

# La microscopie optique

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ANbioΦ

# A quoi ça sert?

**Observer** un objet, un phénomène...

avec une bonne **résolution spatiale** (voire **temporelle**)  
sans trop de perturbations (**innocuité**).

L'instrument doit être **sensible**  
et la mesure (l'image) **contrastée**.

## Le plan

- Les contrastes
- Les microscopies de fluorescence
- La microscopie confocale
- Augmenter la résolution
- Les microscopies non linéaires

# Les 1<sup>er</sup> microscopes

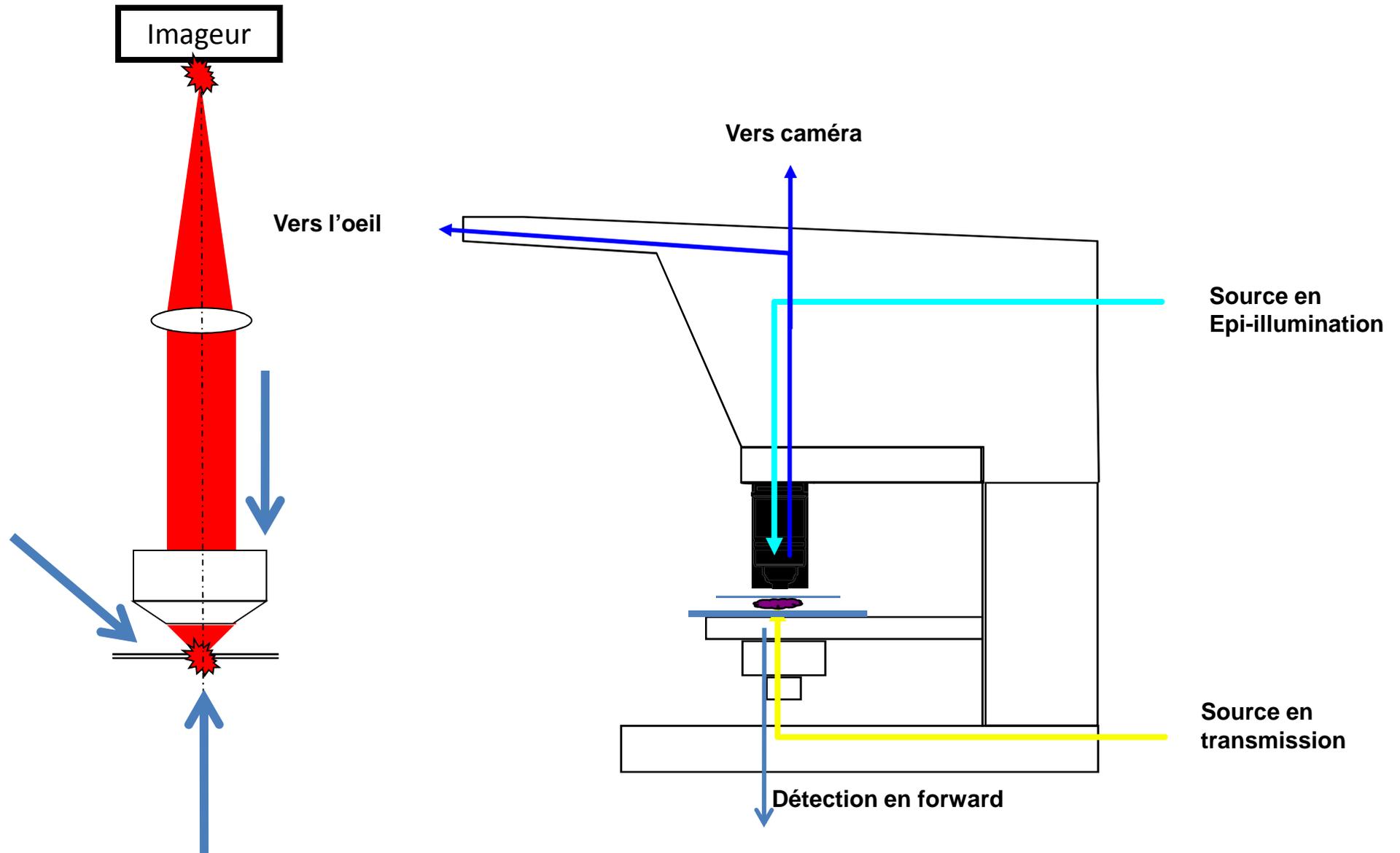


Microscope Carl Zeiss 1891

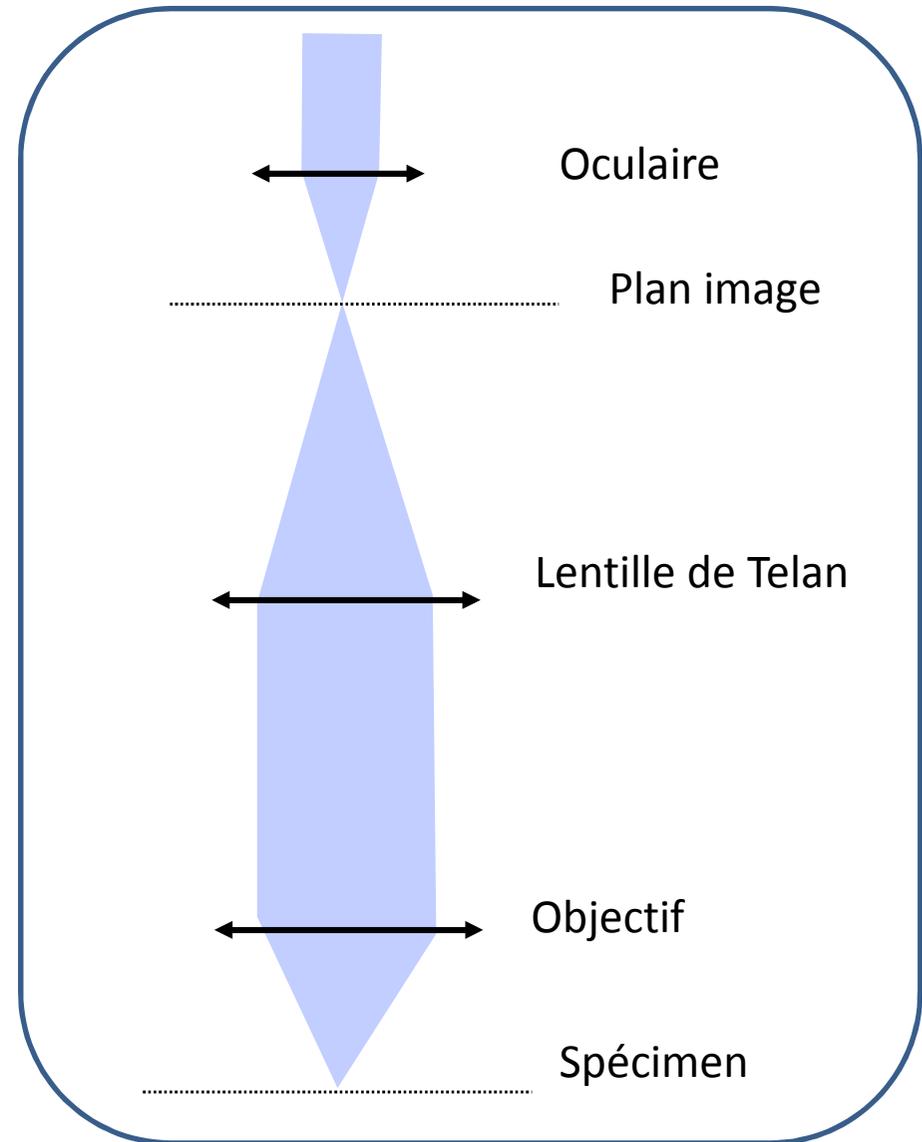
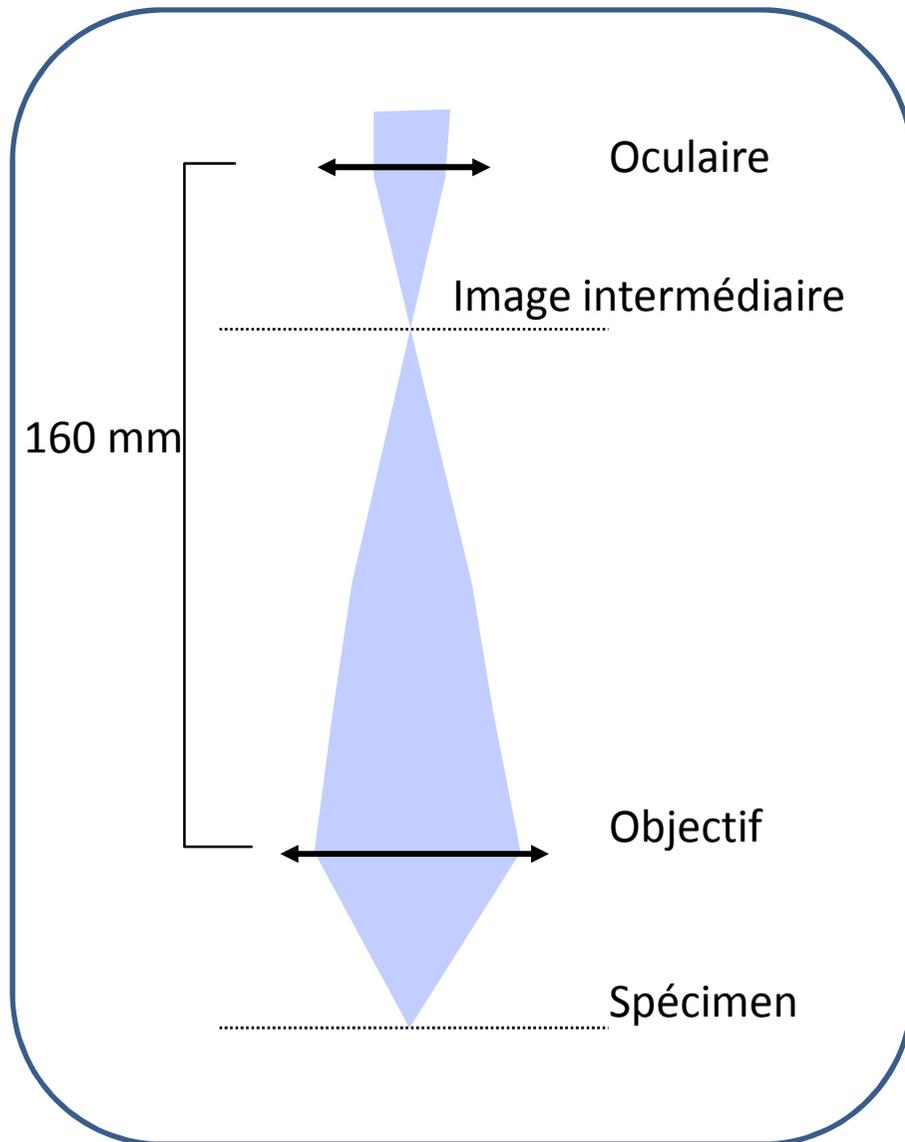
Vers 1880, des détails aussi petits que  $0.2 \mu\text{m}$  sont déjà accessibles.



# Principe du microscope

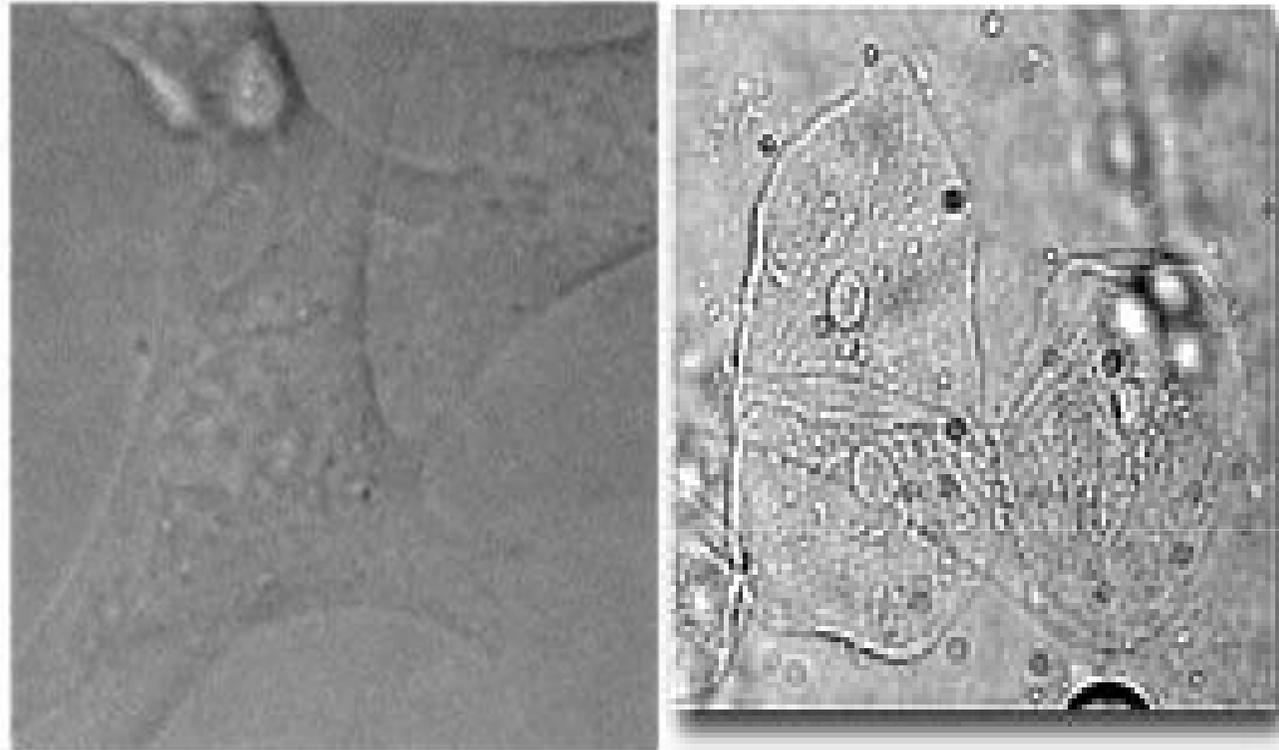


# Principe du microscope



# **Les contrastes en microscopie**

# Contraste en microscopie



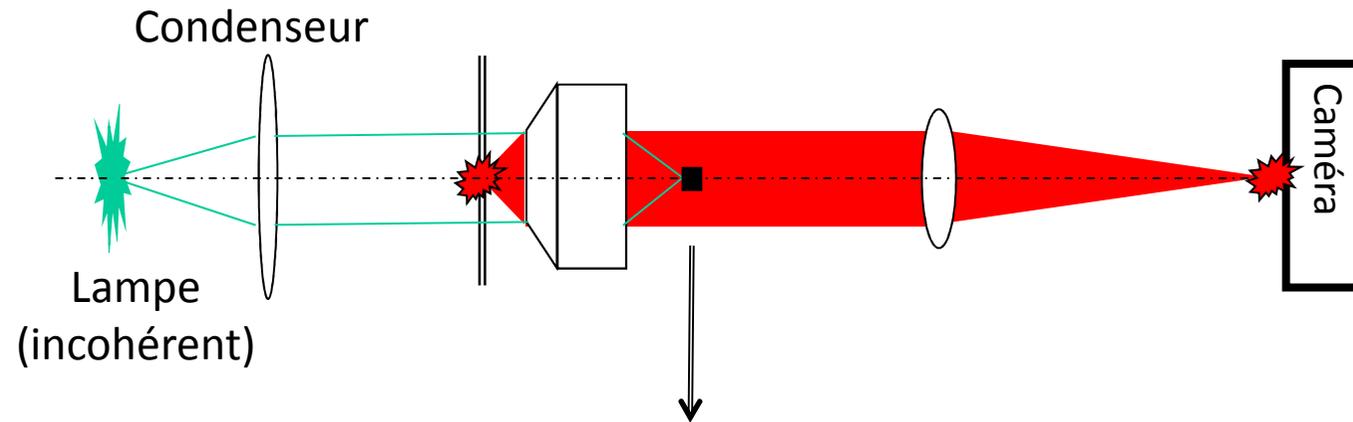
**D'où vient le contraste?**

Absorption, diffusion,  
réfraction, réflexion.

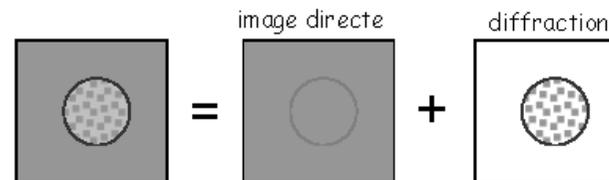


Variations d'intensité,  
de couleurs, de netteté

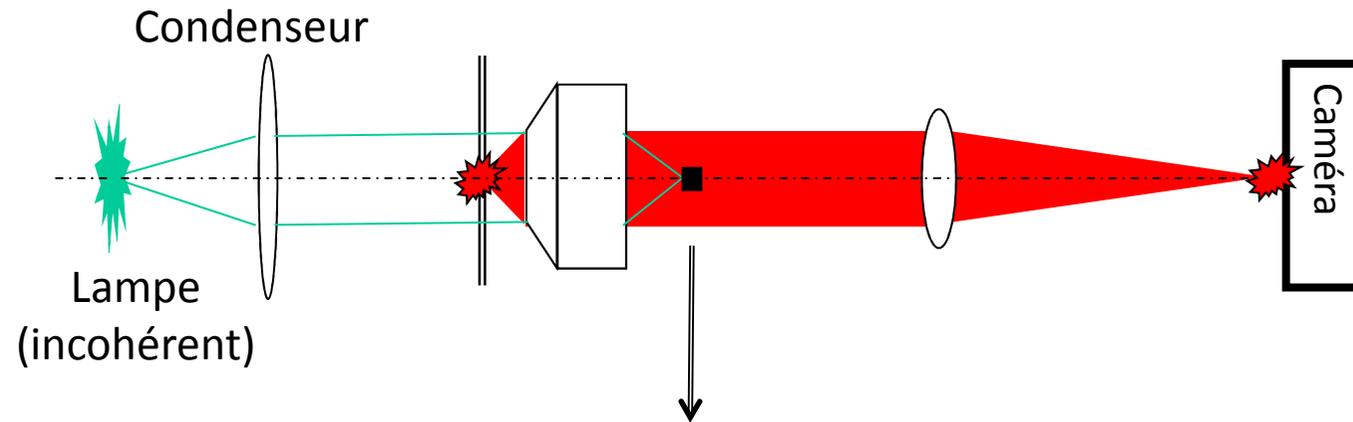
# Contraste en microscopie



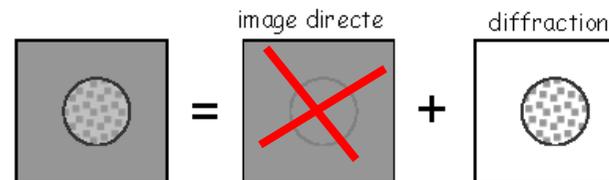
Filtre occultant  $\Rightarrow$  Fond noir  
Filtre déphasant  $\Rightarrow$  Contraste de phase



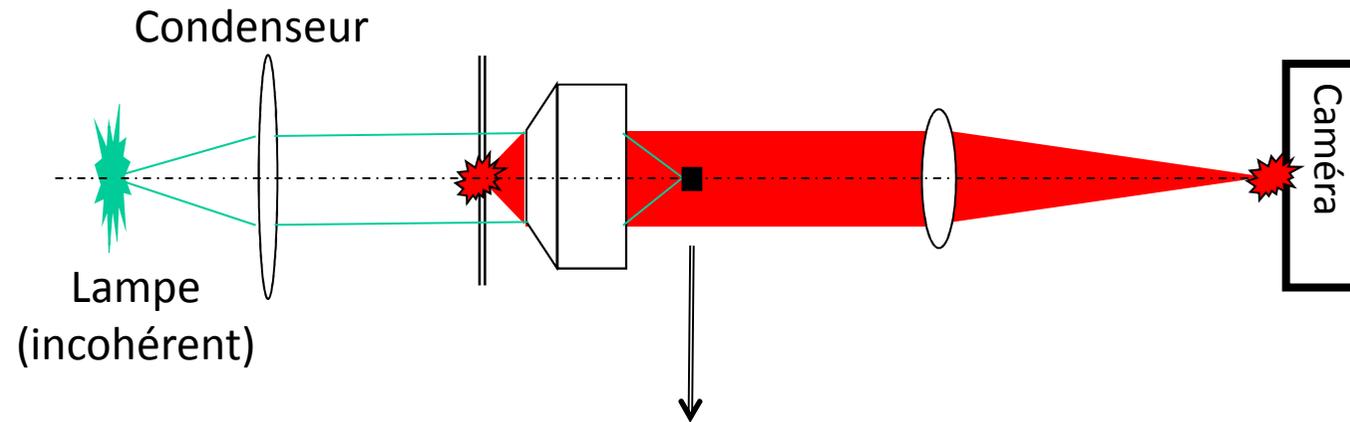
# Contraste en microscopie



Filtre occultant  $\Rightarrow$  Fond noir  
Filtre déphasant  $\Rightarrow$  Contraste de phase



# Contraste en microscopie

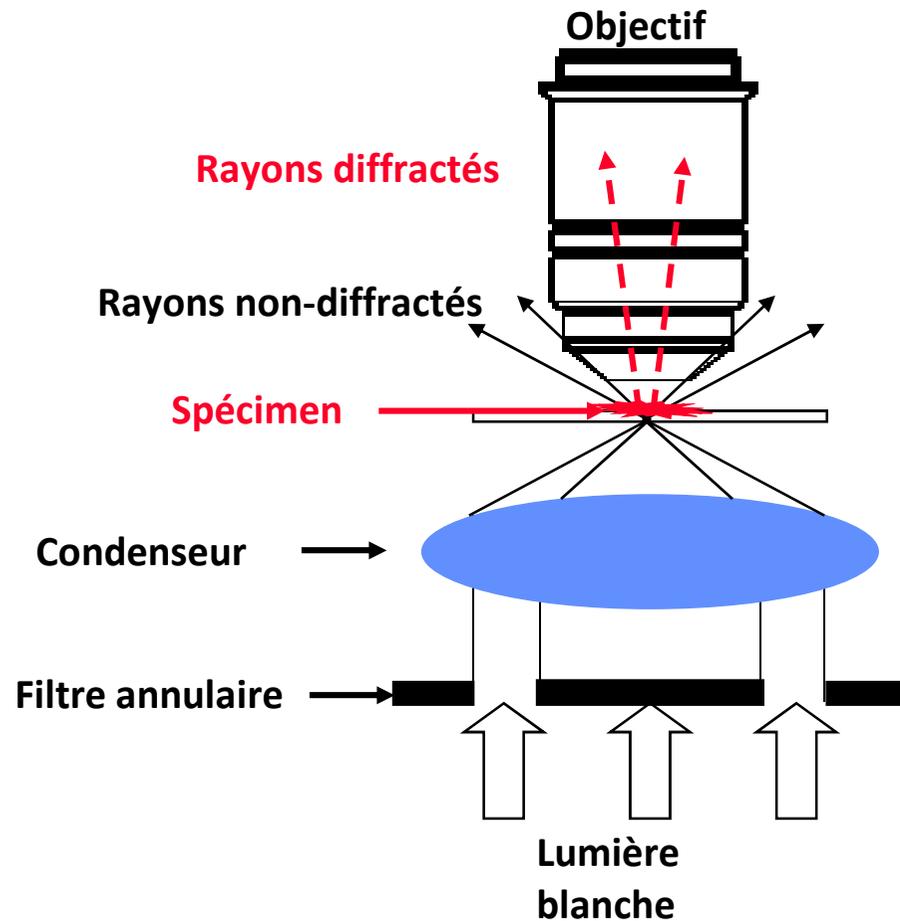


Filtre occultant  $\Rightarrow$  Fond noir  
Filtre déphasant  $\Rightarrow$  Contraste de phase

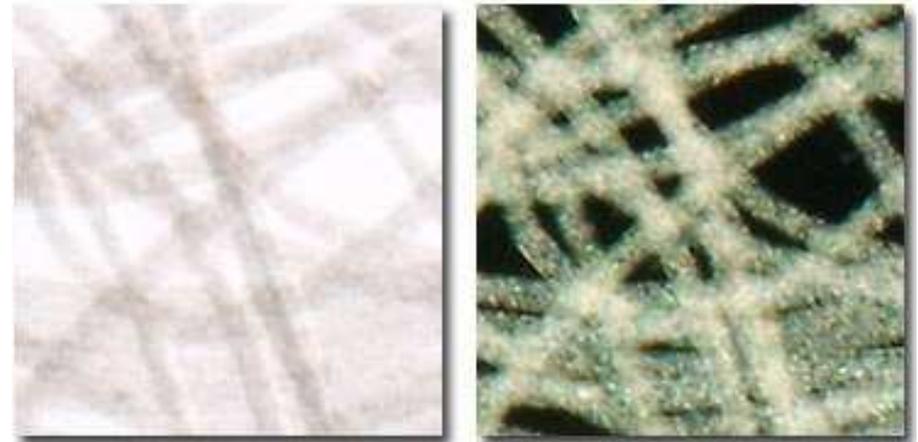
$$\text{Image totale} = \text{image directe} + \text{diffraction}$$

$e^{i\varphi}$

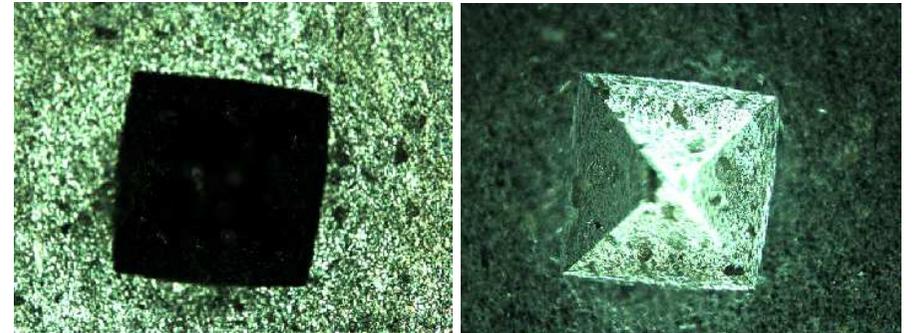
# Microscopie sur fond noir



Fibers in Brightfield and Darkfield Illumination



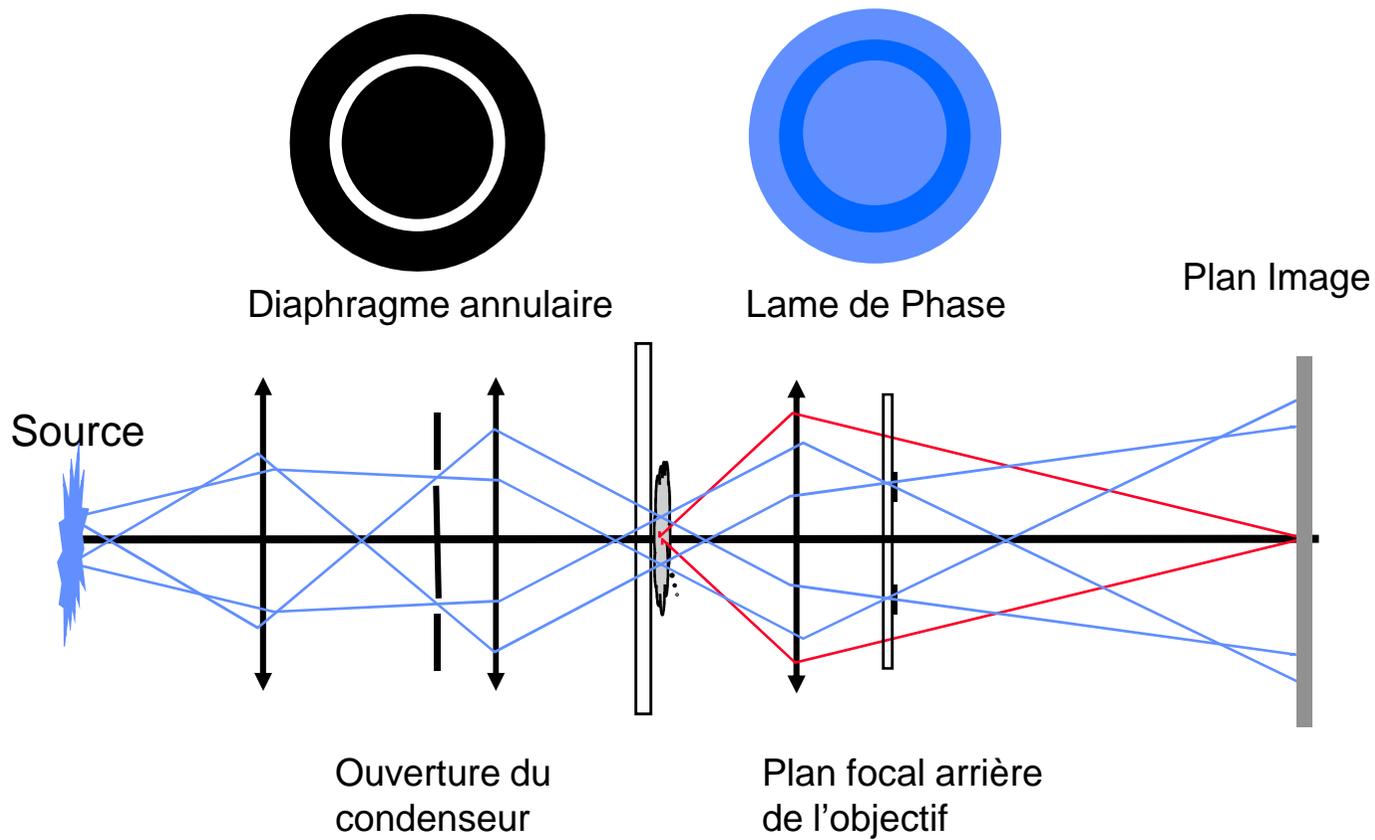
<http://micro.magnet.fsu.edu/primer/techniques/darkfieldgallery>



