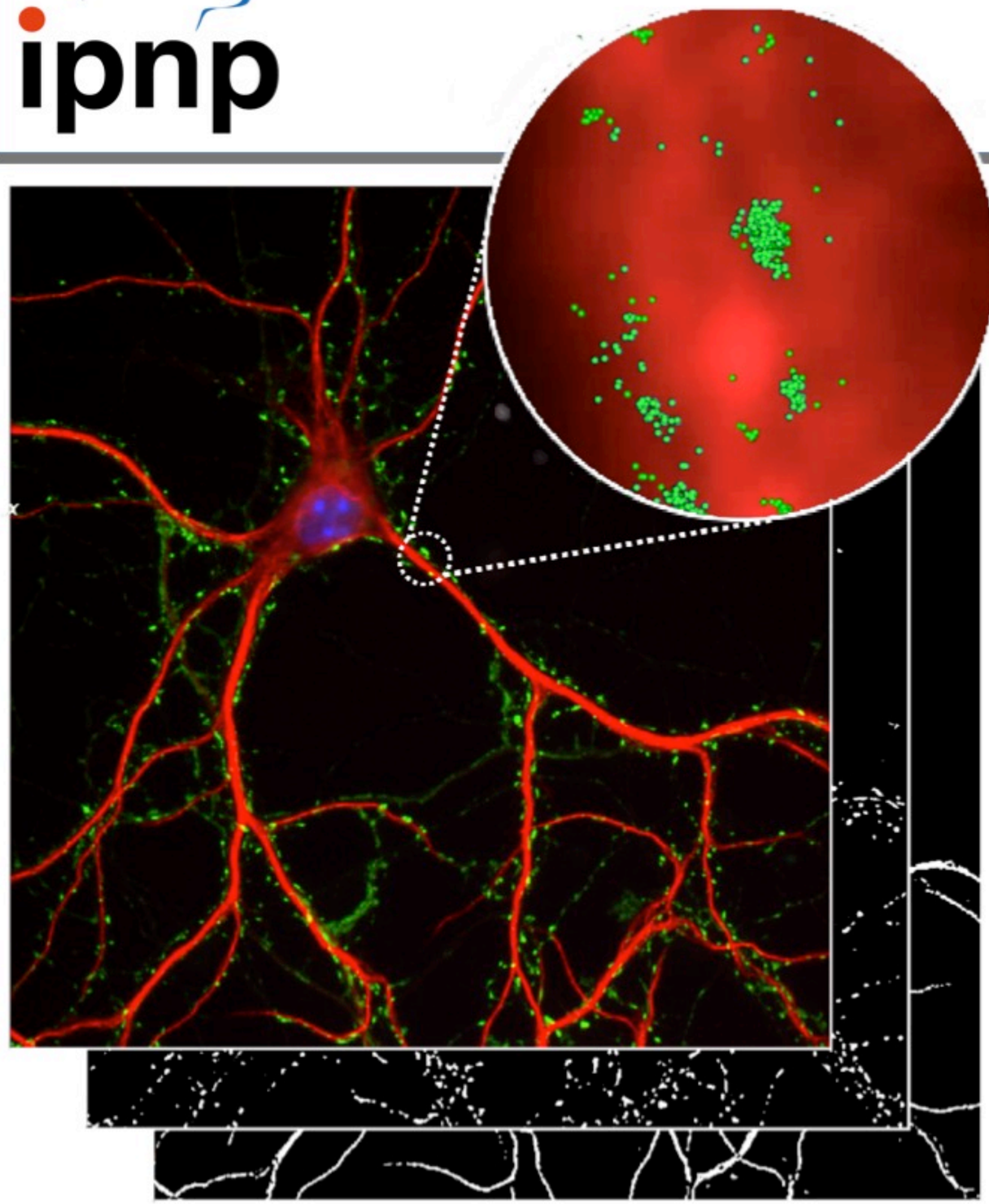
INSERM - UNIVERSITÉ PARIS-DESCARTES
Institute for Psychiatry and Neuroscience of Paris

NeurImag
Imaging facility



Microfludics 2019

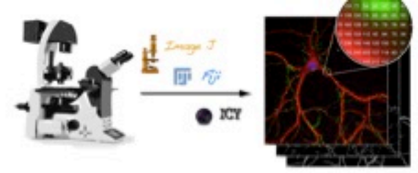
Introduction to microscopy :

from conventional ...
... to Super Resolution

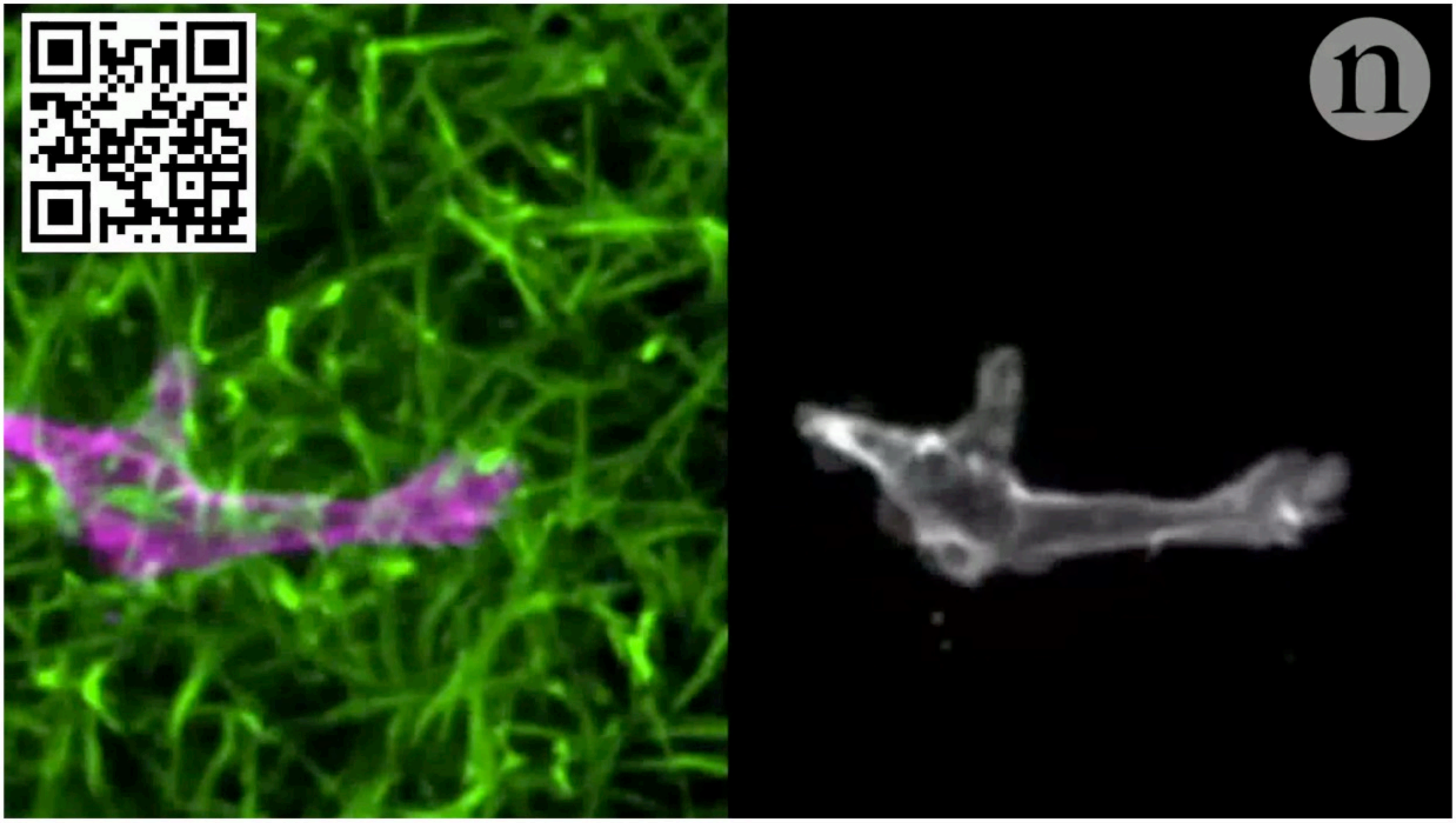
Lydia DANGLOT

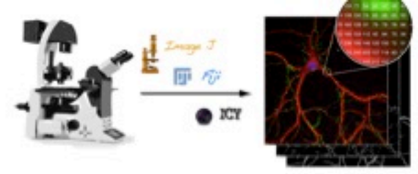
Inserm Researcher, Institute of Psychiatry and Neuroscience of Paris, « Membrane traffic in healthy and diseased brain » Lab.
Université Paris Descartes - Inserm U894- 102 rue de la Santé- 75014 Paris - France
Tel.: +33 (0)1 40 78 86 49 - lydia.danglot@inserm.fr

Website http://lydia.danglot.free.fr



Microfluidics to dissect immune cells dynamics





Introduction to microscopy :

from conventional …

… to Super Resolution

How to choose the better objective for your sample ?
- Refraction index
- Numerical aperture
- Resolution
- Immersion medium
- Mounting medium
- Depth of field
- Working distance

Illumination mode :
- Bright-field microscopy
- Phase contrast microscopy

Fluorescence microscopy
- Matrix, Bit depth, pixel size, histogram, RGB color pictures
- Confocal microscopy
- Spinning-disk microscopy
- Airyscan

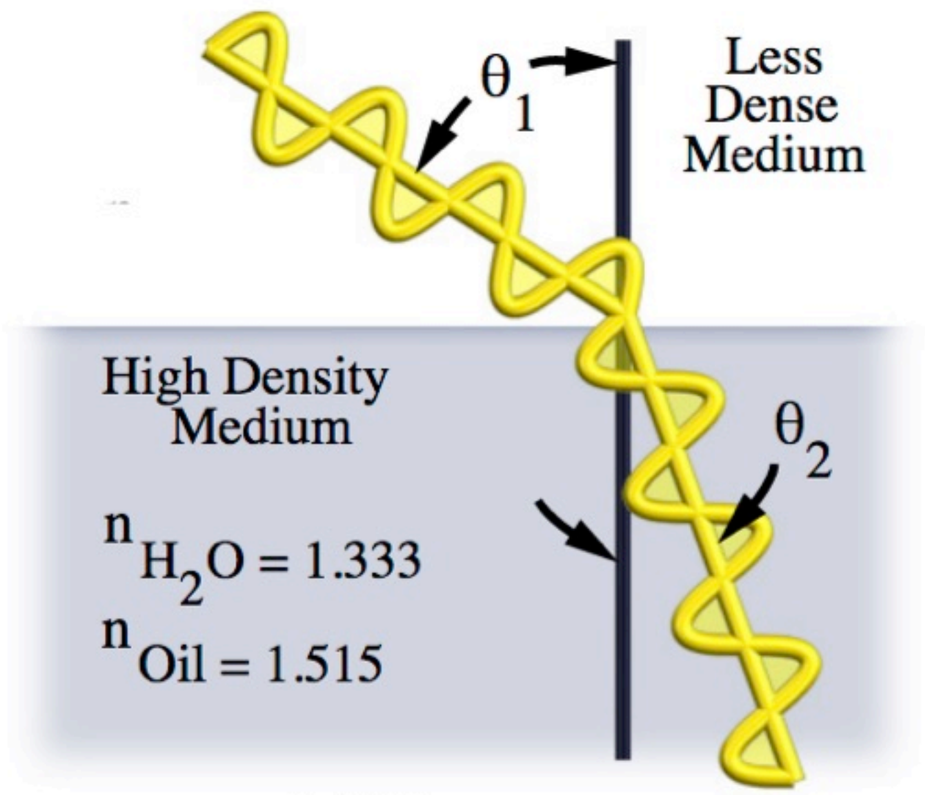
Super-resolution microscopy
- Structured illumination microscopy (SIM)
- Stimulated emission depletion (STED)
- Deconvolution
- STORM, PALM

Refractive index (index de réfraction)

Refractive index: **n**
is a value calculated from the ratio of the speed of light in a **vacuum** (n=1) to that in a **second medium** of greater density.

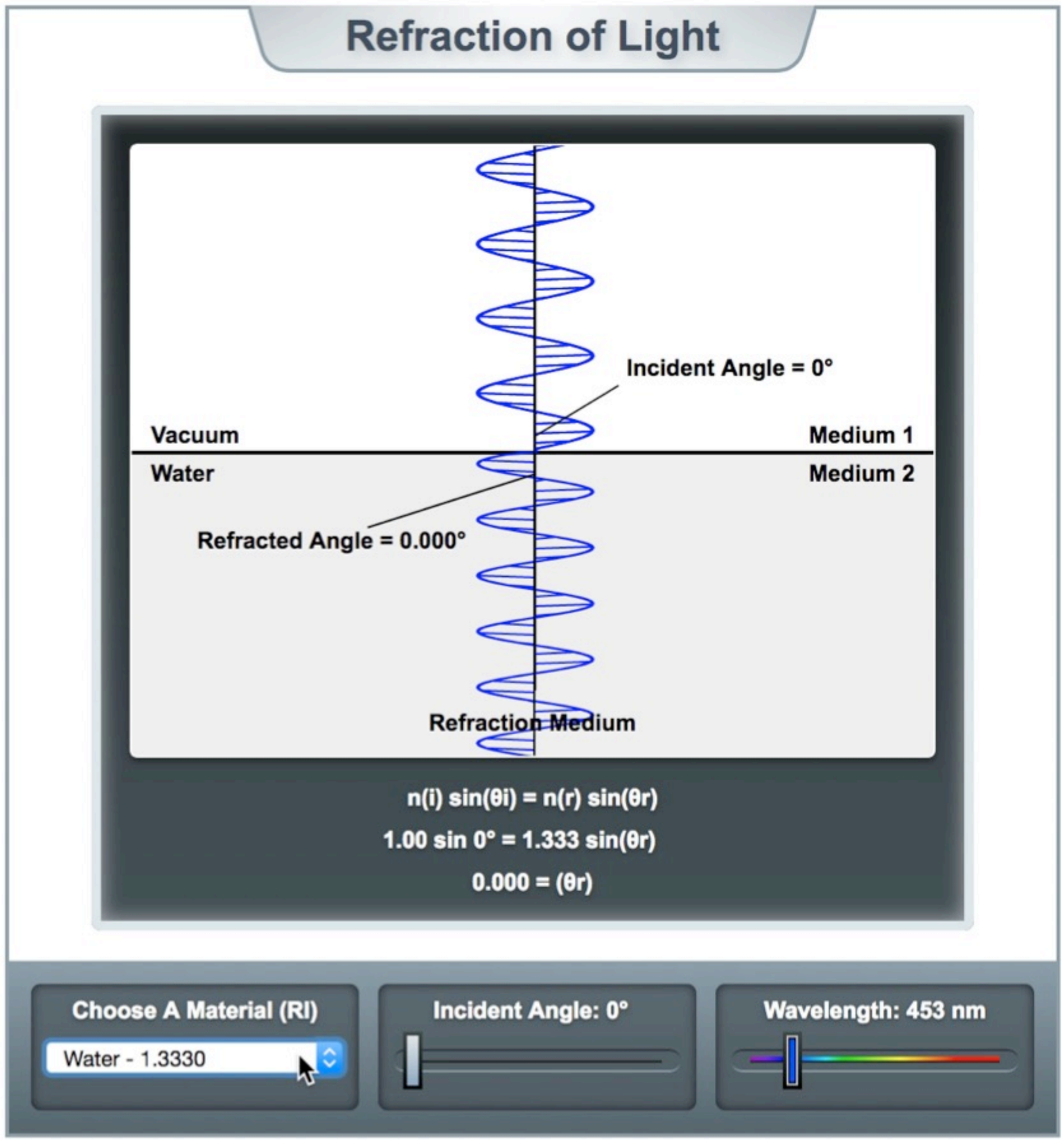
Snell's law :

$n_{vacuum} \times \sin(\theta 1) = n_{Oil} \times \sin(\theta 2)$



$n_{Air} = 1{,}002$

Adapted from http://www.microscopyu.com



Refractive index (index de réfraction)

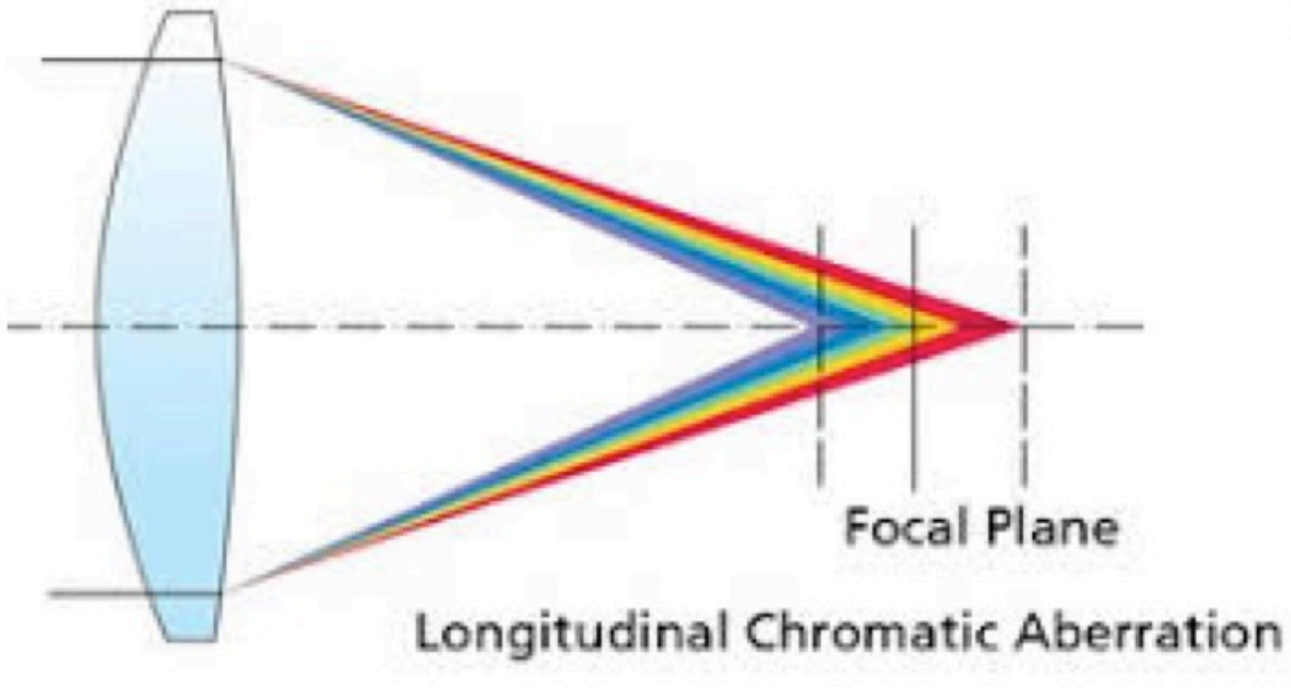


Image from http://www.sony.fr

in in ordinary glass the refractive index for olet light is about one percent greater than that for red light.

n_{Oil} (violet) > n_{Oil} (green)> n_{Oil} (red)

Image from http://www.topoptics.biz

This effect is termed **dispersion** and is responsible for chromatic aberration in microscope objectives.

Numerical aperture (ouverture numérique)

Objective	
Magnification	NA
60x	1.40
40x	1.30
40x	0.55
25x	0.80
4x	0.20

Magnification (grandissement)

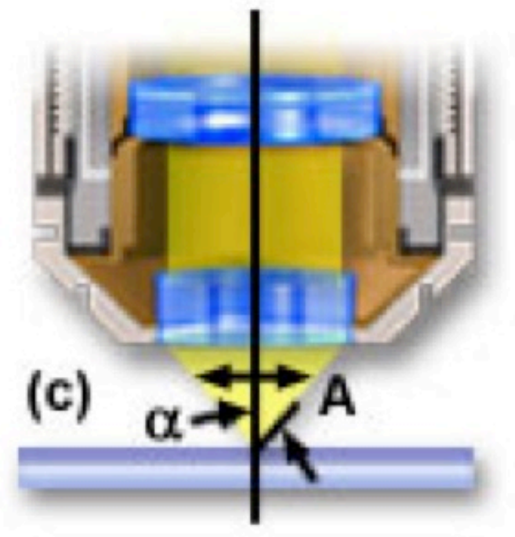
60X

Numerical aperture (Ouverture numérique)

NA= 1,4 - objective using oil

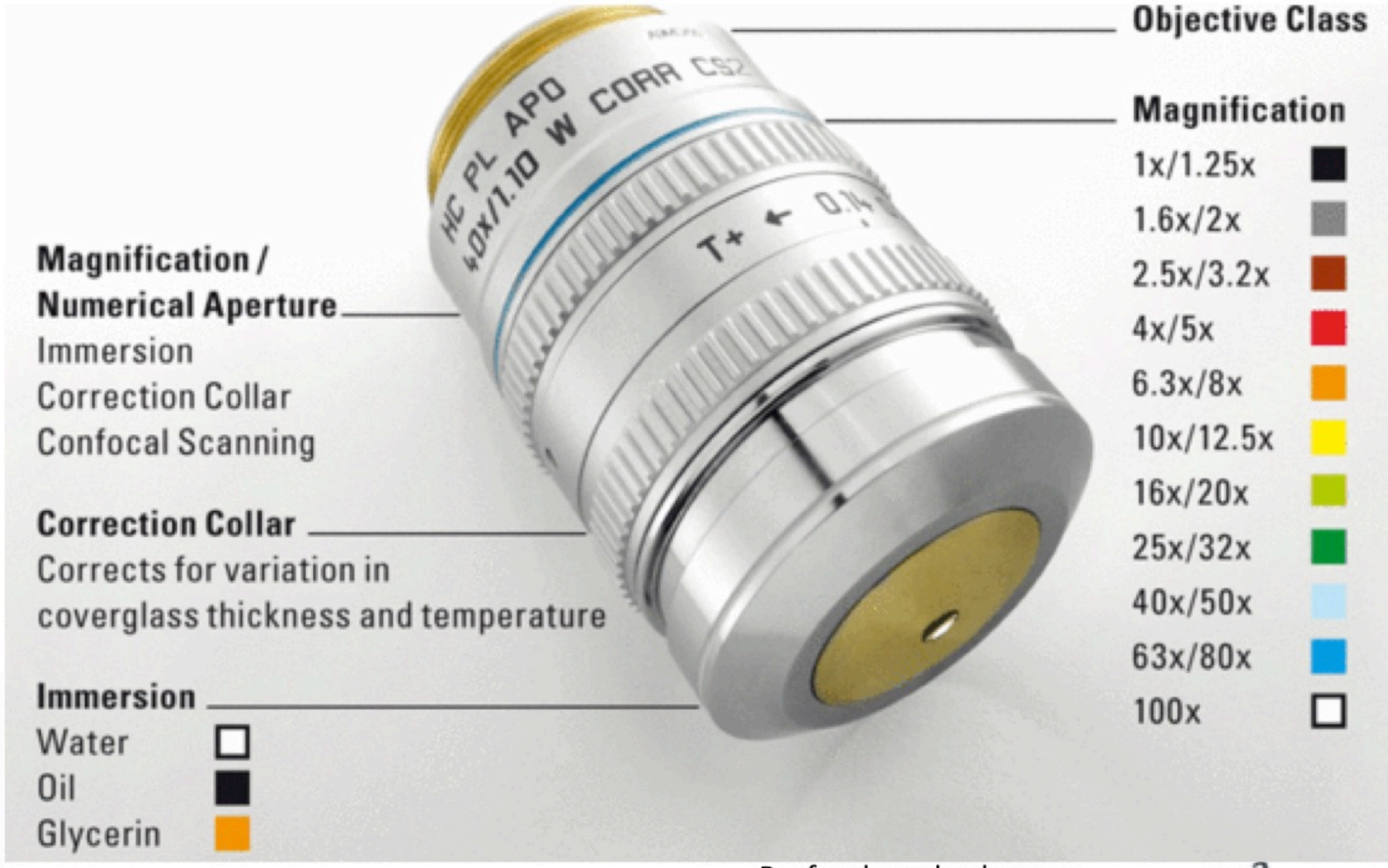
$$NA = n \bullet \sin(\alpha)$$

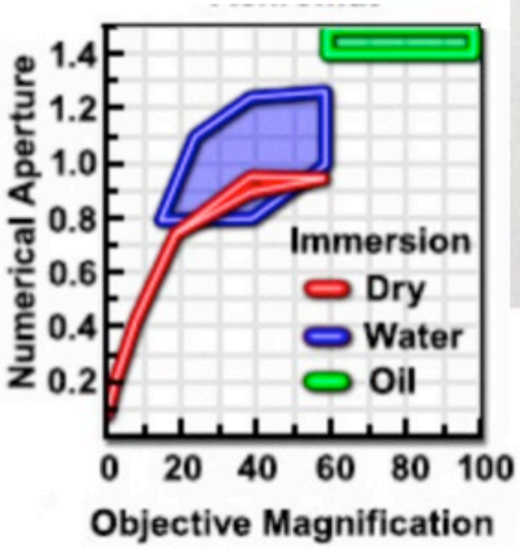
(a) $\alpha = 7°$ NA = 0.12
(b) $\alpha = 20°$ NA = 0.34
(c) $\alpha = 60°$ NA = 0.87



http://www.microscopyu.com

Index matching ? Which immersion media for which mounting media?

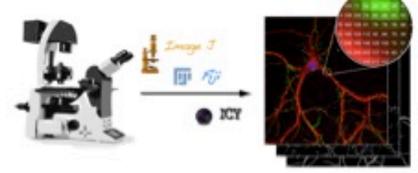




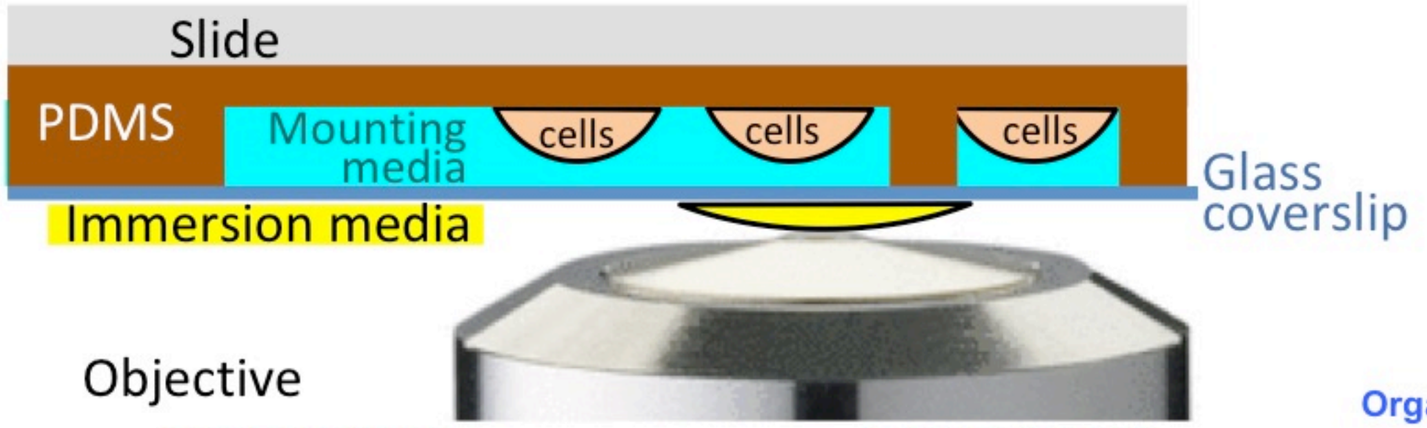
Profondeur de champ :

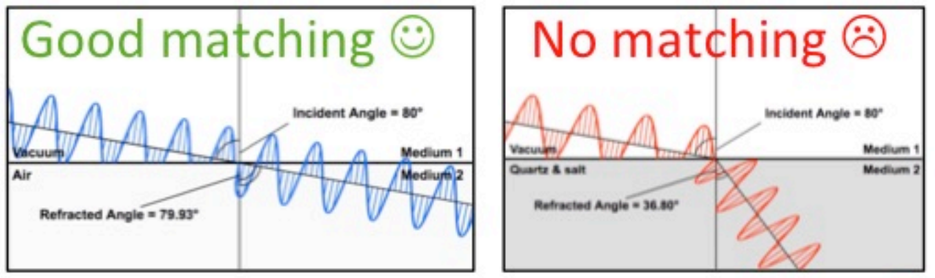
$$\sigma_{ob} = \frac{n\lambda}{2NA^2}$$

Plus l'ouverture numérique de l'objectif est grande et plus les structures d'intérêt de l'échantillon sont profondes, plus il est important de faire correspondre les indices de réfraction de l'échantillon et du milieu d'immersion. Des indices de réfraction différents entraînent des aberrations sphériques et des distorsions géométriques des structures. Cela entraîne ensuite une perte de contraste et de définition ainsi que des structures apparaissant comprimées ou étirées.



Which mounting medium ? : a matter of matching refraction indexes





Bad resolution ☹

Immersion Media	Refractive Index
Oil	1.518
Glycerol	1.46
Silicone oil	1.41
PDMS	1.41
Air	1.002
Vacuum	1

Mounting Media	Refractive Index
Gel/mount (Biomeda)	1.3641
Methyl salicylate (Sigma)	1.5409
DMSO	1,4836
Prolong	1,47 - 1,52
Mowiol	1,46
Vectashield	1.4577
50% glyceroi/PBS/DABCO	1.4159
PBS	1.34
Water	1.3381
Cargille index of refraction liquids	1.460 to 1.700
5% n-propy l gallate/0.0025% p-phenylene gallate (PPD) dissolvcd in glycerol	1.4739
0.25% PPD, 0.0025% DABCO.5% n-propyl gallate dissolved in glycerol	1.4732

Organ Tissue	Refractive Index
Cartilage	1.492
Fat	1.472
Brain Cerebellum	1.470
Brain White matter	1.467
Brain Gray matter	1.395
Liver	1.448
Spleen	1.443
Kidney Cortex	1.444
Kidney Medulla	1.438
Pancreas	1.435
Intestinal wall	1.436
Lung	1.342
Muscle	1.431
Gall bladder wall	1.350
Coagulated blood	1.400
Formed elements	1.432
Serum	1.330
Cells in culture	1.33 to 1.38
Water	1.33
PBS	1.34
From Biswas and Gauta (2002).	

Immersion and Mounting media index should be as close as possible to your sample index

Coverslip thickness (épaisseur des lamelles)

Carl Zeiss™ VERRES DE RECOUVREMENT HP D=0,17MM D=0,17MM

Recommended for applications with high numerical aperture objectives. Carl Zeiss™ come in a box of 100pcs. – VERRES DE RECOUVREMENT HP D=0,17MMD=0,17MM

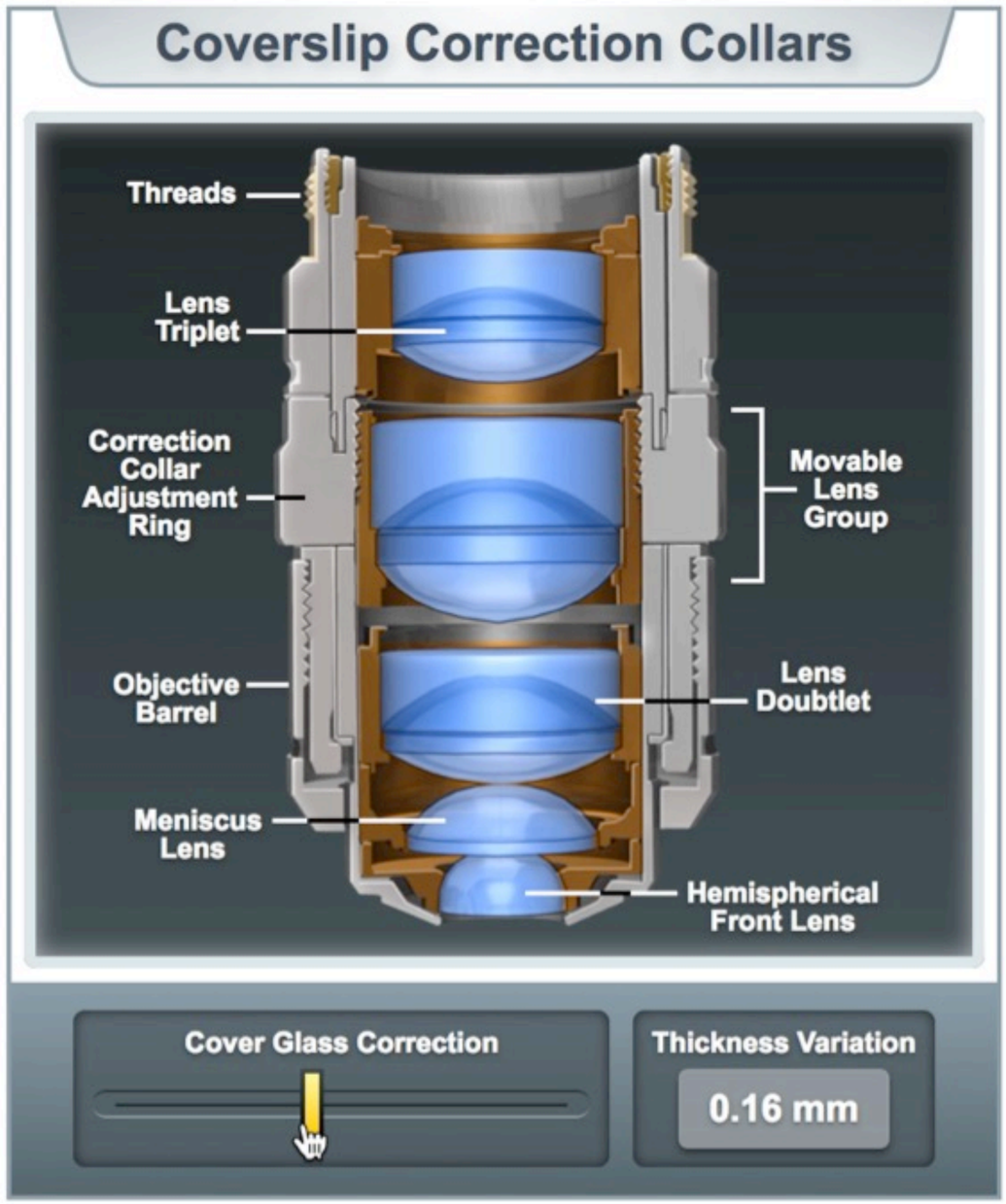
72.70€

The optimal thickness for cover glasses is **0.17 millimeters (#1.5)**, but conventional coverslip varies from 0,13 to 0,16 mm (#1).

Table 1 *- Performance Reduction with Coverslip Thickness Variation*

Numerical Aperture	0.01 mm Deviation	0.02 mm Deviation
0.30	none	none
0.45	none	none
0.70	2 percent	8 percent
0.85	19 percent	57 percent
0.95	55 percent	71 percent

Be carefull :
If the coverslip has a thickness deviation, performance of the objective can be reduced to 29%.



Solution : Compensation for cover glass thickness can be accomplished by adjusting the mechanical tube length of the microscope, or by the utilization of specialized **correction collars**

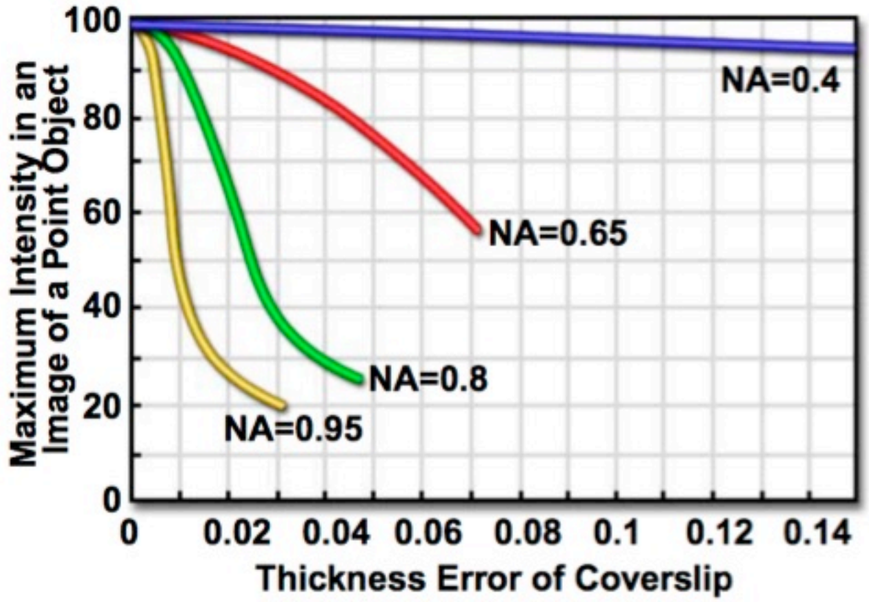
http://www.microscopyu.com

Correction collar (bague de correction)

CORR = Objective with correction collar

The performance of high-resolution objectives is optimal when the refractive indices of the specimen and all intermediate optical media match the values for which the objective is designed.

Water and glycerol immersion objectives are very sensitive to changes in coverglass – introducing a changing thickness of a medium with refractive index mismatch – temperature, and deviations of the immersion medium or the sample itself.



Therefore, immersion objectives with a higher NA have a correction collar to compensate for those differences.

Depth of Field and Depth of Focus

The **axial** (or **longitudinal**) **resolving power of an objective**, which is measured parallel to the optical axis and is most often referred to **as depth of field**.

Depth of field is determined by the **distance from the nearest object plane in focus to that of the farthest plane also simultaneously in focus**.

$$d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} \cdot e$$

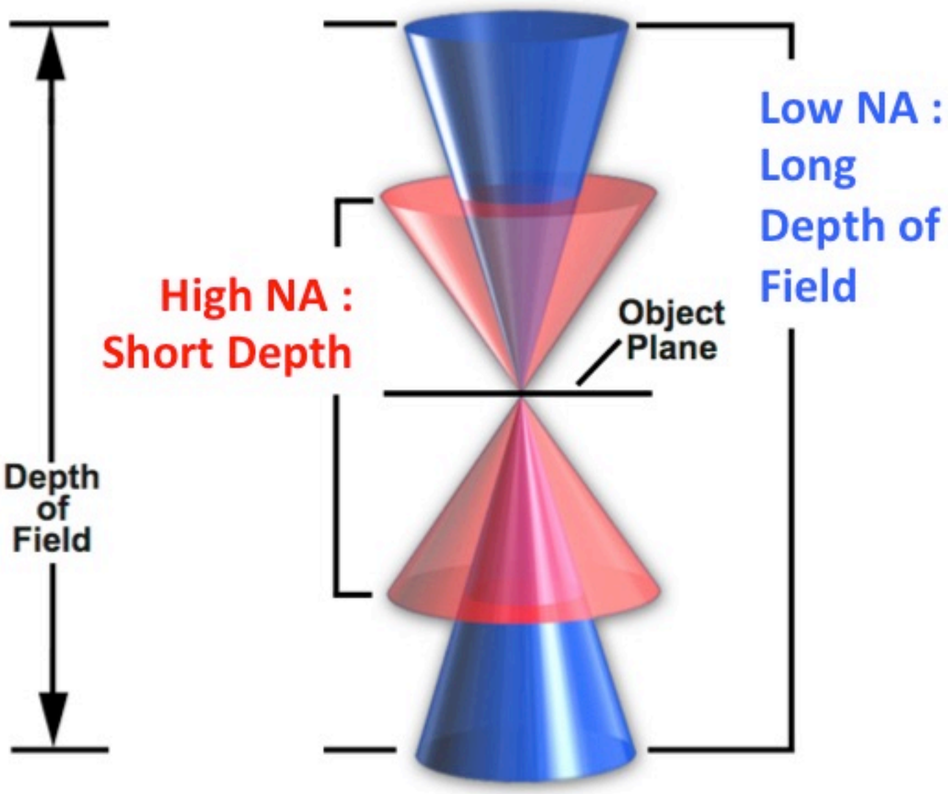
$$0.49727\ \mu m = \frac{550nm \cdot 1.515}{1.40 \cdot 1.40} + \frac{1.515}{60.0x \cdot 1.40} \cdot 4\ \mu m$$

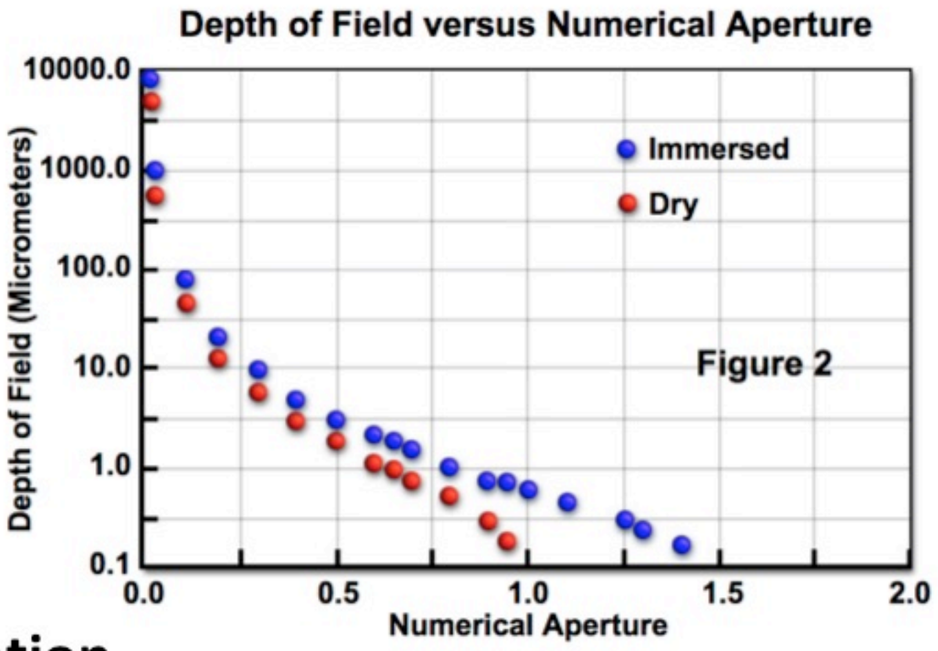
Depth of Field

λ is the wavelength,
n is the refractive index of the medium
NA : numerical aperture
M is obj. magnification
e : smallest distance that can be resolved by a detector that is placed in the image plane of the microscope objective.

