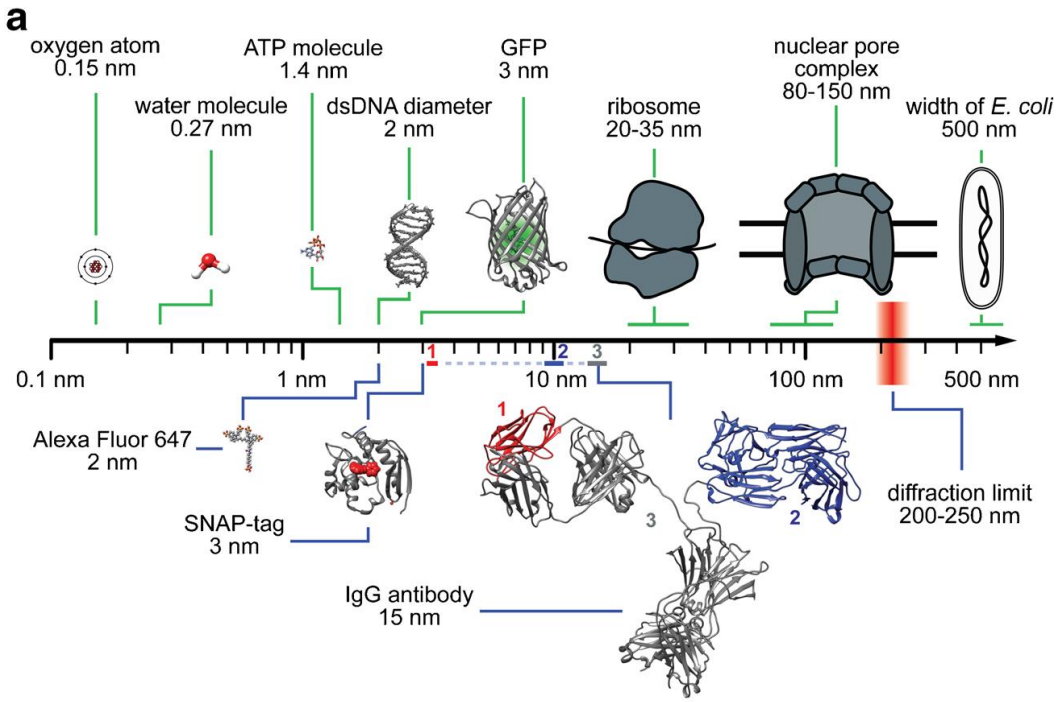
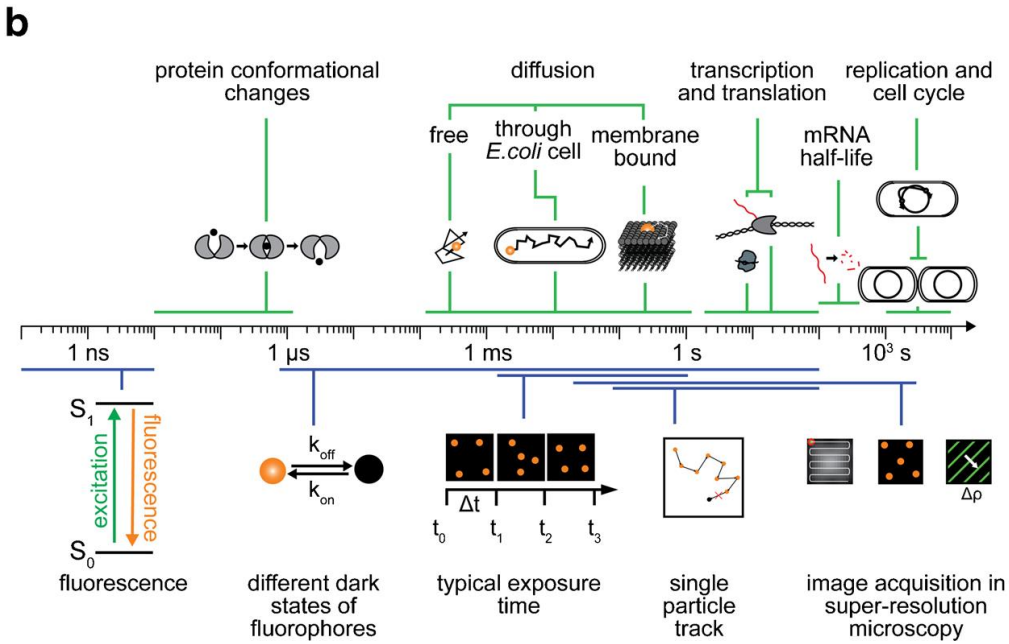
Space



Time



Turkowyd et al ABC 2016

Le microscope

- Les éléments du microscope à fluorescence
- Le microscope (optique, le choix de l'objectif)
- La microscopie plein champ

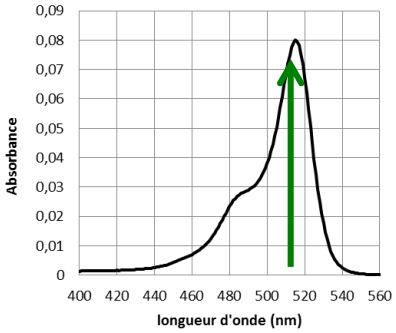
Amélioration de la résolution spatiale

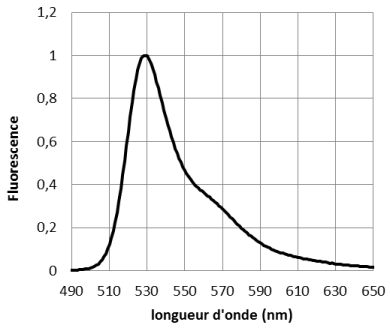
- Confocal (laser scanning, detection)
- Bi-photon
- TIRF
- Super-resolution

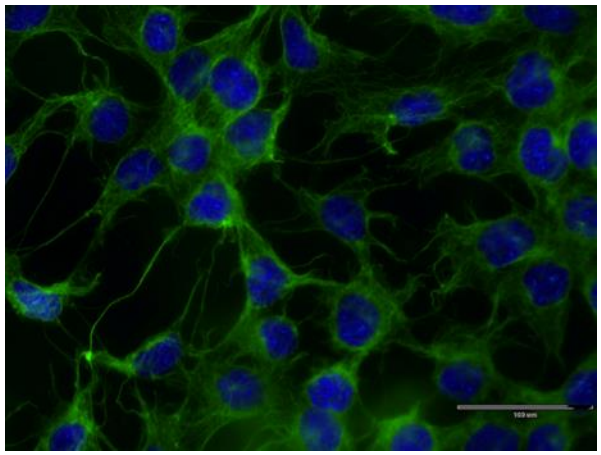
La spectroscopie sous microscope

- FRET
- FLIM
- FCS, FCCS

Les éléments nécessaires

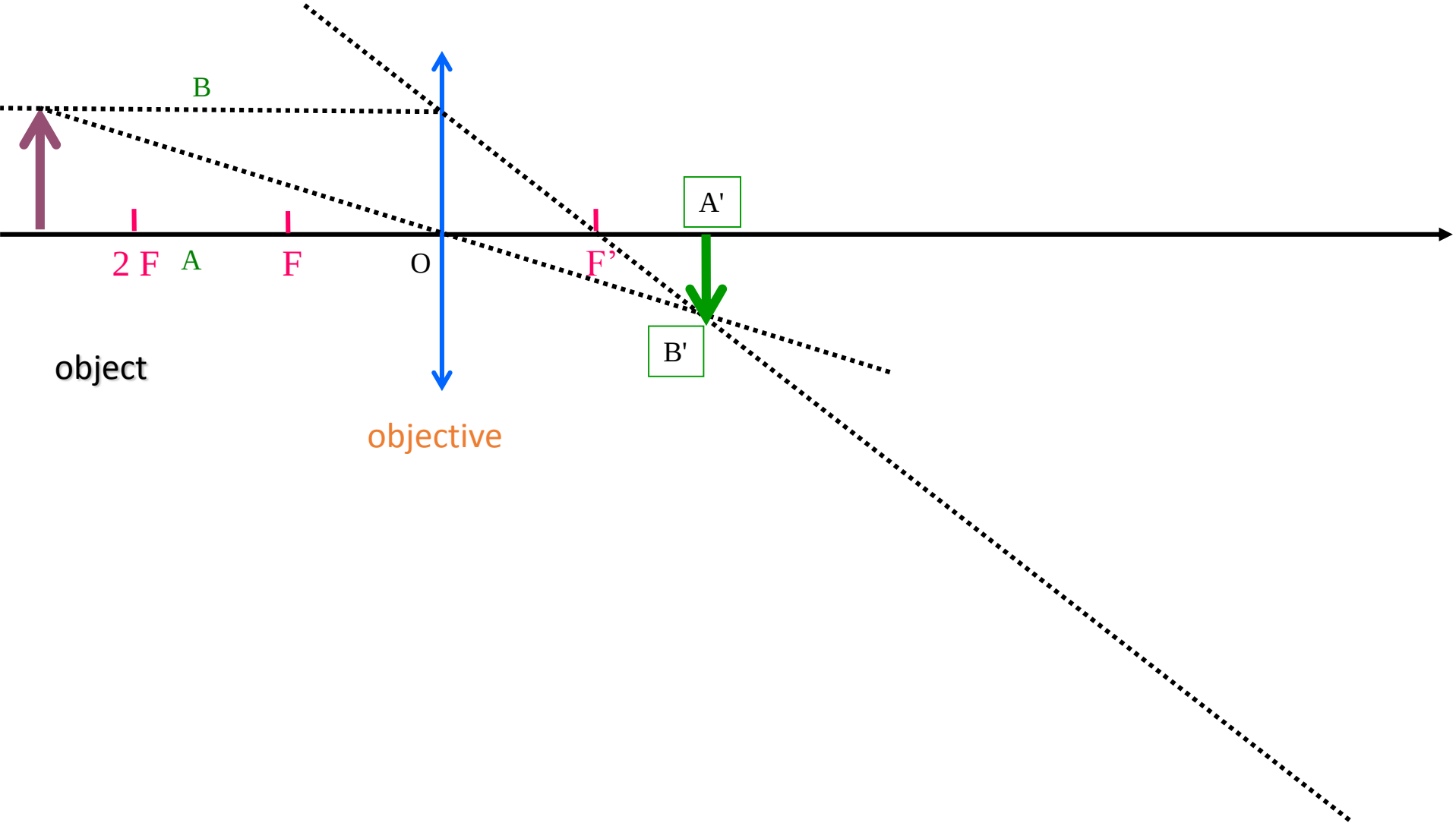


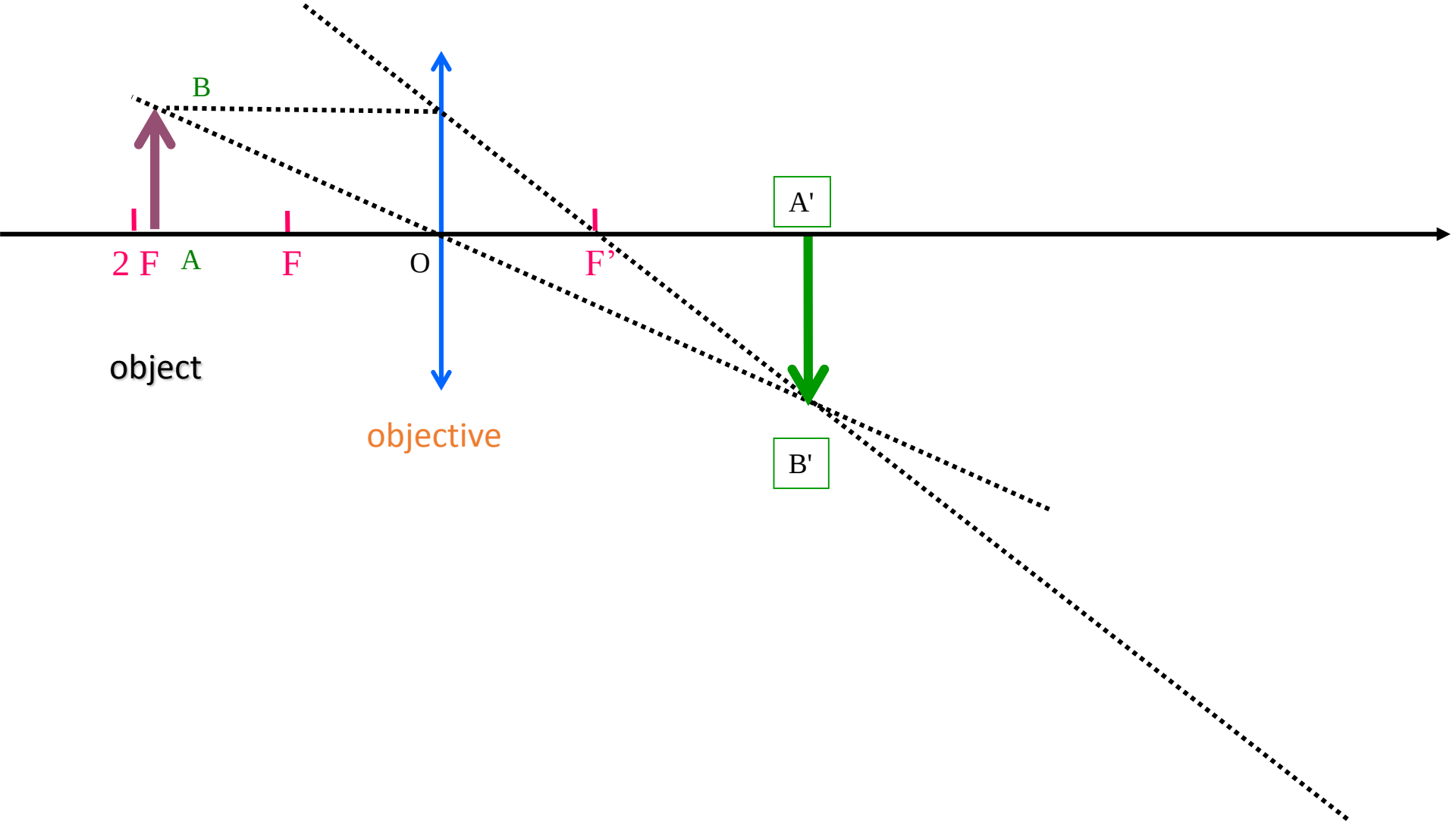


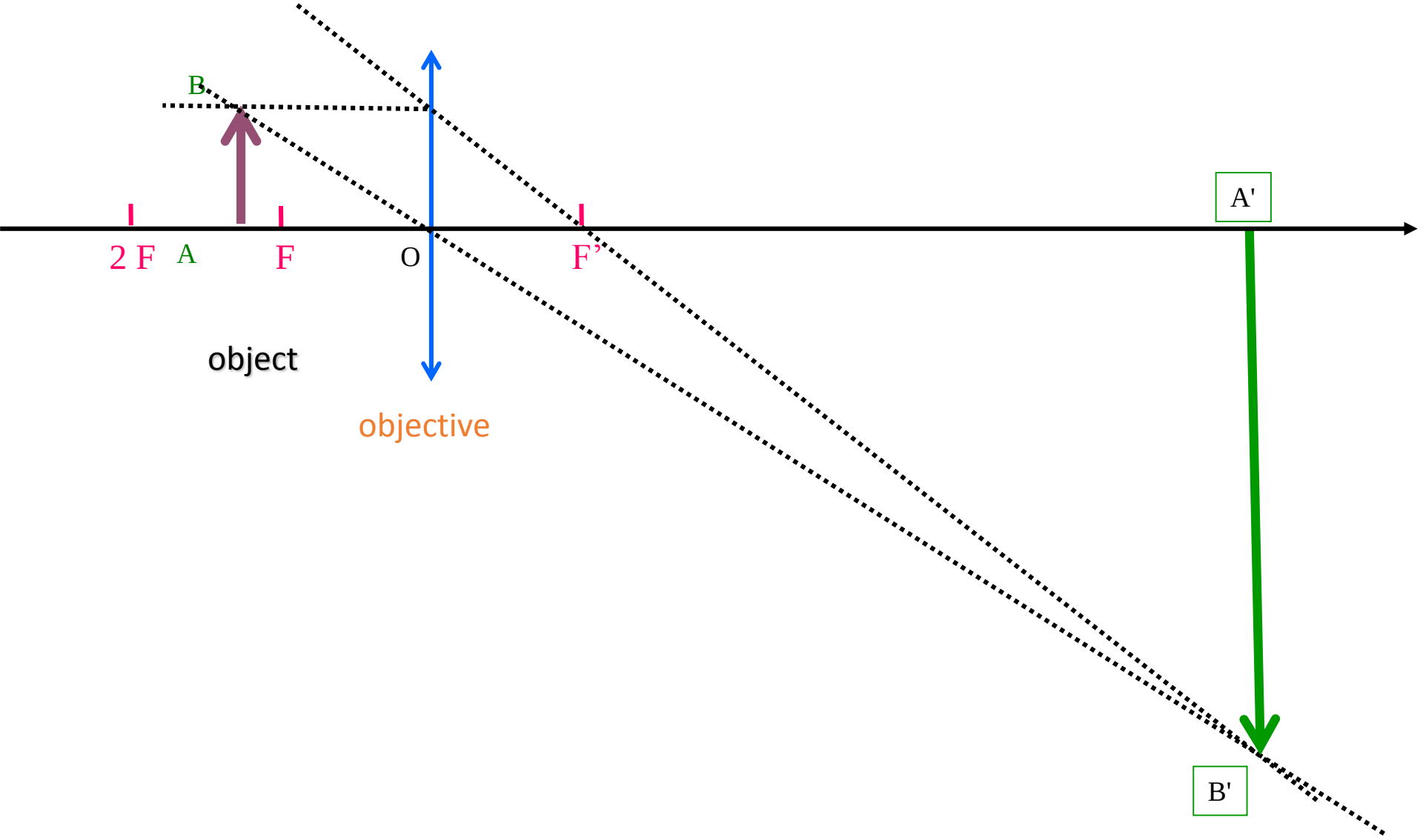


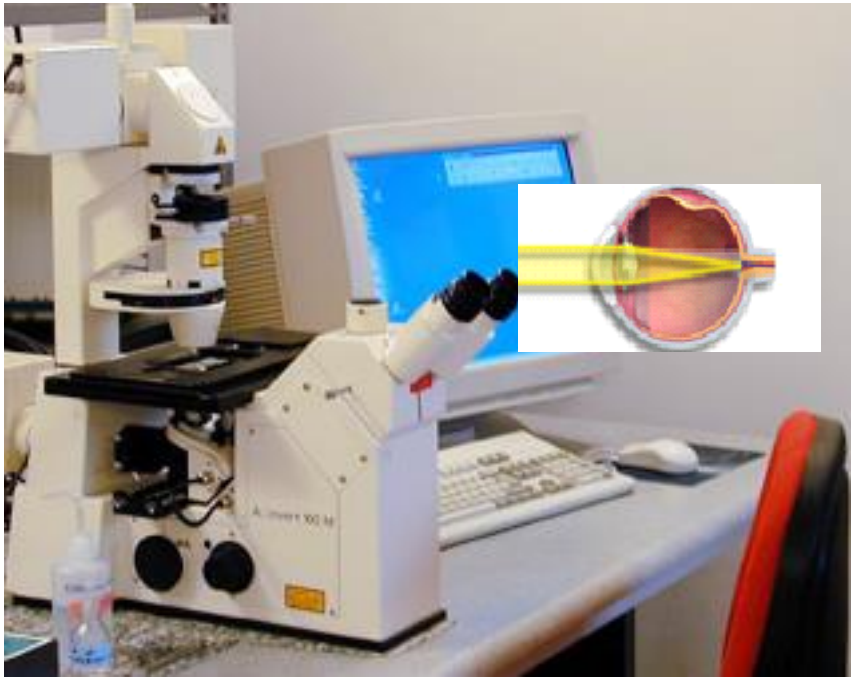
- Source de lumière d'excitation
- Un dispositif qui permet de choisir λ_{ex}
- Un système de transmission de la lumière d'excitation vers l'échantillon / de collection de la lumière d'émission
- Un dispositif qui permet de choisir λ_{em}
- Détecteur

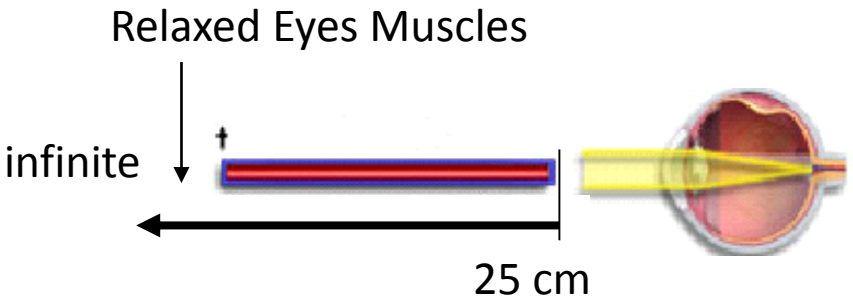
Le microscope




B
A'
2 F
A
F
O
F'
object
objective
B'


B
A'
2 F
A
F
O
F'
object
objective
B'

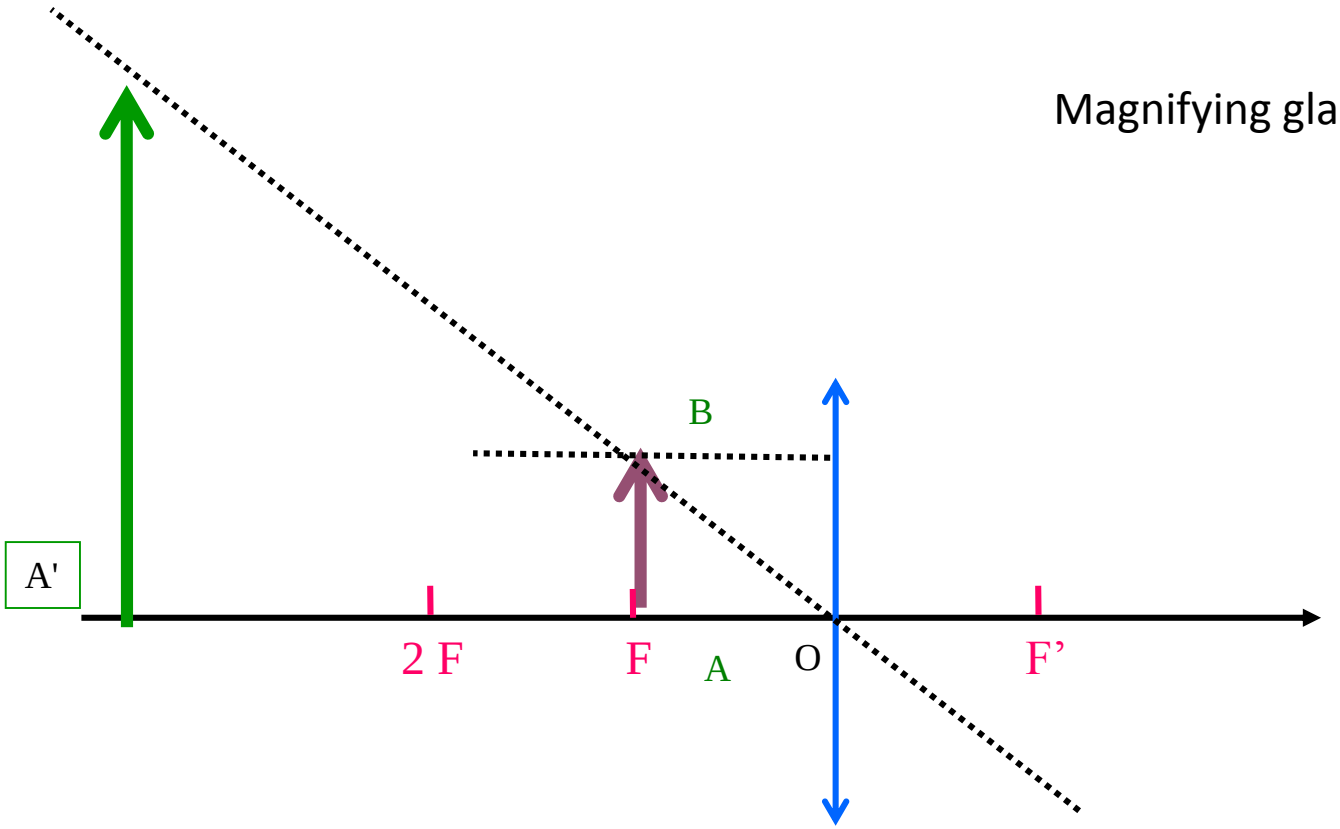




Accommodation of the eye refers to the act of adjusting the crystalline lens element to alter the refractive power and <u>bring objects that are closer to the eye into sharp focus</u>.

