Welcome

Microscopía y Procesamiento de Imágenes

Prof. Dr. Steffen Härtel: www.scian.cl / www.cimt.cl / www.cens.cl

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

|-> 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

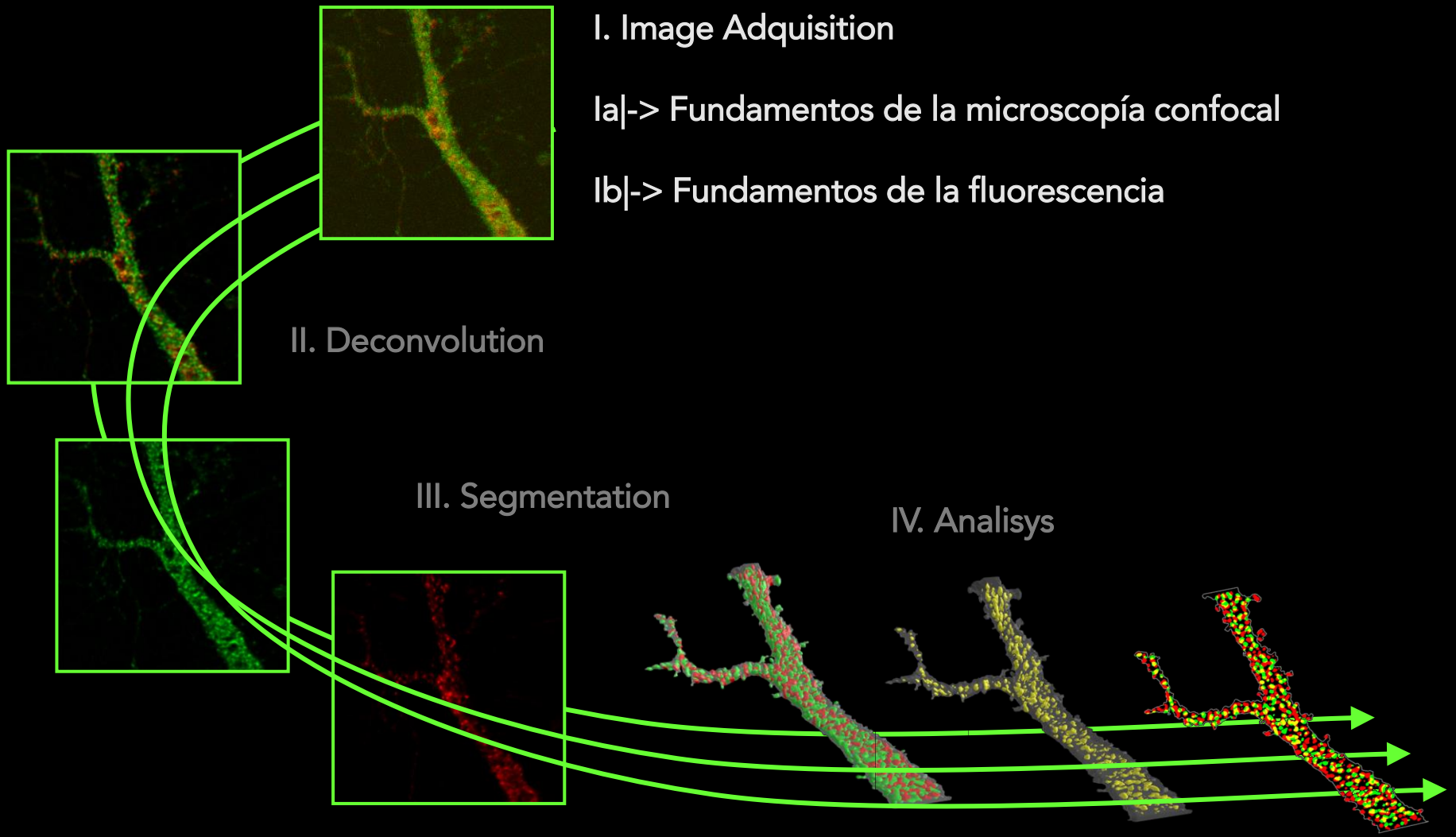
- Fluorescence Microscopy, From Principles to Biological Applications, Ulrich Kubitscheck (Editor), 2nd Edition, June 2017, Hardcover, ISBN: 978-3-527-33837-5
- https://www.zeiss.com/microscopy/int/cmp/edr/21/microscopy-for-dummies.html
- https://www.microscopyu.com/tutorials
- http://zeiss-campus.magnet.fsu.edu/tutorials/index.html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

Laboratory for SCIAN Scientific Image Analysis

Welcome

BIOMEDICAL NEUROSCIENCE INSTITUTE



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

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|-> Microscopy

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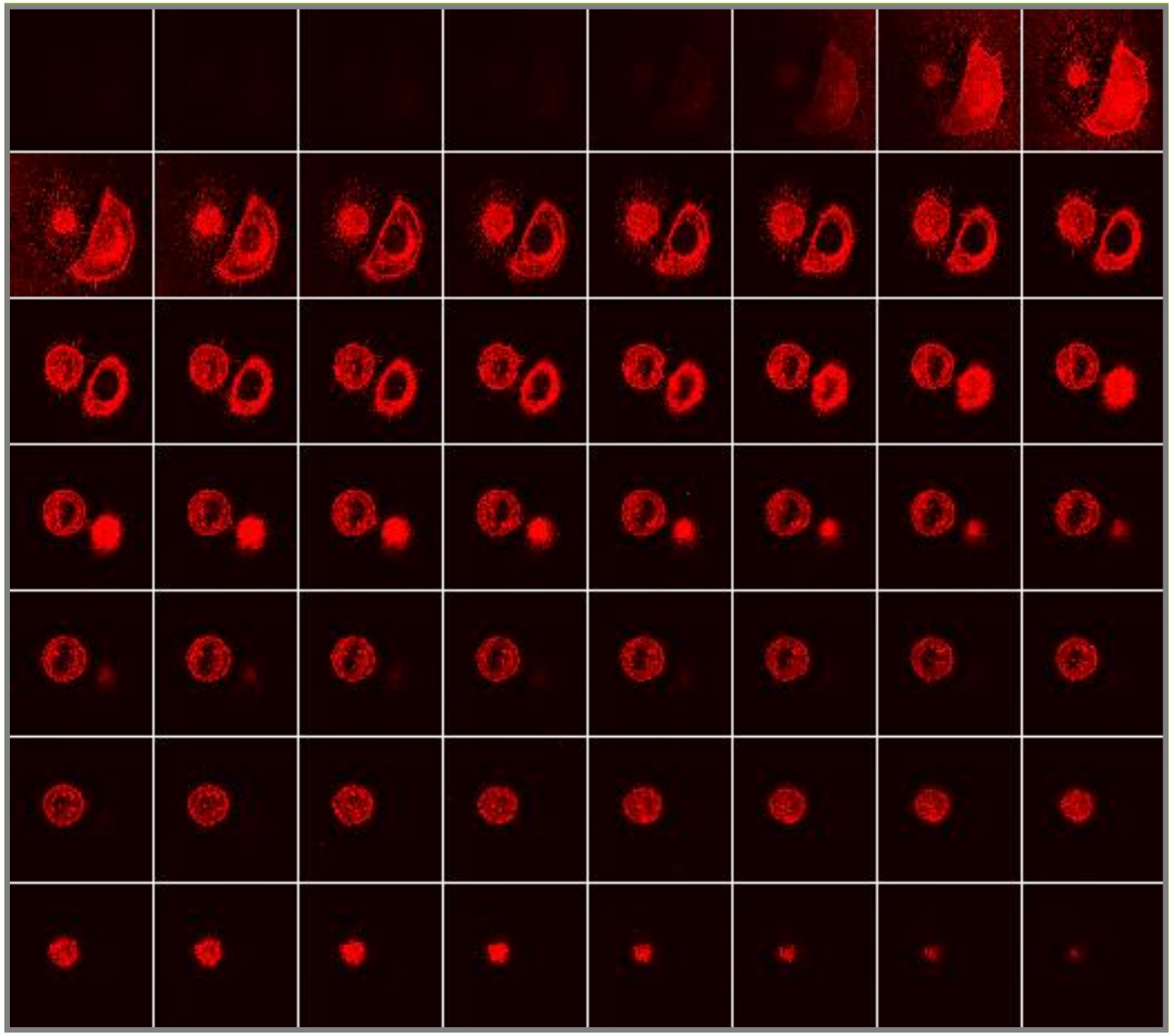

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|-> Microscopy

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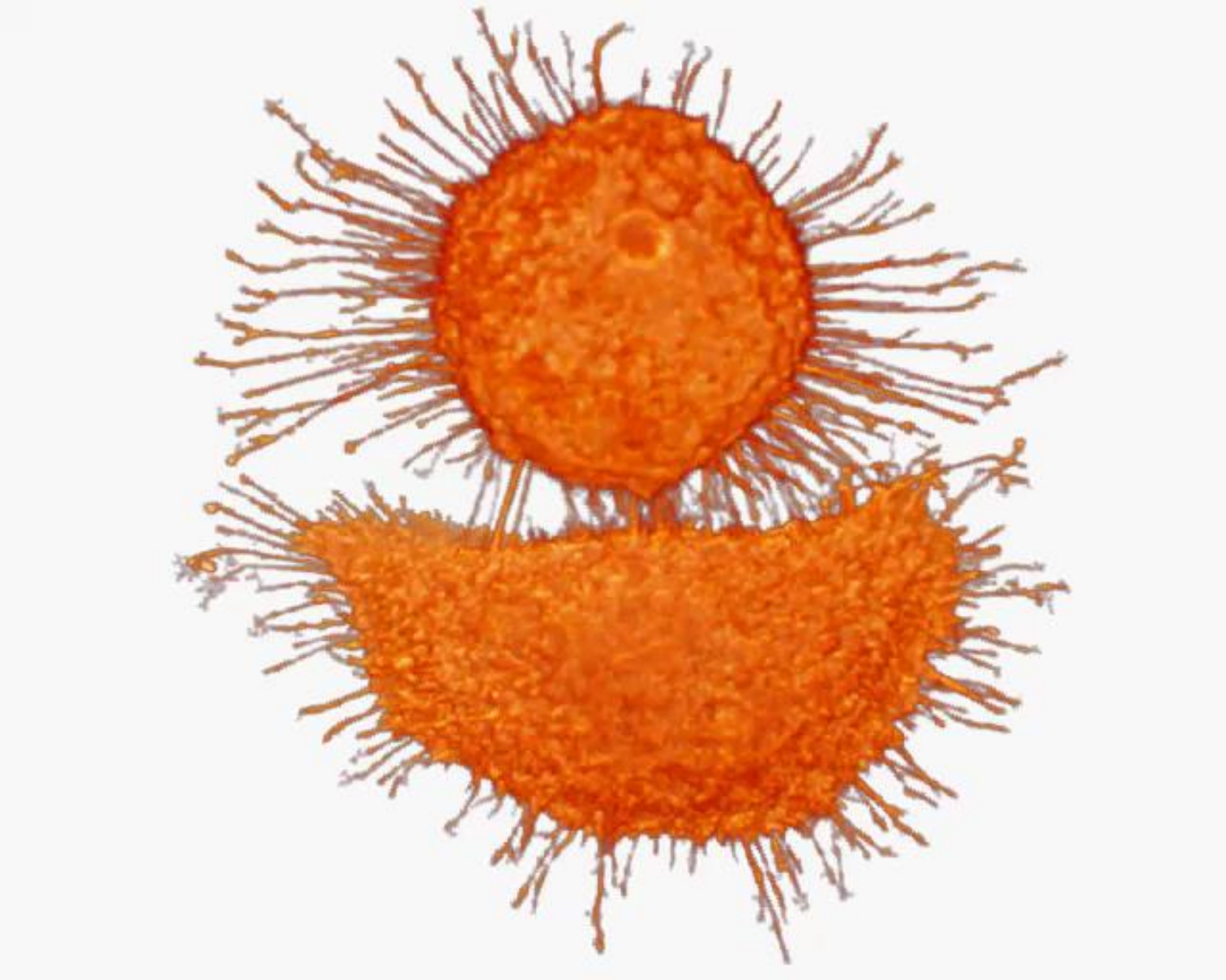


|-> Fluorescencia I

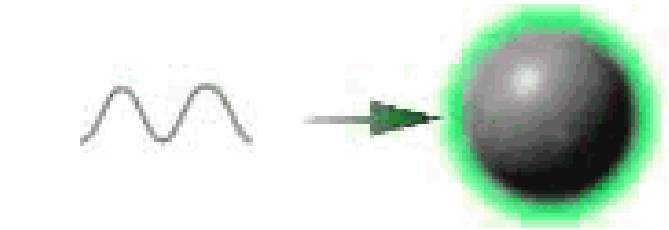
Luminescencia:

