Microscopie et imagerie : enjeu majeur en sciences du vivant

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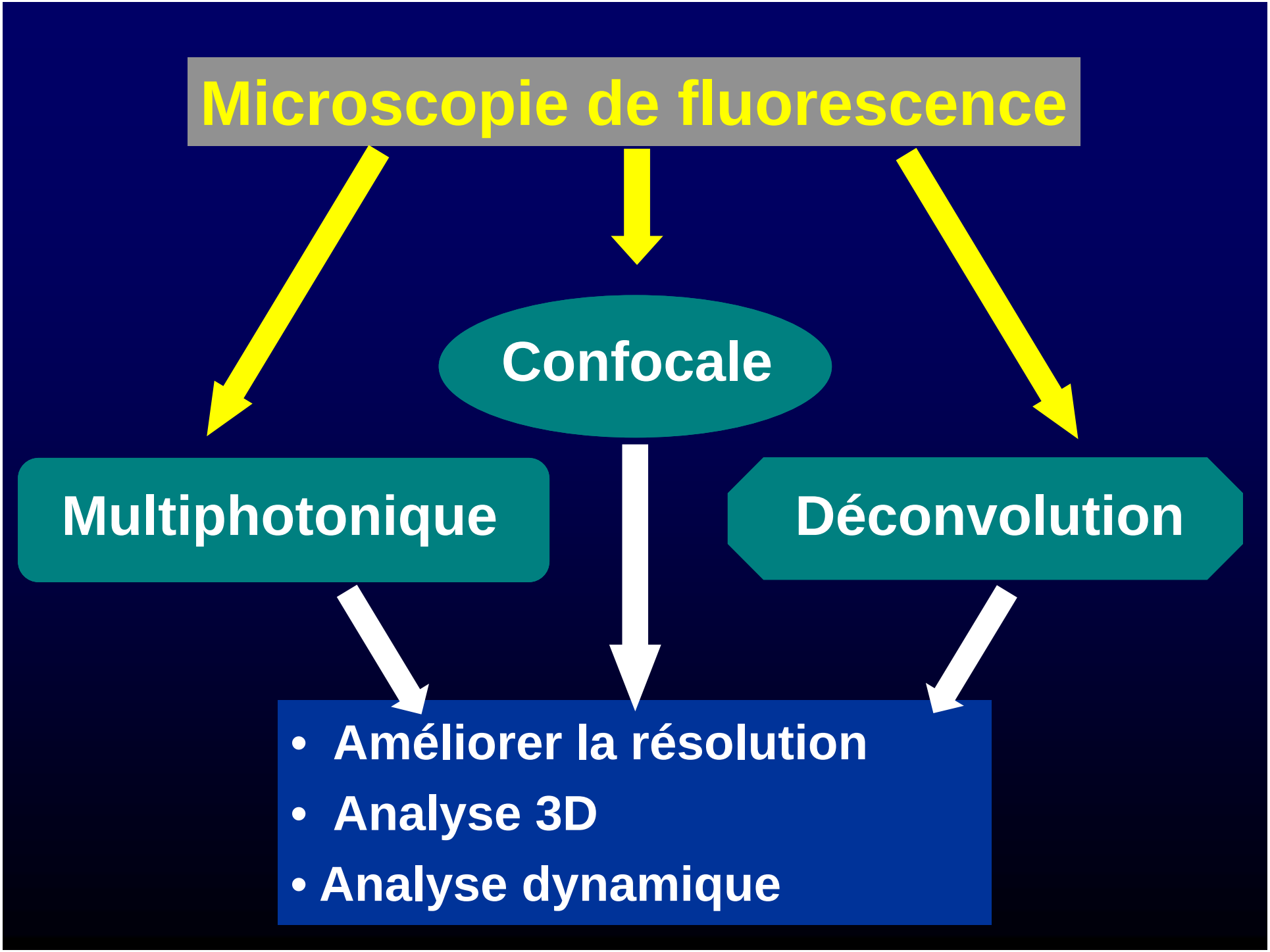
The big picture

Over the past ten years, microscopy has been transformed from slice, stain and fix, to the capacity to view living cells and even whole organisms in real time. Lisa Melton looks at what's on offer.

1. **Instrumentation :**
   Microscopie confocale et multiphoton, déconvolution, caméras EMCCD, super-résolution, sélective plane illumination (SPIM), optical tomography…

2. **Fluorochromes :**
   GFP, CFP, YFP, RFP, Quantum dots….

3. **Méthodologies :**
   FRAP, FRET, FLIM, TIRF, photoactivation, nanochirurgie…


Microscopie de fluorescence
Confocale
Multiphotonique
Déconvolution
• Améliorer la résolution
• Analyse 3D
• Analyse dynamique

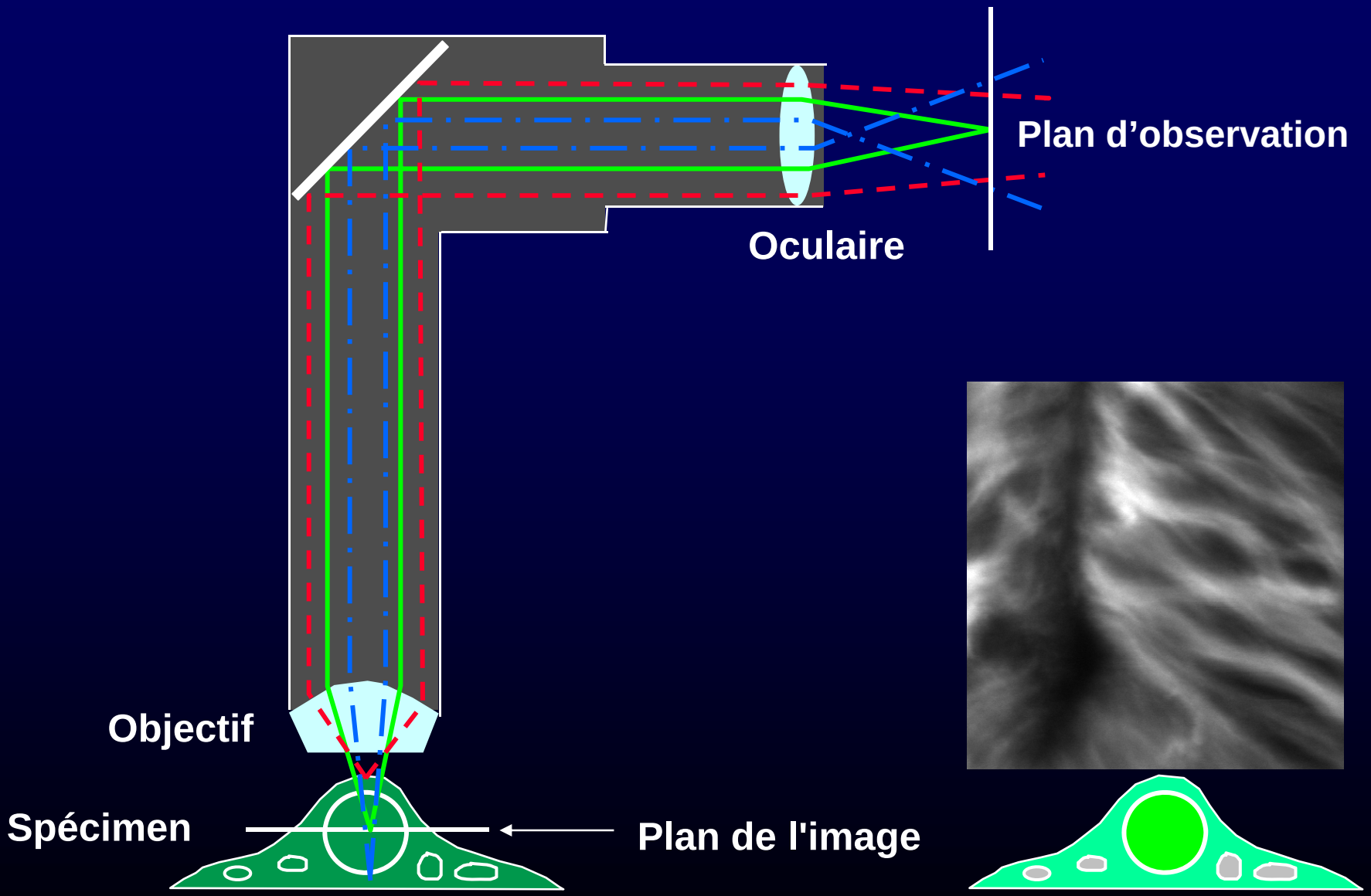
Applications en biologie

- Analyses morphologiques *(3D - Confocal, multiphoton, SPIM)*
- Analyses dynamiques *(4D - Champ large, Confocal rapide, multiphoton, SPIM)*
- Localisations multiples – colocalisations – analyses spectrales *(Confocal)*
- Observation sub-résolutive *(TIRF, TED, PALM, STORM)*
- Mouvements et interactions moléculaires *(FRAP, FRET, FLIM, TIRF…)*
- Mouvements ioniques (Ca^{2+}, pH, …)
- Décageage de molécules actives
- Traçage de cellules ou de compartiments cellulaires
- Nanochirurgie laser

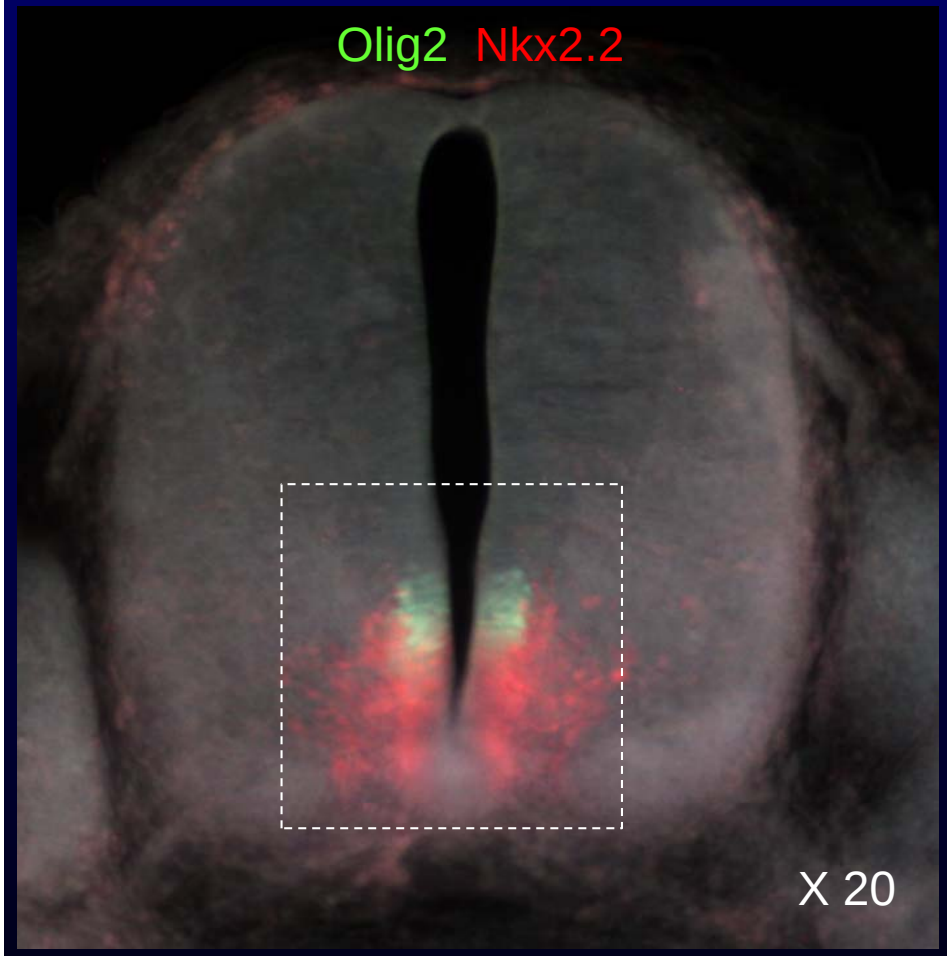
La microscopie confocale

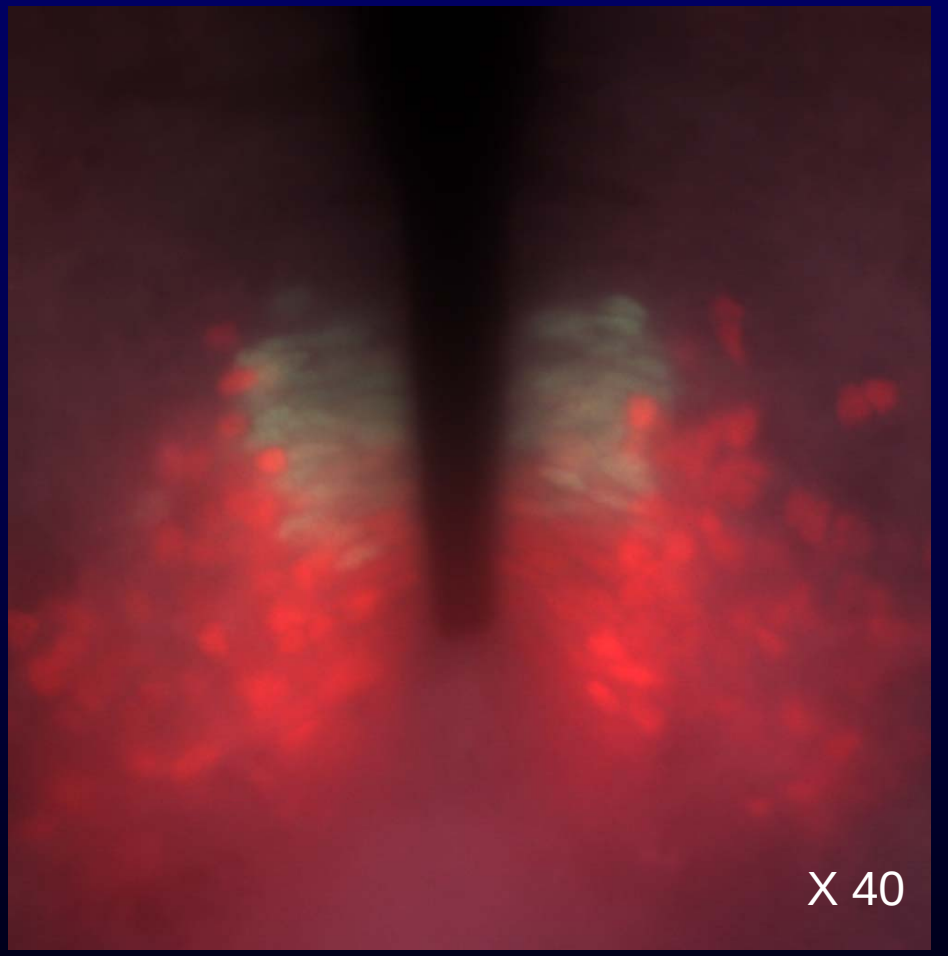
1. Introduction : principes de fonctionnement
2. Numérisation – reconstructions 3D
3. Lasers – Multimarquages
4. Avantages et limites

MICROSCOPIE de FLUORESCENCE CONVENTIONNELLE

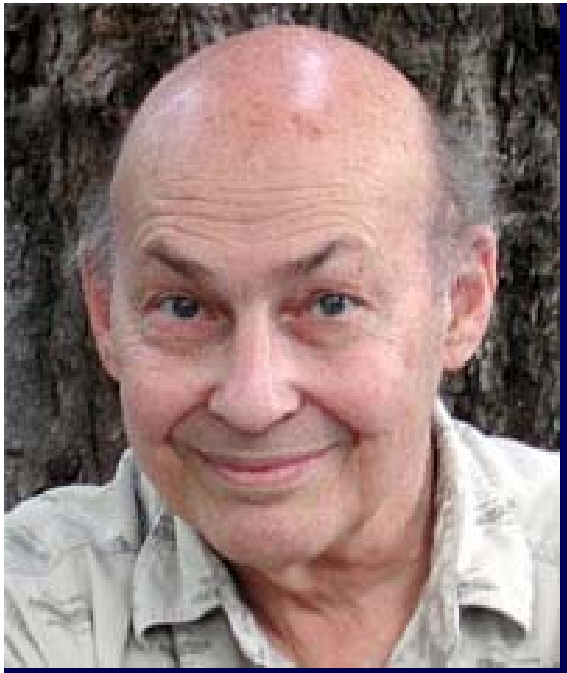


Observations en microscopie à fluorescence conventionnelle

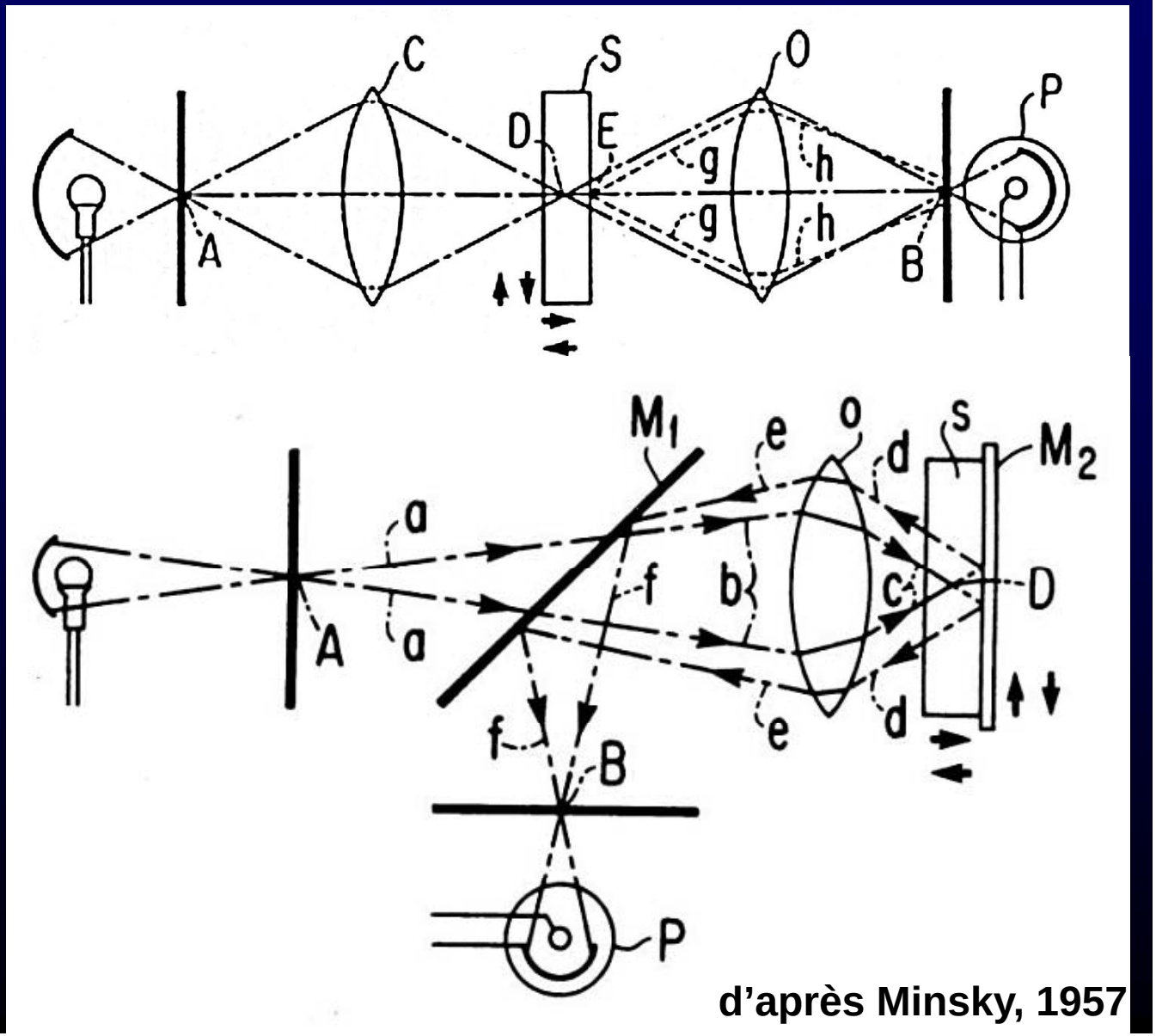




Coupes épaisses (60 à 200 µm)



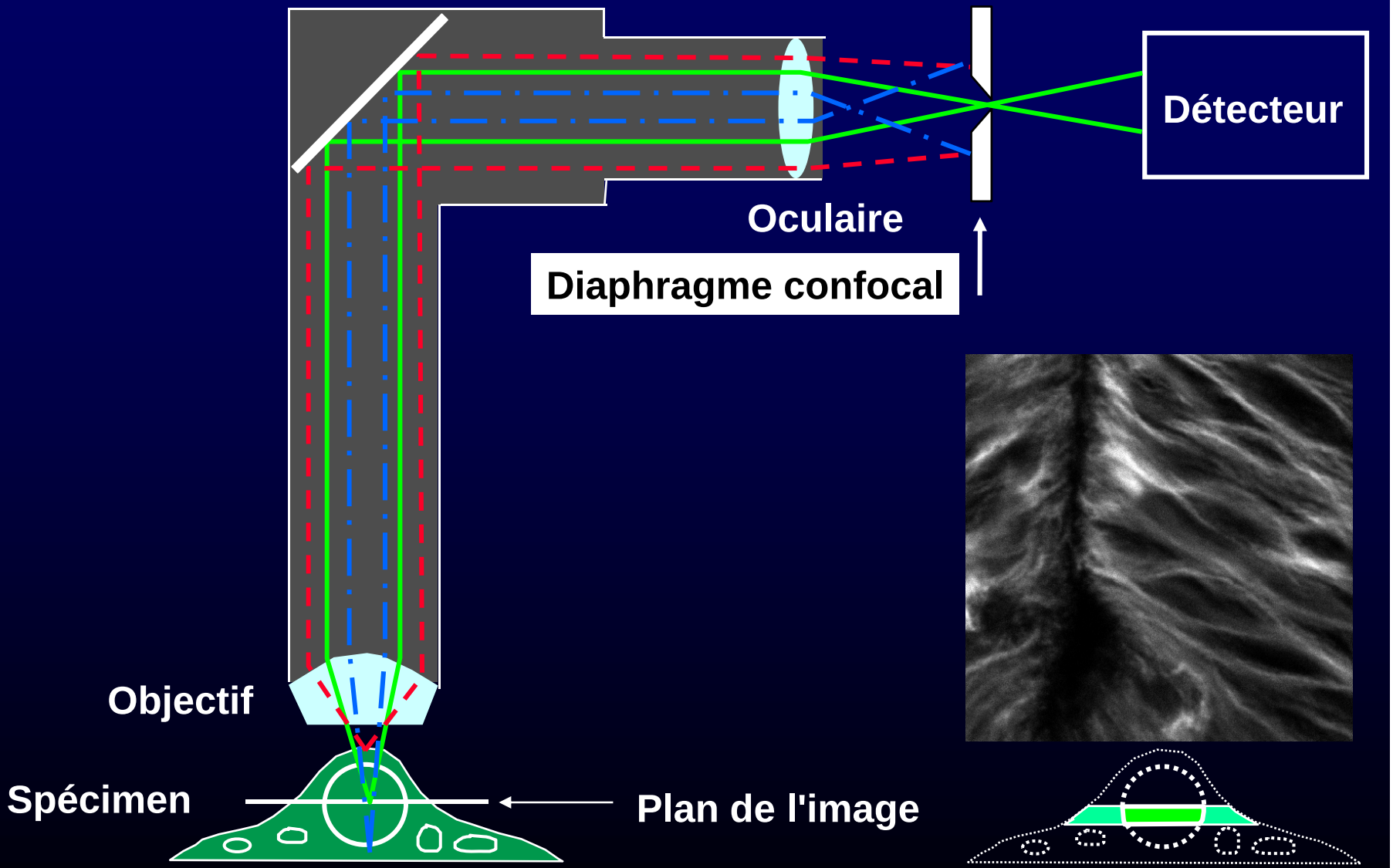
Marvin Minsky :
l‘inventeur du microscope confocal



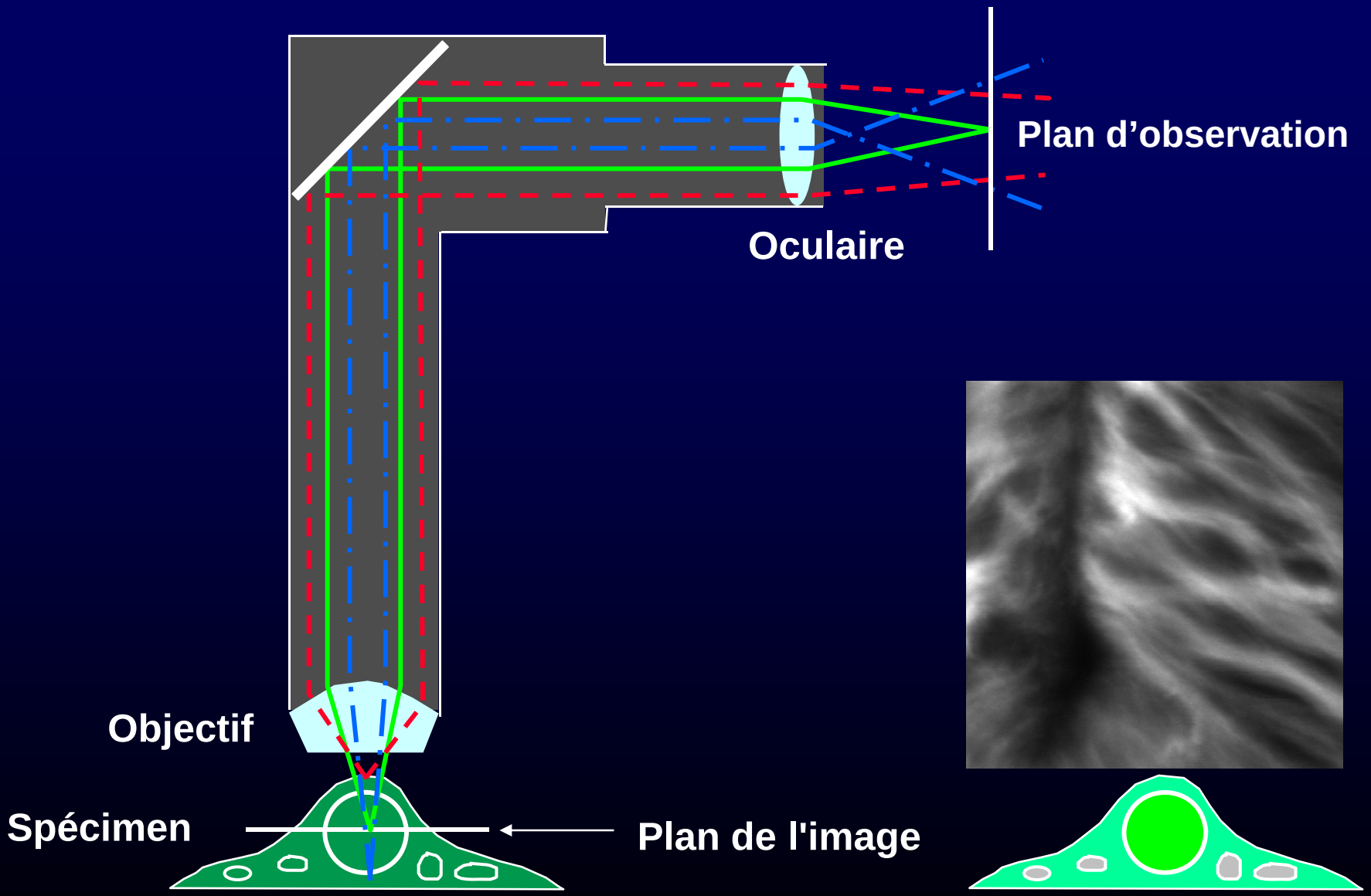
d’après Minsky, 1957


Leica SP2 (2000)

MICROSCOPIE CONFOCALE

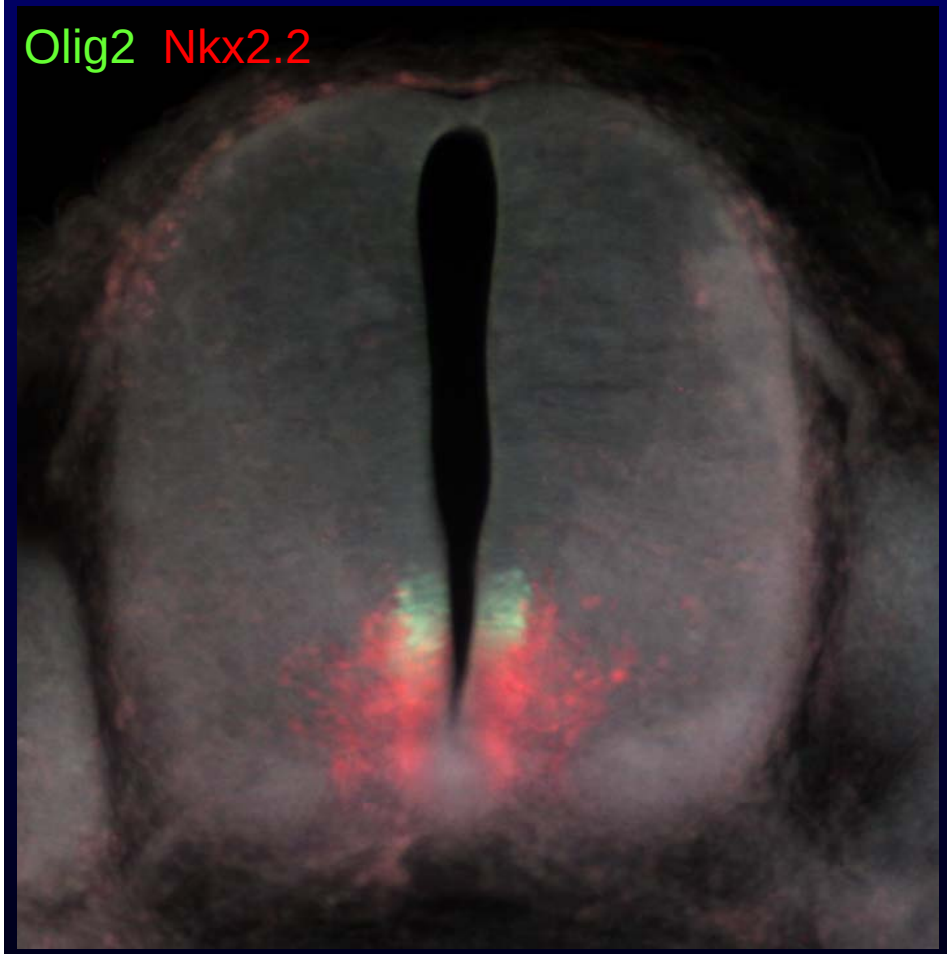


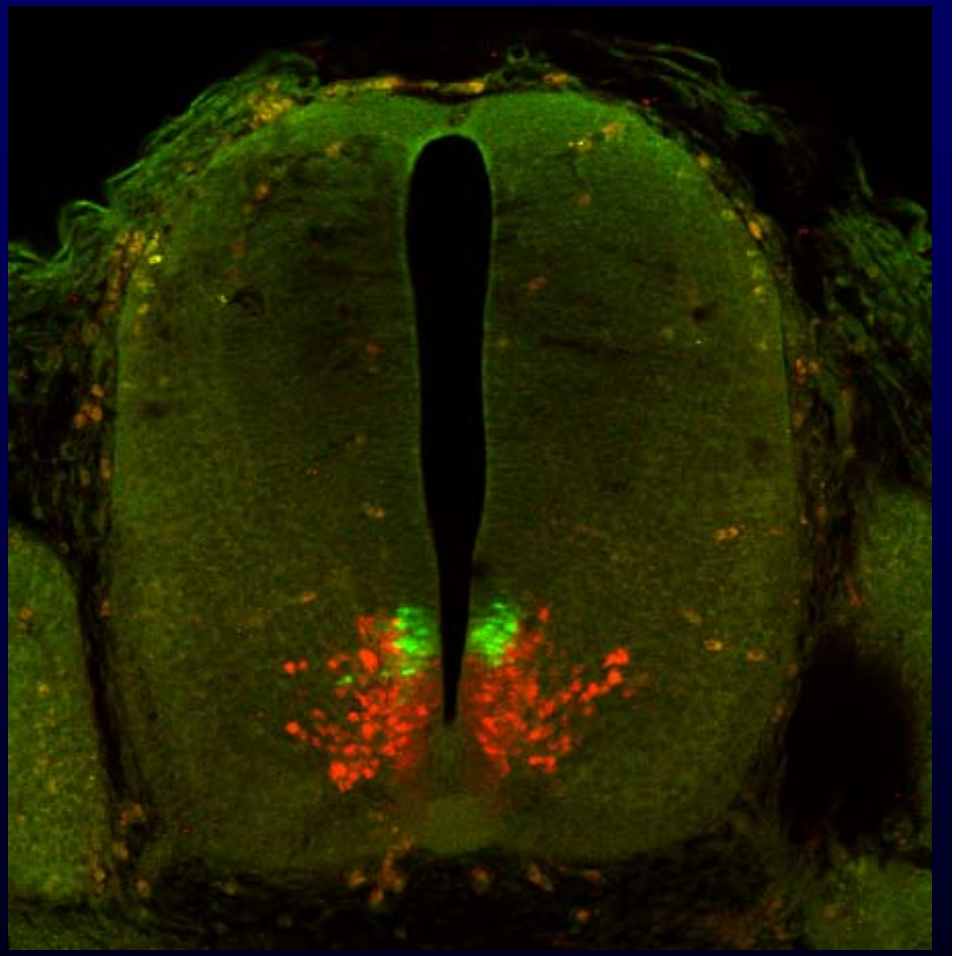
MICROSCOPIE de FLUORESCENCE CONVENTIONNELLE