[http://prn1.univ-lemans.fr/prn1/siteheberge/PublisCours-OPI/OPI\\_fr\\_M03\\_C04/co/Contenu\\_K22.html](http://prn1.univ-lemans.fr/prn1/siteheberge/PublisCours-OPI/OPI_fr_M03_C04/co/Contenu_K22.html)

# Contraste de phase

*(Zernike prix Nobel 1953)*

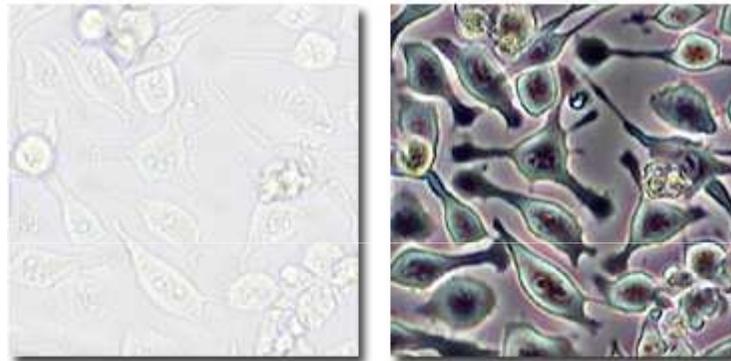


# Contraste de phase

*(Zernike prix Nobel 1953)*

## Image de la phase

Living Cells in Brightfield and Phase Contrast



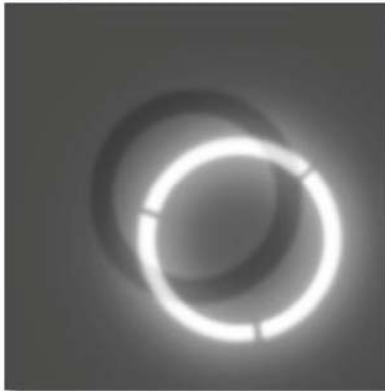
(a)

Figure 2

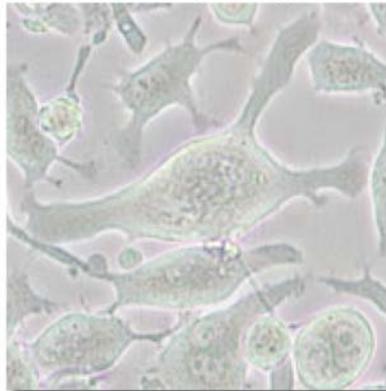
(b)

<http://www.microscopyu.com/articles/phasecontrast/phasemicroscopy.html>

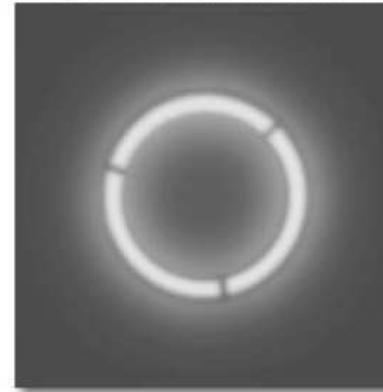
Objective Rear Focal Plane



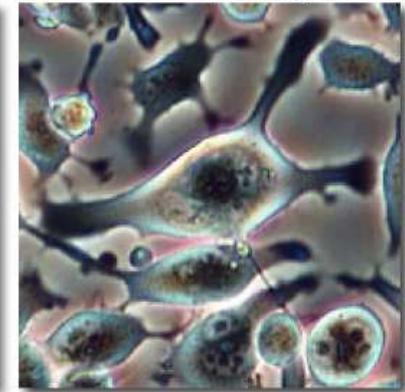
Microscope Image



Objective Rear Focal Plane

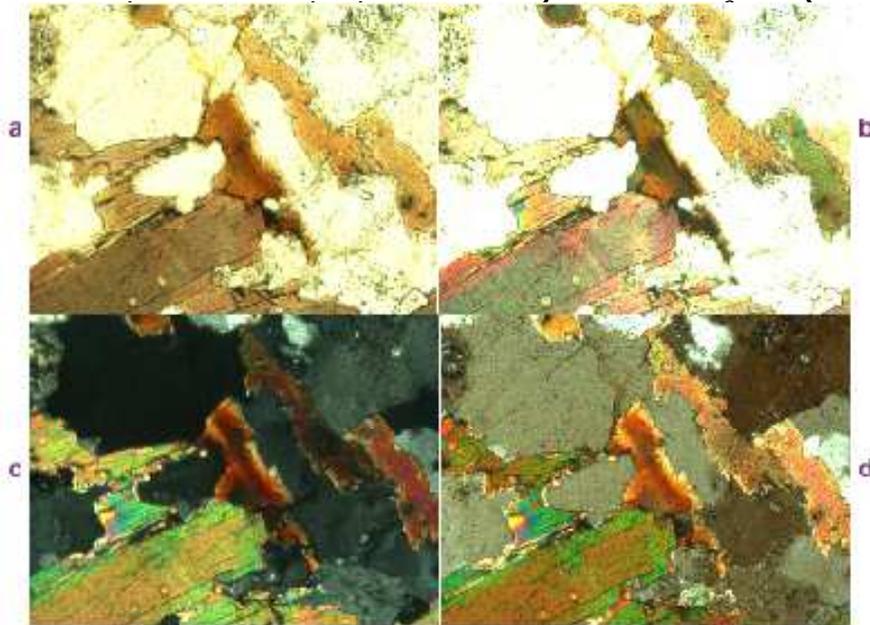
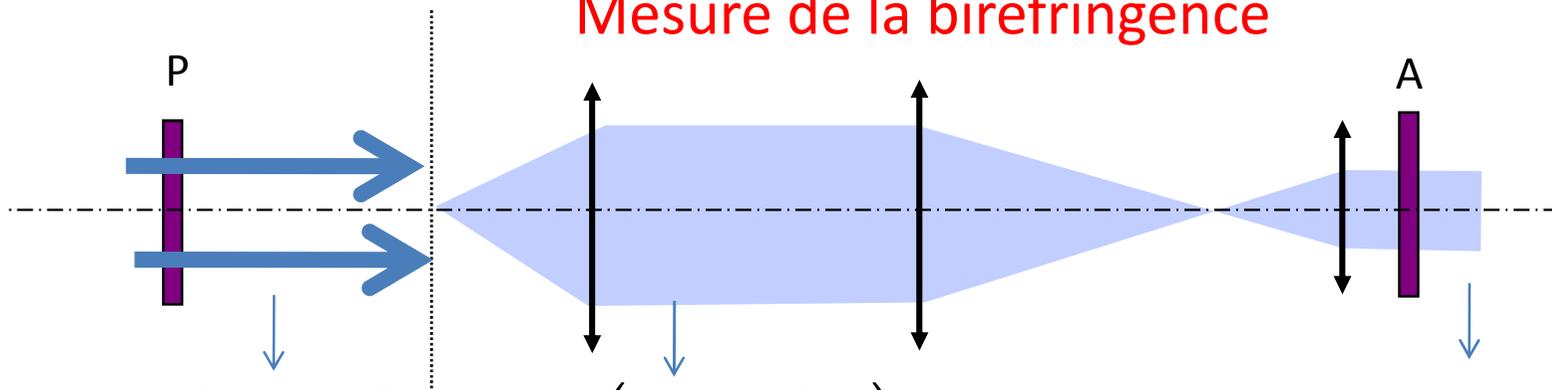


Microscope Image



# Microscope polarisant

Mesure de la biréfringence

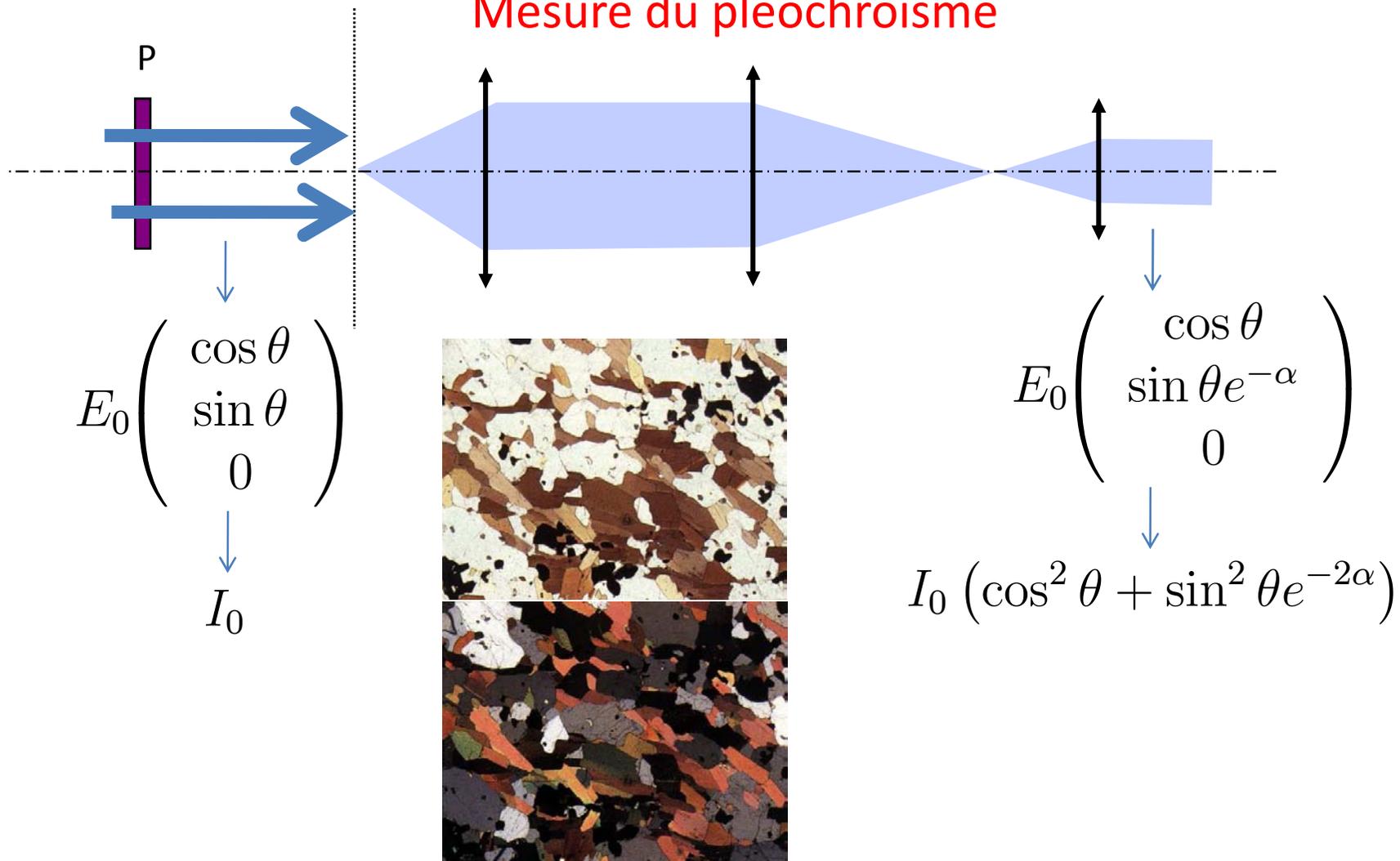


$$\frac{E_0 \sin(2\theta) (e^{i\varphi} - 1)}{2} \begin{pmatrix} -\sin \theta \\ \cos \theta \\ 0 \end{pmatrix}$$

$$I_0 \sin^2(2\theta) \sin^2(\varphi/2)$$

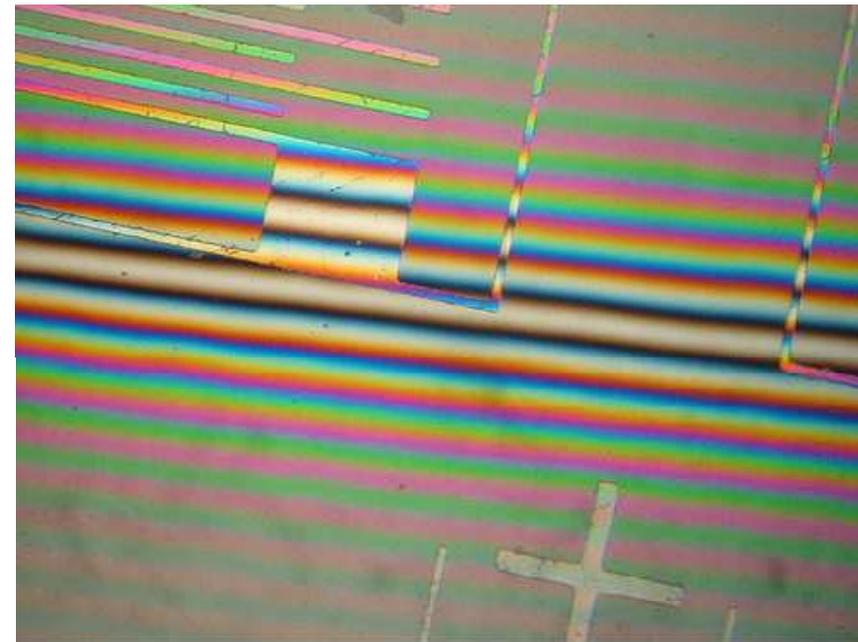
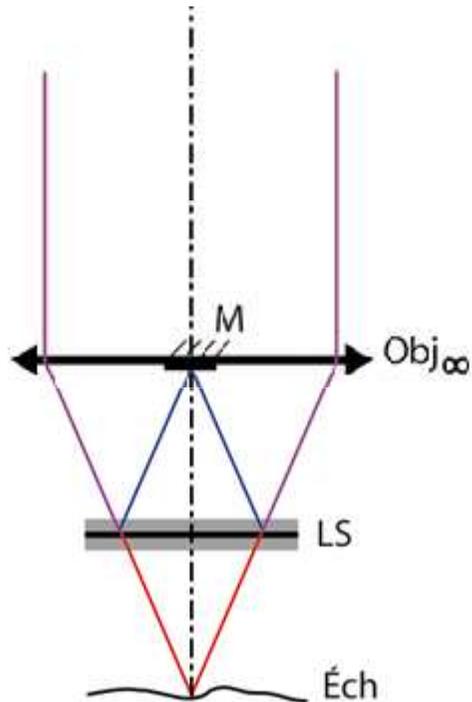
# Microscope polarisant

## Mesure du pléochroïsme



# Contraste par interférométrie

## Image du relief



Microphotographie de pistes (épaisses,  $\sim 0.5\mu\text{m}$ ) d'aluminium sur un substrat plan de silicium.  
Objectif de Mirau 10 $\times$ /0,30  $\infty$ /0.

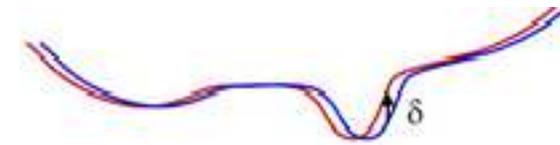
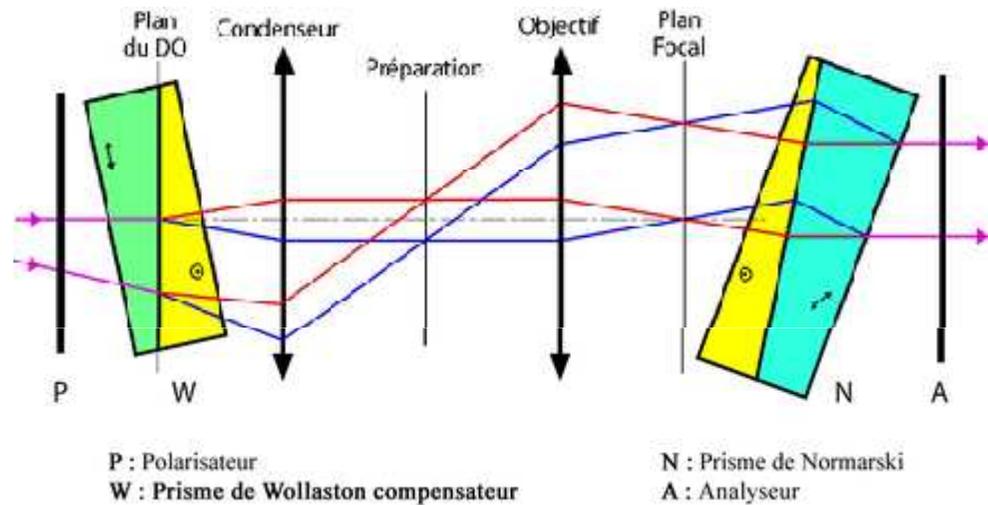
- Obj<sub>∞</sub>** : Objectif de microscope (corrigé à l'infini) à grande frontale
- LS** : lame semi-réfléchissante 50% avec compensatrice symétrique
- M** : Miroir plan de référence
- Éch** : Échantillon à observer

La présence du miroir induit une obturation centrale dans le système optique.

# Microscope DIC

“Differential Interference Contrast”

## Image du gradient de la phase



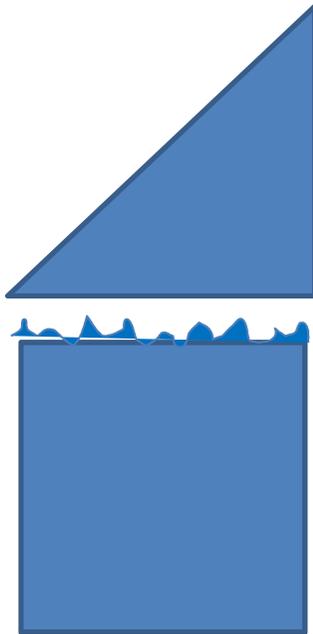
$$\delta < \text{résolution}$$

$$I \propto 1 + \cos(\Delta\varphi + \varphi_0)$$

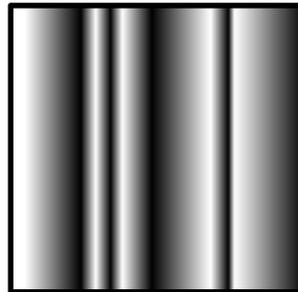
gradient de phase

# DIC ou Contraste de phase?

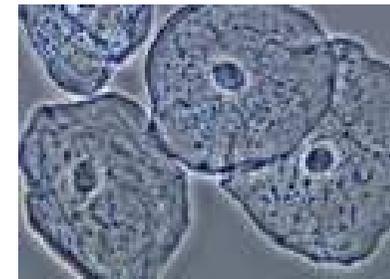
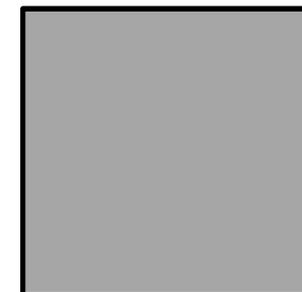
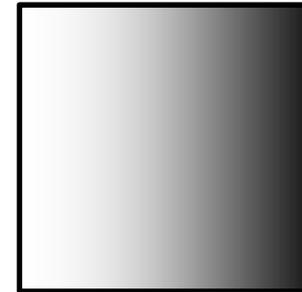
Objets



Images DIC



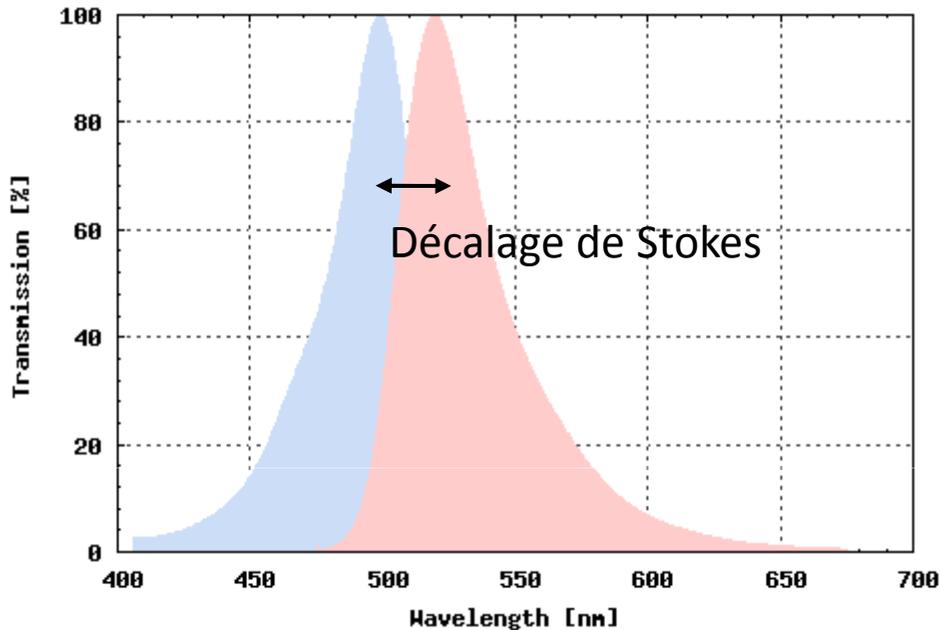
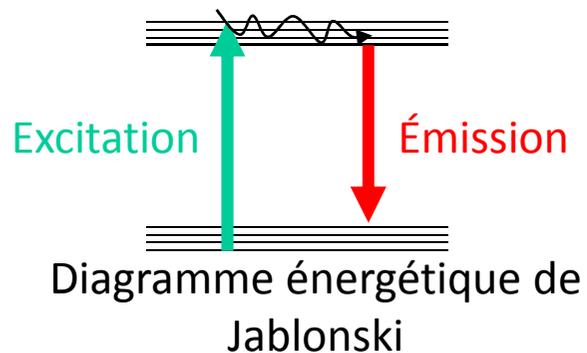
Images de la phase



Contraste de phase insensible aux pbs de polarisation  $\Rightarrow$  boite de Petri  
DIC utilisable avec des objectifs à forte NA  $\Rightarrow$  meilleur résolution

<http://www.microscopyu.com/galleries/dicphasecontrast/index.html>

# Contraste de fluorescence



<https://www.omegafilters.com/curvo2/index.php?dyes=16&xmin=400>

## Intérêts de ce contraste

- Possibilité de filtrer simplement le faisceau d'excitation et ne détecter que l'émission
- Découplage de l'excitation et de l'émission
- Rendement de fluorescence
- Marquage spécifique

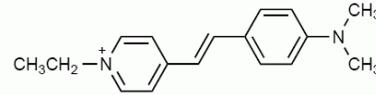
## Défauts

- Photobleaching
- Toxicité

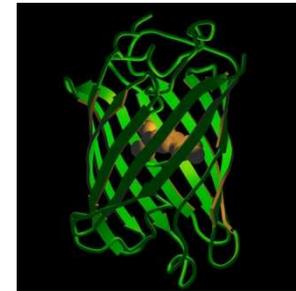
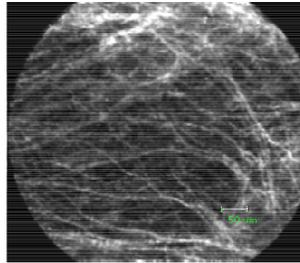
## Particularités

- Temps de désexcitation
- Transfert d'énergie

# Les luminophores

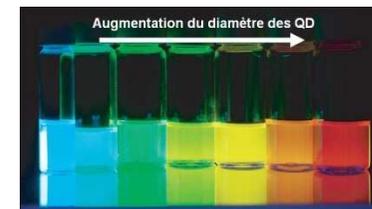


- **Fluorophores exogènes** intercalables, greffables (spécifique)...
- **Protéines chimériques** (GFP...);
- **Billes fluorescentes** (plus lumineux mais plus gros);
- **Fluorophores endogènes** (moins lumineux);

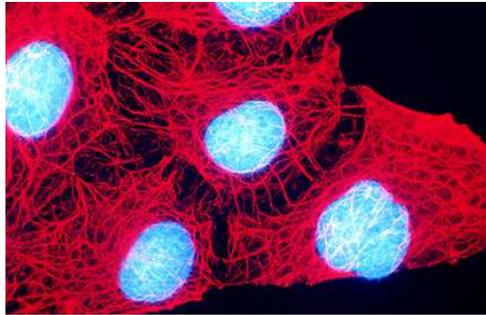


- **Nanocristaux de semi-conducteur** (plus lumineux, moins de photobleaching mais blinking! et pb de greffage et de phototoxicité).

- **Nano-diamants** (plus stables, plus lumineux...)

