



2024

Nouvelles approches en imagerie photonique sur le vivant

"Recherche en Biologie et Santé"

PlaTeforme d'Imagerie et de Biophysique Cellulaire

PTIBC – IBISA Nancy

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PARTIE 1



Les méthodes en imagerie de contraste

A. Le contexte photophysique : la lumière

- I. Le rayonnement électromagnétique/ multimodalités de la lumière
- II. Les aberrations optiques (réfraction et diffraction)
- III. Instrumentation (détecteurs et capteurs)

B. Les méthodes contrastes en imagerie cellulaire (excitation monophotonique)

- I. Microscopie conventionnelle / Déconvolution
- II. Microscopie confocale

C. Exemples de techniques pour caractériser les Interactions et diffusions moléculaires

- I. FRAP: Fluorescence Recovery after Photobleaching
- II. FRET : Fluorescence Resonance Energy Transfer

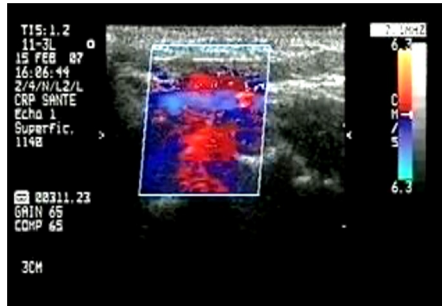
D. Microscopie Multiphotonique et dérivées

- II. Microscopie à excitation multiphotonique
- II. FLIM : Fluorescence Lifetime Imaging Microscopy
- III. SHG: Second Harmonic Generation
- IV. Méthodes Vibrationnelles (Raman-CARS)

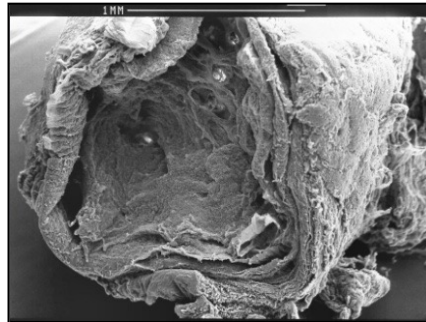
D. Développements

Macroscopie en mode multiphoton

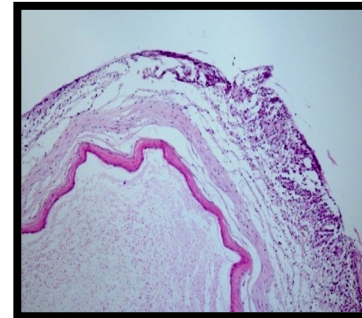
CARTOGRAPHIE 3D EN IMAGERIE / VIVANT / BIOMED IMAGERIES FONCTIONNELLES DE CONTRASTE CORRELATIVES



Doppler - IRM

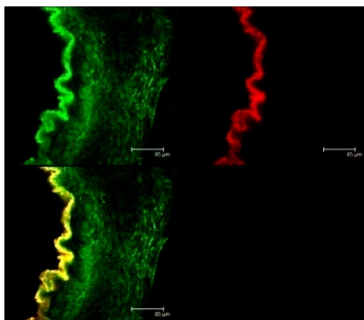
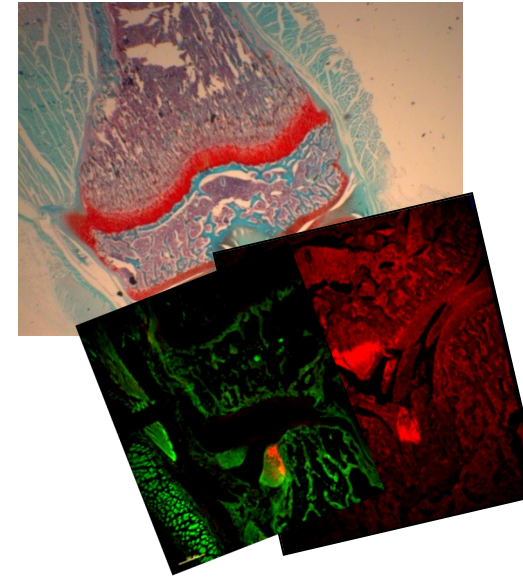


Microscopie Electronique

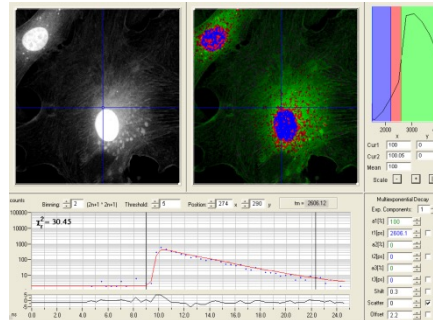


Histochimie

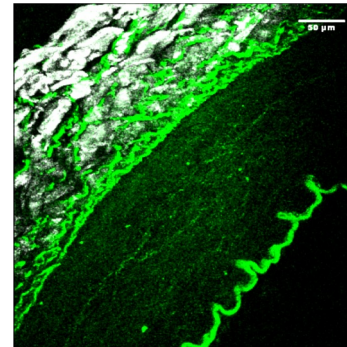
Macroscopie



CONFOCAL



FLIM



SHG + Fluo

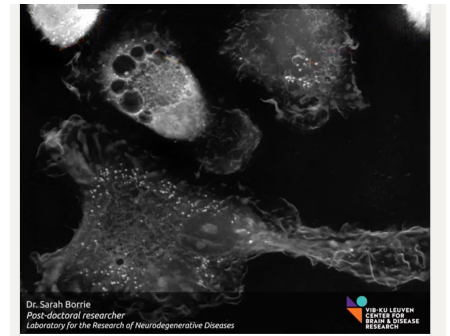


Image de phase

Méthodes Multimodales d'imagerie Médicales et Biomédicales

Agents de contraste :

- Sélectivité des molécules
- Spécificité de détection et résolution
- Invasivité de la méthode



Table of comparison of medical imaging techniques.

Part I: Commonly used radiological imaging modalities

	Conventional X-ray	X-ray Computed Tomography (CT)	Radioisotope Imaging (Nuclear Medicine)	Magnetic Resonance Imaging (MRI)	Ultrasound Imaging (1 to 10 MHz)	Positron Emission Tomography (PET)
Projection/Tomography	Projection	Tomography	Both	Tomography	Tomography	Tomography
Mainly Anatomical / Functional	Anatomical	Anatomical	Functional	Both	Both	Functional
Interventional options?	Yes	No	No	Possible	Yes	No
Maximum imaging depth in soft tissue	Metres	Metres	Body thickness	Body thickness	Body thickness	Body thickness
Spatial resolution in plane of imaging (typical)	0.1 mm	0.25 mm	5 to 10 mm	0.5 mm	0.5 mm	5 to 10 mm
Safety	Ionising radiation leads to a radiation dose	Ionising radiation leads to a higher radiation dose than for conventional x-rays	Ionising radiation leads to a moderately higher radiation dose than for conventional x-rays	A range of hazards arising from strong and time varying electromagnetic fields.	Thermal effects and cavitation	Ionising radiation leads to a moderately higher radiation dose than for conventional x-rays
Examination time	Short	Medium	Long	Long	Medium	Long
Physical property of tissue associated with image formation	Linear attenuation coefficient	Linear attenuation coefficient	Tracer isotope is involved in metabolic process; measure concentration	Proton density and nuclear magnetic resonance relaxation times.	Primarily acoustic impedance: differences lead to reflections at boundaries	Tracer isotope is involved in metabolic process; measure concentration
Relative capital cost*	Low	Fairly high	Fairly high	High	Medium	High
Relative cost per patient study*	Low	Medium	Medium to high	High	Low	High
Comments	Interventional studies such as cardiac catheterisation take longer and are costlier than radiographic studies	May be called CAT (Computerized axial tomography). Current interest in multi-detector CT.	Some gamma camera systems also have PET capability, but these have lower sensitivity than dedicated PET systems	Open systems or special facility needed for intra-operative use. Tailored sequences used for functional measures.	Blocked by bone. The Doppler effect is used for measuring the velocity of blood flow	A cyclotron is used to produce special isotopes; this must be located close by. Current interest in combined CT/PET systems and PET/MR

* Indicative only; medical imaging costs depend strongly upon the healthcare environment and method of economic analysis

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Table of comparison of medical imaging techniques.

Part II: Less commonly used *in vivo* imaging modalities.

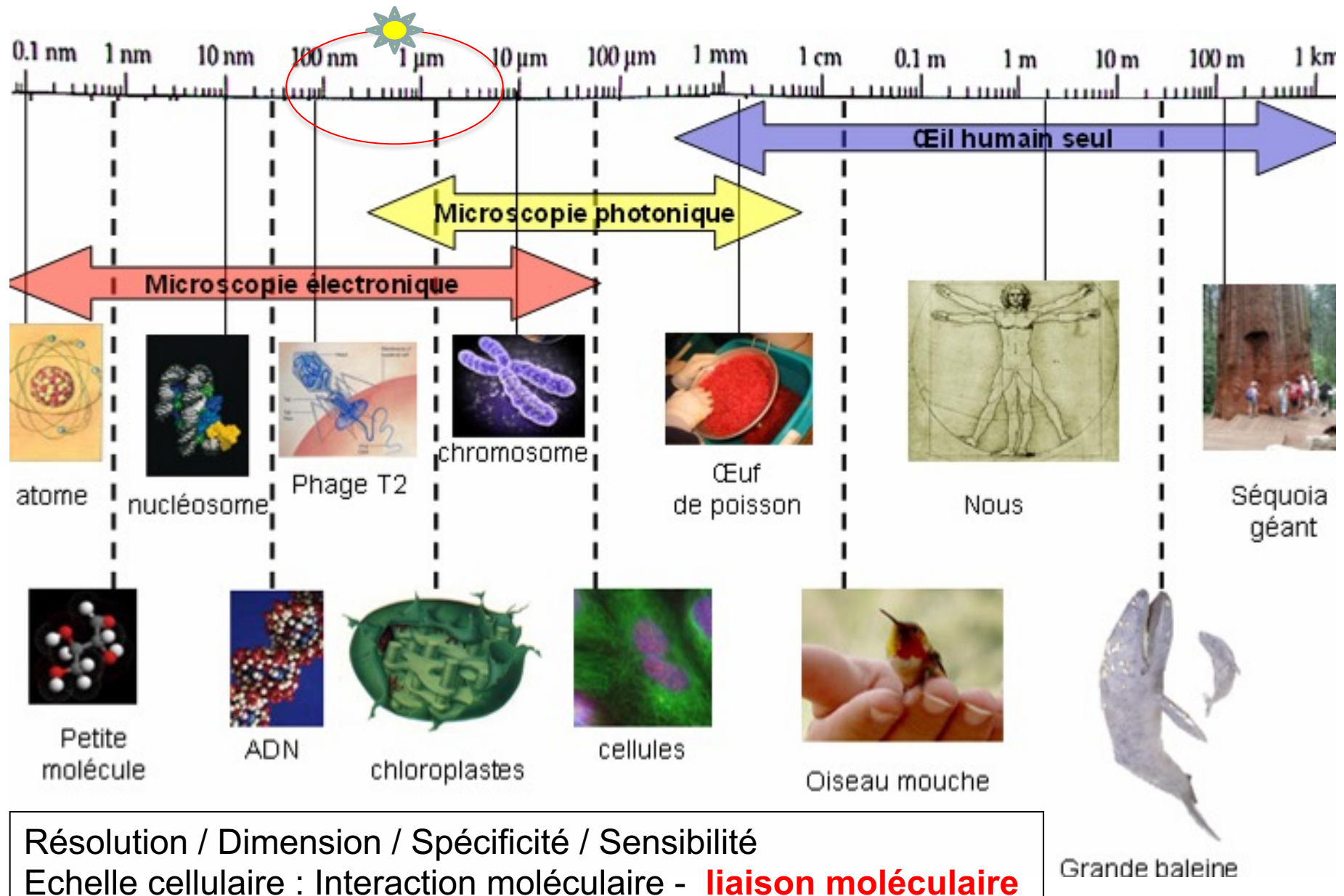
(These have higher spatial resolution than those in Part I, and may be in the early stages of development for *in vivo* use)

	High frequency ultrasound	Fluorescence microscopy	Optical coherence tomography	<i>In vivo</i> confocal microscopy	Spectrophotometric intracutaneous analysis	Diffuse optical tomography	Terahertz pulsed imaging	High resolution MRI
Projection/Tomography	Tomography		Tomography	Tomography	Projection	Tomography	Both	Tomography
Mainly Anatomical / Functional	Anatomical	Functional	Anatomical	Anatomical	Both	Functional	Both	Anatomical
Maximum imaging depth in soft tissue	4 mm	0.5 mm	2 to 3 mm	400 μ m	2 mm	15 cm	Few mm	cm
Spatial resolution in plane	20 μ m	1 μ m	10 μ m	2 to 5 μ m	Up to 10 μ m	1 to 3 mm	350 μ m	100 μ m
Depth resolution (typical)	9 μ m	n/a	5 to 15 μ m	0.5 μ m	n/a	1 to 3 mm	40 μ m	100 μ m
Safety	Thermal effects and cavitations	May involve administration of fluorophores.	Consult special guidelines regarding use in eye	Consult special guidelines regarding use in eye		Thermal effects	Thermal effects	Hazards from strong and time varying electromagnetic fields
Physical property of tissue associated with image formation	Primarily acoustic impedance: differences lead to reflections at boundaries	Decay time of fluorescence induced by laser light, plus spectrum and intensity, give molecular environment	Refractive index. Interferometric techniques used to infer time of flight	Uses reflected light or fluorescence	Effect of the skin's chromophores (haemoglobin, melanin, collagen, dermal melanin)	Refractive index and scattering, chromophore content and absorption	Complex refractive index affecting pulses of radiation.	Proton density and nuclear magnetic resonance relaxation times
Comments	20-200 MHz. High frequency Doppler under development	e.g. FLIM – Fluorescence Lifetime Imaging. Visible light.	OCT. Can be used endoscopically. Visible light.	Accessible tissue surfaces, planes parallel to surface. Visible light.	SIAscopy – analytical version of dermatoscopy or ELM. Visible light & NIR.	Monitor tissue and blood oxygenation levels. Near infrared (NIR).	Also spectroscopy. mm-wave imaging is a passive technique at similar frequency.	Using special small coils in a 1.5-3T whole body scanner.

Note. We might also have included spectroscopic methods such as Fourier Transform Infrared (FTIR) spectroscopy and Raman spectroscopy, which were originally non-imaging laboratory tools. They are now being applied *in vivo* and with data mapping to generate images.

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E&OE. This information is provided as an introductory guide. It is for educational or informational use only, and is not to be used for patient care.
Part I of the table includes the more commonly used radiological imaging modalities. It was the January 2006 download from www.voxelera.co.uk.

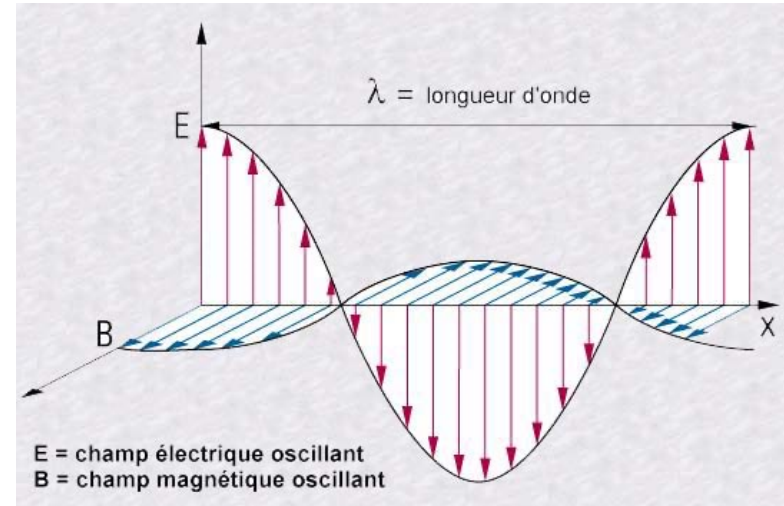
Les méthodes d'investigation : dimension objet



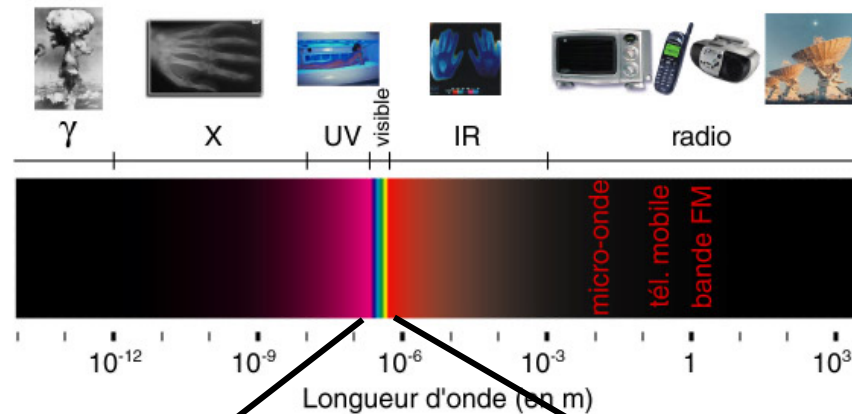
Nature et dualité de la lumière...interaction lumière / matière

La lumière est une
onde électromagnétique

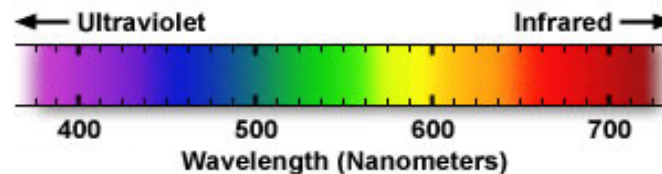
Vitesse de propagation :
 $c = 3.10^8 \text{ m.s}^{-1}$



Longueur d'onde
(couleur)



Spectre visible



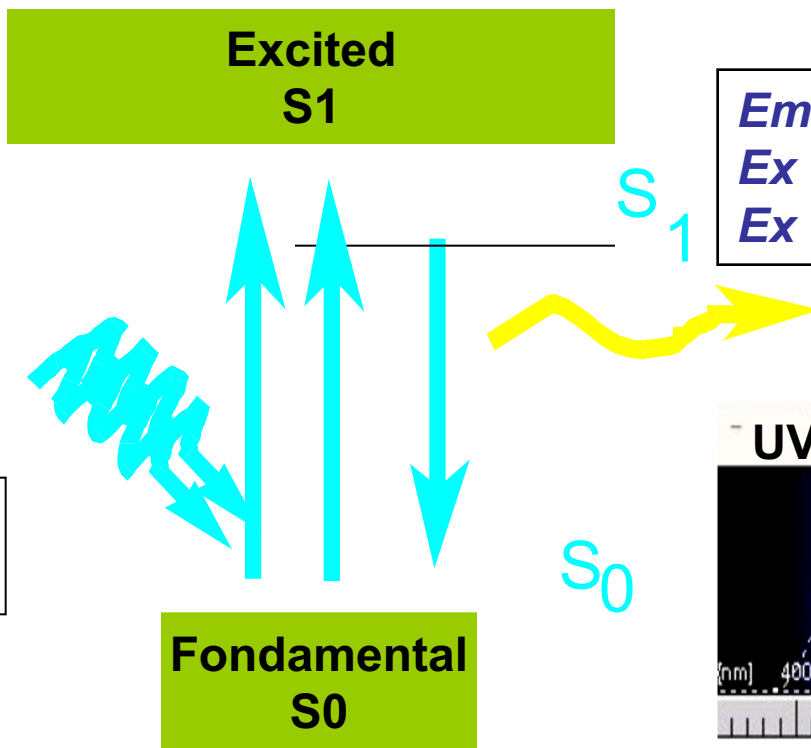
Light / Matter interactions Photon's applications

$$\Delta E = hc / \lambda$$

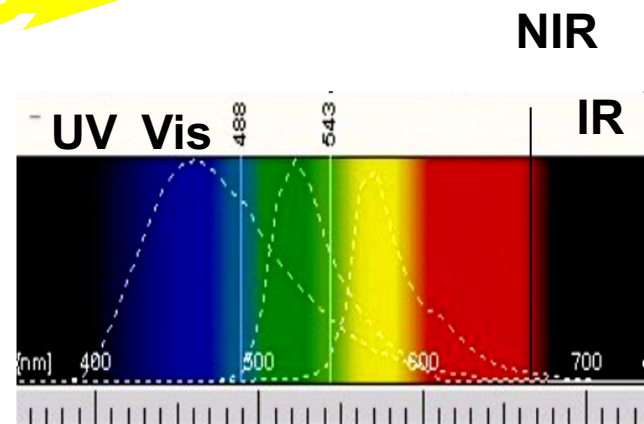
1P : λ (450 nm)

2P : 2λ (900 nm)

Photon Absorption
1P / 2P (NLO)



Emission
 $E_x < E_m$ (1P)
 $E_x > E_m$ (2P)



Spectral
Frequency

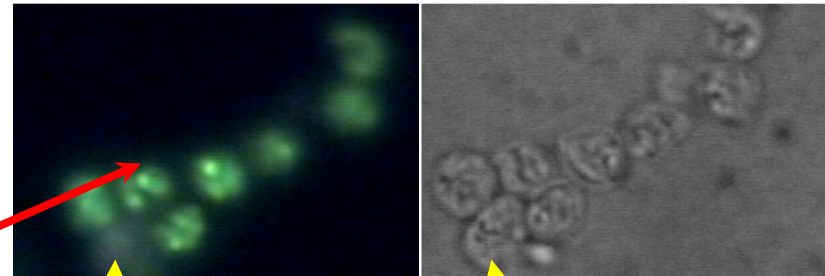
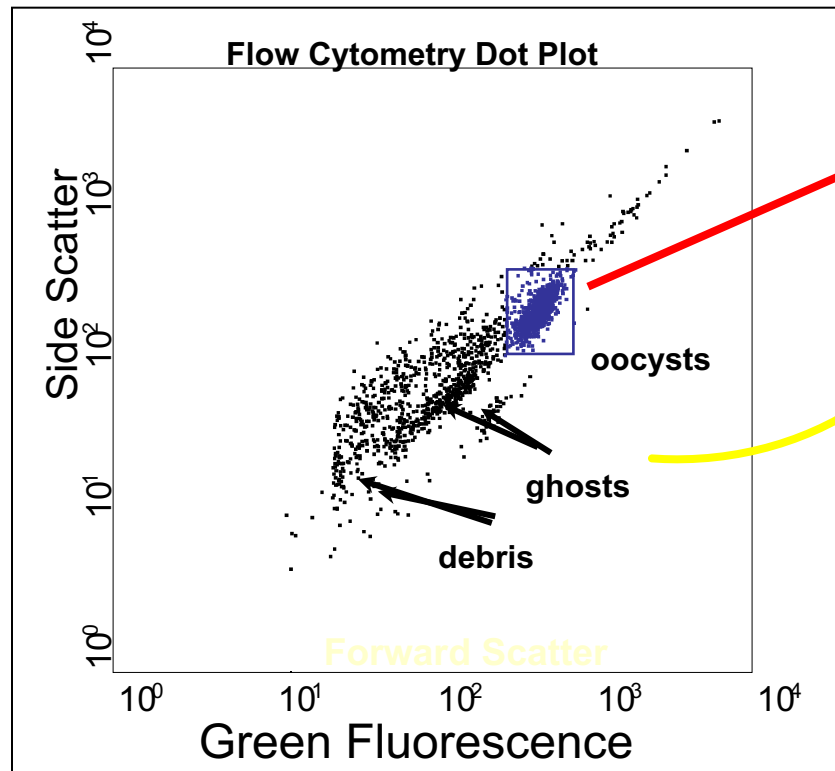
Time-resolved
Lifetime

Spatial
3D

Orientation
Anisotropy

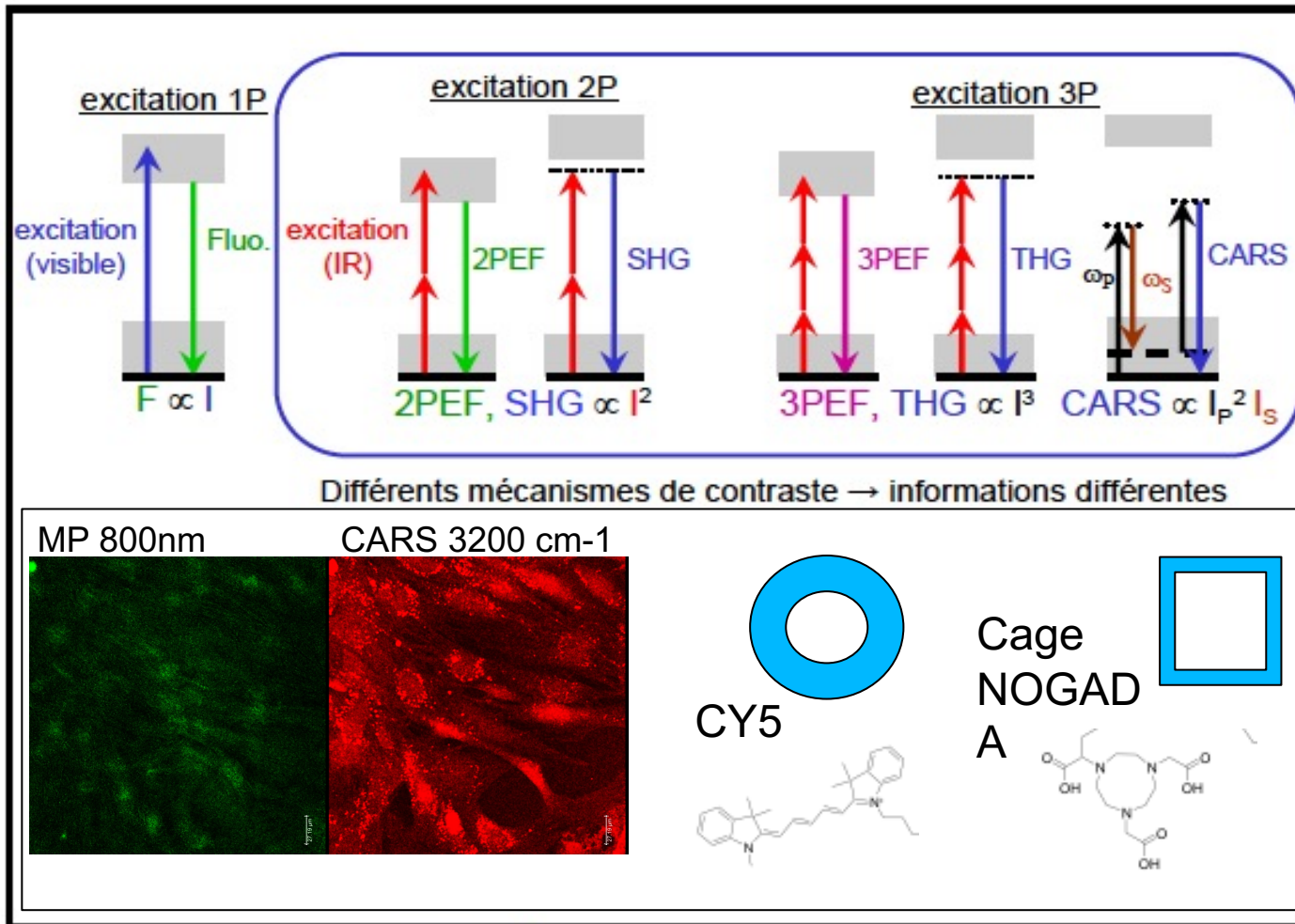
CYTOFLUORIMETRIE EN FLUX ET EN IMAGE (X, IR..Raman)

- Aspects statistiques (reproductibilité)
- Echelle d'analyse (microparticules plaquettaires)
- Nature/conditionnement du matériel biologique (suspension - cellules adhérentes)



Les méthodes pour sonder la matière : processus photophysiques

MICROSCOPIES NON-LINEAIRES – IMAGERIE DE CONTRASTE



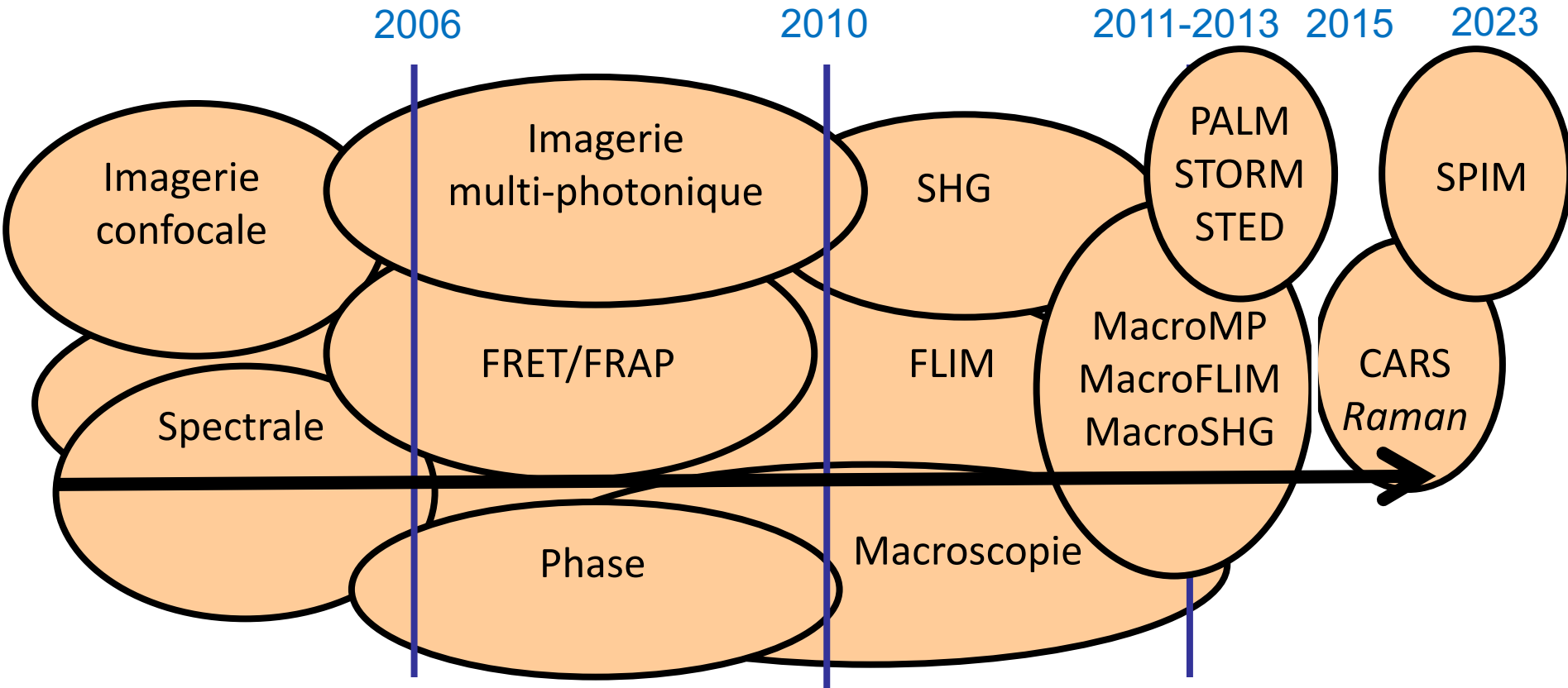
Les modalités en imagerie de contraste

IMAGERIE STANDARD

Modalité simple : Imagerie Confocale

APPLICATIONS AVANCEES

Multi-modalités : Nouveaux outils de détection en microscopie multi-photonique. F-techniques



Spectral : Fluorescence Spectral microscopy
FRET : Förster Resonance Energy Transfer
FRAP : Fluorescence Recovery after Photobleaching
FCS : Fluorescence Correlation Spectroscopy

SHG : Second Harmonic Generation
FLIM : Fluorescence Lifetime Imaging Microscopy (OSEO)
CARS : Coherent Anti-Raman Stocks
SPIM : Selective Plane illumination Microcopy

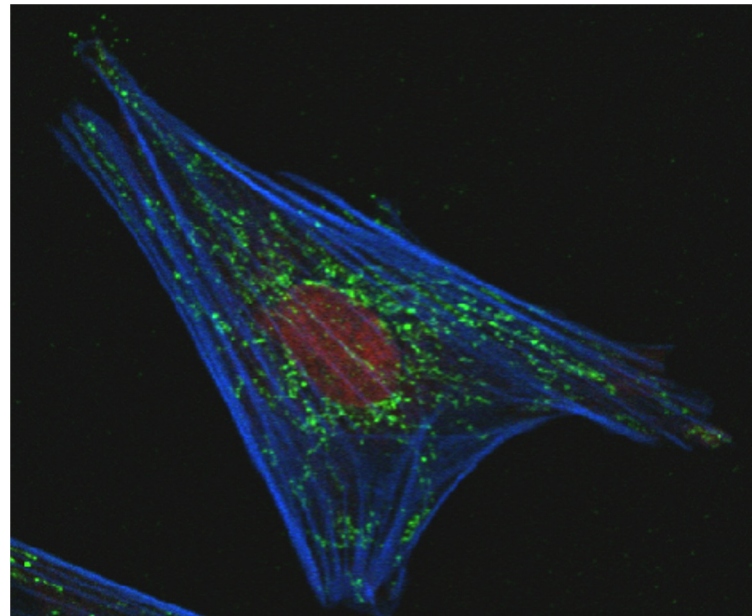
Imagerie de fluorescence.

Avantages :

- Faiblement invasif
- Spécificité de la détection
- Multi marquage

Problèmes :

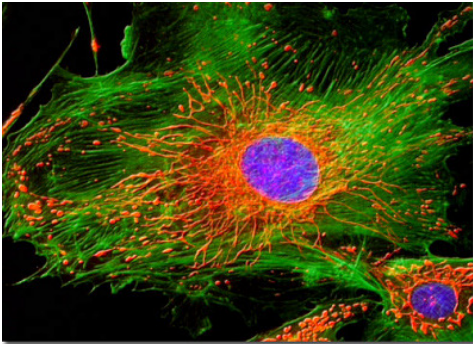
- Bruit de fond
- Fading
usure du fluorophore
- Phototoxicité
espèces réactives de l'oxygène



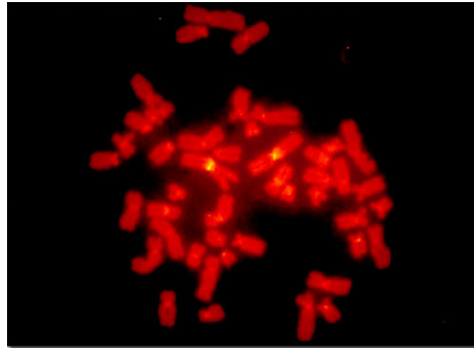
Niveaux d'investigations...la microscopie pour « voir » quoi ?

Question biologique et son périmètre ?

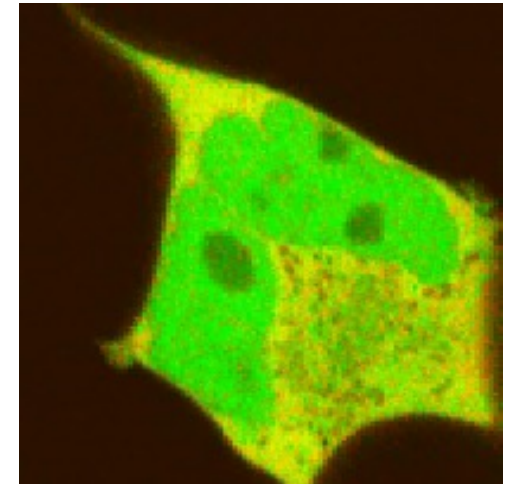
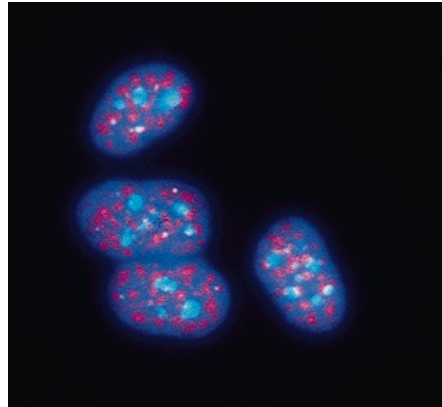
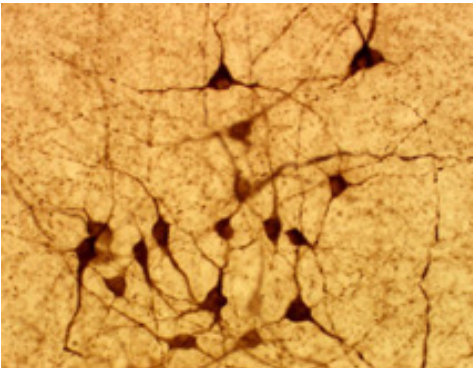
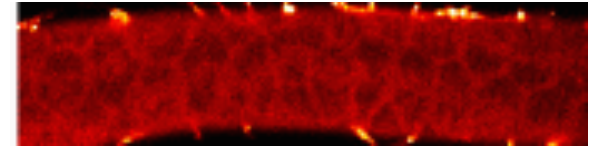
Structures
(mesures...)



Localisations
Numérations

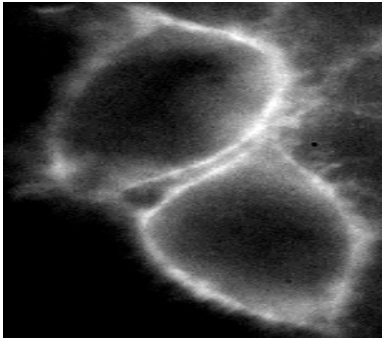
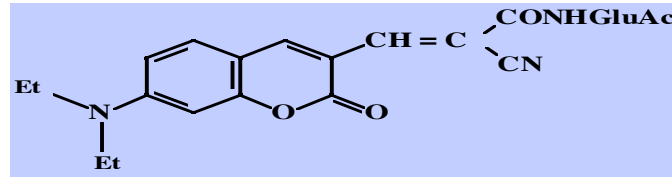


Dynamique
Activité enz

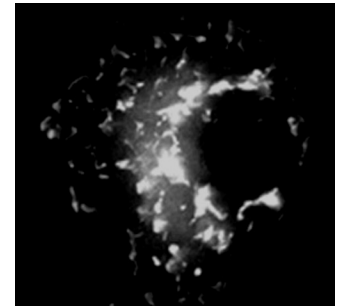
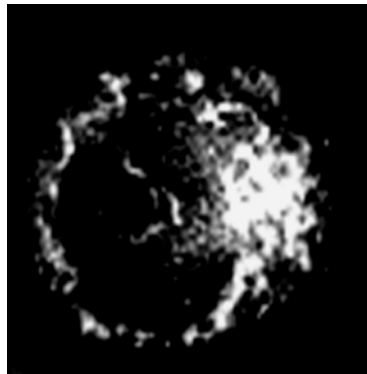


Détection (imagerie/signal): Specificité structurale ou d'activité biologique

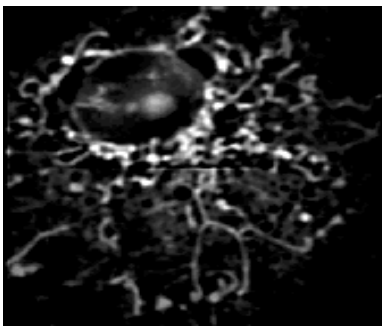
Localisation du rotor dans le réticulum endoplasmique



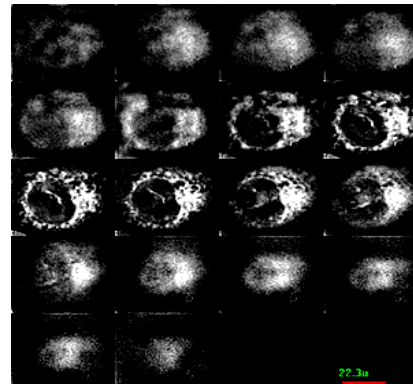
TMA-DPH (membrane)



Rhodamine 123 :
(mitochondries)



Styrylpyridinium (membrane)



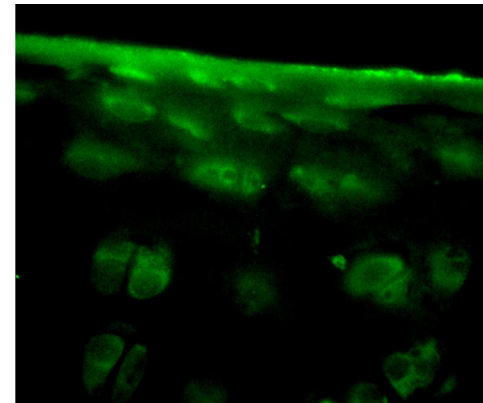
DiOC₆ :
(réticulum)

Détection de protéine recombinante (variants GFP, Yellowship, corail...)

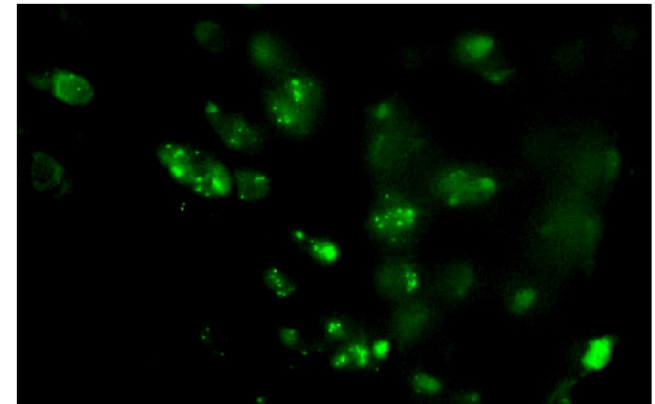
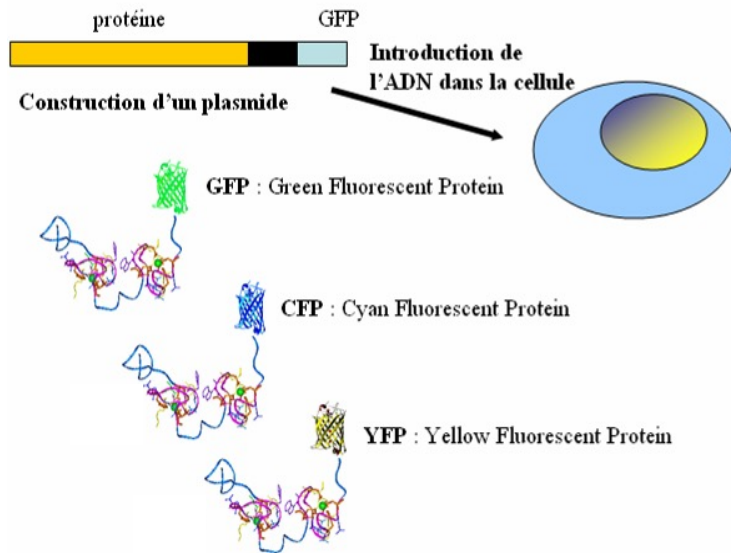
Aequoria victoria



Expression de vecteur GFP cytoplasmique
Chondrocyte de rat



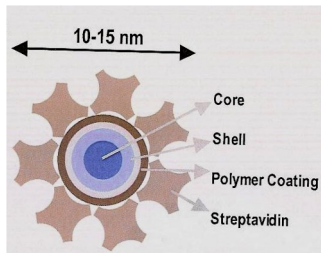
J0



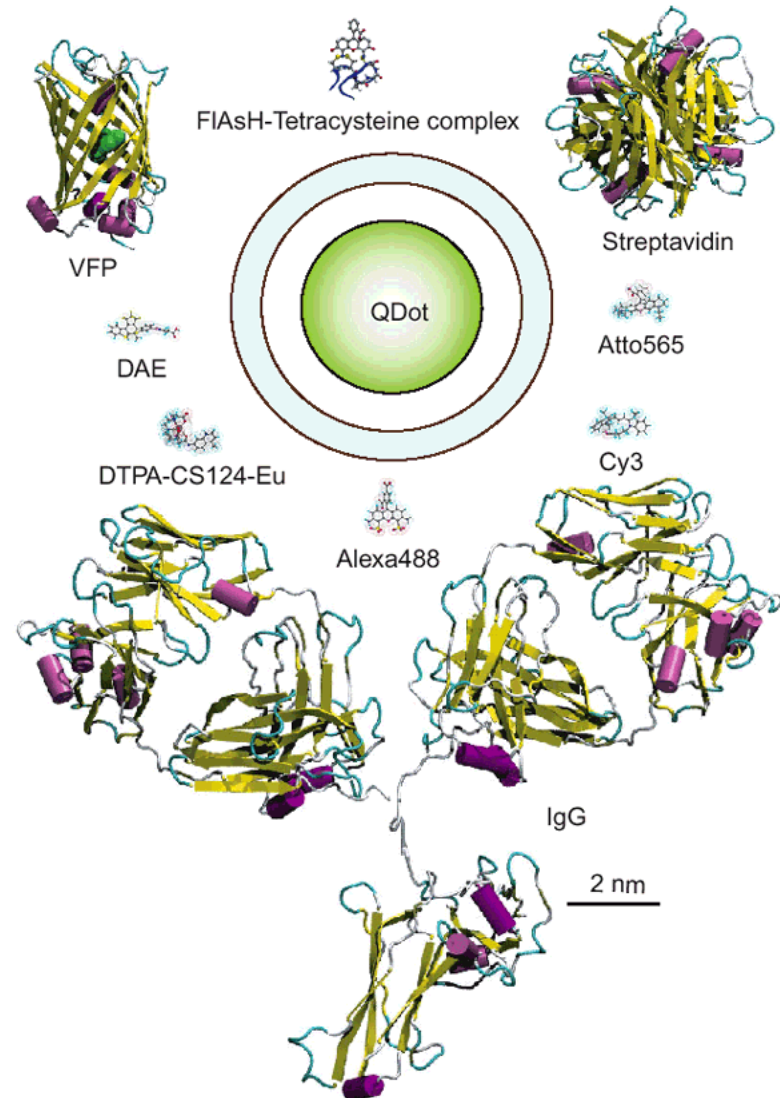
J30

Chromophores / Fluorophores en biologie/encombrement stérique (taille)

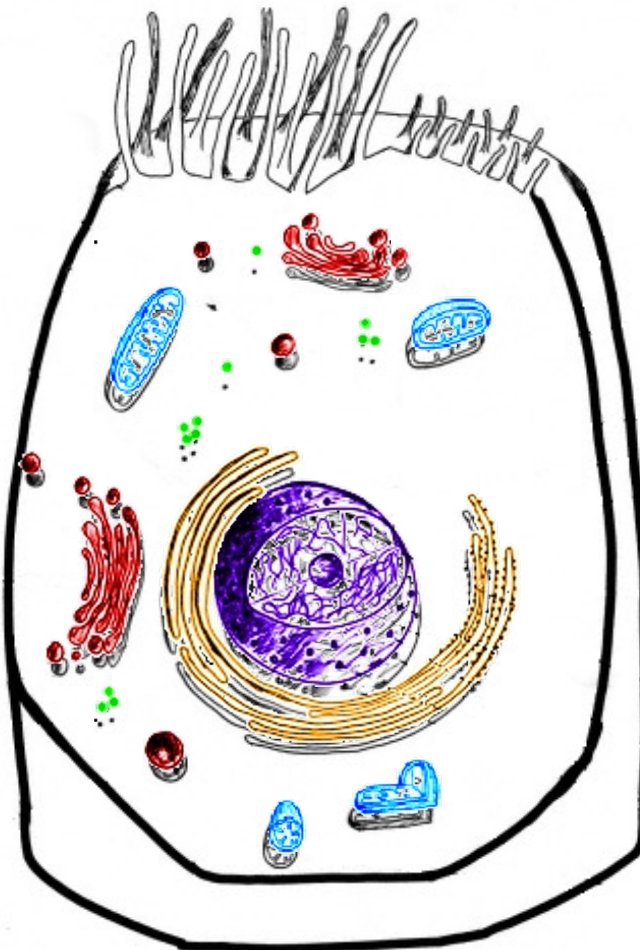
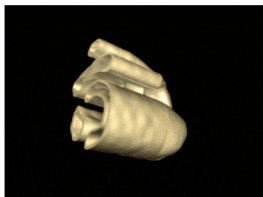
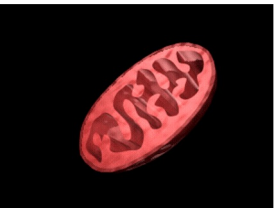
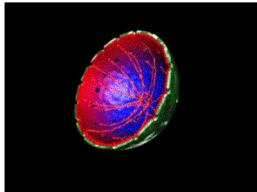
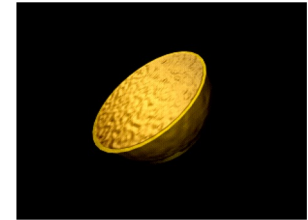
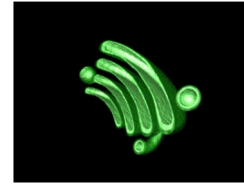
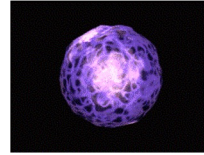
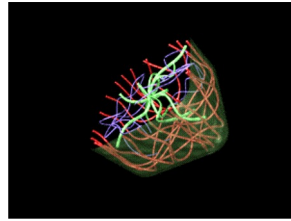
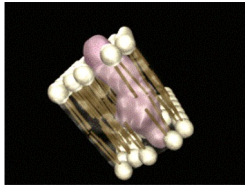
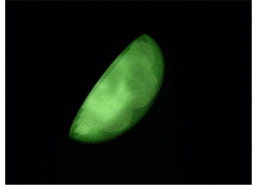
- Organique (chimie carbonée)
- Inorganic : Qdots (semi-conducteur)
- Synthétique (révélateur-histologie)
- Naturel (intrinsèque)



Qdots (semi conductor)



Détection : Spécificité sonde / structure



❖ **Endoplasmic Reticulum RER**

❖ (DiOC6, ER-Tracker[®])

❖ **Golgia** (NBDC6-Ceramids, Bodipy[®])

❖ **Mitochondria** (Mitotracker[®], RH123, JC-1)

❖ **Lysosomes**
(LysoTracker[®], Orange Acridine)

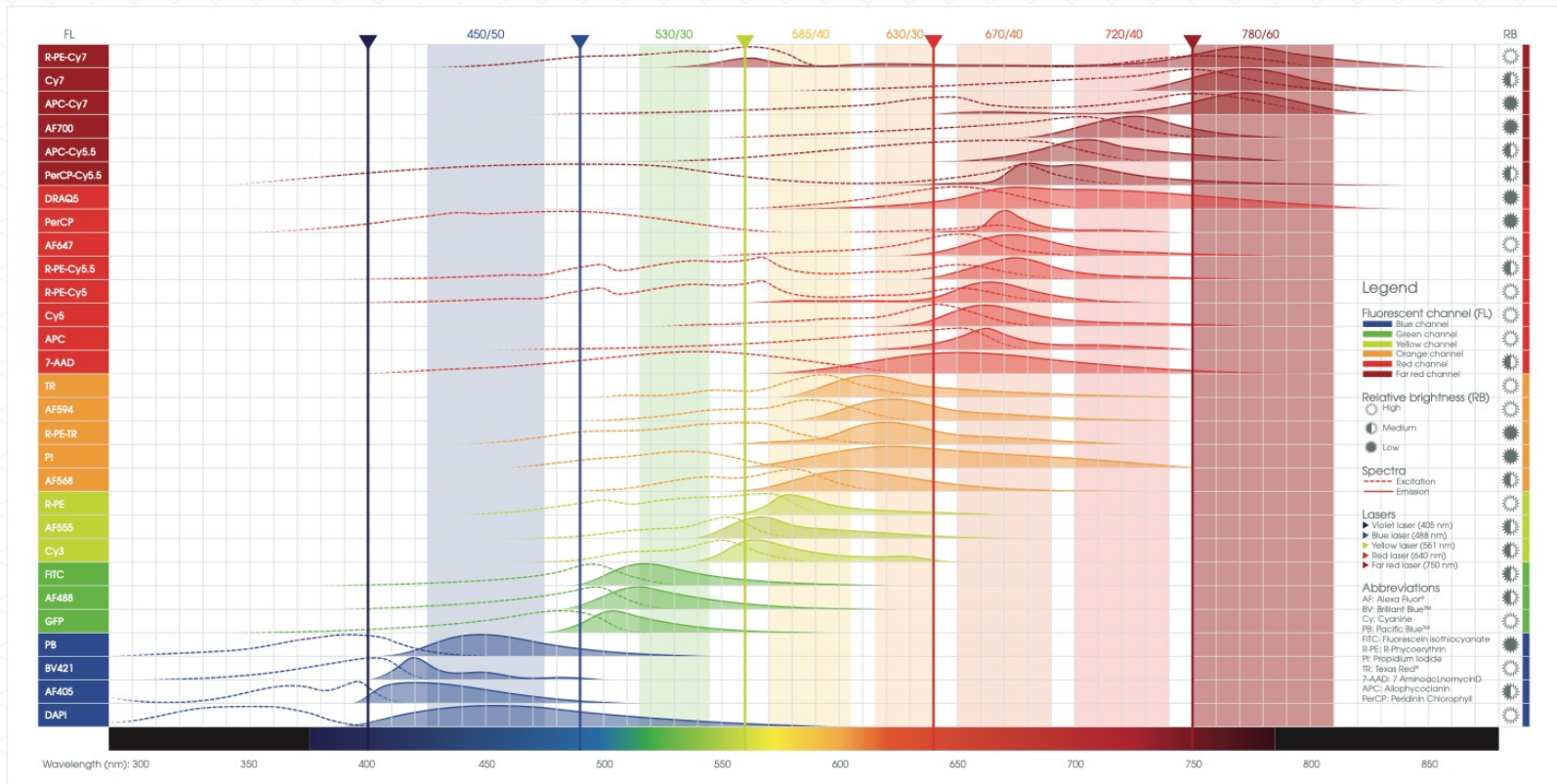
❖ **Nucleus, DNA**
(Hoechst 33342, Orange Acridine)

❖ **Immunology**
(peptide, proteine, oligonucleotide,)

Détection : Sources/ Spécificité sonde / structure

Fluorochrome chart

abcam



How to use this chart

1. Check your instrument

Type, number of lasers, filters and detectors dictate the fluorochromes that can be used.
- Try to choose a fluorochrome for each laser excitation range

2. Select bright dyes

It is possible to rank available dyes according to their brightness on a particular instrument.
- Brightest fluorochromes for dim antibodies and vice versa

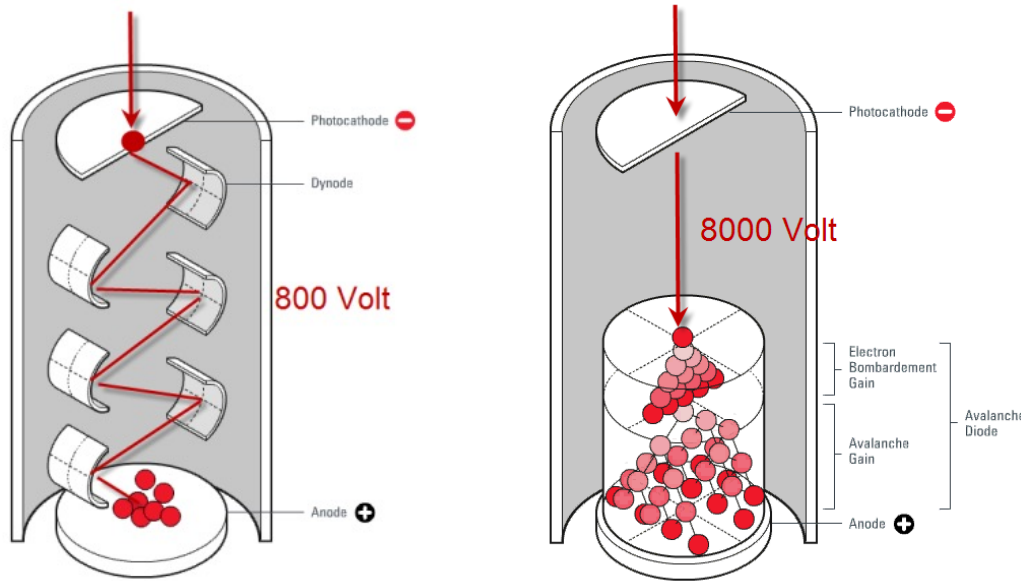
3. Minimize spillover

The amount of spectral overlap will determine whether compensation is required.
- Sacrifice brightness to avoid spillover
- Avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations

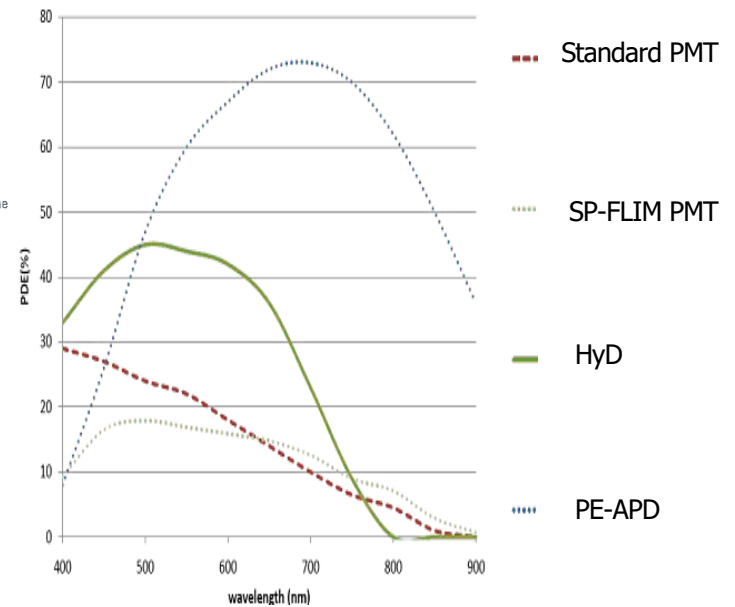
Examples

Fluorochrome	Target Expression	Lasers	Channels	Brightness	Compensation	Combination
FITC APC	High Low	Blue Red	Green Red	Medium High	Mild	Good
FITC PE	High Low	Blue Yellow	Green Yellow	Medium High	Moderate	Medium
PerCP 7-AAD	High Low	Blue Blue	Red Red	Low Medium	Severe	Poor (not recommended)

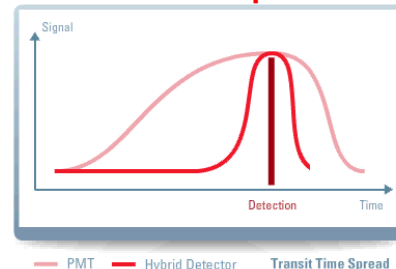
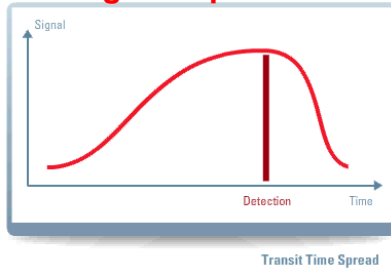
Instrumentation : détecteur PMT-Hyb / APD – CCD ...