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

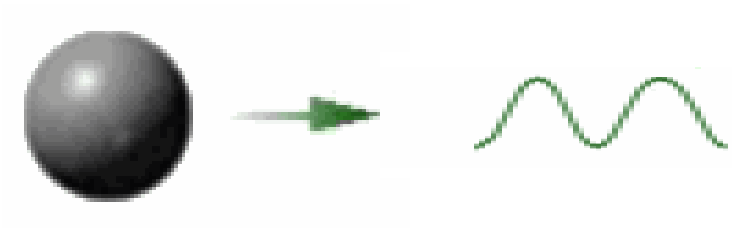
Interacciones ...
- **intra- e inter moleculares ...**

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

$\leftarrow \quad \Delta t \quad \rightarrow$

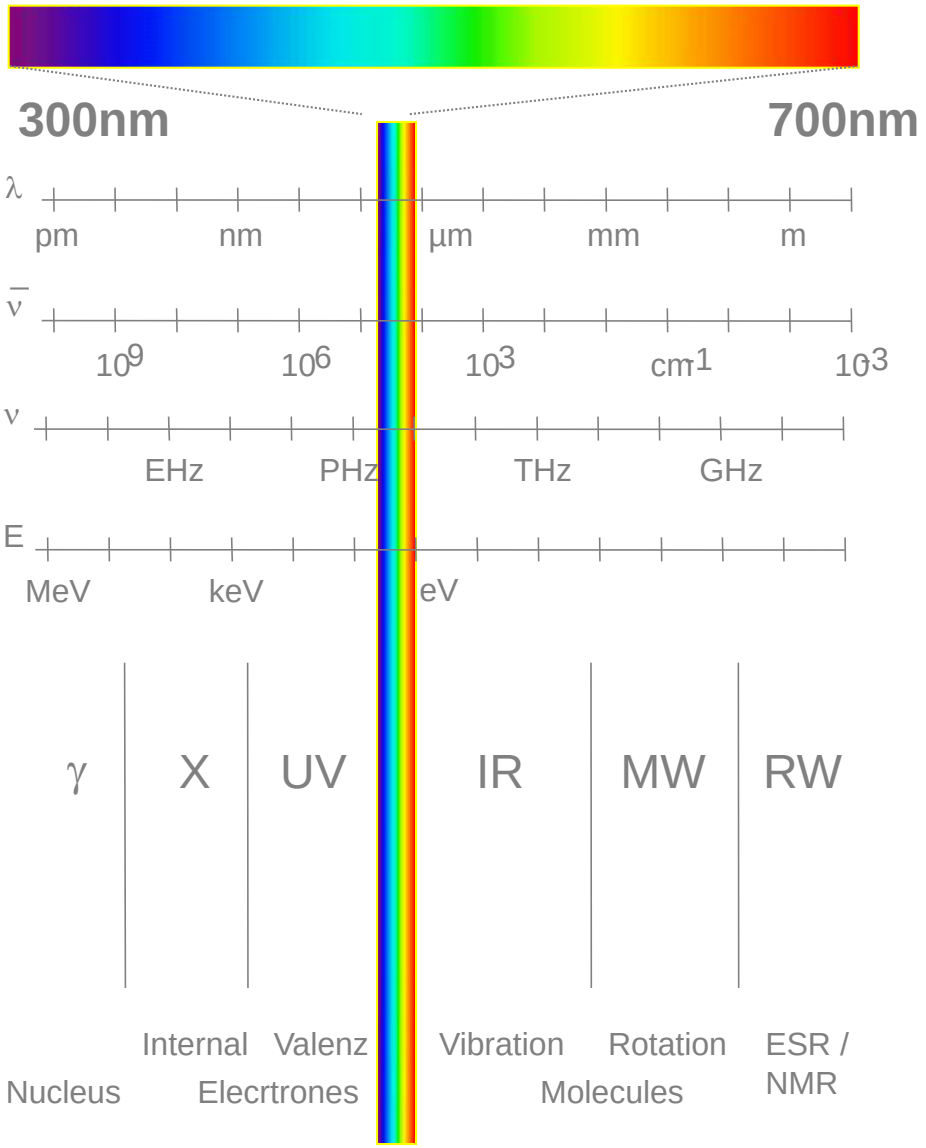


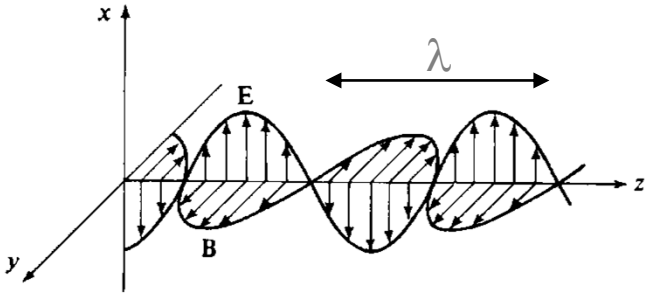
Absorción / Excitación



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 |

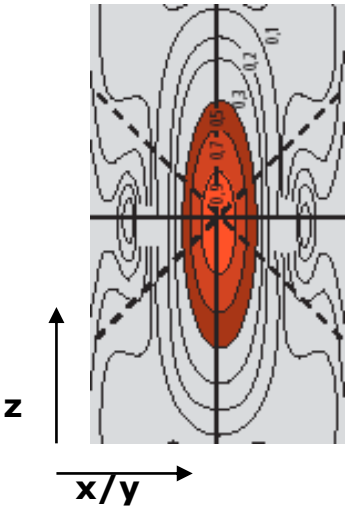
Niveles: —— vibracionales

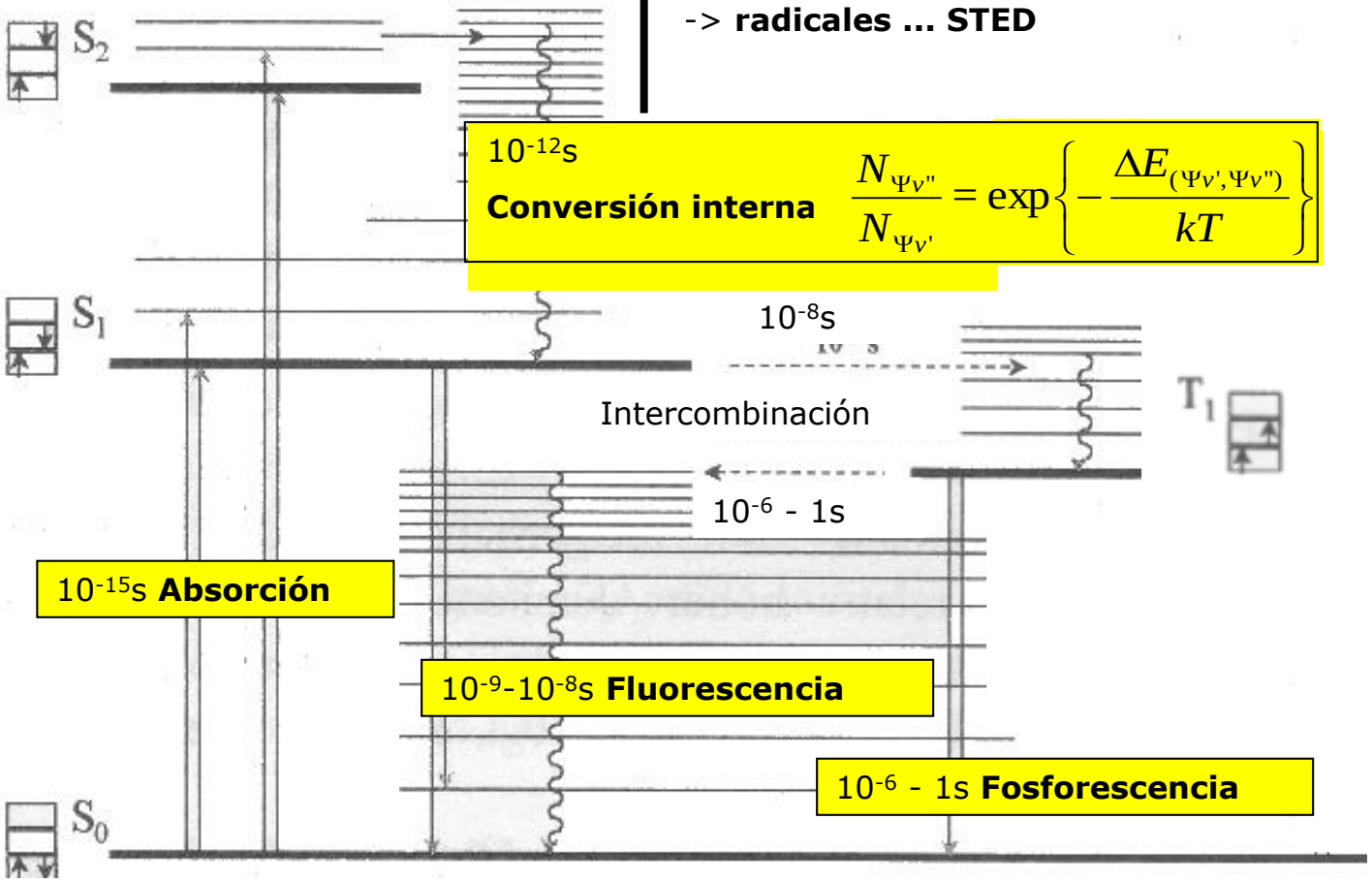
▬ electrónicos

-> **calor**

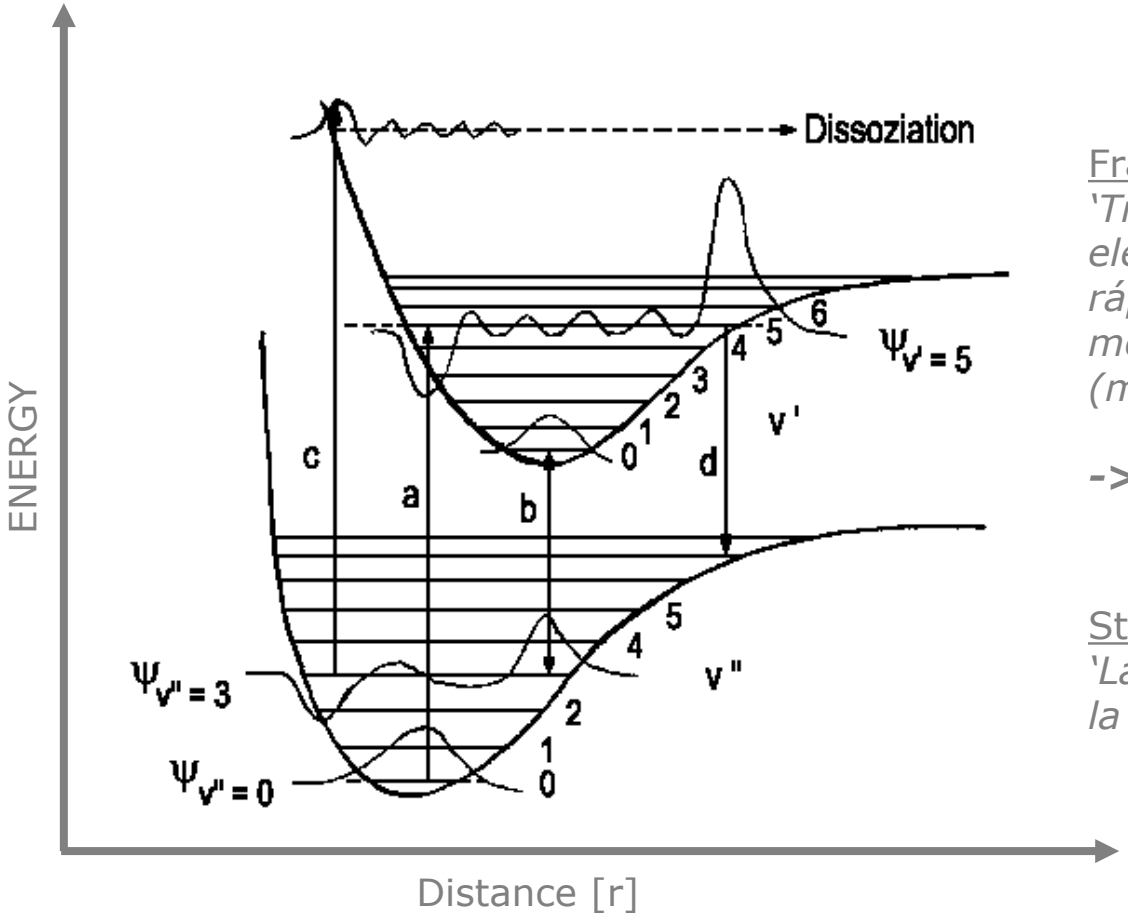
-> **transferencia de energía (FRET)**

-> **radicales ... STED**





