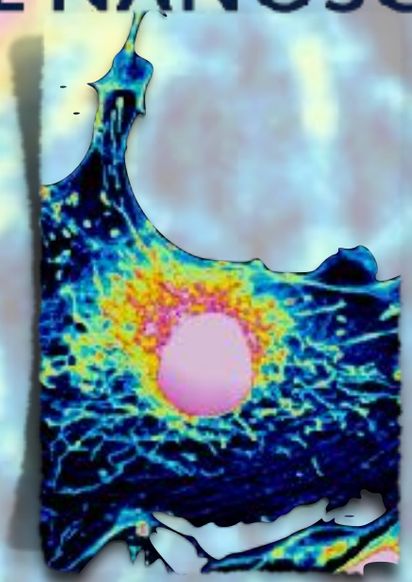
The Abdus Salam
International Centre
for Theoretical Physics

The Extra Microscope

Alberto Diaspro

ISTITUTO ITALIANO DI TECNOLOGIA
OPTICAL NANOSCOPY



EURO-BIOIMAGING

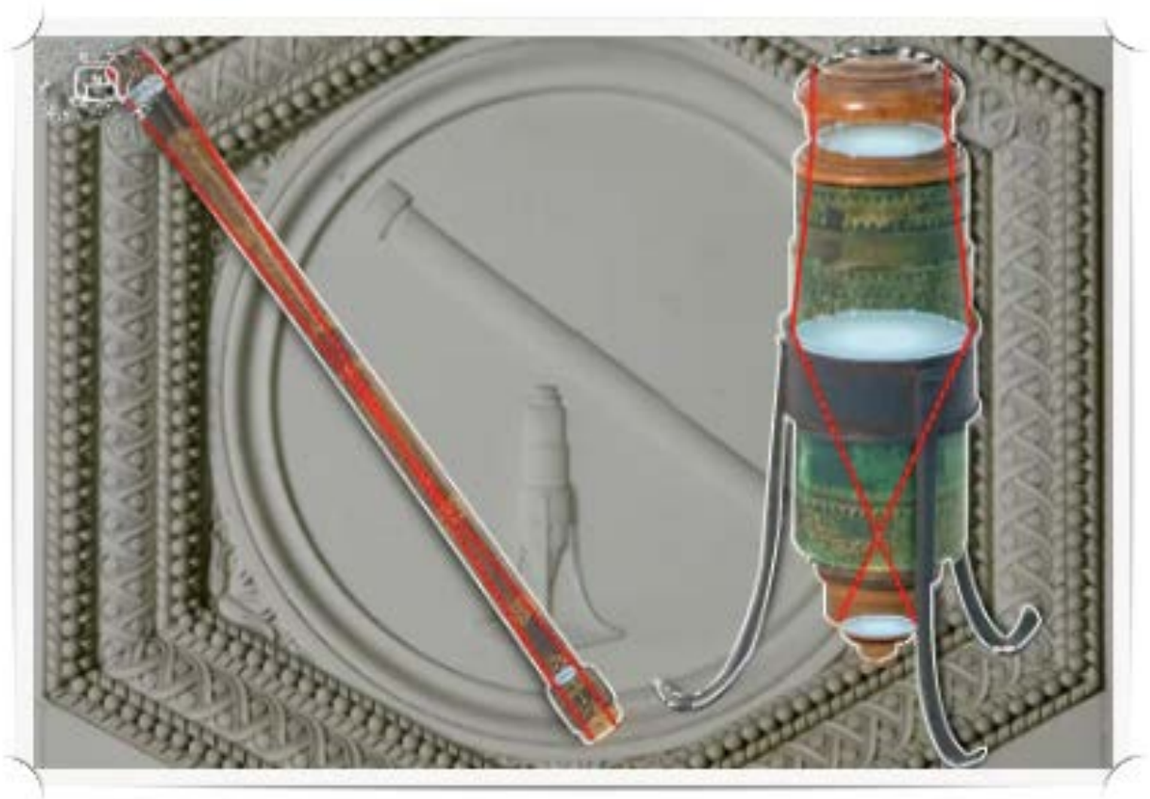
lanir.eu
FP7 Nanoscopy Project

lambs

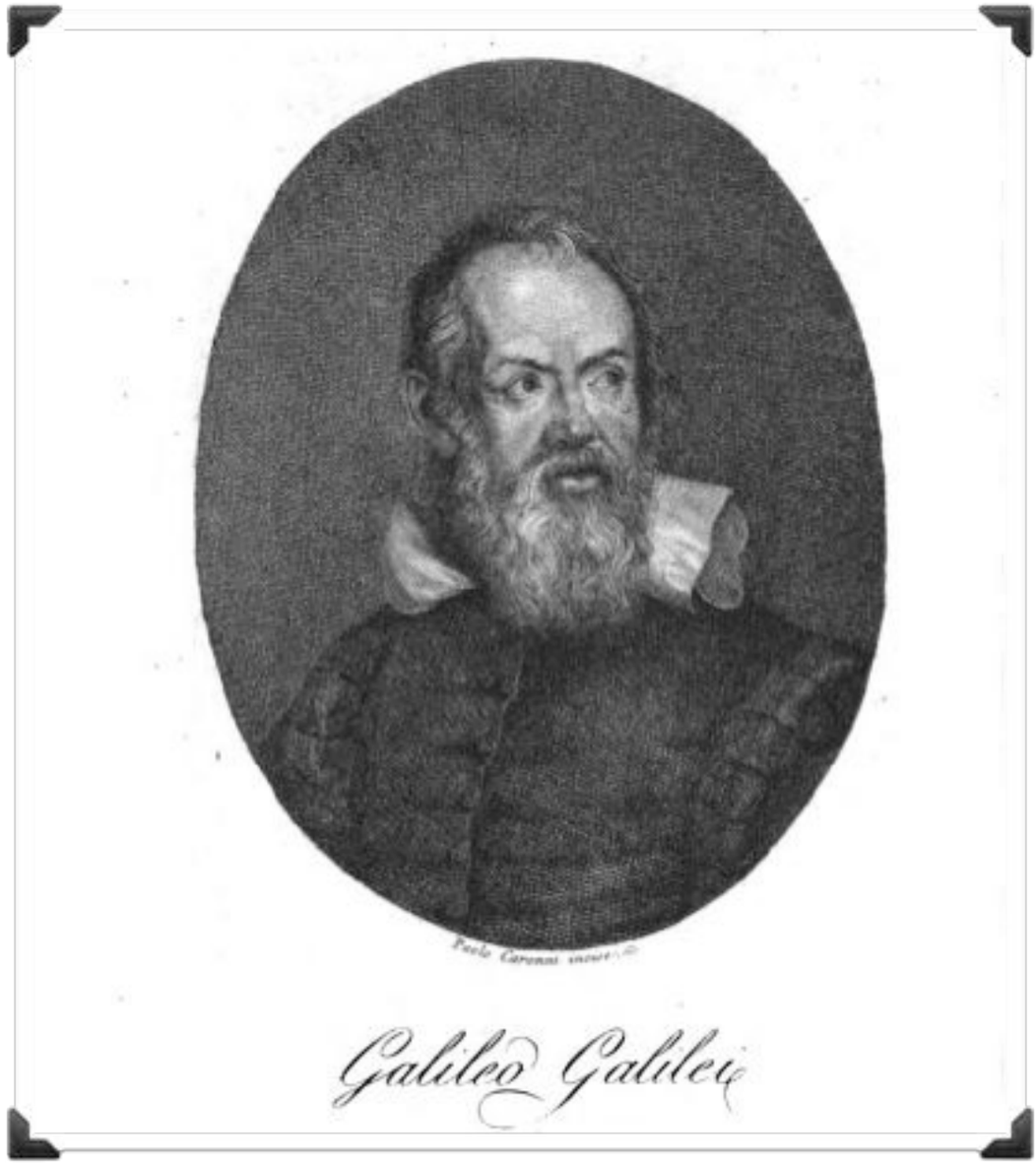
iit NANOSCOPY

MICROSCOPIUM **EXTRAORDINARIUM** NOMINARE LIBUIT…

...microscopium nominare libuit...Faber, Lincei, 1625

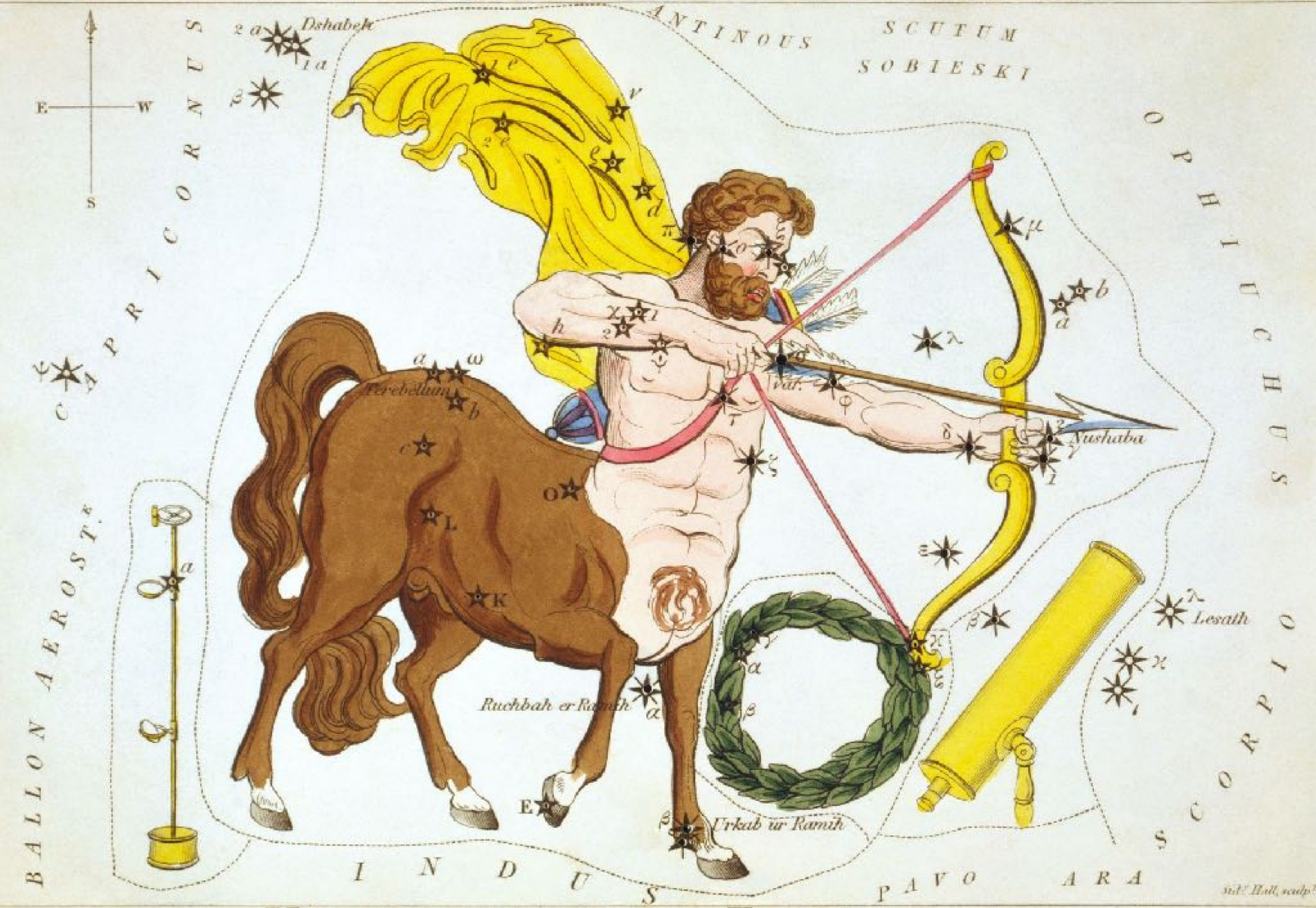


Since the early years of the Galileo Galilei's "Occhialino" - "*un occhialino per veder da vicino le cose minime*" - brillantly renamed, on April 13th 1625 in a letter written to Federico Cesi, as "Microscopium" by Johannes Faber (1574-1629), it was evident the potential of such an optical tool.



Ottavio Leoni, portrait of Galileo Galilei, 1624

SAGITTARIUS AND CORONA AUSTRALIS.



MICROSCOPIUM. AND TELESCOPIUM.

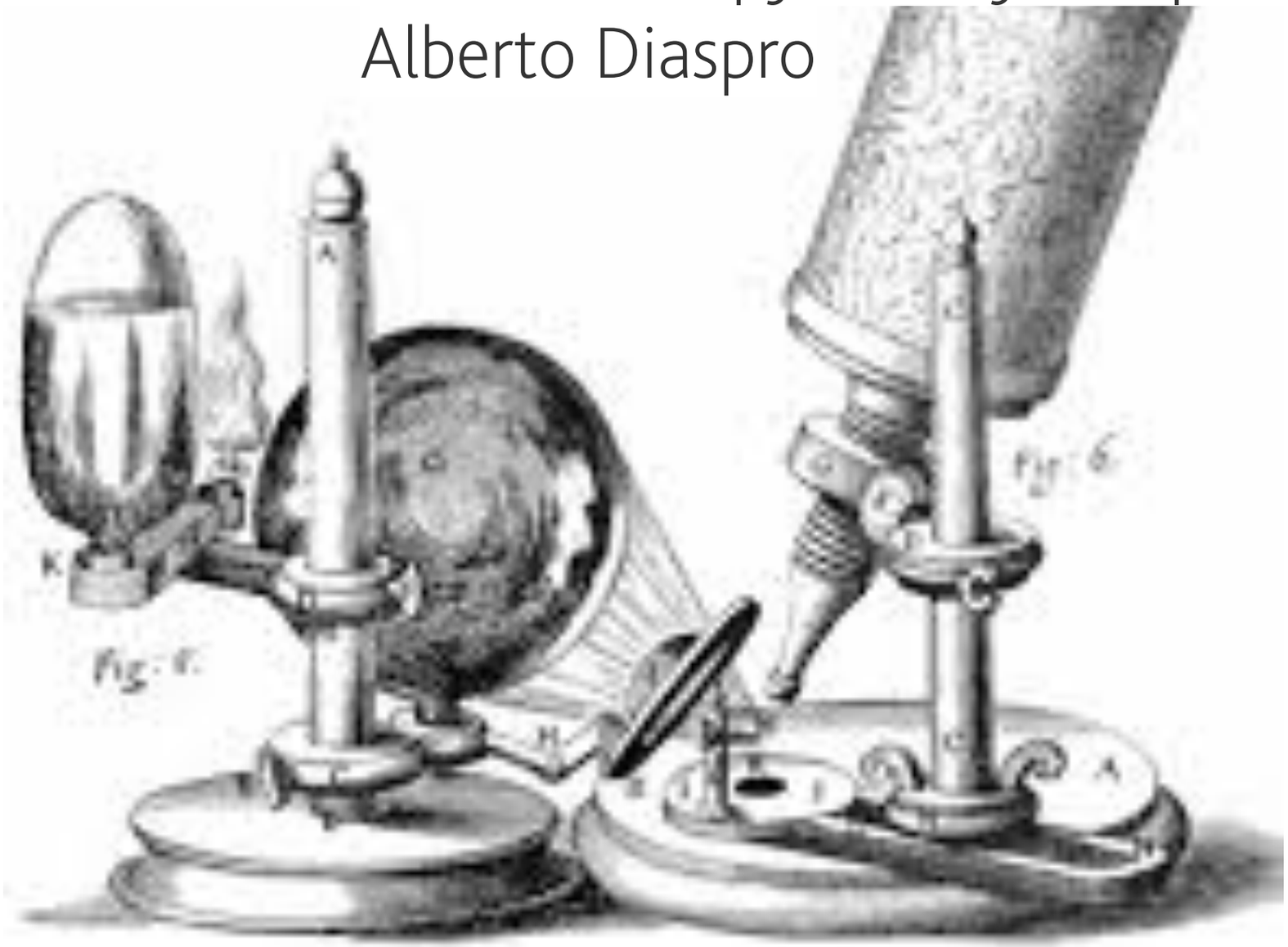
oricornus
Nunki
Sagittarius
Antares
Kaus Australis
Microscopium
Corona Australis
Shaula
Scorpius
Girtab
Telescopium
Lupus
Norma
Ara
Indus
Alnair
Peacock
Pavo
Earth, casa mia, 49m
FOV 55.2°
18.3 FPS
2013-07-06
22:28:48

"Sometimes you can observe a lot by watching."
— Yogi Berra, catcher

The Abdus Salam
International Centre
for Theoretical Physics

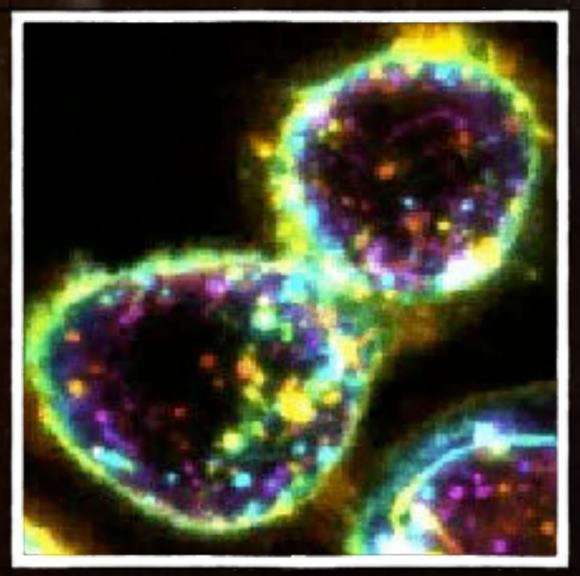
Advanced fluorescence microscopy: theory and practice

Alberto Diaspro

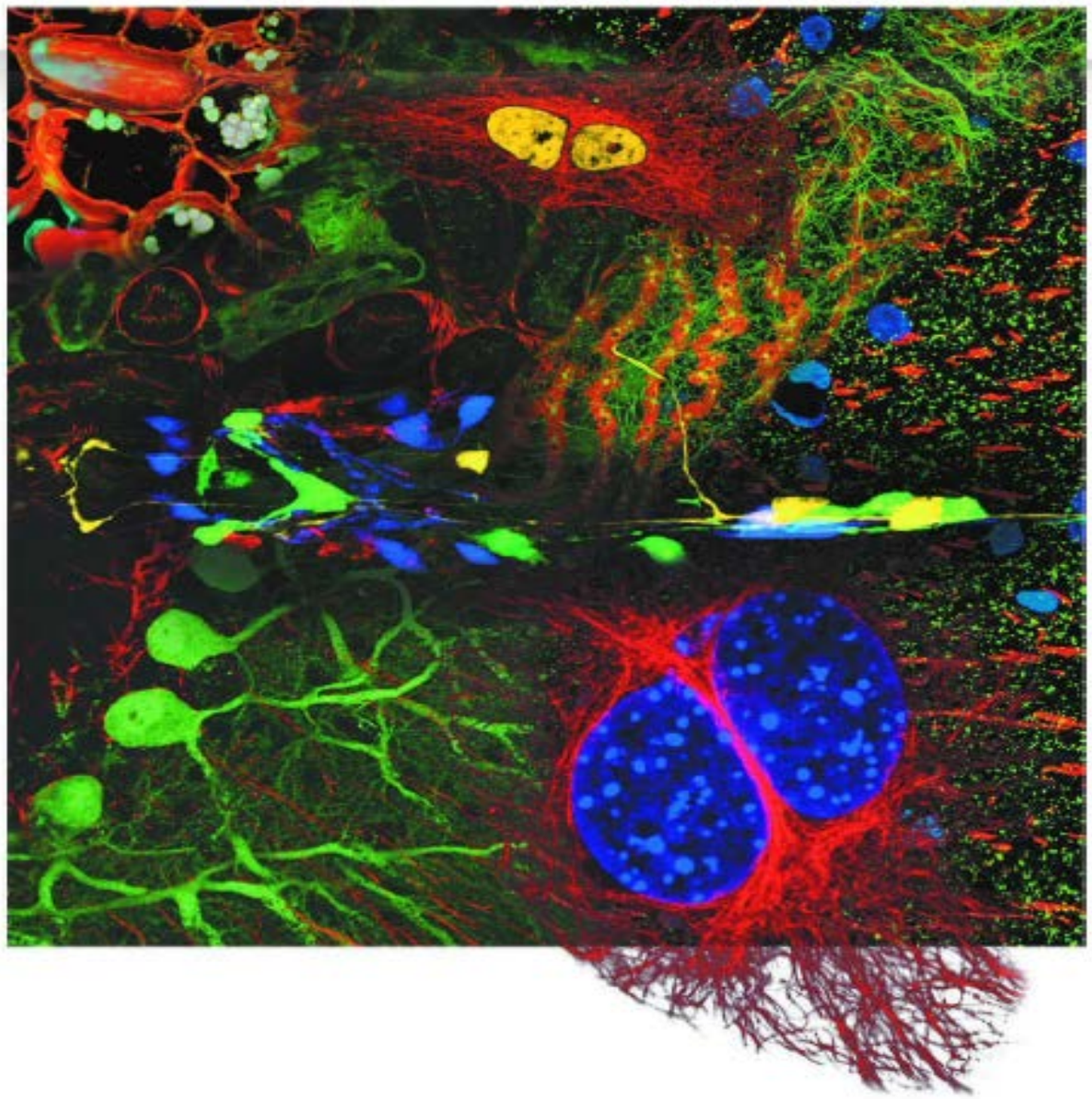


https://www.youtube.com/watch?v=h0jFSHz_5Rc

Light microscopy turning invisible into visible



Light microscopy turning invisible into visible

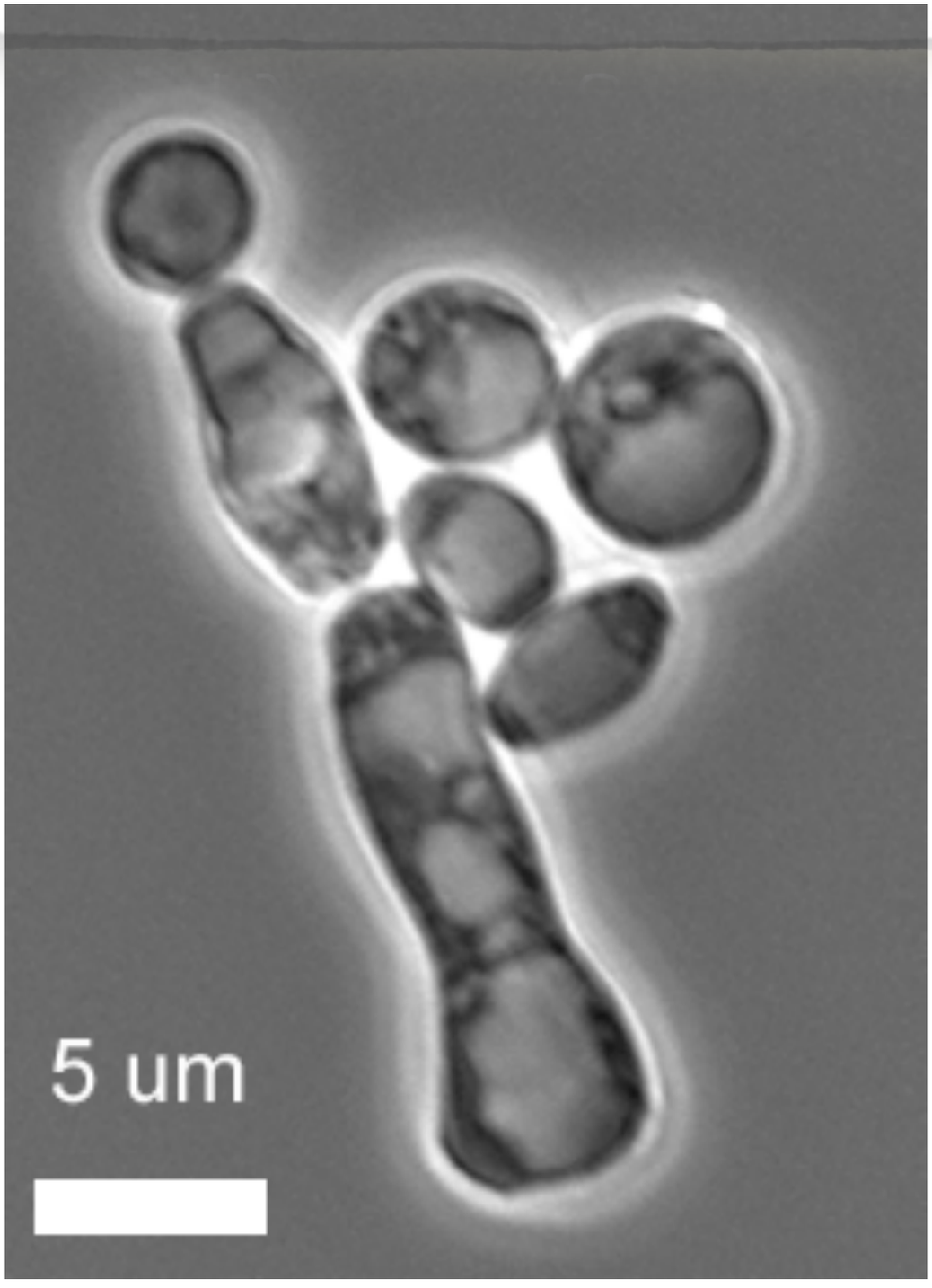


A microscope is an instrument magnifying objects by means of a specific interaction – more commonly by means of lenses – so as to capture details invisible to the naked eye.

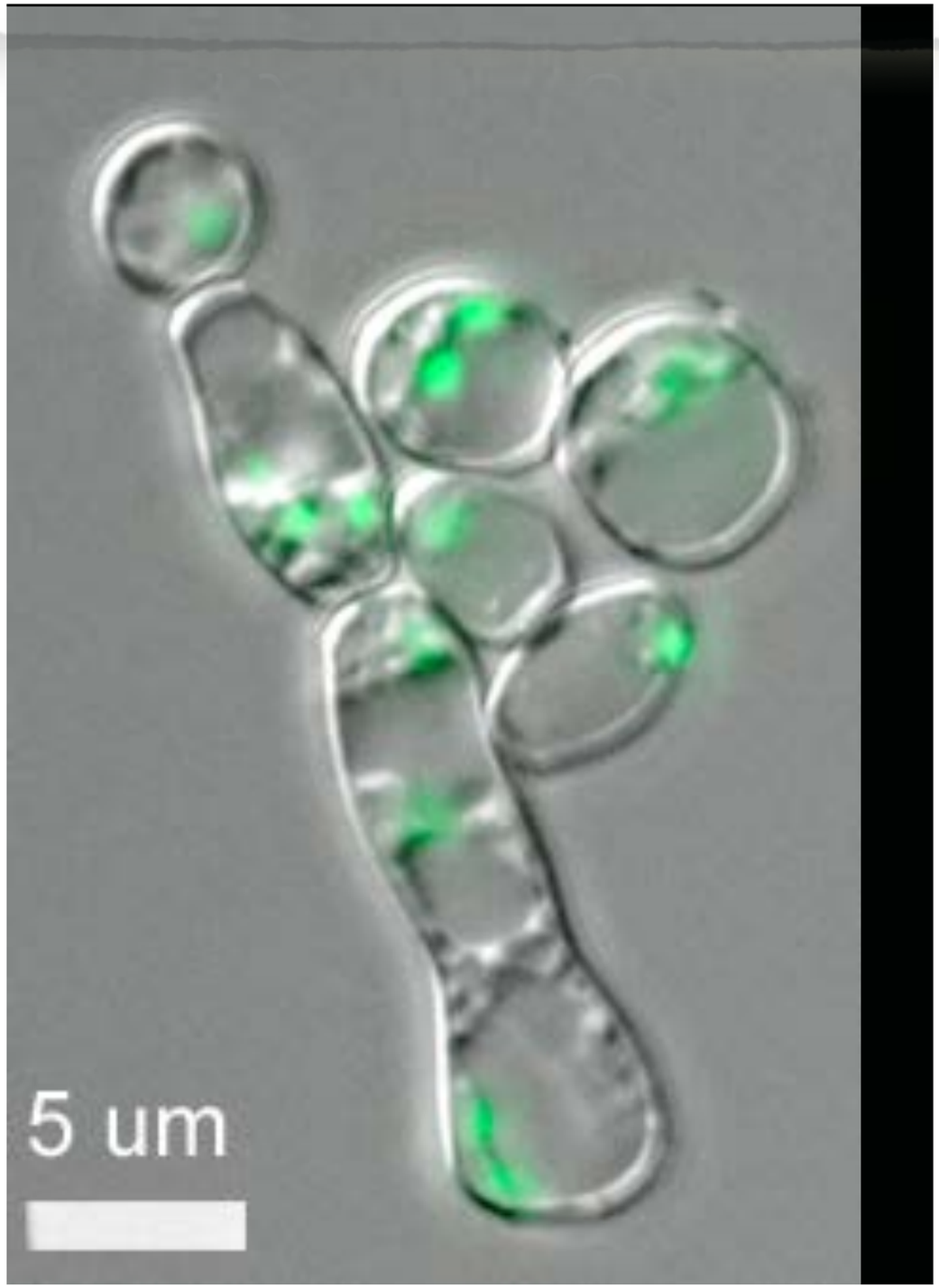
(Oxford dictionary, after Colin Sheppard)

SLIDE CREDIT: leica microsystems

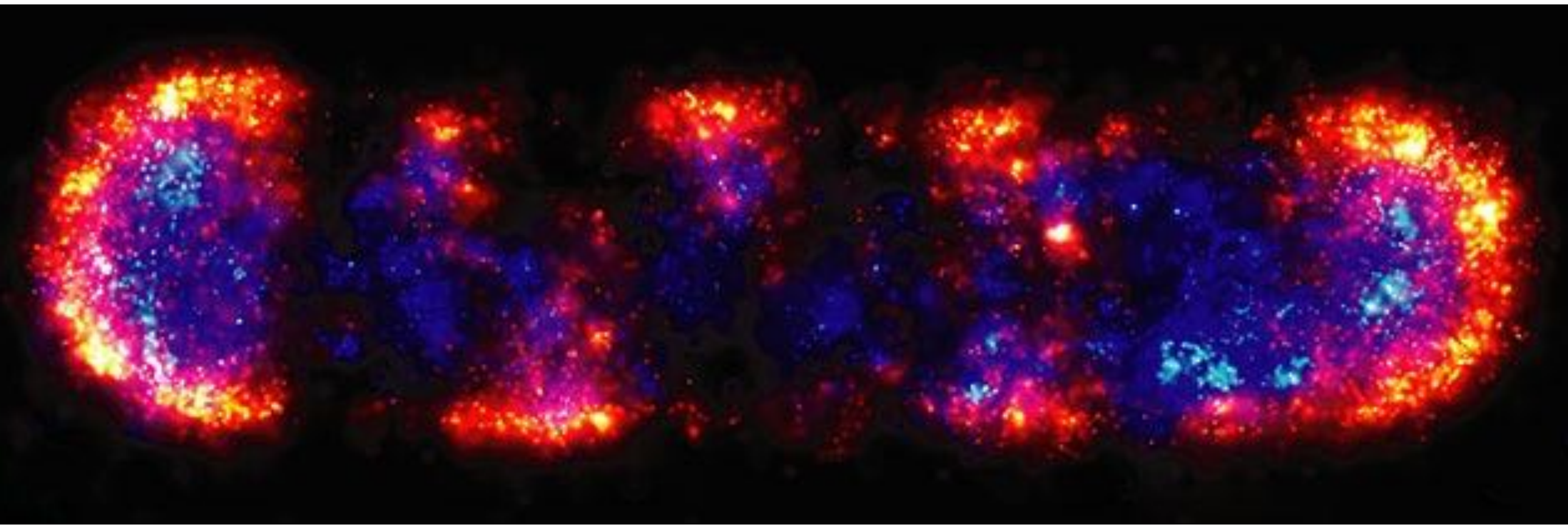
Light microscopy turning invisible into visible



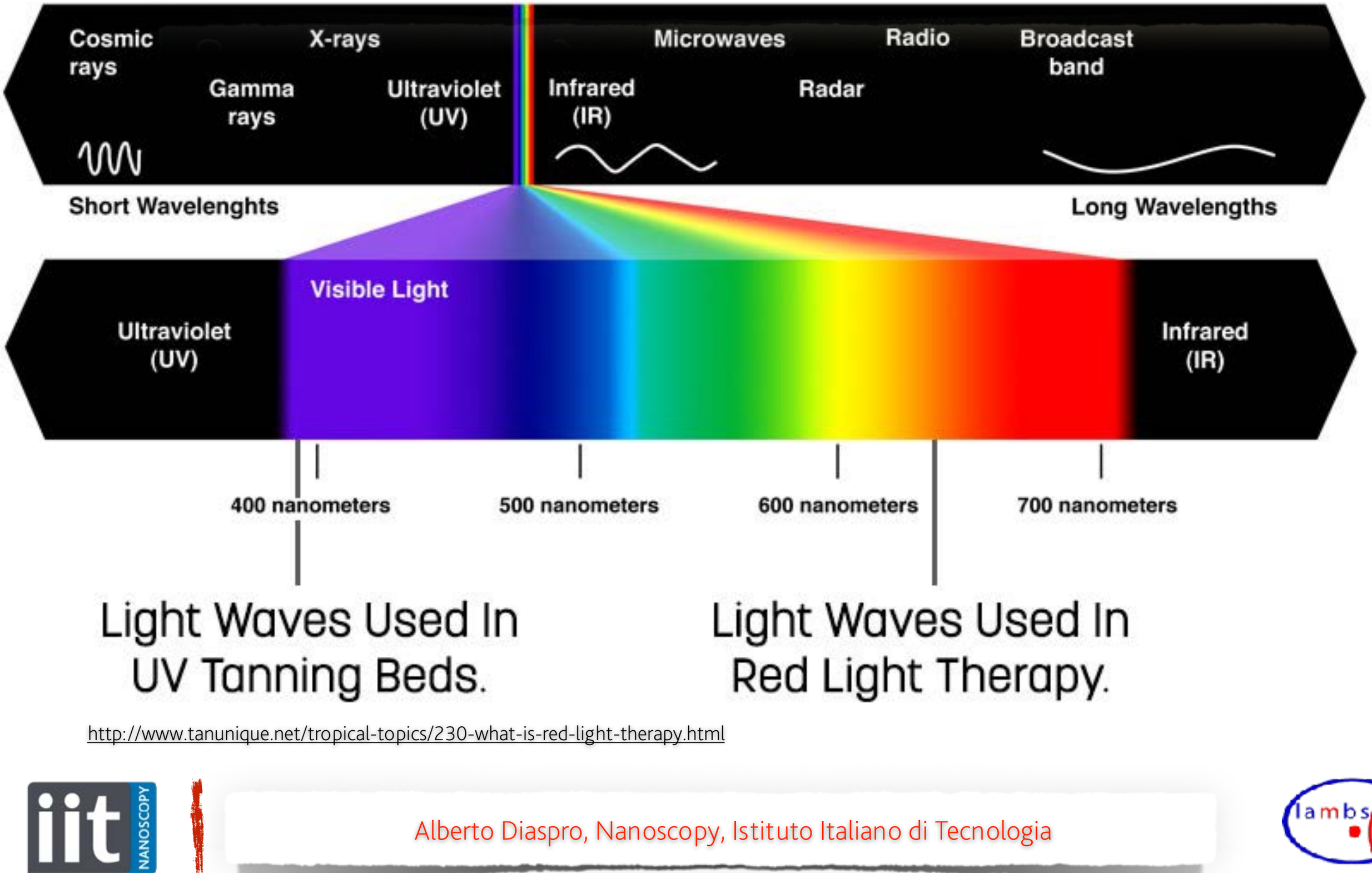
Light microscopy turning invisible into visible



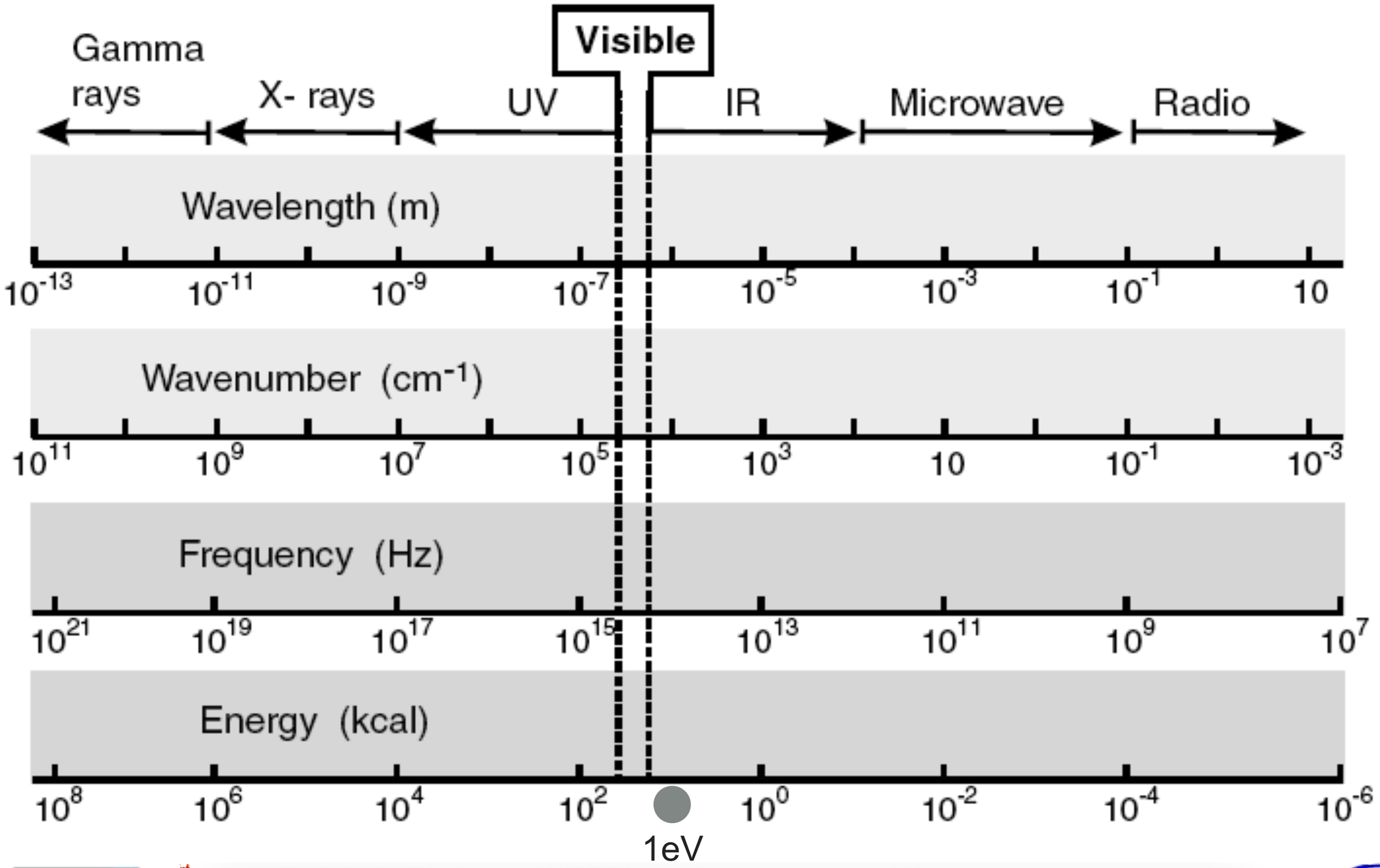
Light microscopy turning invisible into colors



Light microscopy turning invisible into visible

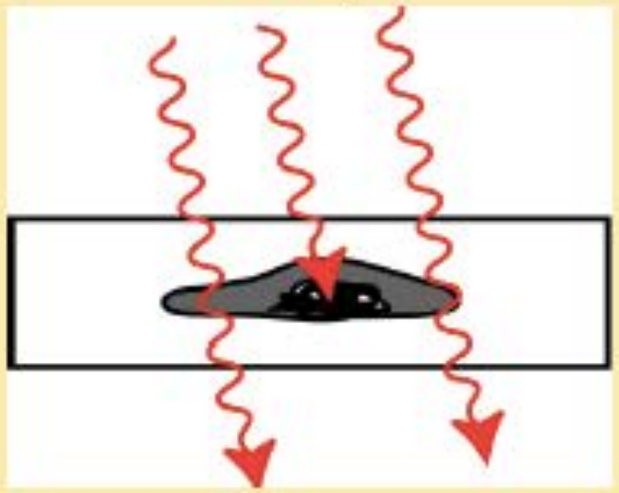


http://www.tanunique.net/tropical-topics/230-what-is-red-light-therapy.html

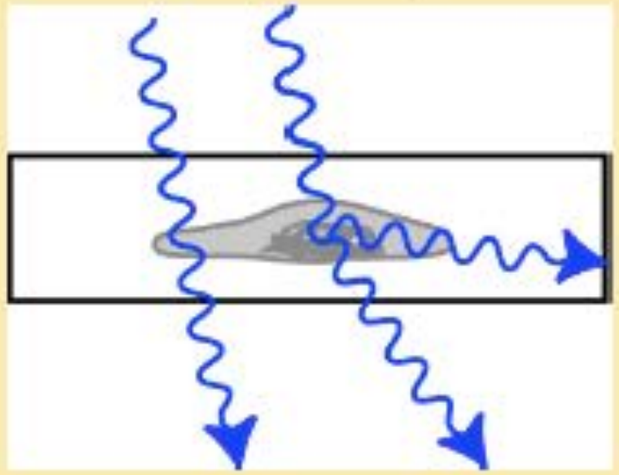

Gamma rays
X- rays
UV
Visible
IR
Microwave
Radio
Wavelength (m)
10^-13 10^-11 10^-9 10^-7 10^-5 10^-3 10^-1 10
Wavenumber (cm^-1)
10^11 10^9 10^7 10^5 10^3 10 10^-1 10^-3
Frequency (Hz)
10^21 10^19 10^17 10^15 10^13 10^11 10^9 10^7
Energy (kcal)
10^8 10^6 10^4 10^2 10^0 10^-2 10^-4 10^-6
1eV

Possible Interactions with Light

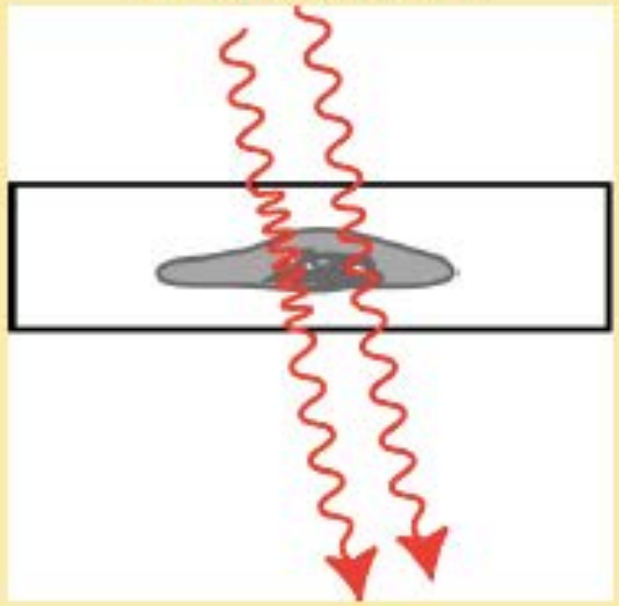
A. Absorption



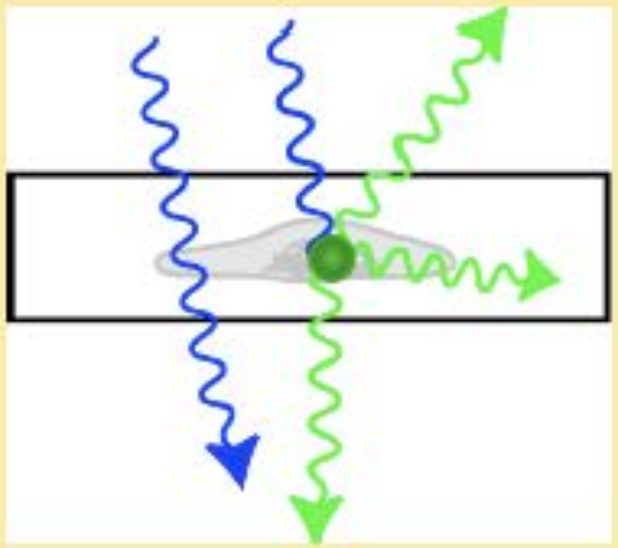
B. Scattering

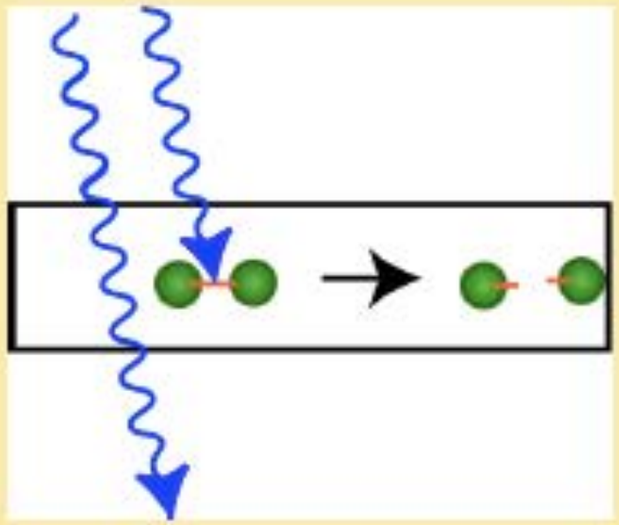


C. Interference



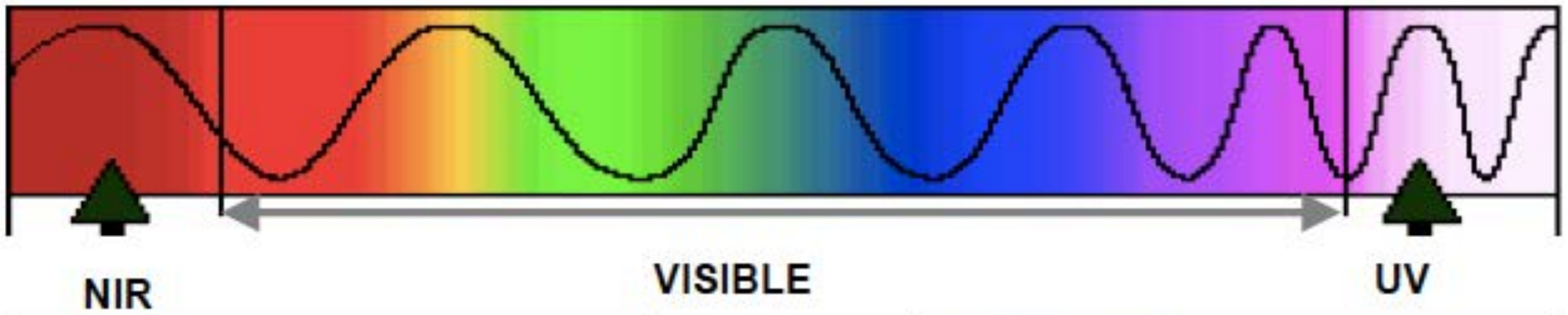
D. Fluorescence





E. Light-Induced Processes

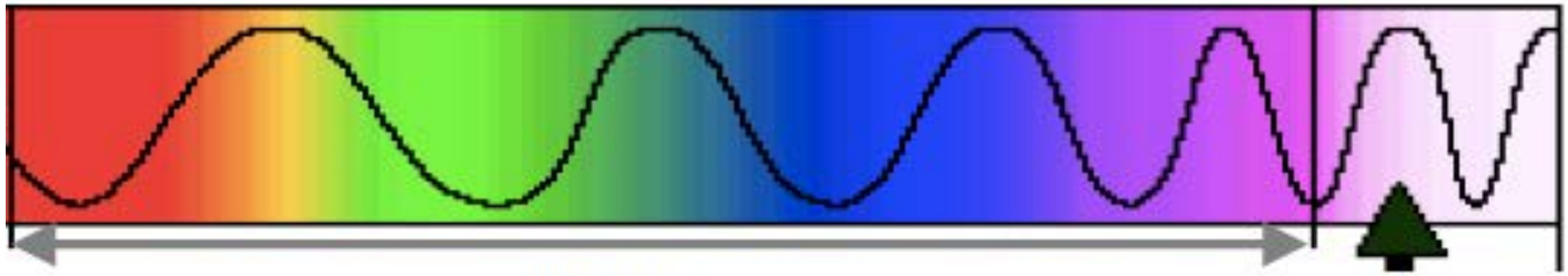
Absorbers in Tissue

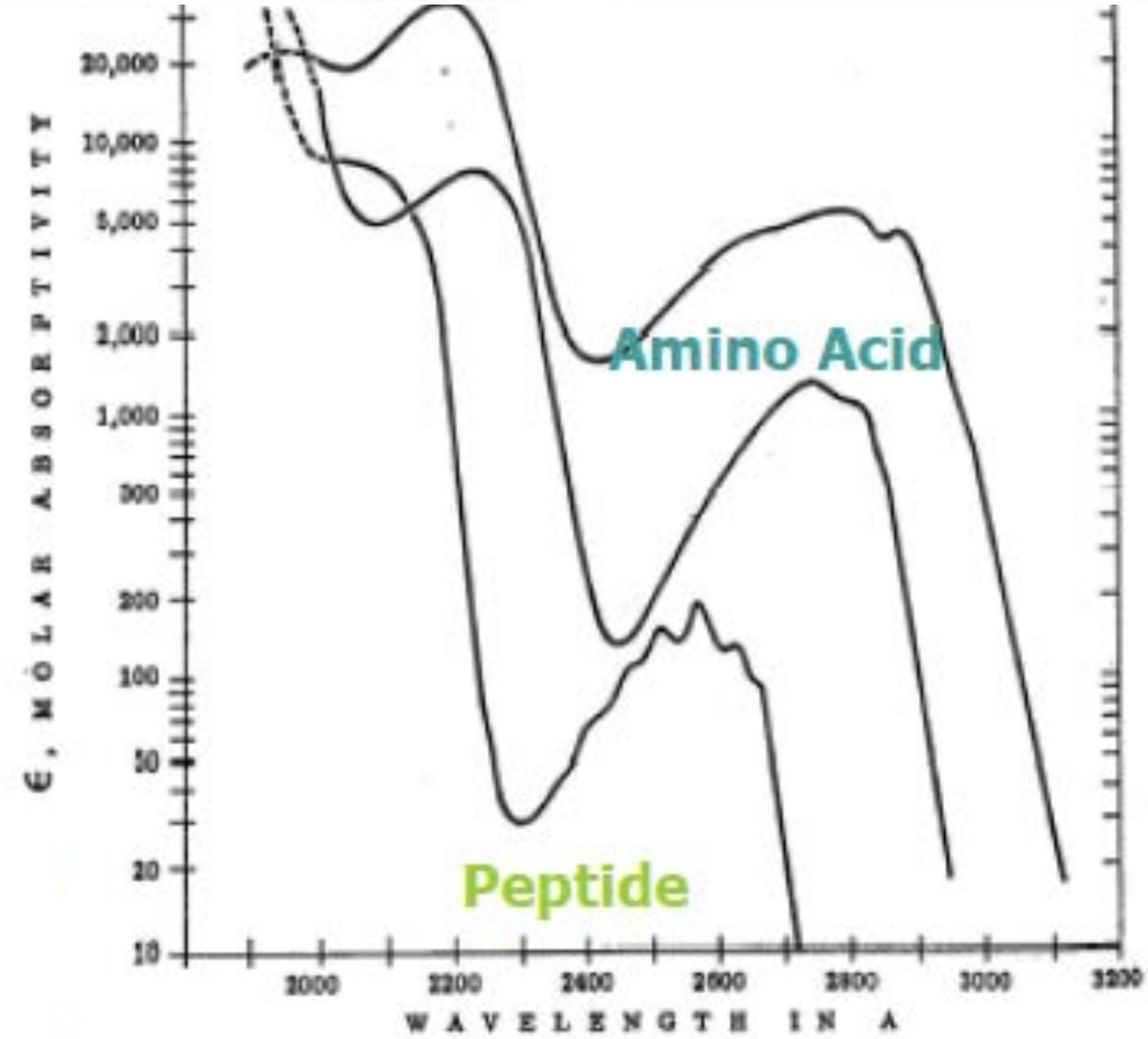


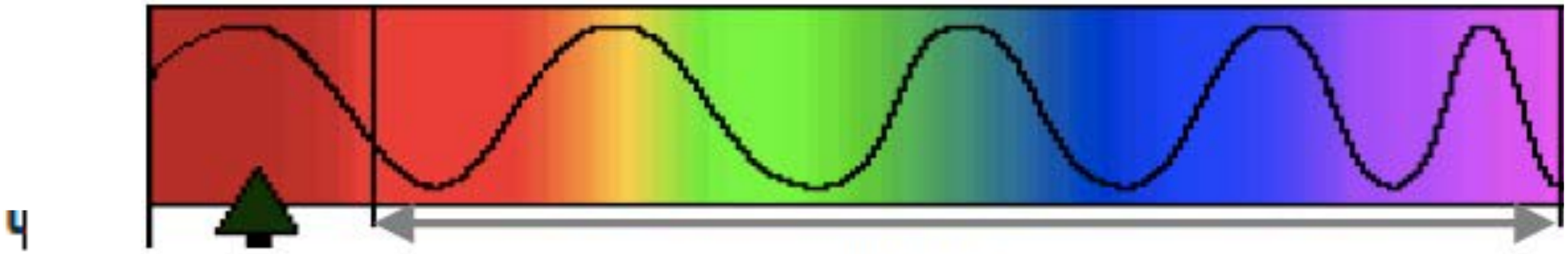
- **NIR**
  - Hemoglobin
  - Lipids
  - Water

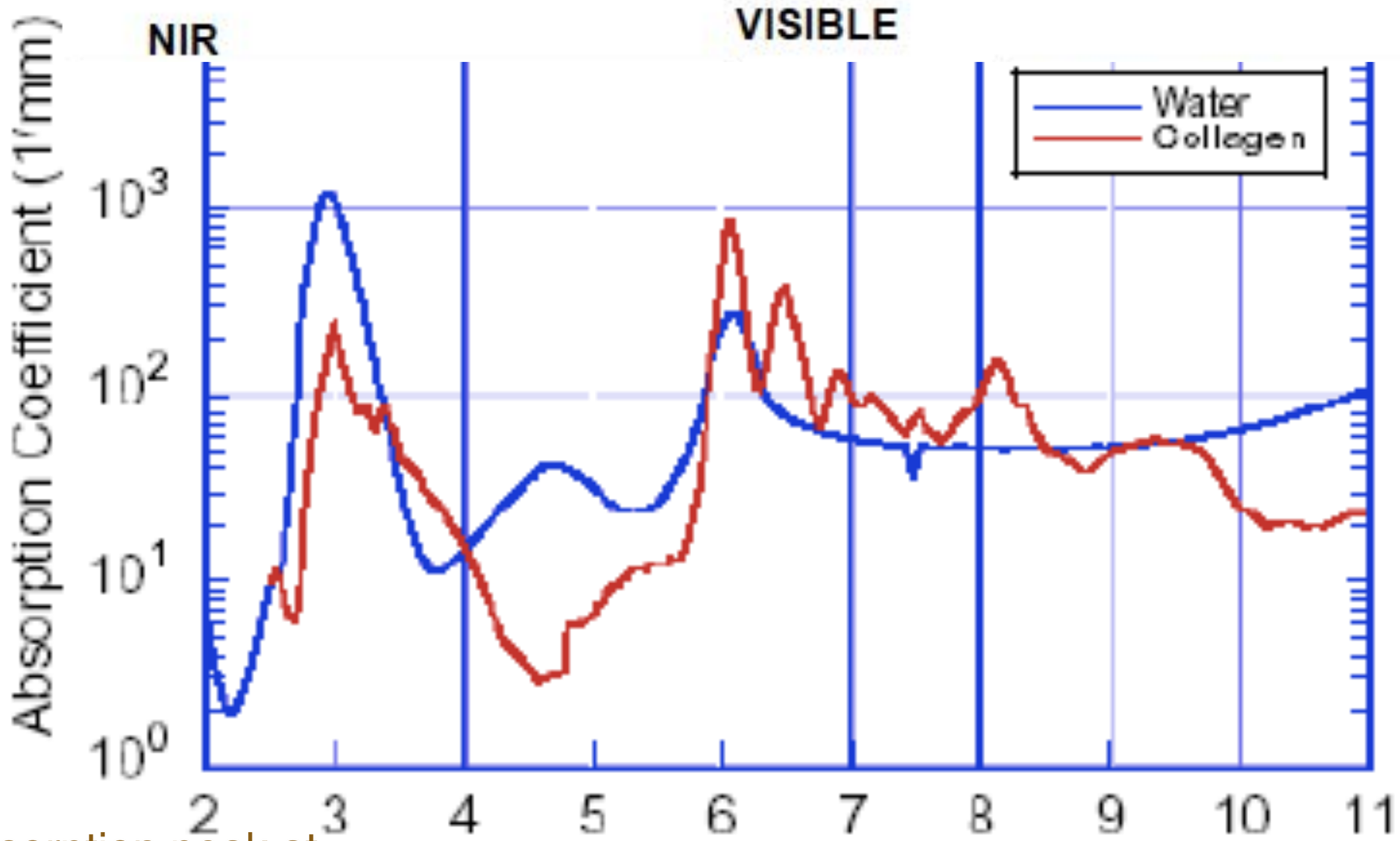
- **UV-VIS**
  - DNA
  - Hemoglobin
  - Lipids
  - Structural protein*
  - Electron carriers*
  - Amino acids*

* Absorbers that fluoresce when excited in the UV-VIS


VISIBLE
UV


Amino Acid
Peptide
ε, MOLAR ABSORPTIVITY
20,000
10,000
5,000
2,000
1,000
500
200
100
50
20
10
2000
2200
2400
2600
2800
3000
3200
WAVELENGTH IN A

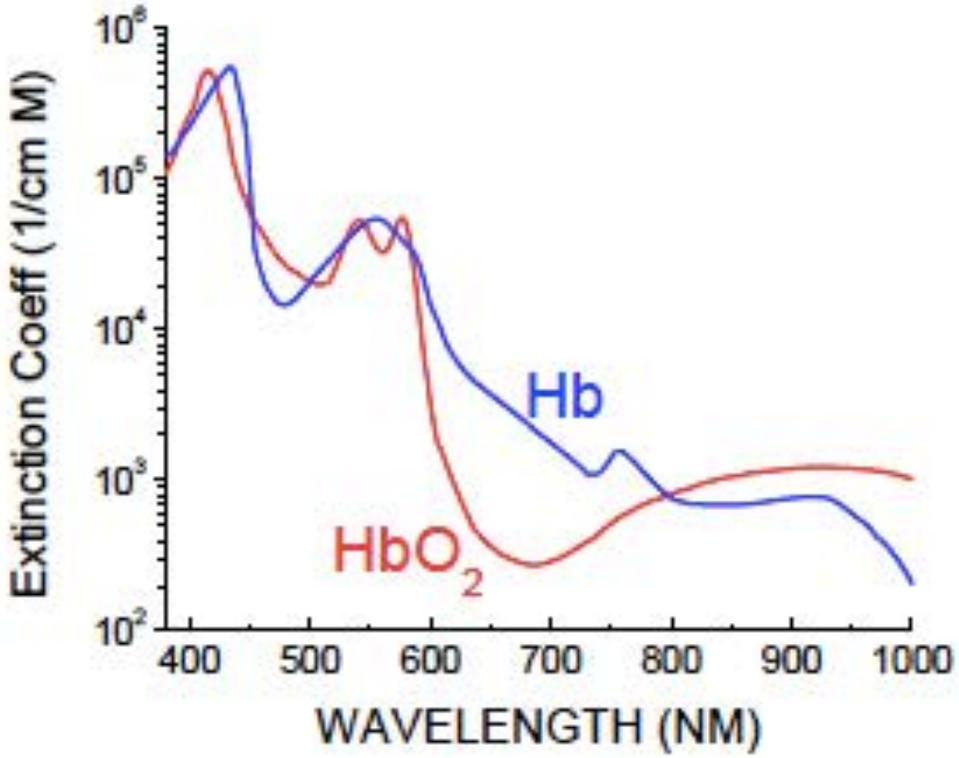


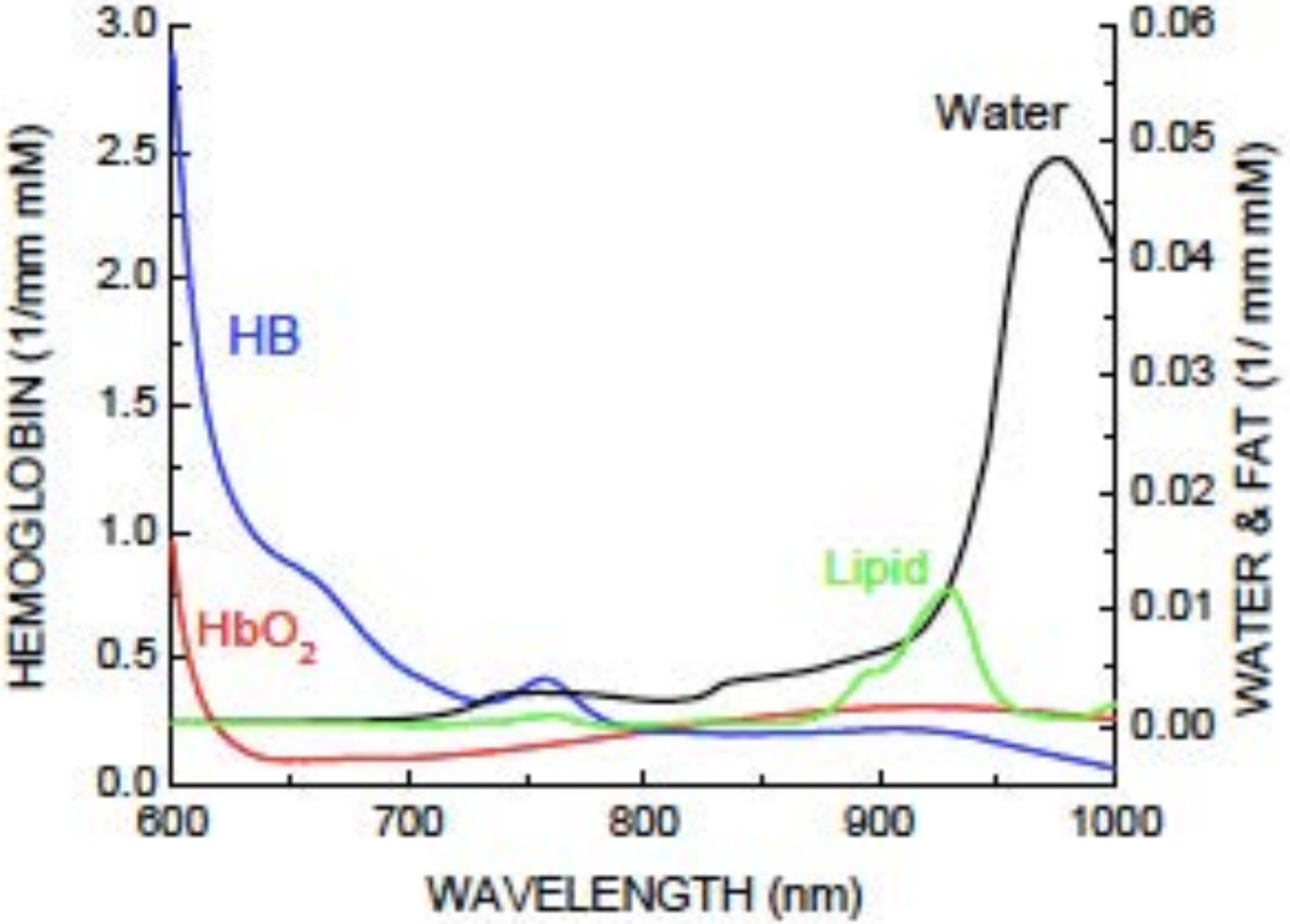


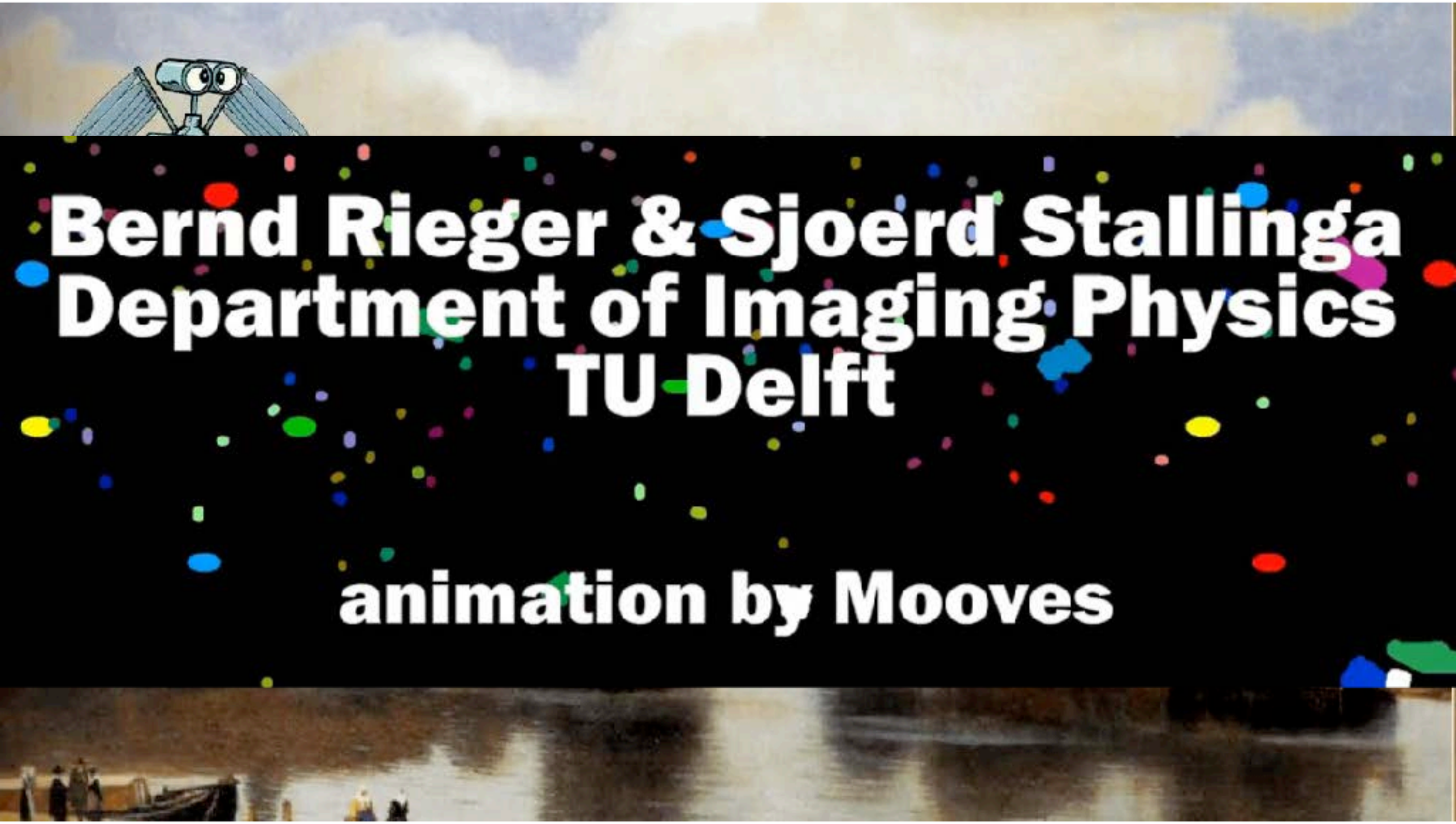
Water absorption peak at 0.96, 1.44, 1.95, 2.94 and 6.1 μm

Hemoglobin

- **Responsible for oxygen transport**
  - HbO2 and Hb
  - oxygen saturation is an indicator of oxygen delivery and utilization as well as metabolic activity
- **Deoxyhemoglobin has lower absorption than oxyhemoglobin in the blue and green**
  - Bright red arterial blood
  - Bluish venous blood
- **Absorption peaks for HbO_2**
  - 418, 542, 577, and 925 nm
- **Absorption peaks for Hb**
  - 550, 758, 910 nm




HEMOGLOBIN (1/mm mM)
WATER & FAT (1/ mm mM)
WAVELENGTH (nm)
HB
HbO2
Water
Lipid
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.06
0.05
0.04
0.03
0.02
0.01
0.00
600
700
800
900
1000


Bernd Rieger & Sjoerd Stallinga
Department of Imaging Physics
TU Delft
animation by Mooves

Optical microscope

- Objective lens
  - Numerical aperture ($n \sin \alpha$)
  - Air / oil immersion / water immersion
  - Corrected for cover slip (No. $1\,^1/_2$ = 0.17mm) or not
  - Corrected for infinity or not
  - e.g 100X 1.4NA Oil 0.17/∞
- Eyepiece
- Illumination system
  - Condenser
  - Aperture stop (diaphragm)
  - Field stop

OPTICAL MICROSCOPY SCENARIO

CORRELATIVE MICROSCOPY

Probes Approach
Photo-Physical Properties

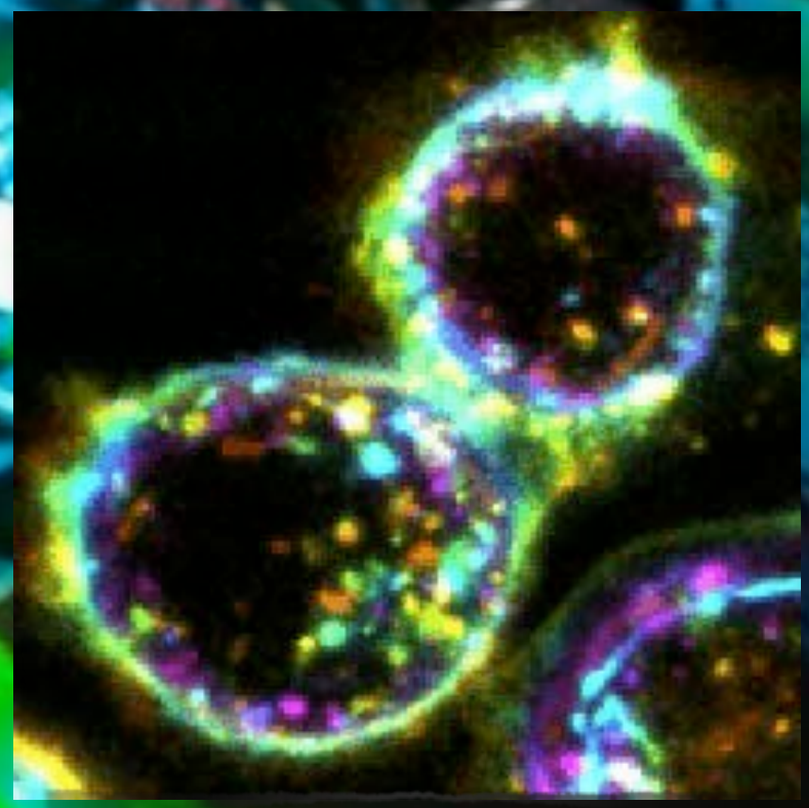
- FRET
- FCS
- FRAP
- FLIM
- Anisotropy

Optics Approach
Optics/Light Properties

- Adaptive Optics
- Deconvolution Methods
- Non-Linear
  - CARS
  - SHG
  - MPE
    - MMM-4Pi
    - SSIM
- Interference Techniques
  - SPIM
    - IML-SPIM
  - Confocal
    - SPEM
    - Saturation
      - GSD
      - STED
      - MPE-STED
        - RESOLFT
        - SW 2PE-STED
  - 4Pi
    - I5S
  - SIM
    - SSIM
  - I5M
  - SWM
- stochastic read out
  - STORM
  - PALM
  - GSDIM

***MUELLER matrix microscopy**

Y. Garini, B. J. Vermolen, I.T. Young, From micro to nano: recent advances in high-res microscopy, Curr.Op. iBiotech. 16, 3 (2005)
A. Diaspro, Circumventing the diffraction limit. Il Nuovo Saggiatore, 30(5) 45 (2014).



Michelangelo Buonarroti, Giudizio Universale(1536-1541) Cappella Sistina, Vaticano

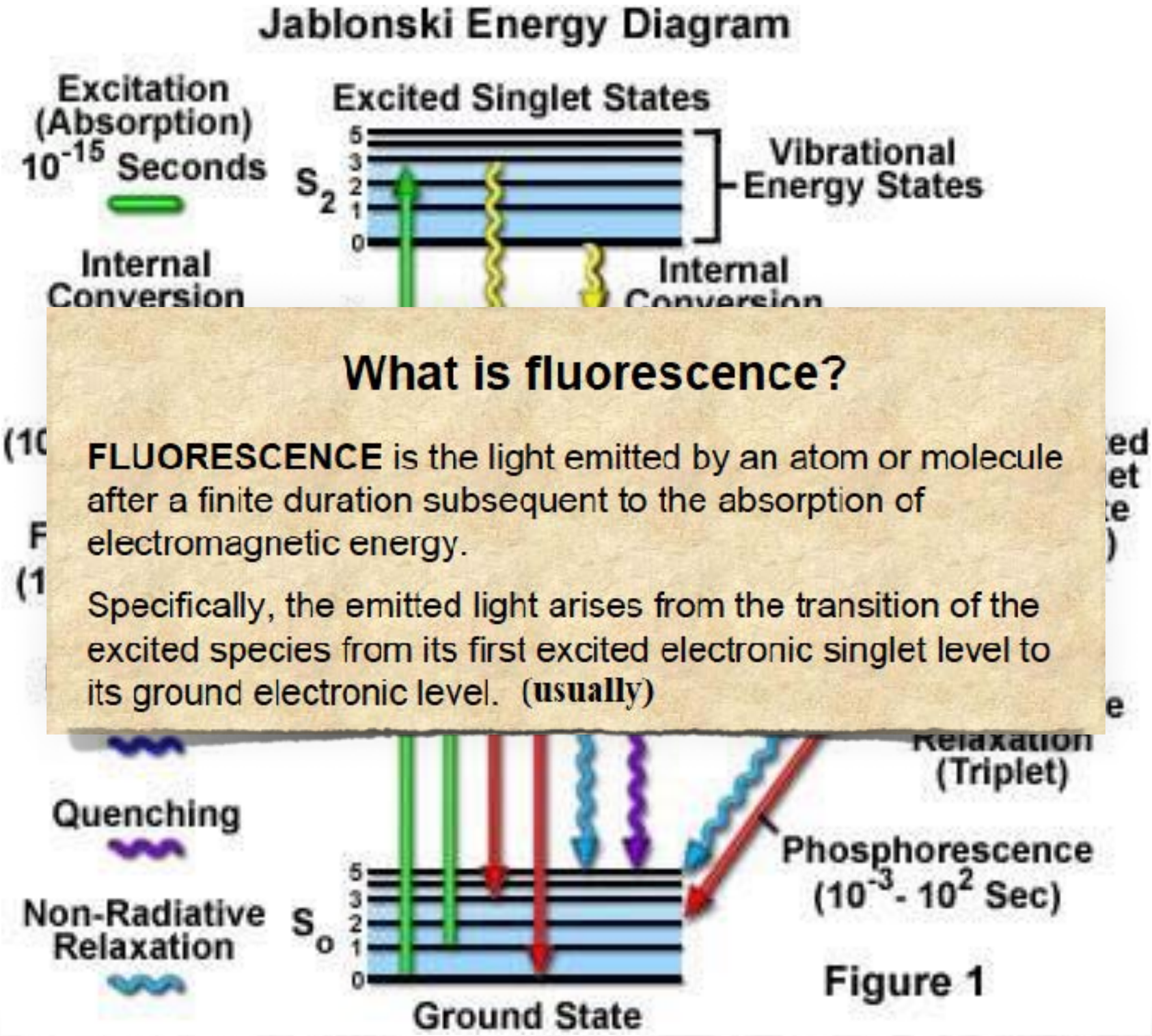
Fluorescence microscopy has become an indispensable tool in cell biology because of its unique advantages: it is a largely non-invasive technique, it can probe the deeper layers of a specimen at ambient conditions and enables spectroscopic diagnosis with chemical sensitivity

5 µm

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

fluorescence

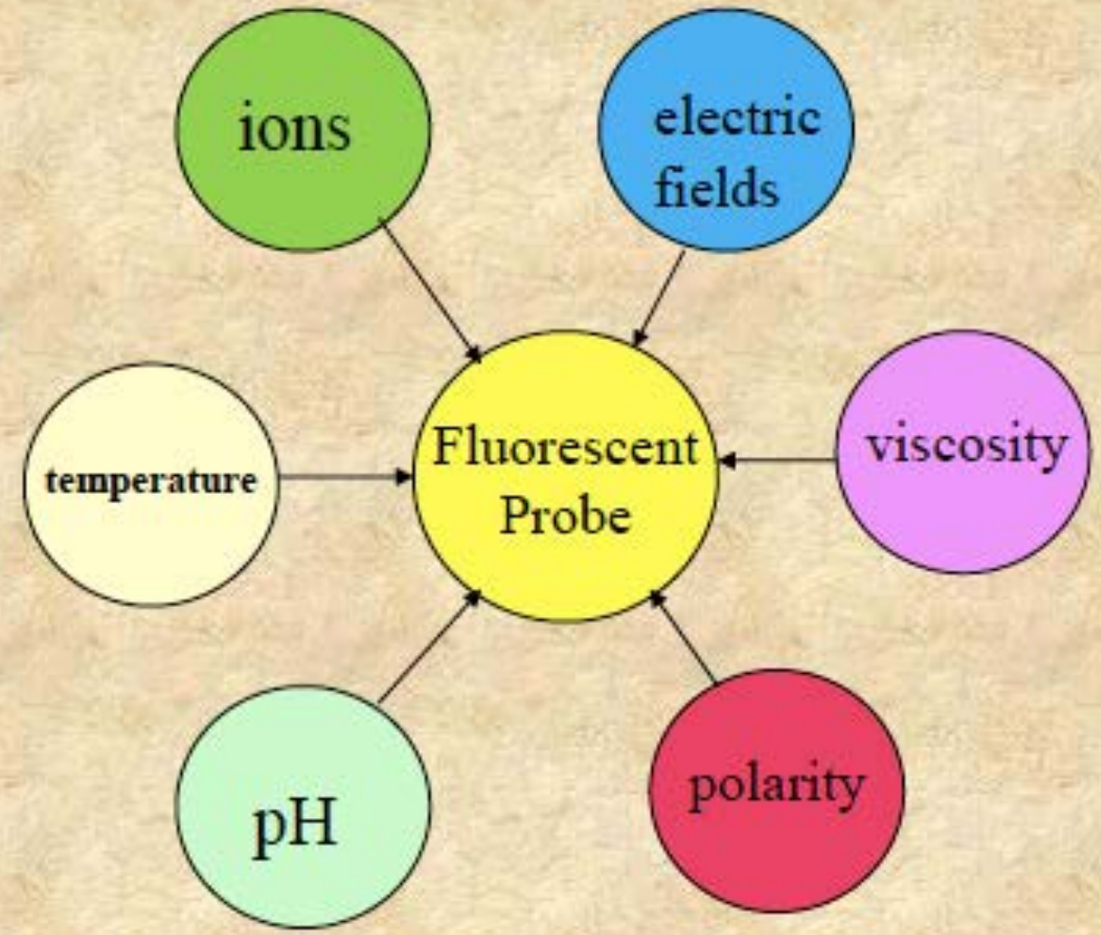
Slide credit for Fluorescence slides(adapted): David Jameson



fluorescence

Why fluorescence?

- its pretty!
- it provides information on the molecular environment
- it provides information on dynamic processes on the nanosecond timescale

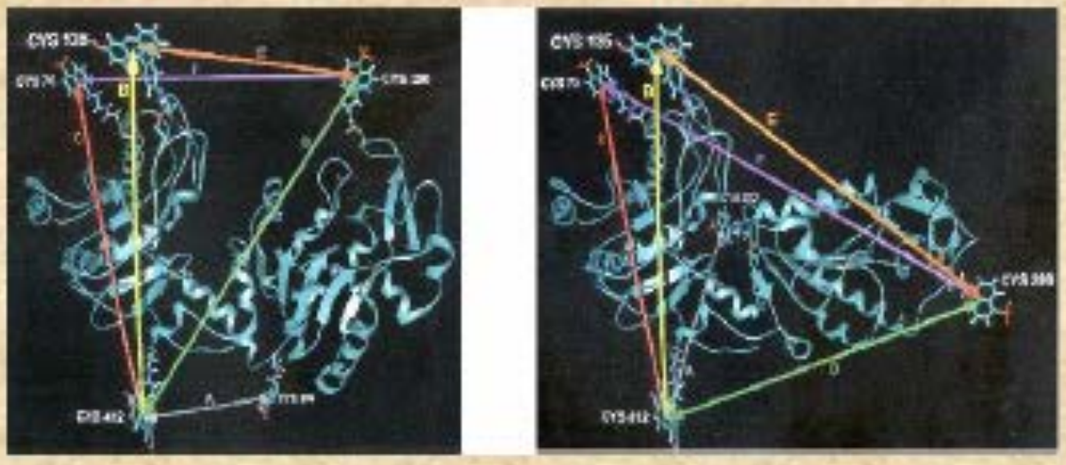


Fluorescence Probes are essentially molecular stopwatches which monitor *dynamic* events which occur during the excited state lifetime – such as movements of proteins or protein domains

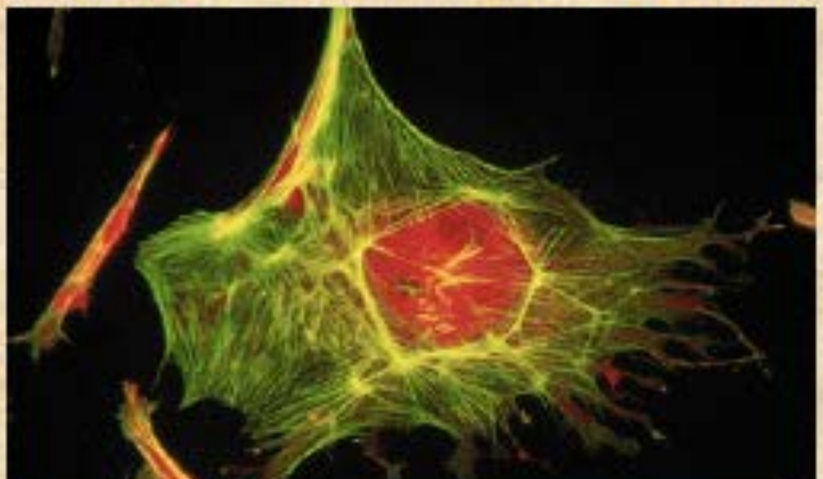
fluorescence

Experimental Systems Accessible to Fluorescence

Molecular structure and dynamics

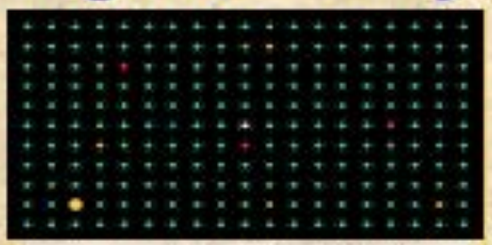


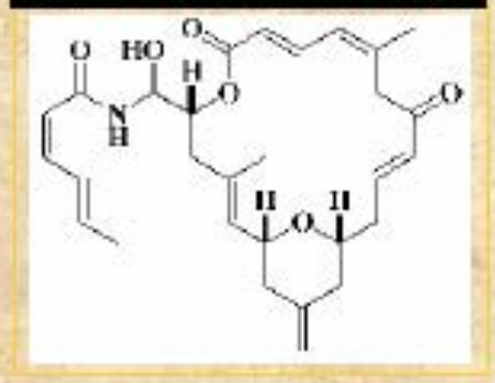
Cell organization and function



Live Animals

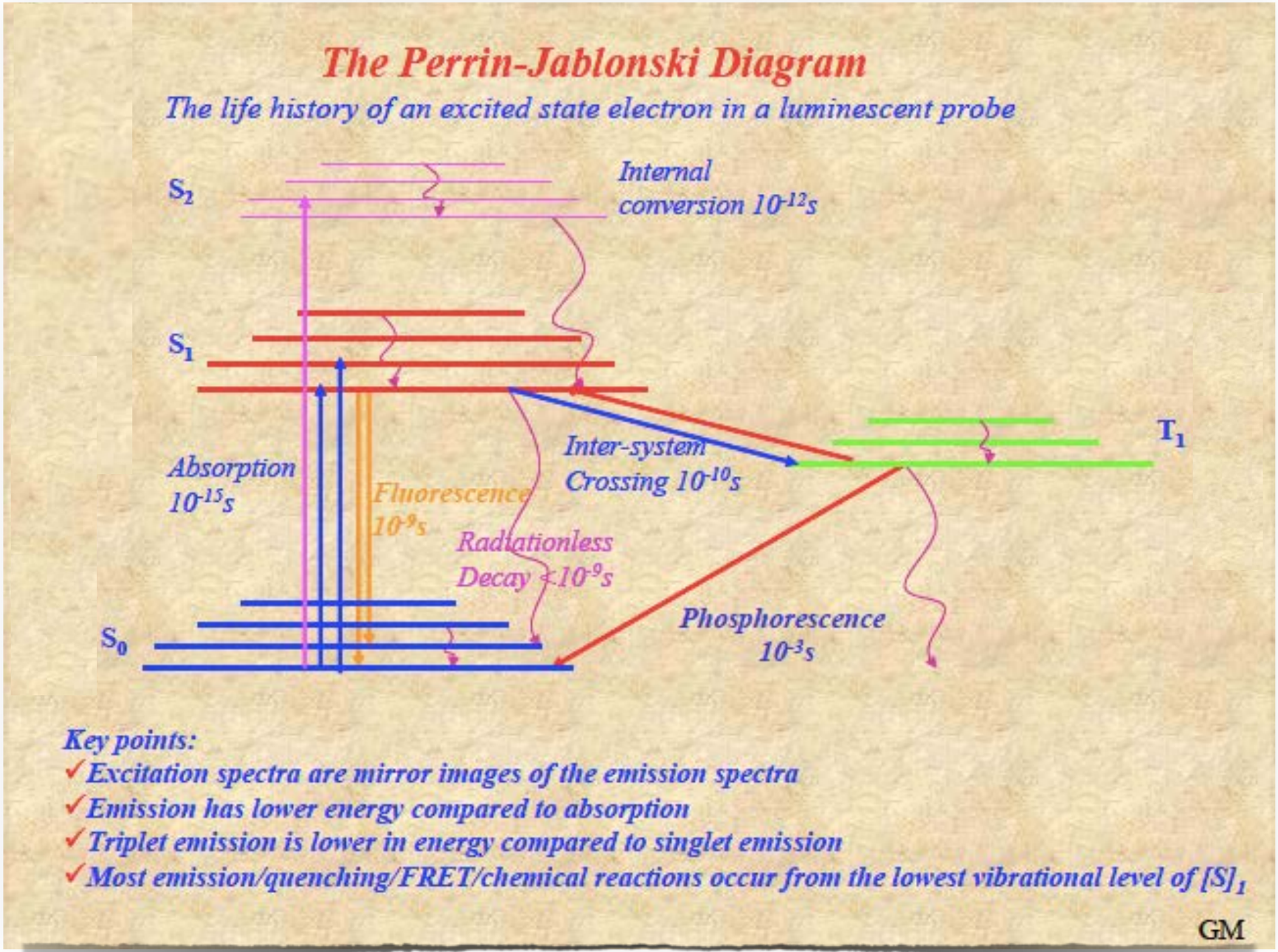
Engineered surfaces





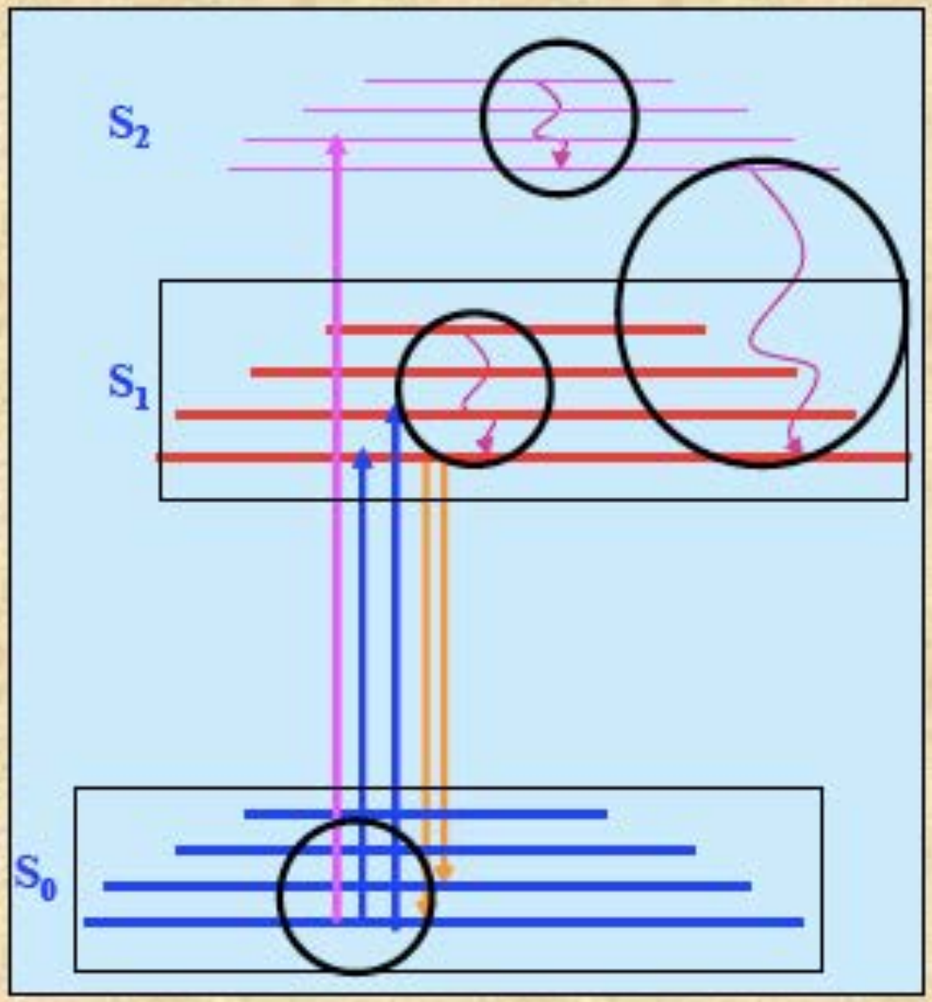
High throughput
Drug discovery

fluorescence



fluorescence

Specifically, although the fluorophore may be excited into different singlet state energy levels (e.g., S_1, S_2, etc) rapid thermalization invariably occurs and emission takes place from the lowest vibrational level of the first excited electronic state (S_1). This fact accounts for the independence of the emission spectrum from the excitation wavelength.



The fact that ground state fluorophores, at room temperature, are predominantly in the lowest vibrational level of the ground electronic state (as required from Boltzmann's distribution law) accounts for the Stokes shift.

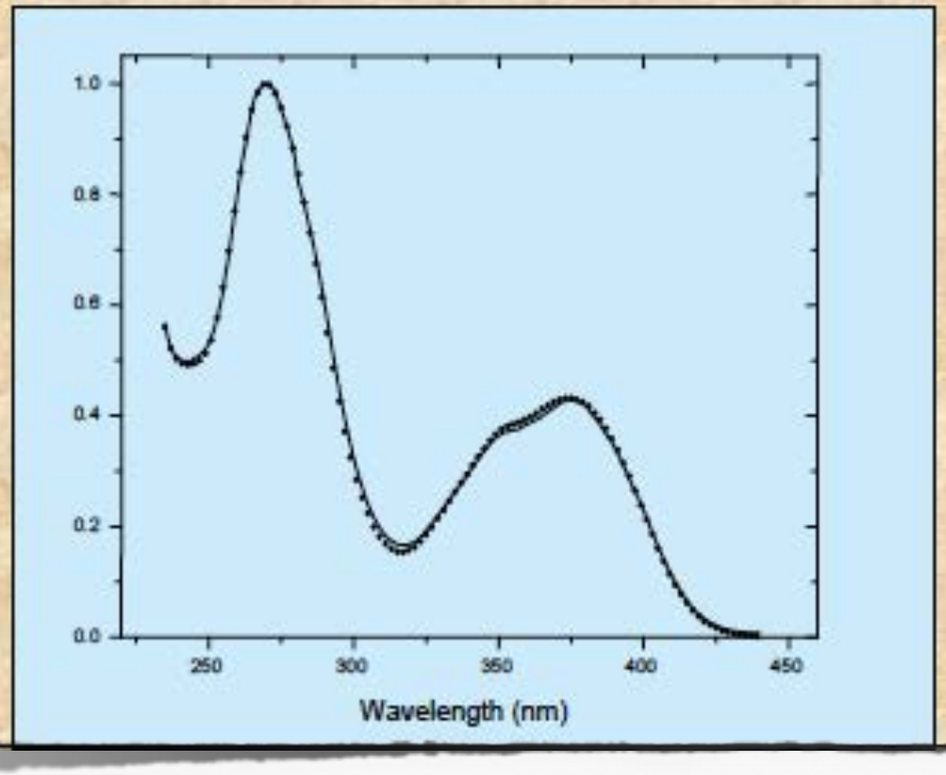
Finally, the fact that the spacings of the energy levels in the vibrational manifolds of the ground state and first excited electronic states are usually similar accounts for the fact that the emission and absorption spectra (plotted in energy units such as reciprocal wavenumbers) are approximately mirror images

fluorescence

The fluorescence excitation spectrum

The relative efficiencies of different wavelengths of incident light to excite fluorophores is determined as the excitation spectrum. In this case, the excitation monochromator is varied while the emission wavelength is kept constant if a monochromator is utilized - or the emitted light can be observed through a filter.

If the system is "well-behaved", i.e., if the three general rules outlined above hold, one would expect that the excitation spectrum will match the absorption spectrum. In this case, however, as in the case of the emission spectrum, corrections for instrumentation factors are required.



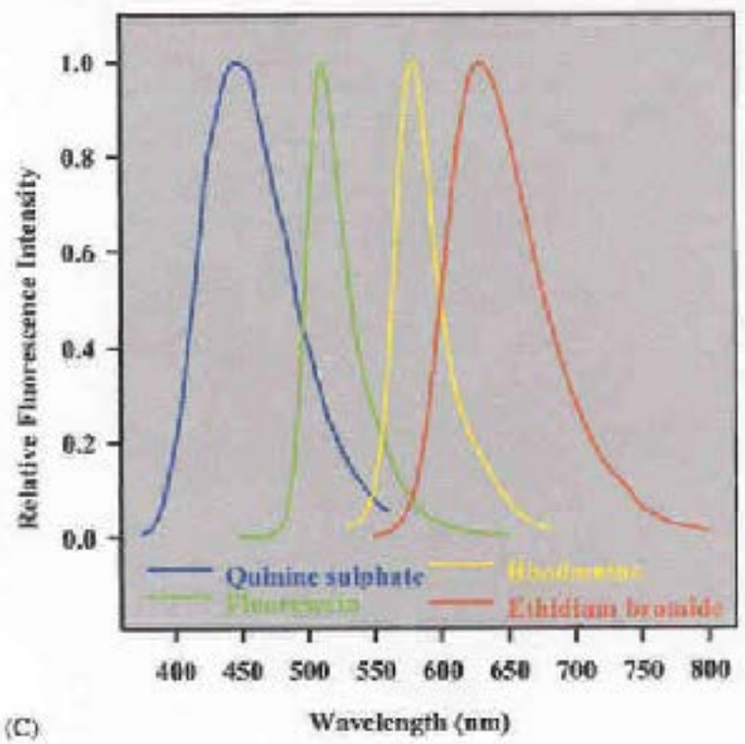
Overlay of Absorption Spectrum and Corrected Excitation Spectrum for ANS in ethanol

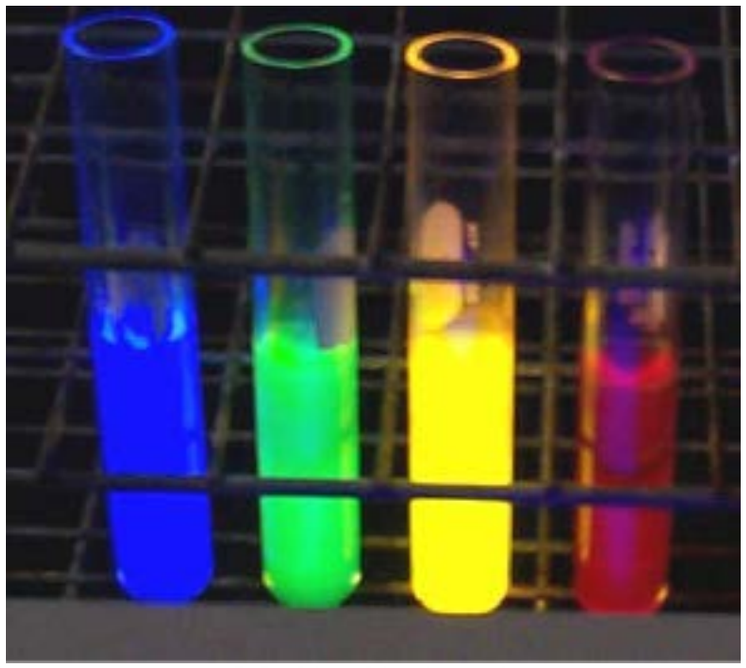
fluorescence

1) In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength

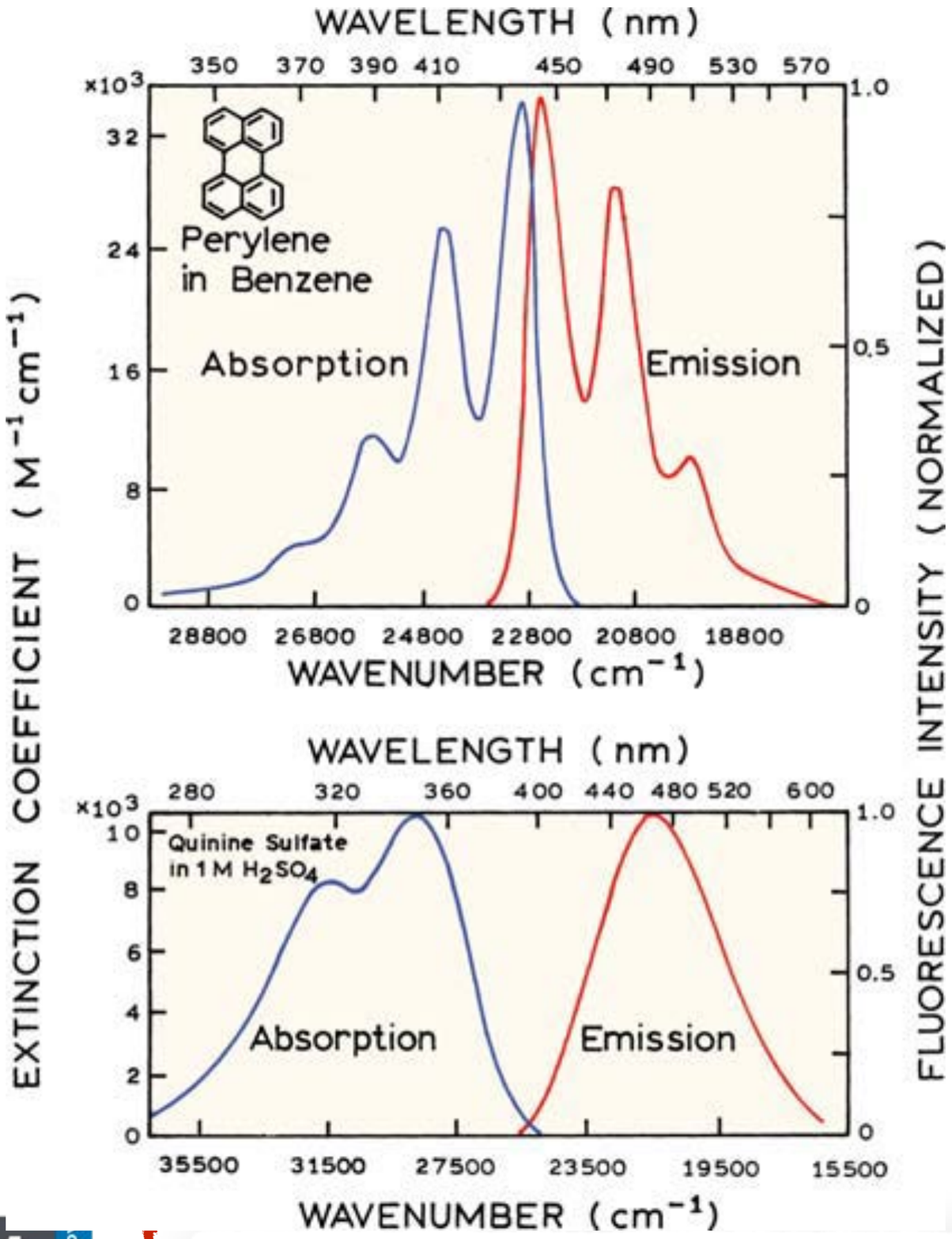
2) The fluorescence spectrum lies at longer wavelengths than the absorption

3) The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency



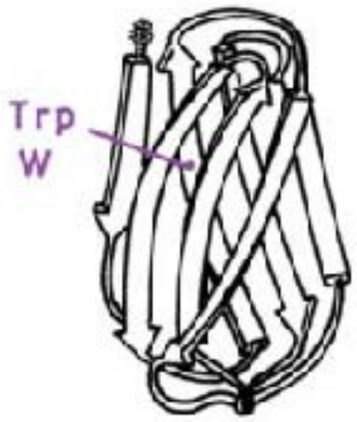


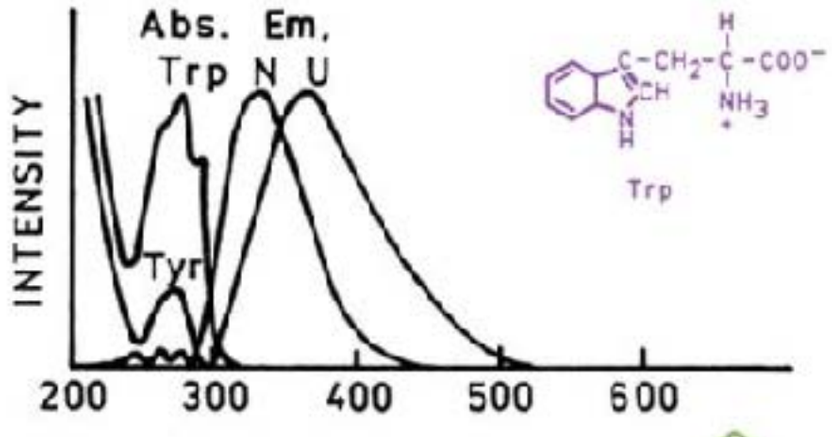
fluorescence

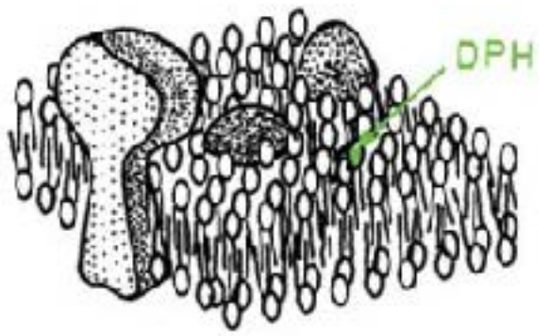


Professor Alexander Jablonski (1898–1980), circa 1935.
Courtesy of his daughter, Professor Danuta Frackowiak.

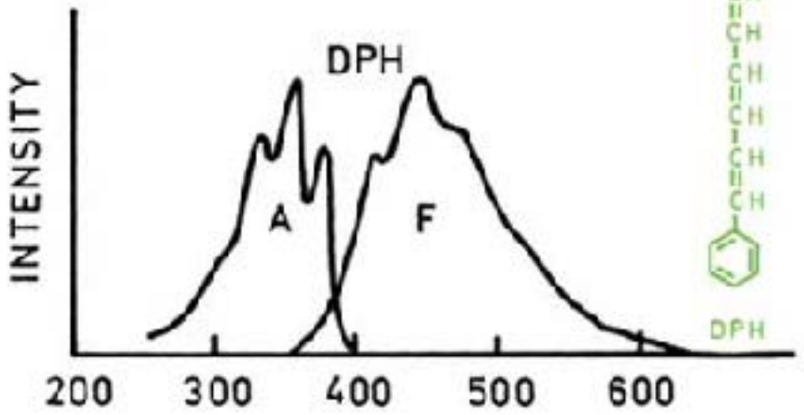
fluorescence

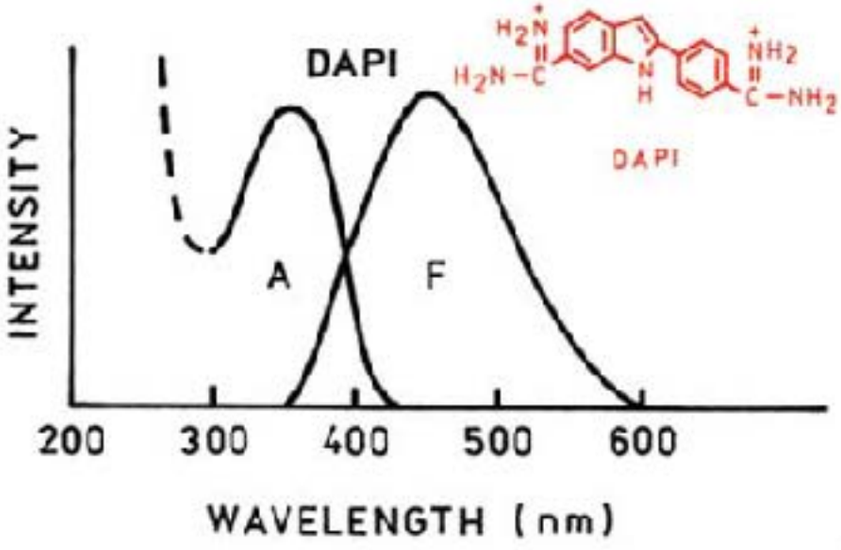




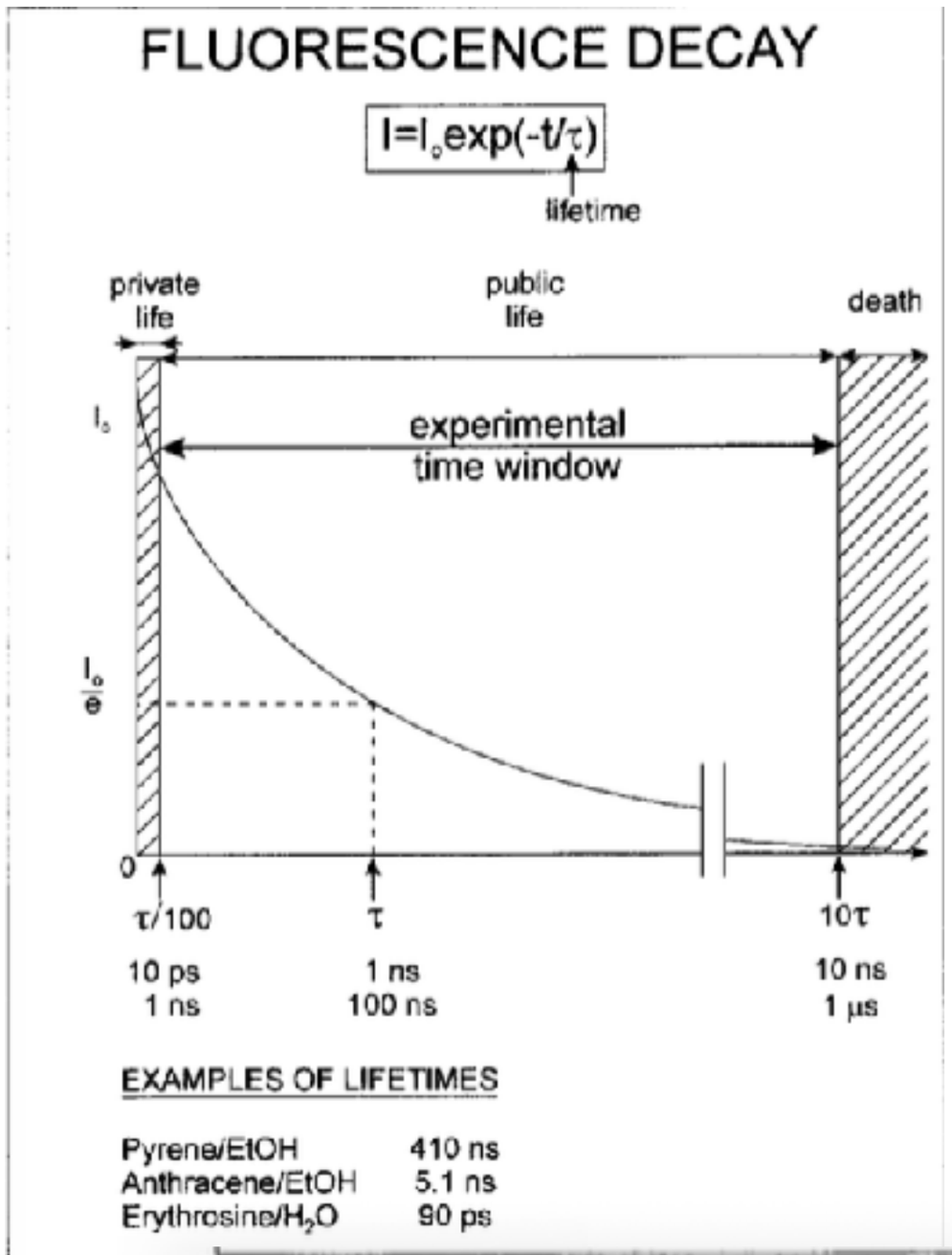


diphenylhexatriene (DPH)





fluorescence



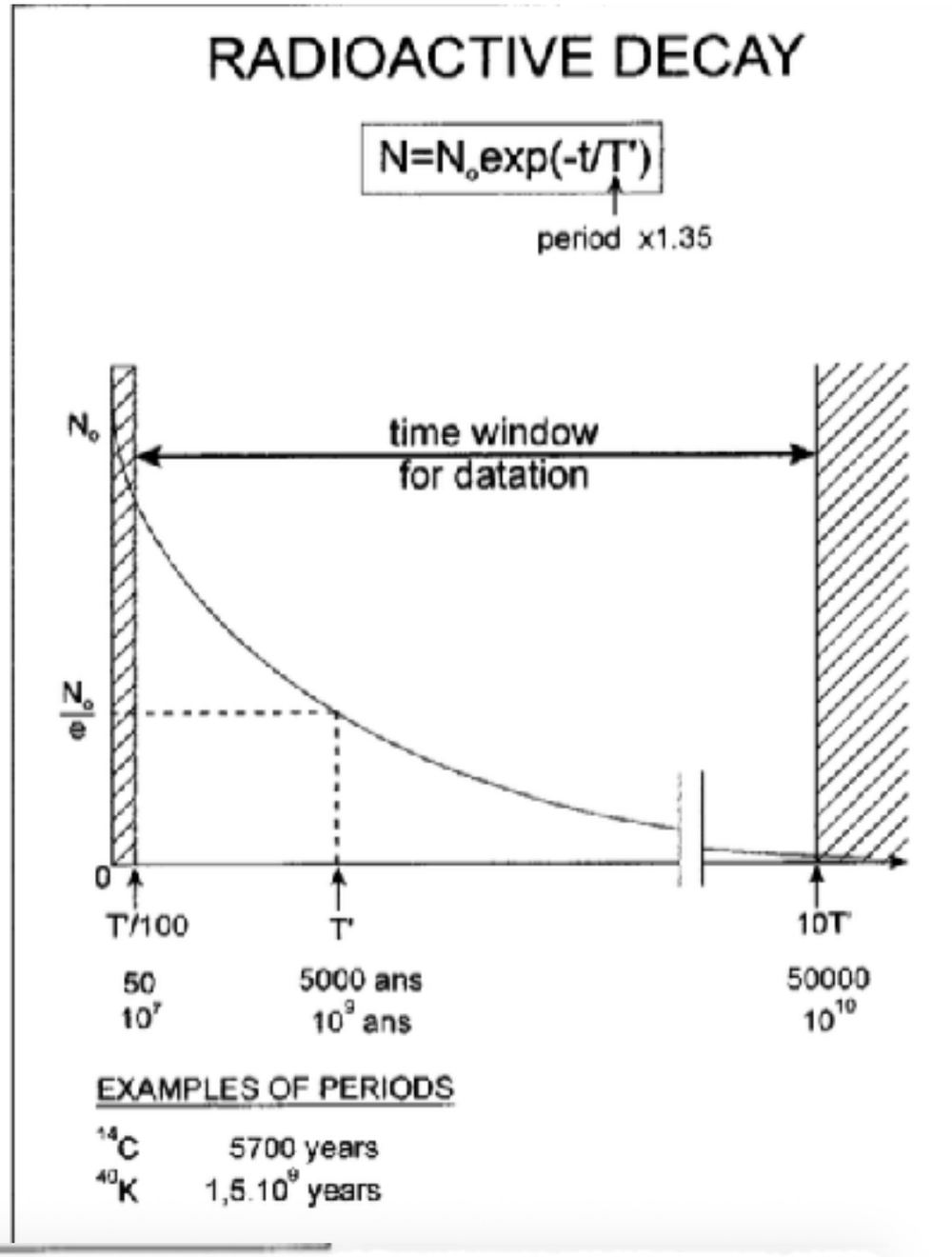


Fig. 3.2. Decay of fluorescence intensity and analogy with radioactive decay. Note that the lifetime τ is the time needed for the concentration of molecular entities to decrease to 1/e of its original value, whereas the radioactive period T is the time needed for the number of radioactive entities to decrease to $\frac{1}{2}$ of its original value. Therefore, T' (the decay time constant equivalent to the lifetime) is equal to $1.35T$.

fluorescence

Quantum Yield

The quantum yield of fluorescence (QY) is dependent on the ***rate of the emission process*** divided by the sum of the ***rates of all other deactivation processes***

$$QY = k_f / k_f + k_i + k_x$$

k_f is the rate of fluorescence, k_i is the rate of radiationless decay and k_x is the rate of intersystem crossing.

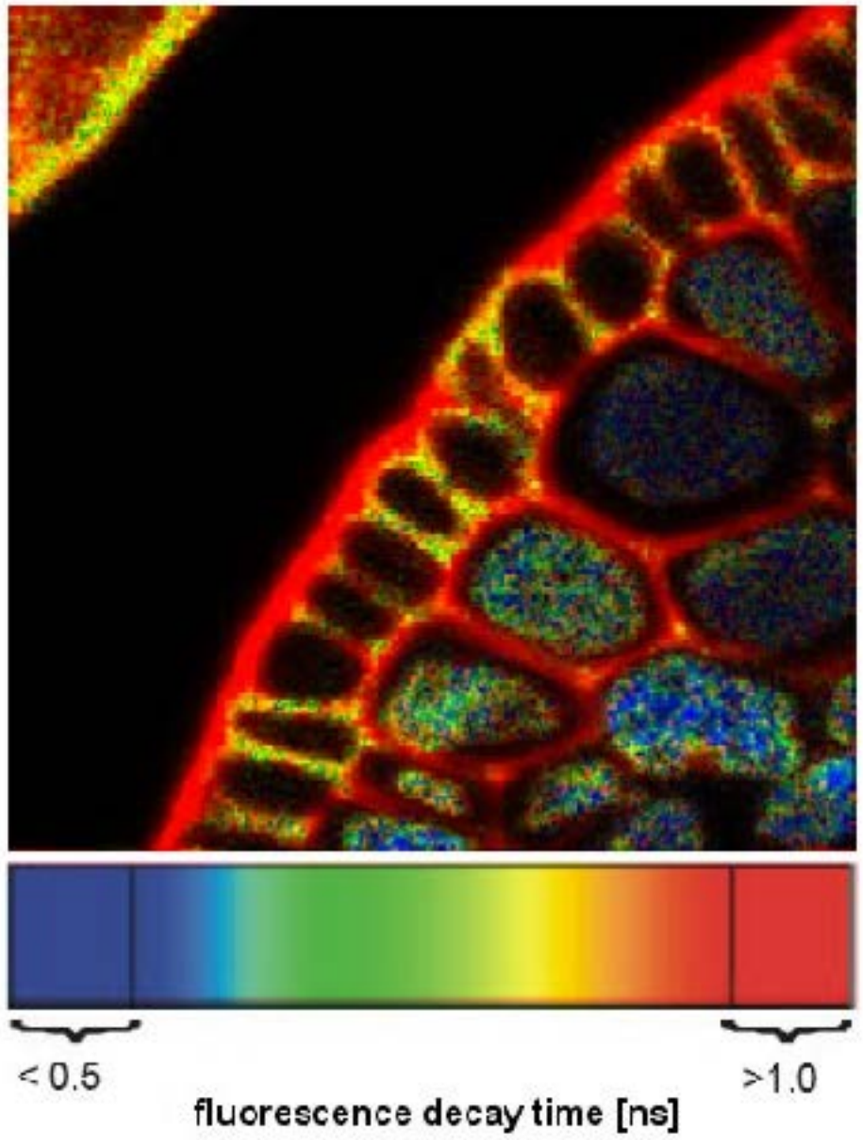
Another way to think about QY is:

QY = Number of emitted photons / Number of absorbed photons

If the rates of the deactivation processes are slow compared to k_f then the **QY** *is high*

However, if the rates of these other processes are fast compared to k_f then ***QY*** is low

fluorescence



$$Q = \frac{k_f}{k_f + k_i}$$

$$\tau = \frac{1}{k_f + k_i}$$

- **Lifetime**

  The average time spent in the excited state before returning to the ground state

fluorescence

Absorption and emission processes are almost always studied on *populations* of molecules and the properties of the supposed typical members of the population are deduced from the macroscopic properties of the process.

In general, the behavior of an excited population of fluorophores is described by a familiar rate equation:

$$\frac{dn^*}{dt} = -n^* \Gamma + f(t)$$

where n^* is the number of excited elements at time t, Γ is the rate constant of emission and f(t) is an arbitrary function of the time, describing the time course of the excitation . The dimensions of Γ are sec^{-1} (transitions per molecule per unit time).

fluorescence

If excitation occurs at t = 0, the last equation, takes the form:

$$\frac{dn^*}{dt} = -n^* \Gamma$$

and describes the decrease in excited molecules at all further times. Integration gives:

$$n^*(t) = n^*(0)\exp(-\Gamma t)$$

The lifetime, τ, is equal to Γ^{-1}

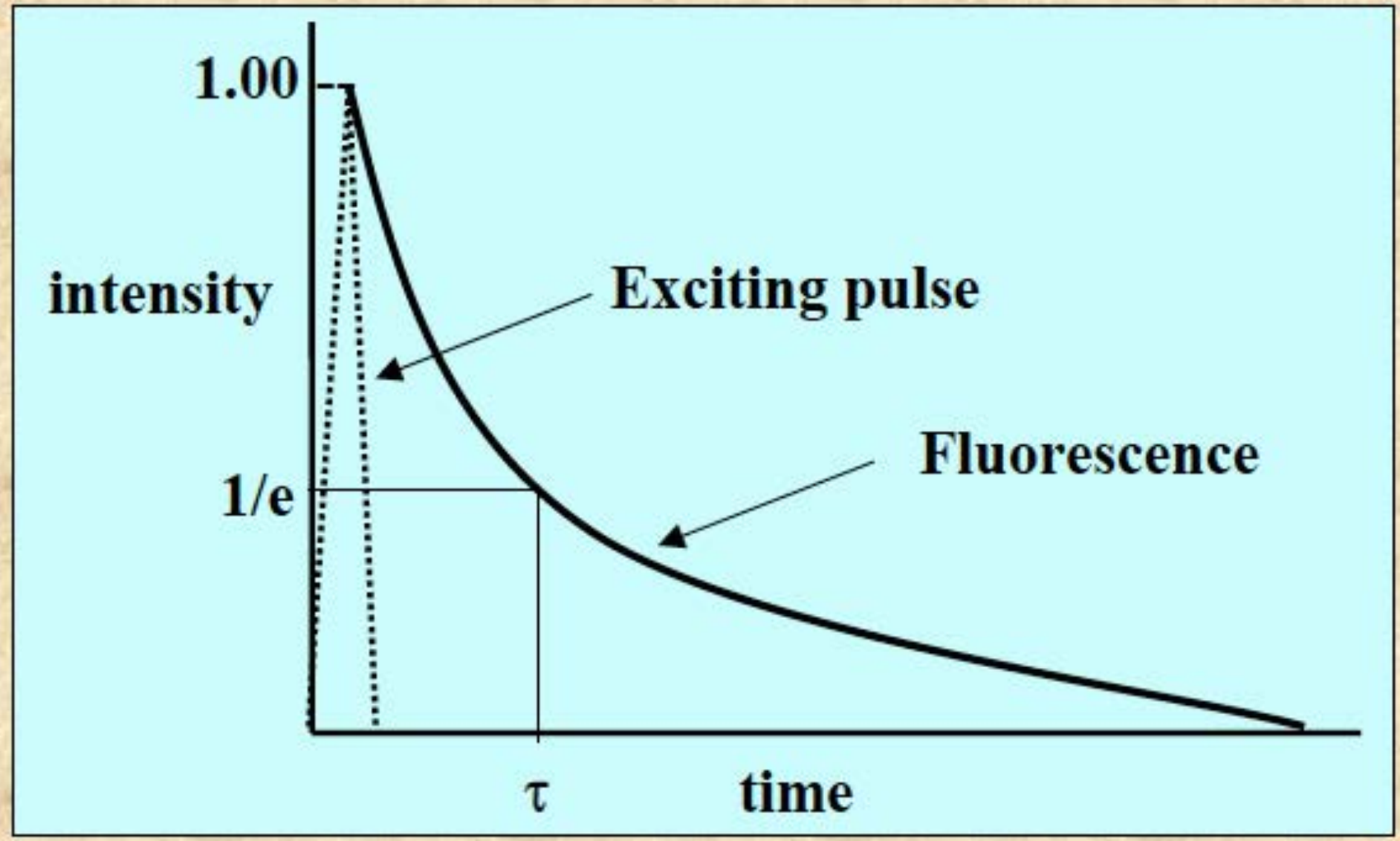
If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to 1/e or 36.8% of the original population according to:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$

fluorescence

In pictorial form:

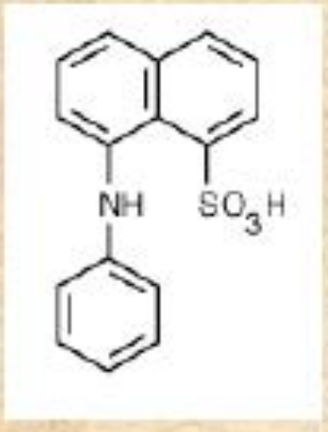
$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$



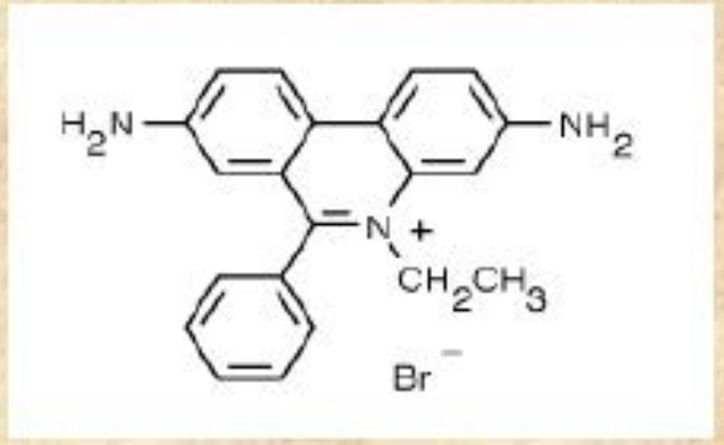
fluorescence

The lifetime and quantum yield for a given fluorophore is often dramatically affected by its environment.

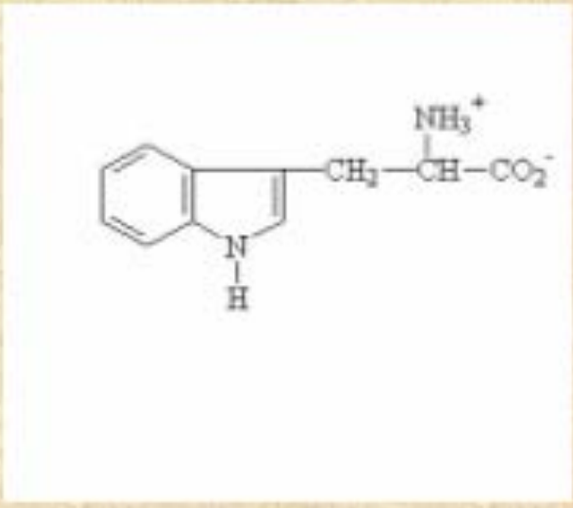
Examples of this fact would be NADH, which in water has a lifetime of ~0.4 ns but bound to dehydrogenases can be a long as 9 ns.



ANS in water is ~100 picoseconds but can be 8 – 10 ns bound to proteins



Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA



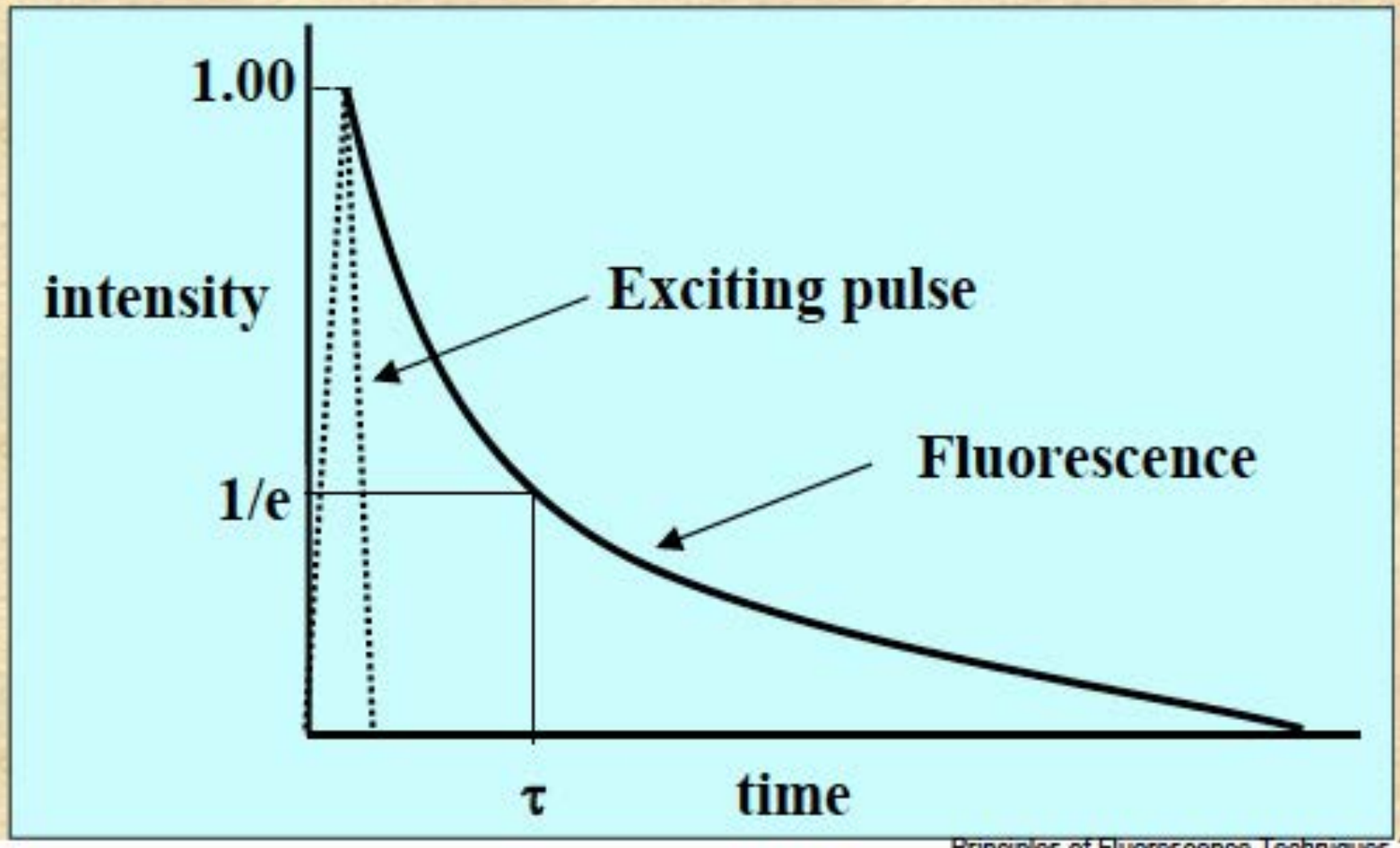
The lifetime of tryptophan in proteins ranges from ~0.1 ns up to ~8 ns

fluorescence

David Jameson

Excited state lifetimes have traditionally been measured using either the *impulse* response or the *harmonic* response method. In principle both methods have the same information content. These methods are also referred to as either the "time domain" method or the "frequency domain" method.

In the *impulse (*or pulse) method, the sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally these short light pulses were generated using *flashlamps* which had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter.



Principles of Fluorescence Techniques 2011

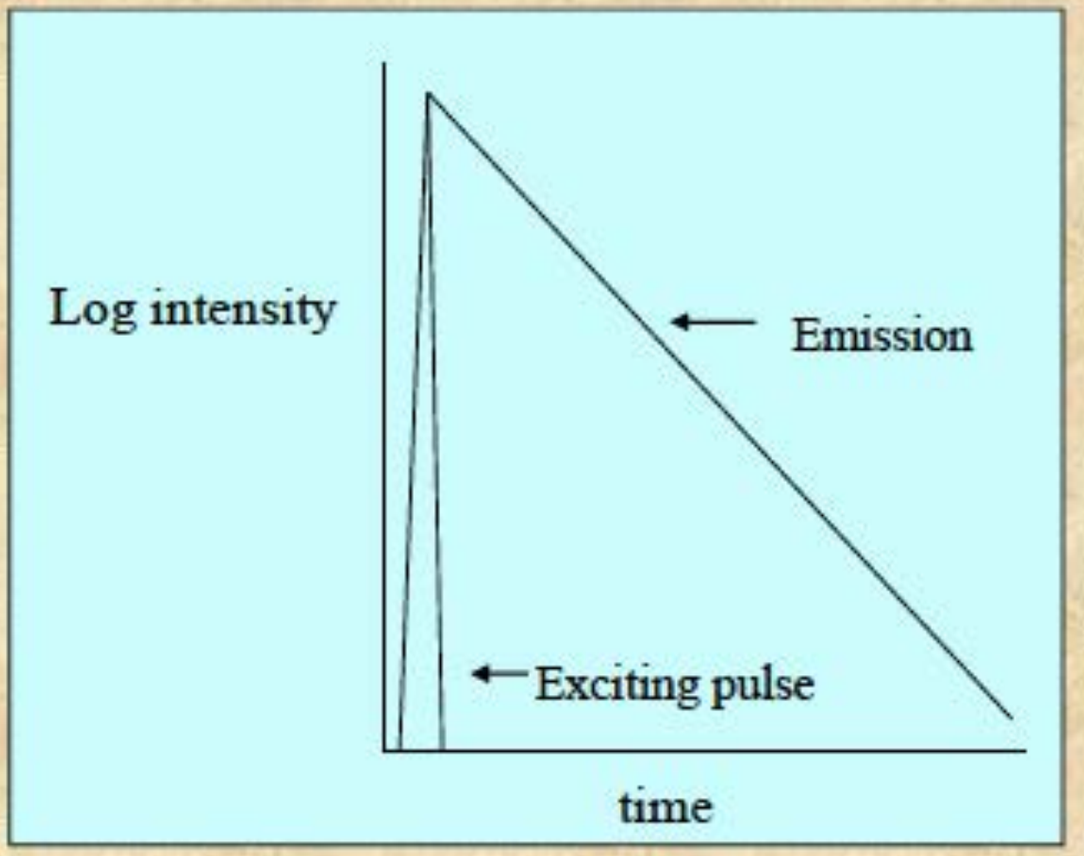
fluorescence

As shown in the intensity decay figure, the *fluorescence* lifetime, t , is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

$$I_t = \alpha e^{-t/\tau}$$

Where I_t is the intensity at time t, α is a normalization term (the pre-exponential factor) and τ is the lifetime.

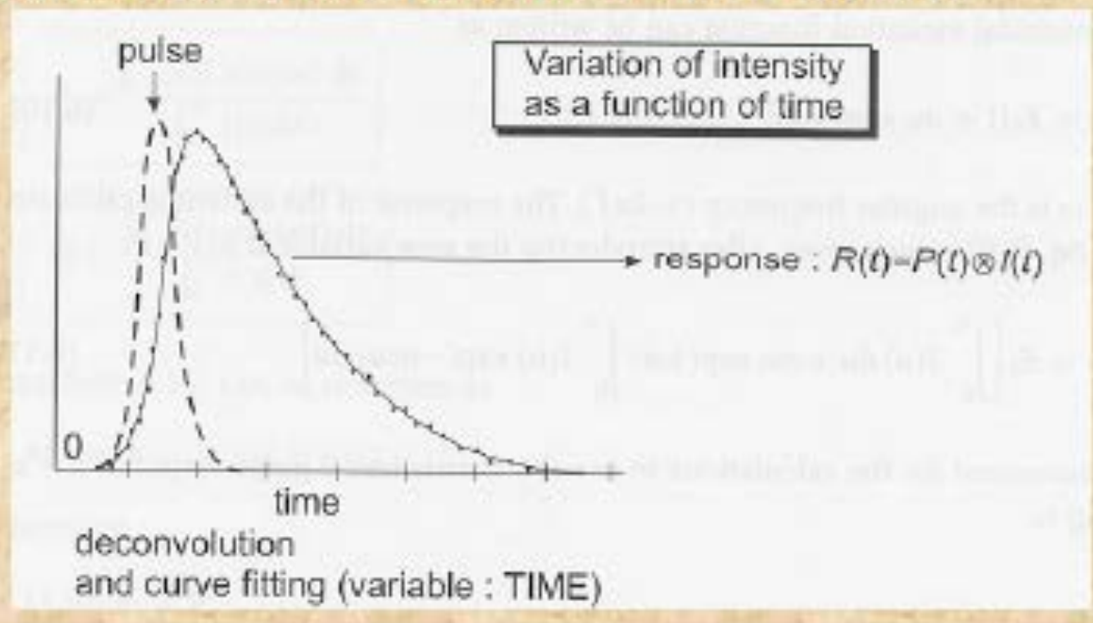
It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.



fluorescence

If the decay is a single exponential and if the lifetime is long compared to the exciting light then the lifetime can be determined directly from the slope of the curve.

If the lifetime and the excitation pulse width are comparable some type of *deconvolution* method must be used to extract the lifetime.



Great effort has been expended on developing mathematical methods to "deconvolve" the effect of the exciting pulse shape on the observed fluorescence decay.

With the advent of very fast laser pulses these deconvolution procedures became less important for most lifetime determinations, although they are still required whenever the lifetime is of comparable duration to the light pulse.

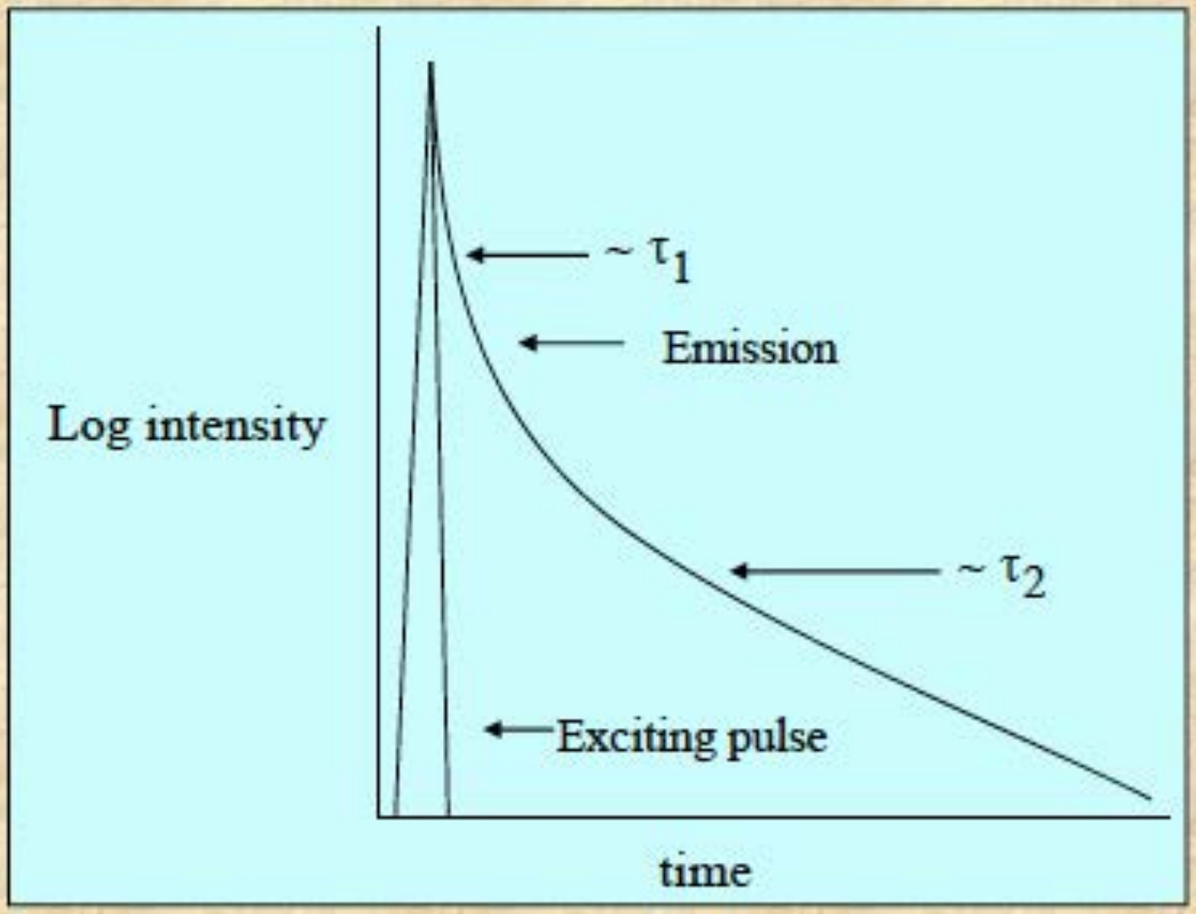
fluorescence

If the decay is multiexponential, the relation between the intensity and time after excitation is given by:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

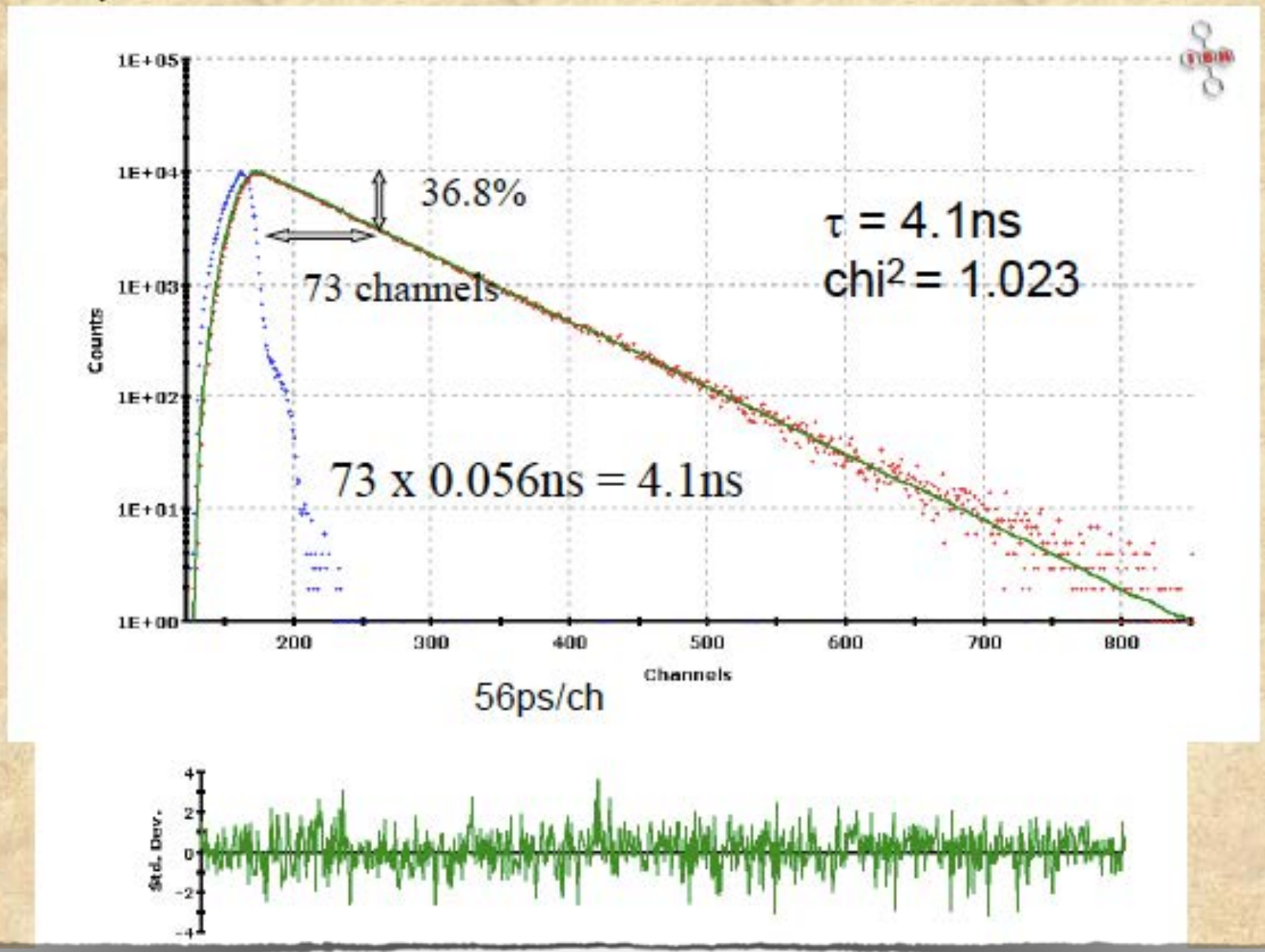
One may then observe data such as those sketched below:

Here we can discern at least two lifetime components indicated as τ_1 and τ_2 . This presentation is oversimplified but illustrates the point.



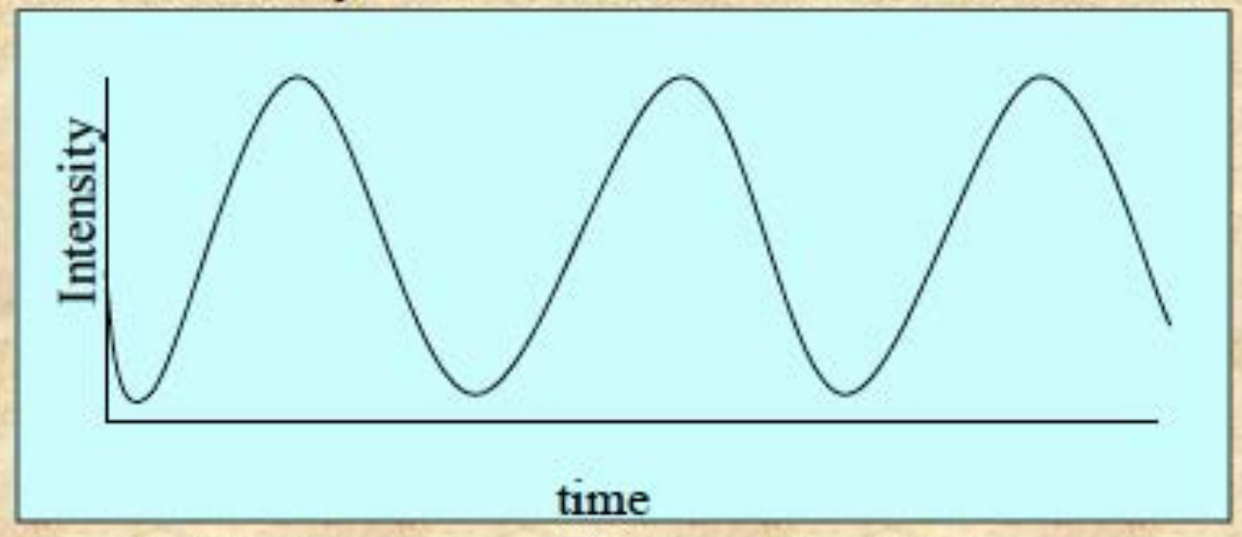
fluorescence

Here are pulse decay data on anthracene in cyclohexane taken on an IBH 5000U Time-correlated single photon counting instrument equipped with an LED short pulse diode excitation source.



fluorescence

In the harmonic method (also known as the phase and modulation or frequency domain method) a continuous light source is utilized, such as a laser or xenon arc, and the intensity of this light source is modulated sinusoidally at high frequency as depicted below. Typically, an *electro-optic* device, such as a *Pockels cell* is used to modulate a continuous light source, such as a CW laser or a xenon arc lamp. Alternatively, LEDs or laser diodes can be directly modulated.



In such a case, the excitation frequency is described by:

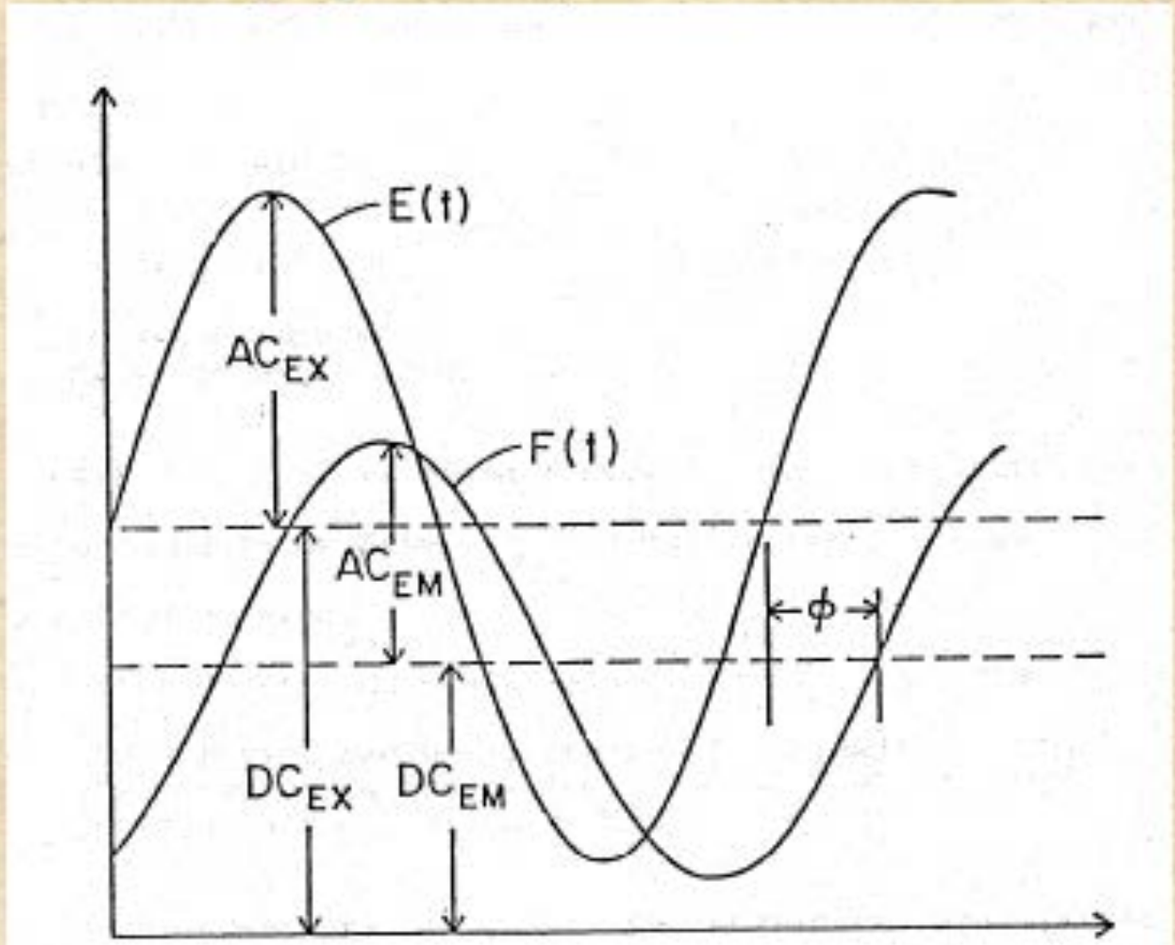
$$E(t) = Eo\,[1 + M_E \sin \omega t]$$

E(t) and Eo are the intensities at time t and o, M_E is the modulation factor which is related to the ratio of the AC and DC parts of the signal and ω is the angular modulation frequency.

$\omega = 2\pi f$ where f is the linear modulation frequency

fluorescence

Due to the persistence of the excited state, fluorophores subjected to such an excitation will give rise to a modulated emission which is shifted in phase relative to the exciting light as depicted below.



This sketch illustrates the phase delay (ϕ) between the excitation, E(t), and the emission, F(t). Also shown are the AC and DC levels associated with the excitation and emission waveforms.

fluorescence

One can demonstrate that:

$$F(t) = Fo\,[1 + M_F \sin(\omega t + \phi)]$$

This relationship signifies that measurement of the phase delay, ϕ, forms the basis of one measurement of the lifetime, τ. In particular one can demonstrate that:

$$\tan\phi = \omega\tau$$

The *modulations* of the excitation (M_E) and the emission (M_F) are given by:

$$M_E = \left(\frac{AC}{DC}\right)_E \quad \text{and} \quad M_F = \left(\frac{AC}{DC}\right)_F$$

The *relative modulation*, M, of the emission is then:

$$M = \frac{(AC/DC)_F}{(AC/DC)_E}$$

τ can also be determined from M according to the relation: $M = \dfrac{1}{\sqrt{1+(\omega\tau)^2}}$

fluorescence

Using the *phase shift* and *relative modulation* one can thus determine a *phase lifetime* (τ_P) and a *modulation lifetime* (τ_M).

If the fluorescence decay is a single exponential, then τ_P and τ_M will be equal at all modulation frequencies.

If, however, the fluorescence decay is multiexponential then $\tau_P < \tau_M$ and, moreover, the values of both τ_P and τ_M will depend upon the modulation frequency, i.e.,

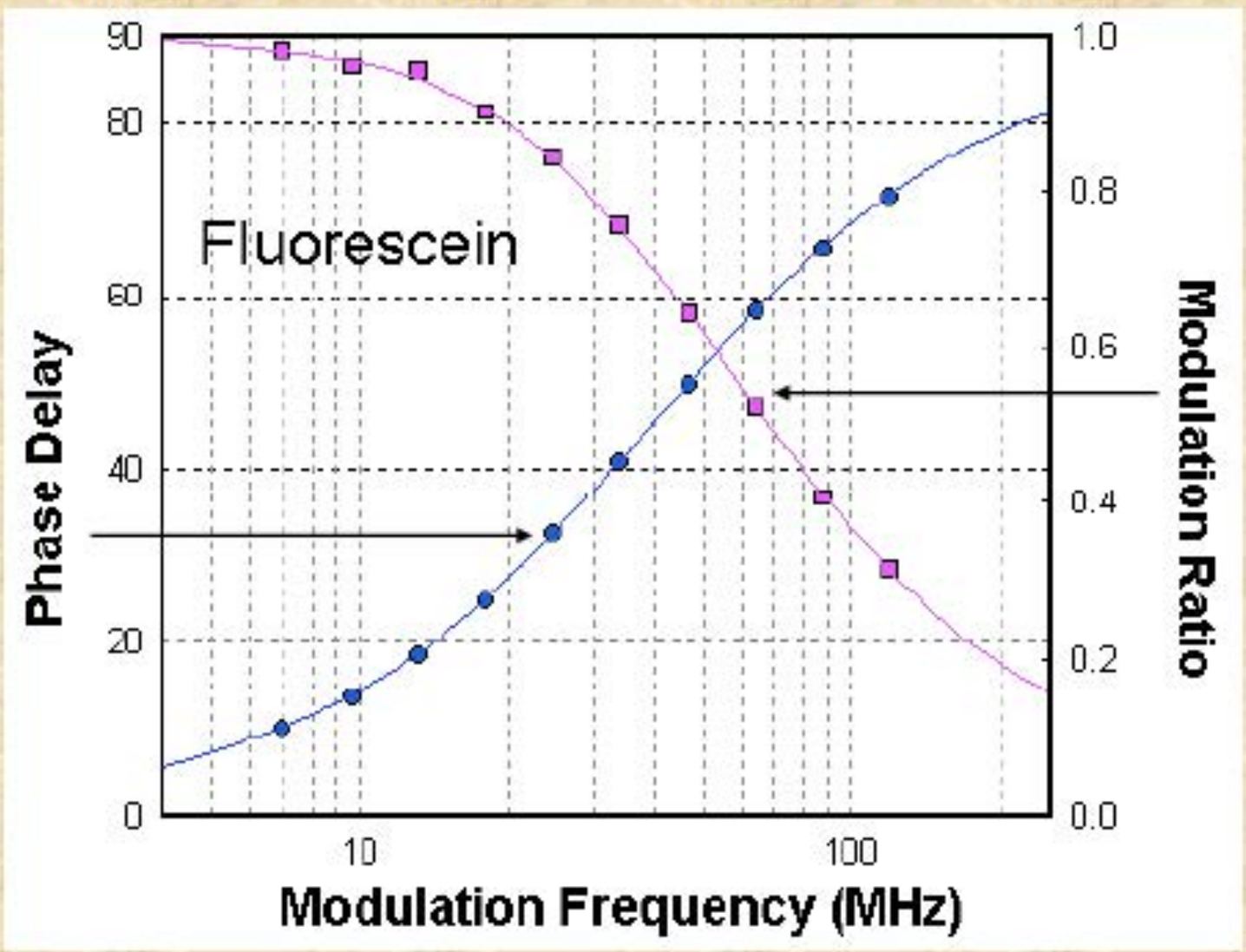
$$\tau_P(\omega_1) < \tau_P(\omega_2) \quad \text{if} \quad \omega_1 > \omega_2$$

To get a feeling for typical phase and modulation data, consider the following data set.