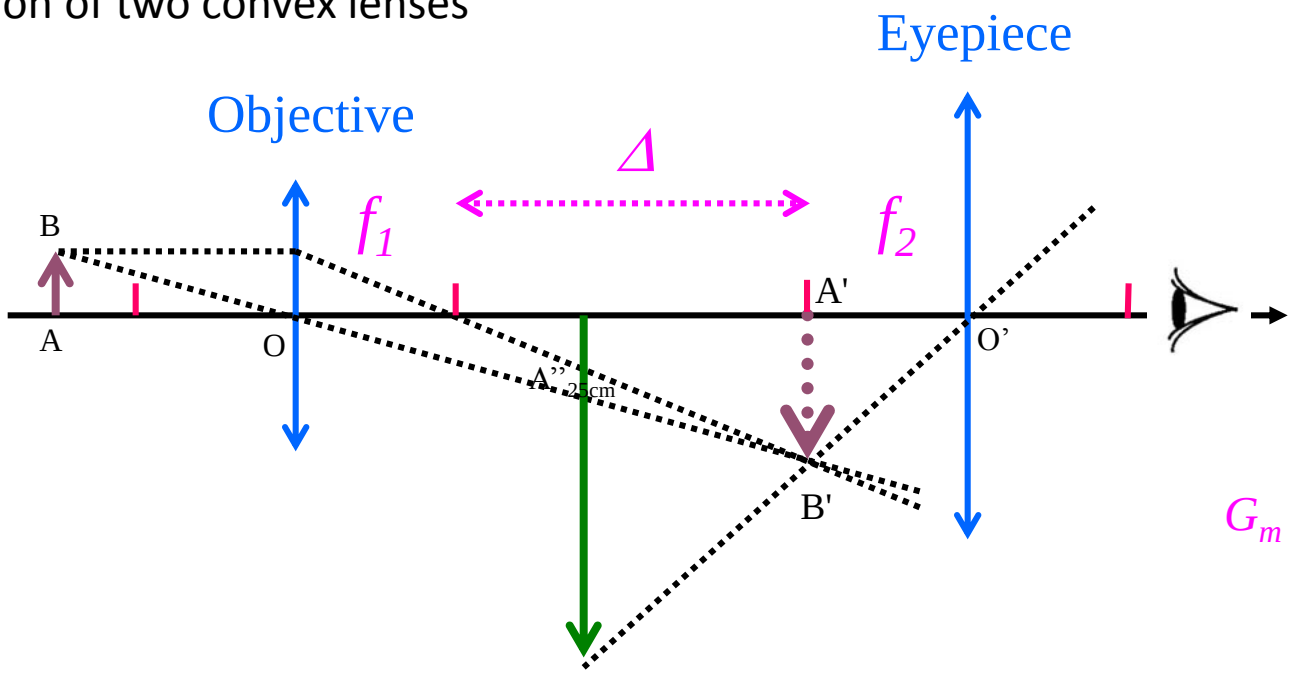
If the object is at the infinite, the eye won't accommodate

Magnifying glass

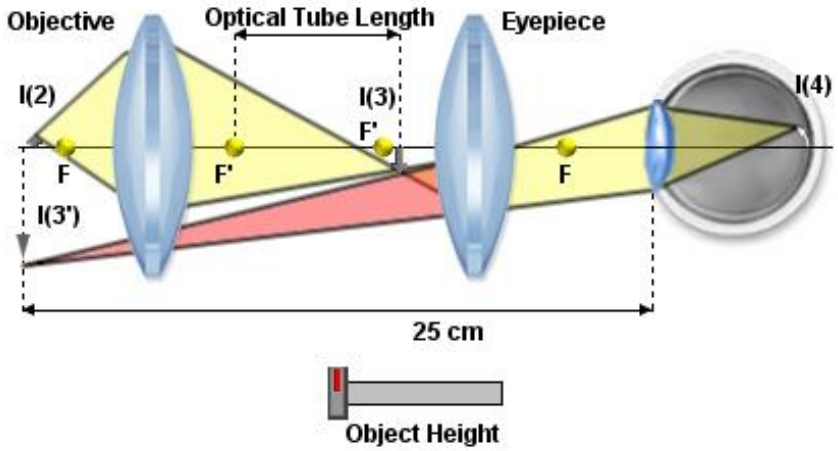


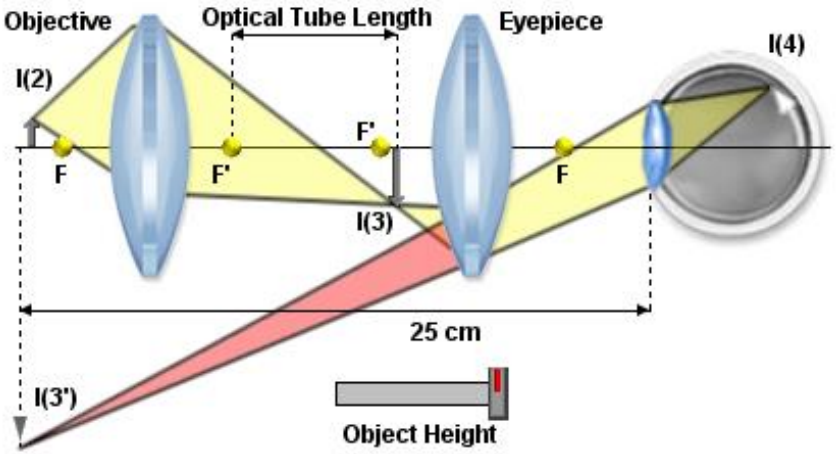
http://www.microscopyu.com/tutorials/java/components/eyepiece/index.html

Combination of two convex lenses

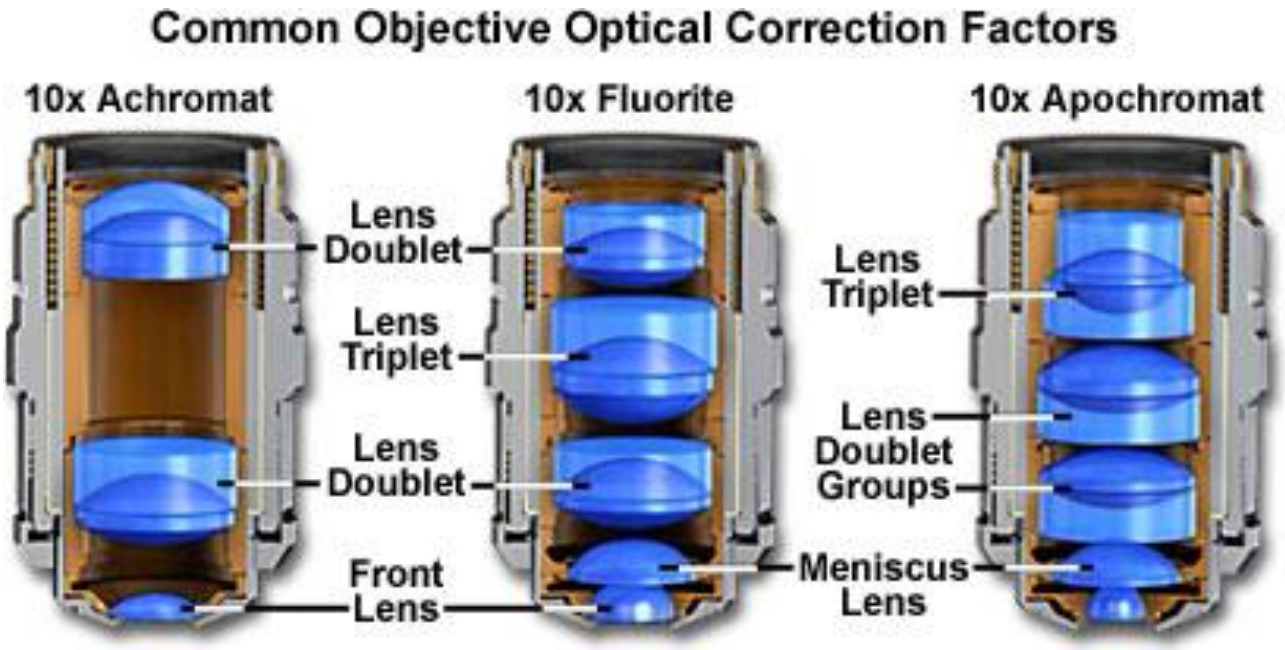


$$G_m = \frac{0.25}{f_2\ f_1}\Delta$$





http://www.microscopyu.com/tutorials/java/components/fixedtubelength/index.html



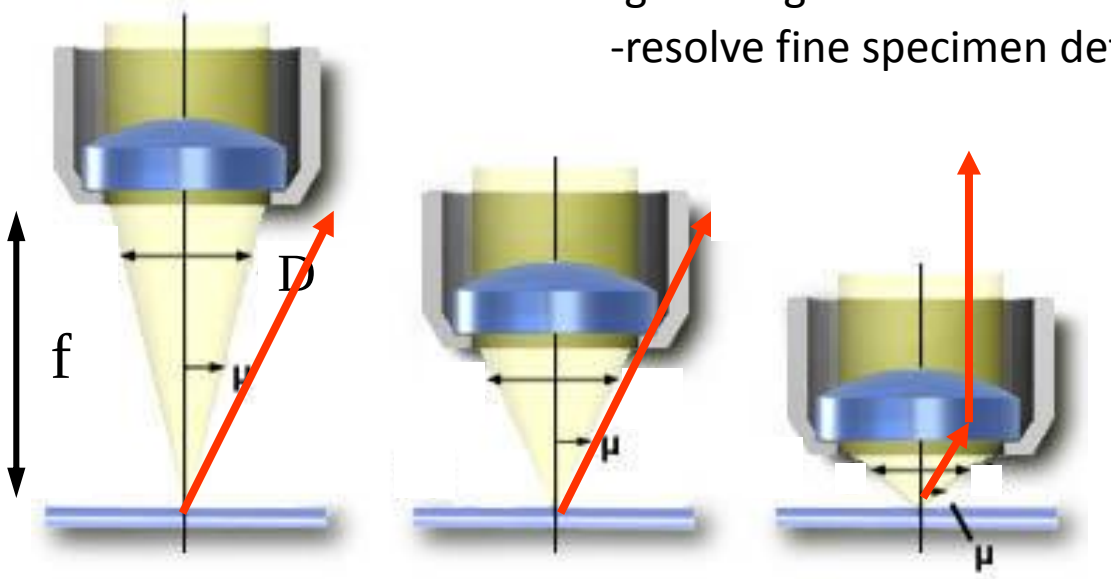
They are not simple convex lens!

Key component of the optical system in the microscope

Correct the chromatic aberration

The most important characteristic of the microscope objective : numerical aperture or NA

N.A. measure the ability of the objective to=
-gather light
-resolve fine specimen detail at a fixed object distance



$n_{verre} = 1.515$
$n_{glycérine} = 1.47$
$n_{eau} = 1.33$

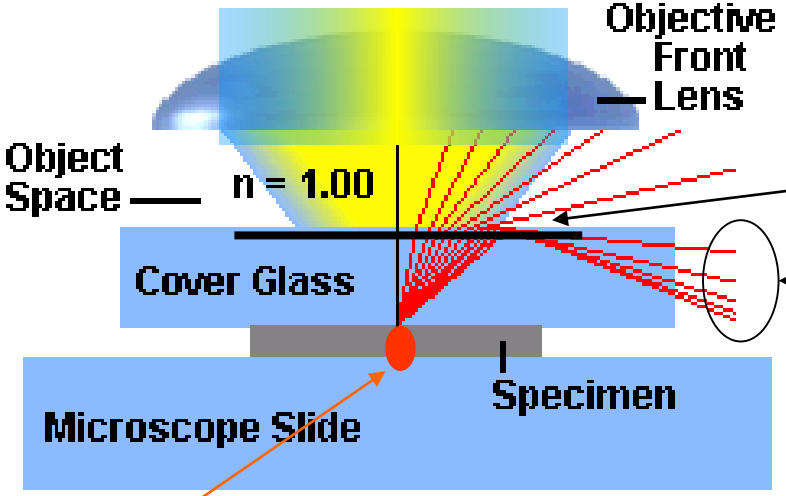
NOT collected

Collected

N.A increases
(like magnification and
amount of collected light)

$N.A. = n \sin \mu$

n is the refractive index of the media in the object space
(between the cover glass and the objective front lens)