Magnification	Numerical Aperture	Depth of Field (µm)
4x	0.10	55.5
10x	0.25	8.5
20x	0.40	5.8
40x	0.65	1.0
60x	0.85	0.40
100x	0.95	0.19

ure 1 - Depth of Field Ranges

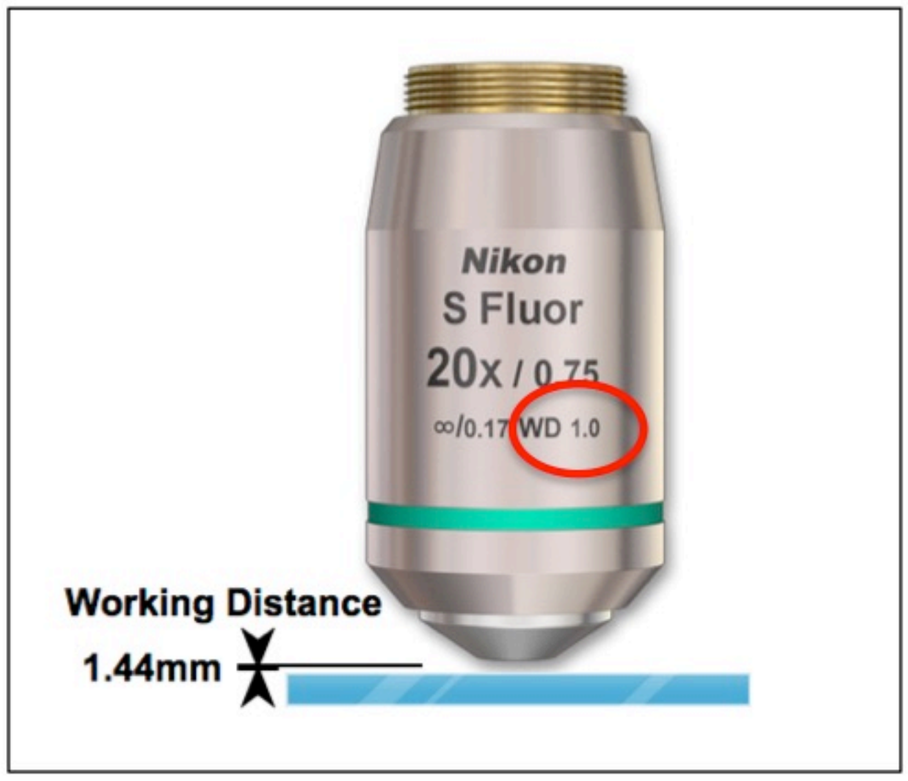


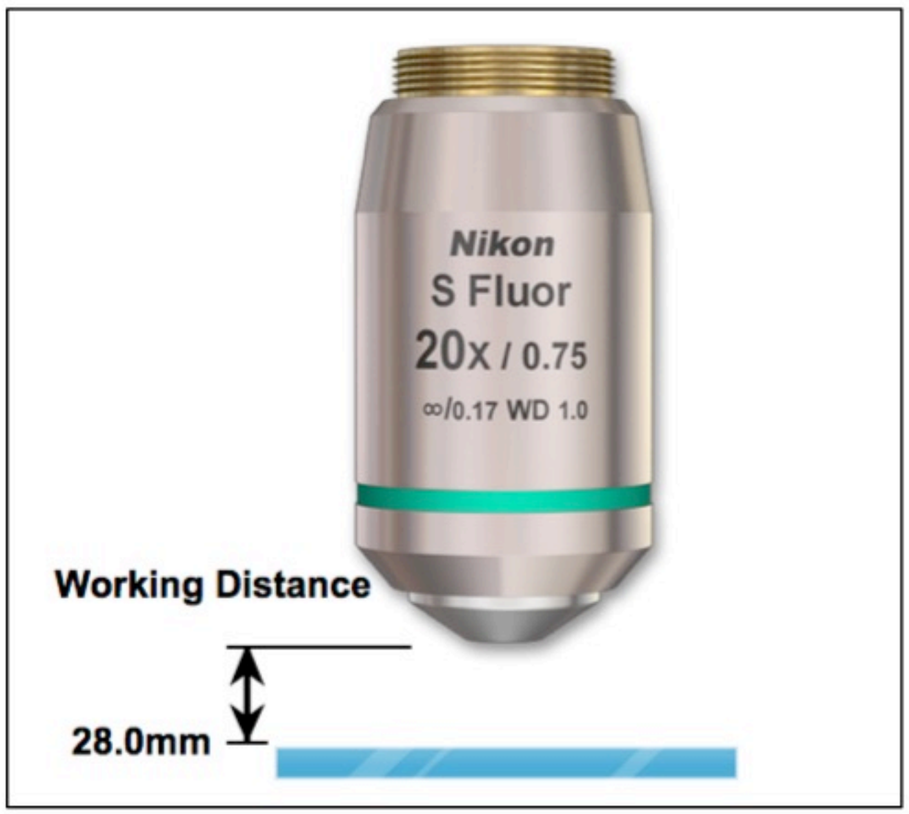


Depth of field decreases with hight NA and high magnification

https://www.microscopyu.com/microscopy-basics/depth-of-field-and-depth-of-focus

Working distance (distance de travail)

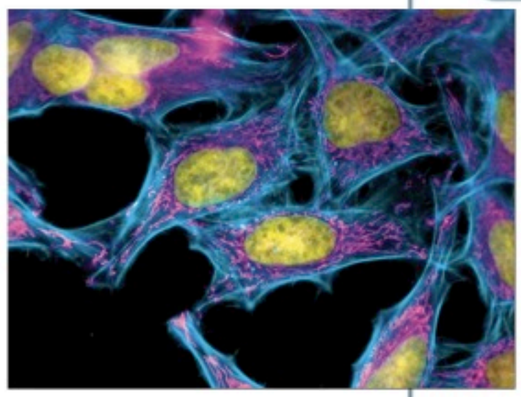


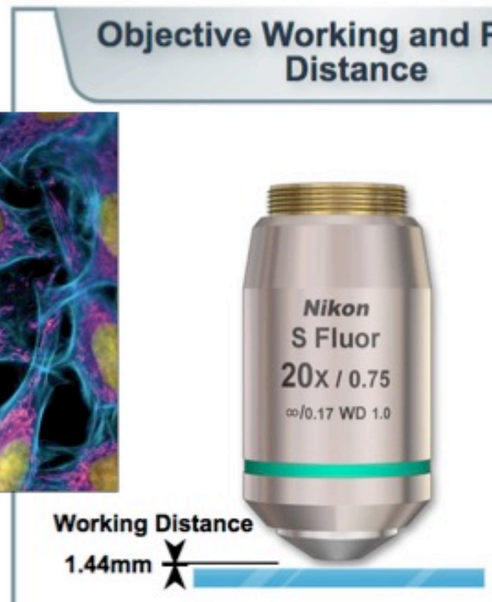


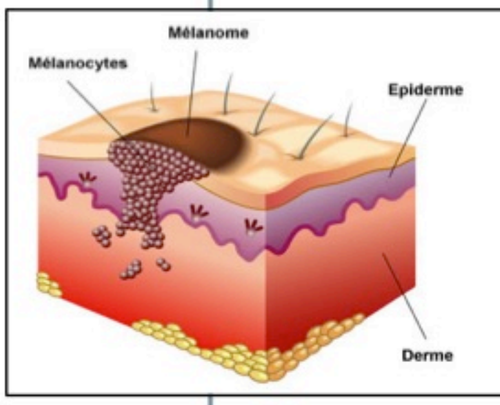
Magnification	Numerical Aperture	Working Distance (mm)
2x	0.10	8.50
4x	0.20	15.70
10x	0.45	4.00
20x	0.75	1.00
40x	0.95	0.14
40x (oil)	1.00	0.16
60x	0.95	0.15
60x (oil)	1.40	0.21
60x (Water Immersion)	1.20	0.22
100x (oil)	1.40	0.13
100x (NCG oil)	1.40	0.17
NCG = No Cover Glass		

From http://www.microscopyu.com

Working distance







Choose the objective adapted to the thickness of your sample.

Manufacturer	Correction	Magnification	Numerical Aperture	Working Distance
Nikon	PlanApo	10x	0.45	4.0 mm
Nikon	PlanFluor	20x	0.75	0.35 mm
Nikon	PlanFluor (oil)	40x	1.30	0.20 mm
Nikon	PlanApo (oil)	60x	1.40	0.21 mm
Nikon	PlanApo (oil)	100x	1.40	0.13 mm

Designation	Magnification	Numerical Aperture	Working Distance
ELWD	20x	0.40	11.0 mm
ELWD	50x	0.55	8.7 mm
ELWD	100x	0.80	2.0 mm
SLWD	10x	0.21	20.3 mm
SLWD	20x	0.35	20.5 mm
SLWD	50x	0.45	13.8 mm
SLWD	100x	0.73	4.7 mm

http://www.microscopyu.com

Numerical aperture and resolution

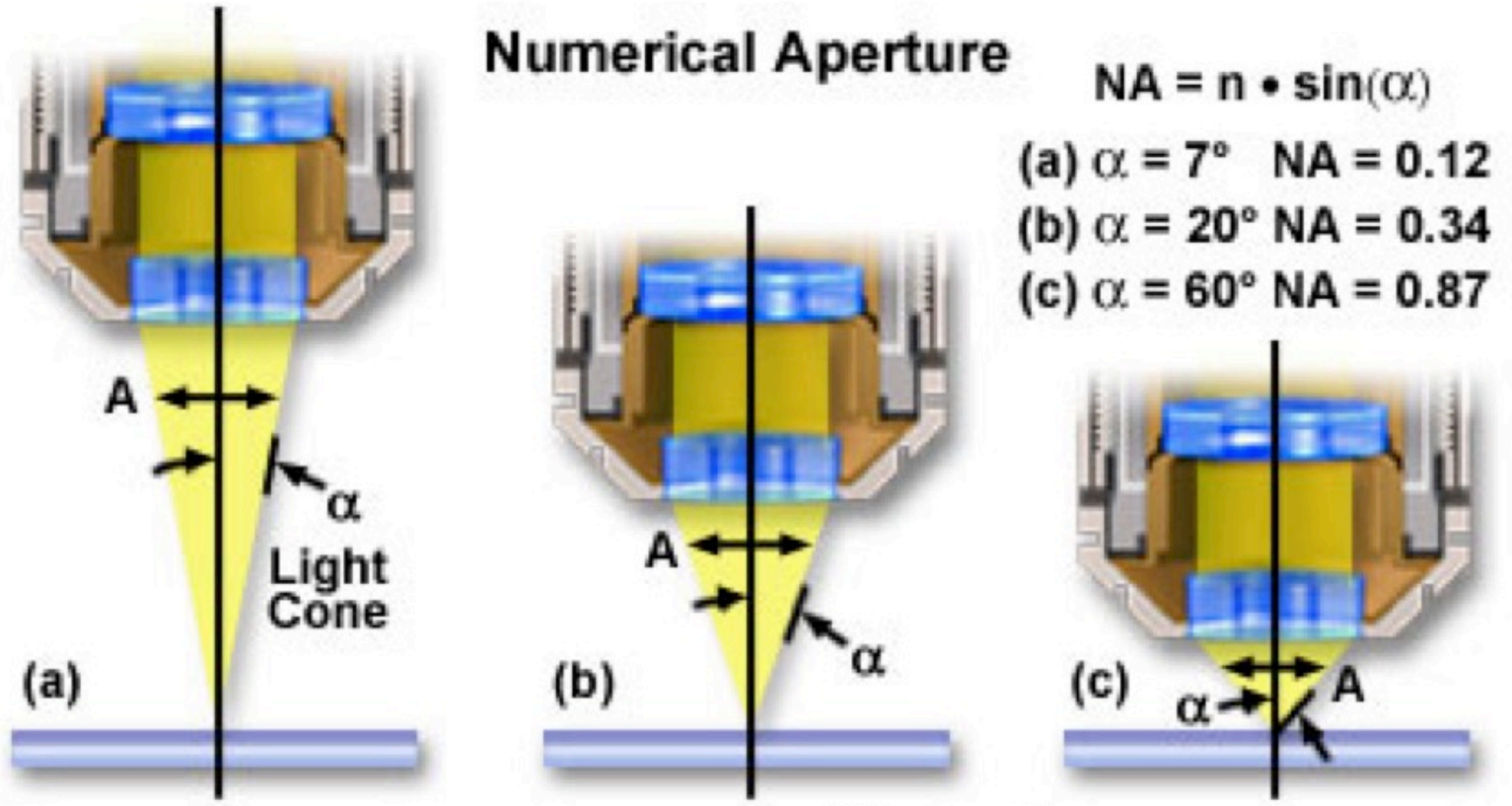
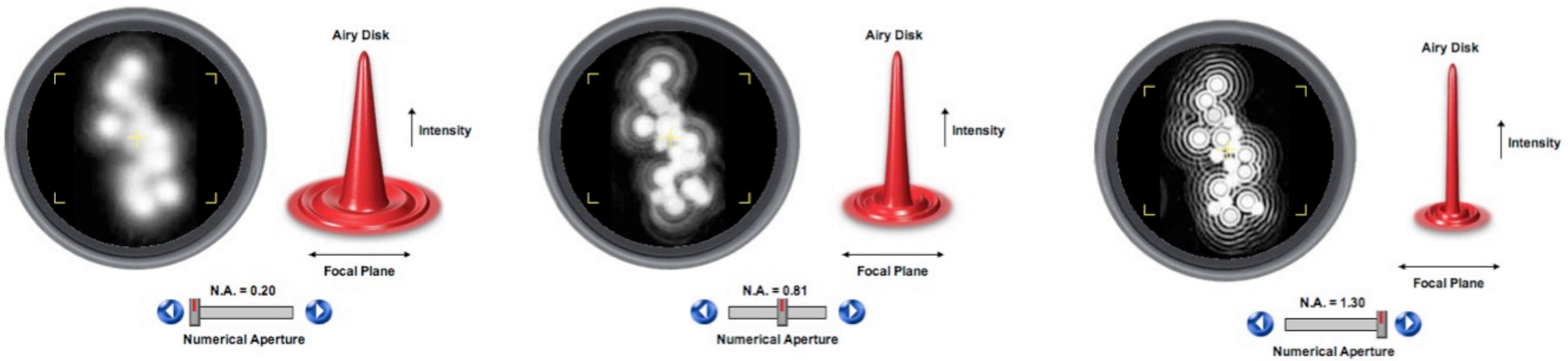


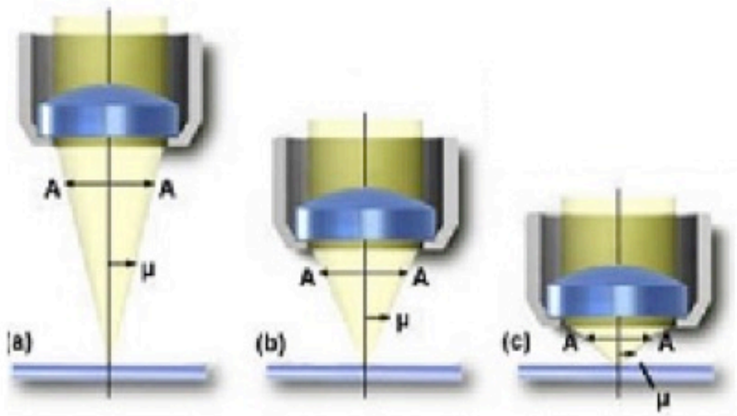
Figure 1

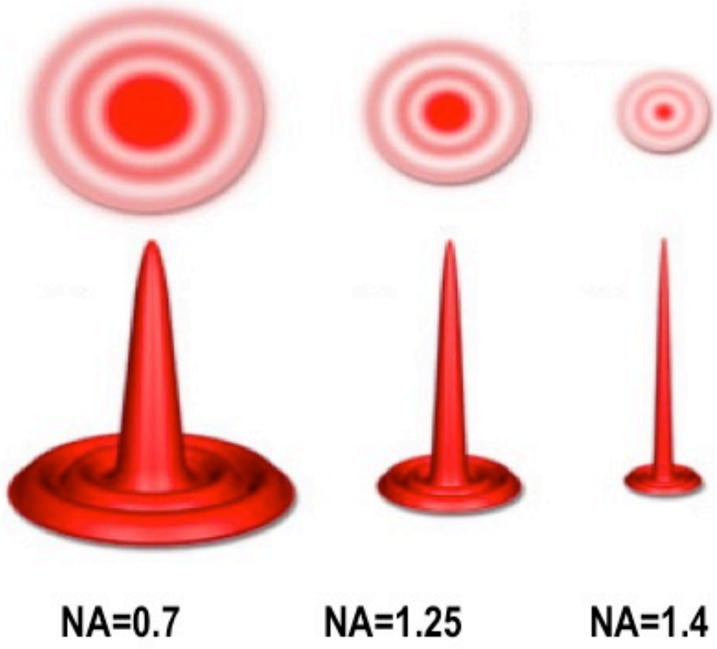


NA = 0,2 NA = 0,81 NA = 1,30

http://www.microscopyu.com

Numerical aperture and resolution (ouverture numérique et résolution)

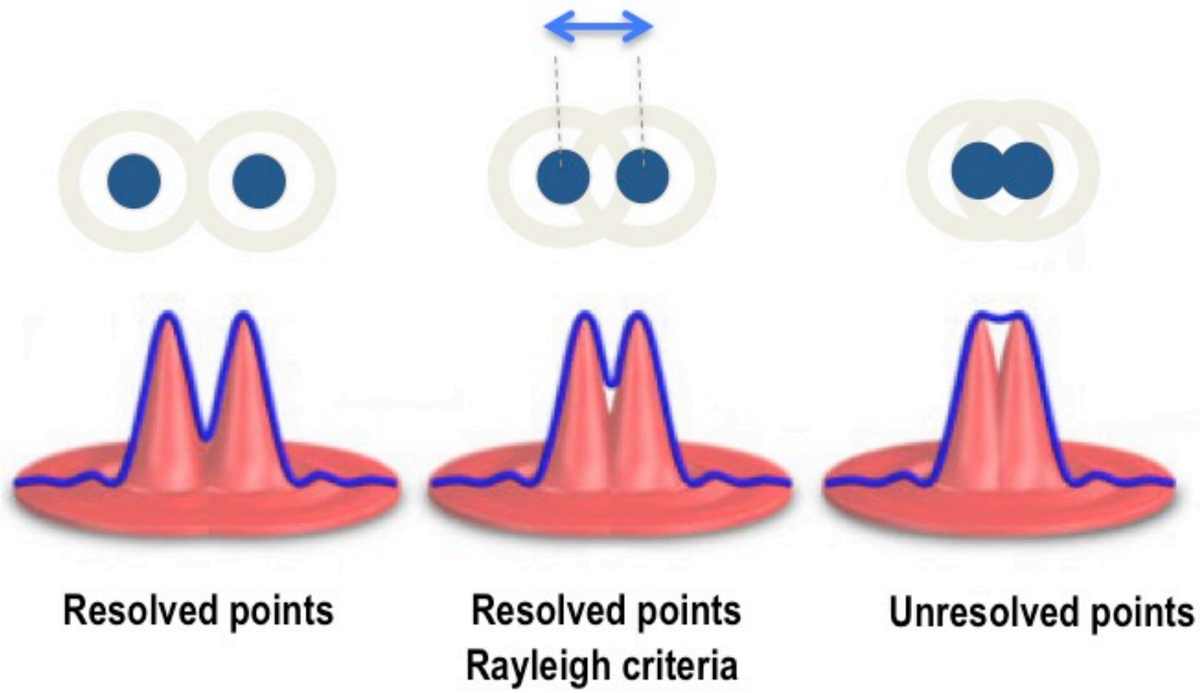




The more the NA is hight, the more the airy disc is sharp, the better is the resolution

Resolution :

is the shortest distance that allow to discriminate 2 dots.



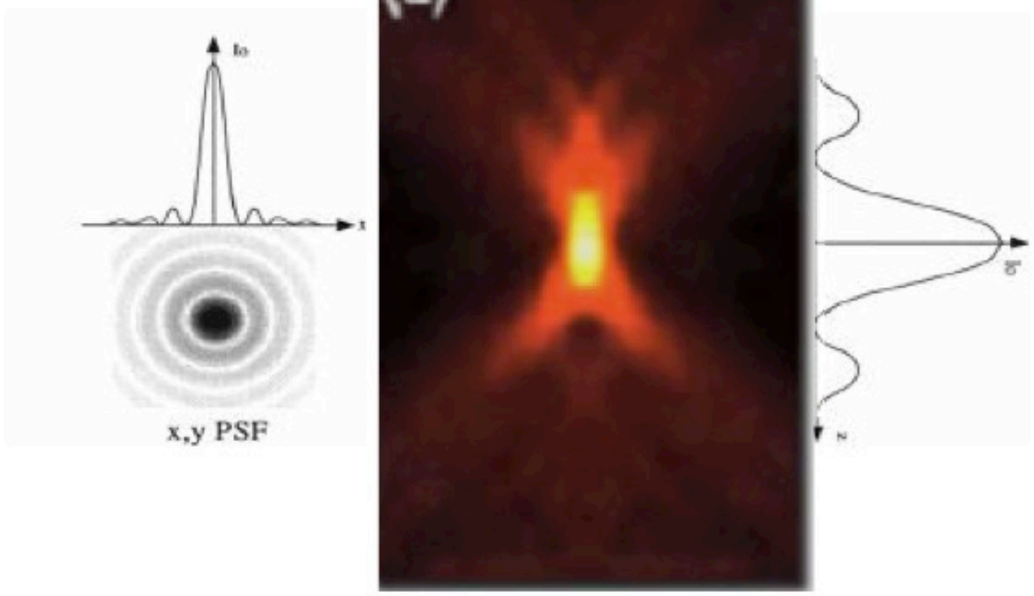
The more the NA is hight, better is the resolution :since picks are sharper the distance dicreases.

Ideal sampling

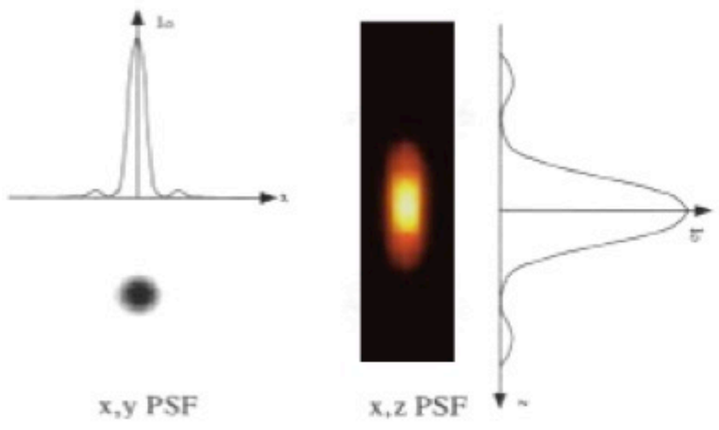
Nyquist criteria :

Image pixel size should be twice smaller than resolution

Plein champ



Confocal



Résolution latérale $d_{xy} = 0{,}61\ \lambda / NA$ — $d_{xy} = 0.46\ \lambda / NA$ + 25 %

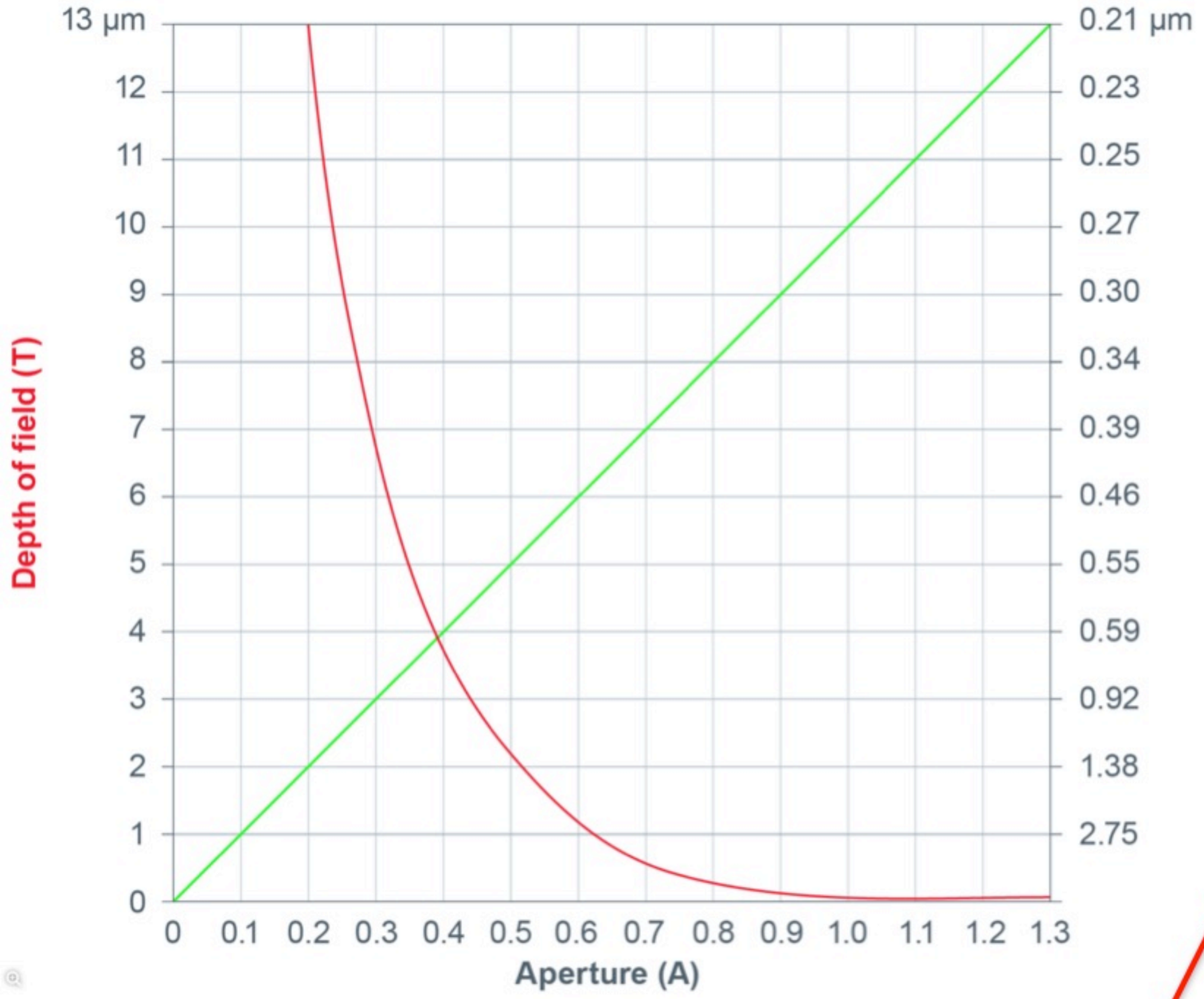
Résolution axiale $d_z = 2\ \lambda\ n / NA^2$ — $d_z = 1.4\ \lambda\ n / NA^2$ + 30%

Ex: with 63xobj, NA = 1,4 at 488 nm, resolution is dxy= 0,46 x 488 / 1,4 = 160 nm

To fulfill Nyquist criteria you should fix your pixel size at 160 / 2 = 80 nm.

At 647 nm, dxy=212 nm so ideal sampling pixel size would be at 106 nm.

Iris diaphragm objective in widefield microscopy

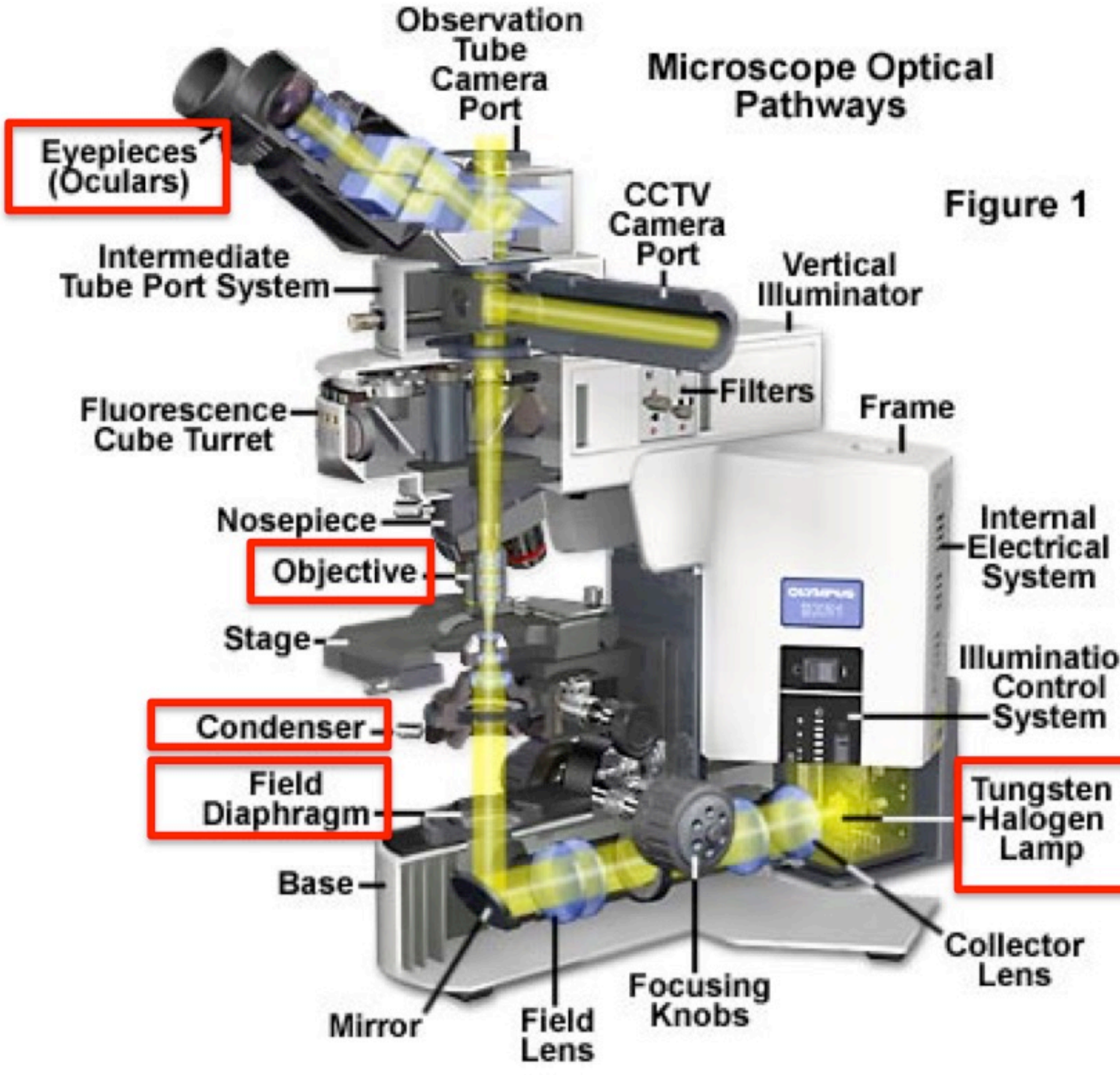


By using iris diaphragm objectives the numerical aperture of the objective can be changed. This is especially useful for widefield microscopy.

When diaphragm is closed: high depth of field but low NA and Resolution

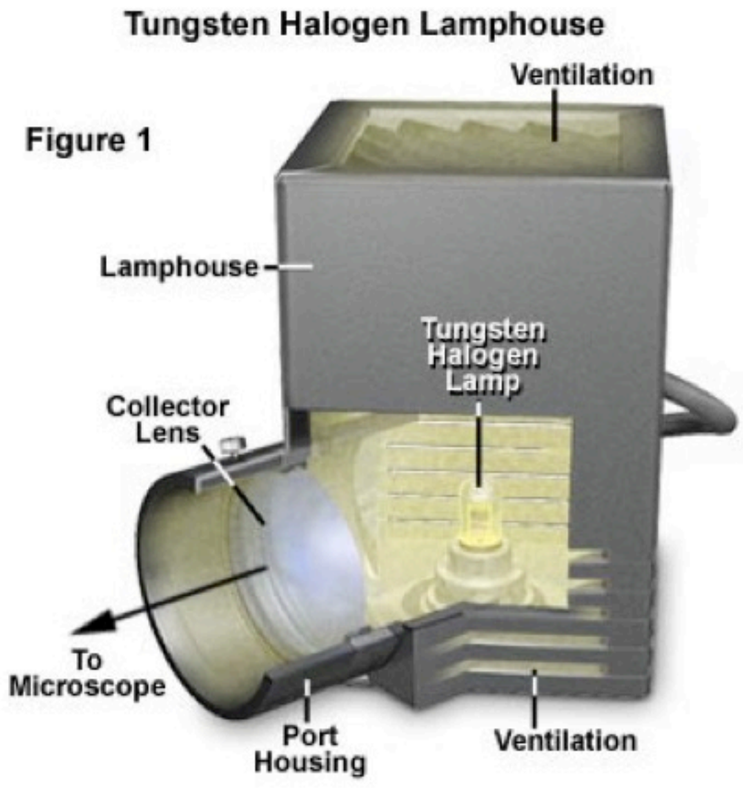
When diaphragm is open: short depth of field but high NA and higher Resolution

Brighield Microscopy, transmitted light



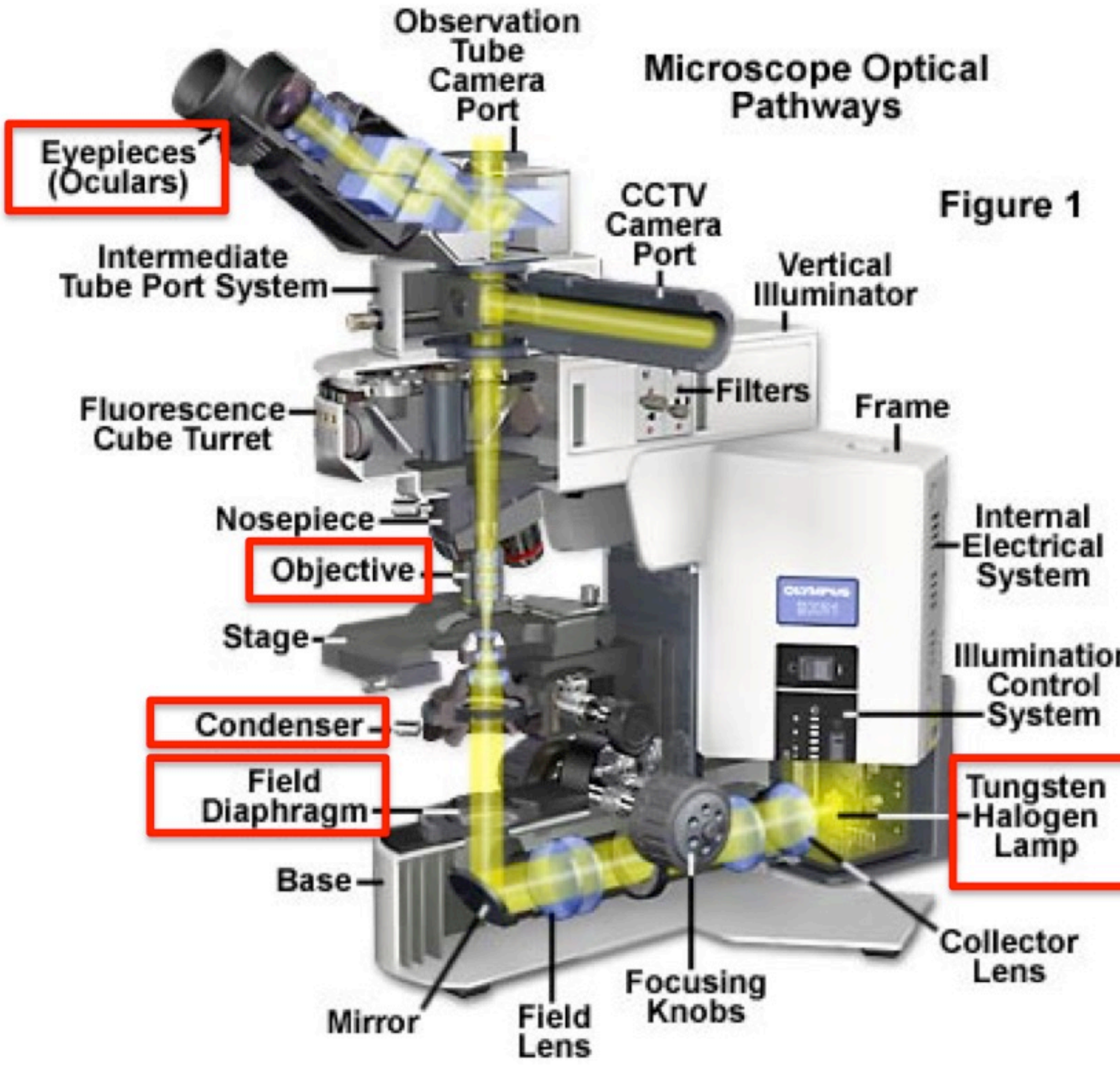
Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light, and contrast in the sample is caused by attenuation of the transmitted light in dense areas of the sample.

Dark sample on a bright background.



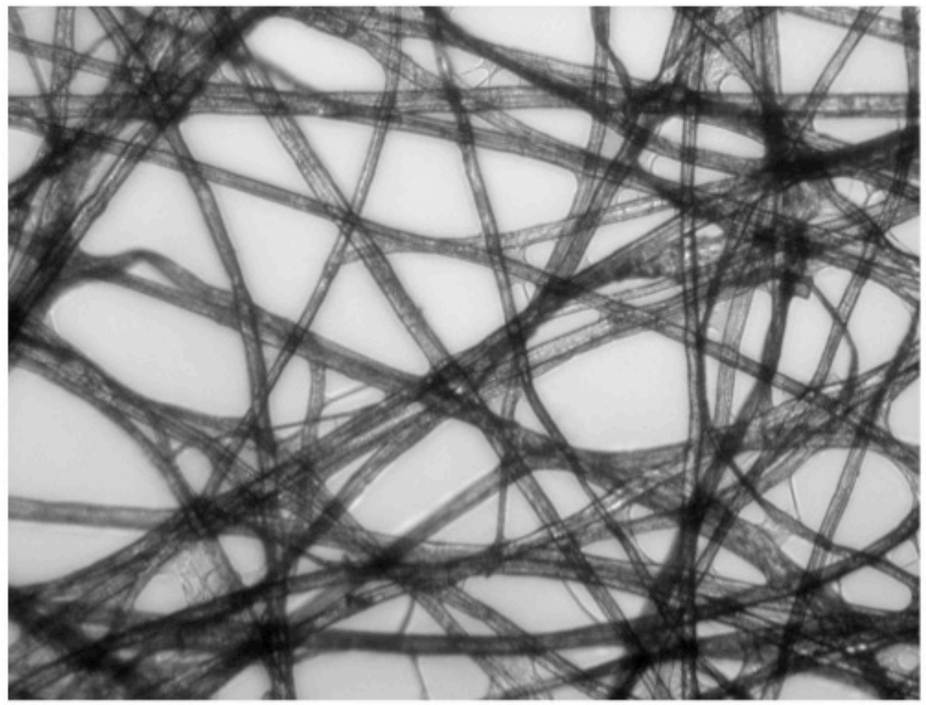
https://www.olympus-lifescience.com/en/microscope-resource/primer/anatomy/illumination/

Brighield Microscopy, transmitted light



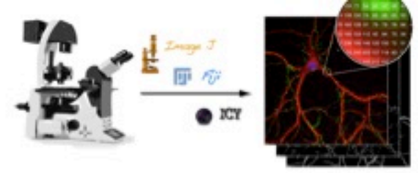
Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light, and contrast in the sample is caused by attenuation of the transmitted light in dense areas of the sample.

Dark sample on a bright background.



Wikibook/Bright-field microscopy - Wikipedia.html

https://www.olympus-lifescience.com/en/microscope-resource/primer/anatomy/illumination/



Brighield Microscopy

Dark sample on a bright background.
Bright-field illumination, sample contrast comes from absorbance of light in the sample

Halogen lamp
Field Diaphragm
Aperture condenser diaphragm
Objective
Oculars

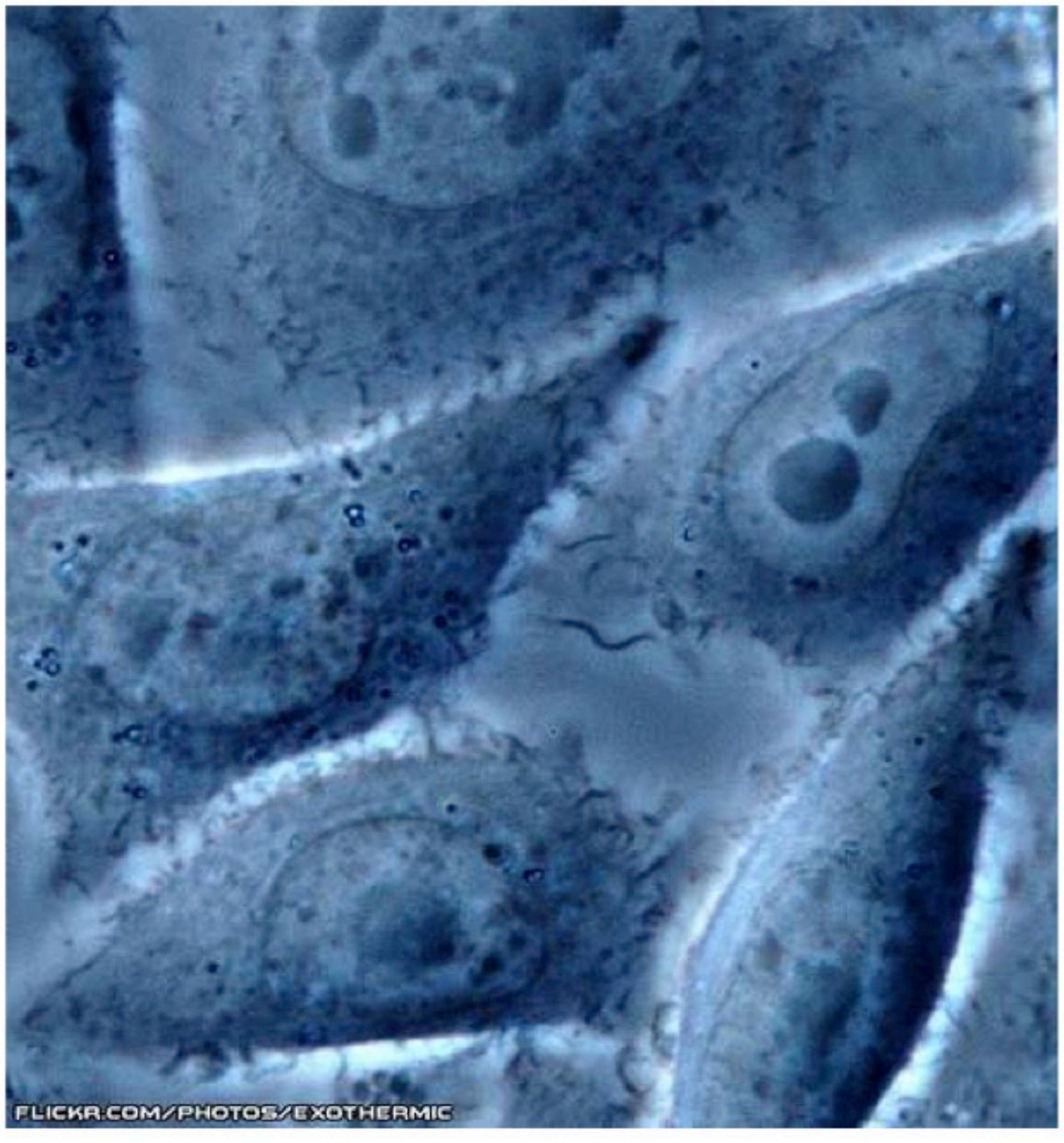
Alignment of the optical components of a microscope to optimize illumination in modern microscopes is carried out following the rules of **Köhler illumination**.

https://www.olympus-lifescience.com/en/microscope-resource/primer/anatomy/illumination/

Phase Constrast Microscopy

A phase contrast microscope is an optical microscope that converts **differences in refractive** indices between two structures **into contrast levels**, which result in phase differences for the light waves passing through them.

It thus visualizes transparent structures when their refractive index differs from that of their neighborhood.



https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/phasecontrast/phase/

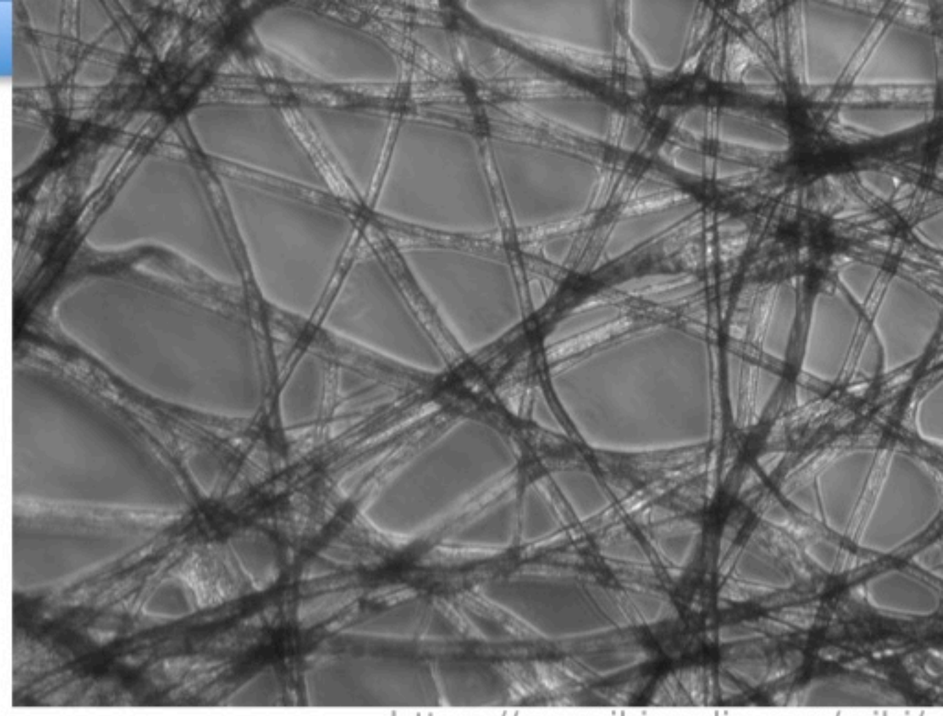
Phase Constrast Microscopy

Phase-contrast illumination, sample contrast comes from interference of different path lengths of light through the sample

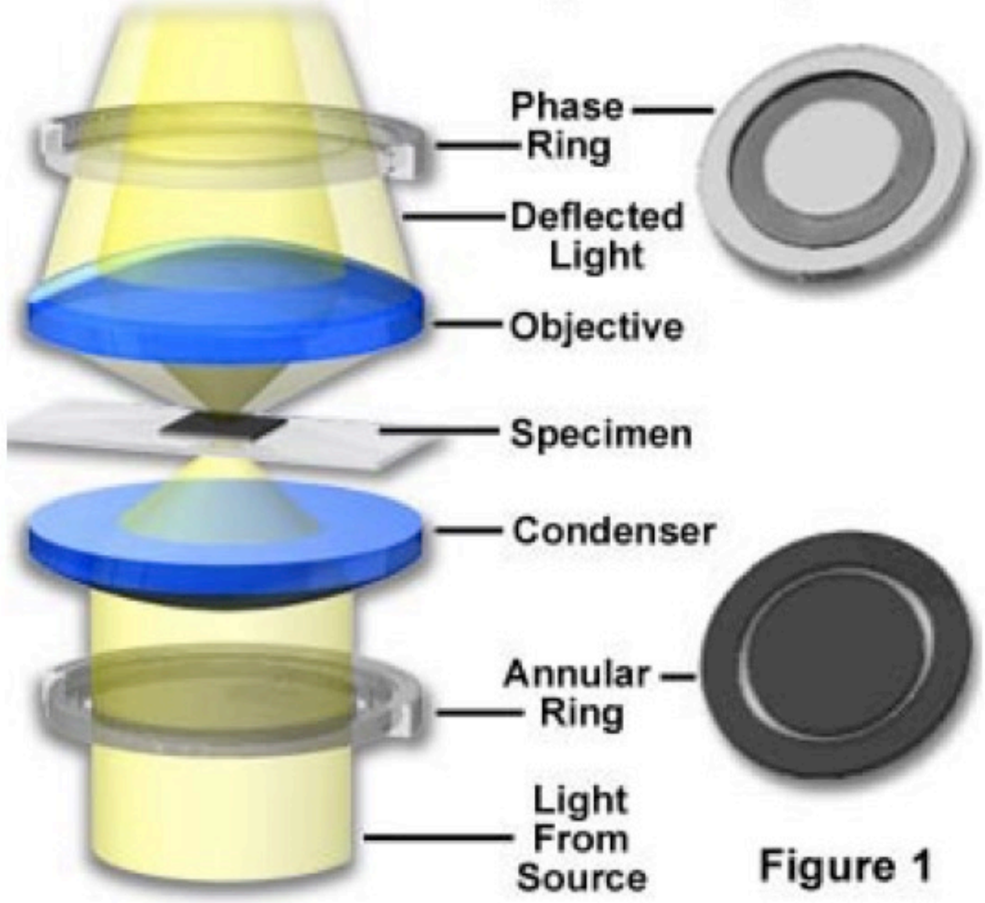
Developed by the Dutch physicist Frederik Zernike in the 1930s(Nobel Prize in physics in 1953).