Microscopie conventionnelle

Microscopie confocale

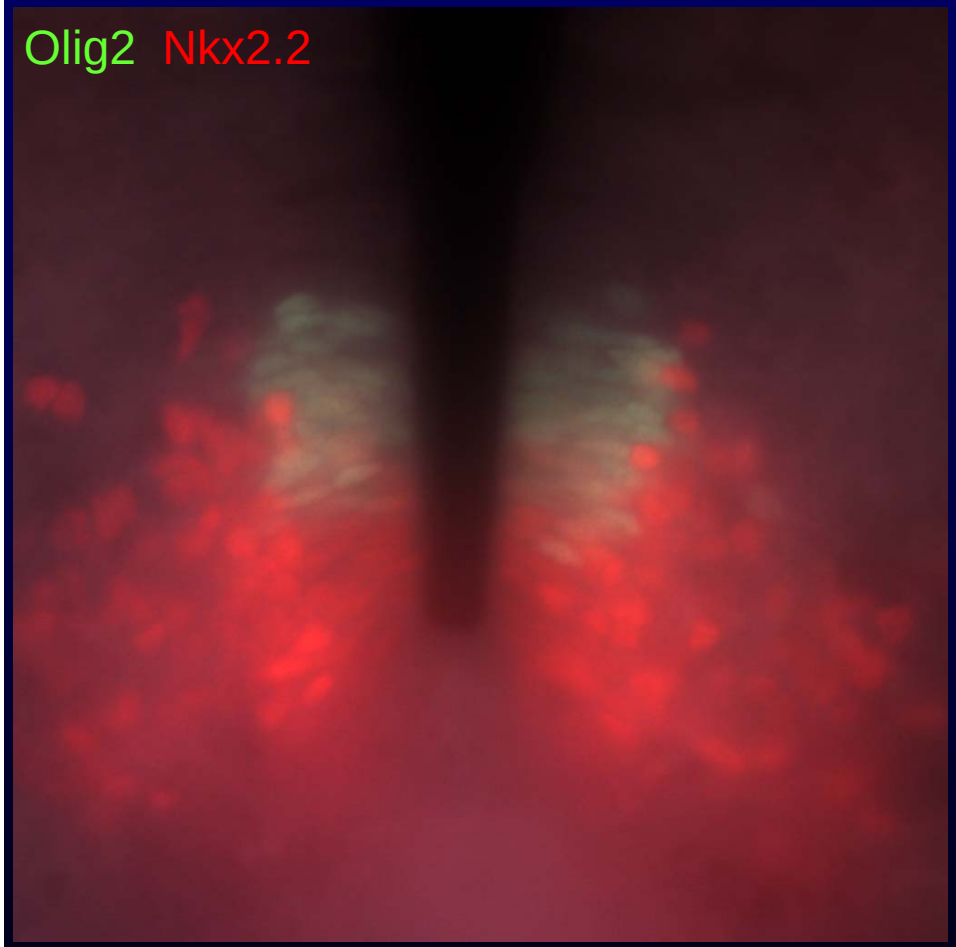


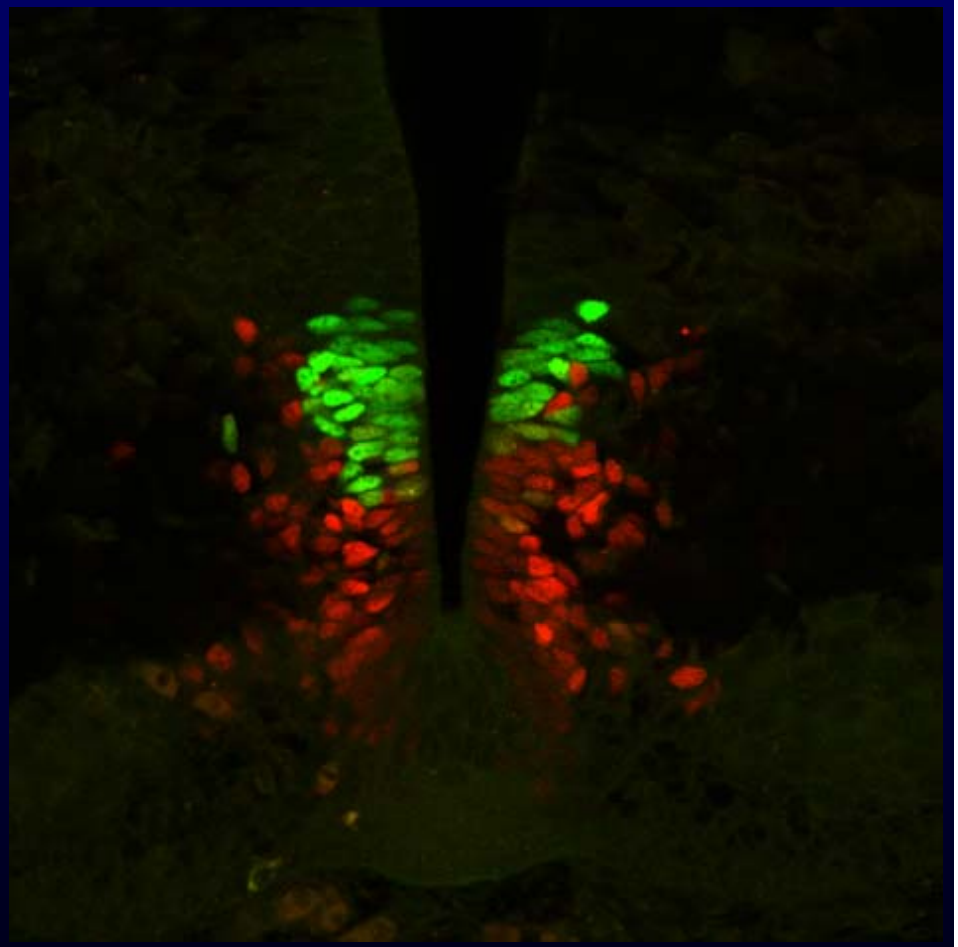


X 20

Microscopie conventionnelle

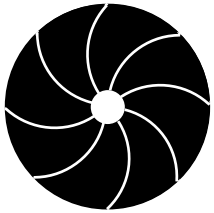
Microscopie confocale



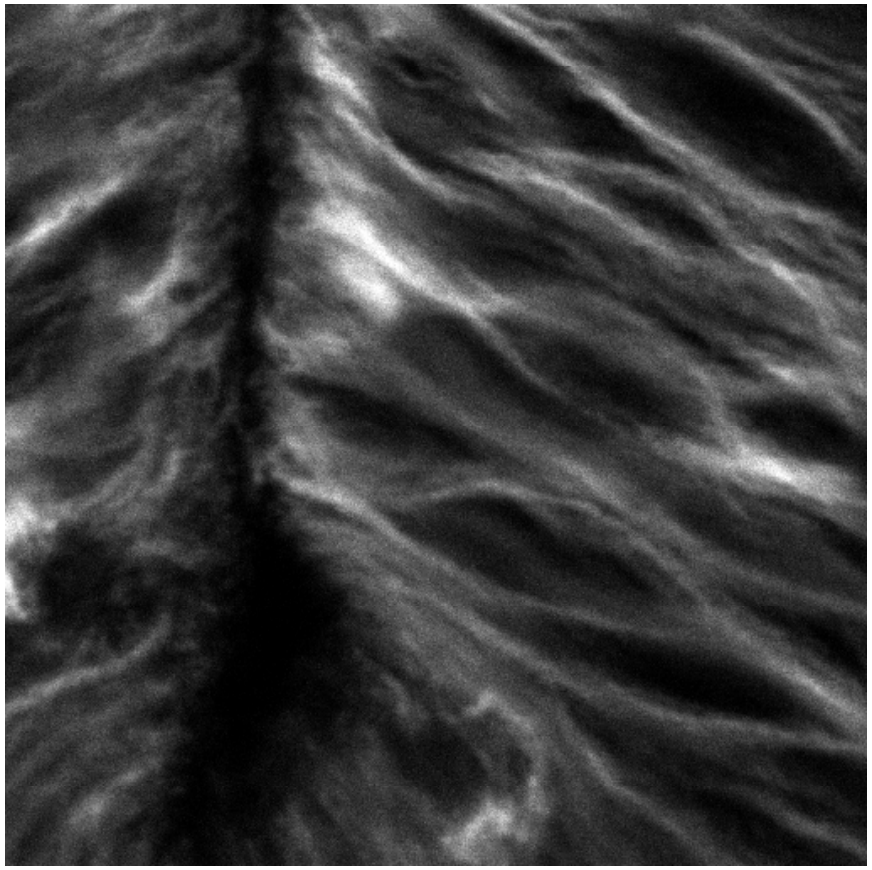


X 40

Importance du diaphragme confocal (pinhole)

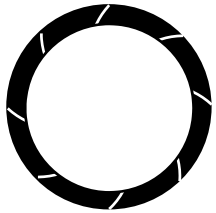


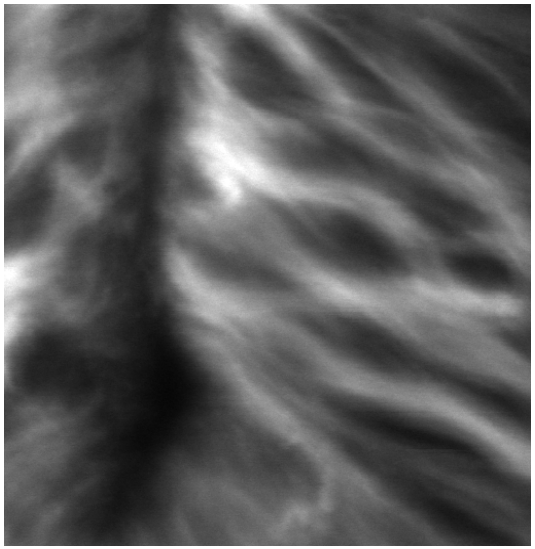
1 Airy

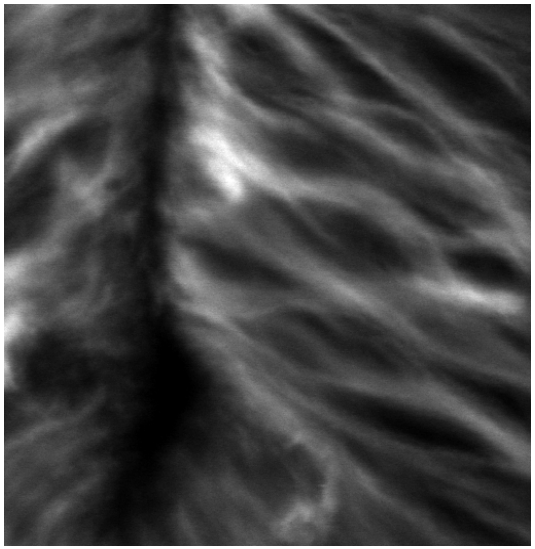


Importance du diaphragme confocal (pinhole)

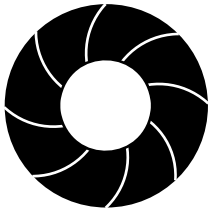
Ouvert



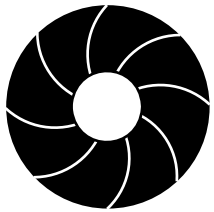


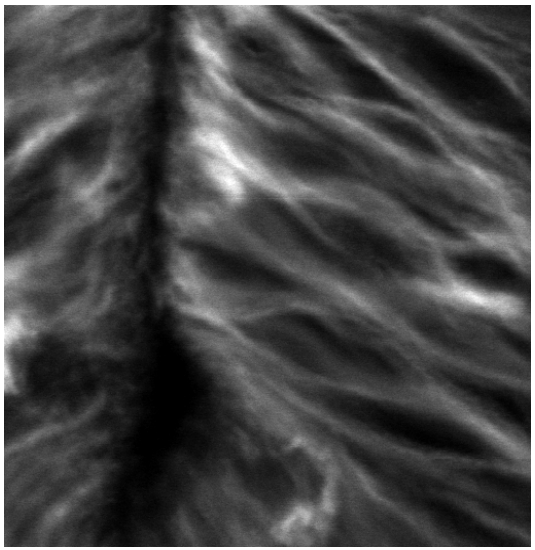


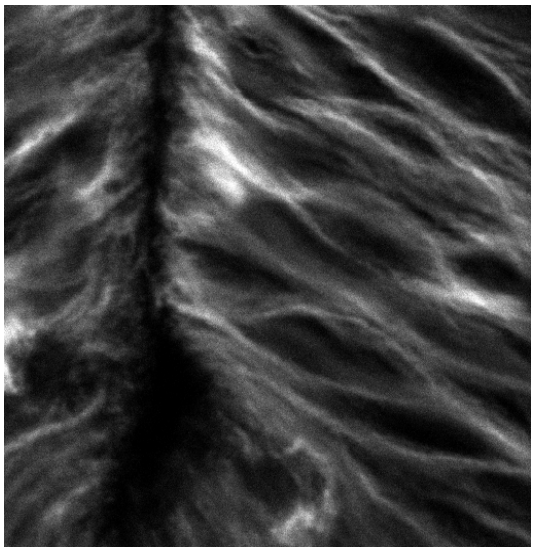
3 Airy



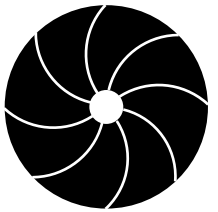
2 Airy

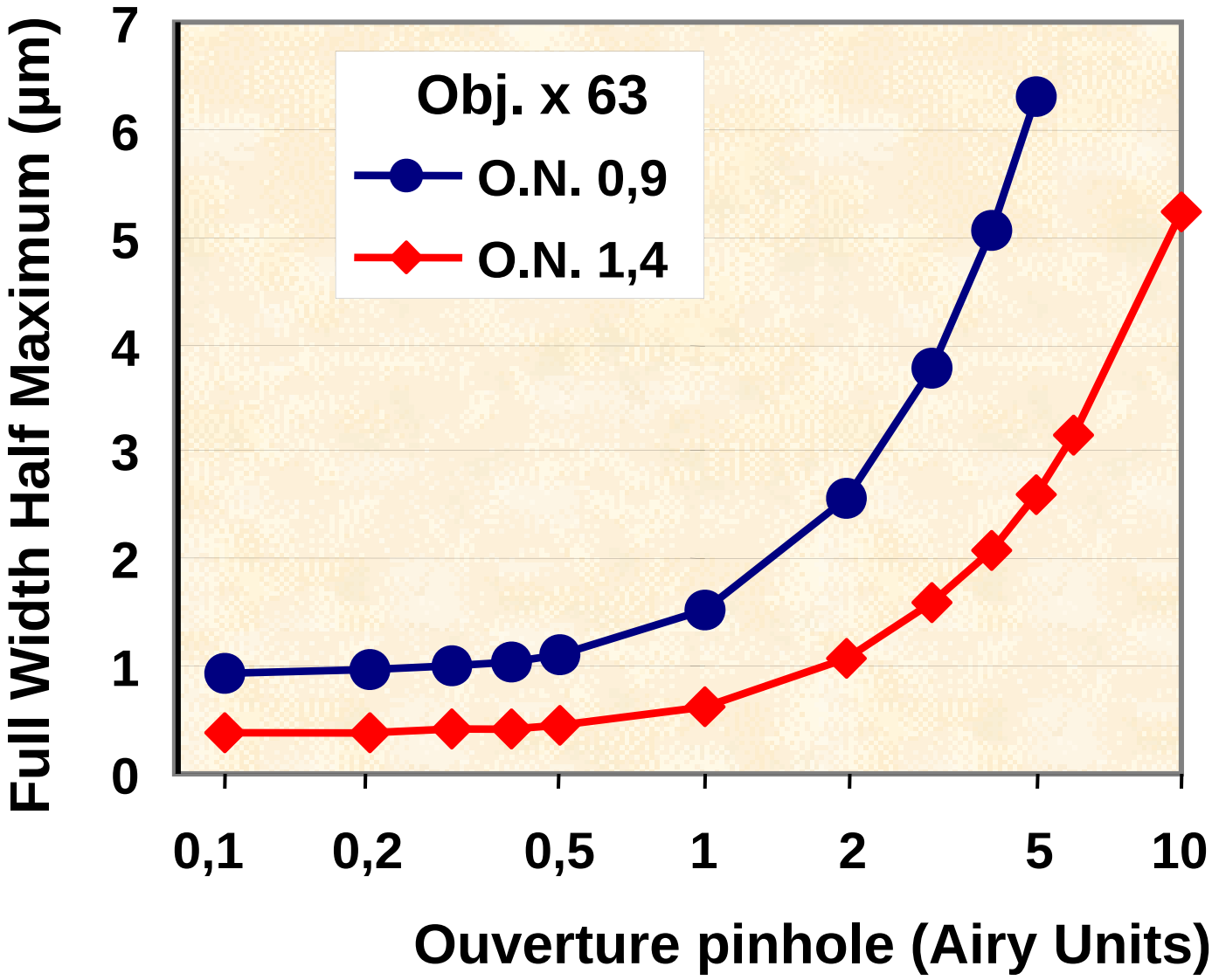






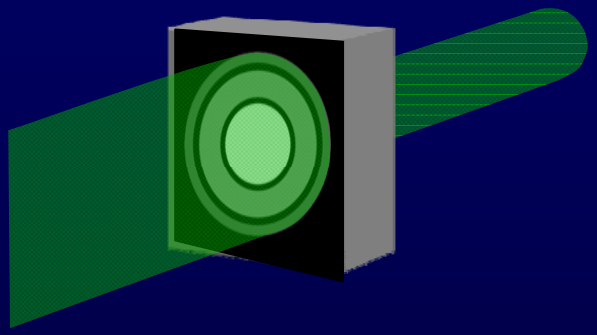
1 Airy




Full Width Half Maximum (µm)
7
6
5
4
3
2
1
0
Obj. x 63
O.N. 0,9
O.N. 1,4
0,1
0,2
0,5
1
2
5
10
Ouverture pinhole (Airy Units)

Résolution en microscopie confocale

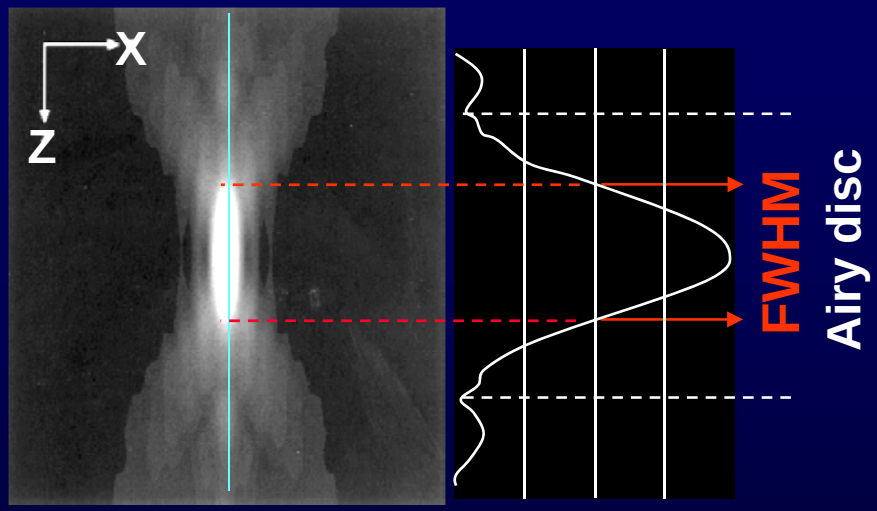
Résolution latérale :



Si le diamètre du pinhole
= taille du disque d'Airy

$$\delta_{xy} = \frac{1}{\sqrt{2}} \cdot \frac{0{,}61\lambda}{ON} = 0{,}16\ \mu m$$

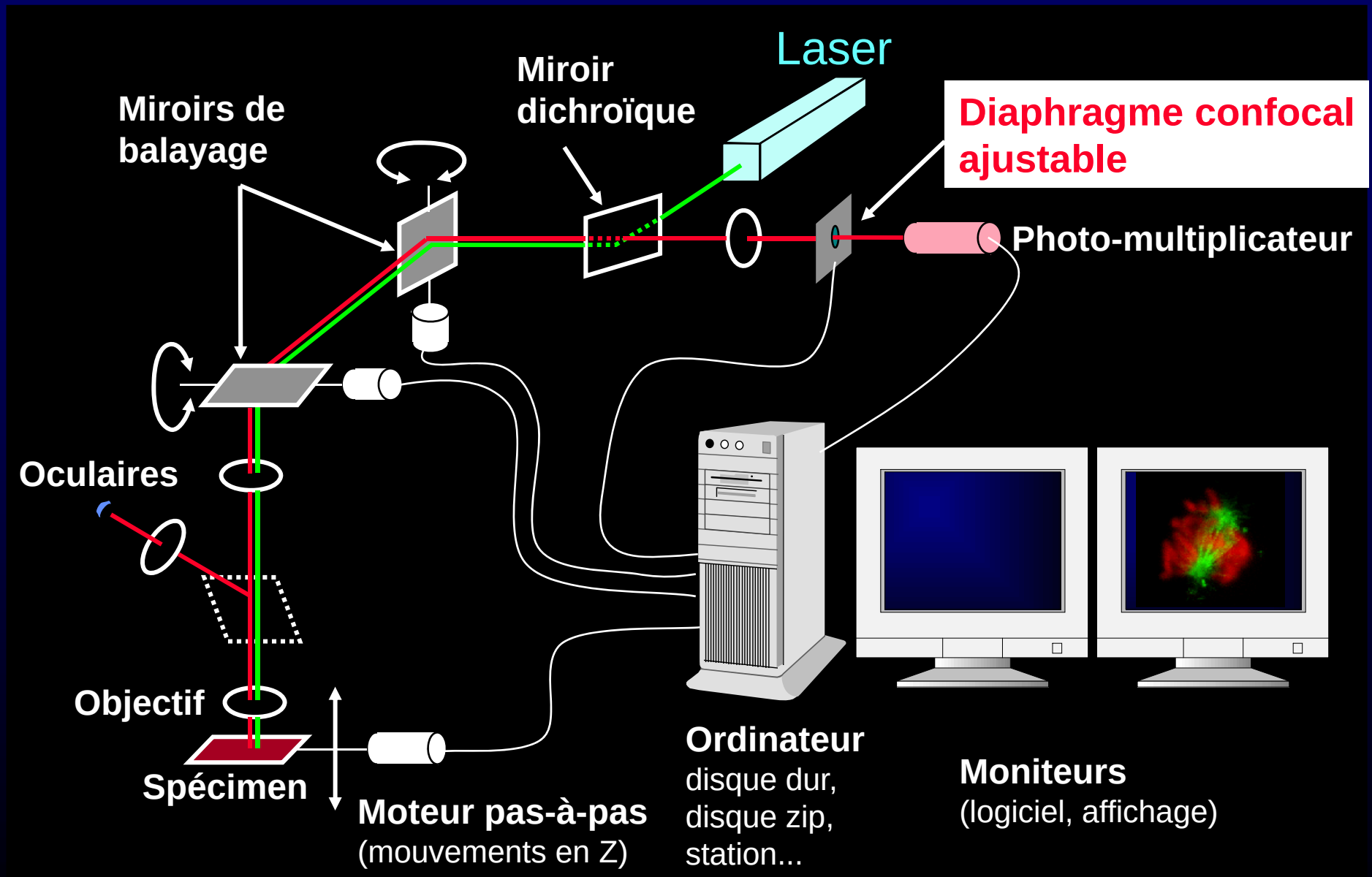
Résolution axiale :



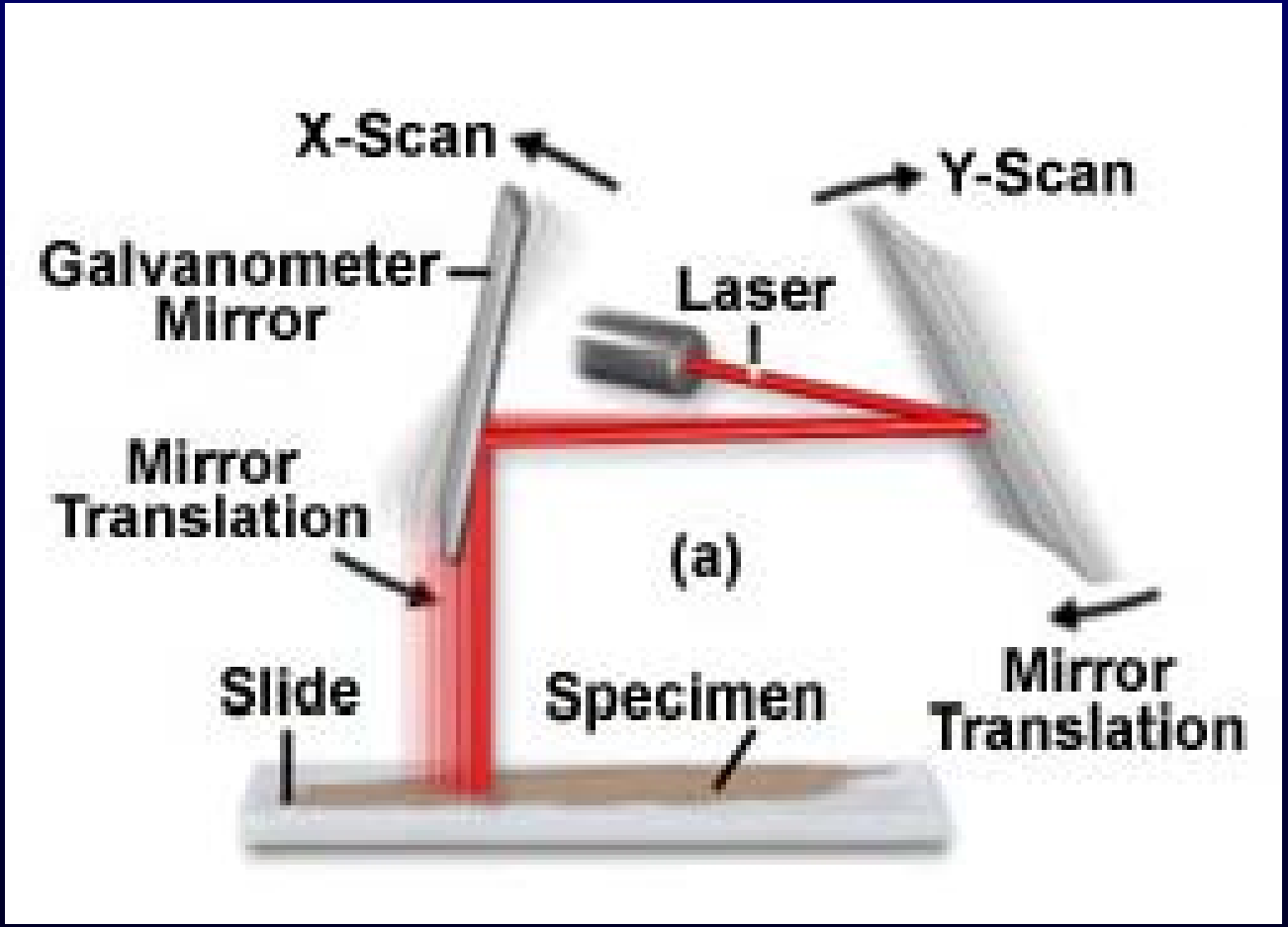
δ_z = FWHM
= Full width at half maximum

$$\delta_z = \frac{1{,}4.n.\lambda}{ON^2} = 0{,}4 \text{ à } 0{,}6\ \mu m$$

Fonctionnement du microscope confocal



Balayage laser conventionnel point par point



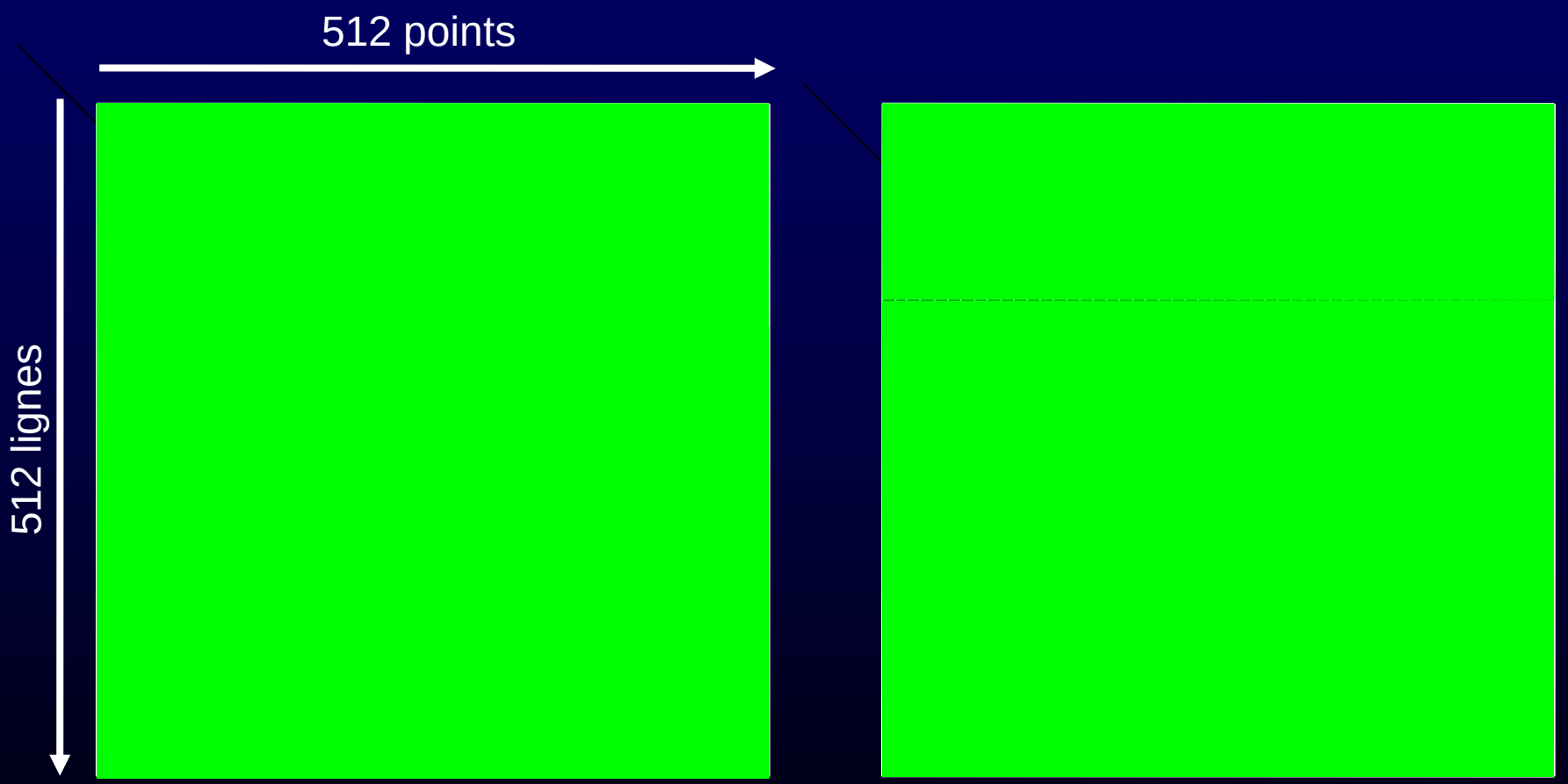
Limite : acquisition lente (1 à 2 images/sec)

