

Curso Binacional 2023
Uruguay - Chile

Microscopía para el Estudio de Biofilms Bacterianos

Instituto de Investigaciones Biológicas Clemente Estable (IIBCE)
Instituto de Neurociencia Biomédica (BNI), ICBM, F-Med, U-Chile

2 - 6 de octubre - Teórico
9 - 13 de octubre - Práctico Uy,
10 de octubre - 3 de noviembre - Práctico Cl.



Prof. Dr. Steffen Härtel

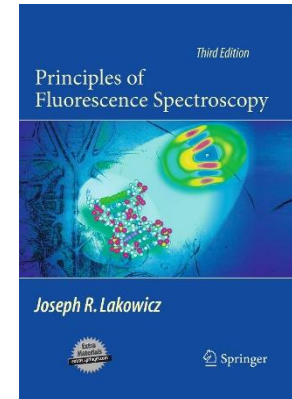
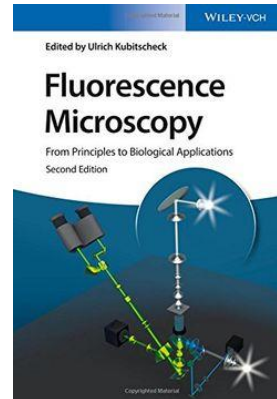
www.scian.cl / www.cimt.cl / www.cens.cl www.bni.cl / www.rsdue.cl

Laboratory for Scientific Image Analysis (SCIAN-Lab)
Centro de Informática Médica y Telemedicina (CIMT)
Centro Nacional en Sistemas de Información en Salud (CENS)
Biomedical Neuroscience Institute (BNI)
Red de Salud Digital de Universidades del Estado (RSDUE)
Institute of Biomedical Sciences (ICBM)
Anatomy and Developmental Biology Program
Escuela de Postgrado
Facultad de Medicina, Universidad de Chile



FACULTAD DE
MEDICINA
UNIVERSIDAD DE CHILE

• Links & Literatura



- 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 Kubitschek (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>
- https://www.youtube.com/playlist?list=PLG8B8Uyfh7-Azddns5nv_kZU1YQGD4bBg
- 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>

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 non-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 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 lookout 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 microscopes. 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 in electron microscopy (Hayat, 2010), 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 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 Recalcan et al., 1995; Alder, 1999), although glutaraldehyde fixation often reduces antigenicity and increases background and 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, Triton treatment produces smaller holes in

Cytoskeletons merge during fixation

JCB

© The Rockefeller University Press 0022-0919/06/172(1):9-18
DOI: 10.1083/jcb.172.1.9-18
http://www.jcb.org/cgi/doi/10.1083/jcb.172.1.9-18

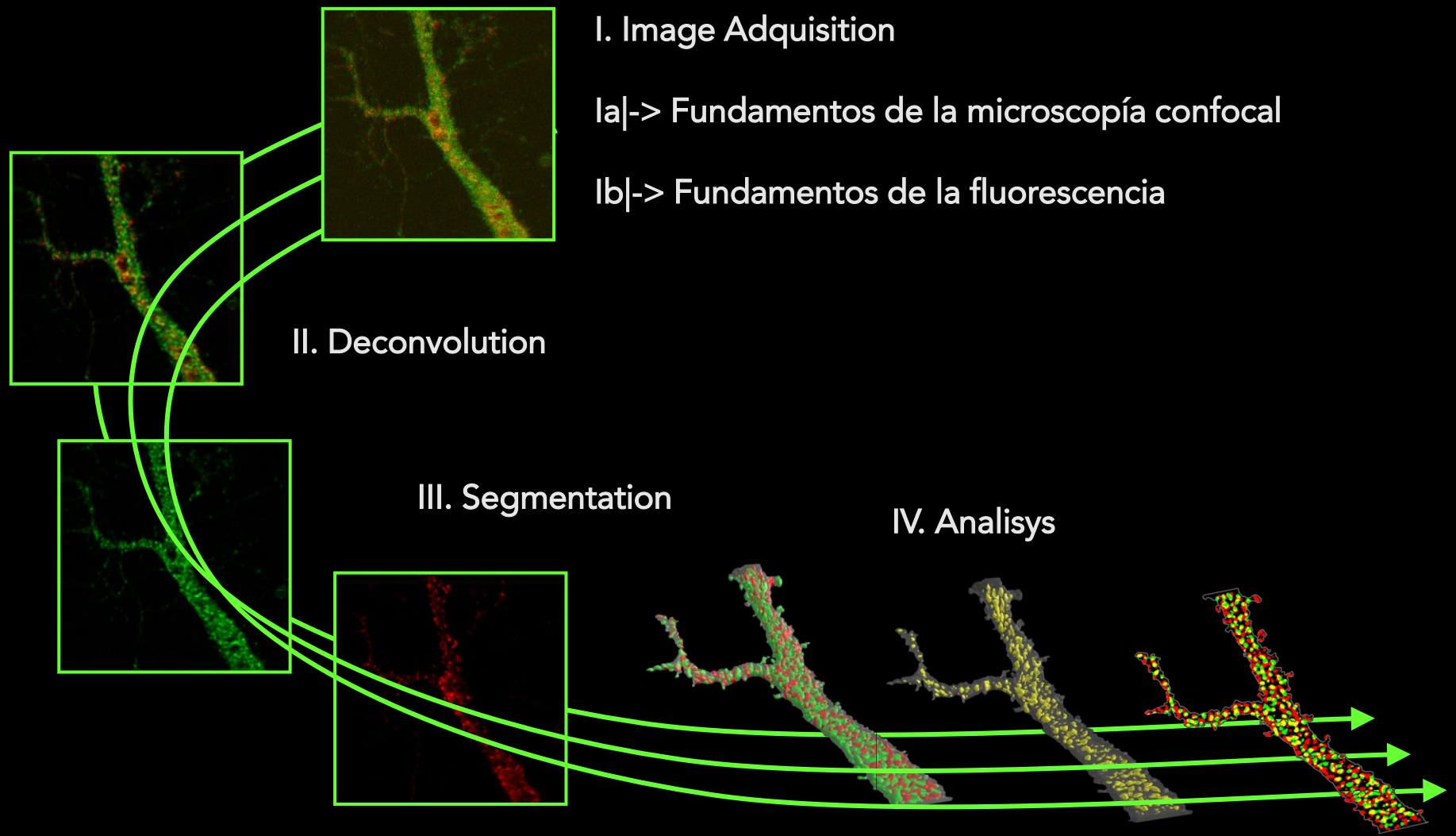
The objective lens is the most critical component of a microscope and yet few researchers grasp the differences between specific objective classes.

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*.

- 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





Joaquín Torres García 1874-1949



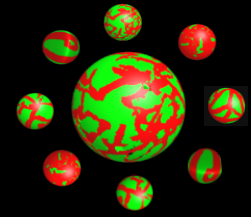
Richard Feynman (1918-1988)



María Goeppert-Mayer 1906-1972



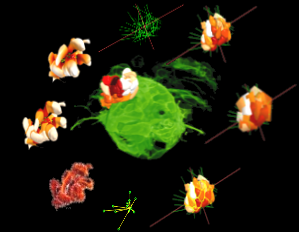
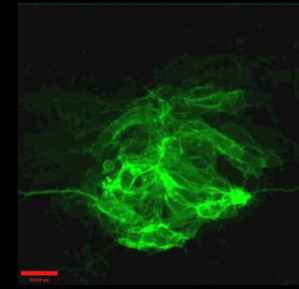
Mats Gustafson 2006 - 2011



René Descartes (1596-1650)



Ernst Abbe 1840- 2005



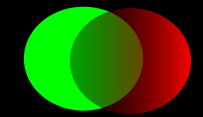
E Betzig



S Hell



W Moerner



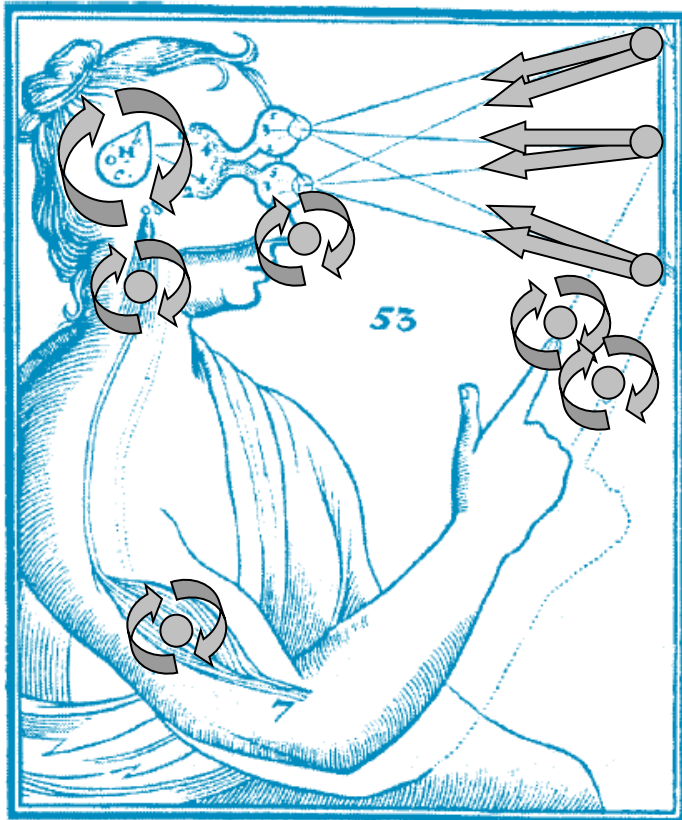


René Descartes (1596-1650)

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

Treatise of man (~ 1637)

Passions of the soul (~ 1649)



A combination of ...

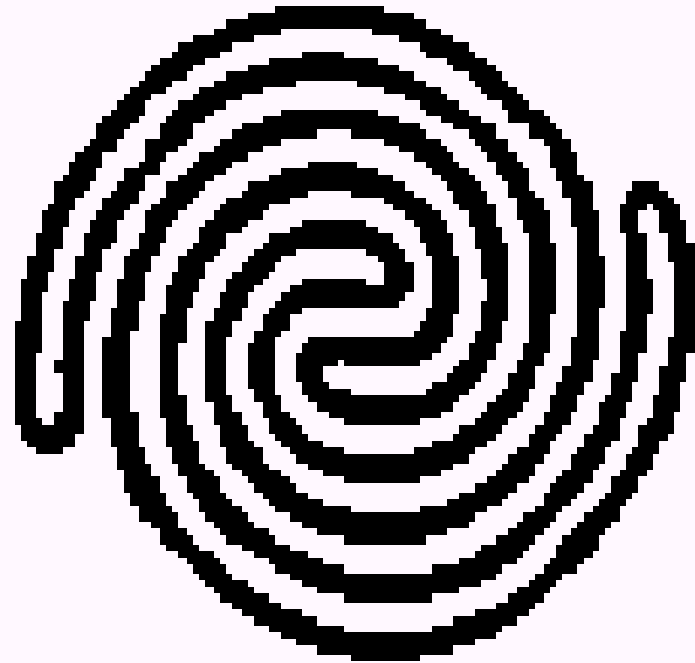
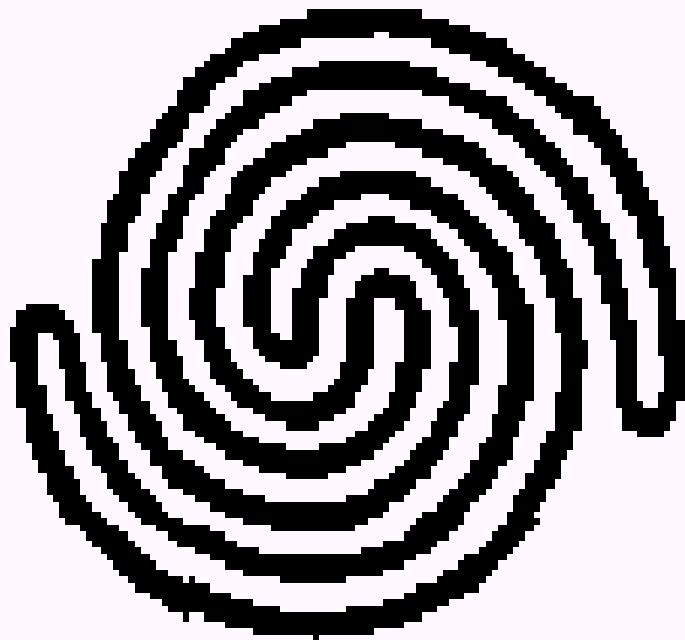
1| direct signals ...