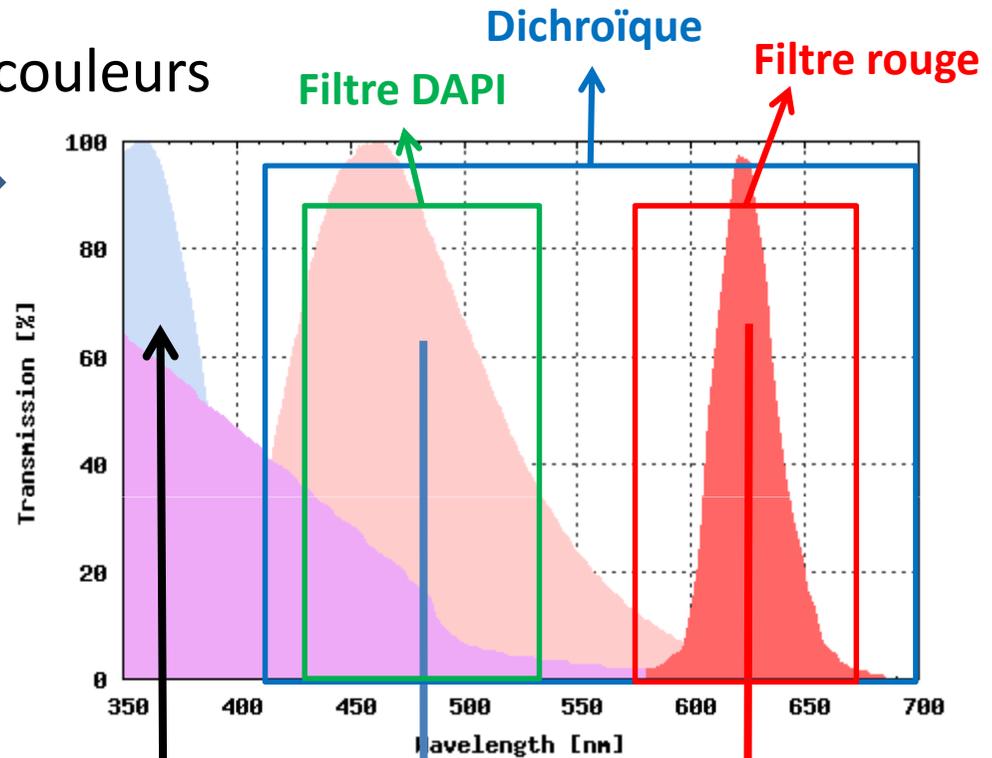


# Contraste de fluorescence (spectral)



Cellules de foie [www.zeiss.de](http://www.zeiss.de)

2 (fausses) couleurs

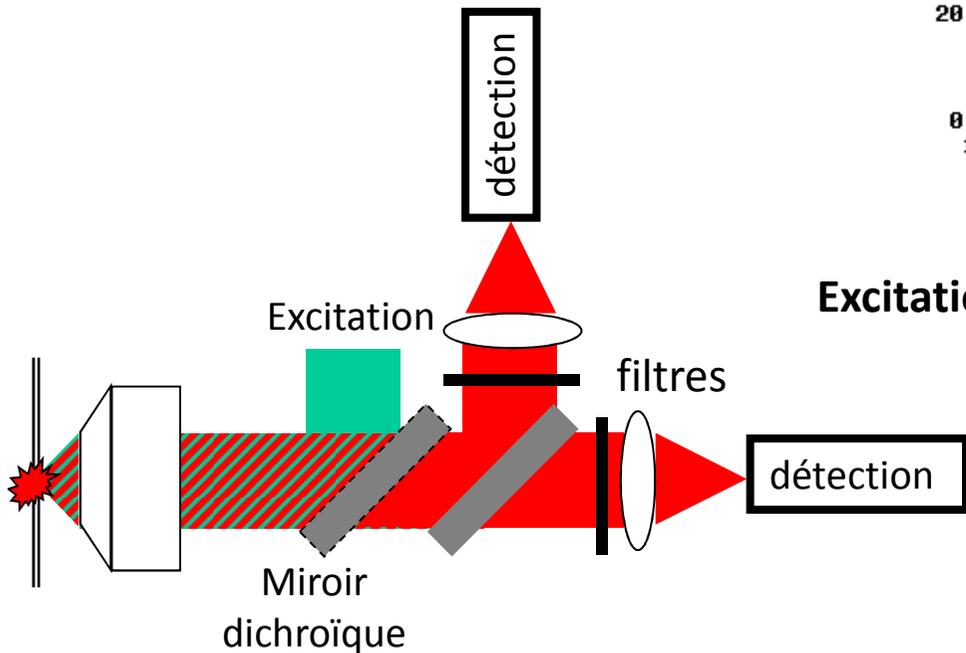


Excitation à 360nm

Émission

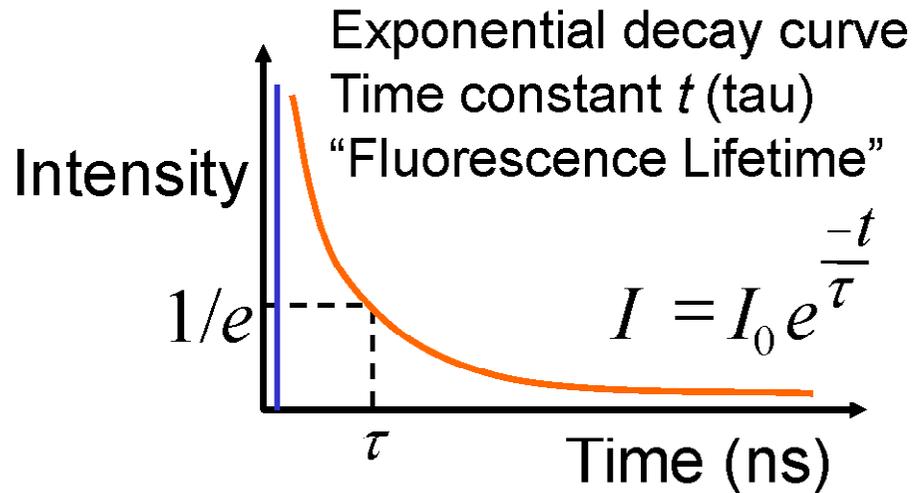
Noyau (DAPI)

Cytosquelette



# Contraste de fluorescence (temps de vie)

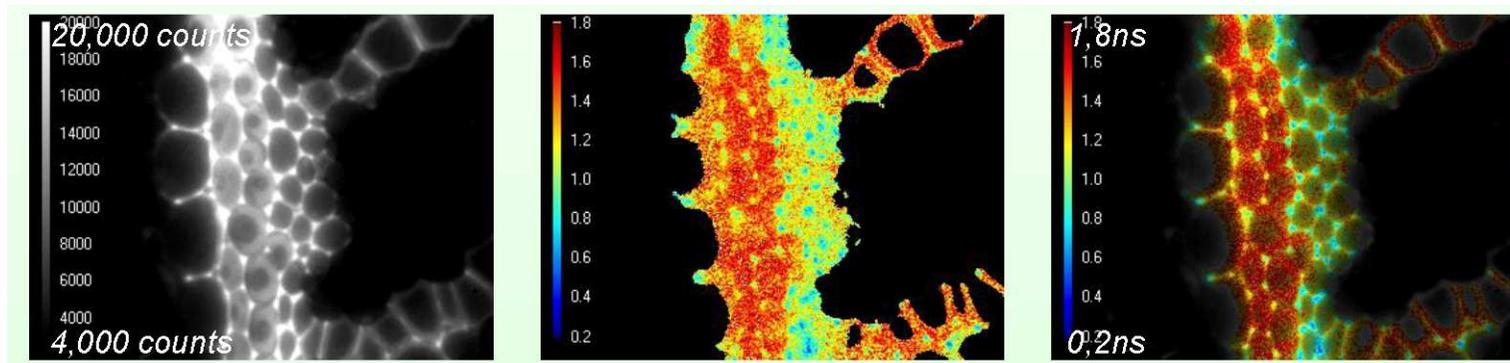
## FLIM (*Fluorescence Lifetime Imaging Microscopy*)



Deux méthodes (Cf F. Sureau):

- Time domain
- Frequency domain

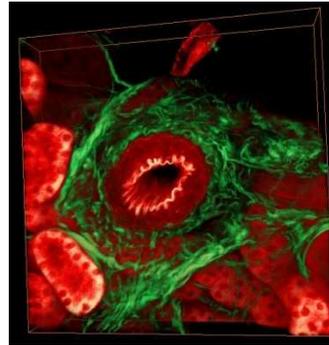
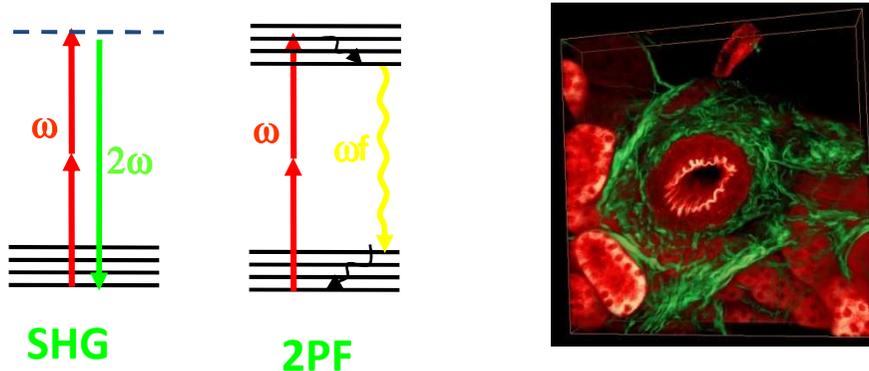
Permet de différencier 2 fluorophores qui ont même spectre d'émission



Images Sandrine Lévêque-Fort - Lab.de Photophysique Moléculaire

# Contraste en microscopies cohérentes

- **Génération de Second Harmonique (SHG)**  $\vec{P}^{2\omega} = \chi^{(2)} \vec{E}^\omega : \vec{E}^\omega$   
 $\Rightarrow$  uniquement sur des matériaux non-centrosymétriques!



Artère de rein fibrotique  
 Rouge:2PEF  
 Vert: SHG=>collagène  
<http://www.lob.polytechnique.fr>

- **Génération de Troisième Harmonique (THG)**  
 $\Rightarrow$  efficace sur les interfaces!

$$\vec{P}^{3\omega} = \chi^{(3)} \vec{E}^\omega : \vec{E}^\omega : \vec{E}^\omega$$

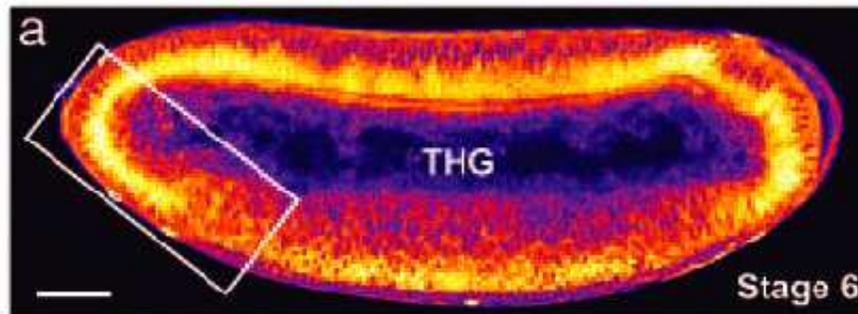
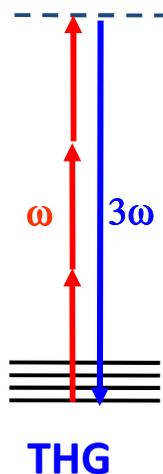
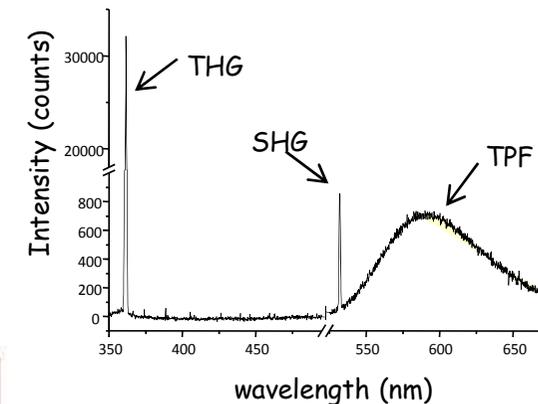


Image d'embryon de drosophile  
 W. Supatto et al., PNAS (2005)



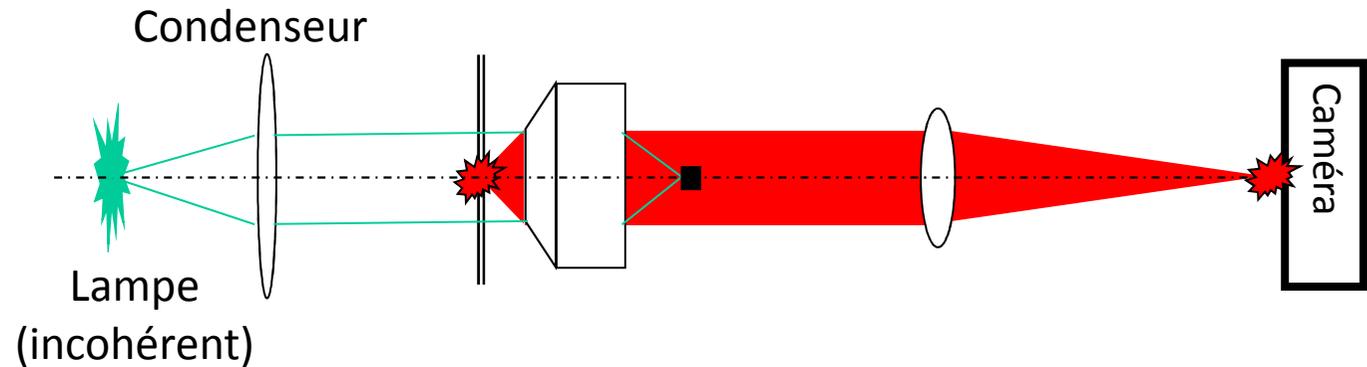
# **Les microscopies de fluorescence**

# Microscopies de fluorescence

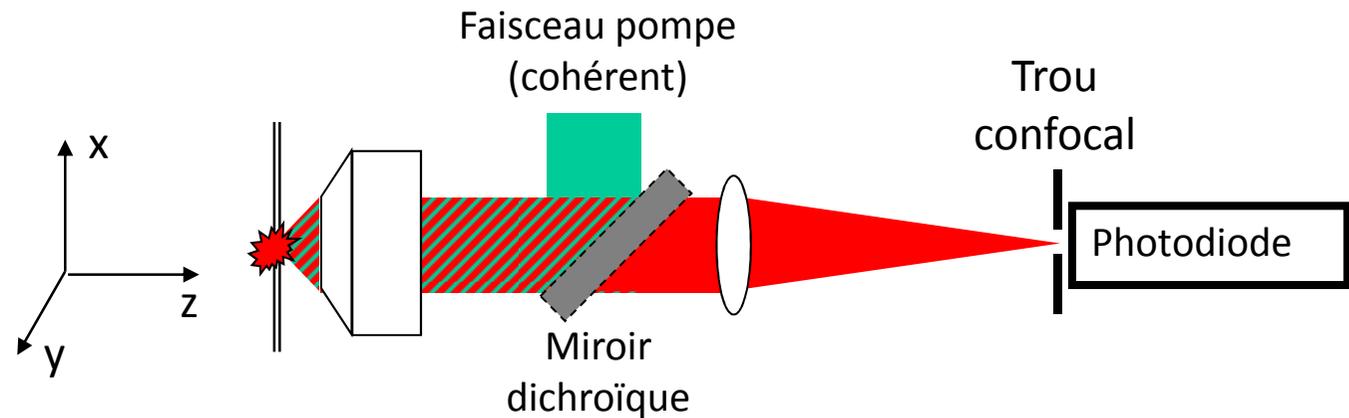
*Échantillons spatialement incohérents!*

Deux modes d'imagerie :

**Imagerie en  
champ large**



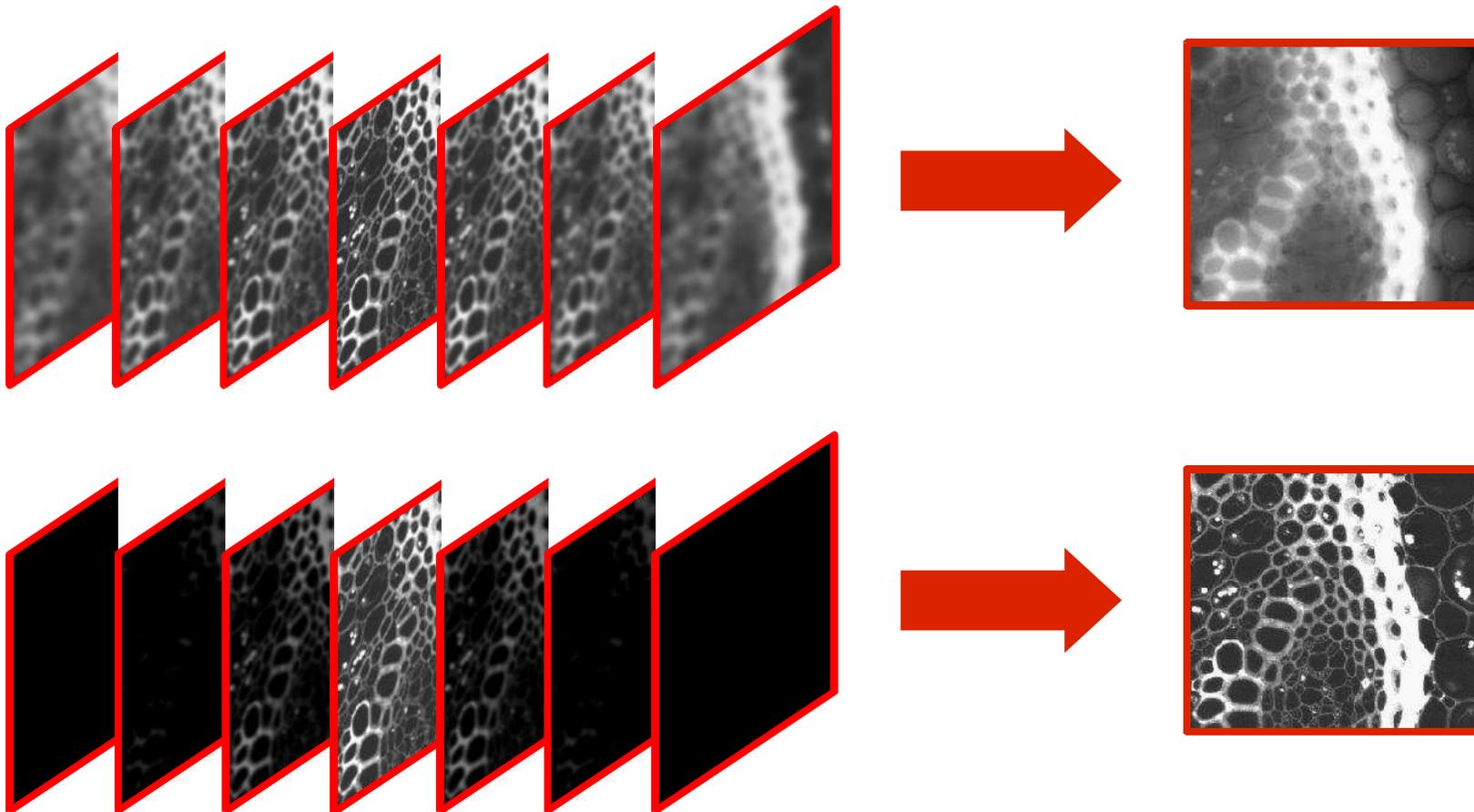
**Imagerie en  
mode confocal**



# Microscopies de fluorescence

*Échantillons spatialement incohérents!*

Deux modes d'imagerie : La profondeur de champ  $d_z = \frac{n\lambda}{NA^2}$



# Microscopies de fluorescence

## Résolution vs Localisation

La localisation latérale est limitée par le bruit

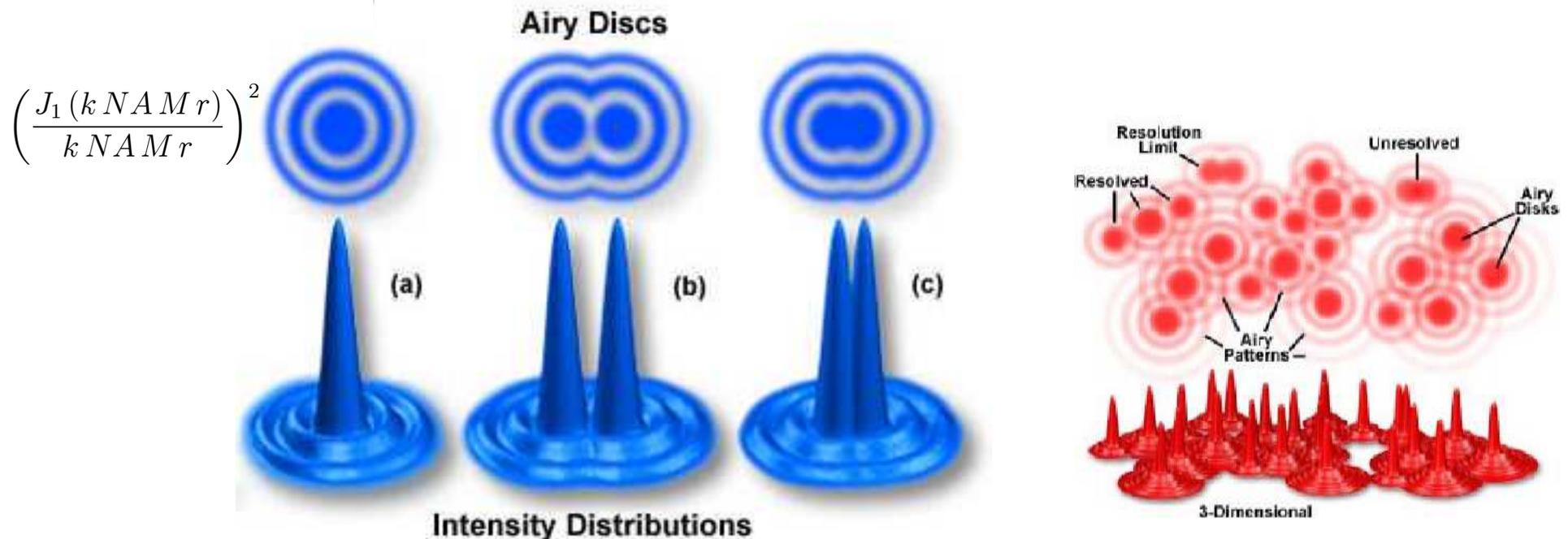
⇒ de l'ordre du nm avec 1 émetteur!

⇒ Single Particule Tracking (SPT)

*New directions in single-molecule imaging and analysis, W. E. Moerner, PNAS 104, 12596–12602, 2007*

La résolution latérale est limitée par la diffraction

⇒ de l'ordre de  $\lambda/2$ .



# Microscopies de fluorescence

## Résolution vs Grandissement

Image à faible grandissement



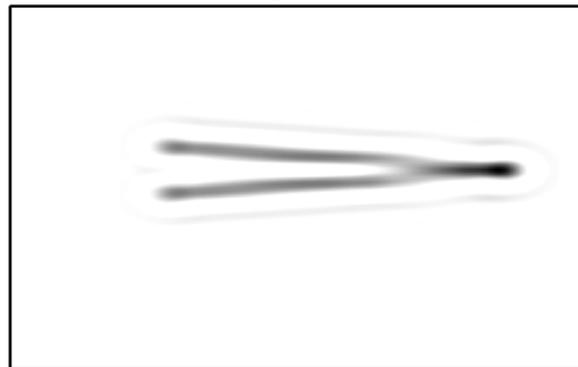
Image grandie 4 fois



Le grandissement  $M = \frac{f_t}{f_o}$

La résolution  $\frac{1.22\lambda}{2NA}$

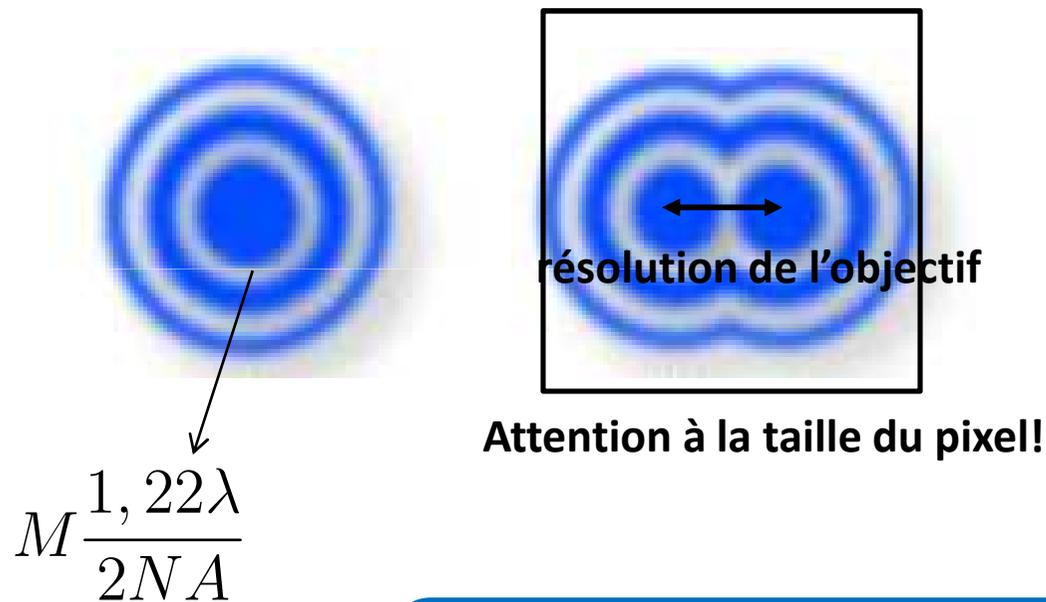
Image grandie 4 fois  
et 2 fois plus résolue



**Le grandissement est important  
mais l'ouverture numérique de  
l'objectif l'est beaucoup plus!**

# Microscopies de fluorescence

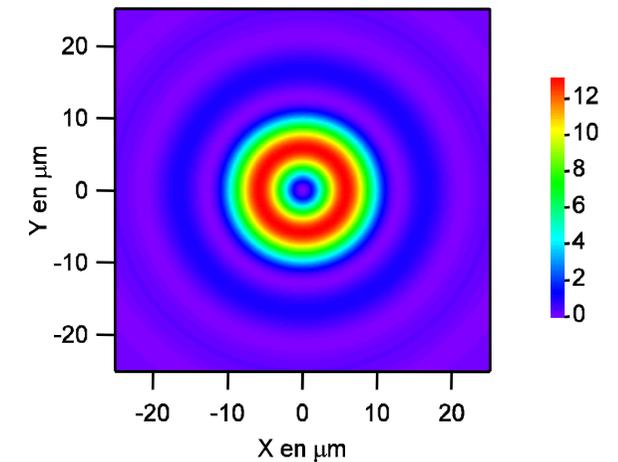
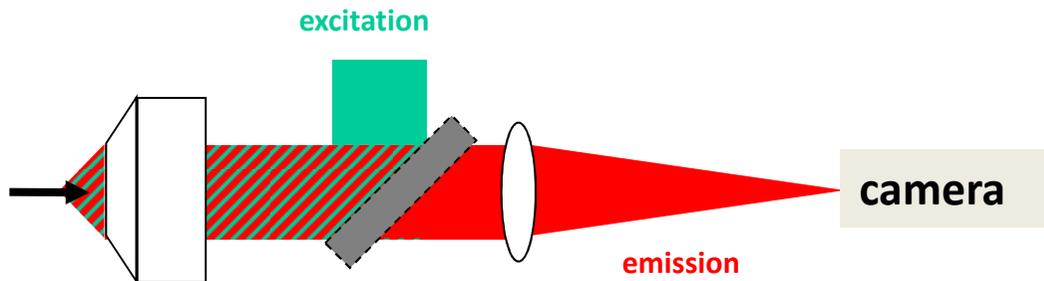
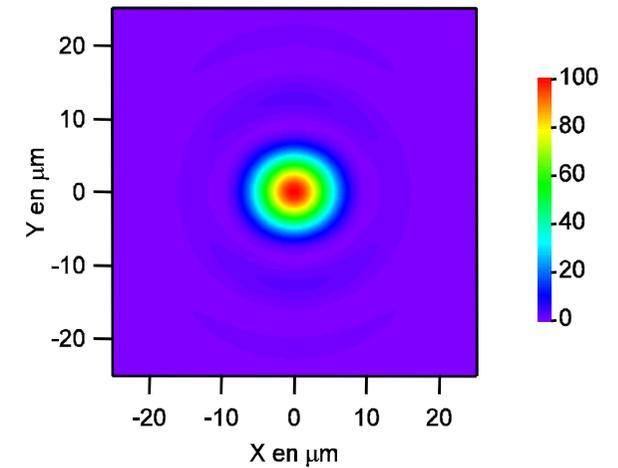
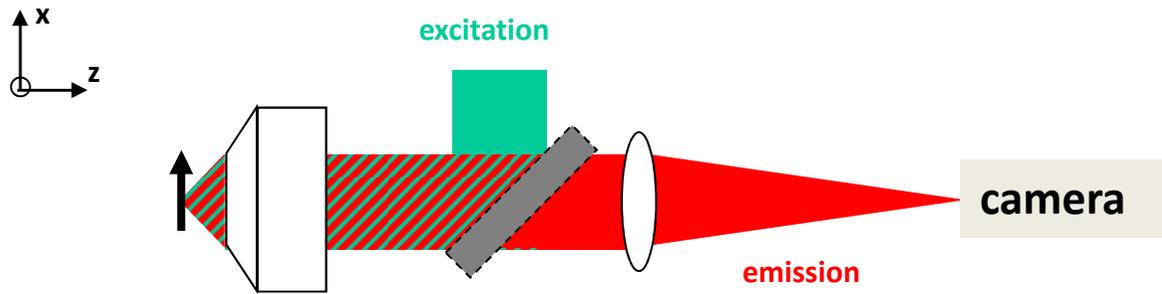
## Résolution vs Grandissement



**Il faut adapter le grandissement  
à la taille du détecteur!**

# Microscopies de fluorescence

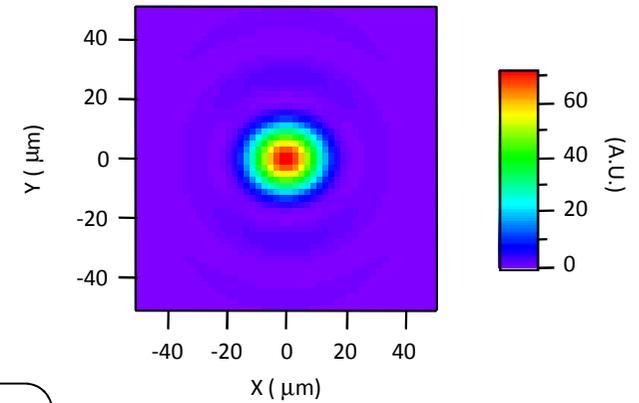
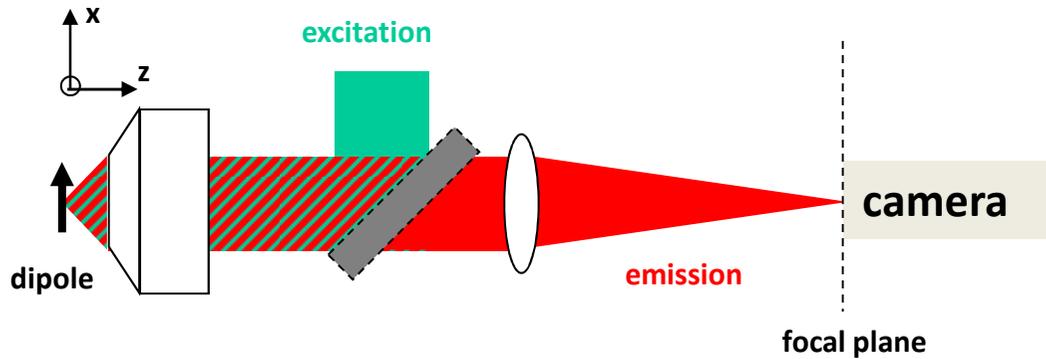
## Mesure de l'orientation 3D



