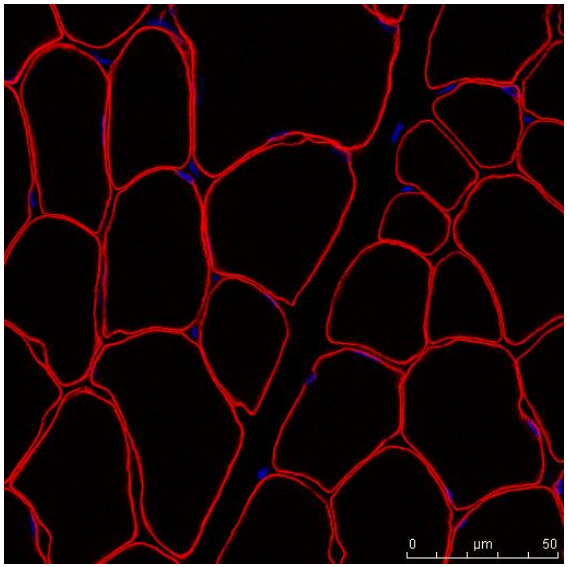
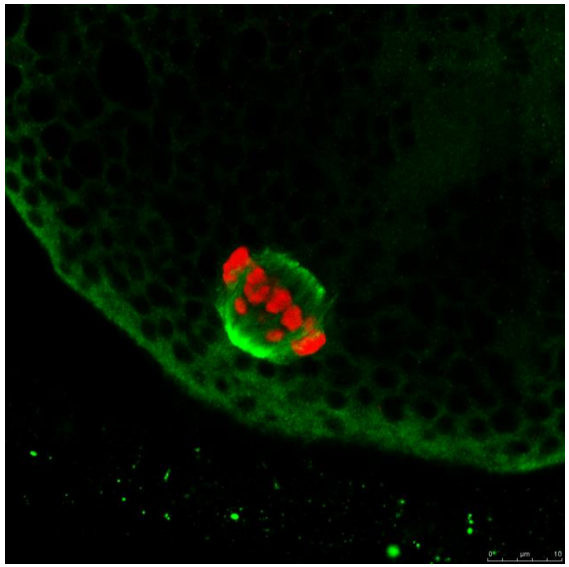
Microscopie confocale - 2010





Microscopie Confocale

- Introduction
  - Historique
  - Fluorescence
  - Résolution
  - Microscopie Confocale
- Applications et exploitation des données
  - Immunomarquages
  - Réflection
  - Time-Lapse
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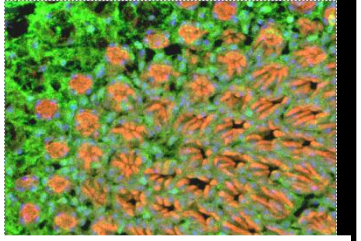
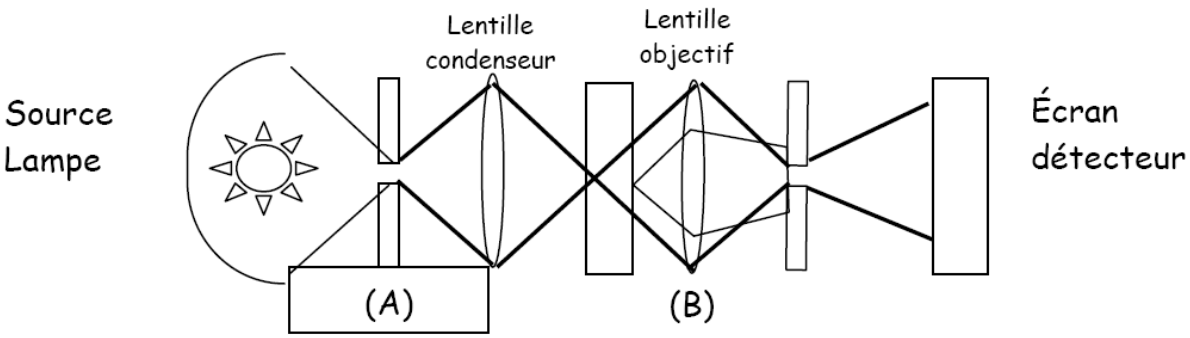


Historique

XVII : naissance de la microscopie photonique (découverte d'agents pathogènes : tuberculose, peste..)

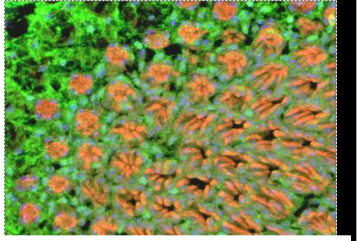
1957 : Naissance de la microscopie confocale avec Marvin Minsky

conjuguer le plan de la source lumineuse et celui de l'image filtrée



1980 : Émergence de la microscopie confocale

besoins en biologie cellulaire + progrès technologiques (source laser, développement de l'électronique, informatique…)



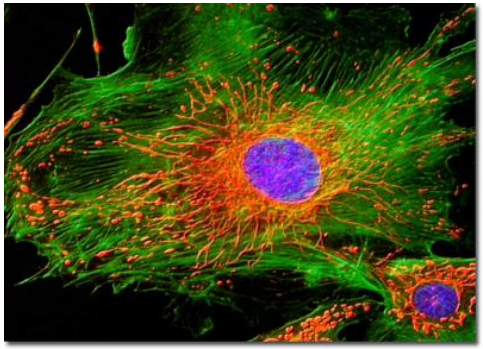
Historique

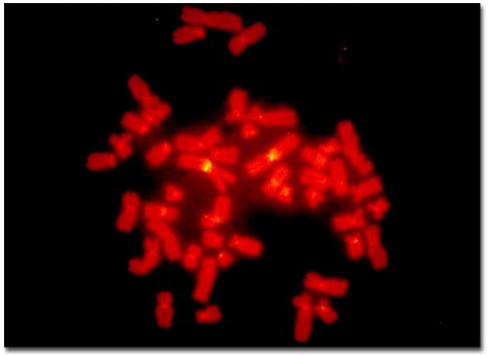
Question biologique ?

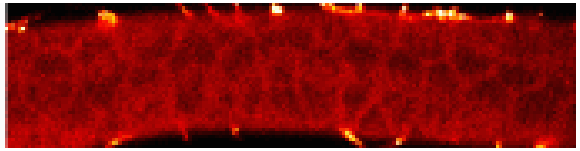
Structures

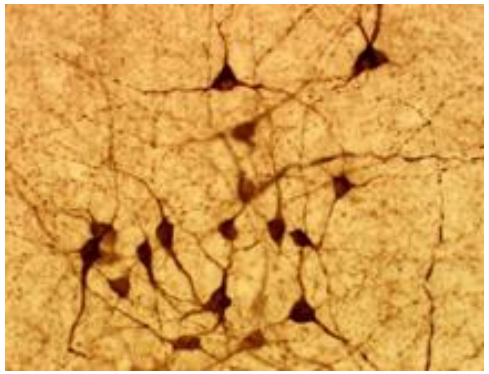
Localisations

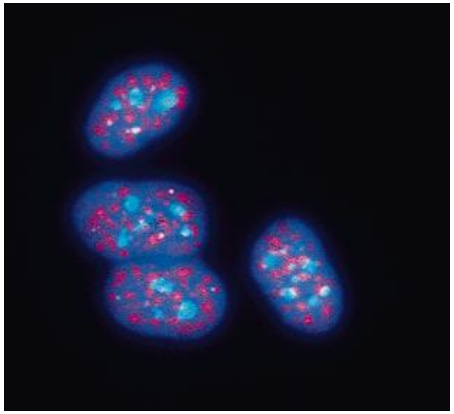
dynamiques

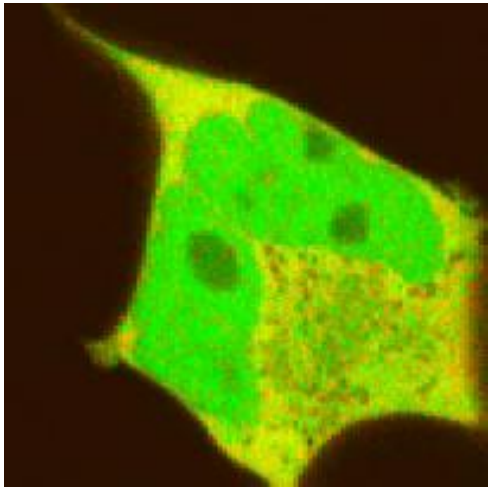


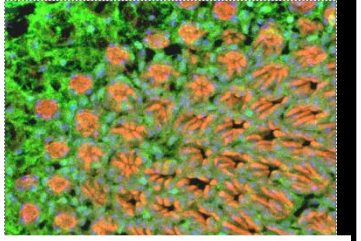




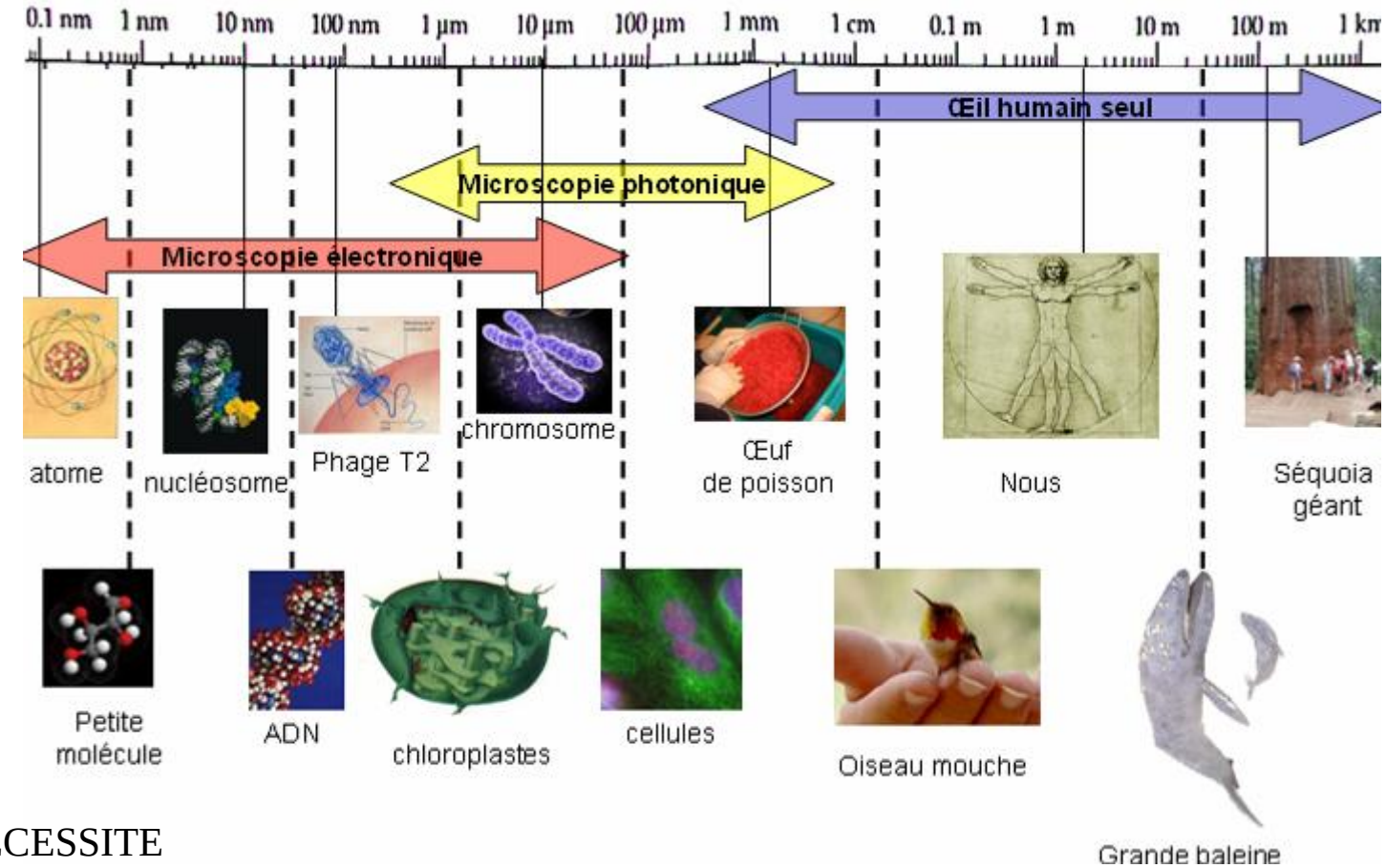






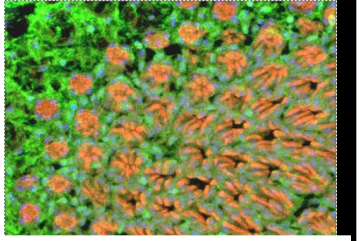


Historique



NECESSITE

- Grossissement: voir des petits détails
- Résolution : pouvoir distinguer 2 éléments très proches
- Contraste : possibilité de disinguer un élément d'un autre



Historique

- **Microscopes de fluorescence à champ large**

(Wide field fluorescence microscopes)

Capteurs CCD à haute résolution et sensibilité

Déconvolution possible pour éliminer les signaux hors plan focal

Fondamentale, video-microscopie rapide

- **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

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

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

Avantages : excitation restreinte au volume focal, résolution temporelle

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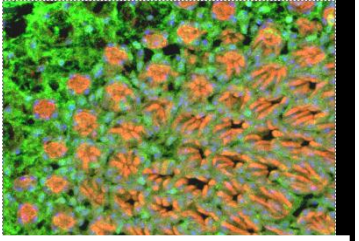
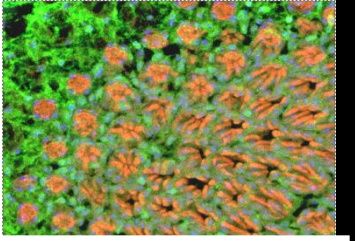


