction

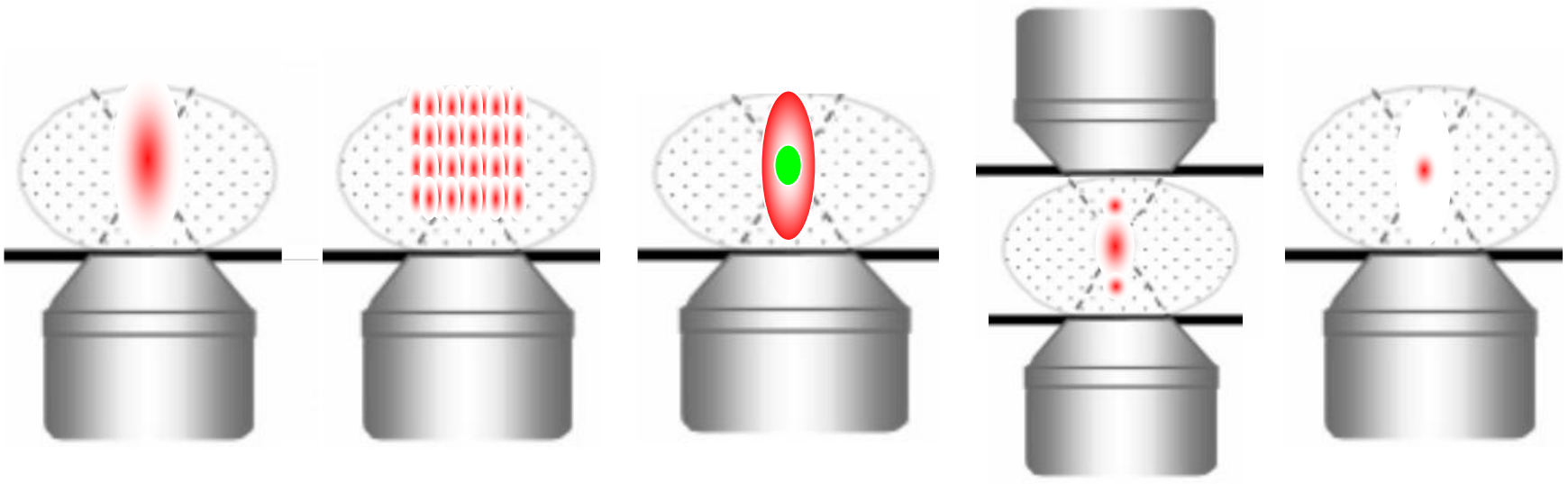
M Goeppert-Mayer
1906-1972

M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

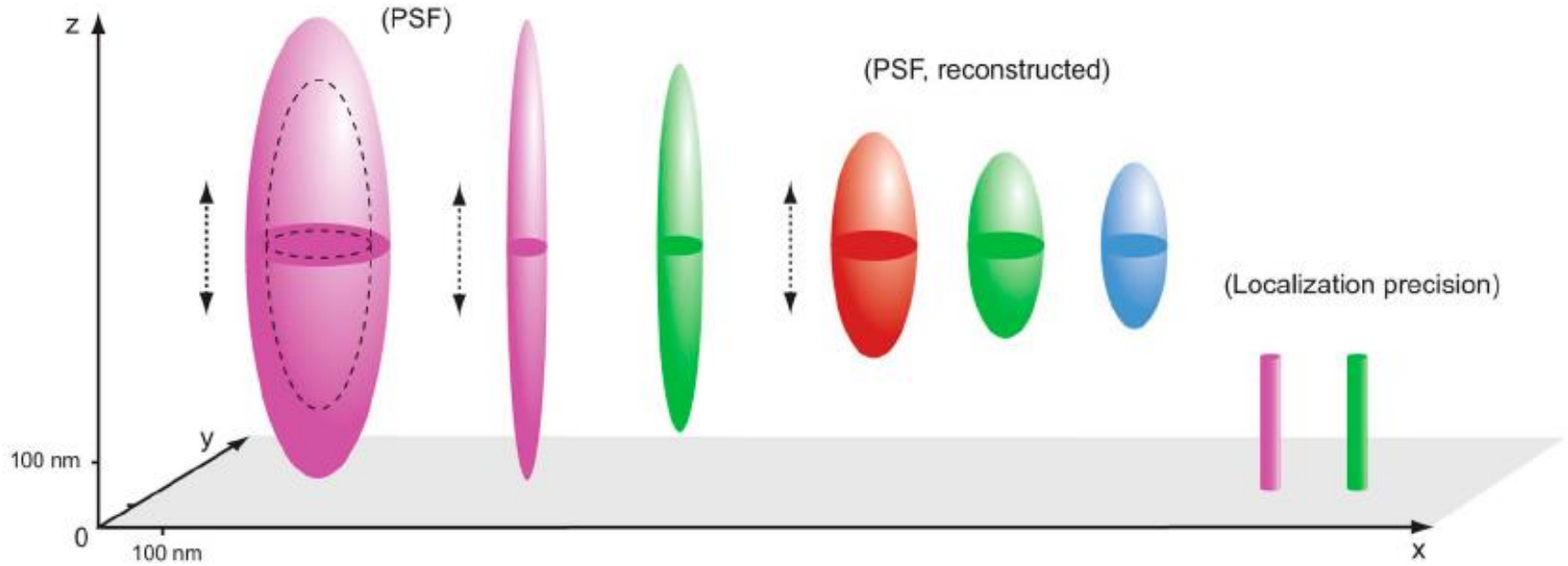
FWHM(xy) ~ λ/2 | ~ λ/4 | ~ λ/∞ | ~ λ/4 | ~ λ/100



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



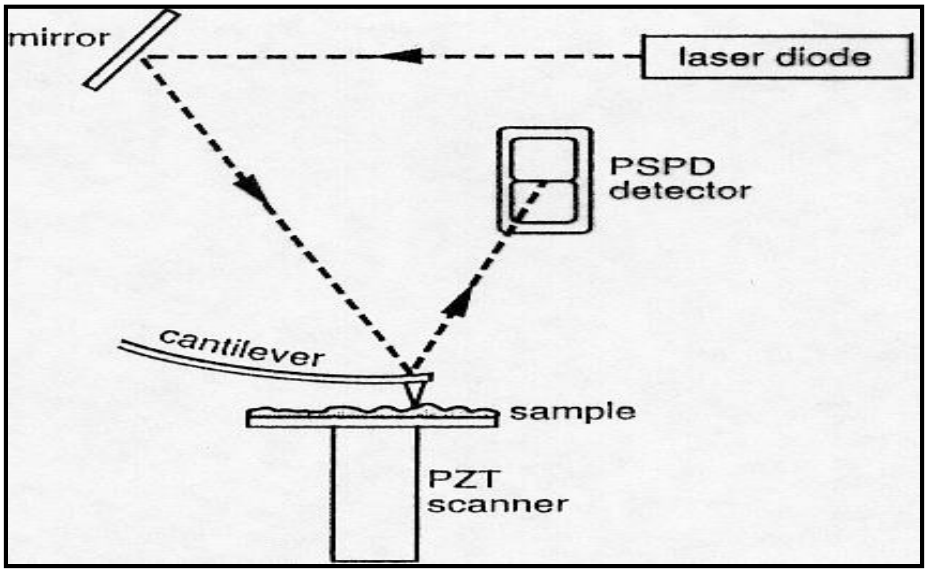
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

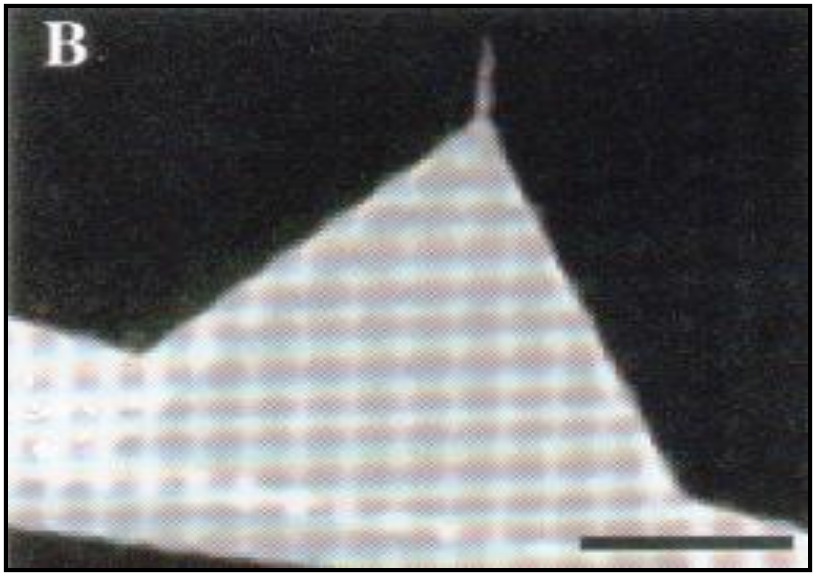
Schermelleh et al 2010 JBC

|-> Atomic Force Microscopy

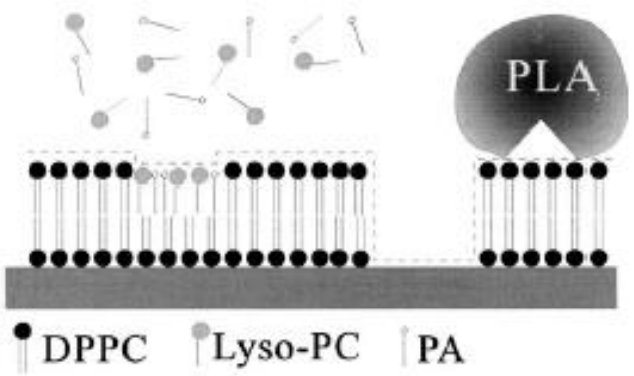
AFM allows the investigation of structural and functional properties of biomolecules in liquid environments, by a unique combination of :