Sensibilité/gamme spectrale



Time of flight dispersion in PMT's Short transit time spread in HyD



- Gain d'amplification : Photon-Photoélectron-Tension
- Offset (bruit de fond électronique)

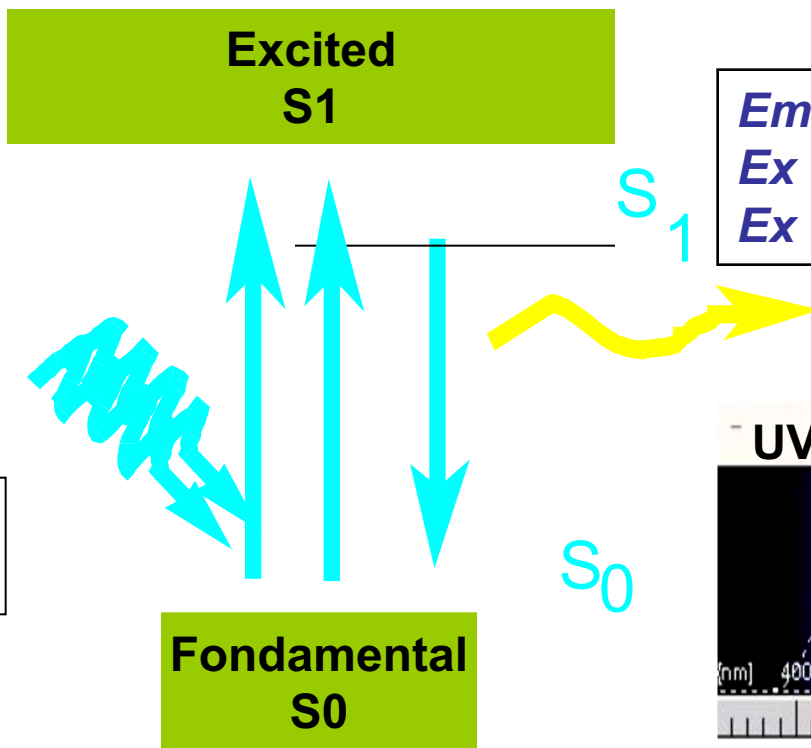
Light / Matter interactions Photon's applications

$$\Delta E = hc / \lambda$$

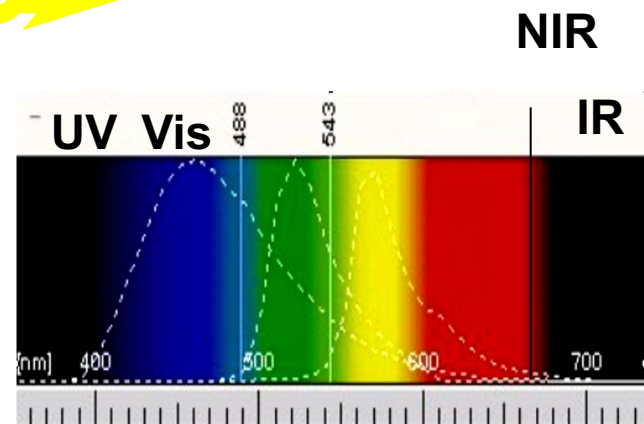
1P : λ (450 nm)

2P : 2λ (900 nm)

Photon Absorption
1P / 2P (NLO)



Emission
 $E_x < E_m$ (1P)
 $E_x > E_m$ (2P)



Spectral
Frequency

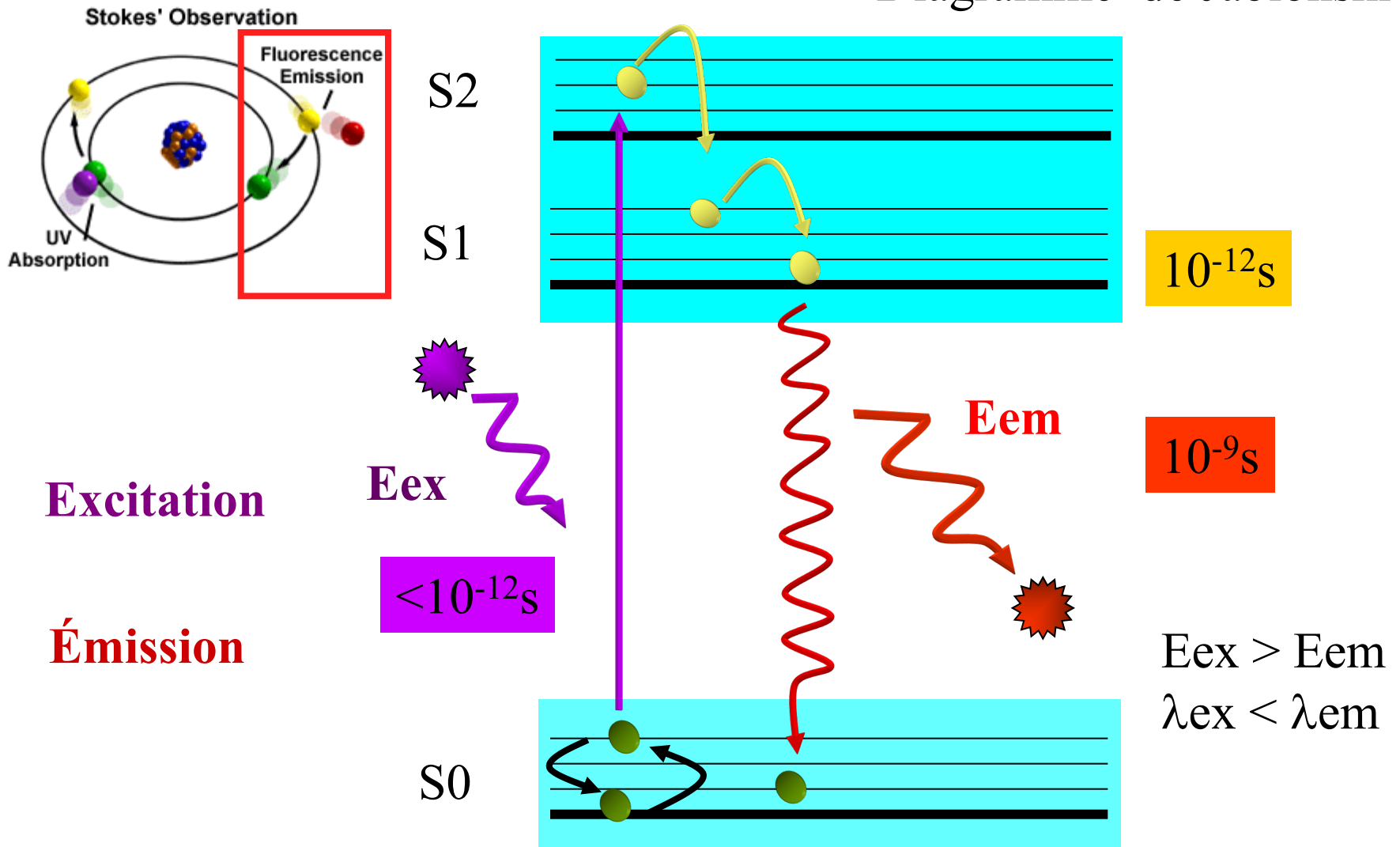
Time-resolved
Lifetime

Spatial
3D

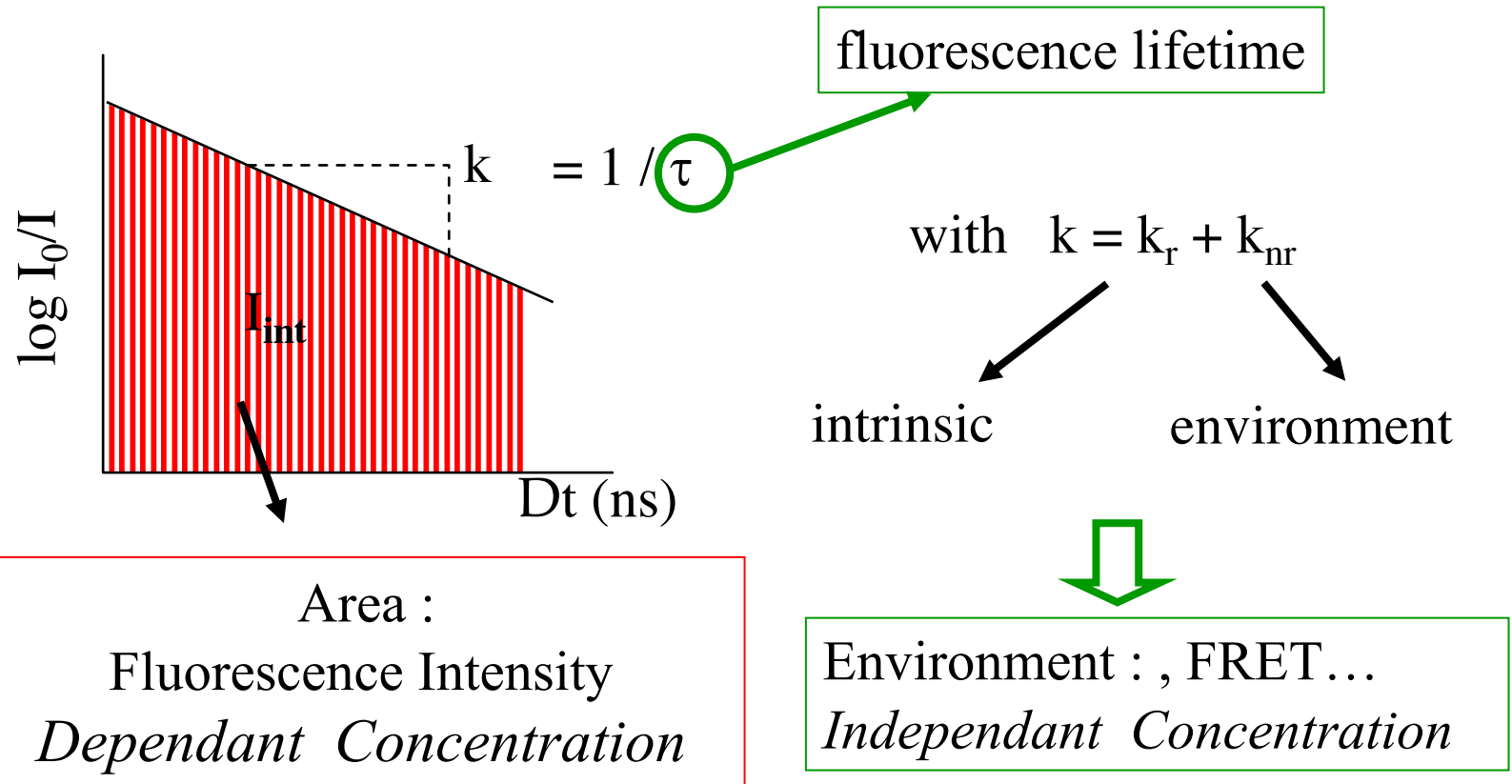
Orientation
Anisotropy

La fluorescence comme moyen de détection : fluorophore (contraste)

Diagramme de Jablonski



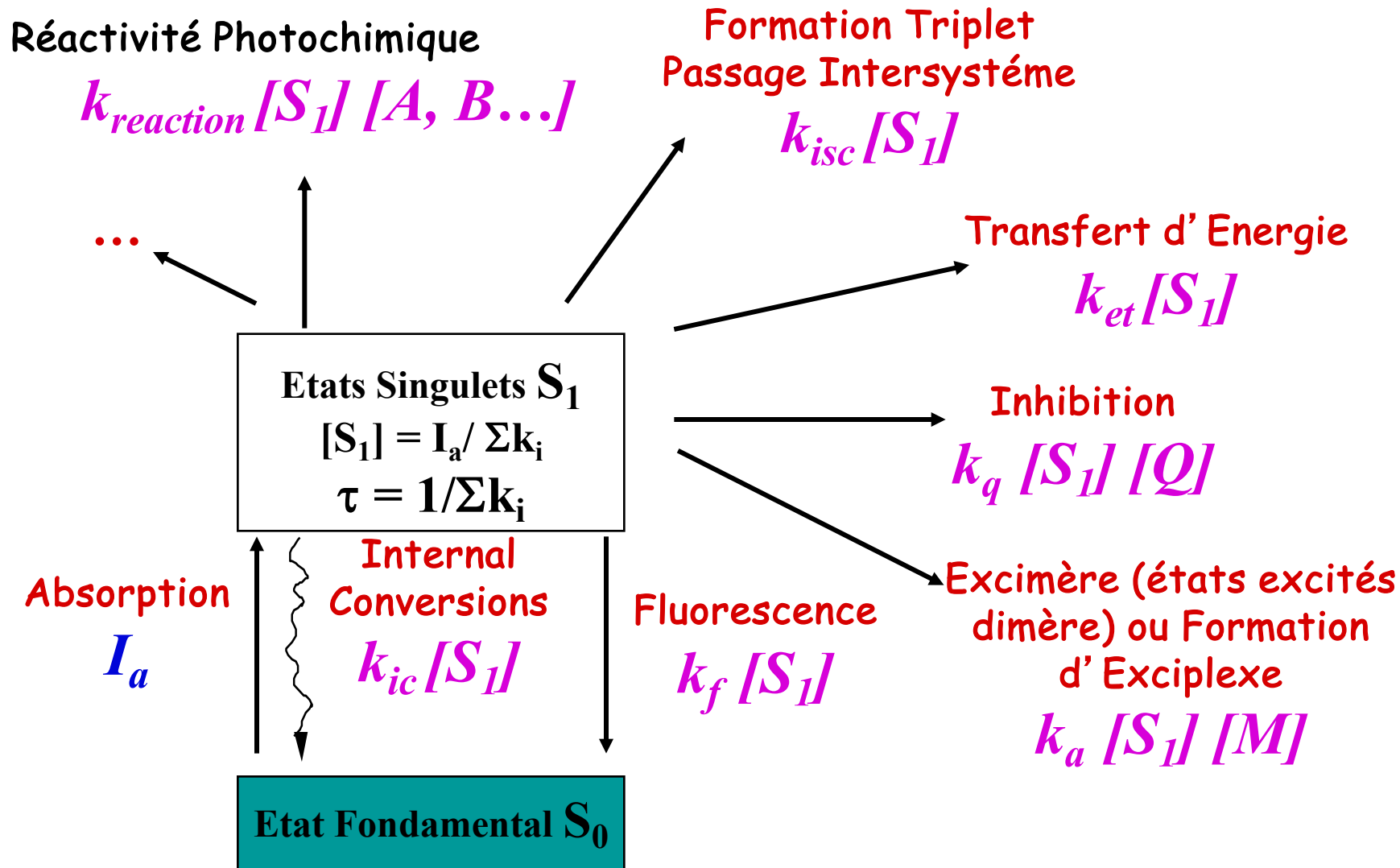
Désactivation de l'état singulet S1 : sensible à l'environnement



**SPECTRAL IMAGING
POLARIZATION**

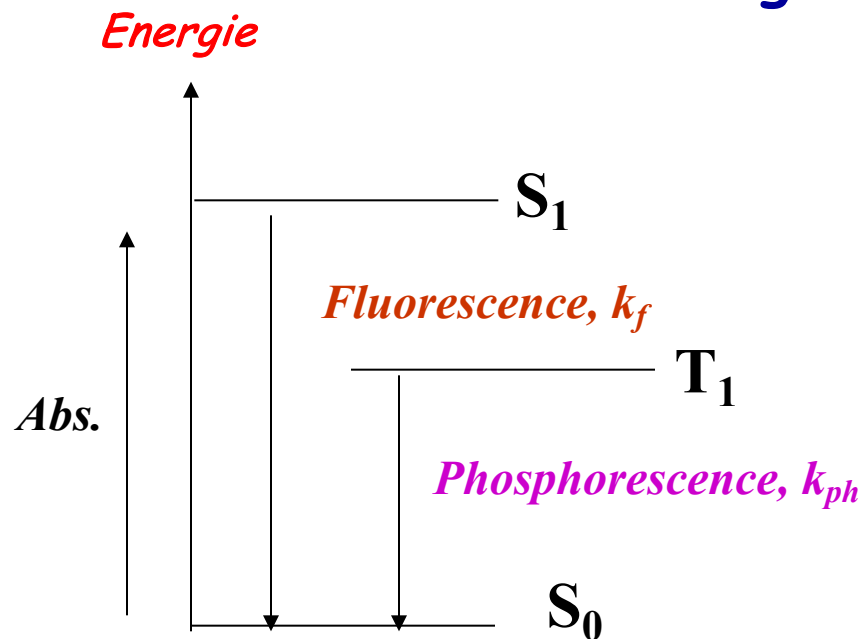
LIFETIME IMAGING

Compétition pour le processus de désactivation de l'état singulet : la boîte noire



Durée de vie des états excités singulets (précurseurs de la fluorescence)

Etats Singulet et Triplet



$$\tau_{\text{nat}} = 1 / k_F$$

τ_{nat} : temps nécessaire à I_F pour atteindre $1/e$ de sa valeur initiale $I_{t=0}$ en absence de tout processus de désactivation autre que la fluorescence.

Signal

$$I_F = I_{t=0} e^{-t/\tau}$$

Relaxation
Exp $[-t/\tau]$

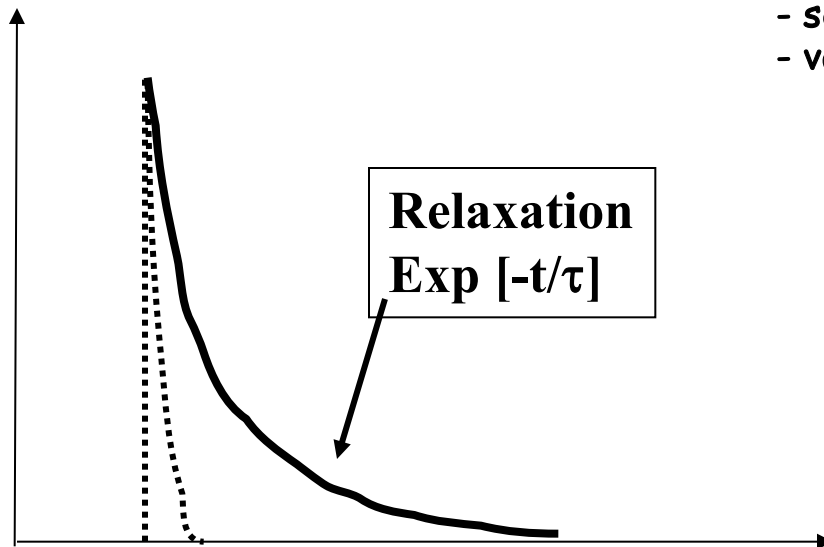
Temps

$$I_F = I_{t=0} e^{-K_f t}$$

I_F : décroissance selon un processus monomoléculaire
 $I_{t=0}$: intensité initiale à un temps zéro
 K_F : constante de vitesse d'émission de fluorescence

FLIM : durée de vie des états excités TCSPC

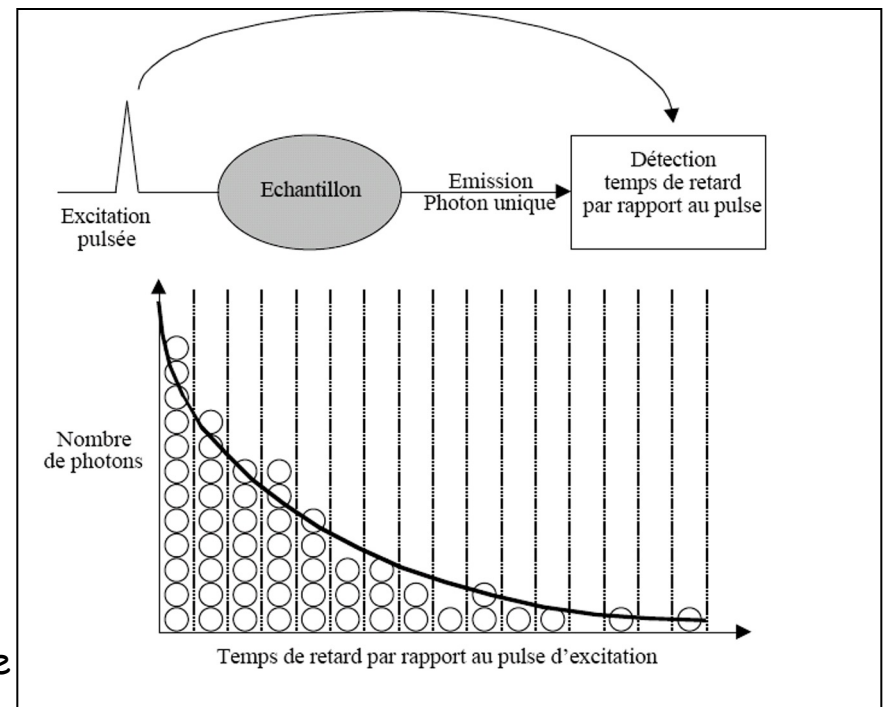
$$I_F = I_{t=0} e^{-t/\tau}$$



$$I_F = I_{t=0} e^{-K_f t}$$

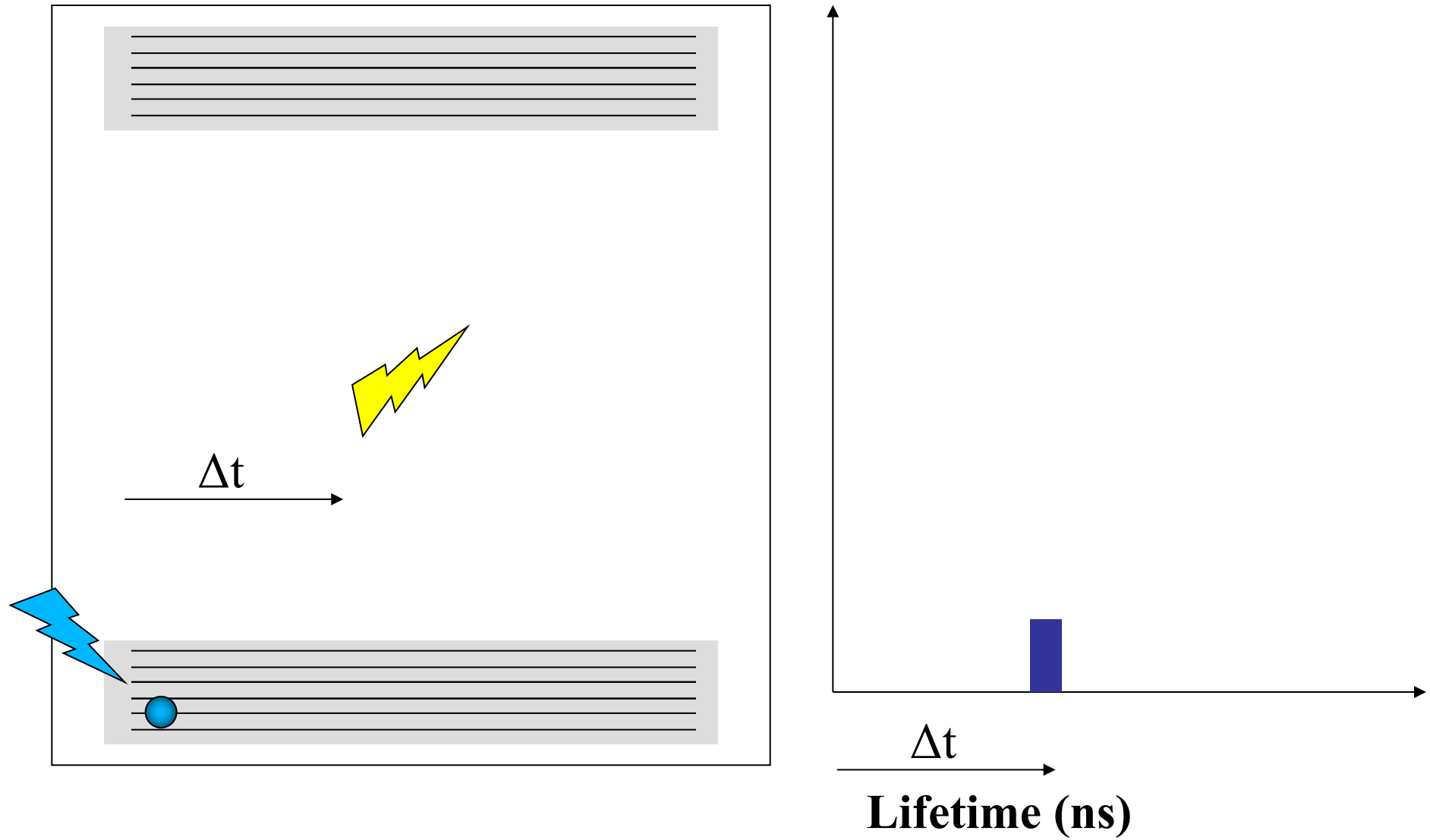
I_F : décroissance selon un processus monomoléculaire
 $I_{t=0}$ intensité initiale à un temps zéro
 K_f : constante de vitesse d'émission de fluorescence

- approche quantitative / fluorescence stationnaire
- discrimination / bruit de fond
- imagerie de concentration (ions, protéines...)
- segmentation (contraste) / multimarquages
- variations environnementales

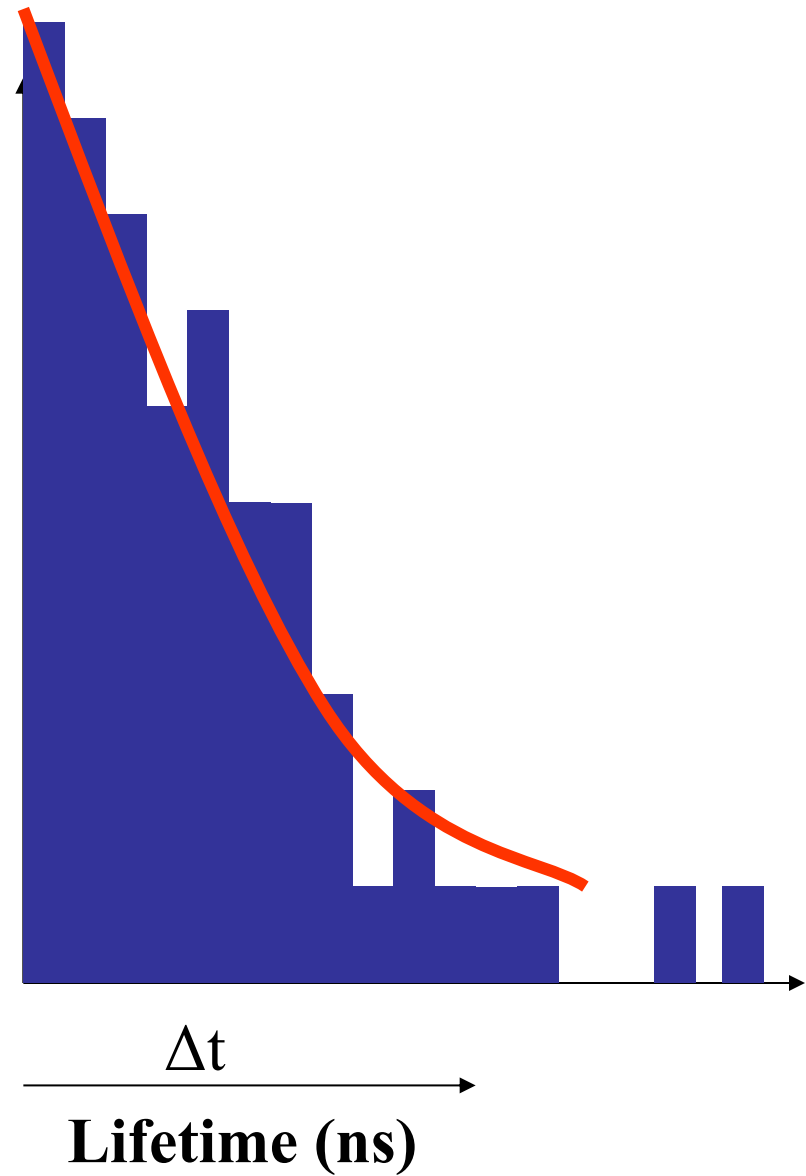
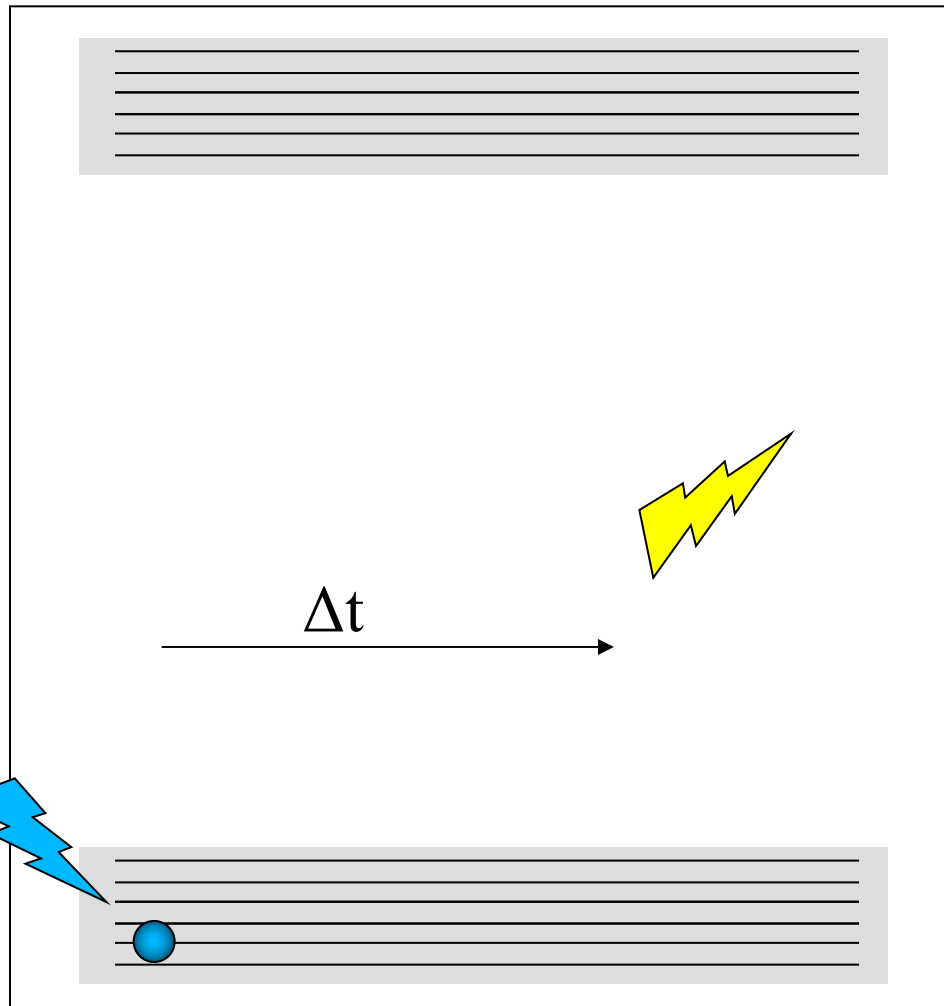


Histogramme des retards des photons : uniques comptés.
L'histogramme de ces retards correspond au déclin de fluorescence

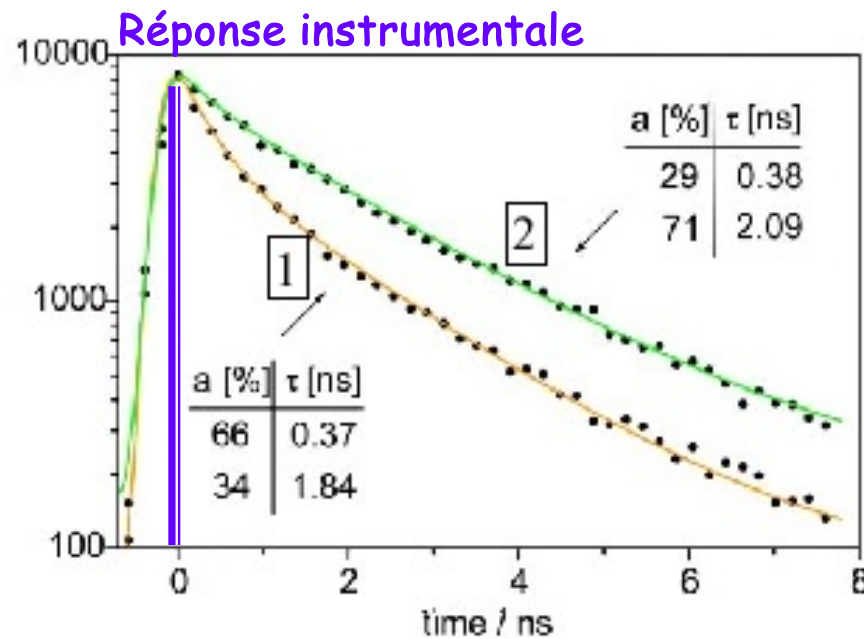
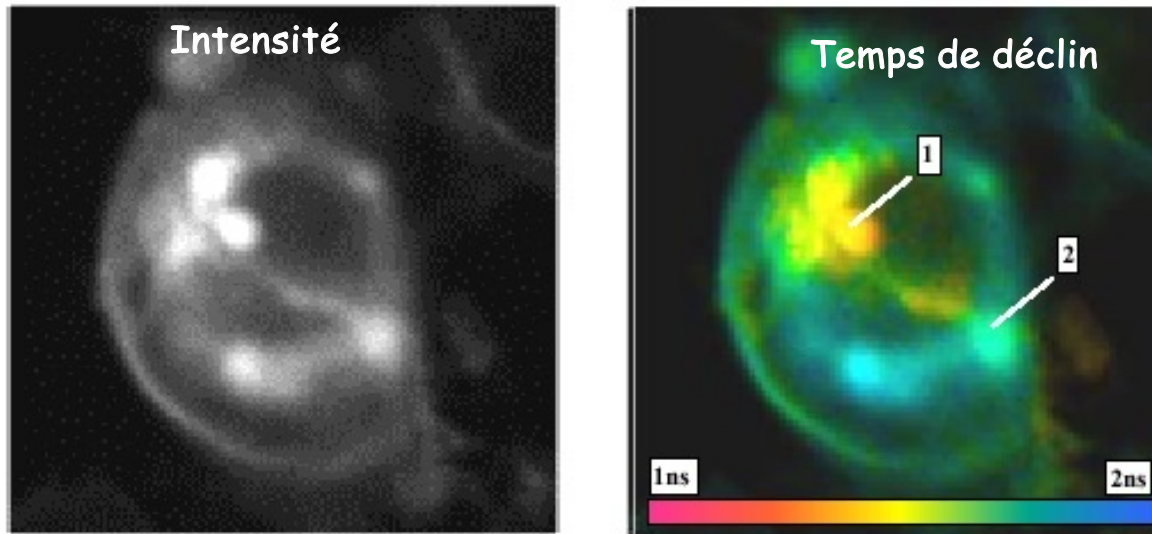
III. Fluorescence Lifetime Imaging Microscopy : TCSPC



III. Fluorescence Lifetime Imaging Microscopy : TCSPC

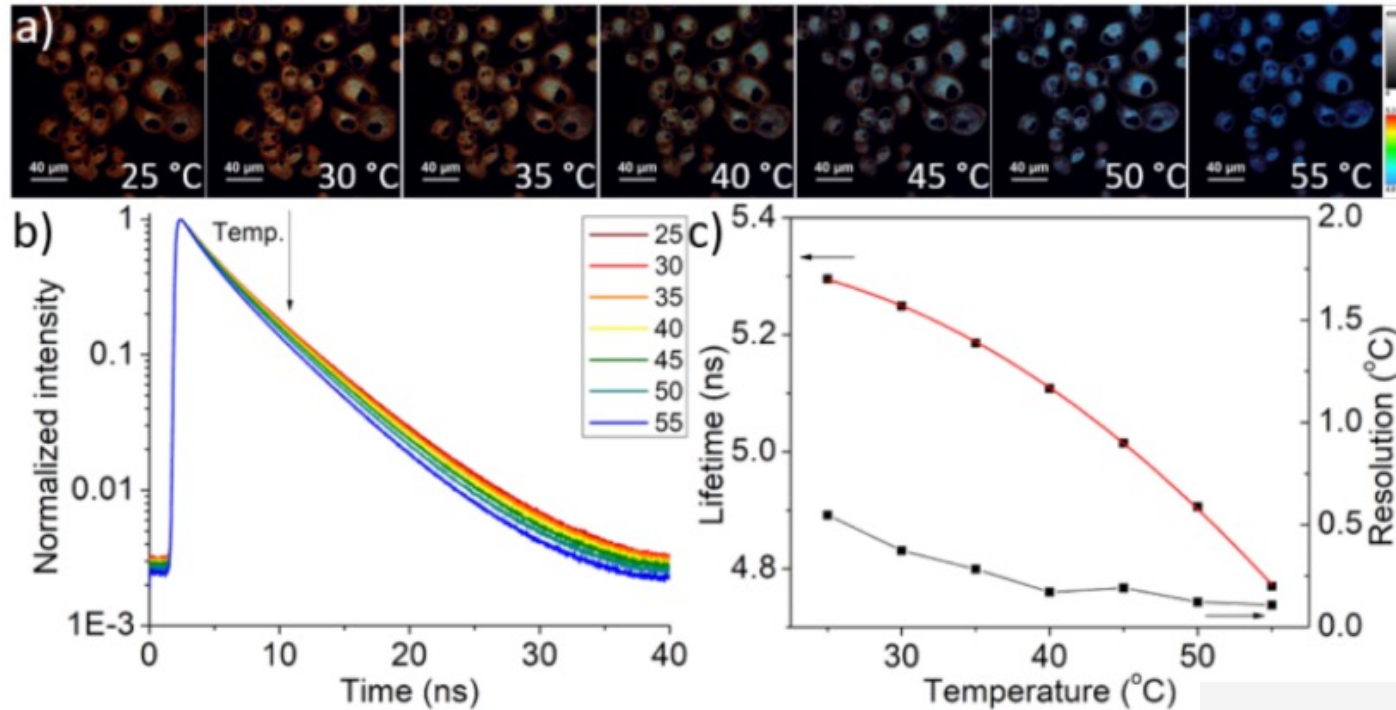


III.FLIM : Fluorescence Lifetime Imaging Microscopy



Durée de vie naturelle dépendante du Microenvironnement

"Sensing Temperature in Vitro and in Cells Using a BODIPY Molecular Probe" by Marti et al.



PEG-BODIPY

$$\tau_{\text{nat}} = 1 / k_F$$

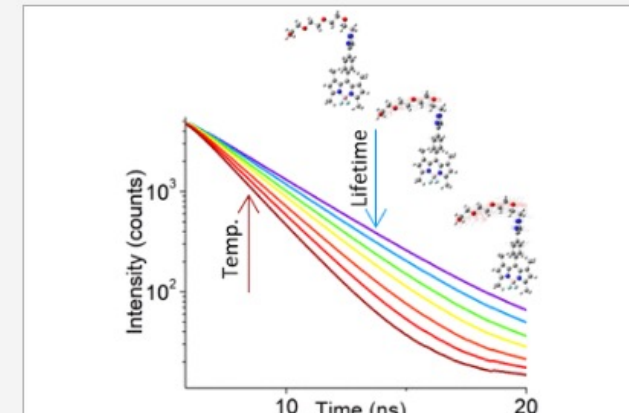
Indépendante :

- de la concentration en fluorophore
- De la puissance d'illumination de la source

Dépendante :

de l'environnement:

- Température
- viscosité



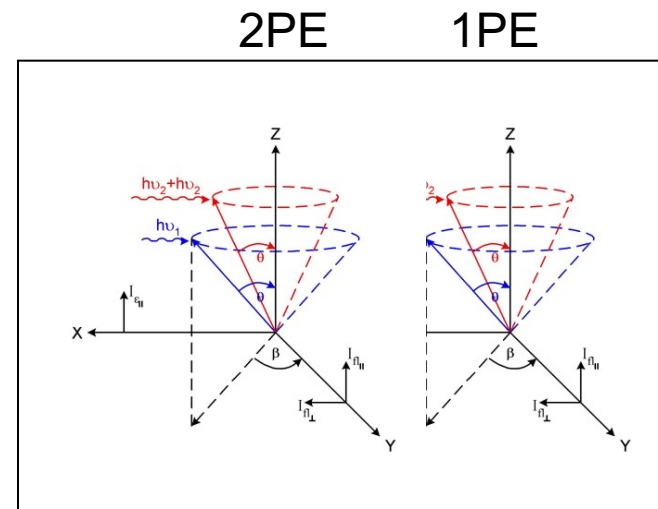
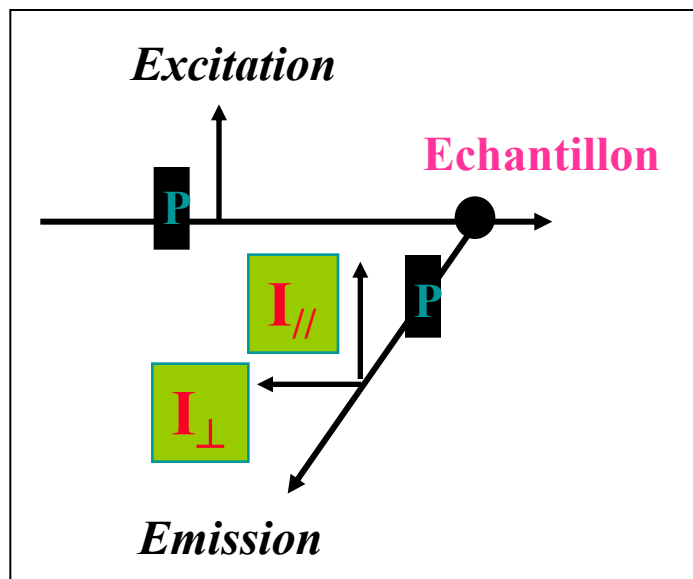
La fluorescence comme moyen de détection...et de quantification relative

La découverte ...



Francis et Jean-Baptiste Perrin

Amplitude/intensité/Polarisation de fluorescence
mesure la corrélation angulaire entre
l'axe de polarisation des photons émis / photons incidents



I_{pol} ↘ quand les molécules sont séparées (50 à 80 Å)

- > Ø molécules
- > Distance parcourue pendant τ (temps de déclin)

$$\langle r \rangle = (I_{//} - I_{\perp}) / (I_{//} + 2 I_{\perp})$$

$$\frac{1}{\bar{r}} = \frac{1}{r_0} \left(1 + \frac{\tau R T}{V \eta} \right)$$

...à l'origine de la découverte du FRET

if τ , V and T are constant,
 $\langle r \rangle$ is related to the local viscosity (η)

Caractéristiques de la fluorescence comme détection

$$I_f; \lambda_f; \tau; \Phi_f; \langle r \rangle$$

Φ_f rendement quantique de fluorescence
nombre de photons émis / nombre de photons absorbés
 $\Phi_f = I_f / I_a$; valeur absolue

$\Sigma \Phi_i = 1$ (excepté pour les réactions radicalaires)

I_f Intensité de fluorescence ; dépendant de l'appareil
 $I_f = \Phi_f I_a$

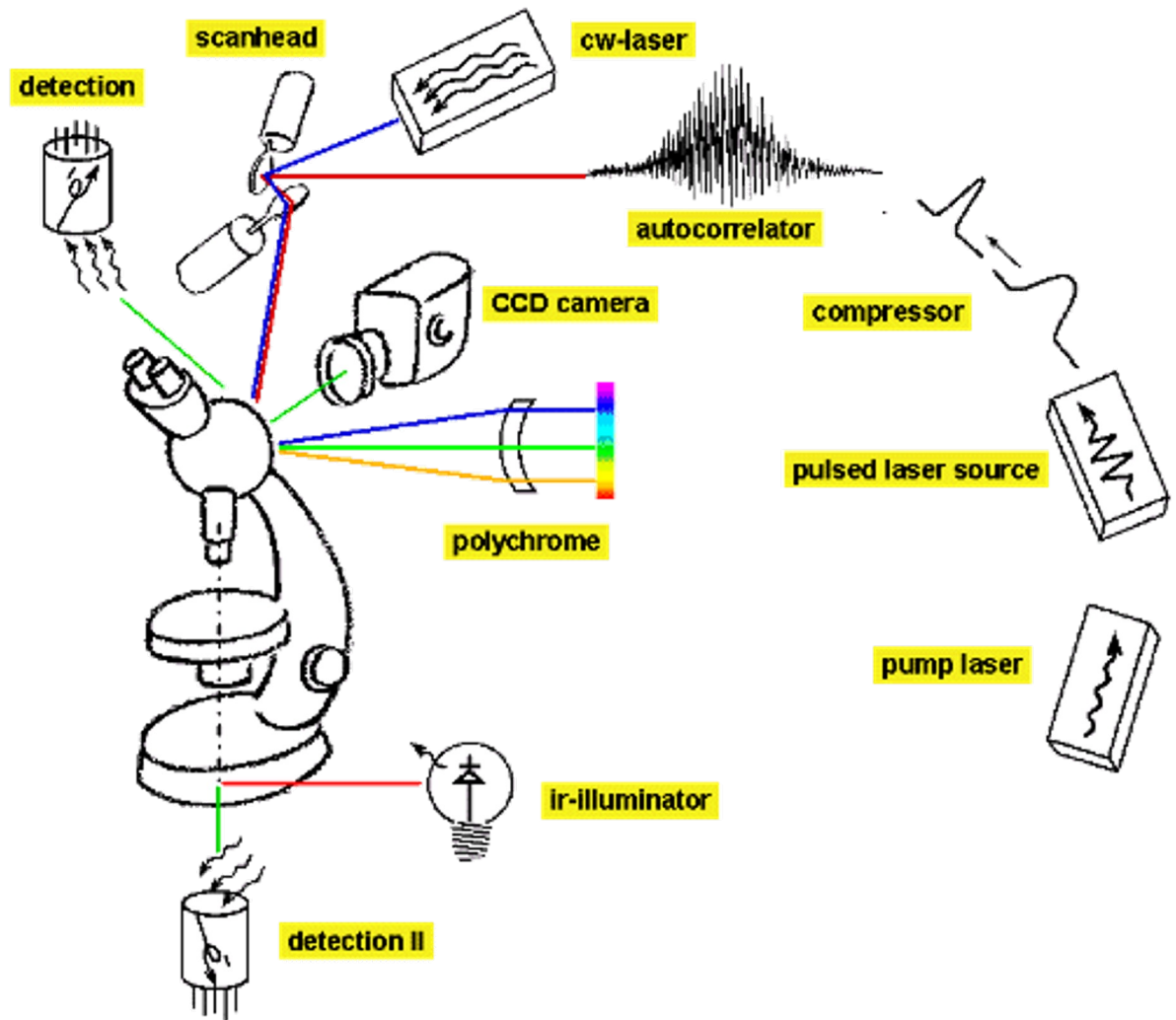
λ_f longueur d'onde d'émission ($\lambda_{f \max}$: au maximum)

τ temps de vie de l'état excité ; $\tau = 1/\Sigma k_i$

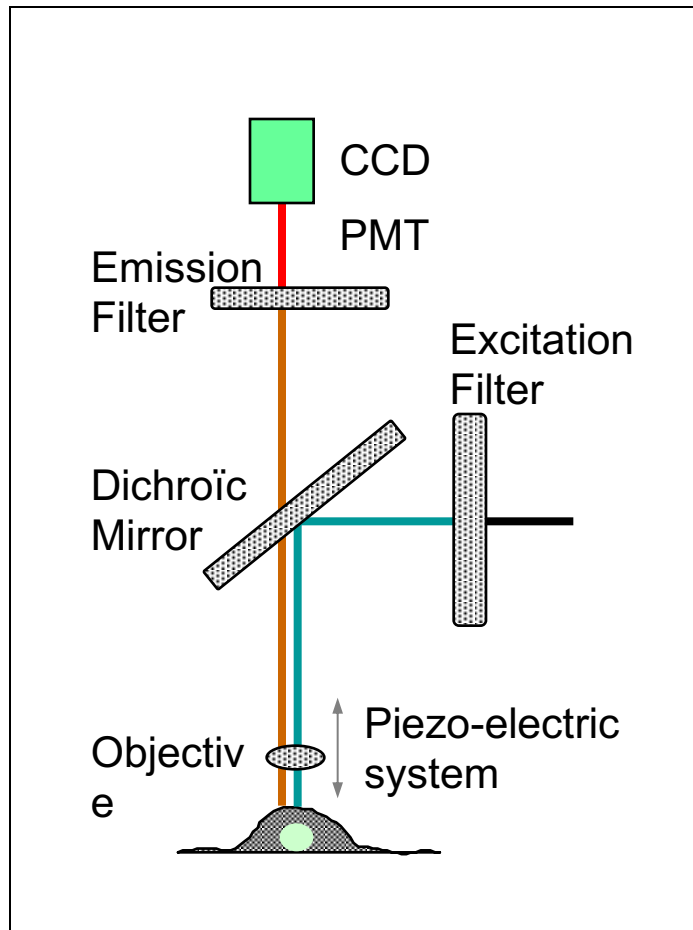
$\langle r \rangle$ anisotropie (axe polarisation photon émis/ photon incident)

Instrumentation

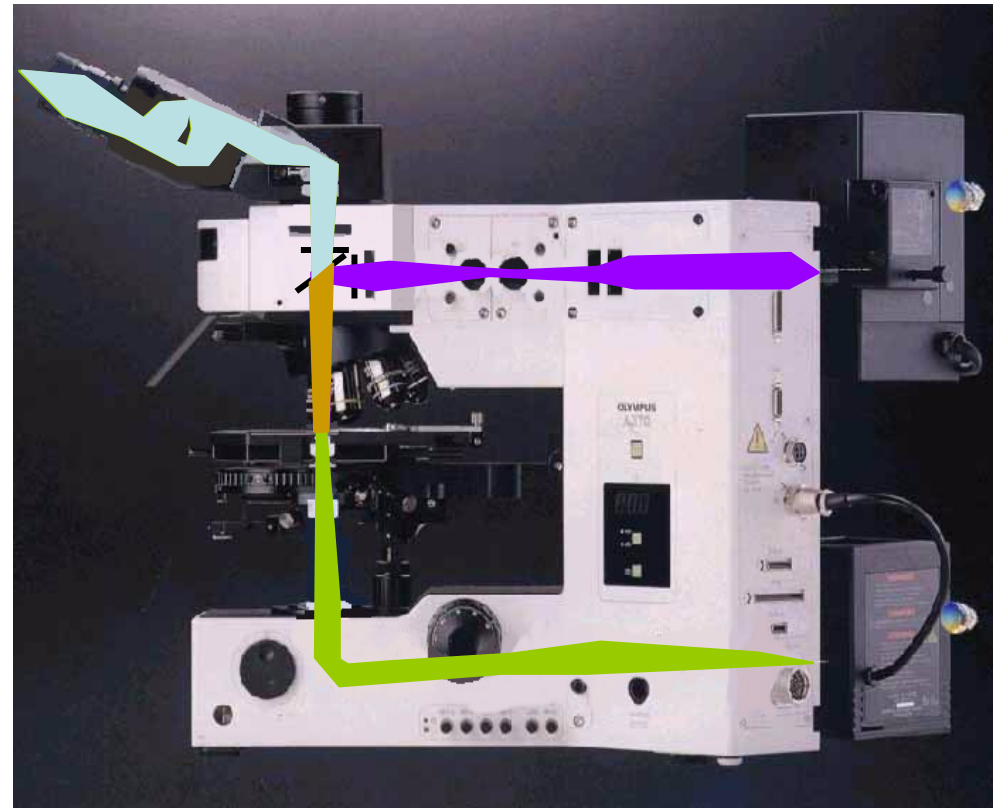
- Biologie
- Electronique
- Optique
- Informatique



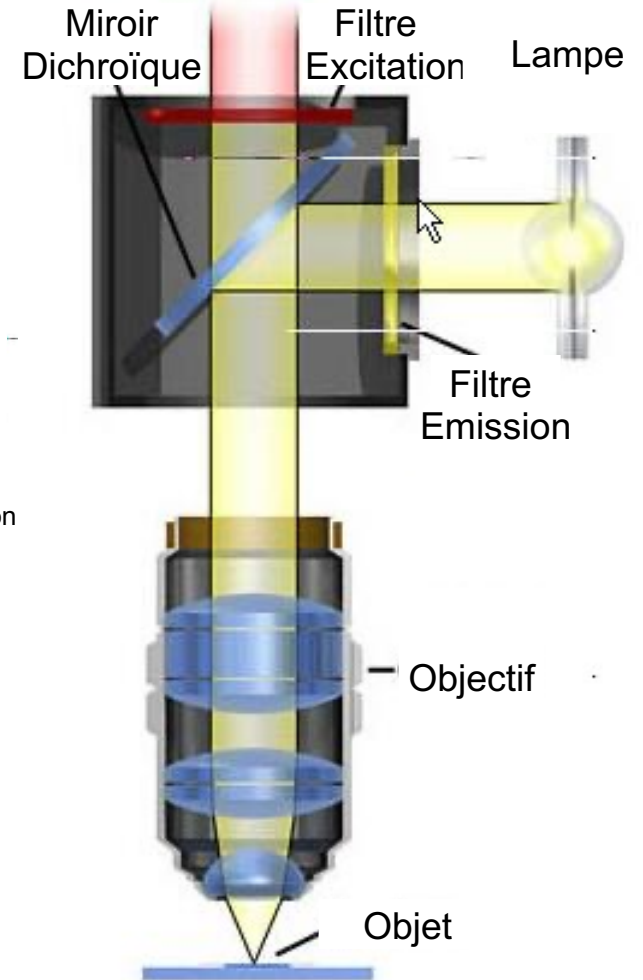
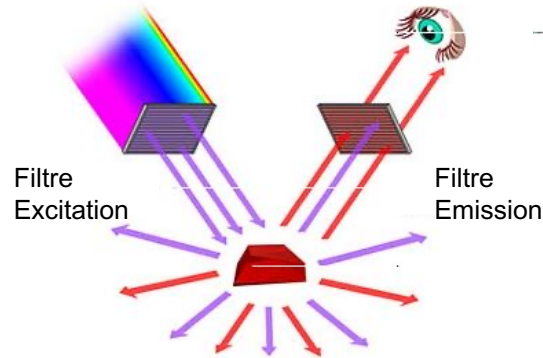
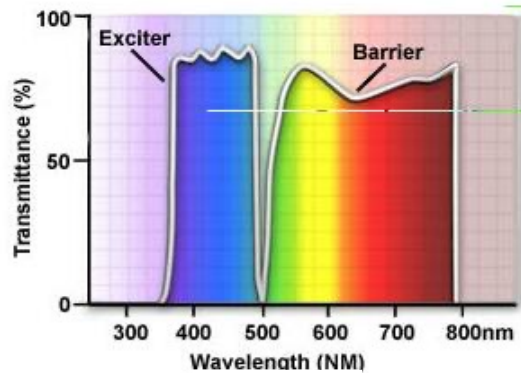
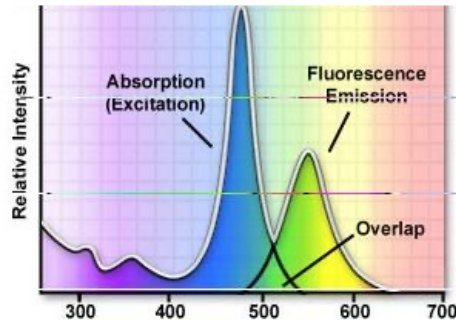
Microscopie Conventionnelle à épifluorescence



PROVIS AX70 (OLYMPUS)



Microscopie Conventiennelle à Epifluorescence ...optique



Microscopie Conventionnelle à Epifluorescence ...optique

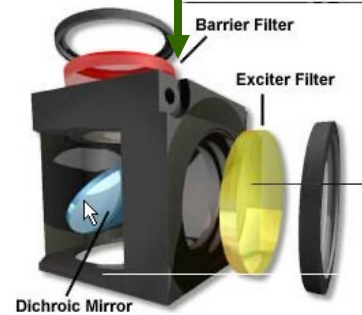
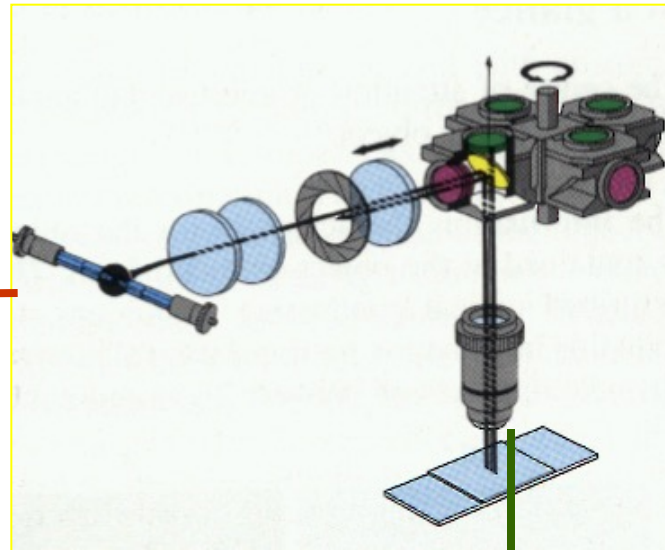
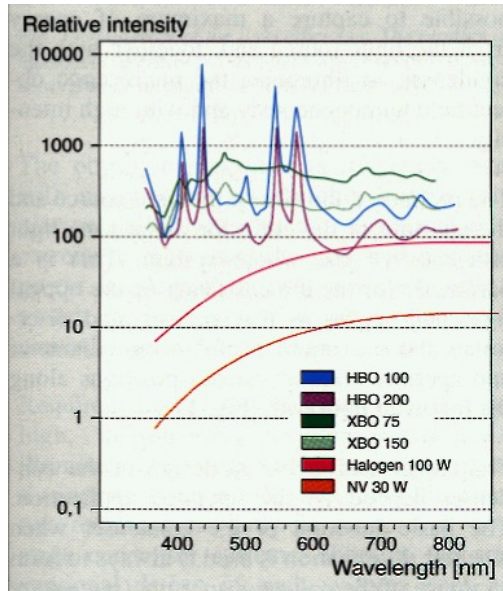


Figure 1

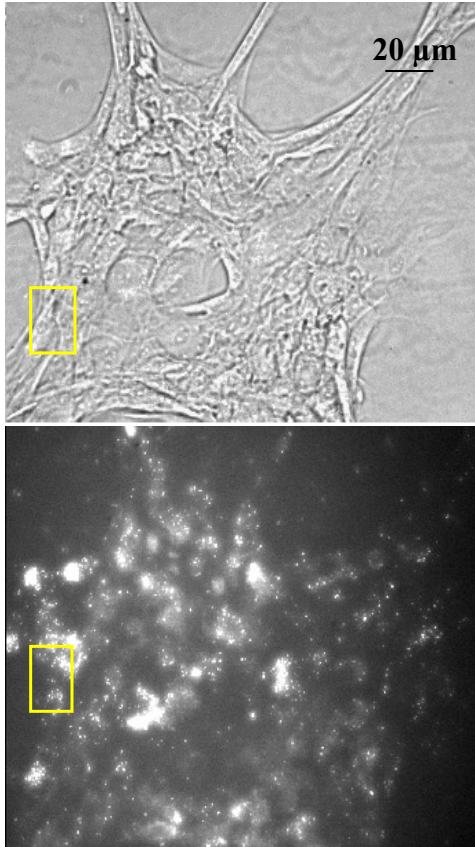


Objective Specifications



Intérêt de la 3D

2D

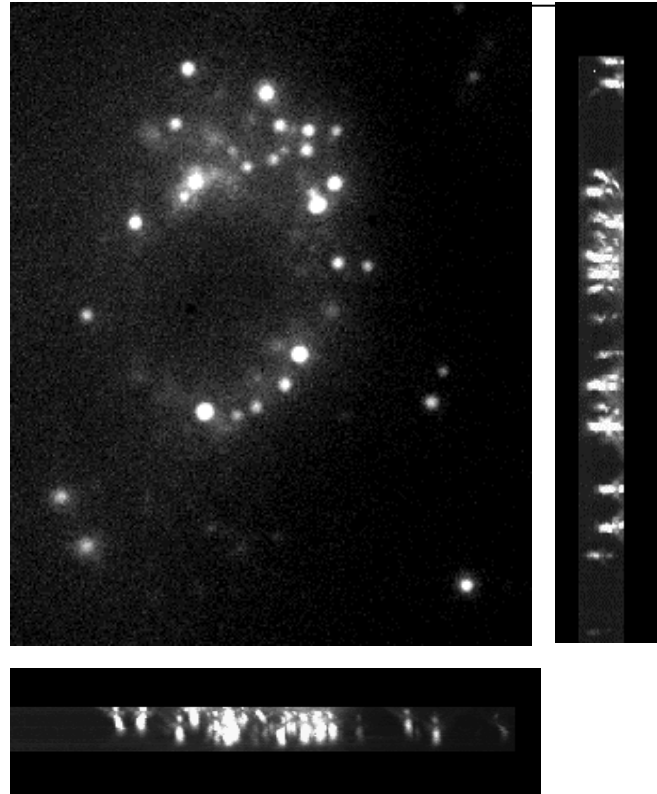


x20
Adhèsion

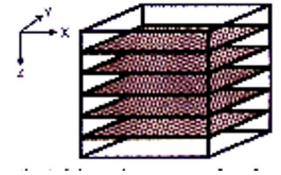
3D

4 µm

20 µm

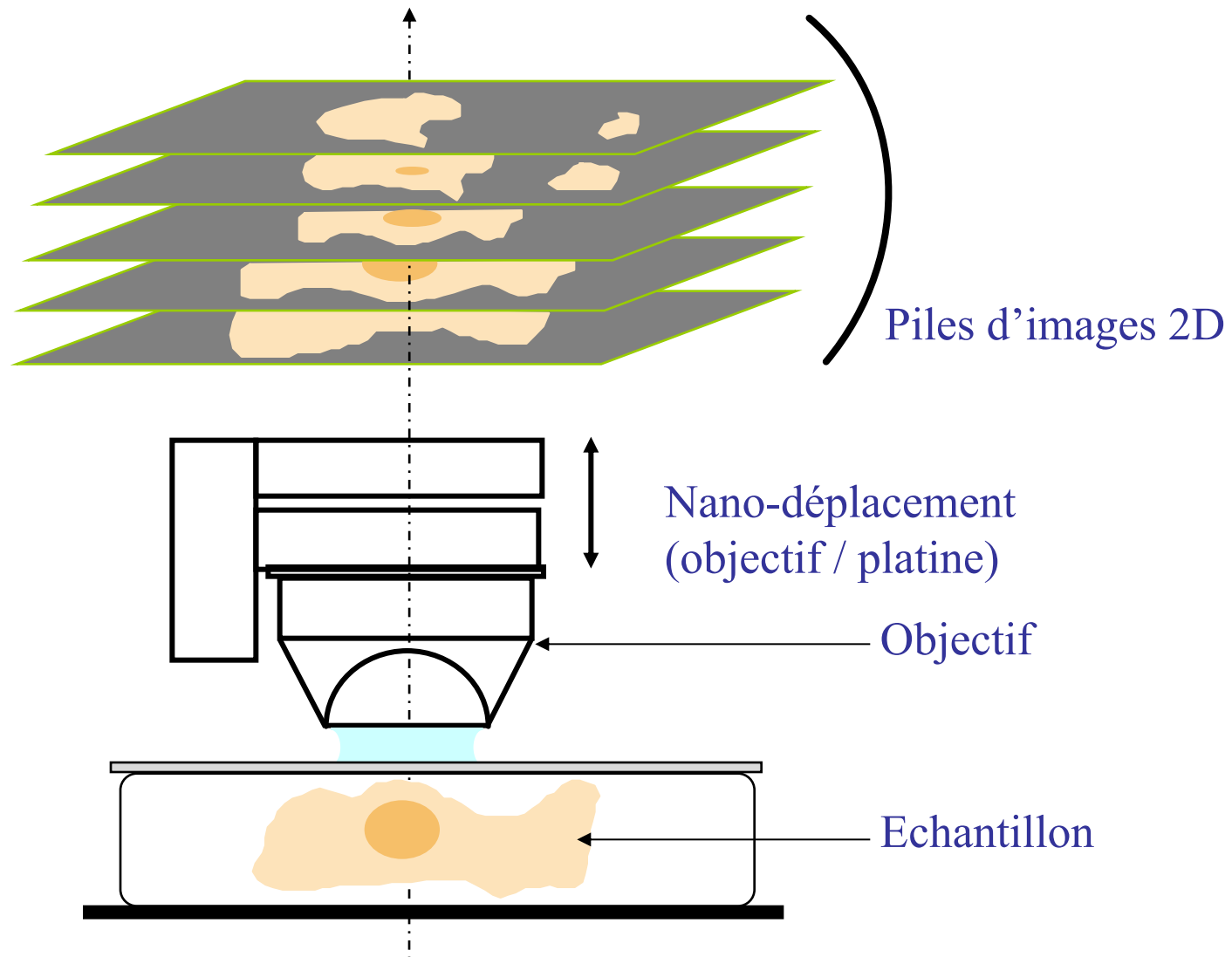


XZ
x100
Internalisation

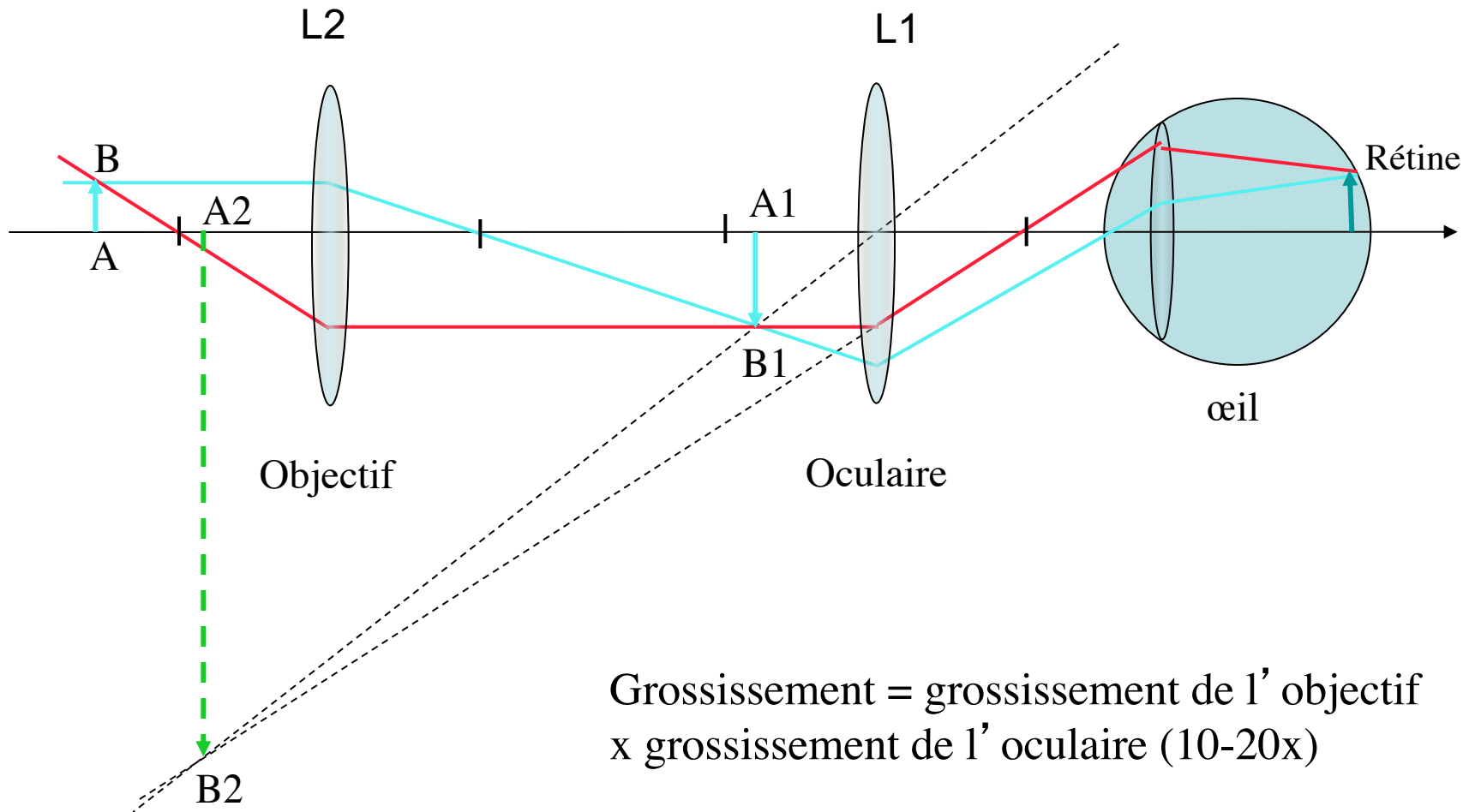


Nanoparticules (HA) à la surface d'une monocouche de chondrocytes (cellules du cartilage)

Microscopie à sectionnement optique..coupes sériées virtuelles



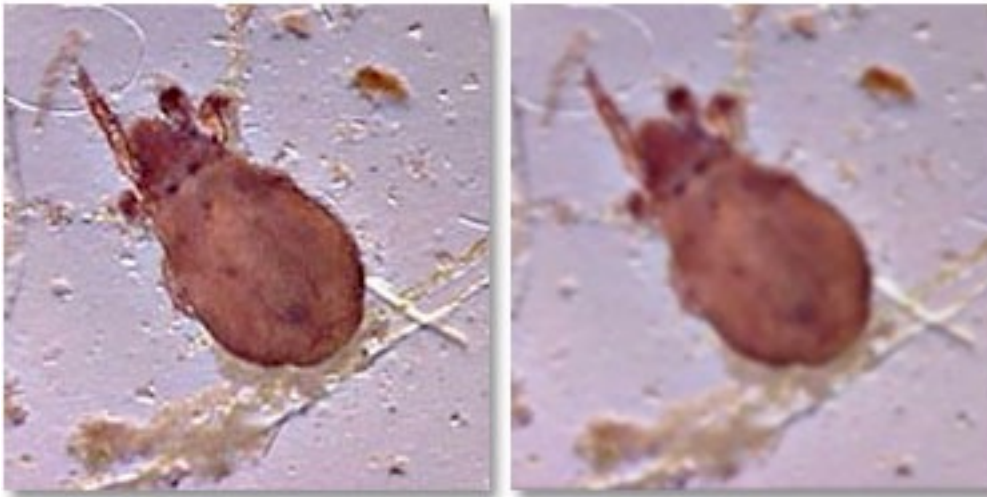
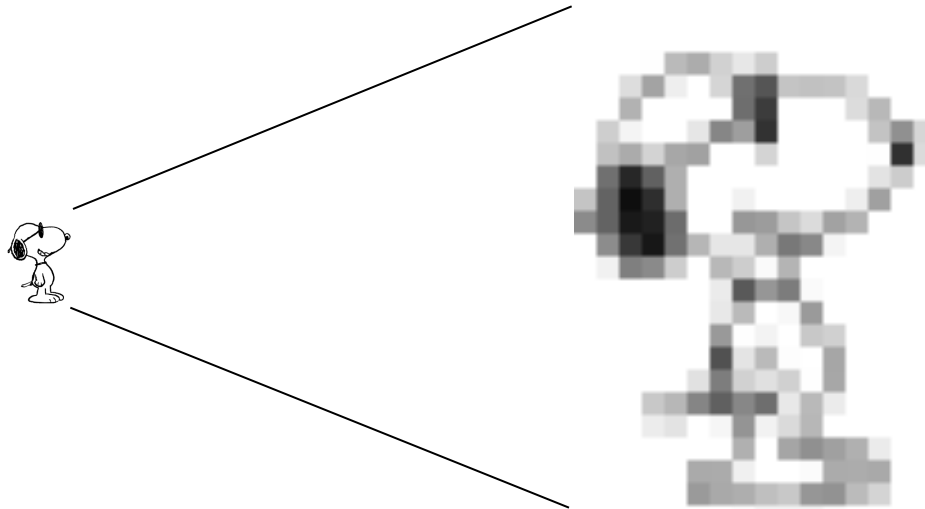
Agrandir plus : le microscope pour agrandir



Grossissement = grossissement de l'objectif
x grossissement de l'oculaire (10-20x)

➡ agrandissement jusqu' à 2000x !