Exception : Leica *resonant scanner*, jusqu'à 25 images/sec

Balayage laser conventionnel point par point

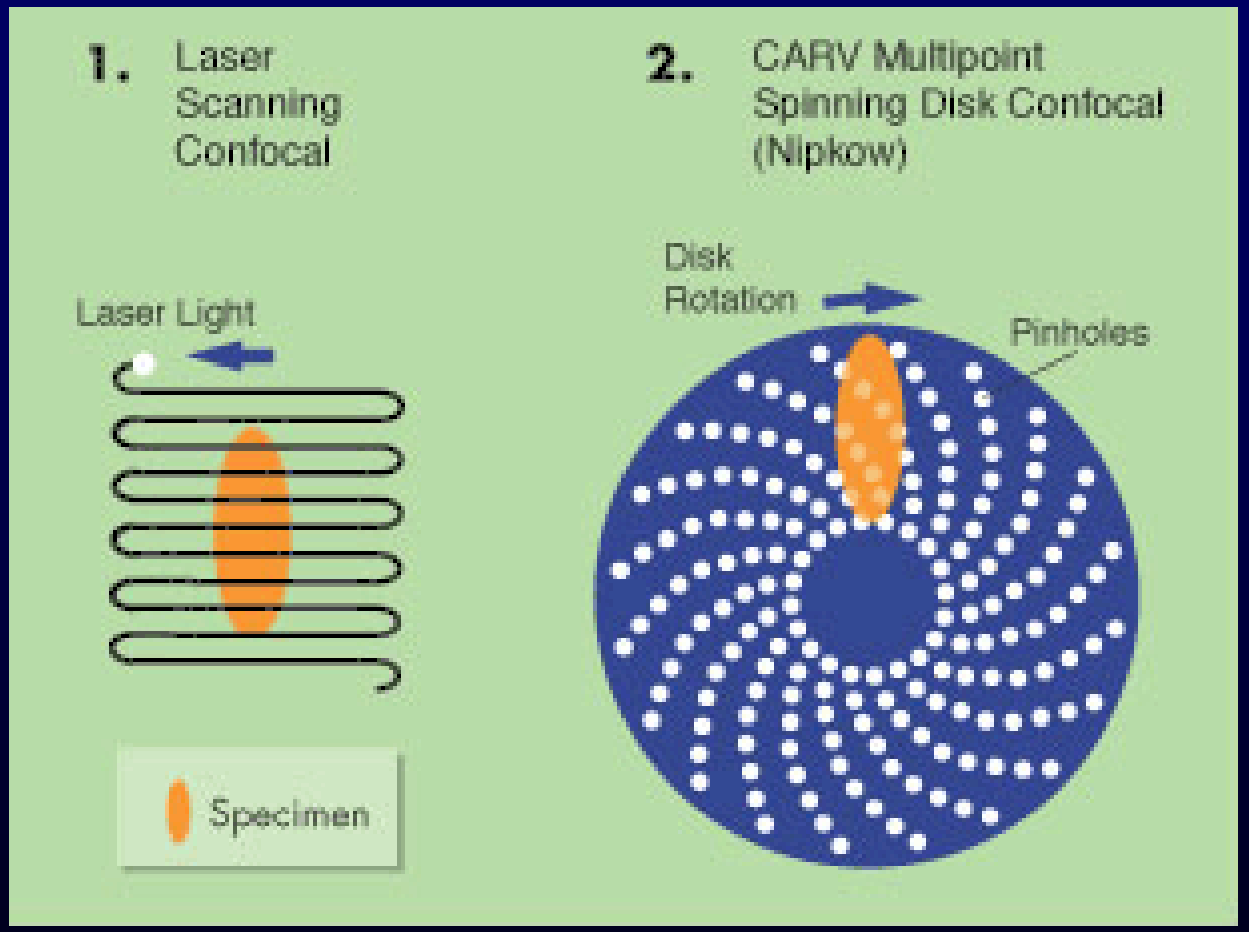
Mono-directionnel

Bi-directionnel



Deux méthodes différentes :

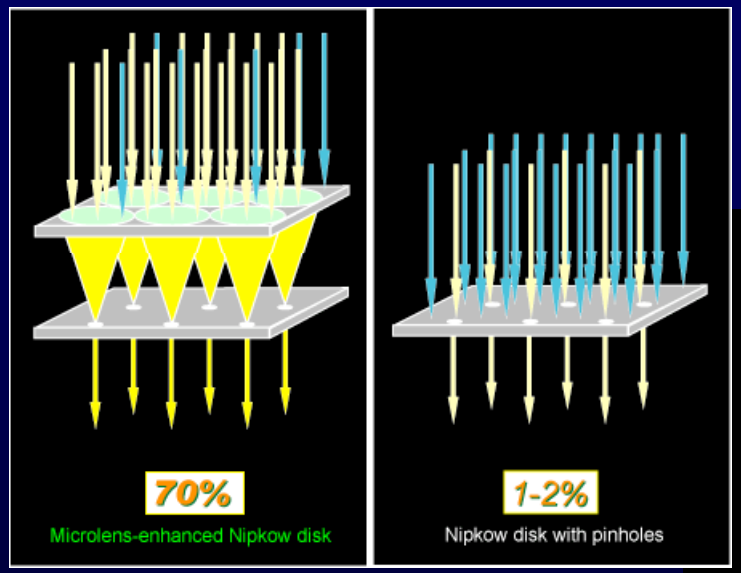
1. Balayage mono-point **2. Eclairage multipoints**

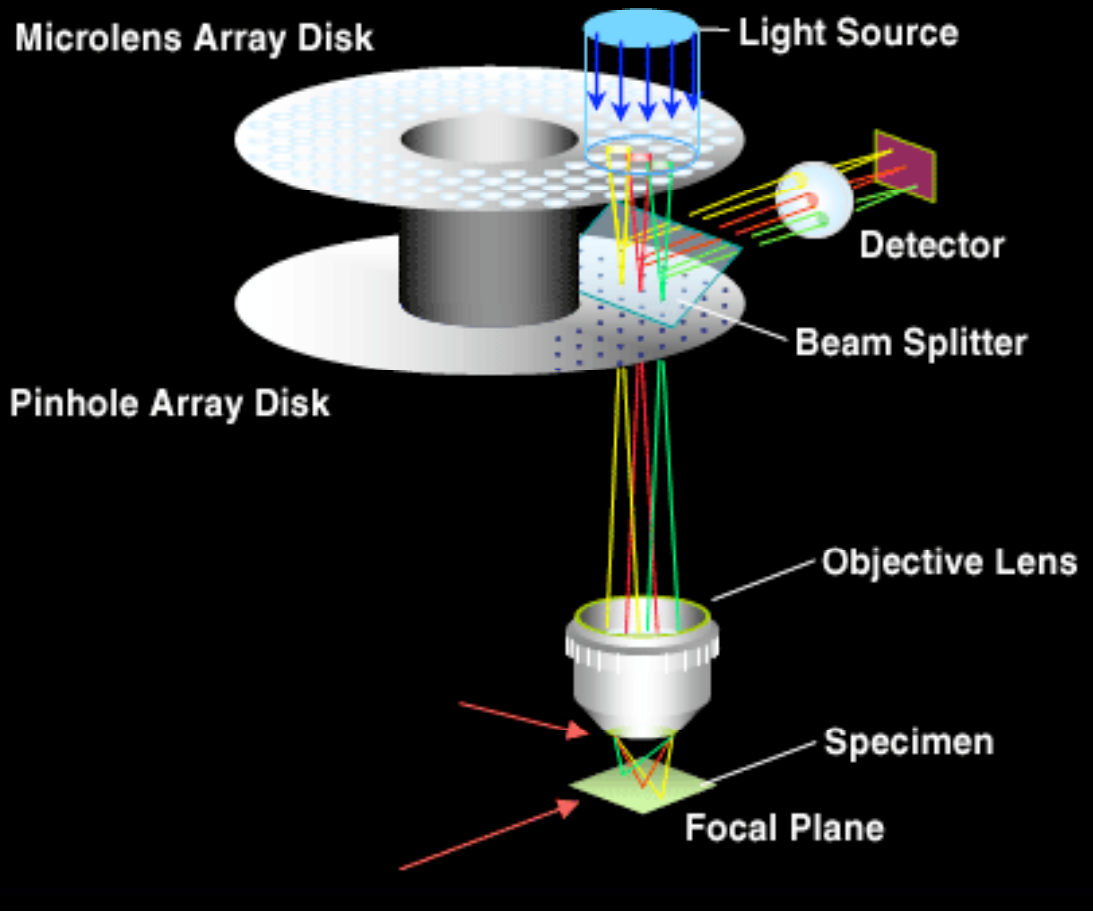


Balayage lent :
1 à 2 images /s

Balayage rapide : 80 images/s
Limite : taille du pinhole fixe

Confocal rapide à « spinning disk »



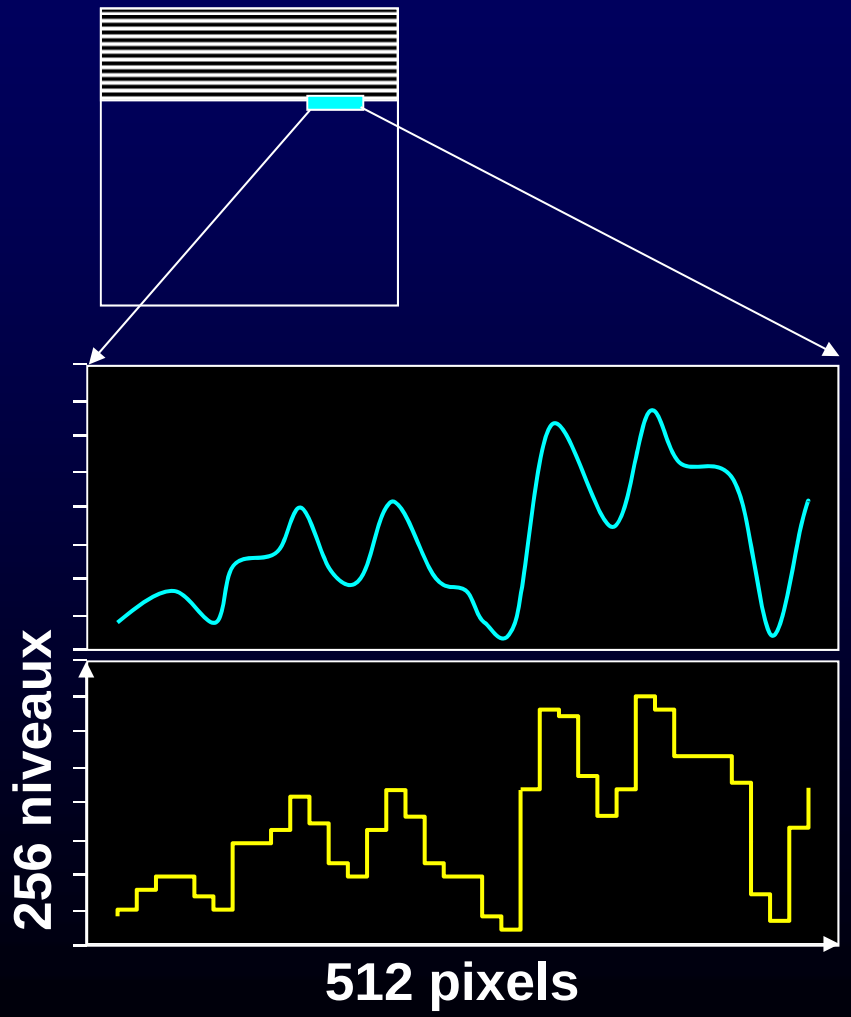


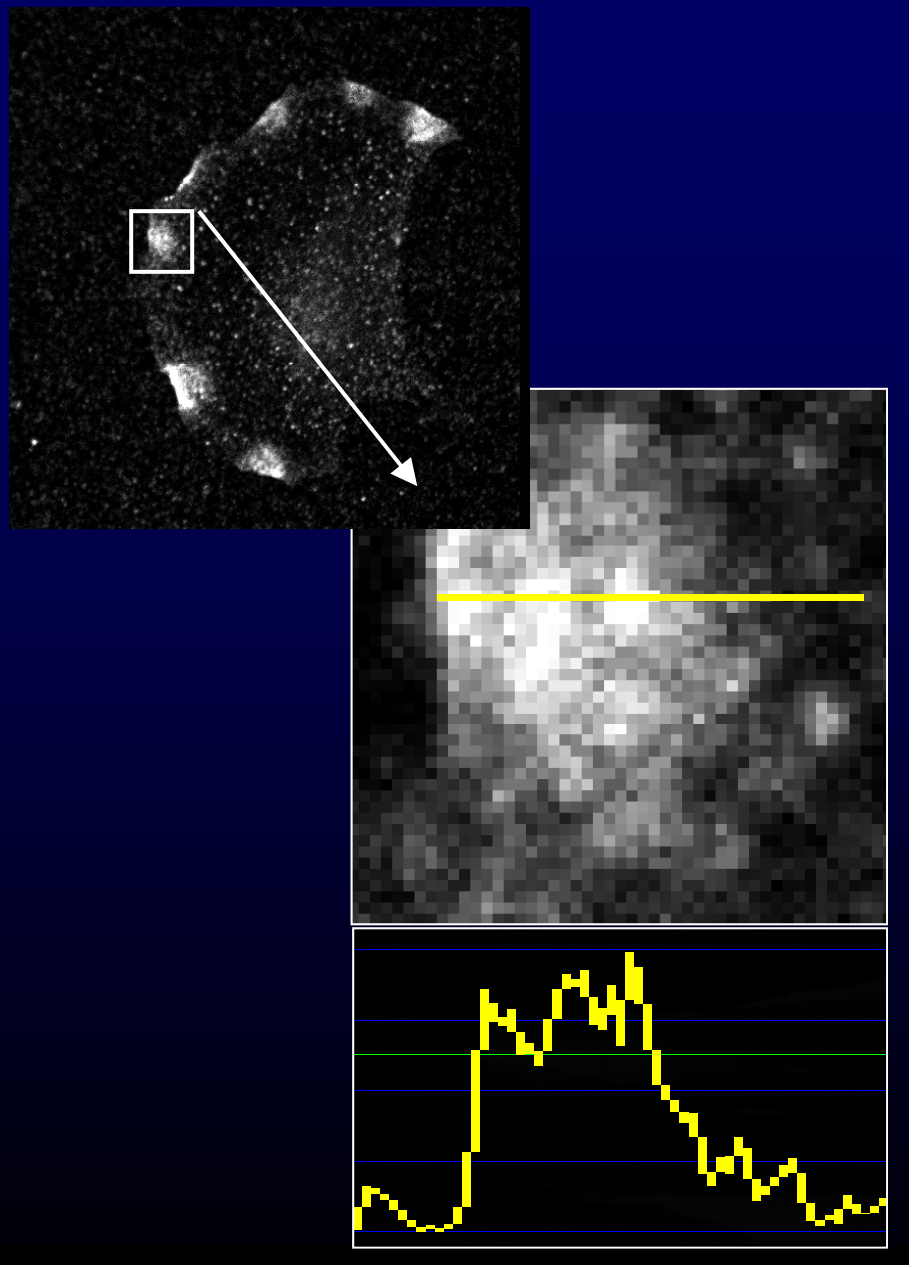
La microscopie confocale

1. Introduction : principes de fonctionnement
2. **Numérisation – reconstructions 3D**
3. Lasers – Multimarquages
4. Avantages et limites

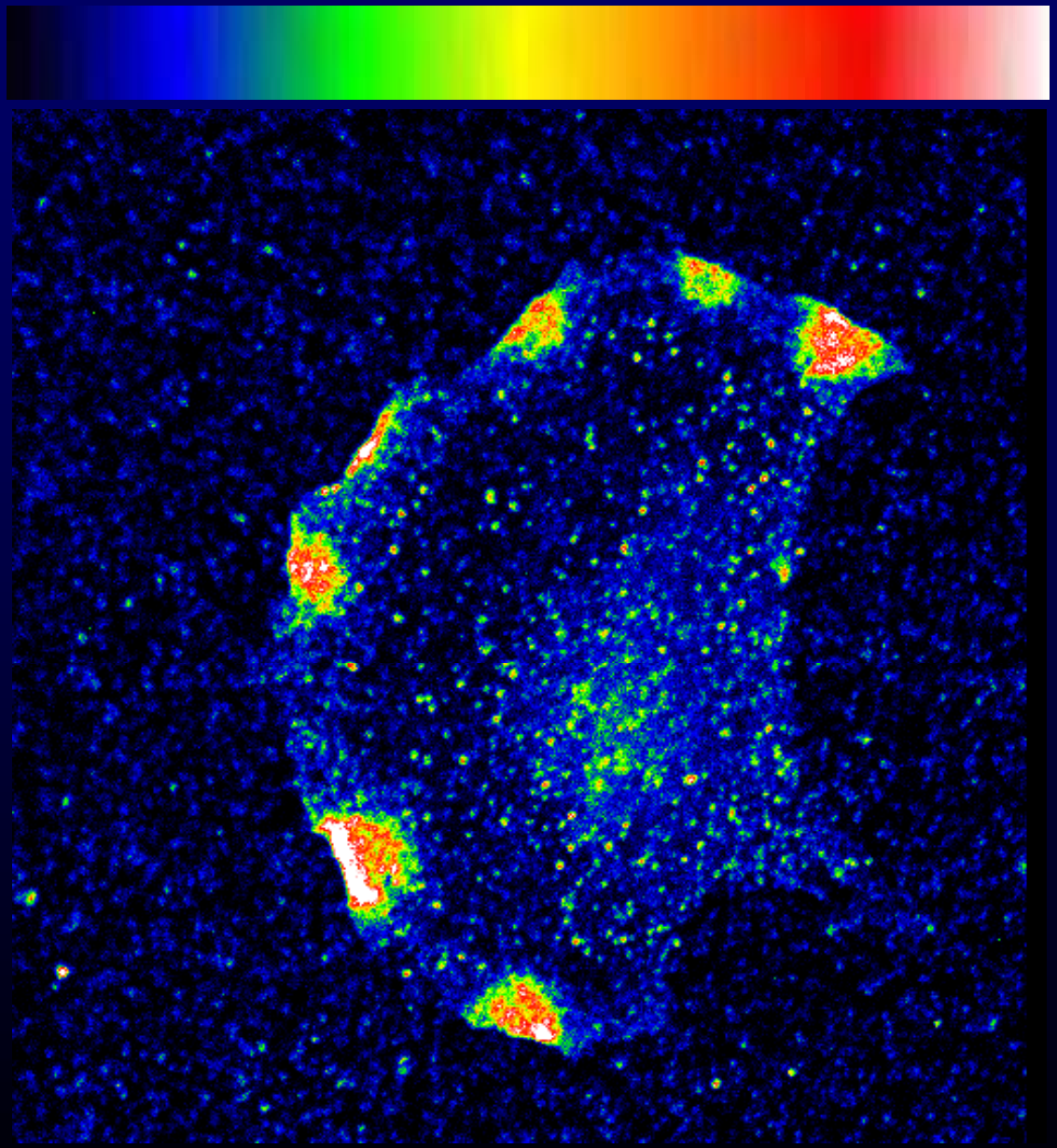
Numérisation des images

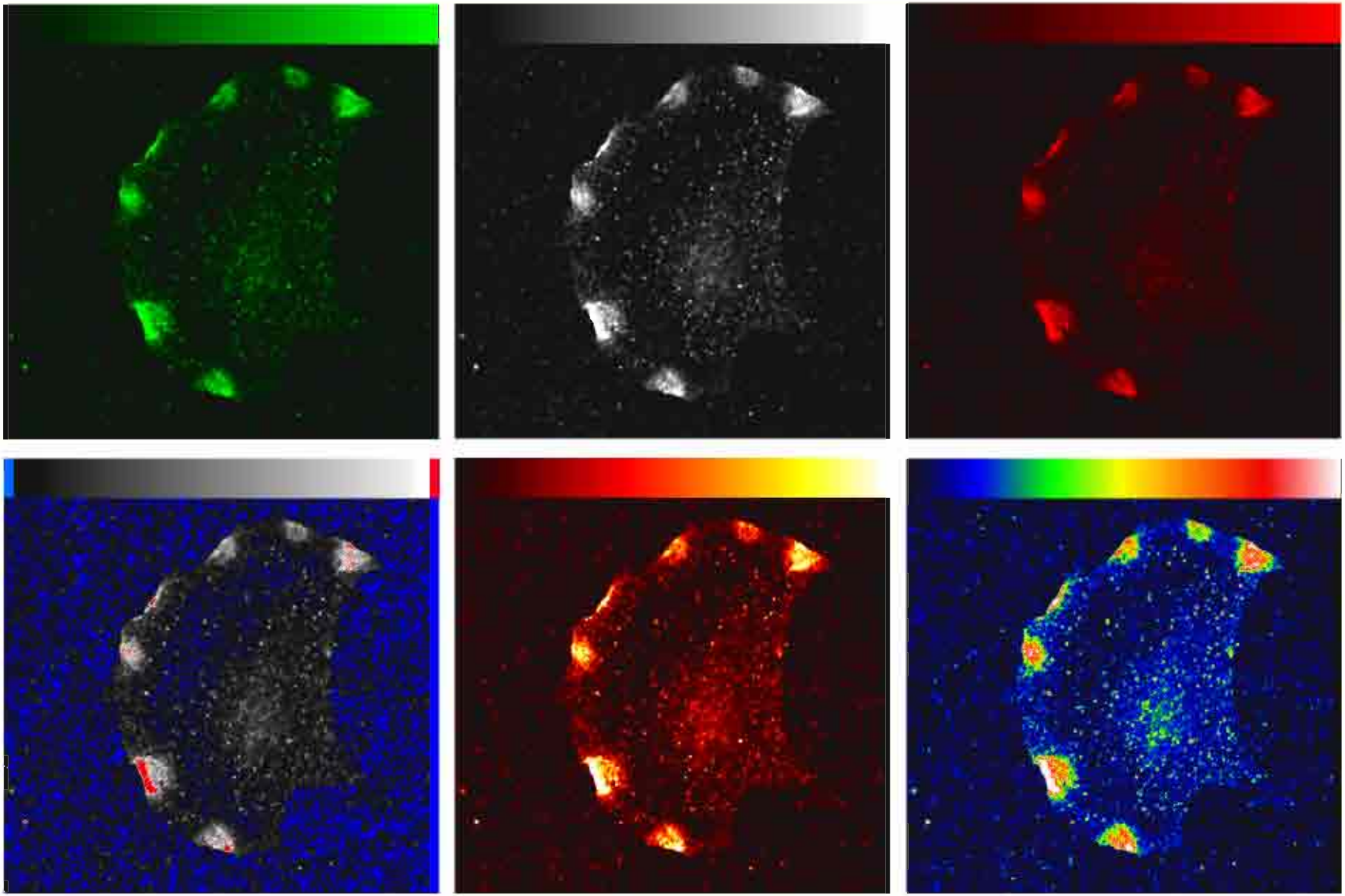
1 - échantillonnage (ex. 512 x 512)
2 - quantification (en 8 bits = 2^8 = 256 niveaux de gris)

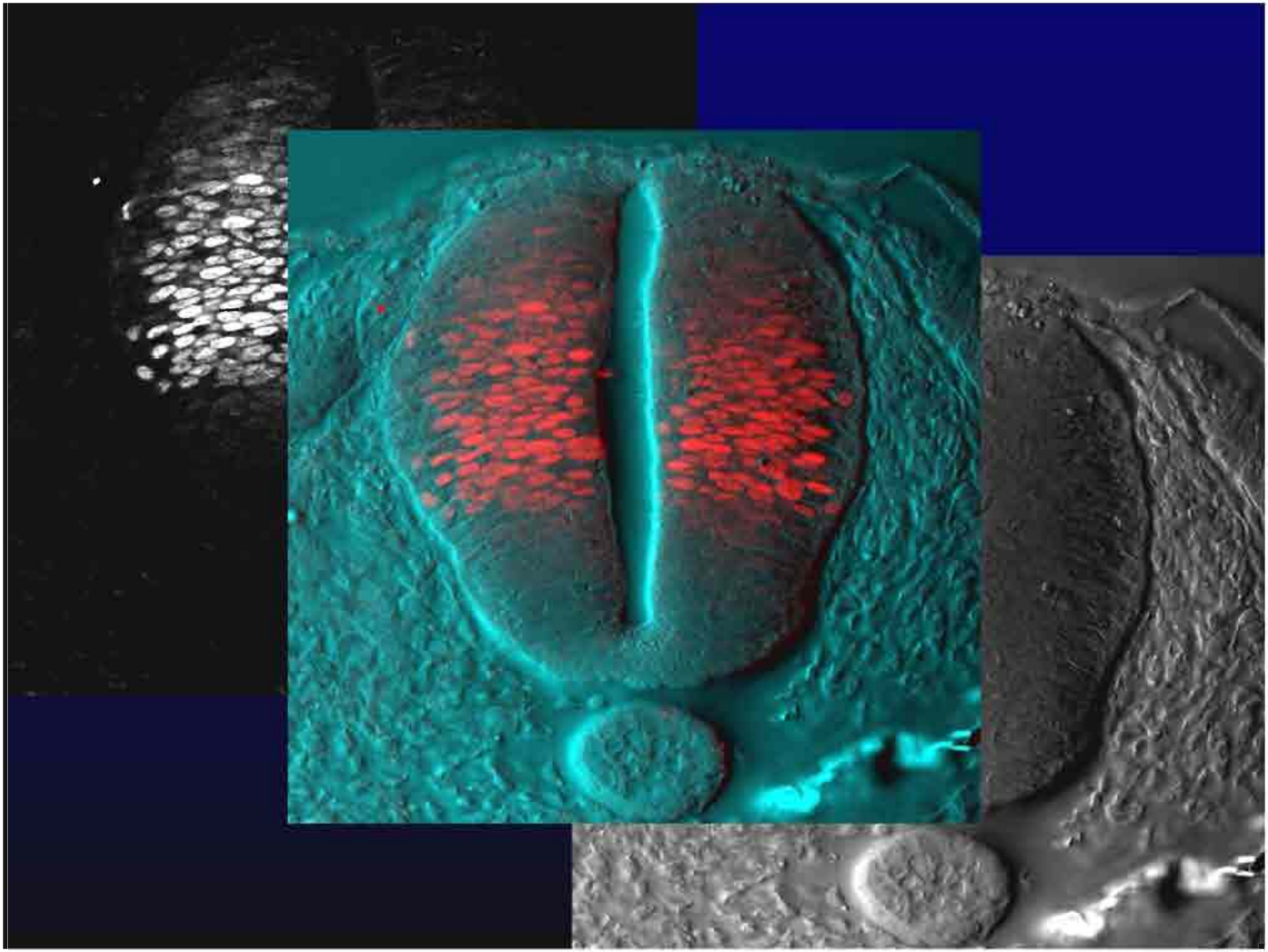


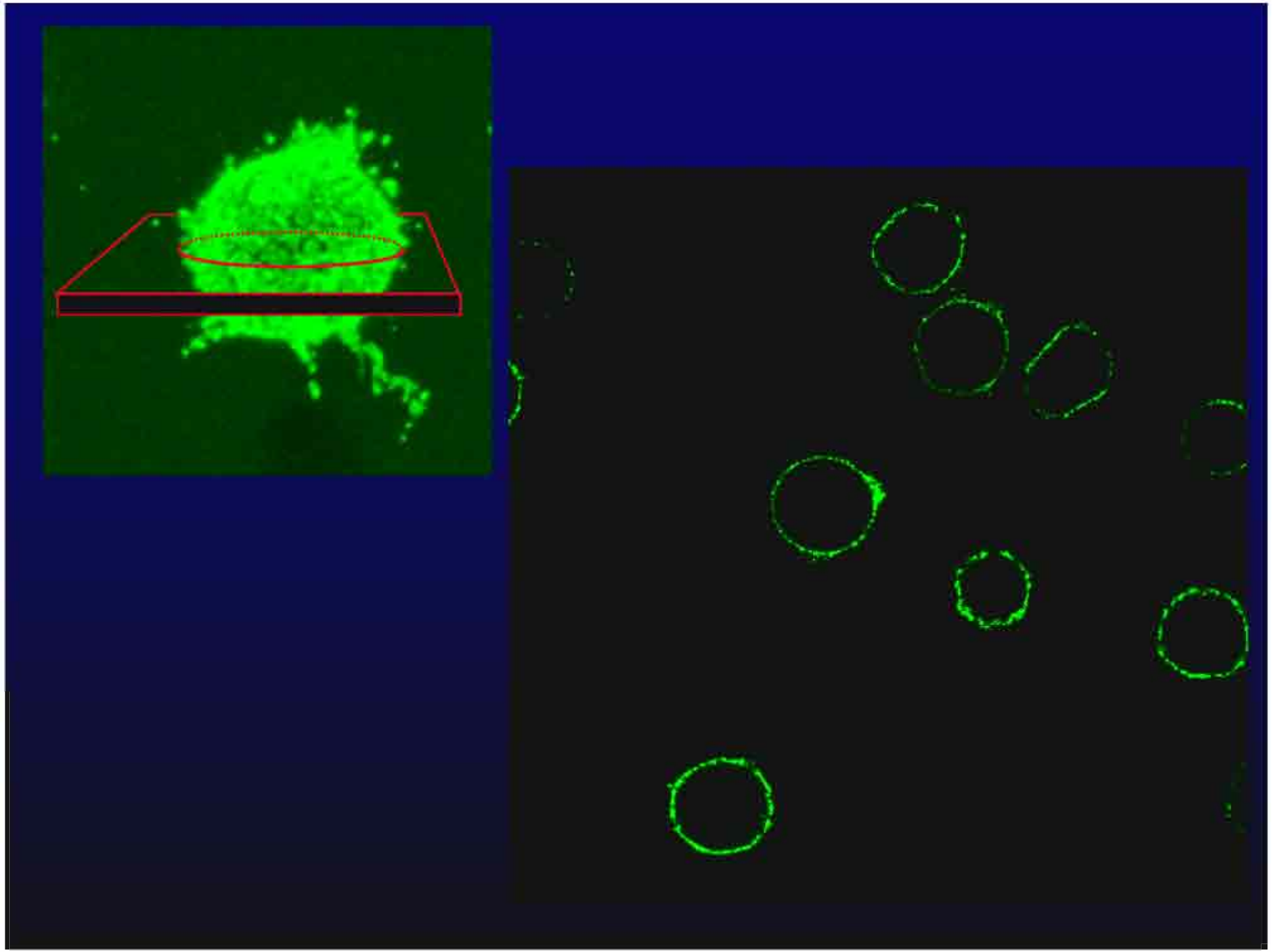


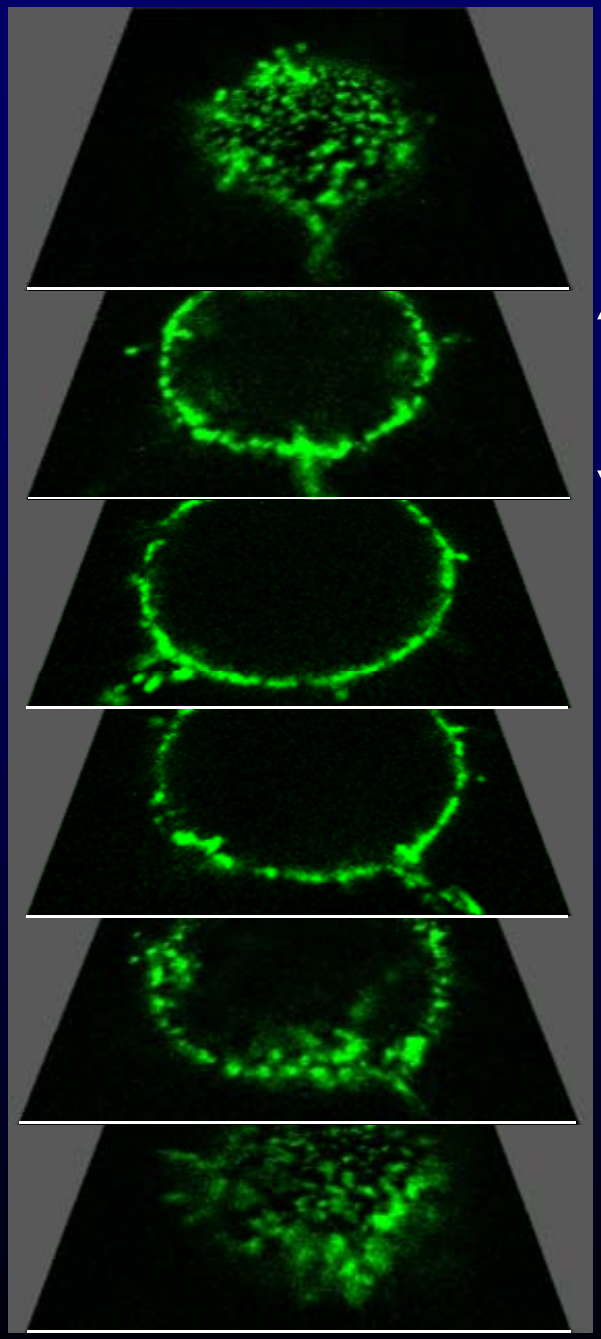
Pseudo-couleurs : Look-Up Tables (LUT)