Fluorescence

Principe d'excitation / émission

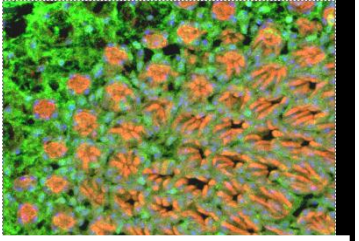
$$E = h\nu = hc/\lambda$$

Prof. Alexander Jablonski, 1935



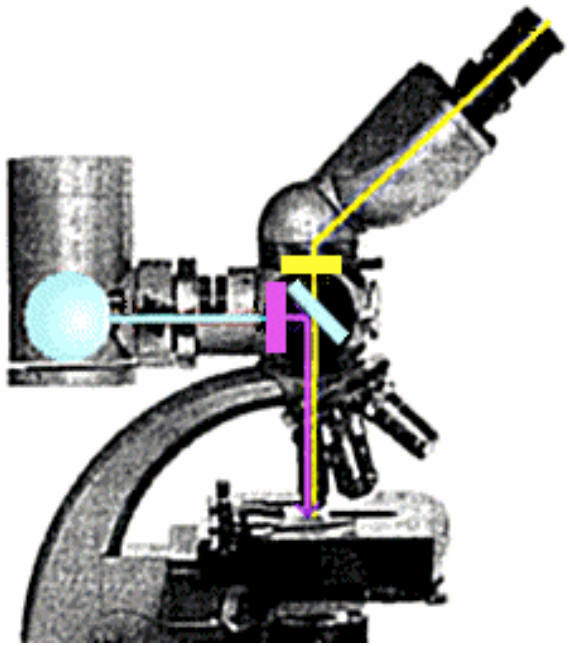
Fluorescence

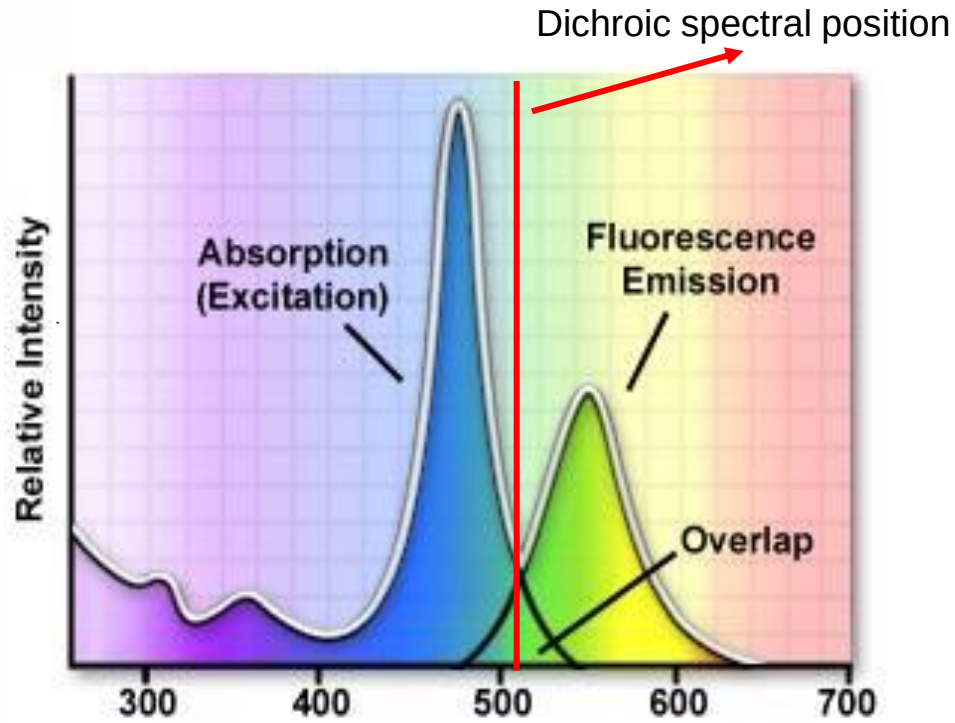
Principe du filtrage de la lumière

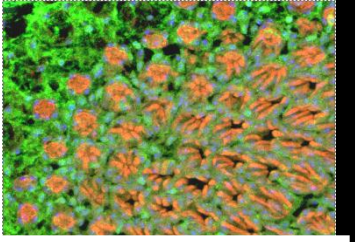


Fluorescence

Application au microscope : dichroïque

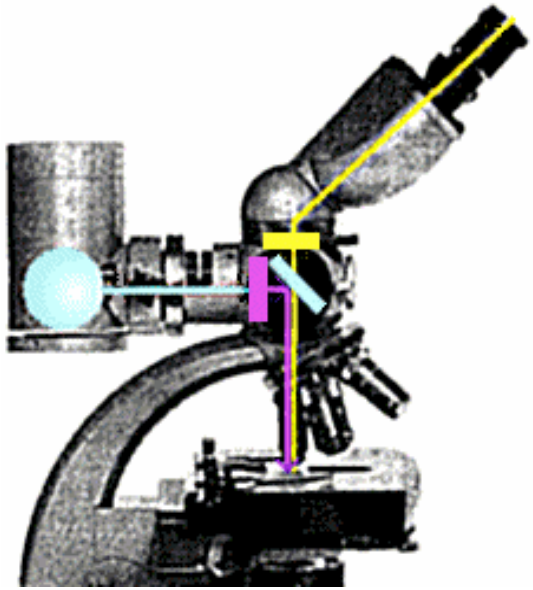


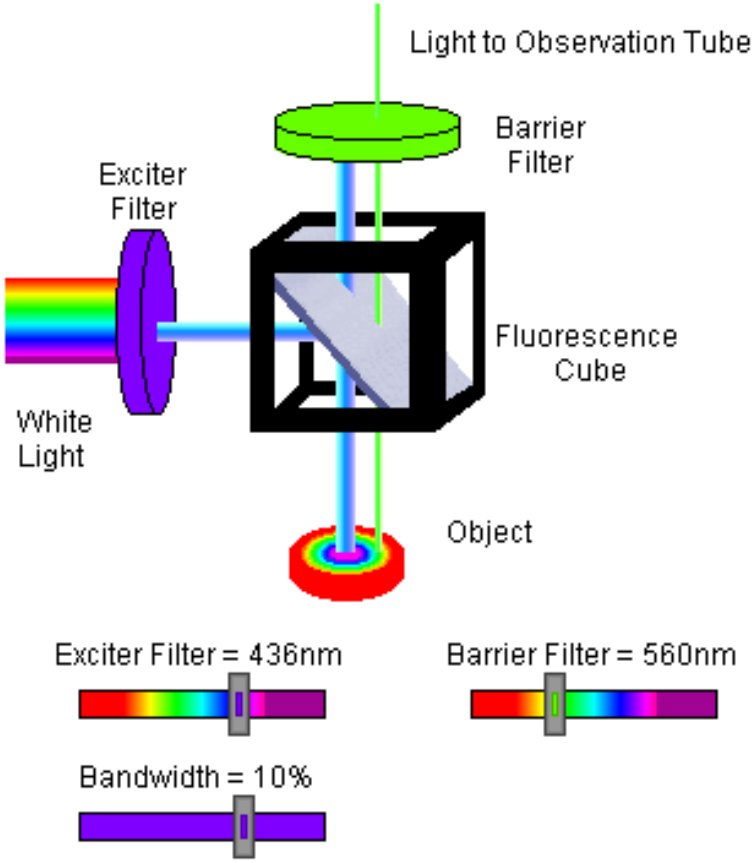


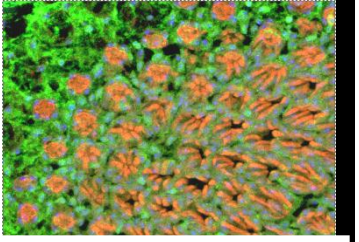


Fluorescence

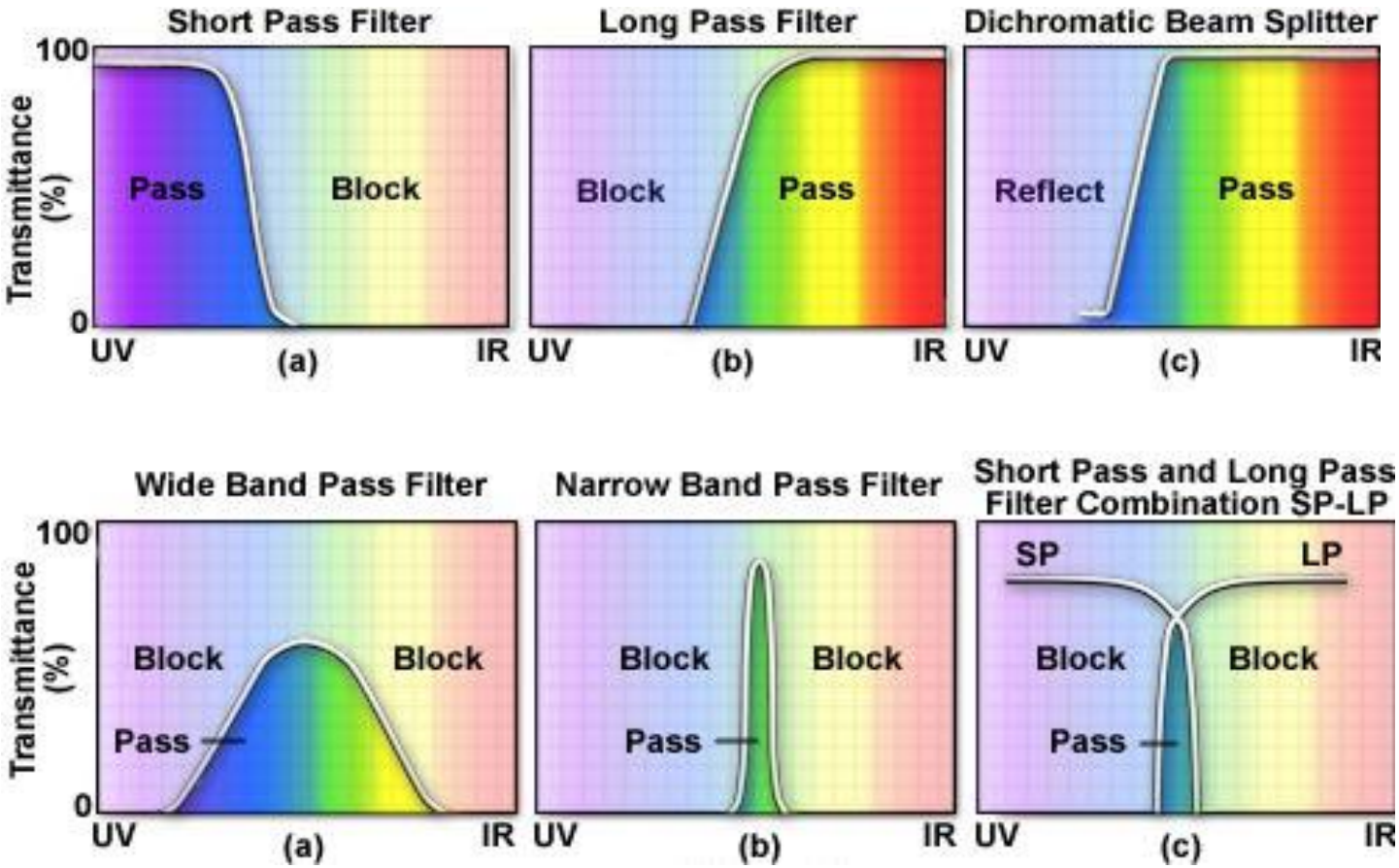
Prof. Ploem's invention

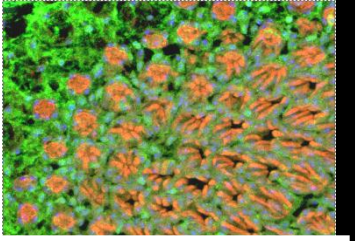






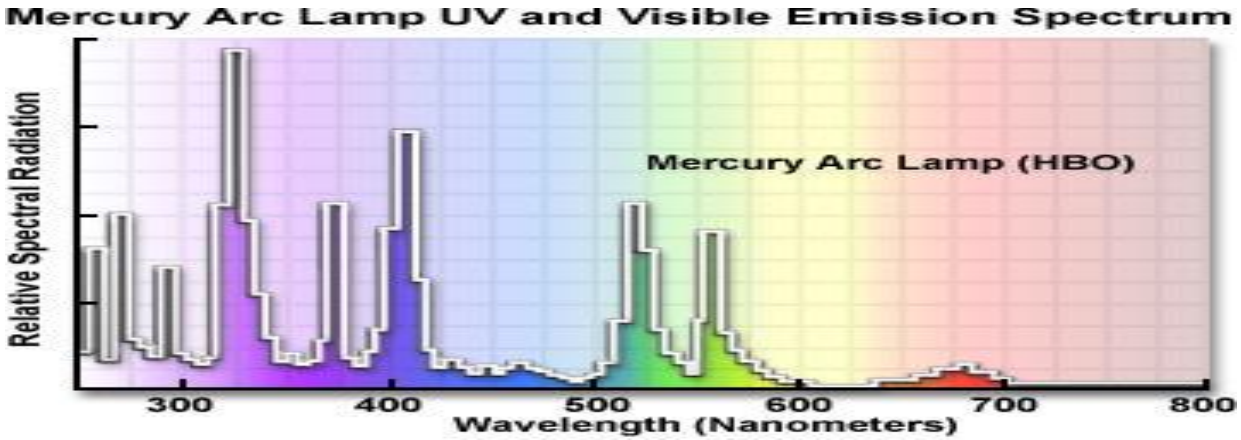
Fluorescence

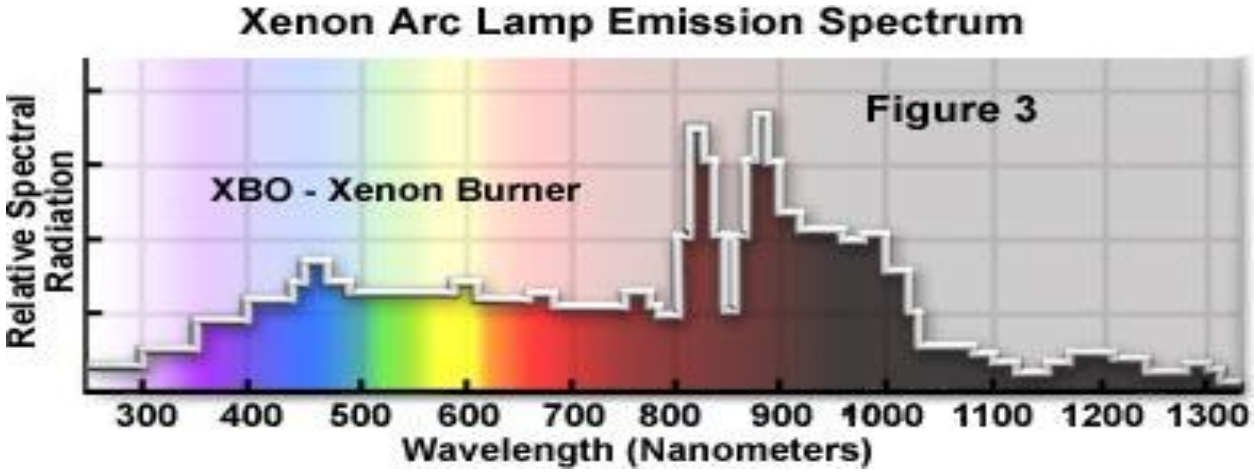




Fluorescence

Exemple de lampes à fluorescence





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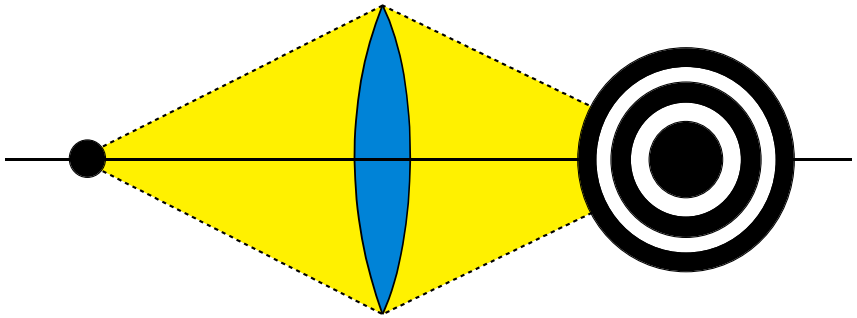
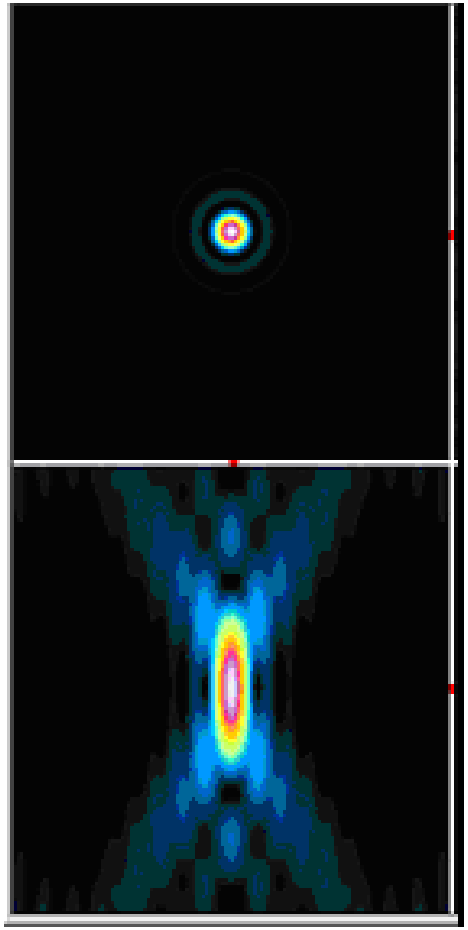
Résolution



Airy disc

$D_0 = 1.22 * \lambda / NA$ (lateral)

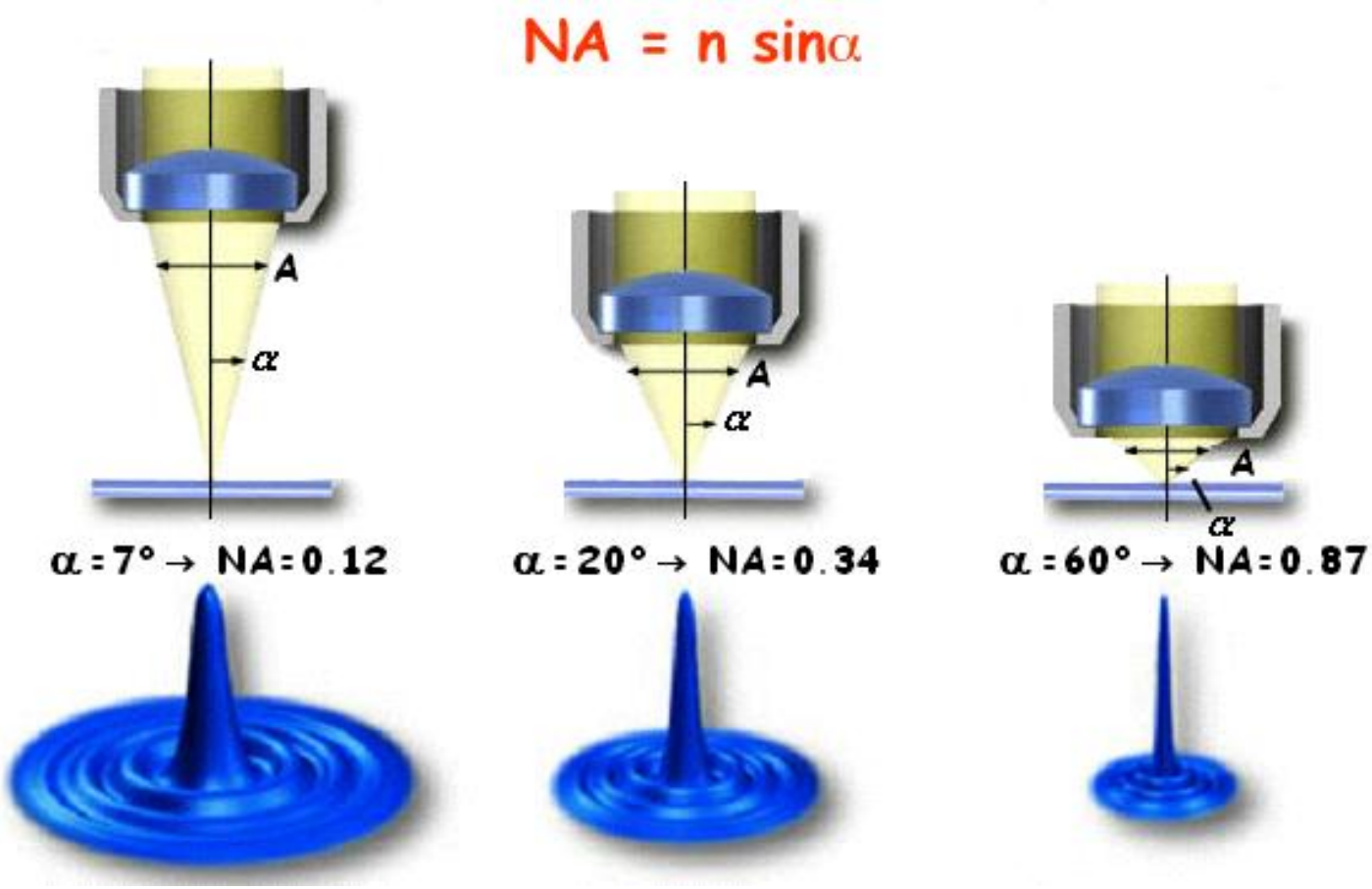
Limited by Diffraction



XY Plane

XZ Plane

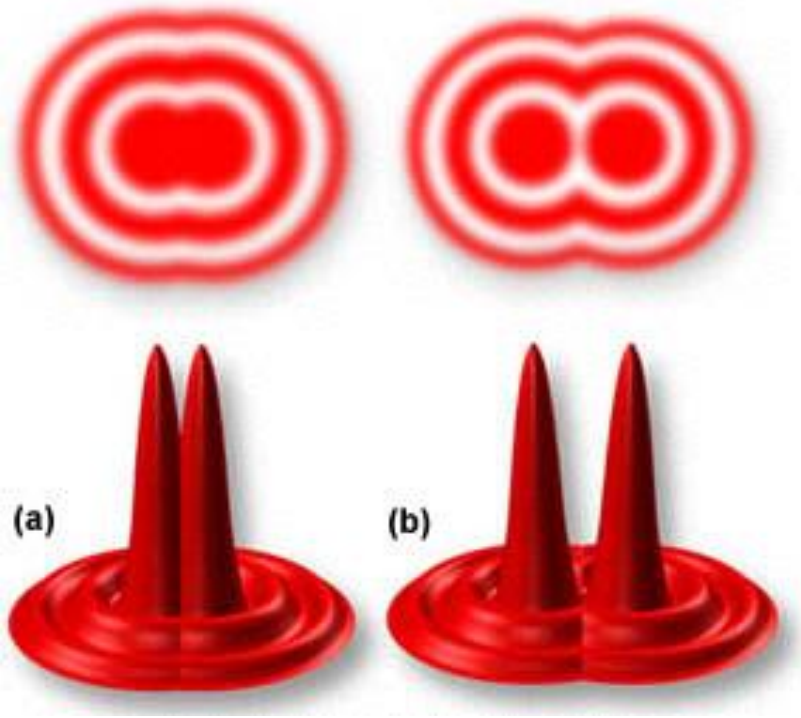
Résolution



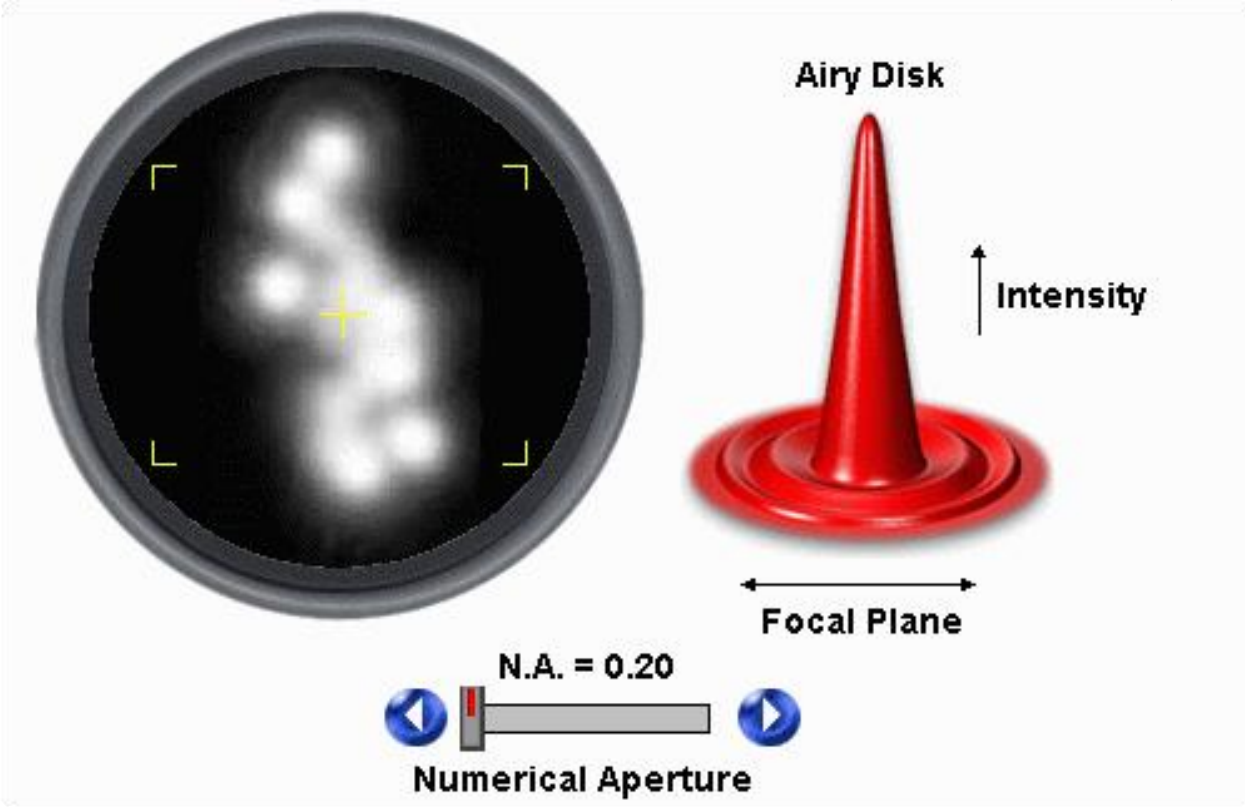
Point Spread Function (PSF)

Résolution

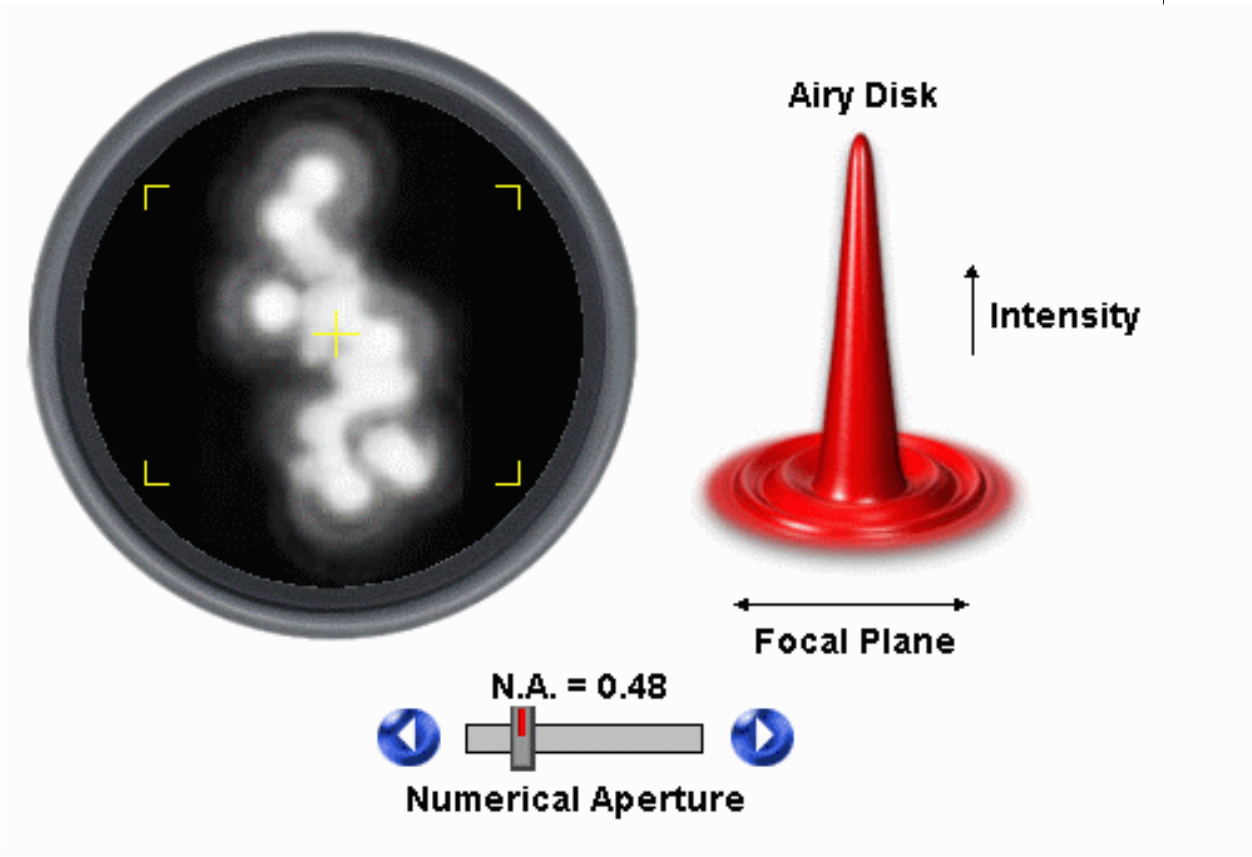
Res = 0.61*λ / NA



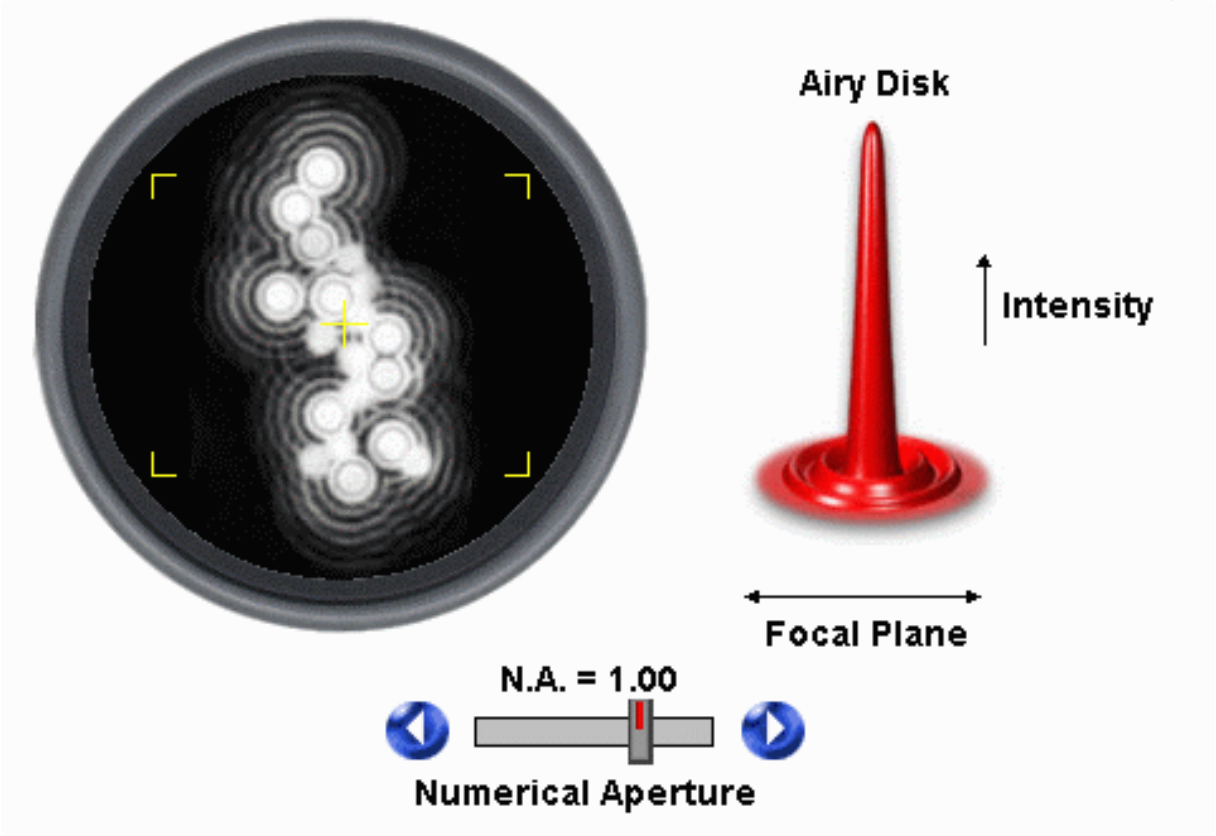
Résolution



Résolution



Résolution

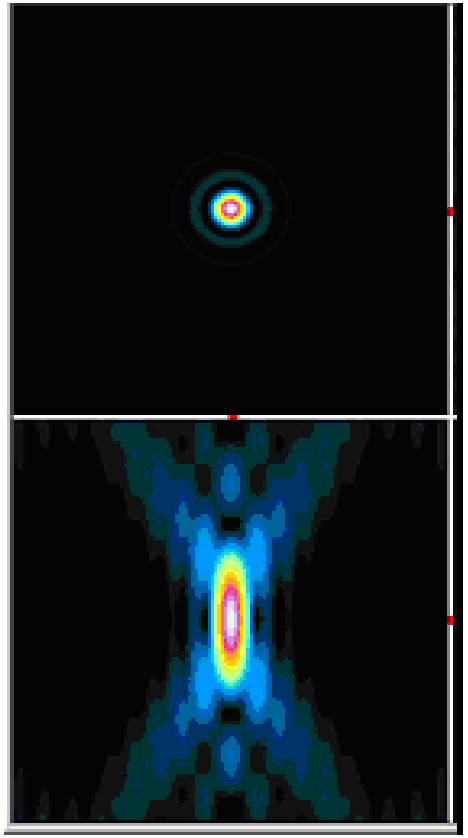


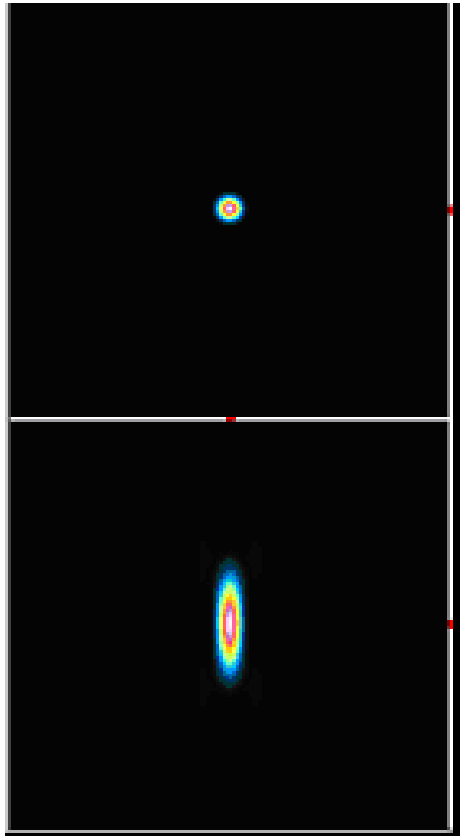
Résolution

Conventional

Confocal

Res = 0.61*λ / NA



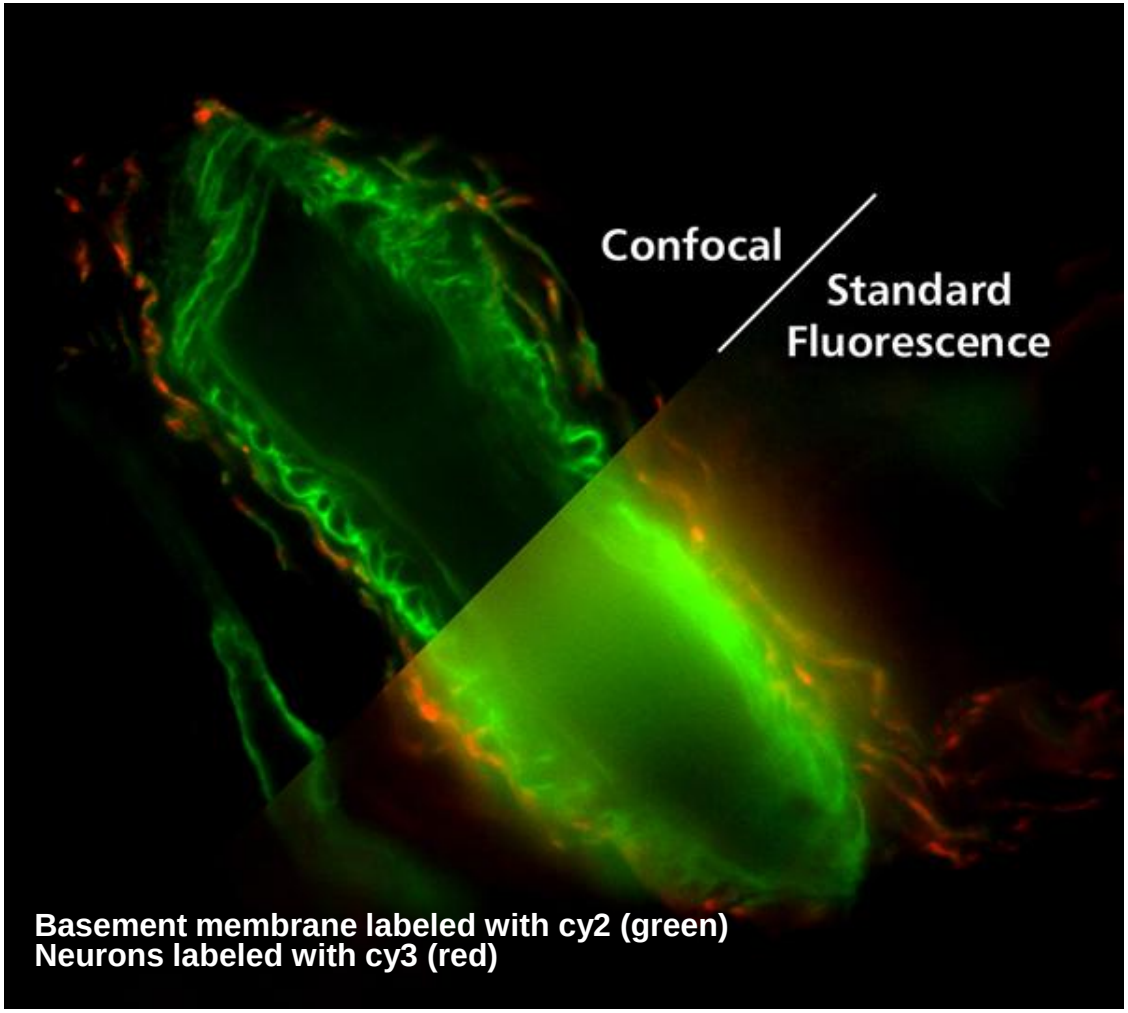


Res(xy) = 0.4*λ / NA

Res = 2*λ / NA^2

Res(xz) = 1,4λ /NA^2

Résolution



Basement membrane labeled with cy2 (green)
Neurons labeled with cy3 (red)