2| signals from other senses ...

3| feedback loops ...

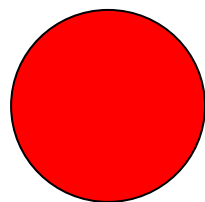
**... produce a symbolic
representation of an object.**

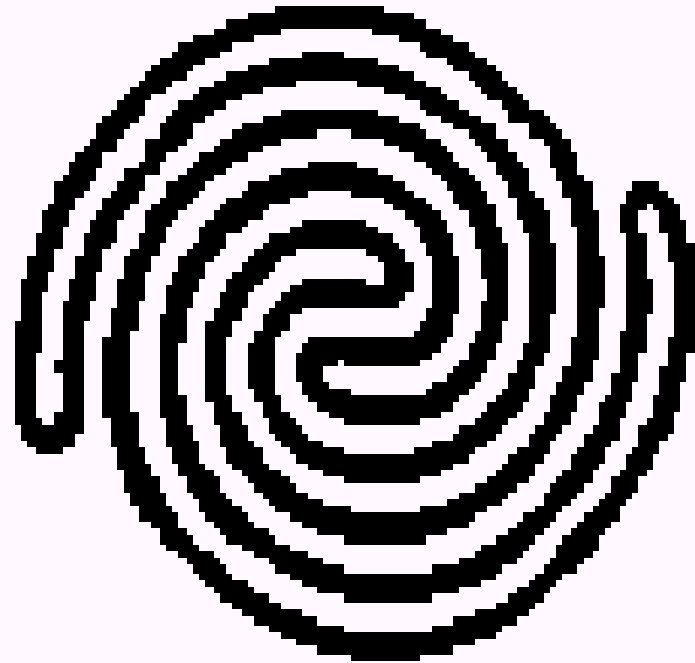
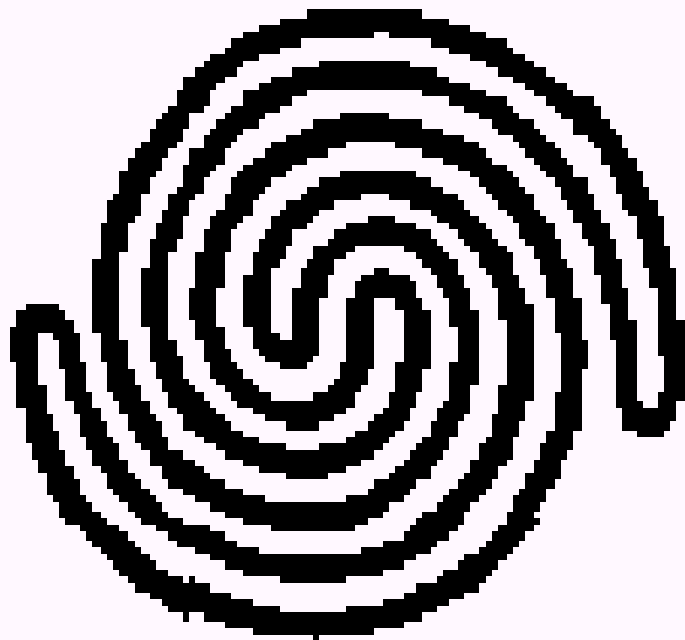
glandula pinealis / pineal organ













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

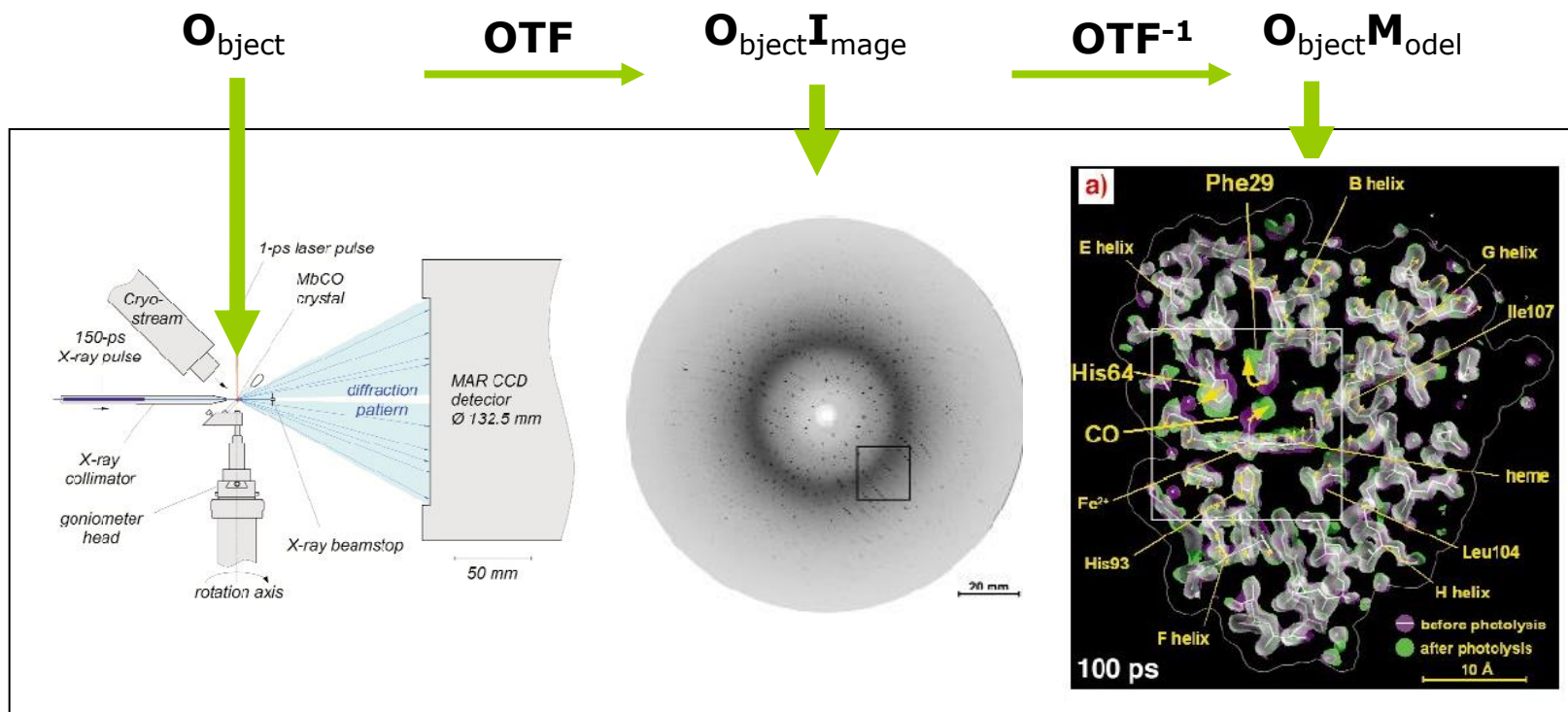
Make microscopes a hundred times more powerful and many problems of biology would be made very much easier.”

Richard Feynman (1918-1988)

OTF: Object/Optical Transfer Function

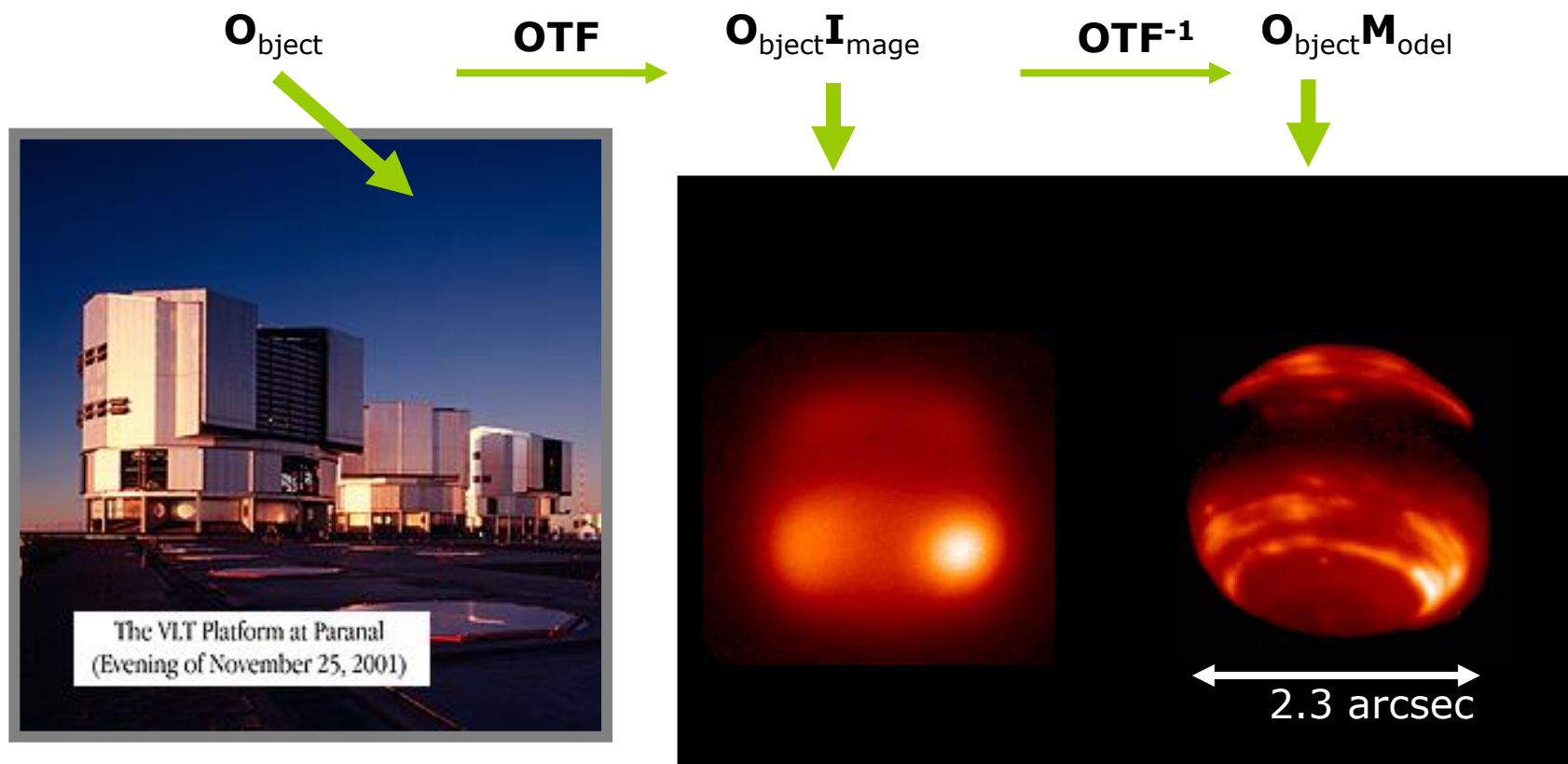
Myoglobin in Action | Picosecond Laue Crystallography Diffraction Data