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

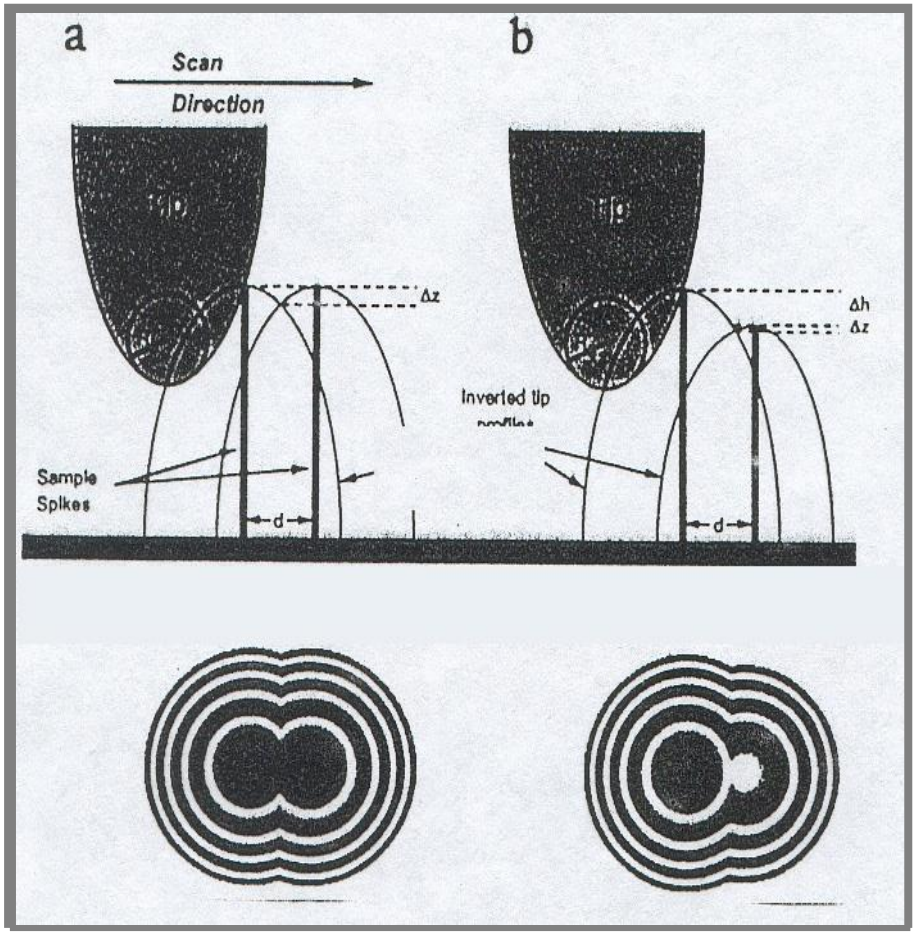




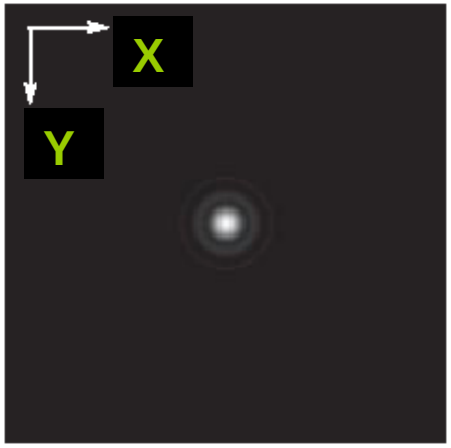
|-> Atomic Force Microscopy

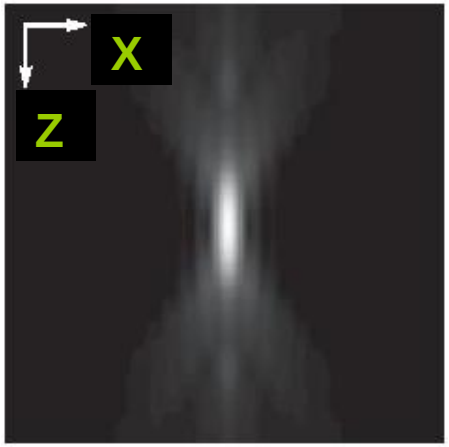


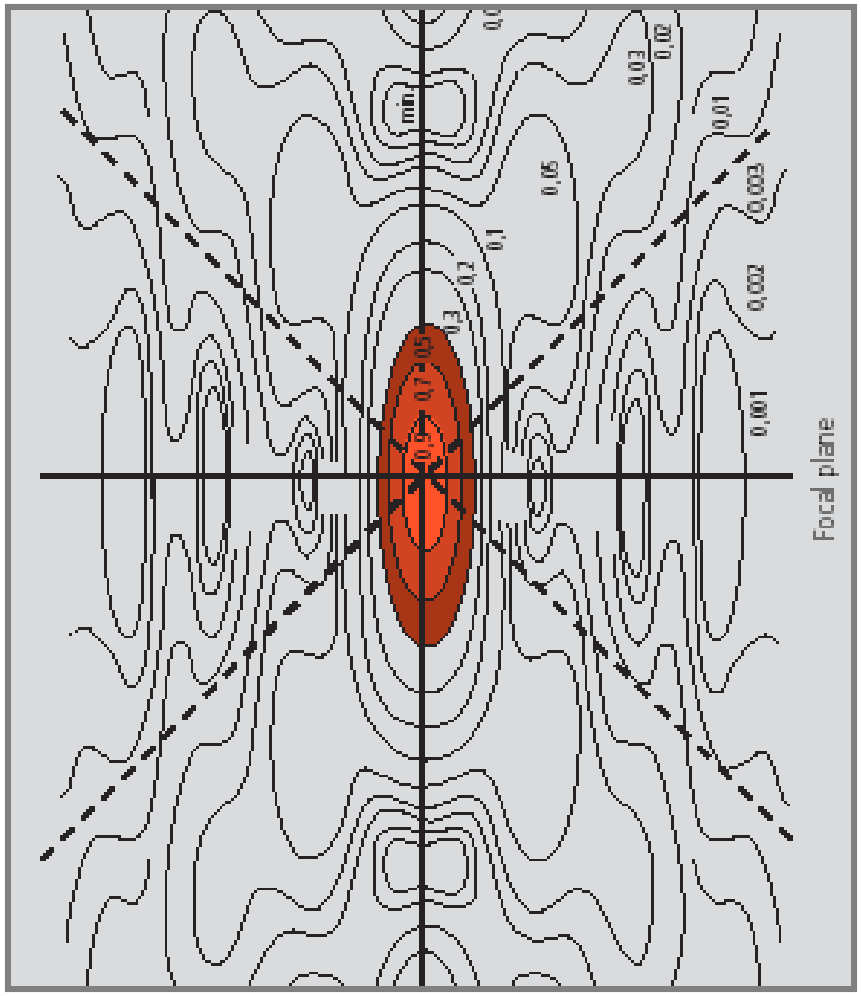
M Grandbois et al. (1998) Biophys J.



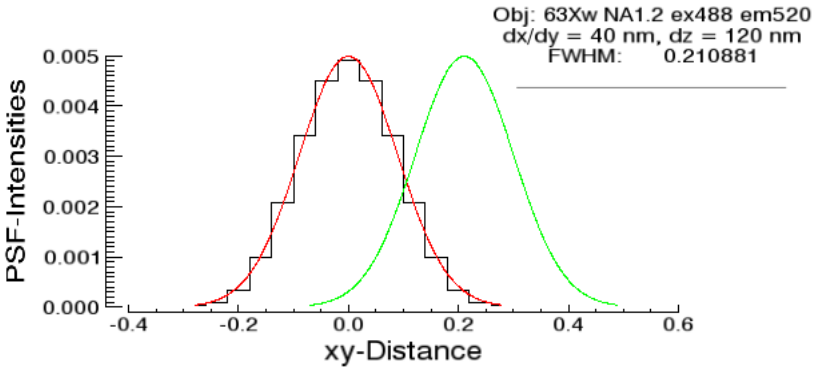
|-> PSF

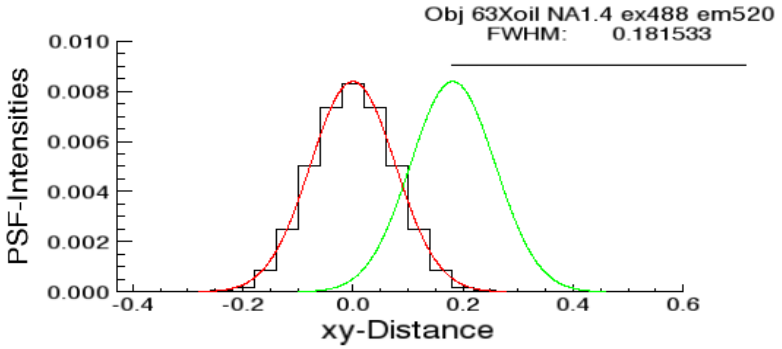


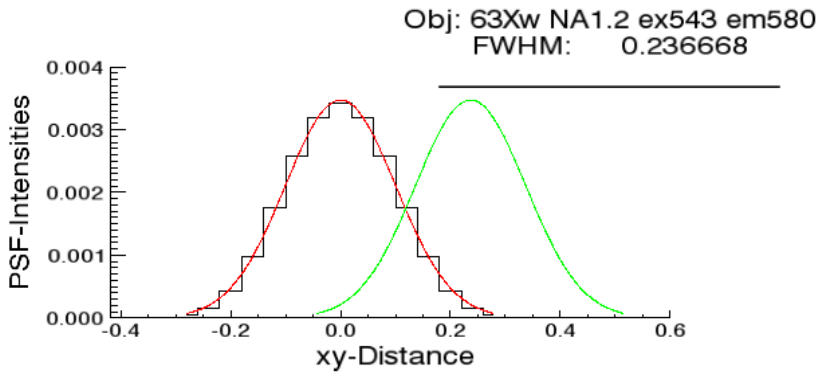




|-> PSF


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


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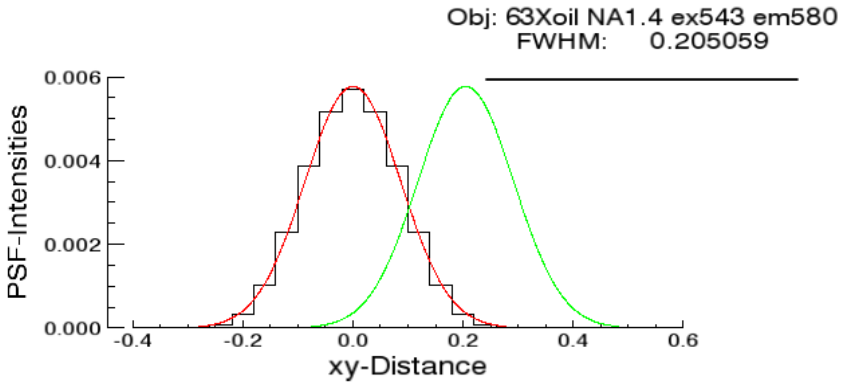
0.003
0.002
0.001
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance


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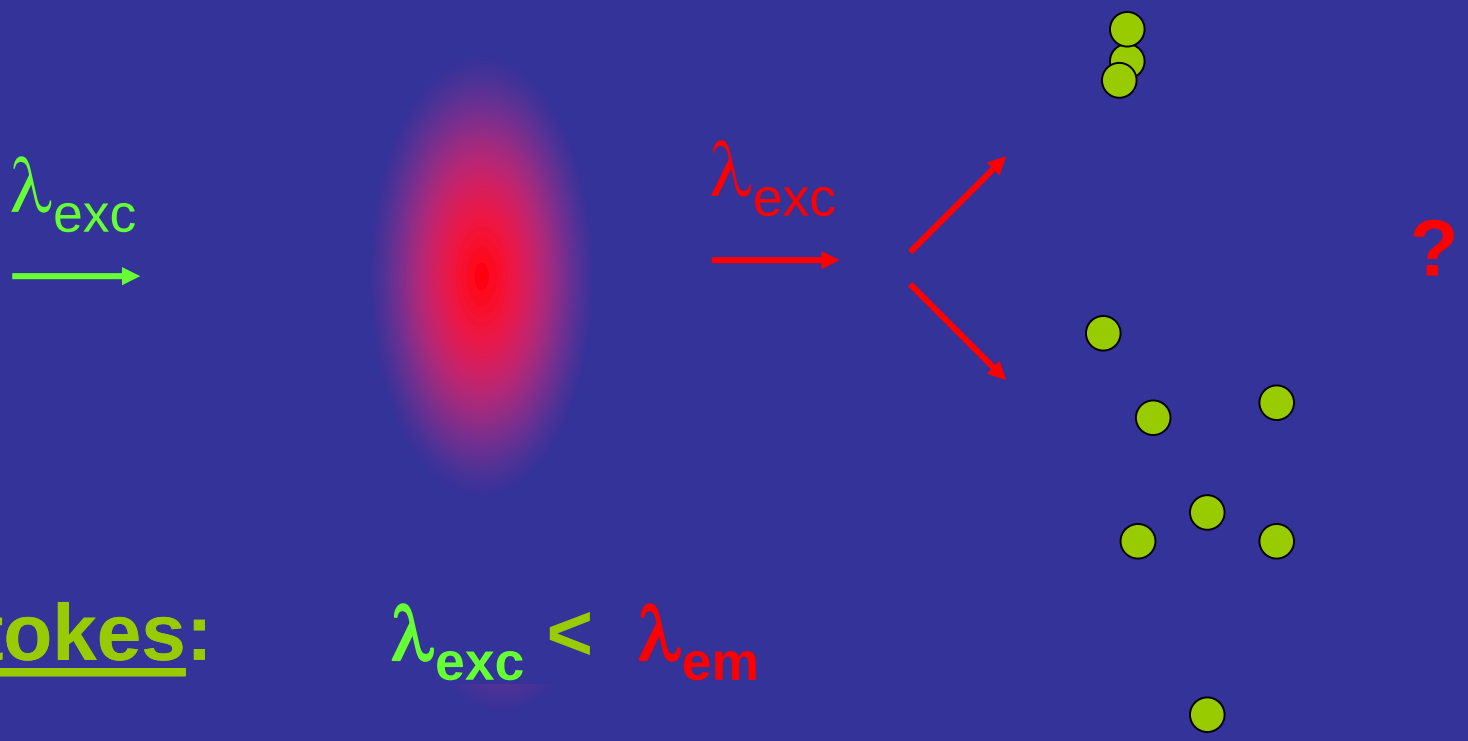
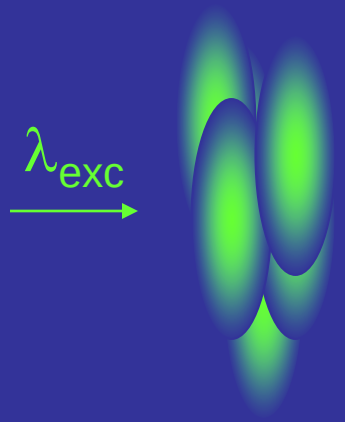
0.004
0.002
0.000
-0.4
-0.2
0.0
0.2
0.4
0.6
xy-Distance

|-> Convolution



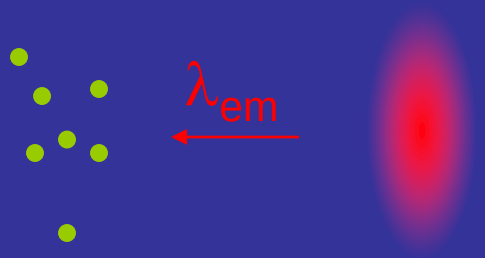
Stokes: $\lambda_{exc} < \lambda_{em}$

|-> Convolution



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

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



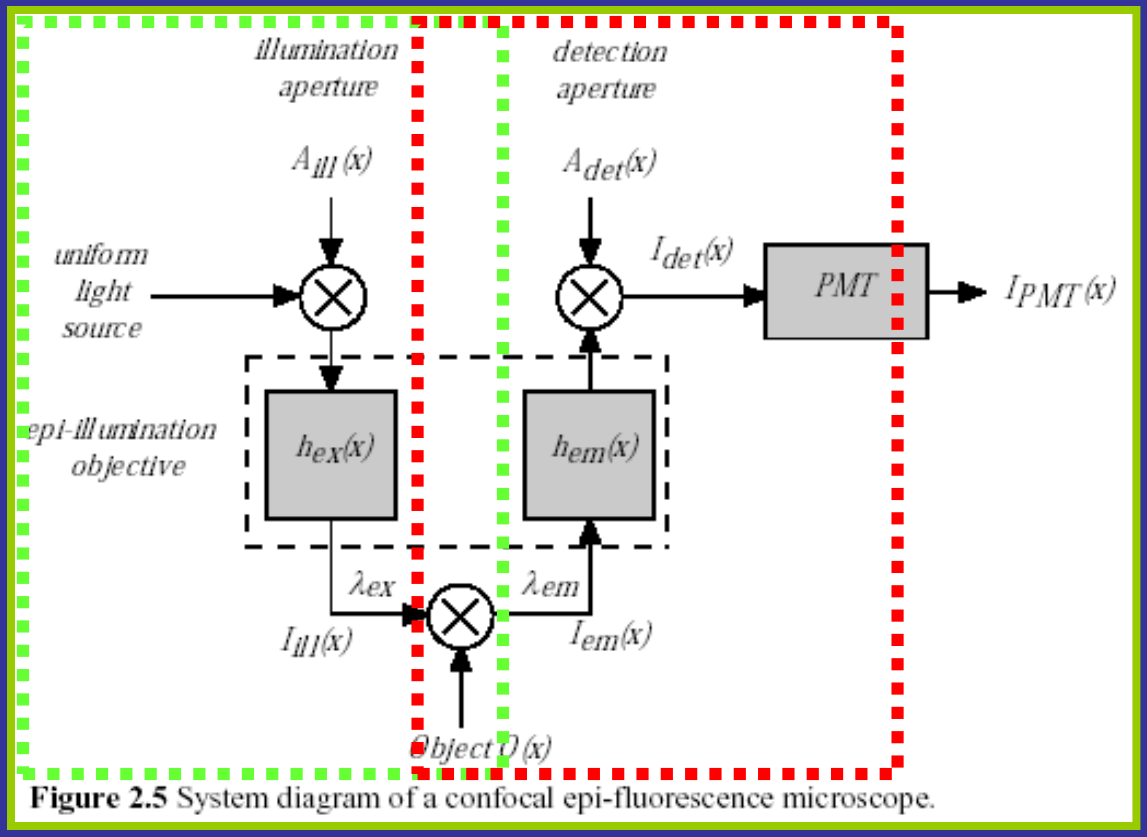
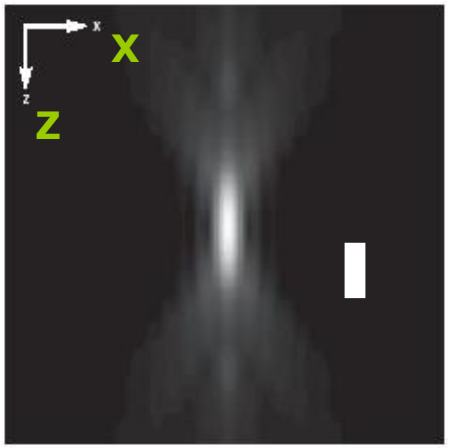
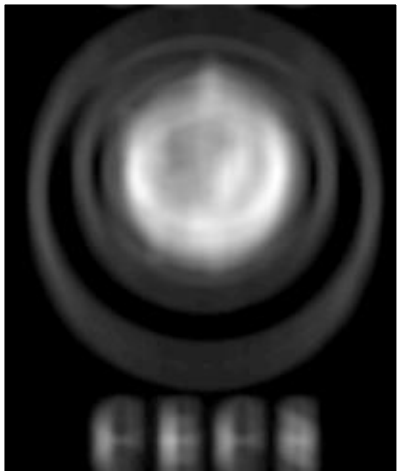


Figure 2.5 System diagram of a confocal epi-fluorescence microscope.

|-> Convolution





PSF:

Δxy ~ 500 nm | Δz ~ 1500 nm

|-> Deconvolution

PSF: *Point Spread Function*

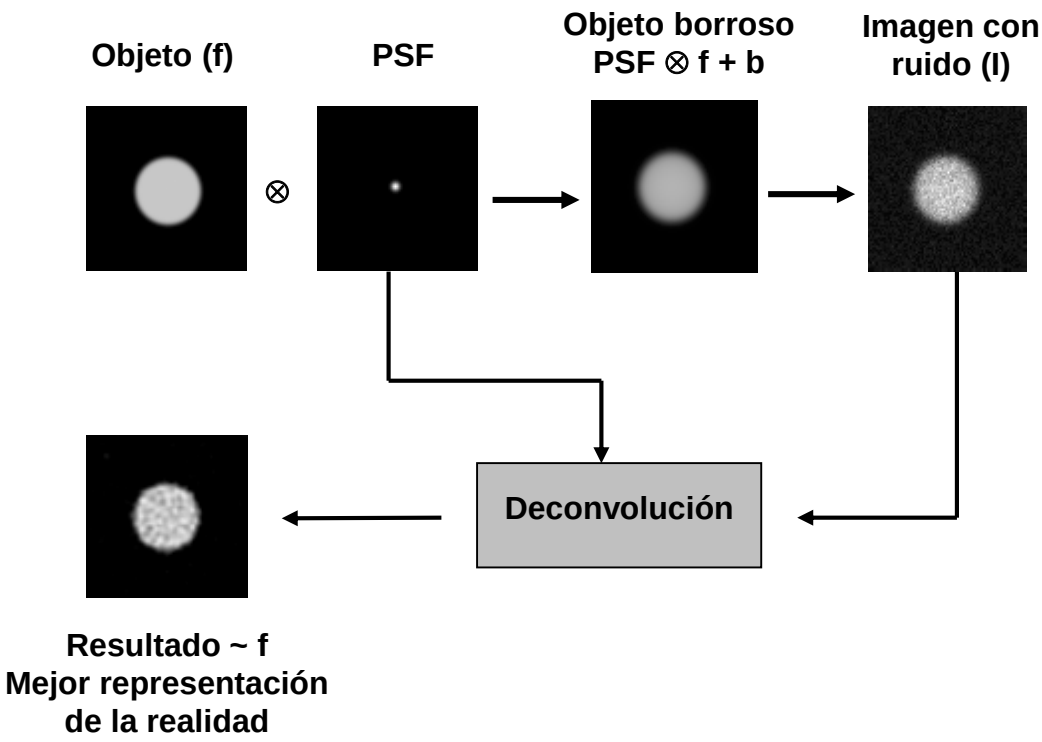
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

