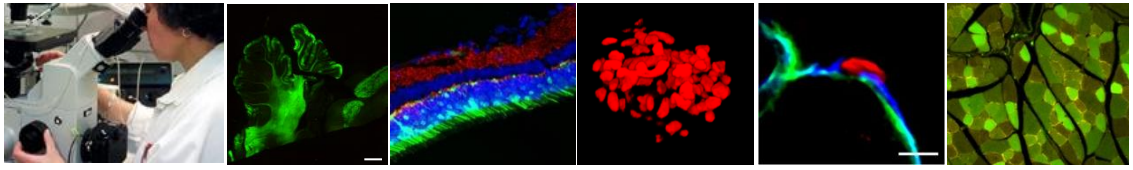
New approaches in fluorescent Bio-imaging for tissue & cell investigations

Dr Laurence Dubreil, Plateforme APEX UMR 703 INRA ONIRIS
Dr Steven Nedellec Plateforme MicroPicell



Microscopy of fluorescence

Wide-field
Confocal microscopy
Spectral confocal microscopy
Biphotonic microscopy

→ High Sensitivity and contrast

→ Multilabeling

→ Molecular interactions

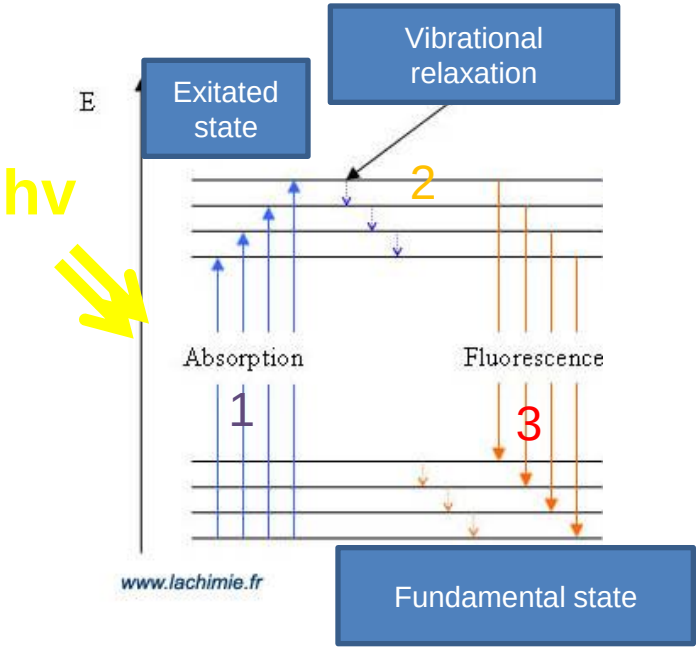
→ Thick specimens and exploration *in vivo*

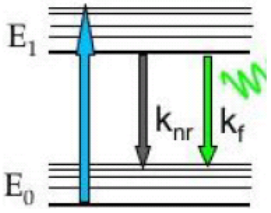
TIRF
SIM
STED
PALM and STORM

→ High resolution

Fluorescence : emission of photons

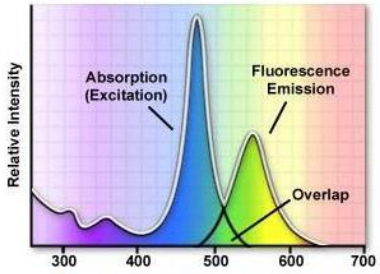
Jablonsky Diagramme





Loi de stokes

$E = hc/\lambda$

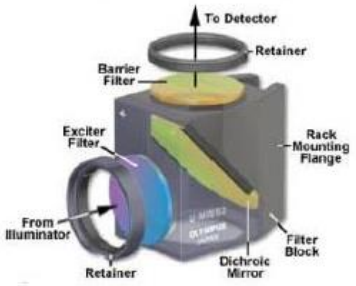


Exemple de spectres d'excitation et d'émission

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

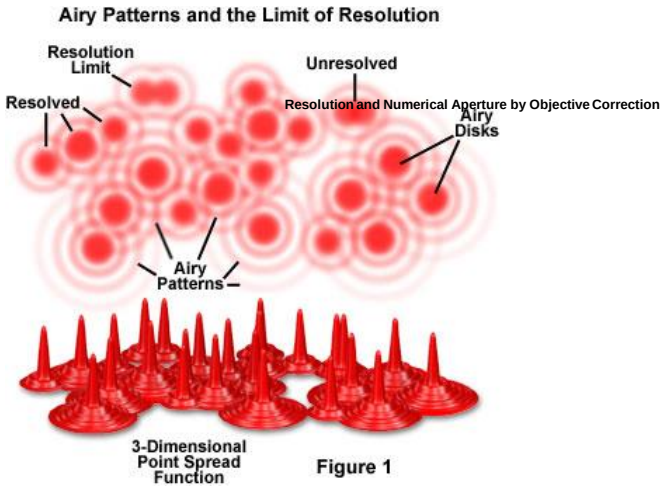
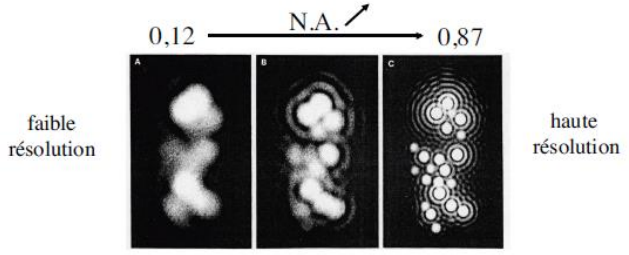


Figure 1



Rayleigh criterion

$Rxy = 0,6\lambda/NA$

$Rz = 2\lambda/NA^2$

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

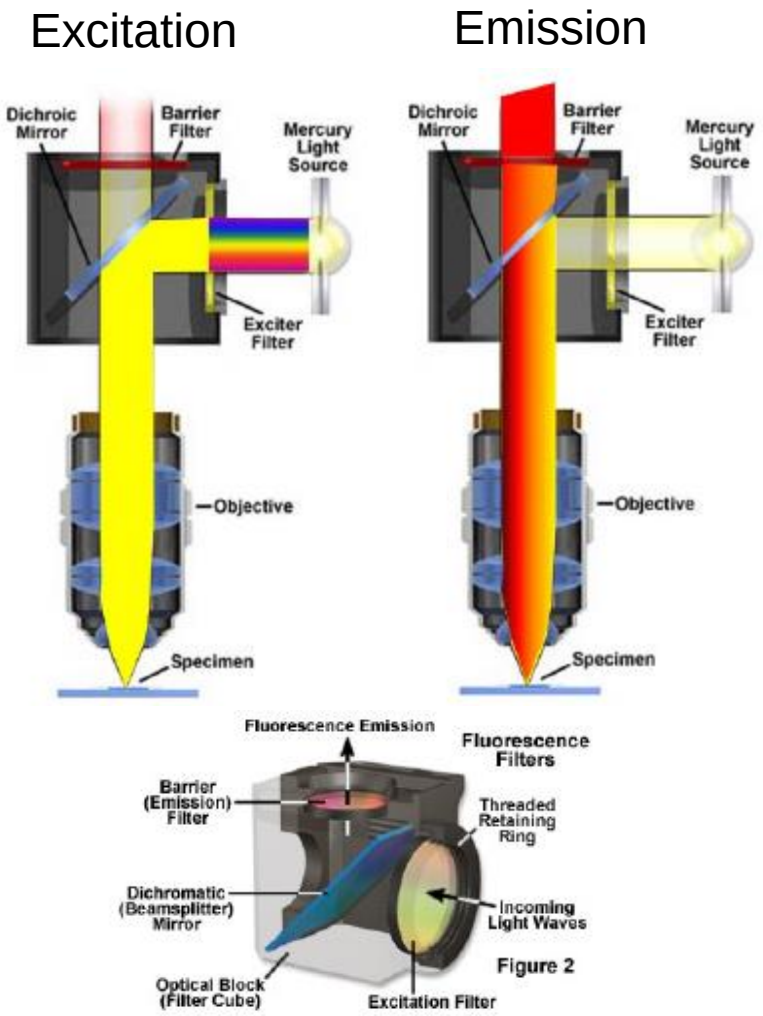
dxy = 212,62 nm
dz = 498 nm

Resolution and Numerical Aperture by Objective Correction

	Objective Type					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A.	Resolution (µm)	N.A.	Resolution (µm)	N.A.	Resolution (µm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20

N.A. = Numerical Aperture

Wide-field fluorescence microscopy

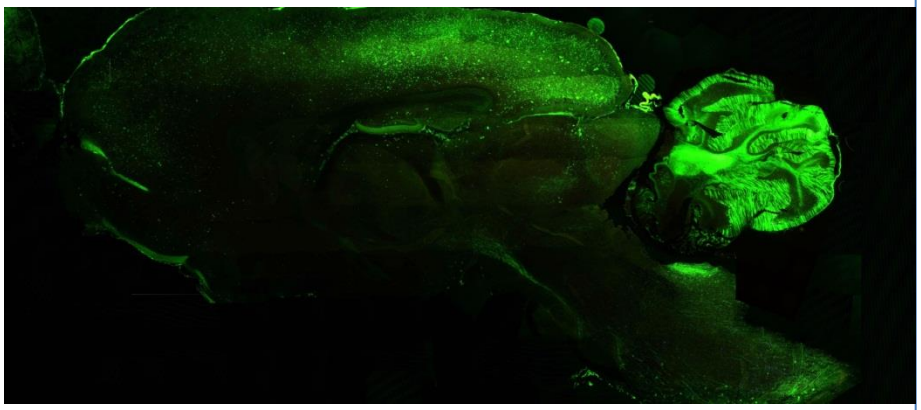


Applications of the wide-field fluorescent microscopy

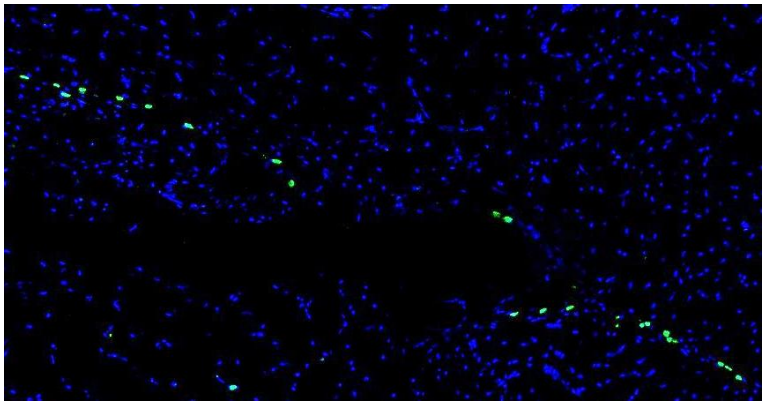
Major input : Large scale analysis, tissular and cellular analysis

Tissular investigation

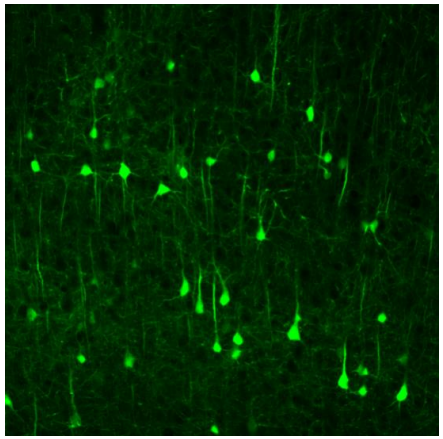
Gene therapy, brain



Cell therapy, muscle

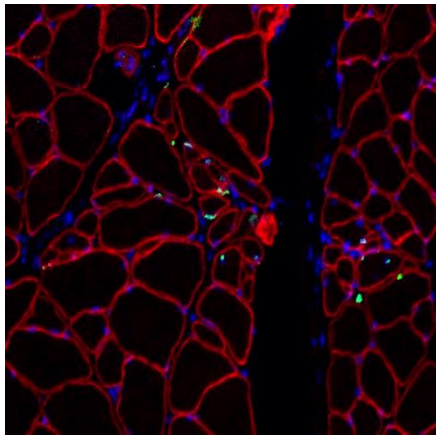


Cellular investigation



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

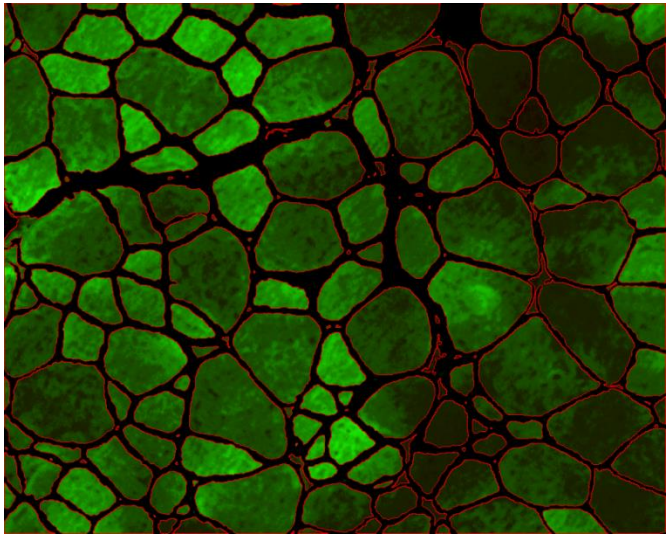
Human lamin A/C immunolabeling on muscle sections



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

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

Quantification of fluorescence



L. Dubreil, APEX UMR 703

Cryosections of muscle injected with AAV2/8 RSV-eGFP

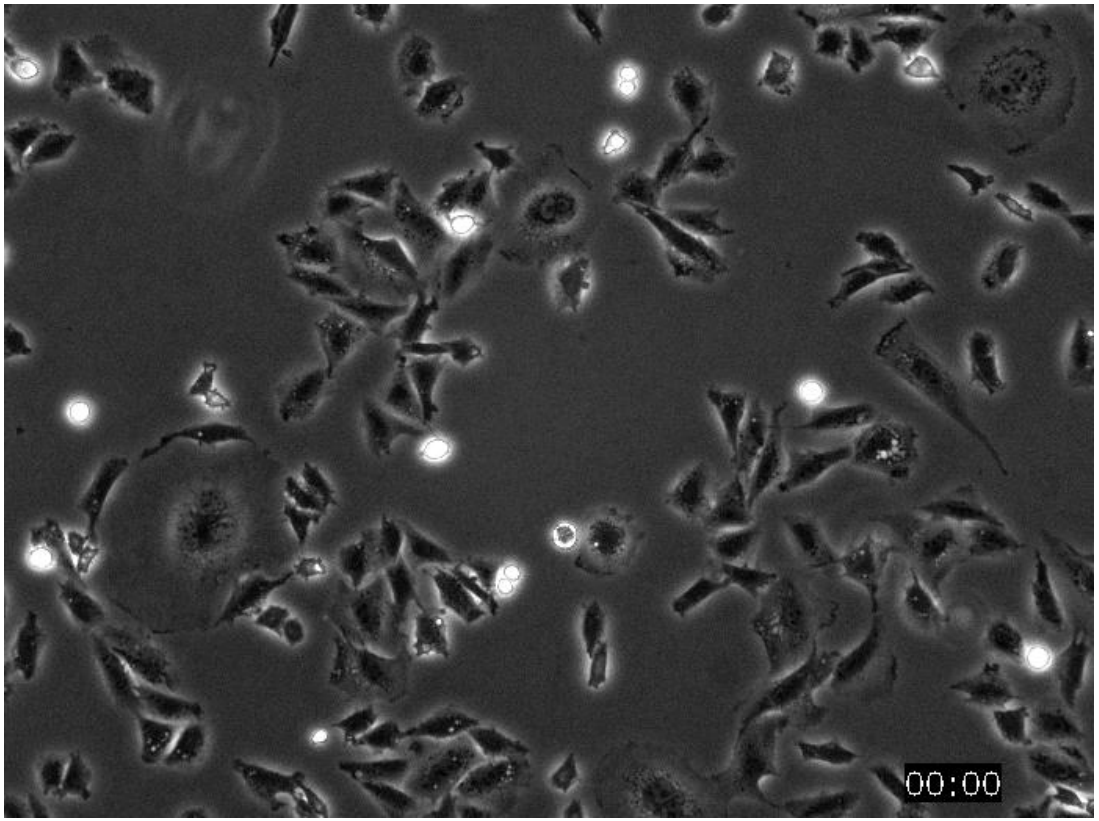
- Number of transduced fibers

- Fluorescence Intensity

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

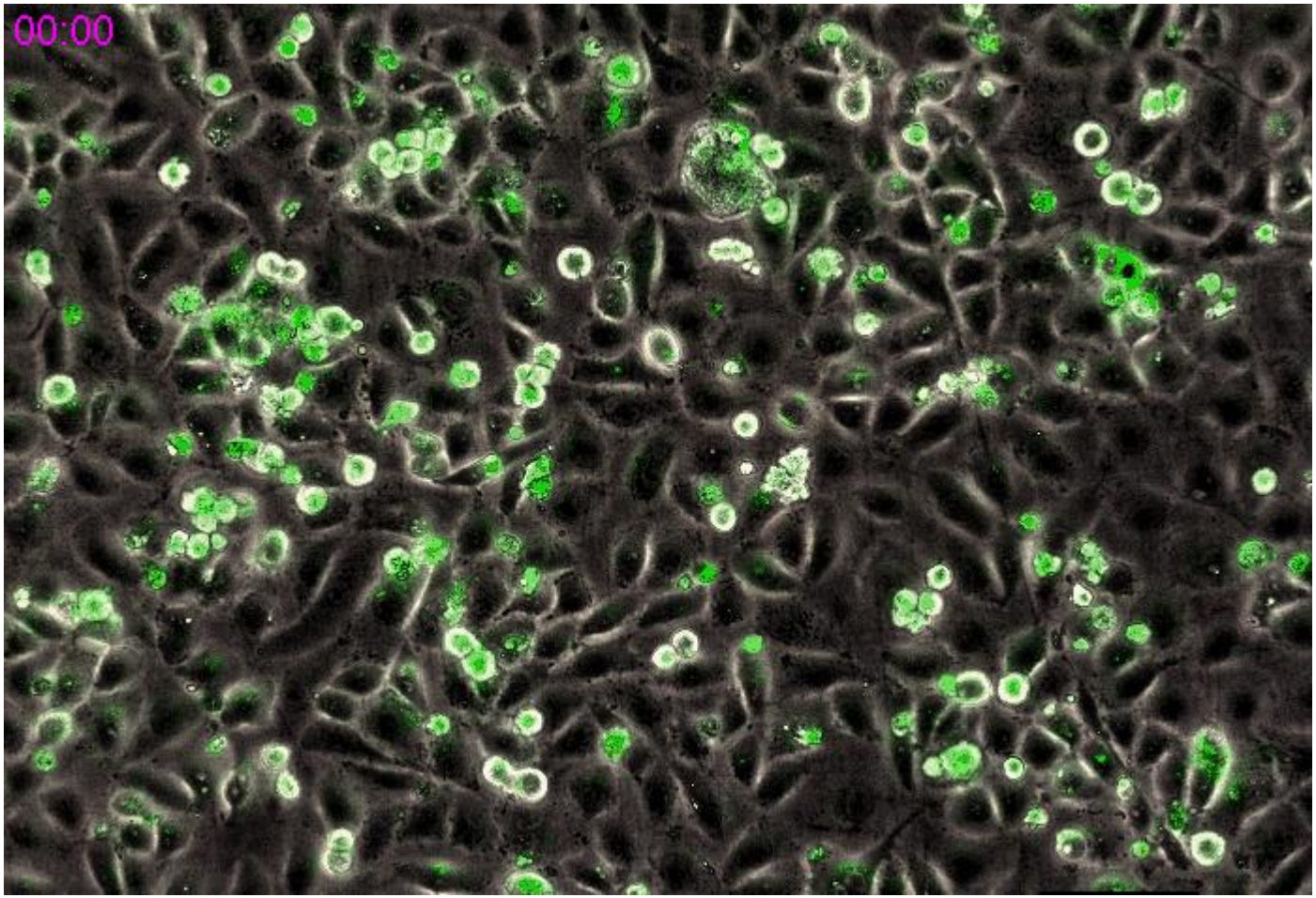
Cellular « ethology » by video microscopy

