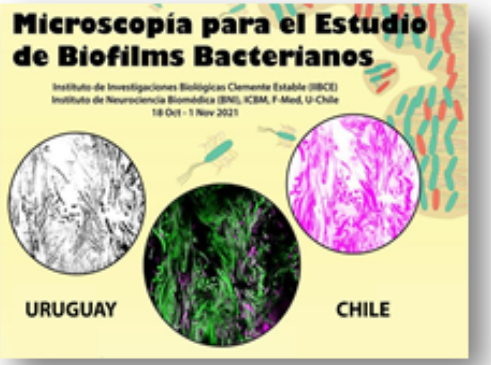
Welcome

Microscopía y Procesamiento de Imágenes



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Biomedical Neuroscience Institute (BNI)
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|-> Microscopy

- The Good, the Bad and the Ugly. Helen Pearson. 2007 Nature 447:138-140
- Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. Alison J. North. 2006 The Journal of Cell Biology, 172(1):9-18
- V Castañeda, M Cerda, F Santibáñez, J Jara, E Pulgar, K Palma, ... Computational methods for analysis of dynamic events in cell migration, Current molecular medicine 14 (2), 291-307

- Fluorescence Microscopy, From Principles to Biological Applications, Ulrich Kubitscheck (Editor), 2nd Edition, June 2017, Hardcover, ISBN: 978-3-527-33837-5
- https://www.zeiss.com/microscopy/int/cmp/edr/21/microscopy-for-dummies.html
- https://www.microscopyu.com/tutorials
- http://zeiss-campus.magnet.fsu.edu/tutorials/index.html
- https://www.leica-microsystems.com/science-lab/topics/basics-in-microscopy

- Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz 4.1 Introduction to Fluorescence
- A global view of standards for open image data formats and repositories JR Swedlow, P Kankaanpää, U Sarkans, W Goscinski, G Galloway, ..., Nature Methods, 1-7
- Jonkman, J., Brown, C.M., Wright, G.D. et al. Tutorial: guidance for quantitative confocal microscopy. Nat Protoc 15, 1585–1611 (2020), https://doi.org/10.1038/s41596-020-0313-9

|-> Microscopy

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 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. 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 subcellular 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; 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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http://www.jcb.org/cgi/content/full/jcb.200507103/DC1

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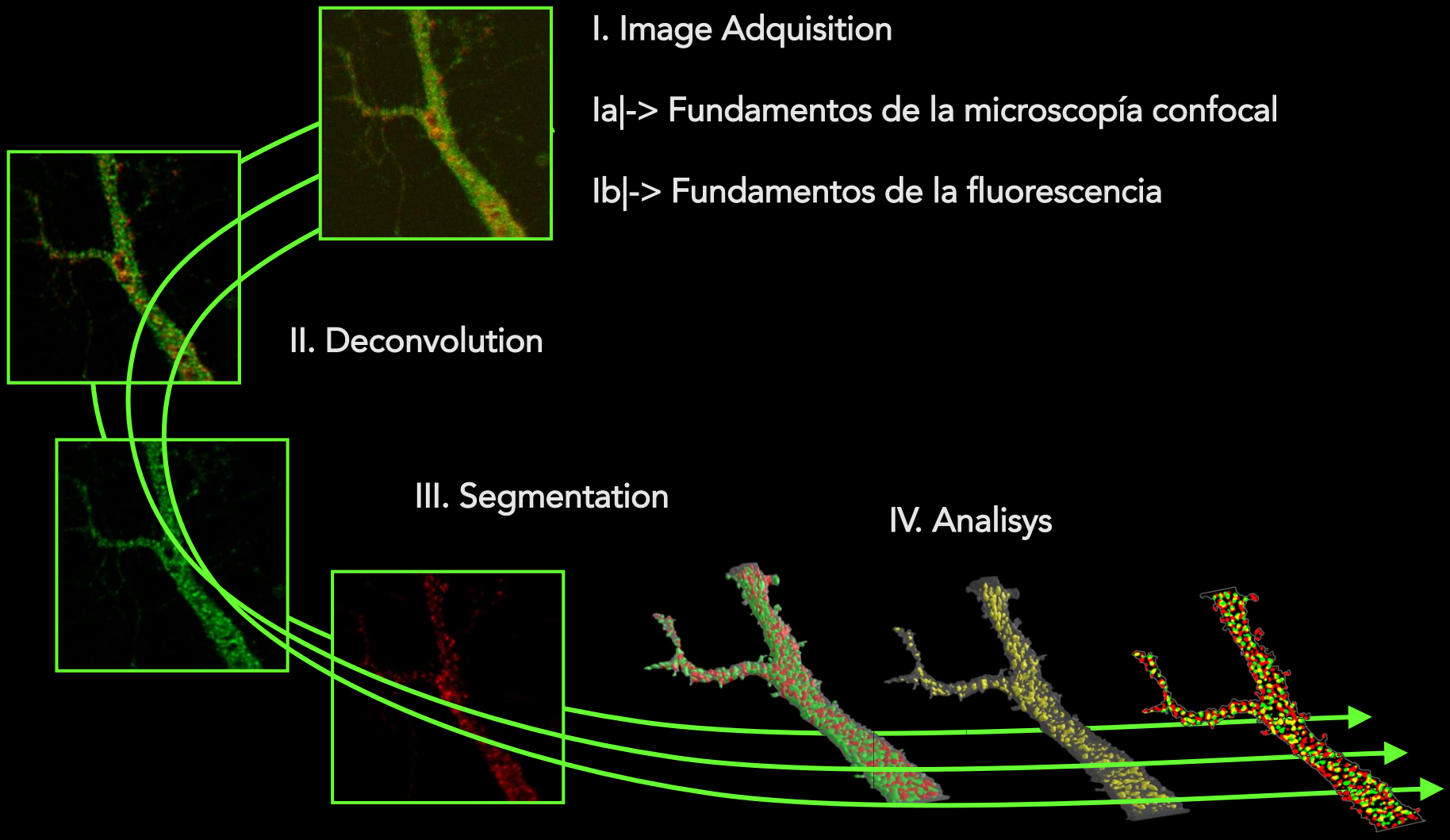
The objective lens is the most critical component of a microscope and yet few researchers grasp the differences between specific objective classes.

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.

Welcome


I. Image Adquisition
Ia|-> Fundamentos de la microscopía confocal
Ib|-> Fundamentos de la fluorescencia
II. Deconvolution
III. Segmentation
IV. Analisys

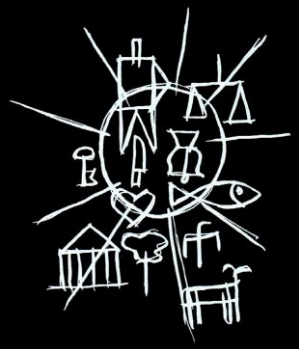
Laboratory for SCIAN Scientific Image Analysis

Welcome

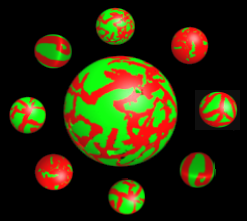
BIOMEDICAL NEUROSCIENCE INSTITUTE

Joaquín Torres García 1874-1949

Richard Feynman (1918-1988)

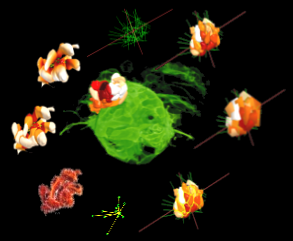


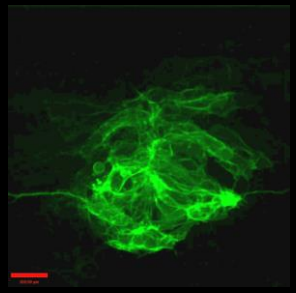
Mats Gustafson 2006 - 2011



Maria Goeppert-Mayer 1906-1972

René Descartes (1596-1650)