Agrandir ne suffit pas !



⇒ **Résolution !**

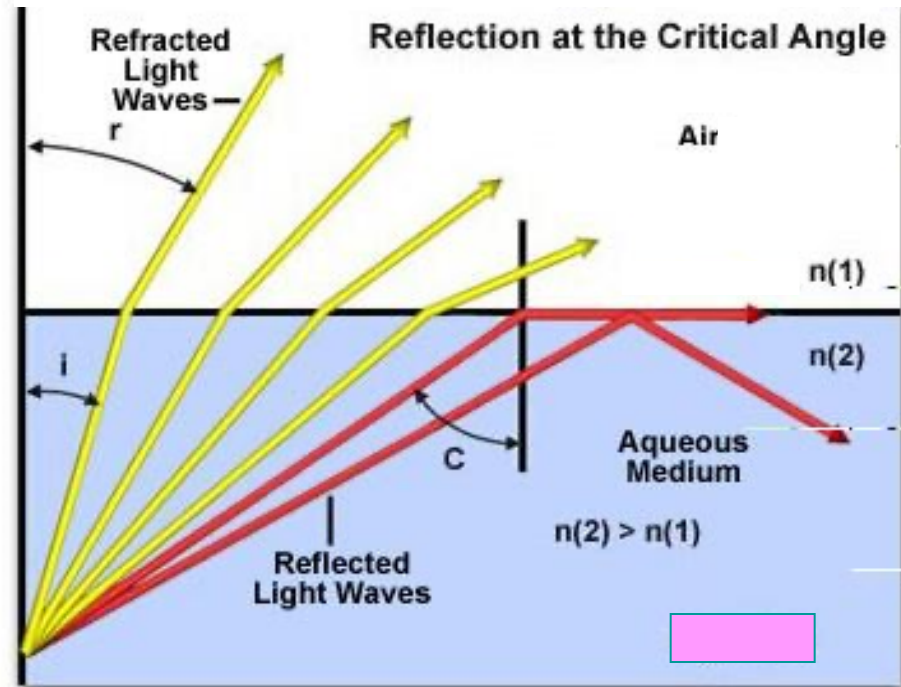
Grossissement identique !

Microscopie à Epifluorescence ...limitations

Réfraction de la lumière

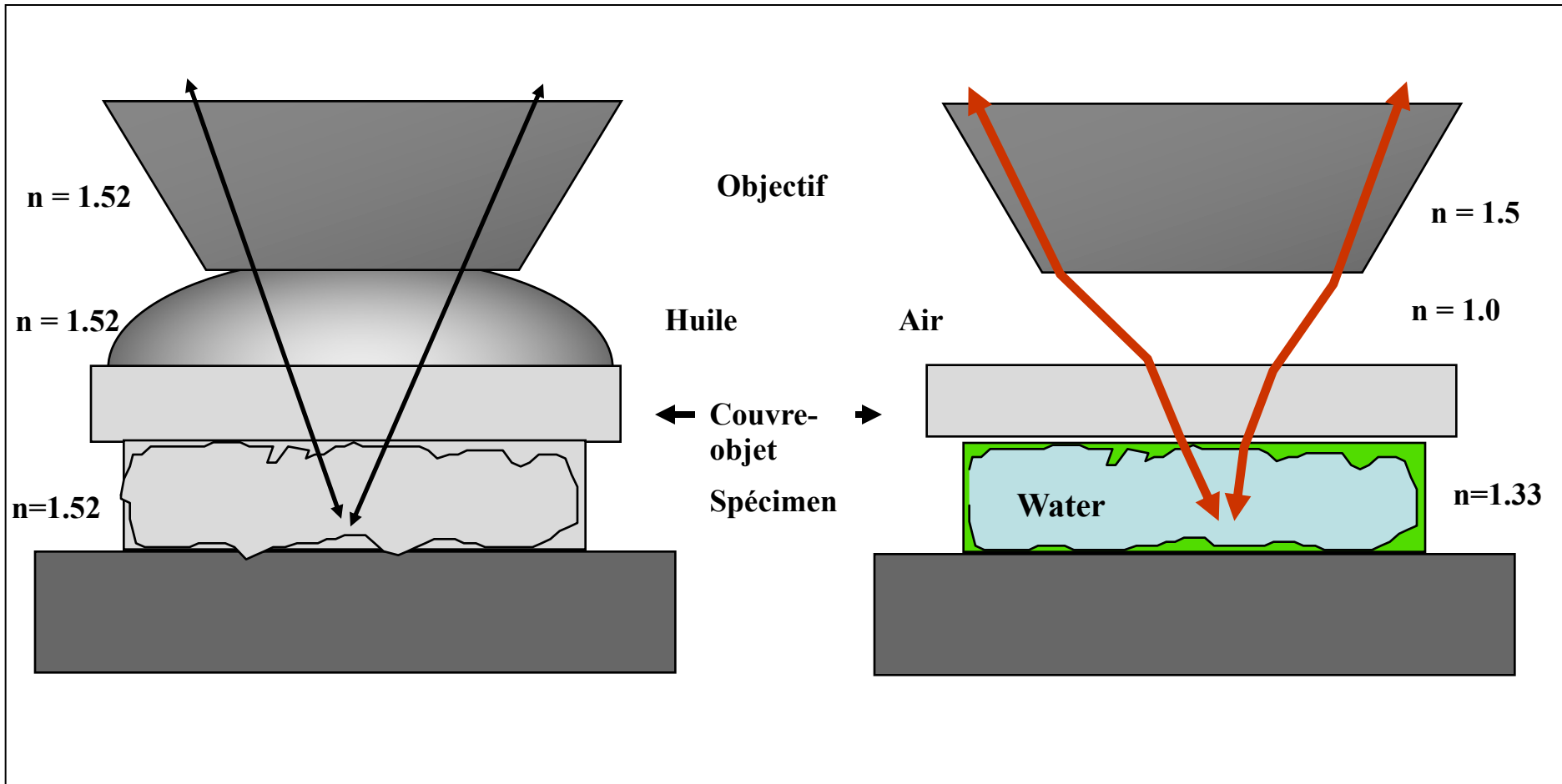


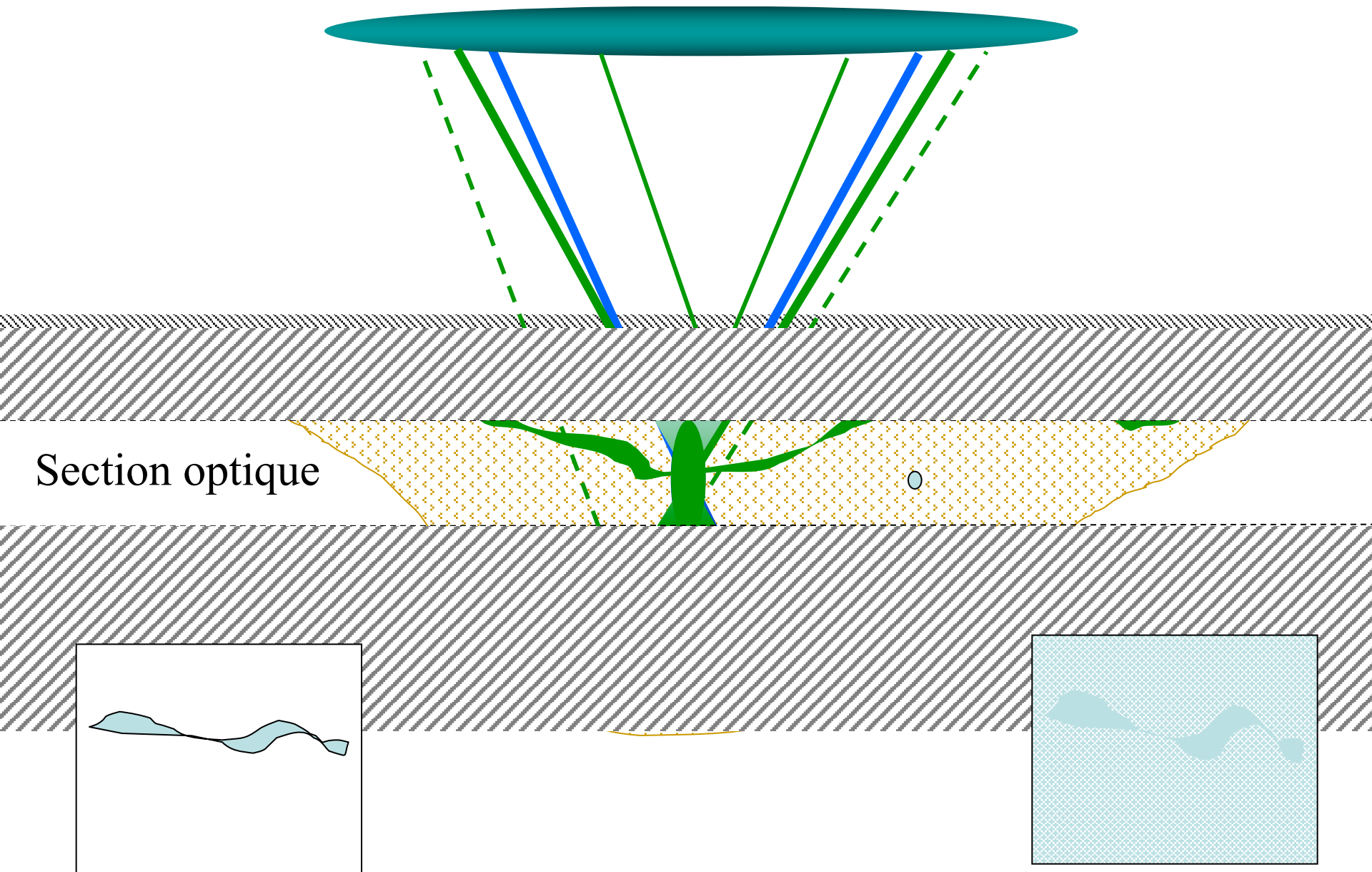
Phénomène de réfraction
de la lumière dans l'eau



Indice / cytoplasme / organites —————> Liquide immersion
($n >$ fluide interstitiel)

Indice Réfraction (n)



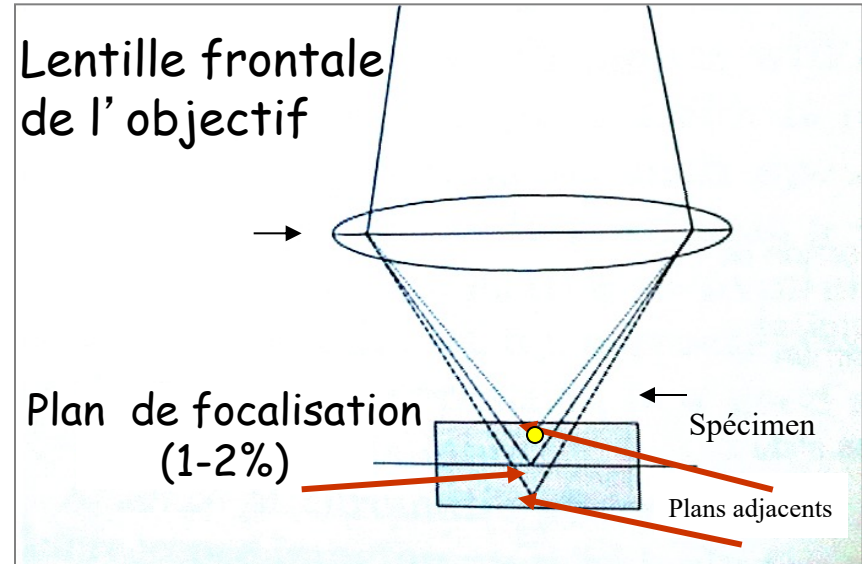
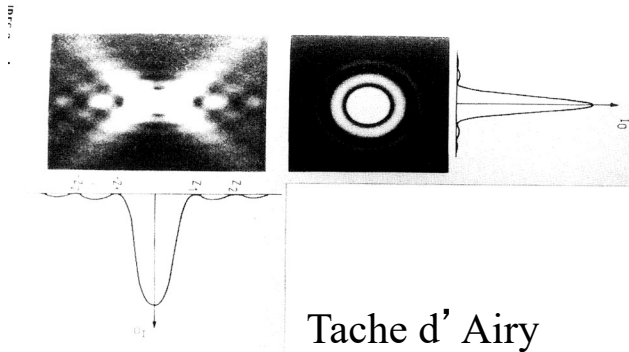


Microscopie à Epifluorescence ...limitations

Microscopie optique :

Une image contient des informations provenant des plans inférieurs et supérieurs au plan de focalisation.

Il a été déterminé que seule 1% à 2% de l'information provient effectivement du plan de focalisation.



Flou généré par les plans adjacents

supprimer l'information hors plan de focalisation

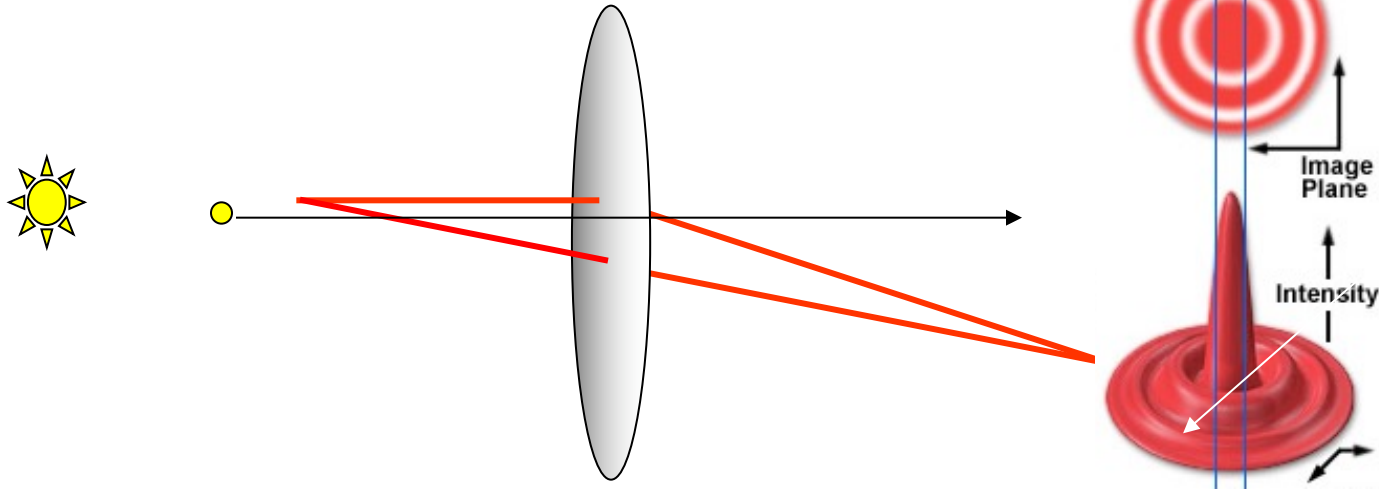
—————> déflouer

Diffraction de la lumière : interférence en optique

Cliché de diffraction d'ordre multiple : Airy

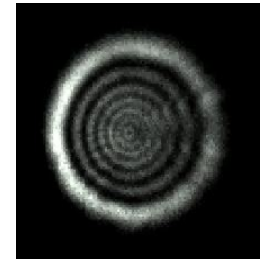
Source
ponctuelle

lentille

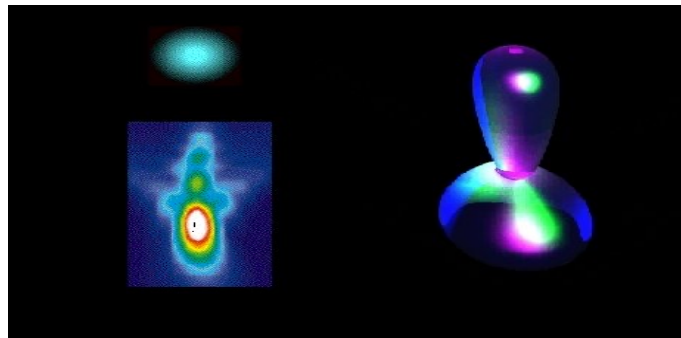


Abbe (1873)

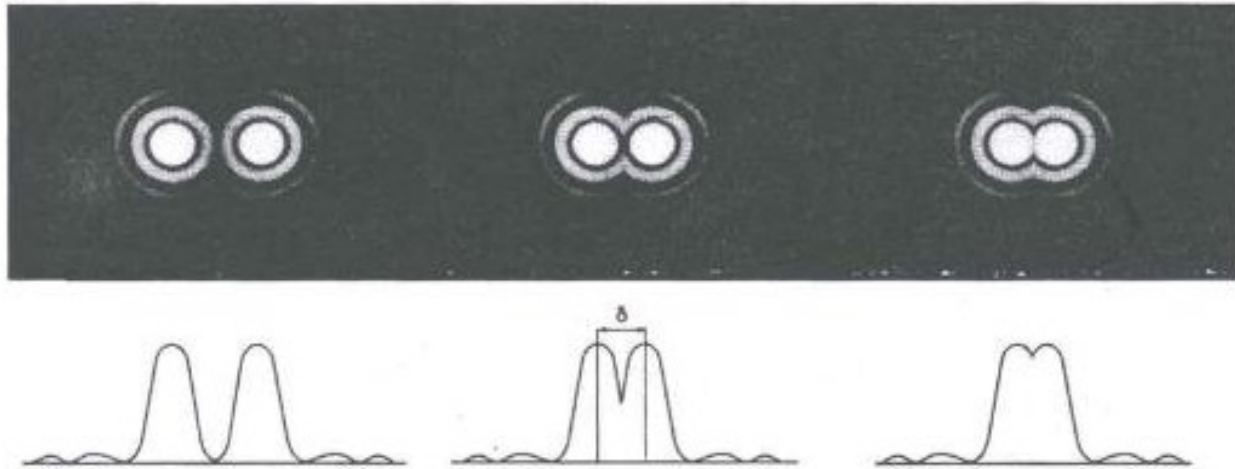
X100 / NA 1.65



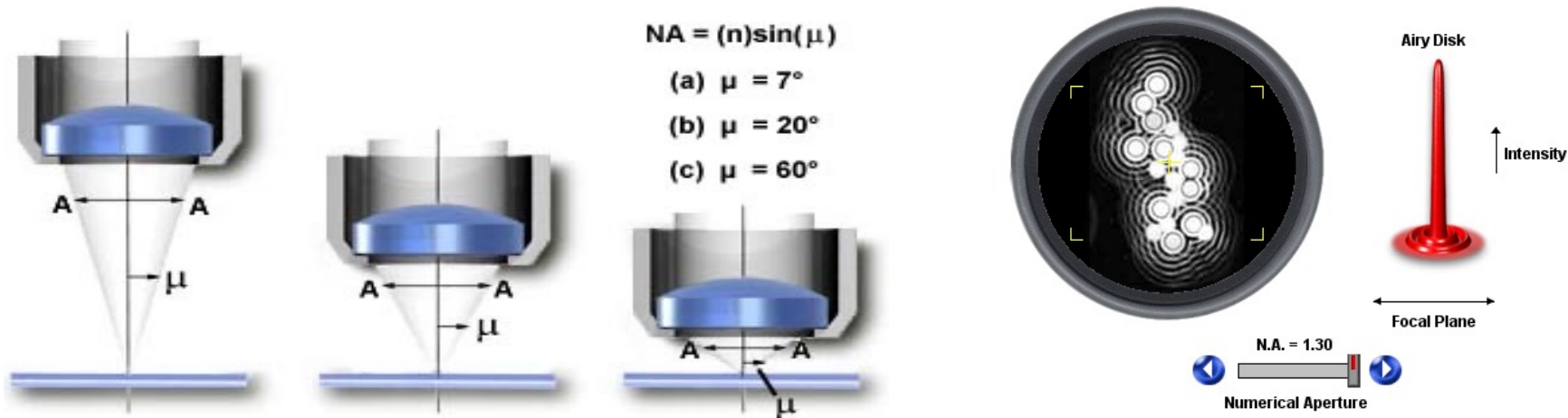
Tâche d' Airy



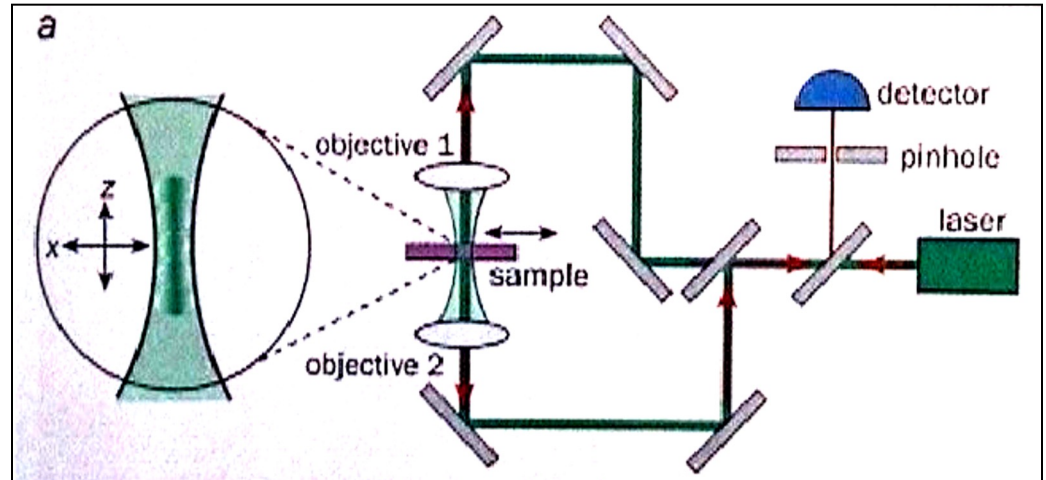
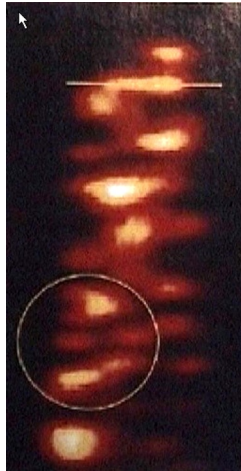
La résolution latérale dans le plan focal et l' ON de l' objectif



$$d_{xy} = 0,61\lambda / \text{ON} \quad \text{avec} \quad \text{ON} = (n)\sin(u)$$

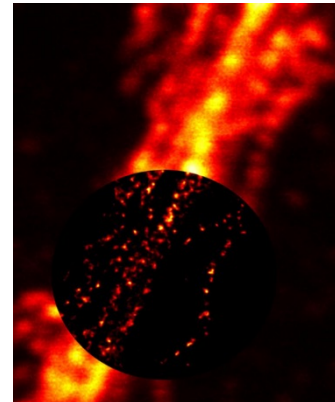
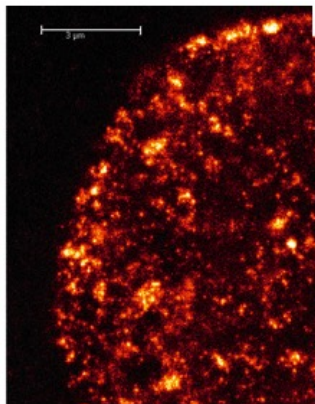
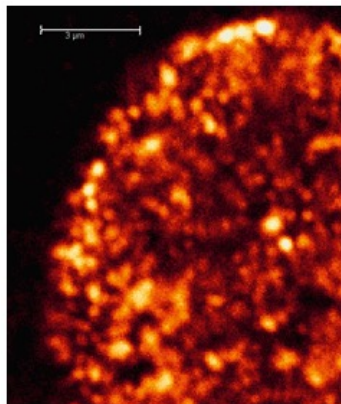


4Pi : Manheim 2008: 10 nm



Microtubules in mouse fibroblast cells in the x-z plane

STED : Bordeaux 2008 : 25nm



Les méthodes en imagerie de contraste

A. Le contexte photophysique : la lumière

- I. Le rayonnement électromagnétique/ multimodalités de la lumière
- II. Les aberrations optiques (réfraction et diffraction)
- III. Instrumentation (détecteurs et capteurs)

B. Les méthodes contrastes en imagerie cellulaire (excitation monophotonique)

- I. Microscopie conventionnelle / Déconvolution
- II. Microscopie confocale

C. Exemples de techniques pour caractériser les Interactions et diffusions moléculaires

- I. FRAP: Fluorescence Recovery after Photobleaching
- II. FRET : Fluorescence Resonance Energy Transfer

D. Microscopie Multiphotonique et dérivées

- II. Microscopie à excitation multiphotonique
- II. FLIM : Fluorescence Lifetime Imaging Microscopy
- III. SHG: Second Harmonic Generation
- IV. Méthodes Vibrationnelles (Raman-CARS)

D. Développements

Macroscopie en mode multiphoton

Les microscopies optiques dites « à déflouage »

- **Microscopes de fluorescence – Méthode de déconvolution**

(Wide field fluorescence microscopes)

Capteurs CCD à haute résolution et sensibilité

Déconvolution possible pour éliminer les signaux hors plan focal

Fondamentale, video-microscopie rapide ! En temps réel !

Psuedo-Quantification

- **Microscopes confocaux à balayage laser**

(Confocal laser scanning microscopes)

Détecteurs : photomultiplicateurs

Diaphragme (pinhole) confocal pour sélectionner la fluorescence émise dans le plan focal, colocalisation, 3D

Quantification relative

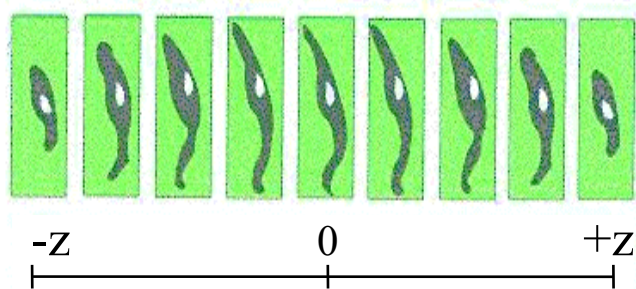
- **Microscopes à balayage laser et excitation bi- (multi-) photonique**

(Two- (or multi-) photon laser scanning microscopes)

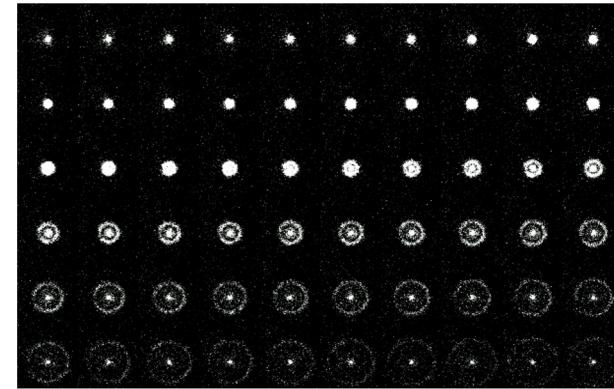
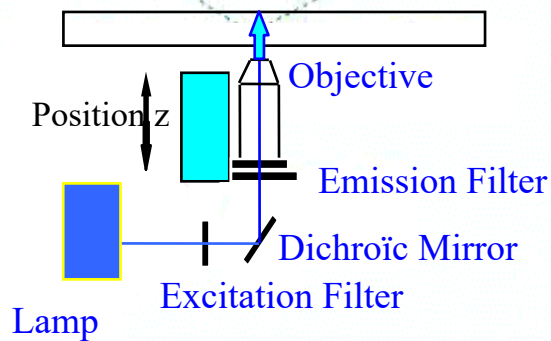
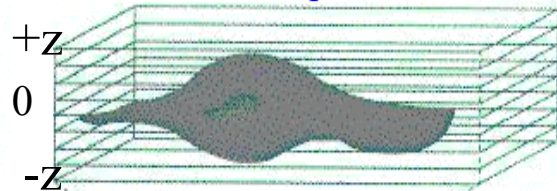
Avantages : excitation restreinte au volume focal, résolution temporelle

Quantification correcte dans le volume de collection

I. Microscopie Conventionnelle à Déconvolution



Series of optical sections



PSF. Bead ($0.17 \mu\text{m}$). Step : $0.25 \mu\text{m}$

OTF/PSF

Function of convolution



Function of deconvolution

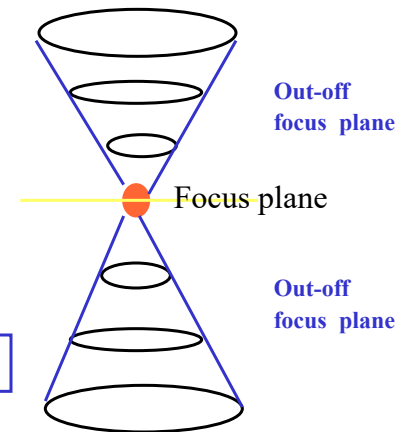
$$g = h \otimes f + n$$

f : real object

n : noises

h : Point Spread Function (PSF)

$\otimes$: deconvolution symbol



Mathematical reversion

I. Conventional Optical Scanning Microscopy: Deblurring

Description

$$g = h \otimes f + n$$

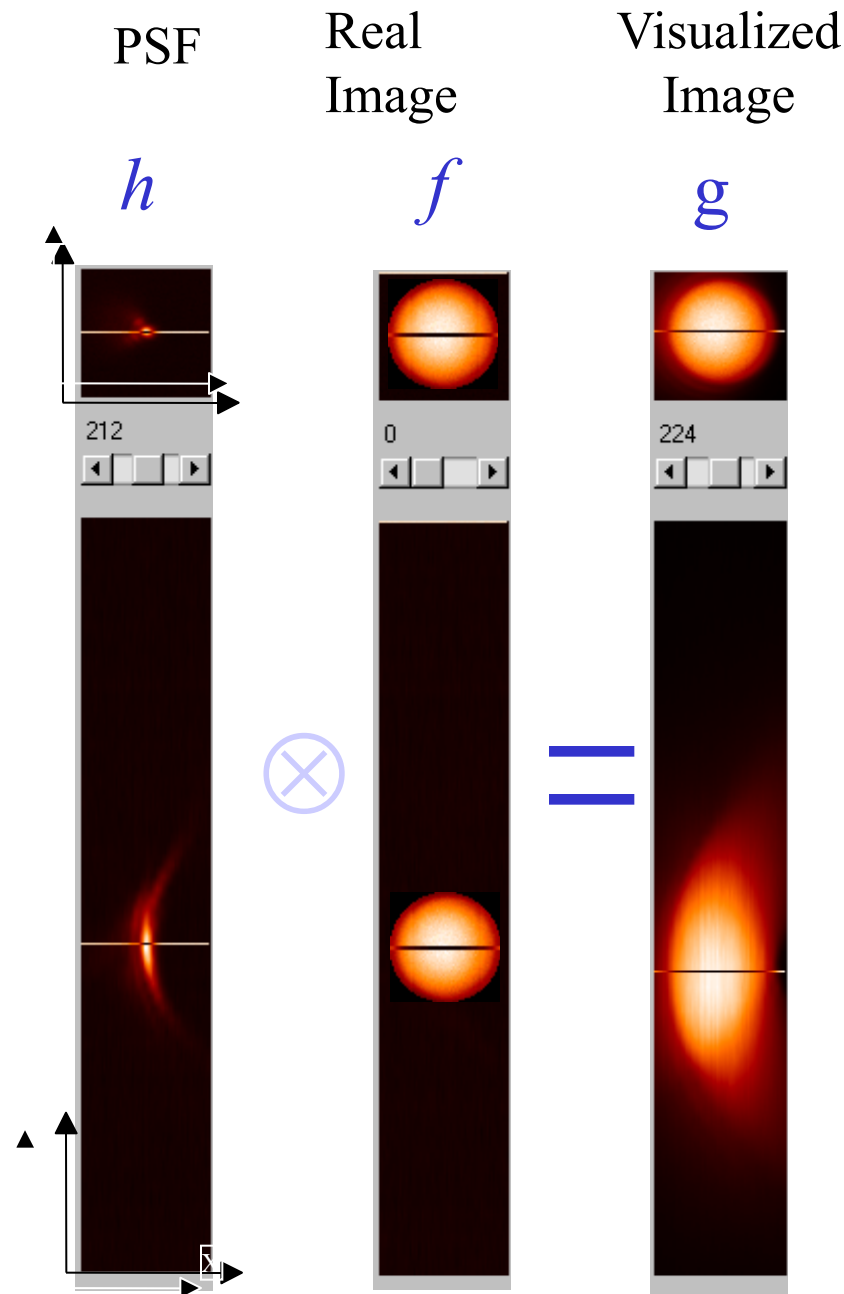
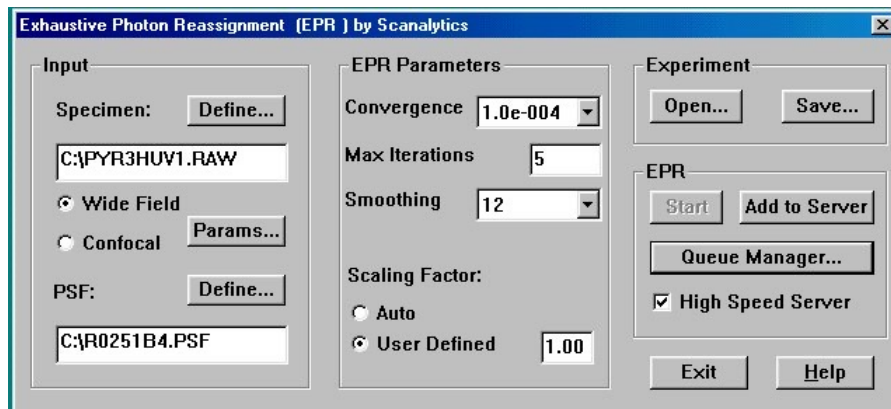
Convolution

noiset

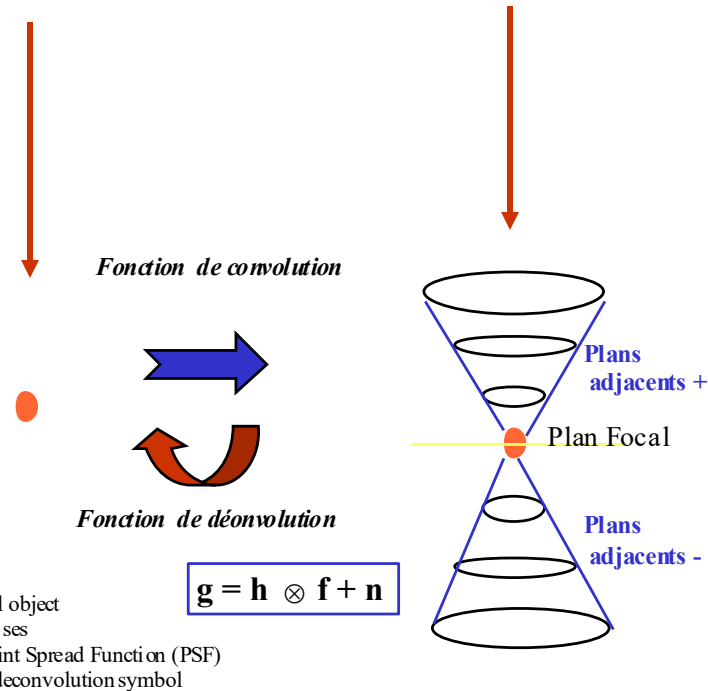
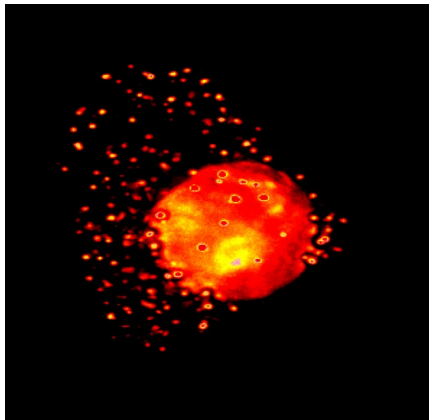
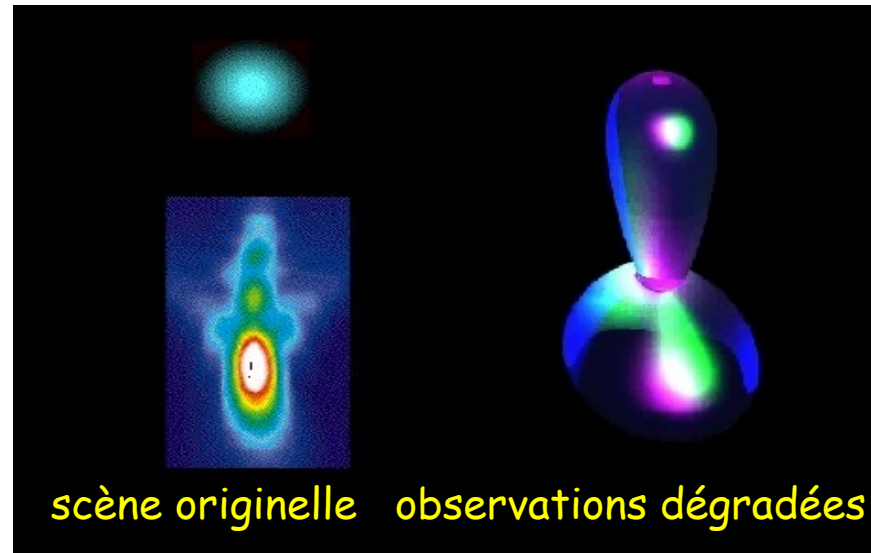
g : Blur image observed via microscope

h : PSF (point spread function)

f : Real distribution of the fluorescent probe



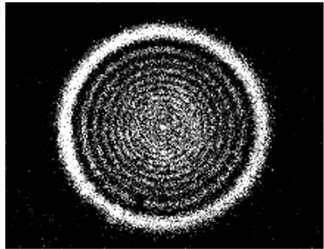
I. Restauration d'Images : déconvolution



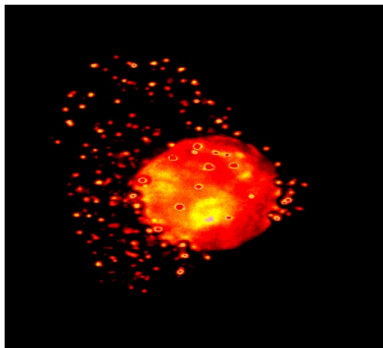
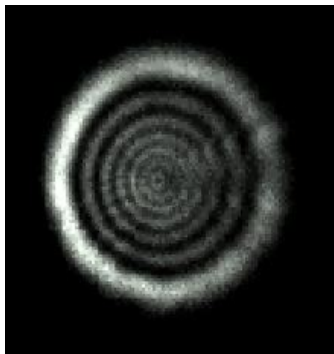
I. Restauration d'Images : déconvolution ou pseudo-confocale

PSF

X100 / NA 1.30



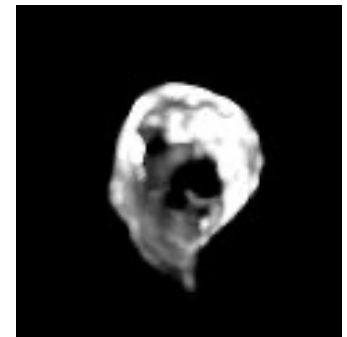
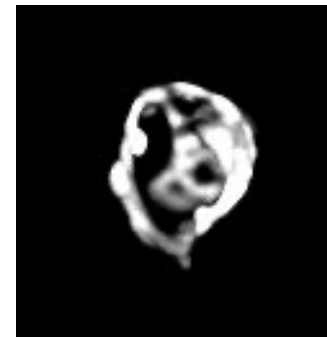
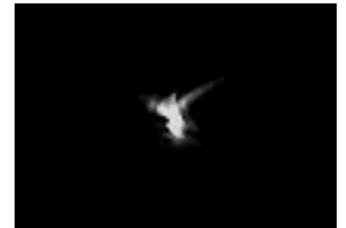
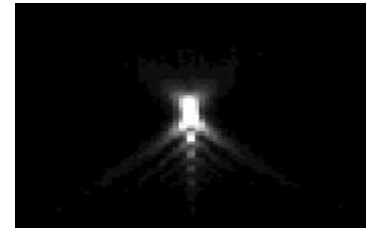
X100 / NA 1.65



Fonction de Transfert Optique

PSF et déflouage

PSF

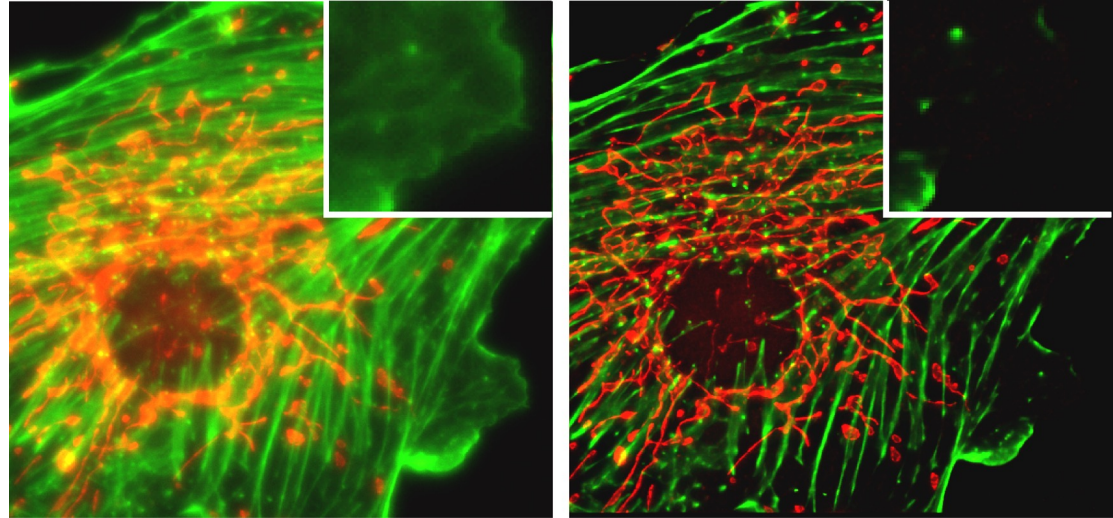


Chondrocyte Rat cultivé en alginate/éponge. 60x/1.25NA

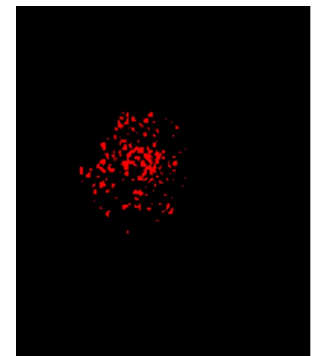
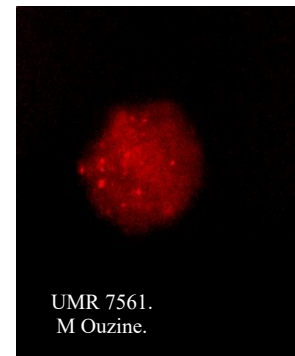
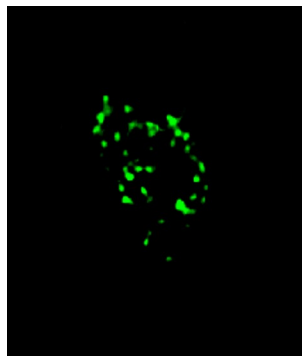
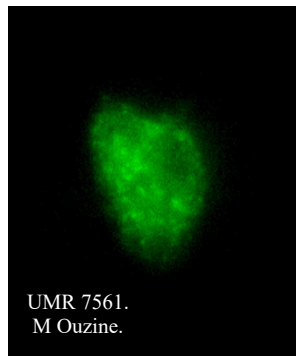
I. Restauration d'Images : déconvolution ou pseudo-confocale

EPR™ System
Deconvolution
(iteration : 15))

Microtubules

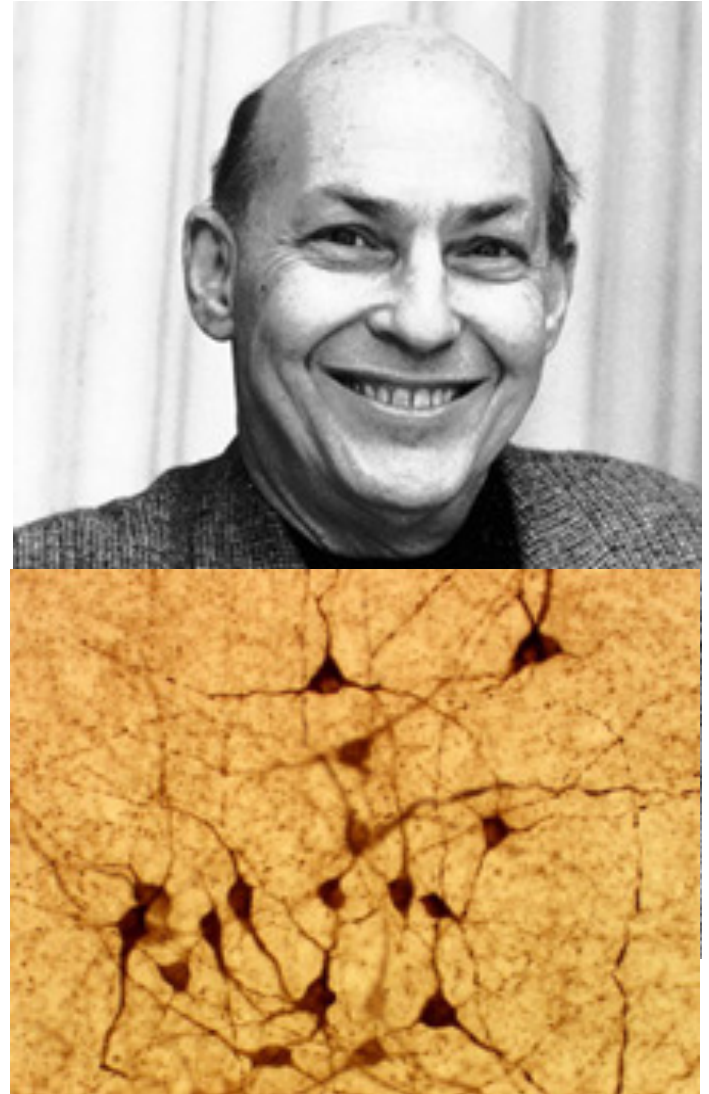


Vert : GFP
Rouge : Mitochondrie

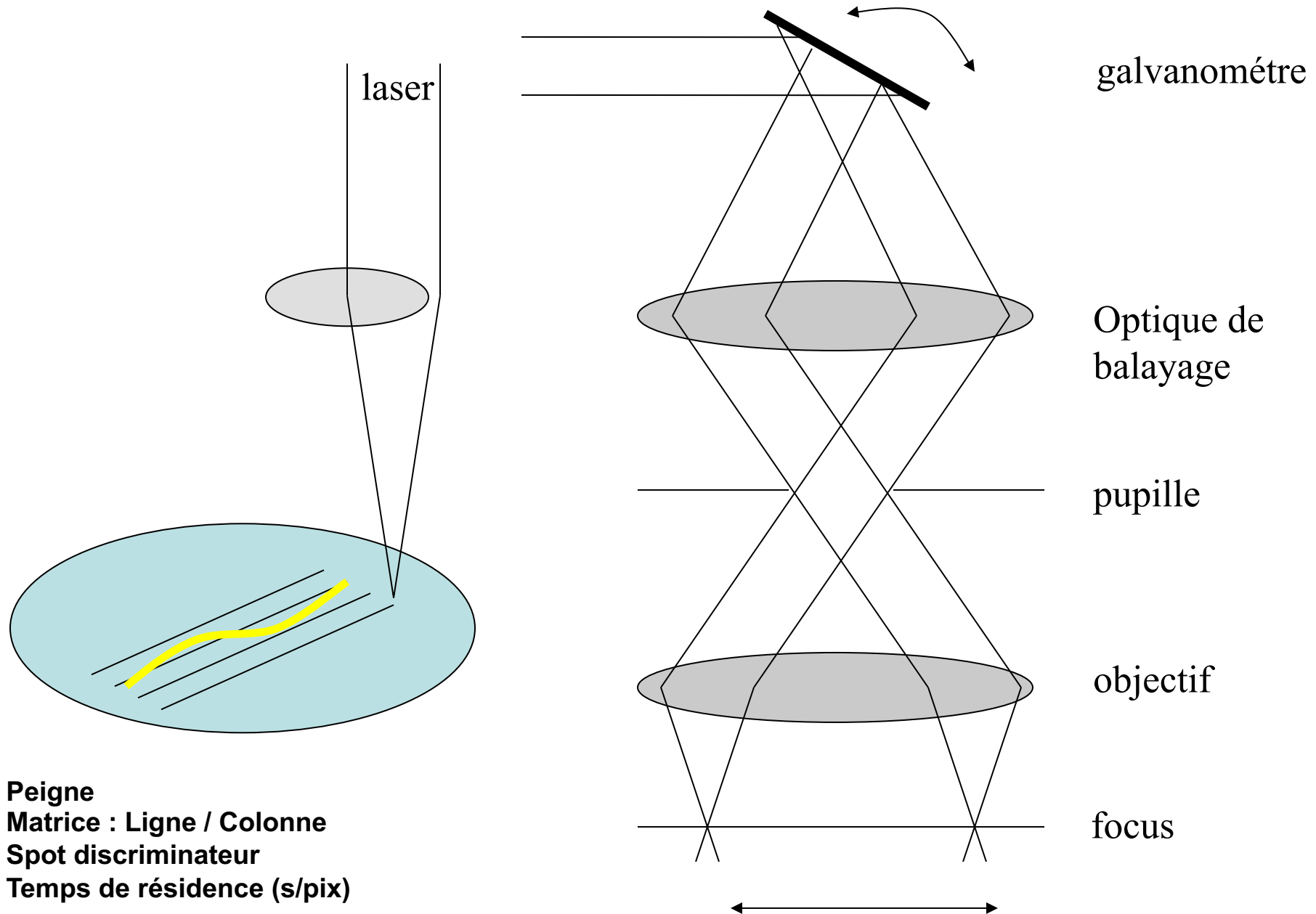


Marvin Minsky (1957)

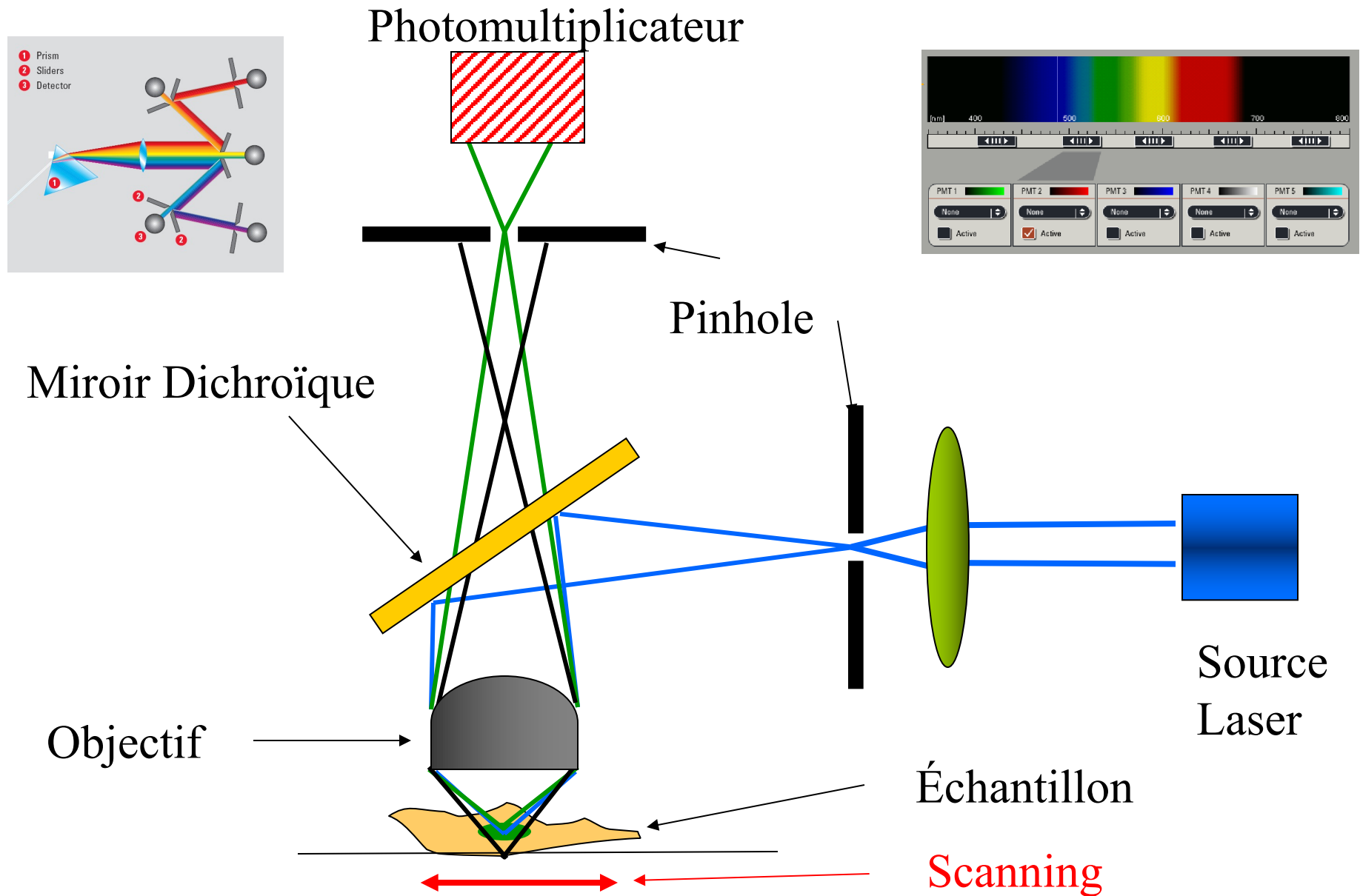
Informaticien et Roboticien américain
En 1958, il fonde le Laboratoire
d'intelligence artificielle au
Massachusetts Institute of Technology. Il
est le créateur de l'intelligence artificielle
et père de la révolution informatique et
du confocal...