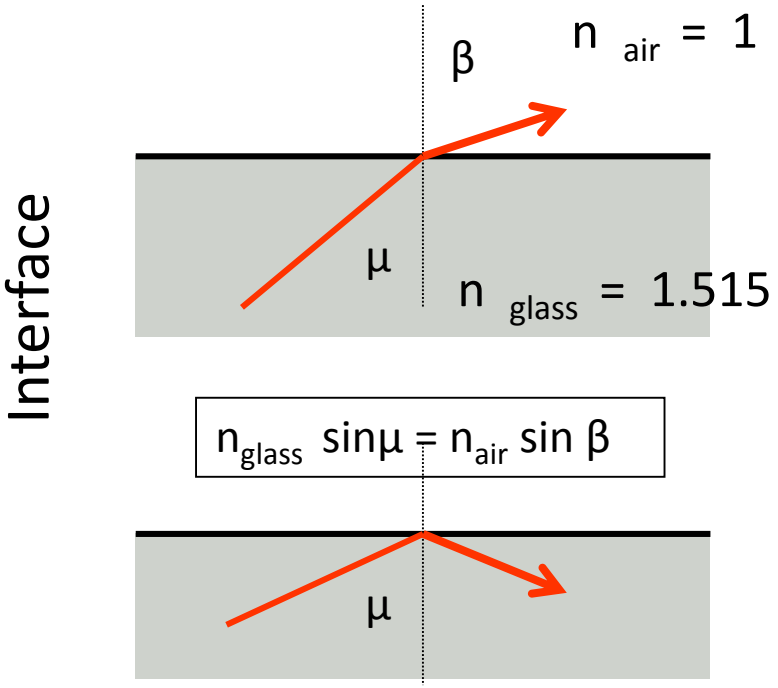


Interface

Excluded rays of light

Fluorescent object

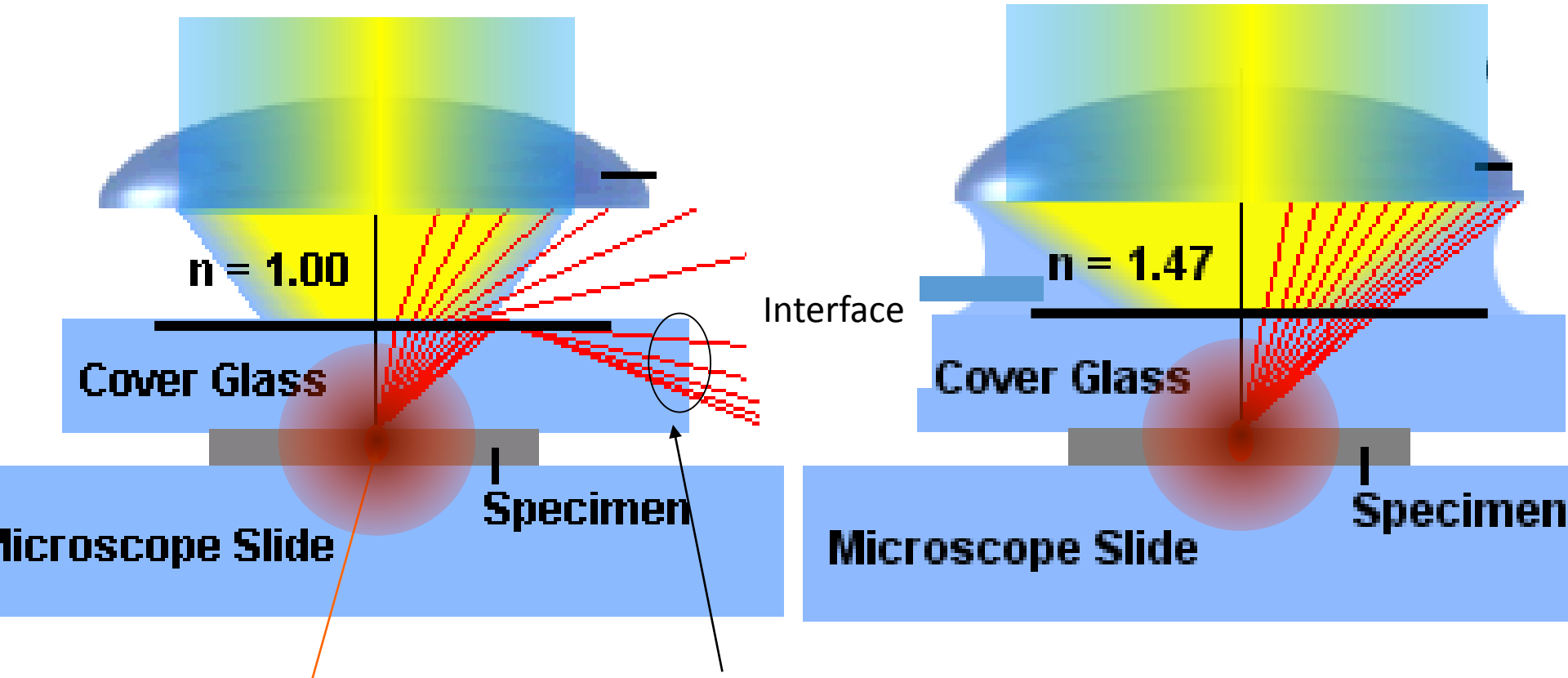
Total internal reflection



$$n_{glass} \sin\mu = n_{air} \sin\beta$$

Collected cone angle = 37°

Collected cone angle = 76°



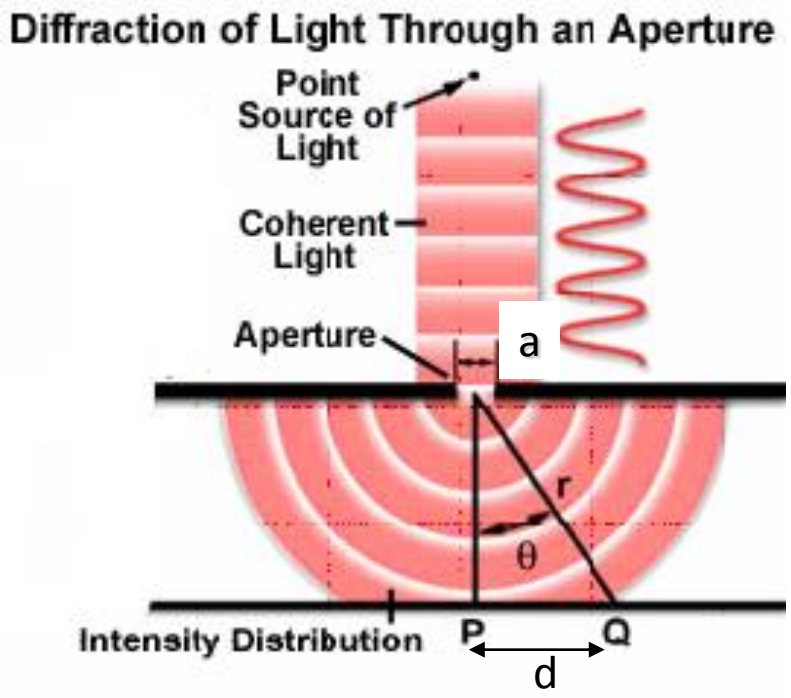
Excluded rays of light

Fluorescent object

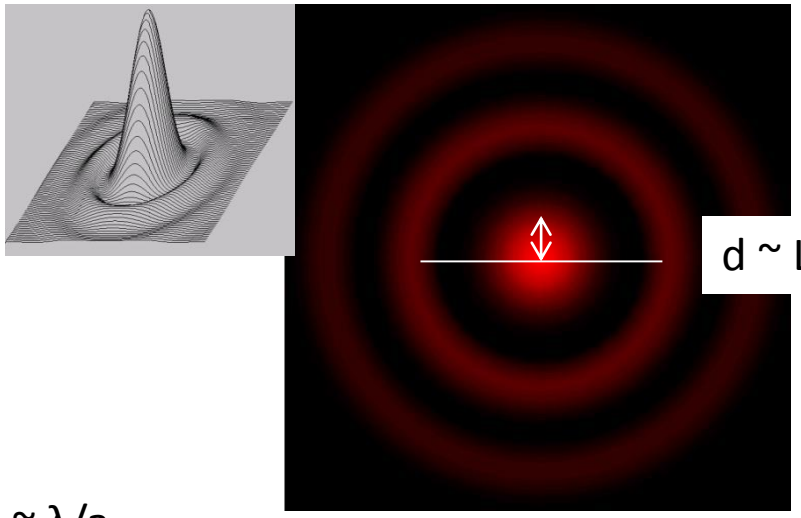
Fluorescence emission is isotropic

Immersion objective have an increased NA

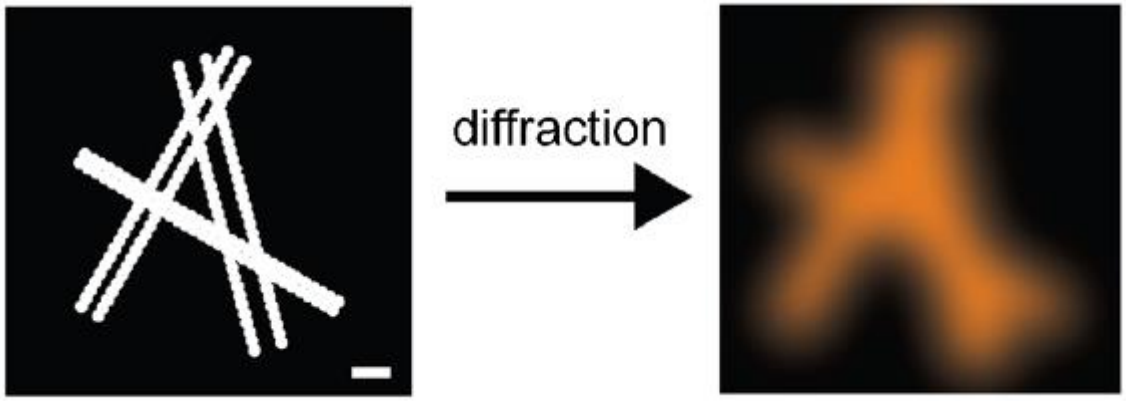
NA is important for the resolution



θ ~ λ/a

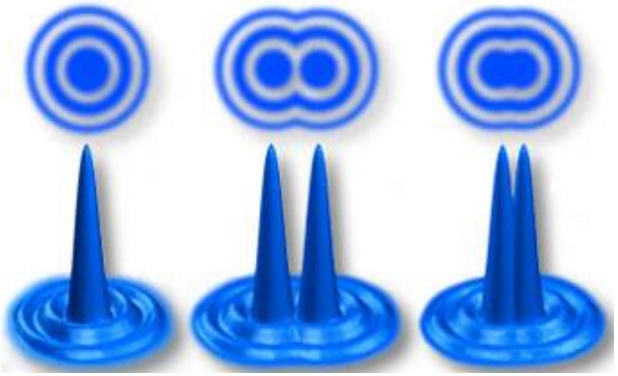


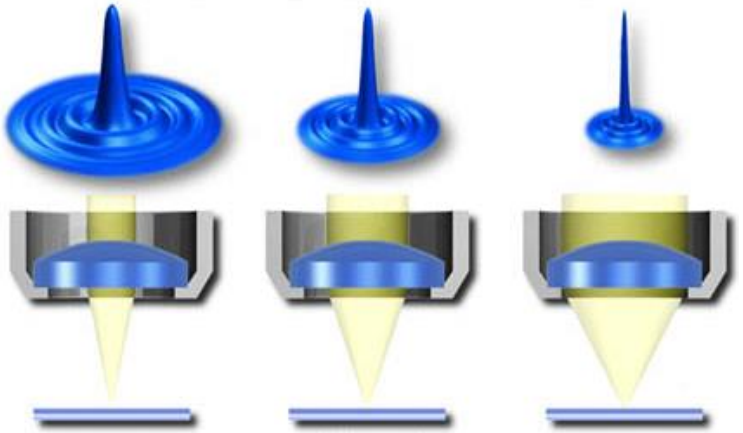
d ~ Lx θ ~ L x λ/a



Turkowyd et al ABC 2016

NA is important for the resolution …..

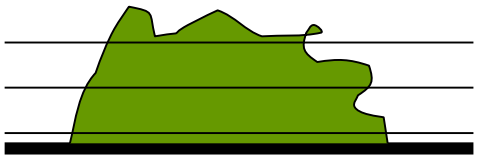


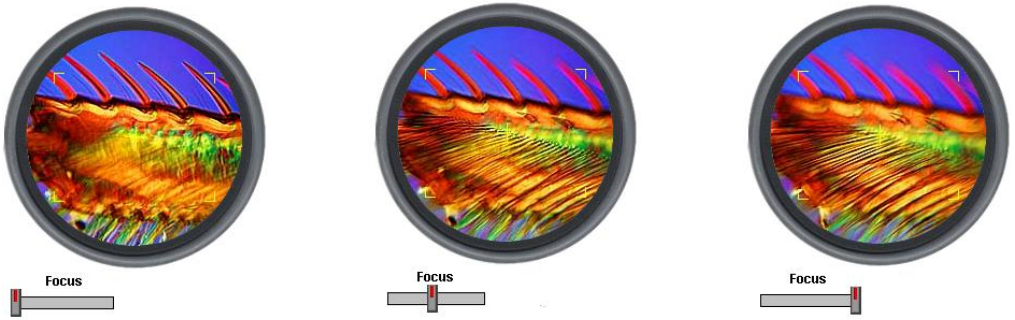


resolution is the smallest resolvable distance between two objects

$$r_{résolution} = \frac{\lambda}{2\ N.A.} \qquad N.A. = n \sin \mu$$

… and the depth of field





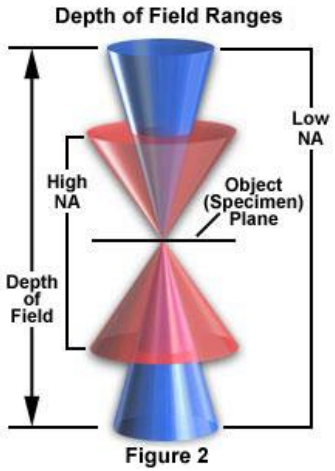


Figure 2

Le microscope

- Les éléments du microscope à fluorescence
- Le microscope (optique, le choix de l'objectif)
- La microscopie plein champ

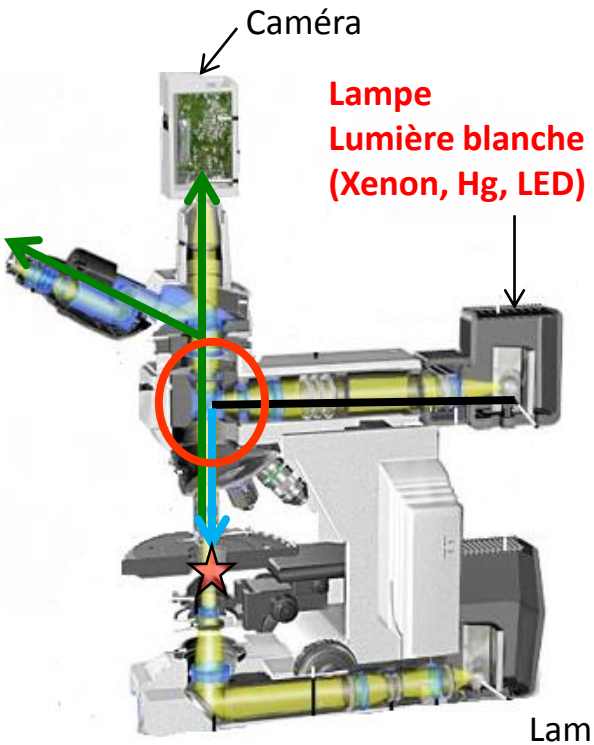
Amélioration de la résolution spatiale

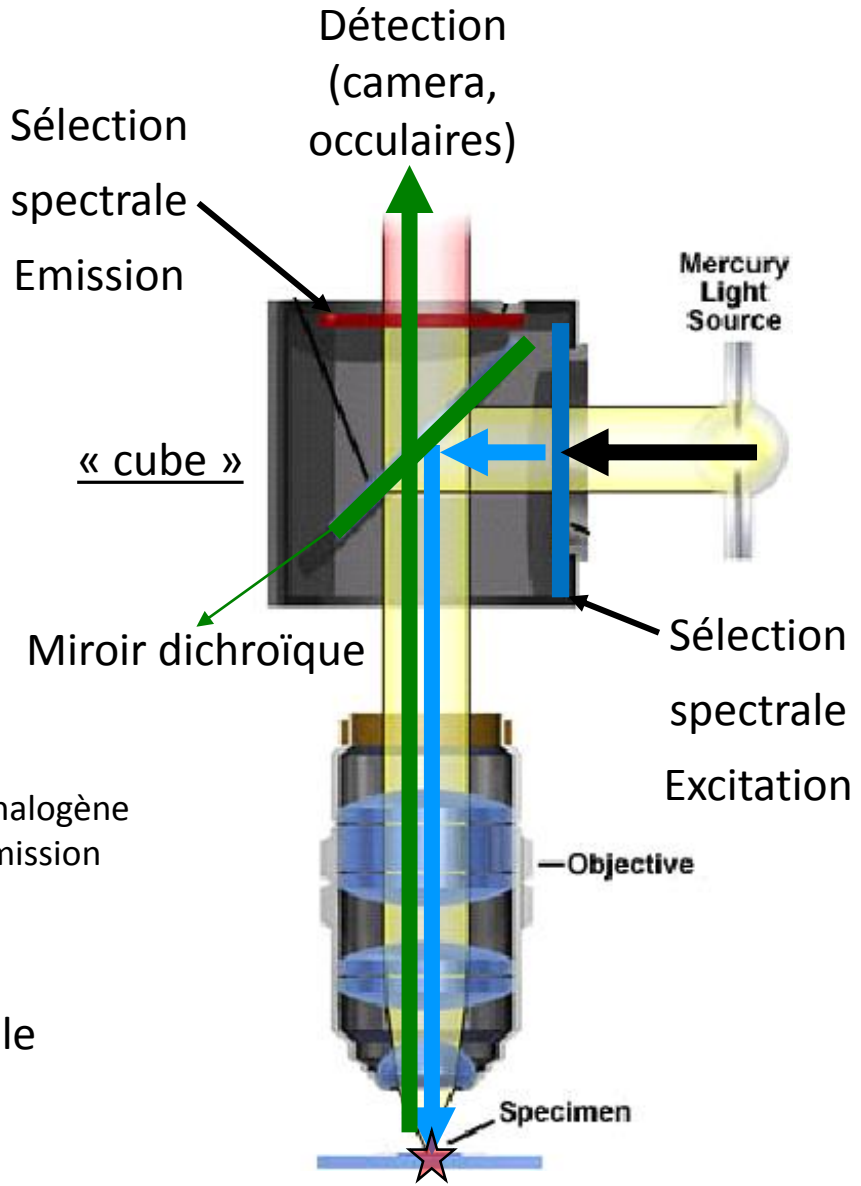
- Confocal (laser scanning, detection)
- Bi-photon
- TIRF
- Super-resolution

La spectroscopie sous microscope

- FLIM
- FCS, FCCS
- FRAP et photoconversion

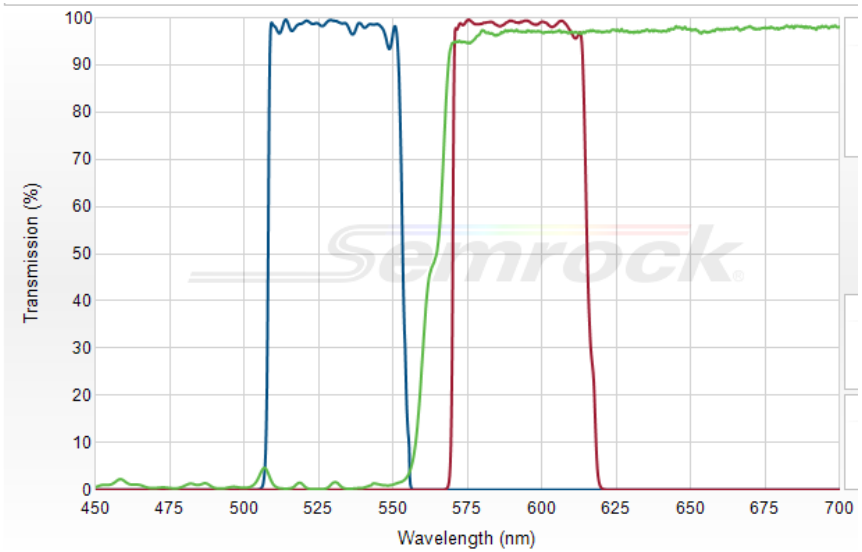
Le cas plein champ

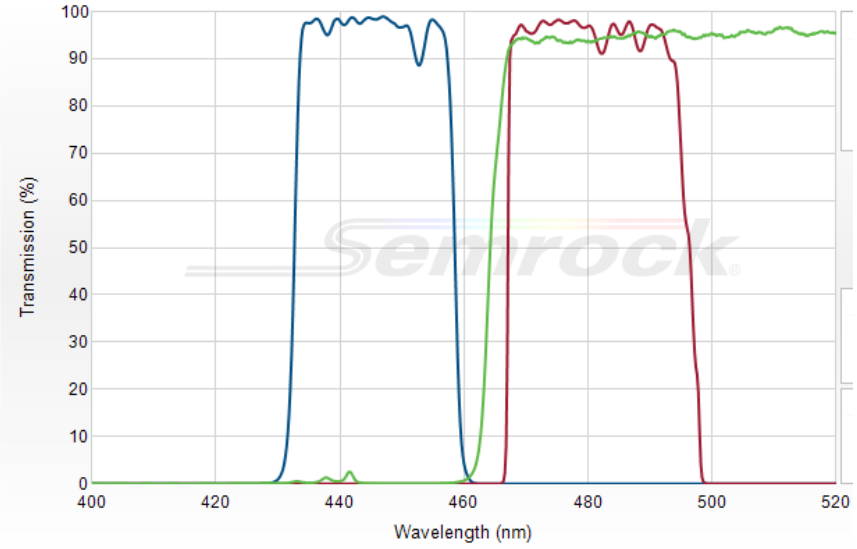


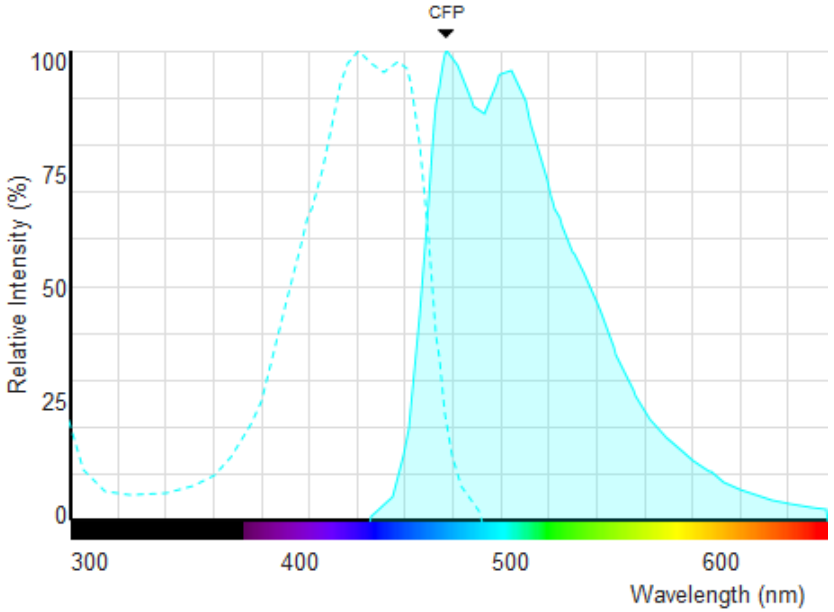


- Microscope droit ou inversé
- Excitation et collecte de la fluorescence sur le même chemin optique = epifluorescence

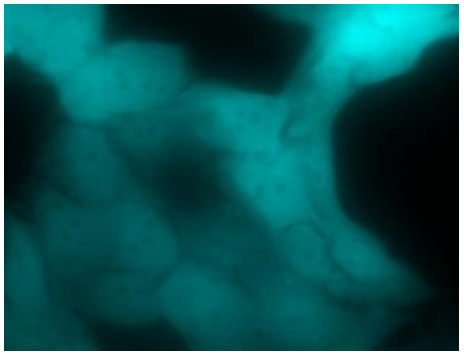
Quelle combinaison de filtre faut il choisir pour observer de la ECFP exprimée par des cellules eucaryotes?



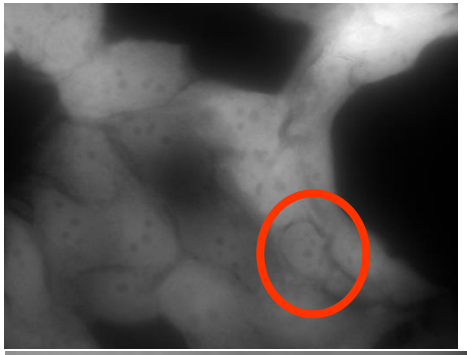




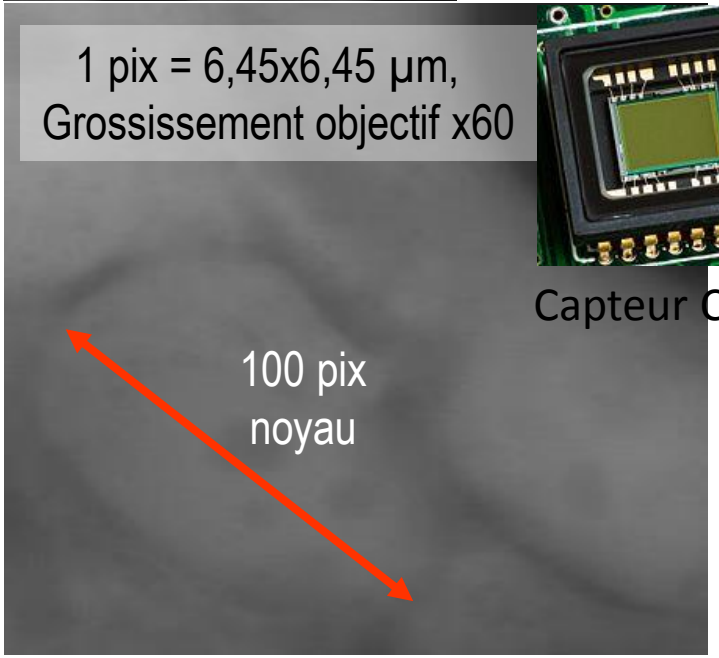
Ce qu'on voit aux oculaires:



Ce qu'on voit à la caméra:

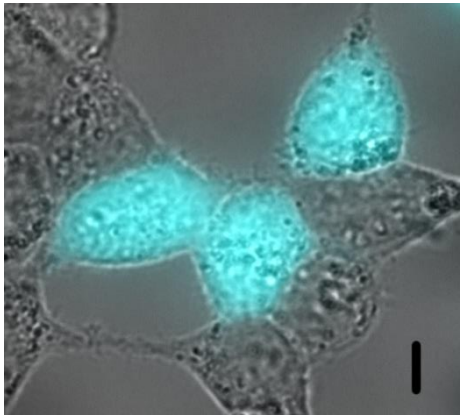


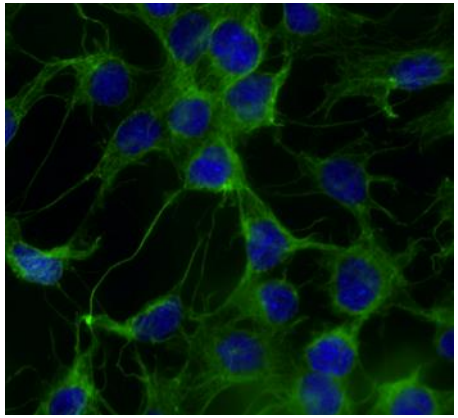
Niveaux de gris du <u>blanc</u> (bcp de photons) au <u>noir</u> (pas de photons)



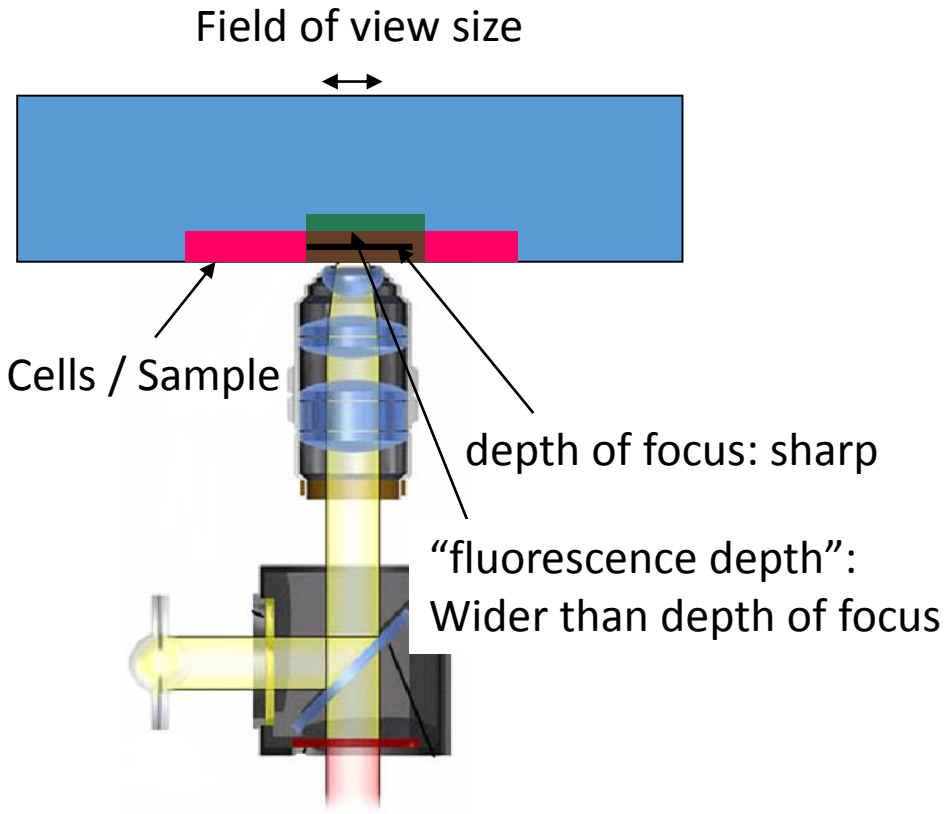
Capteur CCD

Ce qu'on peut faire en traitant les image:





Calculer la taille réelle du noyau?