-Time lapse



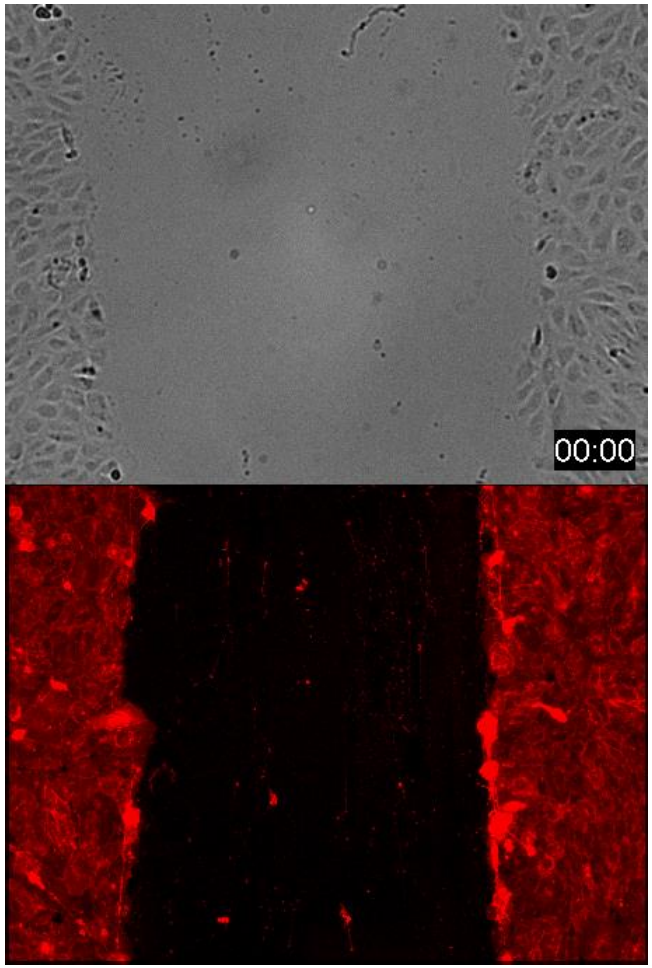
P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy



P. Hulin, MicroPIcell

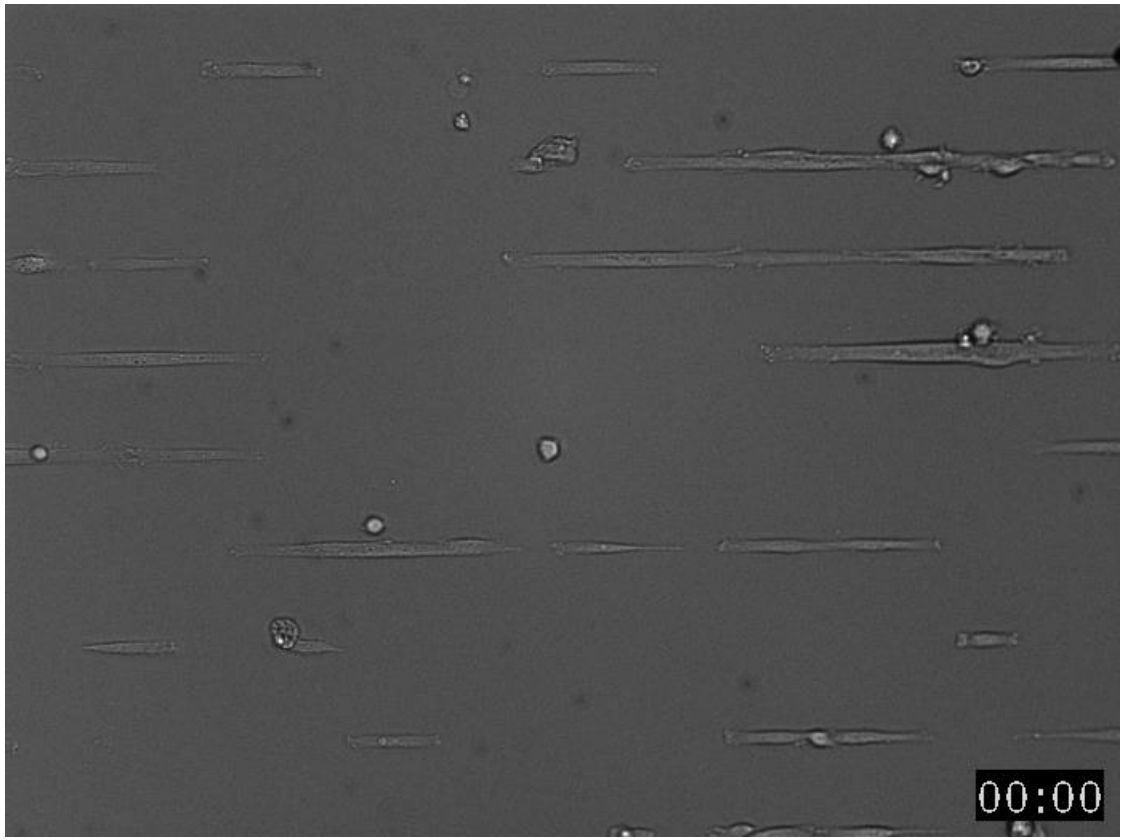
Applications of the wide-field fluorescent microscopy



P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy

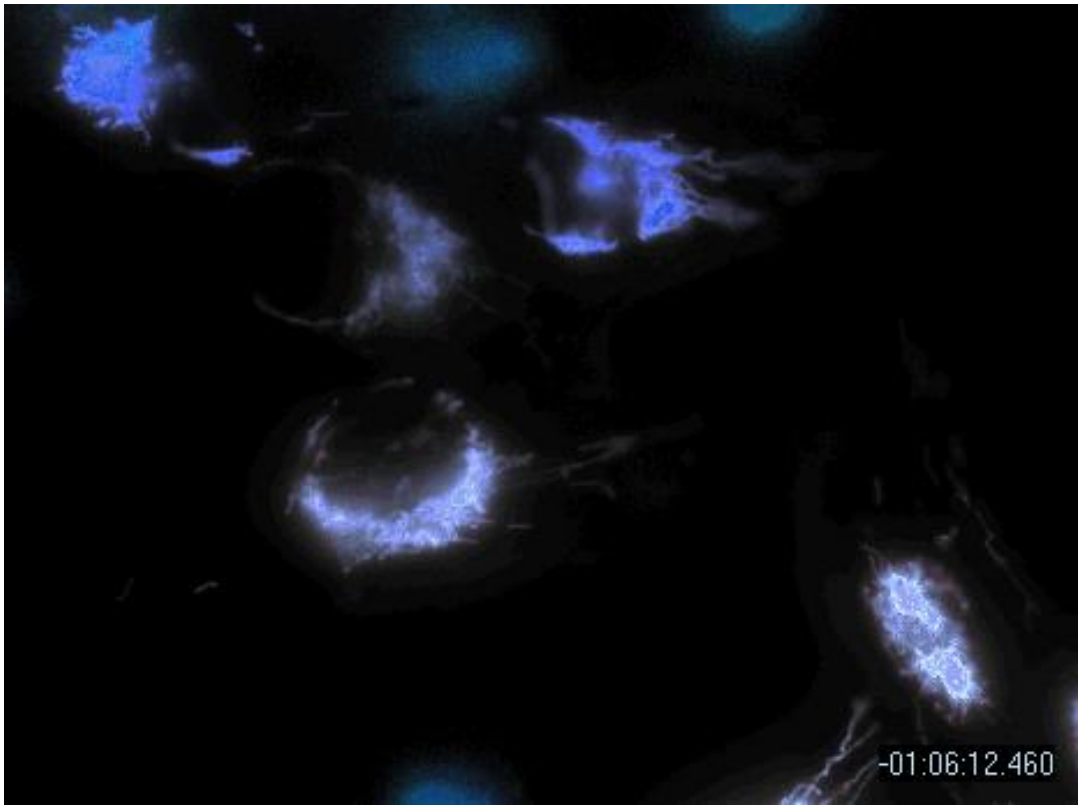
-Cytoo motility



P. Hulin, MicroPIcell

Applications of the wide-field fluorescent microscopy

-Calcium



P. Hulin, MicroPIcell

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 = 0,6λ/NA

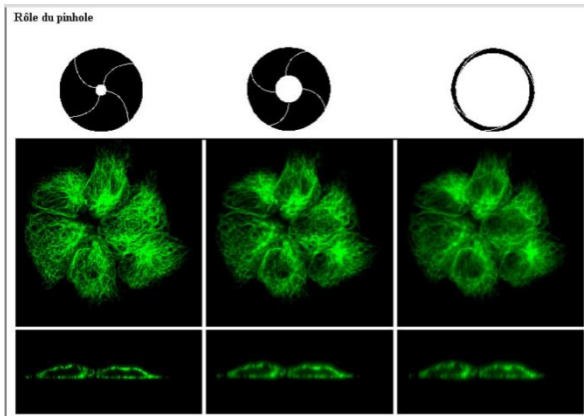
Major input : Increasing of the resolution and monochromatic excitation

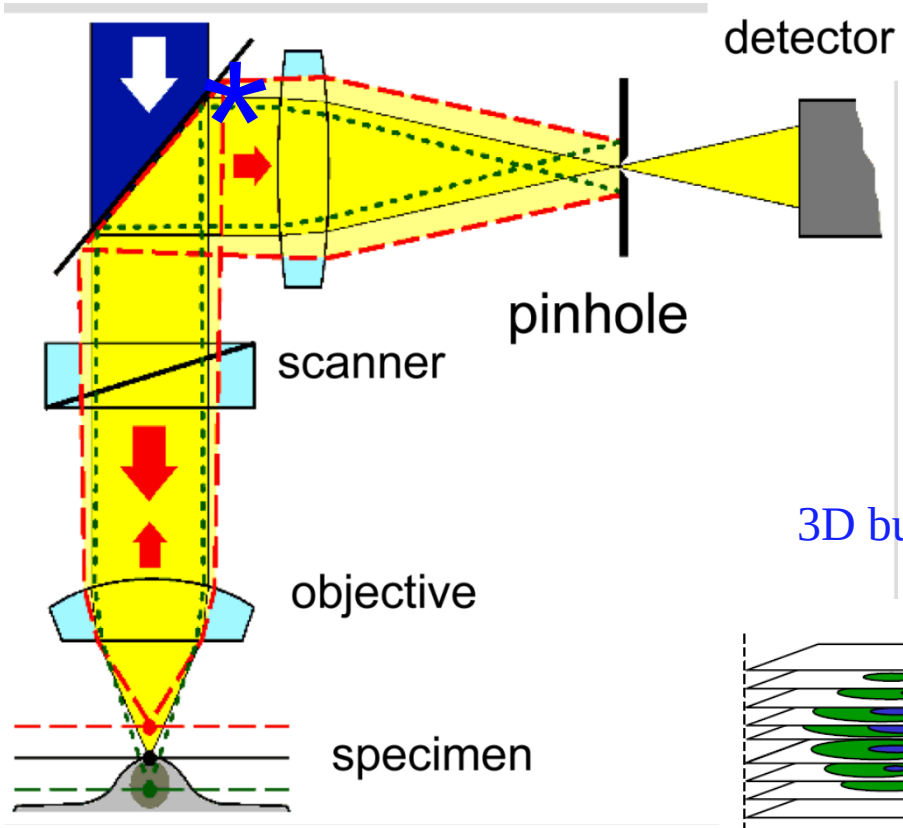
$Rxy = 0,4\lambda/NA$

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

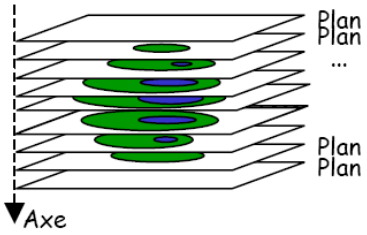
NA : Numerical Aperture : 1,4
λ: 488 nm

dxy = 140 nm
dz = 348 nm





3D building

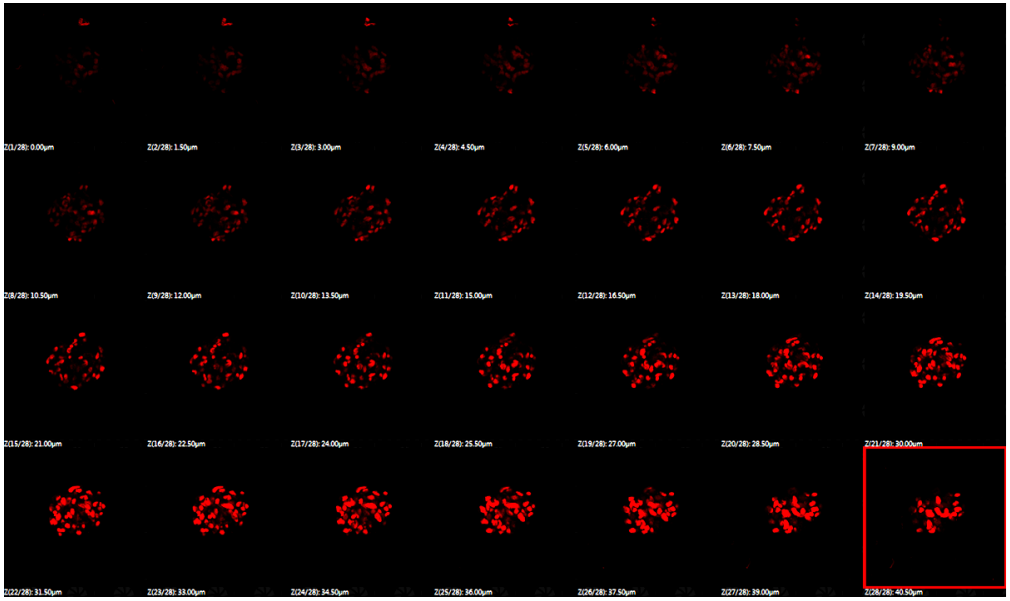


* Monochromatic excitation : Lasers (HeNe), (Arg) ou Diodes

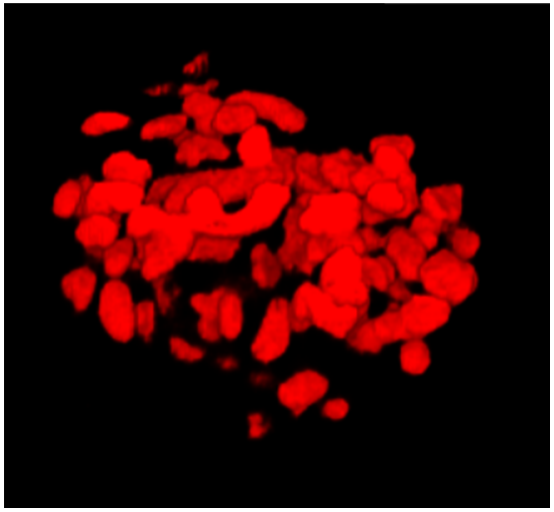
Applications of the confocal microscopy in cell therapy

3D cellular imaging

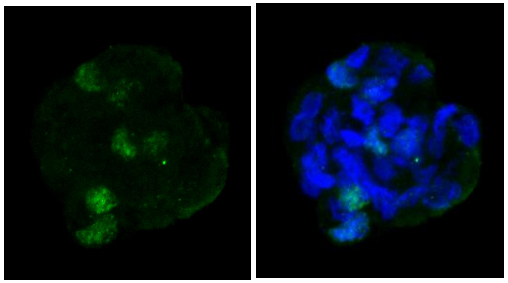
Z stack from cultured Mustem Cells



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

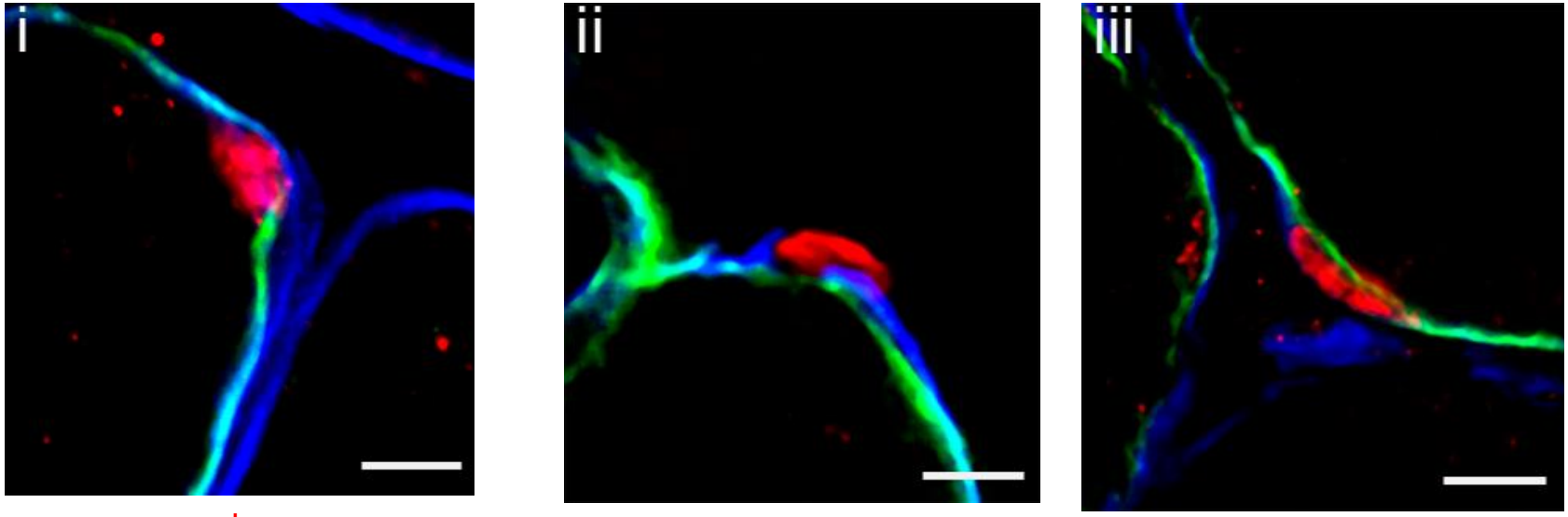
Detection of Mustems after their injection in the muscle

Plasma membrane

Cell

Basal lamina

Muscle section, Z projection



under

above

between

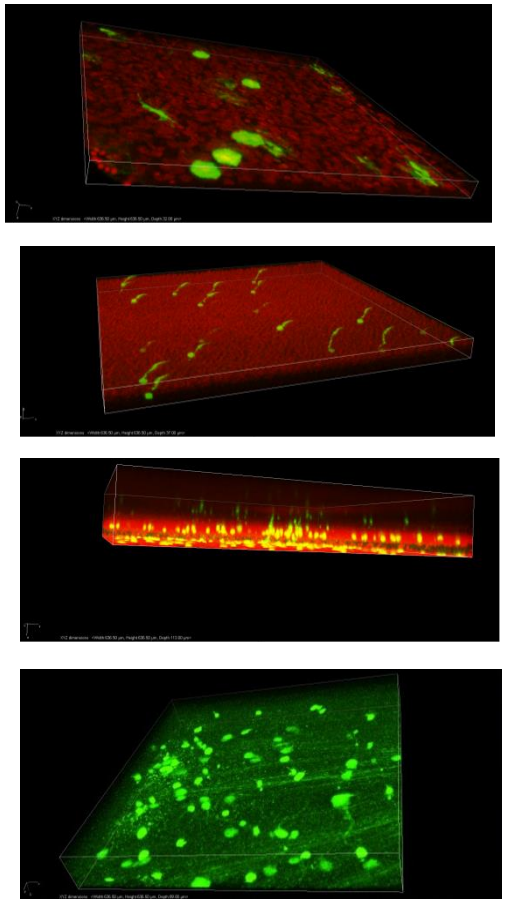
Triple immunolabeling, dystrophine (green), bgal (red), laminine(blue).

