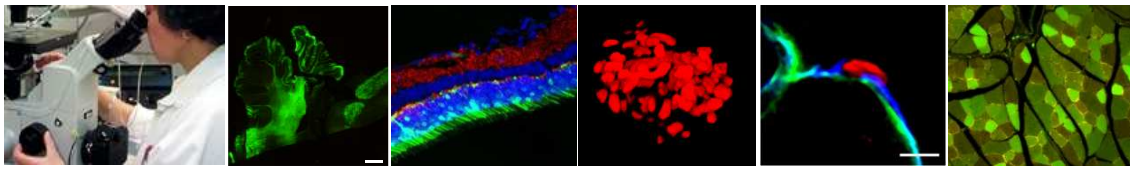
Séminaire SFR santé

11 Décembre, 2012 -

Les nouvelles approches en microscopie pour l'exploration cellulaire et tissulaire

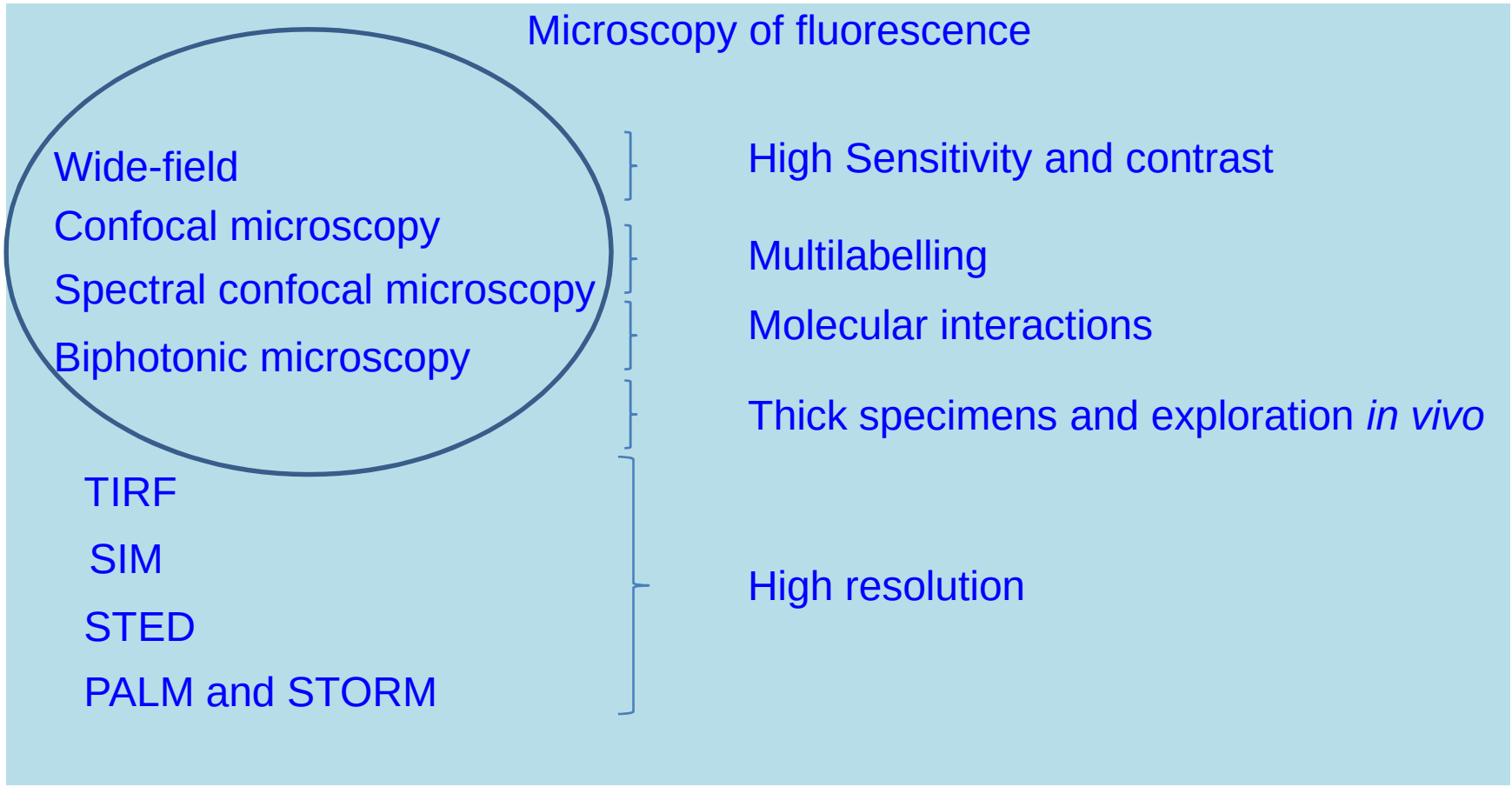
Laurence Dubreil, Plateforme APEX UMR 703 INRA ONIRIS
Philippe Hulin, Plateforme MicroPicell
Steven Nedelec Plateforme MicroPicell



Oniris École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique

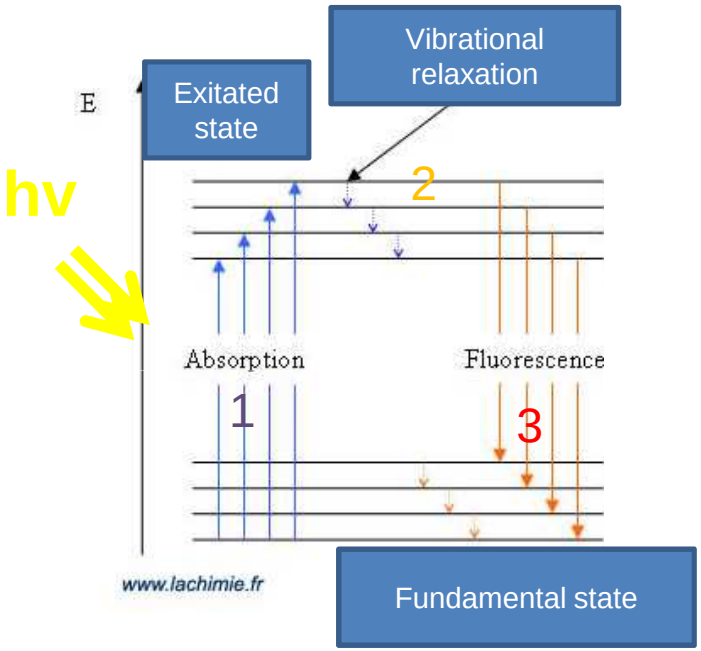
INRA

Structure Fédérative de Recherche - SANTÉ François Bonamy


Microscopy of fluorescence
Wide-field
Confocal microscopy
Spectral confocal microscopy
Biphotonic microscopy
TIRF
SIM
STED
PALM and STORM
High Sensitivity and contrast
Multilabelling
Molecular interactions
Thick specimens and exploration *in vivo*
High resolution

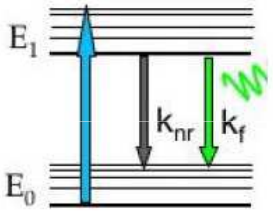
Fluorescence : emission of photons

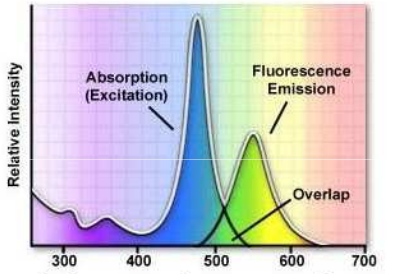
Jablonsky Diagramme



Loi de stokes

$E = hc/\lambda$



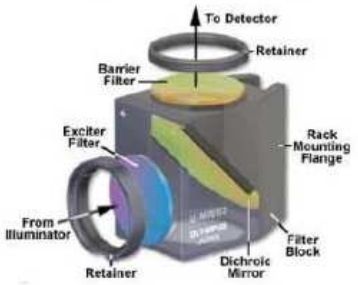


Exemple de spectres d'excitation et d'émission

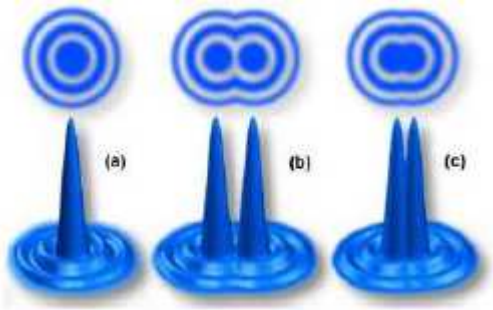
1- stable state to excitated state following the absorption of light energy

2- vibrational relaxation

3- come back to fundamental state S0 with light energy emission



Resolution limit of an optical system in wide-field



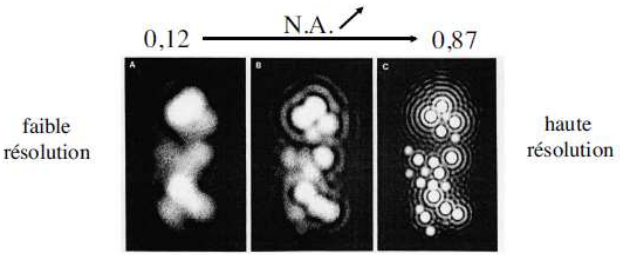
Rayleigh criterion

$Rxy = 0,6\lambda/NA$

$Rz = 2\lambda/NA^2$

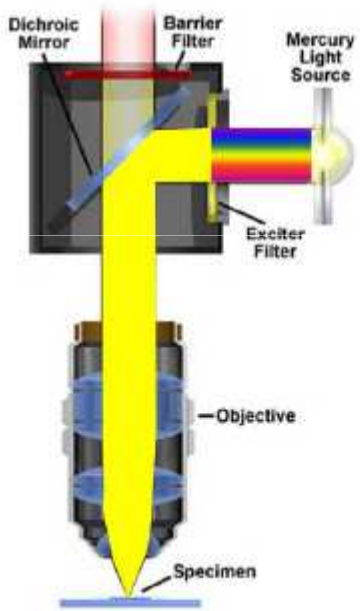
NA : Numerical Aperture : 1,4
λ: 488 nm

dxy = 212,62 nm
dz = 498 nm

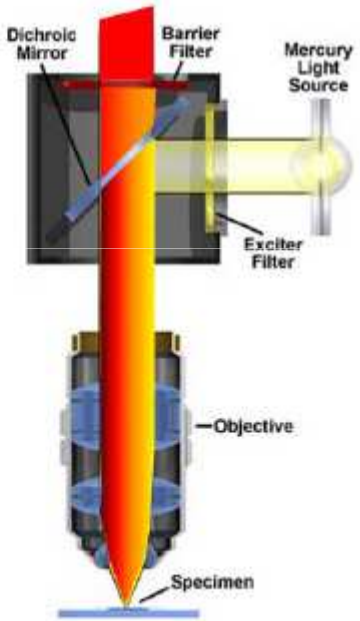


Wide-field fluorescence microscopy

Excitation



Emission

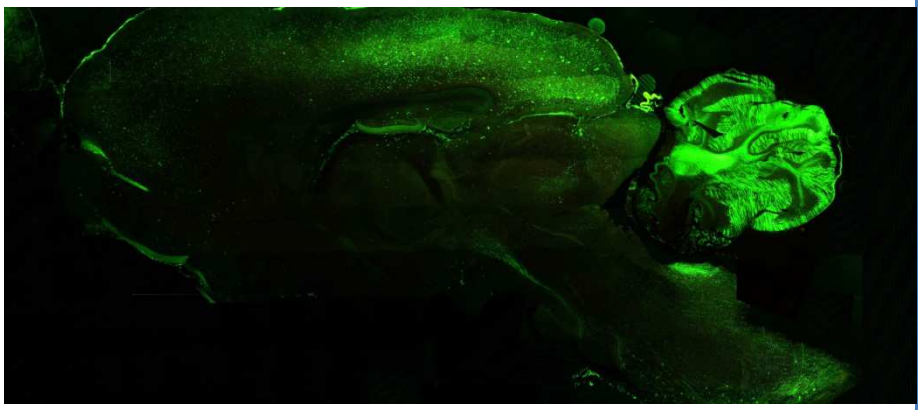


Applications of the wide-field fluorescent microscopy

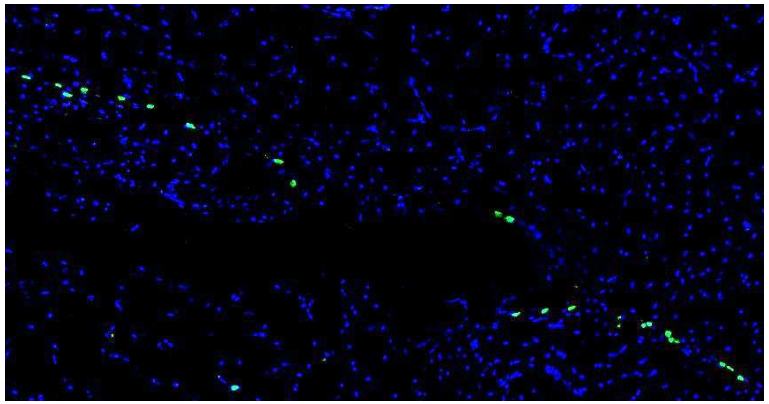
Major input : Large scale analysis, tissular and cellular analysis

Tissular investigation

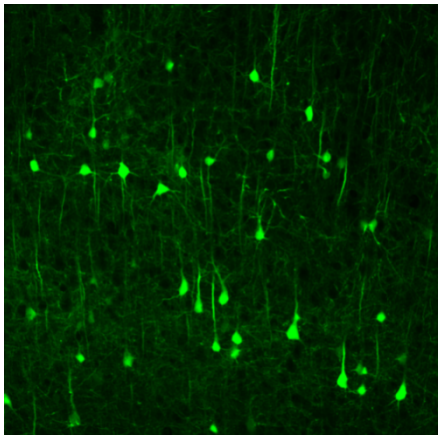
Gene therapy, brain



Cell therapy, muscle

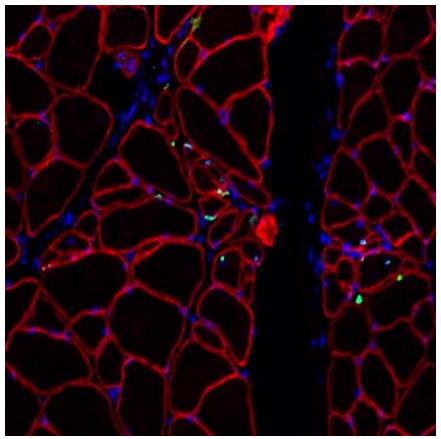


Cellular investigation



J. Hordeau et al., SFTC 2011, Nantes, France.

Human lamin A/C immunolabeling on muscle sections

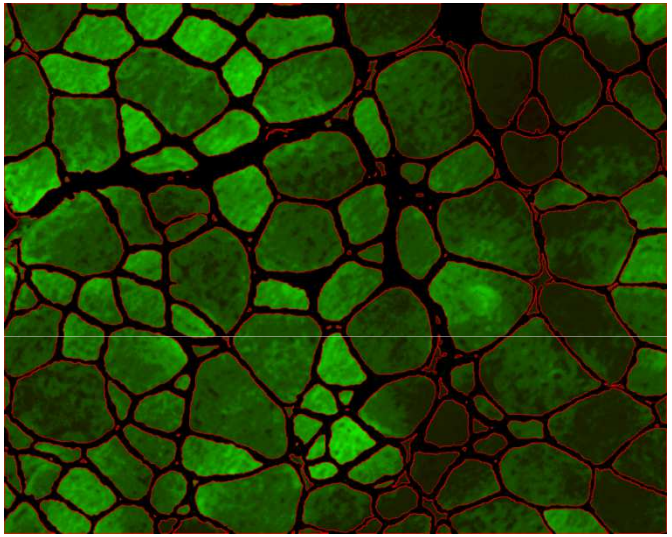


Lamin A/C (green), dystrophin (red)

F. Robriquet et al, ESGCT and FSCGT 2012, Versailles, France

Applications of the wide-field fluorescent microscopy

Quantification of fluorescence



- Number of transduced fibers

- Fluorescence Intensity

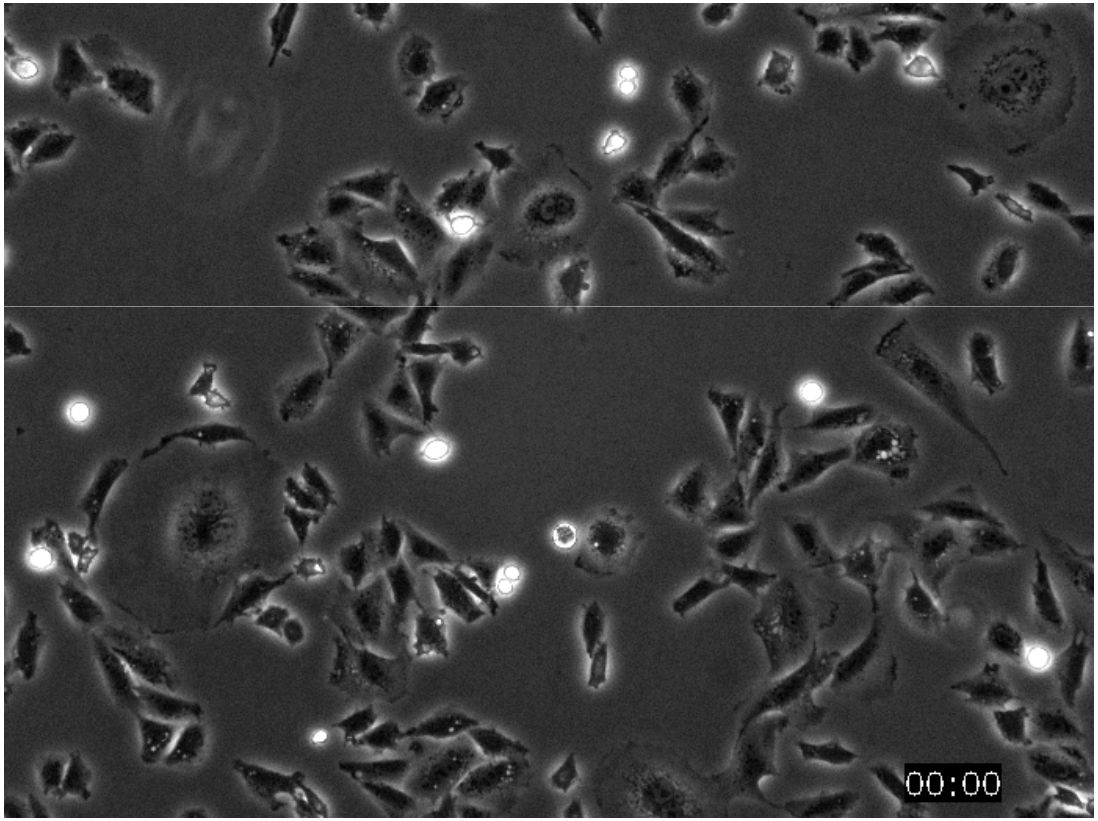
Cryosections of muscle injected with
AAV2/8 RSV-eGFP

Difficulties : Heterogeneity of the GFP fluorescence intensity, saturation of signal, same threshold for all the specimens, quality of the sections.

Applications of the wide-field fluorescent microscopy

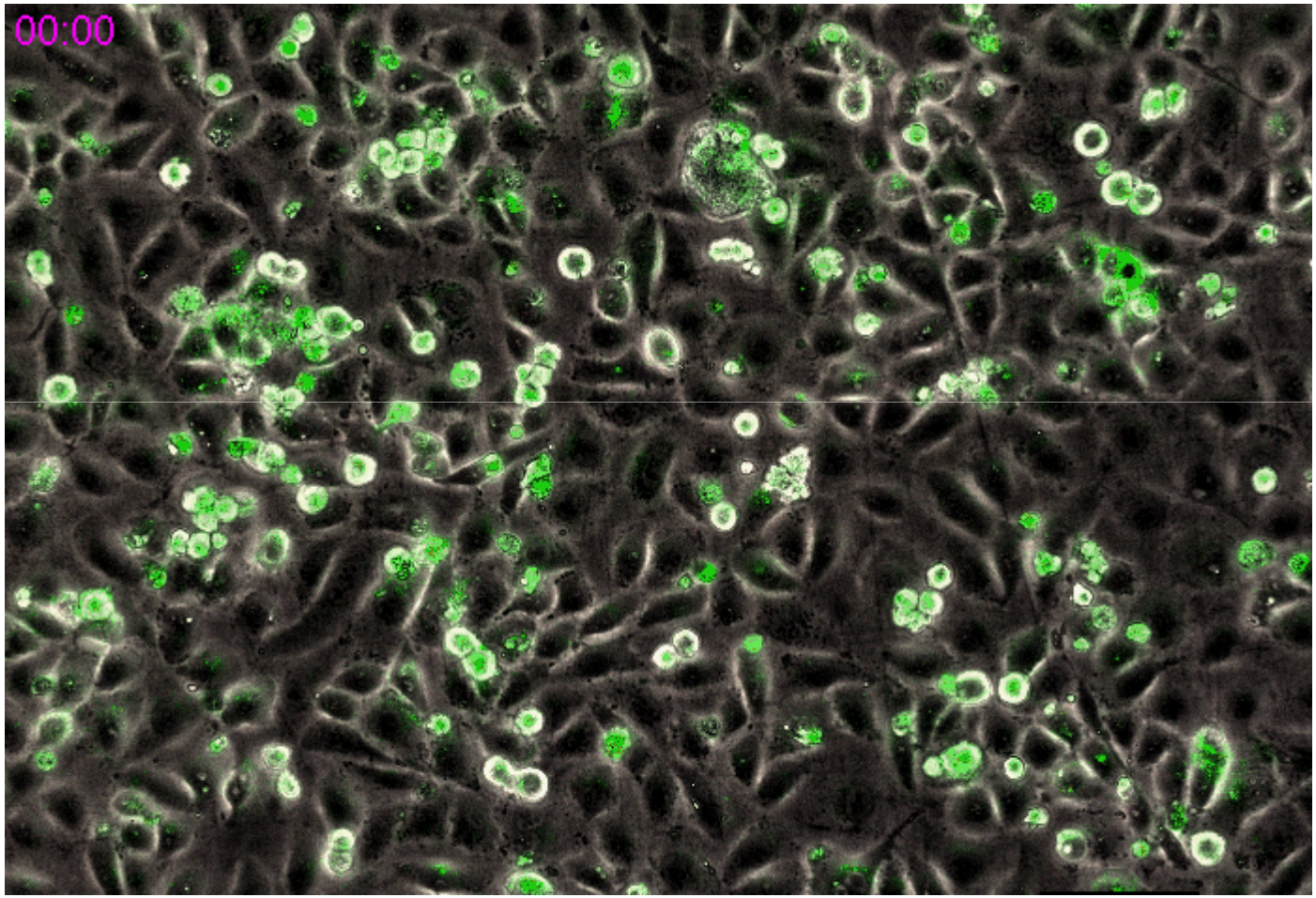
Cellular « ethology » by video microscopy

-Time lapse



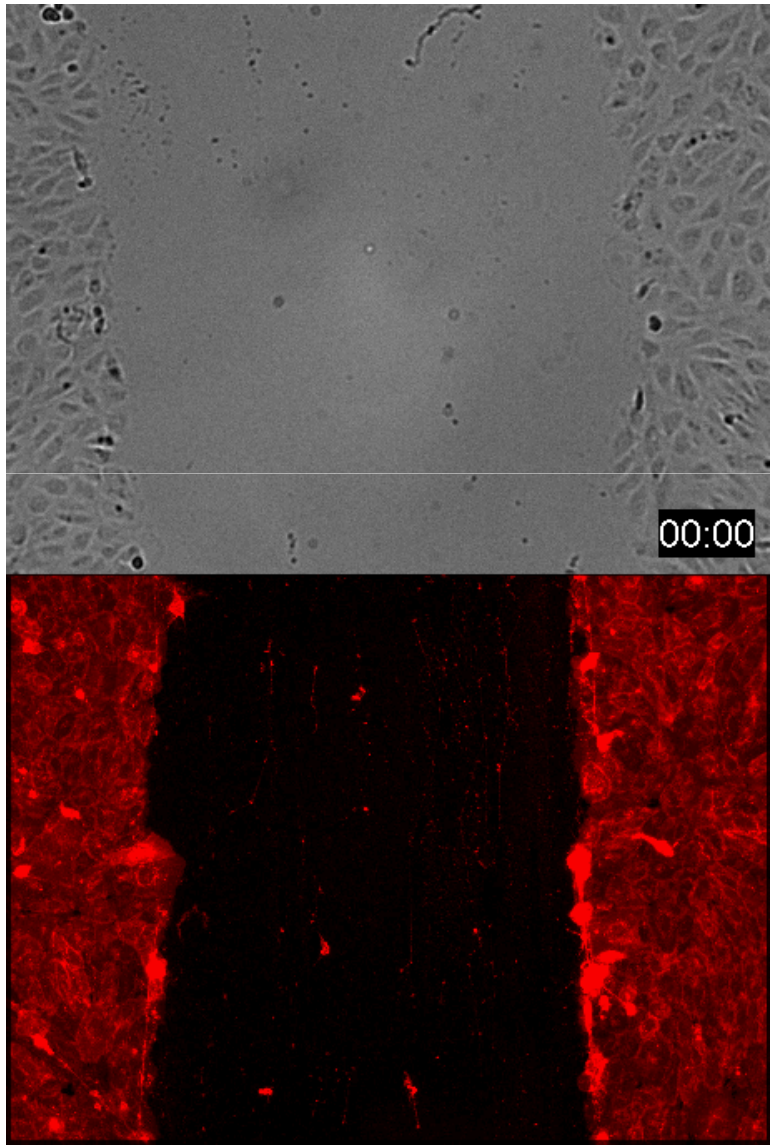
P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy



P. Hulin, MicroPIcell

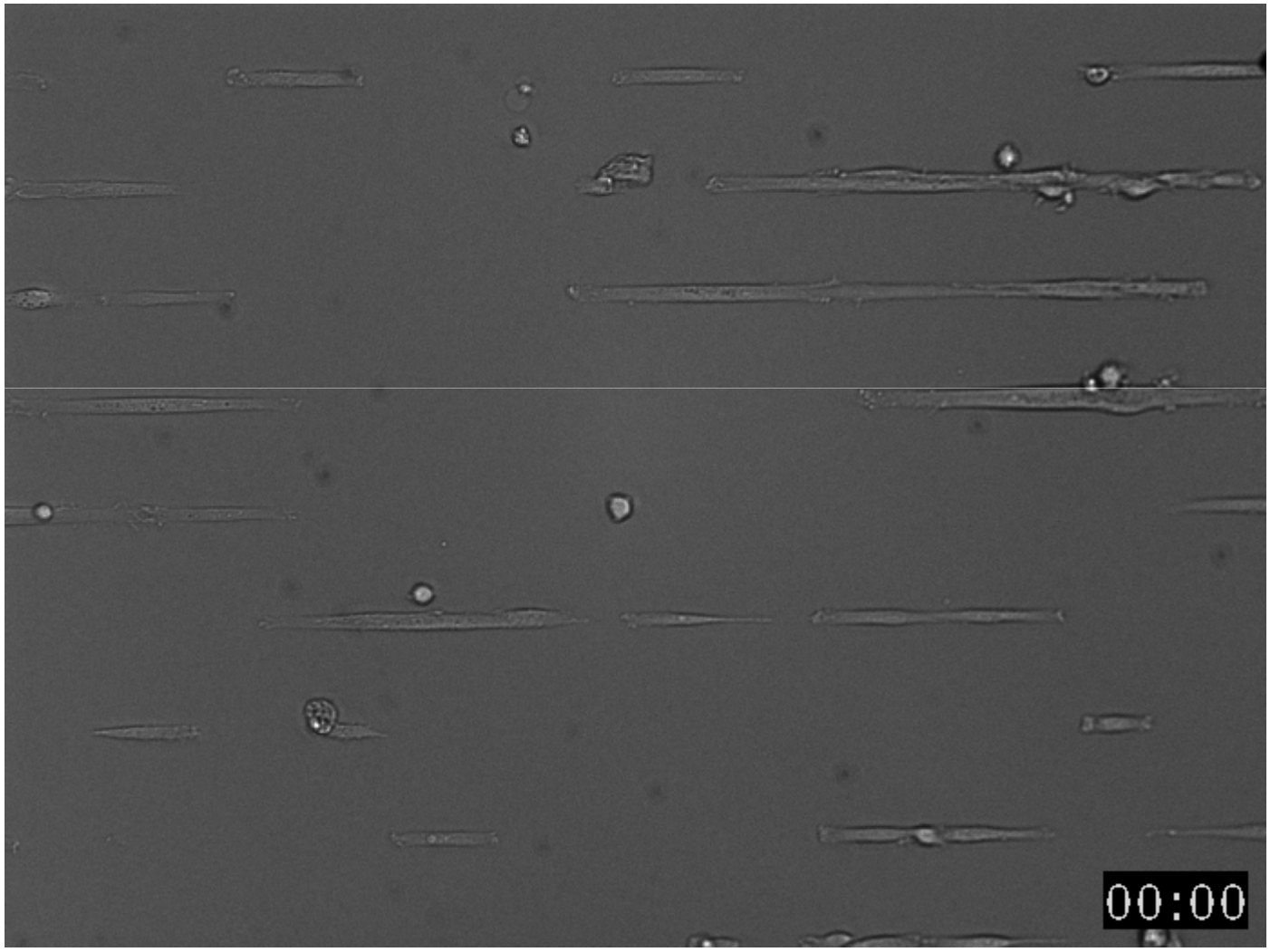
Applications of the wide-field fluorescent microscopy



P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy

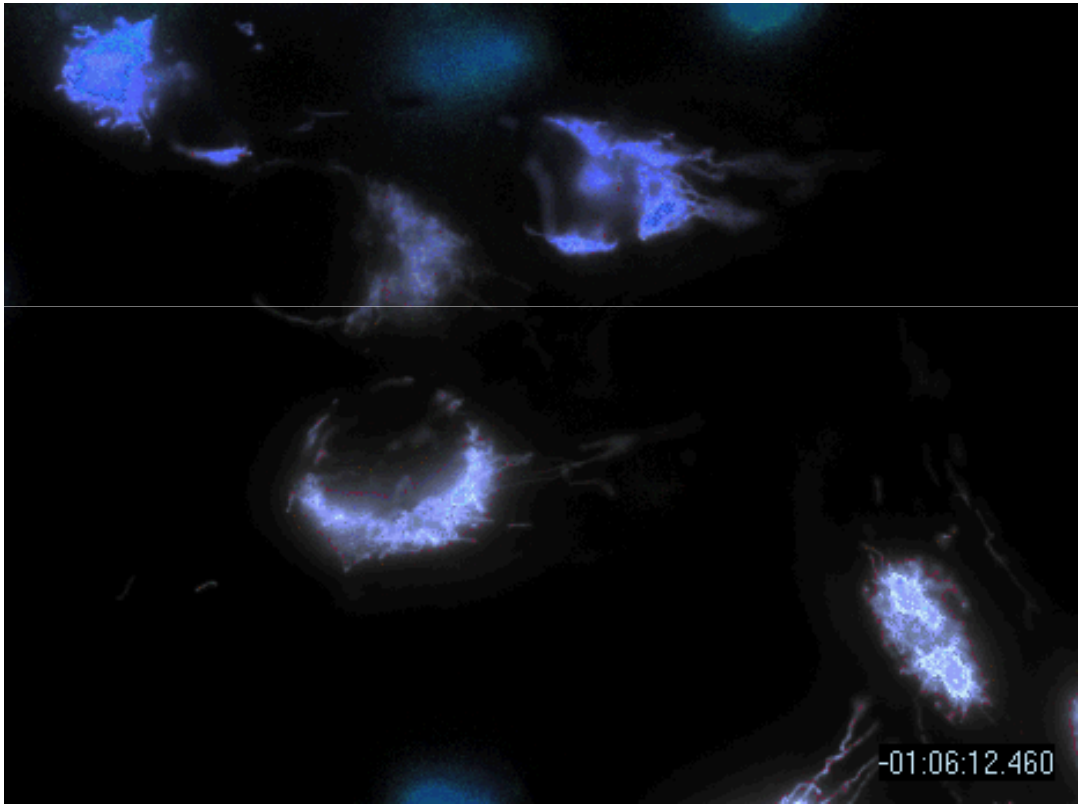
-Cytoo motility



P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy

-Calcium



Wide-field fluorescence microscopy

High sensitivity

Mercury Light source less energetic than Laser (less photobleaching)

Less expensive than confocal

Multilabeling

Fast acquisition

Video microscopy

Fluorescent emission from all the specimen and not from the focal plane

Limited by the thickness of the specimen

Resolution = $0{,}6\lambda/NA$

Confocal microscopy

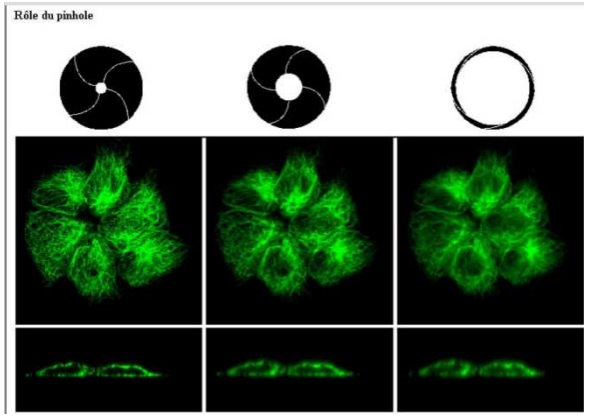
Major input : Increasing of the resolution and monochromatic excitation

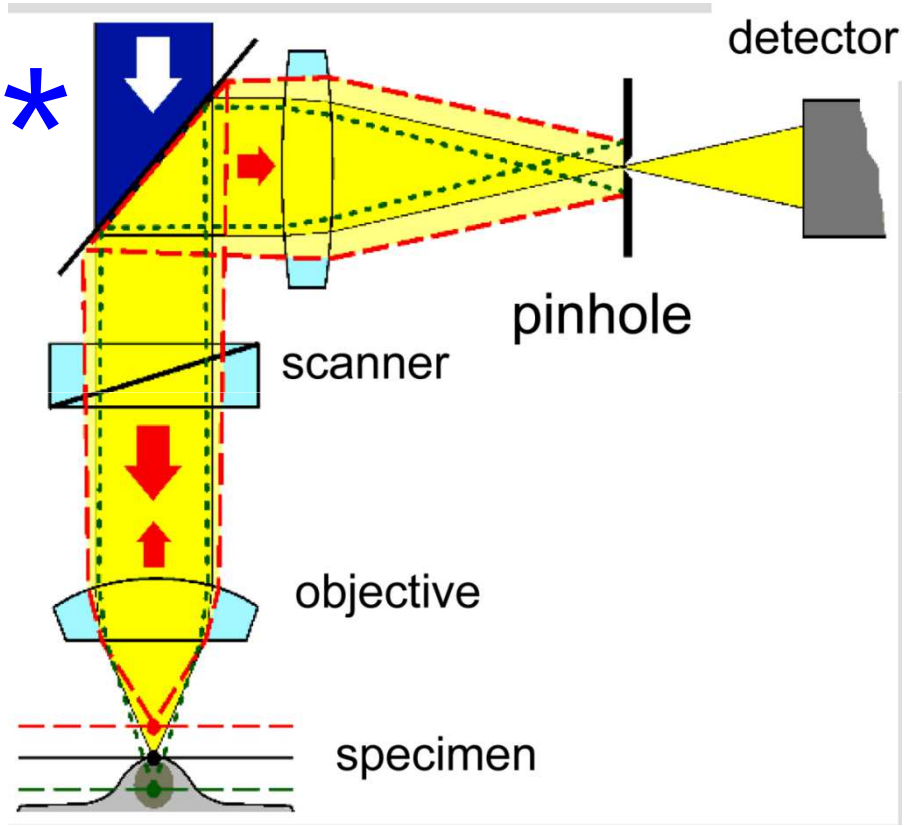
$Rxy = 0,4\lambda/NA$

$Rz = 1,4\lambda/NA^2$

NA : Numerical Aperture : 1,4
λ: 488 nm

dxy = 140 nm
dz = 348 nm

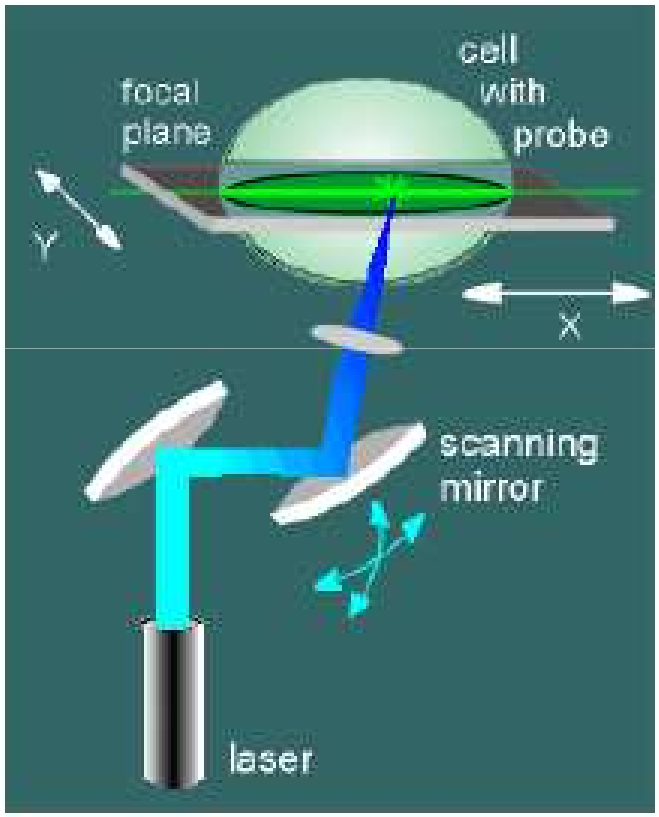




* Monochromatic excitation : Lasers (HeNe), (Arg) ou Diodes

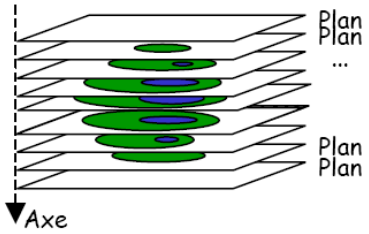
Scanning mirrors

Major input : rate of scanning and 3D imaging



Resonant scanning

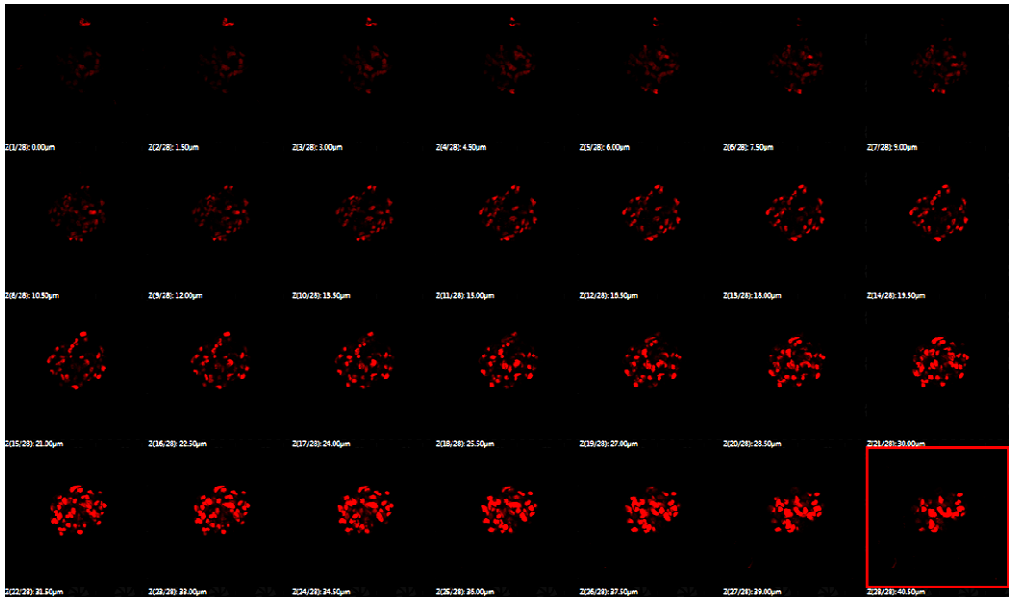
3D building



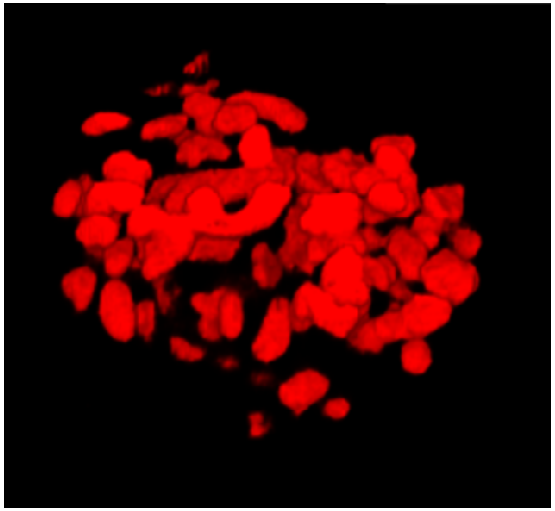
Applications of the confocal microscopy in cell therapy

3D cellular imaging

Z stack from cultured Mustem Cells

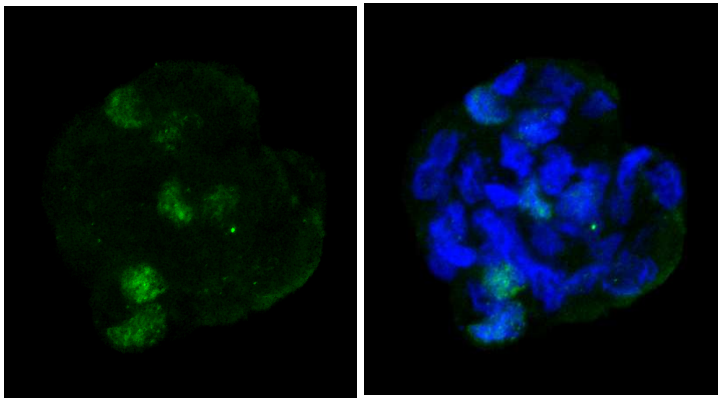


Confocal 3D imaging of cultured cells



Myosphere, Z projection

Blue : Nuclei
Green : Myod1



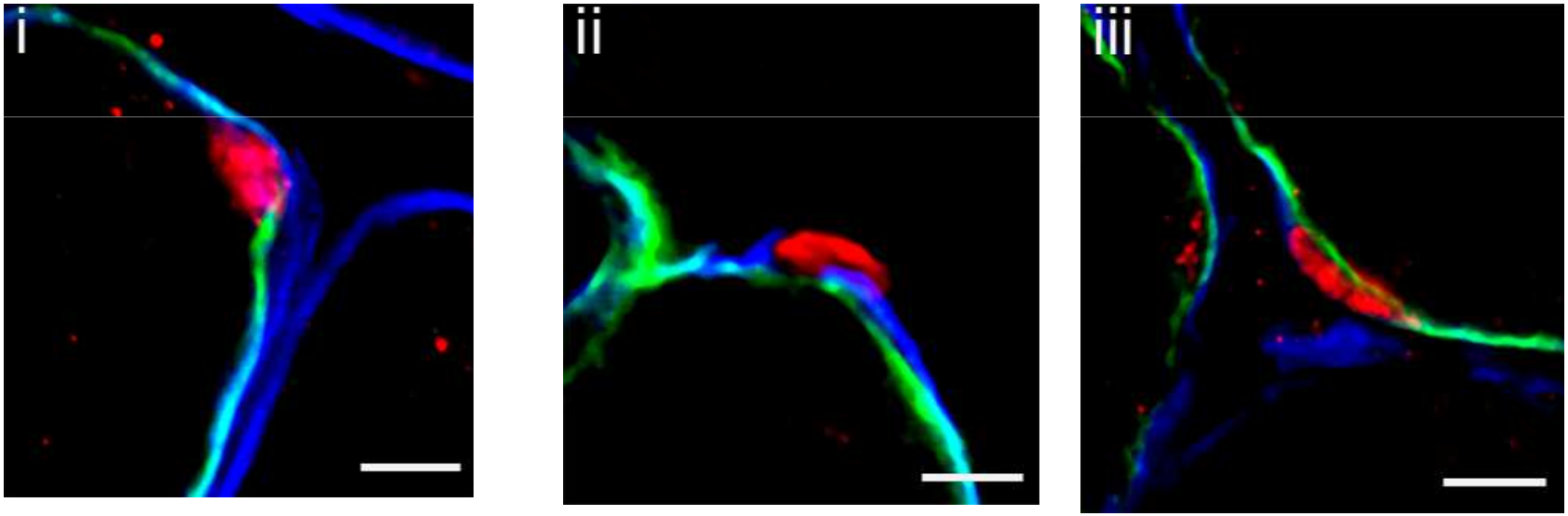
K. Rouger et al., Am J Pathol. 2011, 179(5):2501-2518

Applications of the confocal microscopy in cell therapy

Subcellular localisation

Detection of Mustems after their injection in the muscle

Muscle section, Z projection



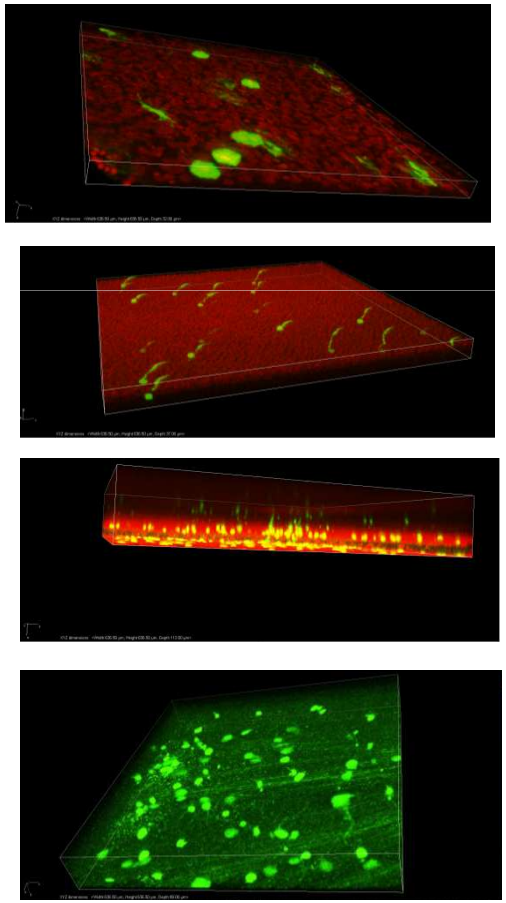
Triple immunolabeling, dystrophine (green), bgal (red), laminine(blue).

K. Rouger et al., Am J Pathol. 2011, 179(5):2501-2518

Applications of the confocal microscopy in gene therapy

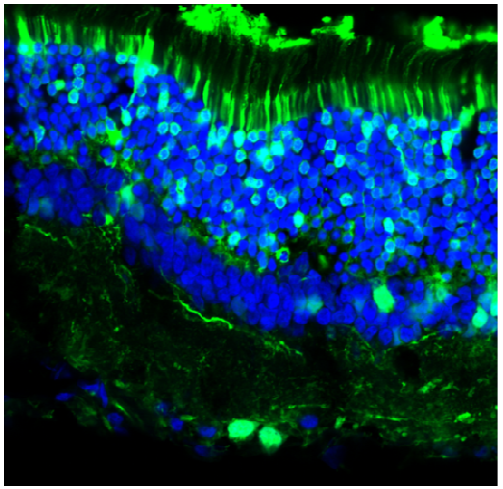
Investigation on wholemount retina

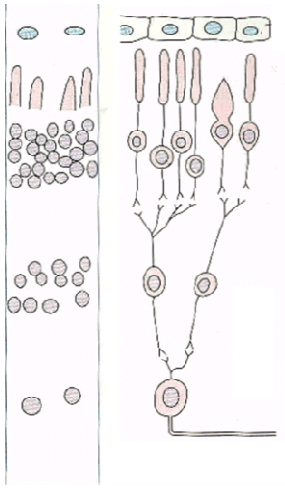
Confocal 3D imaging of retina



Retinal wholemount

Cartography of GFP expression in the retina after IV injection of AAV10-egfp (newborn rats)

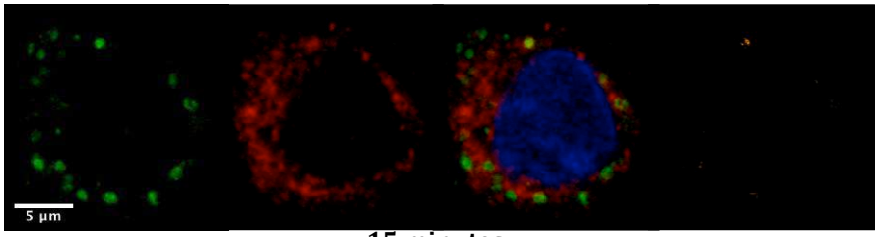




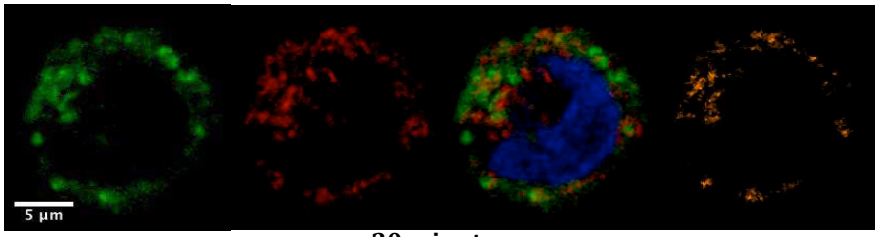
Cryosections of retina : Transduced ganglion cells are underestimated compared to the numerous transduced ganglion cells observed on retinal wholemount (non invasive method).

Applications of the confocal microscopy

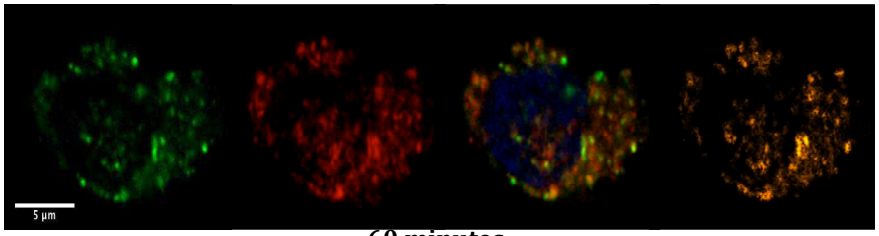
SLP$_{16-40}$-FITC Lysosomes Overlay Colocalization