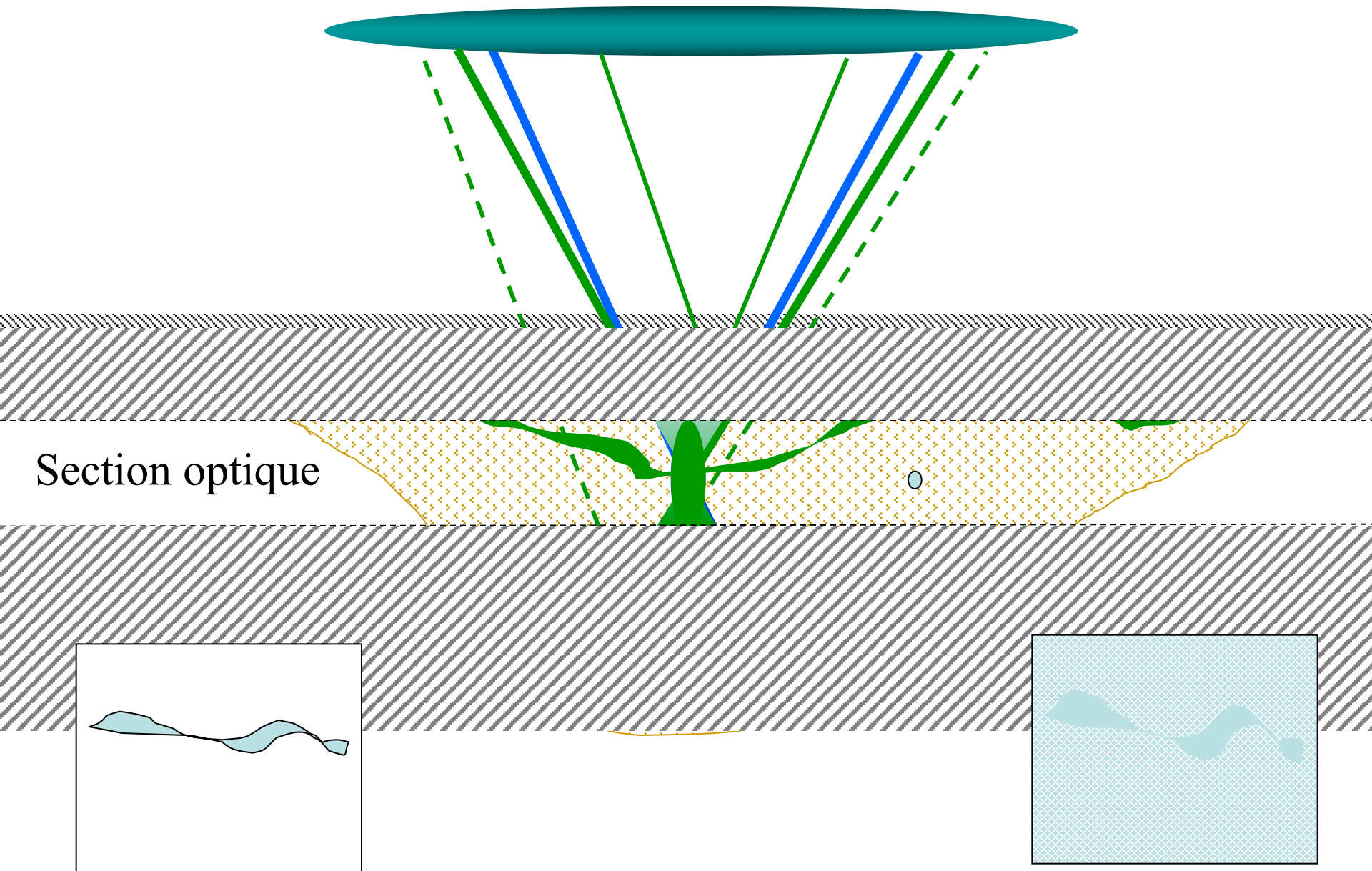
II. Le principe de confocalité : le Confocal



II. Microscope confocal : le diaphragme pour rejeter la lumière parasite



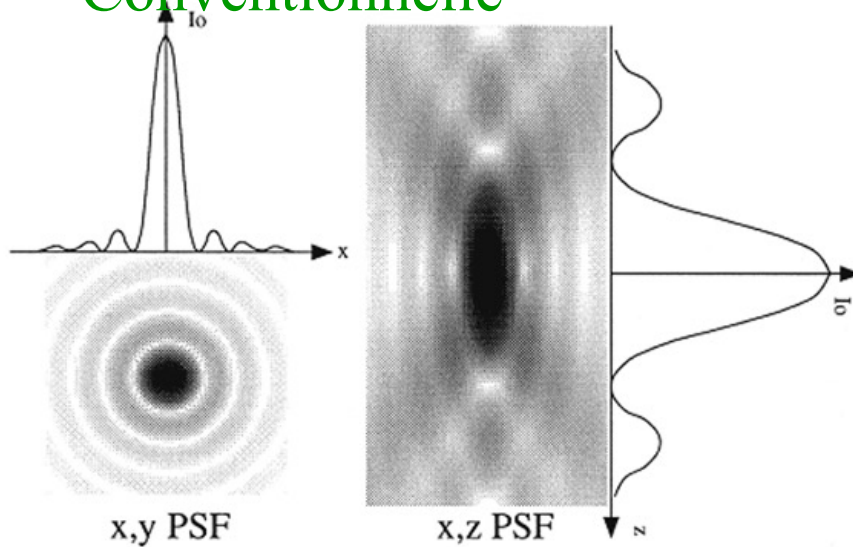
II. Le plan de focalisation et la section optique en mode Confocal



II. Diffraction et résolution en mode confocal

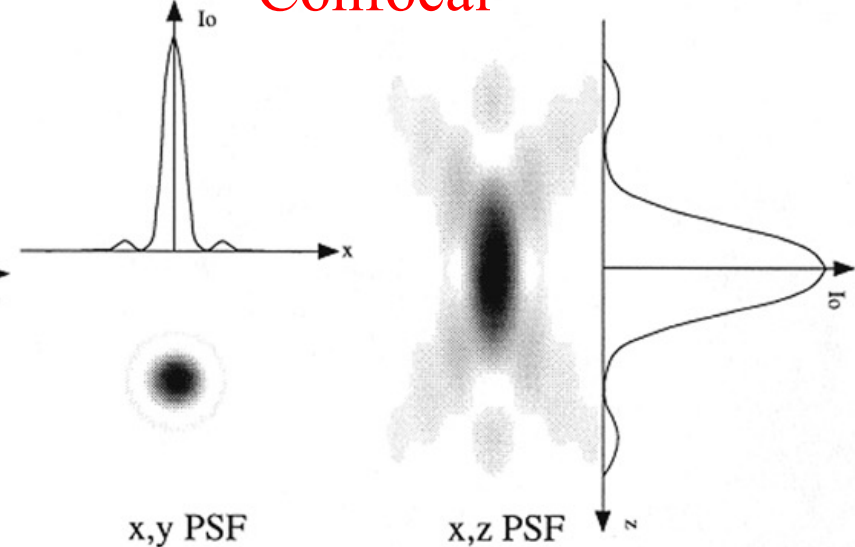
$R_{x,y} = 400 \text{ nm}$

Conventionnelle



$R_{x,y} = 250 \text{ nm}$

Confocal



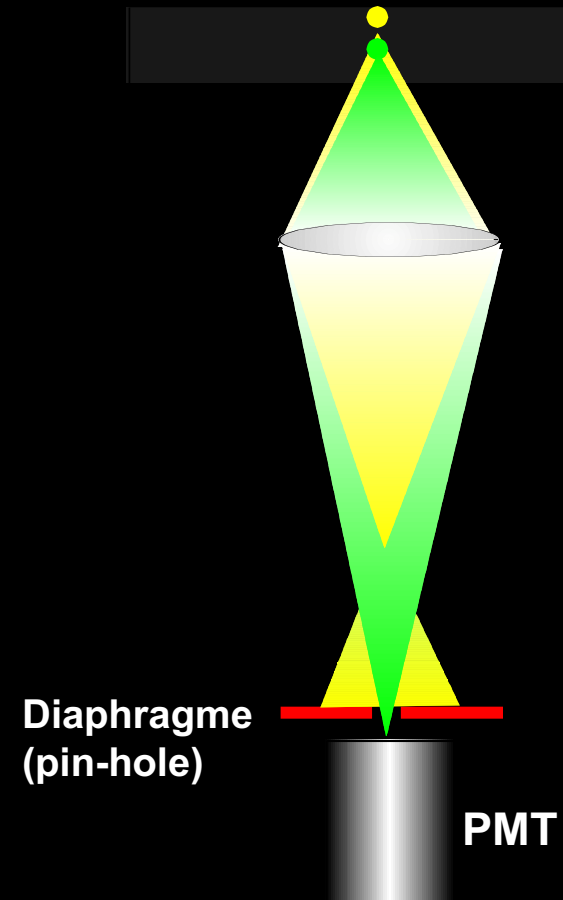
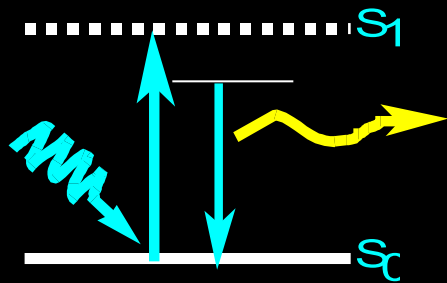
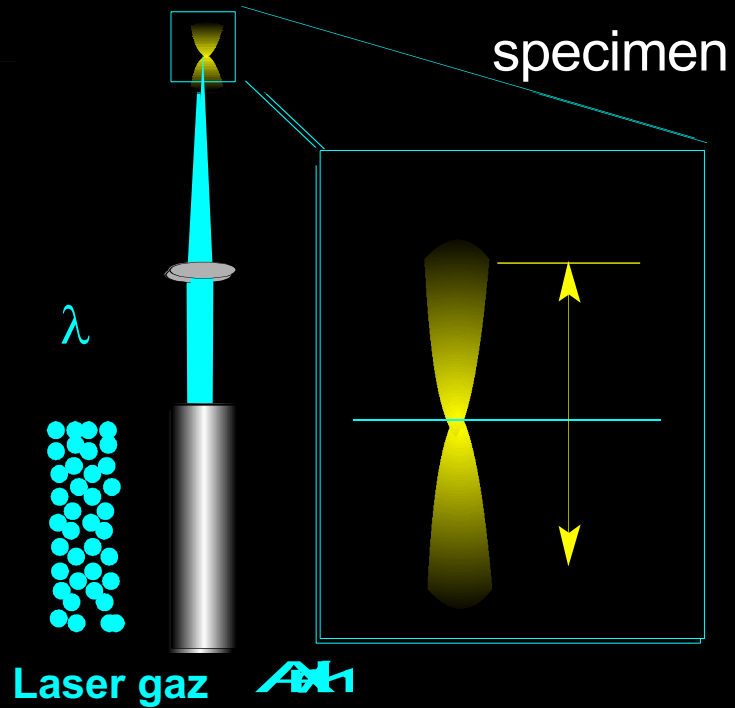
$$d_{xy} = 0,61 \cdot \lambda_{em} / (n \sin \alpha)$$

$$d_{xz} = 2 \cdot \lambda_{em} / (n \sin \alpha)^2$$

$$d_{xy} = 0,4 \cdot \lambda_{em} / (n \sin \alpha)$$

$$d_{xz} = 1,4 \cdot \lambda_{em} / (n \sin \alpha)^2$$

II. Microscopie Confocale à balayage Laser

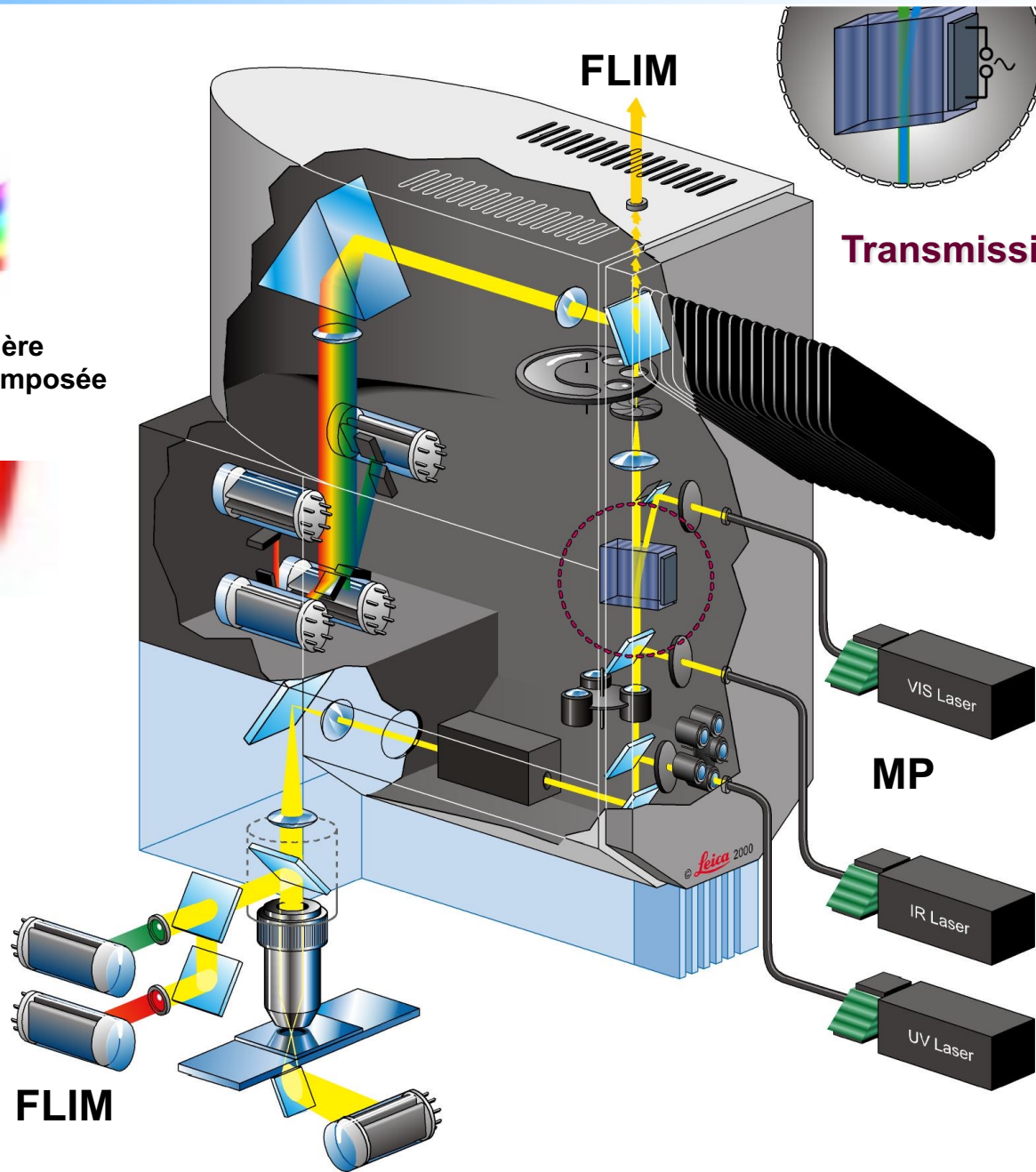
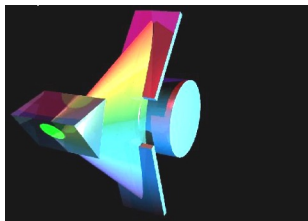


II. Microscopie Confocale à balayage Laser

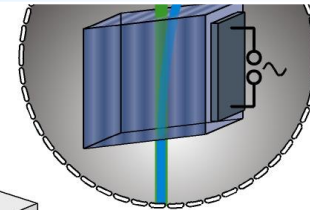
Prisme



Accordabilité :nm



FLIM



AOBS

Transmission : 98 %

MP

VIS Laser

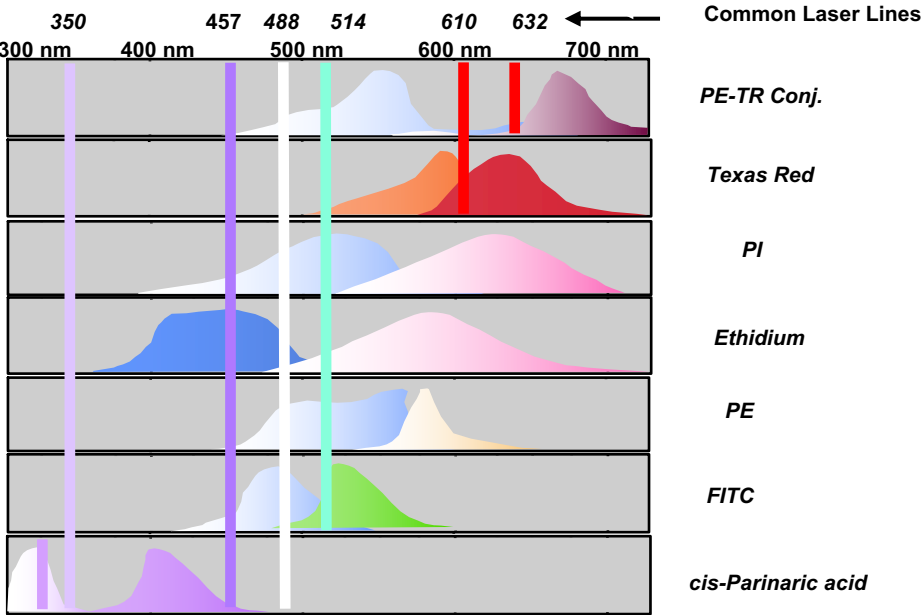
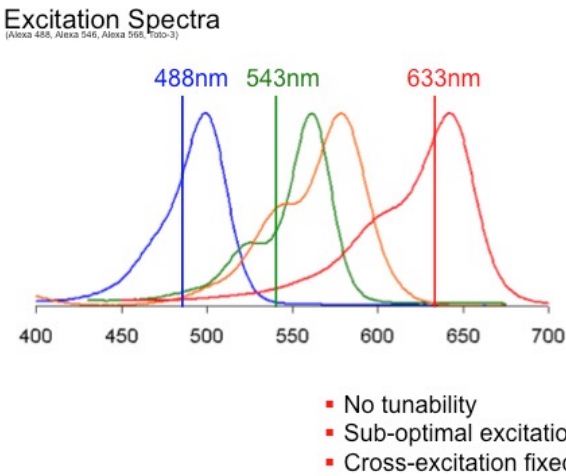
IR Laser

UV Laser

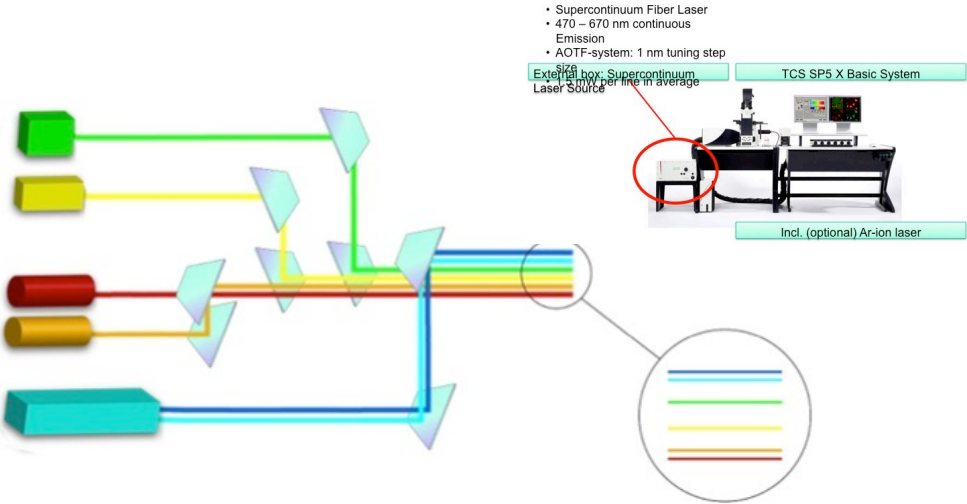
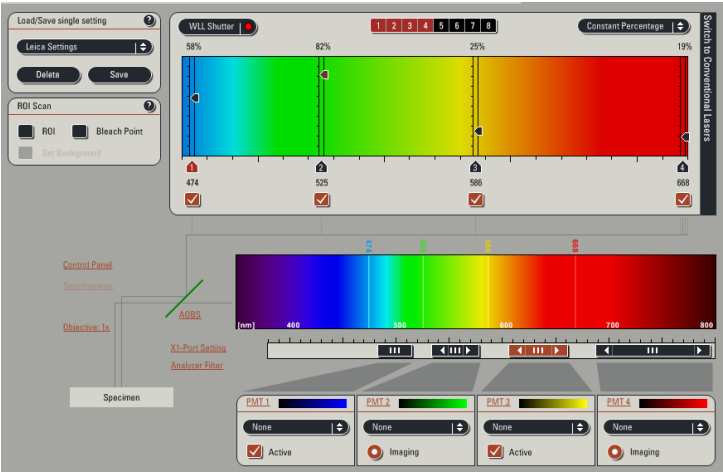
FLIM

II. Microscopie Confocale à balayage : type d'illumination Laser

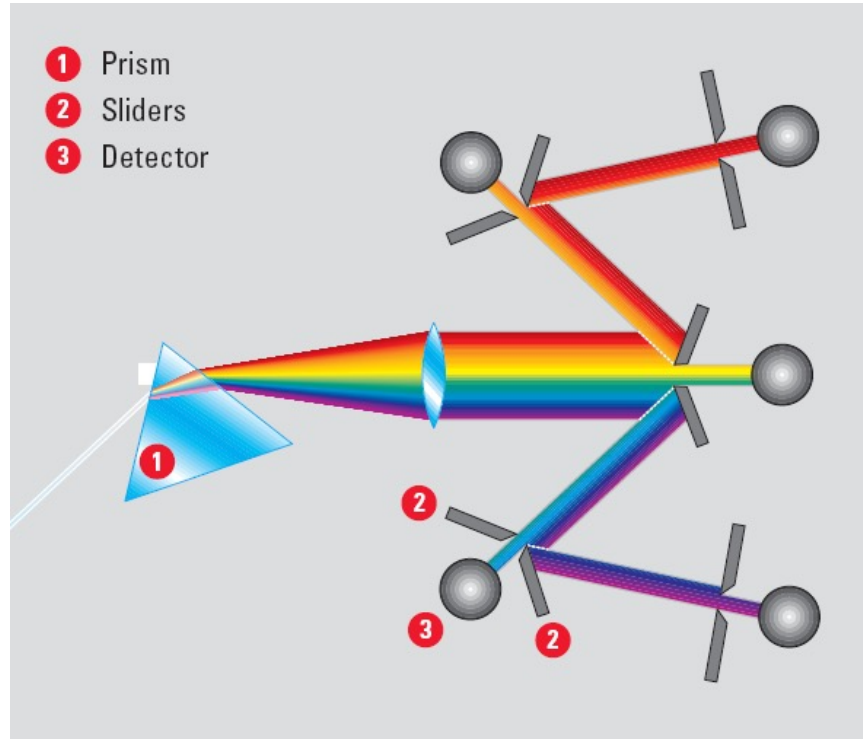
Lasers solides continus conjugués



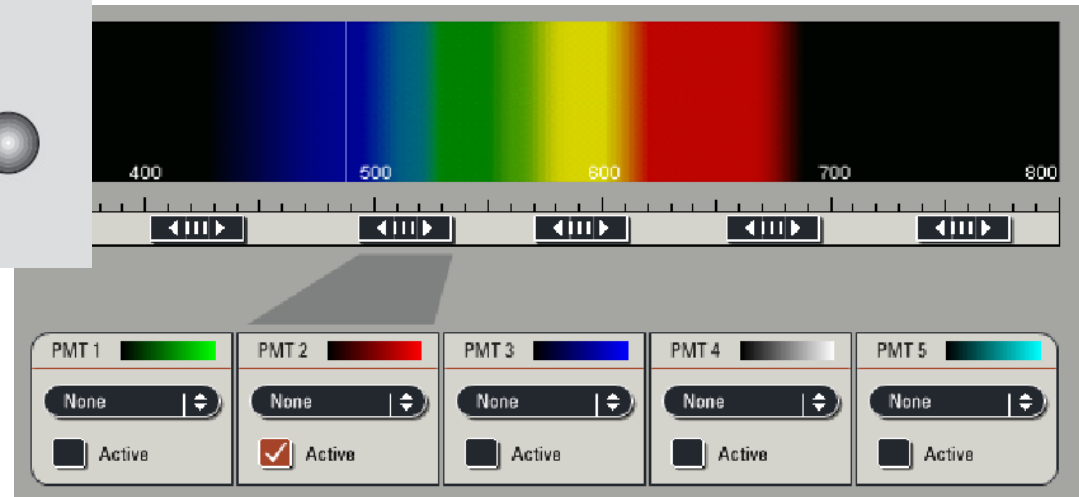
Lasers Blancs pulsés (470-670nm) :



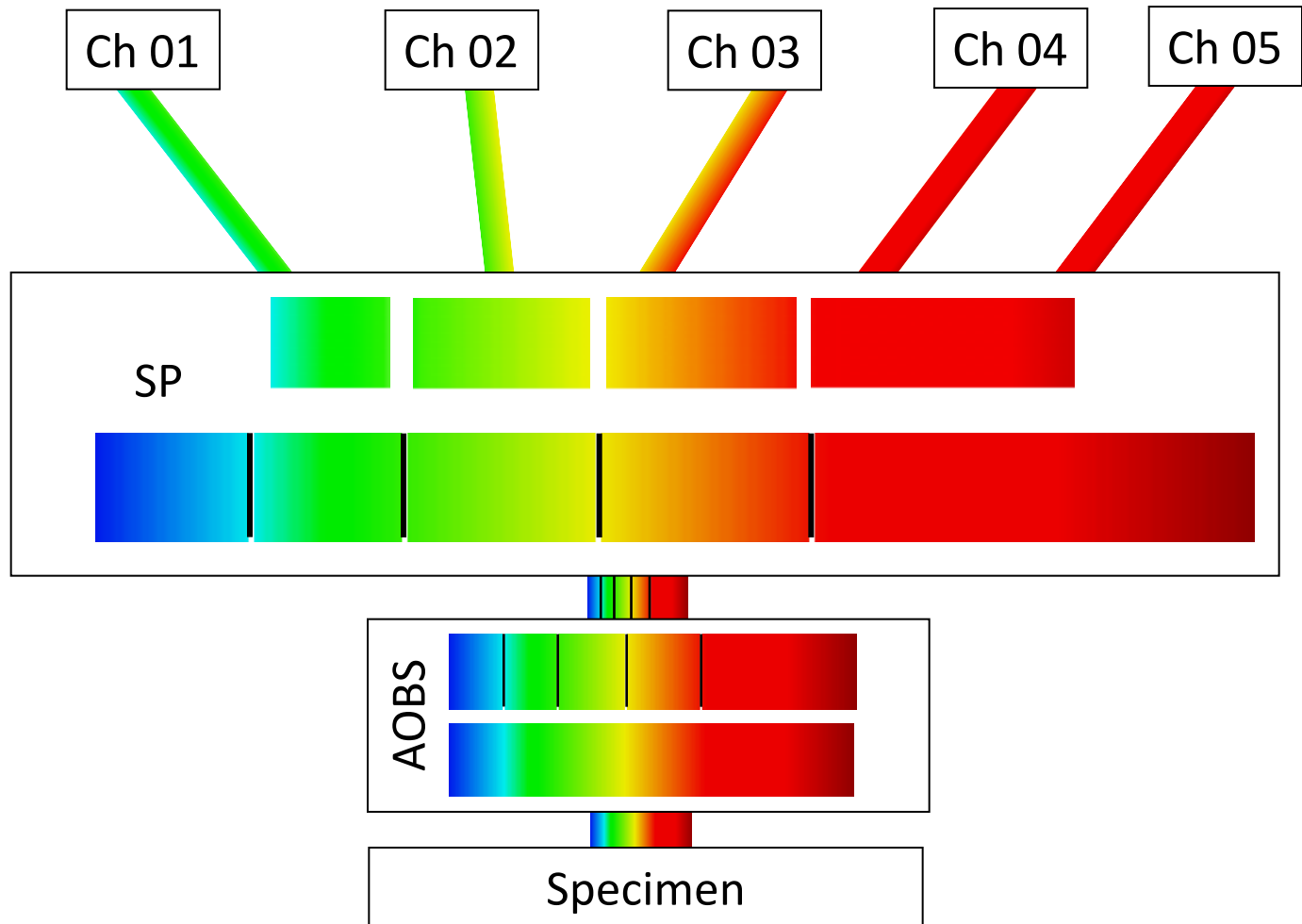
II. Microscopie Confocale à balayage Laser



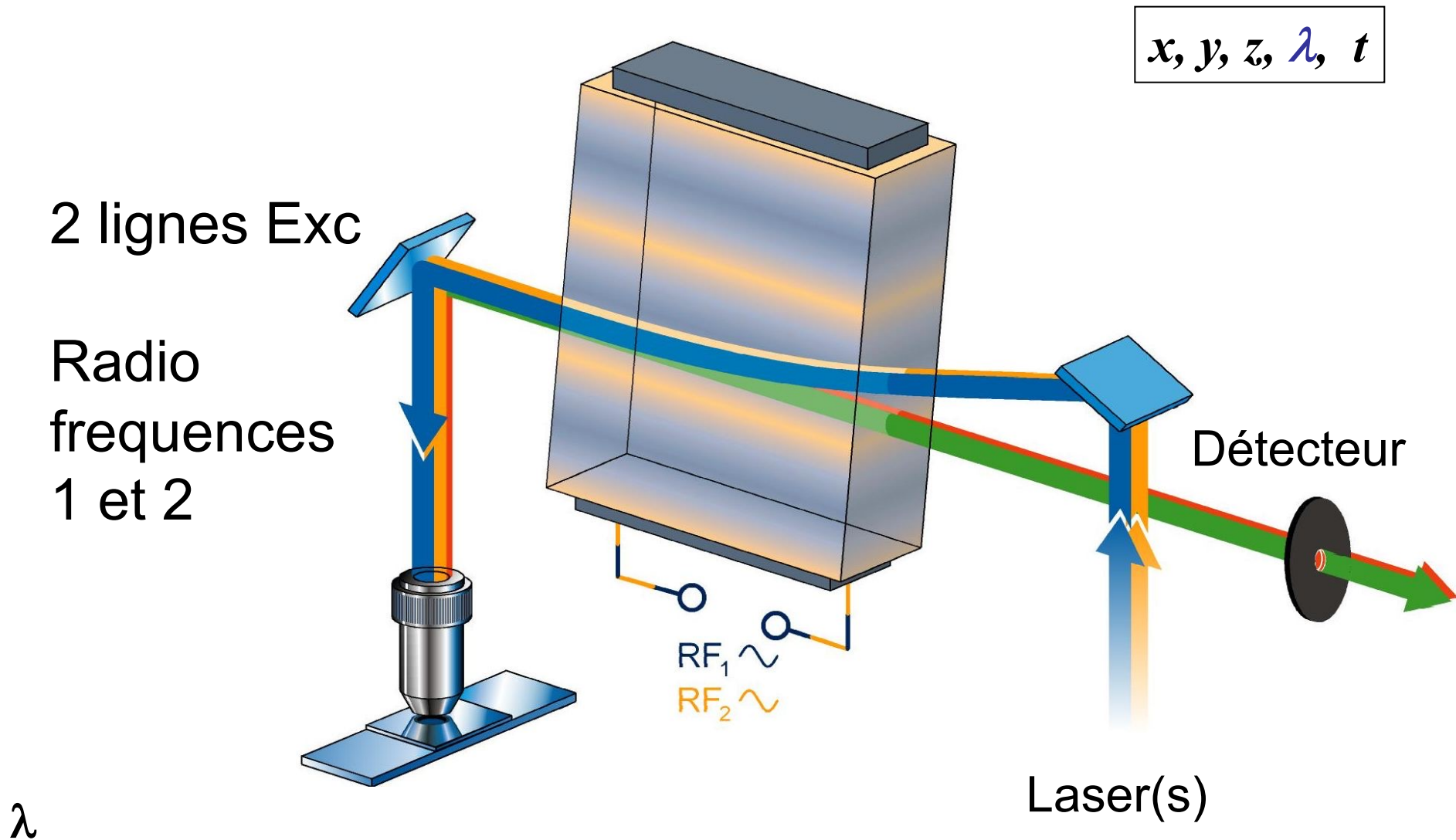
SP-Detector: Leica 1997



II. Microscopie Confocale à balayage Laser: séparation spectrale



II. Microscopie Confocale à balayage Laser: séparation spectrale



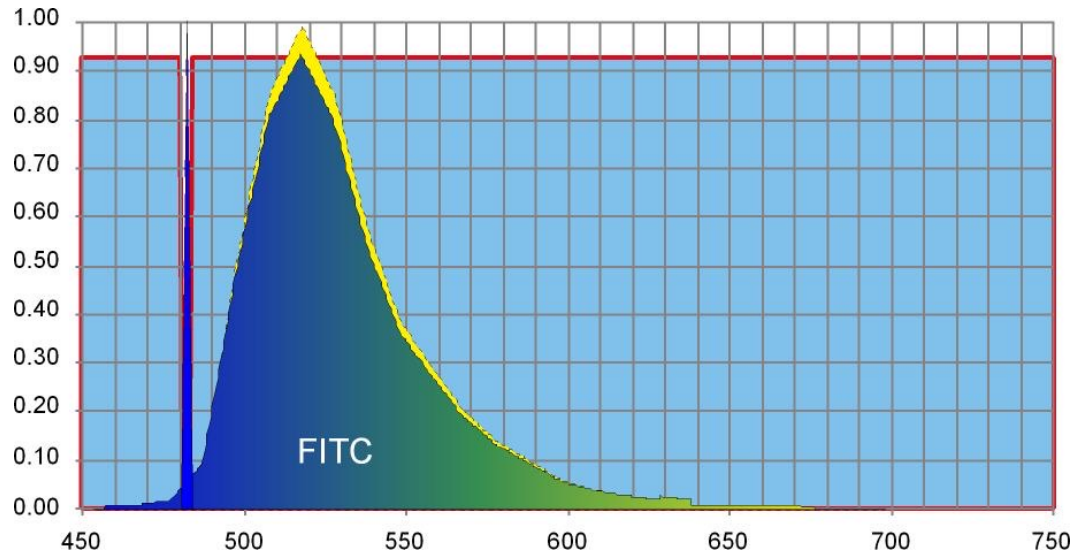
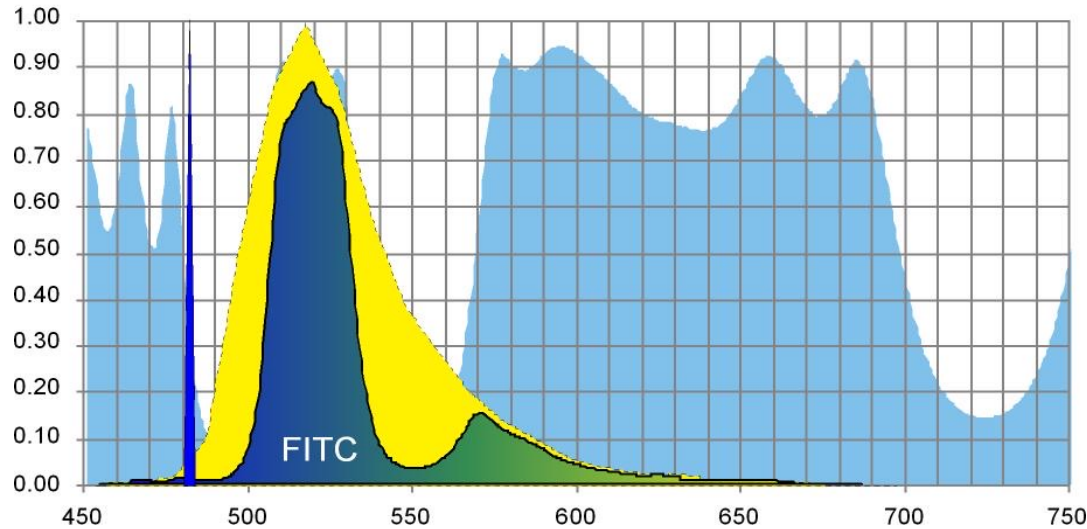
II. Microscopie Confocale à balayage Laser: séparation spectrale

Double Dichroic 488/543
(measured curve)

Jaune= perte de fluorescence

FITC

Beam splitter



AOBS

➤ + 30%

II. Multimarquage et illumination Laser

Image MultiCouleur (3)

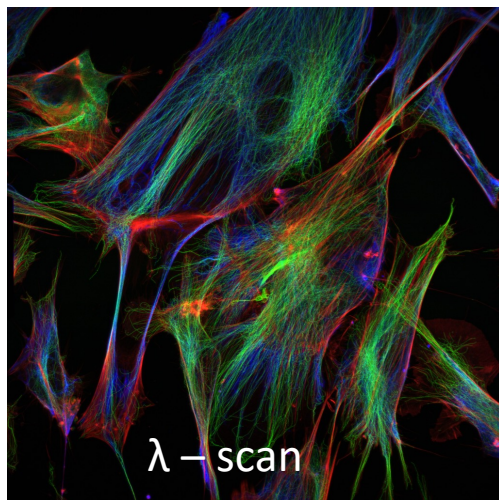


Image Spectrale

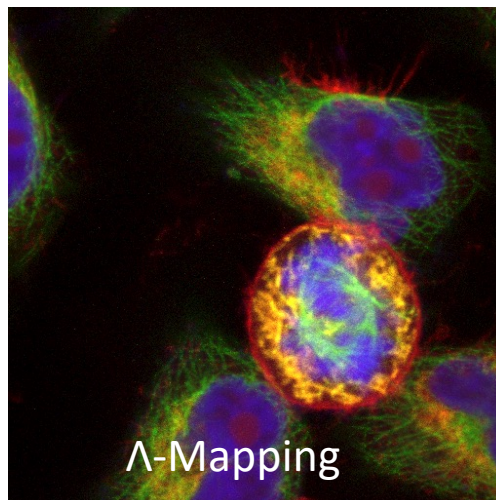
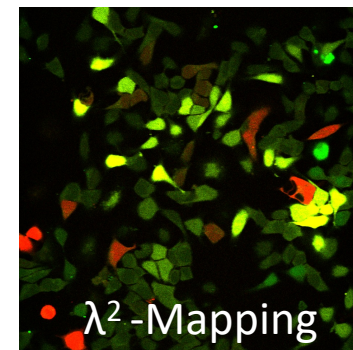
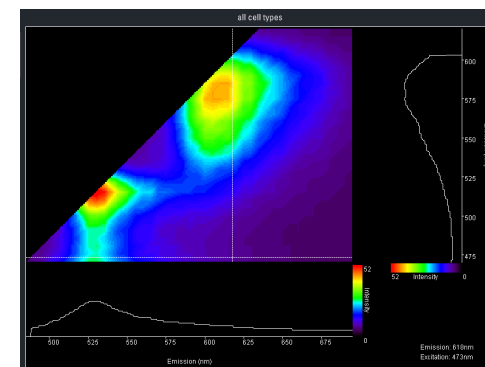
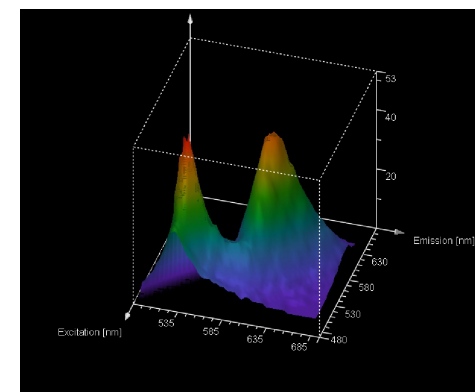
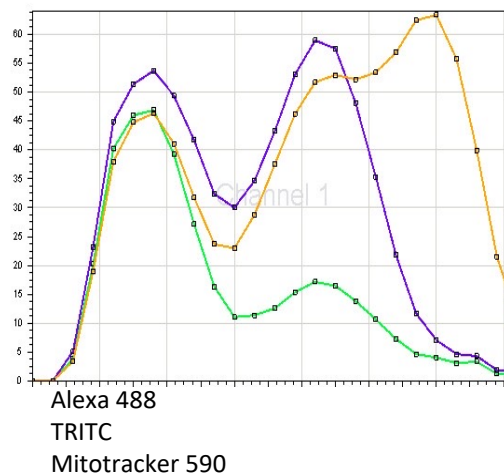


Image MultiSpectrale

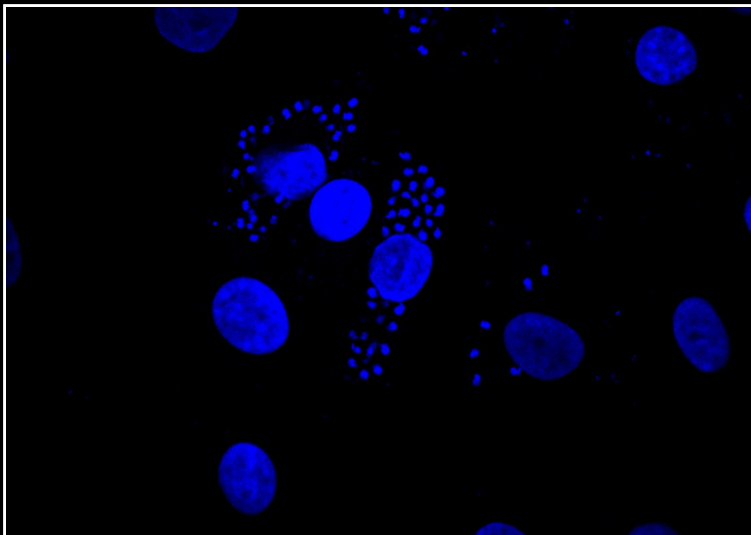
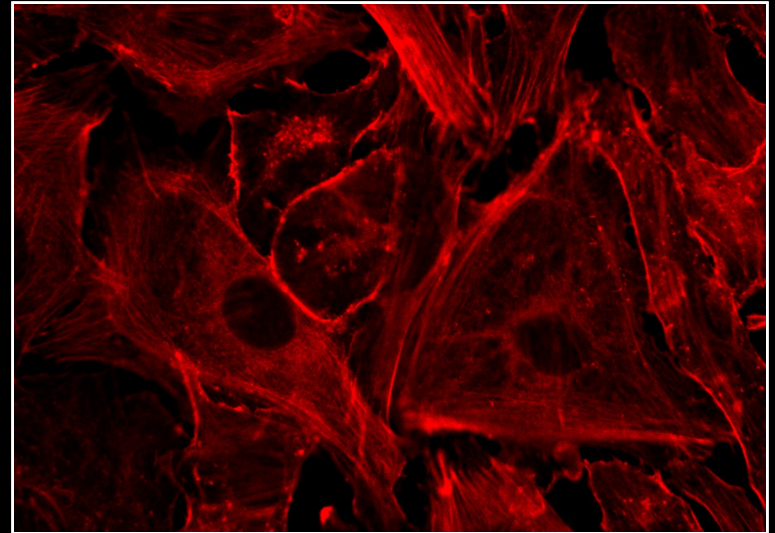
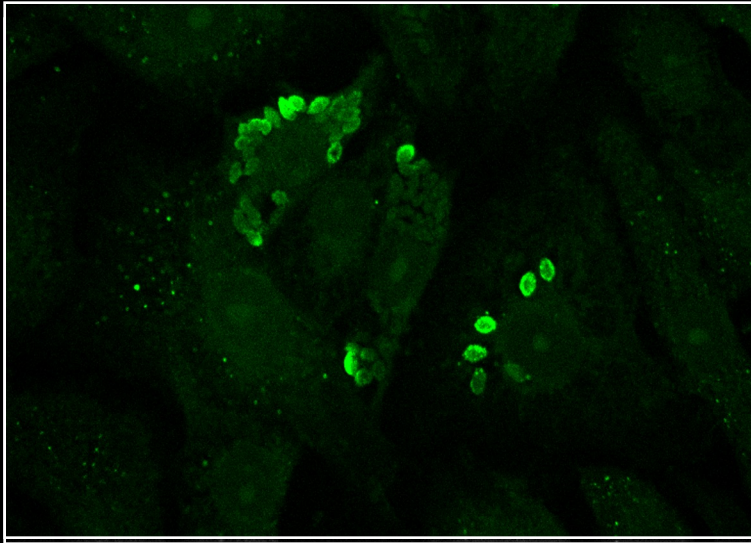


Combine 405 & WLL: HeLa cells, quadruple staining

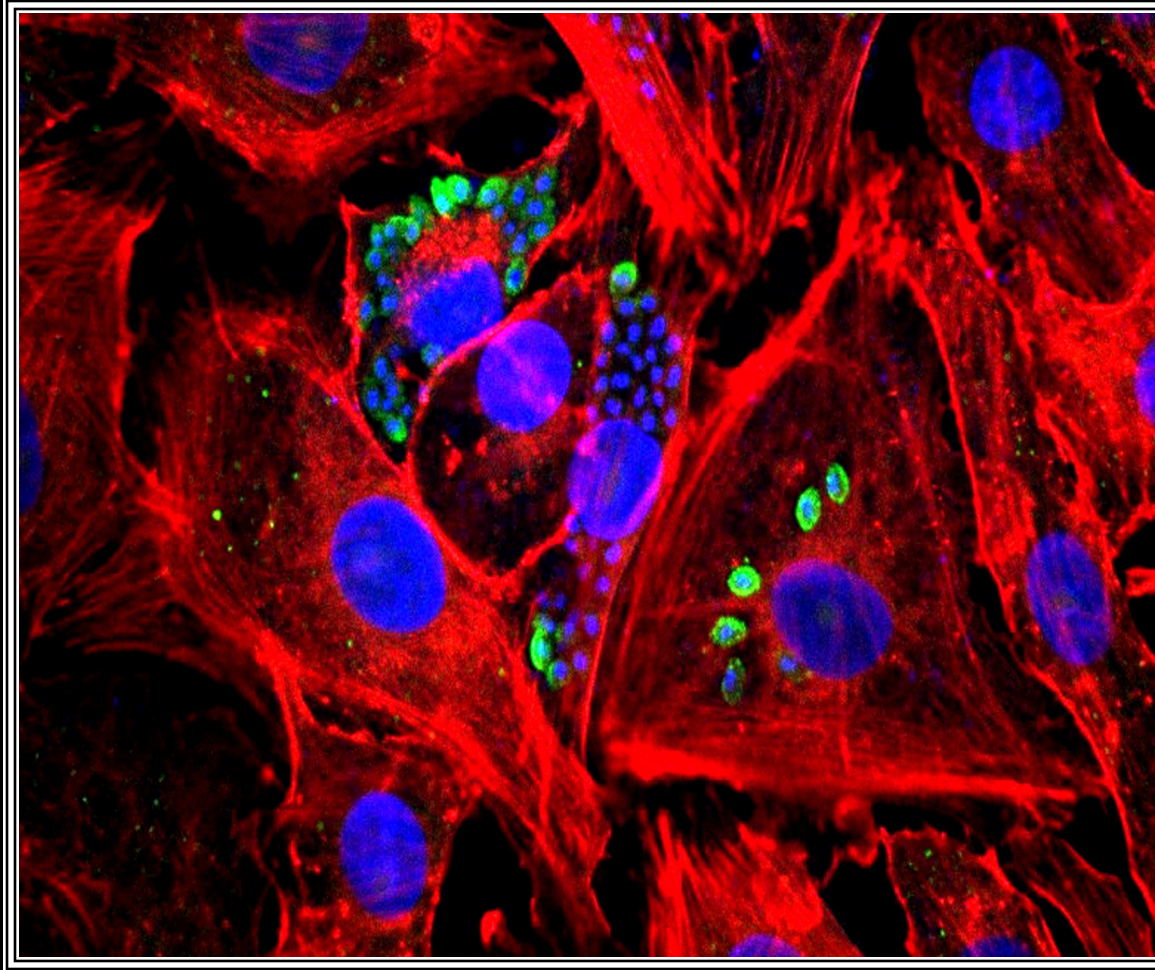
	Excitation [nm]	Emission [nm]
Nuclei (DAPI)	405	410-450
Microtubules (Alexa 488)	490	496-540
F-Actin (TRITC)	548	555-590
Mitochondria (Mitotracker 590)	600	608-800



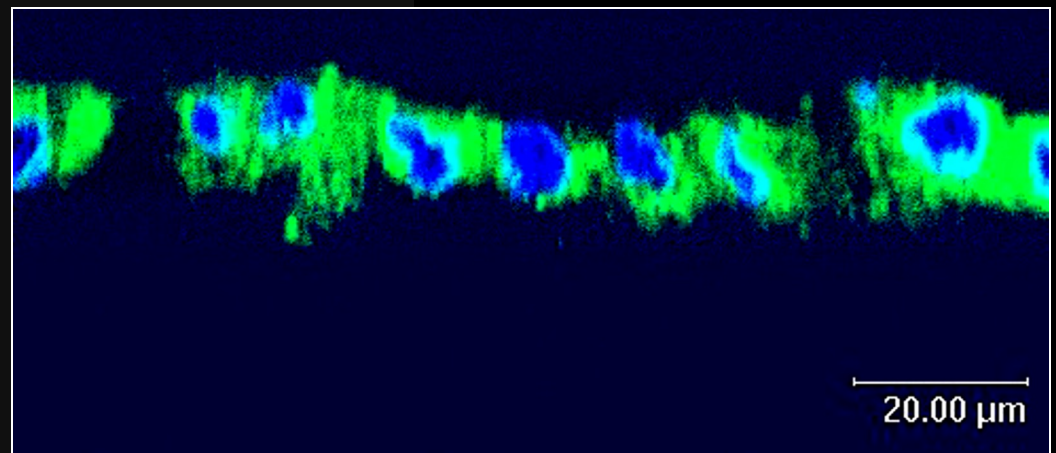
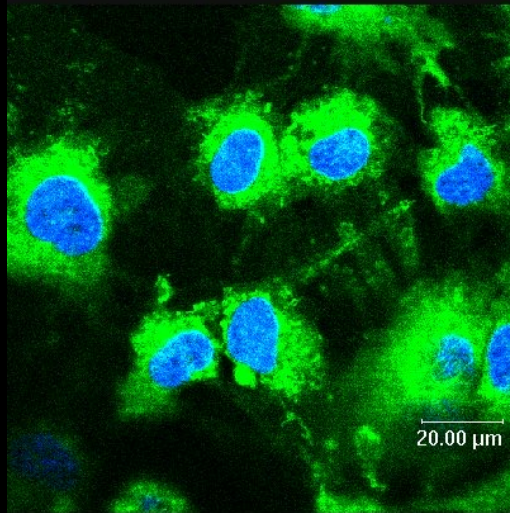
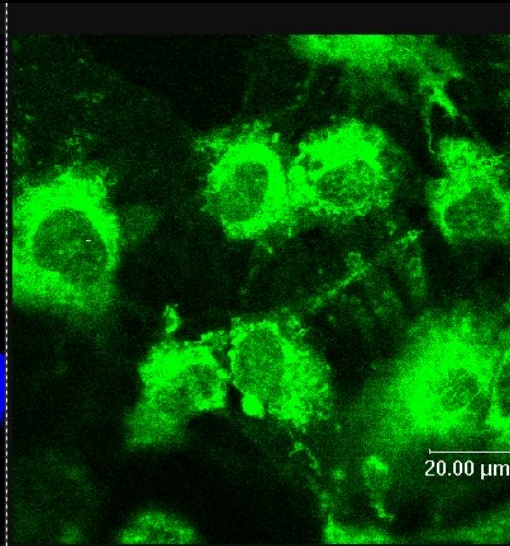
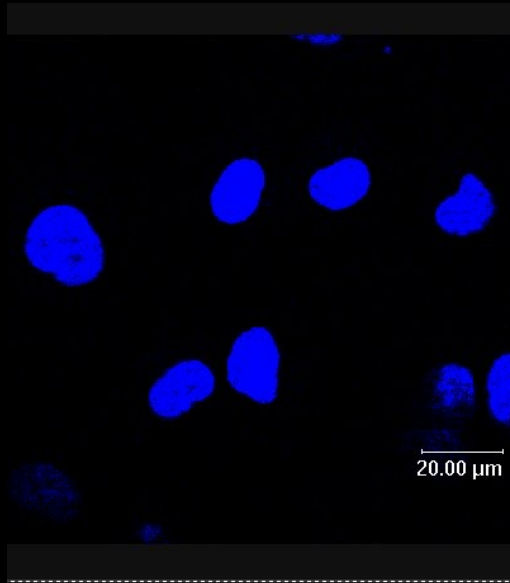
II. Microscope Confocal : Multi-voies



II. Microscope Confocal : Multi-voies

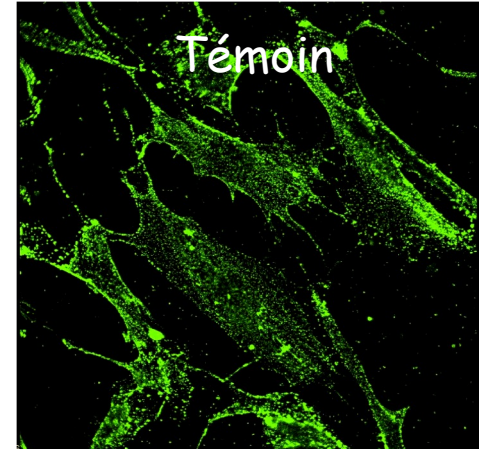
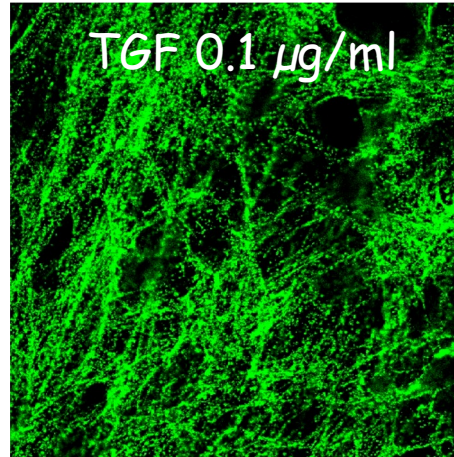
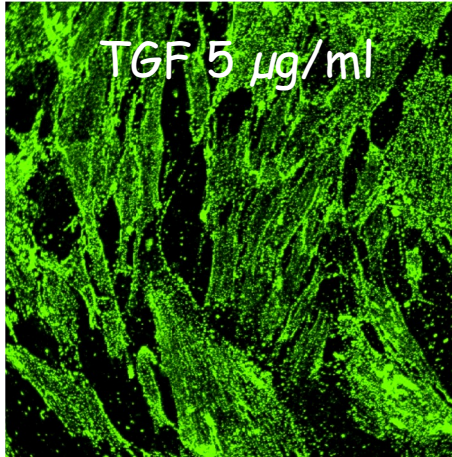


II. Microscope Confocal : Multi-modes (x, y, z,t, ...)

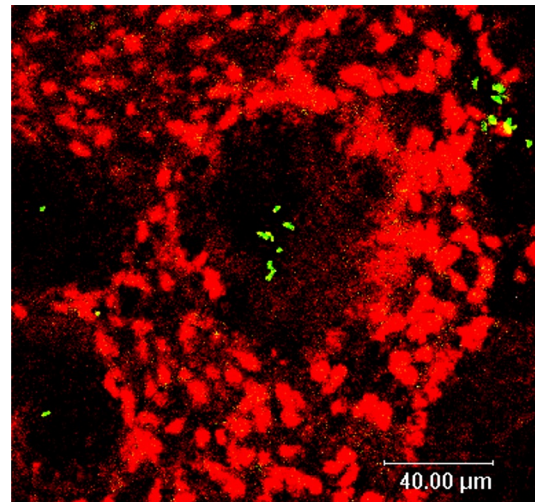
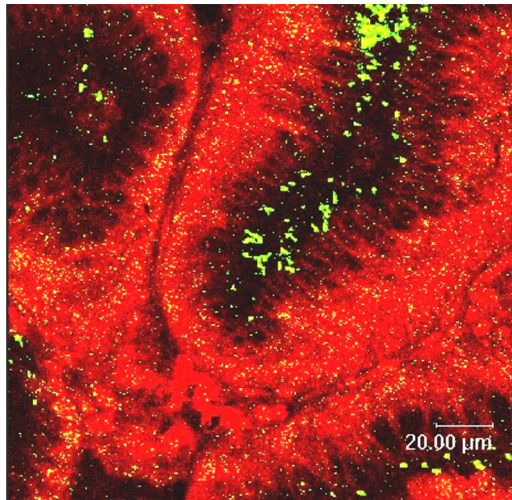


II. Microscope confocal / Multi-applications

Biomatériaux sur Films PLGA - Réseau Collagène Type 3
Fibroblaste (tendons)



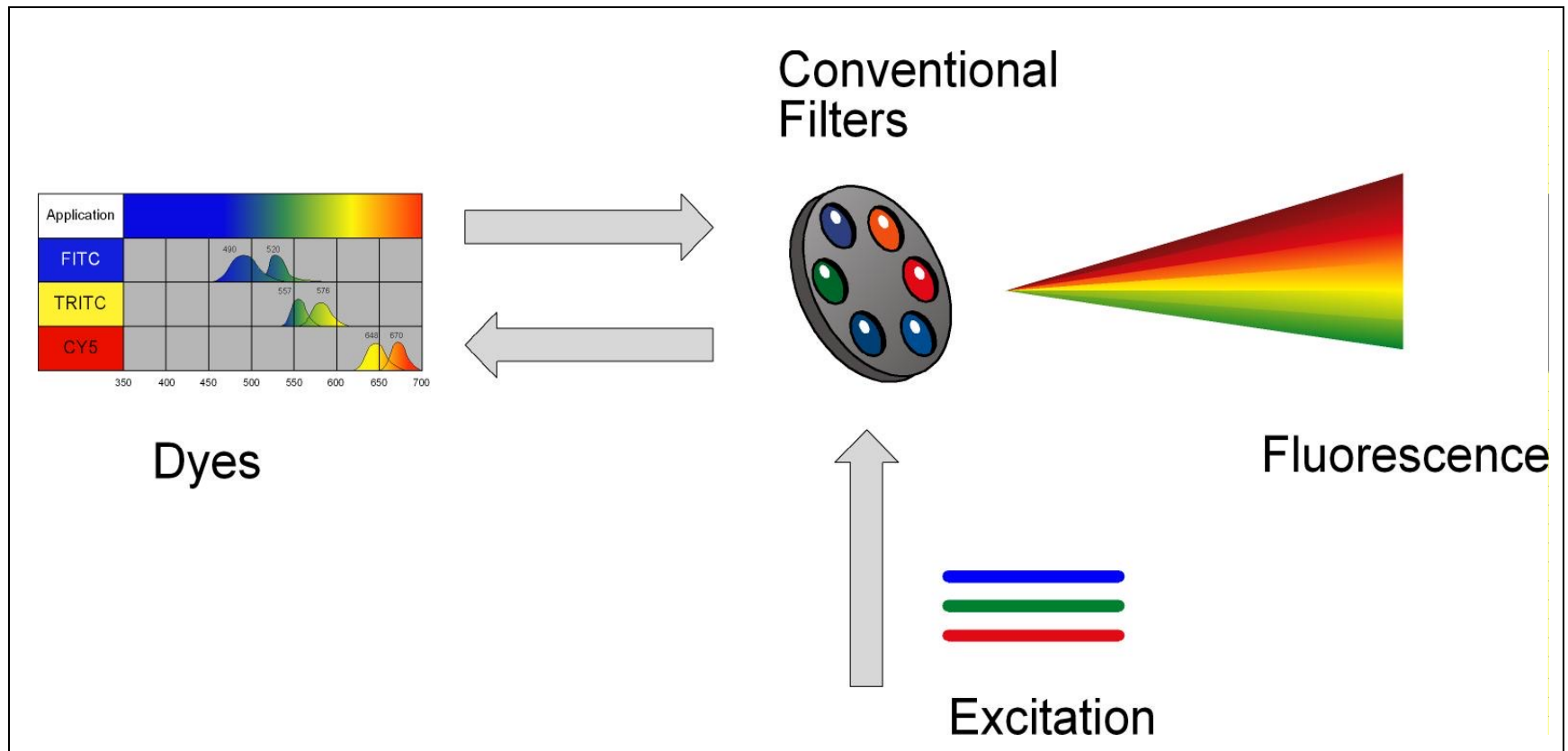
Helicobacter Pylori - Coupe de poumon Humain