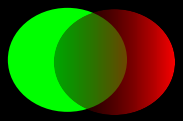


Ernst Abbe 1840- 2005

E Betzig

S Hell

W Moerner

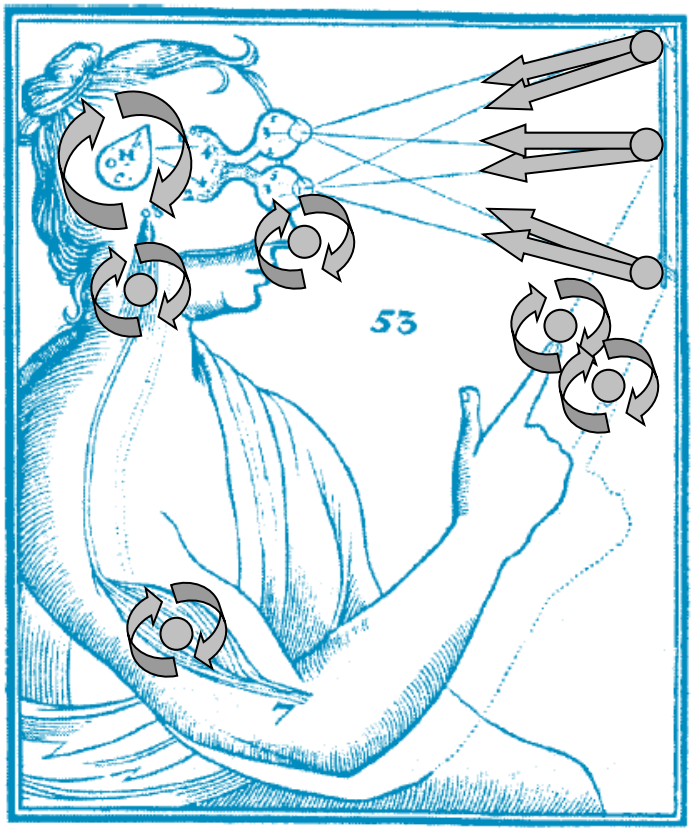


René Descartes (1596-1650)

... just look at the thing ...
¿ Human visual perception ?

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

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



glandula pinealis / pineal organ

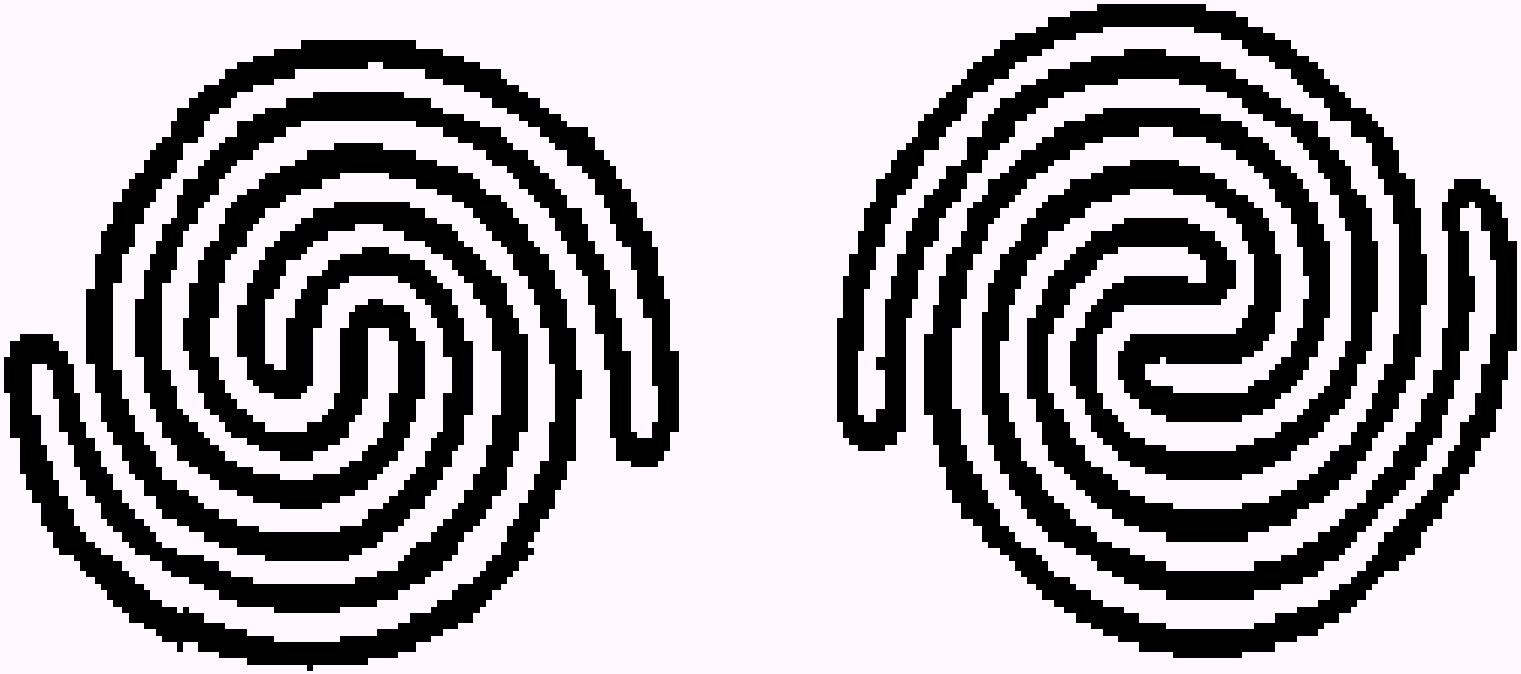
A combination of ...

1| direct signals ...

2| signals from other senses ...

3| feedback loops ...

... produce a symbolic representation of an object.



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

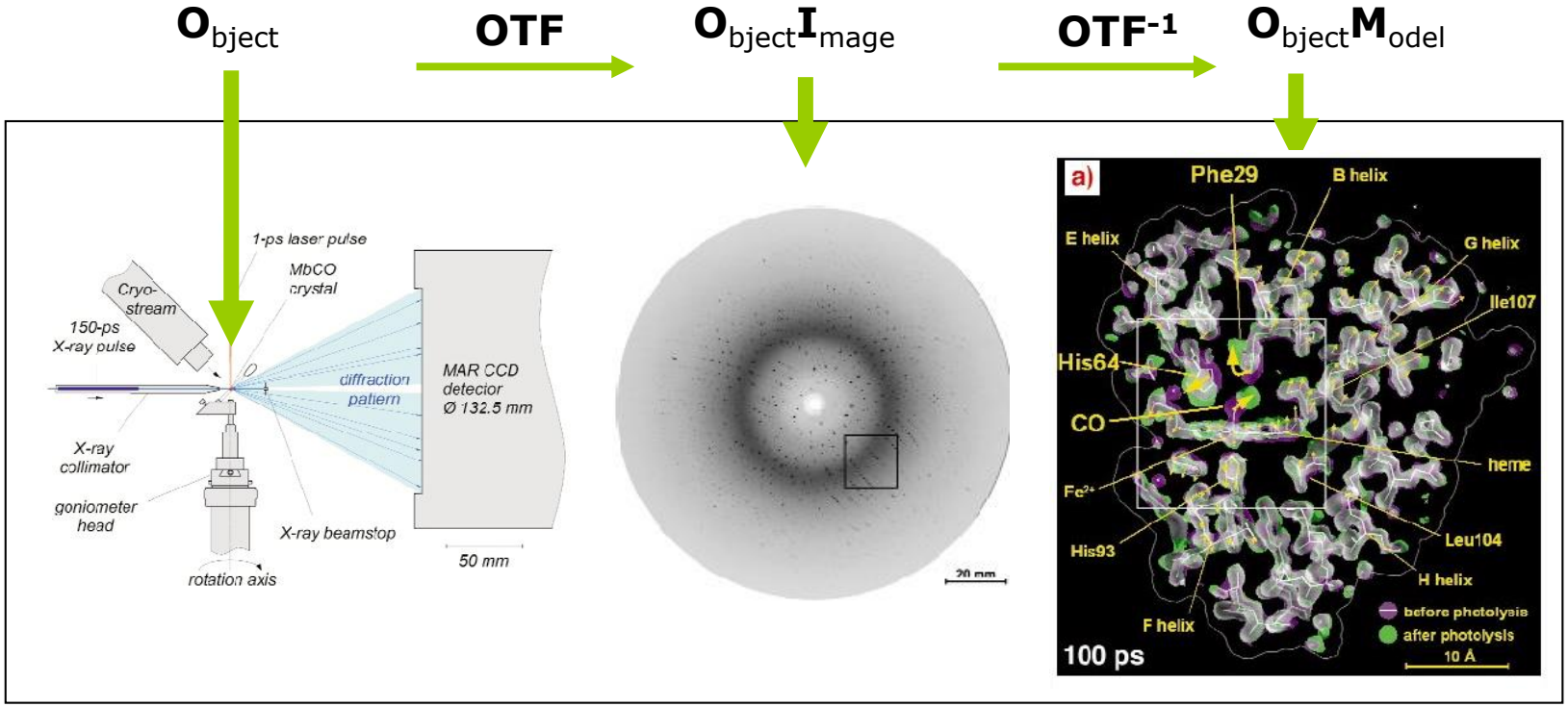
Make microscopes a hundred times more powerful and many problems of biology would be made very much easier."

