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Inmed
Institut de neurobiologie de la méditerranée

# Fluorescent microscopy

Microscopie for dummies (or not)
Part 2

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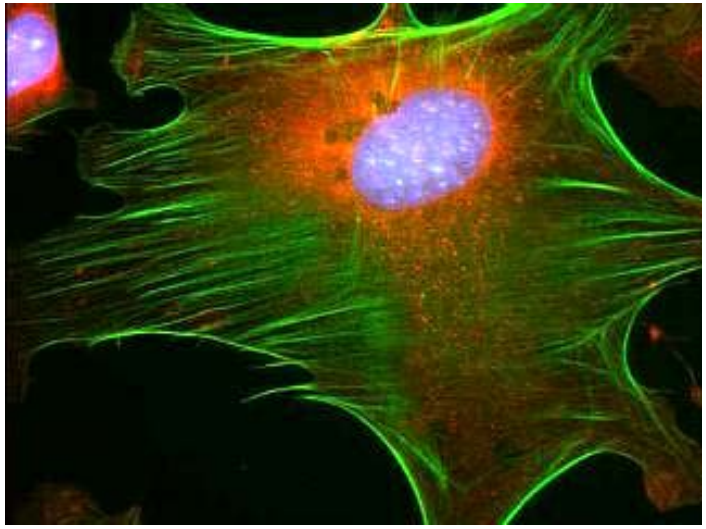
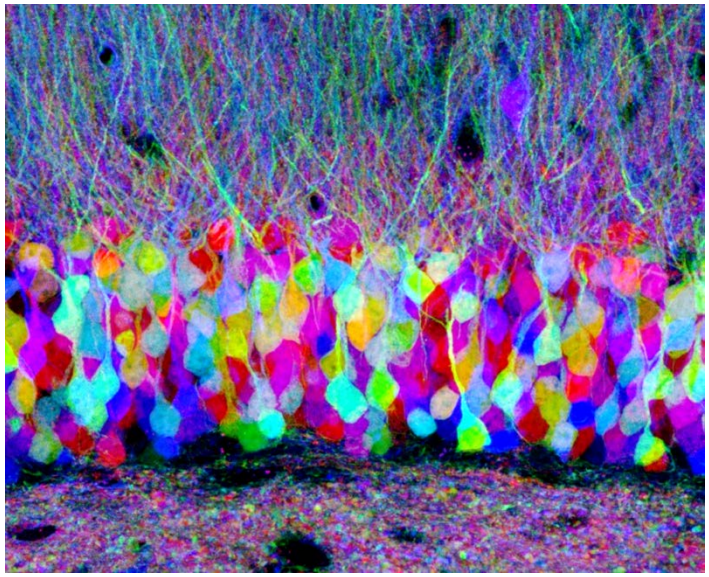
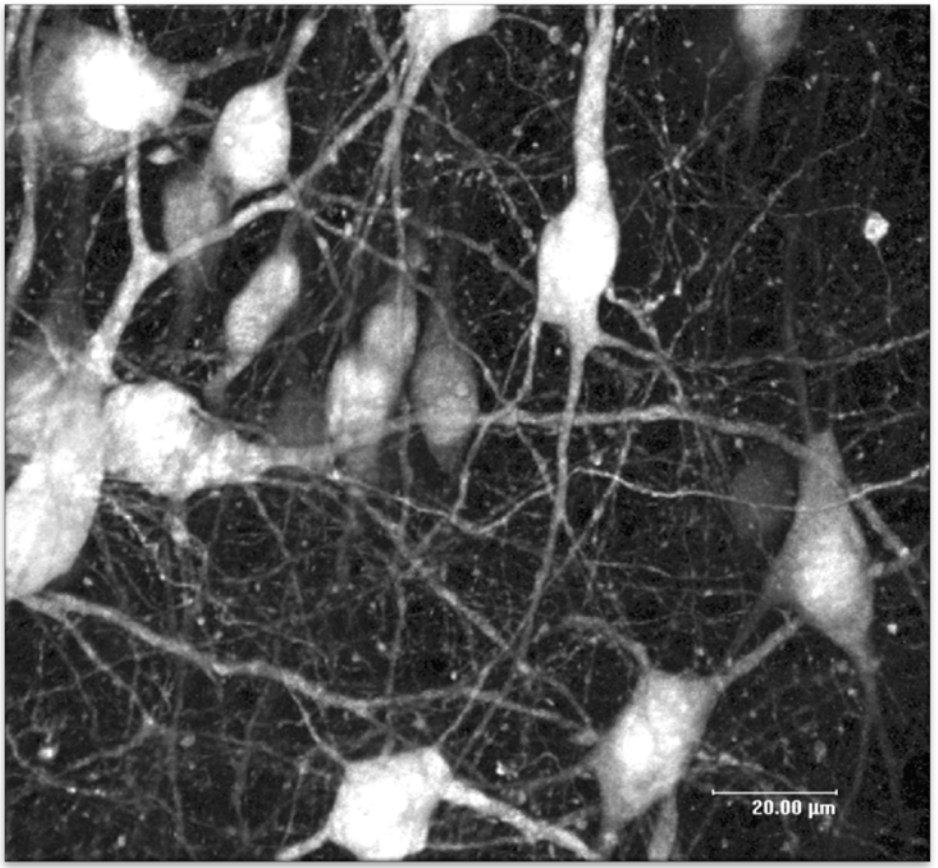
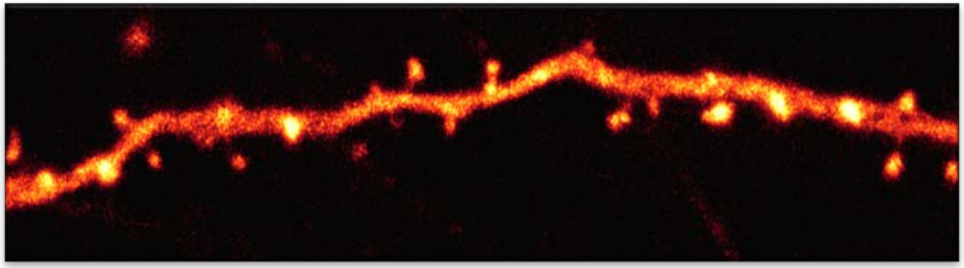
# Some fluorescent images







# What's going on

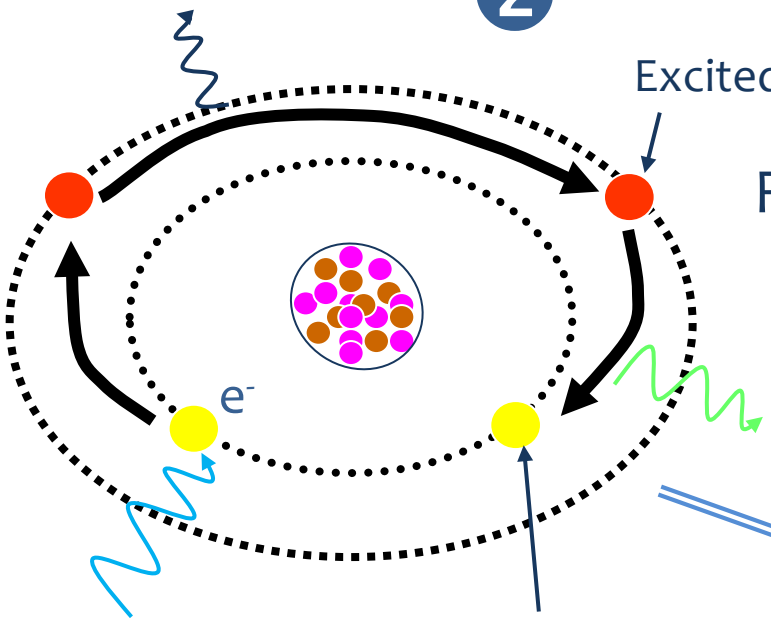
- Fluorescence ??
- Epi-fluorescent microscopy
- Welcome in the third dimension
- Two-photons and others…

# Fluorescence principles

Energy loss (potential, thermal, kinetic…) : **relaxation** 2

Excited state ($S_1$) : external electronic orbital

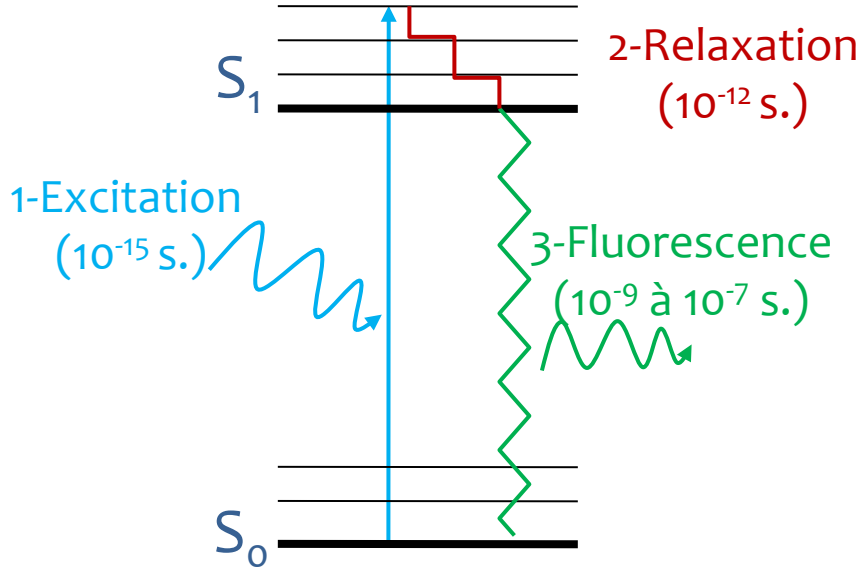
Fluorescence emission 3




Stable state ($S_0$)

**Excitation**

= Give energy E1 1




Energetic diagram of Jablonski (very simplified)

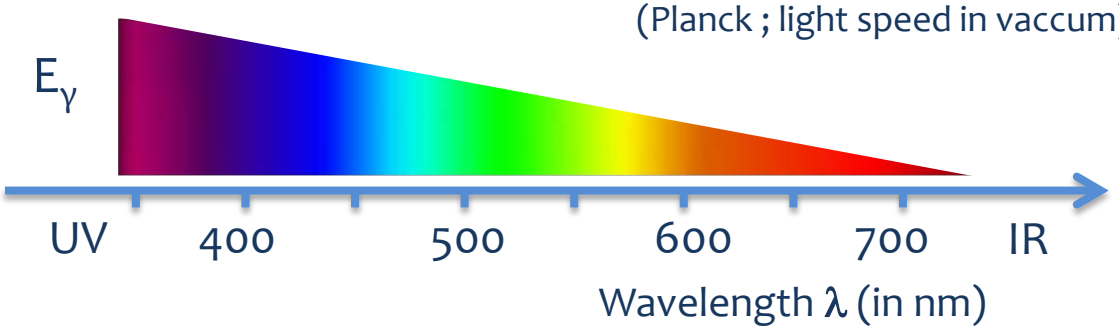
Fluorescent molecule = Fluorophore

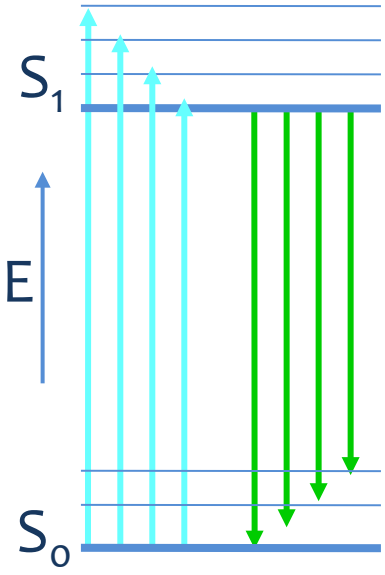
# Fluorescence principles

Excitation and emission are quantum phenomenon: the molecule accept a certain energy amount, neither too much nor too little, to be excited and reemit this energy around a privileged value (emission peak).

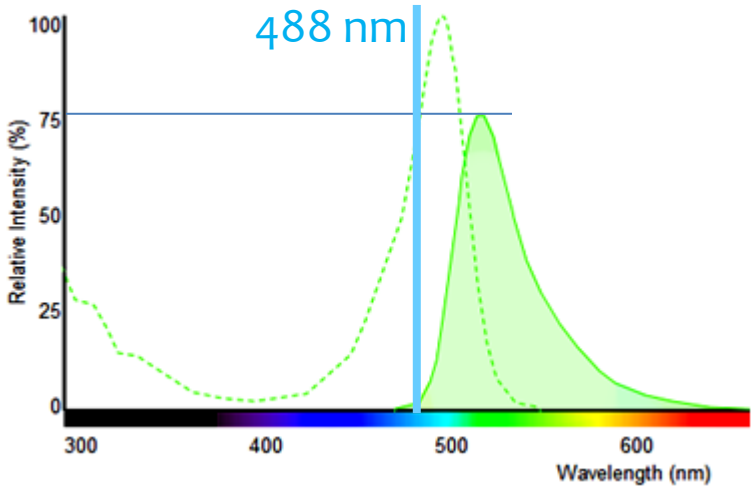
The energy of a photon is: $E_\gamma = hc/\lambda$

Where h and c were constants (Planck ; light speed in vaccum)



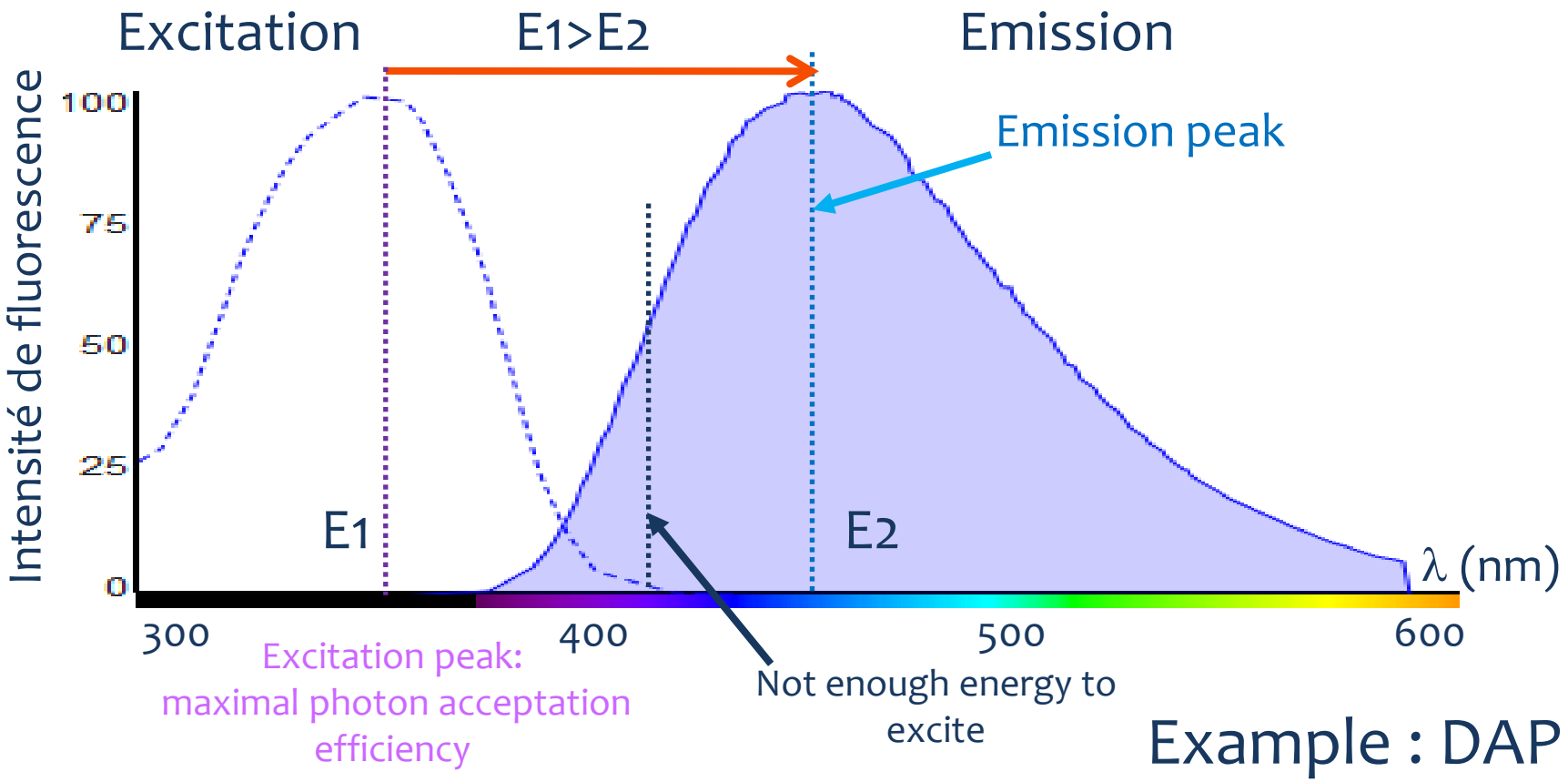

The different vibrationnal states of each energy levels reflected themselves in the excitation and emission spectra



# Fluorescence principles

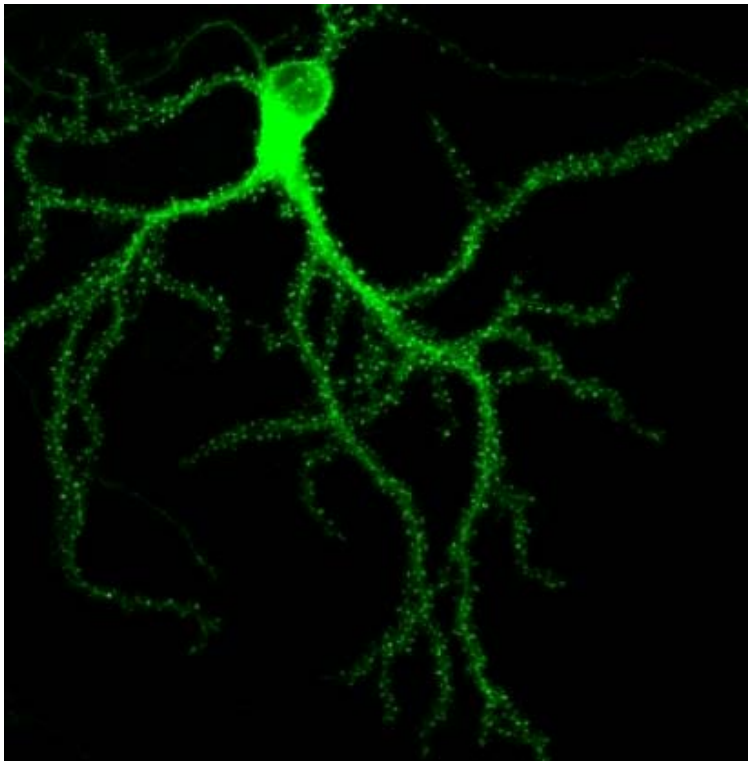
Each fluorescent molecule is characterized by an excitation and an emission spectra (influenced by its own chemical structure and physical environment (pH, T… )).