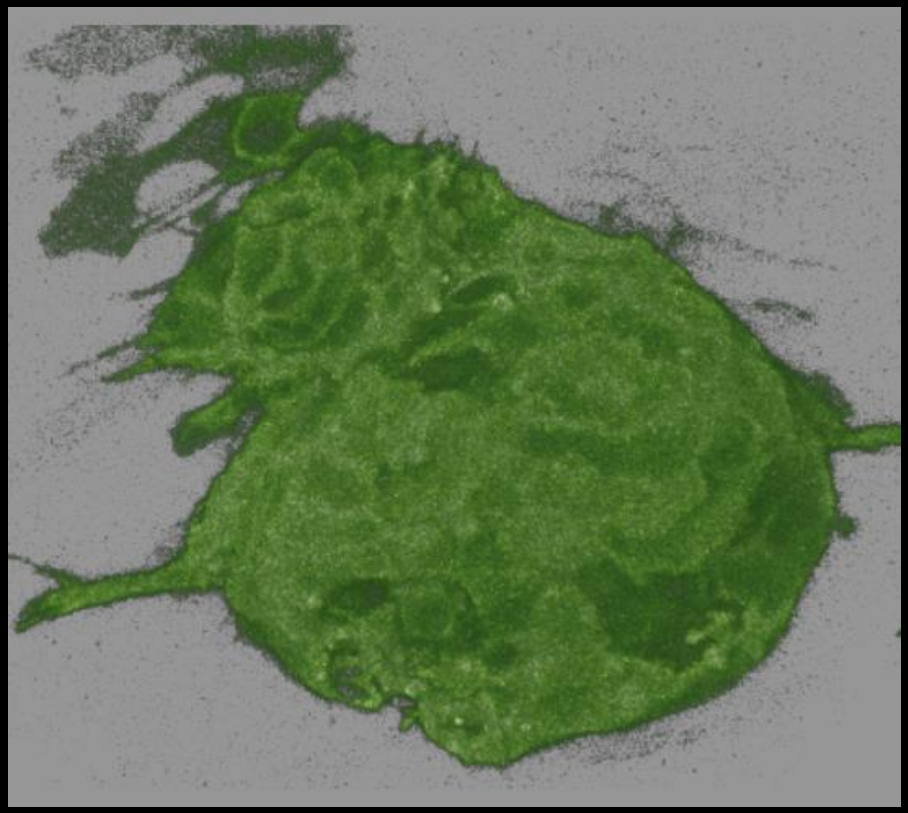
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