“Fluorescence depth” wider than depth of focus

Le microscope

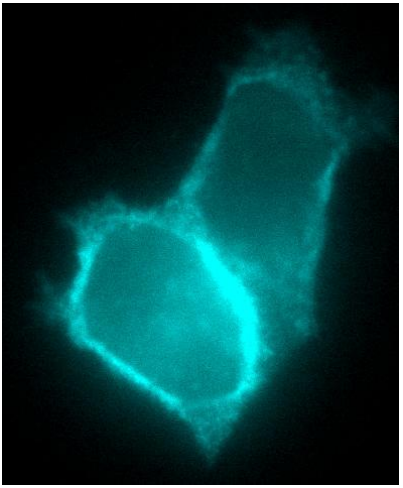
- Les éléments du microscope à fluorescence
- Le microscope (optique, le choix de l'objectif)
- La microscopie plein champ

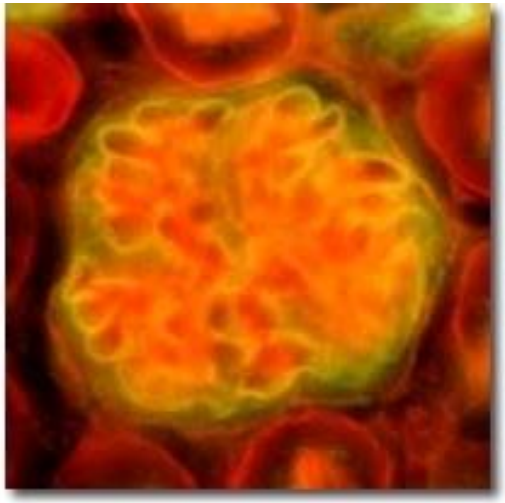
Amélioration de la résolution spatiale

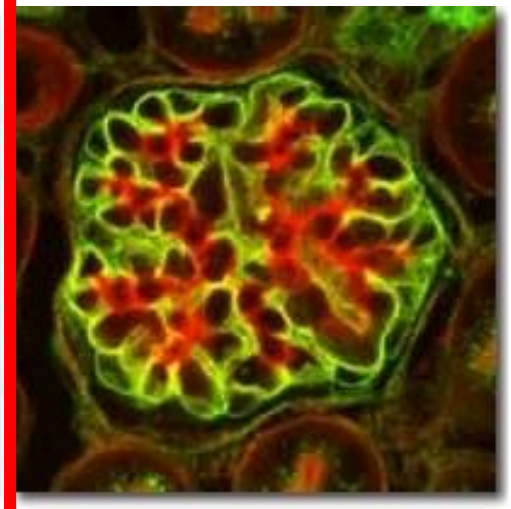
- Confocal (laser scanning, detection)
- Bi-photon
- TIRF
- « Super-resolution »

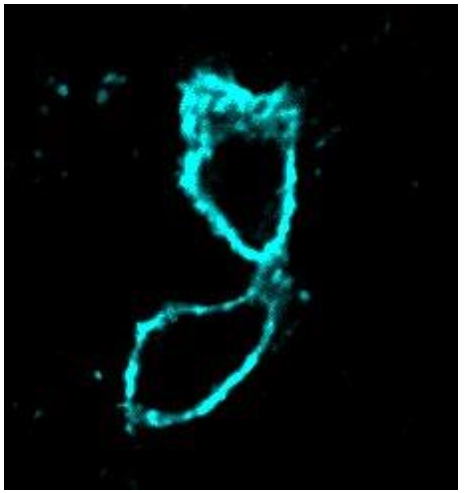
Le cas confocal

Images plein champ | **Microscopie confocale**

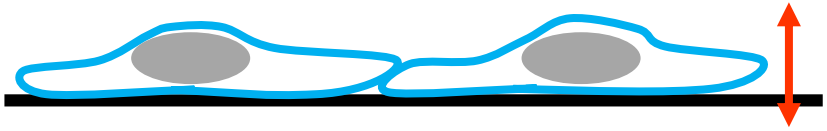




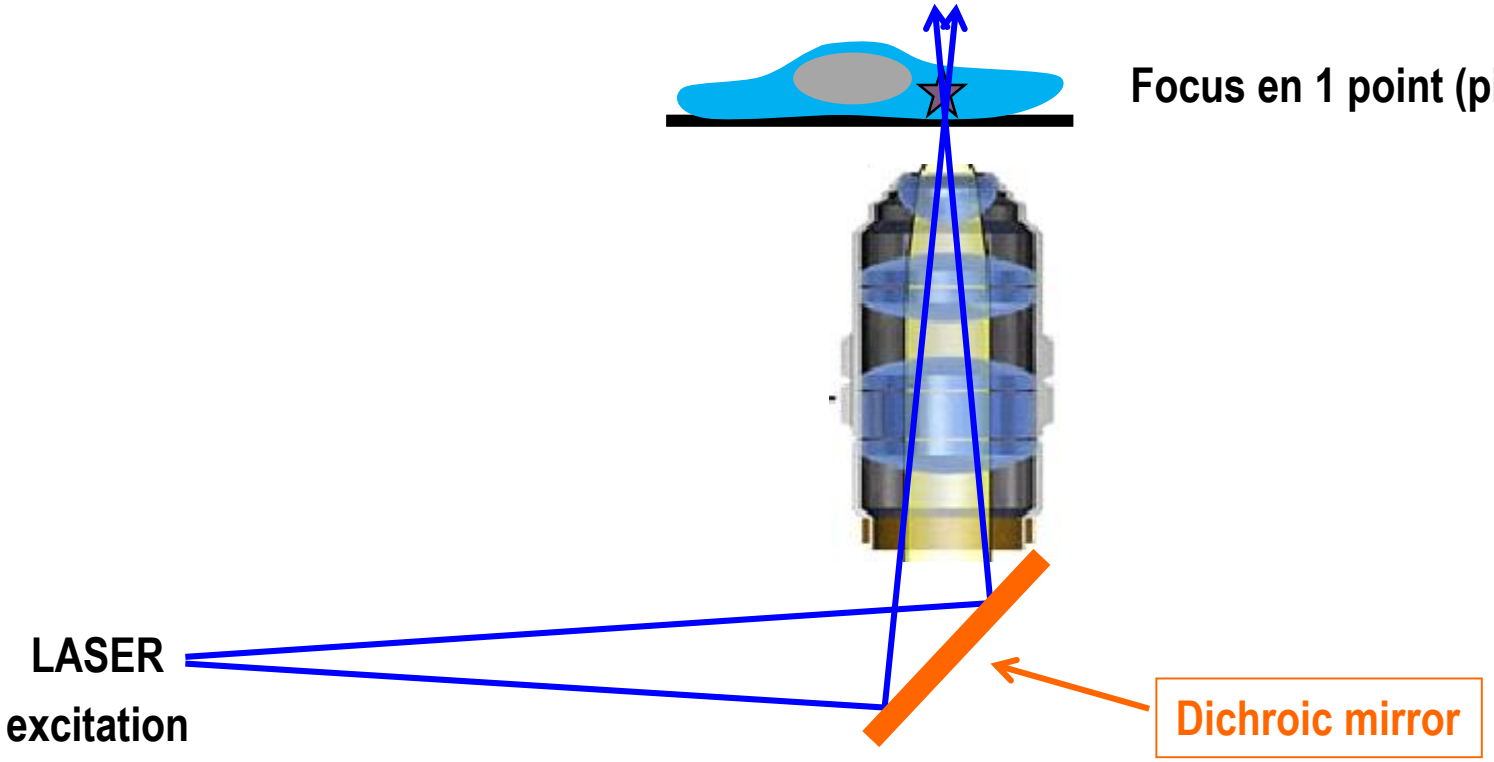


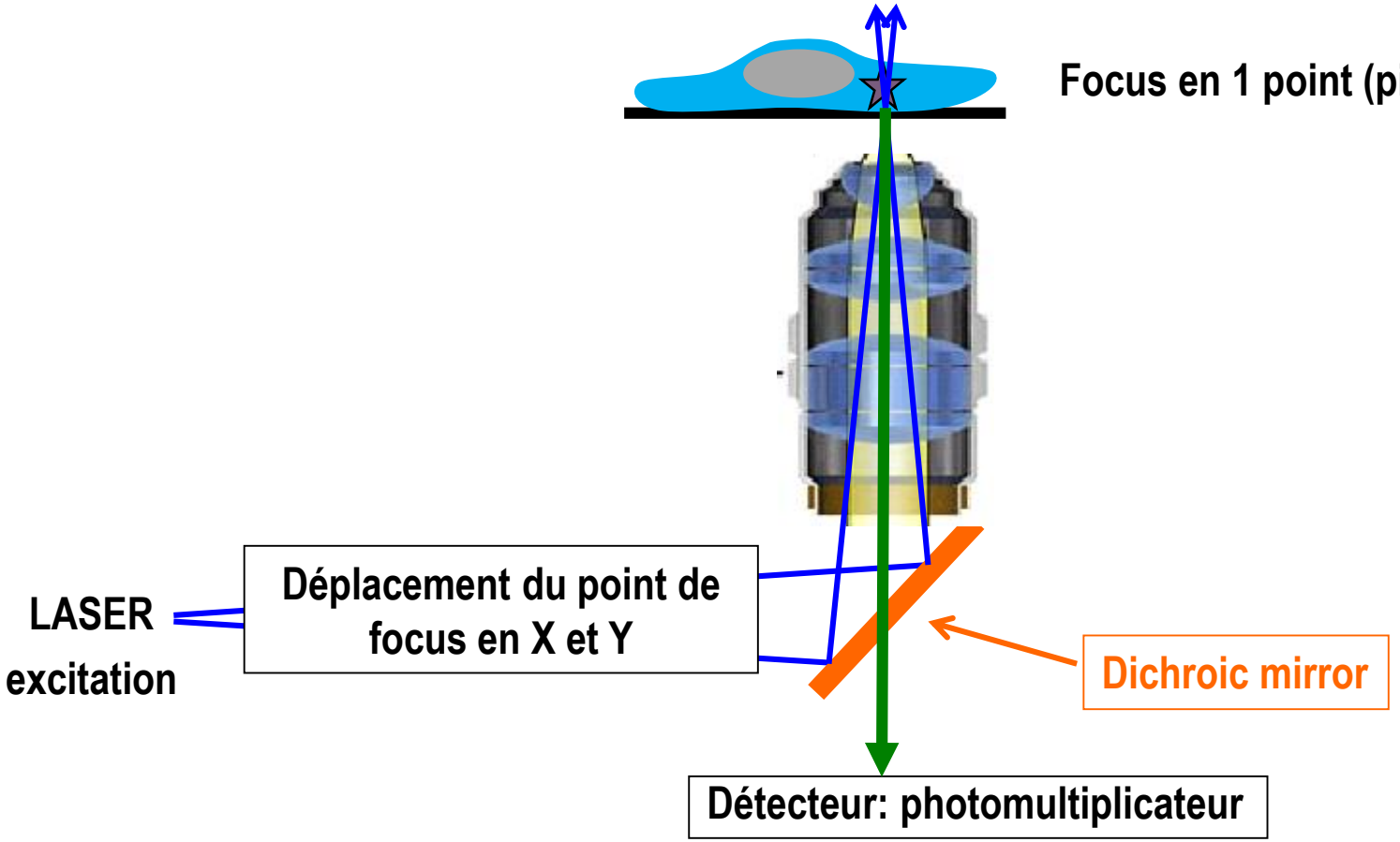


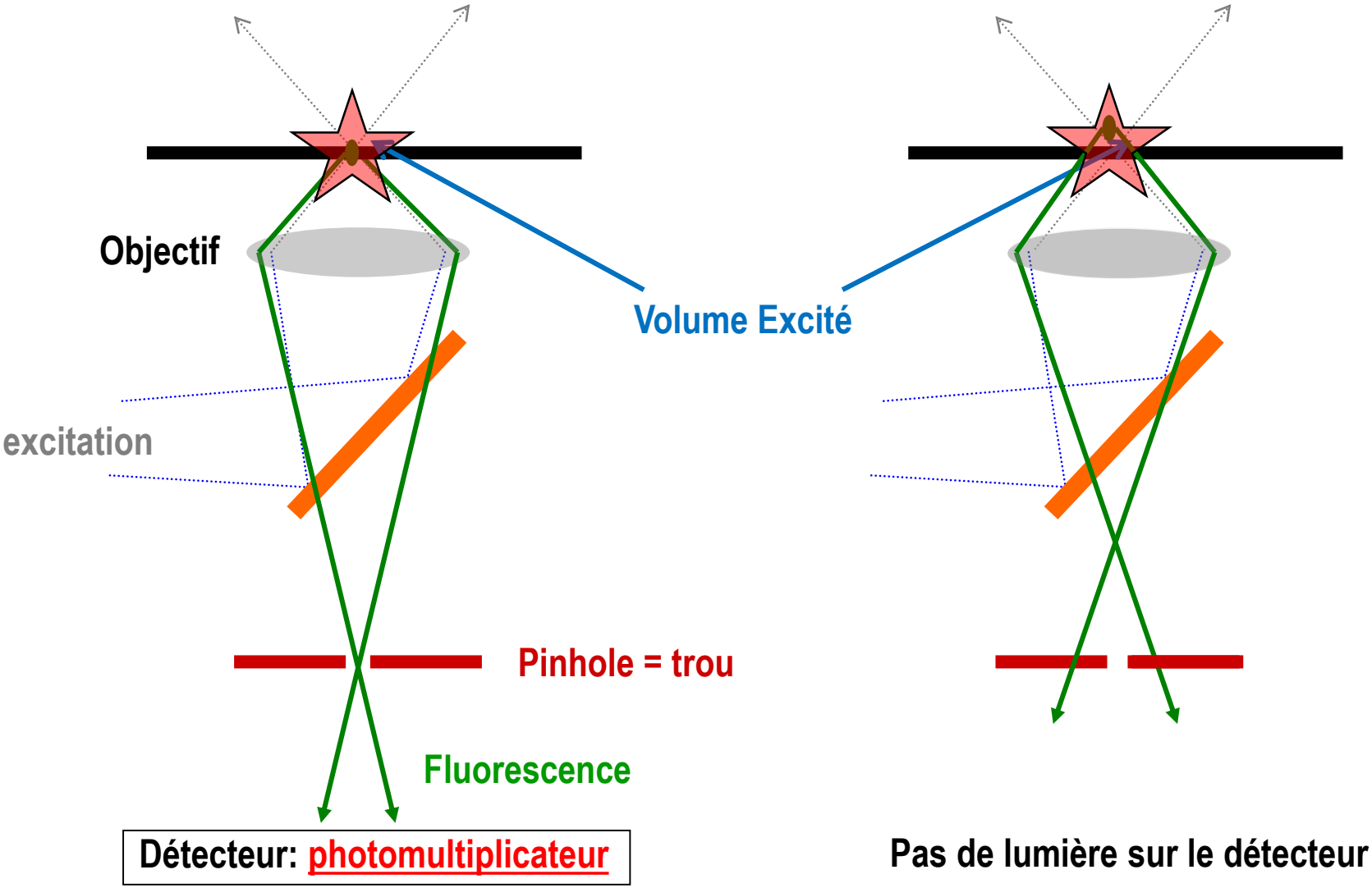
Marquage membranaire



Microscopie plus complexe et donc plus couteuse
Permet de collecter la fluorescence d'un seul plan


Focus en 1 point (pixel)
LASER
excitation
Dichroic mirror

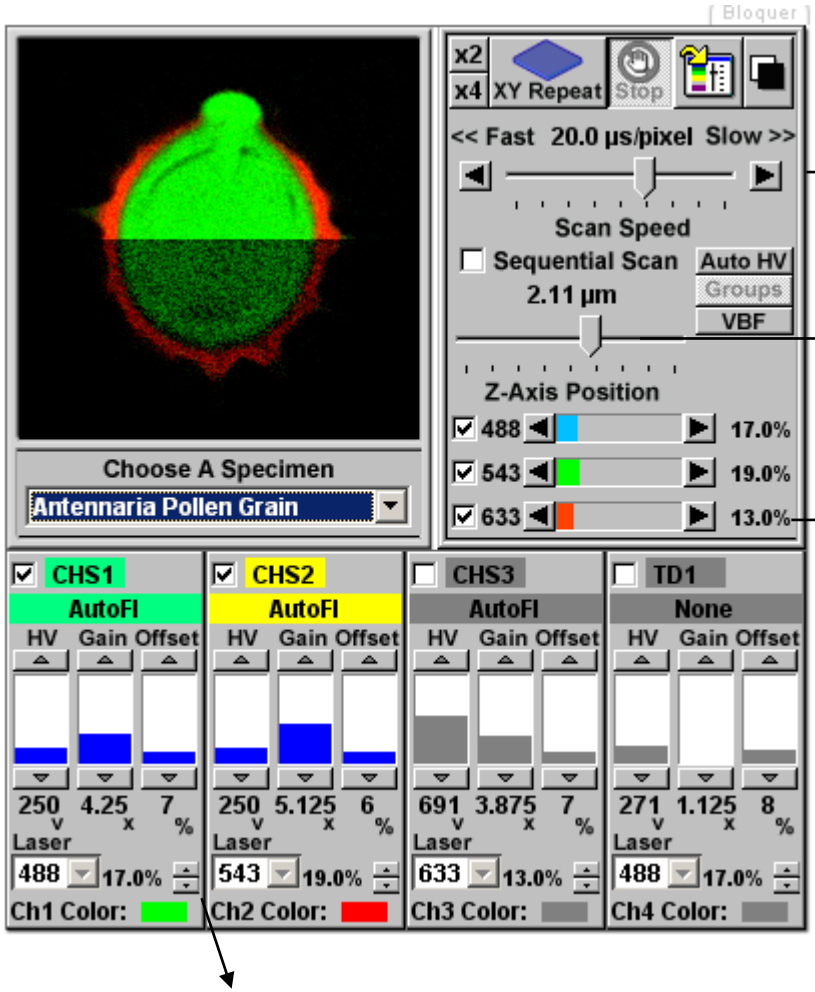

Focus en 1 point (pixel)
LASER
excitation
Déplacement du point de
focus en X et Y
Dichroic mirror
Détecteur: photomultiplicateur