|-> Franck Condon



Frank Condon **:**

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

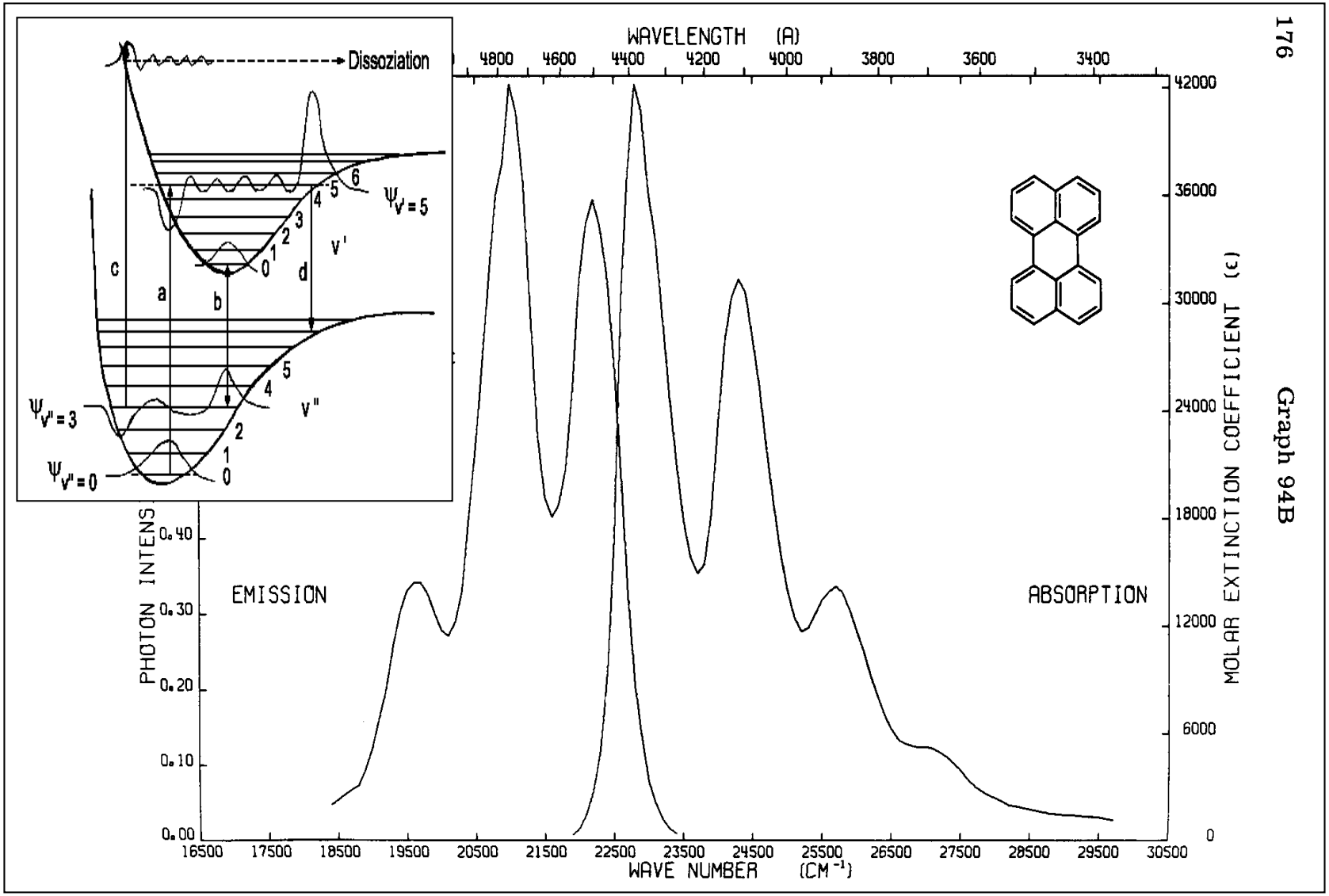
($masa_{electron}$ / $masa_{atom}$: 1 : 2000)

-> Mirror Image Rule

Stokes (Shift) **:**

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

|-> Mirror Image Rule



|-> Joseph R. Lakowicz

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

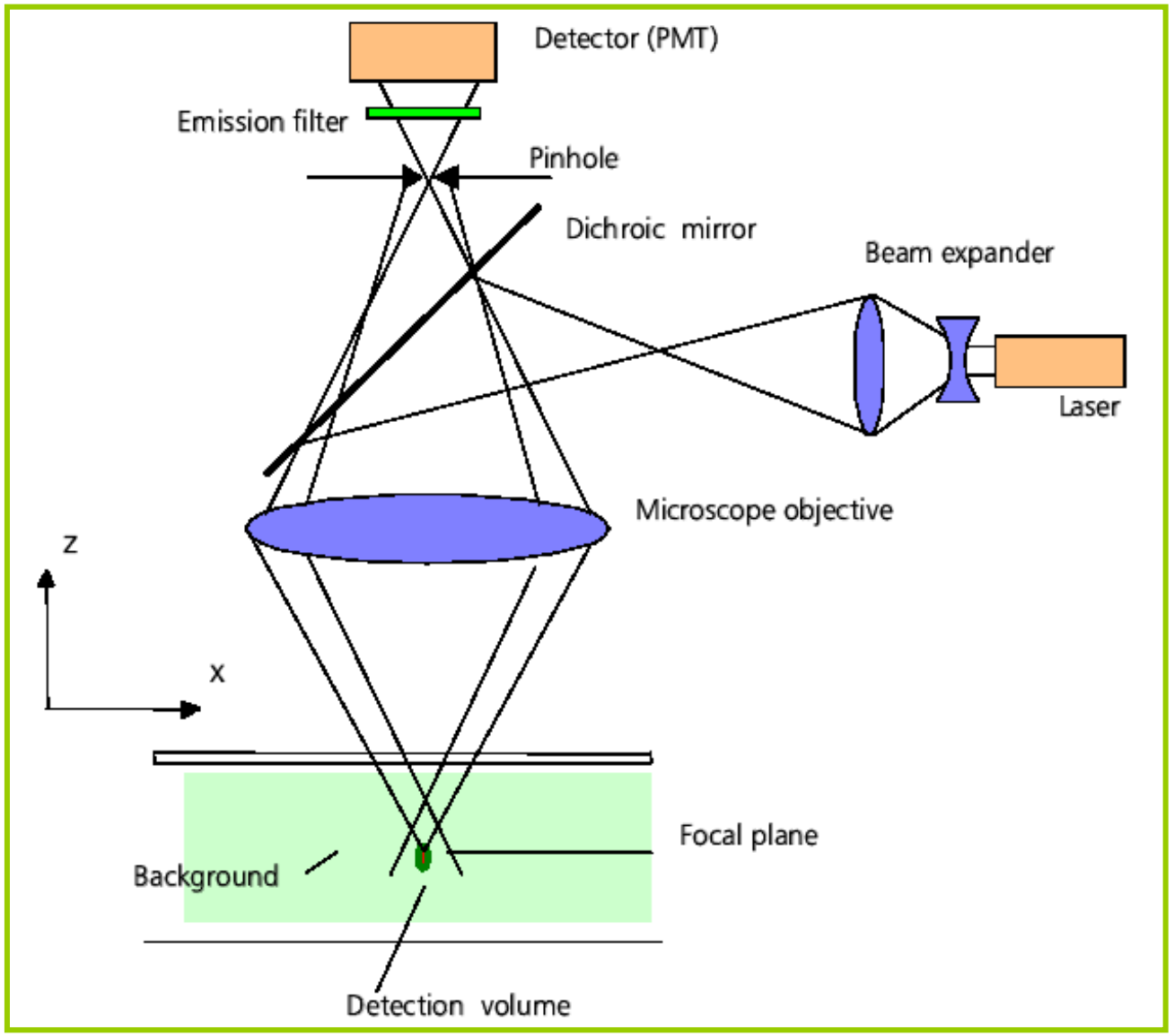
|-> Quenching, Bleaching ...

|-> Polarisation ...

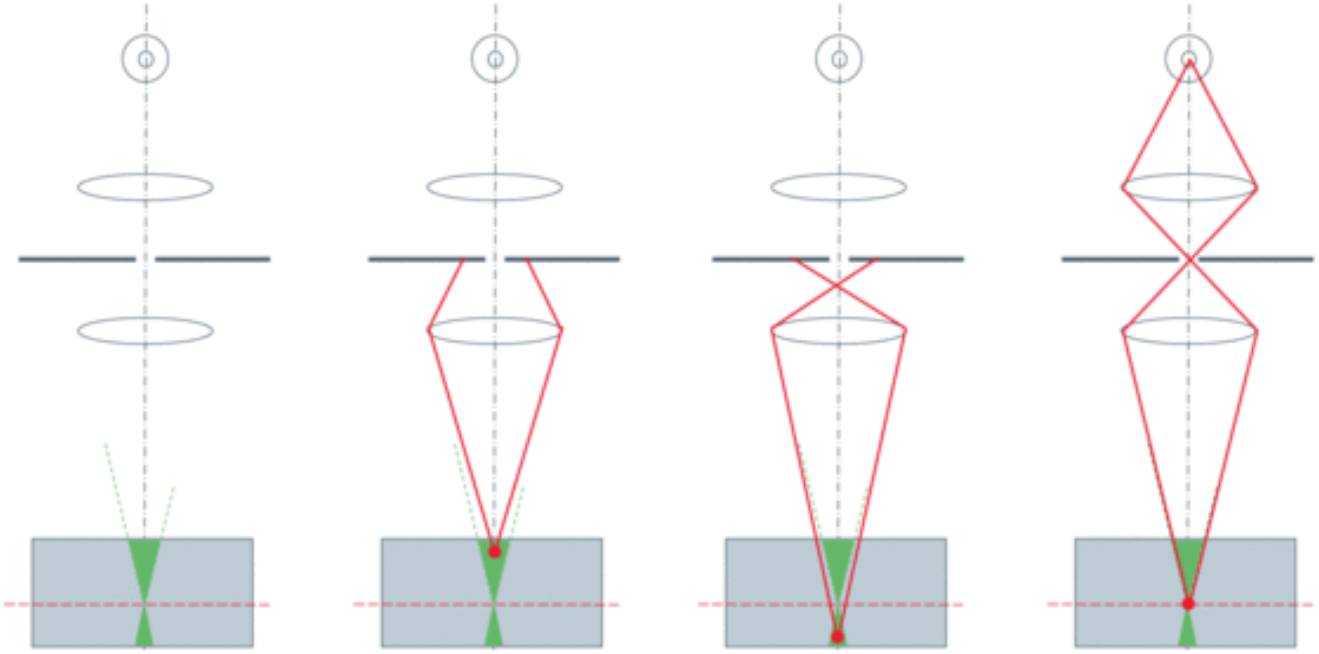
|-> Steady-State and Time-Resolved Fluorescence...

|-> Förster Resonance Energy Transfer ...

|-> 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.