In order to convert a phase difference into an observable **contrast**, that is to say a **difference in intensity**, interference is formed between the light rays of the object and a reference ray. We say that we transform a "phase object" into an "amplitude object".

This is achieved by means of a **circular** ring in the condenser which produces a **cone of light**. This cone is superimposed on a **ring of similar size in the lens**. The latter reduces the intensity of the direct light and creates a **phase difference** of a quarter of a wavelength.

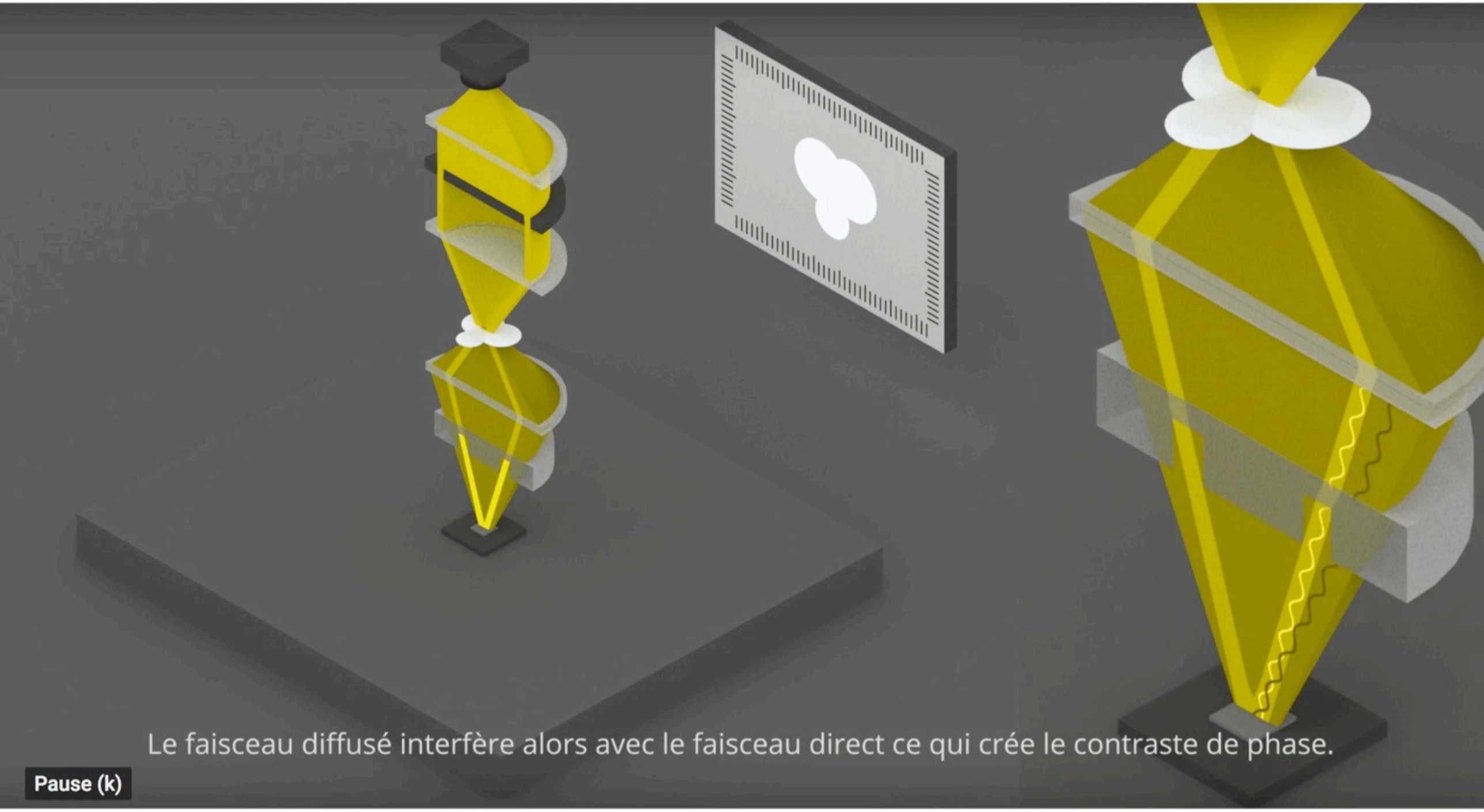


https://en.wikipedia.org/wiki/File:Paper_Micrograph_Phase.png



https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/phasecontrast/phase/

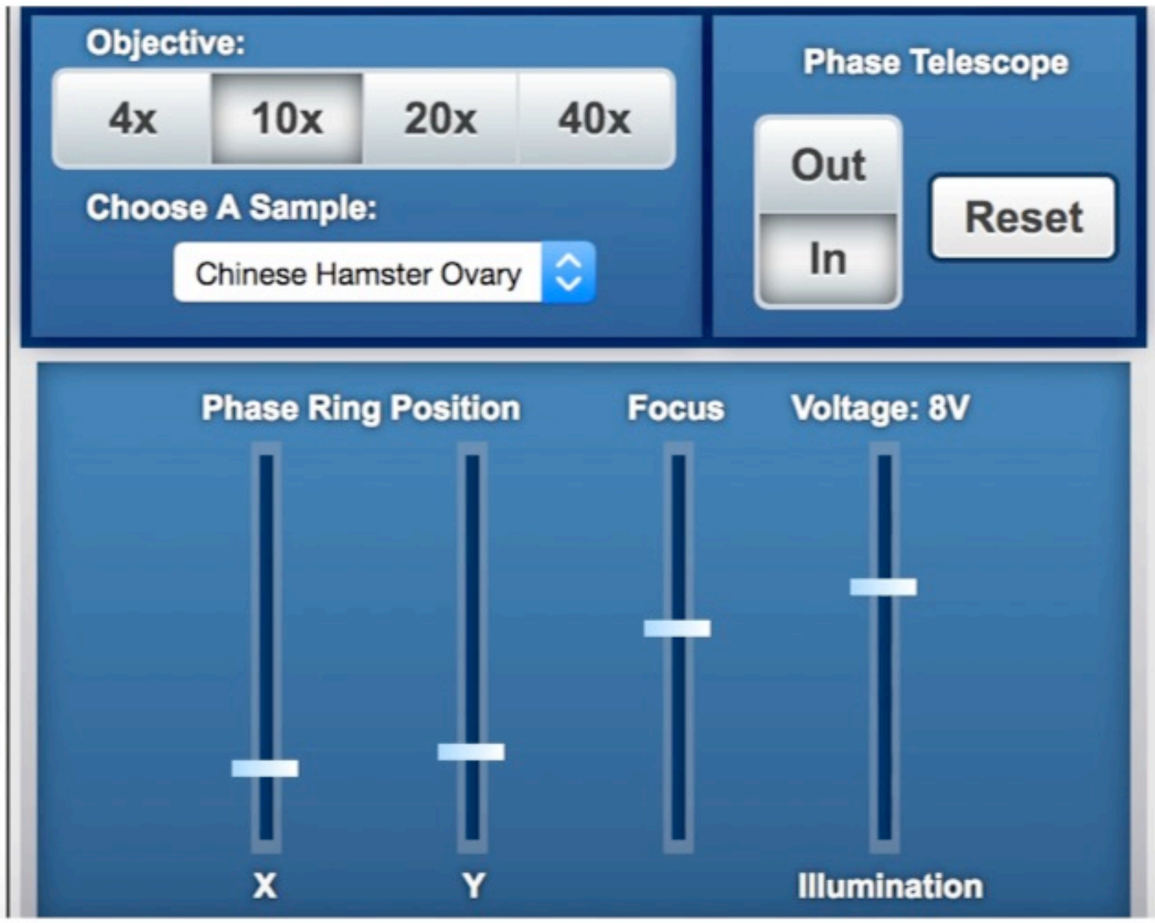
Phase Constrast Microscopy



From www.toutestquantique.fr

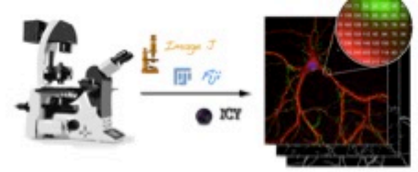
Phase Plate – Ring Alignment

Concentric alignment of the condenser phase plate slits with the phase ring, positioned inside the objective, is of paramount importance in phase contrast microscopy. This tutorial explores phase plate/ring alignment.



- Adjust **Focus** and **Intensity** sliders to achieve the best possible image.
- Use the **Phase Ring PositionX** and **Y** sliders to maximize specimen contrast.

https://www.olympus-lifescience.com/en/microscope-resource/primer/java/phasecontrast/phasemicroscope/



Luminescence

La luminescence : spontaneous emission of light after being excited

Excitation can result from:
- electromagnétique radiation (**photoluminescence**)
- chemical reaction (chimioluminescence)
- mecanical stimulation.

Photoluminescence

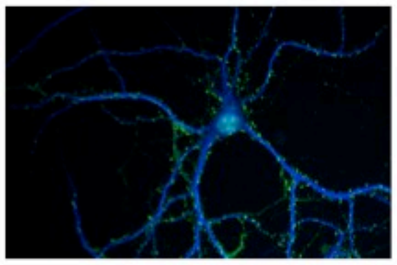
Molecule → Photons absorption → **Excited molecule** → Photoluminescence → **Molecule**

Back to basal state: Emission of lower energy photon

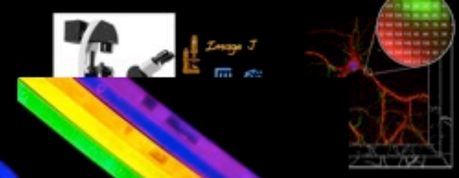
One fluorescent molecule (fluorophore or fluorochrome) can absorb light energy (excitation light) and quickly restore it through fluorescent light (emission light).

Photoluminescence →

Fluorescence
Instaneaous emission



Phosphorescence
Réémission prolongée



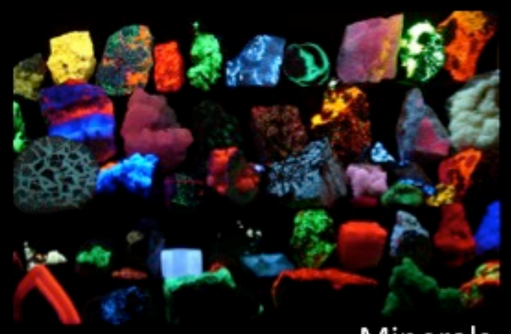
Excited molecule → **Luminescence** (Light emission) → **Molecule** (Back to fondamental state) → Luminescence

Chemiluminescence
(chemical activation)

$C_8H_7N_3O_2$ (luminol) + H_2O_2 → 3-APA + light

Photoluminescence
(Activation by light)

Fluorescence
Instantaneous emission

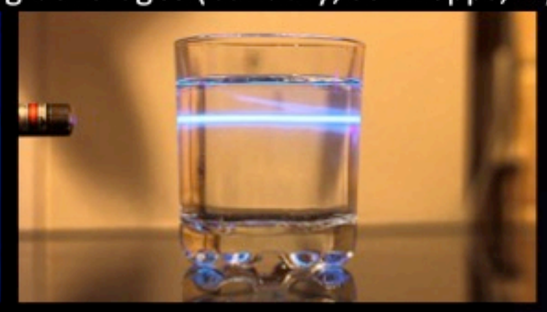


Minerals

Corals

BODY Painting

Quinine containing beverages (Canadry, schwepps, …)

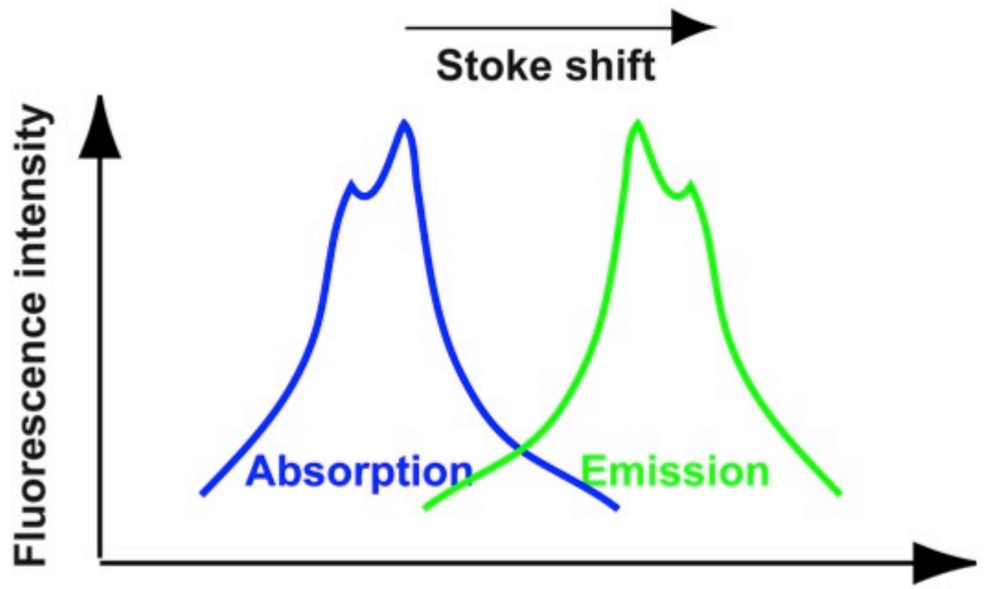


Phosphorescence
Longer emission (even in the dark)

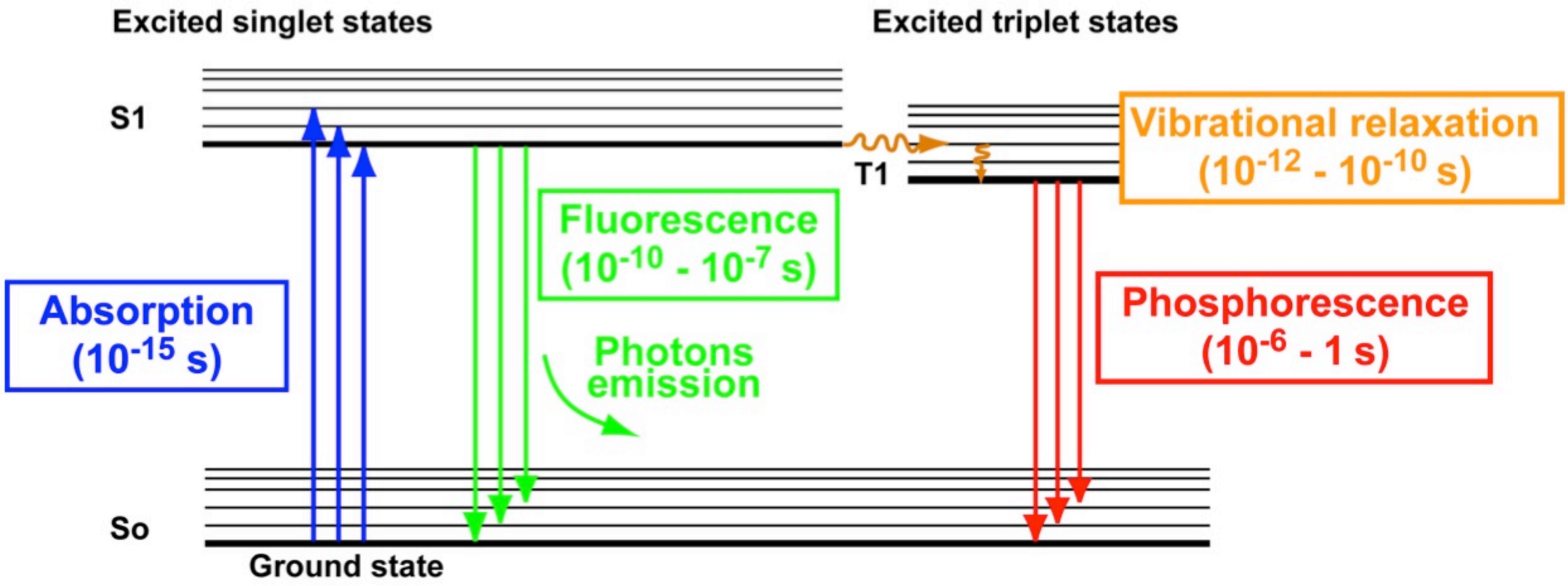
La Fluorescence

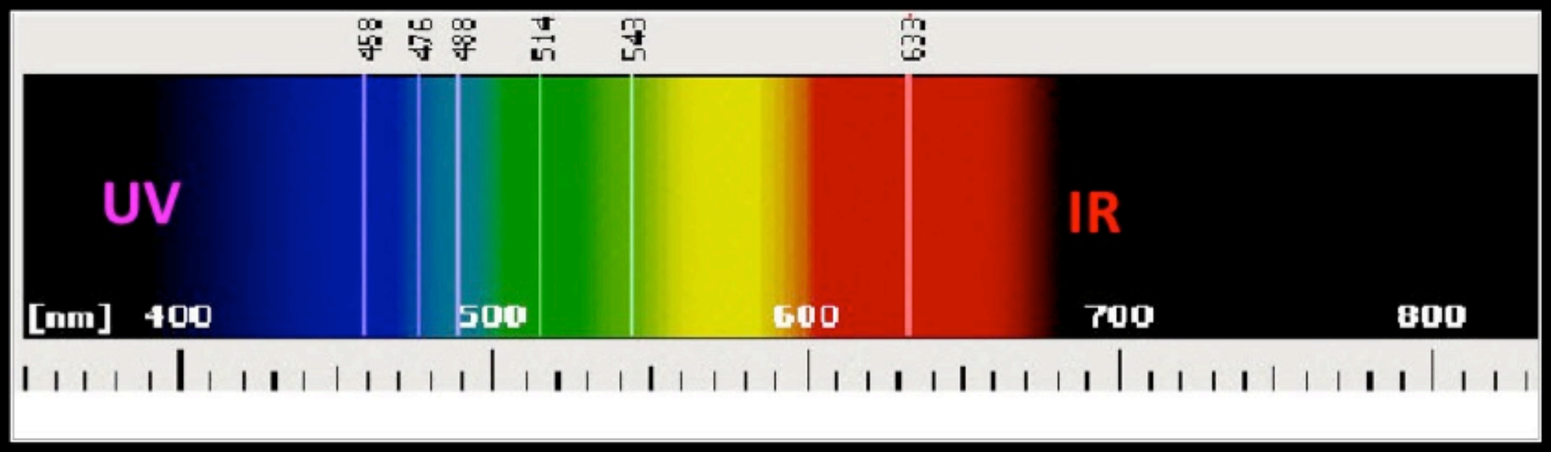
Fluorescence:
after photon absorption, the fluorophore is excited and restitutes by emitting photons of lower energy.

The shift between absorption and fluorescence emission is called « Stoke shift »

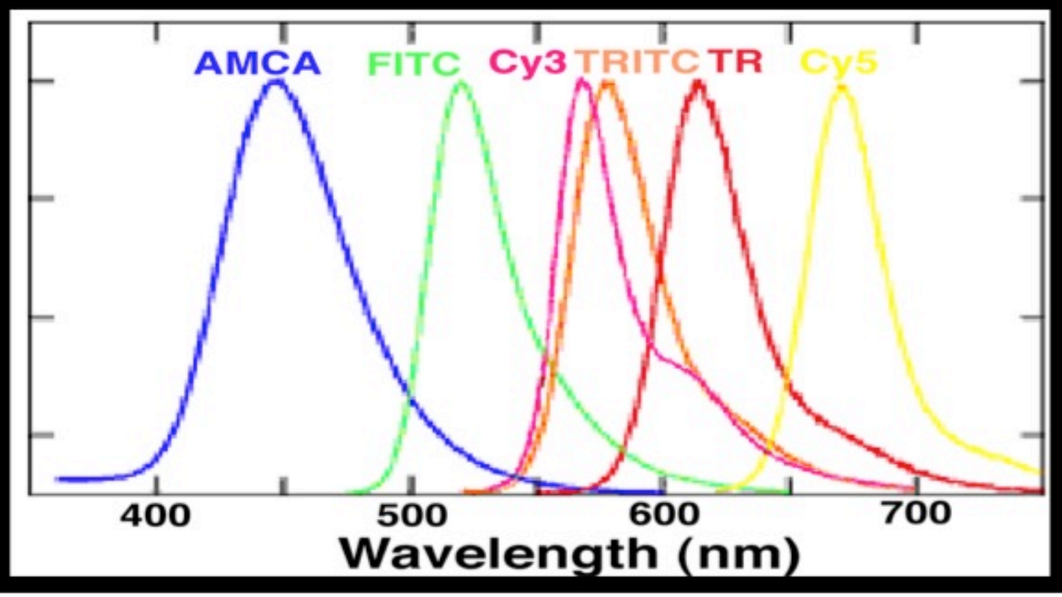


Return to basal state produces **fluorescence** (from singlet state) or phosphorescence (from a triplet state).





Fluorescent probes



Fluorophore	Absorption Peak (nm)	Emission Peak (nm)
Aminomethylcoumarin, AMCA	350	450
Cyanine, Cy2	492	510
Fluorescein, FITC	492	520
Indocarbocyanine, Cy3	550	570
Tetramethyl Rhodamine, TRITC	550	570
Rhodamine Red-X, RRX	570	590
Texas Red, TR	596	620
Indodicarbocyanine, Cy5	650	670

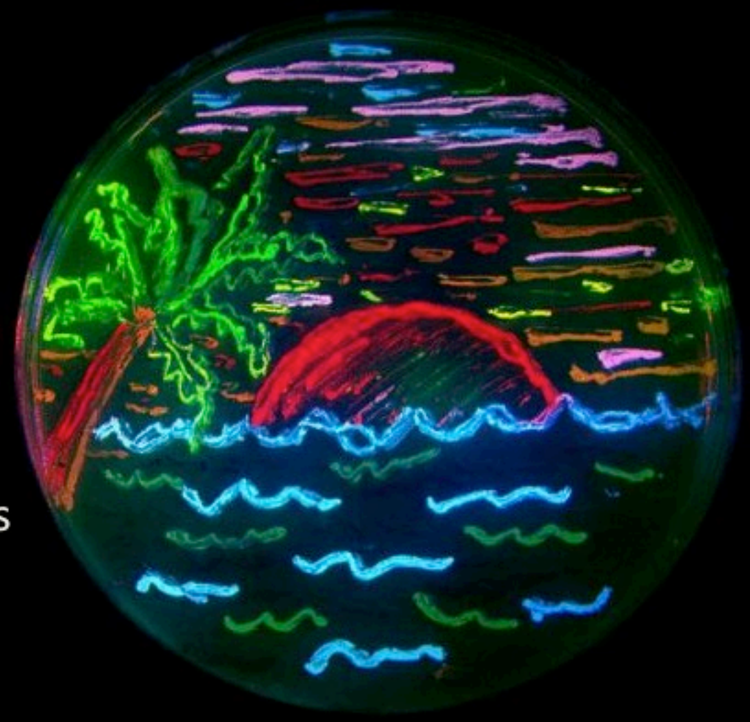
Color	Alexa Fluor Dye	Abs *	Em *
1	Alexa Fluor 350	346	442
2	Alexa Fluor 405	401	421
3	Alexa Fluor 430	433	541
4	Alexa Fluor 488	495	519
5	Alexa Fluor 532	532	553
6	Alexa Fluor 546	556	573
7	Alexa Fluor 555	555	565
8	Alexa Fluor 568	578	603
9	Alexa Fluor 594	590	617
10	Alexa Fluor 633	632	647‡
11	Alexa Fluor 647	650	665‡
12	Alexa Fluor 660	663	690‡
13	Alexa Fluor 680	679	702‡
14	Alexa Fluor 700	702	723‡
15	Alexa Fluor 750	749	775‡

GFP

Green Fluorescent Protein

Roger Tsien

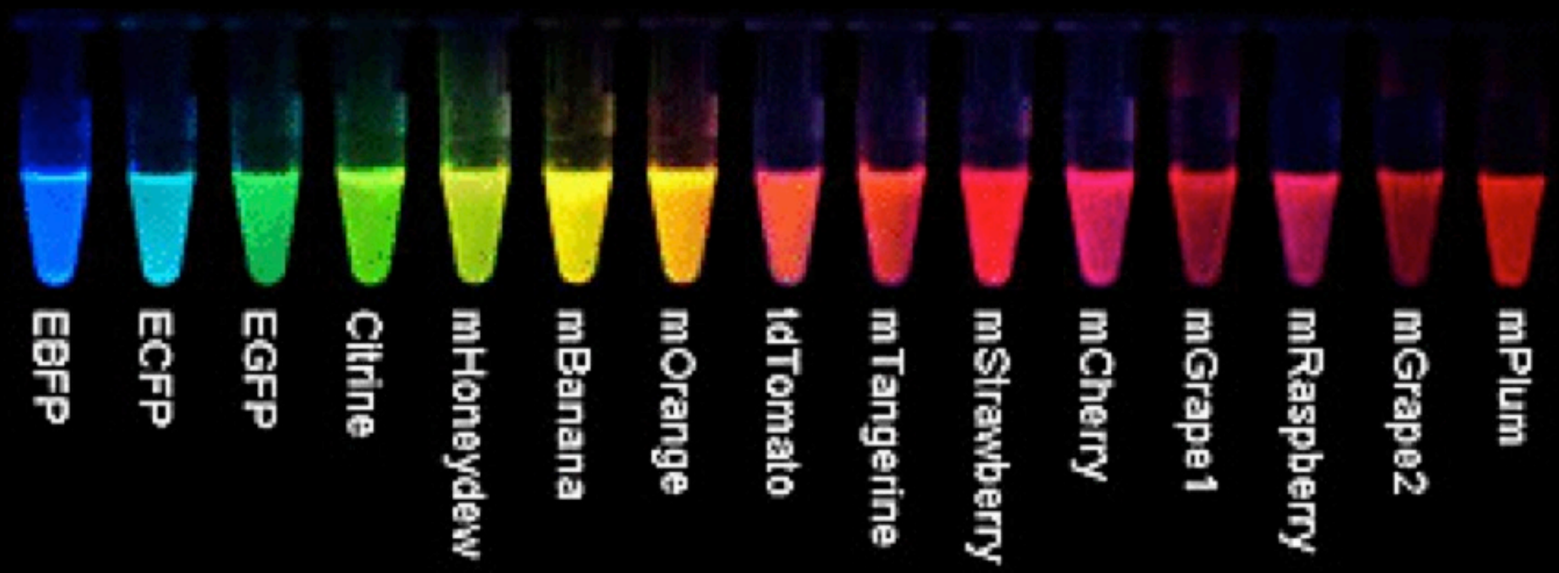
His group has developed mutants that start fluorescing faster than wild type GFP, that are brighter and have different colors (see below, the E stands for enhanced versions of GFP, m are monomeric proteins and tdTomato is a head-to-tail dimer).



R Heim, DC Prasher, RY Tsien: Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA 91 (1994) 12501-04.

R Heim, A Cubitt, RY Tsien: Improved green fluorescene. Nature 373 (1995) 663-64.

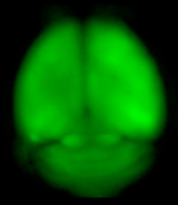
M Ormo, AB Cubitt, K Kallio, LA Gross, RY Tsien, SJ Remington: Crystal structure of the Aequorea victoria green fluorescent protein. Science 273 (1996) 1392-95.



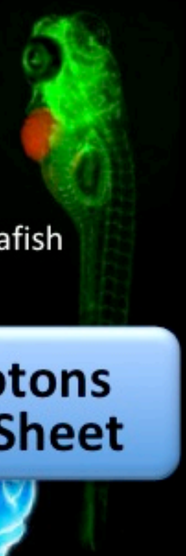
Adapted after http://www.conncoll.edu/ccacad/zimmer/GFP-ww/instCD.html, ***Marc Zimmer, University of Connecticut.***

Main Goal

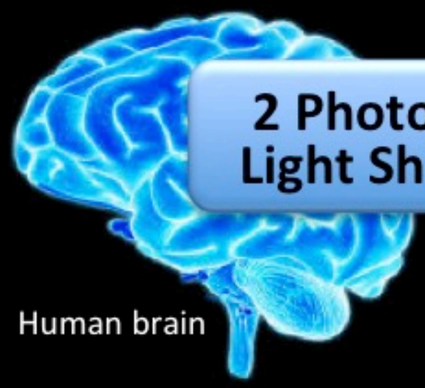
→ To cover imaging from whole organism to tissular, cellular and molecular levels



Mouse brain

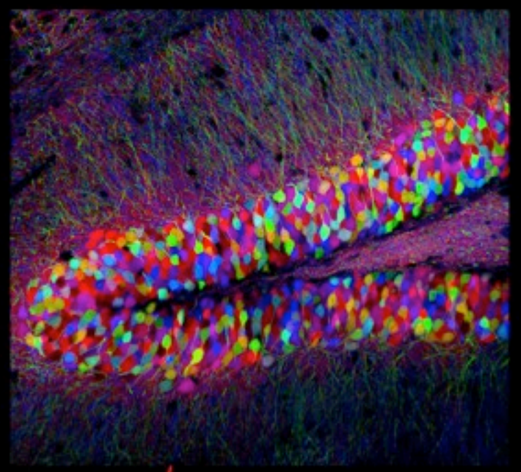


Zebrafish

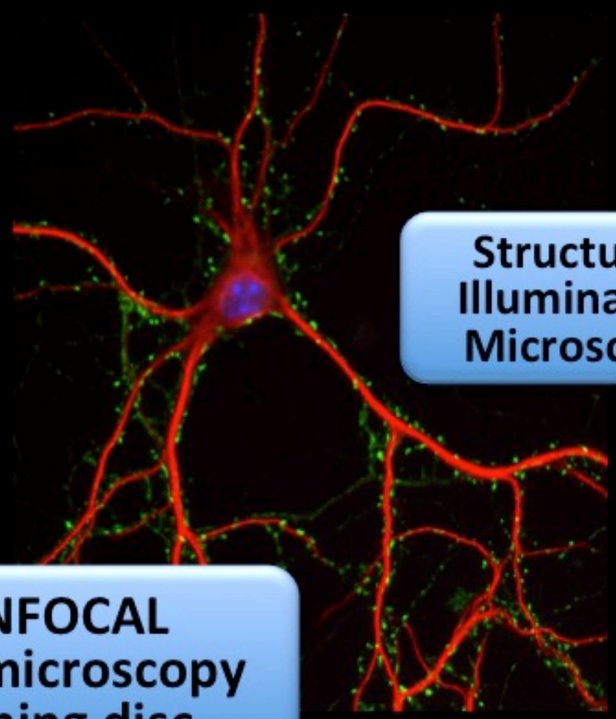


Human brain

2 Photons Light Sheet



Tissue : brain slices



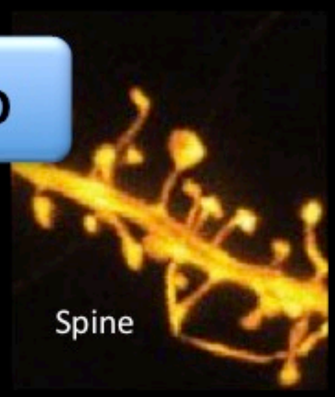
Neurons

CONFOCAL Videomicroscopy Spinning disc

Structured Illumination Microscopy

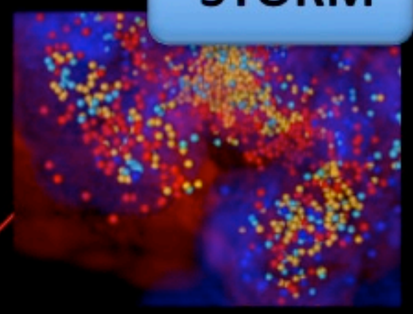
Synapse

STED

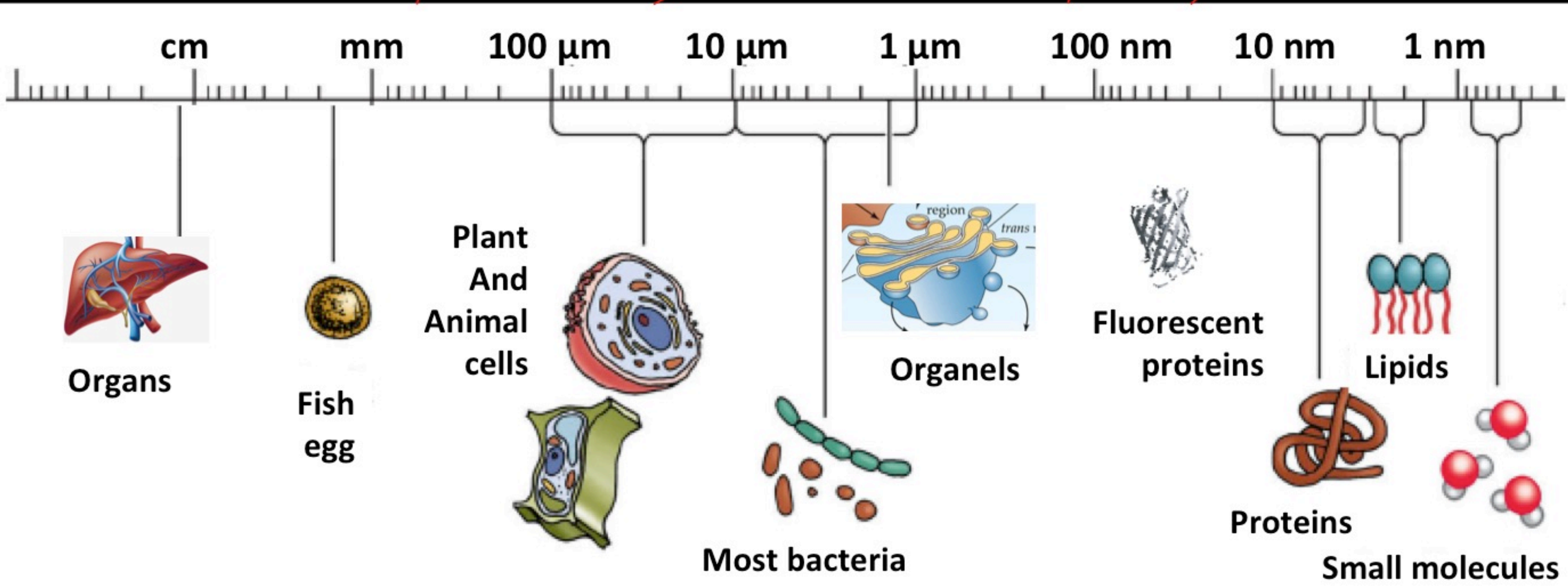


Spine

STORM



Synaptic molecules



Fluorescence Microscope

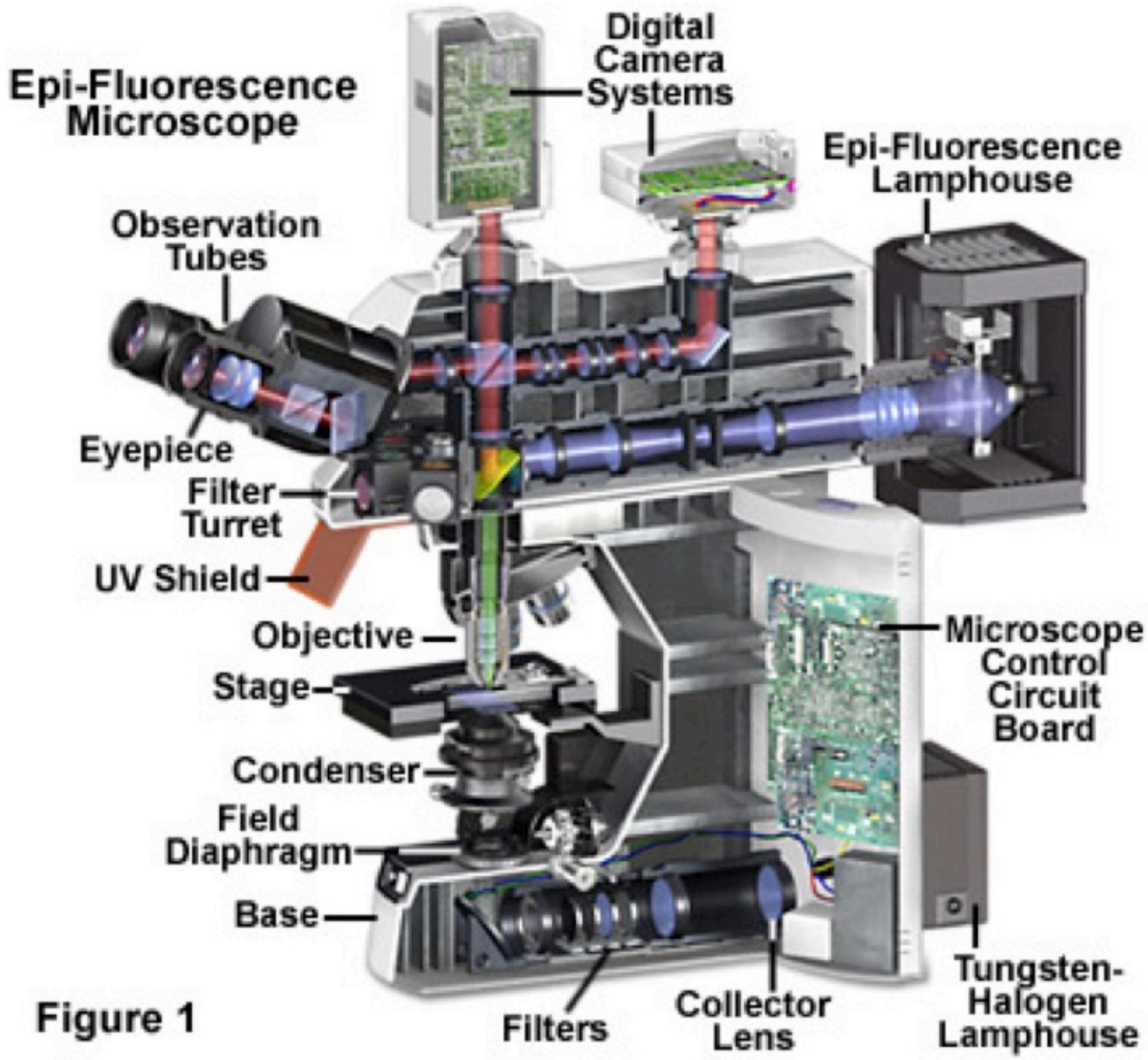
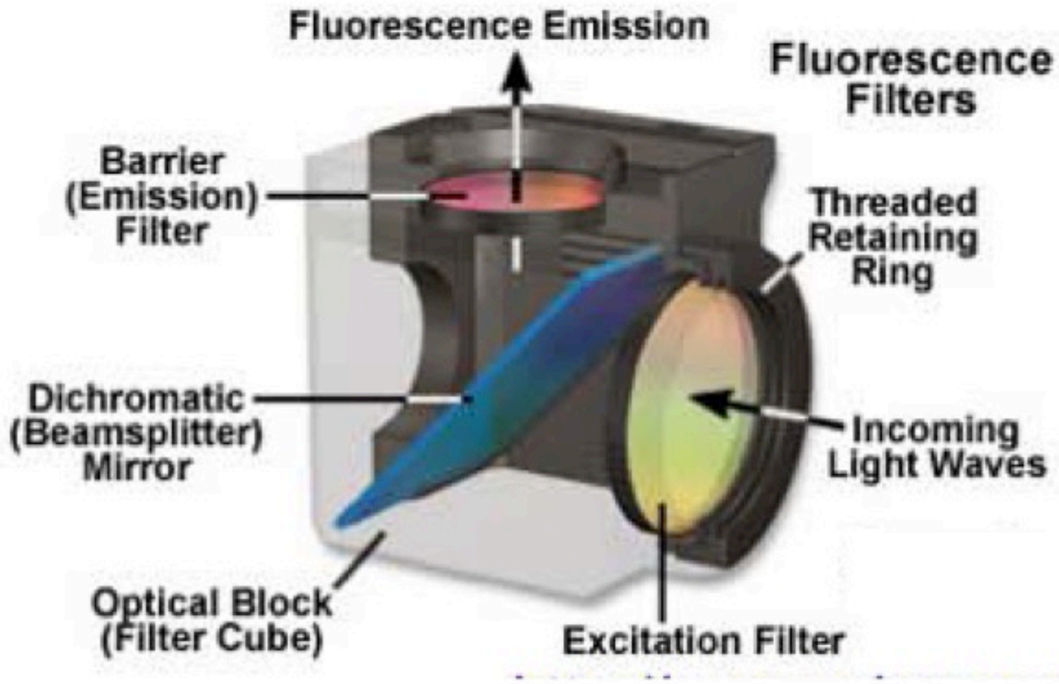