Richard Feynman (1918-1988)

OTF: Object/Optical Transfer Function

Myoglobin in Action |Picosecond Laue Crystallography Diffraction Data

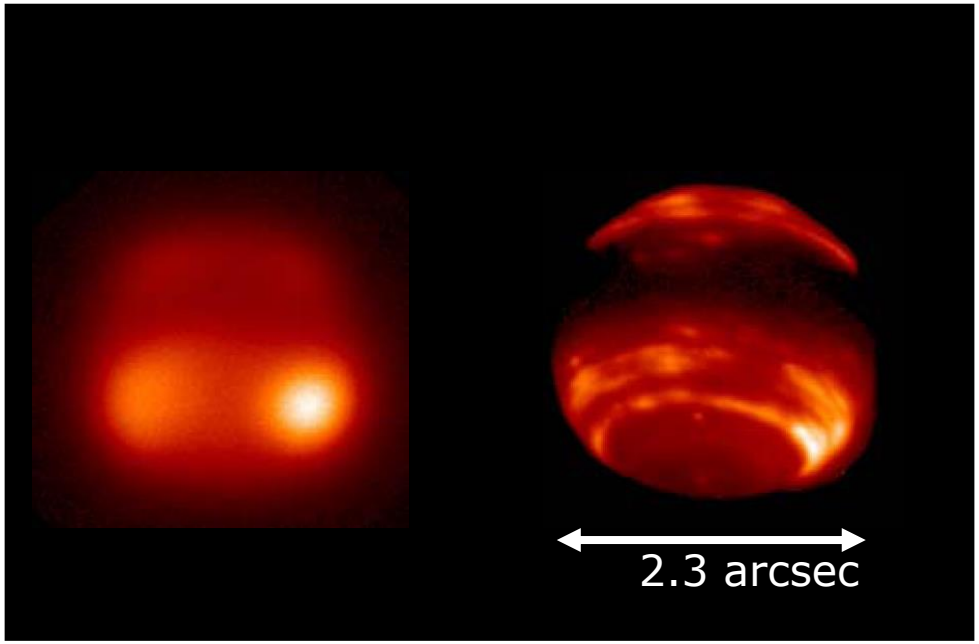
Schotte et al (2003) Science



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

Object → **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.

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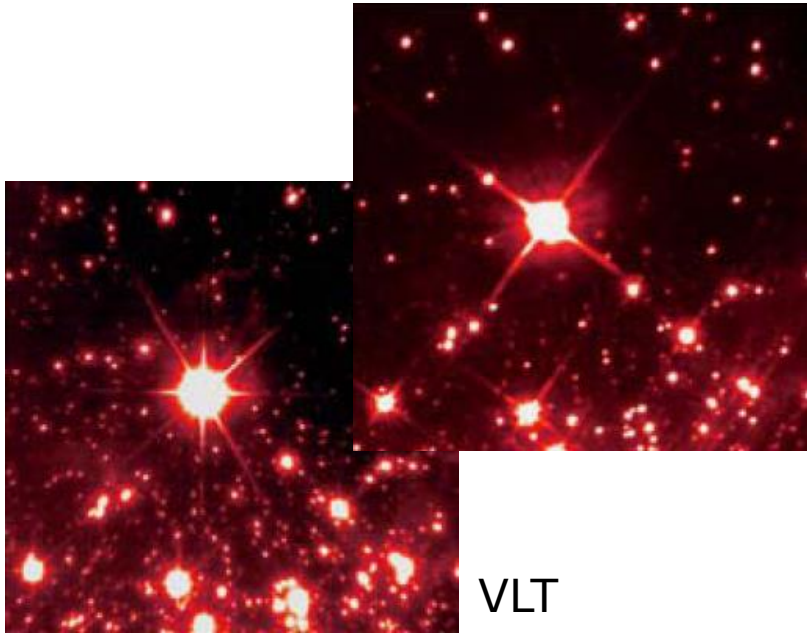


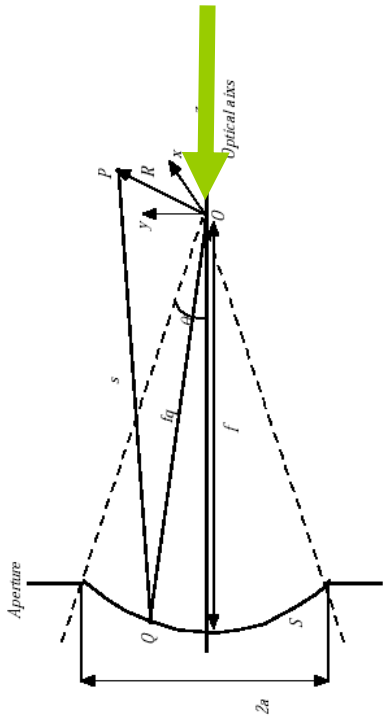
Hubble

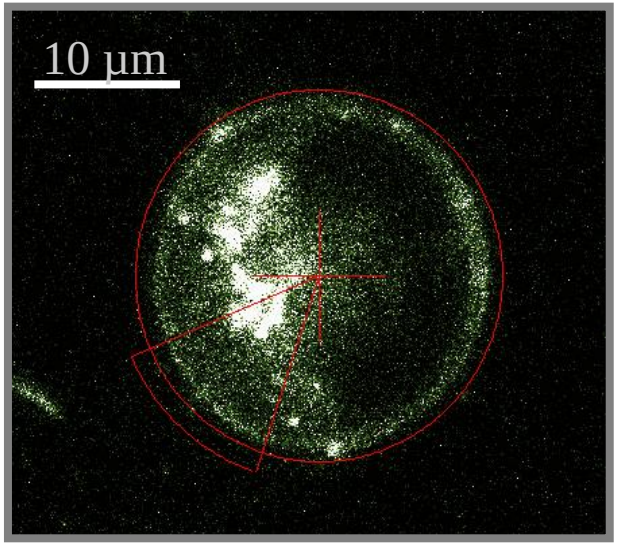
VLT

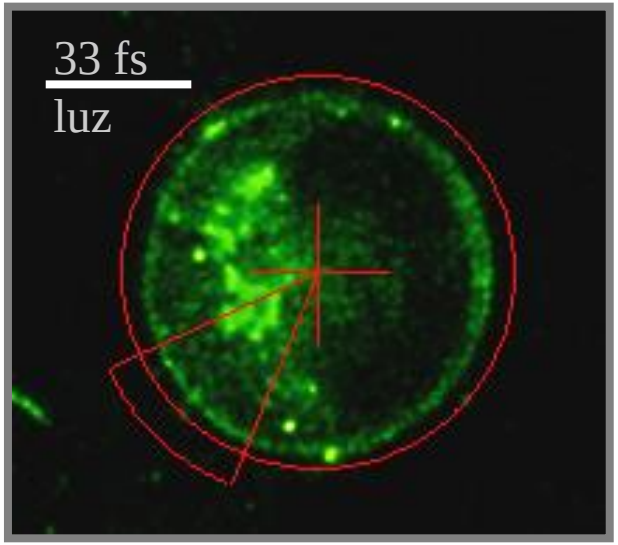
Confocal Microscopy | From Geometric Optics to Diffraction Theory

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.