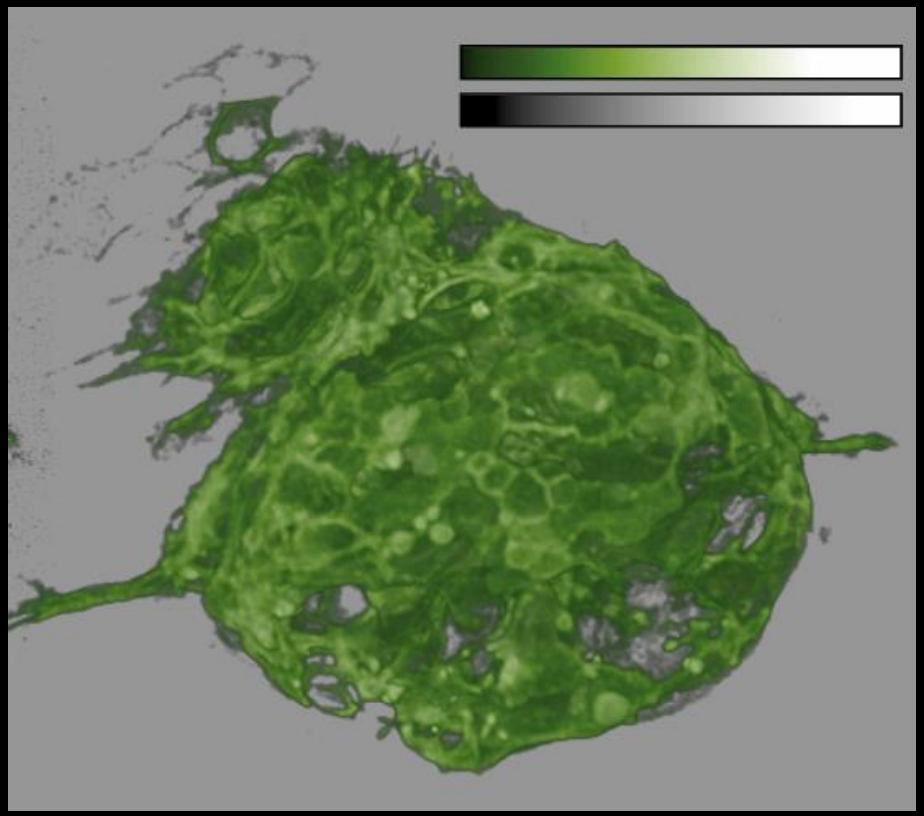


Laboratory for SCIAN Scientific Image Analysis

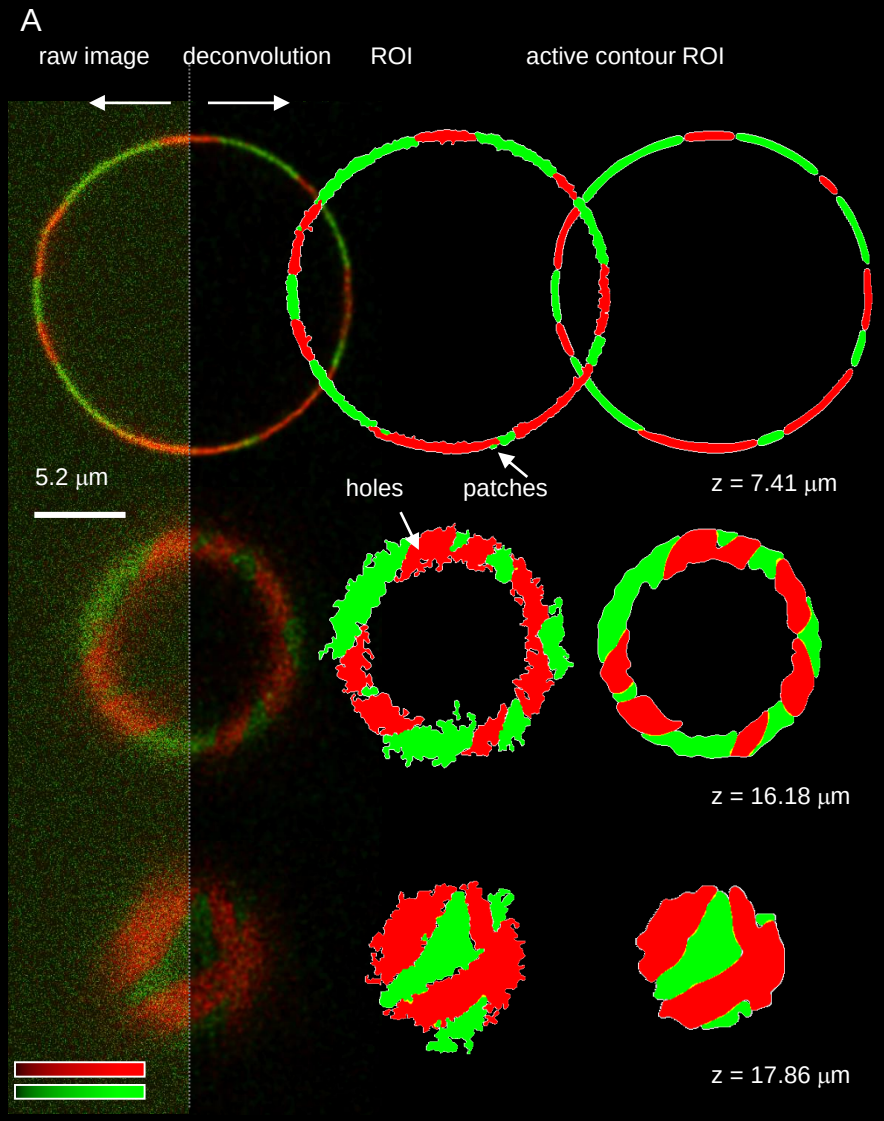
|-> Deconvolution

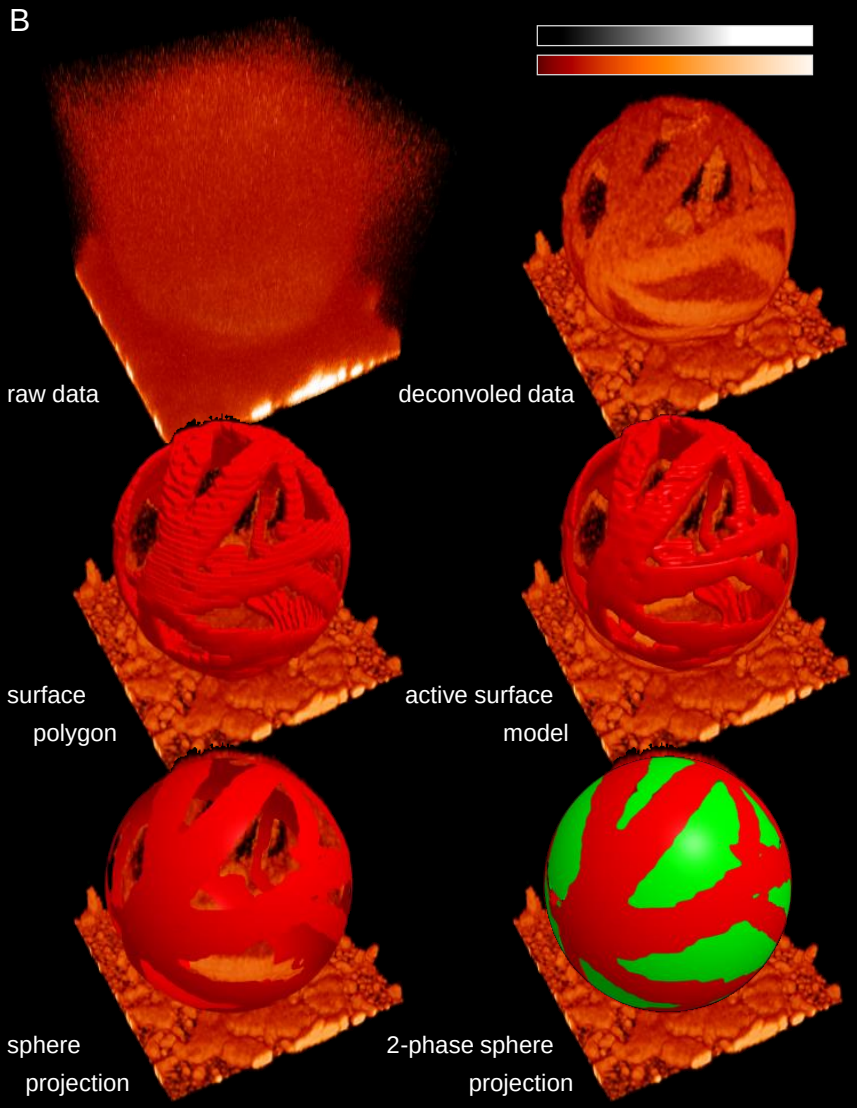
BIOMEDICAL NEUROSCIENCE INSTITUTE





|-> Deconvolution





|-> Deconvolution

PSF: *Point Spread Function*

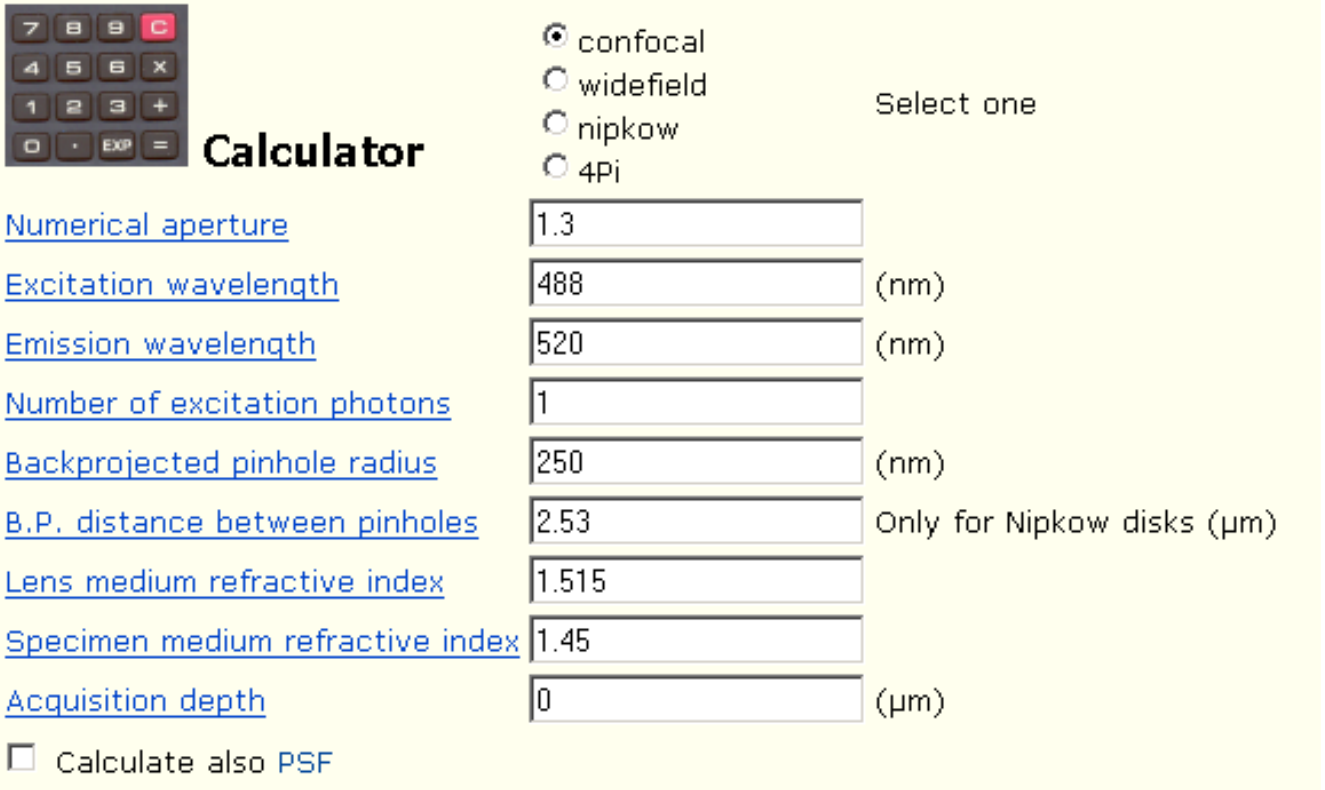
f: *Object Function*

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*

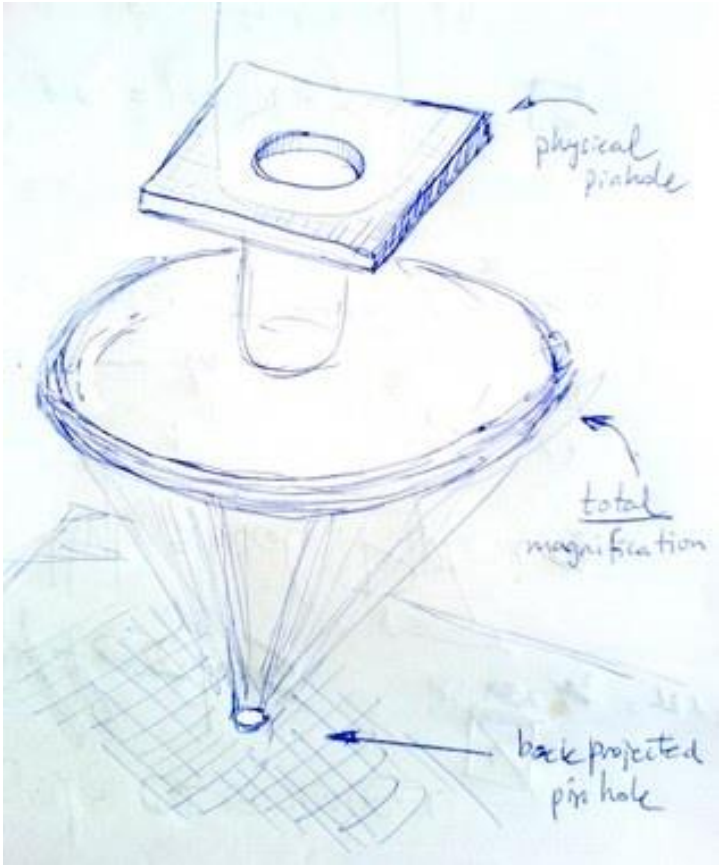
b: *Offset Function*

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)*

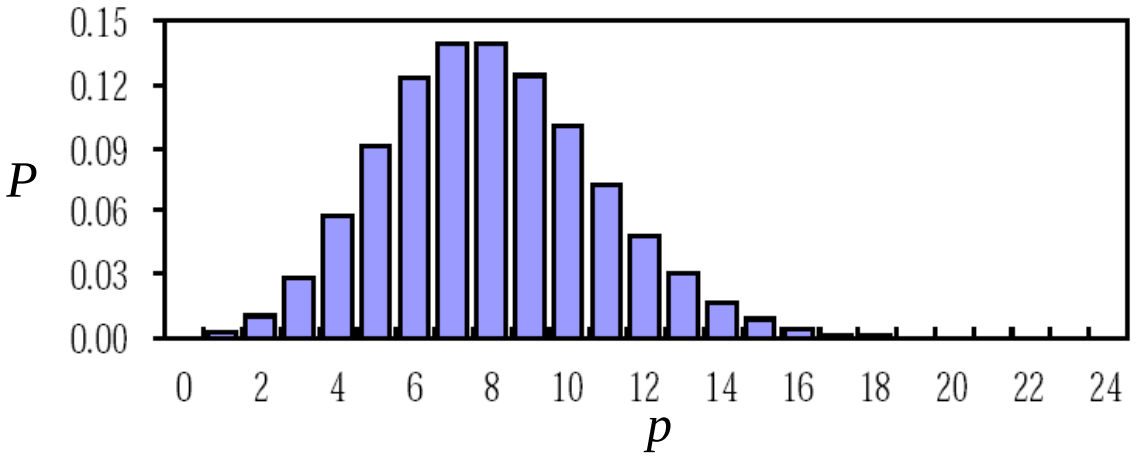
$$N(PSF(x, y, z) \otimes f(x, y, z) + b(x, y, z)) = I(x, y, z)$$

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

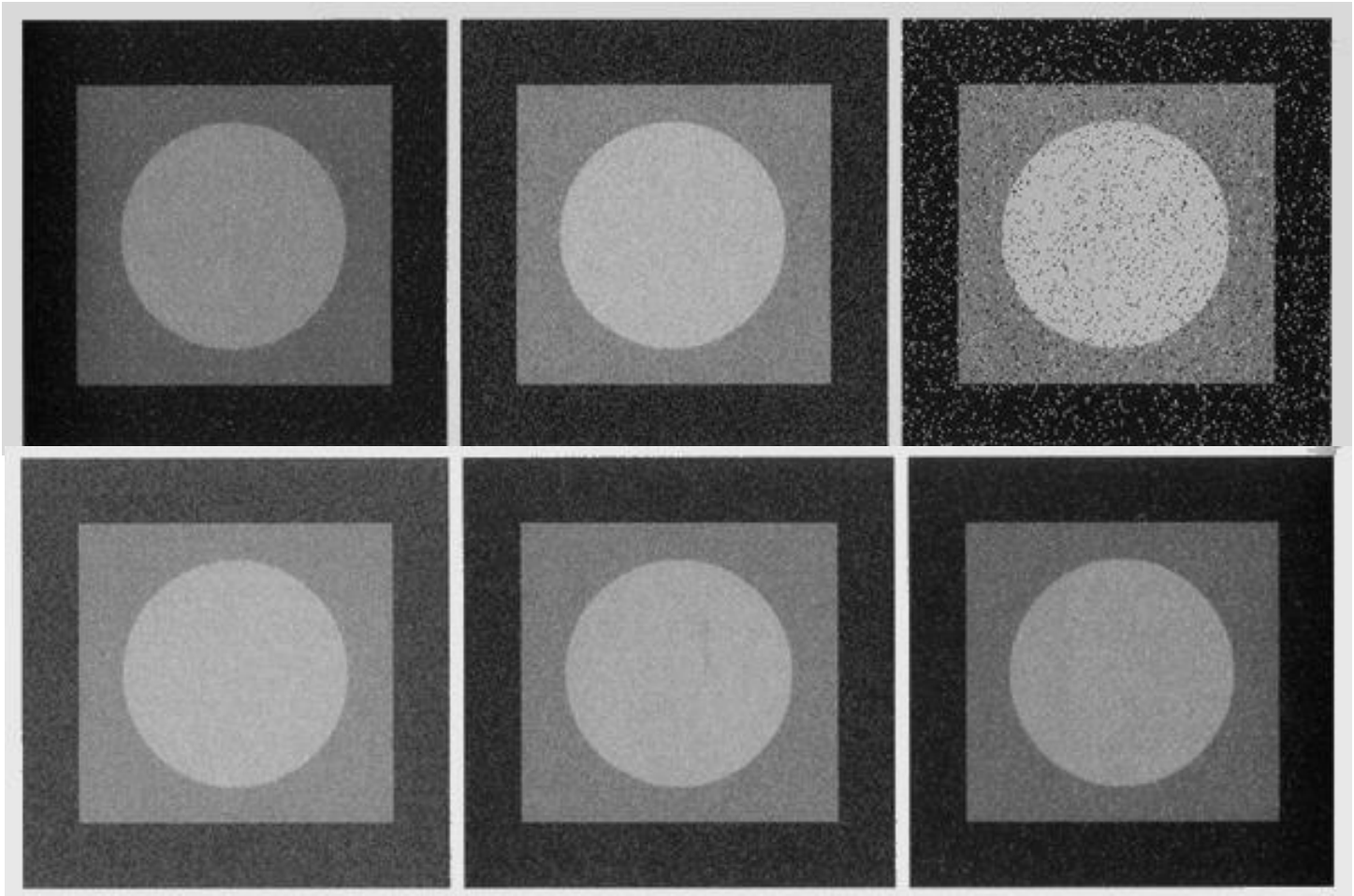
$$1.\ \bar{p} = \mu = \sigma^2,\ sd = \sigma = \sqrt{\bar{p}} = \sqrt{\mu}$$