**Conservation de l'information  
sur l'orientation de l'émetteur à travers le microscope**

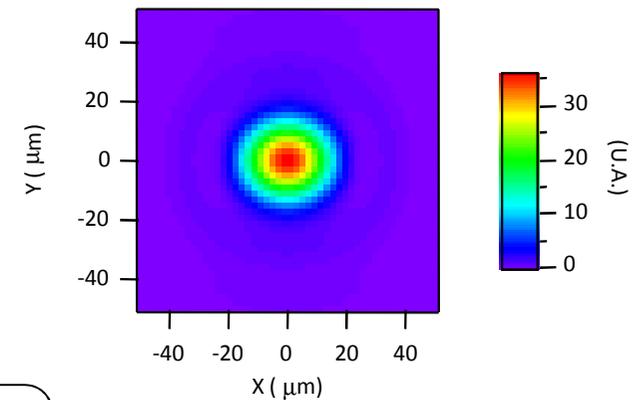
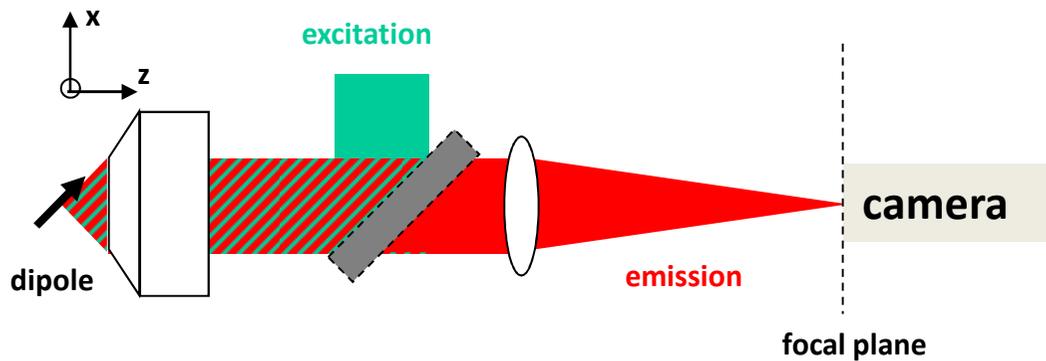
$\lambda=525 \text{ nm}$   
 $NA=1,3 \ n=1.518 \ m=40$

# Imagerie défocalisée



Conservation de l'information  
sur l'orientation de l'émetteur à travers le microscope

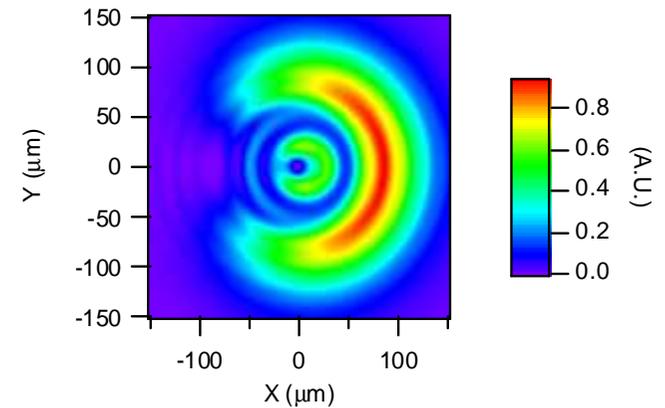
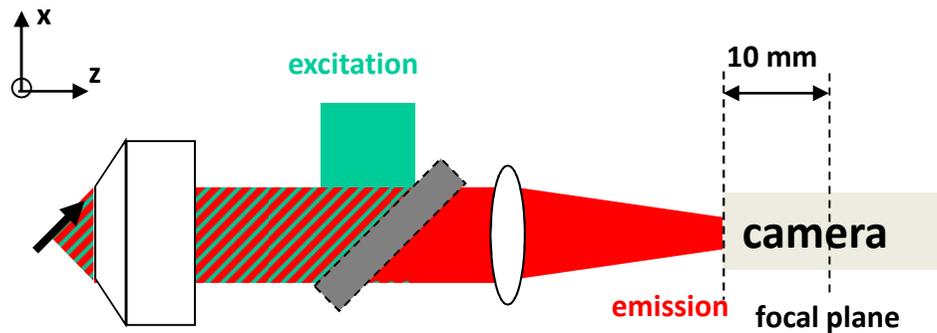
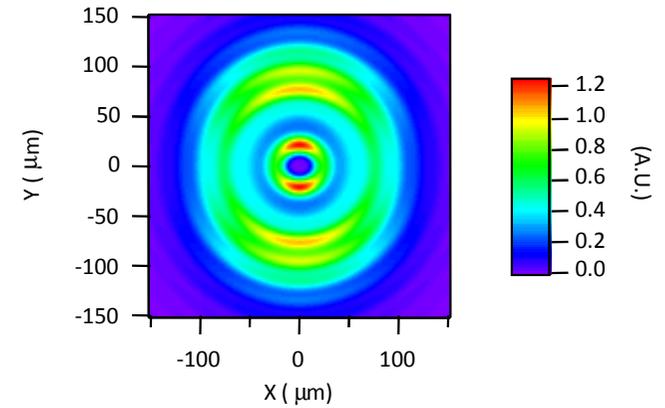
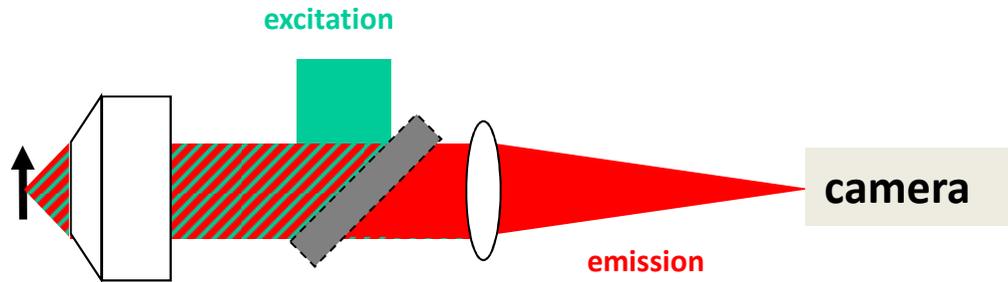
Image du dipole



Mais information difficile à lire!

Image du dipole

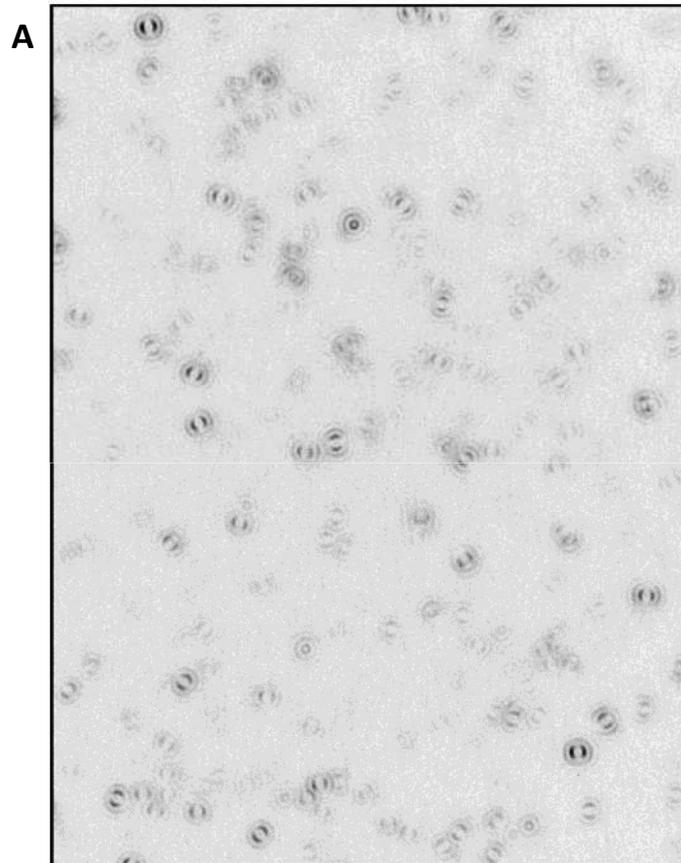
# Imagerie défocalisée



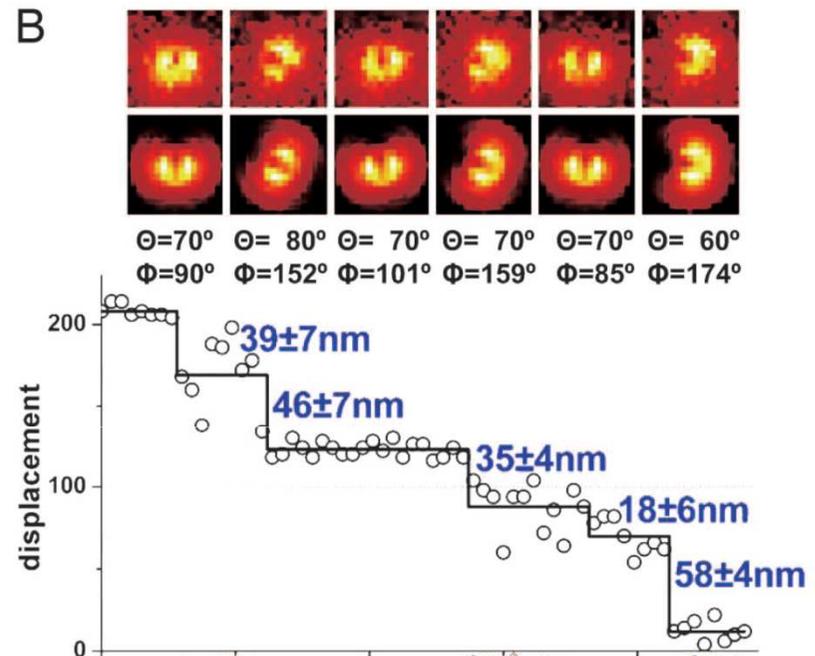
L'imagerie défocalisée pour lire cette information!

« image défocalisée » du dipole

# Imagerie défocalisée



**Figure 2.** Measured defocused image of Cy5 molecules embedded in poly(vinyl alcohol) on glass near the air/polymer interface. Defocusing was achieved by moving the objective 1  $\mu\text{m}$  toward the sample.

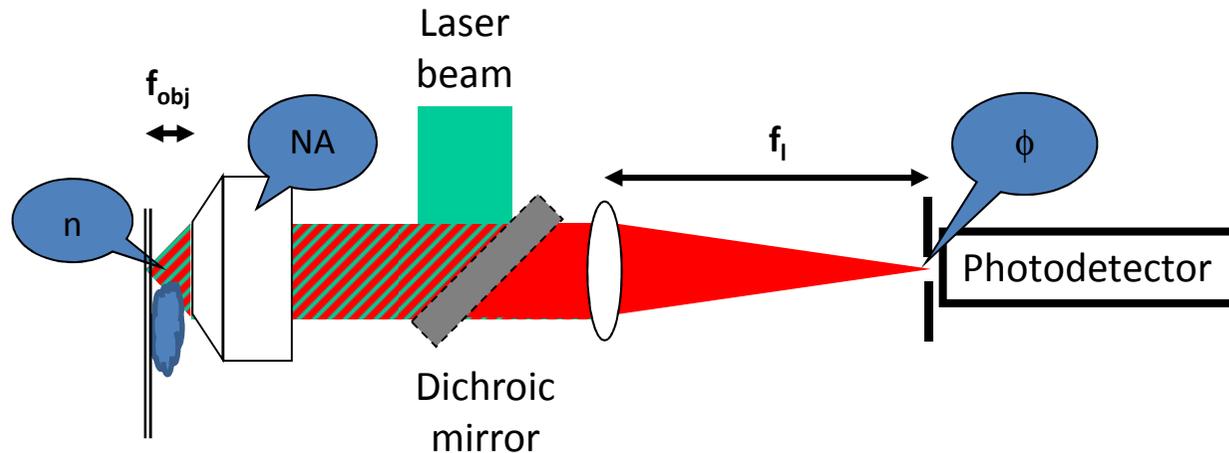


A D. Patra, I. Gregor, J. Enderlein, *J. Phys. Chem. A* **108** (33) p. 6836-6841 (2004)

B E. Toprak et al., *PNAS* **103** (17) p. 6495-6499 (2006)

# **Les microscopies de fluorescence à balayage**

# Microscopie confocale de fluorescence



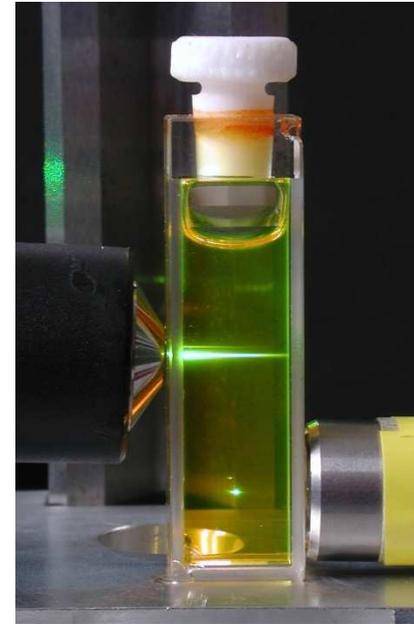
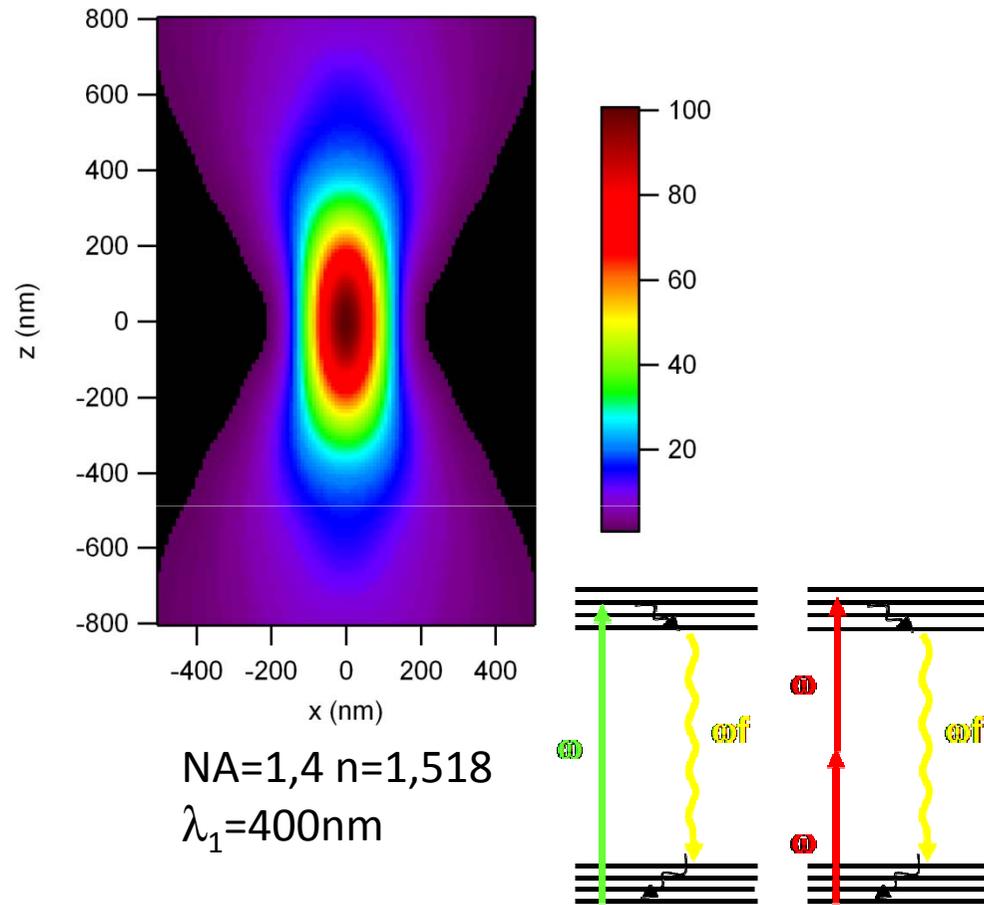
Main parameters

- of the microscope : **NA** and the ratio  $\phi/M$ ...
- of the excitation : **mode, wavelength, polarization, shape of the beam**...
- of the emission : **wavelength**...

**3D-Resolution** is defined by a **volumetric function** depending on the **excitation, emission** and **collection** parameters.

# Volumes d'excitation (EEF)

## Excitation Efficiency Function (EEF)

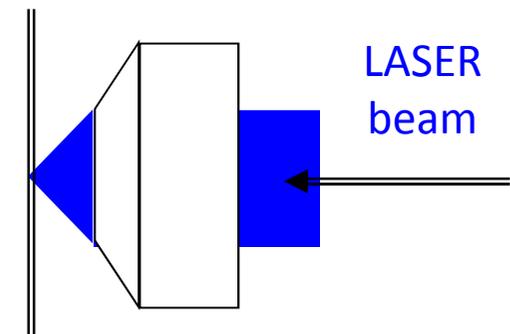


E. Guiot, PhD Orsay Univ. (2001)

EEF = Carte 3D de l'intensité focalisée (1PF)

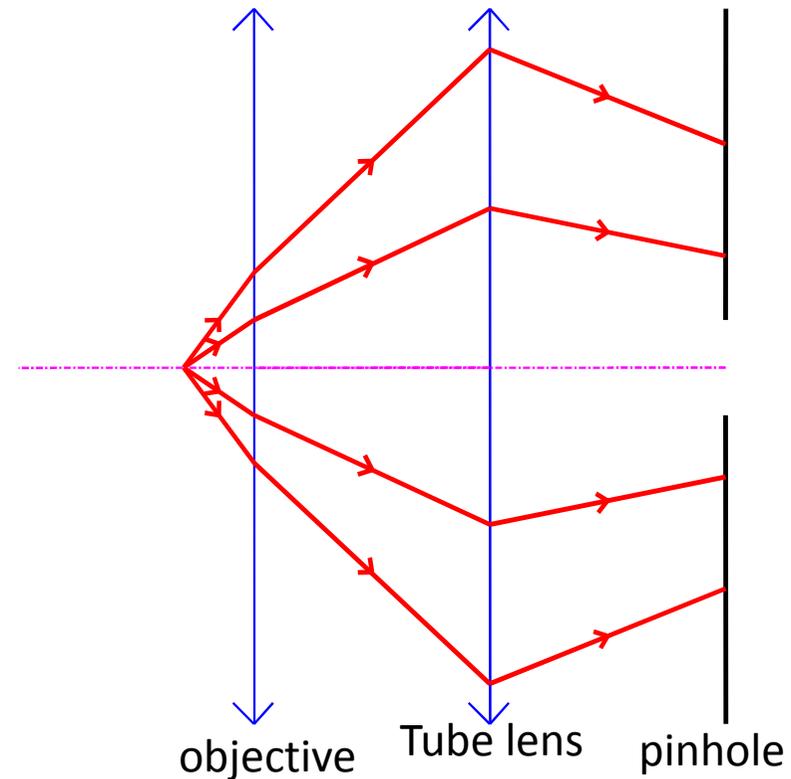
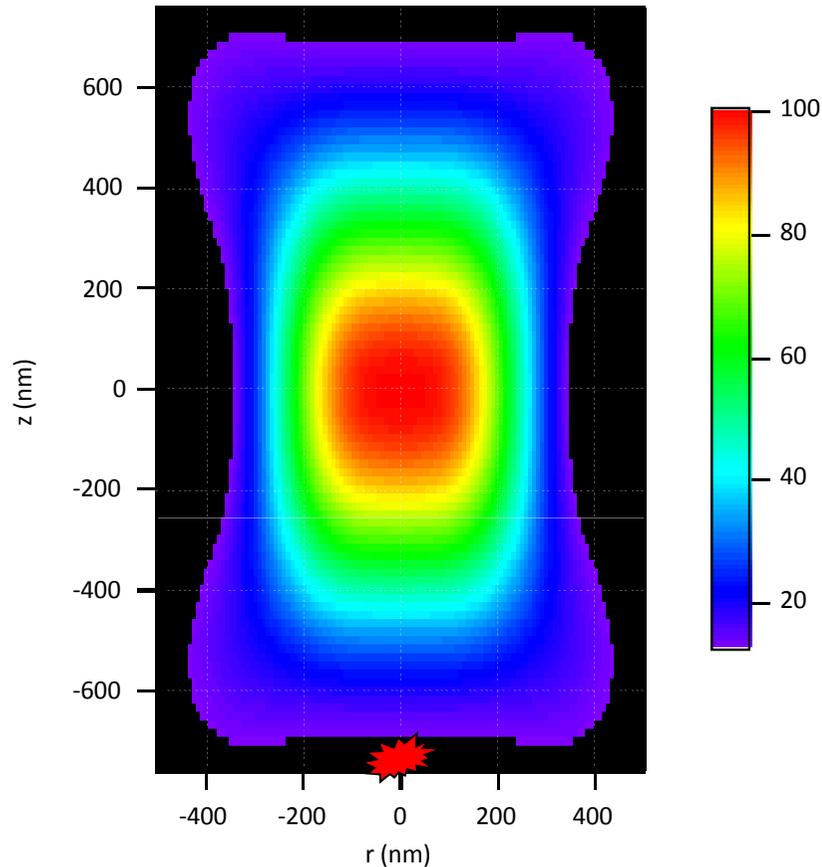
= Carte 3D de  $I^2$  (2PF)

meilleure confocalité



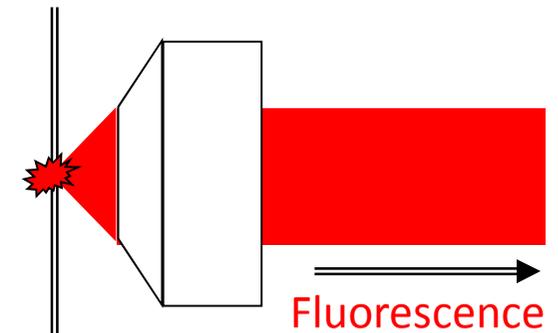
# Volumes de collection (CEF)

## Collection Efficiency Function (CEF)



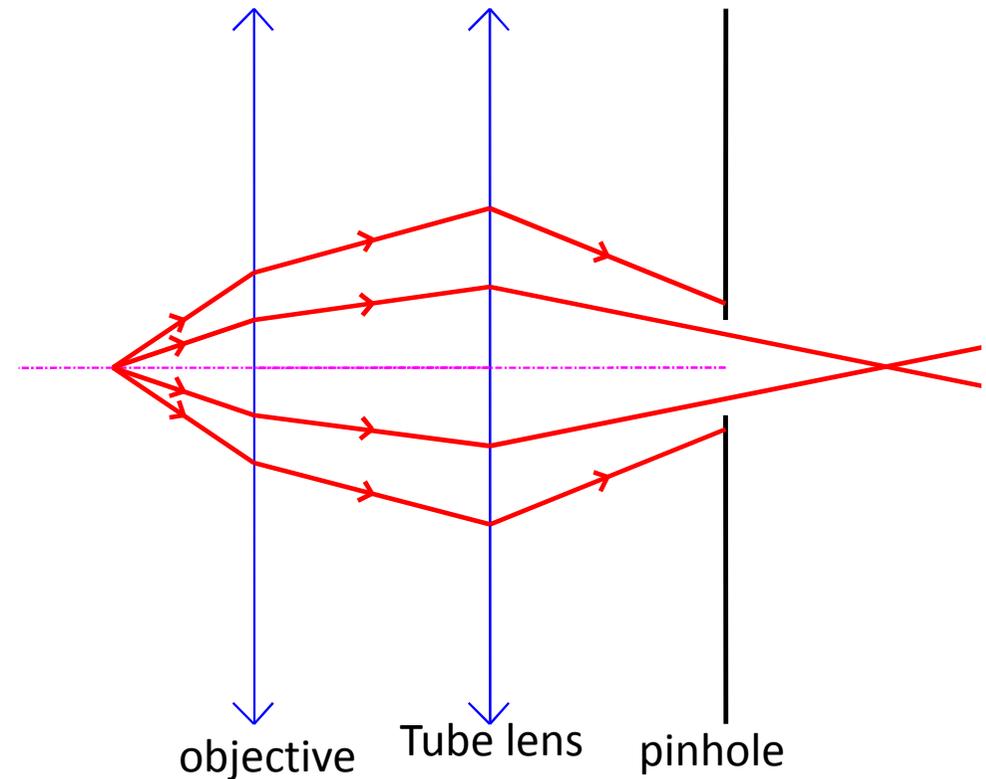
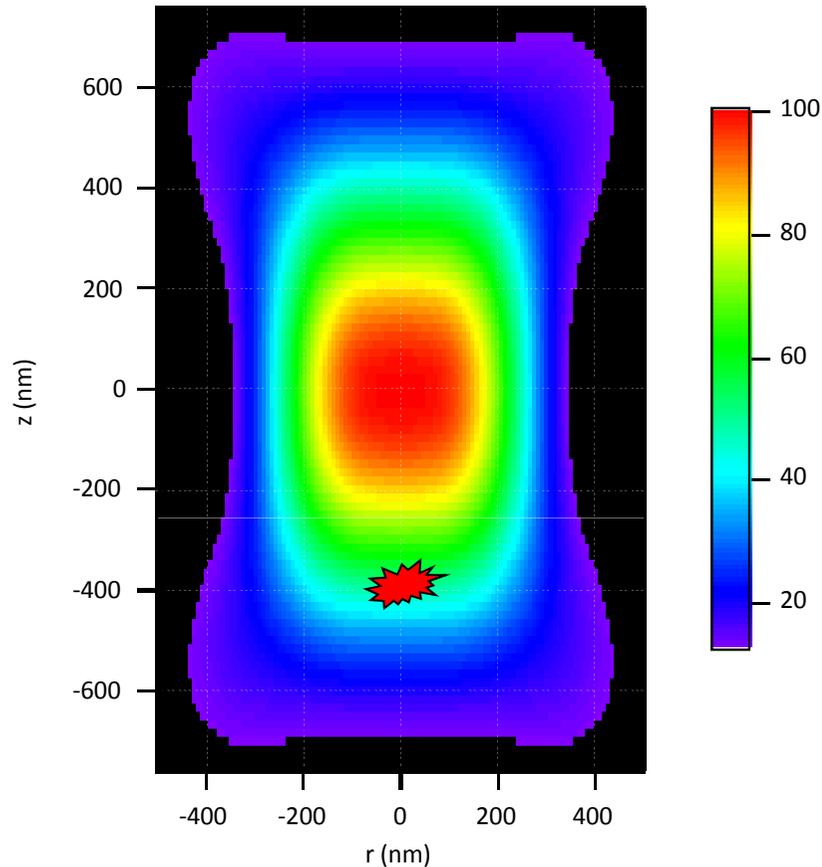
**CEF = Image 3D du trou confocal**

Carte 3D de l'intensité détectée en  
= fonction de la position 3D de l'émetteur  
individuel uniformément éclairé



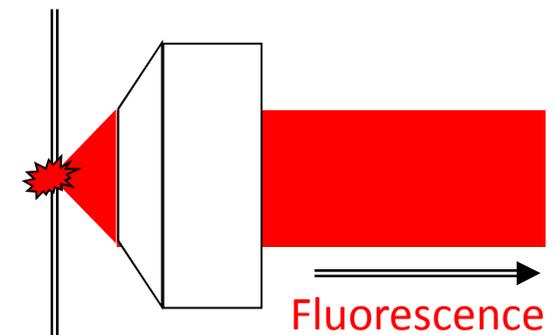
# Volumes de collection (CEF)

## Collection Efficiency Function (CEF)



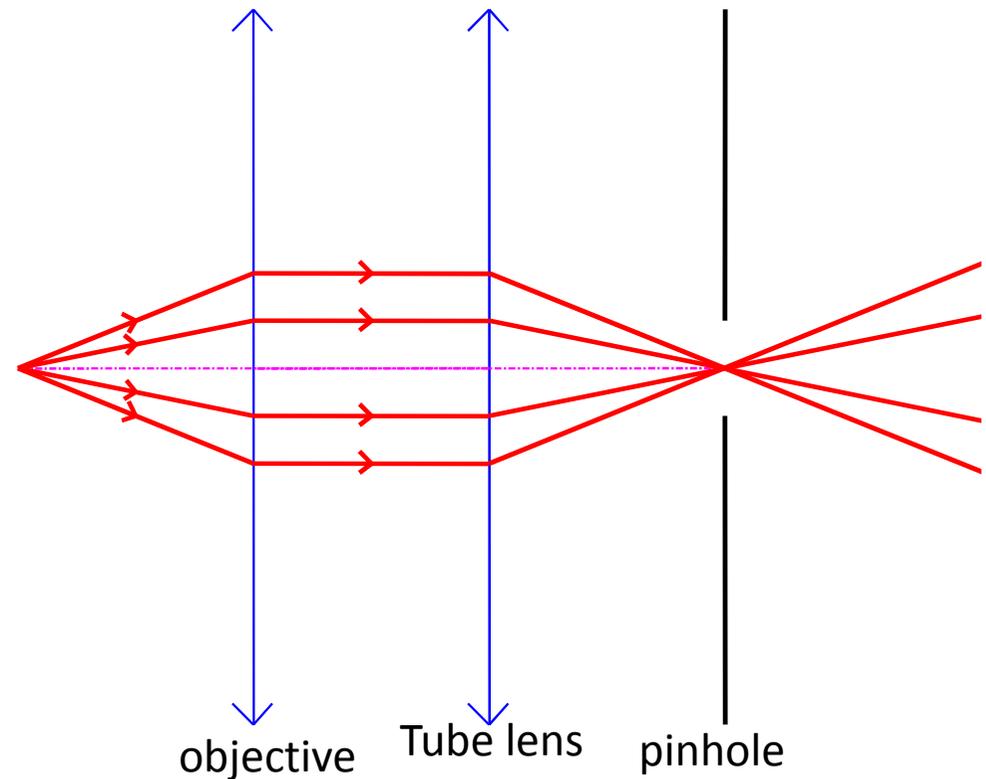
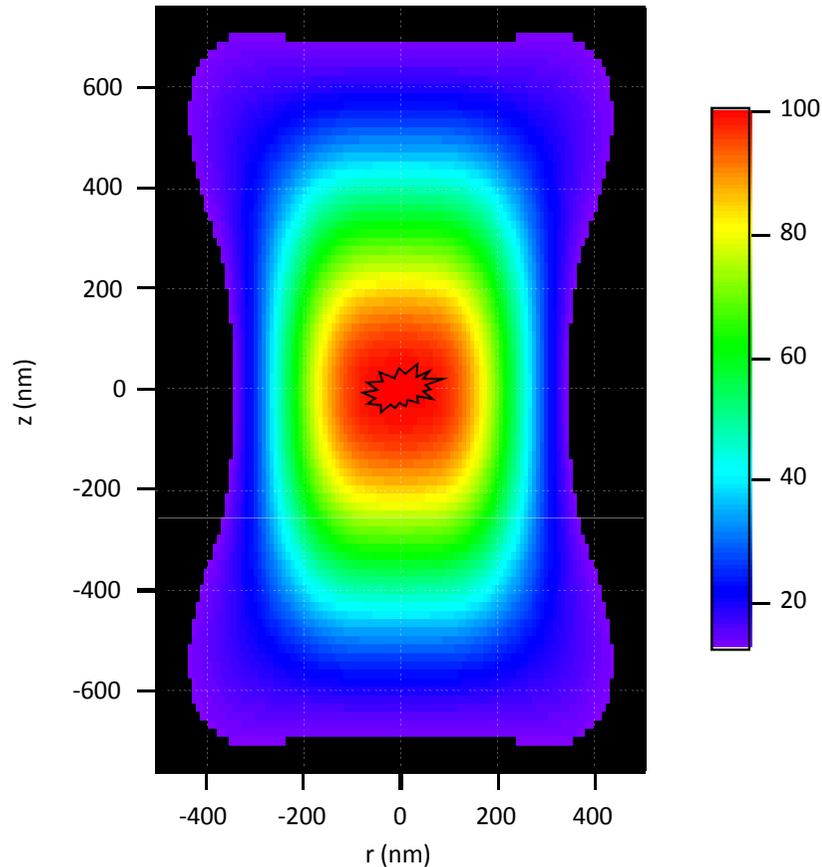
**CEF = Image 3D du trou confocal**