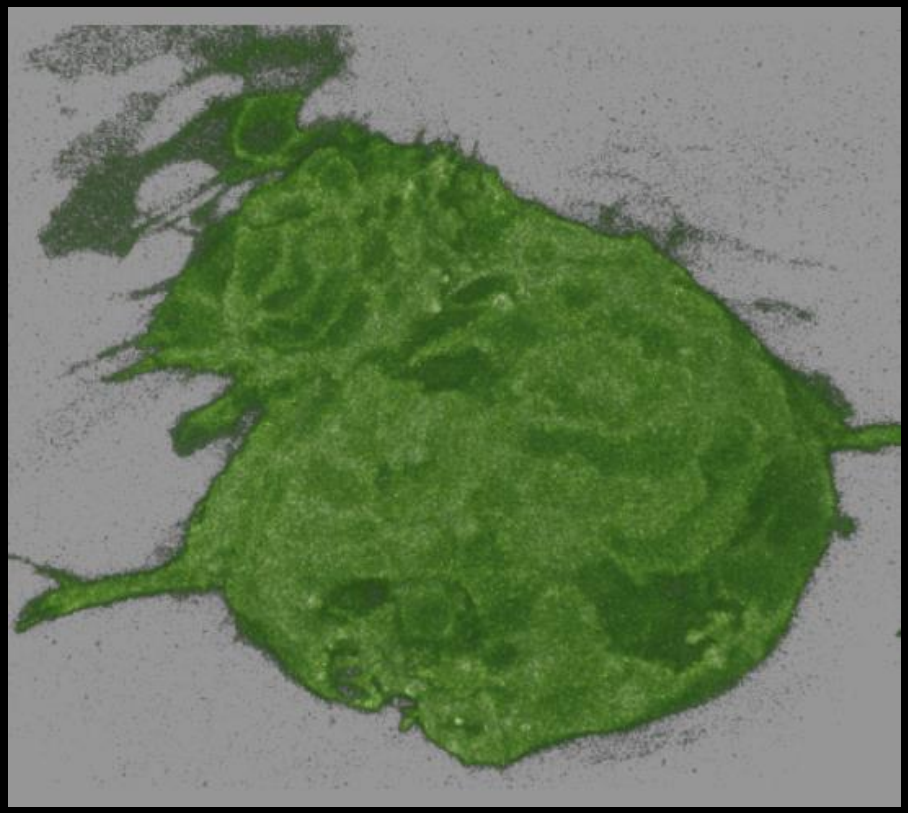
Object → **OTF** → **O**bject**I**mage → **OTF**$^{-1}$ → **O**bject**M**odel