Energy loss between excitation and emission
Red shift : (Stokes shift)




Example : DAPI

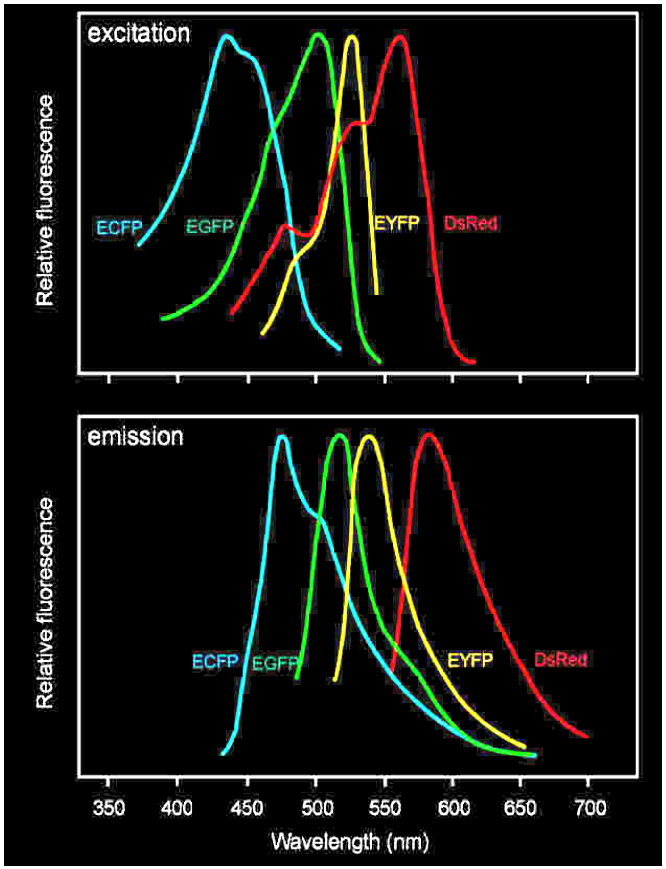
# Fluorescence principles



The main interest of fluorescence is the high level of signal/noise ratio.
We can also add other advantages such as:

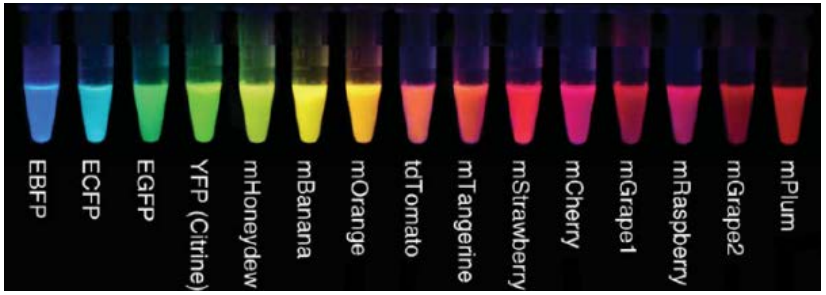
- Label specificity
- *In vivo* compatibility and non-toxicity
- Easy to use
- Cheap
- Versatility…

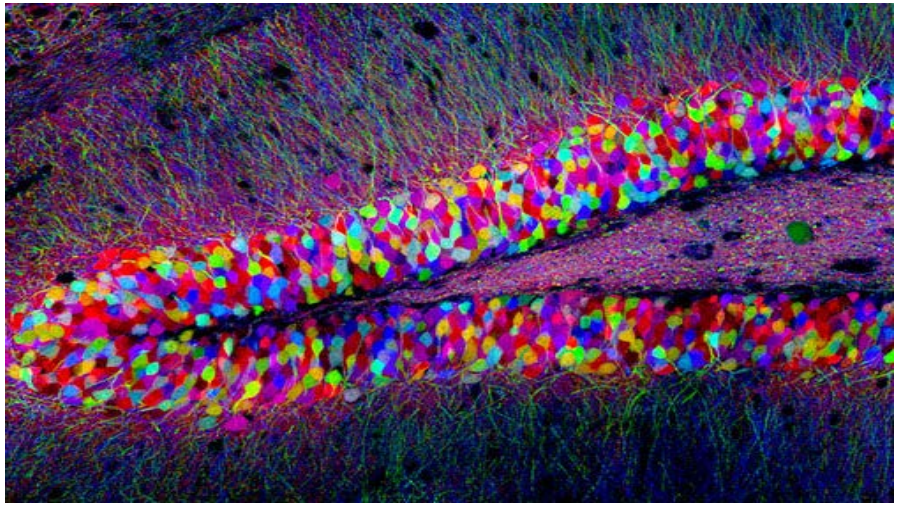
# Fluorescent proteins




They come from jellyfish or from corral genes. Fluorescent proteins combine the advantages of fluorescence and genetic engineering to help us in biological processes analysis. They were optimized for different characteristics: spectral, quantum yield, stability, expression speed, sensitivity to pH, $Ca^{2+}$, $Cl^{-}$ …

| | Excitation Max. (nm) | Emission Max (nm) |
|---|---|---|
| **CFP** | 434 | 477 |
| **GFP** | 489 | 508 |
| **YFP** | 514 | 527 |
| **Ds Red** | 558 | 583 |



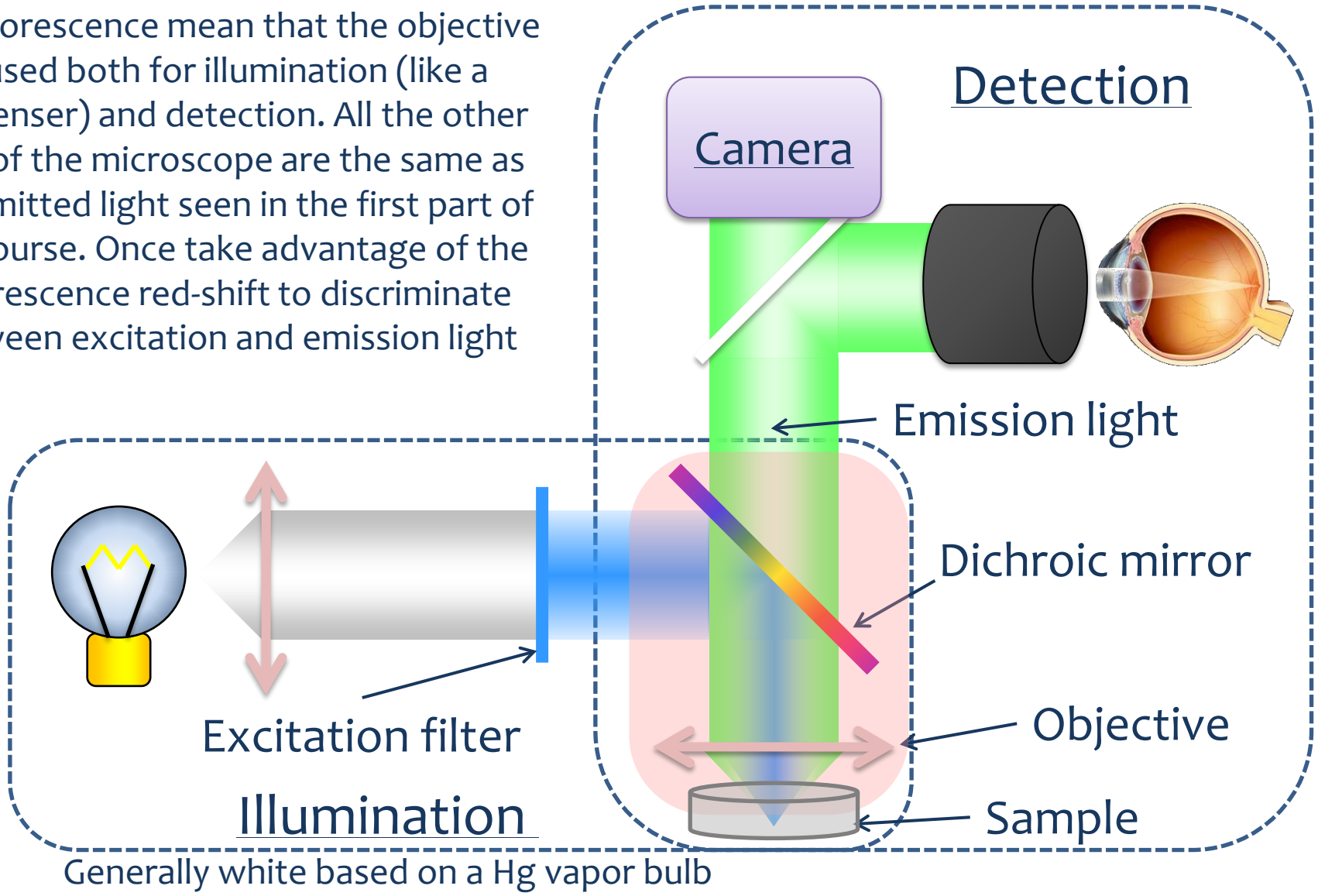
*« Brainbow mouse » Livet et al. Nature 2007*

# What's going on (again)

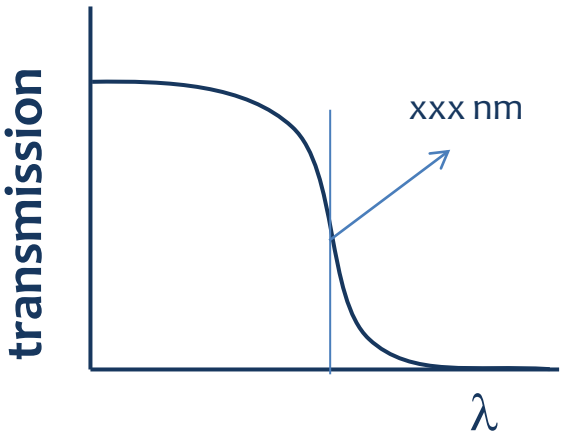
- ✓ Fluorescence ??
- ➢ Epi-fluorescent microscopy
- ➢ Welcome in the third dimension
- ➢ Two-photons and others…

# Epi-fluorescence microscopy

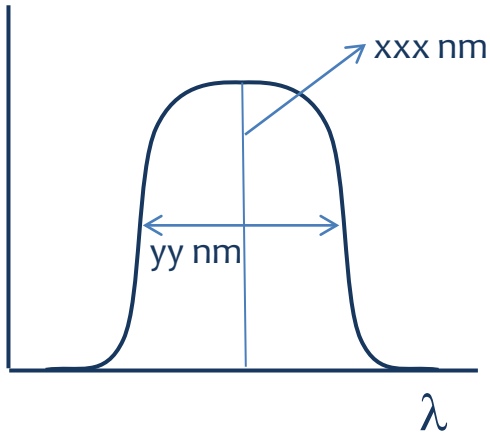
Epi-fluorescence mean that the objective is used both for illumination (like a condenser) and detection. All the other part of the microscope are the same as transmitted light seen in the first part of the course. Once take advantage of the fluorescence red-shift to discriminate between excitation and emission light



# Filters typology

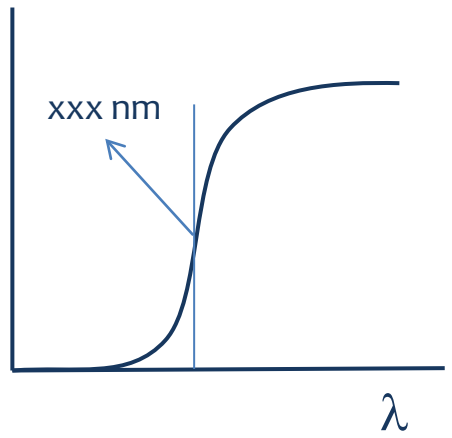



**Low-pass (LP xxx)**

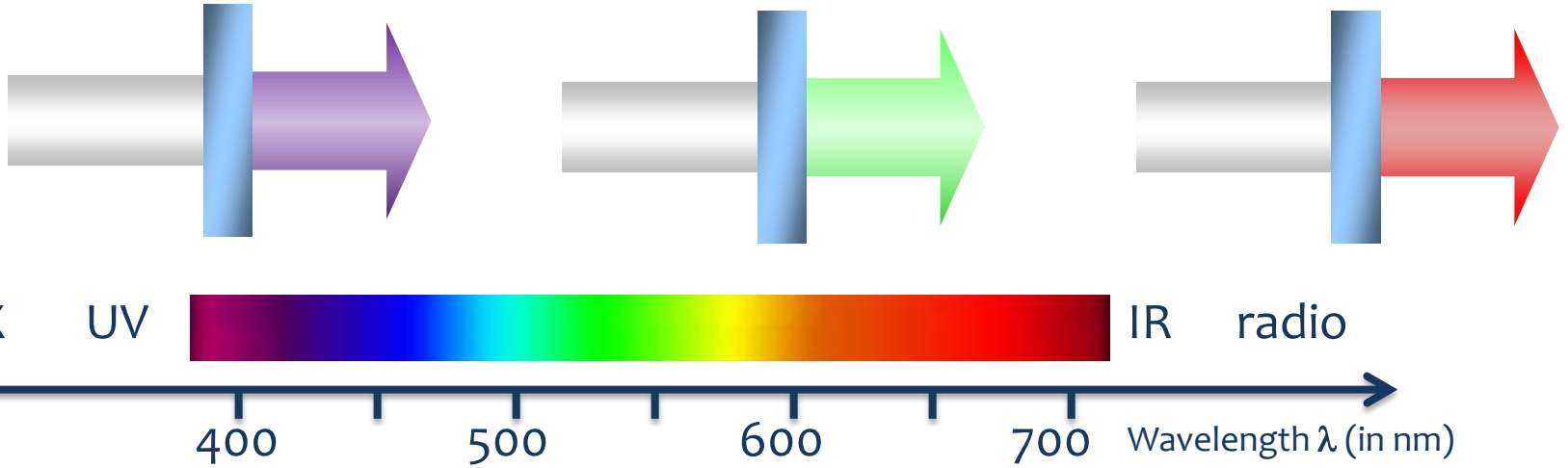



**Band pass (BP xxx/yy)**

**BP 525/50 mean a filter that transmit from 500 to 550 nm**



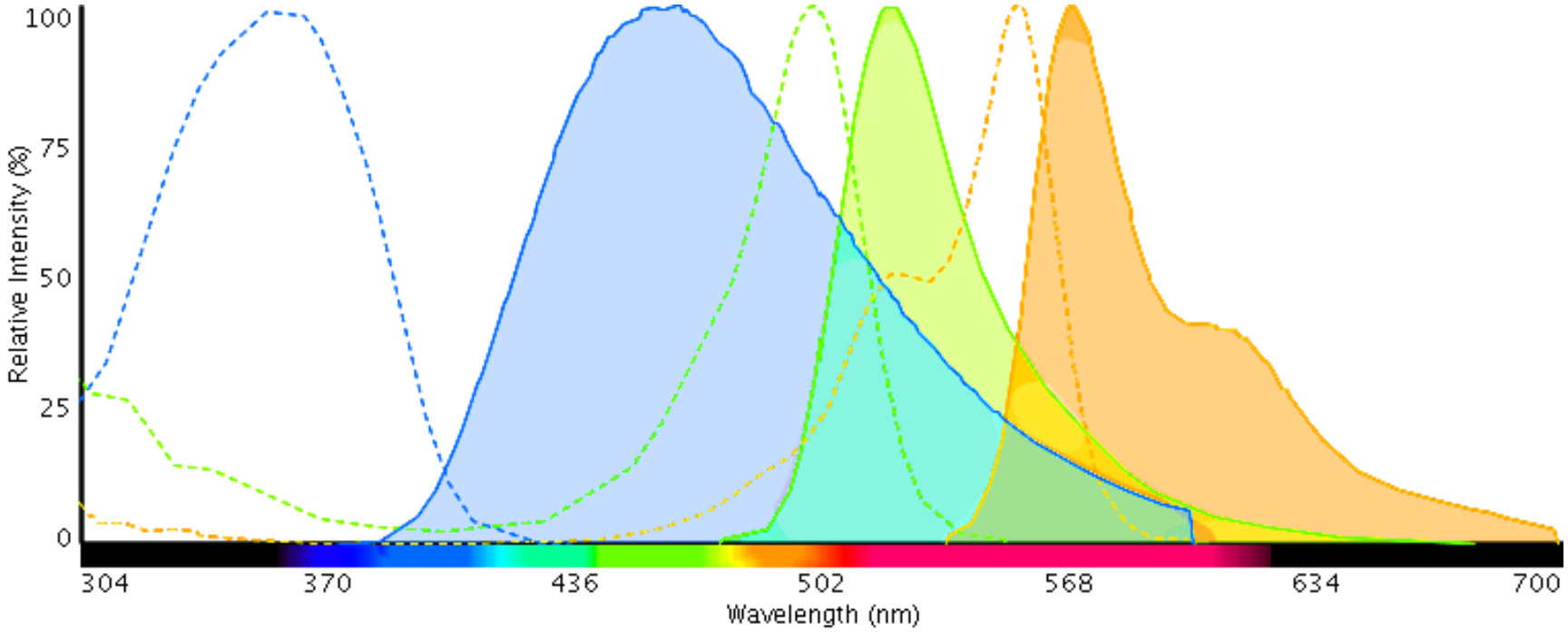

**High pass (HP XX)**



# Filter typology

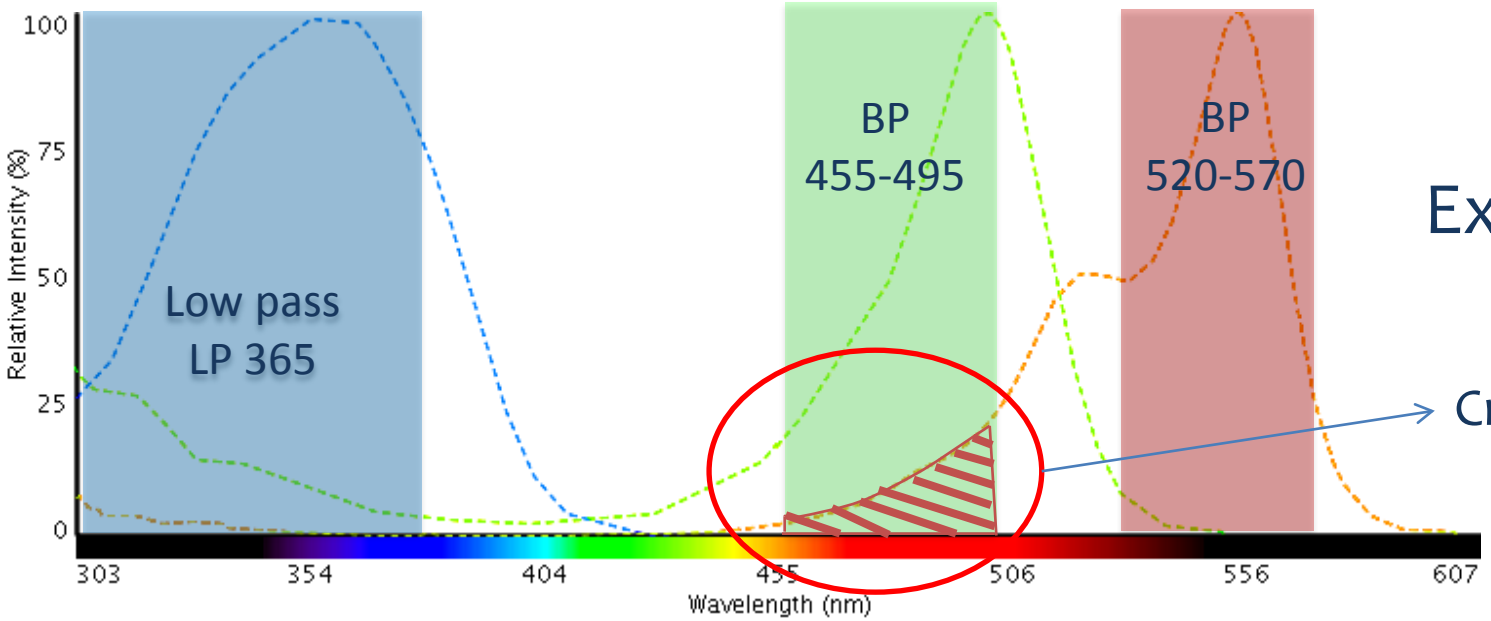
Multi-labeling need smart choice in the filters and fluorophores. Indeed, excitation and emission spectra could overlap and induce artifacts. For example, excitation spectrum of A555 have a shoulder in excitation spectrum of A488 : We have to discriminate both signal with emission even if we lose part of the signal and if we need to make sequentially one color after the other.