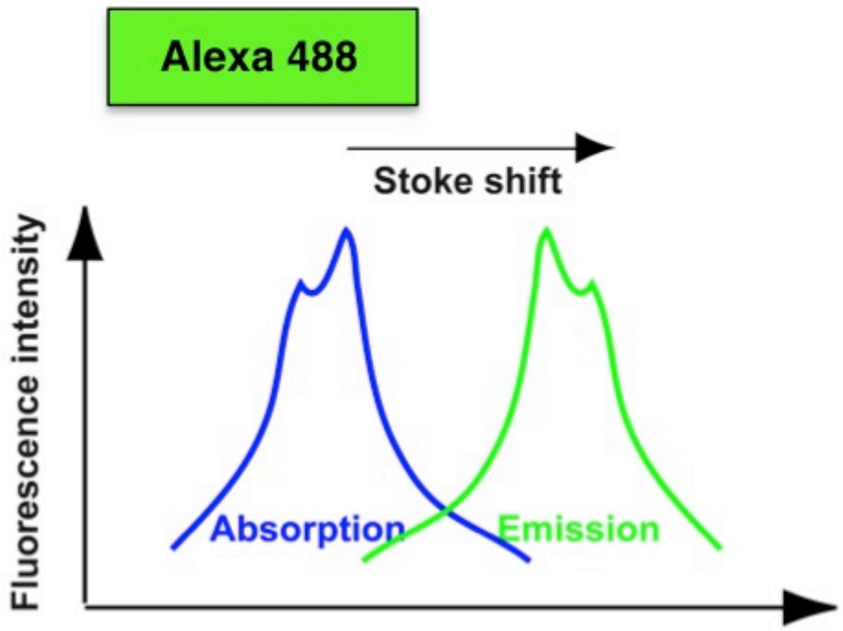
Figure 1

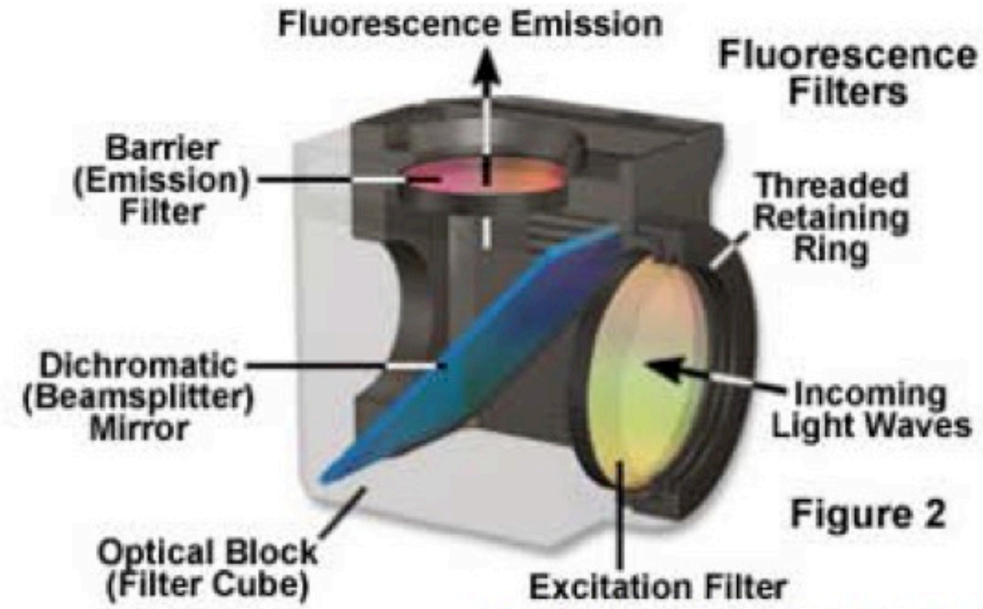
Dichroïc filter

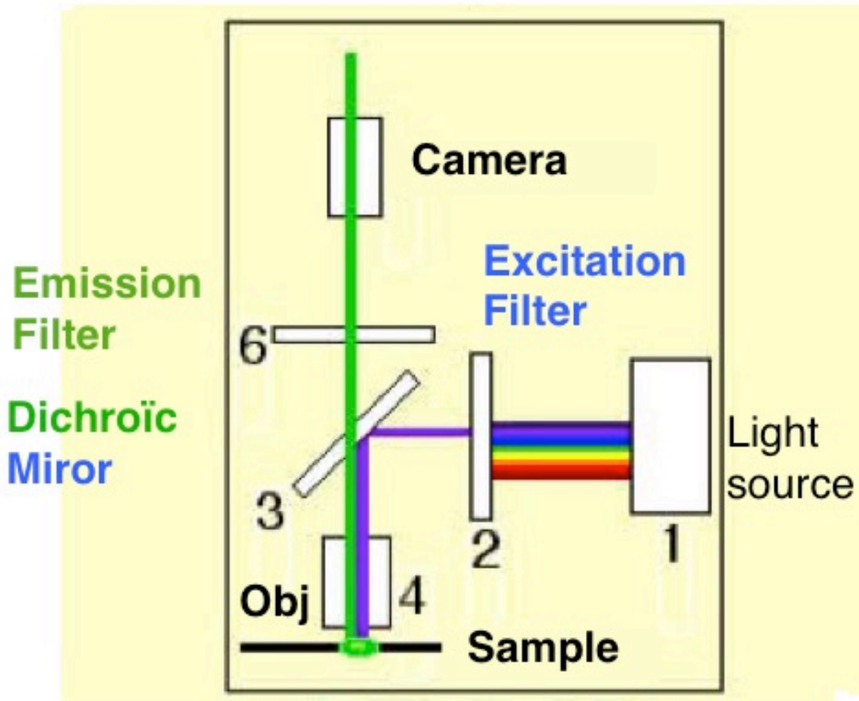


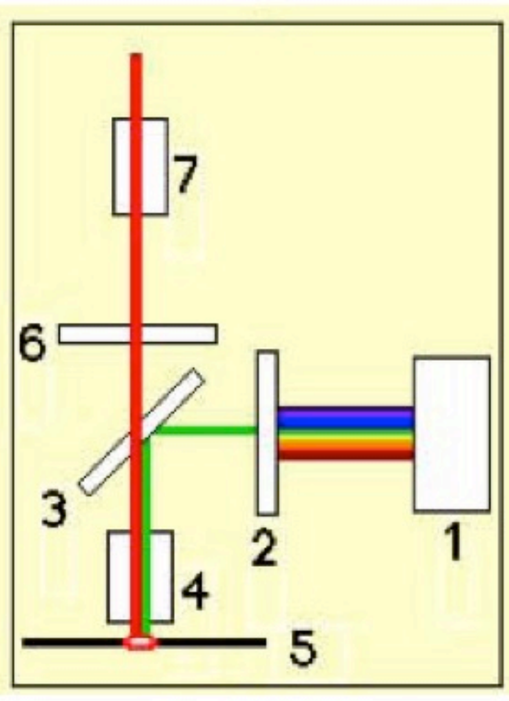
http://www.microscopyu.com

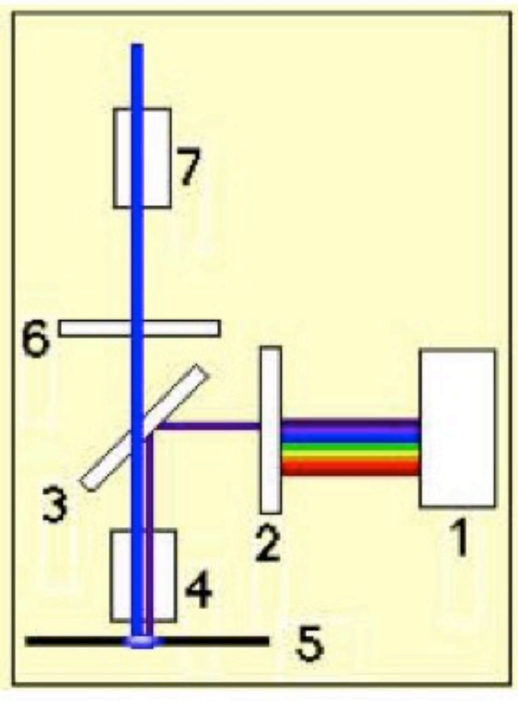
Dichroïc Filter anatomy





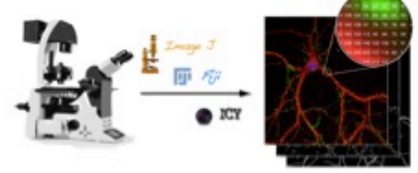






Fluorescence Microscope

https://toutestquantique.fr/fluorescent-et-confocal/

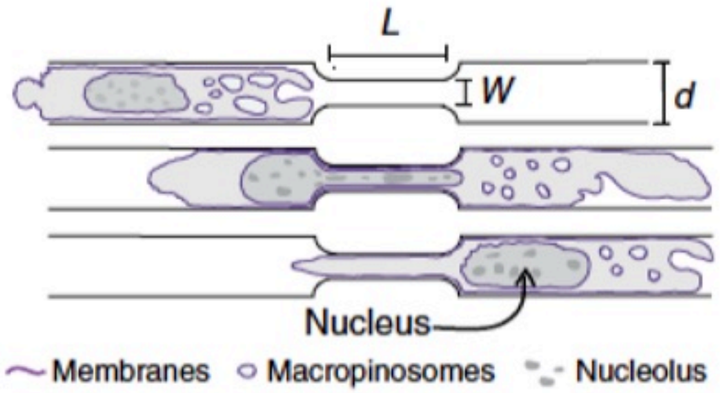


Phase & Fluorescence Microscopy

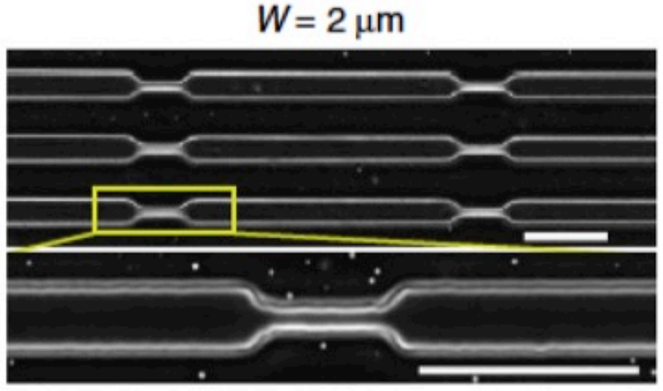
Phase & Fluorescence Microscopy

Hawa-Racine Thiam, Pablo Vargas, Nicolas Carpi, Carolina Lage Crespo, Matthew Raab, Emmanuel Terriac, Megan C King, Jordan Jacobelli, Arthur S Alberts, Theresia Stradal, Ana-Maria Lennon-Dumenil, Matthieu Piel (2016 Mar 16) **Perinuclear Arp2/3-driven actin polymerization enables nuclear deformation to facilitate cell migration through complex environments.** *Nature communications* : 10997 : DOI : 10.1038/ncomms10997

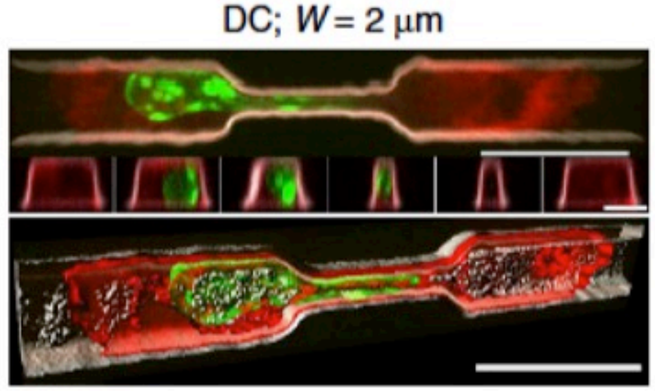
Migratio trough constrictions

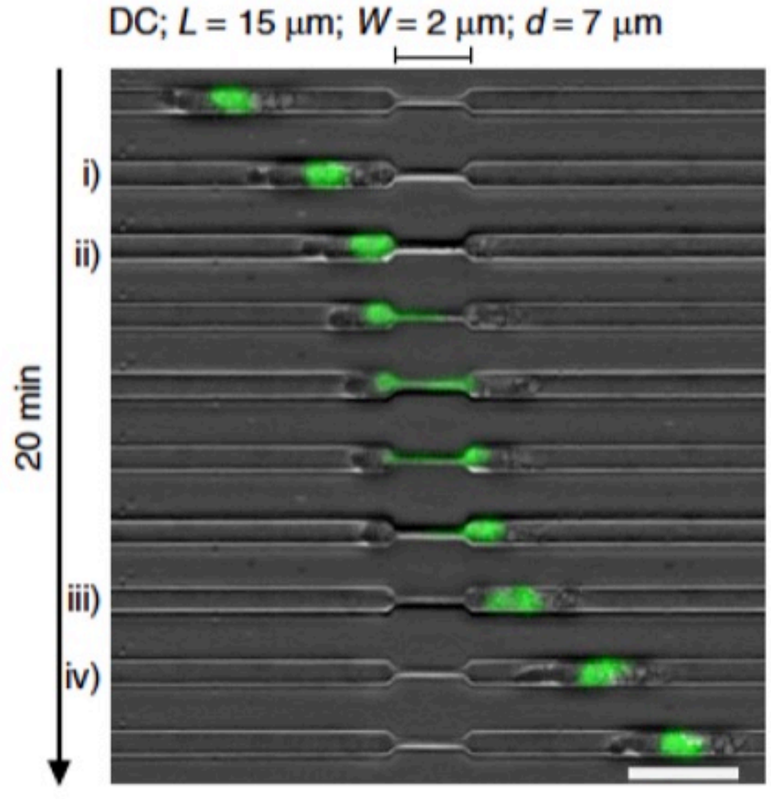


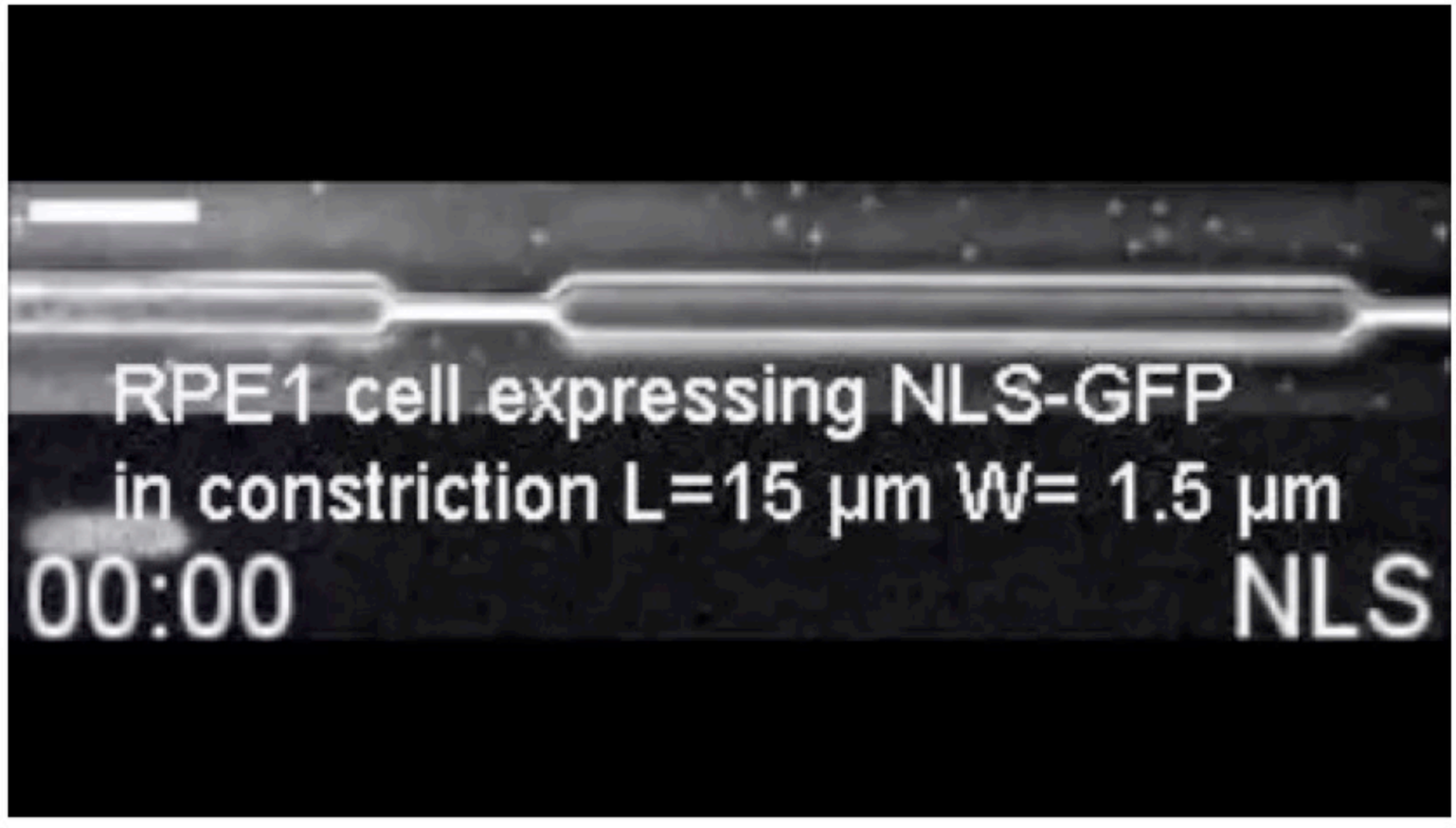
Phase contrast



Epifluorescence



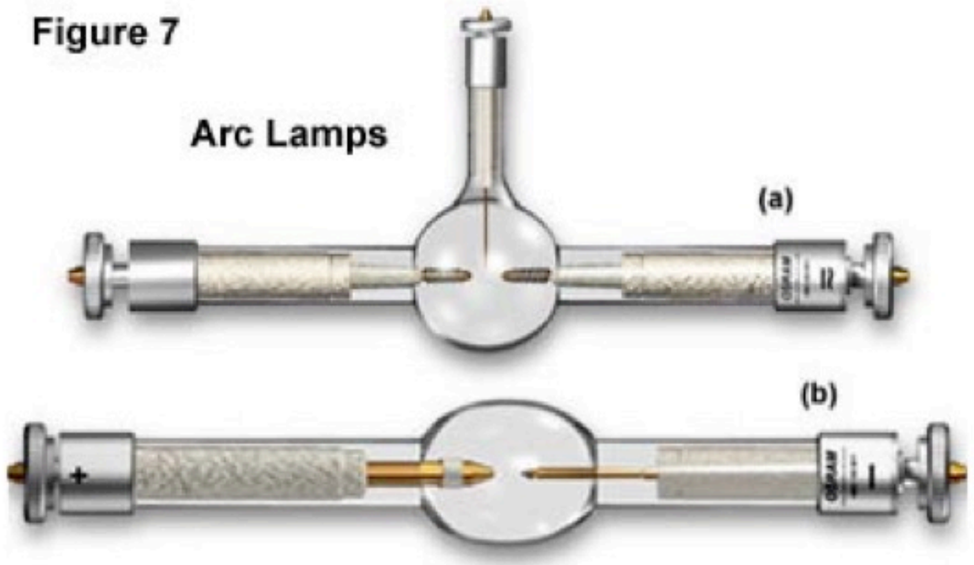




Fluorescent Light source

Figure 7

Arc Lamps



Mercury Arc Lamp UV and Visible Emission Spectrum

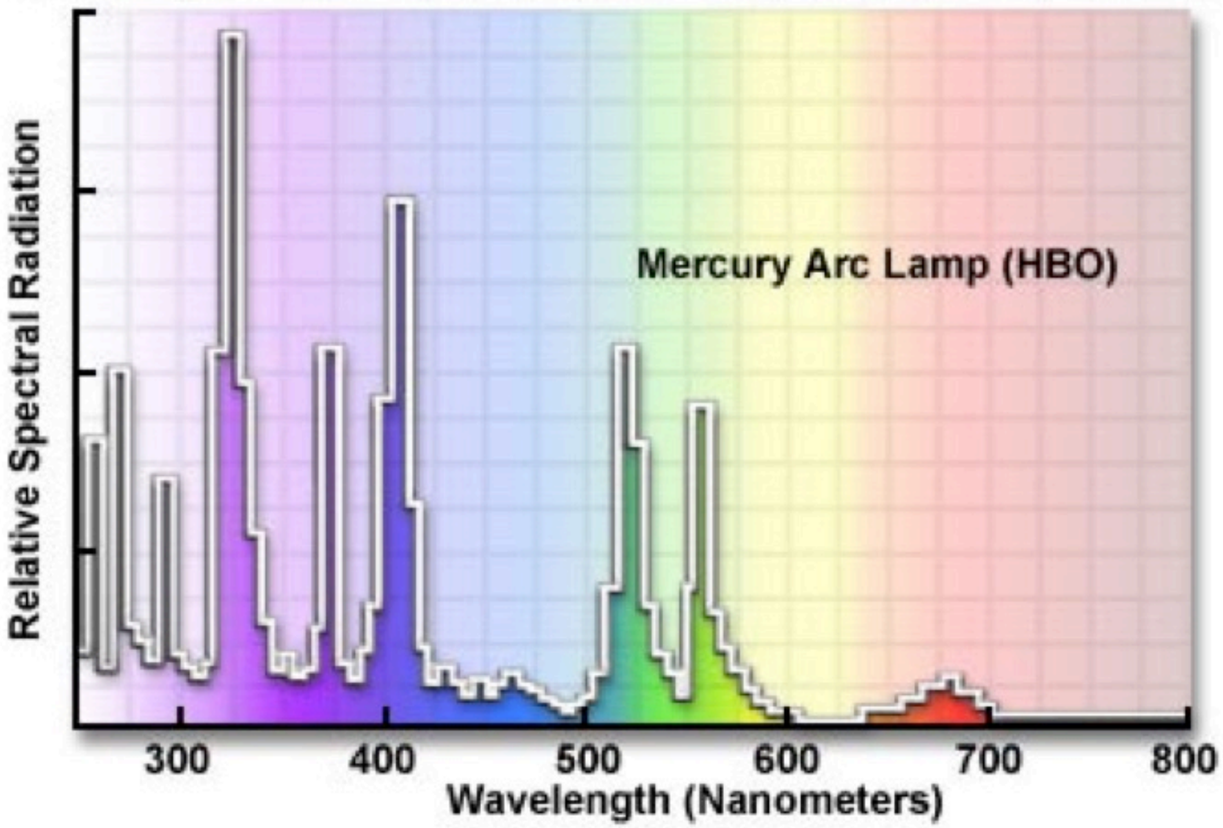


Figure 8

Laser Illumination Source Emission Spectra

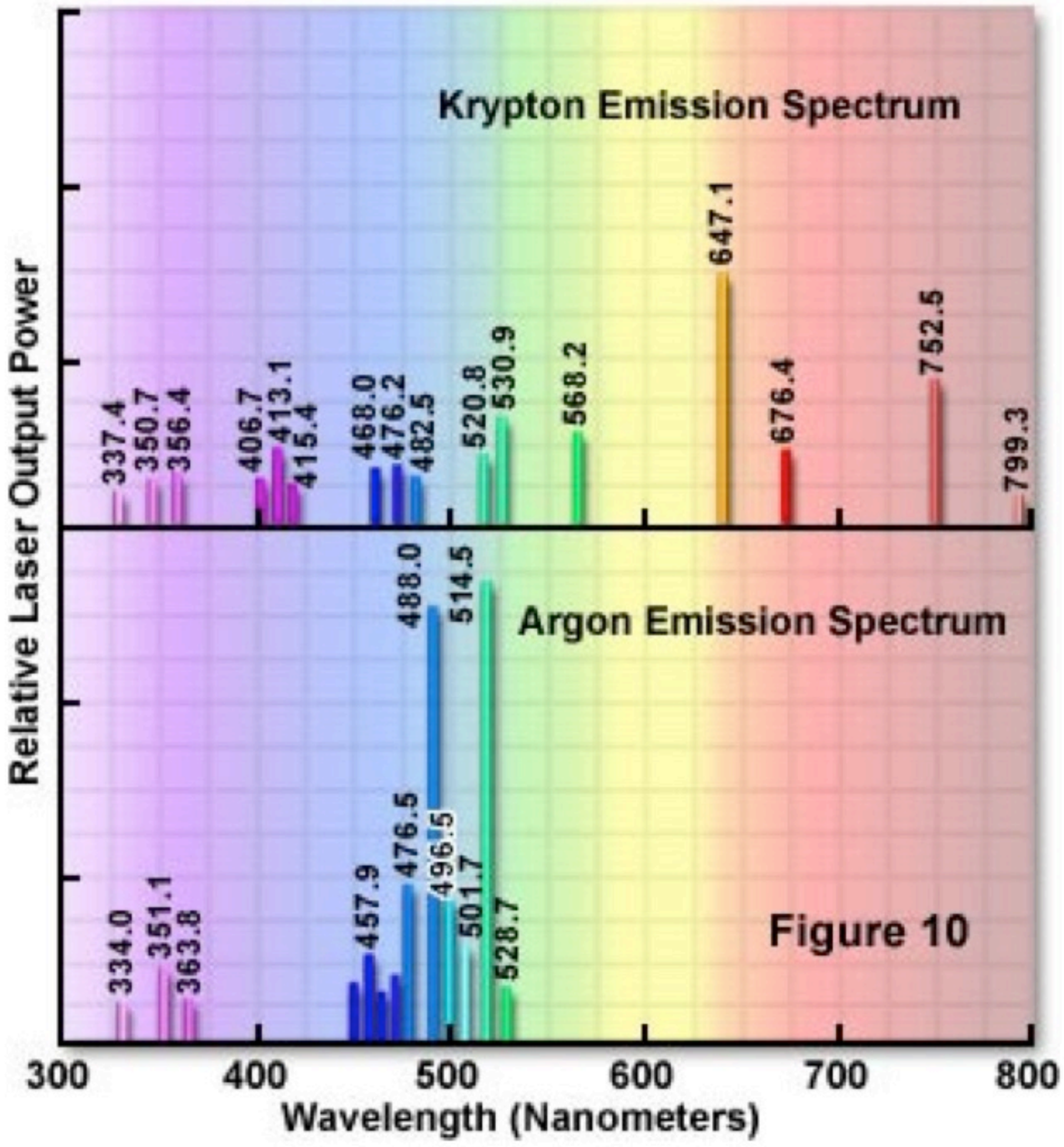


Figure 10

https://www.olympus-lifescience.com/en/microscope-resource/primer/anatomy/illumination/

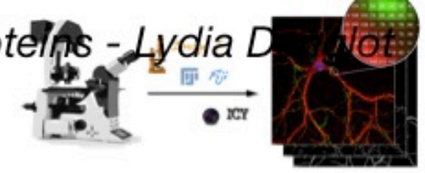
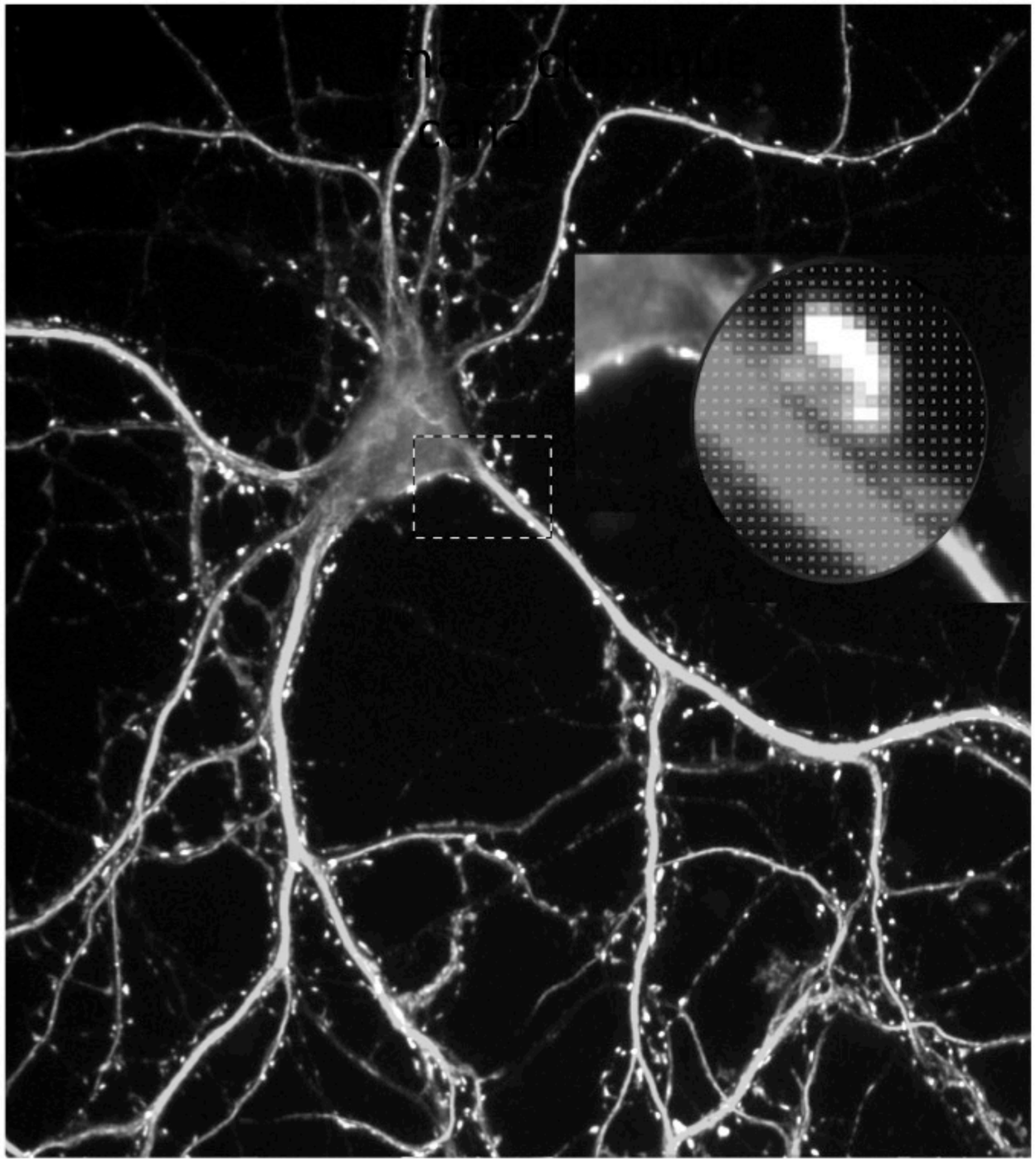
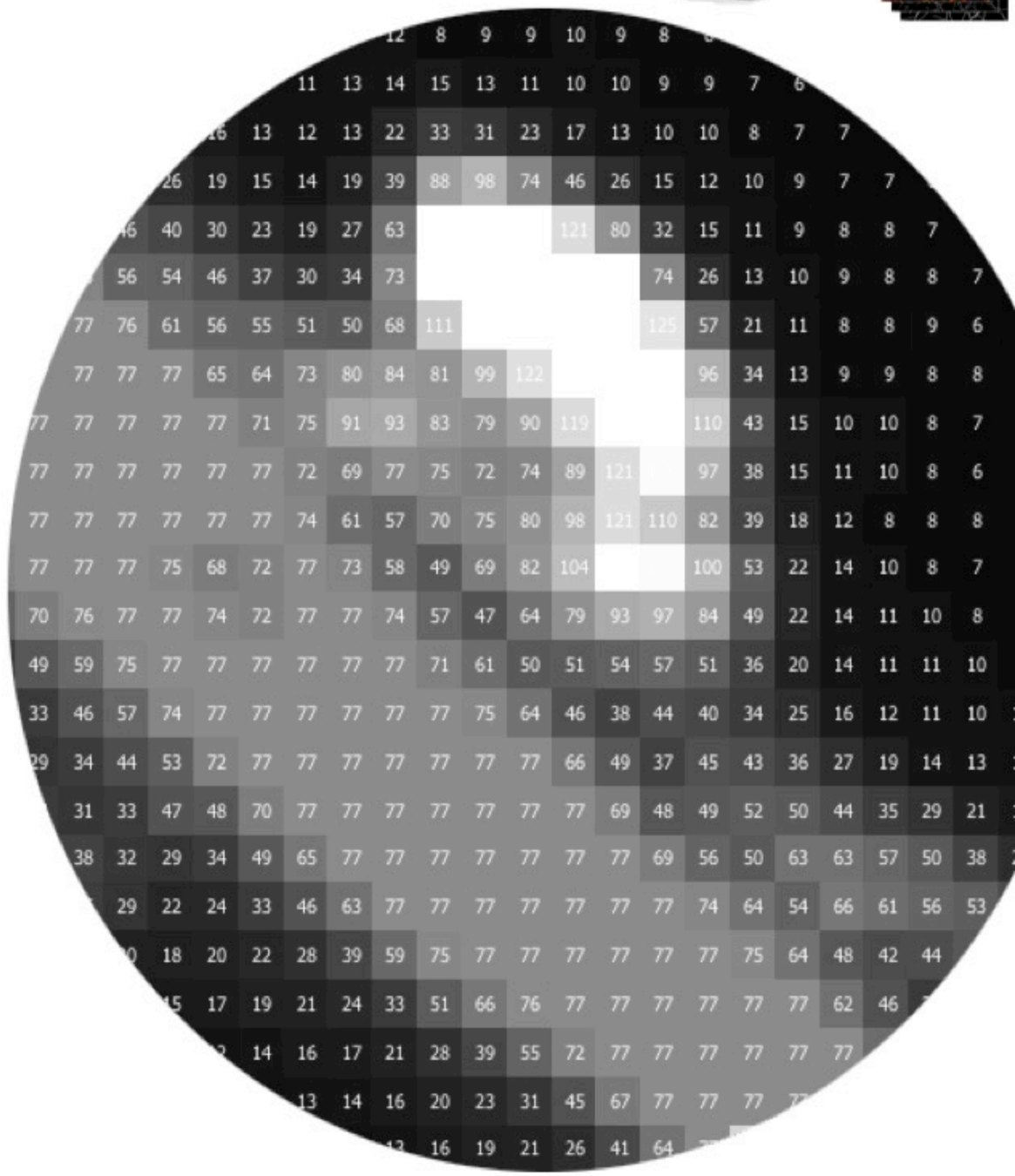


Image anatomy

matrix, histogram, bit depth and RGB colors

Pictures are composed of pixels



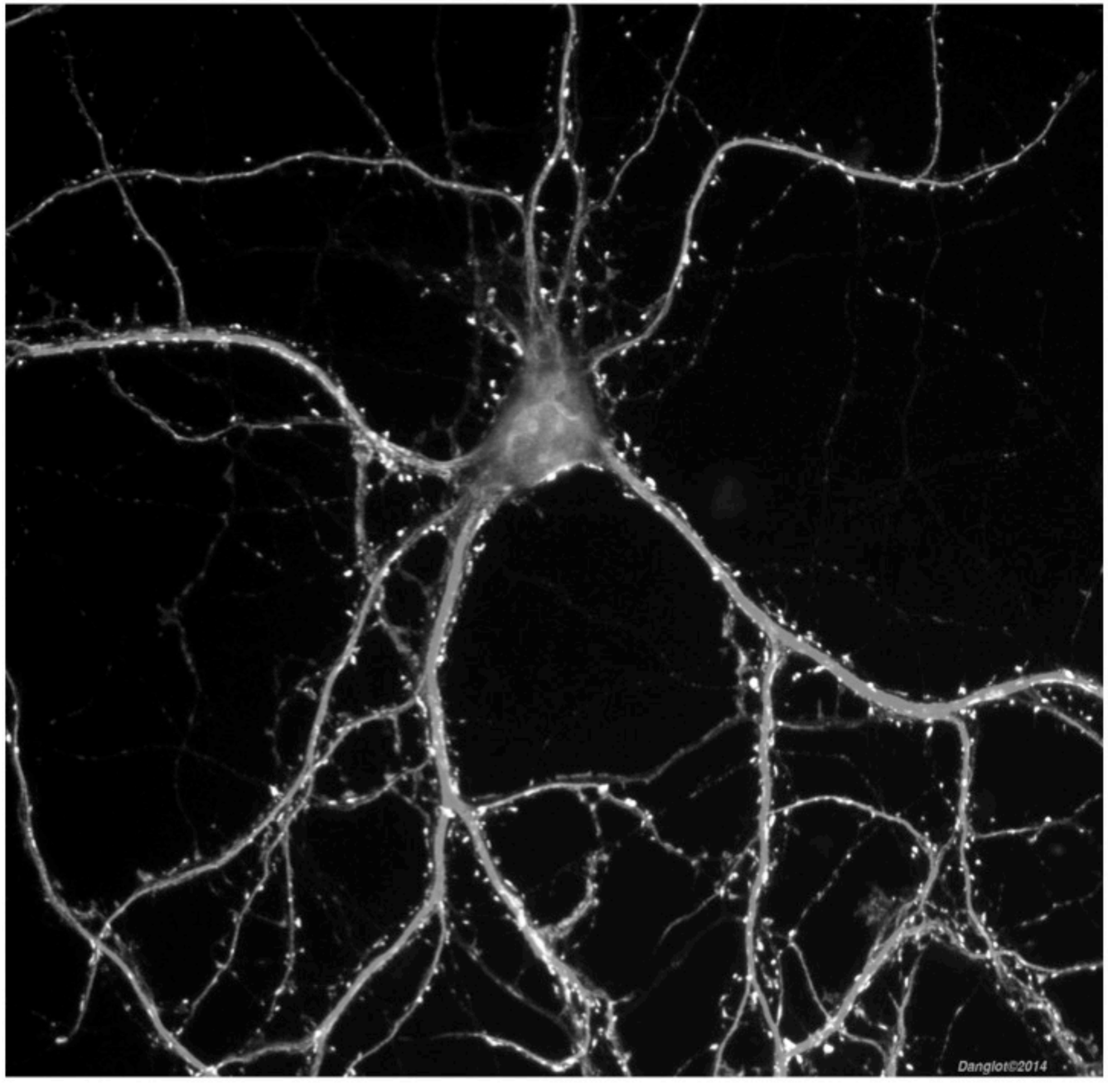


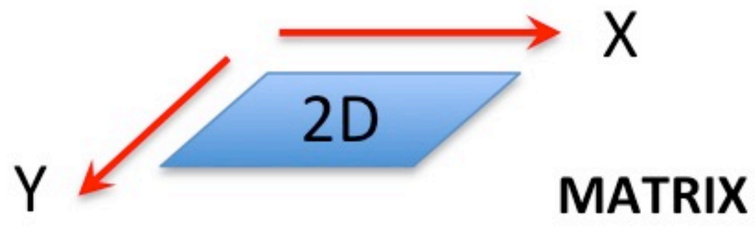
Each pixel has an intensity
Bright : 255
Black : 0

Pictures are composed of pixels

Number of pixels in X axis

Number of pixels in Y axis

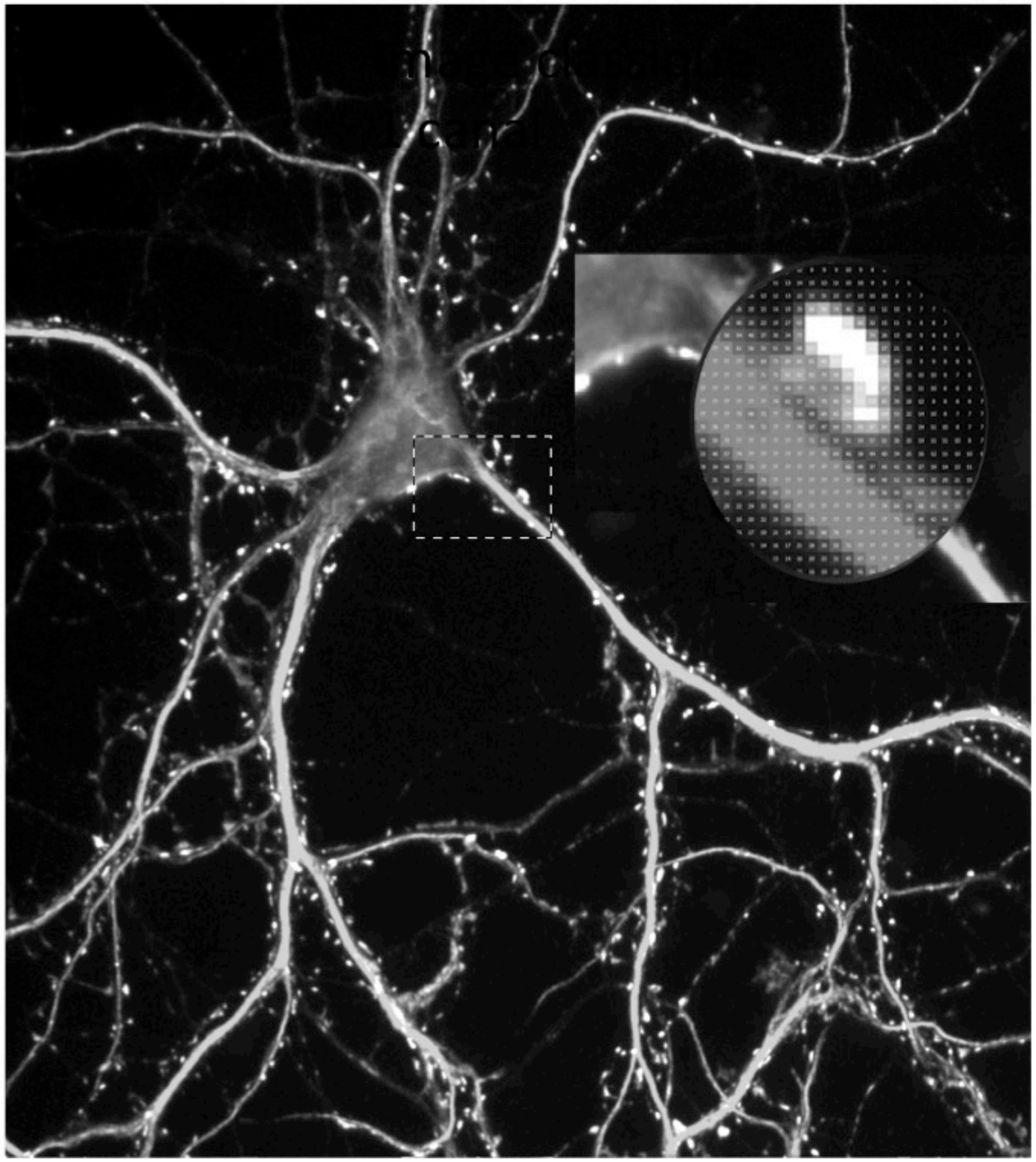


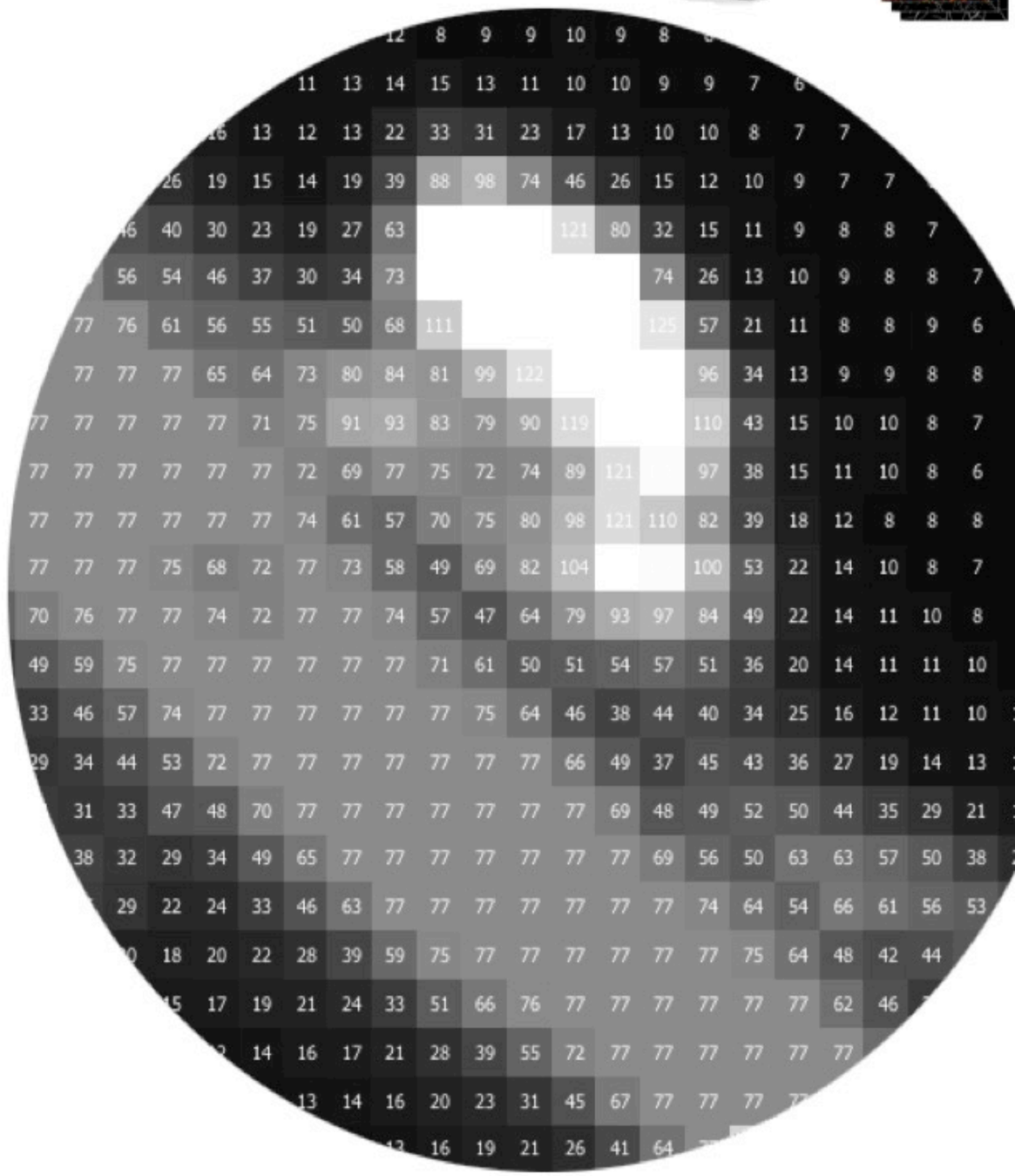


Picture size (matrix) is corresponding to the number of pixels in X and Y

ex: 1024 pixels x 1024 pixels
Pictures of 1 048 656 pixels
Ie 1,048 Mega pixels

Pictures are composed of pixels



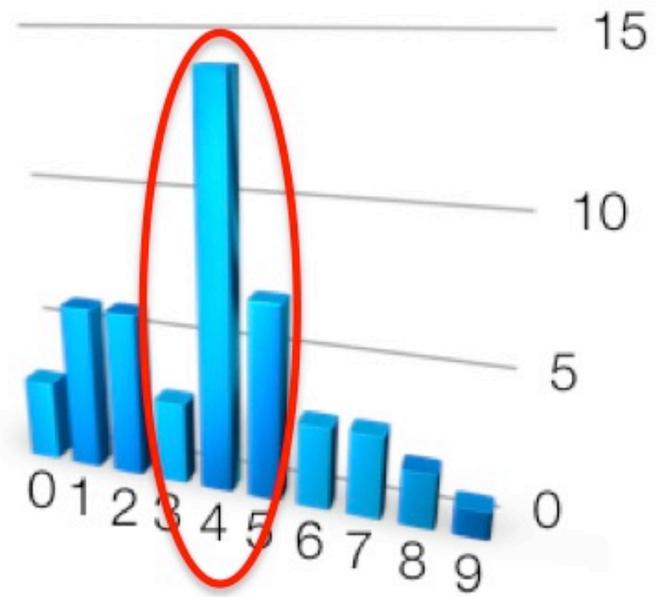


Numbers corespond to fluorescence intensity

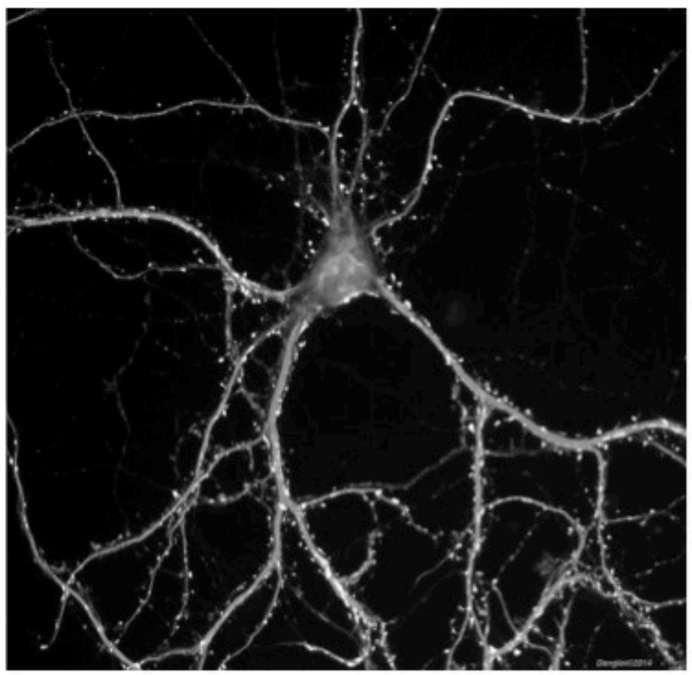
- Histogram : how to visualize intensity dynamics

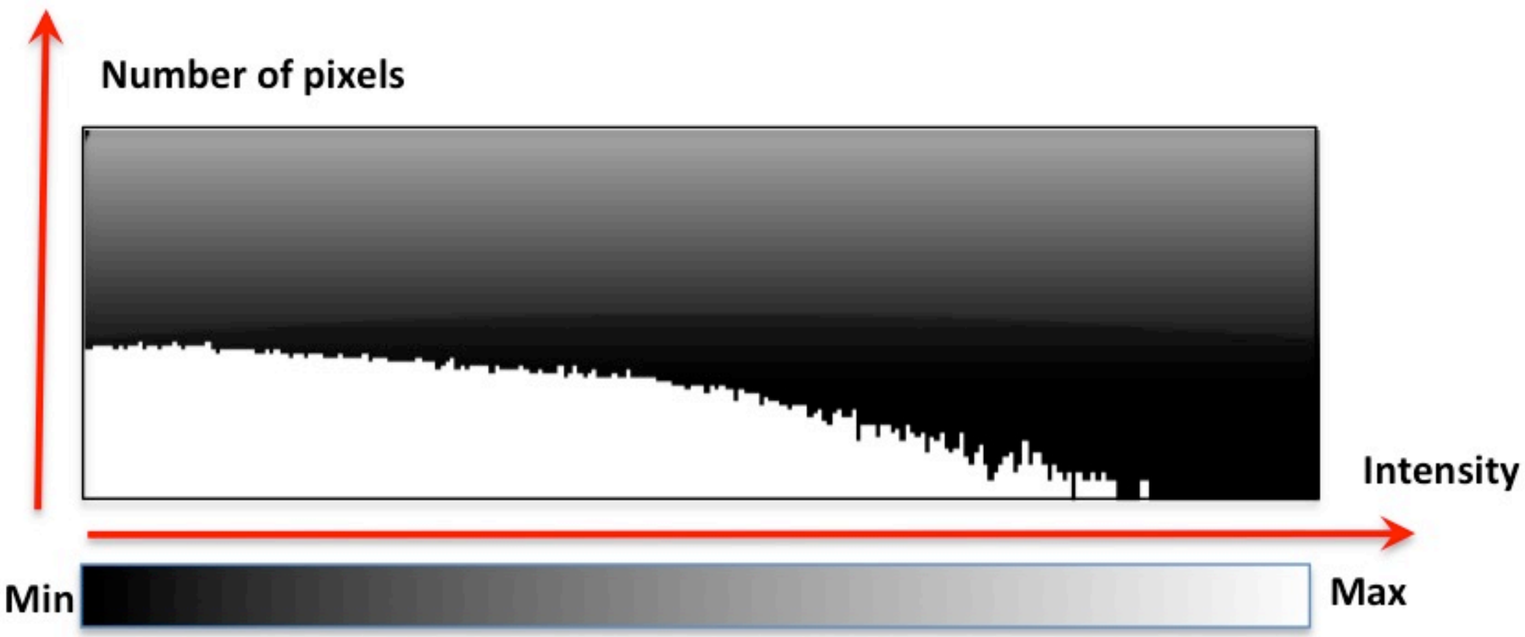
1	4	1	0	1	0	1
3	5	4	4	4	3	4
4	5	4	4	4	4	5
5	5	2	2	2	4	6
4	2	1	0	1	6	7
4	2	2	3	4	7	8
5	4	5	6	7	8	9

Intensity	# of pixels
0	3
1	6
2	6
3	3
4	14
5	7
6	3
7	3
8	2
9	1



14 pixels have a grey value of 4.