Frequency (MHz)	τ_P (ns)	τ_M (ns)
5	6.76	10.24
10	6.02	9.70
30	3.17	6.87
70	1.93	4.27

fluorescence

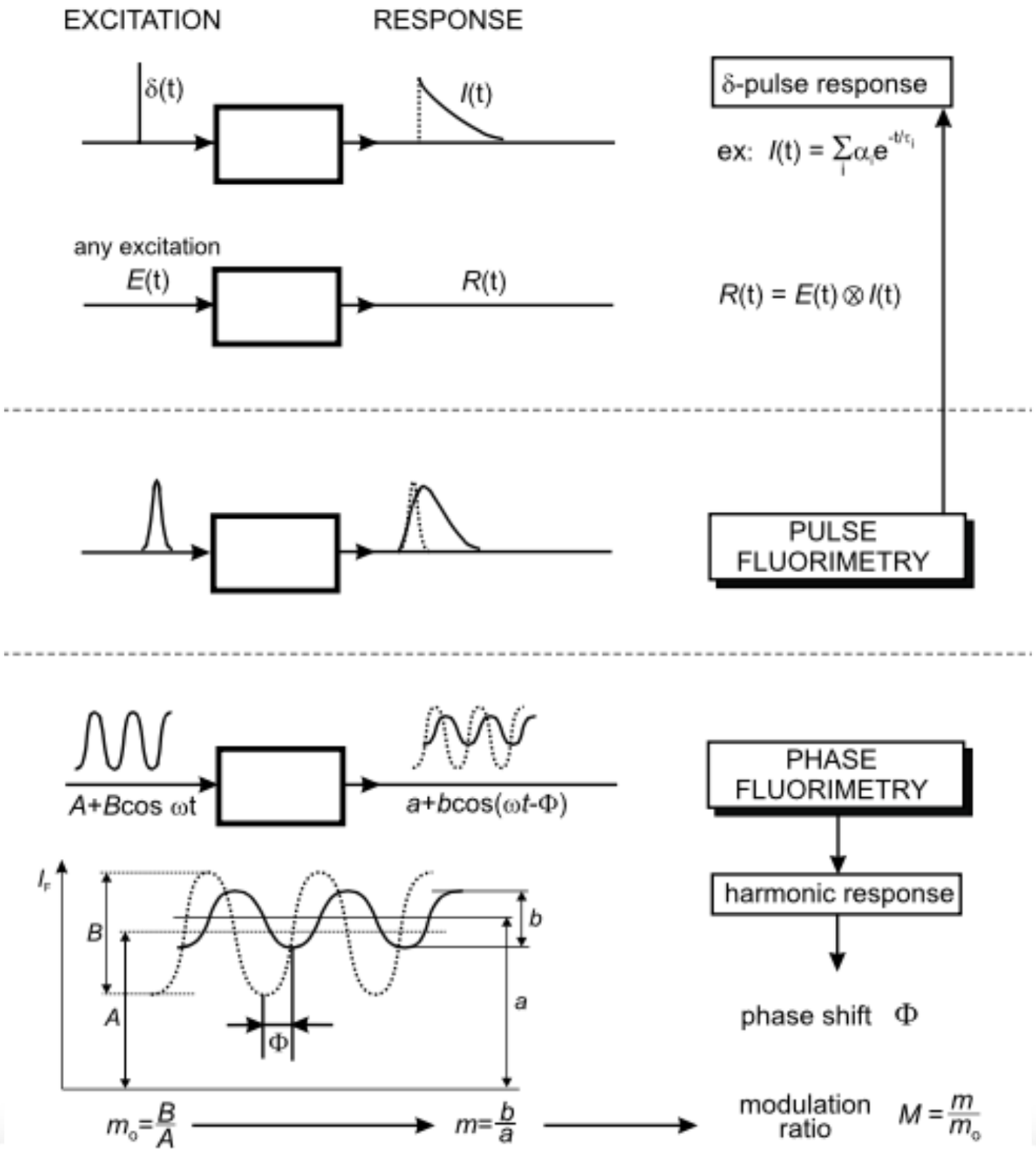
Multifrequency phase and modulation data are usually presented as shown below:



The plot shows the frequency response curve (phase and modulation) of Fluorescein in phosphate buffer pH 7.4 acquired on an ISS Chronos using a 470 nm LED. The emission was collected through a 530 high pass filter. The data is best fitted by a single exponential decay time of 4 ns.

fluorescence

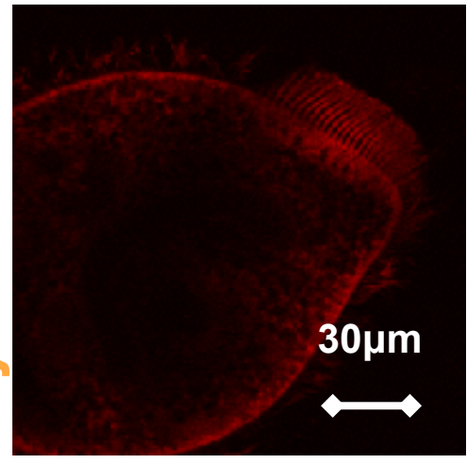
Slide credit for Fluorescence slides(adapted): David Jameson

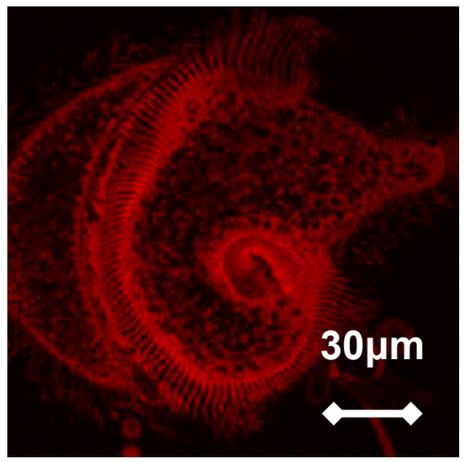


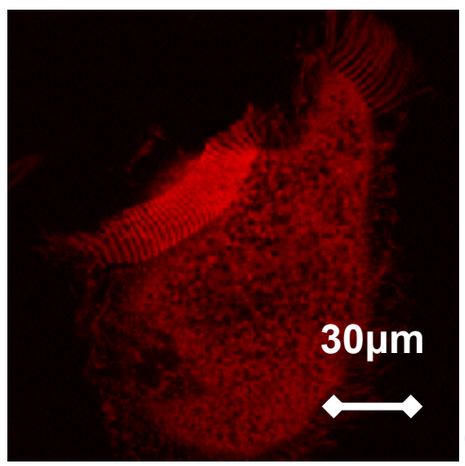
FLIM on Biological system

Giuliano Colombetti, IBF CNR, Pisa

Fluorescence intensity

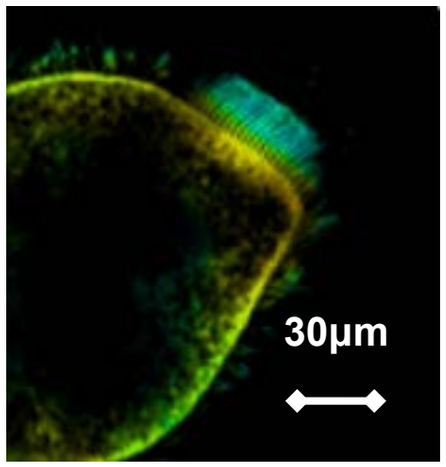


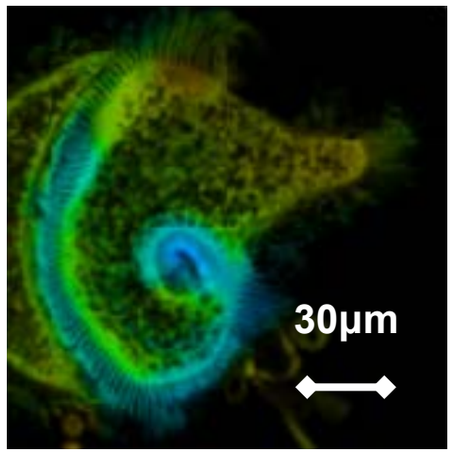


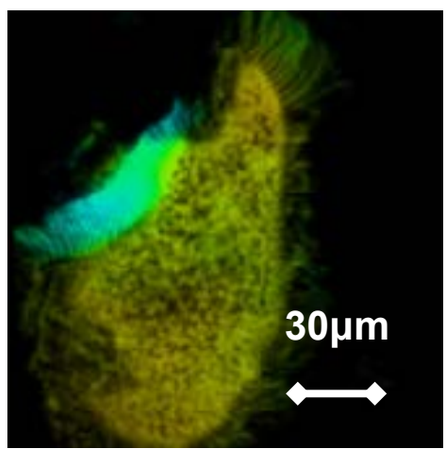


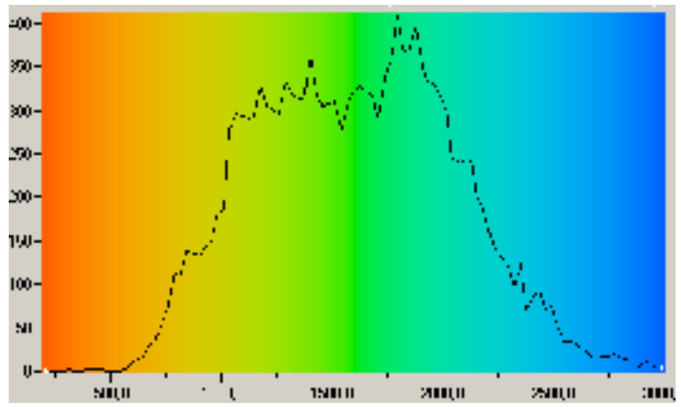
Credits: Mattia Pesce

Fluorescence Life time imaging









200ps

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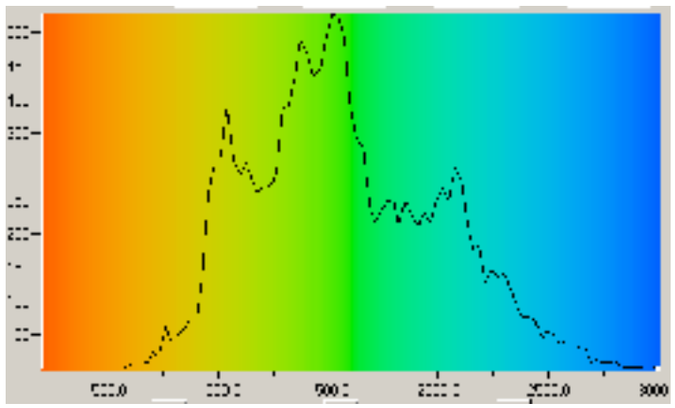

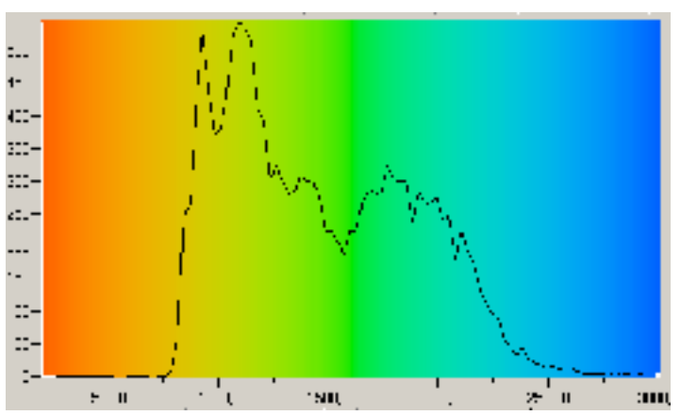

Credits: Mattia Pesce

Ciliated Protozoa (Fabrea Salina)

Colombetti G., Checcucci G., Lucia S., Usai C., Ramoino P., Bianchini P., Pesce M., Vicidomini G., Diaspro A. (2007).Microsc Res Tech. 70(12), 1028–33

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia


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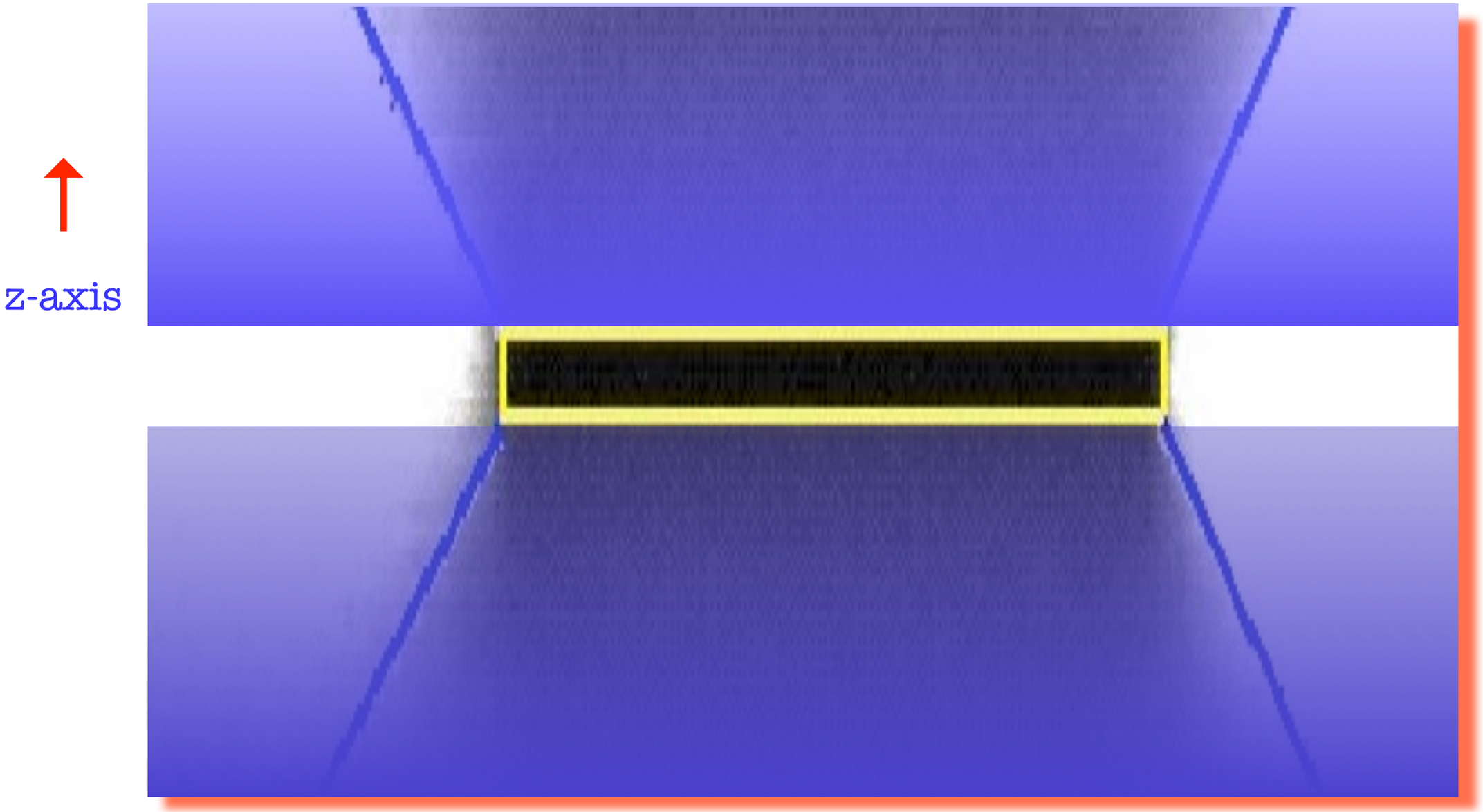
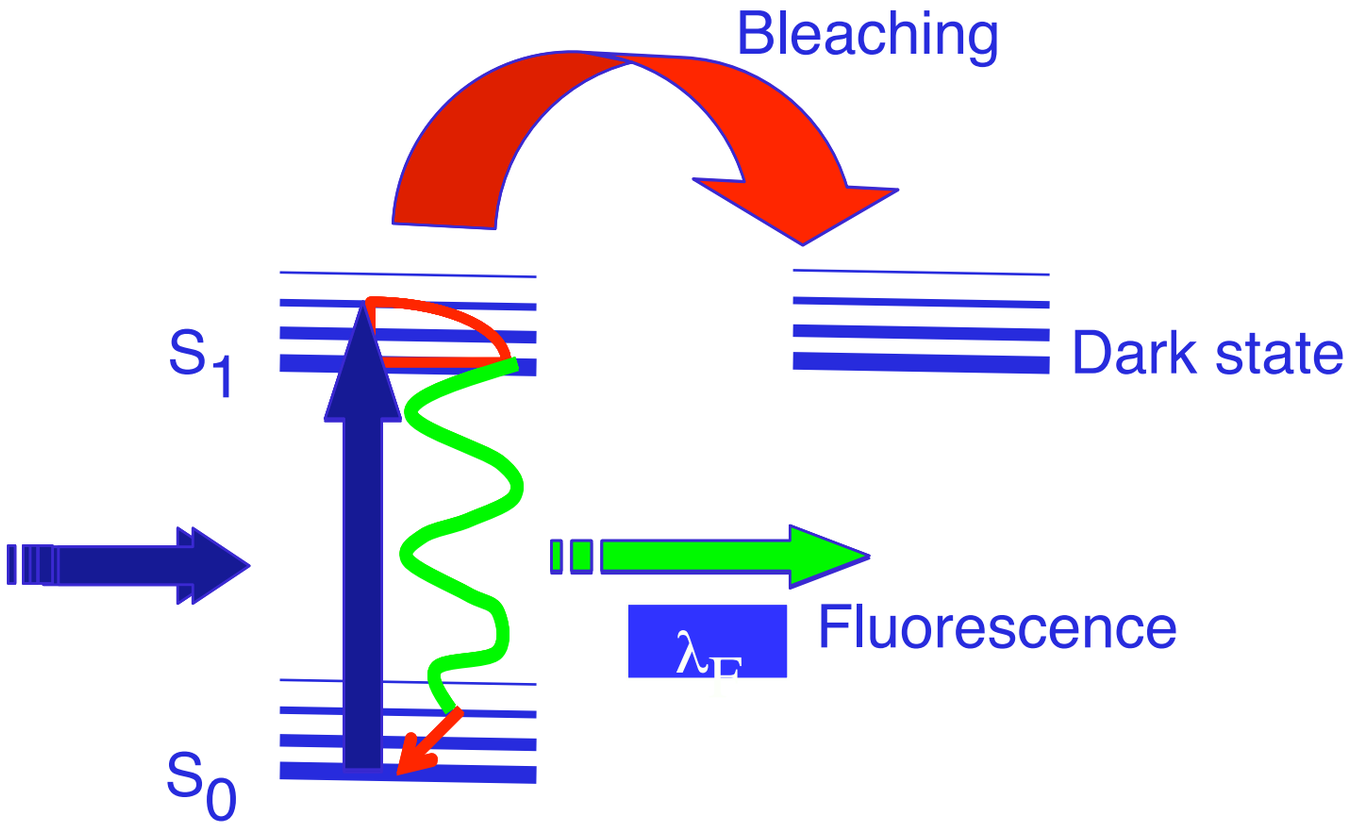
photobleaching

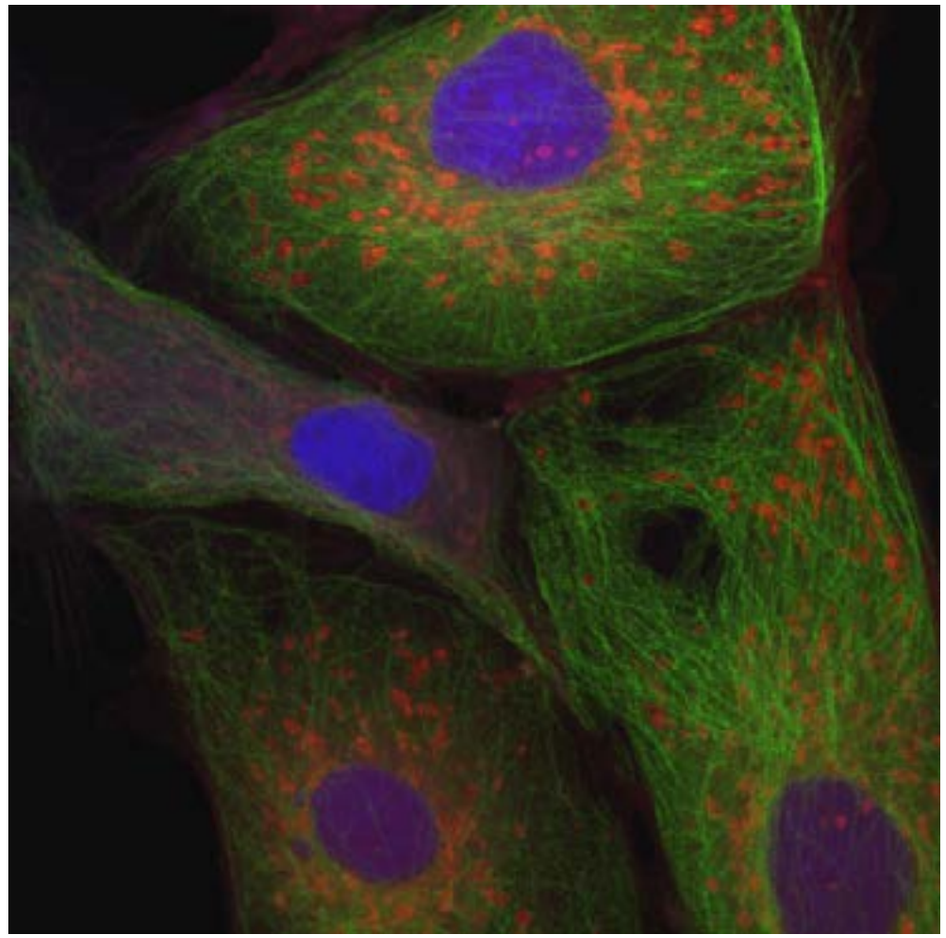


Diaspro A. et al., Photobleaching, in Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

Image credit:David Piston, Vanderbilt University

photobleaching

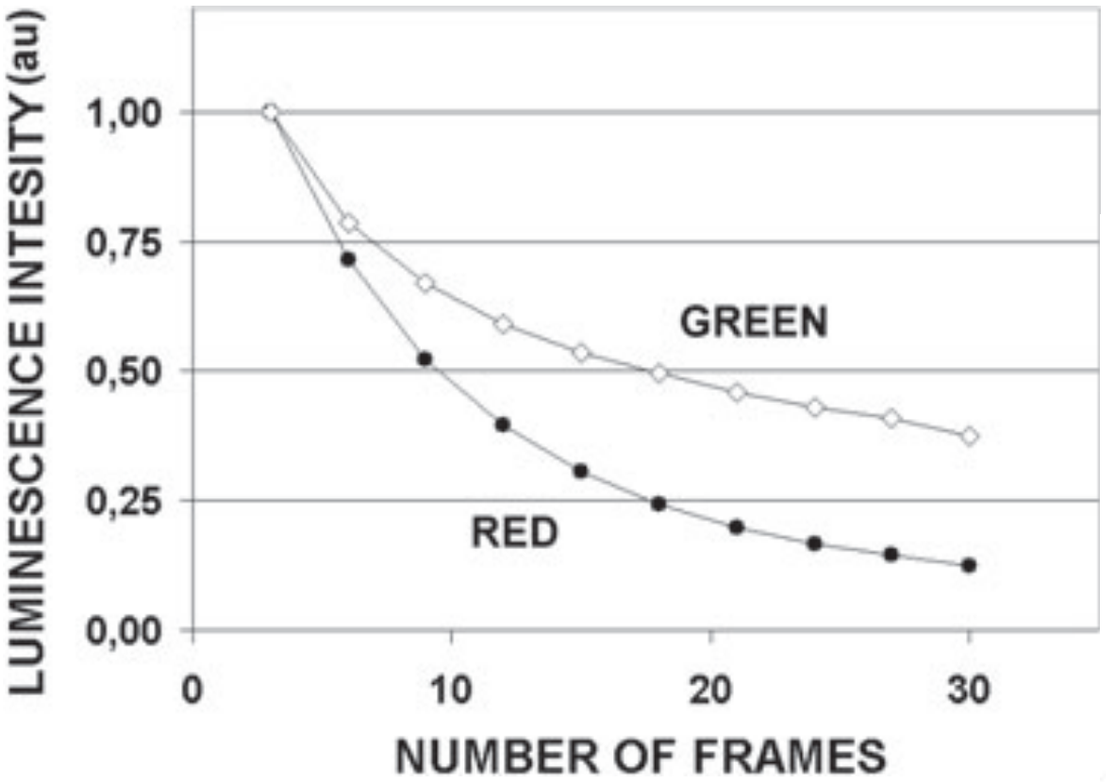




Diaspro A. et al., Photobleaching, in Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

Image Credit: Giovanni Giozzet Olympus

photobleaching



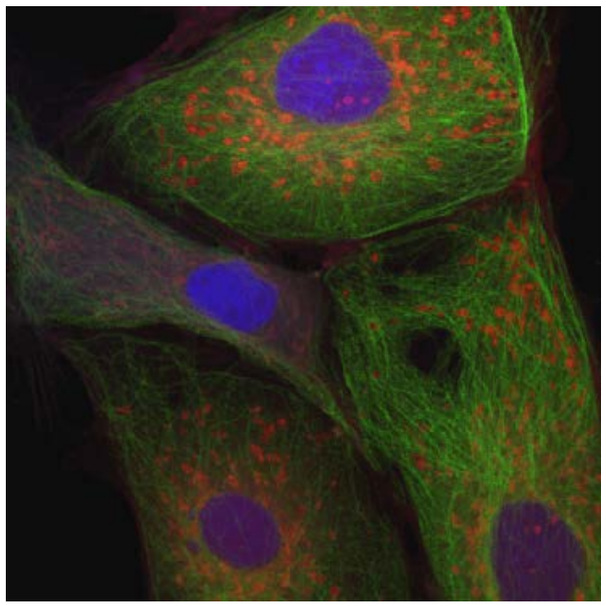
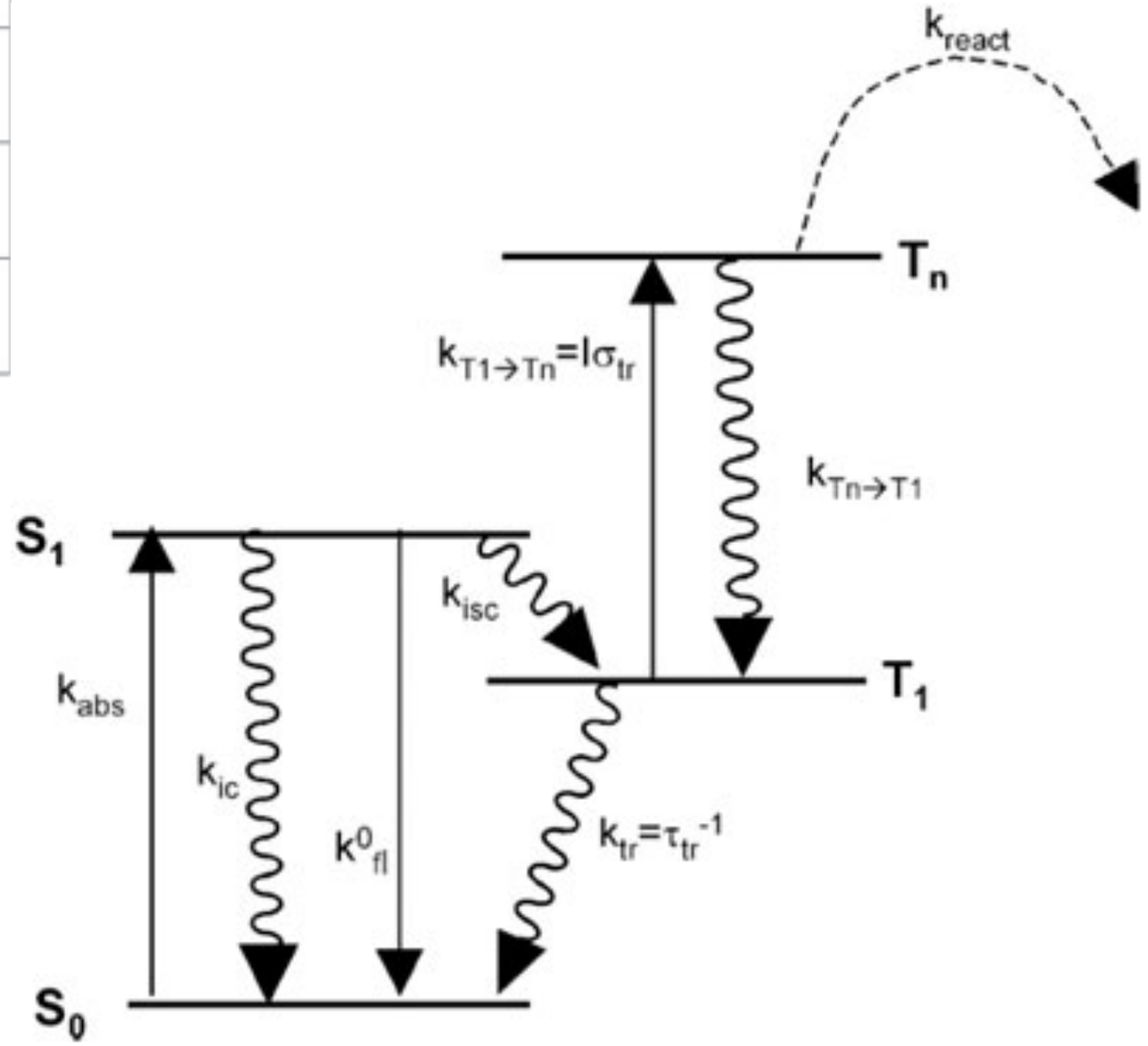
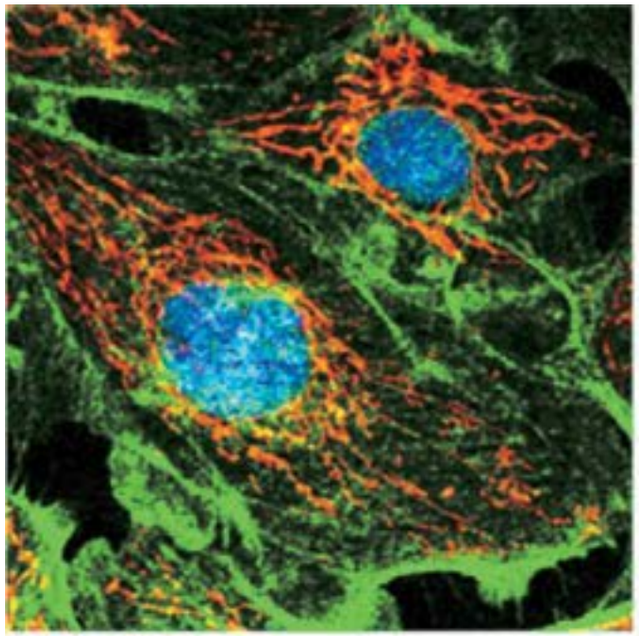


Image Credit: Giovanni Giozzet Olympus



photobleaching



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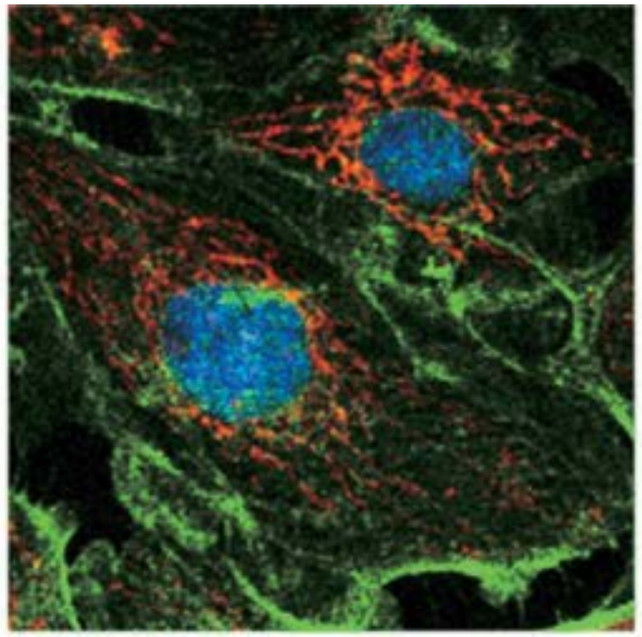

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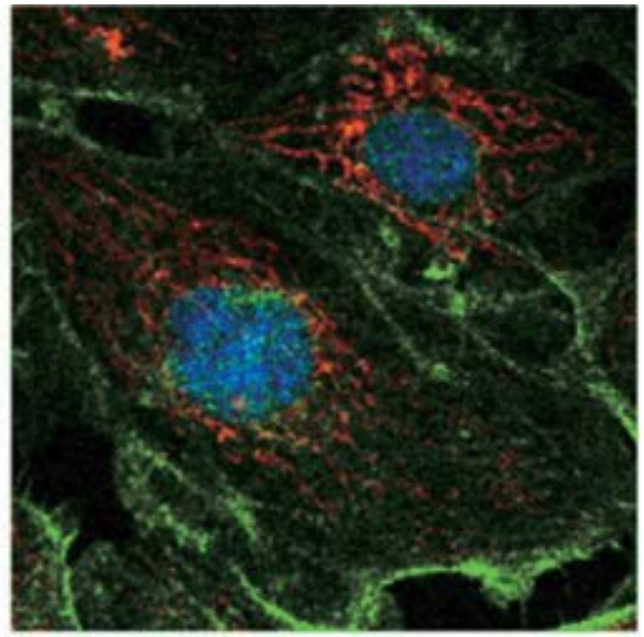

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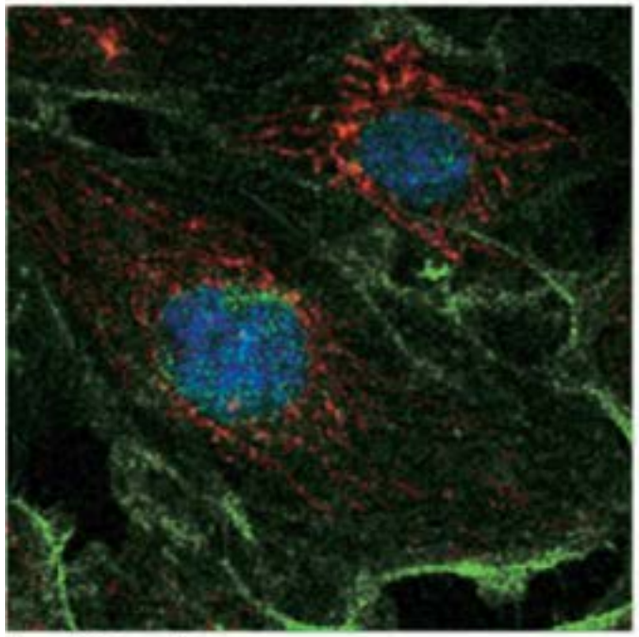

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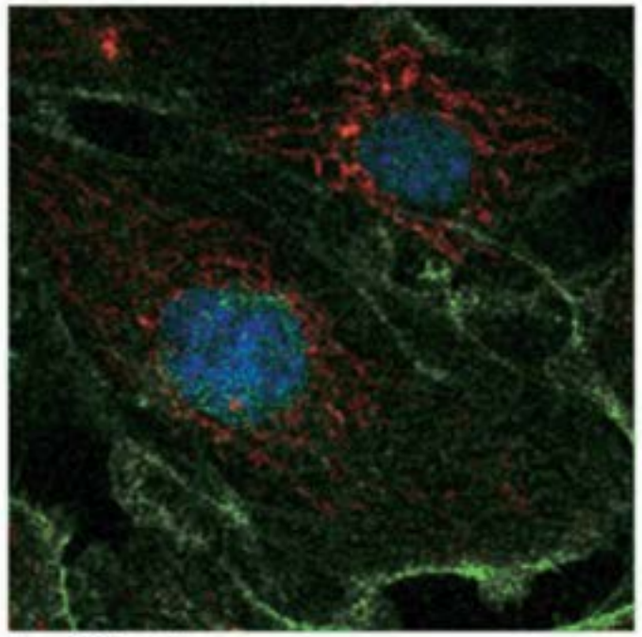

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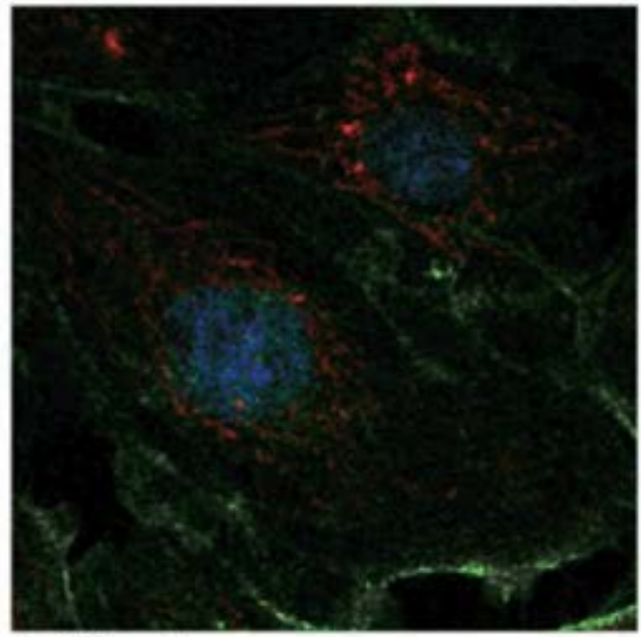
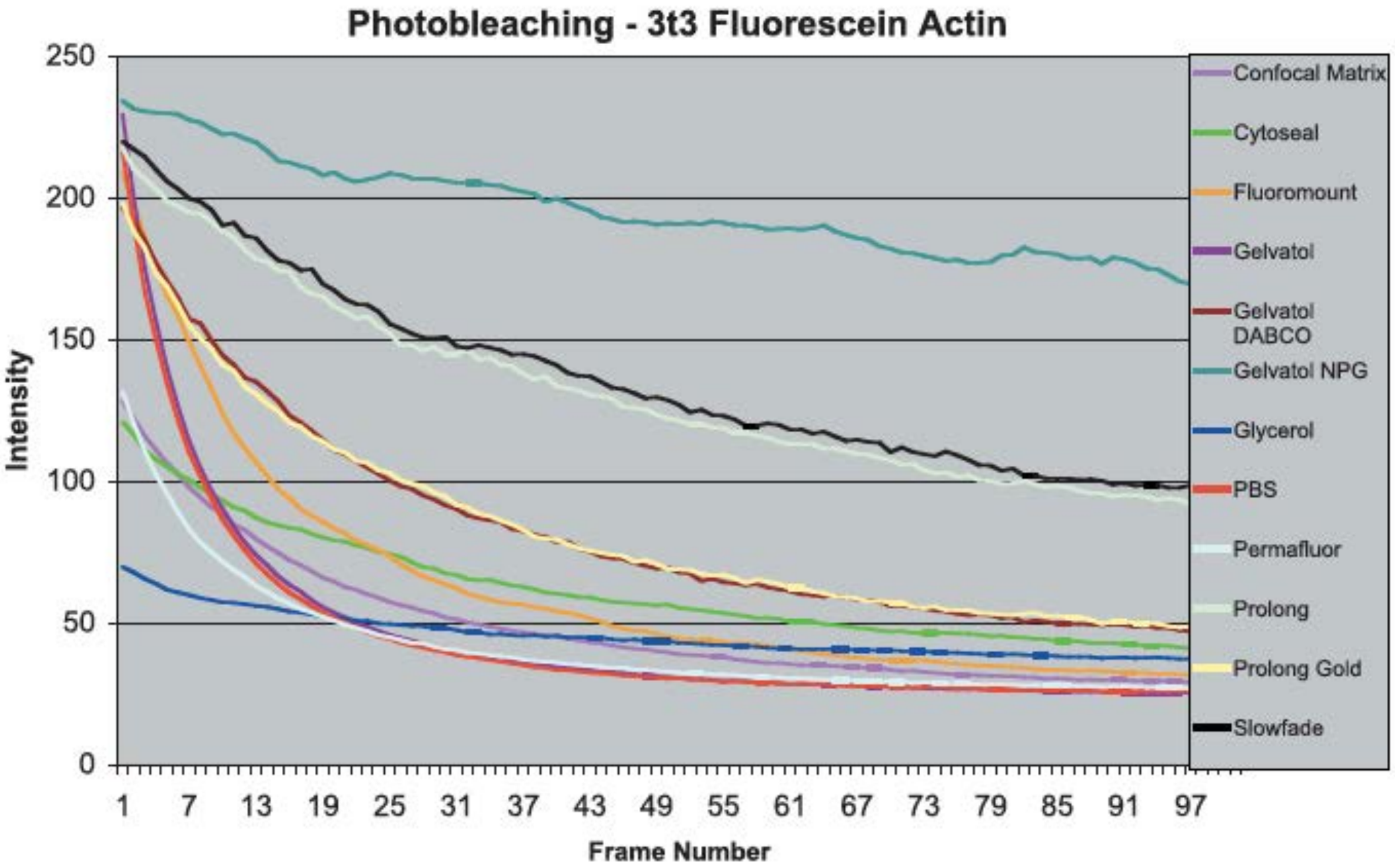
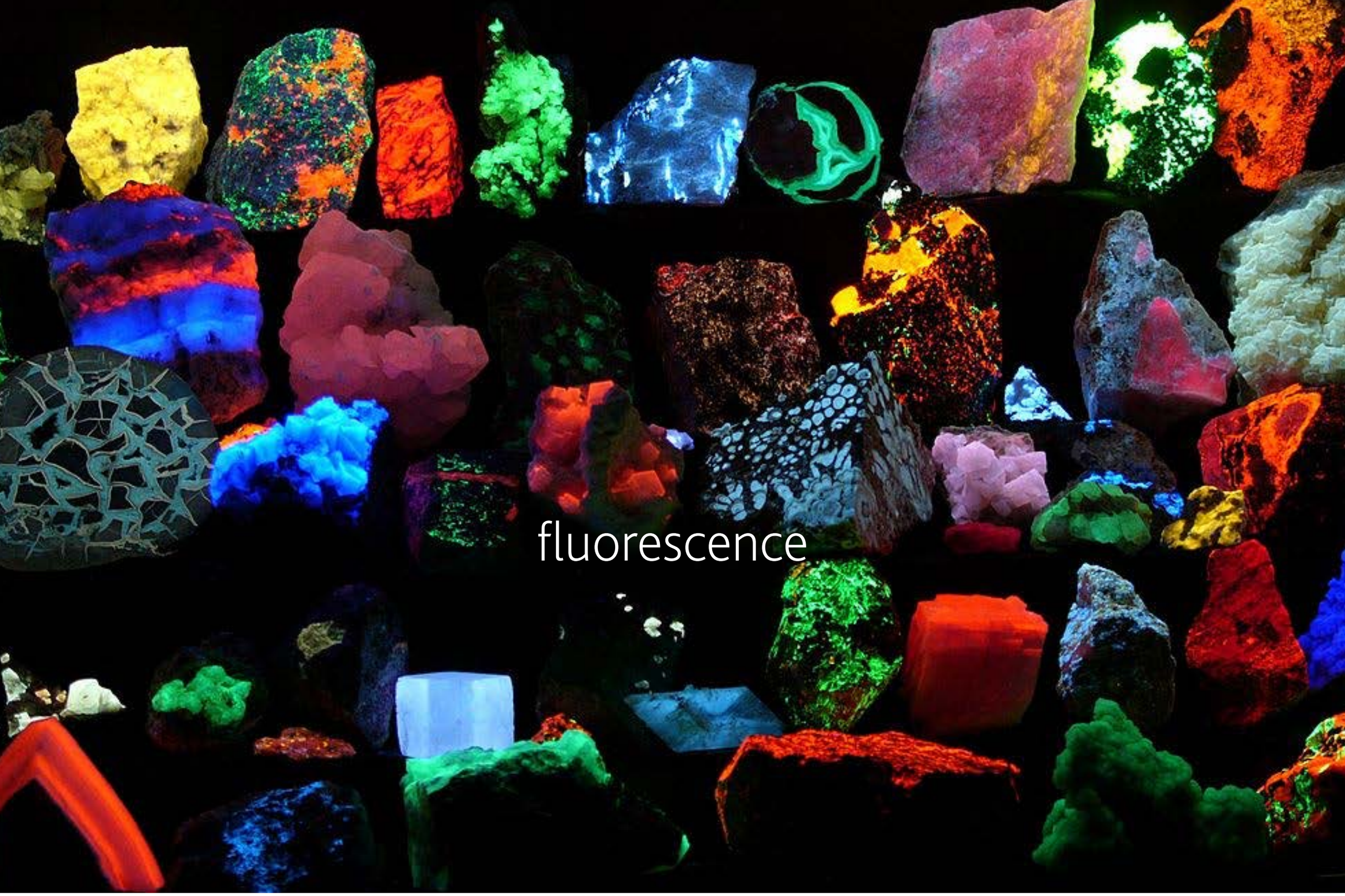


t=20min

Diaspro A. et al., Photobleaching, in Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

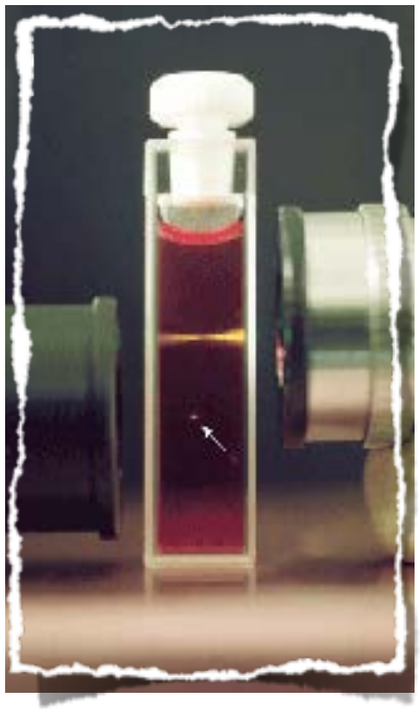
photobleaching



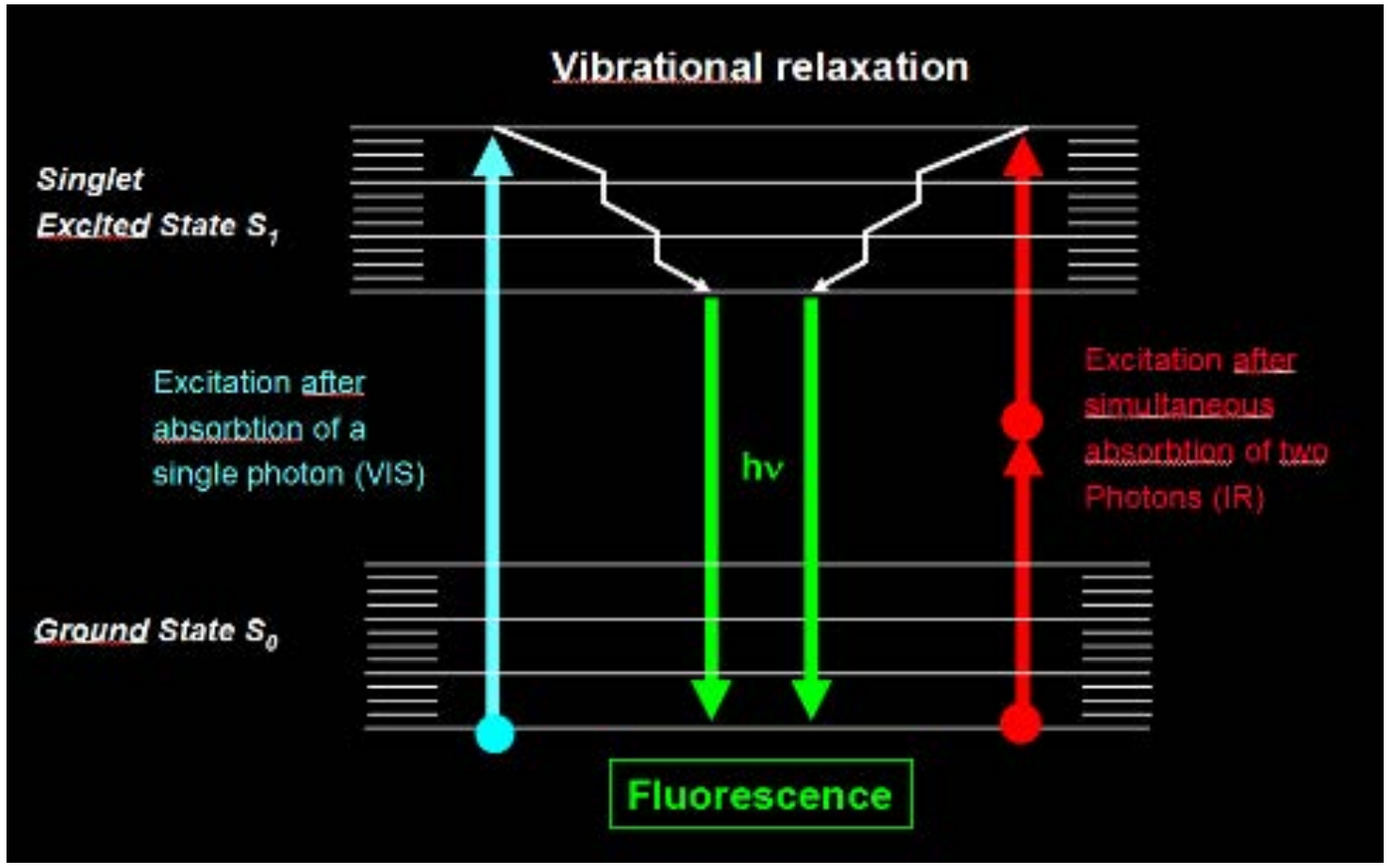


Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

non linear excitation



Credit:BRAD AMOS
MRC UK



Maria Göppert-Mayer predicted that an atom or a molecule could interact with two photon(s) simultaneously by absorbing them in the very same quantum event (1929).

Diaspro et al., Quart. Rev. .Biophys. (2005) 38 (2): 1-72 .

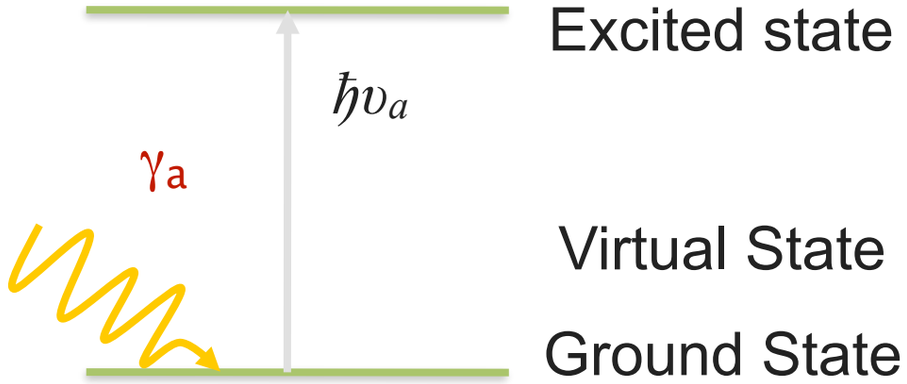
Sheppard CJR and Kompfner R. Applied Optics .17: 2879–2882 (1978)
Denk, W., Strickler, J.H. & Webb, W.W. Science 248, 73–76 (1990).

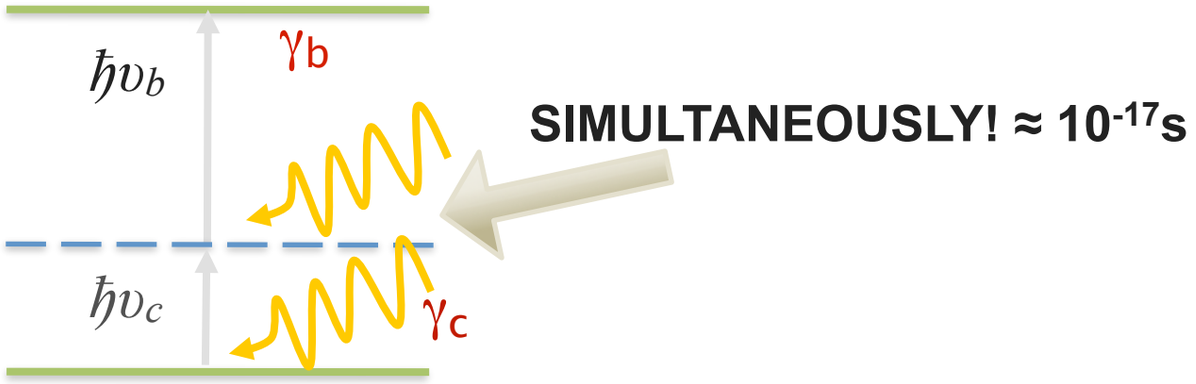
Movie credit: P.Sapuppo, E.Cocchia Leica MIcrosyastems

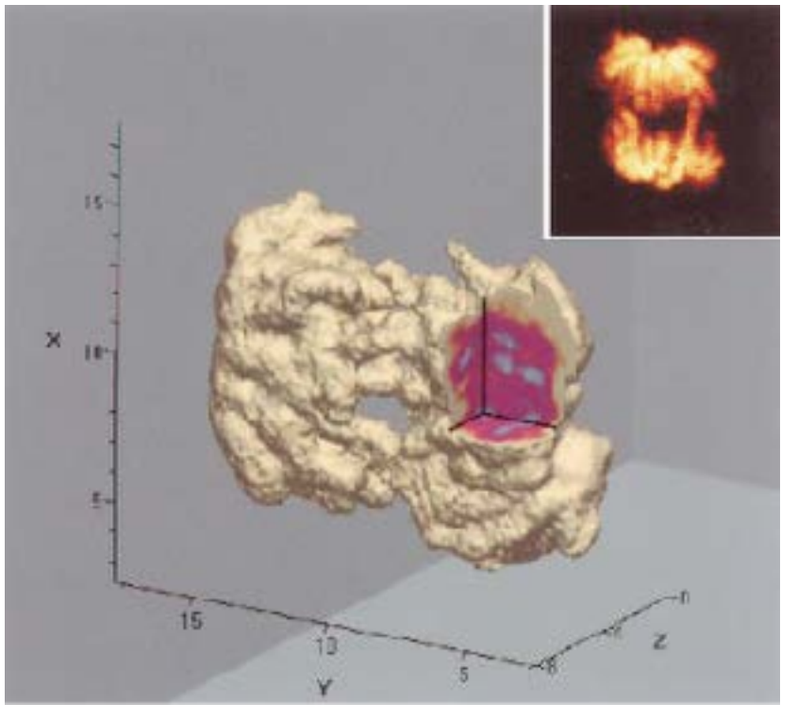
non linear excitation

Denk, W., Strickler, J. H., & Webb, W. W. (1990). Science, 248(4951), 73-76

Sheppard CJR, Kompfner R (1978). Appl. Opt. 17, 2879-2882







$$I_f \propto n_a \propto \langle P \rangle^2$$

$$n_a \approx \frac{\sigma \langle P \rangle^2}{\tau_p f_p} \left(\frac{N.A.^2}{2 \hbar c \lambda} \right)^2$$

n_a = number of absorbed photons per molecule

σ = two Photon cross section 10^{-48} - 10^{-50} cm^4 $photon^{-1}$ $molec^{-1}$

τ_p = pulse width 100 fs

f_p = pulse repetition rate ~80 Mhz

N.A. = lens numerical aperture ~1.2/1.4

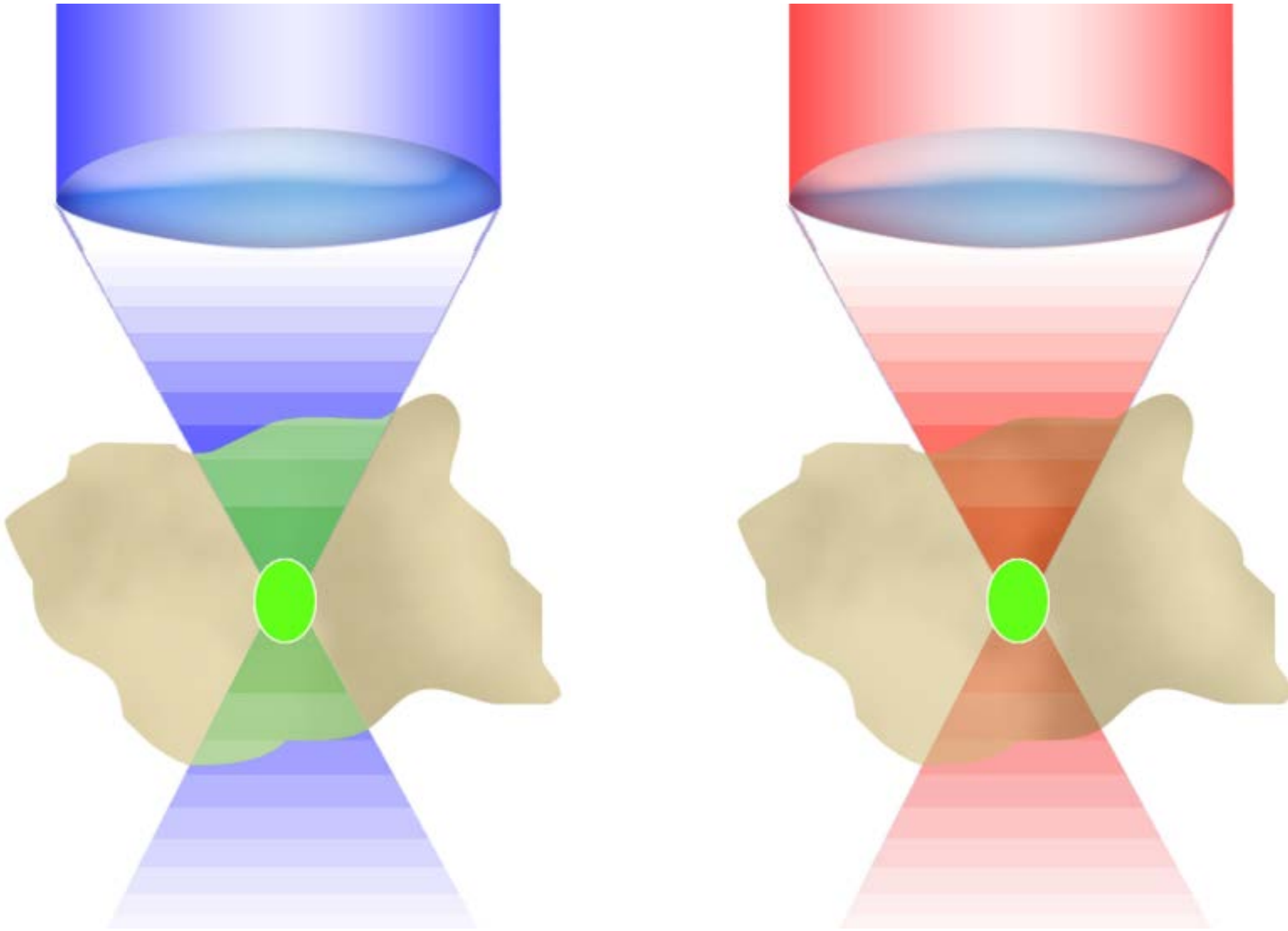
$\langle P \rangle$ = average incident power ~20mW

CW-two-photon excited 3D-data stack after volume rendering taken of DAPI-stained mouse fibrobalst DNA undergoing mitosis taken with 210mw.

Booth, M. J., & Hell, S. W. (1998) “Continuous wave excitation two-photon fluorescence microscopy exemplified with the 647-nm ArKr laser line”, Journal of Microscopy 190(Pt 3), 298-304.

P. Bianchini and A. Diaspro, (2012) “Fast scanning STED and two-photon fluorescence excitation microscopy with continuous wave beam,” Journal of Microscopy 245(3), 225–228

non linear microscopy



Diaspro et al., Quart. Rev. .Biophys. (2005) 38 (2): 1-72 .

two-photon excitation (2pe) microscopy

Simply Winnie Pooh Philosophy: Two are better than one!

A.Diaspro (ed.), (2001) "Confocal and Two-photon Microscopy. Wiley, NY, 1-567.

two-photon excitation (2pe) microscopy

A.Diaspro (ed.), (2001) "Confocal and Two-photon Microscopy. Wiley, NY, 1-567.

Diaspro A: **Two-photon fluorescence excitation. A new potential perspective in flow cytometry.** *Minerva Biotecnologica* 1998, **11**: 87-92.

two-photon excitation (2pe) microscopy

Giuseppe Pellizza da Volpedo (1868–1907)

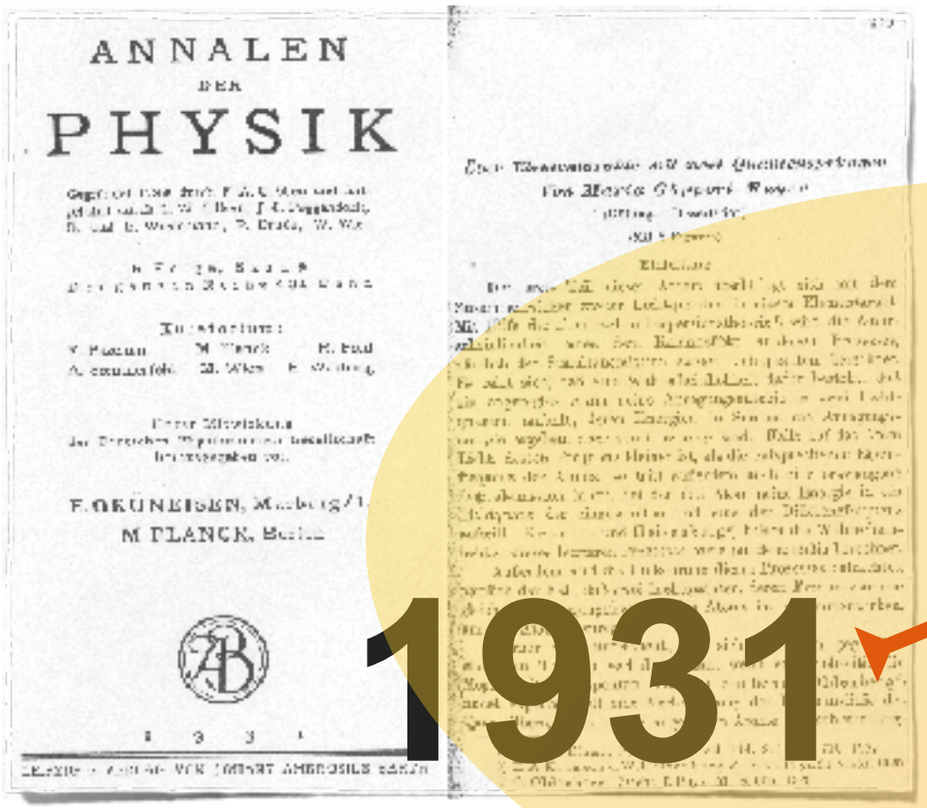
Il quarto stato (1901), olio su tela, 293x545 cm, Milano, Civica Galleria d'Arte Moderna

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

two-photon excitation (2pe) microscopy

A.Diaspro et al., (2006) Biomedical Engineering On Line (BMEOL), 5(1):36..

This article can be downloaded for free from: http://www.biomedical-engineering-online.com/content/5/1/36


ANNALEN
DER
PHYSIK

F. GRÜNEISEN, Marburg/L.
M. PLANCK, Berlin

1931

Resonant scanning optical microscope

15 September 1978 / Vol. 17, No. 18 / APPLIED OPTICS 2879

C. J. R. Sheppard and R. Kompfner

1977

Two-Photon Laser Scanning Fluorescence Microscopy

WINFRIED DENK,* JAMES H. STRICKLER, WATT W. WEBB

Molecular excitation by the simultaneous absorption of two photons provides intrinsic three-dimensional resolution in laser scanning fluorescence microscopy. The excitation of fluorophores having single-photon absorption in the ultraviolet with a stream of strongly focused subpicosecond pulses of red laser light has made possible fluorescence images of living cells and other microscopic objects. The fluorescence emission ... nonlinearly with the excitation intensity so that fluorescence and photobleaching were confined to the vicinity of the focal plane as expected for cooperative ... two-photon excitation. This technique also provides unprecedented capabilities for ... three-dimensional, spatially resolved photochemistry, particularly photolytic release of caged effector molecules.

1990

Two-photon excitation STED microscopy

Gael Moneron and Stefan W. Hell*

Department of Nanobiophotonics, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

Abstract: We report sub-diffraction resolution in two-photon excitation (TPE) fluorescence microscopy achieved by merging this technique with stimulated-emission depletion (STED). We demonstrate an easy-to-implement and promising laser combination based on a short-pulse laser source for two-photon excitation and a continuous-wave (CW) laser source for resolution enhancement. Images of fluorescent nanoparticles and the immunostained transcription regulator NFκB in mammalian cells with cellular resolutions of <90 nm and ~70 nm in the focal plane, corresponding to a 4–5.4-fold improvement over the diffraction barrier.

OCIS codes: (180.1790) Confocal microscopy; (180.2520) Fluorescence microscopy; (180.4315) Nonlinear microscopy; (180.6900) Scanning microscopy; (180.6940) Three-dimensional microscopy; (110.2350) Microscopy.

References and links

1. W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," Science 248(4951), 73–76 (1990).
2. S. W. Hell, and J. Wichmann, "Breaking the diffraction resolution limit by stimulated emission: stimulated

2009

Neuron
Neurotechnique

Supraresolution Imaging in B... using Stimulated-Emission D... Two-Photon Laser Scanning

Juan B. Ding,[1] Kevin T. Takasaki,[2] and Bernardo L. Sabatini[1,*]

[1]Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
[2]Program in Neurosciences, Harvard University, Cambridge MA 02138, USA
*Correspondence should be addressed to B.L.S. (bsabatini@hms.harvard.edu)

DOI 10.1016/j.neuron.2006.07.021

PNAS

2012

Single-wavelength two-photon excitation–stimulated emission depletion (SW2PE-STED) superresolution imaging

Paolo Bianchini[a], Benjamin Harke[a], Silvia Galia..., Giuseppe ...licidomini[a], and Al... Diaspro[a,b,1]

[a]Department of Nanophysics, Istituto Italiano di Tecnologia, 6100... Italy; [b]D... of Physics, ...ersity of Genoa, 16100...

Edited by David A. Weitz, Harvard University, Cambridge, MA, ...pp... M... 15, 201... ...ceived fo... ...w November 22, 2011)

We developed a new class of two-photon excita... ...lat... ...ical Pr... ...ples... ...p
emission depletion (2PE-STED) optical microscope. ... ...e key point ... ...es on finding a wave
we show the opportunity to perform superresolved fluorescence nonzero cross-section for 2PE and STED proces
imaging, exciting and stimulating the emission of a fluorophore 1PE excitation of a given dye. Then one has the

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

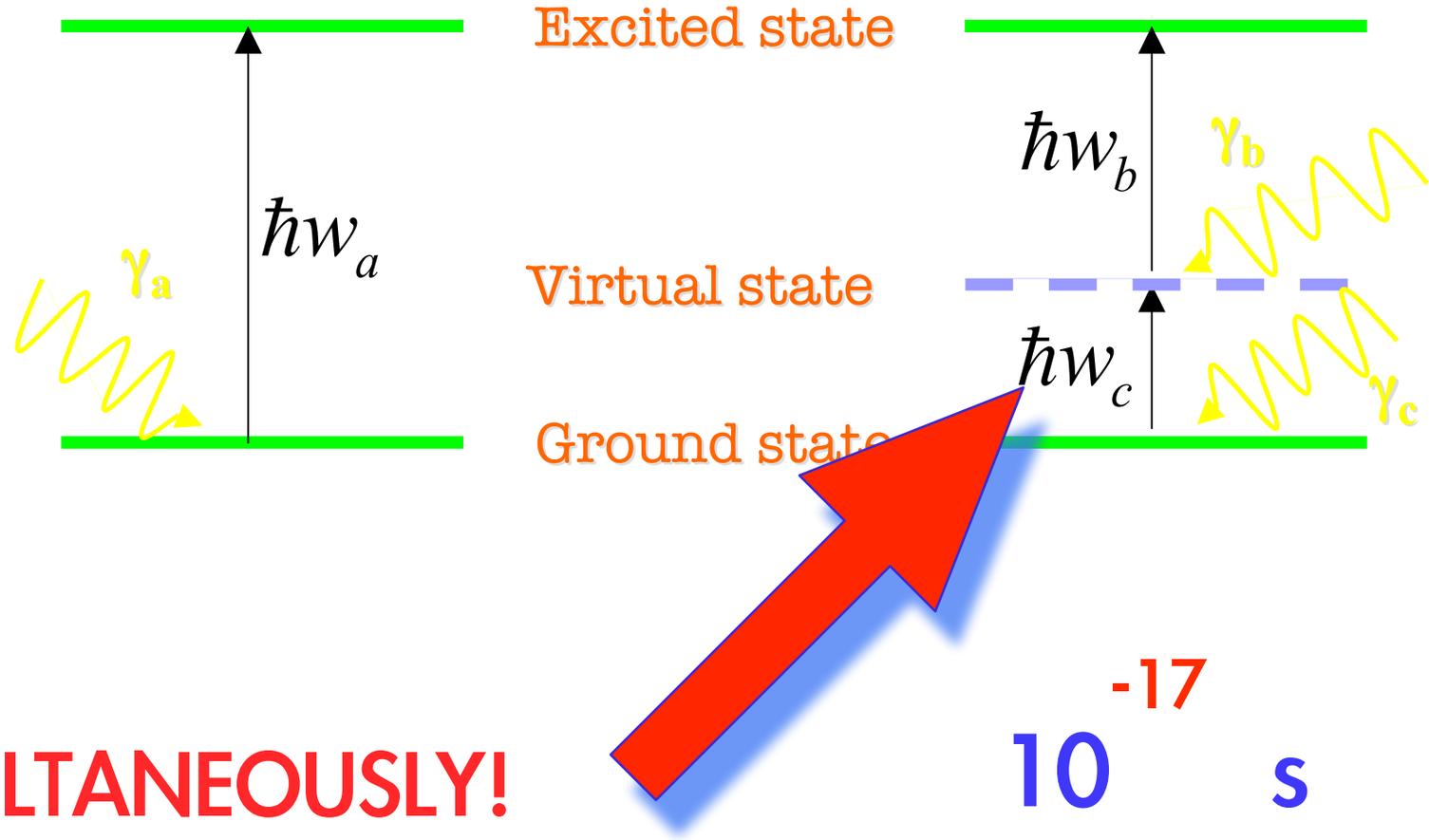
two-photon excitation (2pe) microscopy

MARIA GOEPPERT MAYER

The shell model

Nobel Lecture, December 12, 1963

(credit: EMILIO SEGRE' archive)



SIMULTANEOUSLY!

10^{-17} s

Maria Göppert-Mayer predicted that an atom or a molecule could interact with two photons simultaneously by absorbing them in the very same quantum event.

two-photon excitation (2pe) microscopy

History

- 1931: Prediction of two-photon absorption by Maria Göppert-Mayer
- 1961: Experimental observation of two-photon excitation in $CaF_2:Eu^{2+}$
- ***1978: Proposal of application of TPE and other NLE to scanning microscopy***

(Colin Sheppard, Oxford)

- 1990: First successful realization of two-photon laser scanning fluorescence microscopy

(Winfried Denk at W.Webb Lab, Cornell University)

Physics background

- Description by means of perturbation theory:

e.g. $H' = e\,\boldsymbol{r} \cdot \boldsymbol{E}$ (spatially odd function)

A.Diaspro (ed.), (2001) "Confocal and Two-photon Microscopy. Wiley, NY, 1-567.

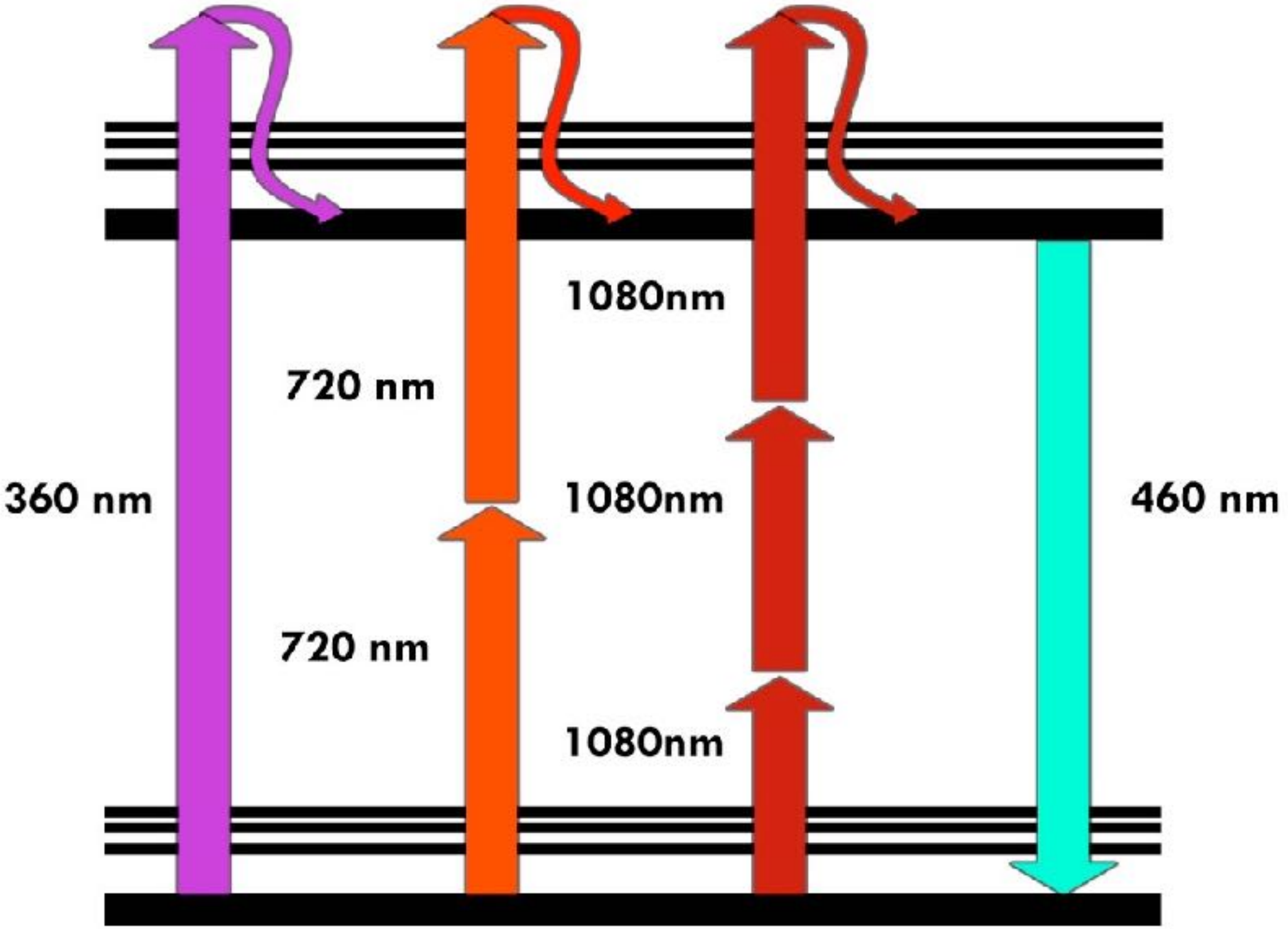
two-photon excitation (2pe) microscopy

$E_a = E_b + E_c$

$$\lambda_a = \left(\frac{1}{\lambda_b} + \frac{1}{\lambda_c} \right)^{-1}$$

Movie Credit: Elio Cocchia Fabrizio Papalia, Paolo Sapuppo - LEICA Microsystems

two-photon excitation (2pe) microscopy

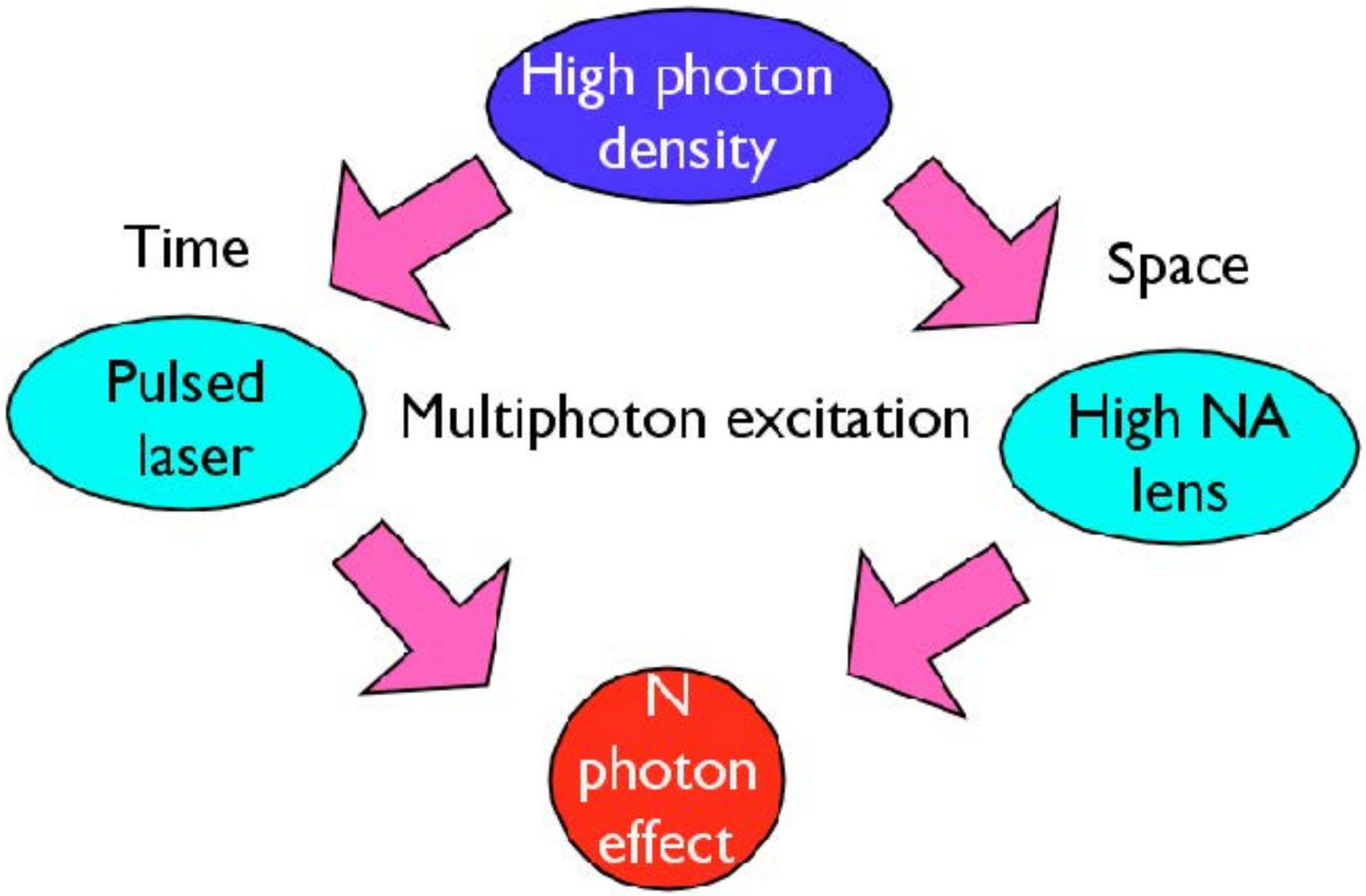


two-photon excitation (2pe) microscopy

In bright sunlight, a molecule of rhodamine B, an excellent 1- or 2- photon absorber, absorbs a photon through a 1-photon process about once a second, a photon pair by 2-photon absorption every 10 million years...

Denk and Svoboda (1997) Neuron, 18:351

two-photon excitation (2pe) microscopy

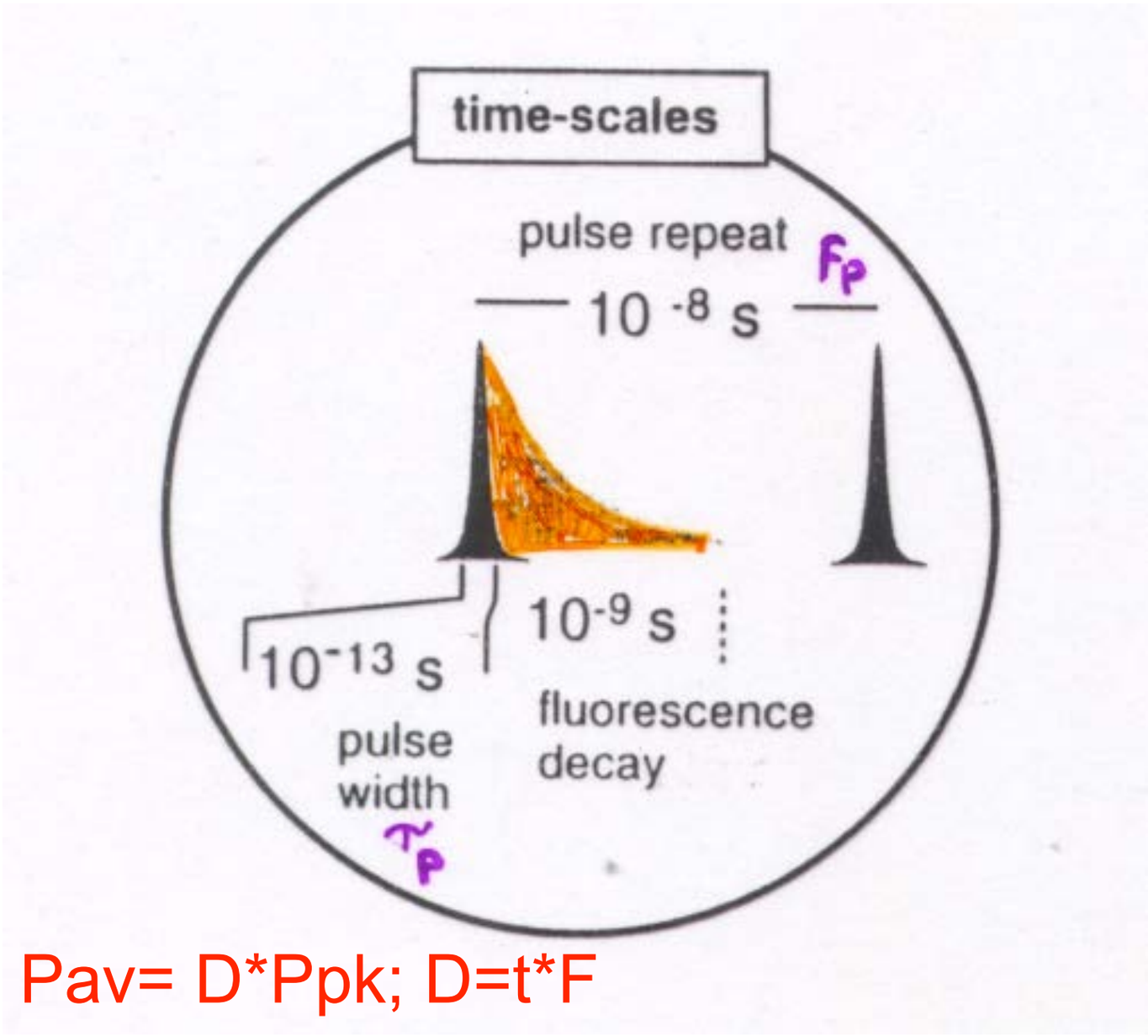


A.DIASPRO, ET AL. (2005) QUART. REV. .BIOPHYS.,VOL.38, NR.2, PP.1-72.

Diaspro A: **Two-photon fluorescence excitation. A new potential perspective in flow cytometry.** *Minerva Biotecnologica* 1998, **11**: 87-92.

two-photon excitation (2pe) microscopy

CORE ELEMENTS ARE LASER SOURCES



Pav= D*Ppk; D=t*F

two-photon excitation (2pe) microscopy

$$n_a = \frac{\sigma < P >^2}{\tau_p f_p^2} \left(\frac{N.A.^2}{2\hbar c \lambda} \right)^2$$

$I_f \alpha n_a \alpha < P >^2$

n_a = no. of absorbed photons per molecule

σ = two-photon cross-section 10^{-48}-10^{-50} cm^4 s $photon^{-1} molec^{-1}$

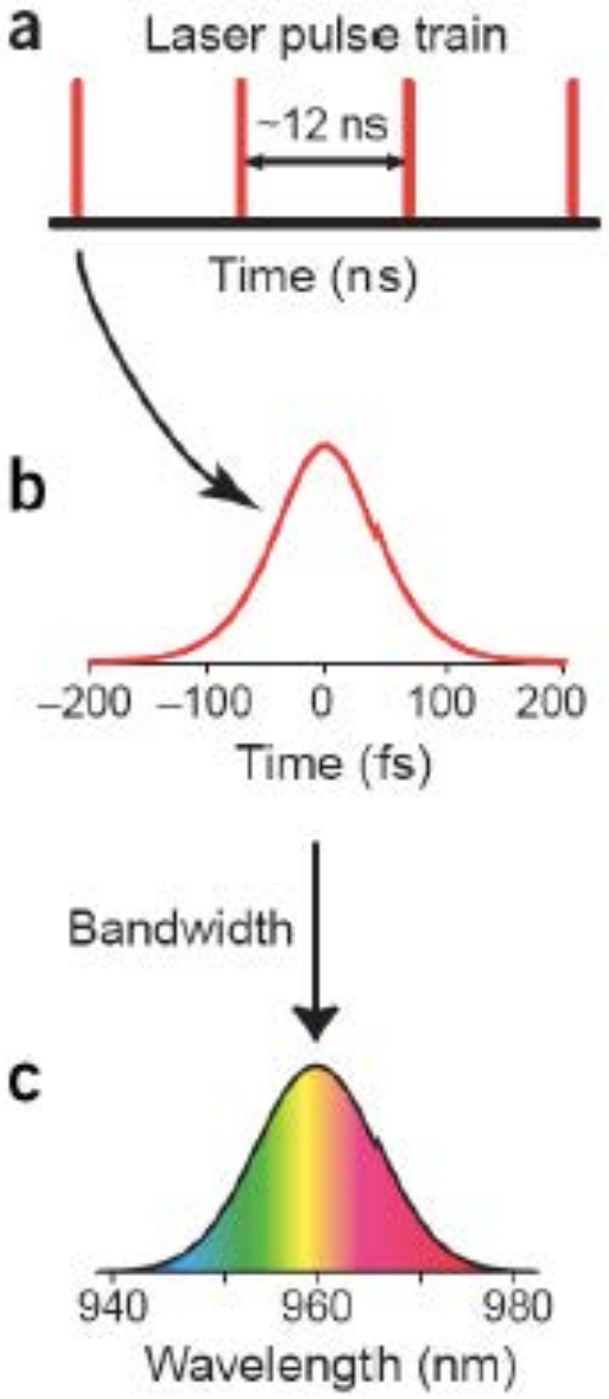
τ_p = pulse width ~ 100 fs

f_p = pulse repetition rate ~ 80 MHz

NA = lens numerical aperture ~ 1.2 – 1.4

<P>= average incident power ~ 20 mW

Denk, W., Strickler, J.H. & Webb, W.W. *Science* **248**, 73–76 (1990).



80 MHz repetition rate

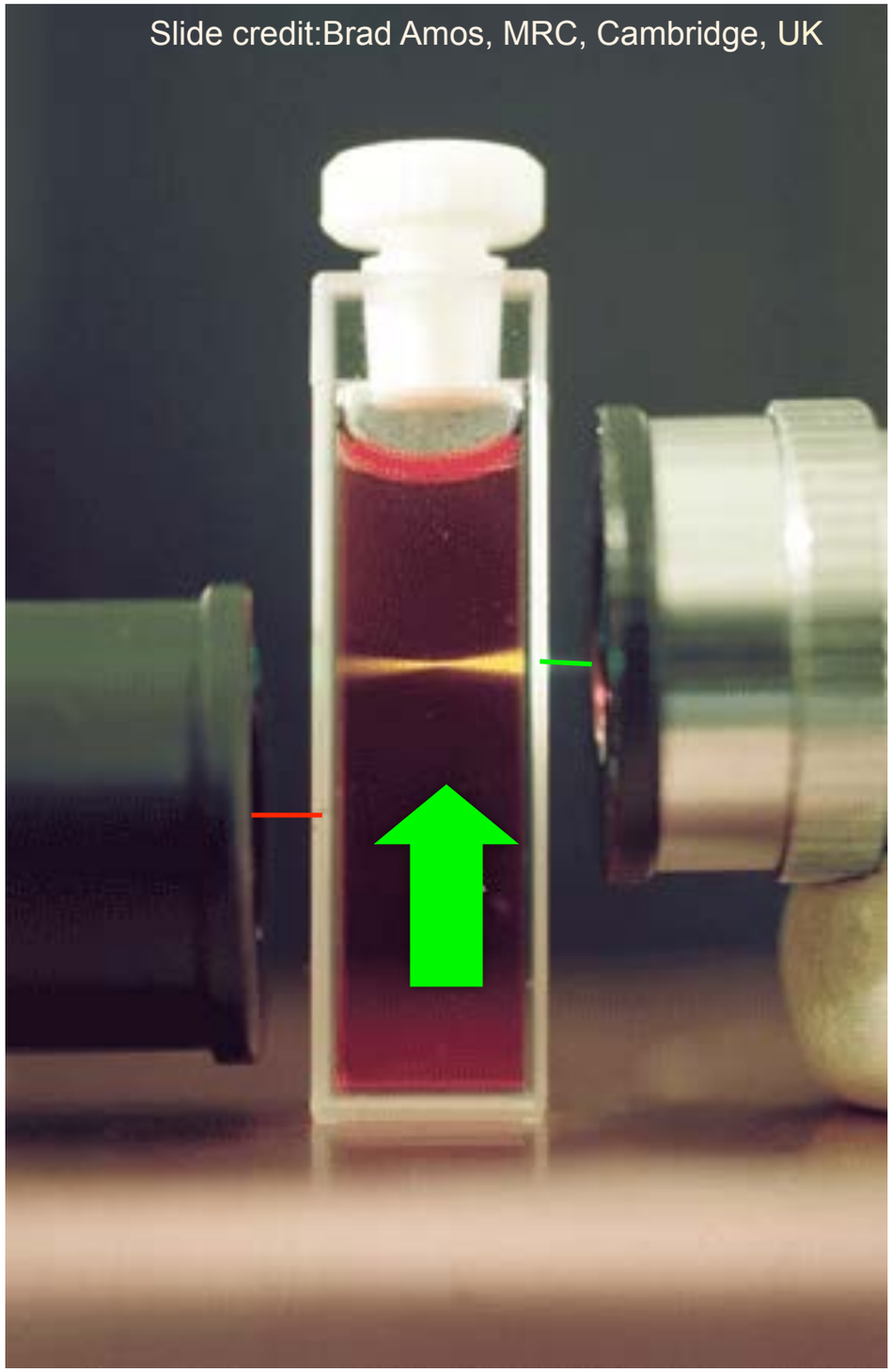
FWHM 100 fs – time

FWHM 10 – 15 nm

Short pulses of monochromatic light have colors! - Spectral components

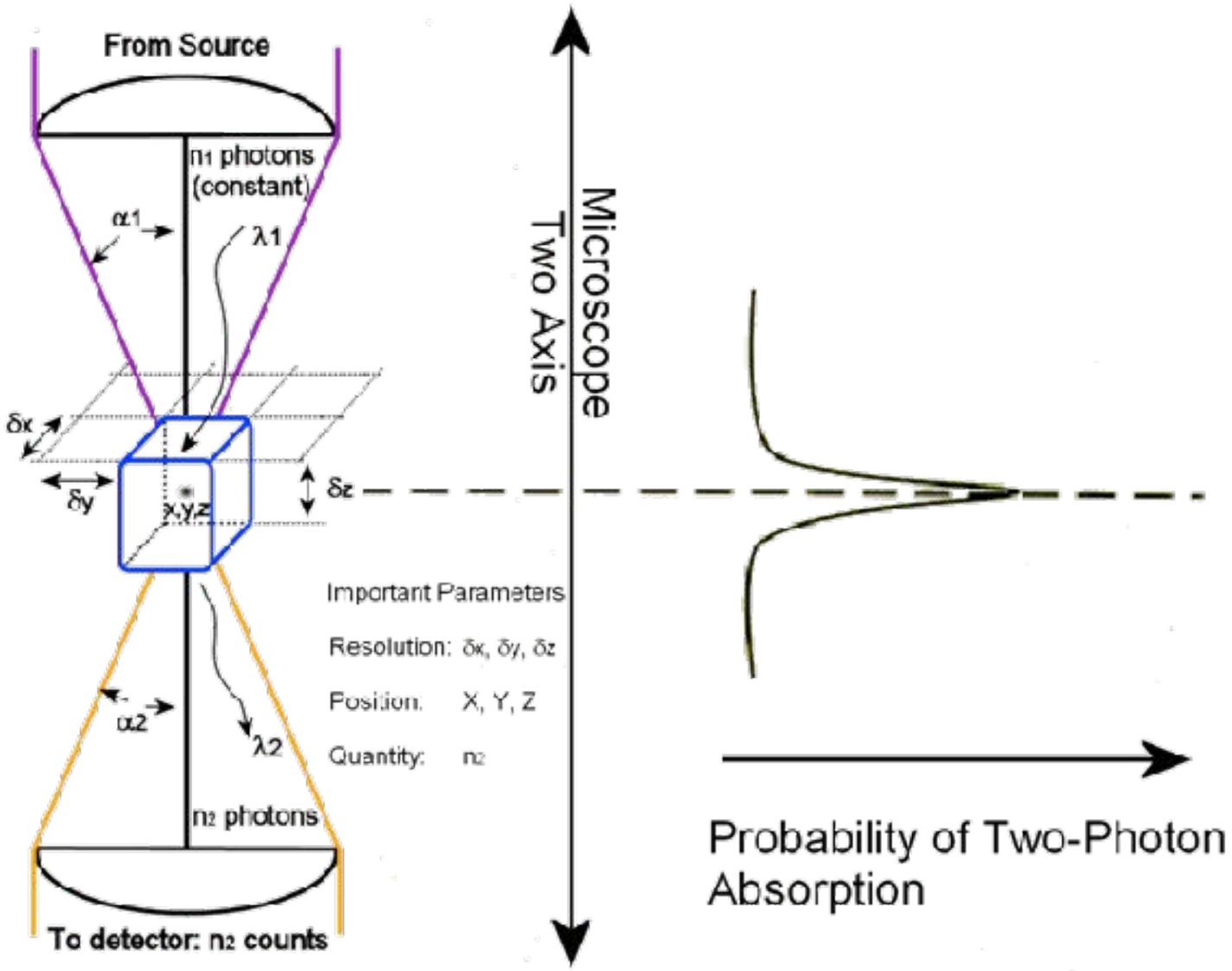
Nat. Biotech. Vol 21 No 11, 2003, Zipfel et al. Nonlinear magic: multiphoton microscopy in the biosciences

two-photon excitation (2pe) microscopy

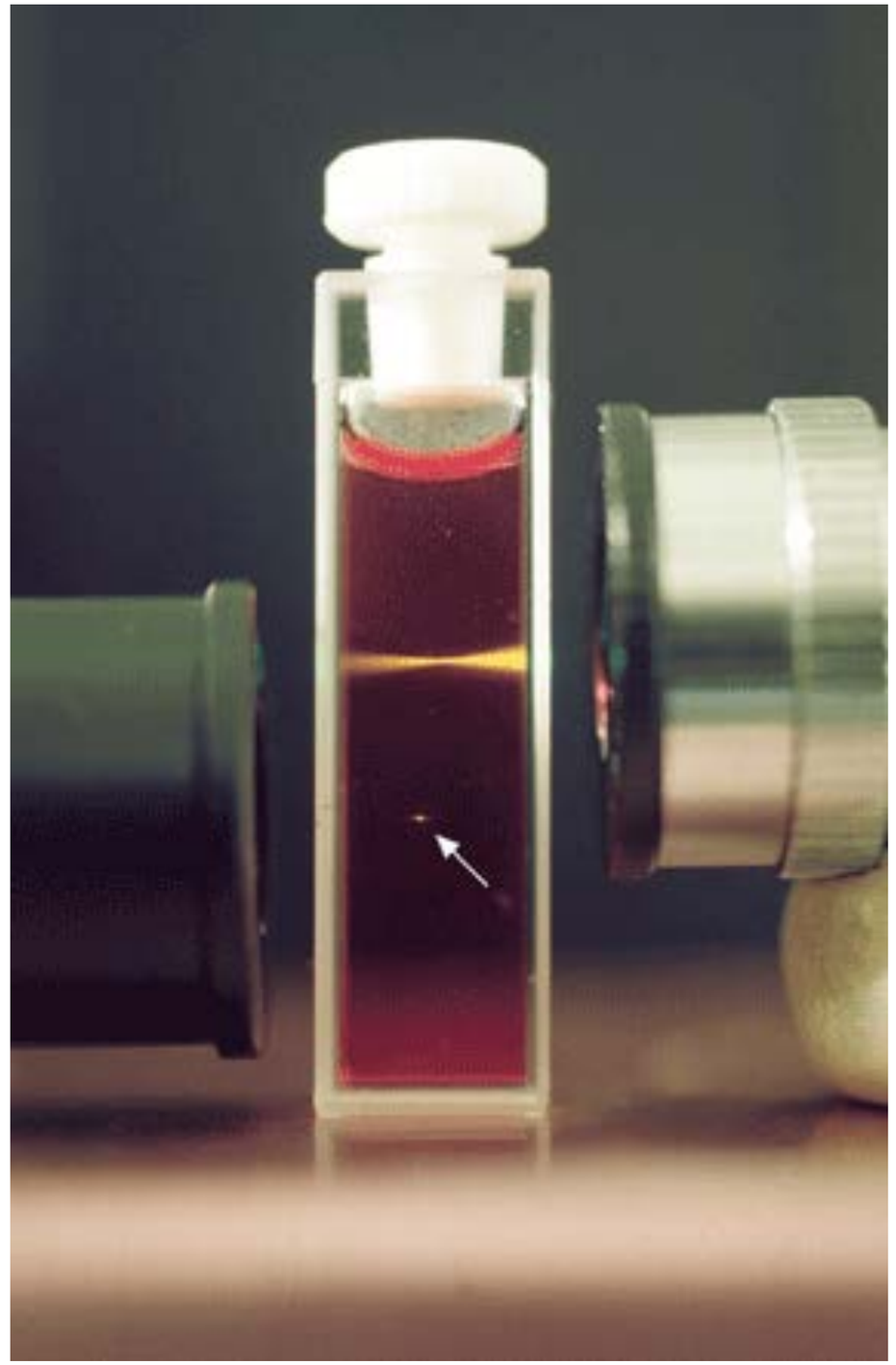


A.DIASPRO, ET AL. (2005) QUART. REV. .BIOPHYS.,VOL.38, NR.2, PP.1-72.

two-photon excitation (2pe) microscopy

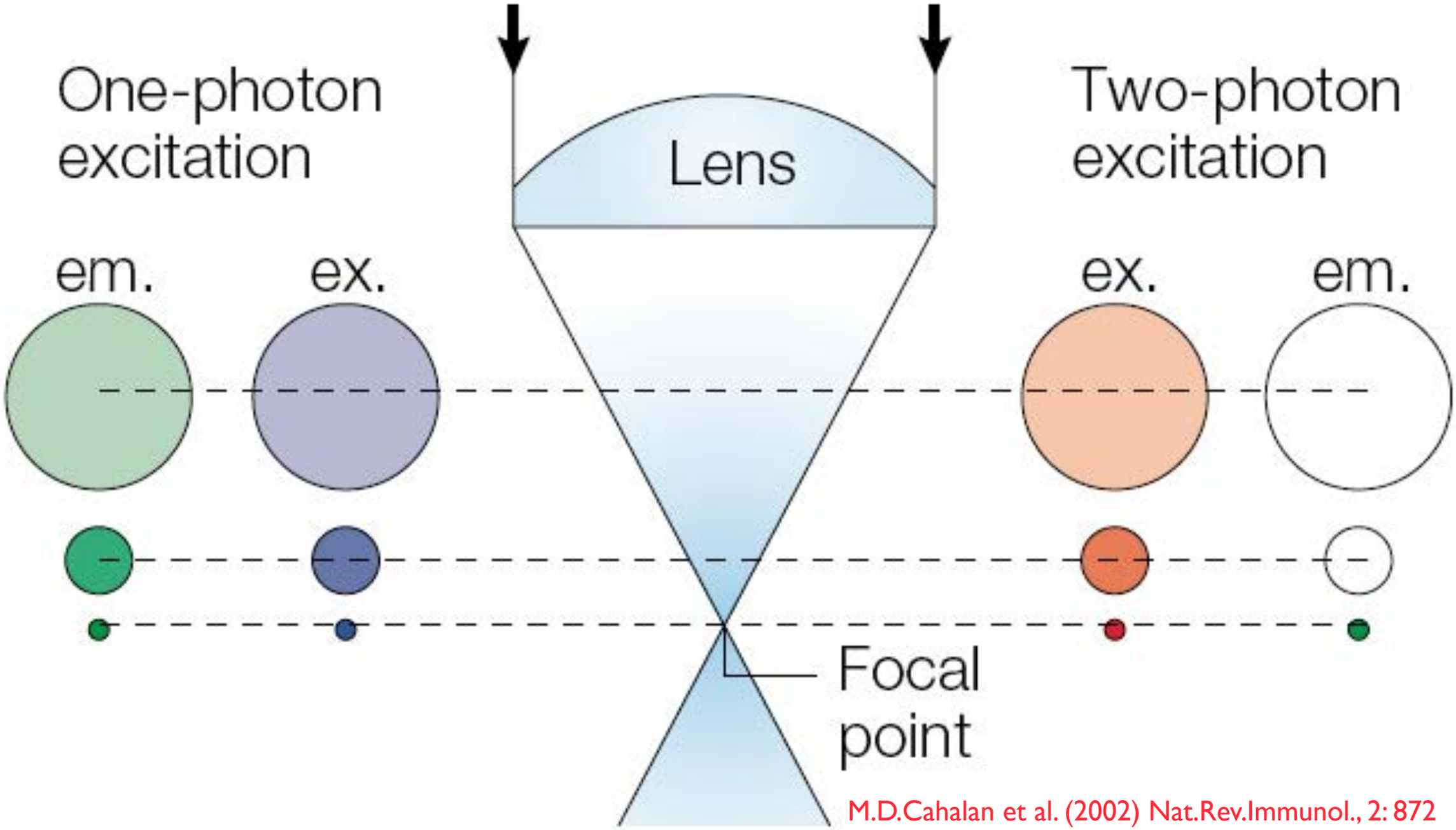


Slide credit:Jim Pawley's Handbook



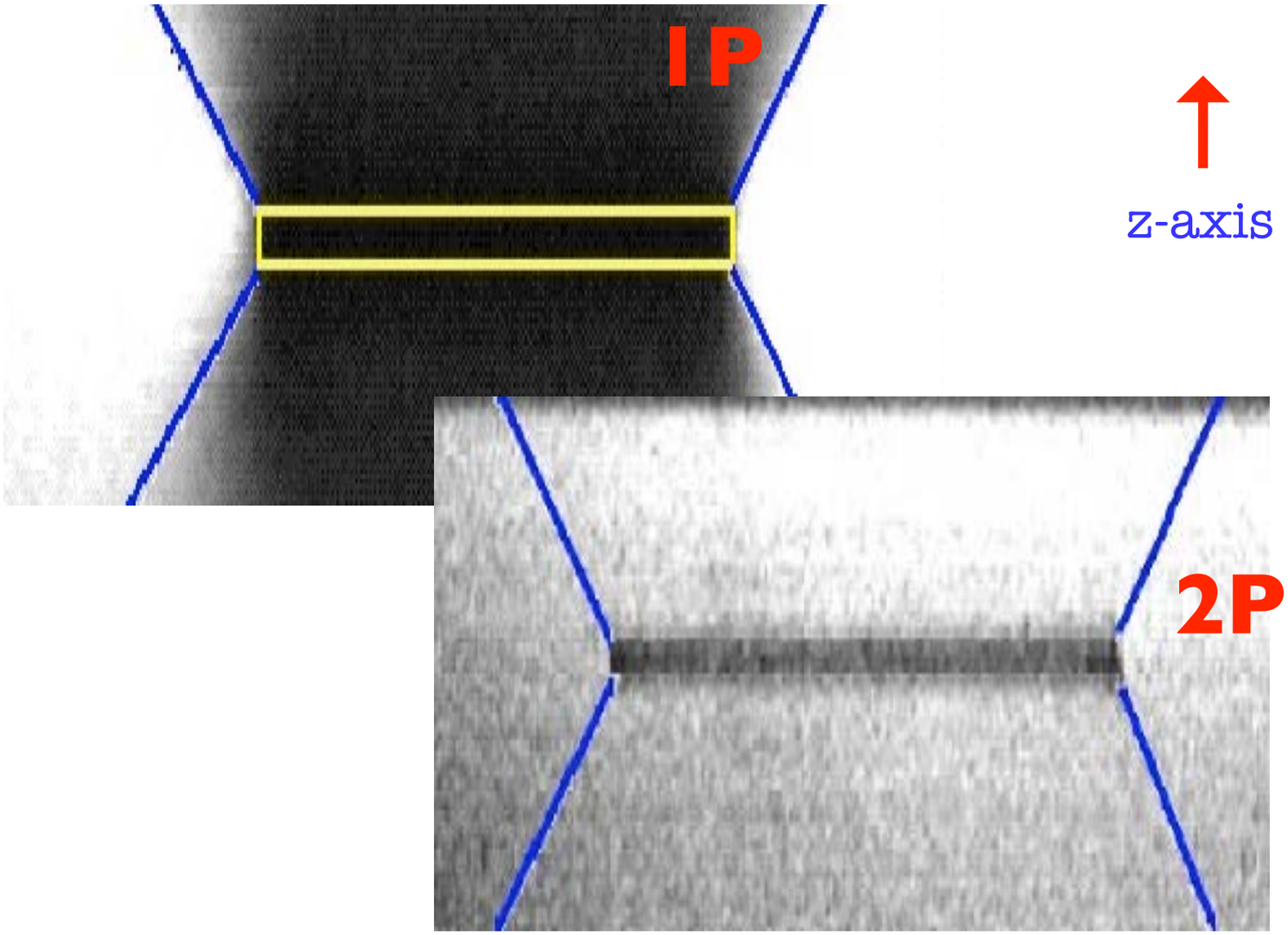
Slide credit:Brad Amos, MRC, Cambridge, UK

two-photon excitation (2pe) microscopy



M.D.Cahalan et al. (2002) Nat.Rev.Immunol., 2: 872

two-photon excitation (2pe) microscopy



Slide credit:David Piston, Vanderbilt University

two-photon excitation (2pe) microscopy

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<P>= average incident power ~ 20 mW

Denk, W., Strickler, J.H. & Webb, W.W. *Science* **248**, 73–76 (1990).

two-photon excitation (2pe) microscopy

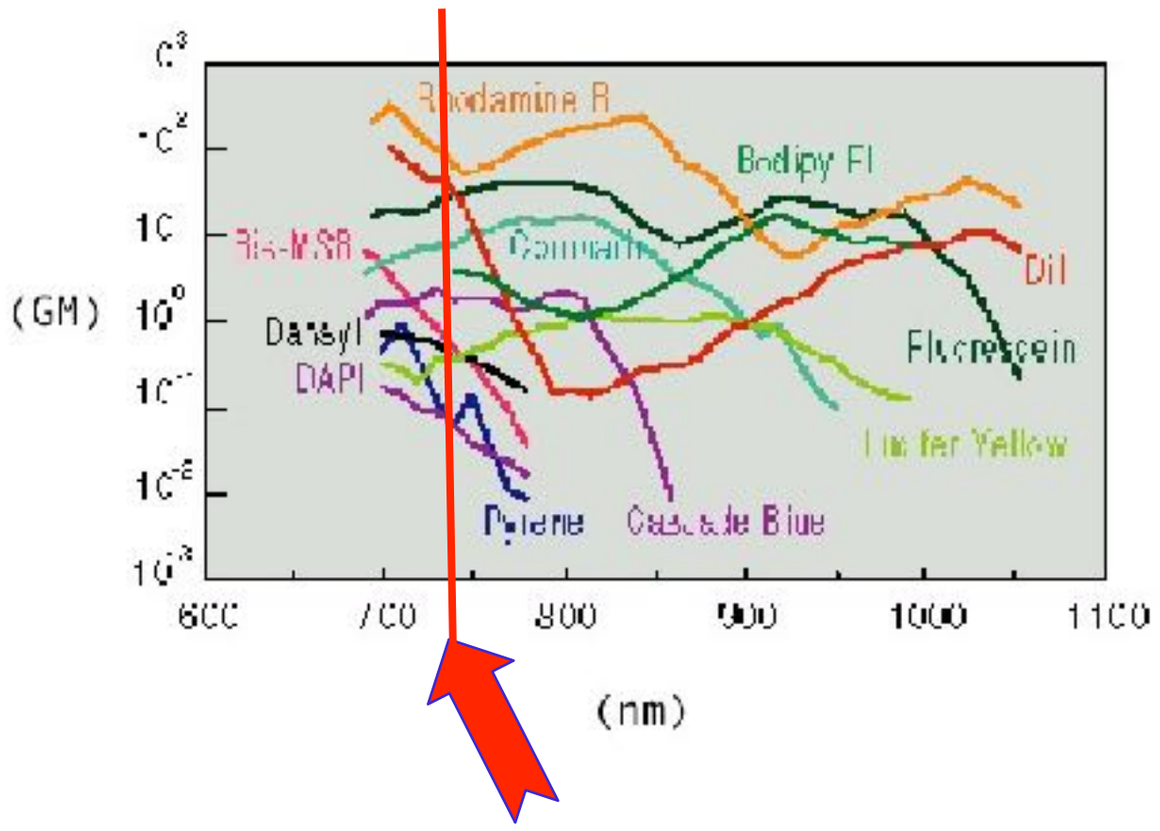
Order of magnitude estimation of MPE cross sections. j,k,l are the intermediate states. τ_j, τ_k, and τ_l represent the corresponding lifetimes of the intermediate states.

$\delta_{1p} = 10^{-16}\ cm^2$

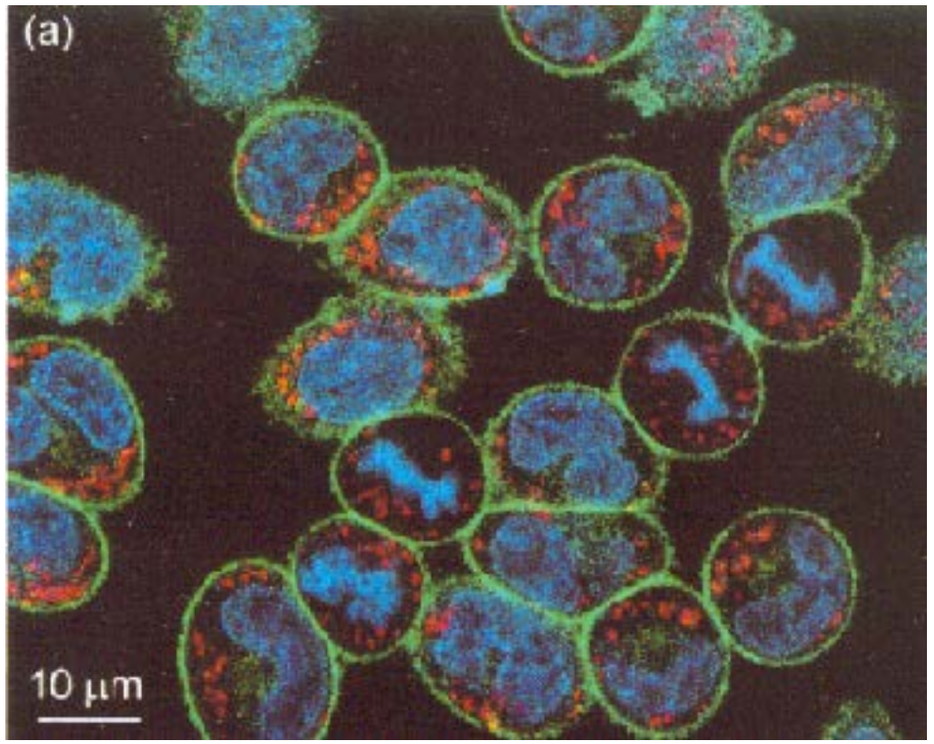
*n*PE	approximate expression	estimated cross section
2PE	$\sigma_2 = \sigma_{ij}\sigma_{jf}\tau_j$	$10^{-49}\ cm^2\ (photons/cm^2 s)^{-1}$
3PE	$\sigma_3 - \sigma_{ij}\sigma_{jk}\sigma_{kf}\tau_j\tau_k$	$10^{-82}\ cm^2\ (photons/cm^2 s)^{-2}$
4PE	$\sigma_3 = \sigma_{ij}\sigma_{jk}\sigma_{kl}\sigma_{if}\tau_j\tau_k\tau_l$	$10^{-115}\ cm^2\ (photons/cm^2 s)^{-3}$

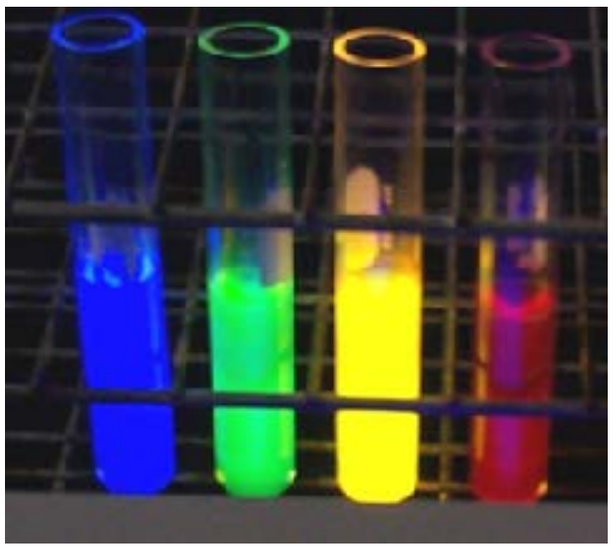
Table credit: Chris Xu

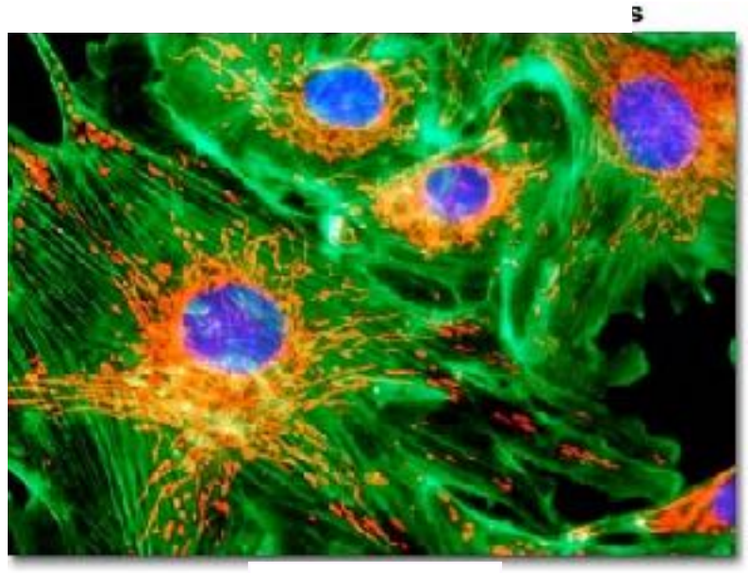
two-photon excitation (2pe) microscopy

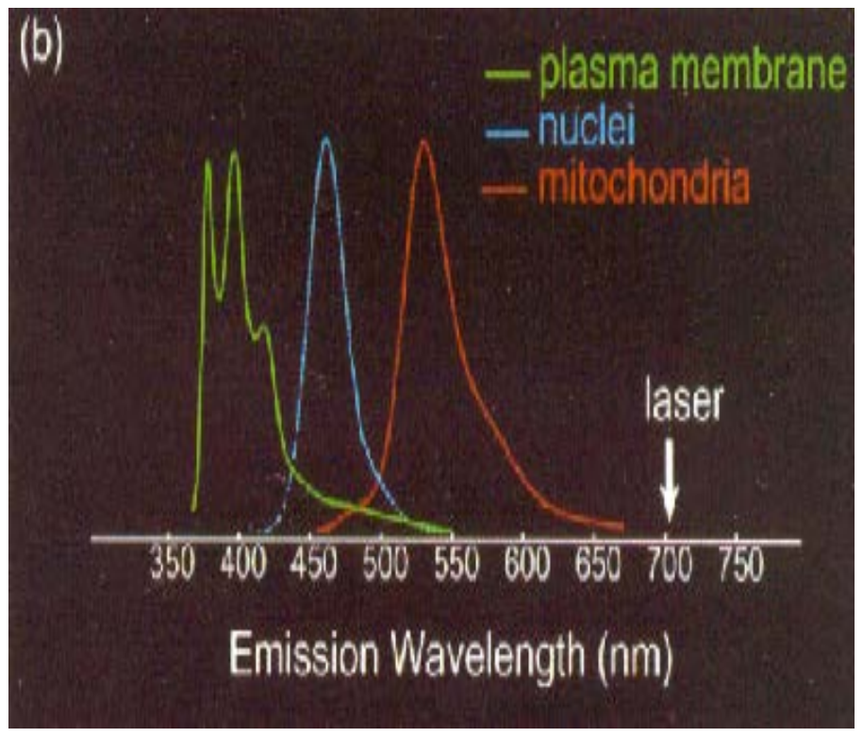


MULTIPLE FLUORESCENCE BY ONE WAVELENGTH EXCITATION





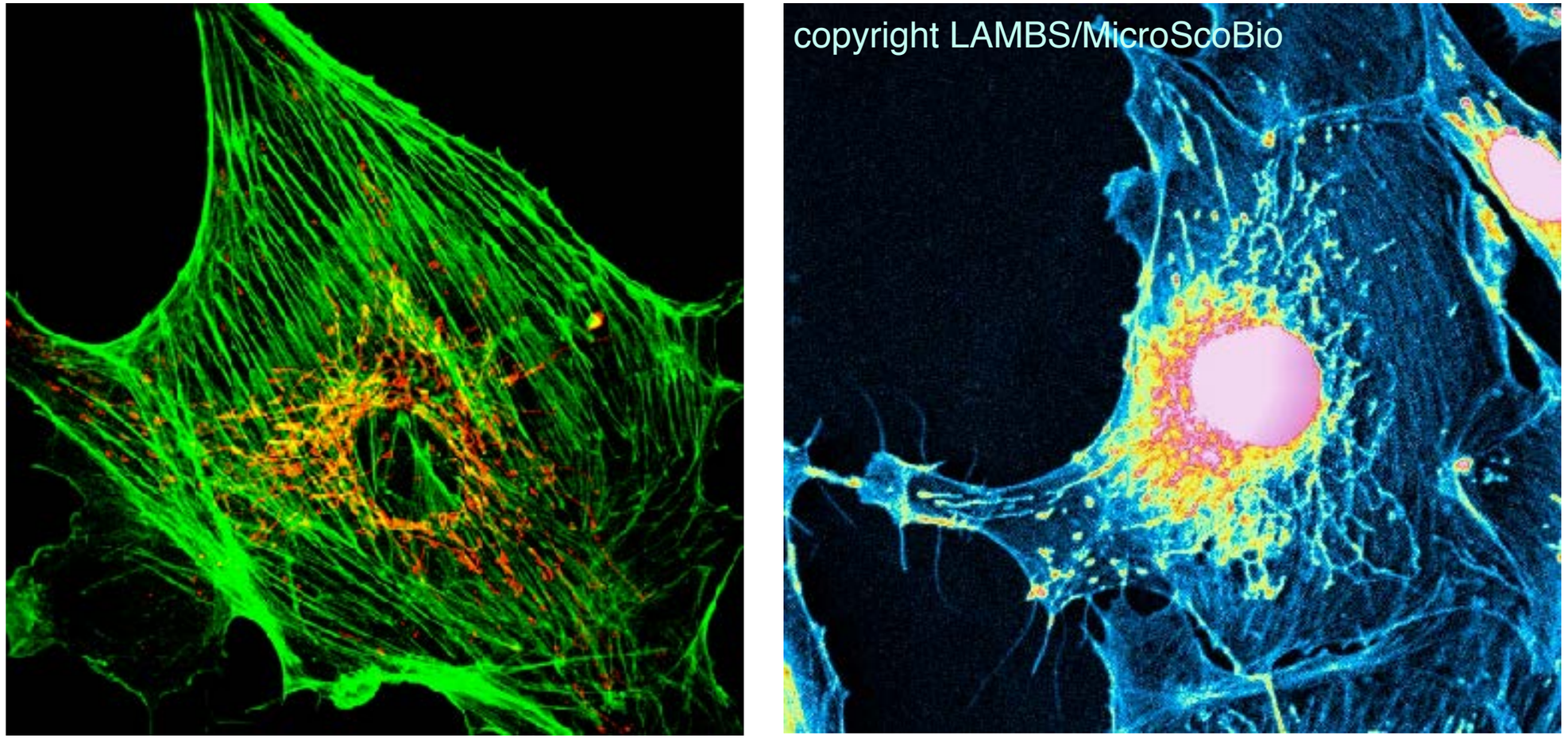




Xu C. et al., Bioimaging, 4, 198-207, 1996

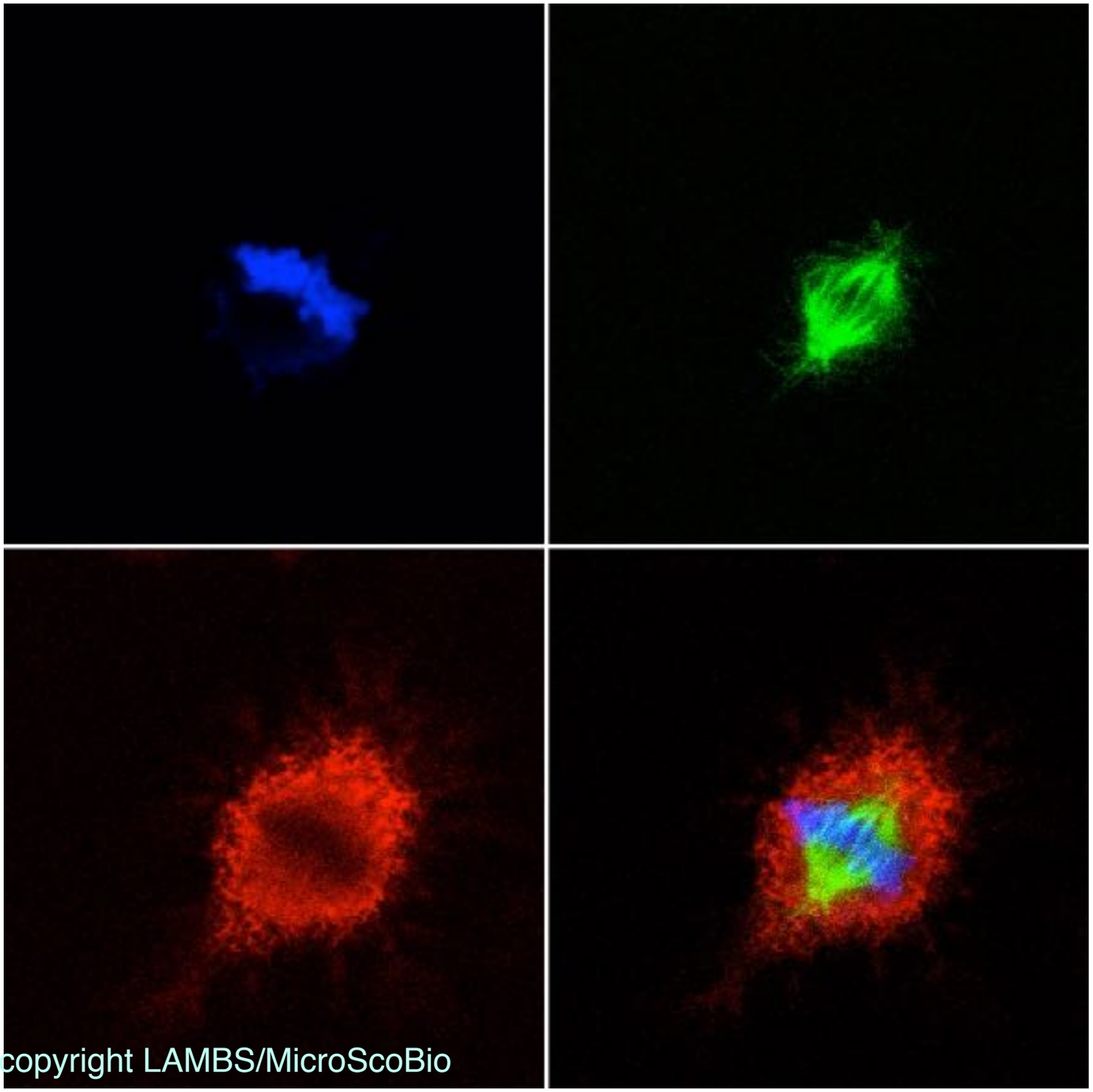
two-photon excitation (2pe) microscopy

Confocal (left) and TPE (right) multiple fluorescence.



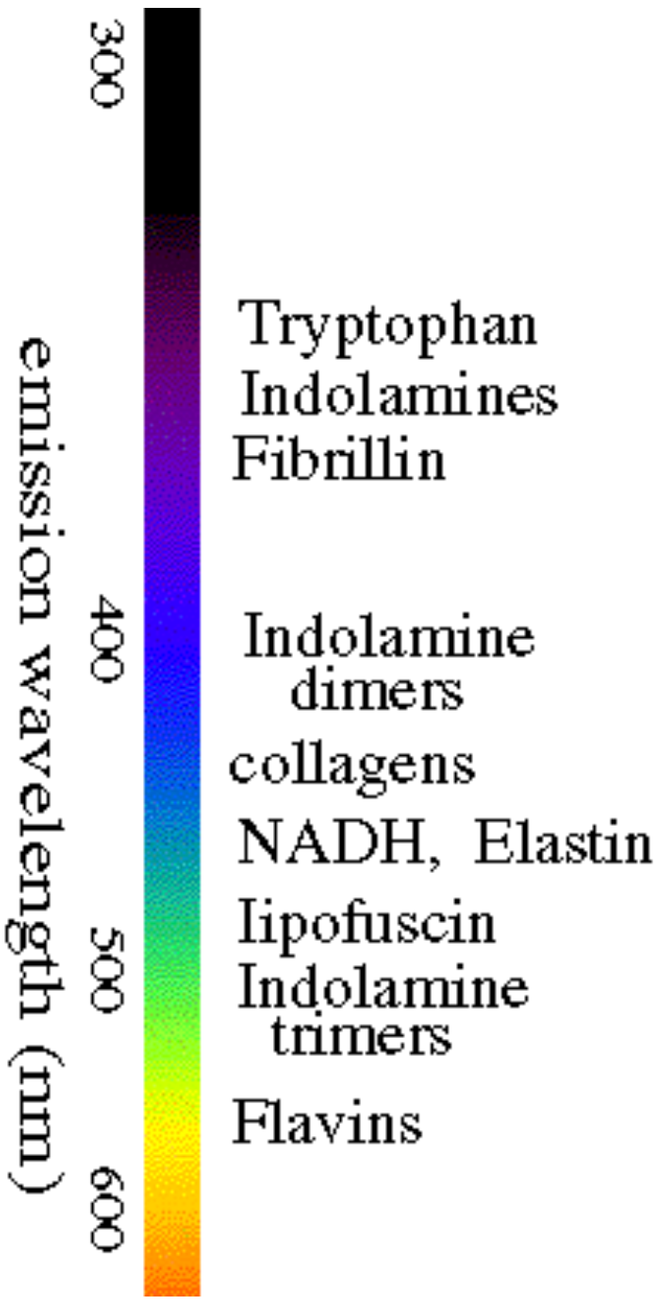
Cells are labelled with DAPI (DNA), mitotracker red (mithocondria) and bodipy (actin); F-14780 Molecular Probes slide.

two-photon excitation (2pe) microscopy



two-photon excitation (2pe) microscopy

INTRINSIC EMITTERS	λ (nm)	δ_2
GFP wt	800-850	~ 6
GFP S65T	~ 960	~ 7
BFP	780/820	-
CFP	780/840	-
YFP	860/900	-
EGFP	940-1000	~ 250
DsRed-Coral Red	960-990	~ 20-110
Citrine-Coral yellow	950	~ 70
Phycoerythrin	1064	~ 300
Flavins	~ 700-730	~ 0.1-0.8
NADH	~ 690-730	~ 0.02-0.09
Retinol	700-830	~0.07
Pyridoxine	690-710	~0.008
Folic acid	700-770	~0.007
Lipofuscin	700-850	-
Collagen, Elastin	700-740	-
Qdots	700-1000	~ 2000-47000

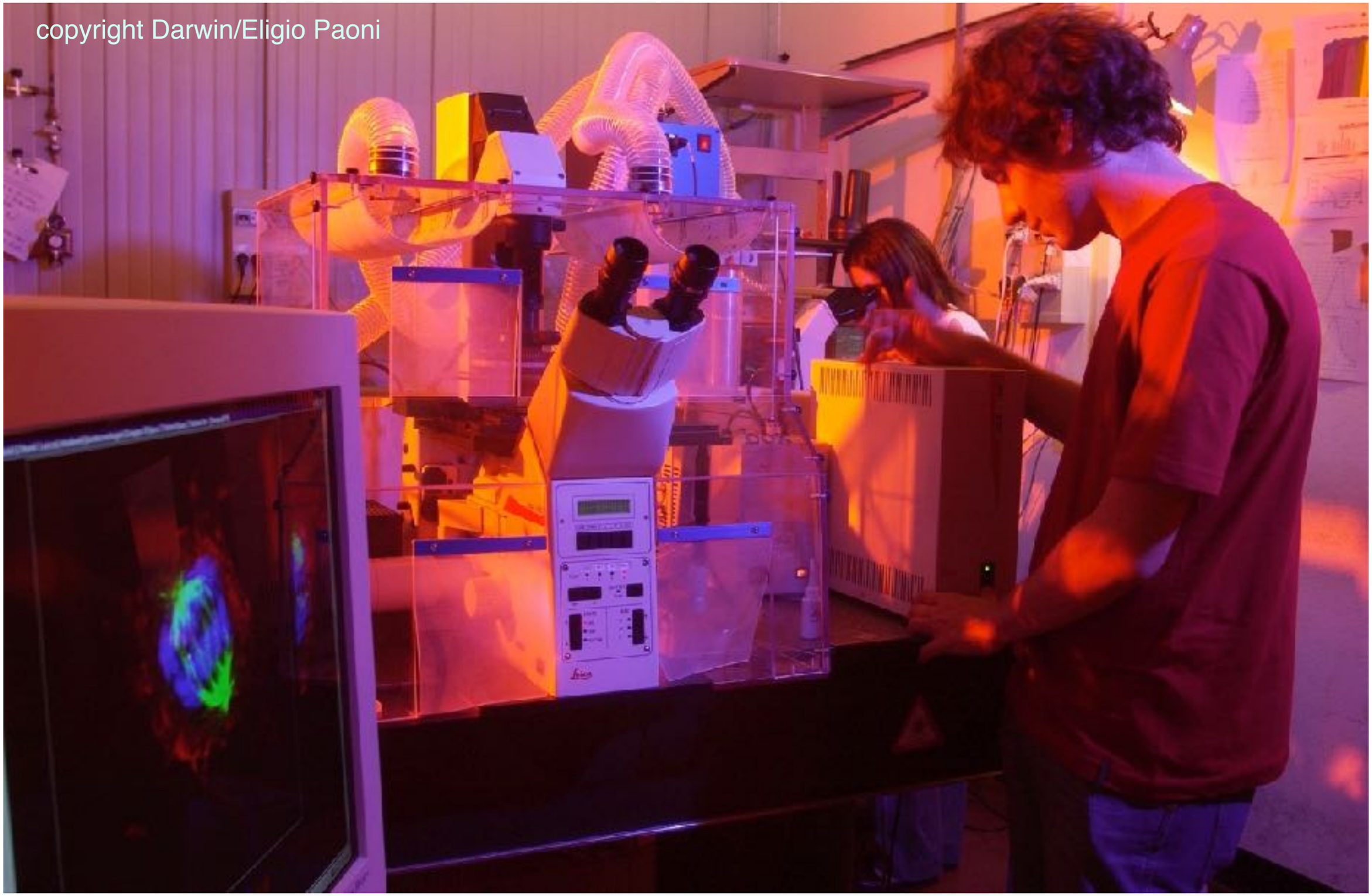


http://www.biomedical-engineering-online.com/home/

A. Diaspro et al. , Multi-photon excitation microscopy.BioMedical Engineering OnLine 2006, 5:36 (6Jun2006)

3D sequence collected at LAMBS, sample courtesy of Paola Ramoino, DIPTERIS

A. Diaspro et al. , Multi-photon excitation microscopy.BioMedical Engineering OnLine 2006, 5:36


copyright Darwin/Eligio Paoni

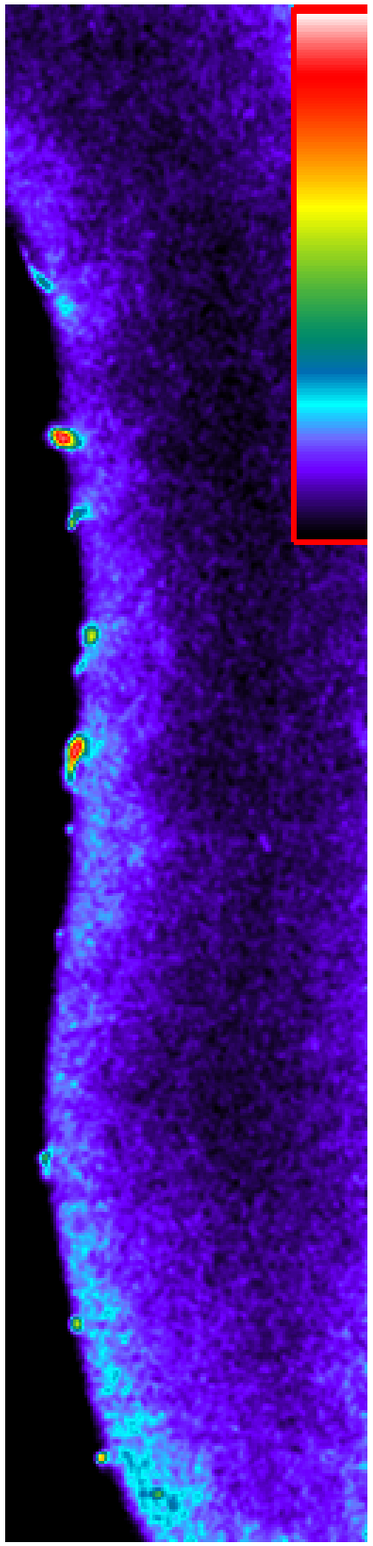
spectral separation

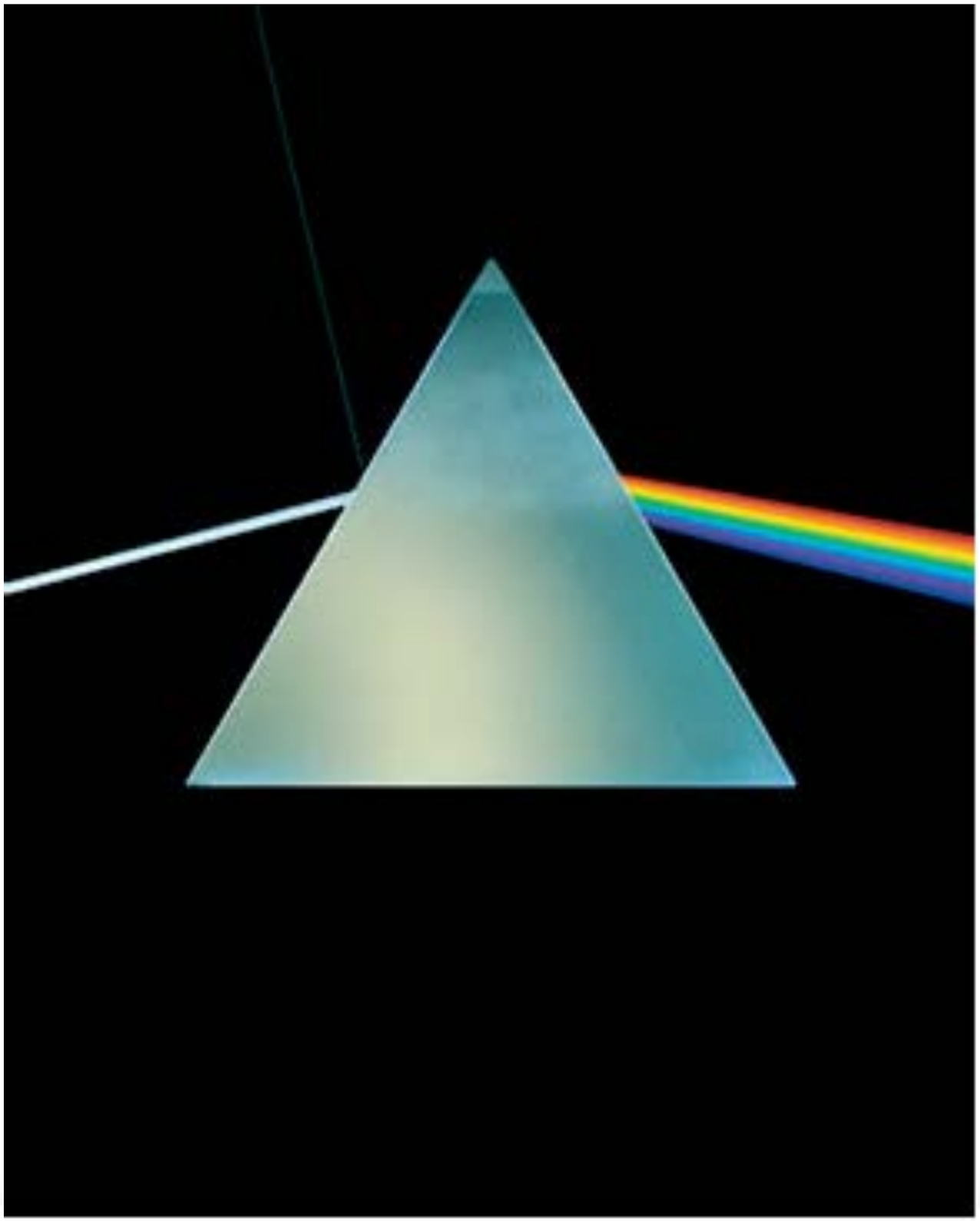
"There were 5 characters…6 considering …ice" (D.Pennac, La Fata Carabina)

Burano, da "laRepubblica.it"

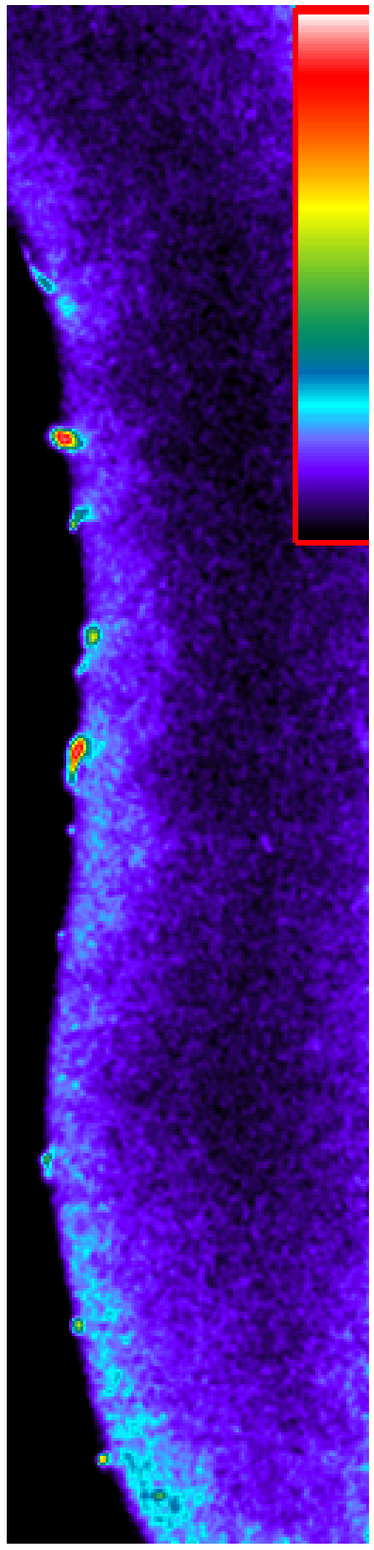
Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

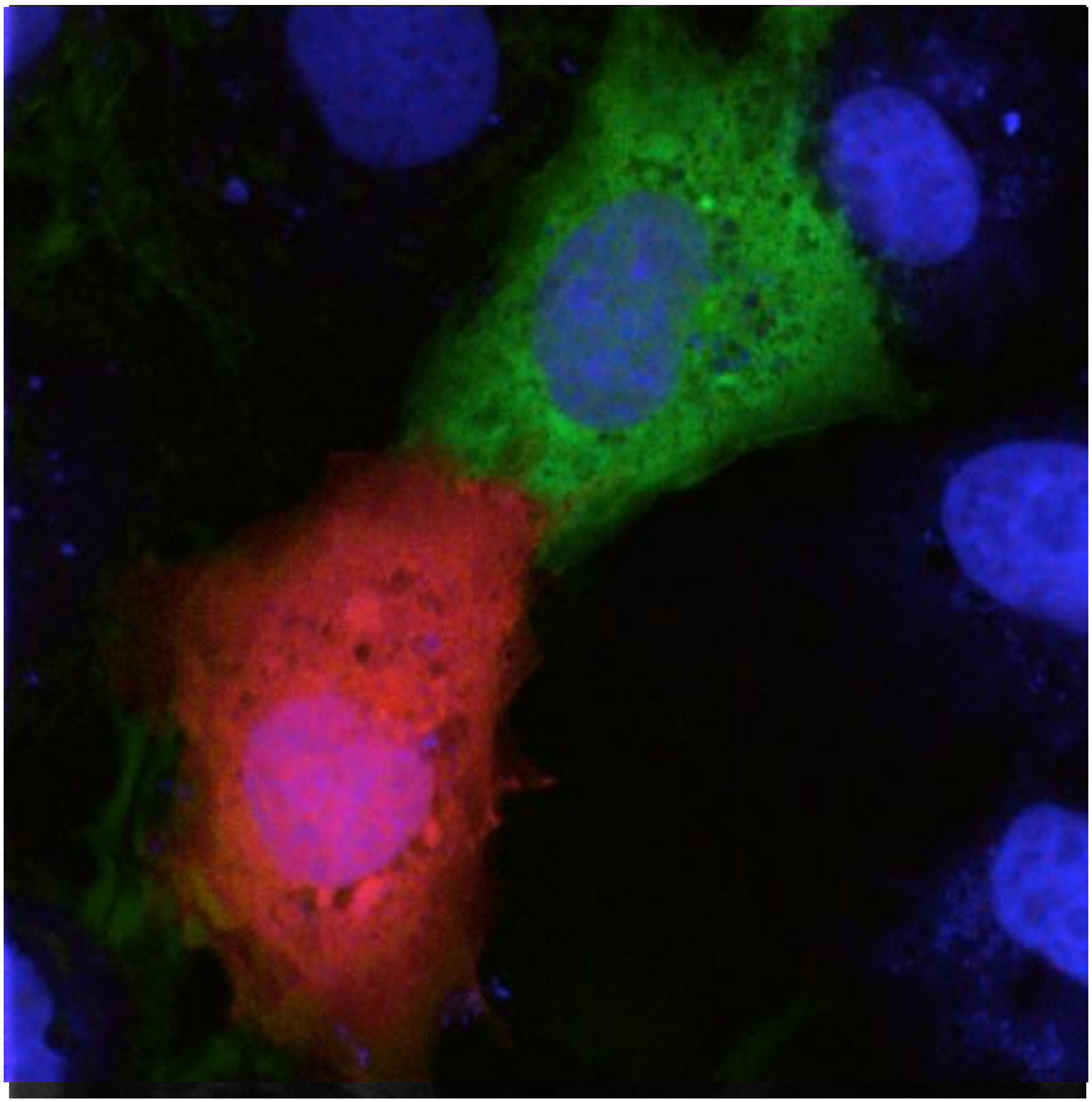
spectral separation



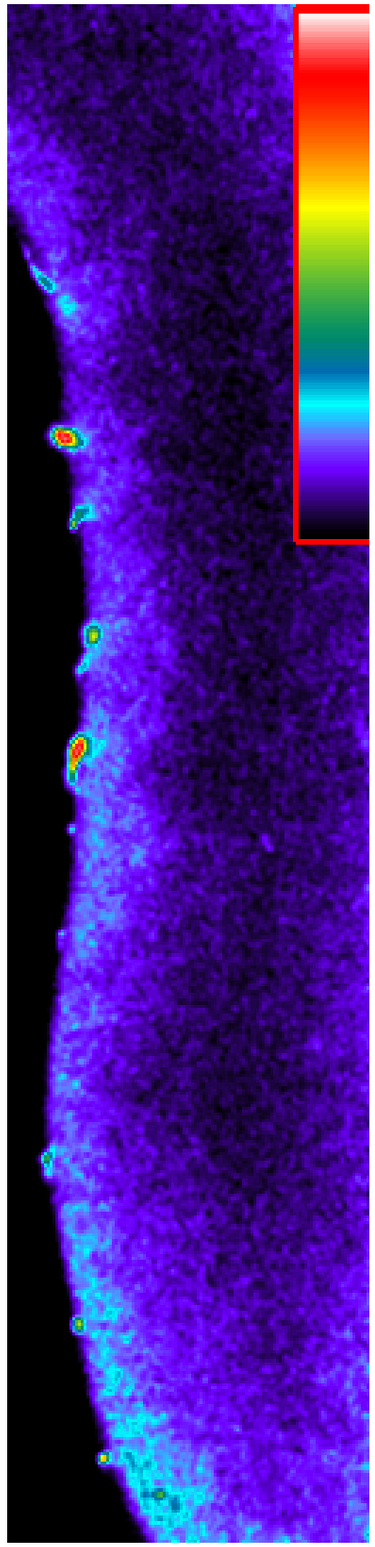


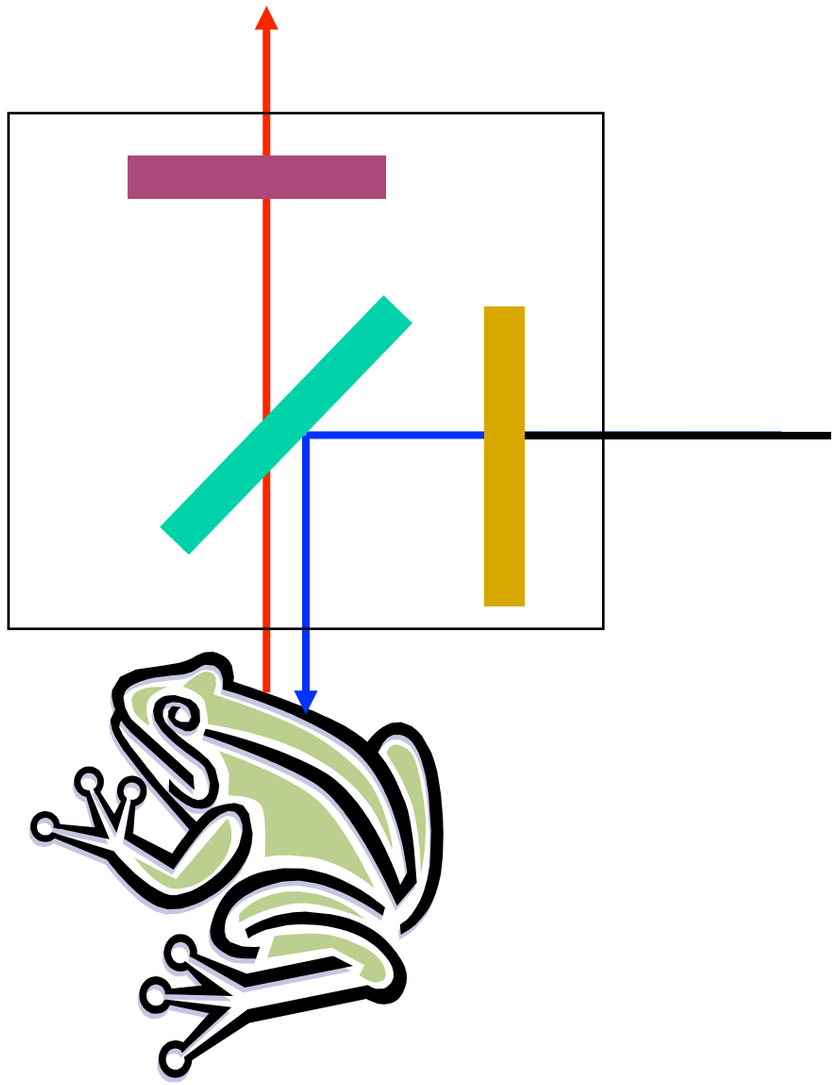
spectral separation

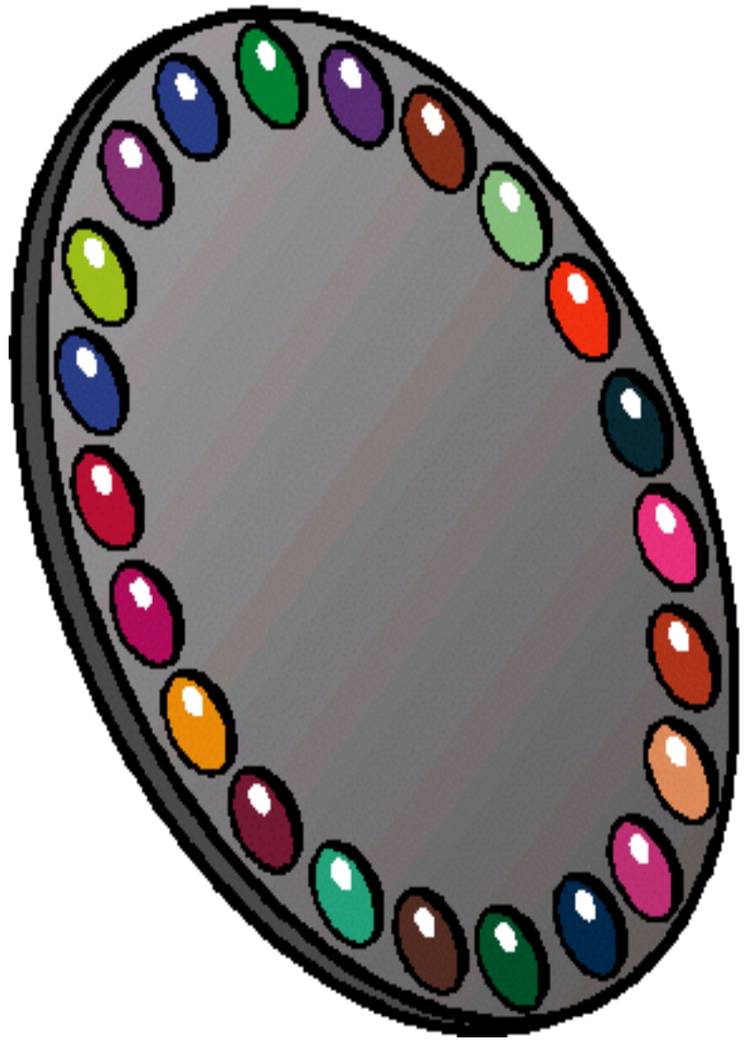




spectral separation

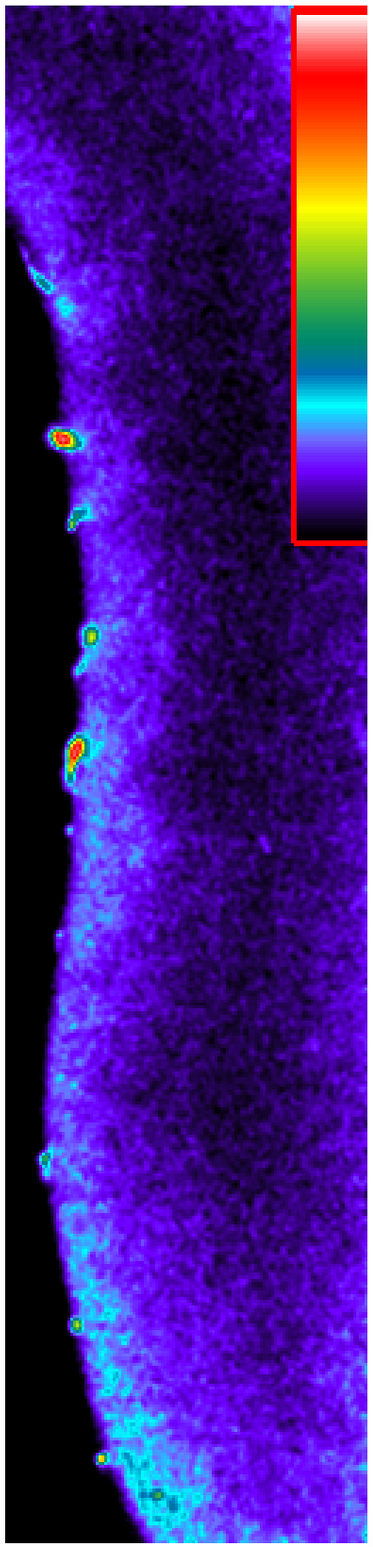


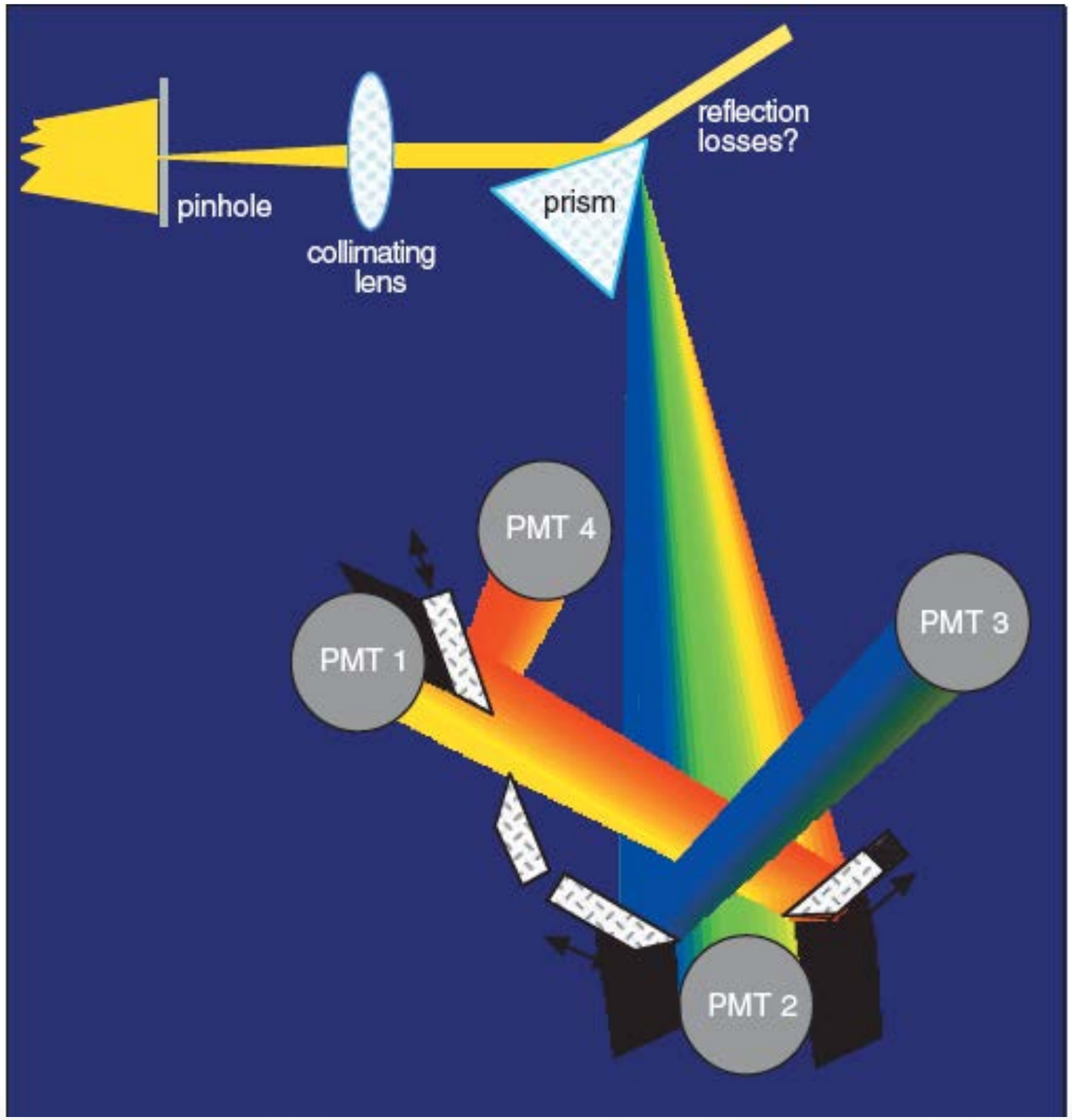




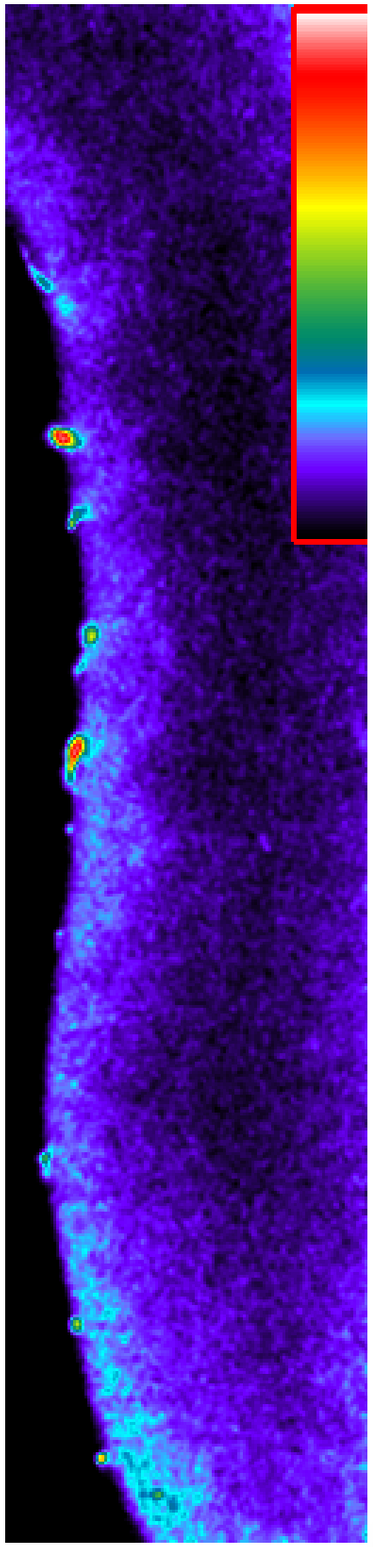
Slide credit: Rolf Borlinghaus, Leica Microsystems