DAPI Alexa 488 Alexa 555




— — — Excitation

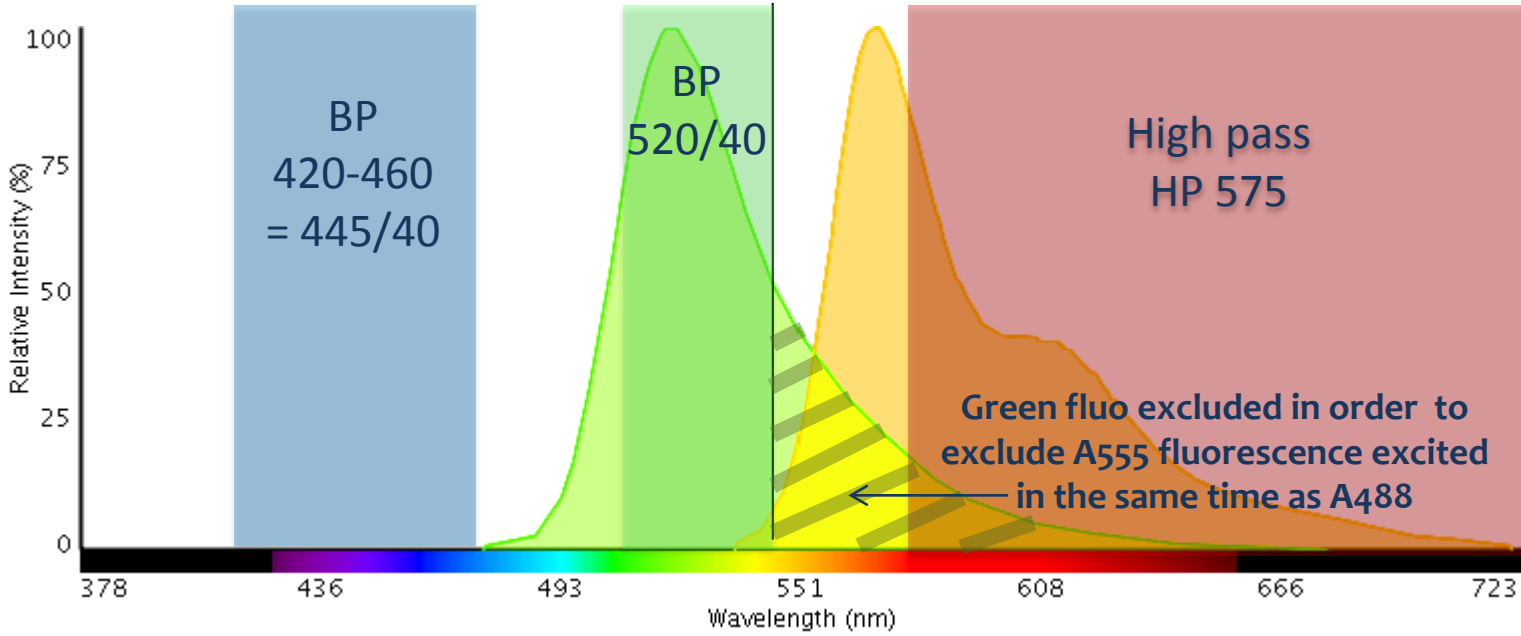
# Filter choice



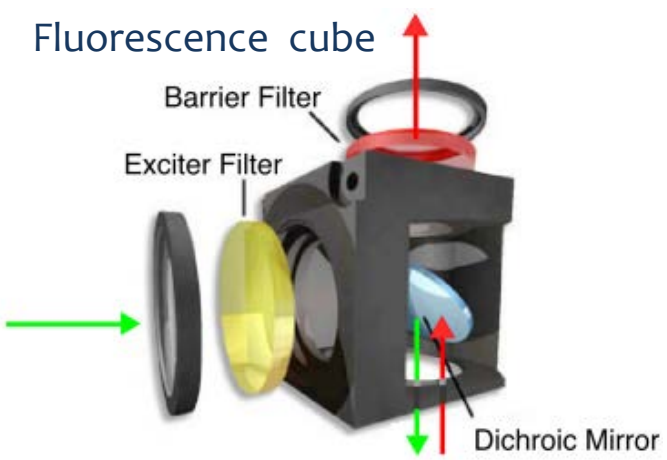

Excitation

Cross-talk

Emission



# Fluorescence microscopy





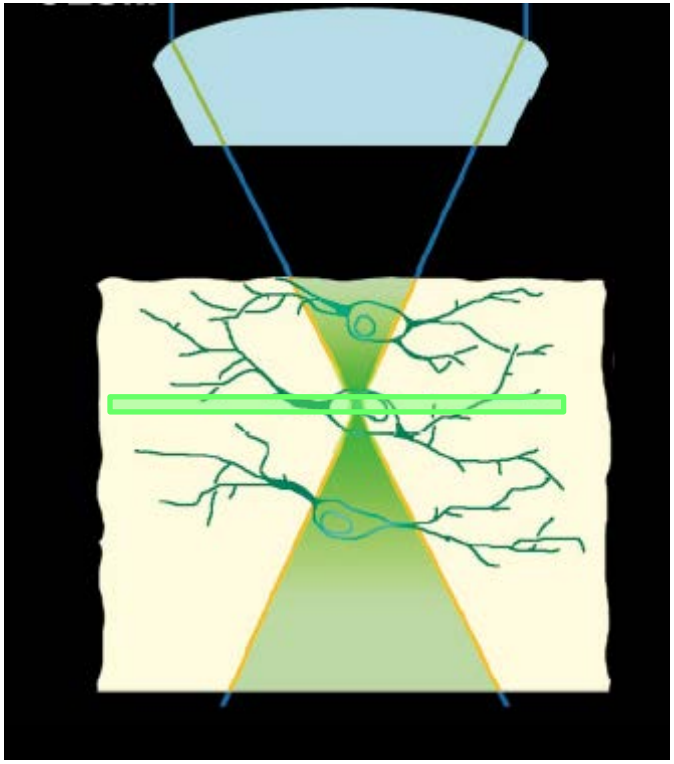


Each trio (cube), Dichroic mirror + filters, depend of each observed fluorophore individually and/or in multi-labeling

# What's going on (again)

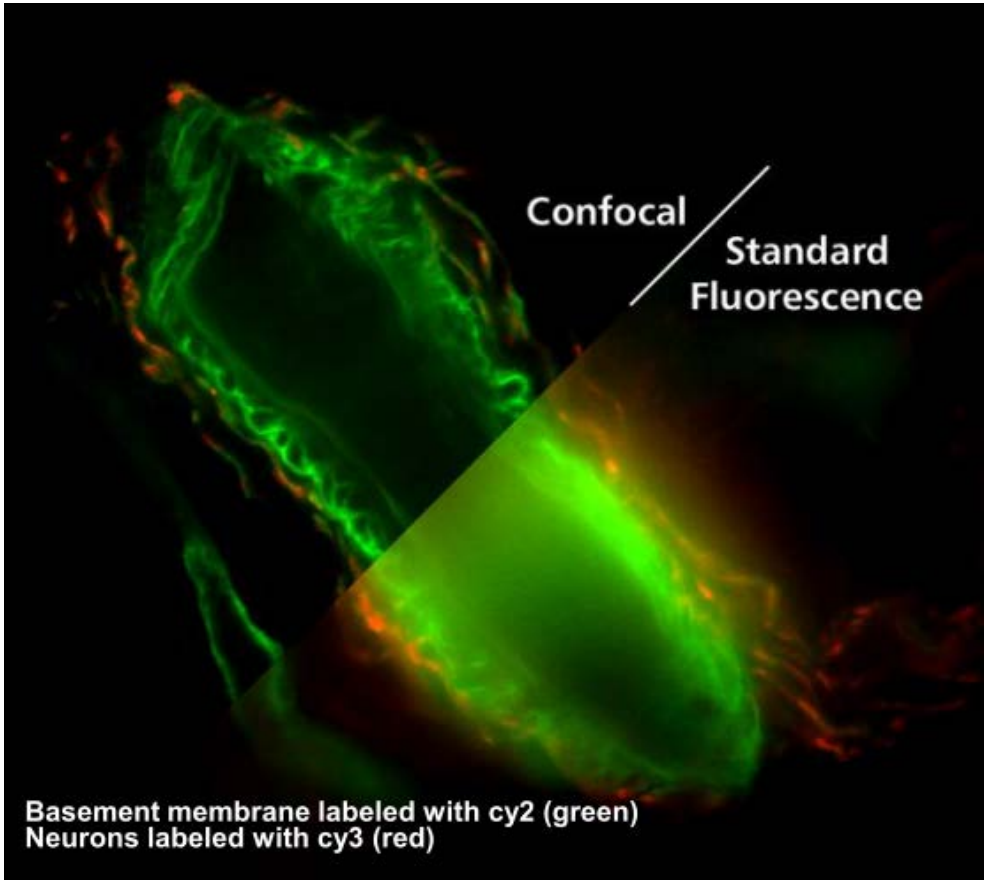
- ✓ Fluorescence ??
- ✓ Epi-fluorescent microscopy
- ➢ Welcome in the third dimension
- ➢ Two-photons and others…

# Welcome in the third dimension

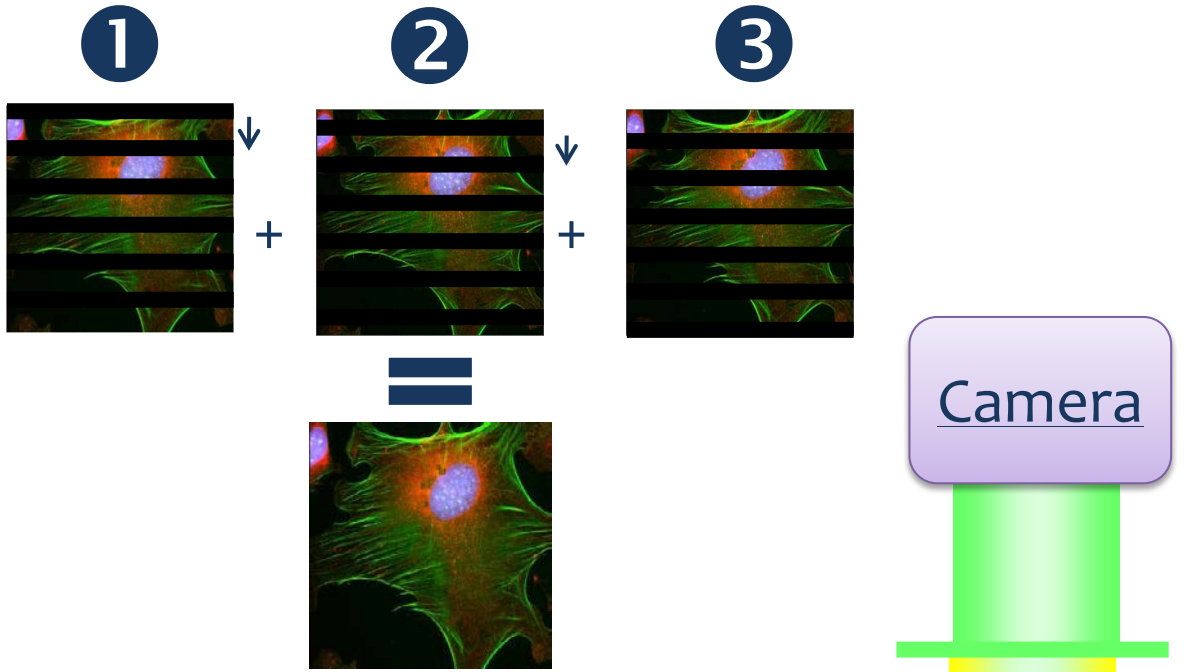


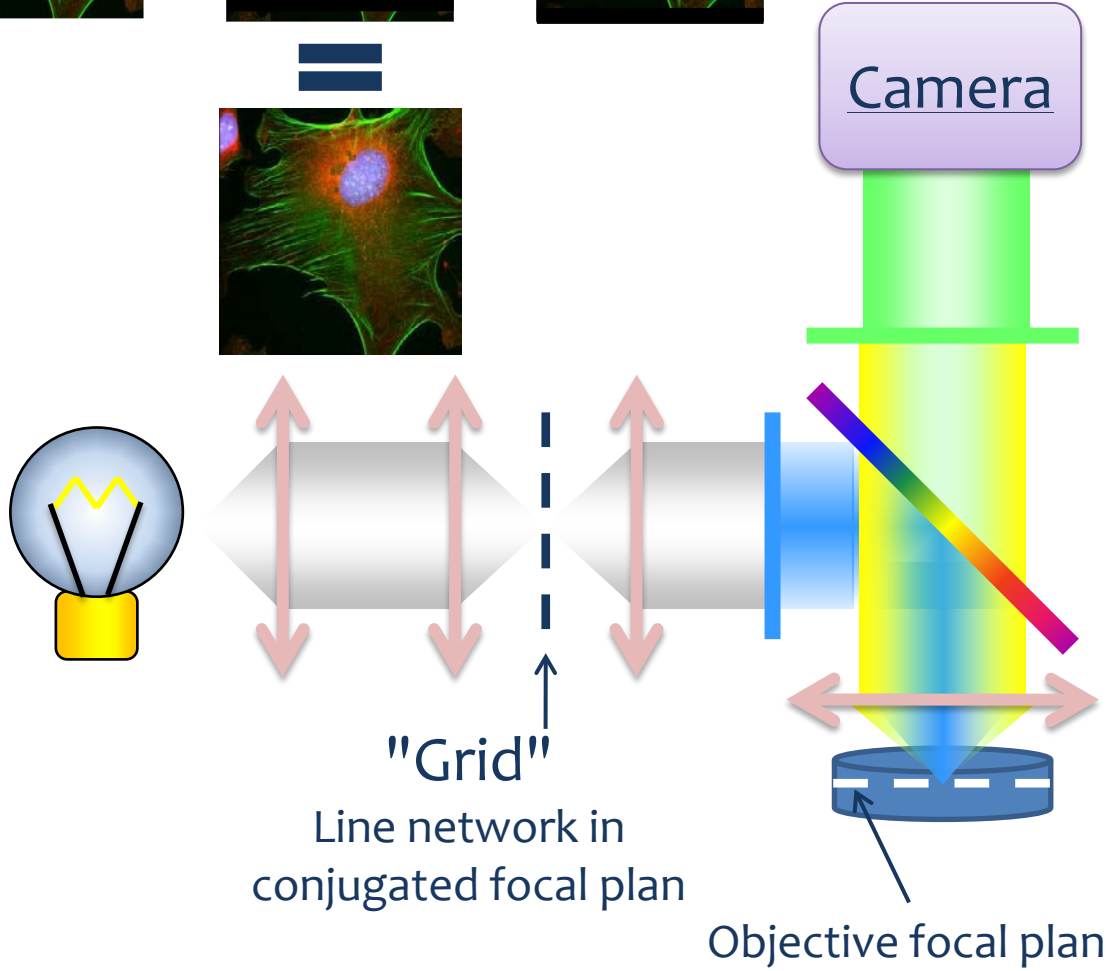
There are several techniques that increase the quality of images, the most famous is confocal microscopy.

Out of focus fluorescence induce blurring that altered images. We can detect all photons that come from the totality of the illumination column (as a diabolo).