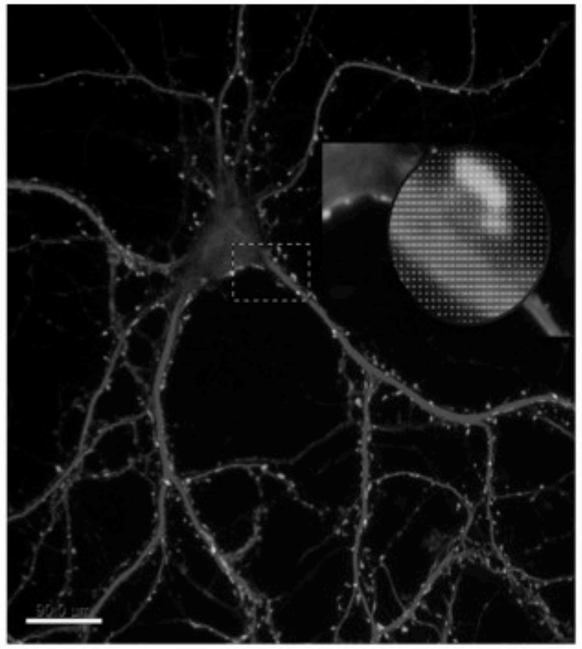


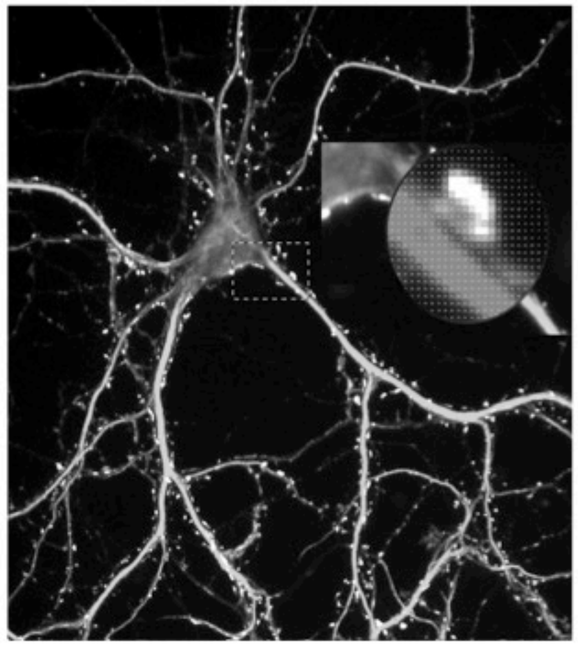


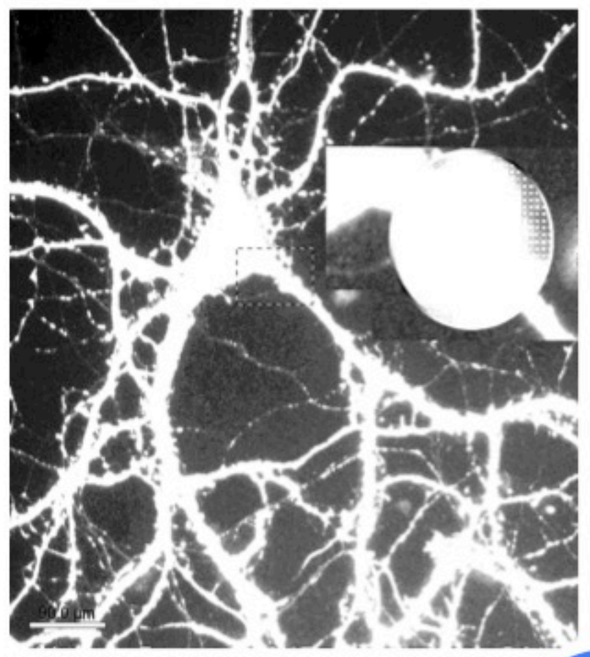
Histogram displays the intensity distributionof black, grey and white pixels.

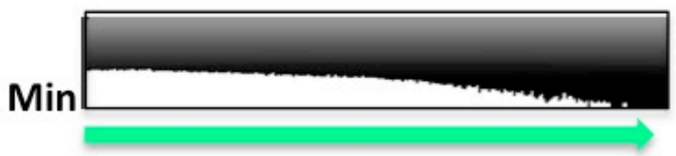
Dynamic must be adjusted:

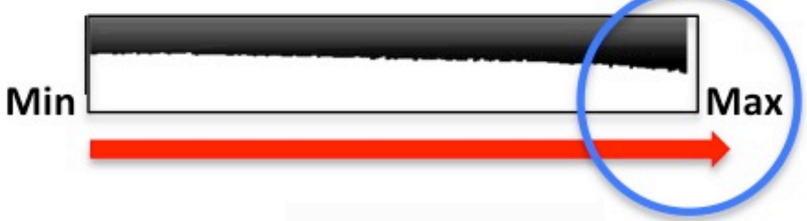
- By increasing exposure time
- By increasing light power











Under exposed

Only hald of the grey level dynamics is used : loss of sensitivity.

Good Dynamic

Expose enough time so the maximum intensity is just below saturation.

Over exposed

Saturated picture: the hight value pixels have all the same value and can not be discriminated anymore. You loose information. PROHIBITED

- ***Black and white dynamics :***

8–bit 16–bit 32–bit ?

Bit: fondamental unit of storage of your computer

Binary : 0 or 1.

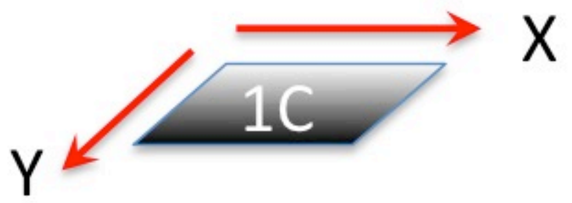


Image 1 bits: can store $2^1 = 2$ grey levels (black or white)

Image 2 bits: can store $2^2 = 4$ grey levels

Image 3 bits: can store $2^3 = 8$ grey levels

Image 4 bits: can store $2^4 = 16$ grey levels

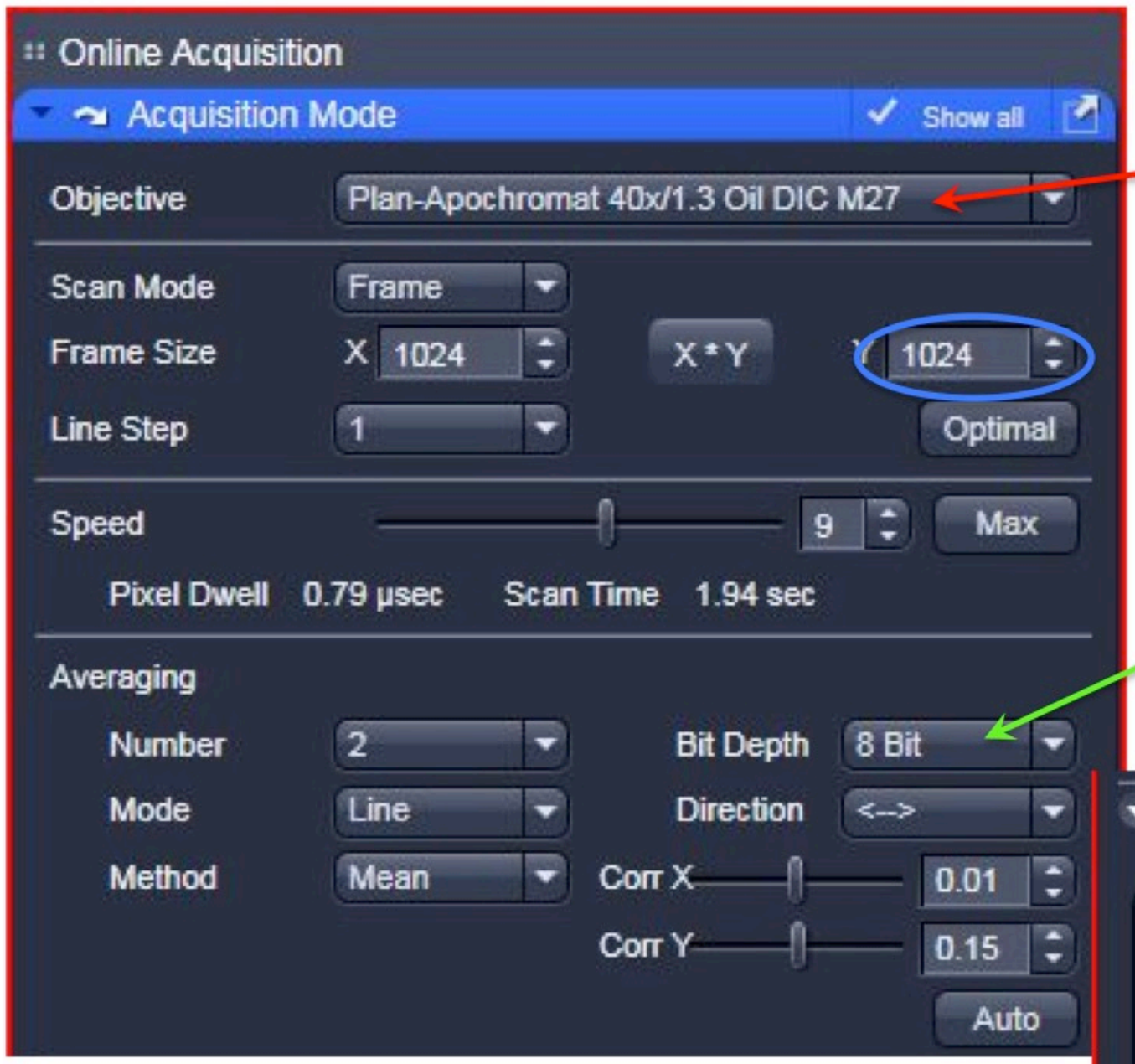
Conventional used dynamics :

8 bits pictures : can store	2^8 = 256	grey levels	from 0 to 255
12 bits pictures : can store	2^{12} = 4096	grey levels	from 0 to 4 095
16 bits pictures : can store	2^{16} = 65 536	grey levels	from 0 to 65 536

16 bits pictures, can store more grey levels so give access to finest differences.

1. Picture format

- ***Confocal ZEISS (710 ou 780)***

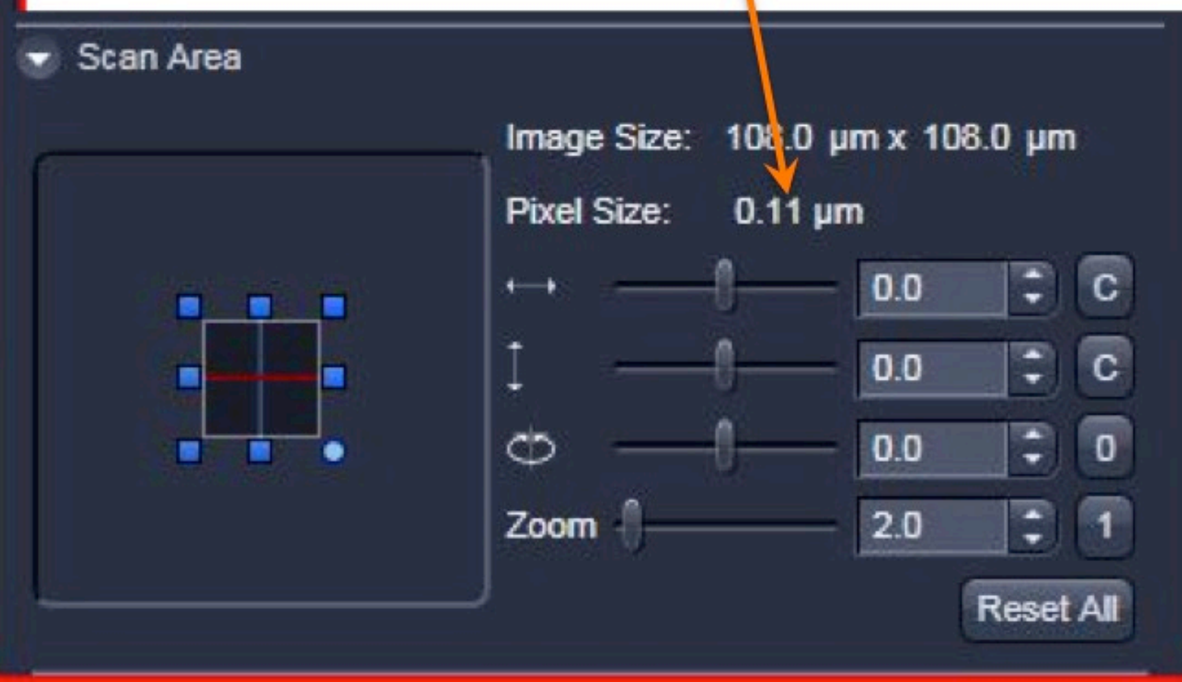


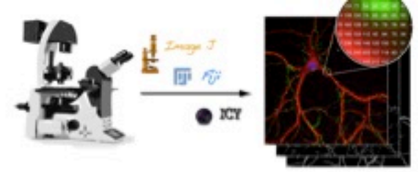
Objective

Matrix size (1024 x 1024 pixels)

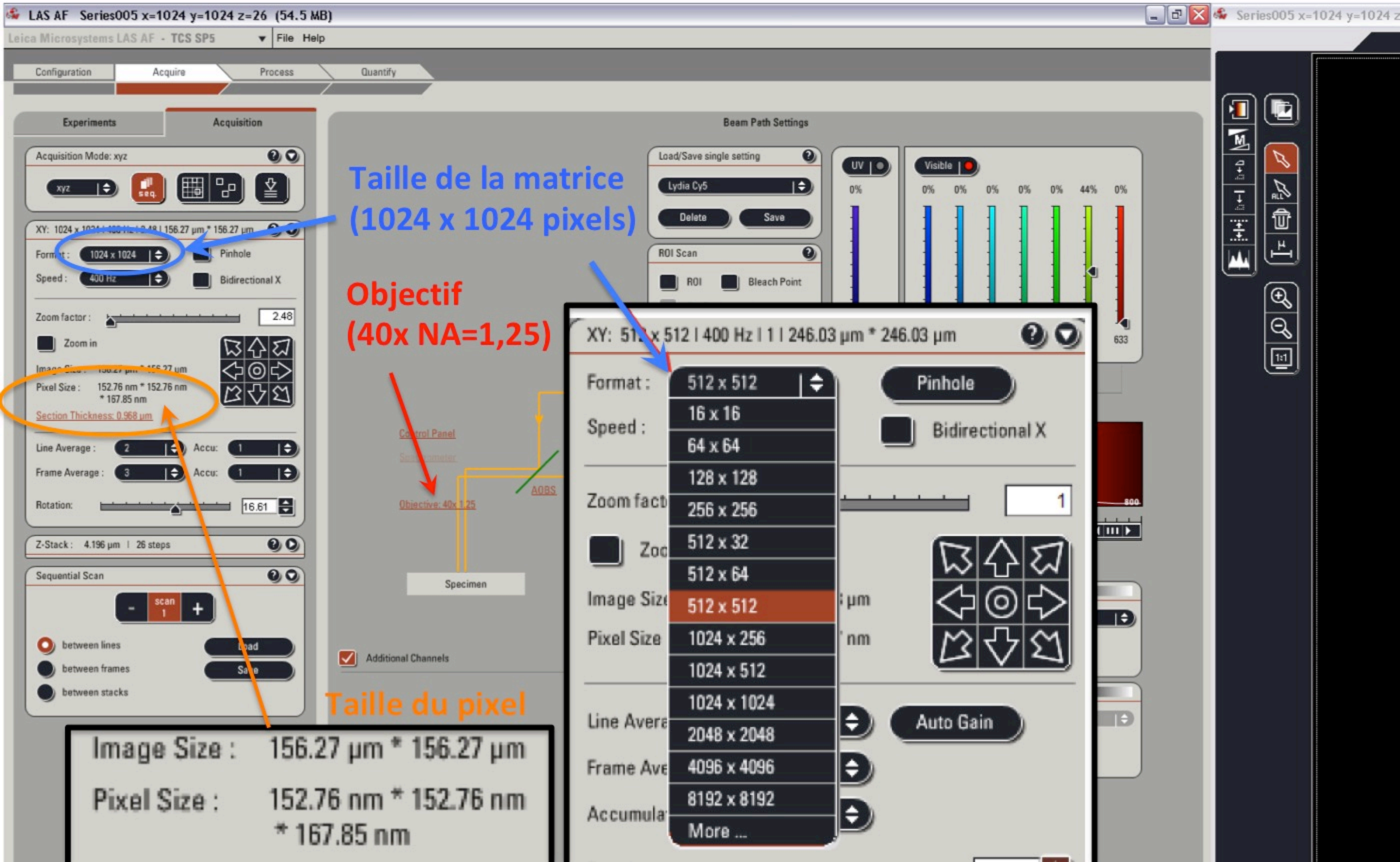
Bit depth (8 bits)

Pixel size

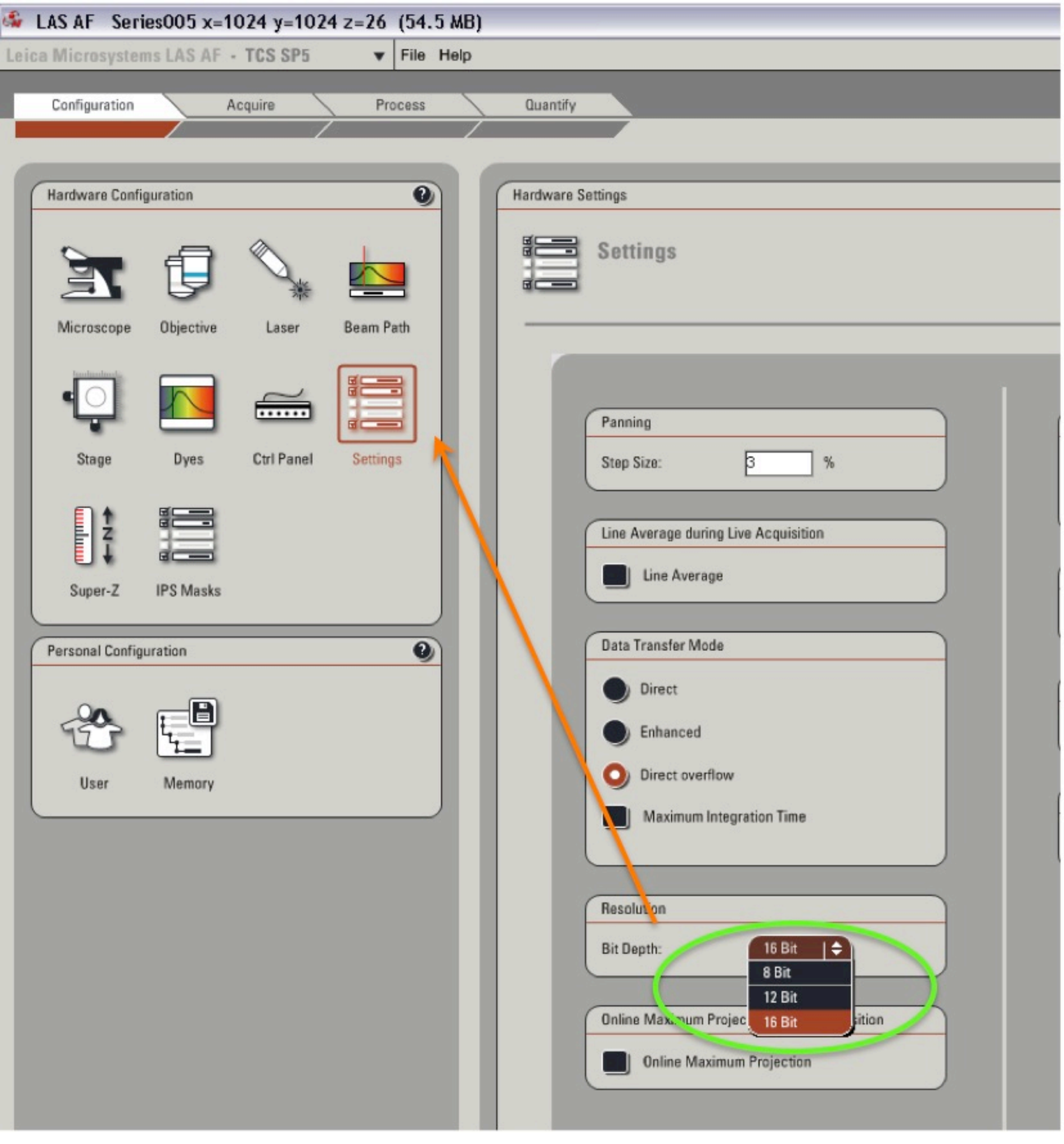




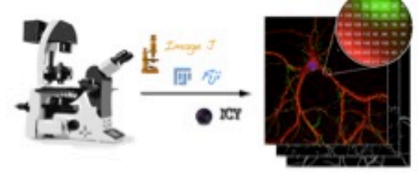
- ***Confocal Leica (SP5))***



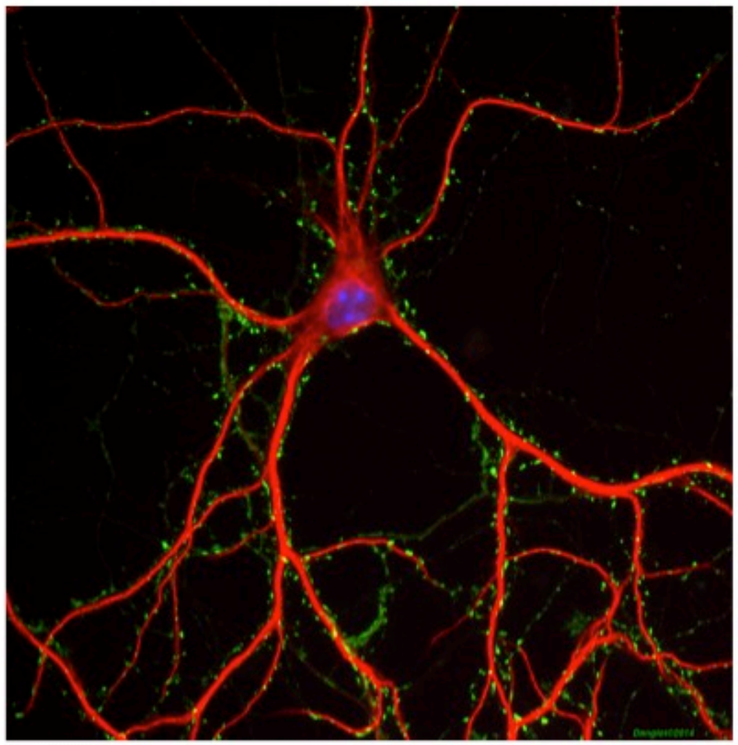
- ***Confocal Leica (SP5)***



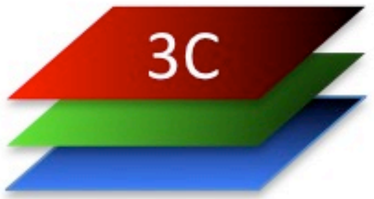
Bit depth (16 bits)



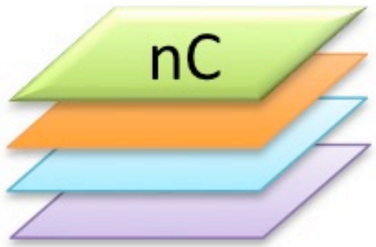
- ***Color image dynamics :***



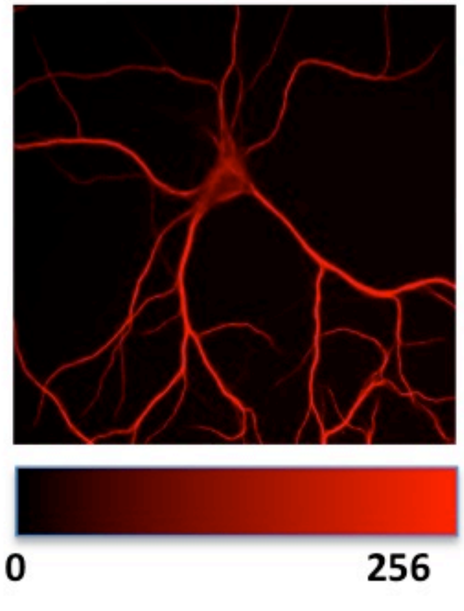
RGB color image :



R G B

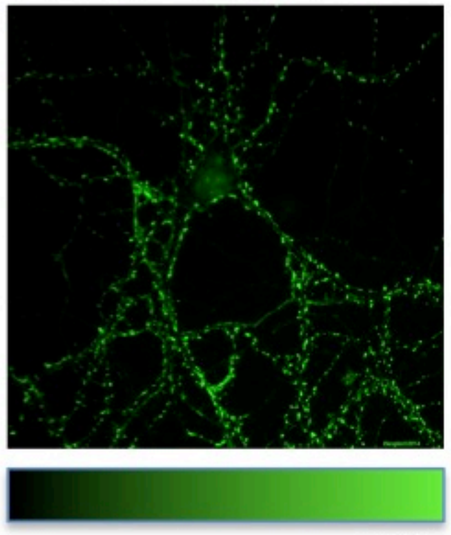


Channel 1 : RED



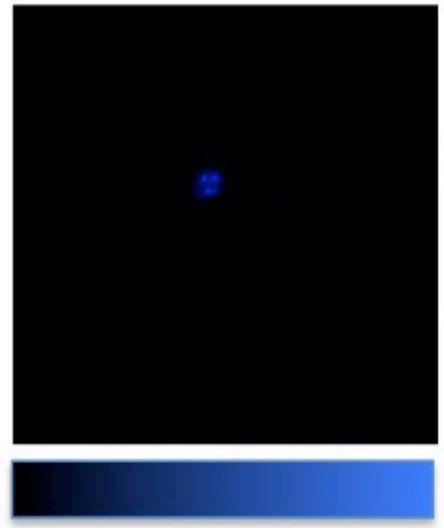
0 256

Channel 2 : GREEN



0 256

Channel 3 : Blue



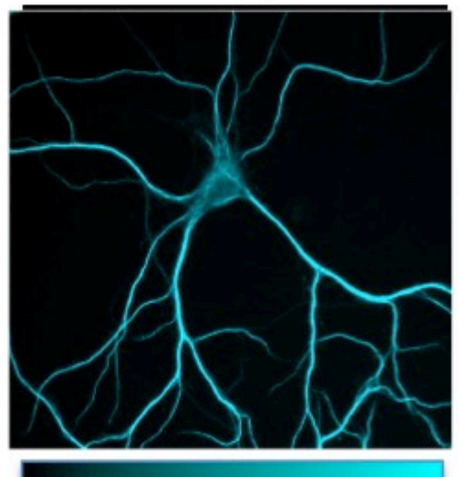
0 256

- 24 bits: 3 chanels :

  Red channel = 256 values
  Green channel = 256 values
  Blue channel = 256 values
  256 x 256 x 256 = 2^{16} = 16 777 216 ie 16 millions colors

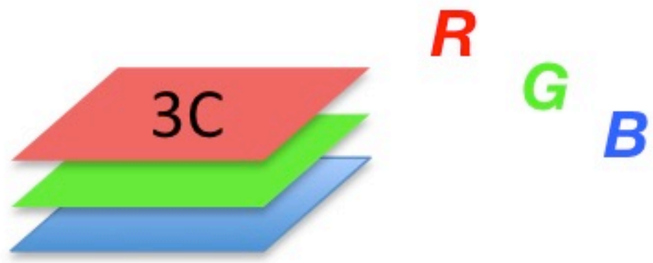
- 32 bits: 3 channels + transparency
  Red channel = 256 values
  Green channel = 256 values
  Blue channel = 256 values
  Alpha channel = 256 valeurs (transparency)

Channel n : cyan



0 65 536

- ***Visulaisation of pictures with RGB colors :***

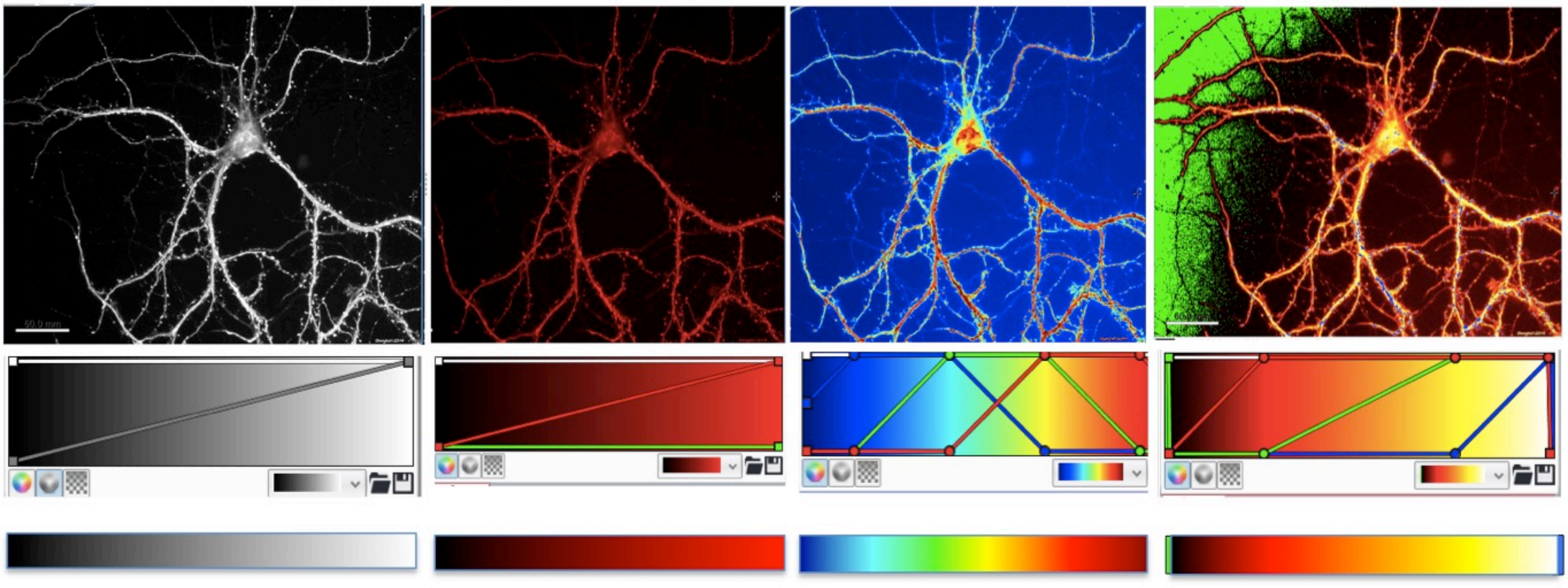


Screens use 3 color channels (Red, Green, Blue, RGB)

Each color is coded in 8 bits (256).

One image is always converted in a display space (16 millions color).

Our eyes are sensitive only to 100 000 tints …

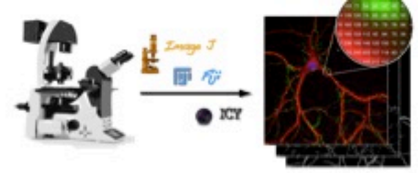


LUT : Black & white

Red gradient

LUT : « JET » :
More sensitive on green

LUT : « GLOW O & U » :
Green: black pixels
Blue: saturating pixels



- ***Les piles d'images ou stack ou multi -tif:***

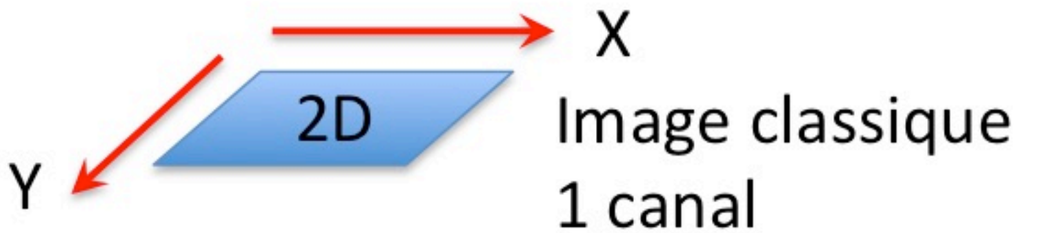
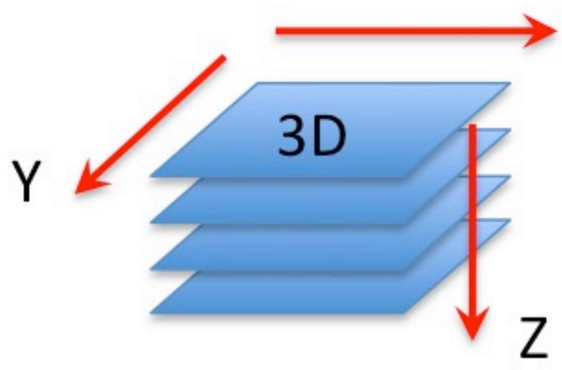
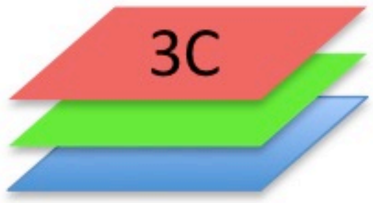


Image classique
1 canal

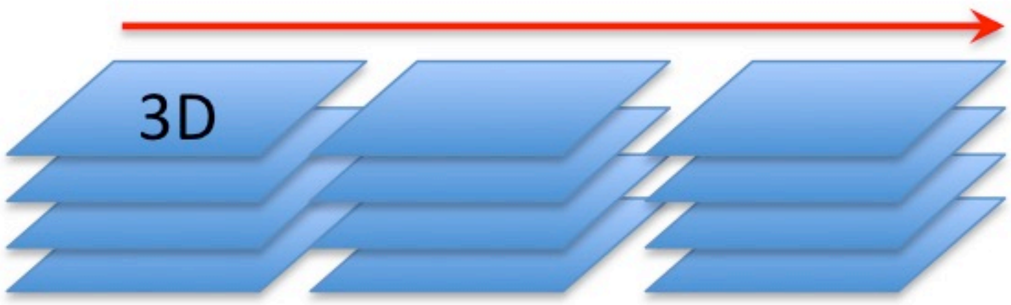


Pile d'image en Z :
stack **3D** au confocal

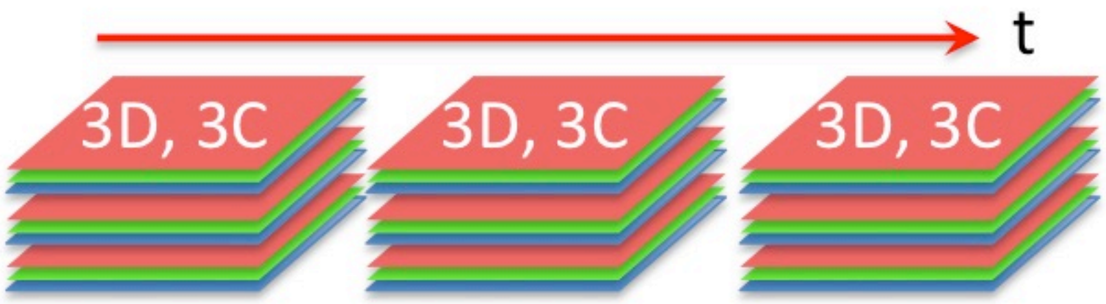


Pile de 3 Canaux RVB :
multi canal

Pile d'image en t:
Succession d'image au cours du temps : **film**

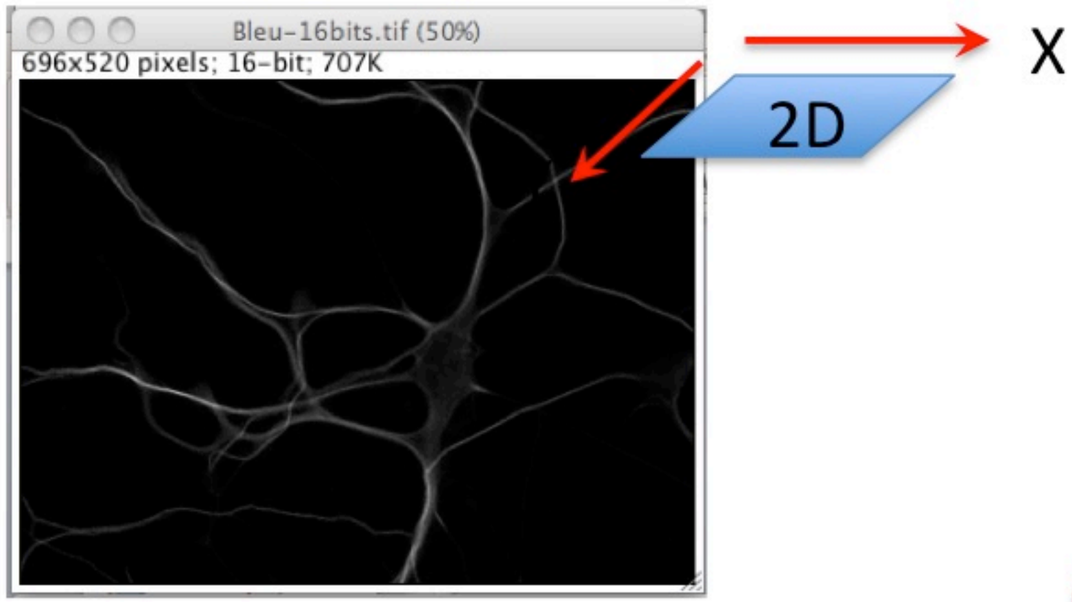


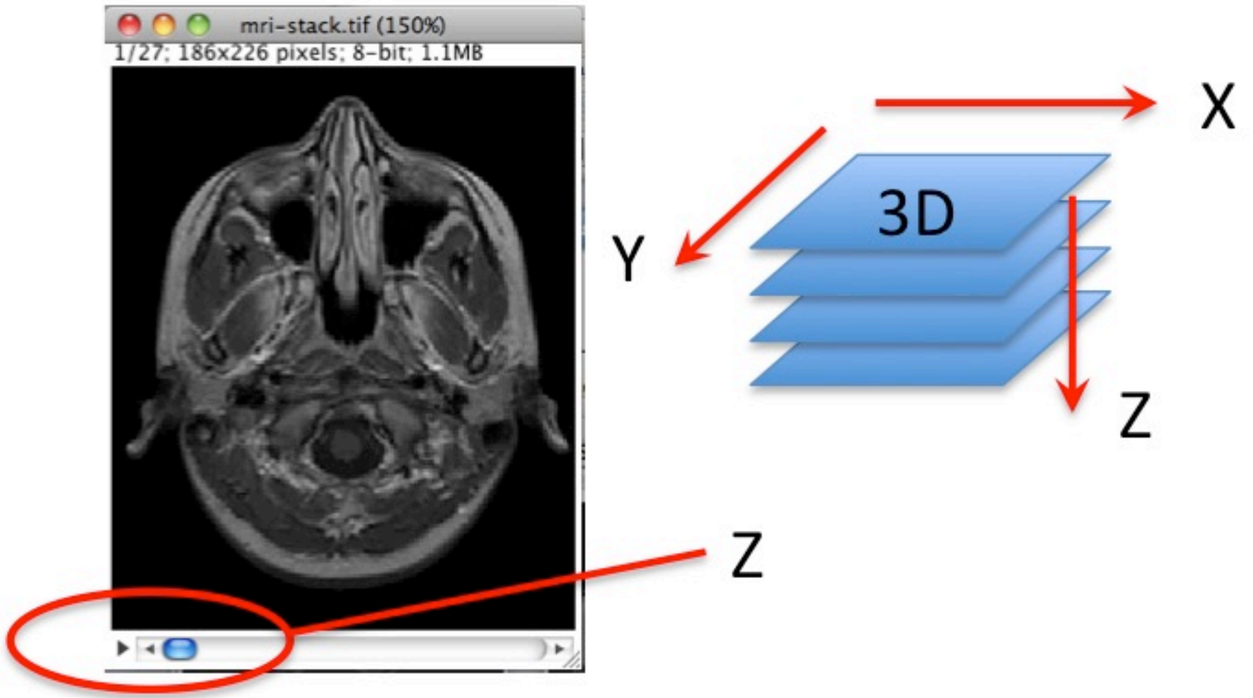
Pile d'image en Z et au cours du temps: **stack 4D**