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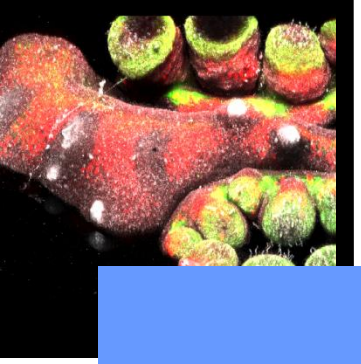
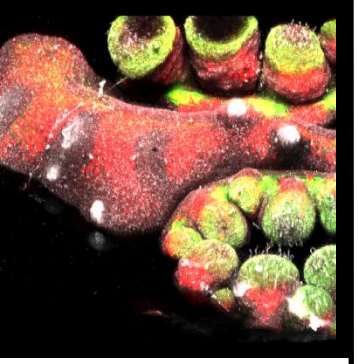


Microscopie confocale

Fundamento



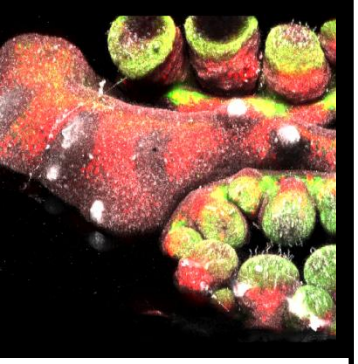
Microscopie confocale

Sectionnement optique de l‘échantillon

Suppression de la fluorescence en dehors du plan focal

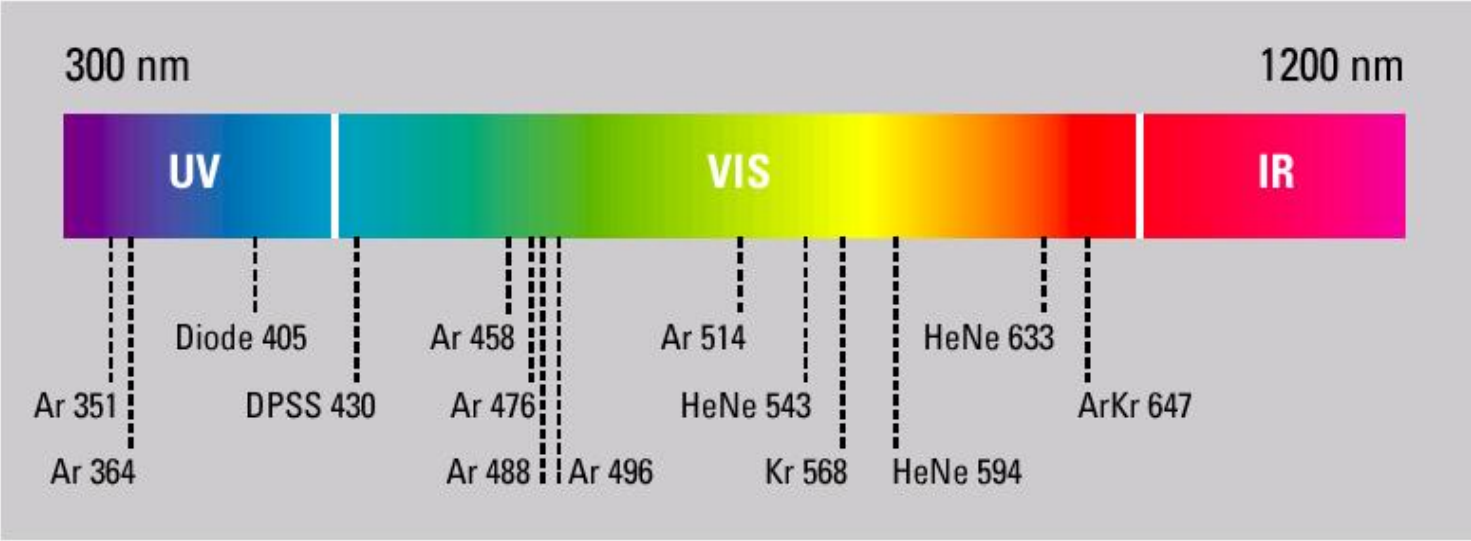
Amélioration de la résolution latérale et axiale

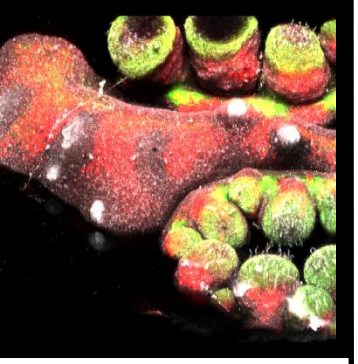
Amélioration du contraste



Microscopie confocale

LASER



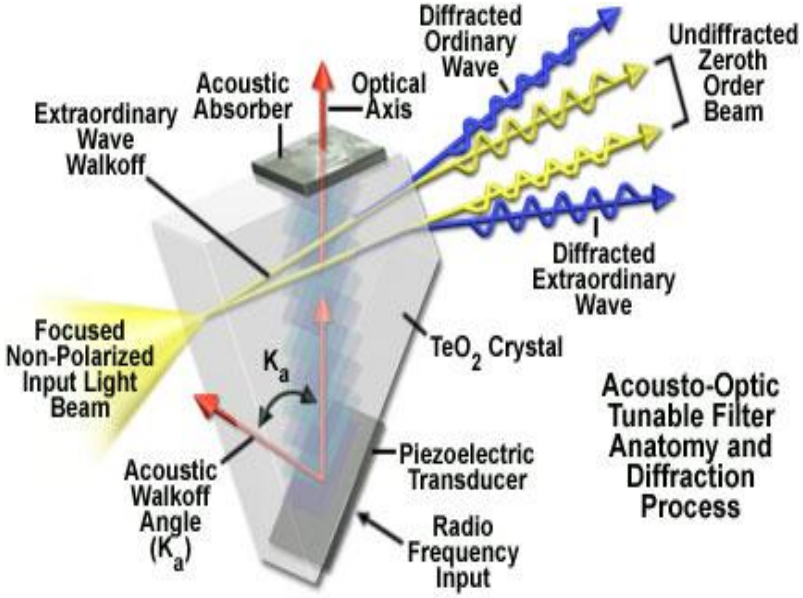


Microscopie confocale

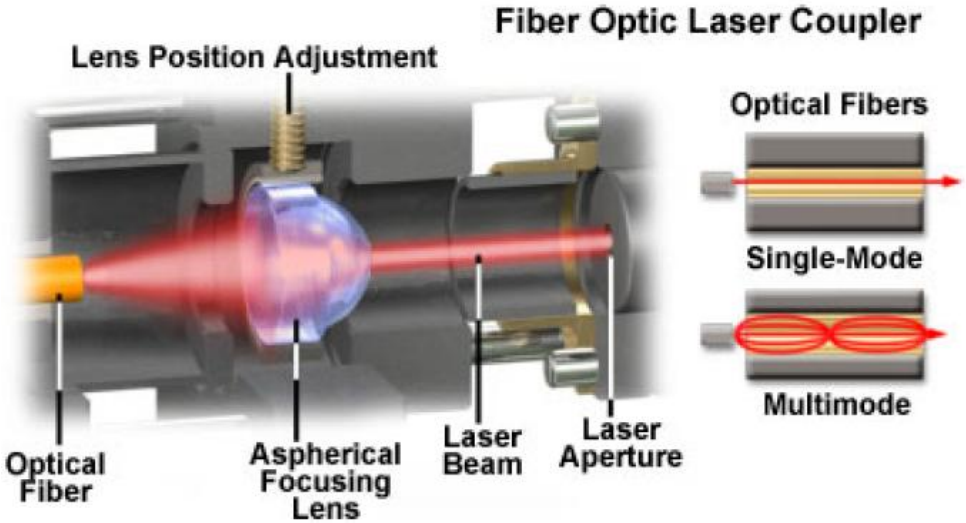
AOTF : Acouto Optical Tunable Filter

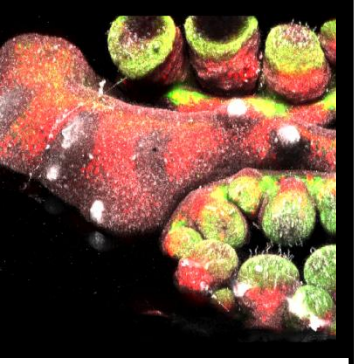
Sélection de la longueur d‘onde d‘émission

Modulation de l‘intensité



Couplage par fibre optique



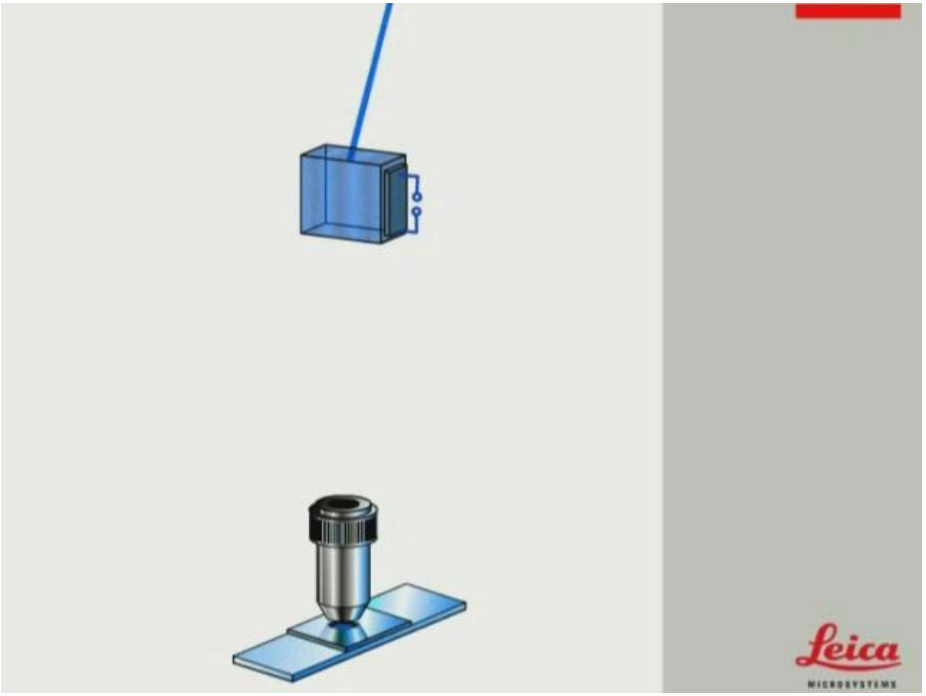


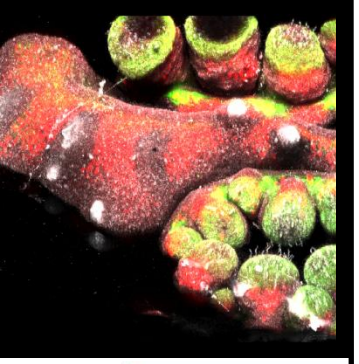
Microscopie confocale

Beam splitter

Miroir dichroïque

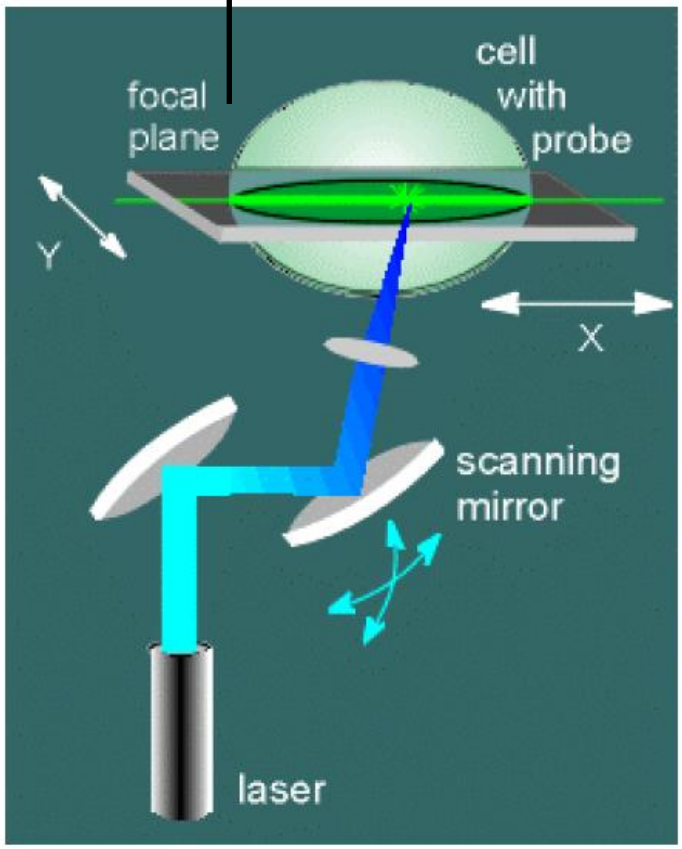
AOBS





Microscopie confocale

Scanner en XY



Comment faire de l'imagerie point par point ?

Miroirs rotatifs (galvanomètres)

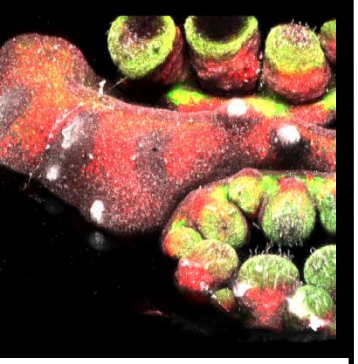
Zoom ?

Amplitude de balayage

Limites sur le vitesse de balayage :

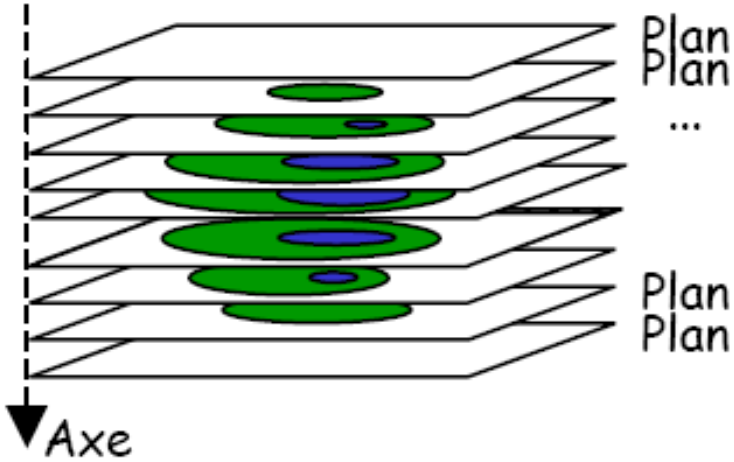
récupération des photons et effets photo-induits