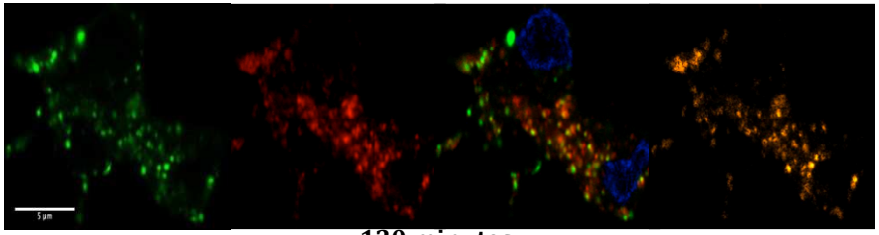
15 minutes



30 minutes



60 minutes

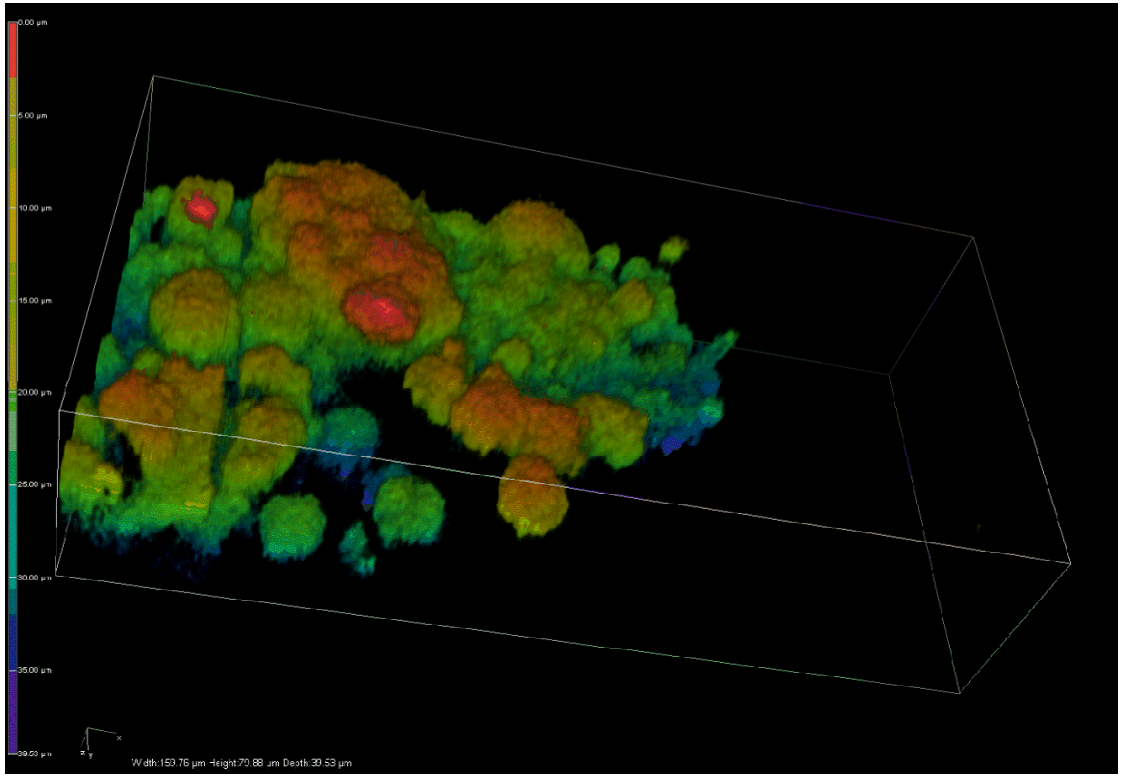


120 minutes

P. Hulin, MicroPIcell

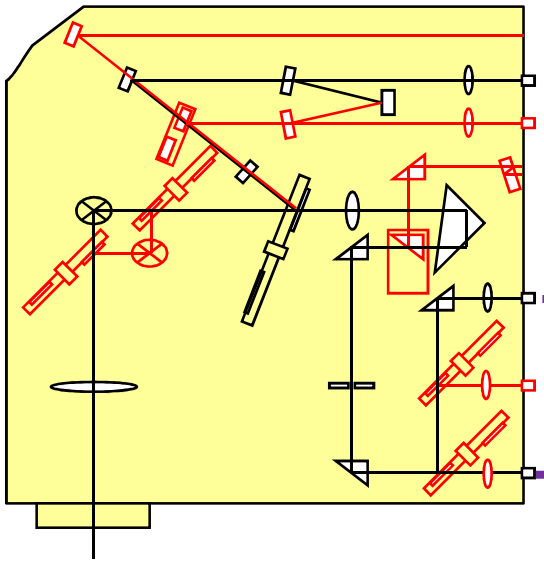
Applications of the confocal microscopy

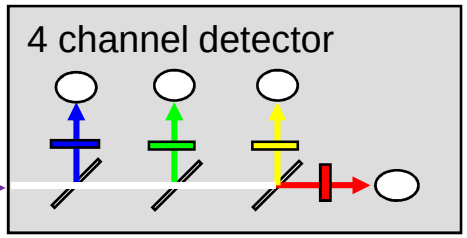
cardiomyocytes



Spectral Confocal microscopy

Major input : spectral separation of emission light





Conventional PMT (4) : bandwidth 50 nm

Système classique PMT

spectral PMT (32) : bandwidth 2,5; 6; 10 nm

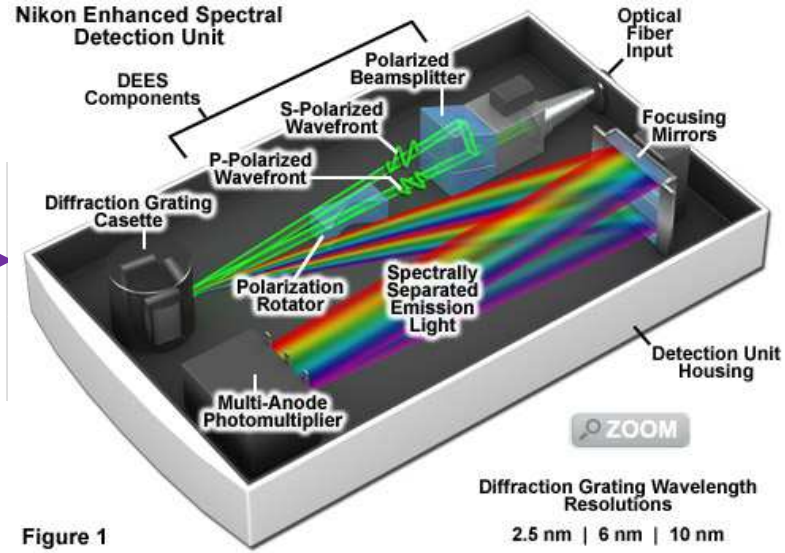
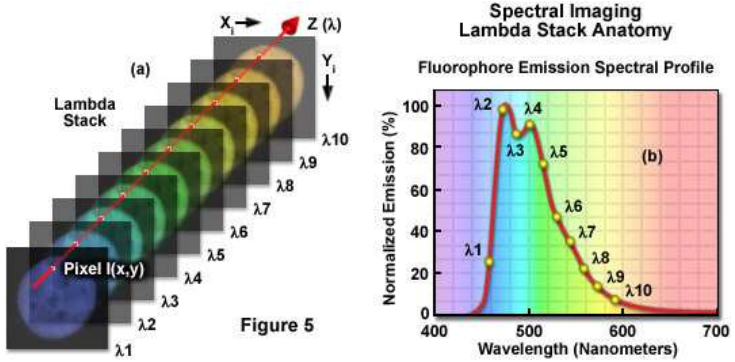


Figure 1

Applications of spectral confocal microscopy

Major Input : specificity of signal by spectral analyses



Separation of fluorescent probe with close emission spectra

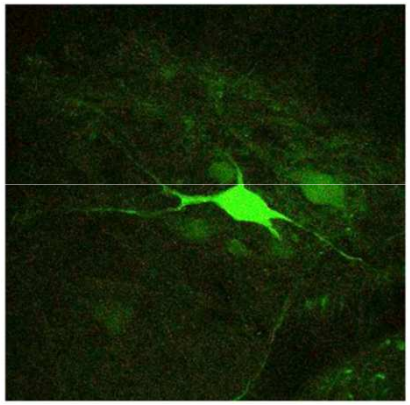
Multilabeling not limited to the 4 PMT

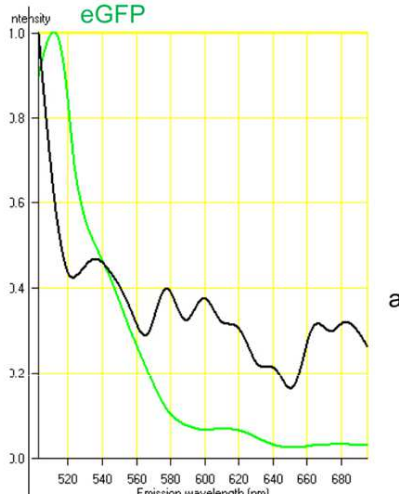
Studies of molecular interactions (ligand and receptor) by transfert of fluorescence (FRET)

Spectral Confocal microscopy

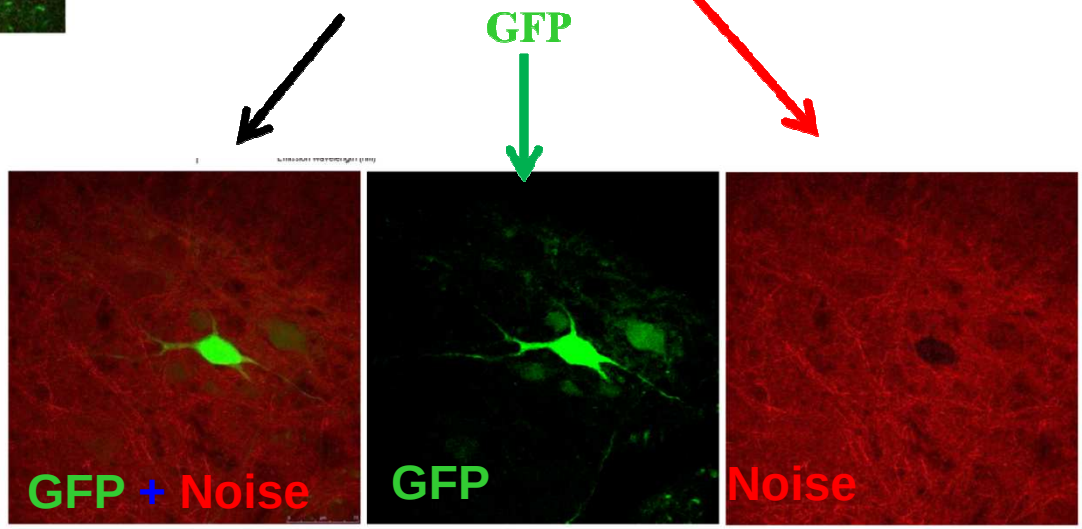
Separation of fluorescent probe with close emission spectra

Image without unmixing





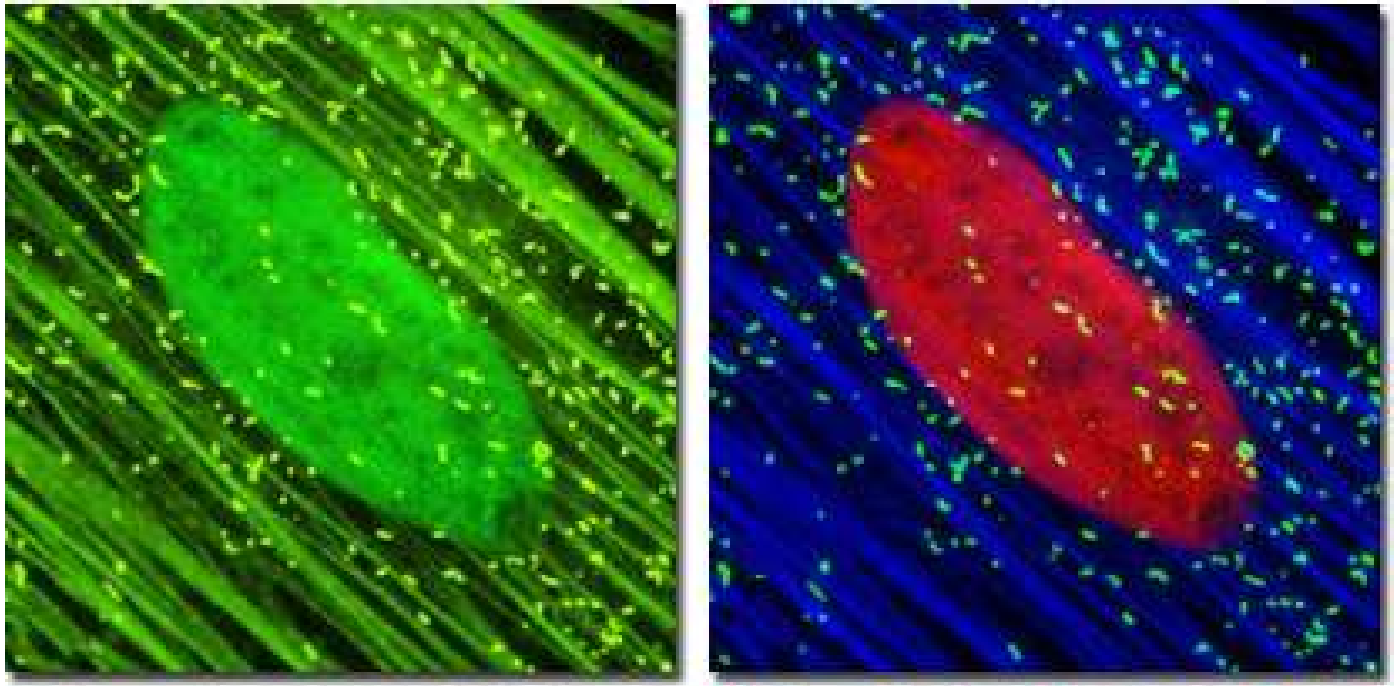
Spectral imaging and linear unmixing separate specific eGFP signal from noise (autofluorescence) generated by chemical fixation of the tissues



L. Dubreil, APEX UMR 703

Spectral Confocal microscopy

Triple immunolabelling with green probes (P. Hulin)



Nucleus-> SYTOX Green (noyau),
Actine -> Alexa Fluor 488
Peroxysomes -> Alexa Fluor 514

P. Hulin, MicroPIcell

Spectral confocal microscopy

Reduction of blurring

Increasing of resolution : 30% xy ; 30% z

Increasing of signal/noise

Observation up to 100 µm (thickness of specimen)

Light source : laser ; decrease of cross talk

3D imaging

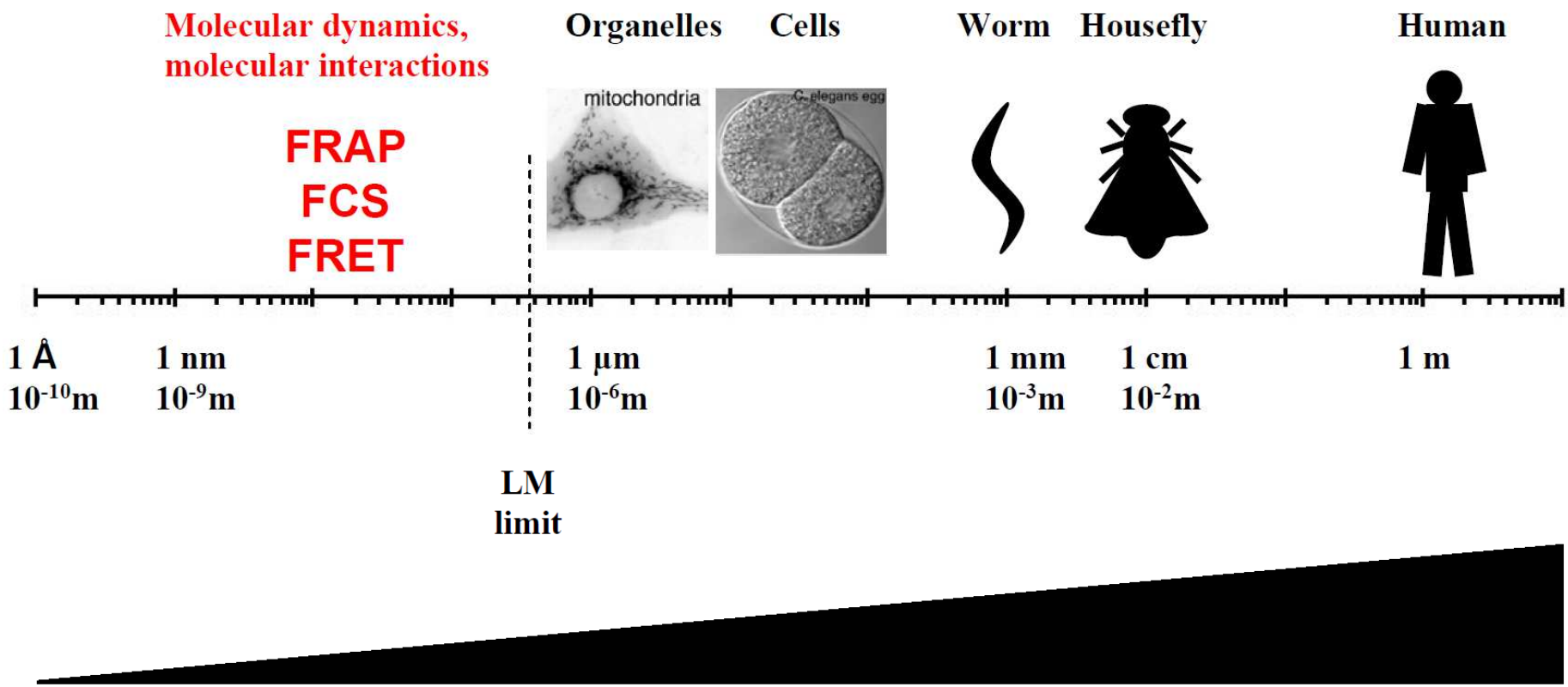
Linear unmixing

Excitation of all specimen planes, photobleaching

Observation above100 µm not possible (thickness of specimen)

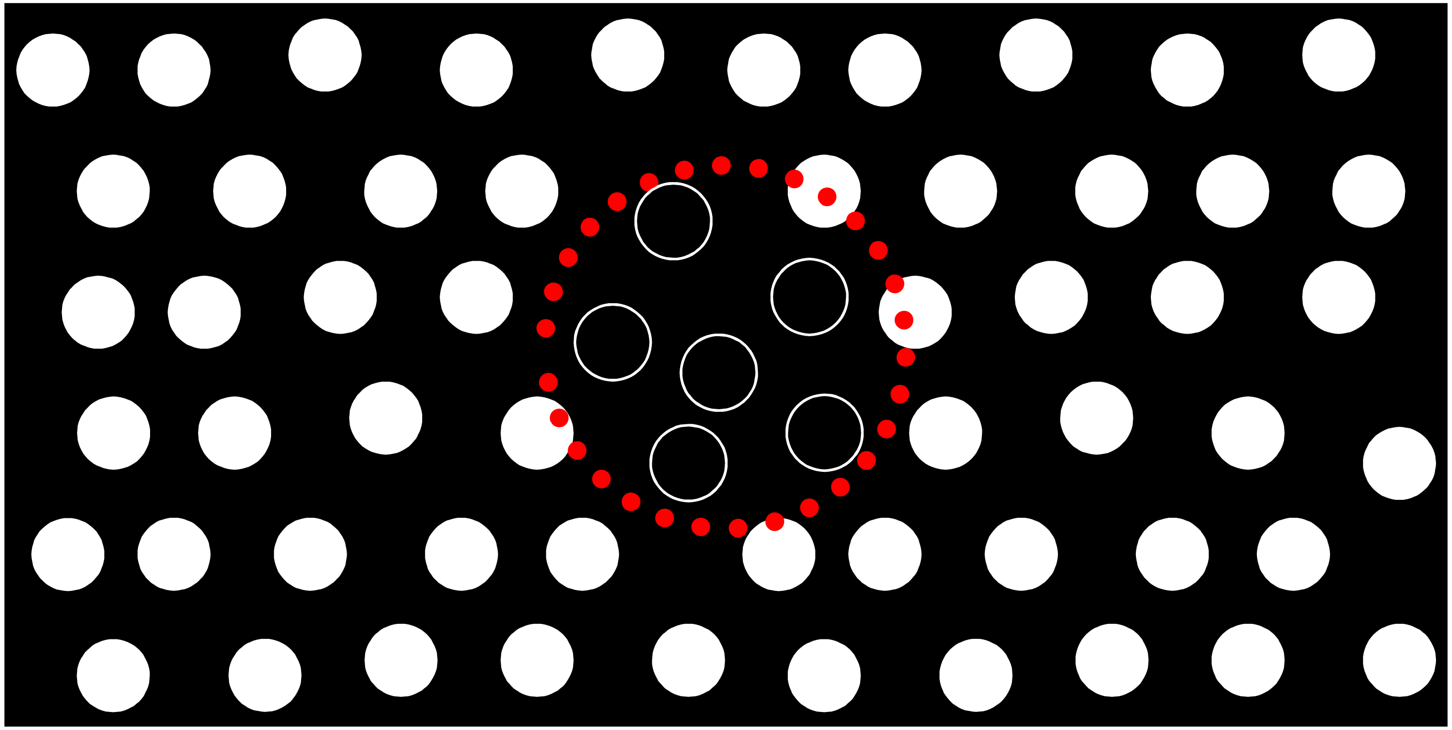
Fast acquisition : resonant scanning

« F words of kinetic microscopy: FRAP, FLIM/FRET, FCS »



Recent developments in advanced microscopy techniques, the so-called F-techniques, including Förster resonance energy transfer, fluorescence correlation spectroscopy and fluorescence lifetime imaging, have led to a wide range of novel applications in biology. The F-techniques provide quantitative information on biomolecules and their interactions and give high spatial and temporal resolution. In particular, their application to receptor protein studies has led to new insights into receptor localization, oligomerization, activation and function *in vivo*.

Fluorescence Recovery After Photobleaching (FRAP)

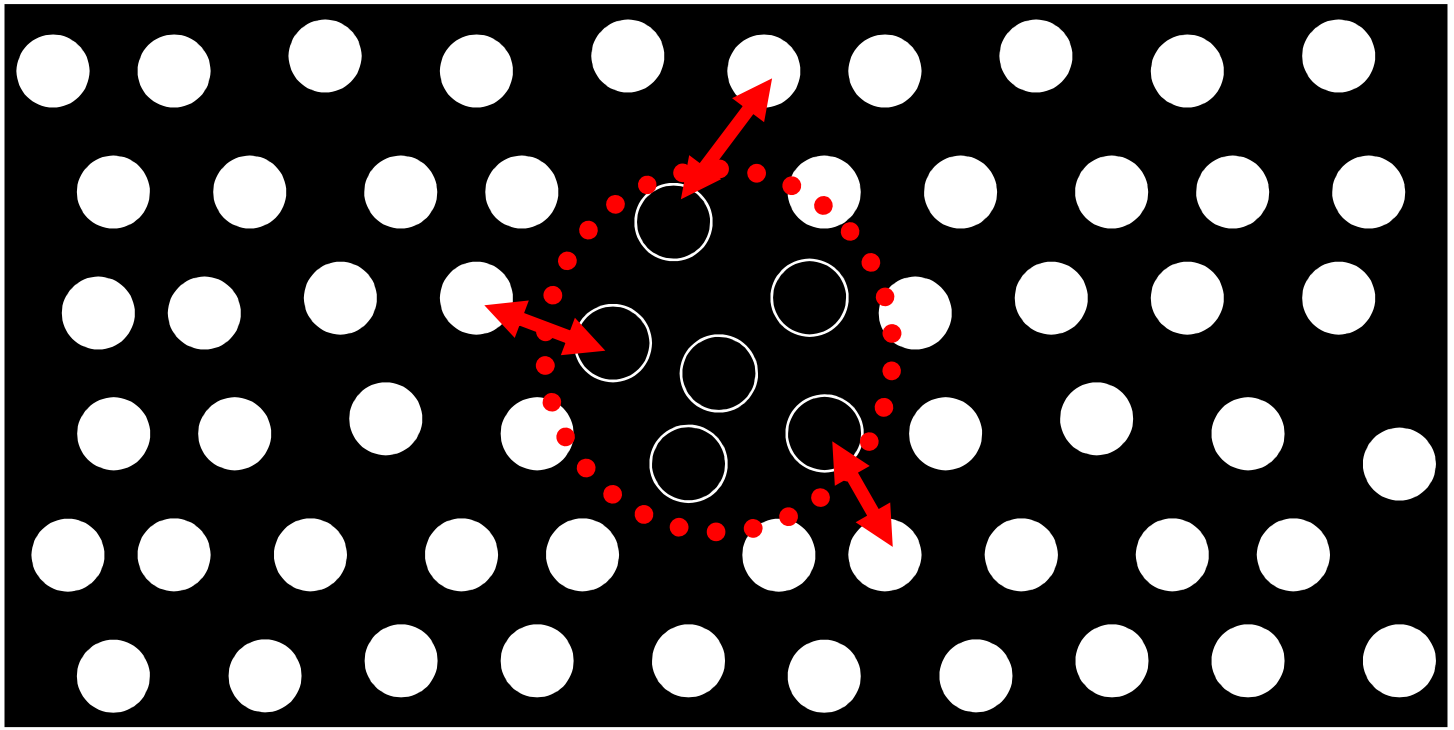


Kota Miura, EMBL Heidelberg, Germany. Based on the EAMNET Practical Course (20, April, 2004)

Let's think of fluorescence molecules dispersed in the field. White circles represent the molecules.

We focus a strong laser and BLEACHES (erase the fluorescence) at that spot.

Fluorescence Recovery After Photobleaching (FRAP)

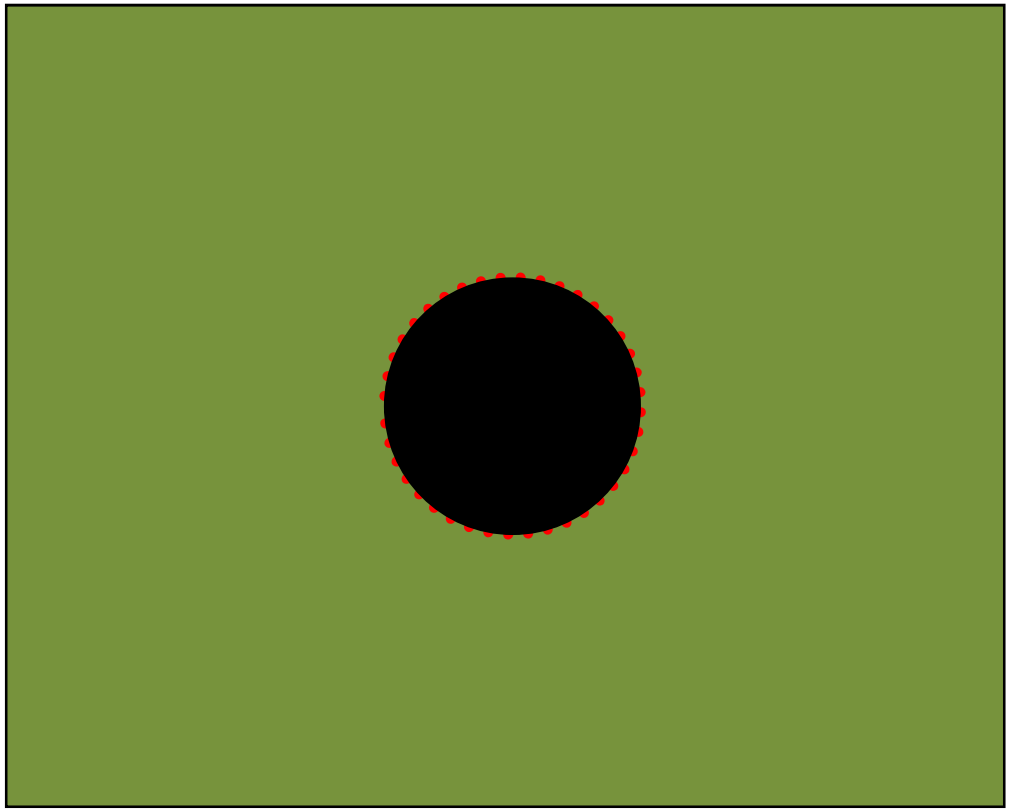


Kota Miura, EMBL Heidelberg, Germany. Based on the EAMNET Practical Course (20, April, 2004)

Since molecules are moving driven by diffusion or active transport,
bleached molecules exchange their place with un-bleached molecules.

Then the average intensity at the bleached spot recovers.

Fluorescence Recovery After Photobleaching (FRAP)

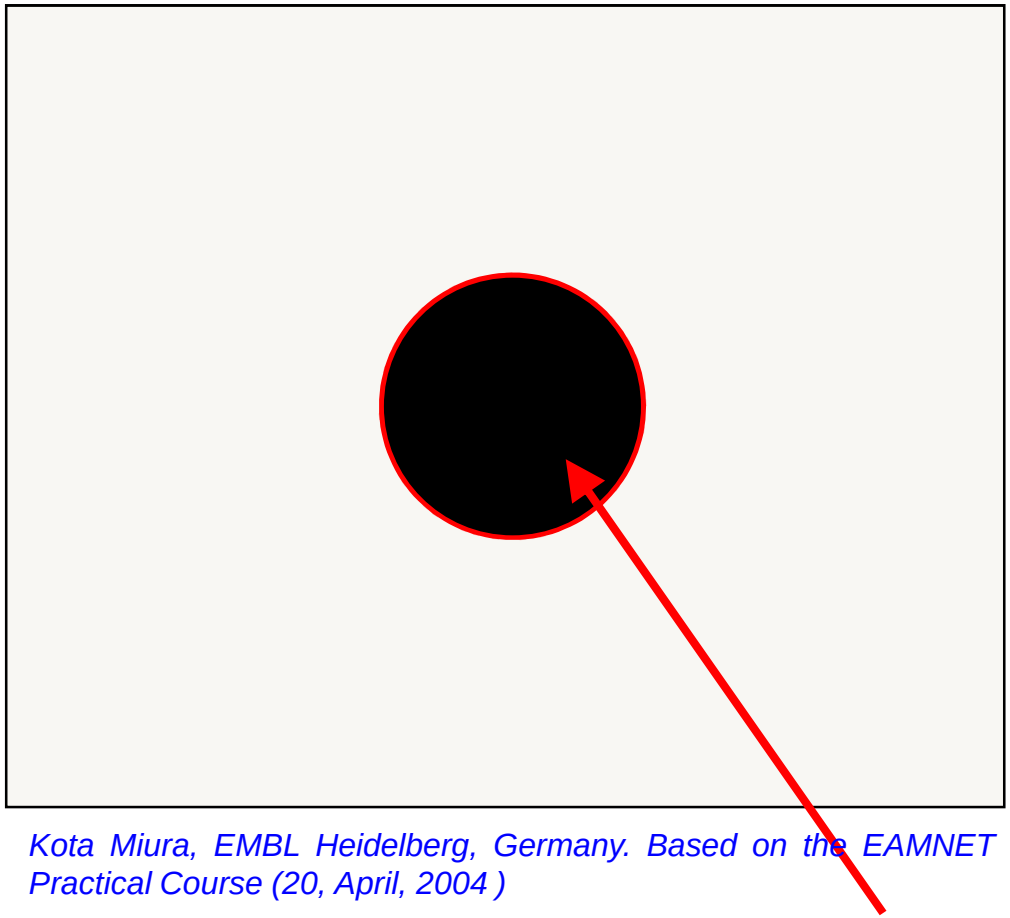


Kota Miura, EMBL Heidelberg, Germany. Based on the EAMNET Practical Course (20, April, 2004)

In practice, the FRAP at first looks like the following. Above is a microscope field filled with fluorophores.