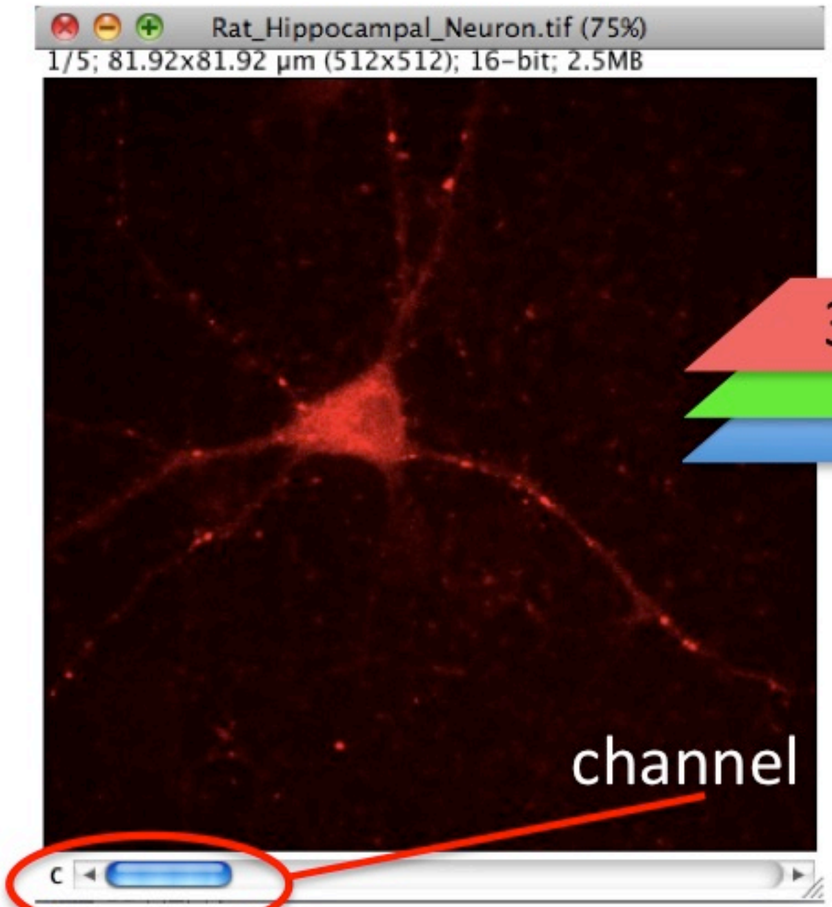


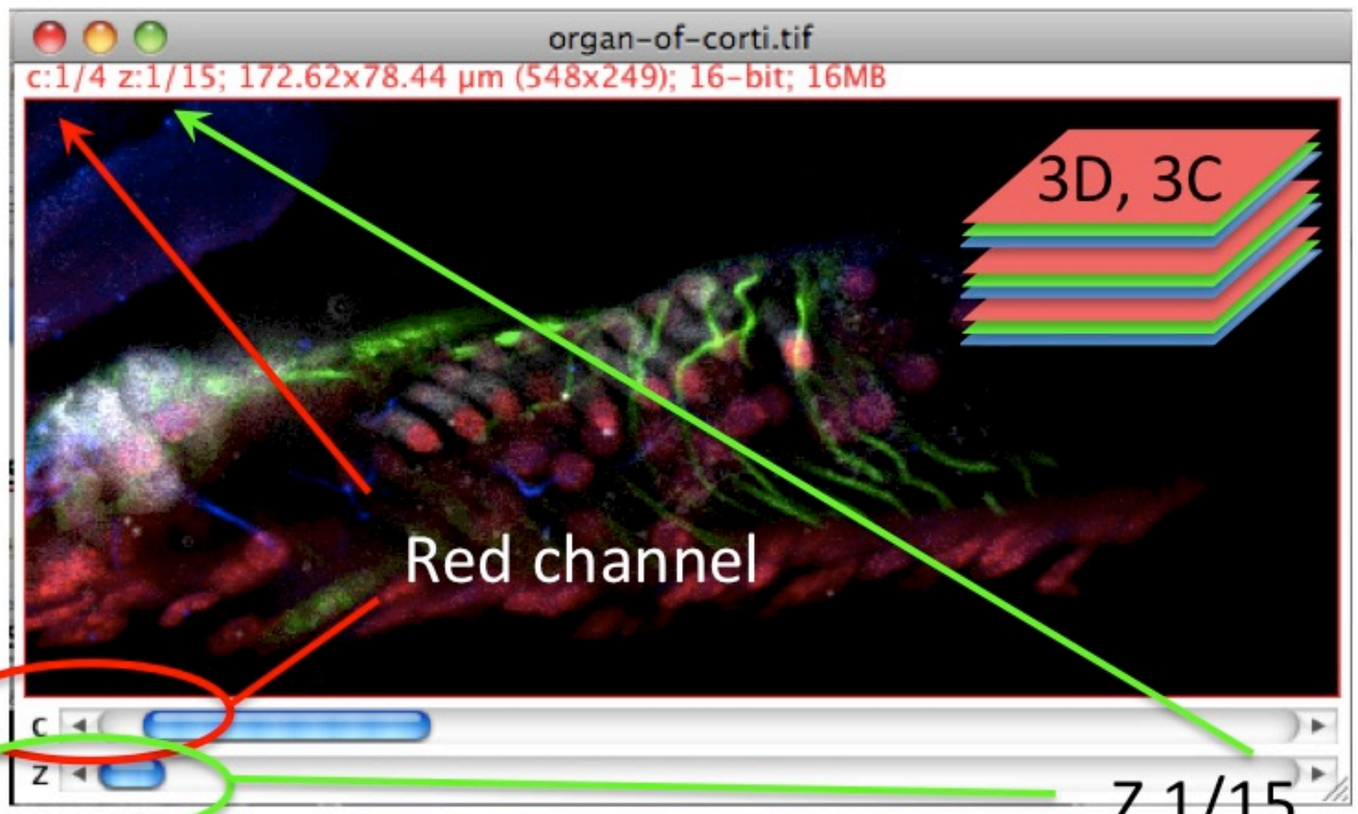
Pile d'image en Z, au cours du temps et multi canal: **stack 5D**

- ***Image stack or multi -tif:***







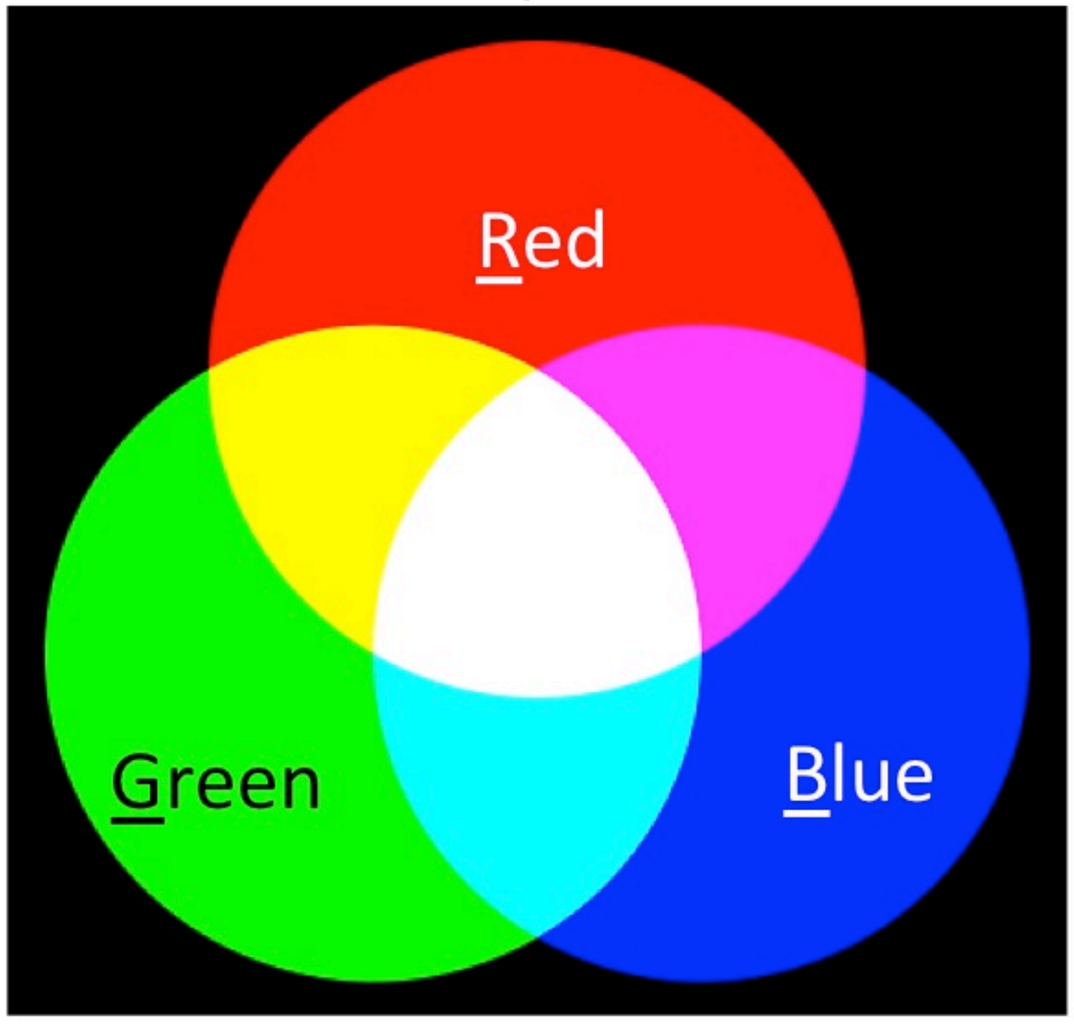


Colorimetric spaces

Additive synthesis

Based on color addition

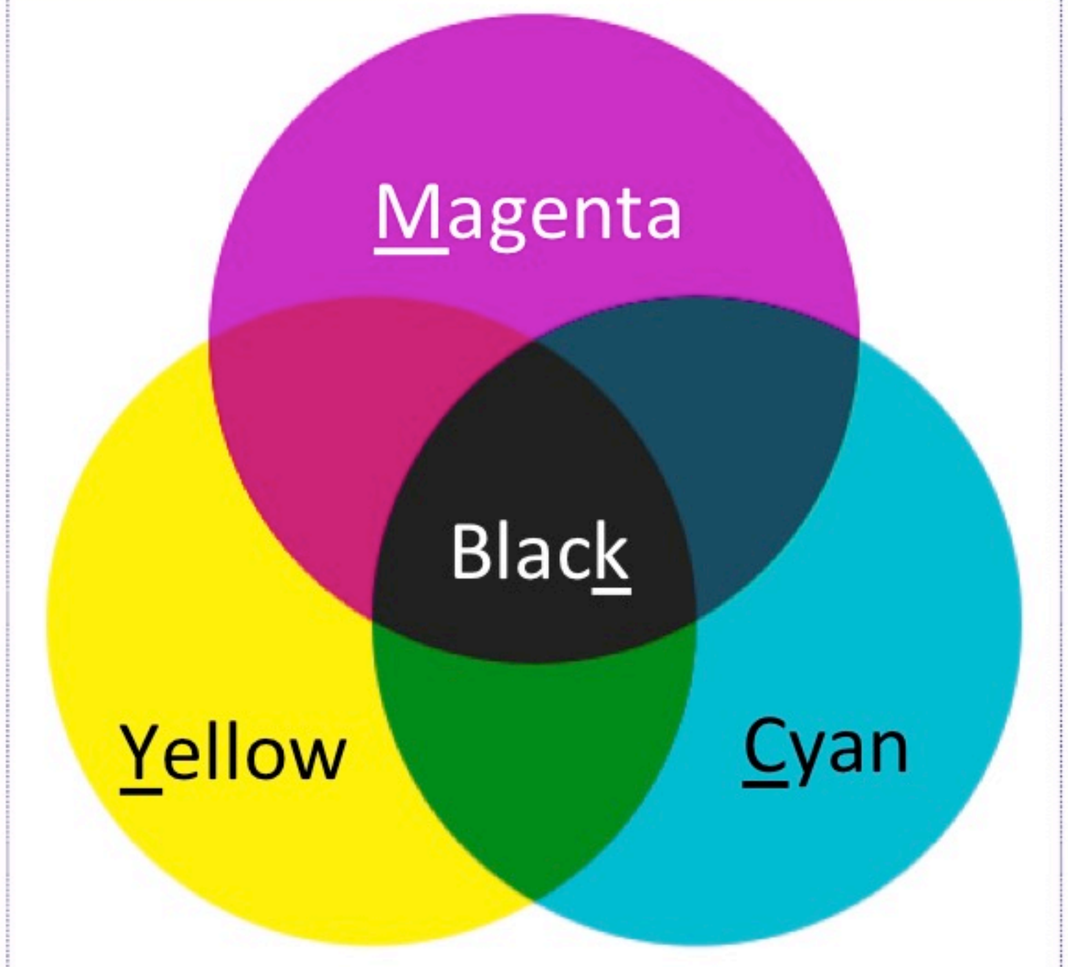
RGB / RVB



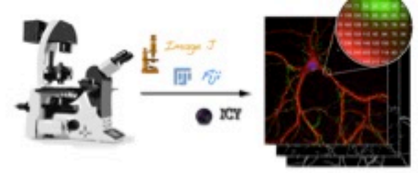
Substractive synthesis

Based on pigment absorption

CMYK / CMJN



- RGB: addition of colors leads to the white.
- CMYK: mixture of colors leads to black.



Colorimetric spaces

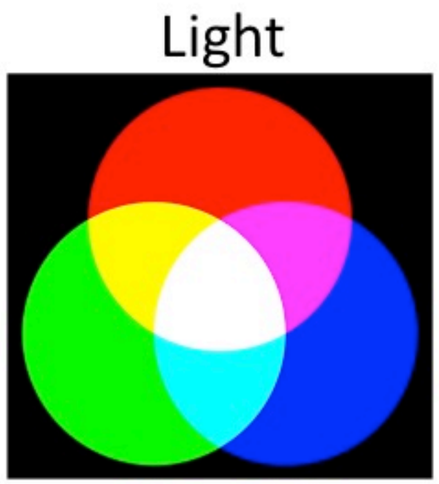
Additive synthesis

Based on color addition

RGB / RVB

screens/ projectors

Light



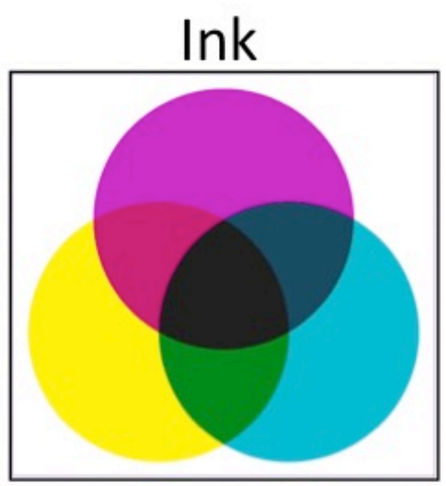
Substractive synthesis

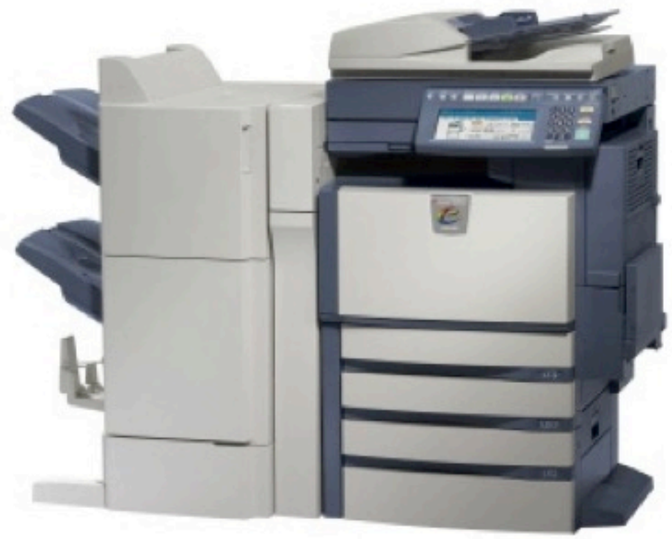
Based on pigment absorption

CMYK / CMJN

Press - printers

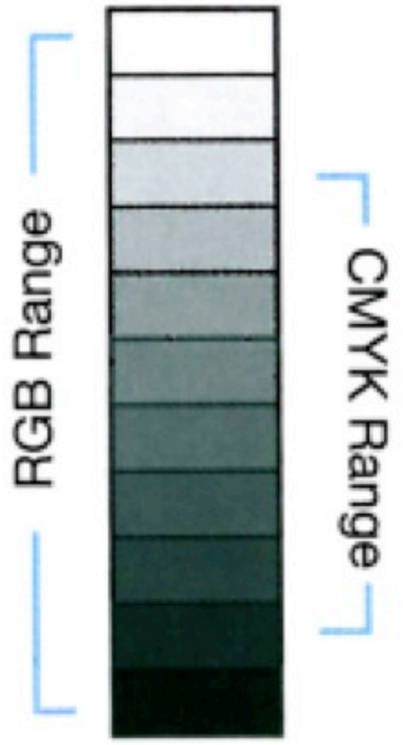
Ink



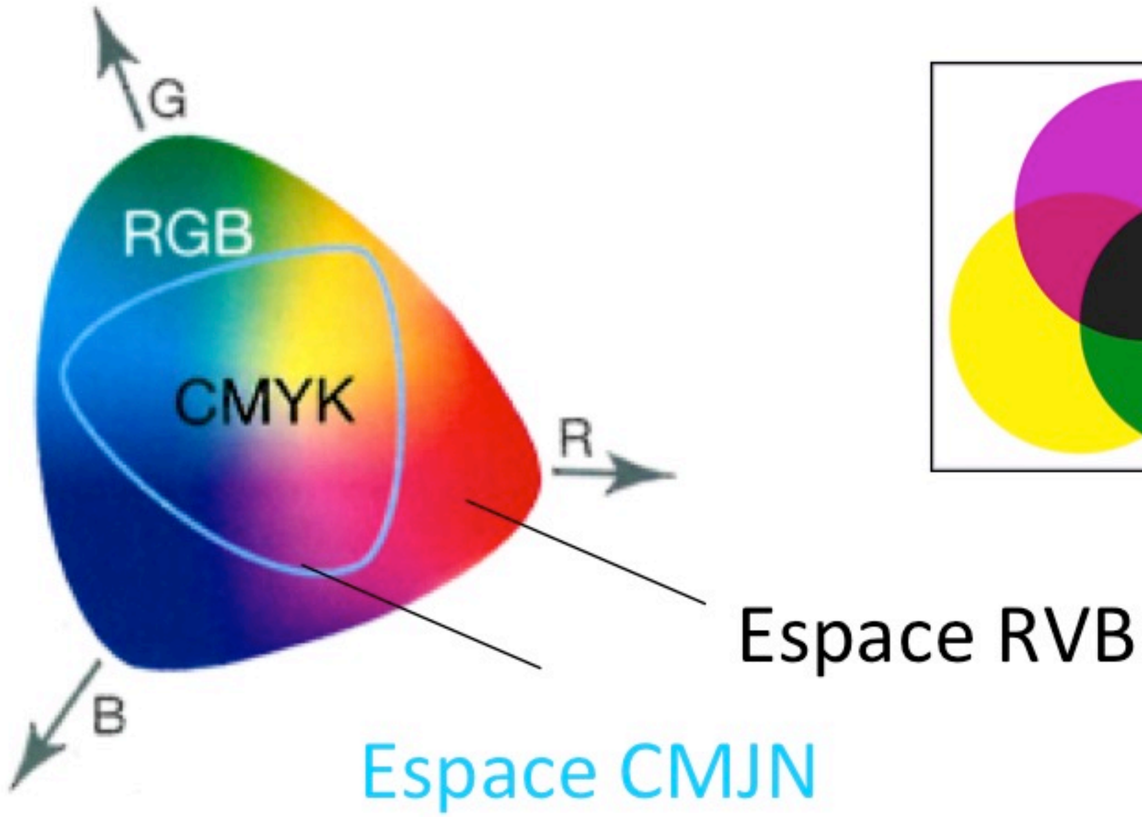


Colorimetric spaces

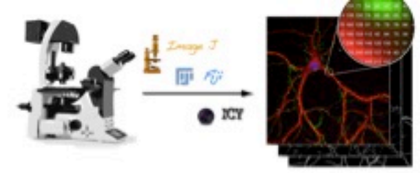
RGB / RVB
screens/ projectors



CMYK / CMJN
Press - printers

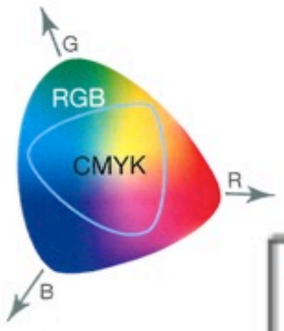


- RGB describes a larger portion of color space than does CMYK
- That the reason why it's hard to convert RGB to CMYK



Colorimetric spaces

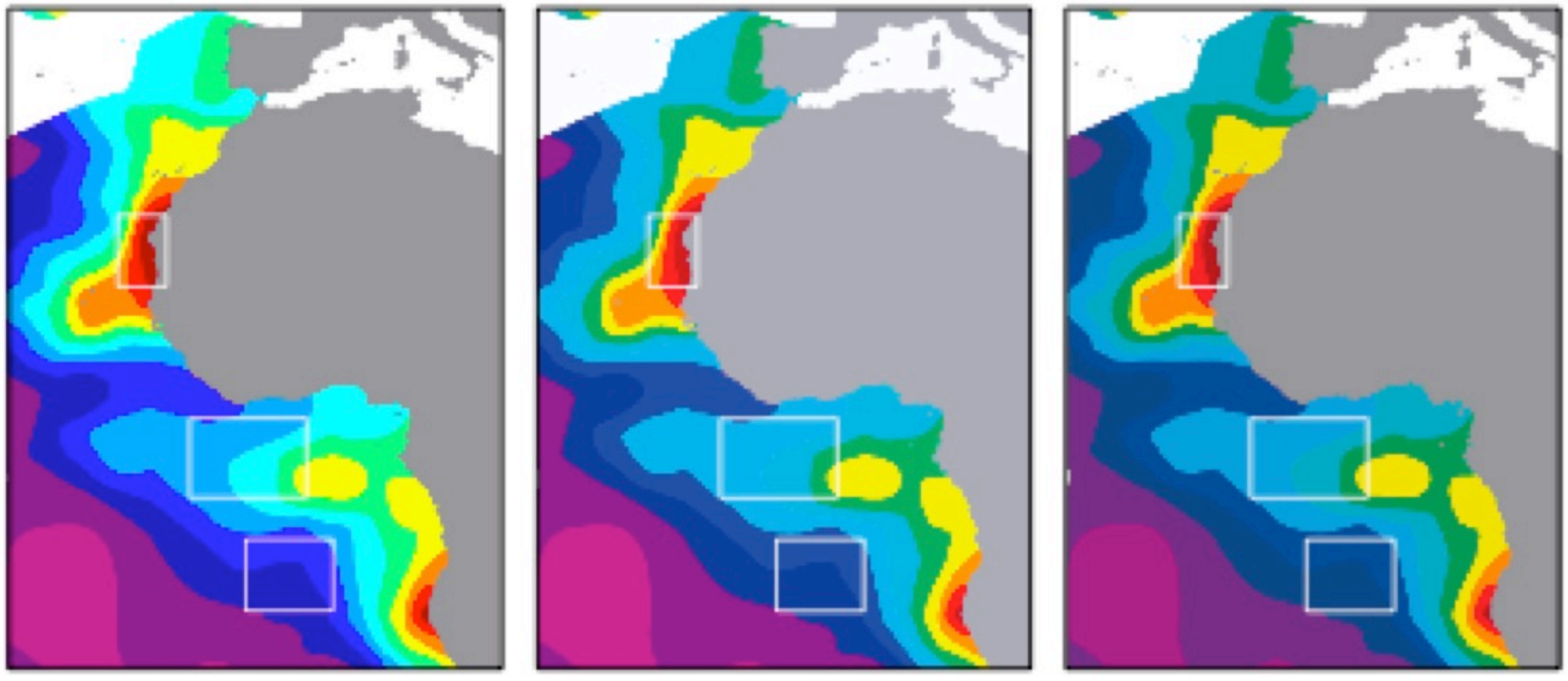
Practical computing** for **biologists



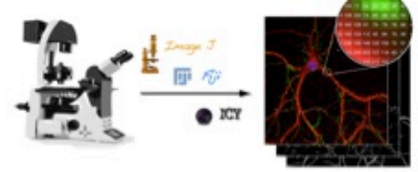
RVB
(screen)

CMJN
Coated paper
(journal)

CMYK
Uncotaed paper
(laser printer)



- RGB space is larger and contain more colors than CMYK.
- Oceanographic datas are lost with CMYK space.



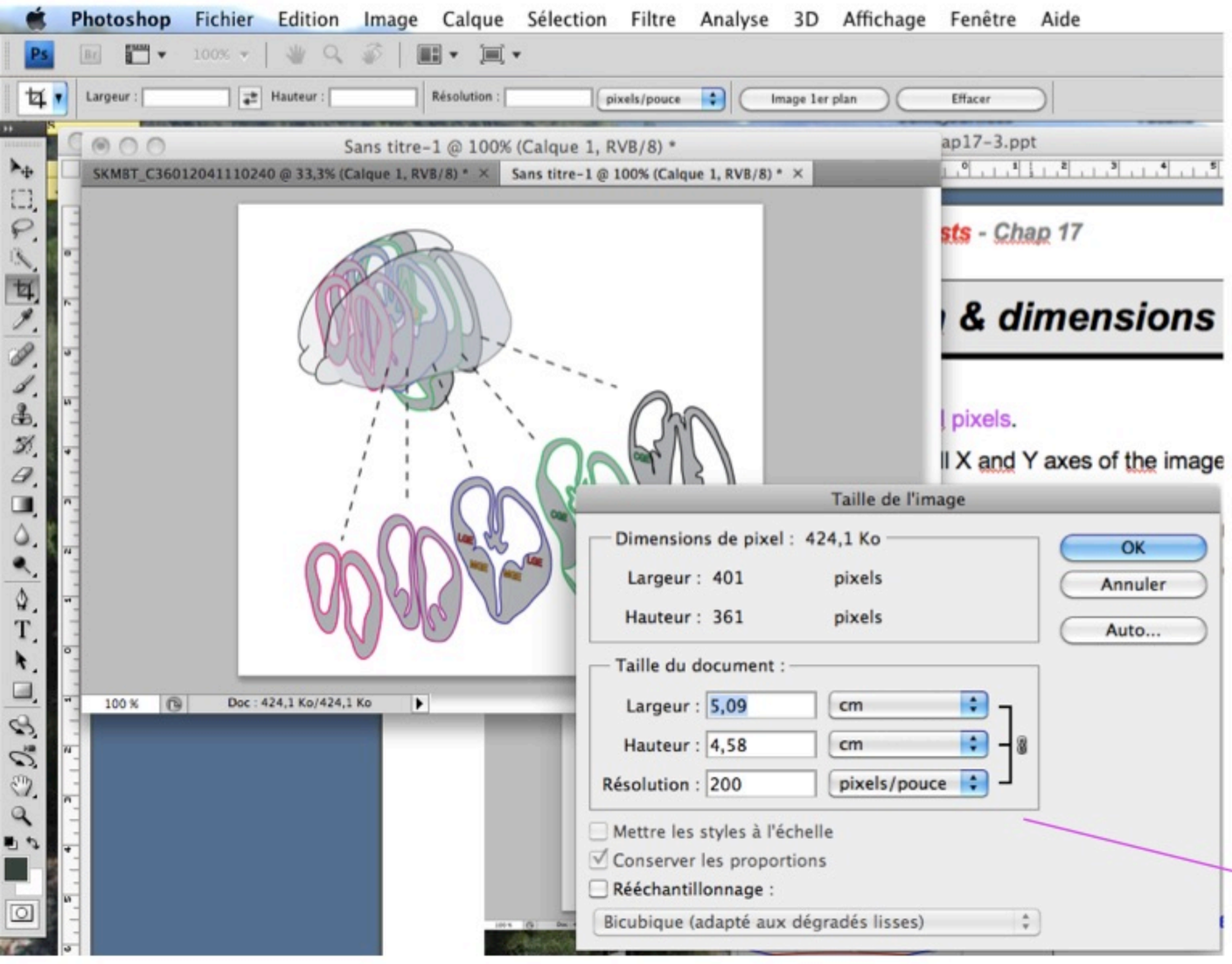
Resolution and image size

Les images pixéllisées sont faites d'une grille de pixels colorisés.

Pixel Dimension en Pixel : number of pixel of the matrix for example 800 x 600 pixels.

Physical size : this is image size on a printed paper, for example 21 cm x 29,7 cm.

Resolution: this is pixel size, expressed as pixel number by unt of physical size, usally named dots per inch (DPI) ou pixel per inch (PPI).



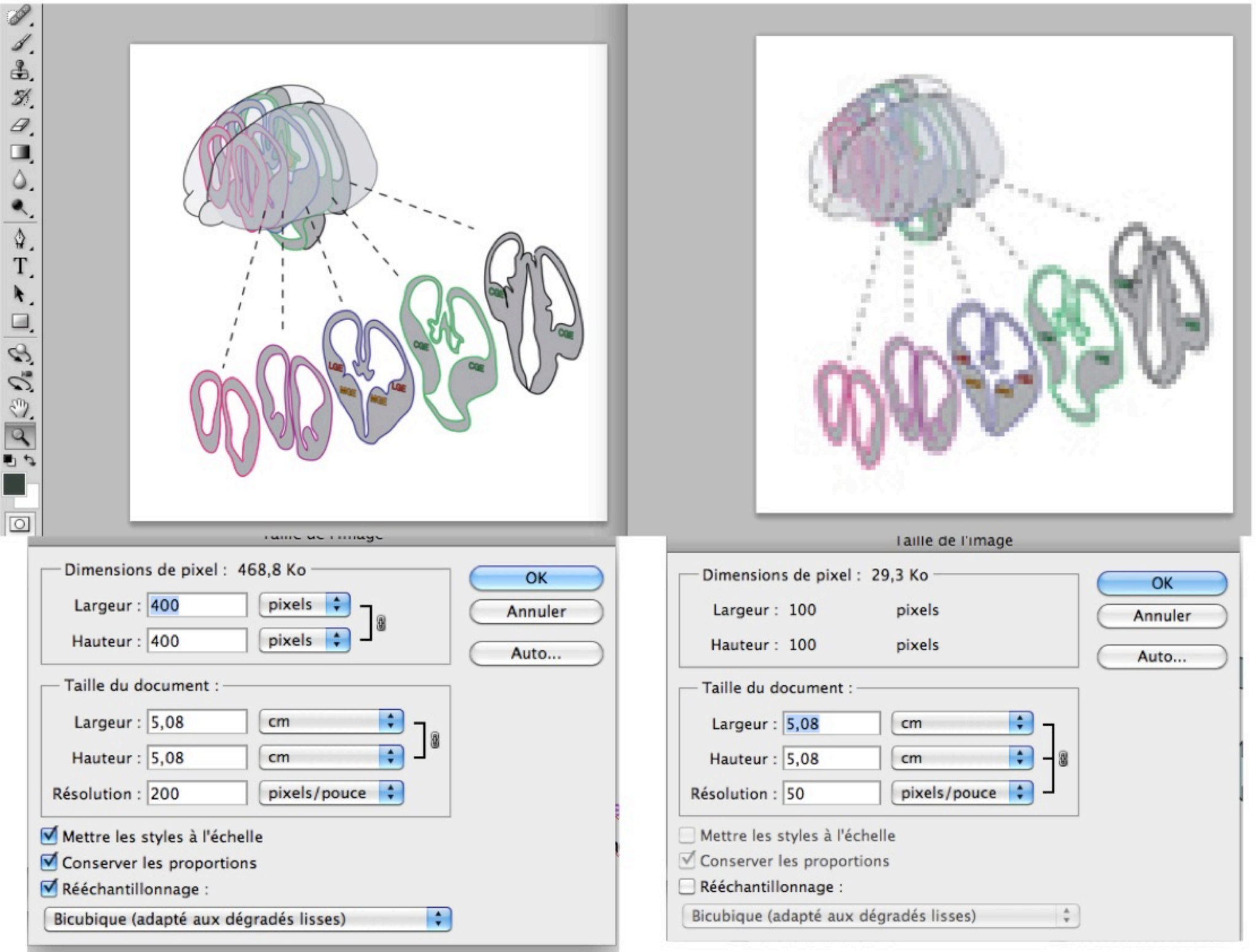
Pixel size

Physical size

Resolution

Parameters are all linked

Resolution and image size

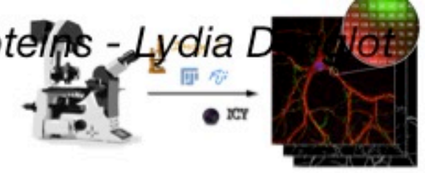


Same physical size

Lower pixels

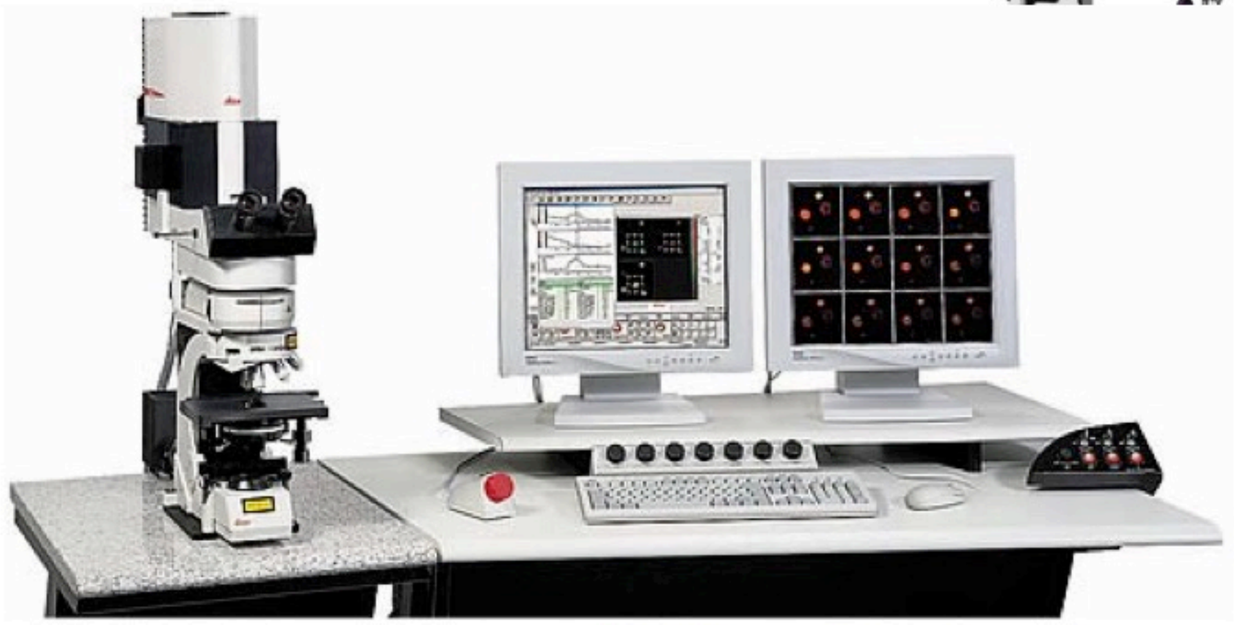
Lower resolution

Pixellised picture

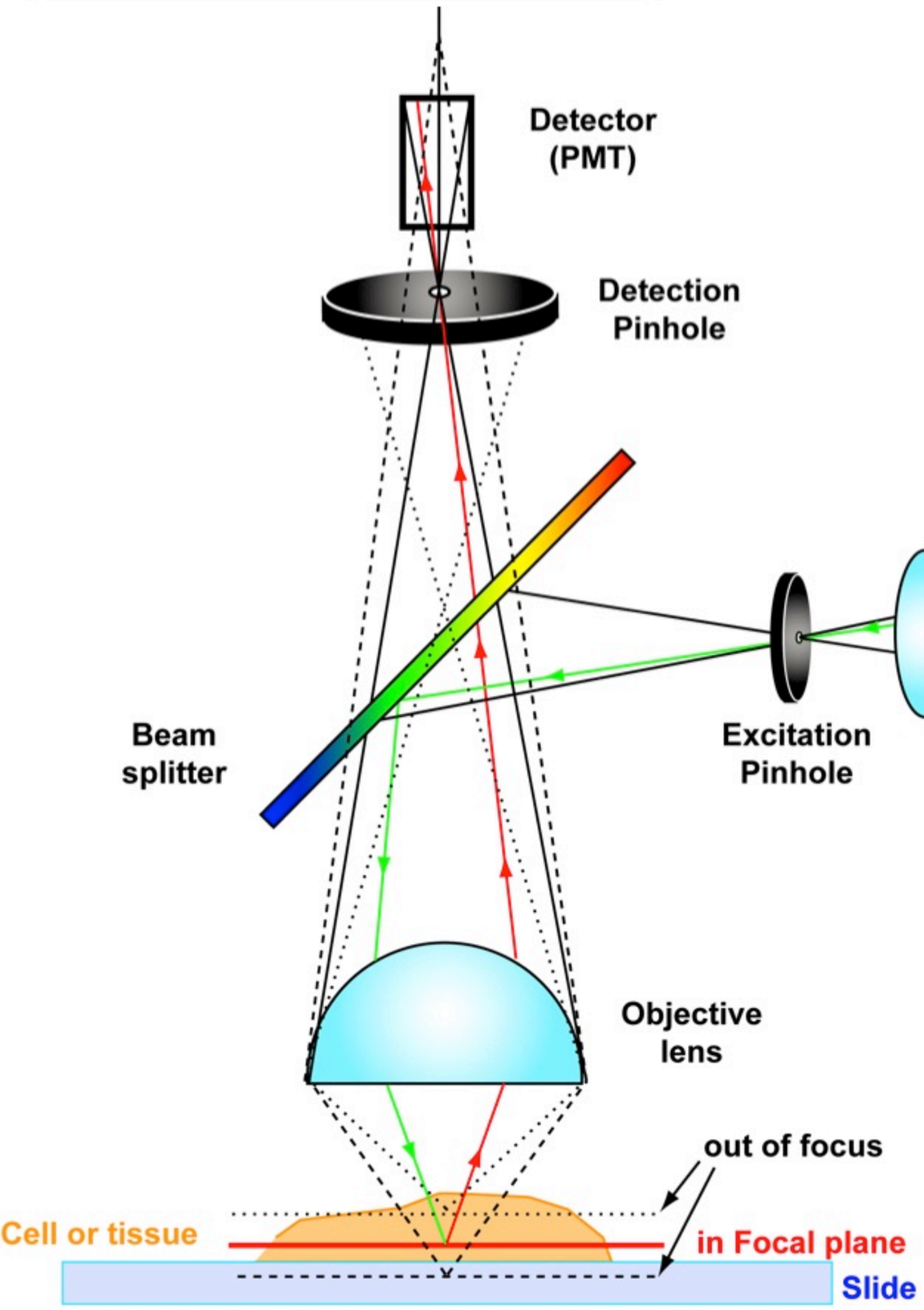


Confocal microcopy

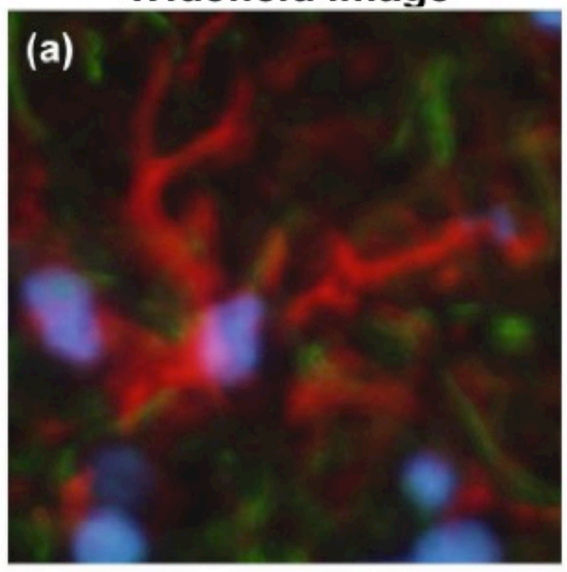
Confocal microscope



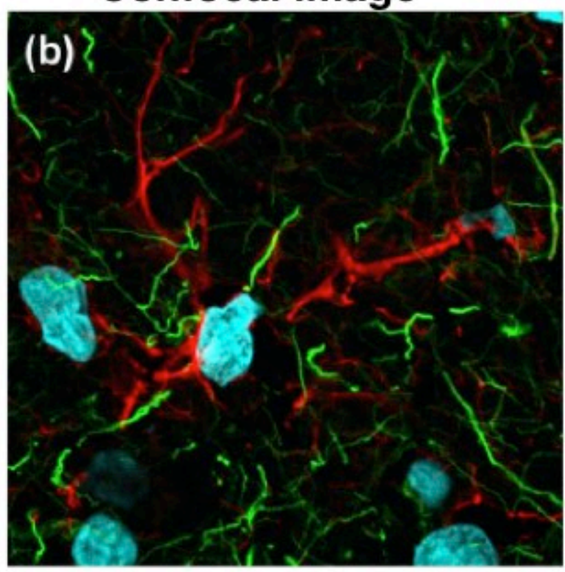
http://www.leica-microsystems.com



Widefield image



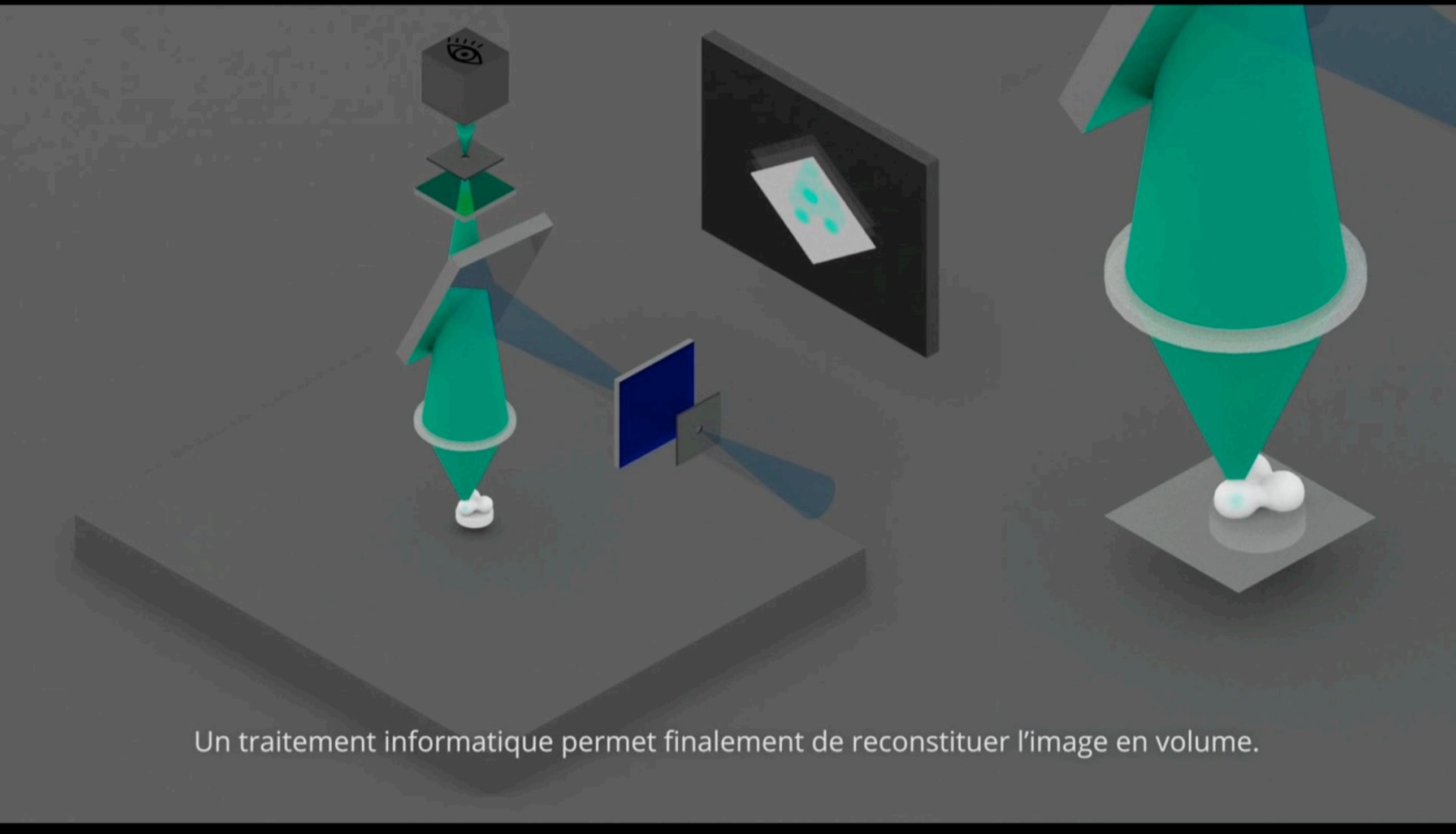
Confocal image



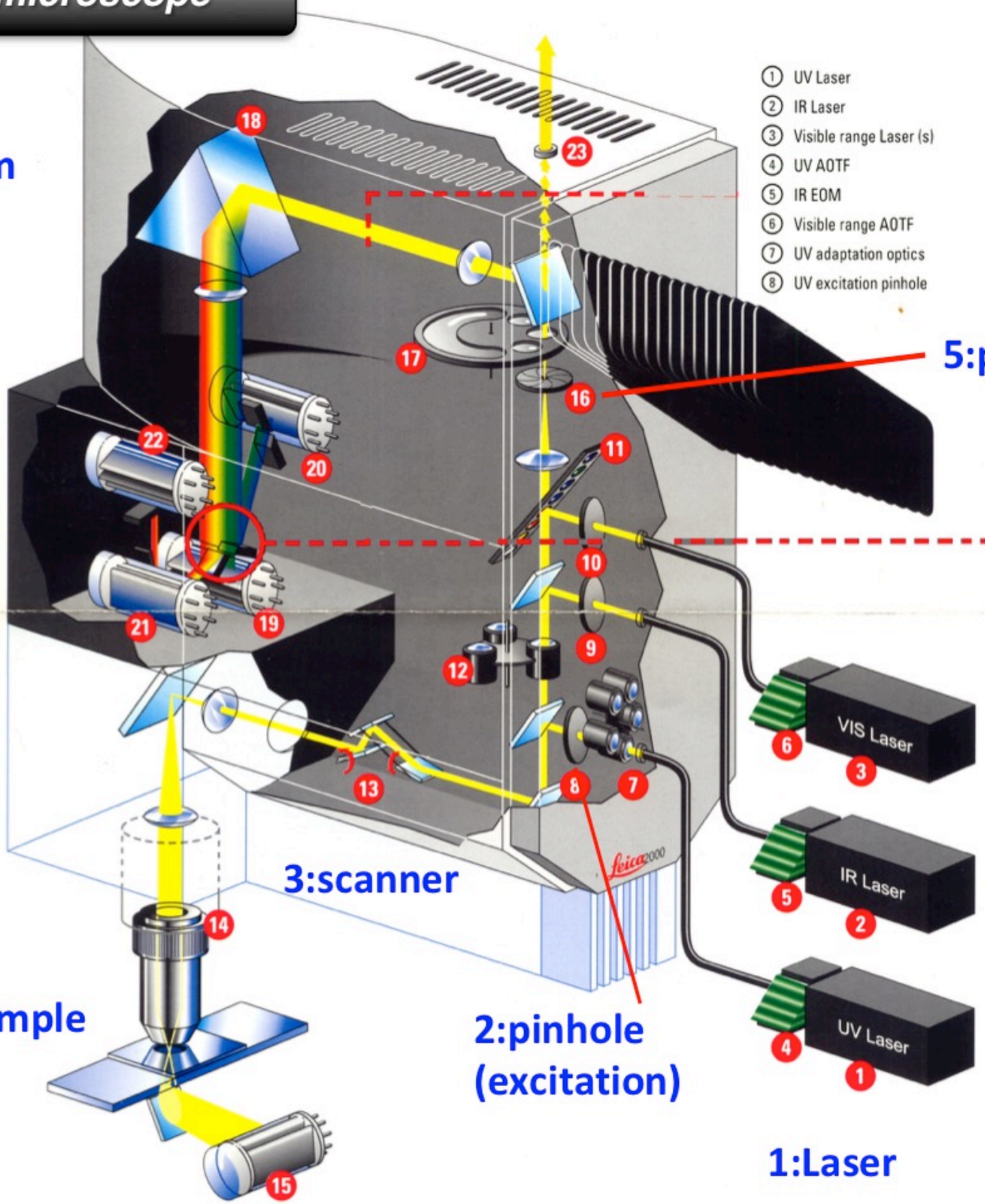
From LASER SCANNING CONFOCAL MICROSCOPY
Nathan S. Claxton, Thomas J. Fellers, and Michael W. Davidson http://www.olympusfluowiew.com