Pas de
déplacement
en Z


0
0.25μm
0.5μm
0.75μm
1μm
1.25μm
1.5μm
1.75μm
2μm
2.25μm
2.5μm
2.75μm
3μm
3.25μm
3.5μm
3.75μm
4μm
4.25μm
4.5μm
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5μm
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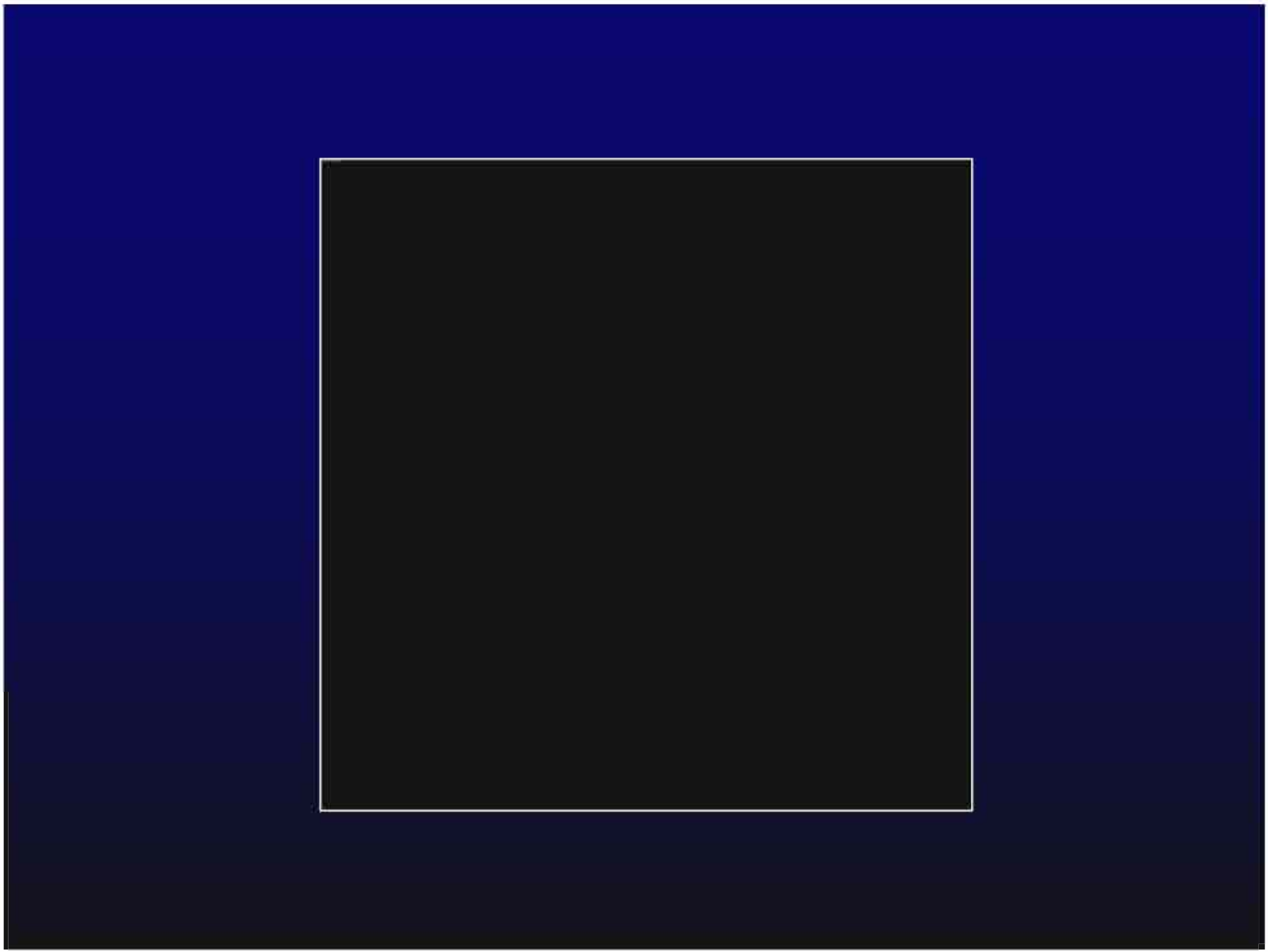
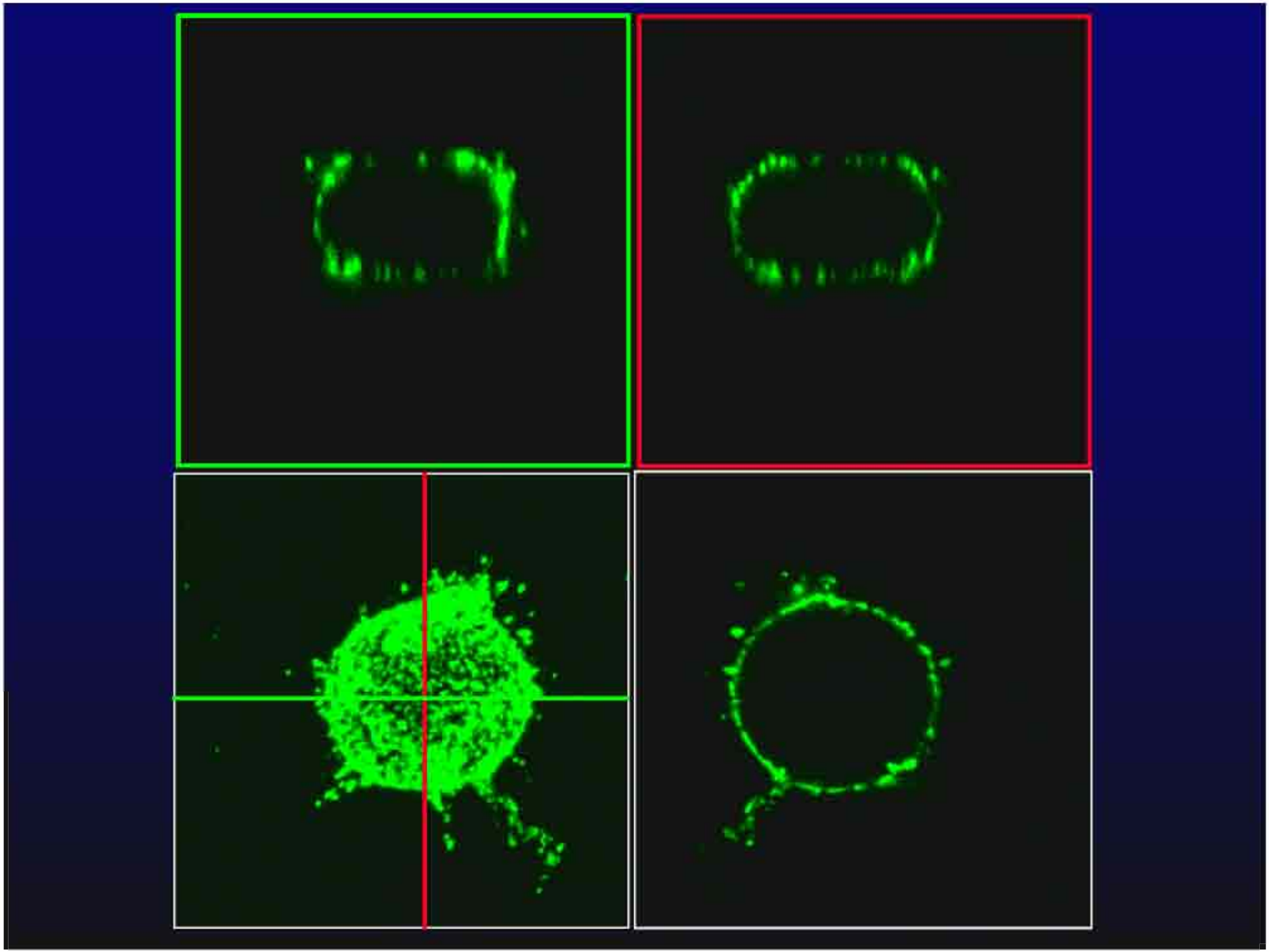
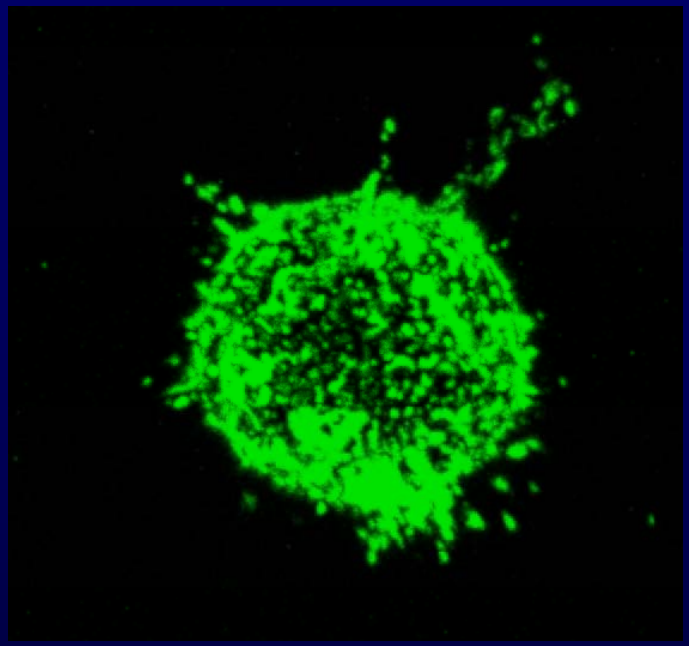
14.75μm
15μm
15.25μm
15.5μm
15.75μm

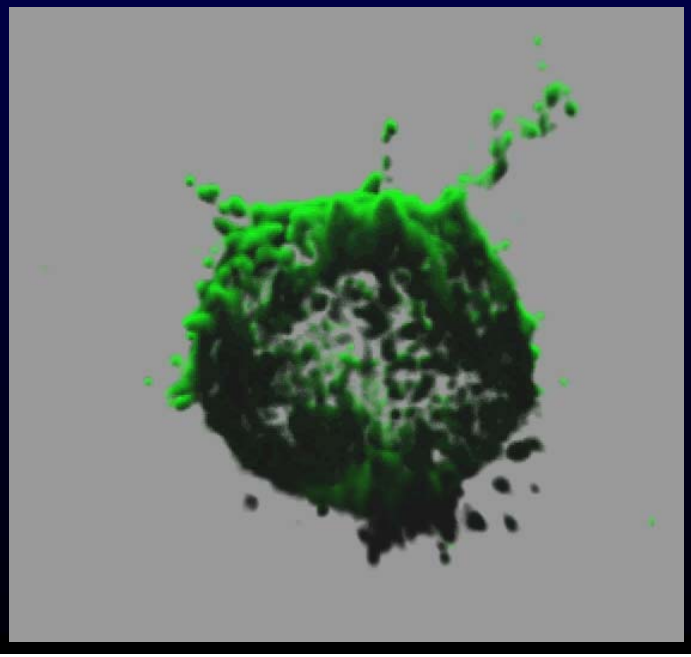




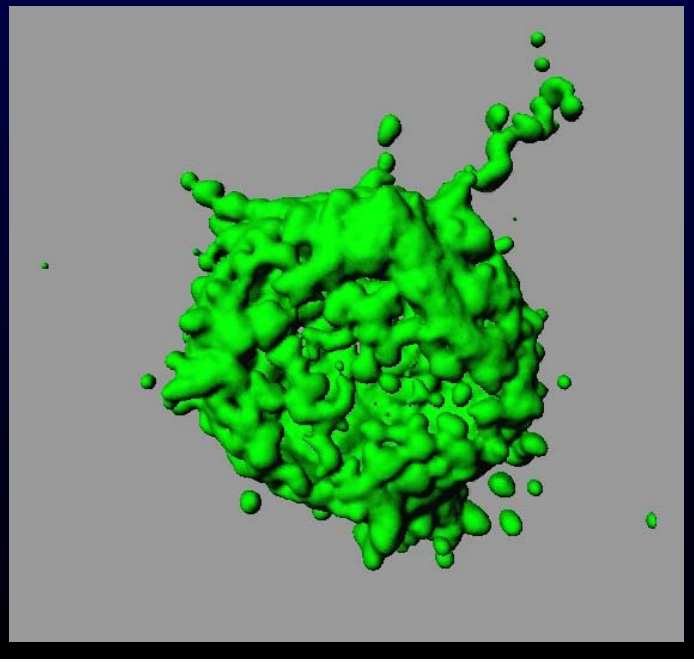
Projection maximum

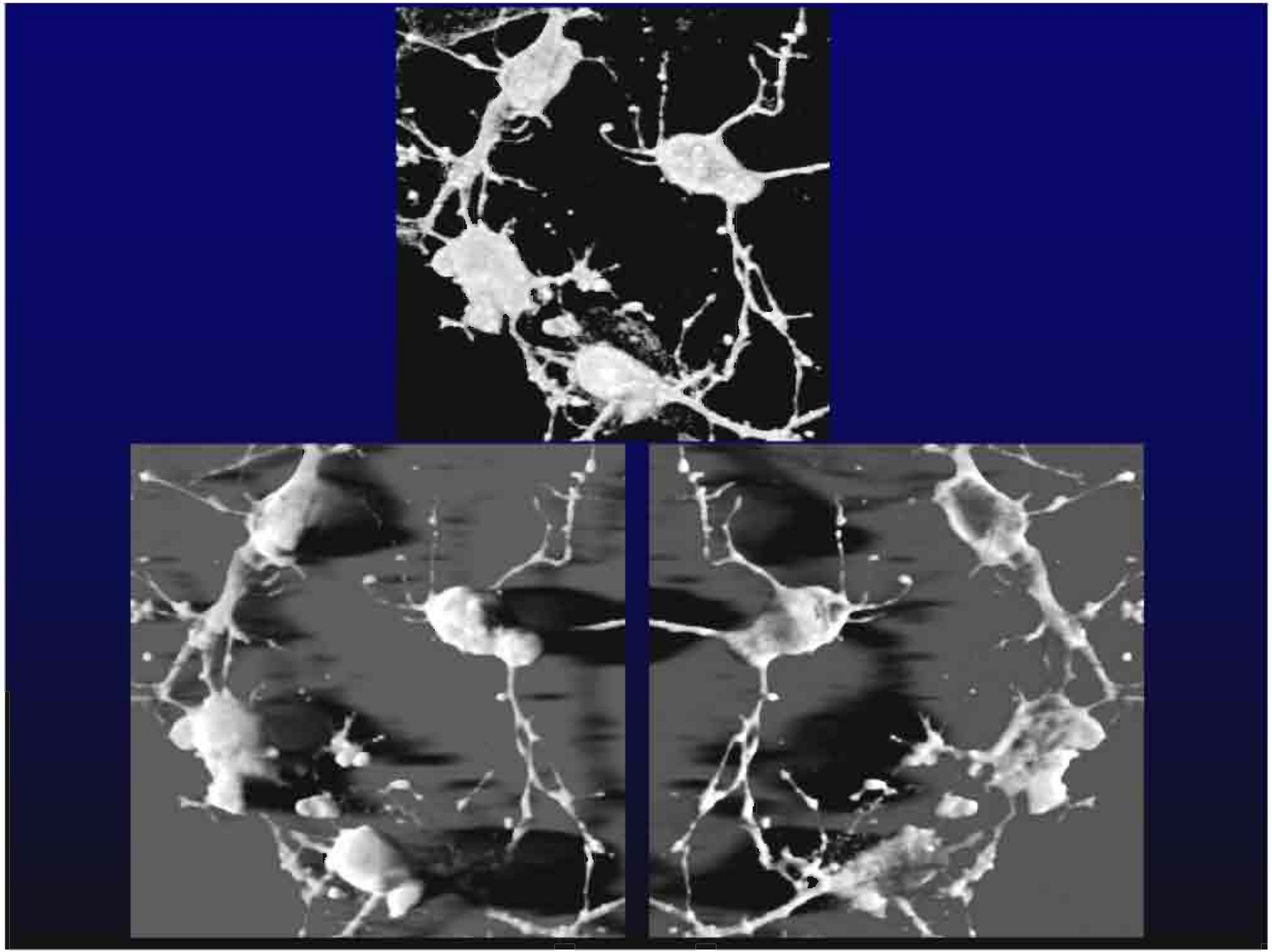


Rendu de volume



Iso-surfaces





La microscopie confocale

1. Introduction : principes de fonctionnement
2. Numérisation – reconstructions 3D
3. **Lasers – Multimarquages**
4. Avantages et limites

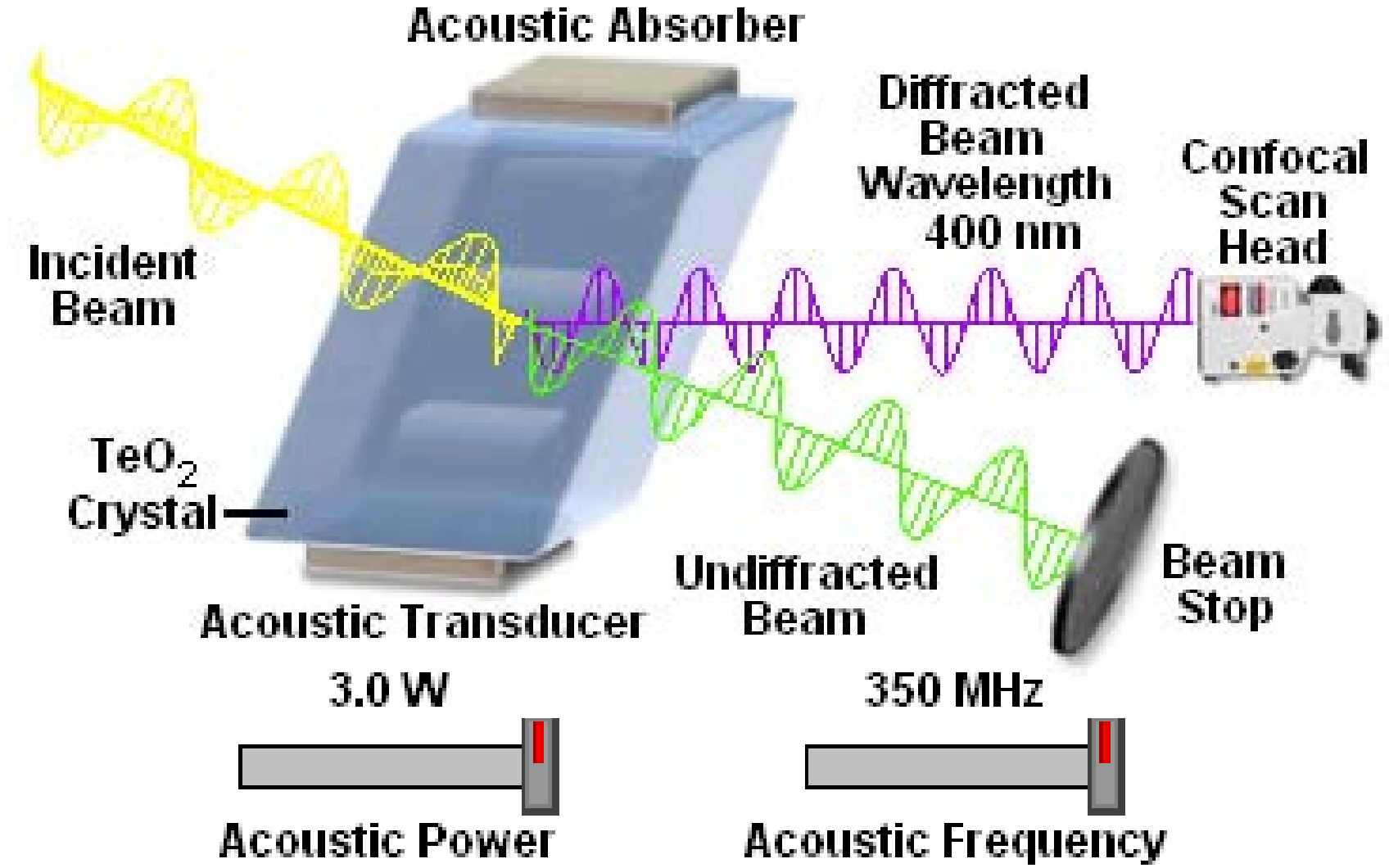
Sources Lasers

Laser	Abrev.	Raies d'excitation (nm)	Puissance (mW)
• Diode UV		405	20
• Argon	Ar	458 476 488 514	5 5 20 20
• Helium-Neon	He-Ne	543	1,2
• Diode	DPSS	561	10
• Helium-Neon	He-Ne	633	10

Spectres utiles en microscopie confocale

Fluorochrome	Excit.	Emiss.	UV laser diode	Argon				Diode DPSS	Helium/ Neon
			406	458	476	488	514	561	633
Hoechst 33258 or DAPI	350	450							
Alexa Fluor TM 430	431	541							
Chromomycin A3 (DNA)	445	580							
Fluo-3	480	520							
Alexa Fluor TM 488	495	519							
Calcein	495	500-550							
Cyanine Cy2 TM	489	505							
DiO (DiOC 18 (3))	484	501							
EGFP	498	510							
Fluoresceinisothiocyanat FITC	490	525							
Ethidiumbromid + DNA	510	595							
Ethidium homodimer	528	616							
EYFP (Enhanced YFP)	520	532							
Alexa Fluor TM 532	531	554							
TOTO-1 ® + DNA	509	533							
YFP (Yellow Fluor. Protein)	520	532							
Alexa Fluor TM 546	556	573							
Cyanine Cy3 TM	550	570							
Cyanine Cy 3.5 TM	580	598							
DiI (DilC 18 (3))	549	565							
Propidium iodide	536	617							
Texas red	595	620							
Rhodamine isothiocyanate TRITC	540	580							
Cyanine Cy5 TM	649	670							
SYTO® 59 red fluorescent nucleic acid stain	640	660							
TO-PRO-3 ® + DNA	642	661							

Principle of AOTF
(Acousto Optical Tunable Filter)



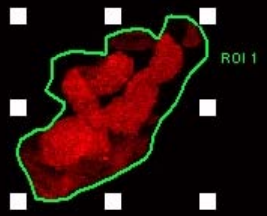
Utilisation de l'AOTF pour scanner des régions d'intérêt (ROIs)