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

Applications of the confocal microscopy in gene therapy

Confocal 3D imaging of retina

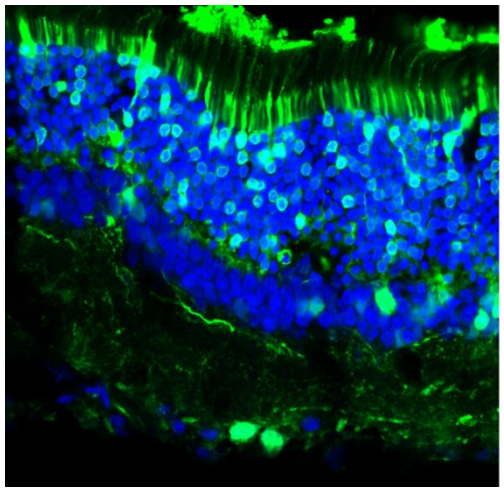
Retinal wholemount

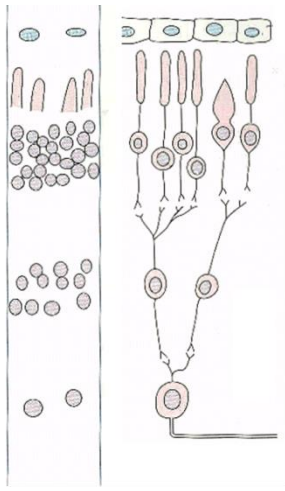


L. Dubreil, APEX UMR 703

Investigation on wholemount retina

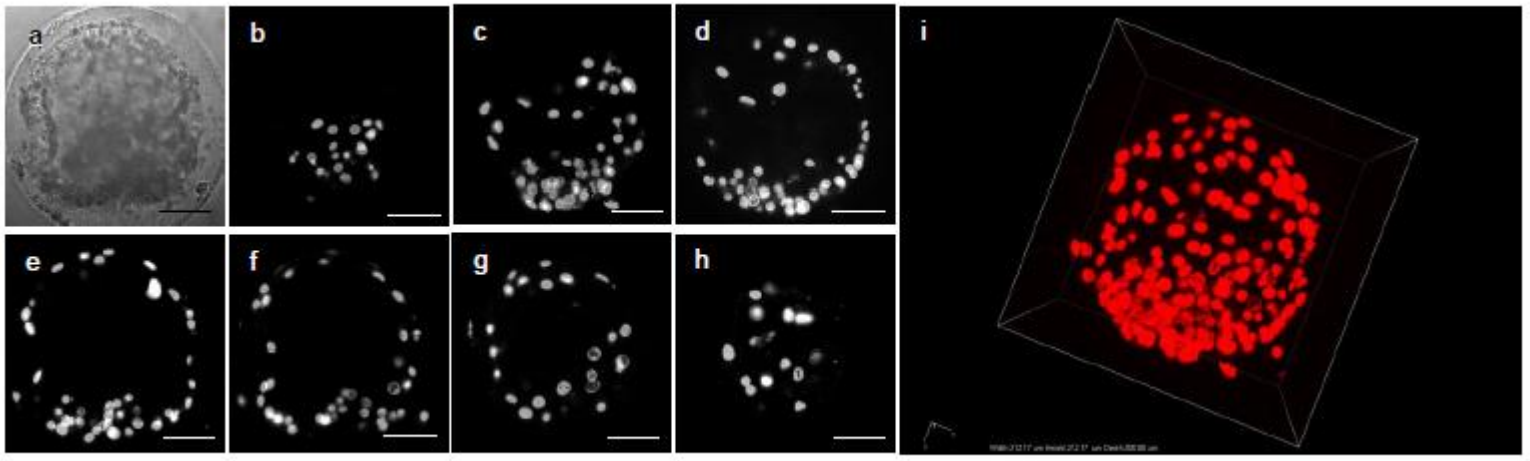
Cartography of GFP expression in the retina after IV injection of AAV10-egfp (newborn rats)





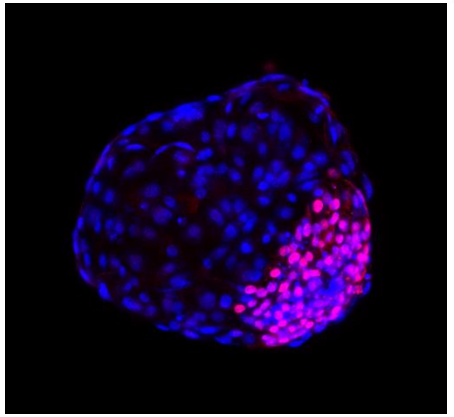
Cryosections of retina : Transduced ganglion cells are underestimated compared to the numerous transduced ganglion cells observed on retinal wholemount (non invasive method).

Confocal microscopy for embyo 3D imaging



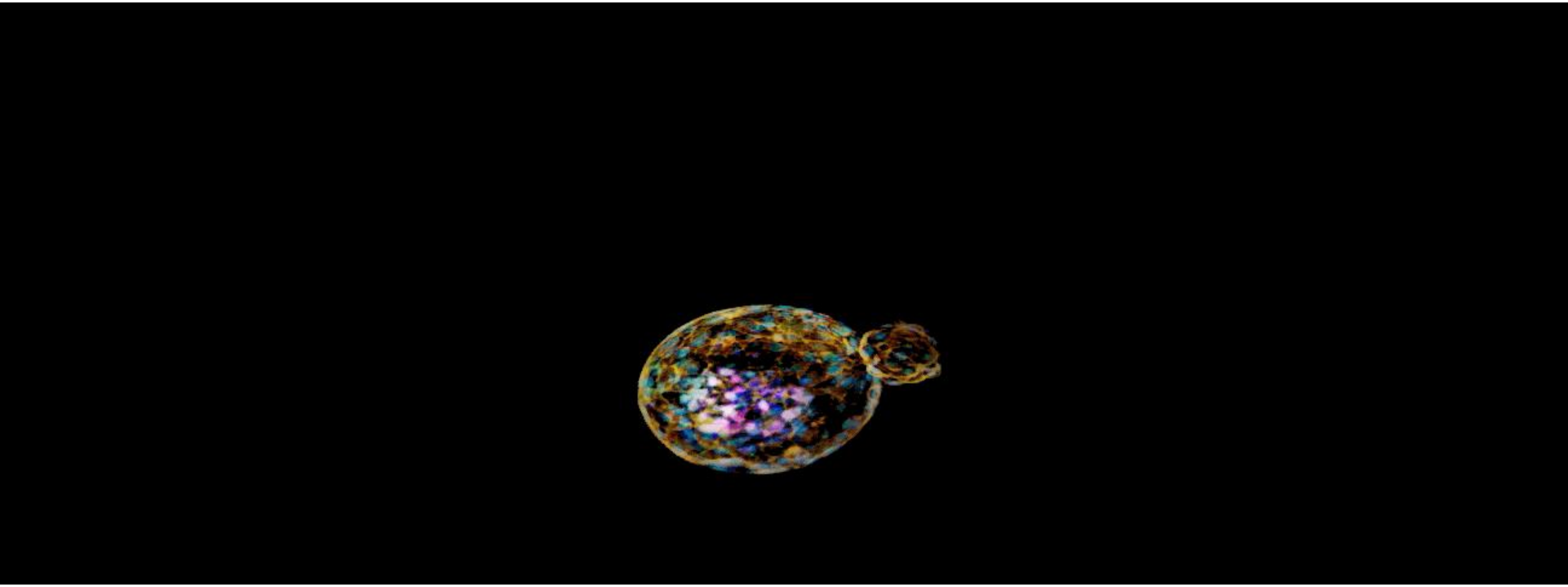
Moreno D and al., Reproduction, Fertility and Development *26(1): 154. January 2014.*

Unit « *sécurité sanitaire des biotechnologies de la reproduction », Oniris*

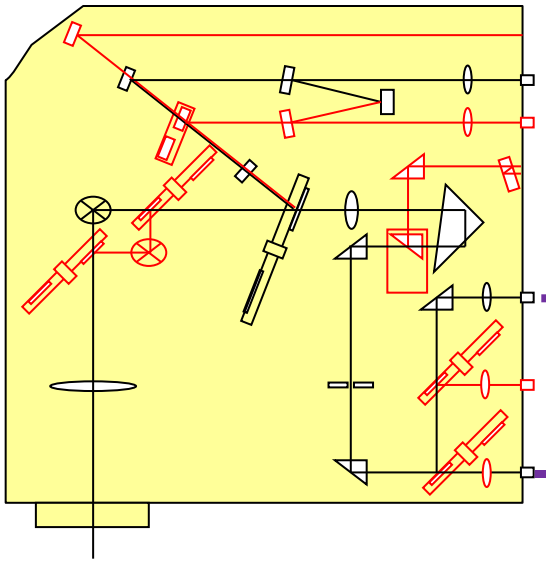


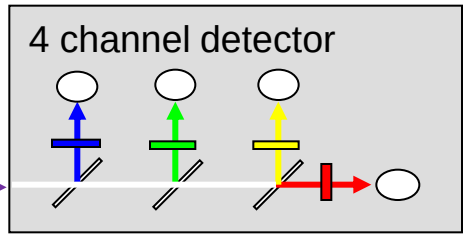
Confocal microscopy

Bovine embryo: Blue-Nuclei/ Red-Actin/Green and Far Red-pluripotent embryonic cells



Major input : spectral separation of emission light

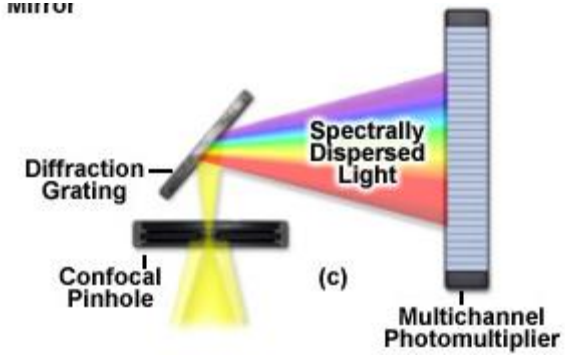




Conventional PMT (4) : bandwidth 50 nm

Système classique PMT

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



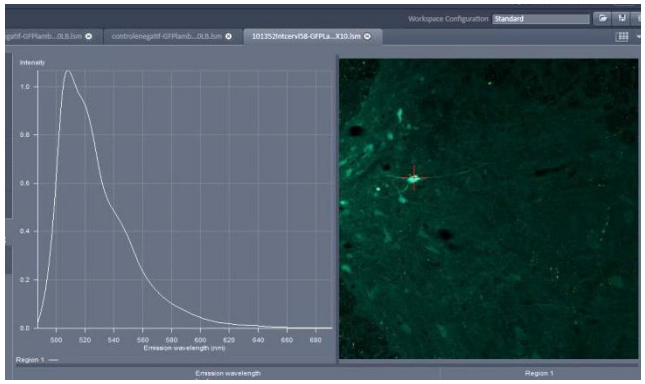
A spectral confocal microscope LSM 780 ZEISS since 11-2013 at APEX

- 6 lasers beam : 405, 458, 488, 514, 561, 633 nm
- 32 spectral detectors GaAsps and 2 PMT
- 1 TPMT/Differential Interference Contrast
- Confocal acquisition FRAP

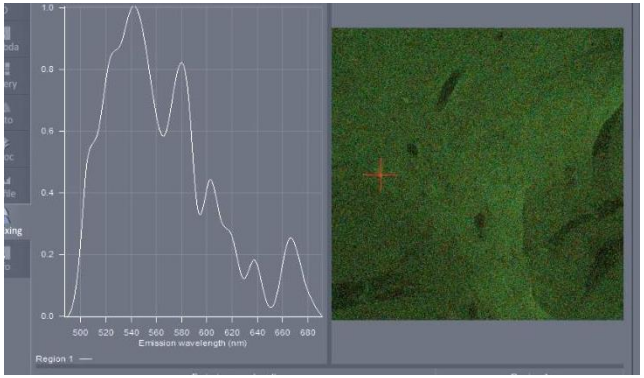
Multilabeling,
Spectral imaging,
Spectral unmixing

Example 1 : separation of GFP signal from autofluorescence in spinal cord

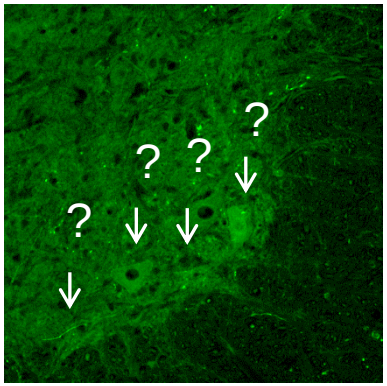
GFP positif sample



GFP negatif sample



Confocal channel mode

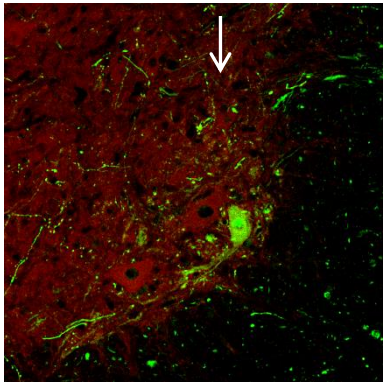


Lambda acquisition from tissue control

___ GFP spectra

___ Autofluorescence

Online fingerprinting



Neurone GFP+

Autofluorescence

Laurence Dubreil, APEX

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

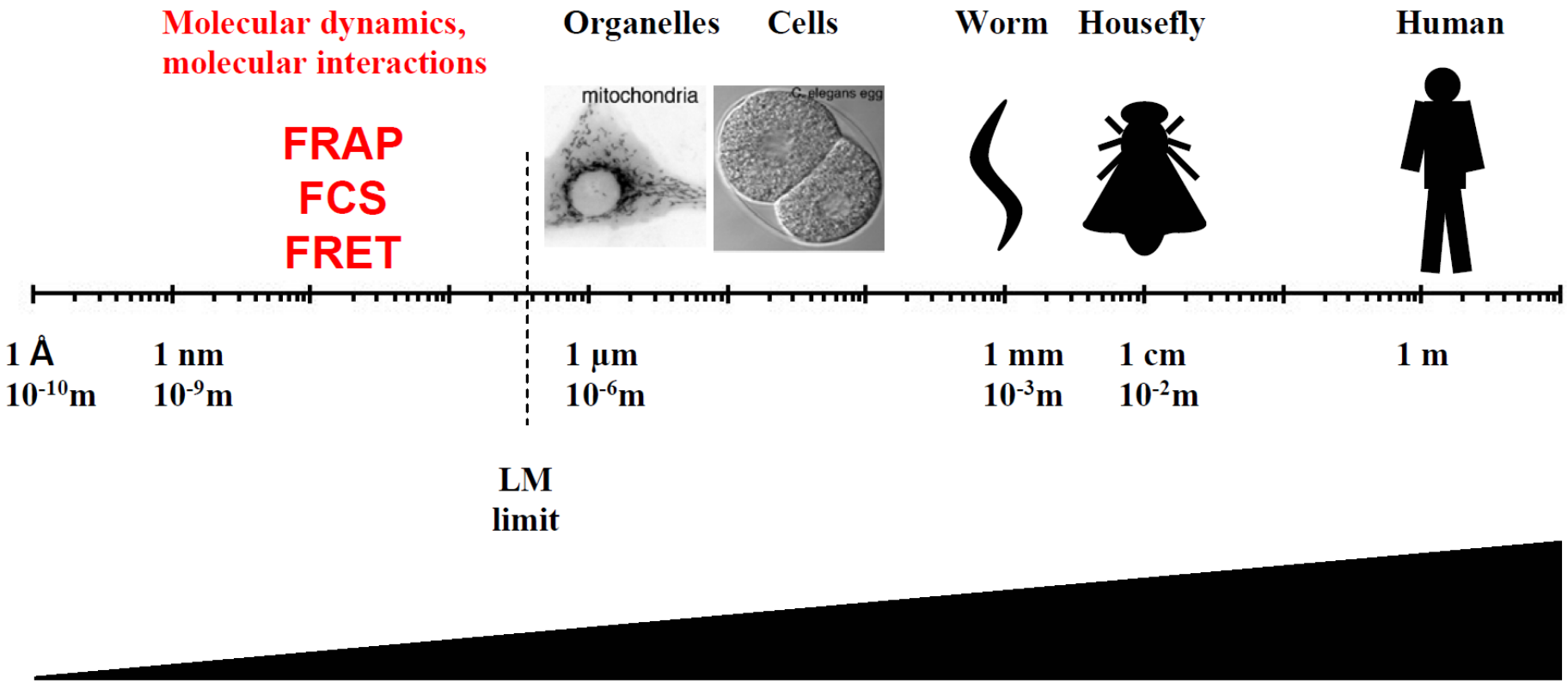
Linear unmixing

Excitation of all specimen planes, photobleaching

Observation above100 µm not possible (thickness of specimen)