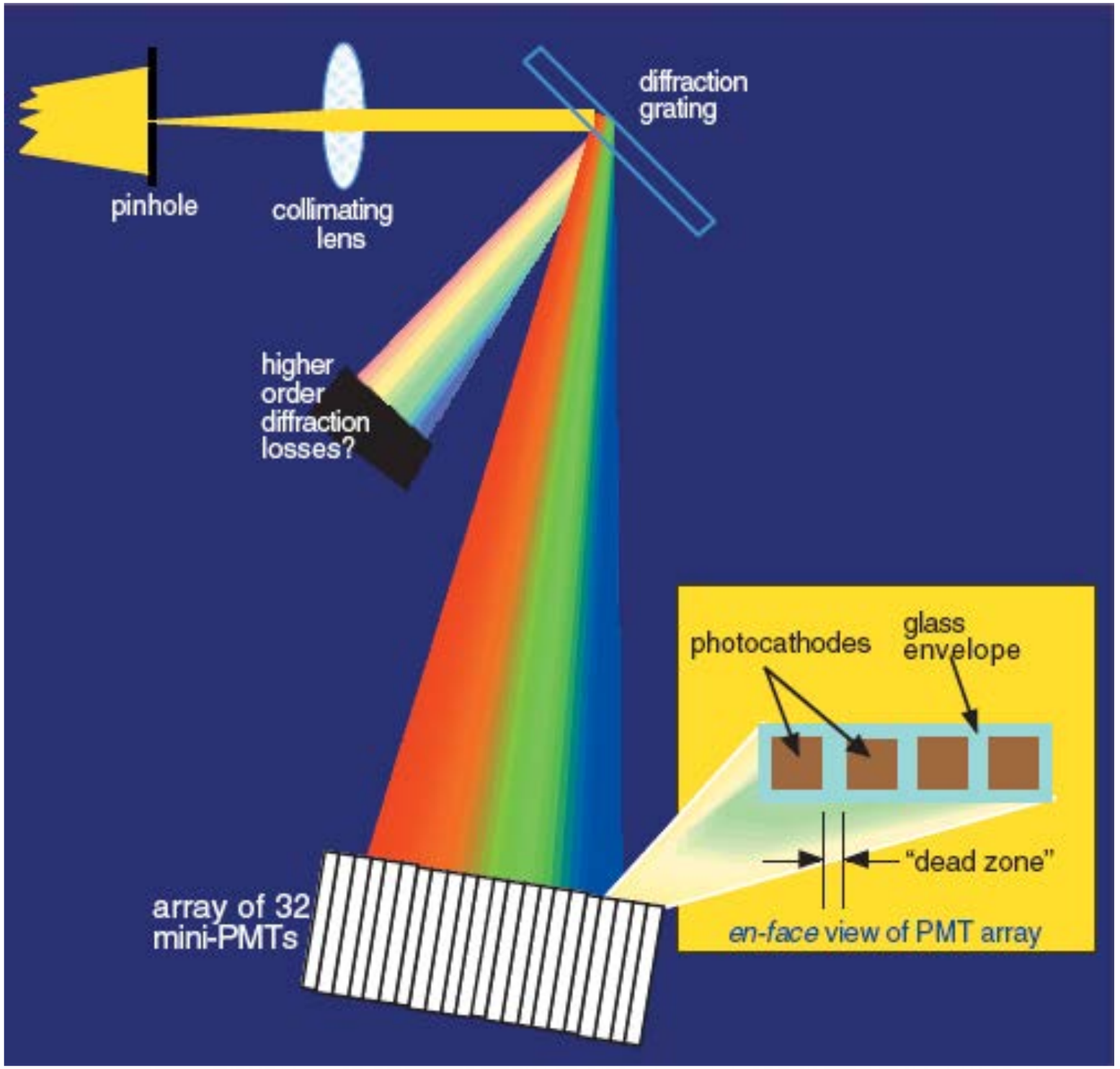
spectral separation





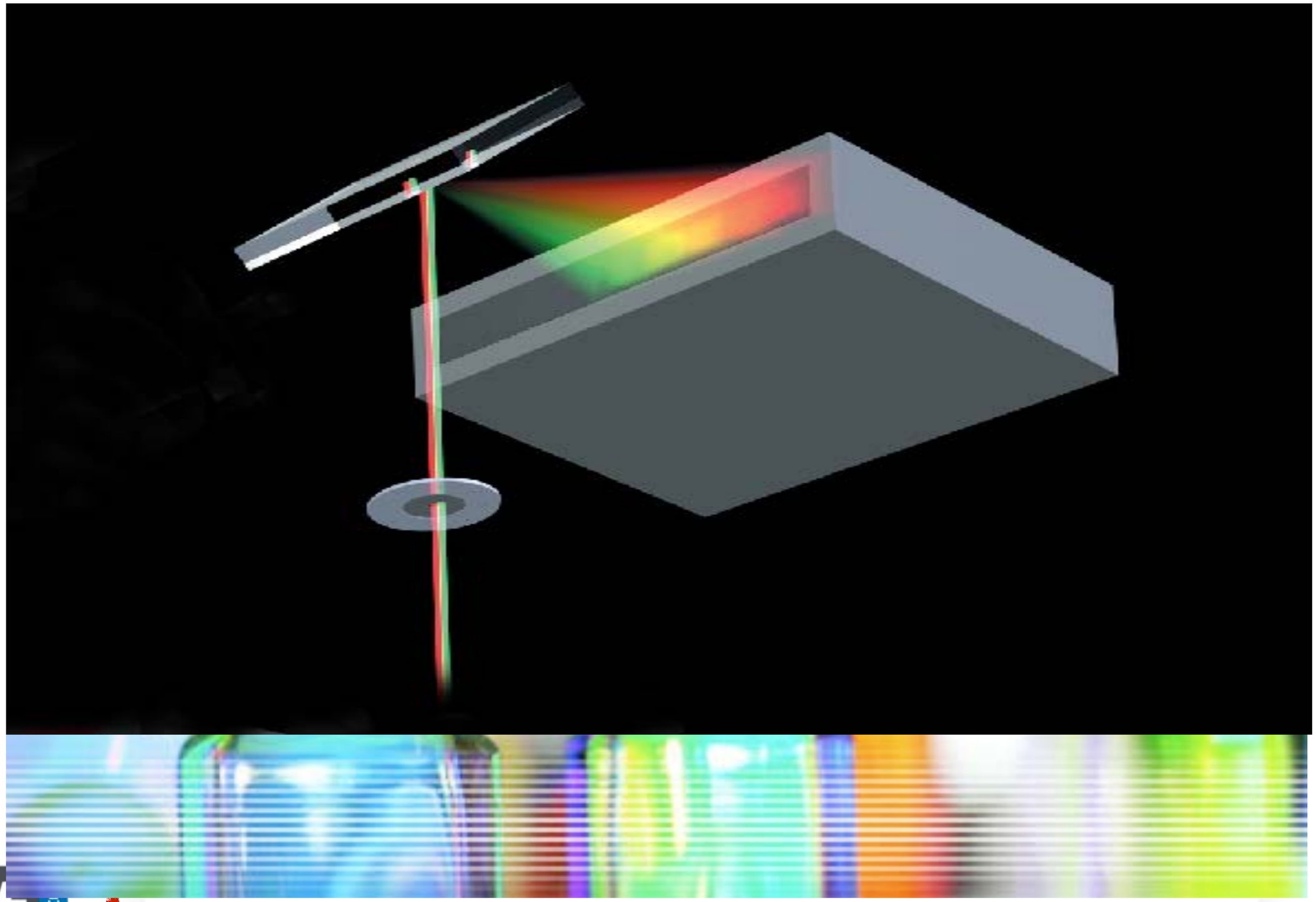
spectral separation



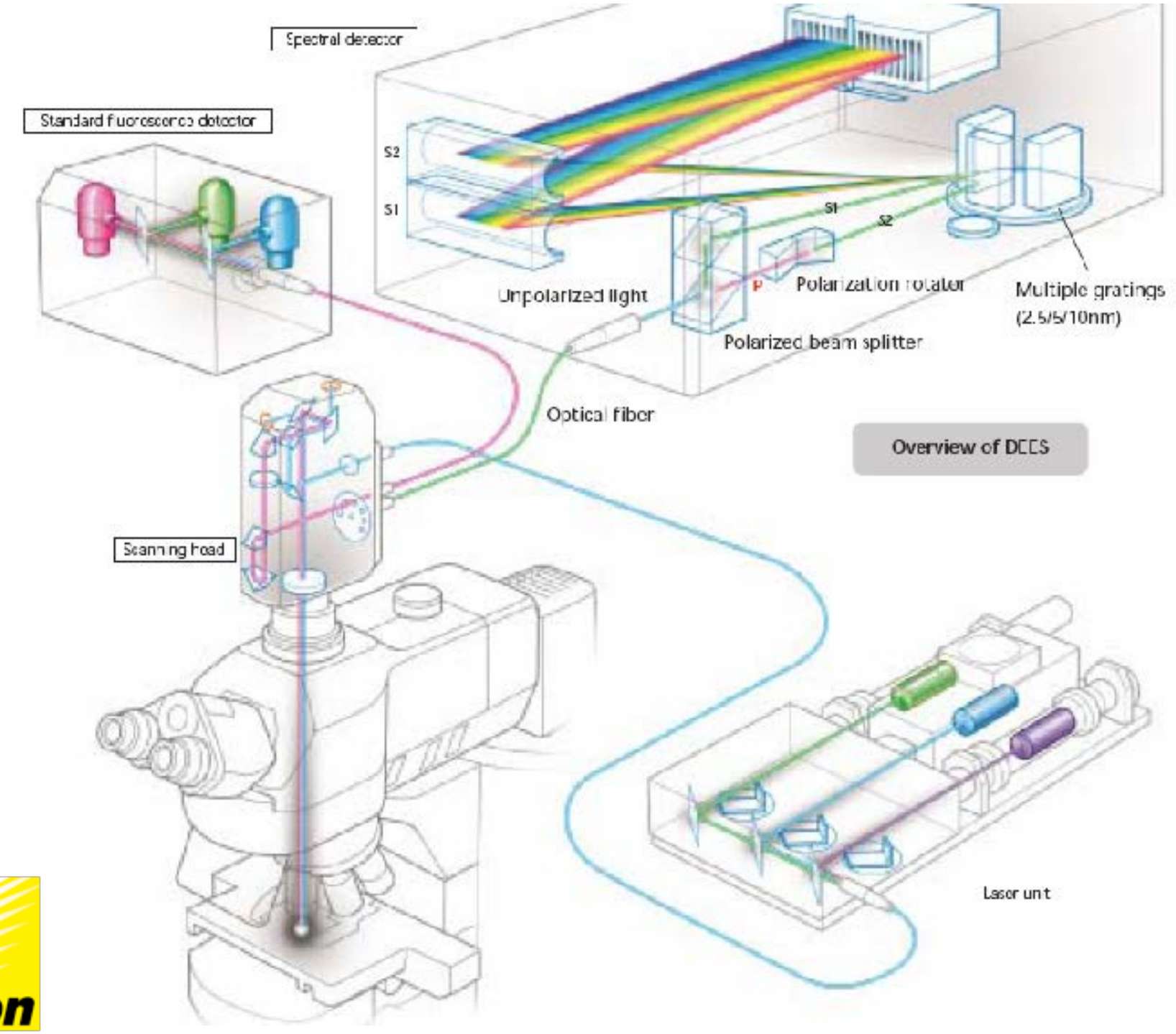


spectral separation

LSM 510 META

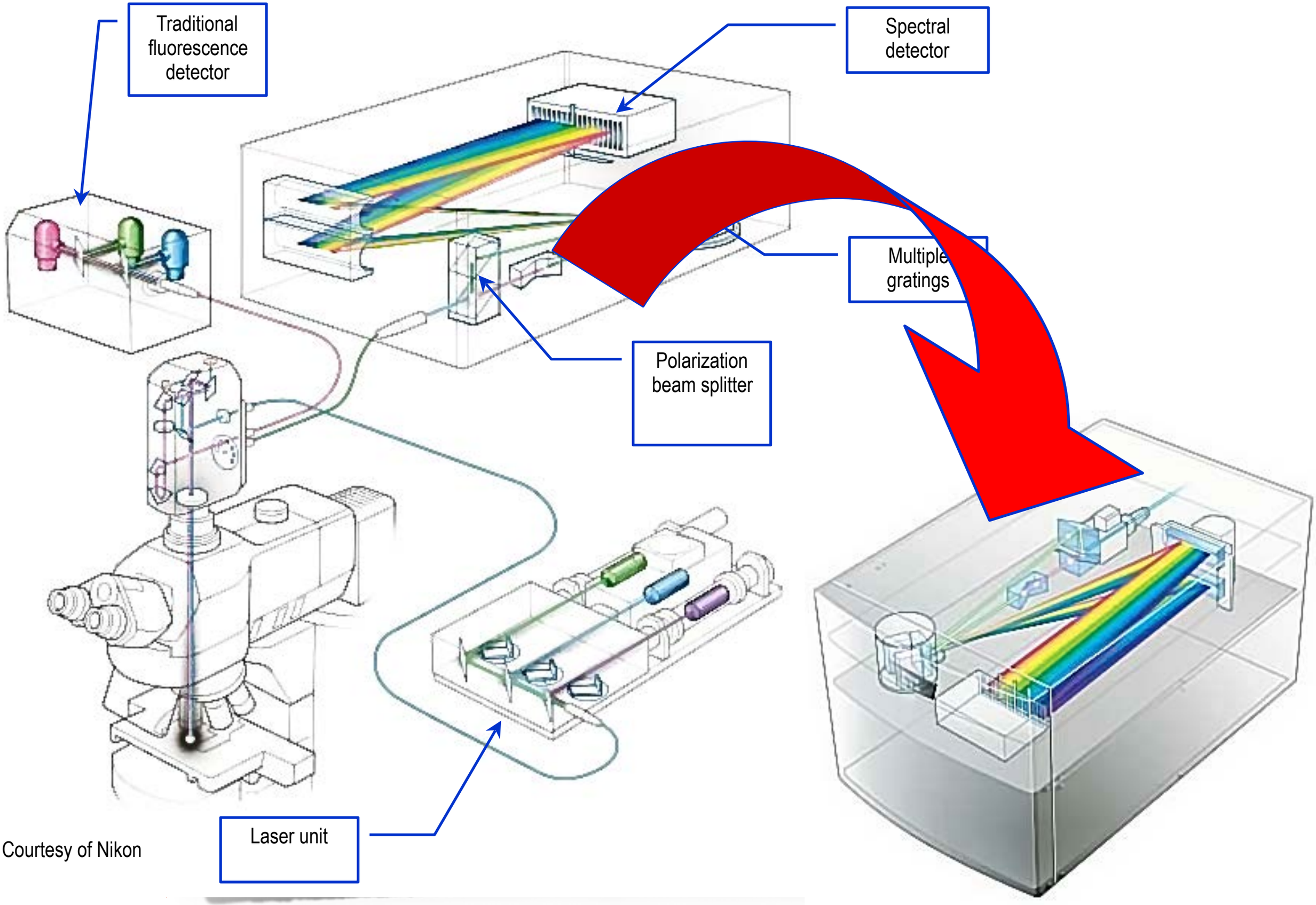


spectral separation



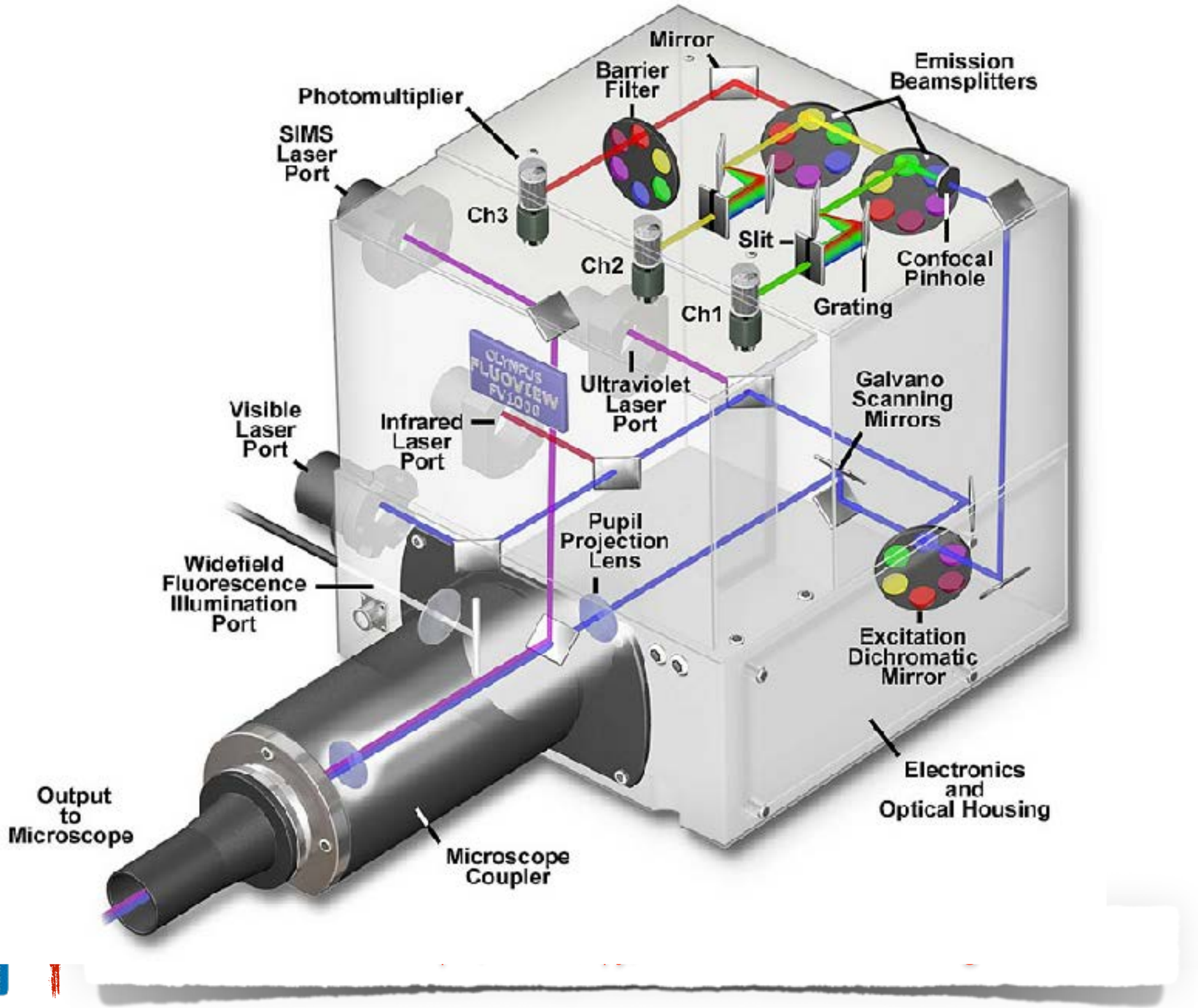
(credit:Nikon)

Spectral confocal systems

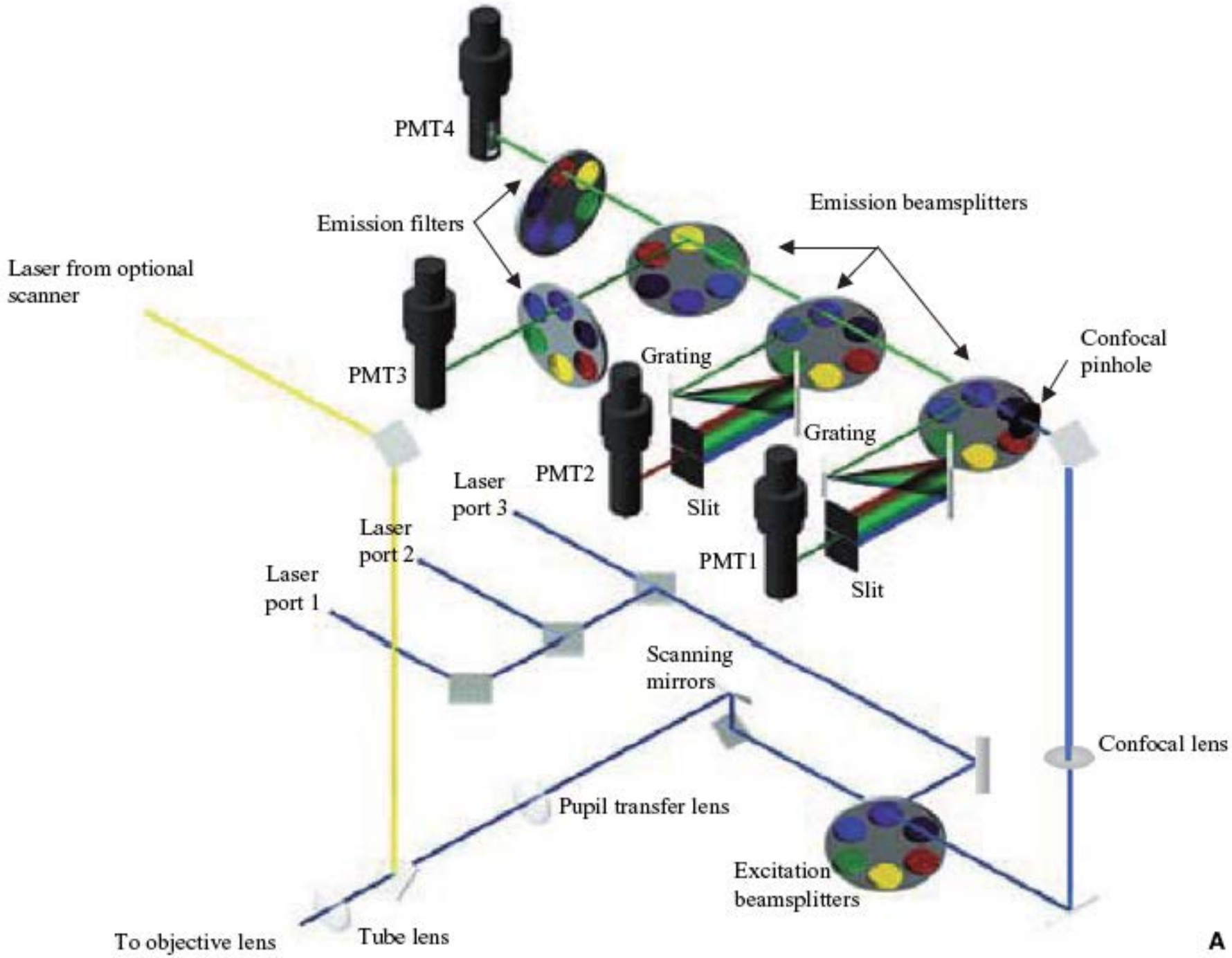


Courtesy of Nikon

Olympus spectral confocal system

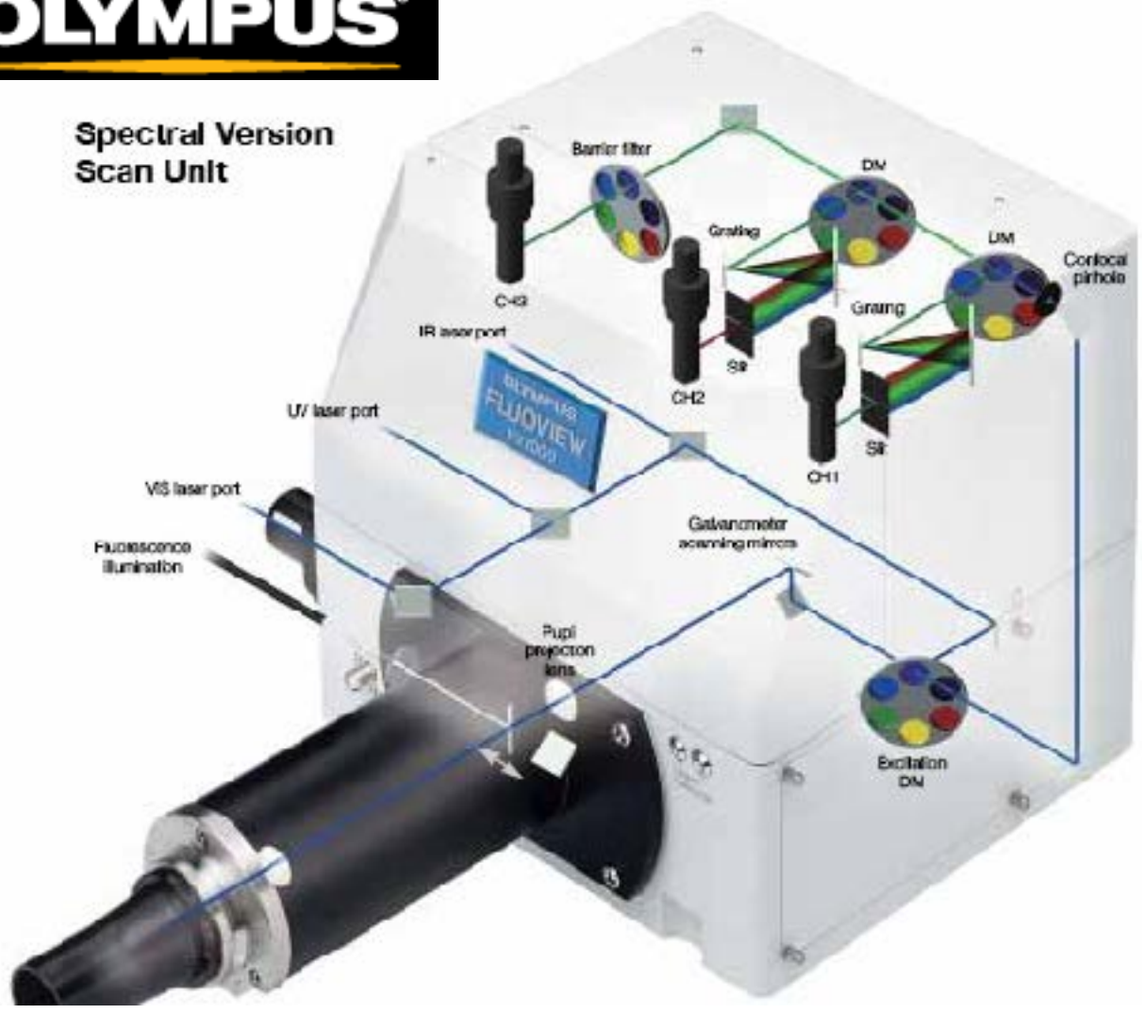


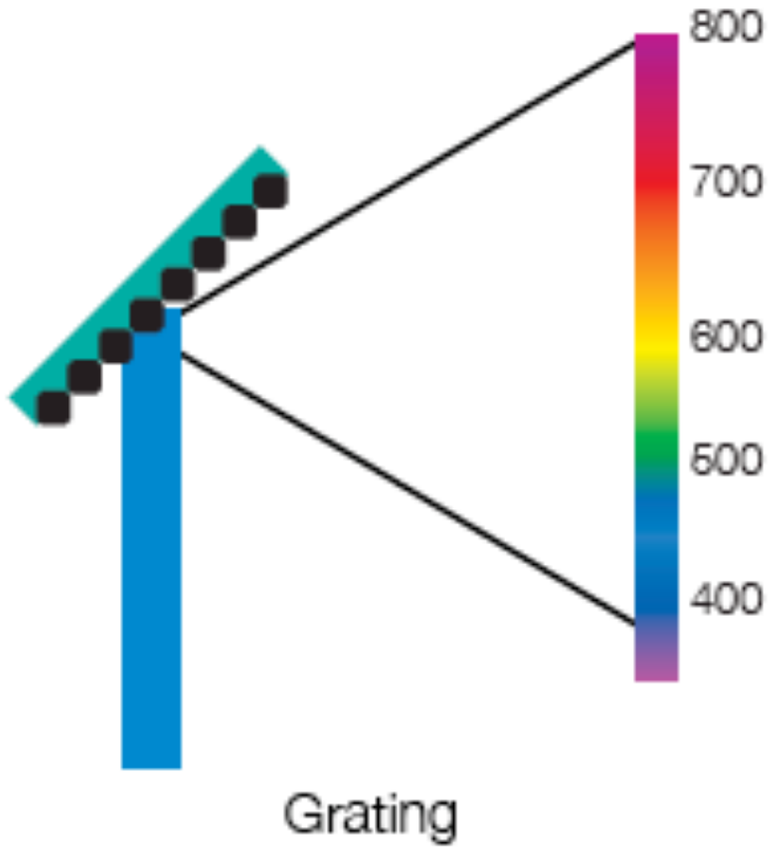
spectral separation



Slide credit: Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

spectral separation

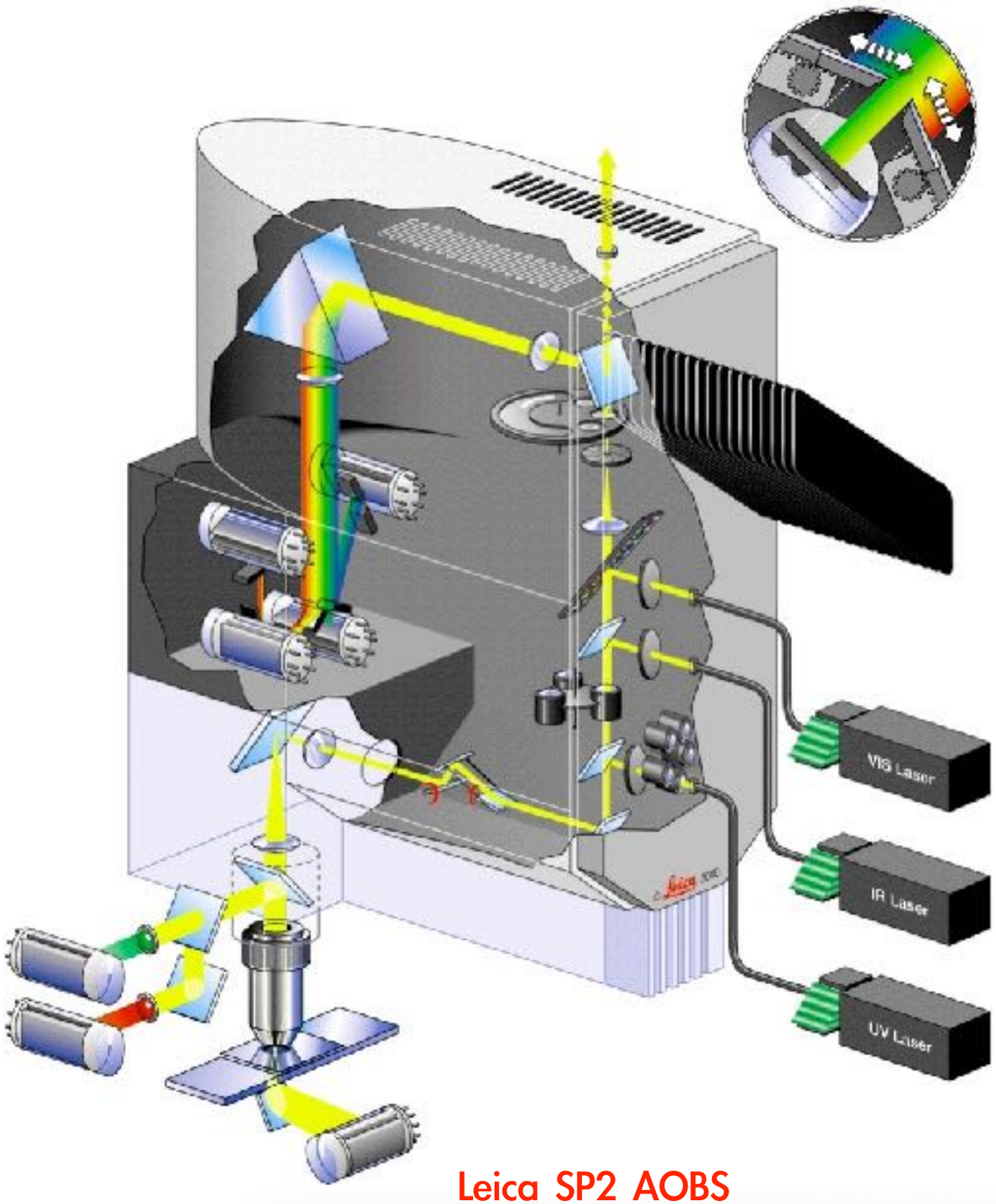




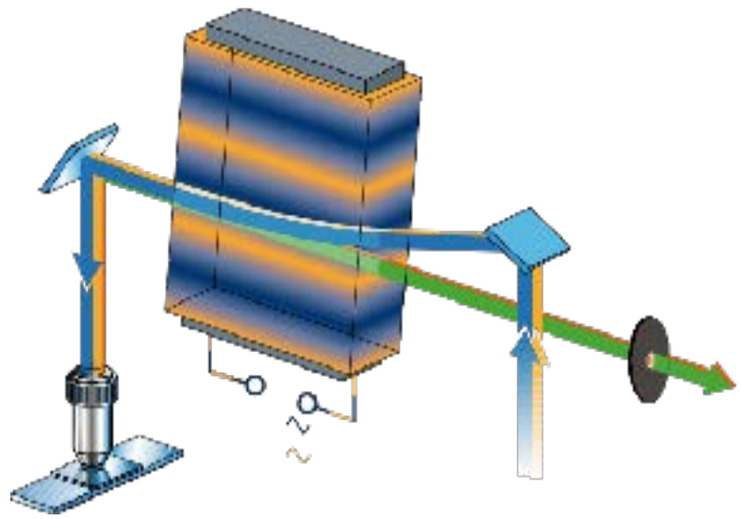
XYλ series, wavelength range 495-695nm

495nm – 515nm | 565nm – 585nm | 575nm – 595nm | 595nm – 615nm | 675nm – 695nm

spectral separation

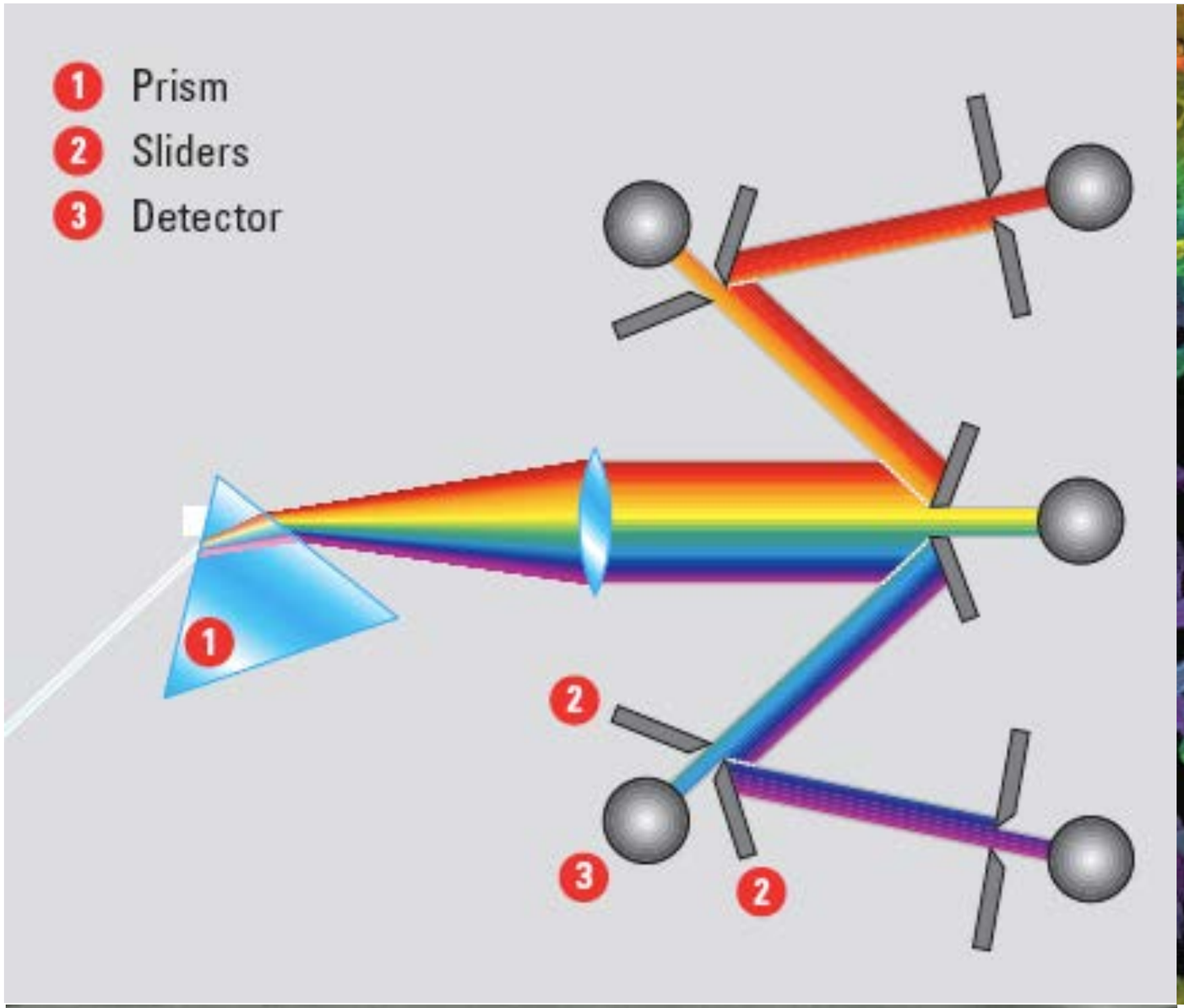


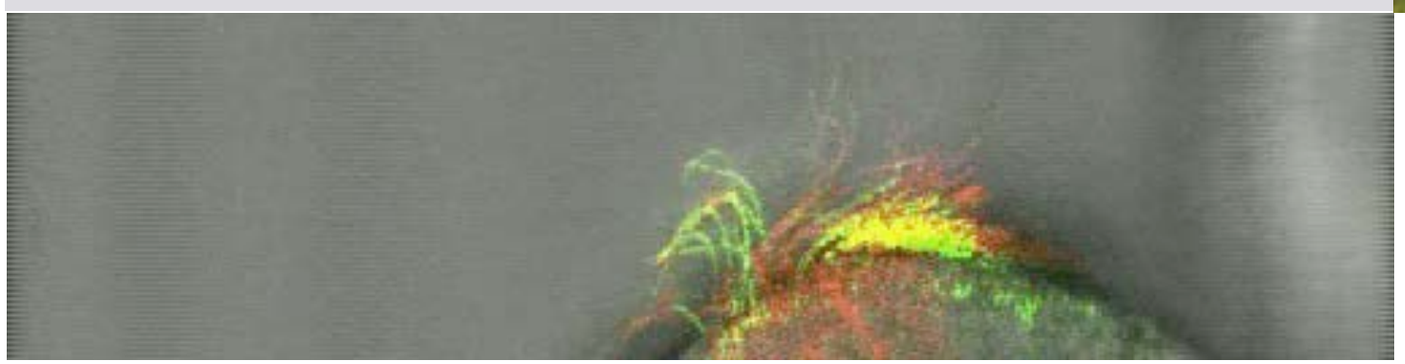
Leica SP2 AOBS



(credit: Leica microsystems)

spectral separation





Larval state of the marine polychaete Platynereis

TCS SP5 resonant scanner 71 Hz framerate.
Beating cilia in reflected light and DiI-Fluorescence together with transmitted light.

Courtesy of Kristin Tessmar und Detlev Arendt
European Molecular Biology Laboratory (EMBL)
Heidelberg, Germany

spectral separation

Cy2, exc 491 nm, emis 499 – 505 nm,
NMDA receptors

Lp, exc 474 nm, 509 – 514 nm,
beads in vacuols

Acridine Orange, exc 484 nm,
emis 520 – 532 nm, nucleus

Cy3, exc 551 nm, emis 555 – 575 nm,
Gaba-B-R1 receptors

TexasRed, exc 562 nm, emis 605 – 612 nm,
vacuoles and endosomes

Alexa 594, exc 594, emis 615 – 625 nm,
nuclear and cellular membrane

Alexa 633, exc 633 nm, emis 655-665 nm,
clathrine vesicles

Cy5, exc 640 nm,
emis 661-675 nm, cilia

fluorescence

FL will provide information related to environmental and boundary conditions.
FRET measurements can be used to monitor protein binding or structural changes.
FRAP measurements can be used to monitor macromolecule diffusion at high spatial localization.
FCS at different time scale provides information on flickering of fluorescent molecules (1-100 micros), diffusion (100 micros-1 ms), and photobleaching (> 1 ms).
FCS allows direct comparison with in vivo condition at single molecule level.
SMD allows single molecule studies.
SHG allows structural studies and does not require any staining.

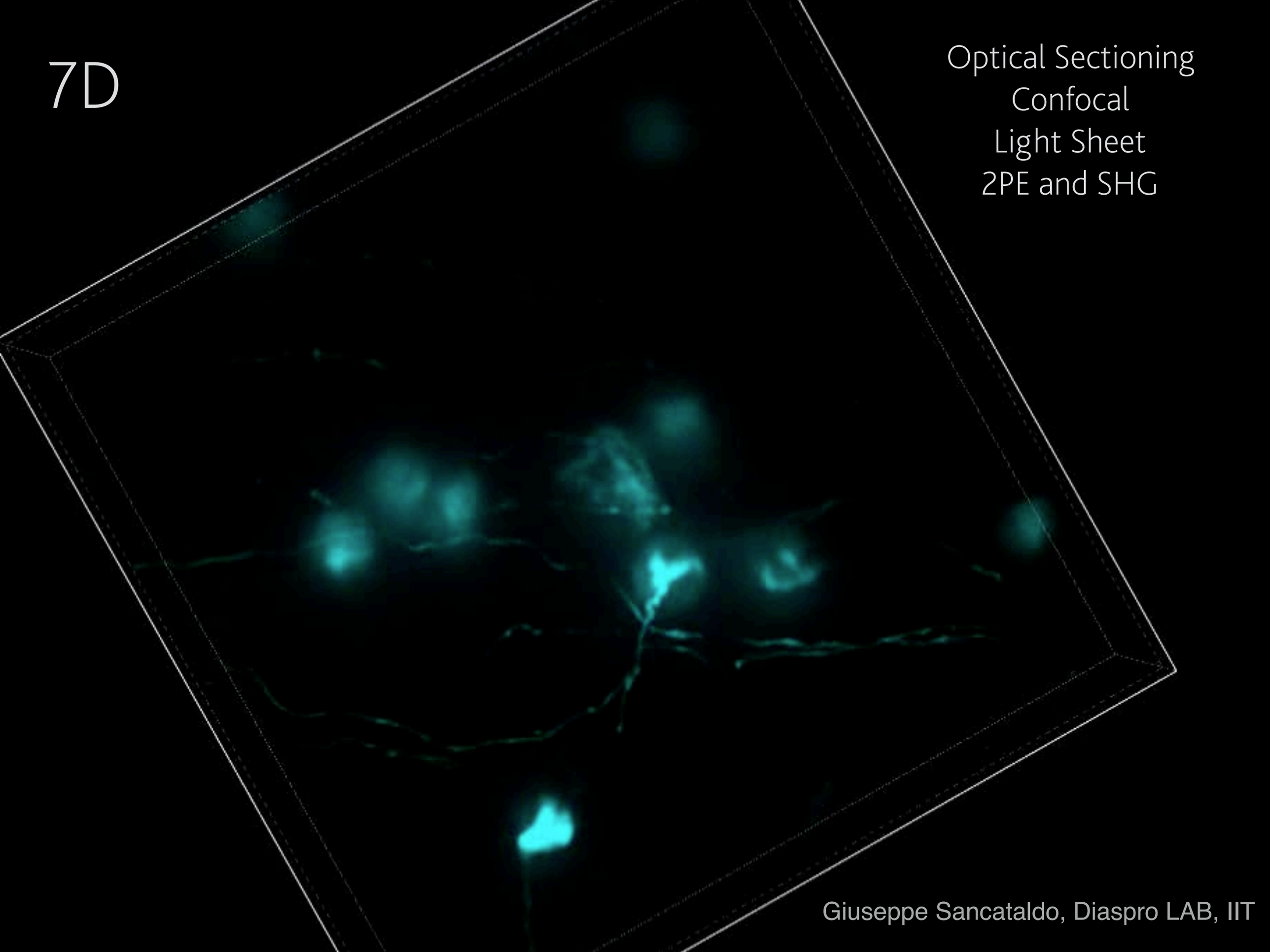
"C'erano 5 personaggi...6 contando la lastra di ghiaccio" (D.Pennac, La Fata Carabina)

7D (x-y-z-t-lambda-tau-chi)

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

7D

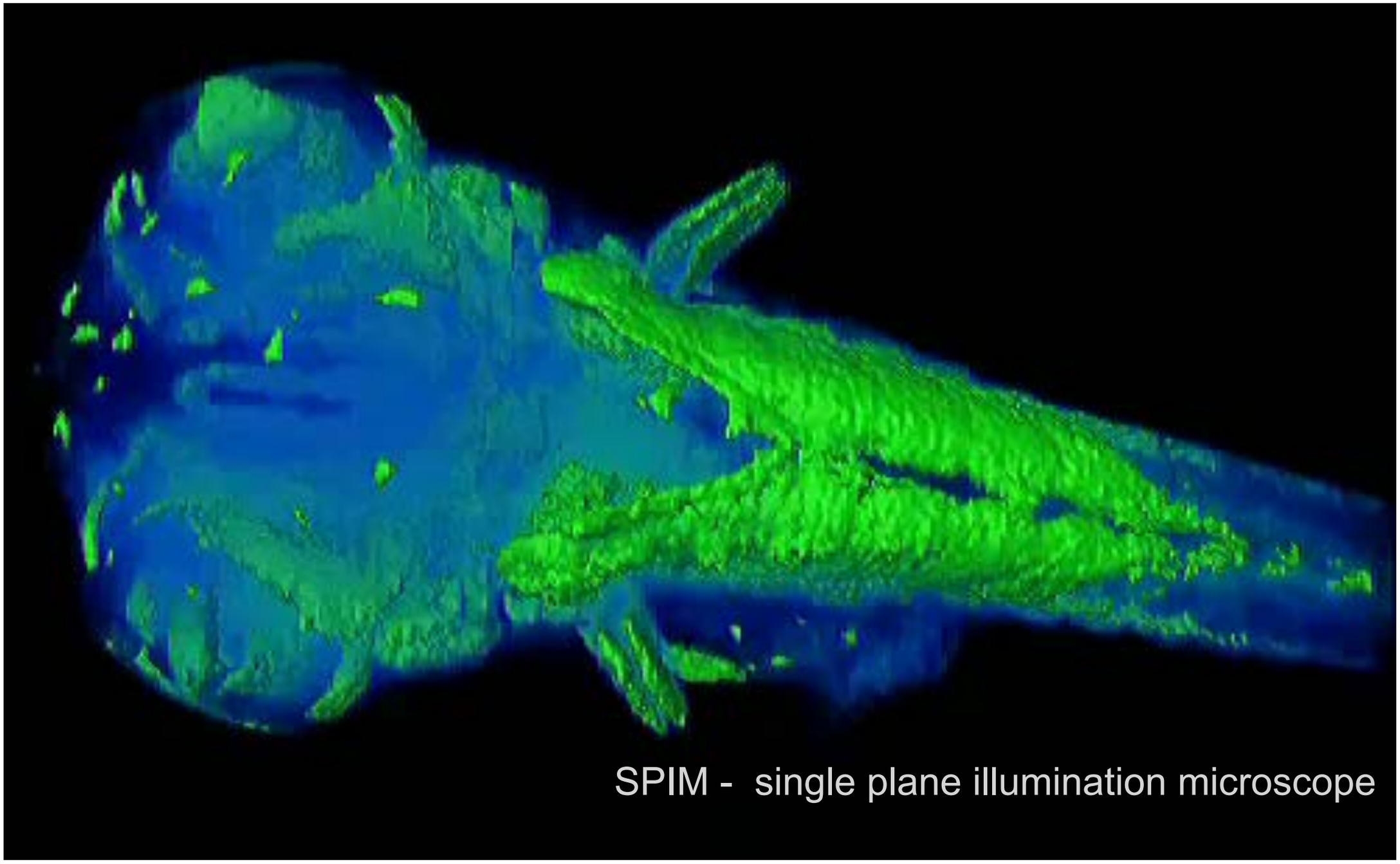
Optical Sectioning
Confocal
Light Sheet
2PE and SHG



Giuseppe Sancataldo, Diaspro LAB, IIT

7D

3D 3D rendered movie of the Medaka embryo.



J. Huisken, J. Swoger, F. Del Bene, J. Wittbrodt and E.H.K. Stelzer, Science 305, 1007 -1009 (2004)

7D

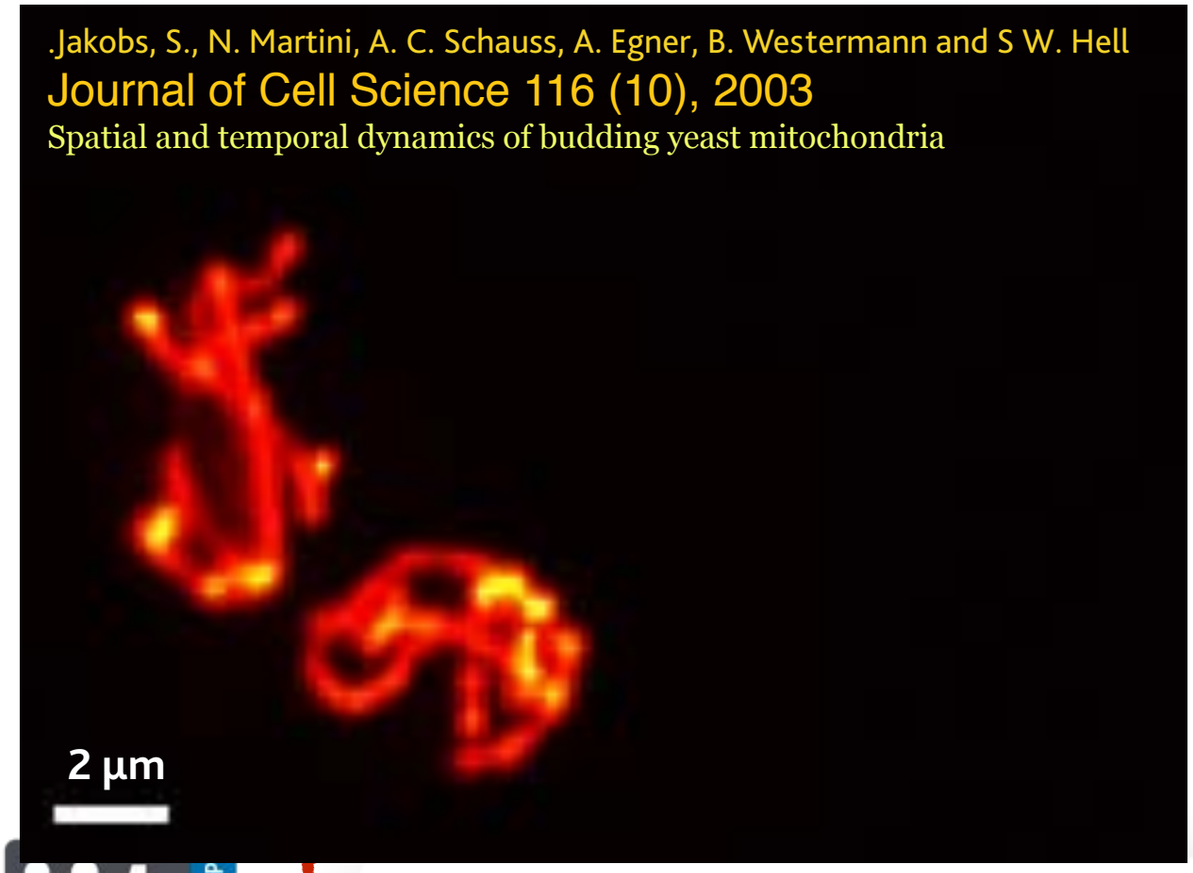
4D

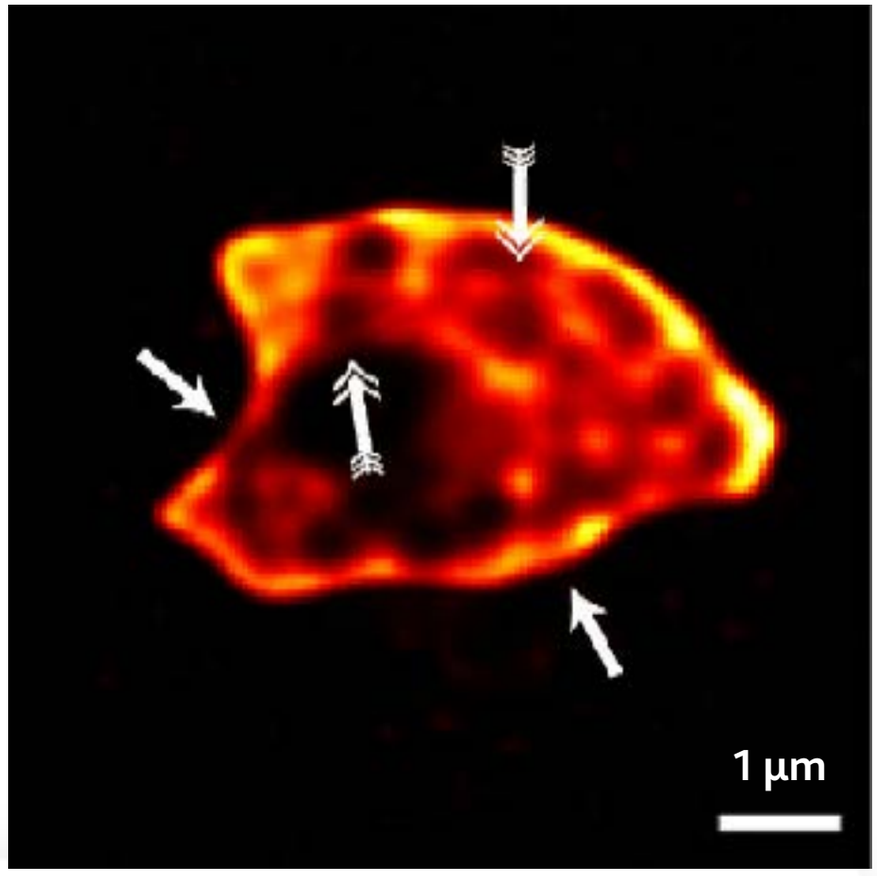
Stefan Hell's lab - http://www.nanoscopy.de/

.Jakobs, S., N. Martini, A. C. Schauss, A. Egner, B. Westermann and S W. Hell

Journal of Cell Science 116 (10), 2003

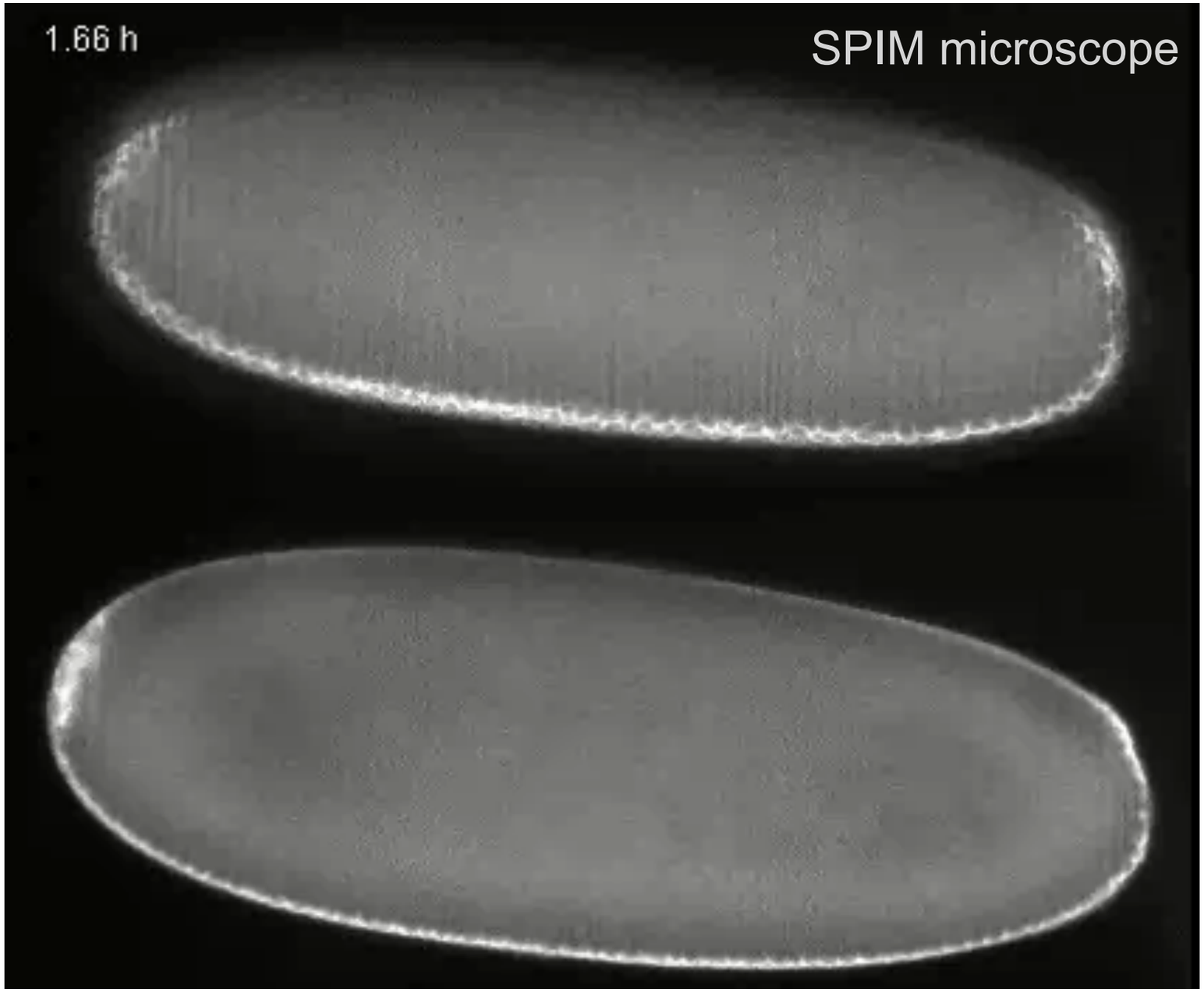
Spatial and temporal dynamics of budding yeast mitochondria





7D

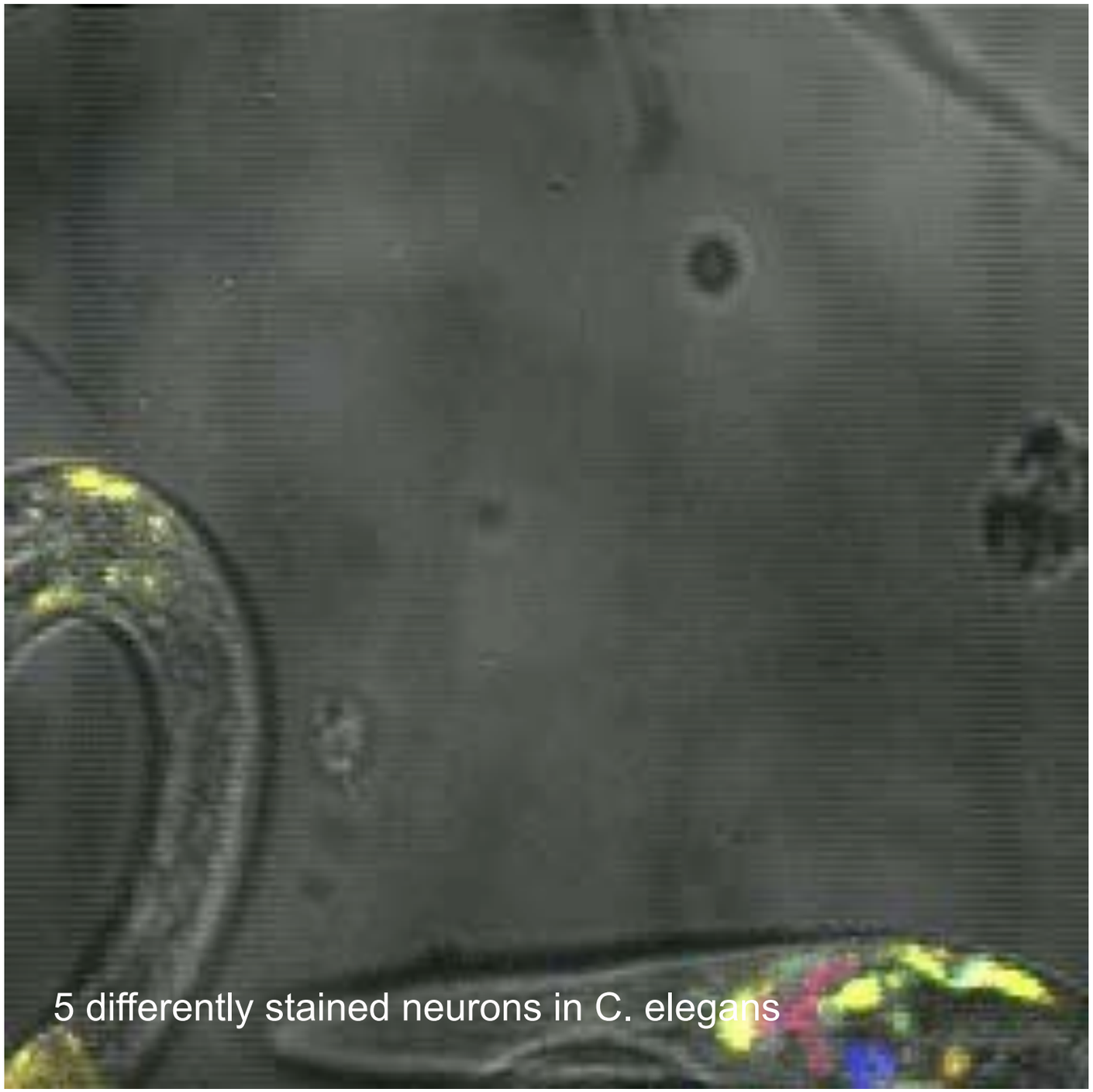
4D Time-lapse imaging of Drosophila melanogaster embryogenesis



J. Huisken, J. Swoger, F. Del Bene, J. Wittbrodt and E.H.K. Stelzer, Science 305, 1007 -1009 (2004)

7D

5D

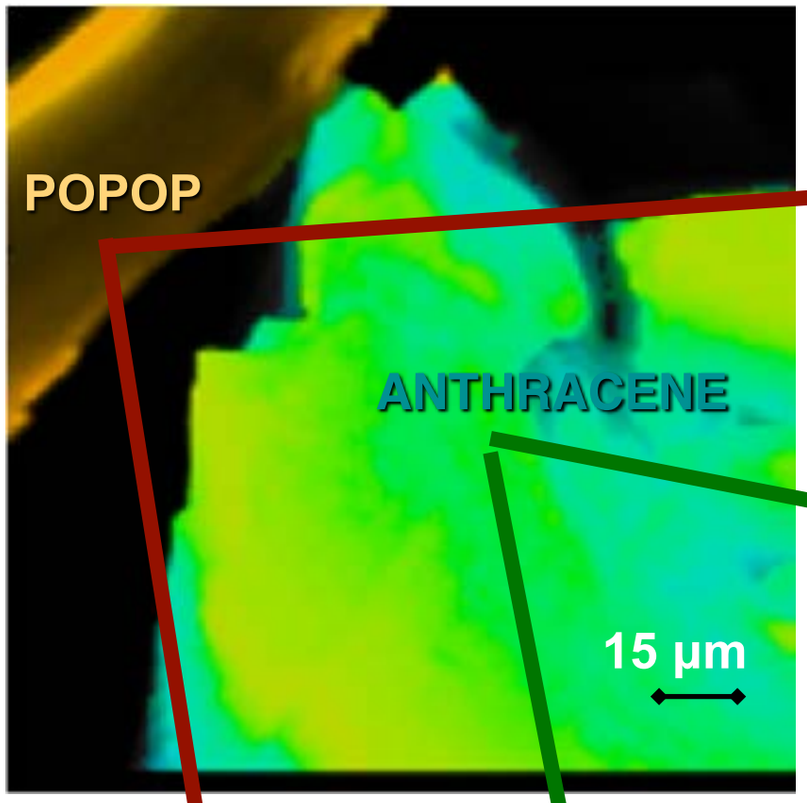


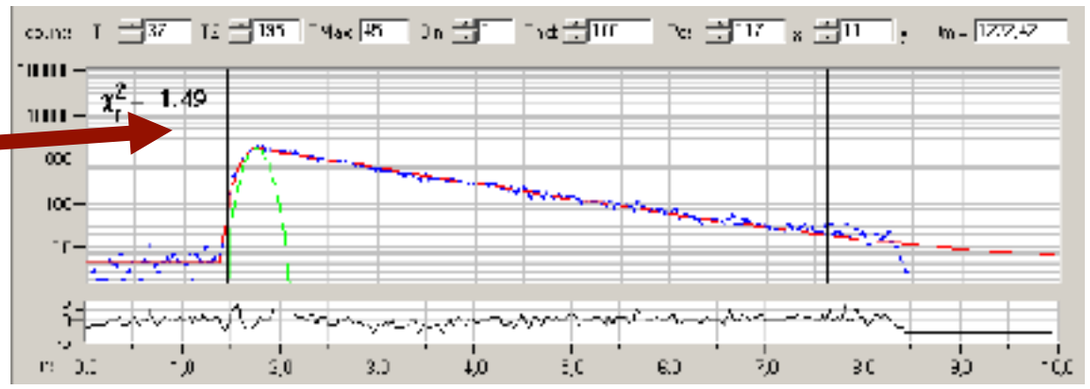
TCS SP5 resonant scanner 50 Hz framerate. Simulataneous record of CFP, GFP, YFP, DsRed, DiD and transmitted light.

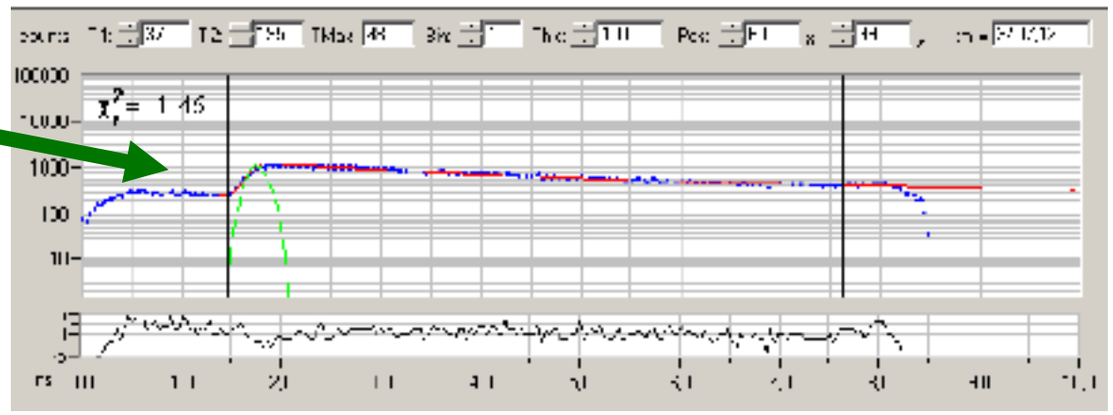
Credits: LEICA microsystems and Dr. Harald Hutter, Max Planck Institute (MPI) for Medical Research, Heidelberg, Germany

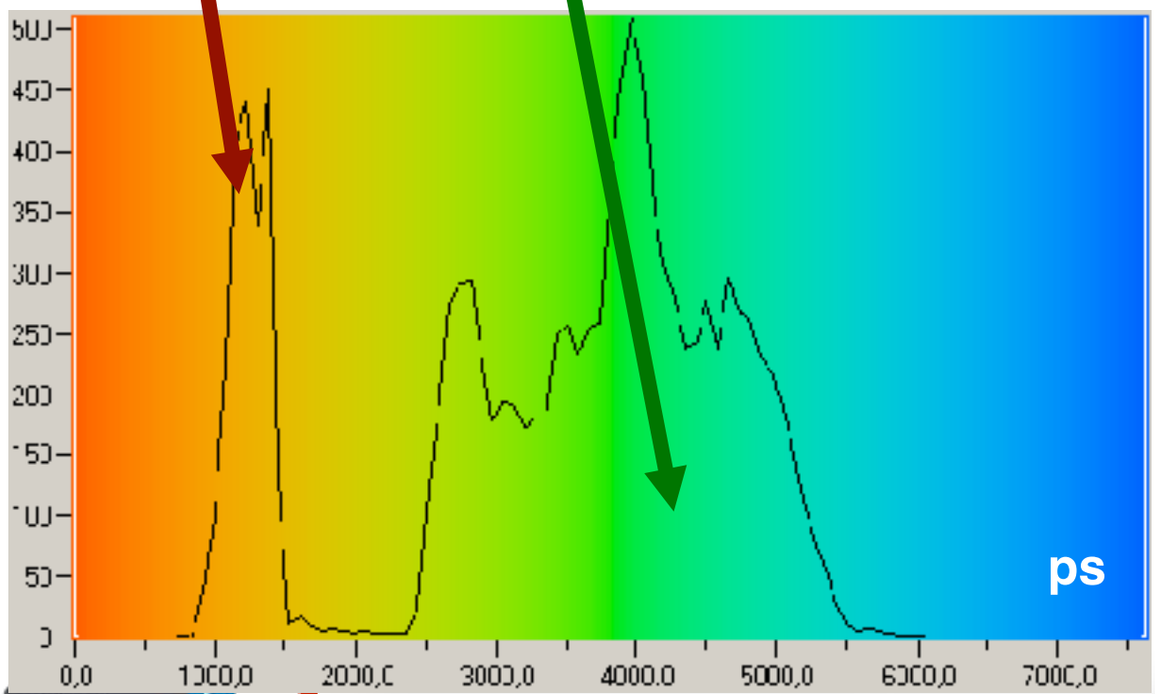
Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

7D

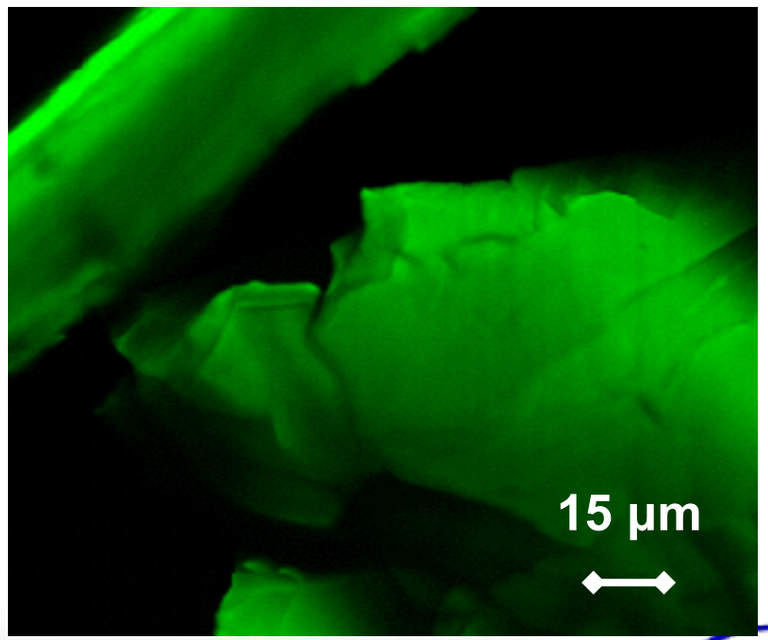






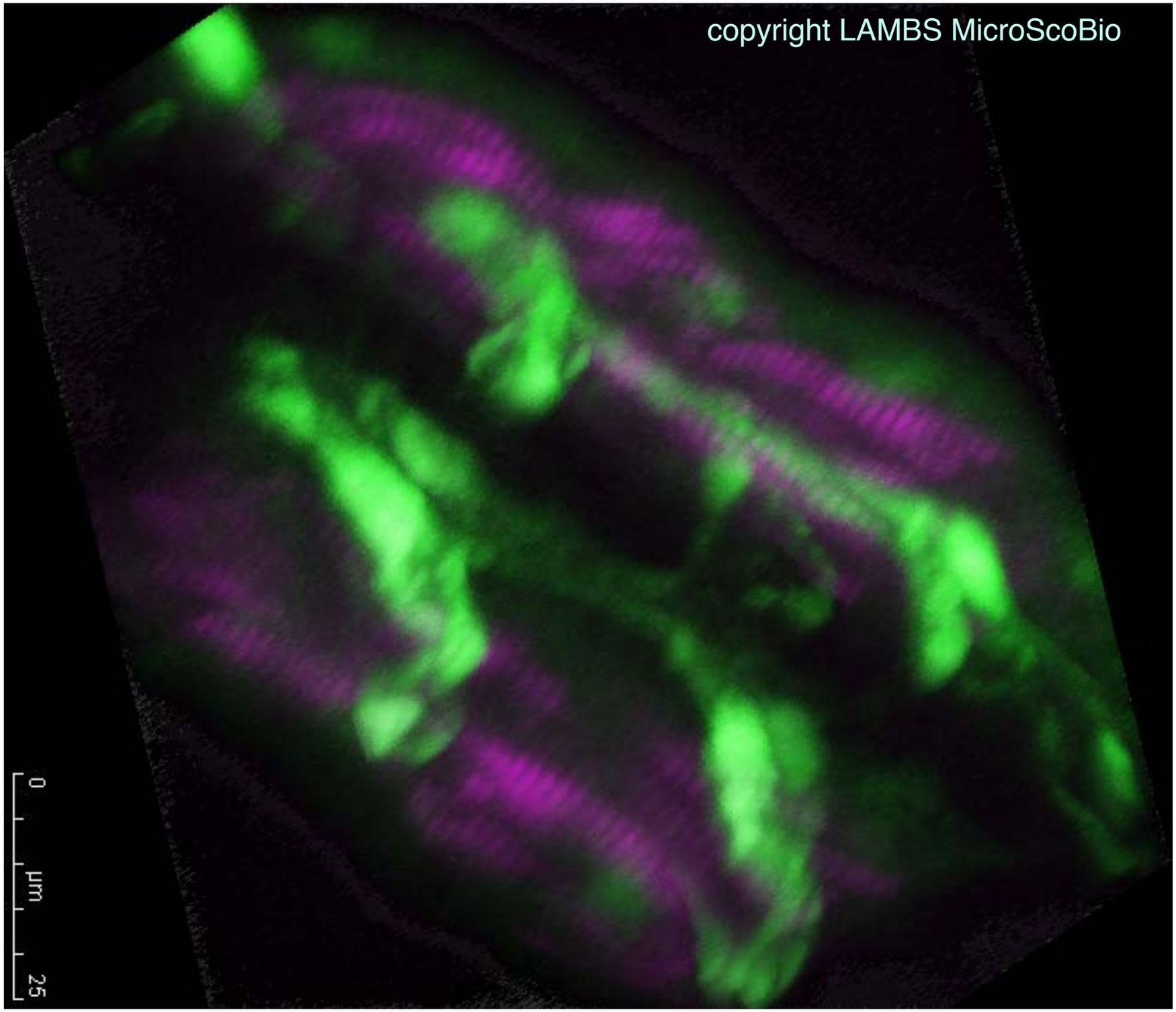


6D



7D

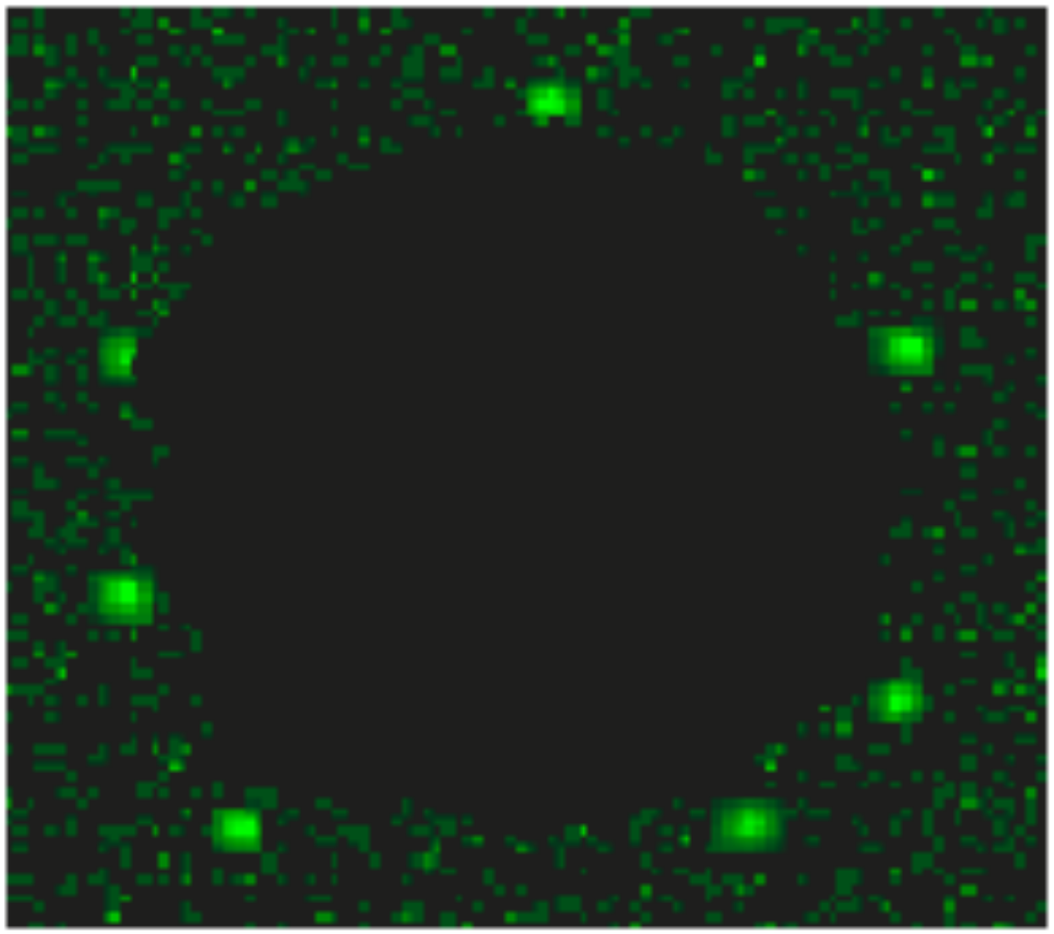
7D

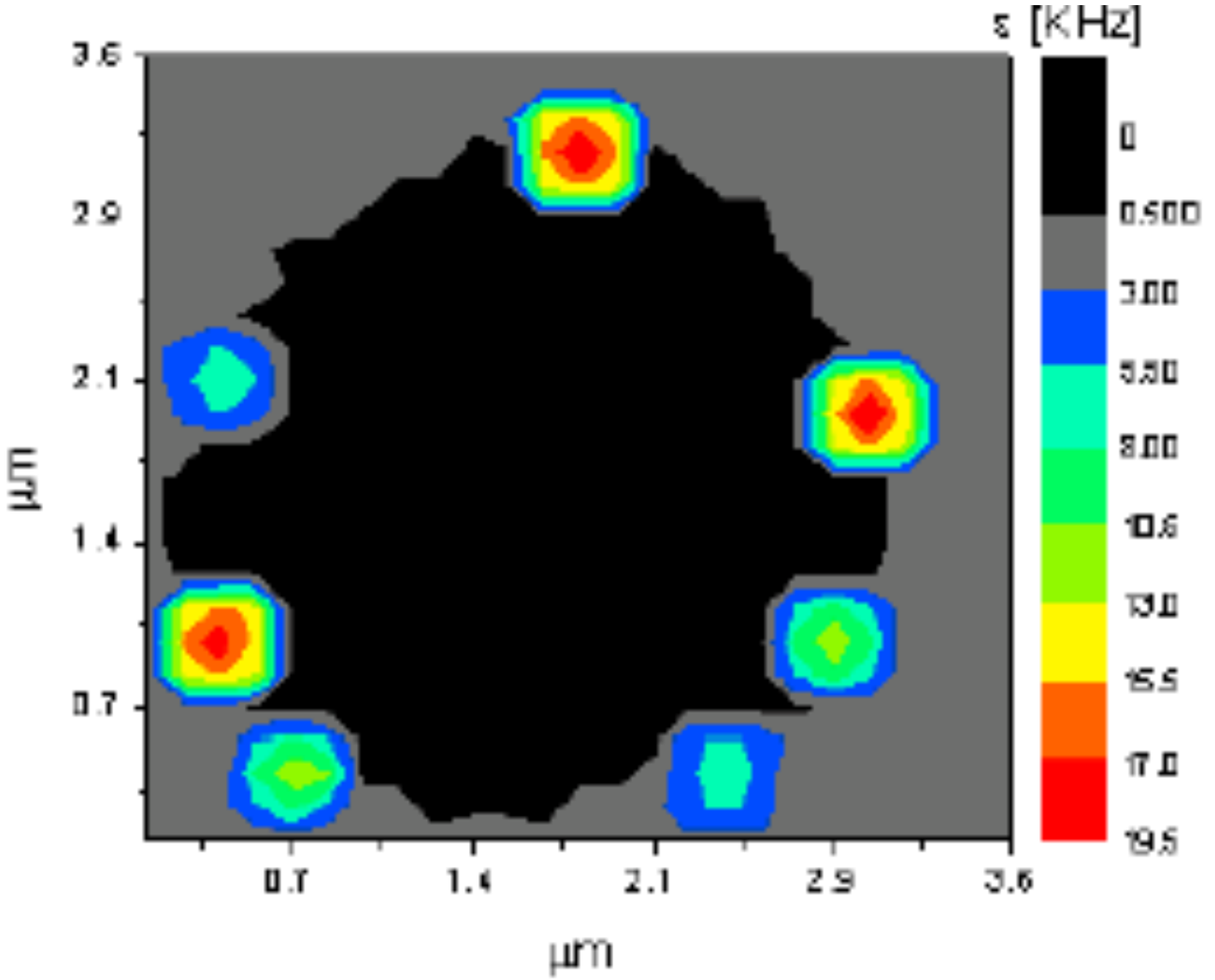


SHG from muscle fibers from the Zebrafish tail and EGFP fluorescence - Paolo Bianchini, LAMBS 2006

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

7D and more

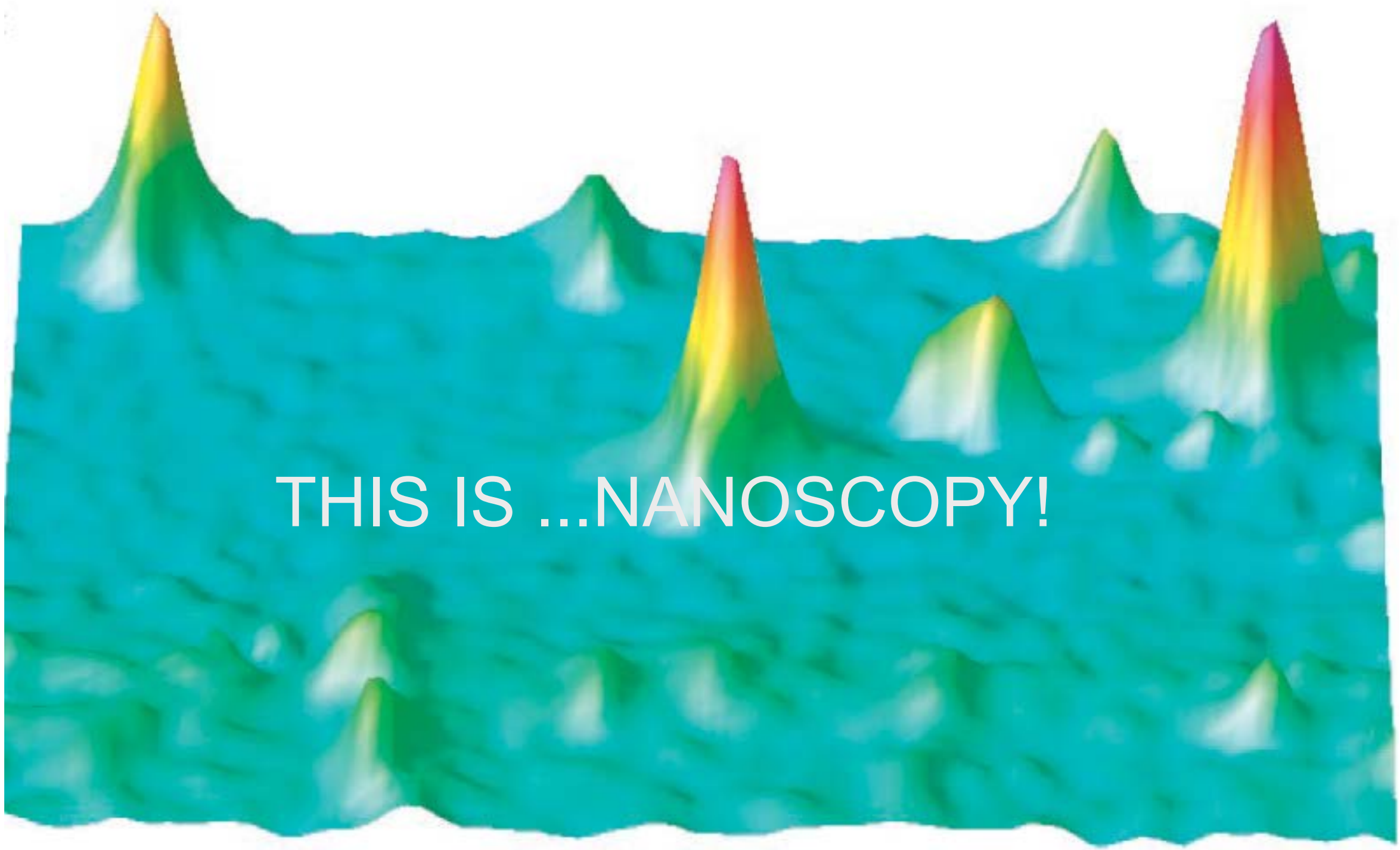




Single GFPmut2 molecules fluorescence (bright green) and brightness after incorporation in a poly-electrolyte matrix built on amorphous calcium carbonate (dark sphere). The field of view is 3.6 x 3.6 µm.

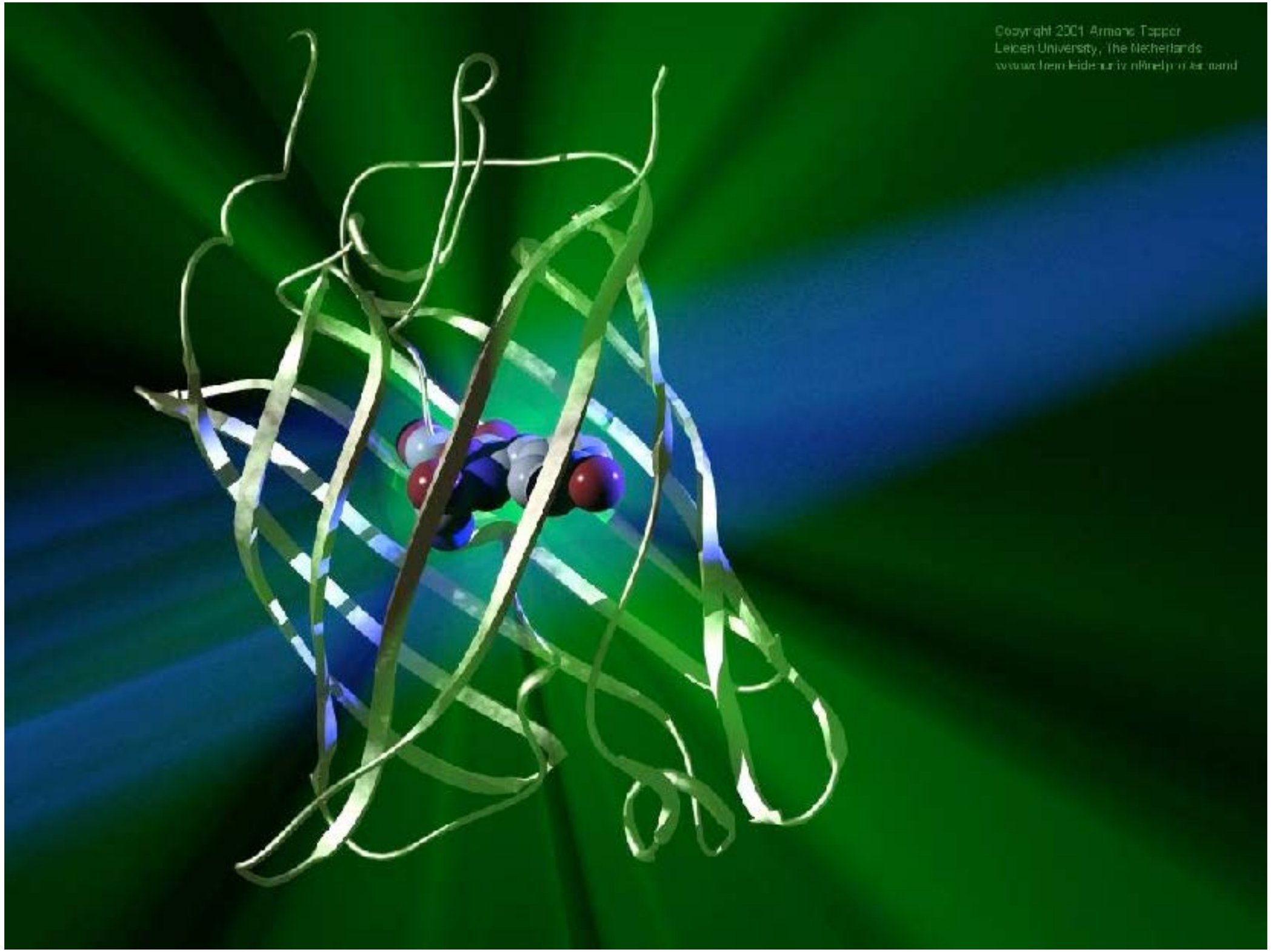
Diaspro A., et al. (2006) Optics Express, Vol. 14(21).

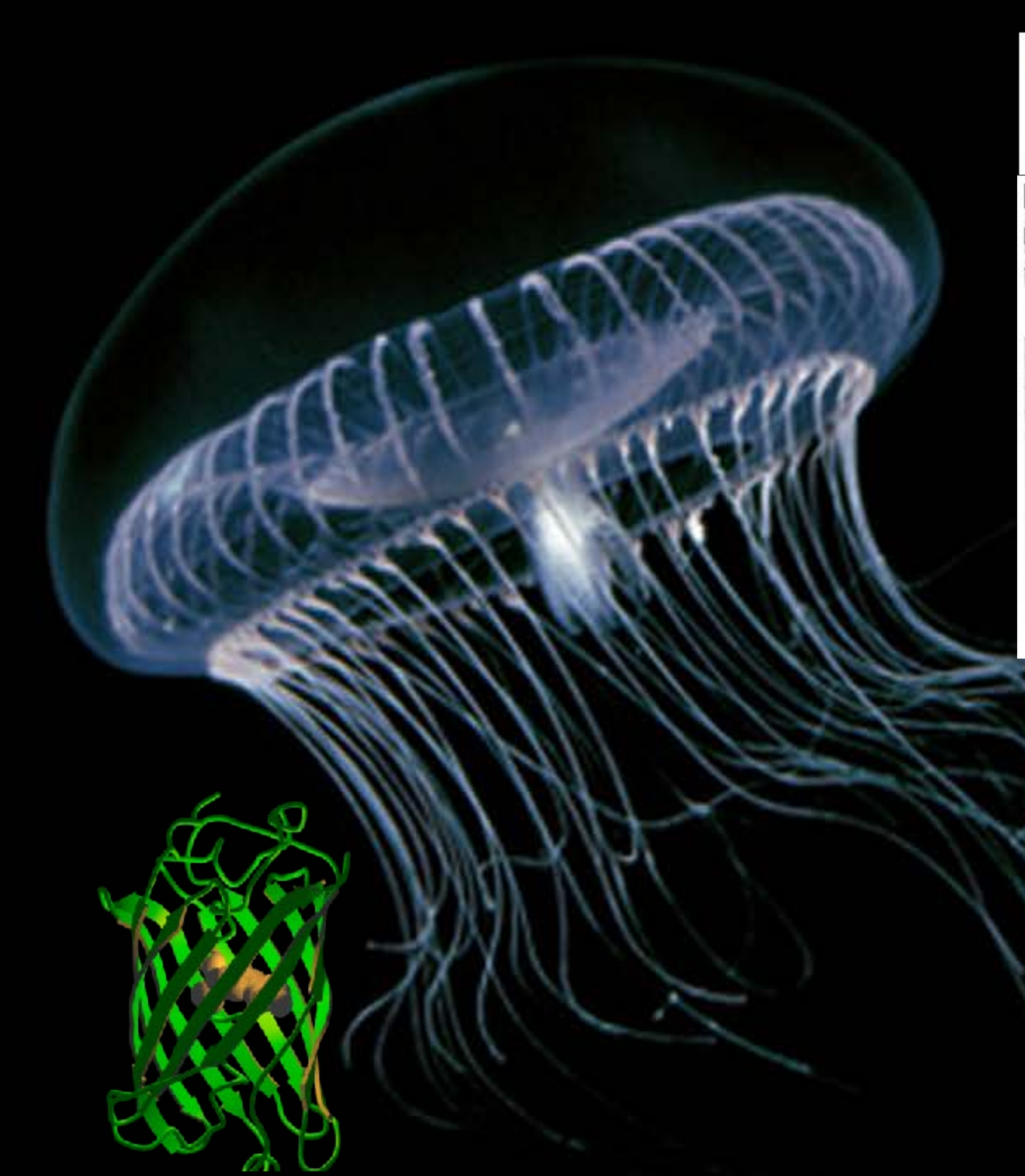
7D and more



Hybrid space-frequency image of single molecules of pentacene. Moerner WE, Orrit M. (1999) Science 283(5408):1670-6

GREEN FLUORESCENT PROTEIN





The Nobel Prize in Chemistry 2008

"for the discovery and development of the green fluorescent protein, GFP"

Photo: J. Henriksson/SCANPIX

Martin Chalfie

1/3 of the prize

USA

Columbia University
New York, NY, USA

b. 1947

Photo: UCSD

Roger Y. Tsien

1/3 of the prize

USA

University of California
San Diego, CA, USA

b. 1952

Photo: J. Henriksson/SCANPIX

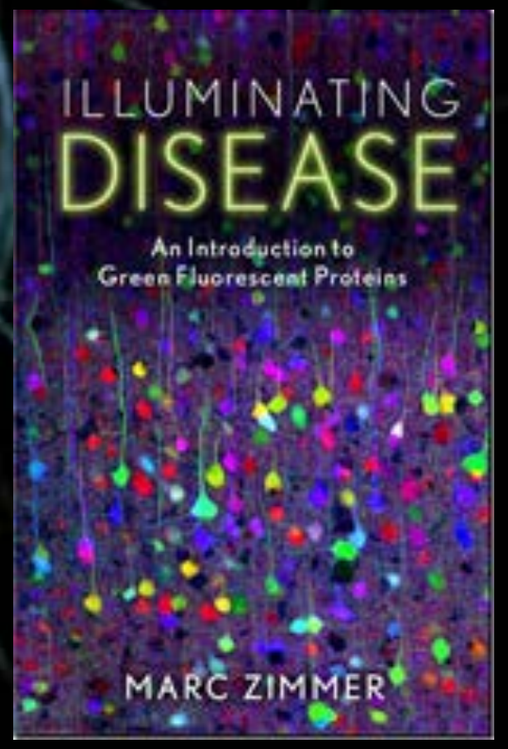
Osamu Shimomura

1/3 of the prize

USA

Marine Biological Laboratory (MBL)
Woods Hole, MA, USA

b. 1928



Credit: M Chalfie - Nobel Laureate 2008

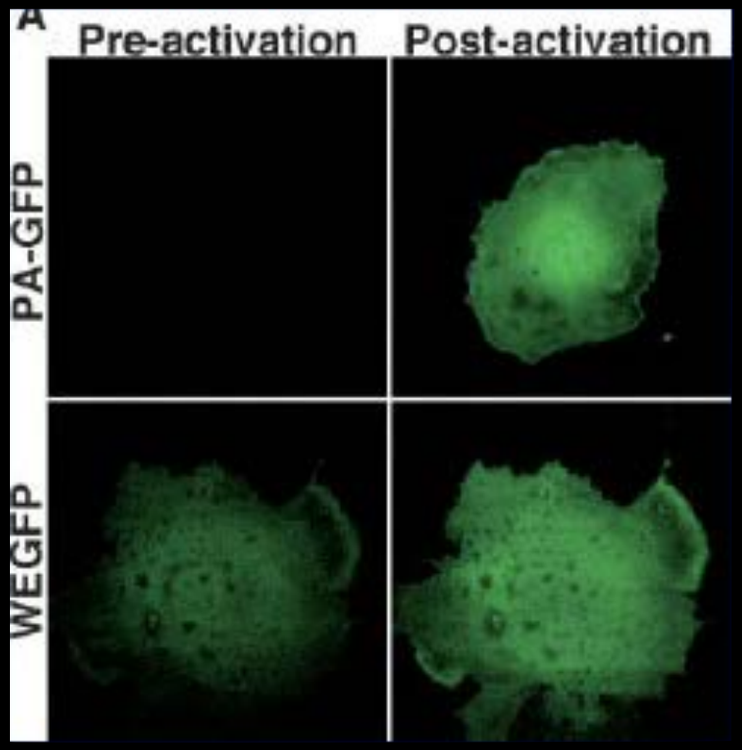
C. Elegans PH-GFP H2B-mCherry

4D

Credit: Eric Betzig - Janelia Farm (2015)

Directed Mutagenesis of Photoactivated Fluorescent Proteins (PA-FPs)

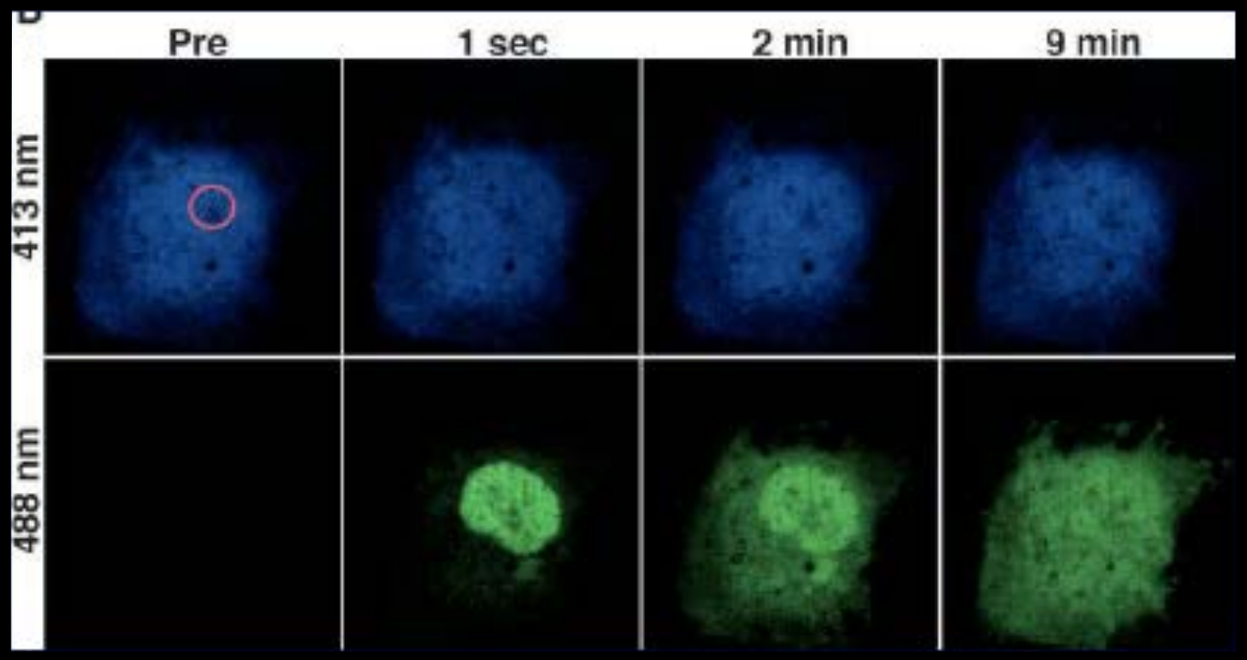
increased on/off contrast of PA-GFP



Jennifer Lippincott-Schwartz

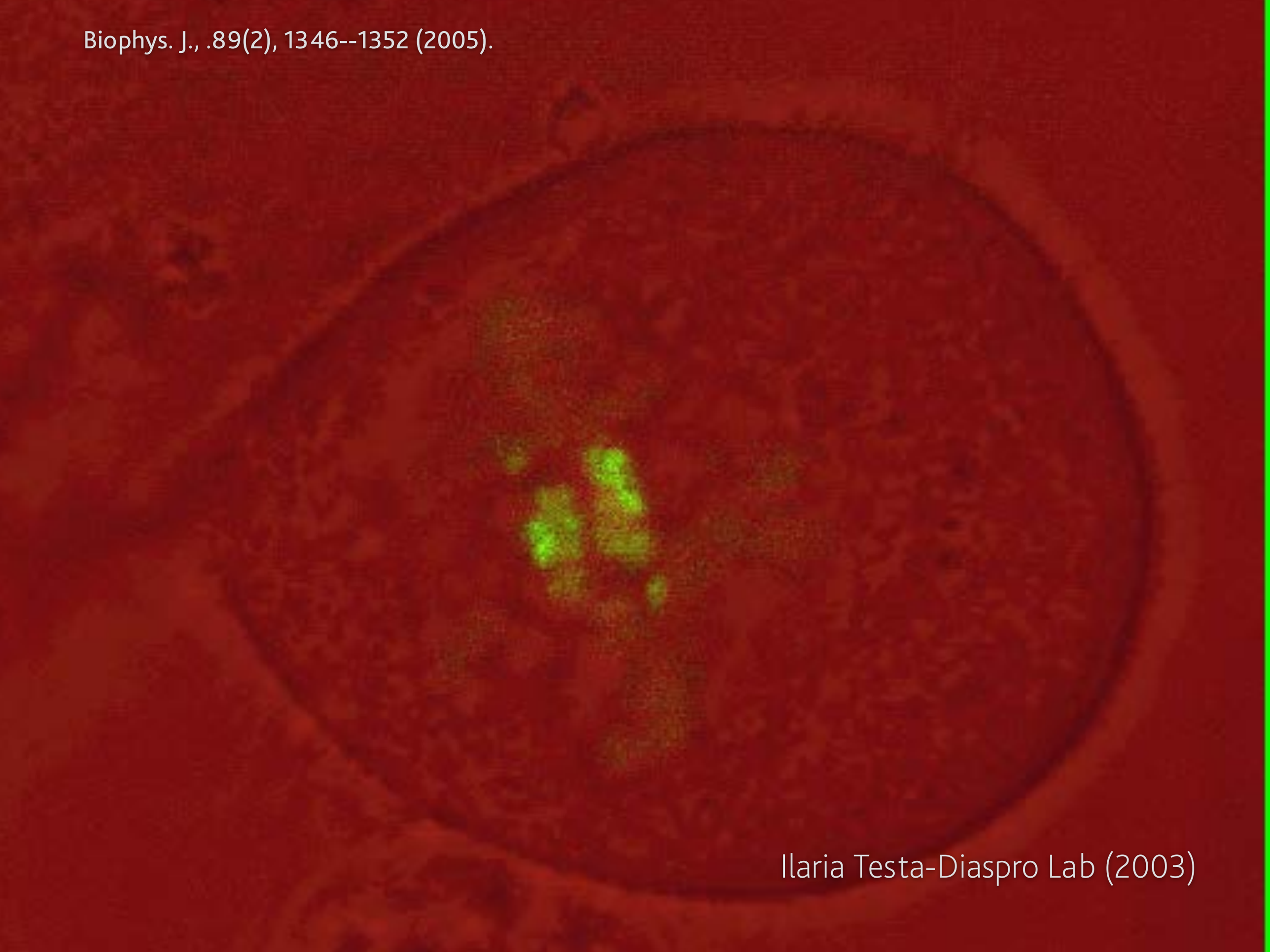
George Patterson

pulse chase: nuclear vs cytosolic diffusion



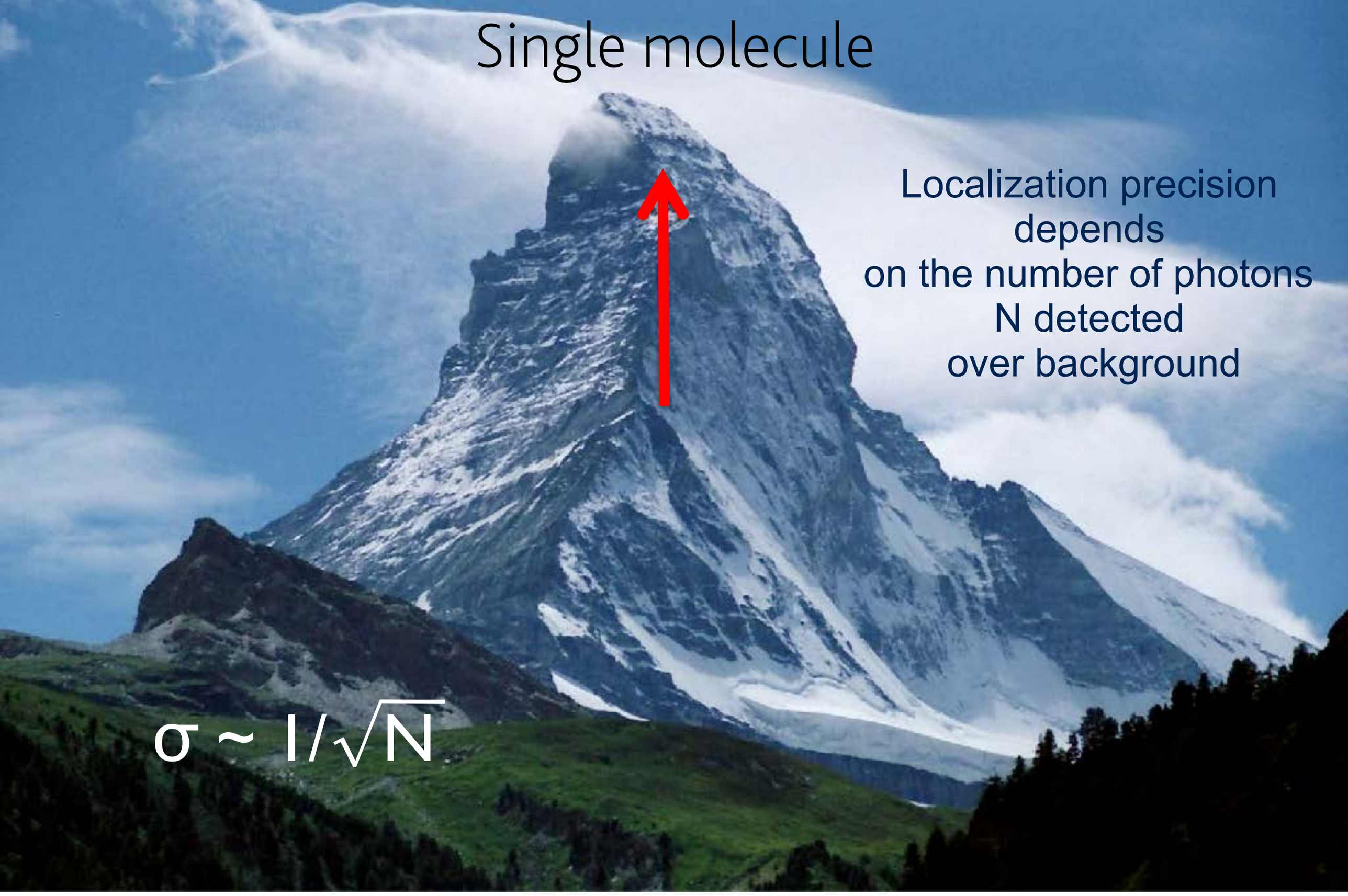
G.H. Patterson, J. Lippincott-Schwartz, *Science* **297**, 1873 (2002)

Biophys. J., .89(2), 1346--1352 (2005).



Ilaria Testa-Diaspro Lab (2003)

Single molecule



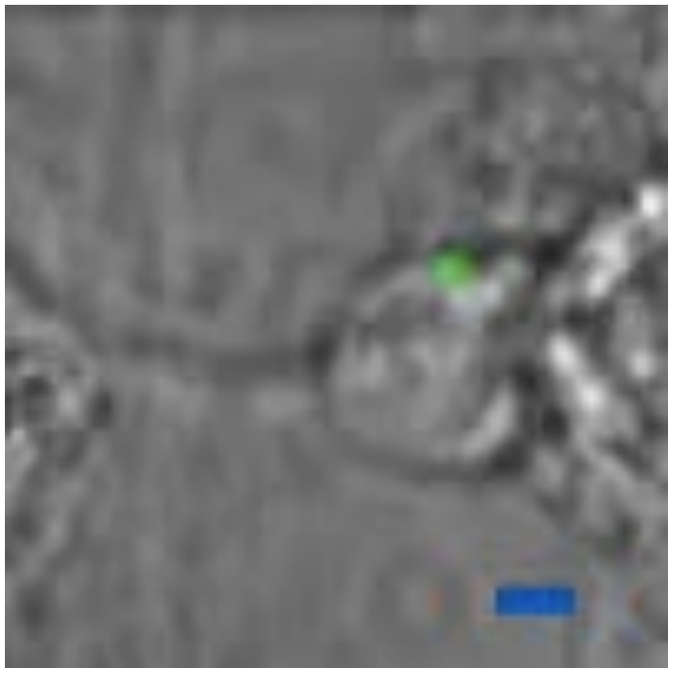
Localization precision
depends
on the number of photons
N detected
over background

$\sigma \sim I/\sqrt{N}$

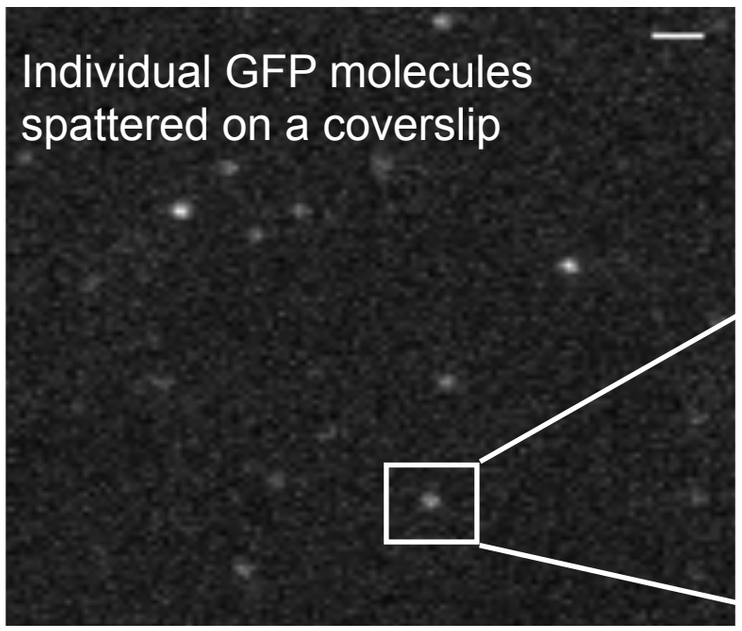
How does a single molecule look like

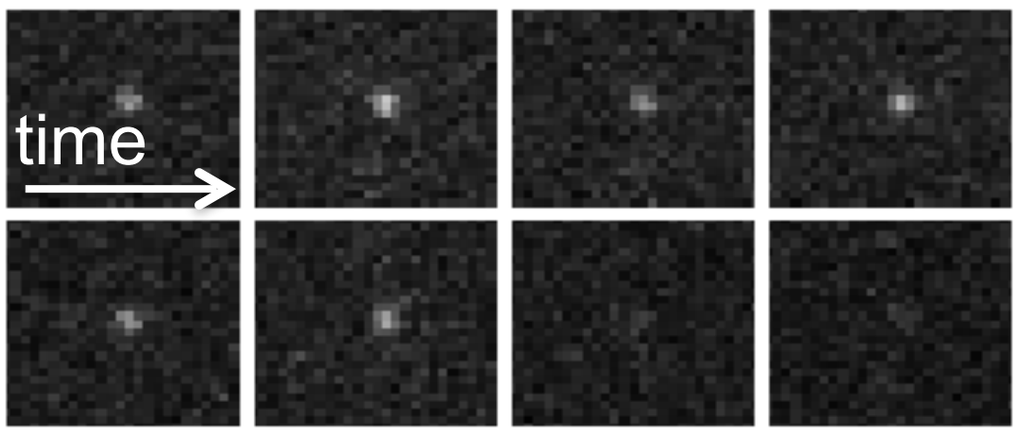
How do we recognize a single molecule?

Single molecules disappear after emitting N photons.



Diffusion of GABA receptor in living neuron

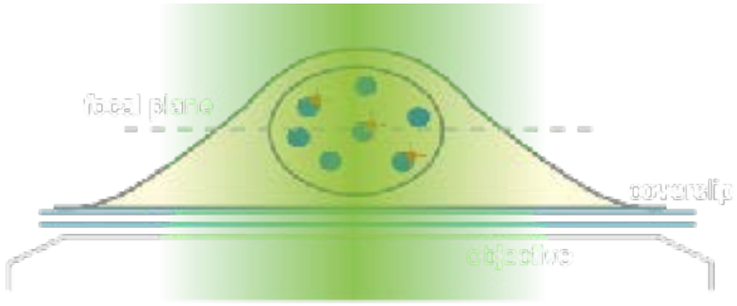




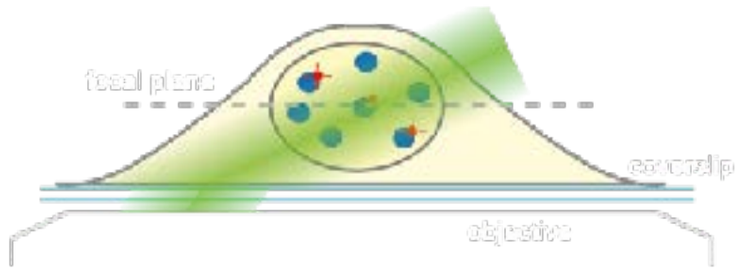
Single molecule

slide credit: Davide Mazza

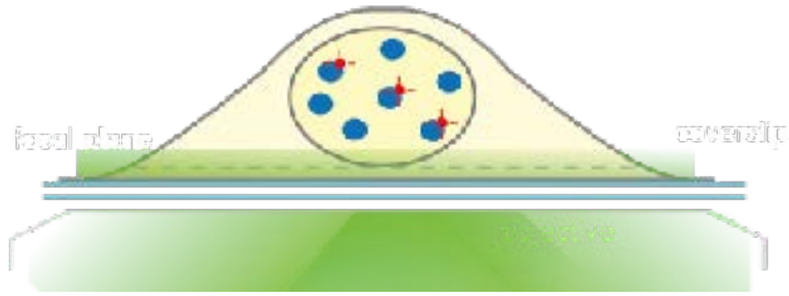
WIDEFIELD ILLUMINATION



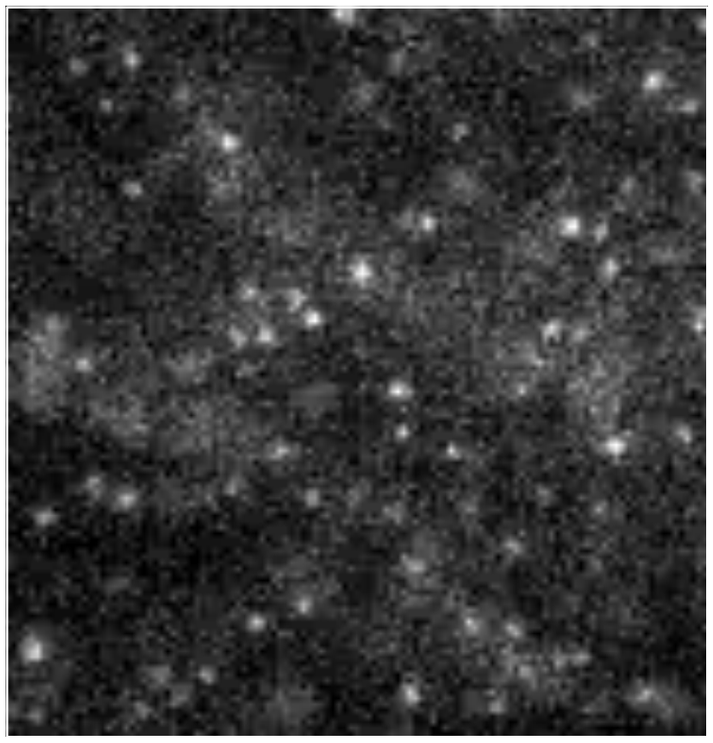
INCLINED (HILO) ILLUMINATION

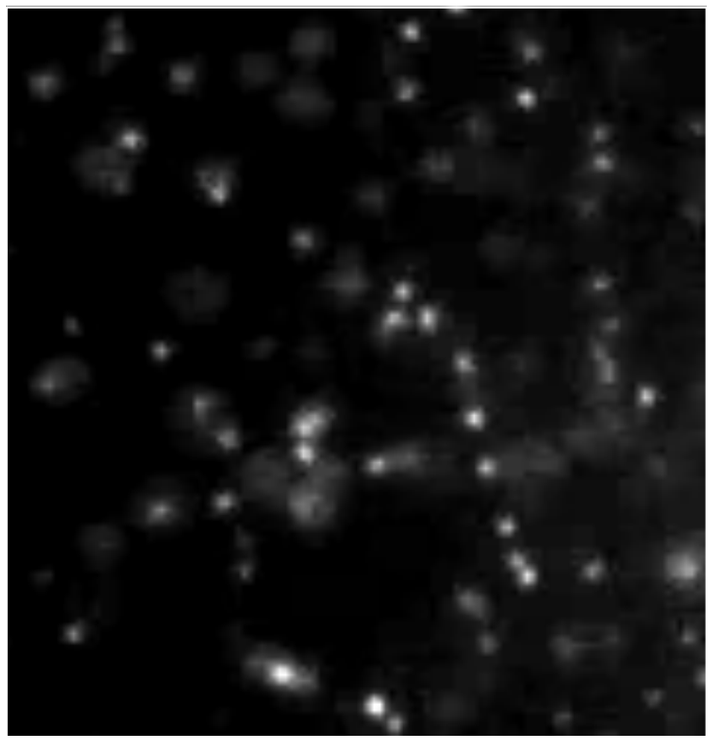


TIRF ILLUMINATION



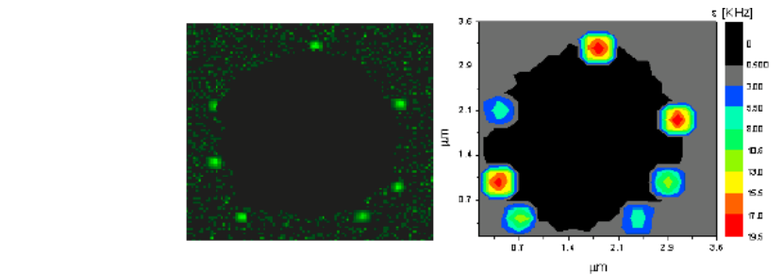
Tokunaga et al., Nat. Methods 2007

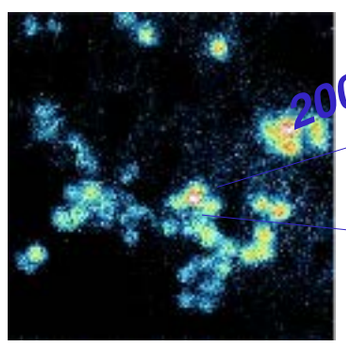


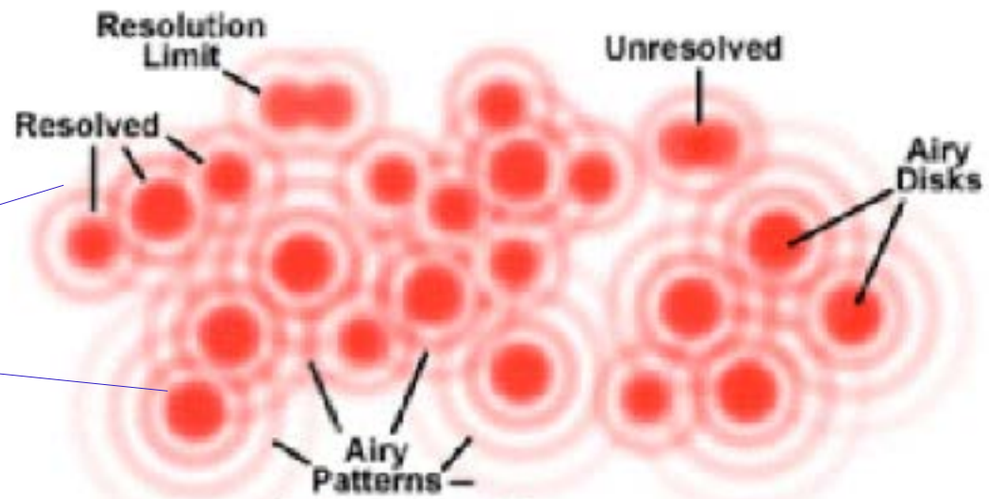


Images of 100nm fluorescent beads in aqueous solution - Frame rate 100fps

Single molecule structure is still not resolved







512 pixels

512 pixels

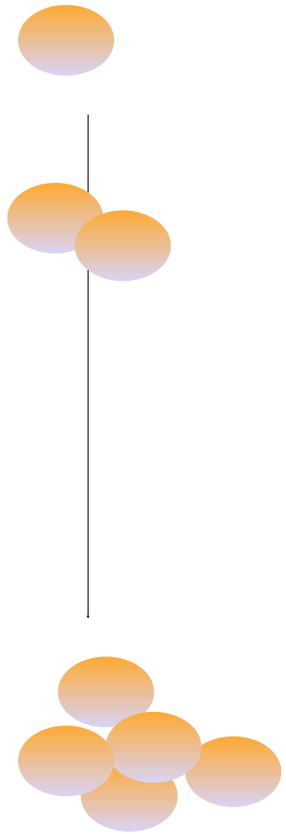
8 μm

Micr. Res. Tech. (2002) 55:359-4

Diaspro Lab -LAMBS - Dept. Physics

Micr. Res. Tech., 55:359-364 (2002)
Biophysical J., 84 (2003).
J.Phys.D,36 (2003.

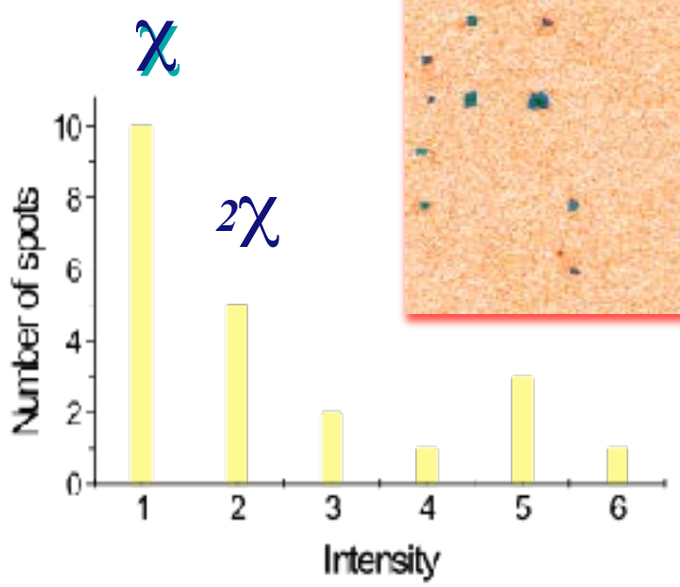
Single Molecule Imaging

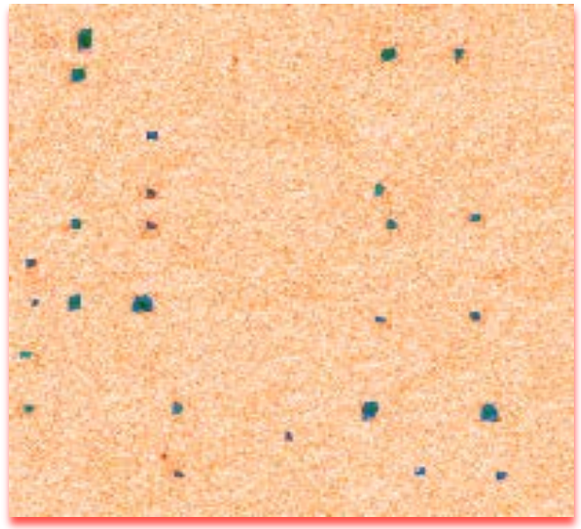


Intensity χ

Intensity 2χ

Intensity $N\chi$



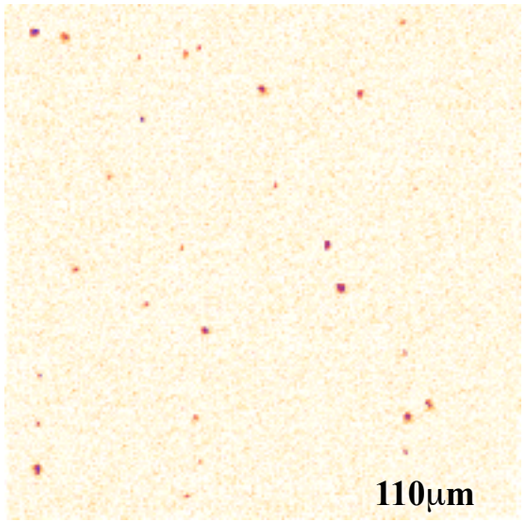


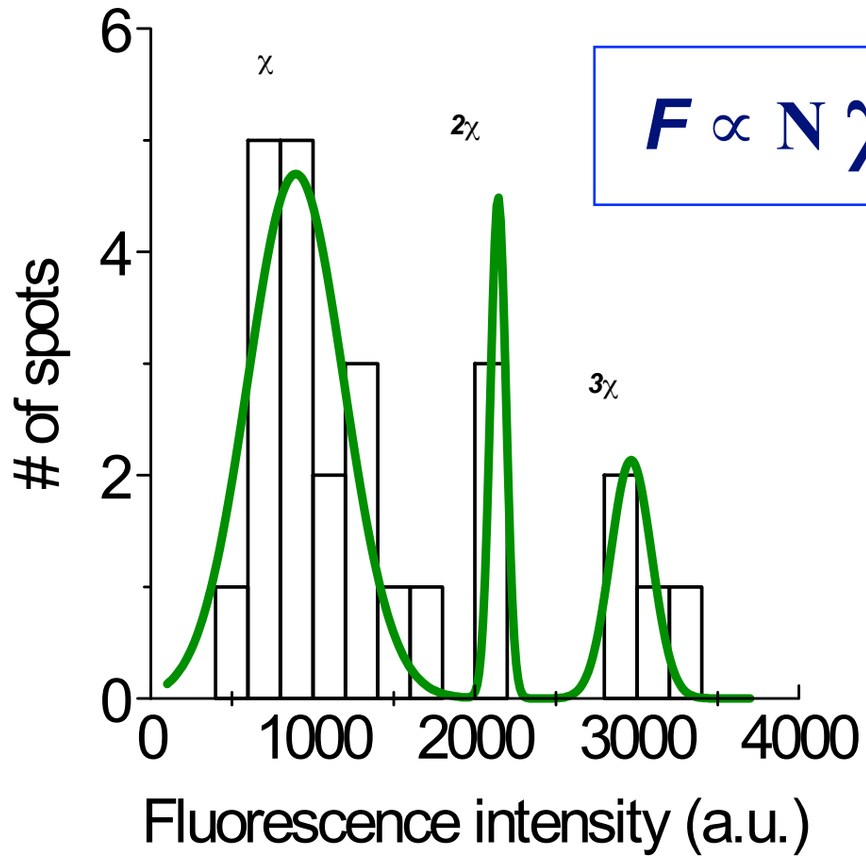
N = number of molecules

Micr. Res. Tech. (2002) 55:359-4

Single Molecule Imaging

Fluorescein ***c=1μM @ 8mW***



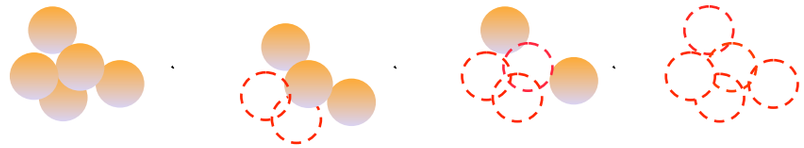


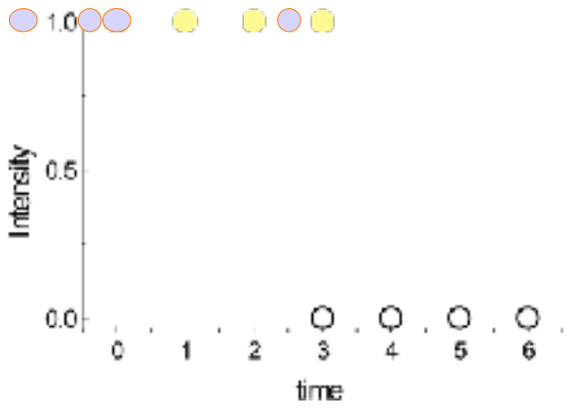
Micr. Res. Tech. (2002) 55:359-4

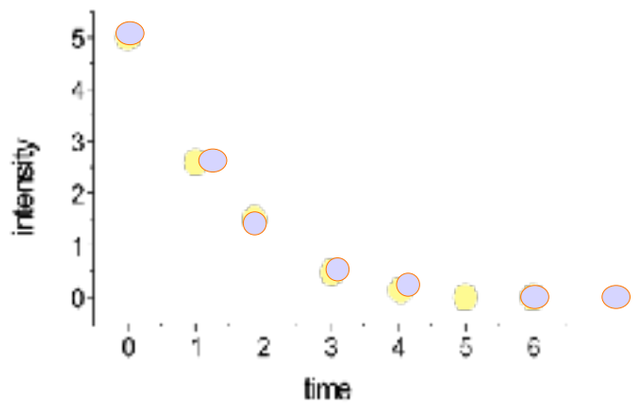
Single Molecule Imaging

Single Molecule

Aggregates



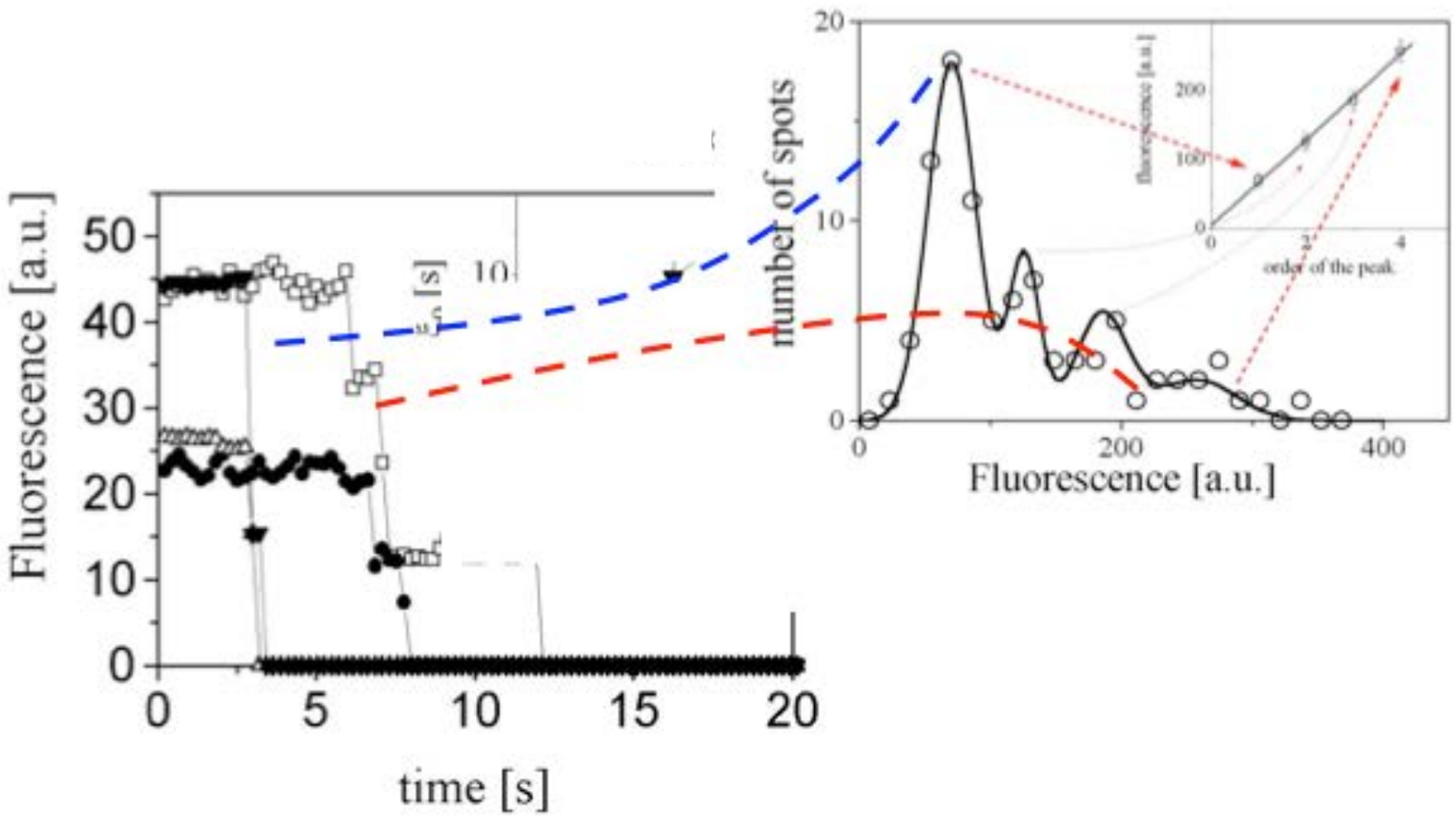




Micr. Res. Tech. (2002) 55:359-4

Single Molecule Imaging

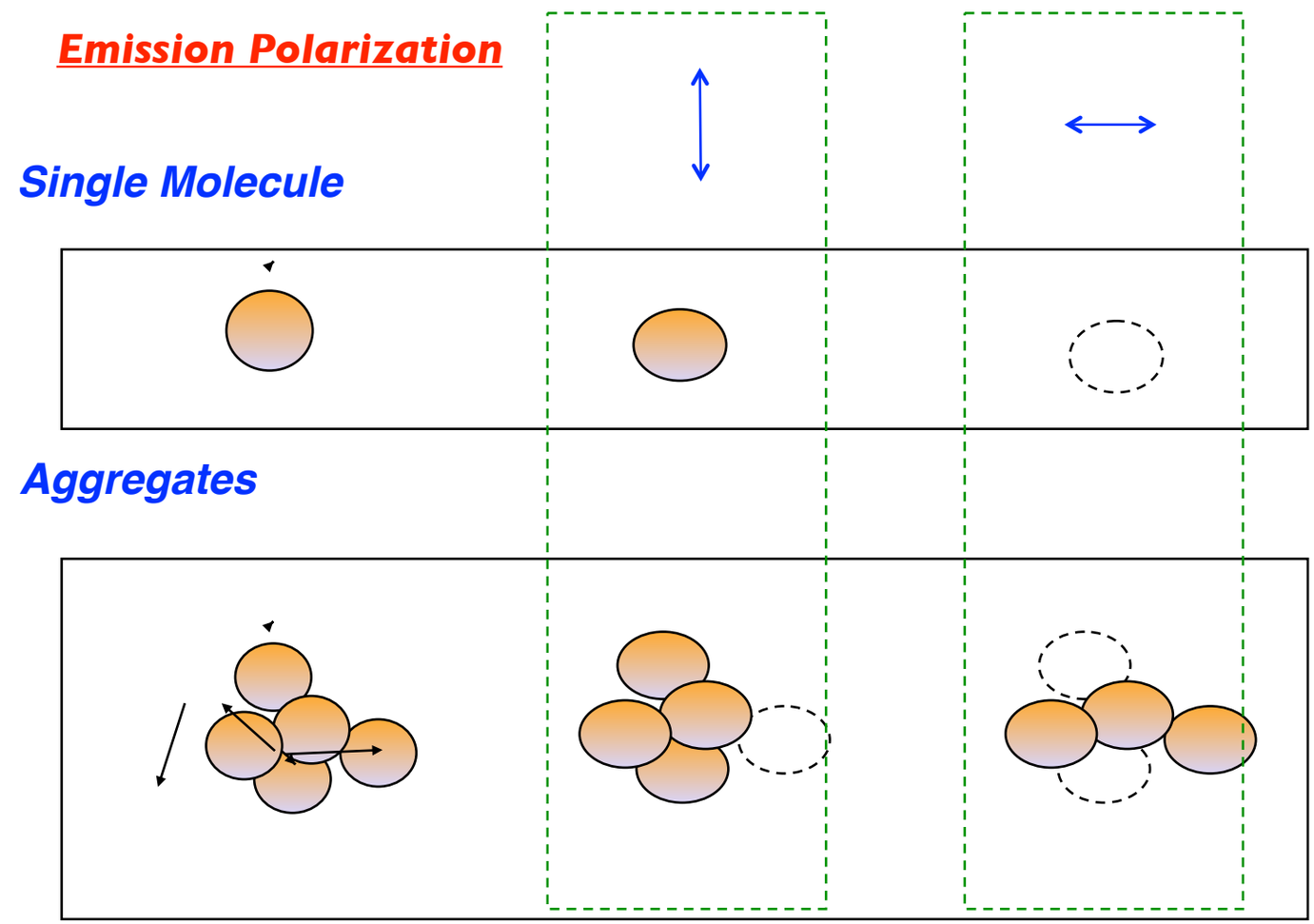
Single Molecule Photobleaching of Sparse Aggregates



Biophys J. 2003 Jan;84(1):588-98

Single Molecule Imaging

Fluorescence Polarization



J Biomed Opt. (2003) 8(3):391-5

2PE and GFP

Multi-photon switching dynamics of single green fluorescent proteins

G. Chirico,[1] F. Cannone,[1] A. Diaspro,[2] S. Bologna,[3] V. Pellegrini,[4] R. Nifosì,[4] and F. Beltram[4]

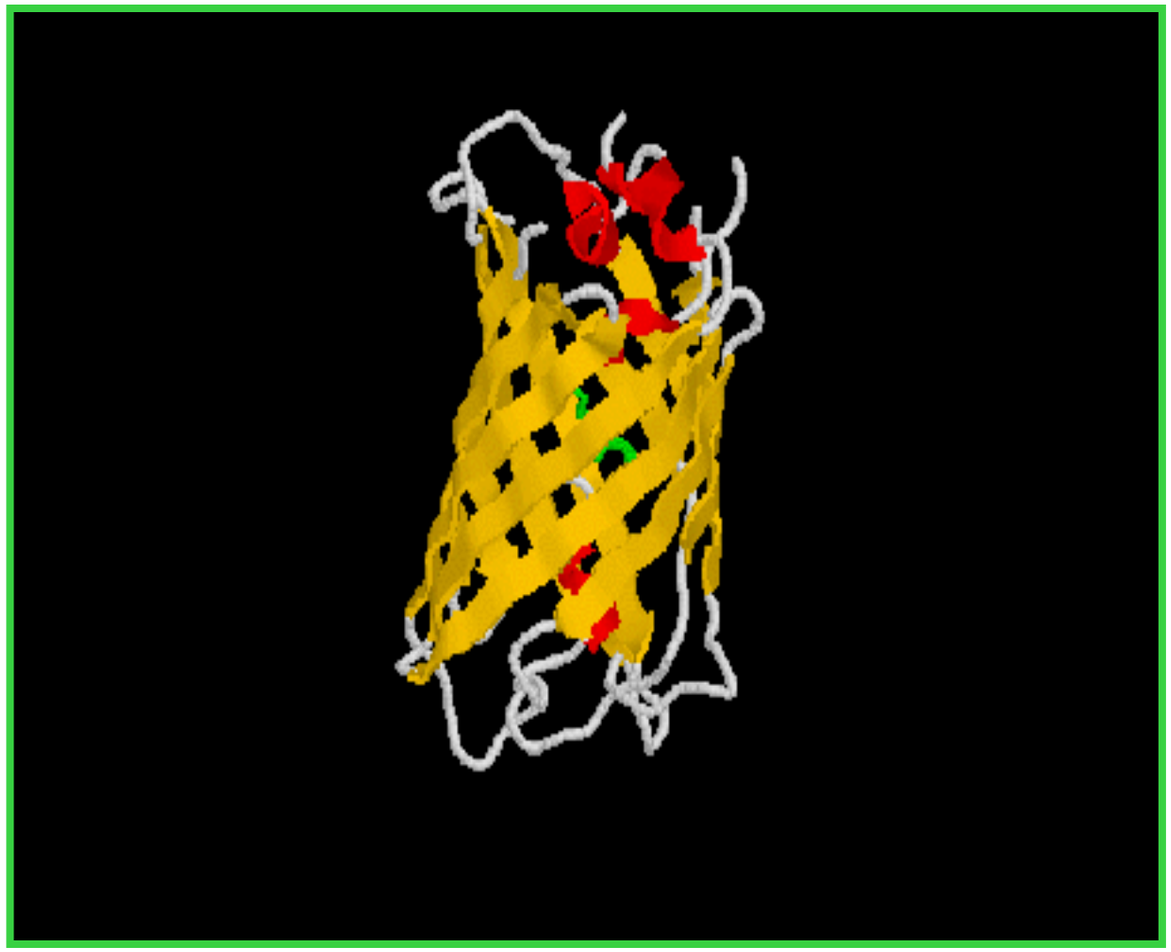
[1]*INFM and Department of Physics, University of Milano Bicocca, Piazza della scienza 3, 20126 Milano, Italy*
[2]*INFM and Department of Physics, University of Genoa, Via Dodecaneso 33, 16146 Genova, Italy*
[3]*Department of Biochemistry and Molecular Biology, University of Parma, 43100 Parma, Italy*
[4]*NEST-INFM and Scuola Normale Superiore, Piazza dei Cavalieri 7, I-56126 Pisa (Italy)*

(Dated: February 19, 2004)

Multi-photon driven photo-switching between dark and bright (fluorescent) states of a green fluorescent protein (GFP) mutant is demonstrated. A single-molecule investigation shows the existence of two distinct bright states that display sharp two-photon cross-section bands peaked at 780nm and at 870nm. Fluorescence of these two species can be independently switched on and off. These results highlight a new photoconversion pathway for photochromic GFPs and can have significant applications in multi-photon confocal microscopy and in optical data-storage architectures.