Confocal Microscope

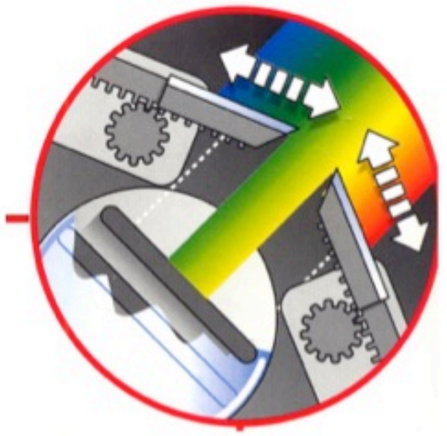
https://toutestquantique.fr/fluorescent-et-confocal/



Confocal microscope



1. UV Laser
2. IR Laser
3. Visible range Laser (s)
4. UV AOTF
5. IR EOM
6. Visible range AOTF
7. UV adaptation optics
8. UV excitation pinhole
9. IR excitation pinhole
10. VIS excitation pinhole
11. Primary beam splitter
12. Adjustable pupil illumination
13. "K"-Scanner with rotator
14. Microscope & objective
15. Transmitted light detector
16. Confocal detection pinhole
17. Analyzer wheel
18. Spectrophotometer prism
19. Photomultiplier channel 1
20. Photomultiplier channel 2
21. Photomultiplier channel 3
22. Photomultiplier channel 4
23. External optical port



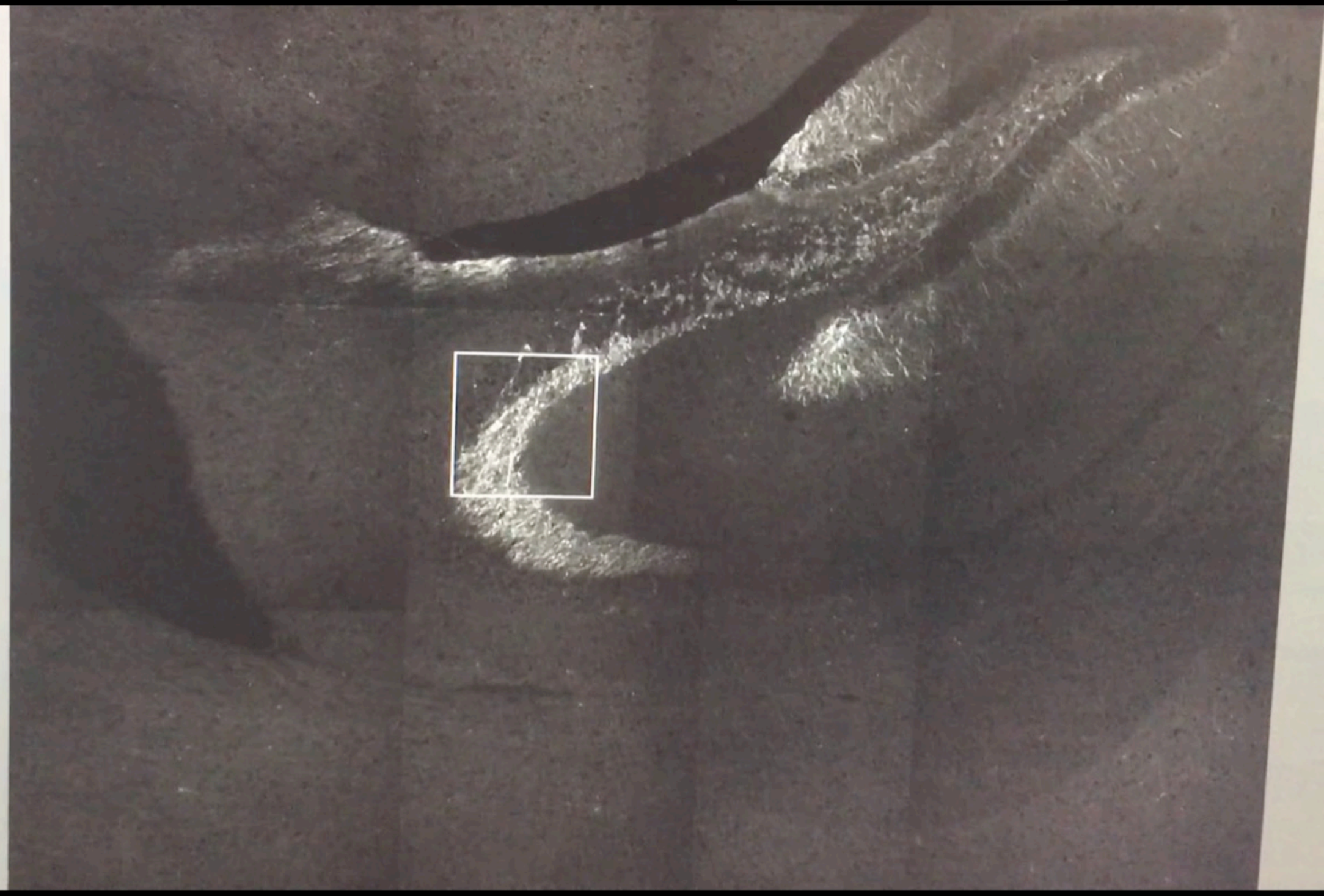
Confocal: the 2 pinholes are <u>conjugated</u> on the same focal plane.

Leica

http://www.leica-microsystems.com

Tile imaging modalities

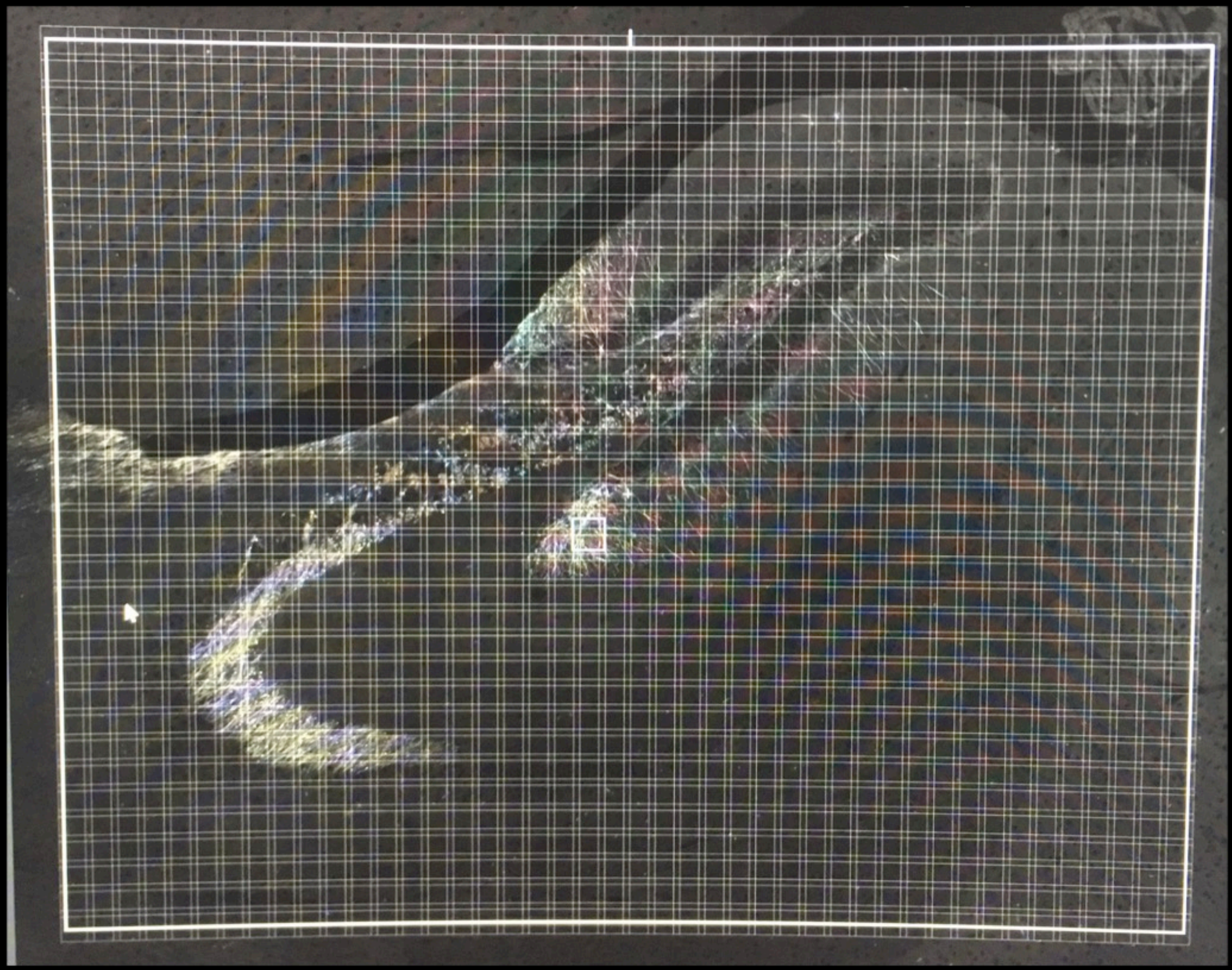
LEICA confocal SP8
20X



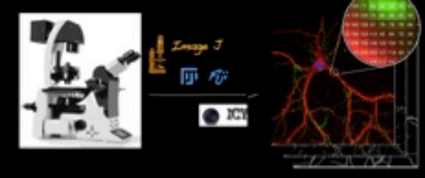
- The inhomogeneity of the picture reveal grids on the picture.
- This artifact can be supressed by zooming on the center of the image (1,6 x)

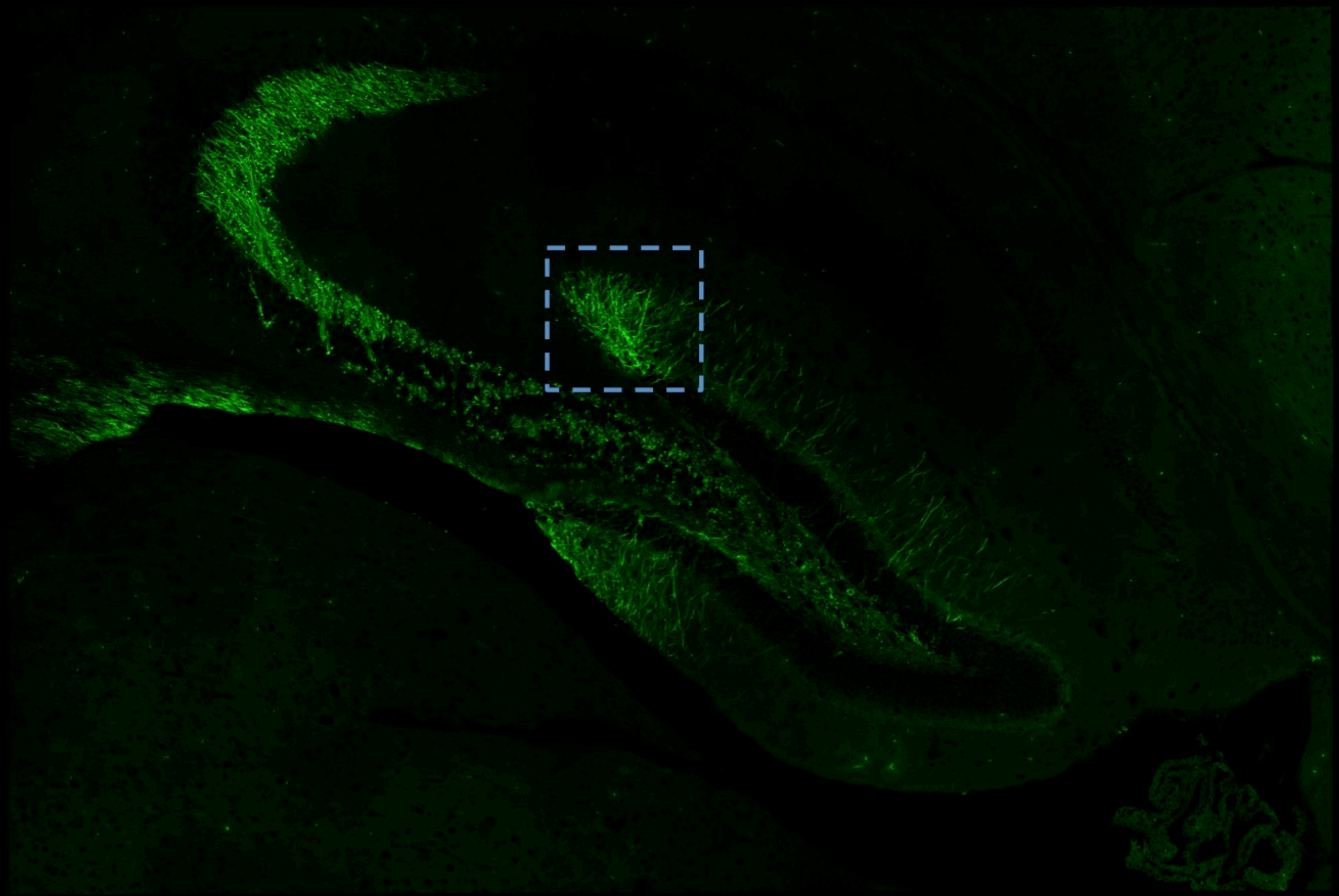
Tile imaging modalities

LEICA SP5, SP8,
93 X

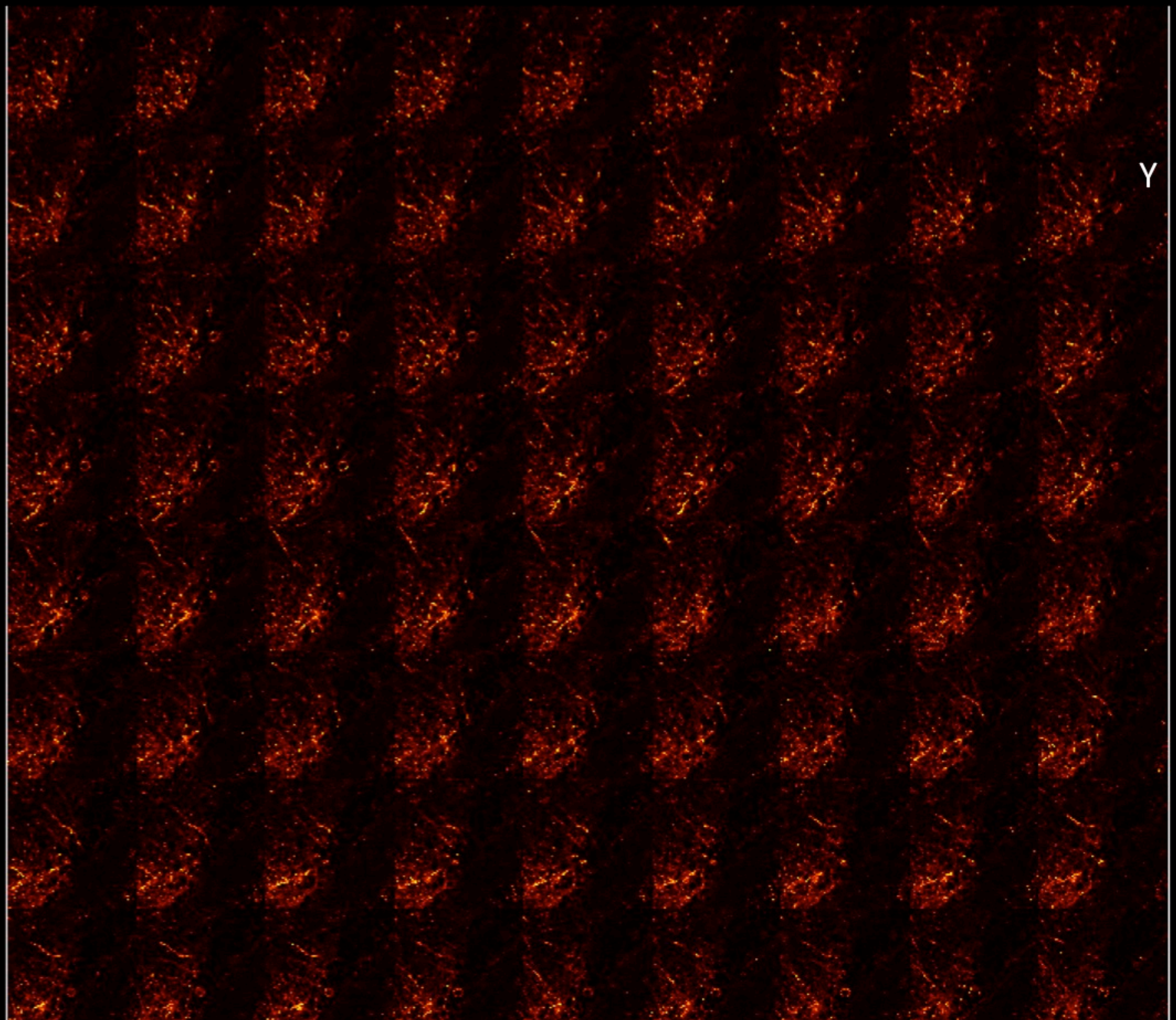


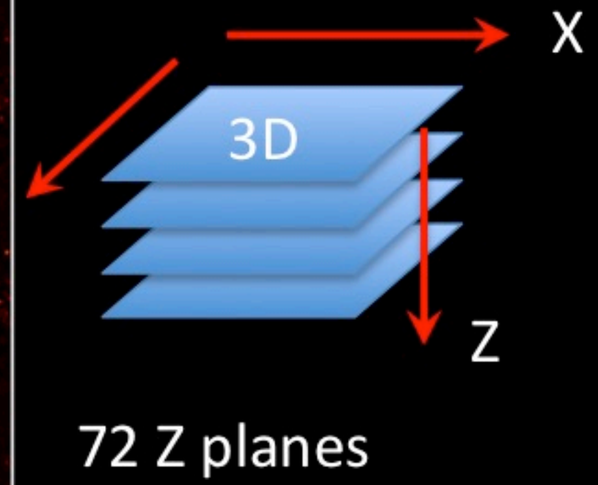
- The same field of view aquired at 93x impose far much more pictures.





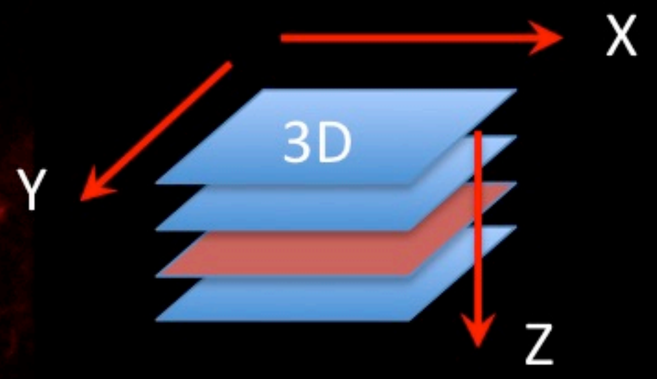
A stack of 72 confocal planes





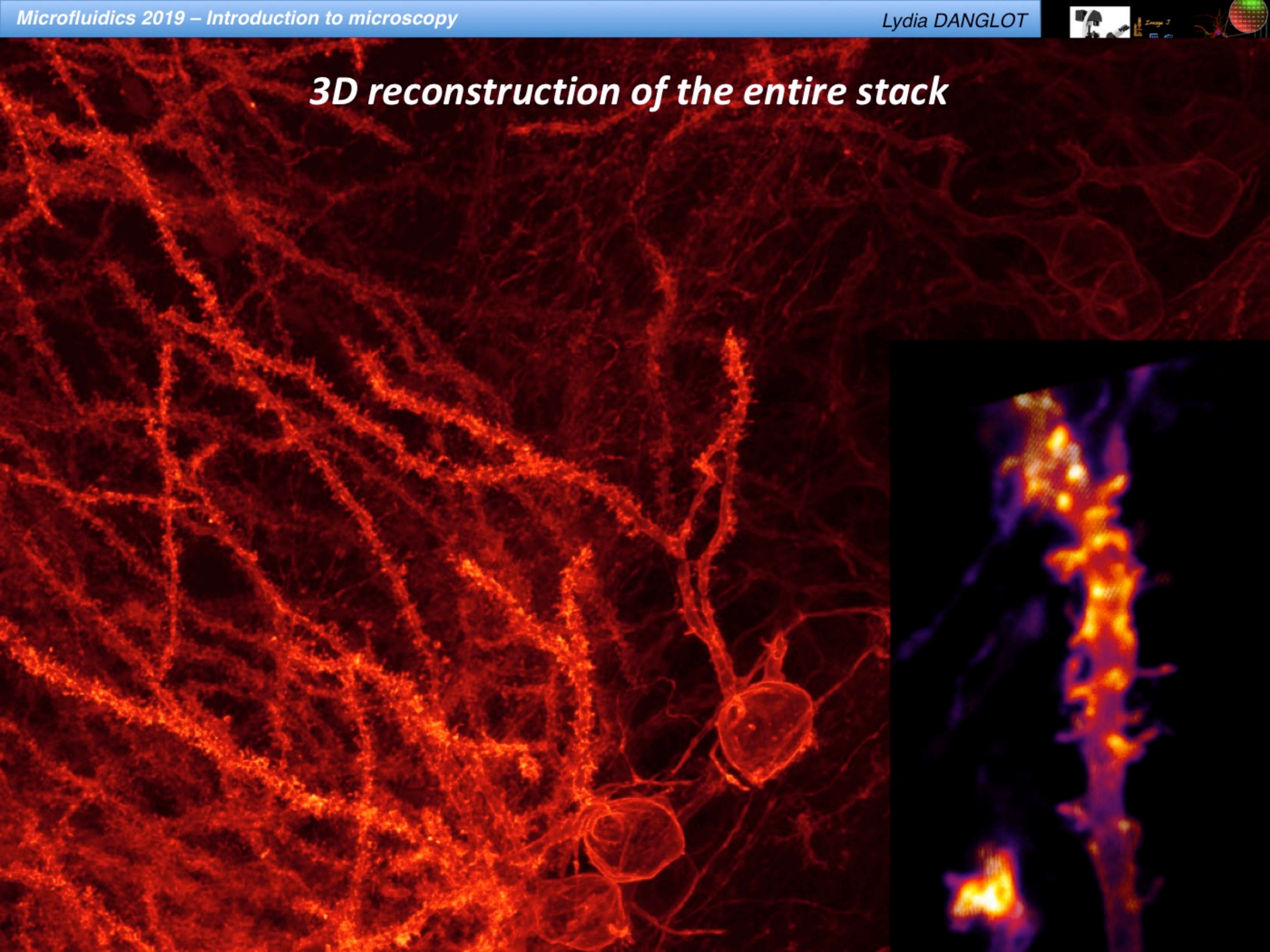
72 Z planes

One plane of a stack of 72 confocal planes

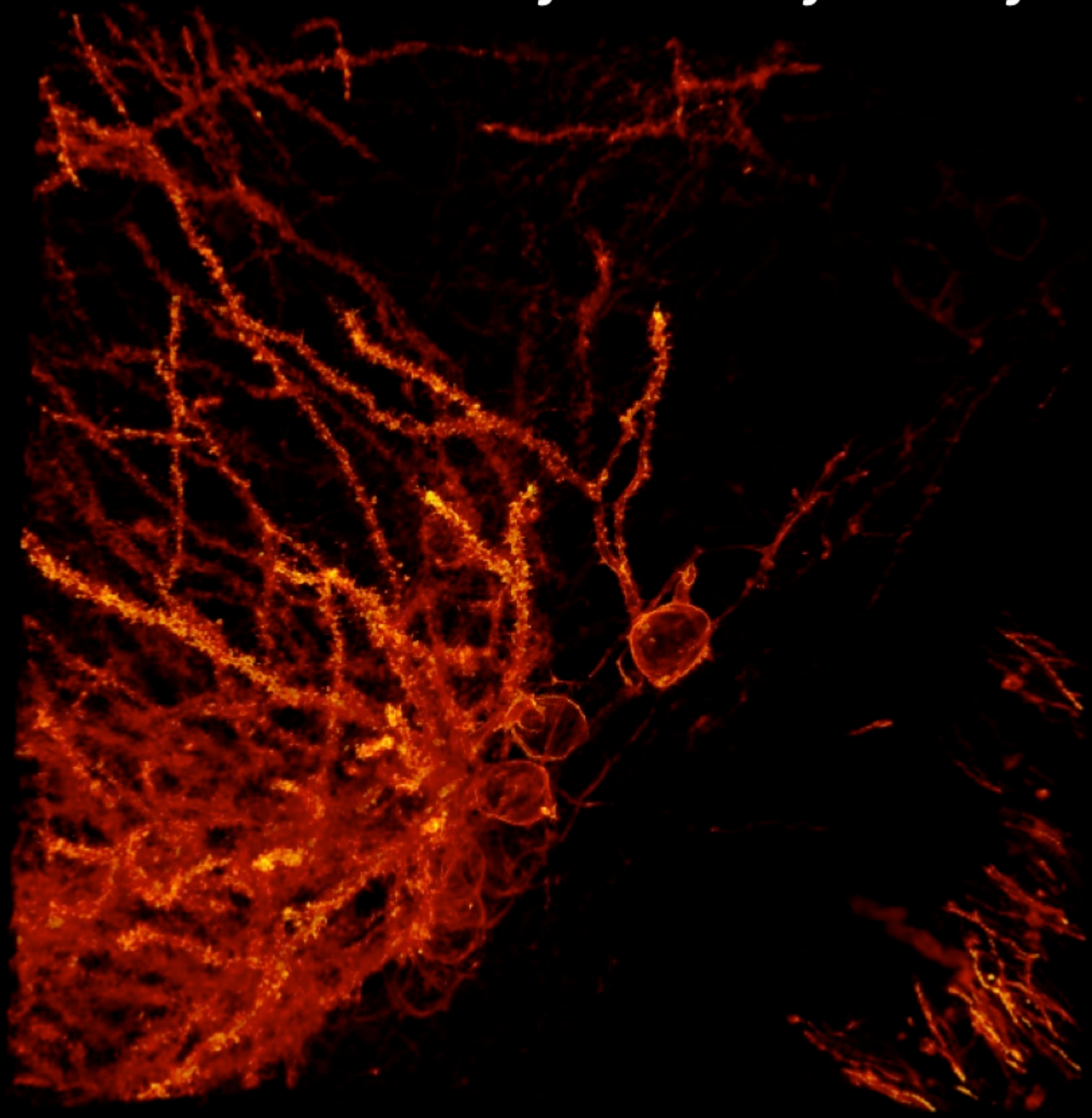


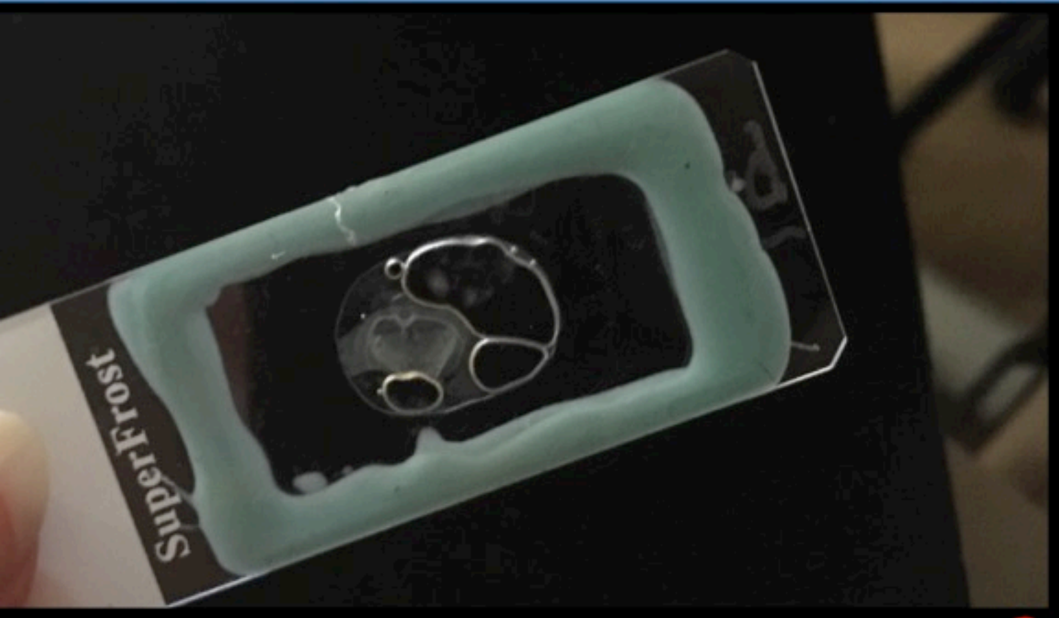
20µm

3D reconstruction of the entire stack



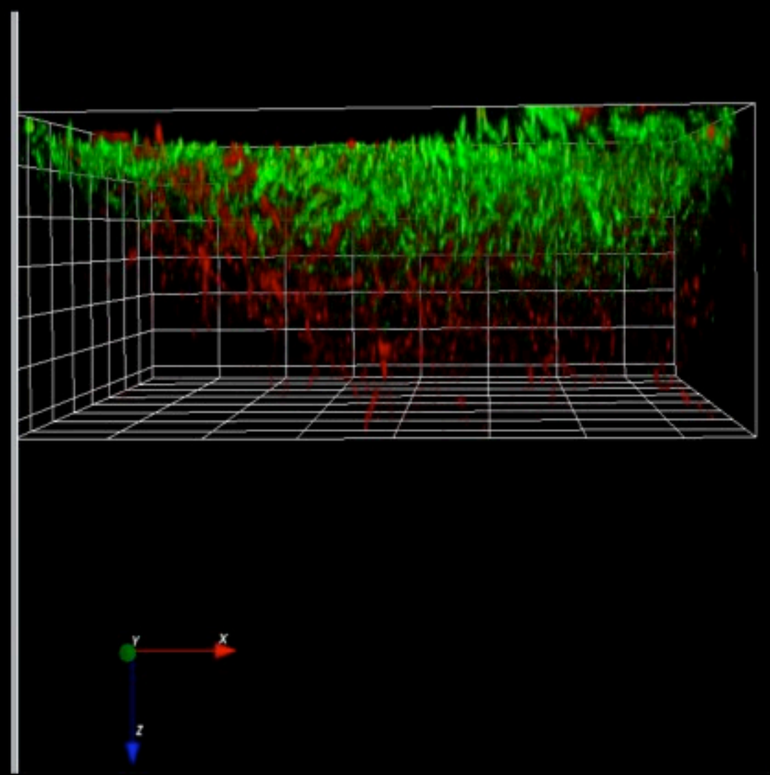
Animated 3D reconstruction of a stack of 72 confocal planes



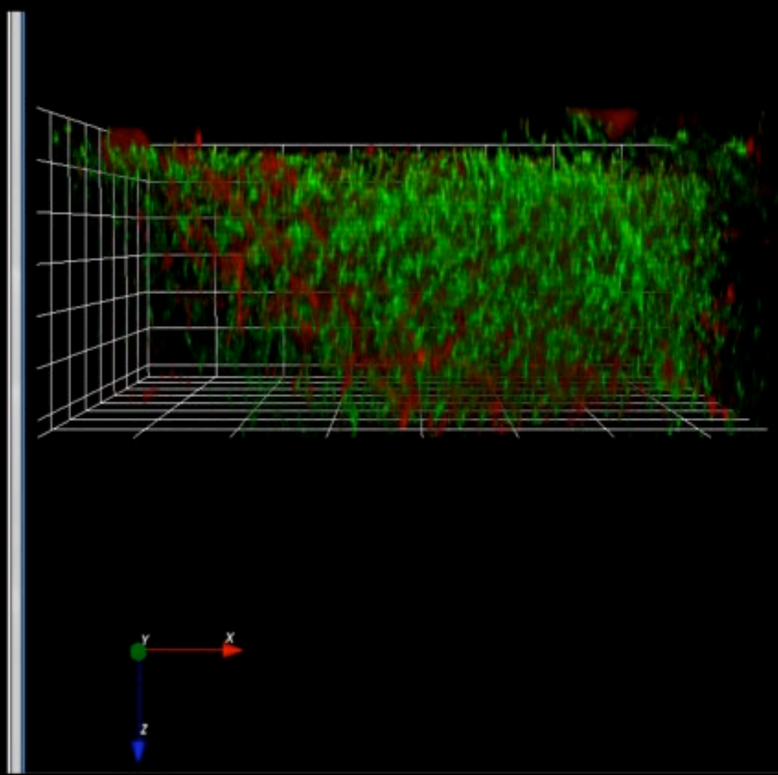


Imaging thick sample : Acquisition on a 500 microns slices mounted within the slide