1. Scan initial

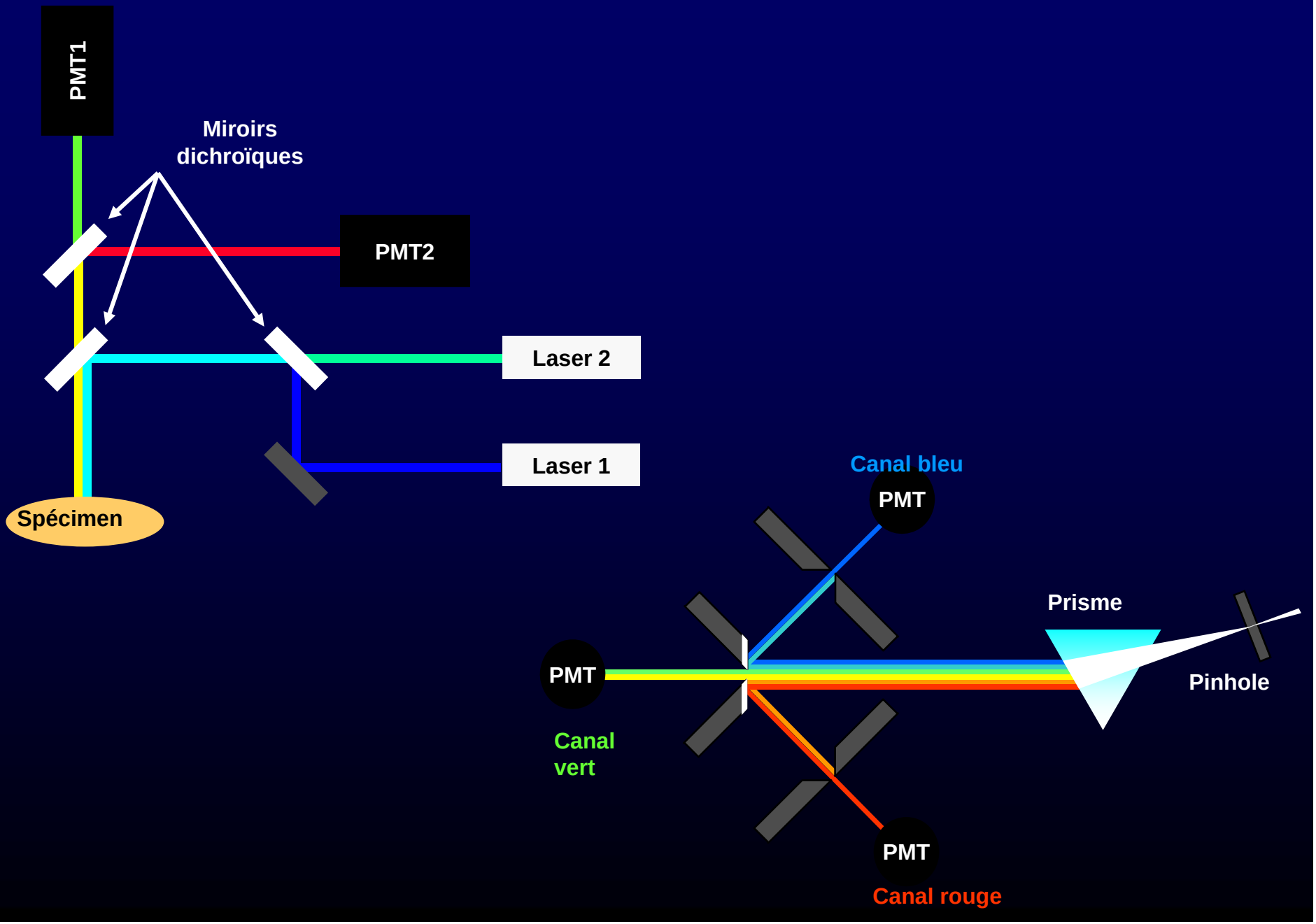
2. Définition de la ROI

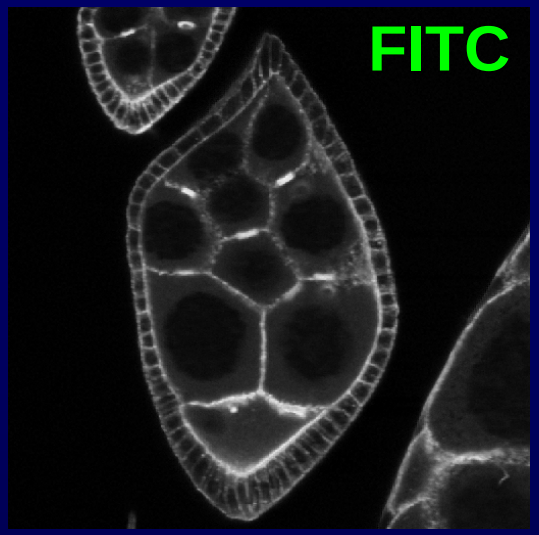
3. Scan de la ROI

4. Image finale

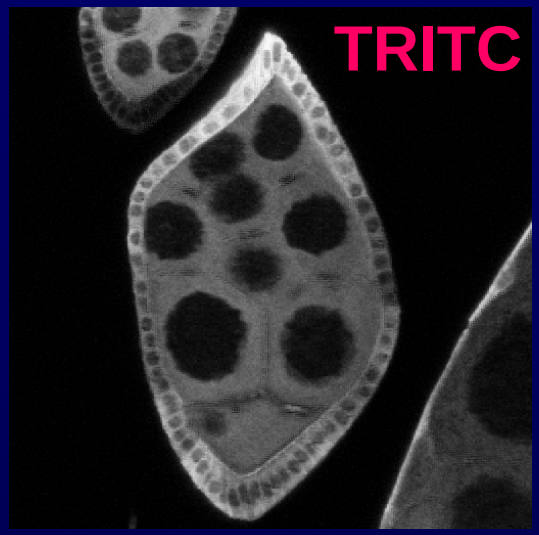


Détection de multi-fluorochromes

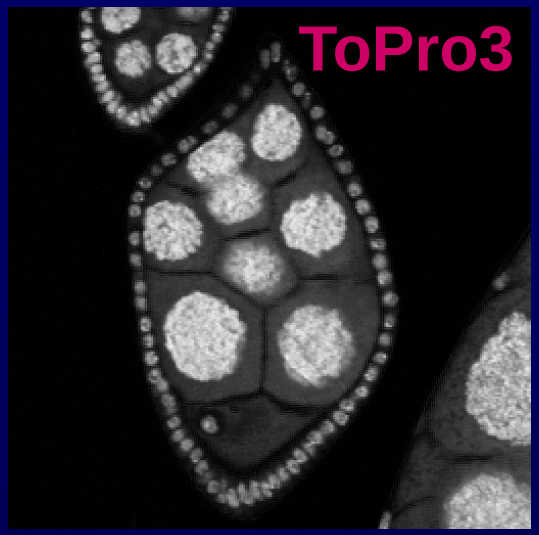




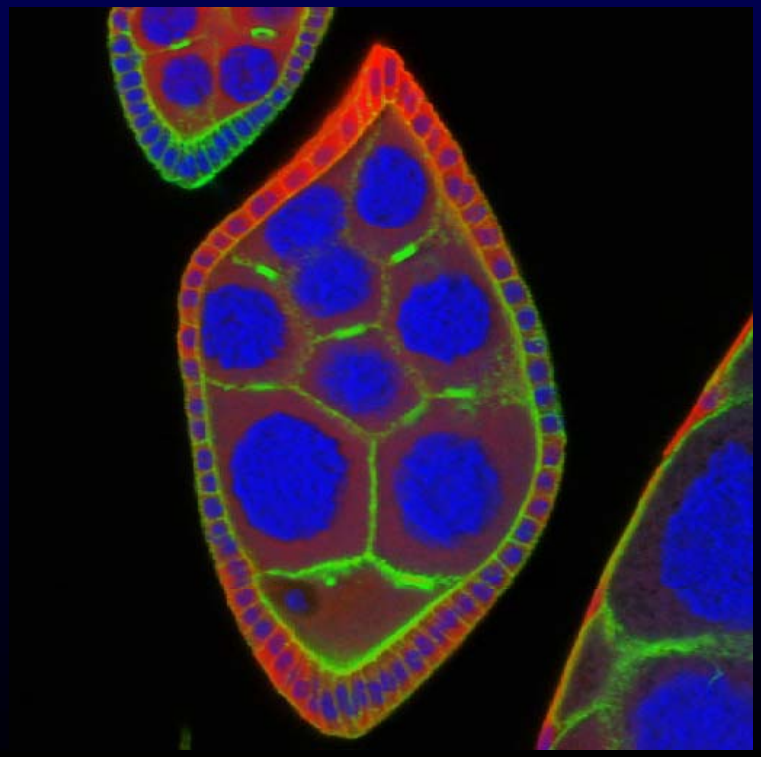
Argon : 488 nm

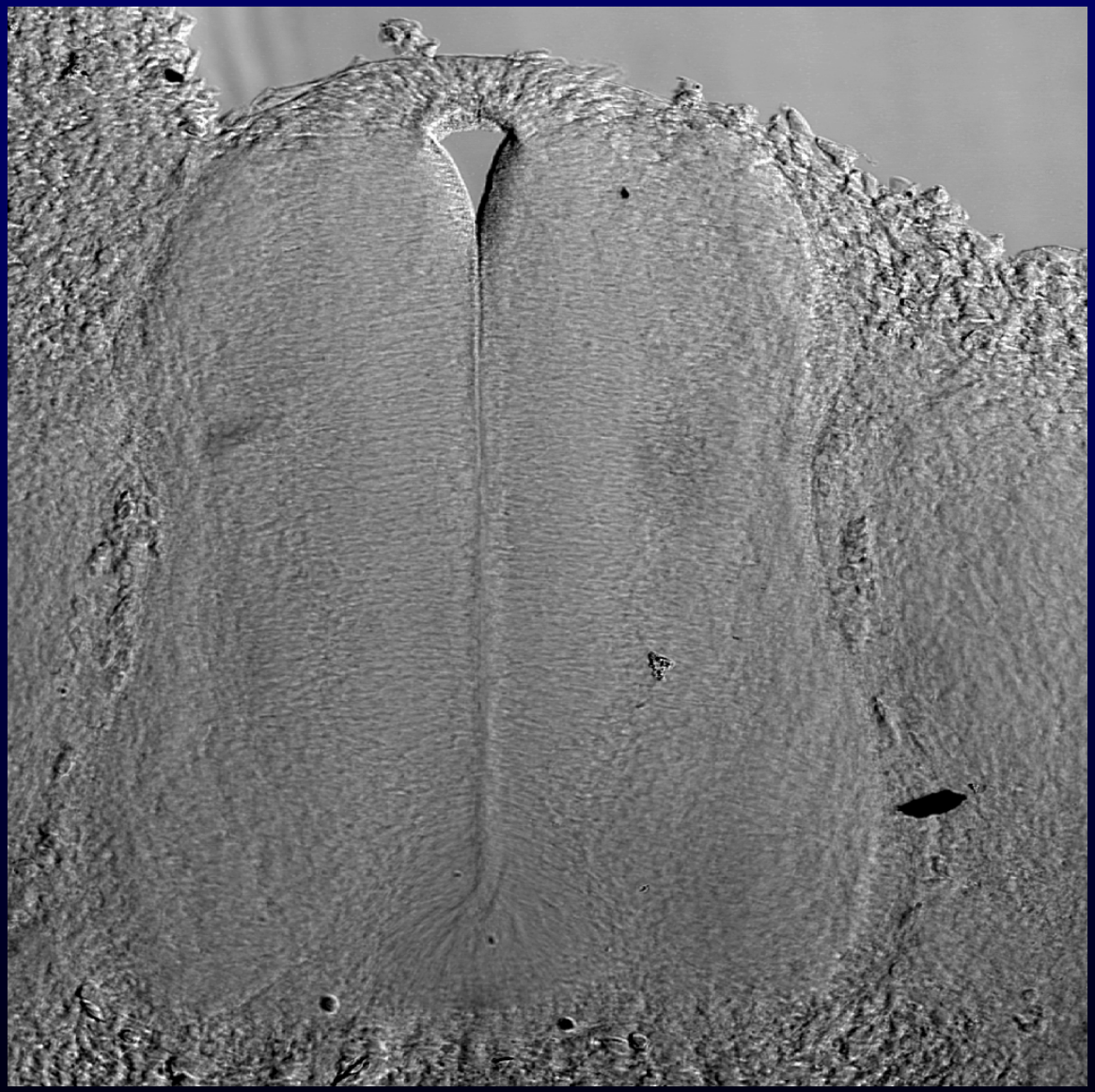


He/Ne : 543 nm



He/Ne : 633 nm



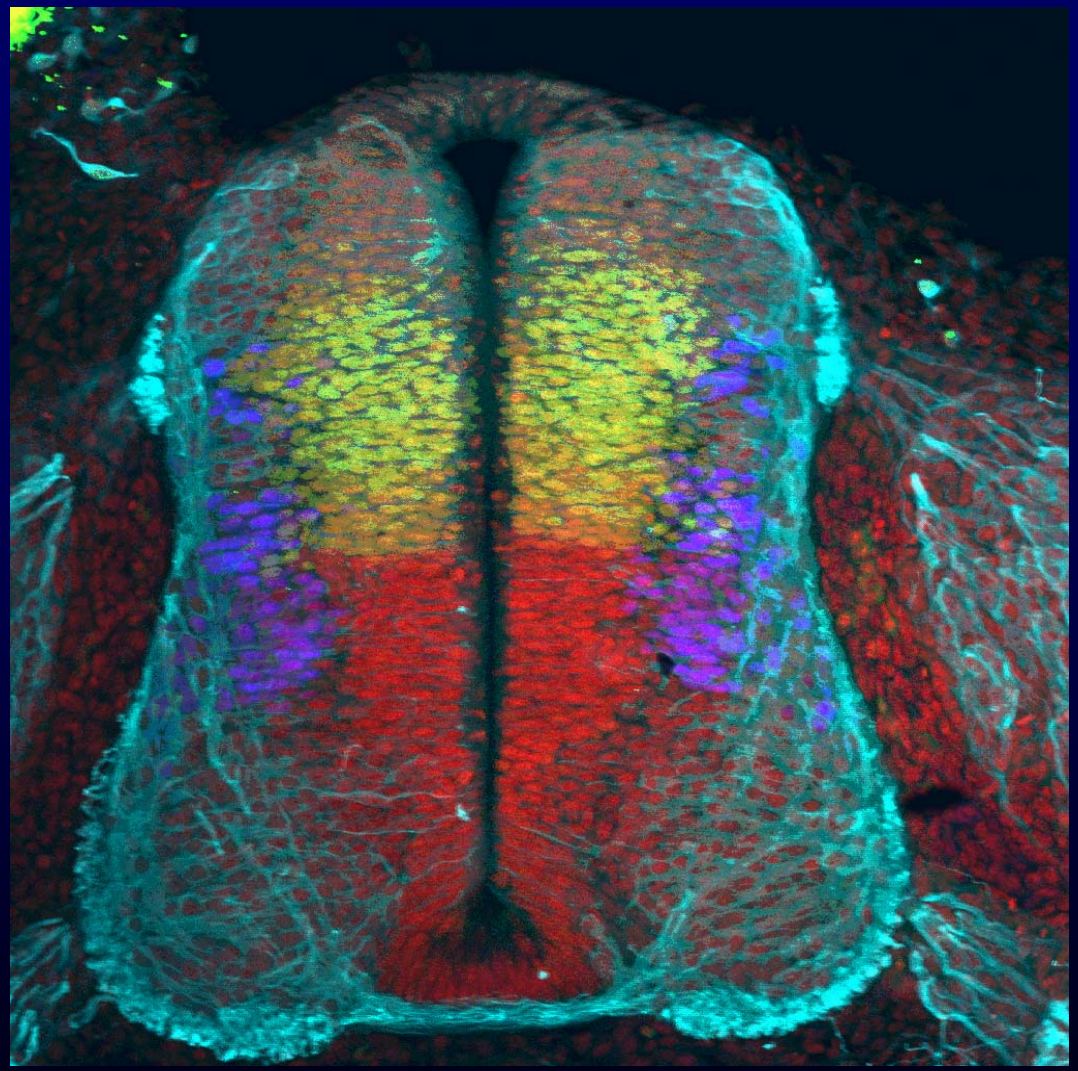


1. Ar 458 nm :
   Chromomycine A3, noyaux

2. Ar 488 nm :
   FITC, Pax7

3. He-Ne 543 nm :
   TRITC, neurones

4. He-Ne 633 nm :
   Cy5, Pax2



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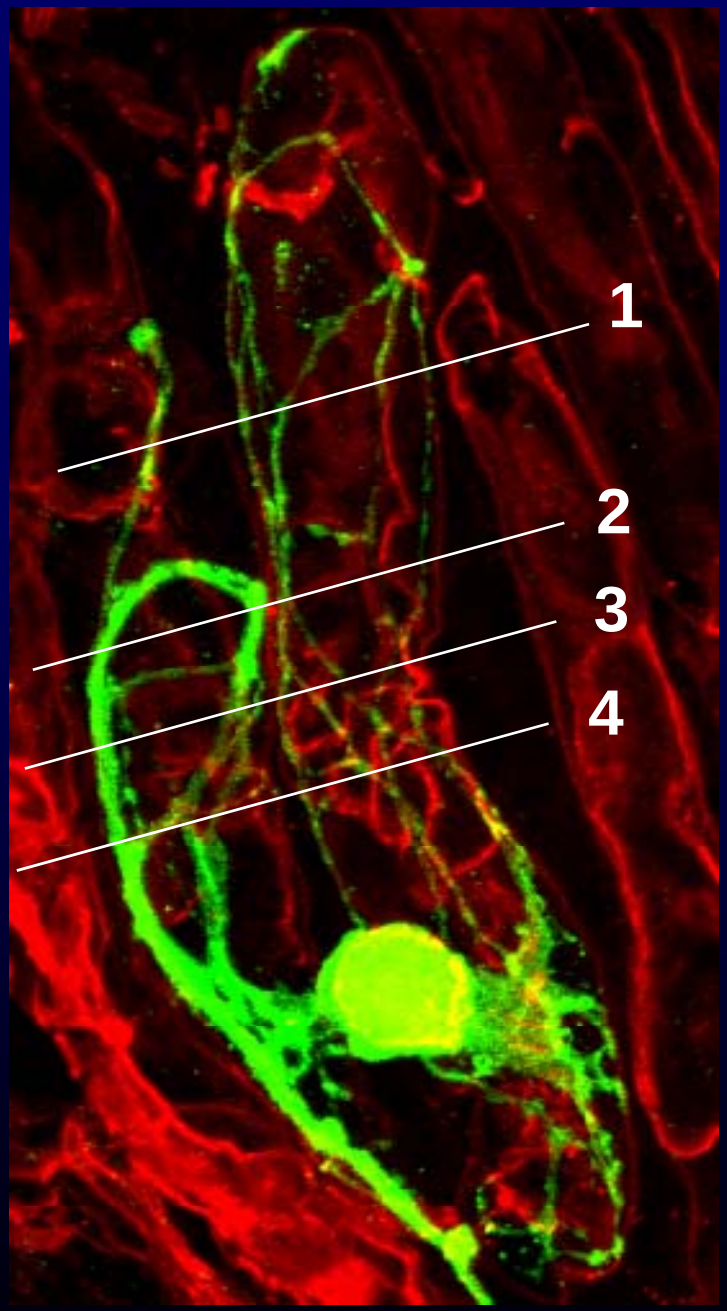
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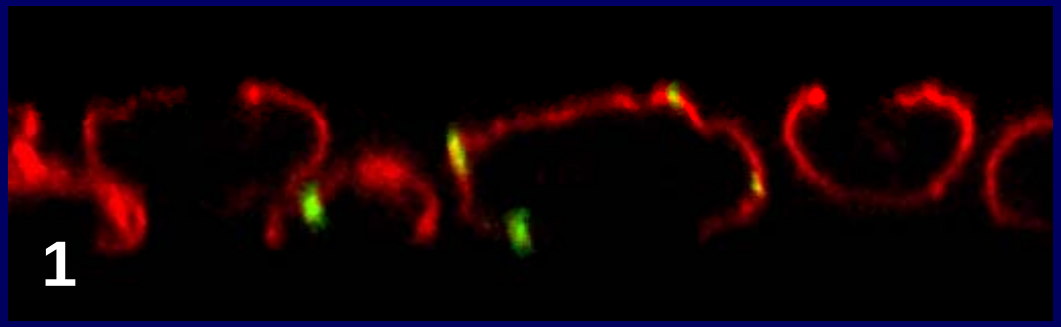
2
3
4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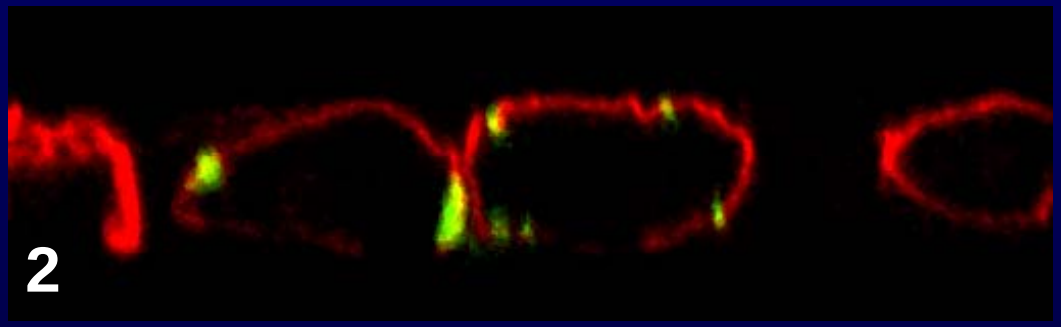
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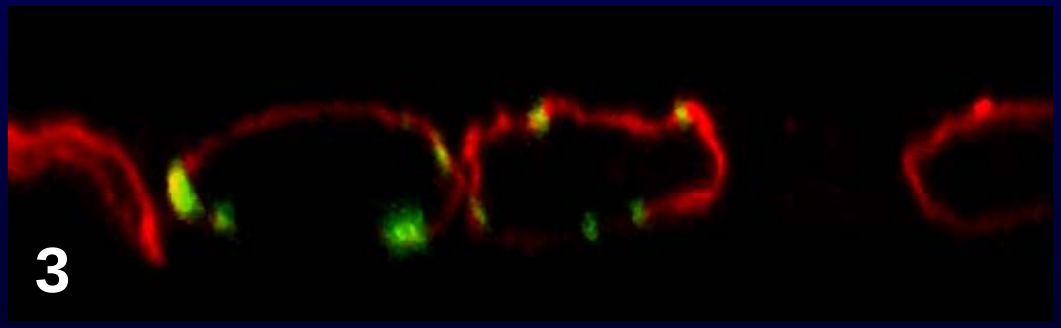
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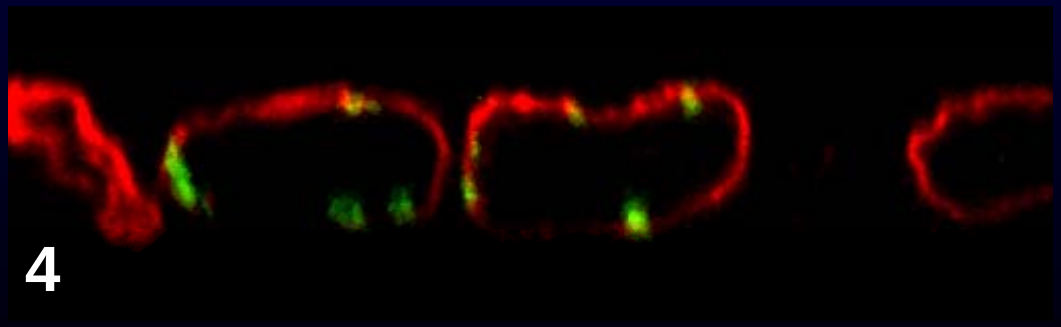
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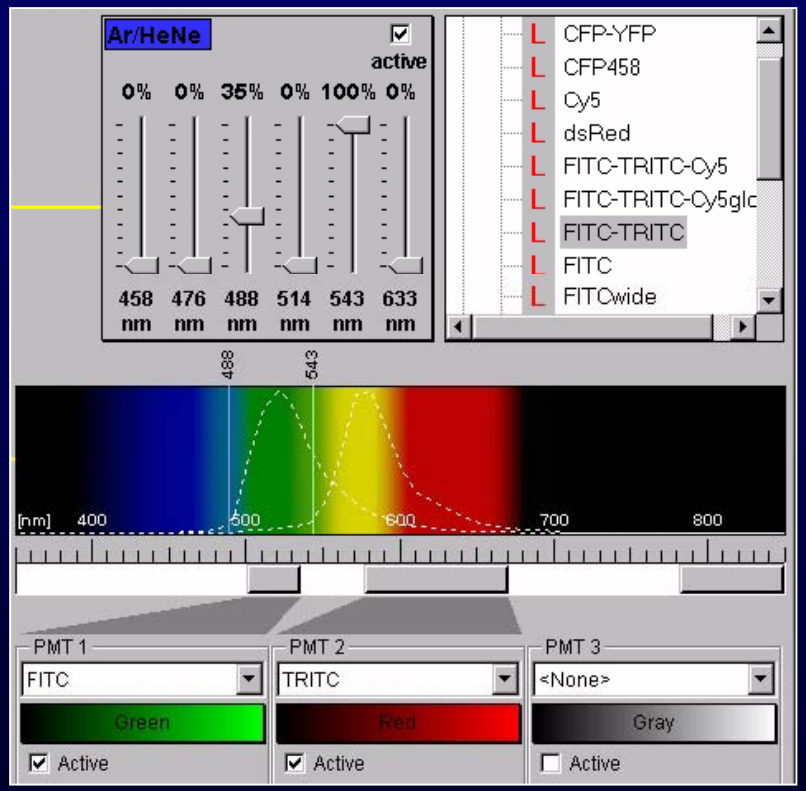
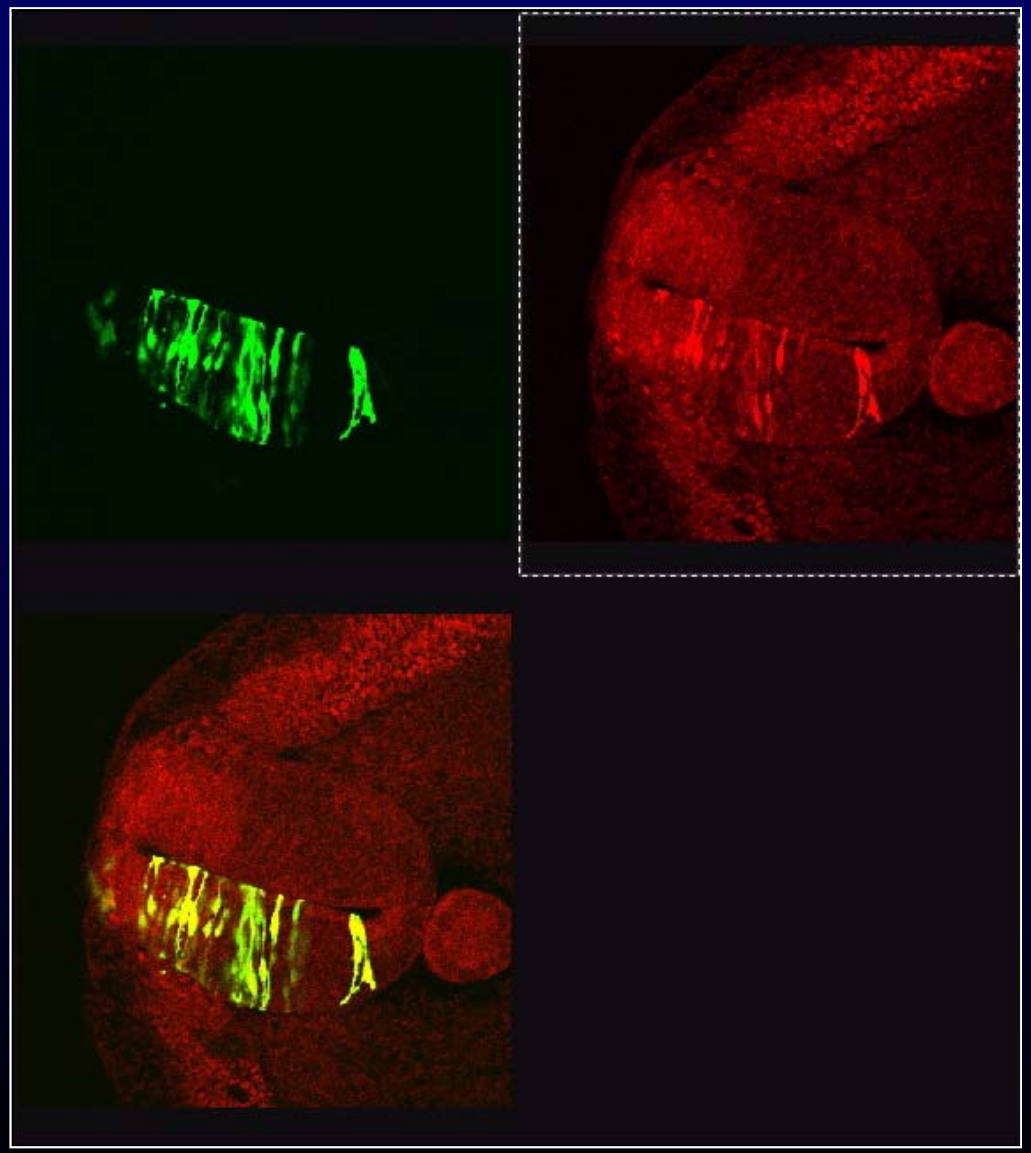
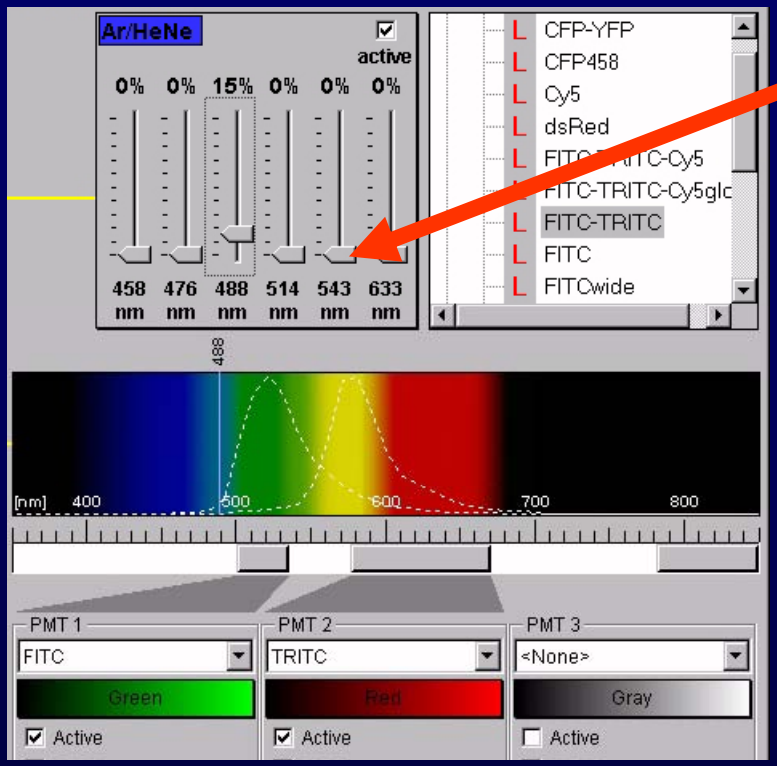
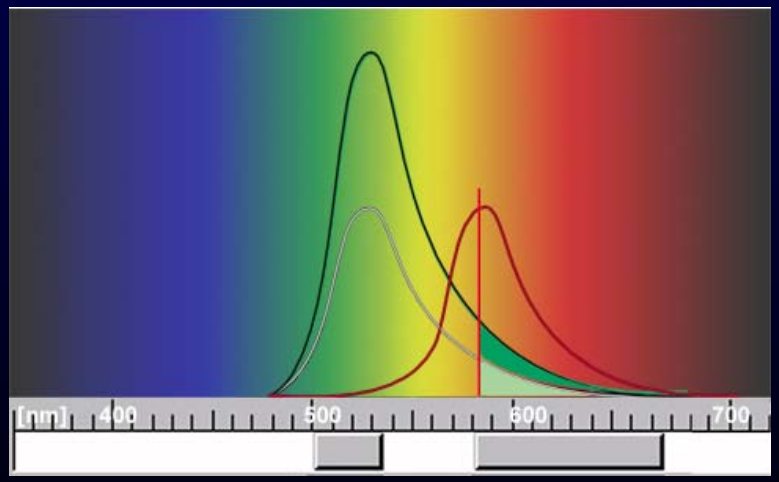
Multifluorescence : le problème du cross-talk des fluorochromes