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= fonction de la position 3D de l'émetteur  
individuel uniformément éclairé



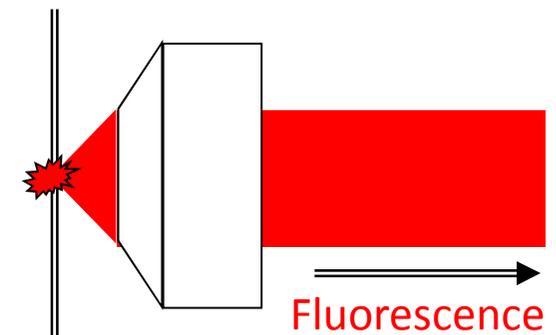
# Volumes de collection (CEF)

## Collection Efficiency Function (CEF)



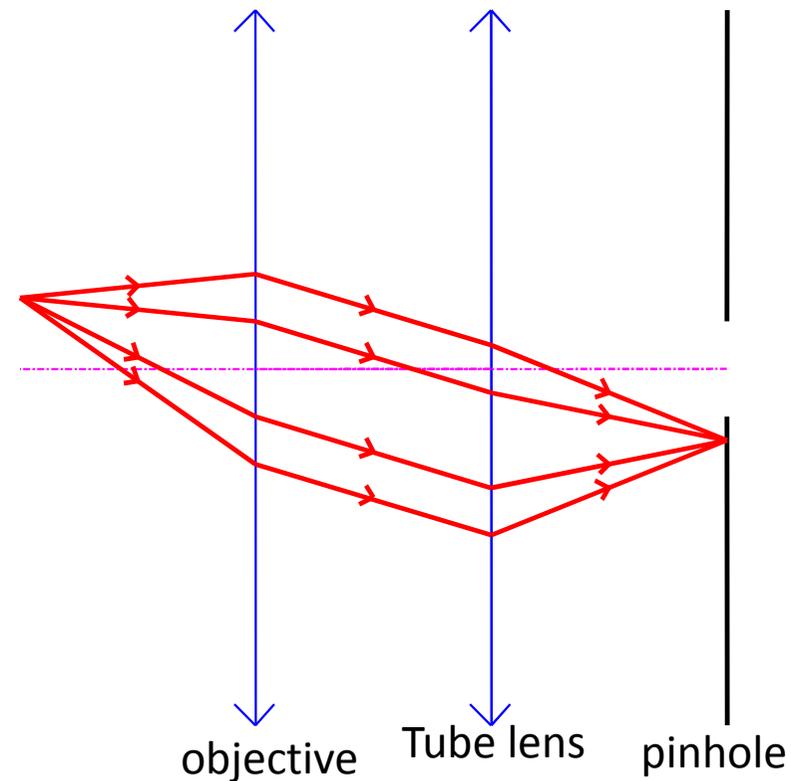
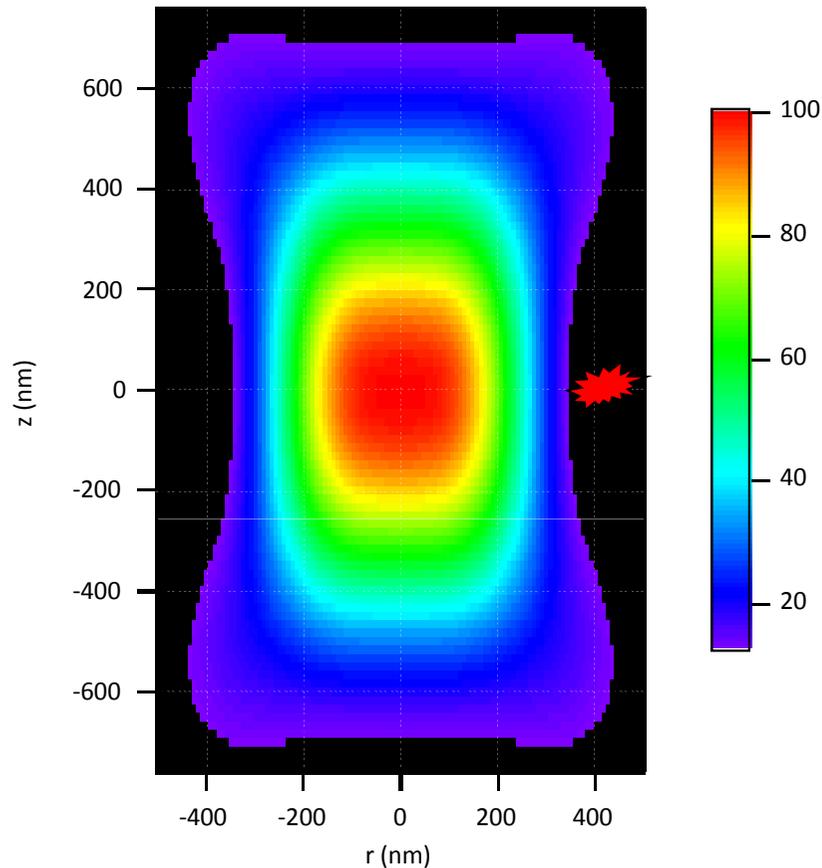
**CEF = Image 3D du trou confocal**

Carte 3D de l'intensité détectée en  
= fonction de la position 3D de l'émetteur  
individuel uniformément éclairé



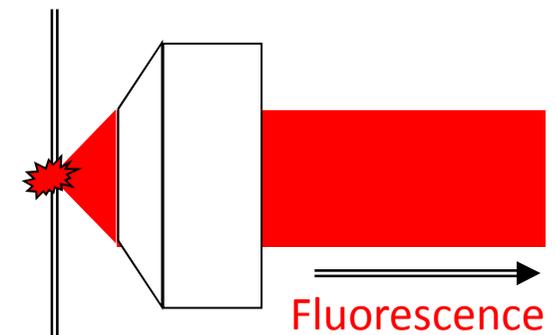
# Volumes de collection (CEF)

## Collection Efficiency Function (CEF)



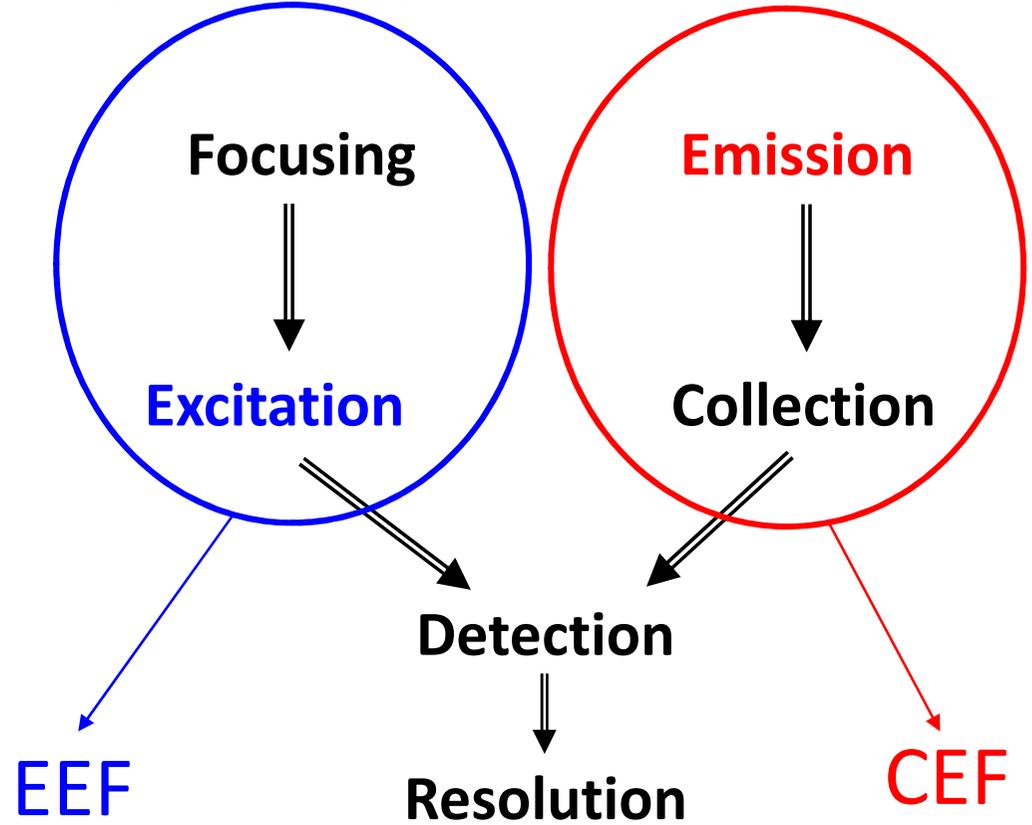
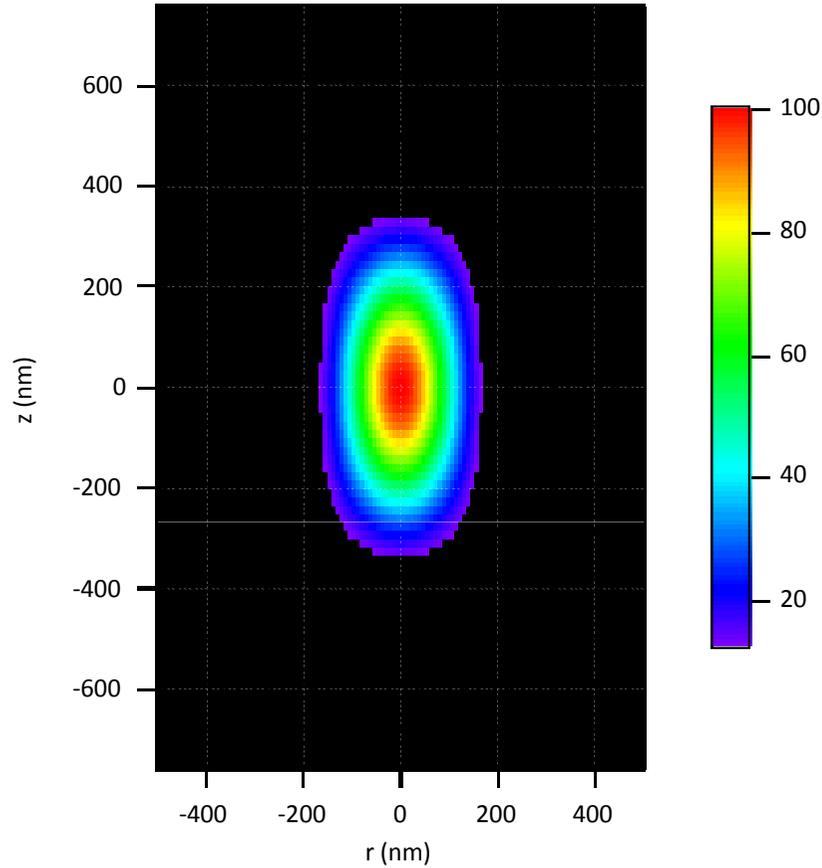
**CEF = Image 3D du trou confocal**

Carte 3D de l'intensité détectée en  
= fonction de la position 3D de l'émetteur  
individuel uniformément éclairé



# Volumes de détection (DEF)

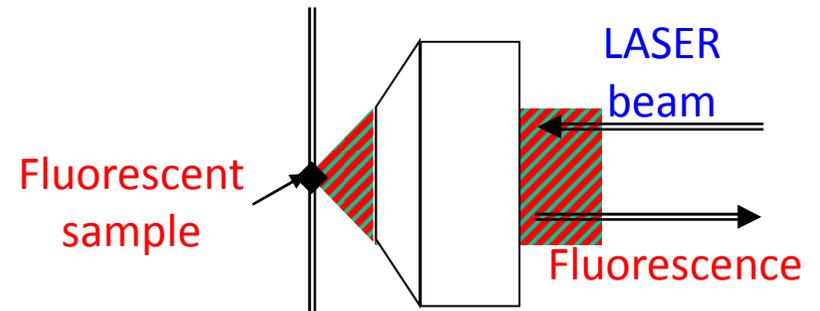
## Detection Efficiency Function (DEF)



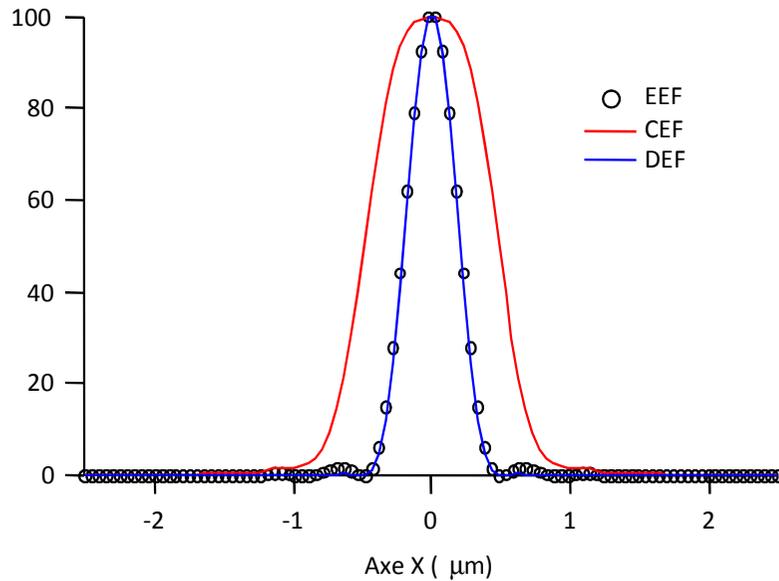
$$DEF = EEF \times CEF$$

Focused beam

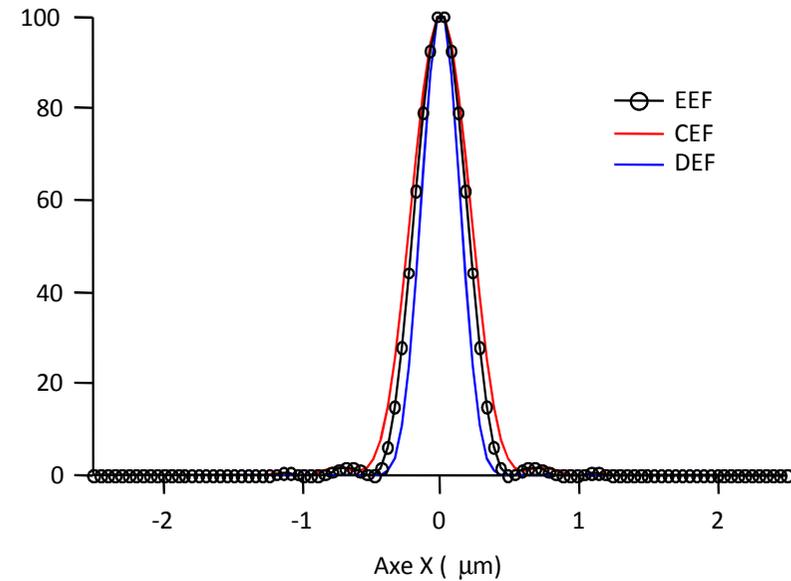
Image of the pinhole



# Augmenter la résolution?



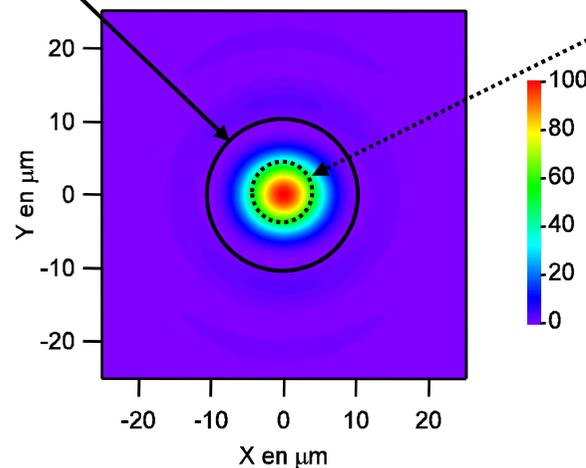
NA=0,6  $\phi/M=1\mu\text{m}$   
 $\lambda_p=488\text{nm}$   $\lambda_f=525\text{nm}$



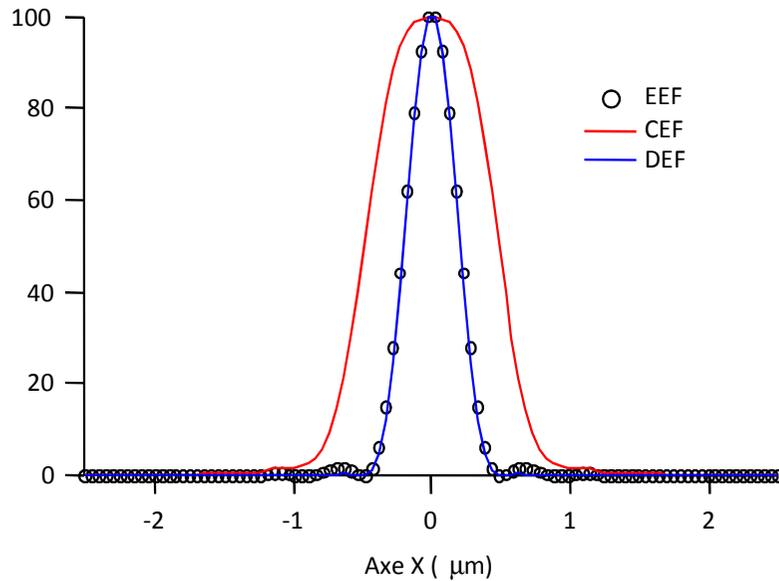
NA=0,6  $\phi/M=0,3\mu\text{m}$   
 $\lambda_p=488\text{nm}$   $\lambda_f=525\text{nm}$

**Le trou confocal  
 doit être adapter à  
 la fonction d'Airy**

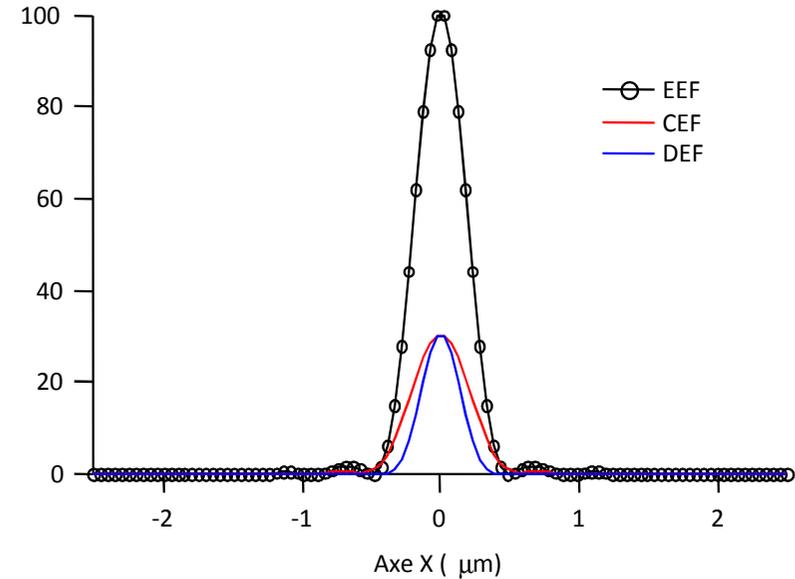
$$R = M \frac{1,22\lambda}{2NA}$$



# Augmenter la résolution?



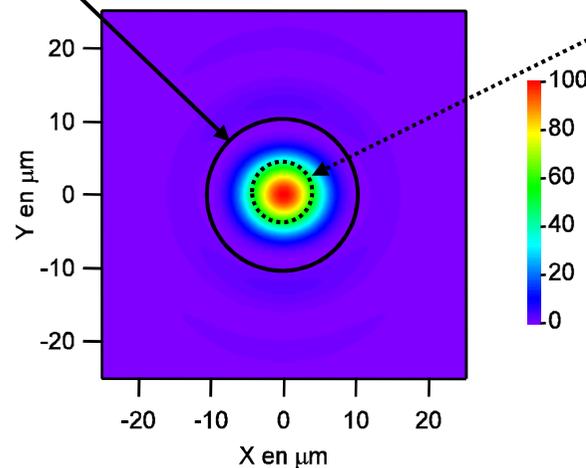
NA=0,6  $\phi/M=1\mu\text{m}$   
 $\lambda_p=488\text{nm}$   $\lambda_f=525\text{nm}$



NA=0,6  $\phi/M=0,3\mu\text{m}$   
 $\lambda_p=488\text{nm}$   $\lambda_f=525\text{nm}$

**Le trou confocal  
 doit être adapter à  
 la fonction d'Airy**

$$R = M \frac{1,22\lambda}{2NA}$$



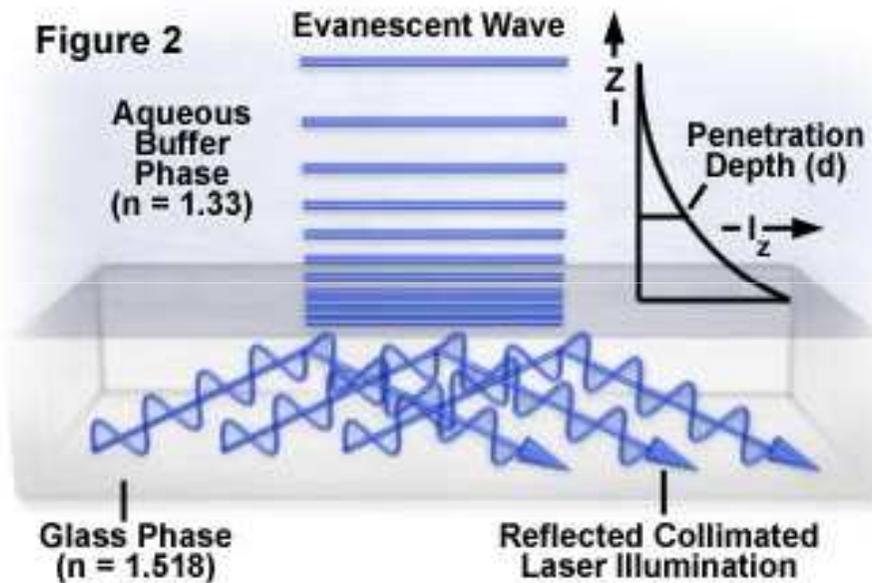
**Augmenter la résolution des  
microscopes confocaux**

# Résolution axiale

## *TIRF microscopy*

(Total Internal Reflection Fluorescence)

### Evanescent Wave Exponential Intensity Decay



$$I(z) = I_0 e^{-z/d}$$

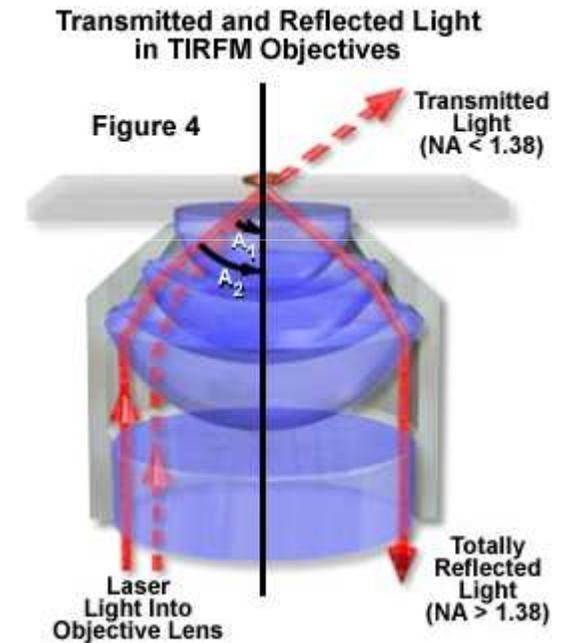
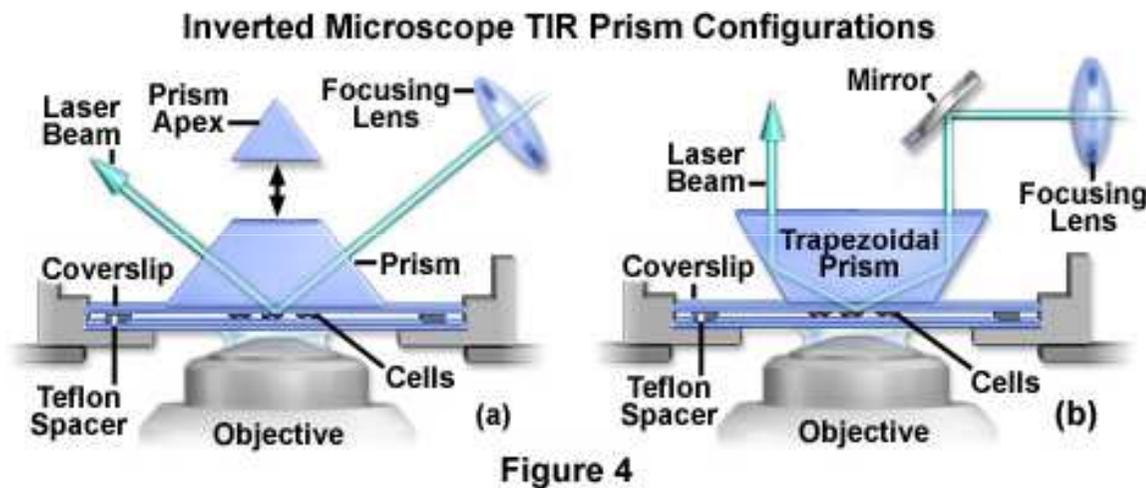
$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}}$$

$d \searrow$  quand  $\lambda \searrow$  ou  $\theta \nearrow$

# Résolution axiale

## *TIRF microscopy*

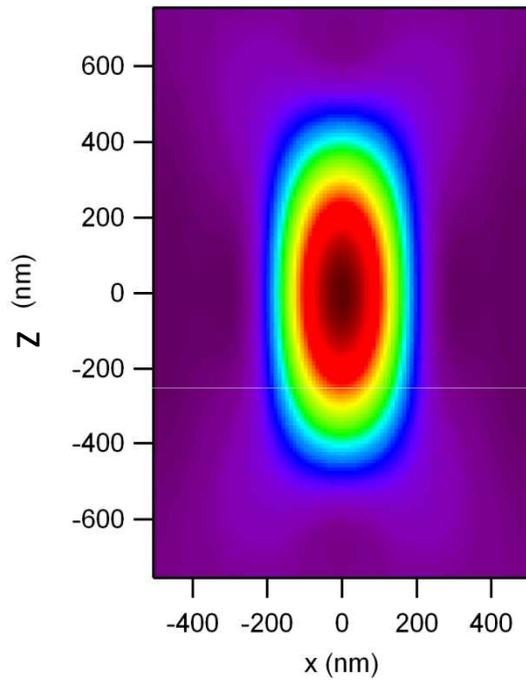
(Total Internal Reflection Fluorescence)