Secciones Ópticas



Microscopie confocale

Sectionnement optique



Imagerie plan / plan en XY

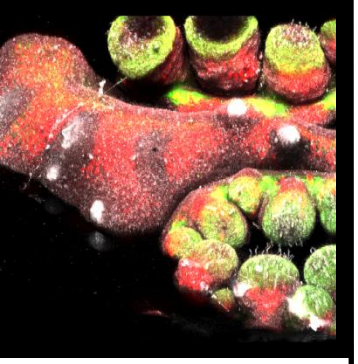
⇩

Echantillonnage : Critère de Nyquist
Taille pixel ≤ 2,3 Res

⇩

Déplacement en Z du microscope

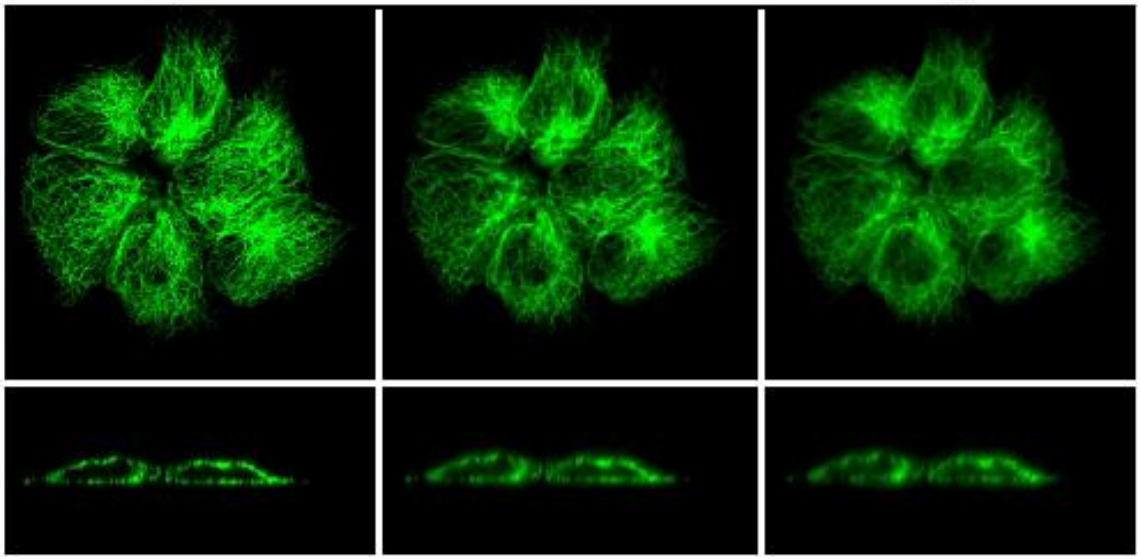
Reconstruction 3D



Microscopie confocale

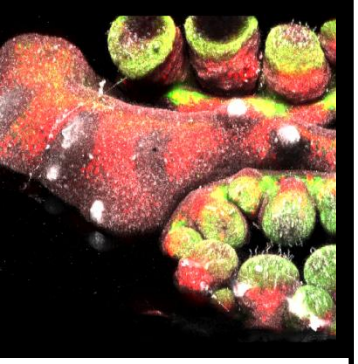
Pinhole

Ouverture fonction de :
λ : longueur d'onde
NA : ouverture numérique



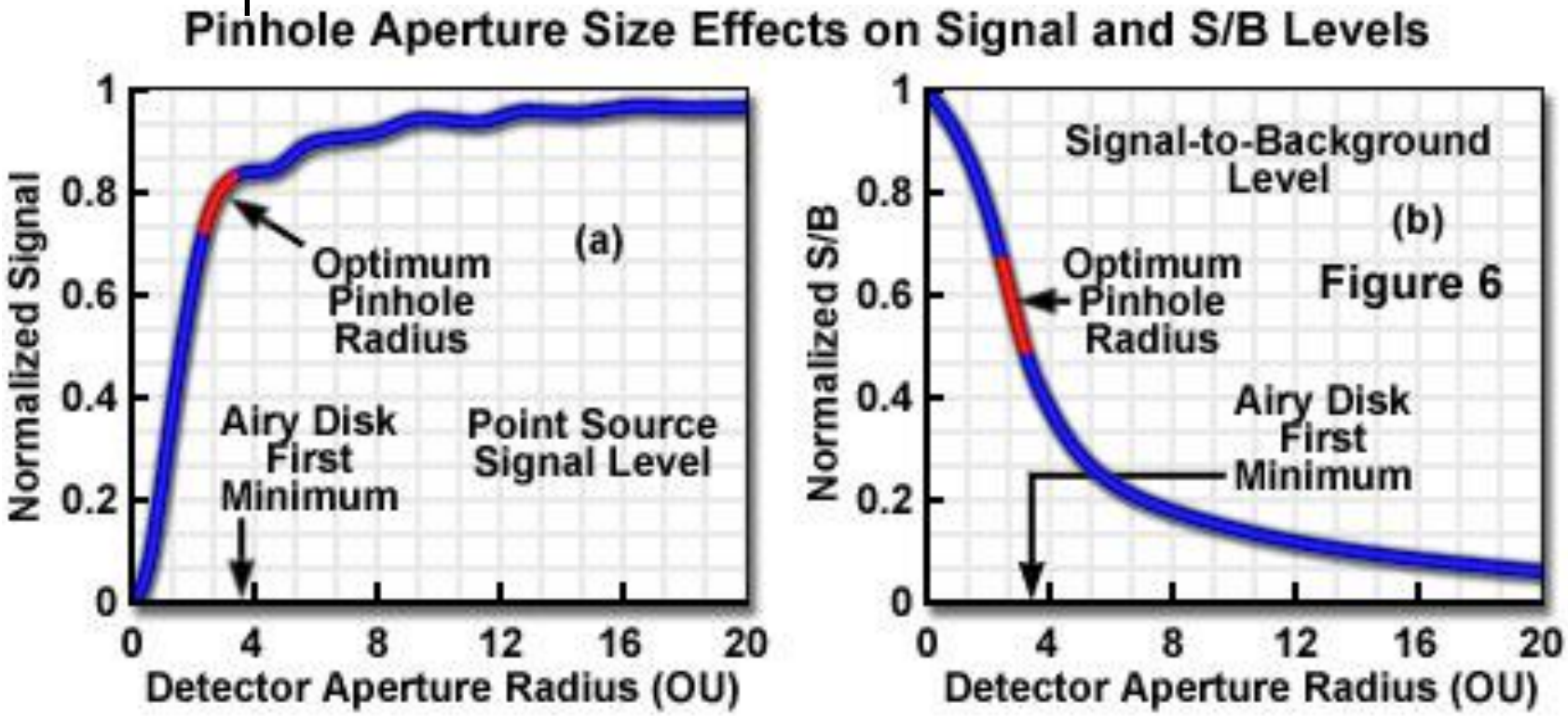
Résolution optimale

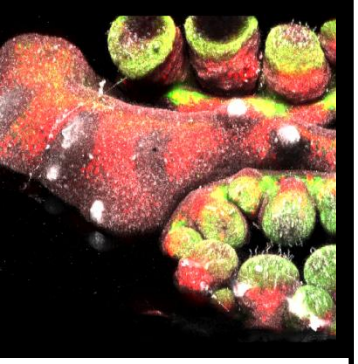
Intensité de fluorescence maximale



Microscopie confocale

Pinhole

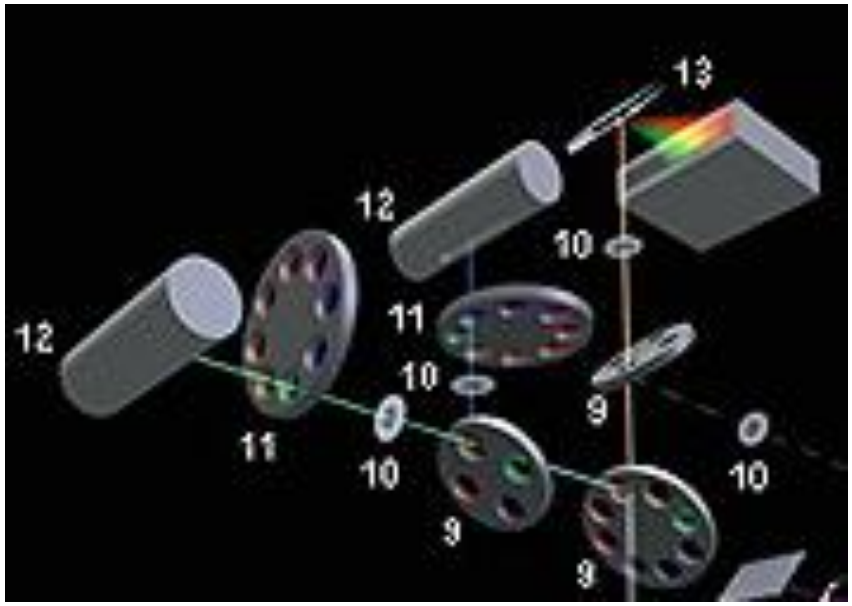




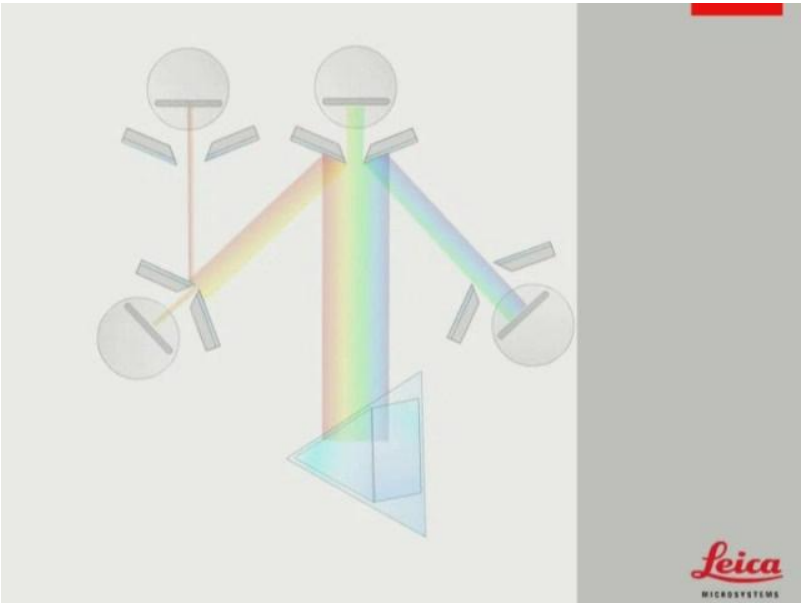
Microscopie confocale

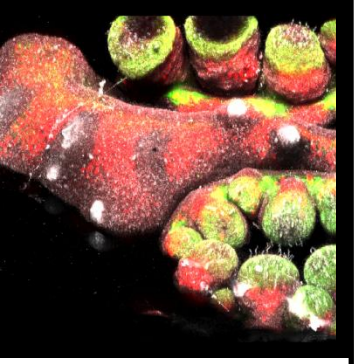
Filtrage spectral

Cascade de dichroïques



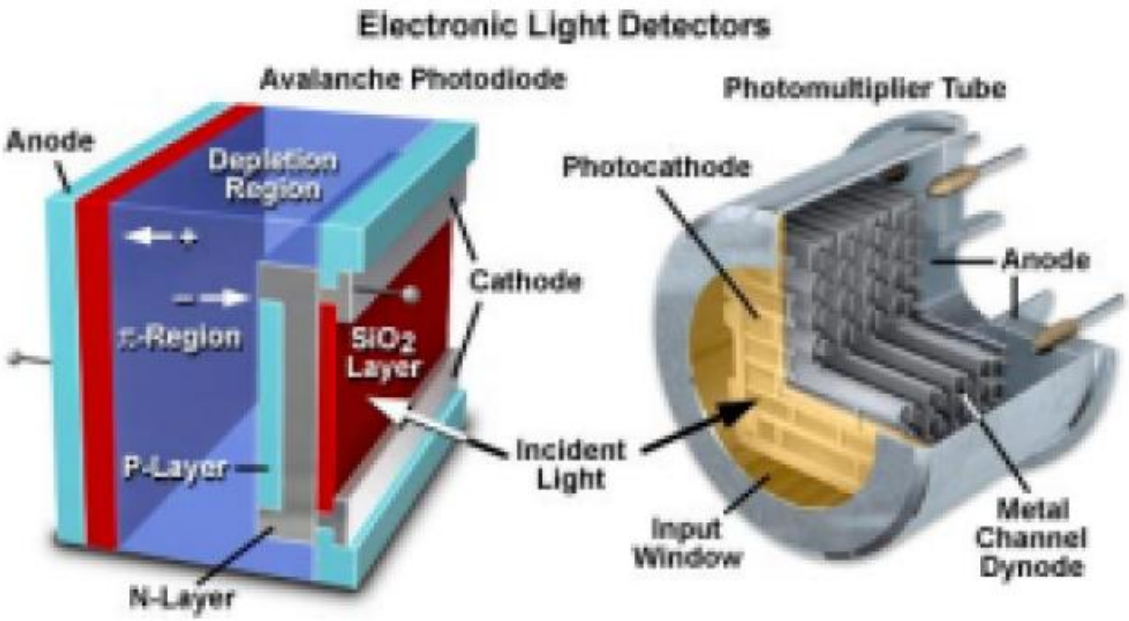
Système spectral





Microscopie confocale

Détecteur

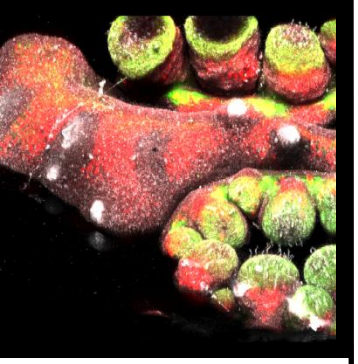


Gain variable 0 à 1250 V

Echantillonage

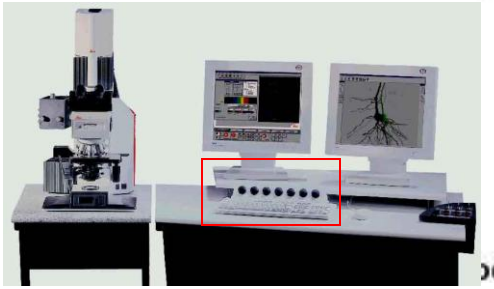
Quantification

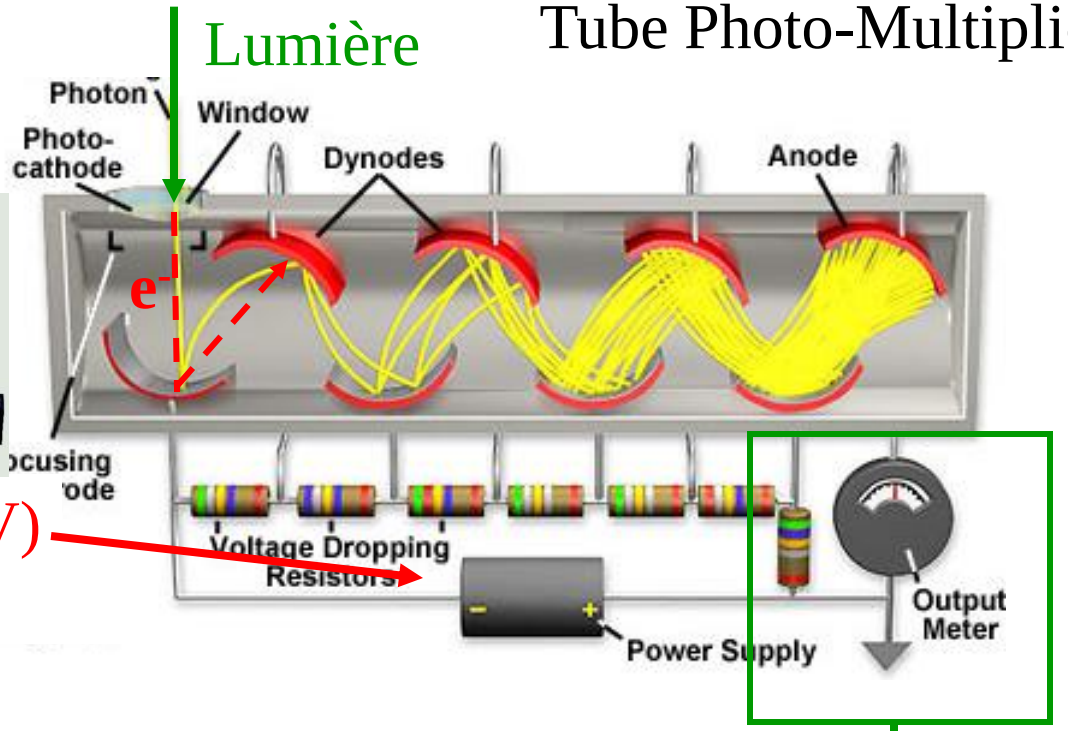
- **Transformation du signal en niveau de gris 8 bits 12 bits...**



Microscopie confocale

Tube Photo-Multiplicateur



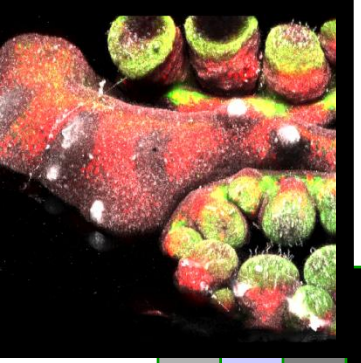


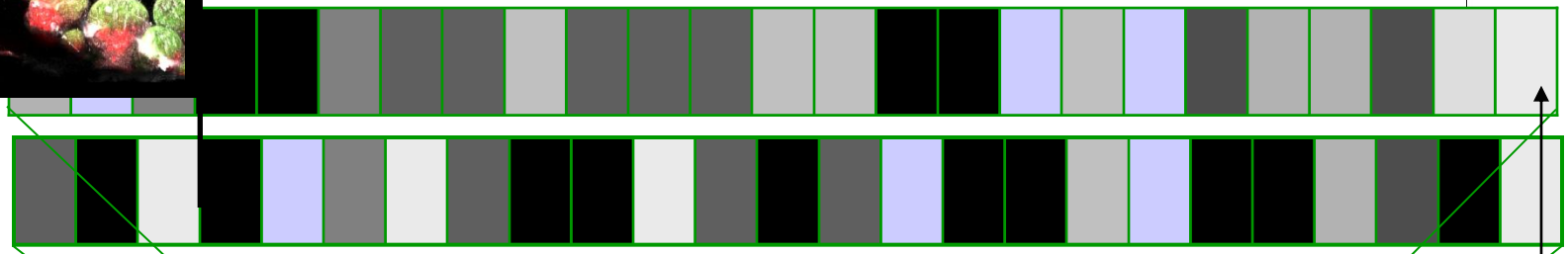
GAIN (mV)

Signal/Bruit

Intensité ← voltage

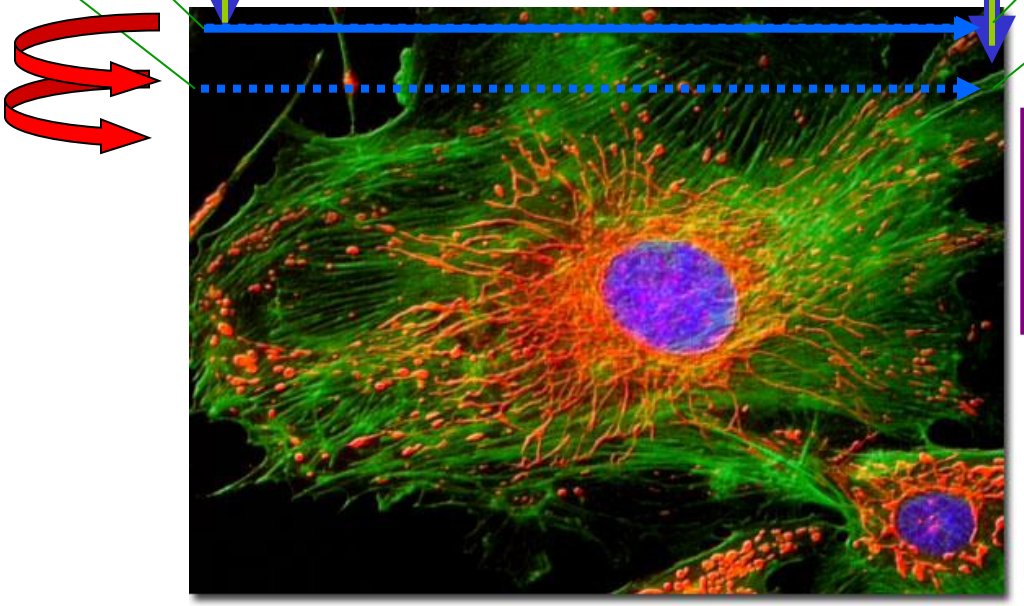
Microscopie confocale : Numérisation





29

Pixel

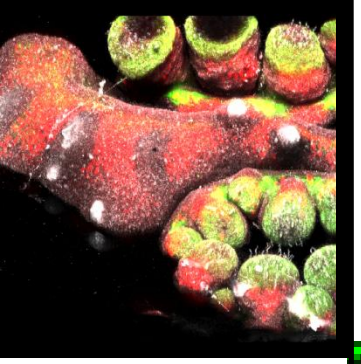


Y
512

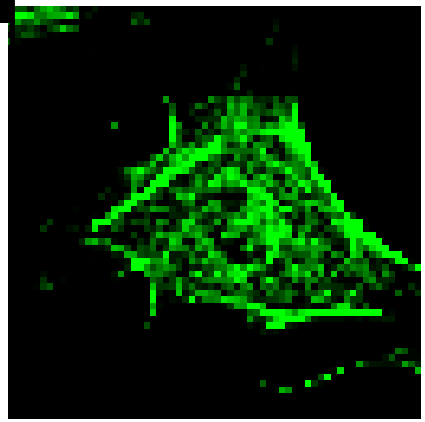
X 512

CAN Converter
8bit / 12 bit (= 256 / 4096 Greyvalues)

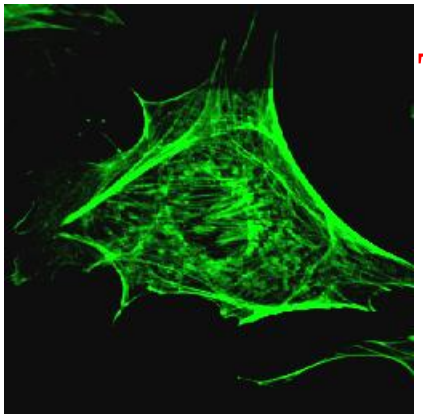
Frame Store
Data Files (e.g. TIF)



Microscopie confocale : pixelisation

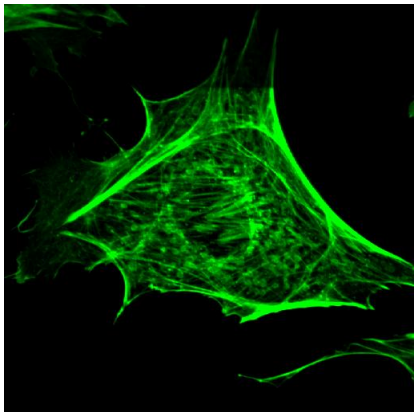


800x800nm

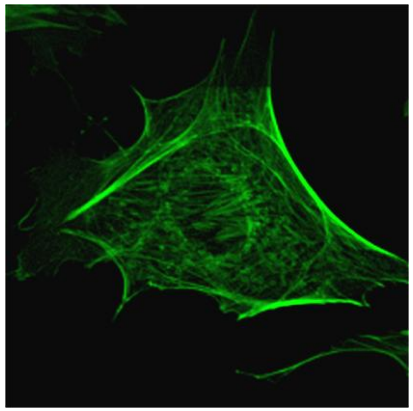


200x200nm

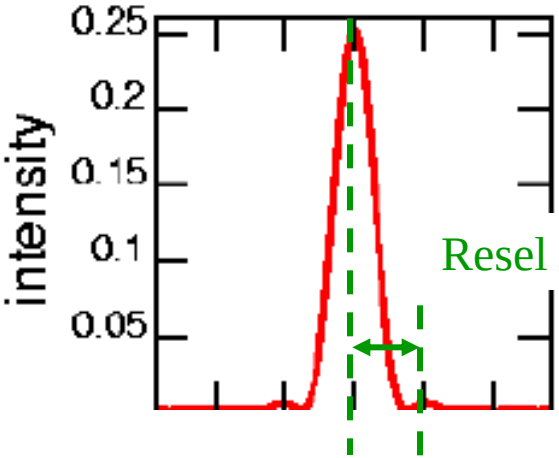
Travailler avec une taille optimal de pixel



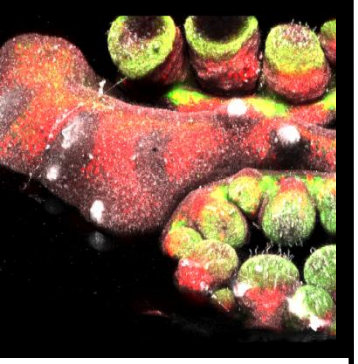
100x100nm



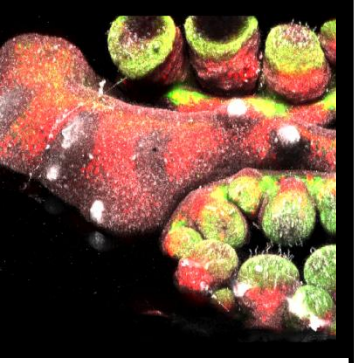
50x50nm



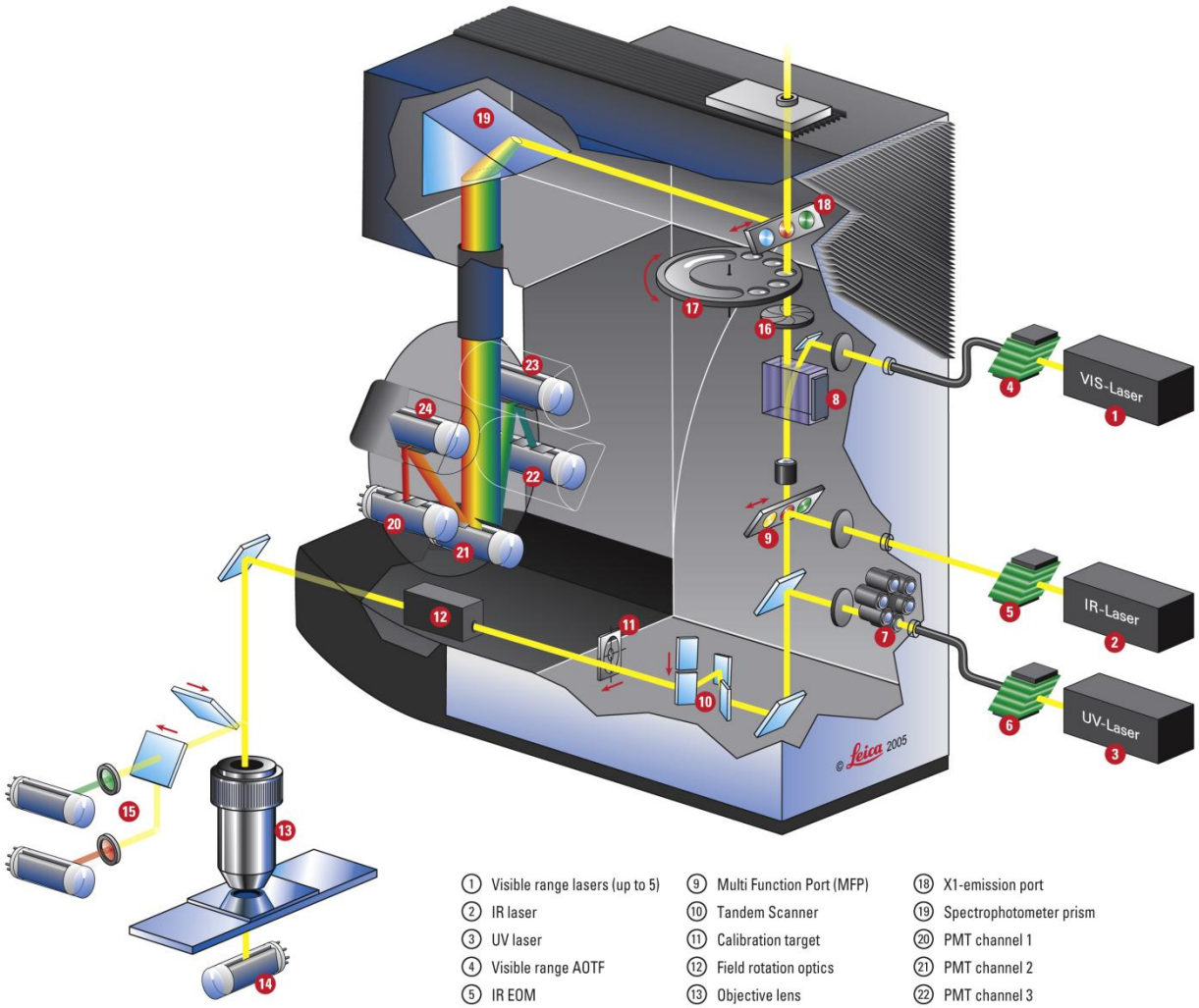
Théorie de Nyquist :
2,3 pixels /resel



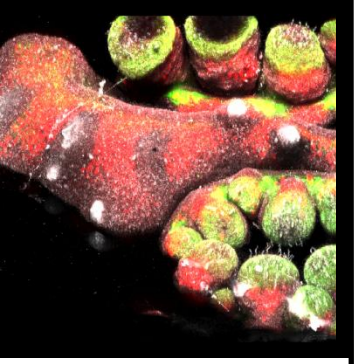
Microscopie confocale



Microscopie confocale



1. Visible range lasers (up to 5)
2. IR laser
3. UV laser
4. Visible range AOTF
5. IR EOM
6. UV AOTF
7. UV optics imaging
8. AOBS Acousto Optical Beam Splitter
9. Multi Function Port (MFP)
10. Tandem Scanner
11. Calibration target
12. Field rotation optics
13. Objective lens
14. Transmitted light dedector
15. Reflected light NDD
16. Confocal detection pinhole
17. Filter- and polarizer wheel
18. X1-emission port
19. Spectrophotometer prism
20. PMT channel 1
21. PMT channel 2
22. PMT channel 3
23. PMT channel 4 (optional FLIM)
24. PMT channel 5 (optional FLIM)



Microscopie confocale

Sectionnement optique de l'échantillon

Suppression de la fluorescence en dehors du plan focal

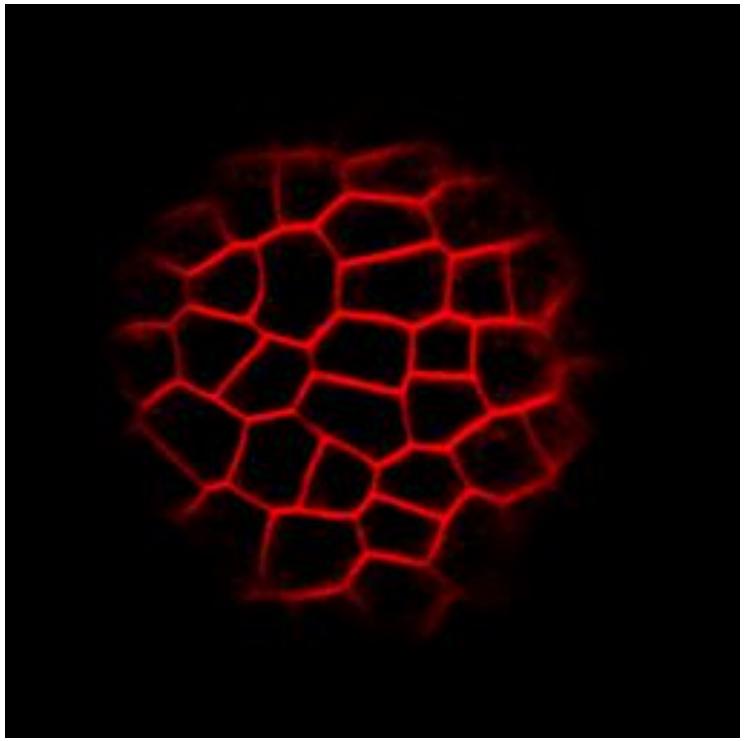
Amélioration de la résolution latérale et axiale