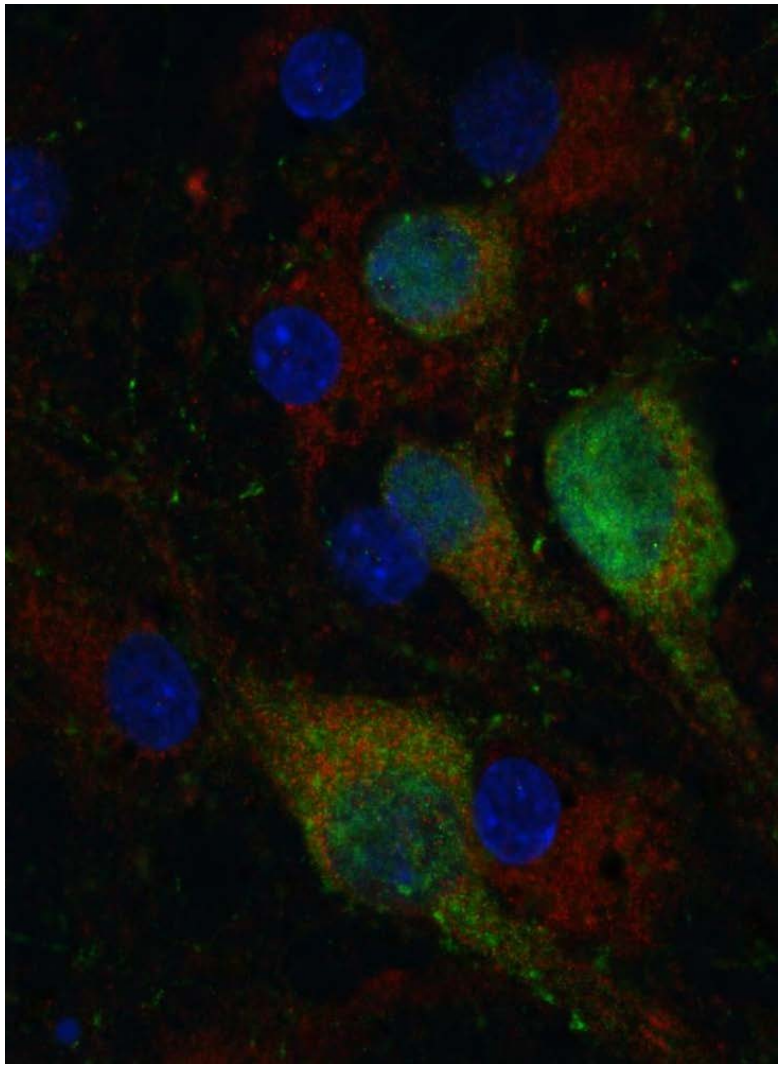
# Structured Illumination microscopy



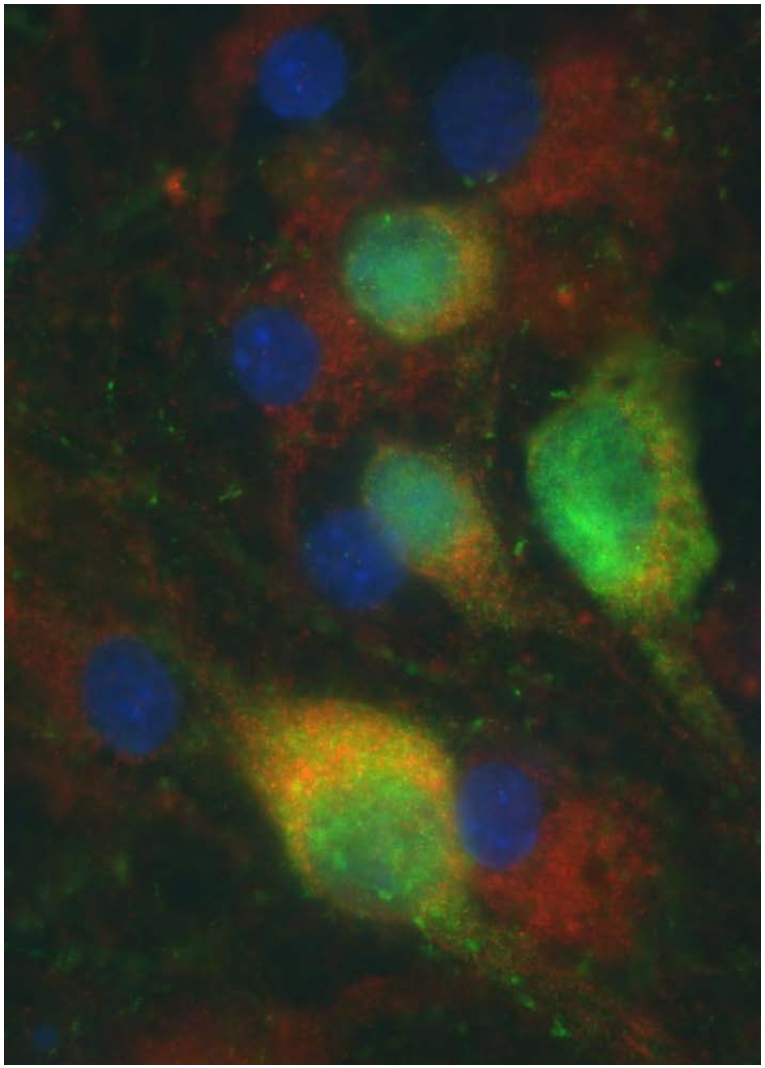




A dark line network (grid) is placed in a conjugated focal plan with the objective one. The lines occult 1/3 of the field. We then obtain a sharp image of the grid in the object focal plan and 1/3 of the field is hidden by black lines and not excited. Diffused/out-of-focus photons that arrived to the camera are removed thanks to the software. Indeed, the computer " know " where are the black lines and non pertinent photons are subtracted. The grid is 1/3 of field translated and a new image is generated. The merge of 3 successive shifted striped images allow the reconstruction of a cleaned of out-of-focus photons image (optical slice).

# Structured Illumination microscopy

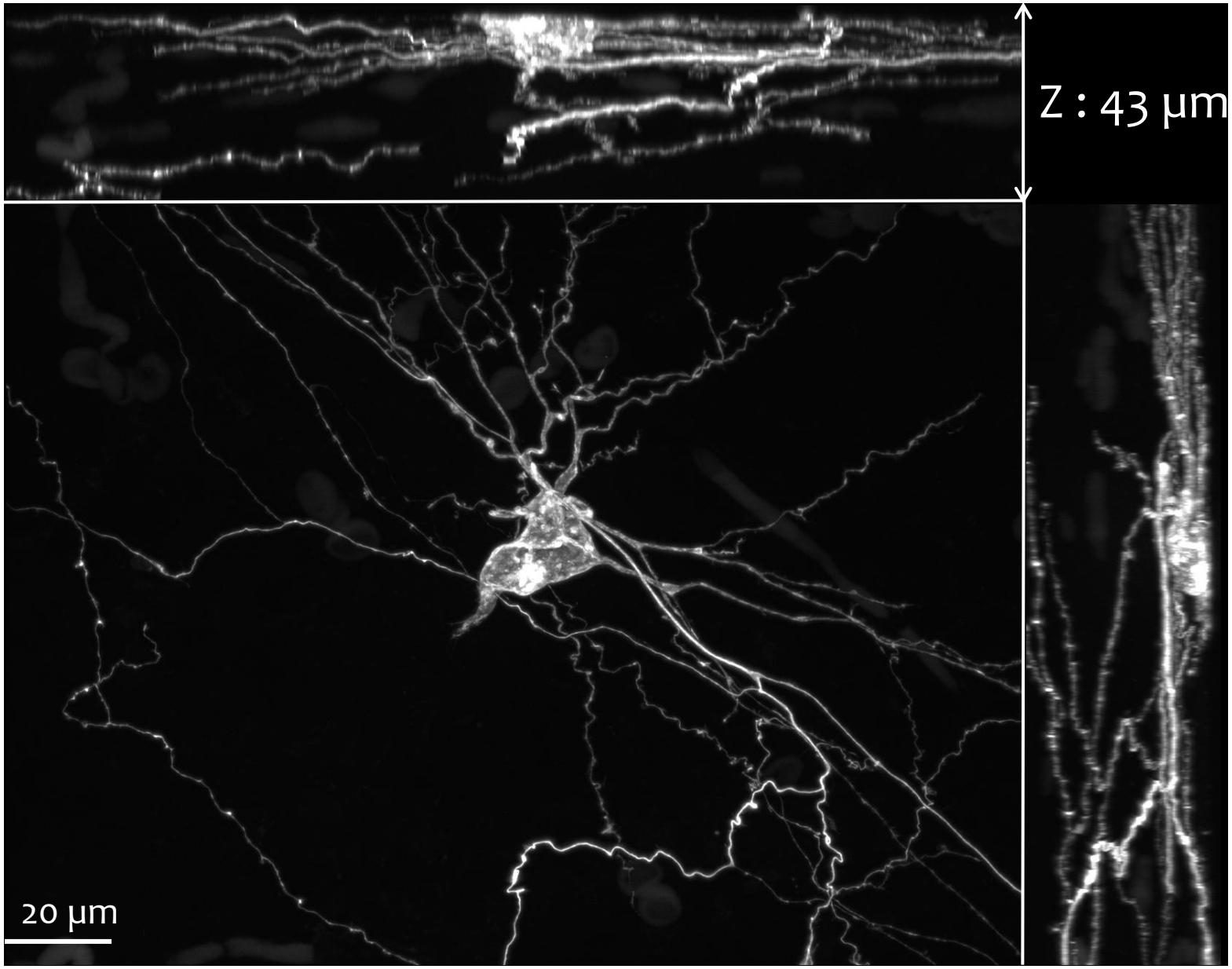


SIM



WF

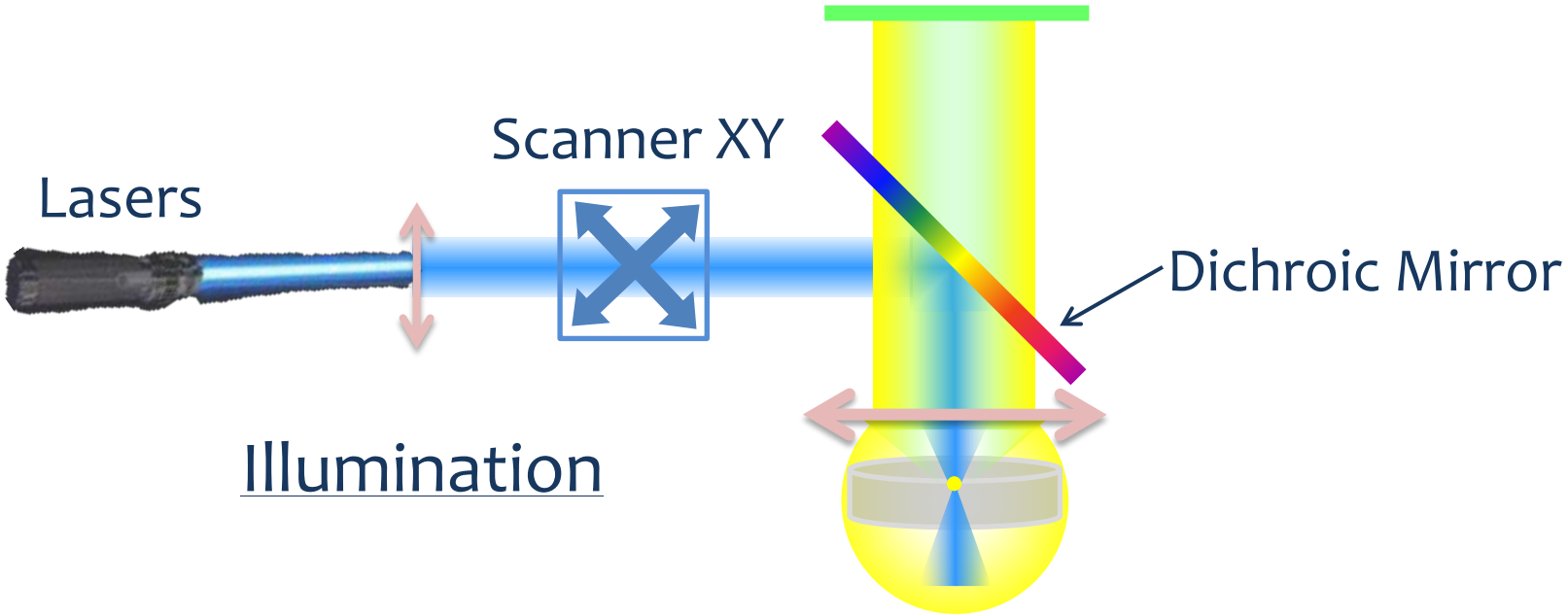
# Structured Illumination microscopy




40 X (NA = 1.3) expo time: 15 ms (87 frames ΔZ=0.5 µm)

# Confocal microscopy

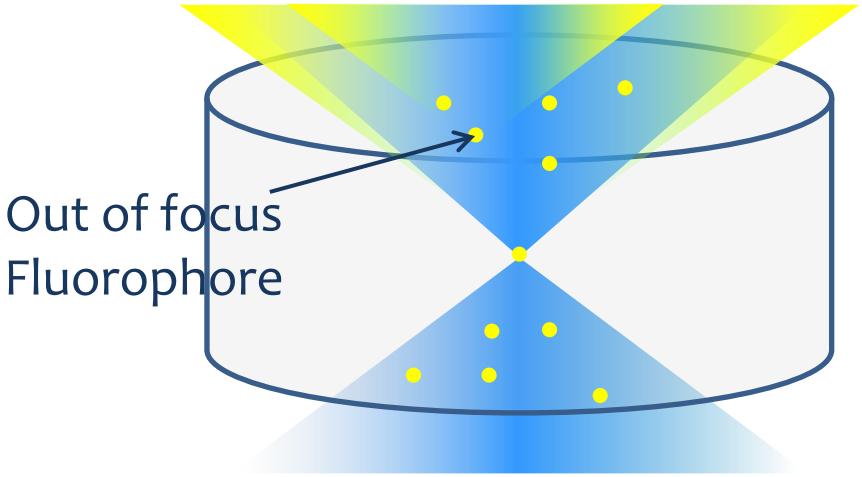
In a confocal microscope, illumination is based on lasers scanning. A laser is a mono-spectral photon source (no more excitation filter). The beam pathway goes in a couple of motorized mirror (XY scanner) and excite the sample by line scanning. The fluorescence goes through the objective and is filtered (dichroic mirror and emission filter) and finally impact a detector. This device give different control level (scan speed, fine tuning of the illumination energy, more power locally available… ). All these parameters increase the image quality and the XY resolution thank to the beam sharp shape.

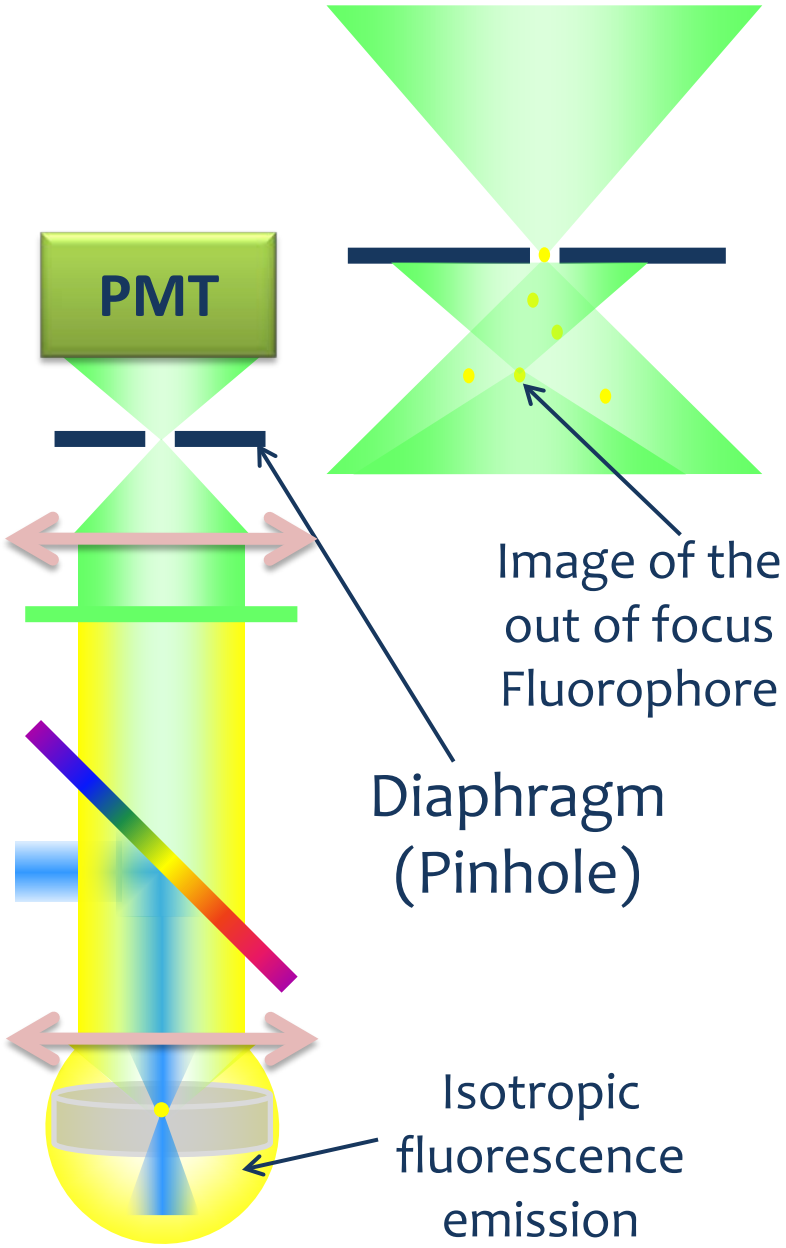


# Confocal microscopy

## Detection

Z resolution improvement is obtain with the creation of a focal point behind the detector, conjugated with the objective focal point. All photons that do not come from objective focal point will be stopped by the diaphragm placed in the conjugated plan: pinhole (spatial filter). It removes out-of-focus photons and generates an optical slice of the sample.

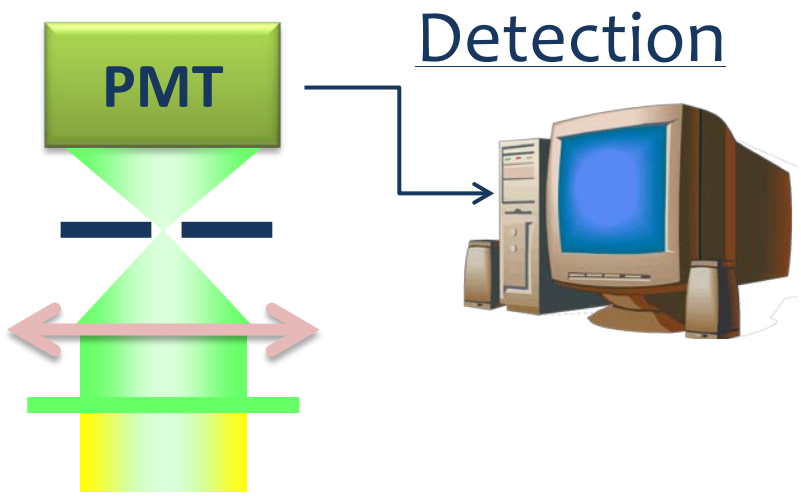


# Confocal microscopy

The detection is operate by a photo-multiplier tube (PMT). It measure the photon quantity (whatever the λ) as a function of time (time sampling) and the pixel size is proportional to the integration time.