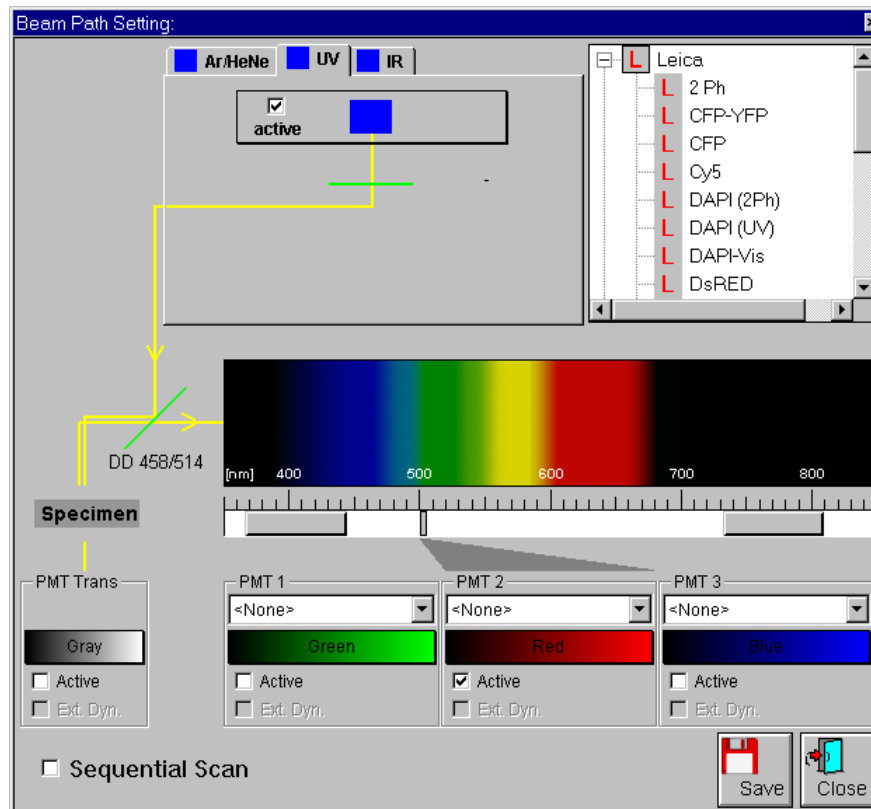
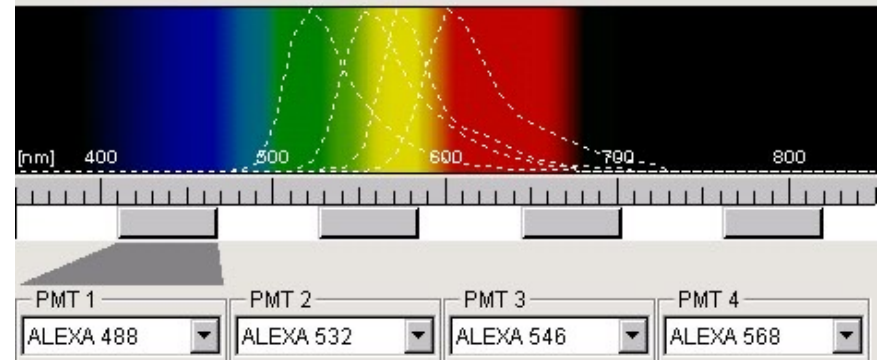
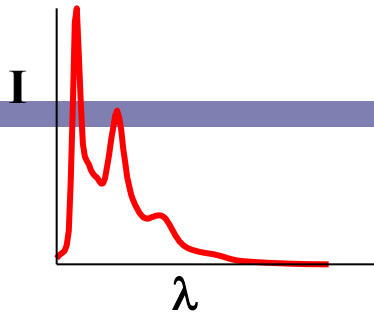
II. Microscopie Confocale à balayage Laser: séparation spectrale

Filtres et miroirs dichroïques :

- sépare l'excitation de l'émission
- fixe la bande passante (λ)



II. Microscopie Confocale à balayage Laser et analyse spectrale



Accordabilité : nm



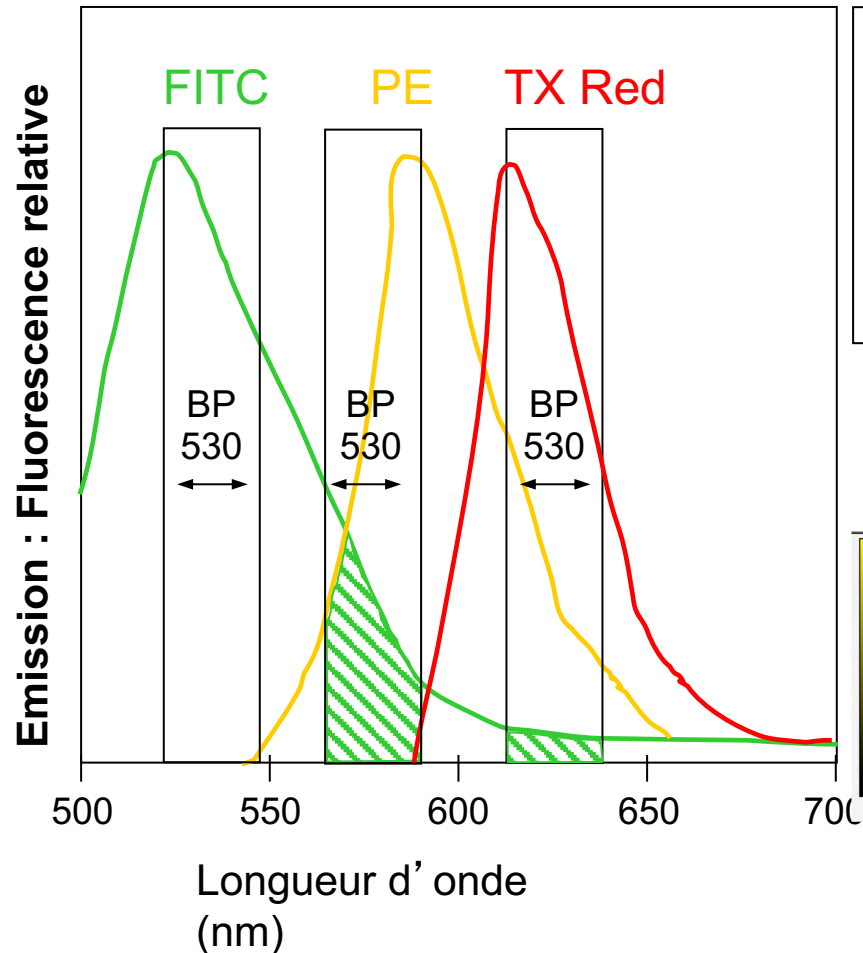
II. Microscope Confocal : contamination spectrale / Multimarquage

Spectres normalisés d'absorbance et d'émission

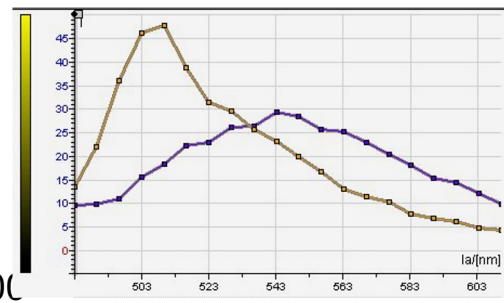
FITC = isothiocyanate de fluorescéine

PE = Phycoérythrine

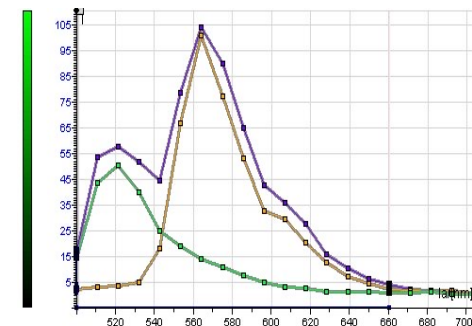
TX Red = Texas Red



- Optimiser la fenêtre spectrale / signature
- Collection du signal (bruit, autofluorescence...)
- Identification dynamique de l'autofluorescence
- Discrimination / multiples marquages
- Caractérisation interactions moléculaires (liée-libre)
- Distance inter-moléculaire (donneur-accepteur)
- Techniques ratiométriques I (FISH, pH, ions, ...)



Autofluorescence /GFP

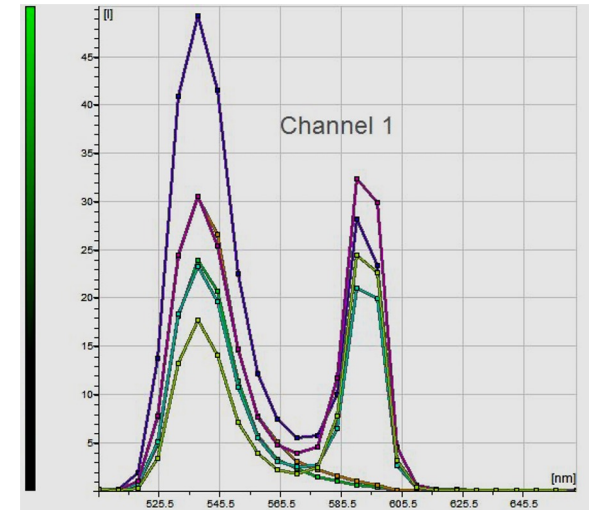
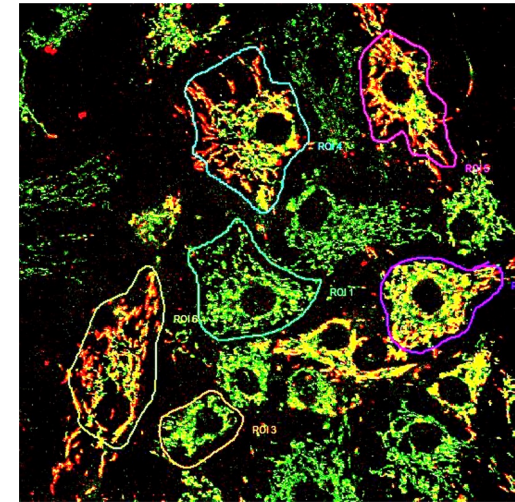
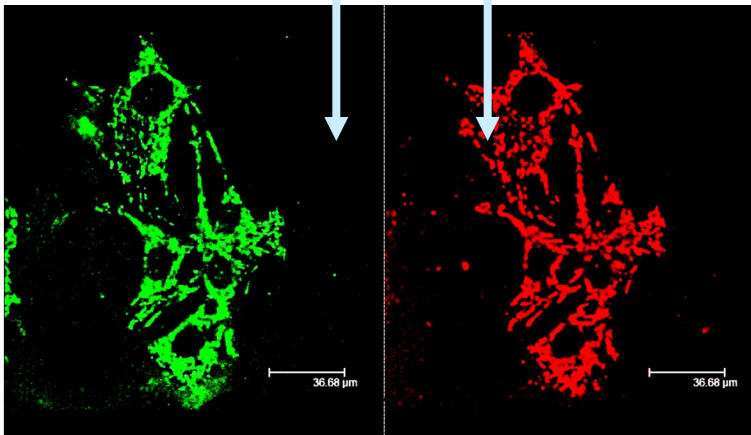
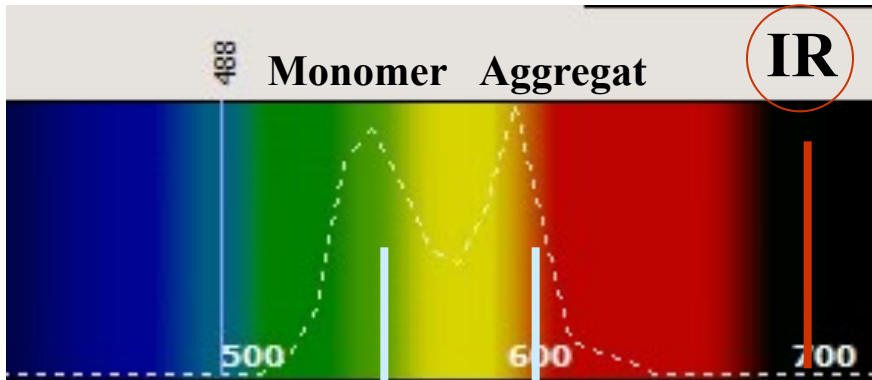


Transfert Energie

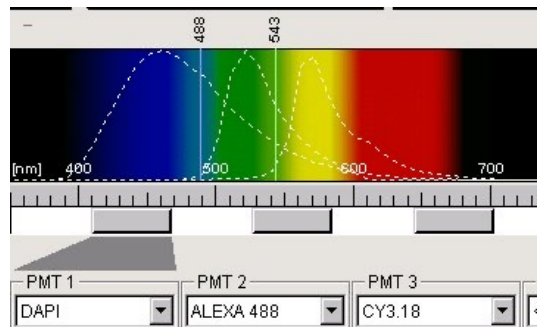
II. Microscope Confocal : mode spectral

JC-1:

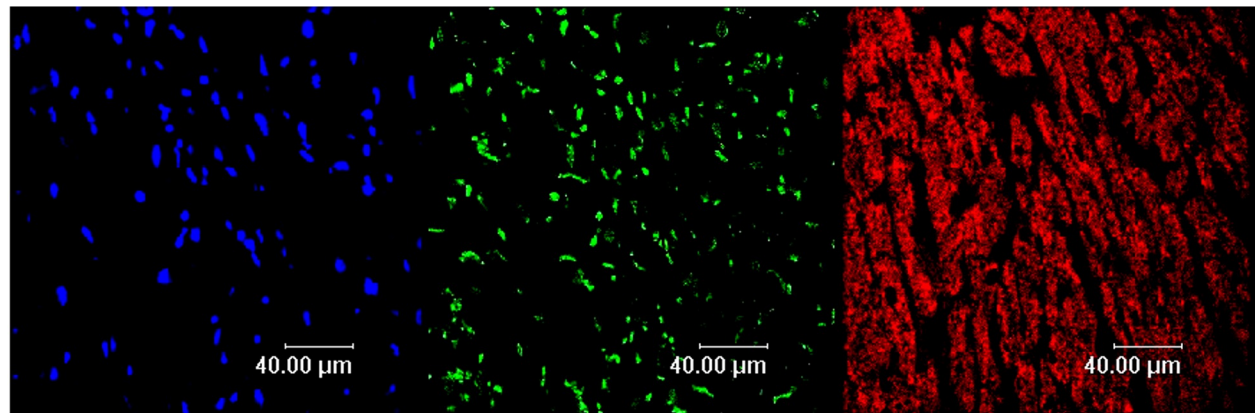
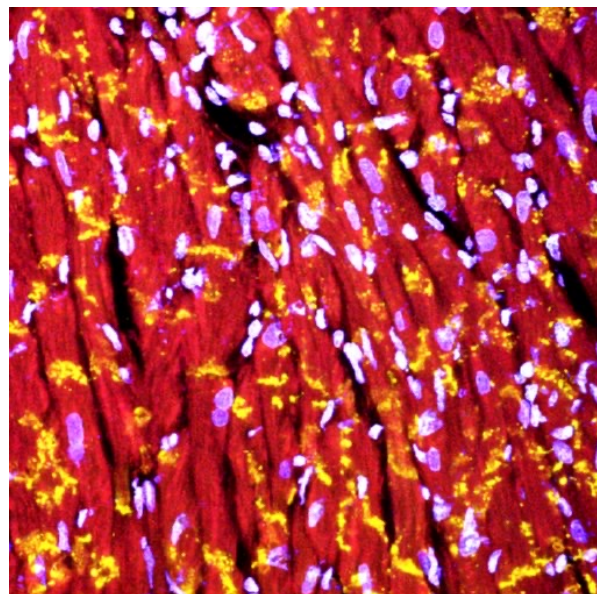
- Lipophilic cationic probe
- Mitochondrial Mb depolarization
- Early Apoptosis



II. Microscope Confocal : déconvolution spectrale



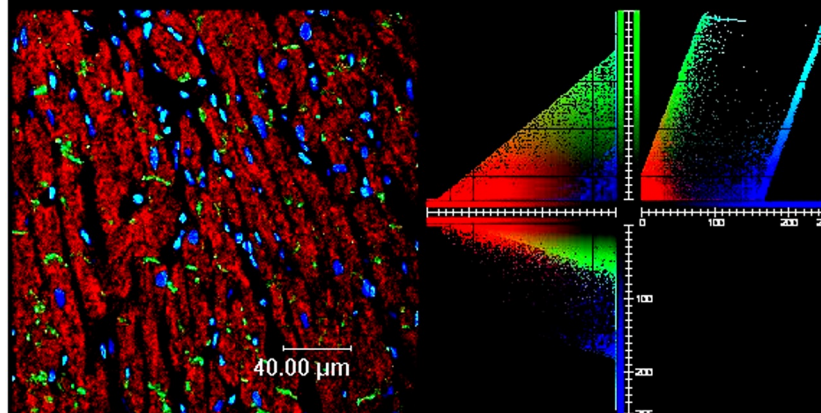
Muscle cardiaque porc
Stack 3D (230 μm)



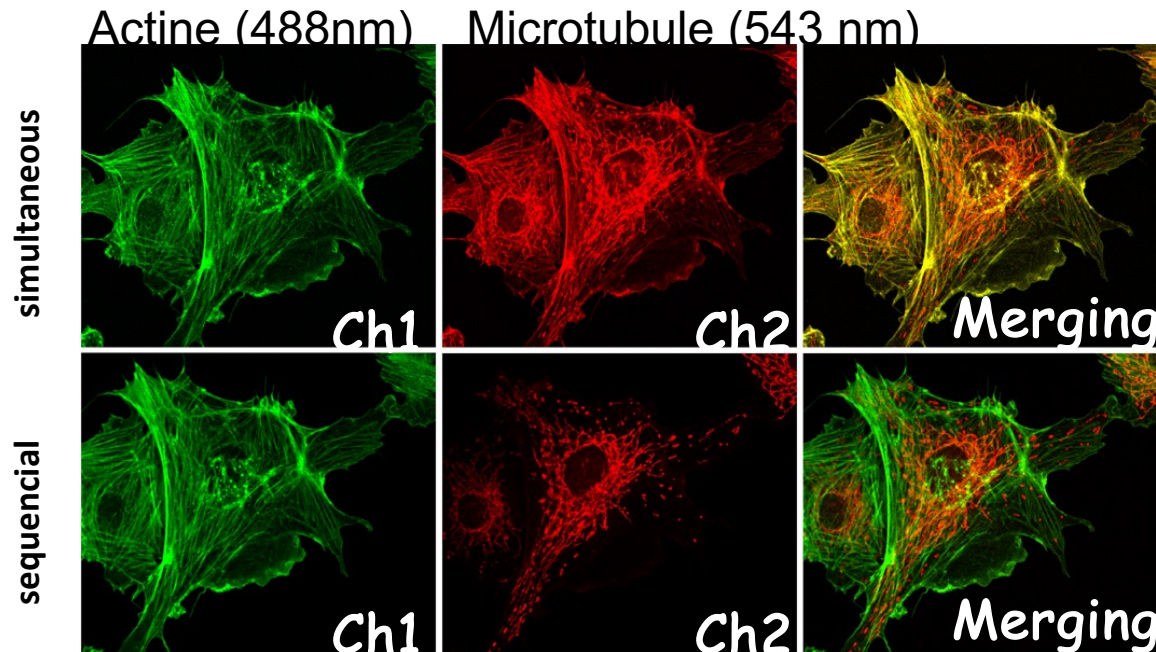
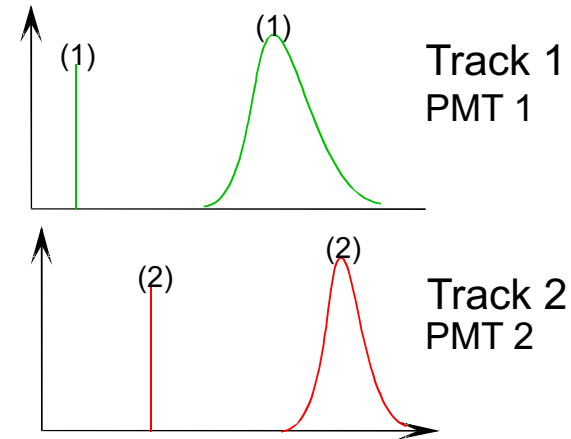
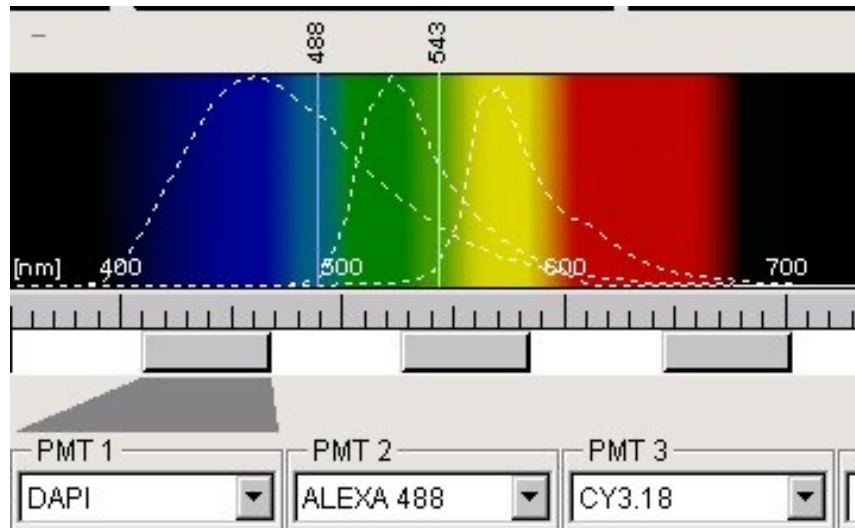
* Déconvolution spectrale

Noyau : Hoecht
Cx43 : Alexa-488
Myosine: Cyanine 3

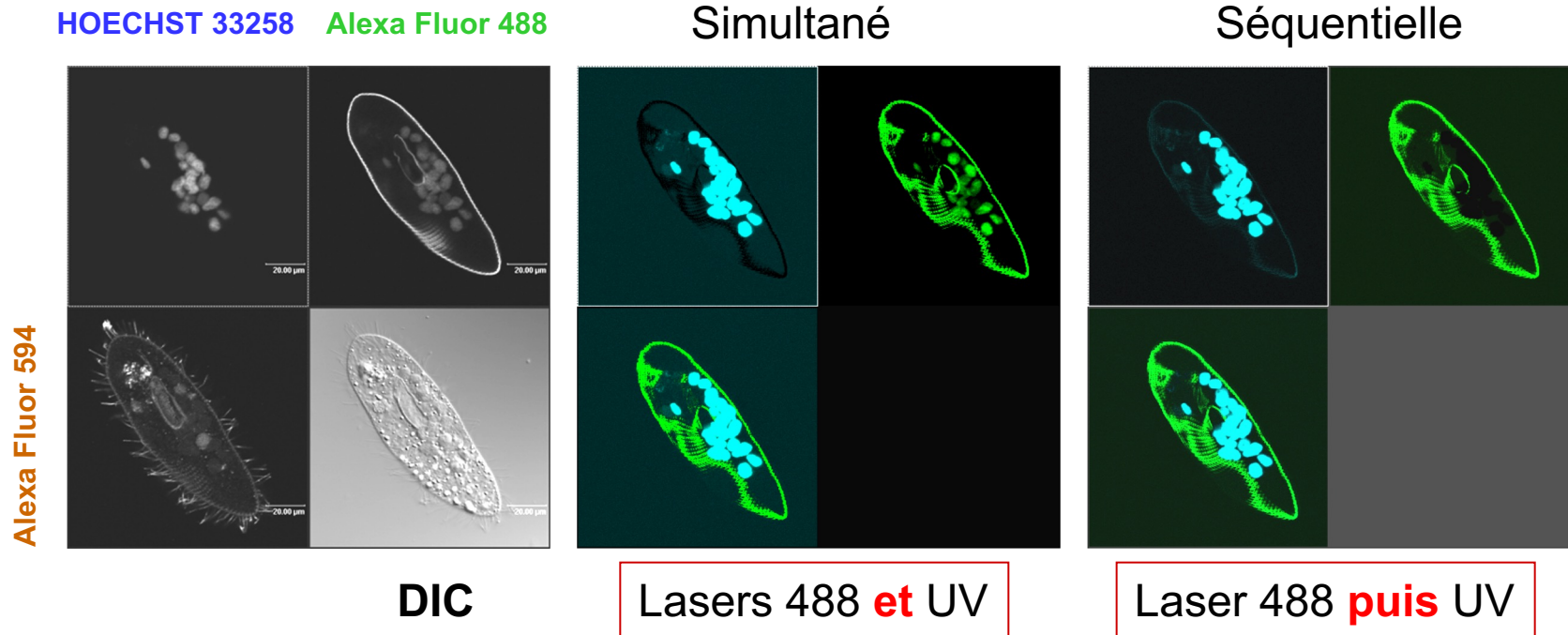
lex : 820 nm



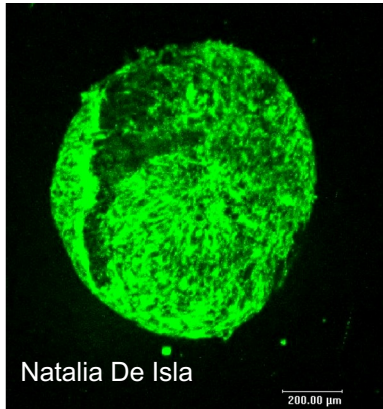
II. Microscope confocal / acquisition séquentielle de la fluorescence



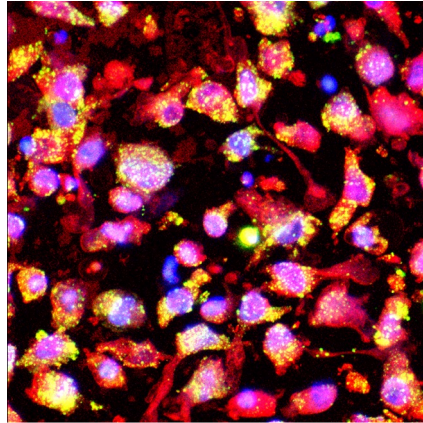
II. Microscope confocal / acquisition séquentielle de la fluorescence



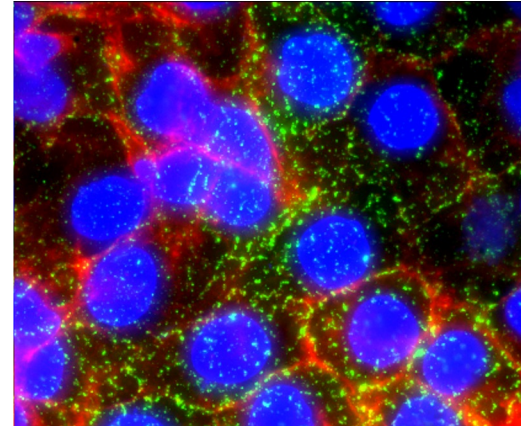
II. Microscope confocal en mode séquentiel / Multi-marquages validés



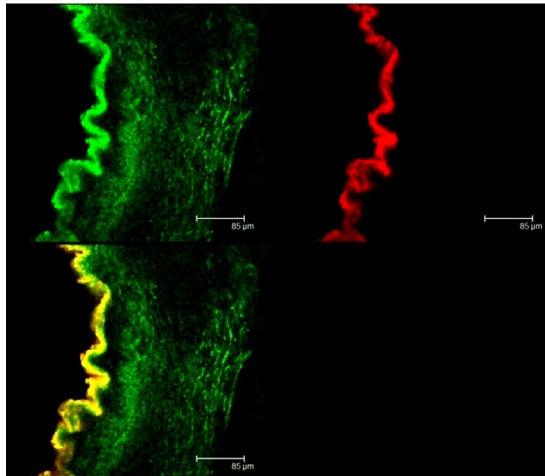
MSC



Live/dead kit

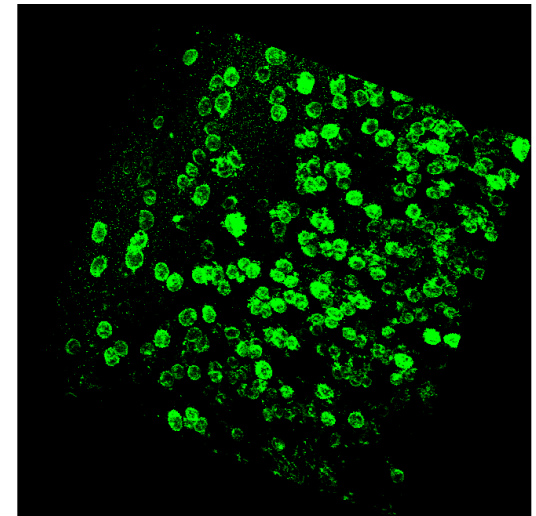
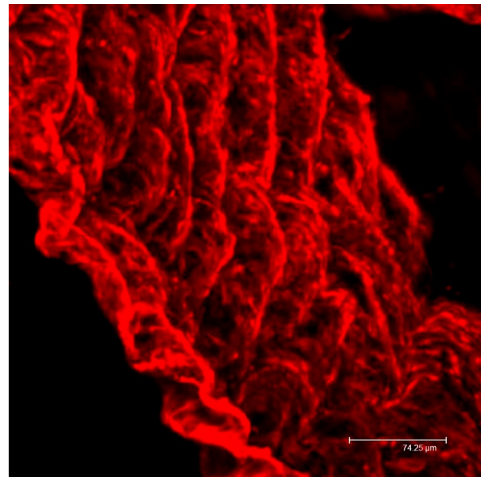


CE



PAH- Rhodamine
Endothelium monolayer

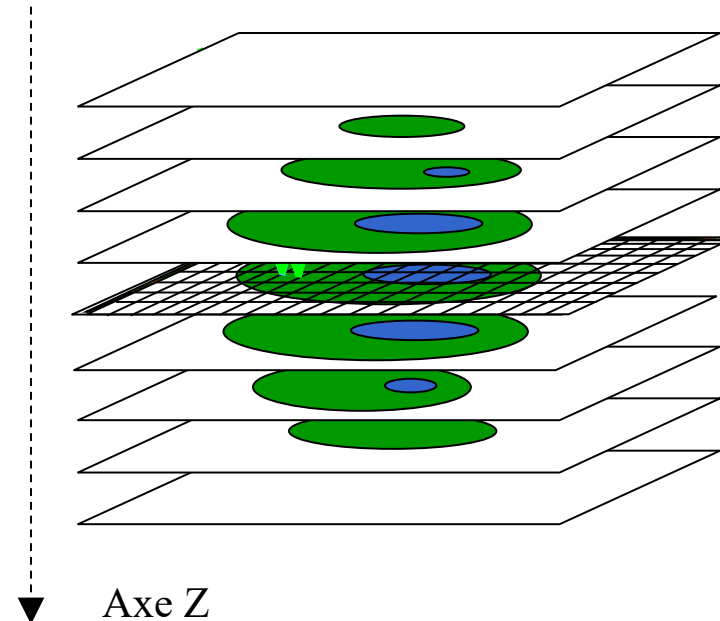
H. Kerdjoudj, C. Boura, D. Dumas



Chondrocyte / Tête Fémorale
Adhésion cellulaire (CD44)

II. Applications au confocal : limitations en micropositionnement

- Moteur du statif
- Surplatine galvanométrique
 - Précision du déplacement
 - Reproductibilité
 - rapidité

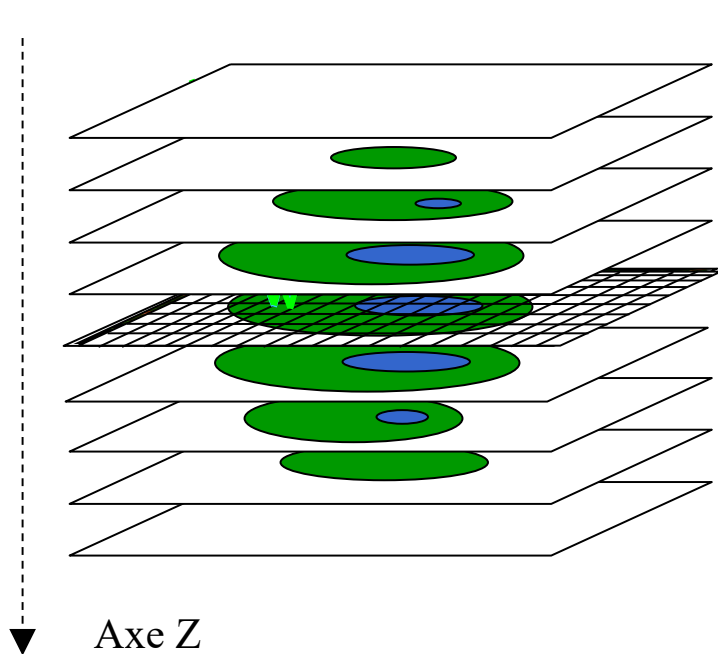


Pas déplacement : 100 nm +/- 10 nm
Echantillonnage optique: 250 nm
Voxel: 250 nm x 100 nm !!

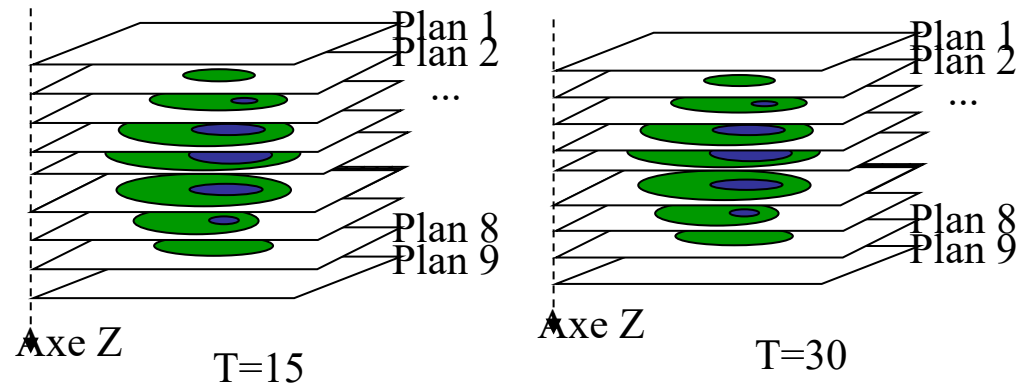
Microscopie confocale

II. Applications au confocal : 3D + temps ... limitations dues au balayage laser

Photodégradation : extinction du signal de fluorescence



De 400 à 4000 Hz

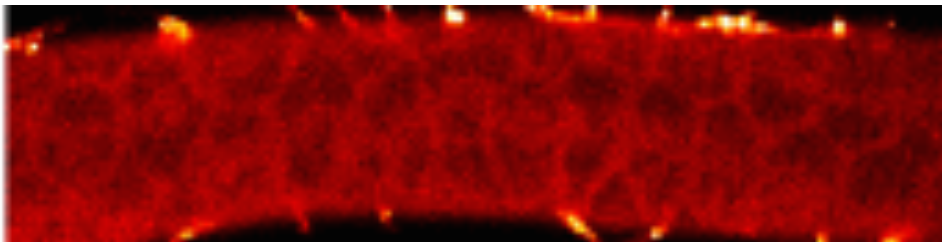


Pas déplacement : 100 nm +/- 10 nm

Echantillonnage optique: 250 nm

Voxel: 250 nm x 100 nm !!

- Fréquence de Balayage / phénomène très fugace (400 Hz)
- 5ms / ligne de 512 points et 3s / frame (512 x 512)
- Temps de résidence / spot discriminateur (6 μ s/pixel)



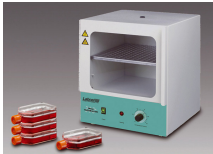
II. Dispositifs pour la culture et observation de matériel d'origine biologique

Enceinte autour de l'instrument d'analyse

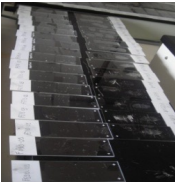
2010

Chambre de culture cellulaire

Cellules



Tissus

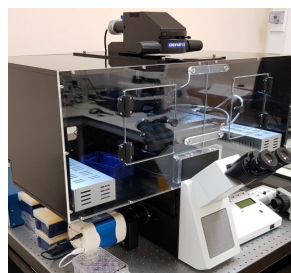
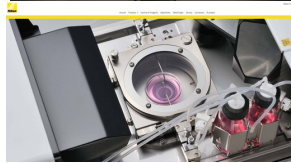
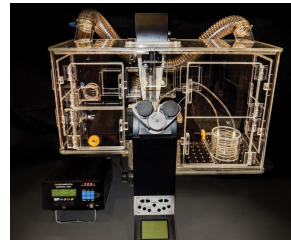


Petits organes



2015

Enceinte d'observation



2020

Pas d'évolution

- **Encombrant/peu mobile**
- **Condensation/non stérile**
- **Besoin énergie T° C**
- **Pas perfusion/viabilité cell**
- **Monovoie / 1 condition exp**
- **Métrieologie/peu pratique**

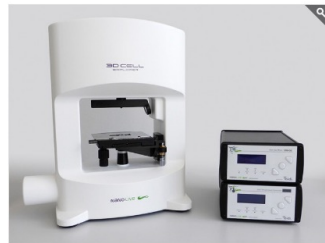


- **45k€**
- **Pas adapté**
- **Peu utilisé**

II. Dispositifs pour la culture et observation de matériel d'origine biologique

Enceinte autour du support de culture.

Nanolive-ibidi Heating System for 1 Chamber



A heating system for live cell imaging on the 3D Cell Explorer

- Fully compatible with the Nanolive 3D Cell Explorer
- Excellent illumination of the sample - no condensation due to heated lid and optimal sample stabilization
- Perfectly suited for live cell imaging applications - full incubator conditions on the microscope
- System includes: Nanolive-ibidi Temperature Controller, Heated Plate in Multi-Well Format for 1 Chamber, 1 Heating Insert, Heated Lid

in cooperation with

NANOLIVE
Looking inside life

System includes:



Nanolive-ibidi Temperature Controller



Heated Plate in Multi-Well Format

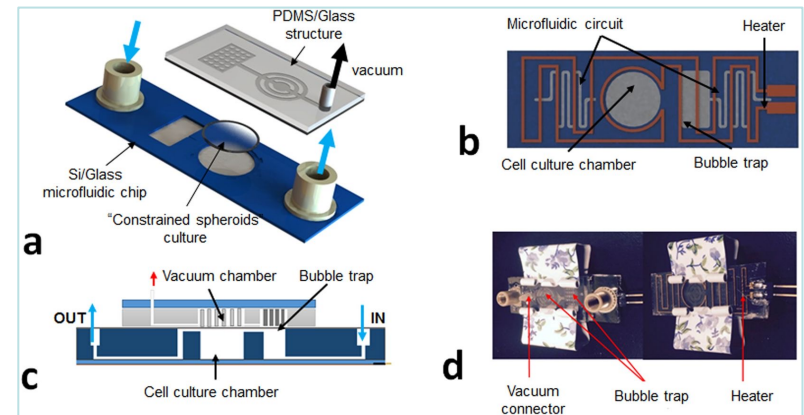


Heated Glass Lid, Universal Fit for ibidi Slide Formats



Heating Insert for ibidi Slide Formats

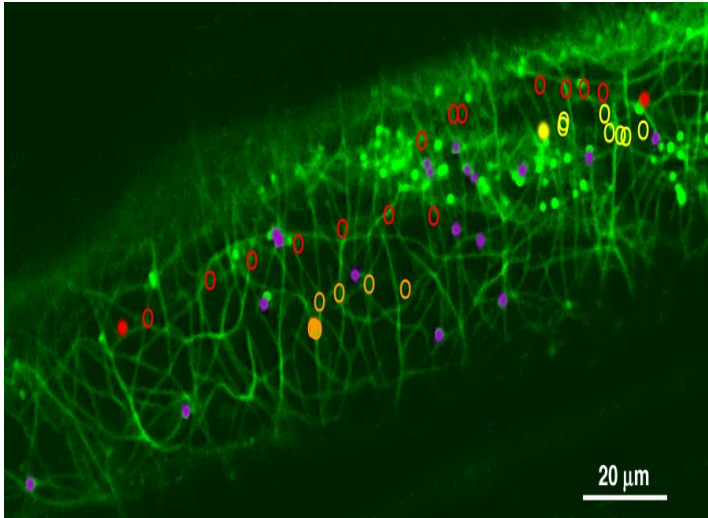
Cat.No: 10918-NAN



- 1 seule voie
- Perfusion de milieu non automatique
- Non transportable

- 1 seule voie
- Perfusion non automatique
- Microfluidique
- Non transportable

II. Confocal : 3D + temps ... MICA Micro Incubateur Cellulaire Avancé

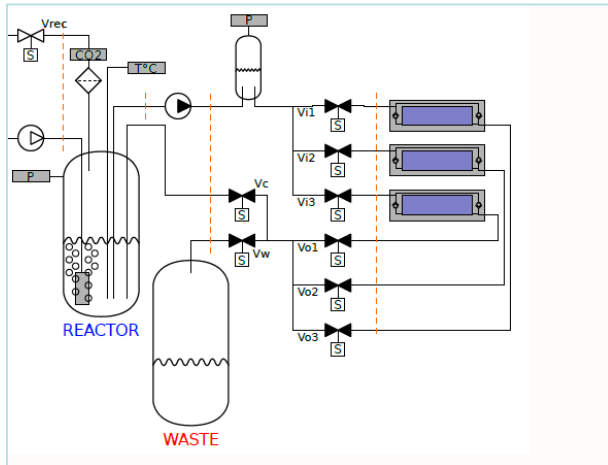
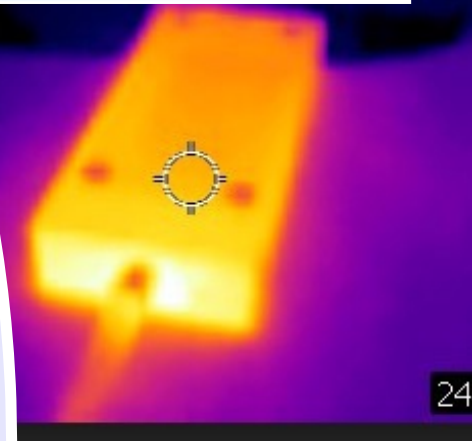
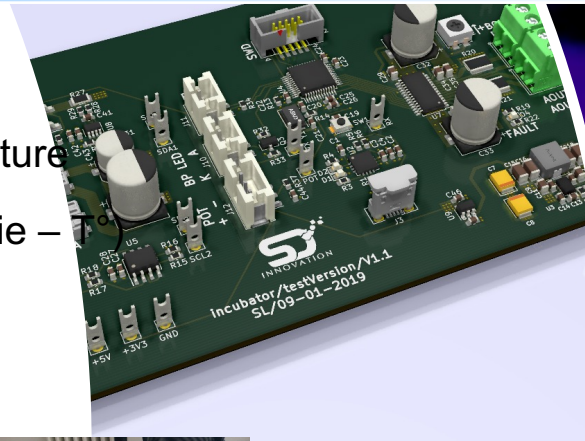


- Imagerie Cellulaire et Tissulaire
- Distribution de fluorophores (co-localisation)
- Imagerie Multicouleur (co-localisation) pour immunomarquage
- Quantification relative (pseudo)
- Observation sur le vivant

II. Dispositifs MICA pour la culture et observation de matériel d'origine biologique

Enceinte autour du support de culture.

- Miniaturisation
- **Mobilité**
- Perfusion et maintien des conditions culture
- Multicanaux en parallèle
- **Conditions multiples** (normoxie/hypoxie –
- Consommables coût unitaire
- **Recueil des déchets /enceinte**
- Universelle (tous supports)
- Transportable (légère et amovible)
- Dynamique (sup. 28 jours)



Les méthodes en imagerie de contraste

A. Le contexte photophysique : la lumière

- I. Le rayonnement électromagnétique/ multimodalités de la lumière
- II. Les aberrations optiques (réfraction et diffraction)
- III. Instrumentation (détecteurs et capteurs)

B. Les méthodes contrastes en imagerie cellulaire (excitation monophotonique)

- I. Microscopie conventionnelle / Déconvolution
- II. Microscopie confocale

C. Exemples de techniques pour caractériser les Interactions et diffusions moléculaires

- I. FRAP: Fluorescence Recovery after Photobleaching
- II. FRET : Fluorescence Resonance Energy Transfer

D. Microscopie Multiphotonique et dérivées

- I Microscopie à excitation multiphotonique
- II. FLIM : Fluorescence Lifetime Imaging Microscopy
- III. SHG: Second Harmonic Generation
- IV. Méthodes Vibrationnelles (Raman-CARS)

D. Développements

Macroscopie en mode multiphoton

III. Microscopie Confocale à balayage Laser: durée de vie (FLIM)

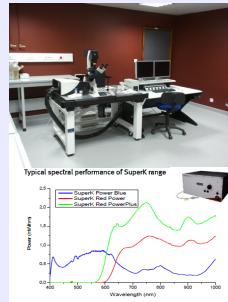
470 – 670 nm

IMAGERIE CONFOCALE ET NON DESCANEE



Applications:

- FRAP (Gap)
- FRET (I, λ, τ)
- Polarisation ($\langle r^2 \rangle$)
- Co-localisation



470
/670 nm

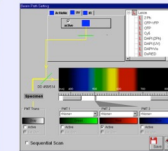
Fluorophores multiples

FRET
FRAP
FCS

Spectres similaires

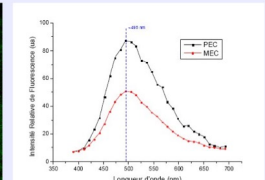
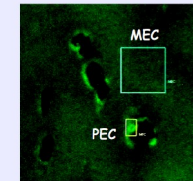
Pulsé

IMAGERIE SPECTRALE



Le spectre d'émission est reconstruit pas par pas (bande passante > 5 nm) en collectant le signal de fluorescence décomposé à l'aide d'un prisme.

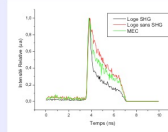
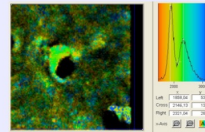
Explant de cartilage Humain
 λ_{exc} : 800 nm en multiphoton



Discrimination spectrale entre les matrices cartilagineuses extracellulaires (MEC) et péricellulaires (PEC)

IMAGERIE DE DECLIN DE FLUORESCENCE FLIM

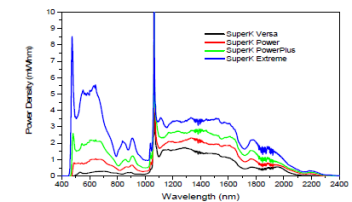
TCSPC-730 Becker-Hickl



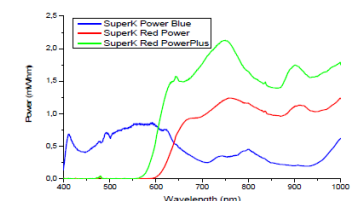
Discrimination de la durée de vie de fluorescence au niveau de la matrice extracellulaire (MEC), des loges chondrocytaires en présence ou non de collagène (pas de signal SHG).

La durée de vie des états excités de fluorescence (temps de déclin τ) est indépendante de la concentration mais reflète le microenvironnement. Le FLIM permet de discriminer des molécules fluorescentes (localisation, caractérisation) alors que leurs spectres d'émission se chevauchent (cross-talk).

Source seed/diode (1064nm/30ps)
Fibre photonique

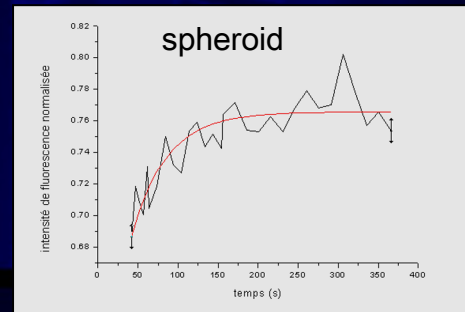
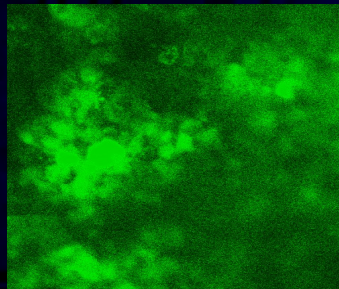
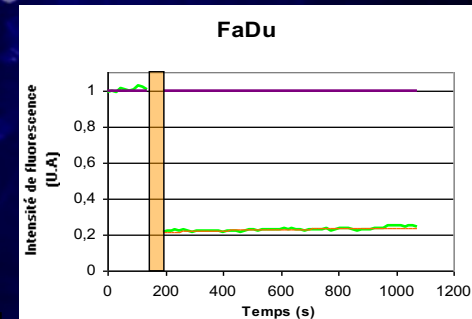
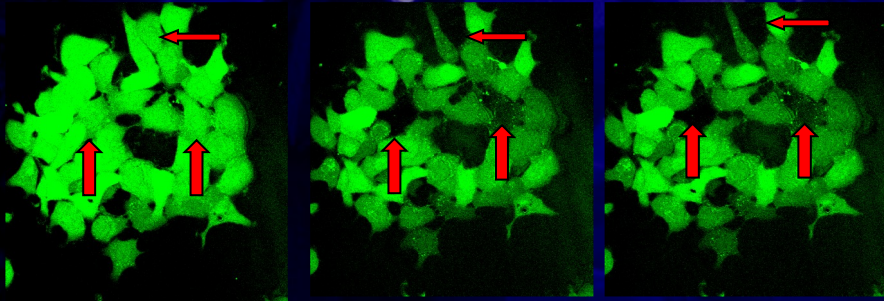
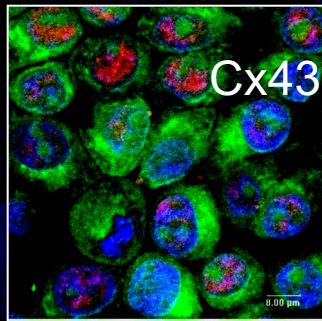
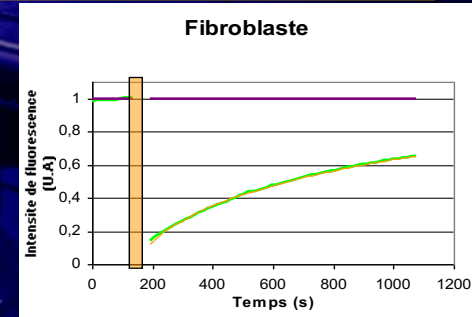
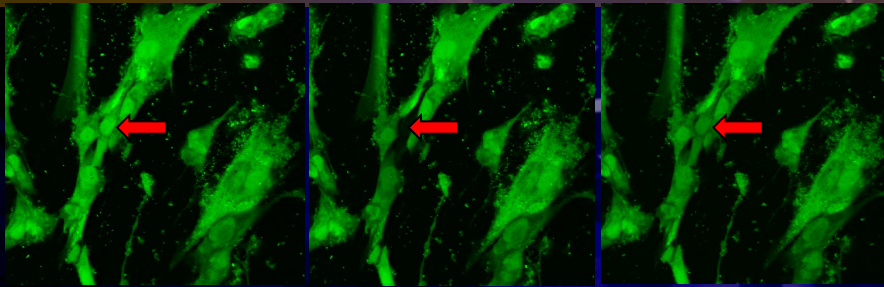
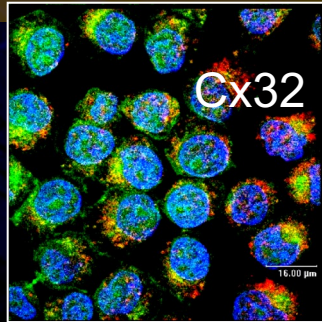


Typical spectral performance of SuperK range



C.I. FRAP: Fluorescence Recovery After PhotoBleaching

« Gap Junctions Intercellulaires »: diagnostic précoce de cancer (connexine)

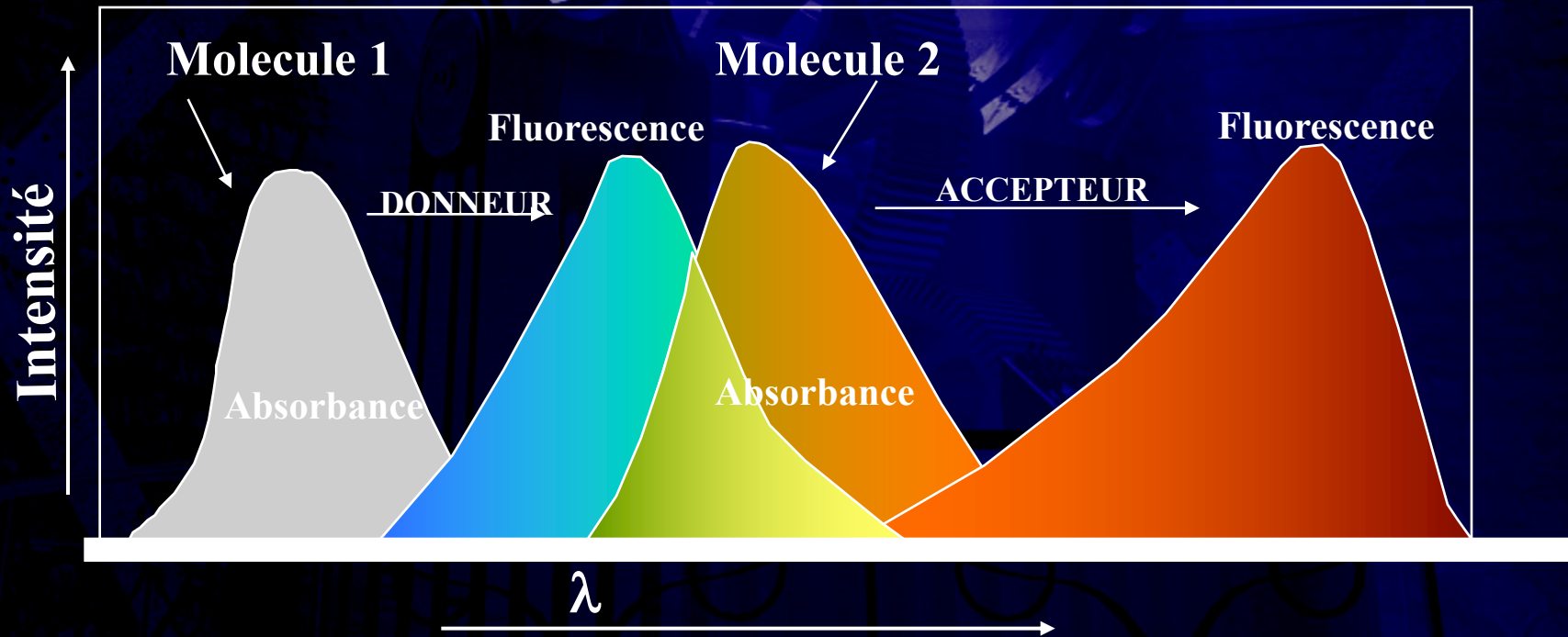
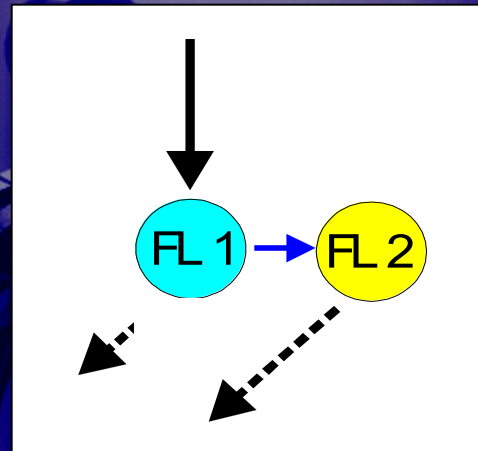
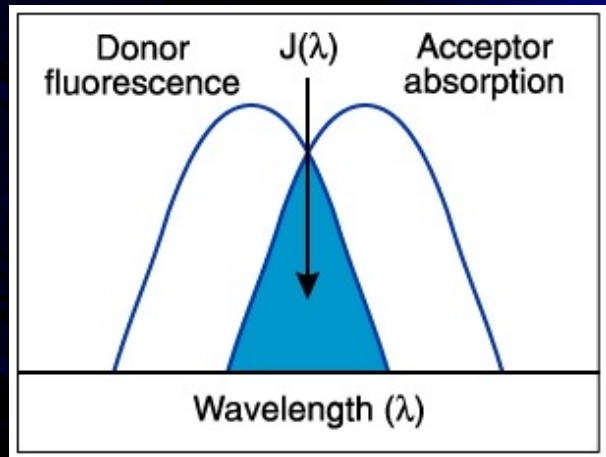


Control	Fibroblast
Cancerous	CasKi
Cancerous	Fadu +/-
Cancerous	KB --
Cancerous	HT29 --

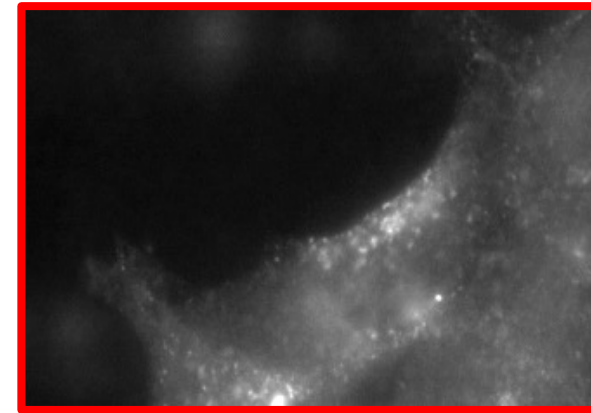
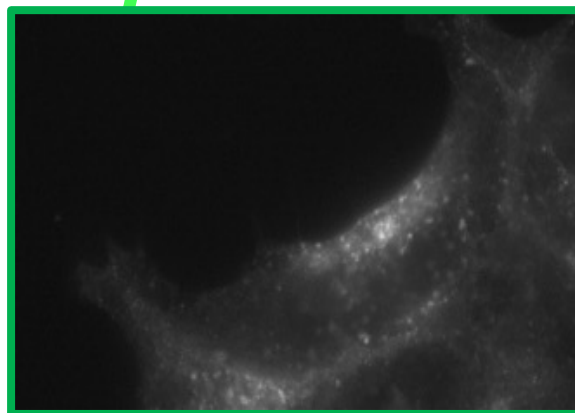
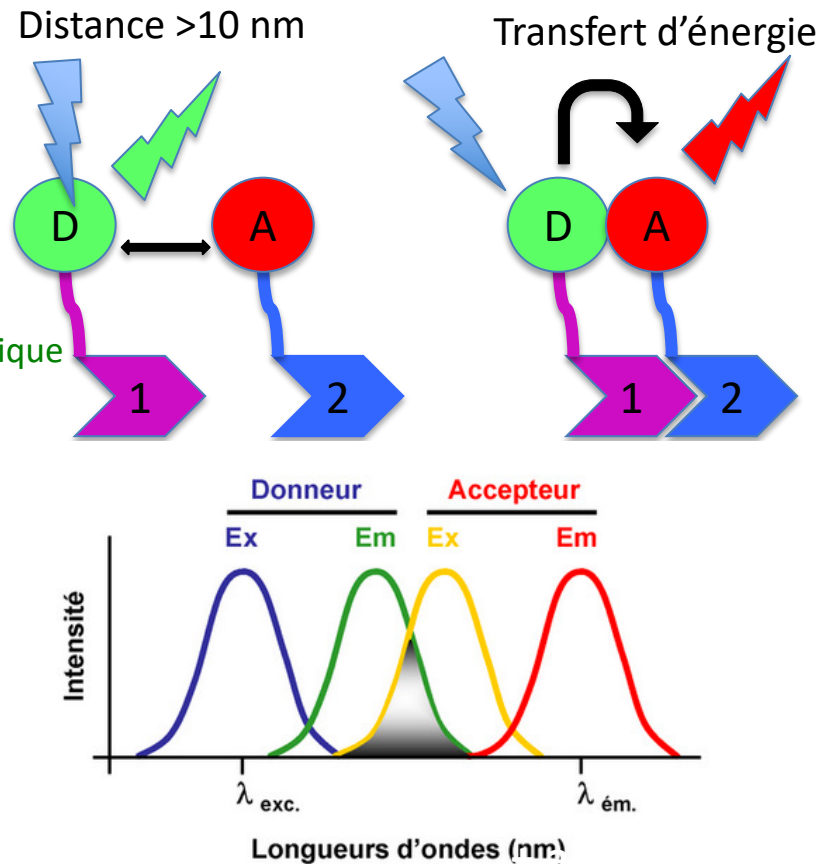
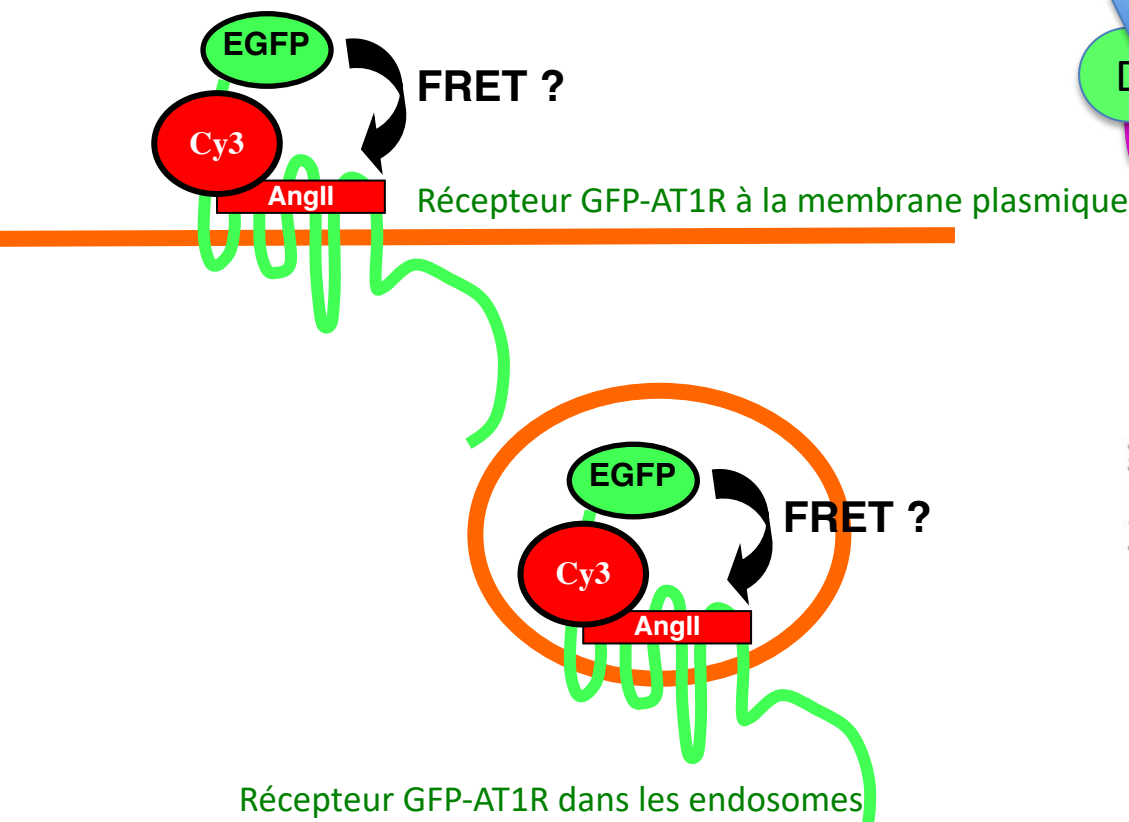
- System identification of the fluorescence recovery after photobleaching in gap junctional intracellular communications. Jean-Baptiste Tylcz. The IEEE Control Systems Society Conference Management System December 10-13, 2013, Firenze, Italy.