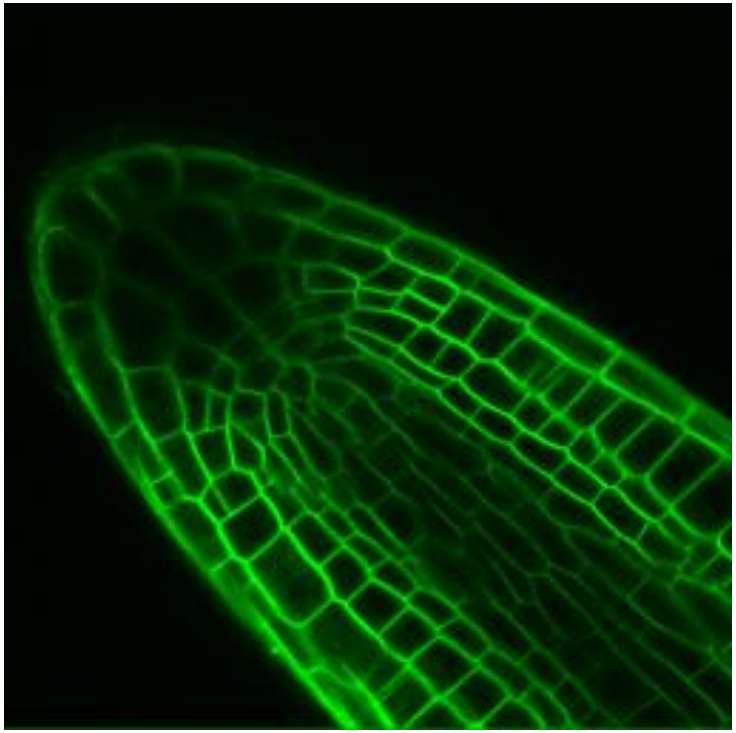
Amélioration du contraste

Microscopie Confocale

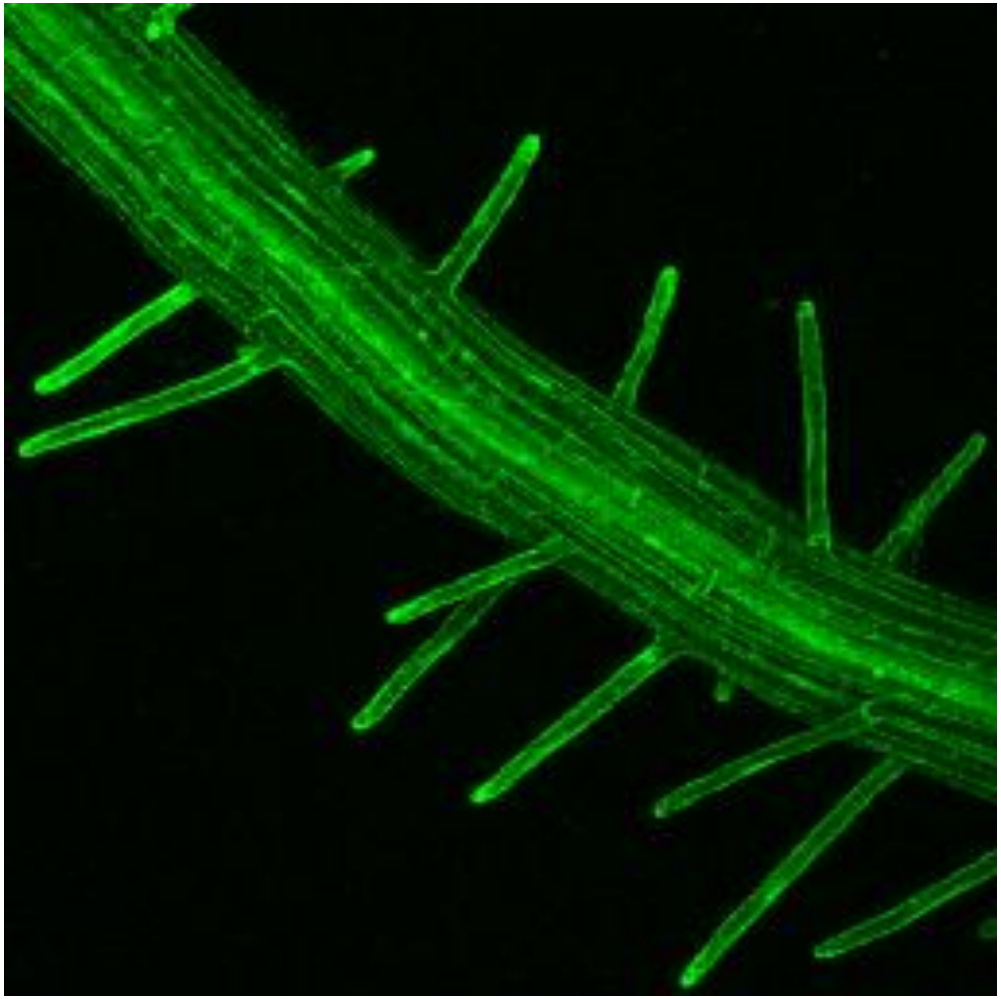
- Introduction
  - Historique
  - Fluorescence
  - Résolution
  - Microscopie Confocale
- **Applications et exploitation des données**
  - Immunomarquages
  - Réflection
  - Time-Lapse
  - Etudes spectrales
  - F-Techniques
  - FCS/FLIM
- Conclusion

Immunohistochimie

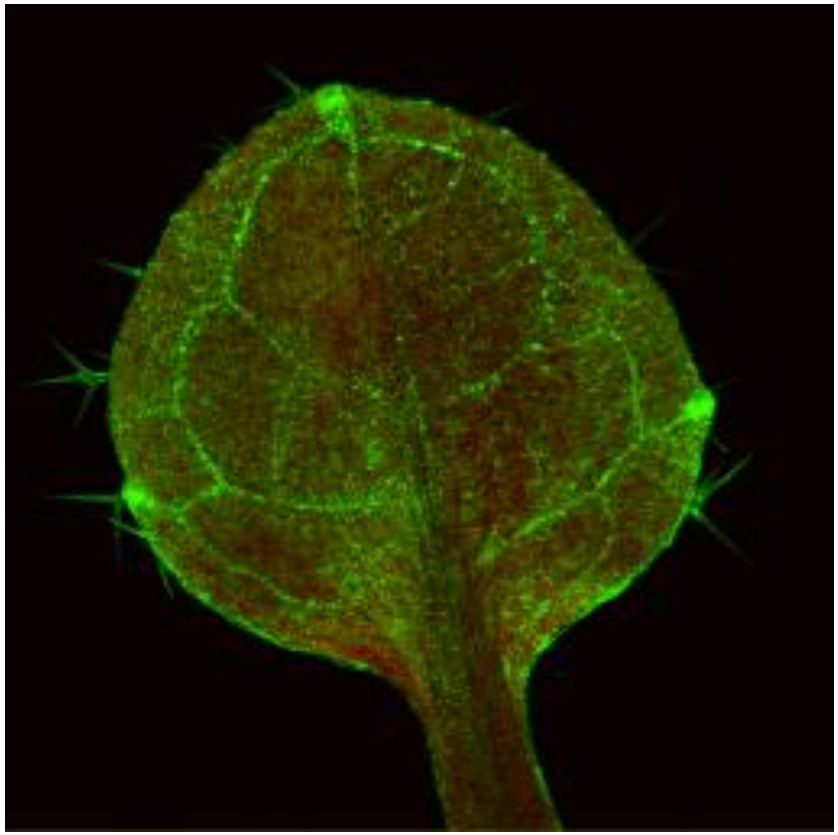


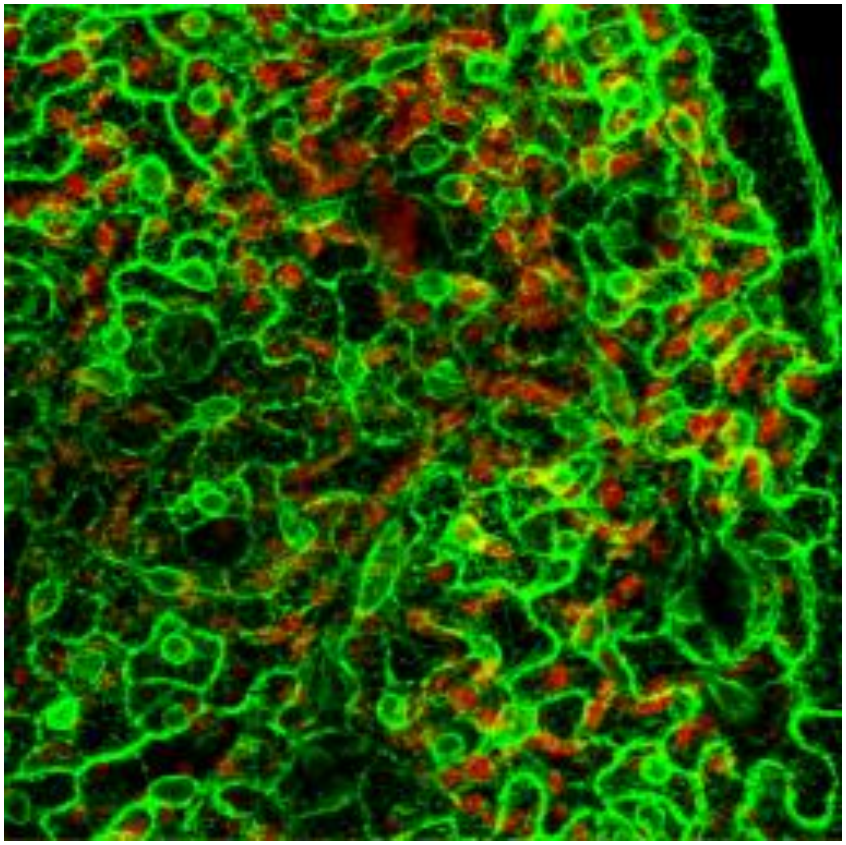


Immunohistochimie

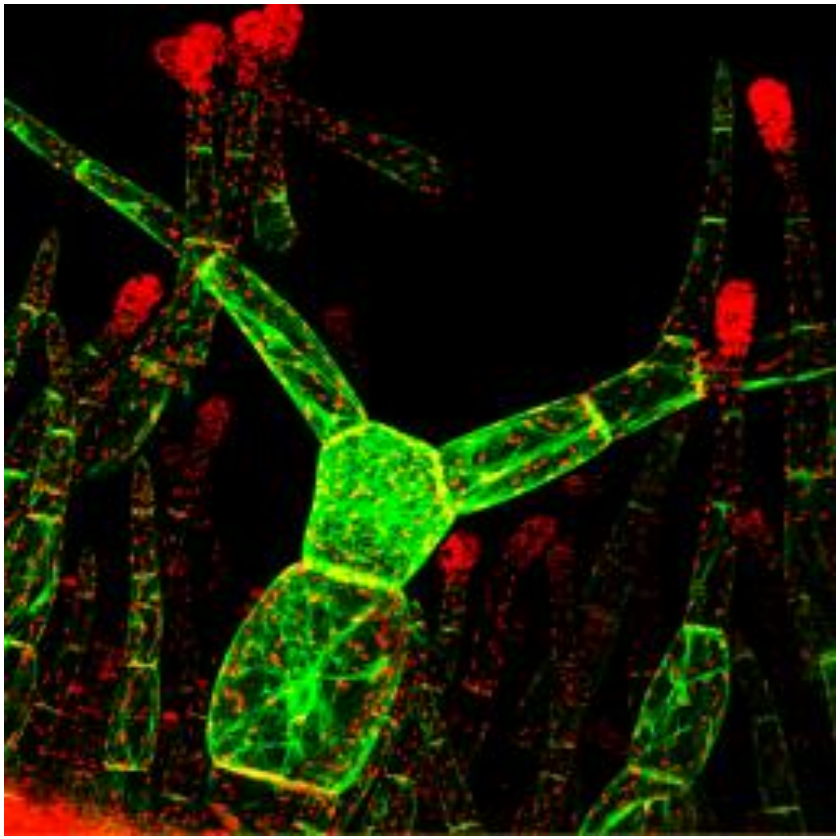


Immunohistochimie



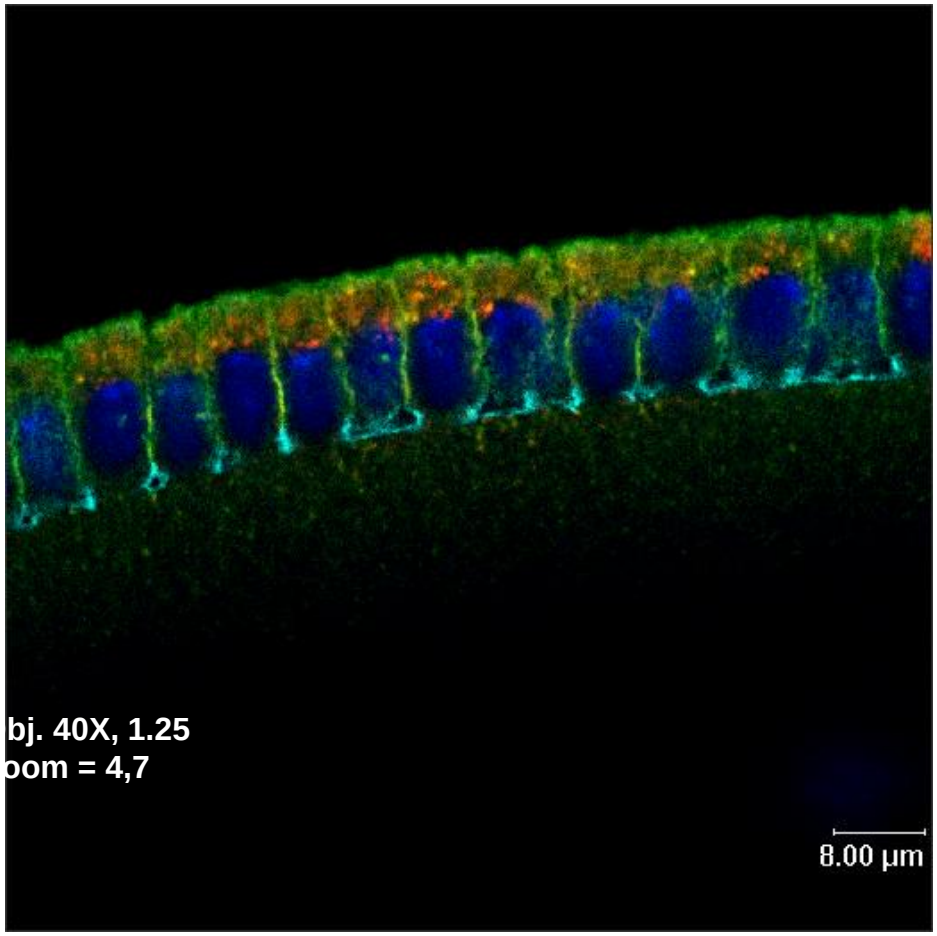


Immunohistochimie



Immunohistochimie

Embryon Drosophile



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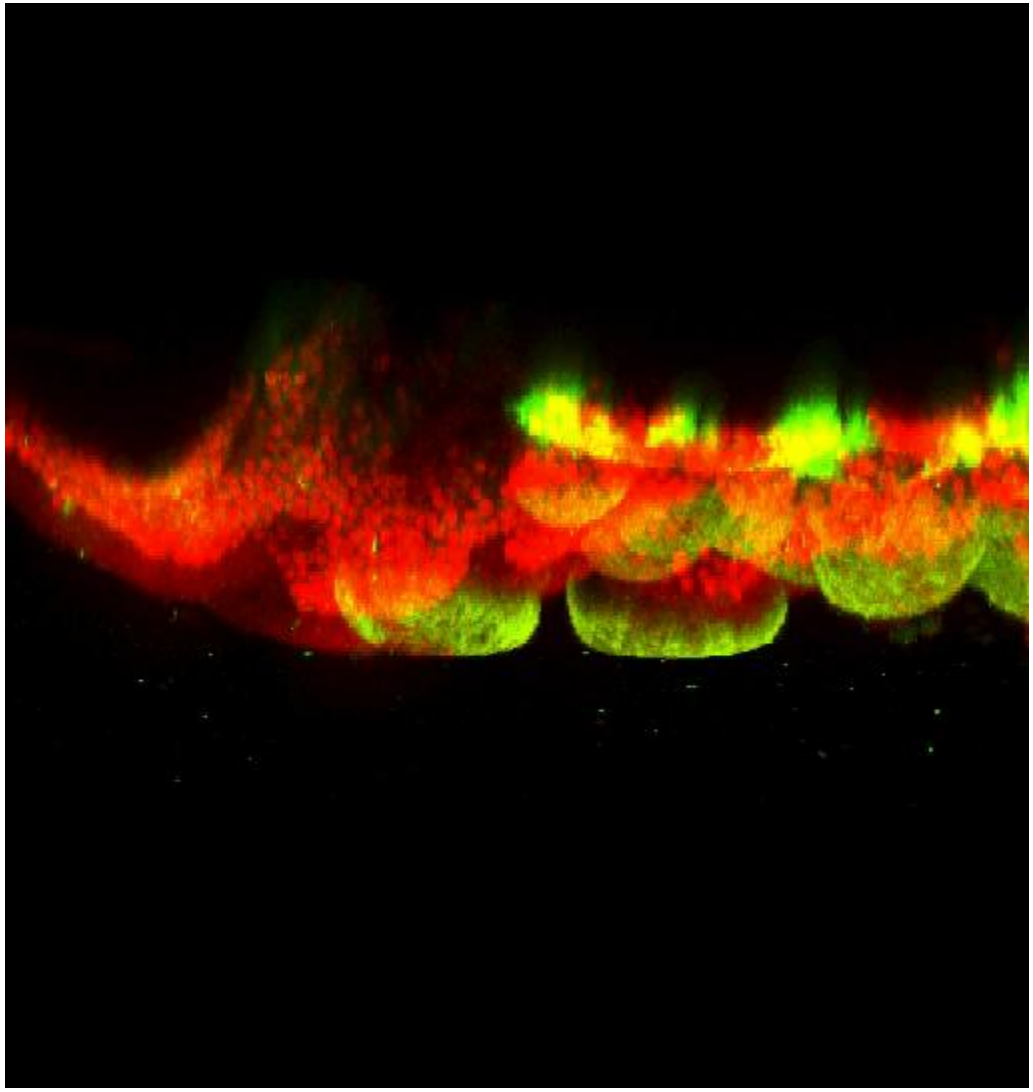
λ exc = 405 nm
λ ém. = 422-496

λ exc = 488 nm
λ ém. = 496-533

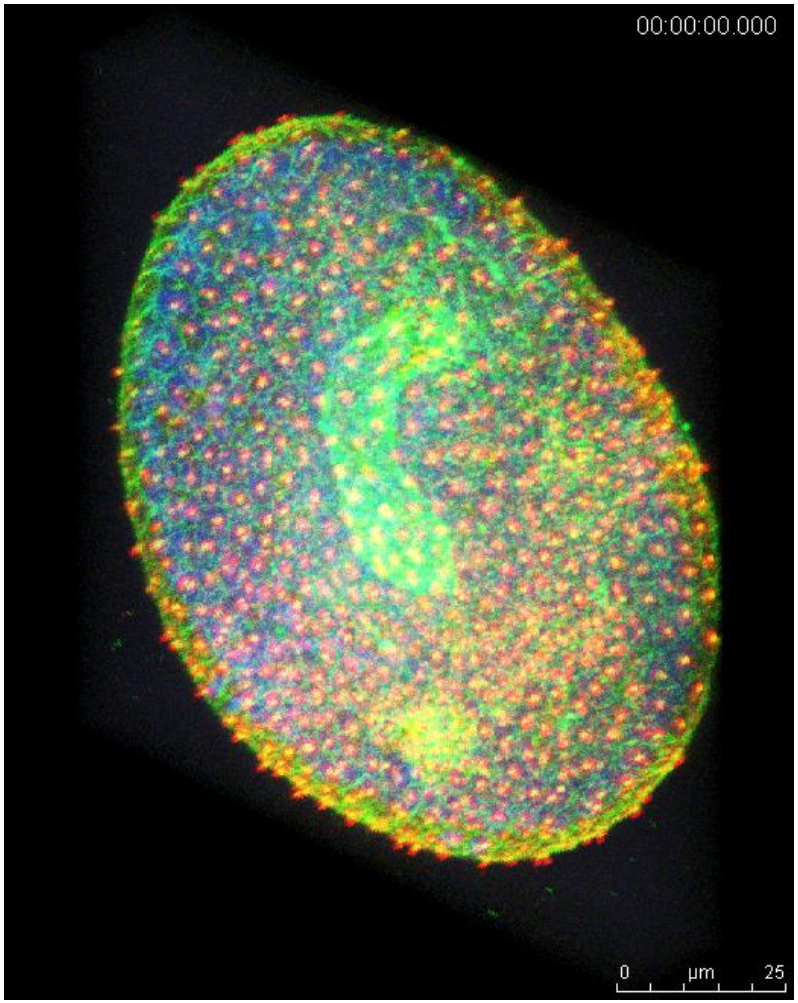
λ exc = 543 nm
λ ém. = 556-628 nm

λ exc = 633 nm
λ ém. = 661-690 nm

Immunohistochimie

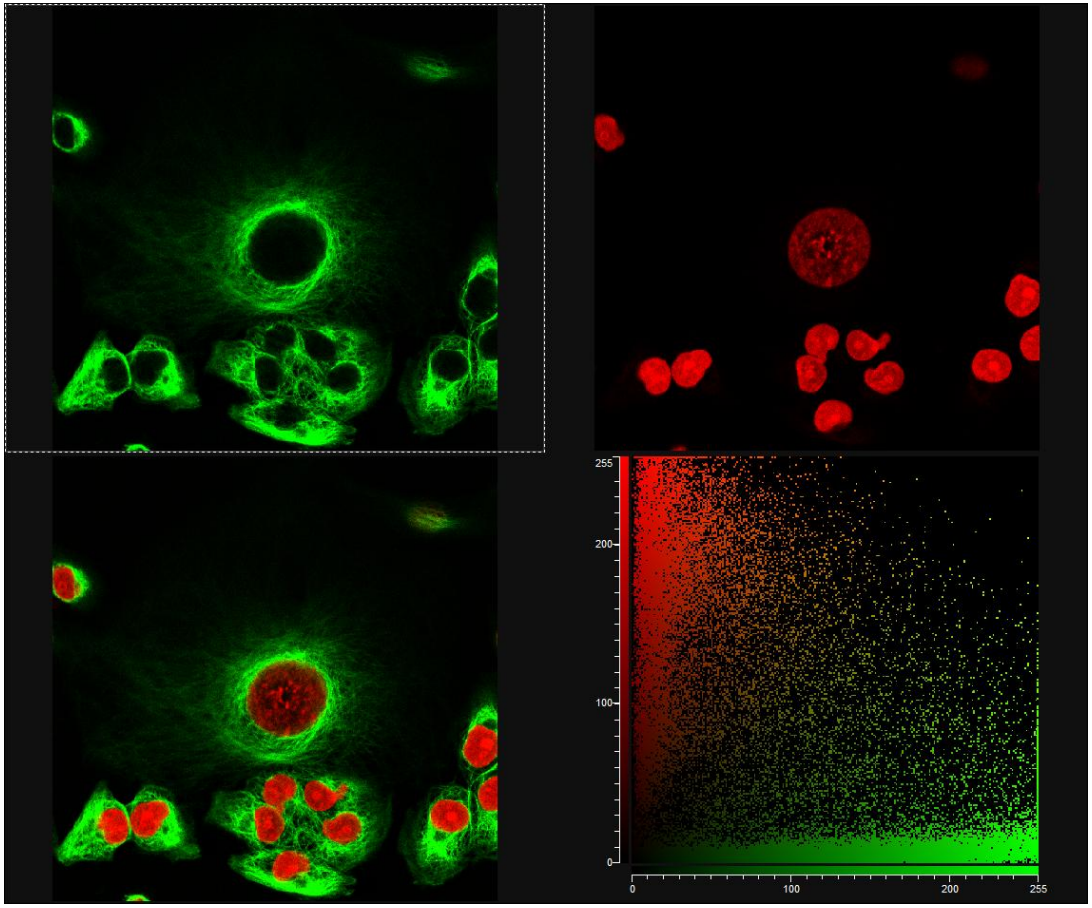


Immunohistochimie



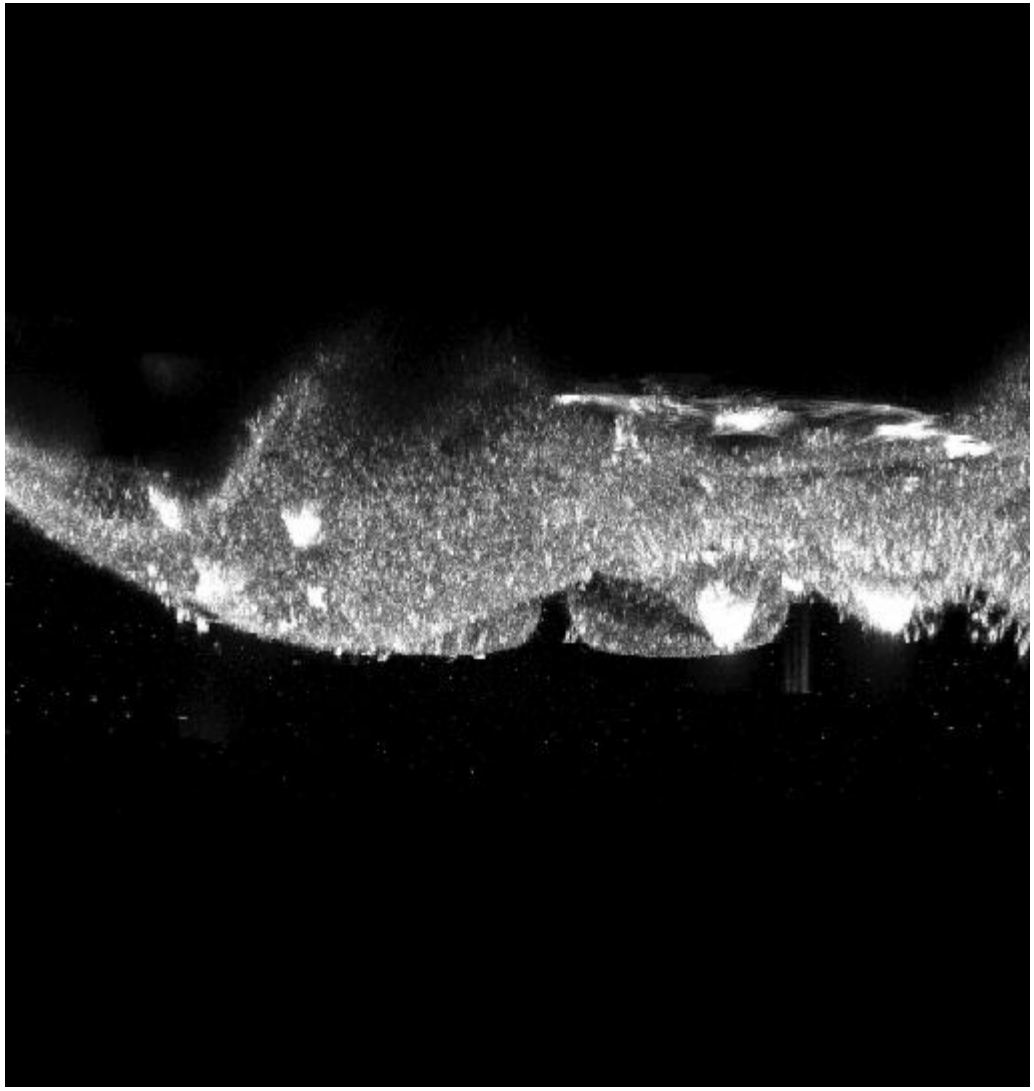
Immunohistochimie

Etude de colocalisation



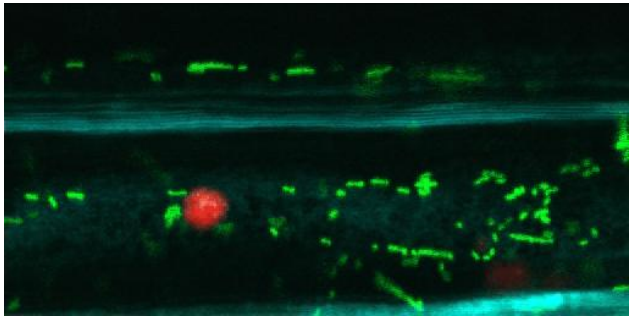
Geometric Analysis	Image
#Pixels	262,144
#Pixels, mask	21,397
Area [µm²]	5,112.01
Area, mask [µm²]	417.26
Mask area rate	8.16%
Densitometric Analysis:	
Channel 1	
Intensity sum	7,744,006
Intensity sum, mask	1,624,968
Mean intensity	29.54
Mean intensity, mask	75.94
Mask intensity rate	20.98%
Channel 2	
Intensity sum	16,348,619
Intensity sum, mask	1,484,046
Mean intensity	62.37
Mean intensity, mask	69.36
Mask intensity rate	9.08%

Réflexion



Réflexion

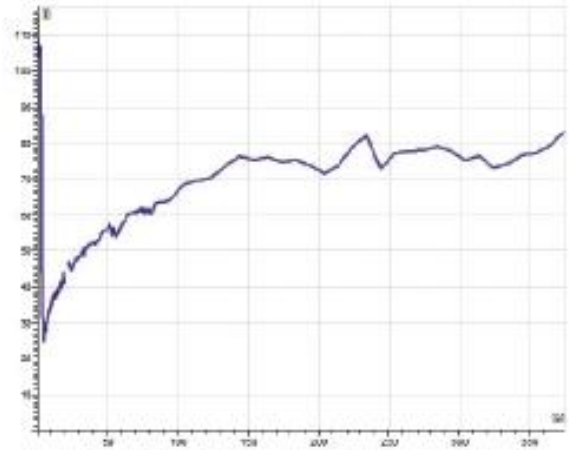
Time-Lapse

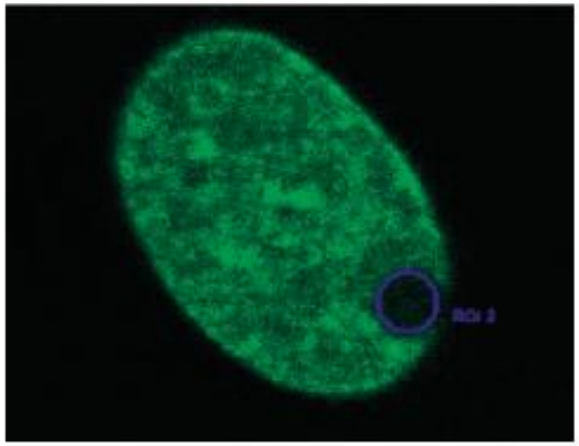


Arabidopsis thaliana
First channel: Cell wall in reflection.
2 & 3 channel: Monitoring mitochondrial (GFP-green) and plastid (autofluorescence-red) movement.