Schotte et al (2003) Science

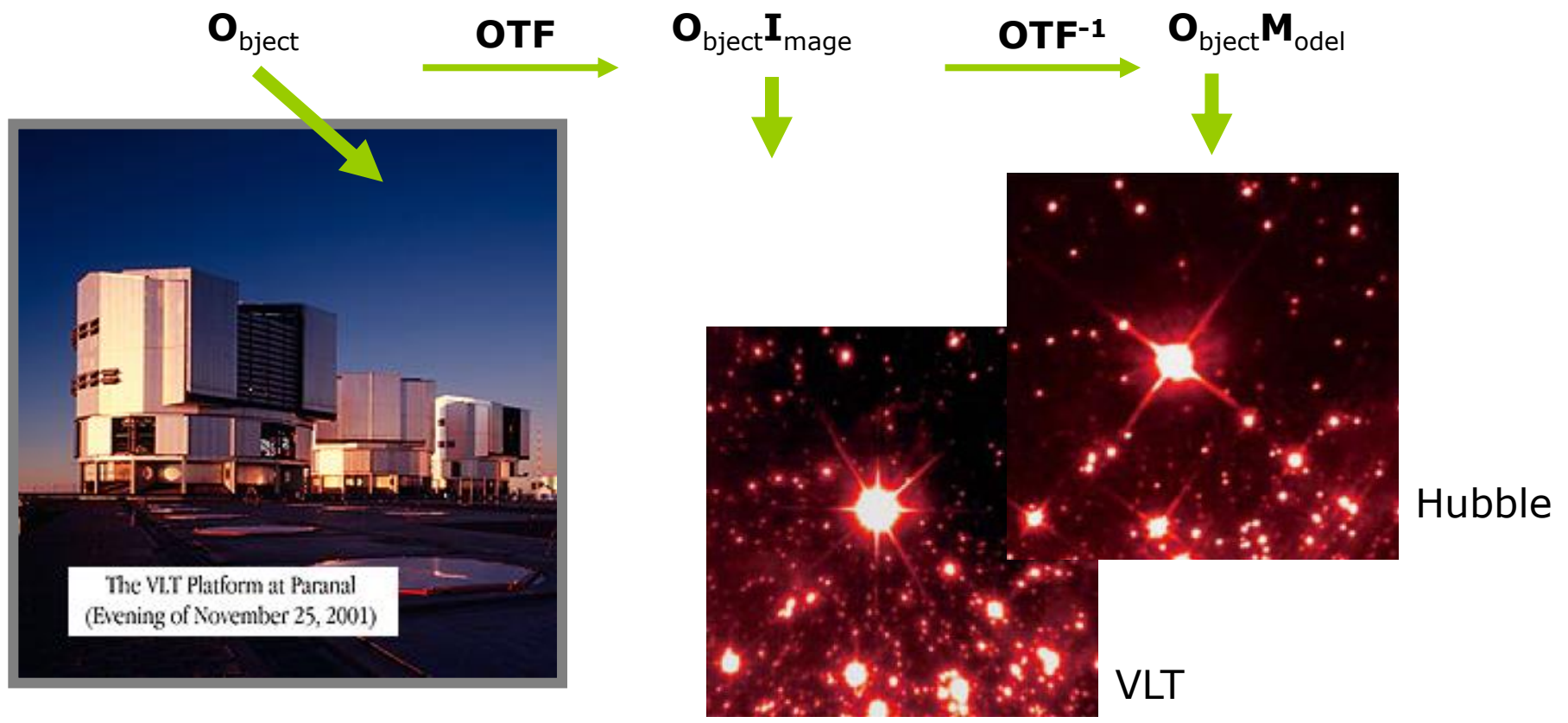


<http://www.youtube.com/watch?v=lnKIBZYarzM>

Diffraction Limited Resolution for a 10m telescope $\sim \lambda/D \sim 0.01$ 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

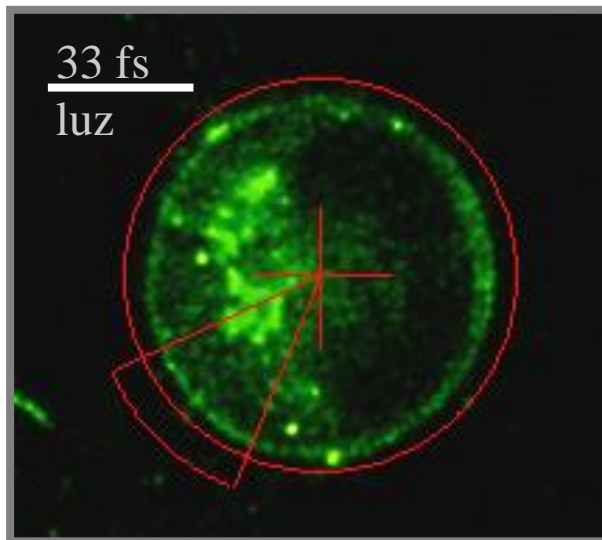
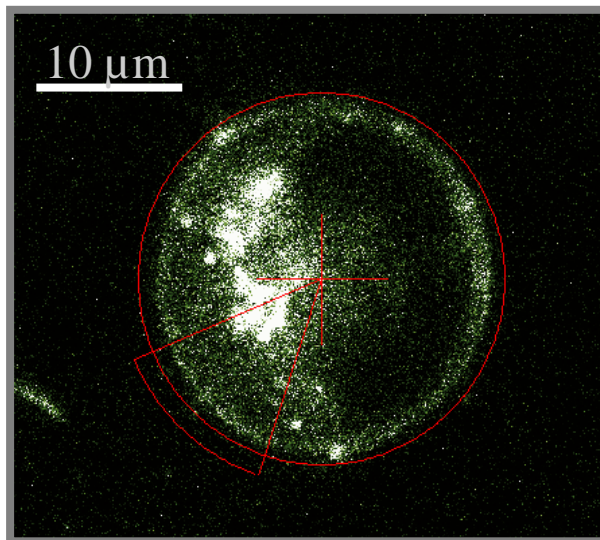
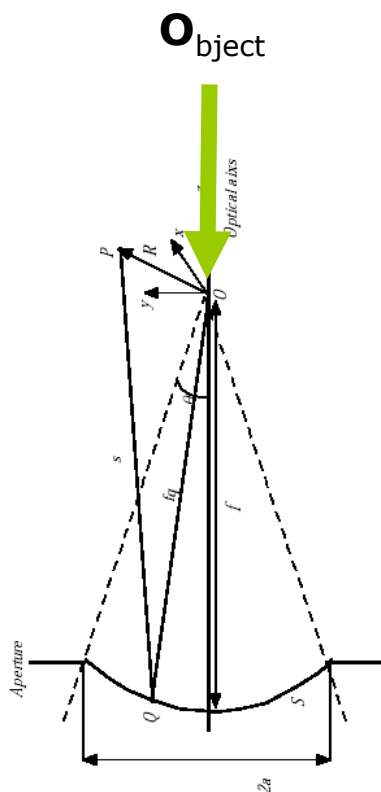


Diffraction Limited Resolution for a 10m telescope $\sim \lambda/D \sim 0.01$ arcsec is limited to ~ 0.5 arcsec by the turbulent atmosphere.

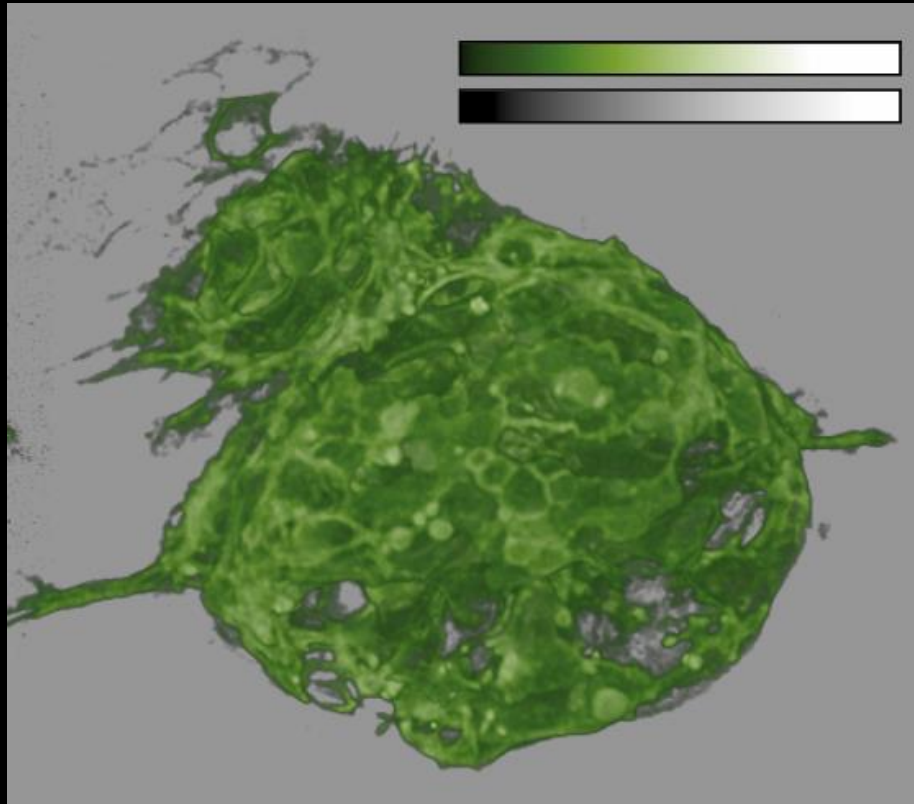
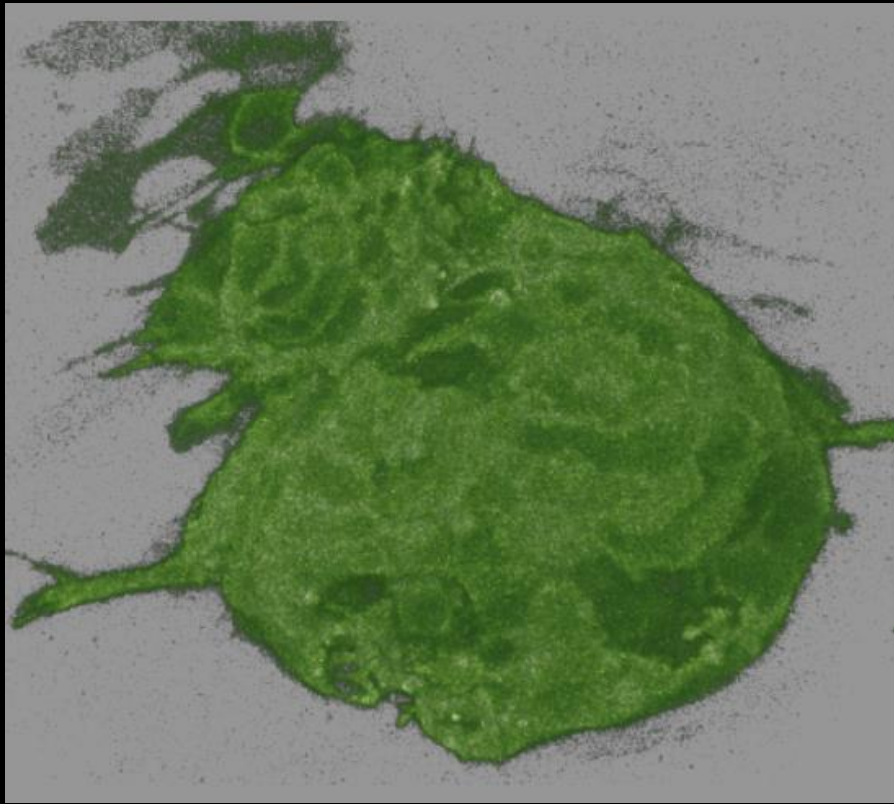