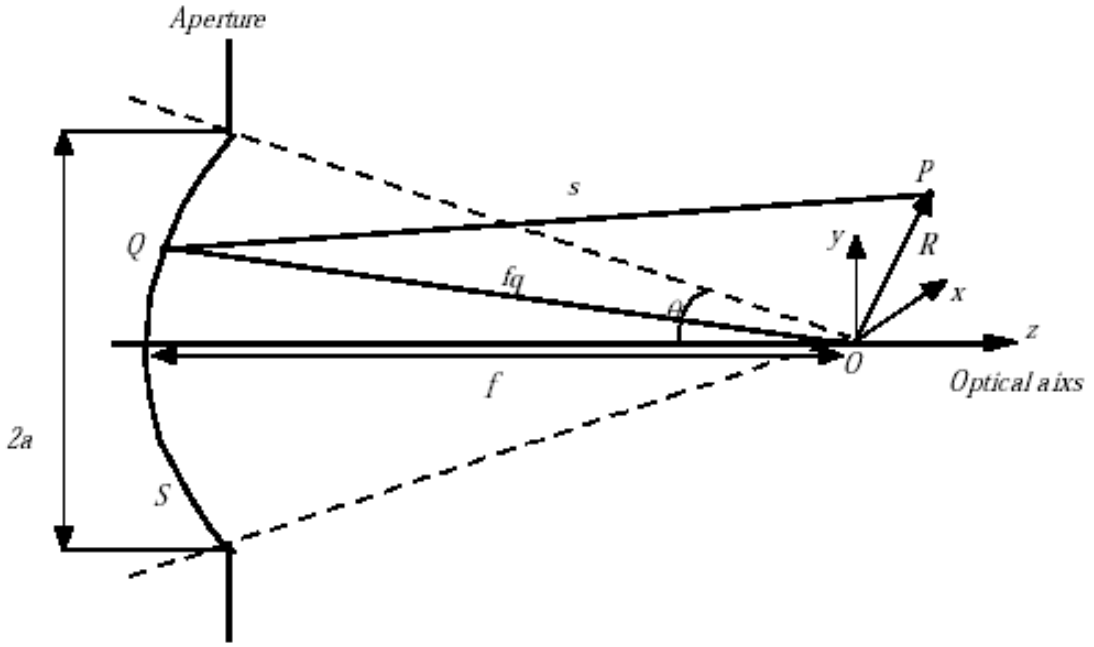


Figure 2.1 Diffraction of a converging spherical wave at a circular aperture

|-> Diffraction limited Microscopy

Óptica no-geométrica /

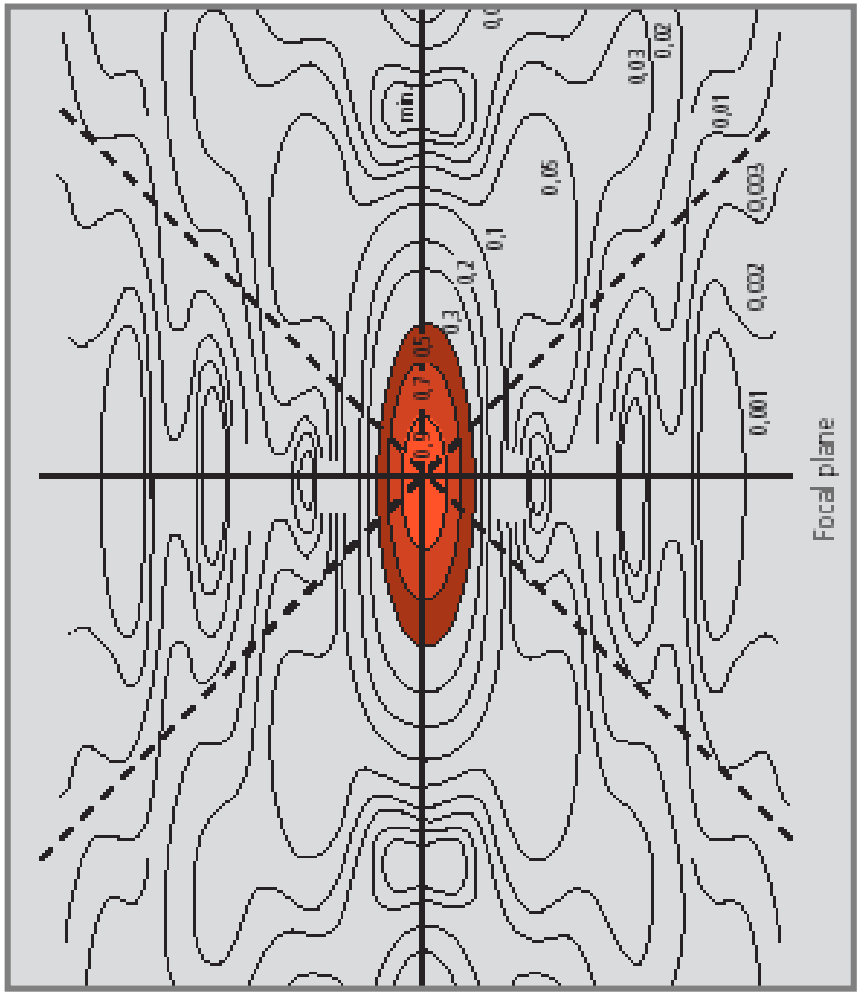
Teoría de difracción

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

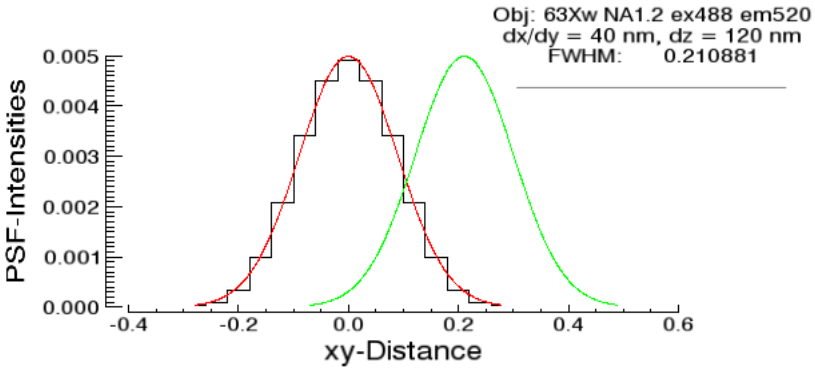
U, Integral de Difracción de Kirhoff

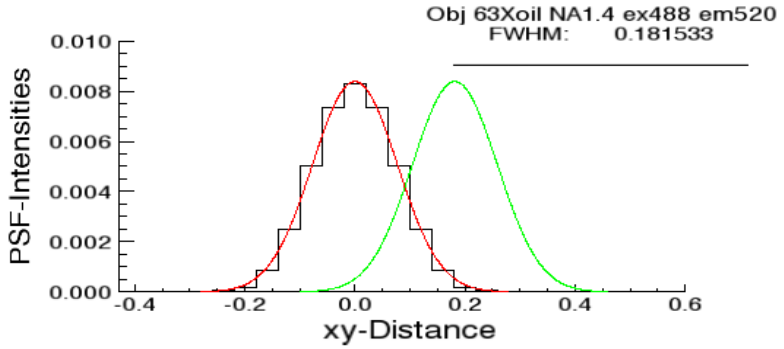
J_0, Serie de funciones de Bessel

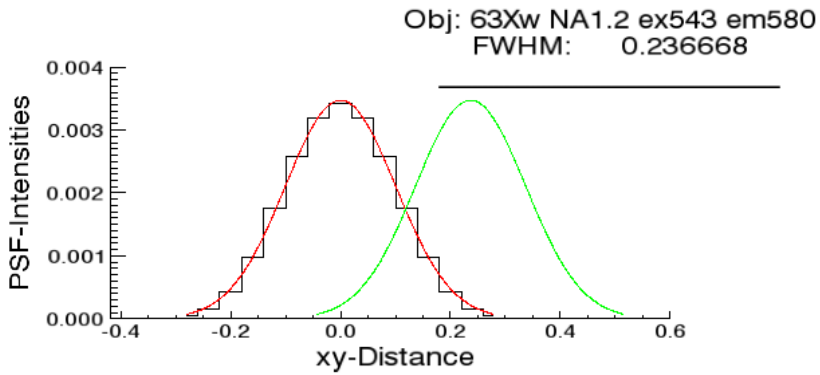
(Born & Wolf, Priniples of Optics, 6th edition 1988, Pergamon Press)