22 fps
Courtesy of Prof. Dr. D. Menzel, Institut für Zelluläre und Molekulare Botanik Zellbiologie der Pflanzen, Bonn University.

Fluorescence Recovery After Photobleaching



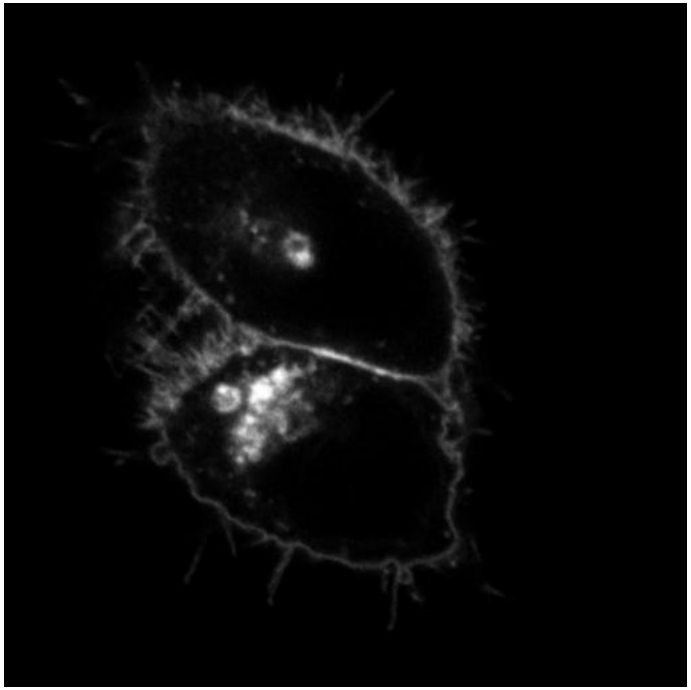


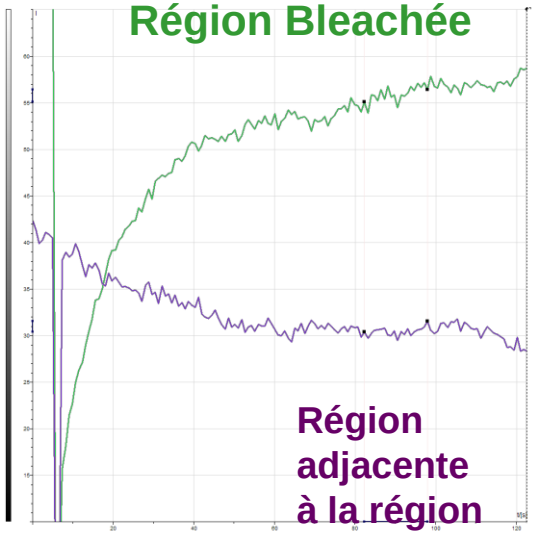
	FD464	$FD464_{theor}$	$H1\text{-}GFP_{arbitrary\ ROI}$	$H1\text{-}GFP_{circular\ ROI}$	$H1\text{-}GFP_{literature}$
M_f [%]	103	100	91	–	~ 90
$t_{1/2}$ [s]	2.3	(1.0)	138.6	59.9	~ 55
τ [s]	3.3	(1.4)	200.9	41.5	–
D_{eff} [$\mu m^2/s$]	1.6	3.7	–	0.01	–

τ: Time constant of recovery (calculated by LCS, circular ROI)

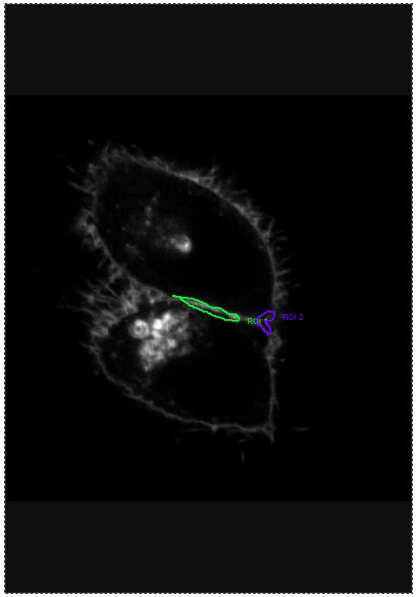
$t_{1/2}$: Half-life of recovery (calculated by LCS, circular ROI)
D_{eff}: Effective diffusion coefficient (Axelrod et al. 1976)

Fluorescence Recovery After Photobleaching





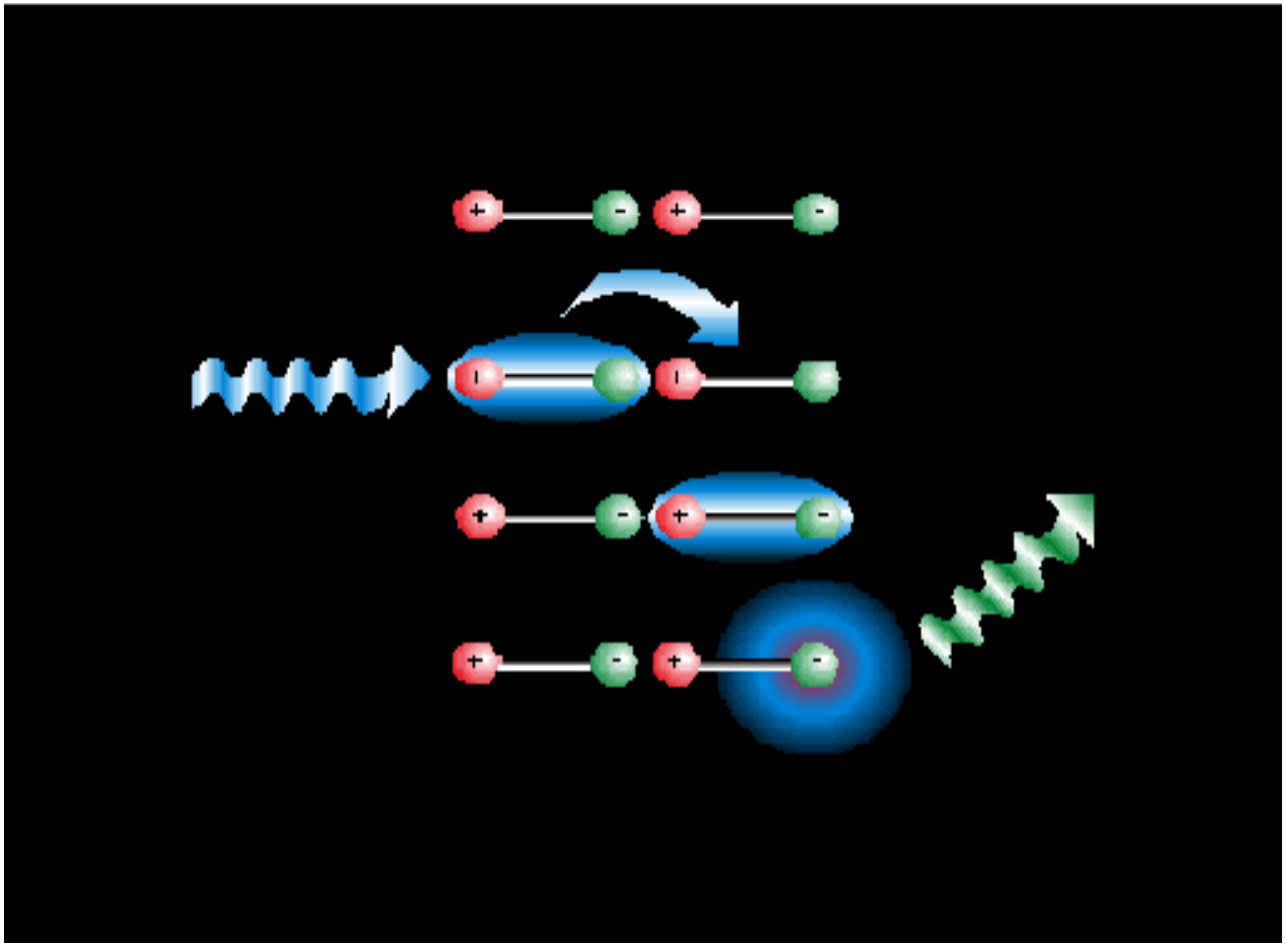
$I = f(t)$



λexc. = 488 nm
Obj. 63X, 1.32
Zoom = 4,6
Δt = 823 ms

T. Lecuit, Luminy, Marseille

Fluorescence Resonance Energy Transfer



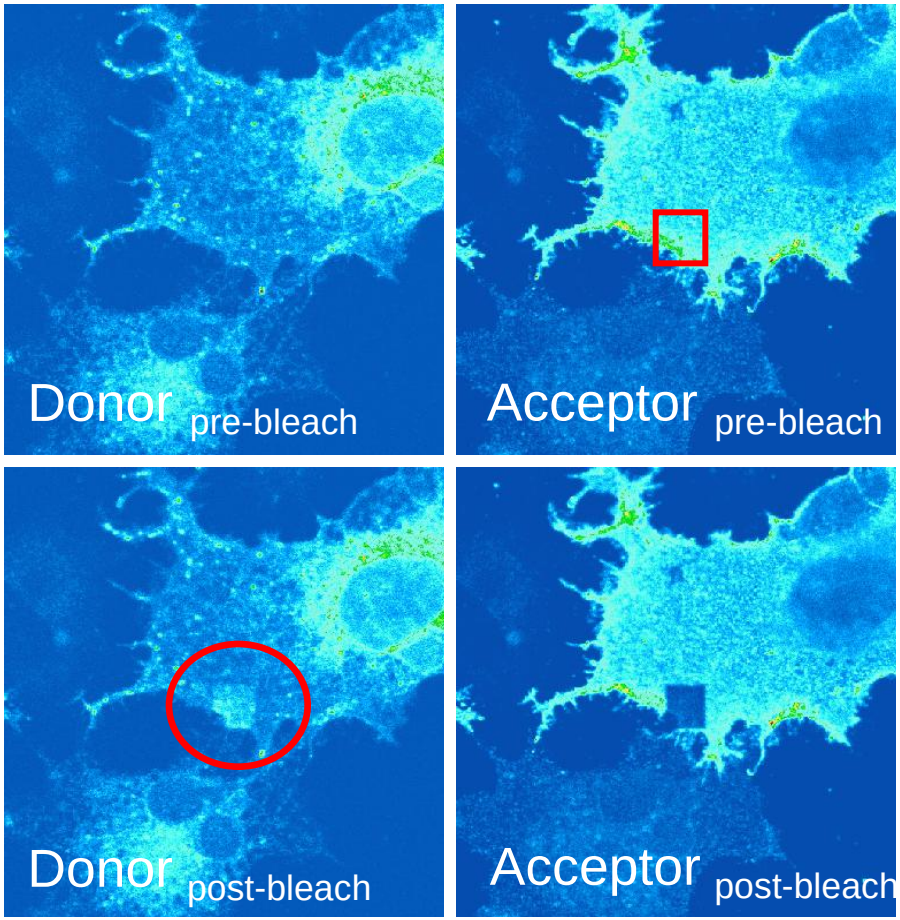
Fluorescence Resonance Energy Transfer

Bleaching de l'accepteur

Etude des modifications des interactions moléculaires

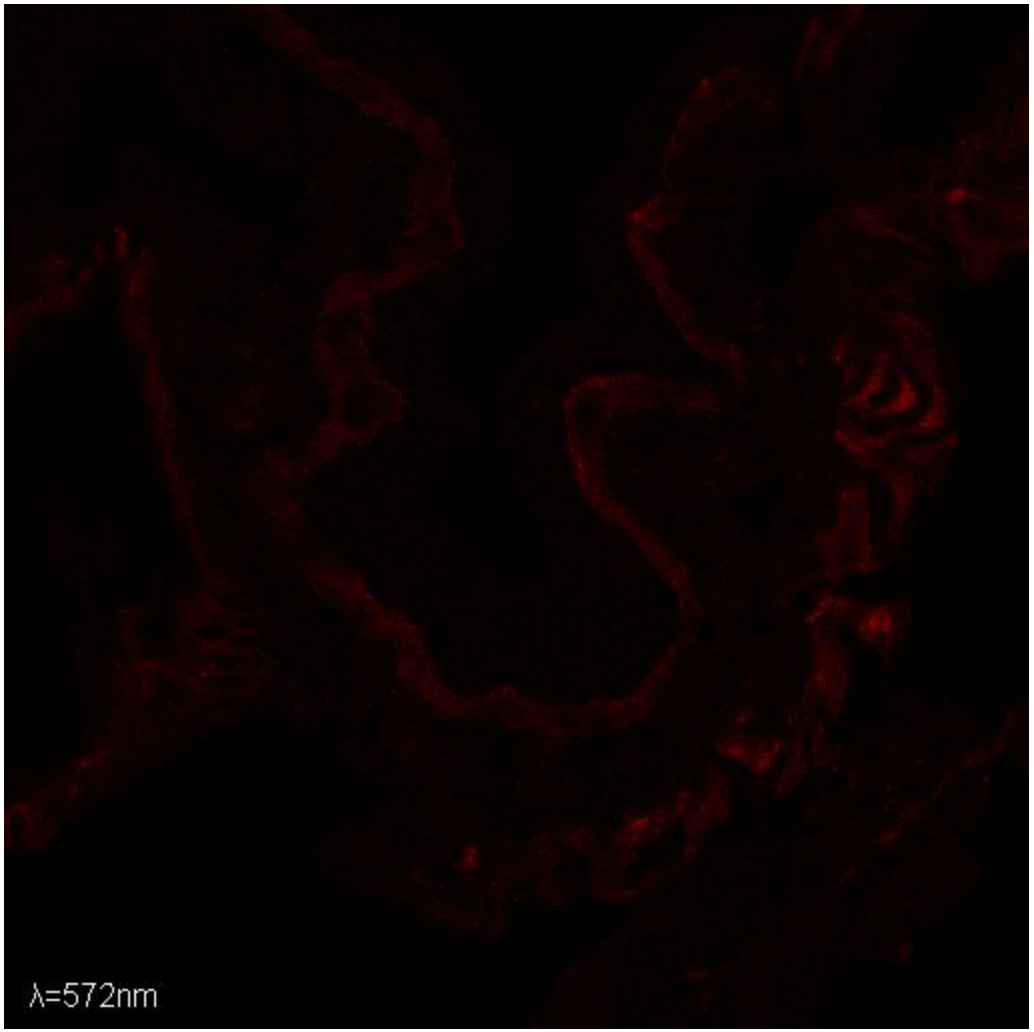
Proximité donneur-accepteur 10–100 Å

Augmentation de l'intensité de fluorescence du donneur



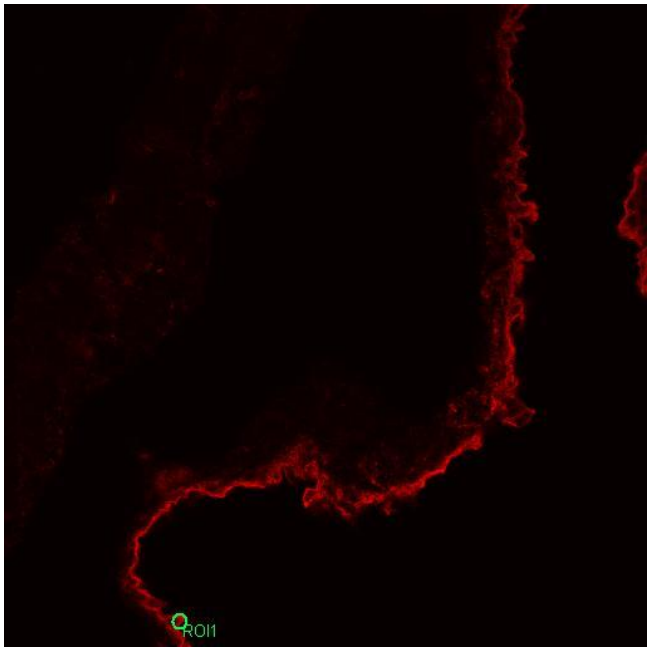
Etudes Spectrales

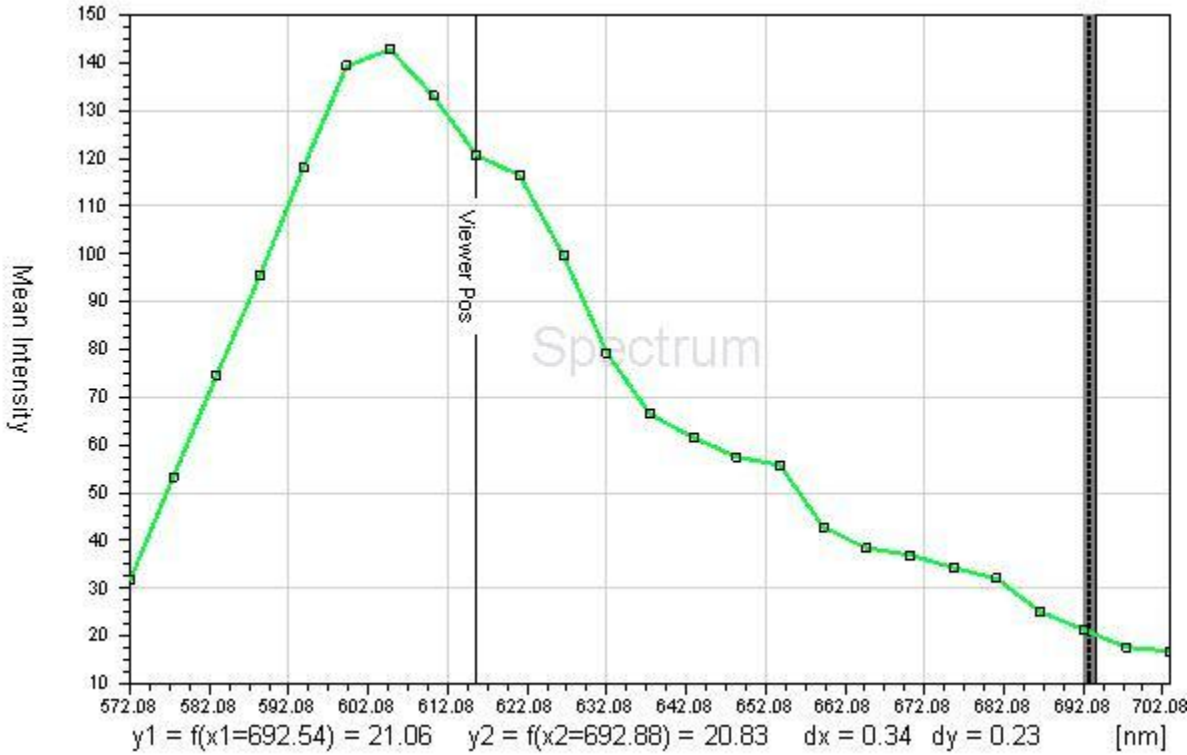
Spectre d'émission de fluorescence



Etudes Spectrales

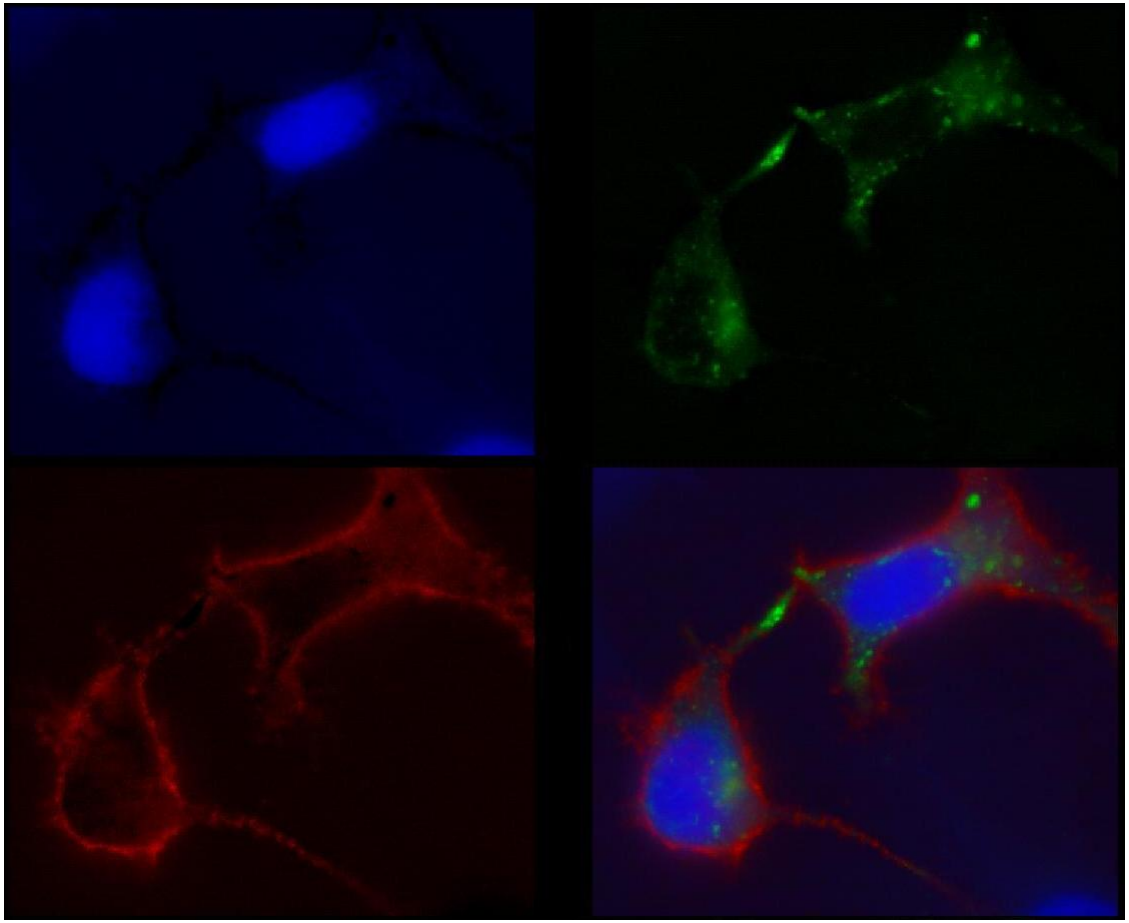
Spectres d'émission de fluorescence





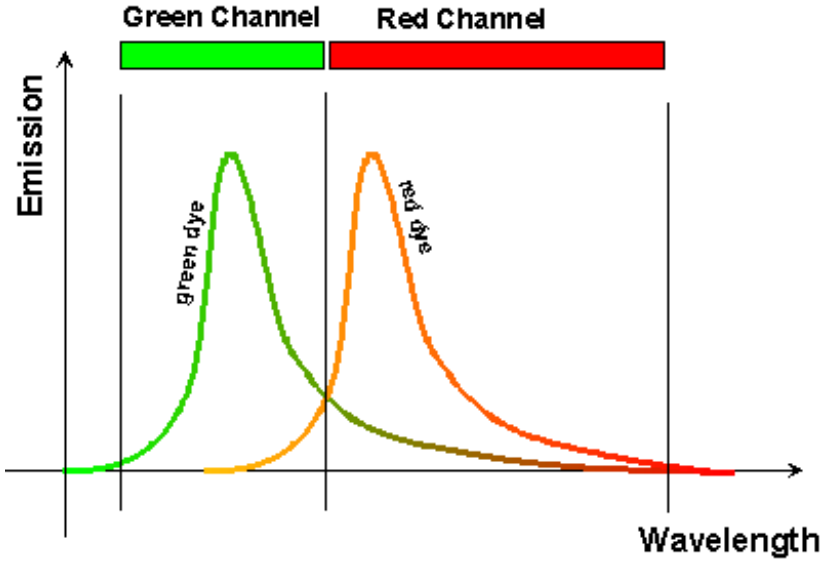
Etudes Spectrales

Séparation spectrale



Etudes Spectrales

Séparation spectrale



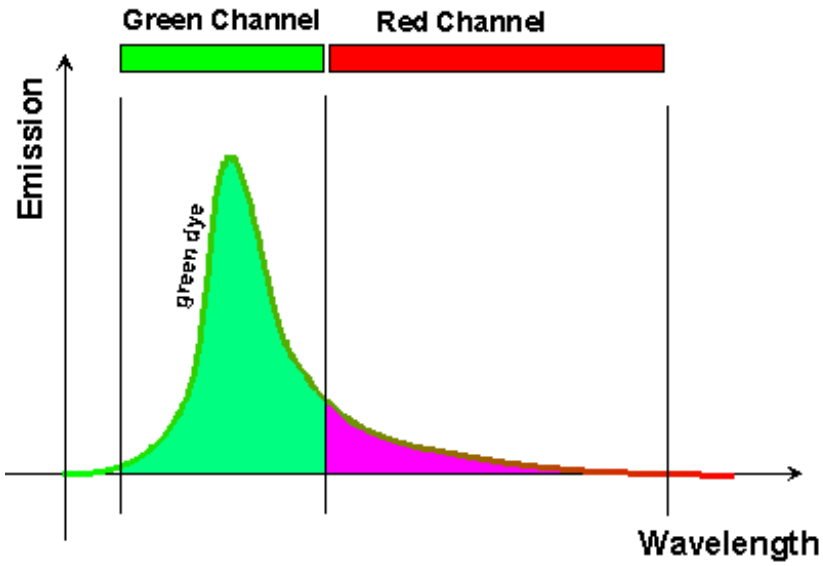
FITC/TxR sample

2 channel recording:

- Detection bands fine tuned
- No gaps between bands
- High efficient prism
- High efficient PMTs
- AOBS® applied

Etudes Spectrales

FITC



The total of all light collected from FITC molecules will be distributed into both channels.