Seule la lumière (fluorescence) qui vient du plan focal est détectée

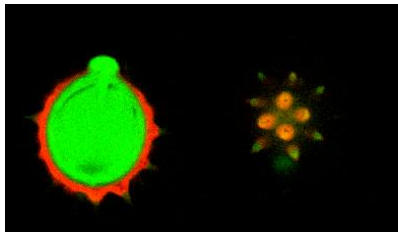
http://olympus.magnet.fsu.edu/primer/virtual/confocal/index.html

http://olympus.magnet.fsu.edu/primer/java/confocalsimulator/index.html

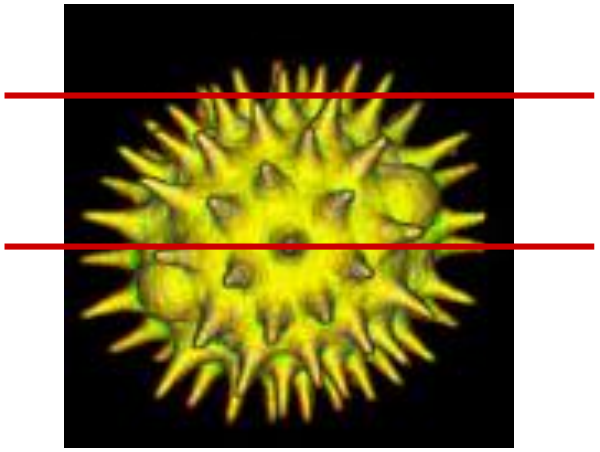


Pixel dwell

Z position



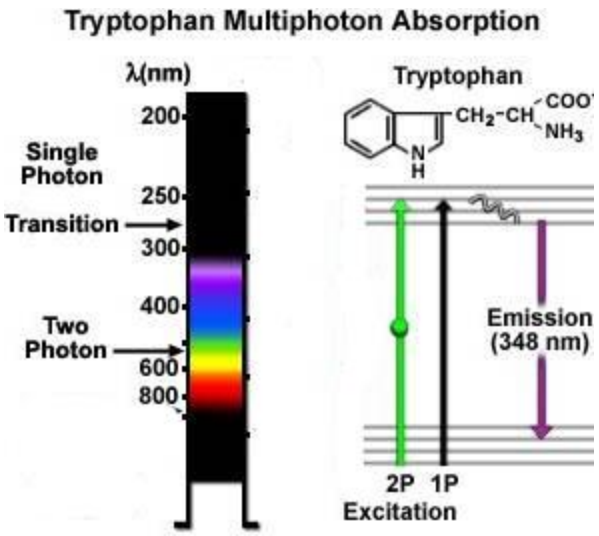
Laser wavelength and power

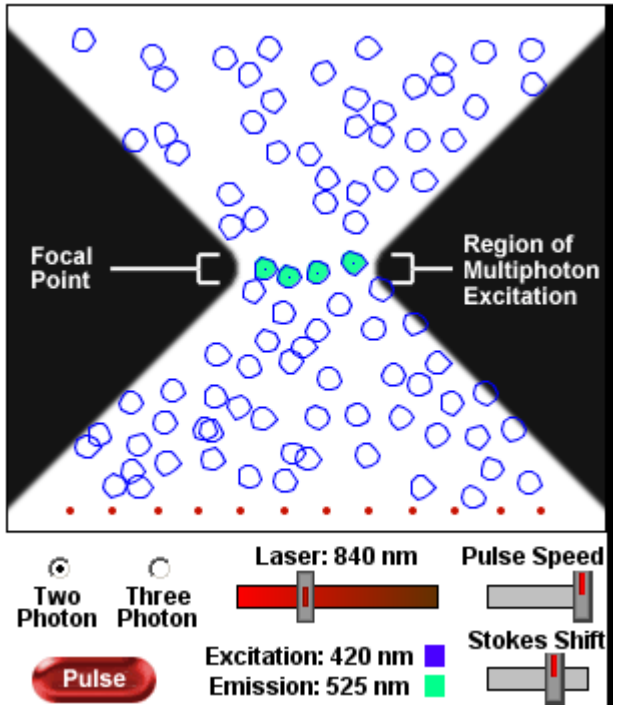


Parameters for the detector

Le cas de l'exciation bi-photonique

Webb et al., 1990





two photons can be simultaneously absorbed at *high photon densities*

Excitation source: pulsed laser

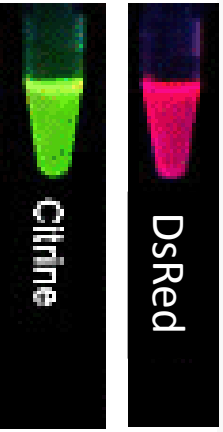
All the emitted photons from two photons excitation are used for imaging

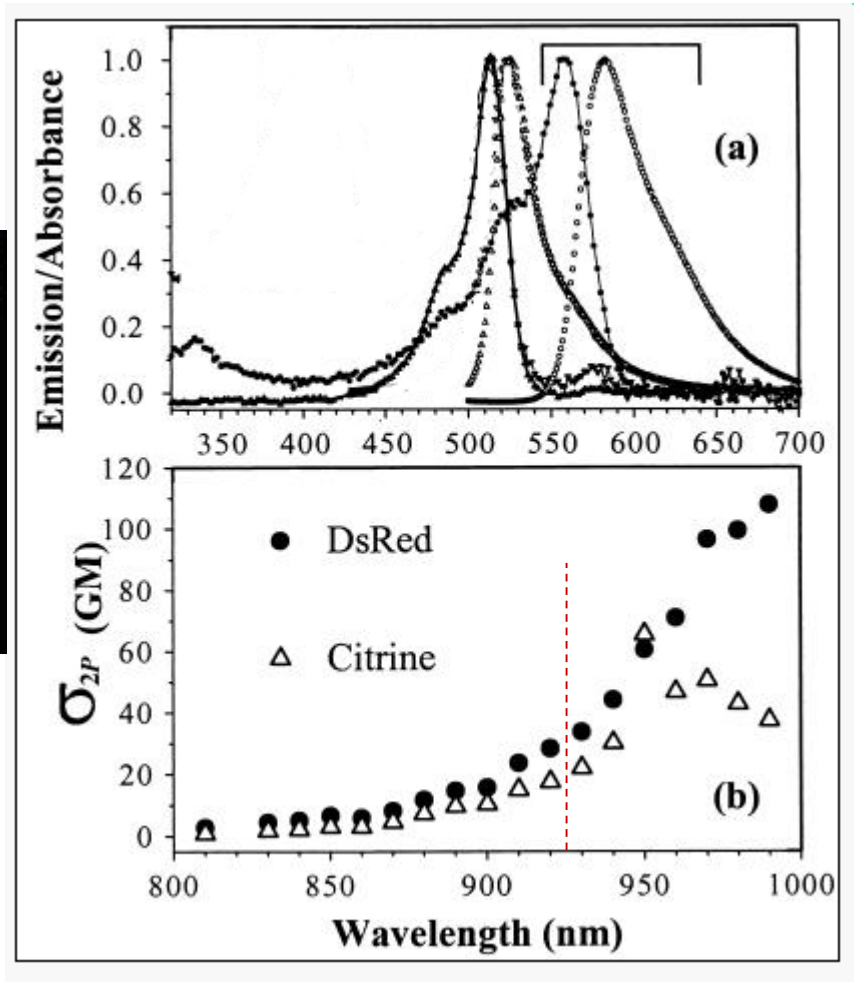
λ_{ex} (IR) → IR excitation wavelengths suffer less scattering in living tissue

→ the excitation light easily discard : λ_{ex}(IR) et λ_{em}(vis)

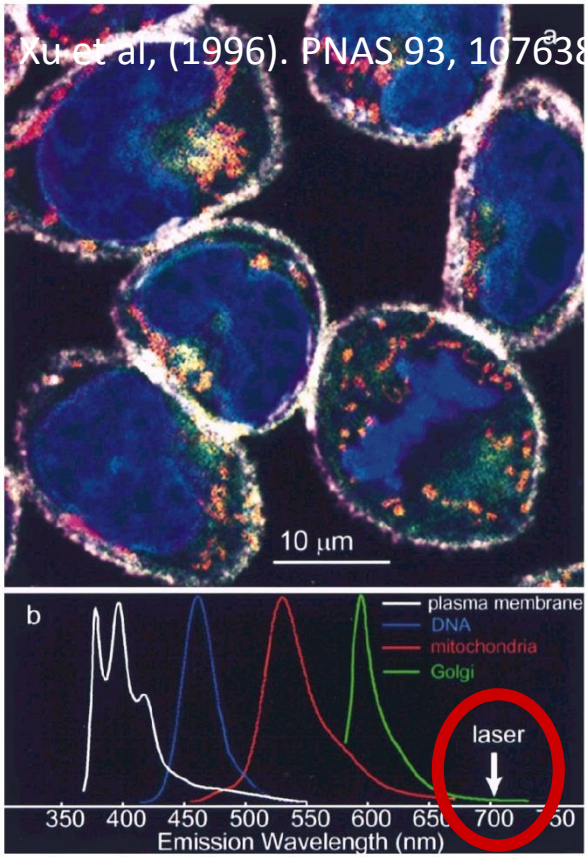
the rate constant of one excitation event depend upon:

- the square of the excitation intensity
- two photons cross section of the molecule





With one excitation wavelength (700 nm), several fluorophores are observed:



Pyrène DAPI Rhodamine Bodipy

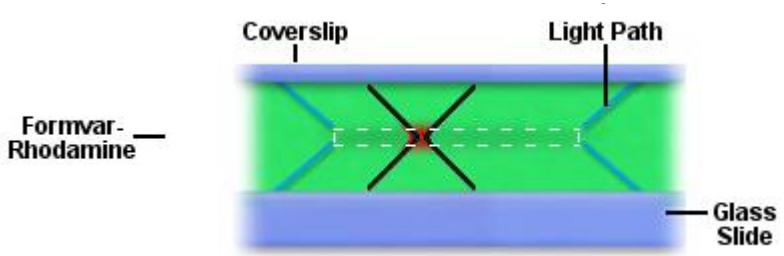
Excitation laser Ti:Saph 120 fs

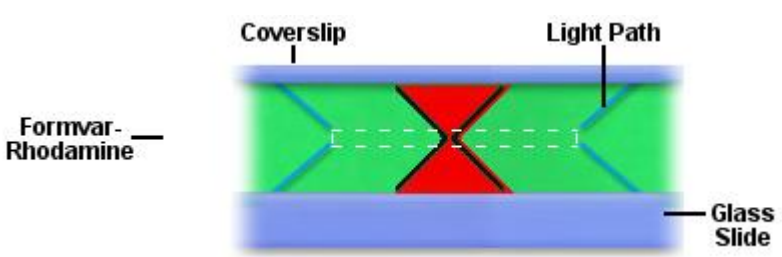
Both techniques:

- pave the way to 3D imaging (stack)

- necessitate high light intensities

- ca ng





Sample scanning is a “slow method” for image formation

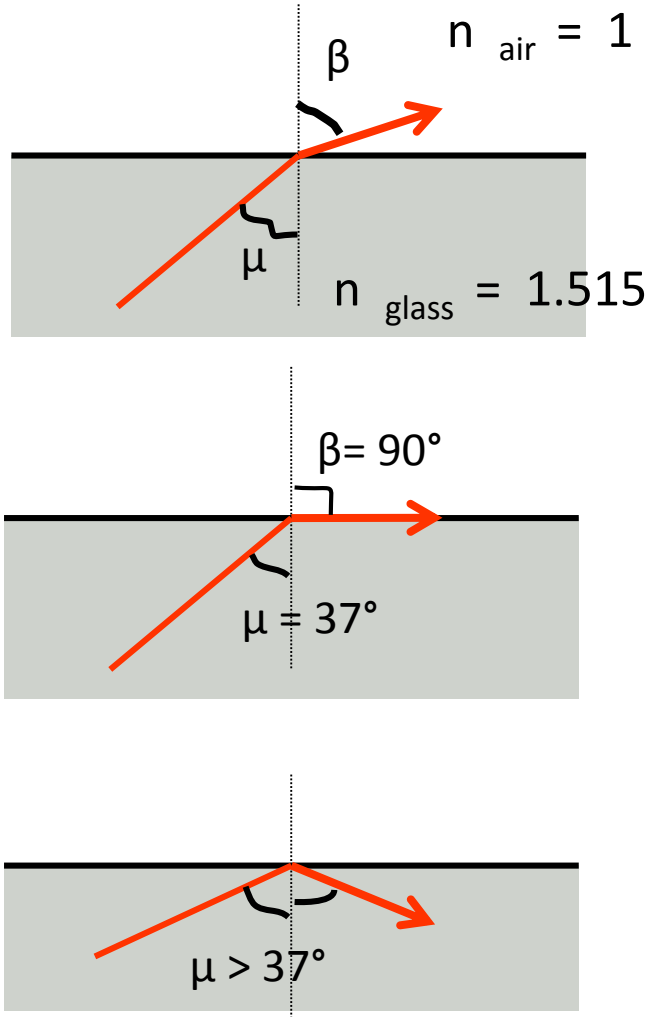
Le cas TIRFM

Techniques used since 80's to study surfaces and cells-surfaces interactions

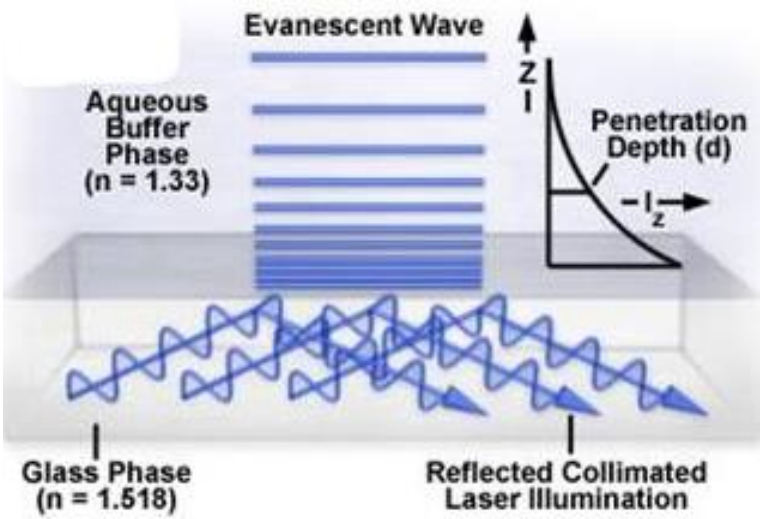
2003-2004: Improvement in optics (objective, miniaturized illumination devices,…)

TIRFM excites fluorescent probes in a restricted specimen region immediately adjacent to a glass-water (or glass-buffer) interface.

$n_{glass} \sin\mu = n_{air} \sin\beta$



Associate evanescent wave

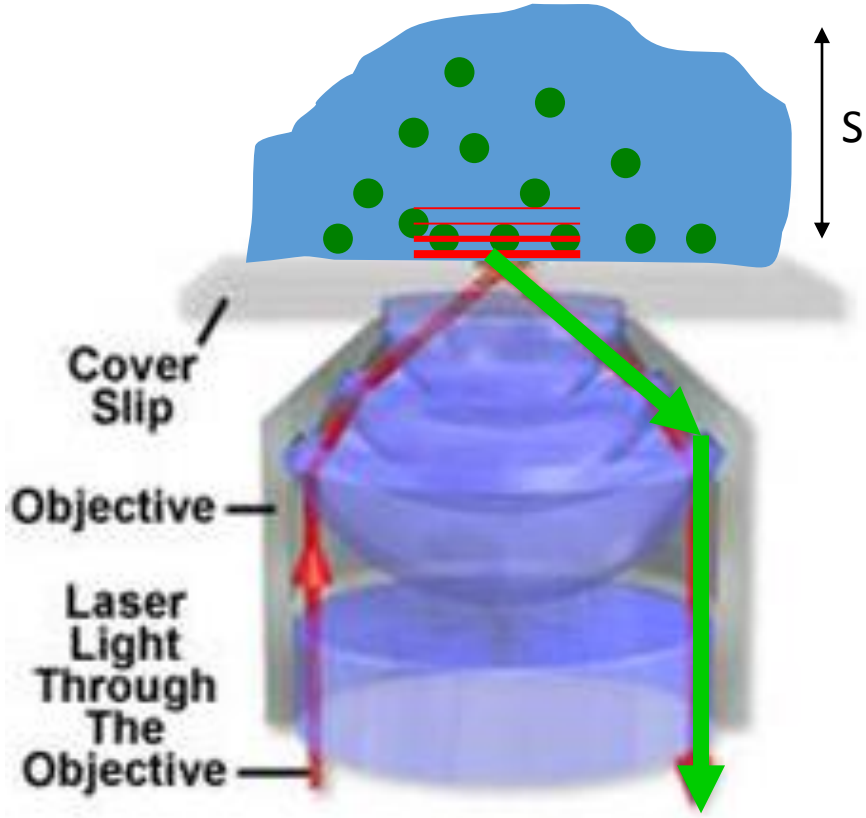


Exponential Intensity Decay

μ_{lim} of glass-water interface (n water = 1.33) = 62°

$$\text{Penetration Depth} = \frac{\lambda / 4\pi}{(n_{glass}^2 \sin^2\mu - n_{water}^2)^{1/2}}$$

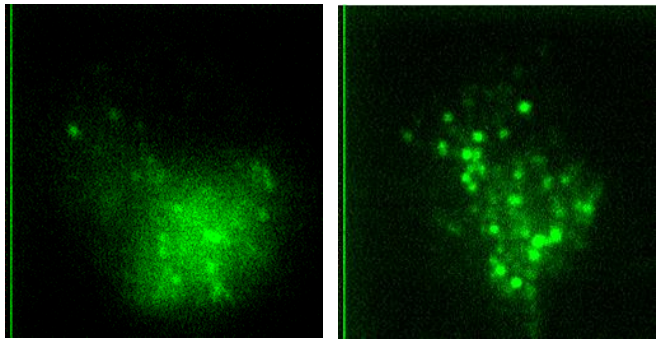
Num. App. => Penetration Depth = 70 à 300 nm



Specimen depth

Non excited fluorophores

Penetration Depth of evanescent wave = excitation depth



wide field

TIRFM

Example:

http://www.cellimagelibrary.org/images/12411

« Super-resolution »

Chemistry Nobel Price 2014

E. Betzig
S. W. Hell
W. E. Moerner

"for the development of super-resolved fluorescence microscopy"

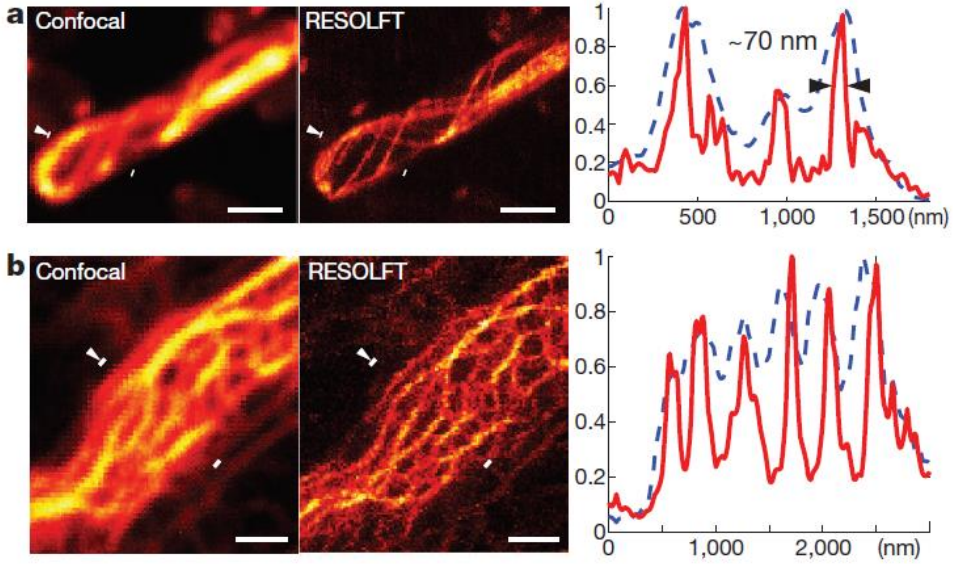
tackle the diffraction barrier of ~200 nm

- stimulated-emission depletion (STED) microscopy
- structured illumination microscopy (SIM)

} Excitation light patterns

- single-molecule localization microscopy (SMLM)

} Modulation of detected emission over time



E. coli bacterium expressing rsEGFP–MreB (=actine des bactéries)

Mammalian cell expressing keratin-19–rsEGFP

https://elifesciences.org/content/1/e00248/media1

Grotjohann, Nature, 2011

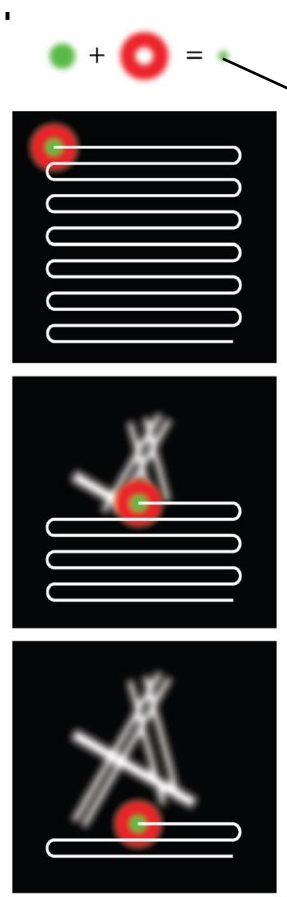
STED

2 lasers, excitation (diffraction limited) et depletion (stimulated emission, far end of fluorescence spectra)

Sub-diffraction scanning beam
Size fct of the intensity of STED depleting beam:

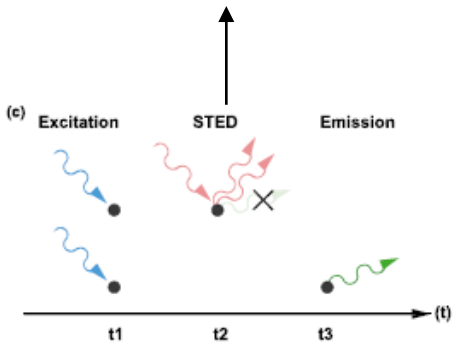
$$d_{STED} = \frac{d}{\sqrt{1 + \frac{I}{I_s}}}$$

Scanning method



No-post processing

Filtration spectrale et temporelle



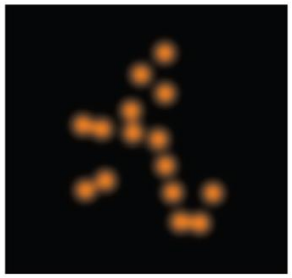
Alternative: RESOLFT
reversible saturable optical (fluorescence) transition
between two states

Turkowyd et al ABC 2016

SMLM

Photoactivated localization microscopy: PALM

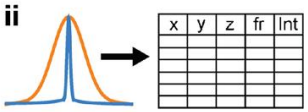
Stochastic photoswitching of fluorophores

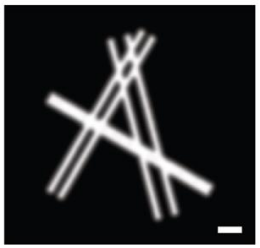


Detection of single spatially separated fluorophores that emit diffraction-limited spots