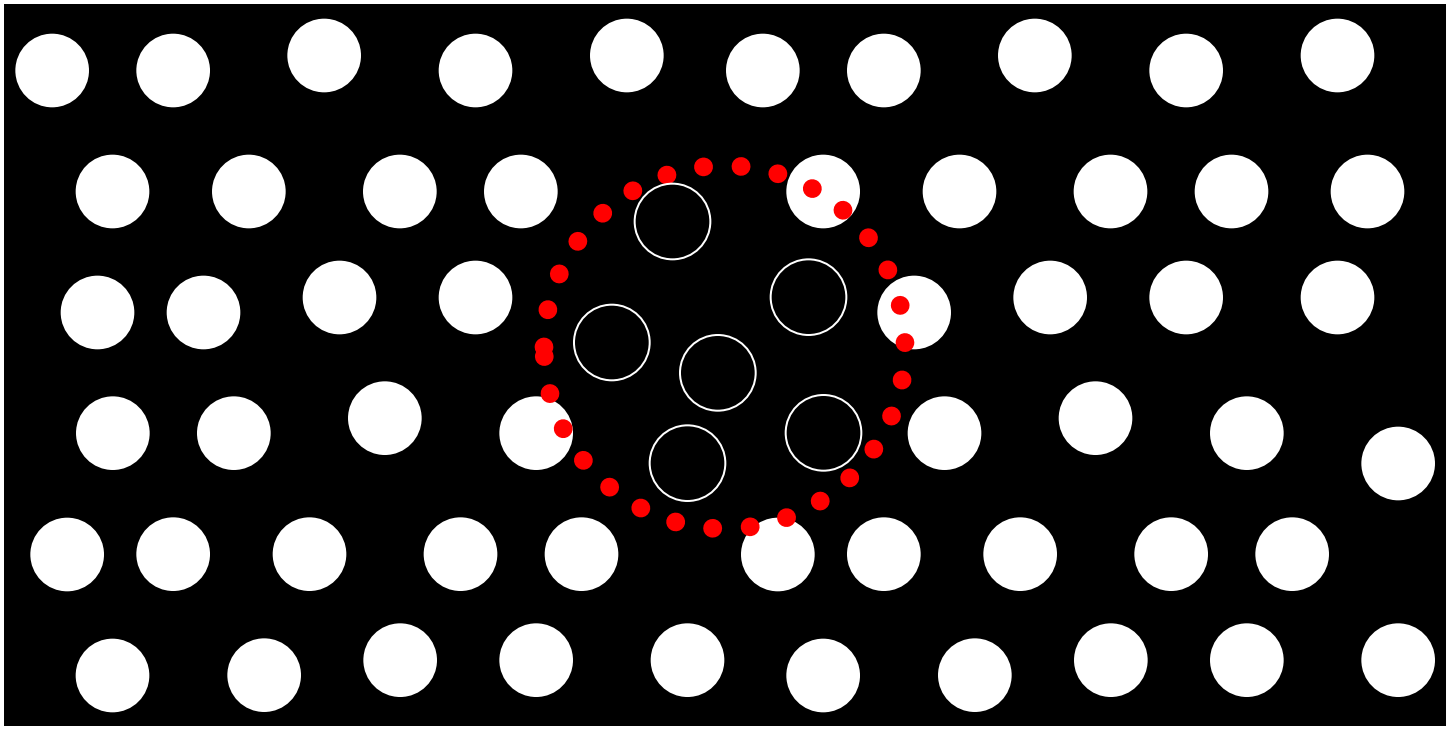
Fast acquisition : resonant scanning

« 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*.

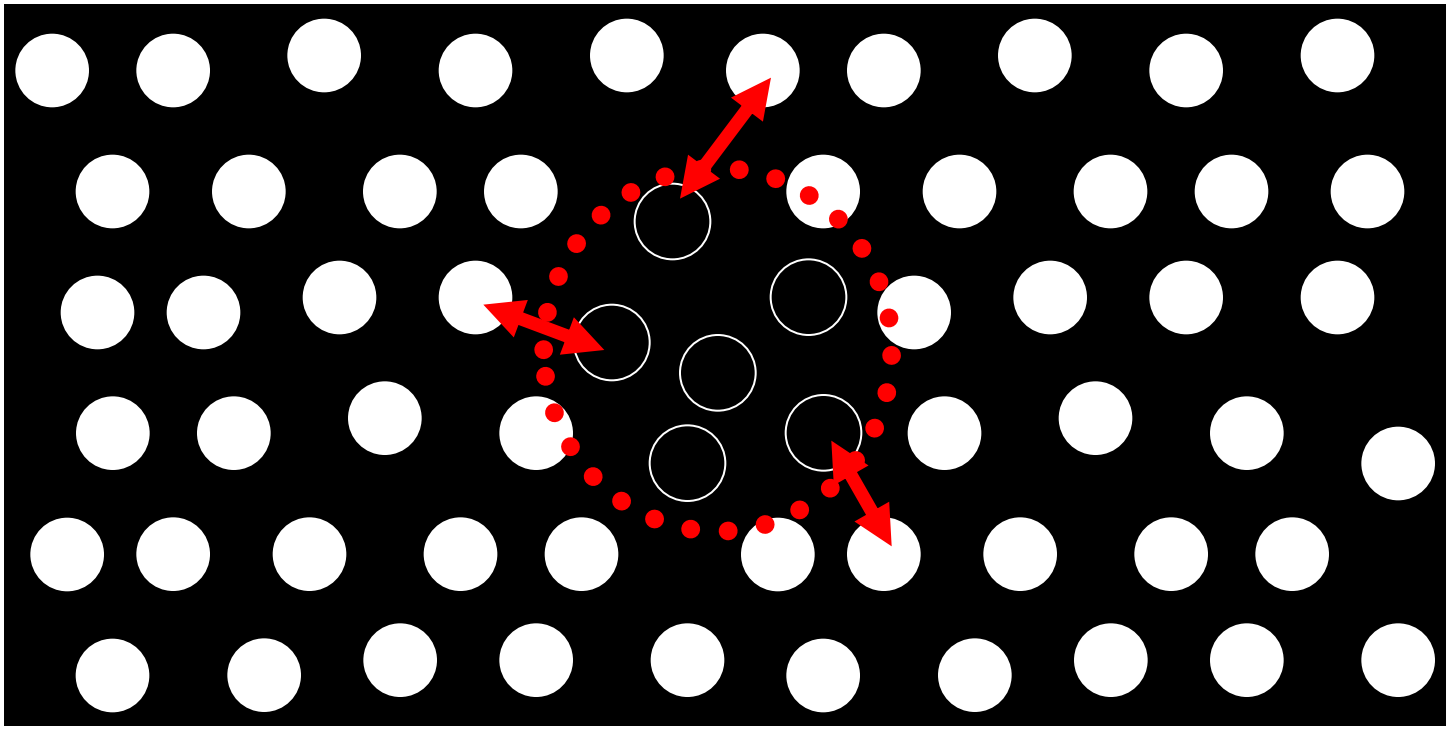
Fluorescence Recovery After Photobleaching (FRAP)



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

We focus a strong laser beam to a spot (red dotted circle).
Then the strong irradiation BLEACHES the fluorescence at that spot.
Let's think of fluorescence molecules dispersed in a field. White circles represent the molecules.

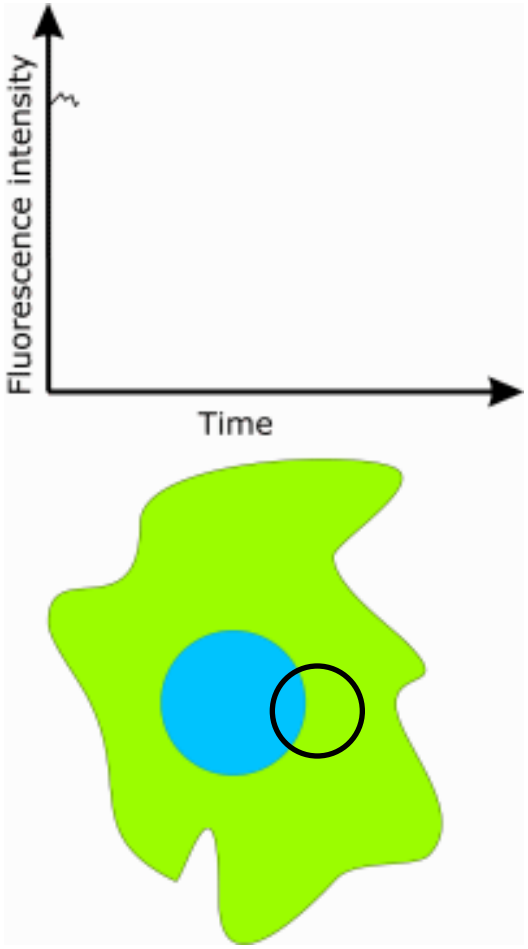
Fluorescence Recovery After Photobleaching (FRAP)

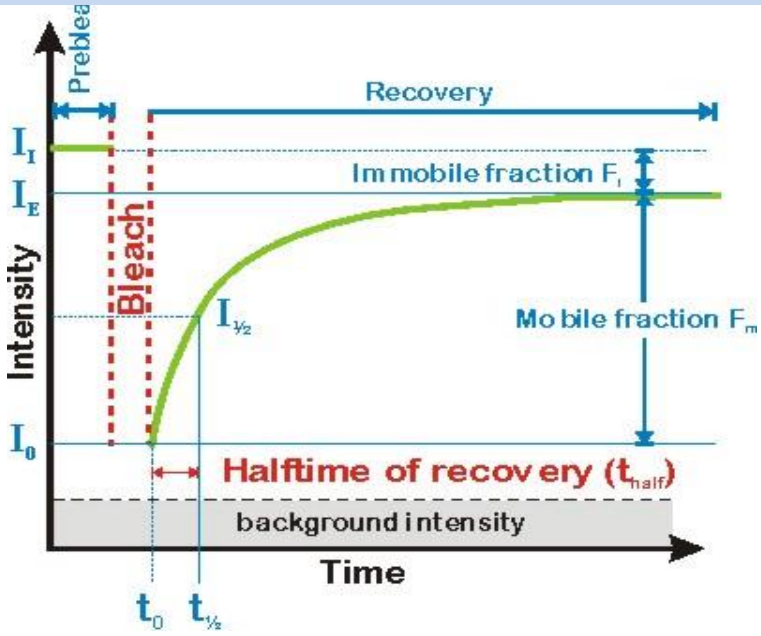


Kota Miura, EMBL Heidelberg, Germany. Based on the EAMNET Practical Course (20, April, 2004)

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)

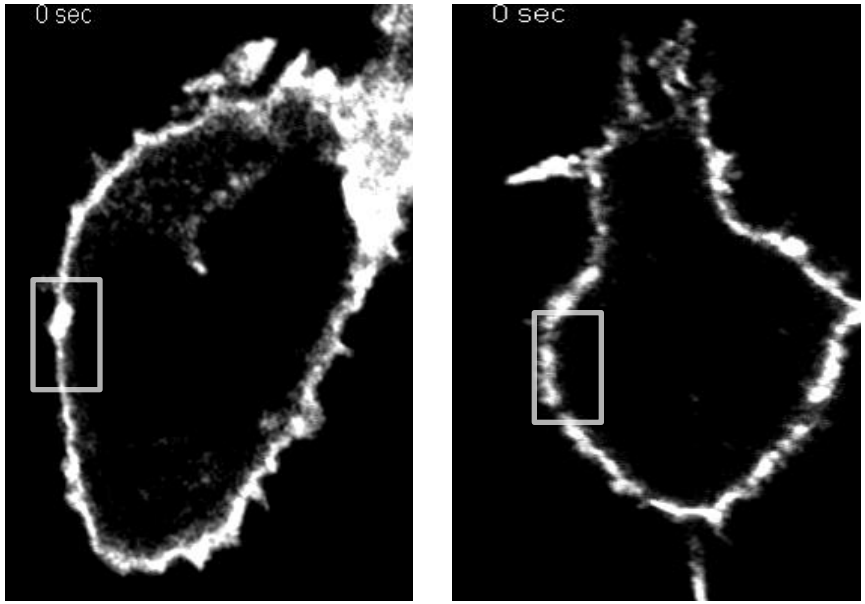




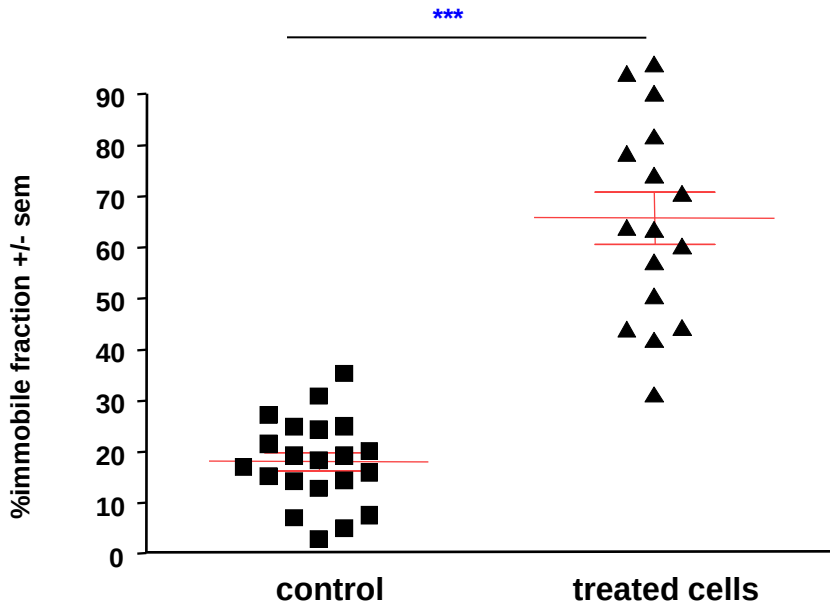
An idealized plot of a FRAP recovery curve.
I_I: initial intensity
I_0: intensity at timepoint t_0 (first postbleach intensity)
$I_{1/2}$: half recovered intensity ($I_{1/2} = (I_E - I_0) / 2$)
I_E: endvalue of the recovered intensity
t_{half}: Halftime of recovery corresponding to $I_{1/2}$ ($t_{1/2}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

Fluorescence Recovery After Photobleaching

control **Treated cell**



S. Nedellec, MicroPIcell



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 Recovery After Photobleaching

Recapitulating, FRAP is generally suitable to study and investigate:

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

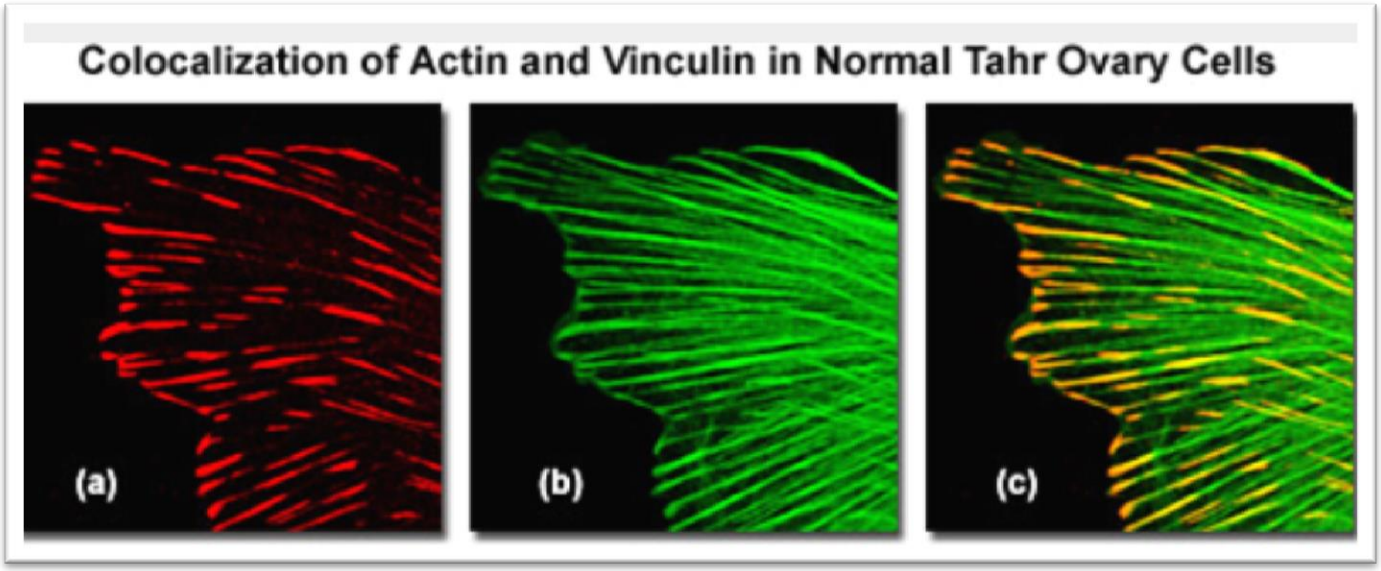
- Compartmentalization and connections between intracellular compartments.