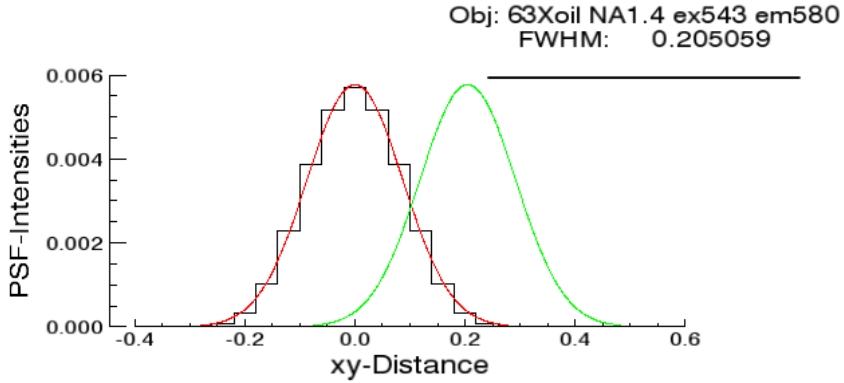


|-> Diffraction limited Microscopy

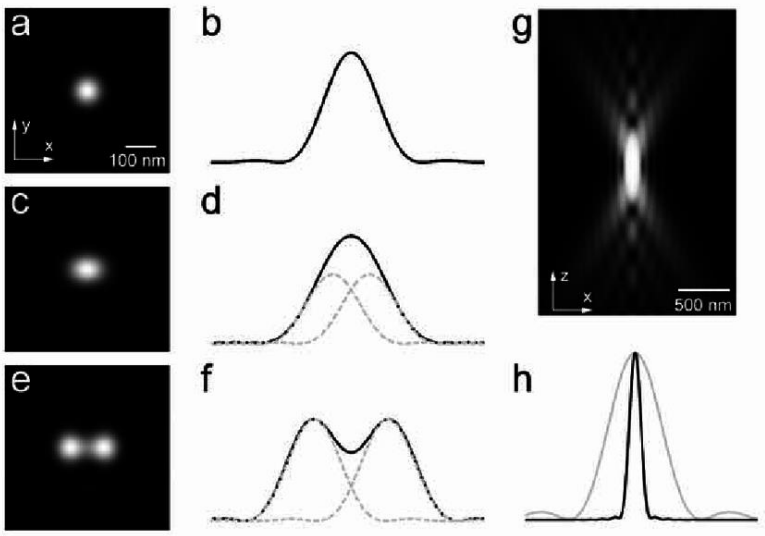




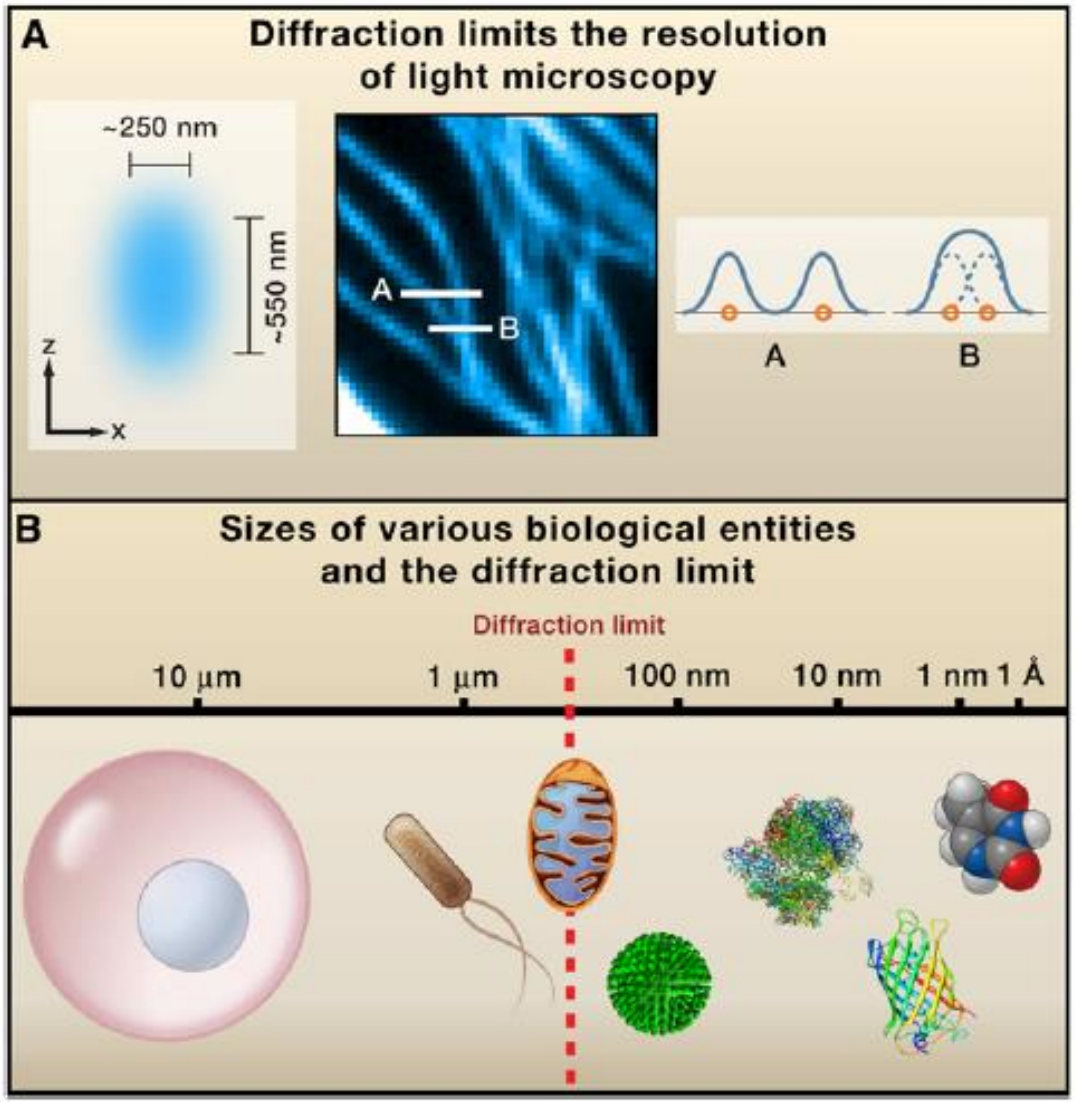




|-> Diffraction limited Microscopy



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.



Bo Huang, Hazen Babcock, Xiaowei Zhuang, Breaking the Diffraction Barrier: Super-Resolution Imaging of Cells, Open Archive Published:December 17, 2010 DOI:https://doi.org/10.1016/j.cell.2010.12.002

|-> Beyond diffraction

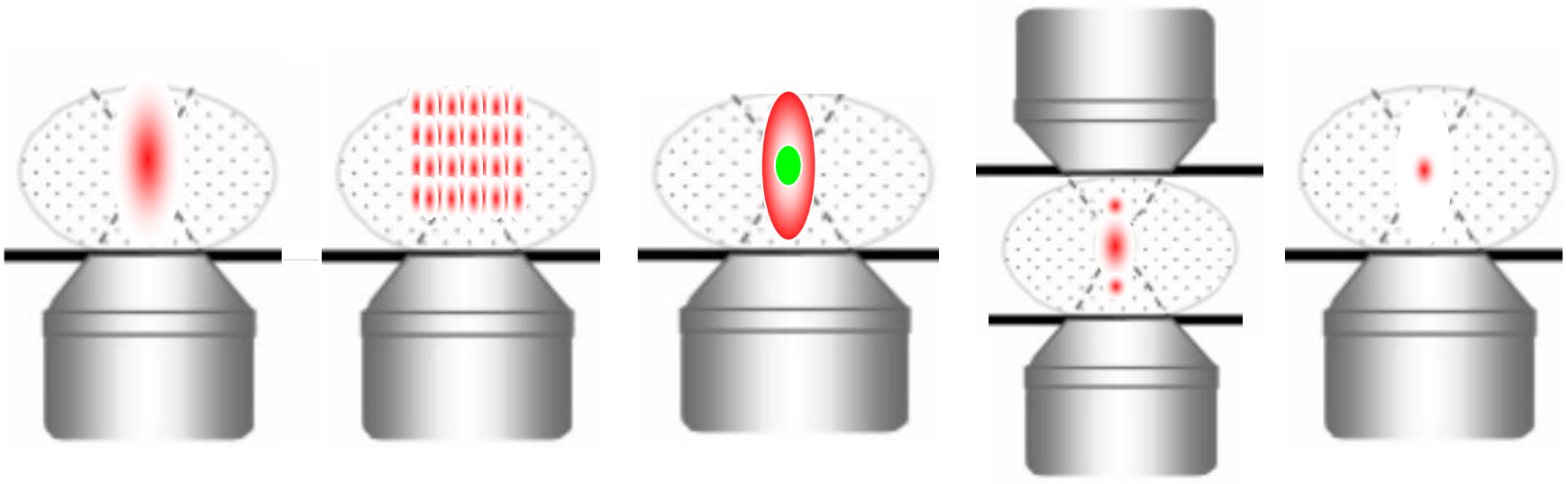
M Goeppert-Mayer
1906-1972

M Gustafson
1960-2011

S Hell
MPI Göttingen
BIOQUANT Hdg

E Betzig
Janelia Farm

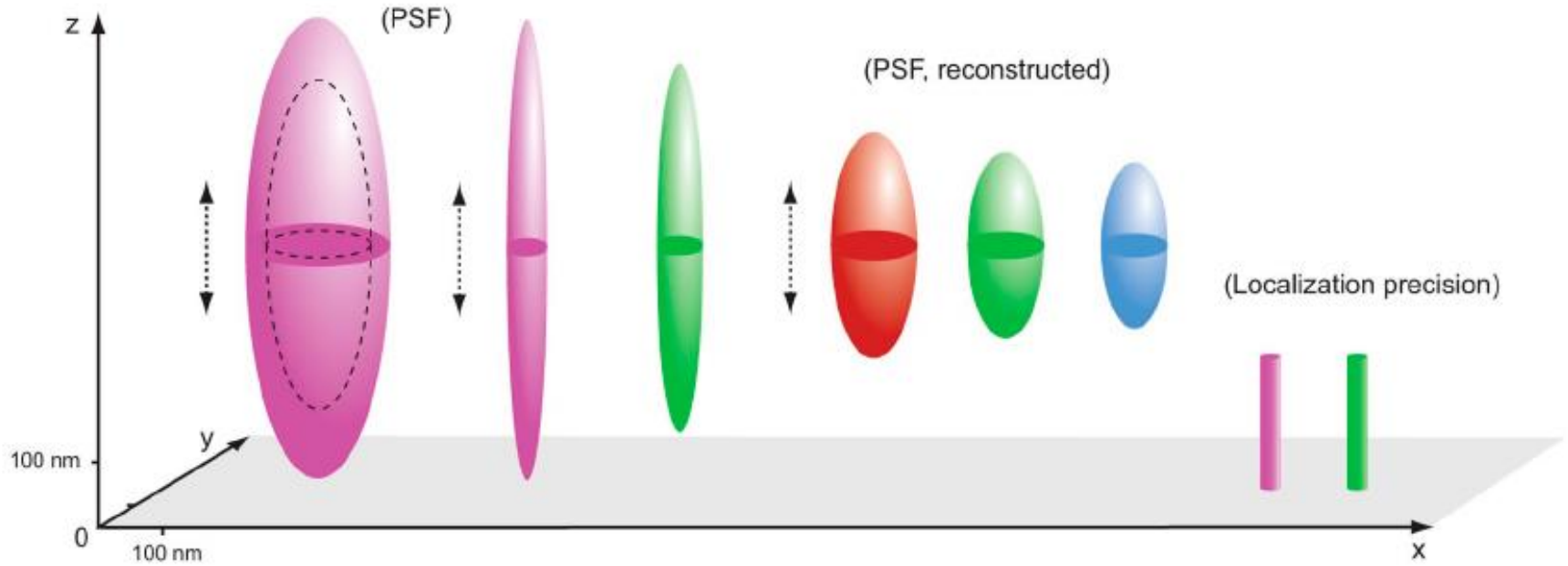
FWHM(xy) ~ λ/2 | ~ λ/4 | ~ λ/∞ | ~ λ/4 | ~ λ/100



2-photon | SIM | STED | 4-π | PALM

|-> PSF overview

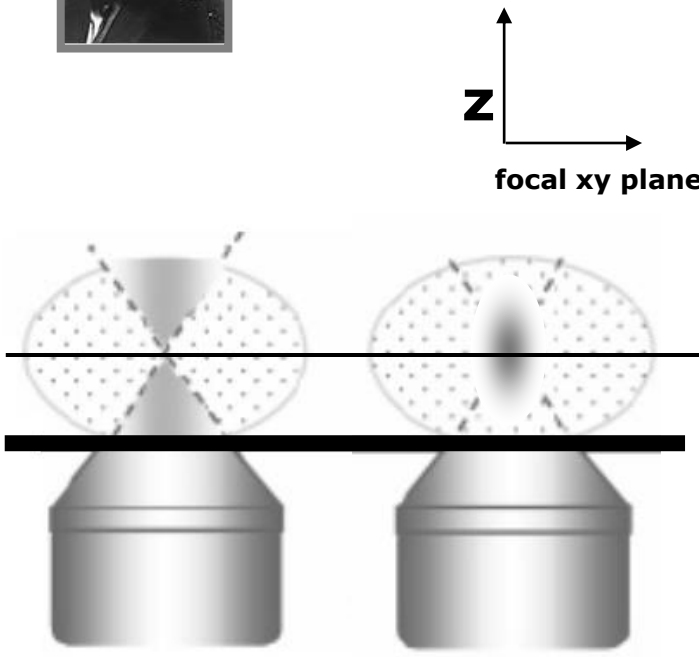
	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520