Fluorescence Recovery After Photobleaching (FRAP)

Quantitative Analysis of FRAP

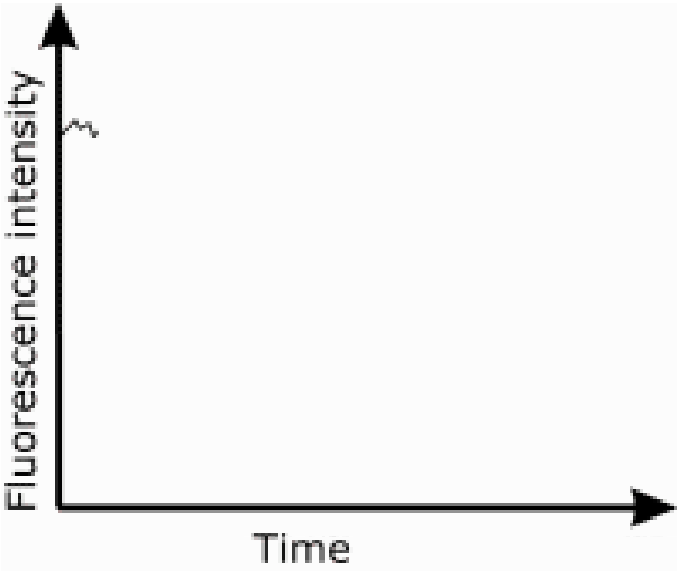


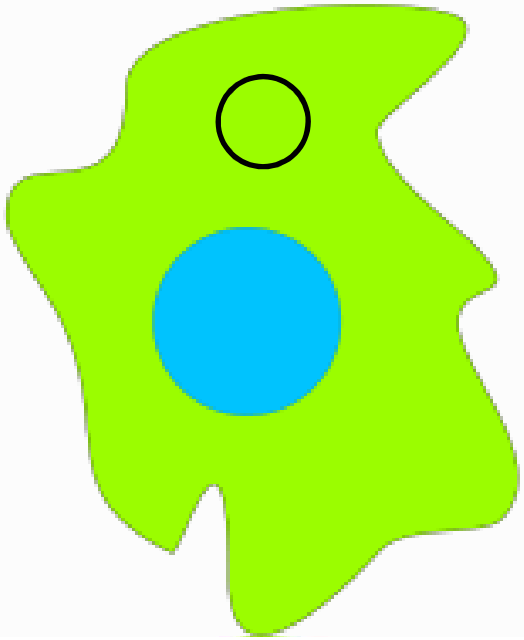
Kota Miura, EMBL Heidelberg, Germany. Based on the EAMNET Practical Course (20, April, 2004)

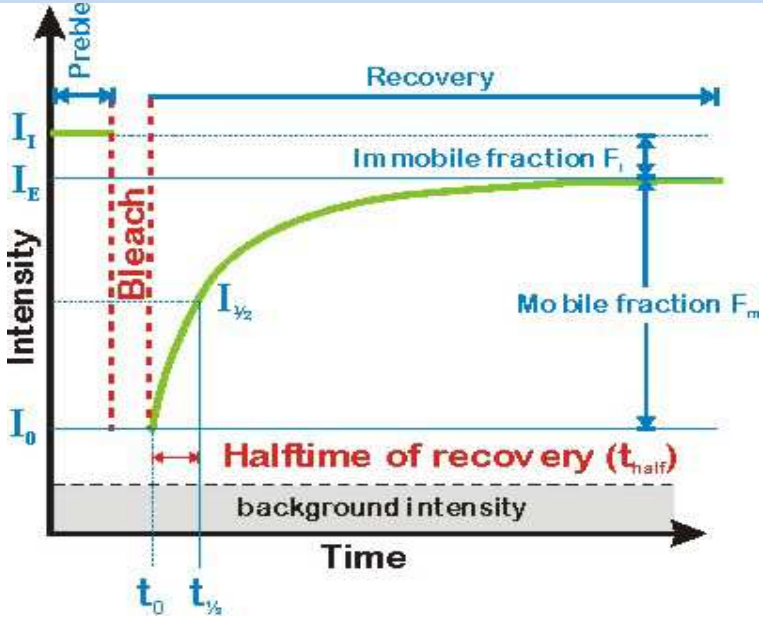
To gain information on molecular dynamics, time-course of the fluorescence recovery must be measured.

Measure the temporal changes of the fluorescence Intensity!

Fluorescence Recovery After Photobleaching (FRAP)

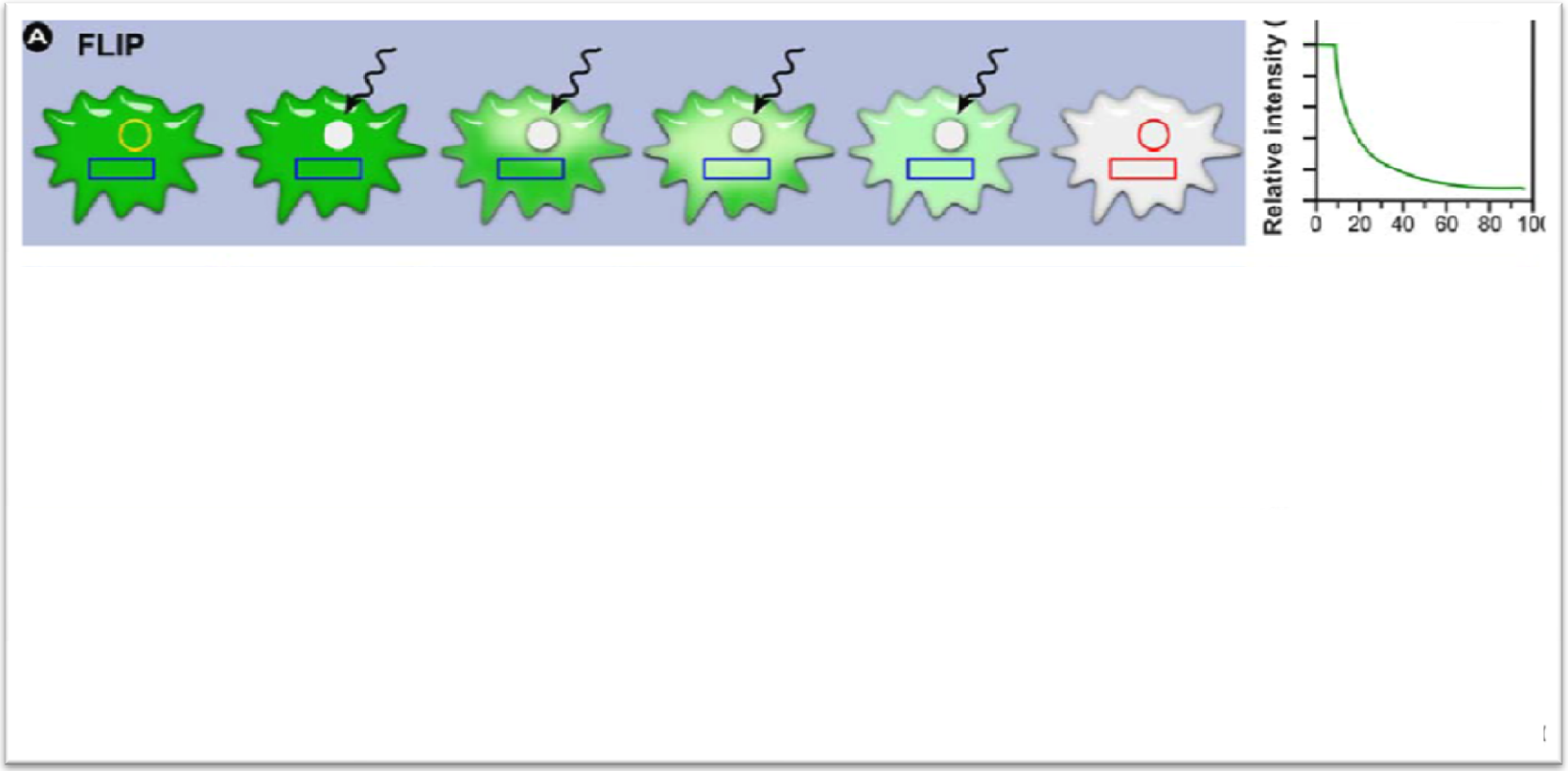






An idealized plot of a FRAP recovery curve.
I_I: initial intensity
I_0: intensity at timepoint t_0 (first postbleach intensity)
$I_{1/2}$: half recovered intensity ($I_{1/2} = (I_E - I_0) / 2$)
I_E: endvalue of the recovered intensity
t_{half}: Halftime of recovery corresponding to $I_{1/2}$ ($t_{1/2} - t_0$)
Mobile fraction **F_m** = $(I_E - I_0) / (I_I - I_0)$
Immobile fraction **F_i** = 1 - Fm

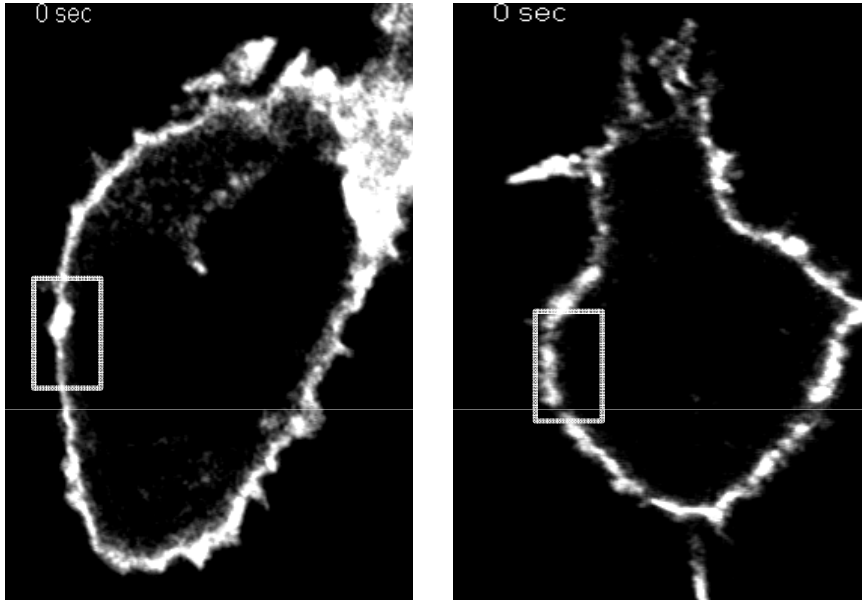
Fluorescence Recovery After Photobleaching



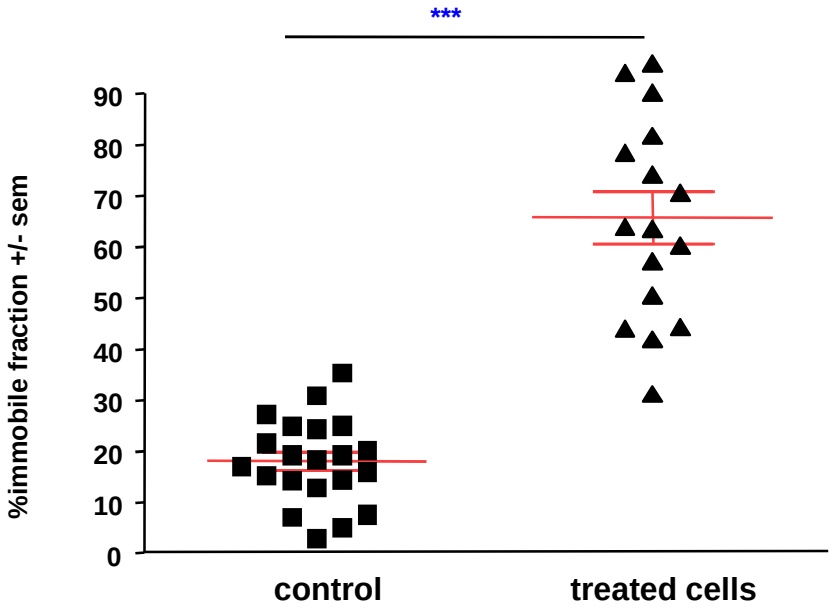
FLIP (fluorescence loss in photobleaching). In this technique, an area within the cell is repeatedly bleached and the loss of fluorescence in areas that are distant from the bleach area is monitored. The decline in fluorescence intensity in the surrounding regions is due to bleaching of fluorochromes that move through the ROI during the repetitive bleaching process.

Fluorescence Recovery After Photobleaching

control **Treated cell**

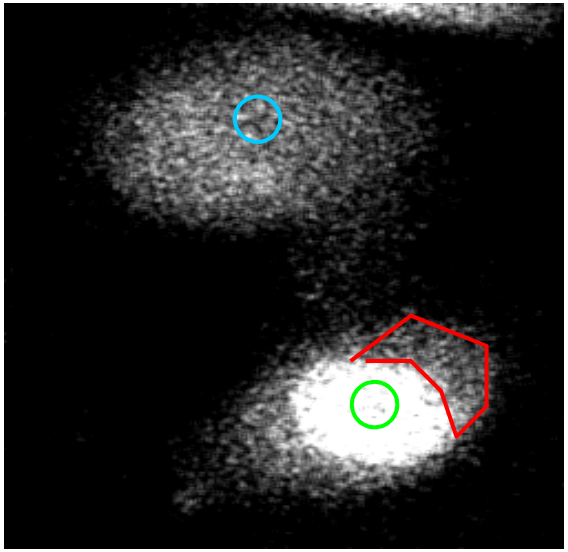


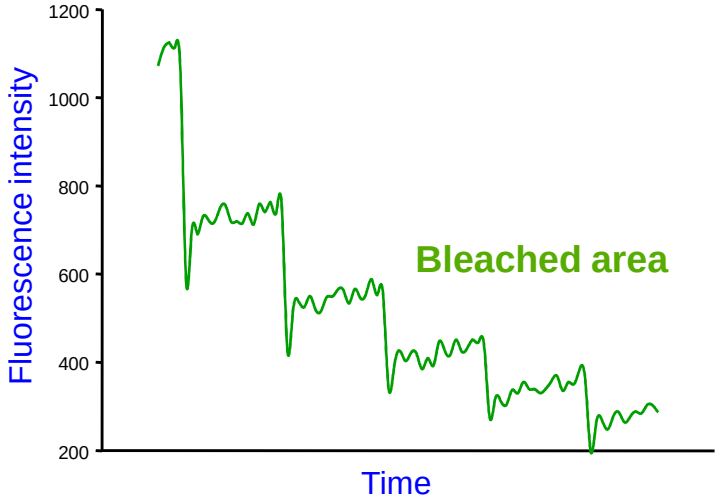
Monitored cells were transduced to express a fusion GFP-CD277 butyrophilin molecule

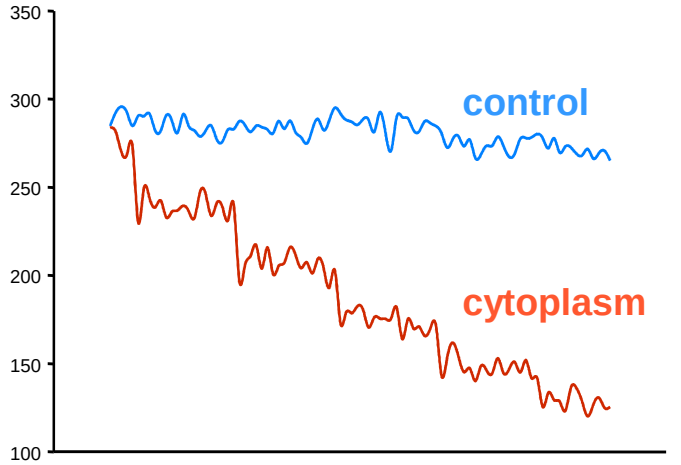


Control and treated cells display different patterns of GFP tagged protein membrane mobility.

Fluorescence Loss In Photobleaching (FLIP)







S. Nedellec, MicroPIcell

Fluorescence Recovery After Photobleaching

Recapitulating, FRAP is generally suitable to study and investigate:

- Protein/molecule movement and diffusion (diffusional speed).

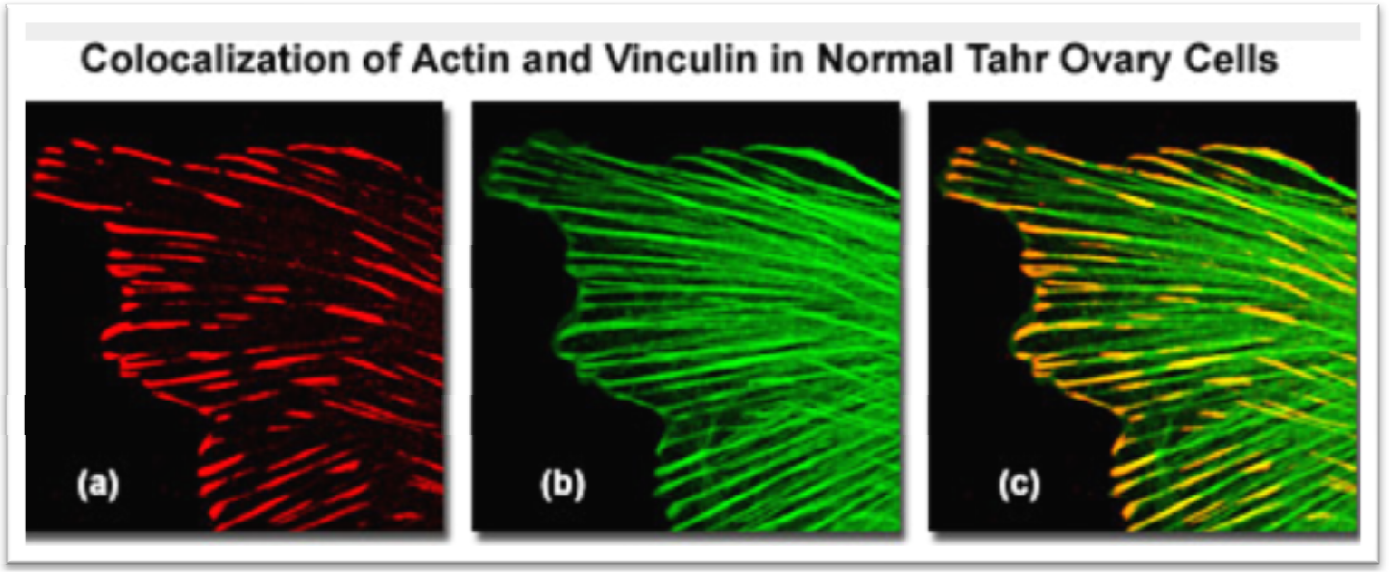
- Compartmentalization and connections between intracellular compartments.

- The speed of protein/molecule exchange between compartments (exchange speed).

- Binding characteristics between proteins. Additionally, the effect of mutations that alter individual amino acids on protein association, and the effect of small molecules, such as drugs or inhibitors, on protein pairs can effectively be studies using FRAP.

- Immobilization of proteins that bind to large structures, e.g., DNA, nuclear envelope, membranes, cytoskeletal elements, etc

Forster Resonant Energy Transfert (FRET) / Fluorescence Lifetime Imaging (FLIM): supplying co-localization

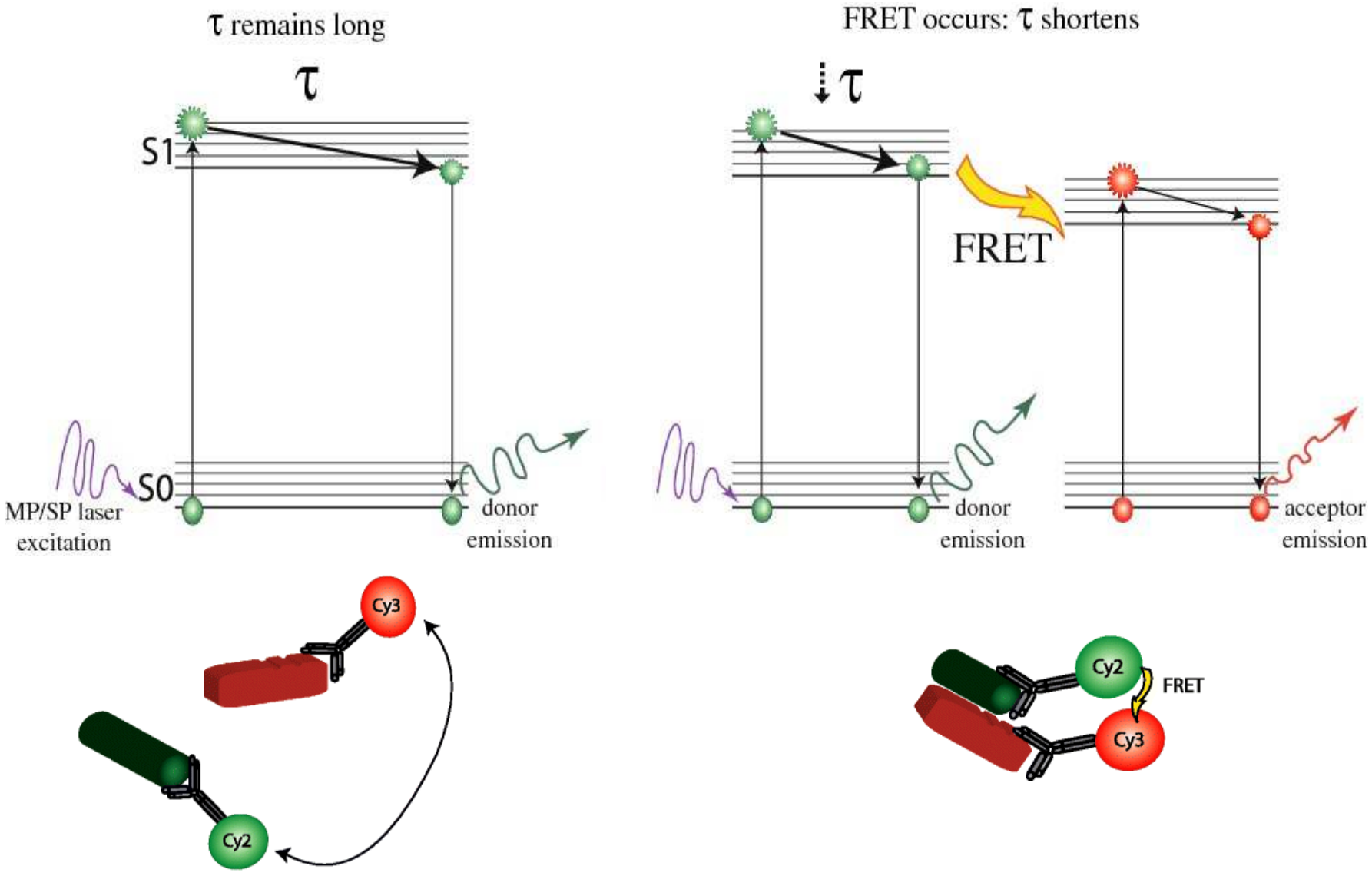


olympusconfocal.com

Colocalization, in a biological manifestation, is defined by the presence of two or more different molecules residing at the same physical location in a specimen. Overlay of both channels (red and green) displays a strong colocalization viewable in yellow.

The ability to determine colocalization in a confocal microscope is limited by the resolution of the optical system and the wavelength of light used to illuminate the specimen. Widefield fluorescence and confocal microscopes have a theoretical resolution of approximately 200 nanometers

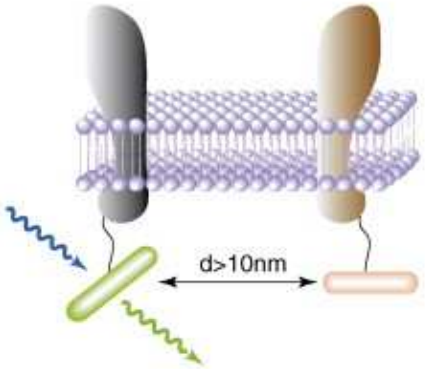
FLIM/FRET: supplying co-localization



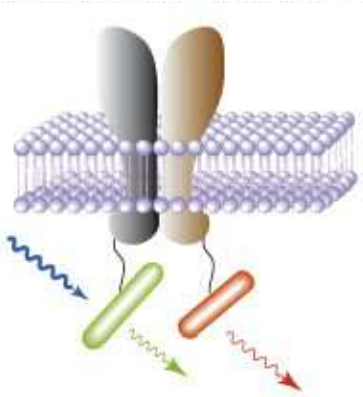
FLIM/FRET: supplying co-localization

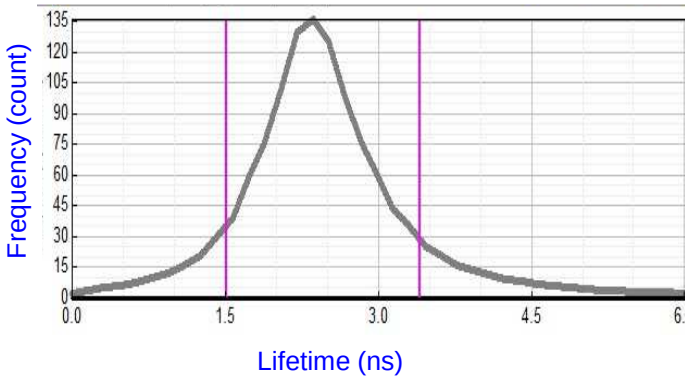
FLIM (Fluorescence Lifetime Imaging Microscopy) is a powerful technique to measure protein-protein interactions, and is based on the FRET (Forster Resonant Energy Transfert) principle, as shown below.