In t1 in the coordinate of the scanning point X1Y1 the fluorescence intensity is F1, in time t2 (X2Y2) the fluorescence intensity is F2… The image is reconstructed pixel by pixel with Fn (XnYn) values.



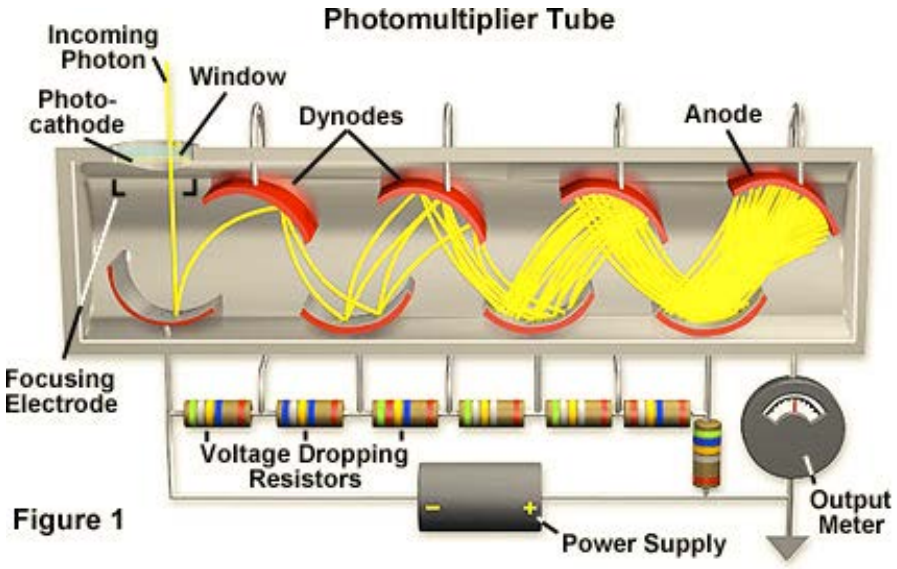



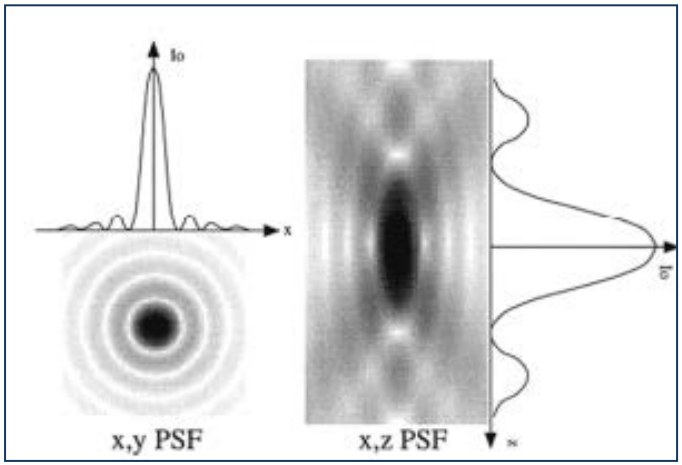

Figure 1

A PMT is a detector that transforms photons in electrons with a photocathode. It generate a current that will be amplified thanks to a large potential difference between the input photocathode and the output anode (more than 1000 V). The amplification is supported by intermediate electrodes (dynodes) with increasing positive potentials that liberate large amount of electrons for each photo-electron impact.

# Confocal microscopy (resolution)

## Fluorescence microscopy

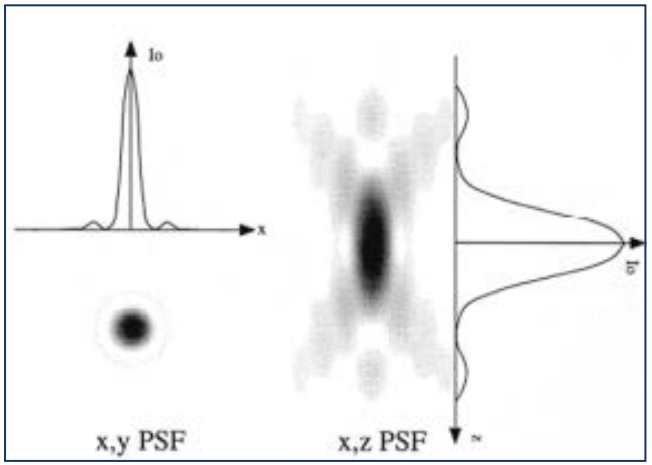
$Res_{xy}=0.61\lambda/NA$
$Res_z=2\lambda/NA^2$




Max xy resolution ≈ 250 nm
Max z resolution ≈ 600 nm

## Confocal microscopy

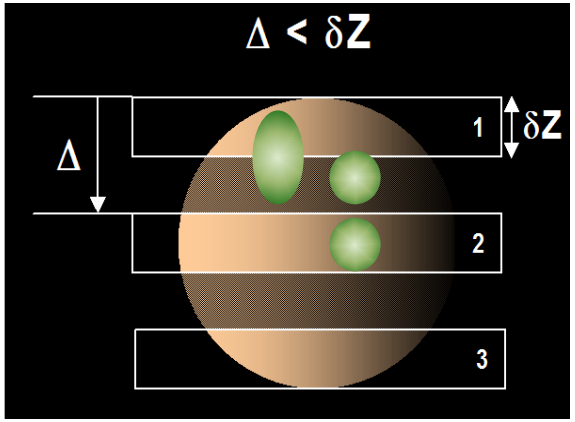
$Res_{xy}=0.4\lambda/NA$
$Res_z=1.4\lambda/NA^2$

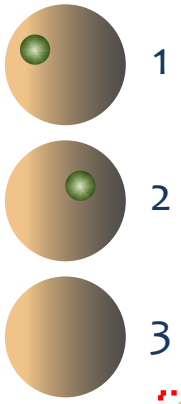


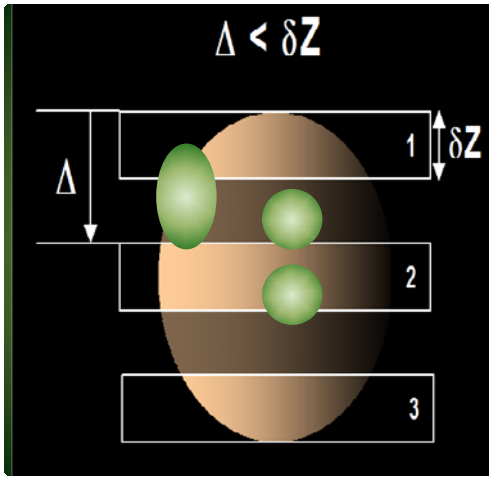

Max xy resolution ≈ 150 nm
Max z resolution ≈ 300 nm

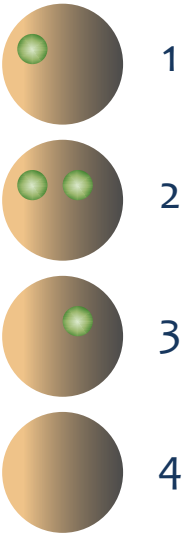
The bigger is NA, the better is the resolution

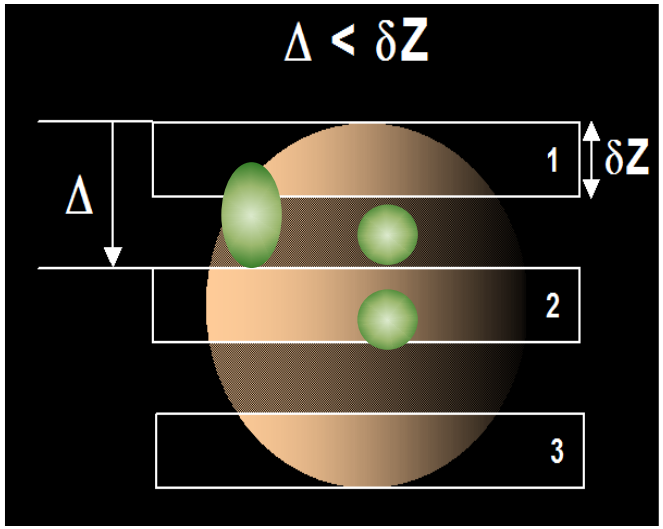
# Confocal microscopy (axial sampling)

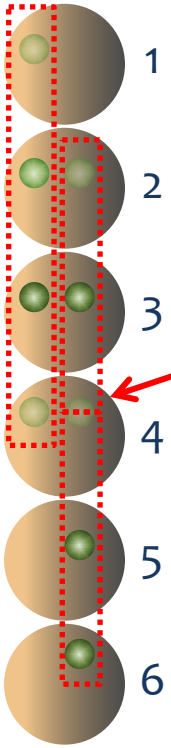


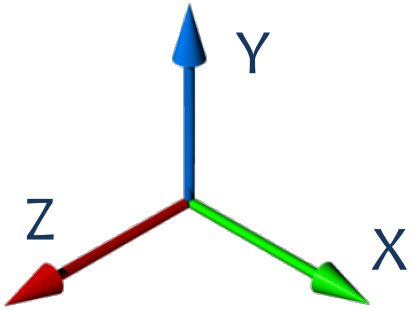

Do not make confusion between resolution and optical slice thickness:
$\delta Z = f(NA, n, \lambda, \text{pinhole}\ldots)$

The optimal step size in Z ($\Delta Z$) depend of the resolution ($Res_z = 1.4\lambda/ON^2$) and of sampling laws (Nyquist-shanon) :
$1/2\ Res_z < \Delta Z < 1/3\ Res_z$

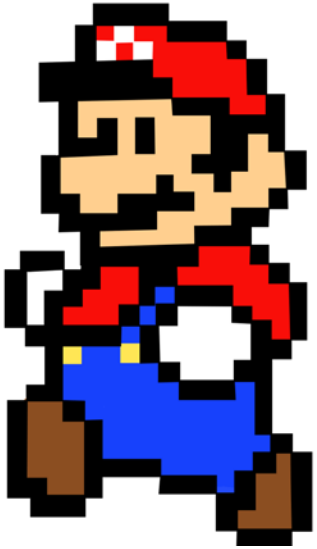
# Pixels and Voxels …

"Picture element" and "volumetric elements" ; scaling

Y>> X=Z

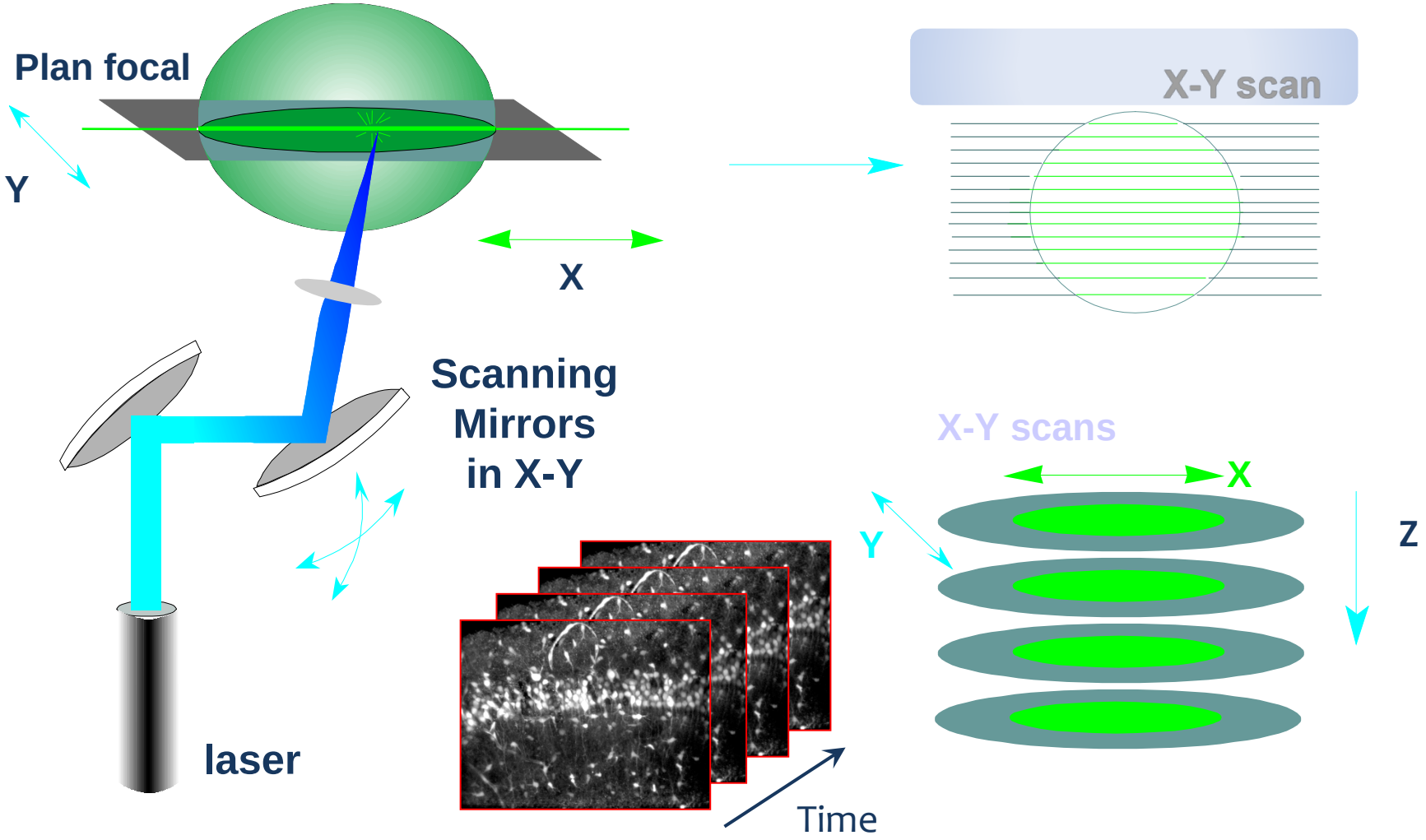



X>> Y=Z



Y=X and Z=0

# Multi-dimensional confocal microscopy




Dimensions may be: the depth Z, Time T, color λ …
We so can make 3, 4 or 5D images.

# Spinning disk (nipkow) confocal microscopy

- Low penetration in thick samples (<40 - 100 µm depending of the tissue and the fluorophore)
- Lower photo-bleaching in and out focal plan Vs regular one beam scaning
- Temporal resolution adapted to in vivo studies (>50 Hz EMCCD camera)