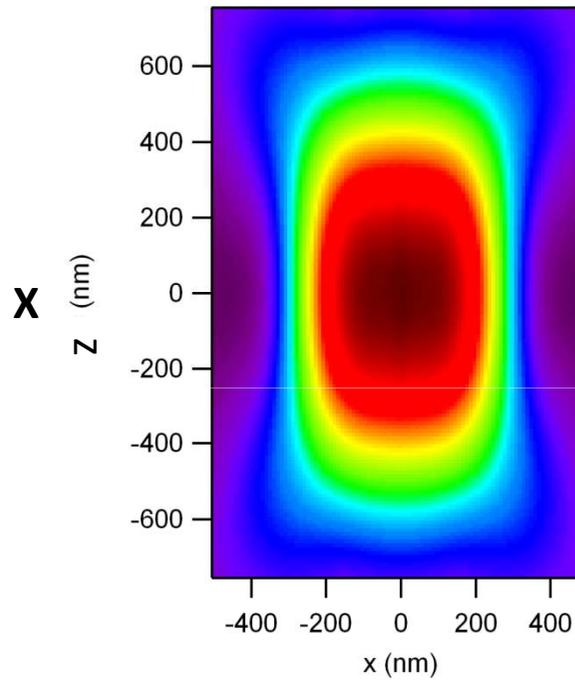
# Résolution axiale

## Le $\theta$ -microscope

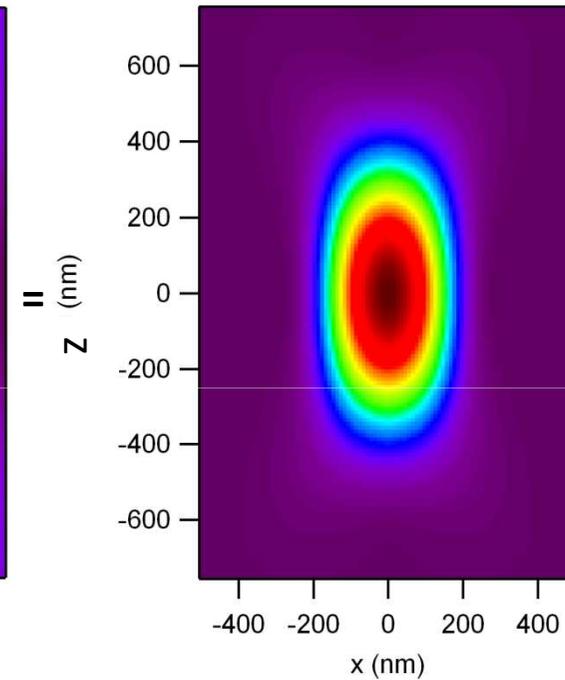
EEF



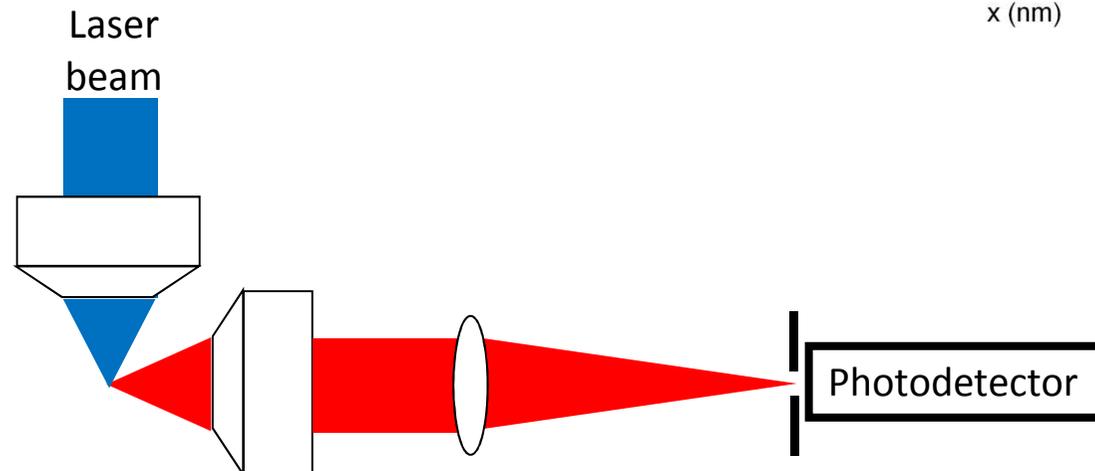
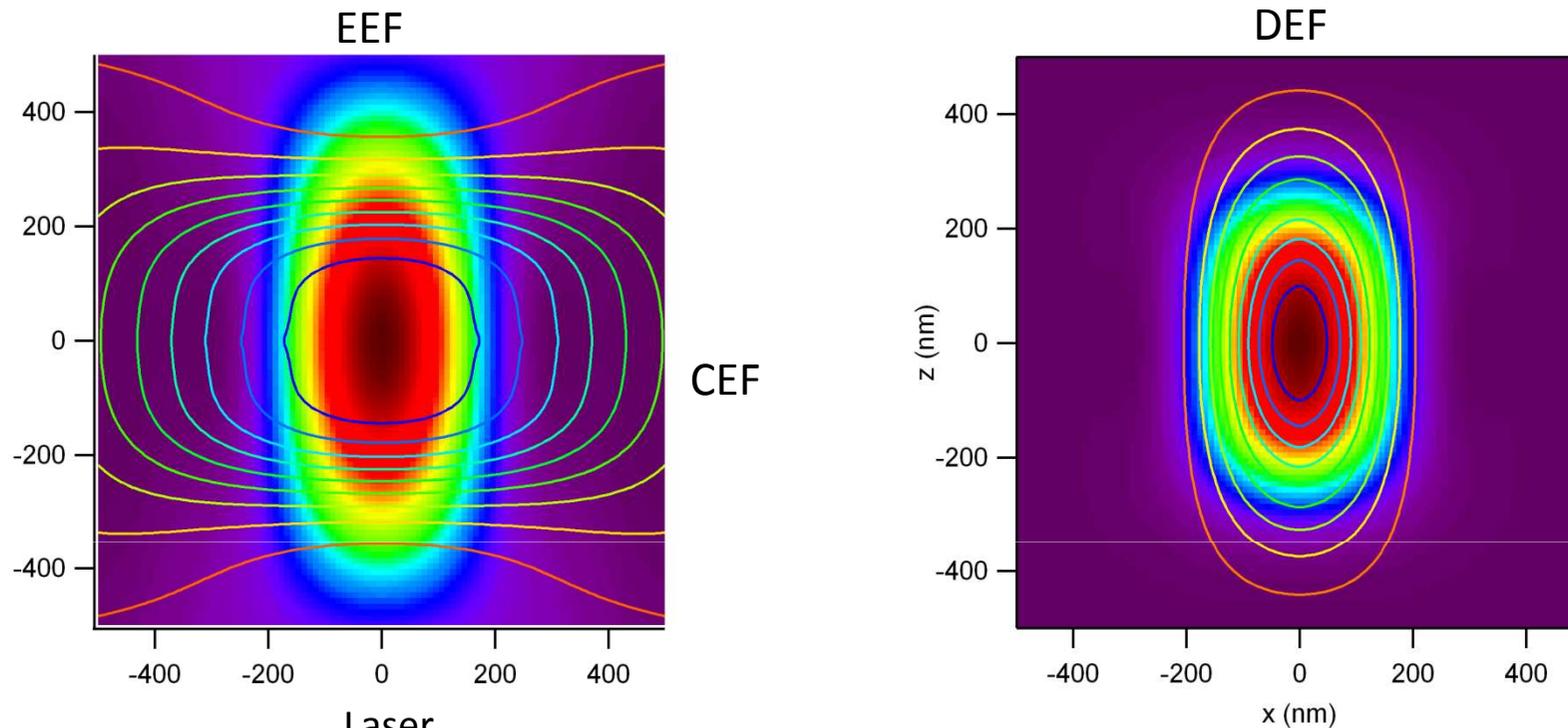
CEF



DEF

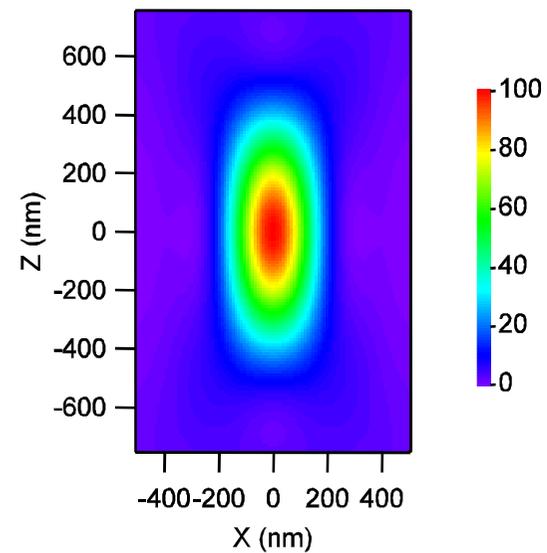
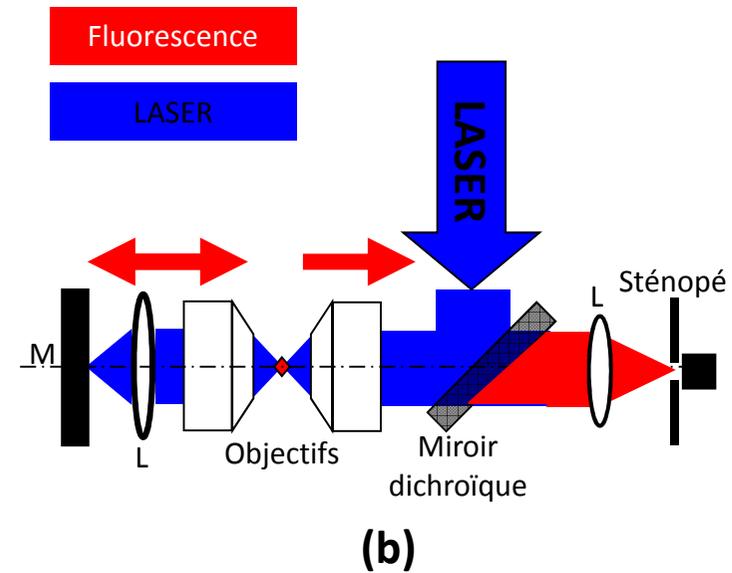
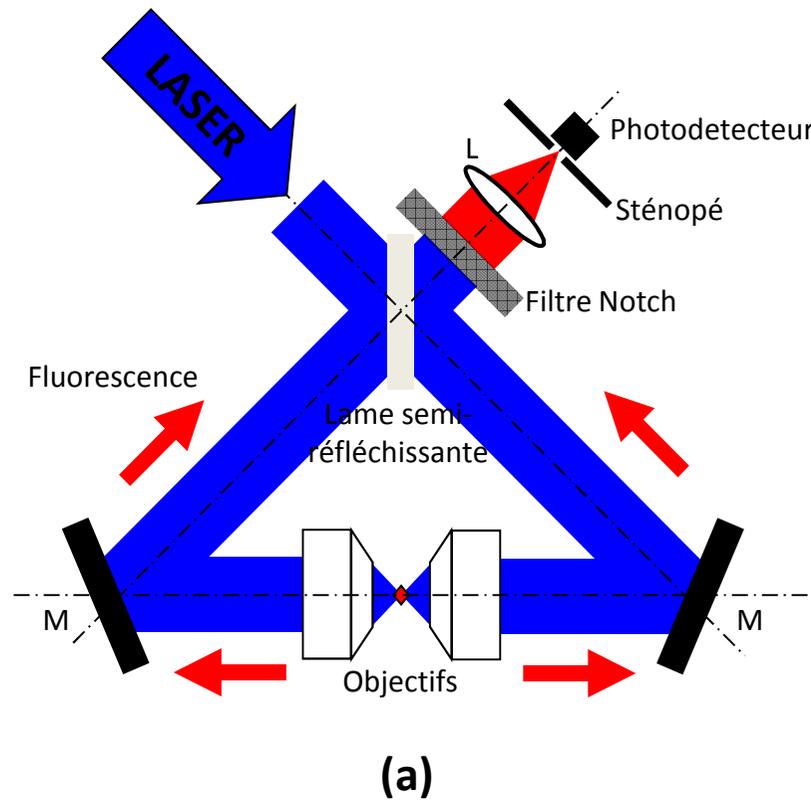


# Résolution axiale: le $\theta$ -microscope



**Pb d'encombrement et de support d'échantillons!**

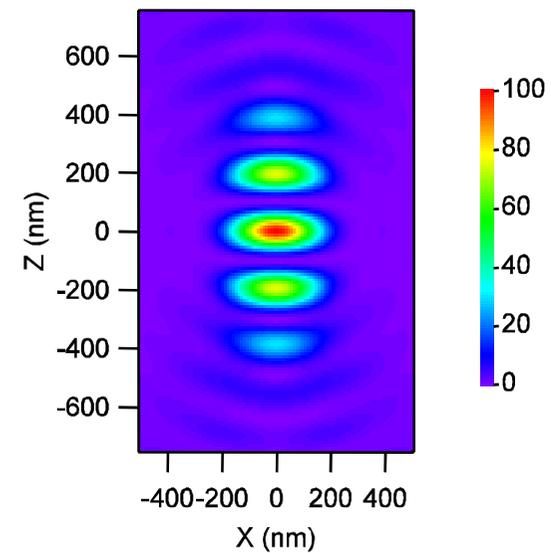
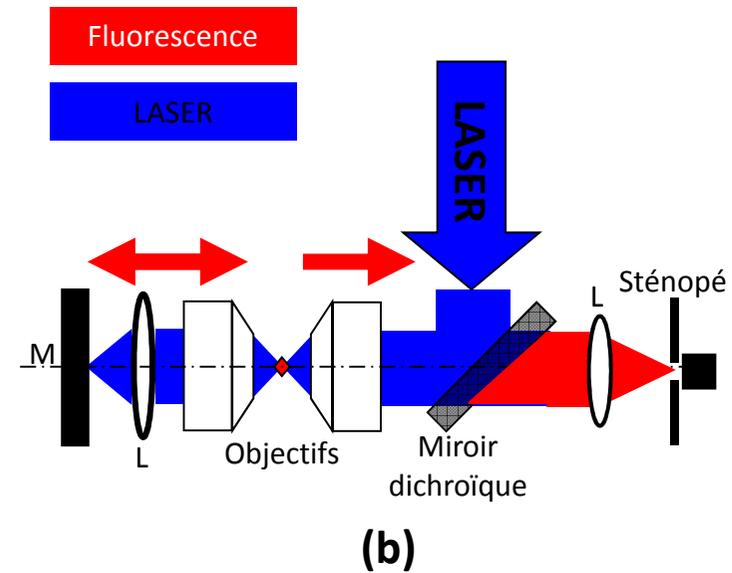
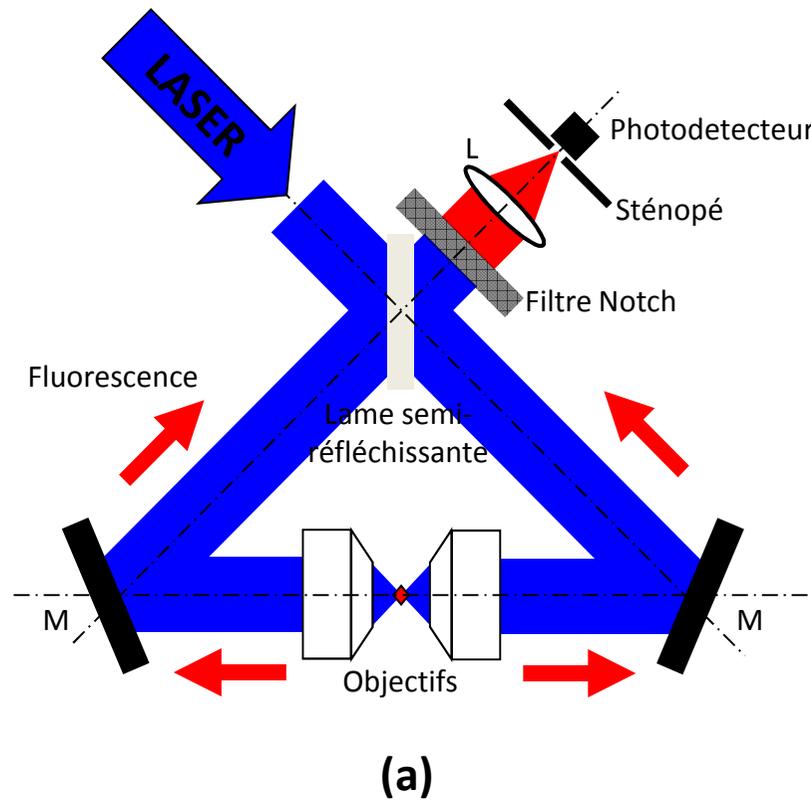
# Résolution axiale: $4\pi$ -microscopie



(a) S. W. Hell, EP0491289 (24-06-1992)

(b) C. J. R. Sheppard et al. *Optik* **87**(3) (1991)

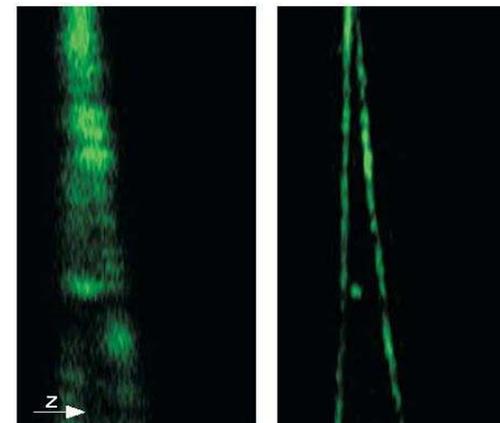
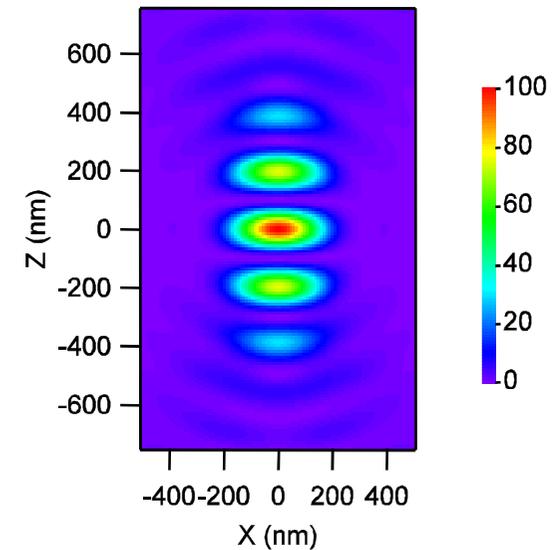
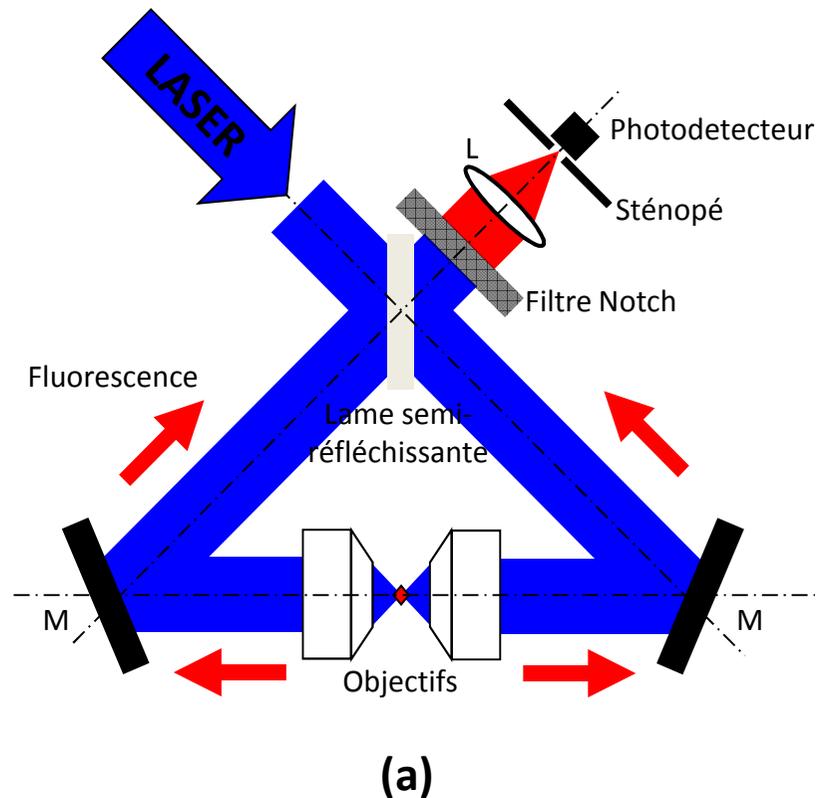
# Résolution axiale: 4 $\pi$ -microscopie



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(b) C. J. R. Sheppard et al. *Optik* **87**(3) (1991)

# Résolution axiale : $4\pi$ -microscopie



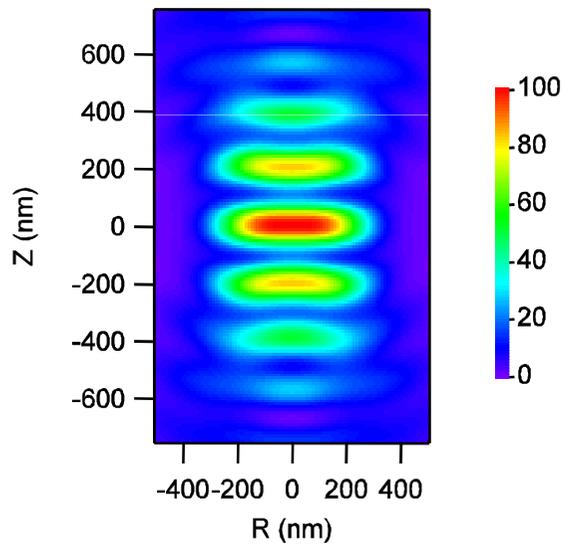
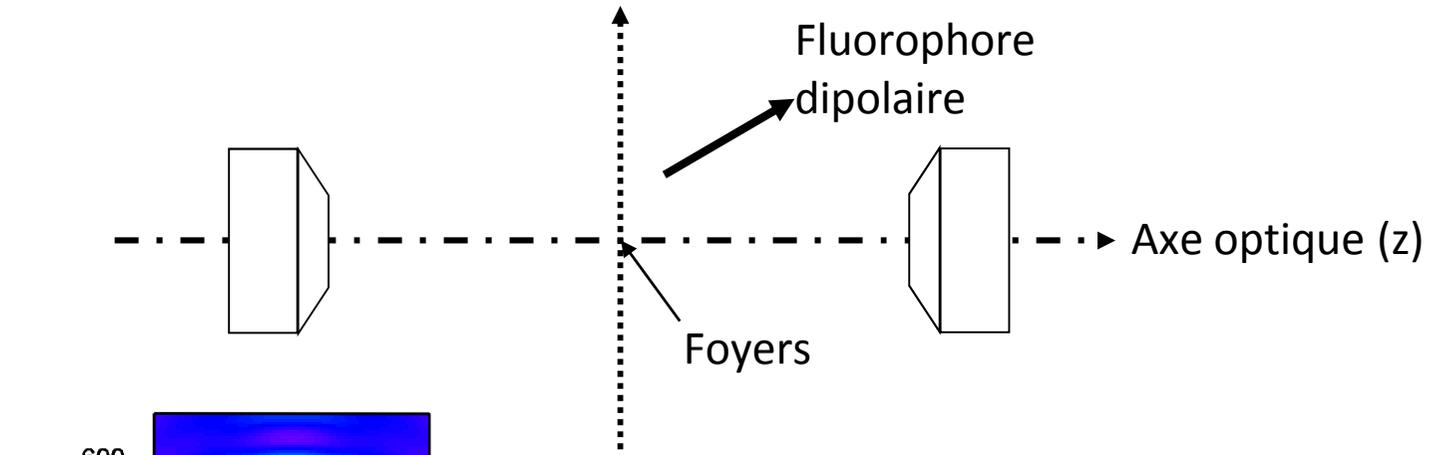
(c) Fibres d'actines

(a) S. W. Hell, EP0491289 (24-06-1992)

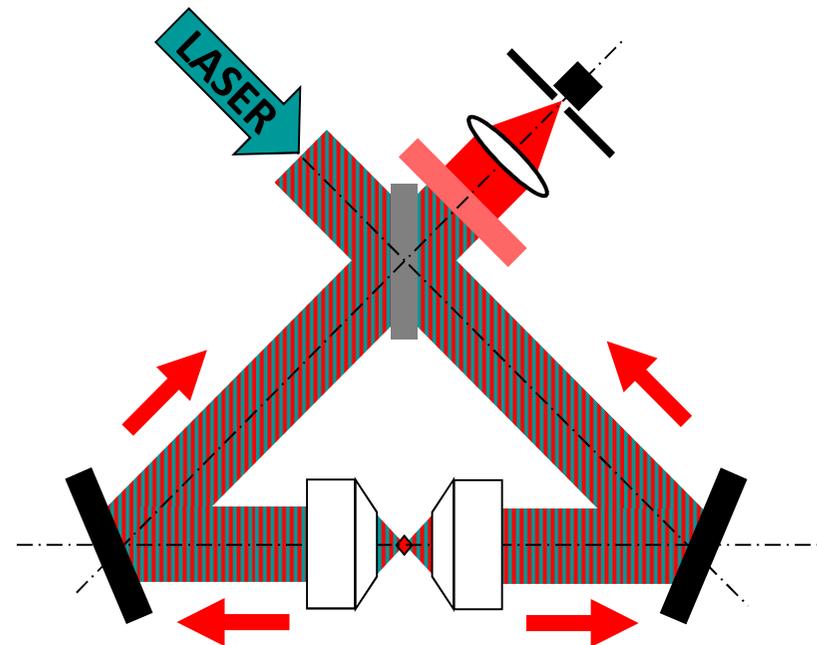
(b) C. J. R. Sheppard et al. *Optik* **87**(3) (1991)