CHIRICO G., CANNONE F., DIASPRO A., BOLOGNA S., PELLEGRINI V., NIFOS R., BELTRAM F. (2004). PHYSICAL REVIEW E, STATISTICAL, NONLINEAR, AND SOFT MATTER PHYSICS. vol. 70, pp. 03-0901

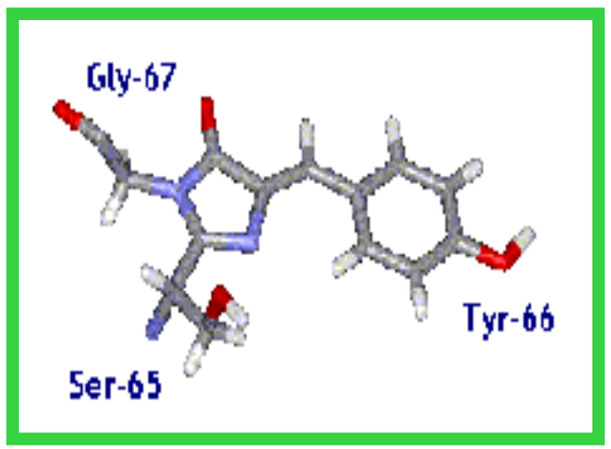
CHIRICO G., DIASPRO A., CANNONE F., COLLINI M., BOLOGNA S., PELLEGRINI V., BELTRAM F. (2005). Selective fluorescence recovery after bleaching of single E2GFP proteins induced by two-photons excitation. CHEMPHYSCHEM. vol. 6, pp. 328-335



238 AA

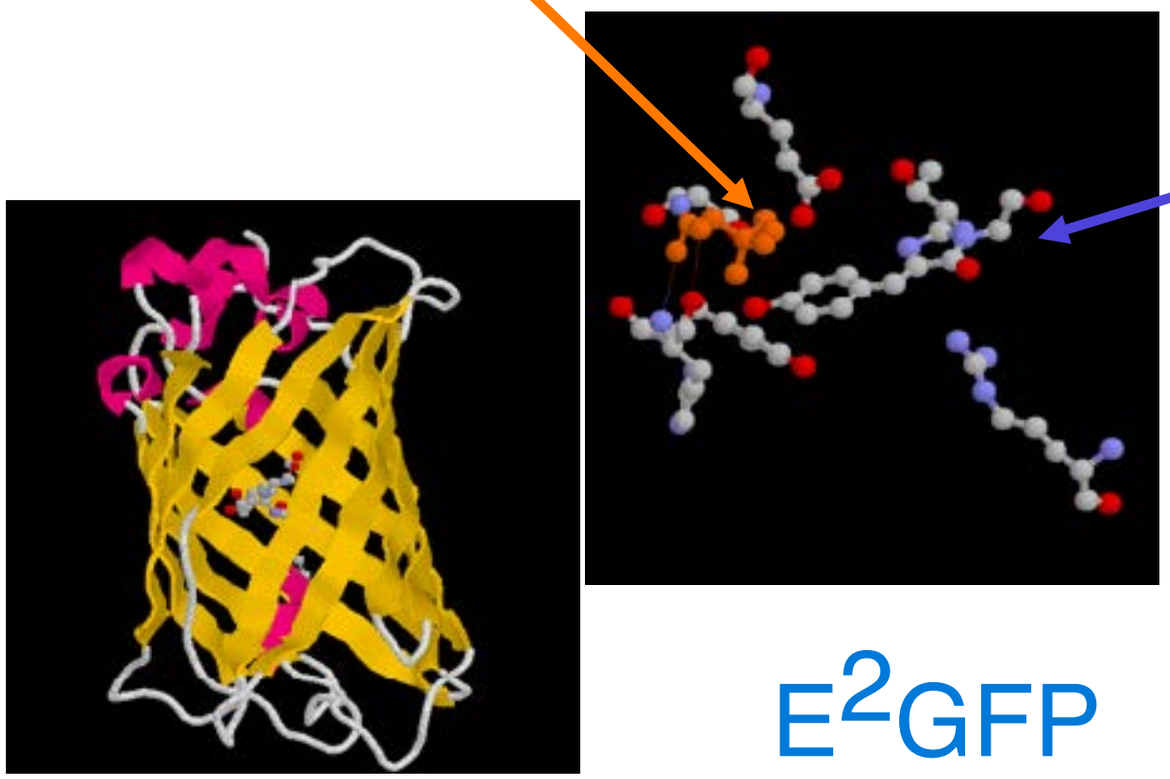
11 β-barrel ; 4 α-helices

Gly-67, Tyr-66, Ser-65



2PE and GFP

Threonine$_{203}$ ⟶ Tyrosine$_{203}$



Chromophore (65)

Fusion of
Ser 65, Tyr 66, Gly 67

E^{2}GFP

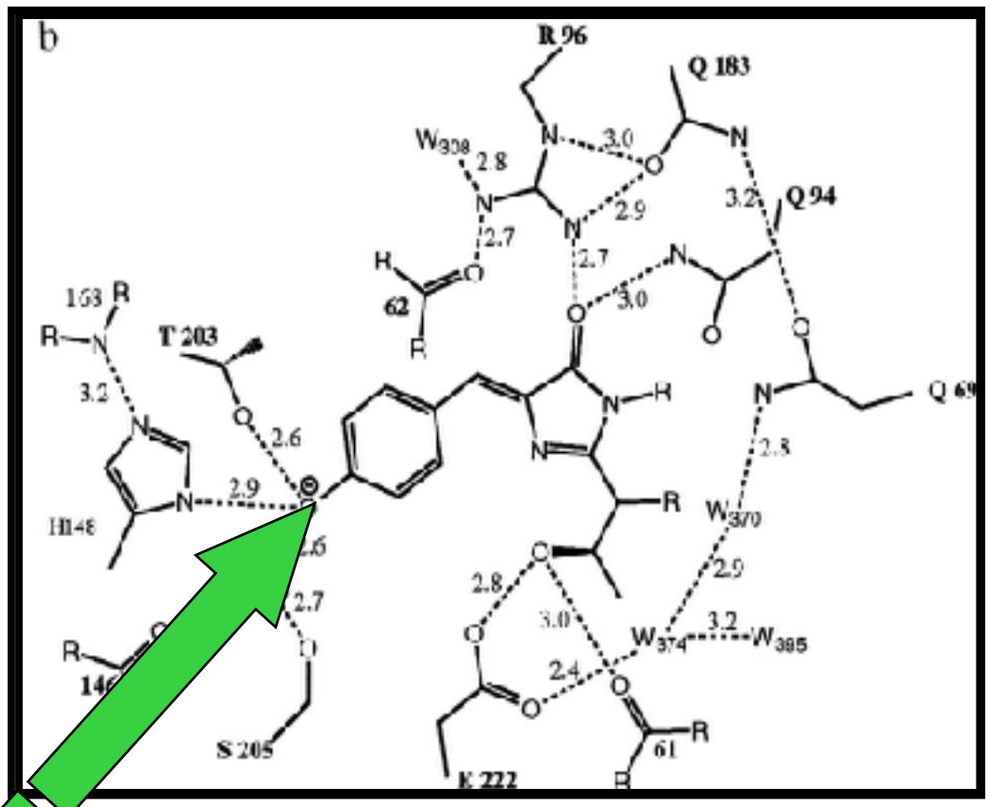
Cinelli RA, Ferrari A, Pellegrini V, Tyagi M, Giacca M, Beltram F., Photochem Photobiol. 2000 Jun;71(6):771-6.

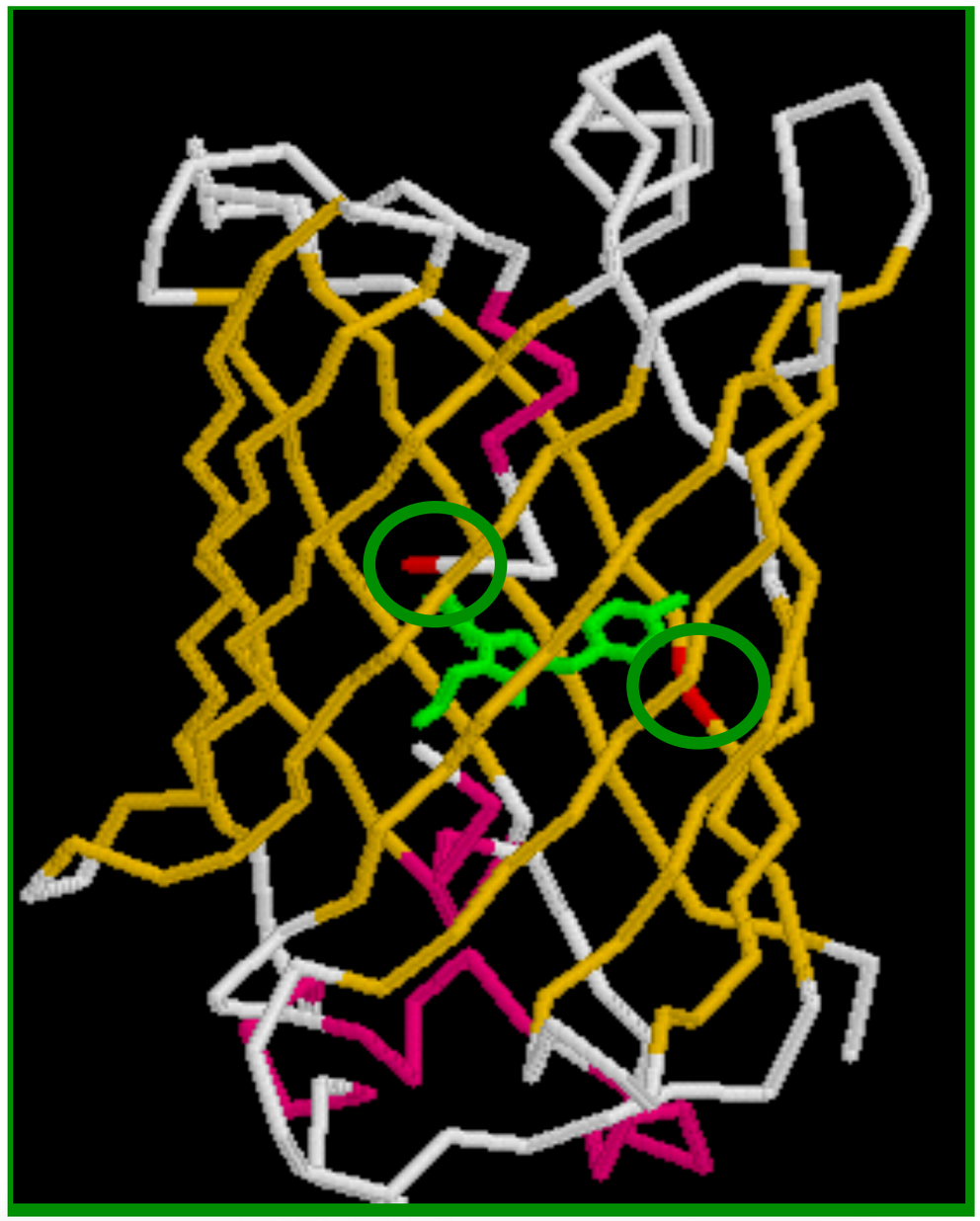
2PE and GFP

Switchable - GFP

E^2GFP

Mutations:
Ser65Thr + Phe64Leu
Thr203Tyr

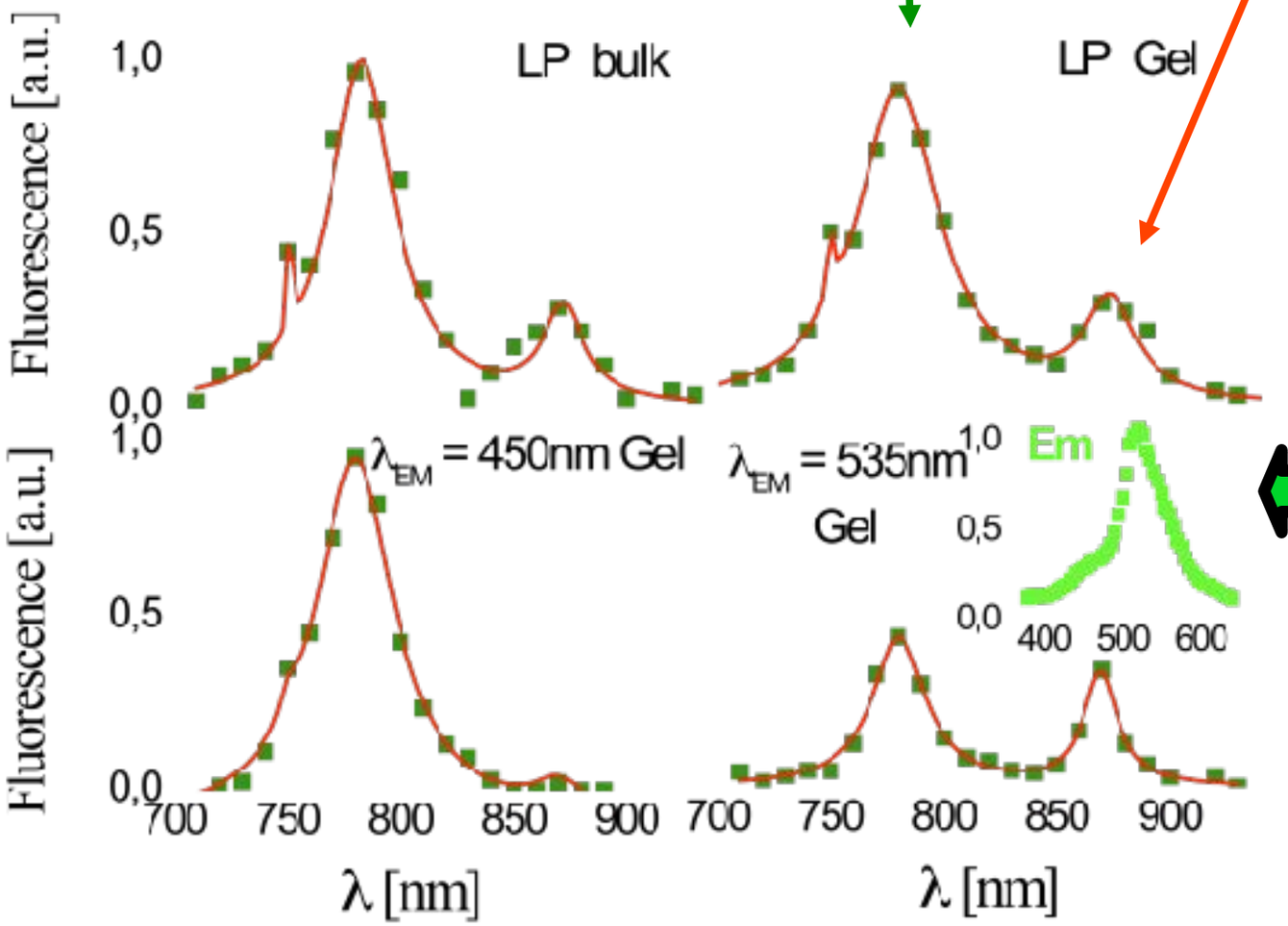




2PE and GFP

Two-Photon Spectra

Excitation Spectra: Maxima at 780 nm and 870 nm



Emission Spectrum

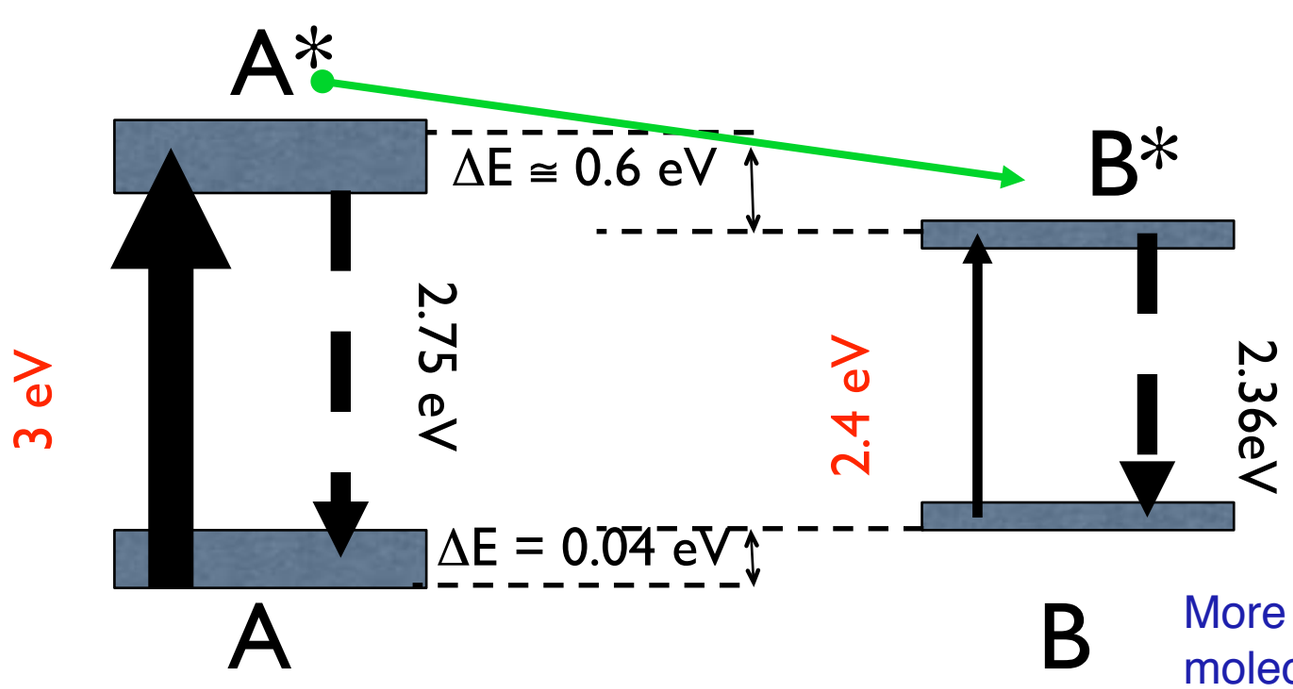
More than 600 molecules

2PE and GFP

Ground States

State A Exc 780 nm
Neutral Em 450 nm
Lifetime 1.0 ns

State B Exc 870 nm
Anionic Em 535 nm
Lifetime 2.8 ns

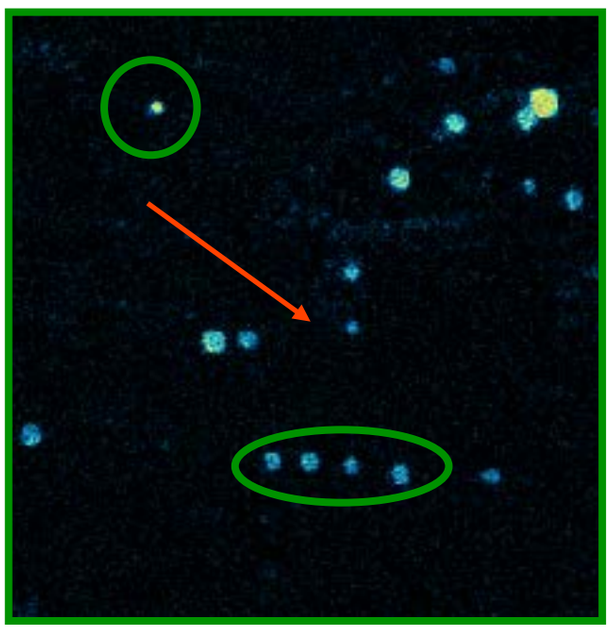


More than 600 molecules

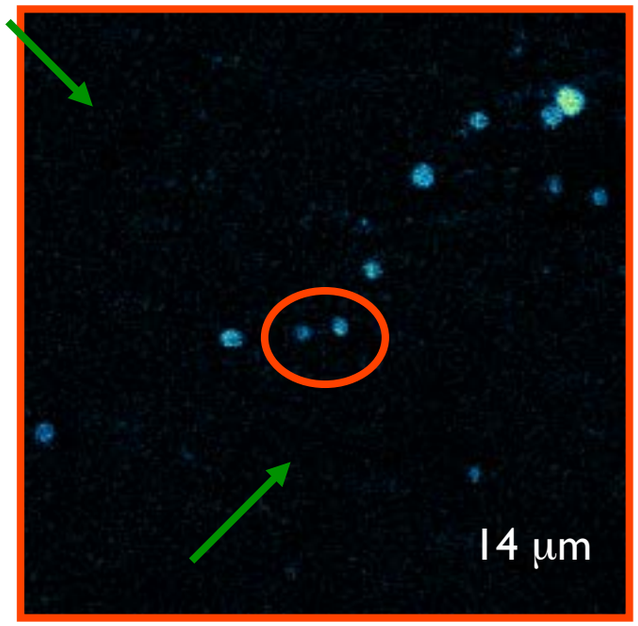
2PE and GFP

Ground States Distribution

$$n_A / n_B = 4.4 \pm 1.5$$



A



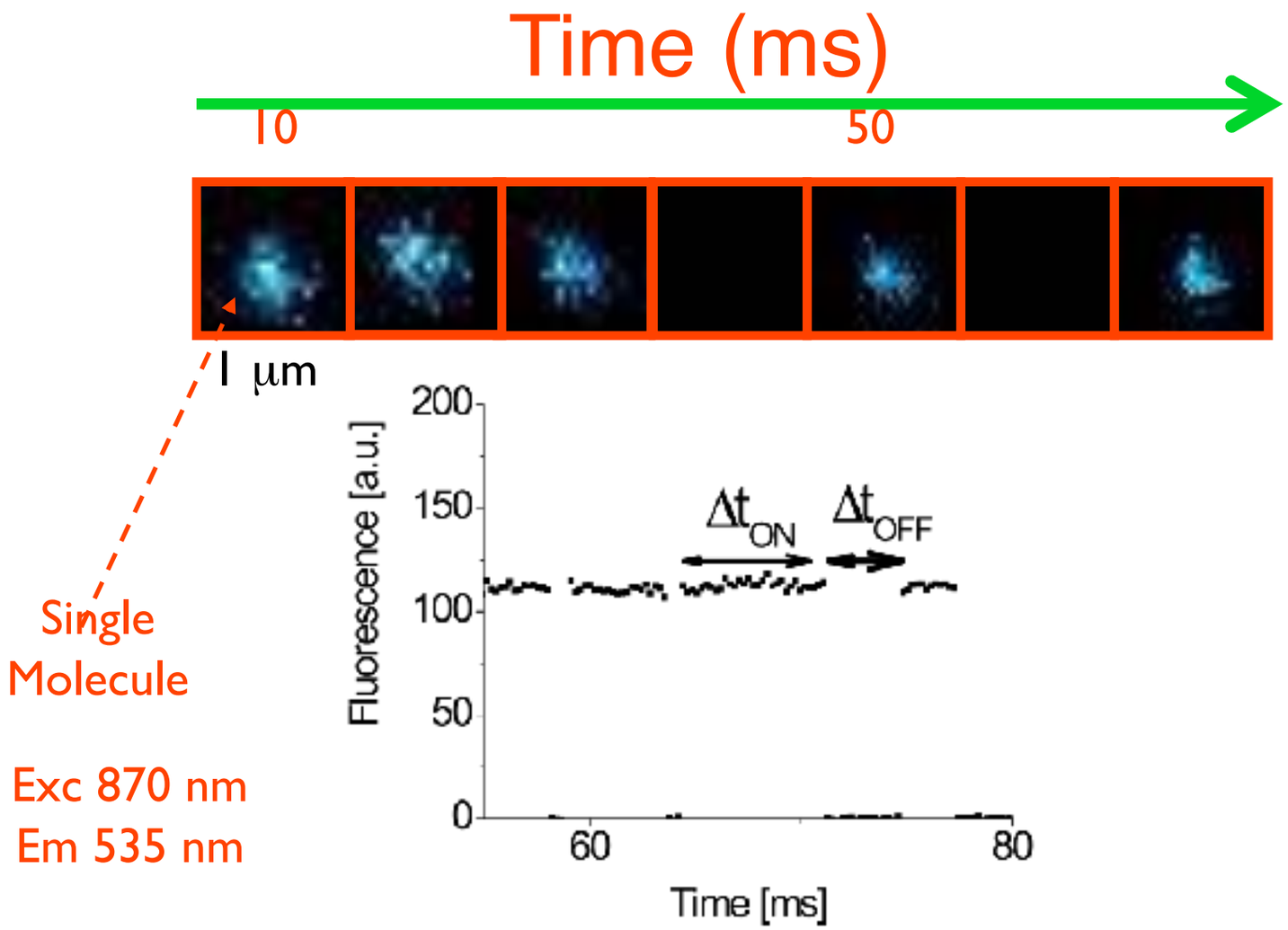
B

Exc 780 nm
Em 450 nm

Exc 870 nm
Em 535 nm

2PE and GFP

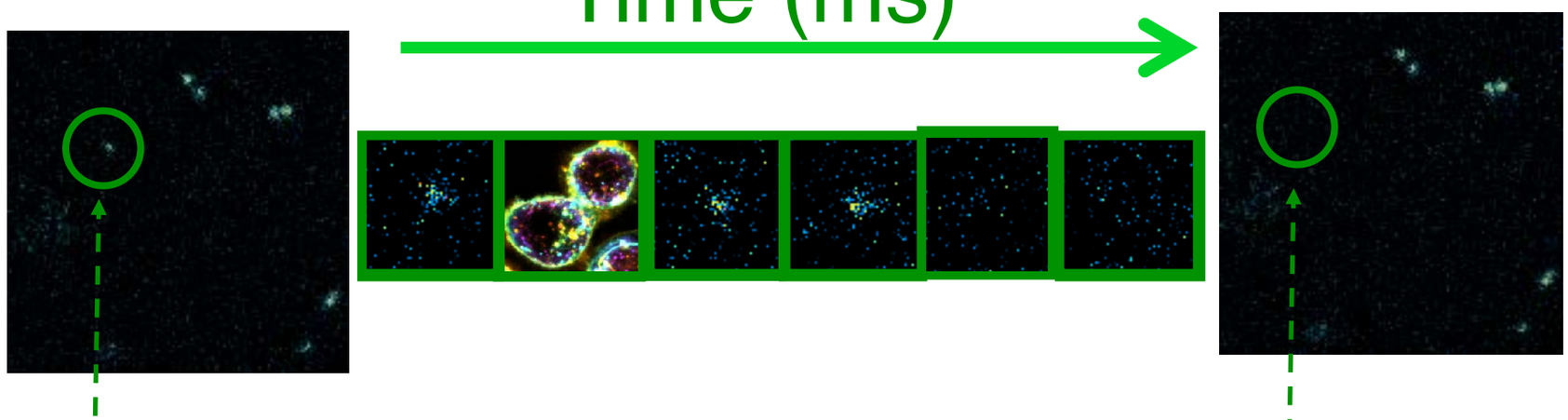
Blinking: State B



2PE and GFP

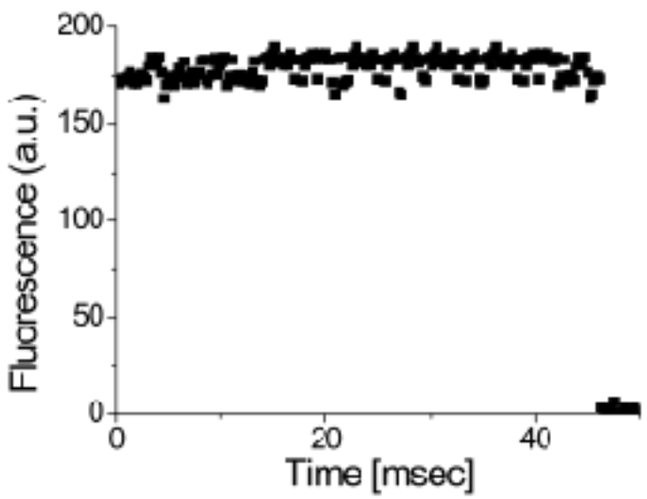
Bleaching: State A and B

Time (ms)



Single Molecule

Exc 780 nm
Em 450 nm



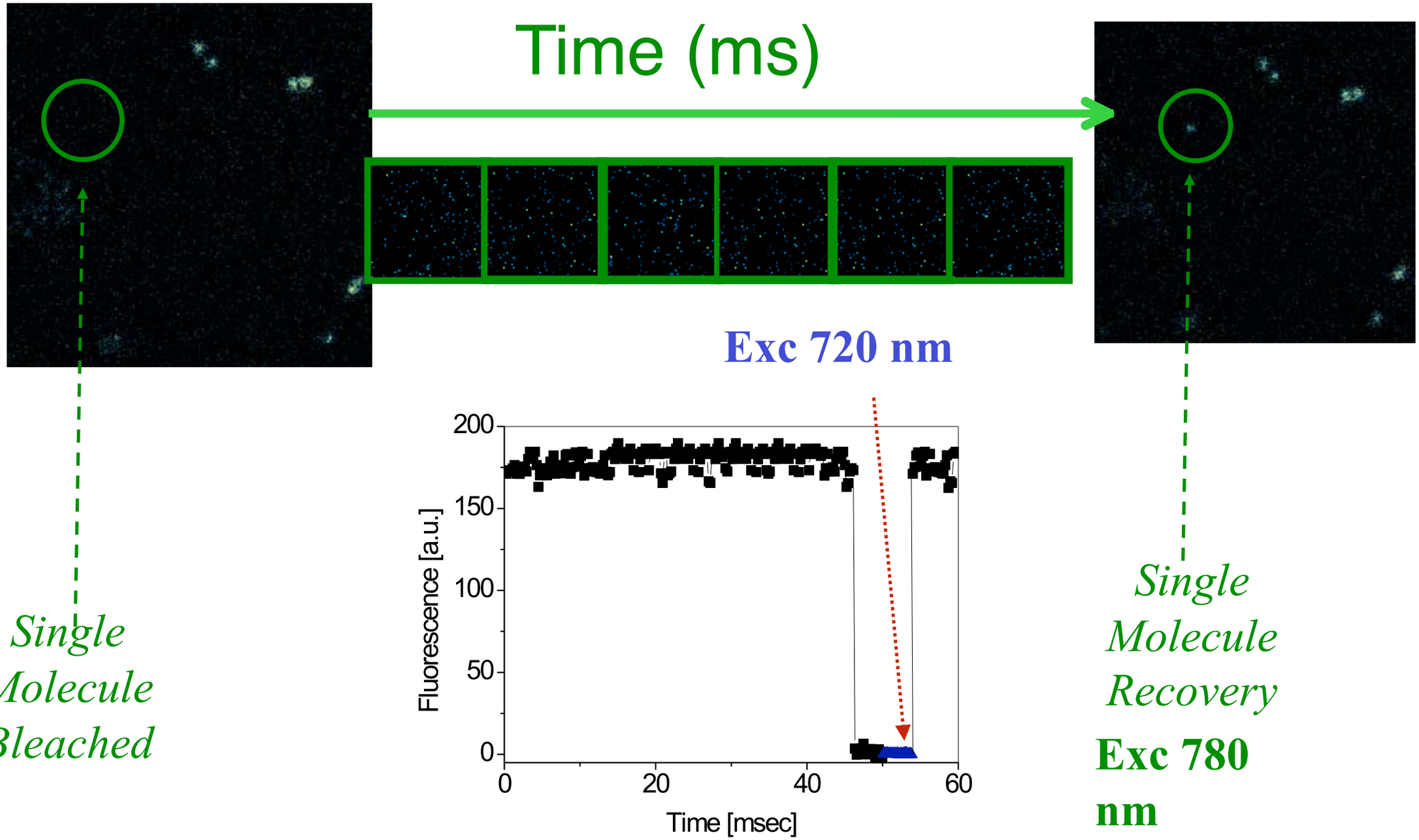
Single Molecule Bleached

reversible ???

Slide credit:Fabio Cannone, Milano-Bicocca

2PE and GFP

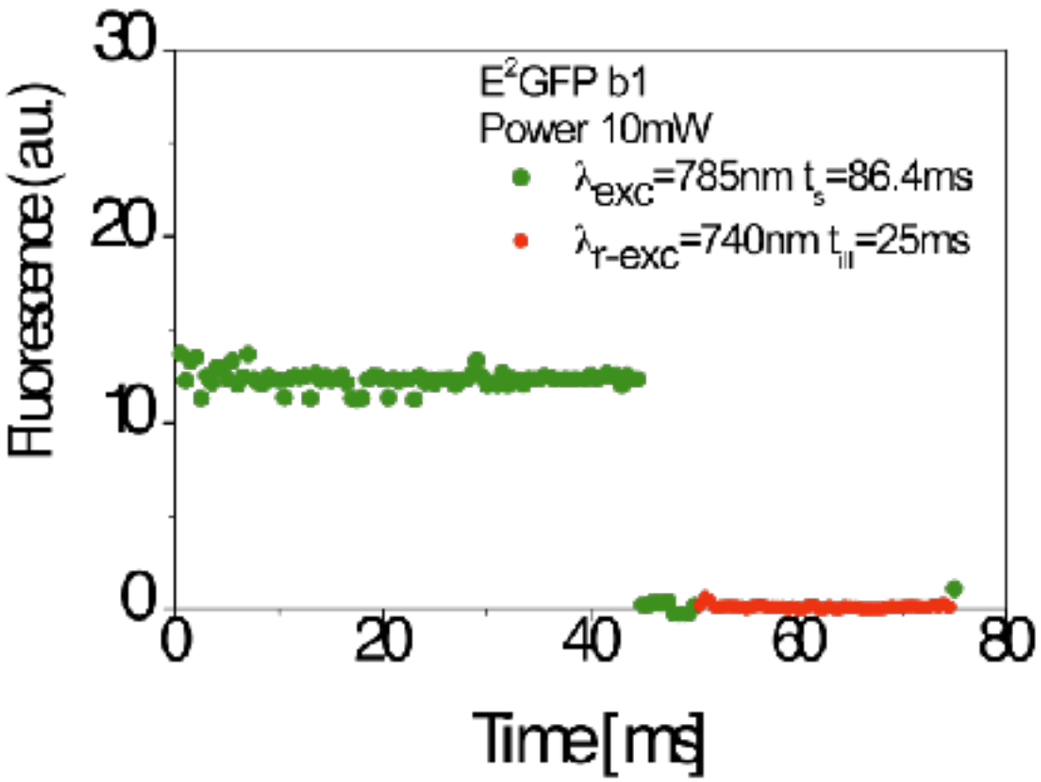
Fluorescence recovery

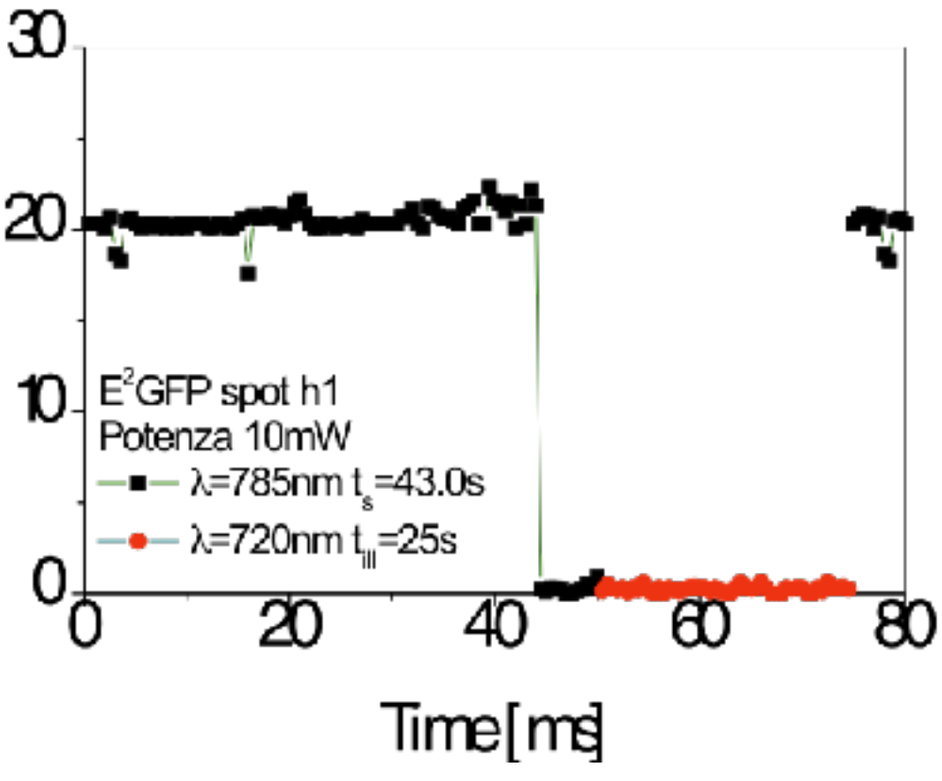


Slide credit:Fabio Cannone, Milano-Bicocca

2PE and GFP

Fluorescence Recovery

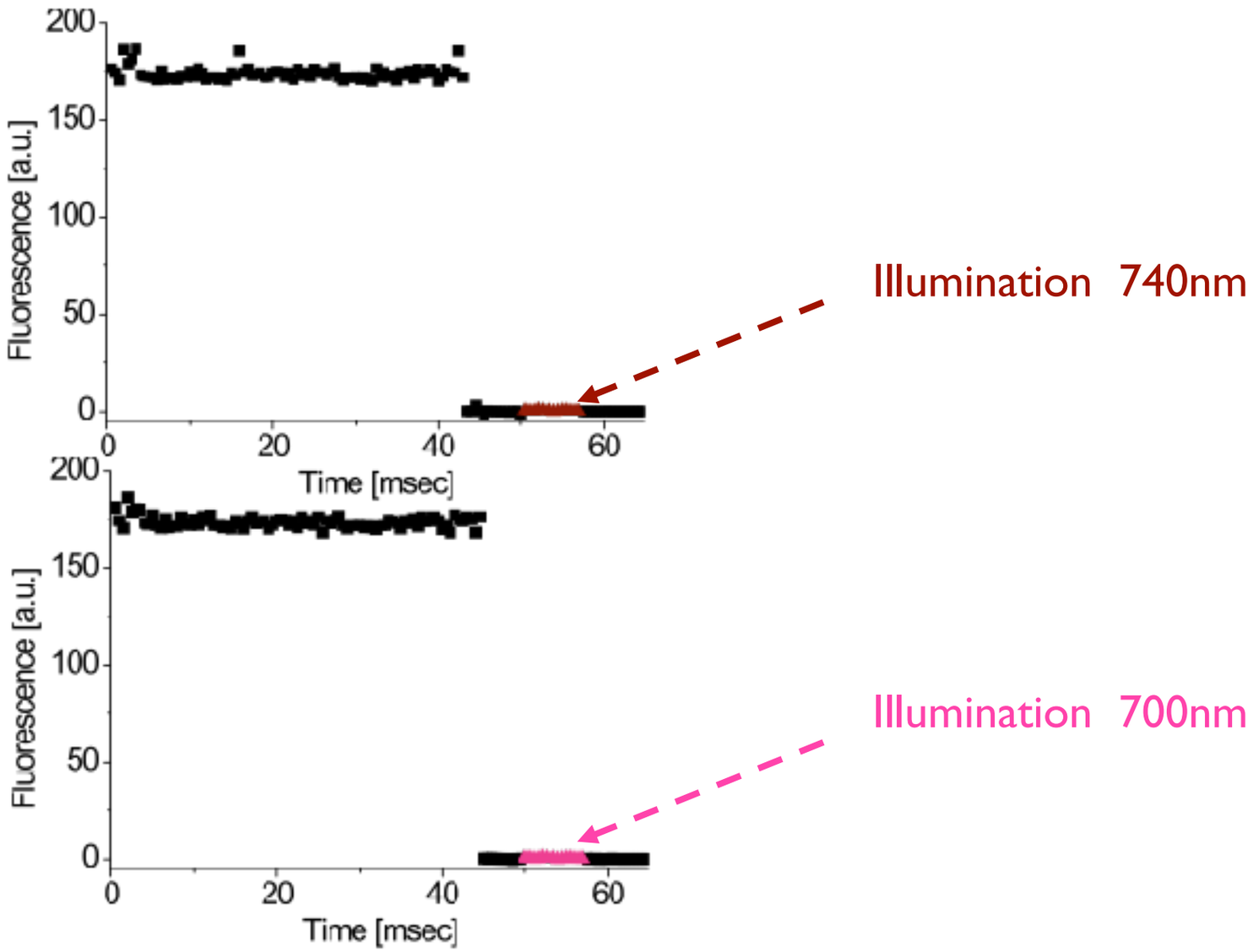




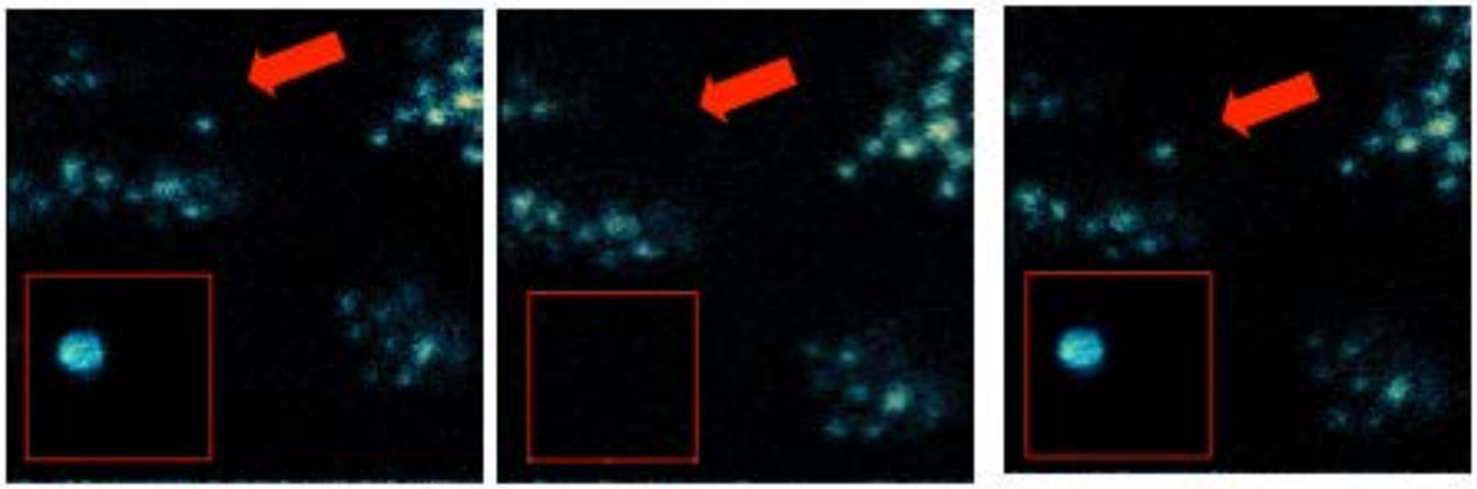
E^2GFP

2PE and GFP

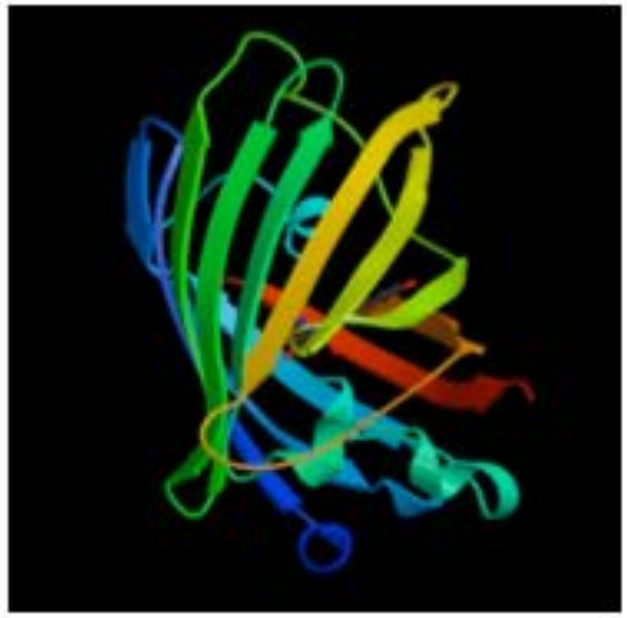
Fluorescence Recovery



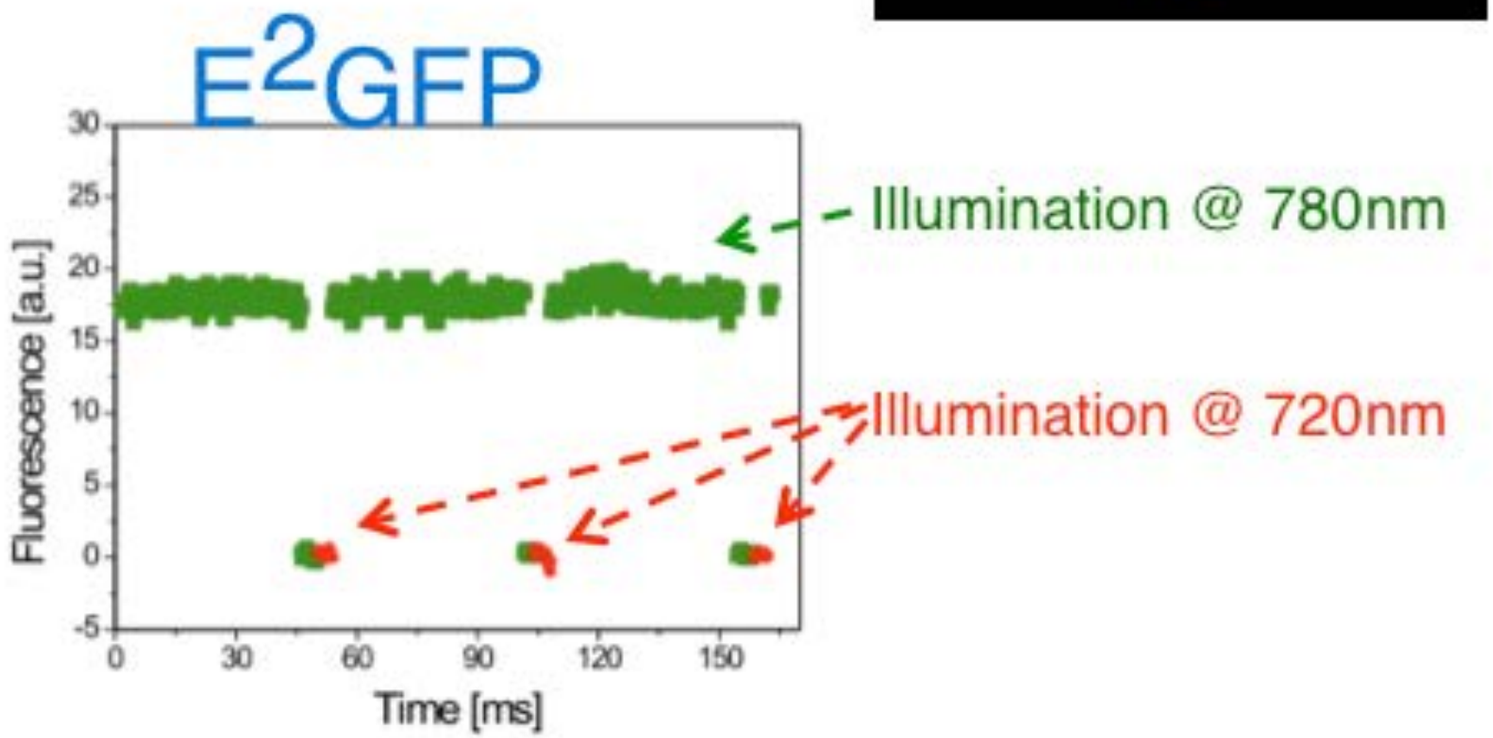
2PE and GFP



TPE @ 785 nm TPE @ 720 nm TPE @ 785 nm



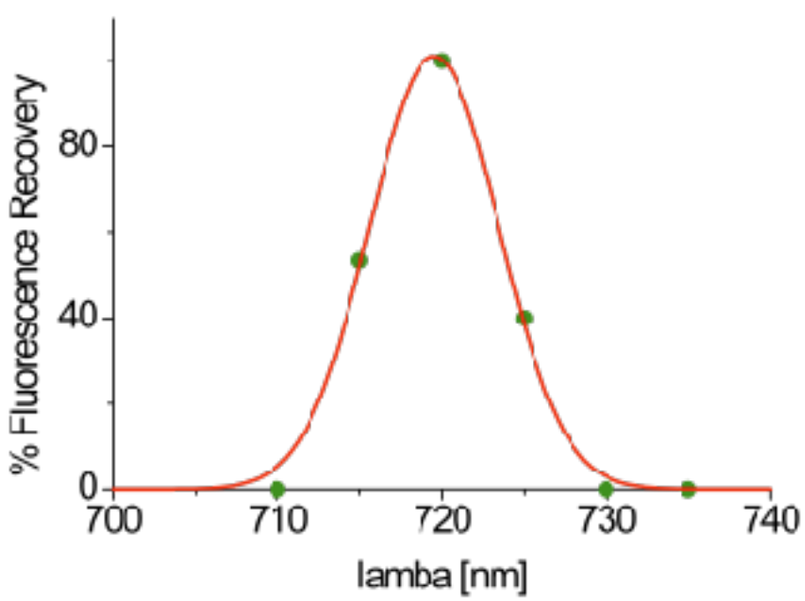
E^2GFP in silica GEL, maximum excitation within 700-800 nm range @ 785; after imaging photobleaching @ 785; photocycling @ 720 nm - no photorecovery @ 710/730 -; final imaging @ 785.

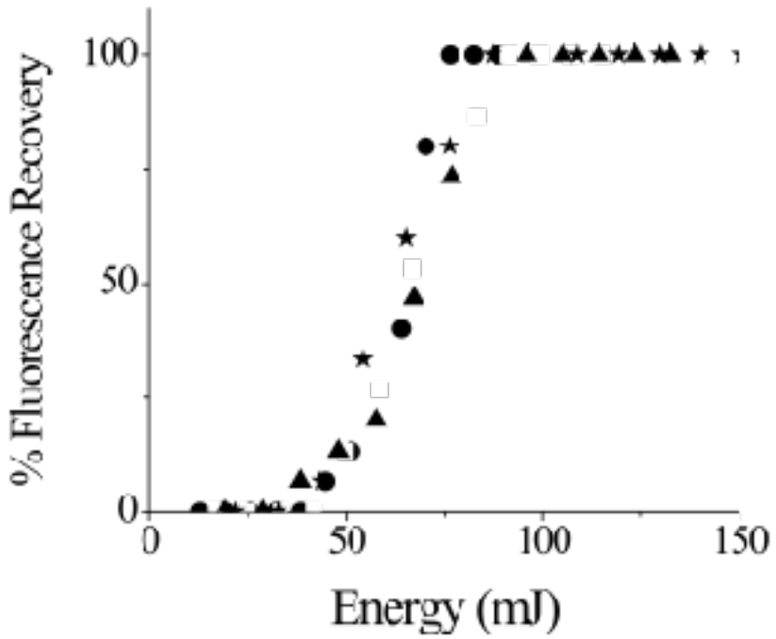


PHYS REV E STAT NONLIN SOFT MATTER PHYS. 2004 SEP;70(3 PT 1):030901

2PE and GFP

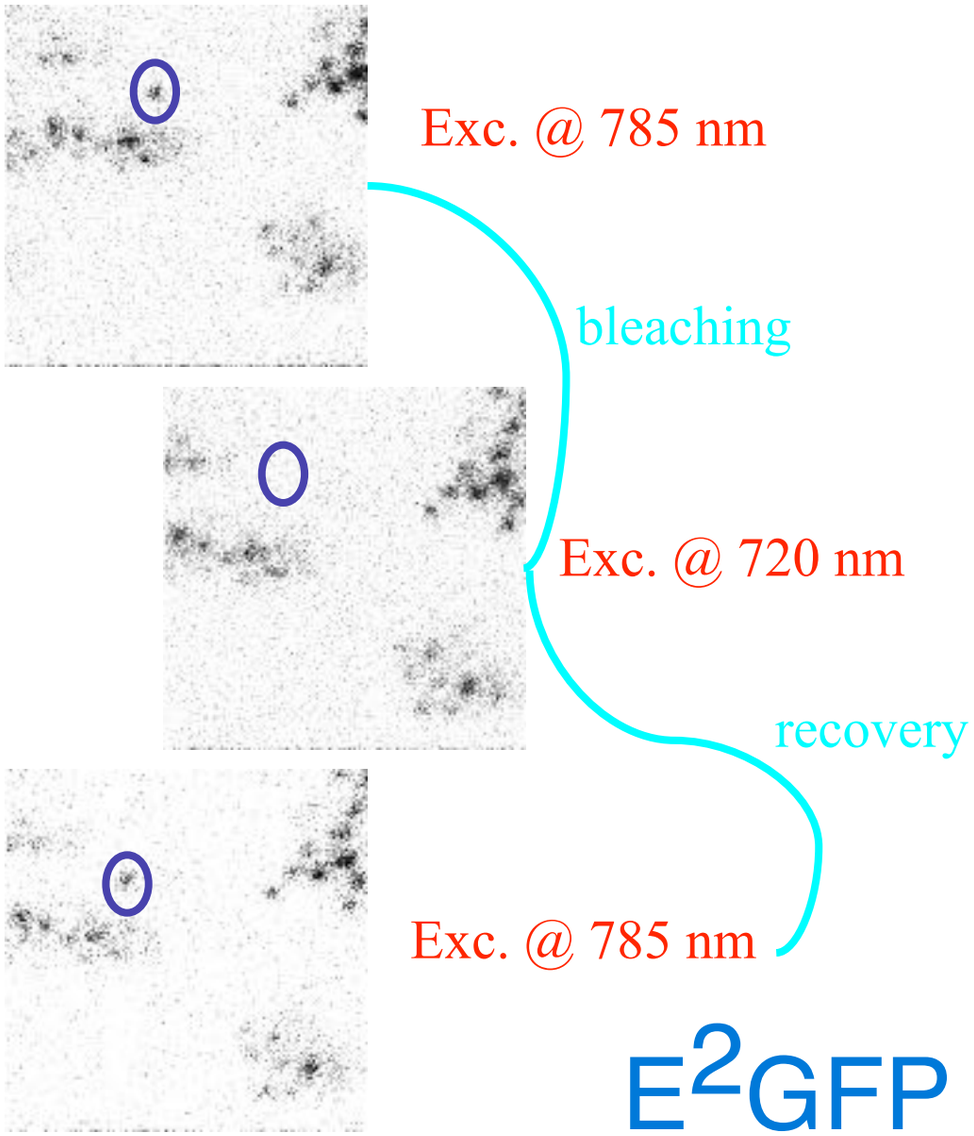
Recovery Threshold Energy

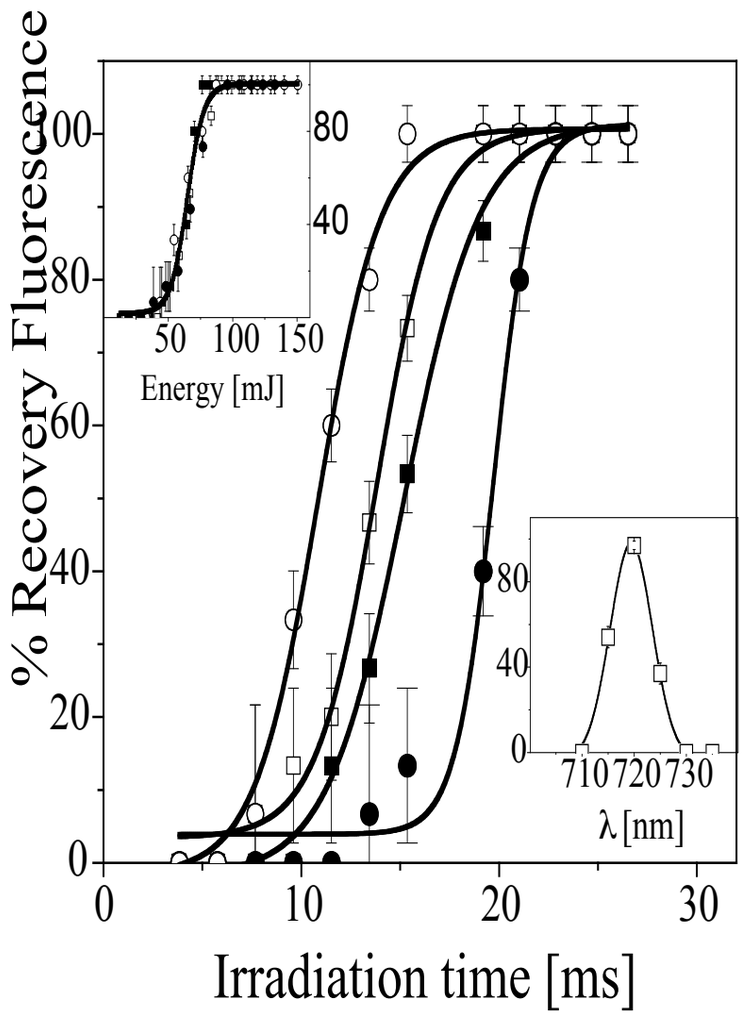




$E = P_{720} \cdot \Delta t$ = power × (illumination time at 720 nm)

2PE and GFP

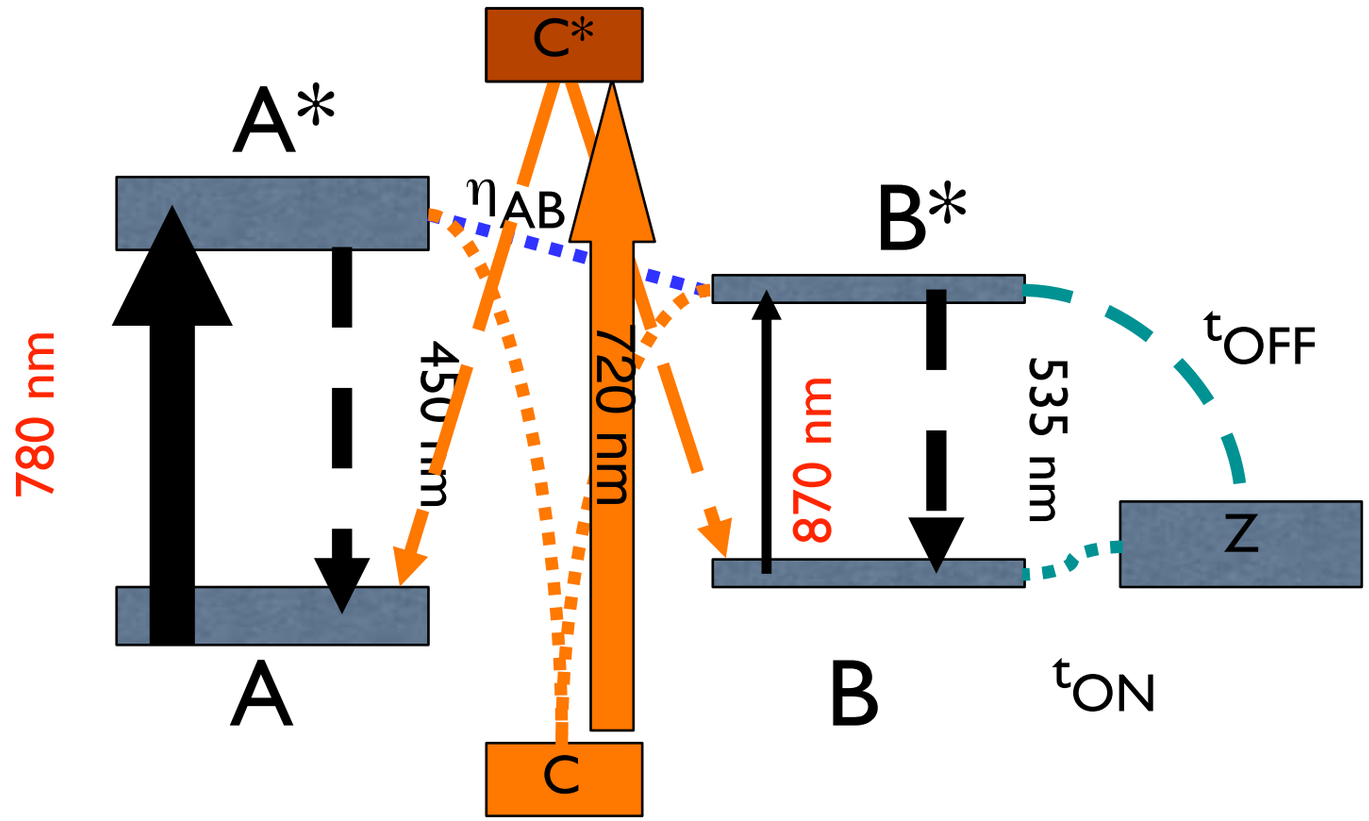




Slide credit:Giberto Chirico, Milano-Bicocca

2PE and GFP

Energy Level Diagram



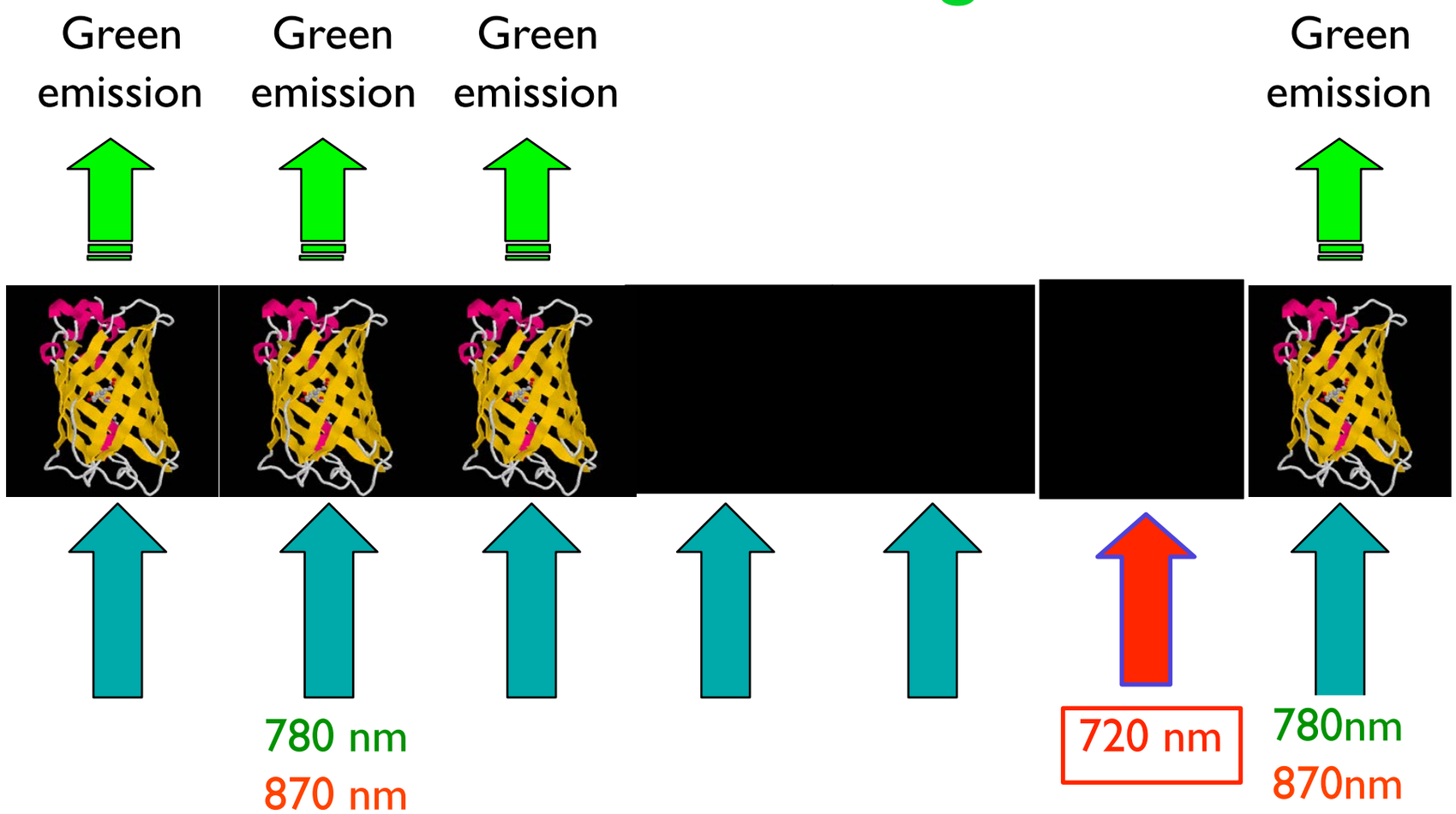
Phys Rev E Stat Nonlin Soft Matter Phys. 2004 Sep;70(3 Pt 1):030901

Slide credit:Giberto Chirico, Milano-Bicocca

2PE and GFP

E^2GFP

Two Photon Switching Protein



Switchable - GFP - Single molecule

Phys Rev E Stat Nonlin Soft Matter Phys. 2004 Sep;70(3 Pt 1):030901

2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

1346 Biophysical Journal Volume 89 August 2005 1346–1352

Two-Photon Activation and Excitation Properties of PA-GFP in the 720–920-nm Region

Marc Schneider,* Sara Barozzi,† Ilaria Testa,*‡ Mario Faretta,† and Alberto Diaspro*‡§

*INFM Genoa, Department of Physics, University of Genoa, I-16146 Genoa, Italy; †European Institute of Oncology, Department of Experimental Oncology, 20141 Milan, Italy; ‡LAMBS-MicroScoBio Research Center, University of Genoa, 16146 Genoa, Italy; and §IFOM, Istituto FIRC di Oncologia Molecolare, 20139 Milan, Italy

ABSTRACT This report covers the two-photon activation and excitation properties of the PA-GFP, a photoactivatable variant of the *Aequorea victoria* green fluorescent protein in the spectral region from 720 to 920 nm. It is known from this special form of the molecule that it has an increased level of fluorescence emission when excited at 488 nm after irradiation at $\lambda \sim 413$ nm, under single-photon excitation conditions. Here, we show that upon two-photon irradiation, PA-GFP yields activation in the spectral region from 720 to 840 nm. After photoactivation, the excitation spectrum shifts maintaining the very same emission spectrum of the single-photon case for the native and photoactivated protein. Additionally, when comparing the conventional photoactivation at $\lambda = 405$ nm with a two-photon one, a sharper and better controllable three-dimensional volume of activation is obtained.

Submitted October 14, 2004, and accepted for publication May 16, 2005.

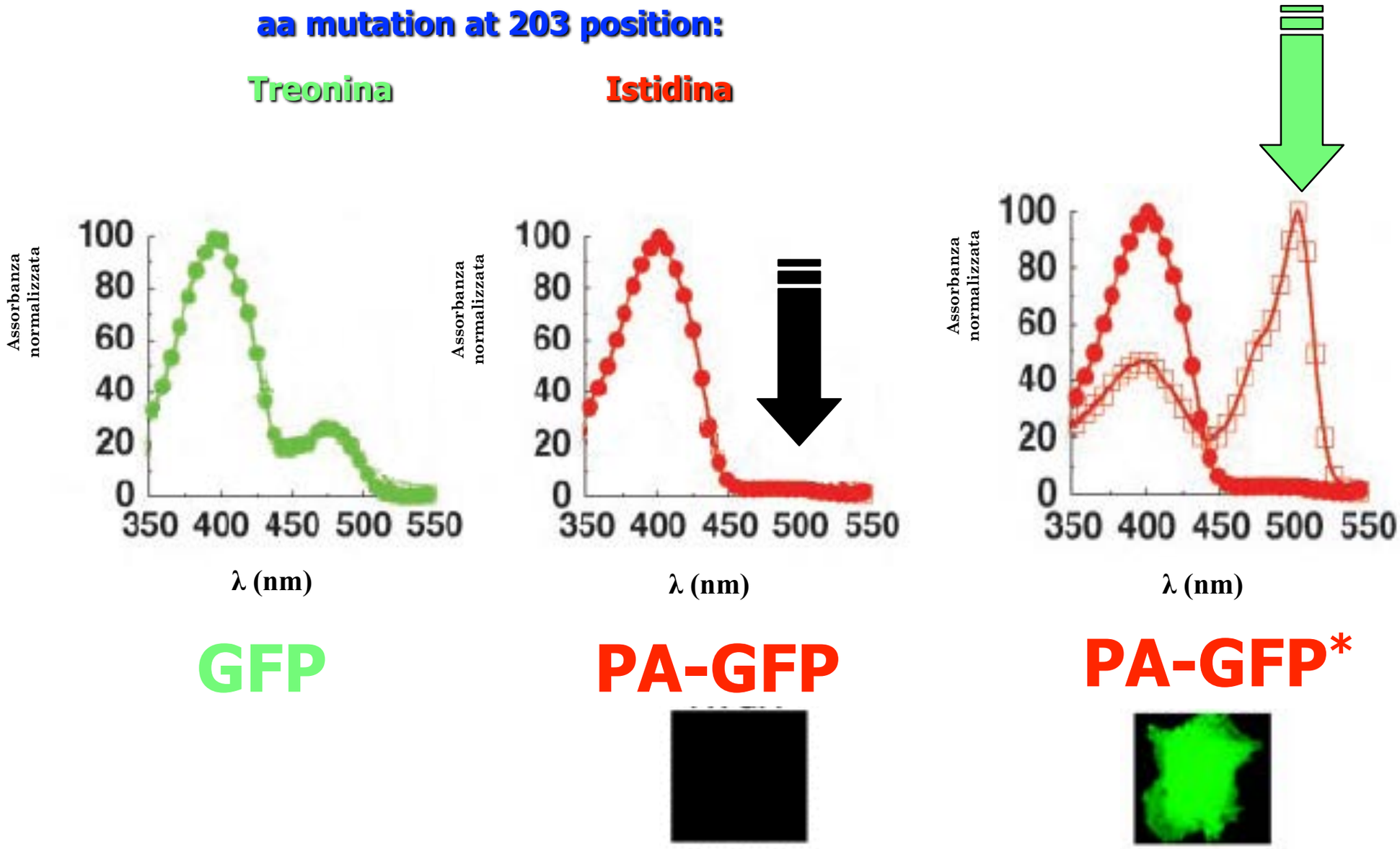
2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

Photoactivatable - Protein

aa mutation at 203 position:

Treonina **Istidina**



GFP **PA-GFP** **PA-GFP***

Patterson GH, Lippincott-Schwarz J. (2002) Science 297:1873-1877.

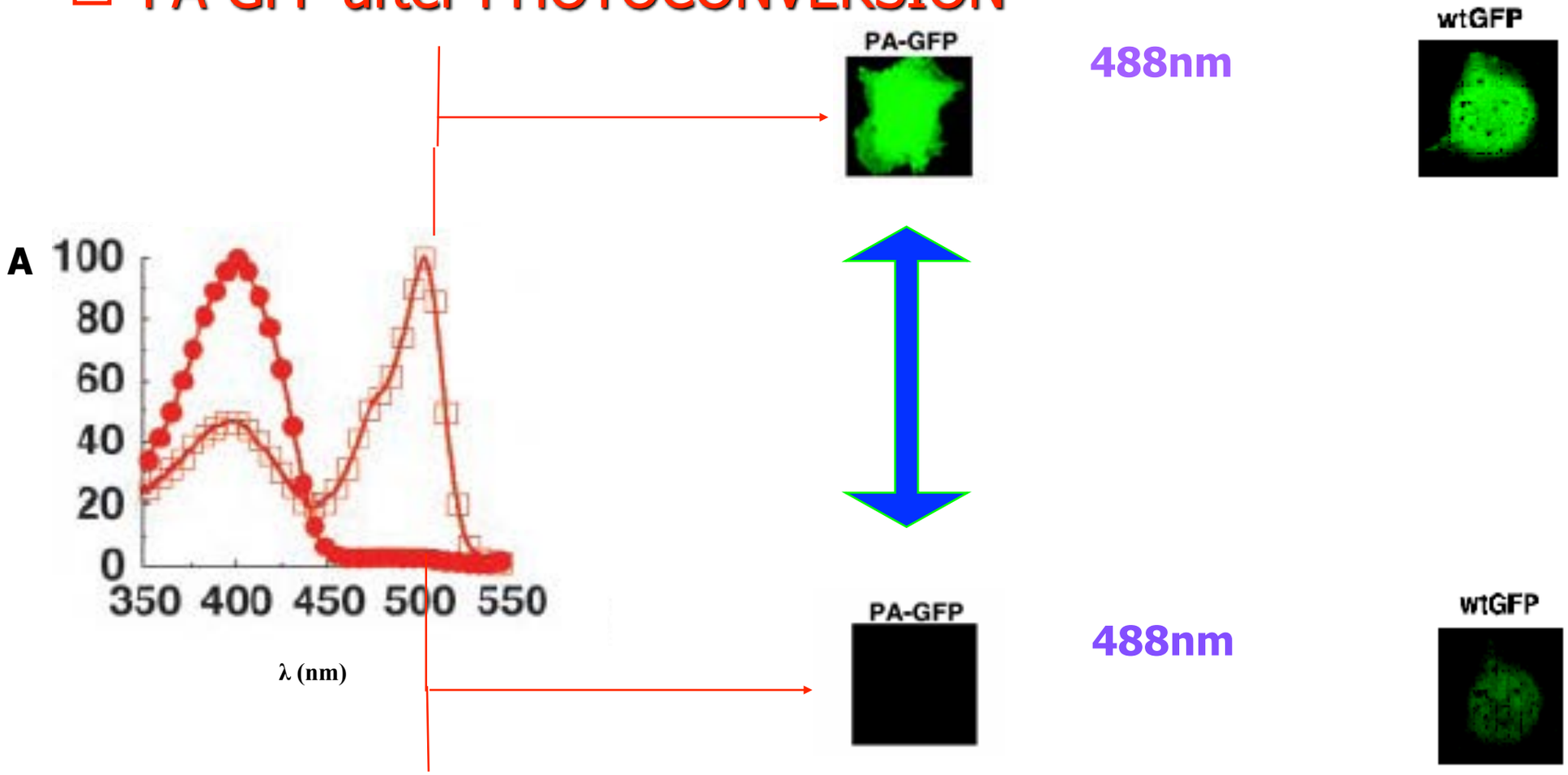
2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

Photoactivatable - Protein

What's new is the extraordinary enhancement achieved: wt-GFP shows a 2-3 fold increase in fluorescence after activation, PA-GFP fluorescence increases 50-100 fold.

- PA-GFP after PHOTOCONVERSION

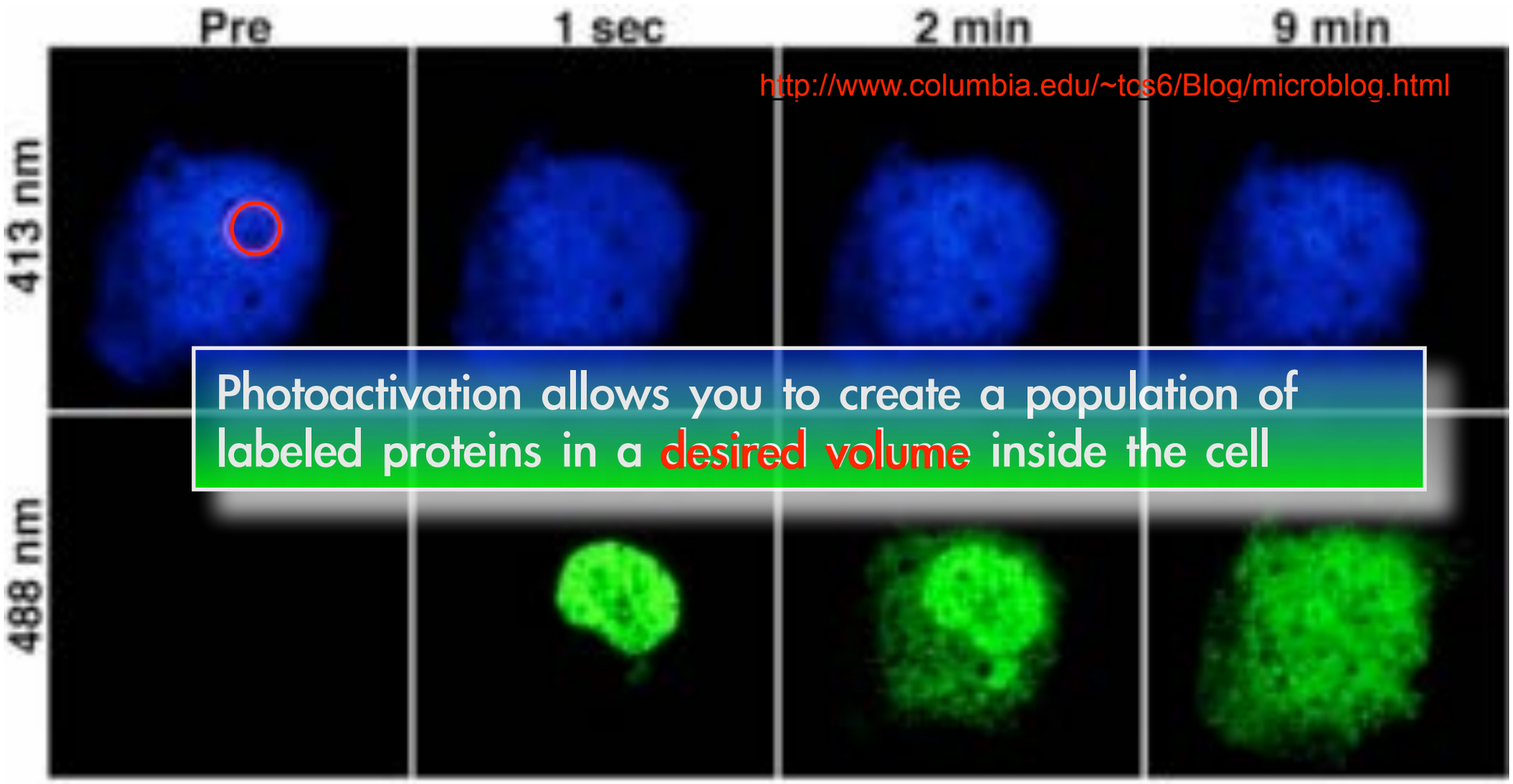


- PA-GFP before PHOTOCONVERSION

2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

Photoactivatable - Protein



The area inside the red circle, which is within the cell nucleus, is photoactivated for ~1 s at 413 nm. After 1 second, GFP is seen throughout the nucleus. Between 2 and 9 minutes later, much of the GFP has distributed throughout the cytoplasm.

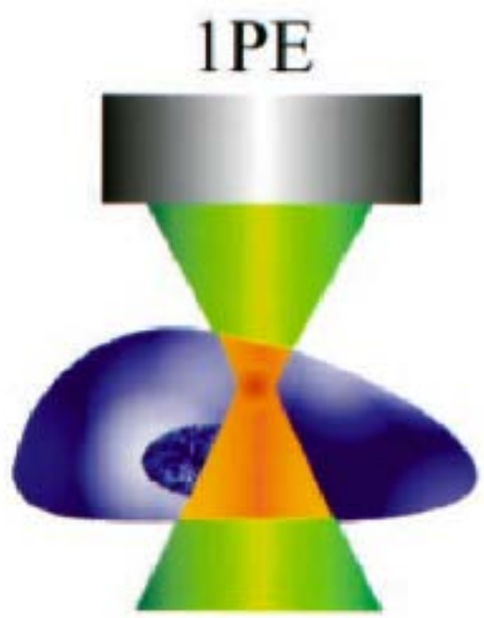
Patterson GH, Lippincott-Schwarz J. (2002) Science 297:1873-1877.

2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

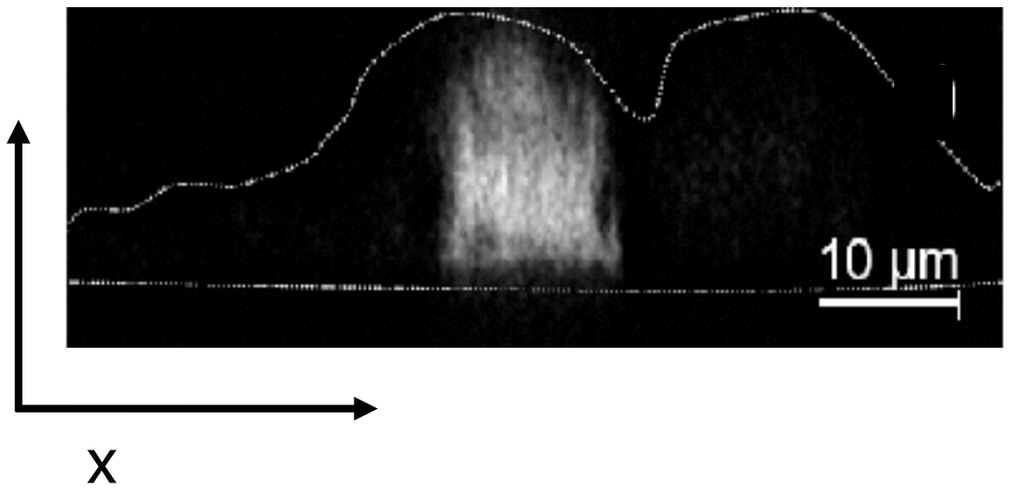
Single-photon interaction

FLUORESCENCE MICROSCOPY



SINGLE-PHOTON ACTIVATION

UV-Vis
405-413 nm



SCHNEIDER M., BAROZZI S., TESTA I., FARETTA M., DIASPRO A. (2005) BIOPHYS. J., vol.89(2)

2PE and GFP PA-GFP

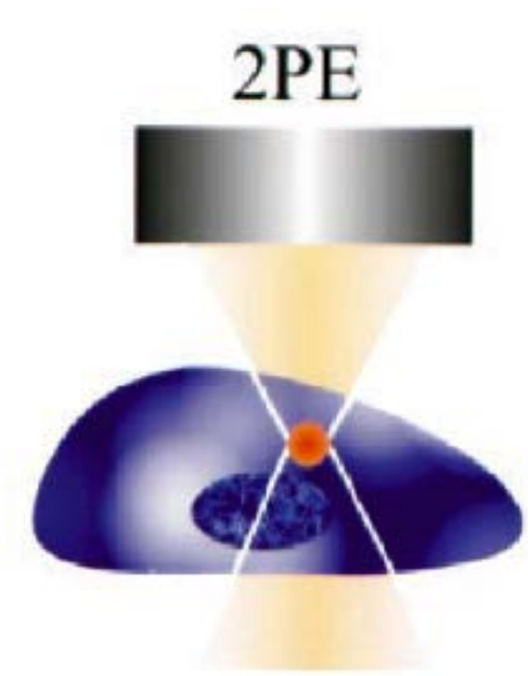
(*green fluorescent protein→photo activatable green fluorescent protein)*

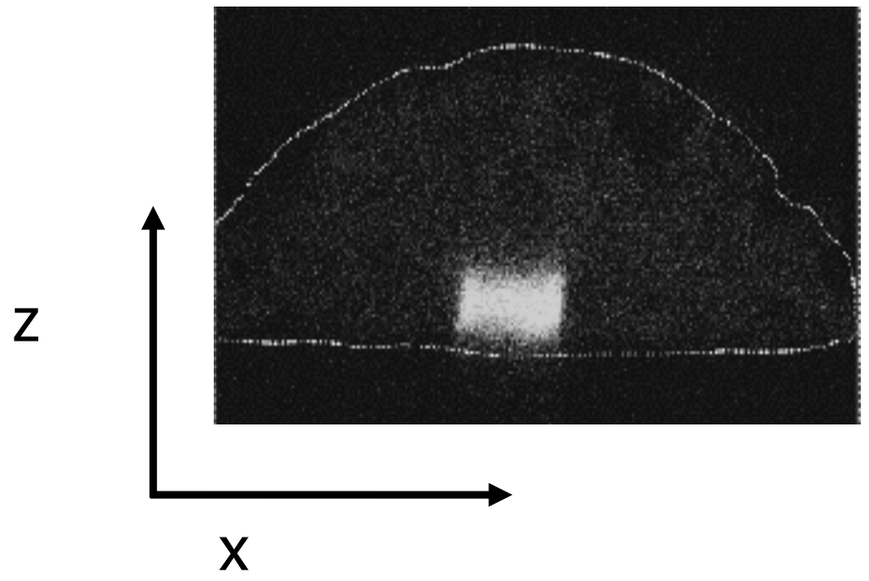
Two-photon interaction

TWO-PHOTON EXCITATION MICROSCOPY

IR-NIR

TWO-PHOTON ACTIVATION





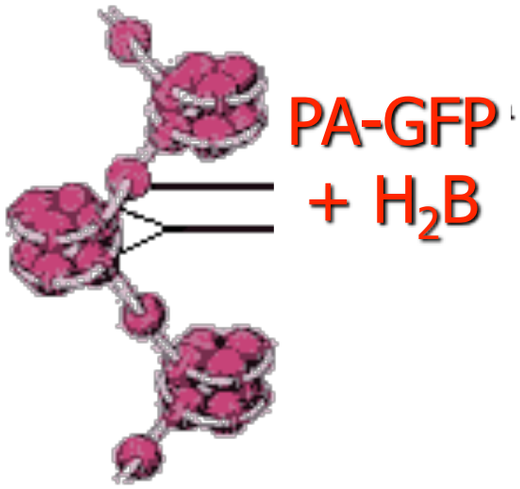
SCHNEIDER M., BAROZZI S., TESTA I., FARETTA M., DIASPRO A. (2005) BIOPHYS. J., vol.89(2)

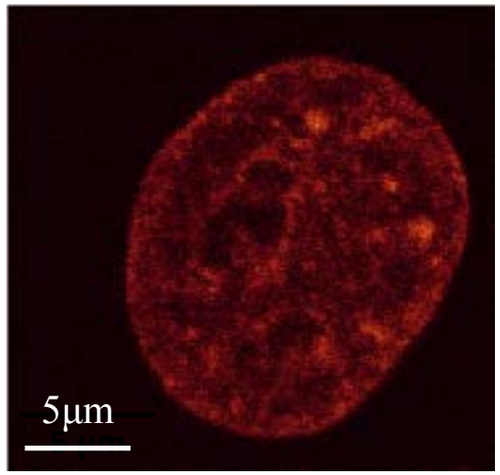
2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

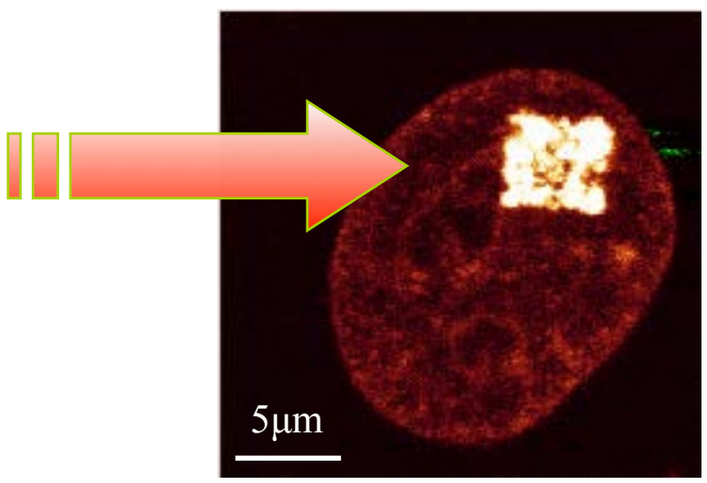
4D (x-y-z-t) switching

TAILOR YOUR OWN SET OF GFPs INTO THE CELL...TRACK IT!





PRE / λ_{vis}= 488 nm



POST / λ_{vis}= 488 nm

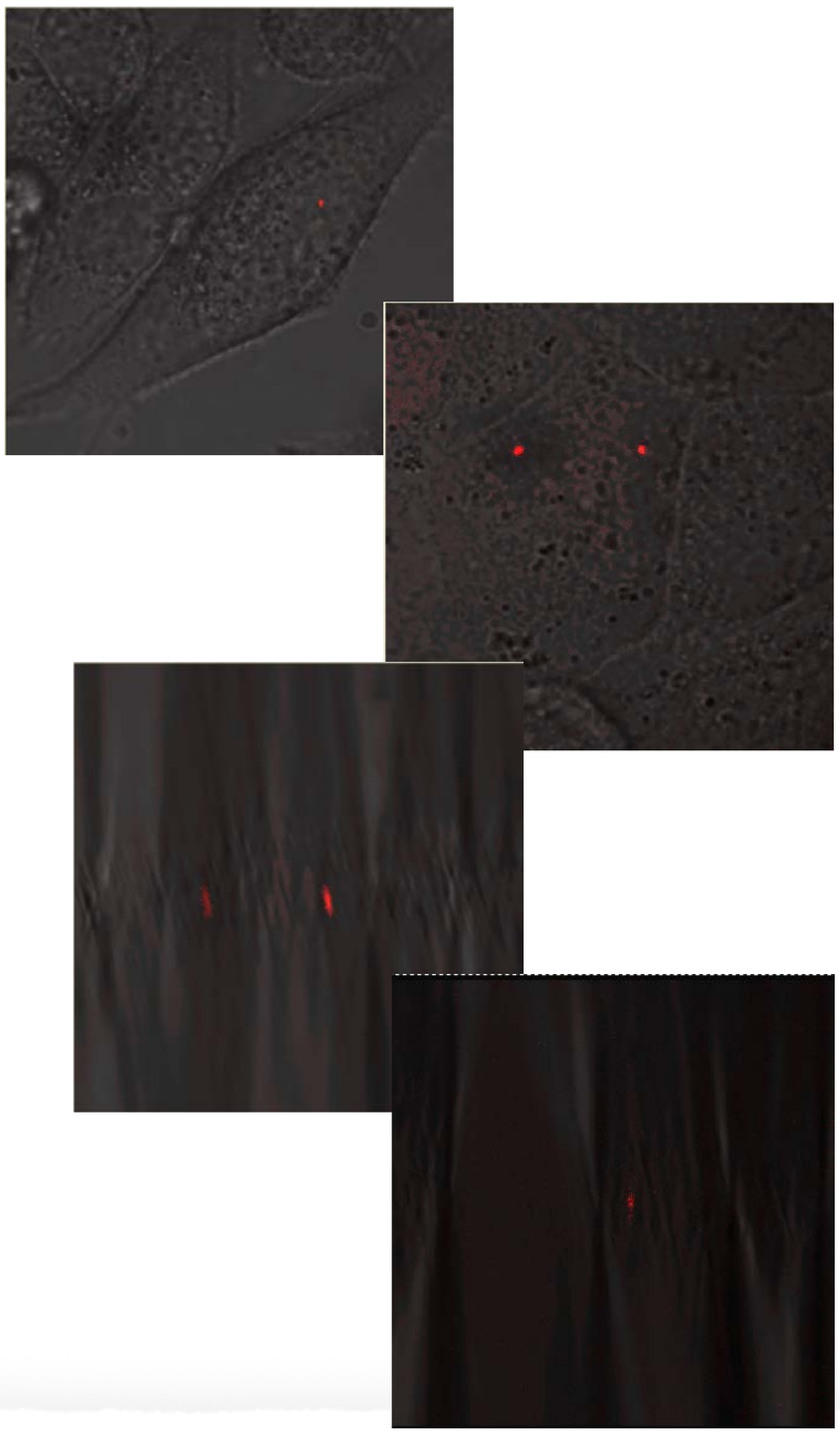
PA into He-La

cell nucleus

2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

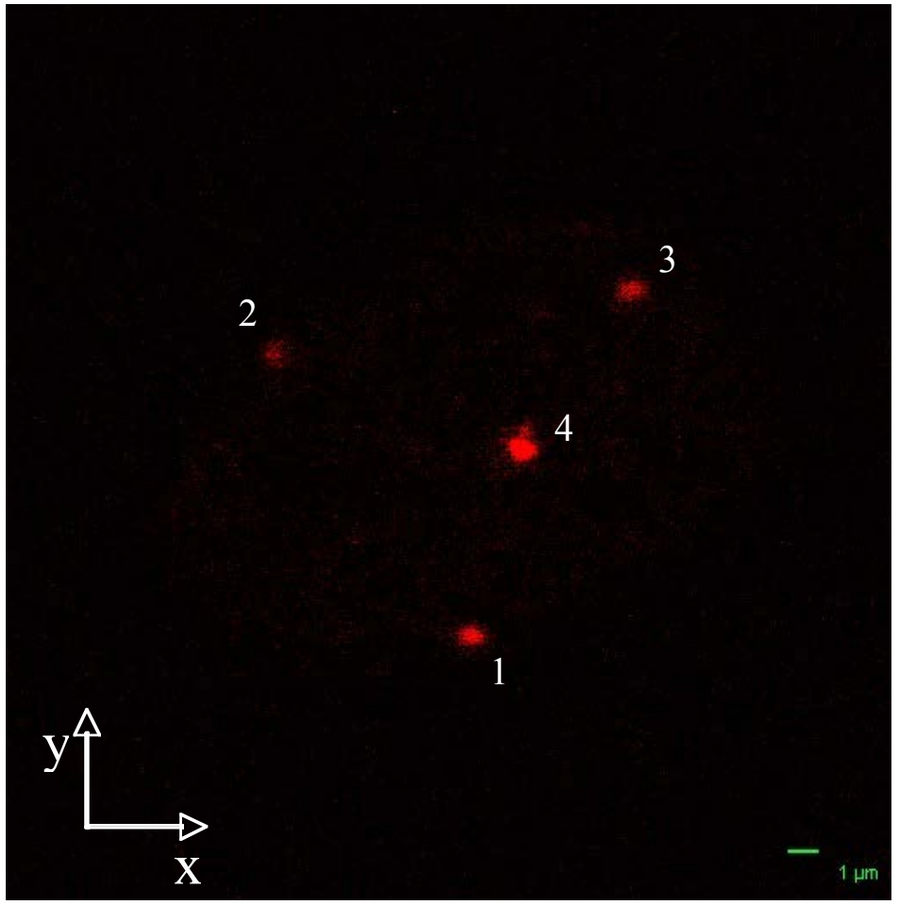
4D (x-y-z-t) switching

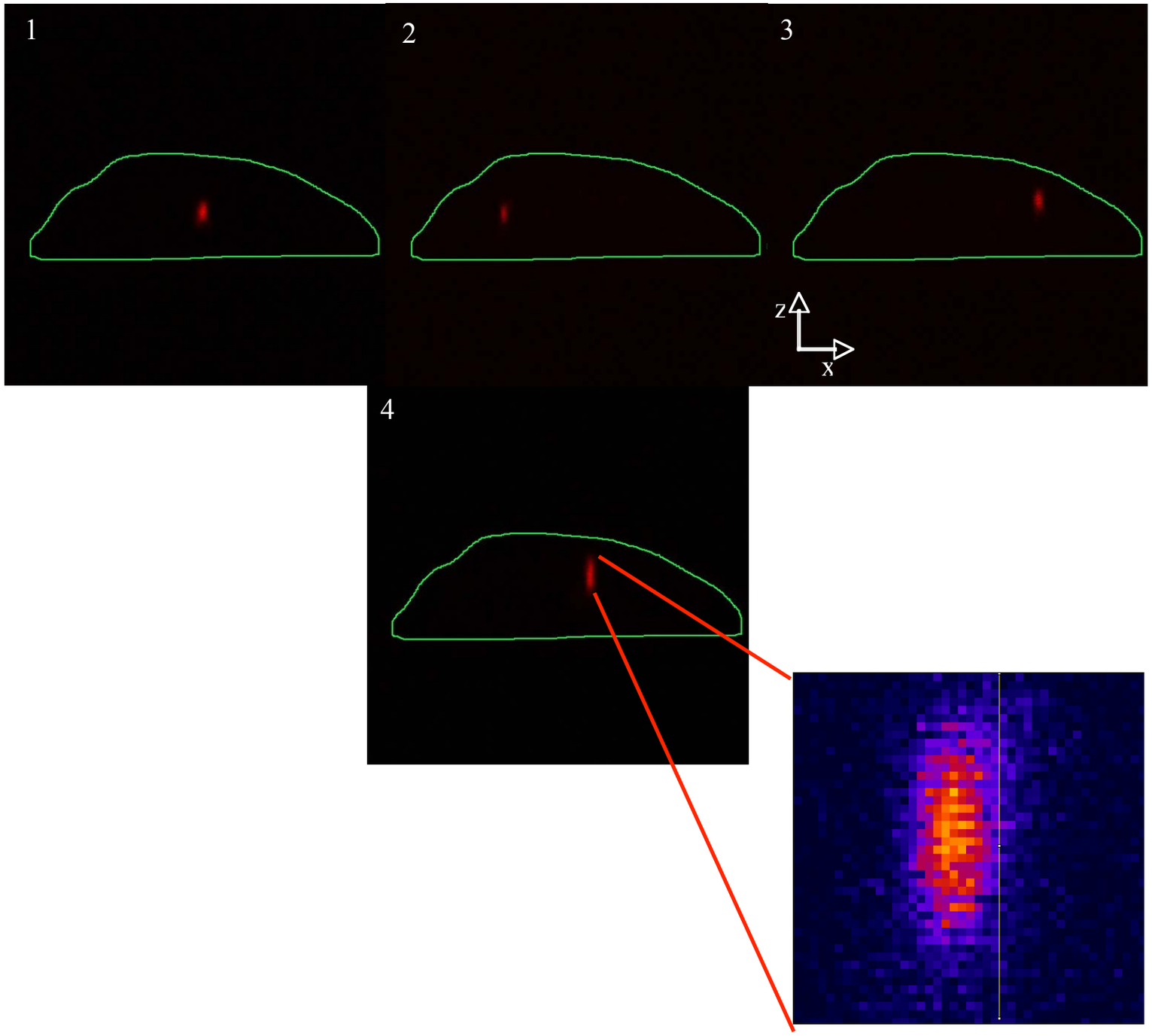


2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

4D (x-y-z-t) switching



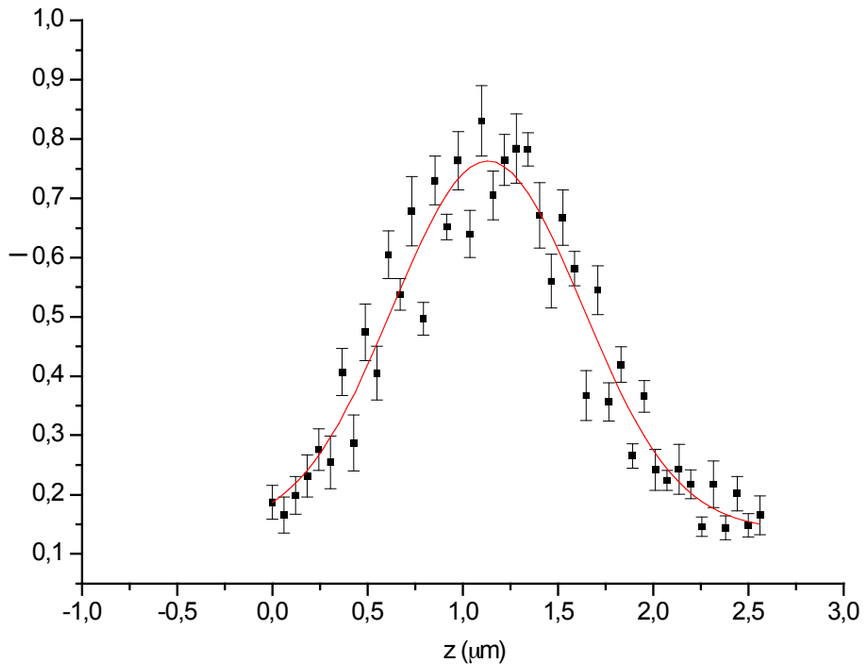


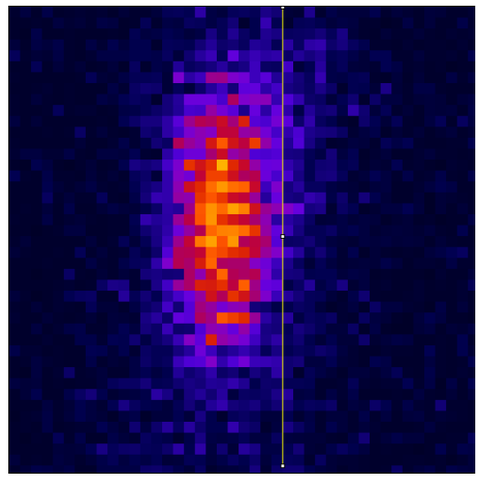
Ilaria Testa - LAMBS 2006

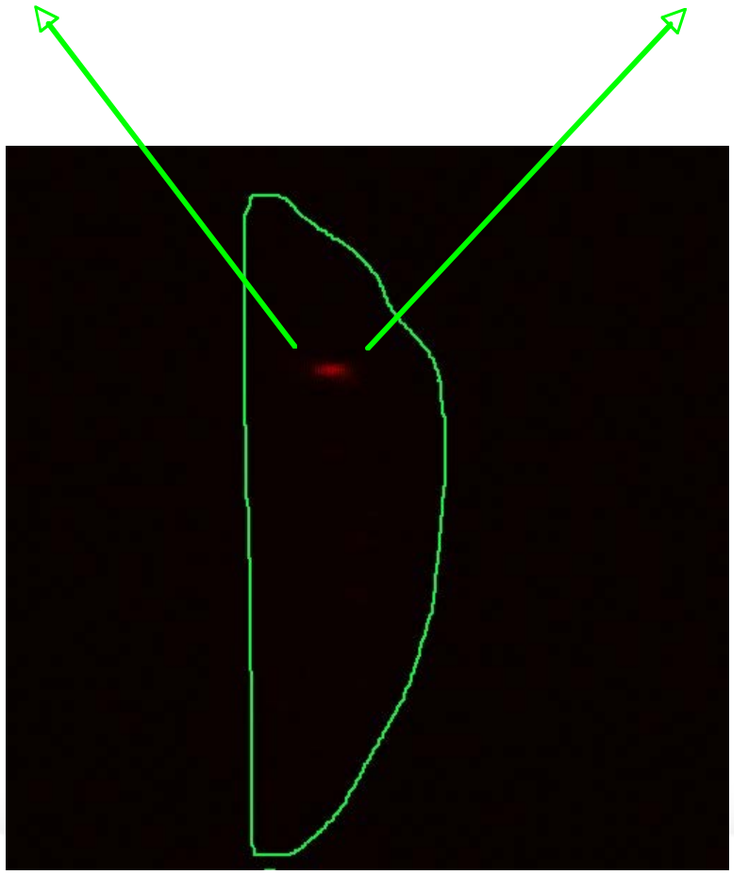
2PE and GFP PA-GFP

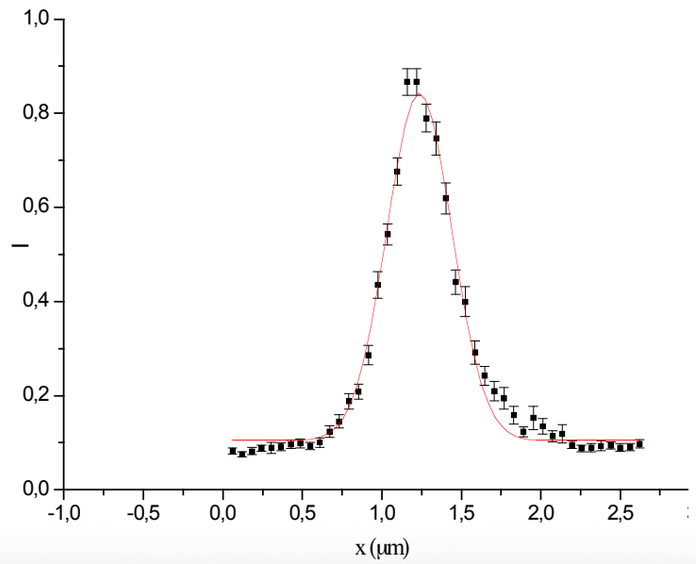
(*green fluorescent protein→photo activatable green fluorescent protein)*

4D (x-y-z-t) switching









2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*

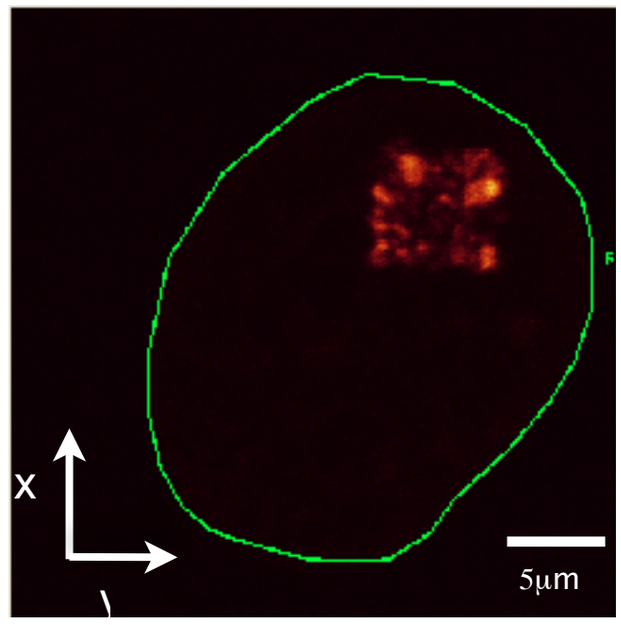
Two photon activation in living cells

PA-GFP + H2B He-La cell

$\langle P_{ACTIVATION} \rangle$= 10mW

$\lambda_{ACTIVATION}$= 720nm

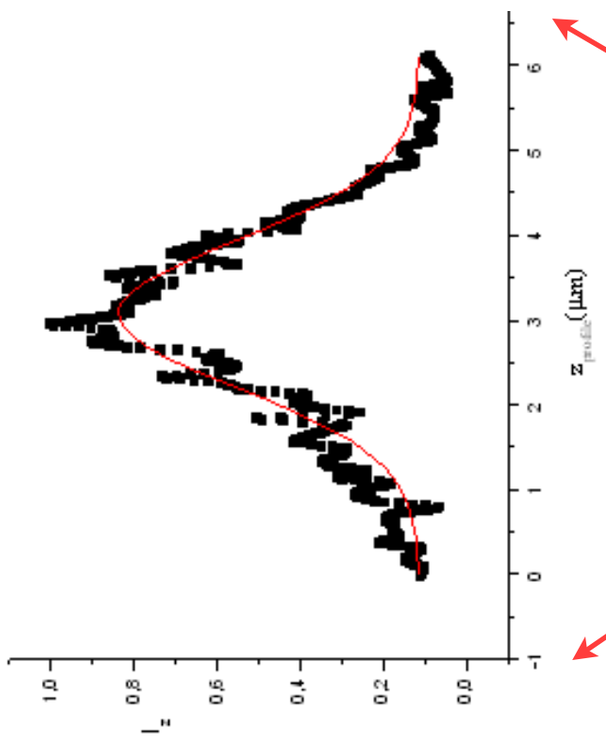
Pixel time= 300µs

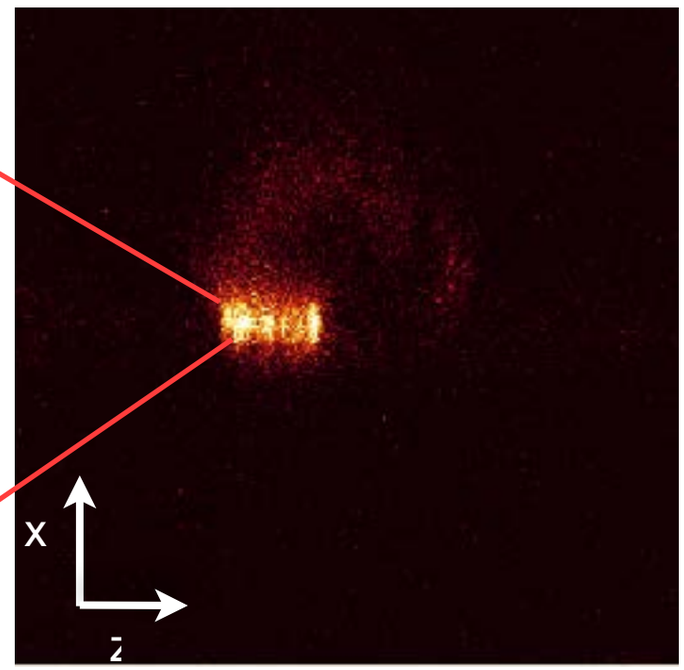


$\langle P_{EXCITATION} \rangle$= 0,04 mW, $\lambda_{EXCITATION}$= 488nm

Pixel time= 4,9 µs, 512*512 pixels

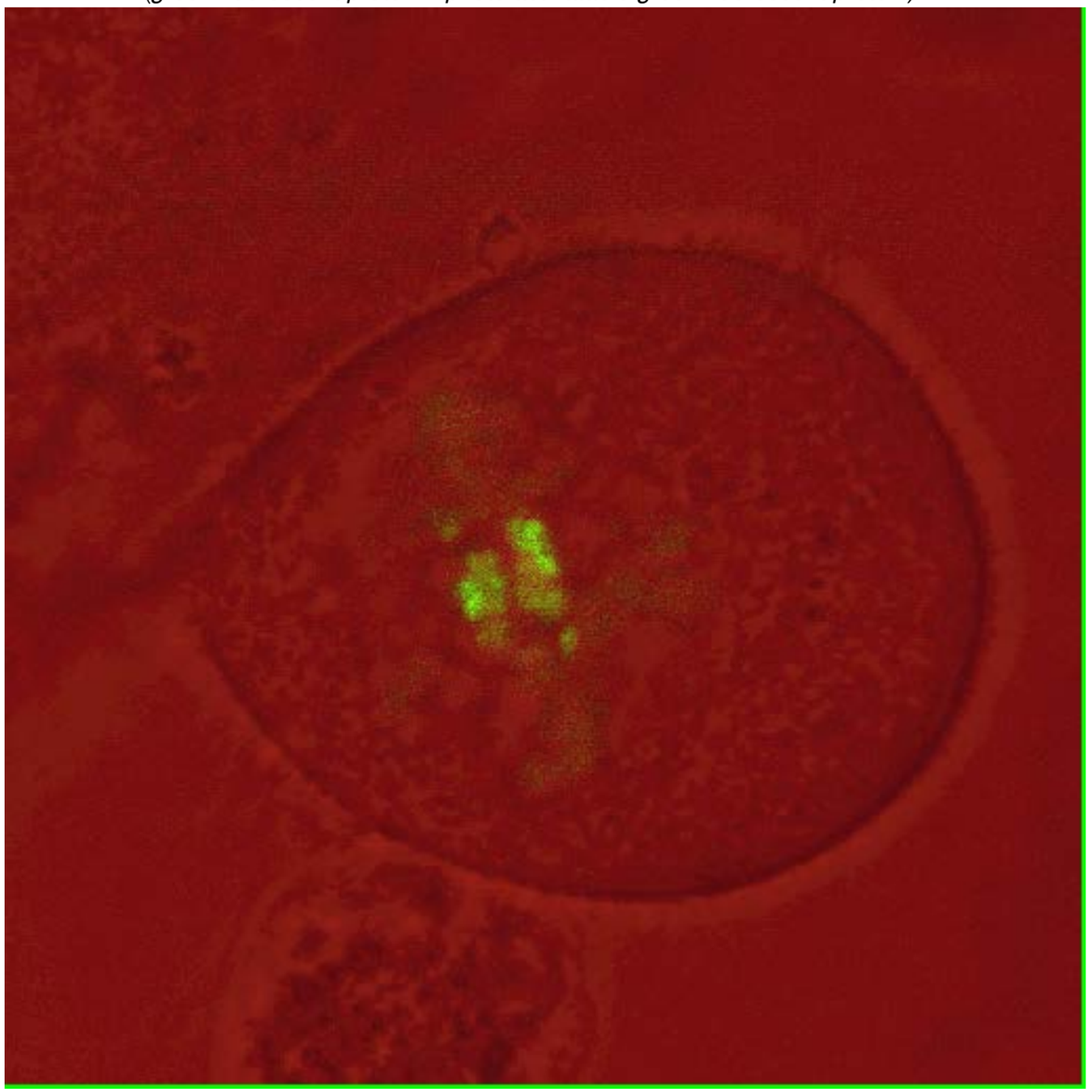
LOCALIZED INTERACTION





2PE and GFP PA-GFP

(*green fluorescent protein→photo activatable green fluorescent protein)*



I. Testa, S. Barozzi, M.Faretta, A.Diaspro (2006)
PA-GFP, LEICA SP2 AOBS spectral system, Chameleon-XR Coherent tunable Ti-Sapphire laser

2PE and GFP PA-GFP

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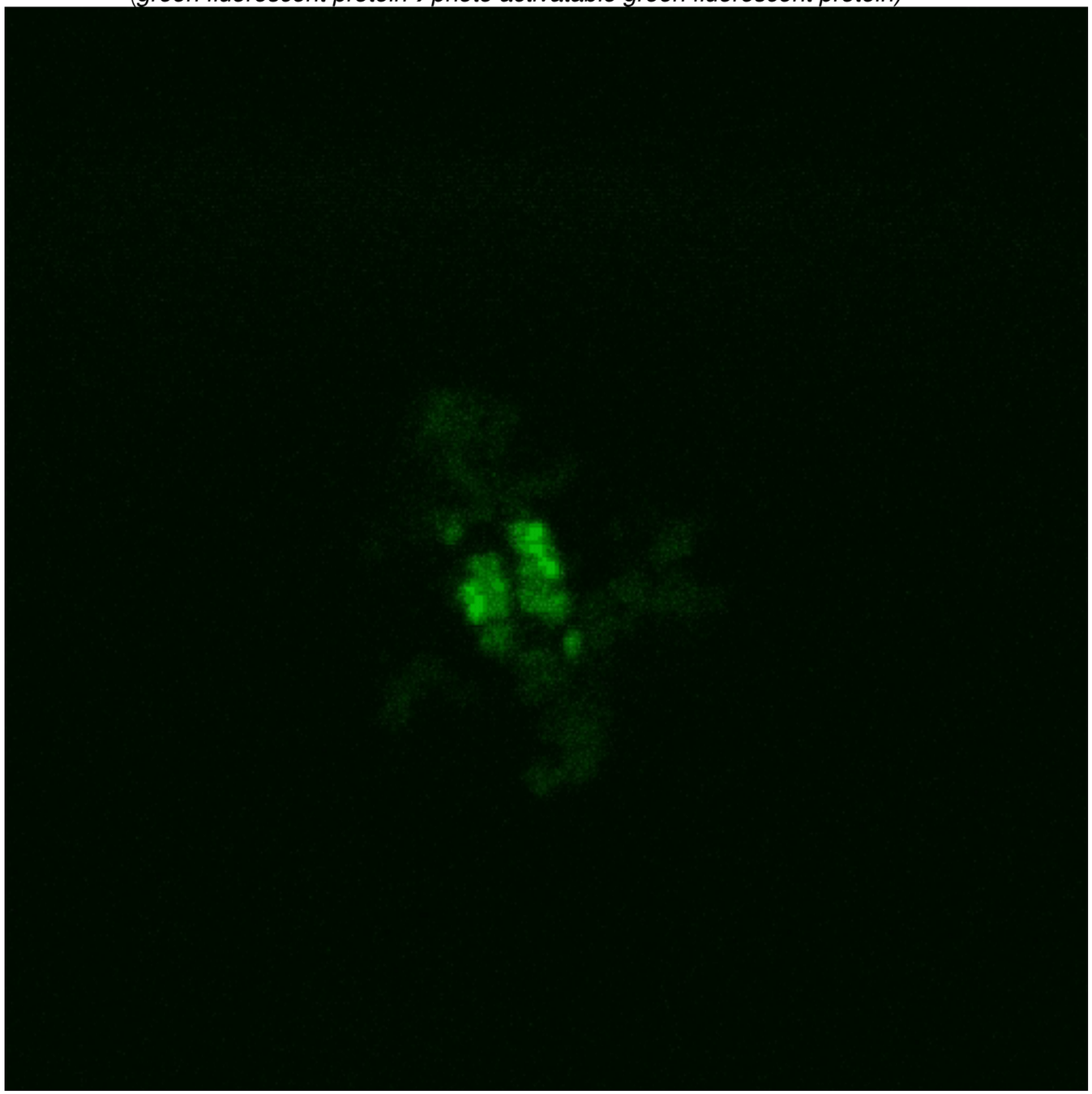

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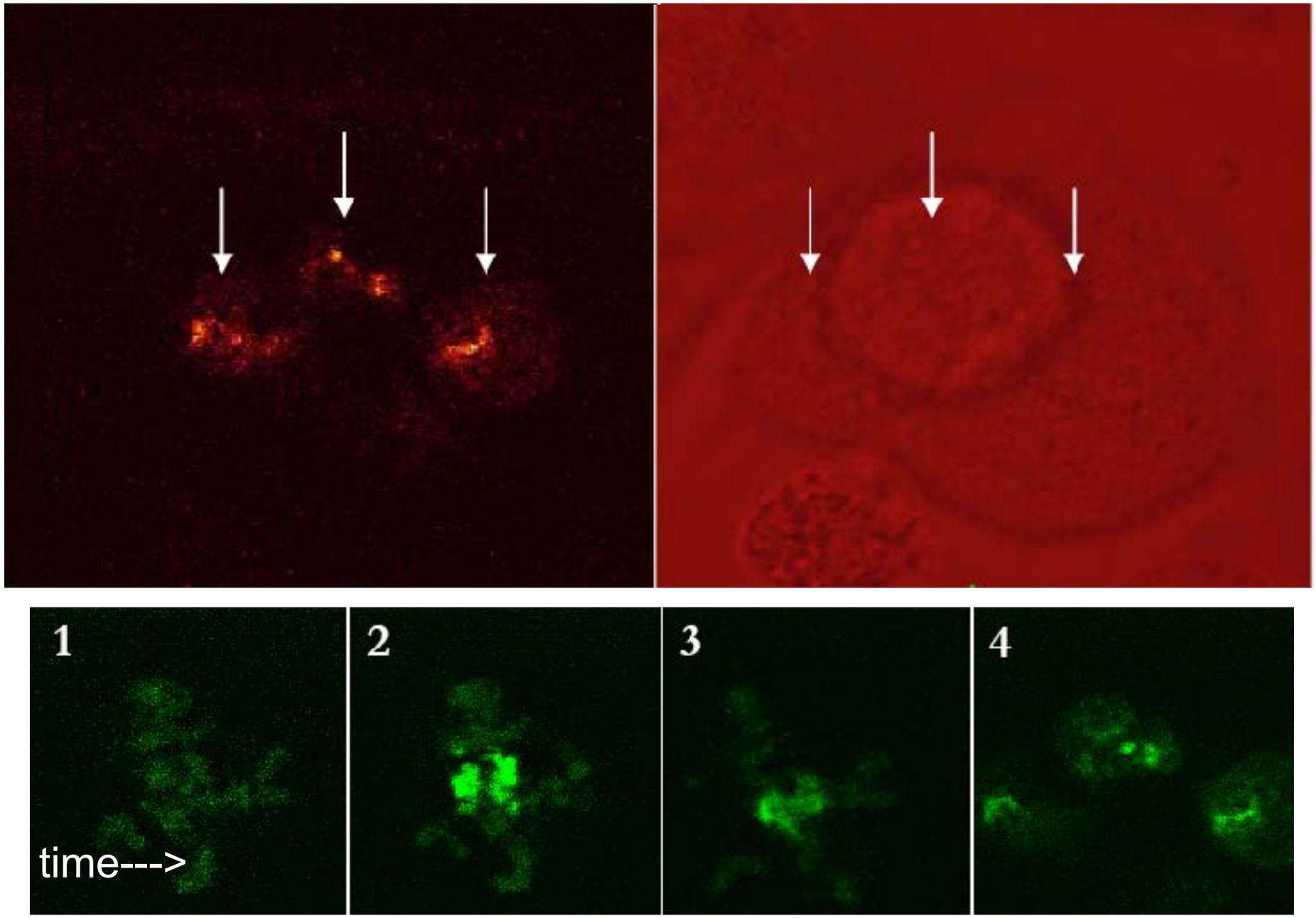


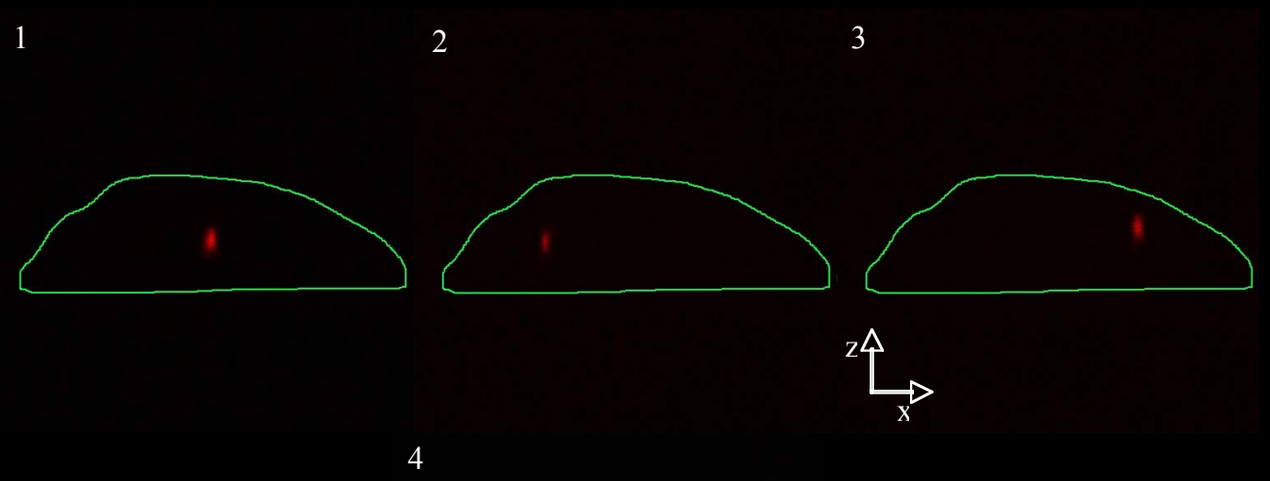
I. Testa, S. Barozzi, M.Faretta, A.Diaspro (2006)

PA-GFP, LEICA SP2 AOBS spectral system, Chameleon-XR Coherent tunable Ti-Sapphire laser

PA-GFP

4D (x-y-z-t) 2PE switching

Biophys. J., .89(2), 1346--1352 (2005).



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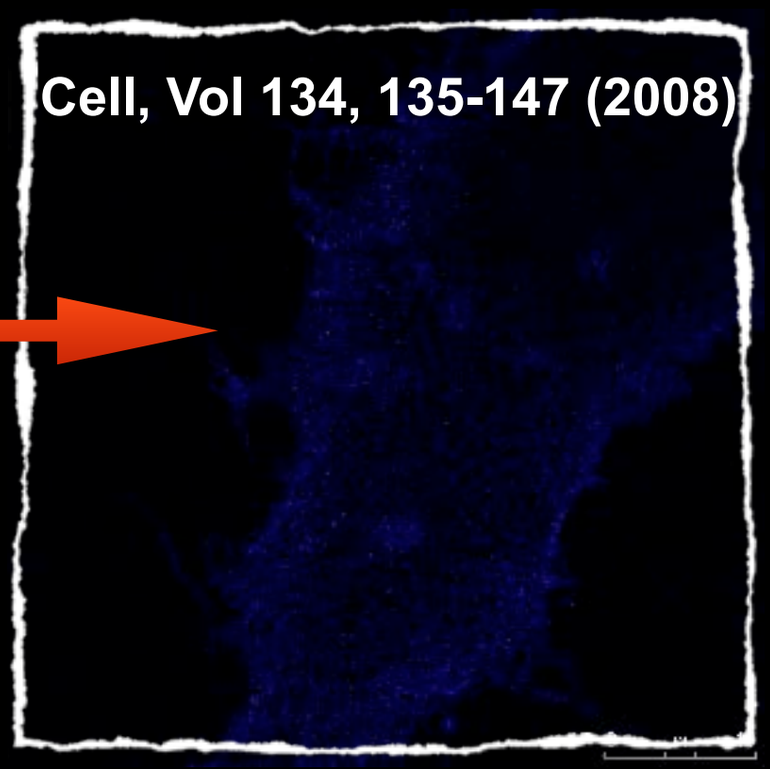
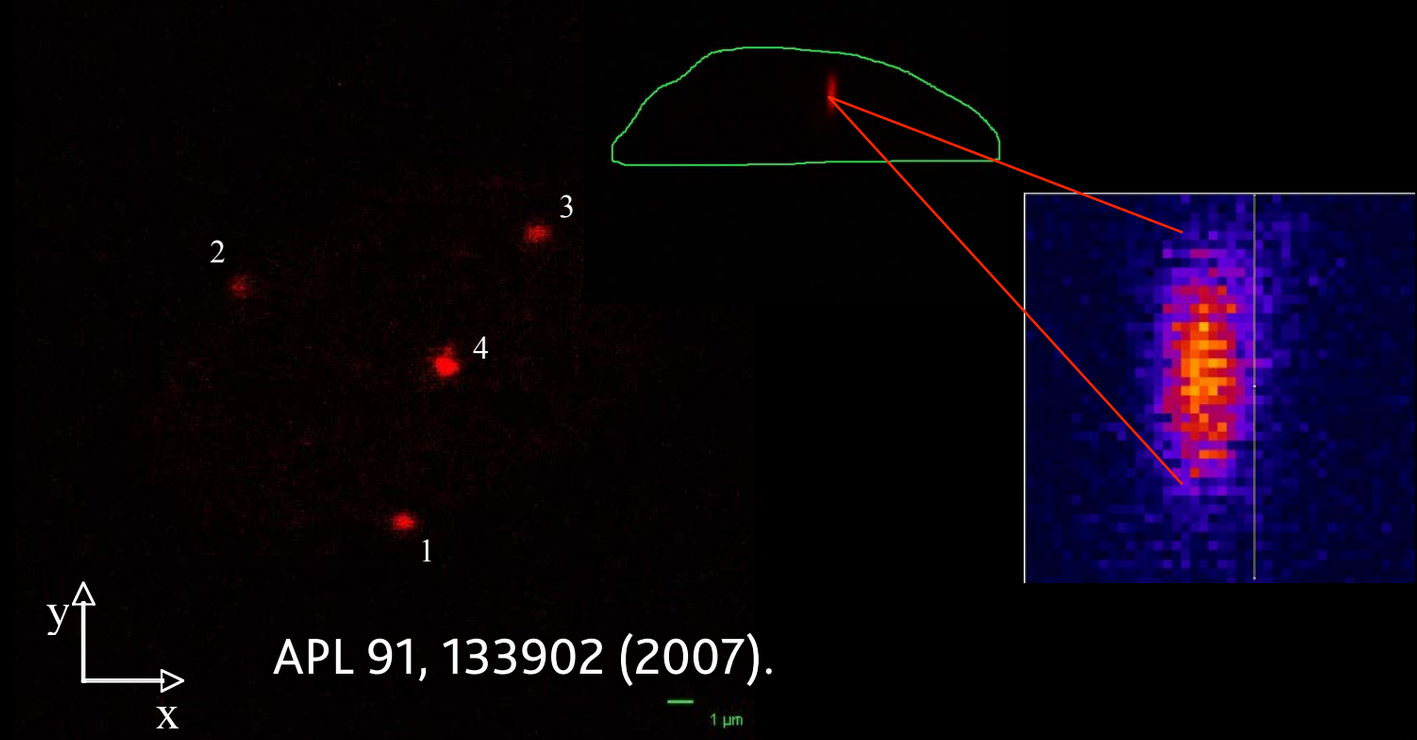


Cell, Vol 134, 135-147 (2008)



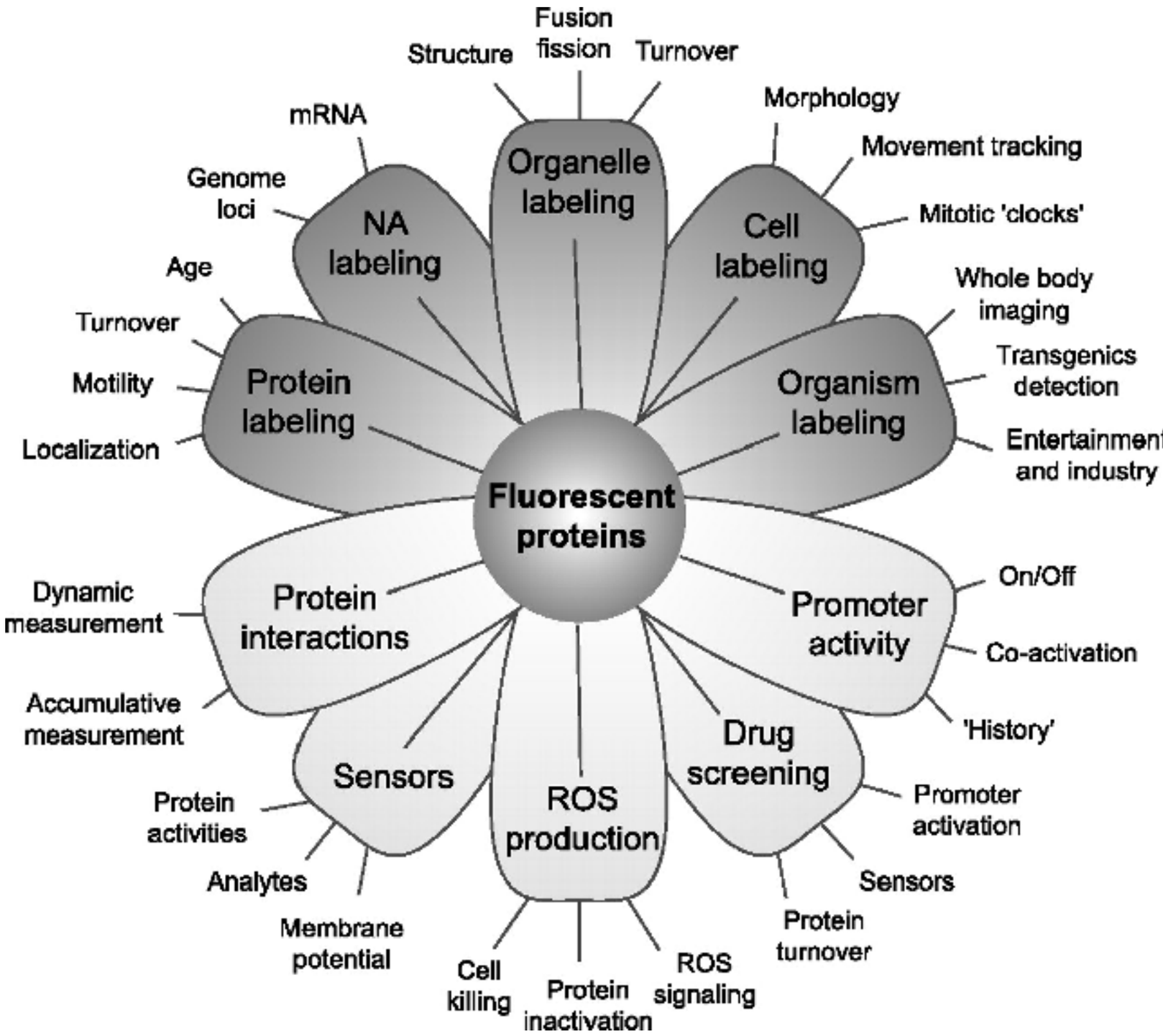
APL 91, 133902 (2007).

Diaspro Lab

Fluorescence is one of the basic processes that we use to access the ‘nanoscale’ information:

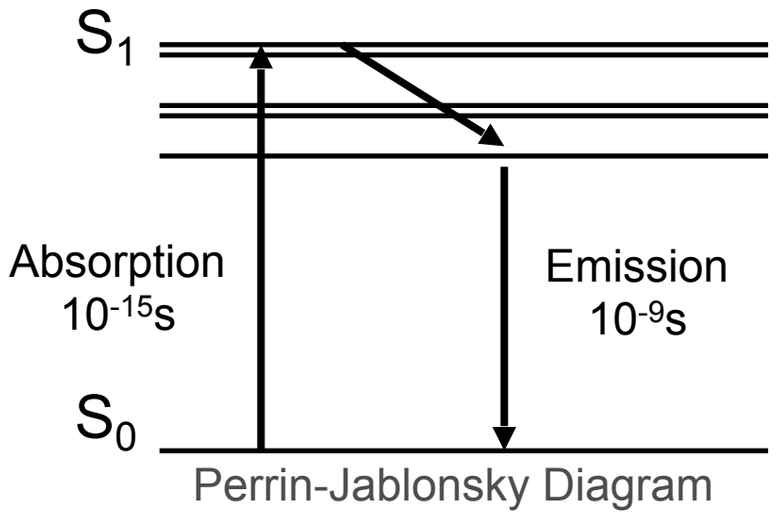
- Fluorescence spectroscopy
- Fluorescence microscopy

fluorescence and its surrounding



Dmitriy M. Chudakov, Mikhail V. Matz, Sergey Lukyanov, Konstantin A. Lukyanov, Physiological Reviews (2010) Vol. 90 no. 3, 1103-1163

fluorescence and its surrounding

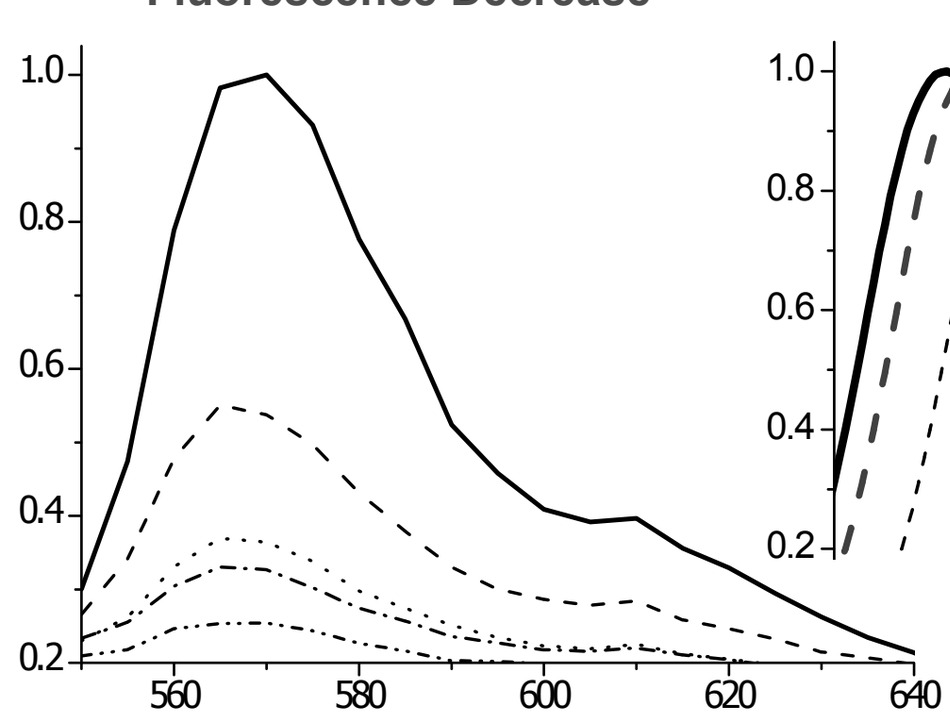


Perrin-Jablonsky Diagram

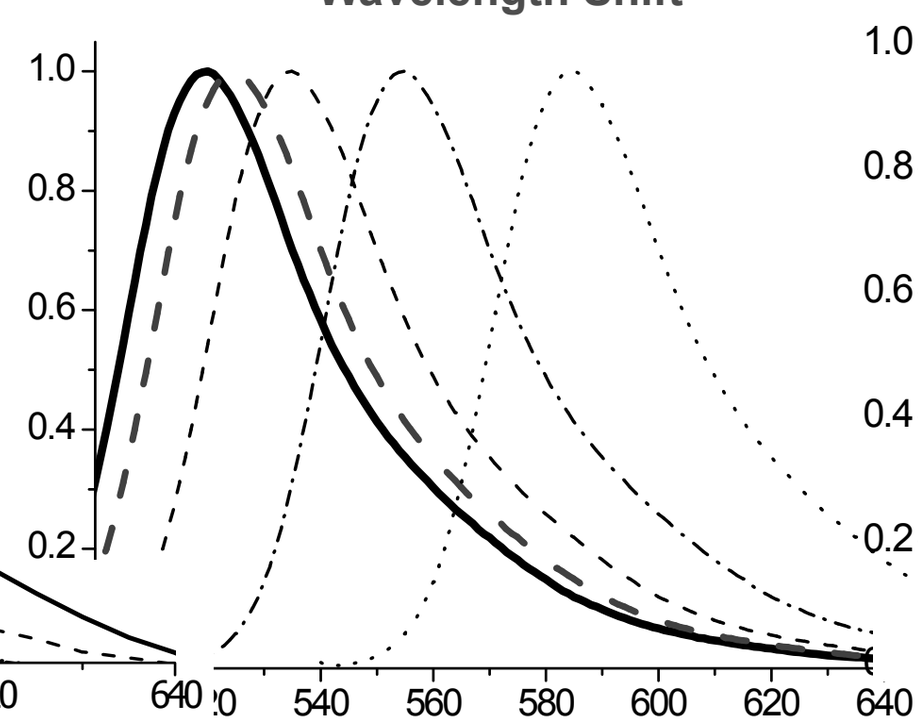
Interaction with surrounding environment

- Molecular interactions
- Molecular binding
- Fluorophore accessibility
- Conformational changes
- Rotational diffusion
- Interactions with solvent molecules
- …

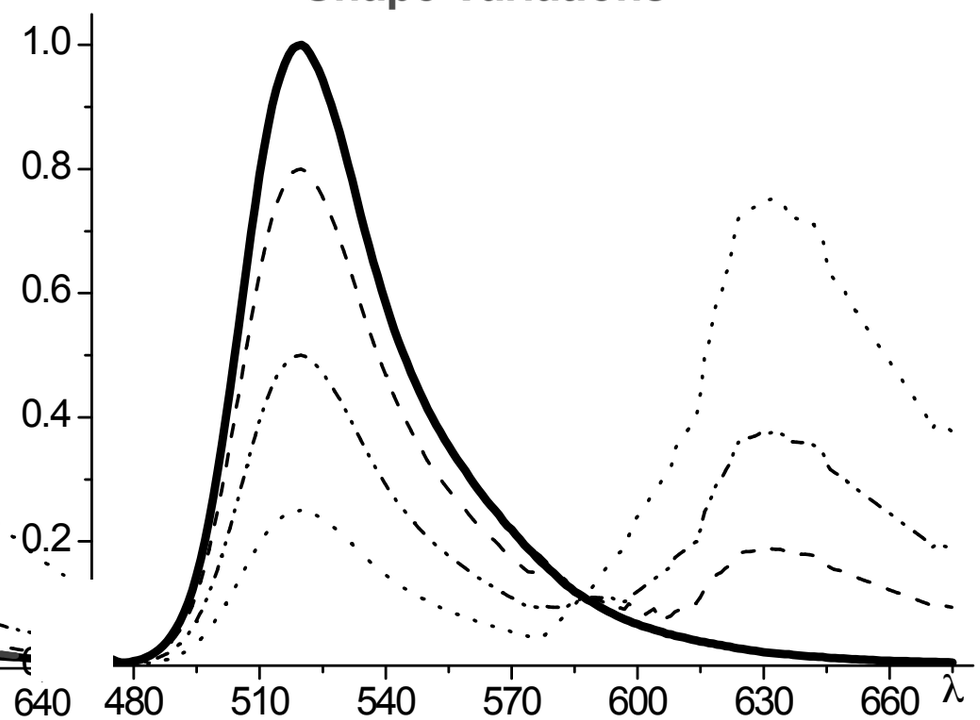
Fluorescence Decrease



Wavelength Shift



Shape Variations

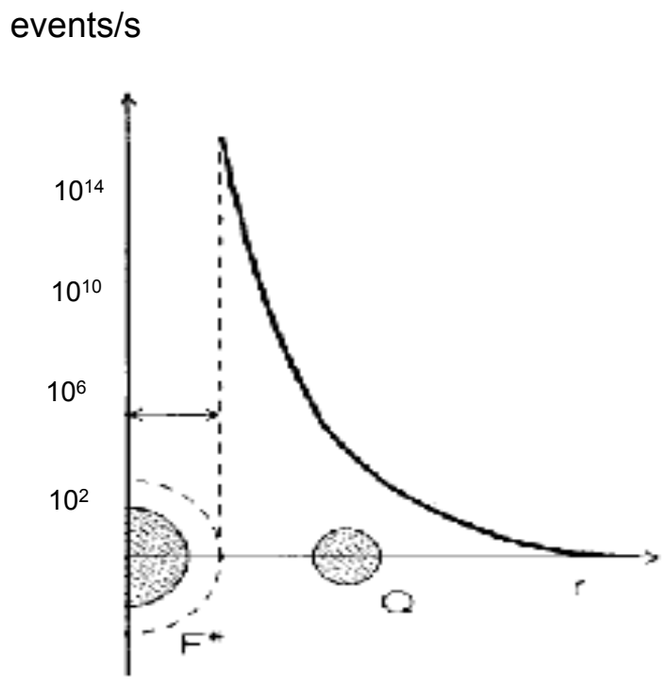


Quenching and FRET

Quenching

Contact with diffusive encounters
Short-range interactions 1Å
Sensitive to steric shielding,
charge-charge interactions…

$$k_Q = \beta e^{-(r-r_c)}$$



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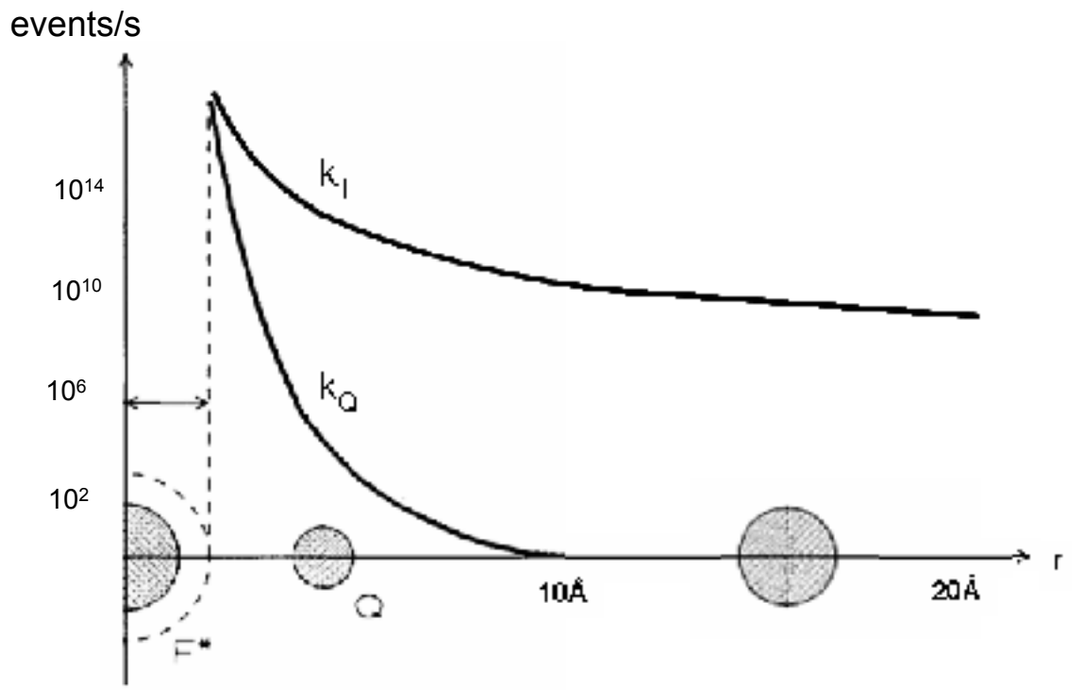

FRET

Resonance Energy Transfer
Long-range interactions 10-100 Å
Insensitive to steric factors,
electrostatic interactions…

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

slide courtesy: Valentina Caorsi

Quenching and FRET

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Phenomenon studies
on model system

Biological Application
for protein-protein investigation

slide courtesy: Valentina Caorsi

Quenching and FRET

Quenching

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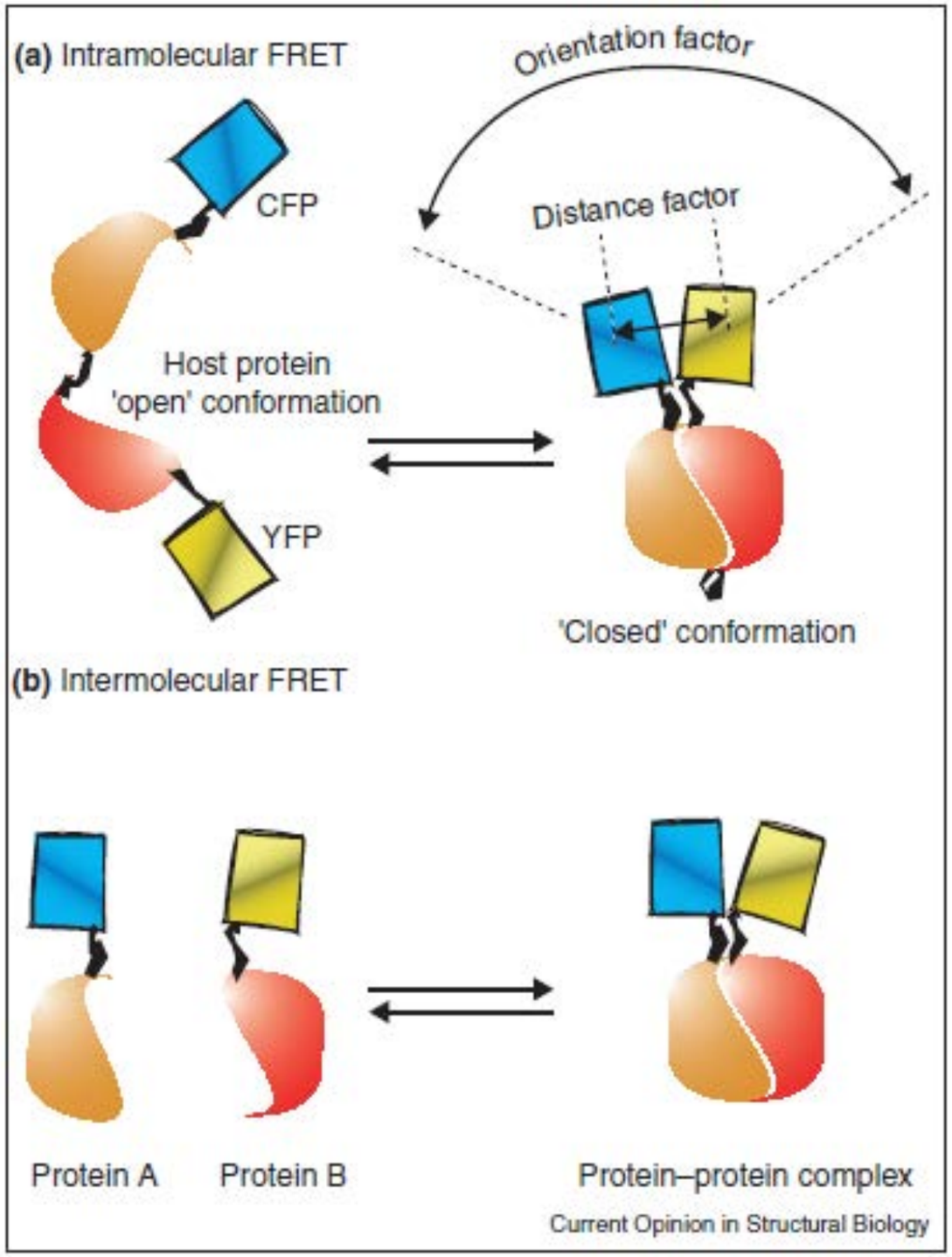
slide courtesy: Valentina Caorsi

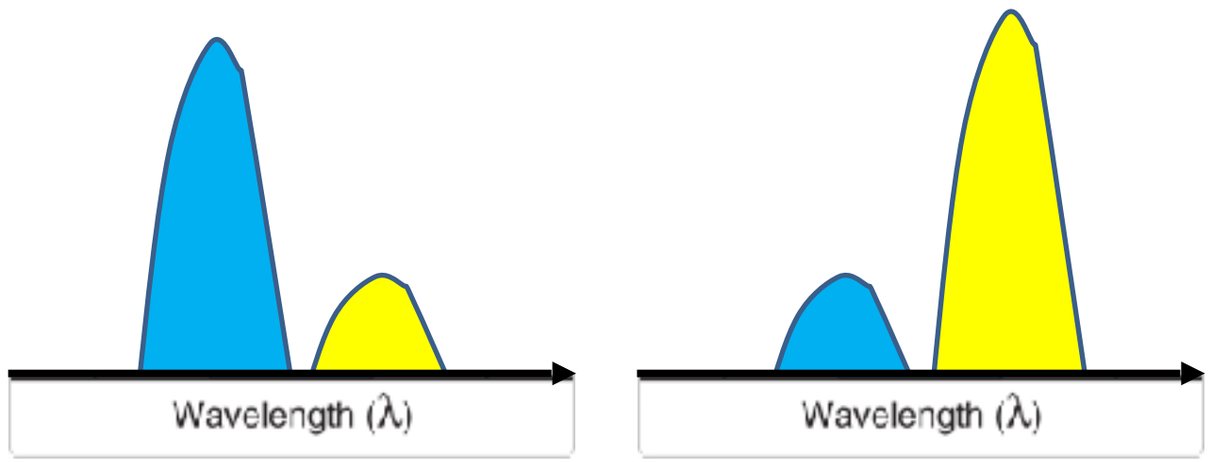
FRET

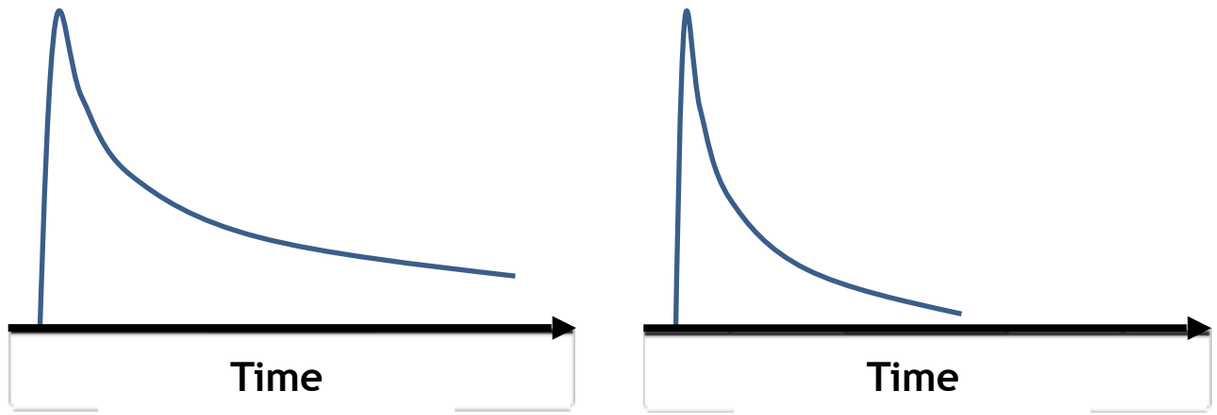


Truong and Ikura, 2001

NO FRET
d>10nm

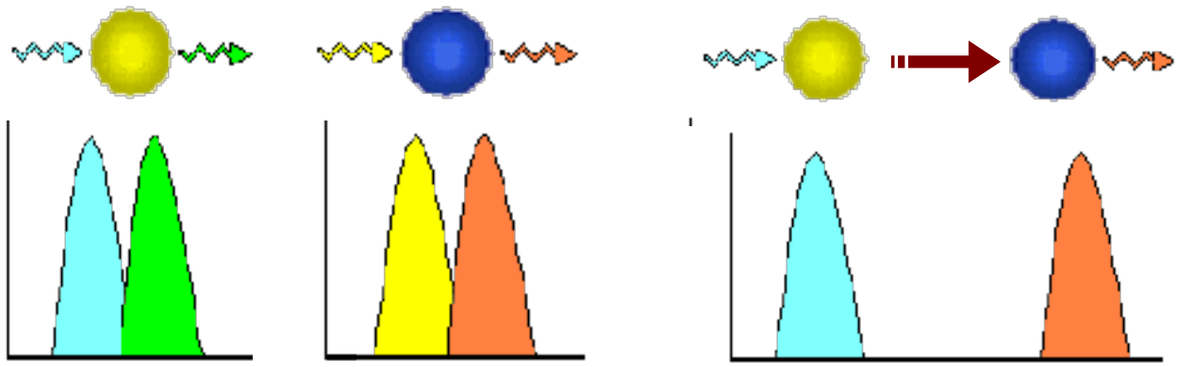
FRET
d<10nm



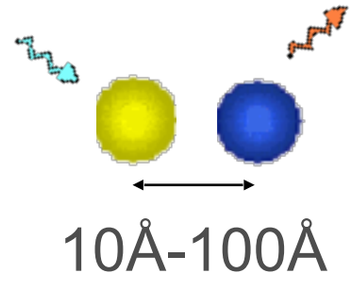


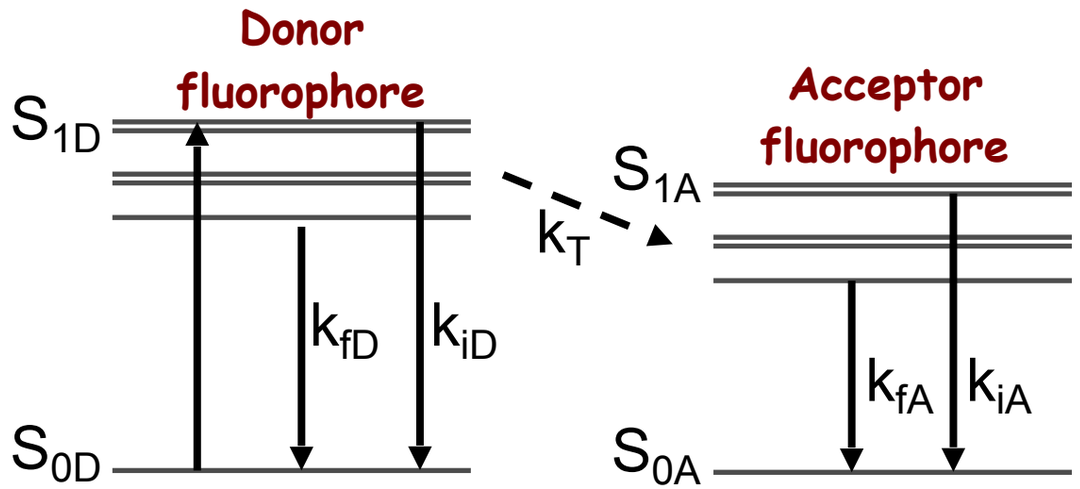
Detected measuring changes in emission spectra or in lifetime.