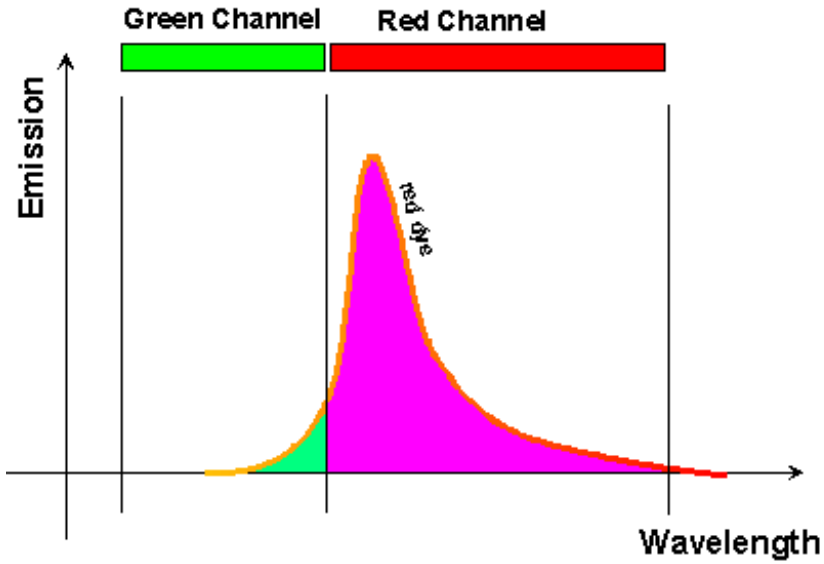
We assume here:

¾ of all FITC emission go into the green channel (G)

¼ of all FITC emission goes into the red channel (R)

Etudes Spectrales

TxR



The total of all light collected from TxR molecules will be distributed into both channels.

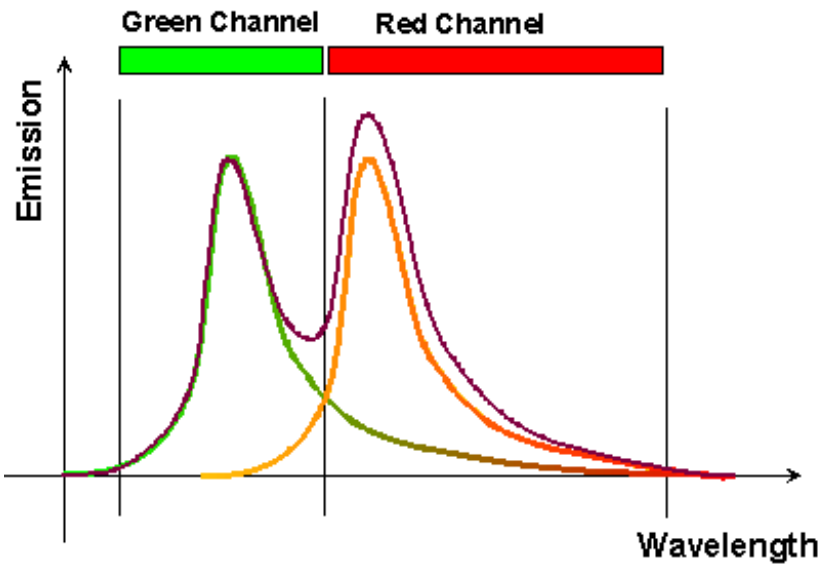
We assume here:

1/5 of all TxR emission goes into the green channel (G)

4/5 of all FITC emission goes into the red channel (R)

Etudes Spectrales

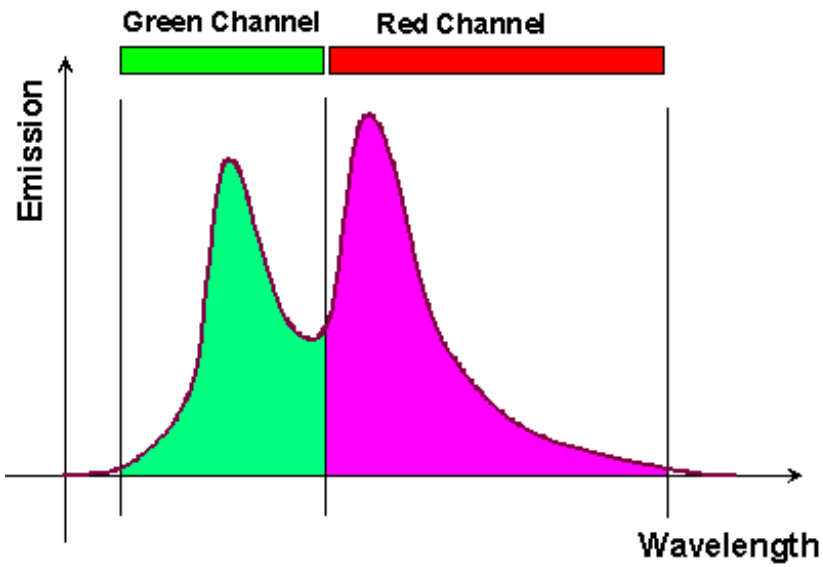
Both dyes



In a real experiment, we will have both dyes simultaneously in the sample and therefore get signals from both dyes in both channels.

Etudes Spectrales

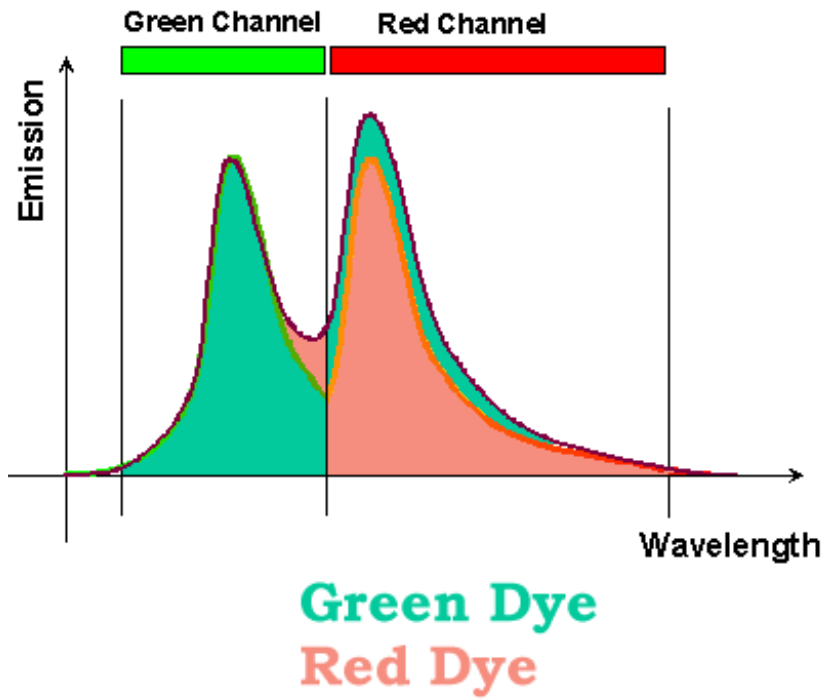
A calculated measurement



$$G = \frac{3}{4}FITC + \frac{1}{5}TxR$$

$$R = \frac{1}{4}FITC + \frac{4}{5}TxR$$

Etudes Spectrales

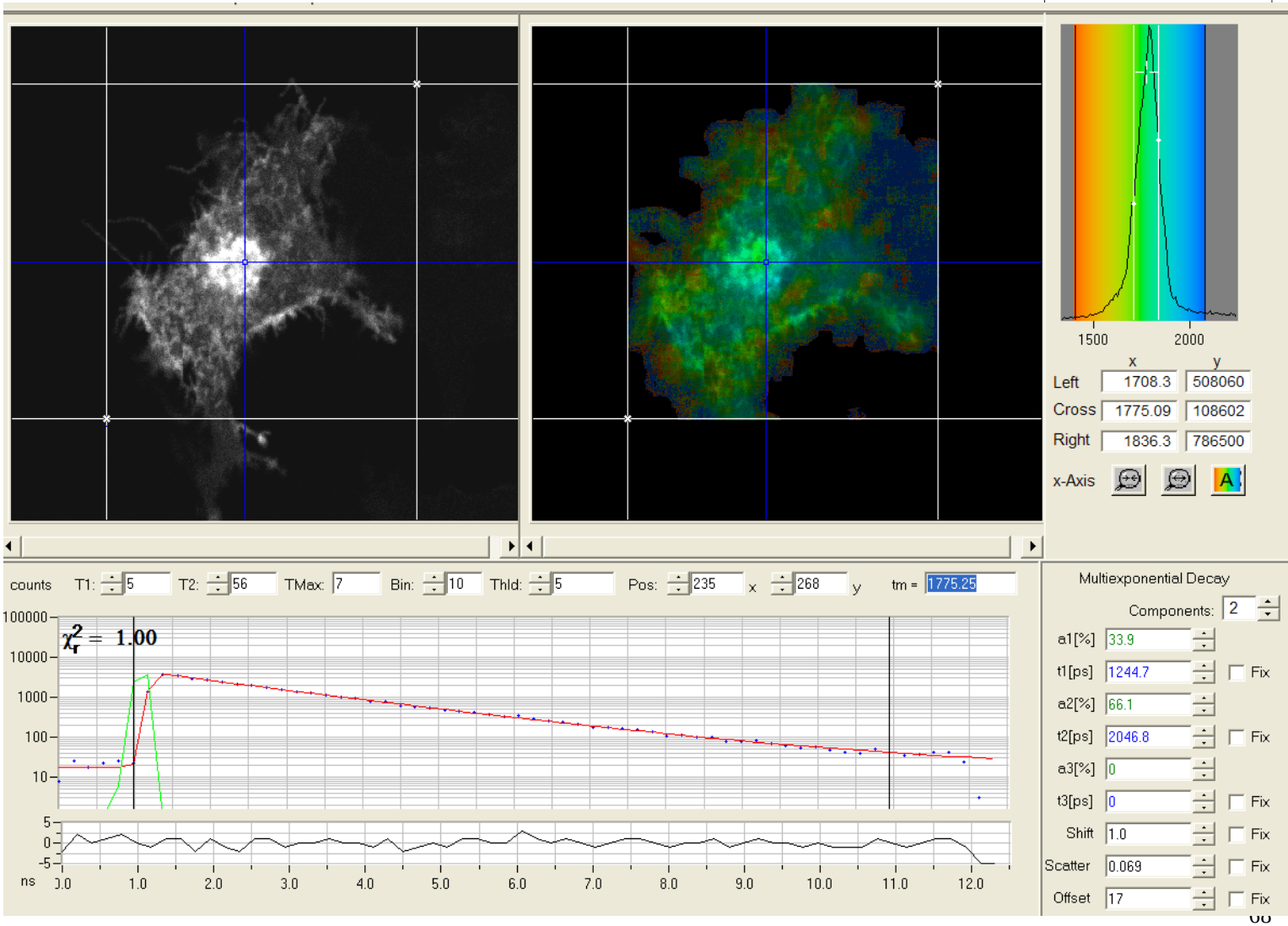


Unmixing is:
Solving sets of *n* linear equations with *n* unknowns.

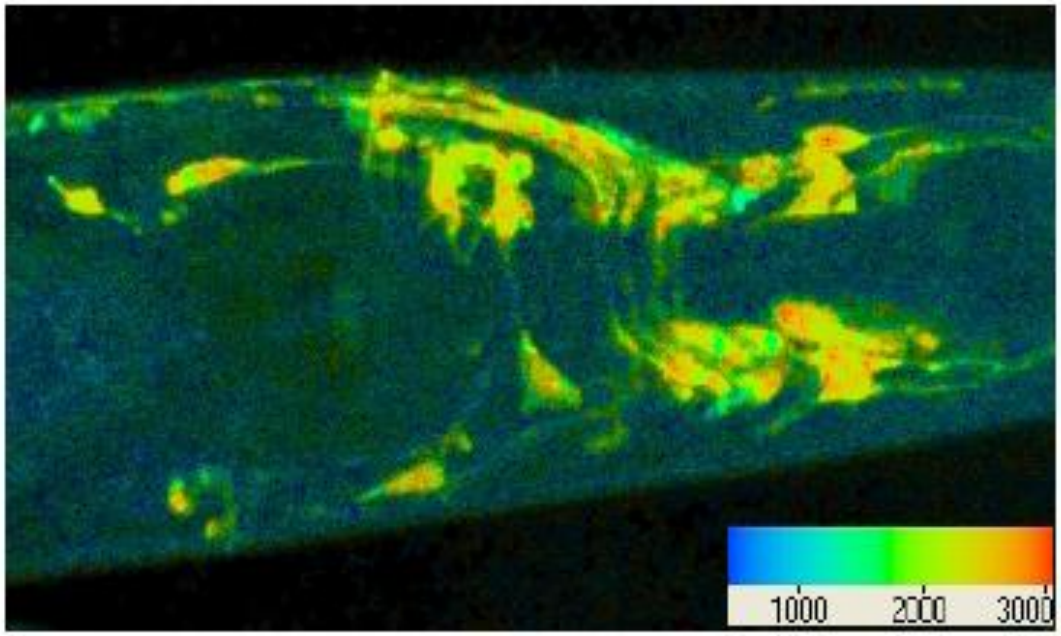
First proven records of solutions go back some 4000 years (Egypt)

For a reference see:
http://www.ETH\EducETH - Mathematik - Leitprogramm Lineare Gleichungssysteme.htm

Fluorescence Lifetime Imaging Microscopy FLIM



Fluorescence Lifetime IMaging FLIM

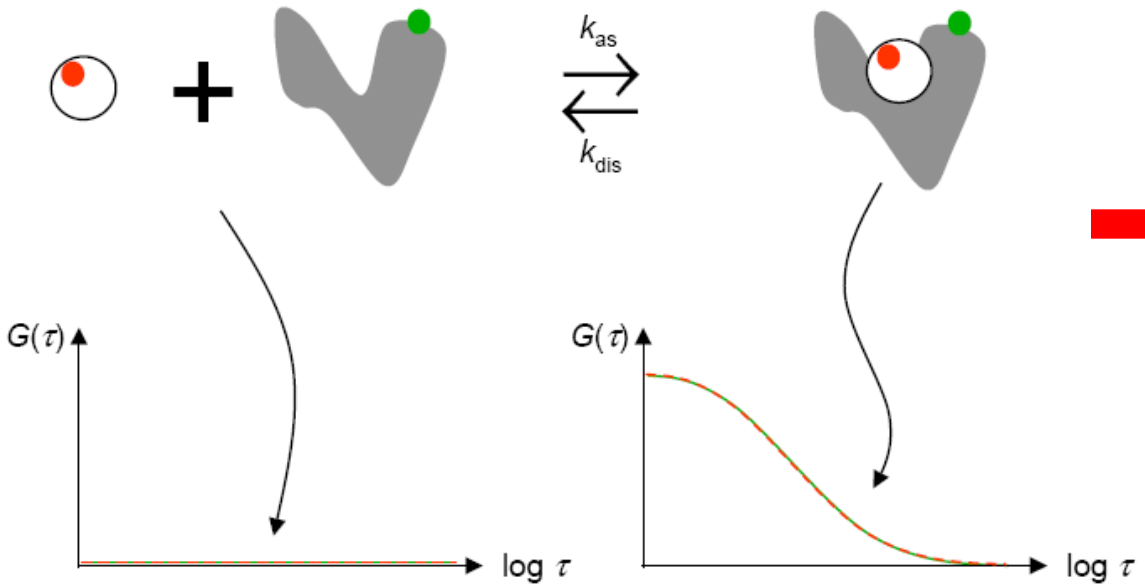


Fluorescence lifetime image of a C. *elegans*. The different lifetime colours derive from various fluorescent proteins (CFP, GFP, YFP). Courtesy H. Hutter.

Fluorescence Correlation Spectroscopy

Lien entre la diffusion de molécules et la fluctuation de l'intensité de fluorescence dans un volume donné

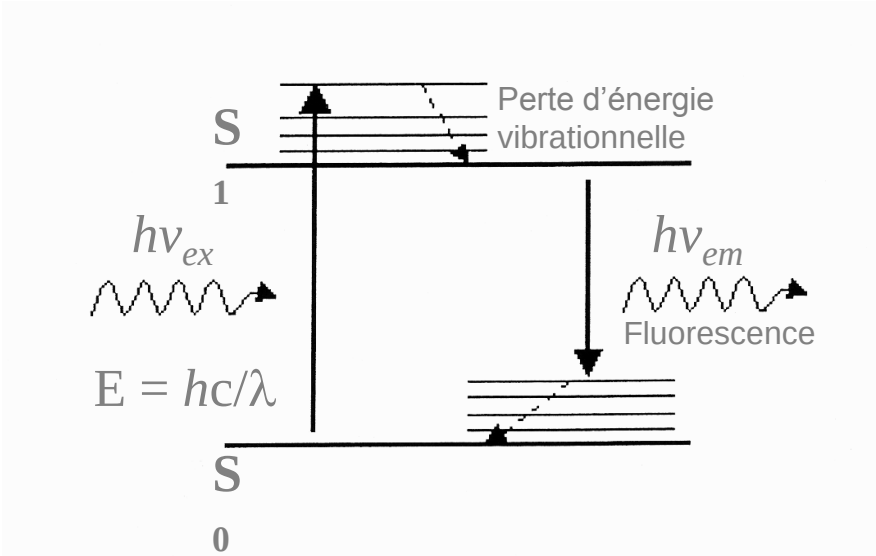
- **Concentration**
- **Coefficient de diffusion**



- **Constante de dissociation**

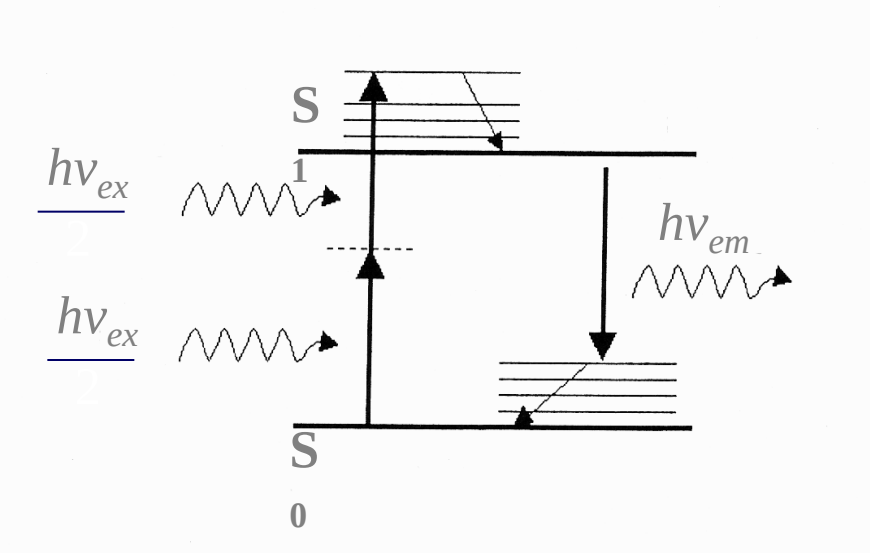
Principe de l'excitation à deux photons

Processus de fluorescence en excitation à un photon



L'énergie d'un seul photon est absorbée par un fluorochrome pour passer d'un état d'énergie basal (S_0) à un état excité (S_1)

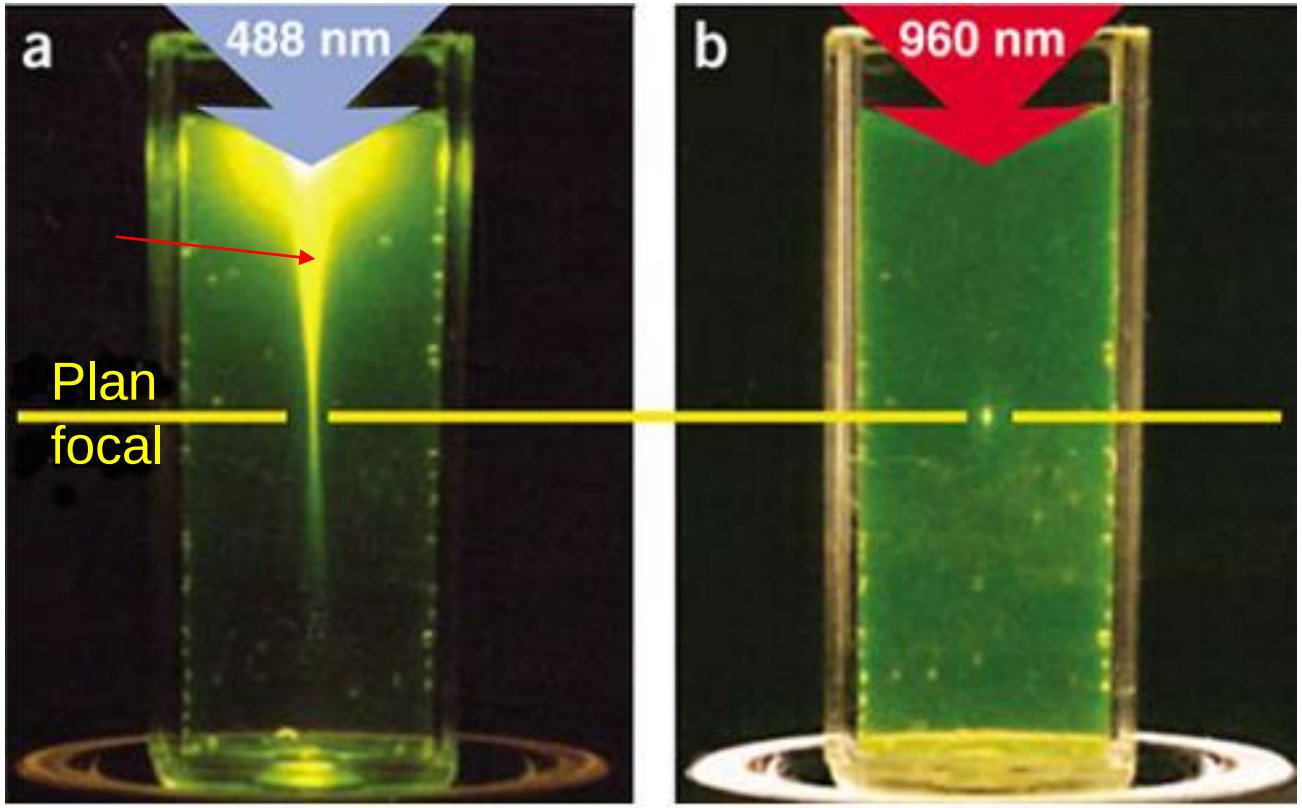
Processus de fluorescence en excitation à deux photons