- Gap junctional intercellular communication capacity by gap-FRAP technique: A comparative study. M Abbaci. Biotechnology Journal 2 (1), 50-61, 2007.

C.II.. FRET: Fluorescence Resonance Energy transfer



QUESTION: voit-on du FRET par FLIM entre GFP-AT1R ou GFP-AT2R et son ligand Cy3-AngII



C.II.. FRET: Fluorescence Resonance Energy transfer

Efficacité :

$$k_T(r) = 1/\tau_D (R_0/r)^6$$

r : distance entre donneur et accepteur

R_0 : distance de Förster (chevauchement spectral)

τ_D : Durée de vie de fluorescence du donneur

Typical Values of R_0

Donor	Acceptor	R_0 (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	DABCYL	33
Fluorescein	Fluorescein	44
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 dye	61

$$R_0 = [8.8 \times 10^{23} \cdot \kappa^2 \cdot n^{-4} \cdot QY_D \cdot J(\lambda)]^{1/6} \text{ Å}$$

where κ^2 = dipole orientation factor (range 0 to 4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)

QY_D = fluorescence quantum yield of the donor in the absence of the acceptor

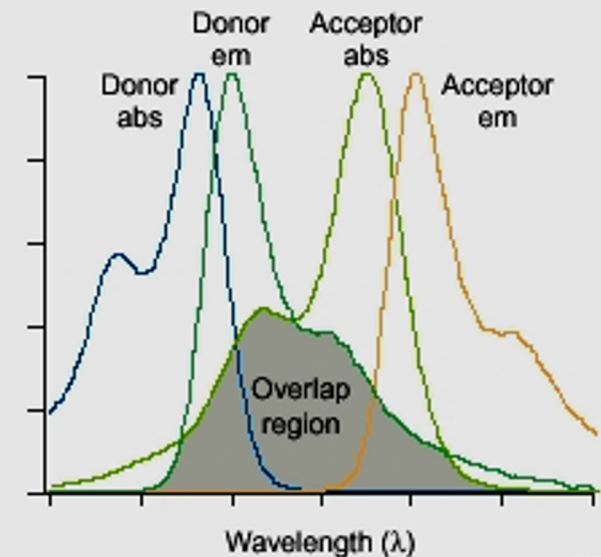
n = refractive index

$J(\lambda)$ = spectral overlap integral (see figure)

$$= \int \epsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \text{ cm}^3 \text{M}^{-1}$$

where ϵ_A = extinction coefficient of acceptor

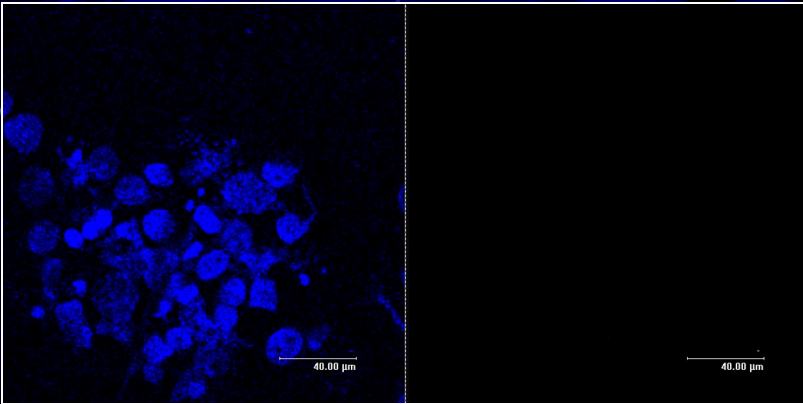
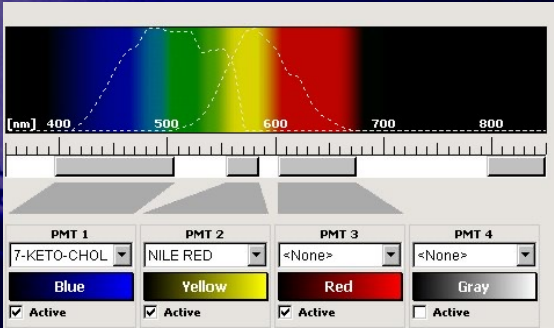
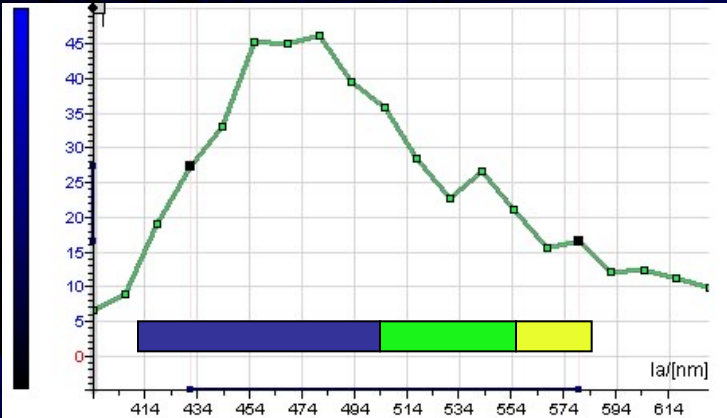
F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity



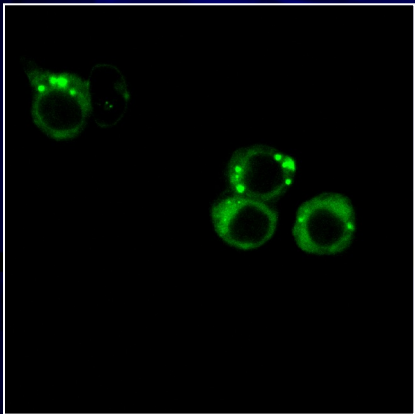
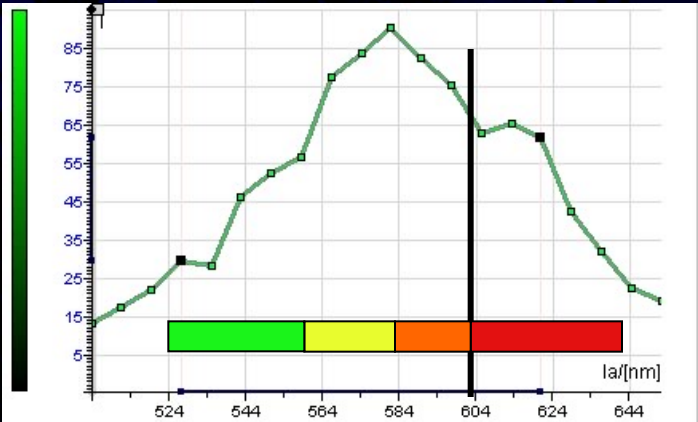
C.II.. FRET: Fluorescence Resonance Energy transfer

Detection of early apoptosis on U937 cell
(human promonocytic leukemia cells)
7-keto-cholesterol (fluo) induces apoptosis (programmed dead)

Donor : 7-keto-cholesterol, λ_{ex} : 750 nm



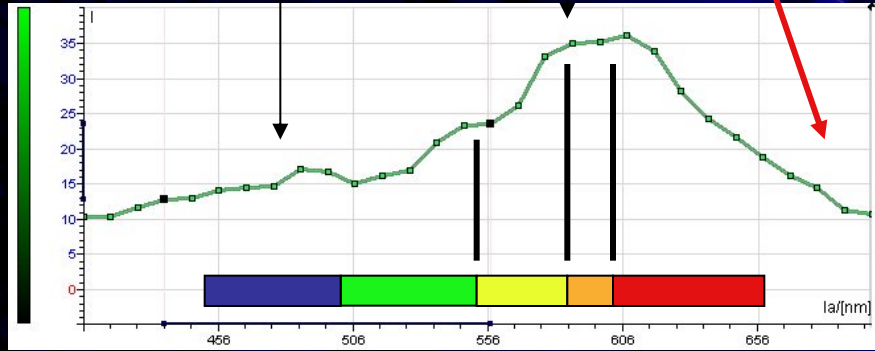
Acceptor : Nile Red, λ_{ex} : 488 nm



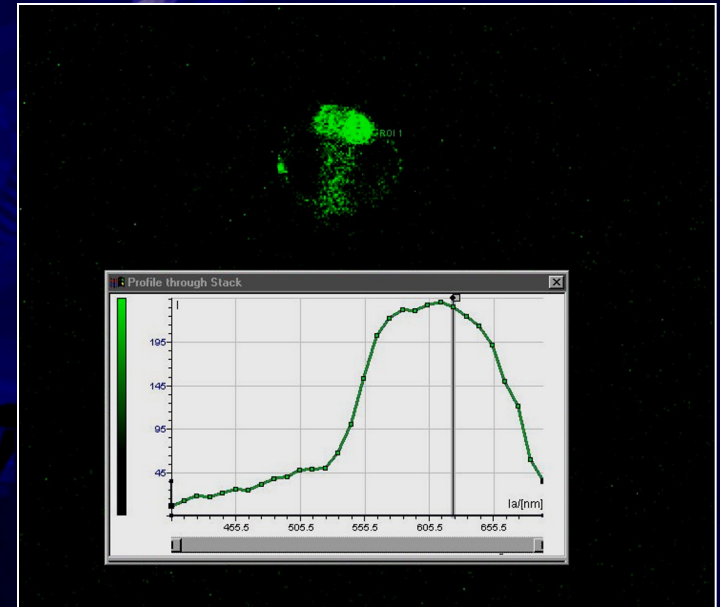
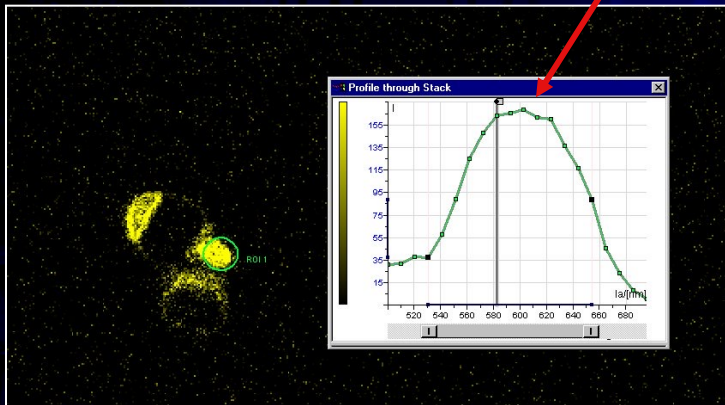
Nile Red
yellow Red
Polarity of SPL

Yellow

C.II.FRET: Fluorescence Resonance Energy transfer

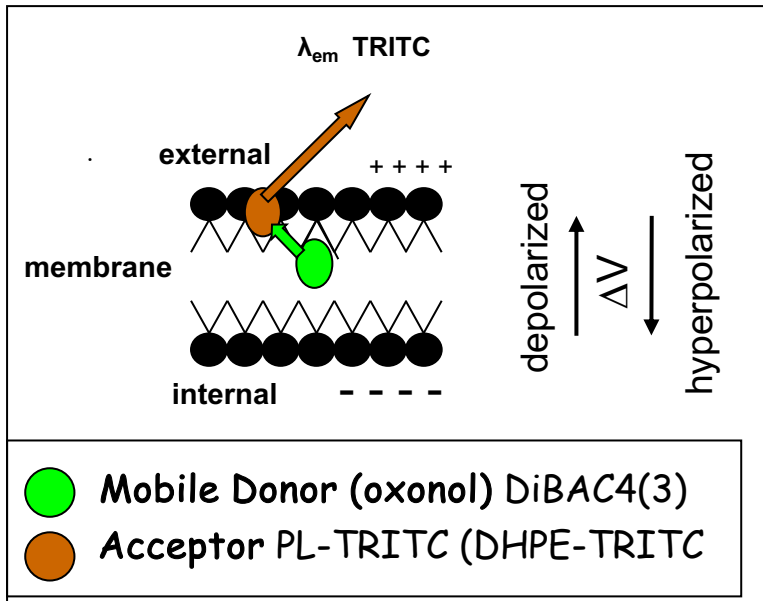


Shift toward the red channel
(polar lipide : 7-kéto-cholestérol/Nile Red)



IV. FRET Spectral/FLIM

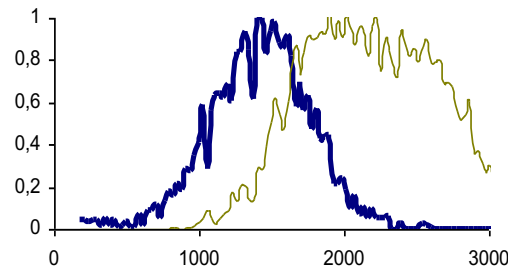
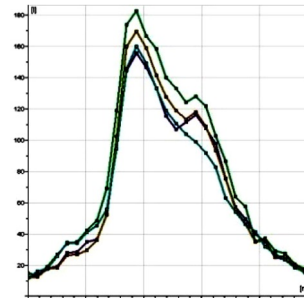
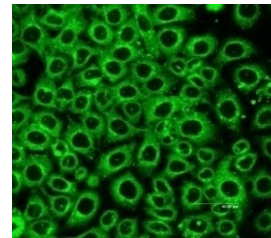
Voltage-sensitive dye (membrane potential)



Control	Fibroblast	-
Cancerous	A949	++
Cancerous	HT29	++
Cancerous	EMT9	+
Cancerous	KB	+

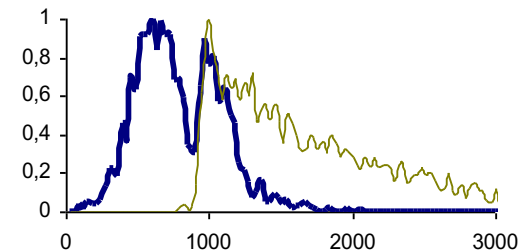
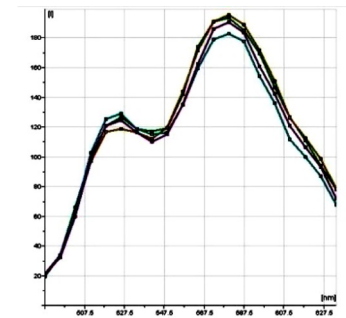
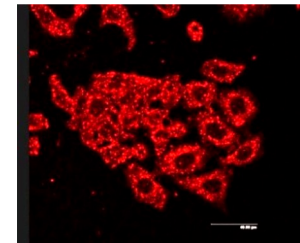
NO FRET

Control



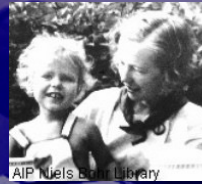
FRET

KCl + valinomycin



D.I. Microscopie à excitation Multiphotonique : principe

Maria Göppert-Mayer
Processus d'absorption 2-photons
(1930)



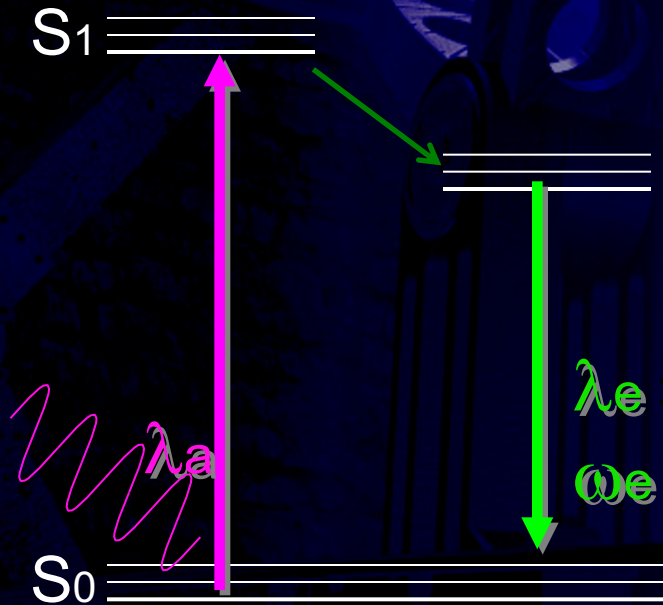
Über Elementarakte mit zwei Quantensprüngen
Von Maria Göppert-Mayer
(Göttinger Dissertation)
(Mit 5 Figuren)
Erscheinung
Der erste Teil dieser Arbeit beschäftigt sich mit dem
Zusammensetzen zweier Lichtquanten in einem Elementarakt.

Excitation monophoton (UV-Vis)

Excitation biphoton (IR)

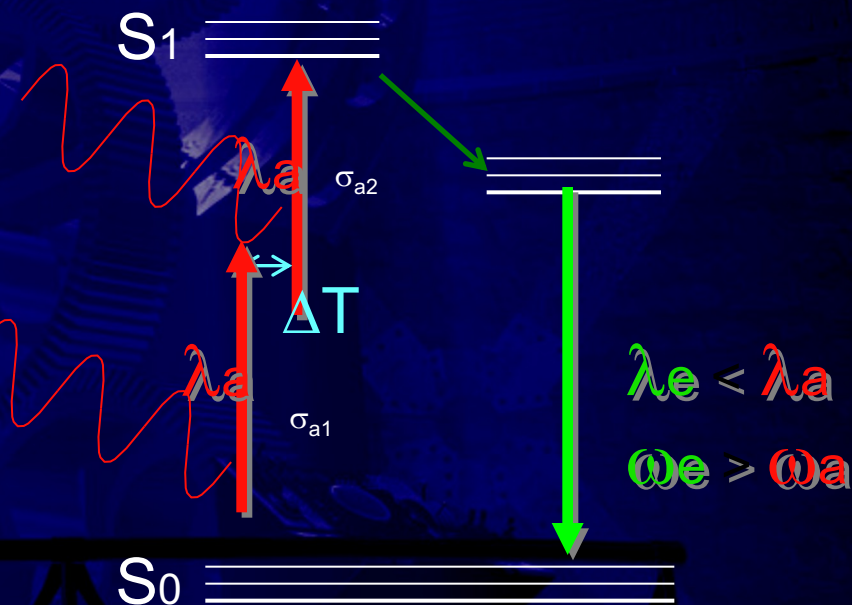
$$\sigma_{\text{SPE}} = \sigma_a = 10^{-16} \text{ cm}^2$$

$$\sigma_{\text{TPE}} = \sigma_{a1} \sigma_{a2} \Delta T = 10^{-34} \text{ cm}^4 \cdot 10^{-15} \text{ s}$$



Absorption Linéaire : 450 nm

Emission dans le visible



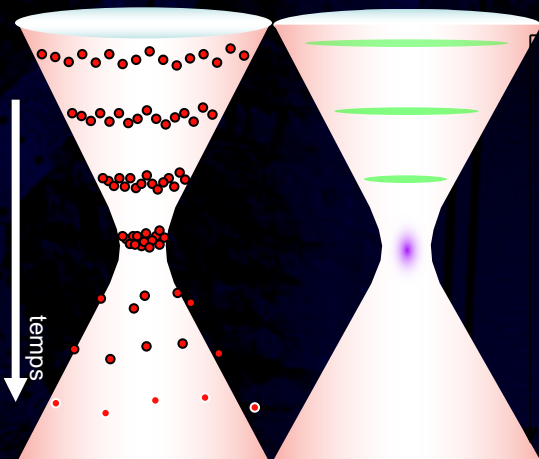
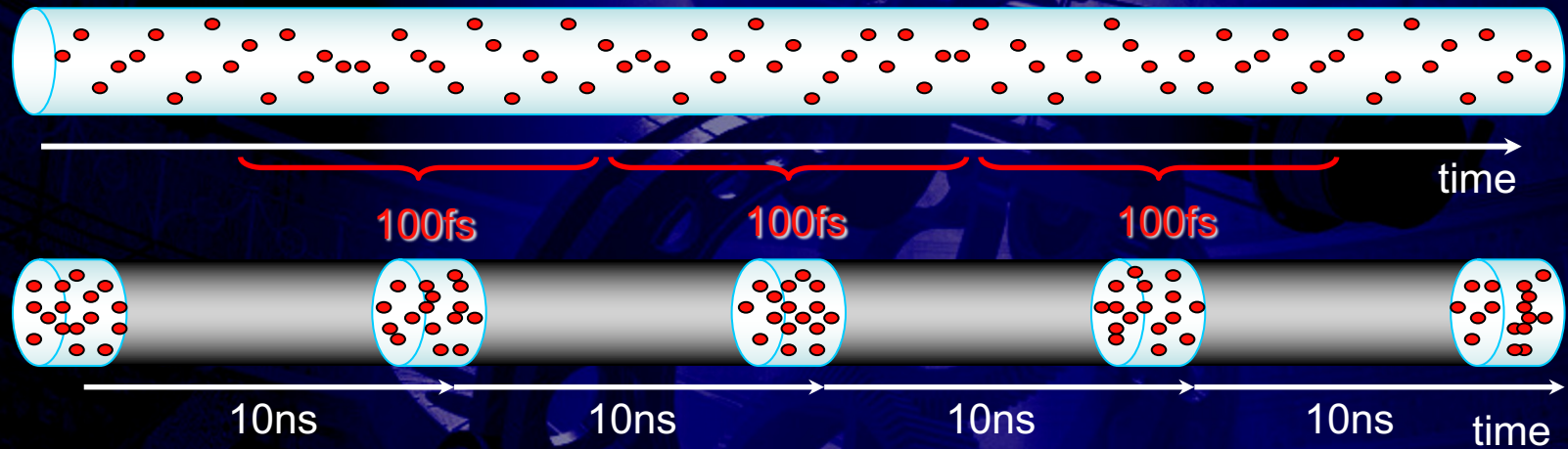
Absorption non Linéaire : 900 nm

Emission dans le visible

D.I. Microscopie à excitation Multiphotonique

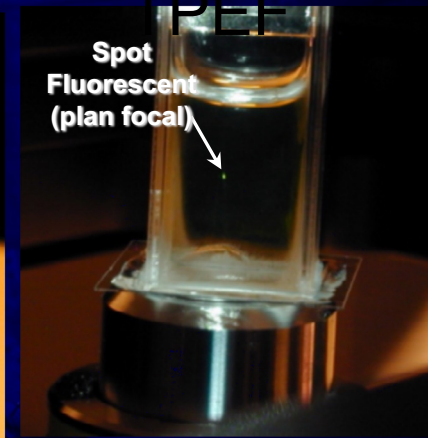
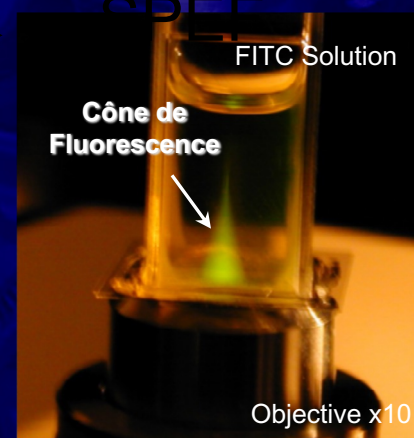
Temporel : Laser IR pulsé

(0.1 - 10 MW/cm²)



Log N

Probabilité



Photobleaching --
Diffusion -- (proche IR)

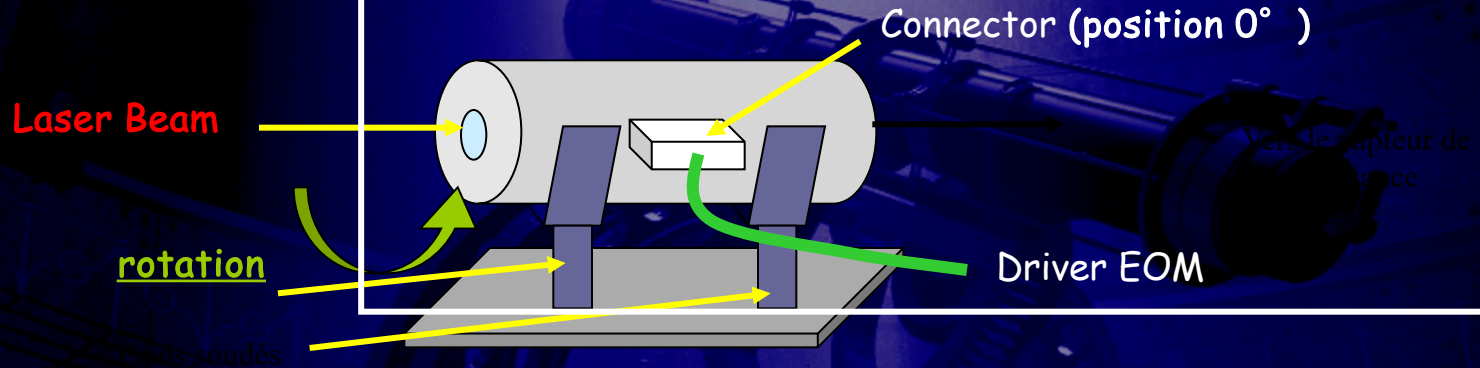
1P - 488nm

Dilution Spatiale
Confinement Temporel

2P - 976 nm

Confinement spatial
(plan focal) et temporel

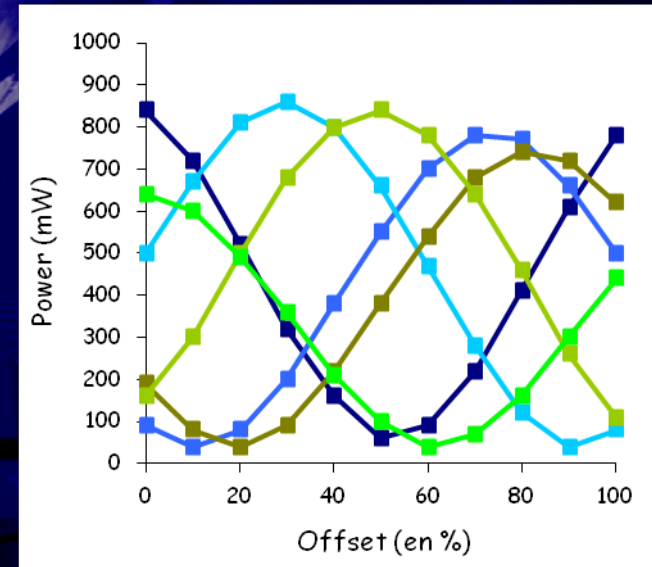
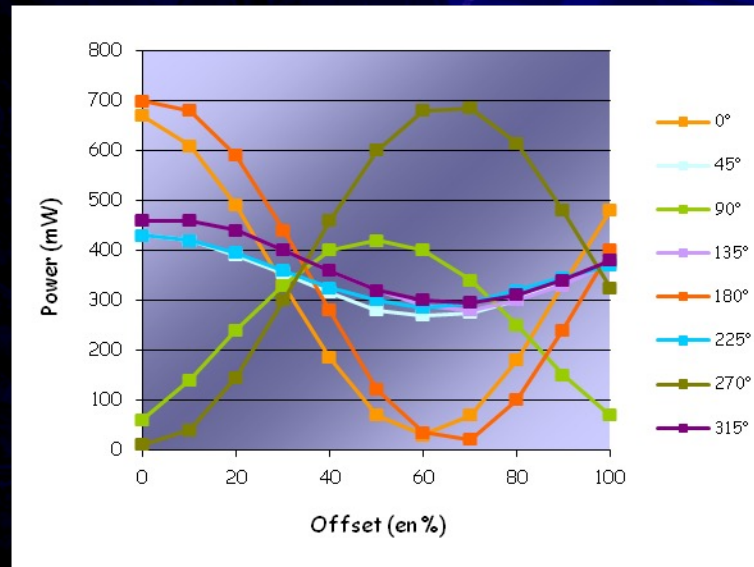
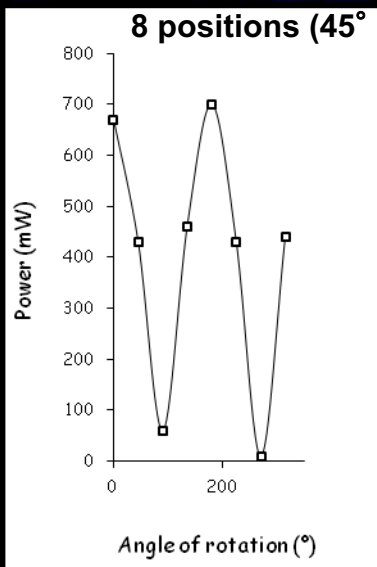
D.I. Microscopie à excitation Multiphotonique : modulation P (EOM)



I : angle q between axes of polarisers
 $\rightarrow I = I_0 \cdot \cos^2 q$

Angle

P_{moy} : 30mW - 193mW
 E : 188pJ - 2,5nJ



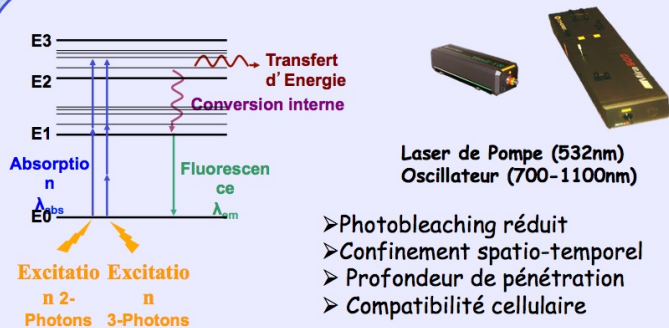
Power delivered (mW) as a function of offset values applied to driver at 780nm.
 Calibration in « High » mode at 780nm, for several angle of rotation values.

Calibration

D.I. Microscopie à excitation Multiphotonique: applications

Fluo (ex700 – 980 nm/ em 350-700nm)

IMAGERIE MULTIPHOTON



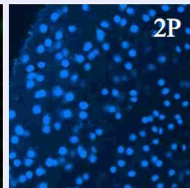
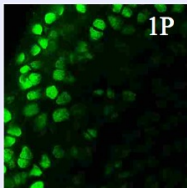
- Photobleaching réduit
- Confinement spatio-temporel
- Profondeur de pénétration
- Compatibilité cellulaire

Tête fémorale de rat (cartilage)

λ_{exc} : 800 nm

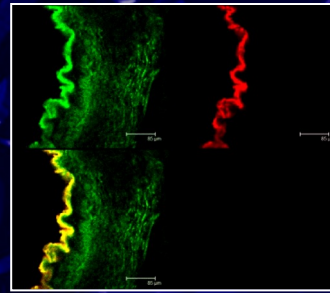
64 μ m

219 μ m

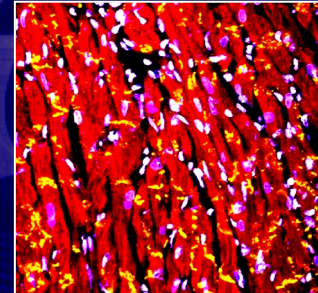


Efficacité de l'illumination en profondeur accrue en mode multiphoton (2P) par rapport au mode monophoton (1P).

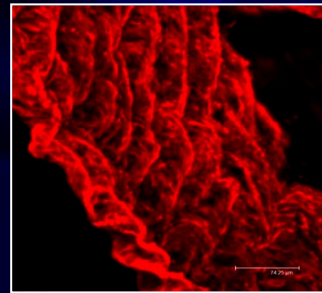
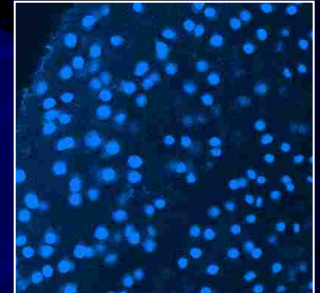
Femoral artery
300 μ m



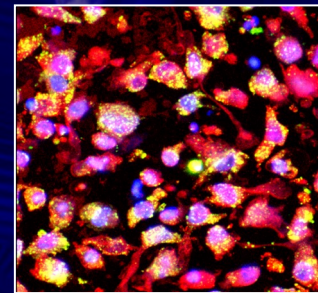
Graft
Cardiomyocyte
450 μ m



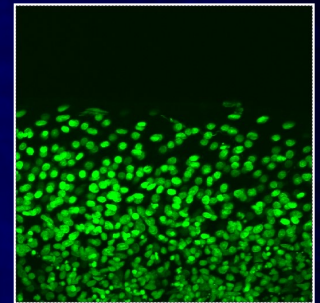
Rat articular cartilage
Chondrocyte (nucleus in blue)
Depth : 219 μ m



Femoral artery
+ film PAH- Rhodamine



Apoptosis
(live/dead)

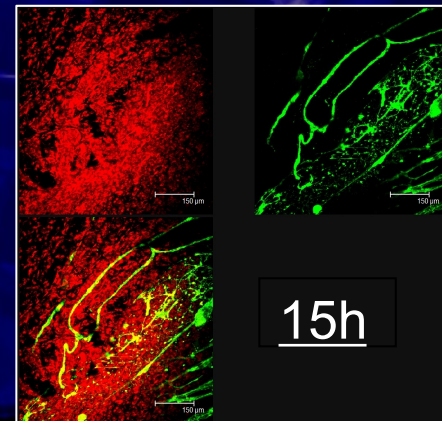
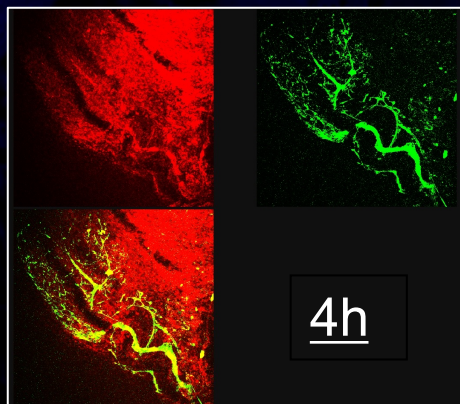
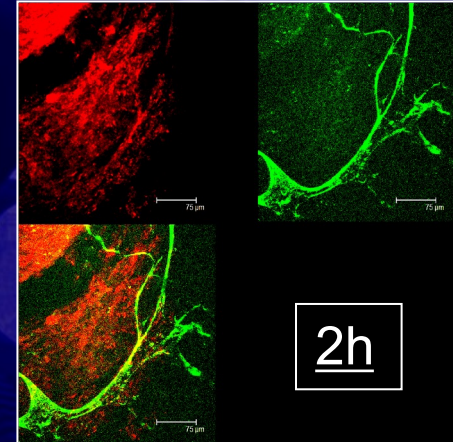
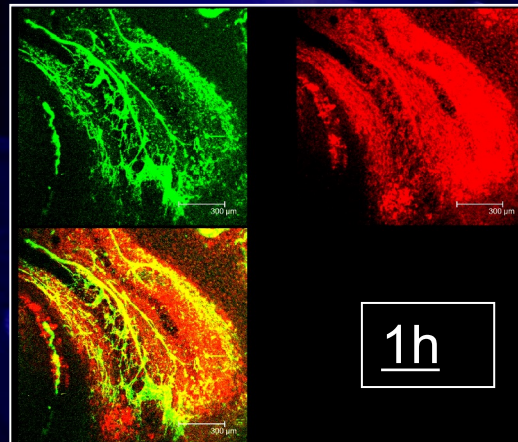
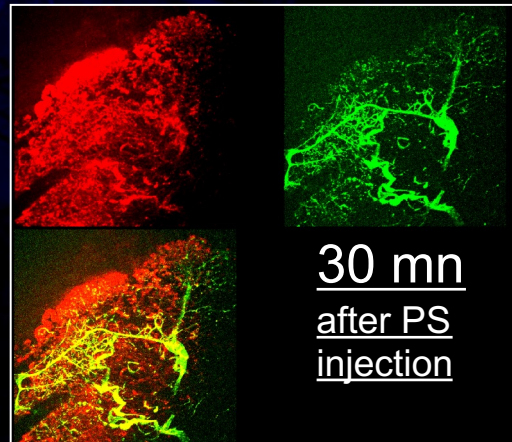


Chondrocyte in
alginate bead
Depth : 1600 μ m

D.I. Microscopie à excitation Multiphotonique : application

Green Channel: Beads (0.22 μm)

Red Channel: mTHPC (PDT)



0-15 mn:
from vessel to tumour
(co-localisation)

≥ 20 mn:
major location in tumor

≥ 1 h:
complete location in tissue

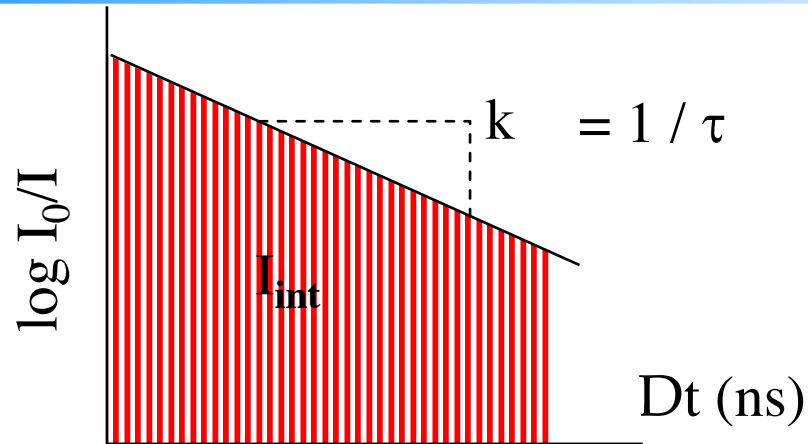
Inconvénients

- * Résolutions xyz inférieures (x2) 1P/ 2P (I_{Exc} , fluorophore...)
- * Temps de mise en oeuvre (mn)
- * Photodégadation (x2,5) / plan focal
- * Effets thermiques IR (eau, [fluorophore]...)
- * Coût d'acquisition et de maintenance
 - Barrette de diode (laser de pompe)
 - Classe IV
- * Stabilité puissance (lasers de pompe)
- * Recul nécessaire
 - effets cellulaires (DNA/3P)
 - caractéristiques spectrales

Avantages

- * Absorption confinée au volume focal
 - pas de déflouage
 - pas de photodégradation / plans adjacents
- * Diaphragmes confocaux ouverts
 - efficacité de collection maximale
 - quantification
- * Mode NDD (Non Descanné)
- * Proche IR
 - pénétration / tissus
 - cellules vivantes (UV)
 - échantillons épais et/ou opaques
- * Modes de résolution de la fluorescence
 - faisceau polarisé
 - mode impulsionnel (FLIM-CARS)

D.II.FLIM : Fluorescence Lifetime Imaging Microscopy



Area :
Fluorescence Intensity
Dependant Concentration

fluorescence lifetime

with $k = k_r + k_{nr}$

intrinsic

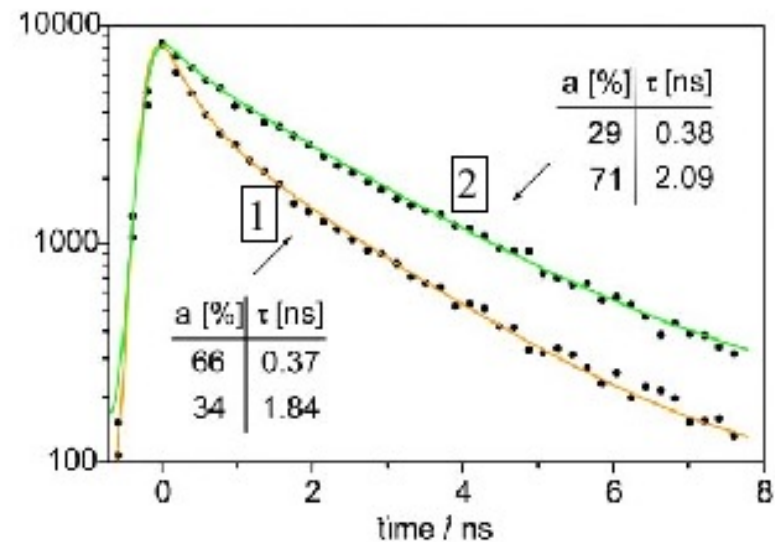
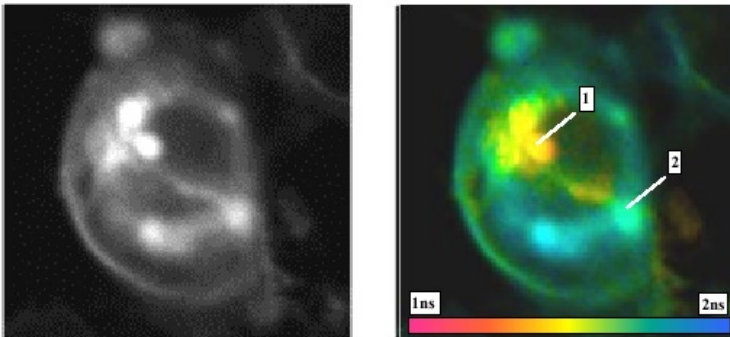


environment

Environment : , FRET...
Independant Concentration

LIFETIME IMAGING

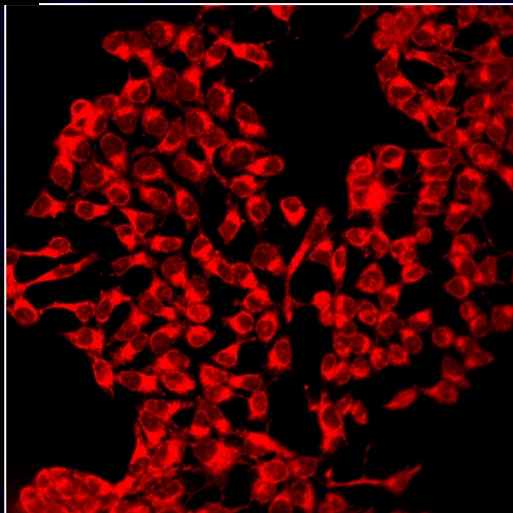
SPECTRAL IMAGING POLARIZATION



D.II.FLIM : Fluorescence Lifetime Imaging Microscopy . Application

MCF-7

Incubation: 12h



PS / Beads

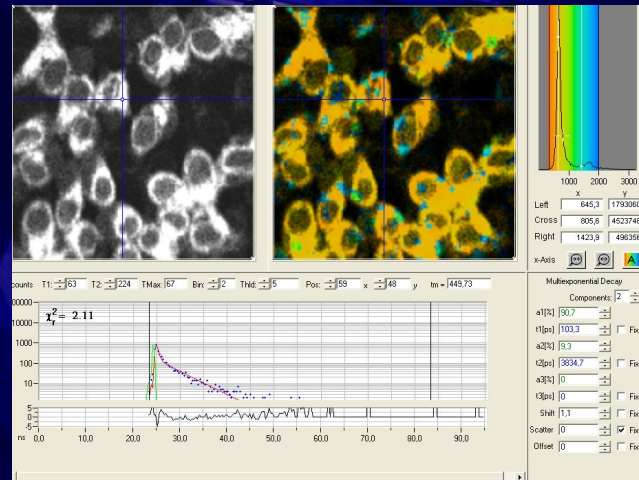
2000 ps / 4000 ps

Intensity

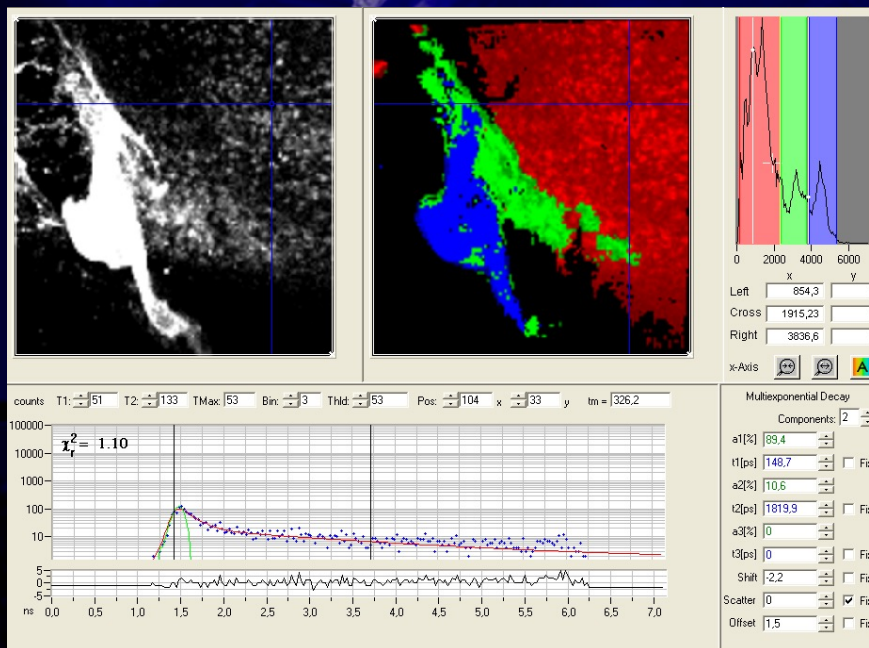
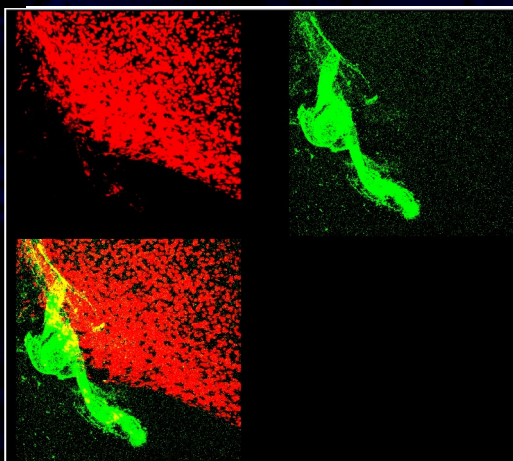
Lifetime

800 ps / 1700 ps

Monomer / Excimer

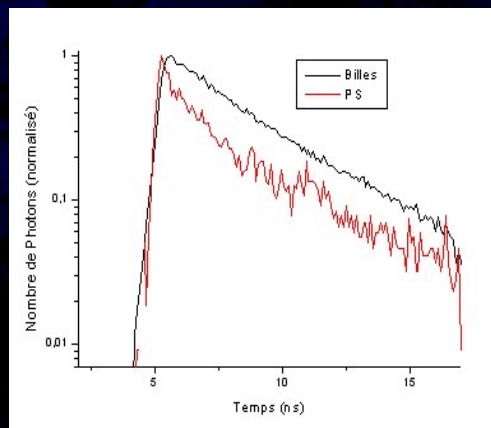
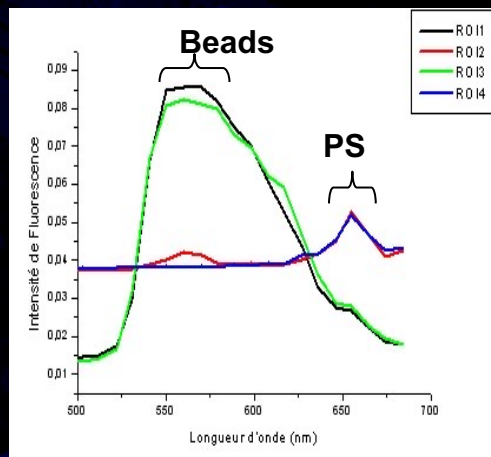


Tumor

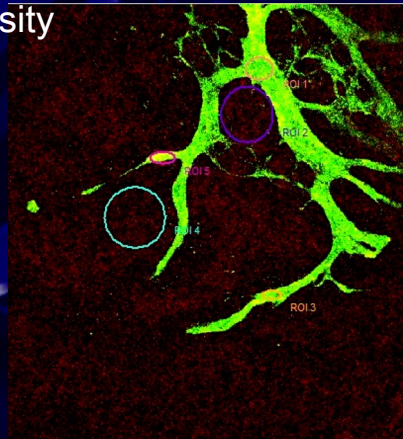


D.II.FLIM : FLIM Multimodalité. Application

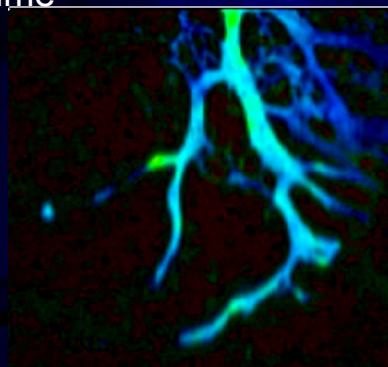
Vascularization and Distribution of PS (depth: 140 μm)



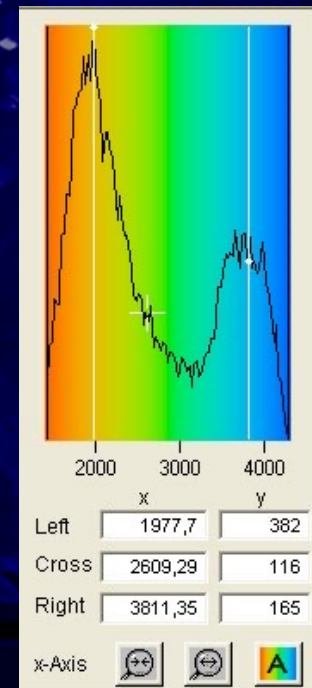
intensity



lifetime

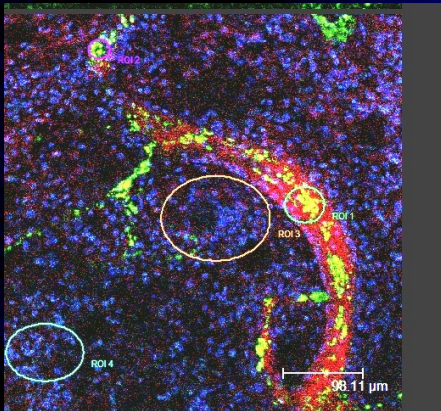


PS : 2,1 ns
Beads : $\tau = 3,6$ ns

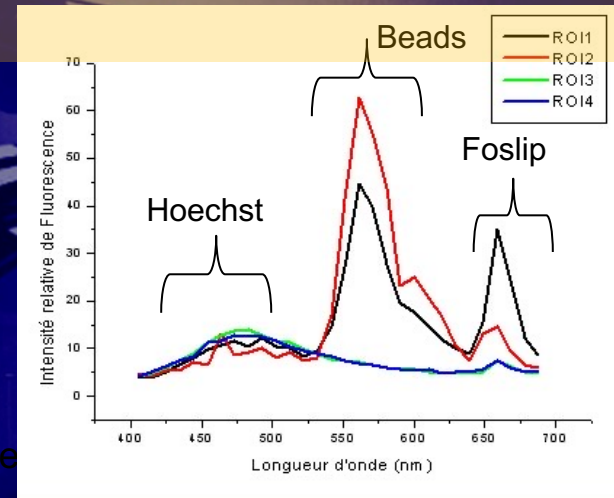
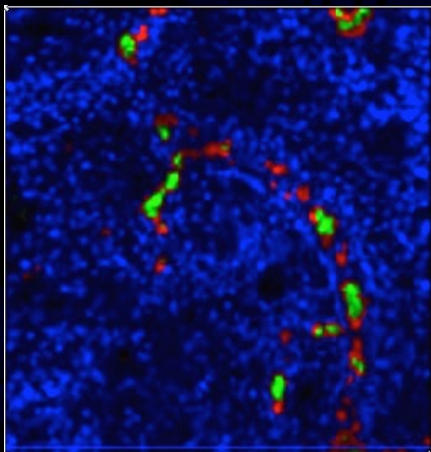


D.II.FLIM : Fluorescence Lifetime Imaging Microscopy . Application

- Foslip/Hoechst/Beads in cancerous cells



- Blue : 2000 – 2800 ps
- Red : 2800 – 3300 ps
- Green : 3300 – 6000 ps

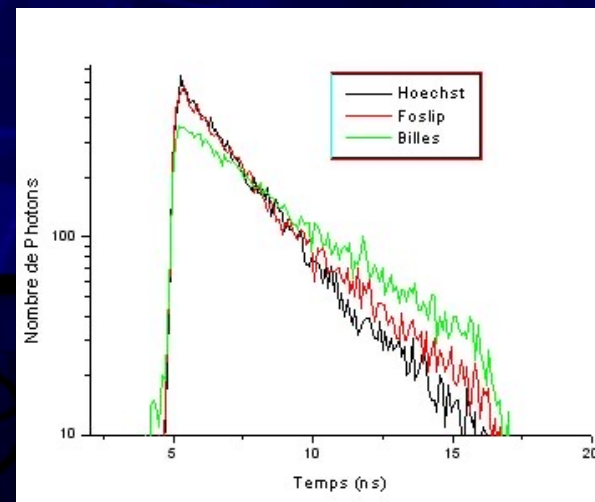


fluorescence decay :

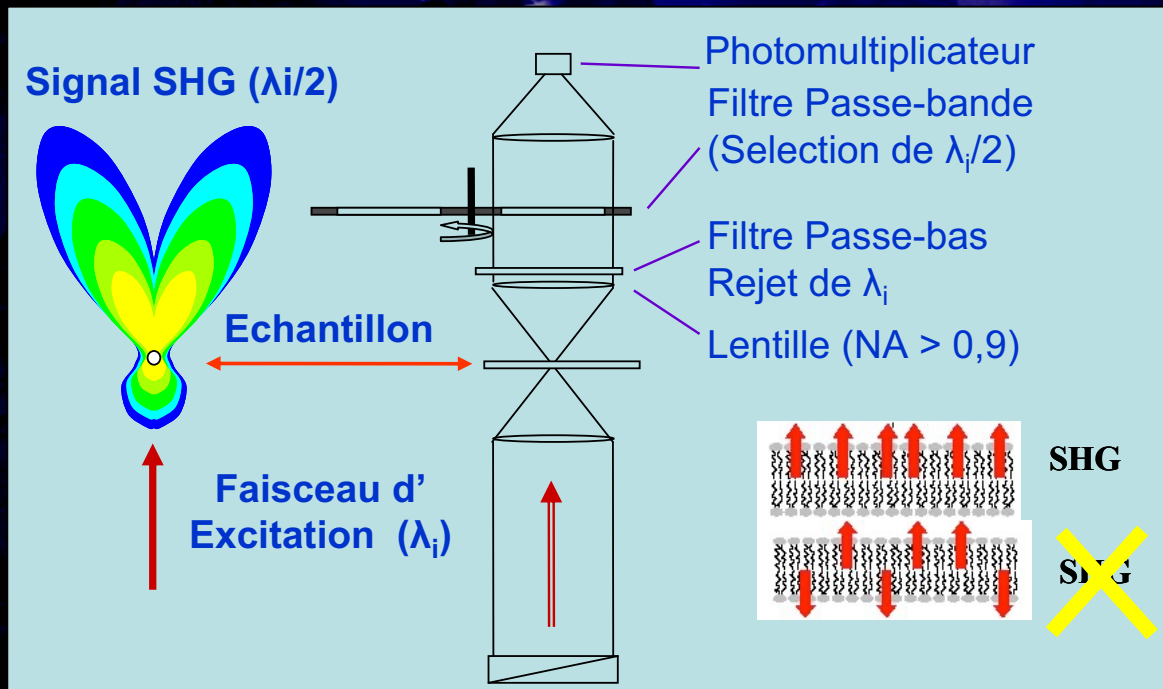
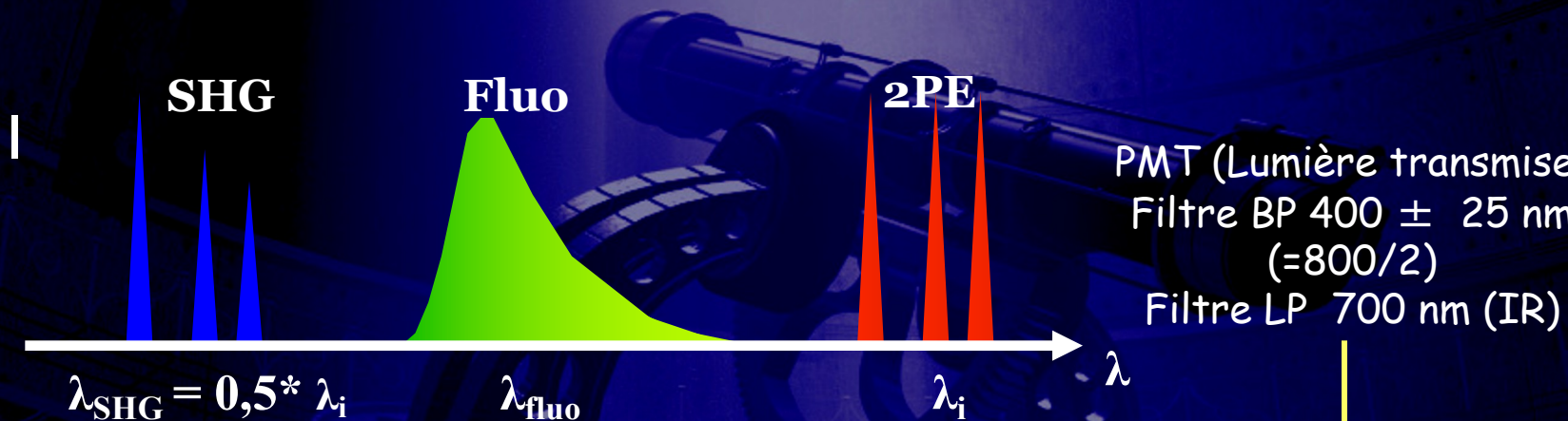
Pix (25,100) → Hoescht : $\tau = 2,3$ ns

Pix (40,7) → Foslip : $\tau = 2,7$ ns

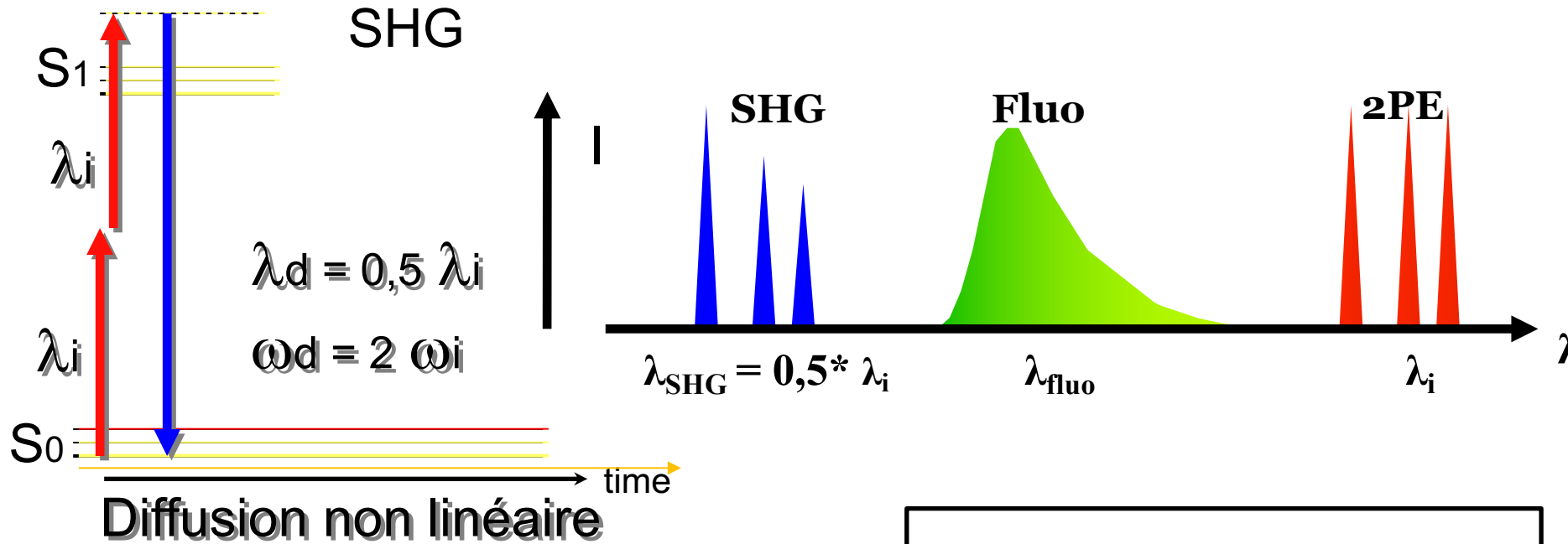
Pix (50,50) → Beads : $\tau = 3,9$ ns



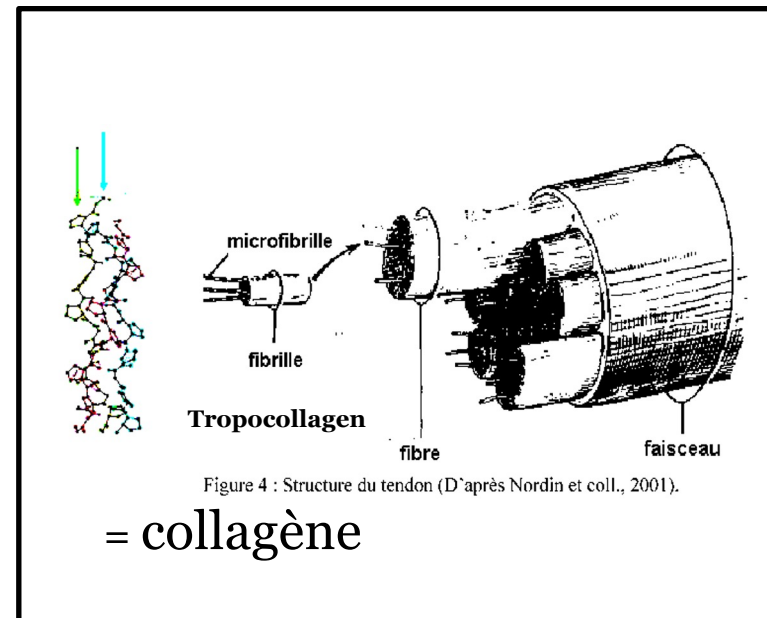
III.b. Second Harmonic Generation - SHG



D.III. Microscopie à excitation Multiphotonique SHG

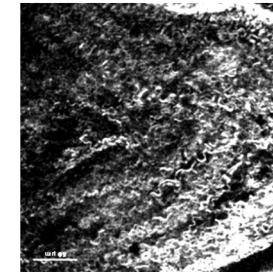
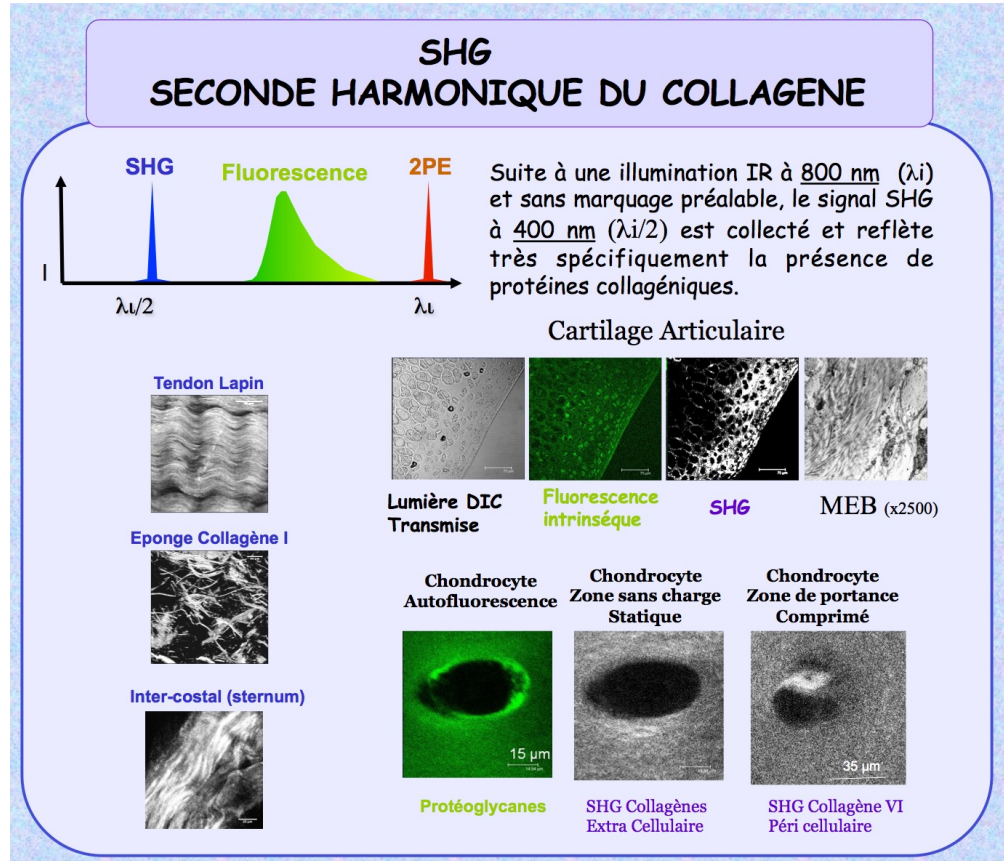


- Génération quasi instantanée (quelques fs)
→ Signal cohérent
- Pas de fluorophore exogène
→ diminution effets cytotoxiques et phototoxiques
- Pas de photoblanchiment ($\neq$ Fluorescence)
- Pas d'absorption
- Pas de perte d'énergie (diffusion élastique)
- Principal composant tissulaire générateur de SHG.