FRET



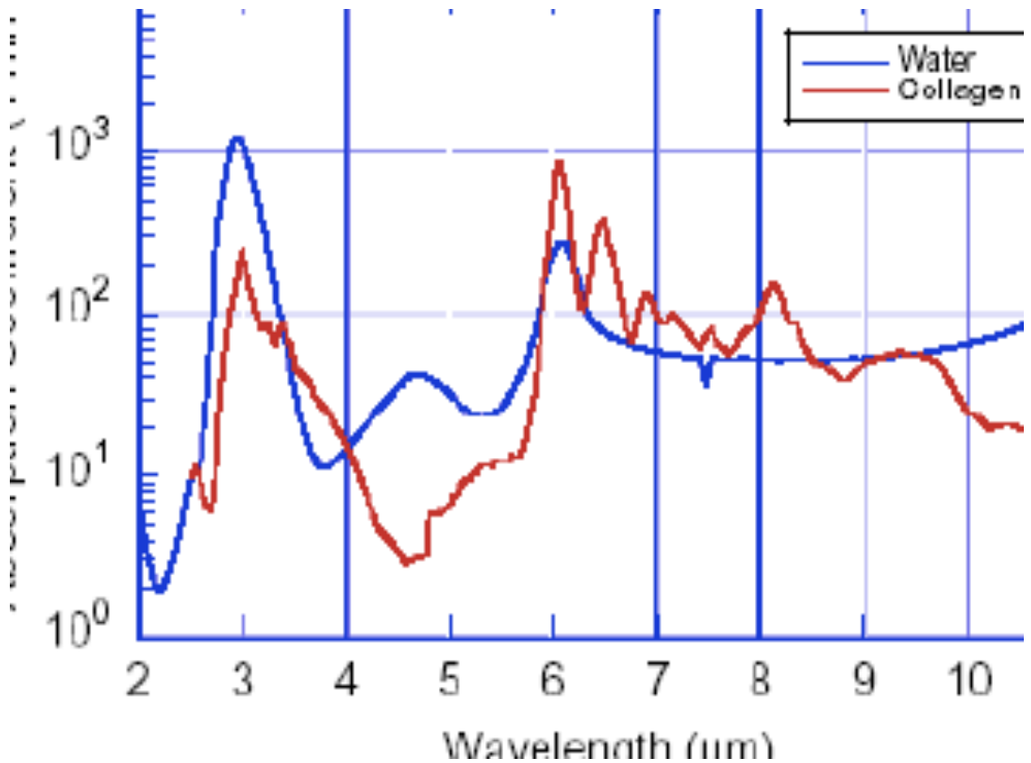
1) Distance



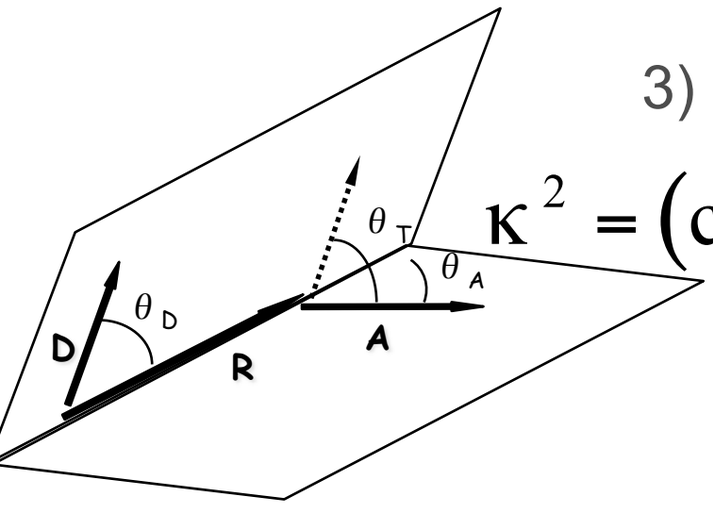


2) Spectral Overlap

$$J(\lambda) = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$



3) Dipole Orientation

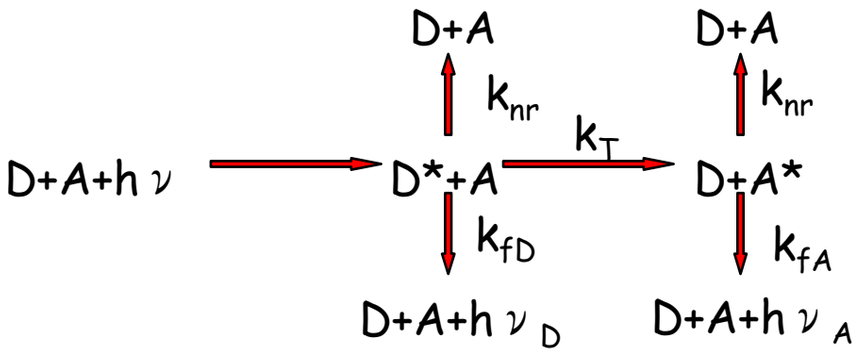


$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$

A. Periasamy and R. Day. Molecular Imaging FRET Microscopy and Spectroscopy Oxford University Press, 2005.

FRET

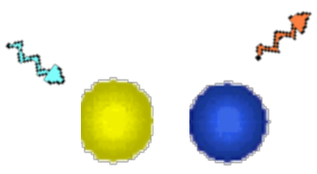
Rate constants for competing events:



$$E = \frac{K_T}{K_T + K_f + K_{nr}}$$

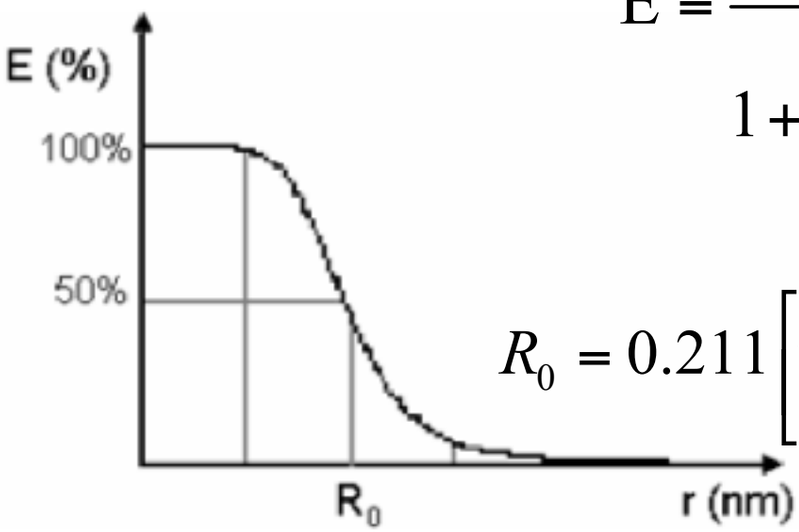
FRET as a nanometric ruler
to investigate cellular process

Single pair



$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

$$E = \frac{1}{1+\left(\frac{r}{R_0}\right)^6}$$



$$R_0 = 0.211\left[\kappa^2 \frac{Q_D}{n^4} J(\lambda)\right]^{\frac{1}{6}}$$

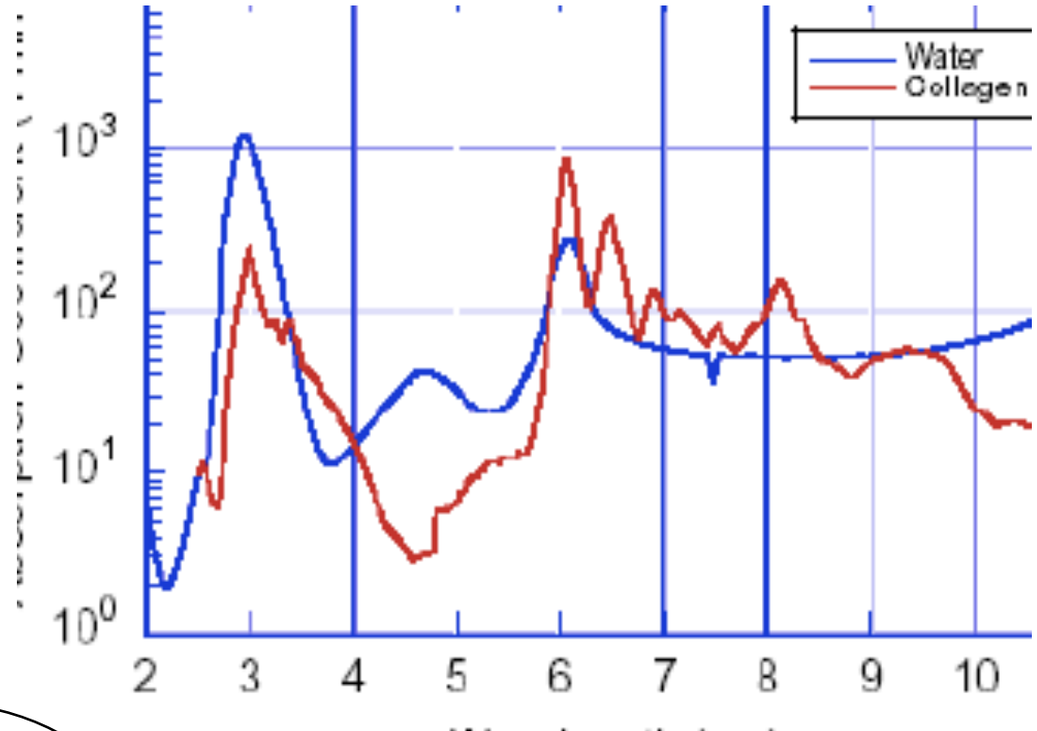
- Protein folding and complexations
- Receptor/ligand interactions
- Conformational Changes
- Structure and conformation of nucleic acids
- Distribution and transport of lipids
- Membrane fusion assays
- Membrane potential sensing

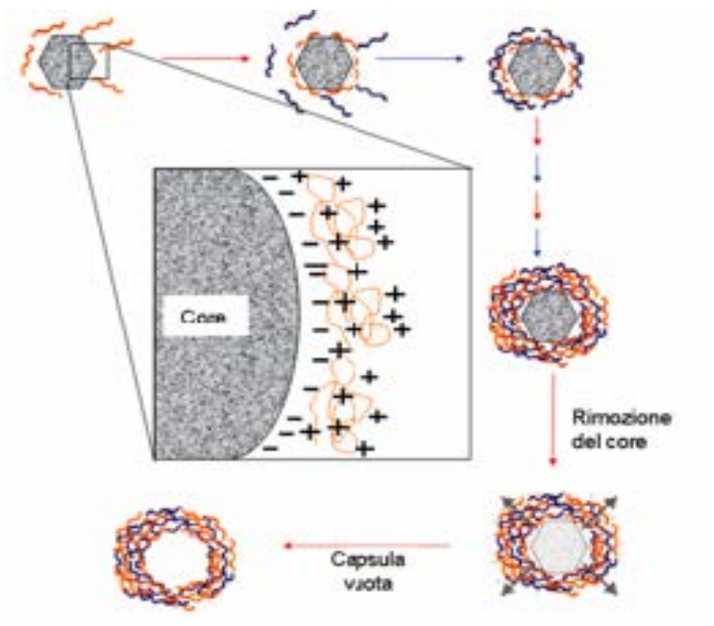
A. Periasamy and R. Day. Molecular Imaging FRET Microscopy and Spectroscopy Oxford University Press, 2005.

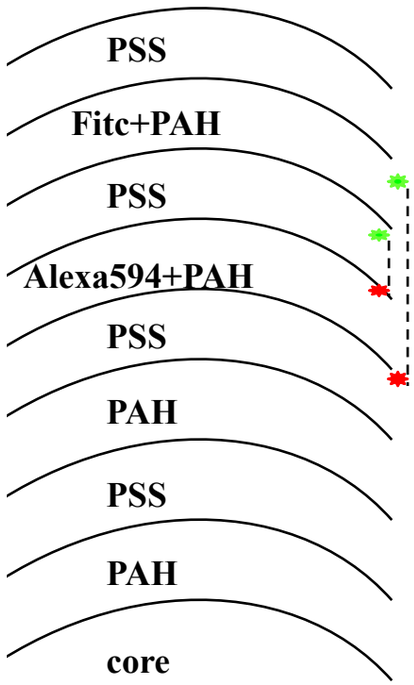
slide courtesy: Valentina Caorsi

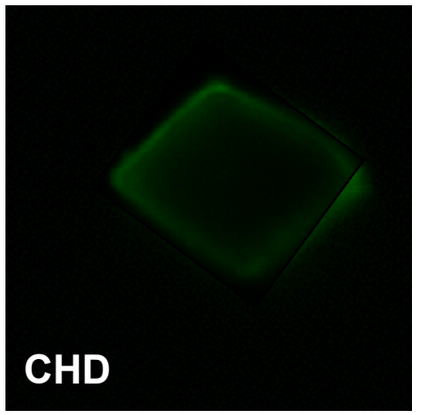
FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved
- Donor Lifetime









The Abdus Salam International Centre for Theoretical Physics

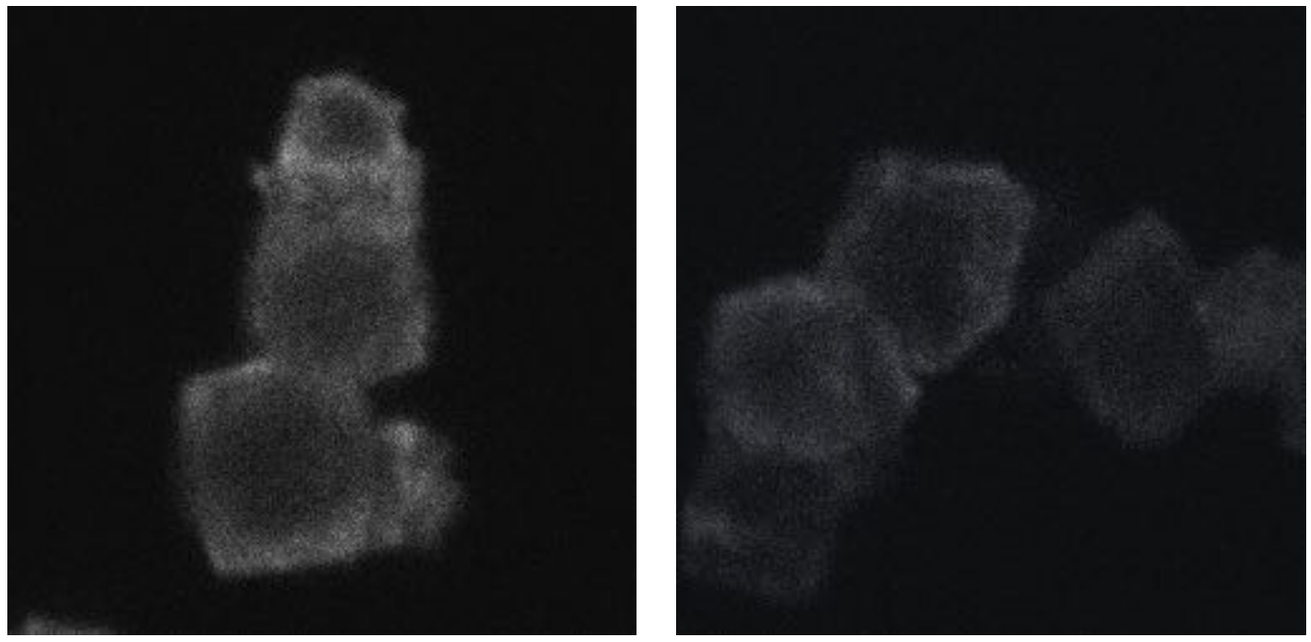
Caorsi V.,Ronzitti E.,Vicidomini G.,Krol S., McConnell G.and A. Diaspro. *FRET measurements on fuzzy fluorescent nano-structures.* MRT 2007 **70** (5):452-458.

slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved
- Donor Lifetime

I_D I_{DA}



$$E = 1 - \frac{I_{DA}}{I_D}$$

FRET couple	E (%)
Fitc-Alexa594	33 ± 5
Alexa488-Alexa594	38 ± 3
Cy3-Cy5	37 ± 6

C.Berney and G.Danuser. FRET or no FRET: a quantitative comparison. Biophysical Journal 2003 **84** 3992--4010

slide courtesy: Valentina Caorsi

FRET

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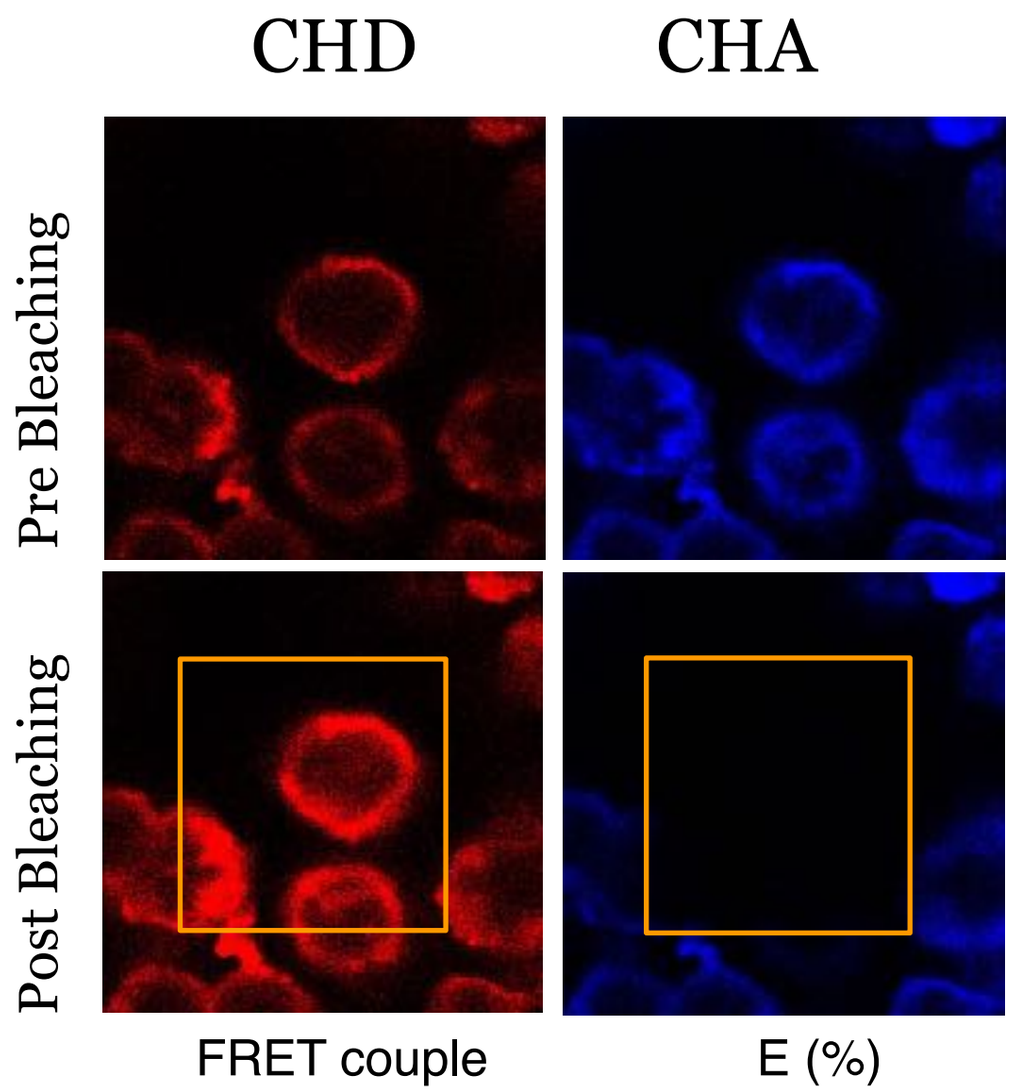

$$E = 1 - \frac{I_{DA}}{I_{DA_{afterphotobleaching}}}$$

T. S. Karpova et Al. Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser. J Microsc 2003 **209** 56--70

slide courtesy: Valentina Caorsi

FRET

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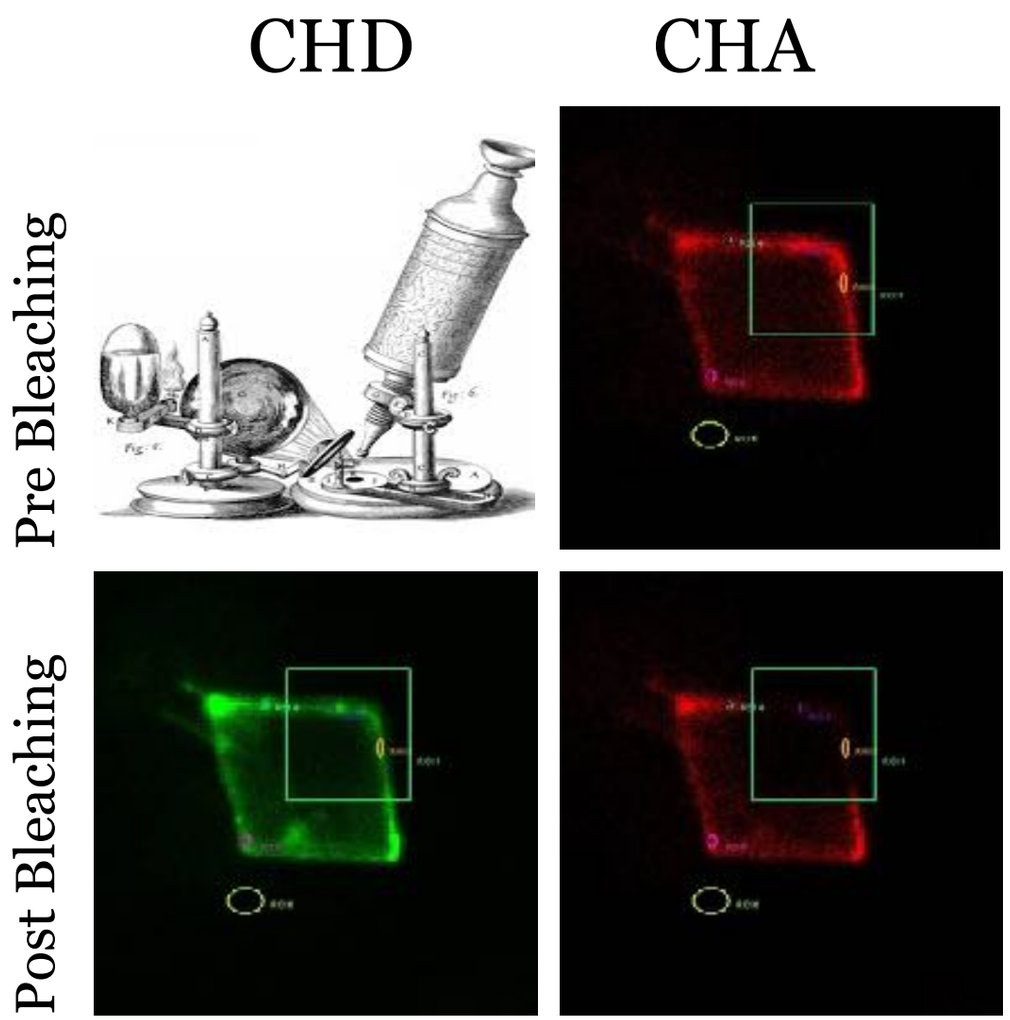


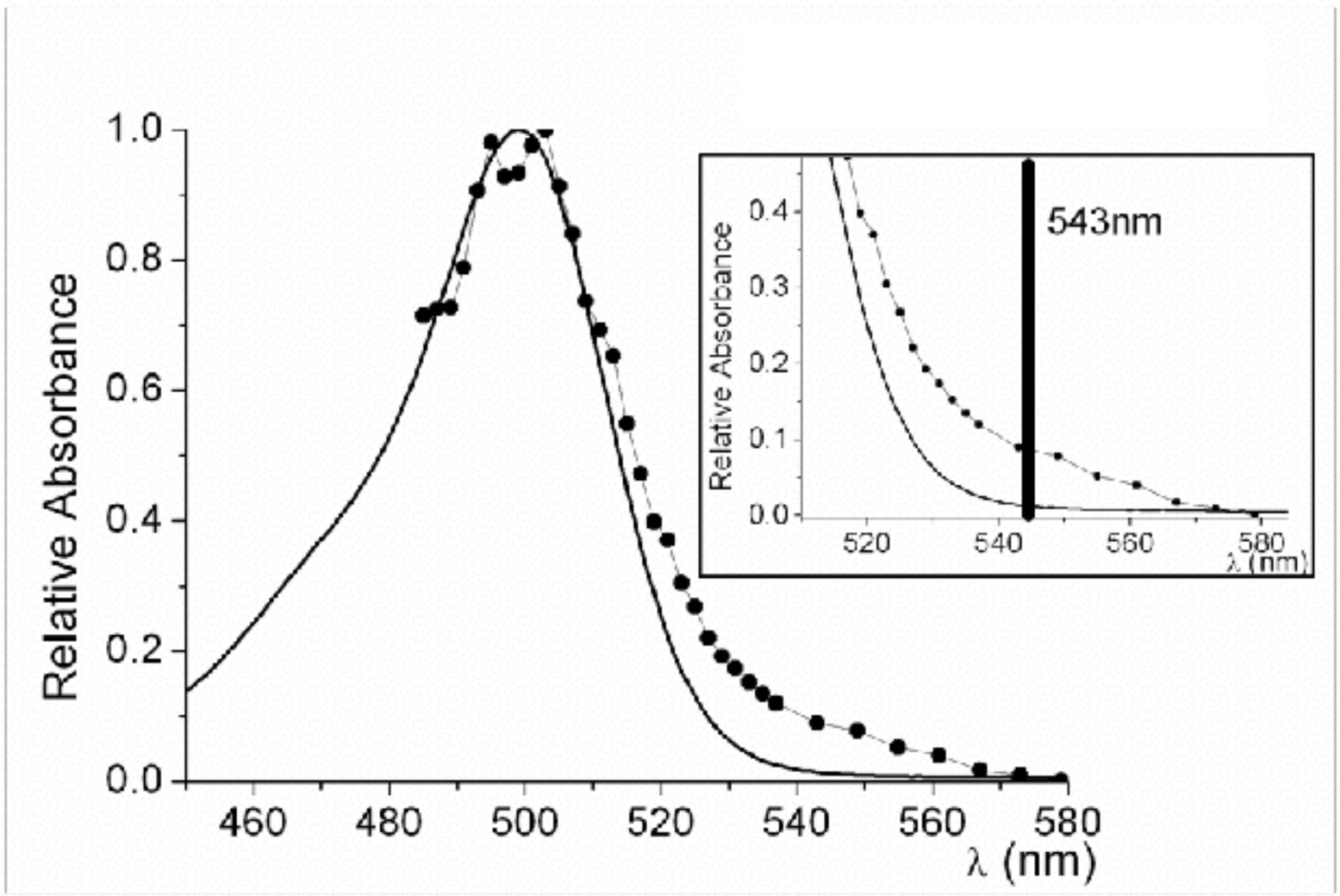
$$E = 1 - \frac{I_{DA}}{I_{DA_{afterphotobleaching}}}$$

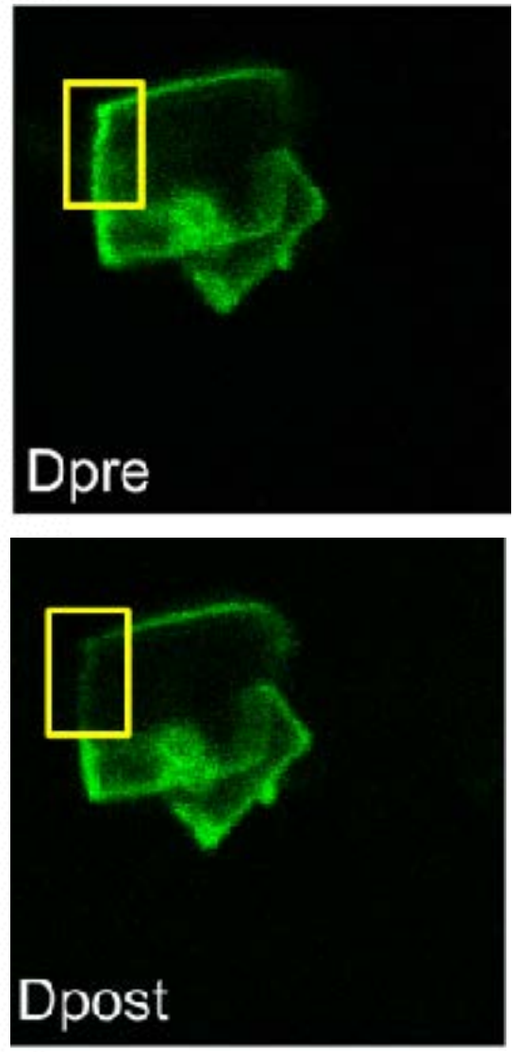
FRET couple	E (%)
Cy3-Cy5	29 ± 5
Fitc-Alexa594	0

T. S. Karpova et Al. Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser. J Microsc 2003 **209** 56--70

slide courtesy: Valentina Caorsi

FRET

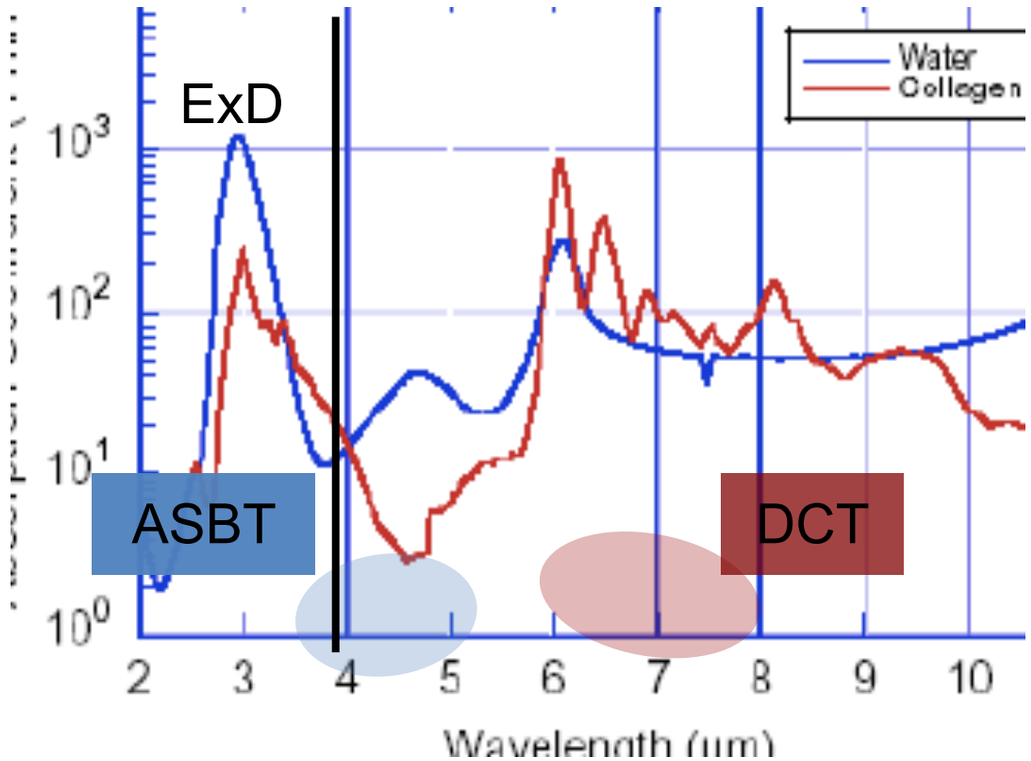




slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved
- Donor Lifetime



M. Elangovan and H. Wallrabe and Y. Chen and R.N. Day and M. Barroso and A. Periasamy. Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. Methods 2003 29 58--73

slide courtesy: Valentina Caorsi

FRET

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- Spectrally Resolved
- Donor Lifetime

$$R_{Dn} = \left(\sum_i \frac{b_i}{a_i} \right)_n \qquad R_{An} = \left(\sum_i \frac{c_i}{d_i} \right)_n$$

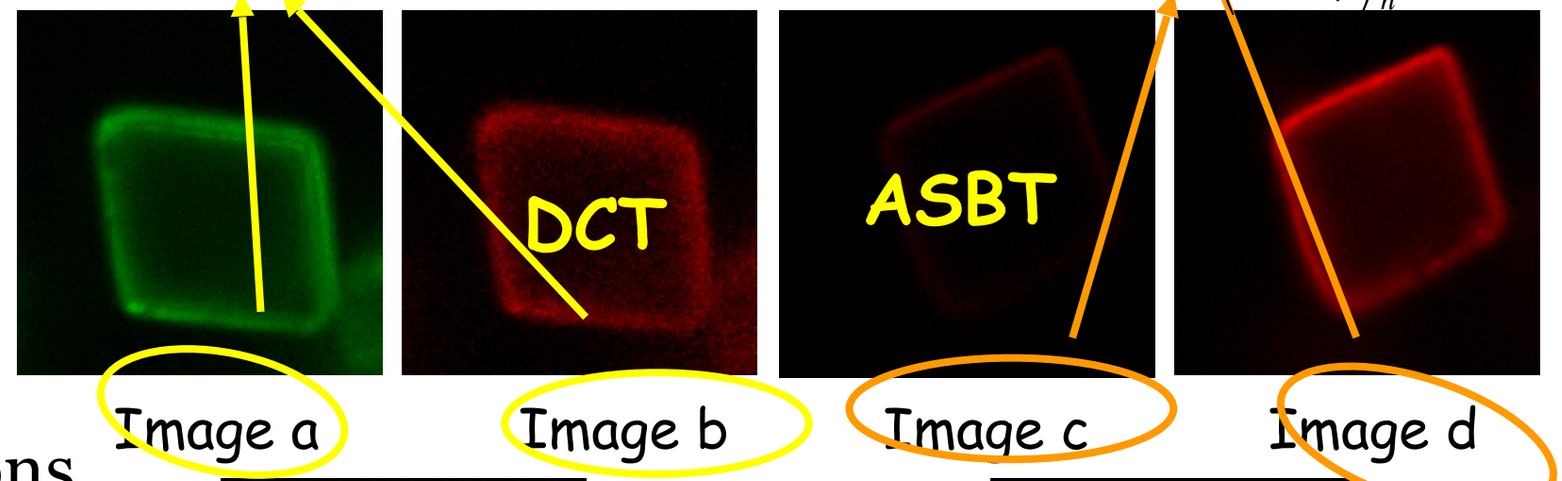


Image e Image f Image g

$$E = 1 - \frac{I_{DA}}{I_{DA} + pFRET \frac{\Psi_{DD}}{\Psi_{AA}} Q_D}$$

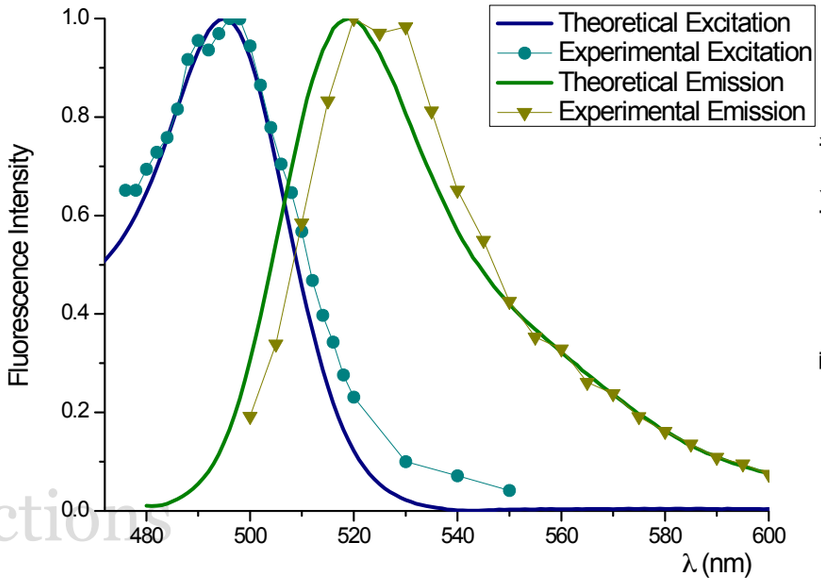
FRET couple	E (%)
Fitc-Alexa594	29 ± 7
Alexa488-Alexa594	24 ± 5
Cy3-Cy5	30 ± 6

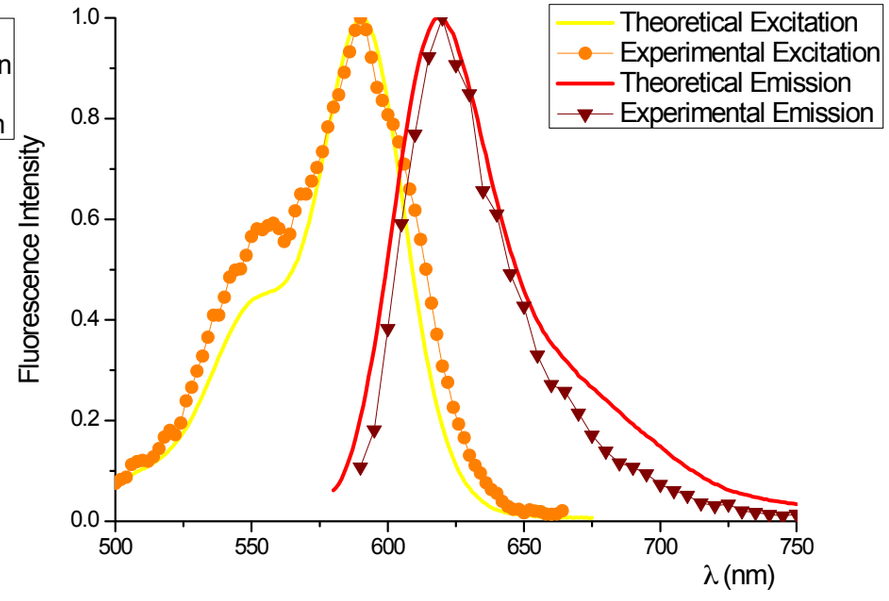
Eliminating Cross Talk and SBT

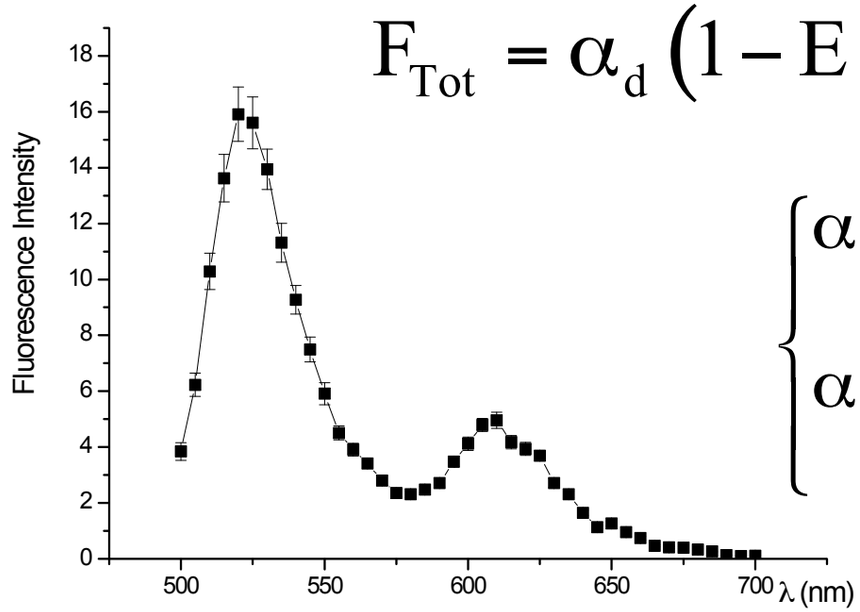
slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved
- Donor Lifetime







$$F_{Tot} = \alpha_d (1-E) F_d + \alpha_a F_a + \alpha_d E \frac{\varepsilon_a}{\varepsilon_d} F_a$$

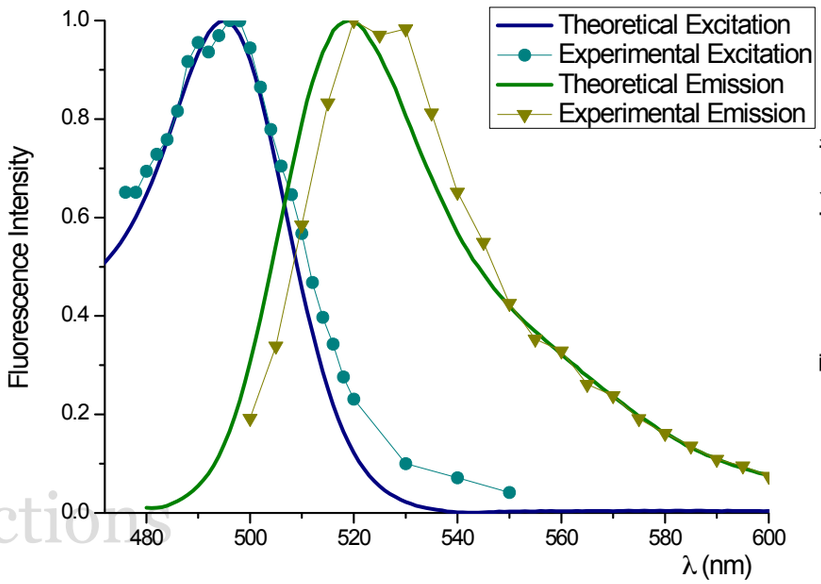
$$\begin{cases} \alpha_1 = \alpha_d (1-E) \\ \alpha_2 = \alpha_a + \alpha_d E \dfrac{\varepsilon_a}{\varepsilon_d} \end{cases}$$

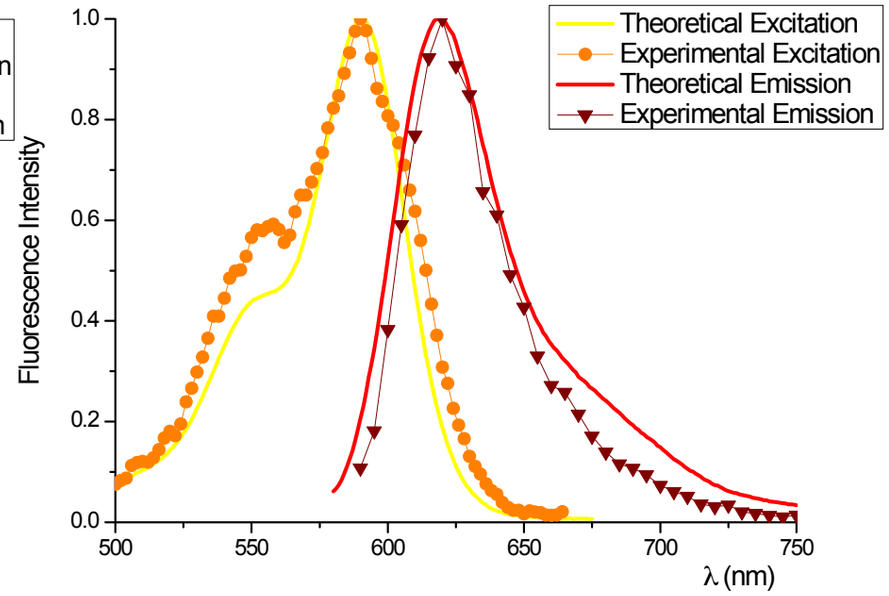
Neher, R.A. and Neher, E. Applying spectral fingerprinting to the analysis of FRET images. Microsc Res Tech. 2004 1;64(2):185-95.

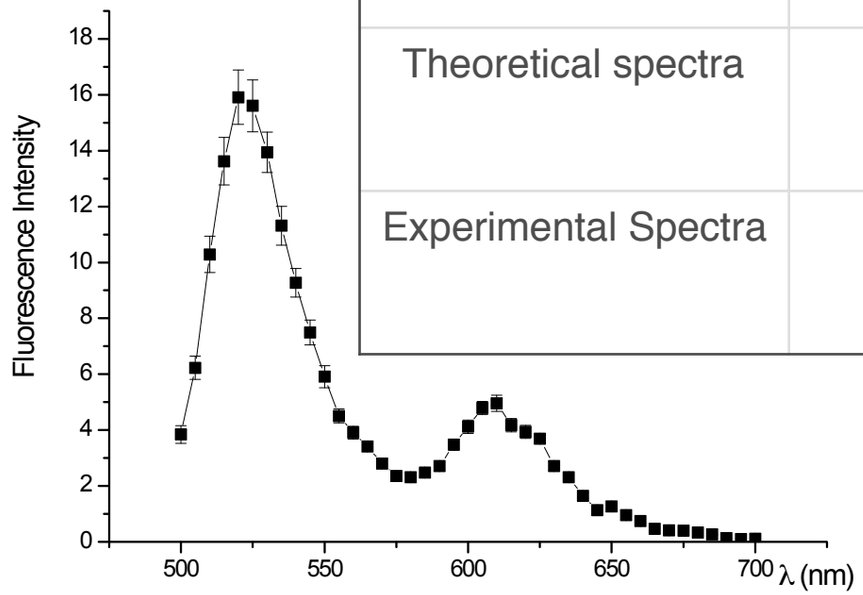
slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- **Spectrally Resolved**
- Donor Lifetime







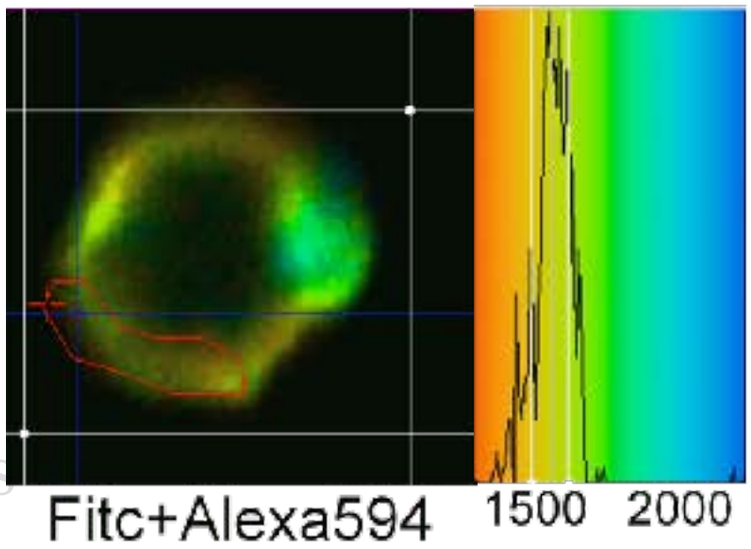
	E	d/a
Theoretical spectra	38 ± 3	3.2 ± 0.4
Experimental Spectra	25 ± 2	4.9 ± 0.9

Neher, R.A. and Neher, E. Applying spectral fingerprinting to the analysis of FRET images.Microsc Res Tech. 2004 **1**;64(2):185-95.

slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved



- Donor Lifetime

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

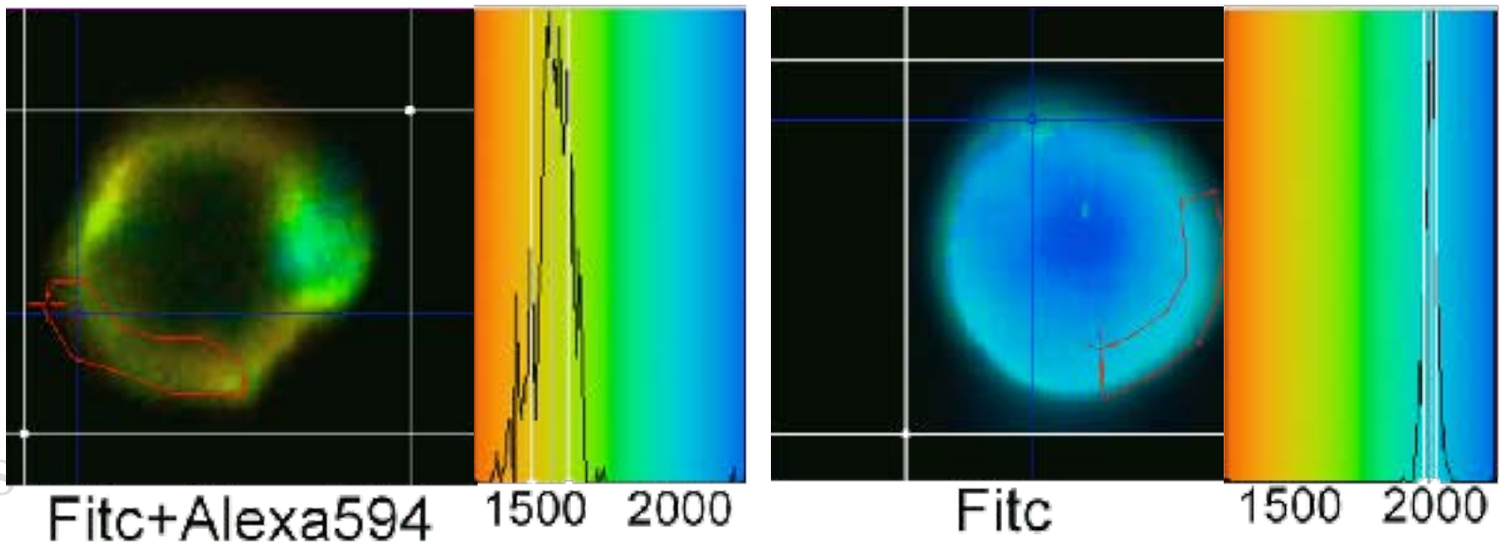
τ_D Fitc	1.96±0.12 ns
τ_{DA}	1.28±0.06 ns
FRET efficiency	35±4 %

W. Becker et al. Fluorescence lifetime images and correlation spectra obtained by multidimensional time-correlated single photon counting. Microsc Res Tech 2006 **69** 186--195

slide courtesy: Valentina Caorsi

FRET

- Donor Emission
- Acceptor Photobleaching
- Cross talk and SBT corrections
- Spectrally Resolved



- Donor Lifetime

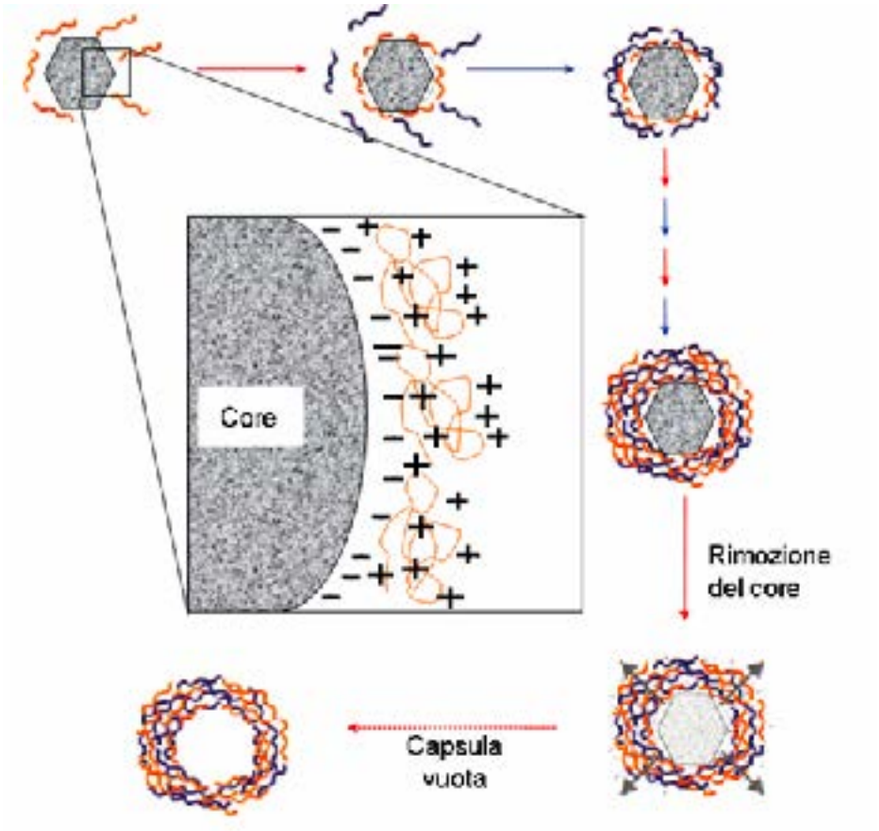
$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

τ_D Fitc	1.96±0.12 ns
τ_{DA}	1.28±0.06 ns
FRET efficiency	35±4 %
τ_D Fitc	2.02±0.04 ns

W. Becker et al. Fluorescence lifetime images and correlation spectra obtained by multidimensional time-correlated single photon counting. Microsc Res Tech 2006 **69** 186--195

slide courtesy: Valentina Caorsi

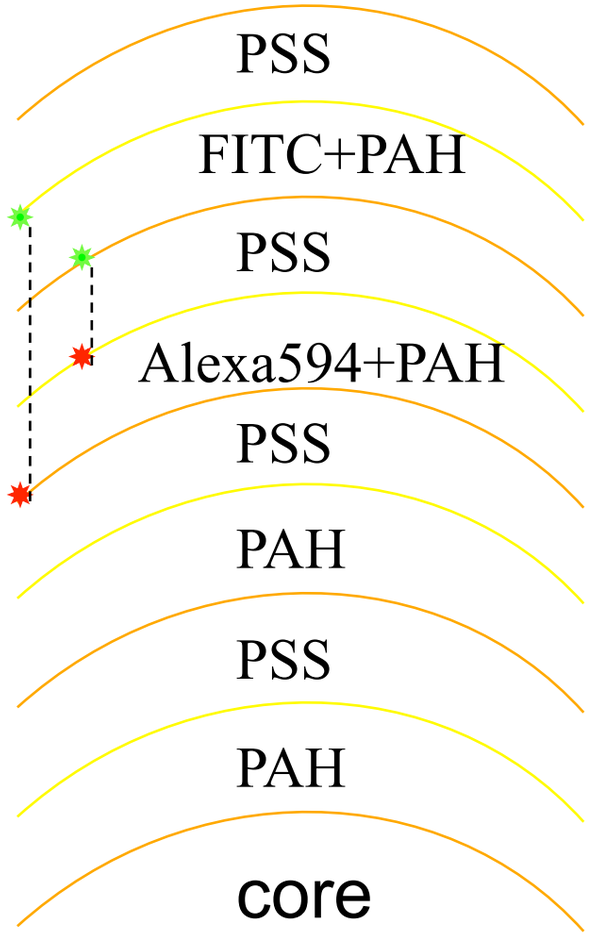
A model system (nanocapsules)



Distance
D-A

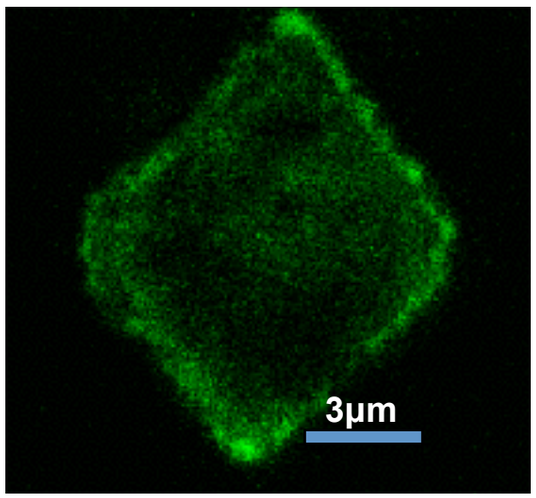
2.5-7.5 nm

Polyelectrolytes with opposite charges



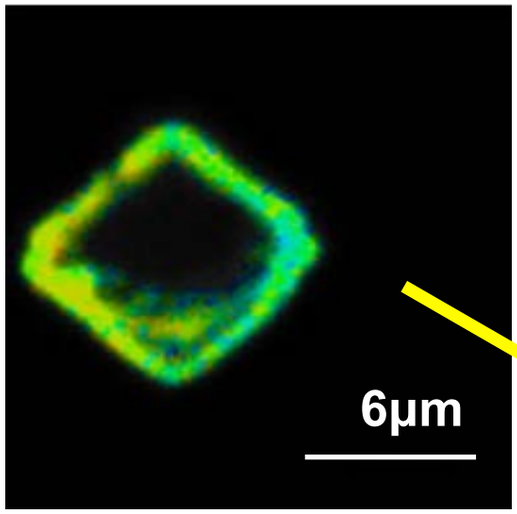
Nanocapsules of 8 layers labelled at 5° and 7°layer

Two photon excitation imaging

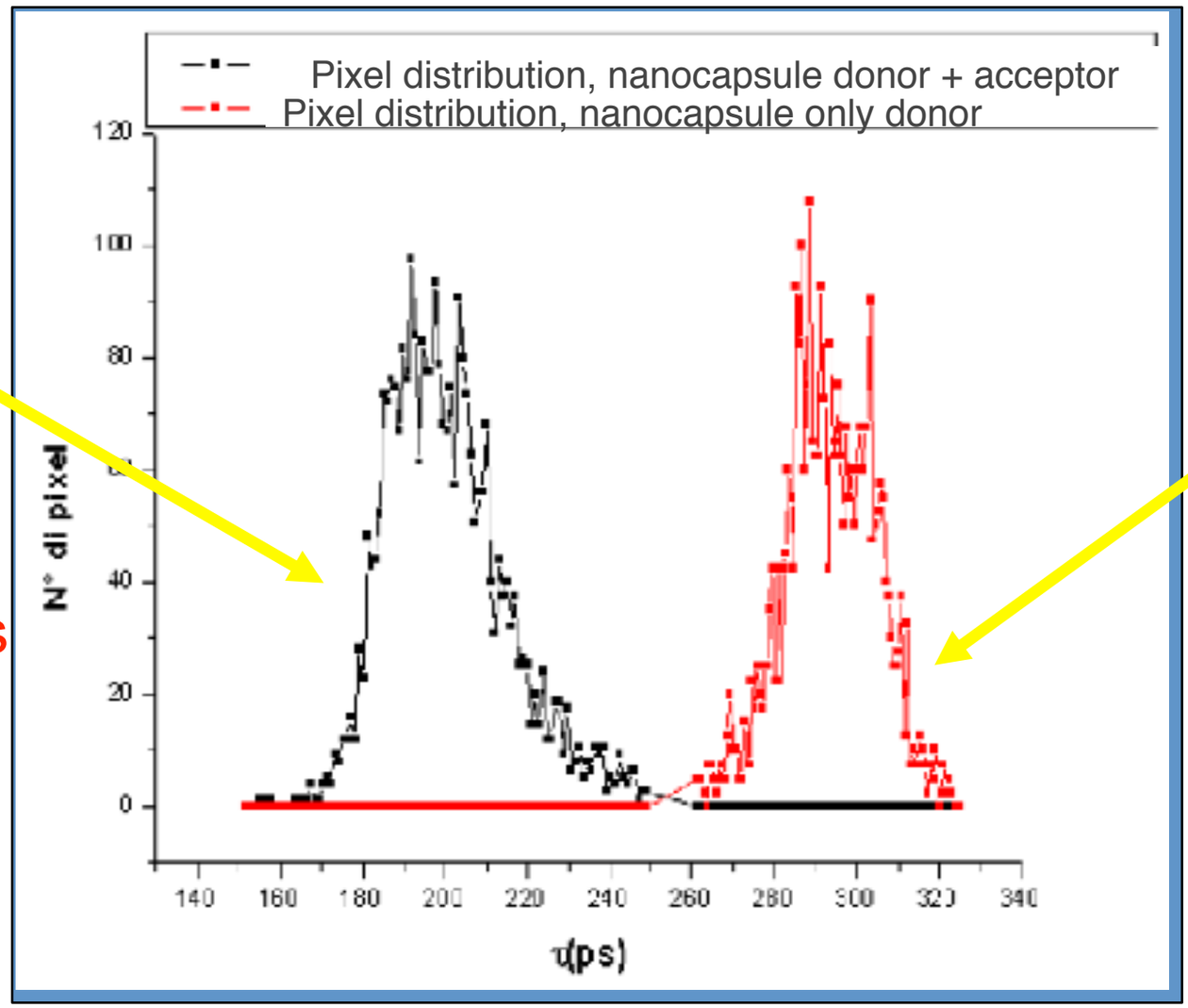


FRET and FLIM on nanocapsules

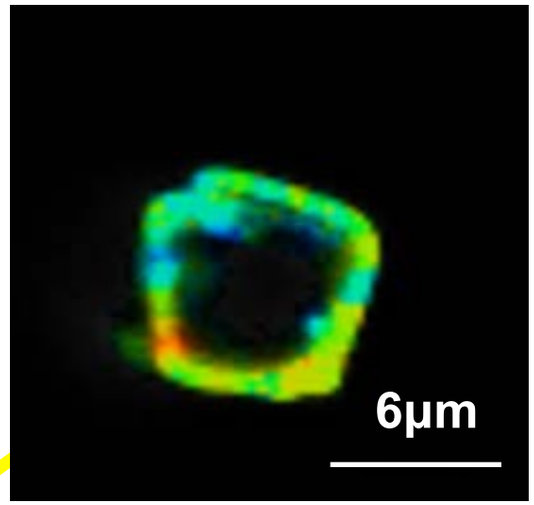
Donor FITC + Acceptor Alexa594



150ps 250ps



Donor FITC



270ps 320ps

Translation due to FRET

Caorsi V., Ronzitti E., Vicidomini G., Krol S., McConnell G., Diaspro A. (2007) Micr. Res. Tech., 70
Nizzari M., Caorsi V., et al., Diaspro A. (2007) JBC

Fluorescence recovery after photobleaching

From ensemble average experiments to single molecule imaging

Slide credit for FRAP slides (adapted): Davide Mazza

Loren…Diaspro et al., **Q Rev Biophys. 2015** Aug;48(3):323-87.

Slide credits: Davide Mazza - mazza.davide@hsr.it

xVivo – The inner life of cell

http://www.xvivo.net/animation/the-inner-life-of-the-cell/

WHY

IS STUDYING THE DYNAMICS OF YOUR PROTEIN IMPORTANT?

I – CARRIES INFORMATION ABOUT UNDERLYING MOLECULAR MECHANISMS.
Interactions, transport processes, oligomerization states…

II – CELLULAR OUTCOMES DEPEND ON PROTEIN DYNAMICS
Eg. Circadian rhythms, Response to inflammation, to DNA damage…

Oscillations in NF-κB Signaling Control the Dynamics of Gene Expression

D. E. Nelson,[1] A. E. C. Ihekwaba,[2] M. Elliott,[1] J. R. Johnson,[1] C. A. Gibney,[1] B. E. Foreman,[1] G. Nelson,[1] V. See,[1] C. A. Horton,[1] D. G. Spiller,[1] S. W. Edwards,[1] H. P. McDowell,[4] J. F. Unitt,[5] E. Sullivan,[6] R. Grimley,[7] N. Benson,[7] D. Broomhead,[3] D. B. Kell,[2] M. R. H. White[1]*

p53 Dynamics Control Cell Fate

Jeremy E. Purvis, Kyle W. Karhohs, Caroline Mock, Eric Batchelor,* Alexander Loewer,† Galit Lahav‡

Optical microscope

In vivo dynamics of RNA polymerase II transcription

Xavier Darzacq[1,2], Yaron Shav-Tal[1,3], Valeria de Turris[1], Yehuda Brody[1], Shailesh M Shenoy[1], Robert D Phair[4] & Robert H Singer[1]

WHAT

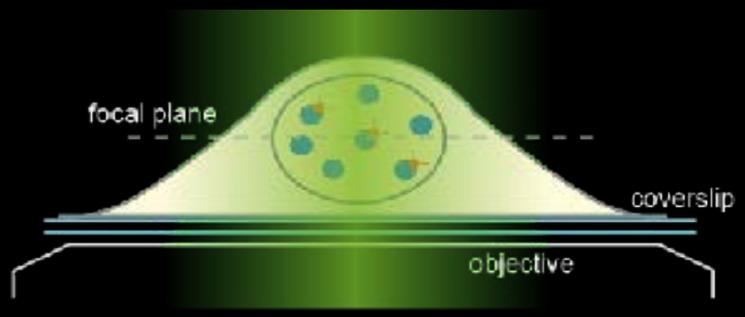
INSTRUMENT TO USE?

OPTICAL MICROSCOPY looks like a good candidate.

Cells are transparent to visible light.
Relatively low phototoxicity (but beware of near UV radiation).
With sufficient resolution (~200nm) to visualize intracellular compartments

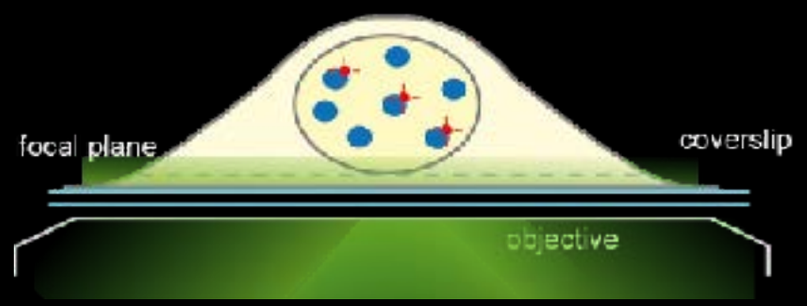
What kind of microscopy?

WIDEFIELD ILLUMINATION



- \+ Sensitive
- \+ Fast
- − Out of focus blur

TIRF ILLUMINATION



- \+ Sensitive
- \+ No out of focus blur
- − Limited to surface

LASER SCANNING (e.g. CONFOCAL)

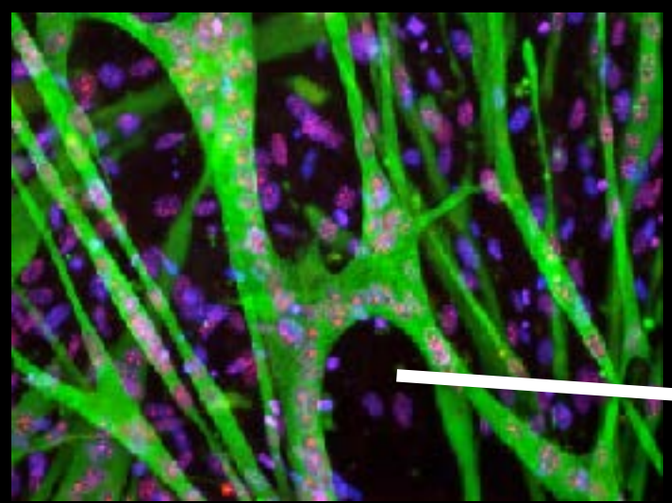
- \+ No out of focus b
- − Not that fast
- − Not that sensitive

The missing link.

Light microscopy

© T. Hatano, NIG, Japan

Immunofluorescence



© M.Neguembor, HSR.

Viability

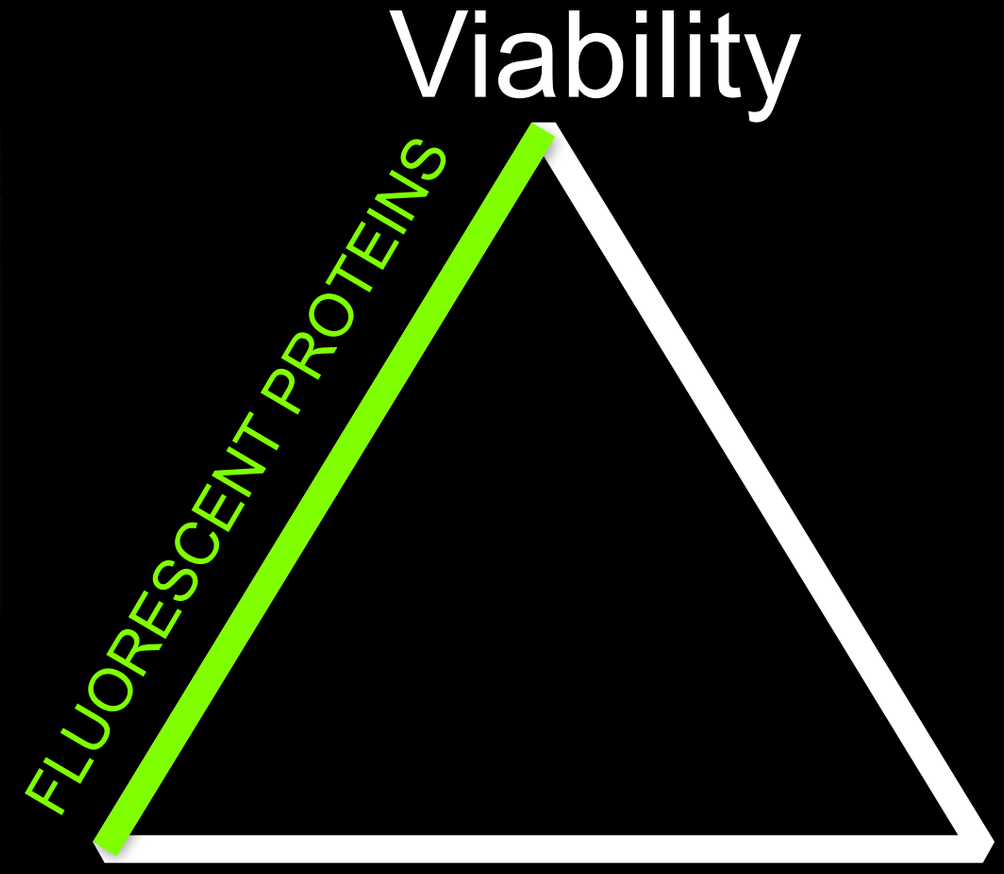
Specificity

Resolution

Electron microscopy

© CBIM, Imperial College, UK

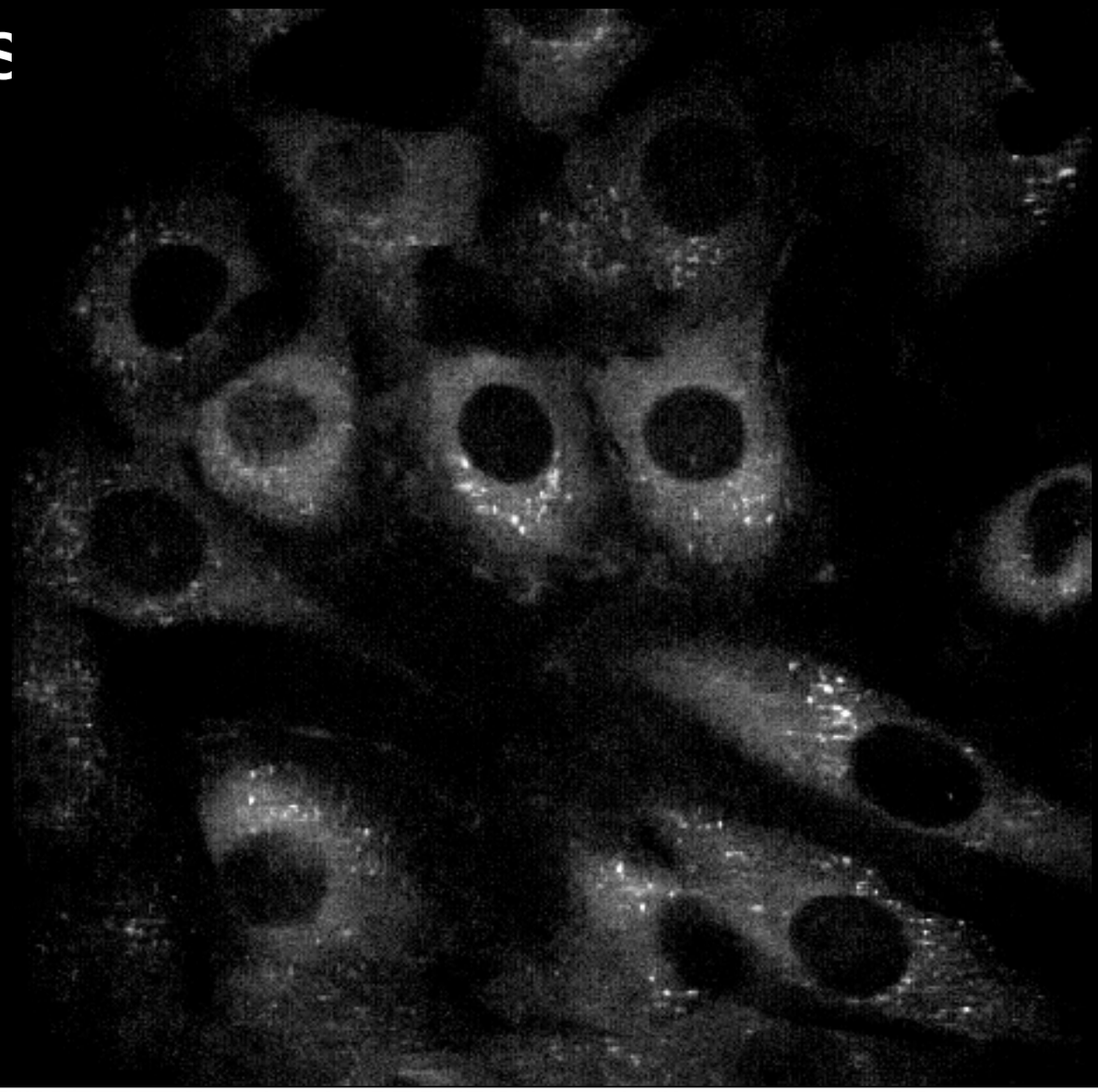
GFP: bridging viability and specificity



Von Dassow, 200

Some dynamics can be measured directly

Example: massive translocations between different compartments

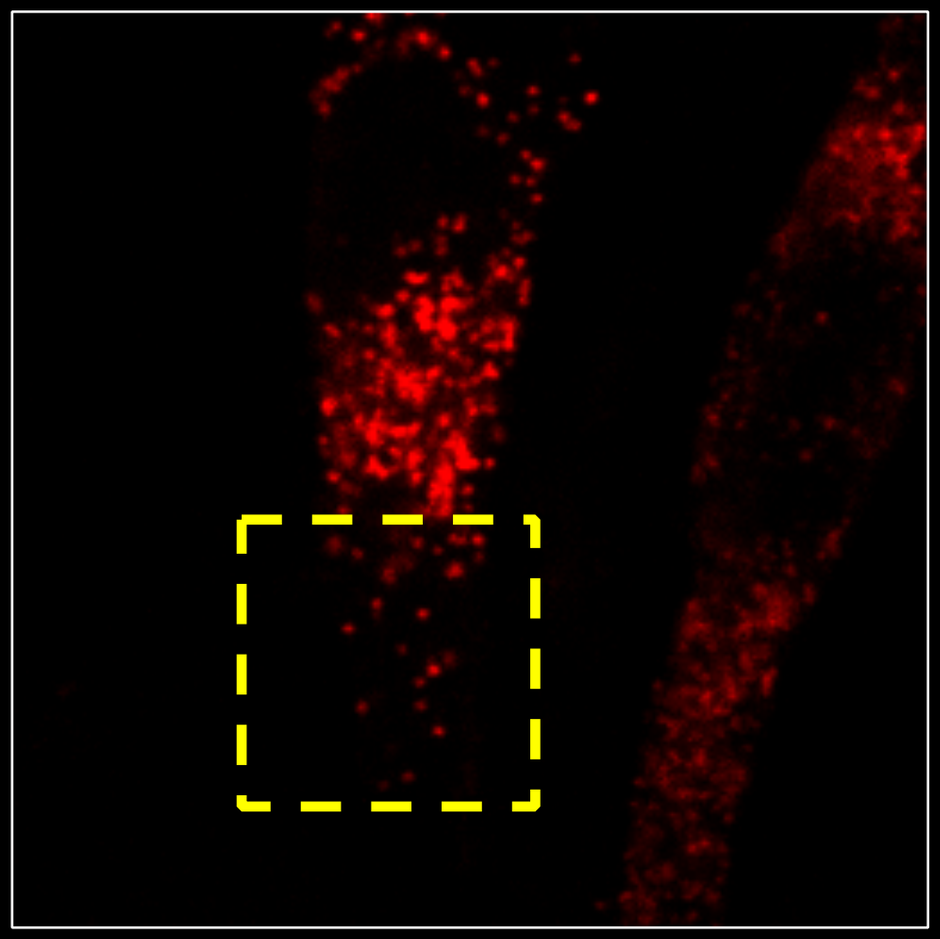


NF-kB translocation

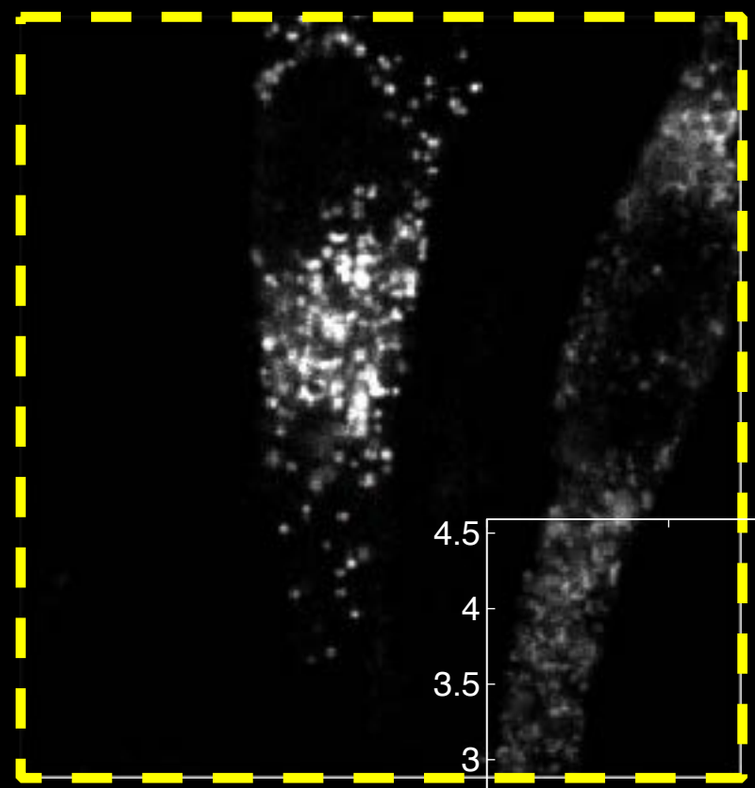
(Sung and Agresti, 2009)

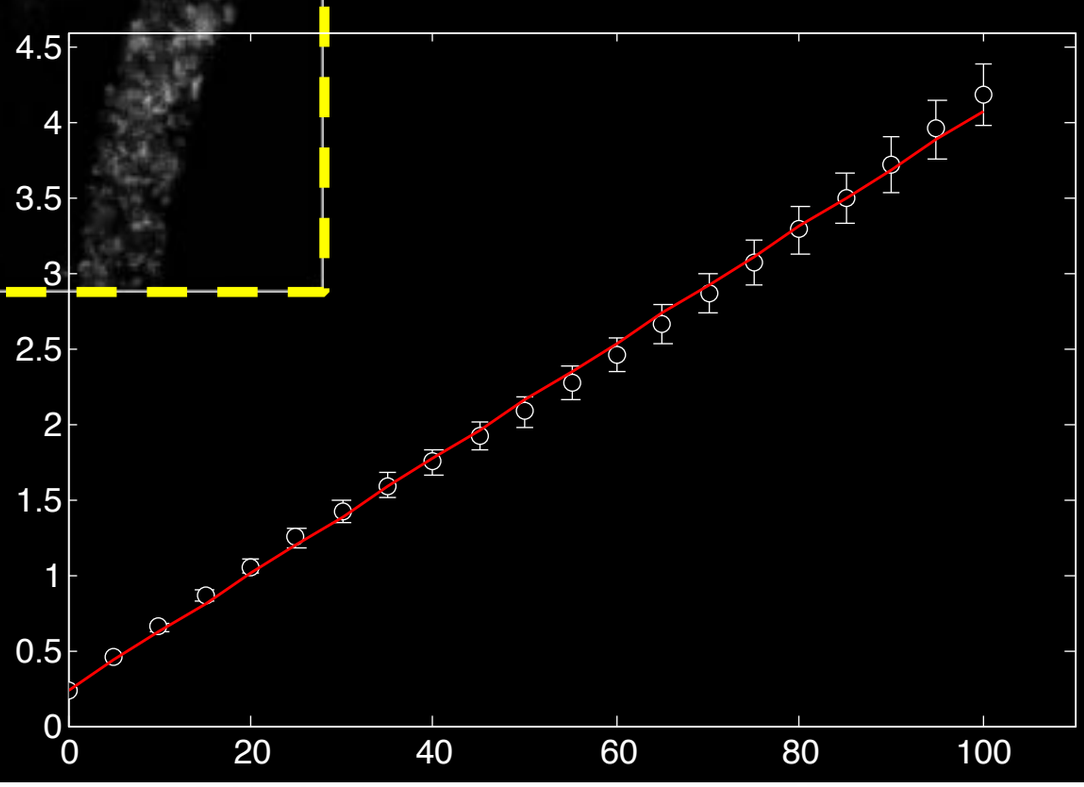
Mobility of dispersed particles

one image every 5 sec
whole movie ~ 500s



Tracked particles



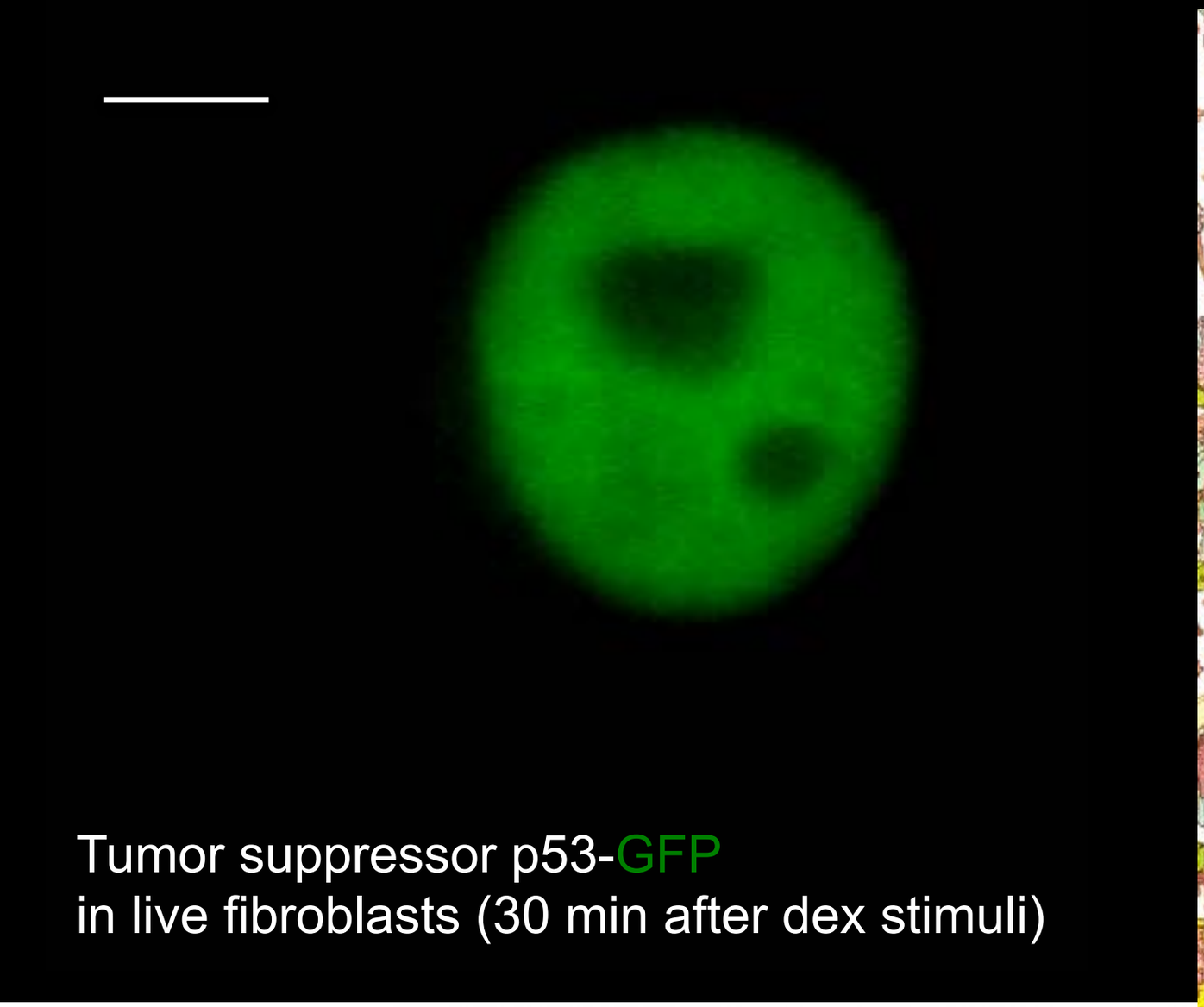


Aggregates of Ab heavy chain.
(w/ M. Mossuto and R. Sitia, HSR)

Equilibrium: a molecular

Where's Waldo

It can be difficult to understand what individual molecules do,
even when selectively labelling your protein of interest.

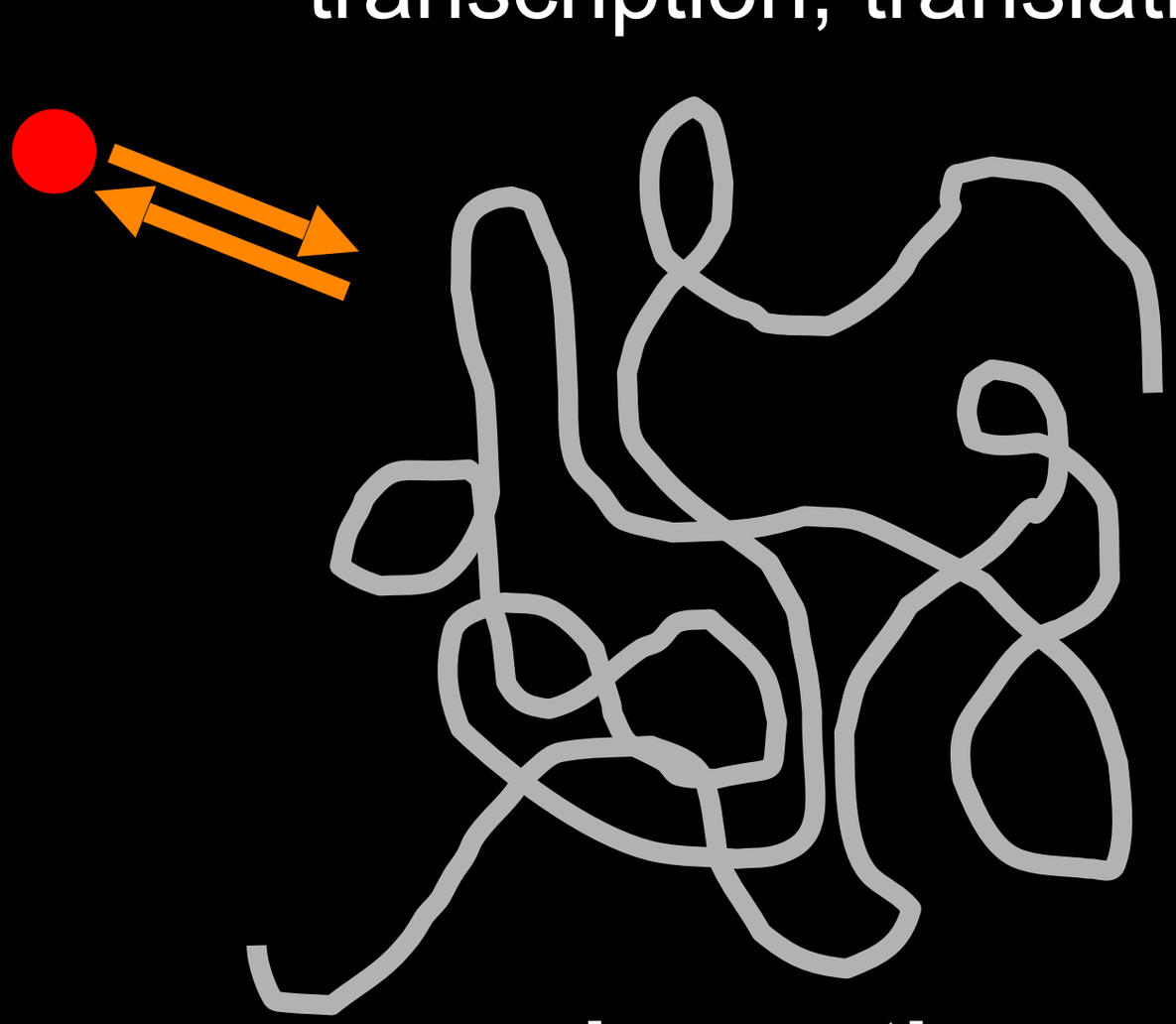


Tumor suppressor p53-GFP
in live fibroblasts (30 min after dex stimuli)

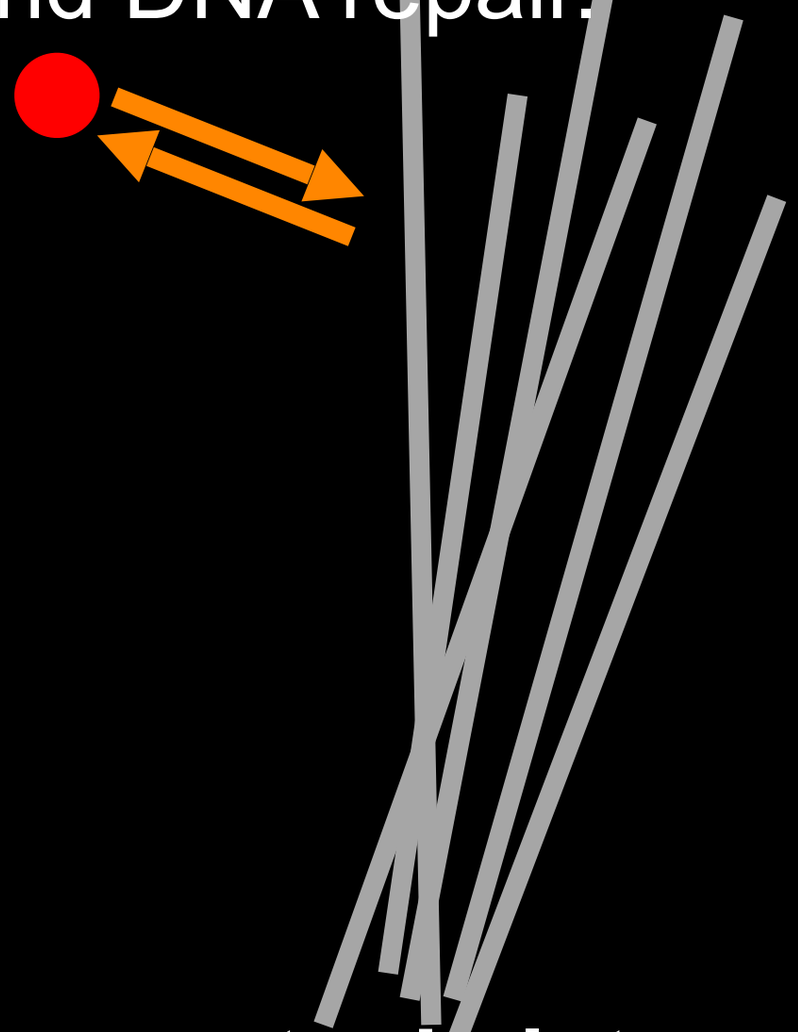
Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

Binding to ~immobile scaffolds is a widespread event in the cells

In the nucleus, these interactions regulate transcription, translation and DNA repair.



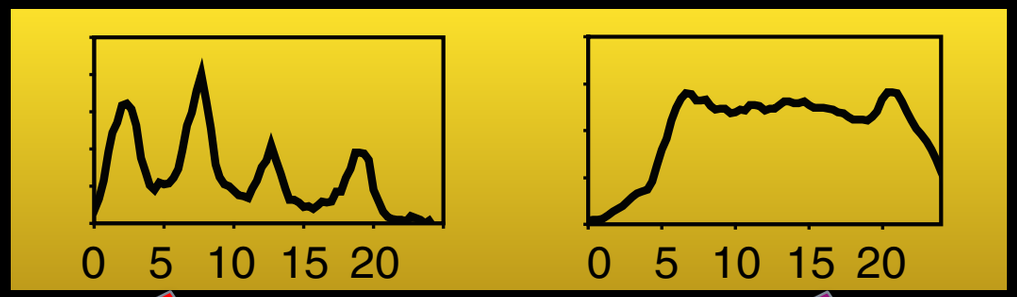
chromatin



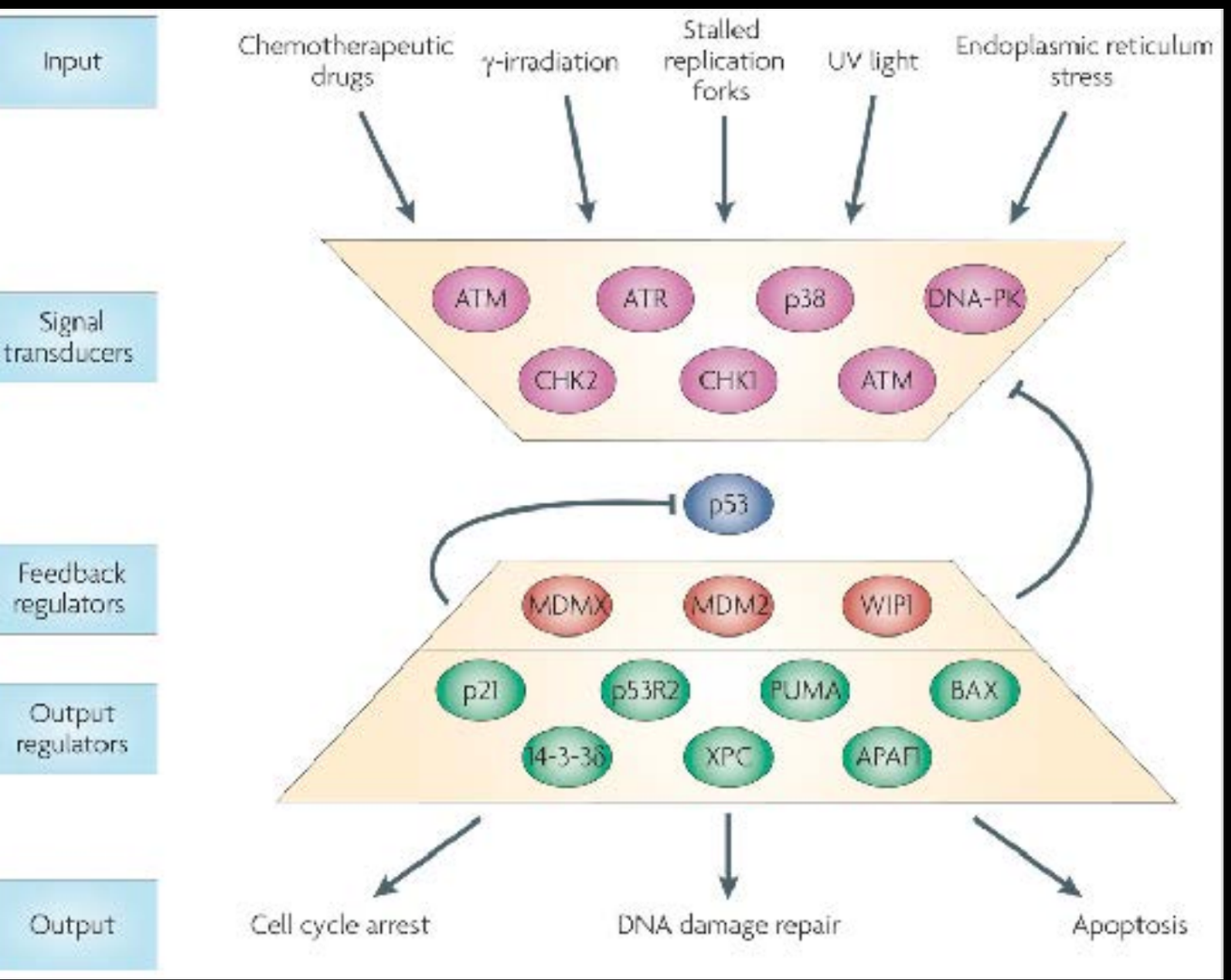
cytoskeleton

Long term experiments?

Interfering with protein dynamics allows interfering with cellular fate.



hours after irradiation → hours after irradiation



(Batchelor et al., Mol Sys Biol, 2011) (Purvis et al., Science, 2012)

DNA DAMAGE REPAIR

CELL DEATH

(Batchelor et al.,Nat. Cancer Rev, 2009)

Perturbation control

Modify the fluorescence properties of a subpopulation of molecules by using a pulse of intense light.

Fluorescence Perturbation techniques (FPT)

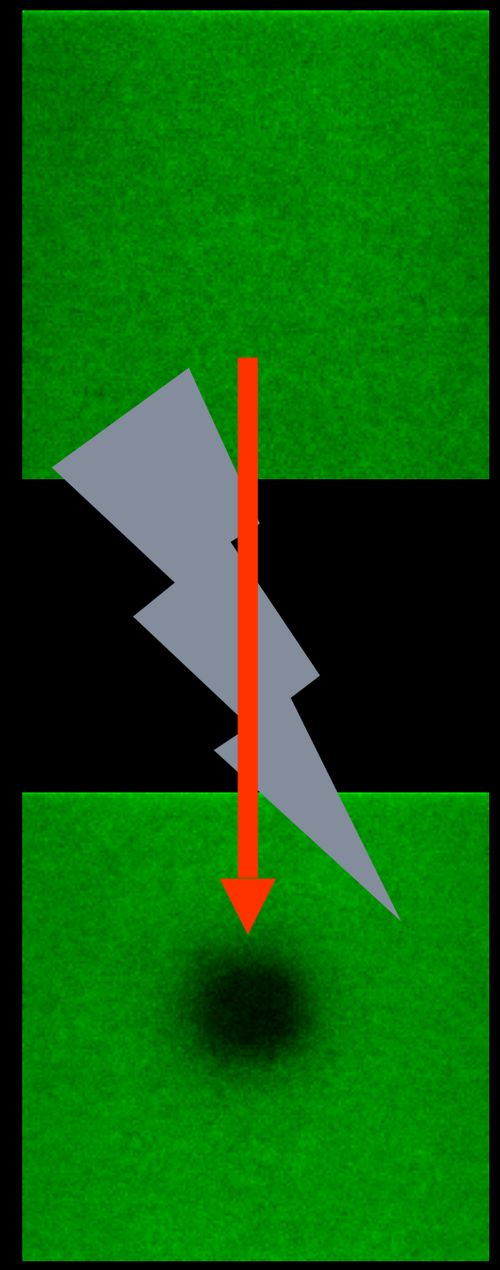
Photobleaching Techniques

Photoactivation Techniques

FRAP
Fluorescence Recovery after Photobleaching

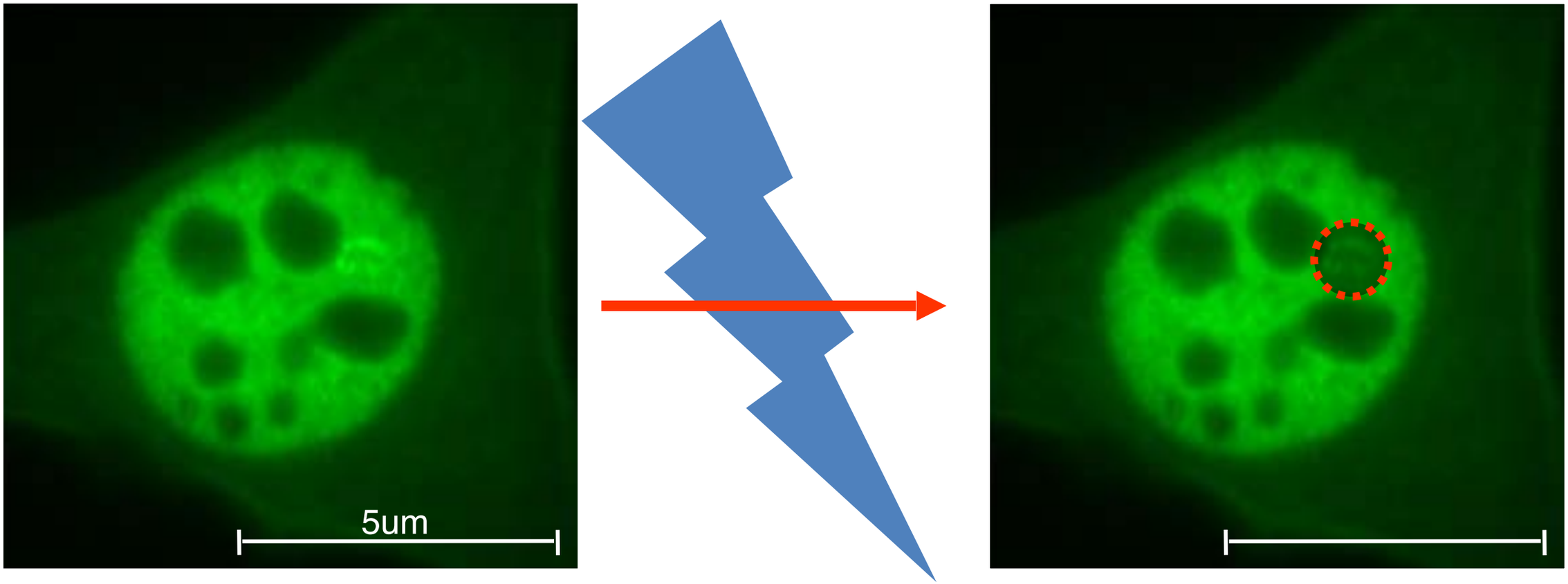
i-FRAP
Inverse FRAP

FLIP
Fluorescence Loss Into Photobleaching



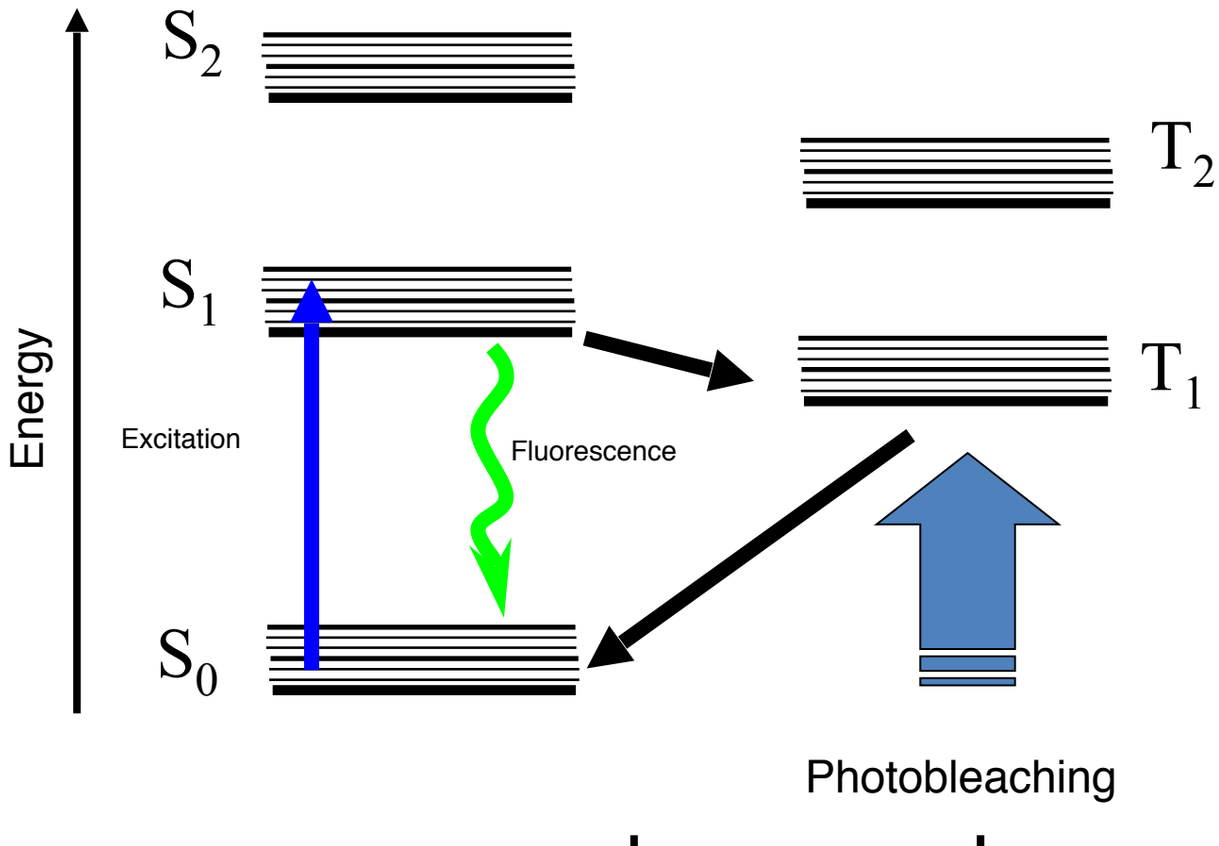
FRAP

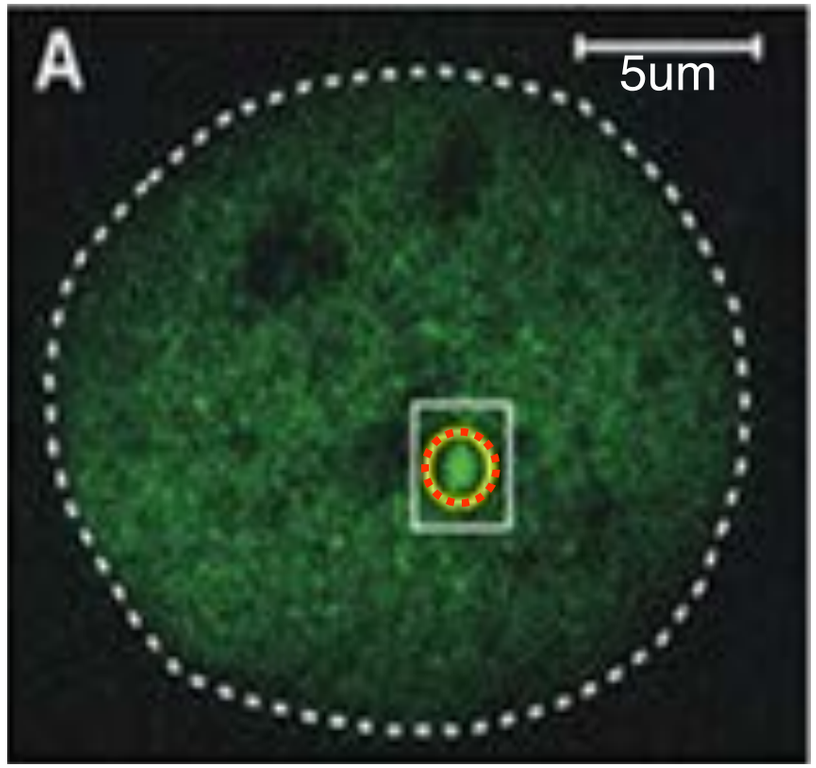
Perturbation control:

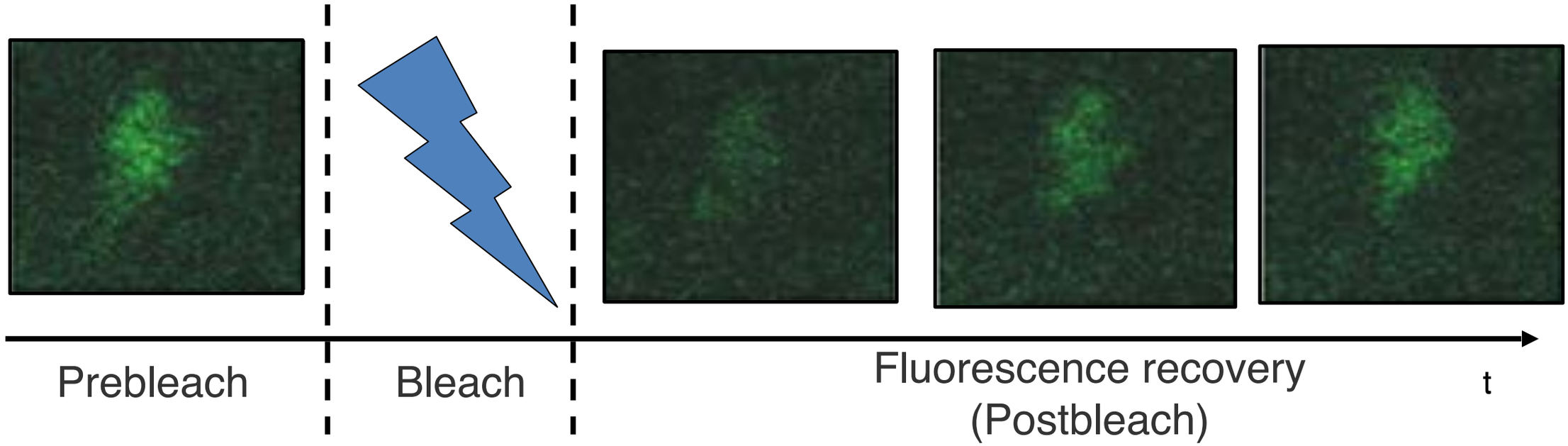


slide credit: Davide Mazza, LAMBS

FRAP



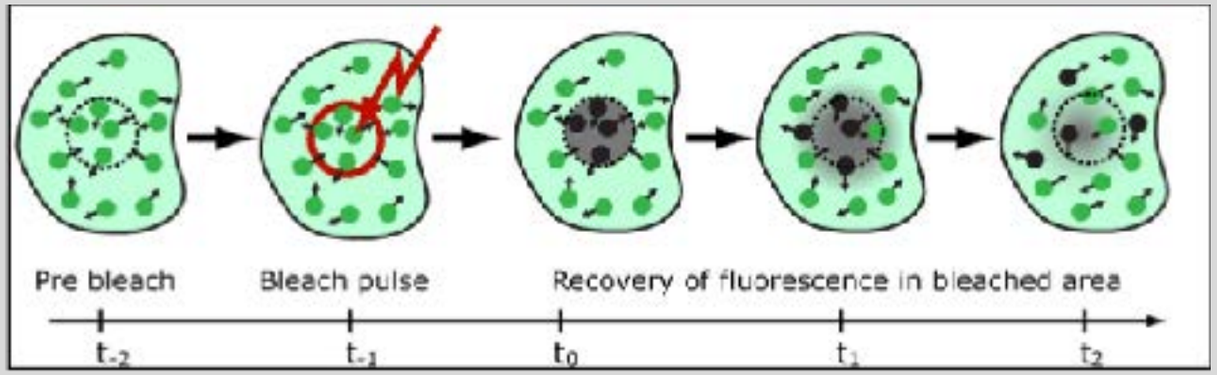


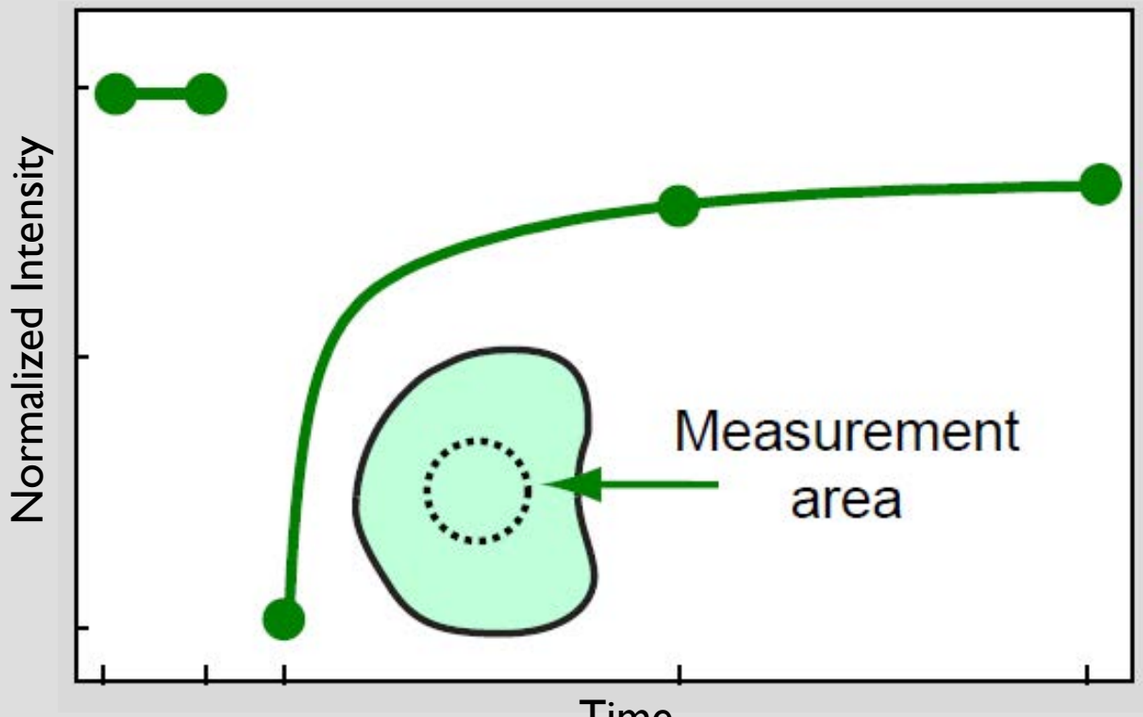


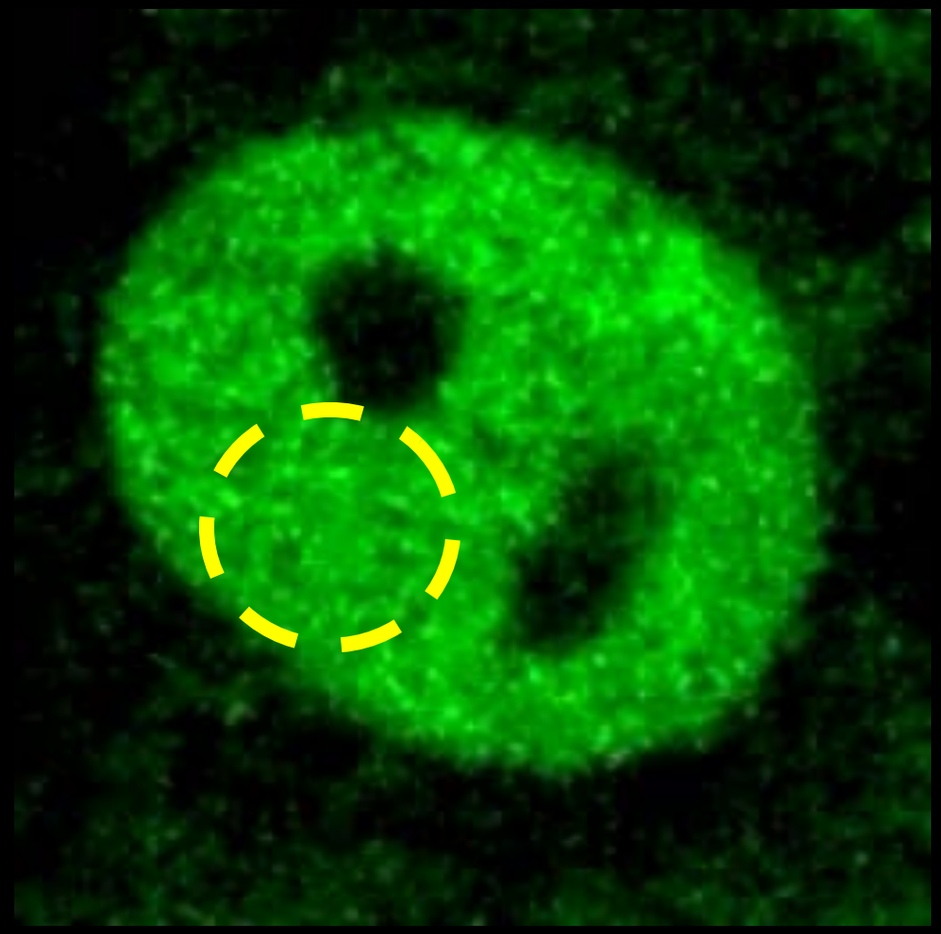
slide credit: Davide Mazza, LAMBS

Fluorescence recovery after photobleaching

Fluorescence Recovery After Photobleaching (FRAP)

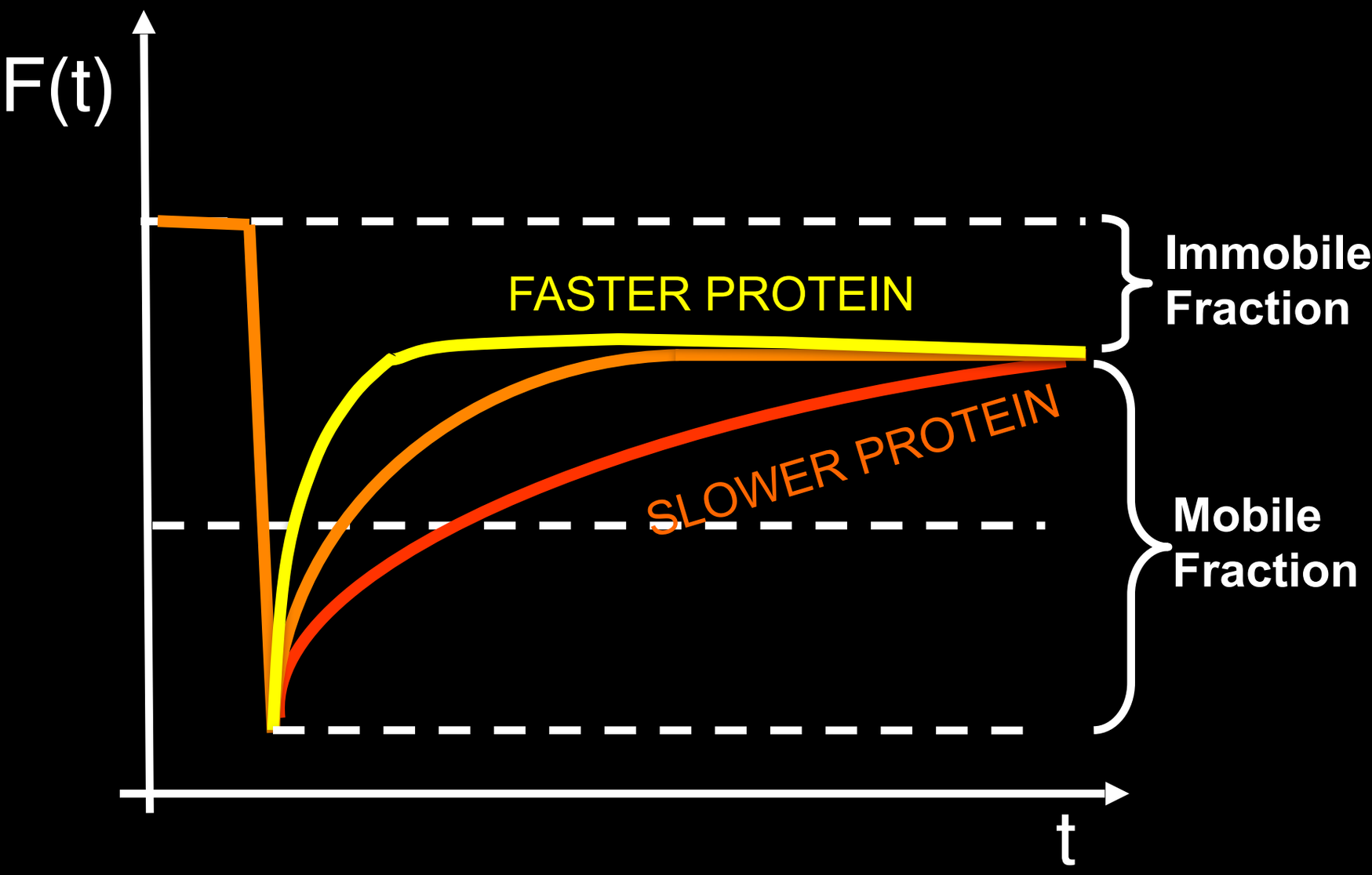






p53 dynamics in living cells
(whole movie 30 s)

Qualitative analysis of FRAP data



Can we be more quantitative?

YES BUT WE NEED SOME MATH

Free diffusion

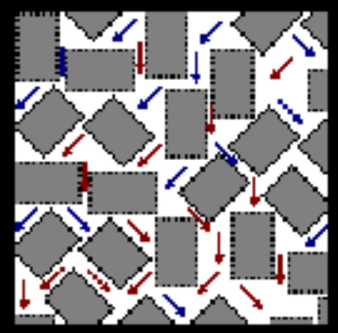
Axelrod, D et al.(1976), Biophys J 16, 1055--1069.
Soumpasis, D. M. (1983), Biophys J 41, 95--97.

Anomalous sub-diffusion

Saxton, M. J. (2001), Biophys J 81, 2226--2240.

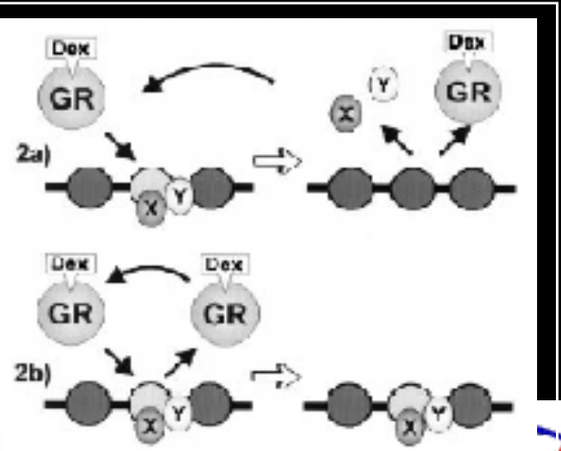
Diffusion in heterogeneous systems

Siggia, E. D. et al. (2000), Biophys J 79(4), 1761--1770.

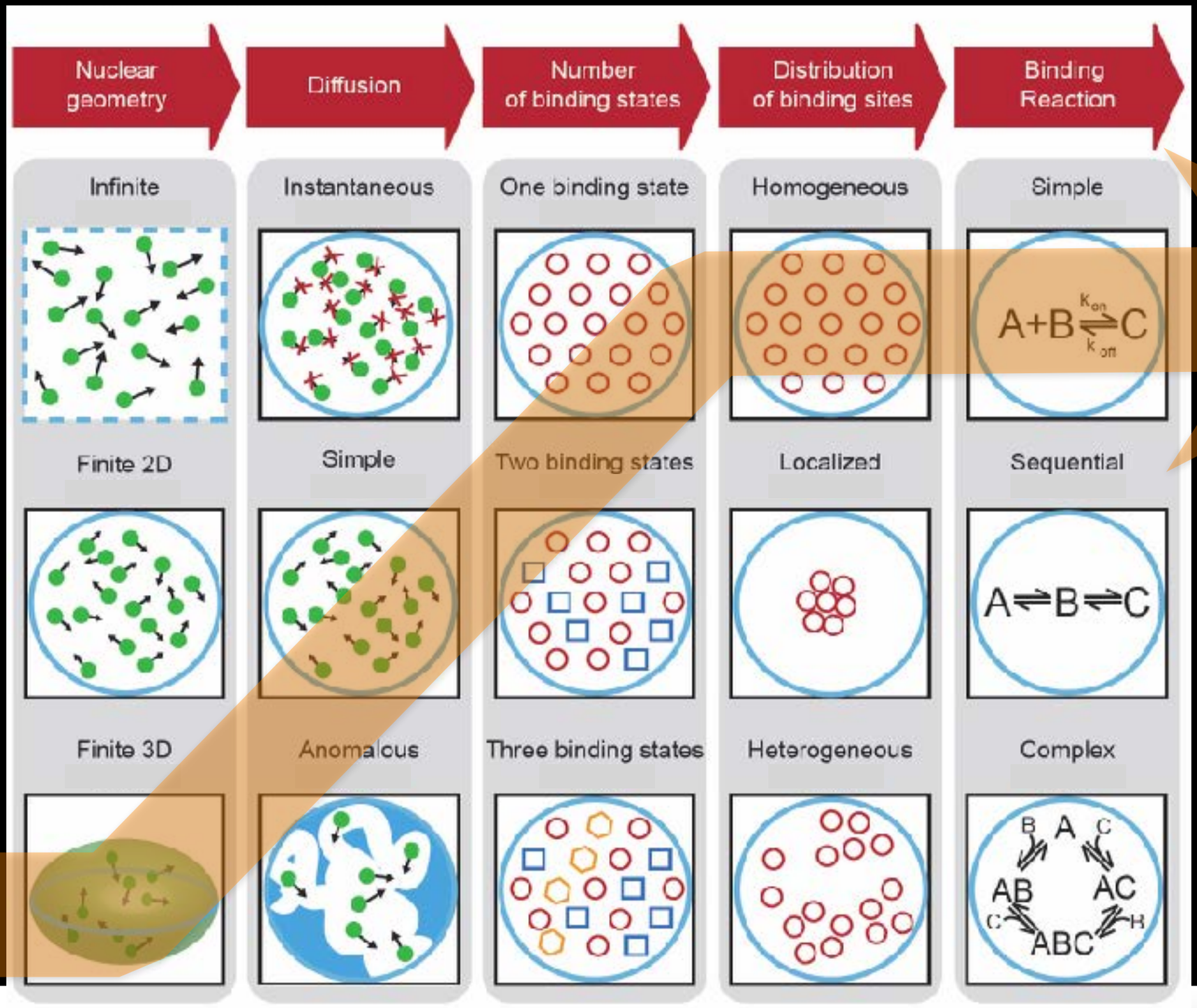


Diffusion and binding problems (hit and run)

Sprague, B. L.; et al. (2004) Biophys J, 86, 3473--3495.
Mueller et al. , B. L. et al. (2008), Biophys J.



Selecting a model for the FRAP experiments

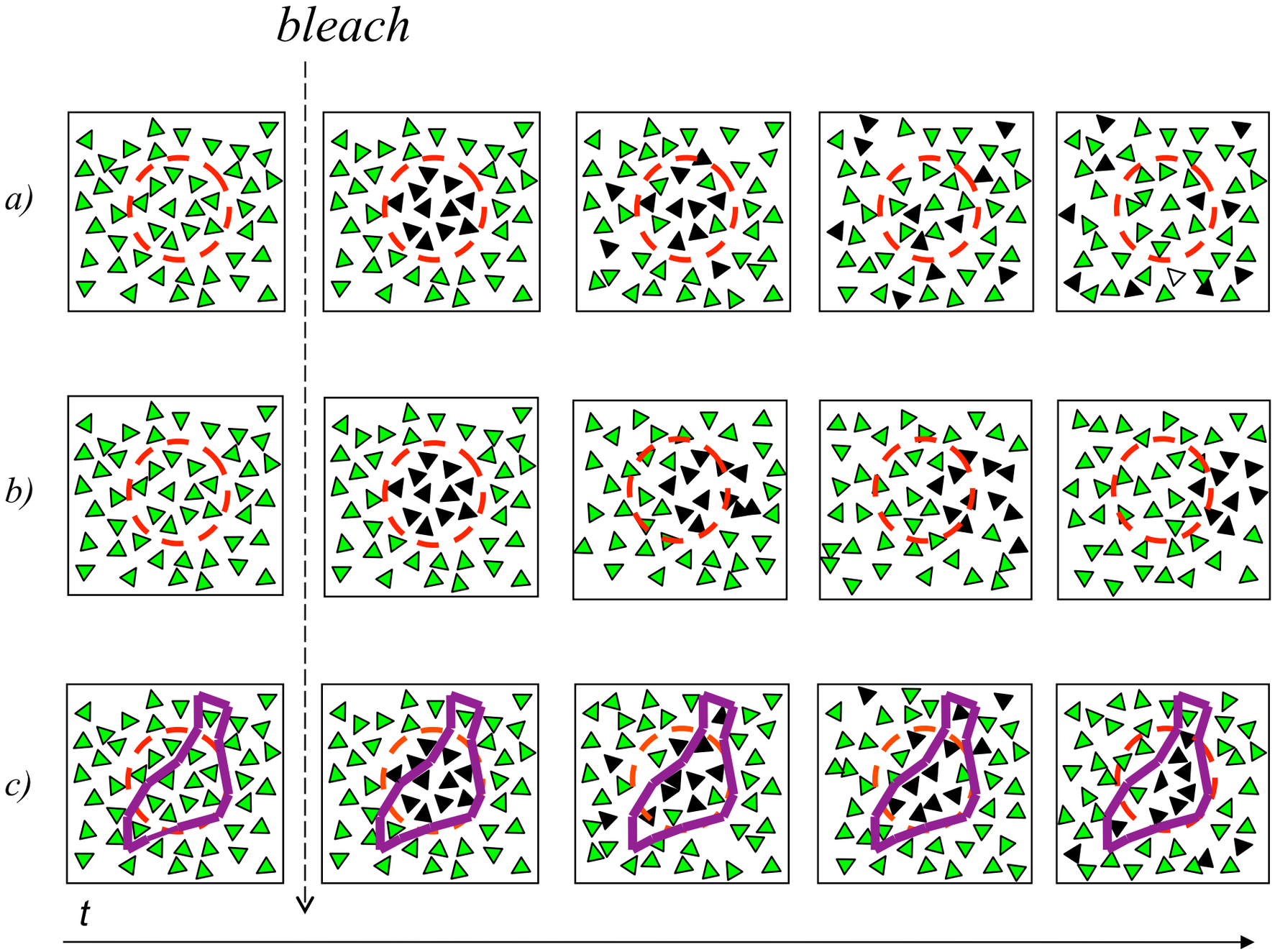


Equations deduced from the choices made

$$\begin{cases} \dfrac{\partial F}{\partial t} = D_F \nabla^2 F - k_{on}^* F + k_{off} C \\ \dfrac{\partial C}{\partial t} = + k_{on}^* F - k_{off} C \end{cases}$$

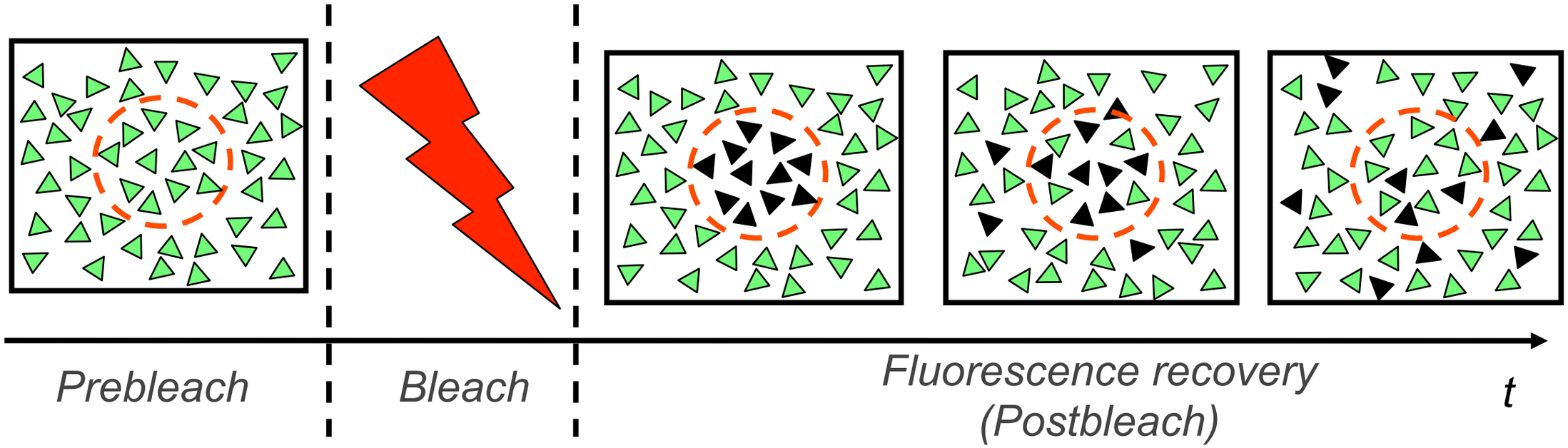
With proper initial and boundary conditions lead to model for experimental data

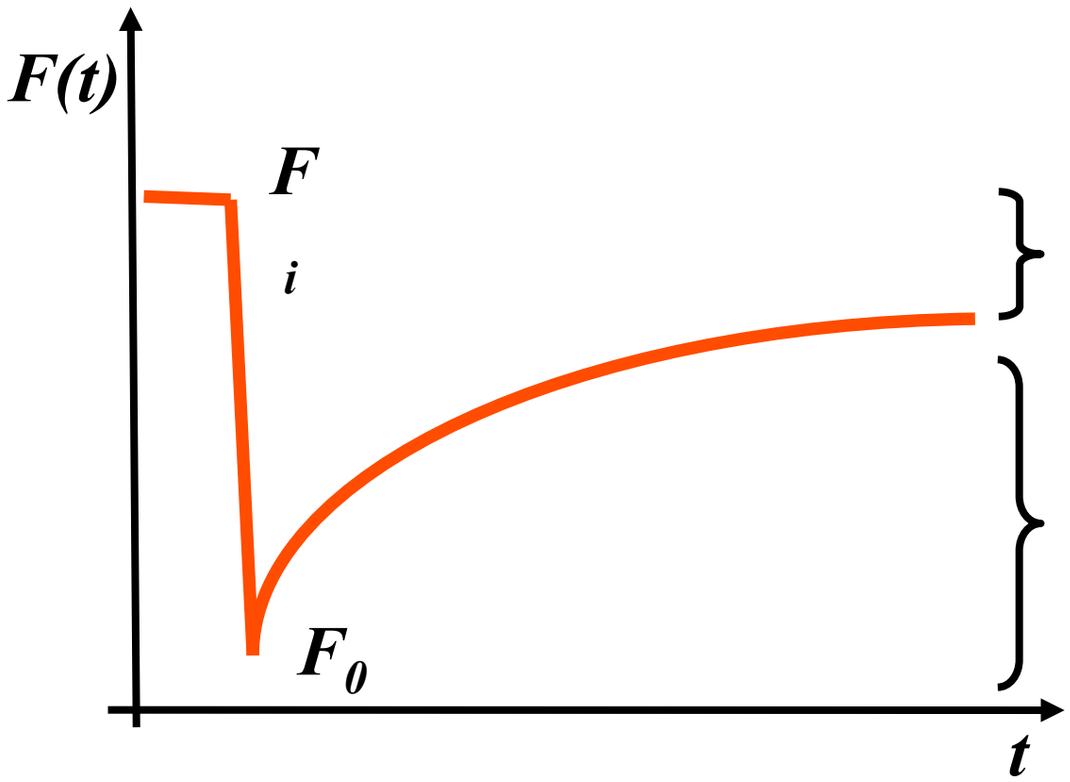
FRAP



Slide credits: Davide Mazza, Francesca Cella, LAMBS

FRAP





Immobile Fraction

$$M_i = 1 - M_f$$

Mobile Fraction

$$M_f = \frac{F_\infty - F_0}{F_i - F_0}$$

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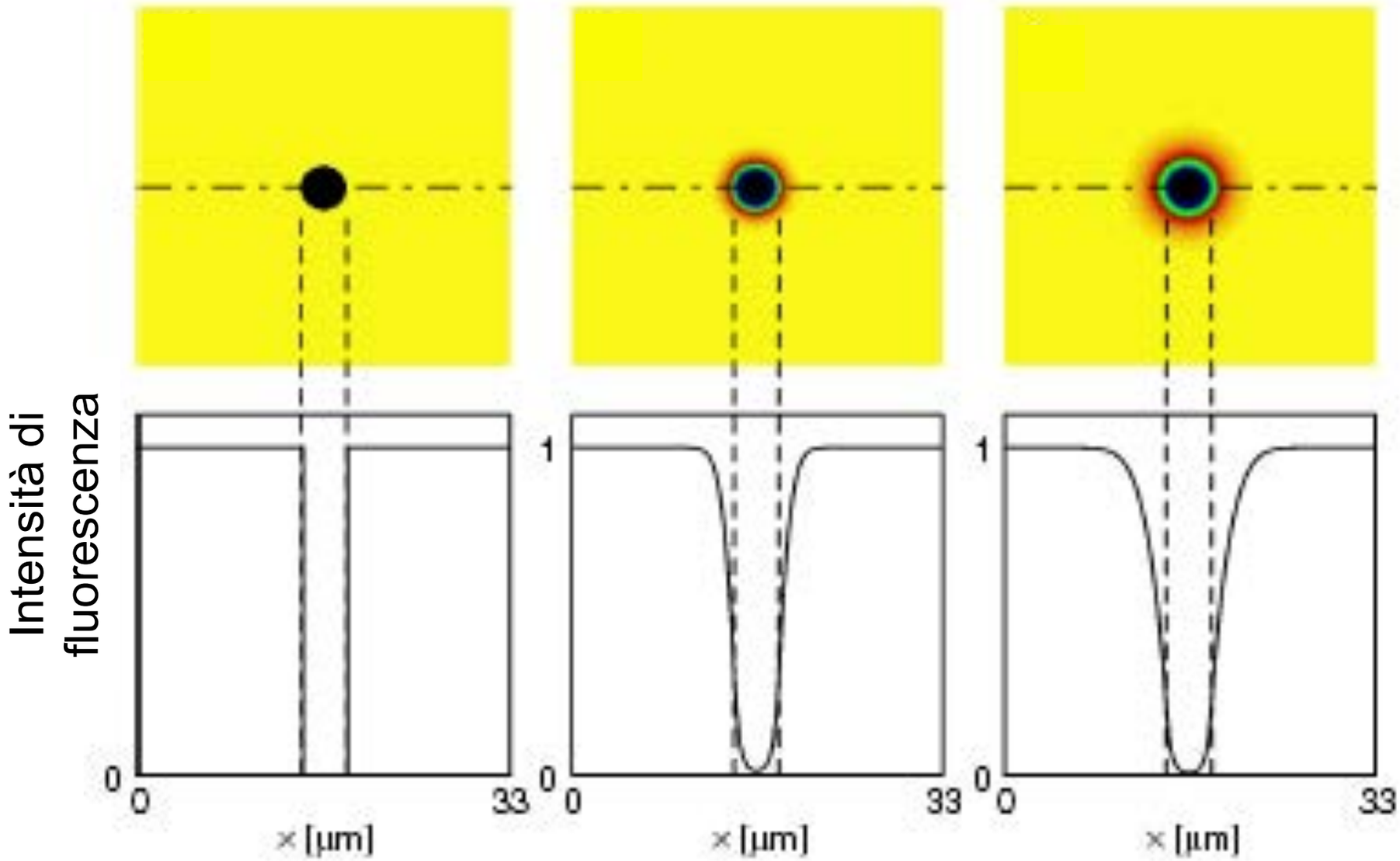
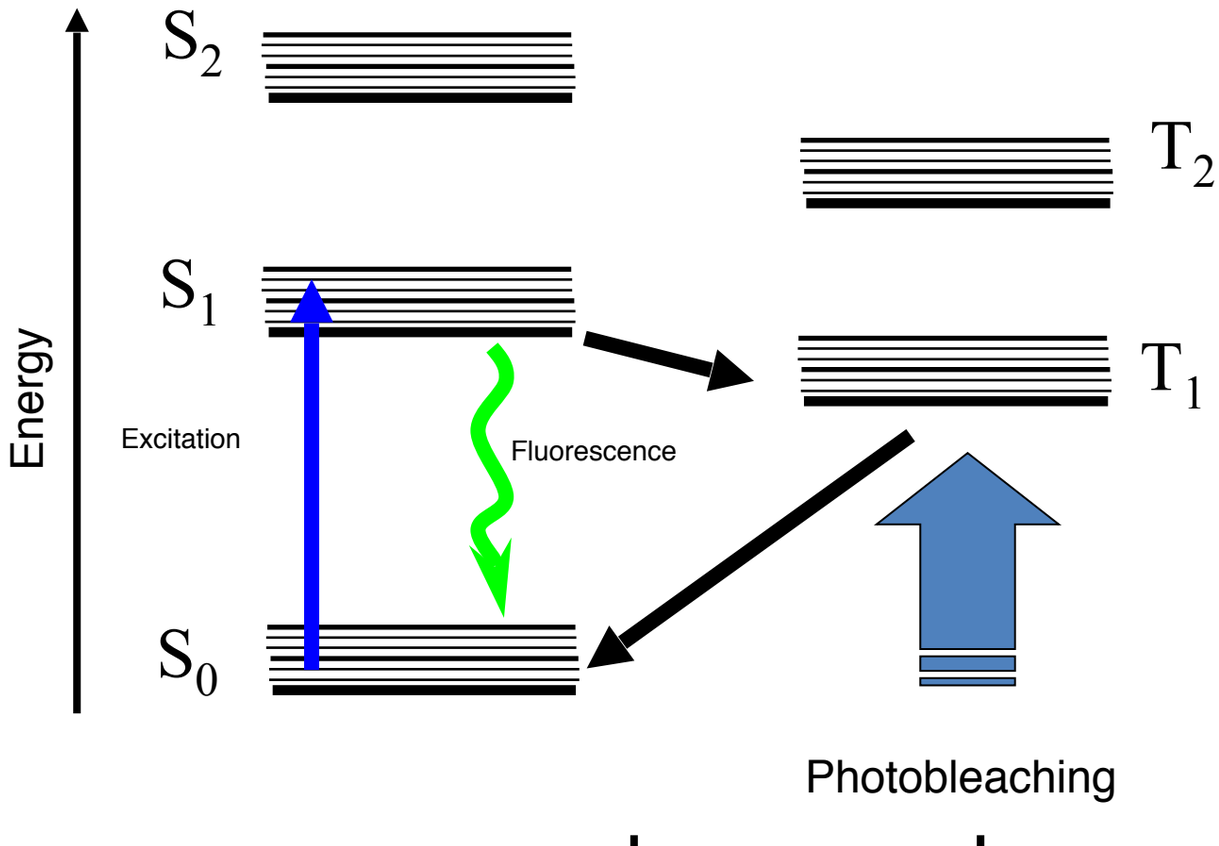
FRAP

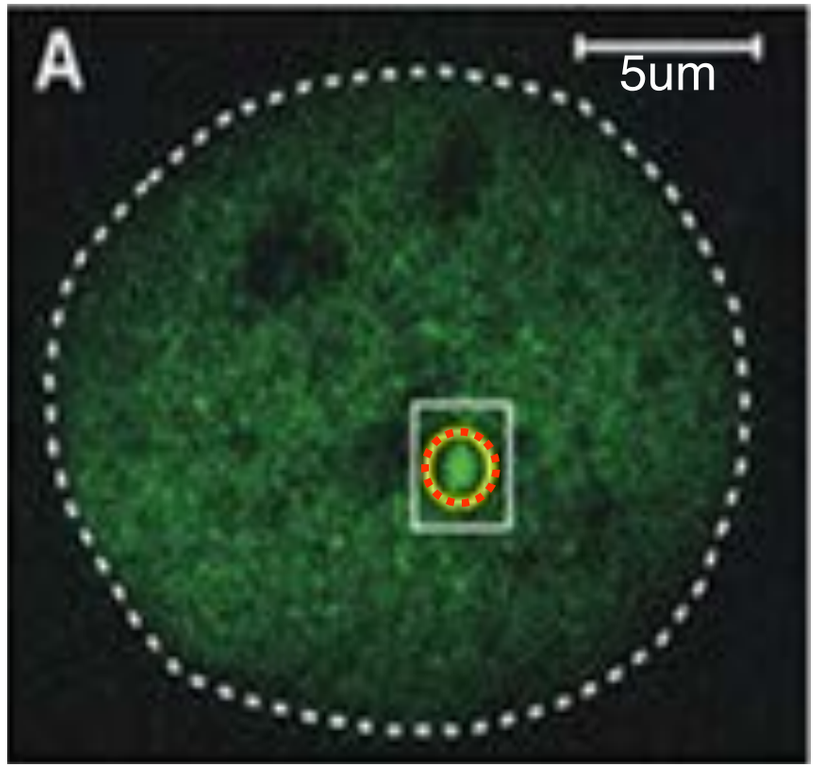
By Increasing the bleach time

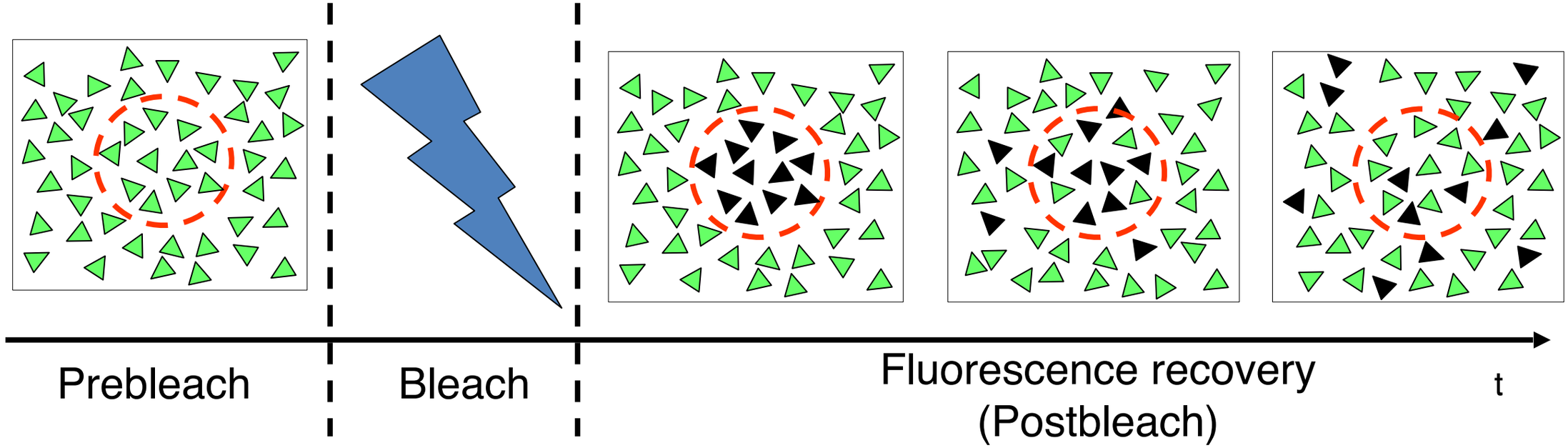


Weiss, M. (2004), *Traffic* 5(9), 662--671.