Stack of time-resolved images until all of fluorophores have been read out

$$d_{SMLM} = \frac{d}{\sqrt{N}}$$

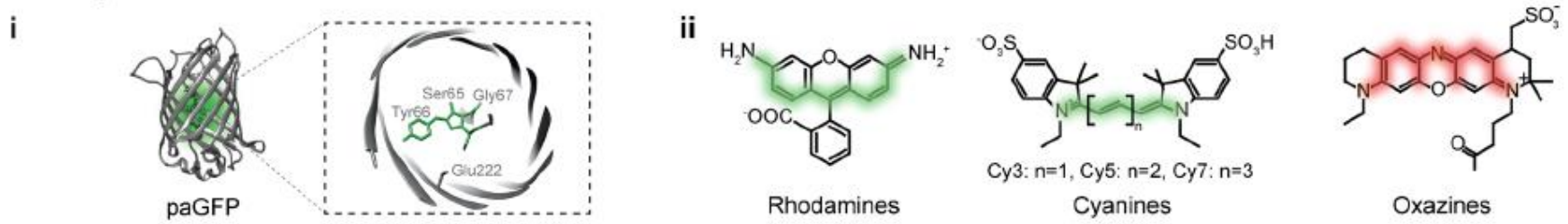




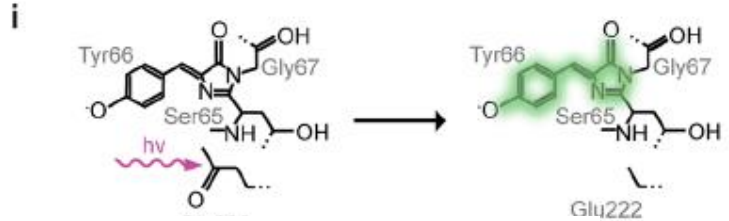
Complex algorithms for data processing

Turkowyd et al ABC 2016

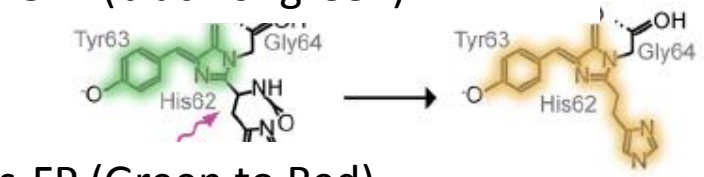
b Fluorophore structures



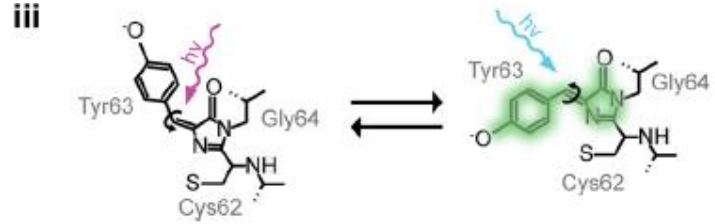
c Photochemical and photoconformational changes



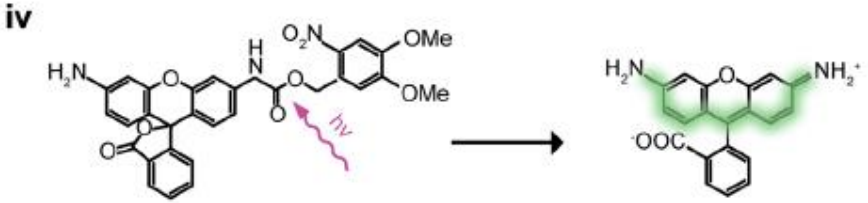
PA-GFP (black of green)



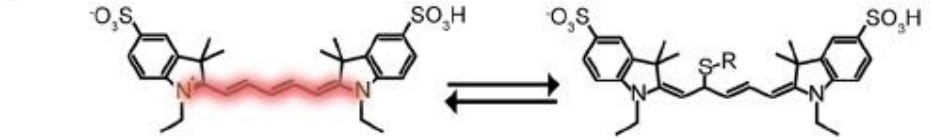
Eos-FP (Green to Red)



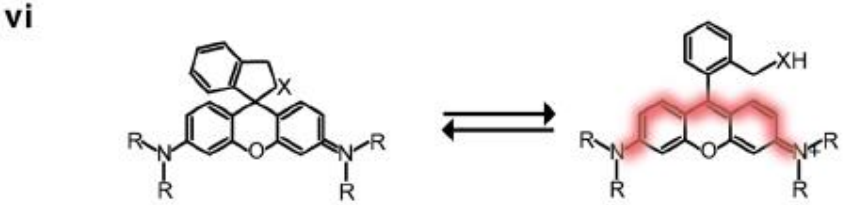
Dronpa (black of green, REV)



Photocaged rhodamine



Reversible quenching by a thiol of Cy5



Reversible cyclization of a rhodamine

Turkowyd et al ABC 2016

Le microscope

- Les éléments du microscope à fluorescence
- Le microscope (optique, le choix de l'objectif)
- La microscopie plein champ

Amélioration de la résolution spatiale

- Confocal (laser scanning, detection)
- Bi-photon
- TIRF
- Super-resolution

La spectroscopie sous microscope

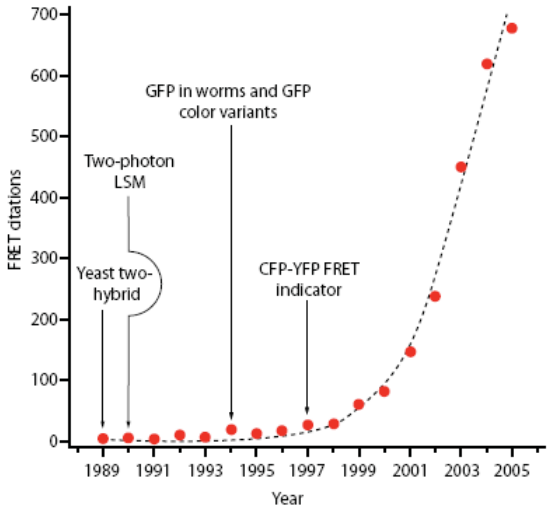
- FRET (interactions, metabolic activities)
- FLIM (for FRET and dyes specific measurments)
- FCS, FCCS (interactions, structure & reactivity)

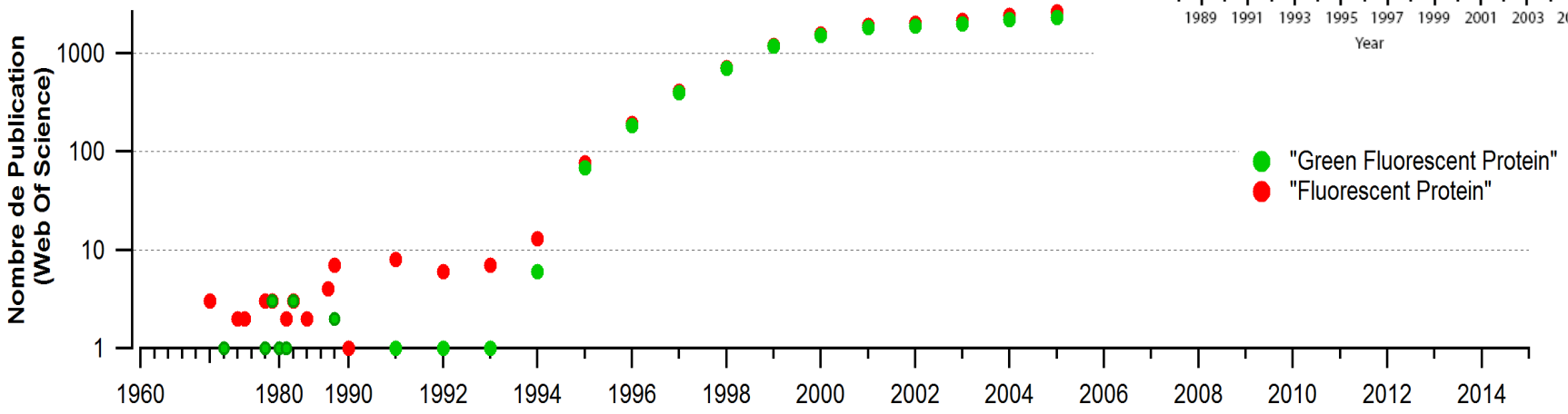
Le cas du FRET : *: Förster Resonance Energy Transfer ou FRET*

Step 1 : Gene <-> Protein identification

Step 2: Proteome Analysis

and Protein-Protein interaction determination



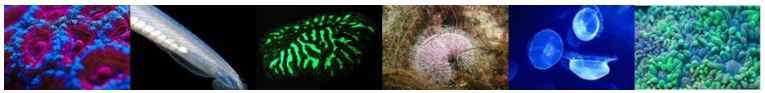


1962
GFP

1992
Clonage gène GFP

1999
Coraux DsRed

Coraux, Crustacés, Lancelet ……



1979
Structure
Chomophore
GFP

1996
Structure
3D
GFP

2000
Structure
Chomo
DsRed

2002
Structure
Chomo
Kaede

2008
PRIX
NOBEL

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

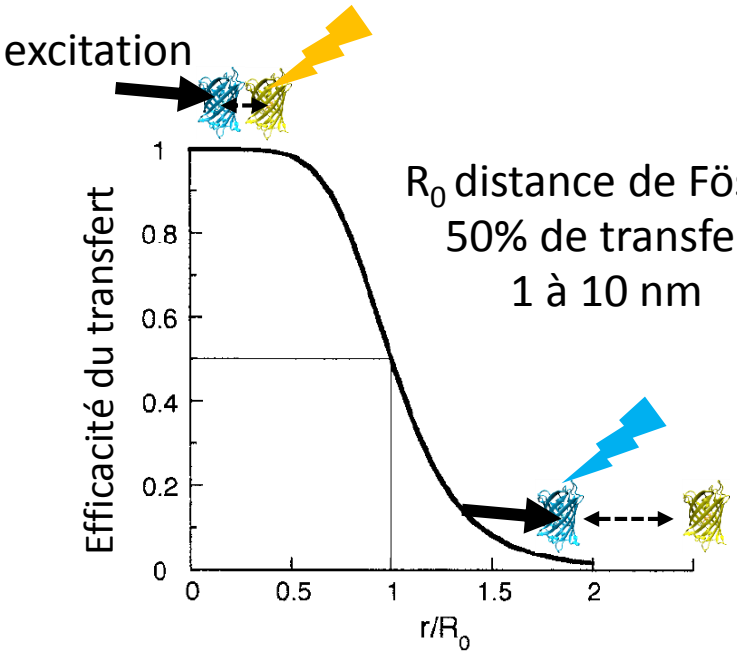
1995-98
CFP, YFP, BFP

1999
cpFP

2002
PA-FP

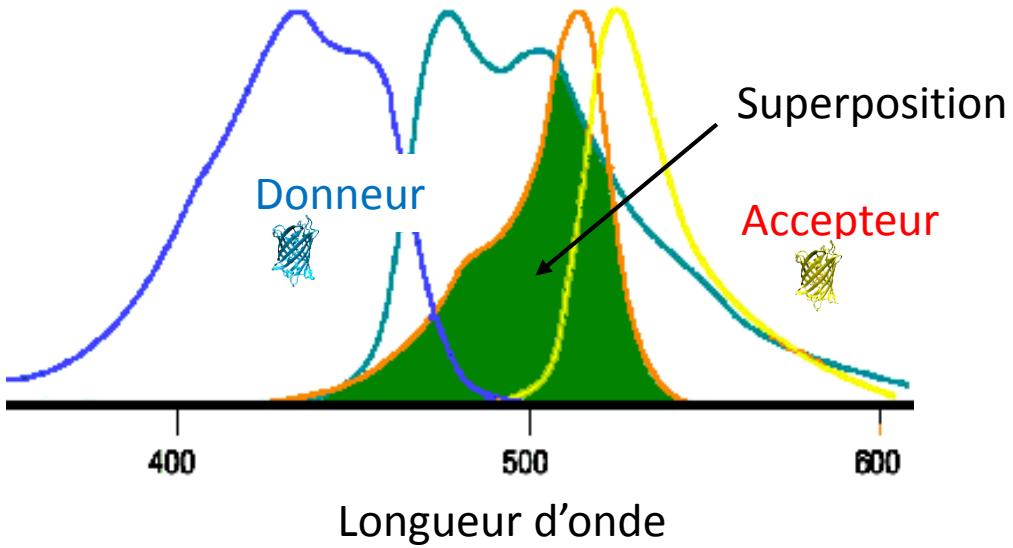
Versions améliorées des protéines monomériques

Il faut 2 fluorophores: donneur et accepteur



R_0 distance de Föster
50% de transfert
1 à 10 nm

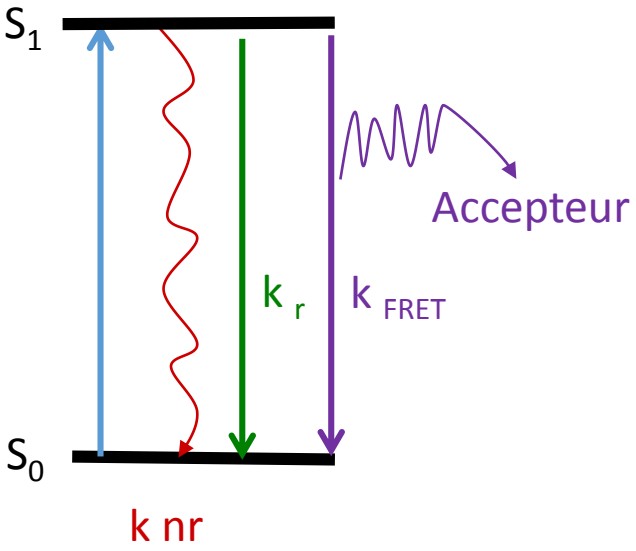
Condition spectrale:



Interaction coulombienne dipôle - dipôle à « longue distance » (1 à 10 nm)
L'efficacité du FRET varie rapidement avec la distance entre le donneur et l'accepteur ($1/r^6$)

Mesure de distances / visualisation des interactions entre de macromolécules

De quoi dépend le transfert d'énergie?



$$k_{FRET} = \left\{ 0.211\ n^{-4}\ \frac{\Phi_D}{\tau_D}\ J_{DA}\ \kappa^2_{DA} \right\} \frac{1}{r^6} = \frac{1}{\tau_D} \left(\frac{R_0}{r} \right)^6$$

D = donneur seul

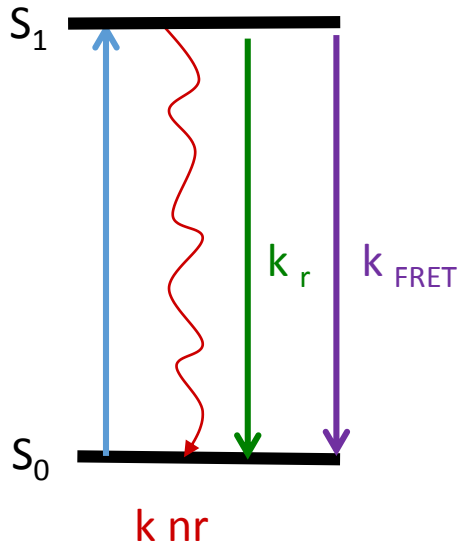
DA= donneur en présence d'accepteur

- Propriétés photophysique du donneur, Φ_D et τ_D
- Indice de réfaction du milieu: *n*
- Surface de recouvrement des spectres: J_{DA}
- Orientation relative du Donneur de l'Accepteur: κ_{DA}

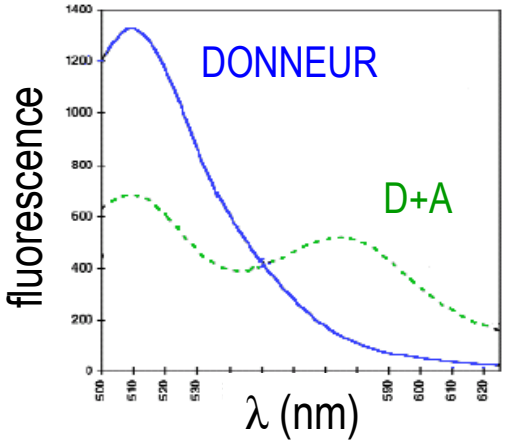
Moments ⊥	$\kappa^2 = 0$
Moments colinéaires	$\kappa^2 = 4$
Moyenne "rapide"	$< \kappa^2 > = 2/3$
Moyenne "statique"	$< \kappa^2 > = 0.476$

Conséquences du FRET?

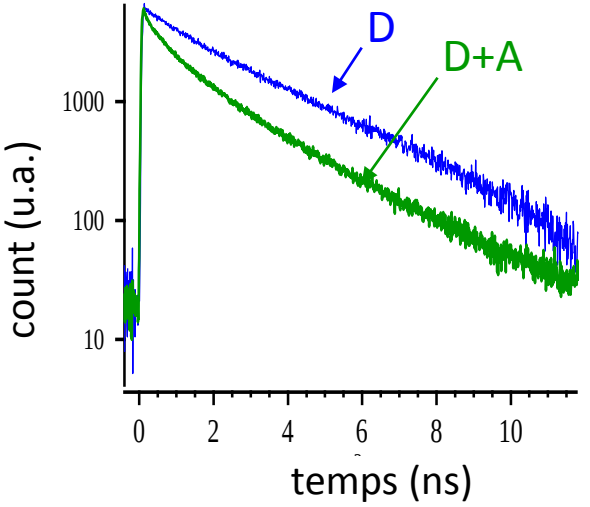
Excitation du donneur



Intensité



Durée de vie

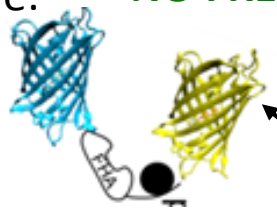


Efficacité du transfert

$$E_{FRET} = \frac{k_{FRET}}{k_r + k_{nr} + k_{FRET}} = 1 - \frac{\phi_{DA}}{\phi_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$

Exemple biosenseur de FRET , détection en ratiométrie

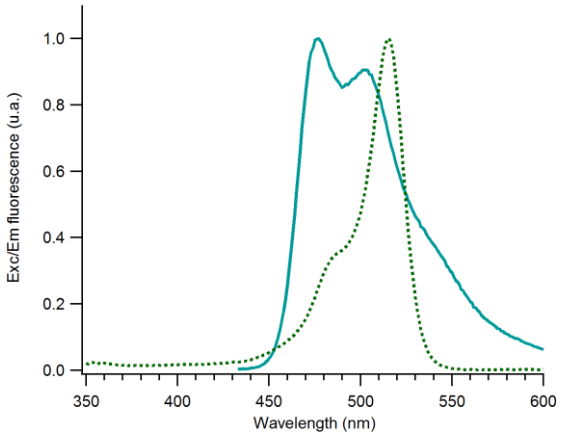
Protéine: **NO FRET**



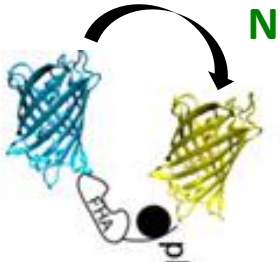
Accepteur

Donneur

Module senseur central
(ici, Phosphorylable)