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

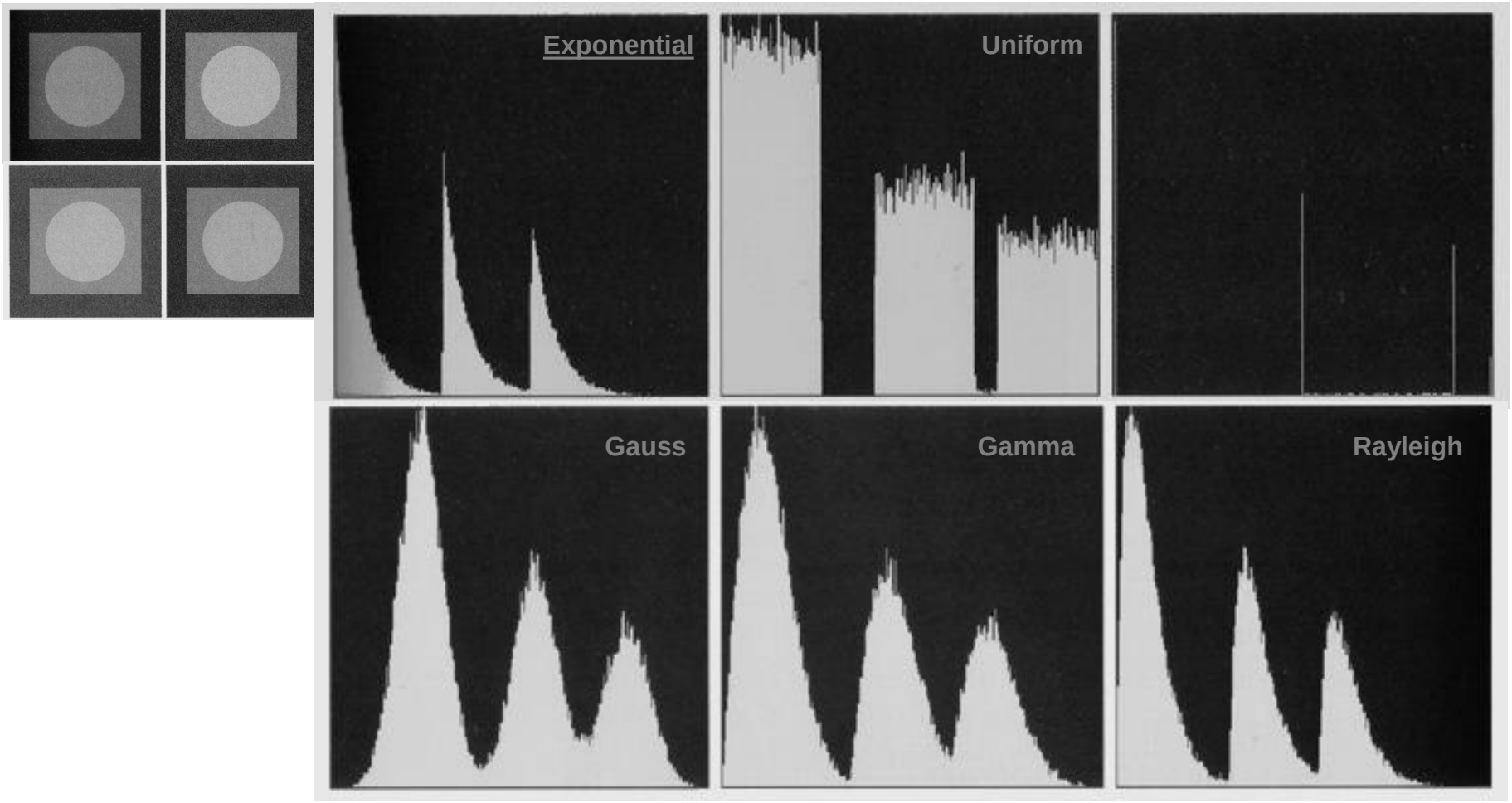
$$3.\ Poisson(discrete) \rightarrow Gauss(continuous) : \mu \rightarrow \infty$$



|-> 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.

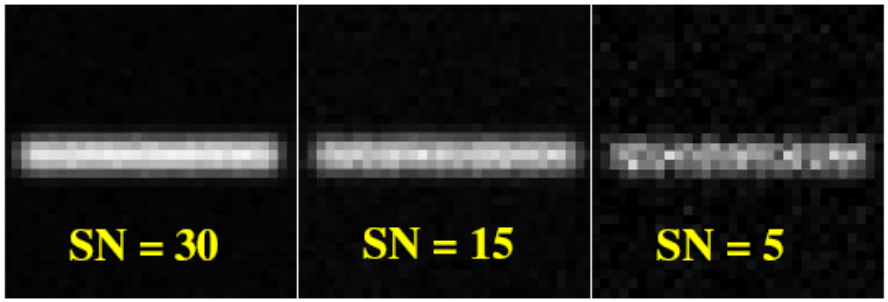
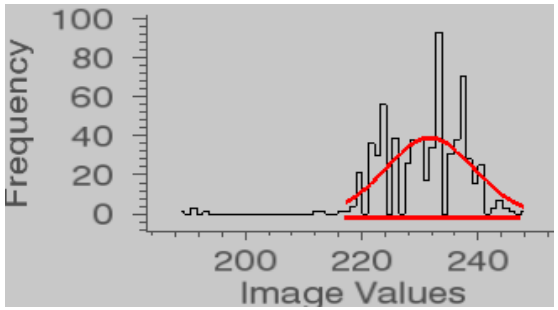
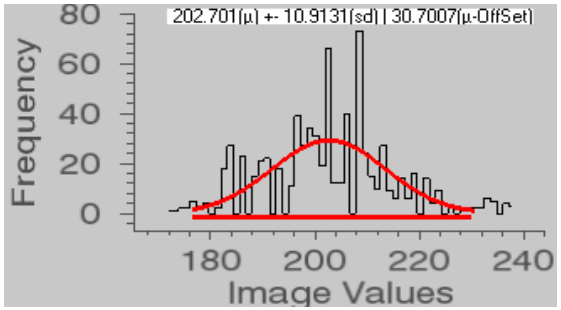
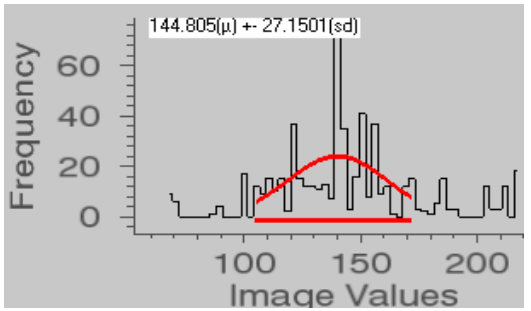


Figure 9. Images with different generated noise levels





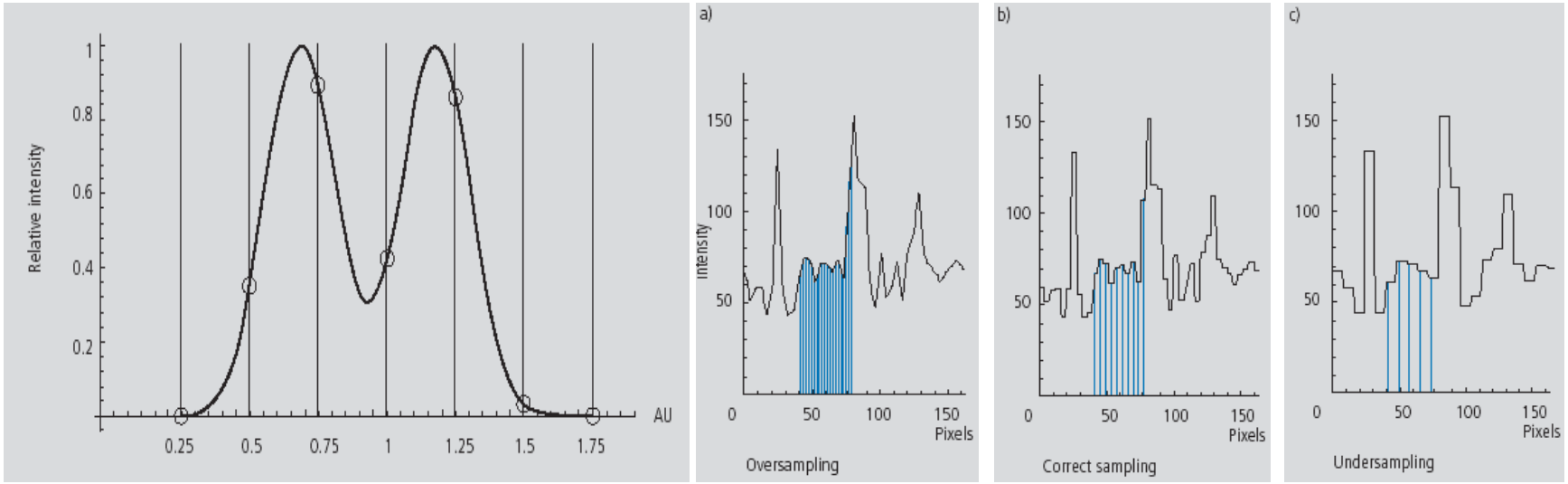


$$SNR = \frac{\bar{I}}{\sigma} = \frac{\bar{I}}{\sqrt{\sigma^2}} = \frac{229}{7.5}$$