(c) A. Egner et al. *Trends in Cell Biology* **15**(4) 2005

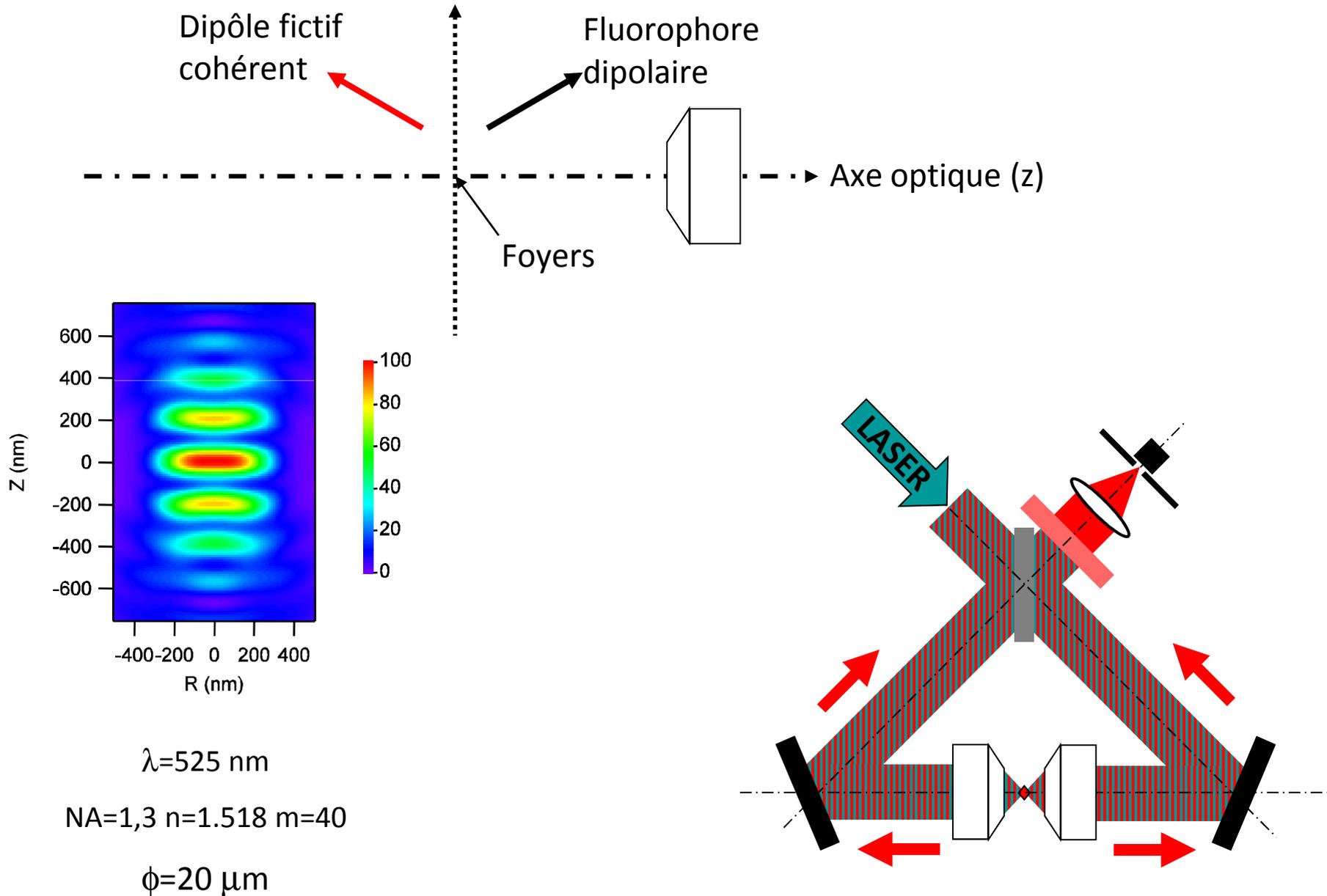
# CEF d'un $4\pi$ -microscope



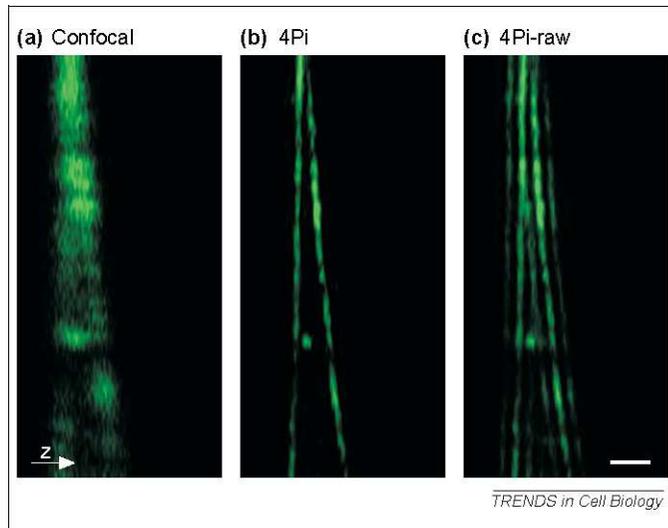
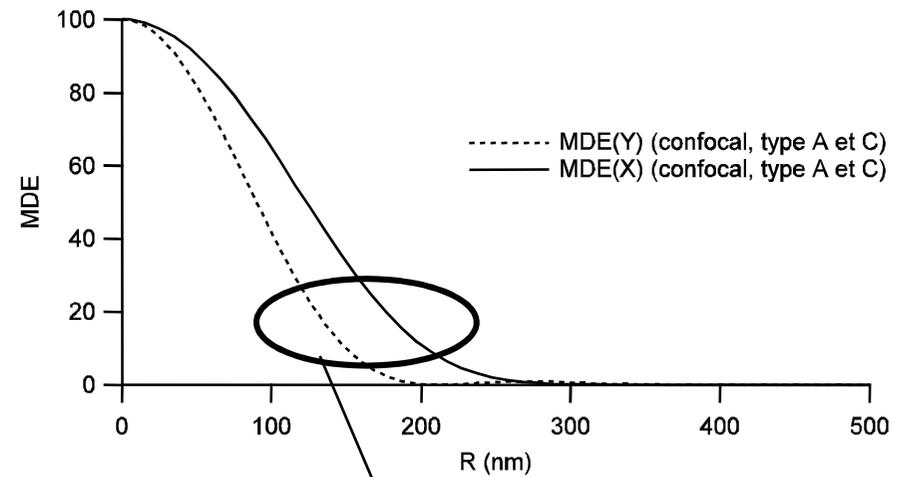
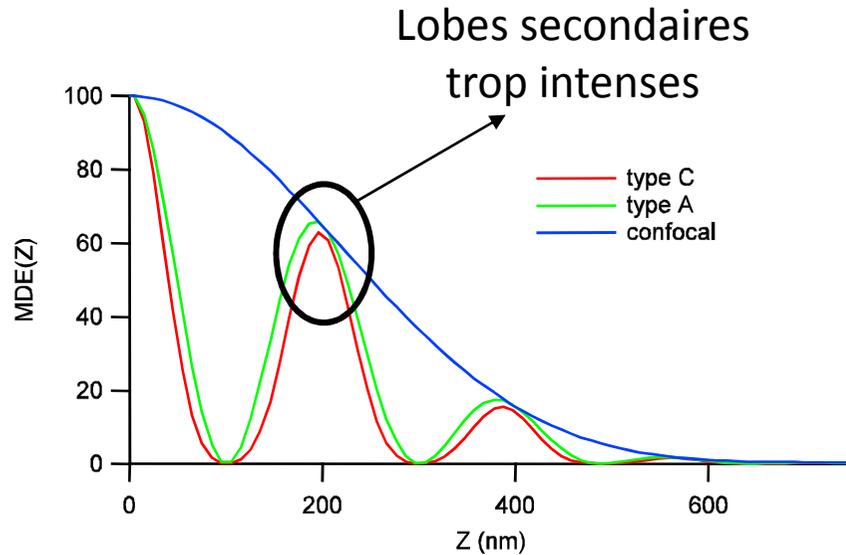
$\lambda=525$  nm  
NA=1,3  $n=1.518$   $m=40$   
 $\phi=20$   $\mu\text{m}$



# CEF d'un $4\pi$ -microscope



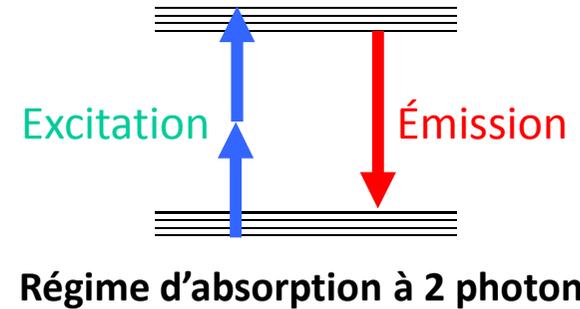
# Résolution axiale: le $4\pi$ -microscope



Images of a pair of actin fibers

# $4\pi$ -microscopie et excitation 2PE

EEF= Carte 3D du carré de l'intensité  
du faisceau pompe focalisé



$$\lambda_{\text{pompe1}} = 488 \text{ nm}$$

$$\lambda_{\text{pompe2}} = 2 \times 488 \text{ nm}$$

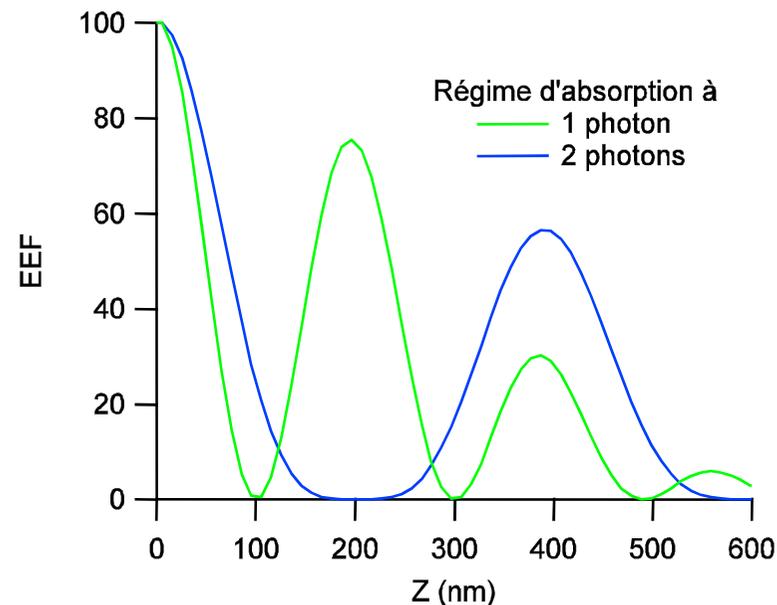
$$\lambda_{\text{fluo}} = 525 \text{ nm}$$

$$\beta = 0.1$$

Polarisation selon X

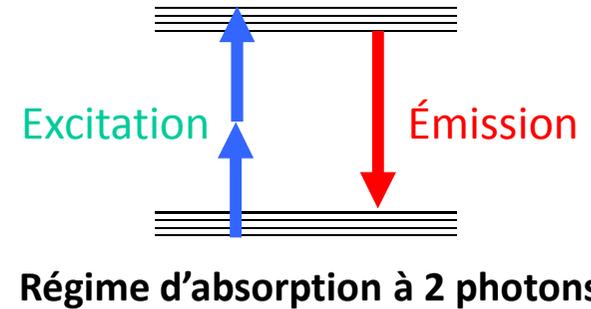
$$\text{NA} = 1,3 \quad n = 1.518$$

$$\Phi = 20 \mu\text{m} \quad m = 40$$



# $4\pi$ -microscopie et excitation 2PE

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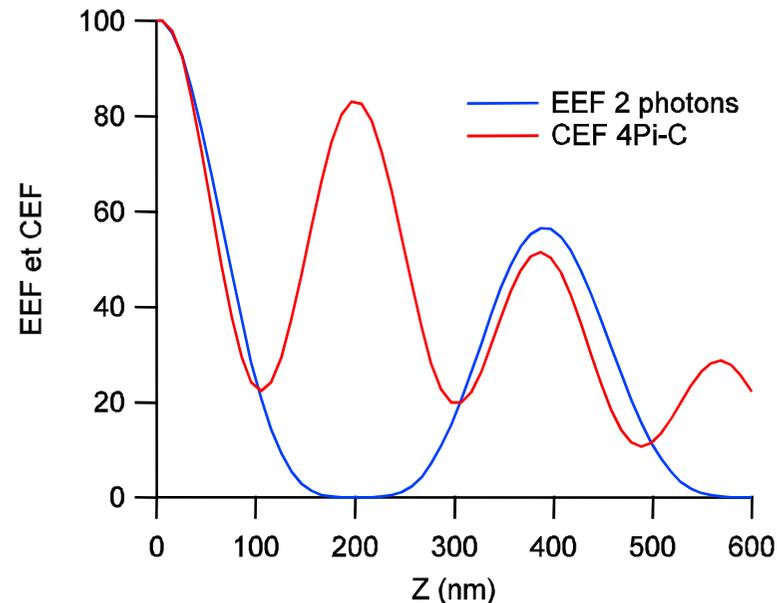
$$\lambda_{\text{fluo}} = 525 \text{ nm}$$

$$\beta = 0.1$$

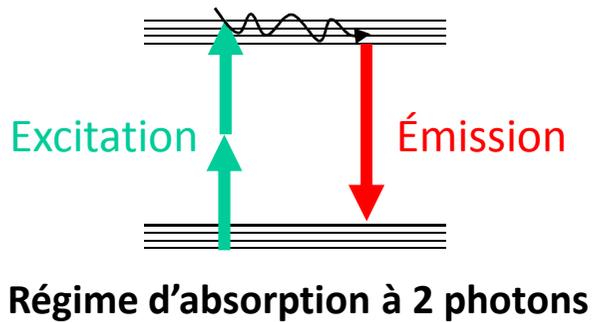
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# $4\pi$ -microscopie et excitation 2PE



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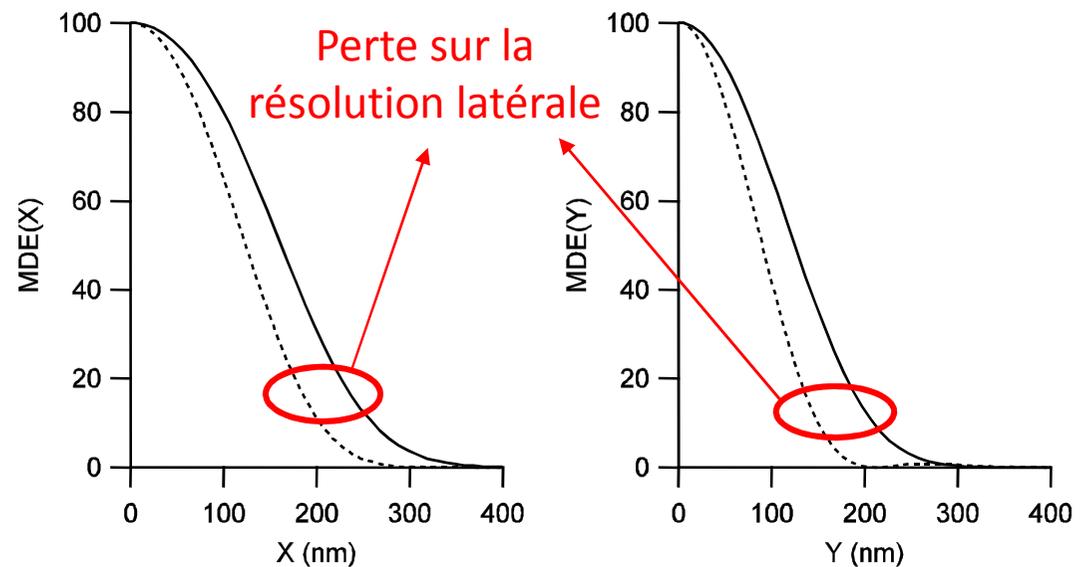
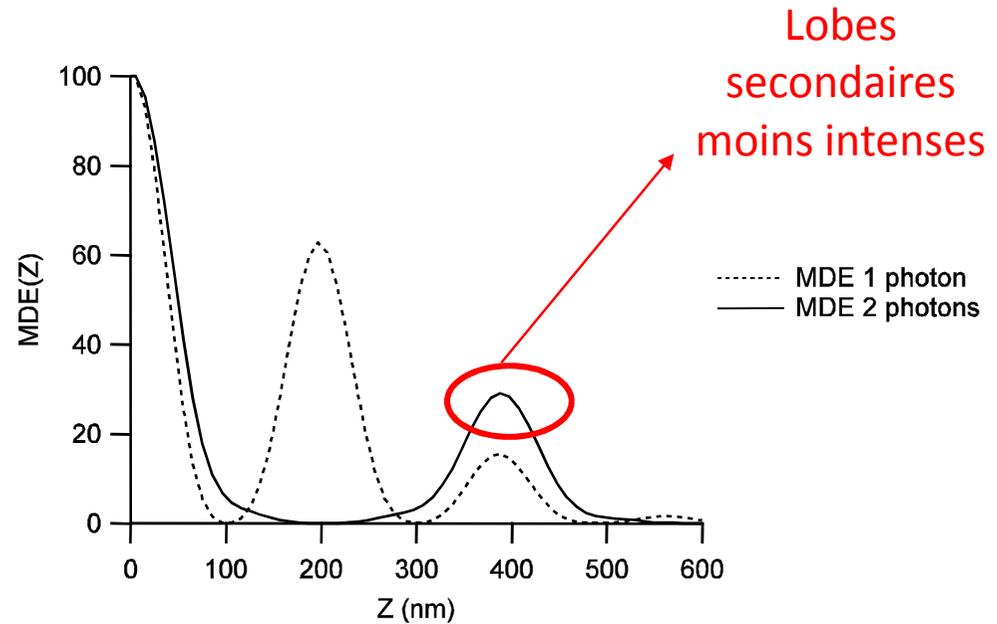
$$\lambda_{\text{fluo}} = 525 \text{ nm}$$

$$\beta = 0.1$$

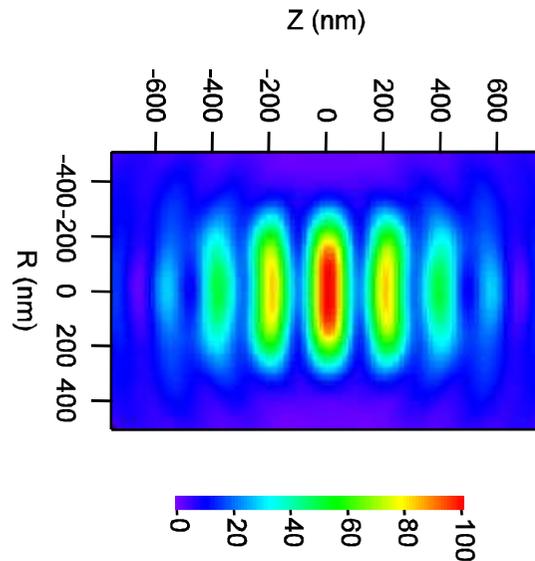
Polarisation selon X

$$\text{NA} = 1,3 \quad n = 1.518$$

$$\Phi = 20 \mu\text{m} \quad m = 40$$

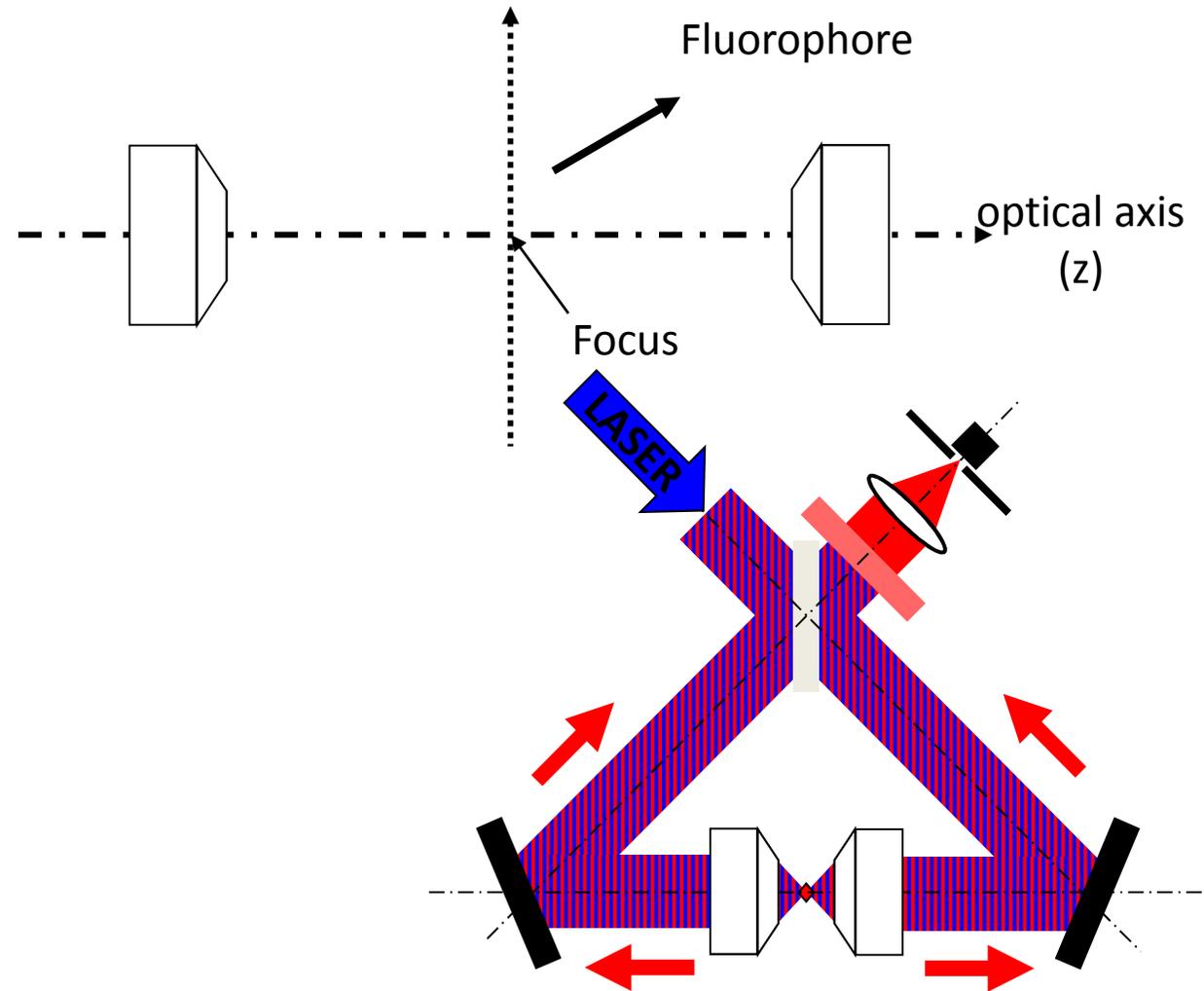


# Résolution latérale: le $4\pi'$



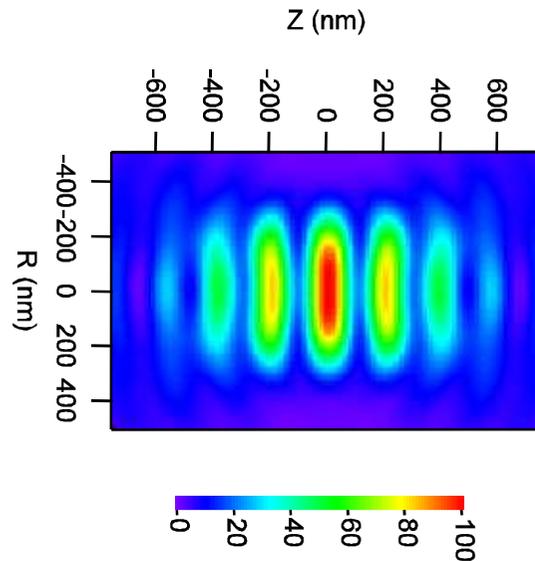
$\lambda=525 \text{ nm } \phi=20 \mu\text{m}$

NA=1,3 n=1.518 m=40



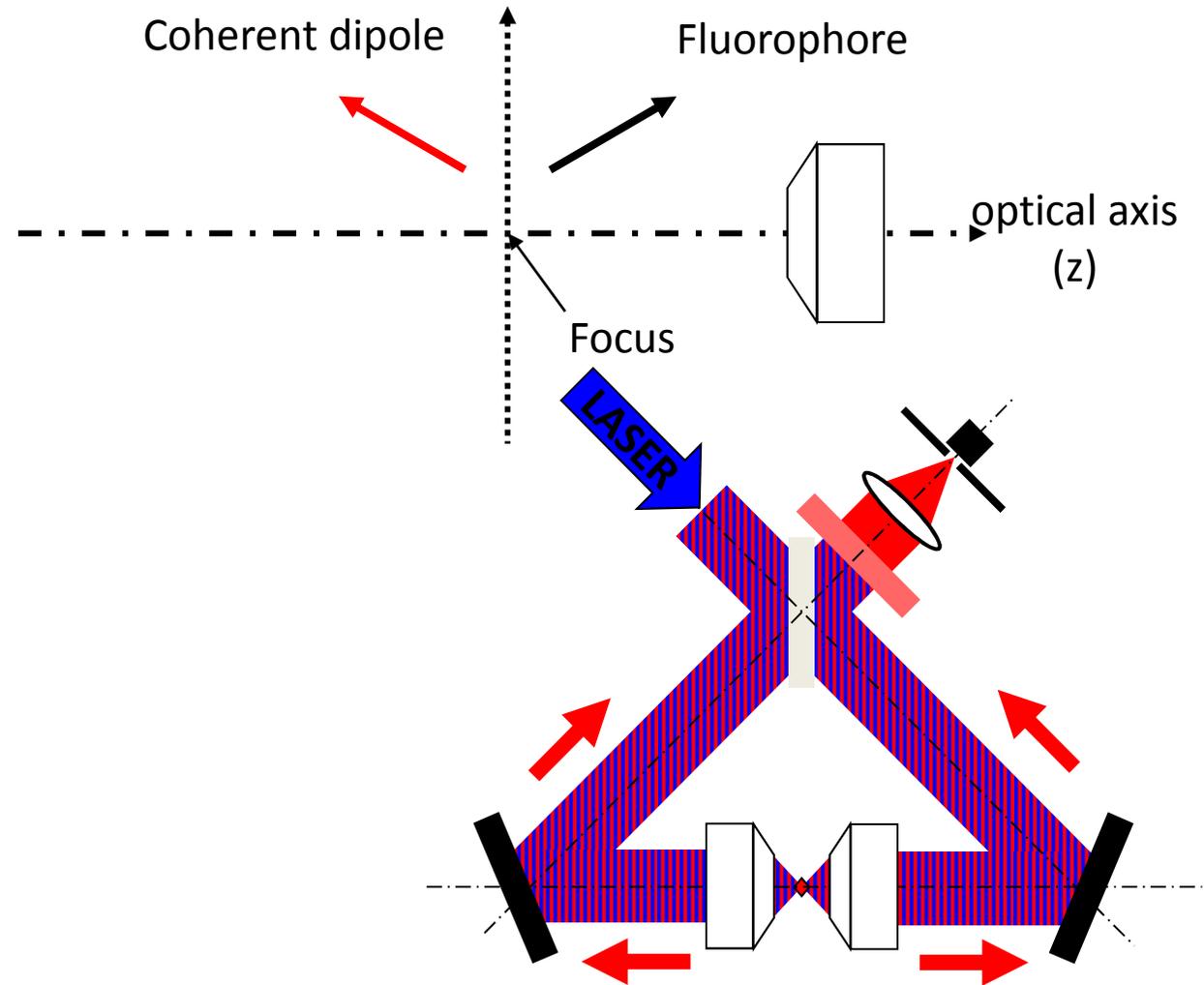
S. Hell, "Double-confocal scanning microscope,"  
European Patent EP0491289 (1992, filed dec. 18 1990).

# Résolution latérale: le $4\pi'$



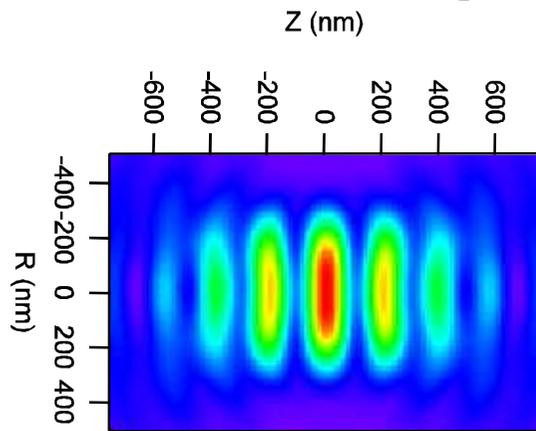
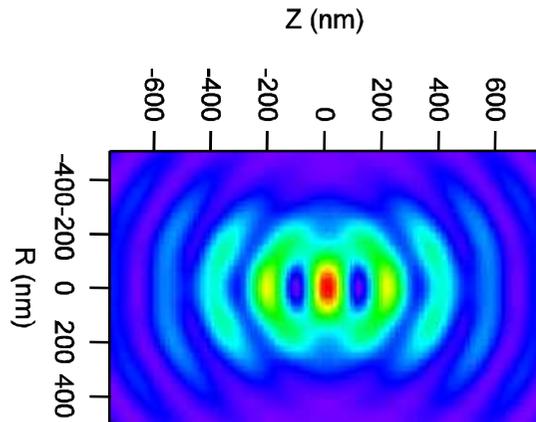
$\lambda=525 \text{ nm}$   $\phi=20 \mu\text{m}$

NA=1,3 n=1.518 m=40



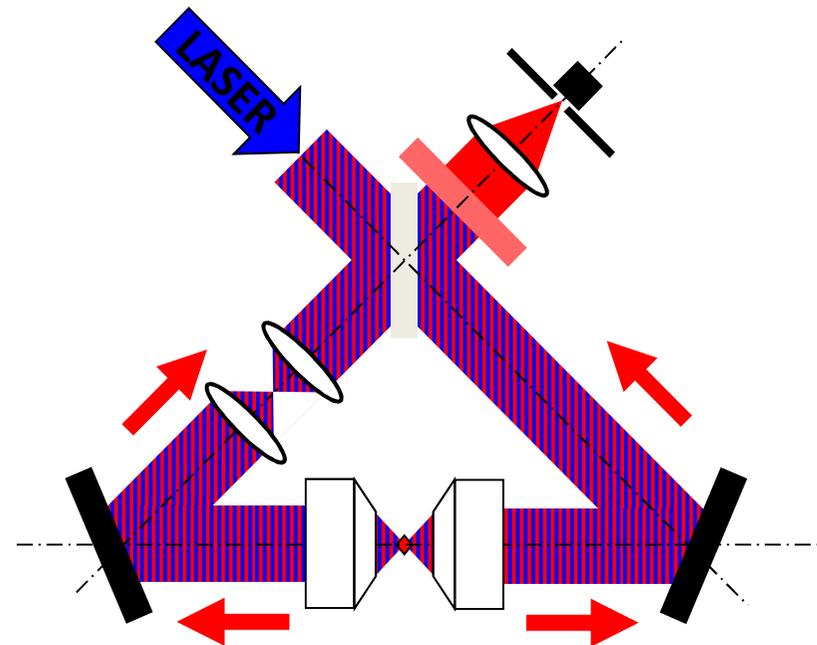
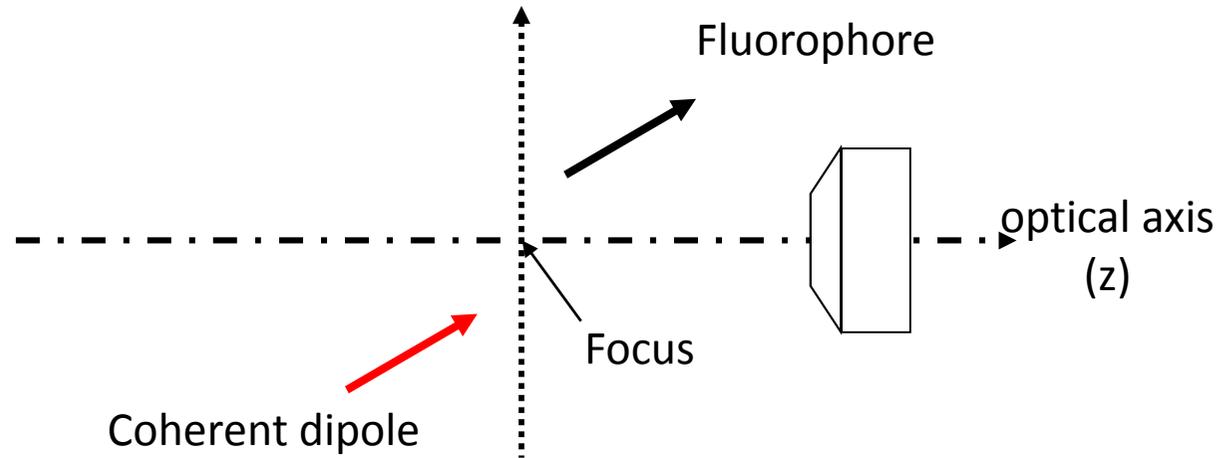
S. Hell, "Double-confocal scanning microscope,"  
European Patent EP0491289 (1992, filed dec. 18 1990).