NO FRET

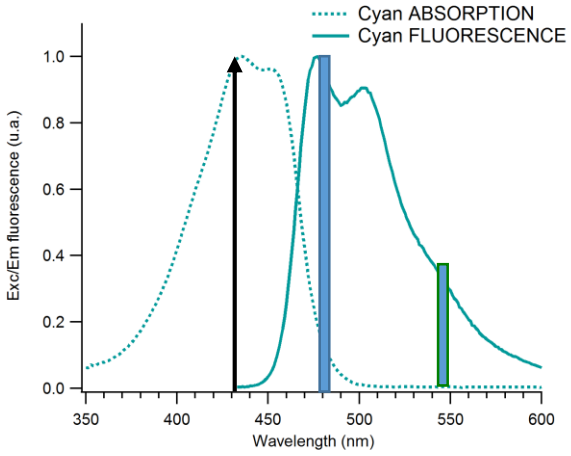


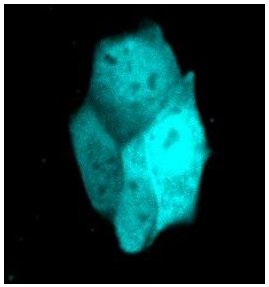
PKA

phosphatase

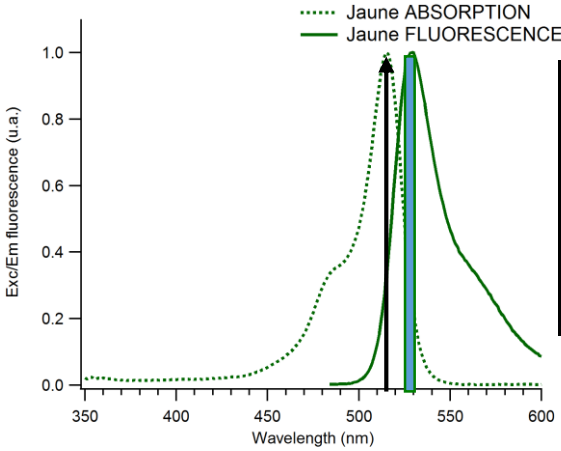
FRET

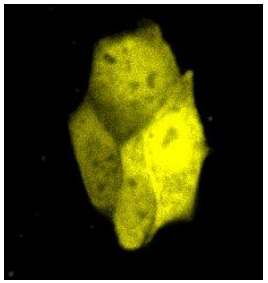
NO FRET





Ex 440 nm
Em 480 nm





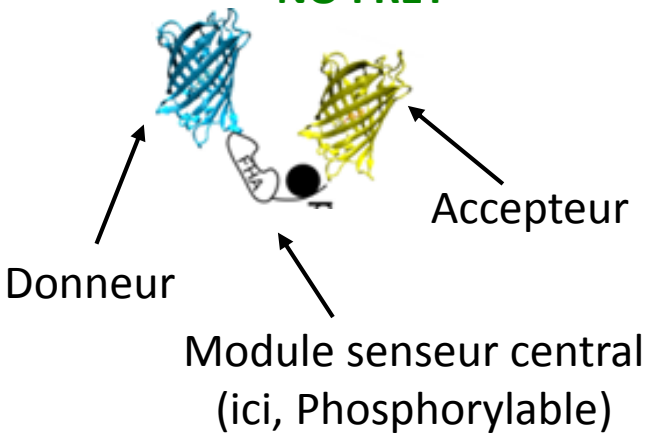
Ex 500 nm
Em 535 nm

Images plein champ

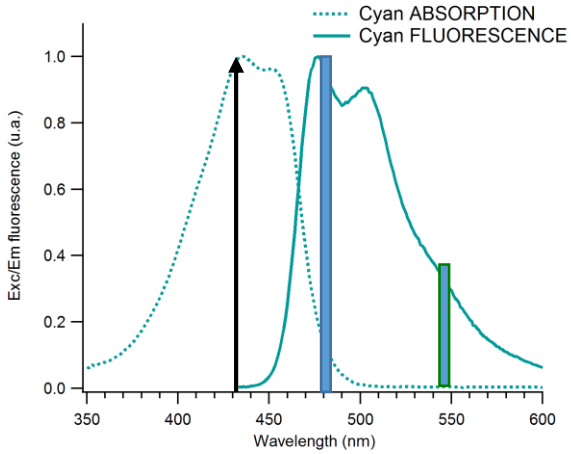
Ex 440 nm
Em 535 nm

Exemple biosenseur de FRET , détection en ratiométrie

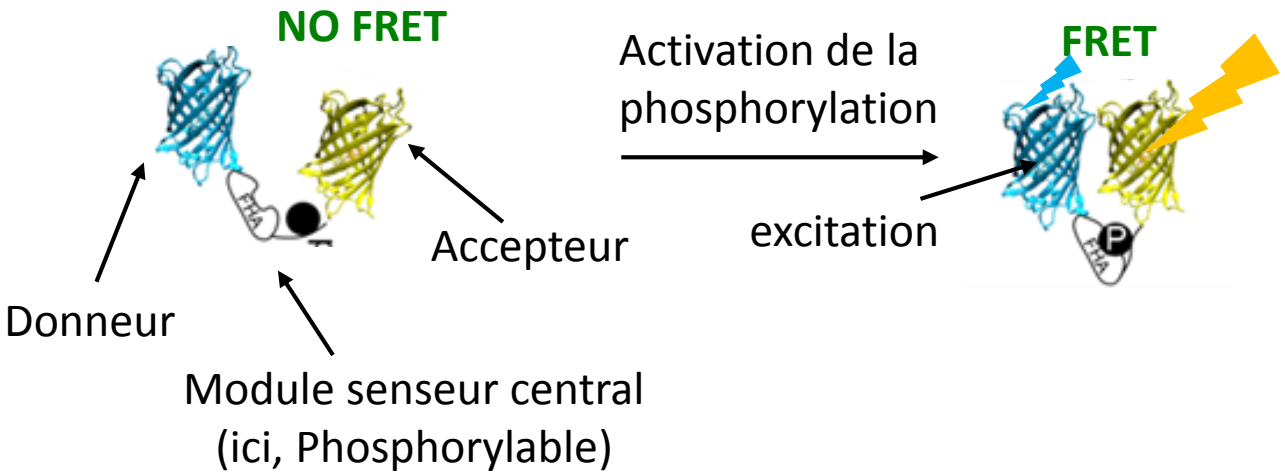
NO FRET



NO FRET



Exemple biosenseur de FRET , détection en ratiométrie



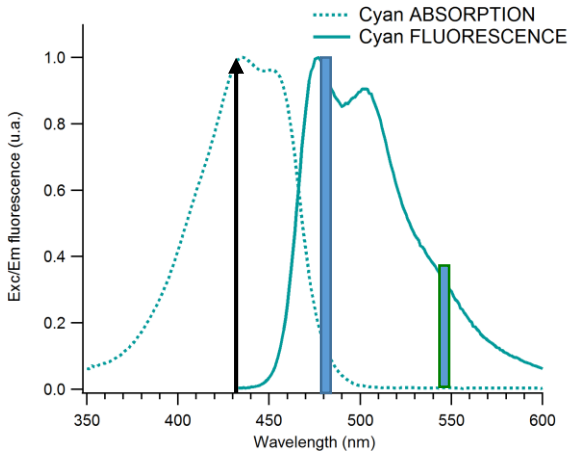
I fluo

Ex 440 nm
Em 480 nm ↓

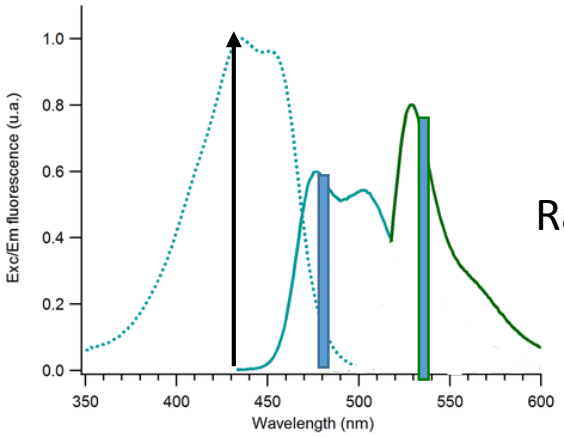
Ex 440 nm
Em 535 nm ↑

Ratio 535 / 480 ↑↑

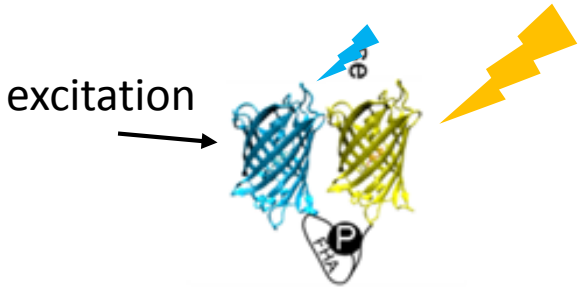
NO FRET

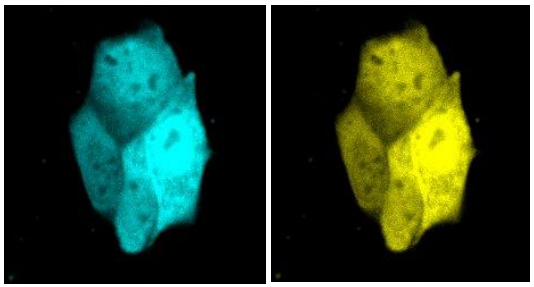


FRET



Exemple biosenseur de FRET , détection en ratiométrie





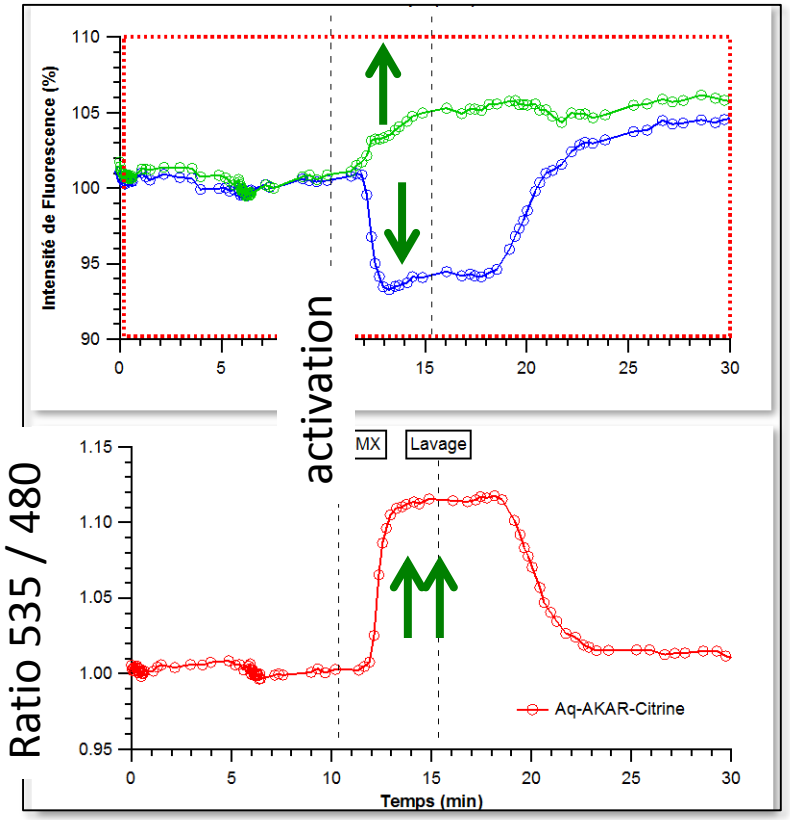
Ex 440 nm
Em 480 nm

Ex 440 nm
Em 535 nm

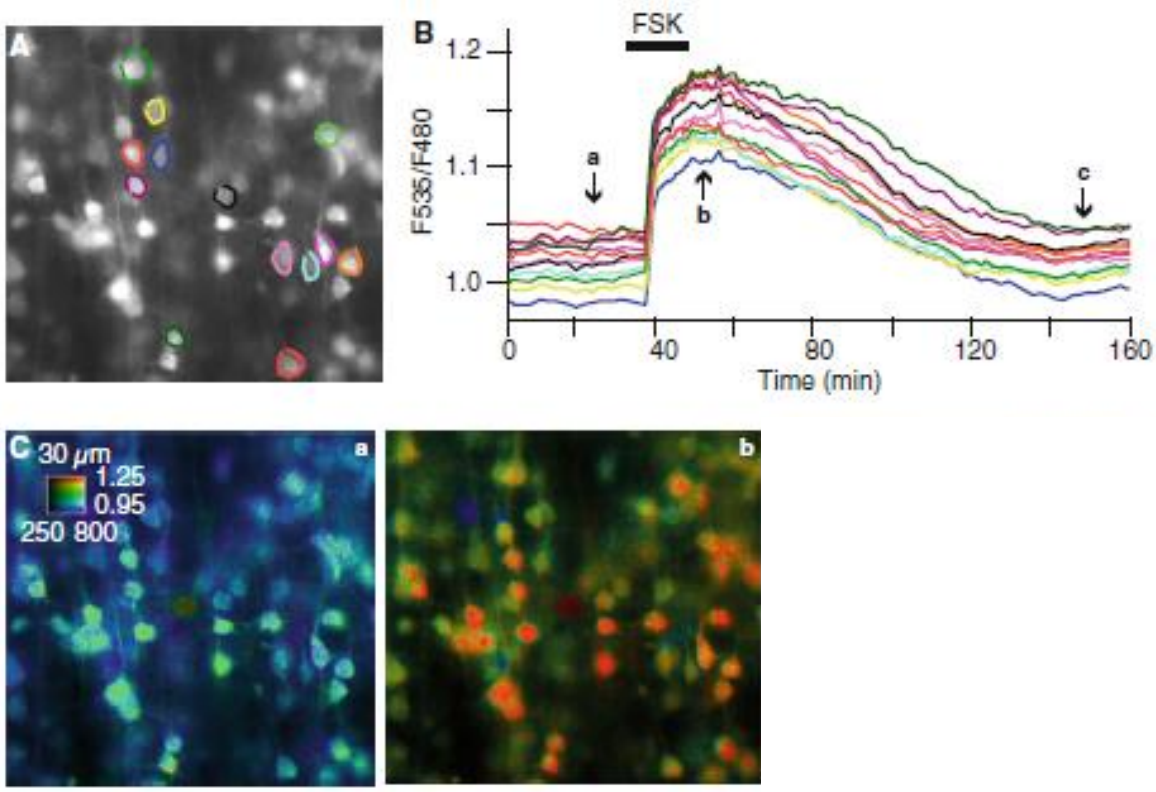
Intensité

Ex 440 nm
Em 480 nm

Ex 440 nm
Em 535 nm



activation

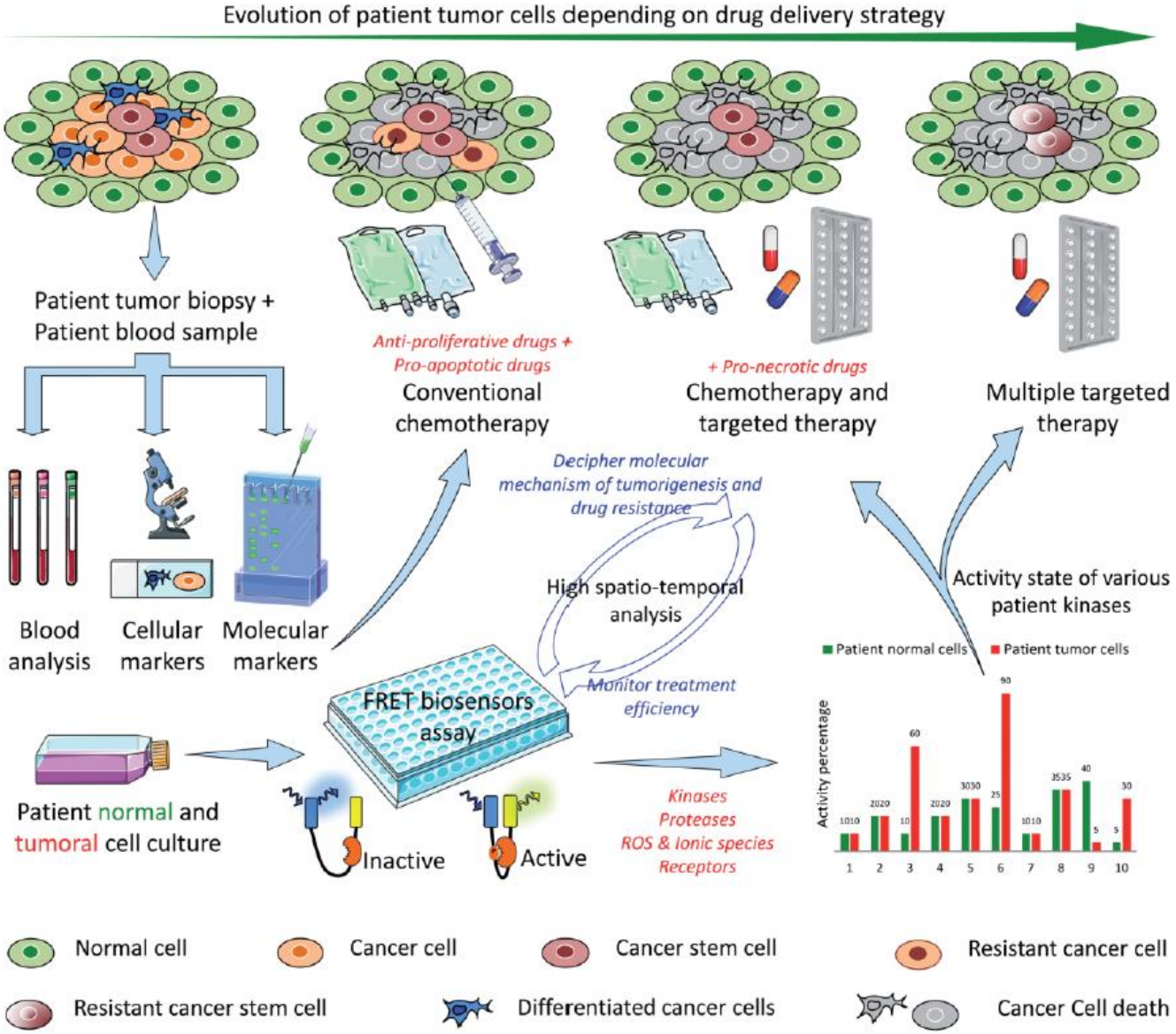


Ratio 535 / 480 ⇈

PKA activity in cortical neurons

Polito et al Methods in Mol Biol 2016

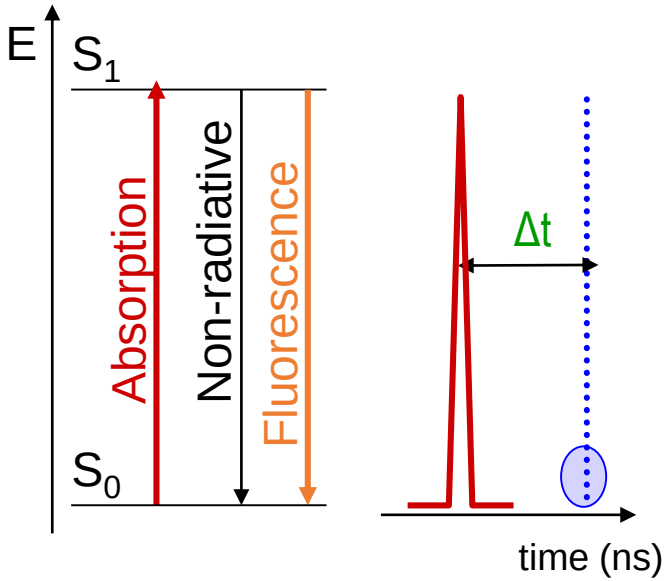
Et dans le futur???

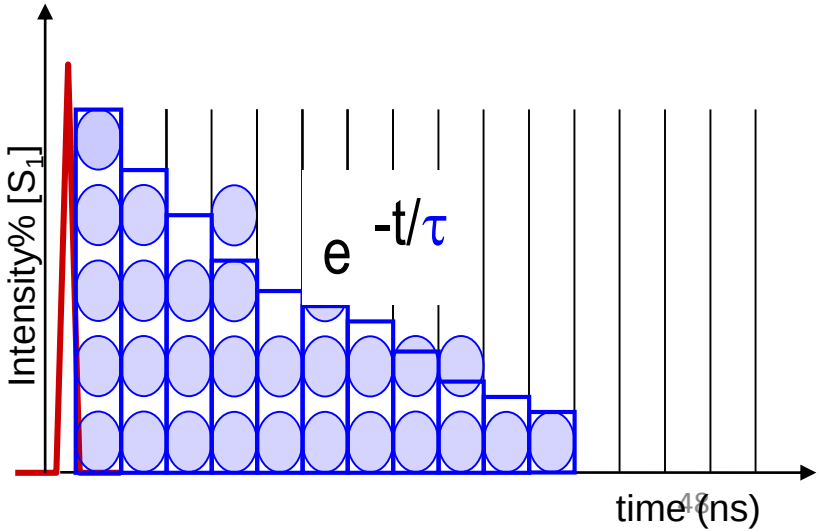


Sipieter Biotech J 2013

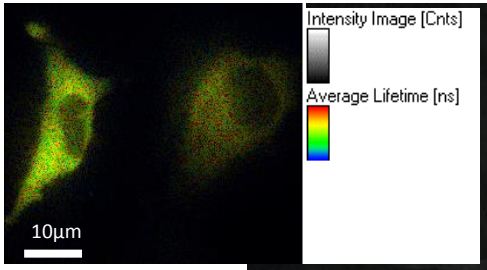
Le cas du FLIM : *: Fluorescence Lifetime Imaging*

Pour l'analyse du FRET mais pas que….

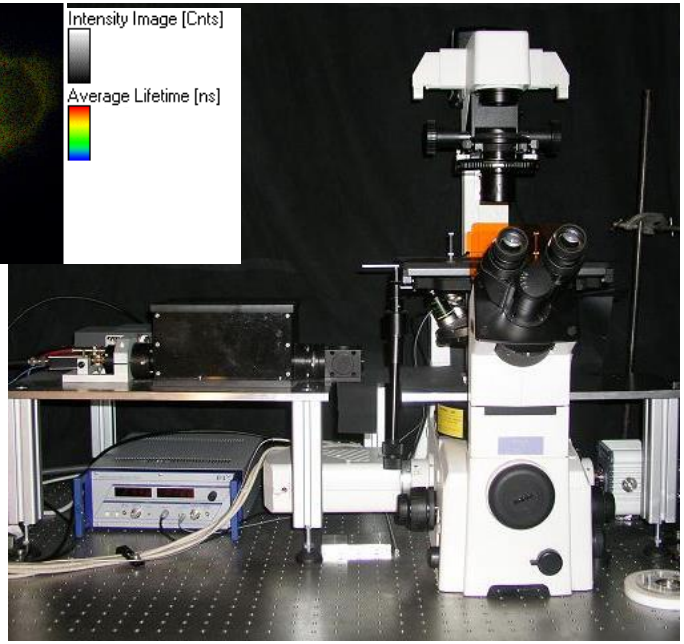


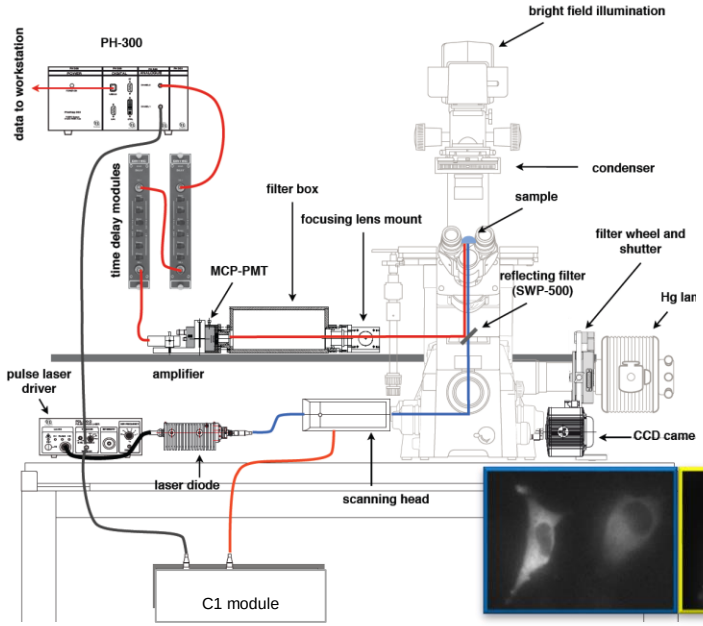


- Independant of concentration
- Strongly dependant of environement
- Highly reliable

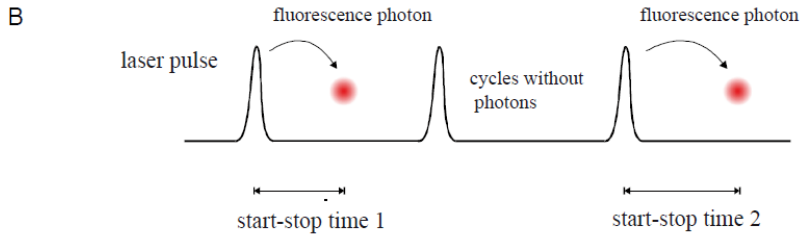


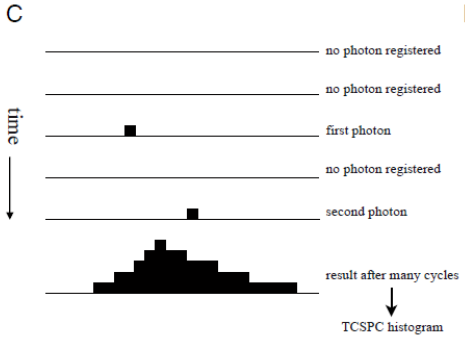
FLIM-TCSPC
ROI: DECLIN

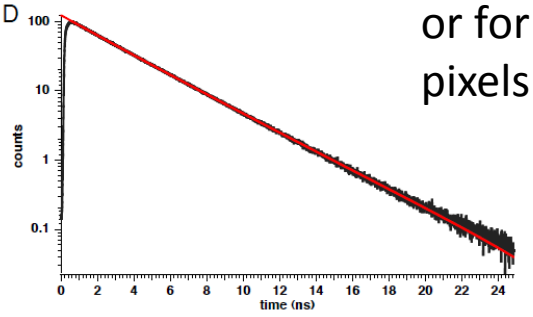




Principe de la mesure:







Decay in 1 pixel or for several pixels ….