- 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



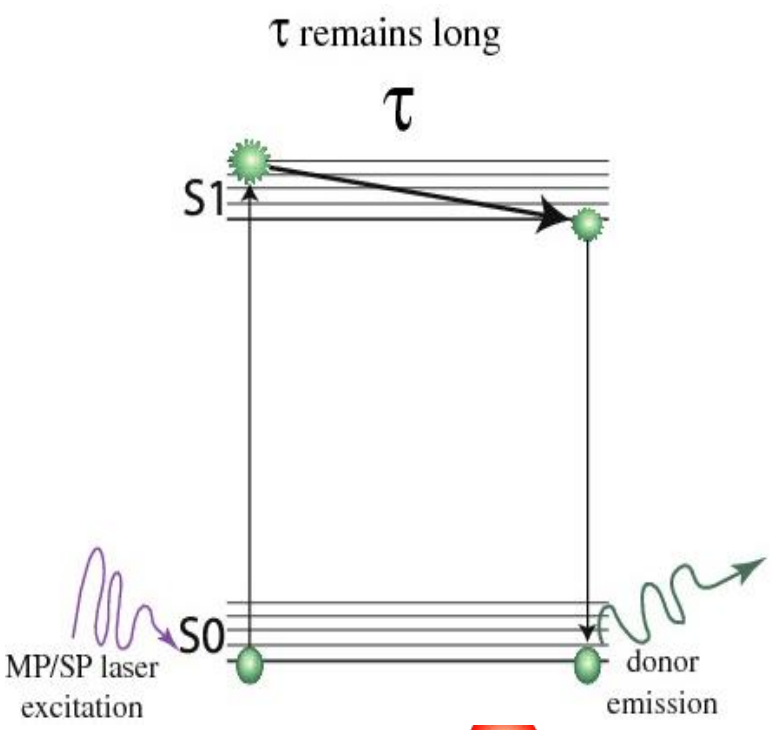
olympusconfocal.com

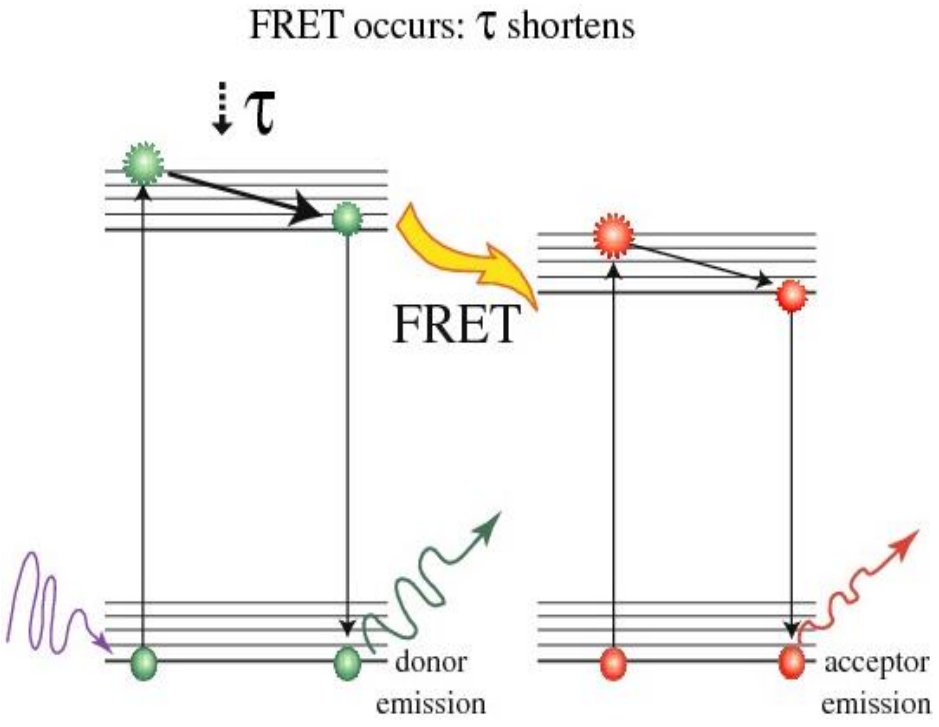
S. Nedellec, MicroPIcell

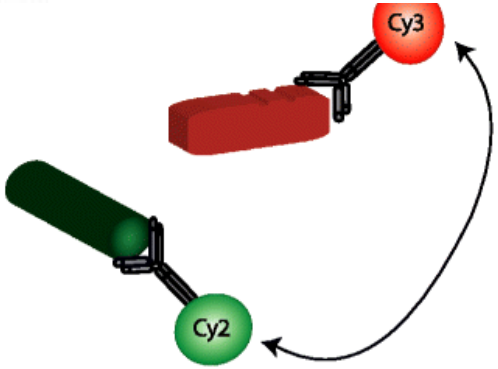
Colocalization, in a biological manifestation, is defined by the presence of two or more different molecules residing at the same physical location in a specimen. Overlay of both channels (red and green) displays a strong colocalization viewable in yellow.

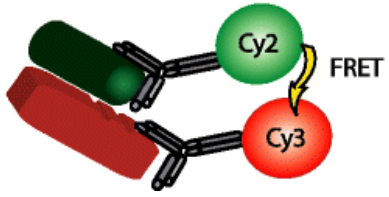
The ability to determine colocalization in a confocal microscope is limited by the resolution of the optical system and the wavelength of light used to illuminate the specimen. Widefield fluorescence and confocal microscopes have a theoretical resolution of approximately 200 nanometers

FLIM/FRET: supplying co-localization





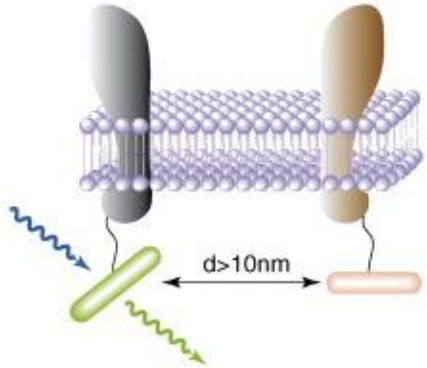




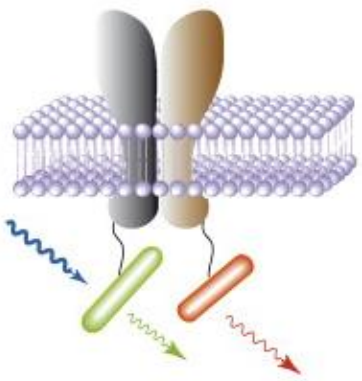
FLIM/FRET: supplying co-localization

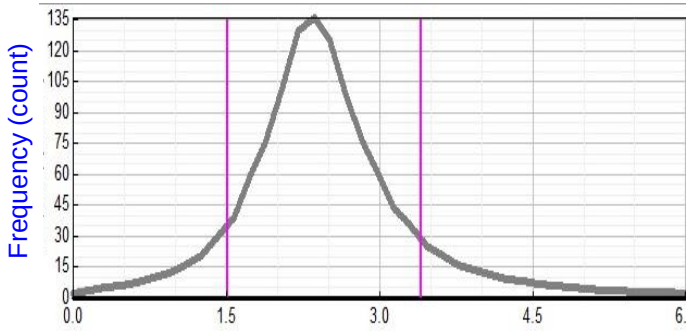
FLIM (Fluorescence Lifetime Imaging Microscopy) is a powerful technique to measure protein-protein interactions, and is based on the FRET (Forster Resonant Energy Transfert) principle, as shown below.

No FRET
d>10nm

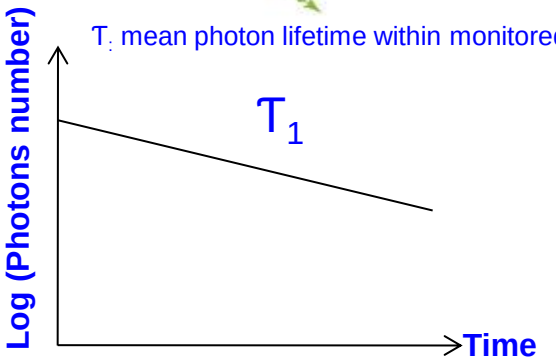


FRET d<10nm

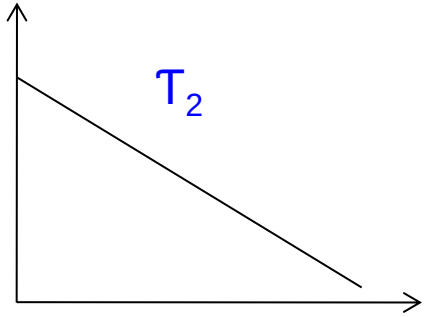




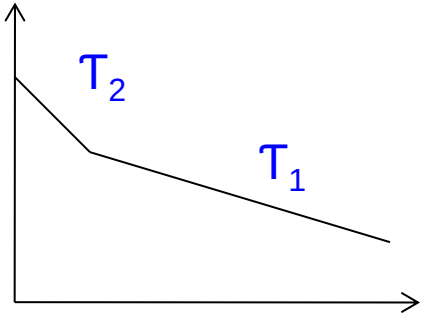
T: mean photon lifetime within monitored area



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



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

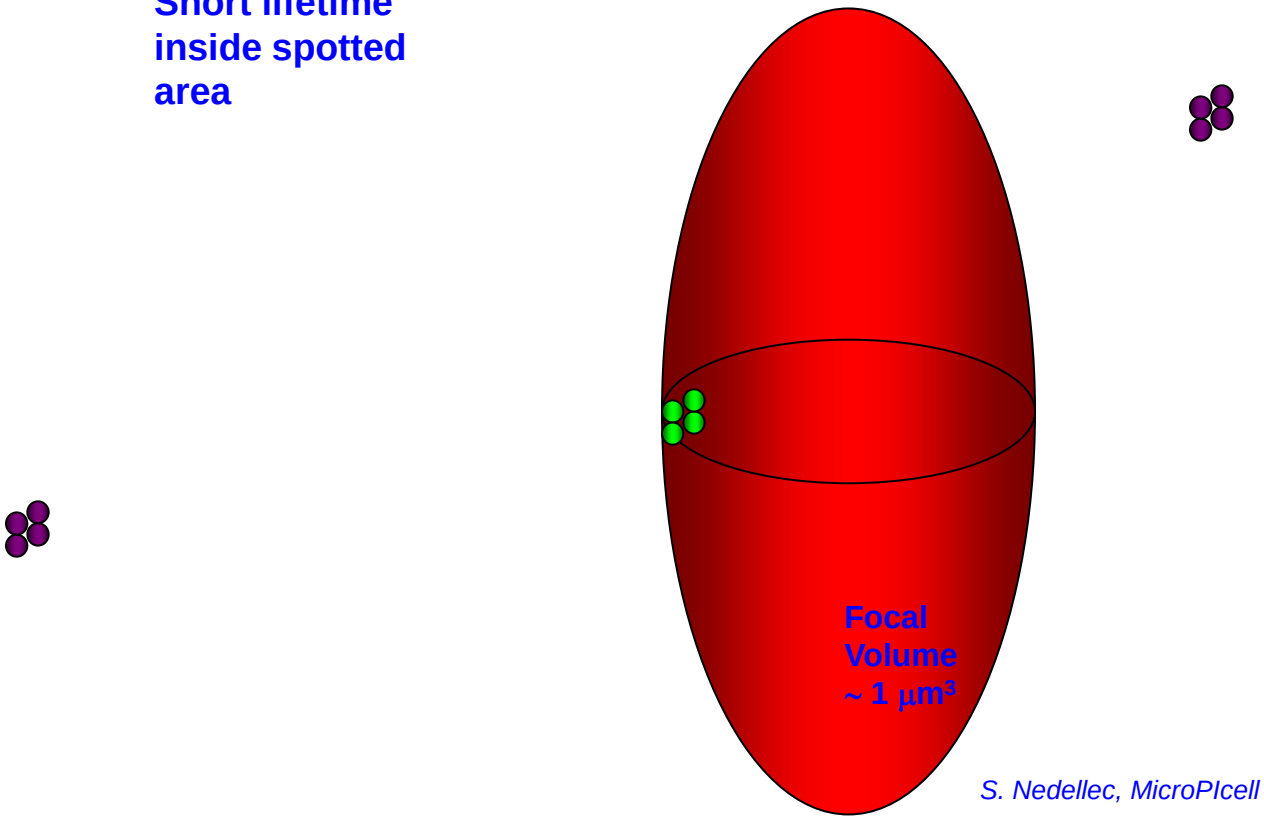


Bi-exponential decline:
33% FRET (T_2)
66% No FRET (T_1)

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

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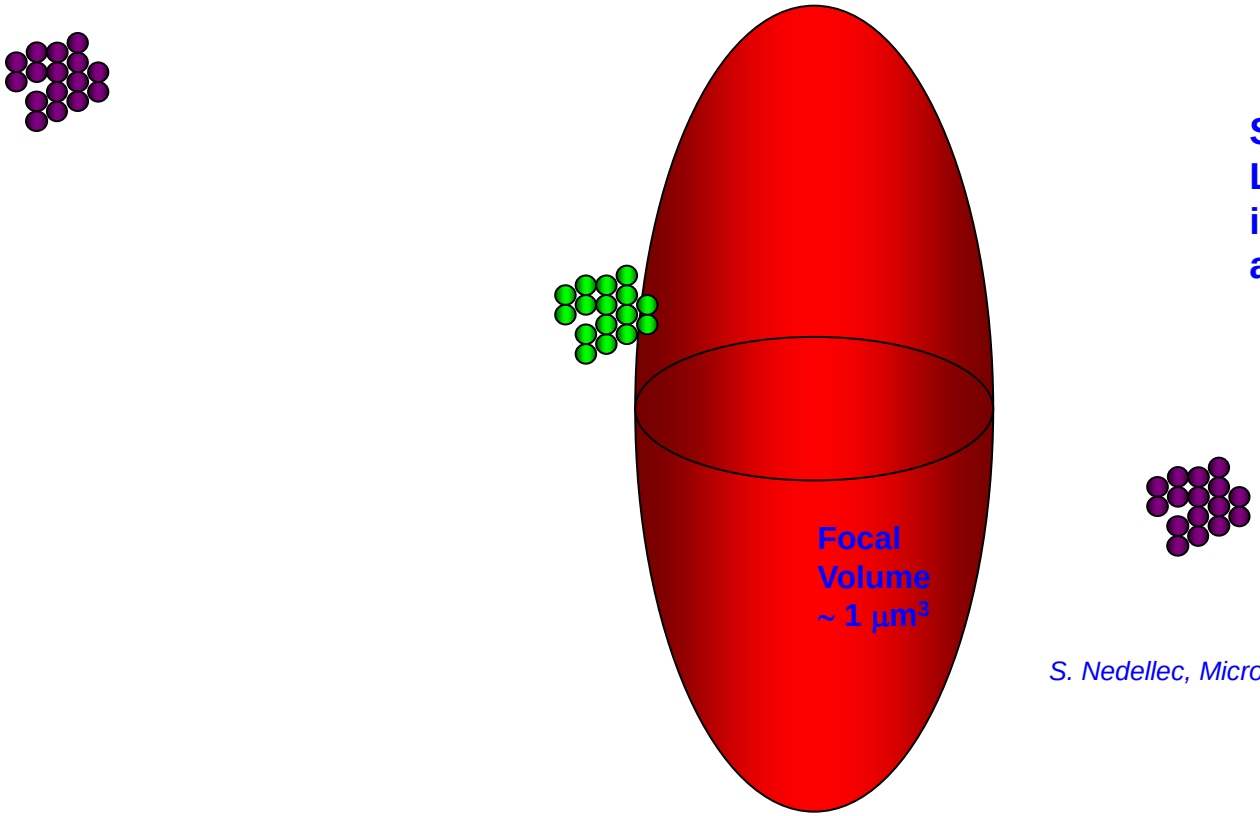
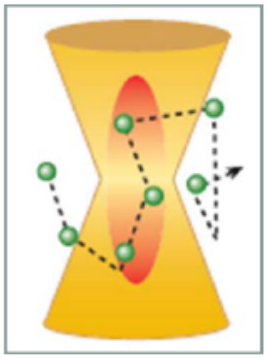
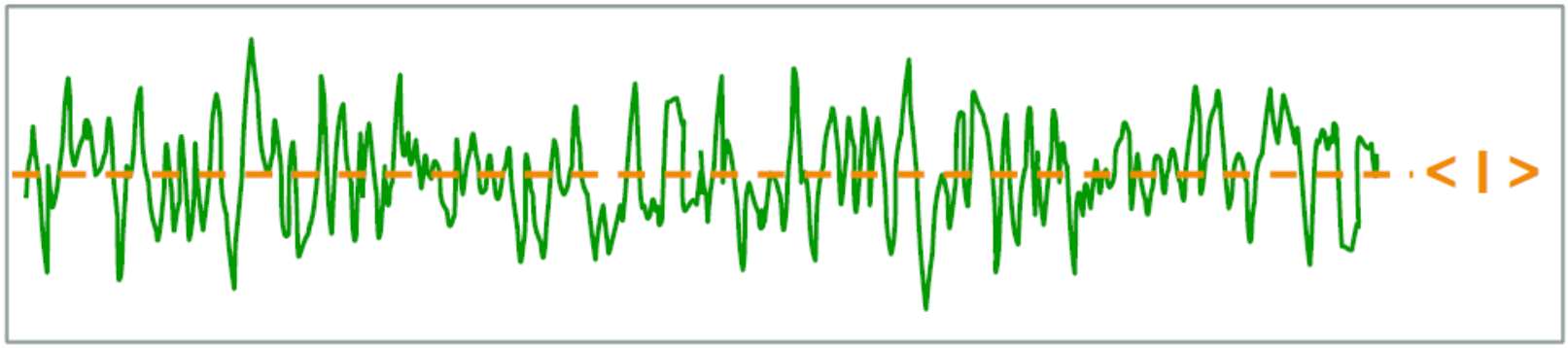