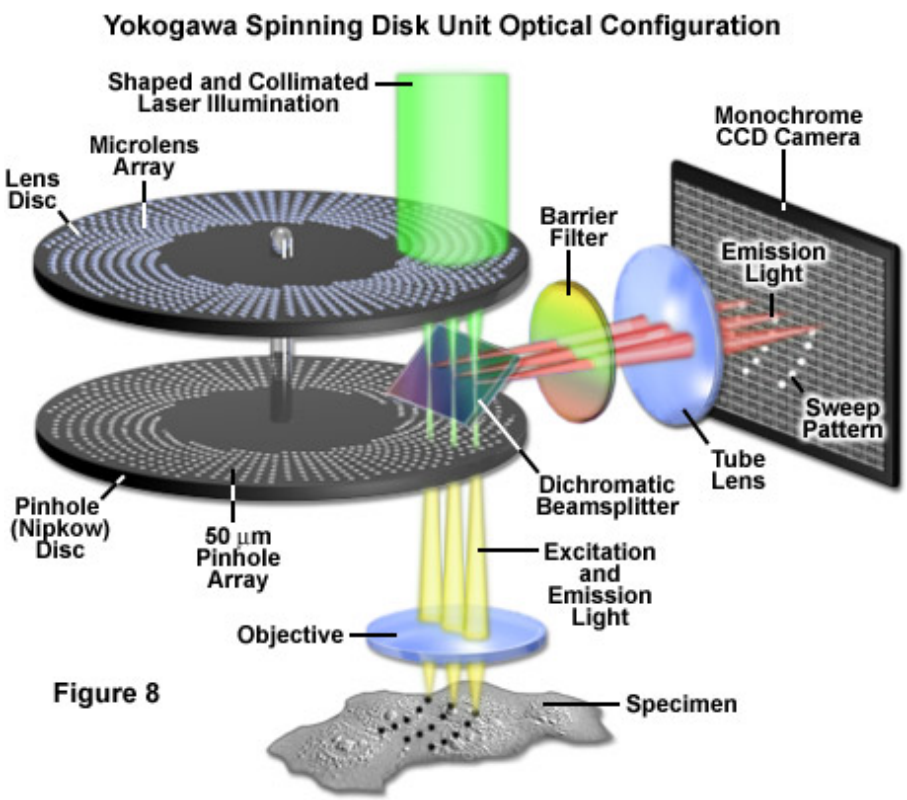
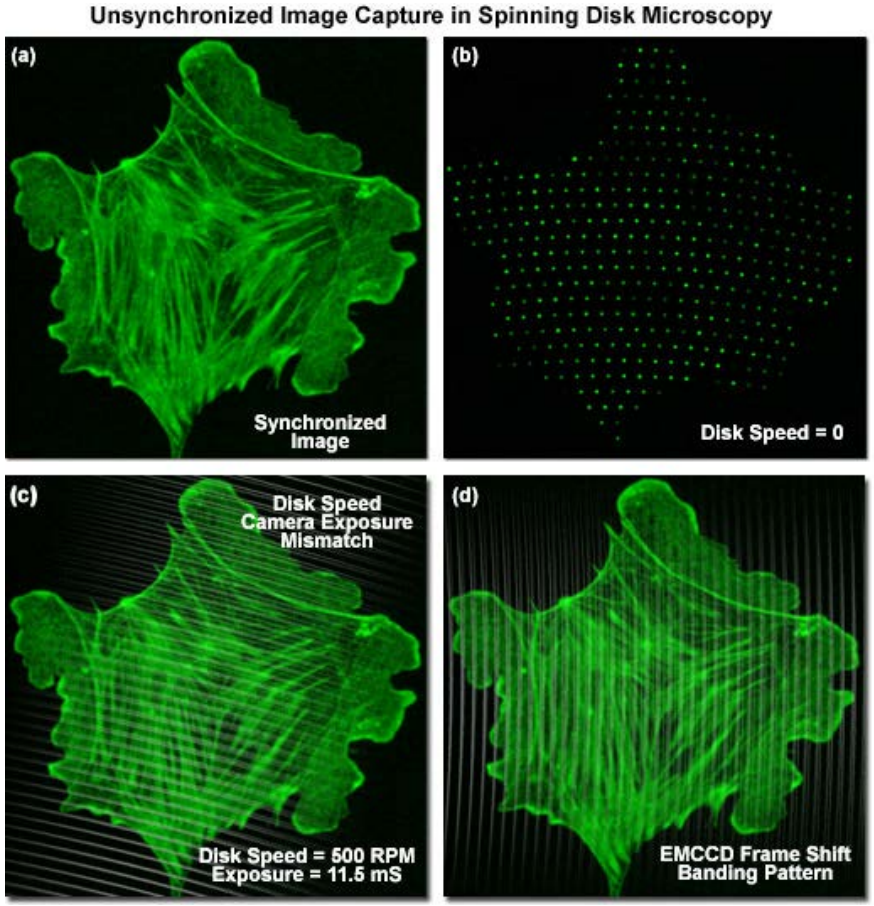
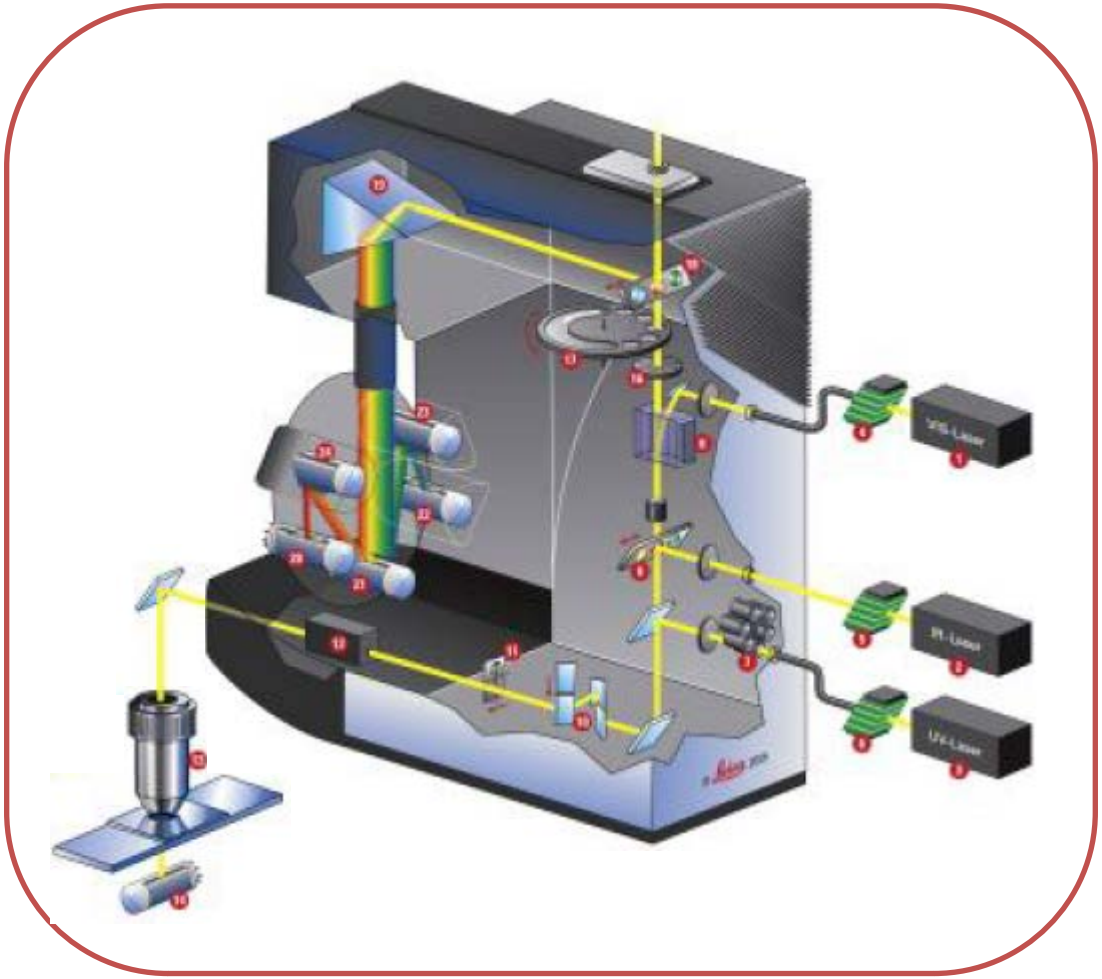



Figure 8



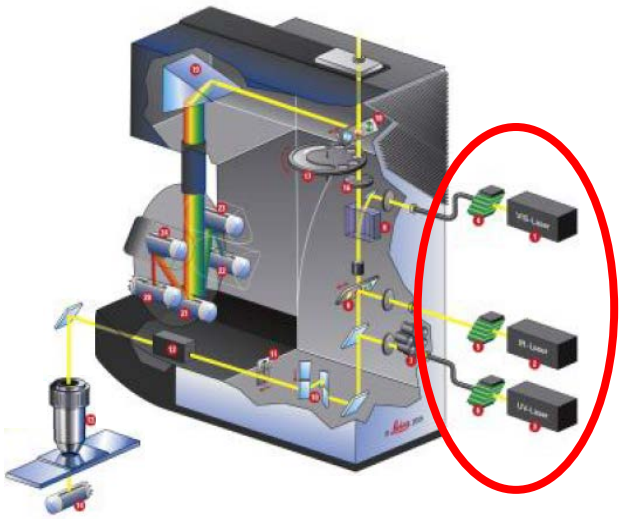
# Leica SP5-X confocal microscope

- White Laser
- Argon Lasers and UV-blue diode
- AOBS
- Spectral detector
- 1 PMT + 2 Hybrides
- Motorised stage

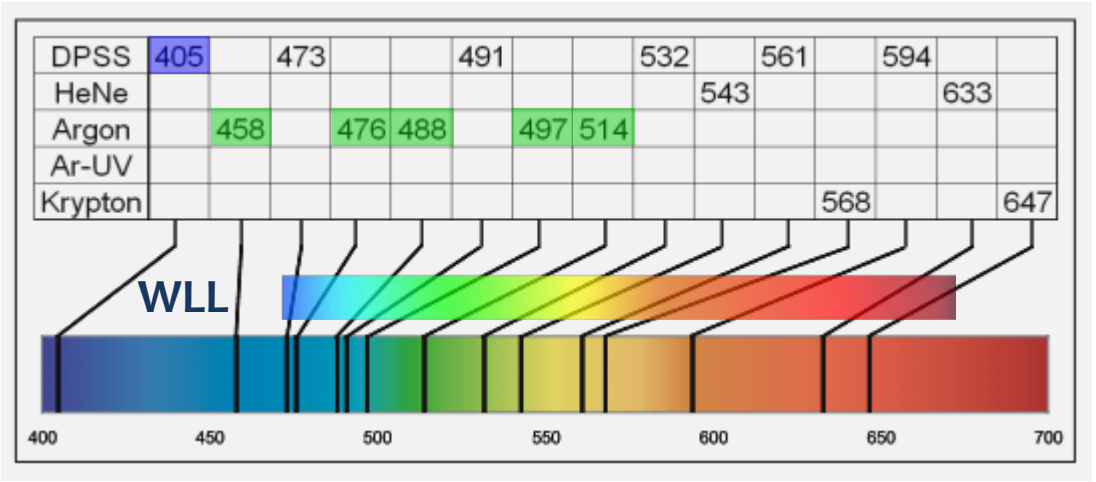


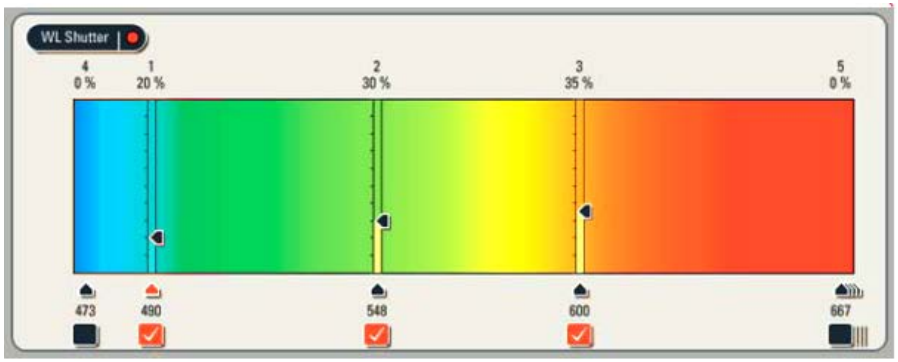
# Leica SP5-X : Lasers

- Argon Lasers (458, 476, 488, 497, 514 nm)
- UV-blue diode (405 nm)
- White Laser (470-670 nm) : 8 possible λ with a minimal gap of 8 nm, 1.5 to 6 mW per beam (@500nm and <600 nm respectively)

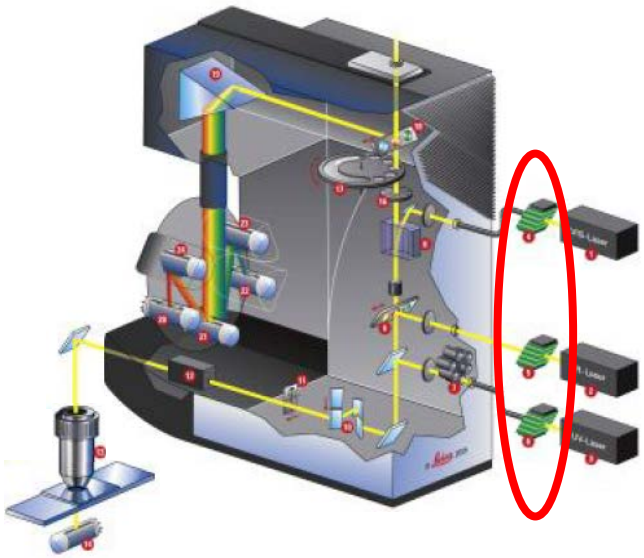


AOTF
(Acousto-Optic Tunable Filter)

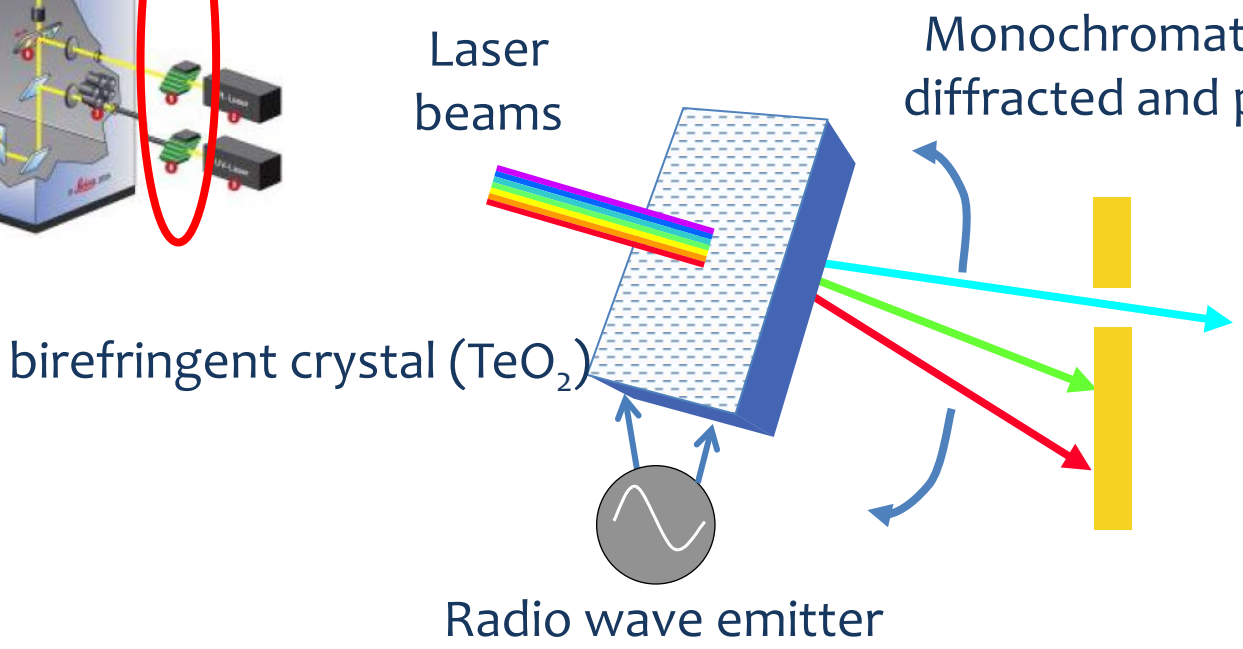


# Leica SP5-X : AOTF



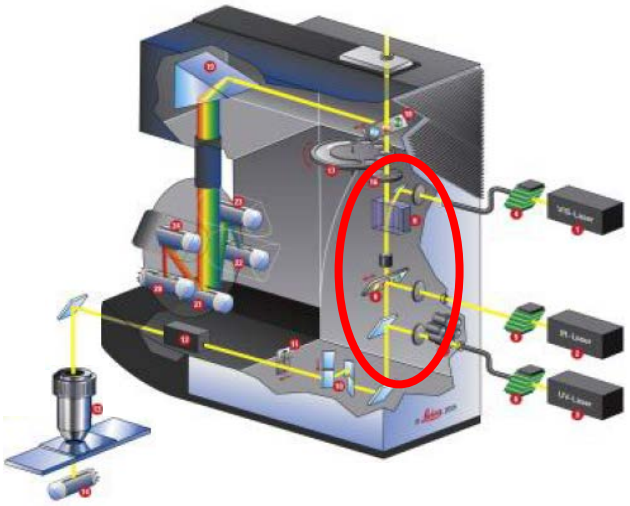
AOTF set power and wavelength of lasers in a µs scale time

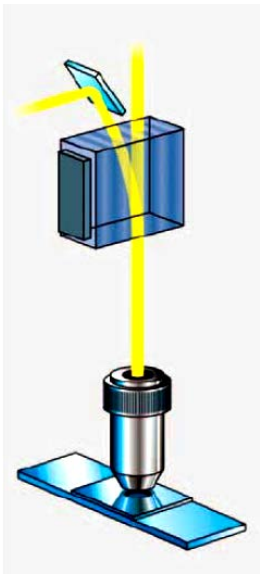


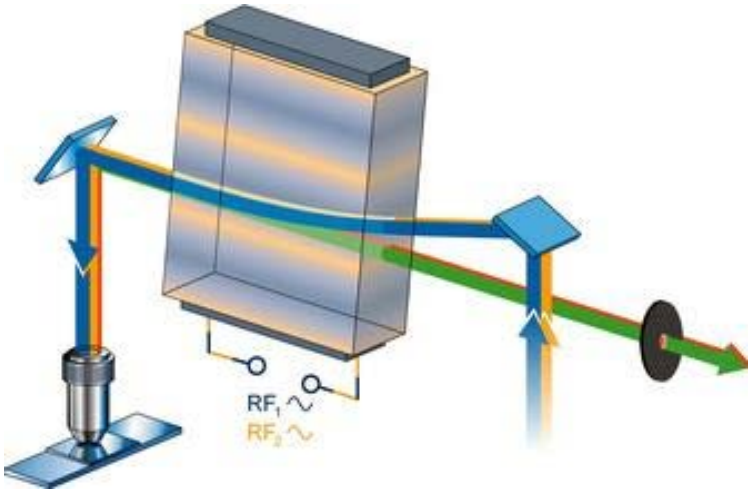

The amplitude of the perpendicular radio wave affects the transmission of the light beam and its frequency on the wavelength