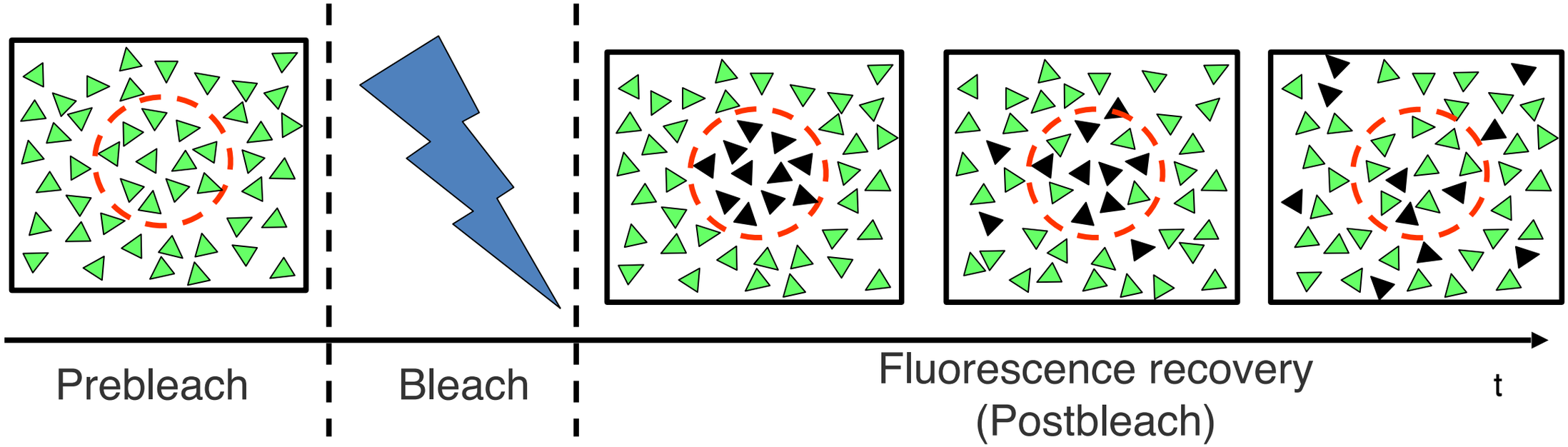
FRAP

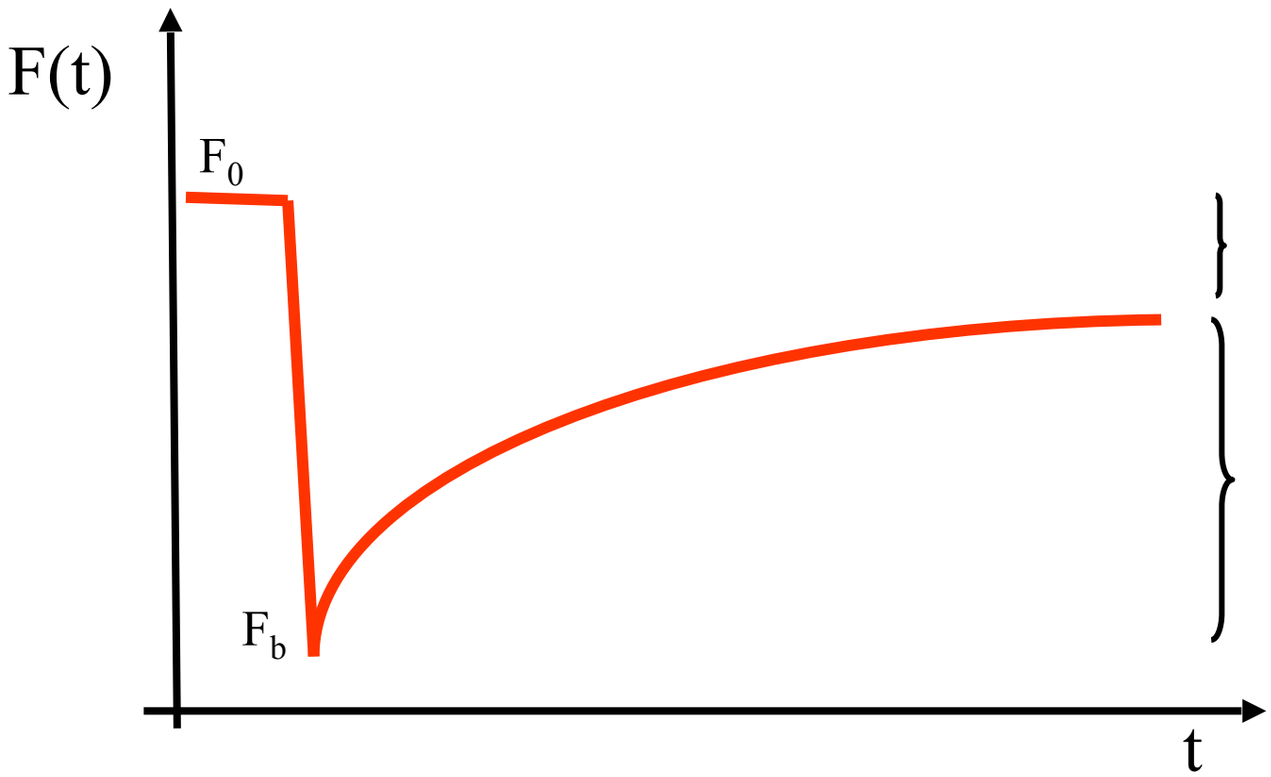






McNally, J. & Smith, C. (2001), in Confocal and two-photon microscopy, ed. A. Diaspro





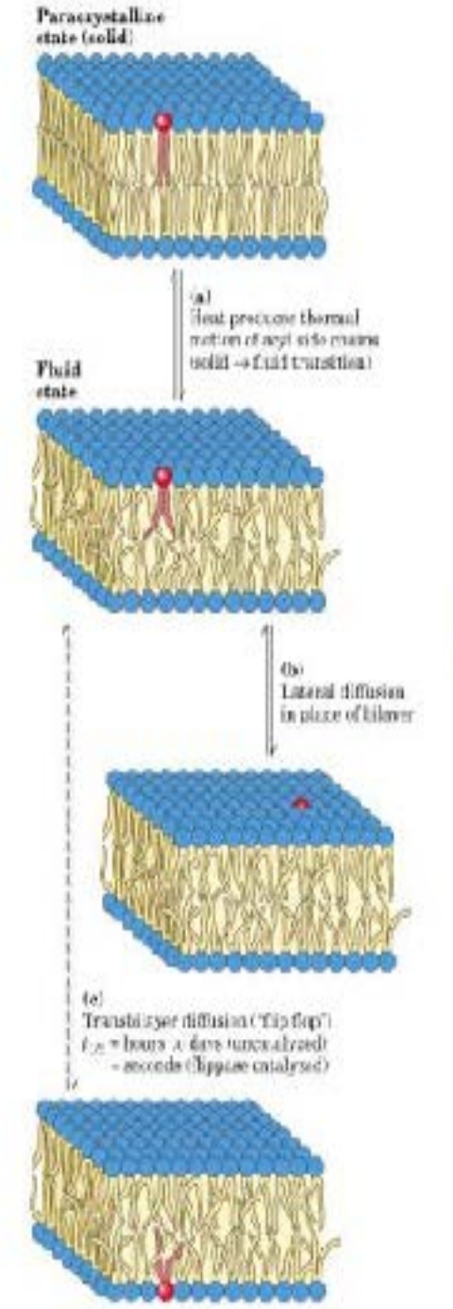
Immobile fraction $1-k$

Mobile fraction $k = \dfrac{F_\infty - F_b}{F_0 - F_b}$

slide credit: Davide Mazza, LAMBS

FRAP

Cell territories are not FLATLAND…

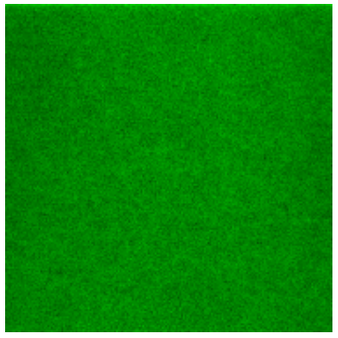


slide credit: Davide Mazza, LAMBS

FRAP

Condizione Iniziale

Distribuzione Uniforme di molecole fluorescenti

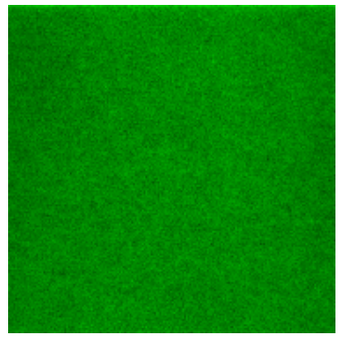


slide credit: Davide Mazza, LAMBS

FRAP

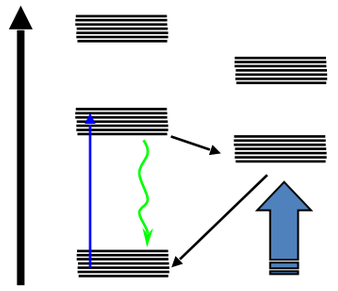
Condizione Iniziale

Distribuzione Uniforme di molecole fluorescenti



Photobleaching

Reazione Irreversibile del primo ordine



$$\frac{dC(x,y,t)}{dt} = -\alpha I_b(x,y)C(x,y,t)$$

Rate di Photobleaching

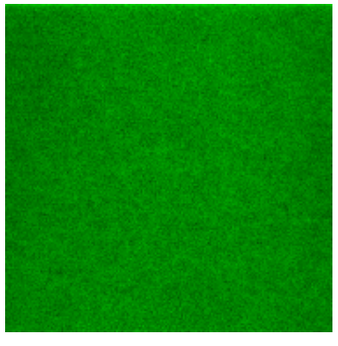
Distribuzione d'illuminazione

slide credit: Davide Mazza, LAMBS

FRAP

Condizione Iniziale

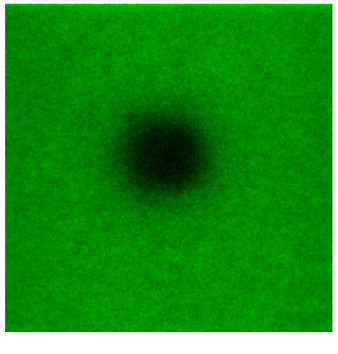
Distribuzione Uniforme di molecole fluorescenti



Photobleaching

Reazione Irreversibile del primo ordine

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$



Fluorescenza finale al termine della fase di bleach

=

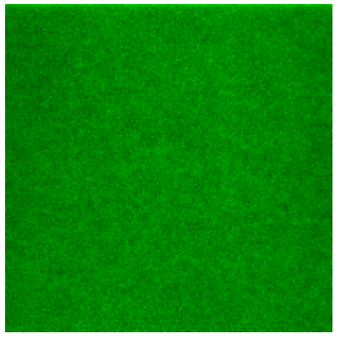
Condizione Iniziale per la fase di Recovery

slide credit: Davide Mazza, LAMBS

FRAP

Condizione Iniziale

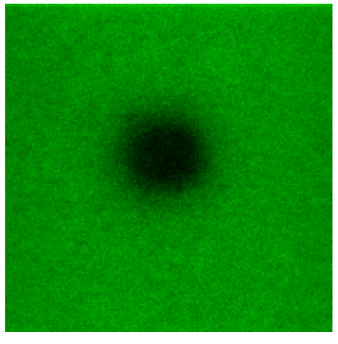
Distribuzione Uniforme di molecole fluorescenti



Photobleaching

Reazione Irreversibile del primo ordine

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$



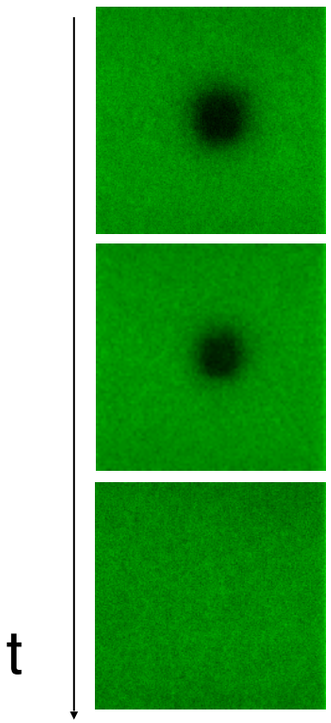
Fluorescenza finale al termine della fase di bleach

Recovery

Equazione che descriva il moto delle molecole

$$\frac{\partial C(x,y,t)}{\partial t} = D\nabla^2 C(x,y,t)$$

(IIa legge di FicK)

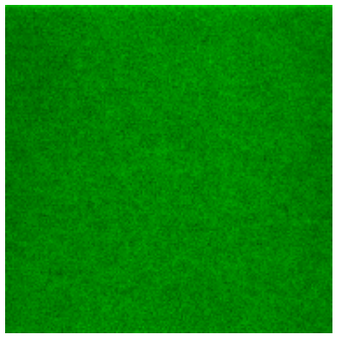


slide credit: Davide Mazza, LAMBS

FRAP

Condizione Iniziale

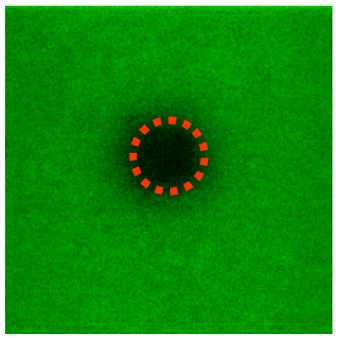
Distribuzione Uniforme di molecole fluorescenti



Photobleaching

Reazione Irreversibile del primo ordine

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$



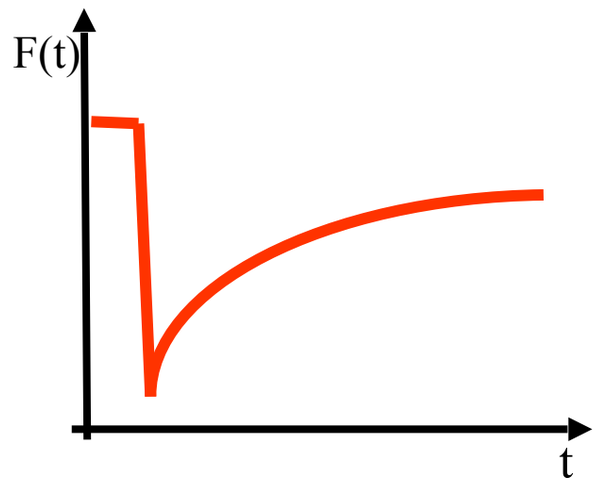
Fluorescenza finale al termine della fase di bleach

Recovery

$$\frac{\partial C(x,y,t)}{\partial t} = D\nabla^2 C(x,y,t)$$

Equazione che descriva il moto delle molecole

Fluorescenza media in funzione del tempo

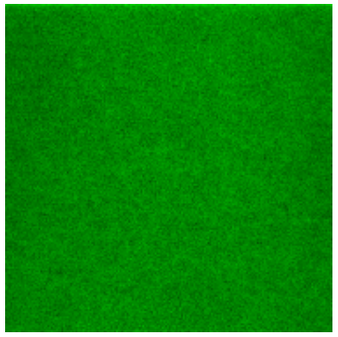


slide credit: Davide Mazza, LAMBS

FRAP

Condizione Iniziale

Questa descrizione considera
bleaching and recovery
disaccoppiati

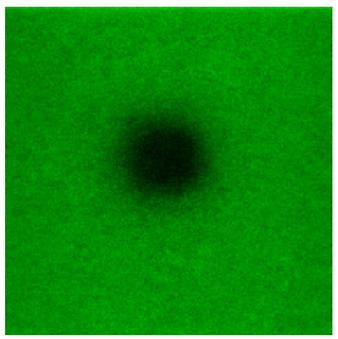


Photobleaching

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$

Fluorescenza finale al
termine della fase di bleach

Niente bleaching durante
la fase di recovery

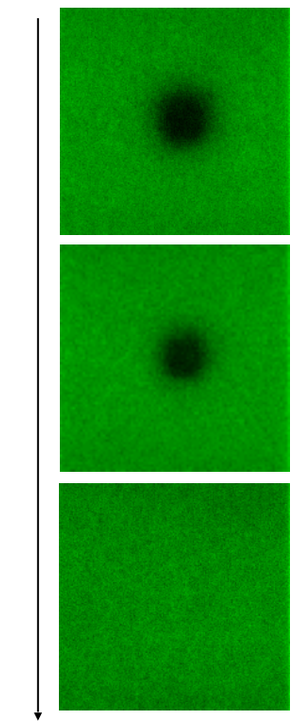


Recovery

$$\frac{\partial C(x,y,t)}{\partial t} = D\nabla^2 C(x,y,t)$$

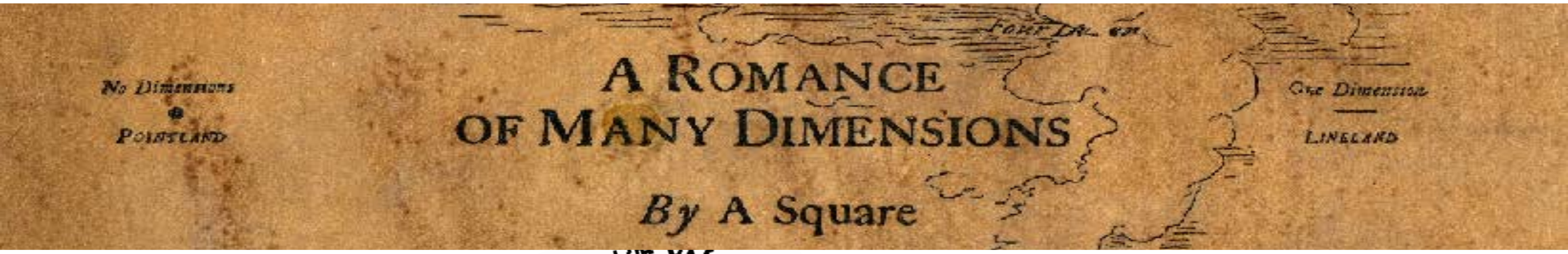
Niente diffusione durante
la fase di bleaching

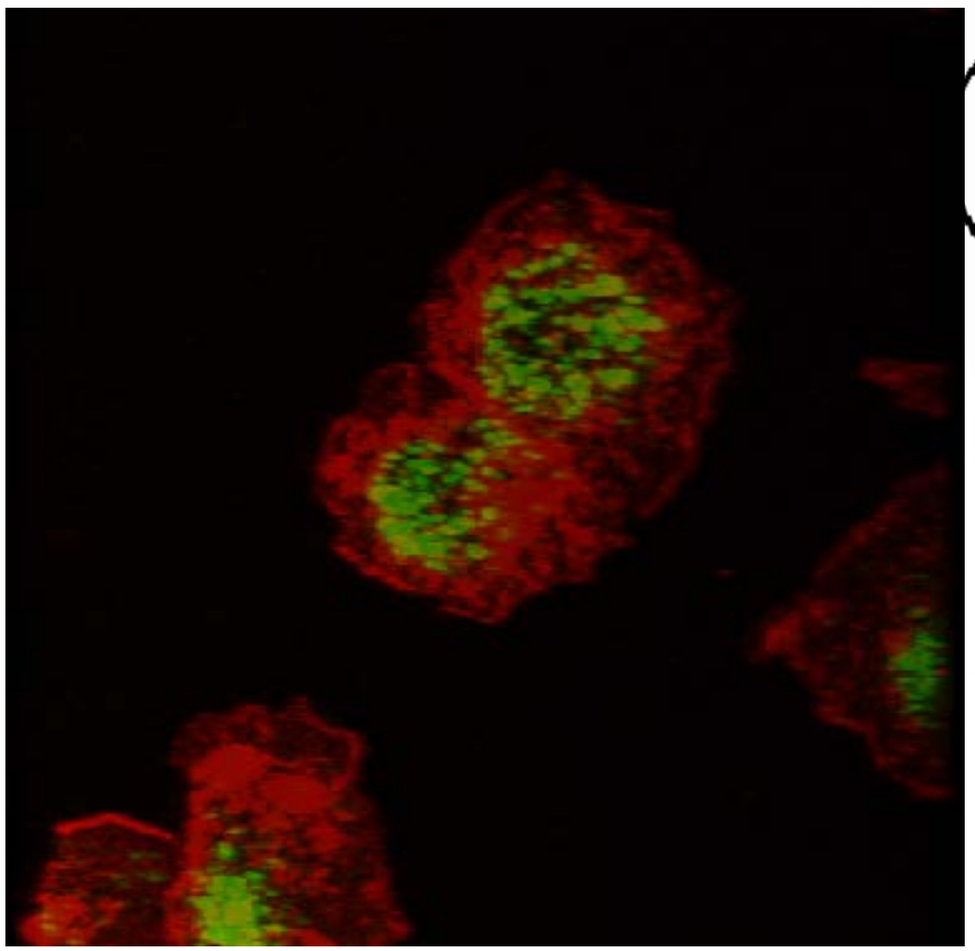
(massimo D misurabile)

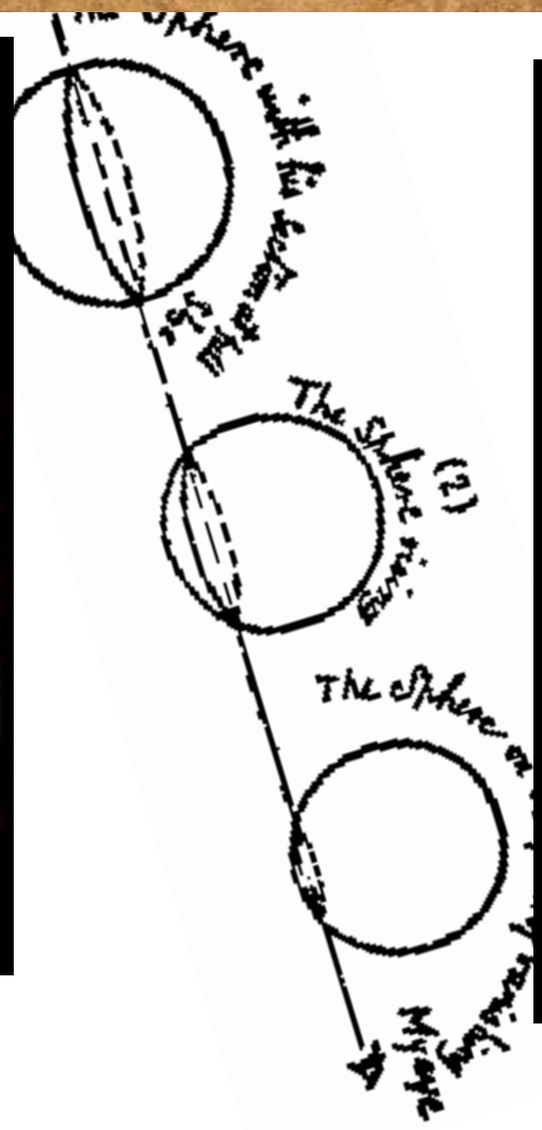


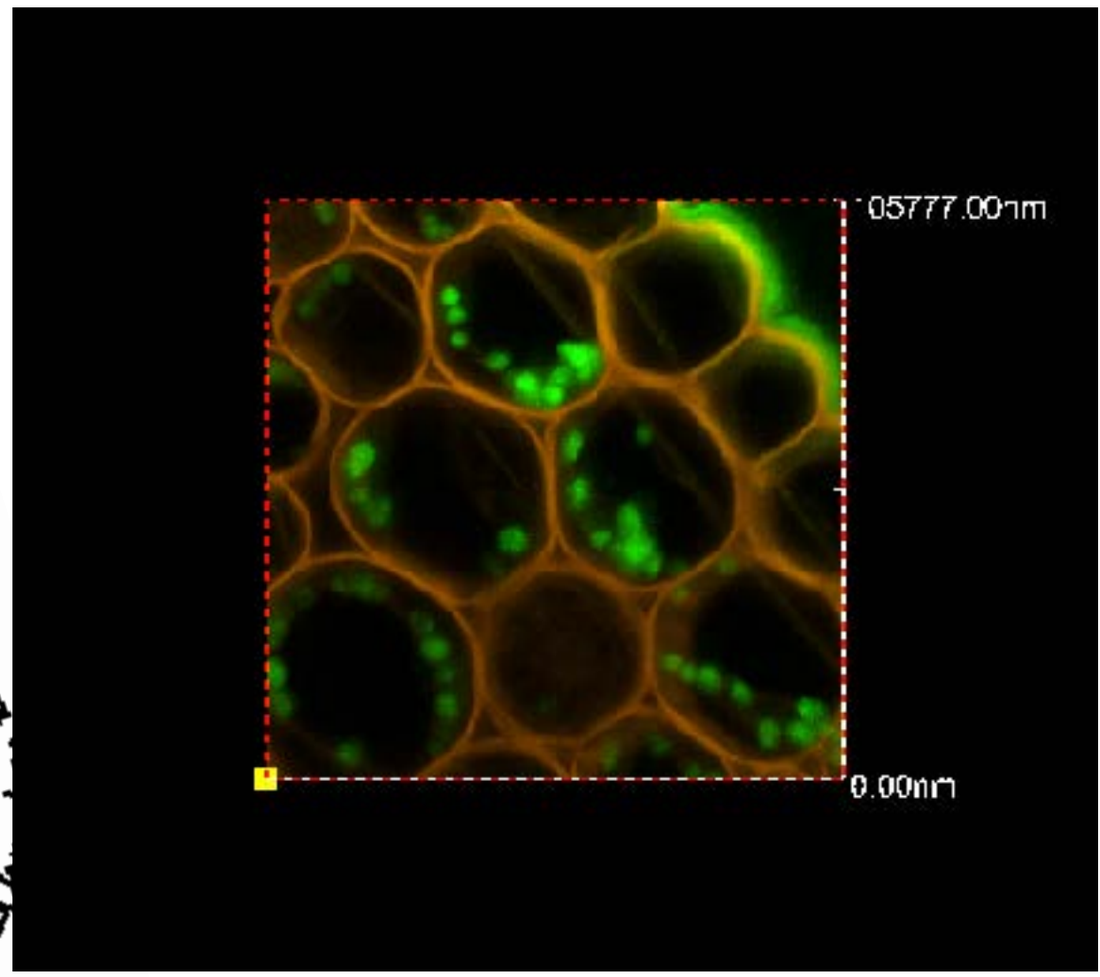
slide credit: Davide Mazza, LAMB

FRAP


No Dimensions
Point-land
A Romance
of Many Dimensions
By A Square
One Dimension
Line-land

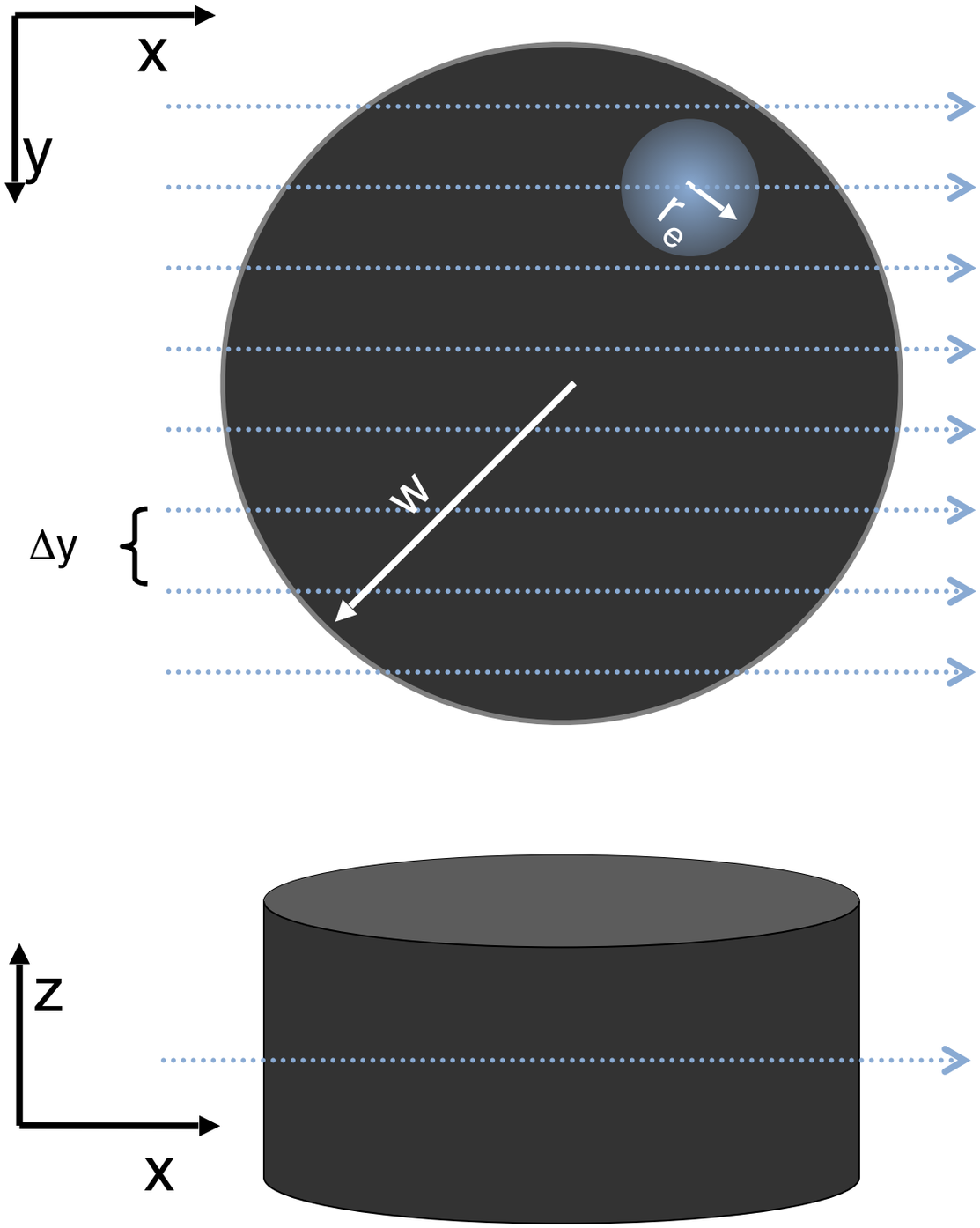


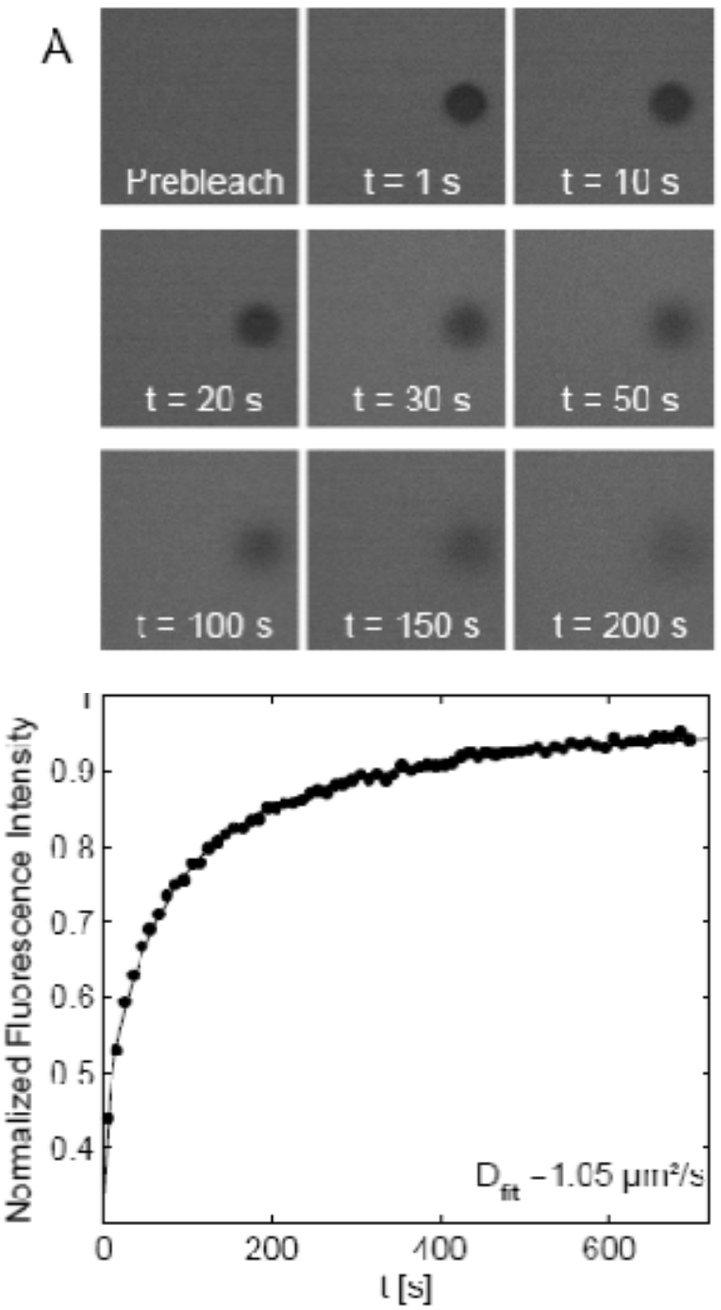



05777.00nm
0.00nm

FRAP

Obiettivo a bassa apertura numerica (Profilo cilindrico)

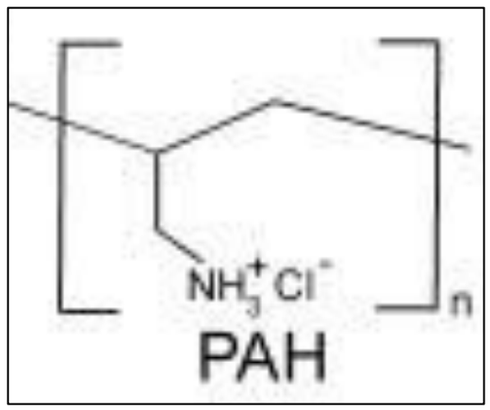




Applicabile per regioni di bleach con w >10 μm

slide credit: Davide Mazza, LAMBS

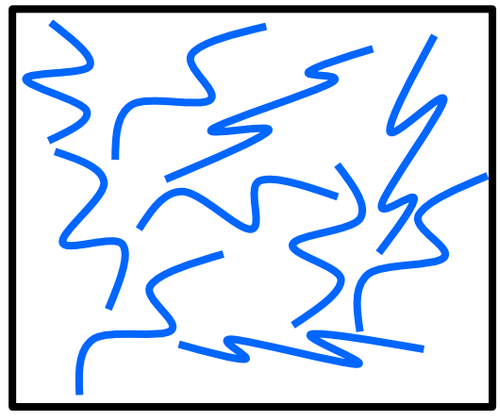
FRAP



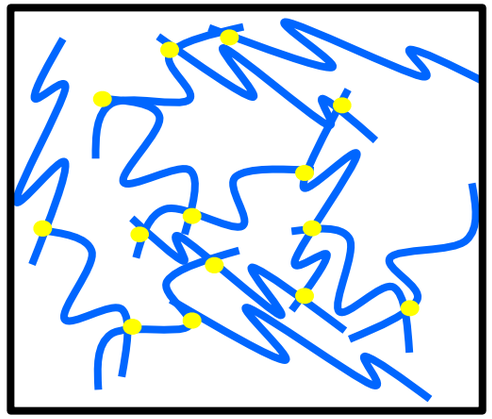
PAH (15KDa, 170mg/ml)

Crosslinker:

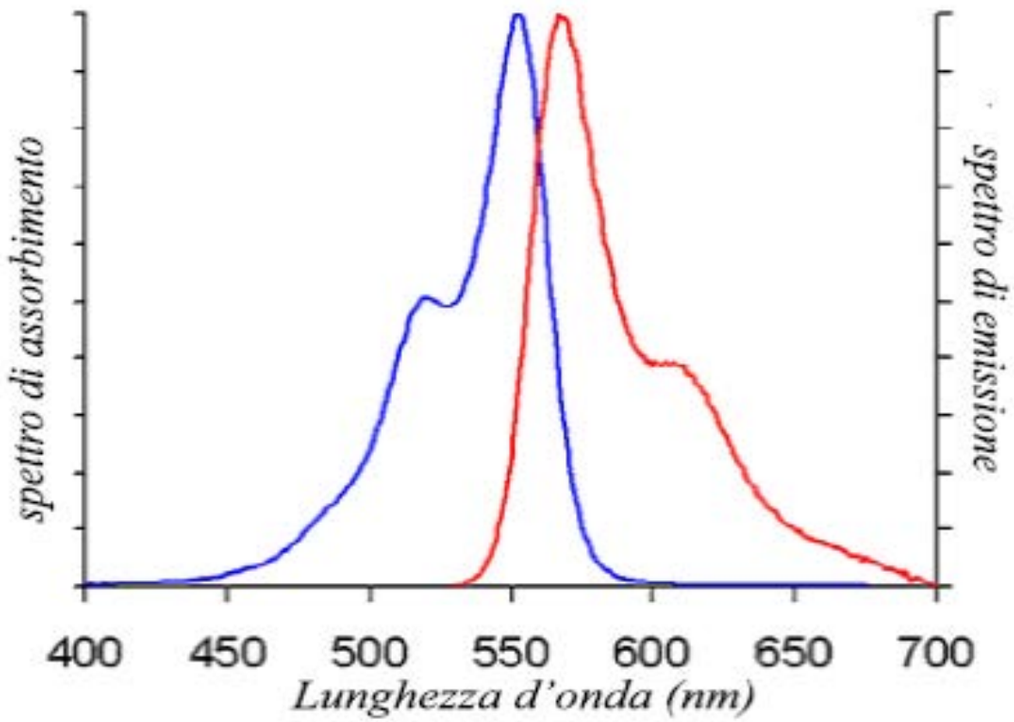
NaH_2PO_4

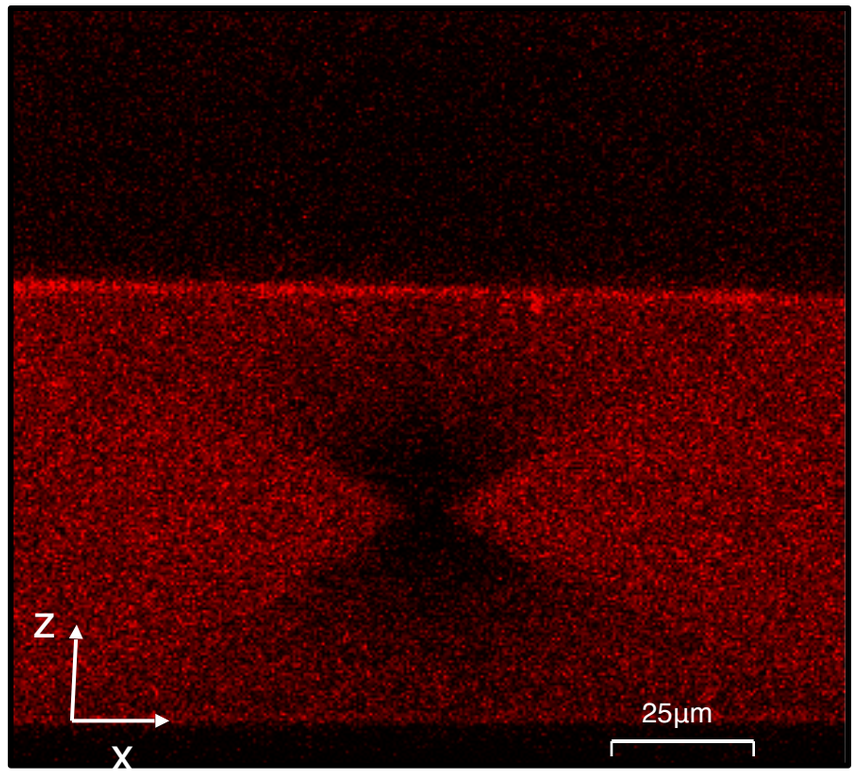


Crosslinking ionico



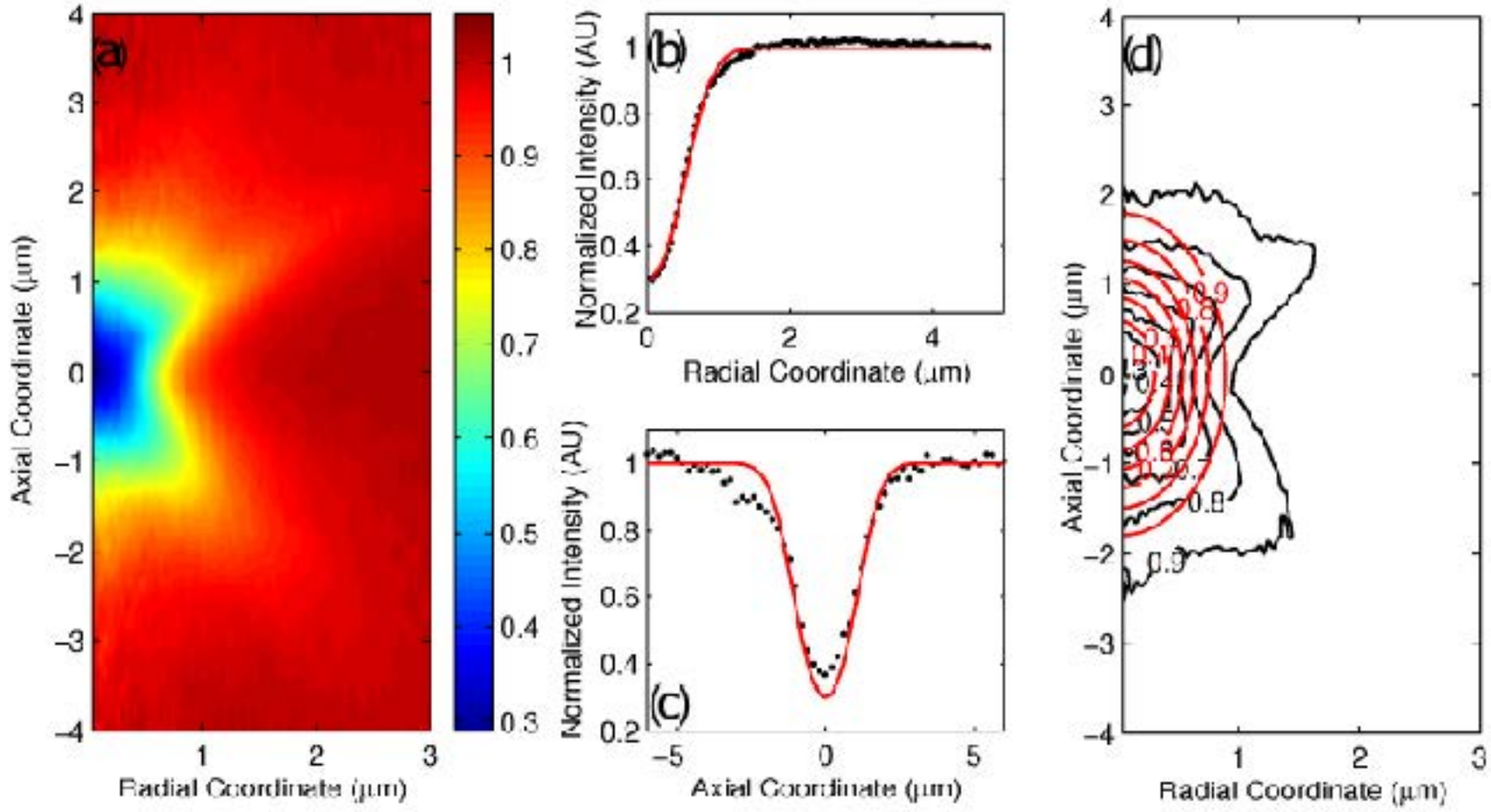
Molecola fluorescente: ALEXA 555





slide credit: Davide Mazza, LAMBS

FRAP



Approssimazione Gaussiana del profilo di Illuminazione:

$$\langle I_b(r,z)\rangle = \langle I_b(0,0)\rangle e^{-\left(\frac{2r^2}{r_e^2}+\frac{2z^2}{z_e^2}\right)}$$

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$

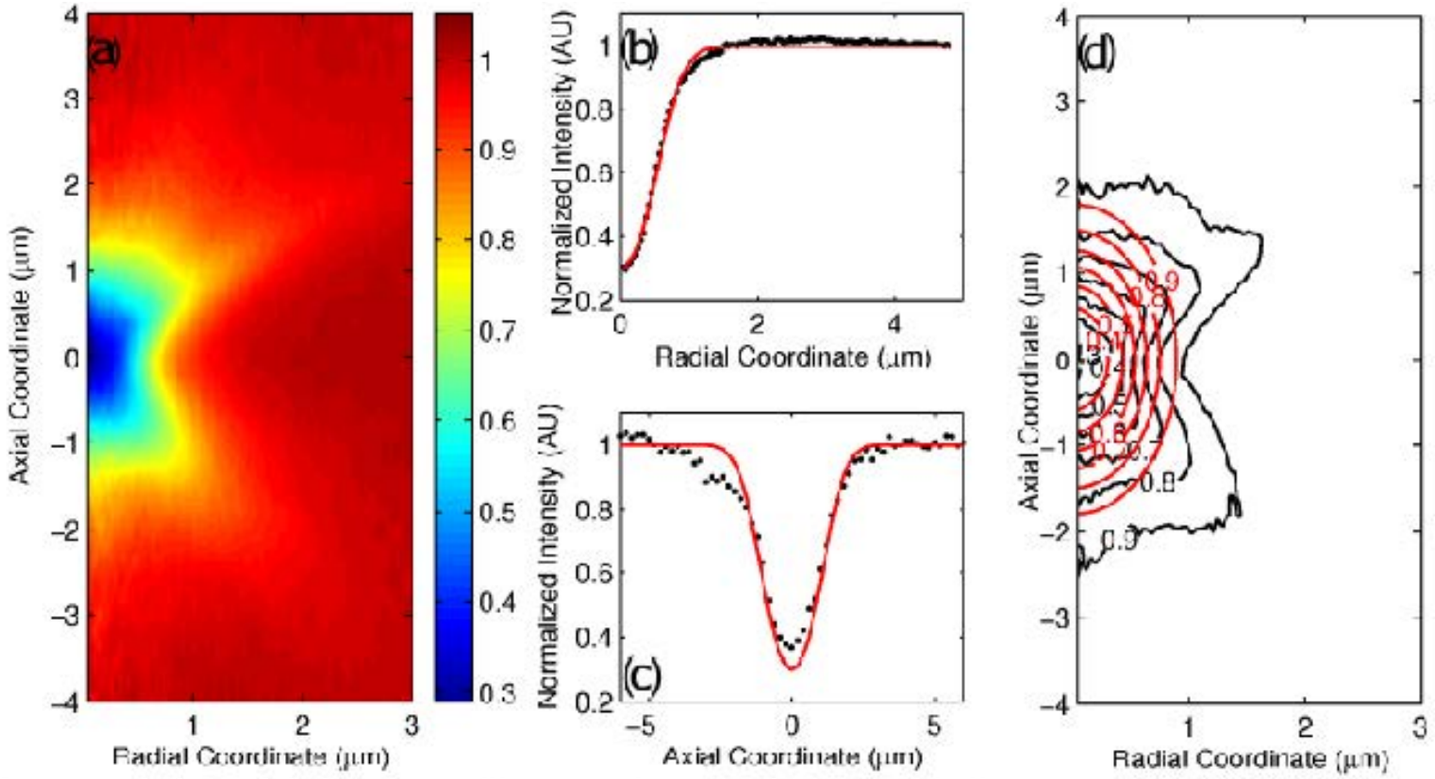
Profilo di Photobleaching

$$C_B(r,z) = C_0 e^{-K_{1ph} e^{-\left(\frac{2r^2}{r_e^2}+\frac{2z^2}{z_e^2}\right)}}$$

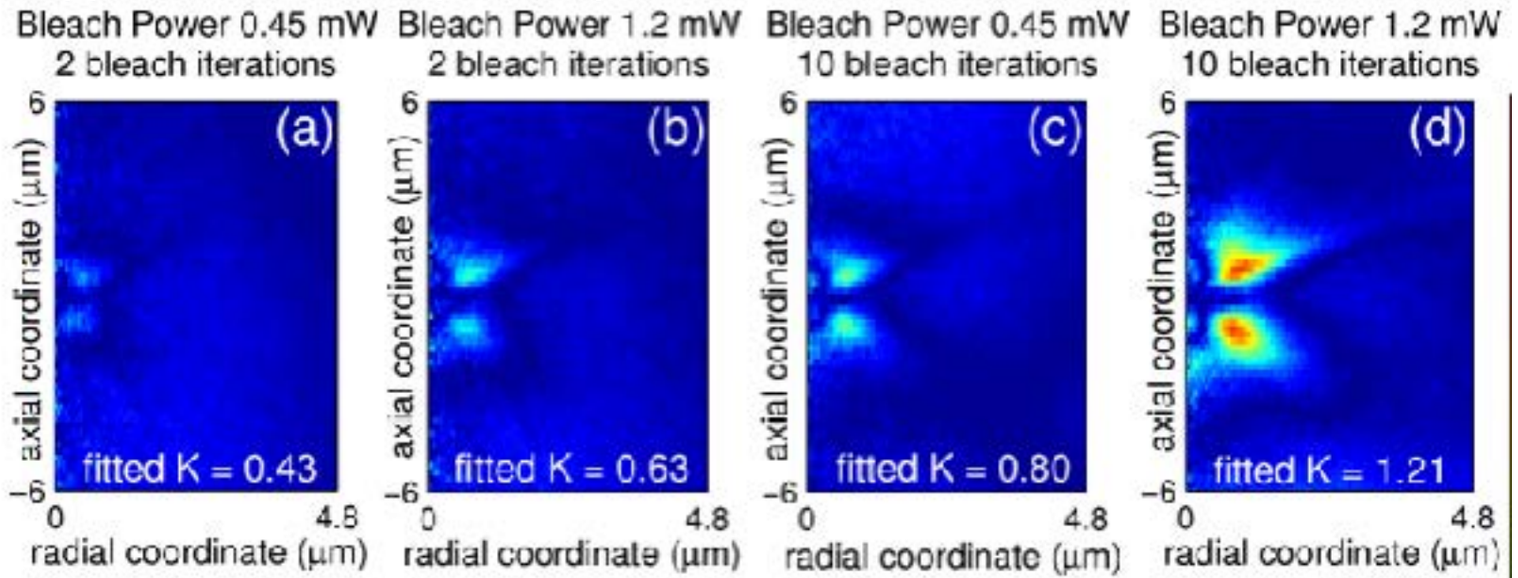
slide credit: Davide Mazza, LAMBS

Mazza D. et al, Applied Optics, 2007

FRAP



Mappa dei residui tra profilo sperimentale e modello

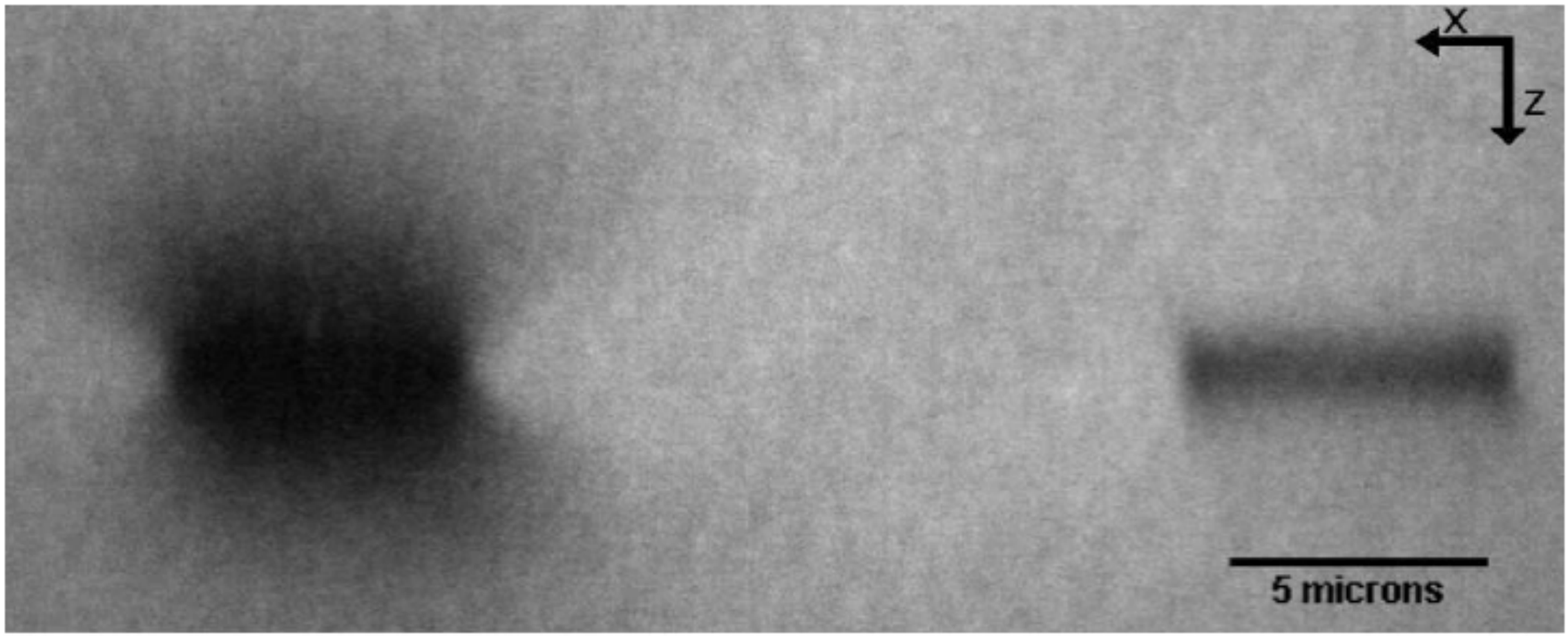


Energy delivered on the sample

slide credit: Davide Mazza, LAMBS

FRAP

Loren…Diaspro et al., **Q Rev Biophys. 2015** Aug;48(3):323-87.



1PE

$$\frac{dC(x,y,t)}{dt} = -\alpha I_b C(x,y,t)$$

2PE

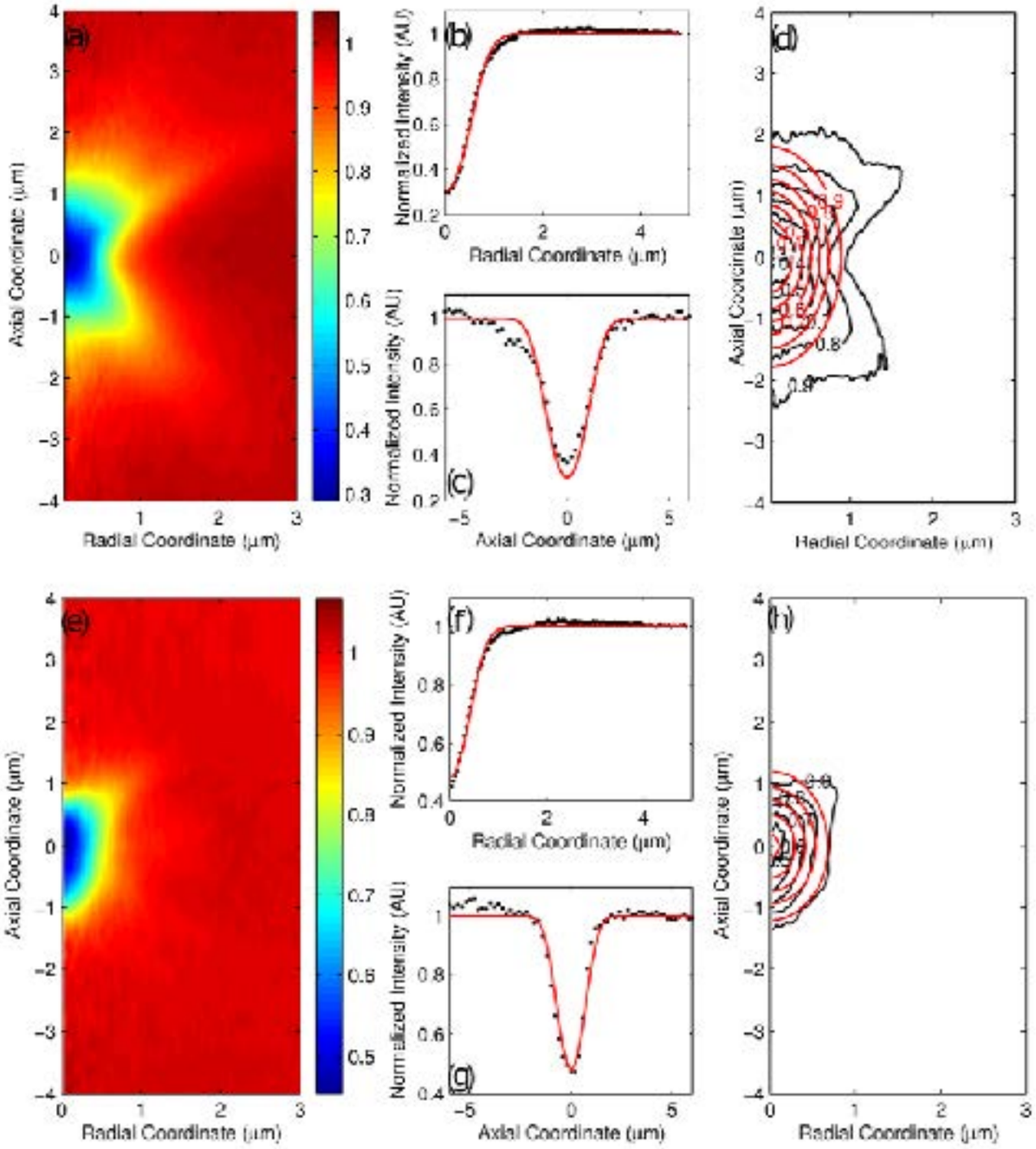
$$\frac{dC(x,y,t)}{dt} = -\alpha < I_b^2 > C(x,y,t)$$

Mazza D, Braeckmans K, Cella F, Testa I, Vercauteren D, Demeester J, De Smedt SS, **Diaspro A**.
Biophys J. 2008 Oct;95(7):3457-69.

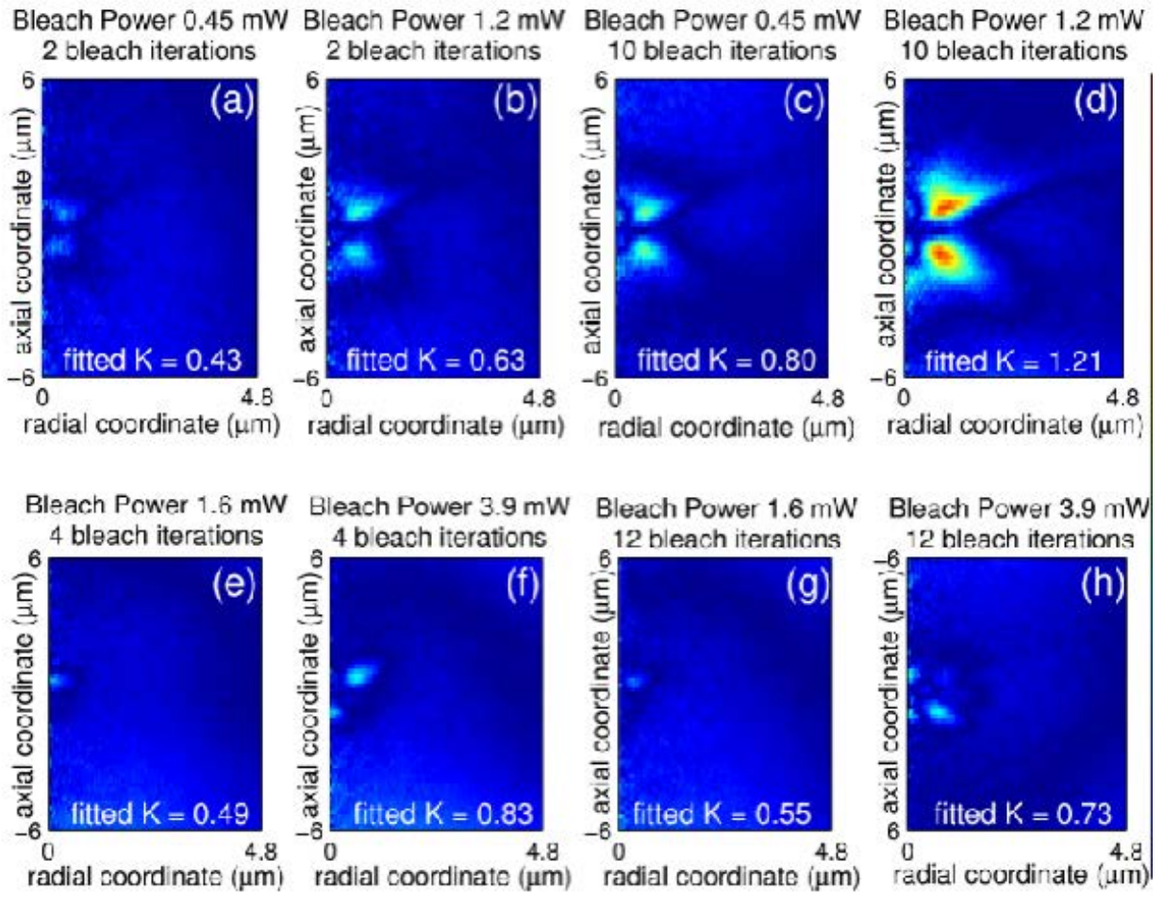
slide credit: Davide Mazza, LAMBS

FRAP

Eccitazione convenzionale



Eccitazione a due fotoni



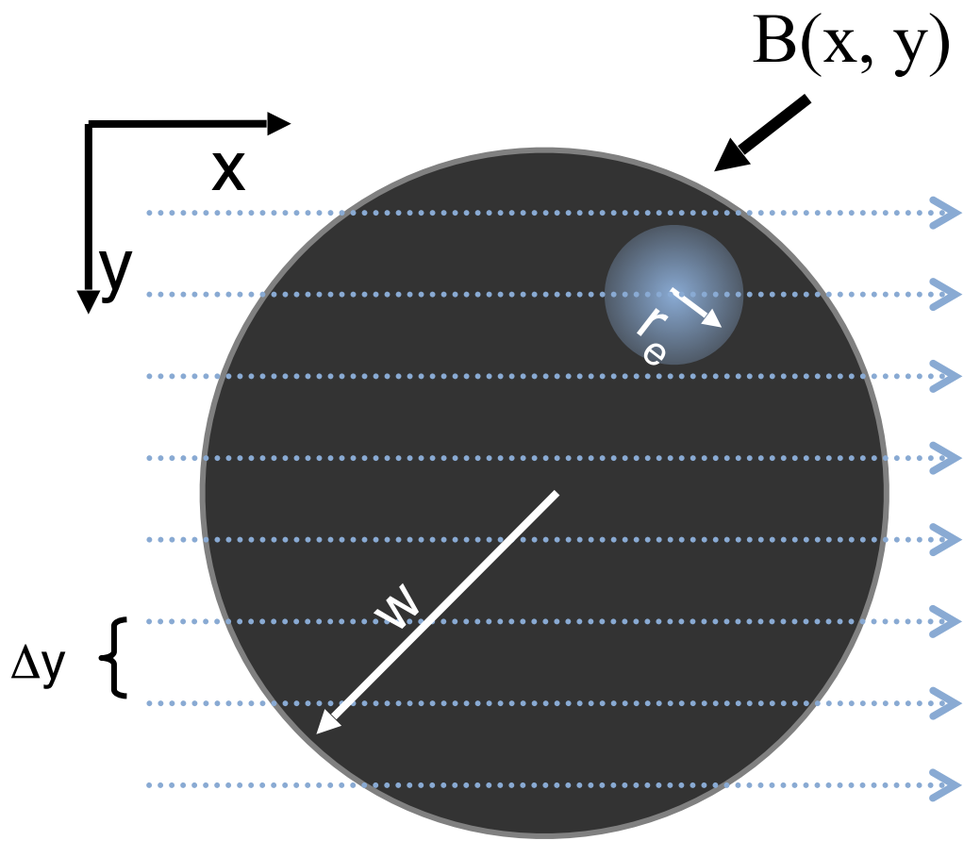
Profilo di Photobleaching

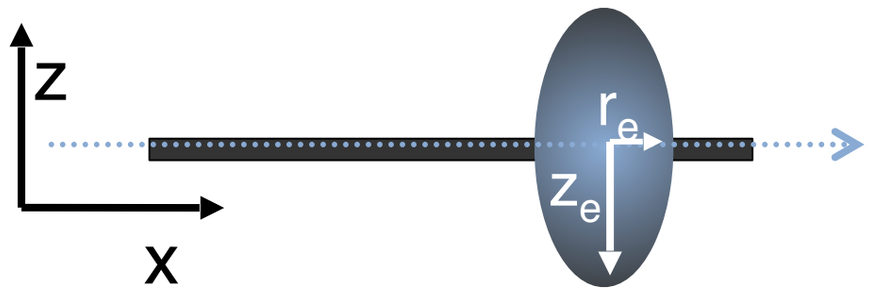
$$C_B(r,z) = C_0 e^{-K_{2ph} e^{-\left(\frac{4r^2}{r_e^2}+\frac{4z^2}{z_e^2}\right)}}$$

slide credit: Davide Mazza, LAMBS

Mazza D. et al, Applied Optics, 2007

FRAP

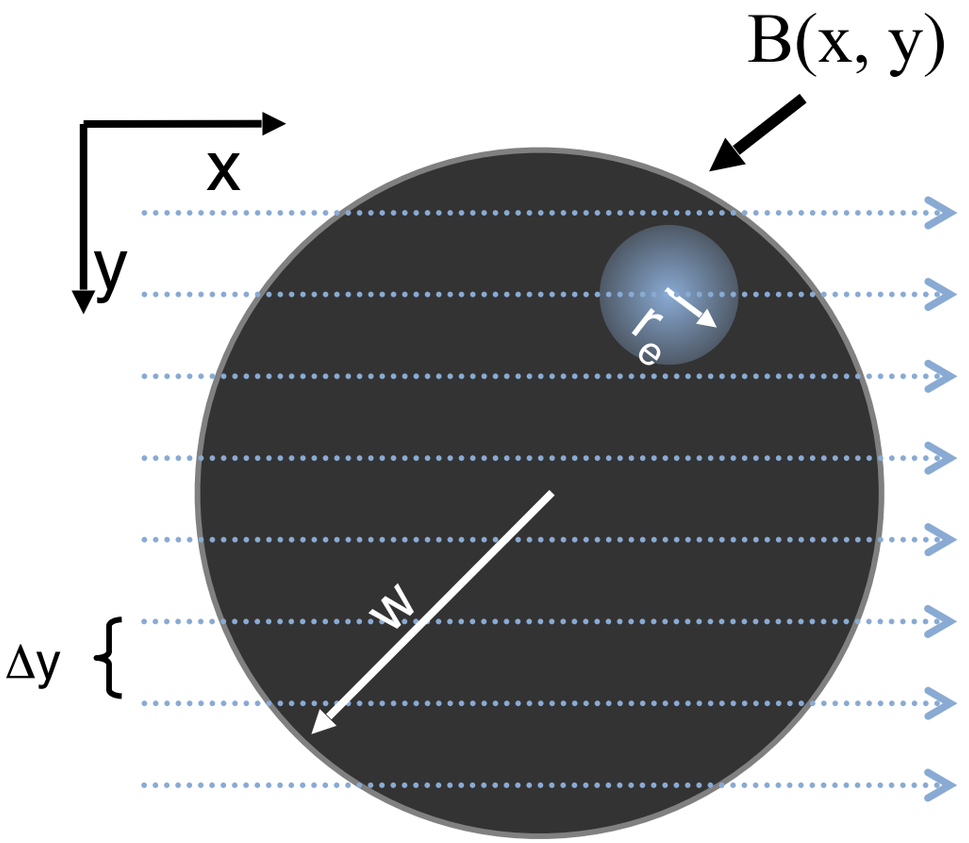




slide credit: Davide Mazza, LAMBS

Mazza D. et al., BJ 2008

FRAP



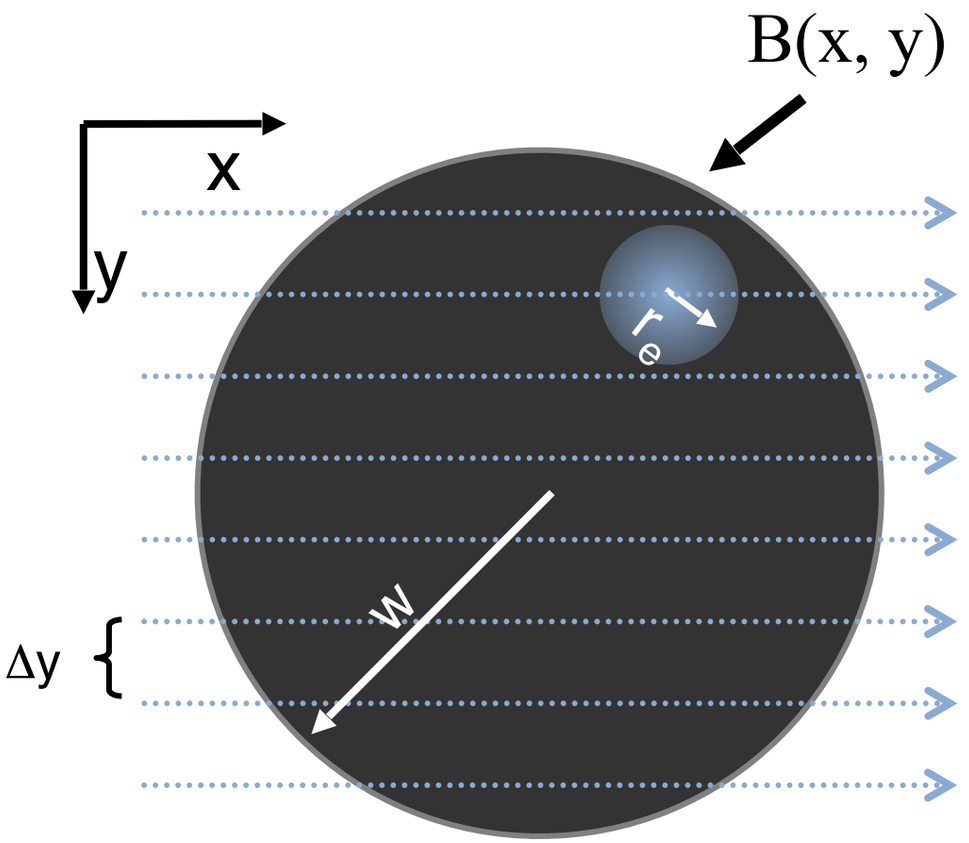
Condizione Iniziale

$$C(r, z) = C_0$$

slide credit: Davide Mazza, LAMBS

Mazza D. et al., BJ 2008

FRAP



Condizione Iniziale

$$C(r, z) = C_0$$

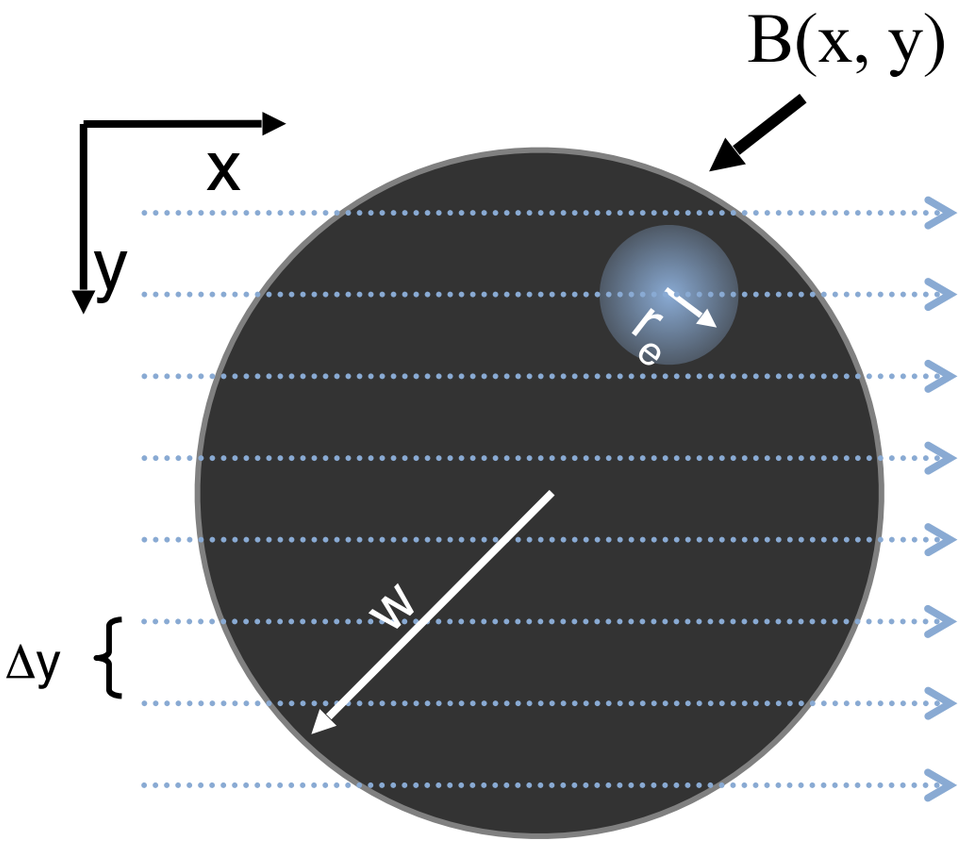
Photobleaching

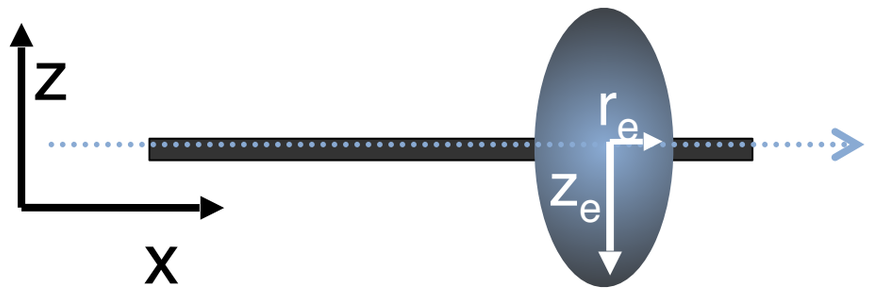
$$\frac{dC(r,z,t)}{dt} = -\alpha < I_b^2(r,z,t) > C(r,z,t)$$

Mazza D. et al., BJ

slide credit: Davide Mazza, LAMBS

FRAP





Condizione Iniziale

$$C(r, z) = C_0$$

Photobleaching

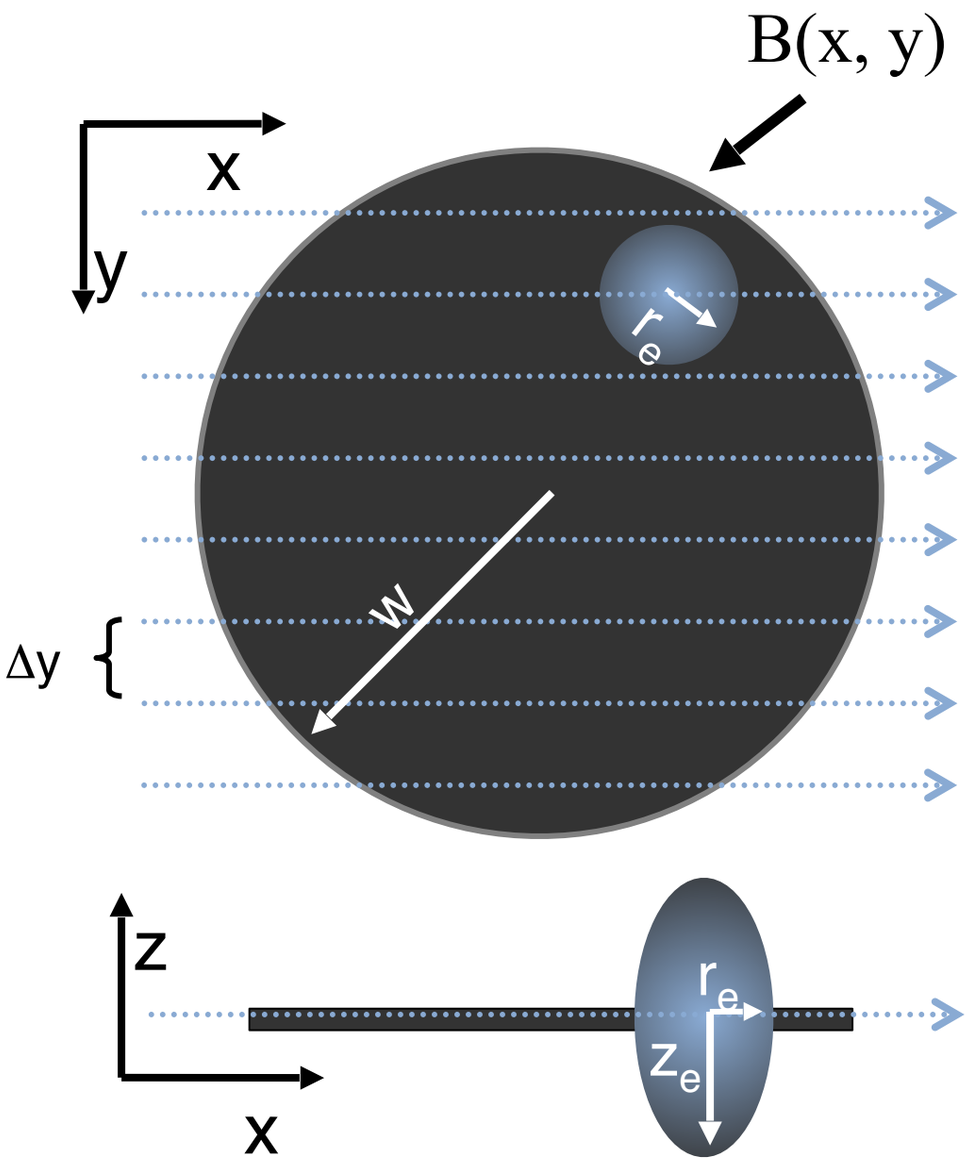
$$\frac{dC(r,z,t)}{dt} = -\alpha < I_b^2(r,z,t) > C(r,z,t)$$

$$C_b(r,z) = C_0 e^{-\frac{\alpha}{v\Delta y}K(r,z)}$$

$$K(x,y,z) = B(x,y) \otimes_{x,y} I_b^2(x,y,z)$$

Mazza D. et al., BJ 2008

FRAP



Condizione Iniziale

$$C(r, z) = C_0$$

Photobleaching

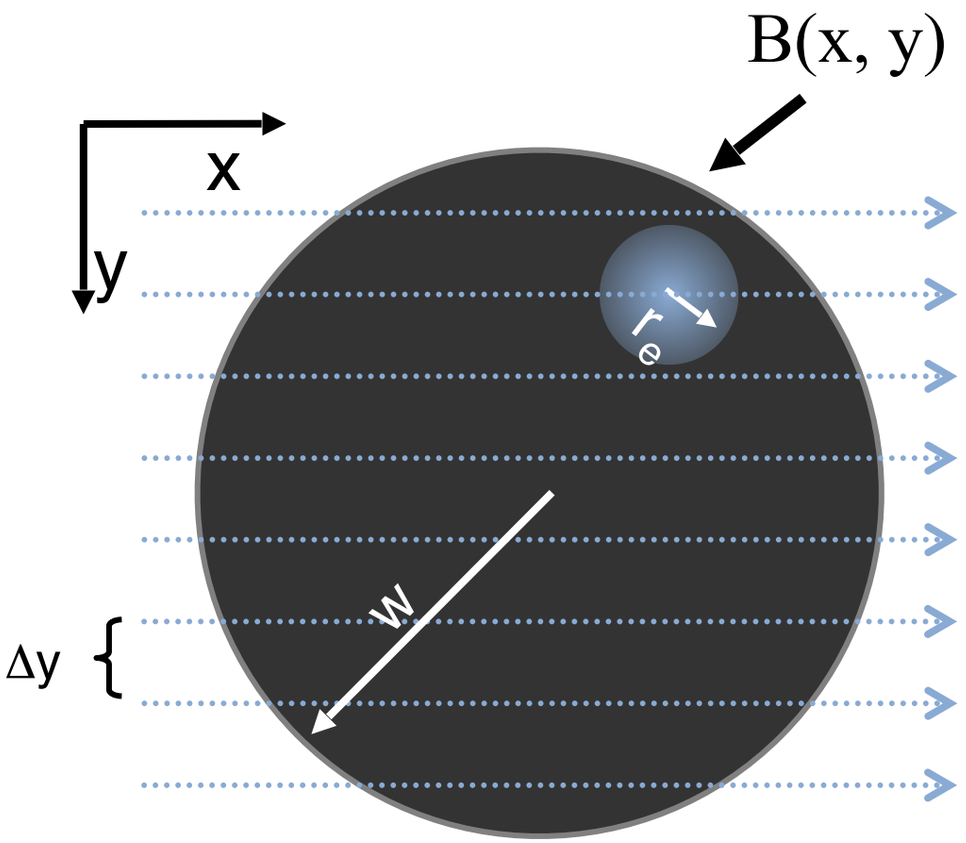
$$C_b(r,z) = C_0 e^{-\frac{\alpha}{v\Delta y}K(r,z)}$$

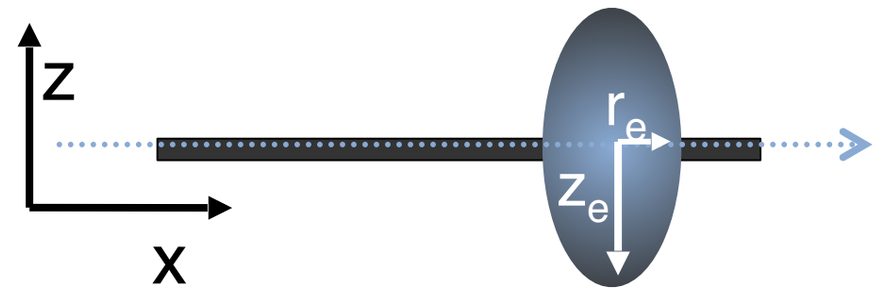
Diffusione

$$\frac{\partial C(r,z,t)}{\partial t} = D\nabla^2 C(r,z,t)$$

Mazza D. et al., BJ 2008

FRAP





Condizione Iniziale

$$C(r, z) = C_0$$

Photobleaching

$$C_b(r,z) = C_0 e^{-\frac{\alpha}{v\Delta y}K(r,z)}$$

Diffusione

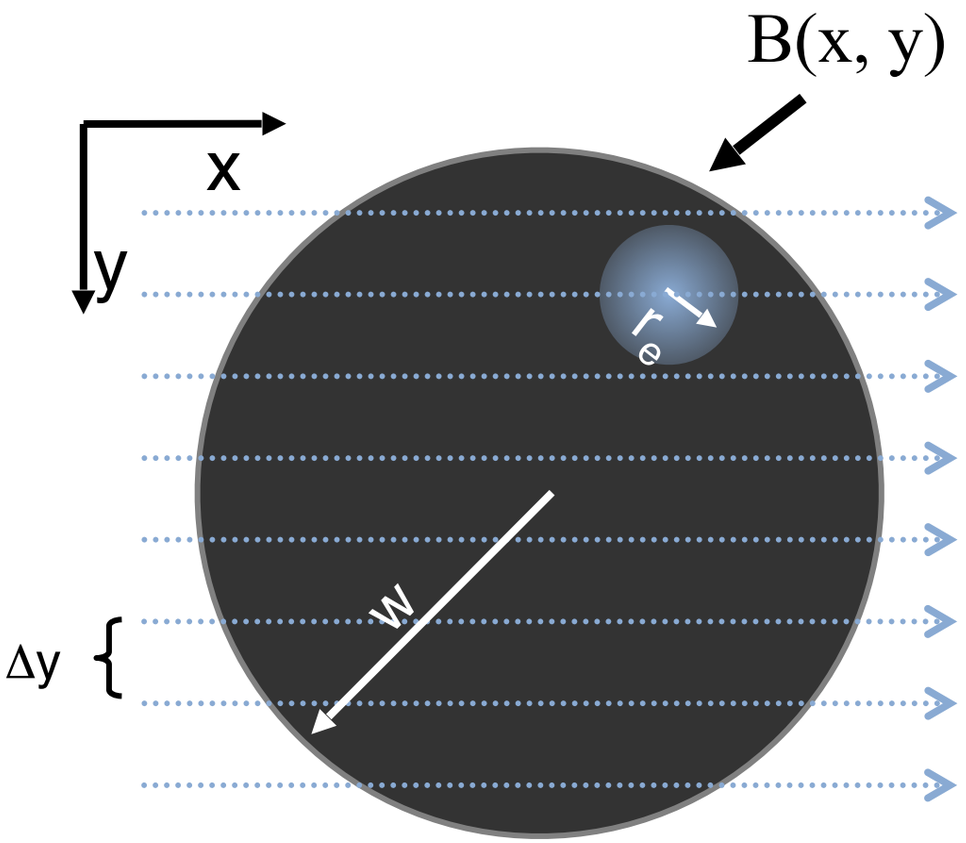
$$\frac{\partial C(r,z,t)}{\partial t} = D\nabla^2 C(r,z,t)$$

$$C(r,z,t) = C_0 - C_0\left[1 - \sum_{i=0}^{+\infty}\frac{(-K_{0n})^i}{i!}\frac{z_e}{\sqrt{z_e^2 + 8iDt}}e^{-\frac{2iz^2}{z_e^2+8iDt}}\right]$$

$$\times\frac{1}{2Dt}e^{-\frac{r^2}{4Dt}}\int_{r'=0}^{w} e^{-\frac{r'^2}{4Dt}}\,\mathrm{I}_0\left(\frac{rr'}{2Dt}\right)r'dr'$$

Mazza D. et al., BJ 2008

FRAP



Condizione Iniziale

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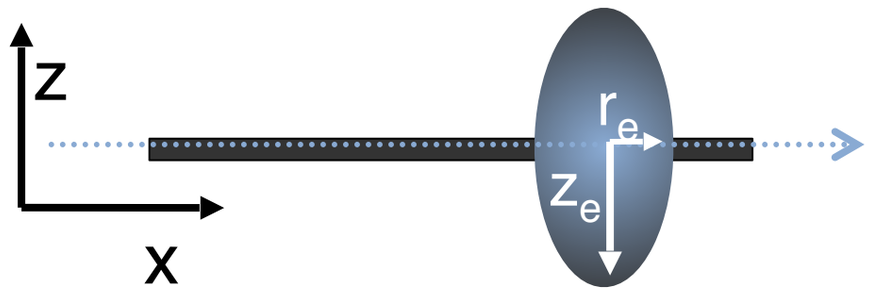
Photobleaching

$$C_b(r,z) = C_0 e^{-\frac{\alpha}{v\Delta y}K(r,z)}$$

Diffusione

$$\frac{\partial C(r,z,t)}{\partial t} = D\nabla^2 C(r,z,t)$$



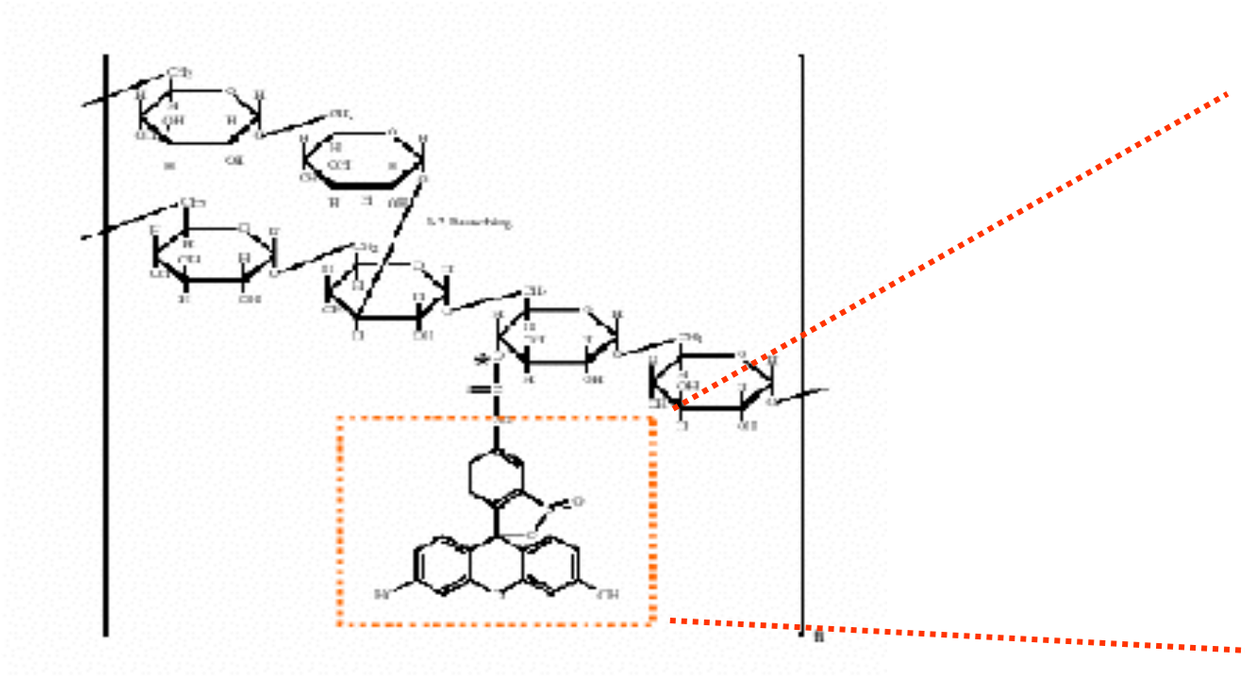
$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}}\left(\mathrm{I}_0\left(\frac{w^2}{2Dt}\right) + \mathrm{I}_1\left(\frac{w^2}{2Dt}\right)\right)\right]$$

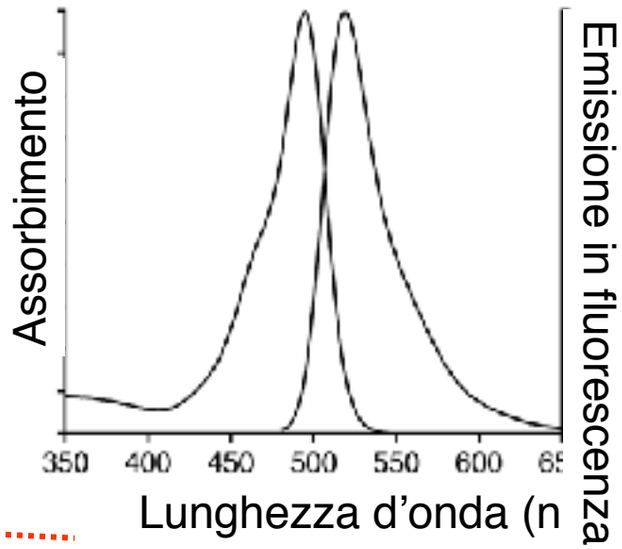
Mazza D. et al., BJ 2008

FRAP

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(\mathrm{I}_0\left(\frac{w^2}{2Dt}\right) + \mathrm{I}_1\left(\frac{w^2}{2Dt}\right) \right) \right]$$

Validazione sperimentale su soluzioni a viscosità controllata di Fluoresceina-Destrani



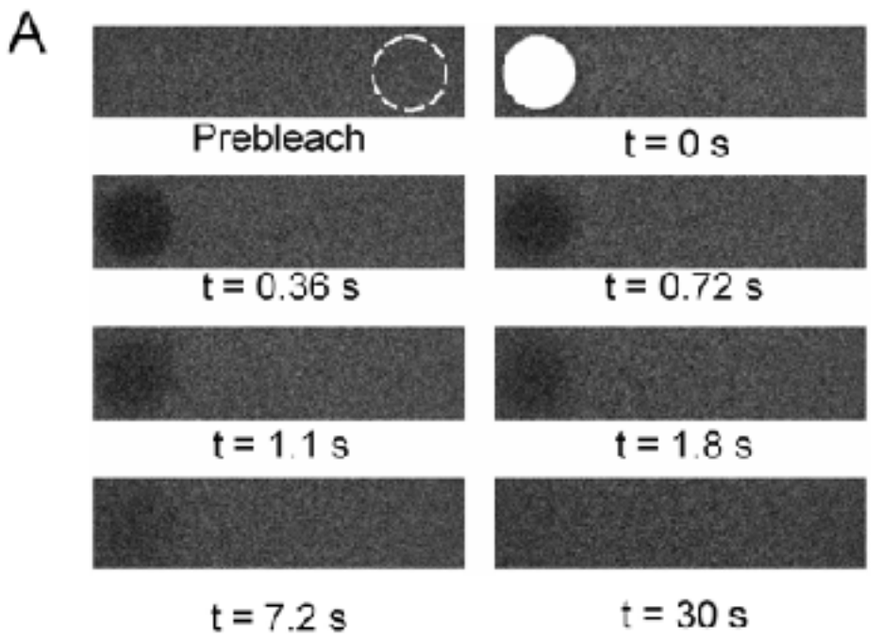


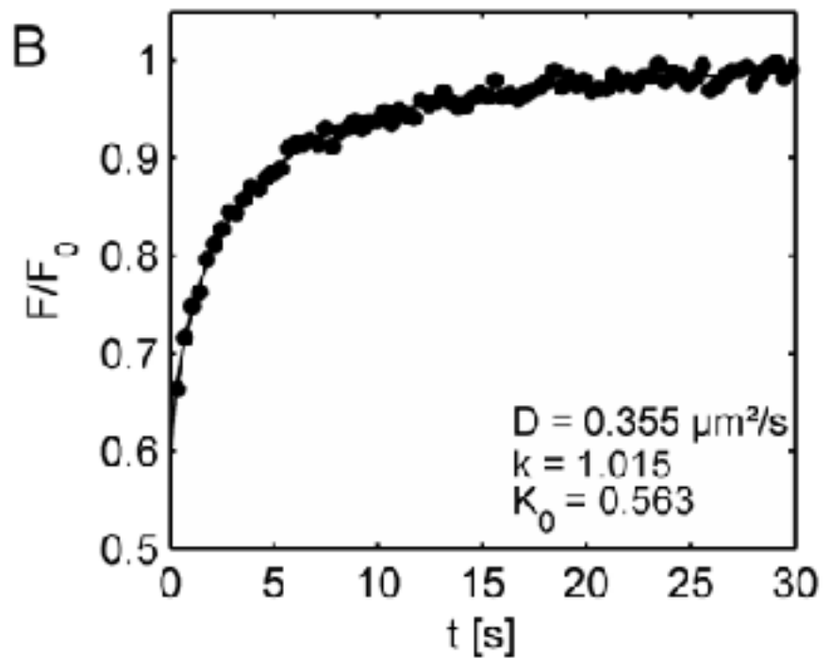
Mazza D. et al., BJ 2008

FRAP

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(\mathrm{I}_0\left(\frac{w^2}{2Dt}\right) + \mathrm{I}_1\left(\frac{w^2}{2Dt}\right) \right) \right]$$

Validazione sperimentale su soluzioni a viscosità controllata di Fluoresceina-Destrani



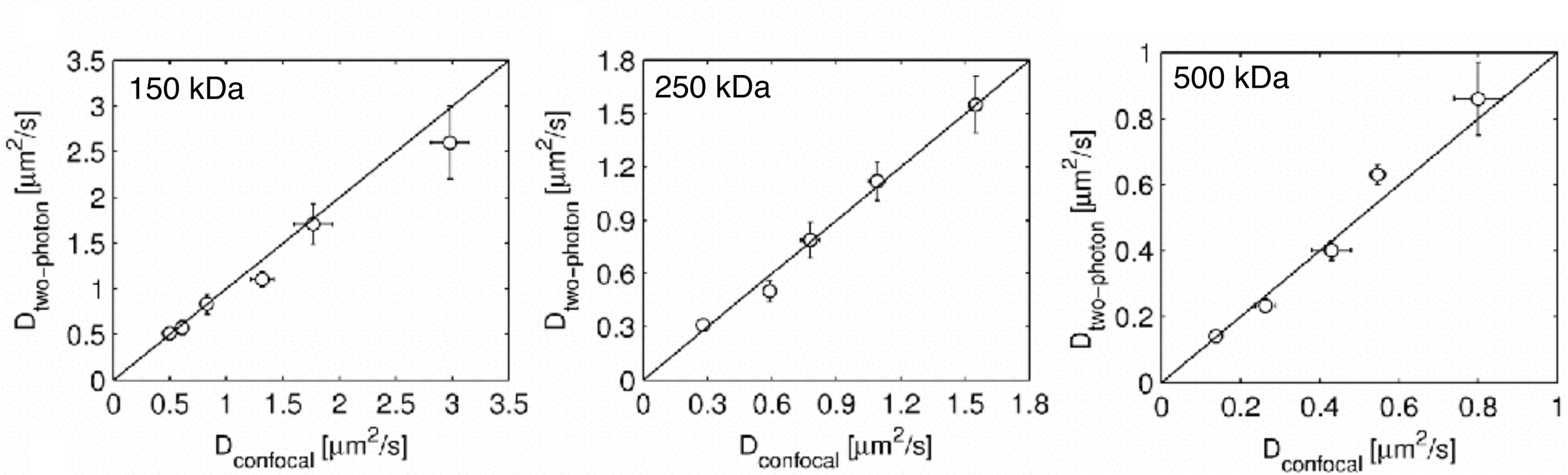


Mazza D. et al., BJ 2008

FRAP

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(I_0\left(\frac{w^2}{2Dt}\right) + I_1\left(\frac{w^2}{2Dt}\right) \right) \right]$$

Confronto tra diffusione 2D (FRAP confocale – bassa apertura numerica)
e diffusione 3D (FRAP a 2 fotoni – alta apertura numerica)



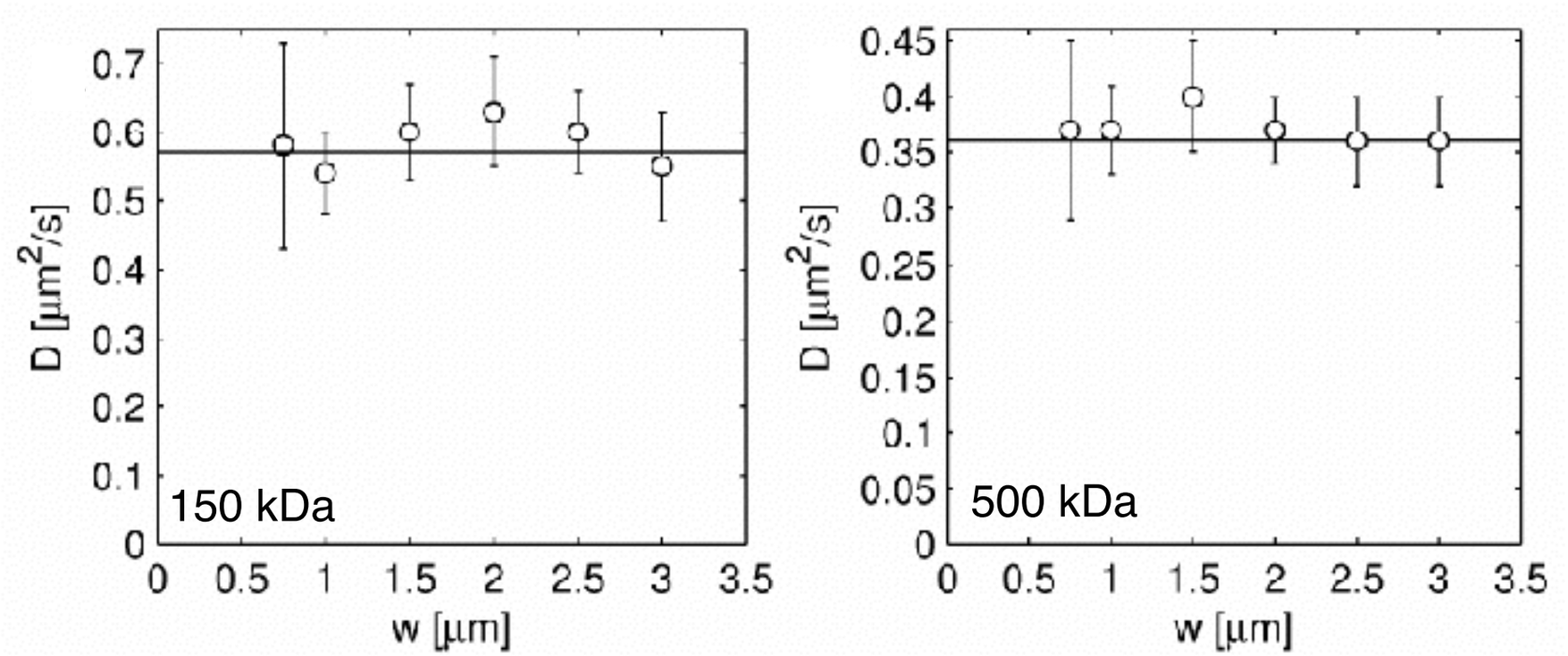
+ Obiettivo ad Alta apertura Numerica

Mazza D. et al., BJ 2008

FRAP

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(I_0\left(\frac{w^2}{2Dt}\right) + I_1\left(\frac{w^2}{2Dt}\right)\right)\right]$$

Qualità del modello in dipendenza della dimensione della regione di bleach



+ Obiettivo ad Alta apertura Numerica

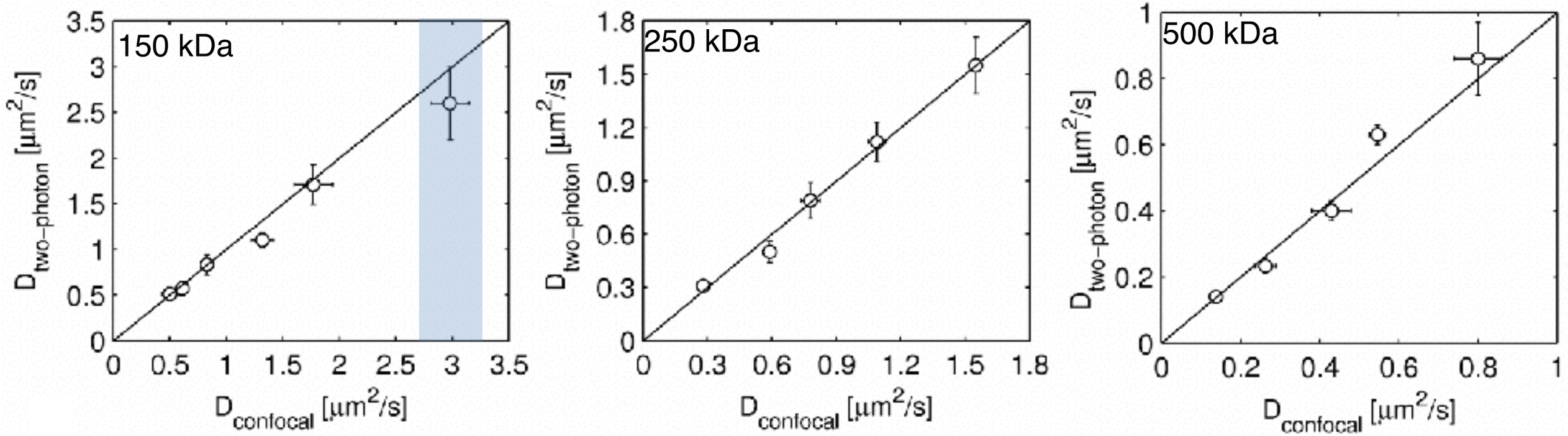
+ Controllo nelle dimensioni della regione di bleach

Mazza D. et al., BJ 2008

FRAP

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(I_0\left(\frac{w^2}{2Dt}\right) + I_1\left(\frac{w^2}{2Dt}\right) \right) \right]$$

Validazione sperimentale su soluzioni a viscosità controllata di Fluoresceina-Destrani



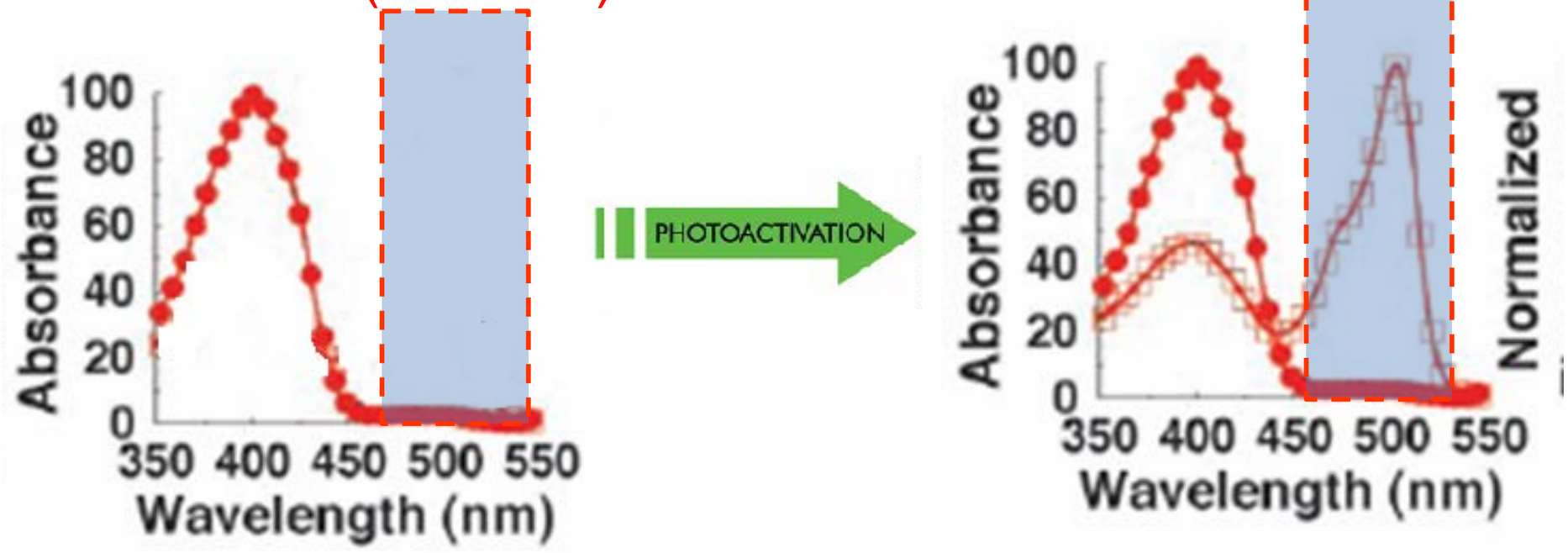
+ Obiettivo ad Alta apertura Numerica

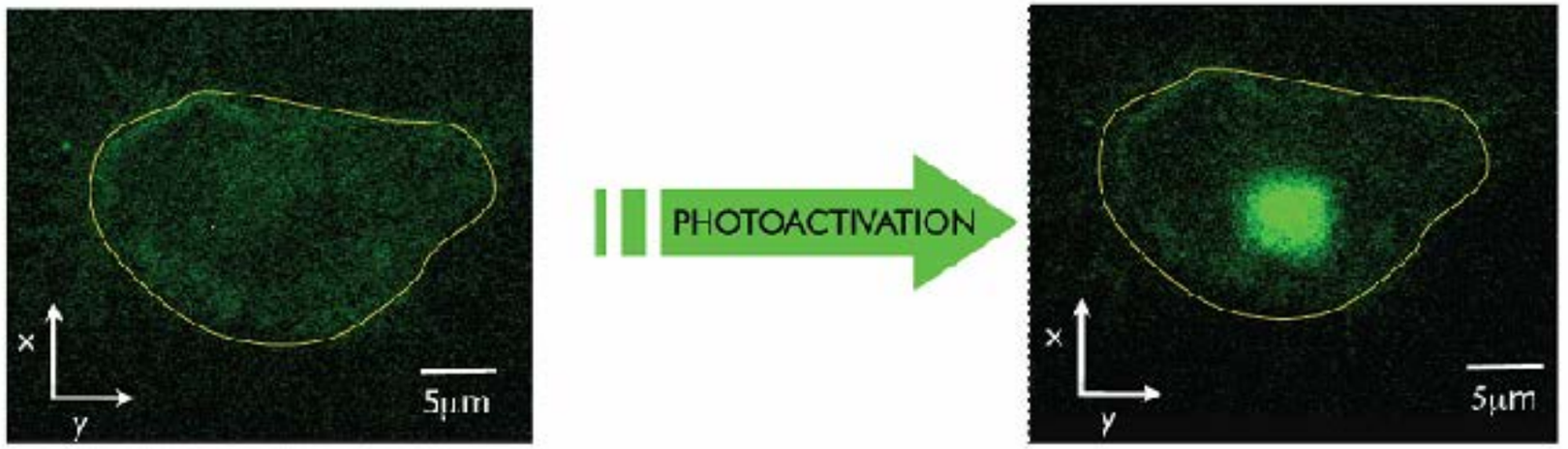
+ Controllo nelle dimensioni della regione di bleach

- Limiti nella misura dei coefficienti di diffusione misurabili

FRAP

Photoactivatable GFP (PA-GFP)





PRE ACTIVATION

⟨P EXCITATION⟩– 0,04 mW, λ EXCITATION– 488nm
Pixel time– 4,9 μs, 512*512 pixels

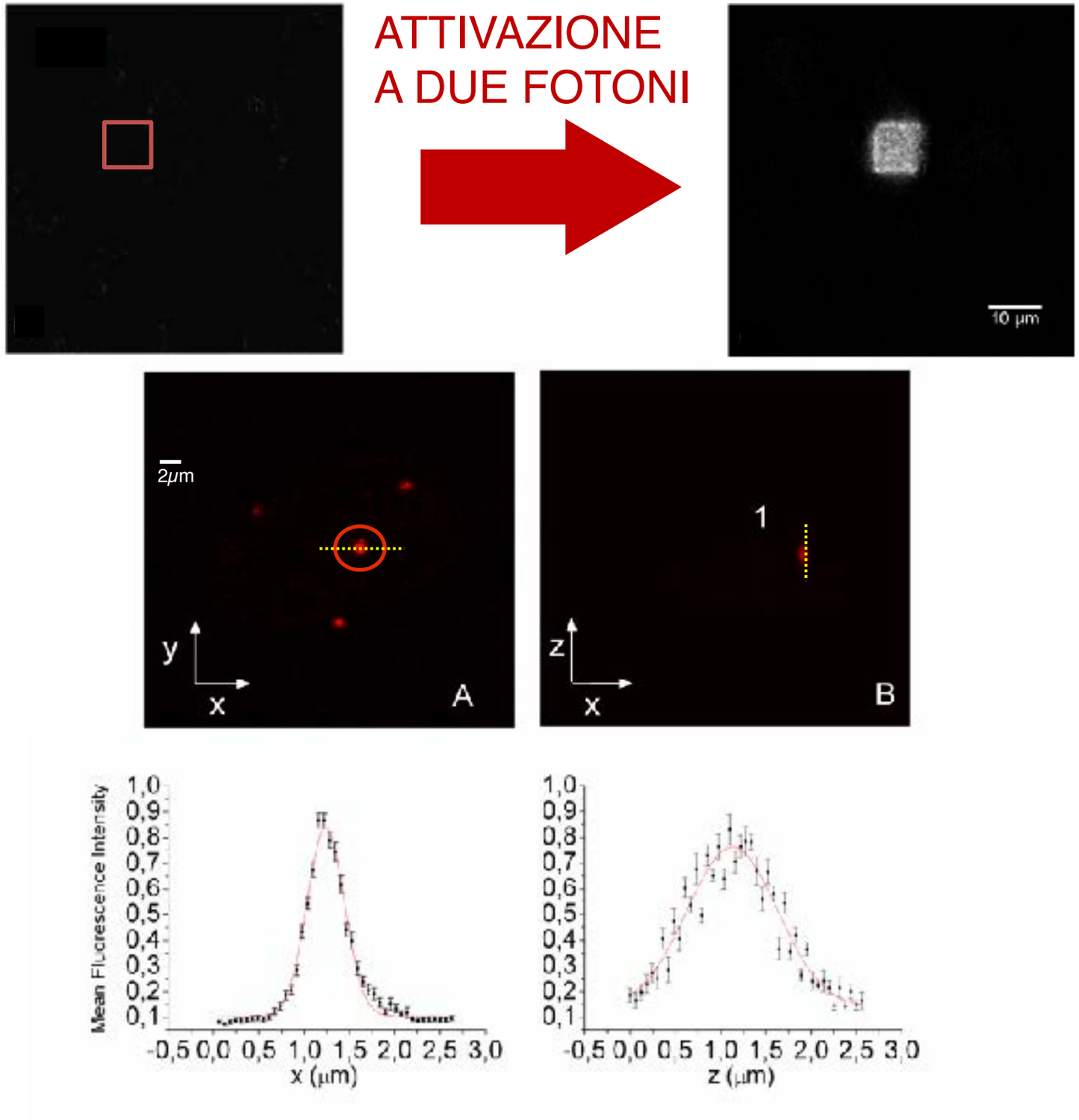
POST ACTIVATION

⟨P EXCITATION⟩= 0,04 mW, λ EXCITATION= 488nm
Pixel time= 4,9 μs, 512*512 pixels

Patterson and Lippincott-Schwartz, Methods, 2003

slide credit: Davide Mazza, LAMBS

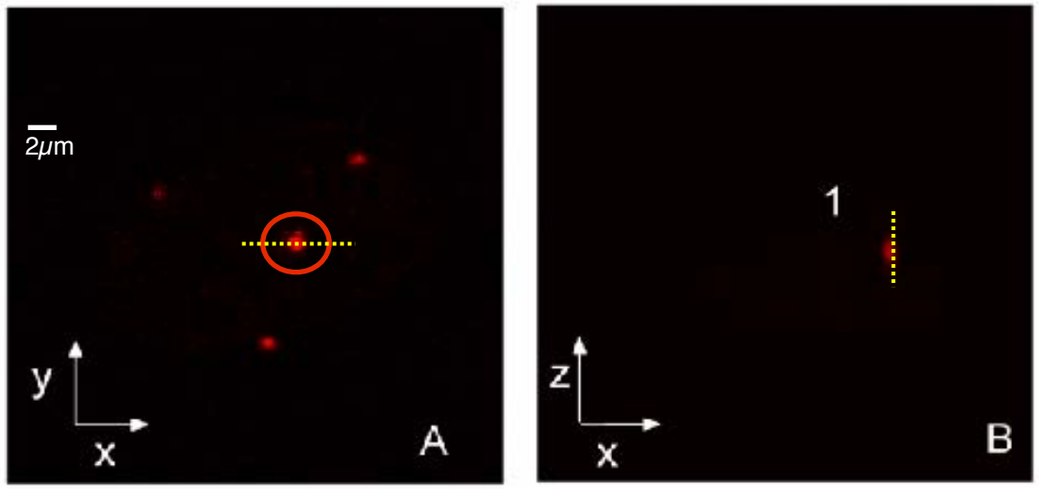
FRAP

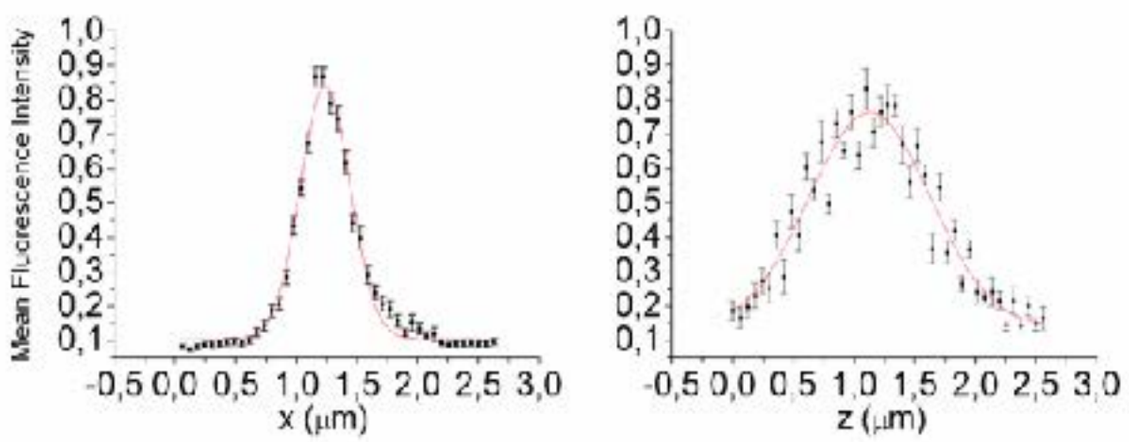


Schneider M, et al., Biophys J, 2004

Testa I, et al., J Microscopy, 2008

FRAP





Schneider M, et al., Biophys J, 2004

Estensione del modello alla fotoattivazione

Diffusione di proteine native
e proteine attivate

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \frac{(\sigma_m - \sigma_{A,m})C_0}{\sigma_m C_0 + \sigma_{A,m} C_{A,0}} \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(I_0\left(\frac{w^2}{2Dt}\right) + I_1\left(\frac{w^2}{2Dt}\right)\right)\right]$$

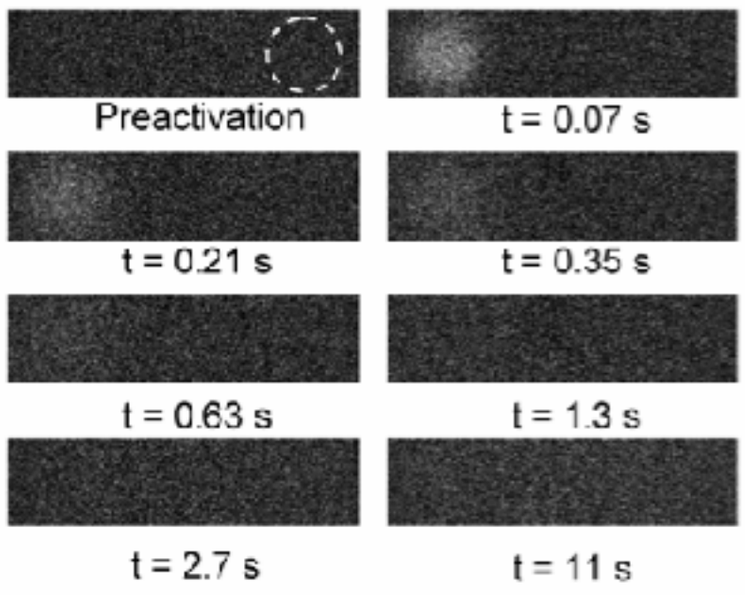
Testa I, et al., J Microscopy, 2008

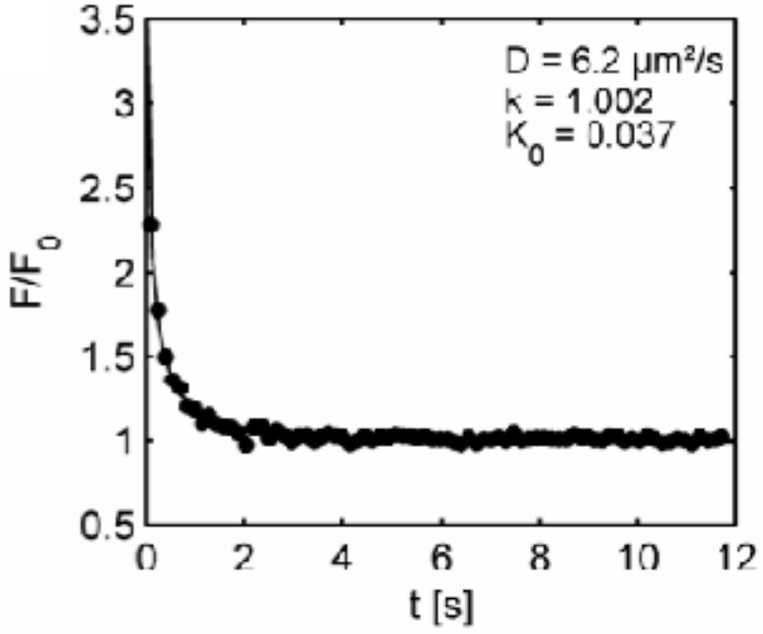
FRAP

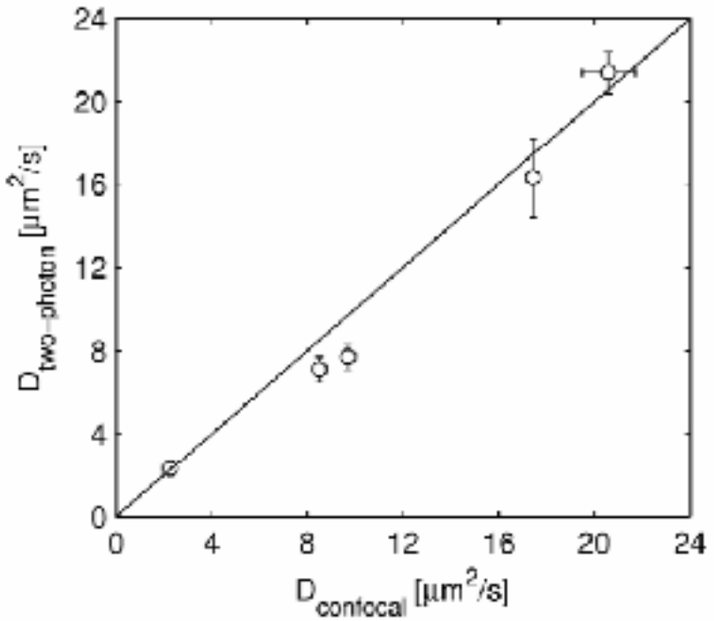
Photoactivatable GFP (PA-GFP)

$$\frac{F_{tot}(t)}{F_{tot,0}} = 1 + \frac{(\sigma_m - \sigma_{A,m})C_0}{\sigma_m C_0 + \sigma_{A,m} C_{A,0}} \sum_{i=1}^{+\infty} \frac{(-K_{0n})^i}{i!} \frac{z_e}{\sqrt{8iDt + z_e^2 + iz_d^2}} \times \left[1 - e^{-\frac{w^2}{2Dt}} \left(I_0\left(\frac{w^2}{2Dt}\right) + I_1\left(\frac{w^2}{2Dt}\right)\right)\right]$$

Validazione sperimentale su soluzioni a viscosità controllata di paGFP

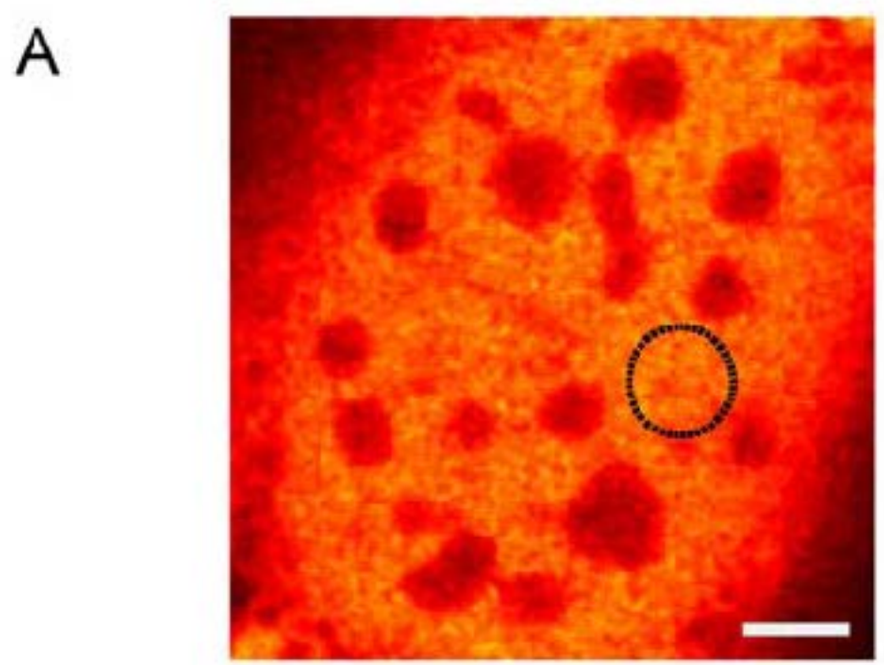


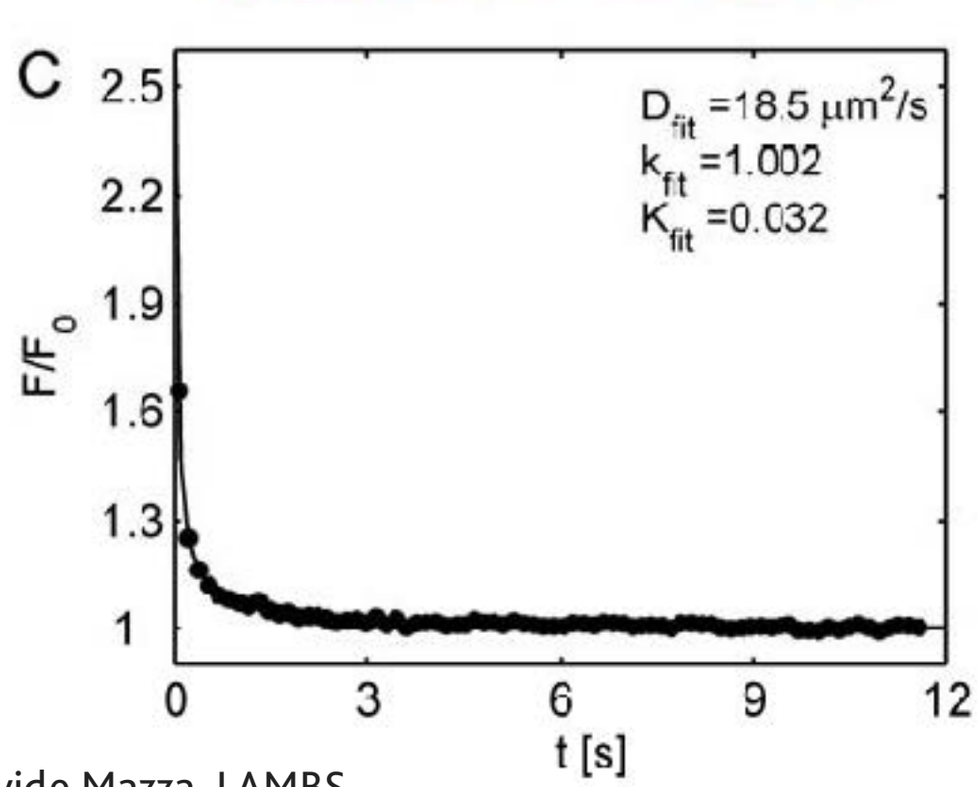


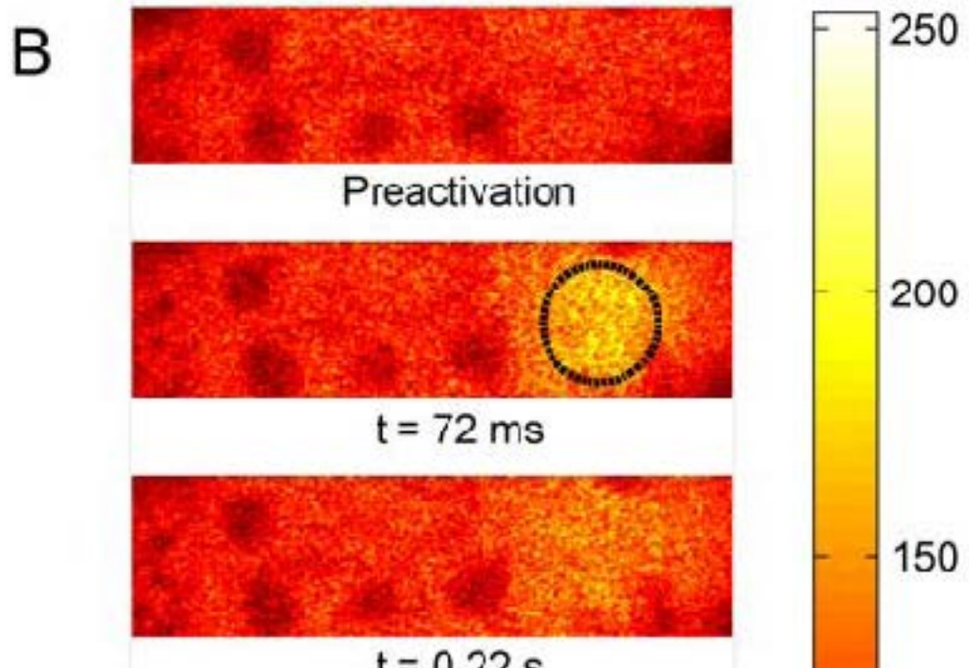


slide credit: Davide Mazza, LAMBS

FRAP







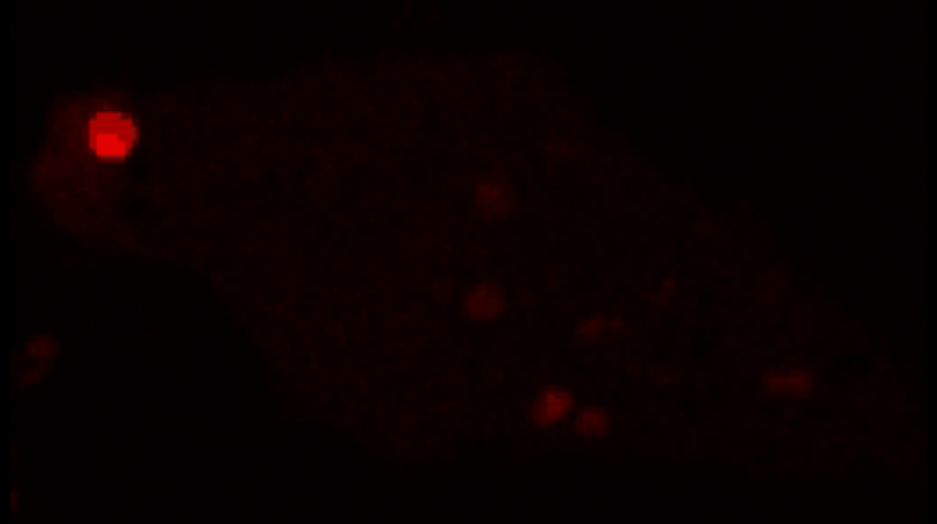
slide credit: Davide Mazza, LAMBS

Mouse Embryo Fibroblasts NIH-3T3

FRAP

Loren…Diaspro et al., **Q Rev Biophys. 2015** Aug;48(3):323-87.

Dendra2 photoactivation
in HEPg2 cells



with Dr. M. Crippa

Fluorescence Perturbation techniques (FPT)

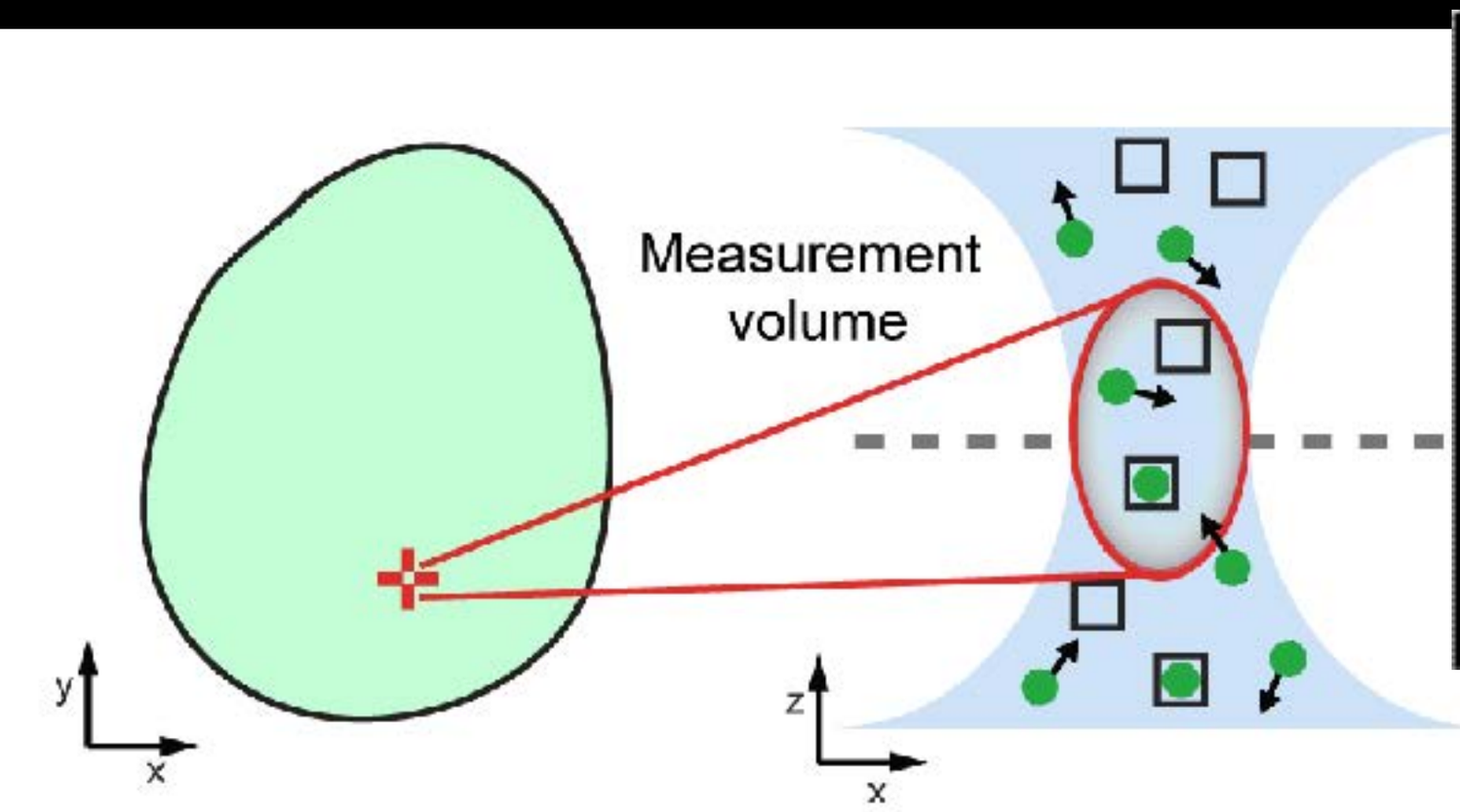
- Photobleaching Techniques
  - FRAP
    Fluorescence Recovery
    after Photobleaching
  - FLIP
    Fluorescence Loss
    Into Photobleaching
- Photoactivation Techniques
  - i-FRAP
    Inverse FRAP

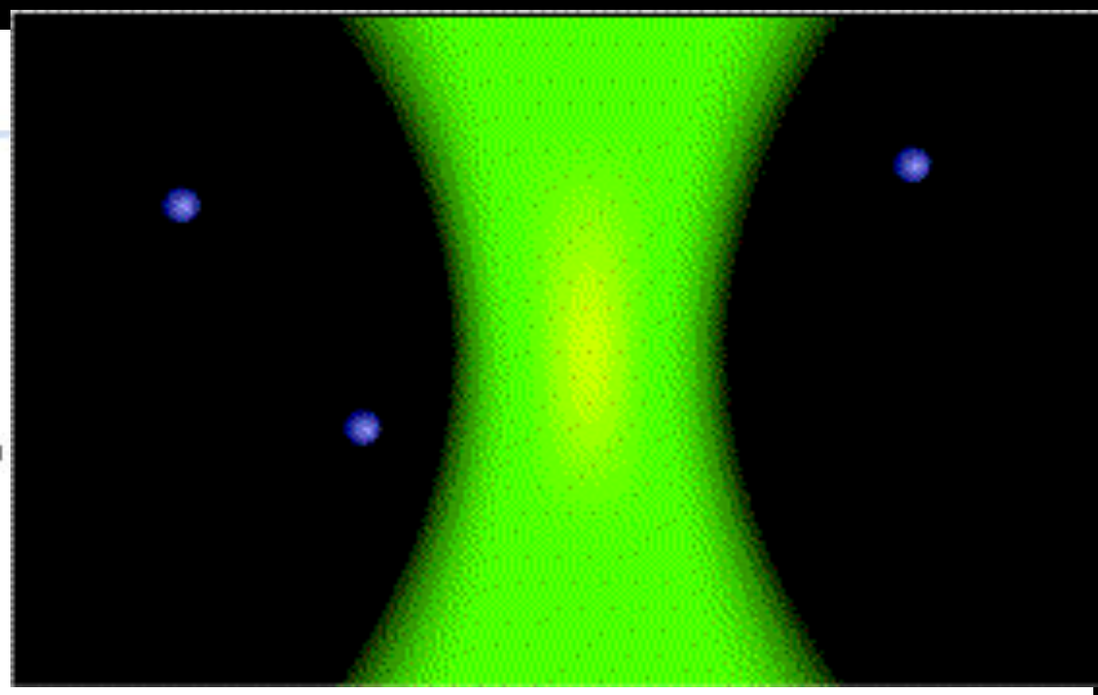
SOMETIMES LESS IT'S BETTER

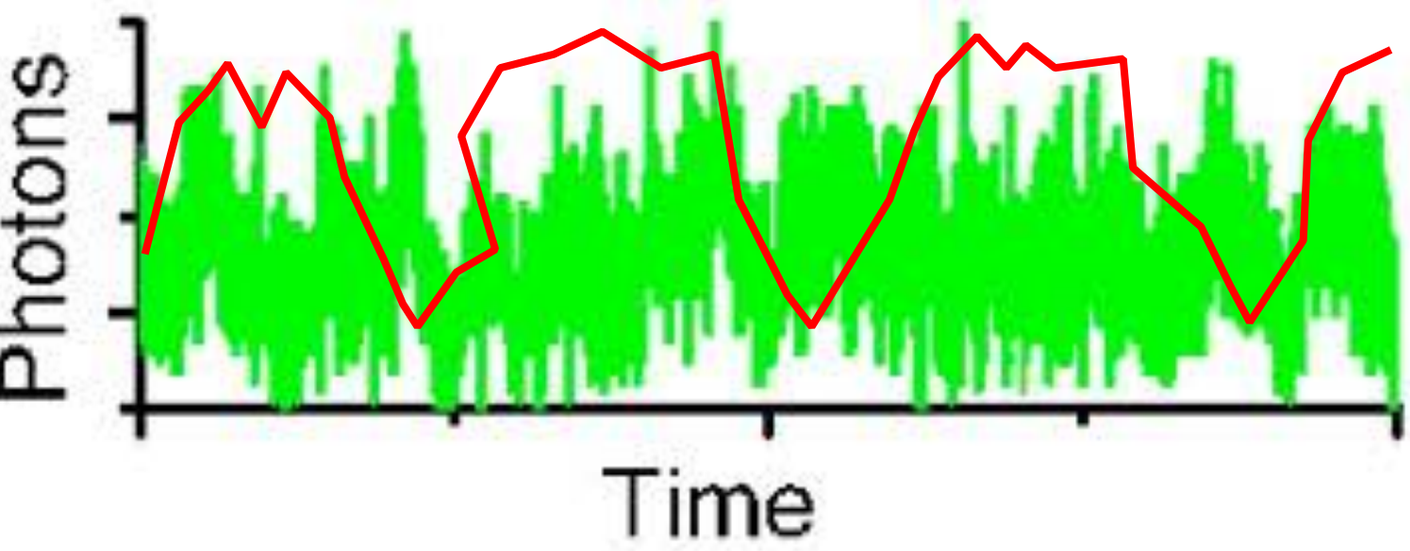
DECREASING CONCENTRATION
OF FLUORESCENT PROBE

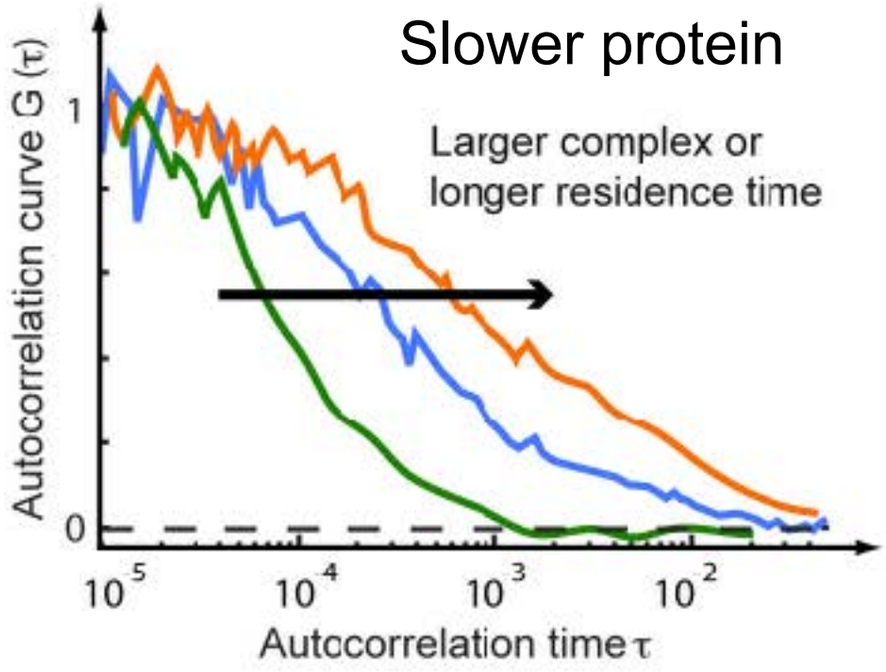
	FRAP	FLIP	Fluorescence fluctuation techniques	Single molecule
Probe concentration	*µM*	*µM*	*nM*	*pM to nM*
Photobleaching	*Yes*	*Yes*	*No*	*No*
Resolution	*1 µm*	*>1 µm*	*0.2 µm*	*0.02 µm*
Local measurement	*Yes*	*No*	*Yes/no*	*No*
Bulk technique	*Yes*	*Yes*	*Yes*	*No*

Fluorescence fluctuation spectroscopy (FCS)









SOMETIMES LESS IT'S BETTER

DECREASING CONCENTRATION
OF FLUORESCENT PROBE

	FRAP	FLIP	Fluorescence fluctuation techniques	Single molecule
Probe concentration	*µM*	*µM*	*nM*	*pM to nM*
Photobleaching	*Yes*	*Yes*	*No*	*No*
Resolution	*1 µm*	*>1 µm*	*0.2 µm*	*0.02 µm*
Local measurement	*Yes*	*No*	*Yes*	*No*
Bulk technique	*Yes*	*Yes*	*Yes*	*No*

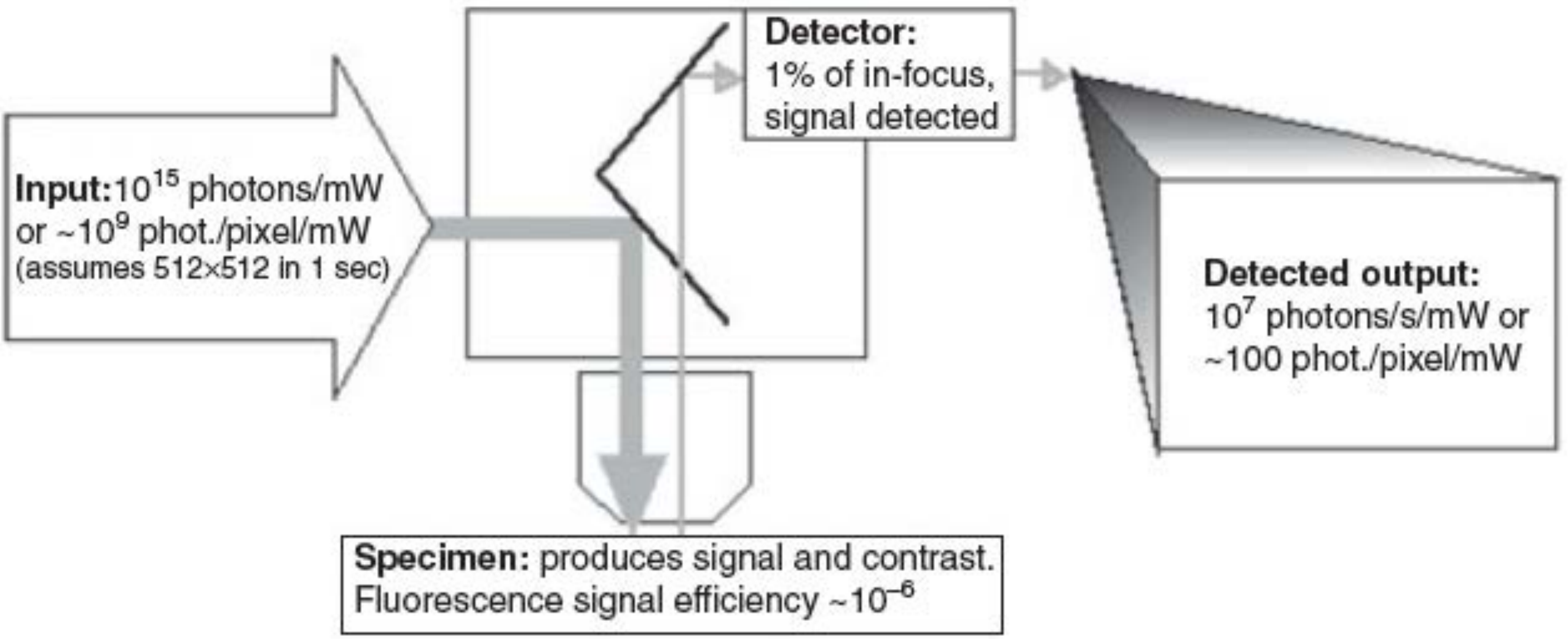
Optical microscopy again

towards

Nanoscale Optical Microscopy

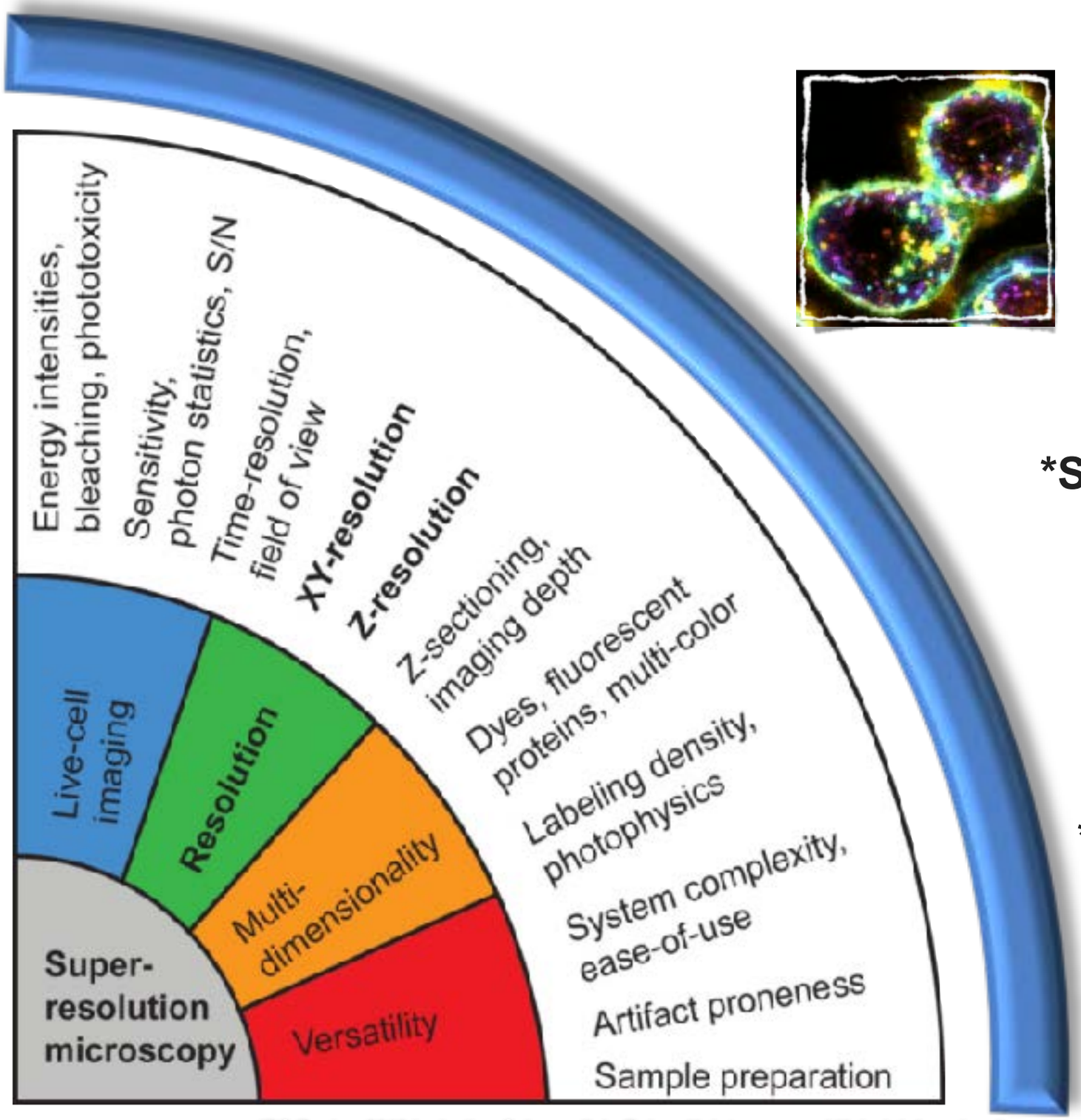
Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

optical microscopy scenario



Slide credit: Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, 2006

Optical Microscopy Framework

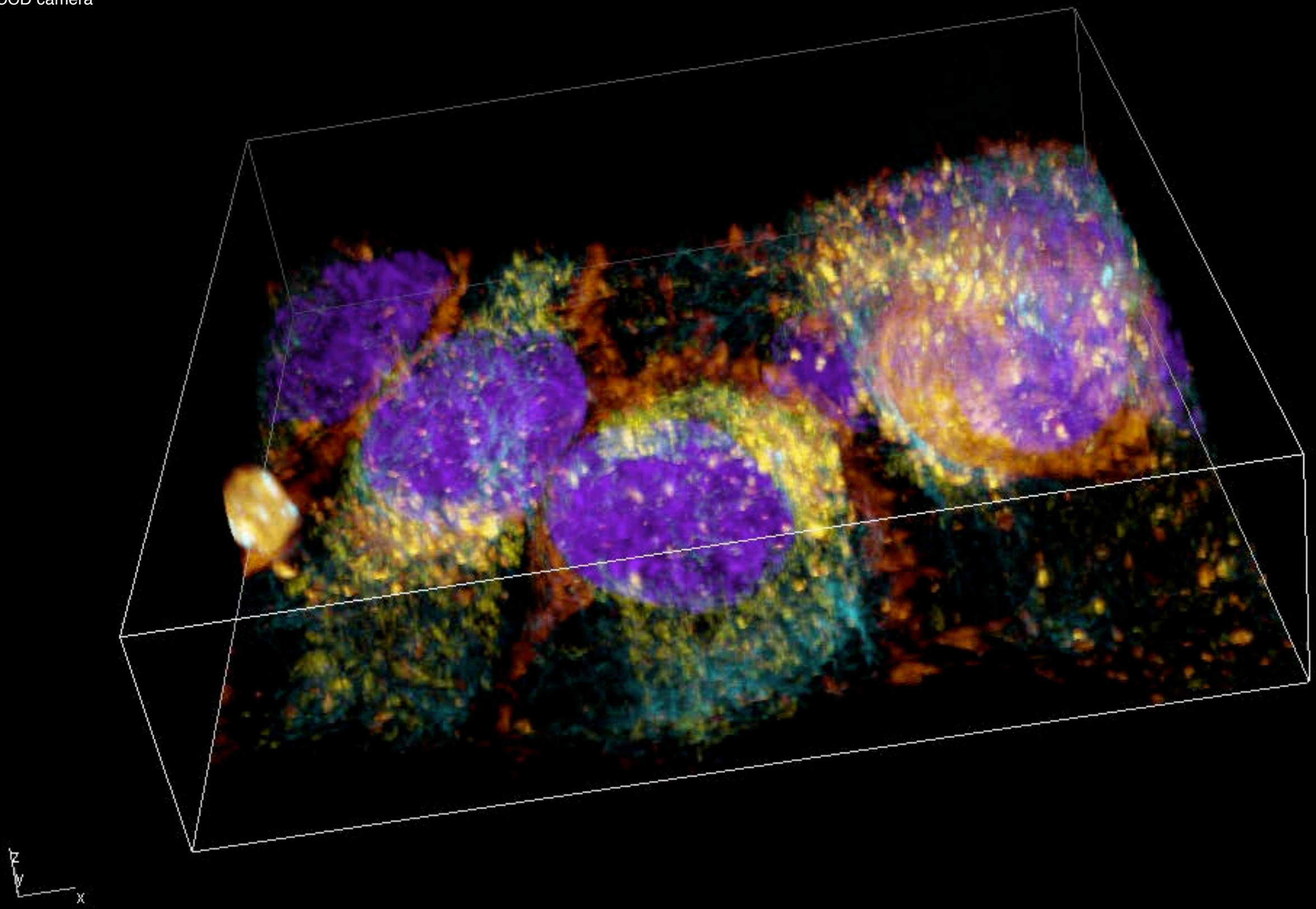


JBC Review 2010 by Lothar Schermelleh, Rainer Heintzmann and Heinrich Leonhardt

***wide field optical sectioning microscopy**
***confocal laser scanning microscopy**
***two-photon excitation microscopy**
***SHG, Raman, Phase, Polarisation**
***light sheet fluorescence microscopy**
***near-field fluorescence microscopy**
…

***optofluidics, origami, lab on a chip microscopy**
***expansion microscopy**

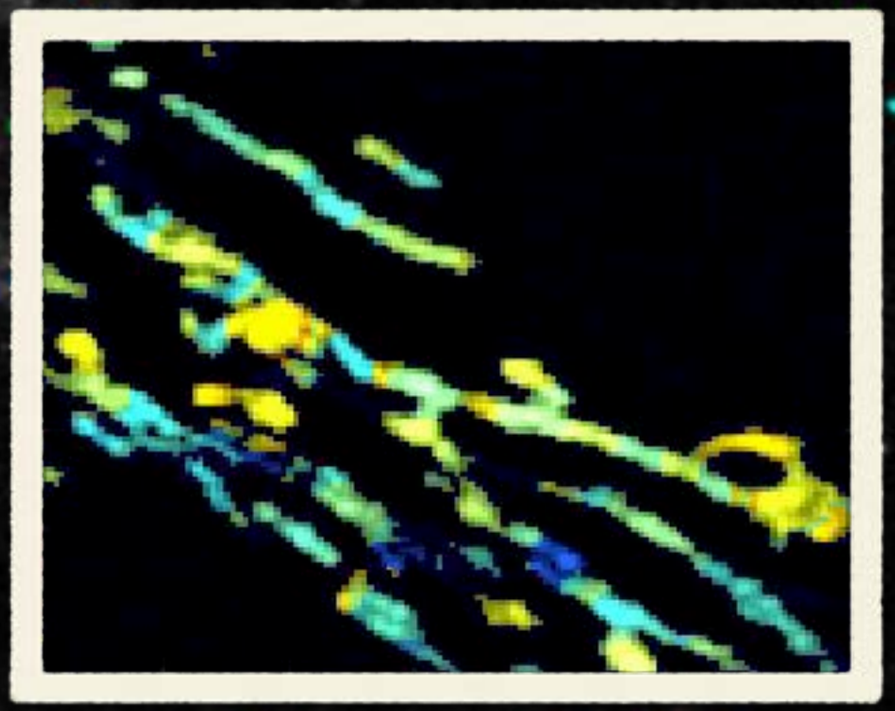
Sample preparation: Luca Pesce
Image Acquisition: Lorenzo Scipioni
Microscope: Nikon Ti Eclipse Spinning Disk, equipped with Andor iXon3 EMCCD camera



Width:71.80 µm Height:71.80 µm Depth:17.00 µm

3D 4-colors confocal microscopy images of live HeLa cells. Cell staining shows DNA (Hoechst 33342 - Magenta), tubulin (Tubulin Tracker - Cyan), mitochondria (Mitotracker Orange - Yellow) and plasma membrane (Cell Mask - Red). image size is 512x512, pixels size is 140 nm/pixel.

Lorenzo Scipioni, Diaspro LAB, IIT

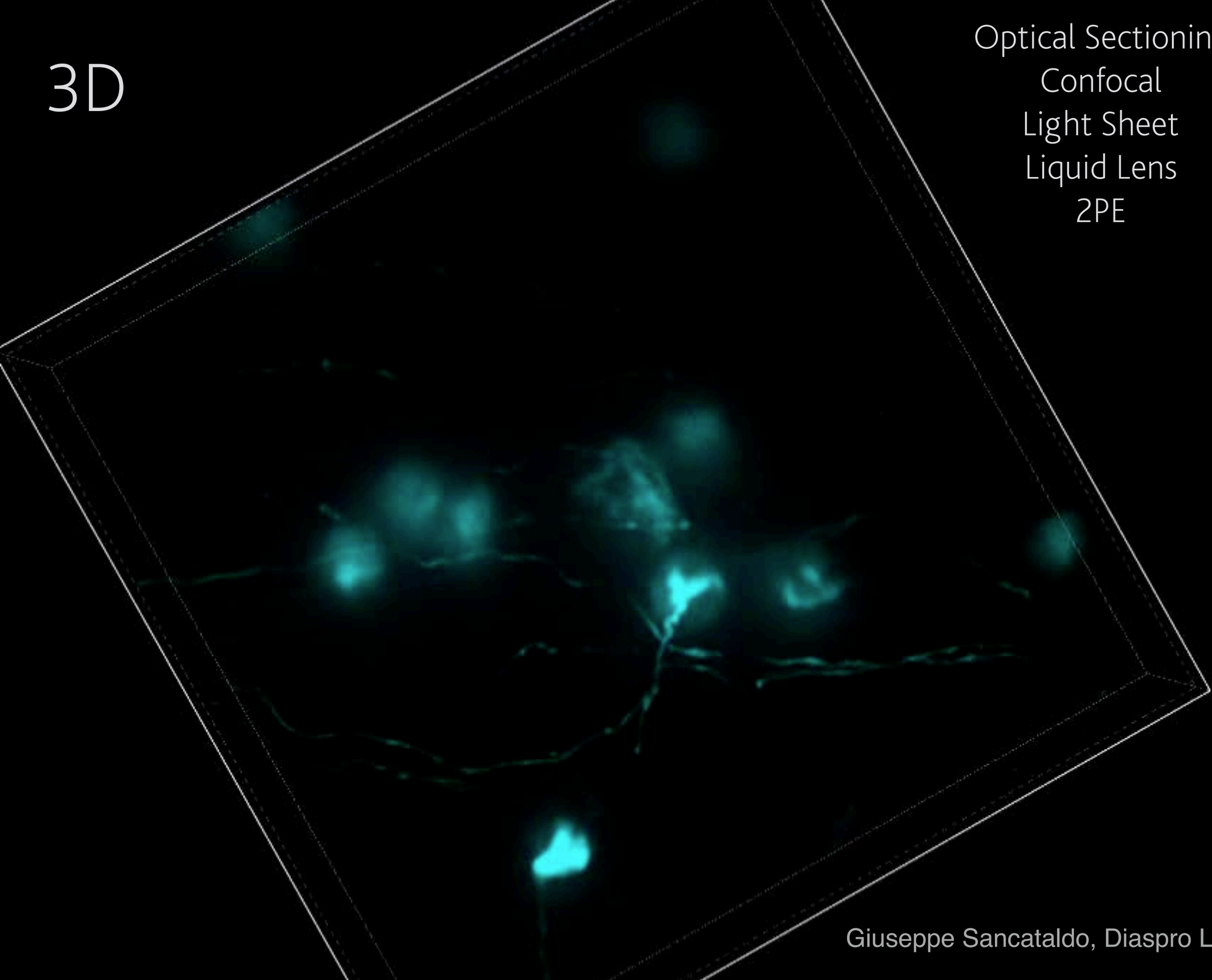


250nm (blu scuro) a 800 nm (rosso scuro).

Scipioni, L., E. Gratton, A. Diaspro, and L. Lanzanò. **2016.** Phasor Analysis of Local ICS Detects Heterogeneity in Size and Number of Intracellular Vesicles. **Biophysical Journal. 111: 619–629**.