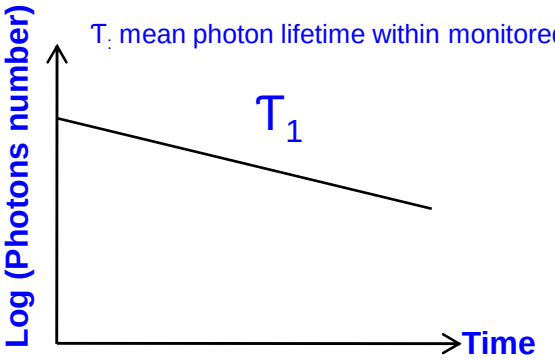
No FRET
d>10nm



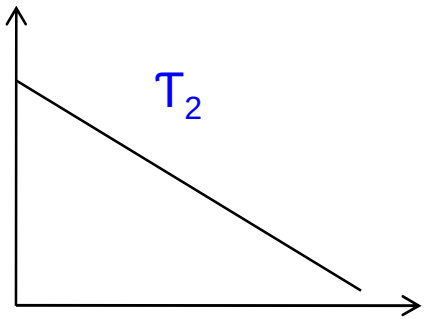
FRET d<10nm



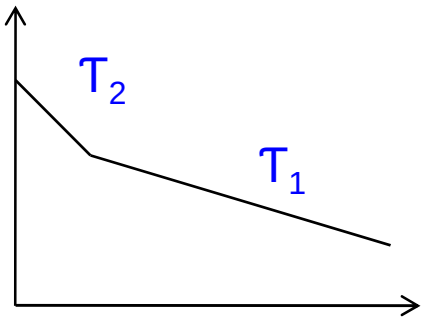




Mono exponential decline:
100% No FRET
(negative control condition)

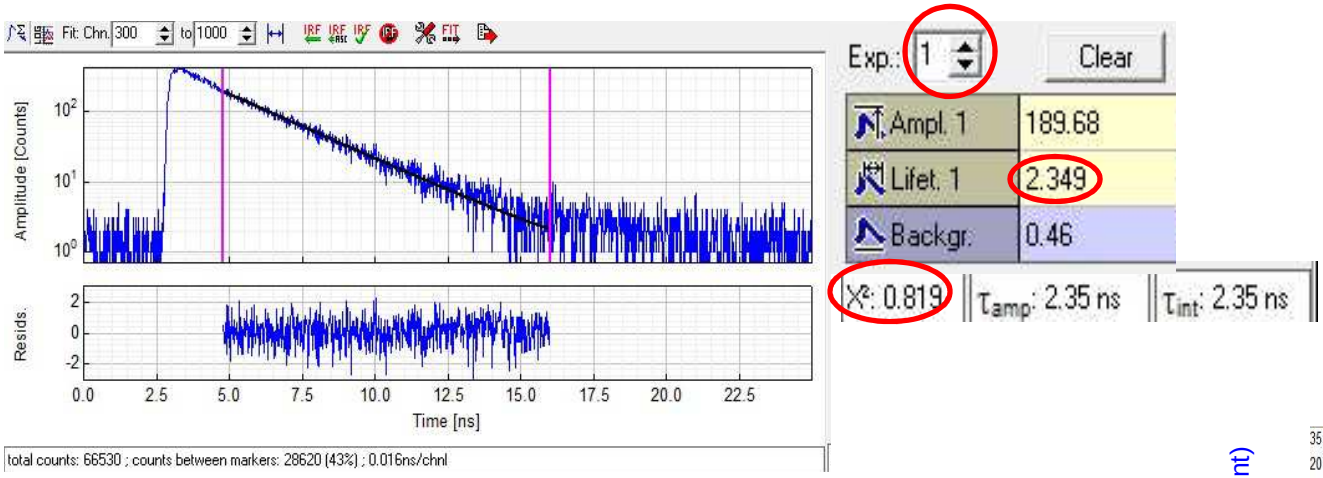


Mono exponential decline:
100% FRET (shorten lifetime)
(positive control condition)

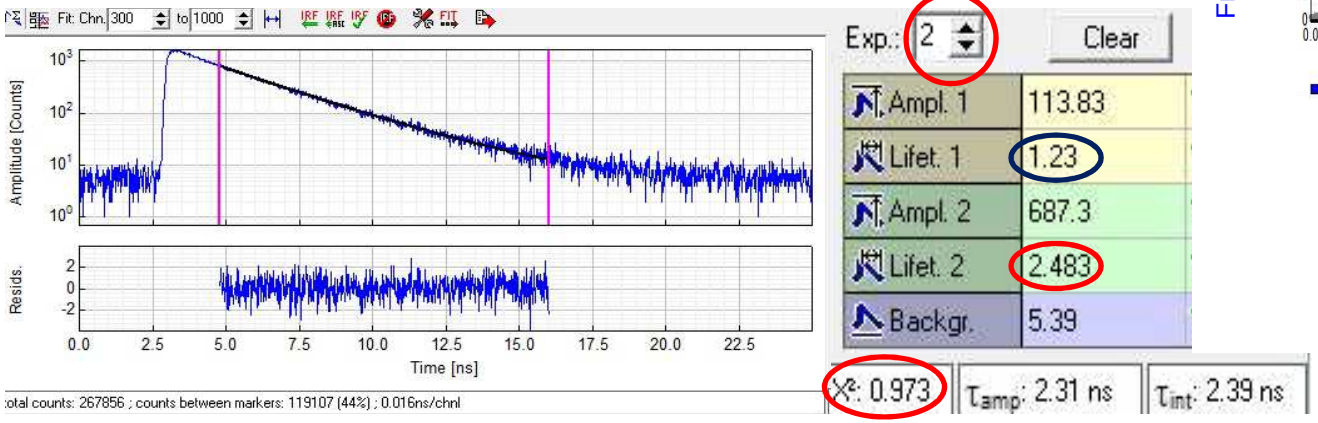


Bi-exponential decline:
33% FRET (T_2)
66% No FRET (T_1)

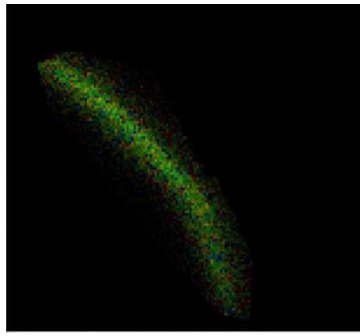
FLIM/FRET: supplying co-localization

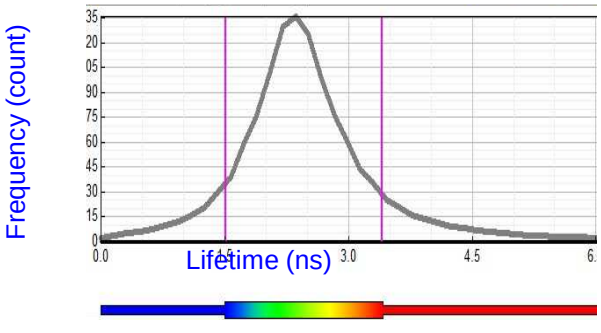


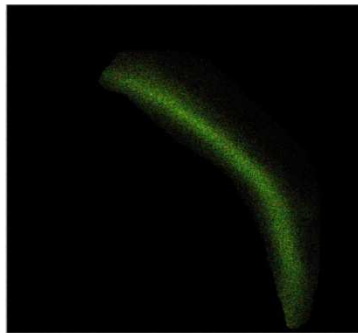
Mono-exponential fitting



Bi-exponential fitting



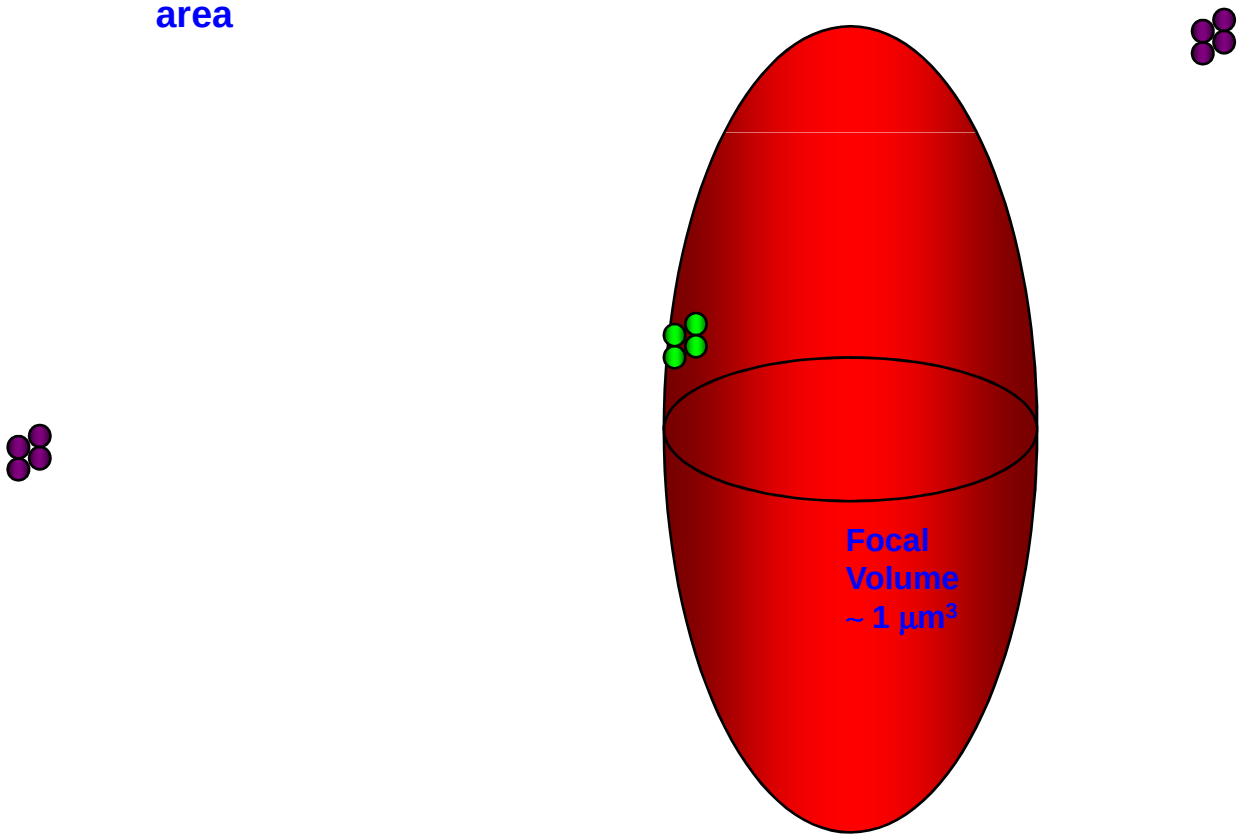




Fluorescent Correlation Spectroscopy

FCS (fluorescence correlation spectroscopy) provides an alternative method for measurements of protein dynamics *in vivo*. A laser beam is focused on a microvolume, typically in the femtolitre range, and fluctuation of the fluorescence signal is measured over a short period of time. The recorded signals reflect the movement of labelled proteins through the sample volume

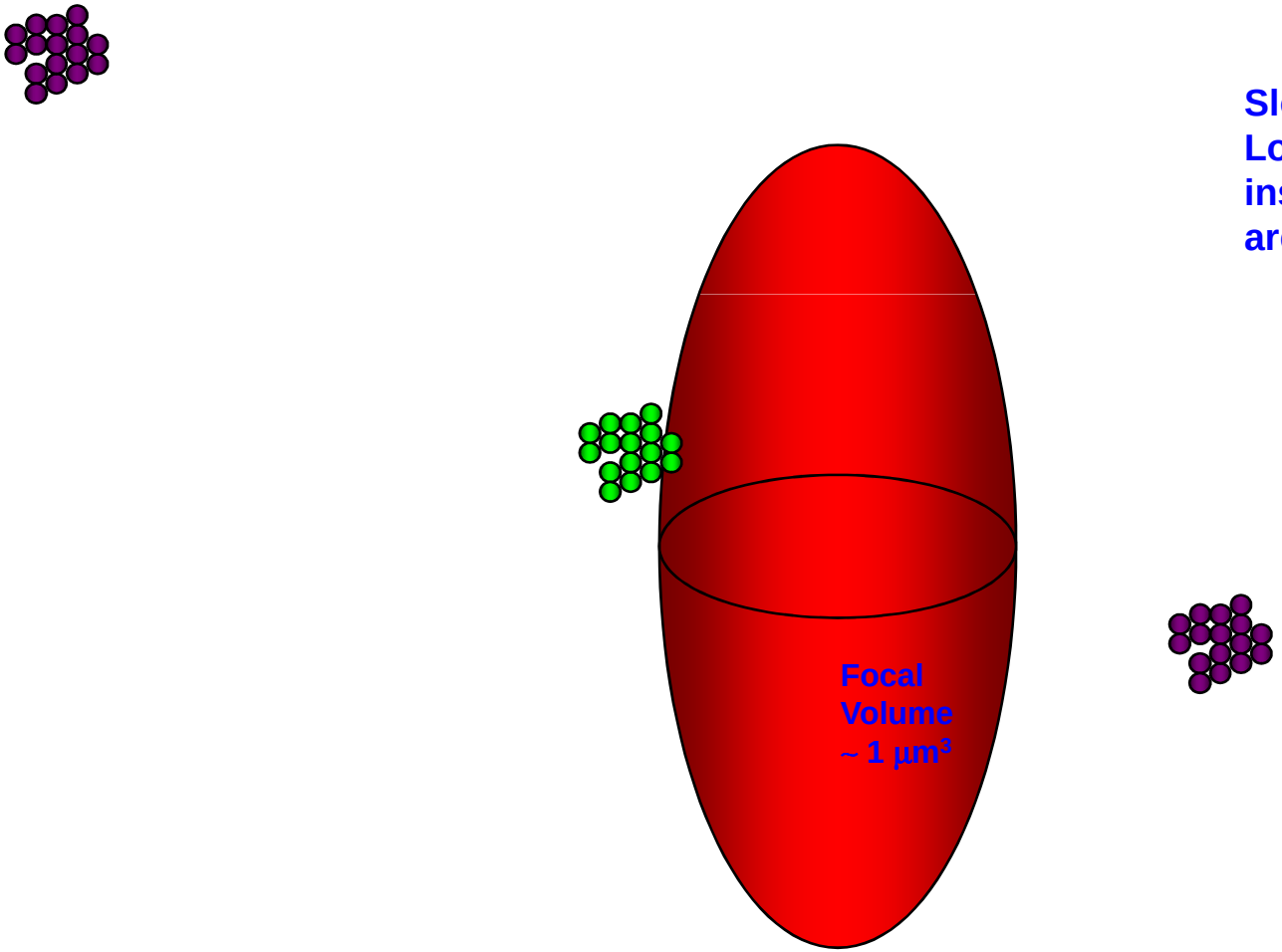
Fast Dynamics
Short lifetime
inside spotted
area



S. Nedellec, MicroPIcell

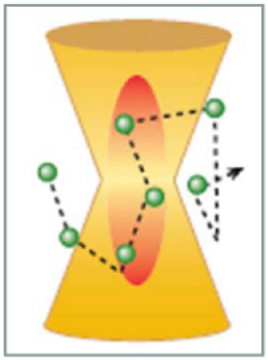
Fluorescent Correlation Spectroscopy

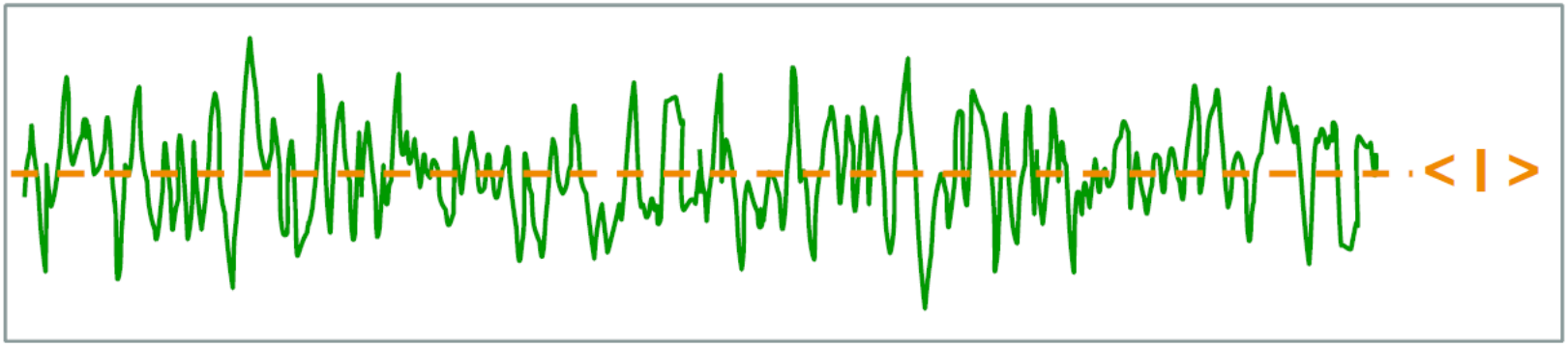
FCS (fluorescence correlation spectroscopy) provides an alternative method for measurements of protein dynamics *in vivo*. In FCS, a laser beam is focused on a microvolume, typically in the femtolitre range, and fluctuation of the fluorescence signal is measured over a short period of time. The recorded signals reflect the movement of labelled proteins through the sample volume

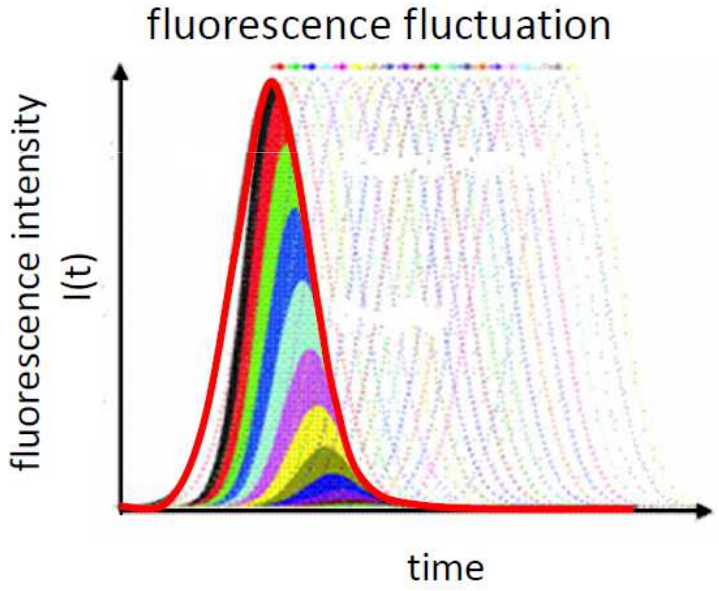


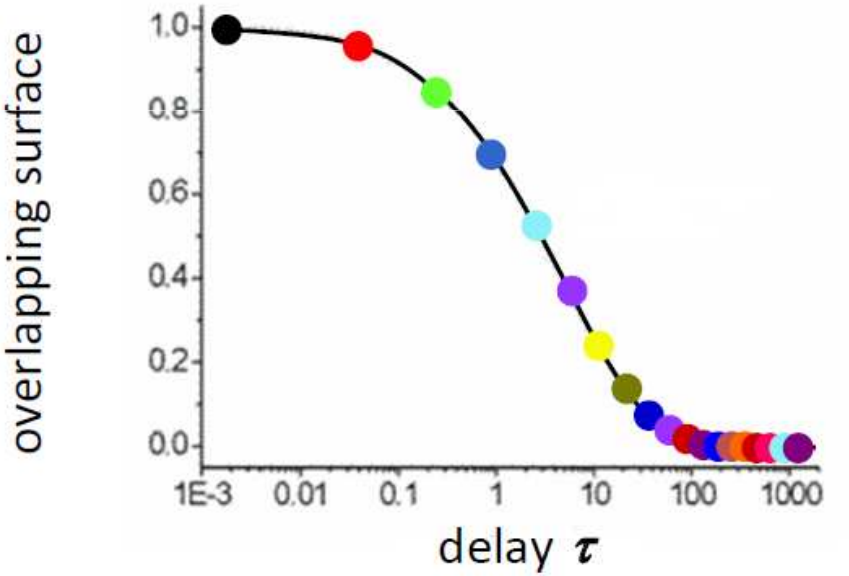
S. Nedellec, MicroPIcell

Fluorescent Correlation Spectroscopy







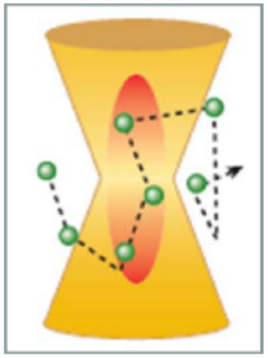


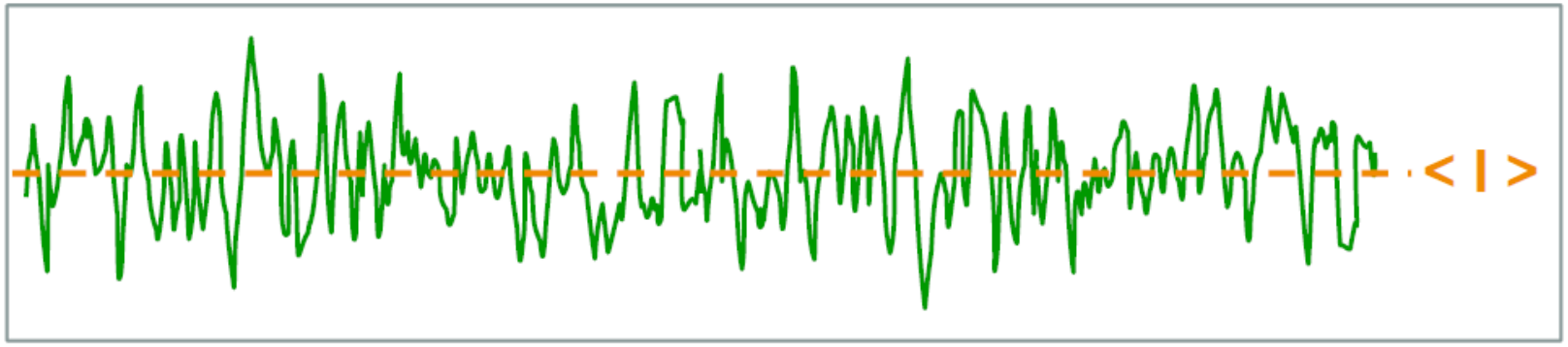
autocorrelation function

$$g^{(2)}(\tau)=\frac{\langle I(t)\, I(t+\tau)\rangle}{\langle I(t)\rangle^{2}}$$

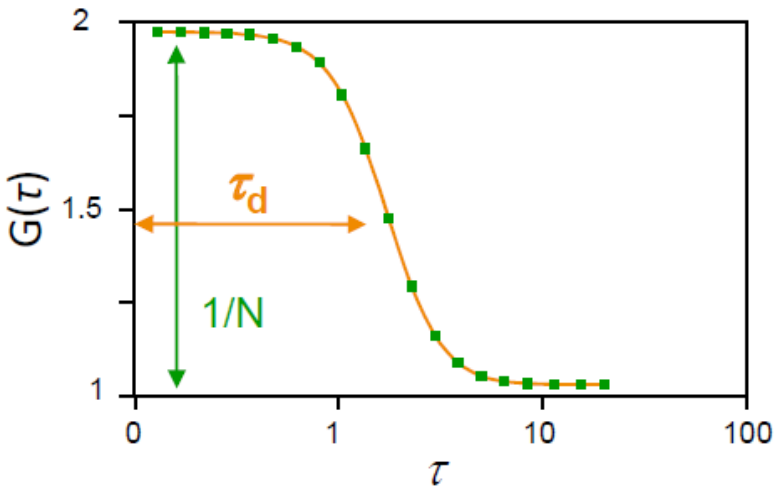
Marguet D, Mifobio 2012

Fluorescent Correlation Spectroscopy





temporal fluctuations



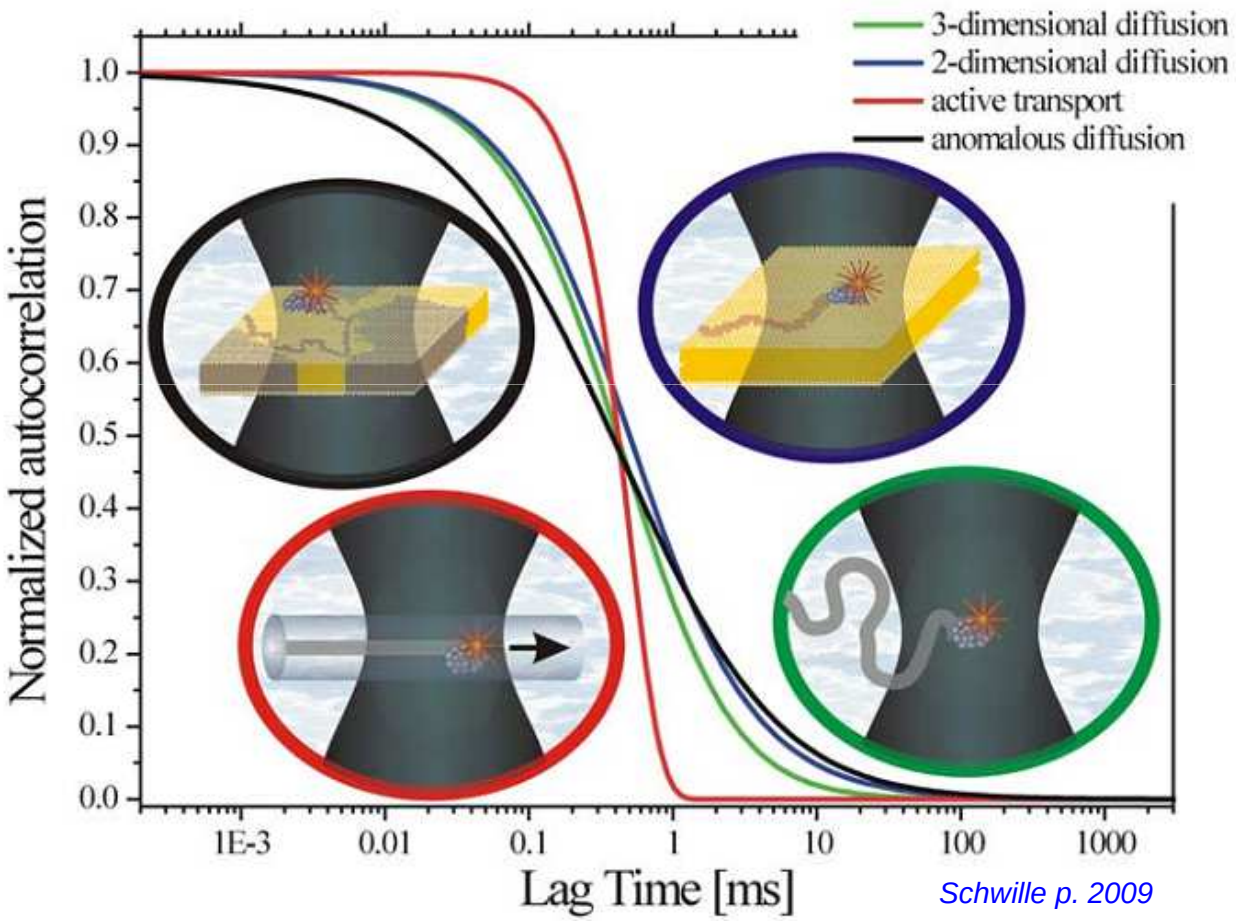
autocorrelation function (ACF)