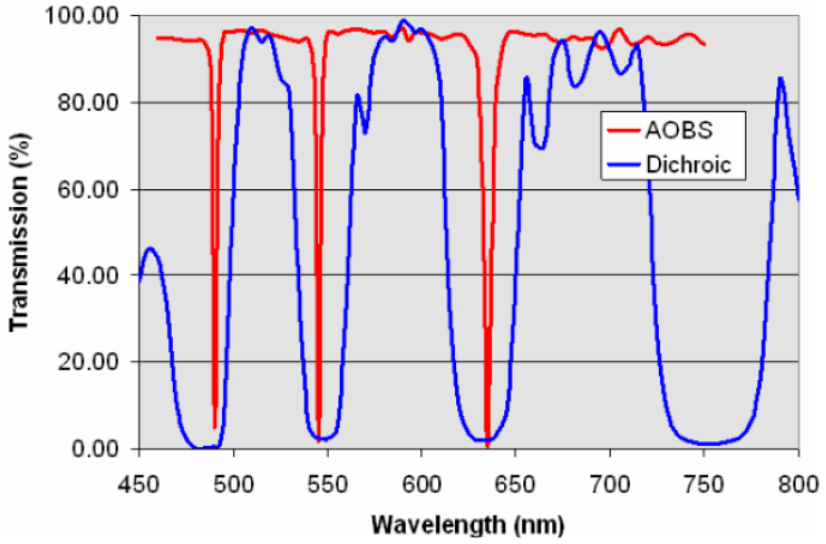
# Leica SP5-X : AOBS

## Acousto-Optic Beam Splitter

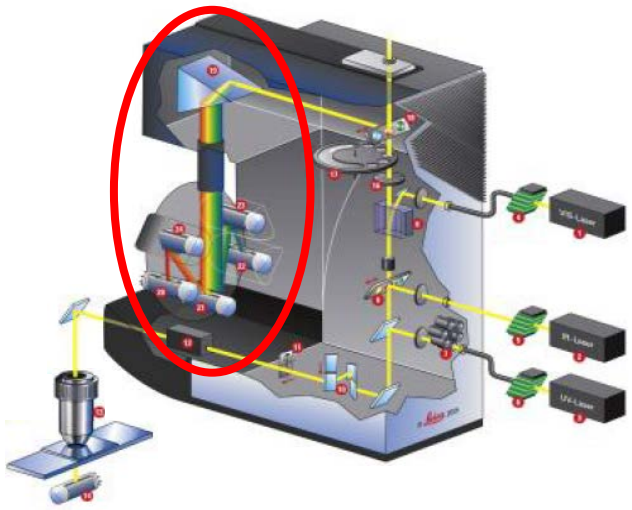


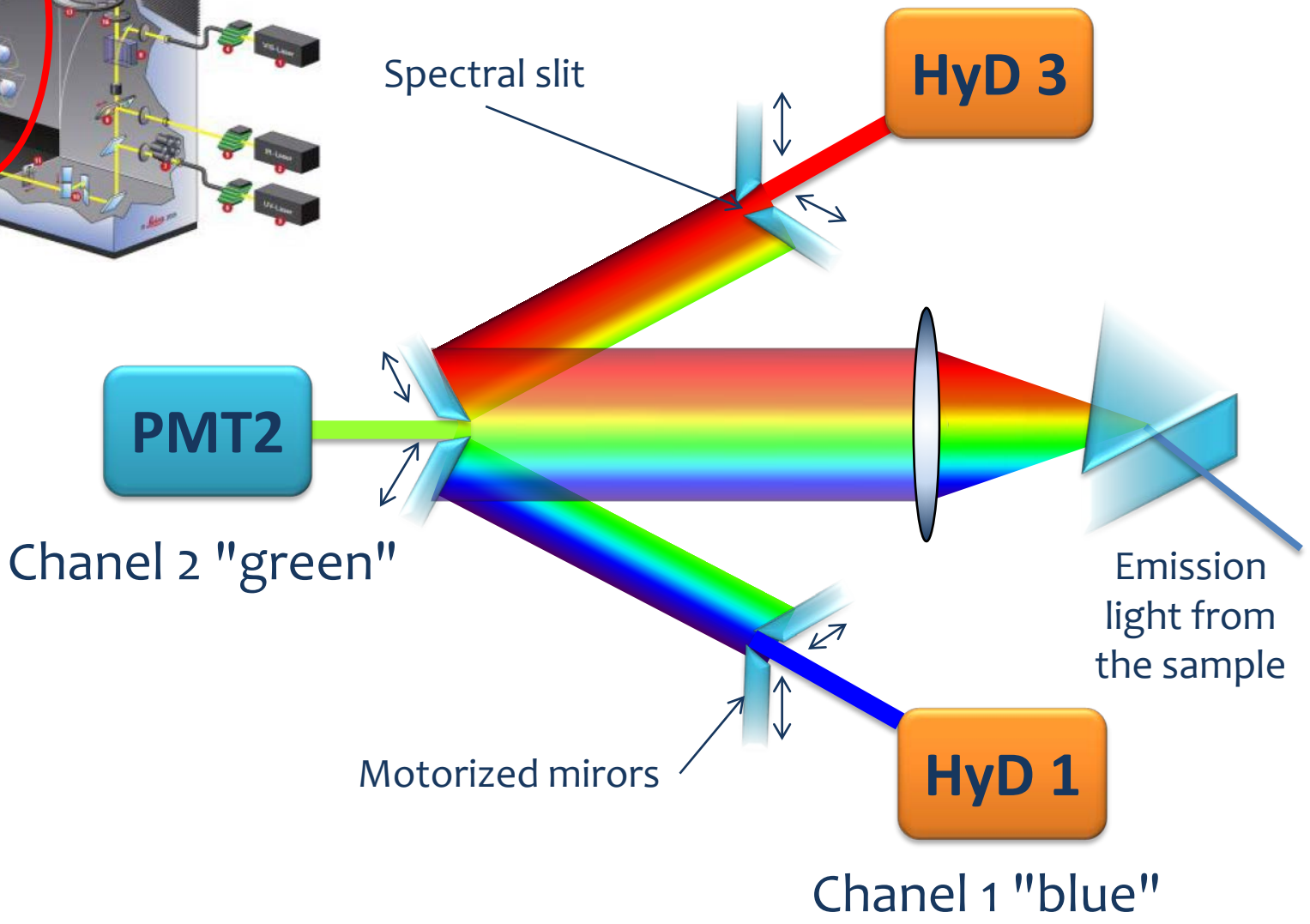




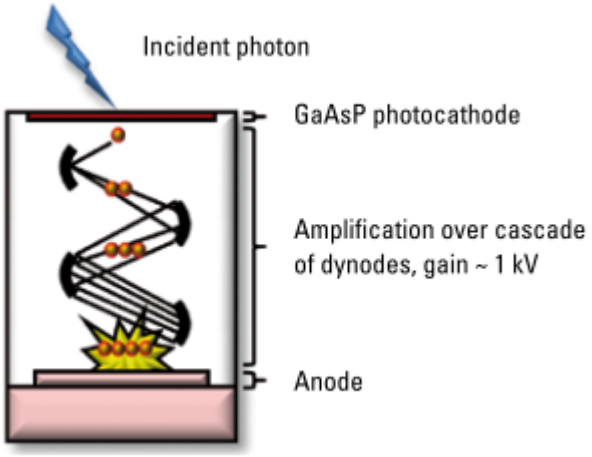
# Leica SP5-X: spectral detector



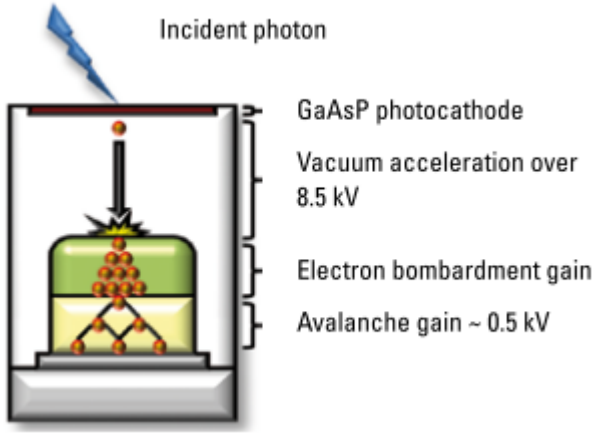


# Leica SP5-X: Hybrides detector

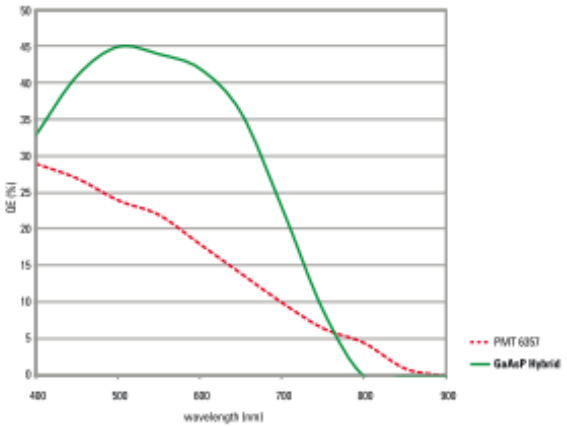
## PMT GaAsP (Gallium-Arsenide-Phosphide)



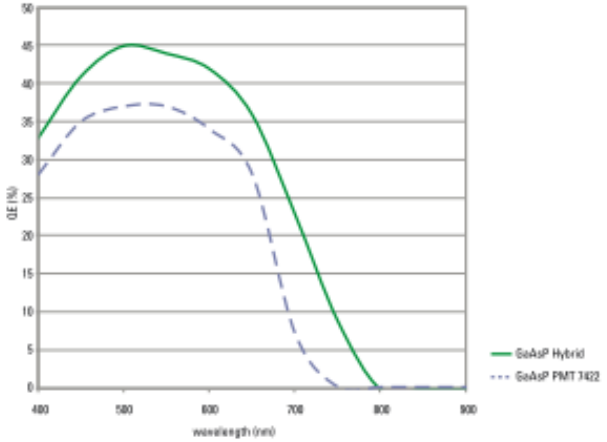

## Hybrides detector: HyD




**HyD's quantum efficiency is superior to standard PMTs ...**

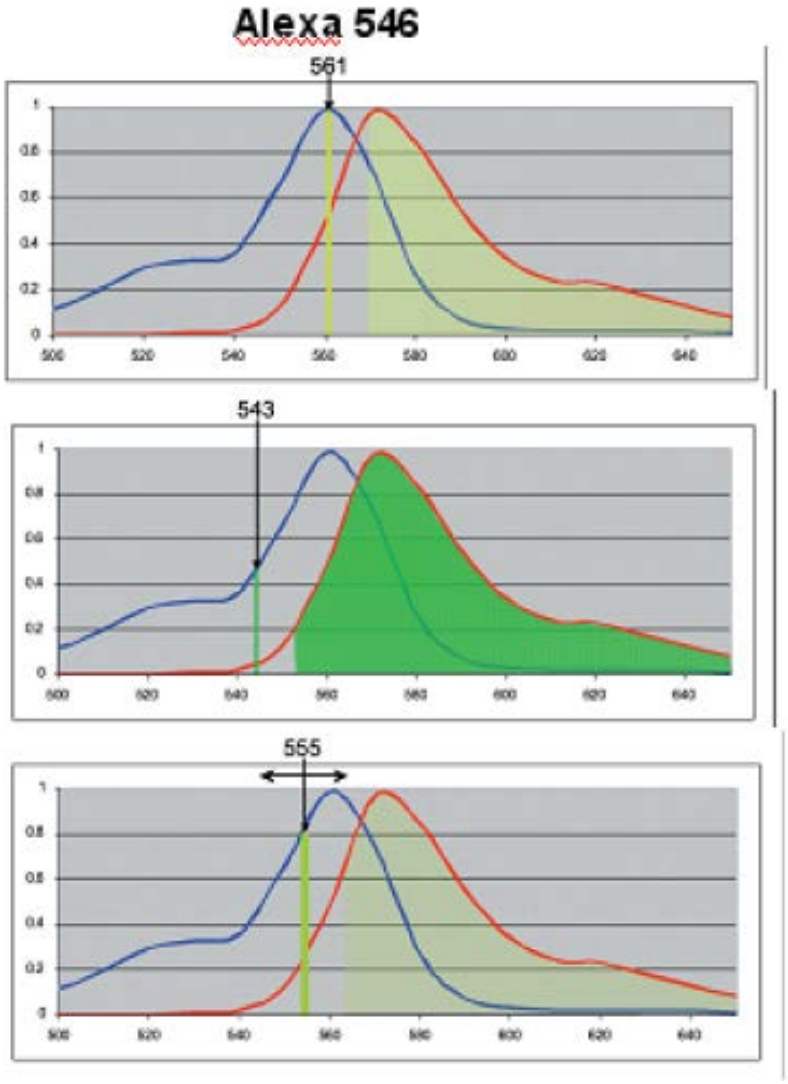



**... and even to GaAsP PMTs**

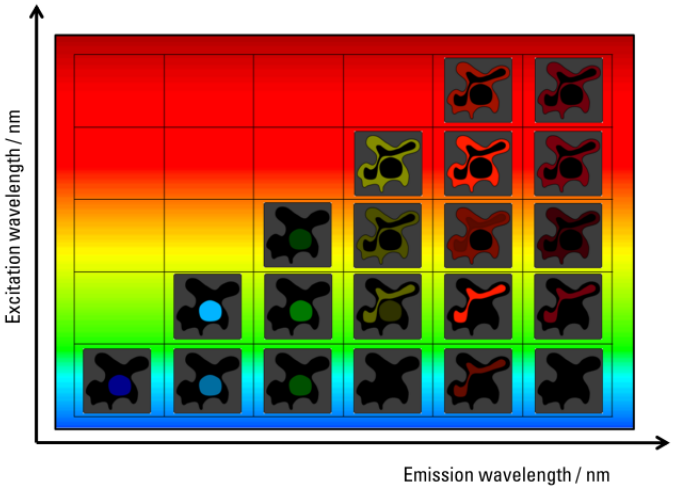


# Leica SP5-X: Spectral excitation/detection

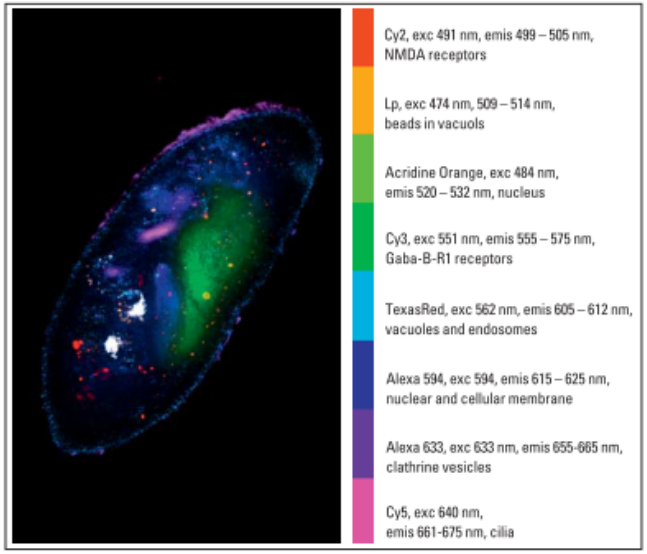
## Excitation Optimization




## $\lambda^2$ map




## Multi - labeling



# Confocal microscopy limitations

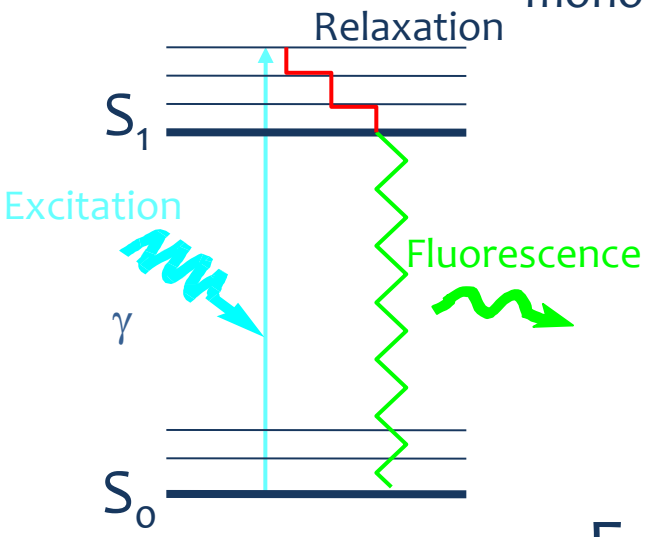
- Deep penetration in thick tissue is limited (<60 - 150 µm depending of the tissue and/or the fluorophore)
- Photo-bleaching in and out the focal plan : limitation of the experiment duration and of the image number per field.
- Photo-toxicity: affect living tissues, due to UV light energy and chemical reactions secondary to bleaching process.
- Temporal resolution do not fit with *in vivo* protocols in a large set of neurobiological studies (action potential 1-5 ms)

# What's going on (again)

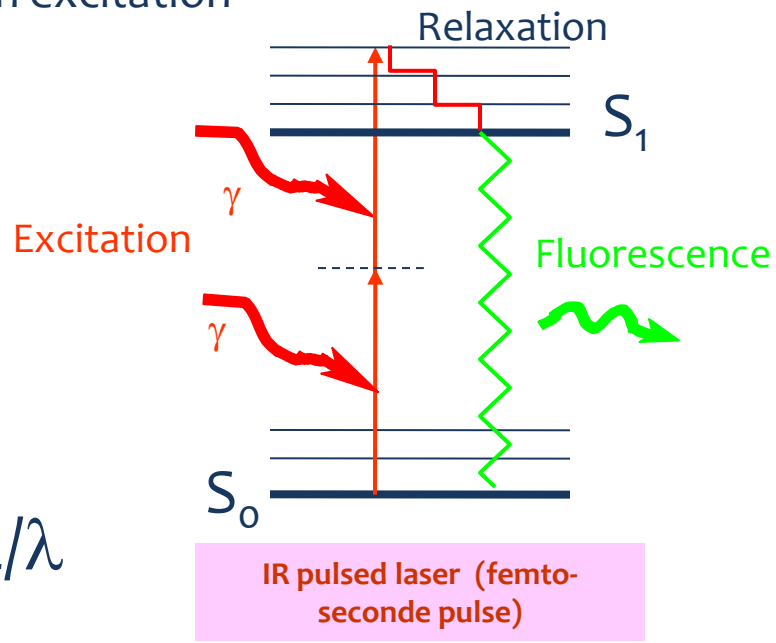
- ✓ Fluorescence ??
- ✓ Epi-fluorescent microscopy
- ✓ Welcome in the third dimension
- ➢ Two-photons and others…

# Physical principle of multi-photon microscopy

Two infrared photons (IR) combine their energy to excite the fluorophore. On the other hand , excited state, relaxation and fluorescence emission are identical as a regular mono-photon excitation




$E_\gamma = hc/\lambda$



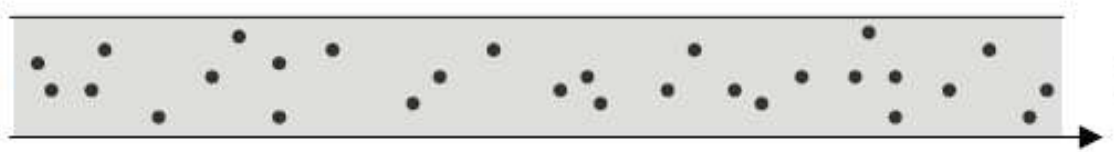

Photons have to arrive in a time window of about $10^{-18}$ seconds (**atto**-second). This time scale is compatible with the time duration of the lifetime of this virtual state ($10^{-17}$ seconds or 0.01 **femto**-second). They have to interact equally with the electronic cloud in a space of about $10^{-12}$ $cm^3$. The two-photon excitation probability is **$10^{-30}$** time lower than one-photon !! (theorized by Maria Goppert-Mayer 1931 (Physics Nobel prize 1963))

# Physical principle of multi-photon microscopy

The photon density that allow us to go beyond this quasi-null probability could only be reach with pulsed lasers (1980) and only in the focal point (convergent point). Each pulse is extremely short (femto-second) and comport an enormous number of photons (pulse power : more than 100 kW) but in a temporal scale are infrequent: 5 orders of magnitude between the duration and the occurrence of pulses.

One femtosecond (fs) is a millionth of a billionth second (1 fs = $10^{-15}$ s).
In one second the light reach the moon from earth while in 10 fs it run through one cell thickness (30 µm).