Laser scanning microscope

Mais à quoi correspond le signal final obtenu ?

Déclin expérimental $I_{exp}(t)$ = le déclin d'émission de fluo de l'échantillon distordu par l'optique et l'électronique du système !!

Causes de distorsions possibles :

- Dérives et instabilités de la source d'excitation ion
- Dispersion et variation des trajets optiques dans les monochromateurs
- Retards induits par la traversée de filtres d'atténuateurs, de polariseurs
- Longueur et dispersion des trajets optiques dans la cuve de l'échantillon dépendantes de la géométrie, de la densité optique, de la turbidité et des réflexions internes
- Réflexions parasites sur tous les dioptres
- Réponses du photomultiplicateur dépendantes de la longueur d'onde
- Dérives et instabilités de l'électronique
- …

Prise en compte de certaines de ces perturbations dans la fonction d'appareil g(t) (= lumière diffusée par une solution diffusante)

g(t) = profil de l'impulsion d'excitation distordue par toute l'électronique et l'optique du système

$$I_{exp}(t) = g(t) \otimes I(t)$$

↓

Déclin d'émission de fluorescence de l'analyte

Ajustement des données

En pratique, $I_{calc}(t) = g(t) \otimes I_{theo}(t)$ comparaison $I_{exp}(t) = g(t) \otimes I(t)$

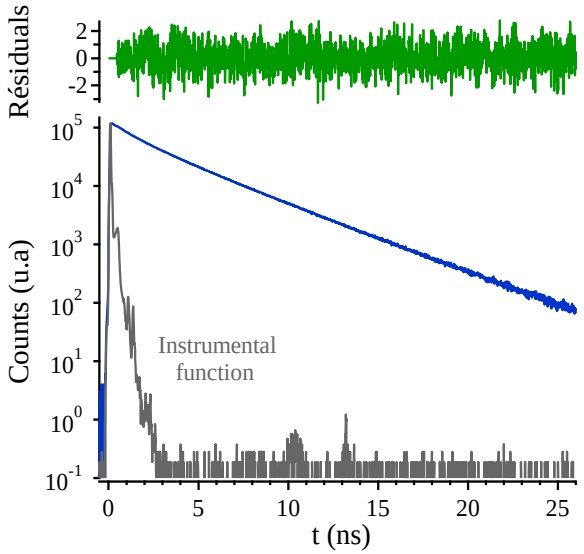
Qualité de l'ajustement :

- $\chi^2 = \frac{1}{N} \sum_i \frac{1}{\sqrt{I_{exp}(t_i)}} \left[I_{calc}(t_i) - I_{exp}(t_i) \right]^2$ Valeur la plus faible, proche de 1

- Courbe des résidus pondérés

$$Residu(t_i) = \frac{\left| I_{calc}(t_i) - I_{exp}(t_i) \right|}{\sqrt{I_{exp}(t_i)}}$$

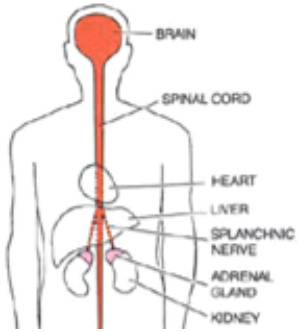
Le plus aléatoirement distribué autour de 0

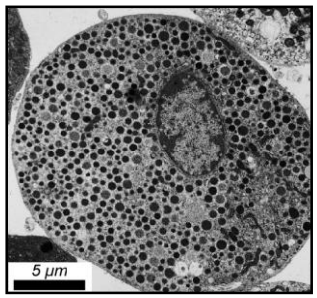


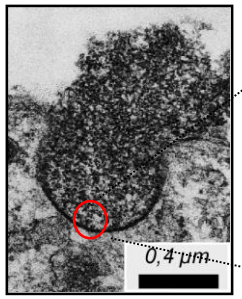
Déclin d'émission de fluorescence de la CFP (bleu). Courbe de résidus obtenue en ajustant le déclin par un modèle multiexponentiel.

H . Pasquier et al., *2008*, Biochemistry

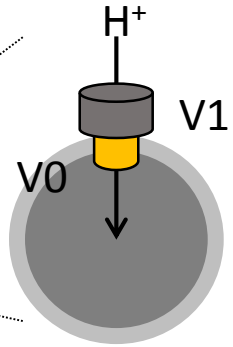
La déconvolution n'est pas toujours possible ou necessaire…



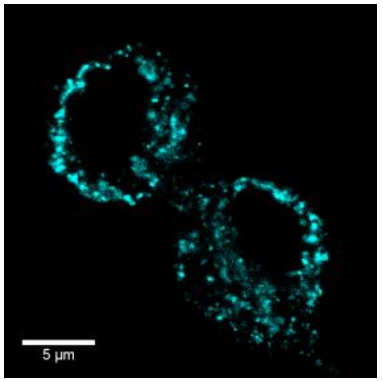




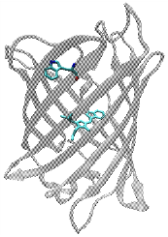
10000 vésicules "dense-core"

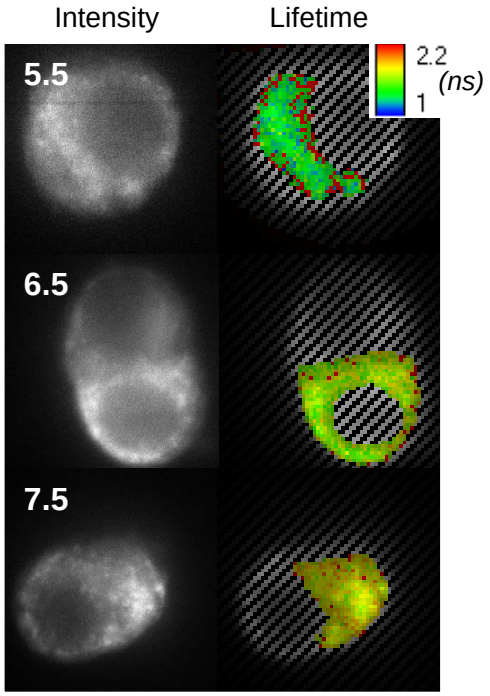


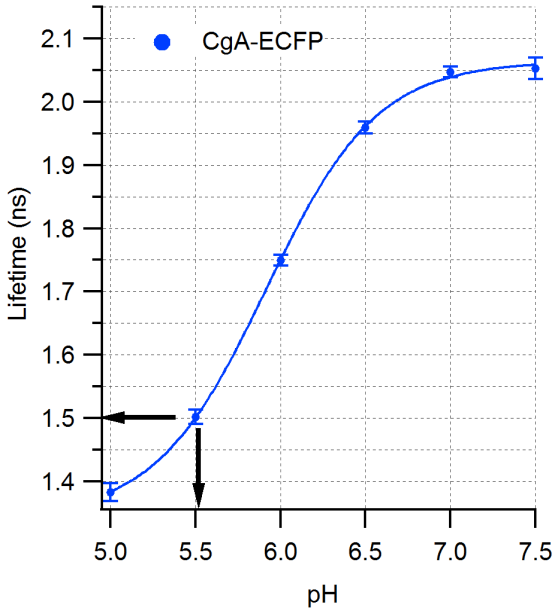
V-ATPase



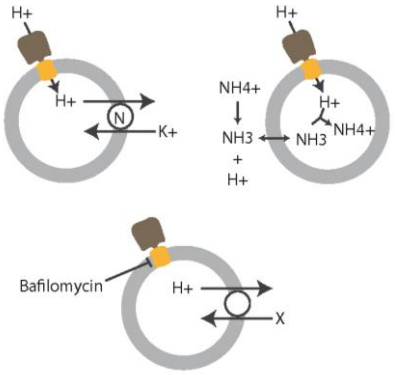
Fox et al *Cell Tissue Res. 1996*

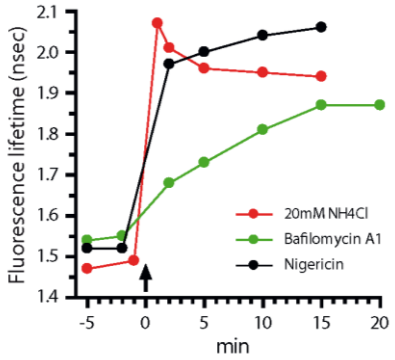


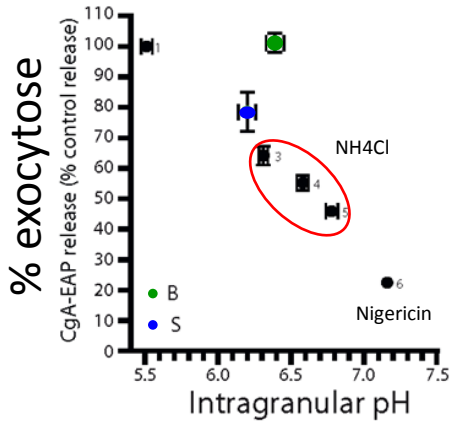


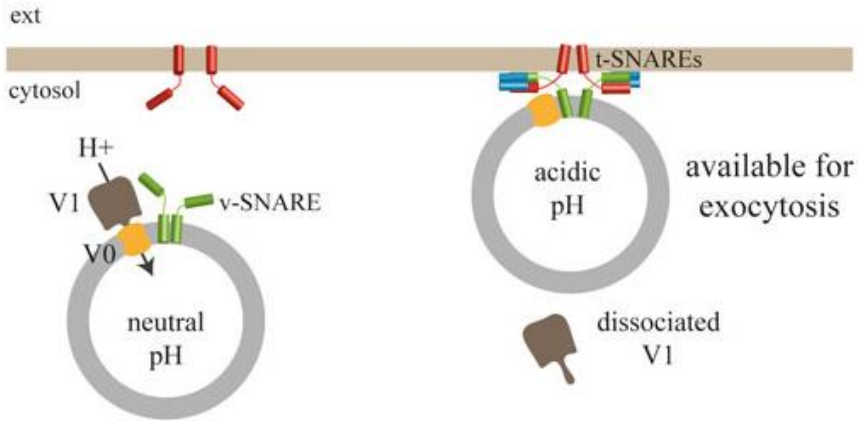


Poea Guyon et al Anal Bioanal Chem 2013





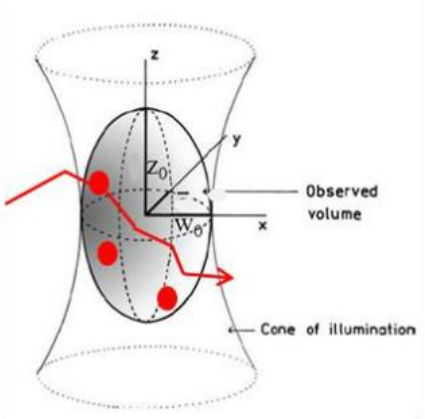




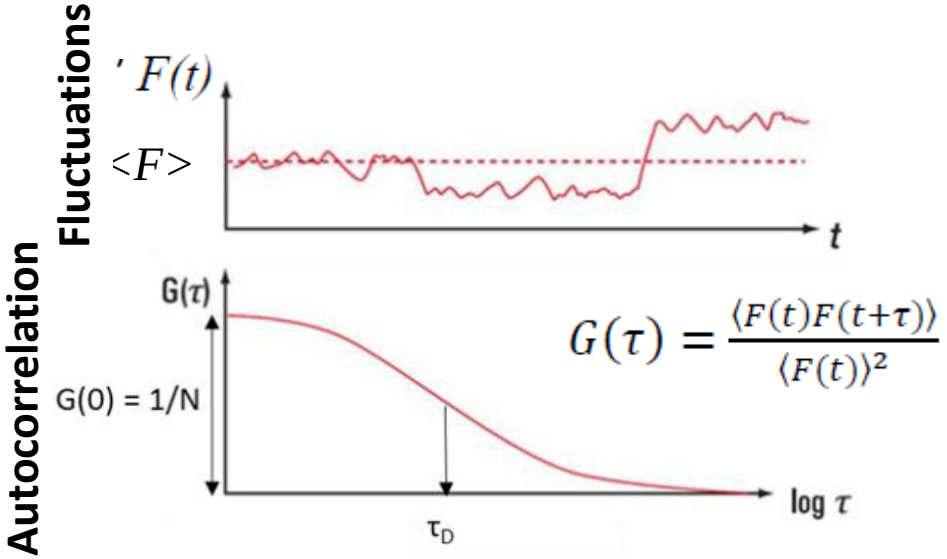
Poea Guyon J Cell Biol 2013

Le cas du FCS, FCCS : *: Fluorescence (Cross-) Correlation Spectroscopy*

- Diffusion (taille, mono-multimères) & concentration
- Réactivité (constantes de vitesse)



0,1 à 0,3 fL



$$C = \frac{N}{N_A \cdot V_{obs}} = \frac{N}{N_A \cdot \pi^{\frac{3}{2}} \cdot z_0 \cdot \omega_0^2}$$

$$D = \frac{\omega_0^2}{4\tau_D}$$

Confocal microscope

Valeur, Lacowicz

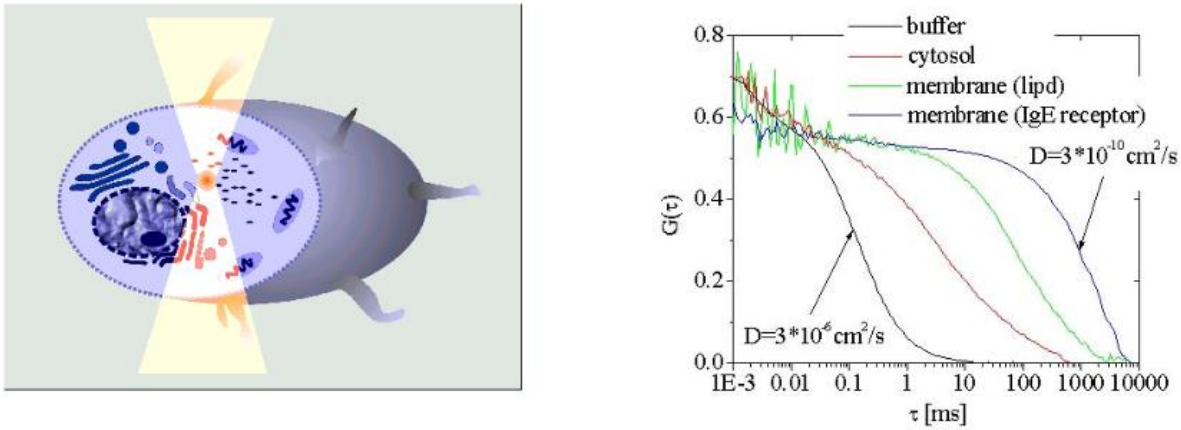


Figure 15: Various autocorrelation curves demonstrating the enormous difference in motility between buffer solution and cytosol

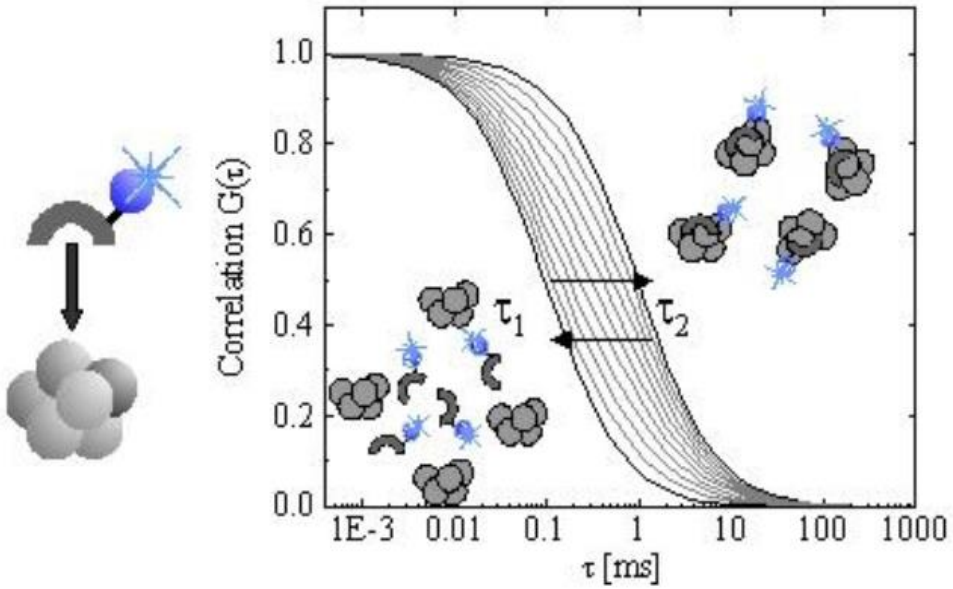
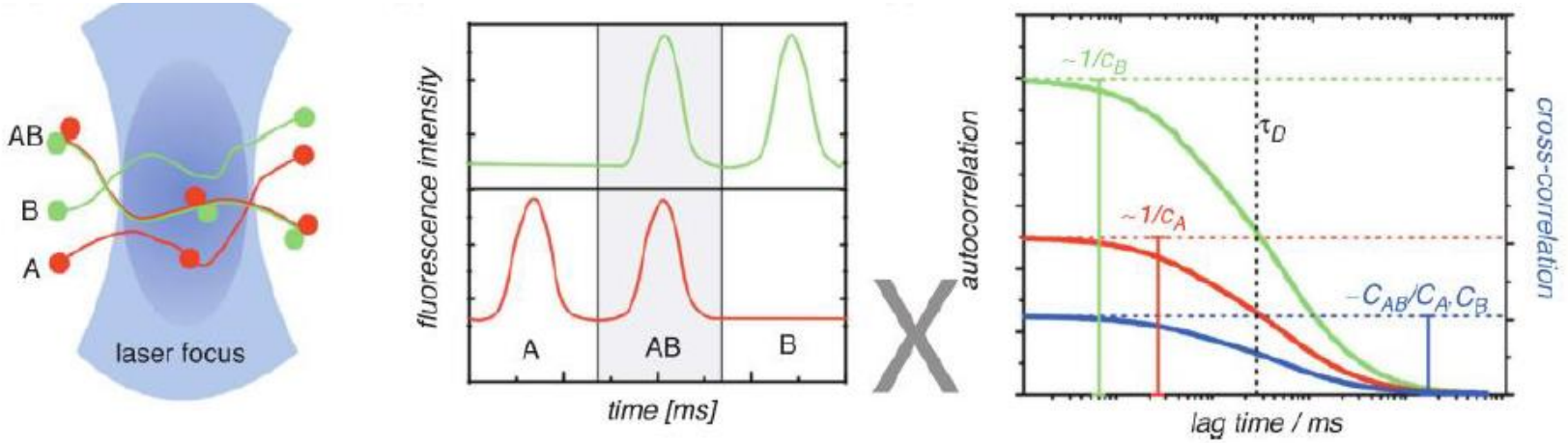


Figure 16: Changes in diffusion time of a small ligand upon binding to a heavy protein



$$C_A = \frac{1}{G_A(0)} \frac{1}{N_A} \frac{1}{V_A} \qquad C_B = \frac{1}{G_B(0)} \frac{1}{N_A} \frac{1}{V_B} \qquad C_{AB} = \frac{G_{cross}(0)}{G_A(0) \cdot G_B(0)} \frac{1}{N_A} \frac{1}{V_{cross}}$$

+ les coefficients de diffusion!!!

=> Permet d'acceder aux constantes d'equilibre dans les cellules, en théorie!

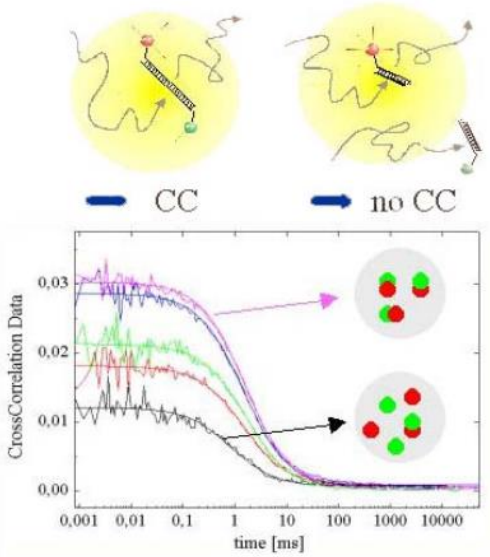


Figure 18: Cross-correlation measurement