Confocal Microscopy | From Geometric Optics to Diffraction Theory

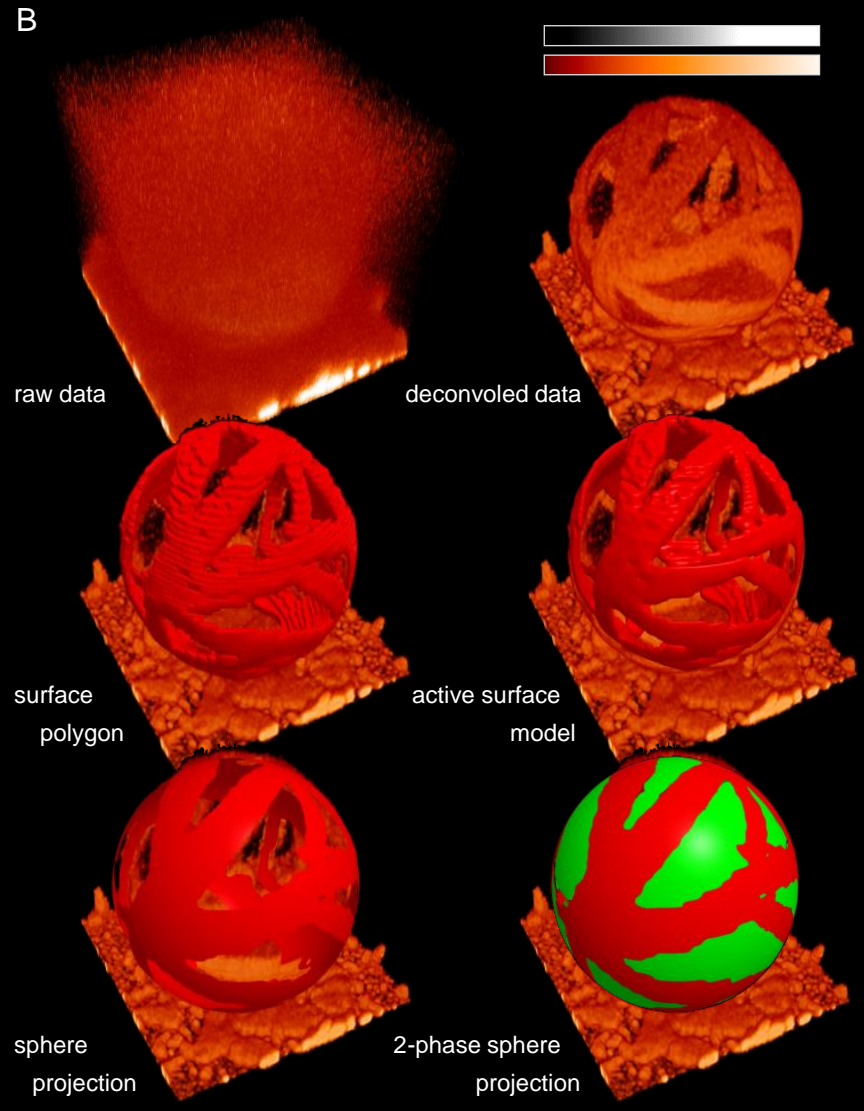
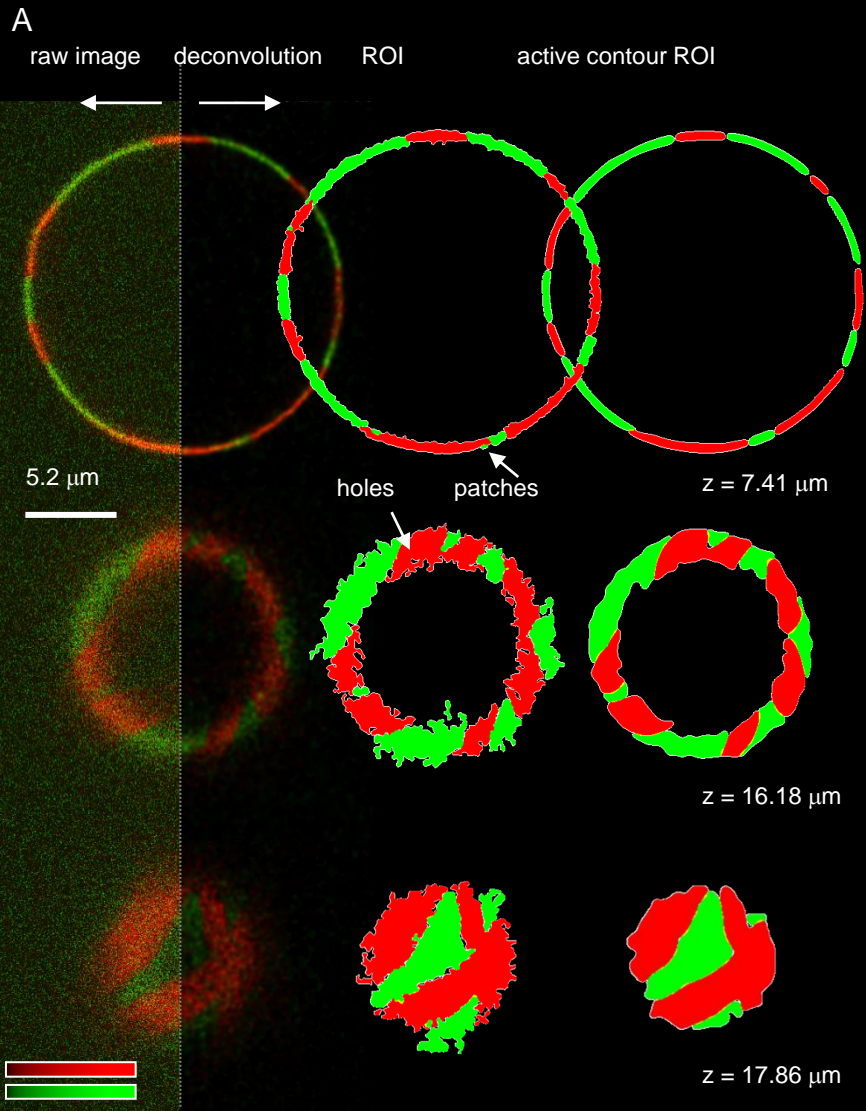
Diffraction: The deviation of an electromagnetic wavefront from the path predicted by geometric optics when the wavefront interacts with a physical object such as an opening or an edge.