3D

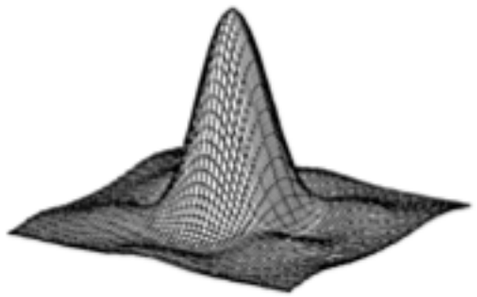
Optical Sectioning
Confocal
Light Sheet
Liquid Lens
2PE

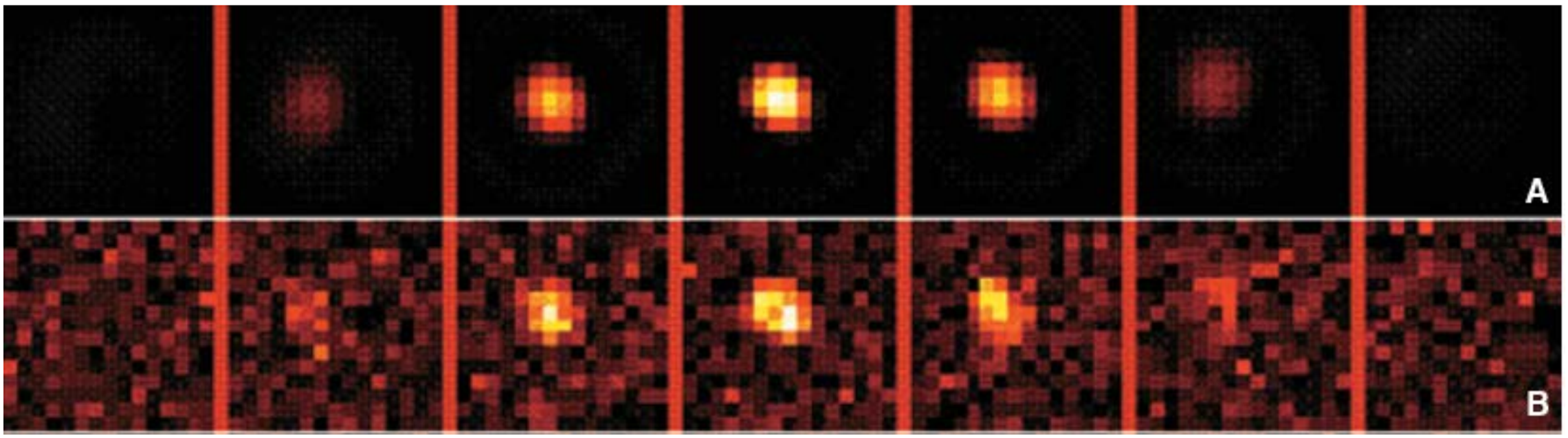


Giuseppe Sancataldo, Diaspro LAB, IIT

Image formation process

Linear and space invariant system fully described by its PSF (point spread function)

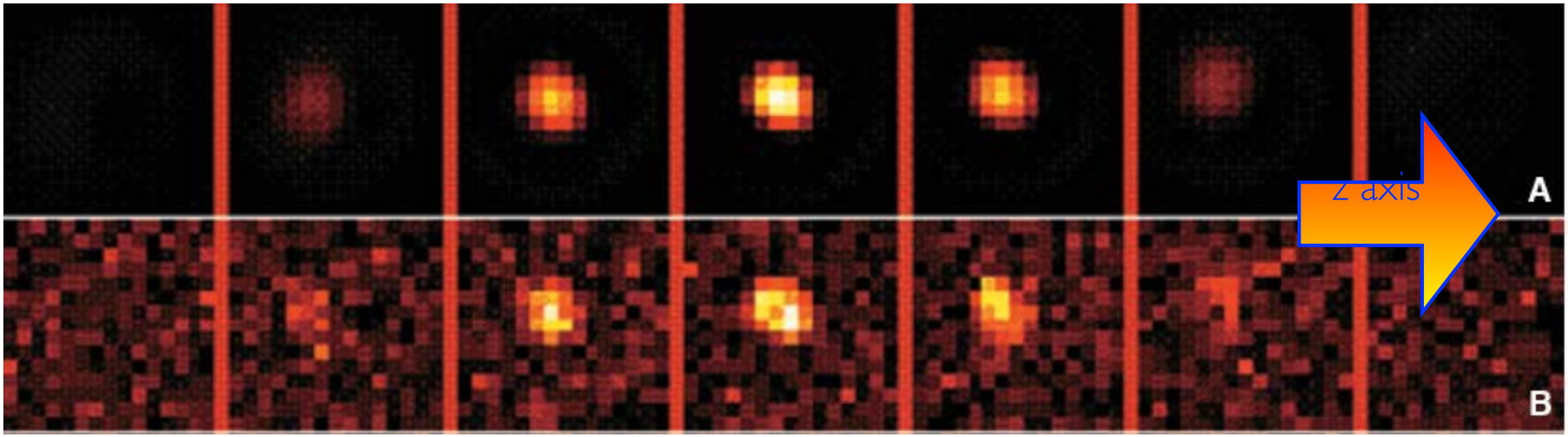


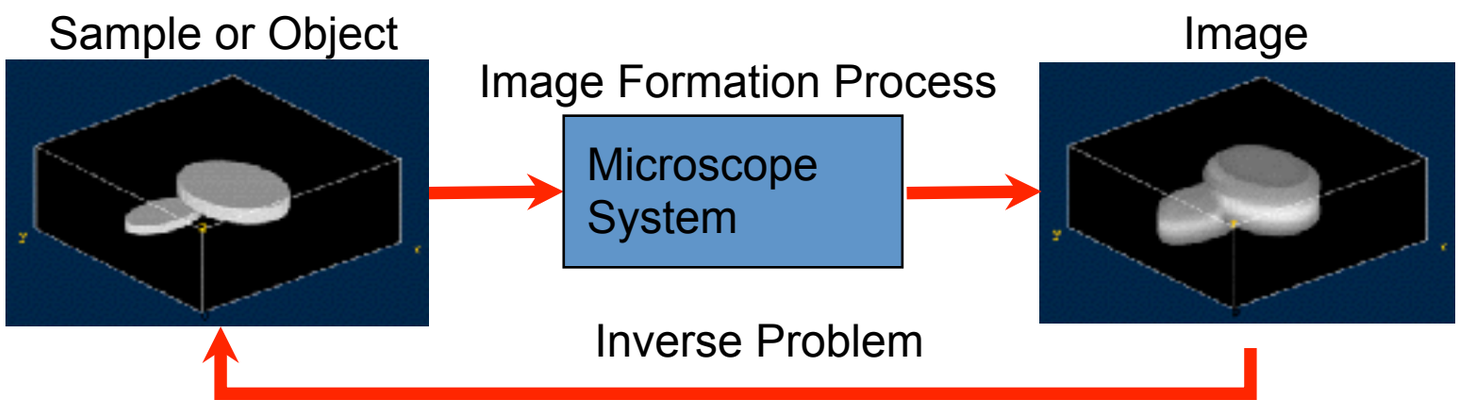


z=0

Slide credit: Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

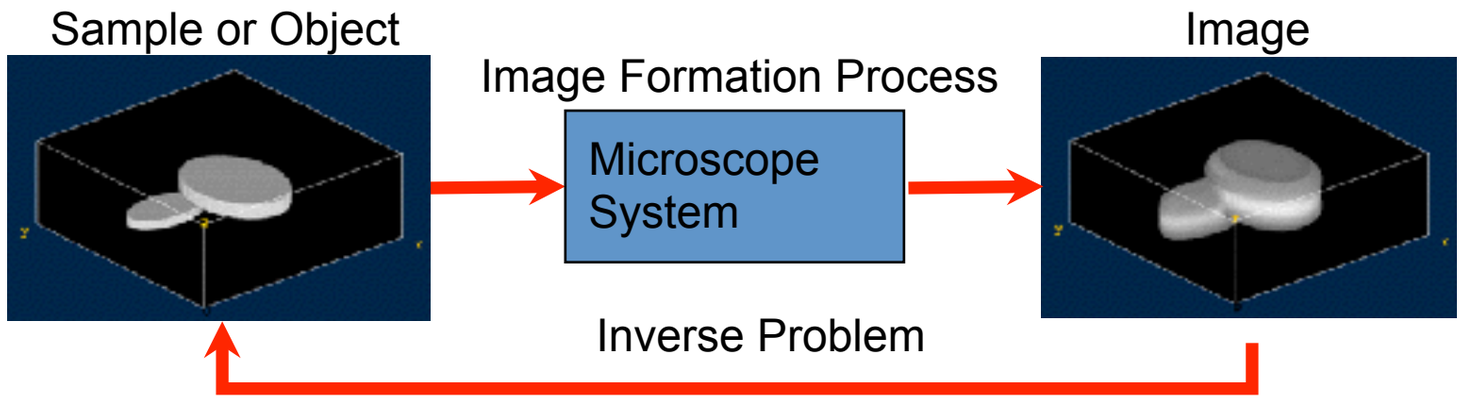
Linear and space invariant system fully described by its PSF (point spread function)



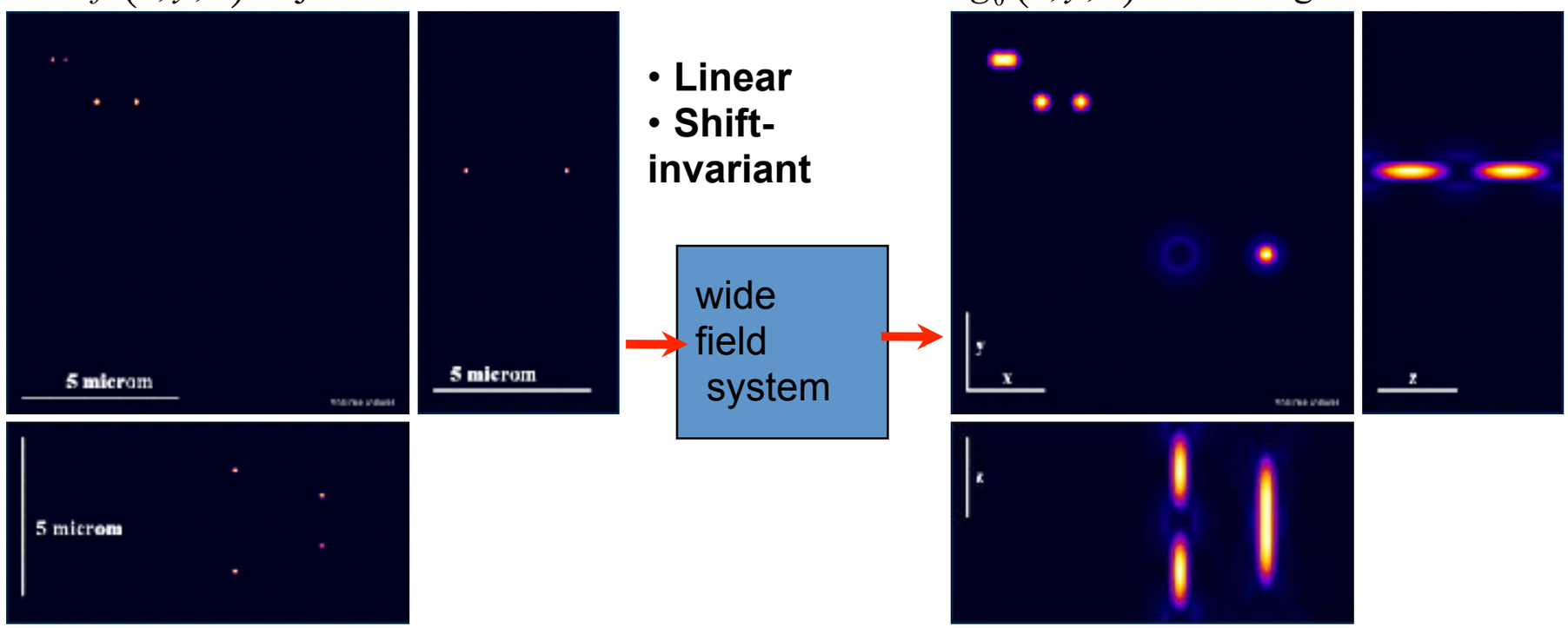


Slide credits: Giuseppe Vicidomini, Jim Pawley

Image formation process



$$g_0(x,y,z) = f(x,y,z) *_{3D} PSF(x,y,z) = \int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty} f(x-\xi, y-\eta, z-\zeta)\, PSF(\xi,\eta,\zeta)\, d\xi\, d\eta\, d\zeta$$



Process image formation simulation of a wide-field microscopy.
Simulation parameters: objective 100x NA 1.3 oil, n=1.518, λex 488nm, λem 520nm.

Image formation process

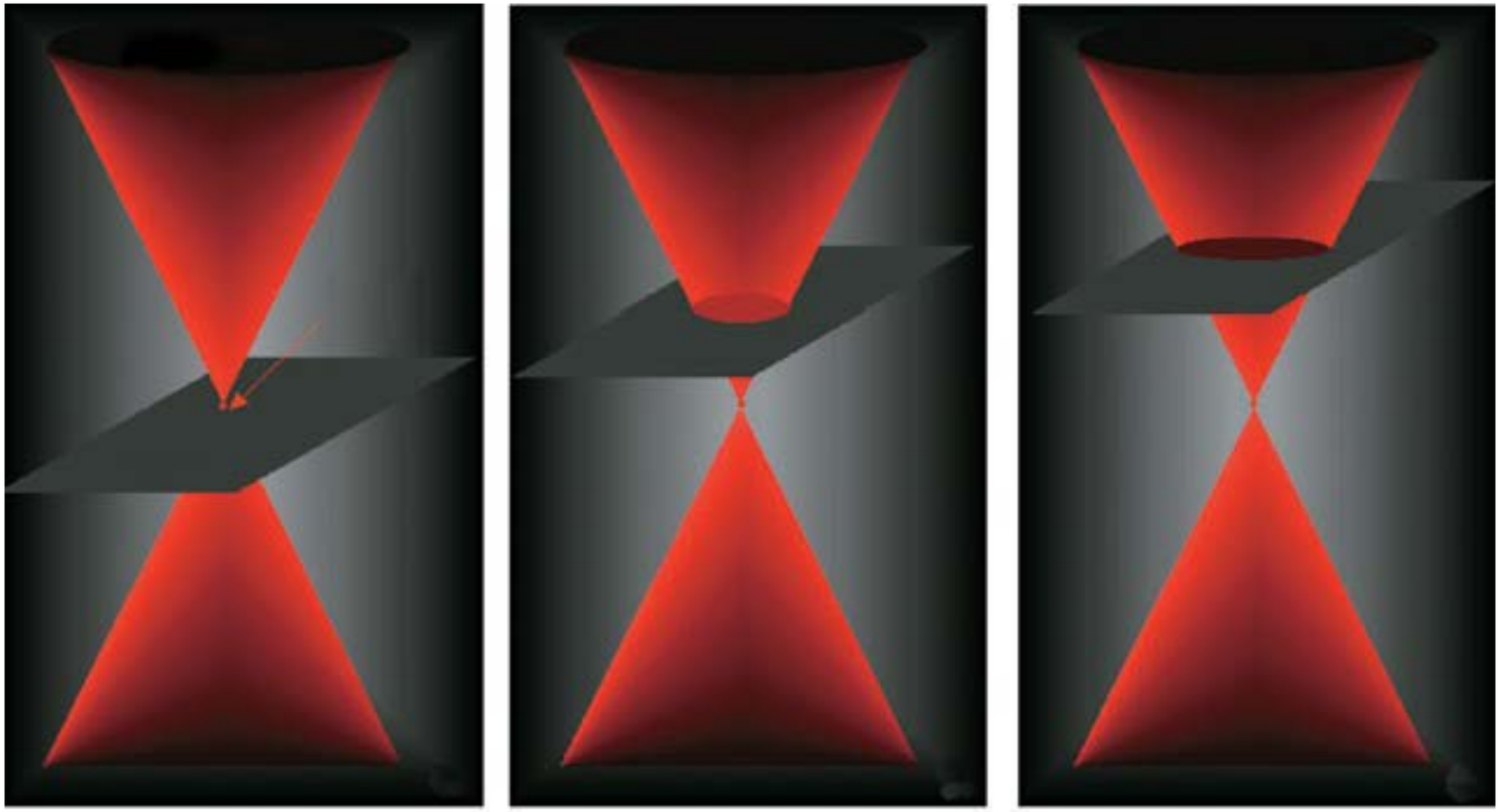


Image formation process

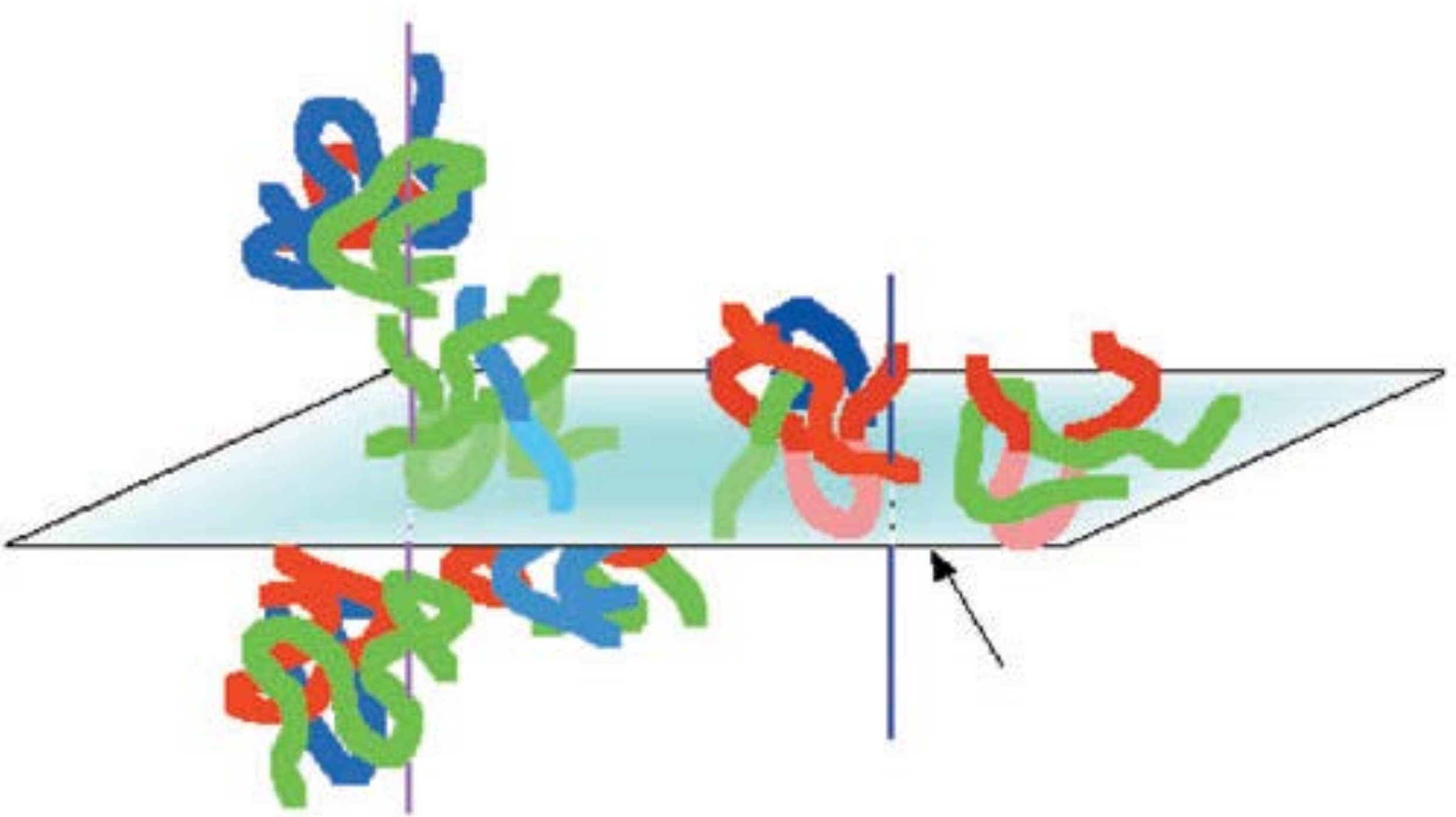
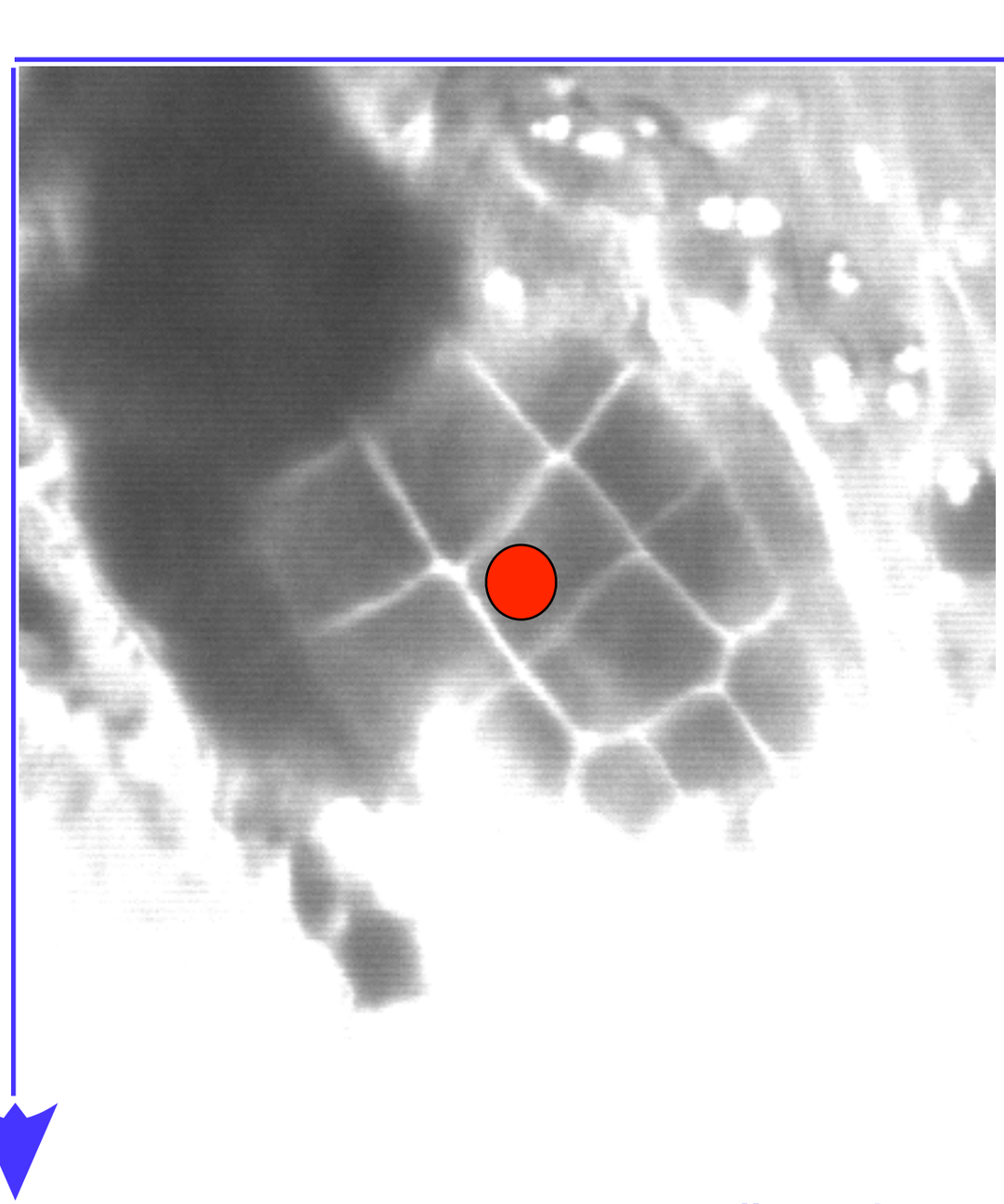
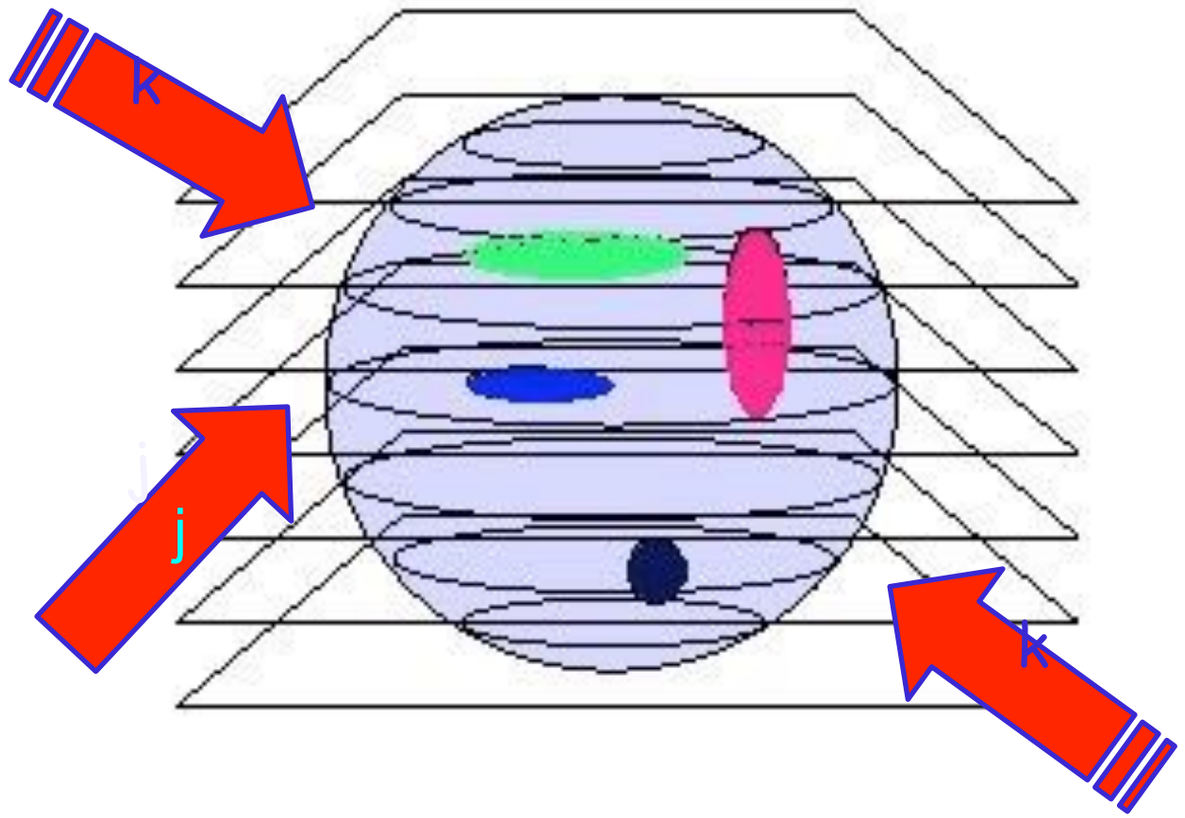


Image formation process



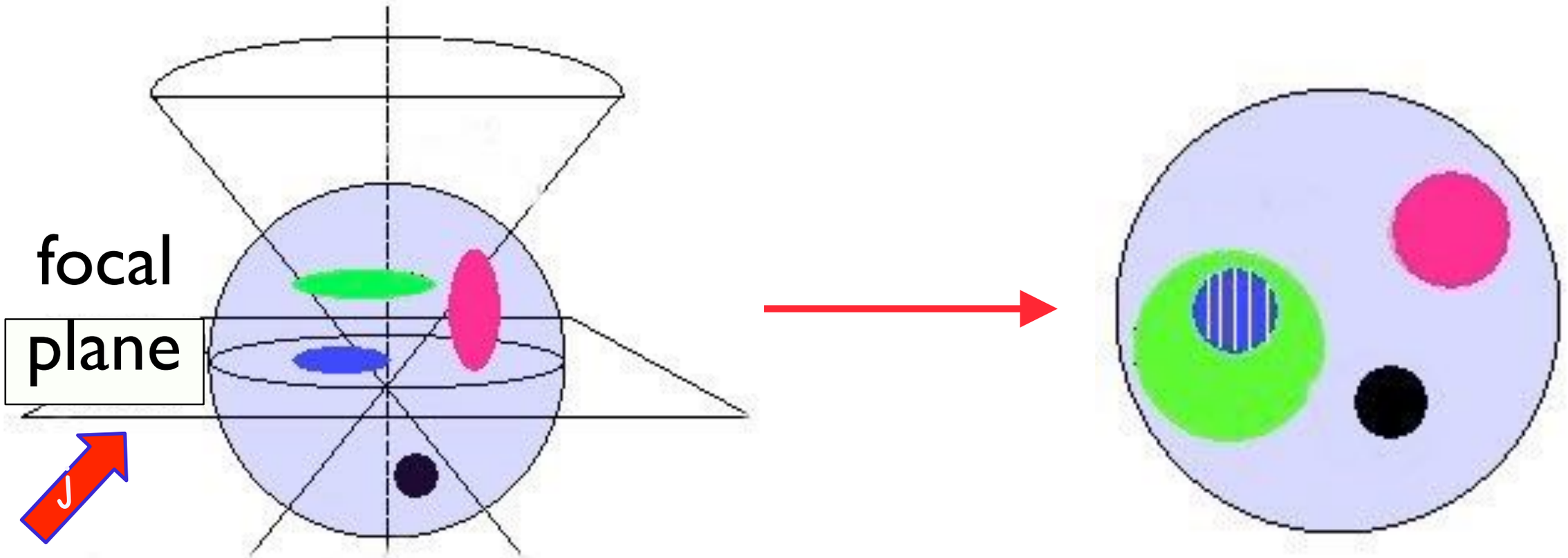
NAVIGATING INTO CELLS AND TISSUES



B. Bianco, A. Diaspro, Cell Biophysics, 15 (3), pp.189-200, 1989.
A. Diaspro, et al., Image Vision and Computing, 8 (2), pp.130-141, 1990.

Image formation process

OPTICAL SECTIONING RESULT



B. Bianco, A. Diaspro, Cell Biophysics, 15 (3), pp.189-200, 1989.
A. Diaspro, et al., Image Vision and Computing, 8 (2), pp.130-141, 1990.

Image formation process

OPTICAL SECTIONING

Within the optical sectioning scheme the situation is that the observed image O at a plane j is produced by the true fluorescence distribution at plane j, distorted by the microscope through S, plus contributions from adjacent k planes and noise N.

$$O_j = I_j S_j + \Sigma_{k \neq j} I_k S_k + N$$

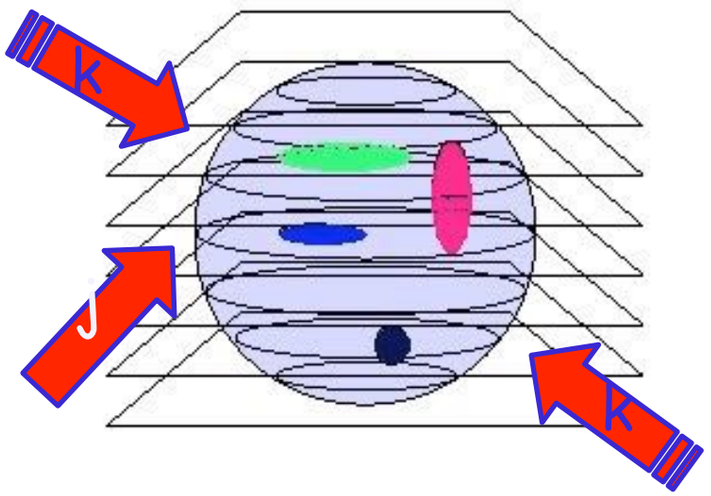
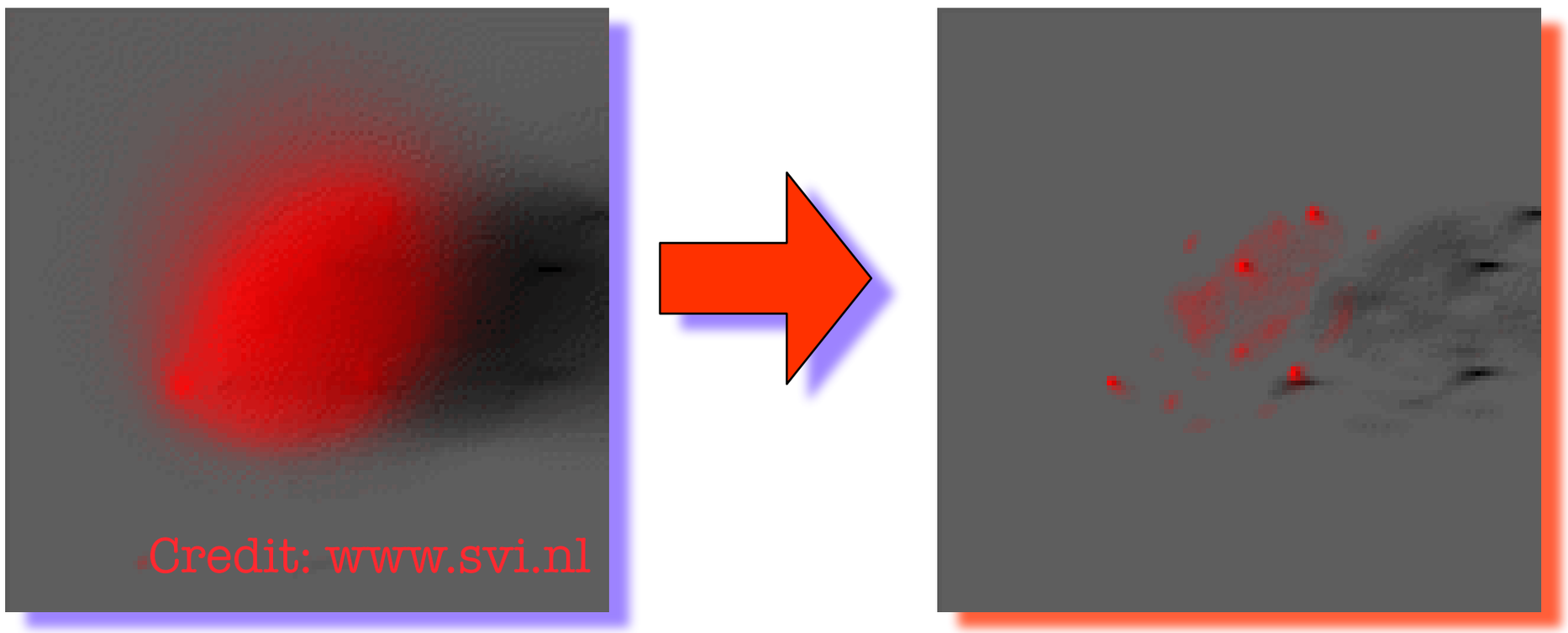


Image formation process

Solving the equation set…

$$O_j = I_j S_j + \Sigma_{k \neq j} I_k S_k + N$$



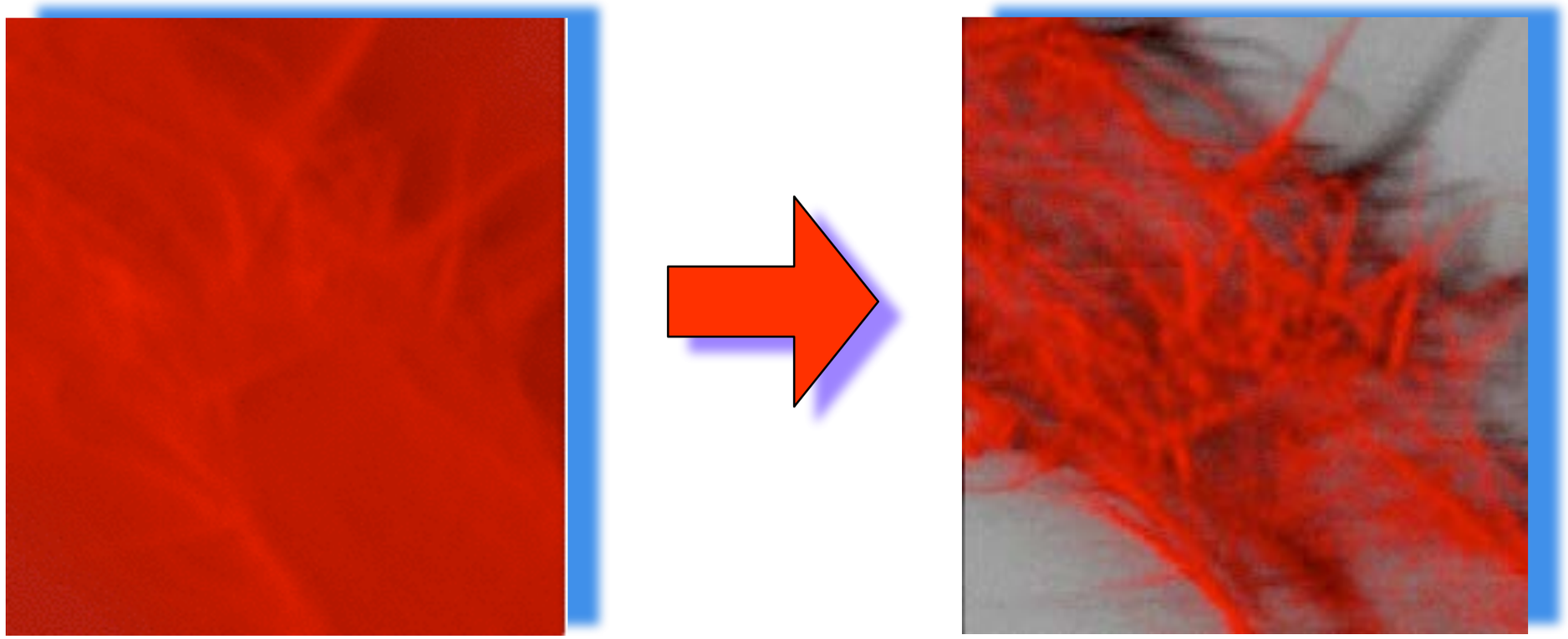
$$h(x,y,z) * f(x,y,z) + n(x,y,z) + b(x,y,z) = g(x,y,z)$$

www.powermicroscope.com

Image formation process

Solving the equation set...

$$O_j = I_j S_j + \Sigma_{k \neq j} I_k S_k + N$$



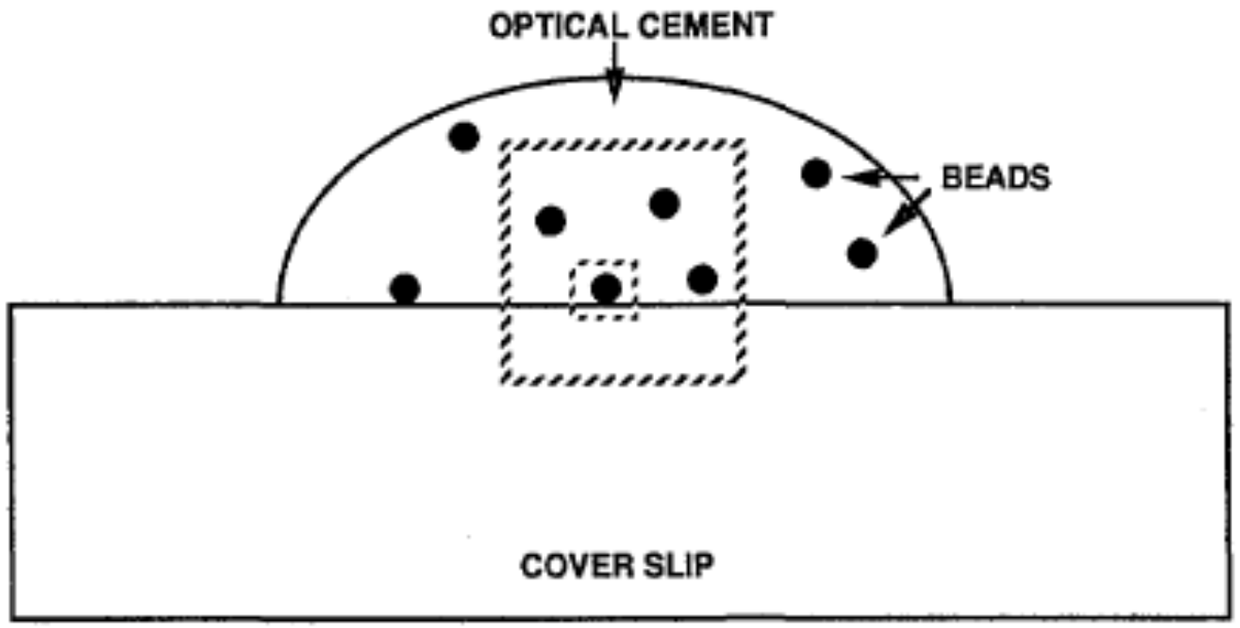
BONETTO P., BOCCACCI P., SCARITO M., DAVOLIO M., EPIFANI M., VICIDOMINI G., TACCHETTI C., RAMOINO P., USAI C., DIASPRO A. (2004) MICROSCOPY RESEARCH AND TECHNIQUE. vol. 64, pp. 196-203.

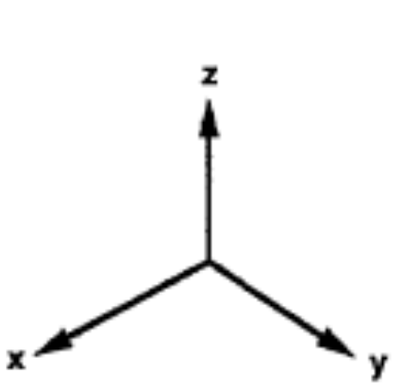
www.powermicroscope.com

OPTICAL RESOLUTION

Artifacts in computational optical-sectioning microscopy

James G. McNally, Chrysanthe Preza, José-Angel Conchello, and Lewis J. Thomas, Jr.





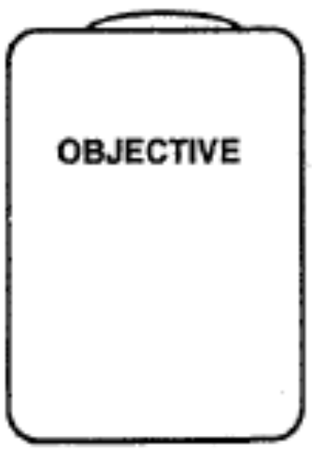
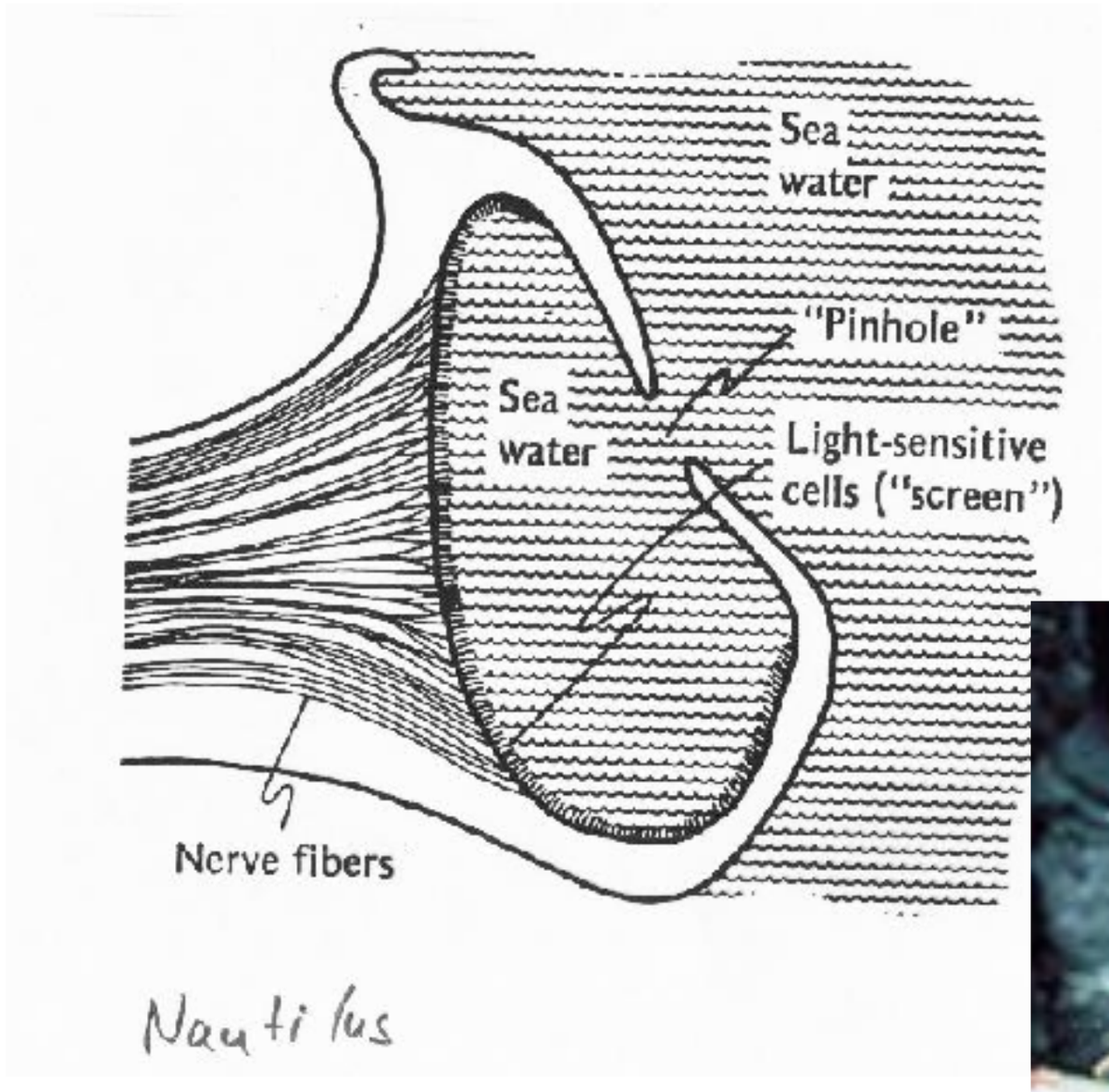
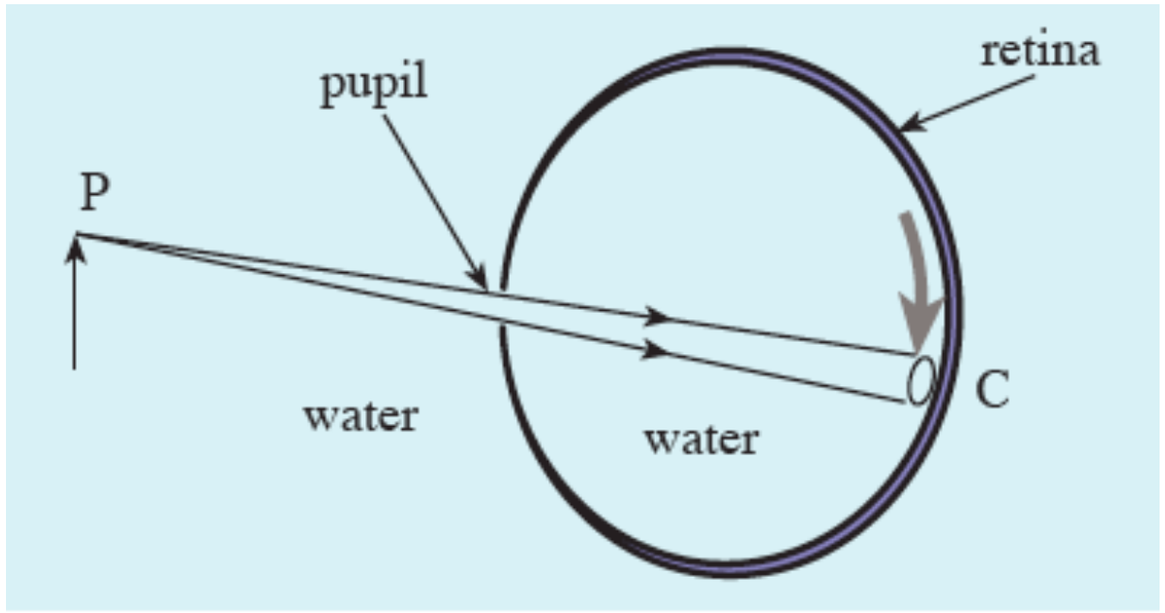


Image formation process





Giuseppe Colicchia 2006 *Phys. Educ.* **41** 15-17

Slide credit idea: Paolo Sapuppo, Leica Microsystems

Image formation process

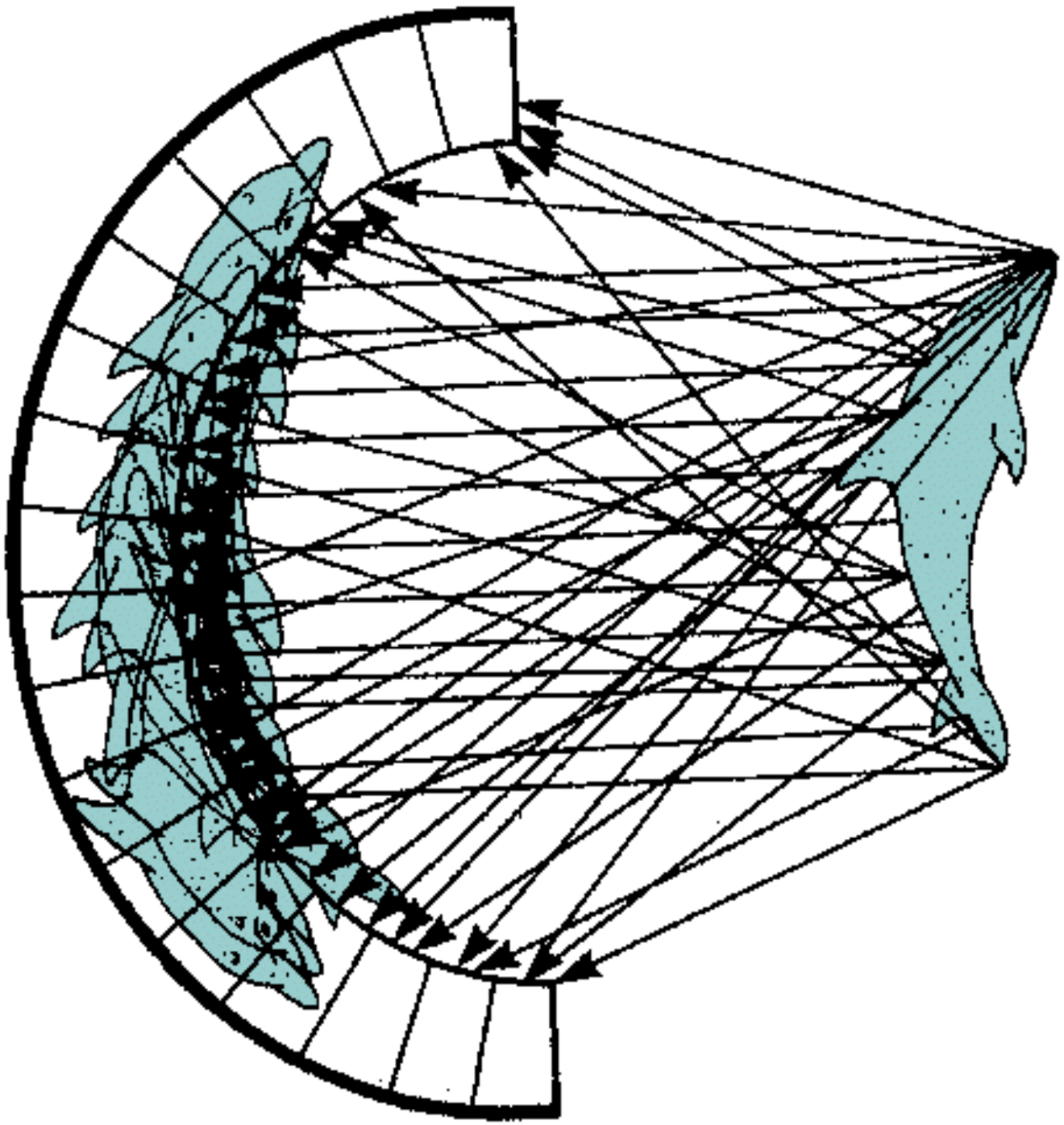


Image formation process

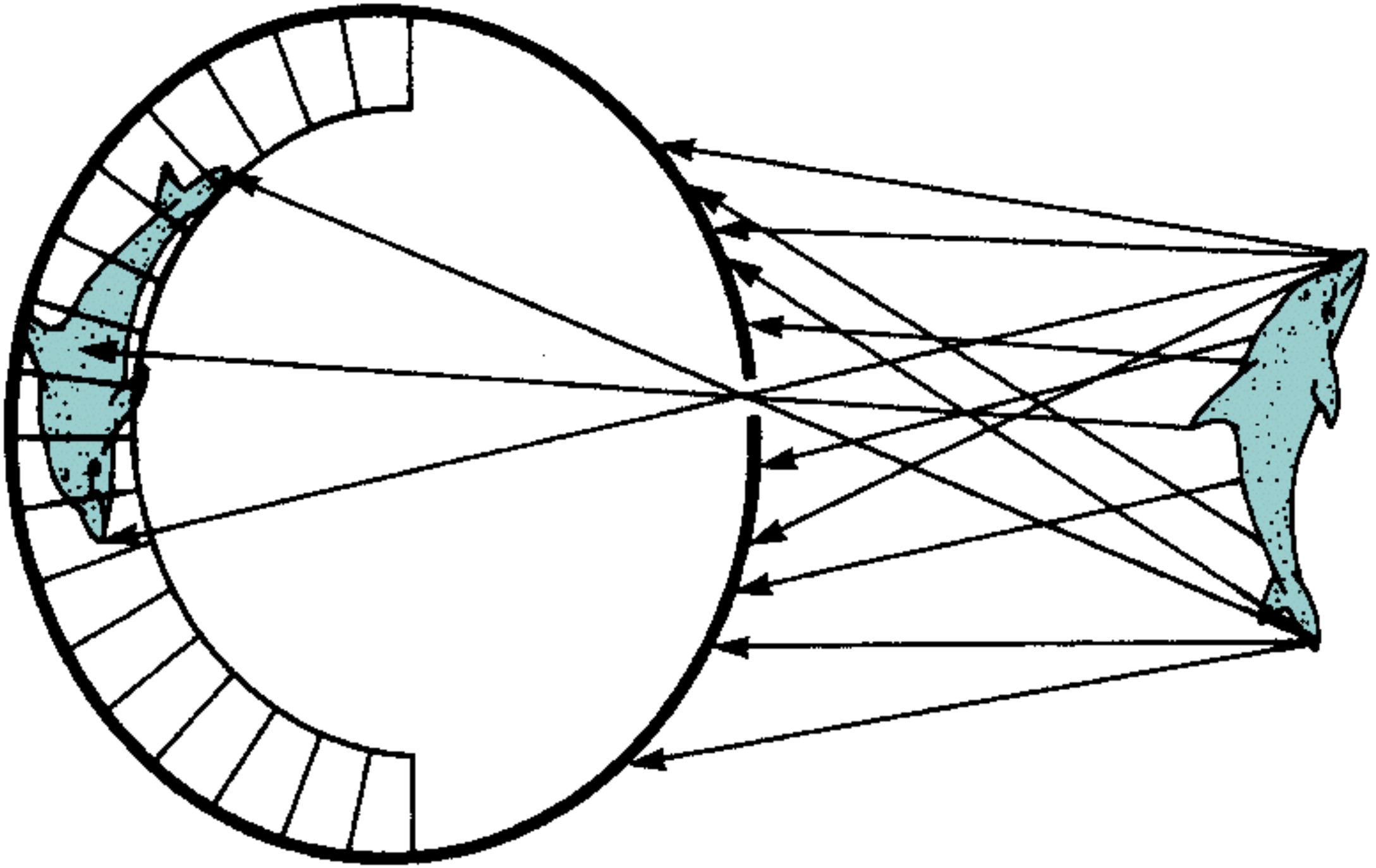


Image formation process

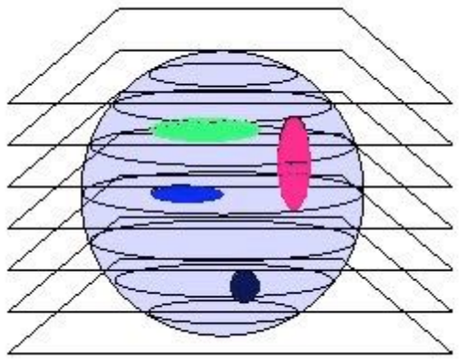
confocal laser scanning microscopy

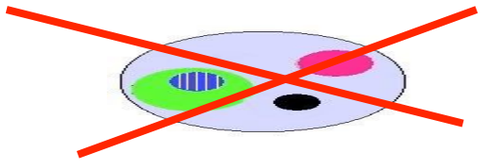
Solving the equation set…by means of a confocal set-up

$\sum I_k S_k \rightarrow 0$

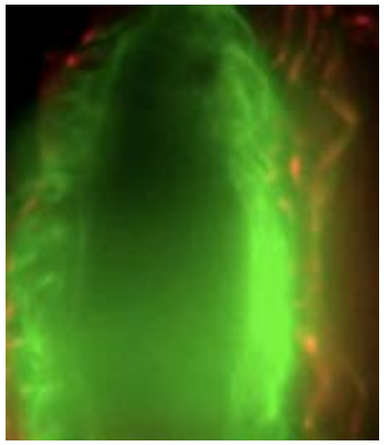
$S_j \rightarrow 1$

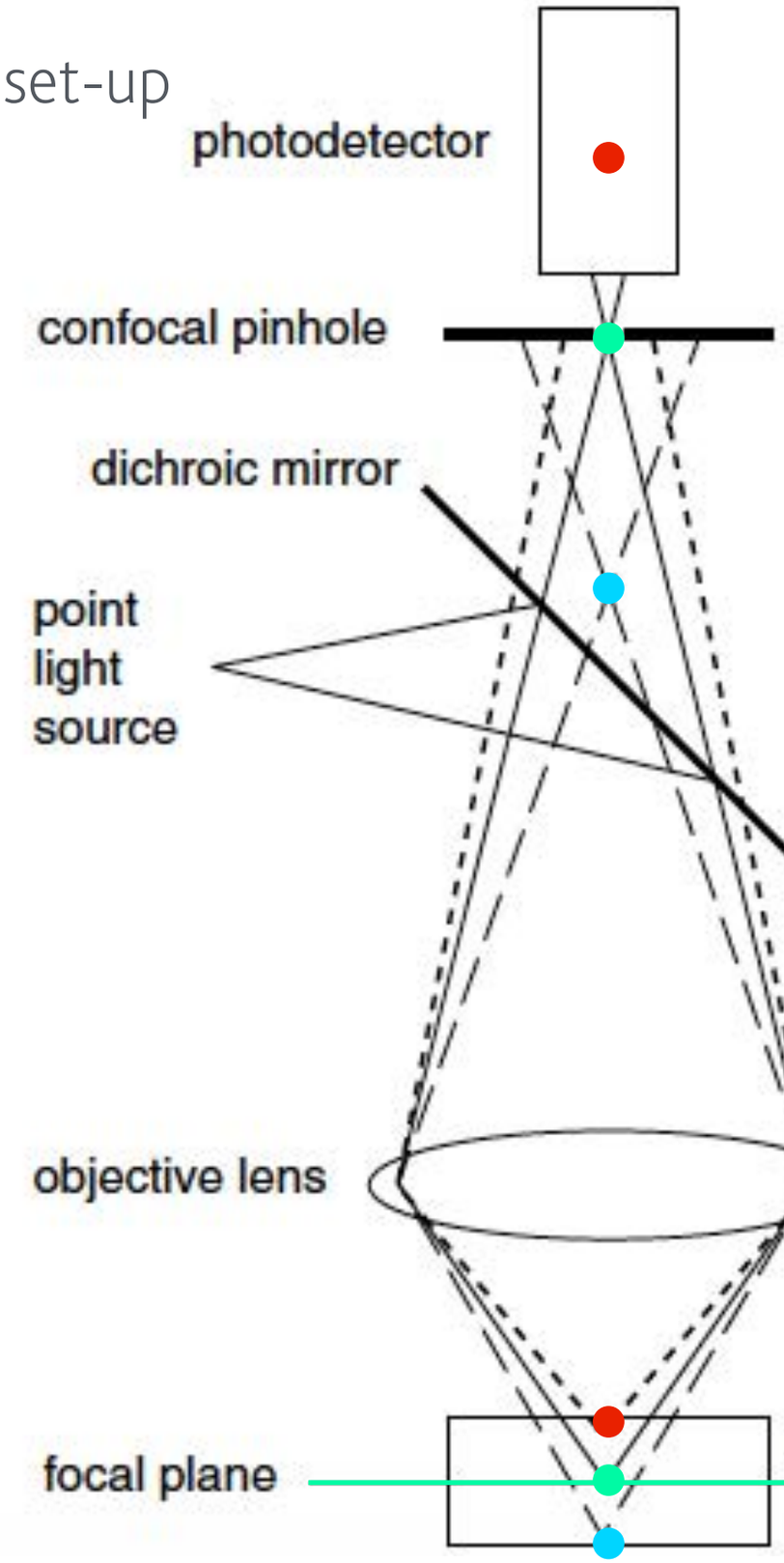
$O_j \rightarrow I_j$



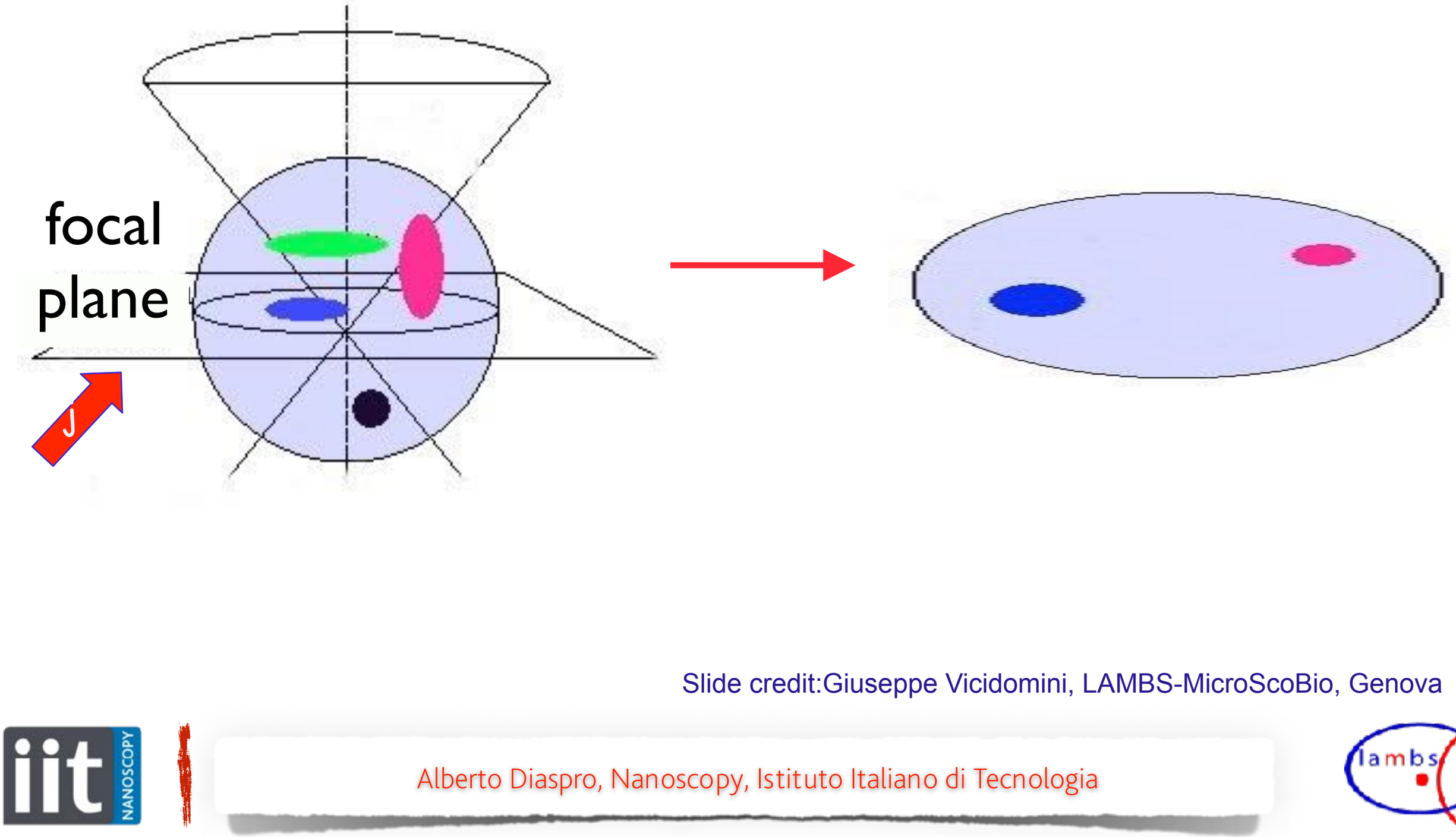


$O_j = I_j S_j + \sum_{k \neq j} I_k S_k + N$





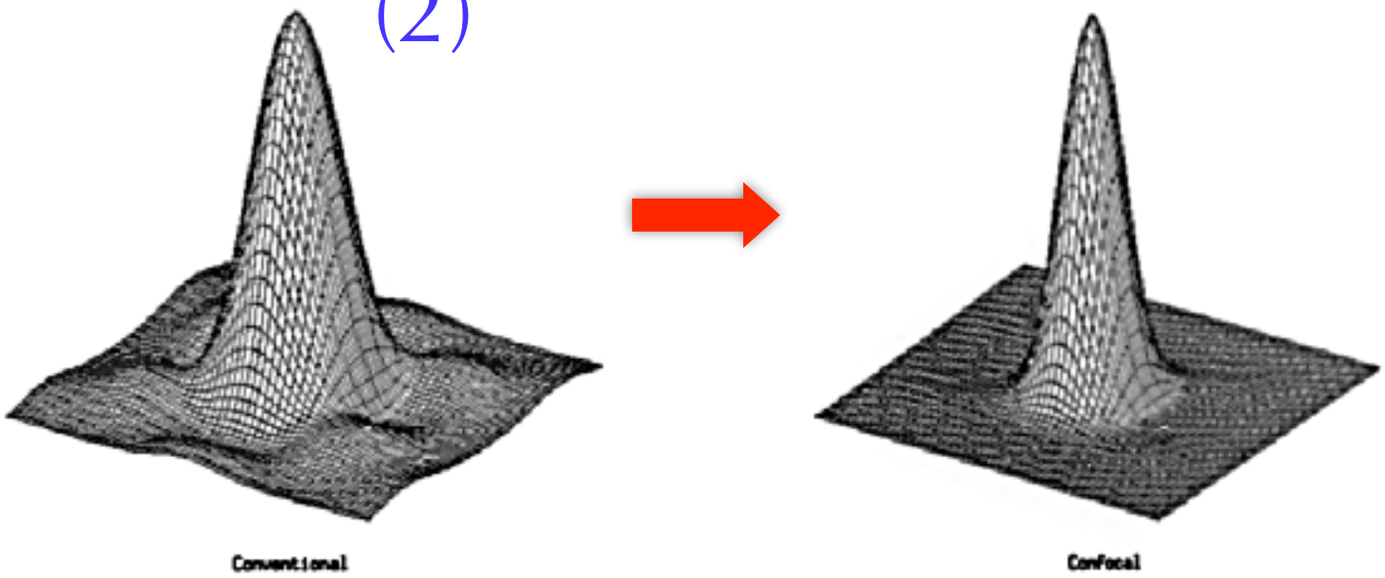
Modified from D.Shotton, 1993 and C.L.Smith, 2011



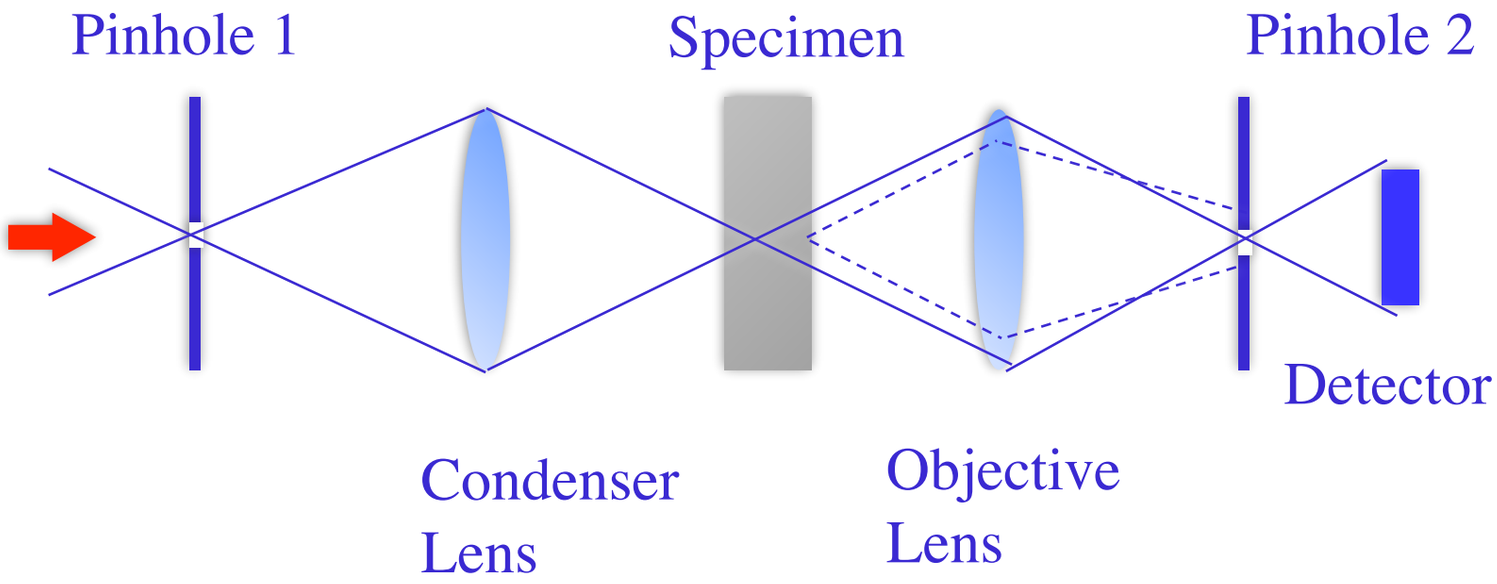
Slide credit:Giuseppe Vicidomini, LAMBS-MicroScoBio, Genova

confocal laser scanning microscopy

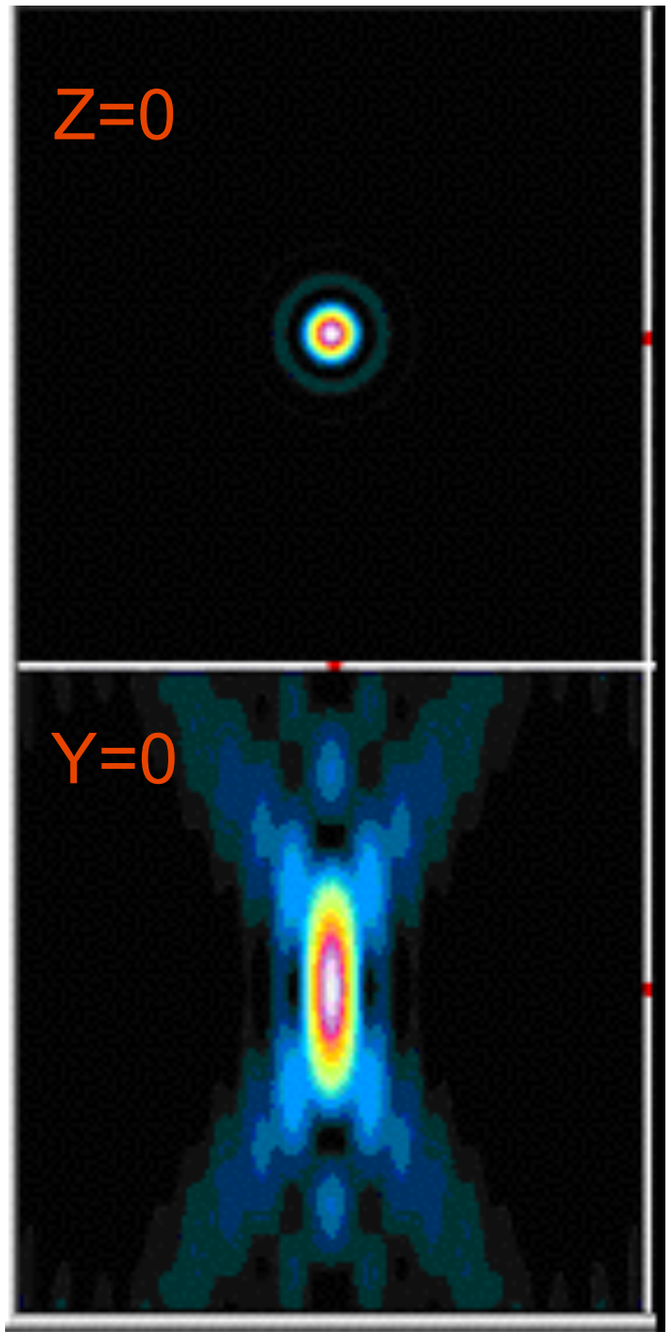
(2)

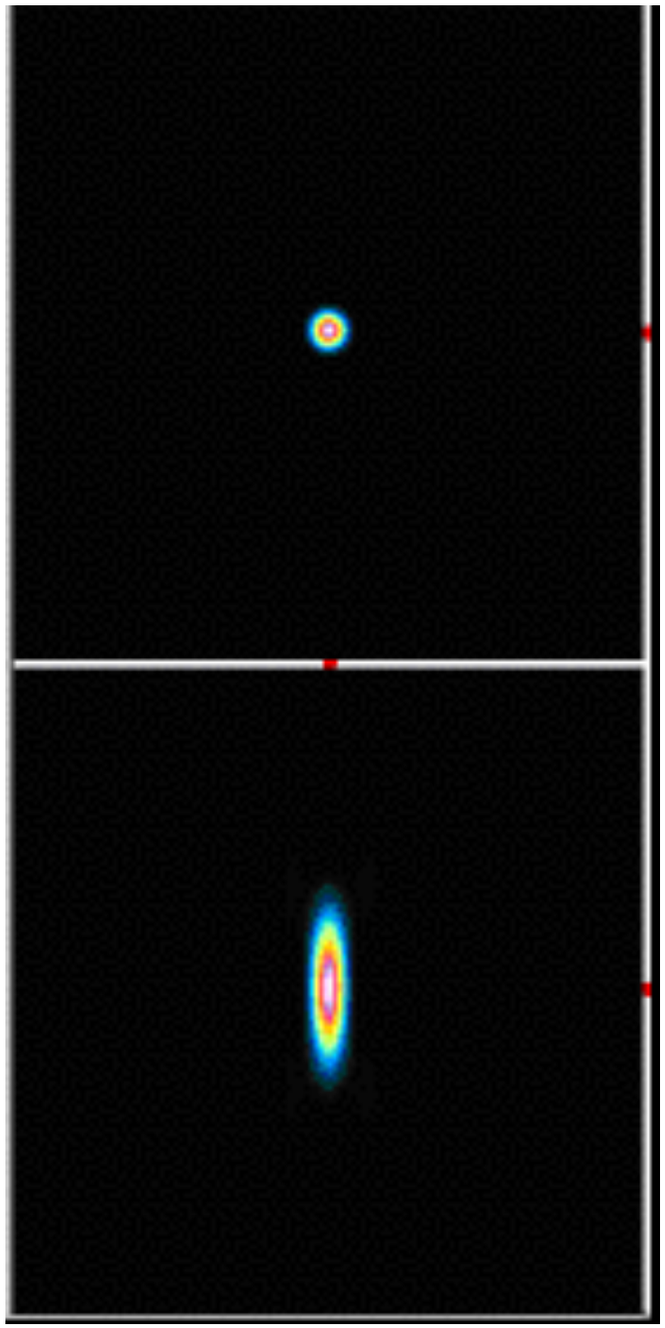


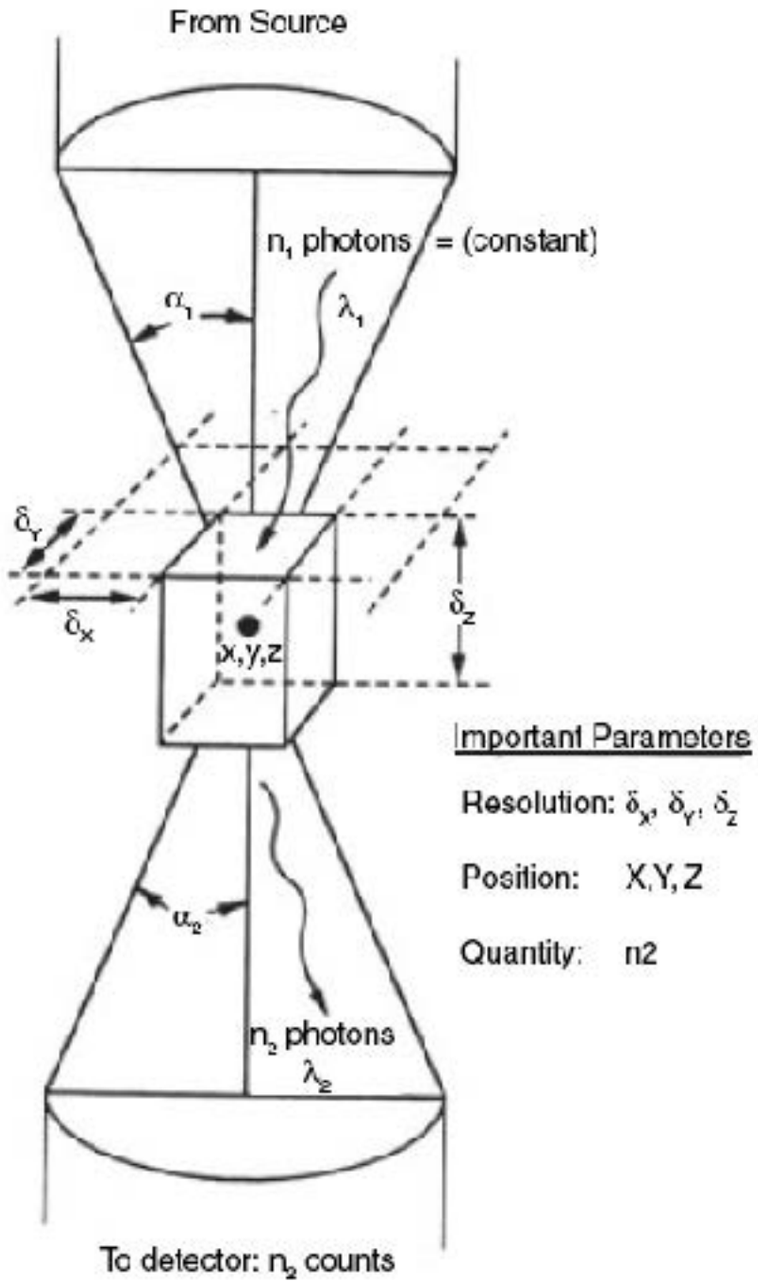
$$I_{fluor-conf} = I_{\det} I_{ill} \rightarrow I^2 \propto \frac{1}{z^4}$$



confocal laser scanning microscopy







POINTLIKE OBJECT VS. QUASI POINTLIKE IMAGE

confocal laser scanning microscopy

ELSEVIER

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How the Confocal Laser Scanning Microscope entered Biological Research

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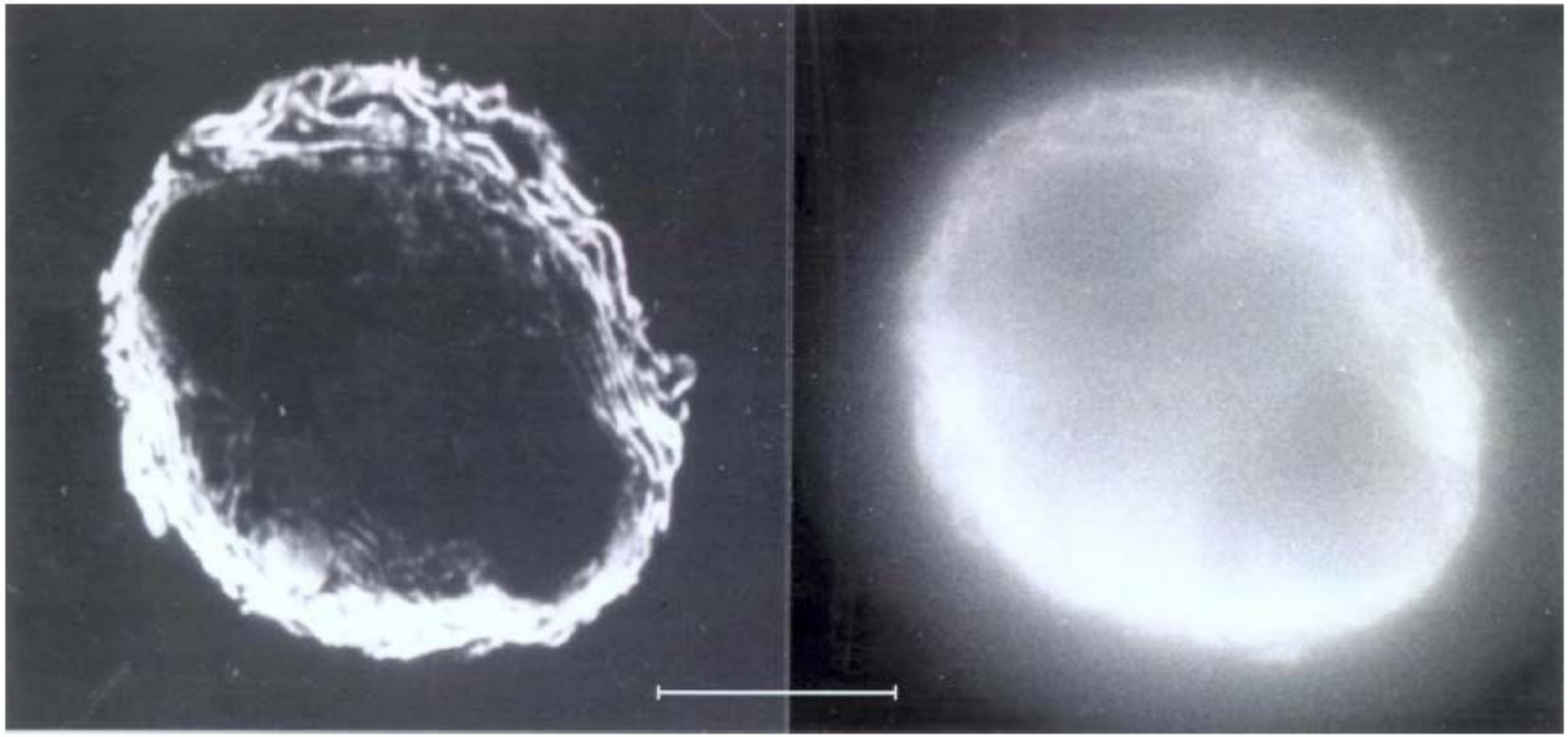
[a] *MRC Laboratory of Molecular Biology, Cambridge, UK*
[b] *Laboratory of Molecular Biology, University of Wisconsin, Madison, USA*

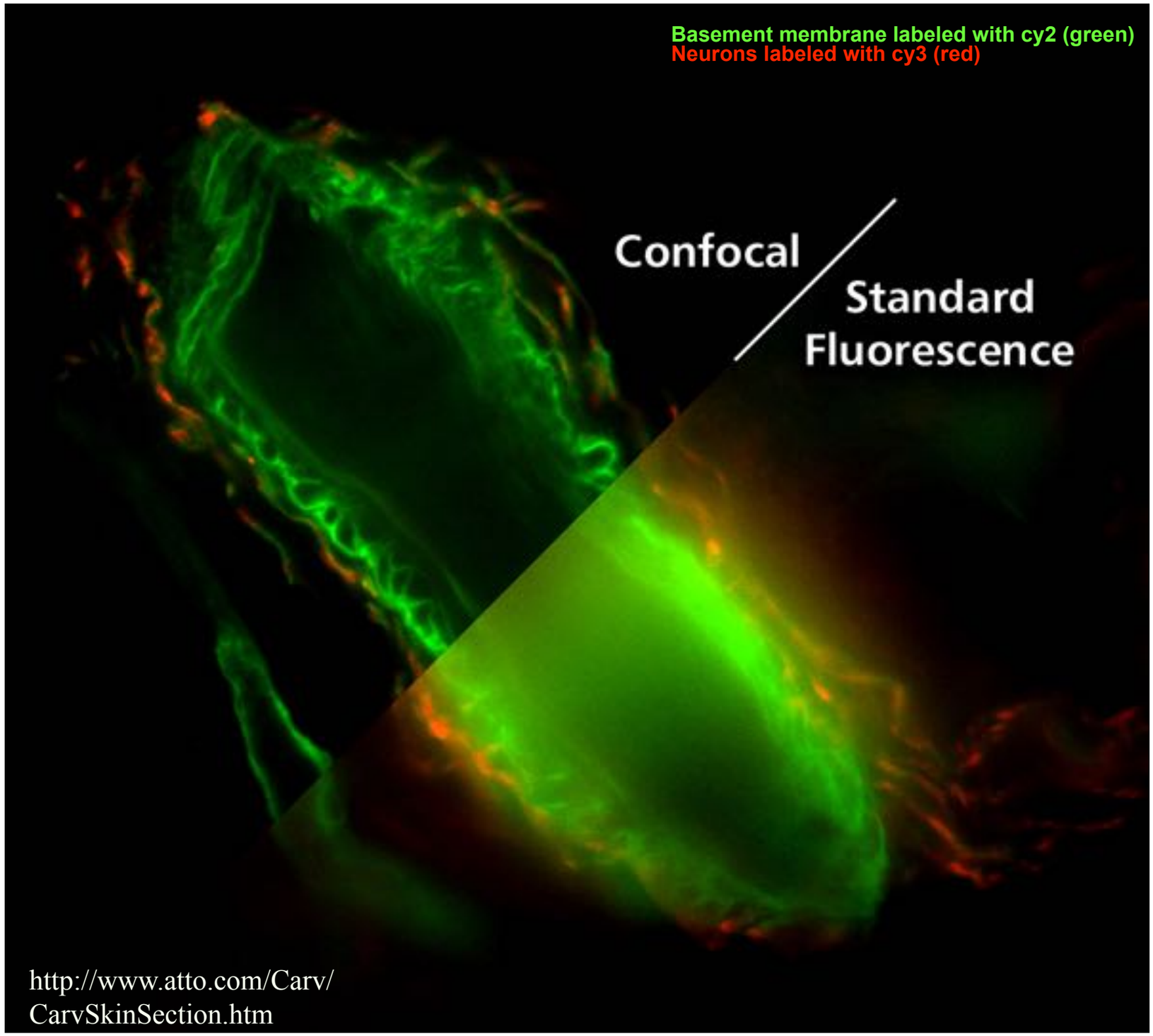
Received 21 May 2003; accepted 27 May 2003

confocal laser scanning microscopy



. An early comparison of a conventional epi-fluorescence image with a confocal one obtained with the polygon scanner. The specimen is a plasmacytoma ... ...belled with anti-endoplasmin, which binds chiefly to cisternae of the endoplasmic reticulum. In the conventional image it is not possible to establish whether the central nucleus lacks endoplasmin and the individual cisternae are unclear. Scale bar = 10 um. (Specimen prepared by Gordon Koch. Image previously published in the Journal of Cell Biology, 105, p44 (1987)).

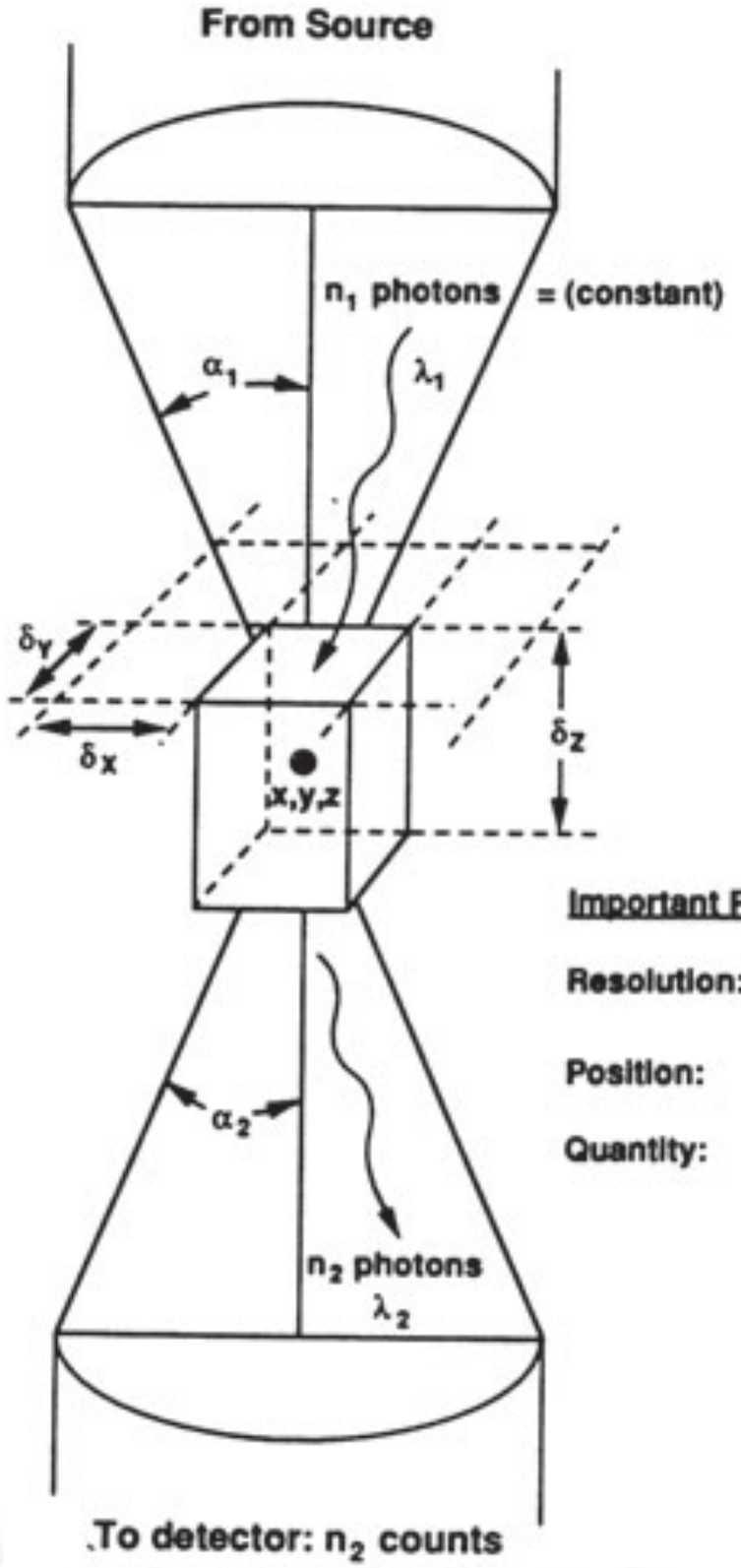
confocal laser scanning microscopy



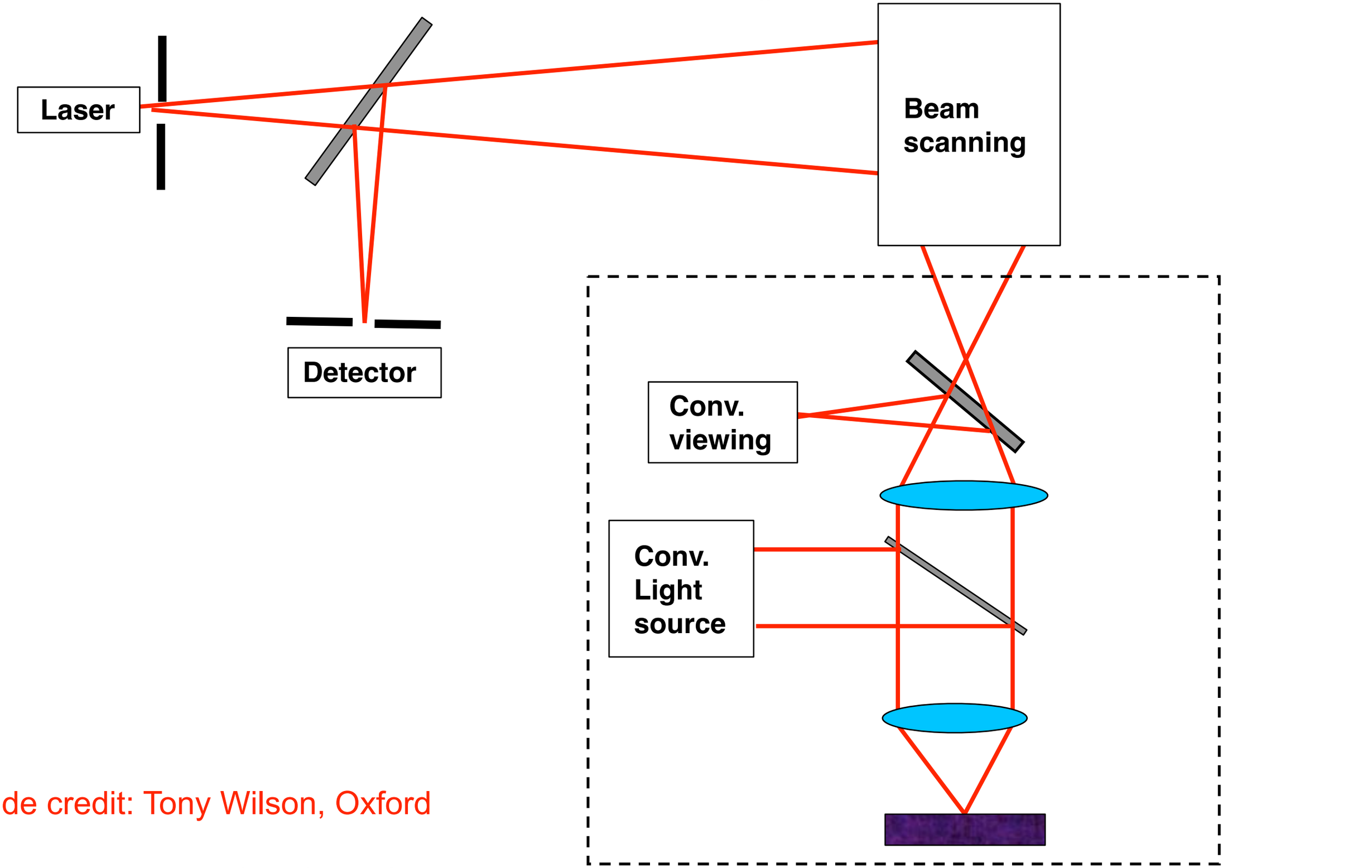
confocal laser scanning microscopy

- Reduced blurring of the image from light scattering
- Increased effective resolution
- Improved signal to noise ratio
- Clear examination of thick specimens
- Z-axis scanning
- Depth perception in Z-sectioned images

Movie credit: Jorge Langowski



confocal laser scanning microscopy



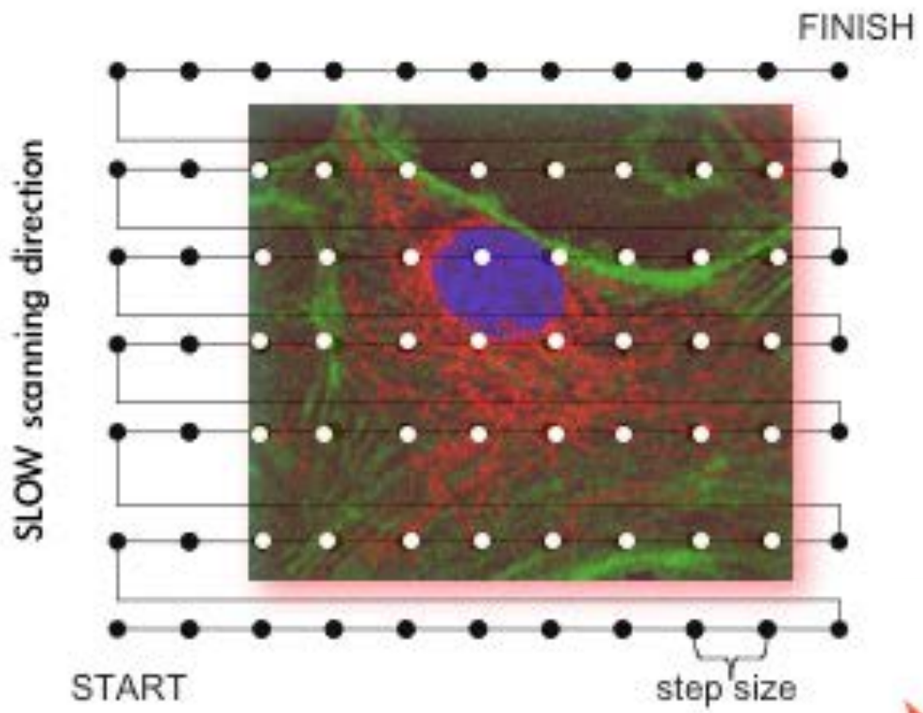
Slide credit: Tony Wilson, Oxford

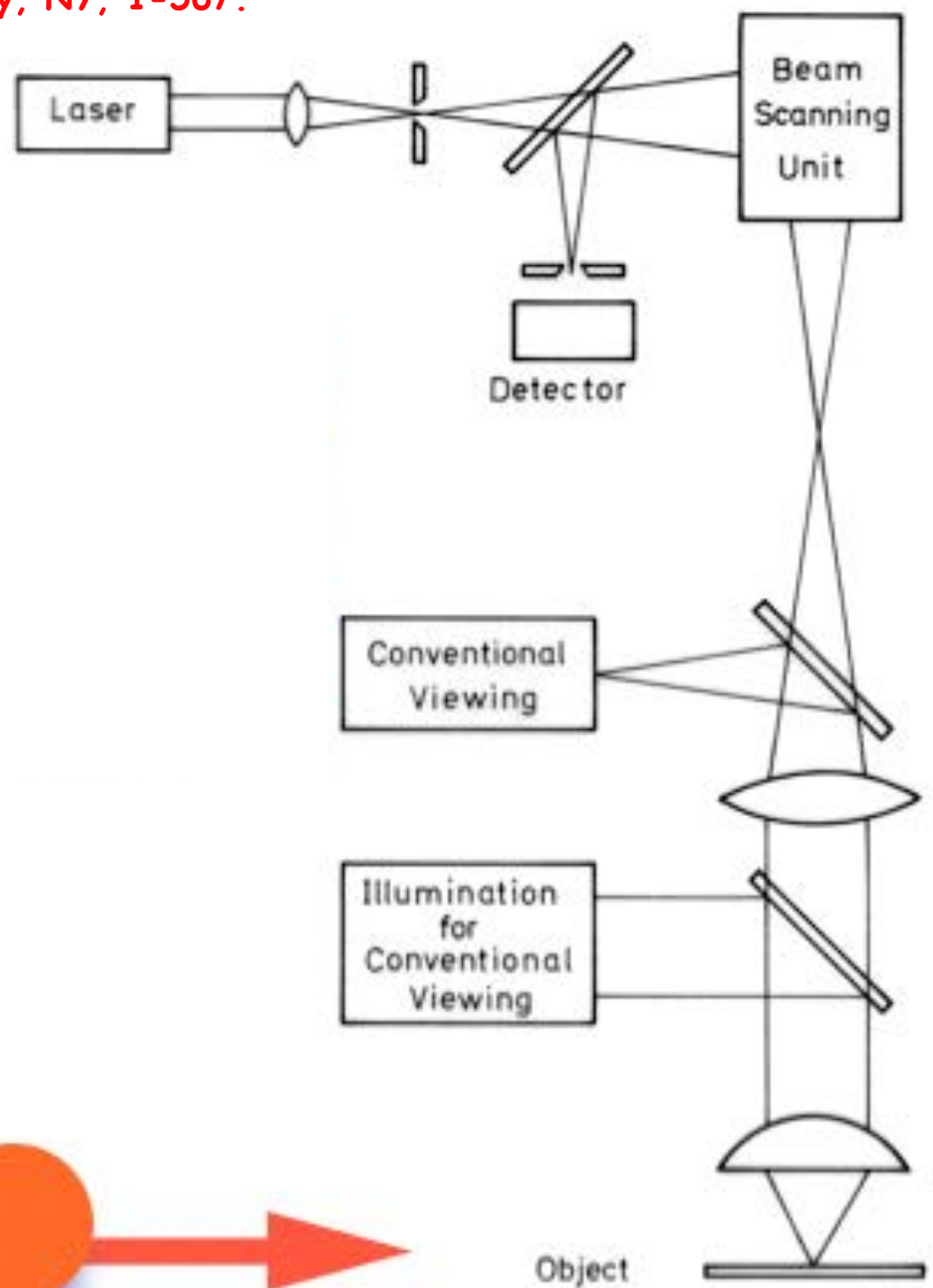
confocal laser scanning microscopy

Giuseppe Pellizza da Volpedo (1868-1907)
Panni al sole (1894), olio su tela, 87x131 cm, Domodossola, collezione privata

confocal laser scanning microscopy

A.Diaspro (ed.), (2001) "Confocal and Two-photon Microscopy. Wiley, NY, 1-567.

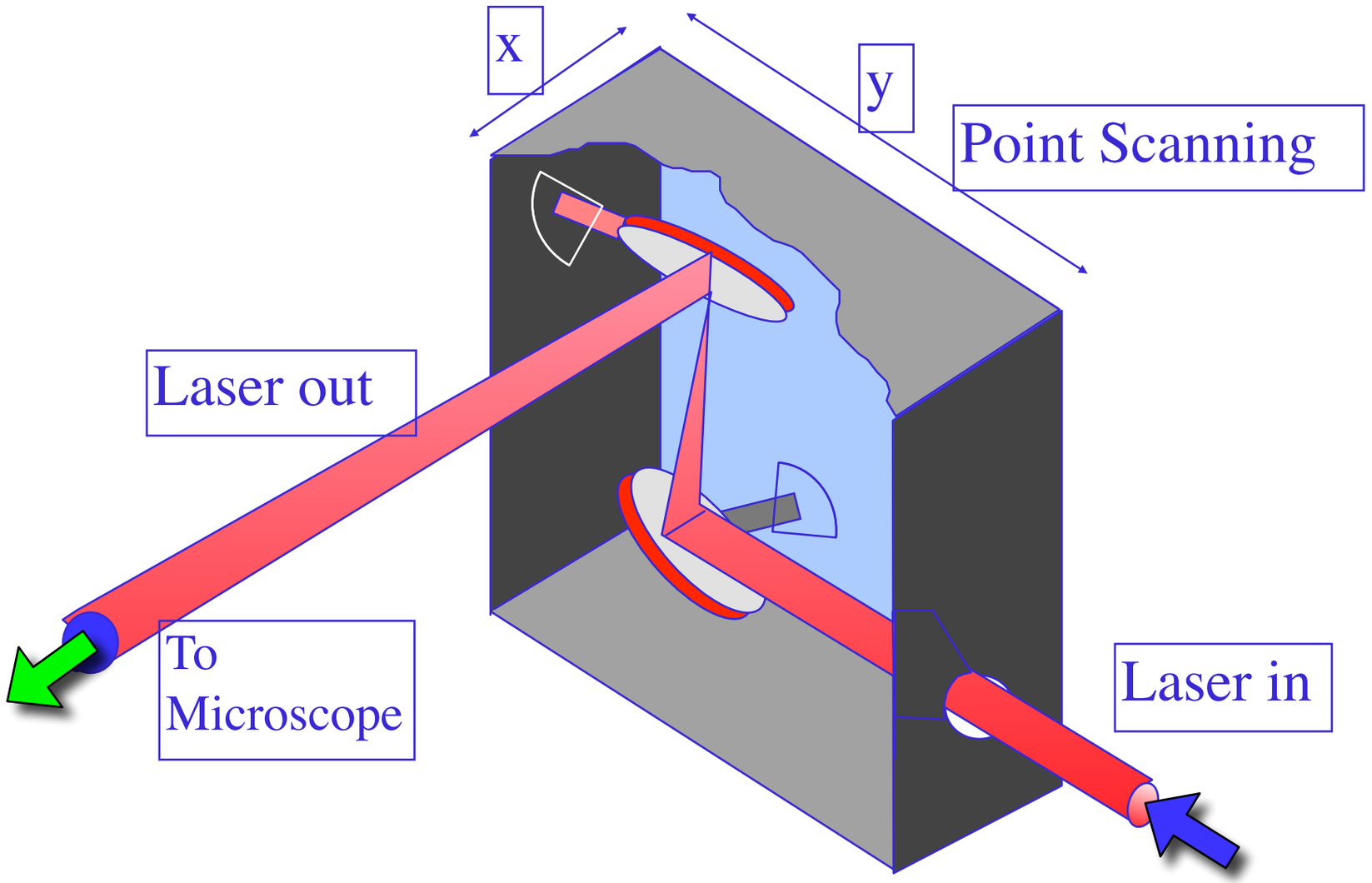


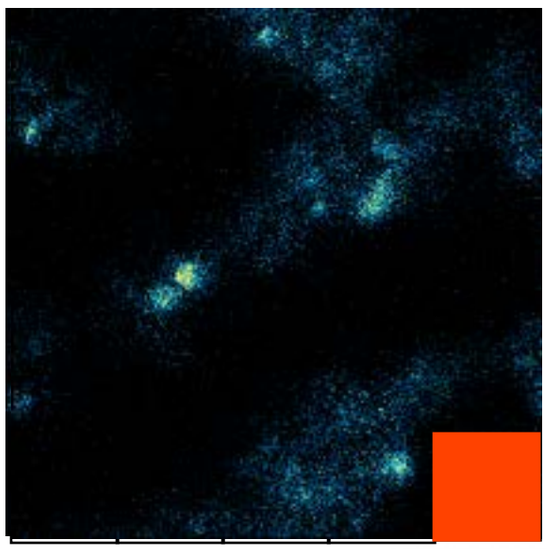


confocal laser scanning microscopy

LIKE A FLORIAN'S COFFE IN VENICE

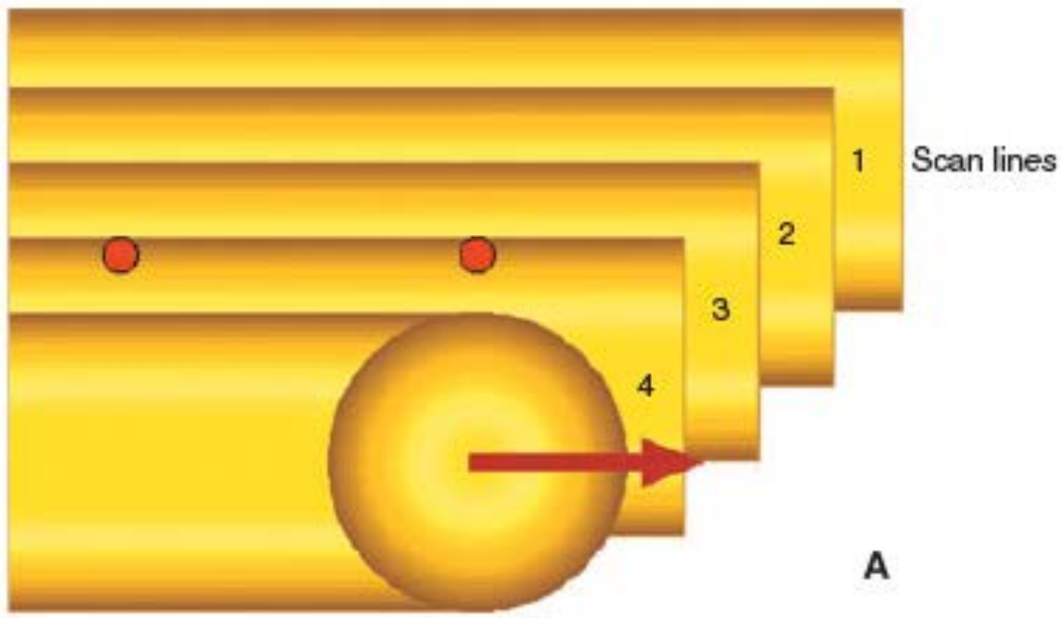
2 ms/line



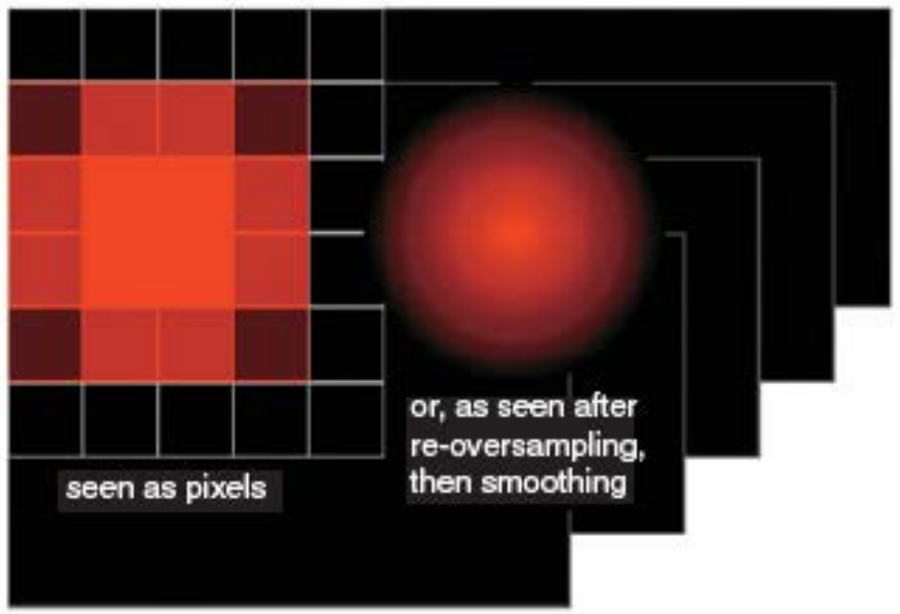


Slide credit: Fabio Cannone

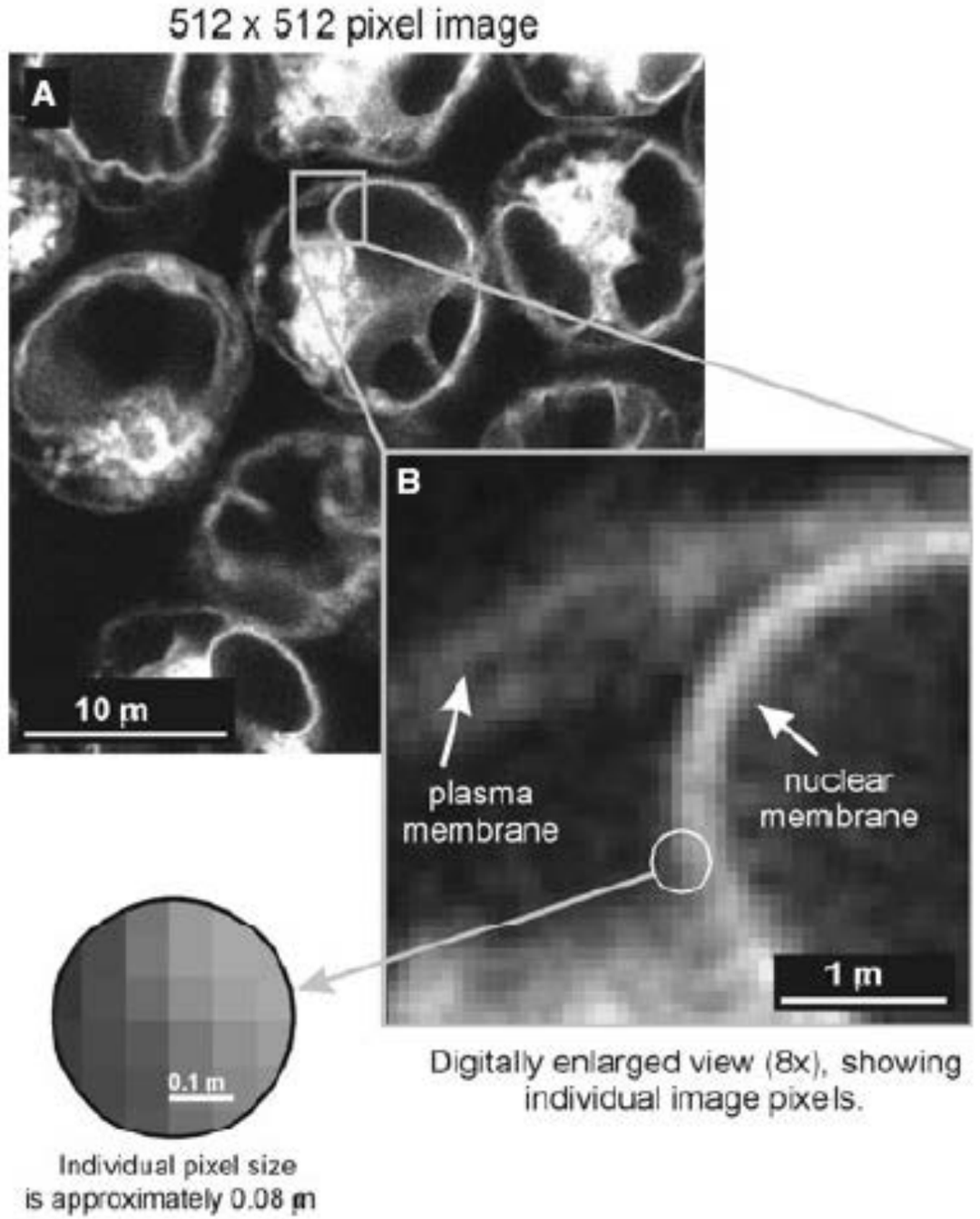
confocal laser scanning microscopy



The geometry of the beam scannning the specimen



The pixels as they appear in red channel of the display C RT B

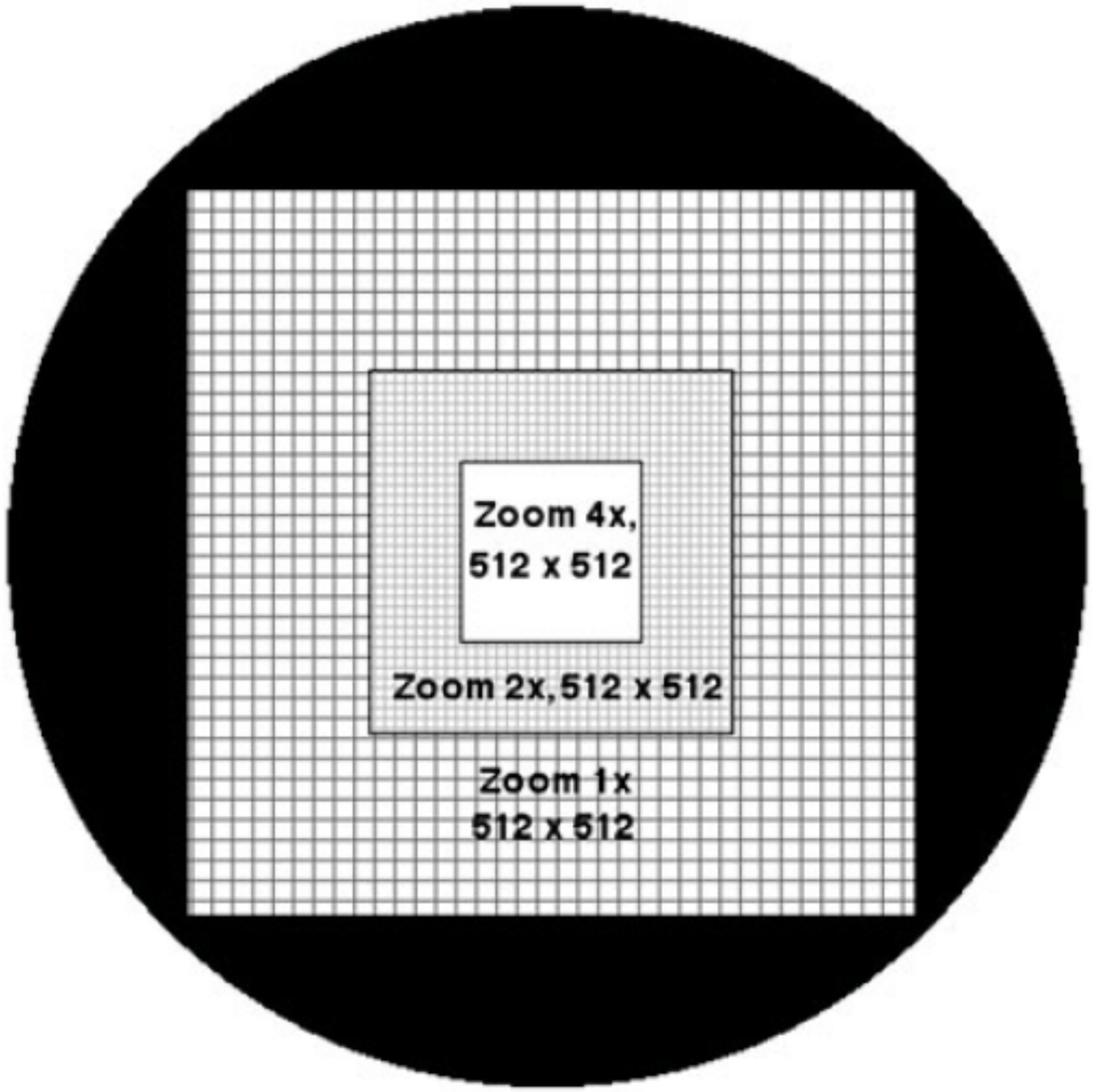


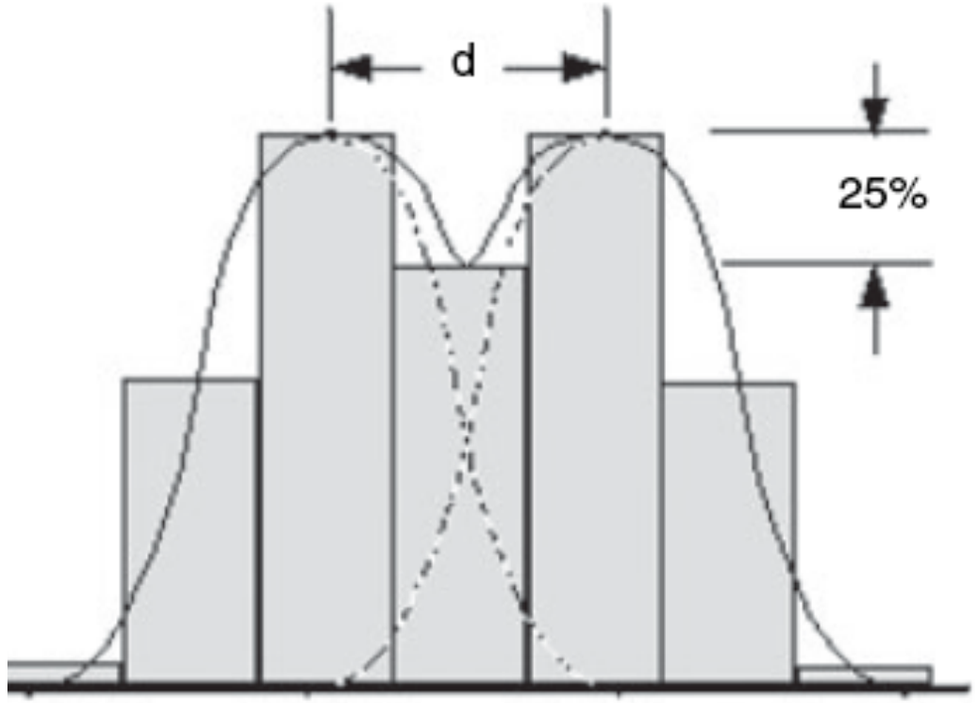
Digitally enlarged view (8x), showing individual image pixels.

Individual pixel size
is approximately 0.08 m

Slide credit: Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

confocal laser scanning microscopy





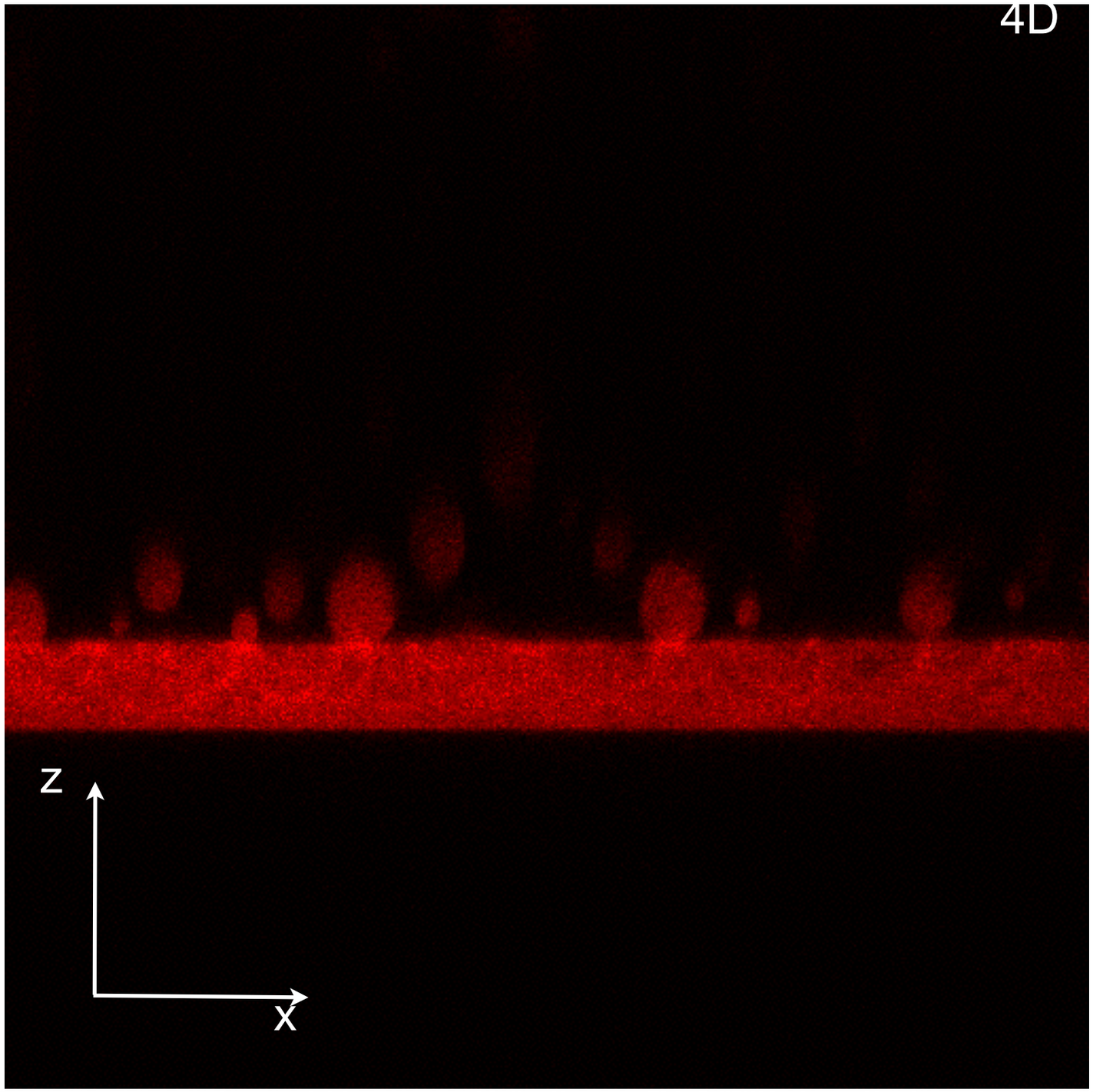
Rayeigh resolution, d,
and Nyquist digitizing

Slide credit: Handbook of Confocal Microscopy (J.Pawley ed.), Plenum, NEW edition, 2006

confocal laser scanning microscopy

POINT BY POINT IMAGING
LIKE A FLORIAN'S COFFE IN VENICE

2 ms/line



Slide credit:Davide Mazza, LAMBS-MicroScoBio, Genova

confocal laser scanning microscopy

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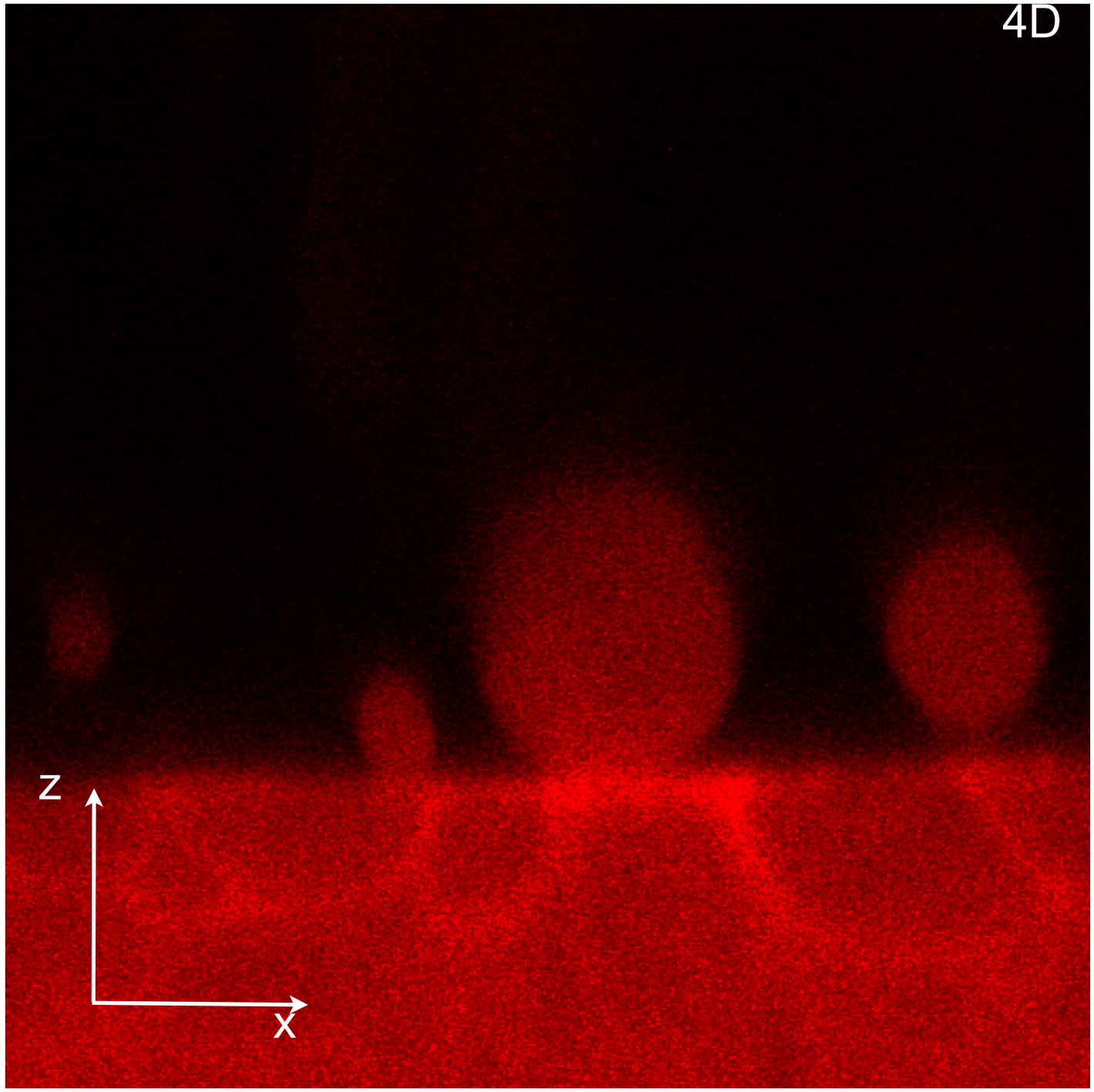
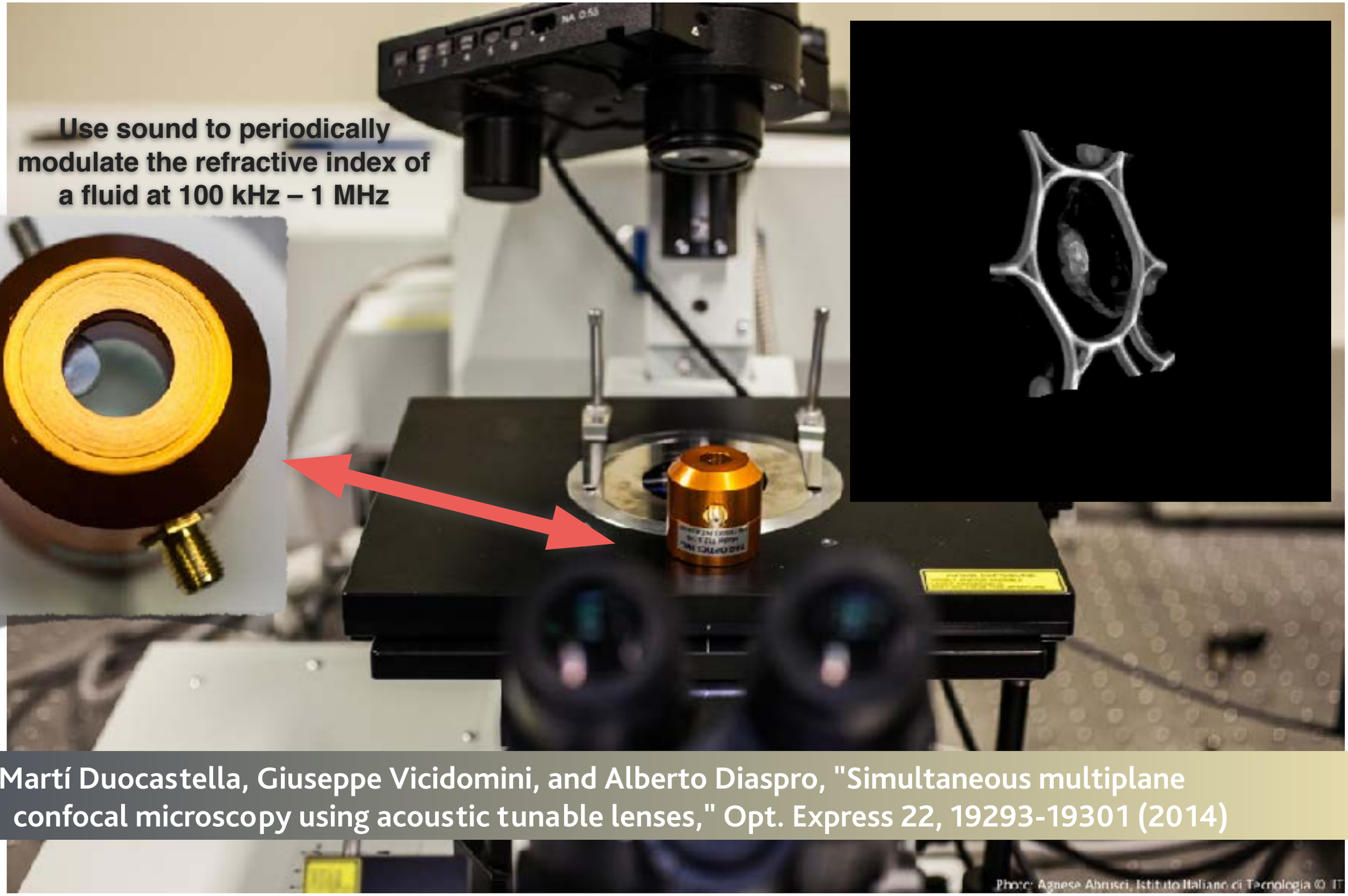


Slide credit:Davide Mazza, LAMBS-MicroScoBio, Genova

3D/4D SUPER FAST Z-SCANNING - liquid lens



Martí Duocastella, Giuseppe Vicidomini, and Alberto Diaspro, "Simultaneous multiplane confocal microscopy using acoustic tunable lenses," Opt. Express 22, 19293-19301 (2014)

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

Ultra-high speed varifocal liquid lens

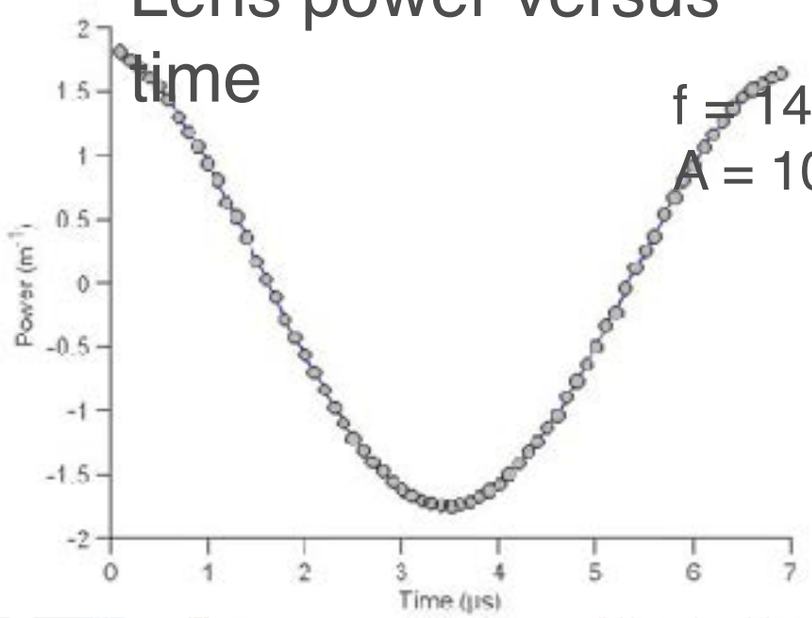
MARTI DUOCASTELLA

TAG lens ➔ Tunable acoustic gradient index of refraction lens

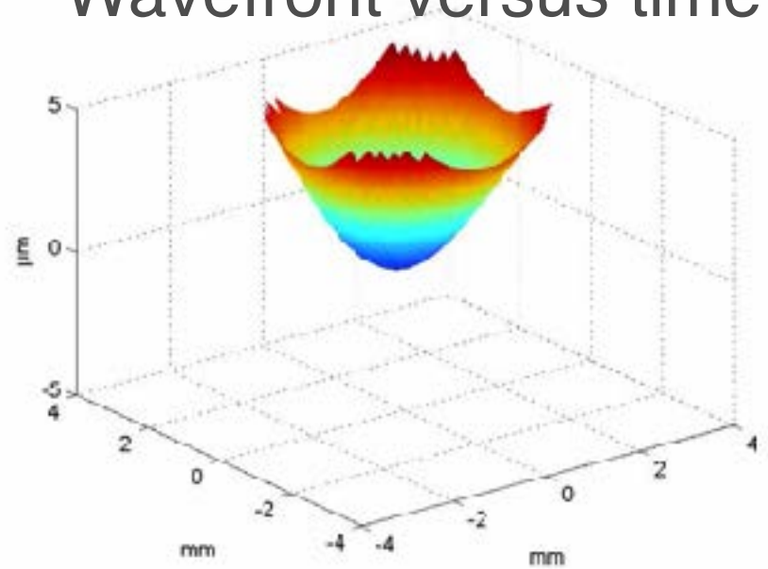
Use sound to periodically modulate the refractive index of a fluid at 100 kHz – 1 MHz

Lens power versus time

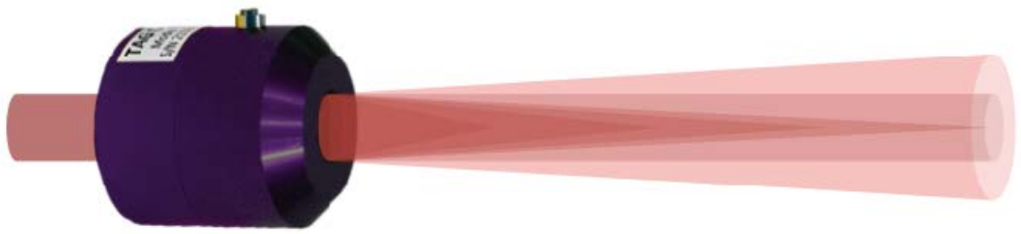
f = 143 kHz
A = 10 V_{pp}



Wavefront versus time



Ultra-high speed varifocal liquid lens



MARTI DUOCASTELLA

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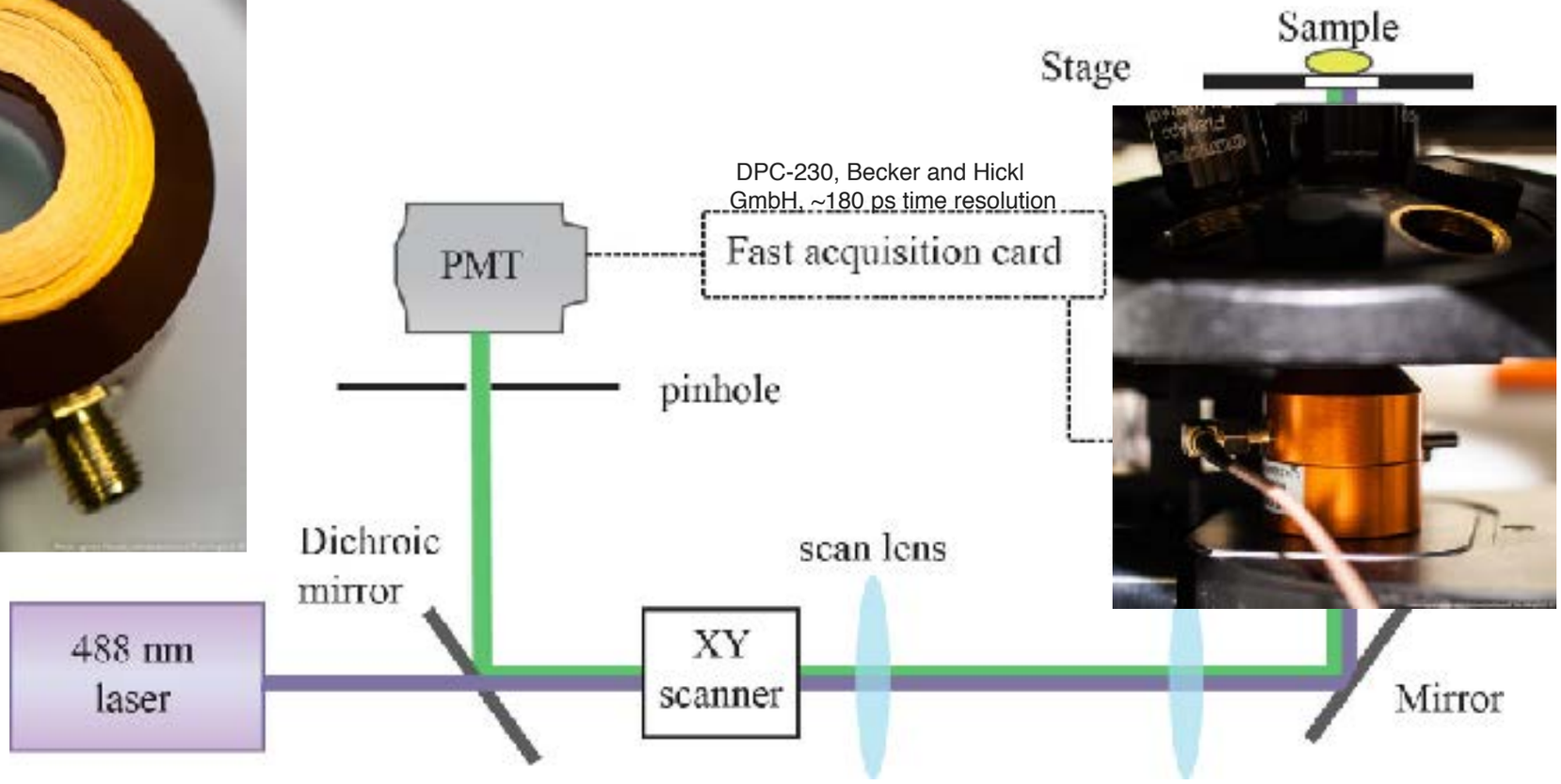
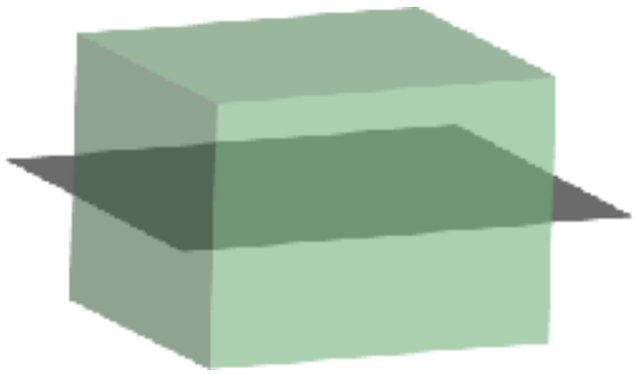


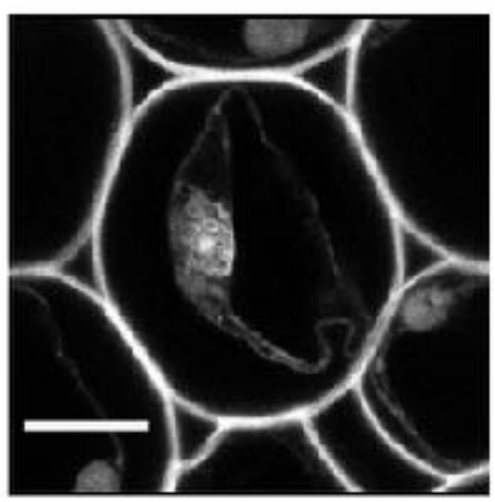
Opt. Express 22, 19293-19301 (2014)

Multiplane imaging

In a single XY scan…

Without the TAG lens

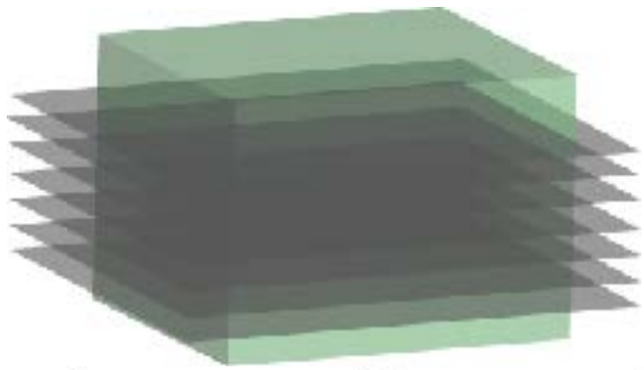


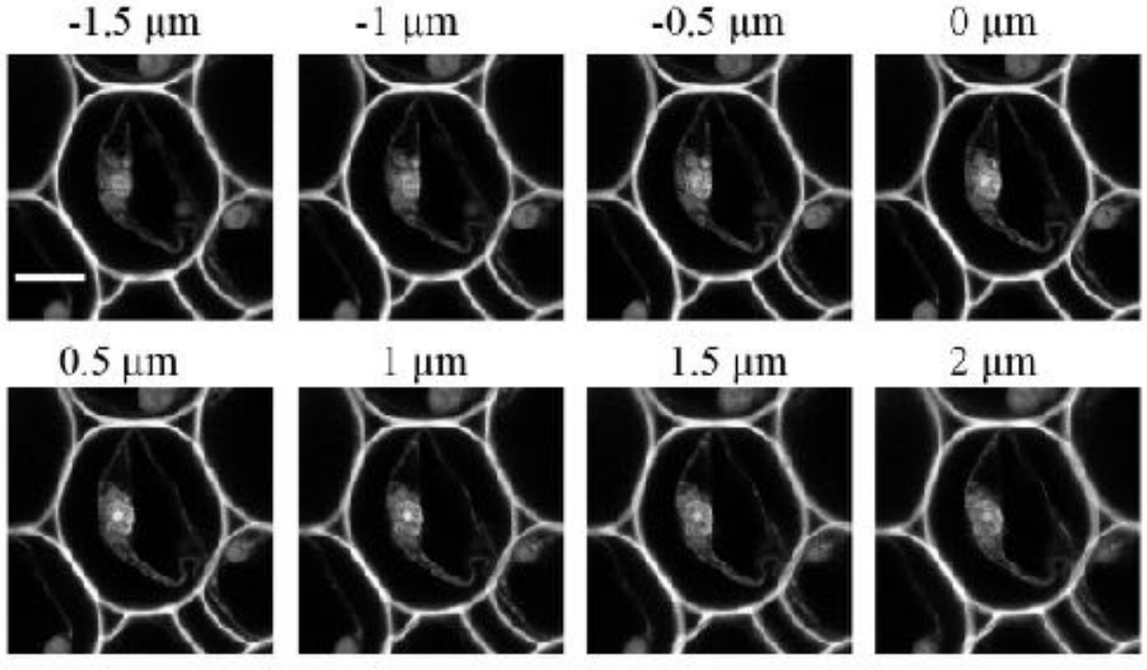


One single plane

With the TAG lens

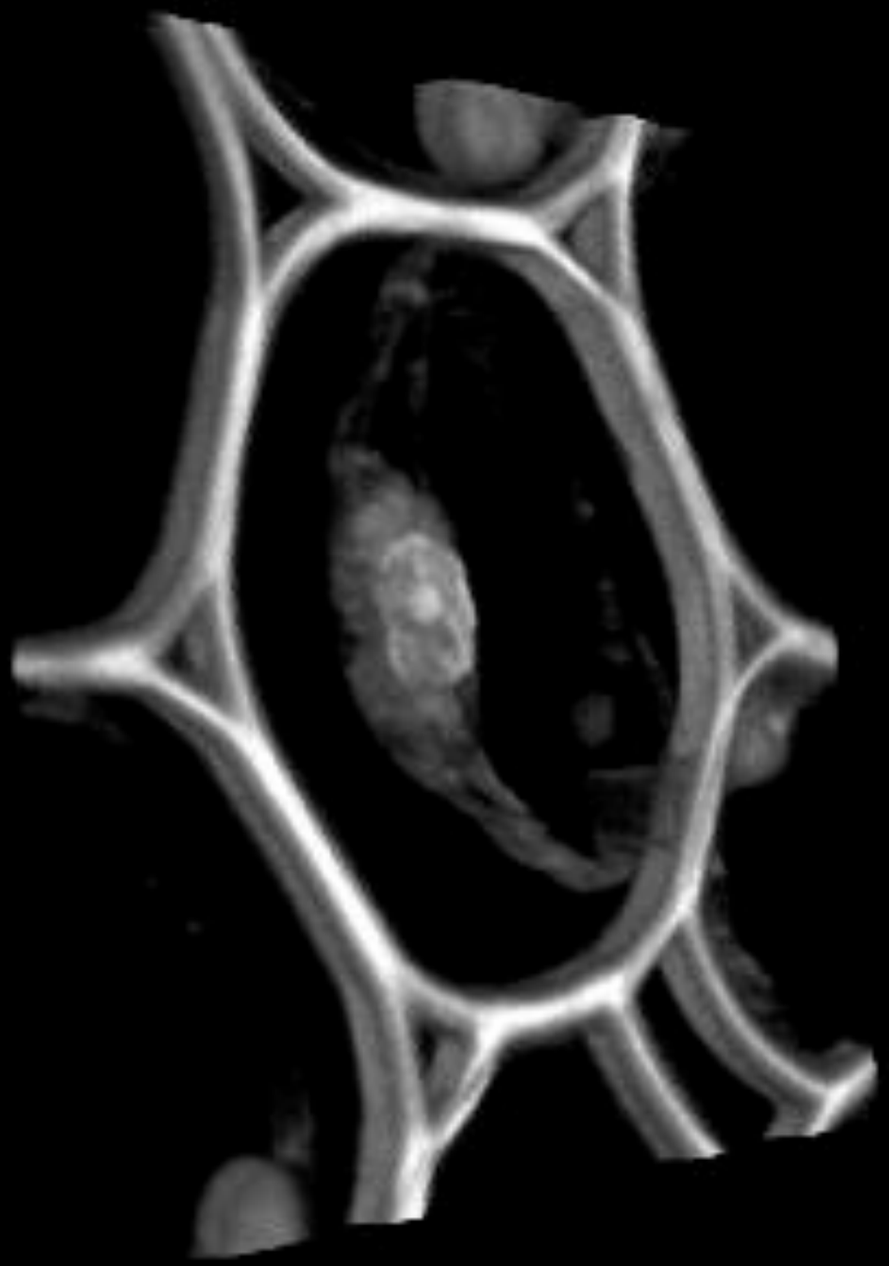
Multiple planes, only limited by SNR





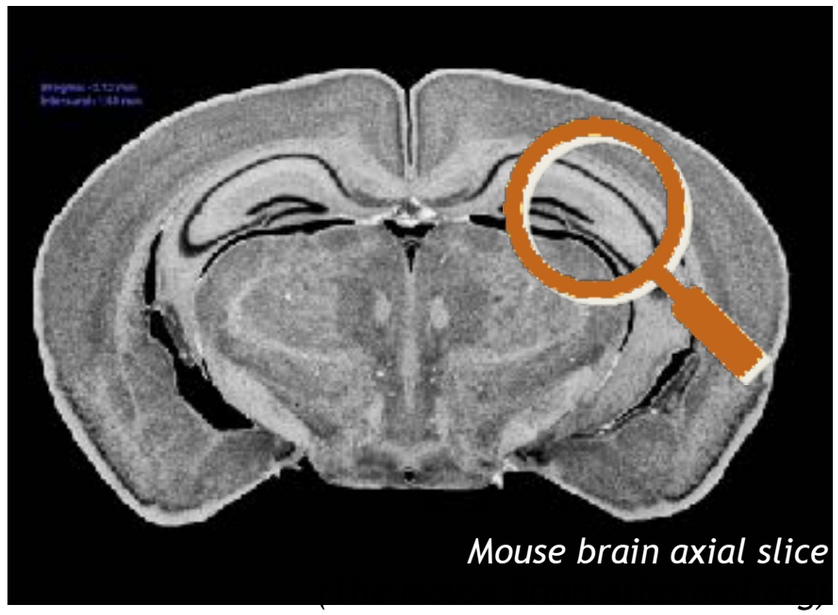
Martí Duocastella, Giuseppe Vicidomini, and Alberto Diaspro, "Simultaneous multiplane confocal microscopy using acoustic tunable lenses," **Opt. Express 22, 19293-19301 (2014)**

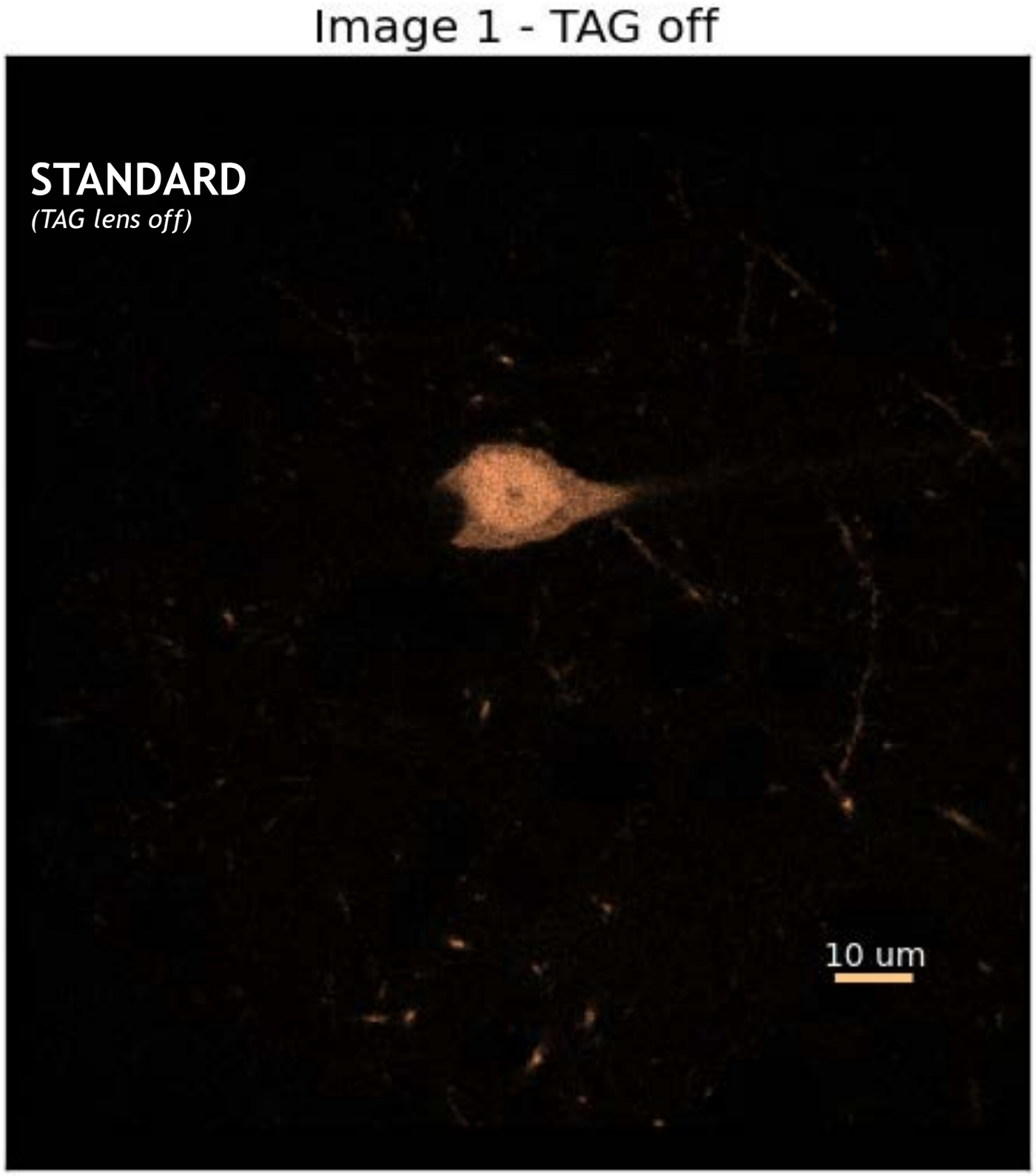
Martí Duocastella, Giuseppe Vicidomini, and Alberto Diaspro, "Simultaneous multiplane confocal microscopy using acoustic tunable lenses," Opt. Express **22**, 19293-19301 (2014)



Volumetric imaging of neurons:

- mouse brain axial slice
  150um thick



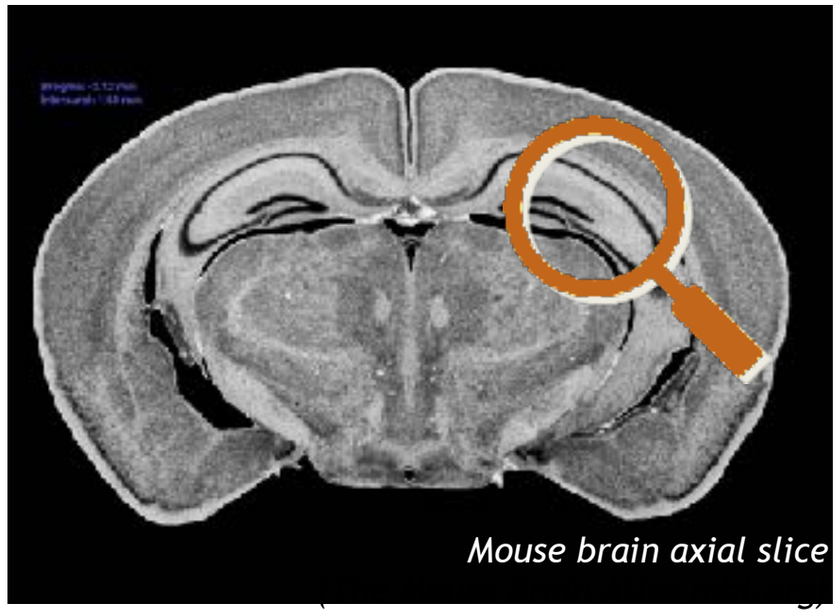


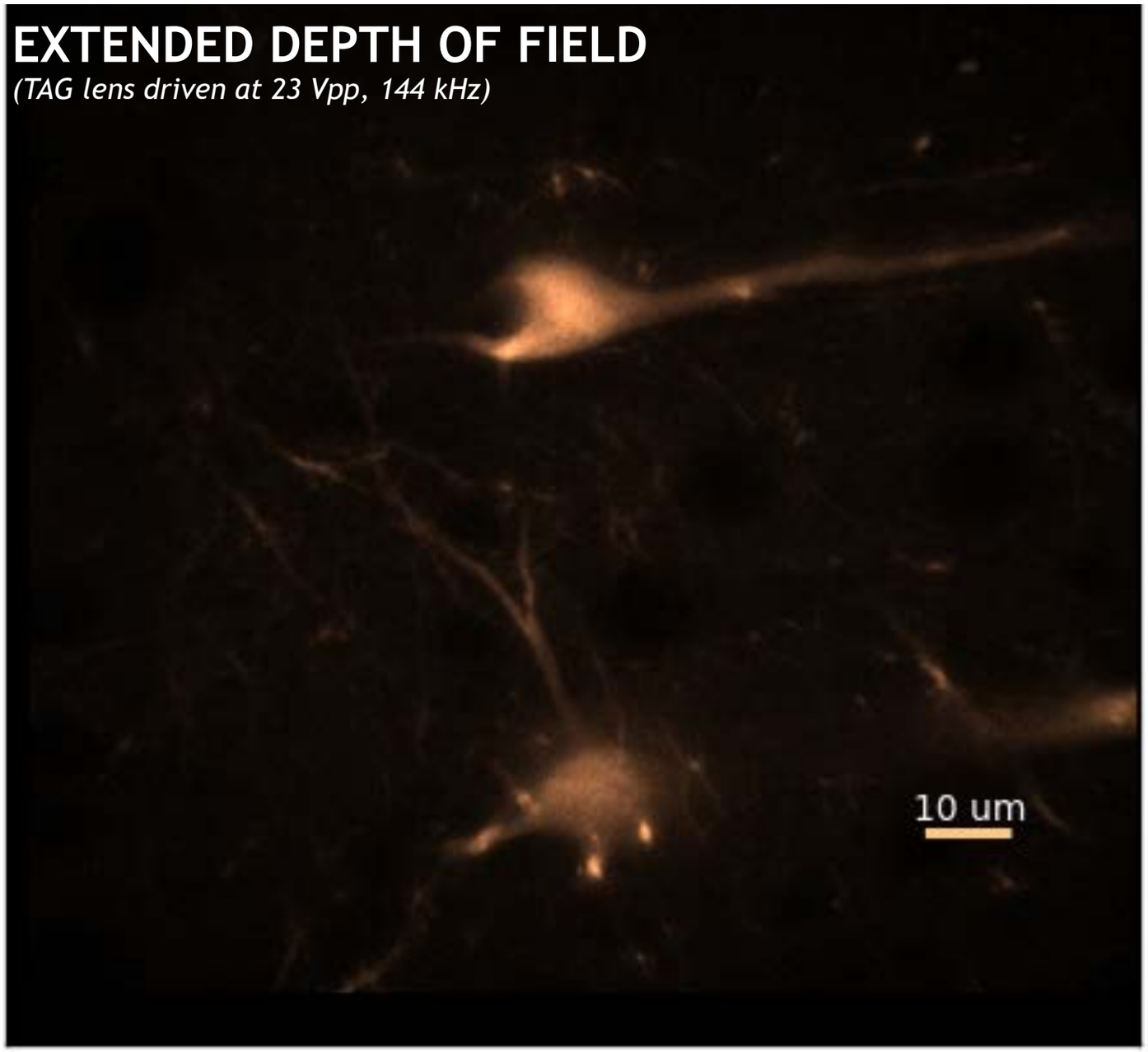
slide credit: Simonluca Piazza, Diaspro Lab, IIT

Image 1 - TAG 23 V*pp*, 144 kHz

Volumetric imaging of neurons:

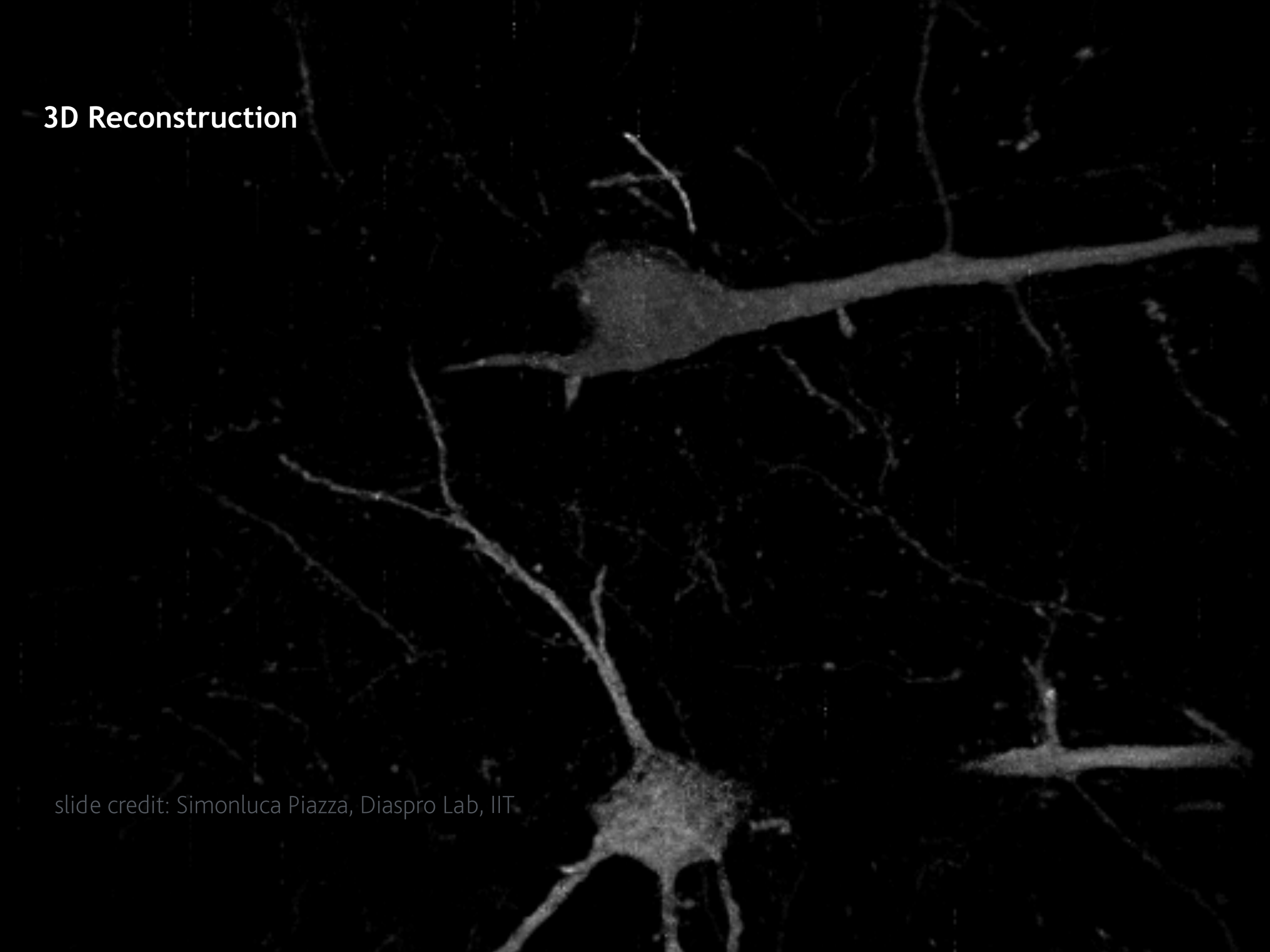
- mouse brain axial slice
  150um thick





slide credit: Simonluca Piazza, Diaspro Lab, IIT

3D Reconstruction



slide credit: Simonluca Piazza, Diaspro Lab, IIT

SPIM - liquid lens

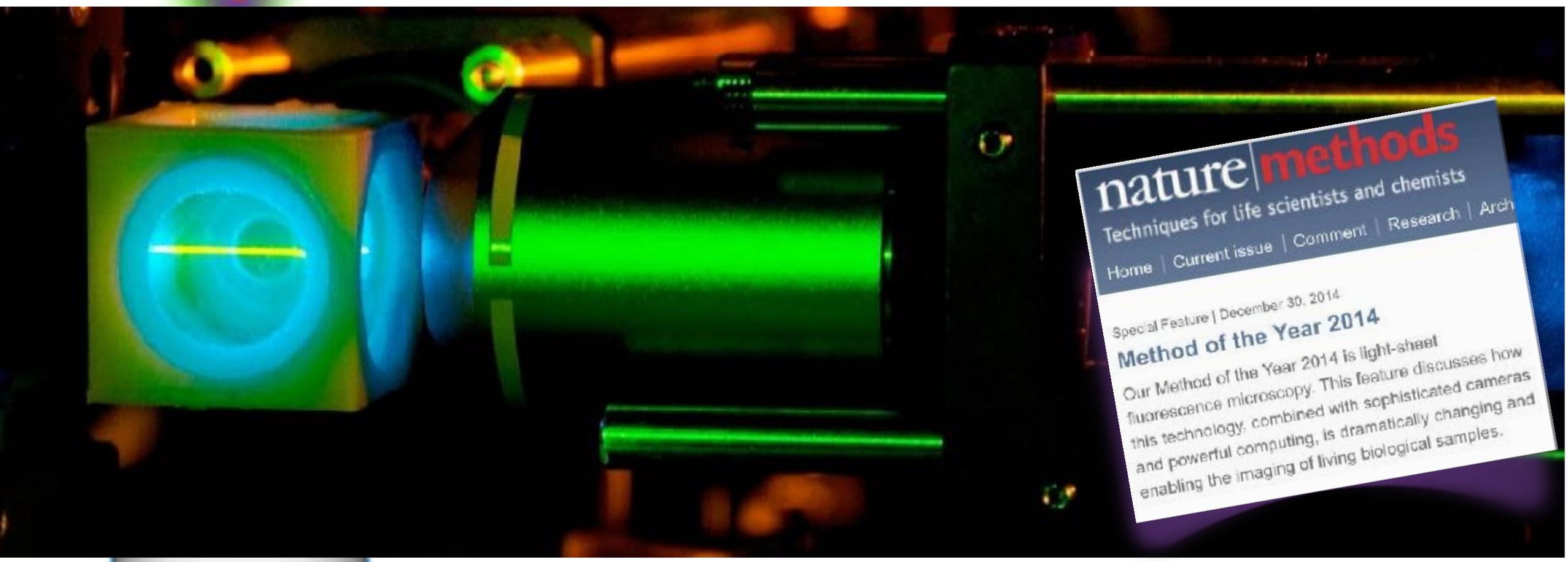
Paolo Bianchini, Diaspro Lab, set-up 2016

Collaboration with Peter Saggau,

ALLEN INSTITUTE for BRAIN SCIENCE

Ducros M et al, (2013) ***Proc Natl Acad Sci U S A*** 110(32):13138-13143.
Diaspro, A. (2013) **Microsc. Res. Tech.** 76: 985–987.