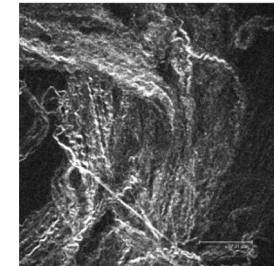


D.III. Microscopie à excitation Multiphotonique SHG

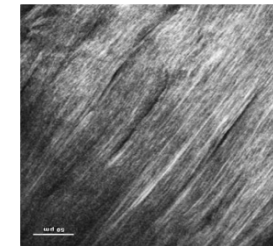
SHG ex800/em400 nm



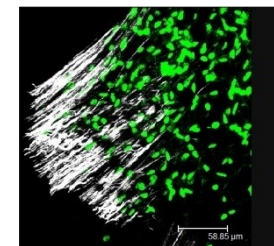
Peau souris



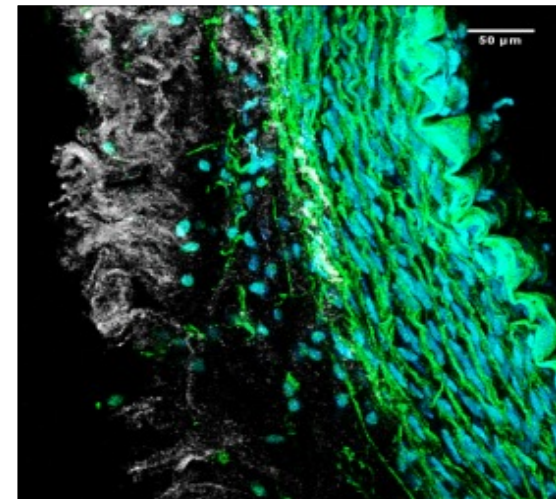
Artère lapin



Muscle lapin



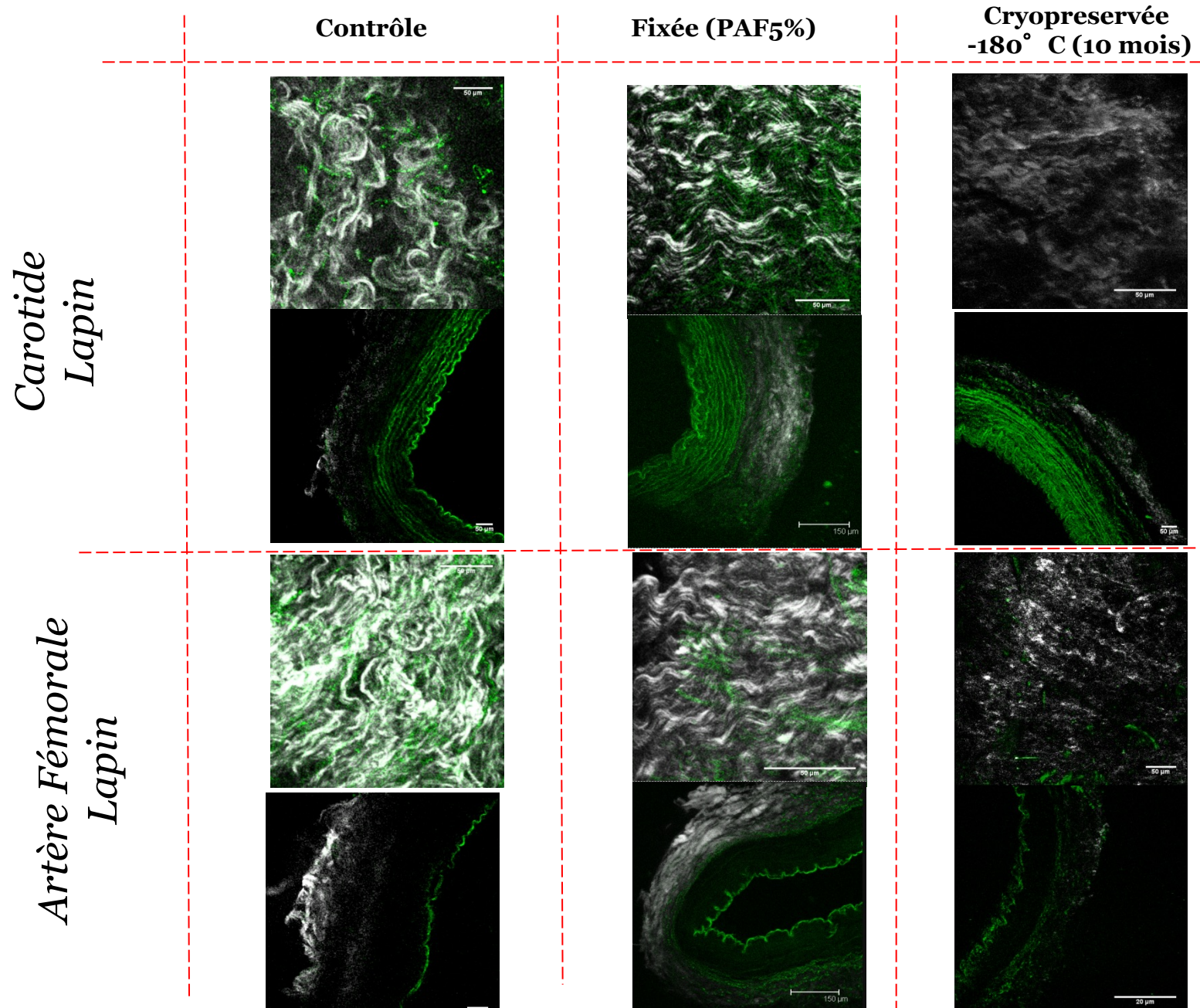
Muscle lapin



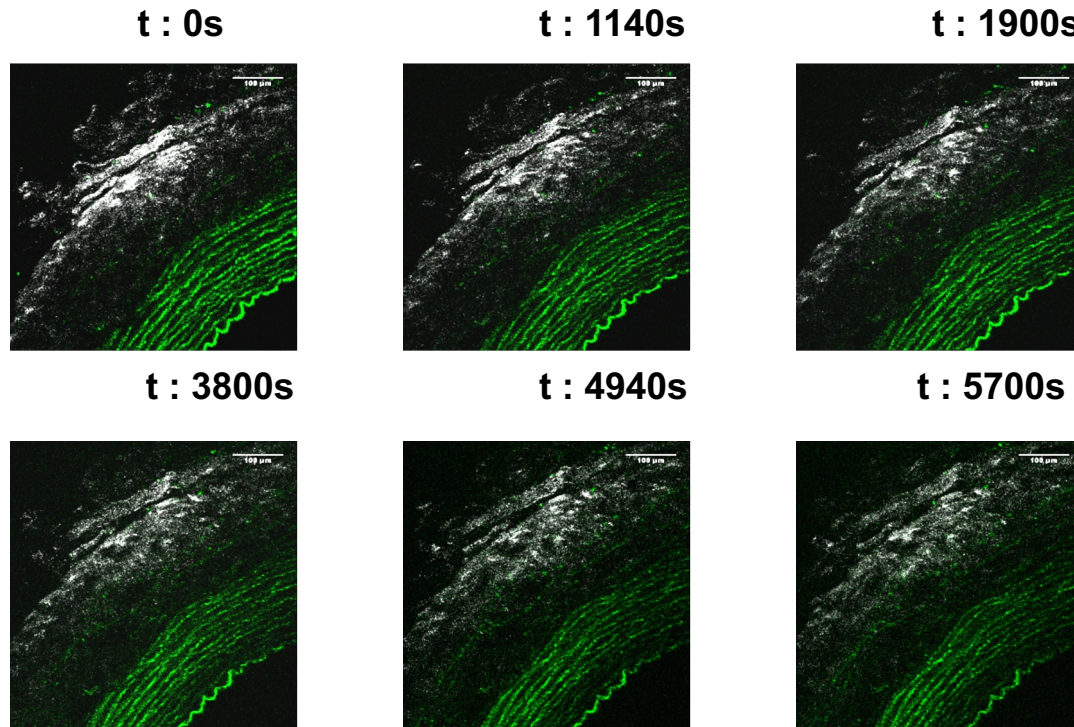
Carotide

- New system for non-invasive exploration of synthesized collagen network inside mesenchymal stem cell functionalized biomaterials dedicated to clinical implantation in cartilage lesion. D. Dumas, Osteoarthritis and Cartilage, Volume 20, S129-S130, 2012.
- Collagenous extracellular matrix of cartilage submitted to mechanical forces studied by second harmonic generation microscopy. Elisabeth Werkmeister, Photochemistry and Photobiology. 86(2):302-10, 2009.

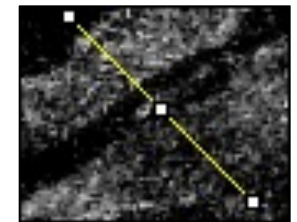
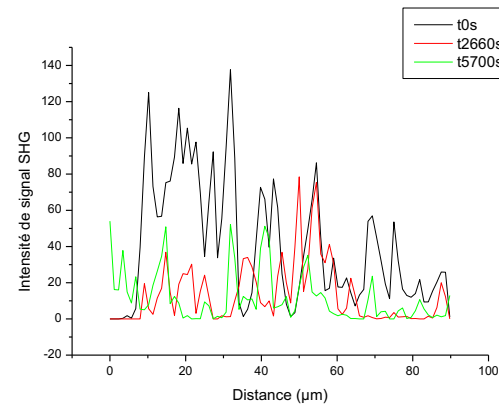
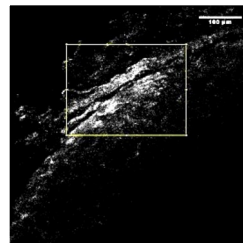
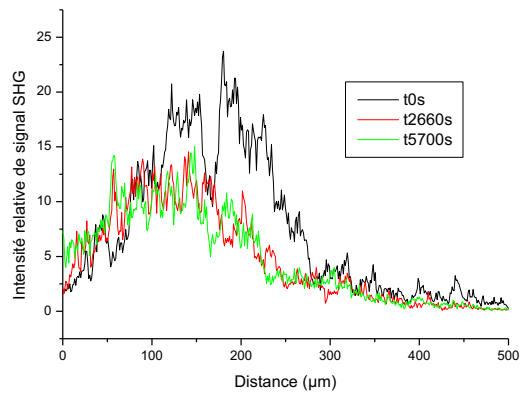
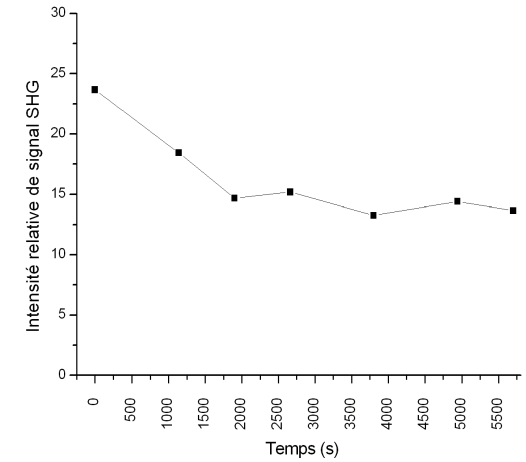
D.III. Microscopie à excitation Multiphototonique SHG



D.III. Microscopie SHG : Spécificité du signal SHG / action de la collagénase



Moyenne du signal SHG sur la ROI1
Artère Fémorale de rat

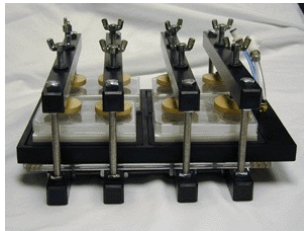


D.III. Microscopie SHG : cartilage articulaire et pathologies dégénératives

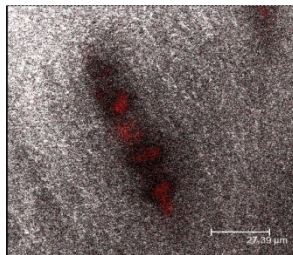
outil de validation en ingénierie tissulaire (propriétés bio- et mécaniques)

Stress mécanique (choc) ou chimique (IL-1 β , hypoxie, MMP...) dans l'arthrose articulaire

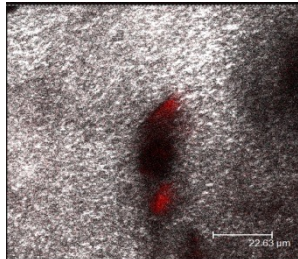
→ caractérisation des organisations 3D et composition matricielle (collagène, PAG..)



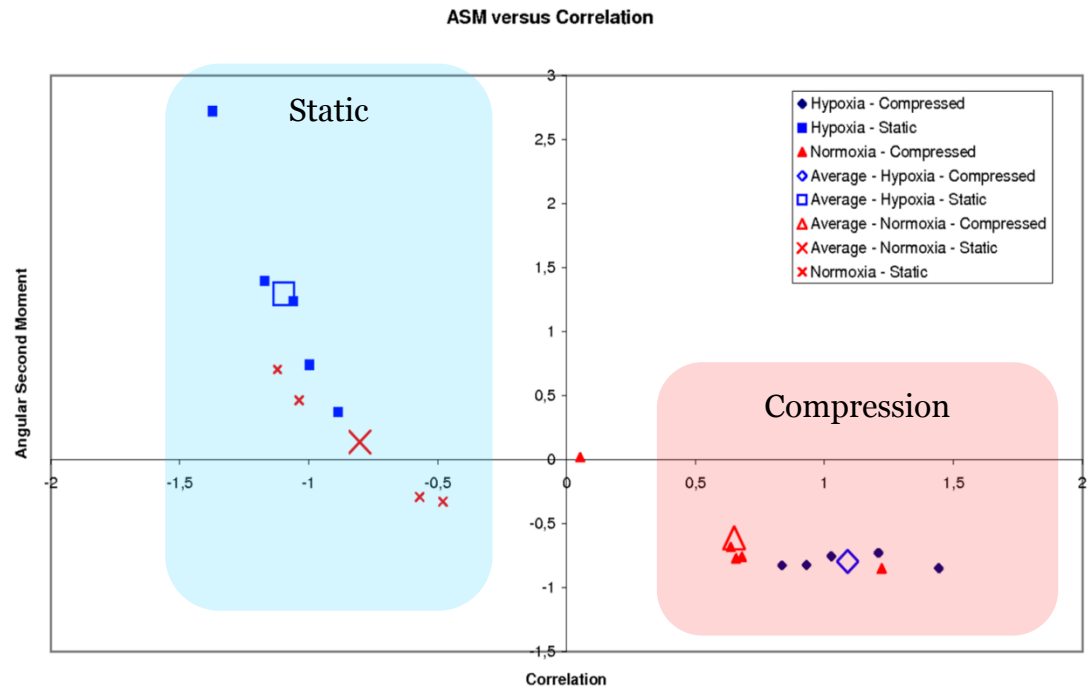
Static



Compression



1MPa ; 1Hz; Intermittent Sinusoïdal
½ h activité; ½ h arrêt; Manip 7 h au total

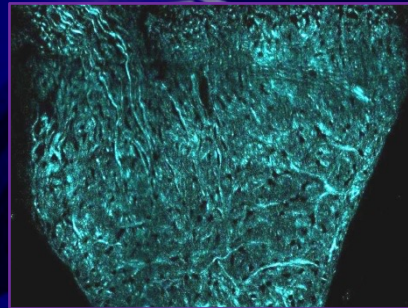
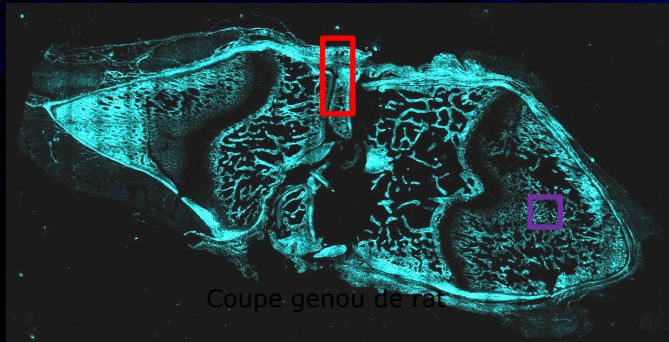


Conclusion : indicateur de reconstruction / désorganisation des matrices tissulaires

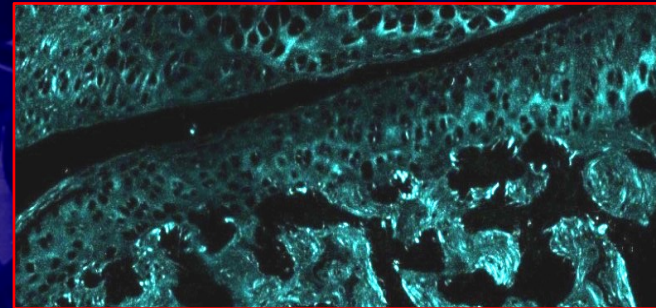
D.III. Microscopie SHG : quantification par analyse de texture

Paramètres de textures : Haralick et al; 1973)

- Matrice de co-occurrence : Moment de la Différence Inverse (IDM), Second Moment Angulaire, Corrélation(ASM), Entropie...



Cartilage articulaire

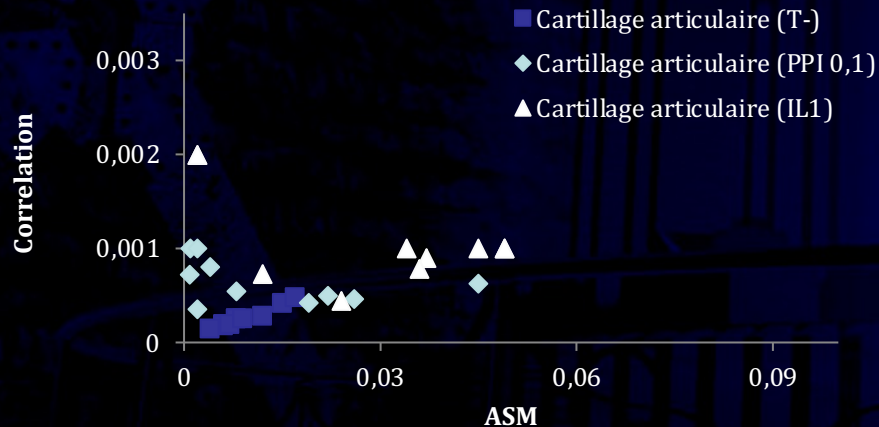


Languelette Sternale

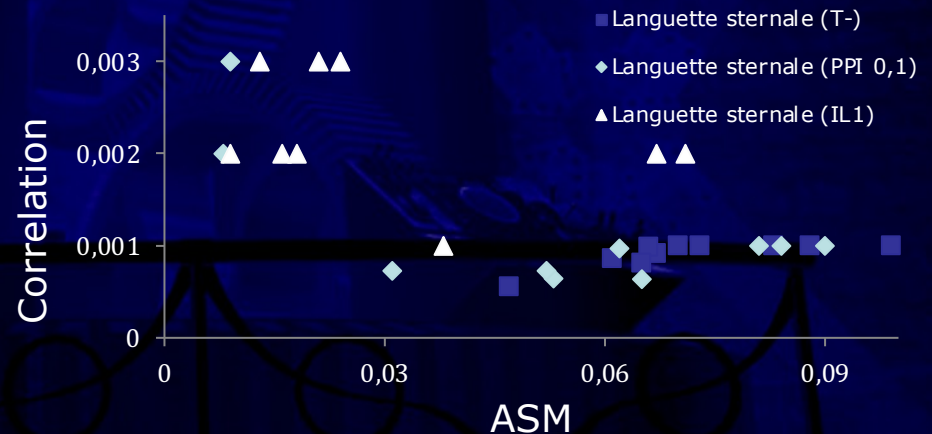
3 types d'échantillon :

- Genou de rat témoin
- Genou de rat traité avec IL1
- Genou de rat traité au PPI 0,1

sur le cartilage articulaire

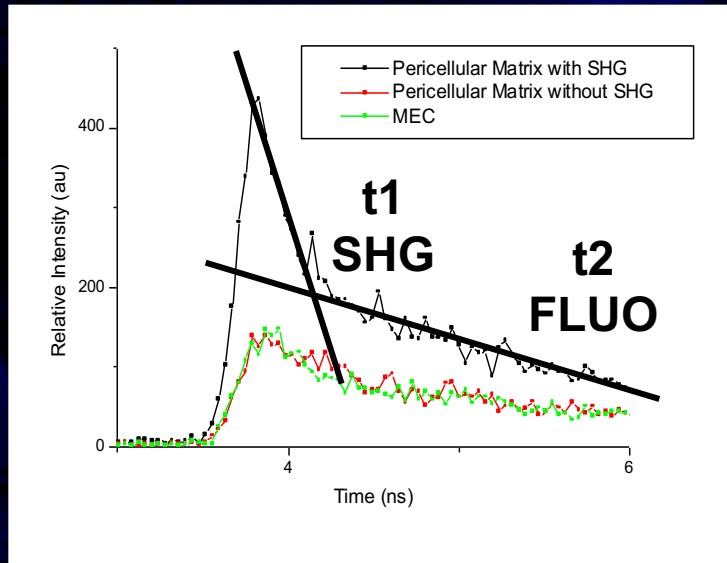


sur la languelette sternale

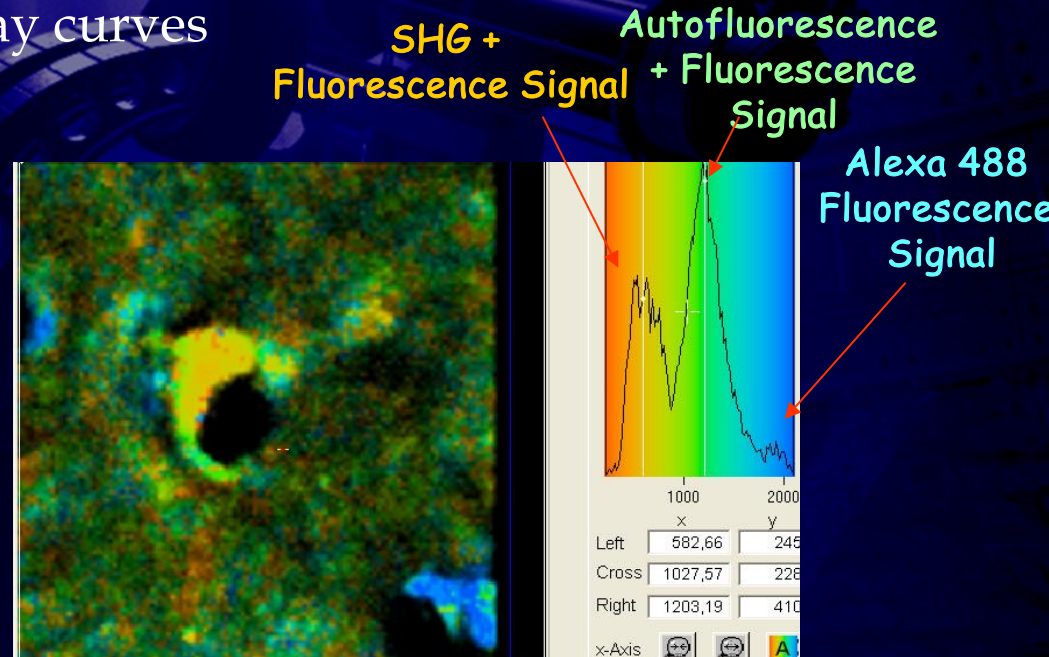


- III. Micro- TCSPC- SHG imaging : **time decay in matrix**

Fluorescence Lifetime Imaging Microscopy
Biexponential adjustment of Decay curves



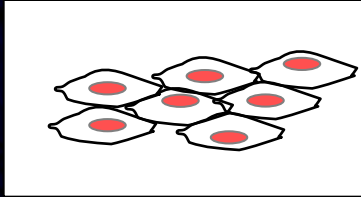
Fluorescence Decay Curves



*Mean Fluorescence lifetime (τ_m)
Color coded image*

Réseau de Collagène en scaffold : **Imagerie 3D non-invasive**

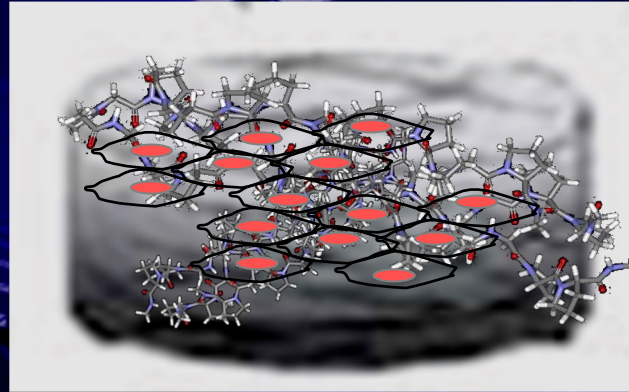
CSM



Scaffold

IMAGERIE DE PHASE
(différenciation)

Expansion
In Vitro



Scaffold + CSM

Pas d'agent de contraste

Implantation
In vivo



Animal

MACROSCOPIE
IMAGERIE SHG
(collagène)

1. Evaluation Qualitative de la différenciation cellulaire
2. Evaluation Qualitative des réseaux de **collagène**
3. Evaluation Quantitative : **prolifération cellulaire** / **synthèse**
4. Observation **Multi-échelle** (de la cellule à l'implant, implant à l'animal)

De la Micro vers la Macro : MULTIPHOTON-SHG

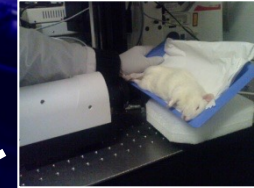
tissu



organe



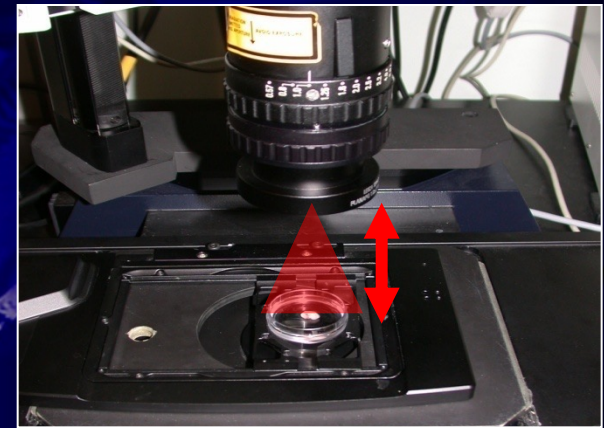
petit animal



Microscopie



Dénaturation du matériel biologique :
- physique (coupes)
- chimique (marquage)

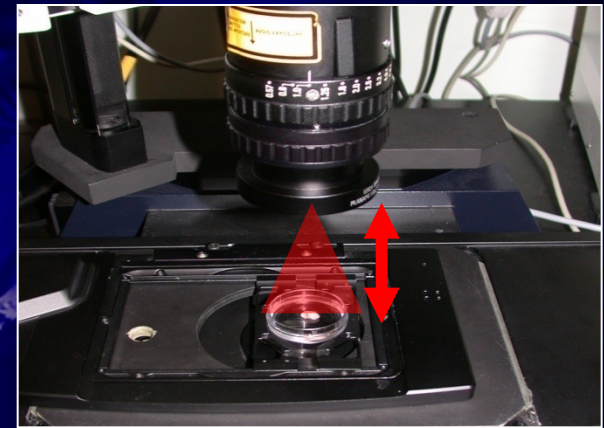
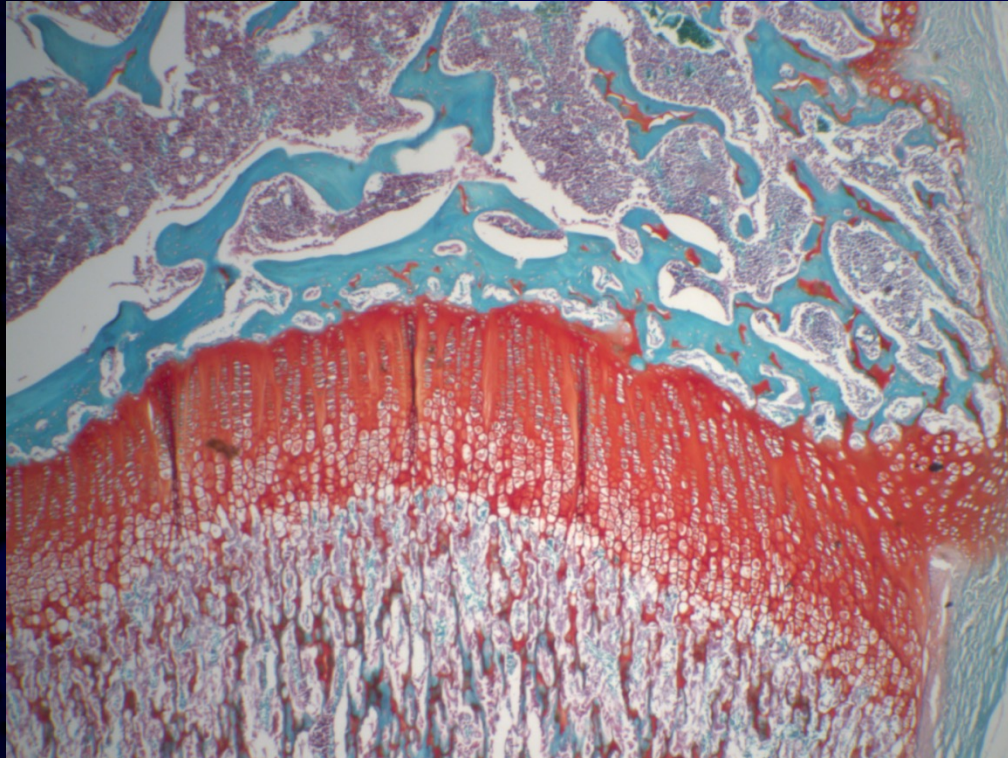


Macroscopie

**Cartographie
non-intrusive**

- * Grande dimension du champs observé : **5 mm** (5x) - **112mm** (x0.32)
- * Grande Distance de Travail (WD) : **19 mm** (5x) – **297 mm** (0.32x)

Imagerie multi-échelle : de la Microscopie à la Macroscopie



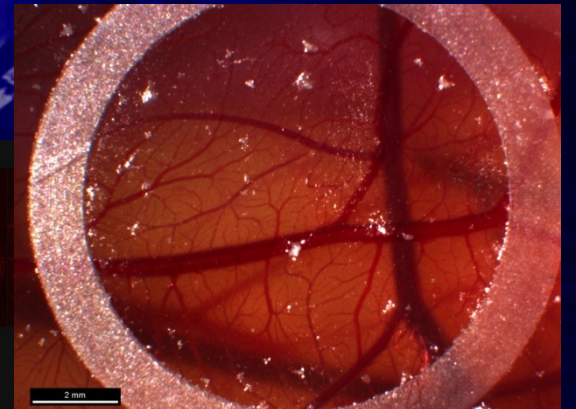
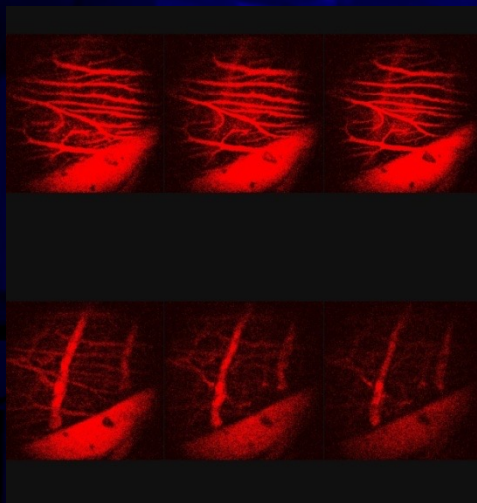
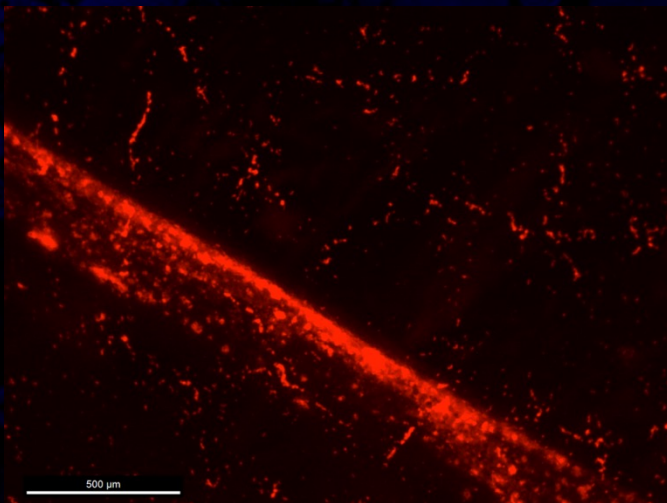
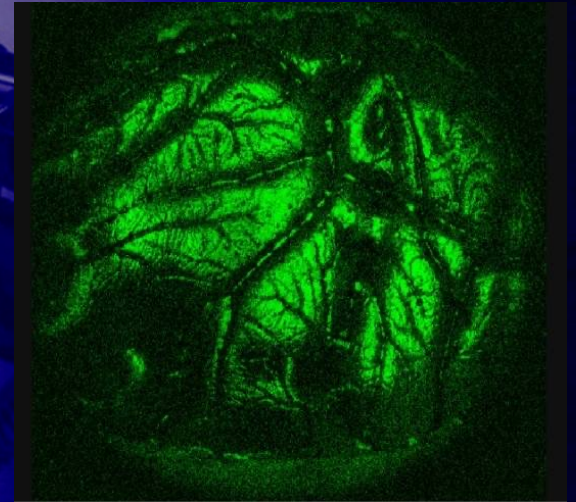
Macroscopie

**Cartographie
non-intrusive**

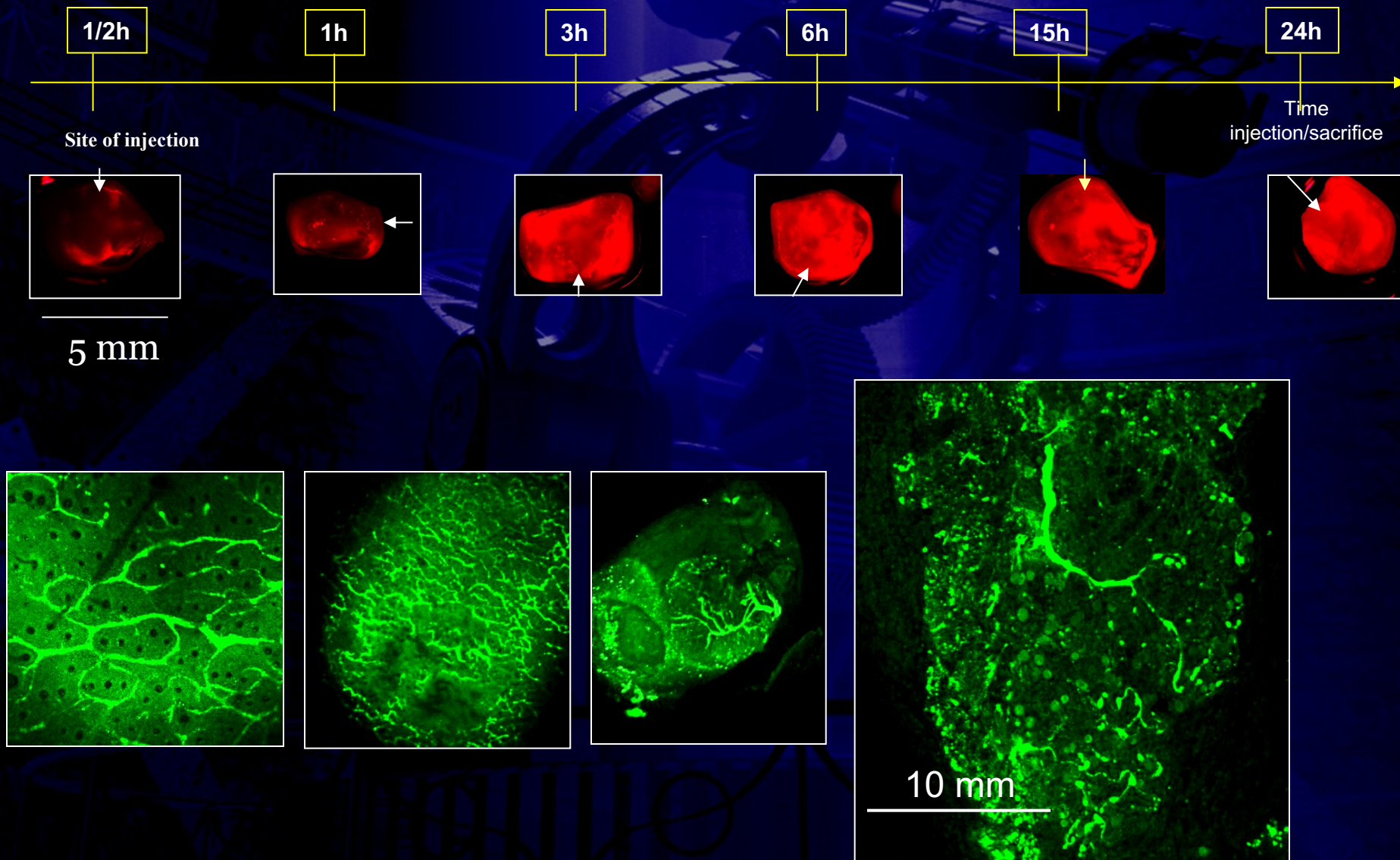
* Grande dimension du champs observé : **5 mm** (5x) - **112mm** (x0.32)

* Grande Distance de Travail (WD) : **19 mm** (5x) – **297 mm** (0.32x)

Imagerie multi-échelle : imagerie in situ (PDT)

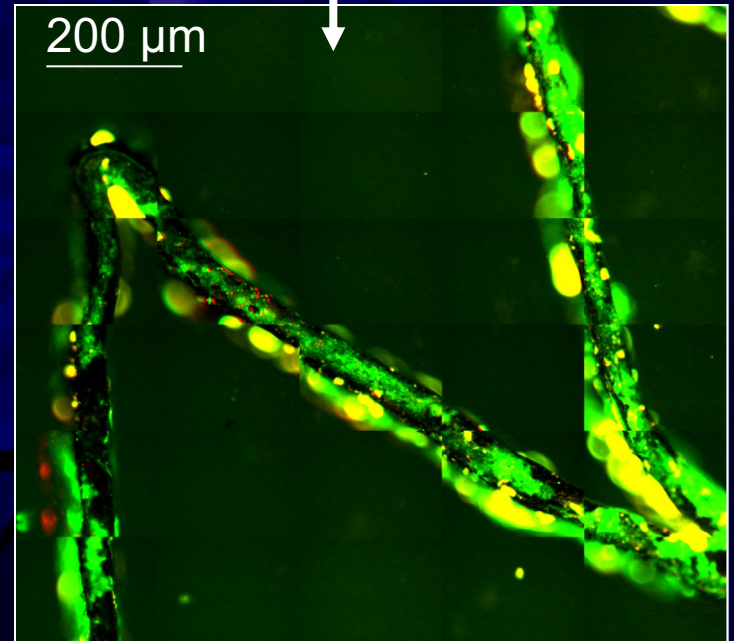
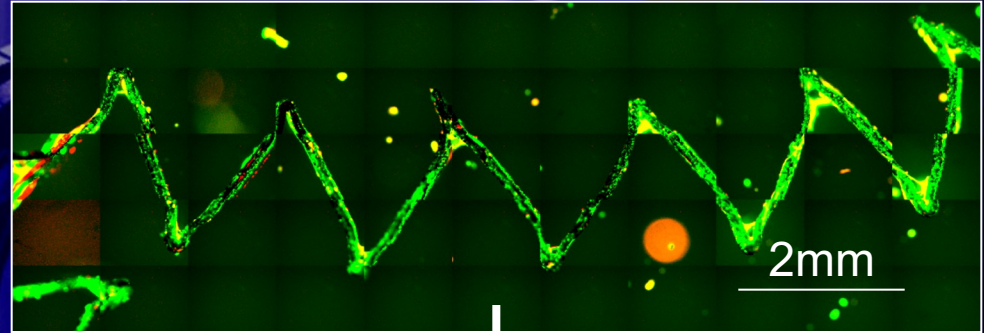
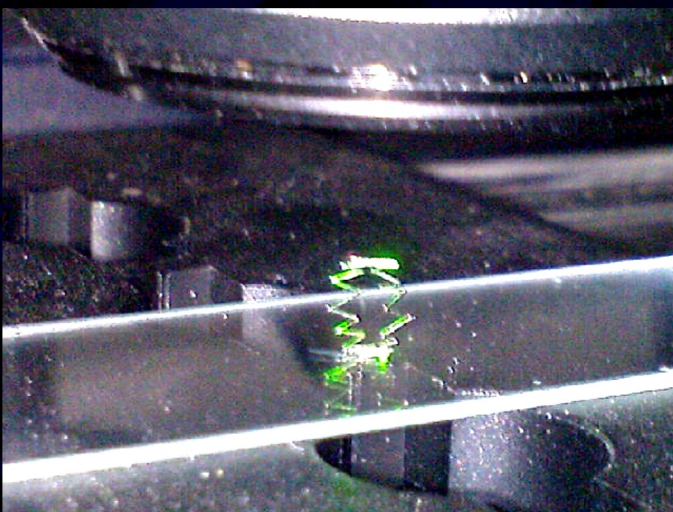
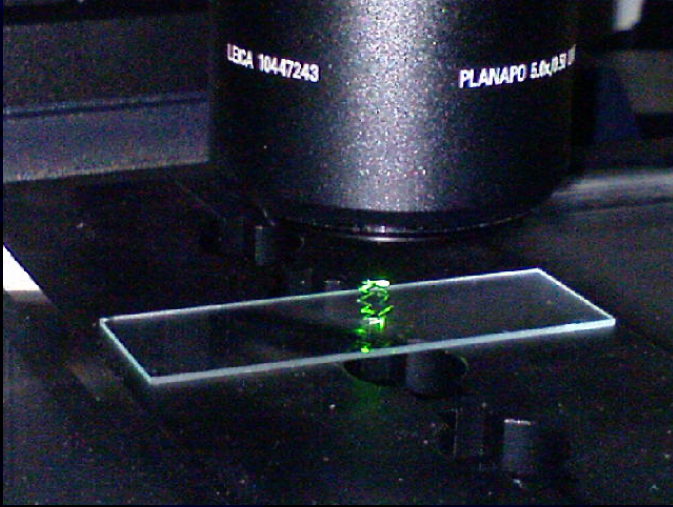


• Cinétique de diffusion intratumorale de PS en PDT



- **Mosaiquage de grands champs : reconstruction 2D/3D**

Stent, film MPE/témoin, Huvec (CD32, vW)

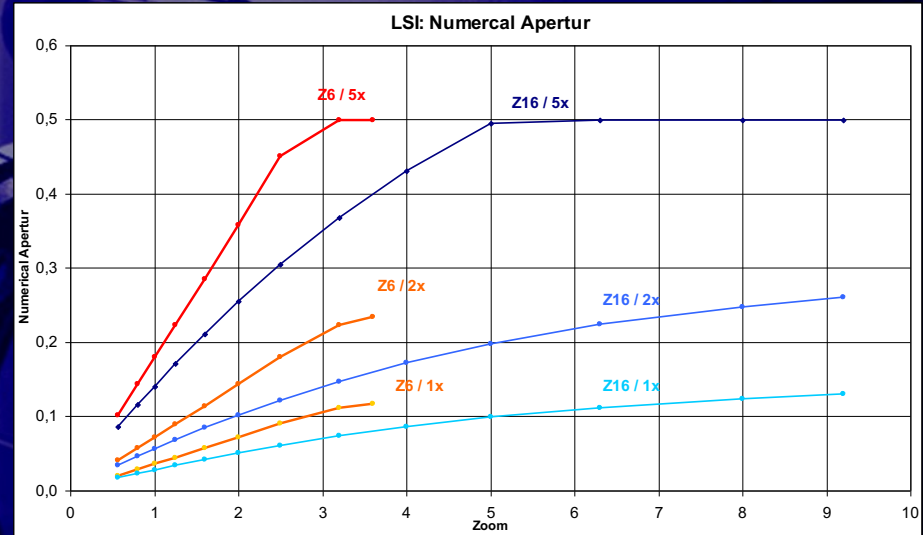
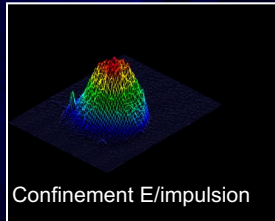
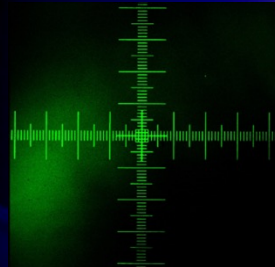




- Bio-construction : sélection de supports de culture optimisé (marquage fluorescent)
- Validation de process qualité (sans agent de contraste)

PTIBC IBISA – BIOPOLE : SP5 Macromultiphoton et Calibration

PCT-UL/CNRS n° PCT/FR2012/051156 (2012)

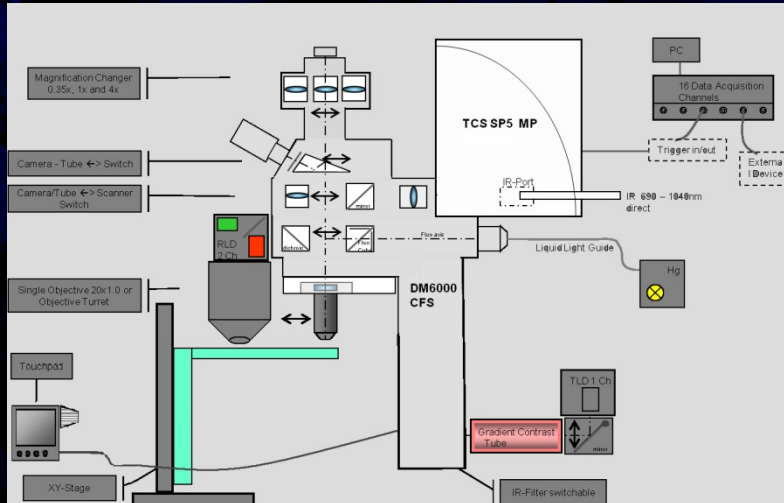


XY resolution : $d = 0.5 \times \lambda / NA$

Z resolution: $t = \lambda / (NA)^2$

Field of View [mm]

edge length			
Obj x1	Obj x2	Obj x5	Mag
21,23	10,61	4,25	0,57
15,12	7,56	3,02	0,8
12,10	6,05	2,42	1
9,68	4,84	1,94	1,25
7,56	3,78	1,51	1,6
6,05	3,02	1,21	2
4,84	2,42	0,97	2,5
3,78	1,89	0,76	3,2
3,36	1,68	0,67	3,6

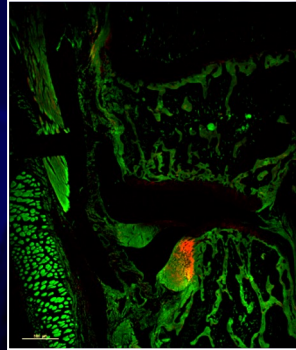


Brevet WO 2012160312 A3 (UL/CNTRS): Improved biomedical imaging with multiphoton excitation Improved biomedical imaging with multiphoton excitation.
(D.Dumas, S. HuPont).

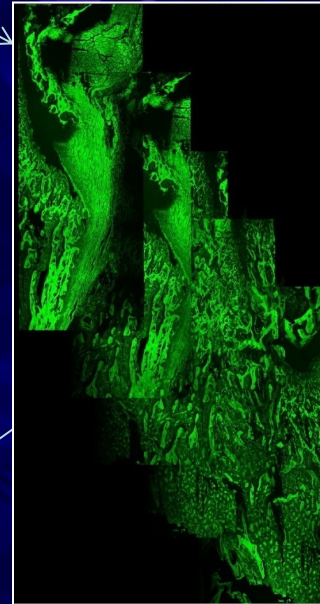
Imagerie « lame entière »



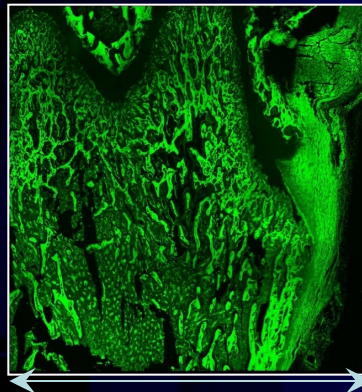
Plan 2D (X80.8)



Coupes sériees

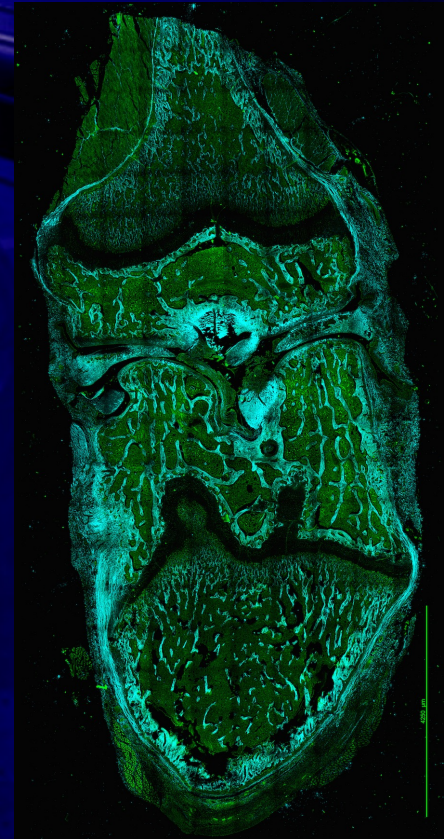


Mosaïque - 50%



2,1 cm

Macroconfocal



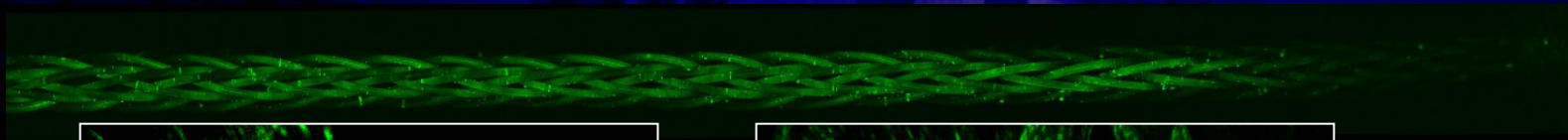
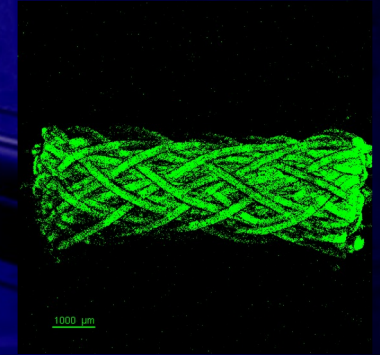
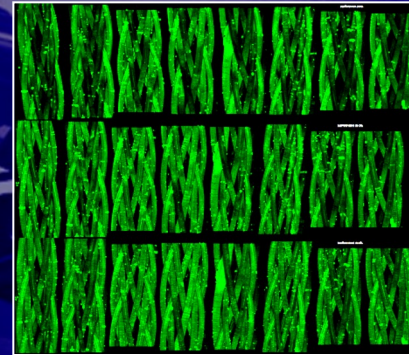
Mosaïque - 10%
1,5 cm²

MacroMP-SHG

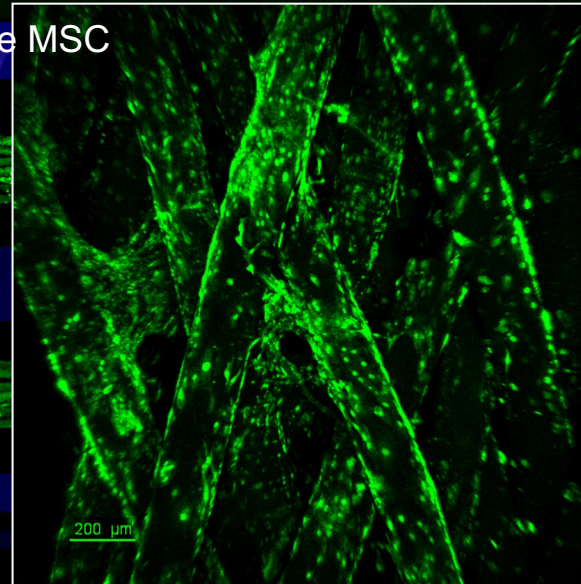
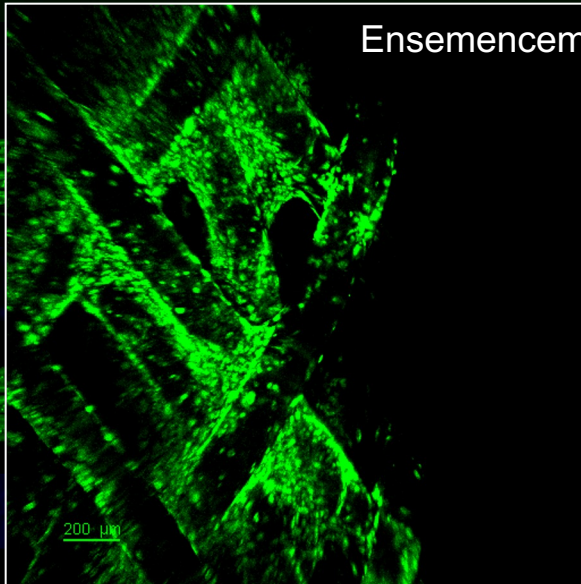
SP5 Macromultiphoton : 3D, mosaïquage et zooming

Scaffold complet 4cm long : acquisition (4h).