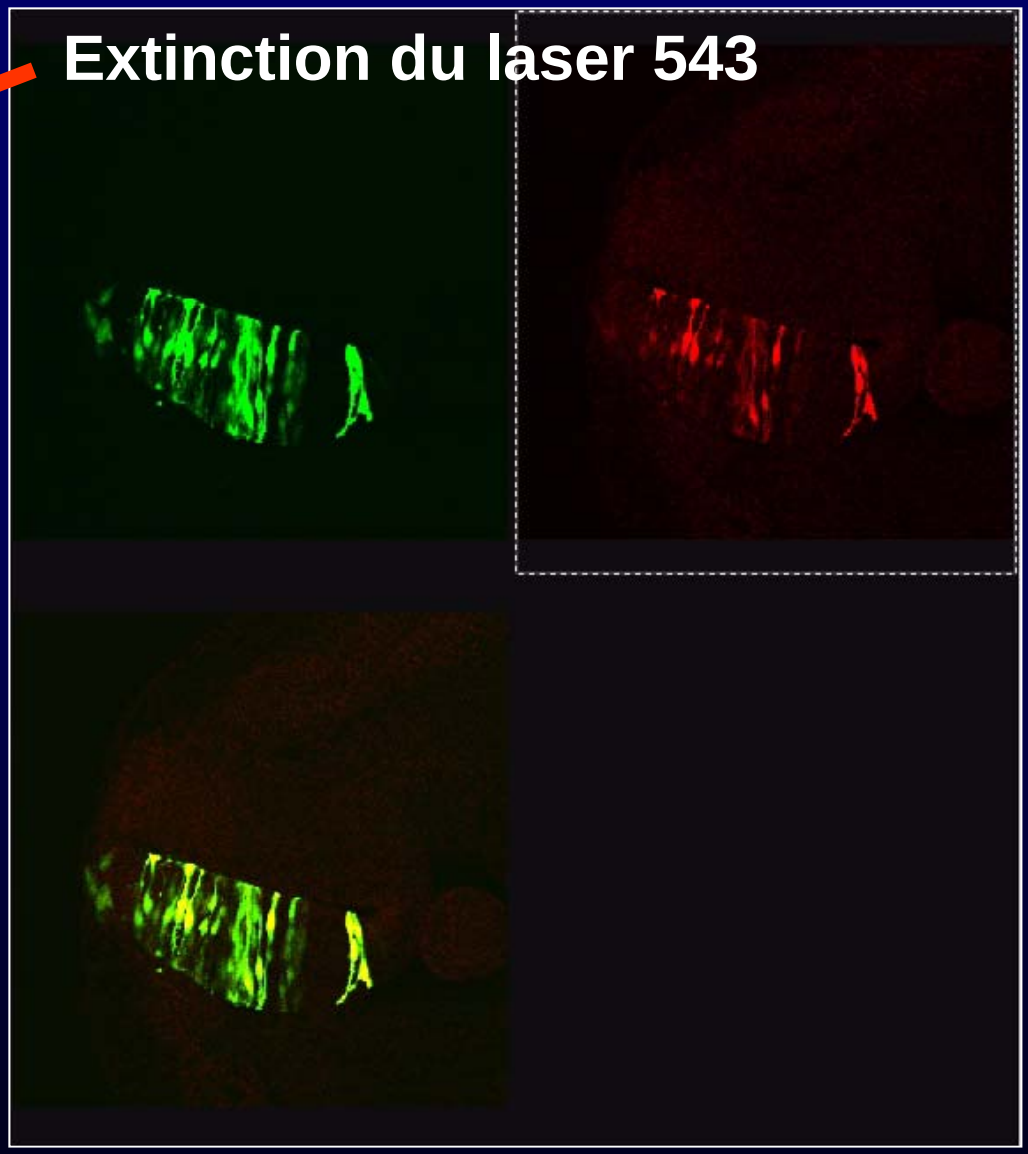




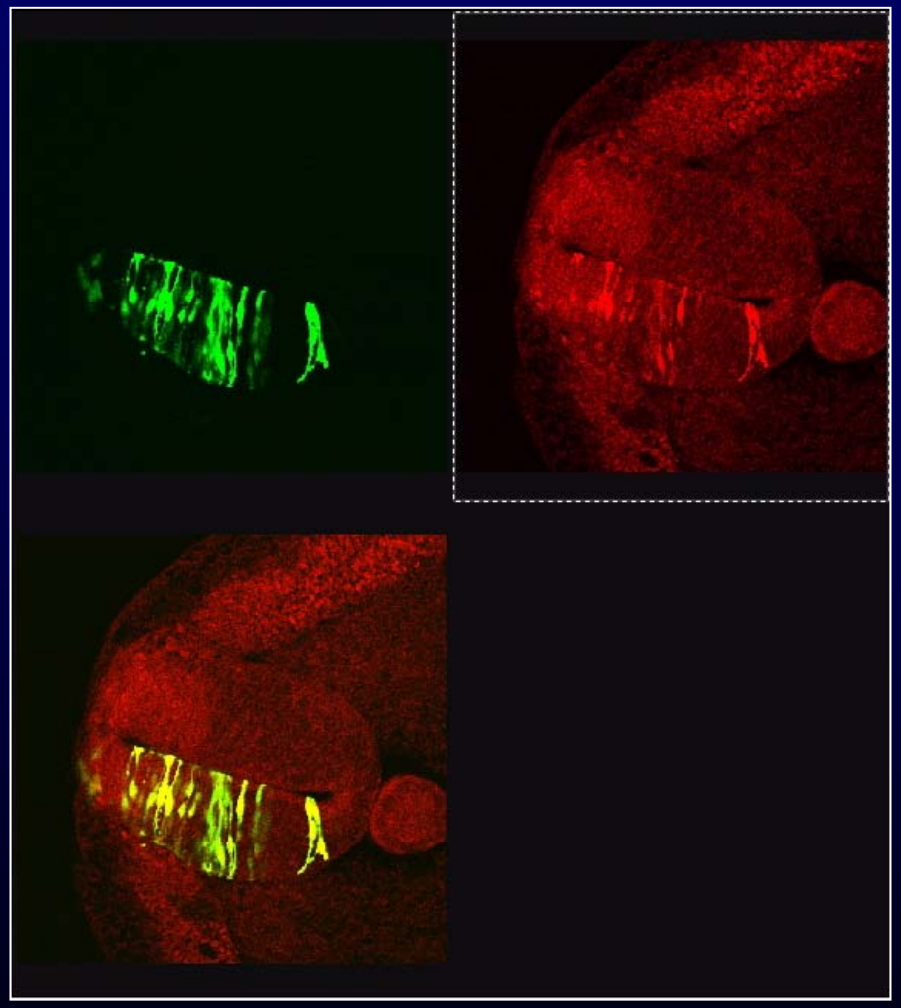




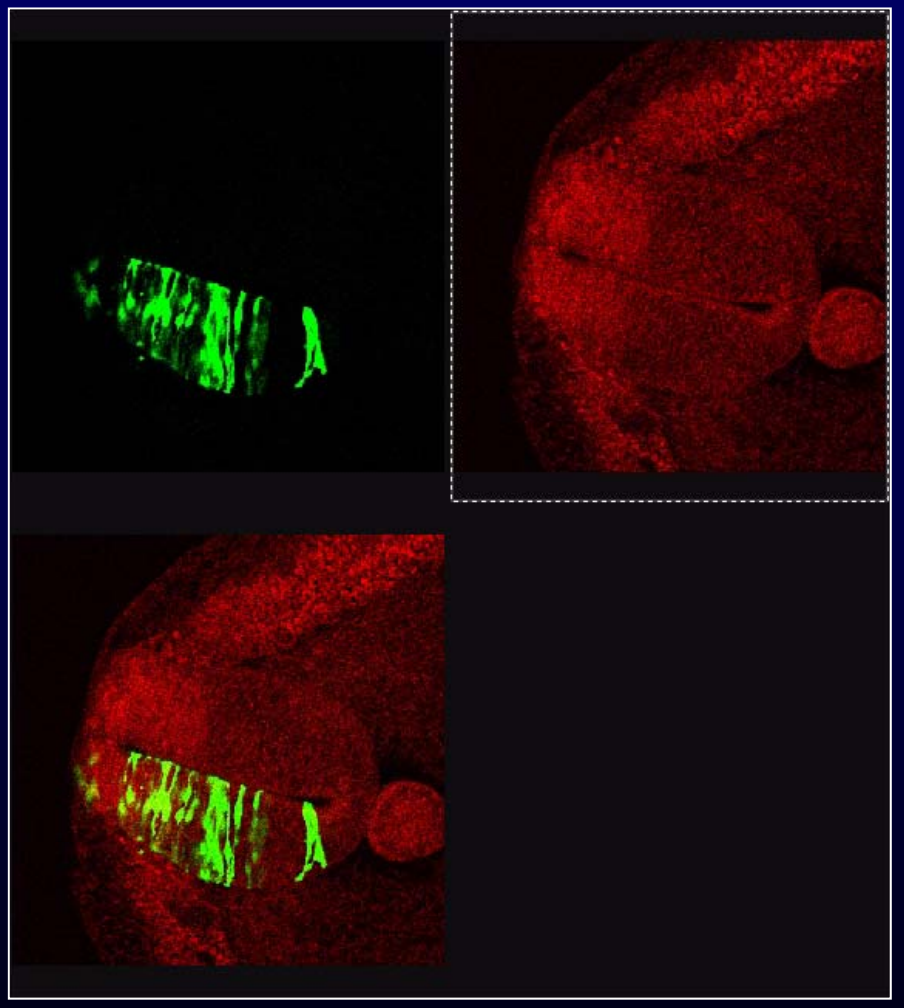
Extinction du laser 543

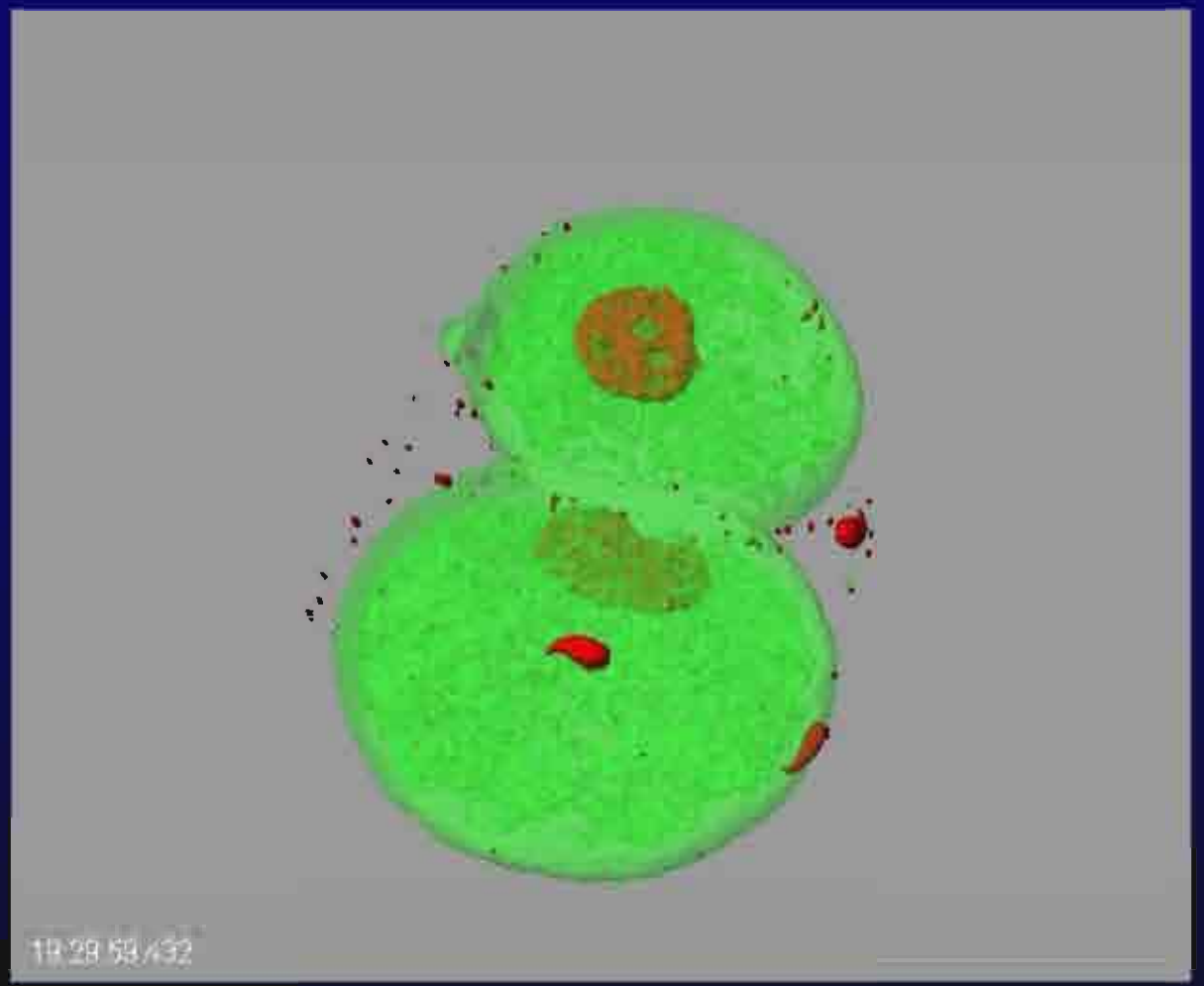


Acquisition simultanée

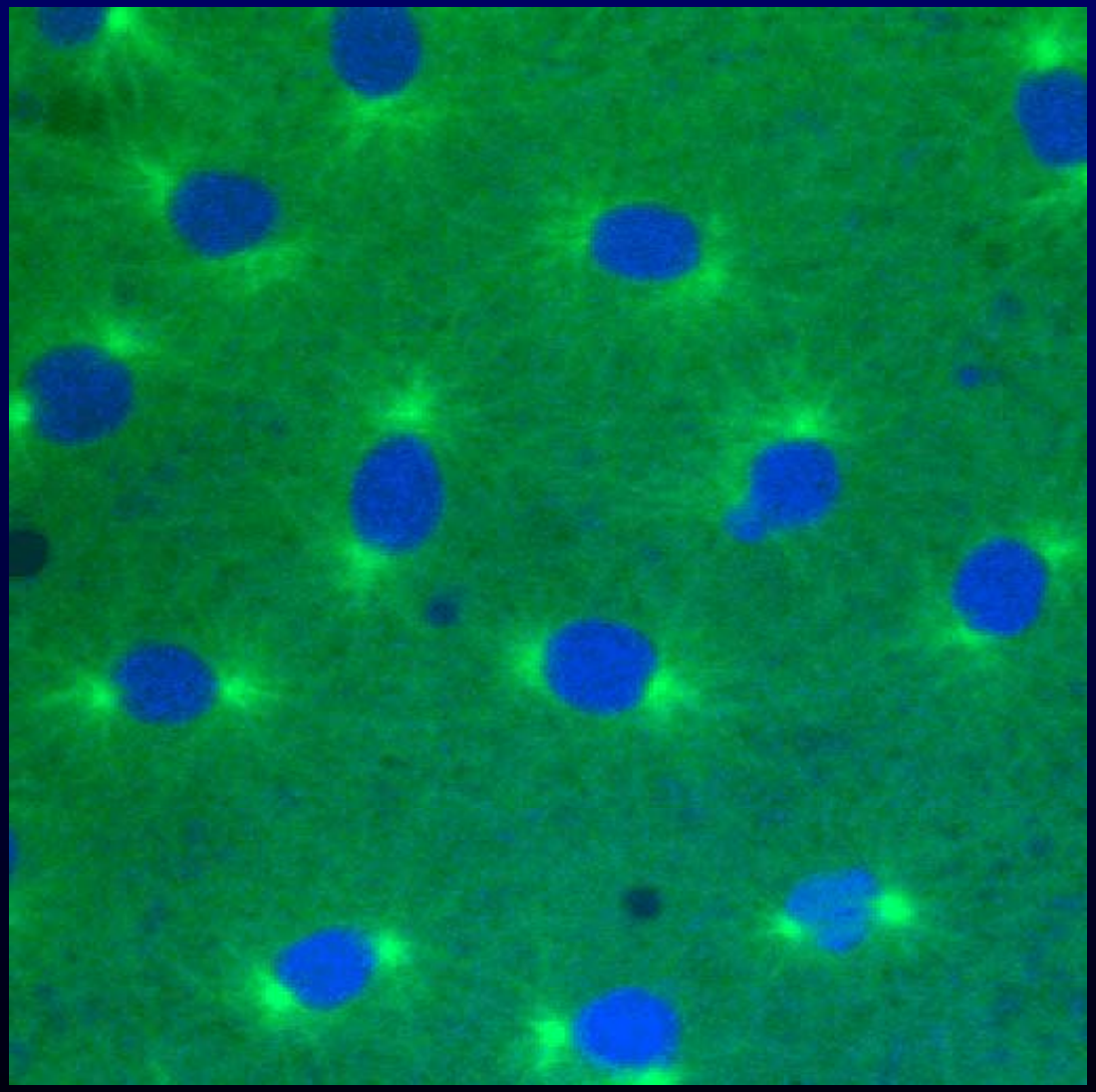


Acquisition séquentielle

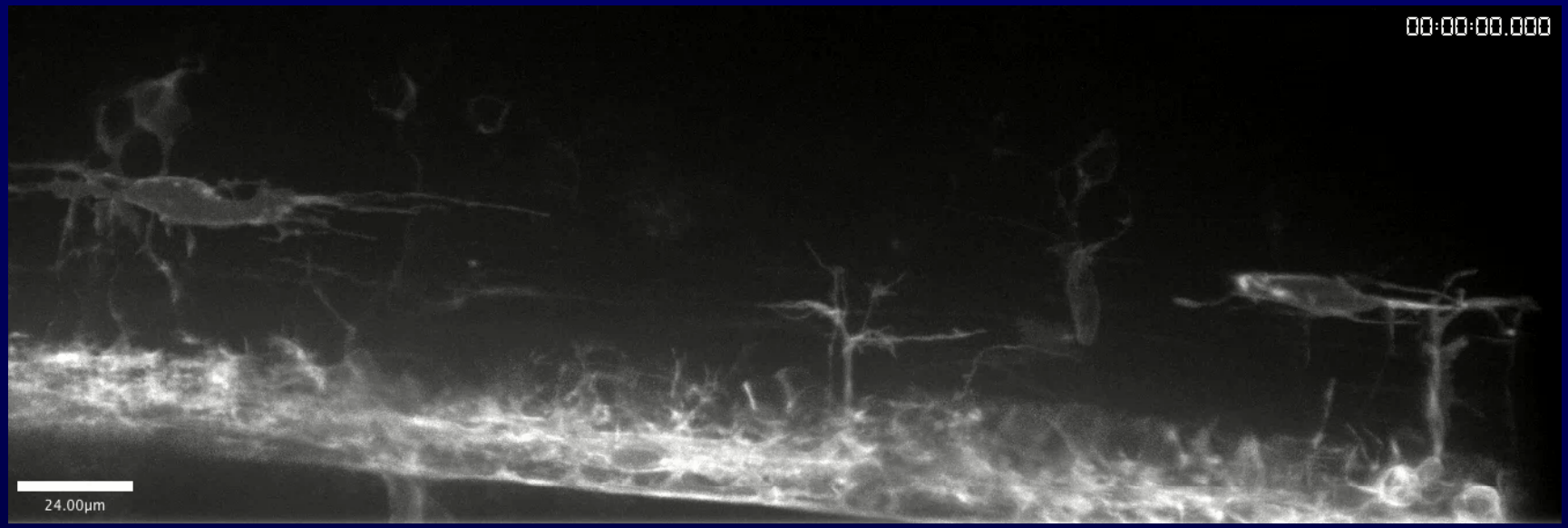


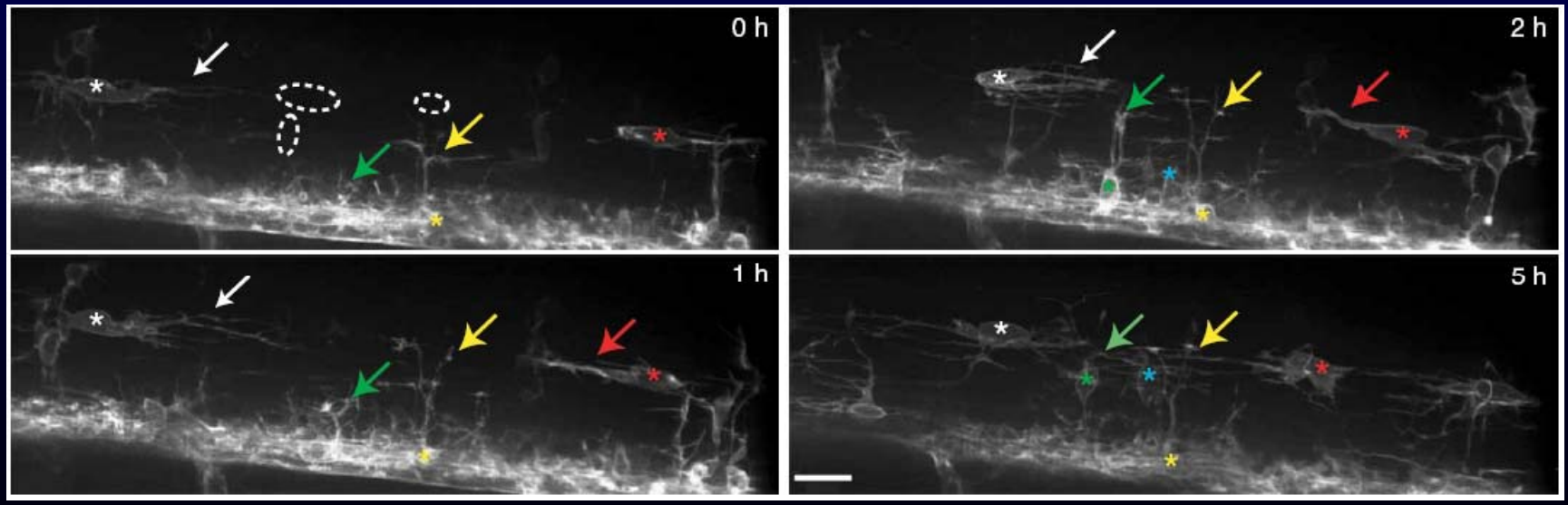

19:28:59.432

Application : mitoses synchrones dans l'embryon de drosophile



Application : plasticité des oligodendrocytes dans l'embryon de poisson zèbre



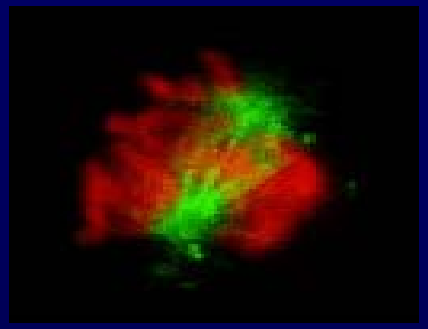


Kirby et al. (2006). Nature Neurosci. 9, 1506-1511

La microscopie confocale

1. Introduction : principes de fonctionnement
2. Numérisation – reconstructions 3D
3. Lasers – Multimarquages
4. **Avantages et limites**

AVANTAGES du MICROSCOPE CONFOCAL

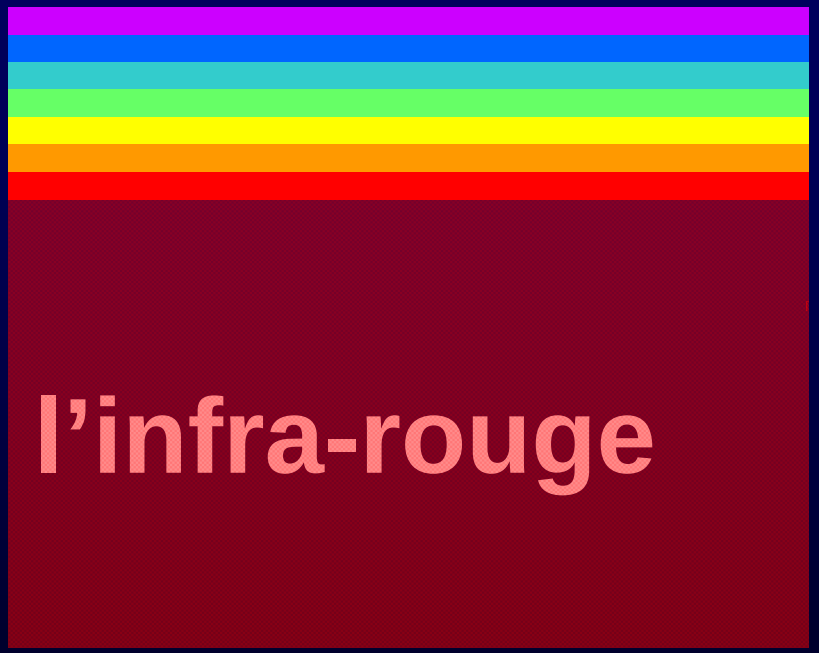


- Résolution accrue
- Permet l’examen d’objet épais
- Permet le balayage en Z
- Obtention d’images 3D à haute résolution
- Séparation des spectres d’émission
- Diminution de la photoatténuation
- Analyse en temps réel de phénomènes dynamiques

LIMITES du MICROSCOPE CONFOCAL

- **Photo-dommage cellulaire dans toute l'épaisseur de la préparation**
- **Photo-atténuation (« bleaching ») dans toute l'épaisseur de la préparation**
- **Pénétration relativement réduite dans l'épaisseur de la préparation (<80µm)**

La microscopie multiphotonique :



l’imagerie dans l’infra-rouge

La microscopie laser en sciences de la Vie

UV, VISIBLE

CONTINU

Microscopie confocale

Observation

PULSÉ

Microchirurgie

Scalpel

PROCHE IR

CONTINU

Pinces optiques

Micromanipulation

PULSÉ

Microscopie multiphotonique

Microscopie 2nde harmonique, triple harmonique

Observation
Nanoscalpel

Principe de la microscopie multiphotonique

Un atome peut être excité par l'interaction de deux quanta de lumière

Cette double excitation procure une résolution tri-dimensionnelle intrinsèque en microscopie de fluorescence à balayage laser.

Maria Göppert-Mayer
Prix Nobel de Physique (1963).

Göppert-Mayer, M. (1931). Über Elementarakte mit zwei Quantensprüngen, ***Ann. Phys.*** **9,** 273-294

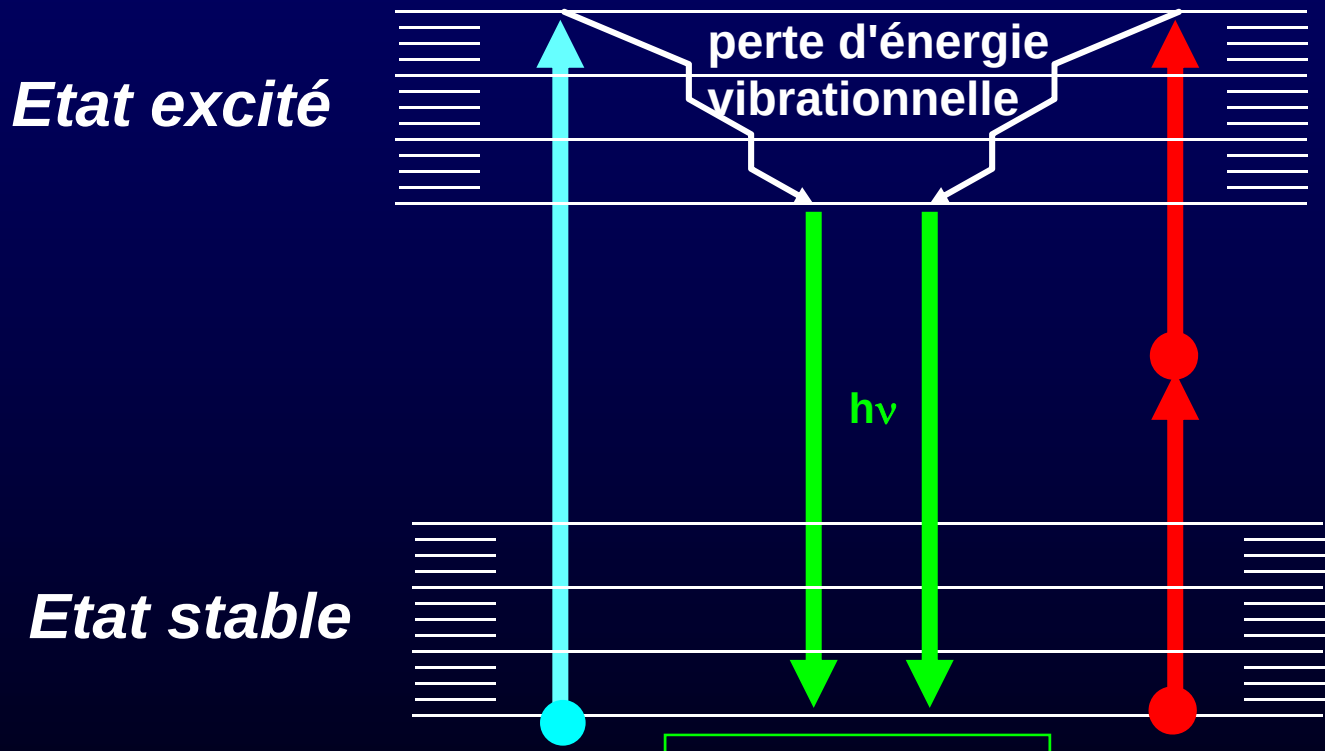
Watt W. Webb

Denk W, Strickler JH, Webb WW (1990). Two-photon laser scanning fluorescence microscopy. **Science 248,** 73-76

Principe de la microscopie multiphotonique

Microscopie conventionnelle

Microscopie à deux photons



Absorption d'un hν, dans l'UV ou le bleu

Fluorescence

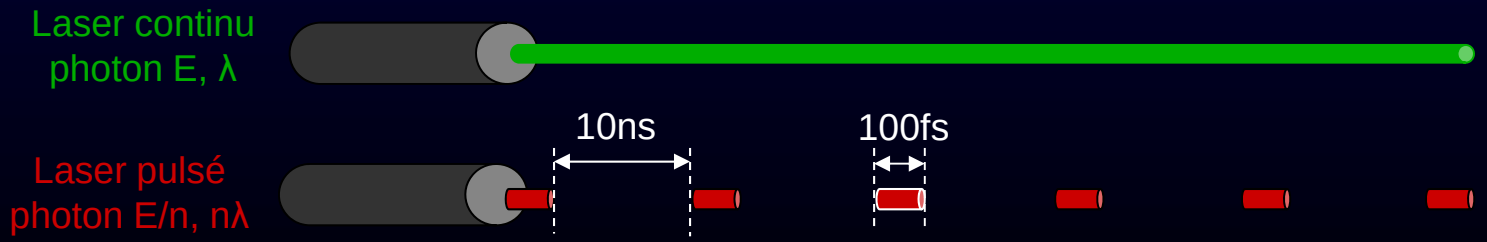
Absorption simultanée de 2 hν, dans le proche IR
⇒ excitation à $\frac{\lambda}{2}$

La probabilité d'absorption de deux photons est extrêmement faible :

Au soleil, une molécule de Rhodamine B absorbe une paire de photons simultanément, une fois tous les 10 millions d'années. L'absorption simultanée de trois photons n'est pas statistiquement envisageable durant l'âge total de l'univers !!!!

Comment augmenter la probabilité d'absorption simultanée de deux ou trois photons ?????

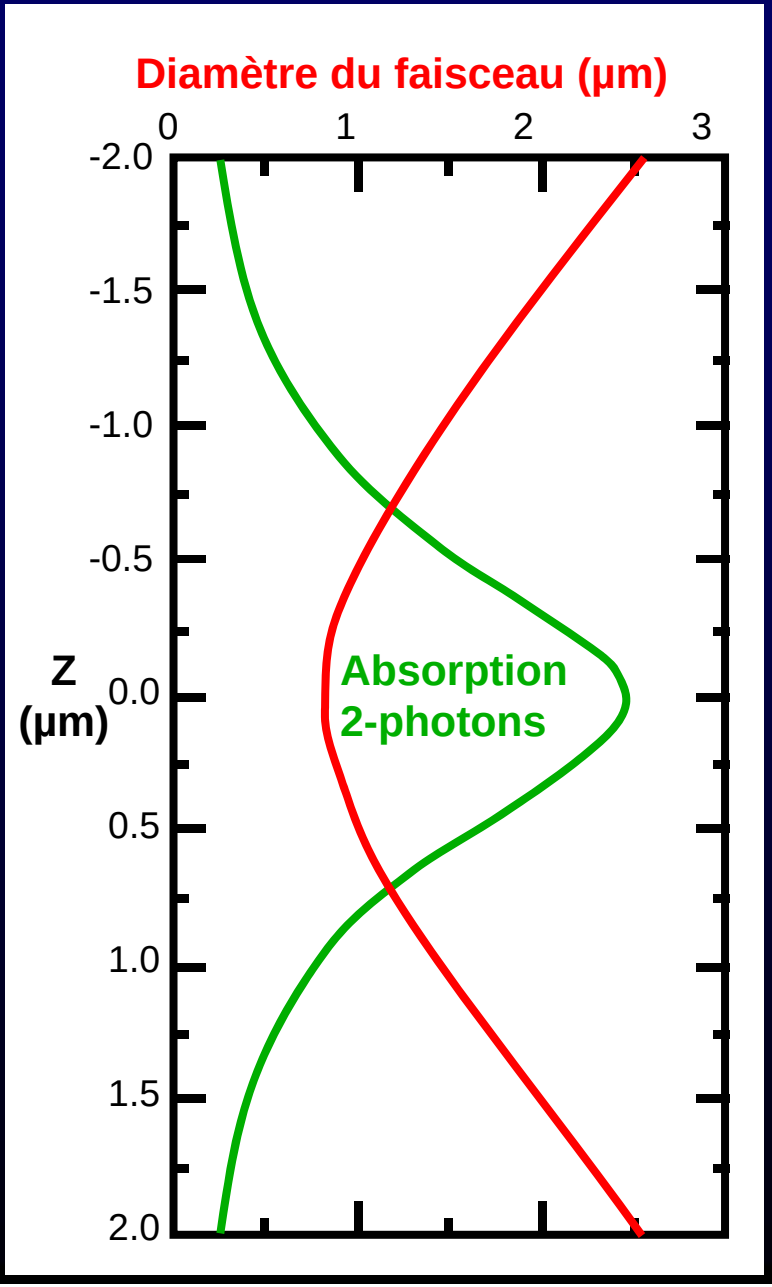
⇒ Utilisation de lasers pulsés : Laser Titane-saphir (Ti:S) : ≈ 80 million pulses par seconde (durée des pulses ≈ 100 fs)



L’excitation efficace est restreinte à la région focale

L’excitation du fluorochrome par absorption simultanée de deux photons procure une résolution tri-dimensionnelle intrinsèque en microscopie de fluorescence à balayage laser.