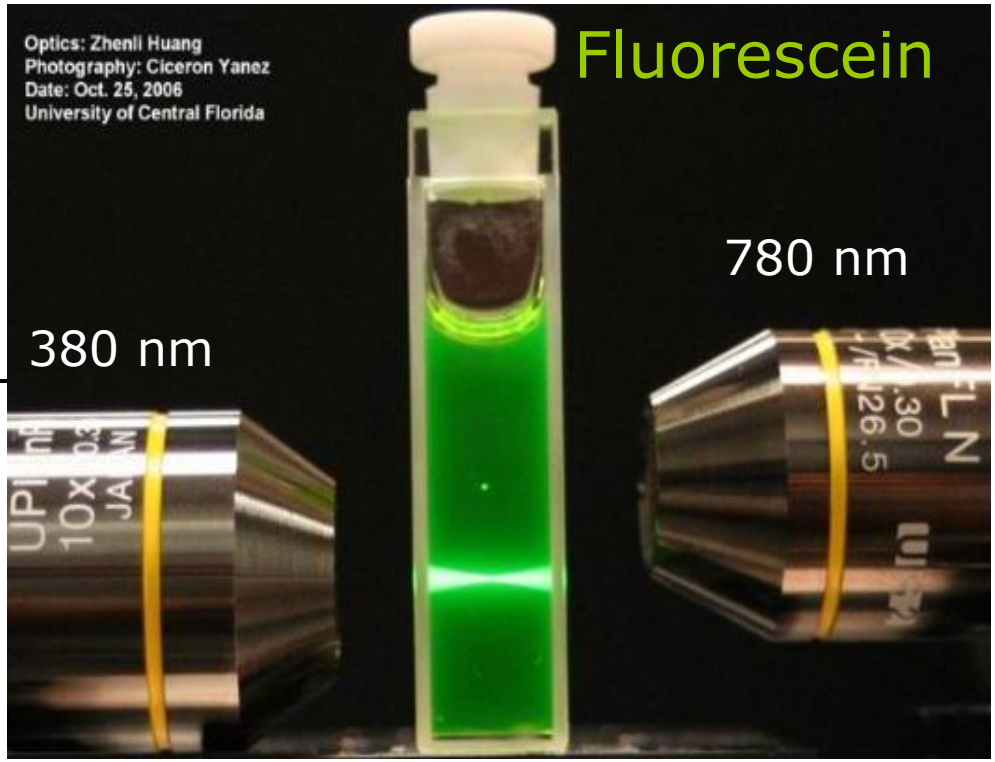
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Schermelleh et al 2010 JBC

| Best localization: confocal microscopy



convencional / confocal

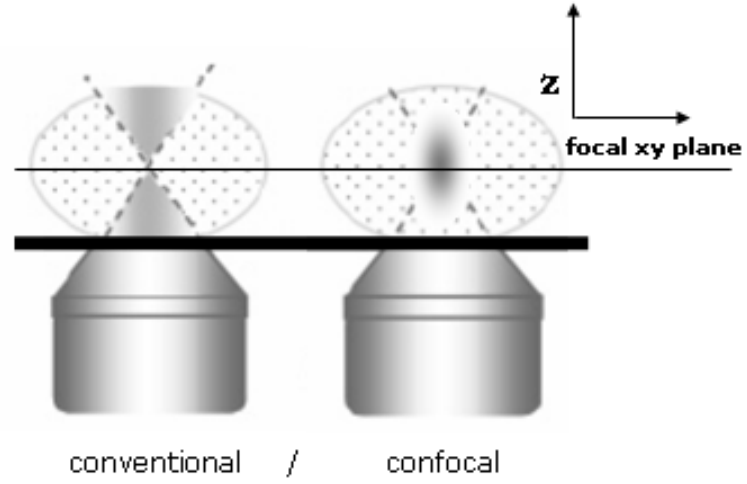


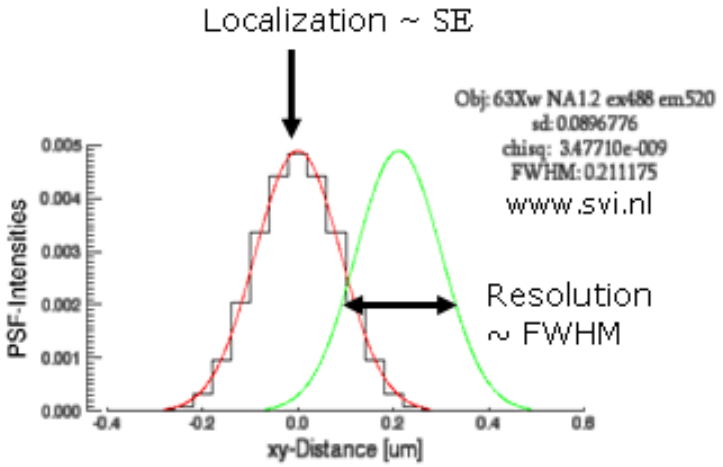
| Diffraction limited microscopy

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





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

KUNGL. VETENSKAPS-AKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

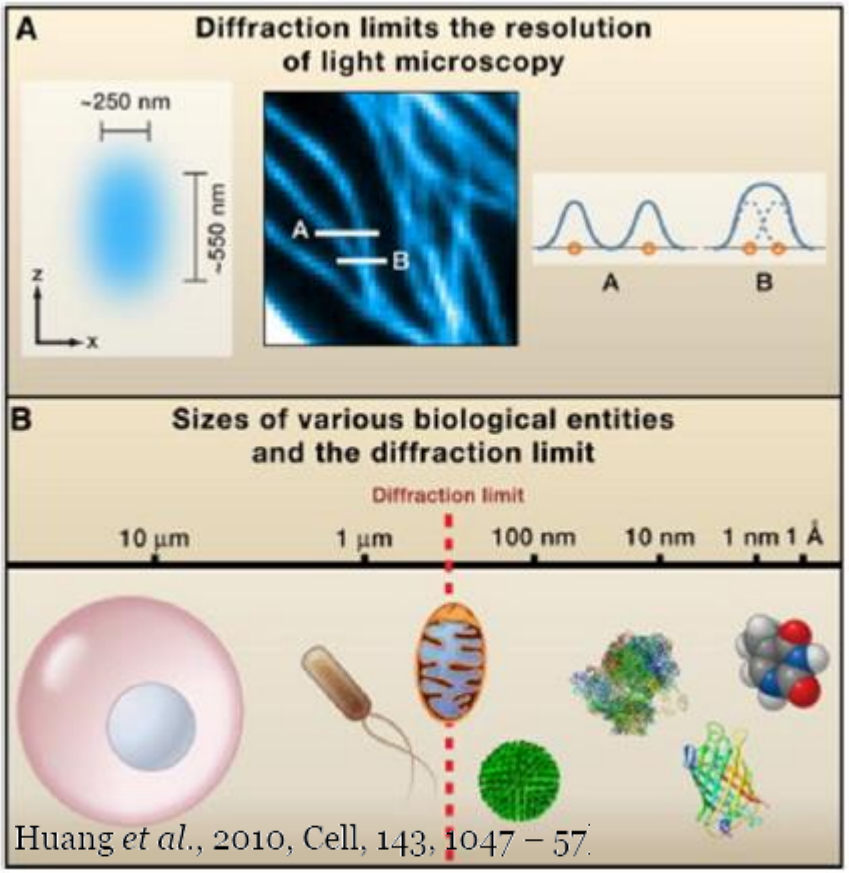
Super-resolved fluorescence microscopy

The Royal Swedish Academy of Sciences has decided to award Erik Betzig, Stefan W. Hell and W. E. Moerner the Nobel Prize in Chemistry 2014 for the development of super-resolution fluorescence microscopy.

|-> Abbe's diffraction limit

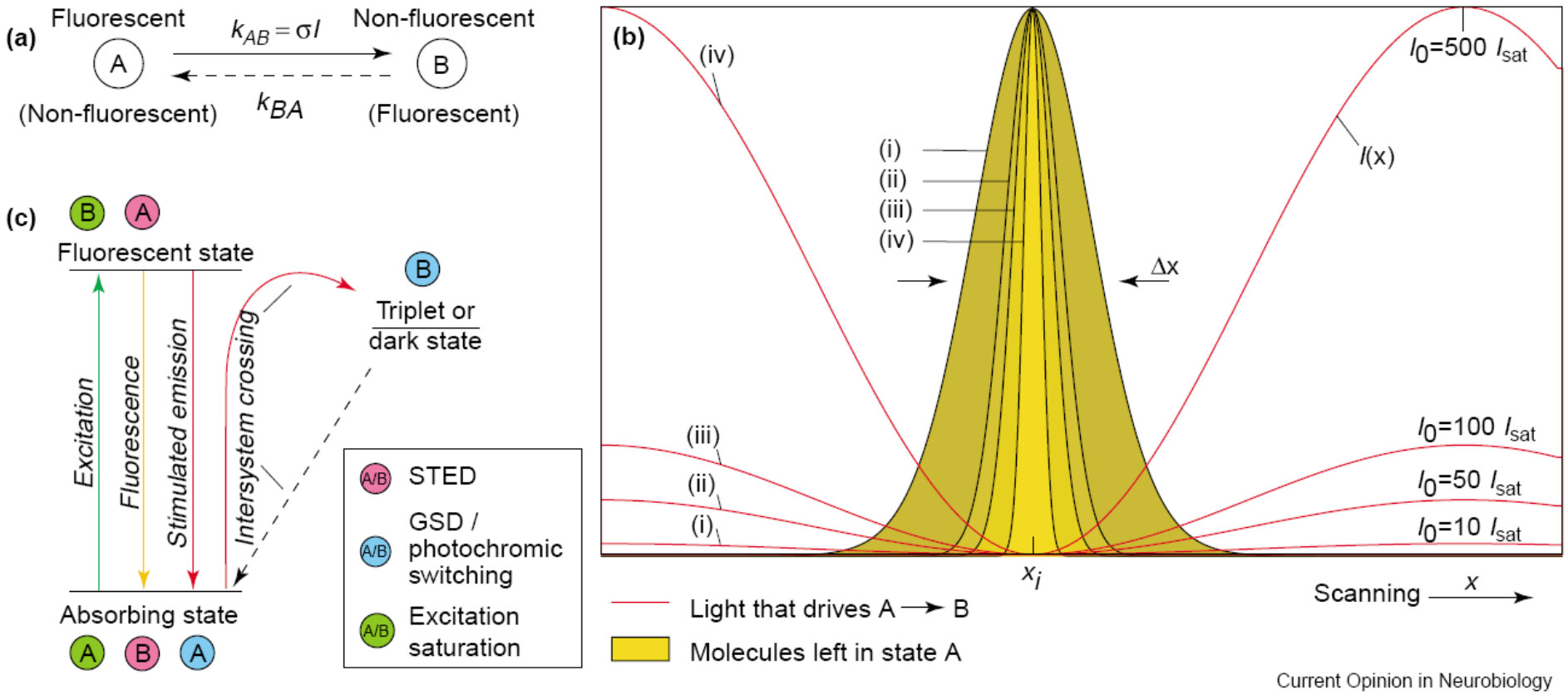
Towards the end of the nineteenth century Ernst Abbe (Abbe, 1873) and Lord Rayleigh (Rayleigh, 1896) formulated what is commonly known as the "diffraction limit" for microscopy. Roughly speaking, this limit states that it is impossible to resolve two elements of a structure which are closer to each other than about half the wave length (λ) in the lateral (x,y) plane and even further apart in the longitudinal plane (z). In other words, the minimal distances (δx_{min}, δy_{min}) that, according to Abbe's criterion, can be resolved in the lateral plane are approximated by

$$\left(\delta x_{\min}, \delta y_{\min}\right) \approx \lambda / 2$$



|-> Stimulated Emission Depletion (STED)

In the beginning of the 1990s, Stefan Hell moved as a post-doc from Germany to the University of Turku in Finland to find space and a possibility to develop his, at the time, controversial idea that it was not only possible but also feasible to transcend Abbe's diffraction limit in far-field light microscopy. In two theoretical papers he demonstrated the principles and outlined, in quantitative terms, the experimental conditions for the ground-breaking novel concept of Stimulated Emission Depletion (STED) microscopy (Hell and Wichmann, 1994) and similar (Hell and Kroug, 1995) techniques.