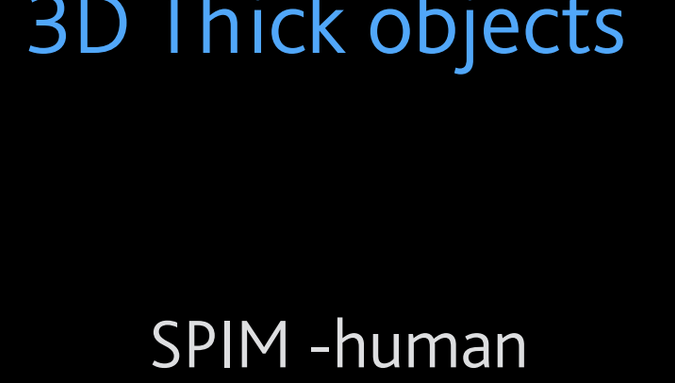
“less lazy”…3D


nature methods
Techniques for life scientists and chemists
Home | Current issue | Comment | Research | Arch
Special Feature | December 30, 2014
Method of the Year 2014
Our Method of the Year 2014 is light-sheet
fluorescence microscopy. This feature discusses how
this technology, combined with sophisticated cameras
and powerful computing, is dramatically changing and
enabling the imaging of living biological samples.

3D Thick objects

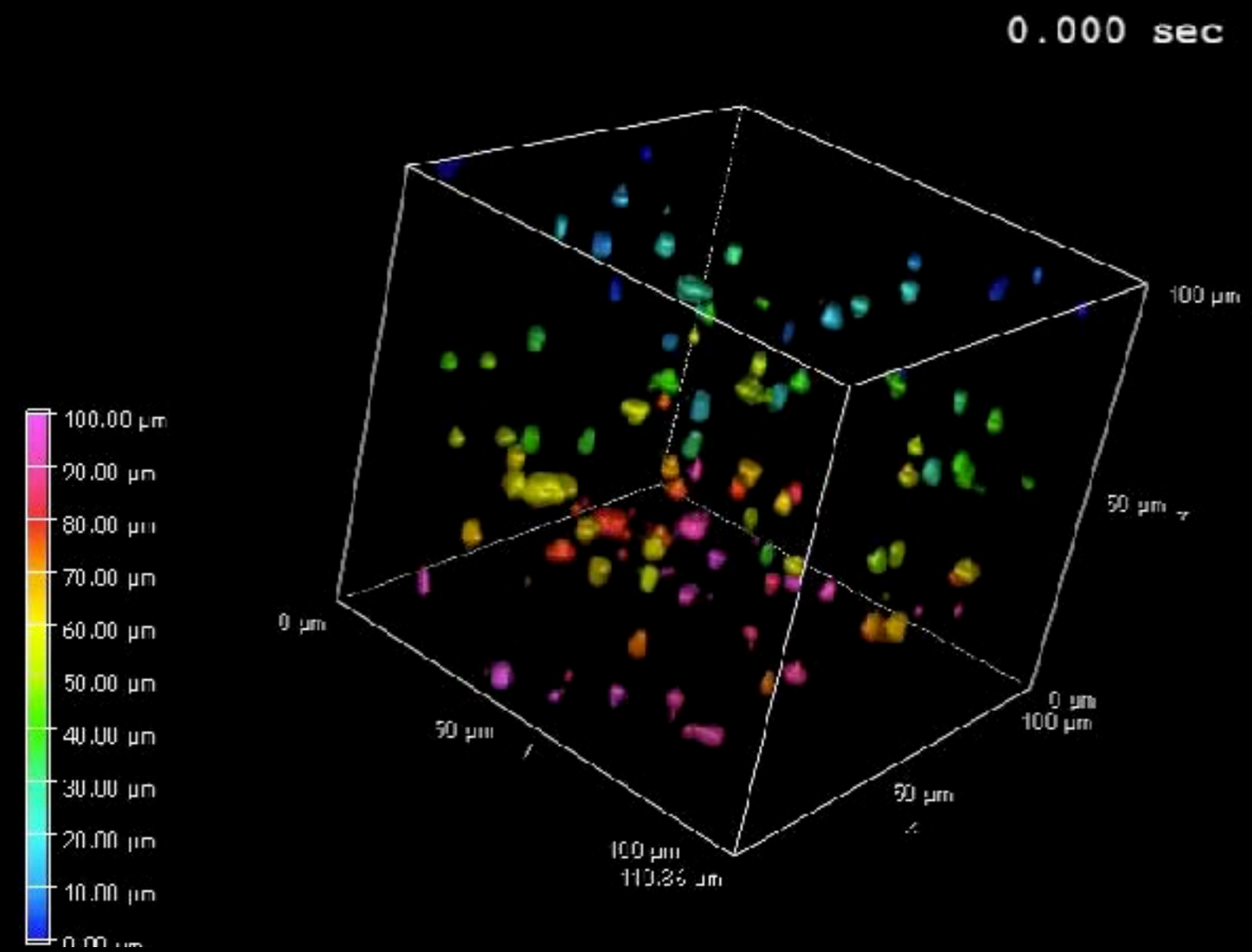
SPIM -human mammary MCF10A cell spheroid

Sample credit: Laura Furia, Mario Faretta - IEO Milan

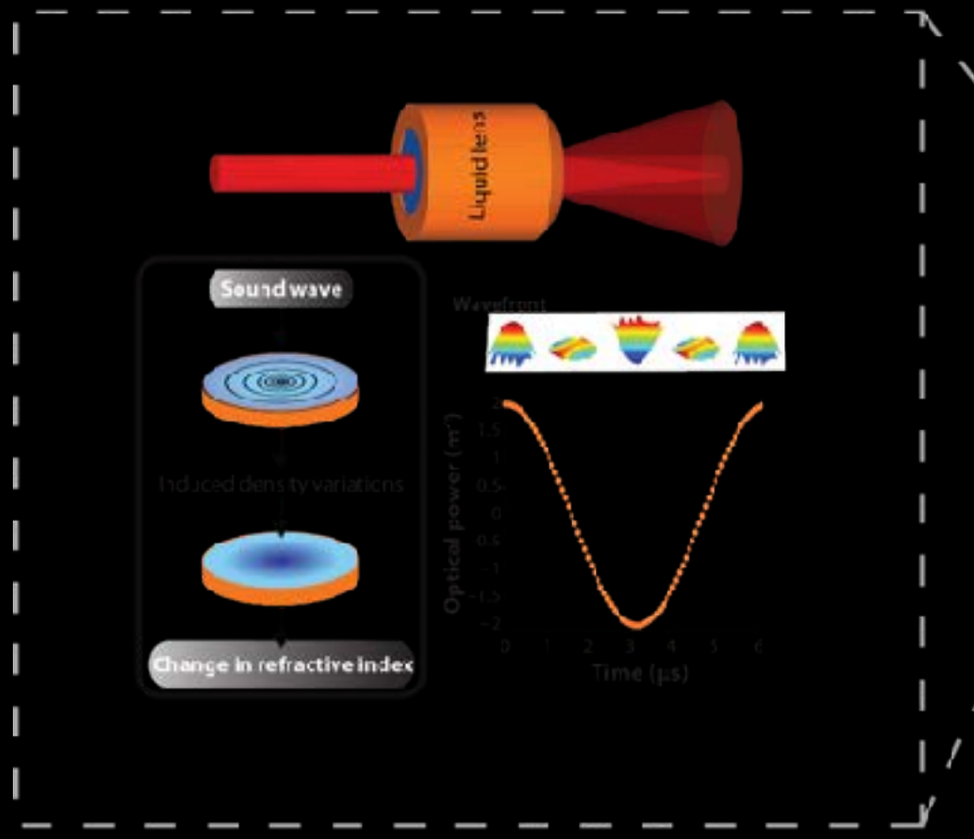


Credits: Zeno Lavagnino, Francesca Cella Zanacchi - Diaspro Lab, IIT

200 Volumes per Second
10000 Frames per Second
600 · 10^6 Voxels per Second

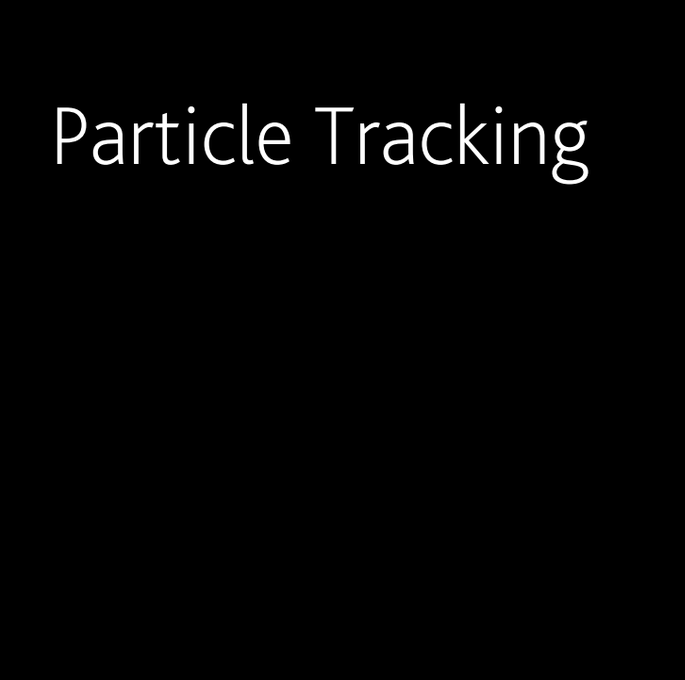


SPIM - liquid lens

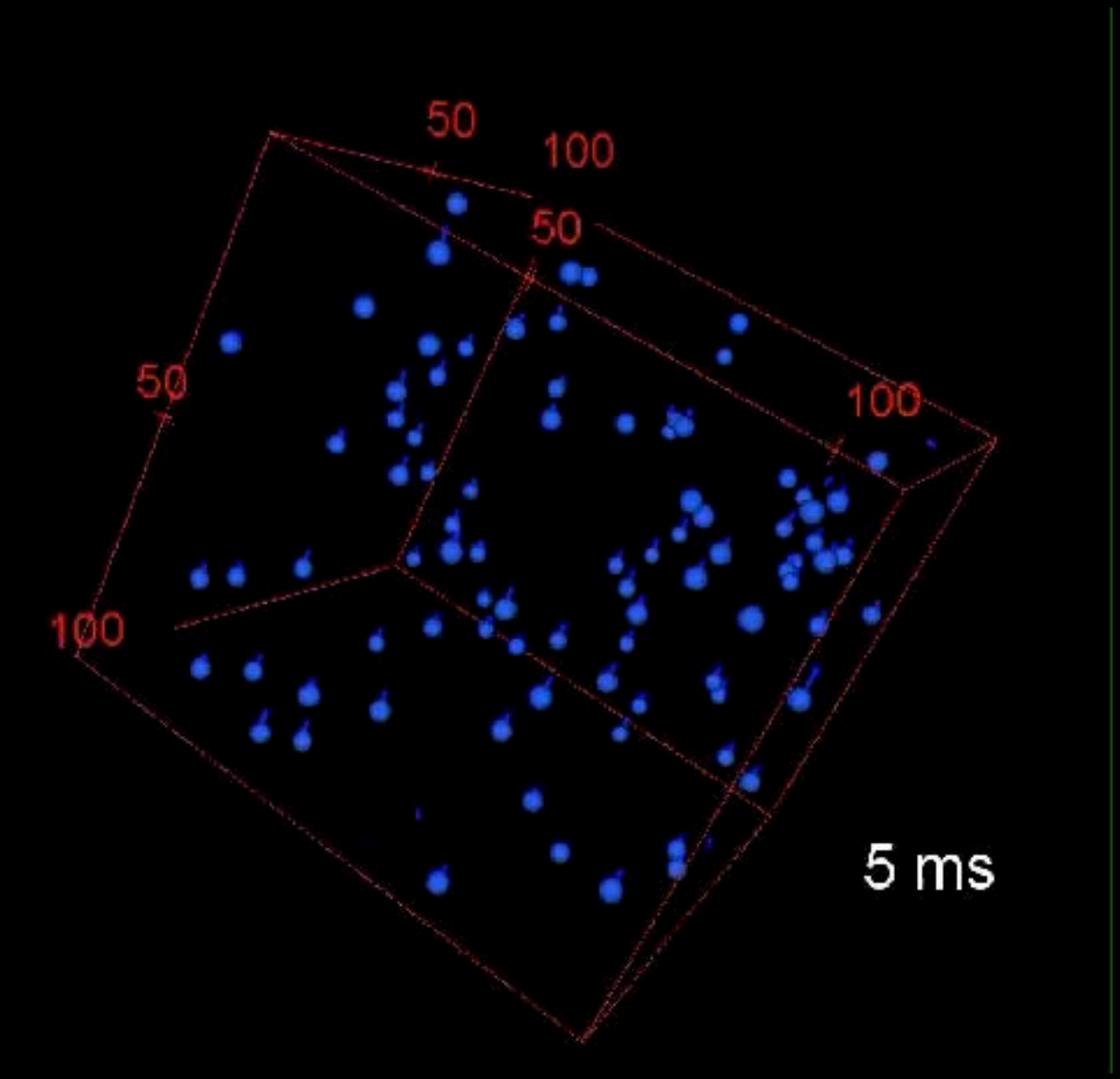


Credits: Giuseppe Sancataldo, Paolo Bianchini, Marti Duocastella - Diaspro Lab, IIT - 2016

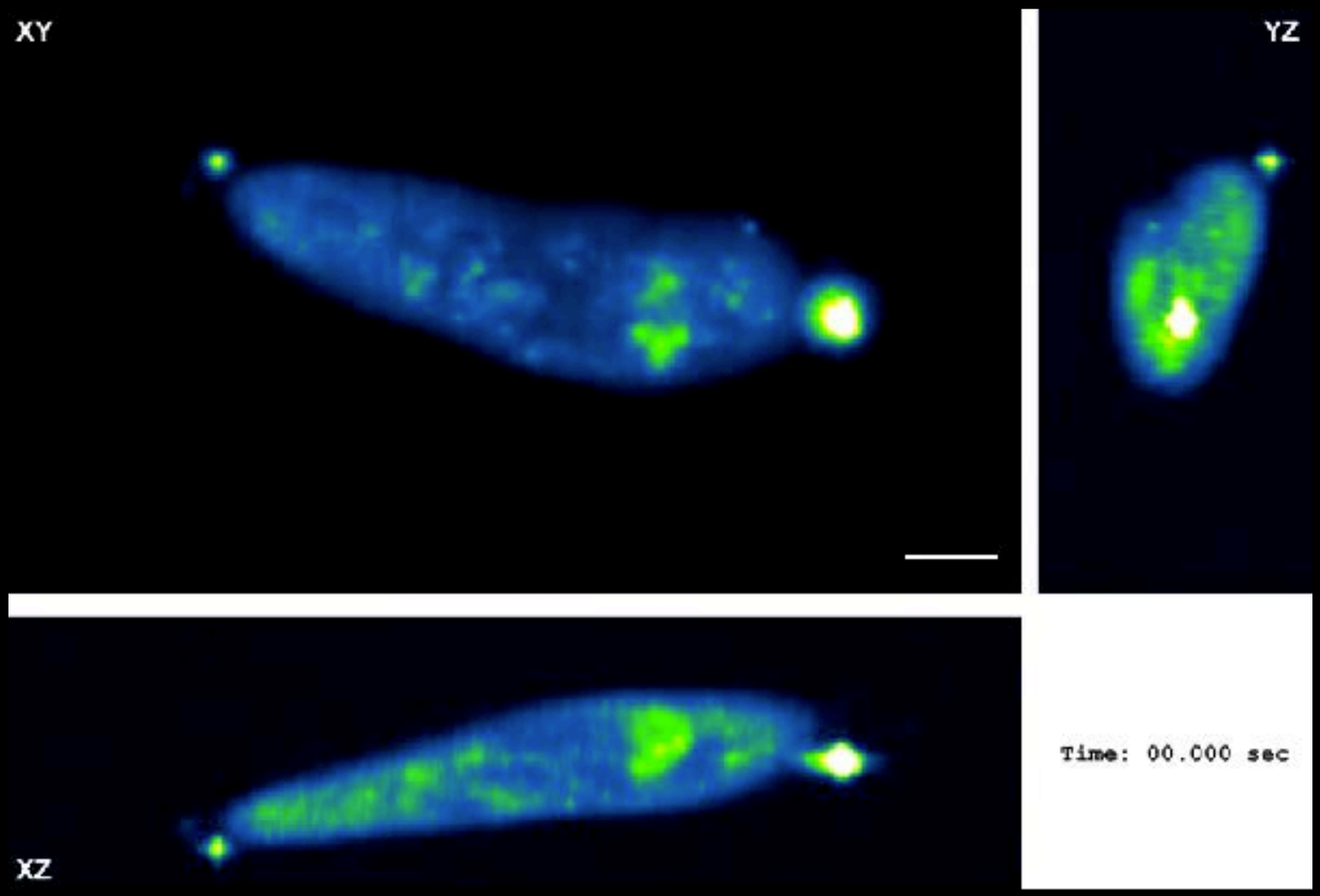
Particle Tracking

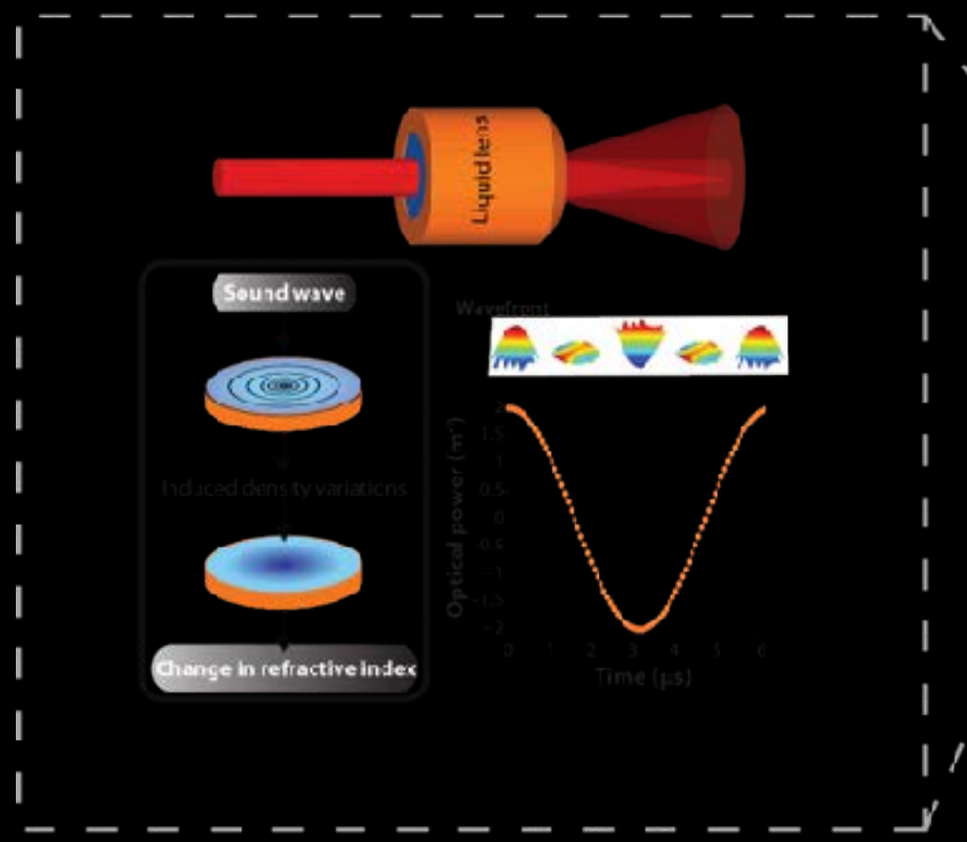


Diffusion of GABA receptor in living neuron



Credit: Giuseppe Sancatldo, Diaspro Lab, 2017





- Volume Size 138×138×60 µm^3 (XYZ)
- 11 Volumes/Second
- 333 Frame/Second
- 3 ms/frame
- 30 planes/Volume
- Nile Red Fluorescence Signal
- Andor NEO 5.5 camera
- Sample credit: Paola Ramoino

Credits: Giuseppe Sancataldo, Paolo Bianchini, Marti Duocastella - Diaspro Lab, IIT - 2016

Simultaneous Planes

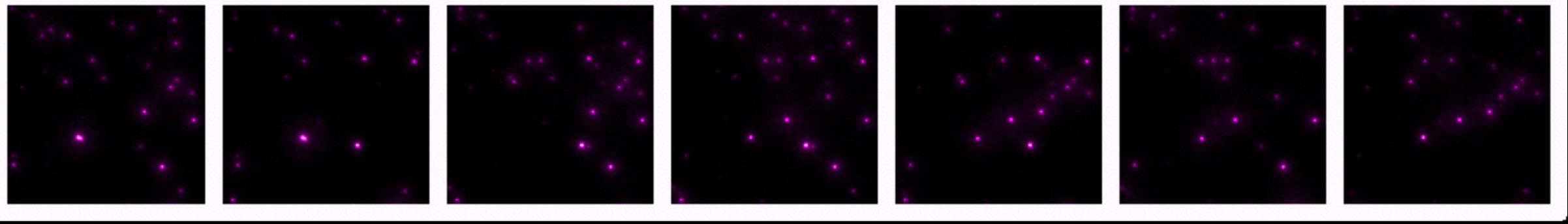
Diaspro Lab - Sancataldo at al., *2017*

Seven Simultaneous Planes

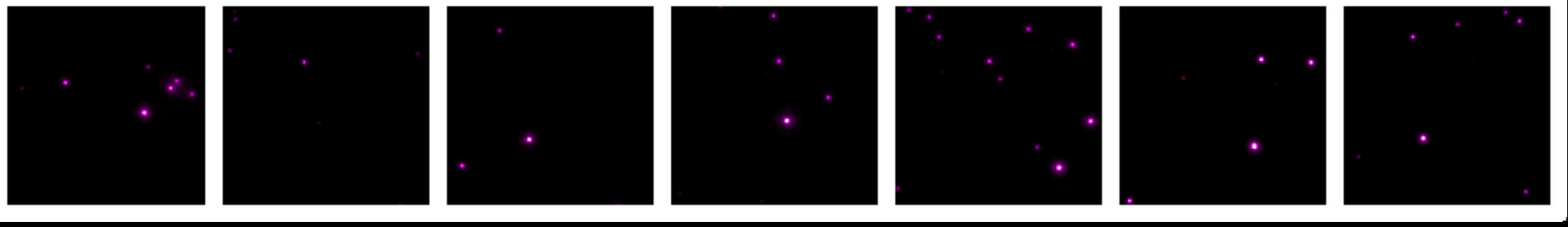
1 LIGHT ON
0 LIGHT OFF

$$\begin{bmatrix} 1 & 0 & 1 & 0 & 1 & 0 & 1 \\ 0 & 1 & 1 & 0 & 0 & 1 & 1 \\ 1 & 1 & 0 & 0 & 1 & 1 & 0 \\ 0 & 0 & 0 & 1 & 1 & 1 & 1 \\ 1 & 0 & 1 & 1 & 0 & 1 & 0 \\ 0 & 1 & 1 & 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 1 & 0 & 0 & 1 \end{bmatrix}$$

ENCODED

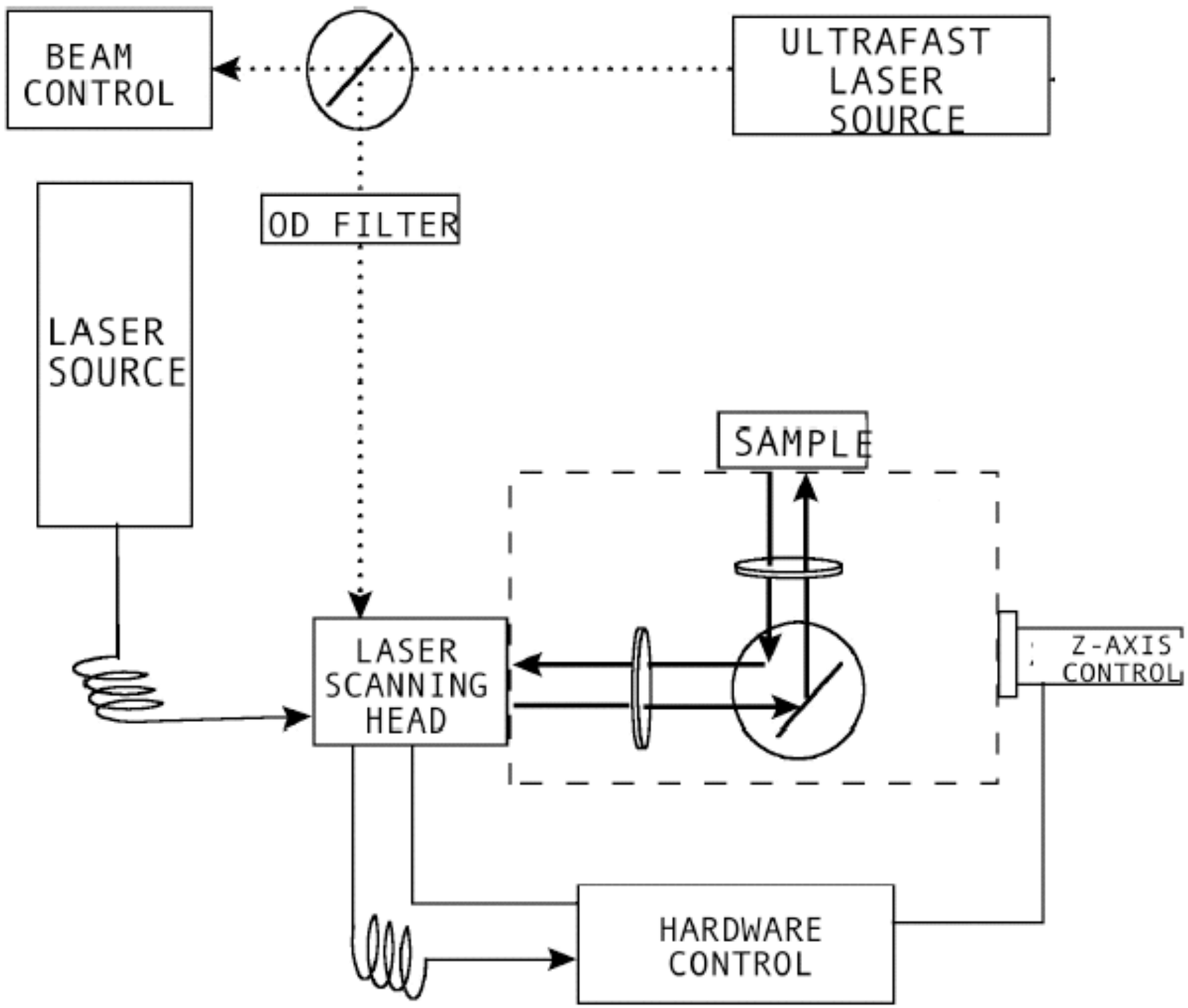


DECODED



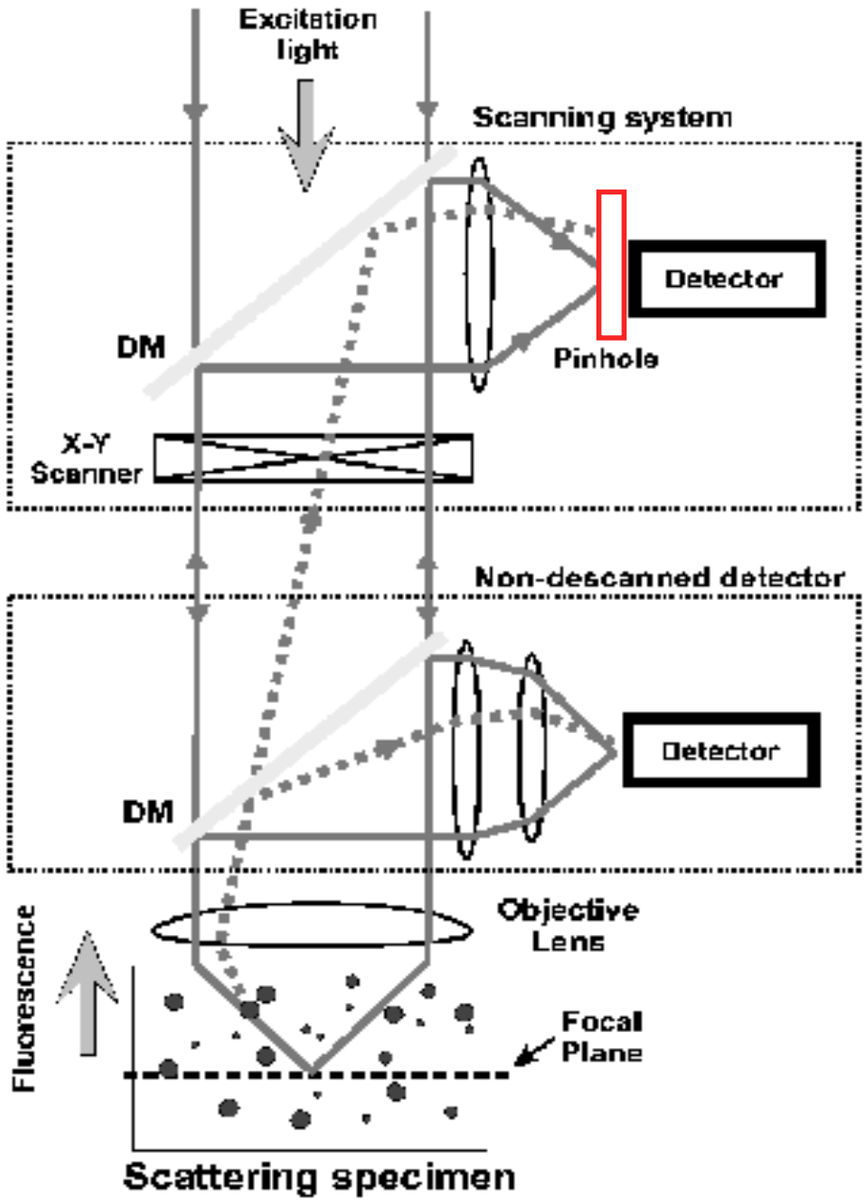
Diaspro Lab - Sancataldo at al., *2017*

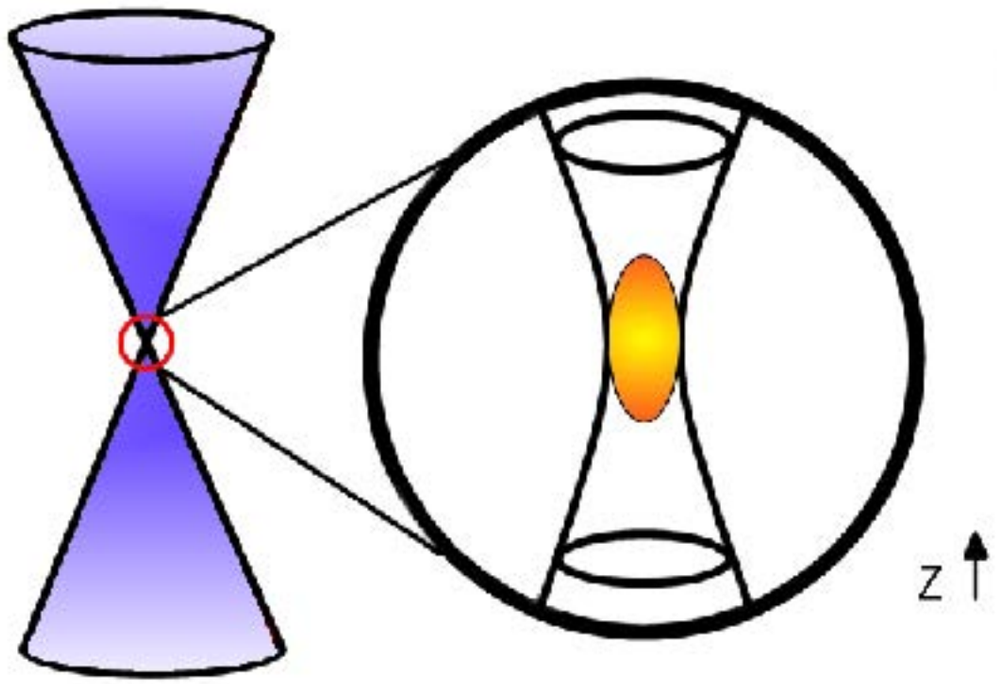
two-photon excitation (2pe) microscopy



DIASPRO ET AL., MICROSC.RES.TECH., 47(3), 196-205, 1999

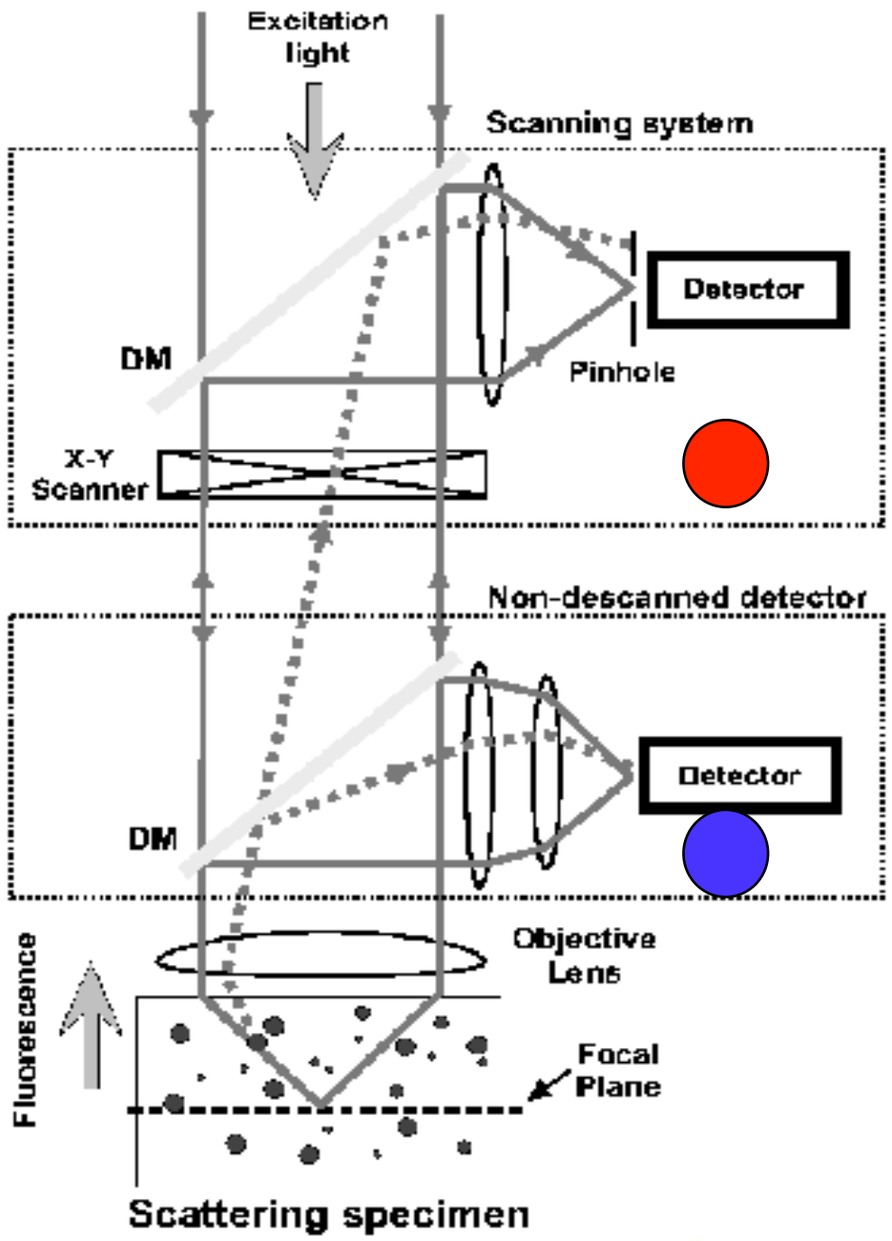
two-photon excitation (2pe) microscopy

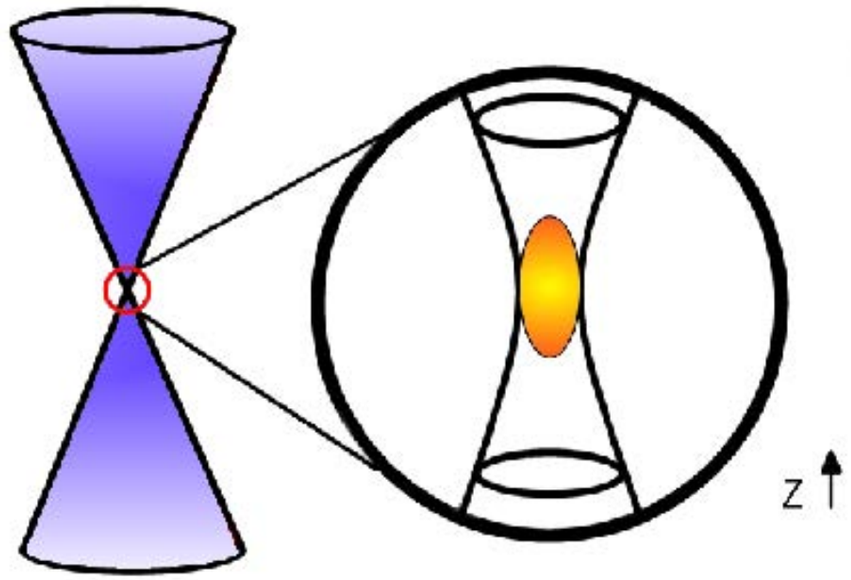


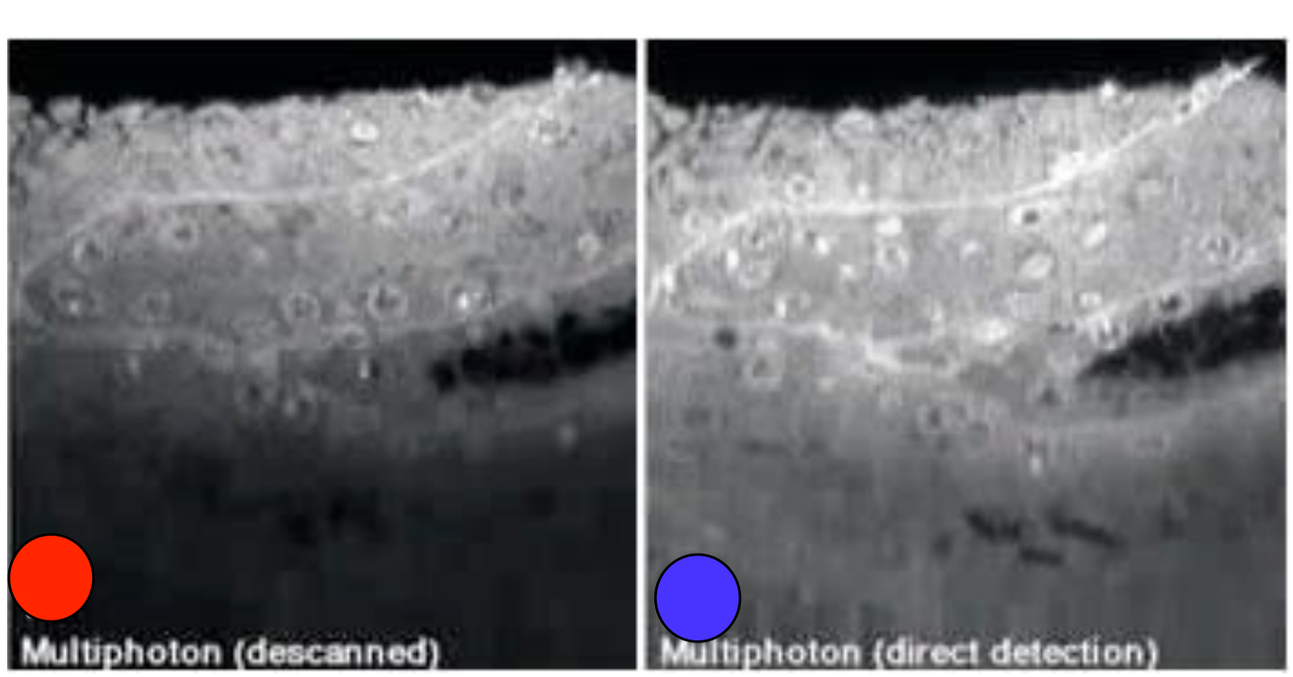


C.SOELLER & M.B.CANNEL, MICROSC.RES.TECH., 47(3), 182-195, 1999

two-photon excitation (2pe) microscopy







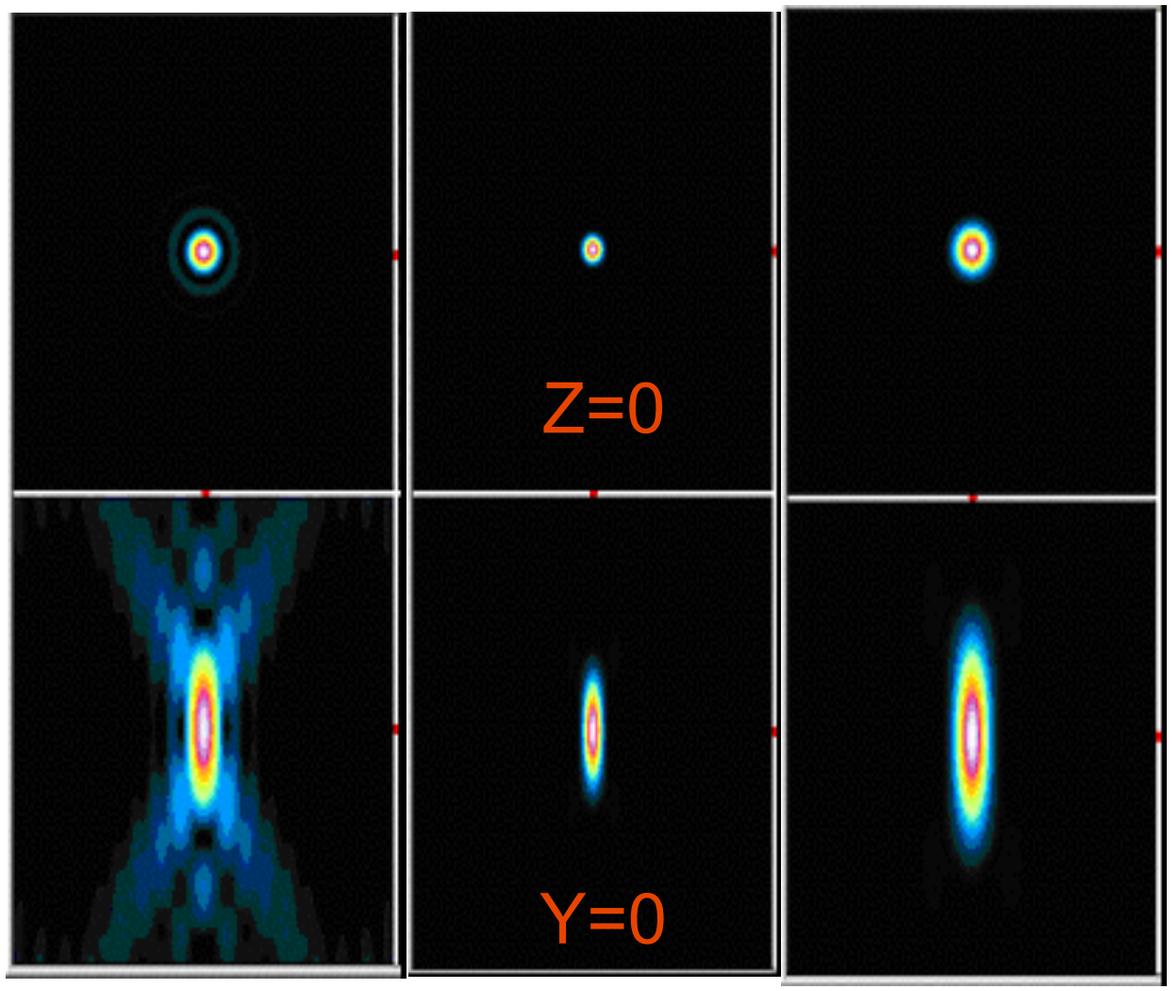
two-photon excitation (2pe) microscopy

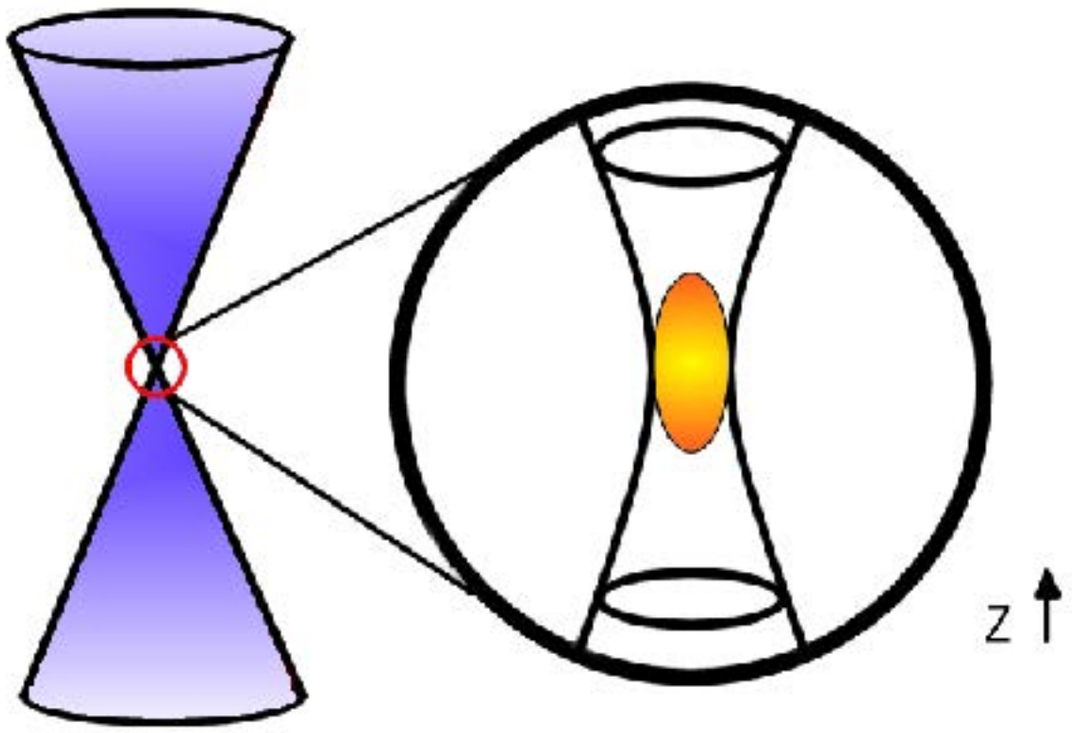
- Confocal PSF:

$$I_{fluor-conf} = I_{det}\, I_{ill} \rightarrow I^2 \propto \frac{1}{z^2}\frac{1}{z^2}$$

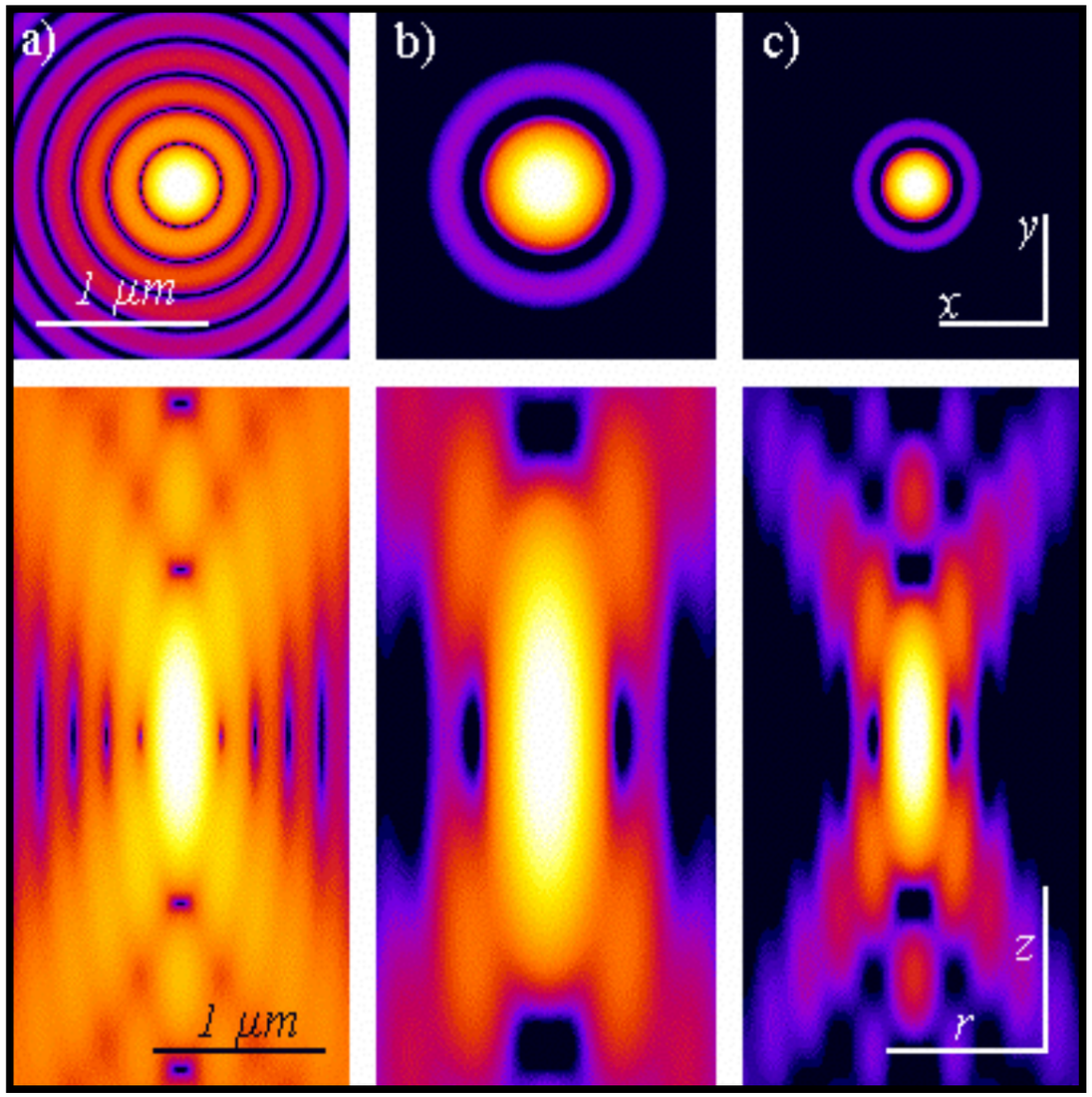
- 2-photon PSF:

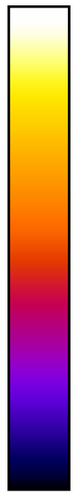
$$I_{fluor-2\gamma} = [I_{ill}]^2 \rightarrow I^2 \propto \frac{1}{z^4}$$





two-photon excitation (2pe) microscopy





$$PSF_{WF}(x,y,z) = h_{em}(x,y,z)$$

$$PSF_{CLS}(x,y,z) = h_{ex}(x,y,z) \times h_{em}(x,y,z) \cong$$

$$\cong \left(h_{ex}(x,y,z)\right)^2$$

$$PSF_{TPE}(x,y,z) = \left(h_{ex}(x,y,z)\right)^2$$

FWHM	WF	CLS	TPE
Axial (μm)	0.83	0.58	1.03
Lateral (μm)	0.20	0.14	0.25

Computed PSFs (a) Wide-field (b) Two-photon (c) Confocal. All PSFs are computed using 100x/ 1.3 NA, n=1.518, single-photon λ_{ex} 488nm, two-photon λ_{ex} 900nm, λ_{em} 520nm. Logarithmic contrast.

Slide credit:Giuseppe Vicidomini, LAMBS-MicroScoBio, Genova

methods

Technique	Three-dimensional resolution	Acquisition speed	Photodamage, photobleaching	Tissue imaging depth
Deconvolution microscopy	Submicron resolution, but algorithms are computationally intensive	Depends on camera; fast acquisition, but slow deconvolution	High (depends on excitation wave-length). Whole sample exposed	Superficial (<40 μm), owing to light scattering
Confocal microscopy	Submicron resolution with pinhole aperture	Limited by scanhead design (typically two frames per second (2 fps), but 30 fps with resonant mirror or spinning disk)	High (depends on excitation wave-length). Whole sample exposed	Moderate (<80 μm), owing to light scattering
Two-photon microscopy	Submicron resolution, but slightly less than for confocal microscopy. Inherent optical sectioning with two-photon effect	Limited by scanhead design (typically 2 fps, but 30 fps with resonant mirror)	Low (long excitation wavelength). Excitat-ion only in focal plane	Hundreds of microns (<500 μm, depends on tissue)

M.D.Cahalan et al. (2002) Nat.Rev.Immunol., 2: 872

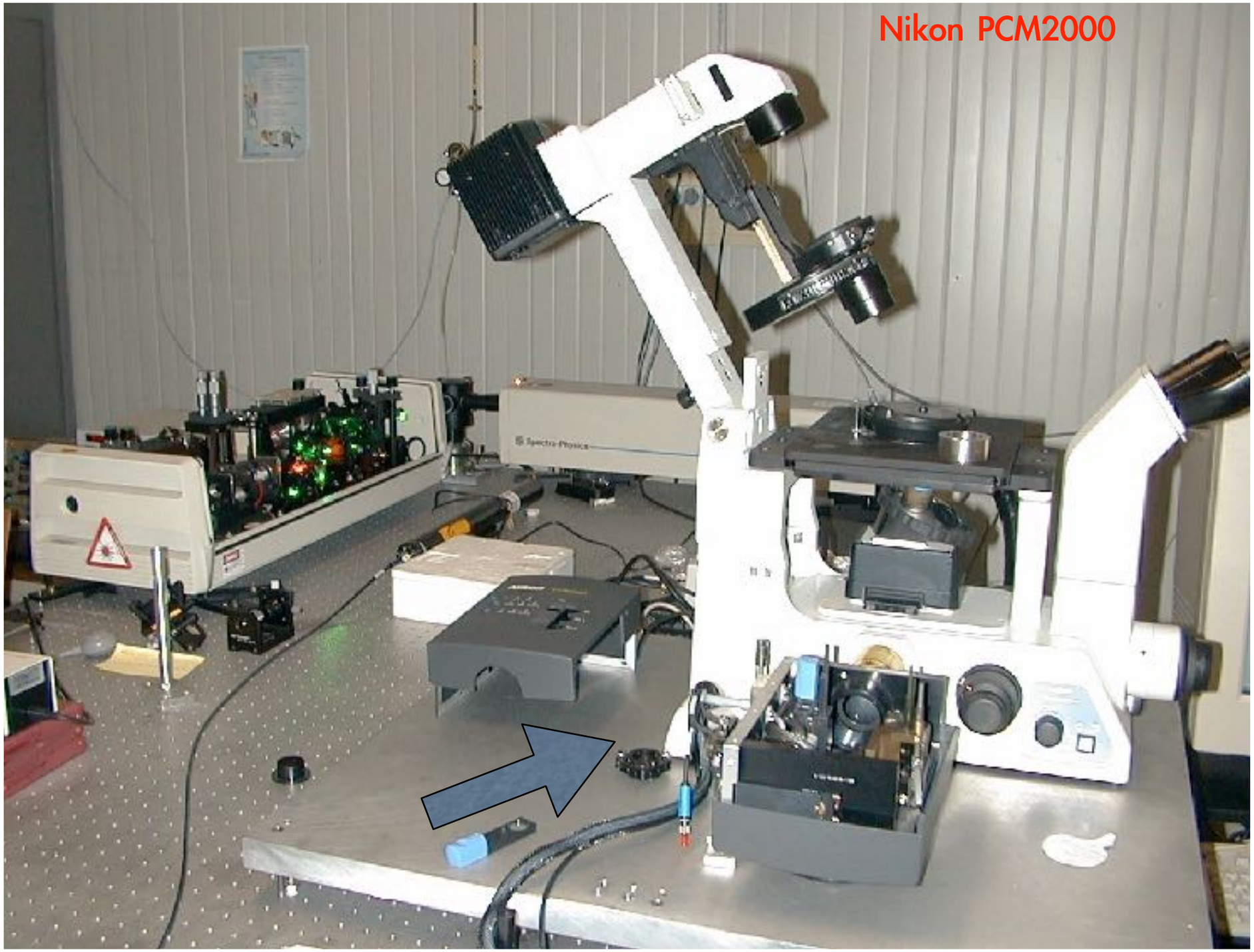
two-photon excitation (2pe) microscopy

This article can be downloaded for free from: http://www.biomedical-engineering-online.com/content/5/1/36

A.Diaspro et al., (2006) Biomedical Engineering On Line (BMEOL), 5(1):36..

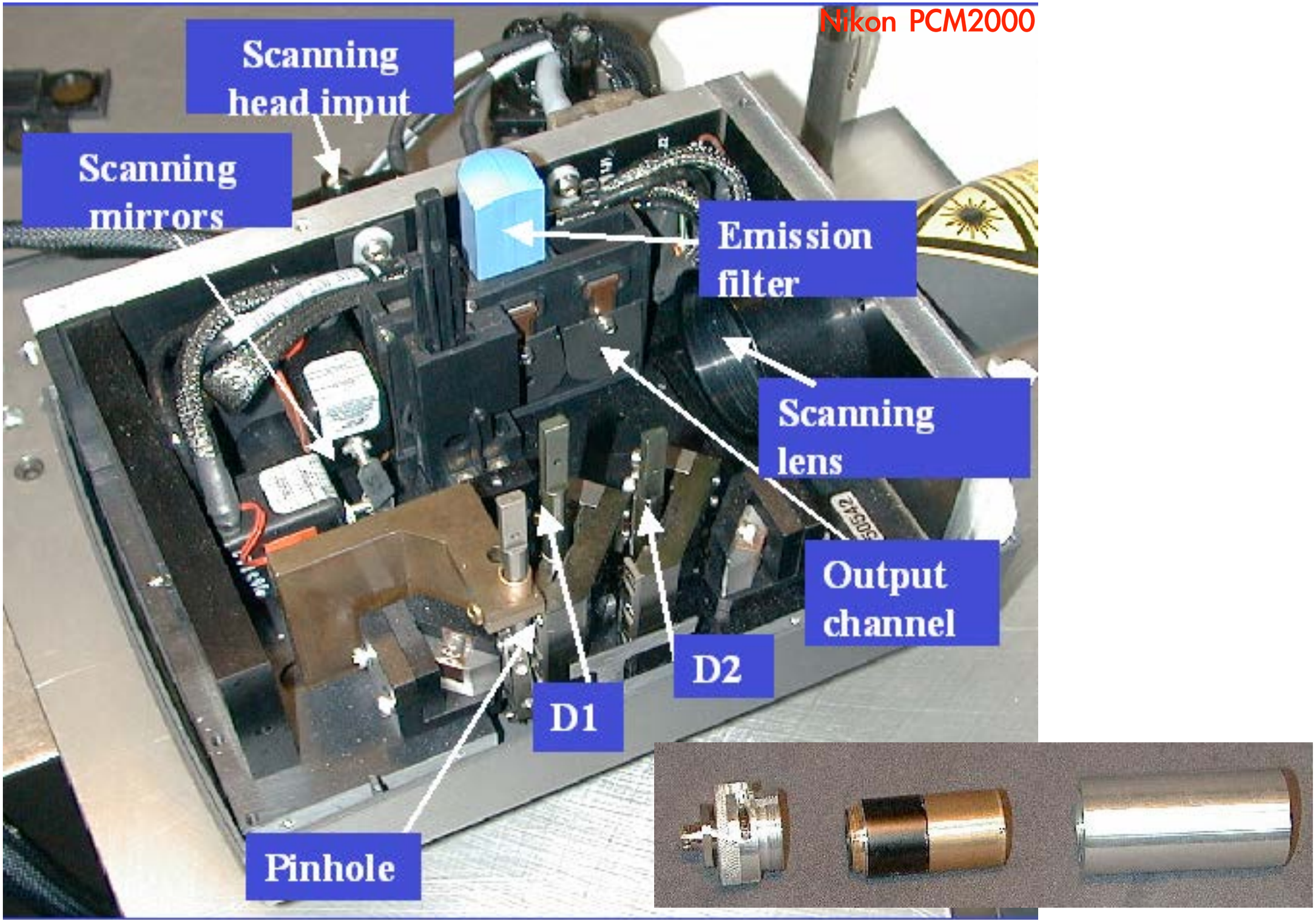
Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

two-photon excitation (2pe) microscopy

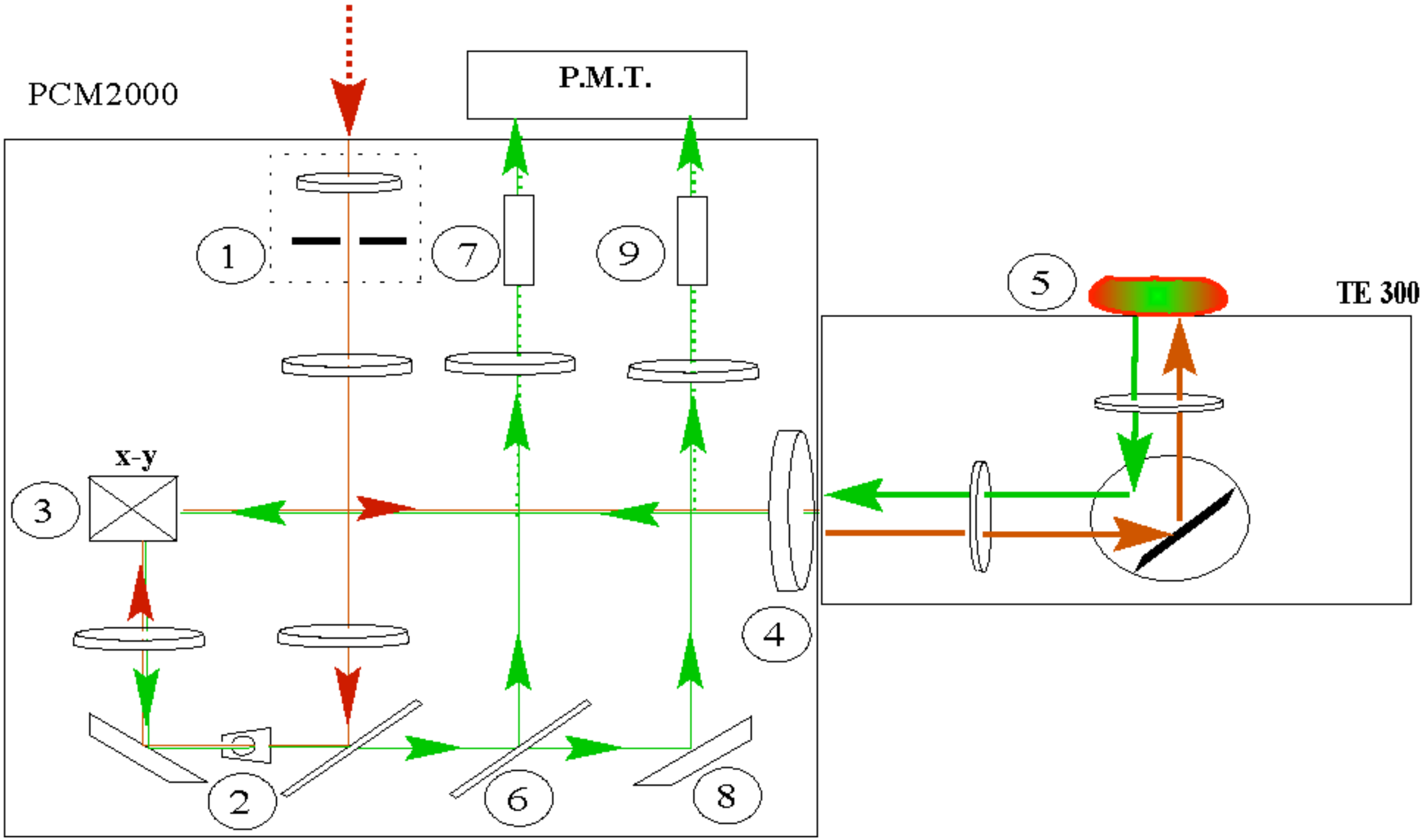


DIASPRO ET AL., MICROSC.RES.TECH., 47(3), 196-205, 1999

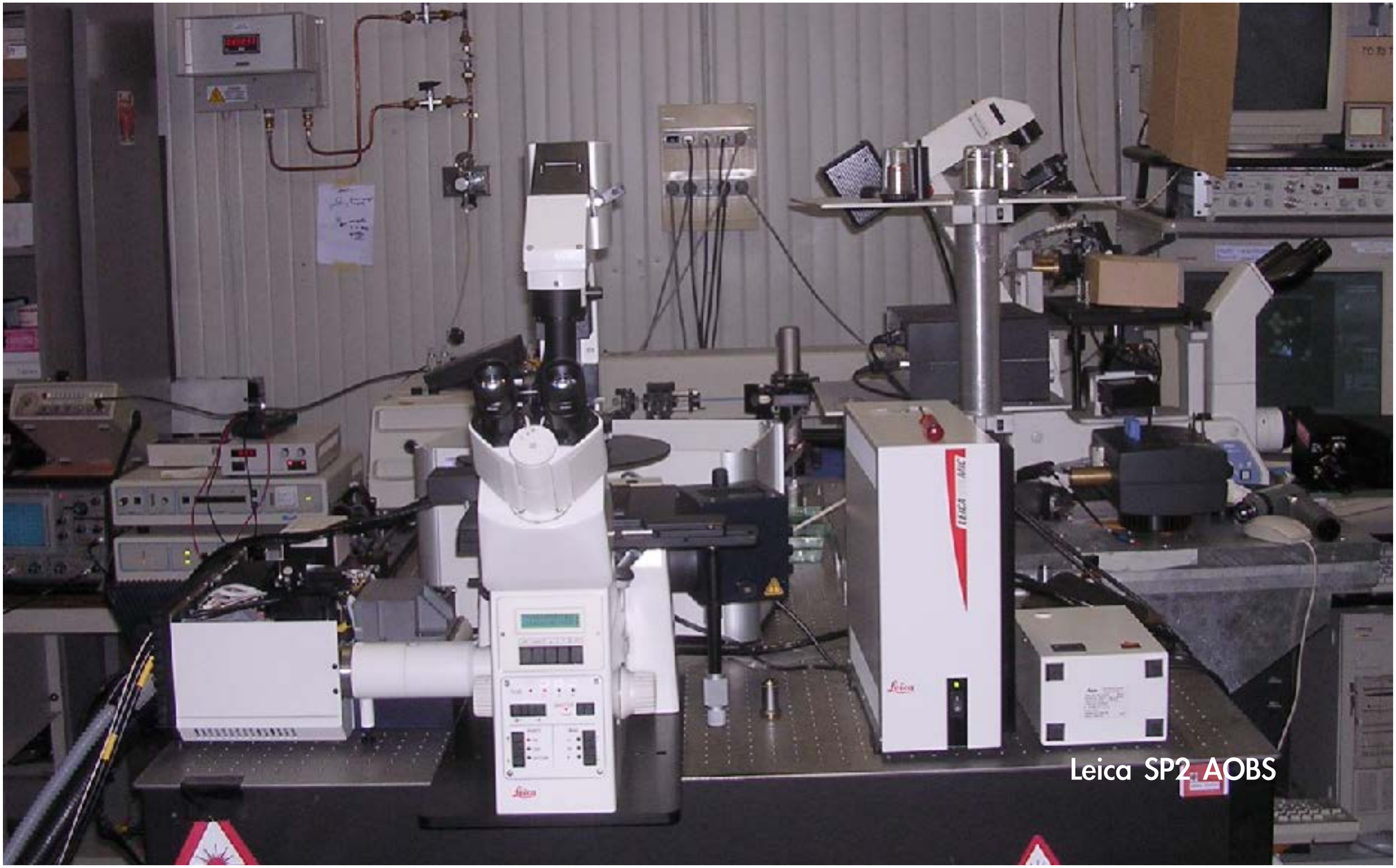
two-photon excitation (2pe) microscopy



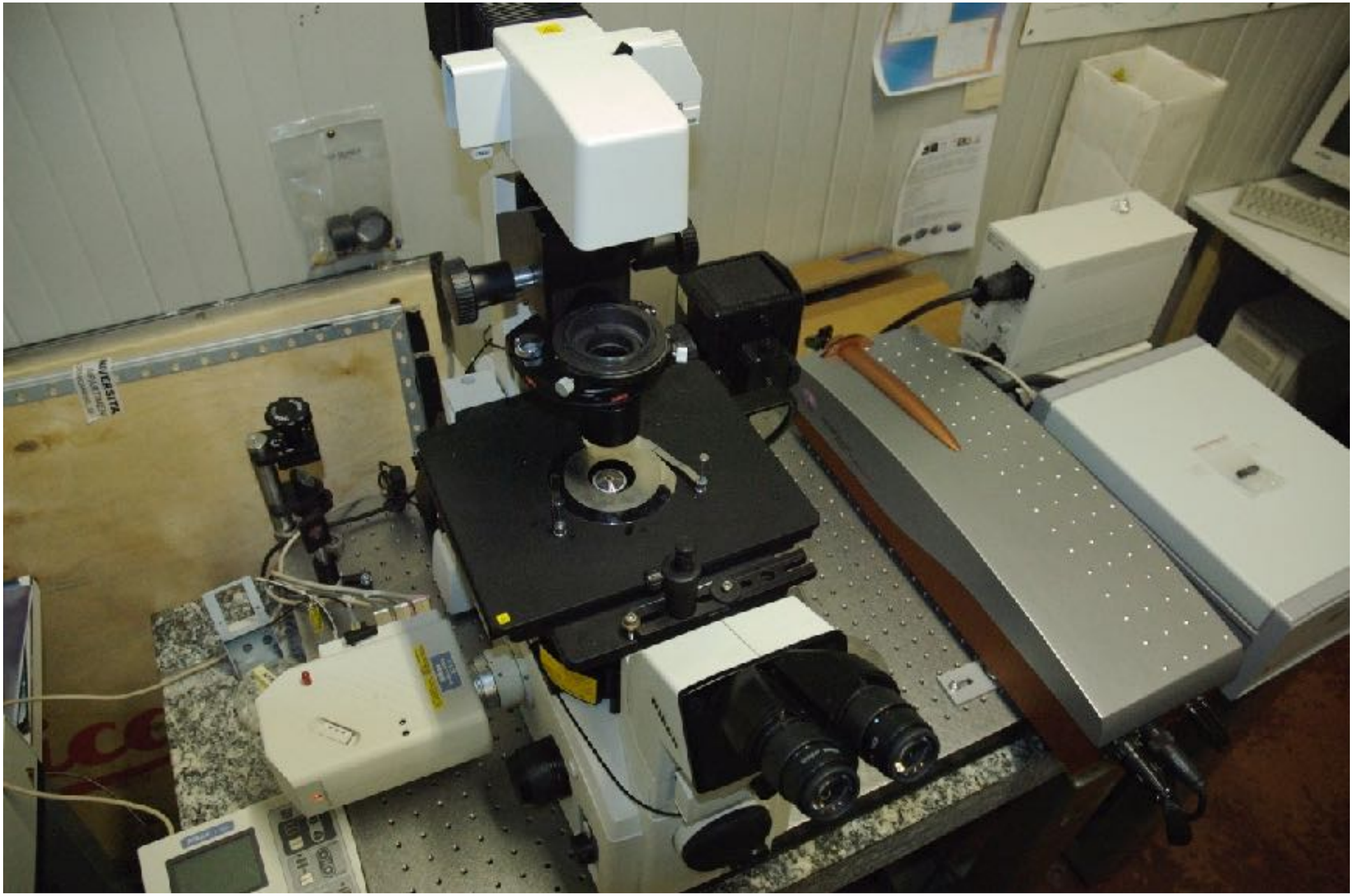
two-photon excitation (2pe) microscopy



two-photon excitation (2pe) microscopy



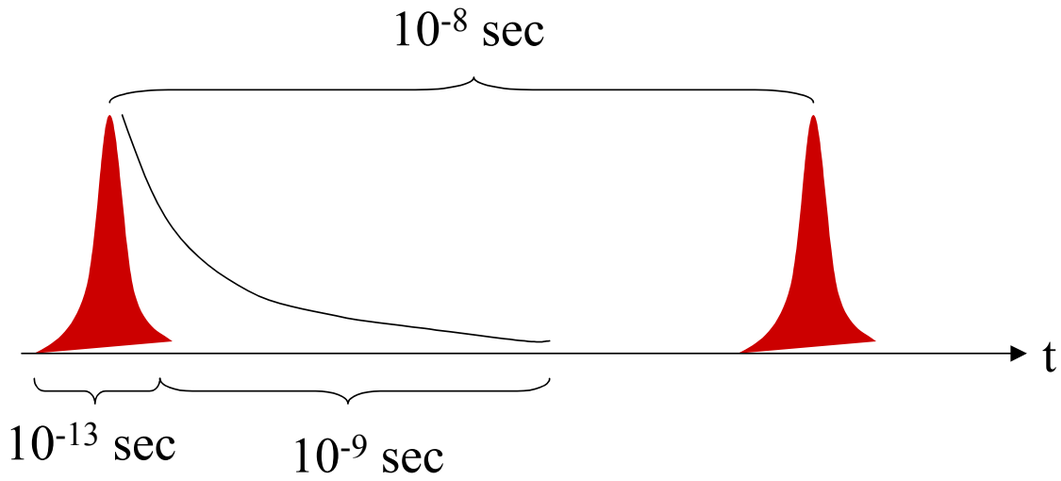
two-photon excitation (2pe) microscopy

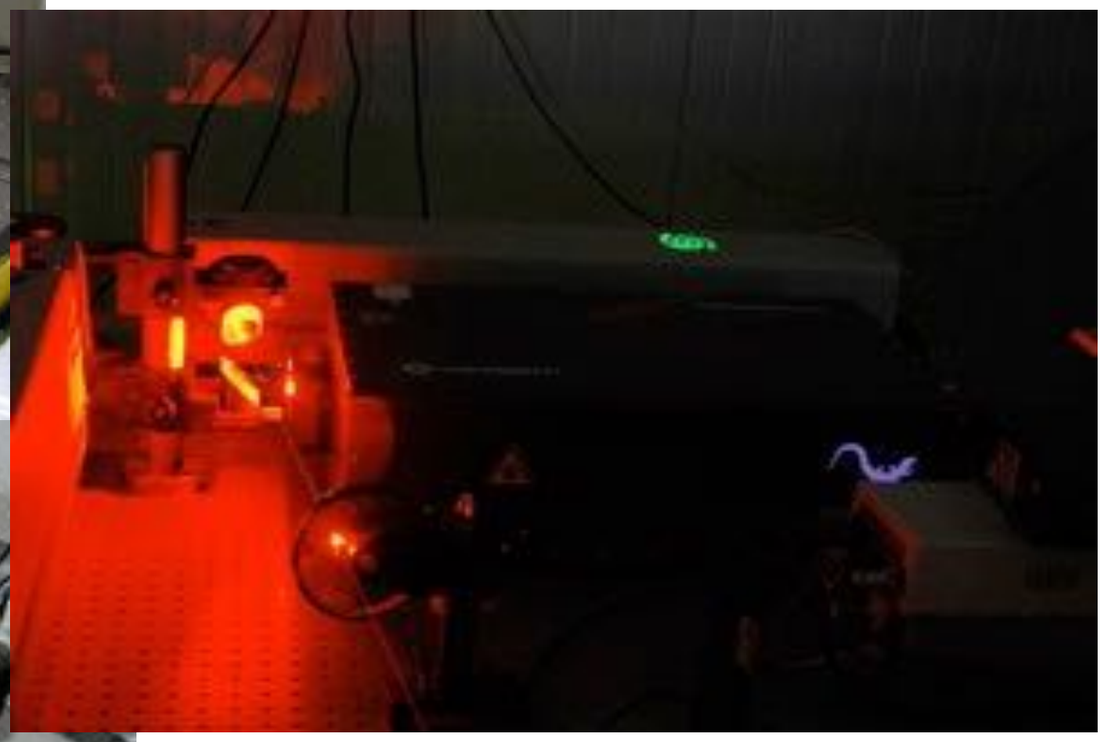


Nikon spectral C1 - Two-photon Amplitude laser

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

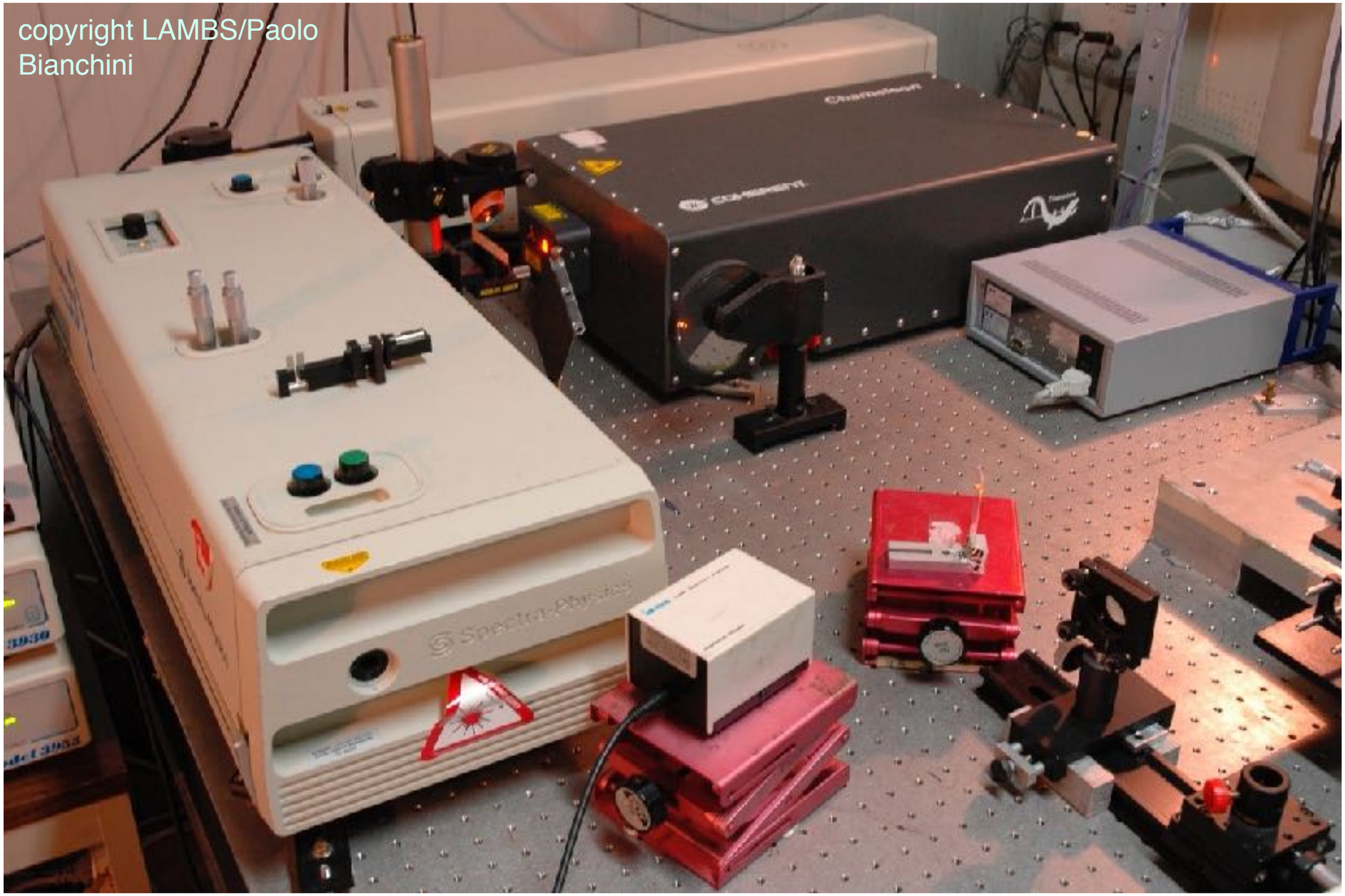
two-photon excitation (2pe) microscopy





Tsunami-Millennia
(680-830/780-930),
Chameleon XR (720-980)

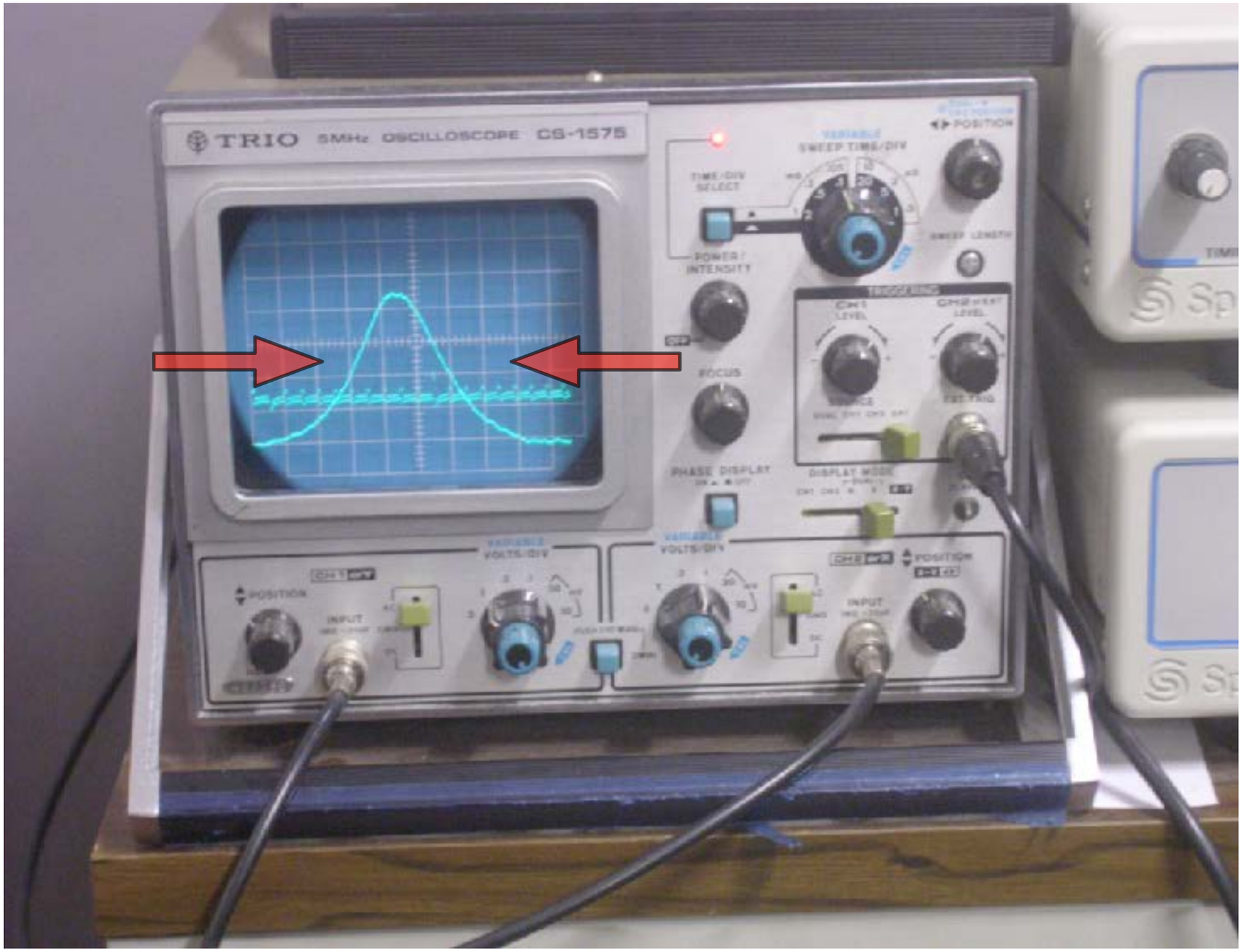
two-photon excitation (2pe) microscopy



Ultrafast Laser Sources for Multiphoton excitation

Alberto Diaspro, Nanoscopy, Istituto Italiano di Tecnologia

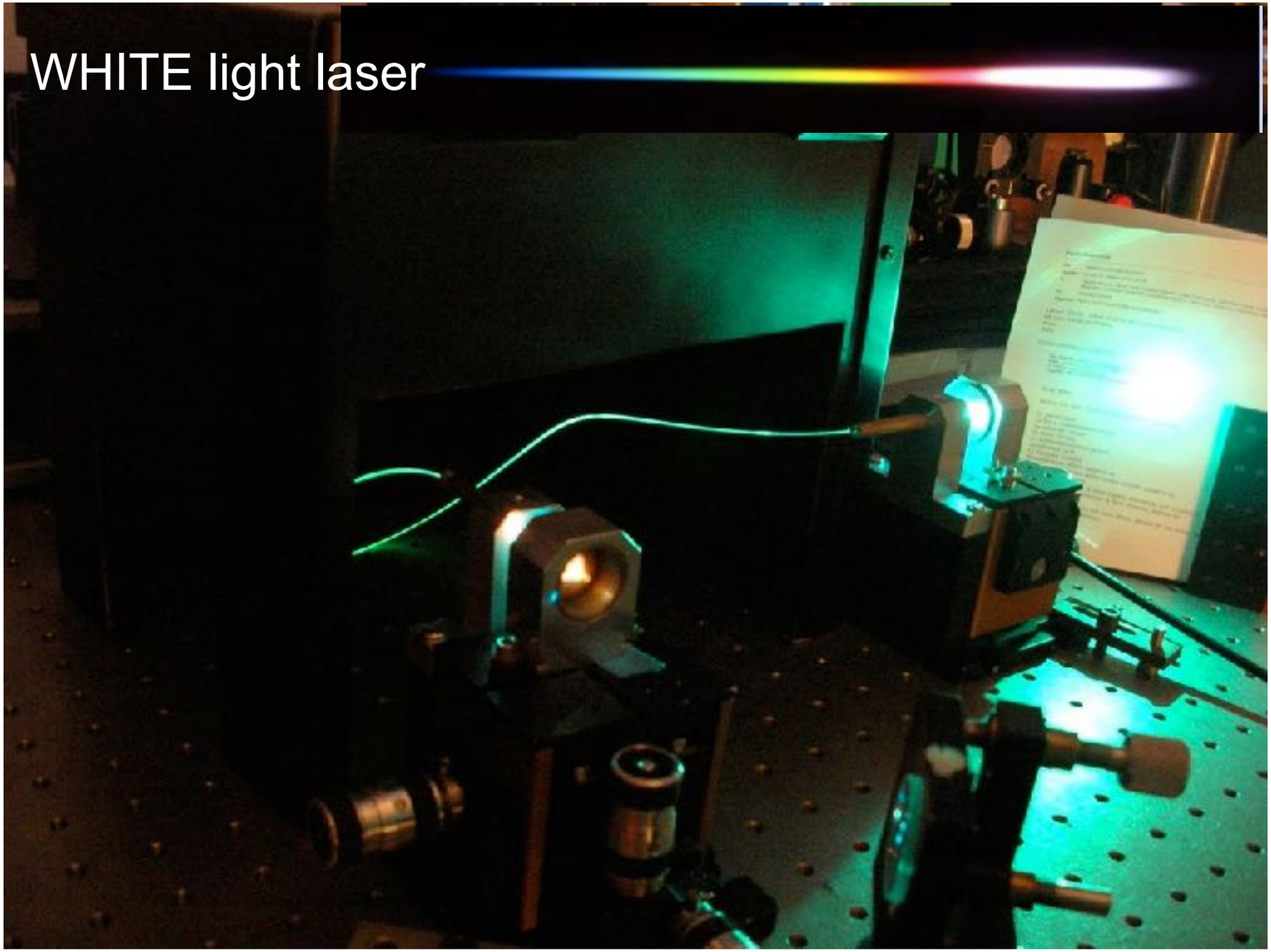
two-photon excitation (2pe) microscopy



A.DIASPRO, ET AL. QUART. REV. .BIOPHYS.,VOL.38, NR.2, PP.1-72 (2005).

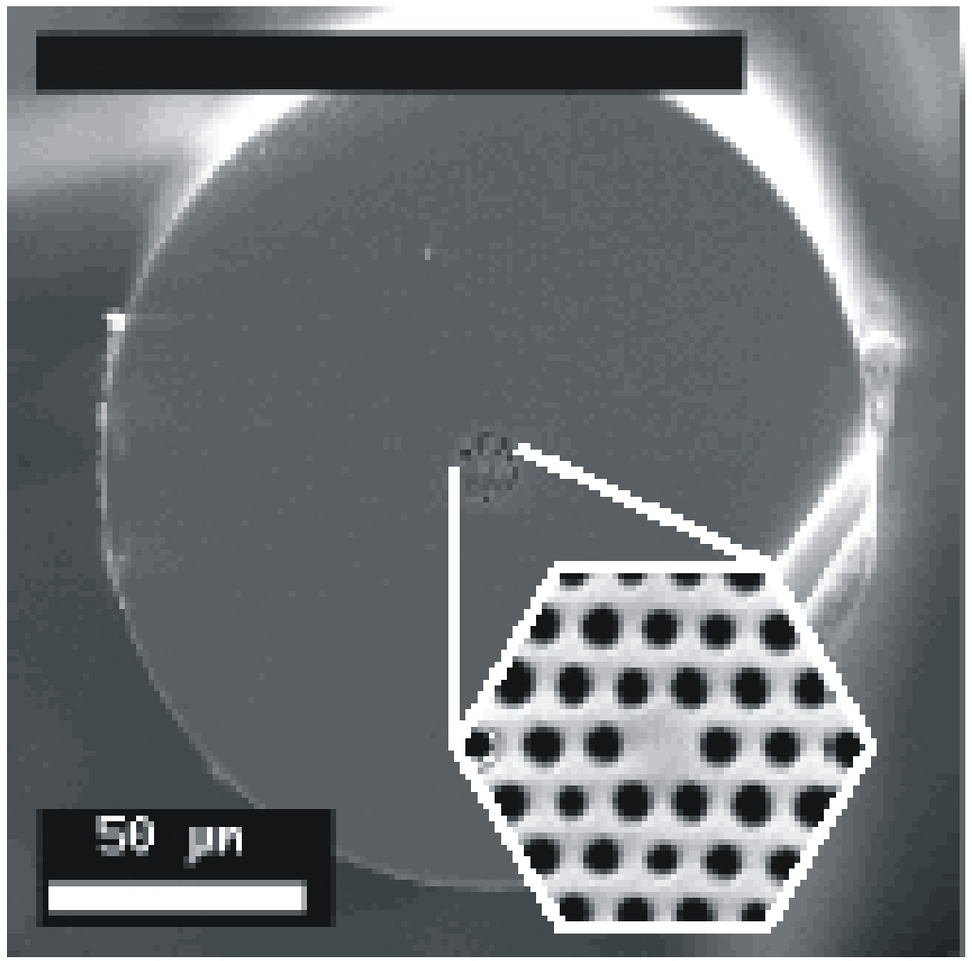
Slide credit:Mario Arace, 1978

two-photon excitation (2pe) microscopy



Caorsi V., Ronzitti E., Vicidomini G., Krol S., McConnell G., Diaspro A. (2007) Micr. Res. Tech., 70

two-photon excitation (2pe) microscopy

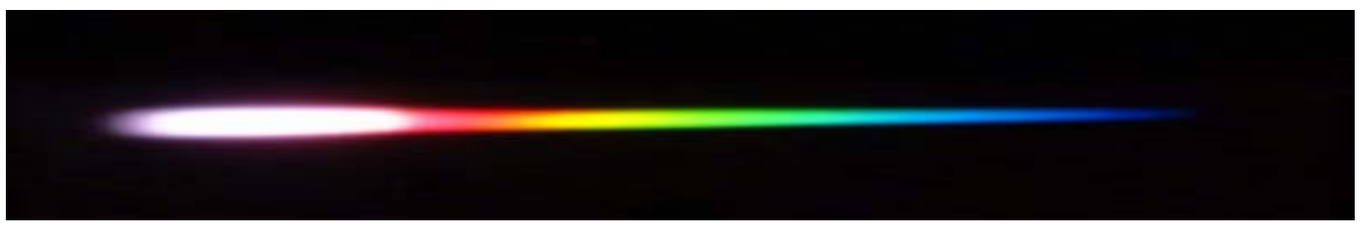


Courtesy of Crystal Fibre A/S

High propagating peak intensity from fs-pulsed sources enhances nonlinear processes:

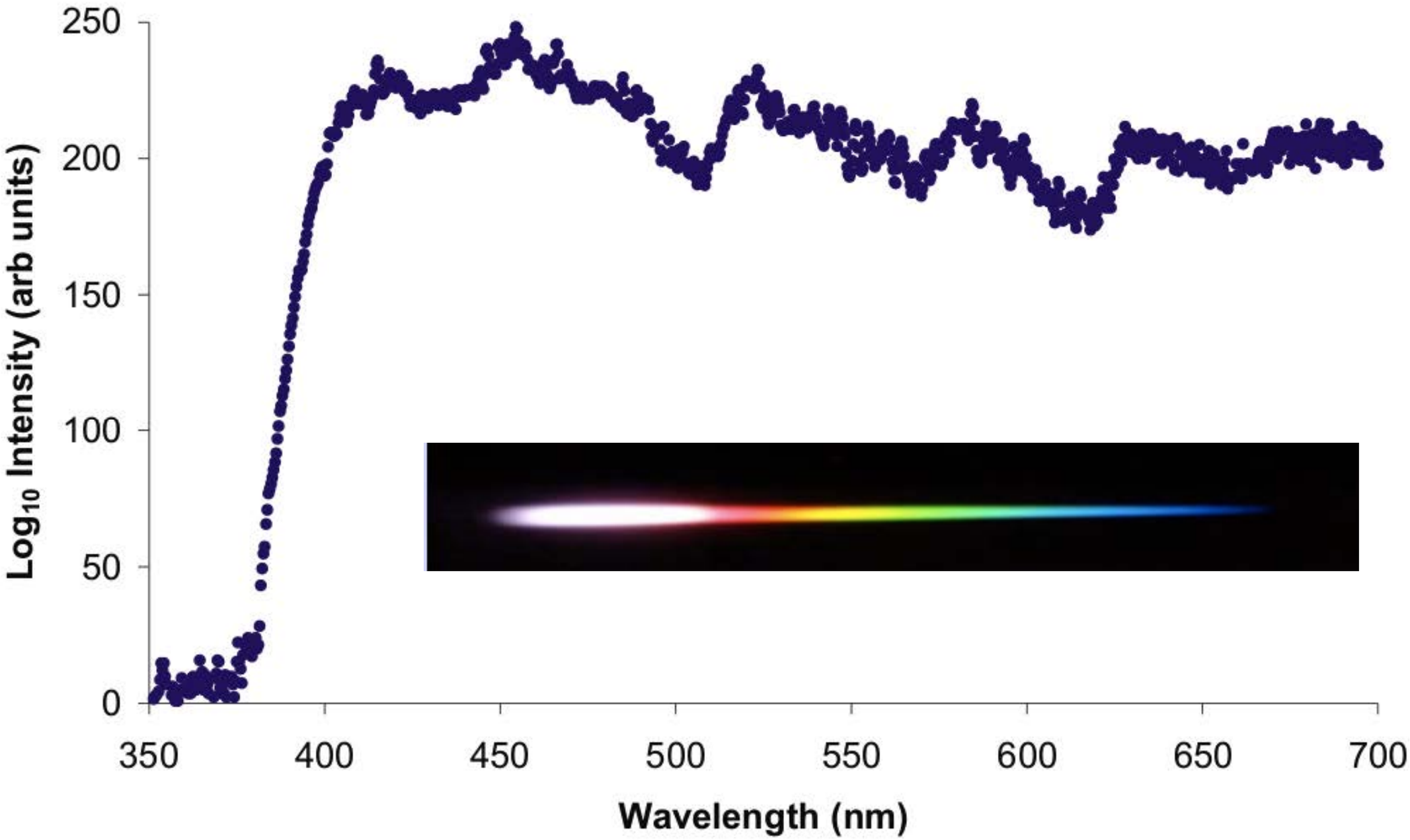
Self-phase modulation + Raman shifting + four-wave mixing + other =

White-light supercontinuum



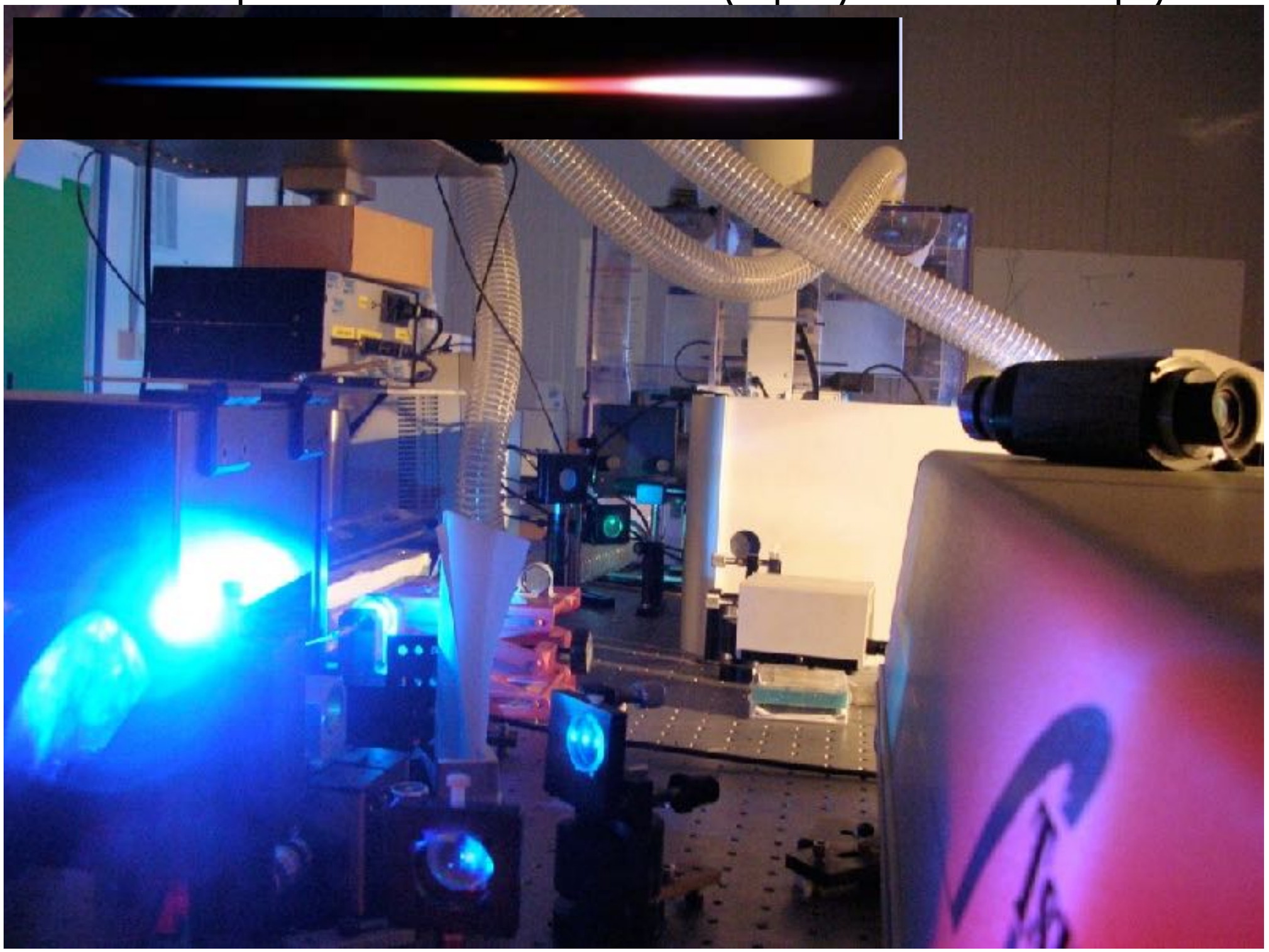
Courtesy Gail Mc Connel, John Girkin

two-photon excitation (2pe) microscopy



Courtesy Gail Mc Connel, John Girkin

two-photon excitation (2pe) microscopy



Caorsi V., Ronzitti E., Vicidomini G., Krol S., McConnell G., Diaspro A. (2007) Micr. Res. Tech., 70

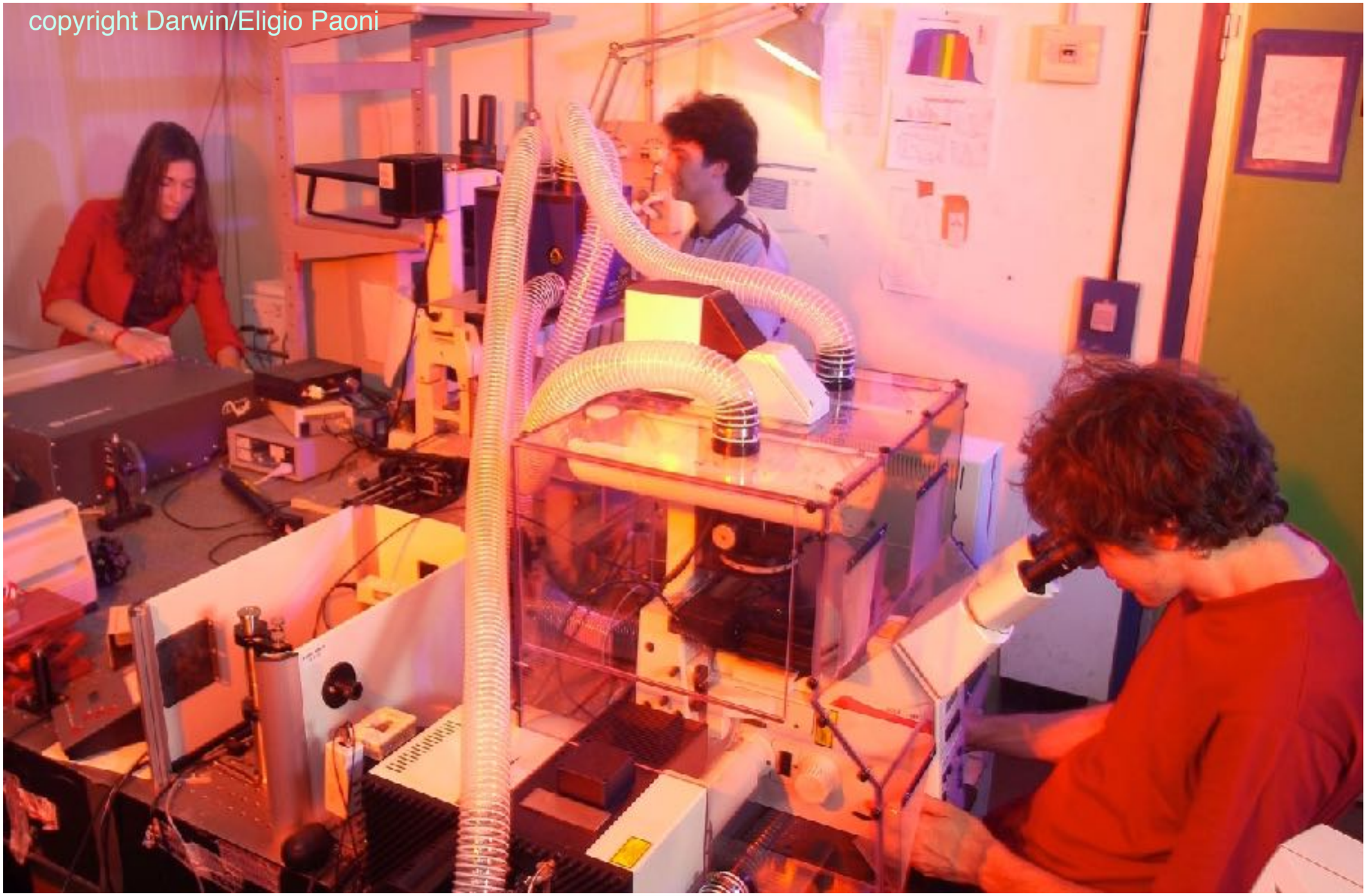
two-photon excitation (2pe) microscopy

Laser Material	Company; Model	Wavelength /nm	Pulse Length	Repetition Rate	Power	Comments
Ti:Sapphire	Coherent; Mira	700-980	<200fs	76MHz	0.7W 1.3W	May require mirror change for full tuning
	Spectra Physics; Tsunami	700-1000	<100fs (or 2ps as option)	80MHz	0.8W 1.4W	May require mirror change for full tuning
	Coherent; Chameleon - XR	705-980	<140fs	90MHz	1.7W	Hands free operation, computer controlled, compact design, single box
	Spectra Physics; Mai Tai Time Bandwidth; Pallas	710-990 780-860	120fs <100fs	80MHz 75MHz	1.5W 500mW	Hands free operation, computer controlled, compact design, single box
	Time Bandwidth; Tiger Femtosource	780-860 750-850	<100fs <12fs	100MHz 75MHz	400mW 400mW 600mW	
Nd:YLF	MicroLase/Coherent Scotland; BioLite	1047	200fs	120MHz	500mW	Not presently available
Nd:Glass	Time Bandwidth; GLX200	1058	<250fs	100MHz	>400mW	
Ytterbium Cr:LiSAF	Amplitude Systems Highqlasers	1030 850nm	<200fs 100fs	50MHz 50MHz	1W >1--mW	Demonstrated on dyes inc Texas Red and GFP; optional 950-1150 selectable
OPO	Coherent and Spectra Physics	350-1200	100fs		~200mW	Exact parameters depends crystal selected. Requires TiSapphire as pump source

A.DIASPRO, ET AL. QUART. REV. .BIOPHYS.,VOL.38, NR.2, PP.1-72 (2005).

Table credit:John Girkin

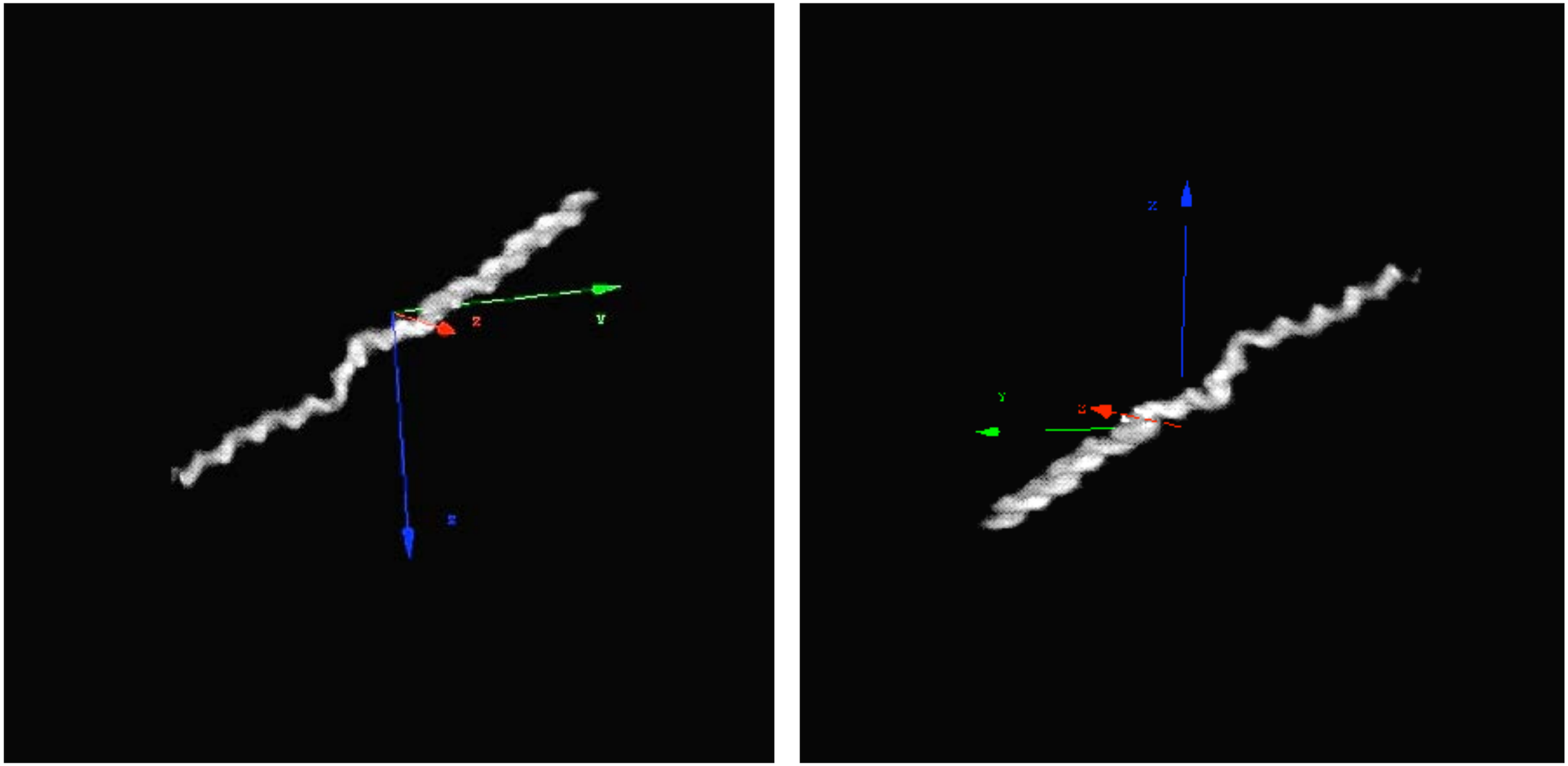
two-photon excitation (2pe) microscopy



two-photon excitation (2pe) microscopy

DIASPRO ET AL., MICROSC.RES.TECH., 47(3), 196-205, 1999

two-photon excitation (2pe) microscopy

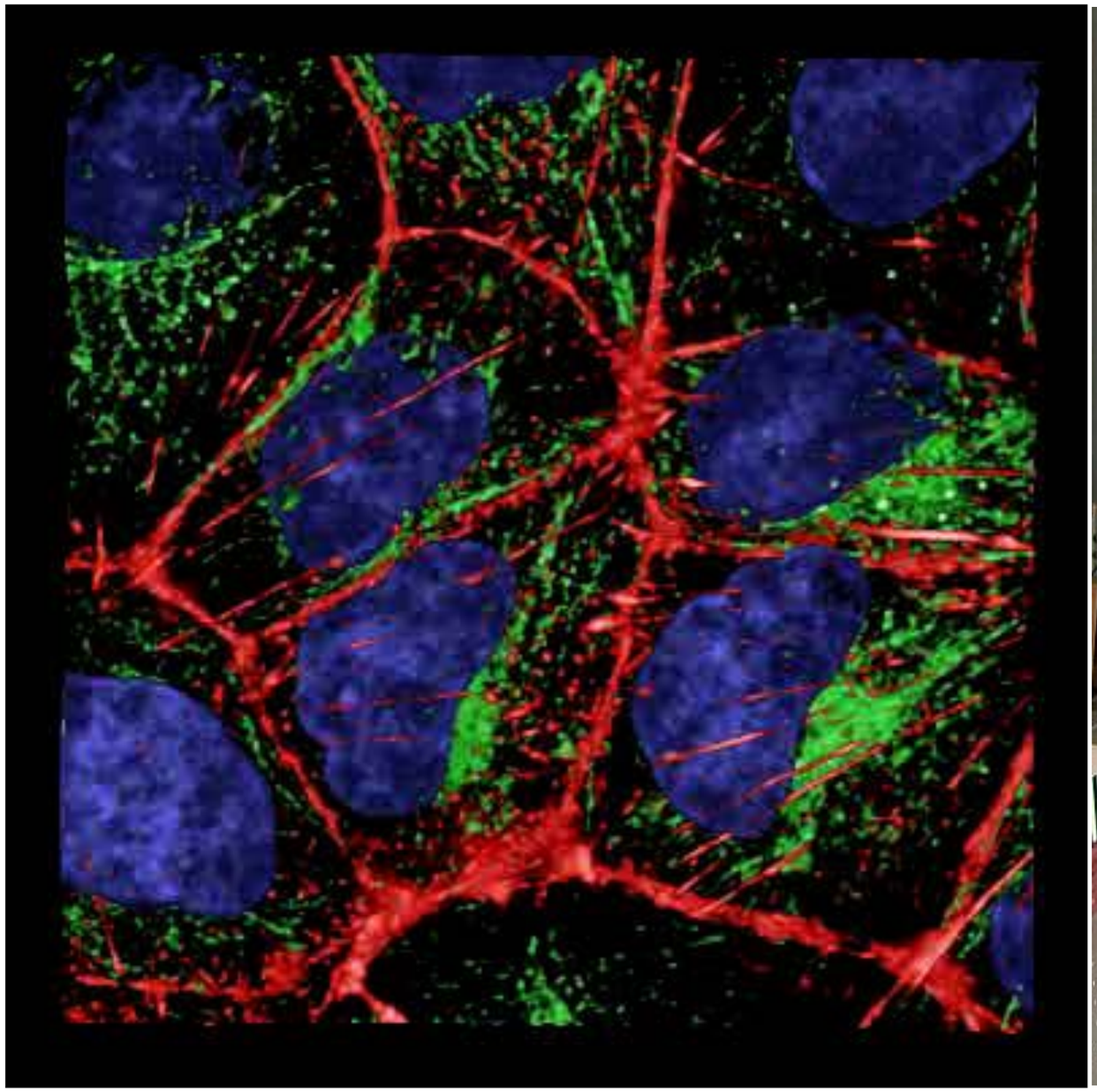


Eledone cirrhosa (octopus) sperm heads

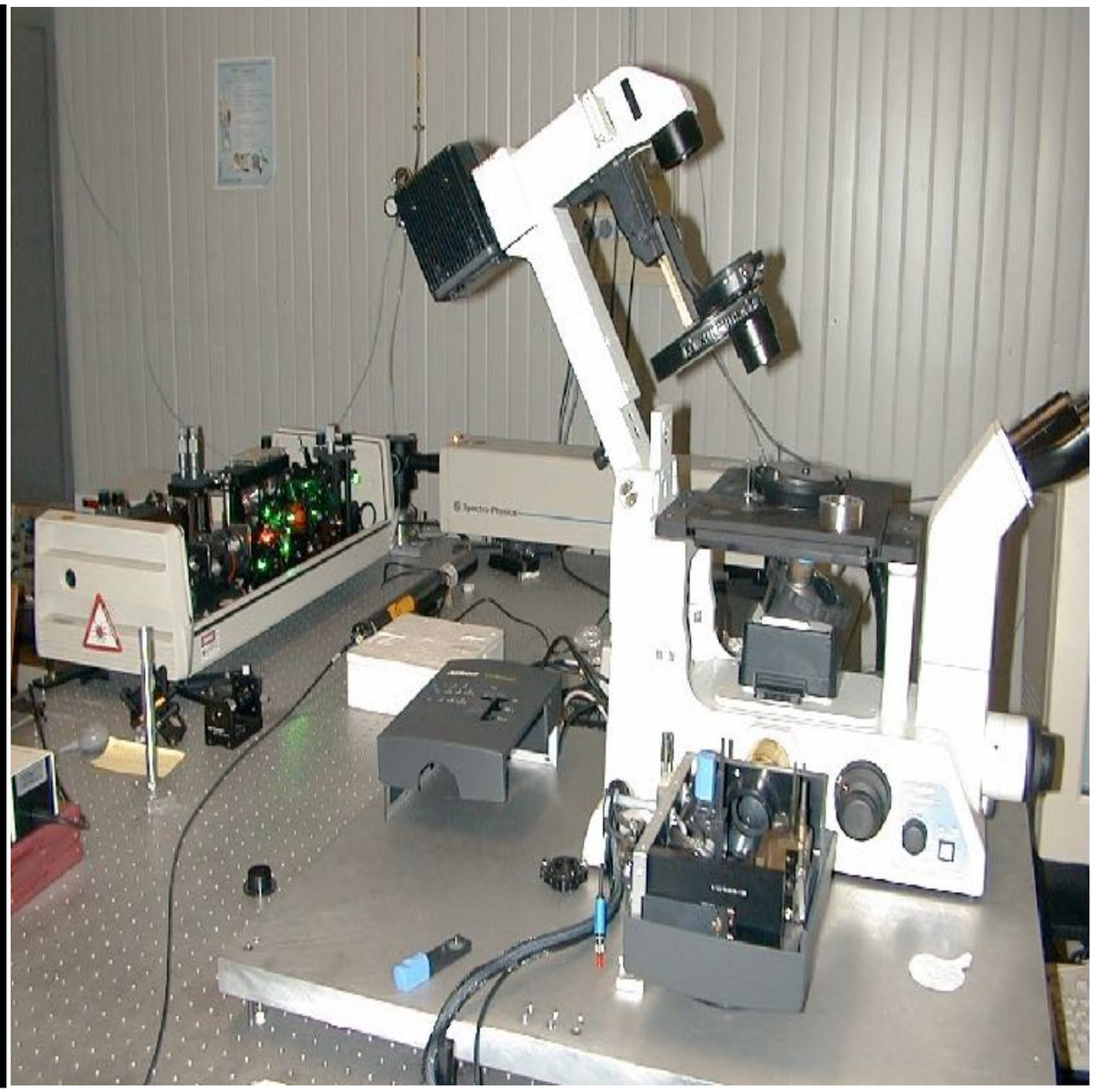
DIASPRO ET AL., MICROSC.RES.TECH., 47(3), 196-205, 1999

Slide credit:Silvia Scaglione, Francesco Difato

two-photon excitation (2pe) microscopy



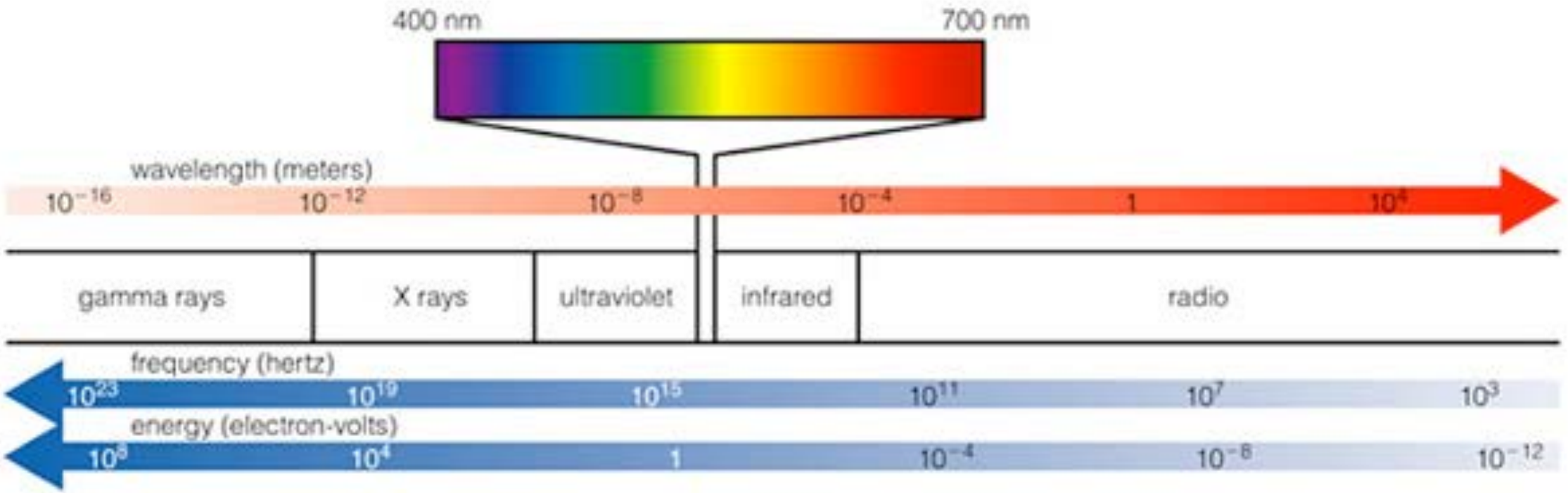
Tissue cultured PTR cells, TexasRed-Phalloidin, FITC-a tubulin, and DAPI. (Drs. Wang and Dunn, ICBM)



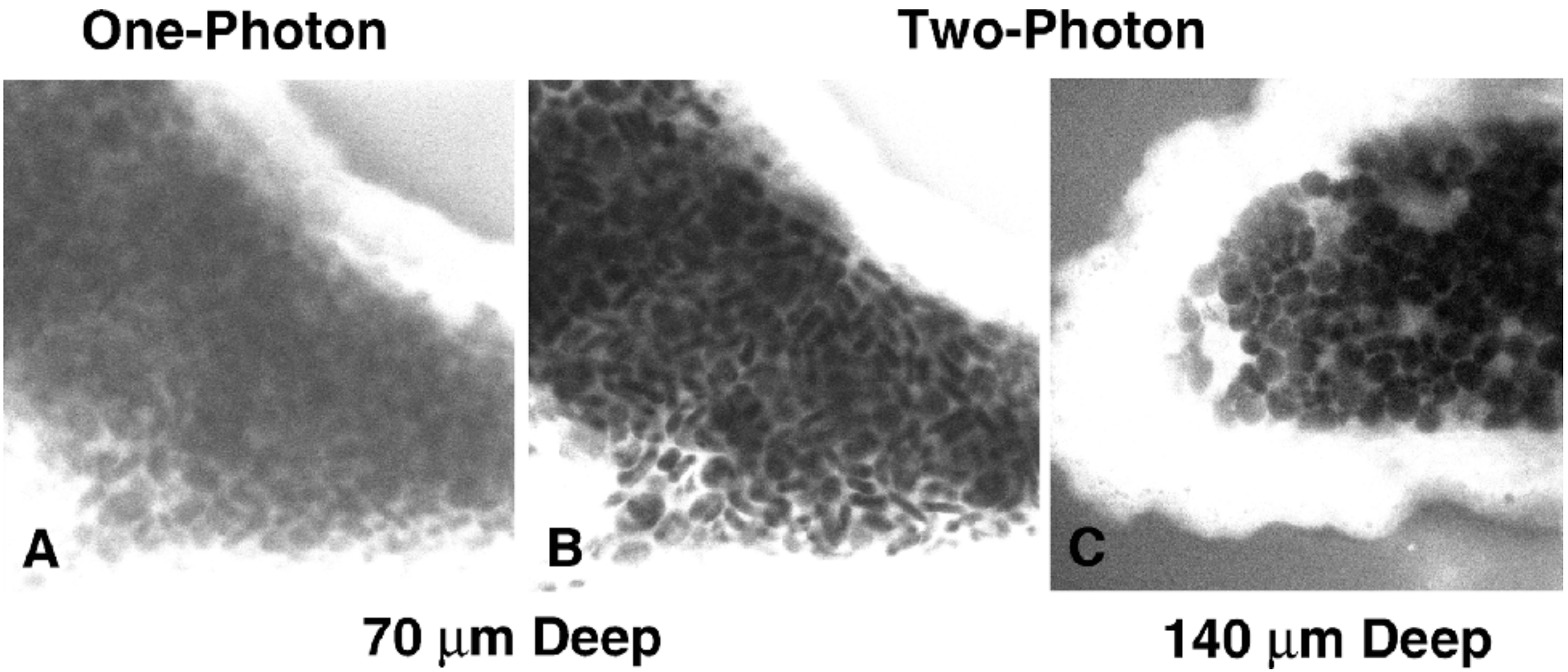
Two photon volume of dapi labeled nuclei and fluorescein labeled anti-cadherin. (Dr. Dunn, ICBM)

two-photon excitation (2pe) microscopy

Wavelengths shifted to the red



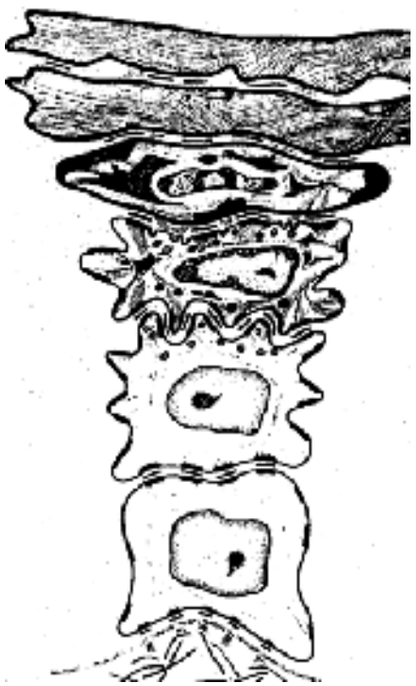
two-photon excitation (2pe) microscopy

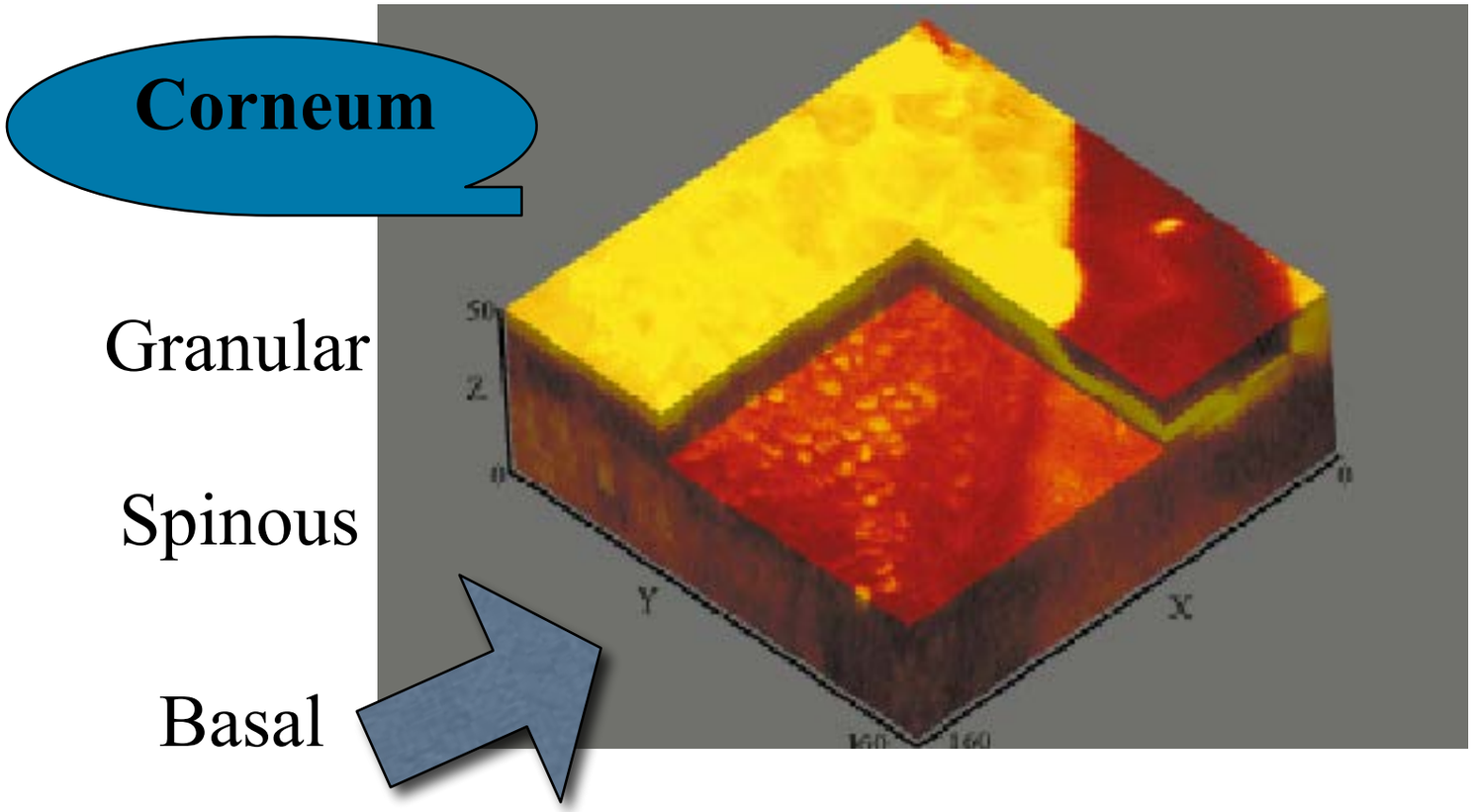


Images of a Shark Choroid Plexus Stained with Fluorescein

Piston DW (2005), PLoS Biol 3(6): e207

two-photon excitation (2pe) microscopy

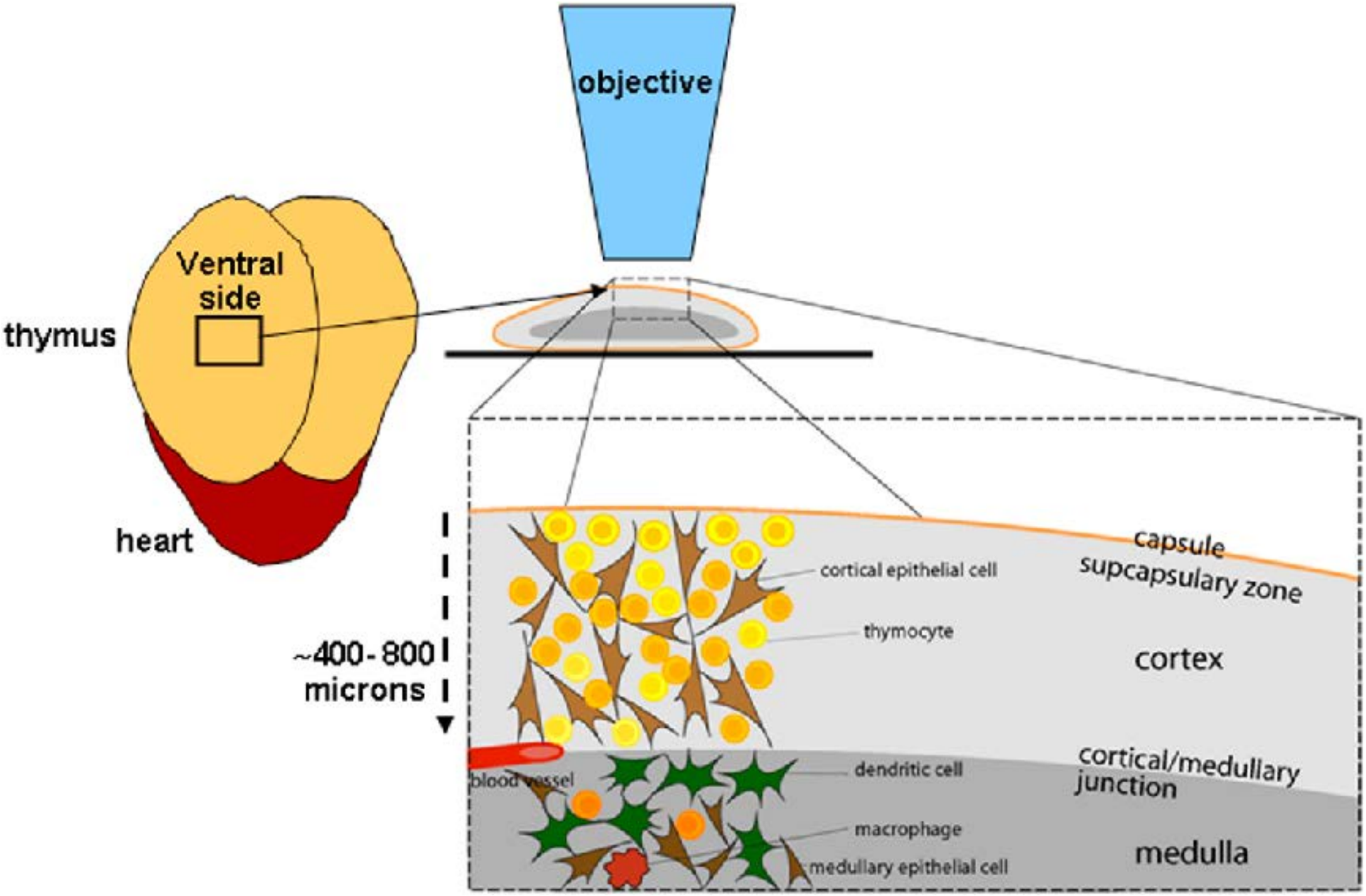




Three-dimensional reconstructed two-photon images of in vivo human skin. The strata corneum and the basal layers are clearly visible.

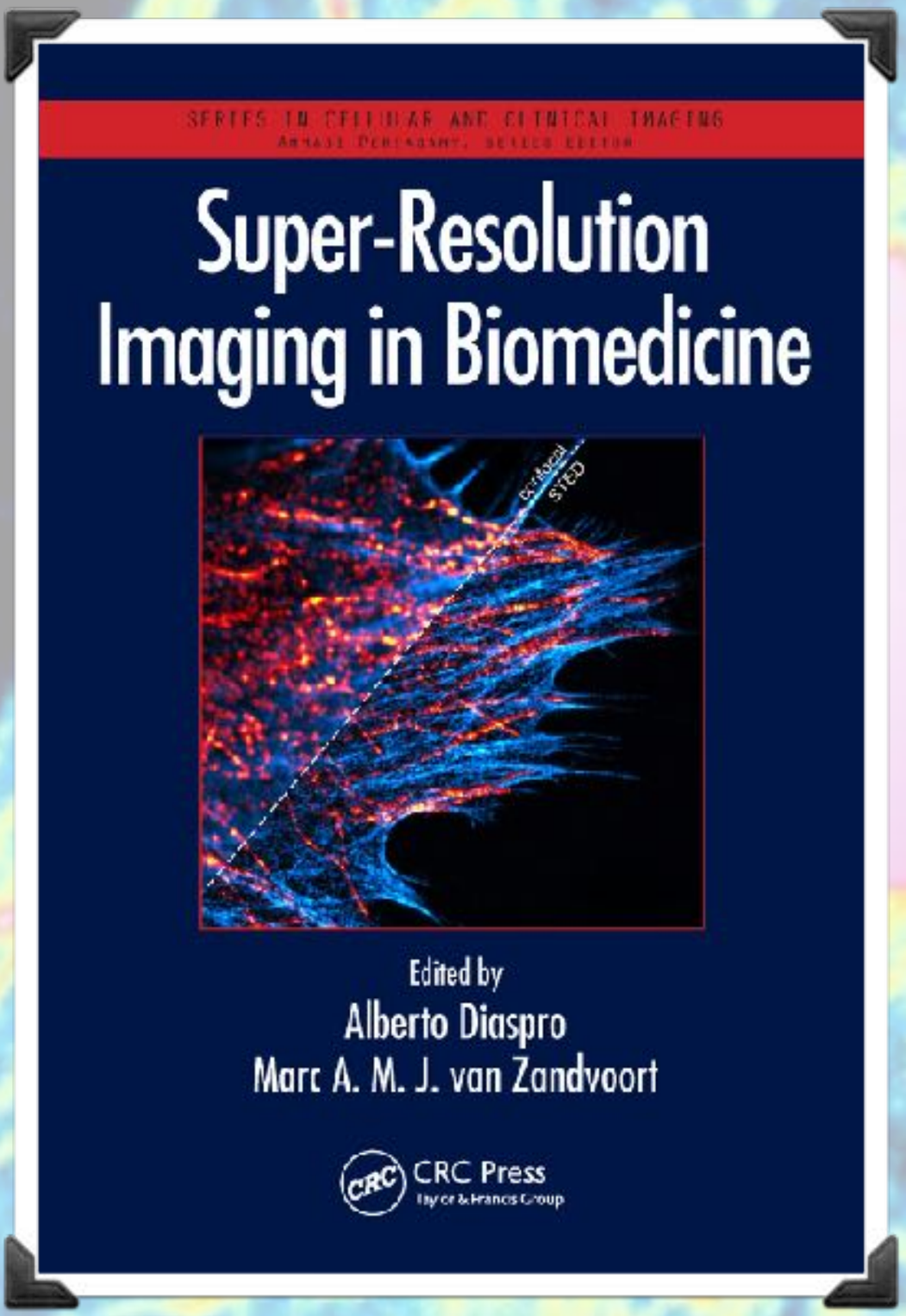
(Image credit: PETER SO, BARRY MASTERS)

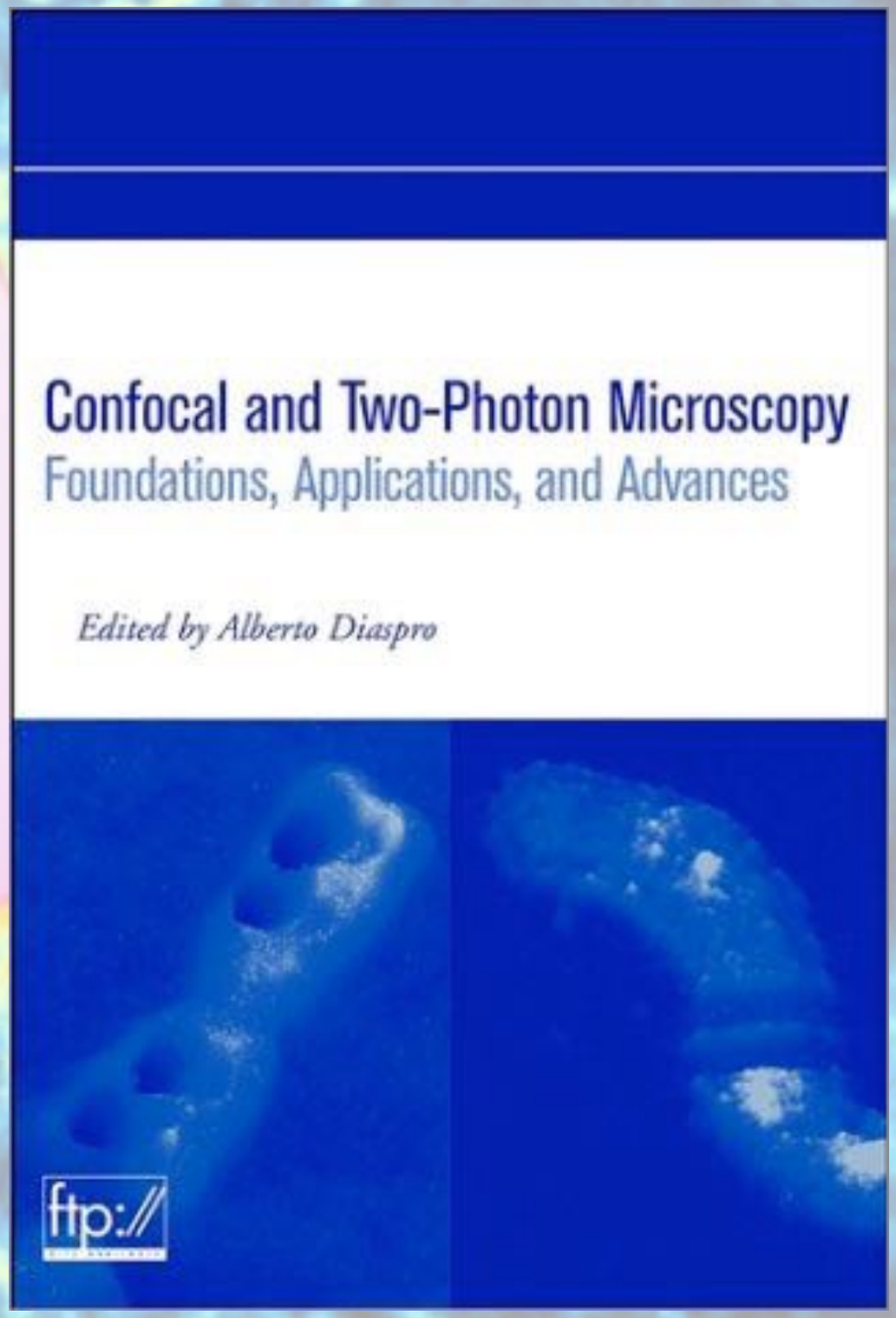
two-photon excitation (2pe) microscopy



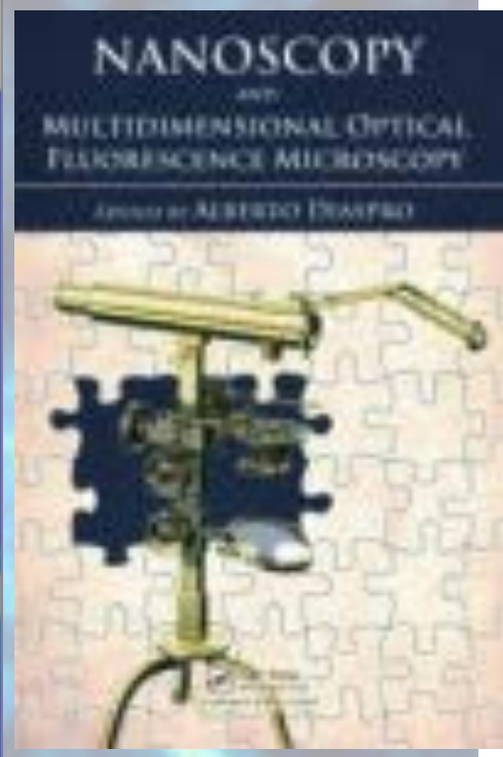
Real-time visualization of thymocytes within intact thymic lobes using two-photon microscopy

From: (2005) Tracking Migrating T Cells in Real Time. PLoS Biol 3(6): e205


Super-Resolution
Imaging in Biomedicine
confocal
STED
Edited by
Alberto Diaspro
Marc A. M. J. van Zandvoort
CRC Press


Confocal and Two-Photon Microscopy
Foundations, Applications, and Advances
Edited by Alberto Diaspro
ftp://


Fundamentals
of Fluorescence
Microscopy


NANOSCOPY
AND
MULTIDIMENSIONAL OPTICAL
FLUORESCENCE MICROSCOPY

Nanoscale Optical Microscopy

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