**Continuous beam**
**Average power 1 to 50 mW**



**Pulsed beam**
**Average power 1 to 5 W**


**τ= $10^{-13}$ s**

# Physical principle of multi-photon microscopy

## Light/mater interactions

### Two-photon absorption

$$E_{2\gamma} = P^2 / (\tau.F)$$

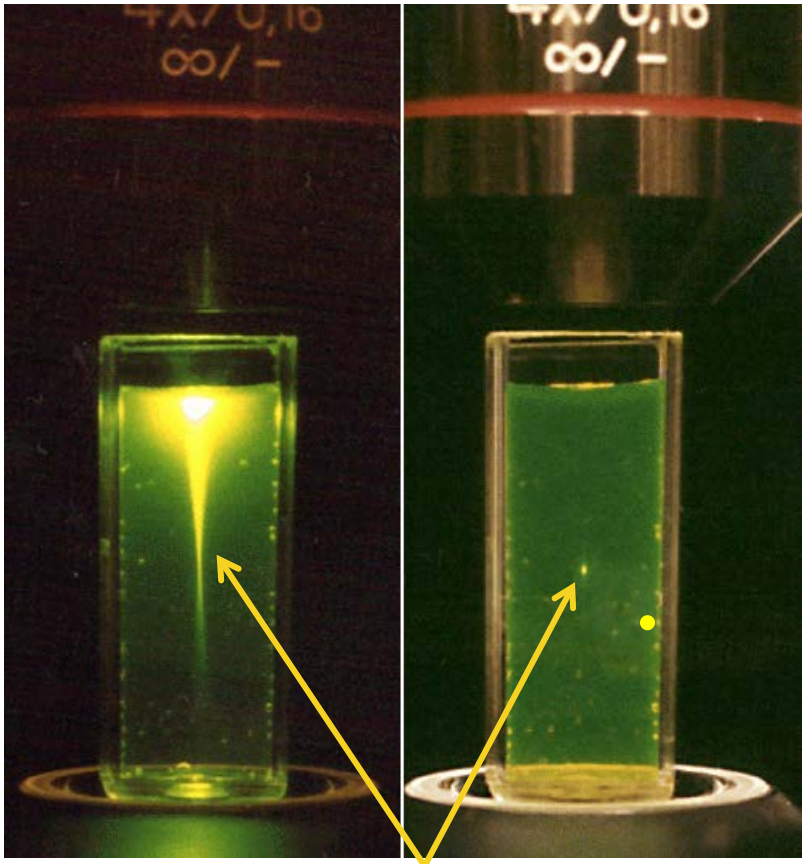
Necessary conditions for two-photon excitation implicate a very high photon density:

### In a spatial dimension
(concentration due to focalization, NA)

### And in temporal dimension
(pulsed laser femtoseconde )

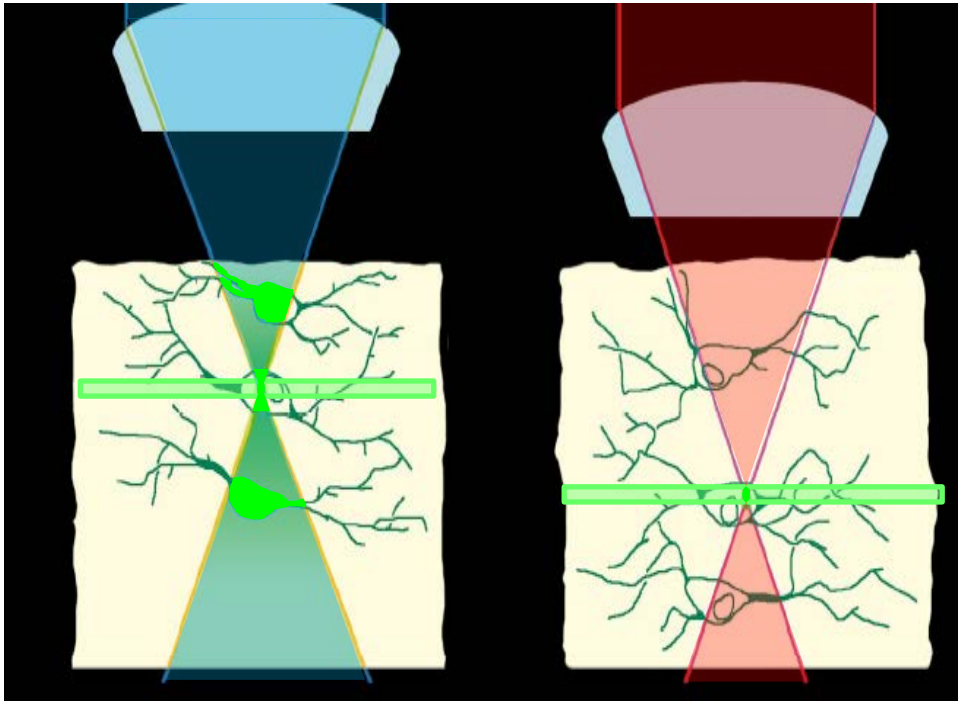
# Two-photon excitation occur only in the focal point

1 photon 2 photons



Focal point

1 photon 2 photons



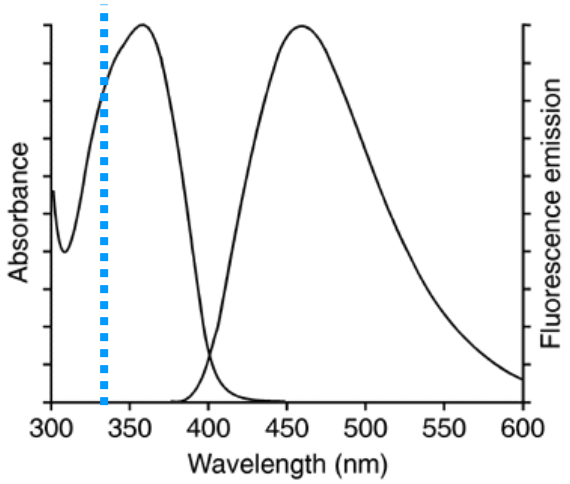
The scanning induce an optical slice intrinsically confocal, we do not need to remove out-of-focus photons (not generated).

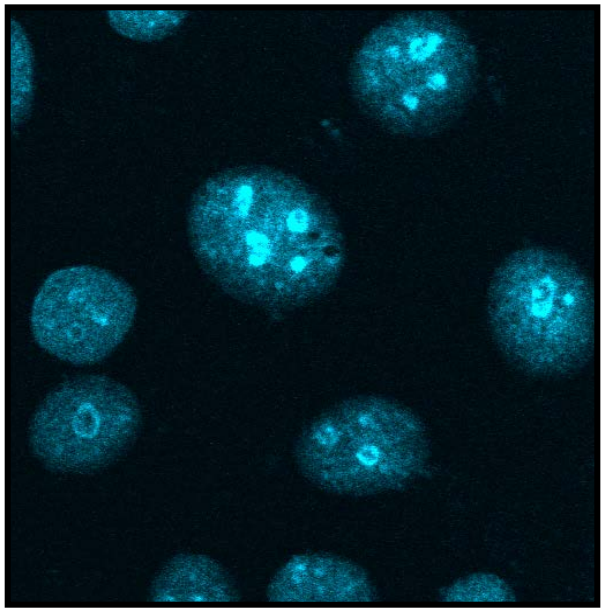
# Optimal absorption wavelength

| | $\lambda_{abs1}$ (nm) | $\lambda_{abs2}$ (nm) |
|---|---|---|
| Bodipy | 503 | 920 |
| Cascade Blue | 400 | 750 |
| DAPI | 350 | 700 |
| Fluorescéine | 492 | 780 |
| Indo-1 | 350 | 700 |
| Lucifer Yellow | 430 | 860 |
| Rhodamine B | 545 | 840 |
| Rhodamine 6G | 526 | 795 |

Two-photon excitation spectra is not directly the double, point to point of mono-photon excitation spectra (non-linear optic).

3- photons excitation
Example : DAPI



T-pulse Laser (Amplitude Systemes): fixed wavelength: 1030 nm

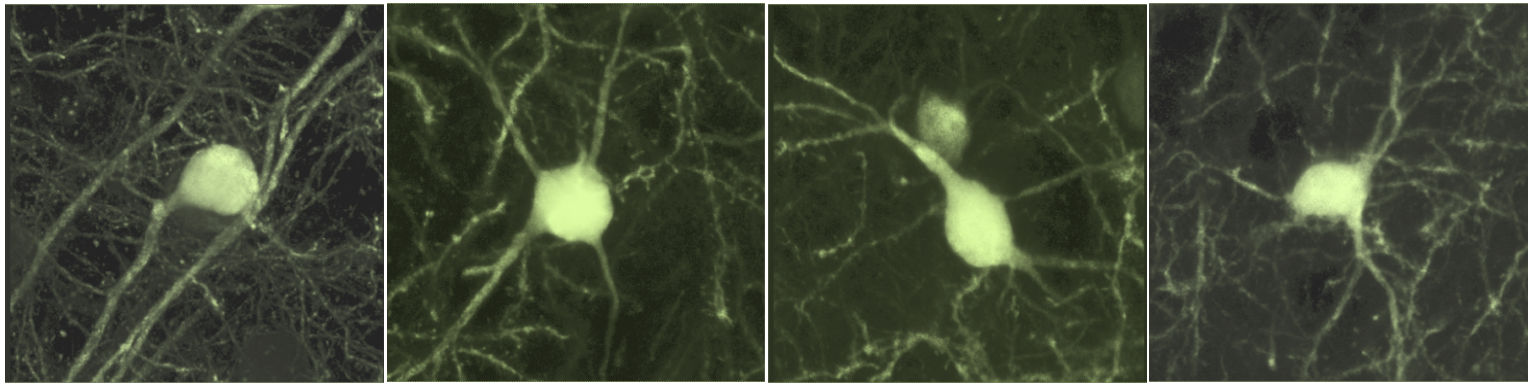
# Two-photon vs confocal images

Two laser lines (488 nm and 920 nm) in the same microscope in a GFP expressing brain slice

***Low depth***: objective 63X oil

**Confocal**

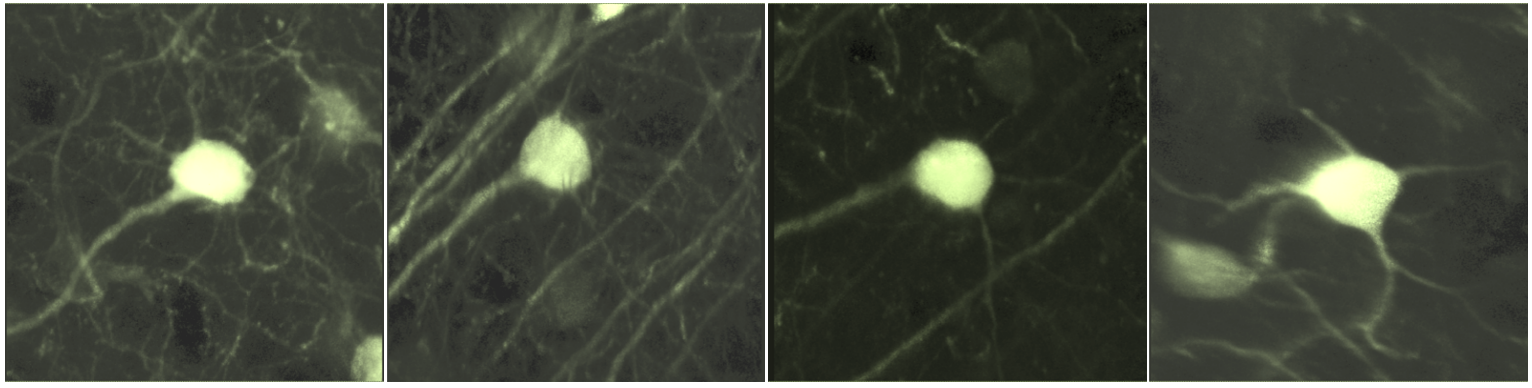
- Better resolution
- Better signal to noise ratio



20 µm | 40 µm | 60 µm | 80 µm

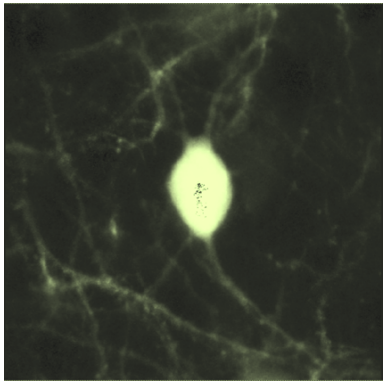
**Two-photon**

- More noise
- Thicker optical slice

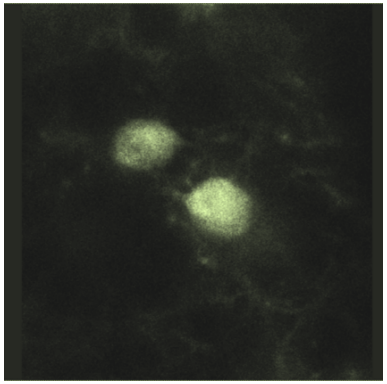


# Two-photon vs confocal images

***Deeper acquisition depth***: objective 63X Water



100 µm



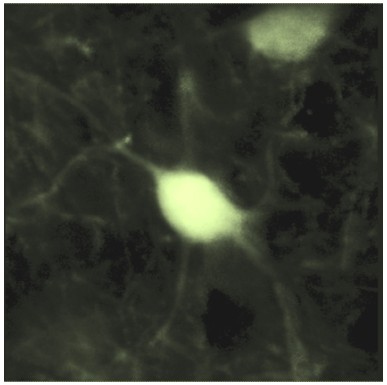
120 µm

## Confocal

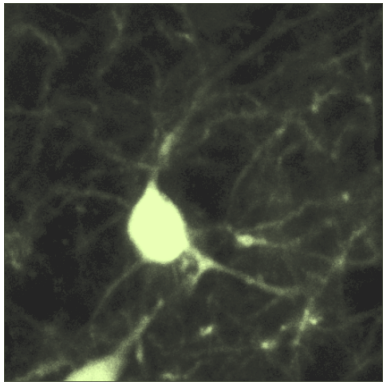
- Maximal laser power
- Increasing noise
- Limit of detection (low signal)

## Two-photon

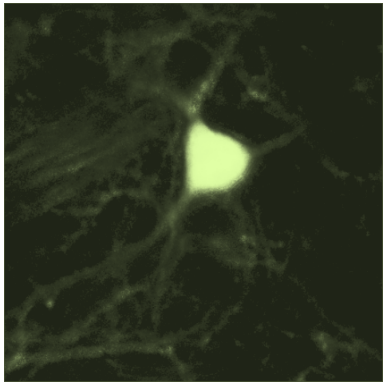
- Increasing laser power
- Easier acquisition than confocal



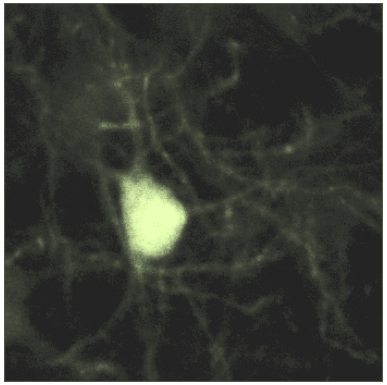
100 µm



140 µm



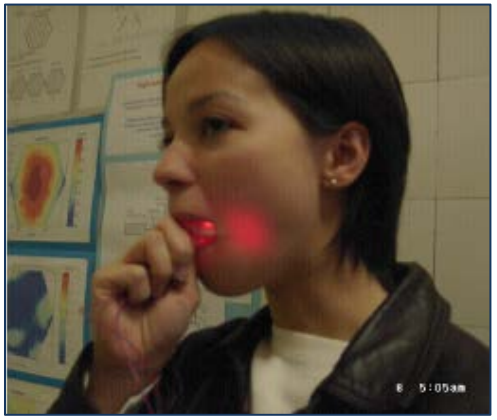
170 µm



200 µm

End of the slice…

# Multiphoton: The pros and the cons

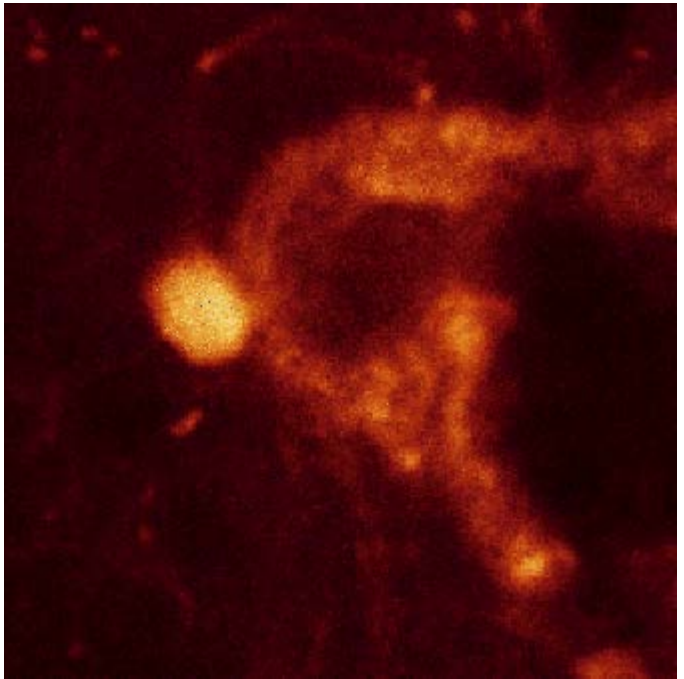


ADVANTAGES

- Intrinsically confocal, small excitation volume
- No out-of-focus fluorescence
- less photo-bleaching and photo-toxicity
- Deeper penetration of IR light in tissues (up to 1 mm)
- UV fluorophores excitation without UV light
- Large gap between excitation and emission light.

LIMITATIONS

- Photo-bleaching at focal point
- Need powerful pulsed laser
- Power balance between damages and detection could be difficult to evaluate
- Expensive to buy and to keep it working



# Sources and Ressources

*WEB divers*

- *Molecular expressions http://micro.magnet.fsu.edu/index.html*
- *Microscopy U (Nikon) : http://www.microscopyu.com*
- *Microscopy resource system (Olympus): http://www.olympusmicro.com/index.html*
- *A whole world of microscopy knowledge (Zeiss): http://zeiss-campus.magnet.fsu.edu/index.html*
- *Wikipédia*

*Plate-formes d'imagerie*

- *PICSL http://www.picsl.univ-mrs.fr/ : MICROSCOPIE OPTIQUE (PDF indisponible)*
- *BIC http://www.bic.u-bordeaux2.fr/ : Microscopie à épi-fluorescence et microscopie confocale (PDF)*
- *Membres du GDR2588 CNRS (microscopie fonctionnelle des systèmes vivants) et du RTmfm (Réseau technique de microscopie de fluorescence multidimensionnelle).*

*Cours*

- *Arnaud Sergé (université de la Méditerranée – Marseille II)*
- *Yves Husson (Université Joseph Fourrier – Grenoble)*
- *Serge Monneret (Institut Fresnel, Marseille)*
- *Maxime Dahan (Laboratoire Kastler Brossel, ENS)*
- *François Waharte (institut Curie, UMR144)*

*Handbooks*

- *Fondamentaux d'optique et d'imagerie numérique à l'usage des microscopistes (C Cibert, ed. Cépaduès)*
- *Handbook of biological confocal microscopy, third edition (JB. Pawley ed. Springer)*
- *Fundamentals of light microscopy and electronic imaging (DB. Murphy, ed Wiley-liss)*

Comments, remarks and improvement (and English corrections ☺ )

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