Current Opinion in Neurobiology

|-> Stimulated Emission Depletion (STED)

Using STED, the normally diffraction-limited focal spot can be made infinitely small. It is clear that there may be other types of problems, such as photo-damage to biological tissues, with highly intense STED beams. It is important, however, that these obstacles are not caused by a hard physical limit and may therefore be successively removed, e.g. by the introduction of other ground state depletion mechanisms than stimulated emission, which do not require such high intensity (Hell and Kroug, 1995). The outcome of Hell's first experimental demonstration of the STED principle (Klar *et al.*, 2000) is illustrated in Fig. 5, showing how the notorious poor axial resolution is improved from about 500 to 100 nm.

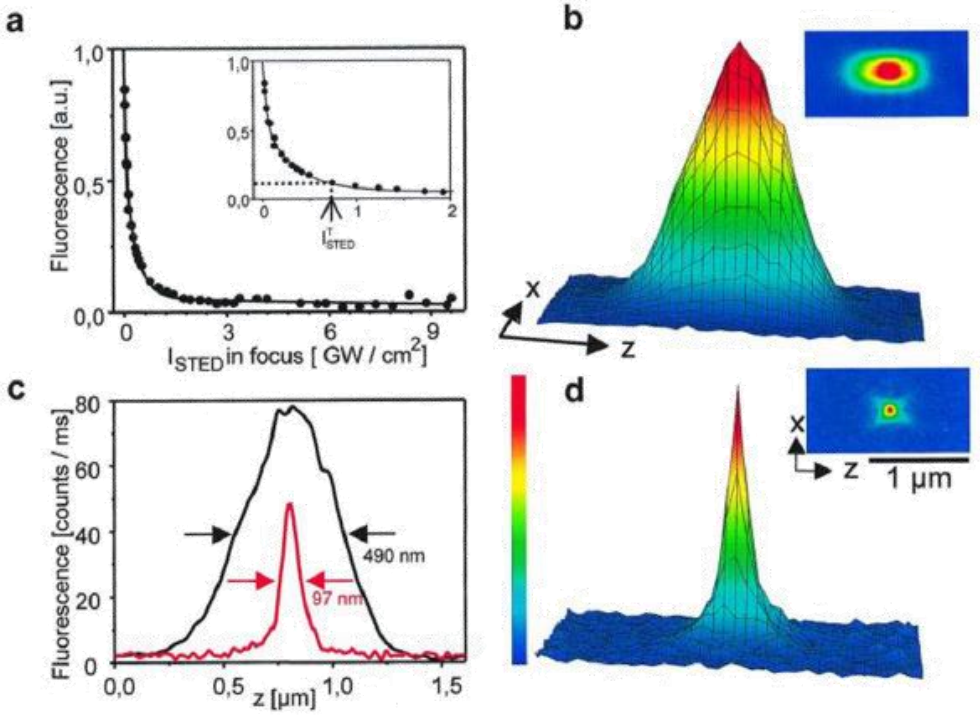
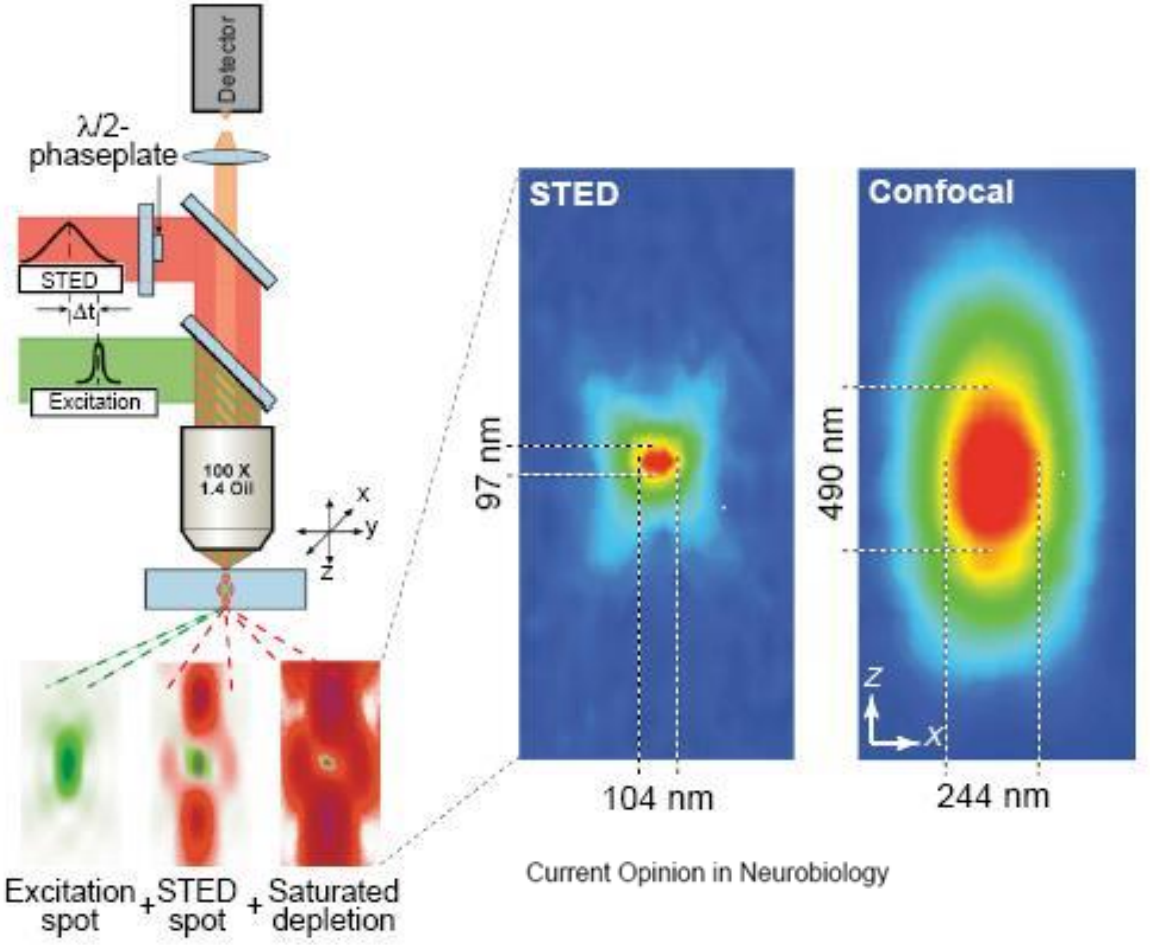


Figure 5: (Klar *et al.*, 2000, *PNAS*, 97, 8206-8210). (a) Nonlinear decrease in fluorescence intensity with increasing STED intensity, ISTED. Illustration of fluorescence intensity spot in x- and z-directions in the absence (b) and presence (d) of STED. Measured fluorescence distribution in the z-direction in the absence (black) and presence (red) of STED.

|-> Stimulated Emission Depletion (STED)

By this arrangement, in conjunction with optimal pulse sequences for the excitation and STED beams, light emission is turned off everywhere except in a small part of the diffraction-limited focal region. The latter region shrinks indefinitely with increasing intensity of the maximal value, I_0, of the STED beam. The width, Δ_{min}, of the effectively fluorescing region is in the lateral plane approximated by (Hell *et al.*, 2004)

$$\Delta_{min} \approx \frac{\lambda}{2n \sin\alpha(\sqrt{1 + I_0 / I_{sat}})}$$



Current Opinion in Neurobiology

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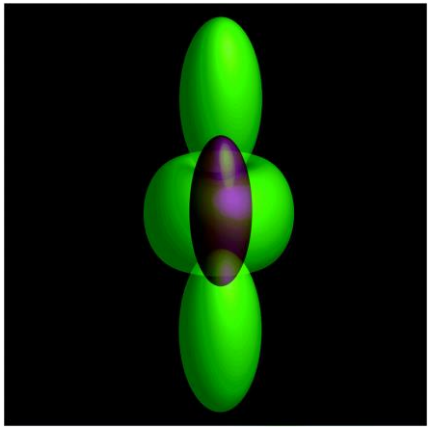
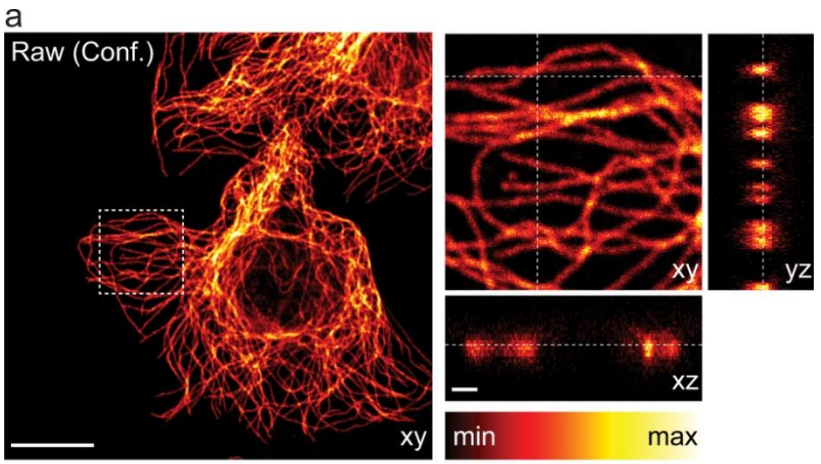
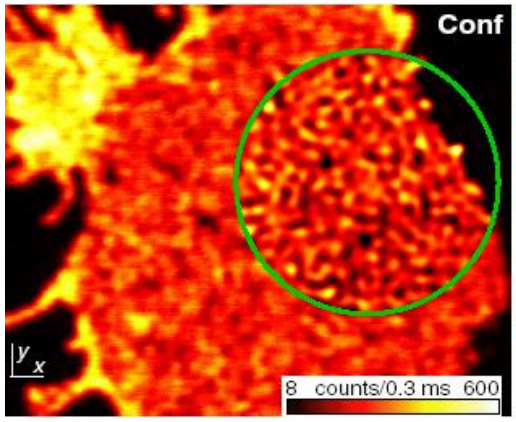
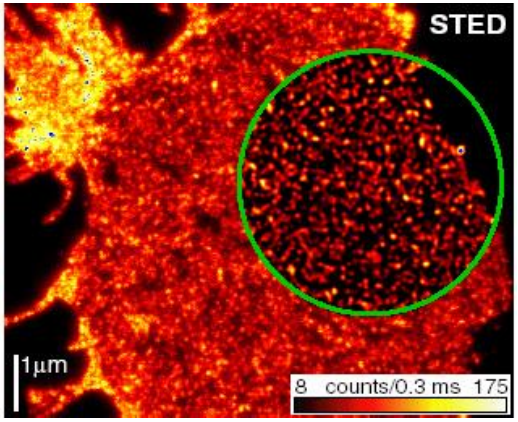
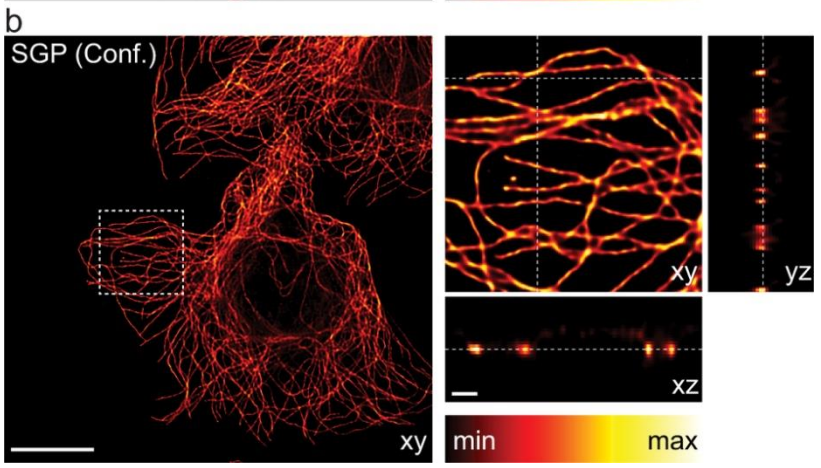