Slow Dynamics
Long lifetime
inside spotted
area

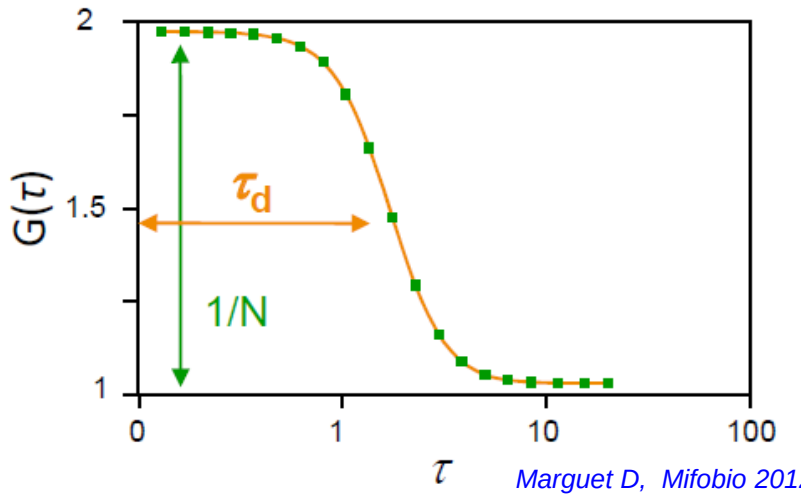
S. Nedellec, MicroPIcell

Fluorescent Correlation Spectroscopy





temporal fluctuations



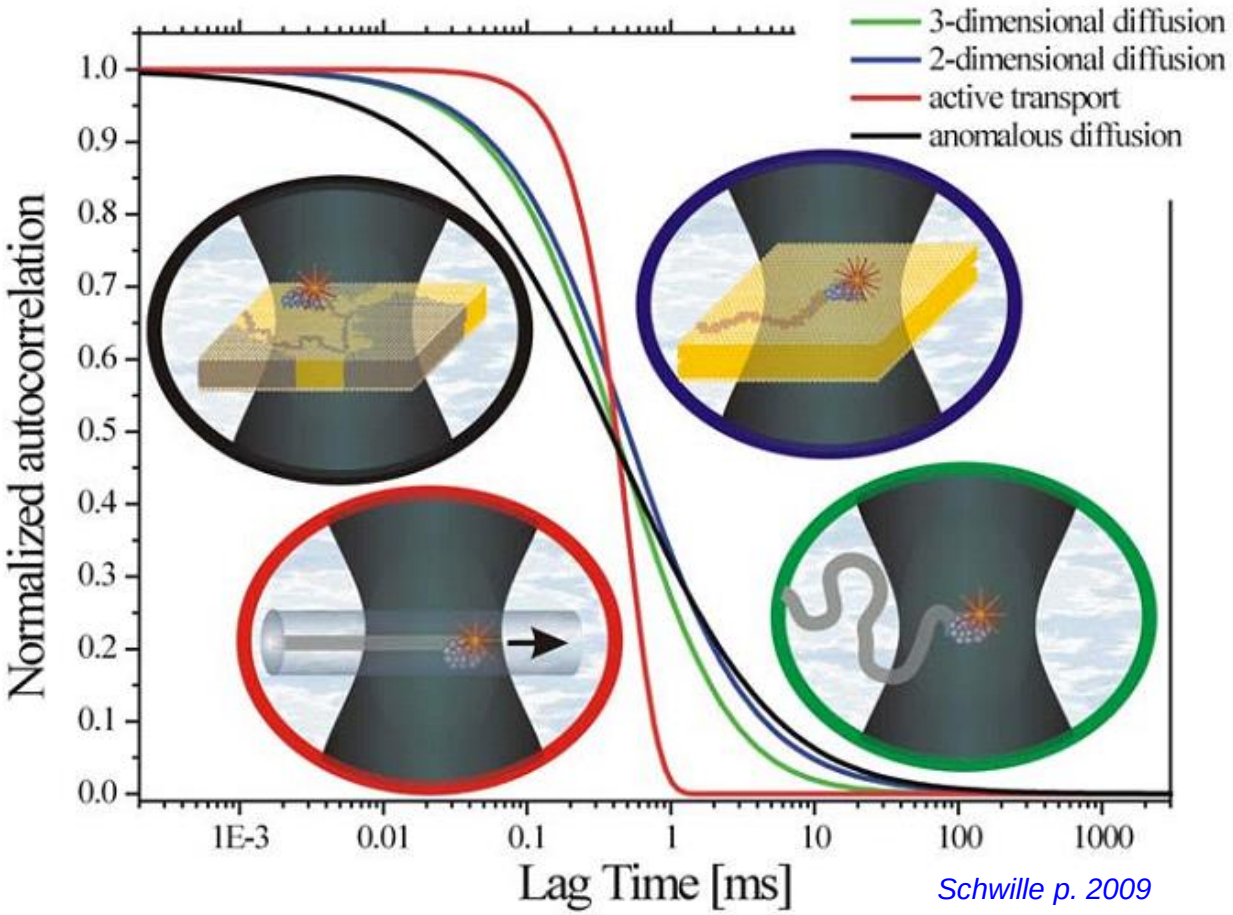
Marguet D, Mifobio 2012

autocorrelation function (ACF)

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

$\Rightarrow$ N the average number of molecules

Fluorescent Correlation Spectroscopy



Schwille p. 2009

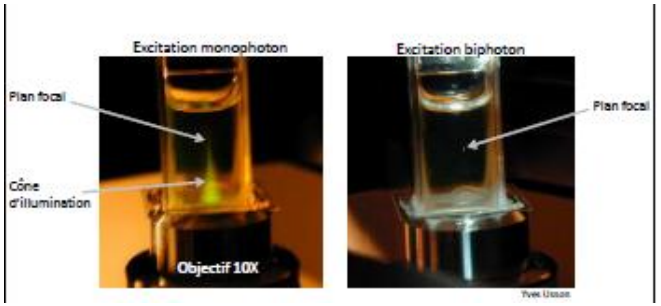
Research question	Live-cell imaging	Fixed-cell imaging
Molecular structure	No	Crystallography, electron microscopy
Conformational changes	FRET, single-molecule FRET	Crystallography, electron microscopy
Mobility of bound species	FRAP, FCCS, SPT	No
Intracellular activity of proteins	FRET sensors, FRET	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

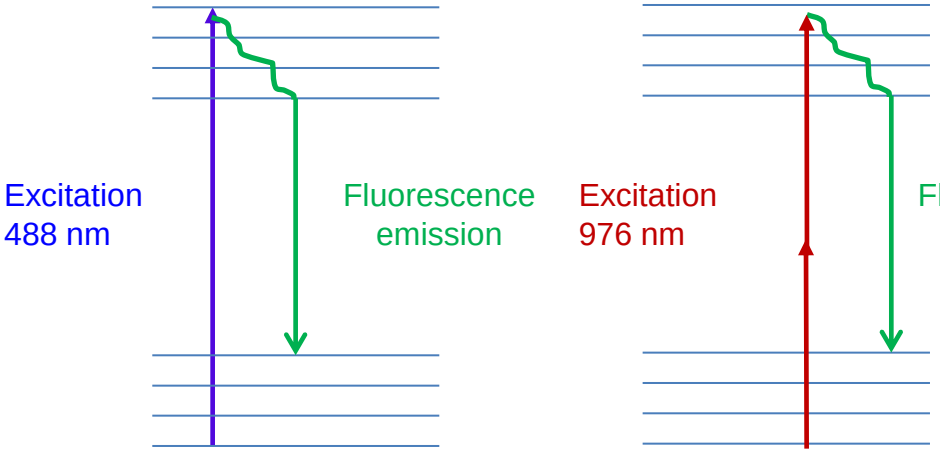
DIC, differential interference contrast; FCCS, fluorescence cross-correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescent resonance energy transfer; IRM, interference reflection microscopy; PALM, photoactivated localization microscopy; SIM, structured illumination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

Principle

1Photon excitation

2Photon excitation





Femtosecond laser (infrared laser beam 680-1300 nm)

Major Inputs of 2Photon excitation:

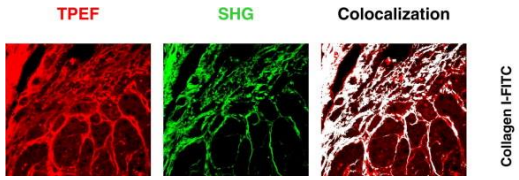
- Deeper tissu penetration
- Reduction of the photoxicity
- Imaging of living tissu
- Imaging of second harmonic signals

Tissular exploration without labeling :

- Tissue structural changing (SHG, THG, CARS):
  - Collagen
  - Microtubule
  - Muscle myosin;
  - Myelin
  - Membranar lipids,
  - Storage lipids

→

- Markers of pathology
- Efficacy of treatment



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]

Tissular exploration with labeling :

- Vasculature imaging (fluorescent probe systemic injection)
- Use of transgenic models expressing fluorescent reporters (GFP, YFP, mCherry …)

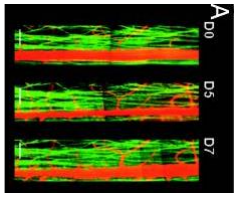
Imaging Single cell in vivo :

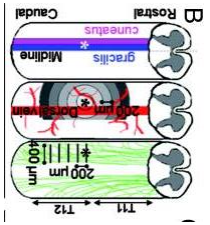
- Track the motility of cells within organs in vivo, long terme imaging in the same animal
- Imaging the cells of the immune system in a live animal by transduce fluorescently tagged genes in specific cell populations
- Progression of virus in live tissue by GFP-expressing virus

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

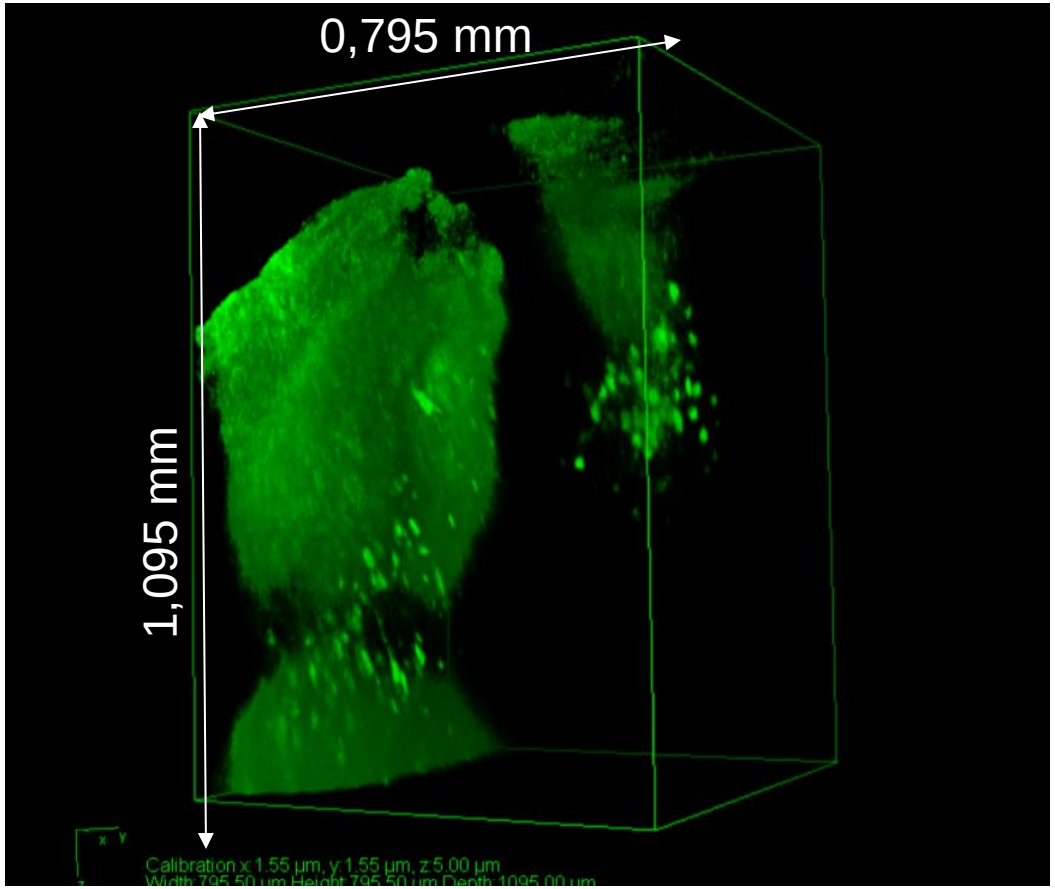




Major Input : deeper tissu penetration (2X with biphotonic microscopy)

Scaled brain GFP expression

A1RPM, Nikon, MiFoBio 2012



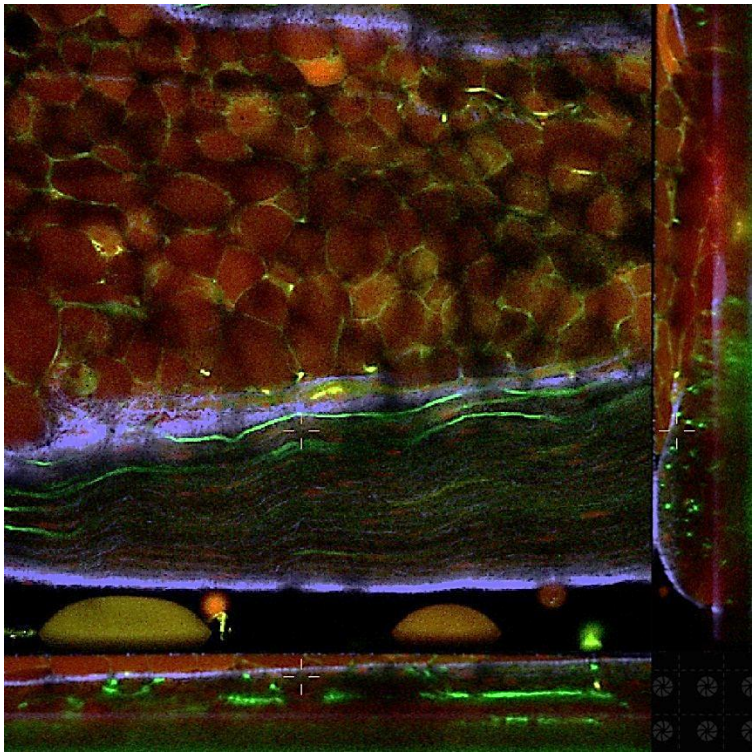
L. Dubreil, APEX UMR 703

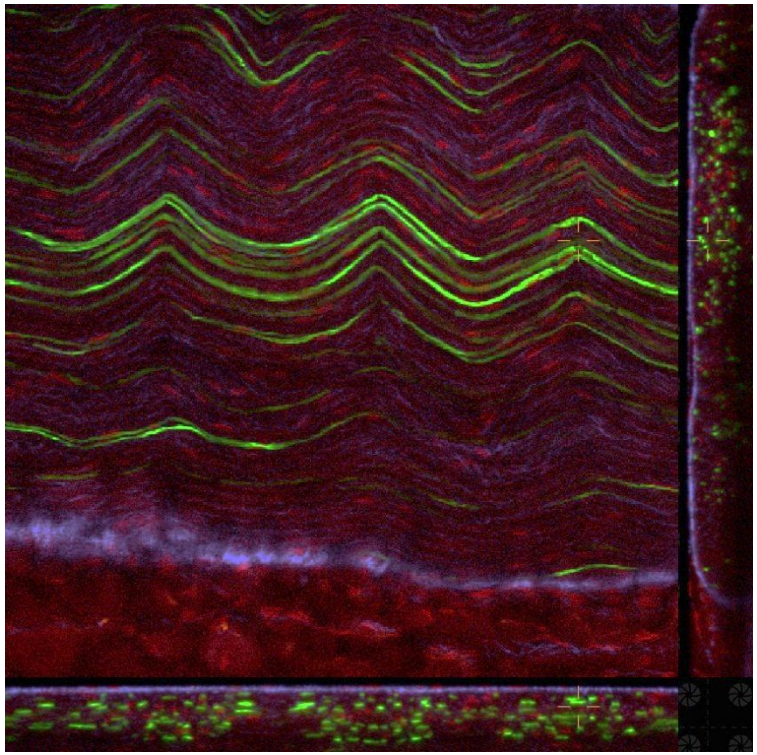
Biphotonic microscopy

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

sciatic nerve from a mice

A1RPM, Nikon, MiFoBio 2012





L. Dubreil, APEX UMR 703

Collagen matrix : second harmonic generation

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	-	-	++

Breaking the limit

Abbe limit : $d = \lambda/2$

TIRF : Total Internal Reflexion Microscopy

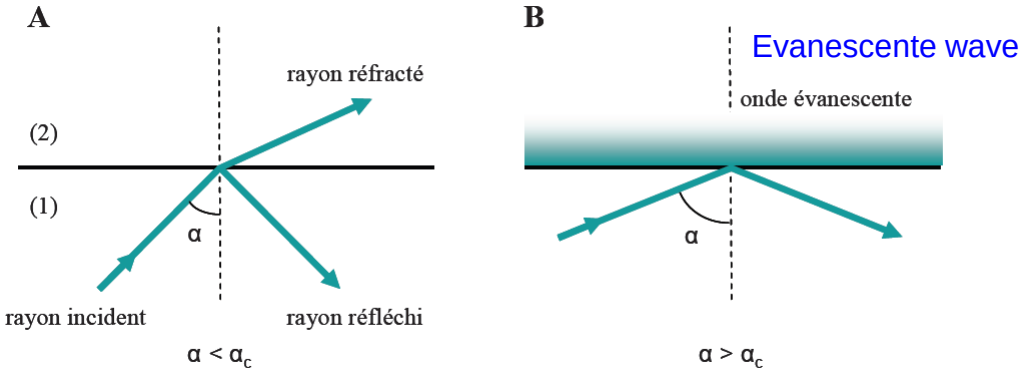
SIM : Structurated Illumination Microscopy

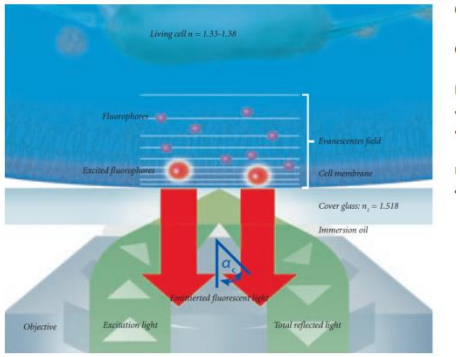
STED : Stimulated Emission of Depletion

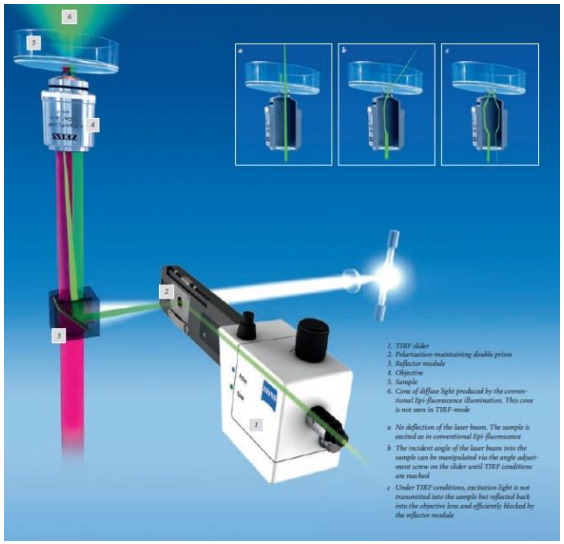
PALM : Photoactivation Light Microscopy

Total Internal reflection fluorescence microscope principle

TIRF : an evanescent wave to selectively illuminate and excite fluorophores in the region immediatly adjacent to the glass water interface. Resolution 80-100 nm