|-> Deconvolution

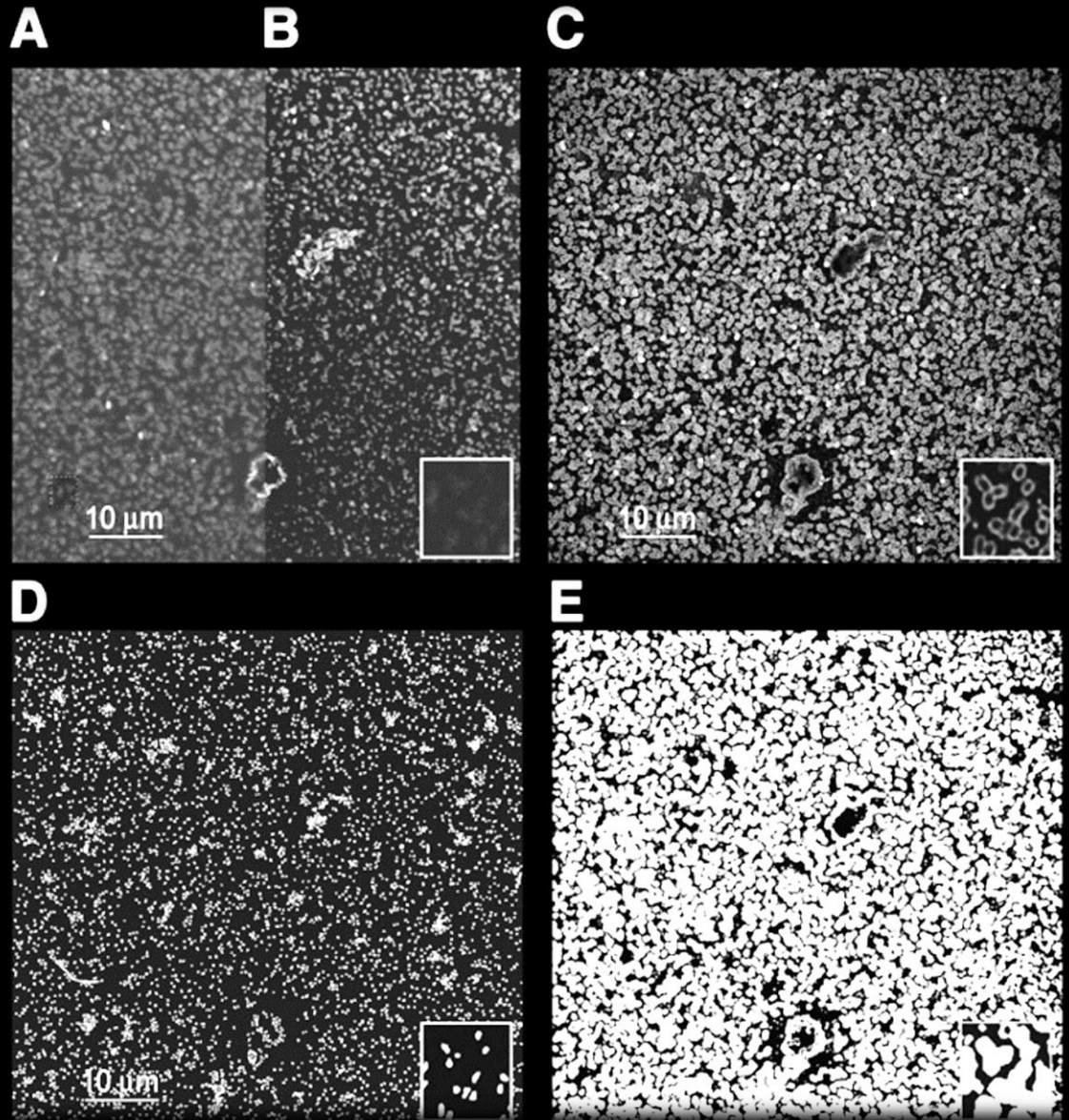


| -> Deconvolution

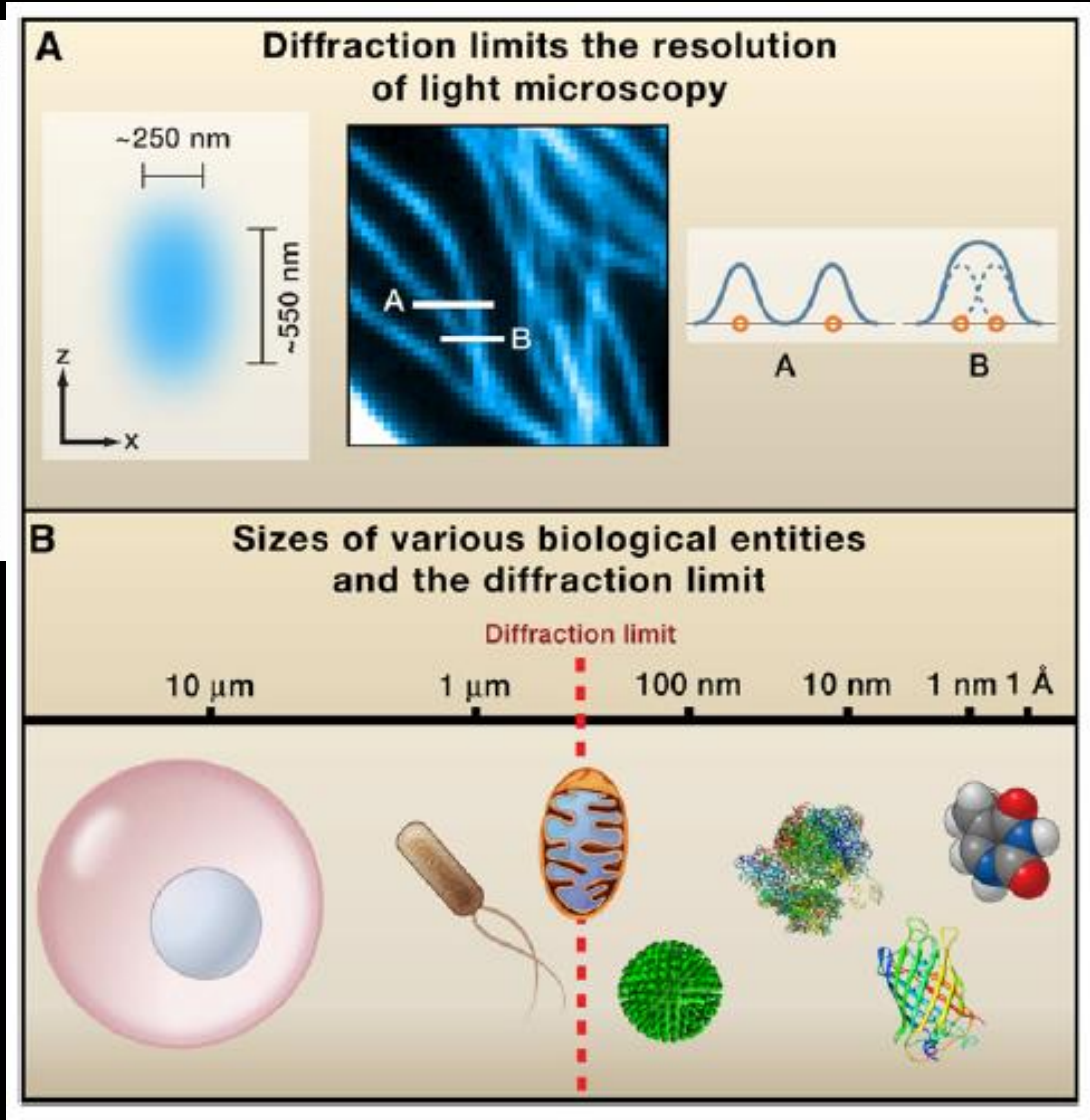
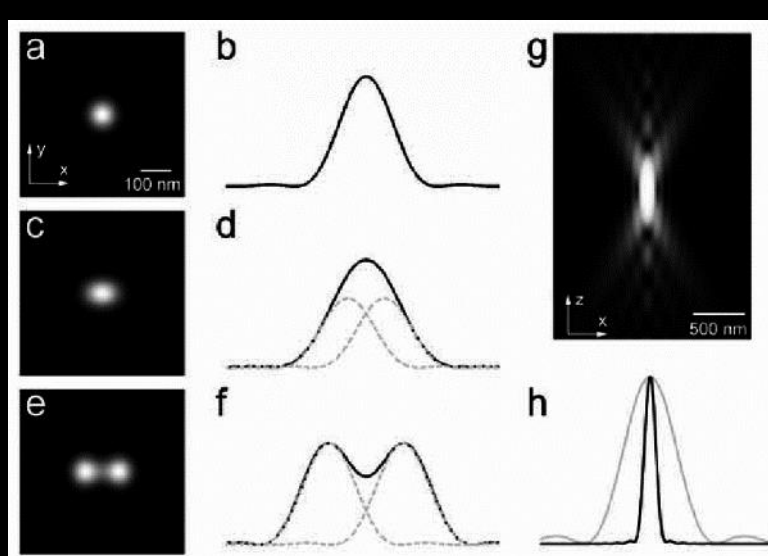