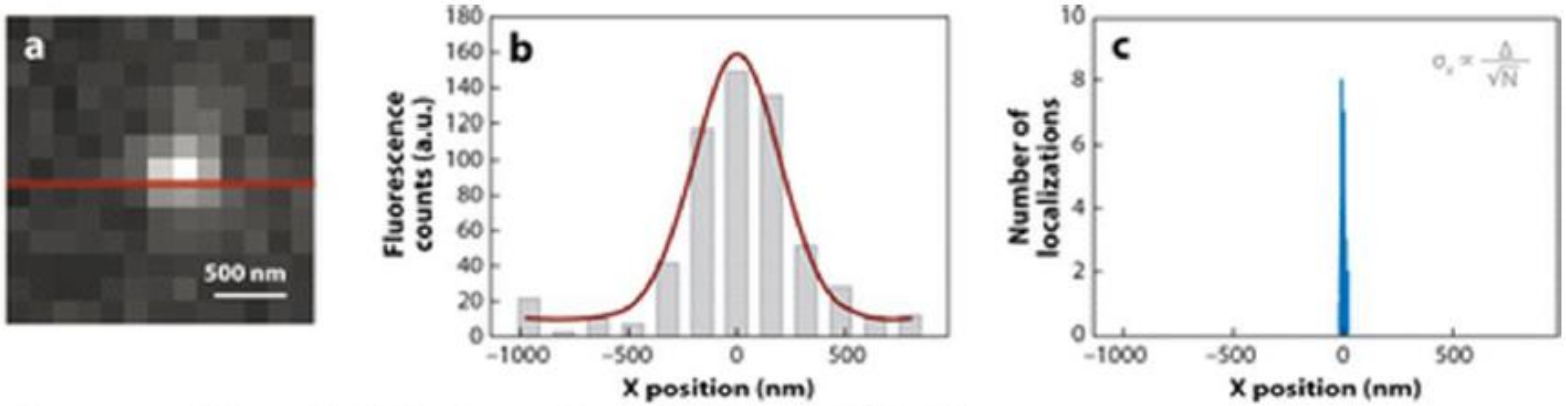








Super-resolved single-fluorophore microscopy should here be understood as a class of techniques for which the super resolution is obtained by the possibility to "super-localize" a point source of photons. .



Thompson MA, et al. 2012. Annu. Rev. Biophys. 41:321–42

Figure 6: (a) Pixelated fluorescence intensity from single fluorophore in x- and y-directions of the detector. (b) Fluorescence intensity in x-direction (blue bars) fitted to a Normal distribution (red line, Eq. 4). (c). Probability density of the center (x=0) of the Normal distribution in (b). The knowledge that there is a single emitter allows the position of the point source to be estimated much more precisely (c) than the width of the PSF (b).

Super-resolved single-fluorophore microscopy should here be understood as a class of techniques for which the super resolution is obtained by the possibility to ”super-localize” a point source of photons. .

Neglecting background and pixelation, the probability density, $p\,(x,y)$, of photon detection in the lateral plane is approximately Gaussian (Thompson *et al.*, 2012):

$$p(x,y) \approx Ce^{\frac{(x-\mu_x)^2}{2\Delta^2}\frac{(y-\mu_y)^2}{2\Delta^2}}$$

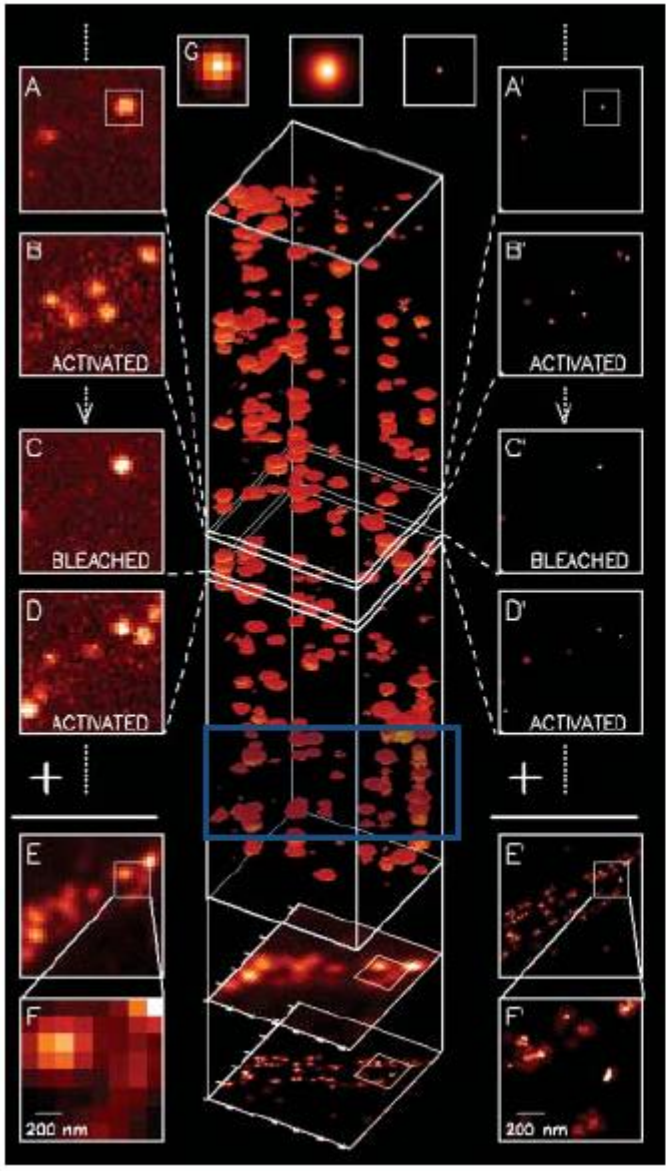
C is a normalisation constant, μ_x and μ_y define the lateral center of the PSF and Δ is equal to Abbe’s diffraction limit in Eq. 1 above. From the knowledge that the photon distribution on the detector stems from a point source, the center of the point spread function can be estimated with a standard error, Δ_{min}, in both the x and y coordinates, given by (Bobroff, 1996; Webb, 2002):

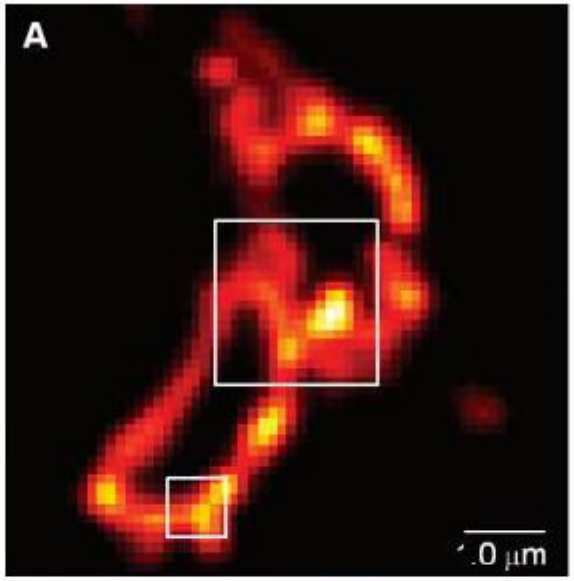
$$\Delta_{\min} = \frac{\Delta}{\sqrt{N}} = \frac{1}{\sqrt{N}}\frac{\lambda}{2n\sin\alpha}$$

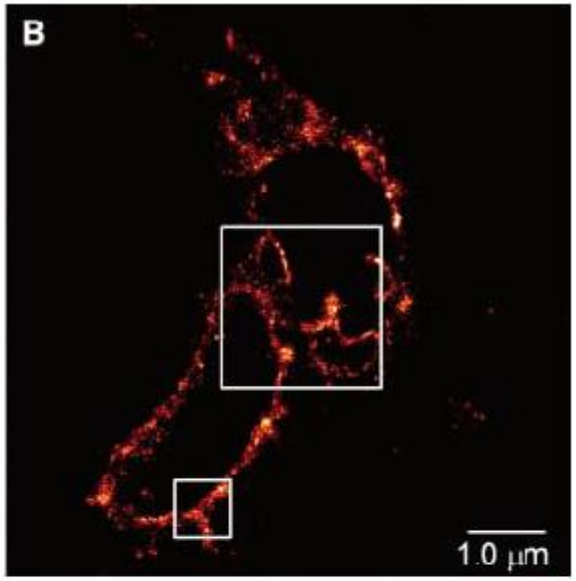
N is the total number of photons registered by the detector, and it is seen that the spatial resolution of the microscope, taken as its ability to localize a point source, Δ_{min} , is improved from Abbe’s original limit by the factor $1/\sqrt{N}$.

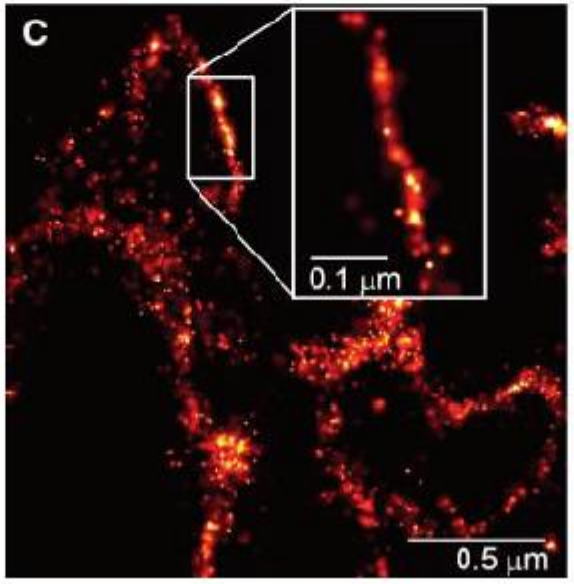
|-> Photo-activated localization microscopy (PALM)

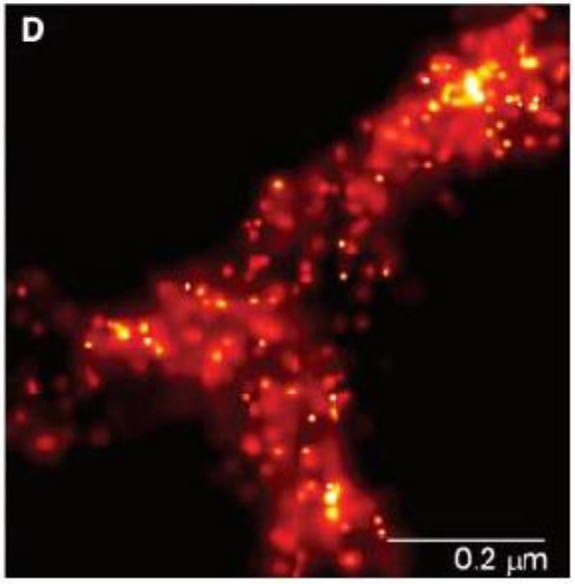
Betzig et al (2006) Science, 313(5793), 1642-5











- Fluorescence Microscopy, From Principles to Biological Applications, Ulrich Kubitscheck (Editor), 2nd Edition, June 2017, Hardcover, ISBN: 978-3-527-33837-5

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