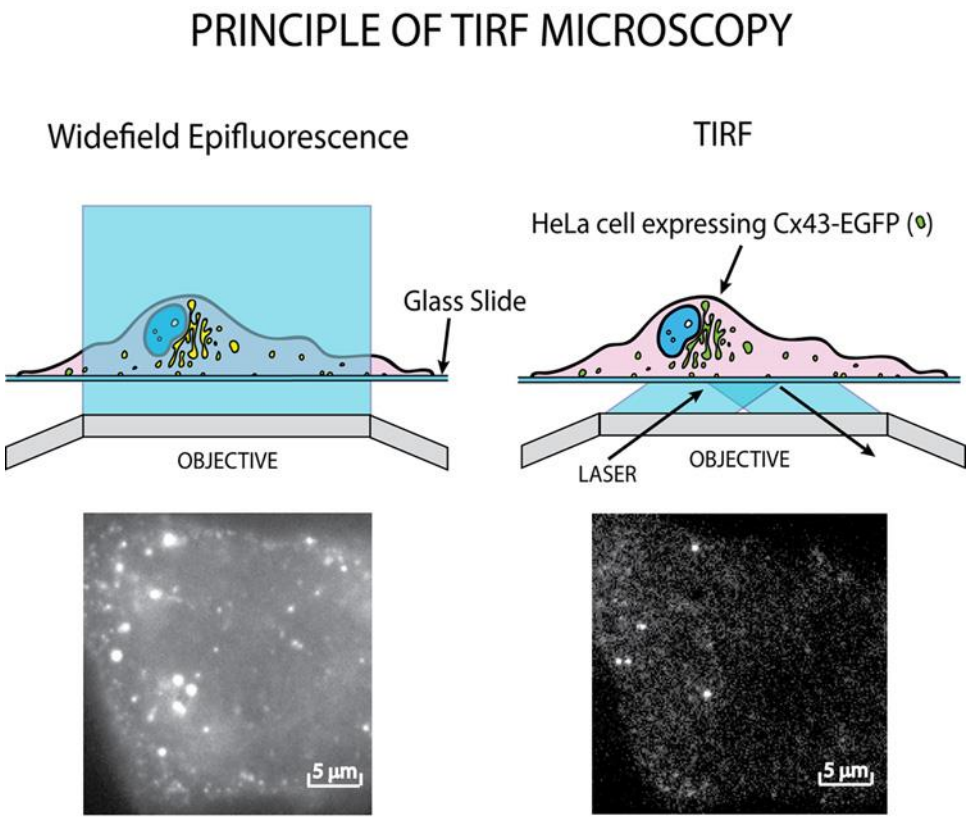
- the module can simultaneously be combined with conventional HBO or other white light.

objX63, NA 1,46

Digital EMCCD Camera

ROLERA em-c²

Illustration TIRF



Connexine 43, protéine membranaire
(teneur réduite cardiomyopathie)

Total Internal reflection fluorescence microscope which applications in gene and cell therapy

- **Entry of viruses or non viral vector labeled with a fluorescent dye into cells on living or fixed cells and colocalization with cellular compartments, analyze route taken. Effect of chemical inhibitor and small interfering RNA to block specific entry pathway**

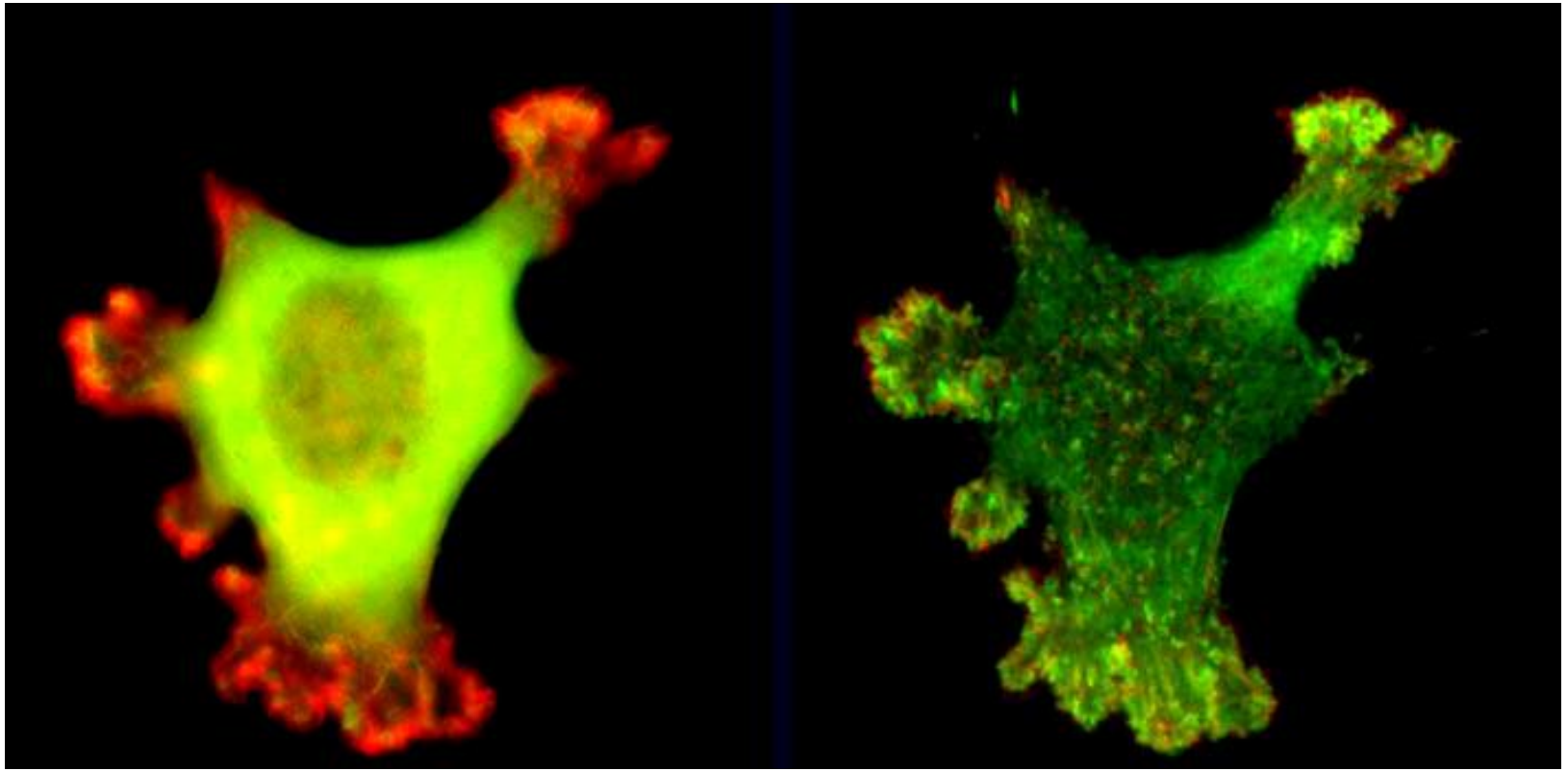
- **Endocytic pathway analysis (transferrin, cholera toxin, dextran, clathrin-GFP, caveolin-GFP), discovery of virus entry pathways provides basic knowledge for potential entry portals into cells and contact points for improvements toward more efficient non viral gene vectors**

- **Investigation of the internalization of nanoparticules into Cells**

Applications of TIRF microscopy analyses

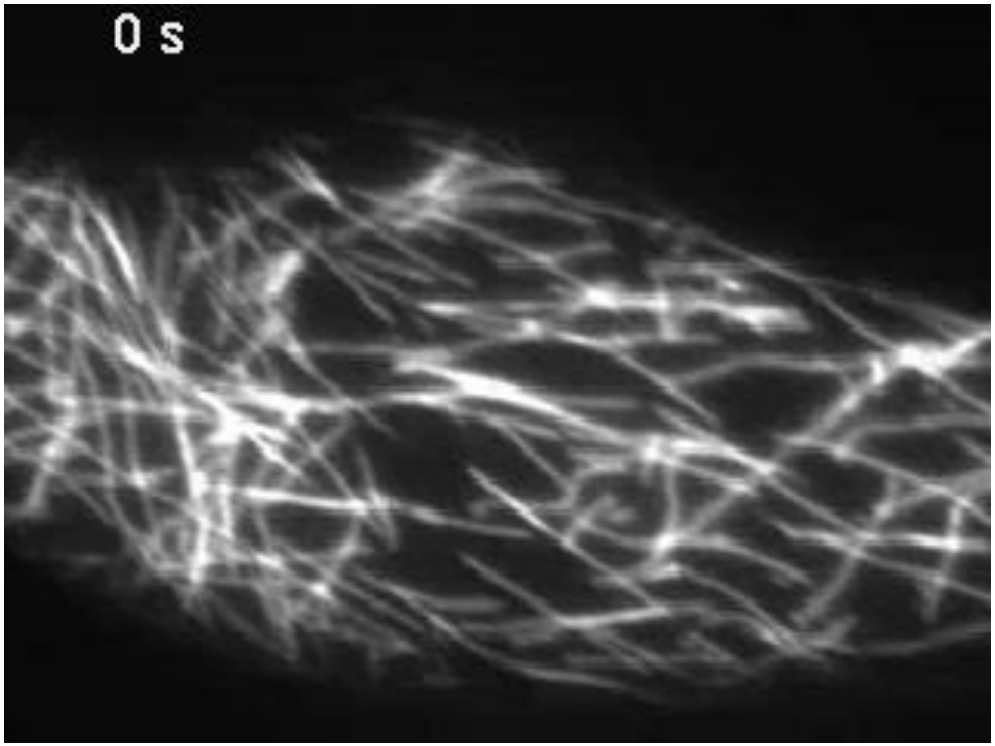
melanoma cells (mouse)

Confocal microscopy | TIRF Microscopy



zeiss

TIRF microscopy

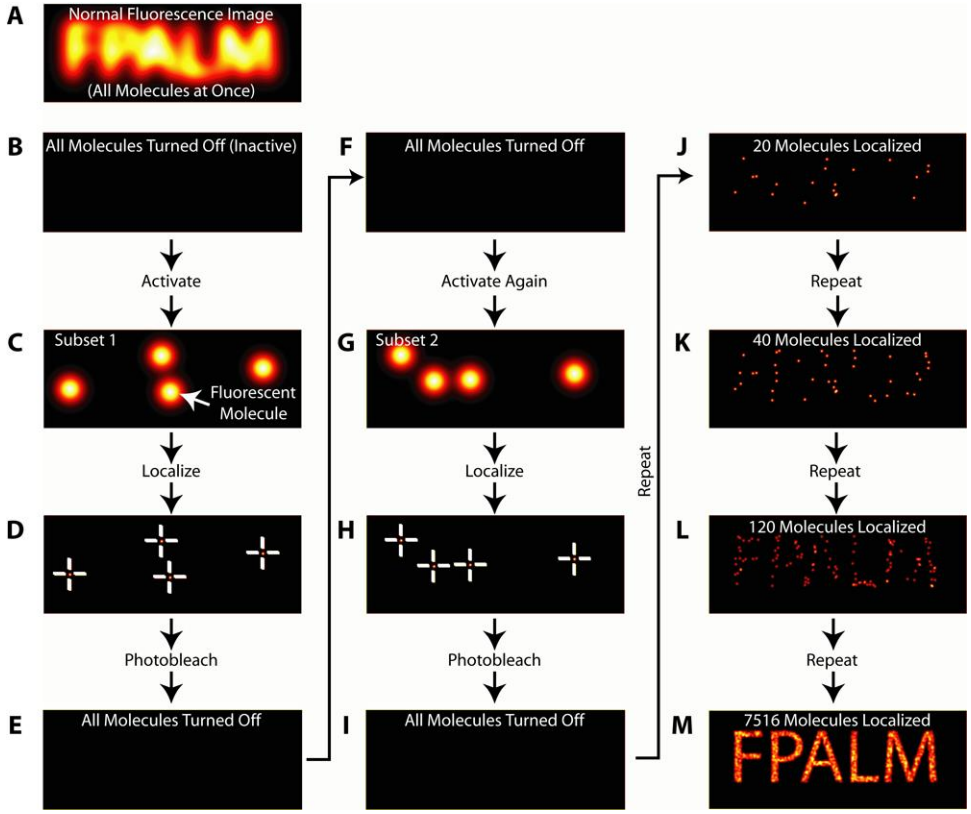


Microtubule GFP Arabipopsis

Fluorescence microscopy with high resolution

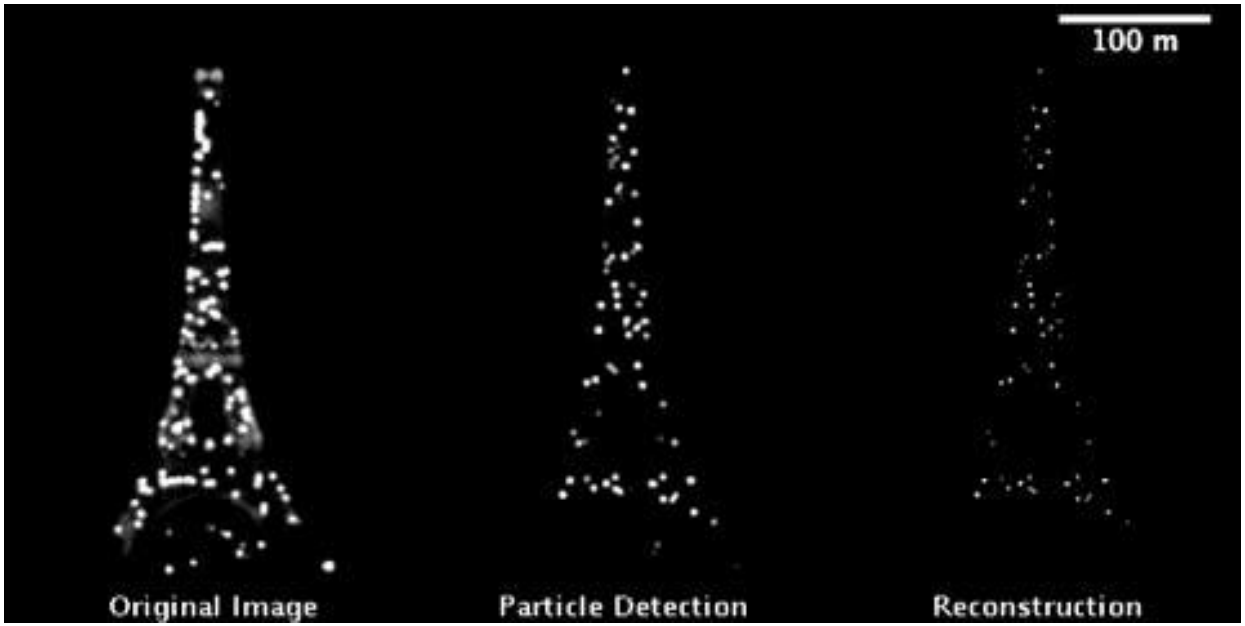
PALM : Photoactivation Localization Microscopy