$\Rightarrow \tau_d$ the average time molecules stay within the spot

$\Rightarrow$ N the average number of molecules

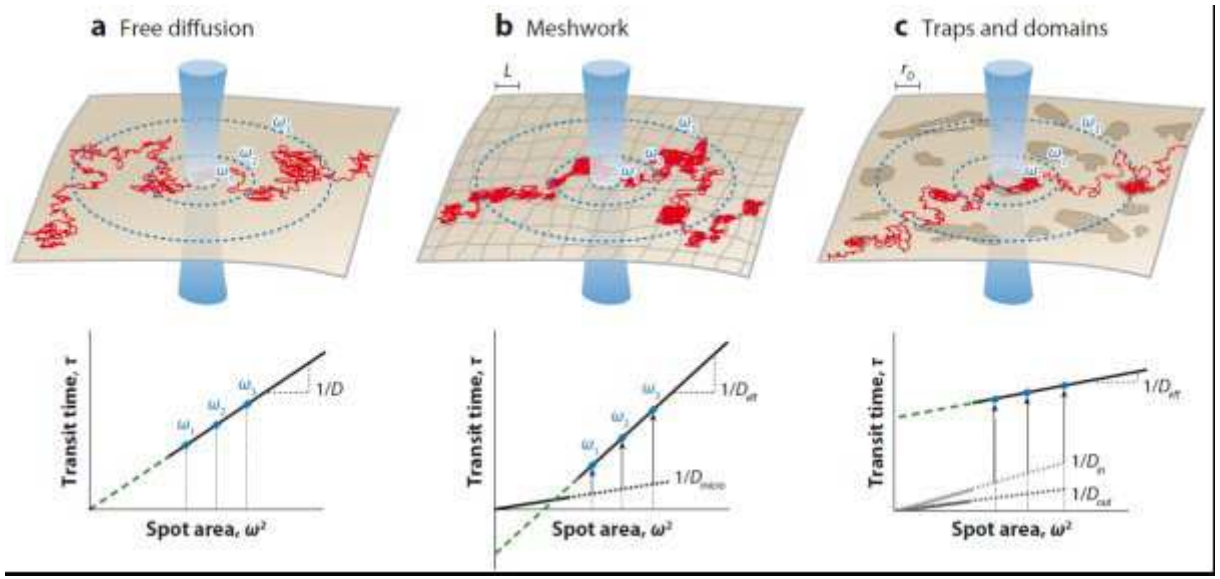
Marguet D, Mifobio 2012

Fluorescent Correlation Spectroscopy



Schwille p. 2009

Fluorescent Correlation Spectroscopy



Research question	Live-cell imaging	Fixed-cell imaging
Molecular structure	No	Crystallography, electron microscopy
Conformational changes	FRET, single-molecule FRET	Crystallography, electron microscopy
Mobility of bound species	FRAP, FCCS, SPT	No
Intracellular activity of proteins	FRET sensors, FRET	No
Intracellular localization	Confocal microscopy, STED microscopy	Confocal microscopy, STED microscopy, SIM, PALM
Aggregation state of receptors	Anisotropy, FRET, PALM, STORM, FCCS and related analyses of molecular brightness	TEM, PALM, STORM, FRET
Mobility at the plasma membrane	TIRF microscopy, FRAP, SPT and sptPALM, confocal microscopy, STED microscopy	No
Cell morphology	Confocal microscopy, epifluorescence microscopy, TIRF microscopy, DIC microscopy	Confocal microscopy, epifluorescence microscopy, TIRF microscopy, DIC microscopy
Cell adherence to a surface	TIRF microscopy, DIC microscopy, IRM	TIRF microscopy, DIC microscopy, IRM, TEM

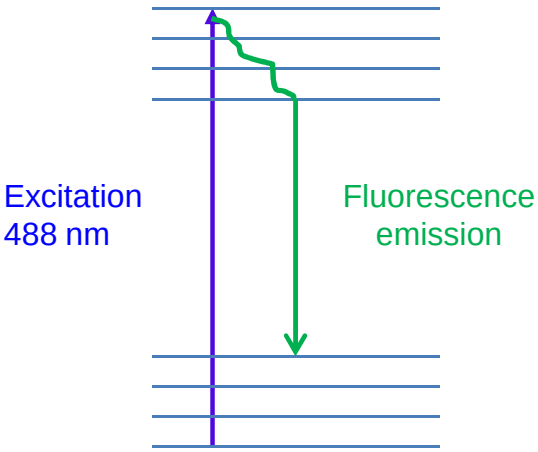
DIC, differential interference contrast; FCCS, fluorescence cross-correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescent resonance energy transfer; IRM, interference reflection microscopy; PALM, photoactivated localization microscopy; SIM, structured illumination microscopy; SPT, single-particle tracking; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TEM, transmission electron microscopy; TIRF, total internal reflection fluorescence.

Biphotonic microscopy

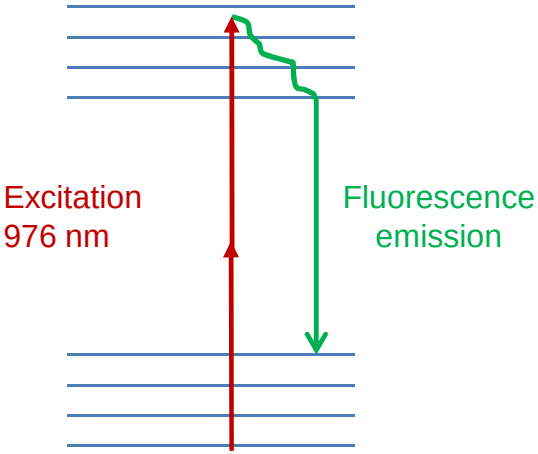
Major Inputs of 2Photon excitation:

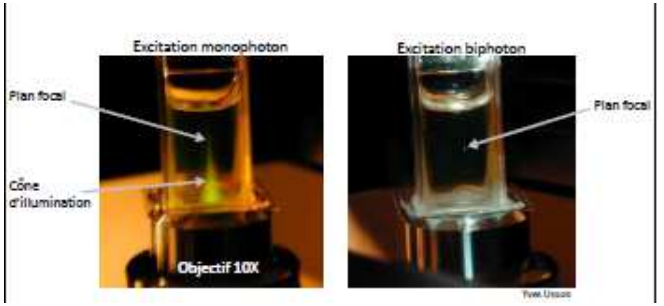
- Deeper tissu penetration
- Reduction of the photoxicity
- Imaging of living tissu
- Studies of FRET by FLIM
- Imaging of second harmonic signals

1Photon excitation



2Photon excitation





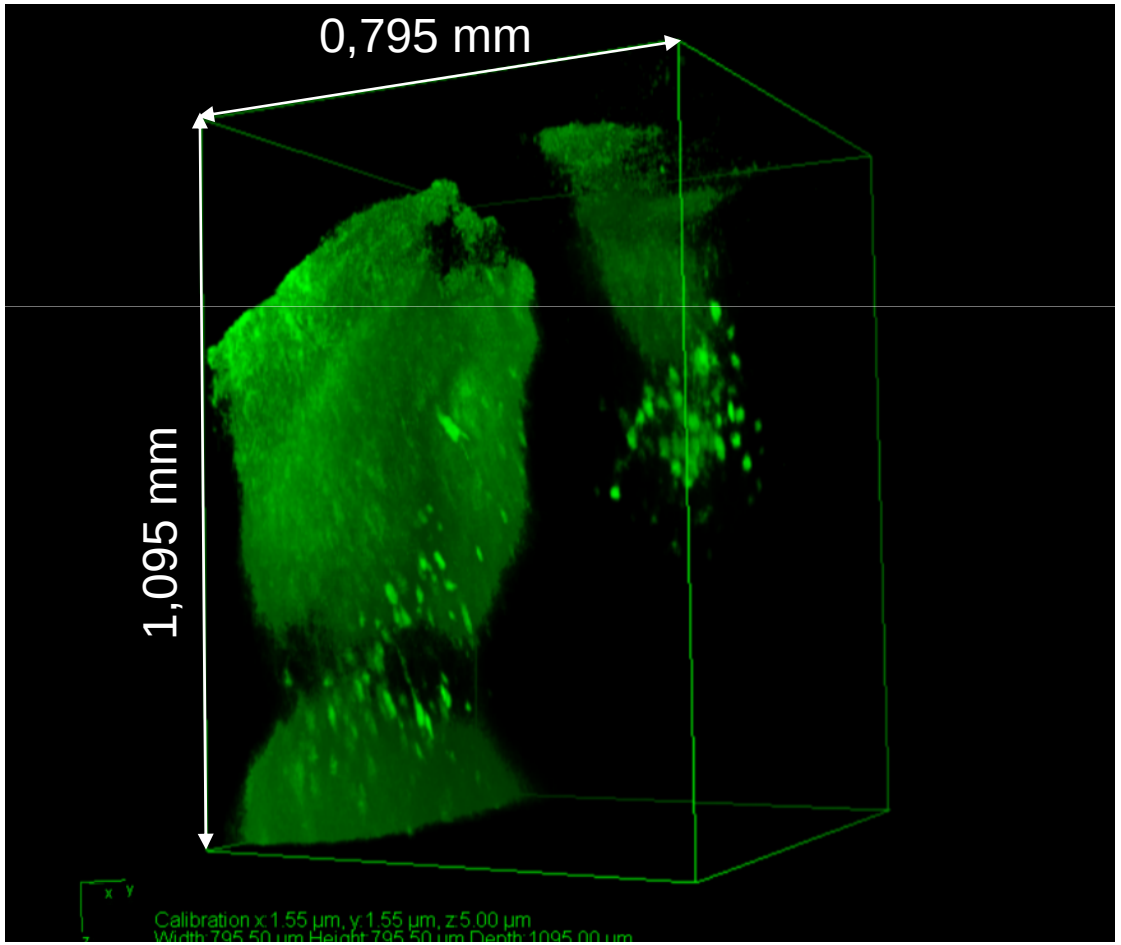
Femtosecond laser (infrared laser beam 680-1080 nm)

Applications of the biphotonic microscopy

Major Input : deeper tissu penetration (2X with biphotonic microscopy)

Sca*l*ed brain GFP expression

A1RPM, Nikon, MiFoBio 2012



Applications of the biphotonic microscopy

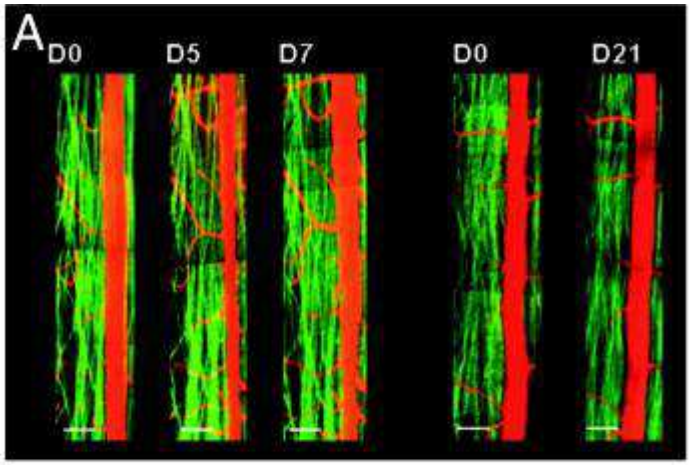
Major input : Imaging of living tissu

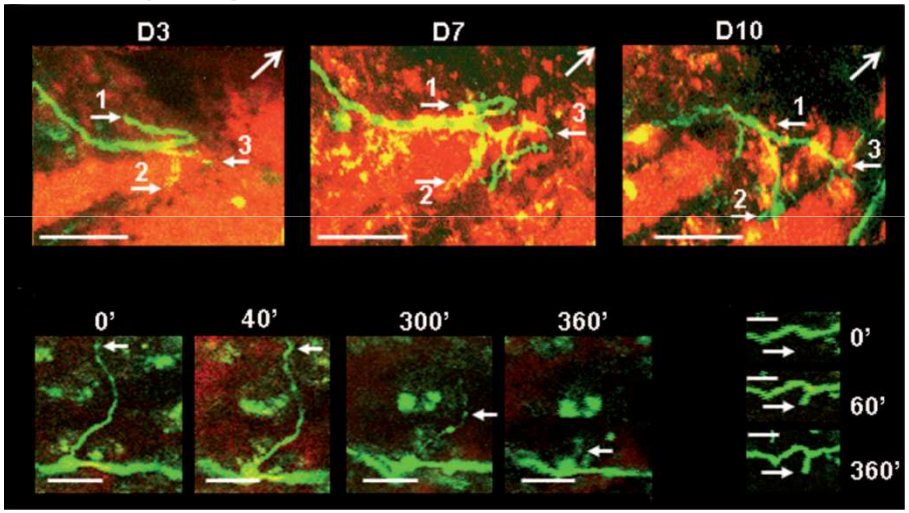
PNAS **June 9, 2009** vol. 106 no. 23 **9459–9464**

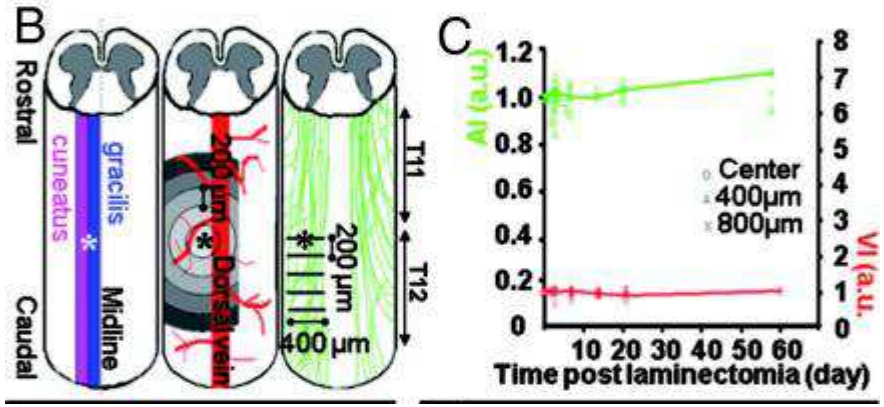
Quantitative analysis by in vivo imaging of the dynamics of vascular and axonal networks in injured mouse spinal cord

Cyril Dray, Geneviève Rougon[1], and Franck Debarbieux

Unité Mixte de Recherche 6216, Centre National de la Recherche Scientifique, Université de la Méditerranée, Institut de Biologie du Développement de Marseille-Luminy, Case 907, Parc Scientifique de Luminy, 13288 Marseille Cedex 9, France







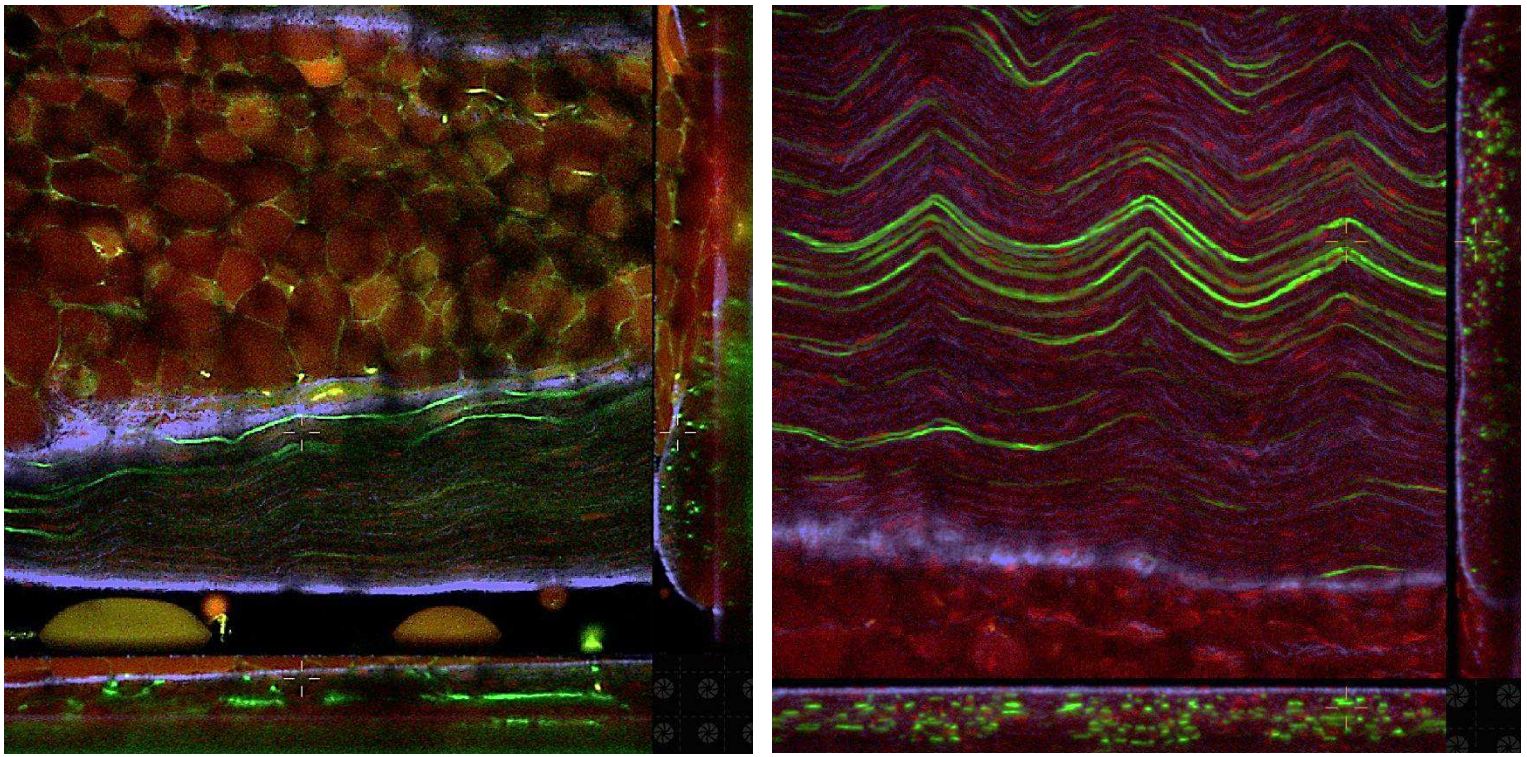
Adult transgenic Thy1GFP-M mice
Rhodamin B isothiocyanate-Dextran

Biphotonic microscopy

Major input : second harmonic generation signal ($\lambda/2$), molecular conformation and orientation (fibrillar collagen).

sciatic nerve from a mice

A1RPM, Nikon, MiFoBio 2012



Collagen matrix : second harmonic generation

Applications of the biphotonic microscopy

Imaging of second harmonic generation signals : A tool to explore fibrosis (collagene type I)

Journal of Hepatology **52**, 3 (2010) 398-406

Fibrillar collagen scoring by second harmonic microscopy: a new tool in the assessment of liver fibrosis.

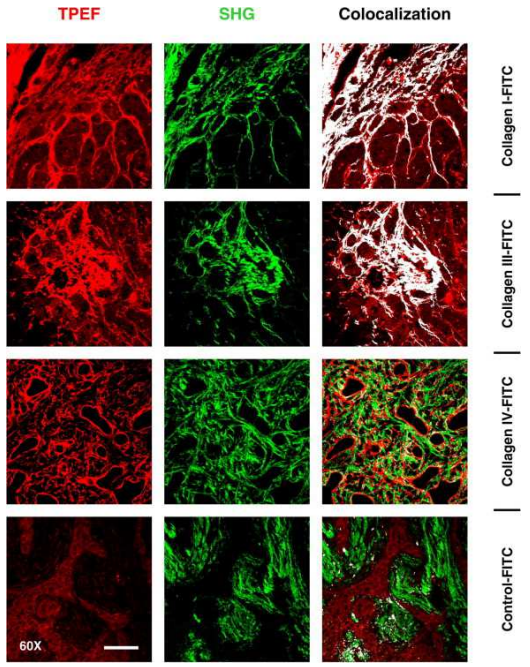
Luc Gailhouste [1, 2], Yann Le Grand [3, 4], Christophe Odin [4], Dominique Guyader [5, 6], Bruno Turlin [7], Frédéric Ezan [1], Yoann Désille [6], Thomas Guilbert [4], Anne Bessard [1, 8], Christophe Frémin [1], Nathalie Theret [9], Georges Baffet [1, 9]

We first validated the selectivity of SHG signals for fibrotic deposits (fibrillar collagen type I and III) in human liver by establishing a strong correlation between SHG and immunochemistry assays.

Both frozen fixed or paraffin embedded tissues can be used

SHG scoring gives the opportunity to quantify fibrosis regression in response to an antifibrotic treatment for validating experimental protocols especially for cirrhotic patients

SHG imaging and fibrillar collagen scoring will be applicable to endoscopic systems by using optic fibers coupled with multiphoton microscopy, thus leading to a less invasive procedure for fibrosis evaluation



Biphotonic microscopy

Depth penetration increased

Background signal strongly suppressed

Imaging of living tissue

Molecular interactions by FLIM

Phototoxicity reduced

Second Harmonic Generation

Femtosecond laser very expensive

Difficult to use two pulsed lasers

Resolution 2P 250 nm

Choice of system

	Wide Field Microscopy	Spectral Confocal Microscopy 1P	Confocal Microscopy 2P
Deconvolution	++	+	Not necessary
Simultaneous acquisition of multilabeling	-	++	+/-
Quantitative Imaging	+	+/-	++
Spectral analyses	-	++	++
FLIM	-	+/-	++
Thick Specimen	-	+/-	++
Imaging in vivo	+/-	+/-	++
Second Harmonic Generation	-	-	++

Photonic microscopy with high resolution

Breaking the limit

Abbe limit : $d = \lambda/2$

TIRF : Total Internal Reflexion Microscopy

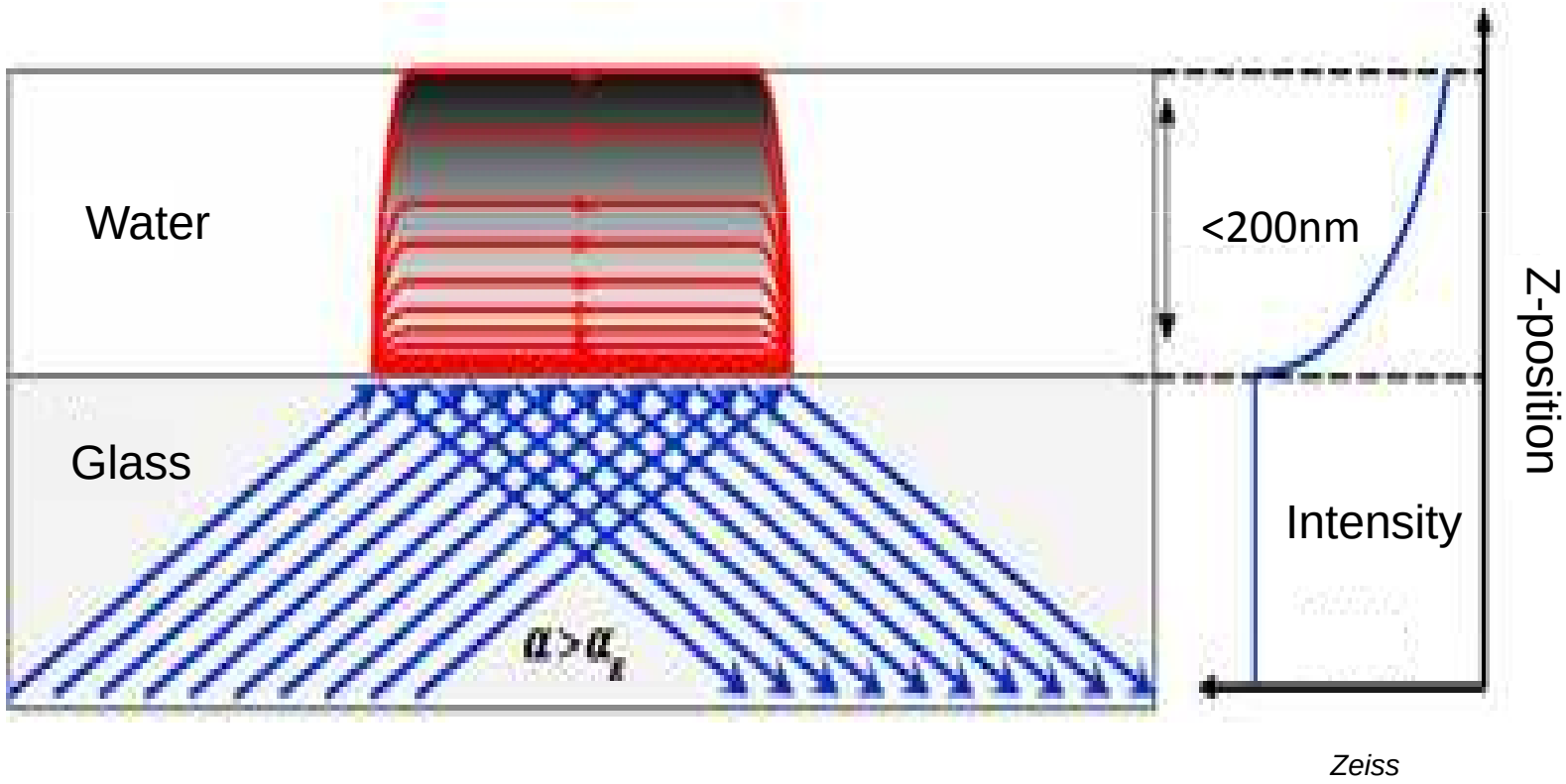
SIM : Structurated Illumination Microscopy