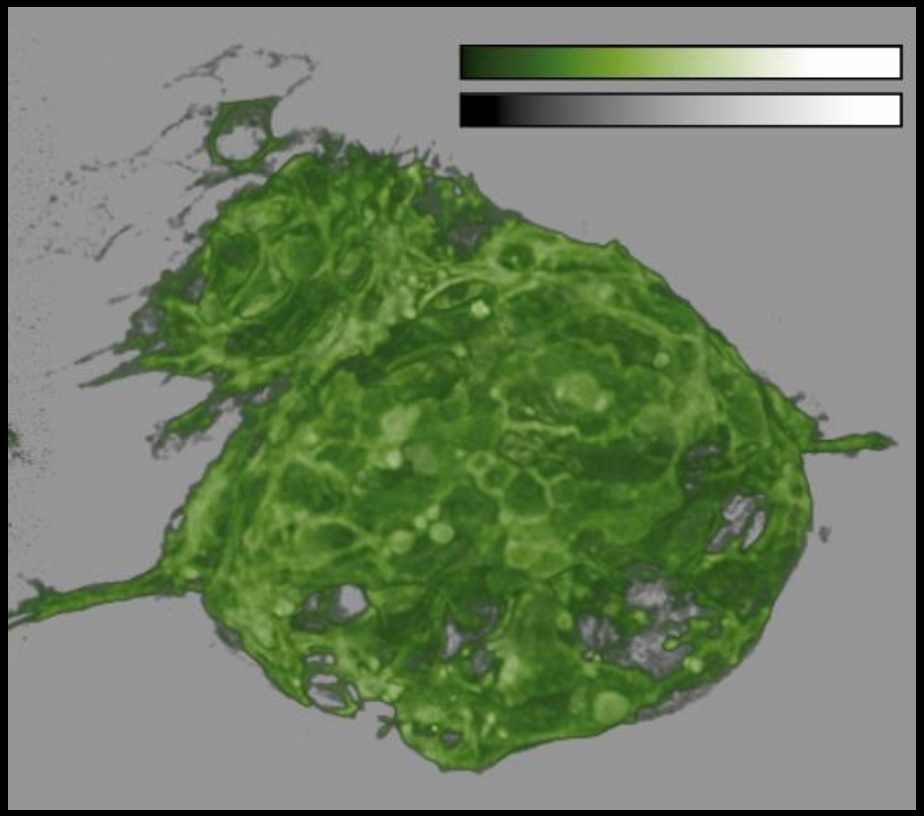






|-> Deconvolution





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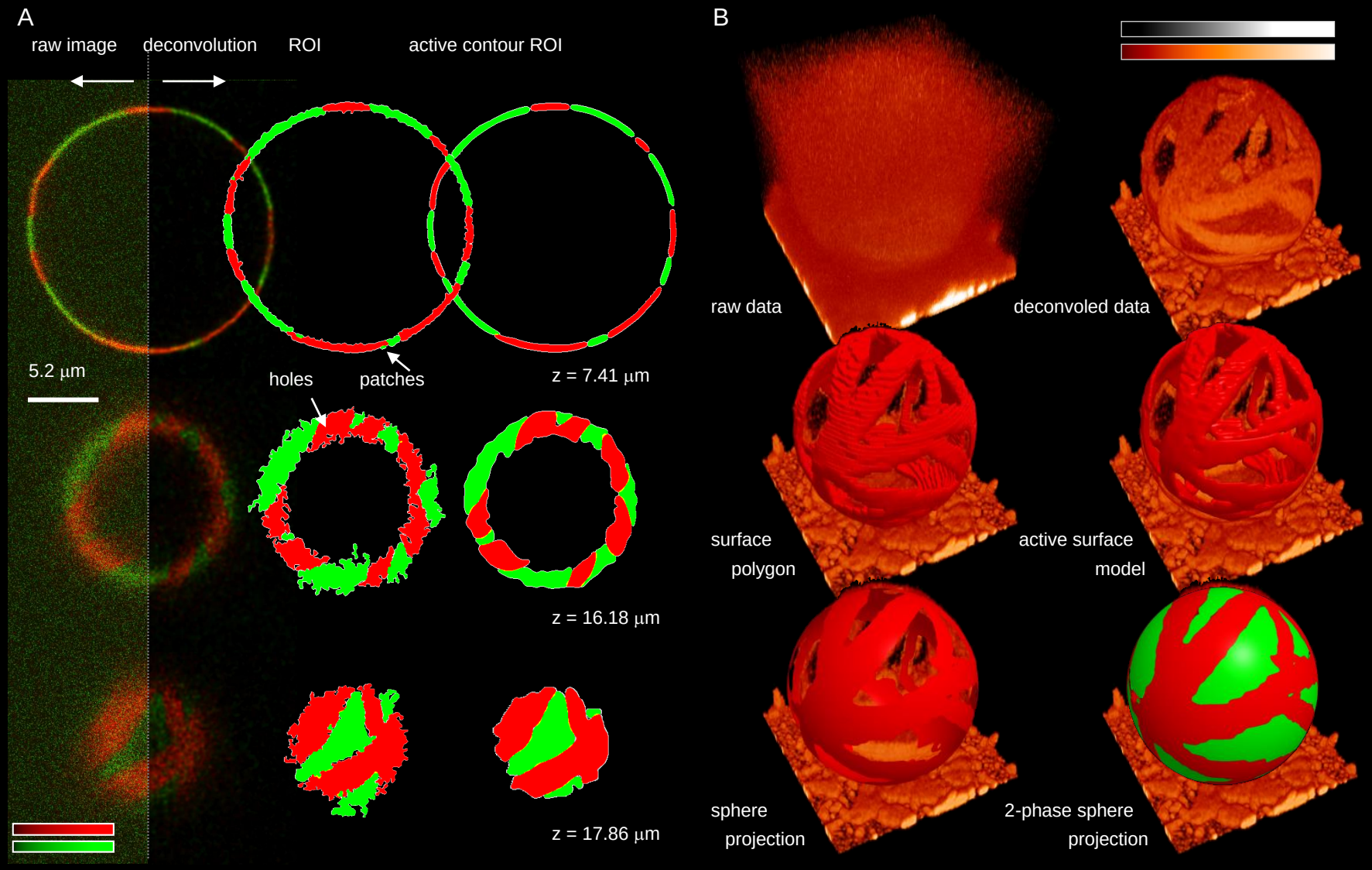
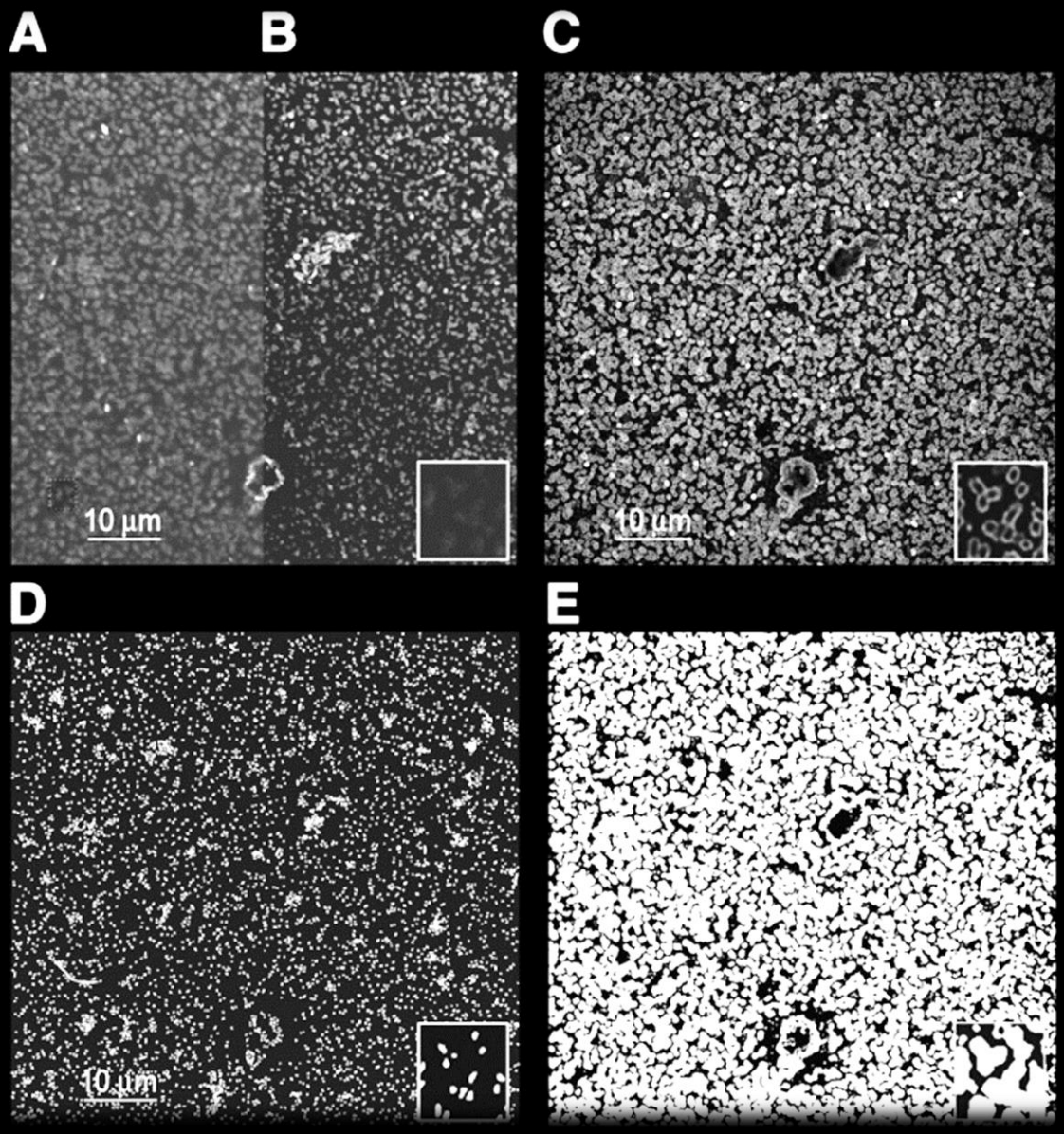
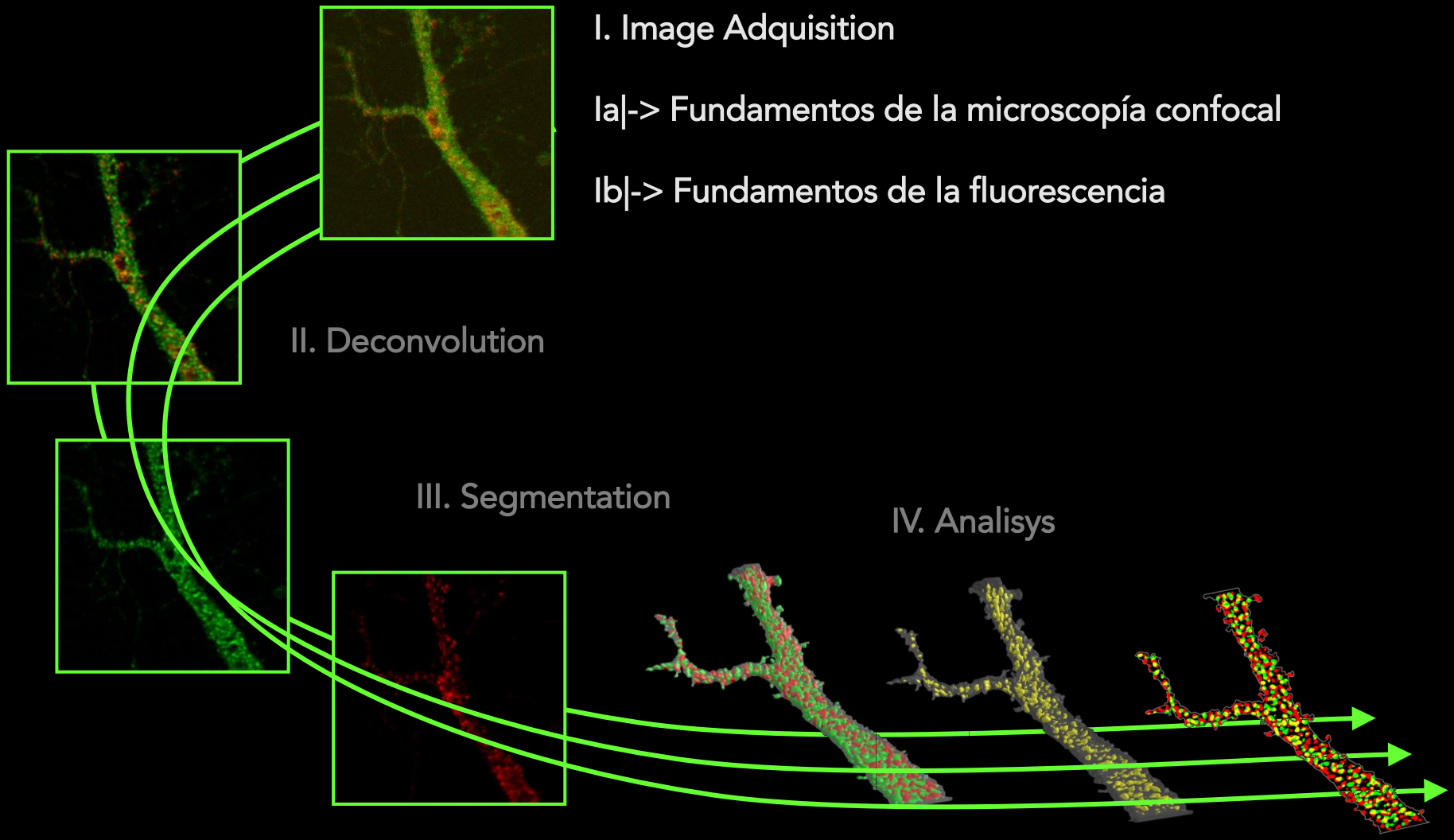