(Watt W. Webb, 1990)



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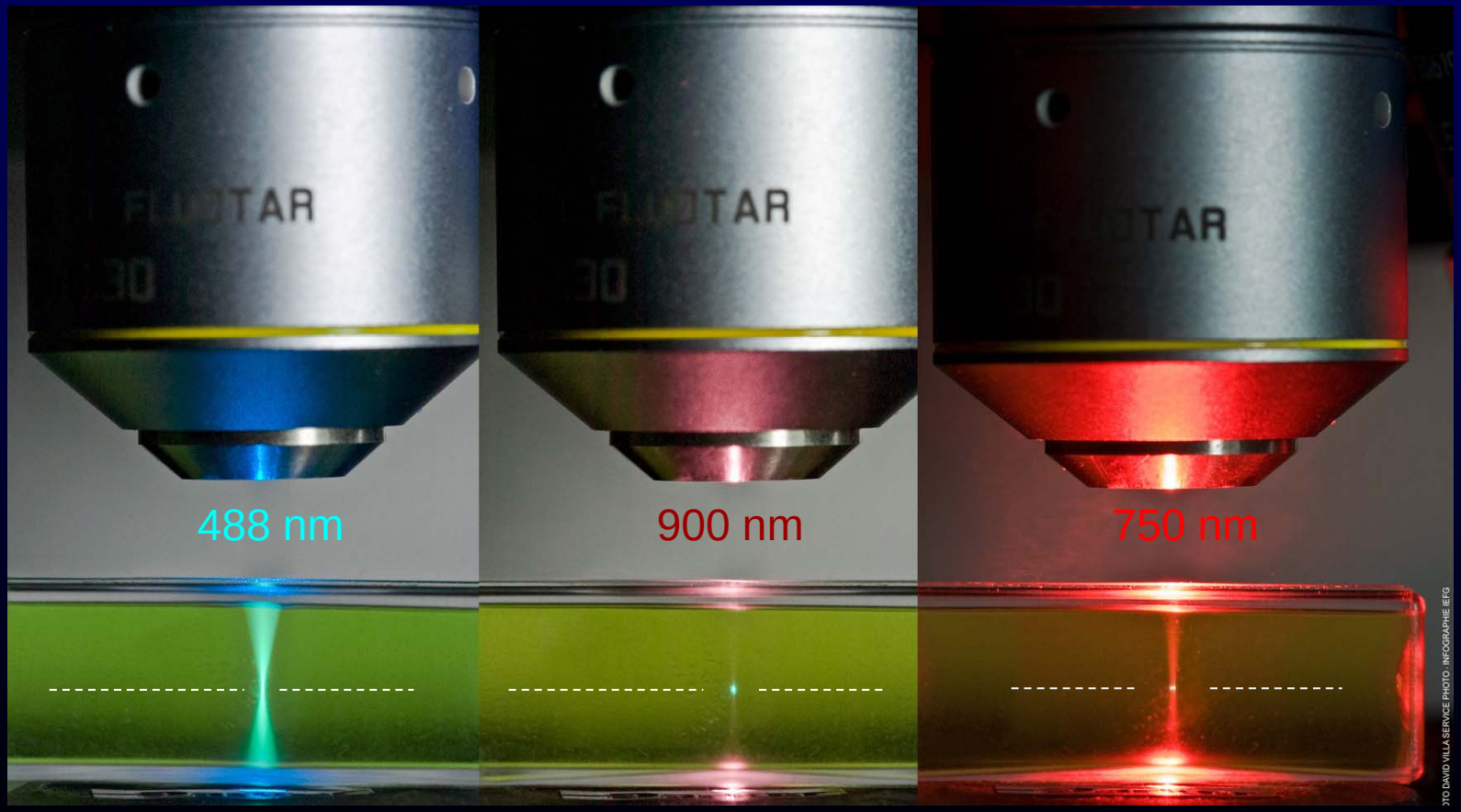
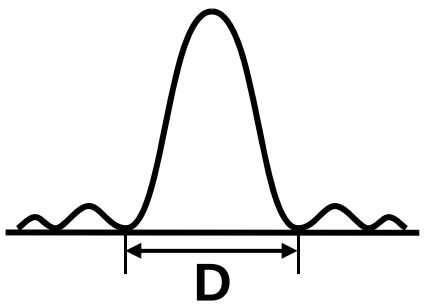
(Watt W. Webb, 1990)



Résolution en microscopie multiphotonique



$$D = \frac{1{,}22\lambda}{n.\sin\alpha} = \frac{1{,}22\lambda}{ON}$$

$$\delta_{xy} = \frac{D}{2} = \frac{0{,}61\lambda}{ON}$$

λ = 488 nm : δ_{xy} = 0,21 µm

λ = 900 nm : δ_{xy} = 0,4 µm

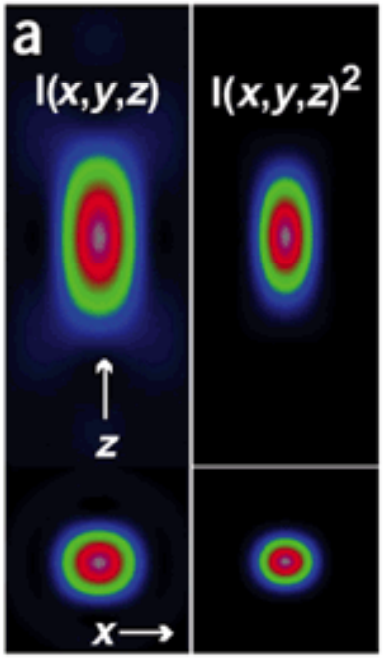
⇒ **Résolution multipliée par 2 !**

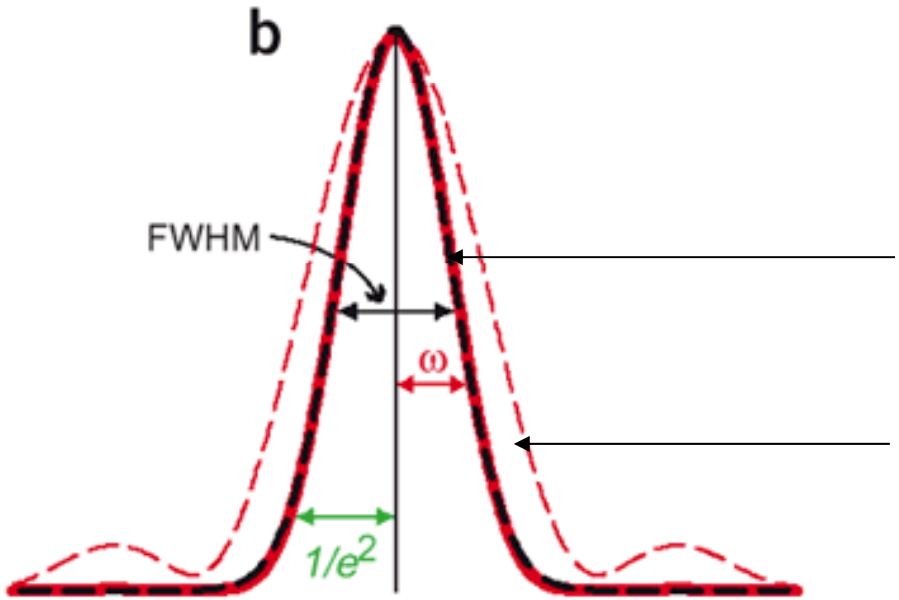
	Longueur d'onde (nm)	Résolution latérale (x-y, µm)	Résolution axiale (x-z, µm)
Confocal	350-500	0,14 – 0,20	0,30 – 0,43
Bi-photon	700-1000	0,20 – 0,29	0,60 – 0,90

En pratique :

- **Résolution confocale jamais optimale (pinhole jamais fermé au maximum)**
- **En multiphoton, fluorescence ne vient que du point focal**
- **⇒ Résolution similaire ou meilleure en MMP**

Focalisation = meilleure résolution





psf axiale 2P

psf axiale 1P

c

$$\omega_{xy} = \begin{cases} \dfrac{0.320\lambda}{\sqrt{2}\ \mathrm{NA}} & \mathrm{NA} \le 0.7 \\ \dfrac{0.325\lambda}{\sqrt{2}\ \mathrm{NA}^{0.91}} & \mathrm{NA} > 0.7 \end{cases} \qquad \omega_z = \frac{0.532\lambda}{\sqrt{2}} \left[\frac{1}{n - \sqrt{n^2 - \mathrm{NA}^2}} \right]$$

Zipfel, W. R., R. M. Williams, et al. (2003). "Nonlinear magic: multiphoton microscopy in the biosciences." Nat Biotechnol **21**(11): 1369-77.

Lasers pulsés

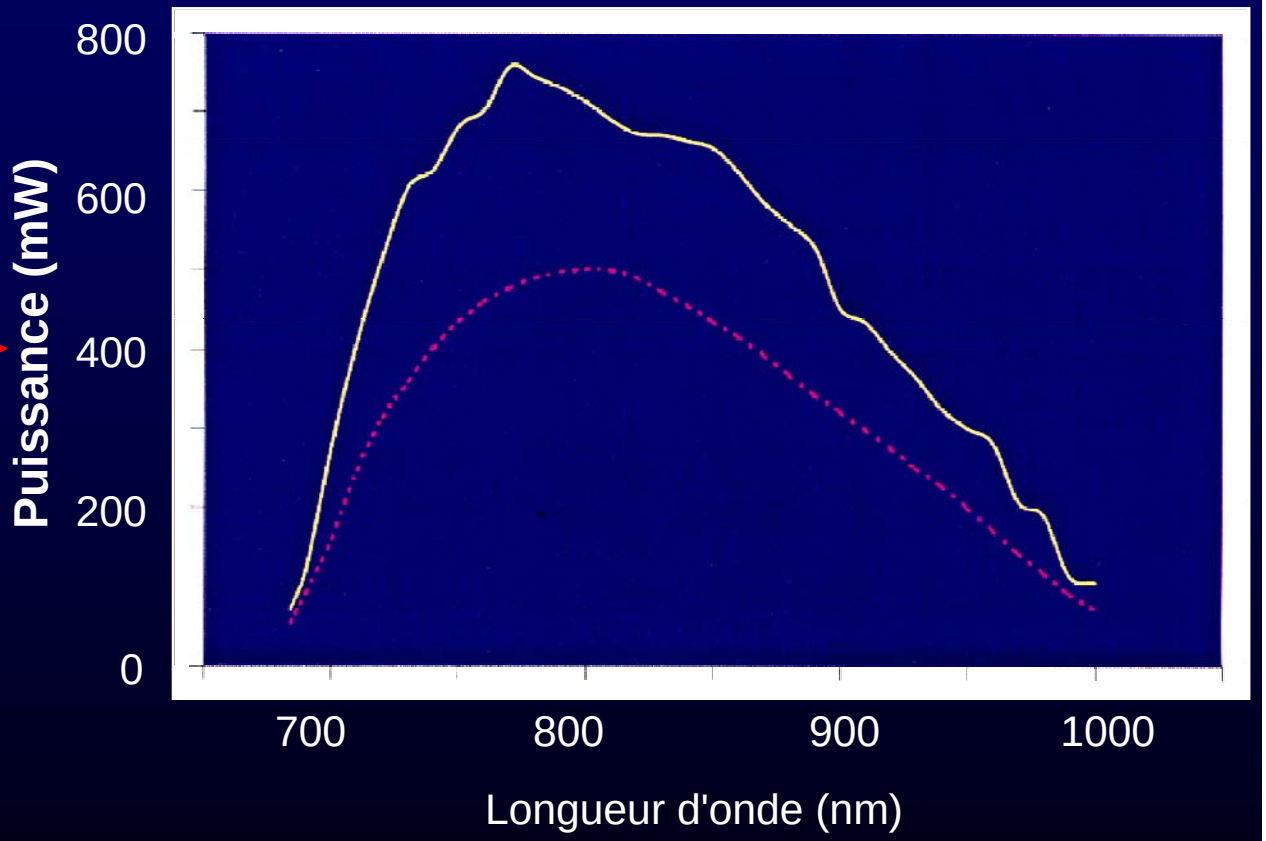
Laser Argon ionisé
8 - 15 W (488, 514 nm)

Laser pulsé à Titane:Saphir femtoseconde

Infra-rouge

Laser à solide
5 W (532 nm)

Courbe d'accordabilité du Laser



Réglage en continu sur toute la gamme 680-1050 nm

Lasers pulsés Titane-Saphir en « mode locked » (1992)

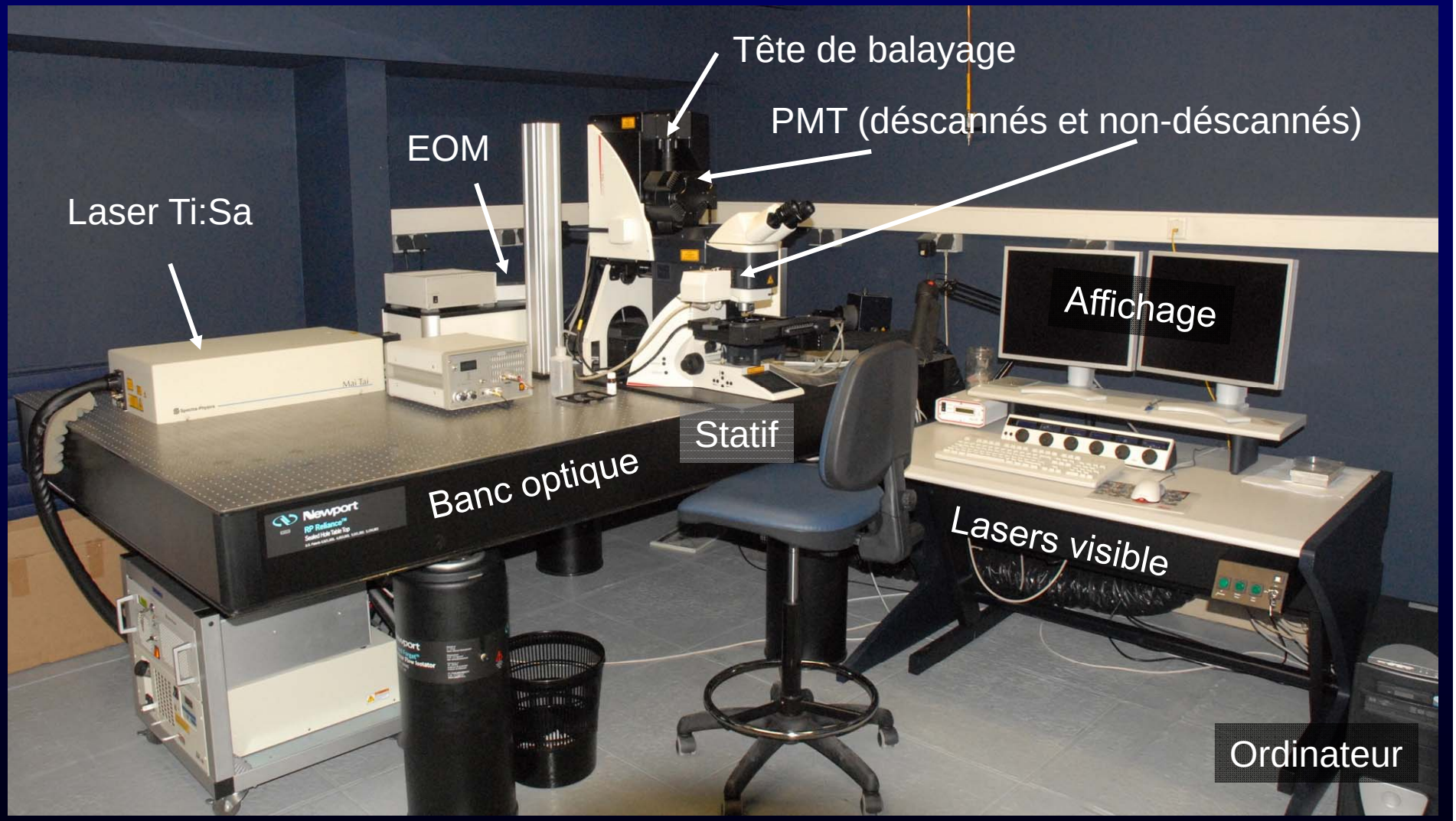
(pulse < 250 femtosecondes, fréquence 80 MHz, puissance moyenne > 200 mW)

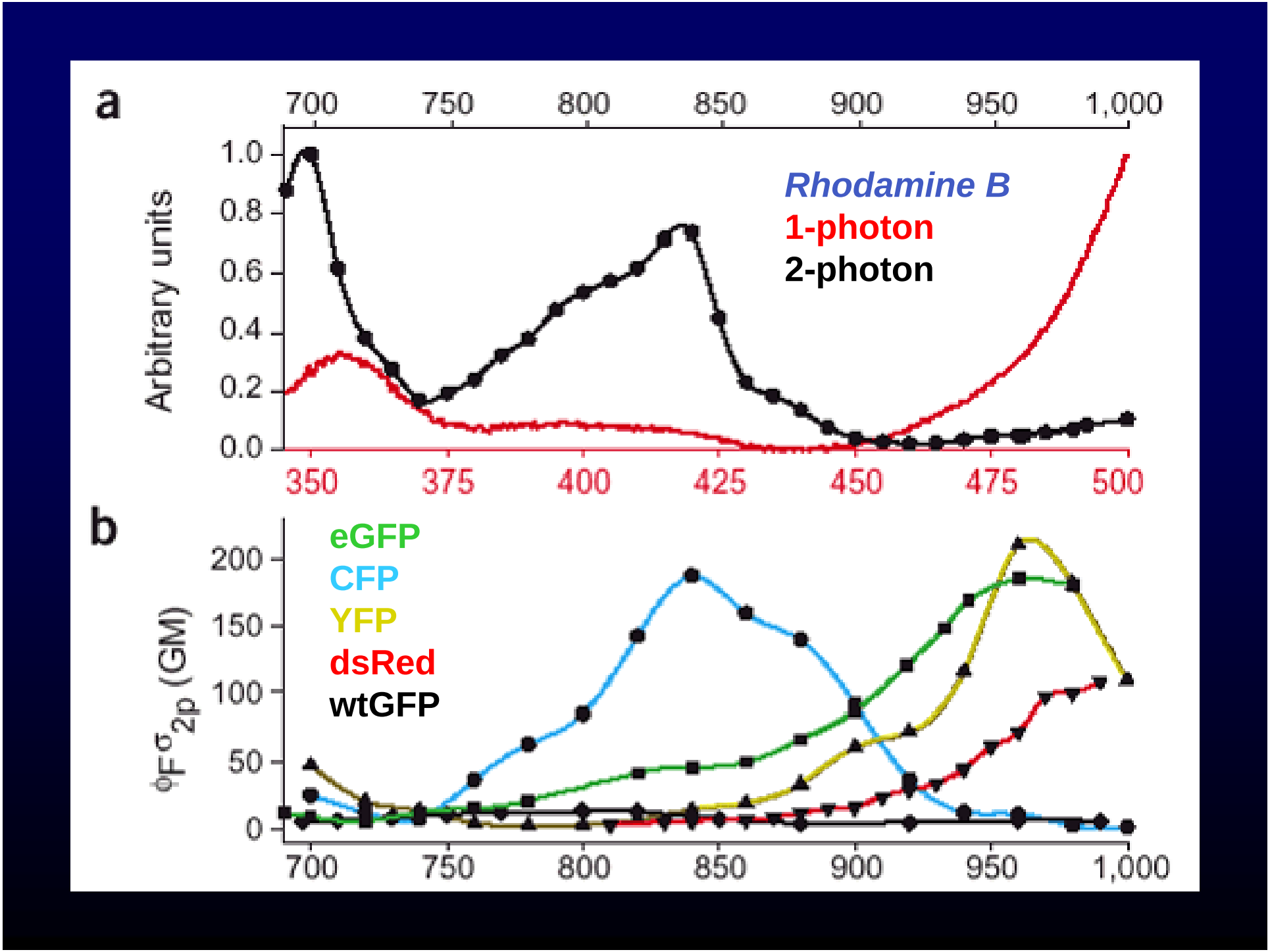
- **Longueur d'onde fixe : 800 ou 1047 nm**
- **Longueur d'onde accordable, bande réduite (700-760 et 760-860)**
- **Longueur d'onde accordable, bande large**

	Lasers pompe et Ti:S séparés		Lasers compacts	
Type Marque	***Verdi-Mira*** (Coherent)	***Tsunami*** (Spectra Physics)	***Chameleon*** (Coherent)	***Mai-Tai*** (SpectraPhysics)
Durée des pulses	**120 fs et ps**	**<50 fs et ps**	**140 fs**	**100 fs**
Cadence de répétition	**76 MHz**	**82 MHz**	**90 MHz**	**80 MHz**
Longueurs d'onde	**690-1080 nm**	**690-1080 nm**	**720 - 930 nm**	**710 - 990 nm**
Puissance maximale	**1,3 W**	**2 W**	**1,2 W**	**1,8 W**

Attention : coût élevé ! (100.000 €)

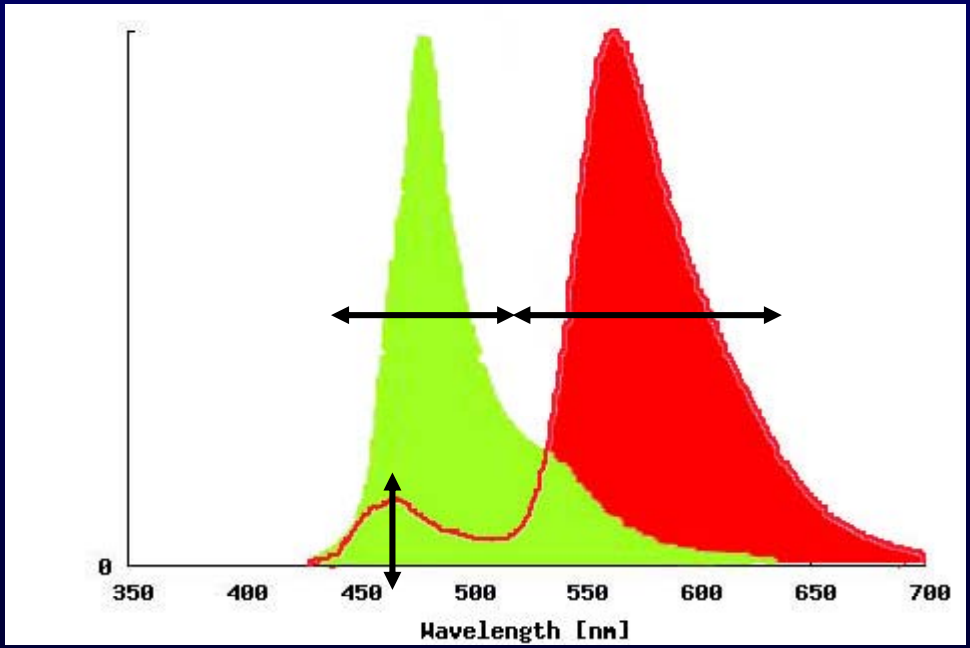
Confocal – Multiphoton Leica SP5




a
700
750
800
850
900
950
1,000
1.0
0.8
0.6
0.4
0.2
0.0
Arbitrary units
Rhodamine B
1-photon
2-photon
350
375
400
425
450
475
500
b
200
150
100
50
0
$\phi F \sigma_{2p}$ (GM)
eGFP
CFP
YFP
dsRed
wtGFP
700
750
800
850
900
950
1,000

Spectres élargis

Inconvénient : détections des multimarquages



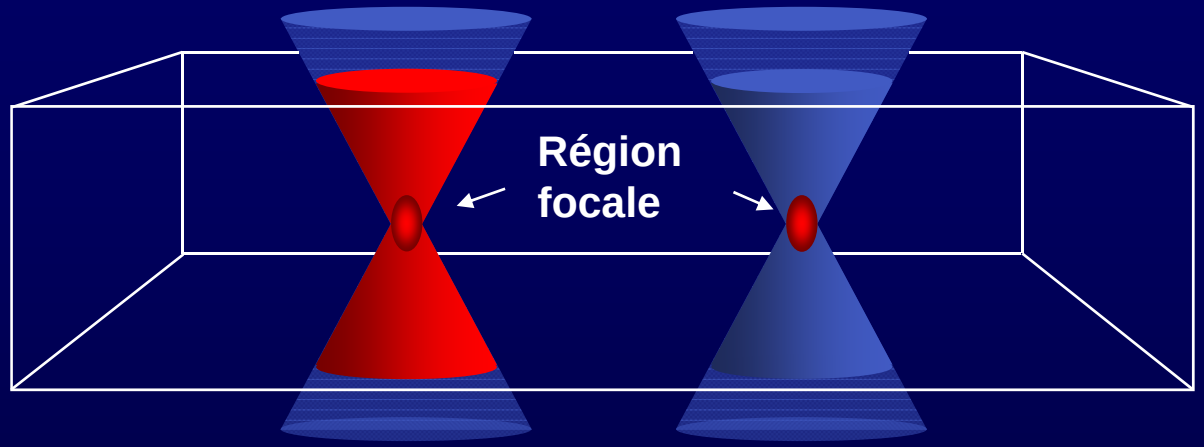
- Augmentation du cross-talk entre fluorochromes
- Une même longueur d’onde peut exciter 2 fluorochromes
- Possibles modifications et amplifications de certains « pic du spectre», en excitation ou émission

Avantages de la microscopie multiphotonique

- **Excitation fortement localisée** :
  - Réduction du photo-dommage cellulaire à la seule région focale : **viabilité accrue**
  - Réduction de la photo-atténuation : **bleaching diminué**
- **Infra-rouge : plus grande profondeur de pénétration : 1000 µm au lieu de 80 µm**
- **Collecte de la lumière plus efficace** : **rapport signal/bruit amélioré**

Photo-atténuation restreinte à la région focale

Microscopie conventionnelle



Microscopie multiphotons

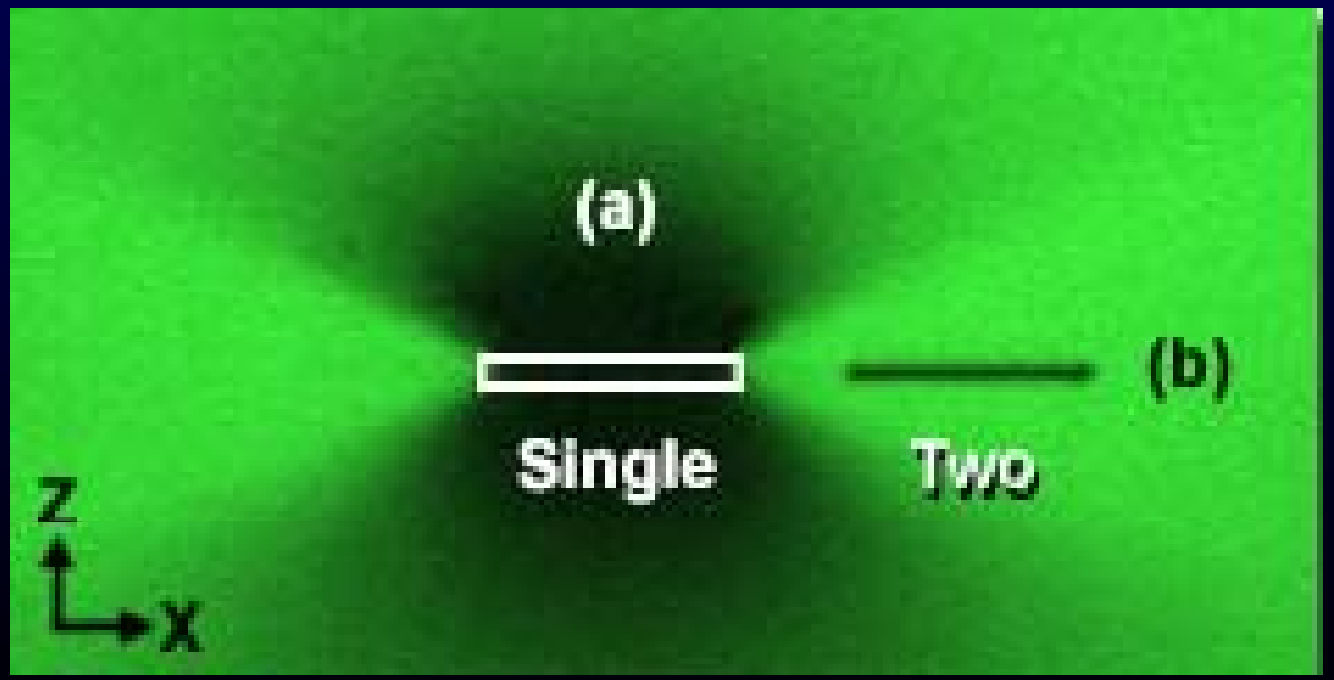
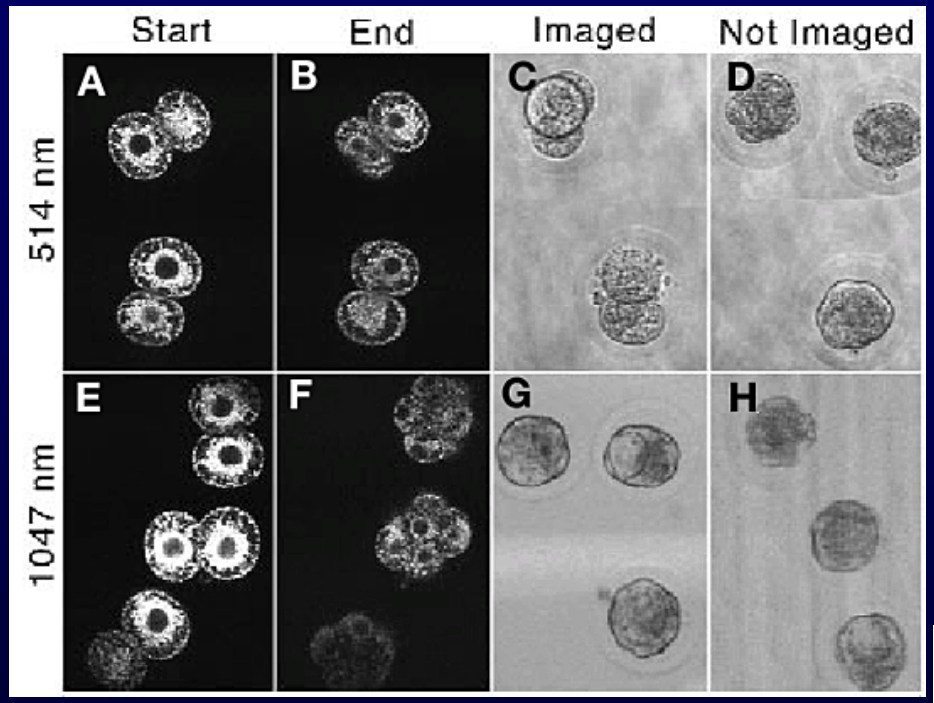


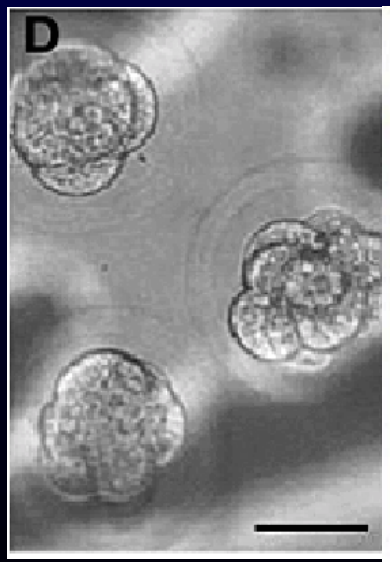
Photo-toxicité réduite : meilleure préservation de la viabilité