| -> Deconvolution

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

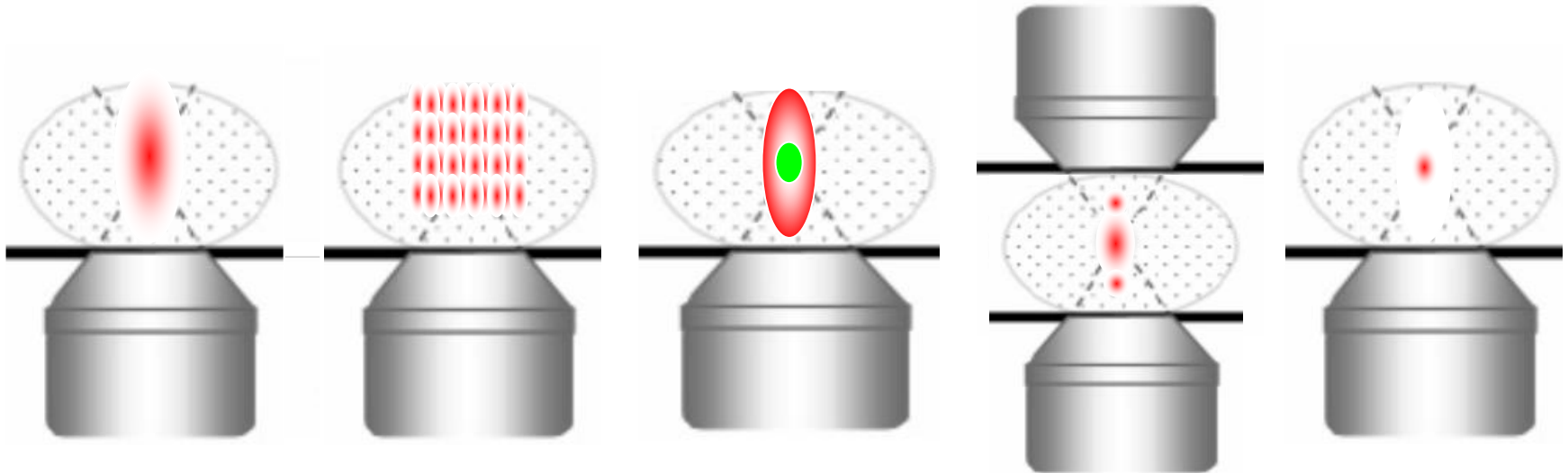


| -> Diffraction limited Microscopy

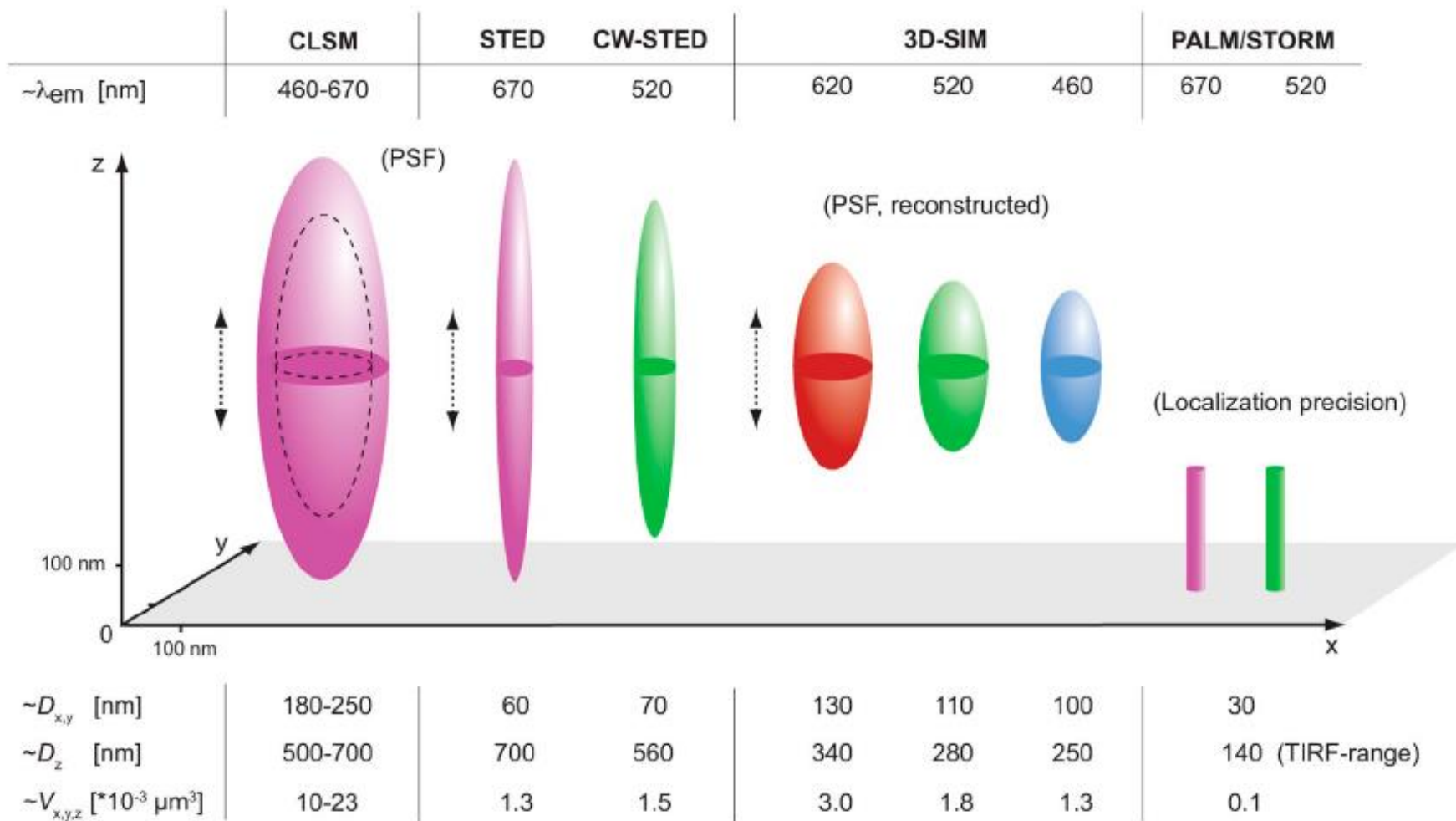


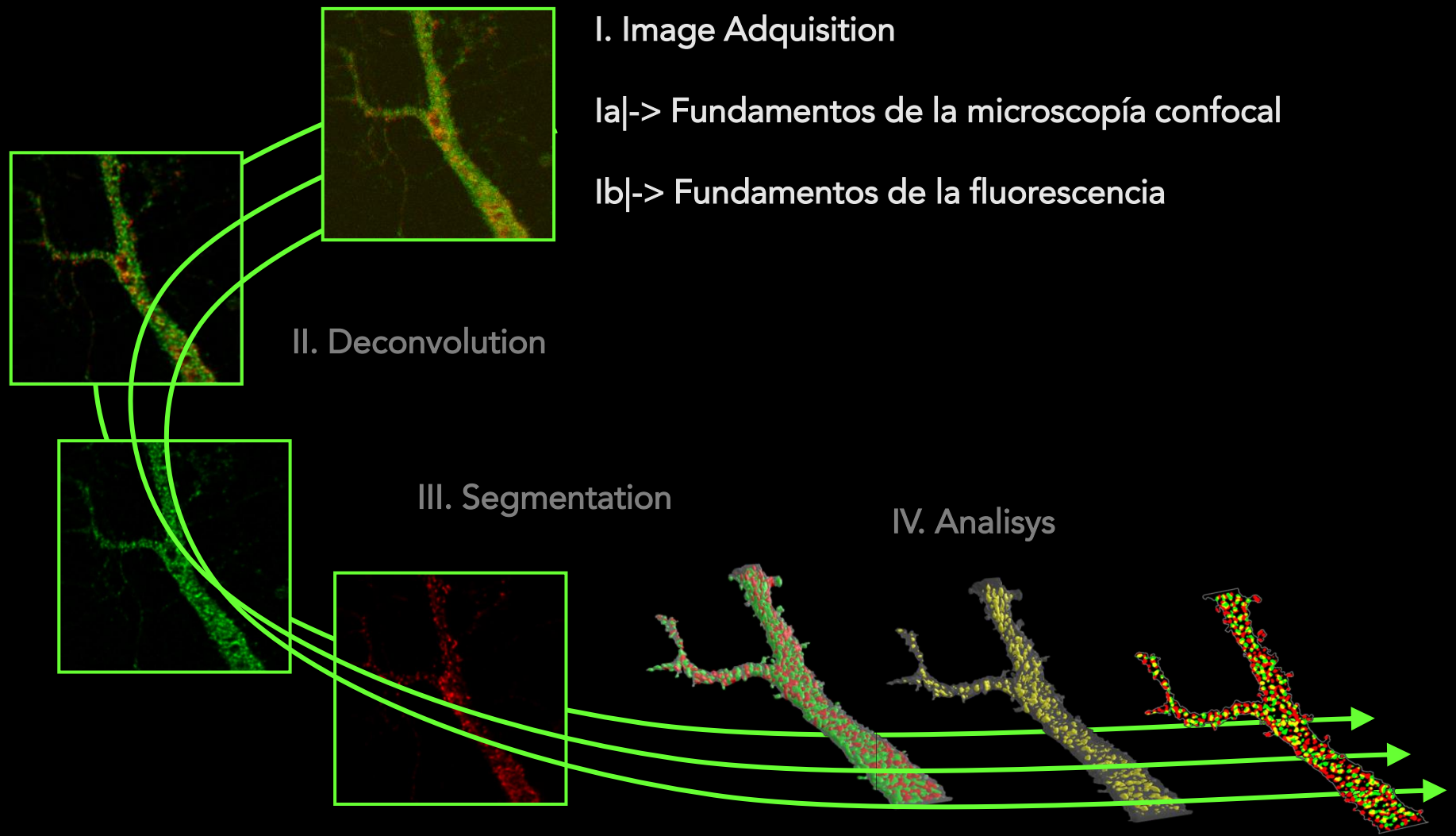
Bertocchi, Cristina & Goh, Wah & Zhang, Zhen & Kanchanawong, Pakorn. (2013). Nanoscale Imaging by Superresolution Fluorescence Microscopy and Its Emerging Applications in Biomedical Research. *Critical reviews in biomedical engineering*. 41. 281-308. 10.1615/CritRevBiomedEng.2014010448.

| -> Beyond diffraction



|-> PSF overview



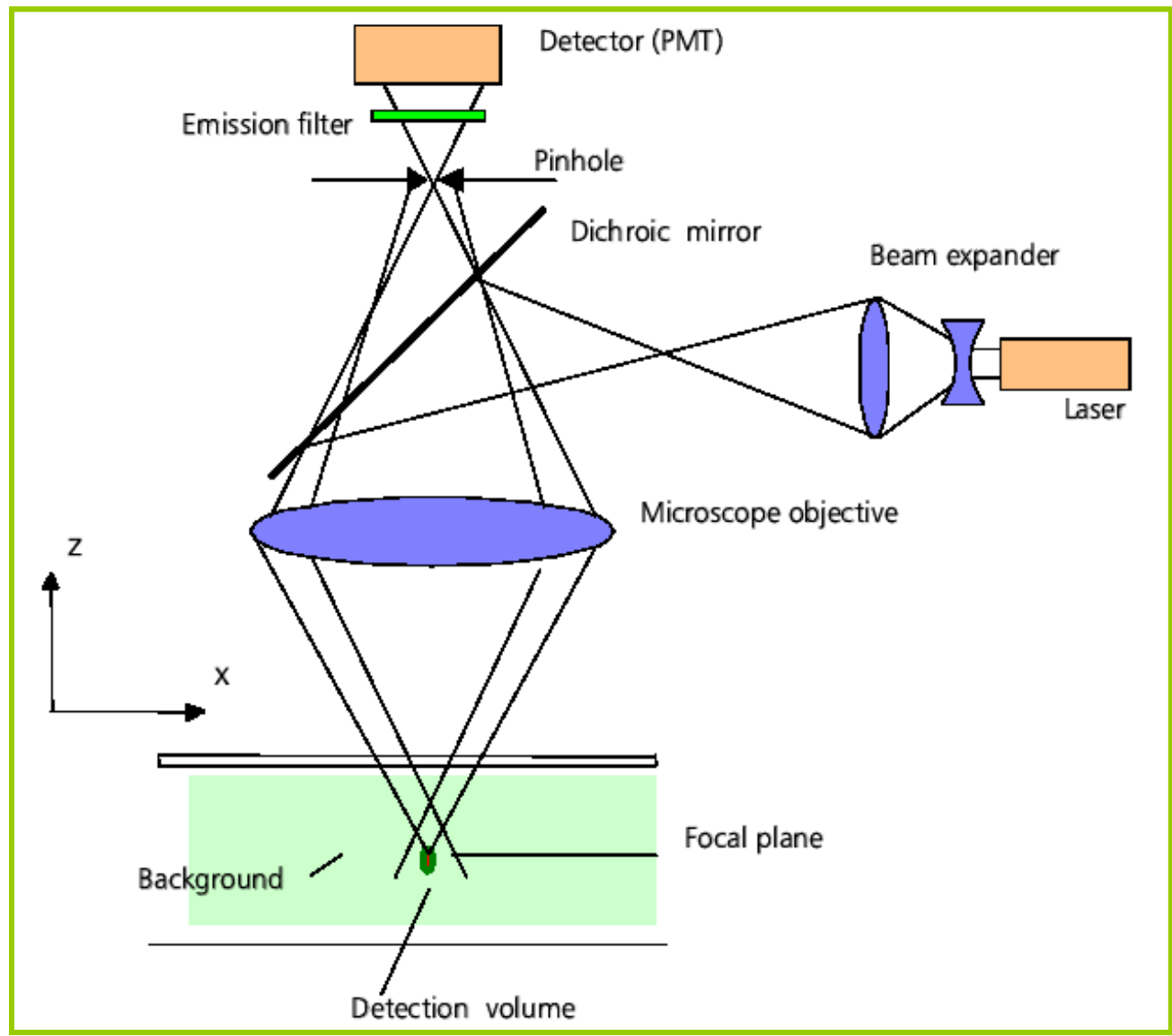


|-> Microscopy

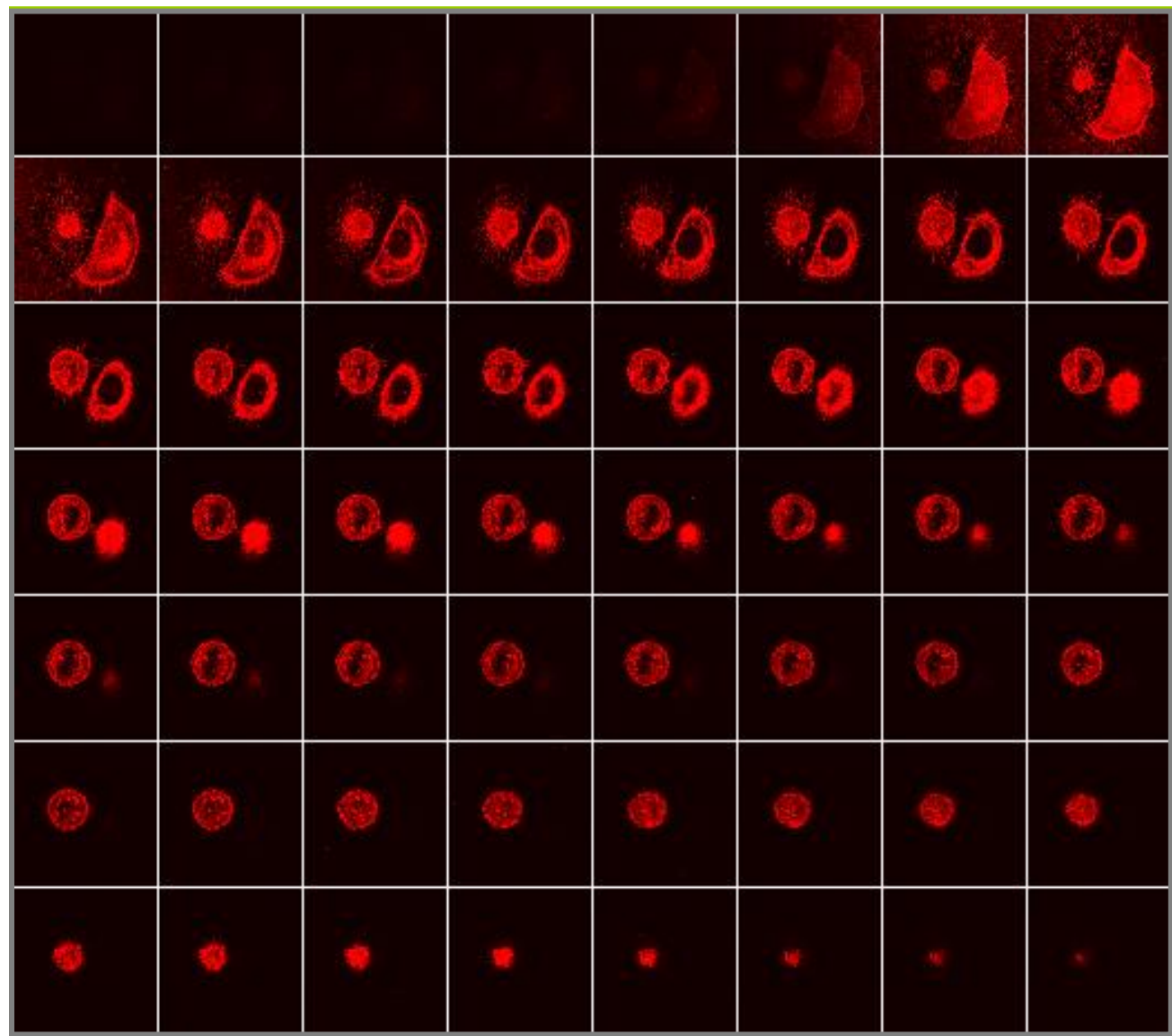
**Hans Janssen (1595),
Galileo Galilei (1610)**