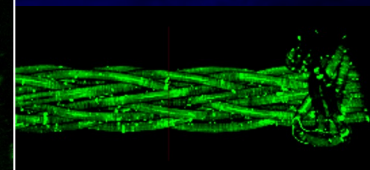
1cm



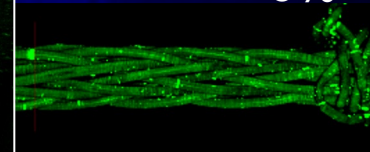
Ensemencement de MSC



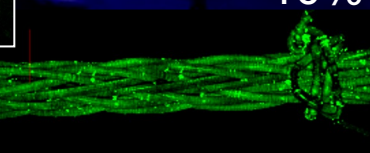
5%



6%

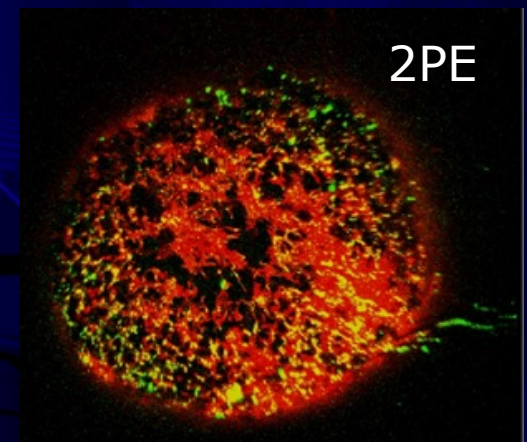
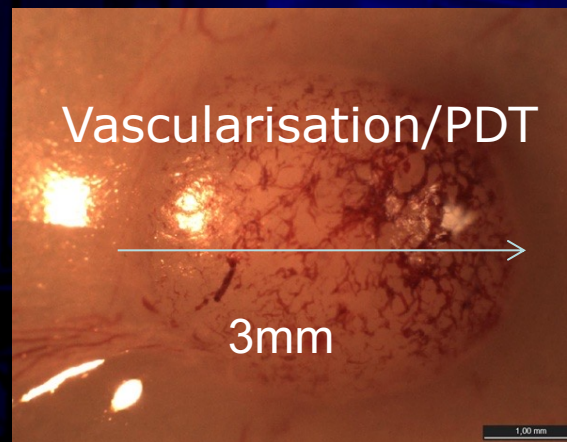
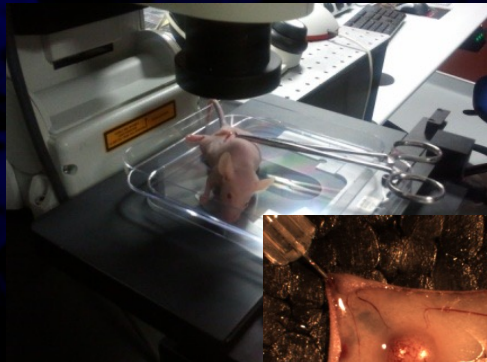
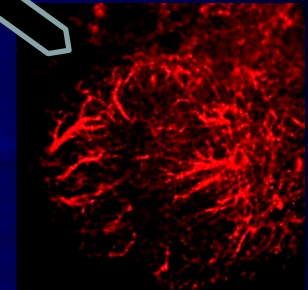
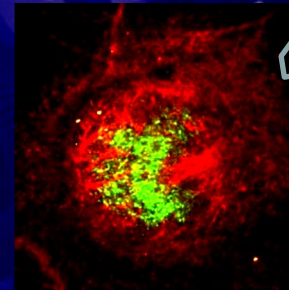
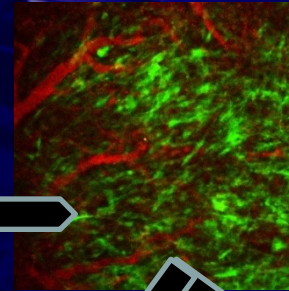
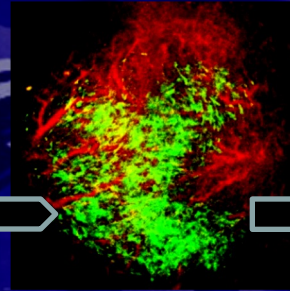
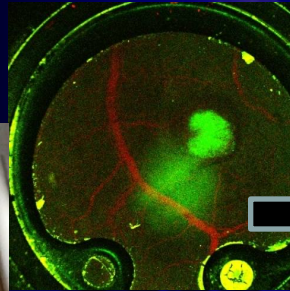
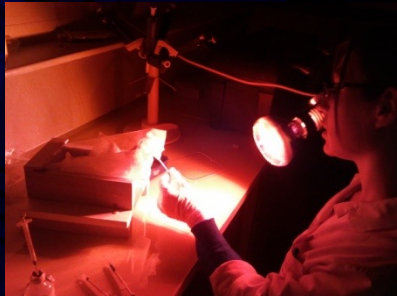


10%



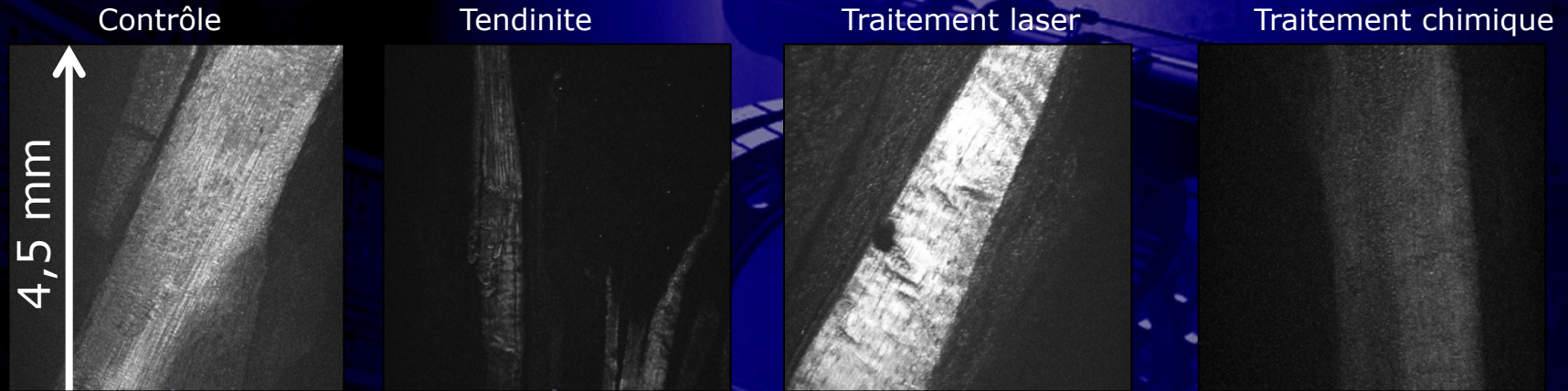
SP5 Macromultiphoton : multi-modalités TPE – AF – SHG

Tumeur dans une fenêtre intravitale (souris)



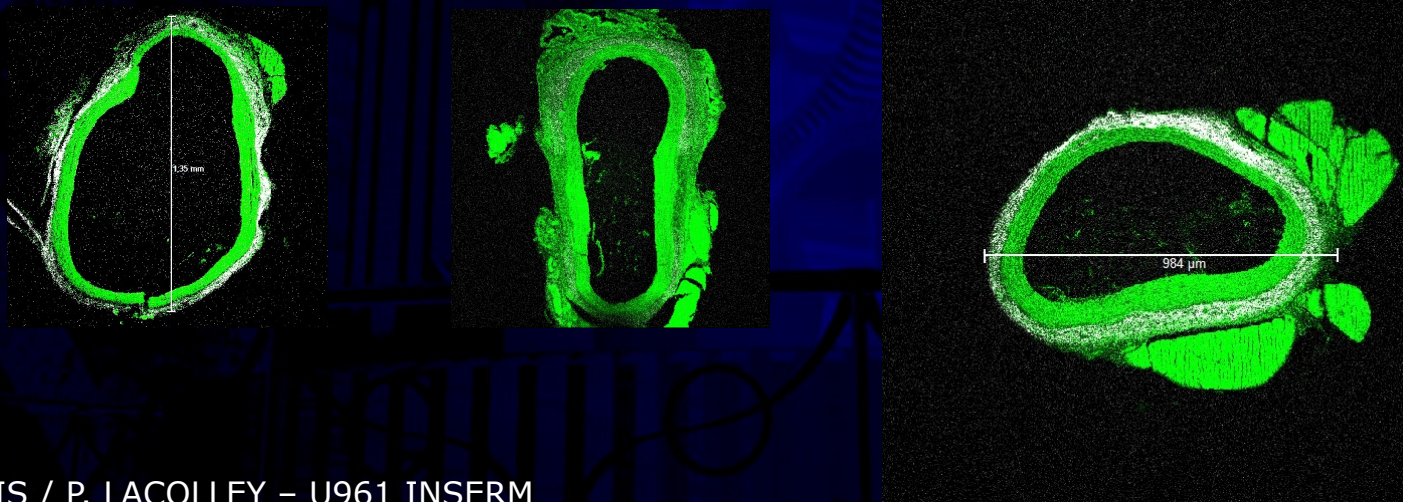
SP5 Macromultiphoton : multi-modalités TPE – AF – SHG

Tendon de rat (contraintes mécaniques)



D.DUMAS / R.LABAT MARCOS – PTIBC Ibisa FR3209 / IMOPA / ICB1 U Sao Paulo, Brasil

Vaisseau de Rat : Alimentation / épaissement de la paroi

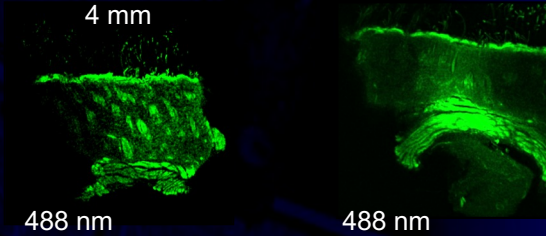


H.LOUIS / P. LACOLLEY – U961 INSERM

SP5 Macromultiphoton : multi-modalités TPE – AF – SHG

Peau de rat

4 mm



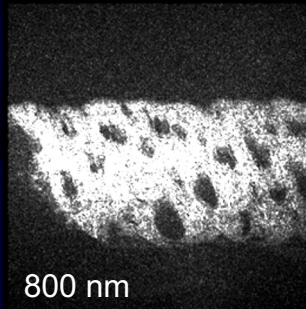
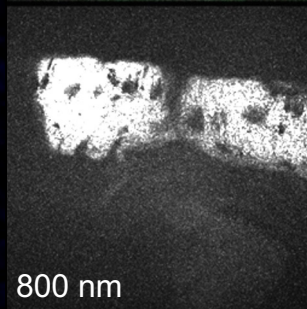
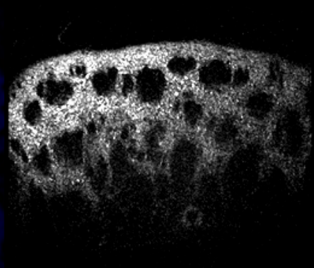
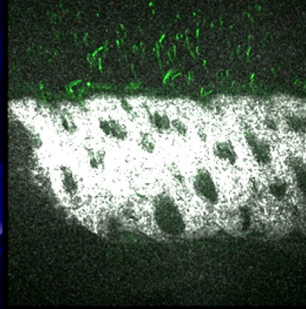
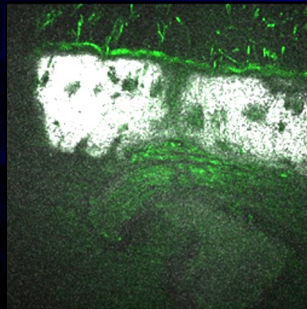
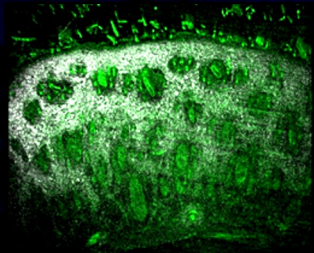
488 nm

488 nm

Contrôle

Cicatrice

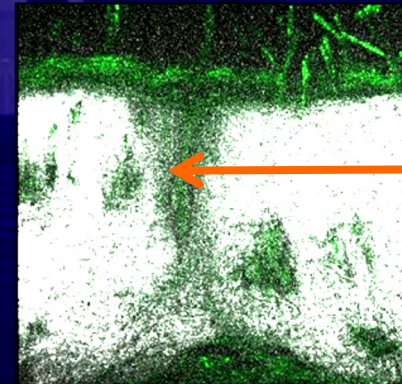
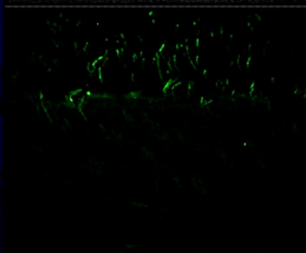
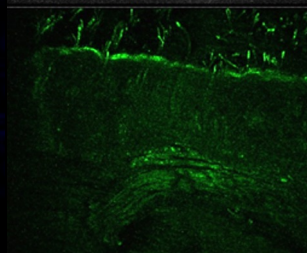
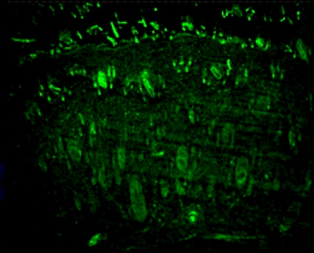
Traitement laser



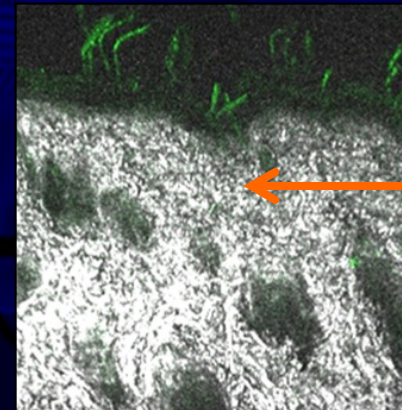
800 nm

800 nm

800 nm



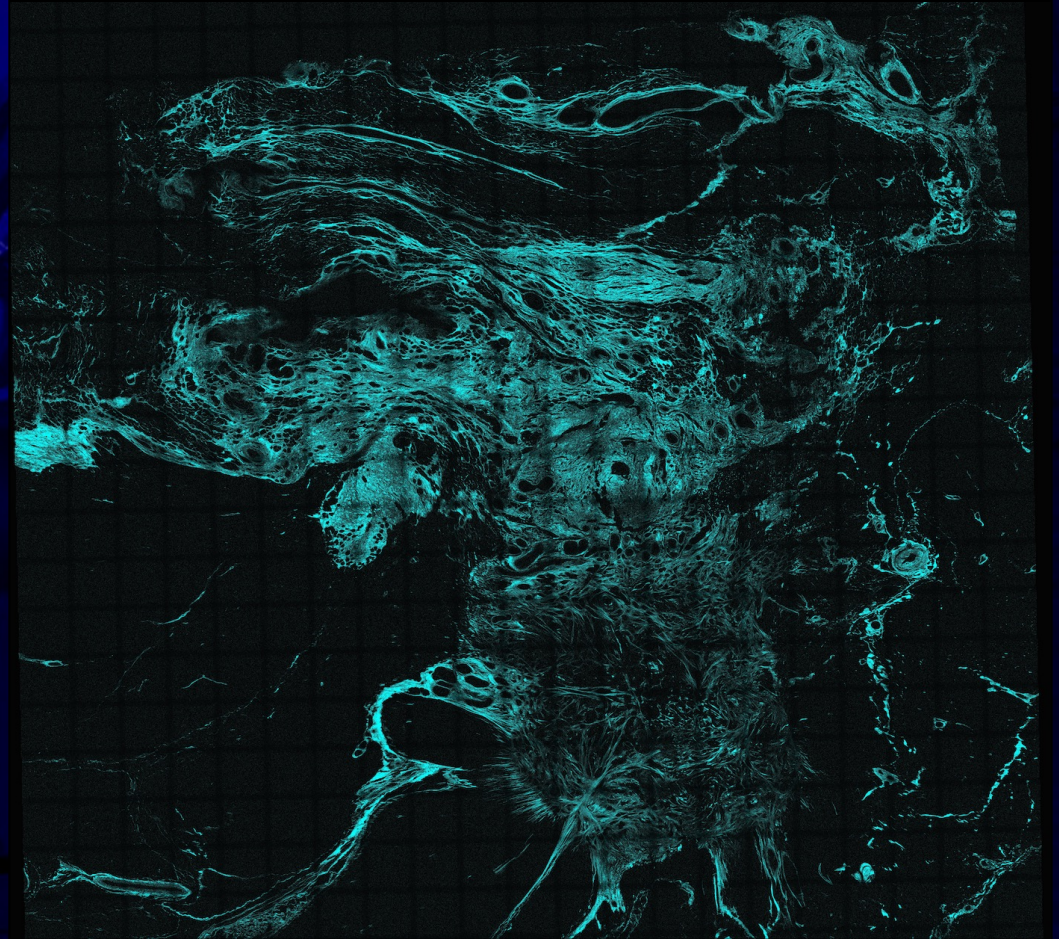
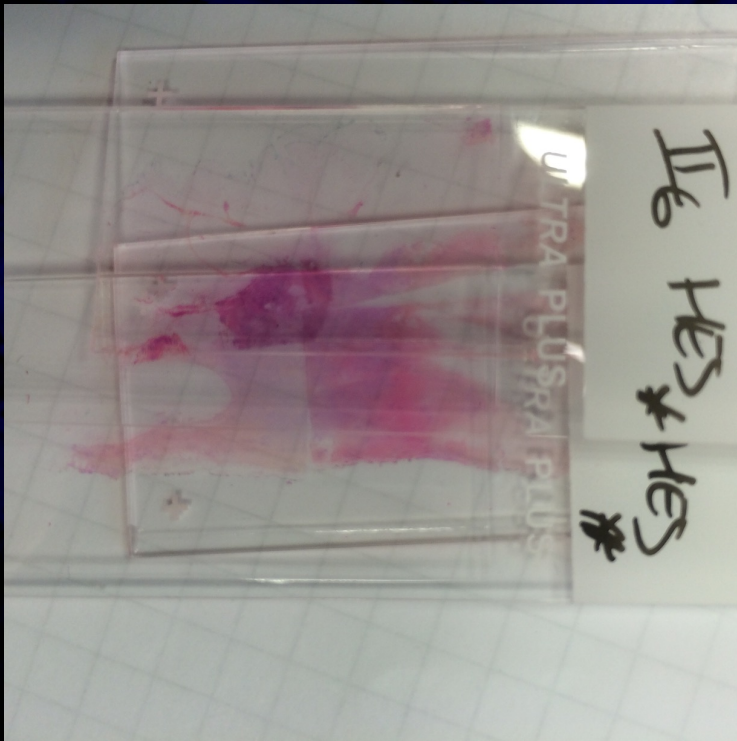
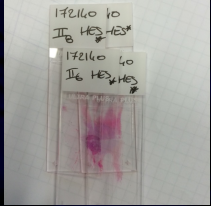
Tissu fibreux



Matrice

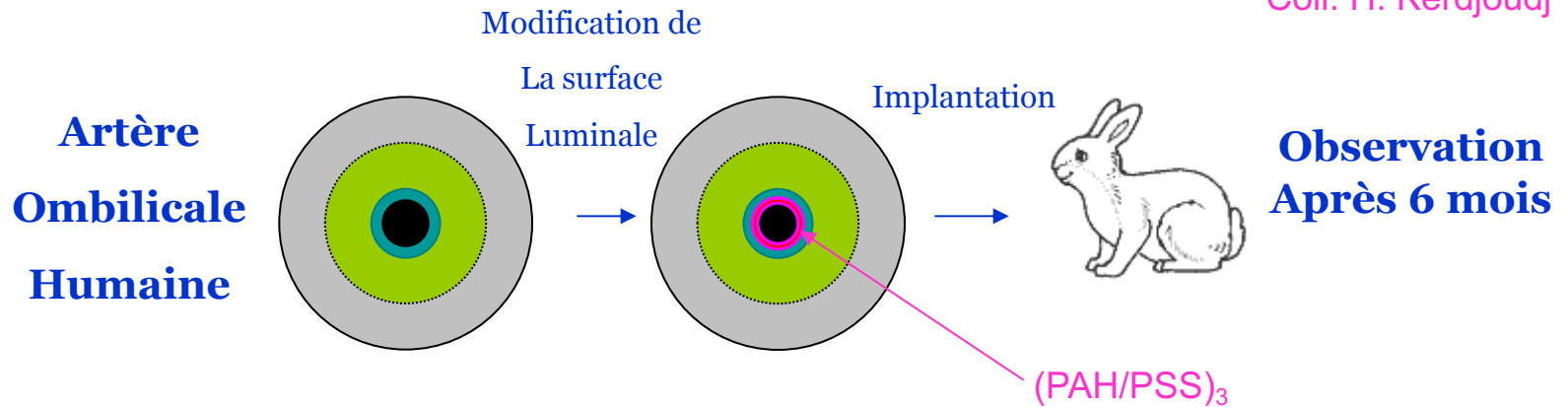
- **Fibrose tissulaire : Path-Image (Caen) / ICL-CRAN**

Tumeur entière (cancer du sein) : dimension 3,5 cm
Reconstruction du Collagène et de l'élastine sans marqueur.

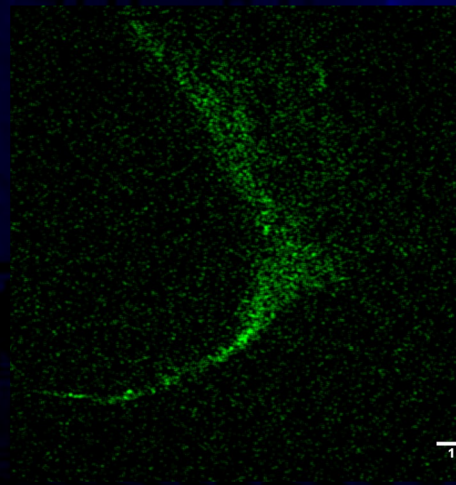


- **Contraste SHG : remodelage vasculaire**

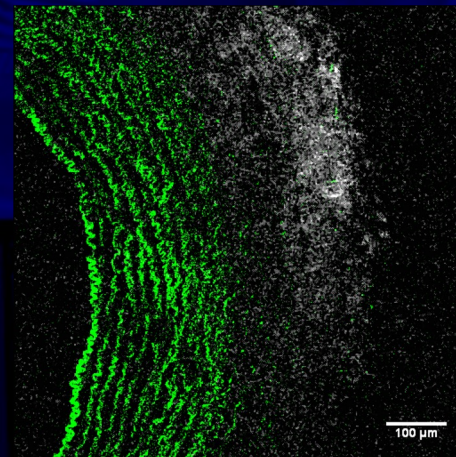
Coll. H. Kerdjoudj



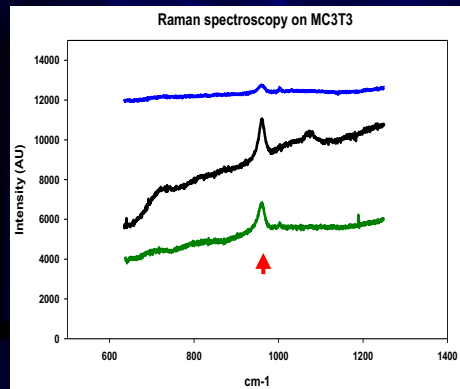
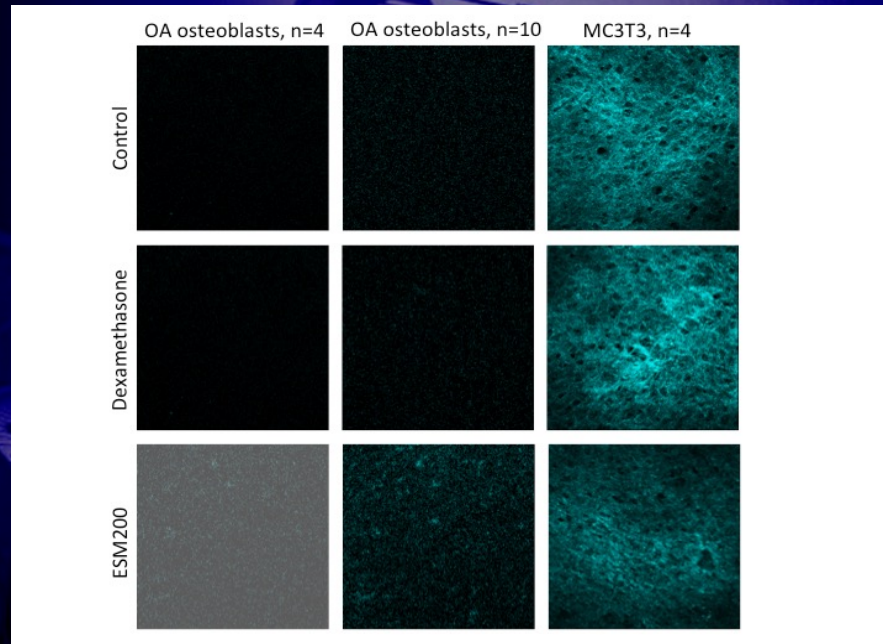
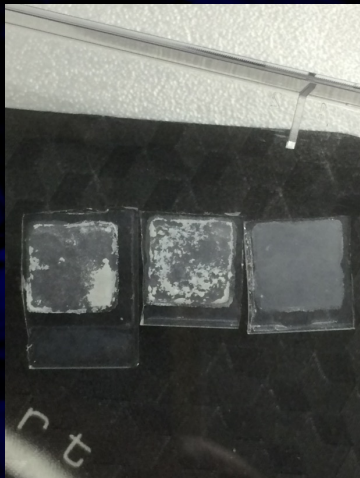
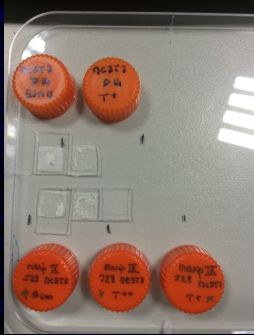
**Artère Ombilicale Humaine
avant implantation**



**Artère ombilicale humaine 6
mois après implantation
(lapin)**



Matrice Extracellulaire et SHG (cellules OA/fibroblaste)



Nanograins de phosphate
(M. DOSSOT)

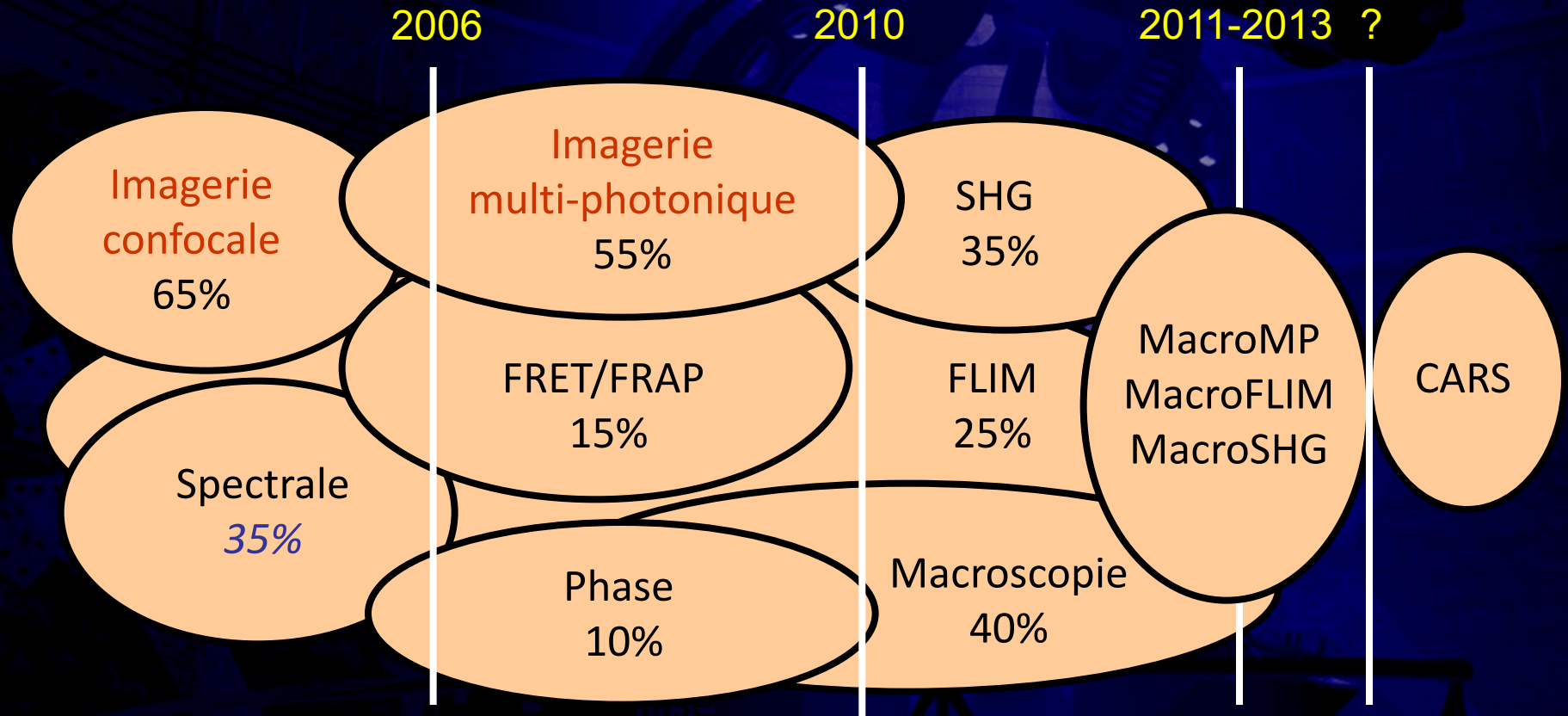
CARS
Cellules Vivantes

IMAGERIE STANDARD

Modalité simple : **Imagerie Confocale**

APPLICATIONS AVANCEES

Multi-modalités : **Nouveaux outils de détection en microscopie multi-photonique. F-techniques**



Spectral : Fluorescence Spectral microscopy
FRET : Förster Resonance Energy Transfert
FRAP : Fluorescence Recovery after Photobleaching

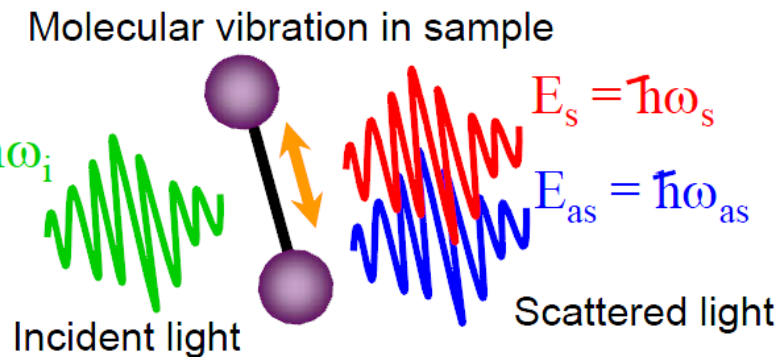
SHG : Second Harmonic Generation
FLIM : Fluorescence Lifetime Imaging Microscopy (OSEO)
FCS : Fluorescence Correlation Spectroscopy

D.III. Imagerie vibrationnelle Raman-CARS

Raman scattering is the interaction of photons and intrinsic molecular bonds



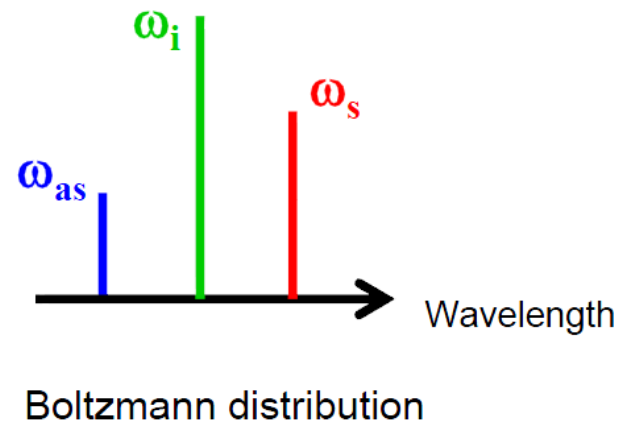
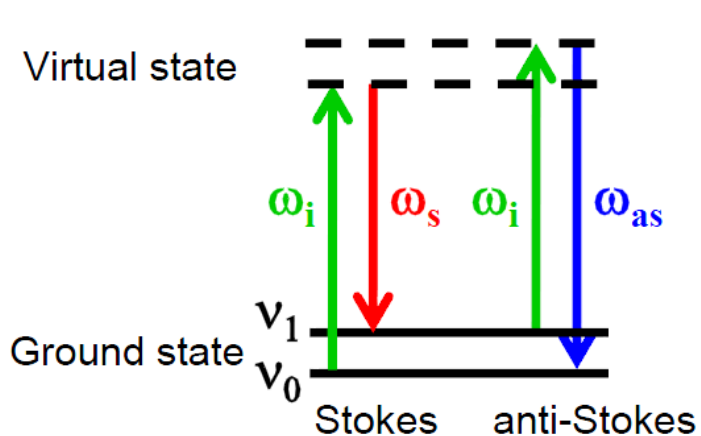
C.V. Raman
1930 Nobel Prize



Excited state

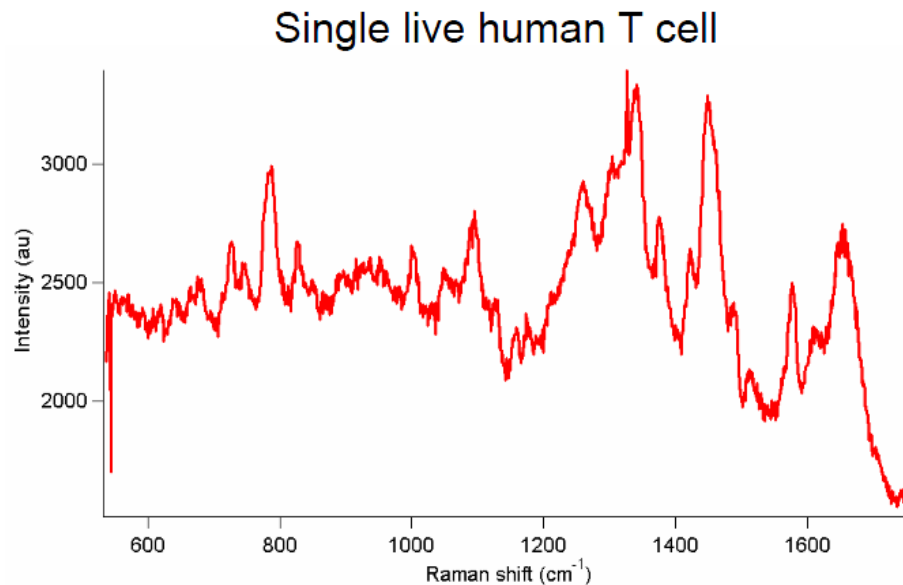


Two horizontal black lines representing the excited state energy levels.



D.III. Imagerie vibrationnelle Raman-CARS

Raman spectra of cells provide a wealth of biological information



$$k = 1/\lambda \text{ (cm}^{-1}\text{) Wavenumber units}$$

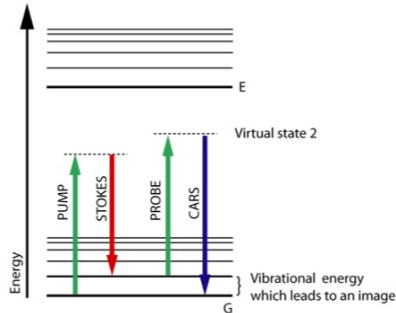
$$\text{Raman shift} = (1/\lambda_{\text{incident}}) - (1/\lambda_{\text{scattered}})$$

Raman frequency in wavenumber units (cm ⁻¹)	Assignment*
618	p: C-C twist
640	p: C-S str., C-C twist Tyr
666	G, T, Tyr, G bk in RNA
678	G (C-2'-endo-anti)
725	A
746	T
785	U, T, C, bk: O-P-O
831	O-P-O asym. str., Tyr
852	Tyr. ring breath.
893	bk, p: C-C skeletal
1003	Phe, C-C skeletal
1031	Phe, p: C-N str.
1053	C-O str., p: C-N str.
1093	O-P-O sym. str., p: C-N
1126	p: C-N str.
1156	p: C-C, C-N str.
1175	Tyr, Phe, p: C-H bend
1208	A, T, p: amide III
1257	A, T, p: amide III
1263	T, A, p: C-H bend
1302	P: amide III
1318	G, p: C-H def.
1337	A, G, p: C-H def.
1373	T, A, G
1421	A, G
1447	p: C-H ₂ def.
1485	G, A
1510	A
1575	G, A
1605	Phe, Tyr, p: C=C
1615	Tyr, Trp, p: C=C
1655-1680	p: amide I, T, G, C

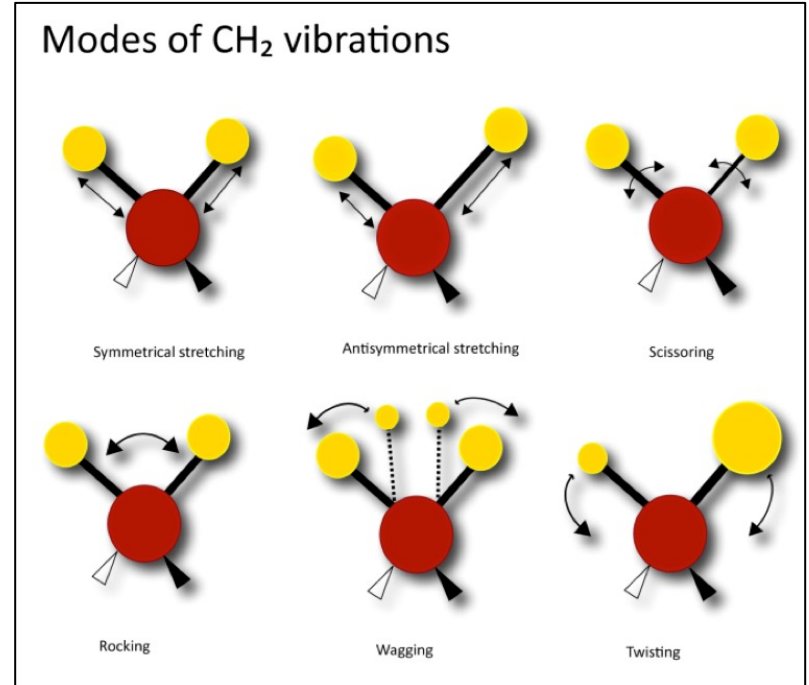
D.III. Imagerie vibrationnelle Raman-CARS

Interaction lumière : **Microscopy CARS** (Coherent Anti-Stokes Raman Scattering)
= Microscopie Vibrationnelle

- Processus non linéaire du 3ème ordre :



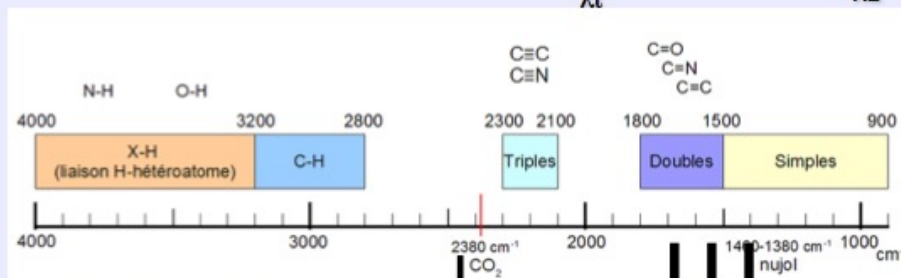
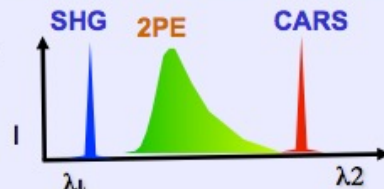
- Faisceau de pompe (fréquence ω_p)
- Faisceau Stokes (fréquence ω_S)
- parfait alignement des faisceaux



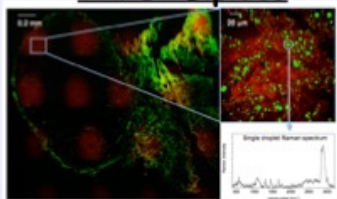
- Différence de fréquence entre ω_p et ω_S
- = fréquence énergie vibrationnelle d'une liaison chimique particulière
- contraste vibrationnel
- → signal Anti-Stokes : $\omega_{AS} = 2 \omega_p - \omega_S$
- → image
- →

D.III. Imagerie vibrationnelle Raman-CARS

Imagerie Moléculaire Vibrationnelle



CARS : Lipides



Imagerie sans agent de contraste
Non invasive
Non intrusive

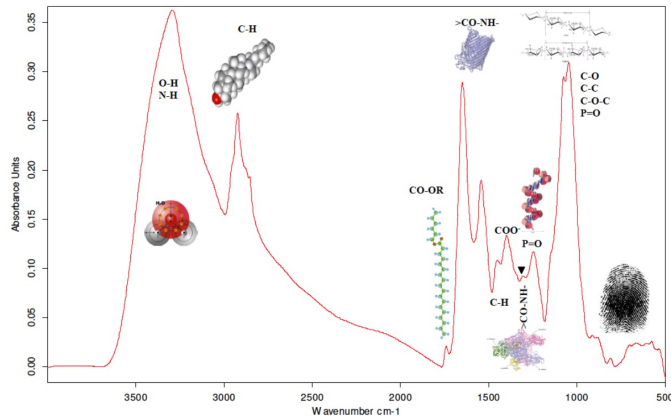
- Balayage / signatures vibrationnelles
- Imagerie CARS/ famille de molécules

TCS CARS - Vibrational Modes - Wave numbers

Wave number [cm ⁻¹]	Vibration	Compound
1205	C6H5-C vibration	alkyl benzenes
1200	ring vibration	para-disubstituted benzenes
1251	in-plane CH deformation	cis-dialkyl ethylenes
1295	CH2 in-phase twist	n-alkanes
1290	in-plane CH deformation	trans-dialkyl ethylenes
1330	CH deformation	isopropyl group
1368	CH3 symmetric deformation	n-alkanes
1370	ring stretch	naphthalenes
1385	ring stretch	anthracenes
1465	CH3 deformation	n-alkanes
1446	CH3, CH2 deformations	n-alkanes
1550	ring stretches (doublet)	benzenes derivatives
1590	NH2 scissors	primary amines
1649	symmetric C=O stretch	carboxylic acids (cyclic dimer)
1700	C=O stretch	aliphatic ketones
1720	C=O stretch	aliphatic aldehydes
2100	C≡C stretch	alkyl acetylenes
2231	C≡C stretch	dialkyl acetylenes
2232	C≡N stretch	aliphatic nitriles
2560	SH stretch	thiols
2849	symmetric CH2 stretch	n-alkanes
2883	symmetric CH3 stretch	n-alkanes
2912	anti-symmetric CH2 stretch	n-alkanes
2965	anti-symmetric CH3 stretch	n-alkanes
3000	aromatic CH stretch	benzene derivatives
3330	bonded antisymmetric NH2 stretch	primary amines

D.III. Imagerie vibrationnelle Raman-CARS

EXEMPLE D'UN SPECTRE INFRAROUGE



Les modes RAMAN

Raman ordinaire (10⁻³M)

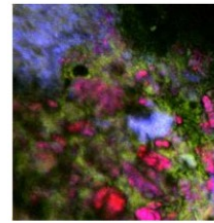
- Caractérisation moléculaire
- Structure des macromolécules
- Caractérisation fonctionnelle
- Domaine λ : UV à proche IR. Choix guidé par les processus de fluorescence. Sur systèmes cellulaires : 785 nm, 830 nm

Raman de résonance (10⁻⁵M)

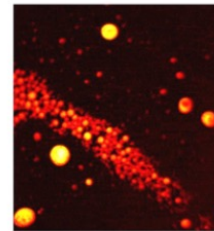
- Sélectivité moléculaire
- Haute sensibilité : **vibrations des chromophores (Intensité 10⁶)**
- Caractérisation fonctionnelle
- Domaine λ : UV - visible. Choix guidé par le/les domaines d'absorption du/des chromophores. Possibilités d'excitation à multi λ

Raman SERS (diffusion Raman exaltée 10⁻¹²M)

- Haute sensibilité
- Sélectivité moléculaire (SERS résonnant)
- Sélectivité spatiale
- Domaine λ : Visible à proche IR. Choix guidé par la nature des substrats SERS

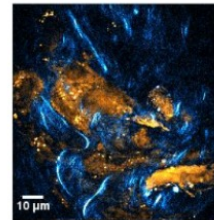


Many new drugs are badly soluble and require complex packaging to ensure their bioavailability at the site of interest. Analysis of the complex oral dosage forms is essential to the understanding of the dissolution behaviour. Using labels for imaging is not possible because it distorts the fabrication process and the dissolution. Coherent Raman imaging provides fast analysis of just a few seconds per image of oral dosage forms with high spatial resolution (200nm). It can also measure static distributions of APIs and their crystalline form, and even show physio-chemical alteration during dissolution. This image shows an extrudate consisting of PEG, Dynasin and Theophylline anhydrate.



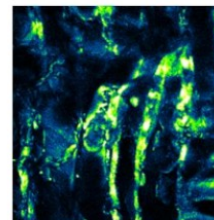
Food technology

Food technology is increasingly molecular, with the size, crystallinity and structure all contributing to the taste perception of food products. Therefore, development of new products requires extensive characterization at high resolution. Furthermore, the analysis of the digestion of foods is essential for the effectiveness of new functional foods and additives. Labelling is not possible as many structured substances would not allow for a homogeneous labeling, and labels would distort the structure. Coherent Raman imaging can provide high resolution imaging with chemical specificity. The image shows the distribution of lipid droplets in water within mayonnaise, demonstrating a wide size distribution of the lipids.



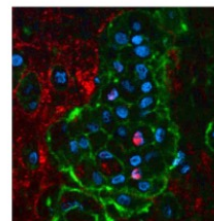
Tissue regeneration

Artificial tissue research has shown that skin, bone and even complex tissue structures can be grown when the right conditions can be simulated. In-depth 3D analysis of the matrix materials and grown tissue is essential for further research and development. Coherent Raman provides this 3D analysis without labeling, or the need for destructive preparation of the tissue. The image shows cartilage growth (brown) in a synthetic collagen matrix (blue).



Chemistry

Conversion of raw materials to biofuels typically starts with a precise spatially-resolved analysis of all the intermediate products involved in the complex process. Labelling is not an option since this inhibits the natural movement of the material involved. Coherent Raman imaging provides speedy 3D analysis with chemical specificity. This reveals the position and composition of all products involved. The image shows analysis of wood fibre, an important possible substitute for oil as a base ingredient in many applications.

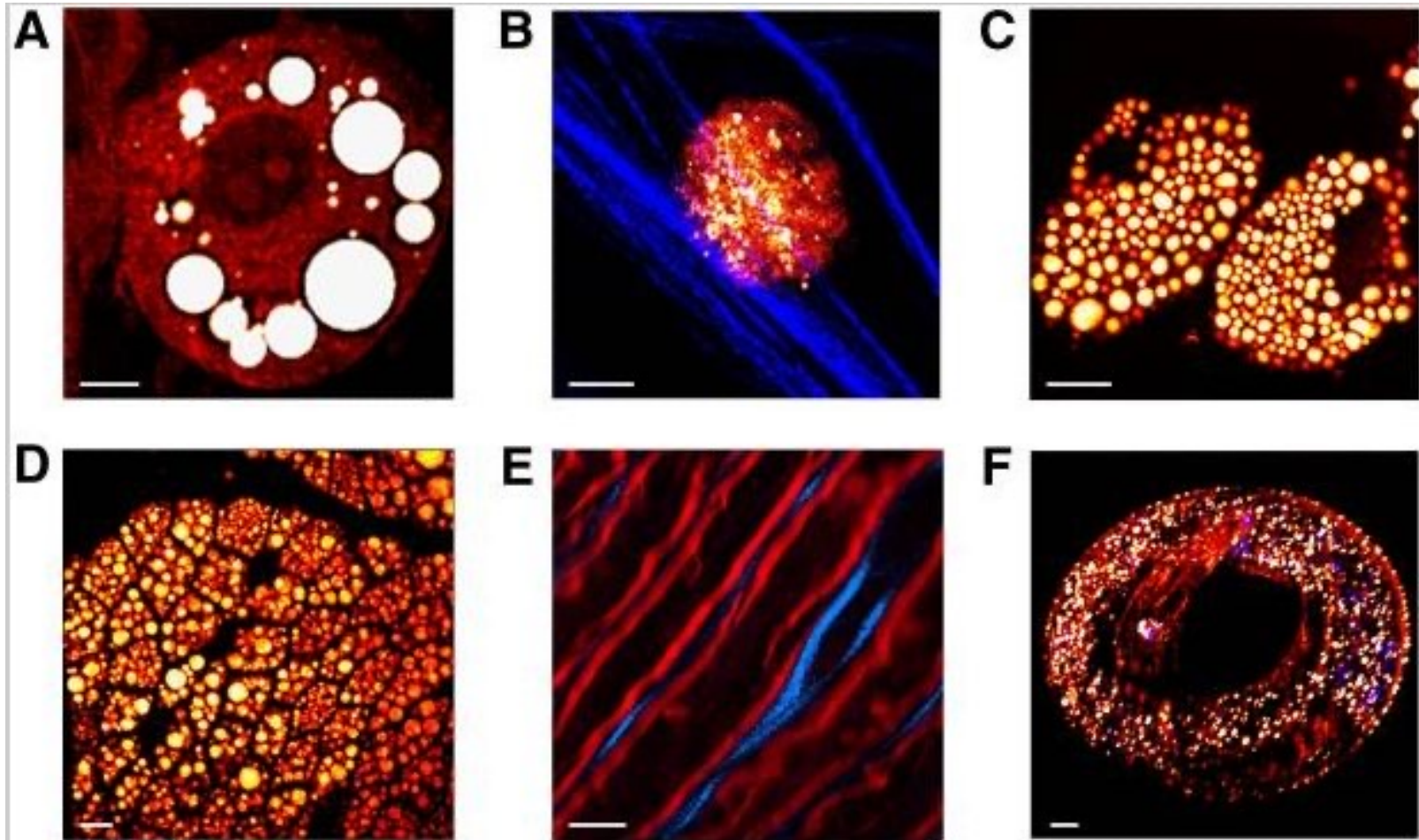


Medicine

Identification of malignant tissue, delineation of melanoma borders and characterization of bioavailability of drugs or skincare products requires a label-free imaging technology capable of tissue sectioning in high-resolution 3D. Coherent Raman imaging provides high-speed label-free imaging over a limited depth range, revealing structure and function without the need for altering or sectioning the tissue. The image shows a cancer lesion with DAPI staining (blue), fluorescent labeling (green) and CARS (red). The CARS demonstrates the changes in the lipid distribution between the malignant and healthy tissue.

D.III. Imagerie vibrationnelle Raman-CARS

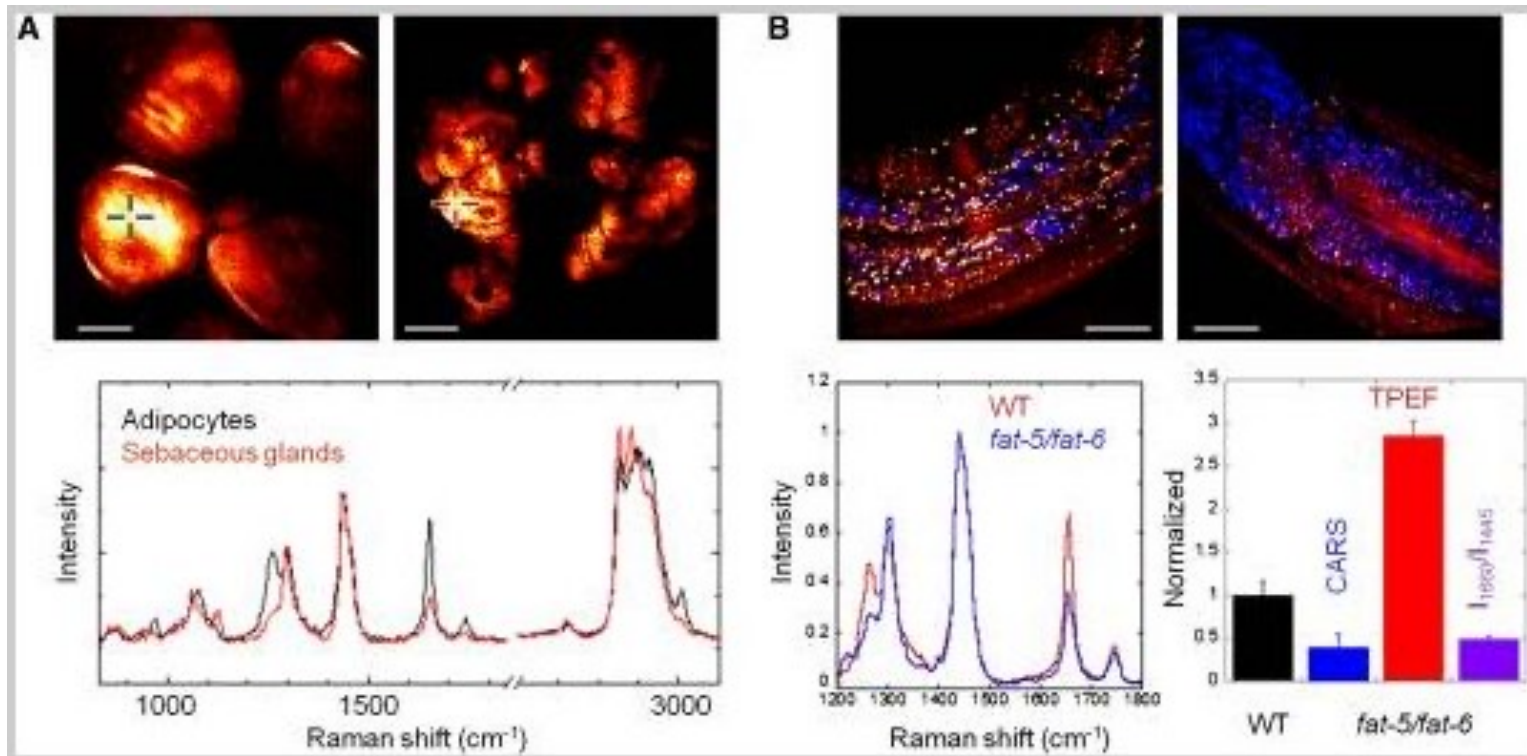
Lipides



CARS-based multimodal NLO imaging of lipid-rich structures. A: CARS image of a differentiating 3T3-L1 cell. B: Overlaid CARS and SFG image of a lipid-rich tumor cell (red) on collagen fibrils (blue). C: CARS image of two lipid-rich foam cells. D: CARS image of lipid-rich intestinal cells of a C57BL/6J mouse after feeding. E: Overlaid CARS and SFG image of myelin sheaths (red) and astroglial processes (blue). Image is a courtesy of Terry B. Huff. F: Overlaid CARS and TPEF image of neutral lipid droplets (red) and autofluorescent lipid species (blue) in a living *C. elegans*. Scale bars: 5 μm. Images adapted from references (48, 59, 90, 98).

D.III. Imagerie vibrationnelle Raman-CARS

Adipocytes - Lipides

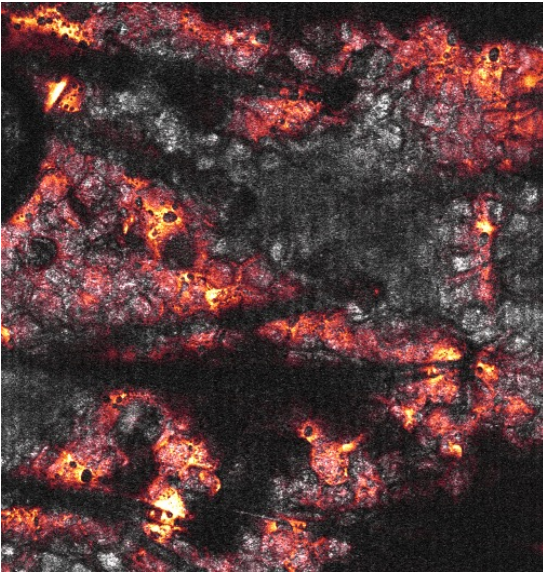


CARS imaging and quantitative analysis of lipid expression and composition. A: CARS images of subcutaneous adipocytes (upper left panel) and sebaceous glands (upper right panel) in the ear of a living Balb/c mouse. The cross-hairs mark the locations for spontaneous Raman spectra acquisition. The bottom graph presents overlaid Raman spectra of subcutaneous adipocytes (black) and sebaceous glands (red). B: Overlaid CARS and TPEF images of neutral lipids (red) and autofluorescent lipids (blue) in a wild-type (upper left panel) and a *fat-5/fat-6* double mutant (upper right panel) *C. elegans*. The *fat-5/fat-6* double mutant has genetic deletions of palmitoyl-CoA desaturase *fat-5* and stearyl-CoA desaturases *fat-6*. Lower left panel: Overlaid Raman spectra of neutral lipids in wild-type (red) and *fat-5/fat-6* double mutant (blue) *C. elegans*. Lower right panel: Quantitative comparison of the expression level of neutral (blue) and autofluorescent (red) lipids and the lipid-chain unsaturation (purple) between wild-type (black) and *fat-5/fat-6* double mutant *C. elegans*. The quantitative values are normalized to one for wild-type and comparatively for *fat-5/fat-6* double mutant *C. elegans*. The lipid-chain unsaturation is measured by the 1160/1445 ratio in Raman spectra. Scale bars: 25 μm . Images adapted from references (47, 48).

D.III. Imagerie vibrationnelle Raman-CARS

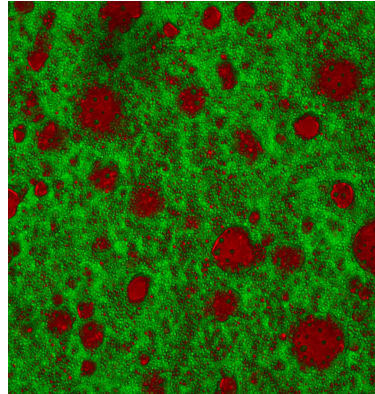
Dermatologie - cosmétologie

Transdermal drug pathway at 1594cm^{-1} :
Diffusion of trans-retinol through skin

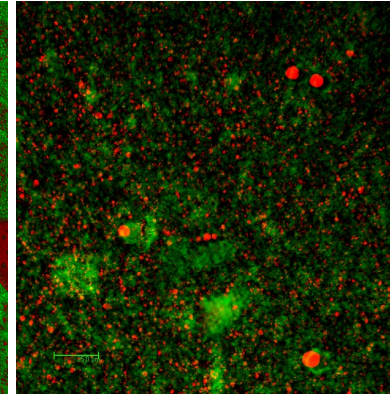


Courtesy of Prof. Sunney Xie,
"Department of Chemistry and Chemical Biology"
Harvard University, Cambridge, USA

HCX PL APO CS 10.0x0.40 DRY



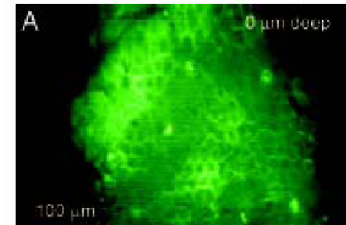
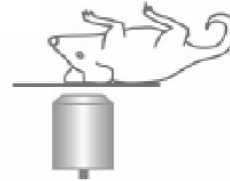
Hand cream



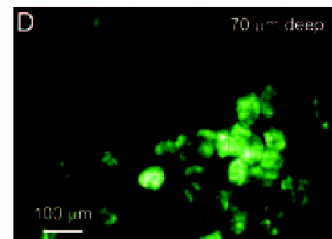
Body lotion

Future applications : CARS *in-vivo* imaging

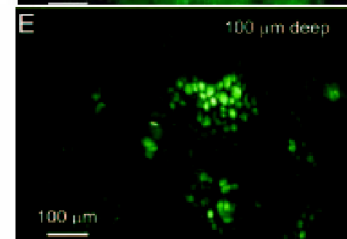
2845 cm^{-1} vibration C-H lipid



Stratum
corneum

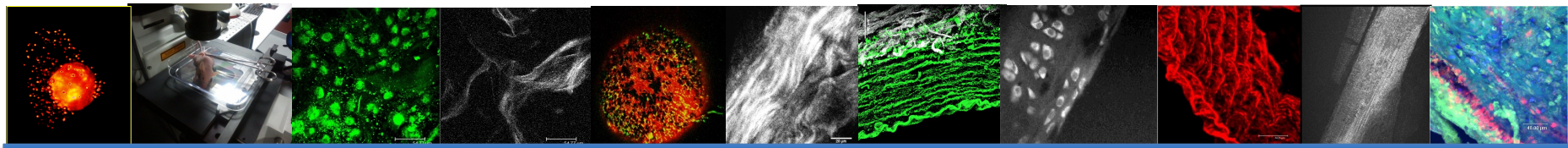


Adipocytes of the dermis



Adipocytes of subcutaneous layer

Evans et. al., PNAS, V102 16807 (2005)



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BIOPÔLE

FR 3209 CNRS/UL/INSERM et UMR 7365 CNRS/UL

Merci pour votre attention !

Prochains Modules de Formation en imagerie confocale et Macroscopie :
Inscrivez vous !!

Session de 10 à 12h

- Stages M2 et bourse de l'école doctorale PTIBC (Imagerie Vibrationnelle).