|-> Deconvolution

G. Schlapp et al. / Journal of Microbiological Methods 87 (2011)



Welcome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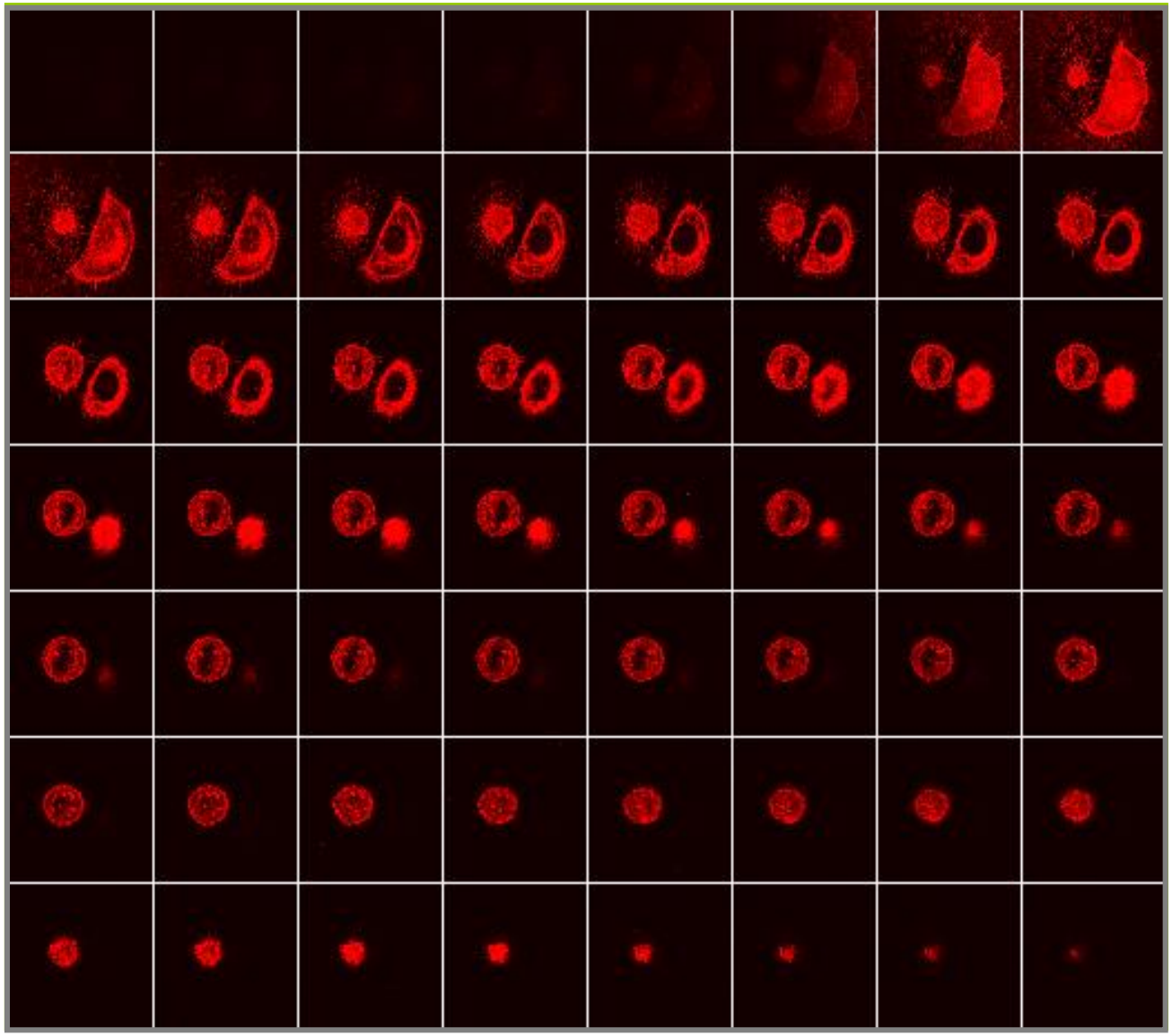
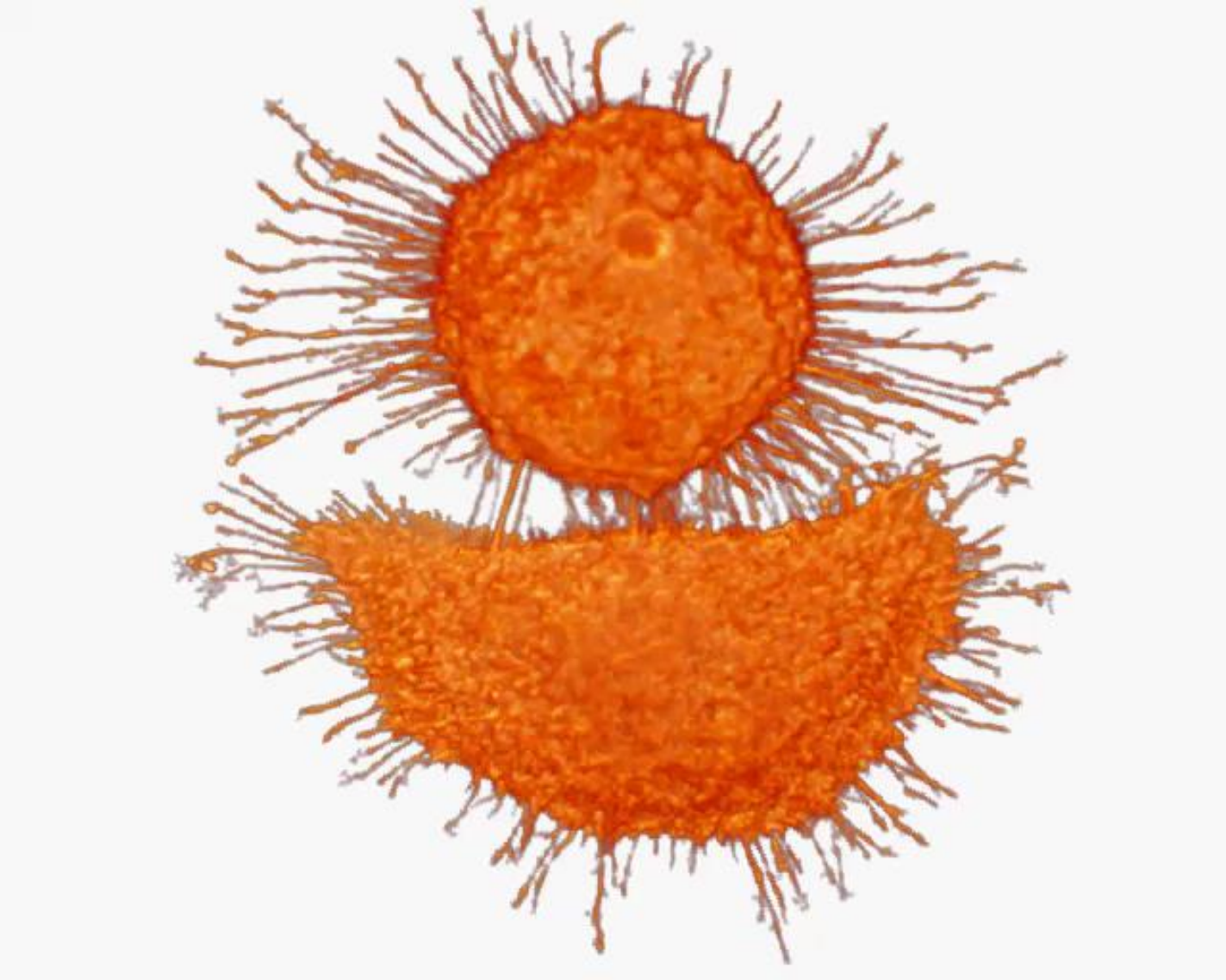
|-> Microscopy

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

|-> Microscopy



|-> Microscopy



|-> Fluorescencia I

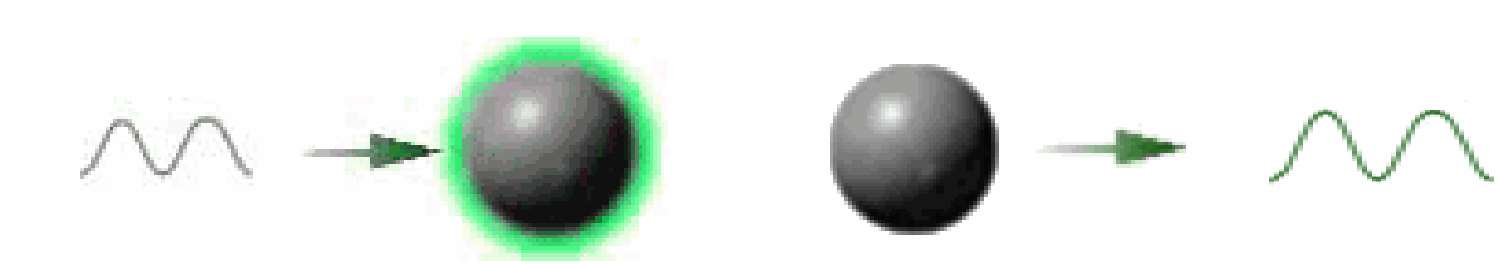
Luminescencia:

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

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

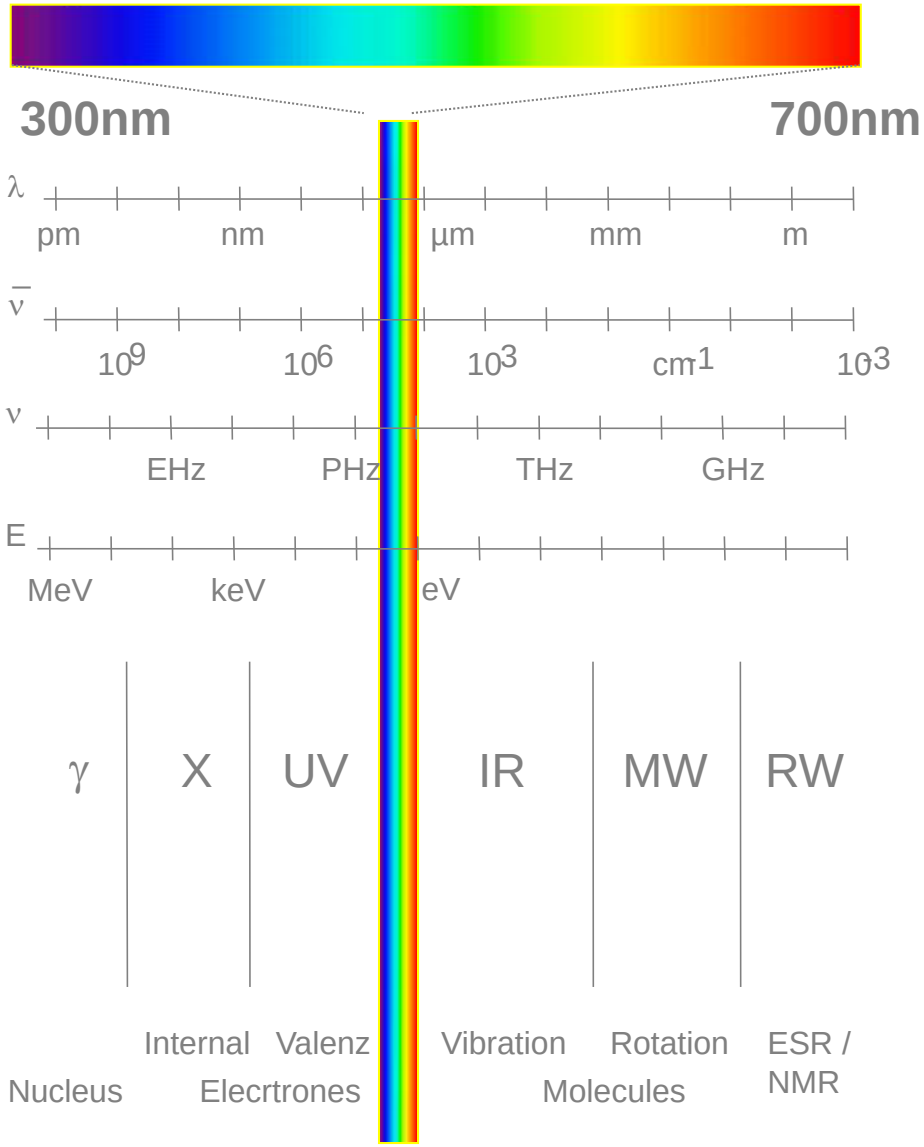
producen cambios ...
- **espectrales**
- **tiempos de vida**
- **polarización**
- **intensidad ...**