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

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

|-> 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*

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

Calculator

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

Select one

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

☐ Calculate also PSF

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

|-> PSF calculator

PSF: *Point Spread Function*

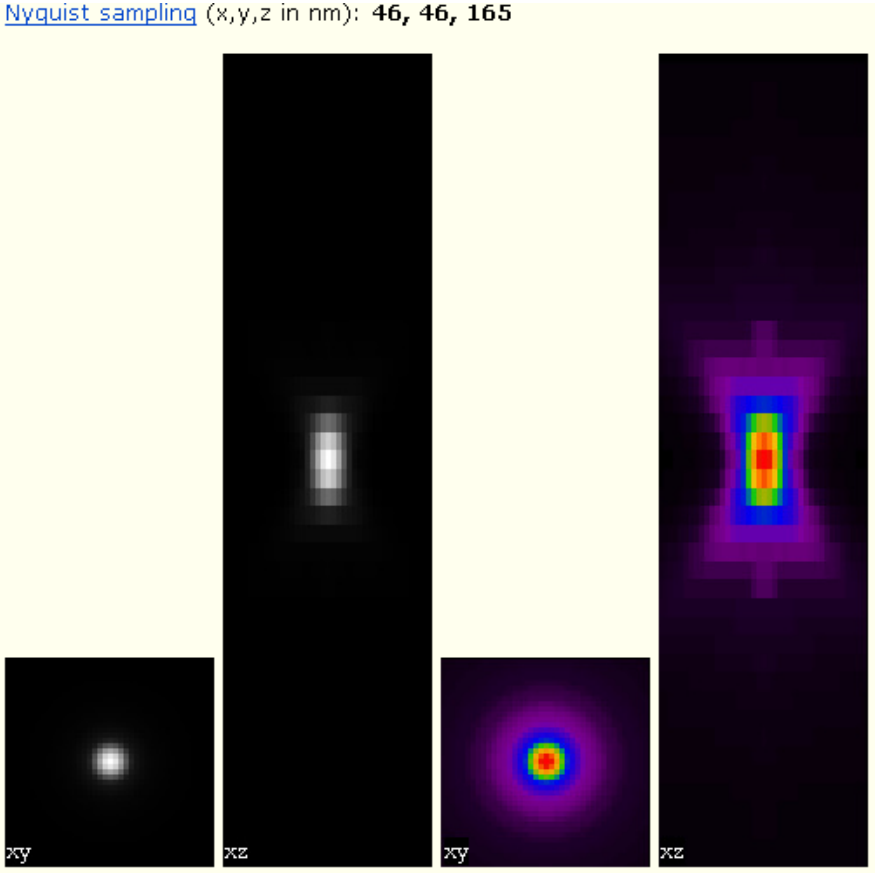
f: *Object Function*

b: *Offset Function*

I: *Image Matrix*

N: *Noise Function*

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



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

|-> Snell's Law

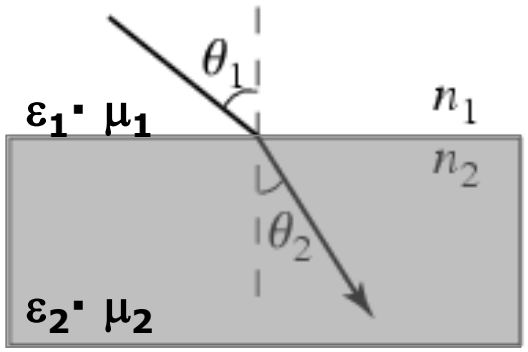
Index of refraction: $n = (\varepsilon \cdot \mu)^{1/2} = c/v$,

ε electric permittivity and μ magnetic permeability.

Snell's Law:

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

- **1.518 [Zeiss Oil]**
- **1.33 [Water]**
- **1.0008 [Air]**



|-> Refractive index

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]

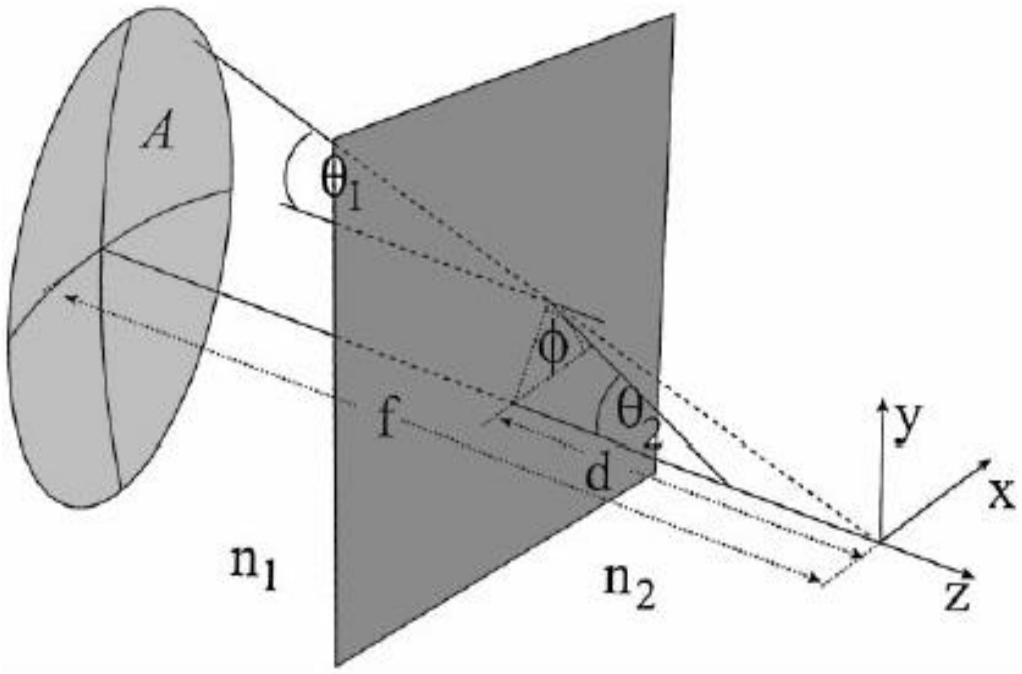
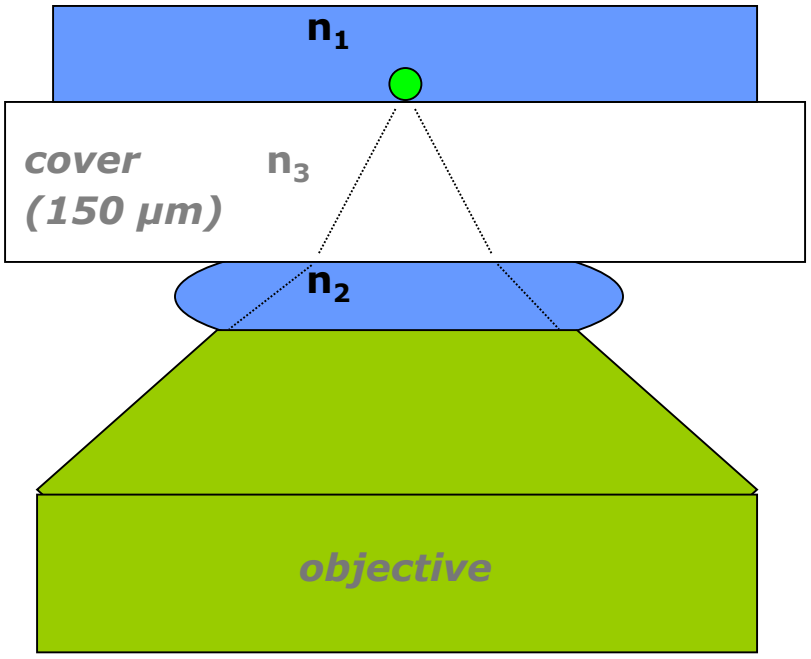


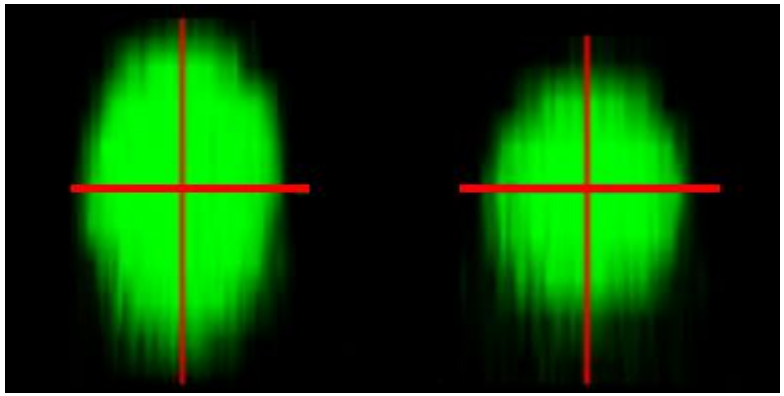
Fig. 1. Geometry when focusing with refractive index mismatch.

(Egner et al 1998)