STED : Stimulated Emission of Depletion

PALM : Photoactivation Light Microscopy

TIRF microscopy

TIRF : Total Internal Reflexion Microscopy Resolution : 80-100 nm

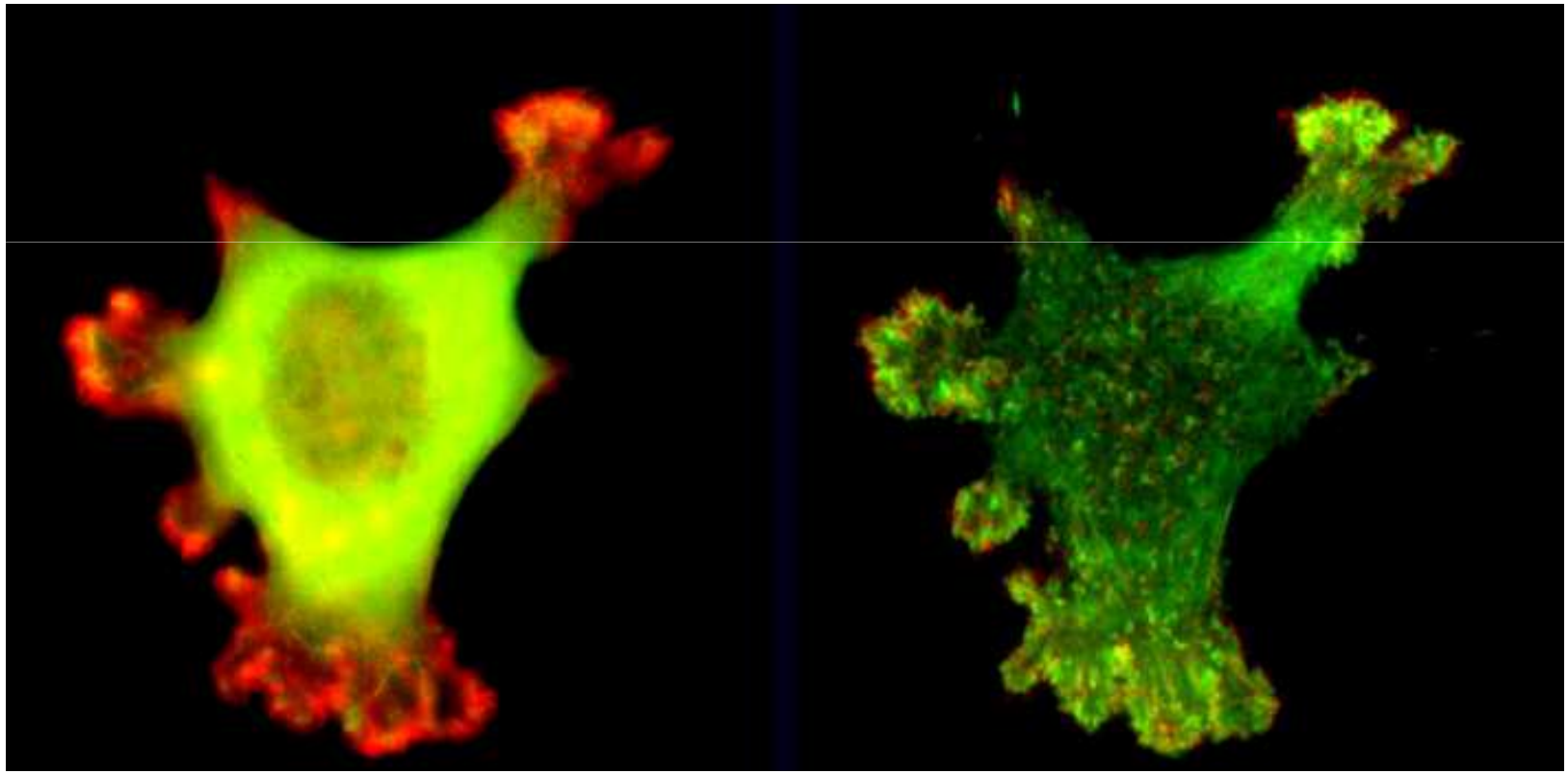


Zeiss

Applications of TIRF microscopy analyses

melanoma cells (mouse)

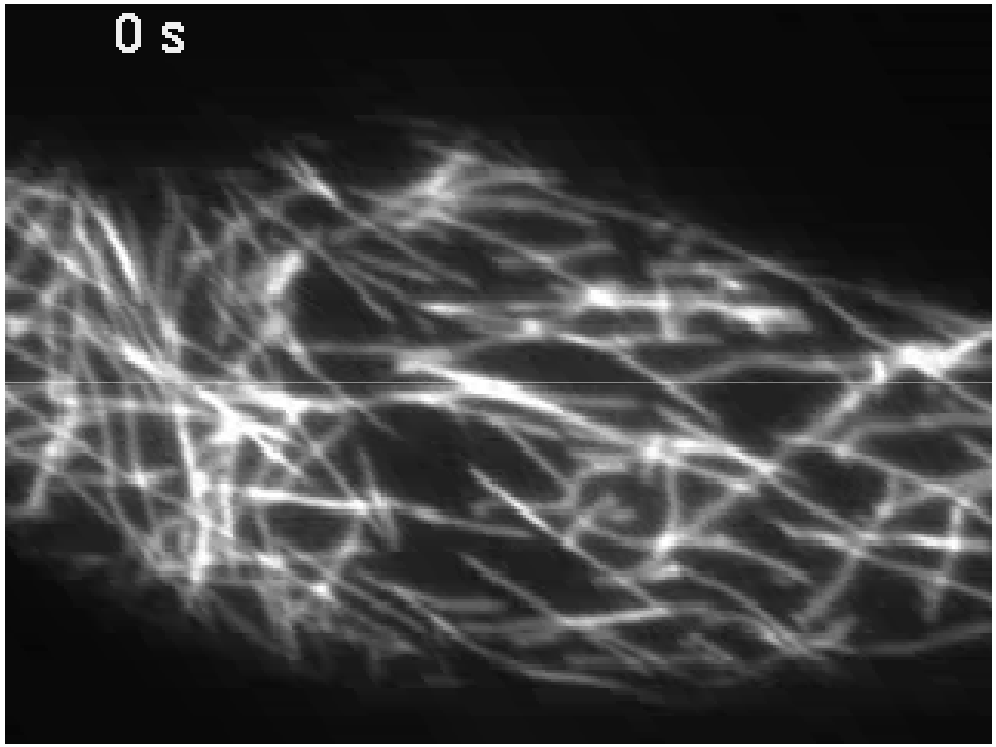
Confocal microscopy TIRF Microscopy



zeiss

Myosin green
Actin red

TIRF microscopy

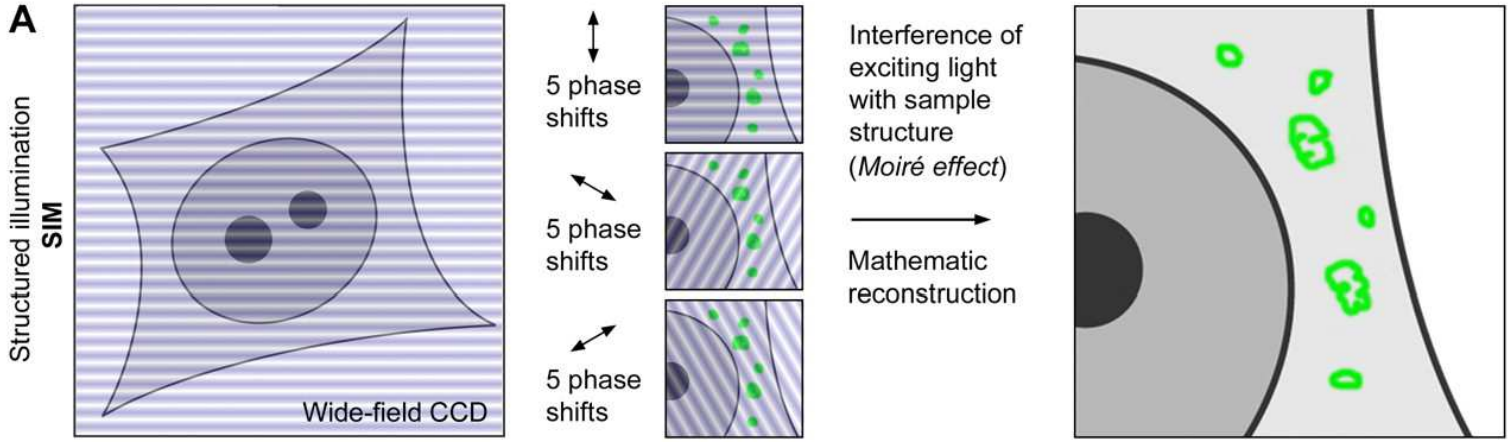


Microtubul eGFP Arabipopsis

SIM : Structurated Illumination Microscopy

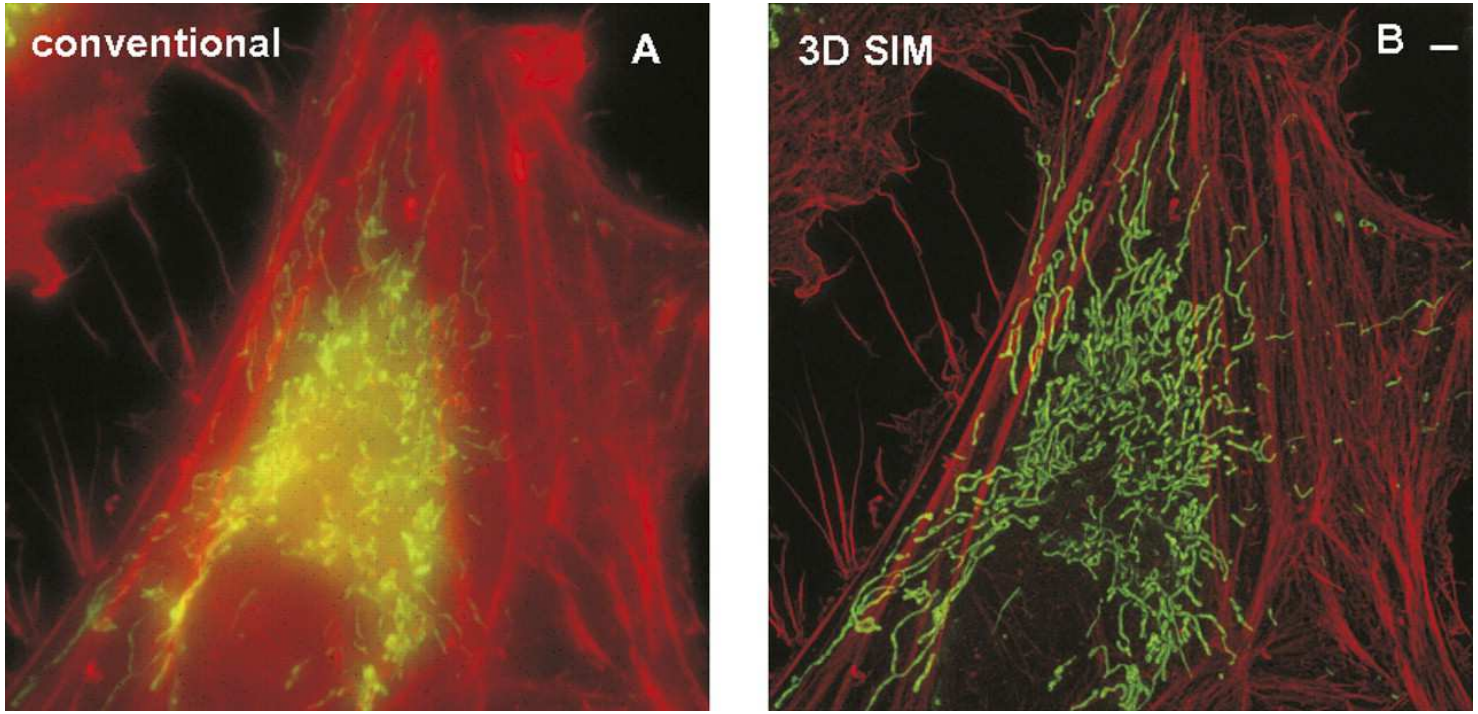
SIM : Structurated Illumination Microscopy

Resolution : 80 - 100 nm



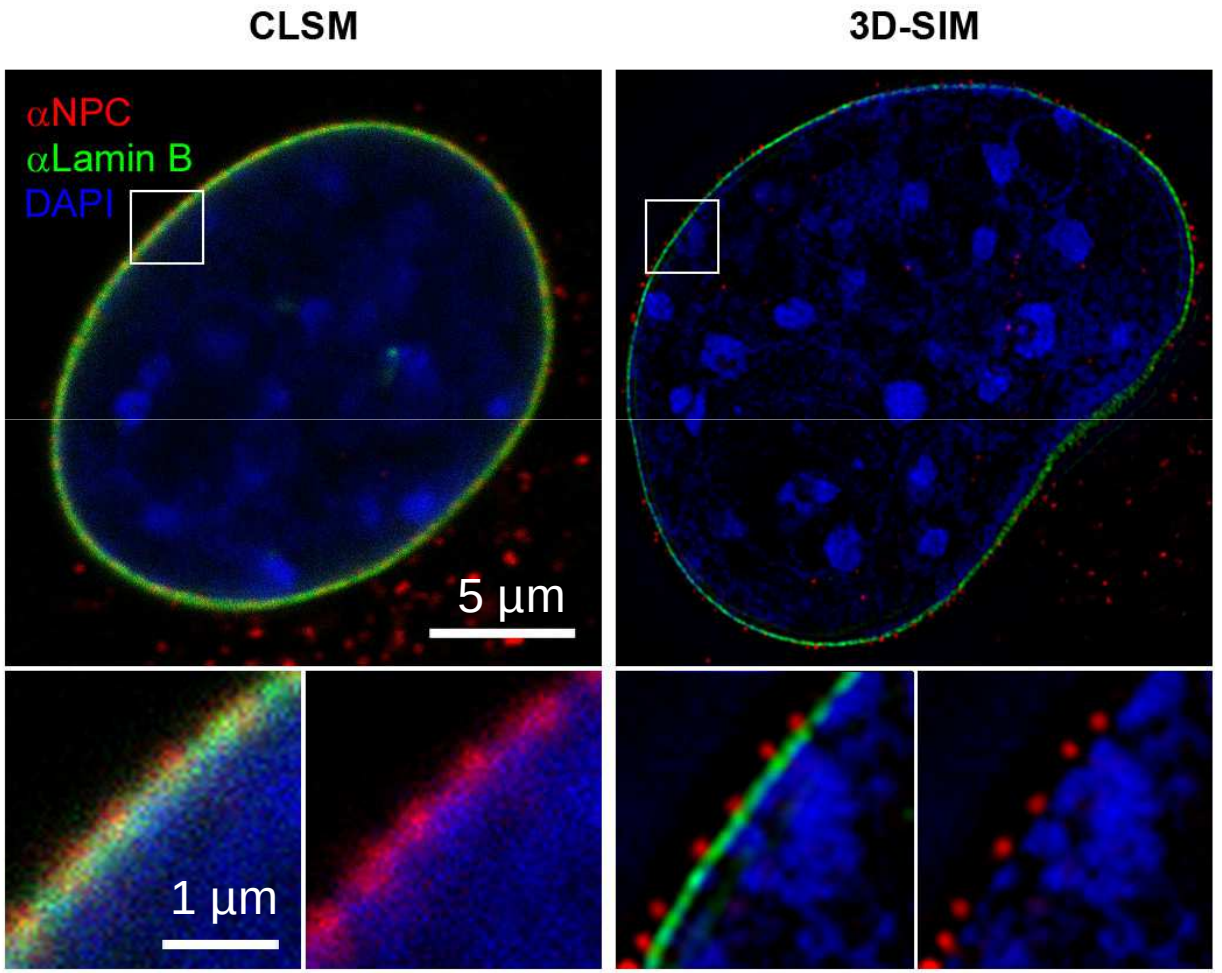
The image detected by the CCD camera thus contains high spatial frequency sample information shifted to a lower spatial frequency band that is transmitted through the objective.

Applications of SIM analyses



Reto Fiolka and al. PNAS 2012

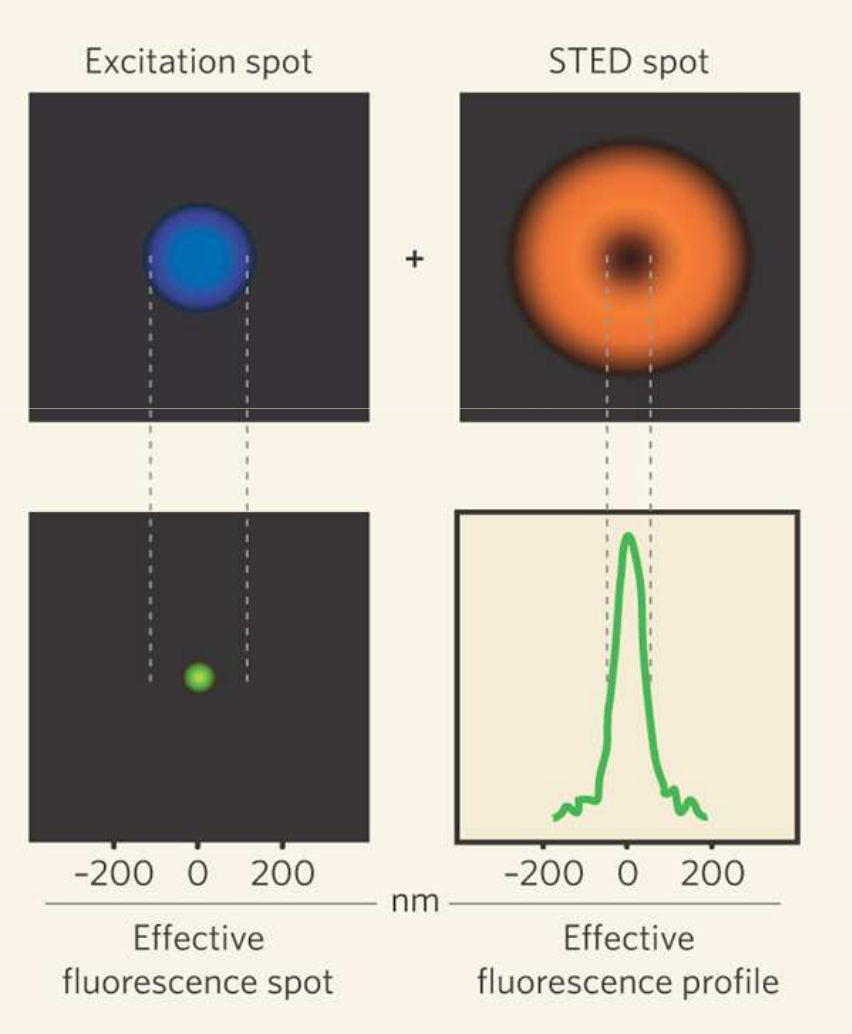
Fluorescence microscopy with high resolution



Schermelleh L and al. *Science 2008*

Fluorescence microscopy with high resolution

STED : Stimulated Emission of Depletion Resolution : 20-40 nm

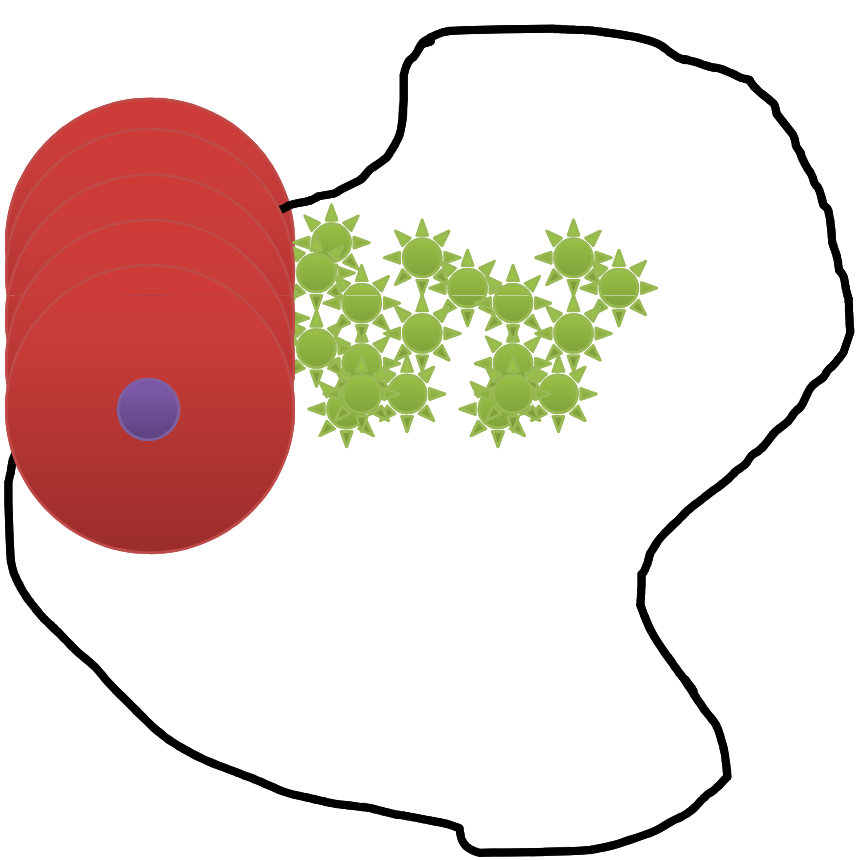


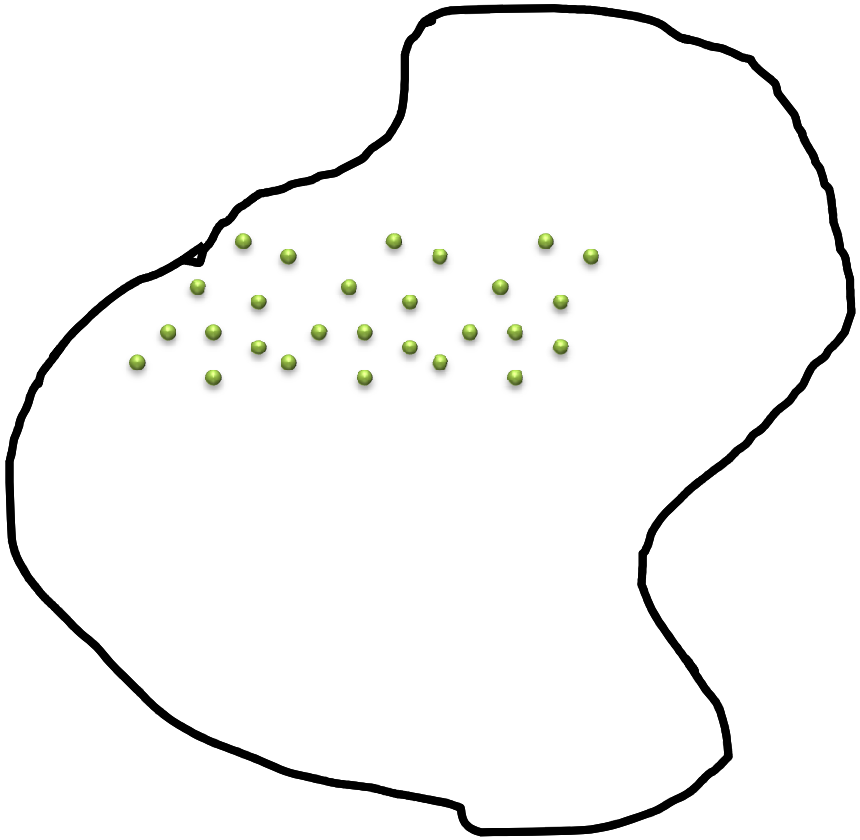
Garth J. Simpson *Nature*. 2006

Fluorescence microscopy with high resolution

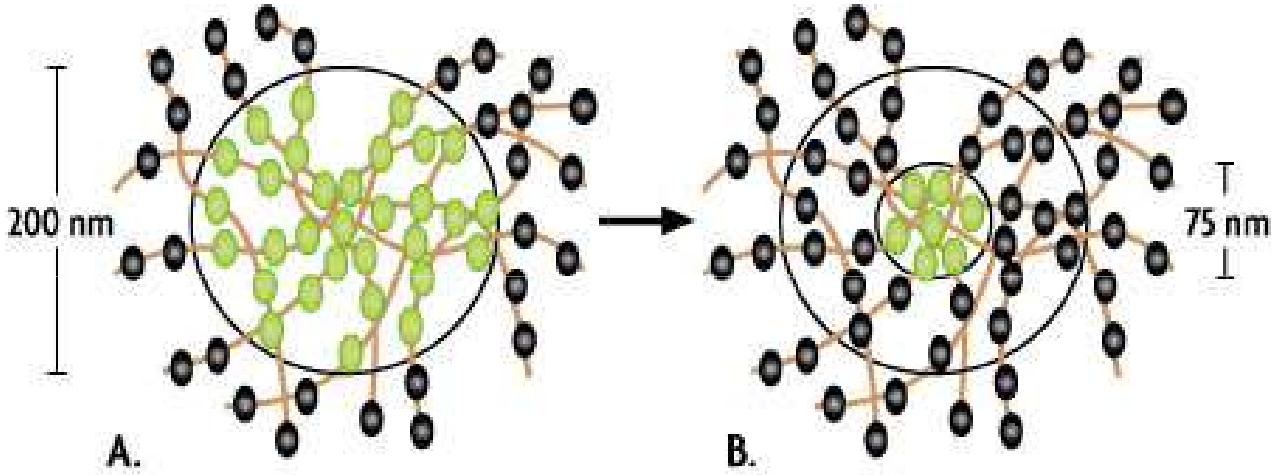
STED : Stimulated Emission of Depletion

Resolution : 20-40 nm





Fluorescence microscopy with high resolution



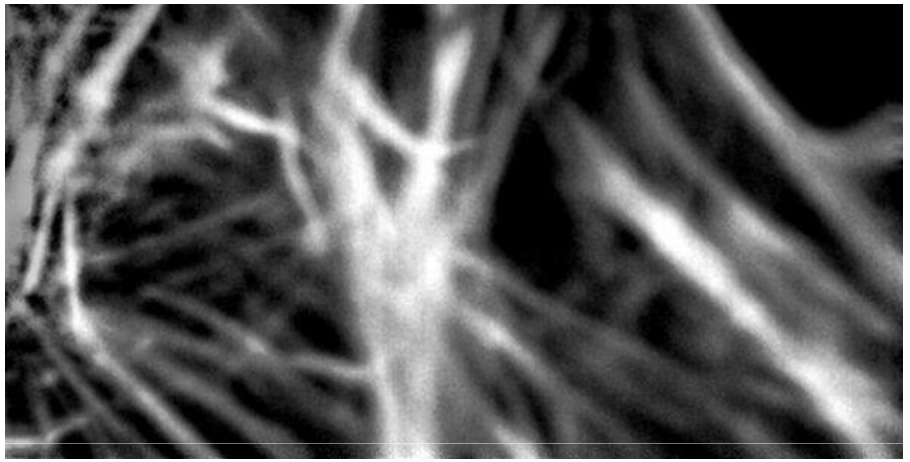
Confocal microscopy

STED microscopy

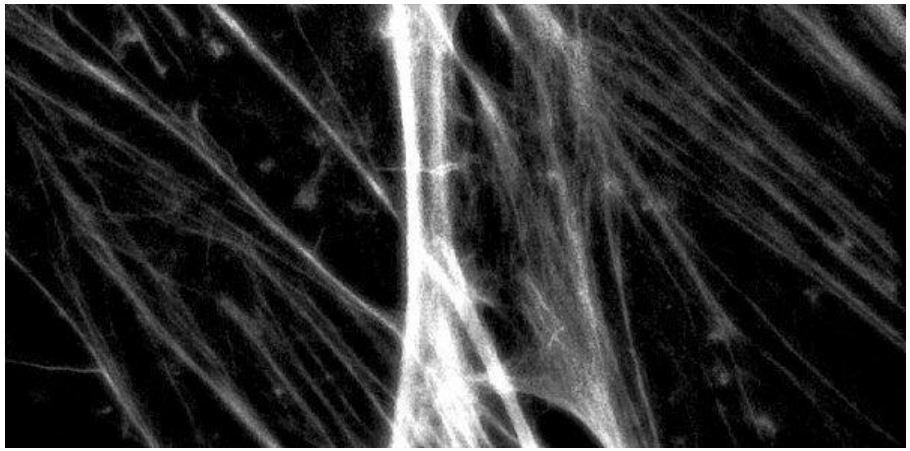
Fluorescence microscopy with high resolution