Deux photons d'énergie deux fois plus faible (et donc de longueur d'onde deux fois plus élevée) sont absorbés par la molécule dans un laps de 10^{-16} s

➢ Les caractéristiques du rayonnement émis par le fluorochrome en excitation à deux photons sont inchangées

En microscopie à balayage laser à deux photons, l'excitation est strictement restreinte au volume focal



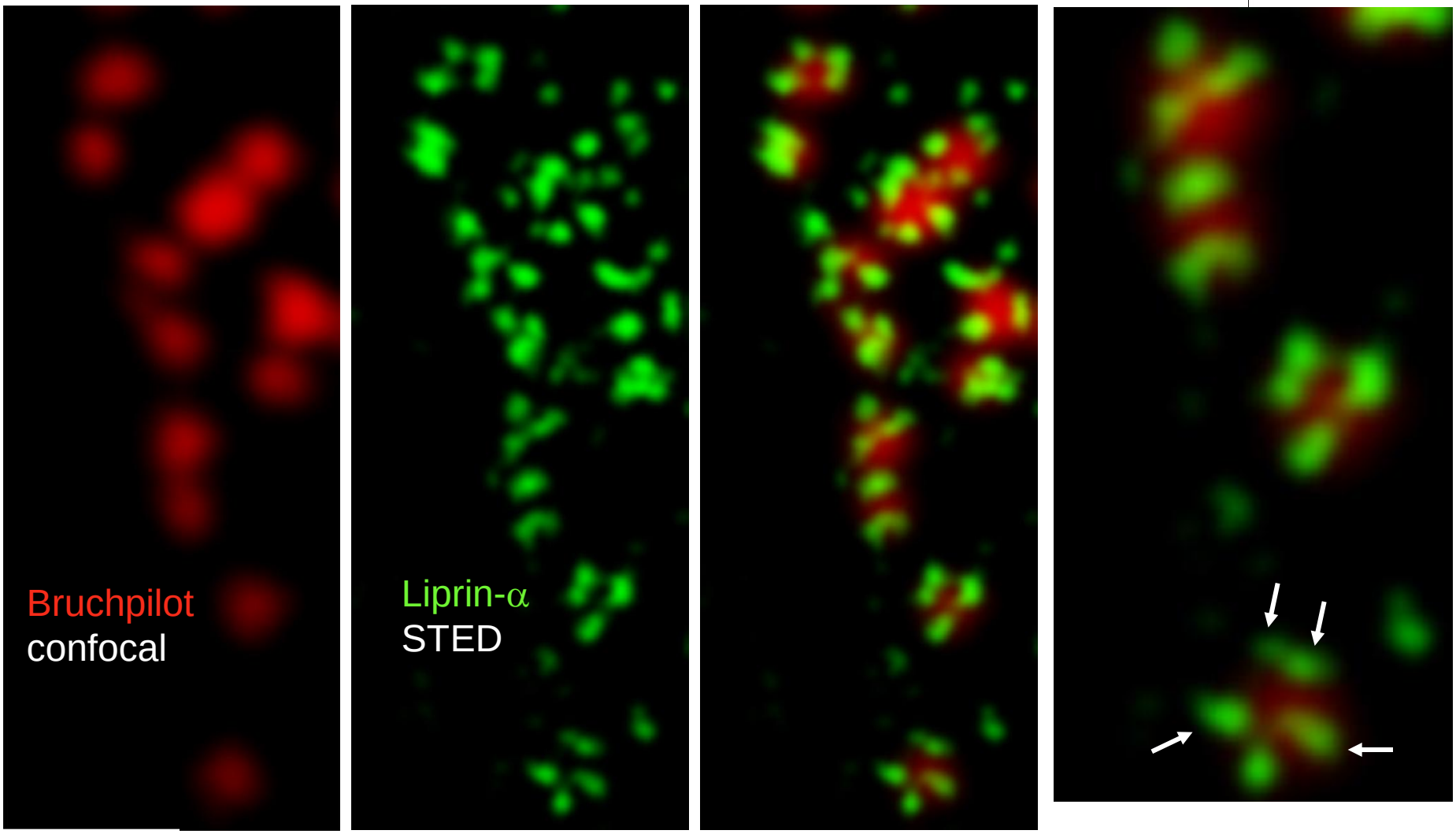
Fluorescence suite à une absorption à un photon

Fluorescence suite à une absorption à deux photons

Leica TCS STED

Leica
MICROSYSTEMS

Multicolor Image of the NMJ



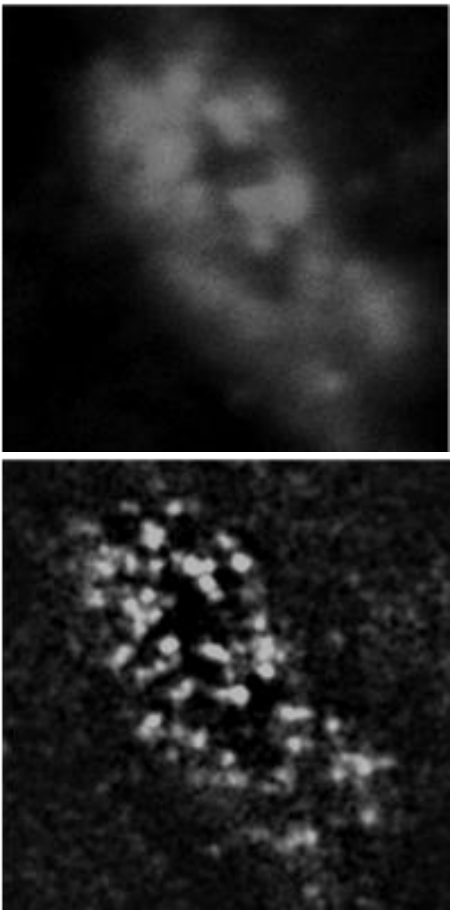
500 nm

Courtesy of Prof. Stephan Sigrist

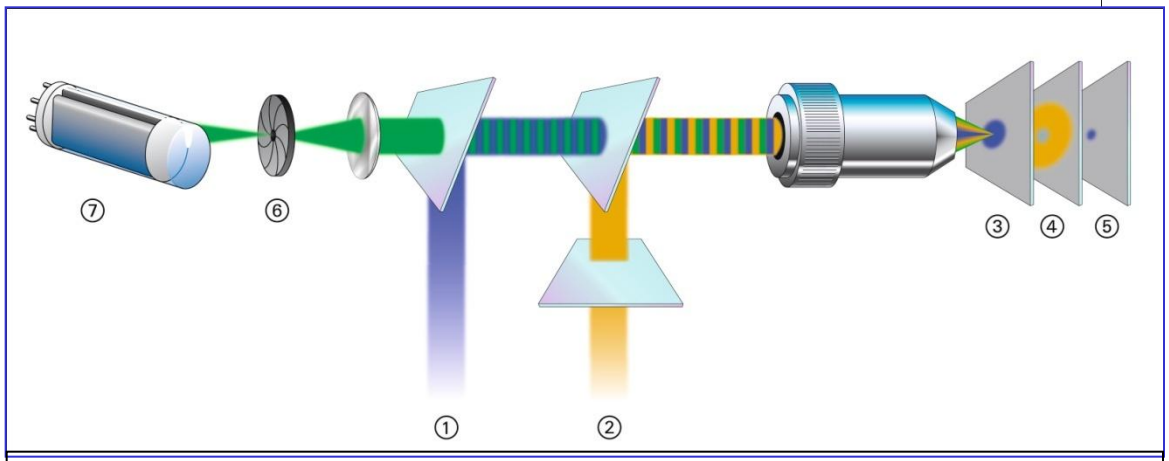
Resolution Increase by STED: Pure Physics!

STED is pure physics! But you can add mathematics on top!

Optical pathway

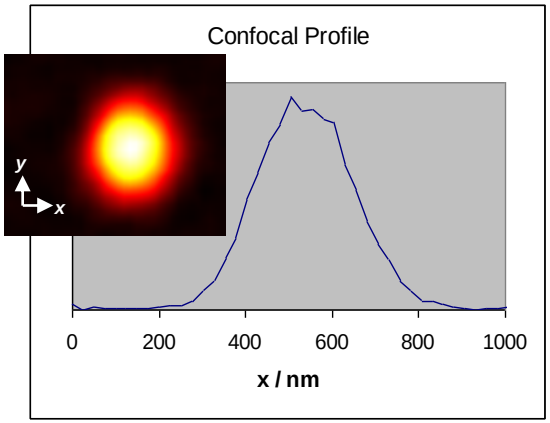


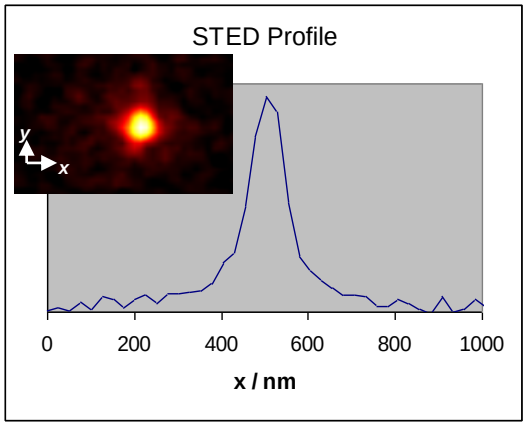
Formation of presynaptic active zone (Liprin)
Courtesy S. Sigrist, Wuerzburg



Principle of STED microscopy

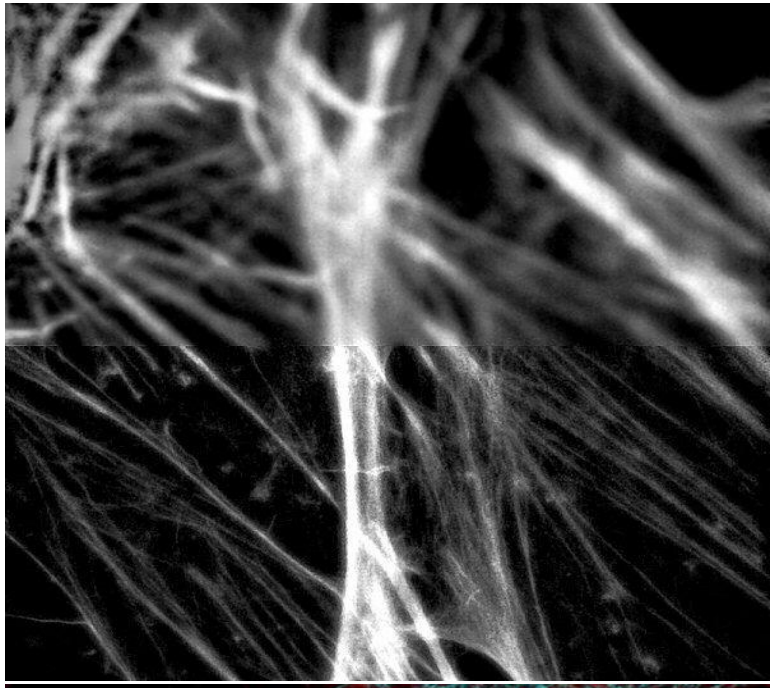
1. Excitation laser
2. Depletion laser
3. Original Excitation spot
4. Depletion (STED) ring
5. Effective excitation spot
6. Confocal pinhole
7. Detector





Typical lateral resolution: **200x200 nm**

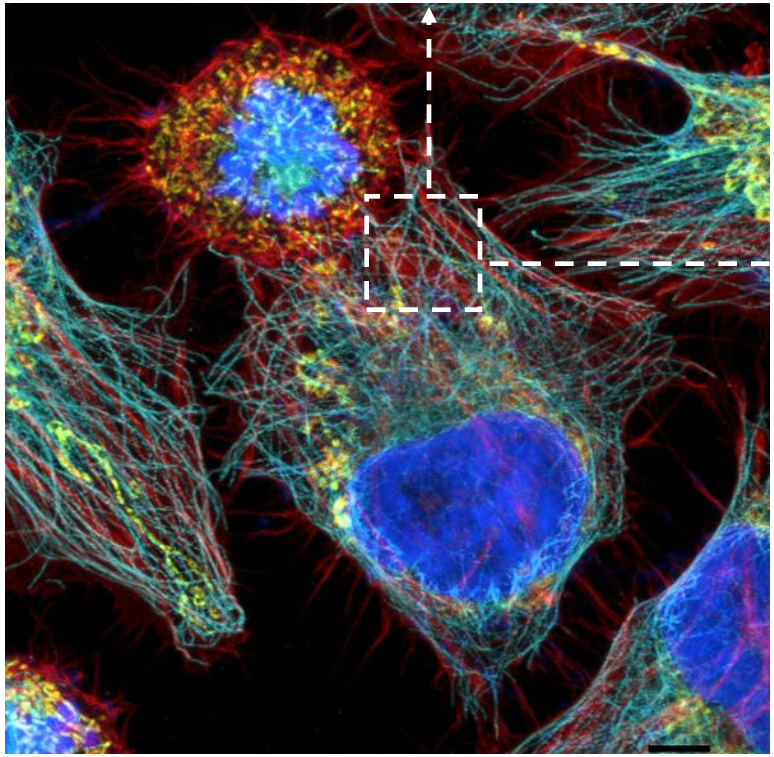
Typical lateral FWHM in STED is **90x90 nm**

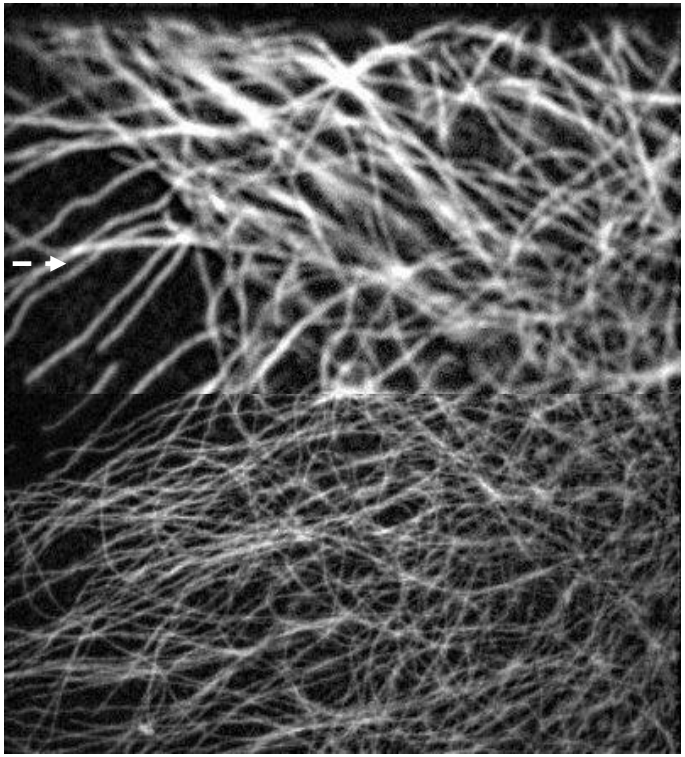


ACTIN

Confocal

STED





MICROTUBULES

Confocal

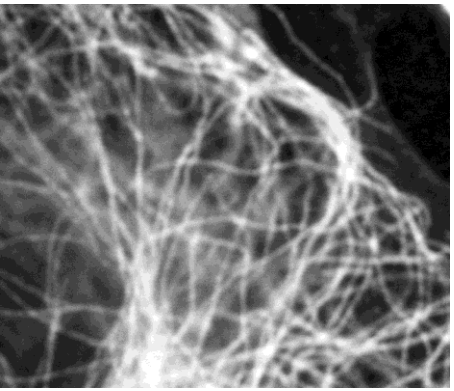
STED

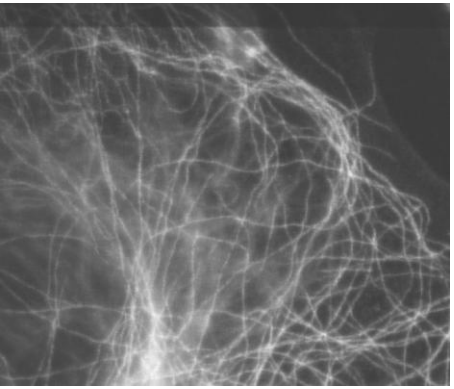
The task

Increase xy resolution in fluorescence microscopy over classical Abbe limits:

$$d_{xy} = \frac{\lambda}{2n \cdot \sin\alpha}$$

FWHM$_{\text{confocal, xy}}$: 200 nm





Microtubules of a Vero cell

BUT: For numerous applications a higher resolution is required *-without the efforts and restrictions of an Electron microscope!-*

SOME EXAMPLES:

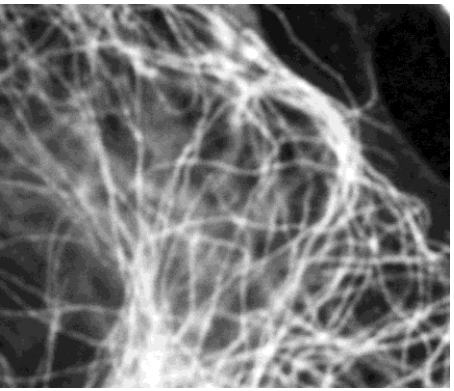
- Neurophysiology (Synapse-cell-interactions, motoneurons etc.)
- Endocytotic processes
- Virus biology (Malaria, AIDS)
- Pathology (Multiple Sclerosis etc.)

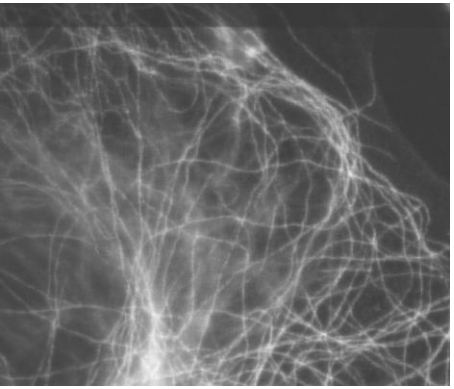
The task

Increase xy resolution in fluorescence microscopy over classical Abbe limits:

$$d_{xy} = \frac{\lambda}{2n \cdot \sin\alpha}$$

FWHM$_{\text{confocal, xy}}$: 200 nm





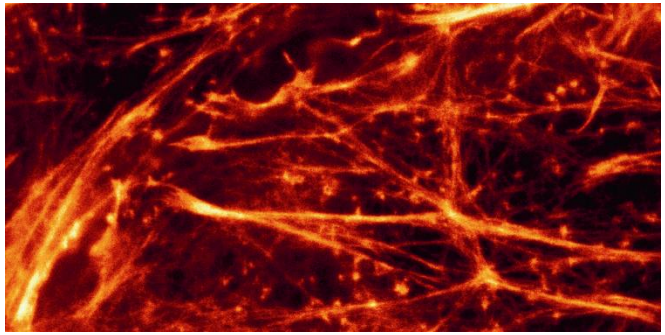
Microtubules of a Vero cell

BUT: For numerous applications a higher resolution is required
-without the efforts and restrictions of an Electron microscope!-

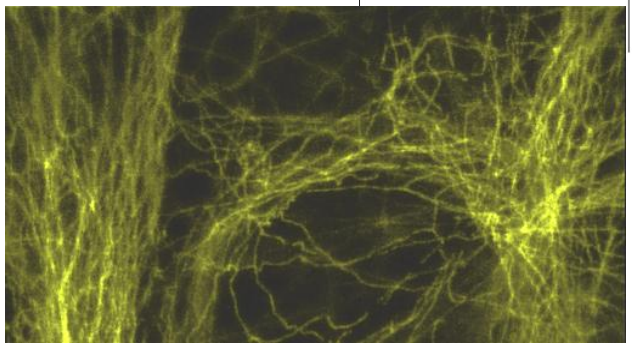
SOME EXAMPLES:

- Neurophysiology (Synapse-cell-interactions, motoneurons etc.)
- Endocytotic processes
- Virus biology (Malaria, AIDS)
- Pathology (Multiple Sclerosis etc.)

Applications Example - Cell Biology

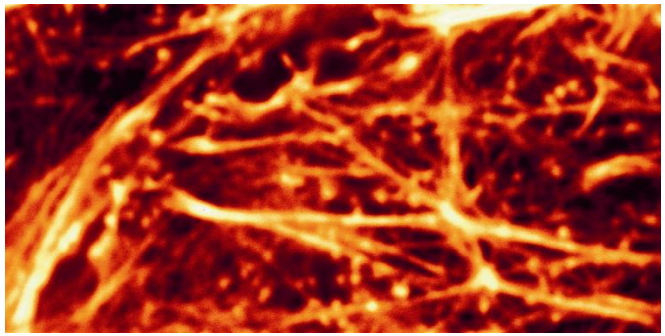


STED

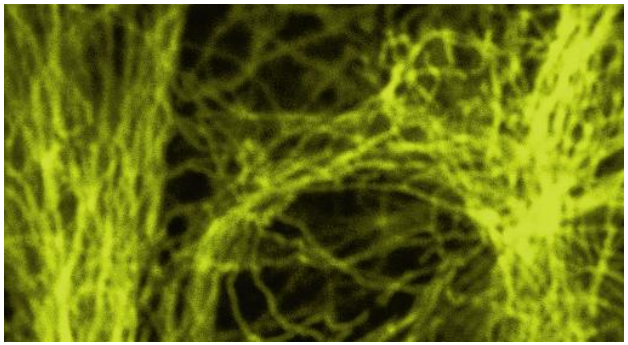


F-Actin

β-Tubulin

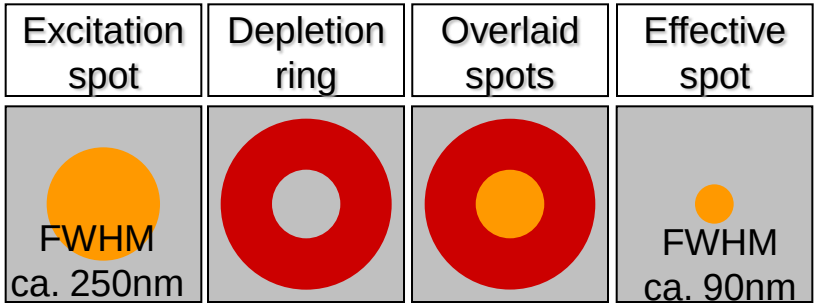


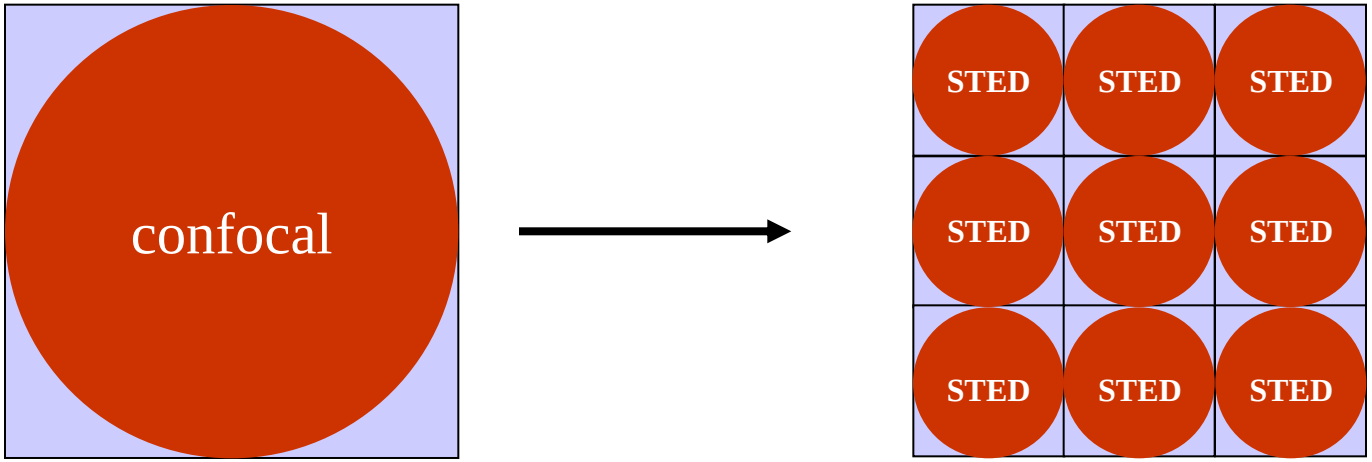
Confocal



Nice for demonstrational purposes

Resolution enhancement by STED:





A threefold improved resolution can make 9 spots out of 1 !

Microscopie Confocale

- Introduction
  - Historique
  - Fluorescence
  - Résolution
  - Microscopie Confocale
- Applications et exploitation des données
  - Immunomarquages
  - Réflection
  - Time-Lapse
  - Etudes spectrales
  - F-Techniques
  - FCS/FLIM
- **Conclusion**

Conclusion

- Technique d’imagerie à haute résolution
- Large domaine d’application
- Exploitation des images brutes
- Système commercial entièrement motorisé
- Système évolutif

Ca y est vous êtes libres !!!