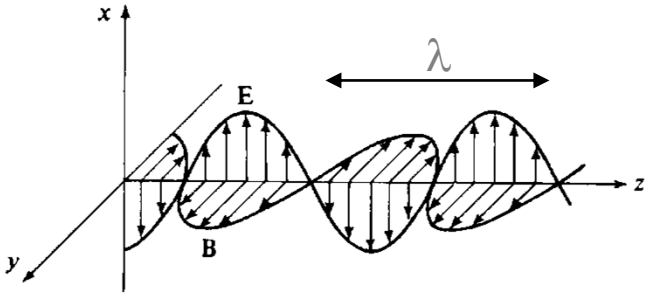
← Δt →



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 |

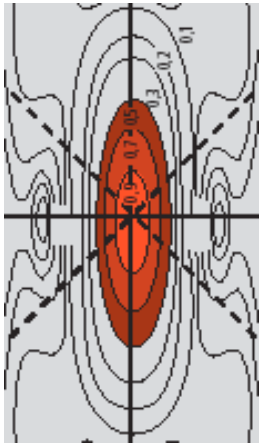
Niveles: —— vibracionales

▬ electrónicos

-> **calor**

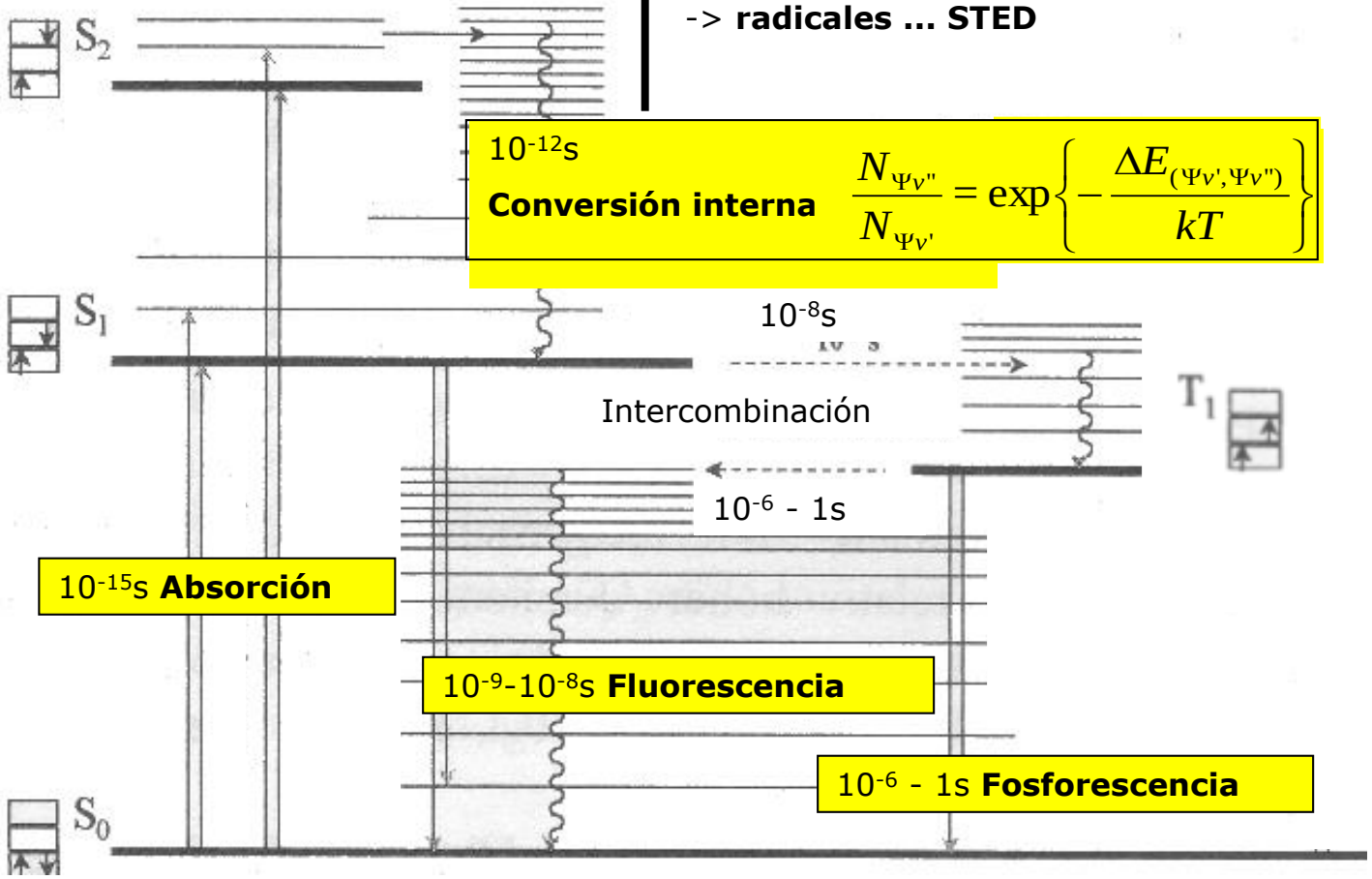
-> **transferencia de energía (FRET)**

-> **radicales ... STED**

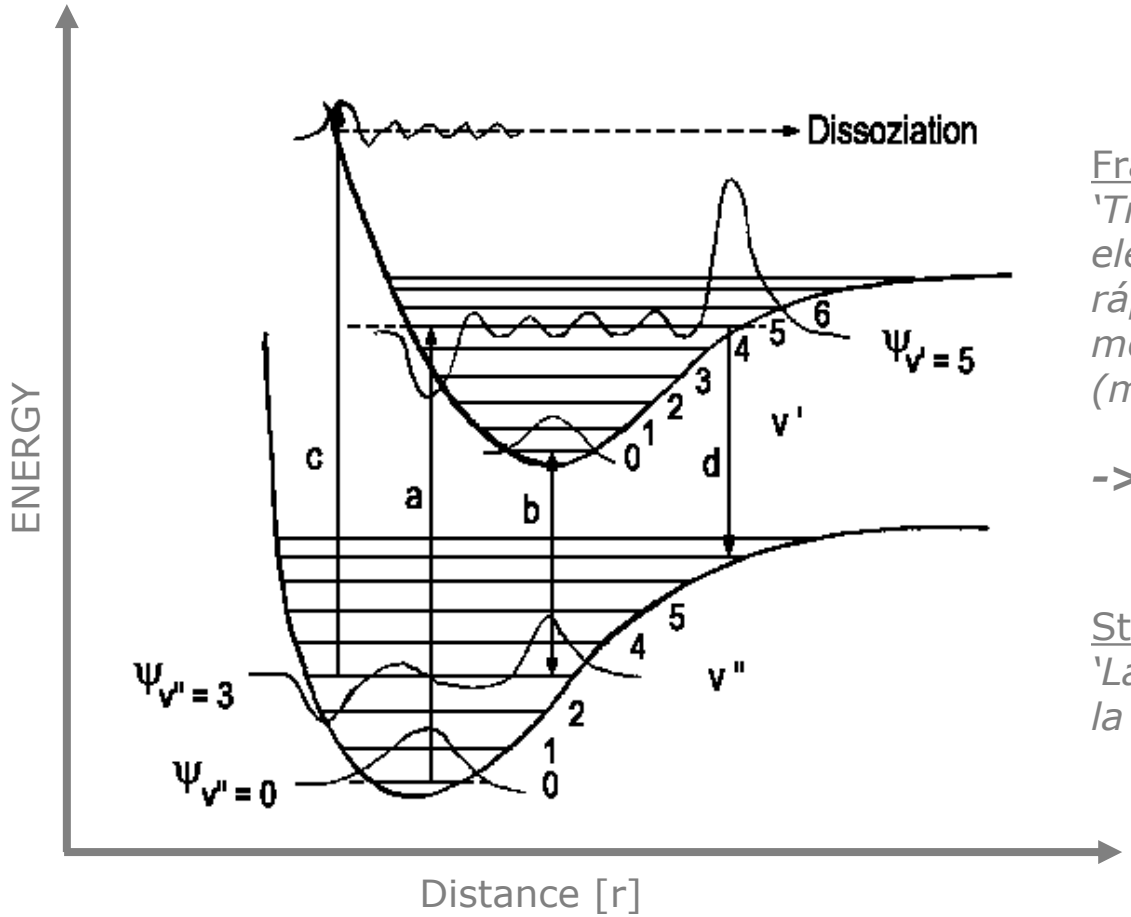


z

x/y



|-> Franck Condon



Frank Condon **:**

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

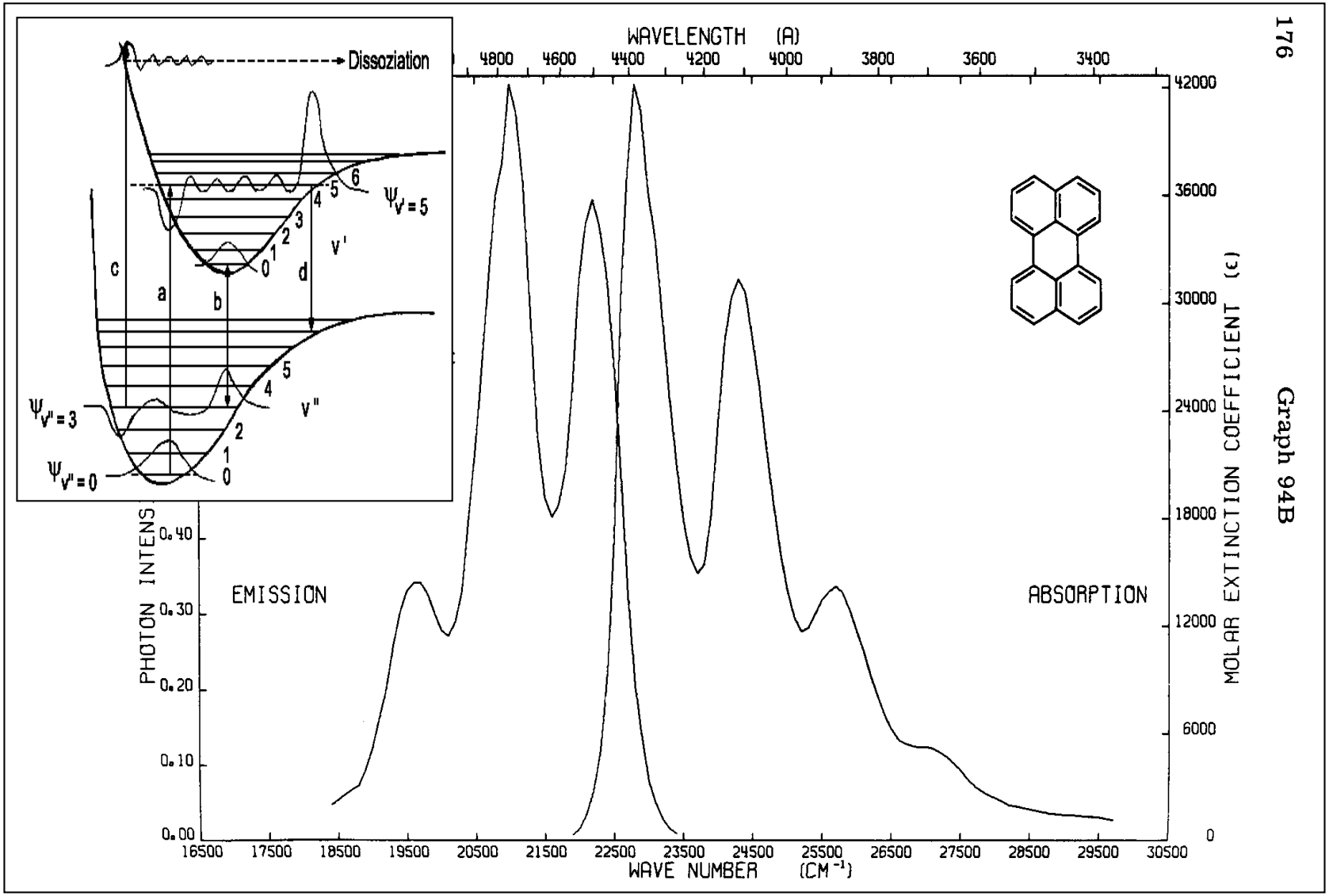
(masa$_{electron}$ / masa$_{atom}$: 1 : 2000)

-> 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, Joseph R. Lakowicz 4.1 Introduction to Fluorescence

|-> Quenching, Bleaching ...

|-> Polarisation ...

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

|-> Förster Resonance Energy Transfer ...