ACTIN

Confocal microscopy



STED microscopy

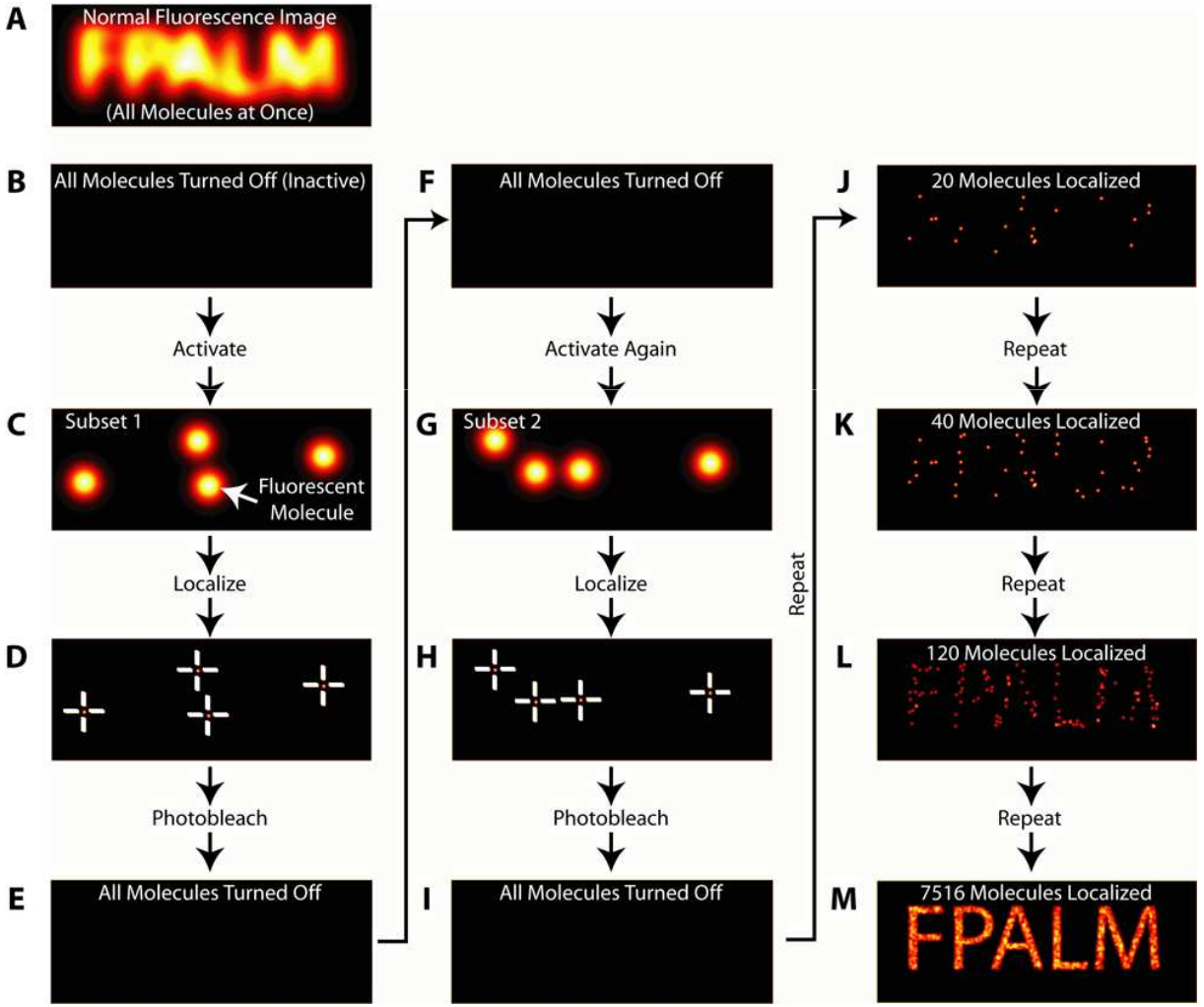


www.leica-microsystems.com

Fluorescence microscopy with high resolution

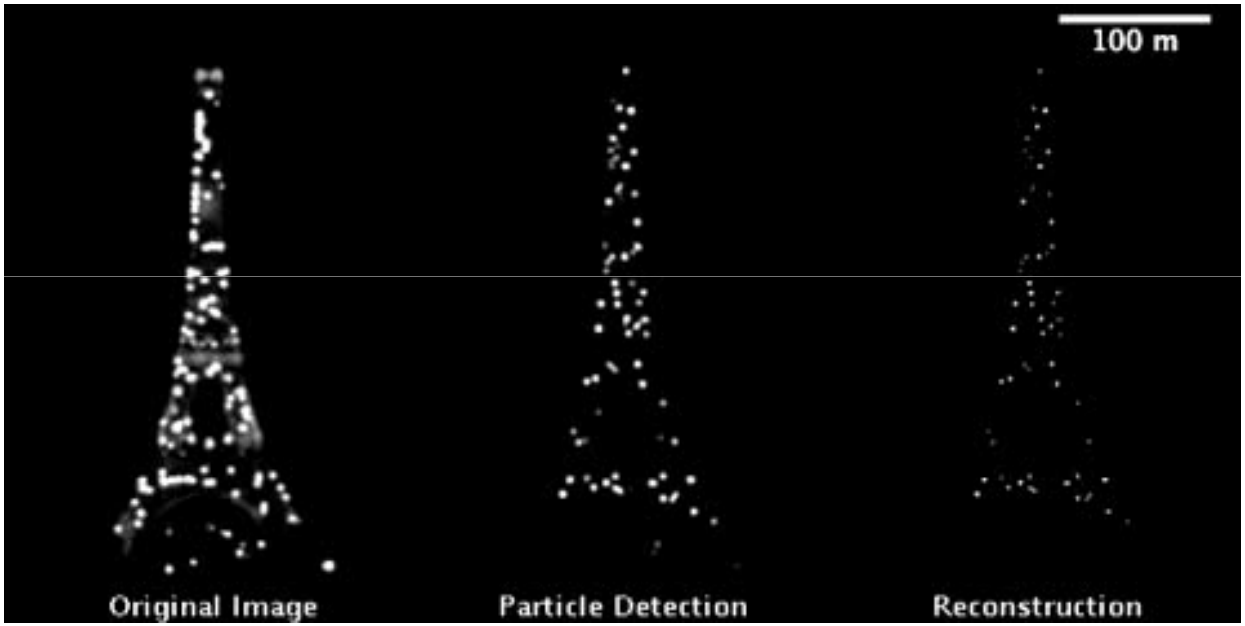
PALM : Photoactivation Localization Microscopy

Resolution : 20-40 nm



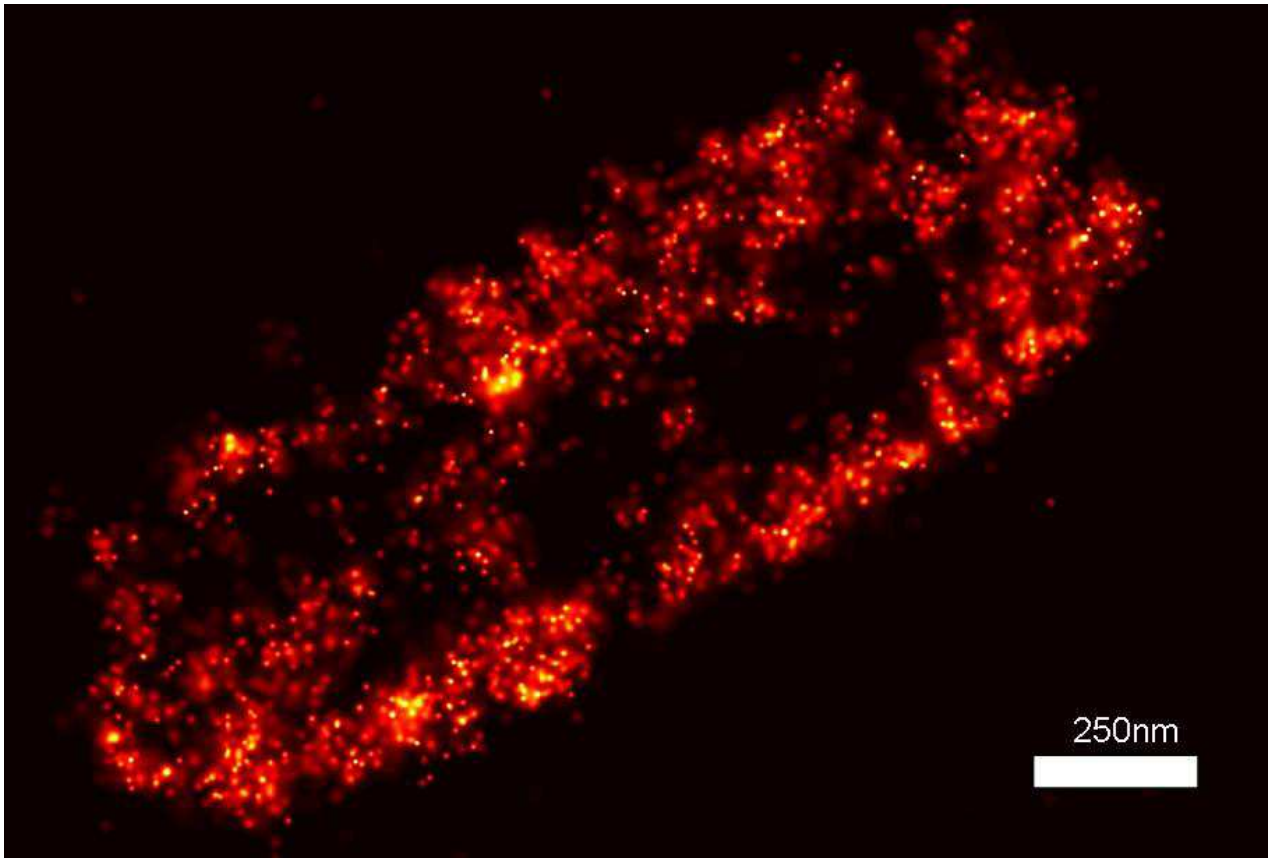
Sam Hess. University of Maine

Fluorescence microscopy with high resolution



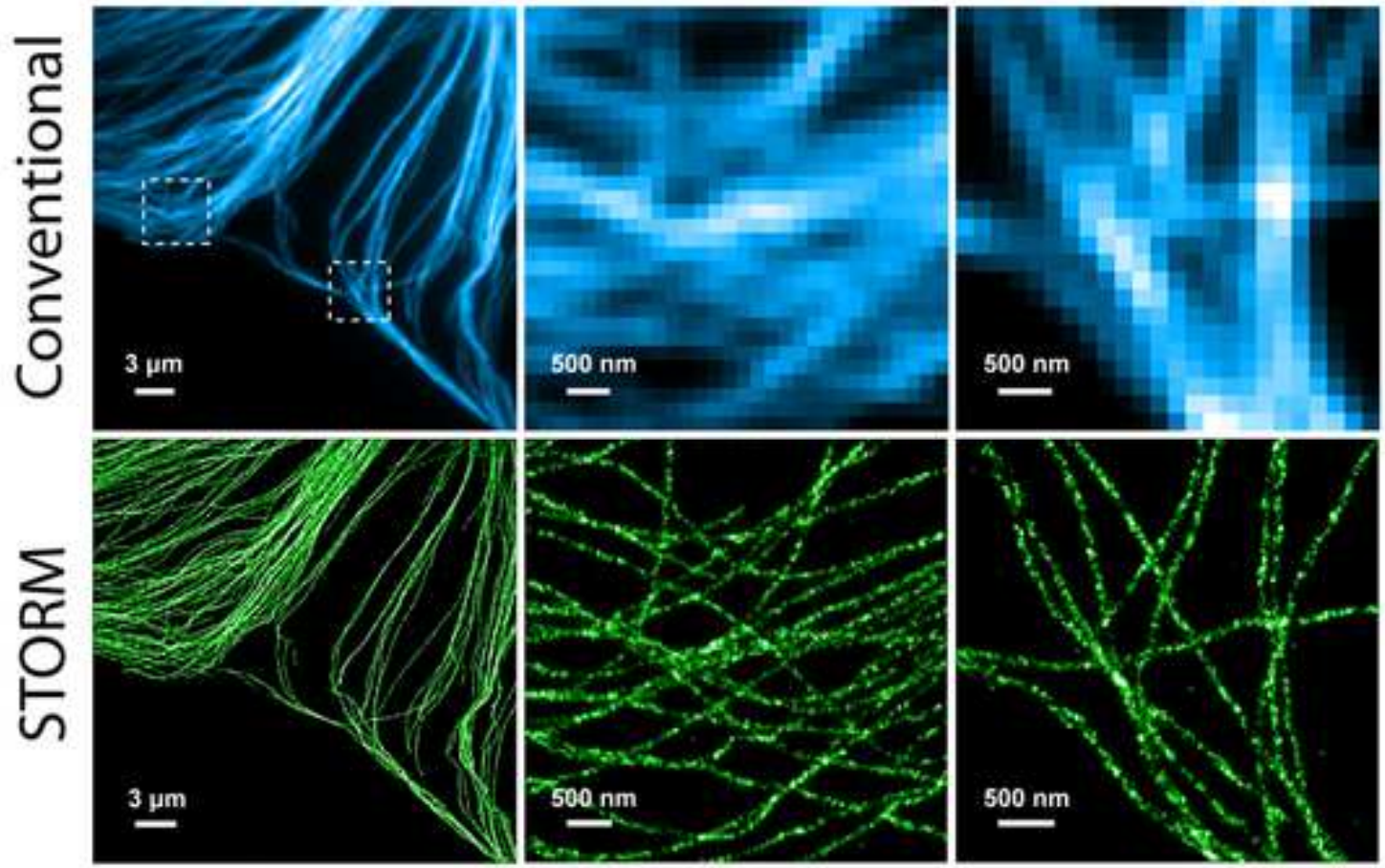
Fluorescence microscopy with high resolution

E Coli Lipo-Poly-Saccharides antibody stained with A647



Stephan Thiberge. Princeton university

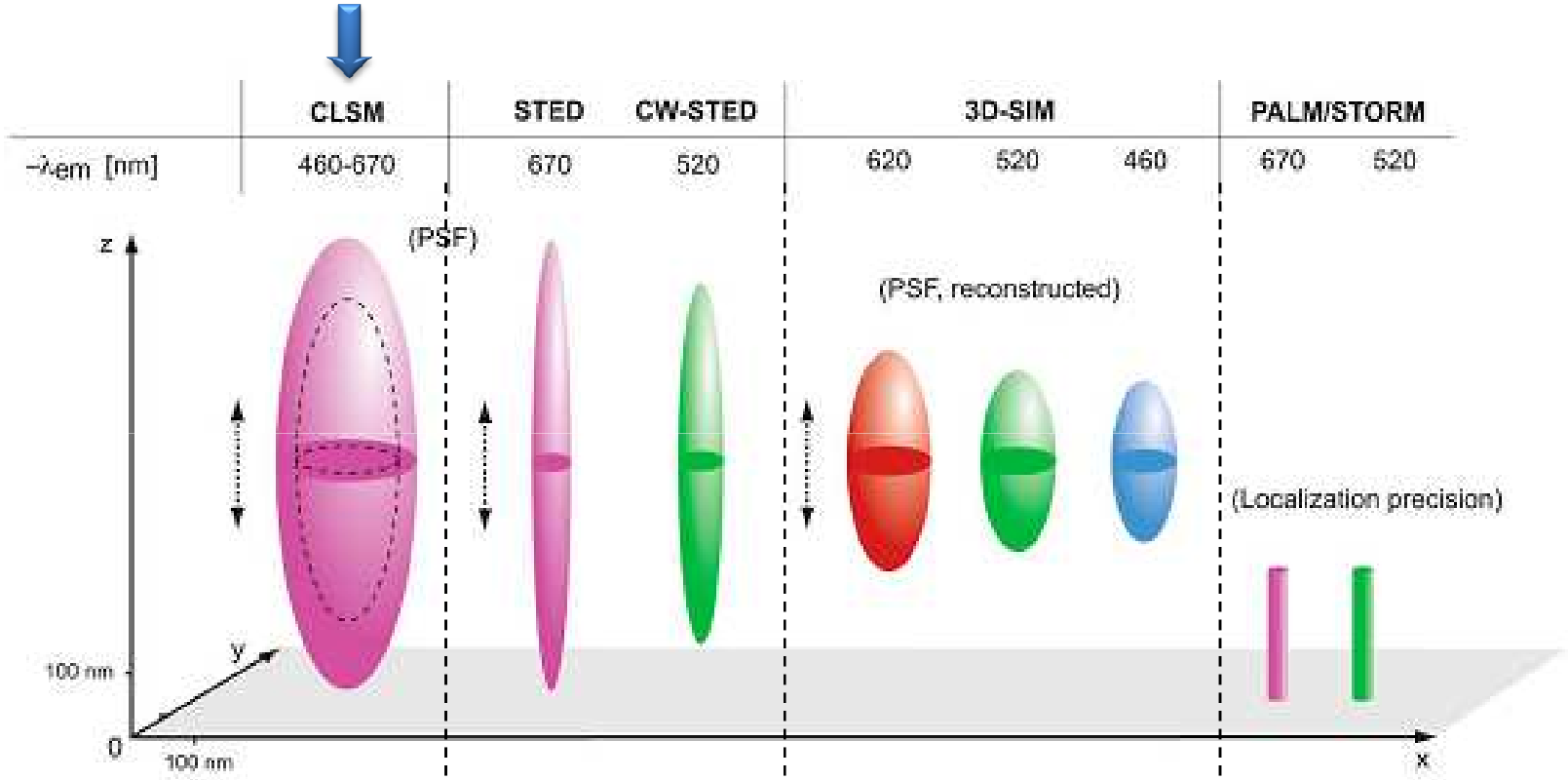
Fluorescence microscopy with high resolution



Science (2007)

Fluorescence microscopy with high resolution

Breaking the limit

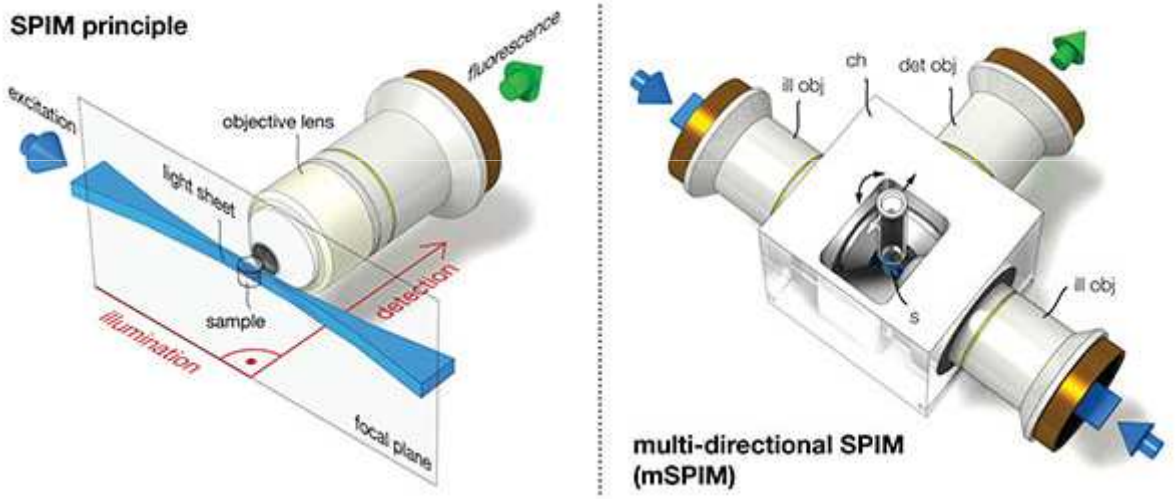


Dyes	any	photoswichable	any	photoswichable

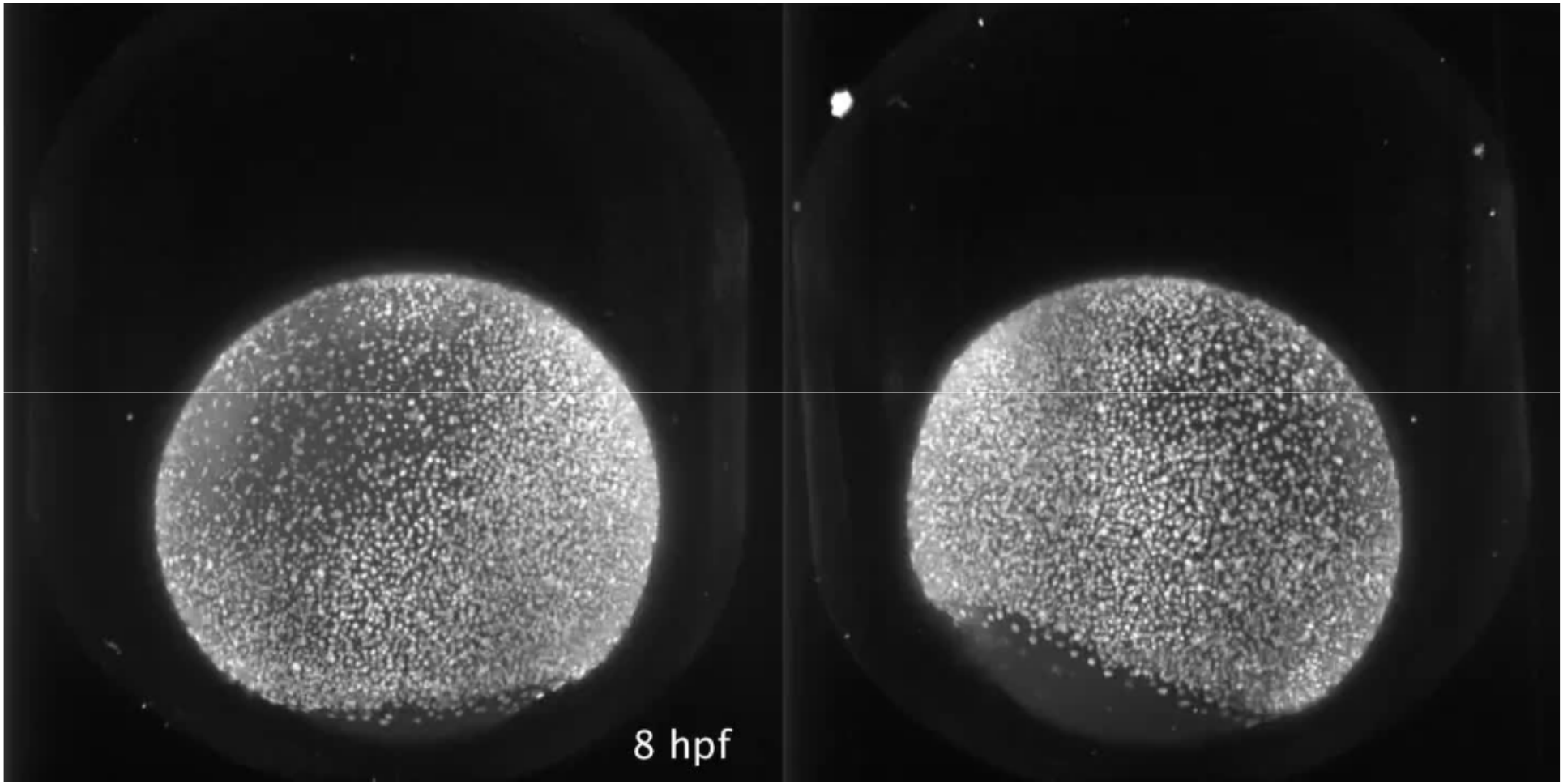
Choice of system

	Confocal	TIRF	SIM	STED	PALM
X-Y resolution	200	80-100	80-100	20-40	20-40
Z resolution	400-500	200	200	400-500	20-40
Dyes	Any	Any	Any	Photo switchable	Photo switchable
Rapidity	+	+++	-	++	-
Simultaneous colors	>3	3	>3	2	2
Post processing	No	No	Yes	Yes	Yes

Selective plane illumination microscope

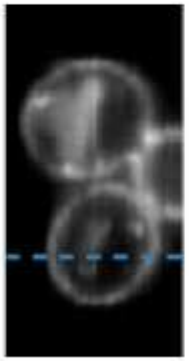


the Huisken Lab, Max Plank Institute



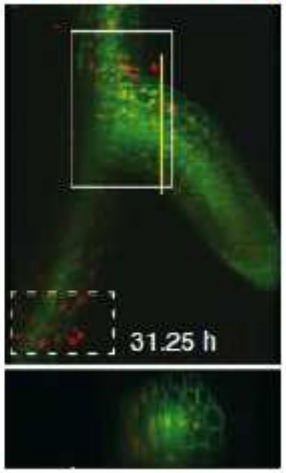
Max Planck Institute
of Molecular Cell Biology and Genetics
Huisken LAB
Quantitative microscopy of
zebrafish organogenesis

Molecular & cellular biology



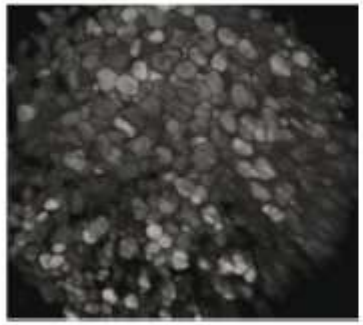
Capoulade & *al.*, 2011

Plant biology



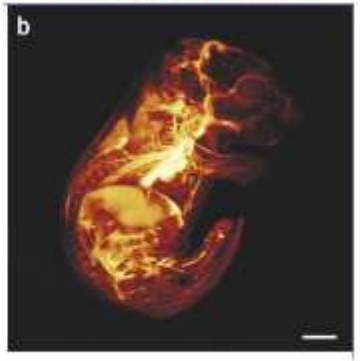
Maizel & *al.*, 2011

Cancer biology



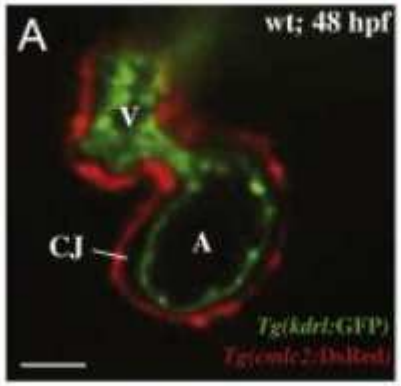
Lorenzo & *al.*, 2011

Neuroscience

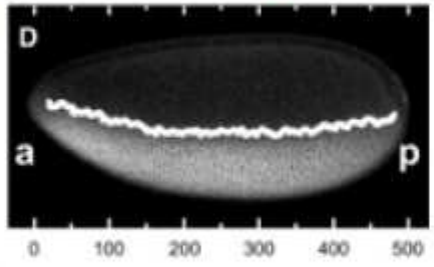


Dodt & *al.*, 2007

Developmental biology

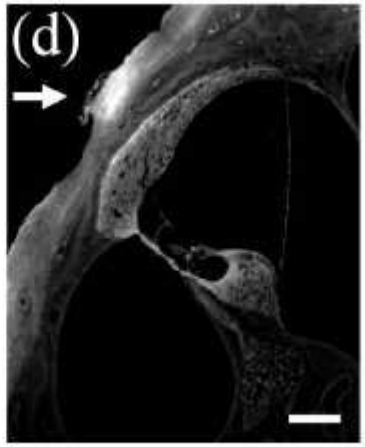


Mellman & *al.*, 2012



Reeves & *al.*, 2012

Anatomical studies



Schröter & *al.*, 2012