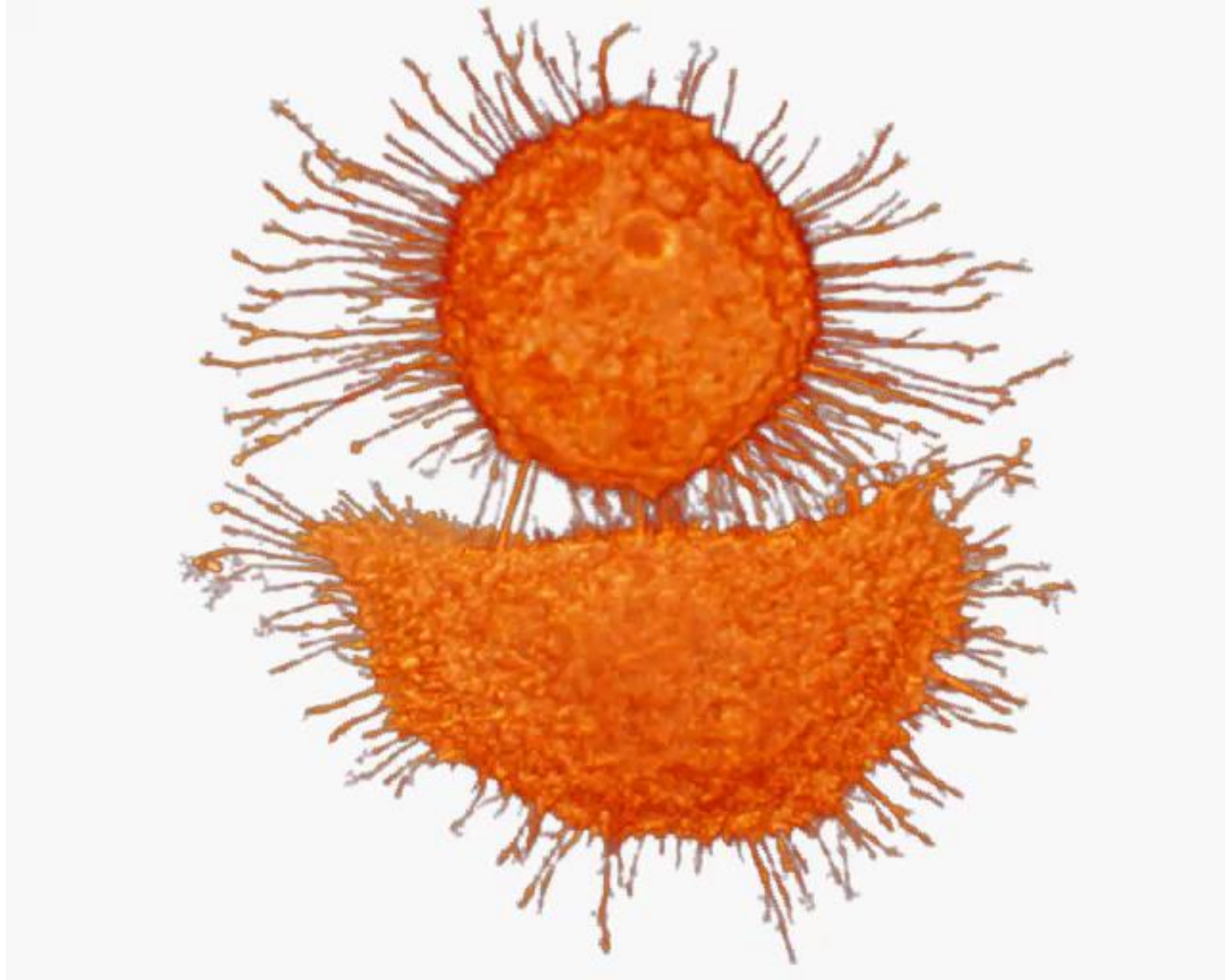
| -> Microscopy



|-> Microscopy



|-> Microscopy



Luminescencia:



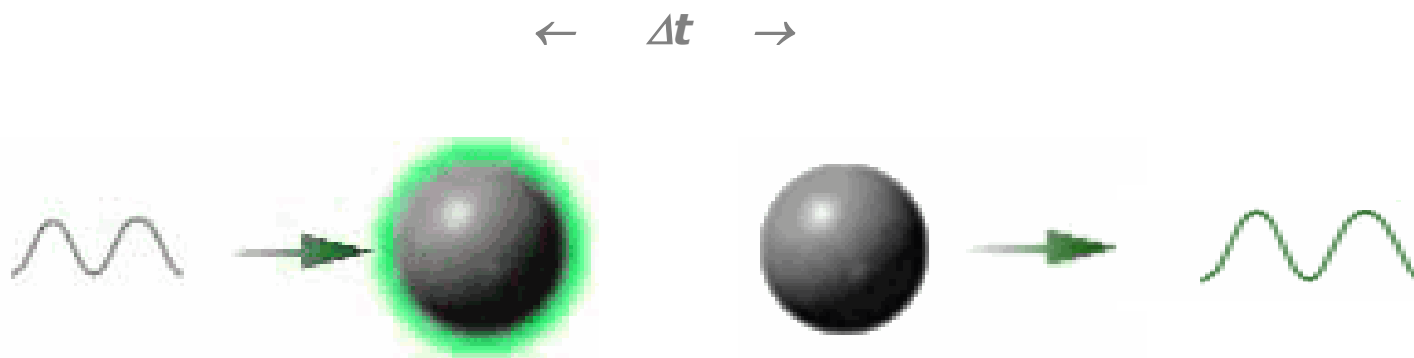
- *Fluorescencia* $\Delta t \sim 10^{-8}s$
- *Fosforescencia* $\Delta t \sim 10^{-3}-10^0s$

Interacciones ...

- intra- e inter moleculares ...

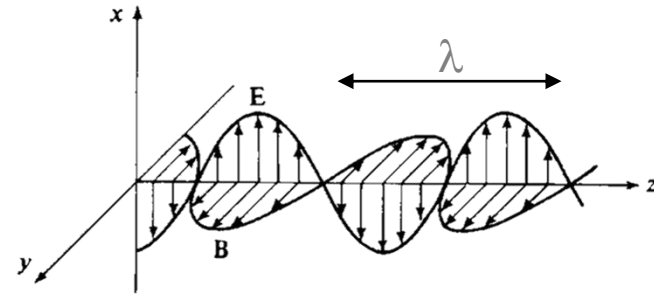
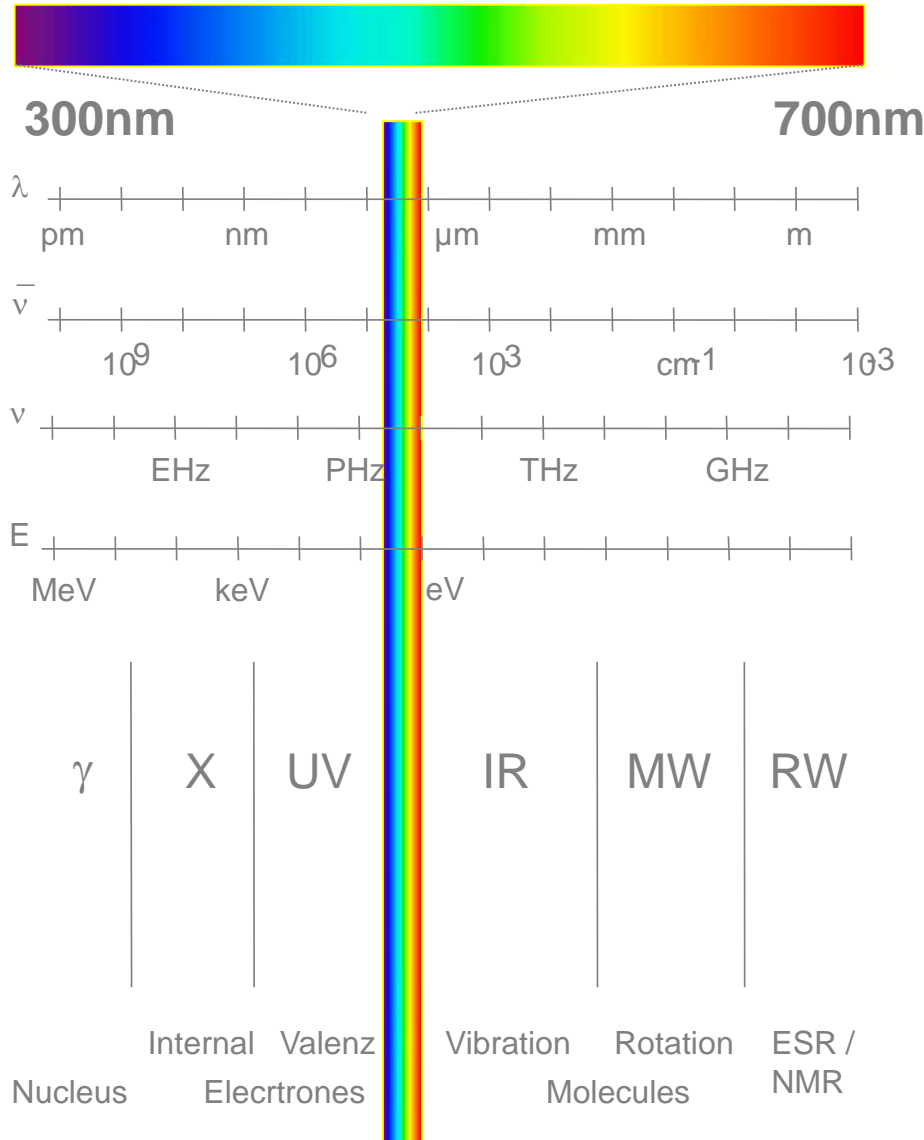
producen cambios ...

- espectrales
- tiempos de vida
- polarización
- intensidad ...