From Squirrel et al., 1999

Développement in vitro d'embryons de hamster, marquage au mitotracker

Imagerie sur 24hr
Intervalles : 2,5 ou 15 min

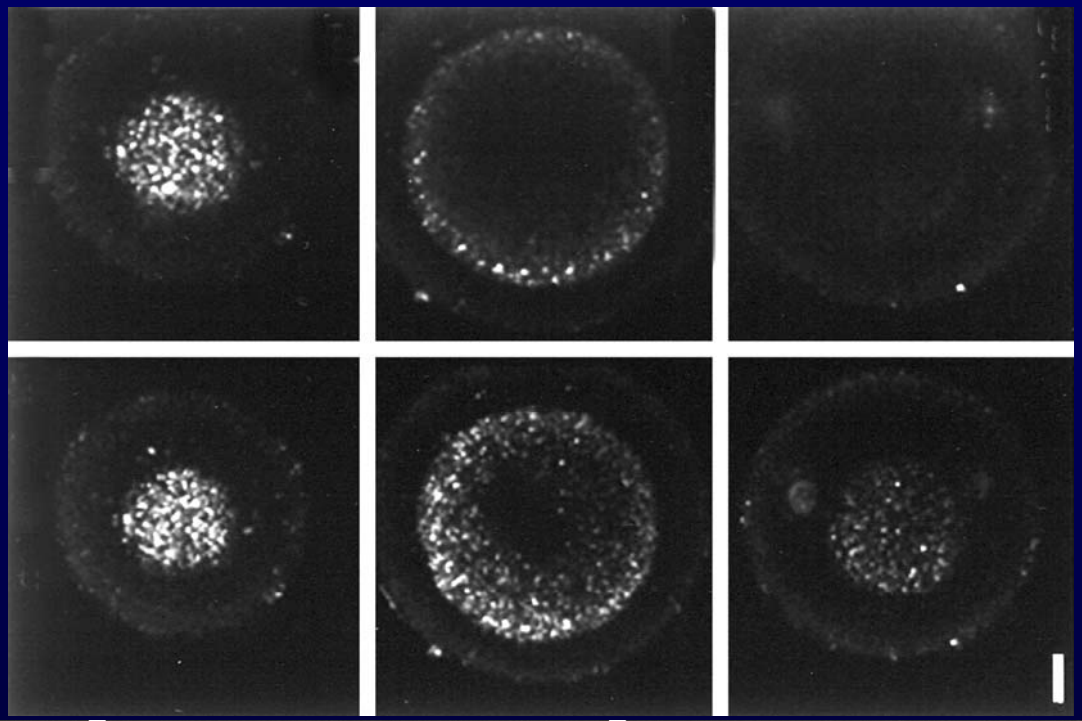


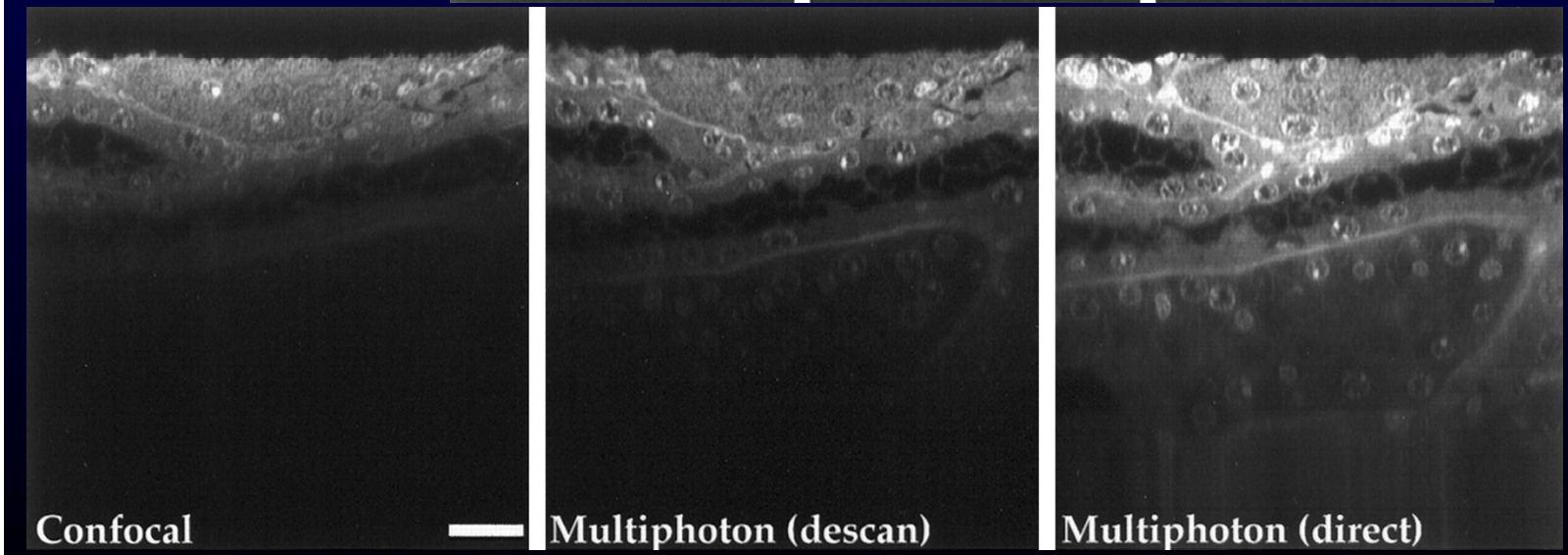
Réimplantation des blastocystes

Infra-rouge : meilleure profondeur de pénétration

Confocal

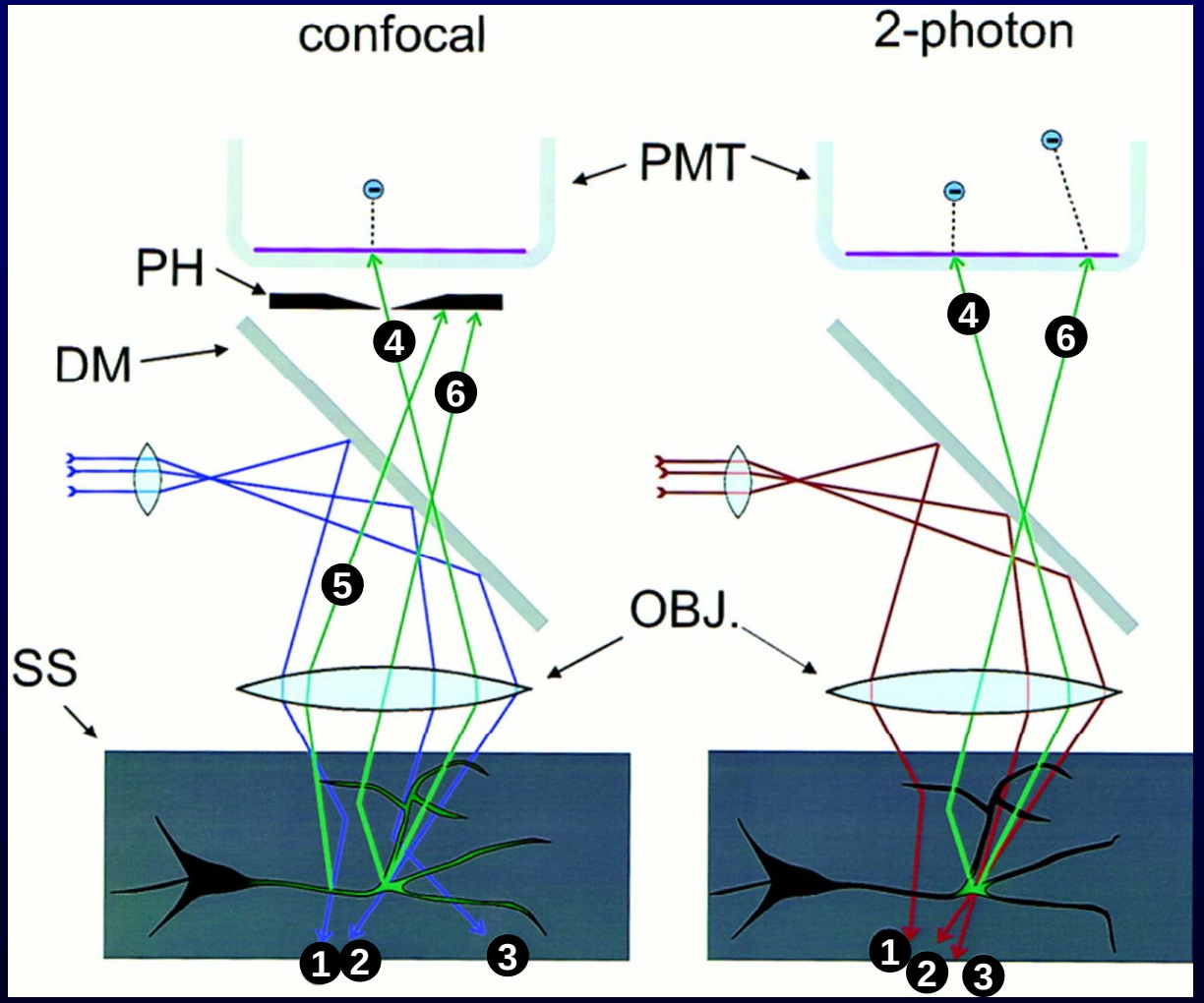
Bi-photon





Images : Victoria Centonze

Collecte de la lumière plus efficace



1-3 : photons d'excitation diffractés
4 : photons d'émission ballistiques (non-diffractés)
5 : photons d'émission hors plan focal
6 : photons d'émission du plan focal mais diffractés

Denk and Svoboda, 1997,
Neuron 18, 351-357

Application : Imagerie in vivo, en profondeur et à haute résolution

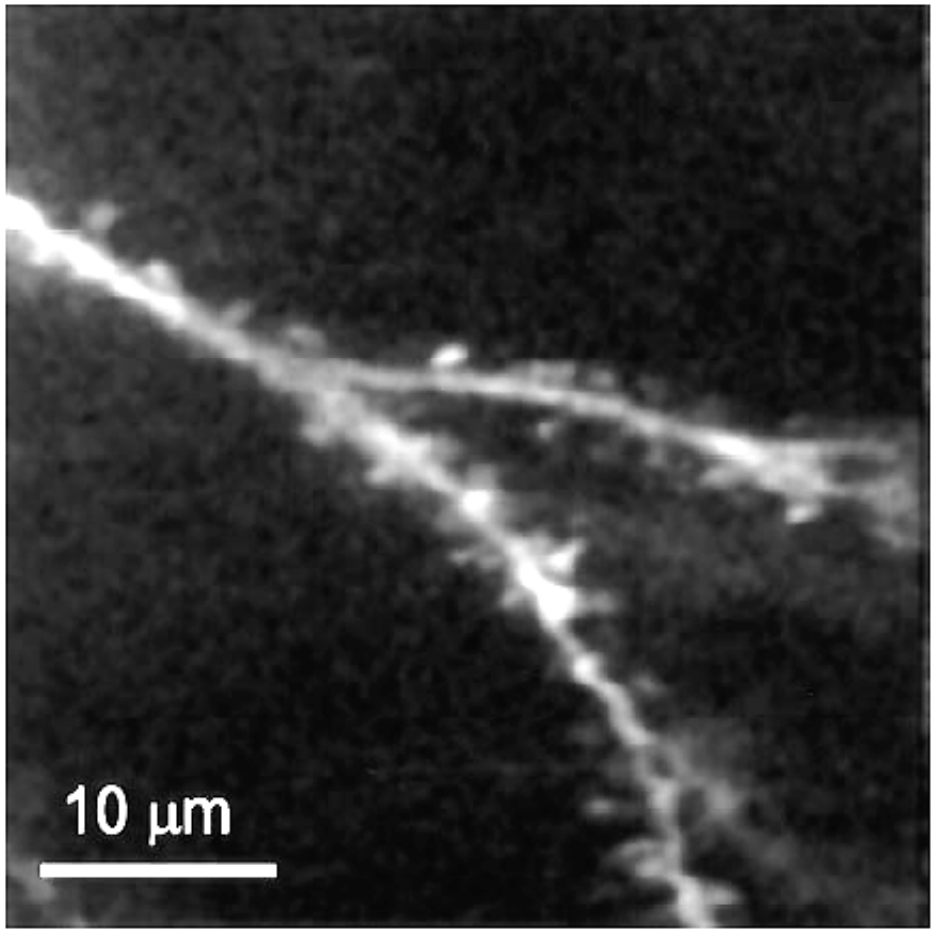


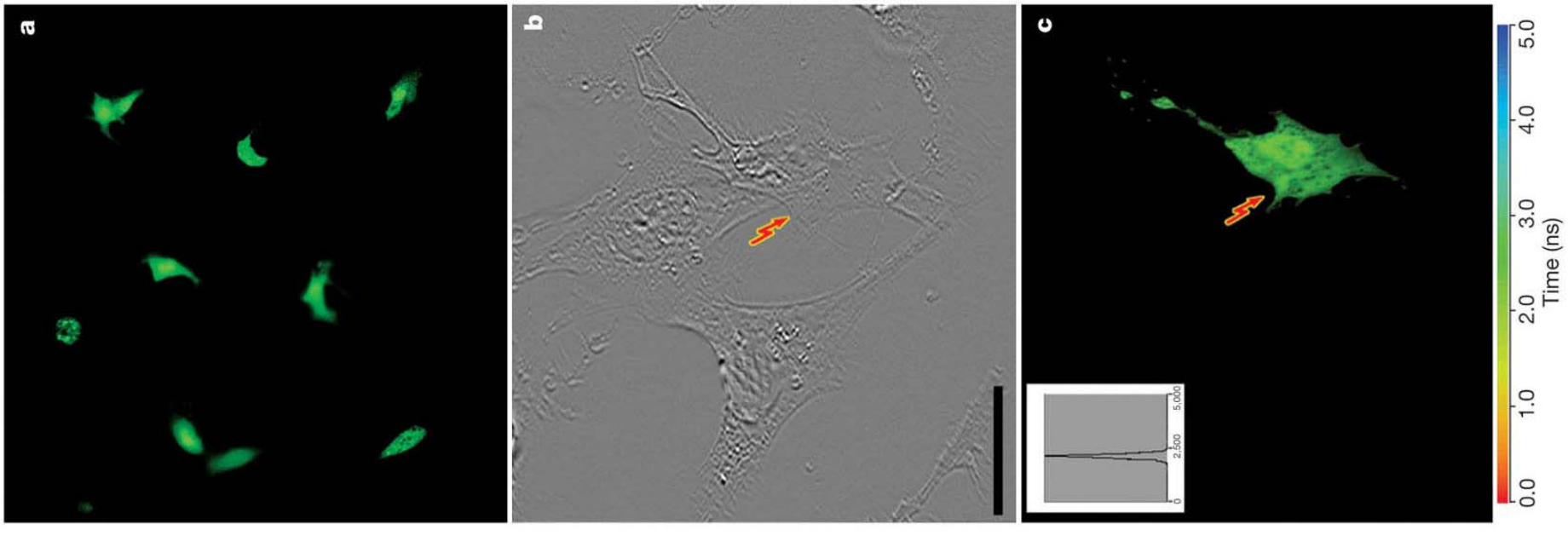
Figure 5. High Resolution Imaging In Vivo

A neocortical pyramidal neuron in layer 2–3 of the rat somatosensory cortex was filled iontophoretically with Calcium Green 1. Shown is a basal spiny dendrite approximately 200 µm below the pial surface (K. S., W. D., D. Kleinfeld, and D. Tank, unpublished data). The excitation source was a Ti:sapphire laser (840 nm wavelength).

Denk and Svoboda, 1997,
Neuron 18, 351-357

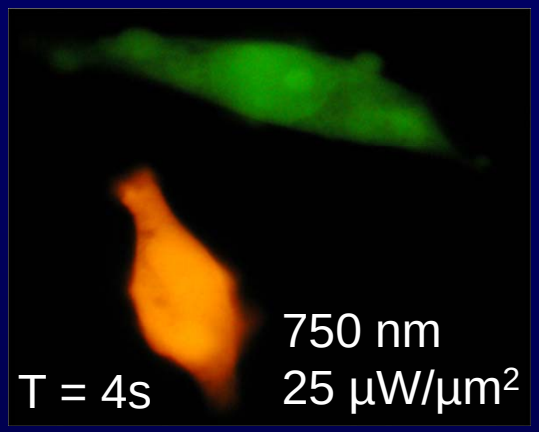
Application : transfection localisée

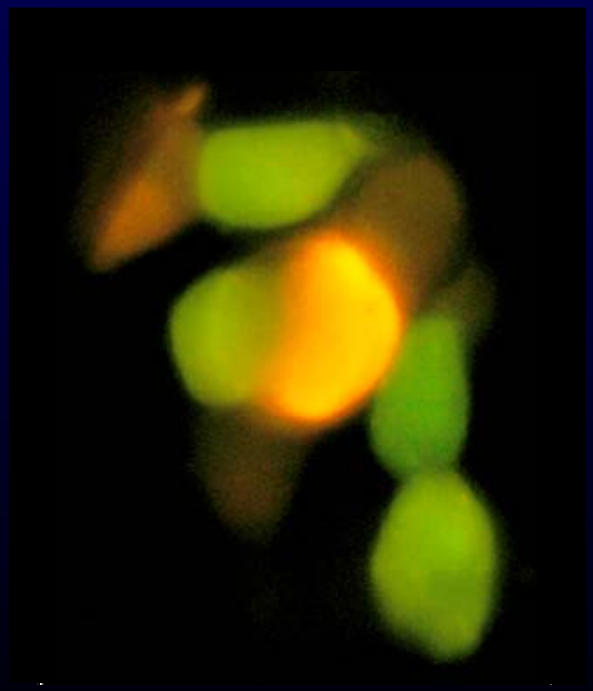
- Cellules CHO, EGFP
- Perforation : 10^{12} W/cm^2, 16 ms, 800 nm, puissance moyenne 50-100 mW
- 10 à 15 s pour chaque cellule

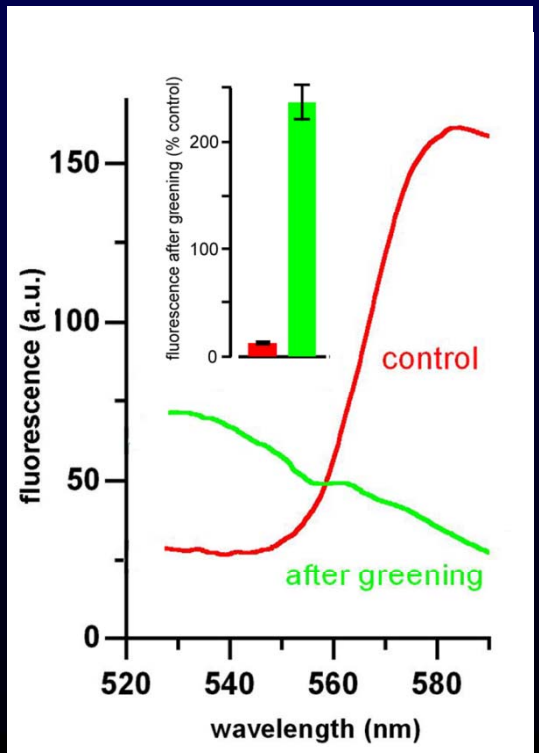


From Tirlapur and König, 2002, Nature 418, 290-291

Imagerie multiphotonique, outil d'intervention in vivo : Traçage de cellules ou de compartiments cellulaires







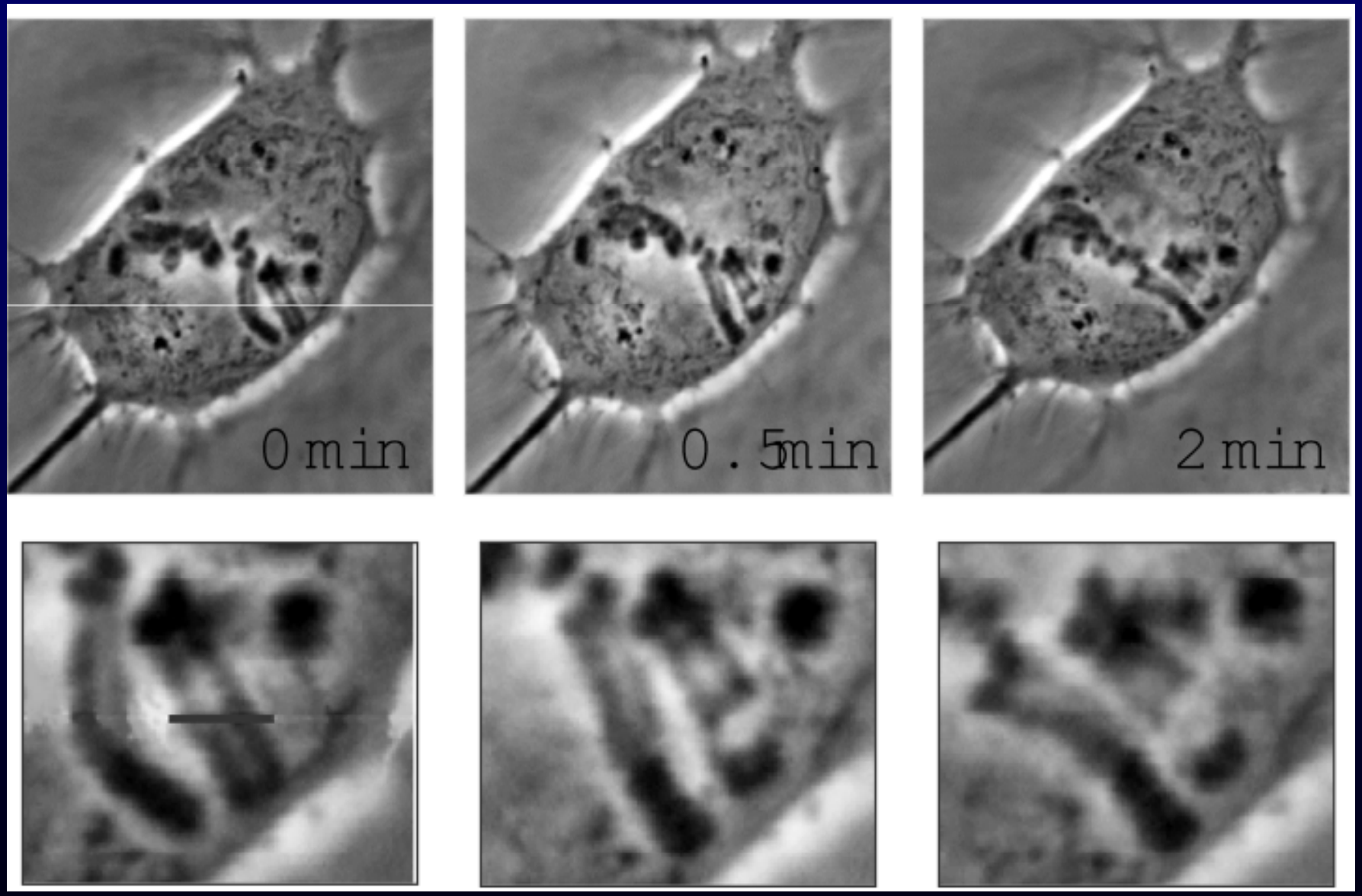
Mécanisme :

1. Bleaching de la forme mature rouge de la ds-red
2. Accroissement de l'émission de la forme immature verte par réduction du FRET

- Persistance : au moins 3 jours
- Pas d'effet sur la viabilité

From Marchant et al., 2001, Nature Biotech. 19, 645-649

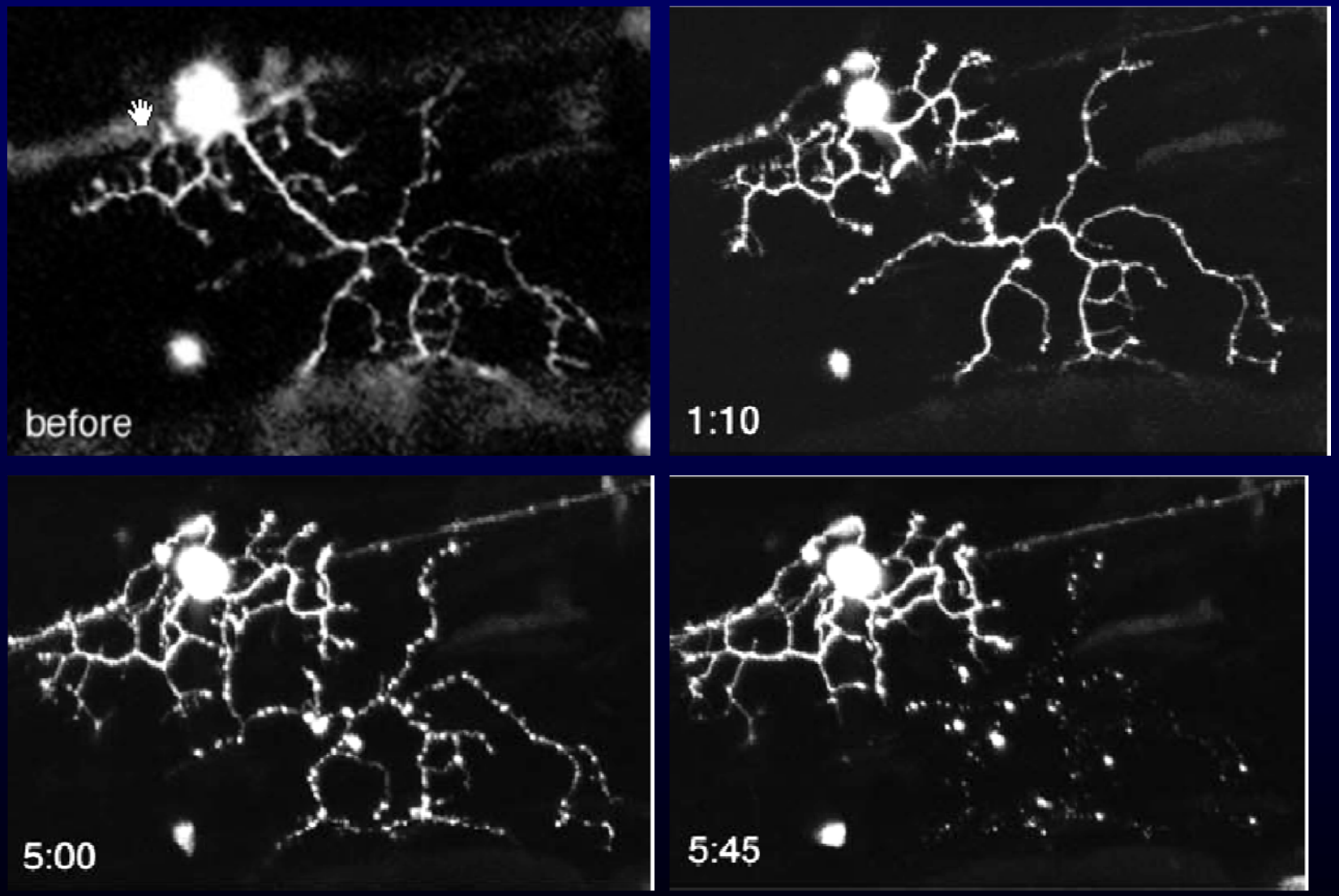
Application : chirurgie laser dans les cellules vivantes



König, 2000, J. Microscopy, 200, 83-104

Application : chirurgie laser dans les cellules vivantes

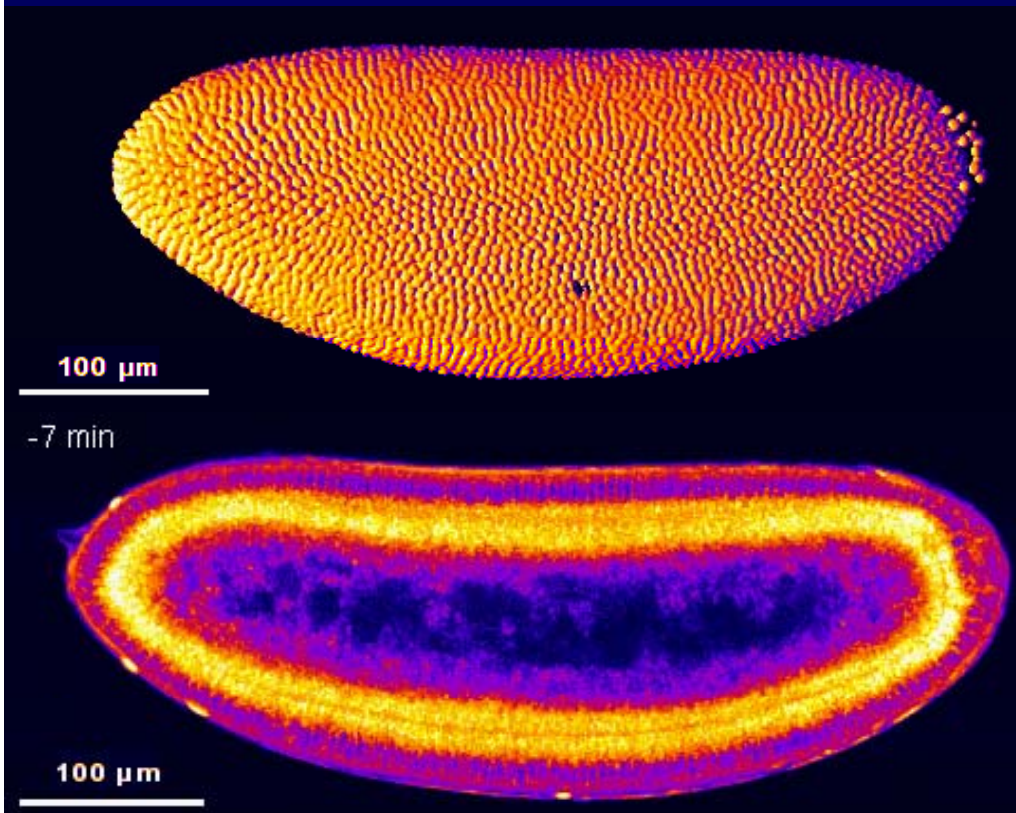
Zebrafish GFP – Neurone de Rohon-Beard



From Galbraith et Terasaki, 2003, Mol. Biol. Cell, 14, 1808-1817

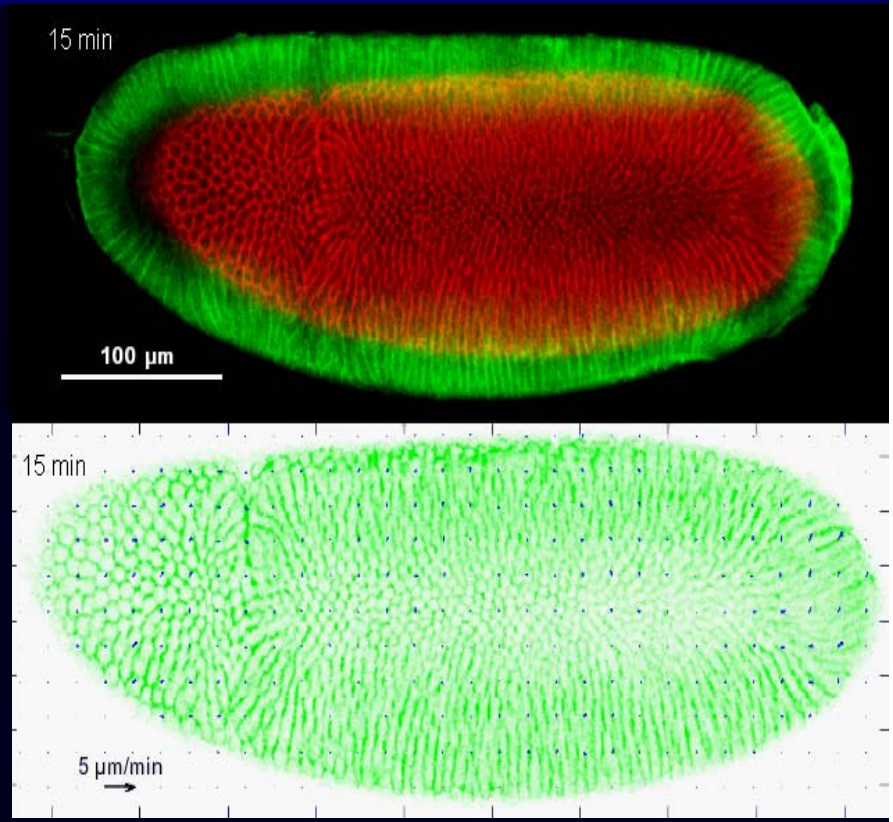
Application : morphogenèse de l'embryon de drosophile

Bi-photon, 4D, 1im/2 min, acquisition 1 min



Troisième harmonique, 1 im/30 sec

Bi-photon – 2 plans



Supatto et al., 2005, PNAS, 102, 1047

Bilan fluorescence biphotonique

Avantages

- Excitation localisée au focus
- Dommages localisés au focus (nano-chirurgie)
- Meilleure résolution
- Plus grande pénétration dans les tissus
- Observation en profondeur
- Excitation multiple par une λ unique

Inconvénients

- Élargissement des spectres
- Laser puissant, potentiellement destructeur (ex : absorption par les pigments → combustion)
- Limitation des fluorochromes et milieux de montage utilisables (problèmes avec les milieux de montages gras, moewiol, glycérol, → ébullition du milieu, cloques…
- Optique adaptée aux infrarouges
- Excitation multiple par une λ unique