|-> Refractive index

- **Micro-esfera*:*** ∅ = 6 µm





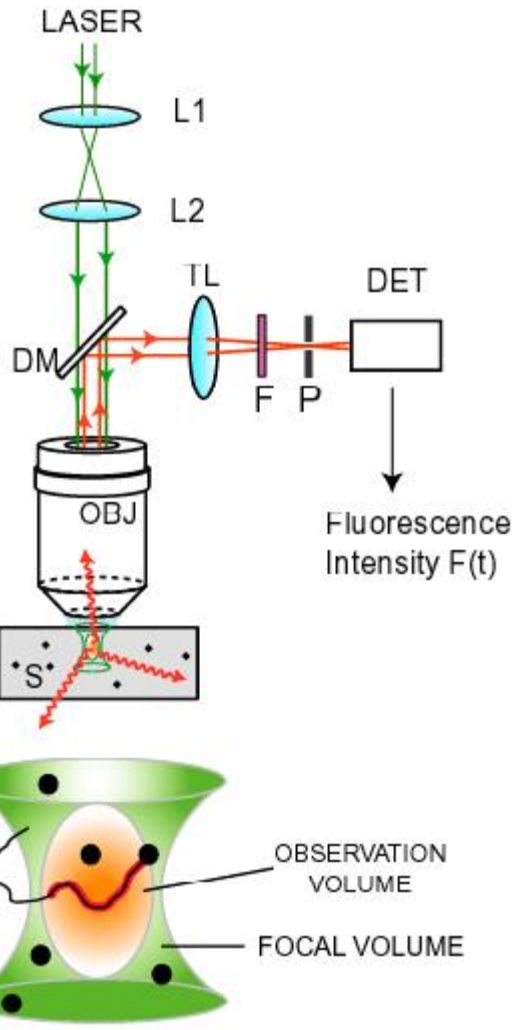
agua/aceite -- *aceite/aceite*

$n_1 \neq n_2$ $n_1 = n_2$

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

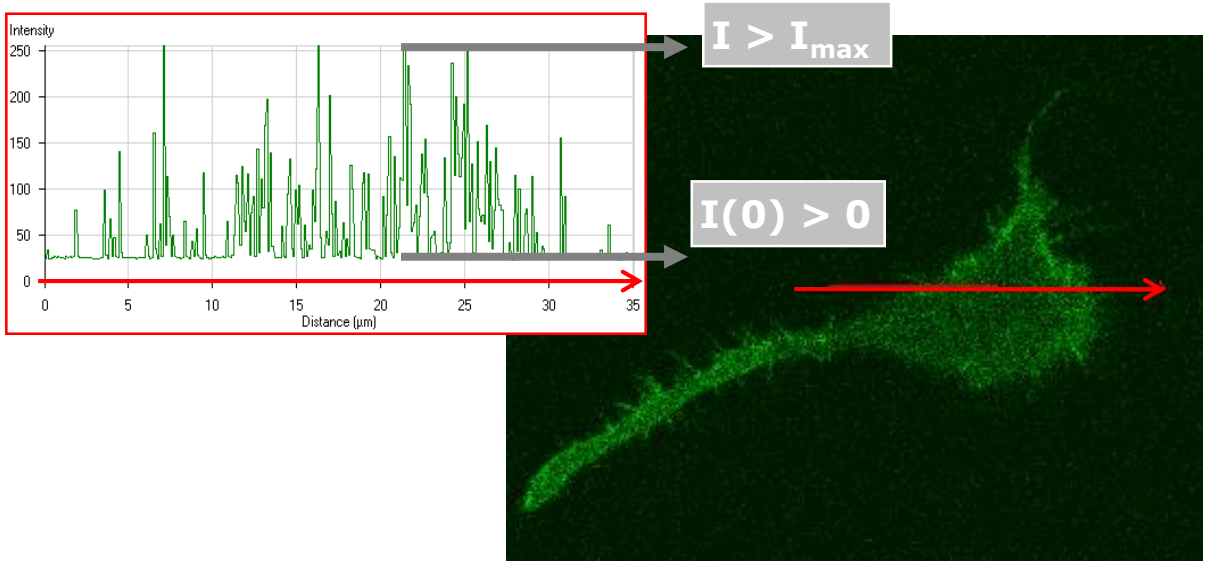
$n = n(\lambda)$!

|-> Resumen I

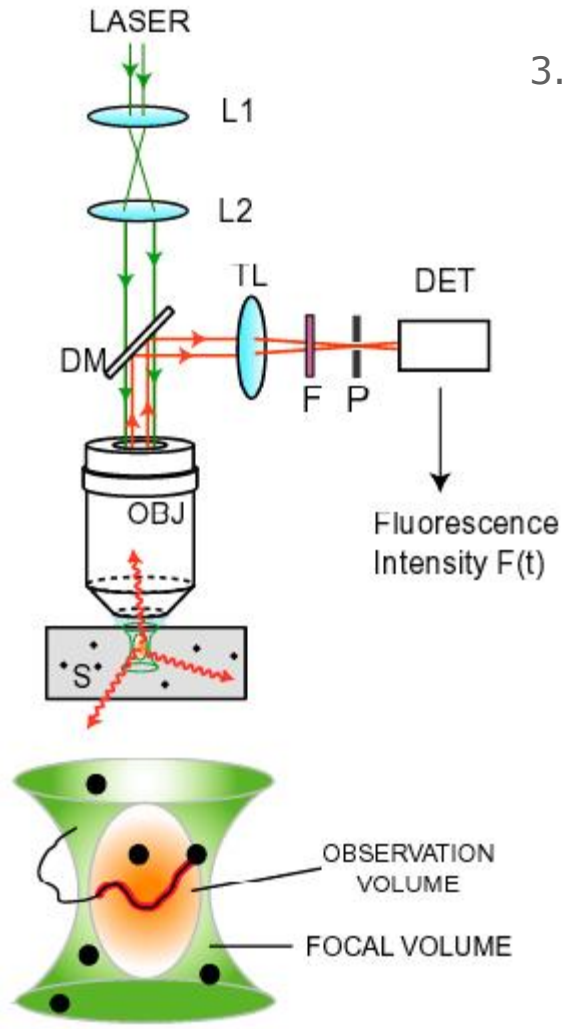


The observation volume (femtoliter) defined by the Point Spread Function must be considered as a mini-sprectrofluorimeter.

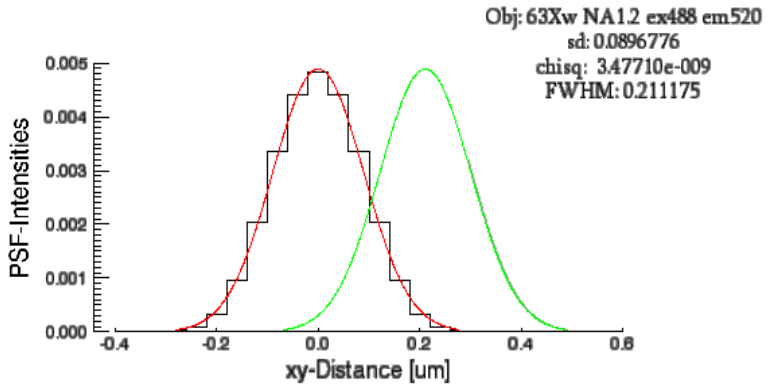
1. You need to consider the Offset I(0) in order to calibrate your signal $I(0) \geq 0$!
2. Never saturate the signal: $I \leq I_{max}$ (255 for 8 bit) !



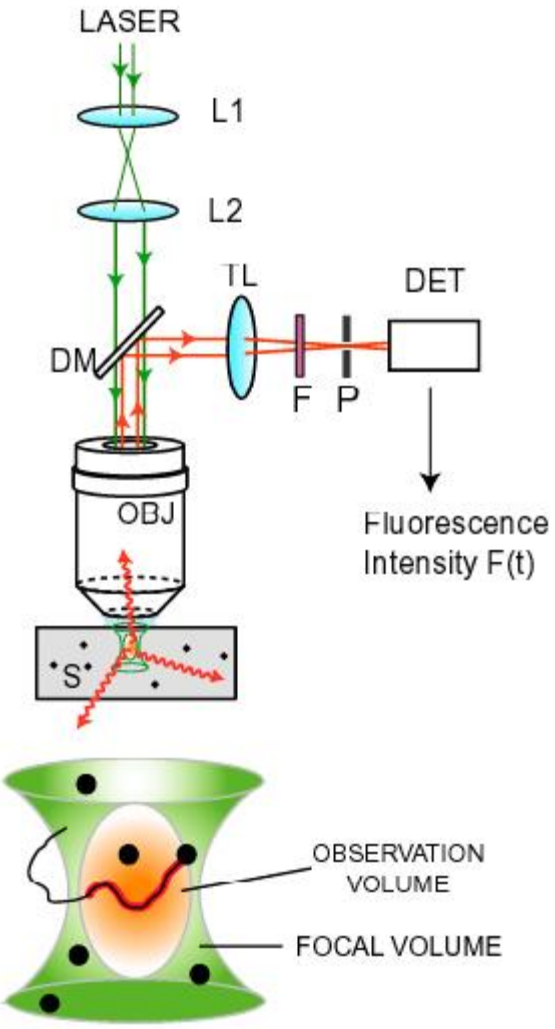
|-> Resumen II



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



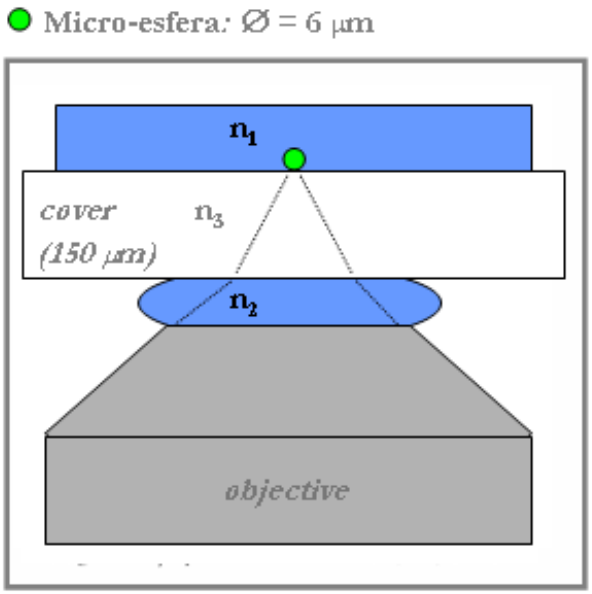
|-> Resumen III

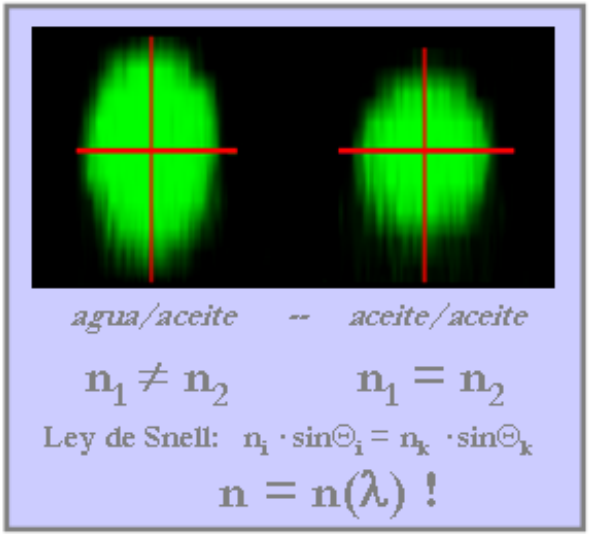


4. Use the right inmersion setup !

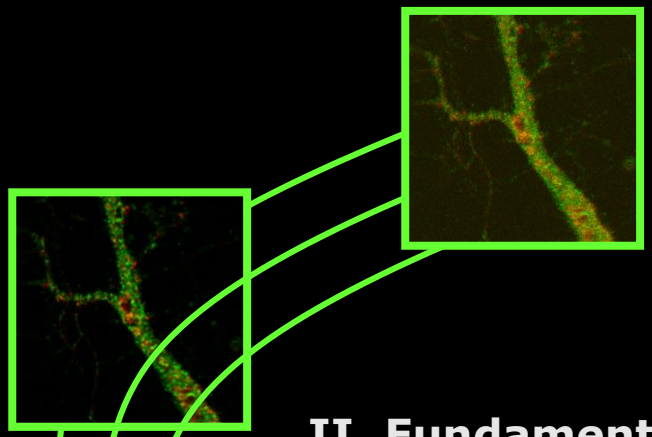
   n1 = n2 !

   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, prepasos y practicos !