Resolution : 20-40 nm



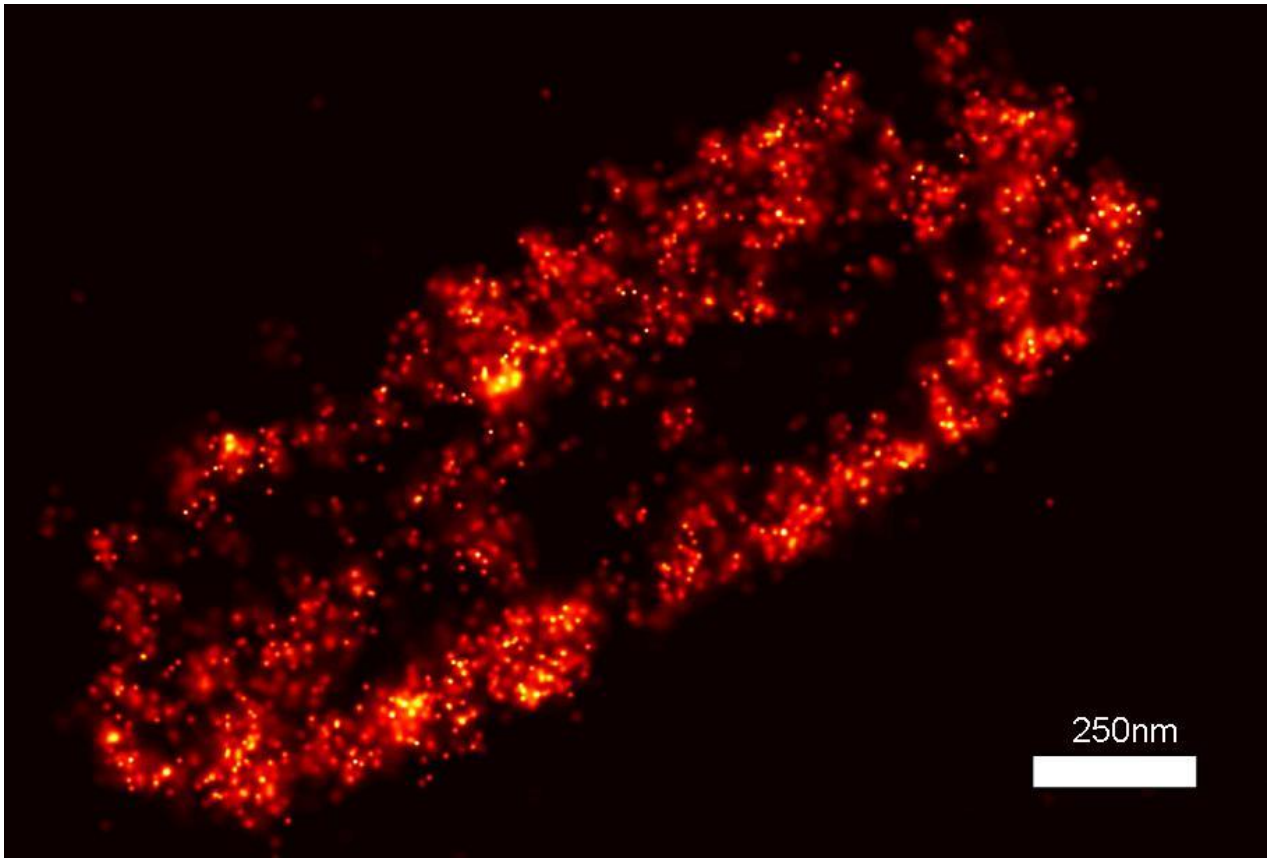
Sam Hess. University of Maine

Fluorescence microscopy with high resolution



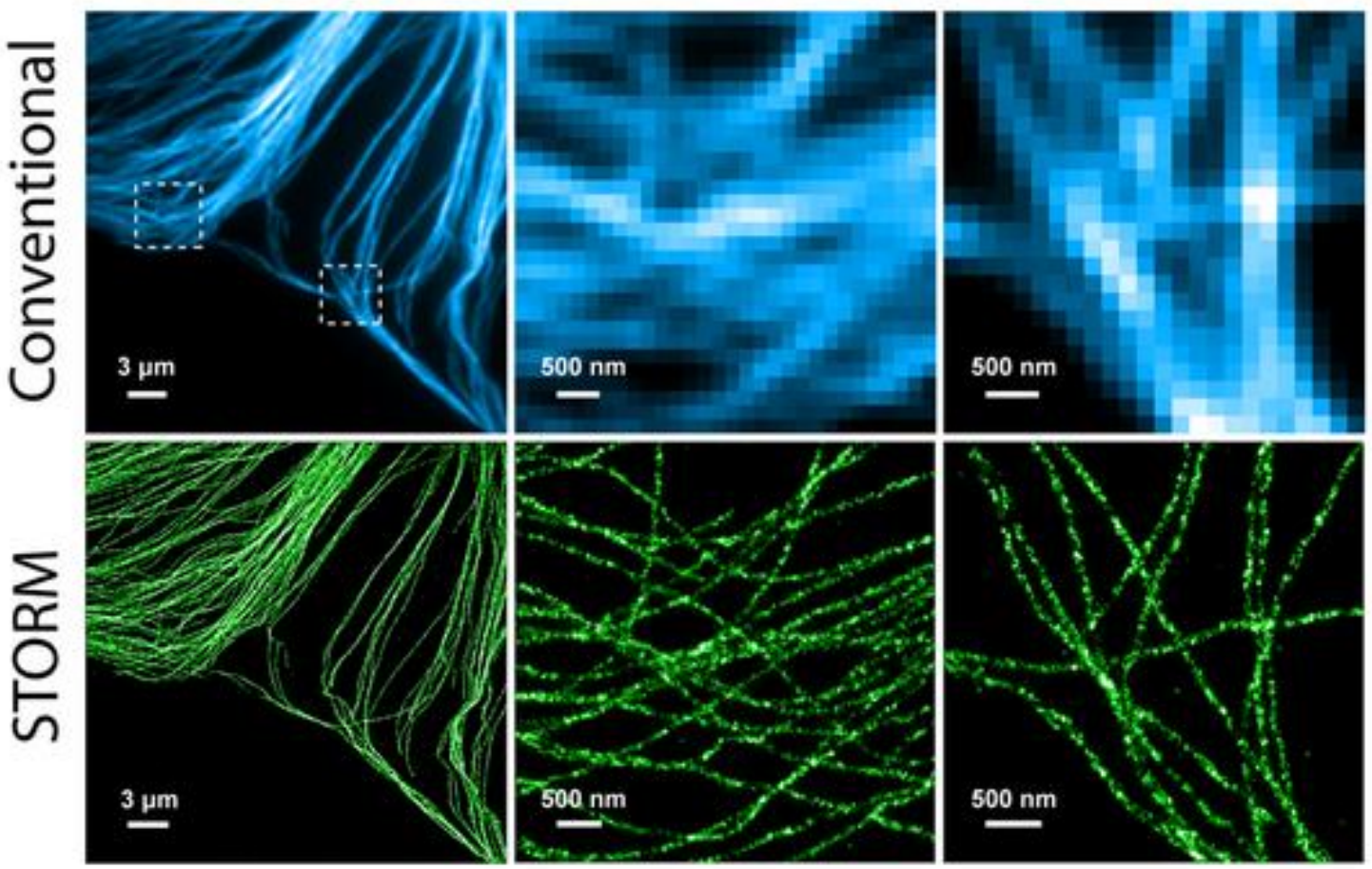
Fluorescence microscopy with high resolution

E Coli Lipo-Poly-Saccharides antibody stained with A647



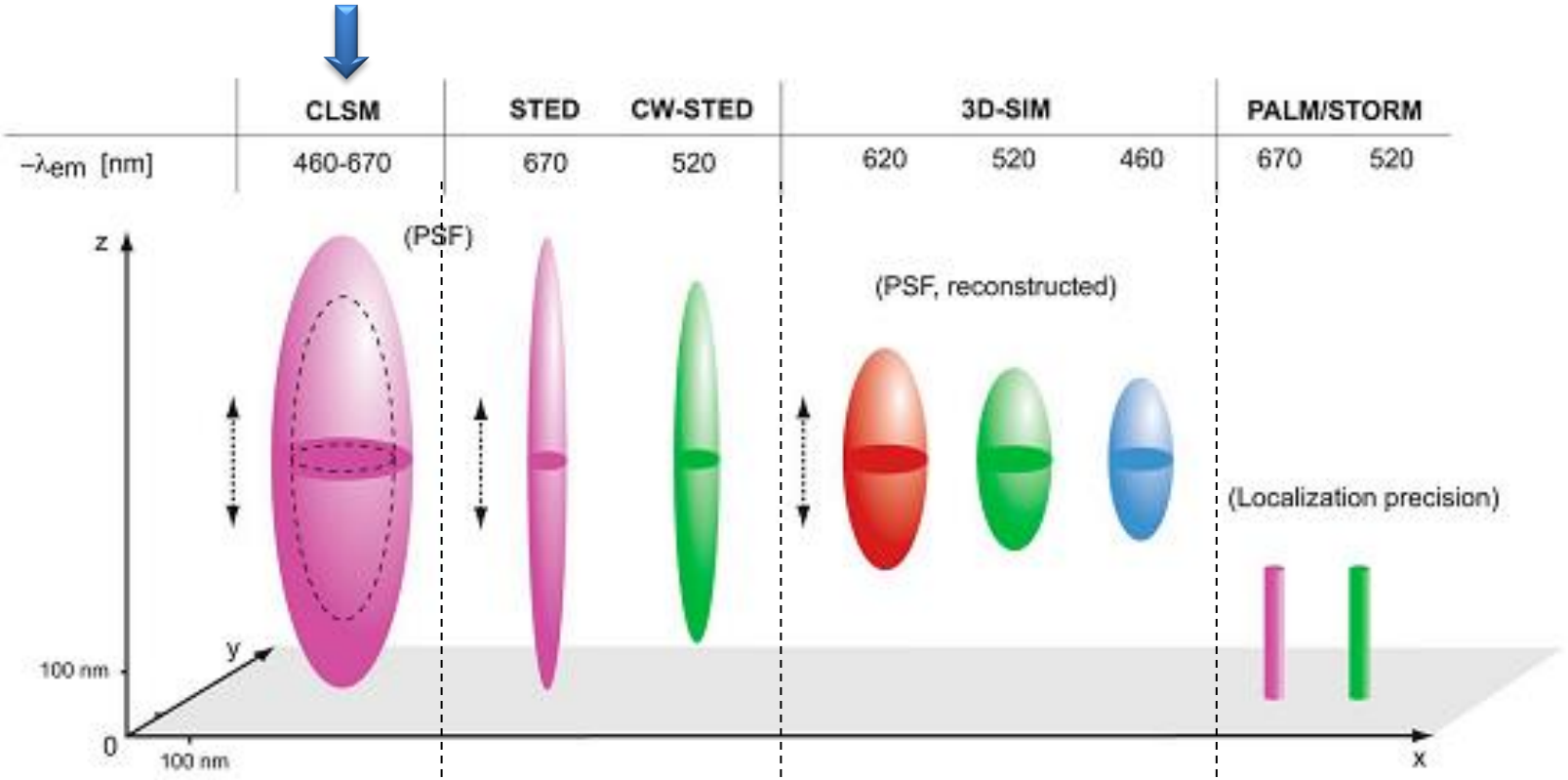
Stephan Thiberge. Princeton university

Fluorescence microscopy with high resolution



Science (2007)

Breaking the limit

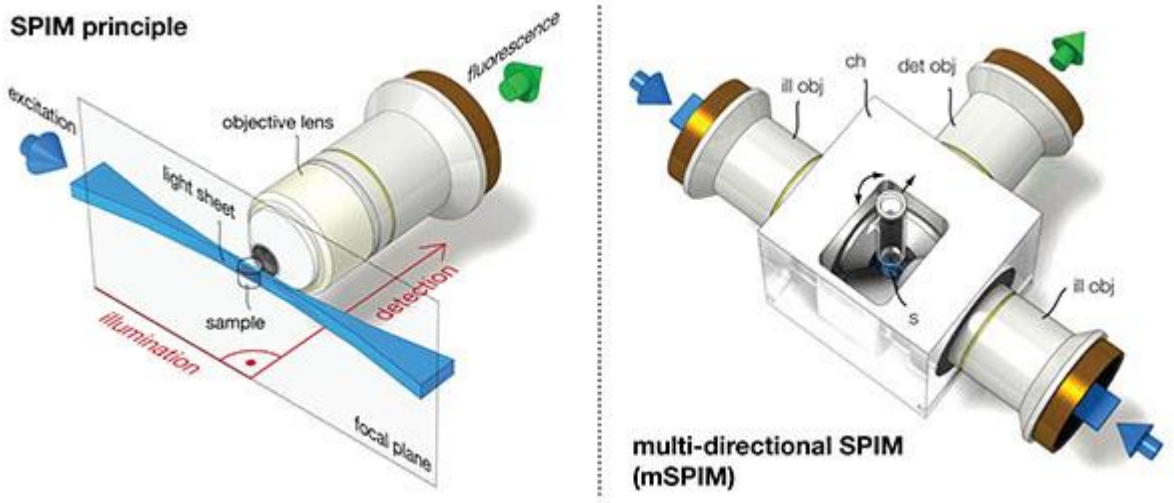


Dyes	any	photoswichable	any	photoswichable

Choice of system

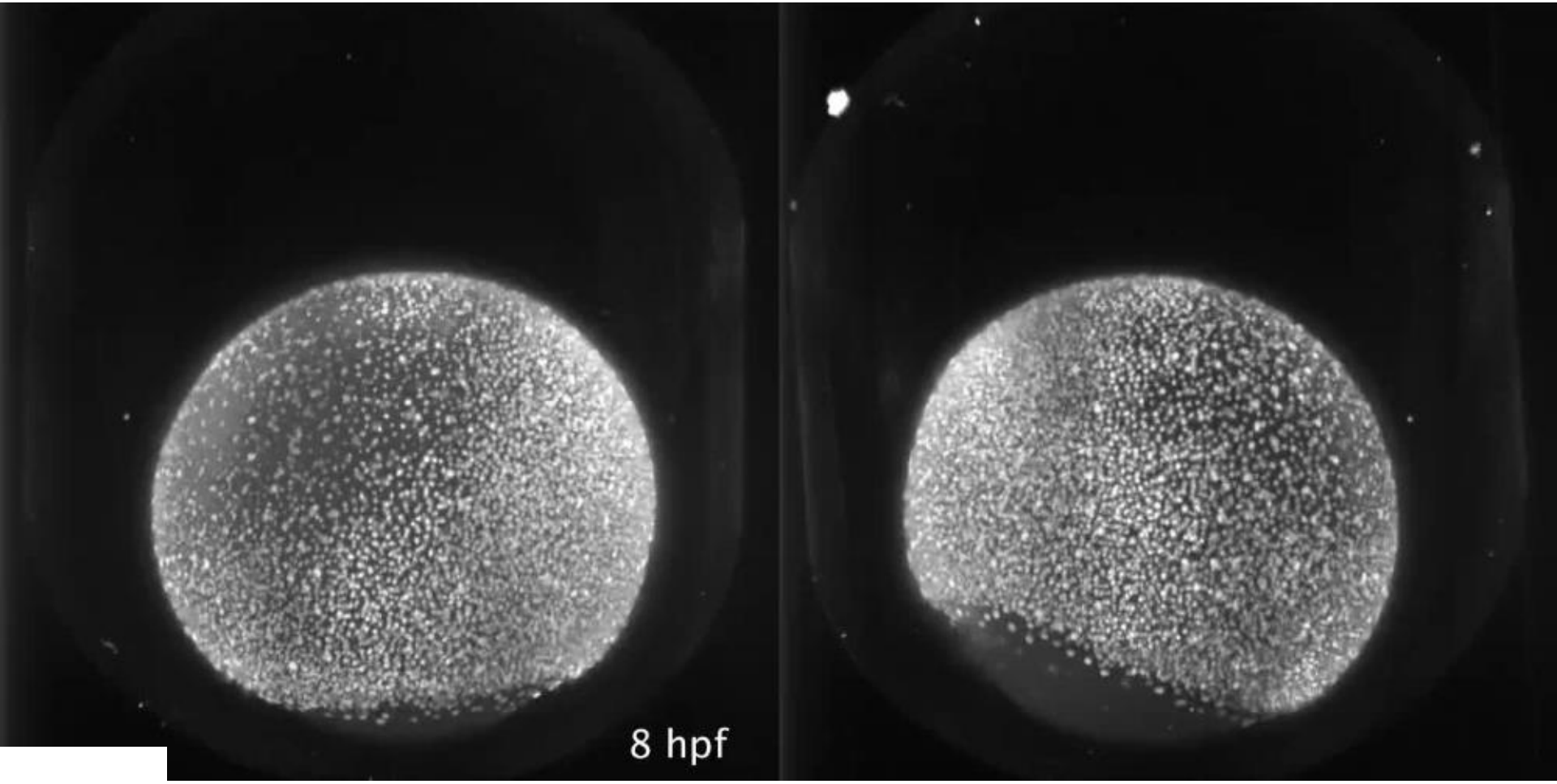
	Confocal	TIRF	SIM	STED	PALM
X-Y resolution	200	80-100	80-100	20-40	20-40
Z resolution	400-500	200	200	400-500	20-40
Dyes	Any	Any	Any	Photo switchable	Photo switchable
Rapidity	+	+++	-	++	-
Simultaneous colors	>3	3	>3	2	2
Post processing	No	No	Yes	Yes	Yes

Light sheet : selective plane illumination microscope



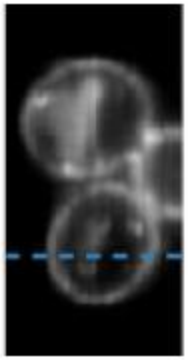
the Huisken Lab, Max Plank Institute

Light sheet : selective plane illumination microscope



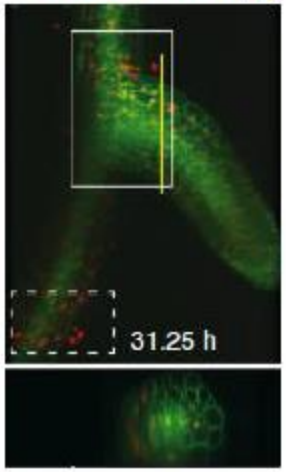
Max Planck Institute of Molecular Cell Biology and Genetics
Huisken LAB
Quantitative microscopy of zebrafish organogenesis

Molecular & cellular biology



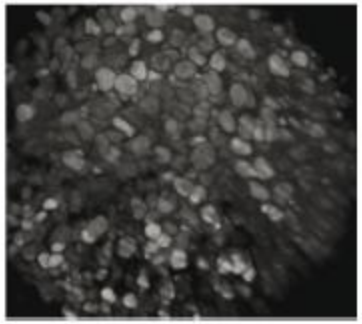
Capoulade & *al.*, 2011

Plant biology



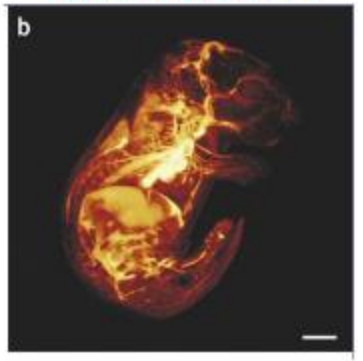
Maizel & *al.*, 2011

Cancer biology



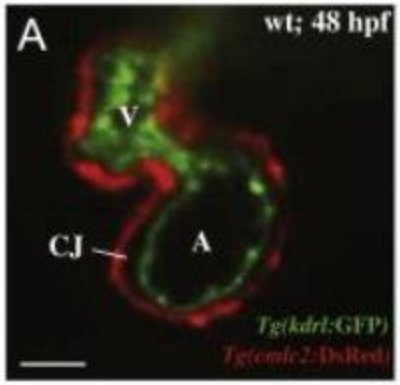
Lorenzo & *al.*, 2011

Neuroscience

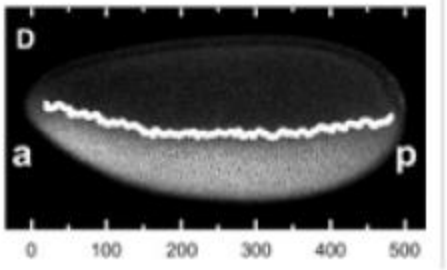


Dodt & *al.*, 2007

Developmental biology

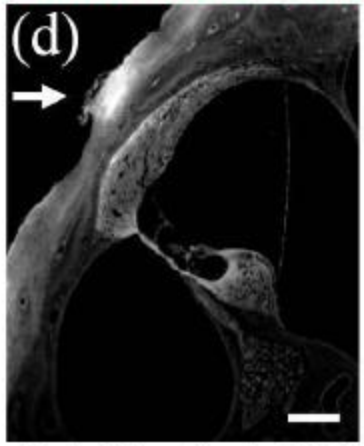


Mellman & *al.*, 2012



Reeves & *al.*, 2012

Anatomical studies



Schröter & *al.*, 2012

Thank for your attention

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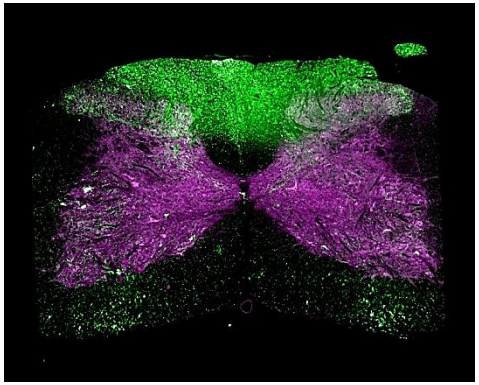
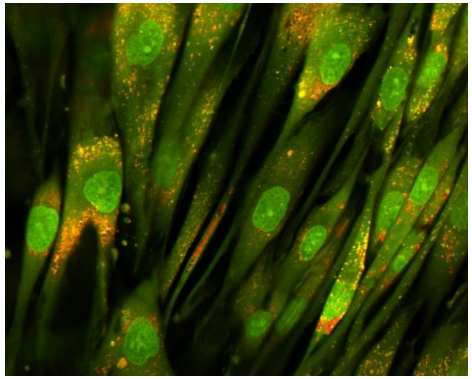
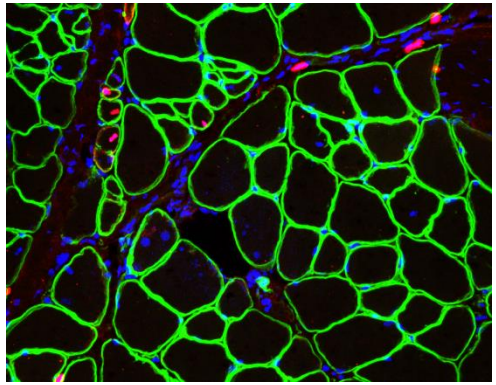
http://www.micropicell.univ-nantes.fr



J. Deniaud



C. Robveille



J. Lorant