Absorción / Excitación

Emisión



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 [$6.626 \cdot 10^{-34} \text{ Js}^{-1}$]

λ , longitud de onda [m]

c , velocidad de luz [$\sim 3 \cdot 10^8 \text{ ms}^{-1}$]

Energía molecular:

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

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


Energía térmica:

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

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

|-> Jablonski Diagram

Transiciones: — con radiación

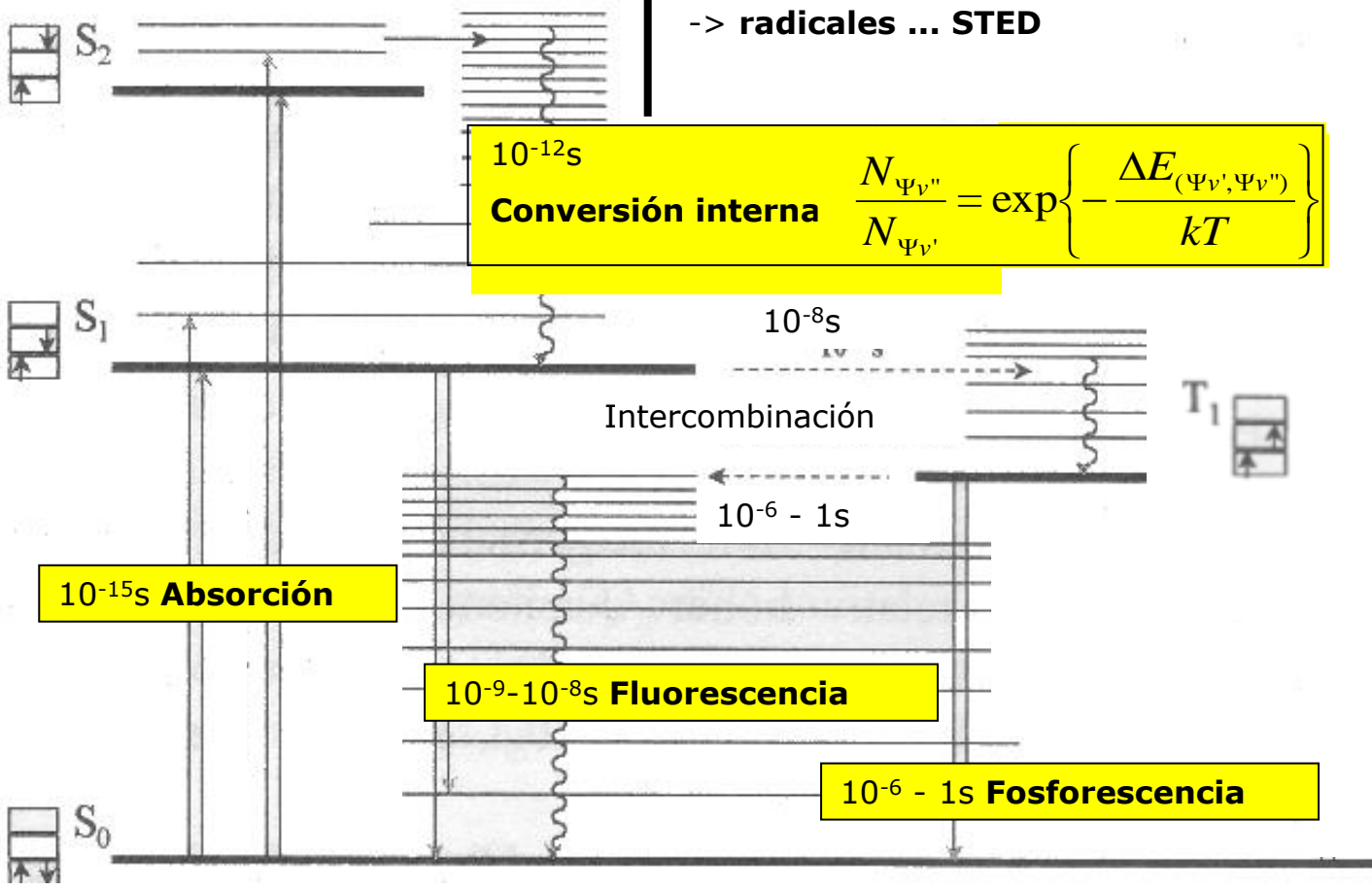
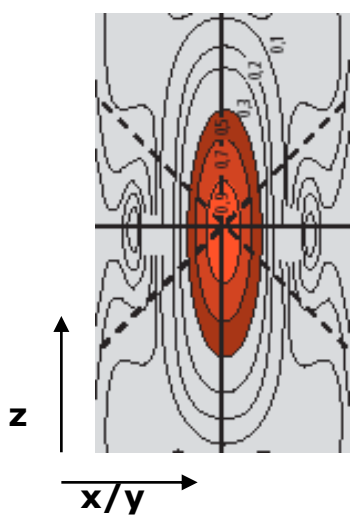
 sin radiación |

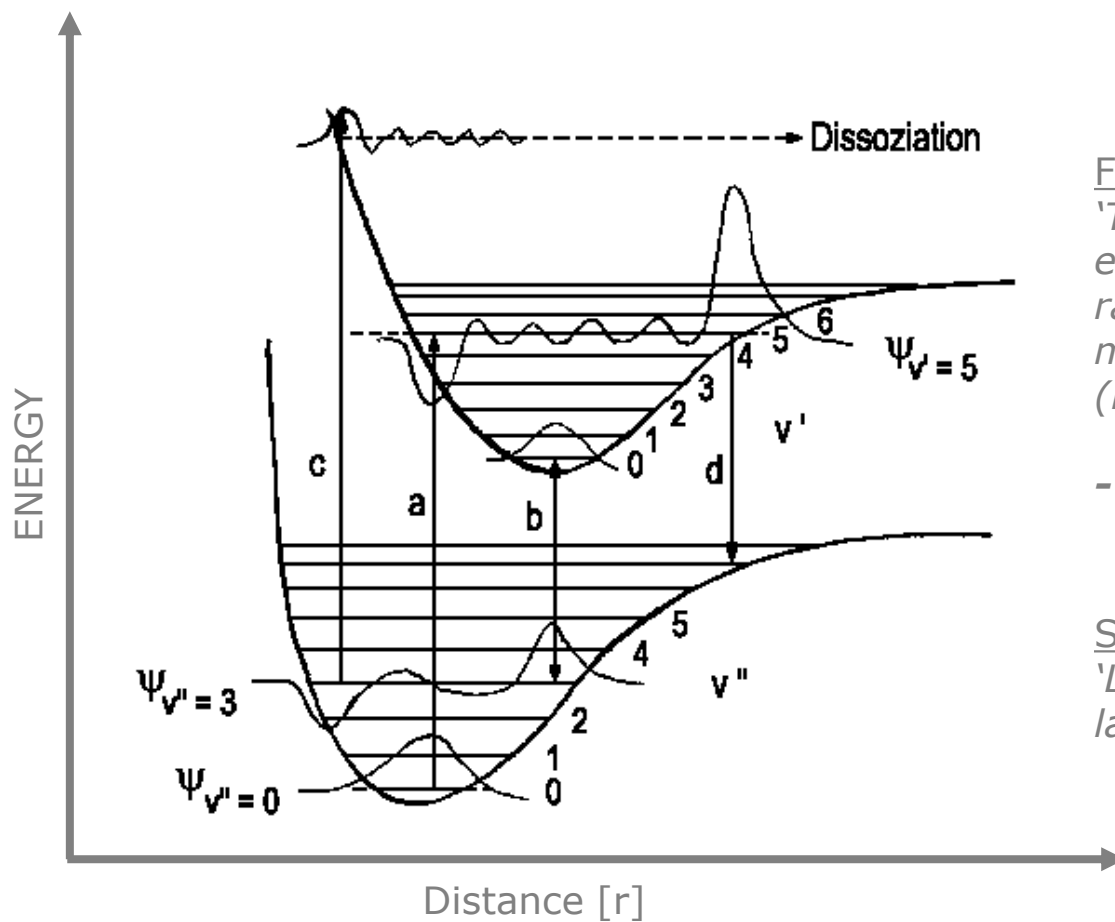
Niveles: — vibracionales

-> calor

-> transferencia de energía (FRET)

-> radicales ... STED



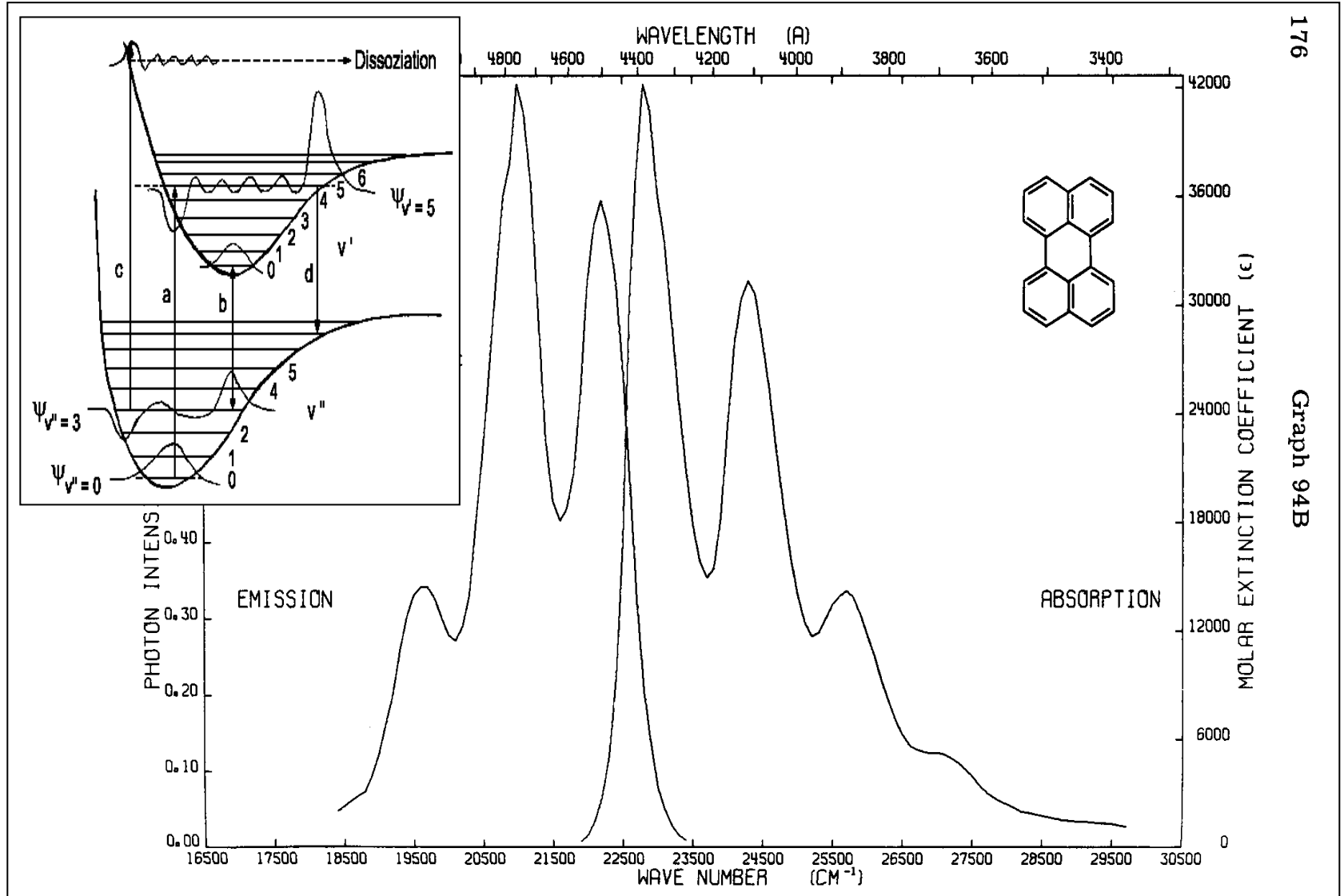


Frank Condon :
 'Transiciones entre niveles electrónicos ocurren mucho más rápido que movimientos de núcleos moleculares.'
 ($masa_{electron} / masa_{atom} : 1 : 2000$)

-> **Mirror Image Rule**

Stokes (Shift) :
 'La energía de emisión es menor a la energía de excitación.'

|-> Mirror Image Rule



- Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz 4.1 Introduction to Fluorescence

|-> Quenching, Bleaching ...

|-> Polarisation ...

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

|-> Förster Resonance Energy Transfer ...

- Photomultiplier www.olympus-lifescience.com/en/microscope-resource/primer/digitalimaging/concepts/photomultipliers
- Chrome Spectra Viewer www.chroma.com/spectra-viewer
- Thermo Fisher Spectra www.thermofisher.com/order/spectra-viewer