# Résolution latérale: le $4\pi'$



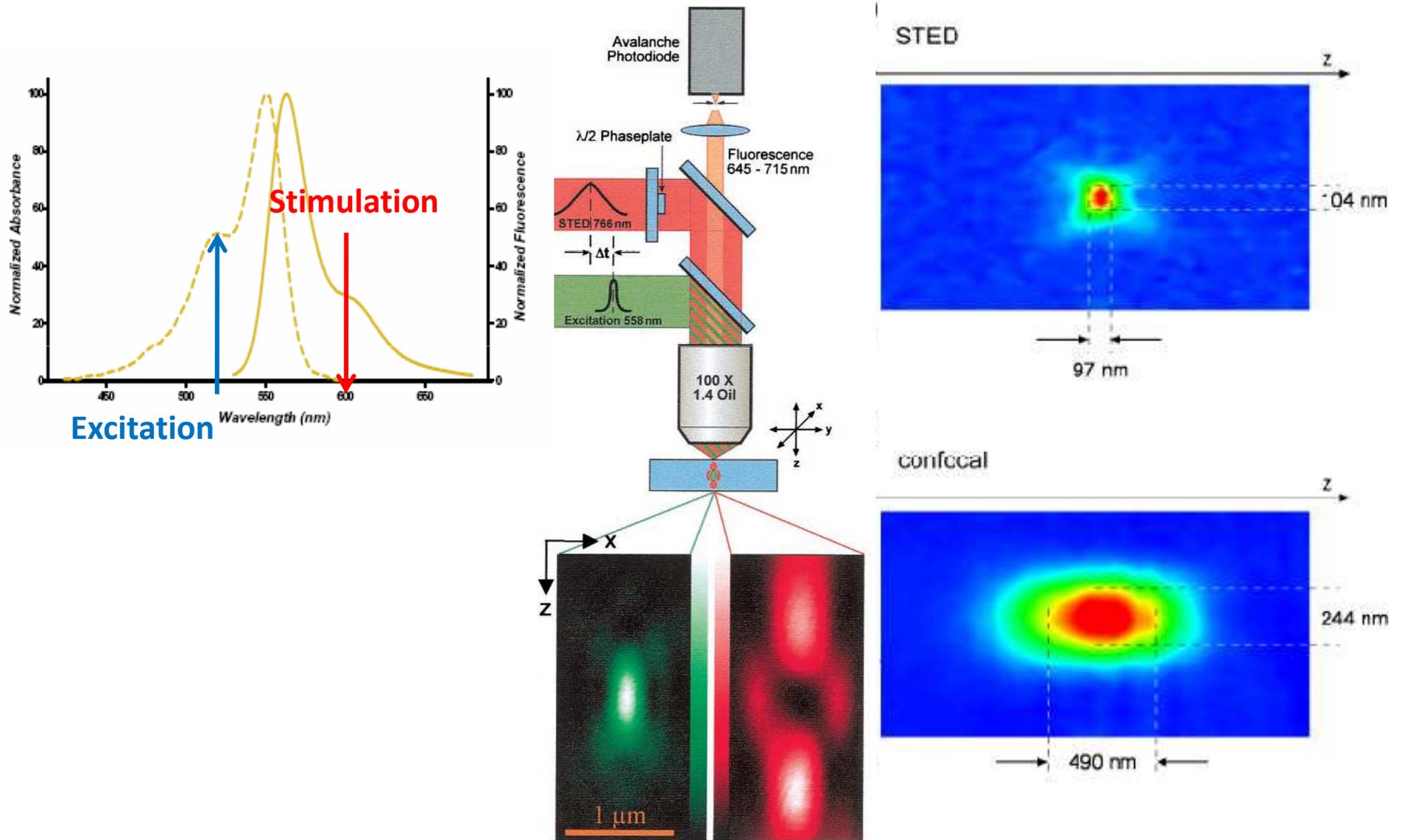
$\lambda=525 \text{ nm}$   $\phi=20 \mu\text{m}$

NA=1,3 n=1.518 m=40



N. Sandeau, H. Giovannini, *J. Opt. Soc. Am. A* **23**, 1089-1095 (2006).

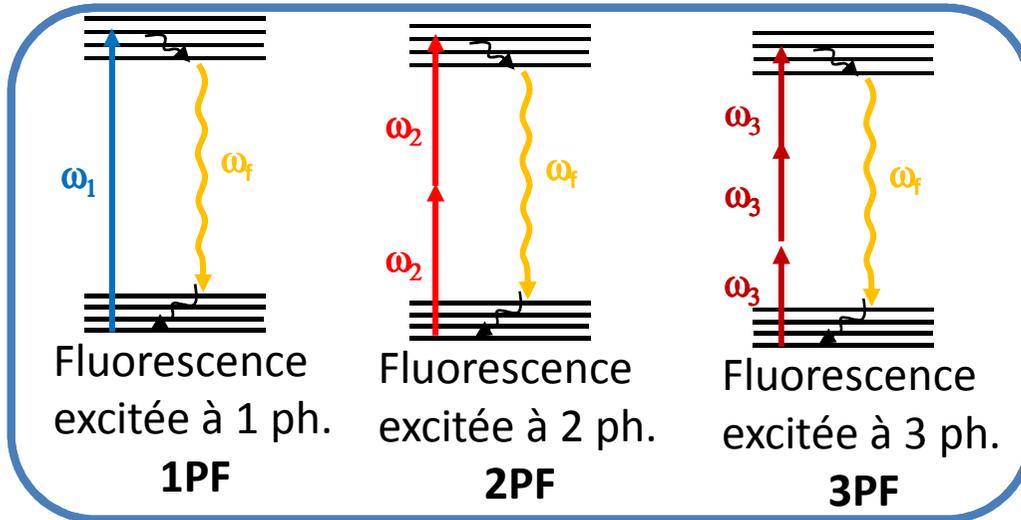
# Résolution 3D: le STED



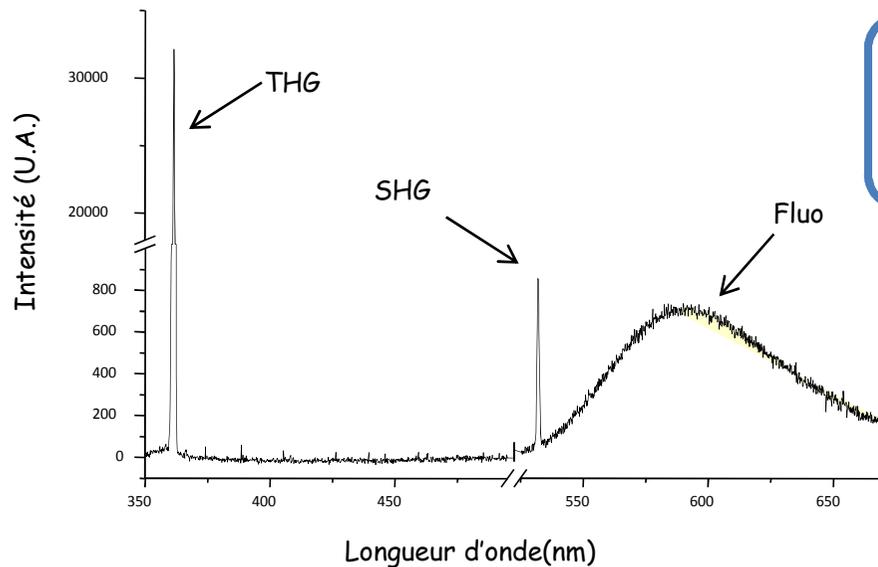
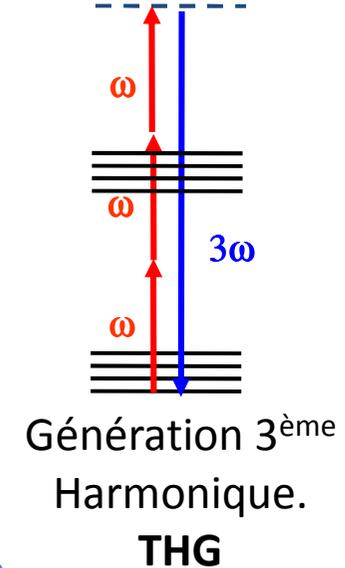
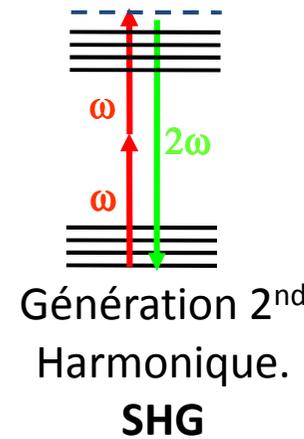
**Principe des microscopies  
non linéaires et/ou  
cohérentes**

# Signaux non linéaires

## Fluorescence



## Signaux cohérents



Uniquement sur les objets non centrosymétriques (**Collagène**)

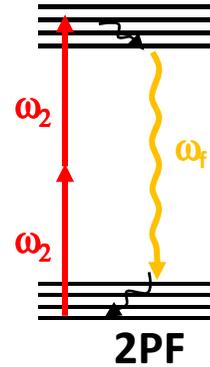
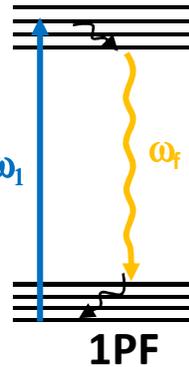
Efficace sur les interfaces (**membranes**)

# Signaux non linéaires

$$I^{1PF} \propto N \sigma^{1PA} \Phi_F I^{\omega_1}$$

avec  $\sigma^{1PA} \approx 10^{-16} \text{ cm}^2$

ex :  $\lambda_1 = 400 \text{ nm}$



$$I^{2PF} \propto N \sigma^{2PA} \Phi_F (I^{\omega_2})^2$$

avec  $\sigma^{2PA} \approx 10^{-49} \text{ cm}^4 \cdot \text{s} \cdot \text{ph}^{-1}$

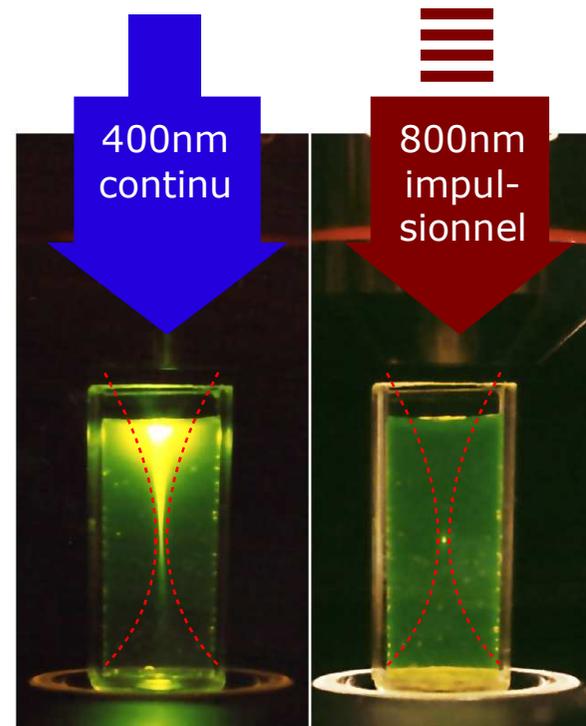
ex :  $\lambda_2 = 800 \text{ nm}$

## Avantages:

- confocalité
- pénétration dans les tissus (- de diffusion)
- + grand écart entre excitation et émission

## Inconvénients:

- sources à impulsions courtes (Prix, spectre...)



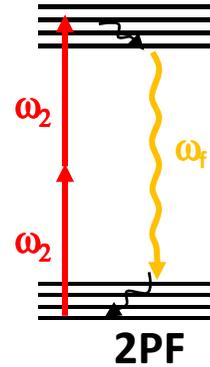
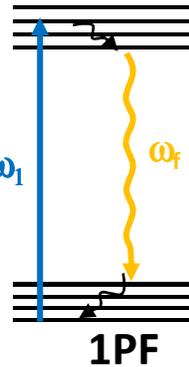
W. R. Zipfel et al. *Nature Biotech.* **21** (2003)

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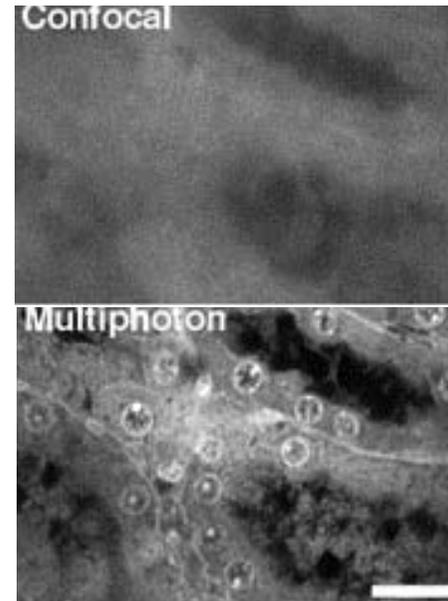
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Rein de singe profondeur 60  $\mu\text{m}$   
exc 750nm, NA 1.2

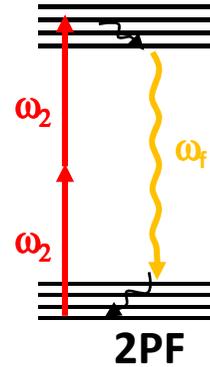
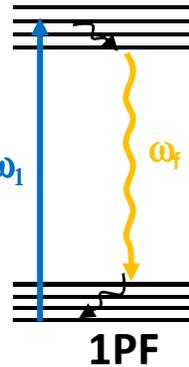
V. E. Centonze et al. *Biophys. J.* **75** (1998)

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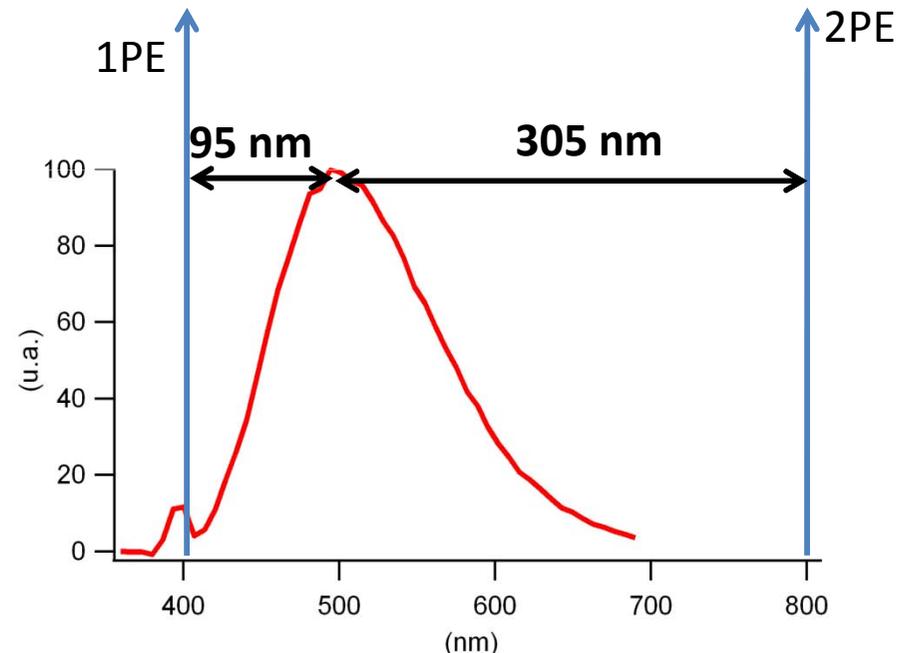
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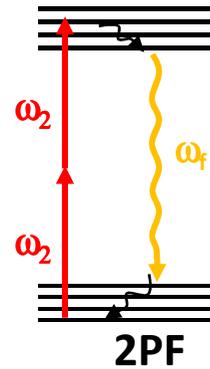
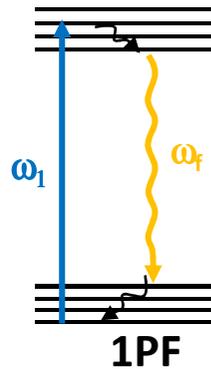
Spectre 2PE sur tissu pulmonaire non marqué  
Excitation à 800 nm

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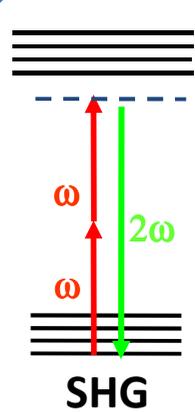
## Avantages:

- confocalité
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## Inconvénients:

- sources à impulsions courtes (Prix, spectre...)

# Signaux Cohérents

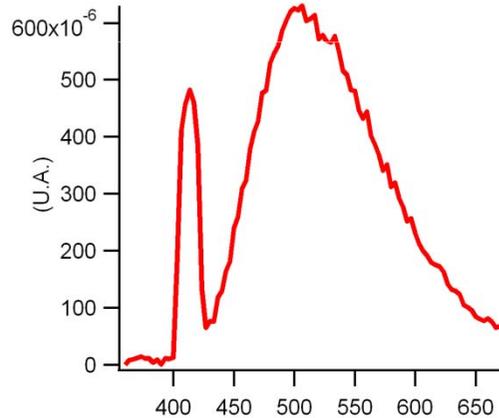


Sur objet non centro-symétrique

$$\vec{p} = \beta : \vec{E}^\omega \vec{E}^\omega \quad (\text{signal cohérent})$$

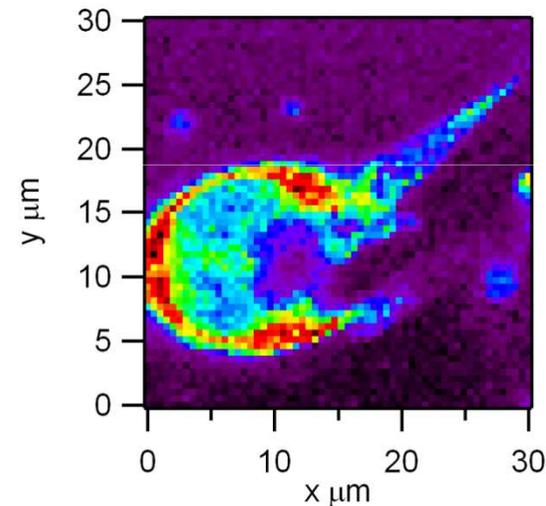
$$I^{\text{SHG}} \longrightarrow N^2 \sigma^{\text{SHG}} (I^\omega)^2$$

avec  $\sigma^{\text{SHG}} \approx 10^{-53} \text{ cm}^4 \cdot \text{s} \cdot \text{ph}^{-1}$



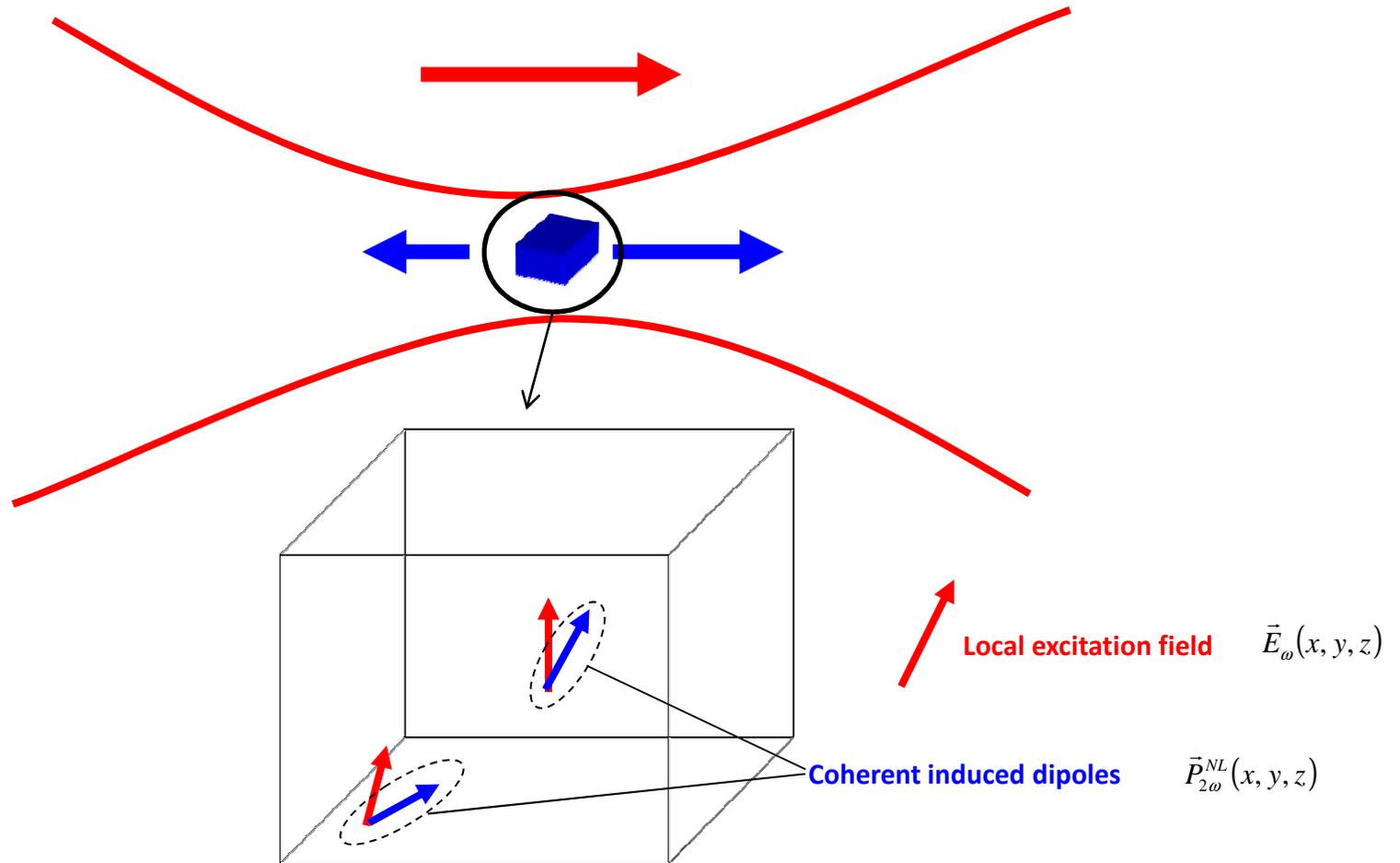
Spectre 2PE sur tissu pulmonaire non marqué  
Excitation à 830 nm

Sensible à l'ordre et à l'orientation des molécules



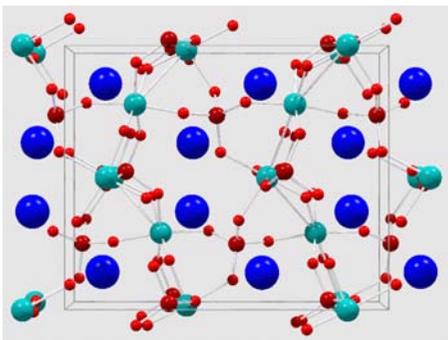
SHG image of fibroblast cell labeled by Di8  
*LPQM Collaboration with The Ben-Gurion University (L. Amire, R. Marx)*

# Microscopies cohérentes : ex la SHG



$$\vec{P}_{2\omega}^{NL}(x, y, z) = \chi_{2\omega}^{(2)} : \vec{E}_{\omega}(x, y, z) : \vec{E}_{\omega}(x, y, z)$$

# Microscopies cohérentes : ex la SHG



Potassium Titanyl Phosphate -  
KTiOPO4

$$\vec{P}_{2\omega}^{NL}(x, y, z) = \chi_{2\omega}^{(2)} : \vec{E}_{\omega}(x, y, z) : \vec{E}_{\omega}(x, y, z)$$

$$\chi_{15}^{(2)} \approx 1.9 \text{ pm/V}$$

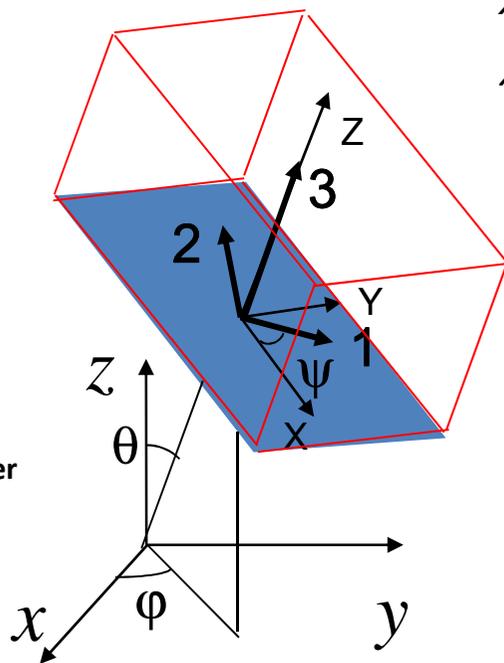
$$\chi_{24}^{(2)} \approx 3.6 \text{ pm/V}$$

$$\chi_{31}^{(2)} \approx 2.5 \text{ pm/V}$$

$$\chi_{32}^{(2)} \approx 4.4 \text{ pm/V}$$

$$\chi_{33}^{(2)} \approx 16.9 \text{ pm/V}$$

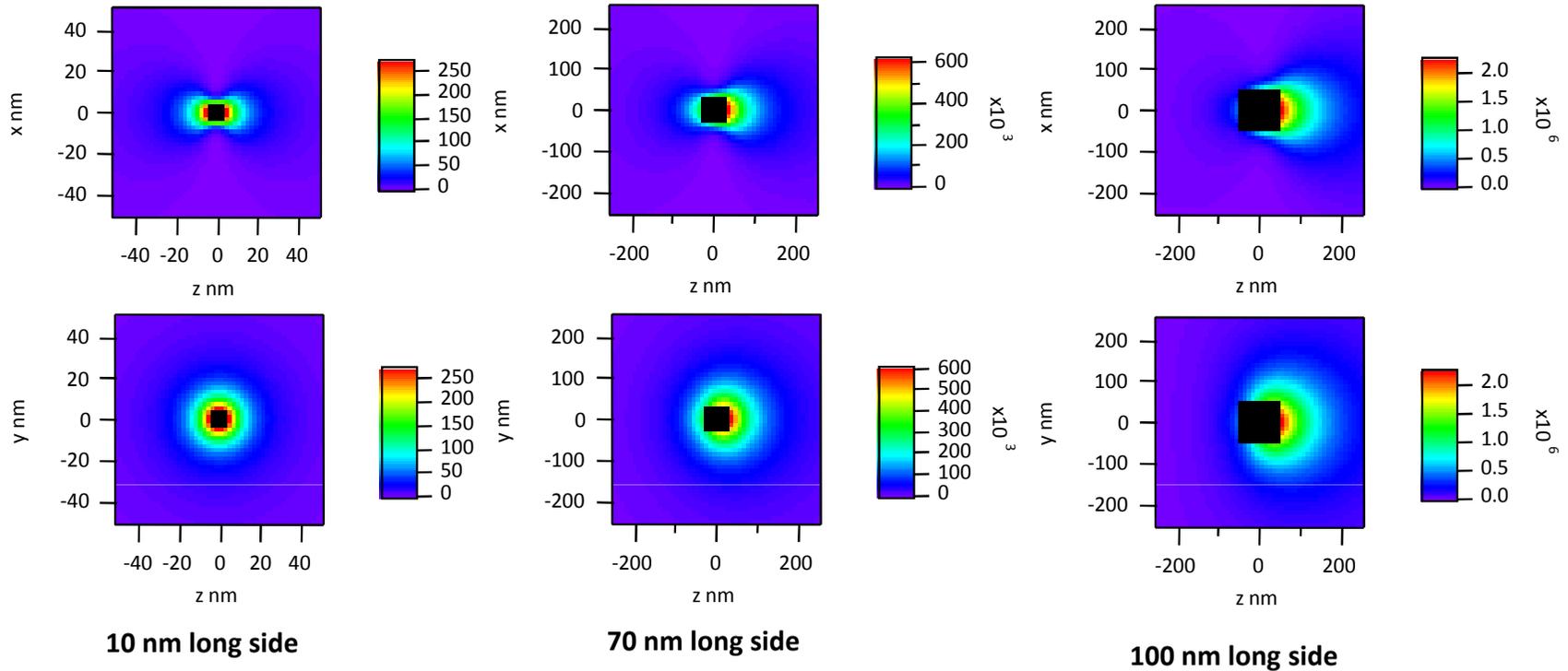
$\chi^{(2)}$  of massive KTP



We define the KTP orientation by the Euler angles  $(\theta, \varphi, \psi)$

**Le signal de SHG dépend du champ d'excitation et de l'objet (orientation, taille...)!**

# Microscopies cohérentes : ex la SHG



KTP along X axis

Euler angles:

$\theta=90^\circ$ ,  $\varphi=0^\circ$ ,  $\psi=0^\circ$