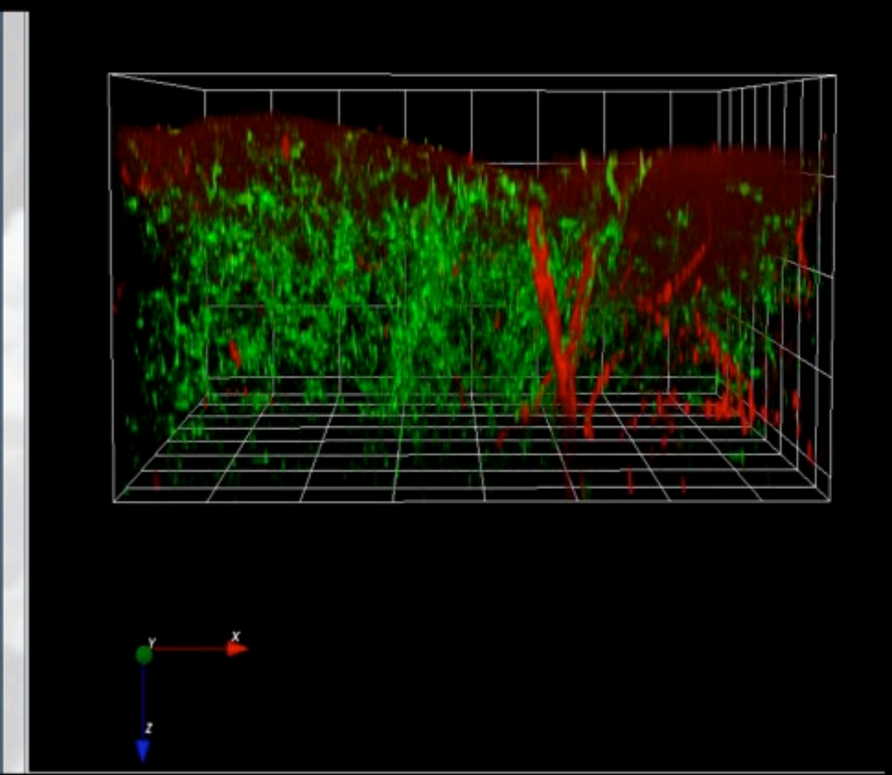
Confocal

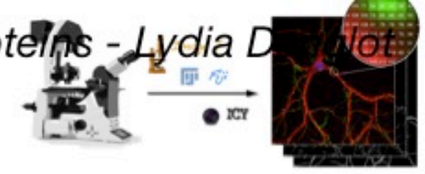


Confocal with laser compensation



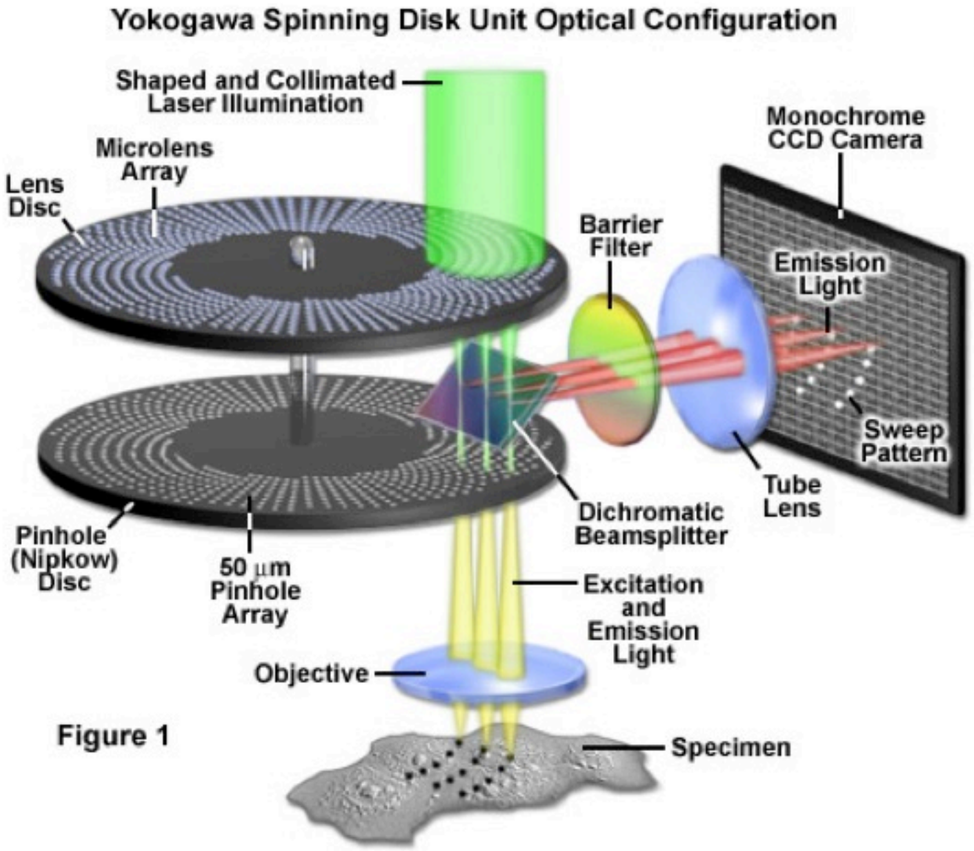
Confocal with CLARITY





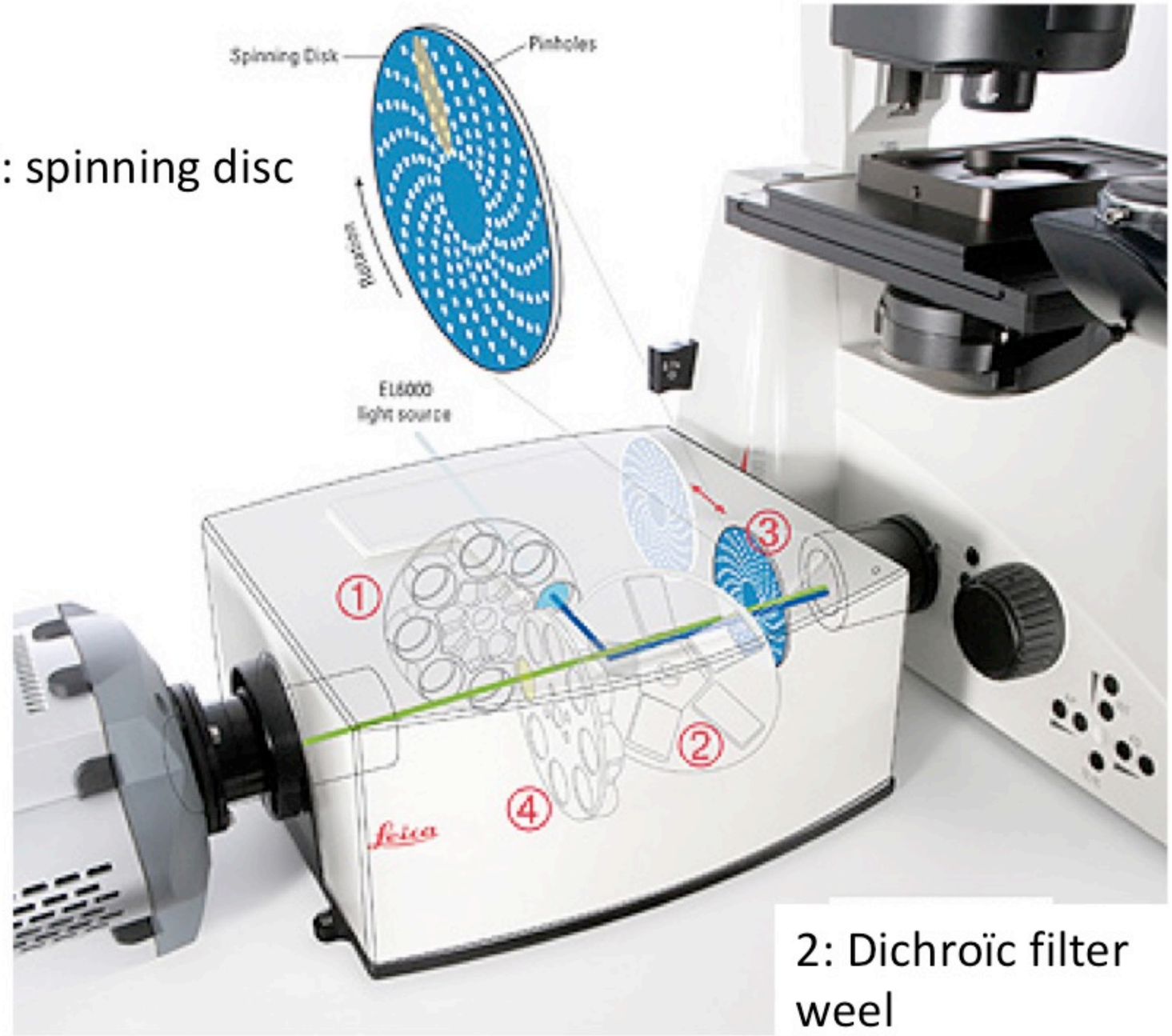
Spinning disc microscopy

Fast confocal microscope : « spinning disc »



http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/indexflash.html

3: spinning disc



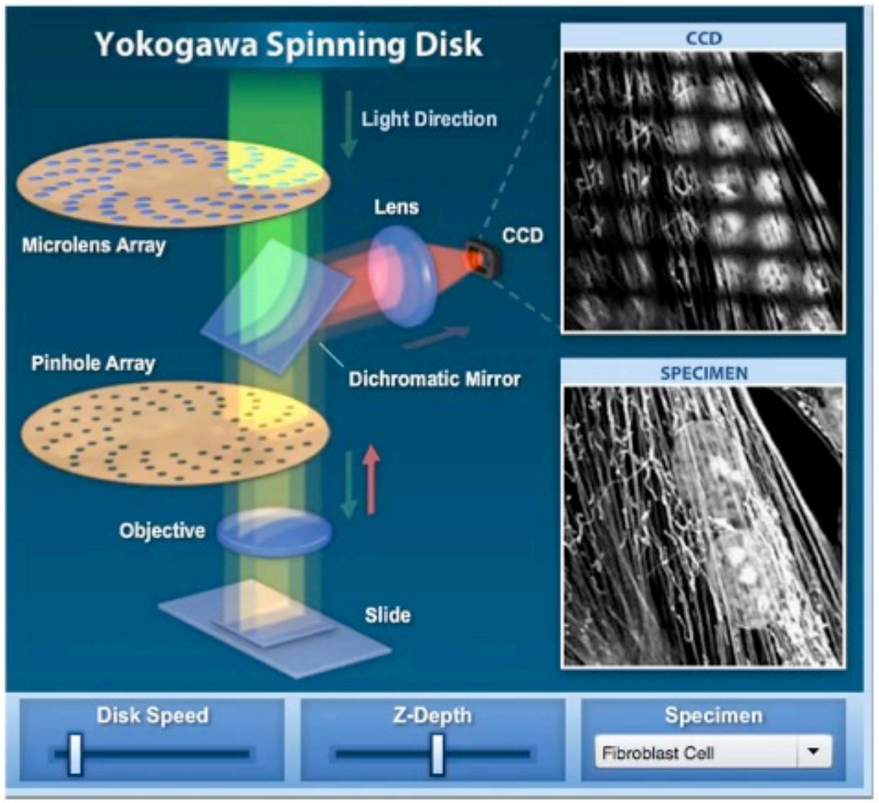
http://www.leica-microsystems.com

2: Dichroïc filter weel

1: excitation filter weel

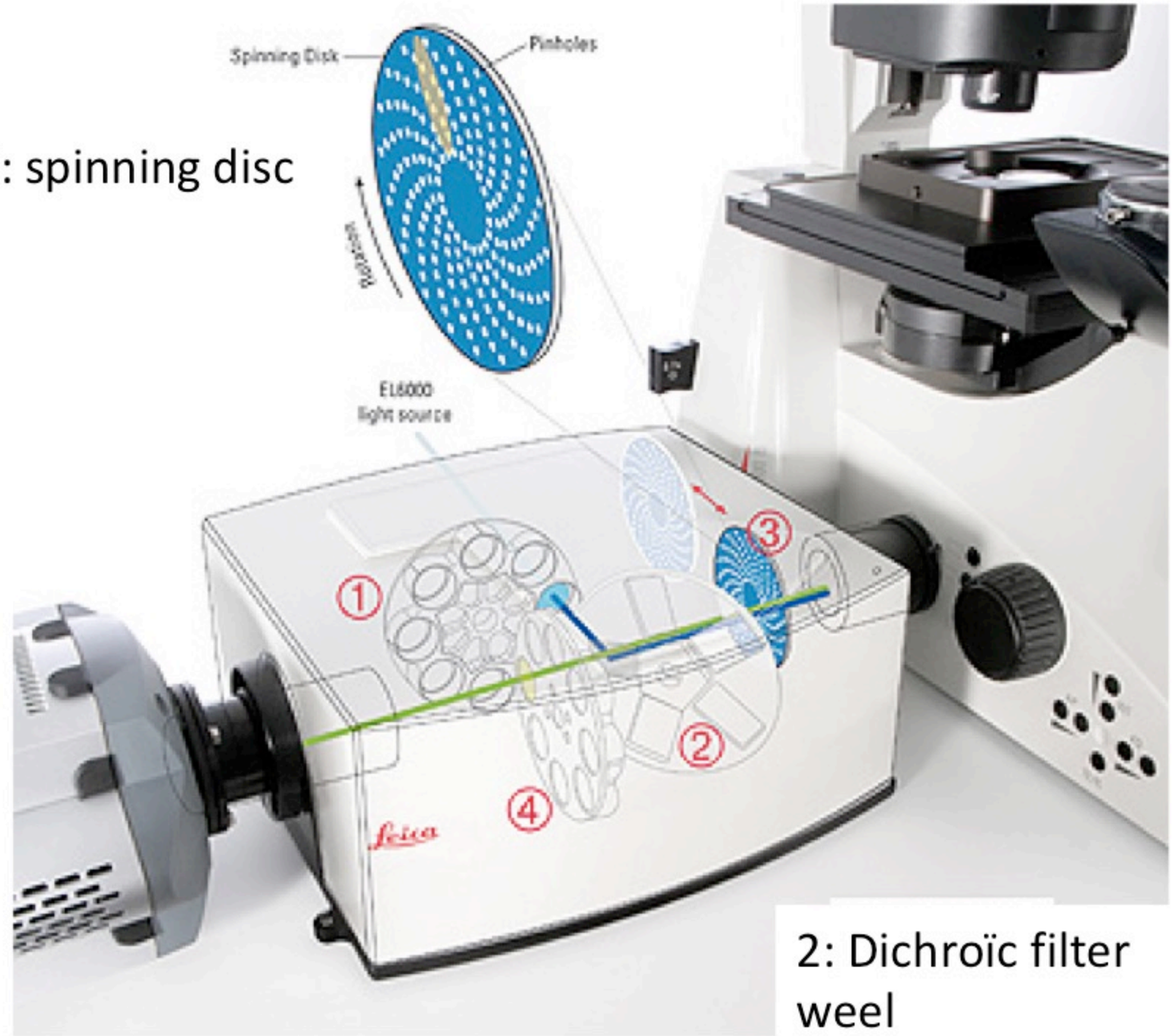
4: emission filter weel

Fast confocal microscope : « spinning disc »



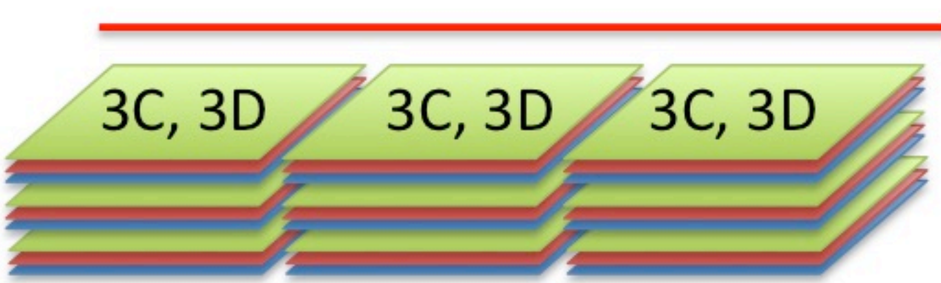
http://zeiss.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/indexflash.html

3: spinning disc



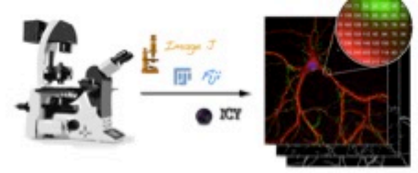
2: Dichroïc filter weel

http://www.leica-microsystems.com

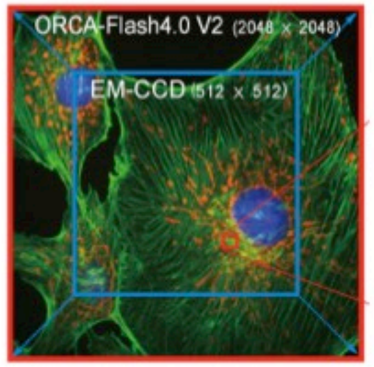


1: excitation filter weel

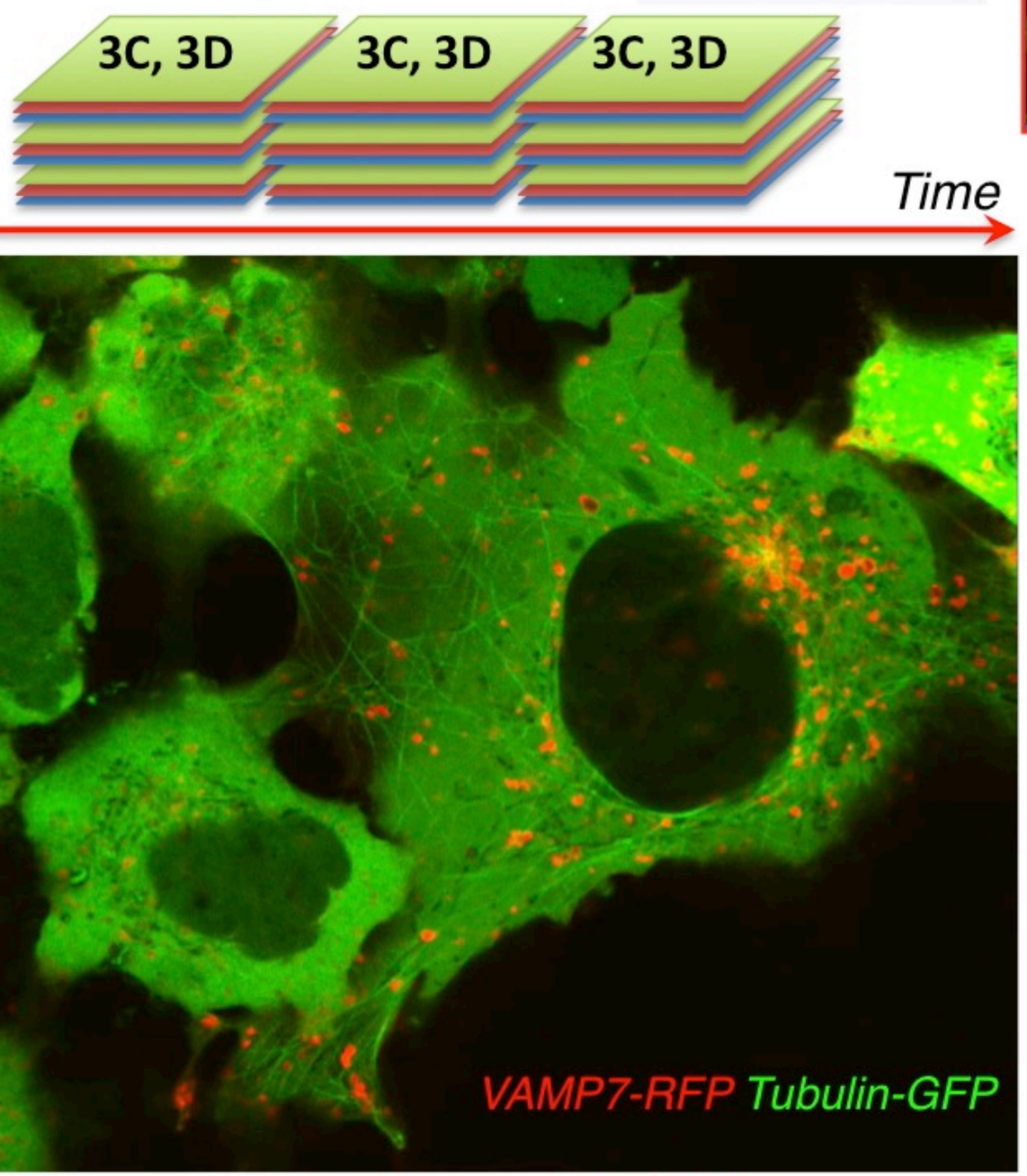
4: emission filter weel



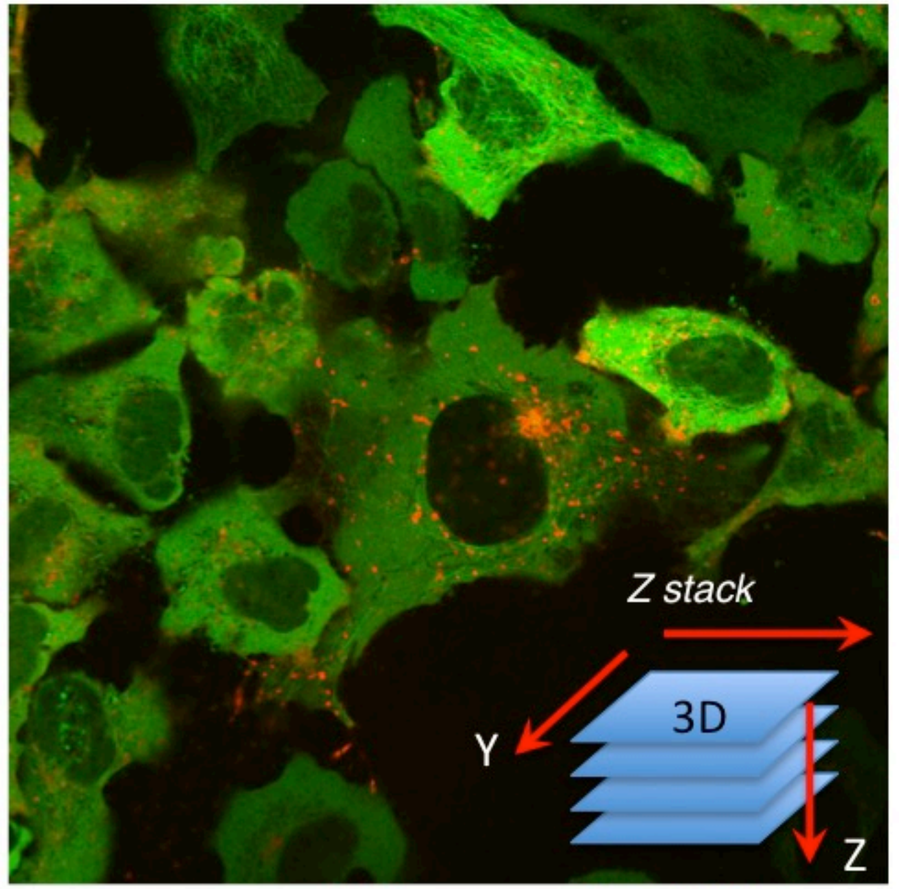
Fast confocal microscope : « spinning disc »



http://www.leica-microsystems.com



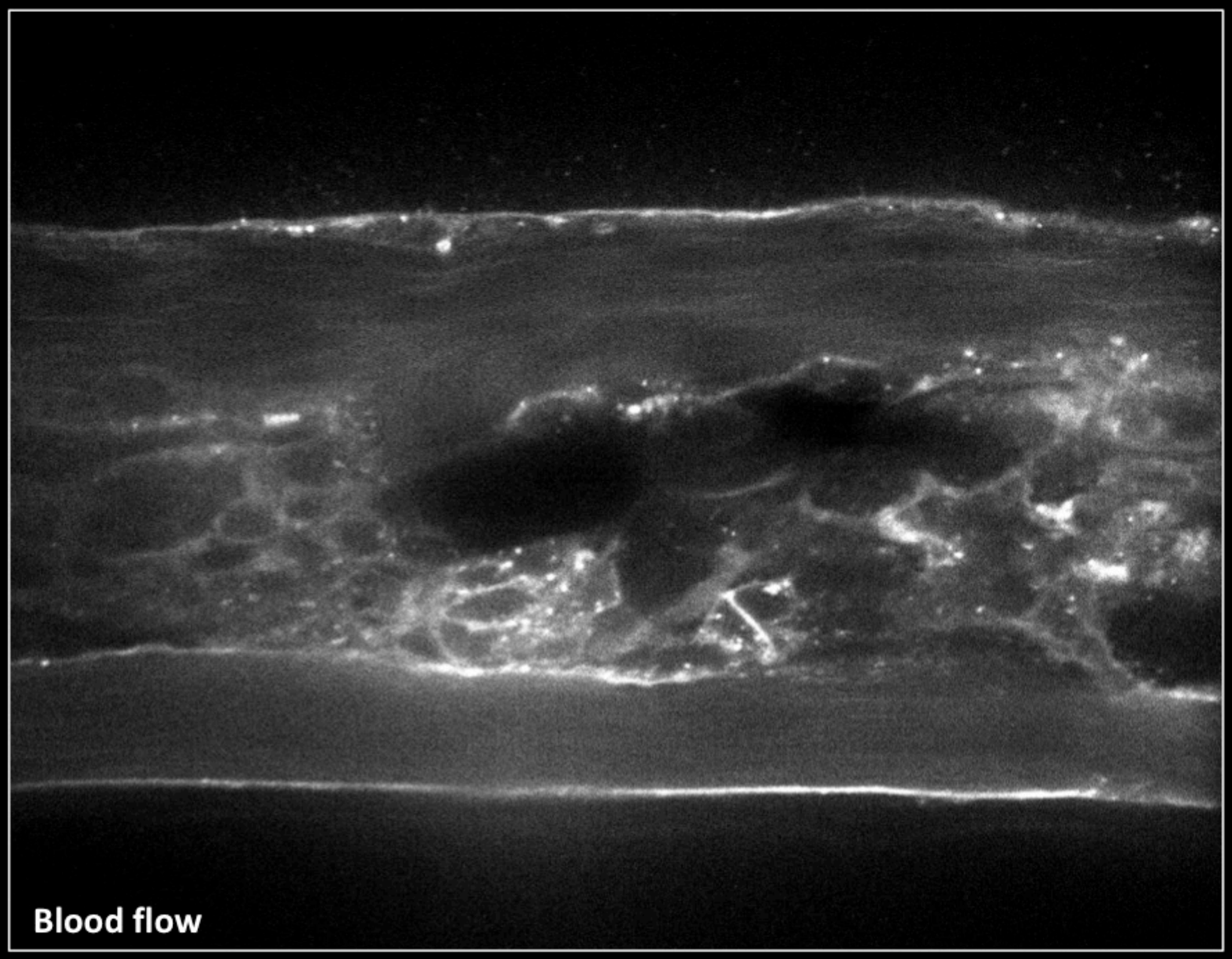
Organel tracking (vesicles) with cytoskeleton along time



Organel tracking (vesicles) with cytoskeleton in 3D

Multiple imaging modalities

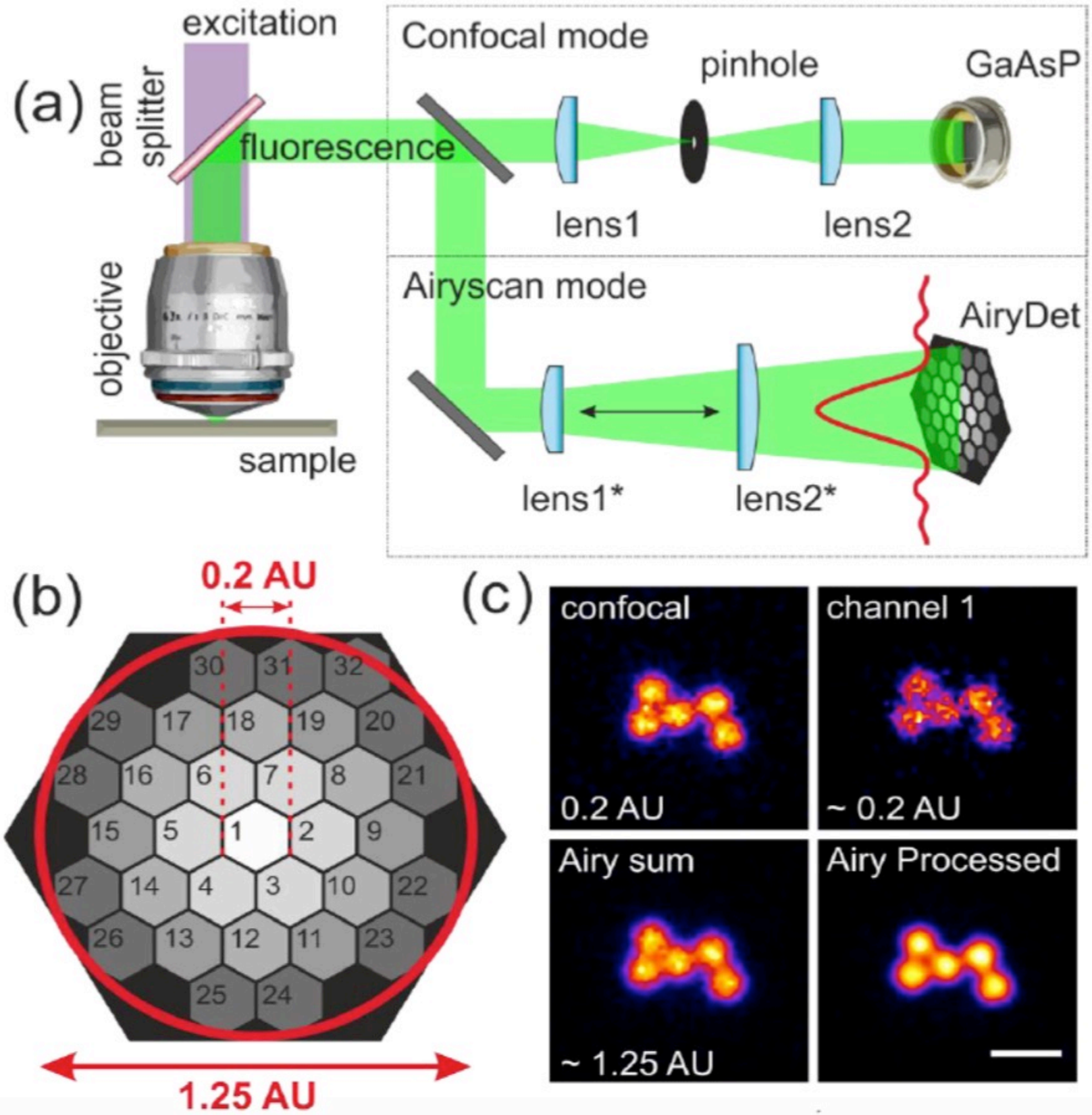
Live biological samples



Leica
Spinning-disk
Yokogawa CSU X1

8ms/image

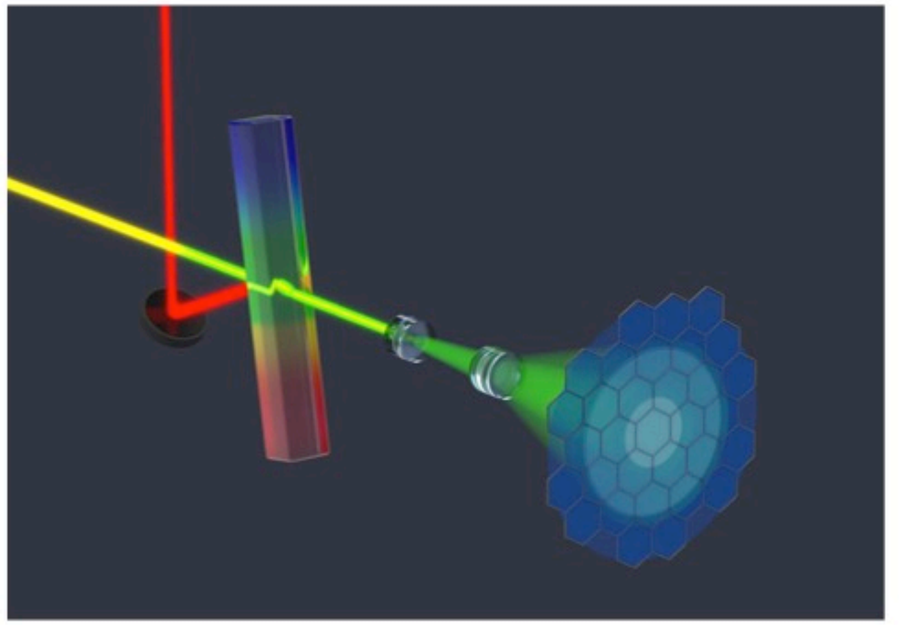
Zeiss Airyscan technology



hv photonics
MDPI
Article

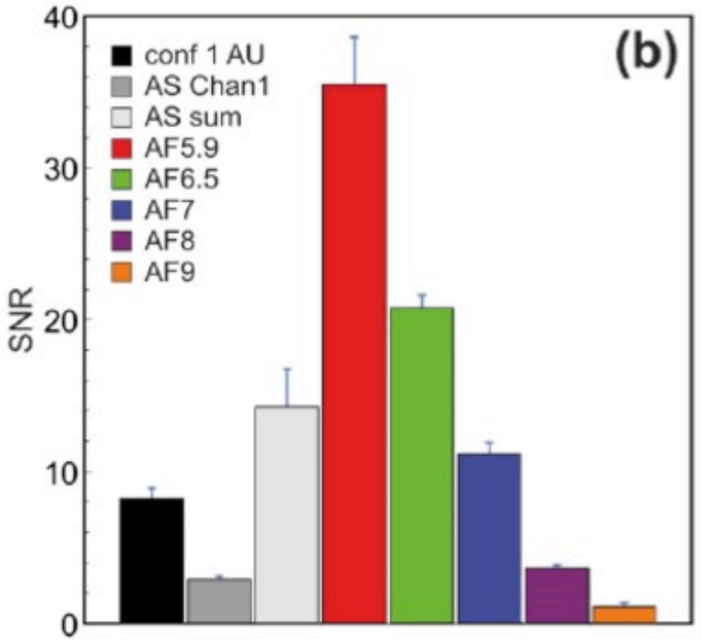
Exploring the Potential of Airyscan Microscopy for Live Cell Imaging

Kseniya Korobchevskaya [1], B. Christoffer Lagerholm [2], Huw Colin-York [3] and Marco Fritzsche [1,3,*]

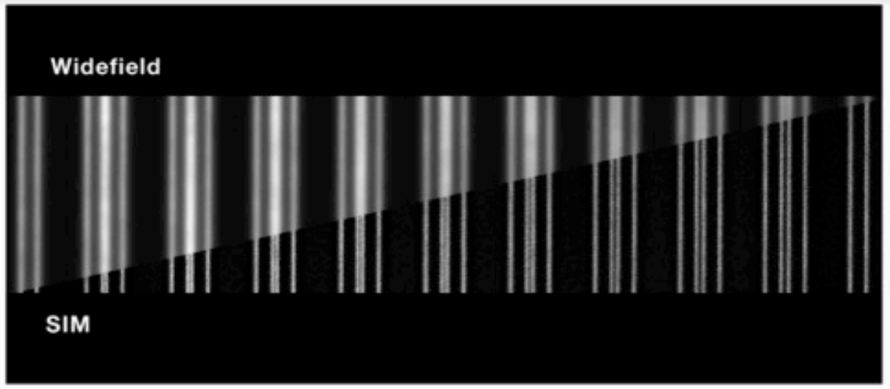


From https://www.zeiss.fr/microscopie/produits/confocal-microscopes/lsm-800-with-airyscan.html#trajet-du-faisceau

Zeiss Airyscan technology measured with Argolight SIM slide



http://argolight.com/products/argo-sim/

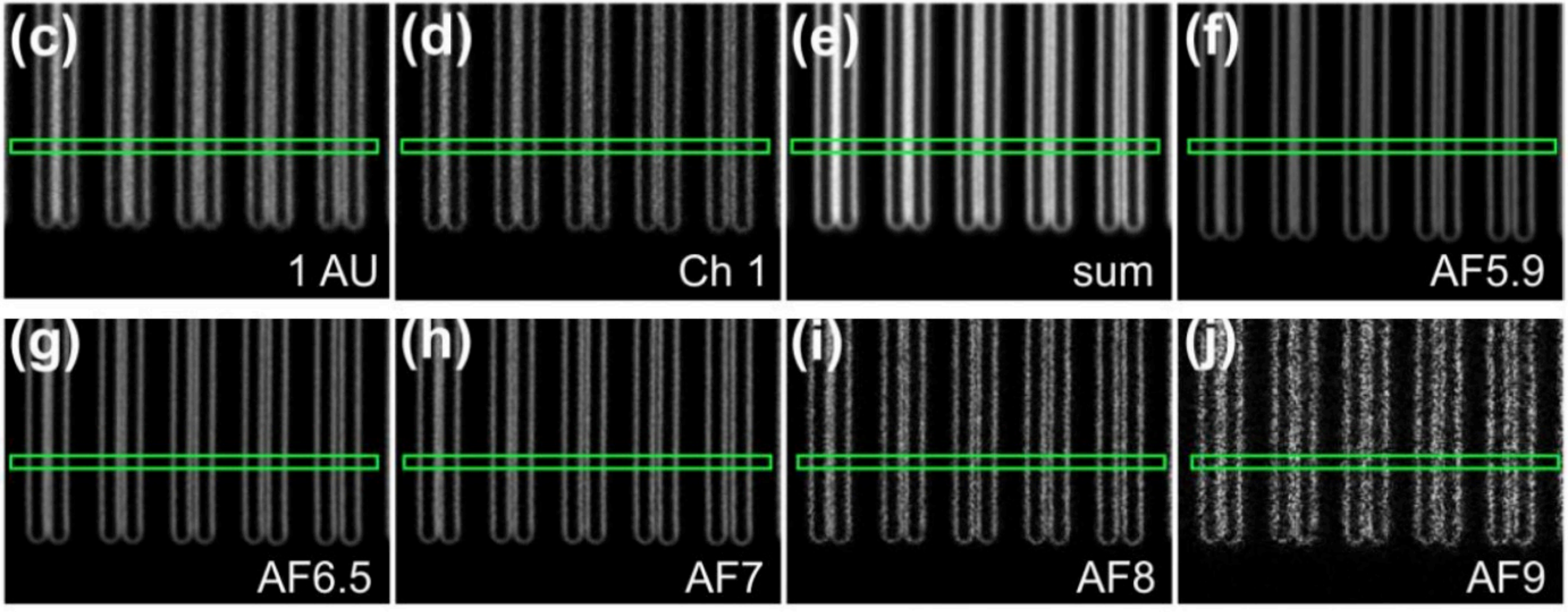


hv photonics MDPI

Article

Exploring the Potential of Airyscan Microscopy for Live Cell Imaging

Kseniya Korobchevskaya [1], B. Christoffer Lagerholm [2], Huw Colin-York [3] and



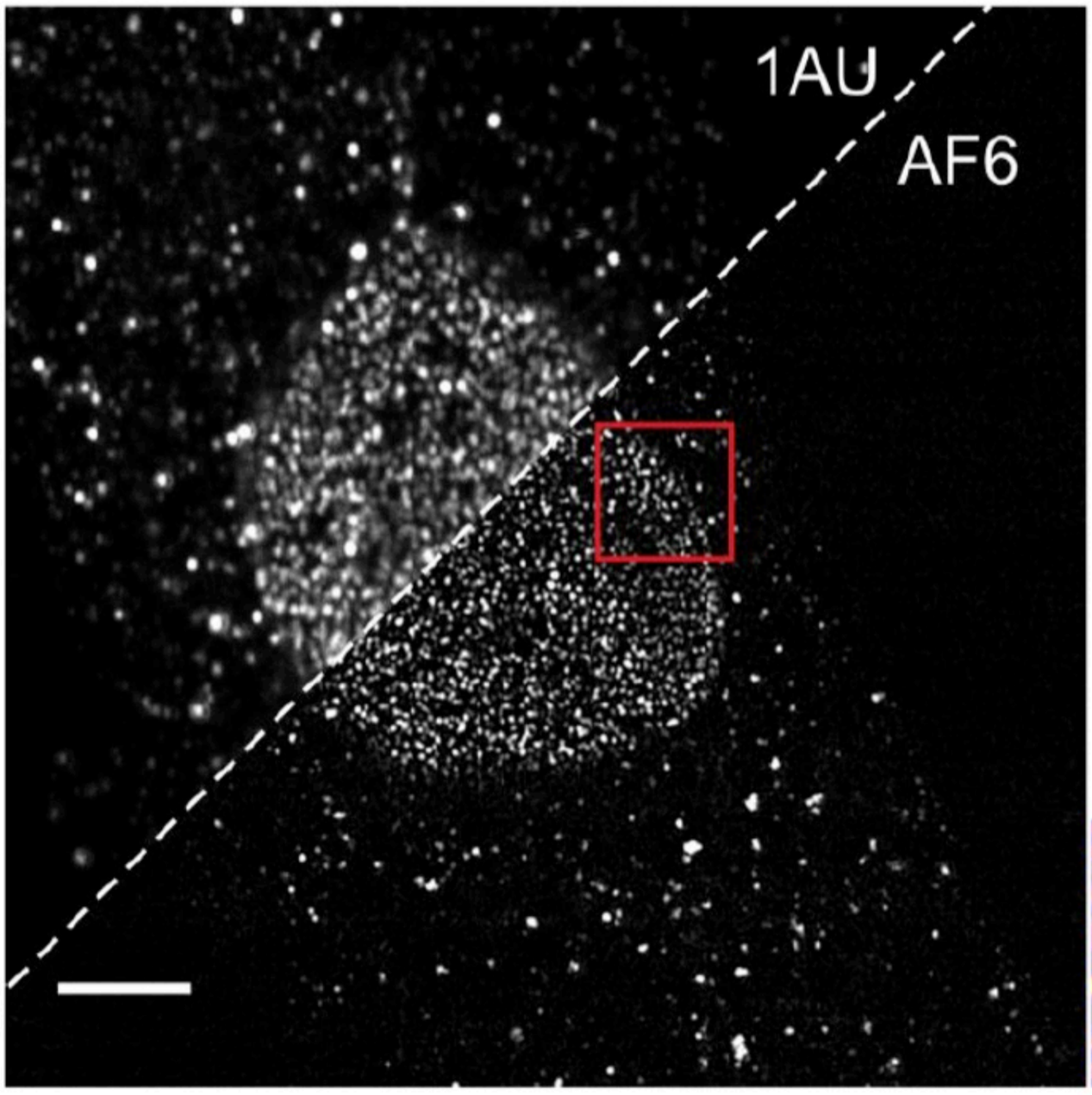
Zeiss Airyscan technology

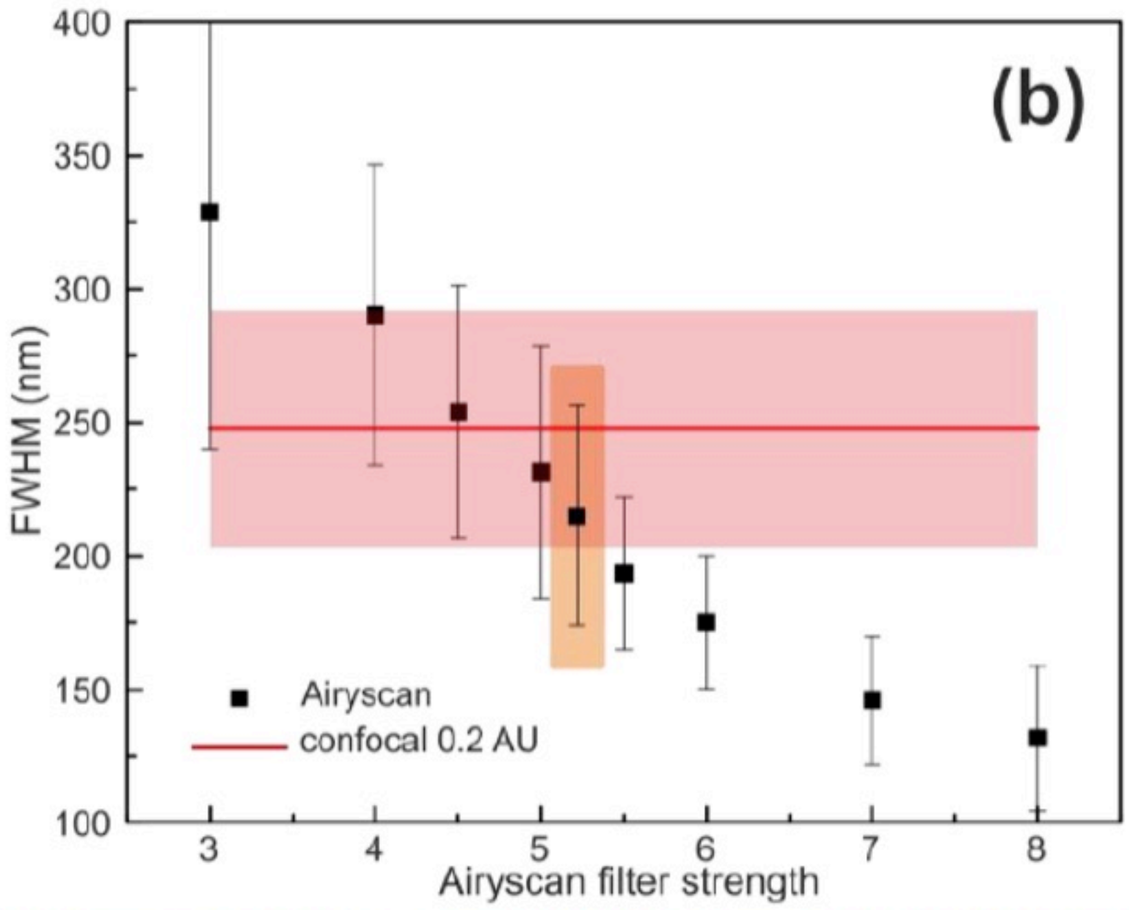
photonics MDPI

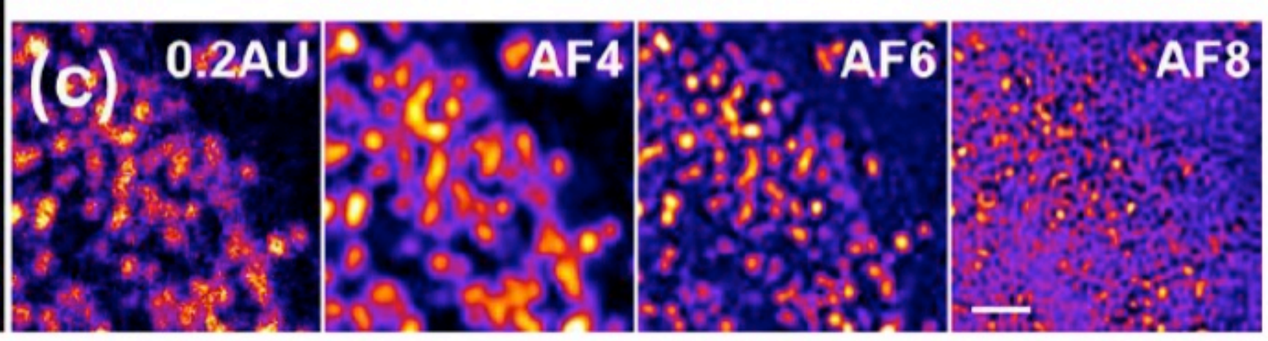
Article

Exploring the Potential of Airyscan Microscopy for Live Cell Imaging

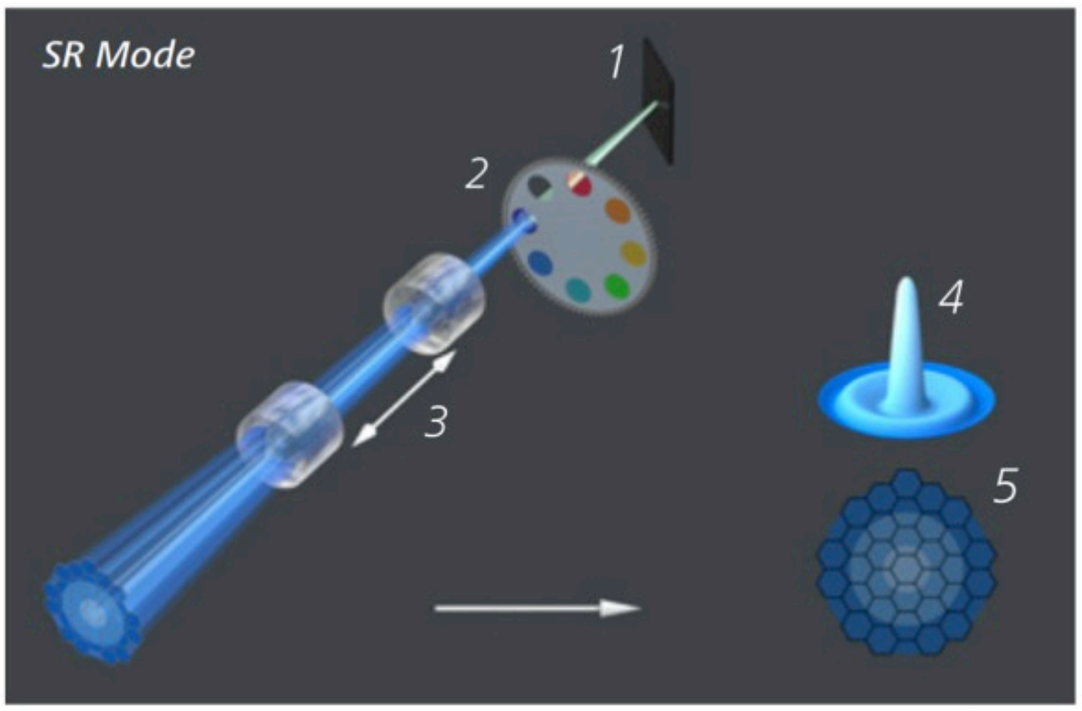
Kseniya Korobchevskaya [1], B. Christoffer Lagerholm [2], Huw Colin-York [3] and Marco Fritzsche [1,3,*]

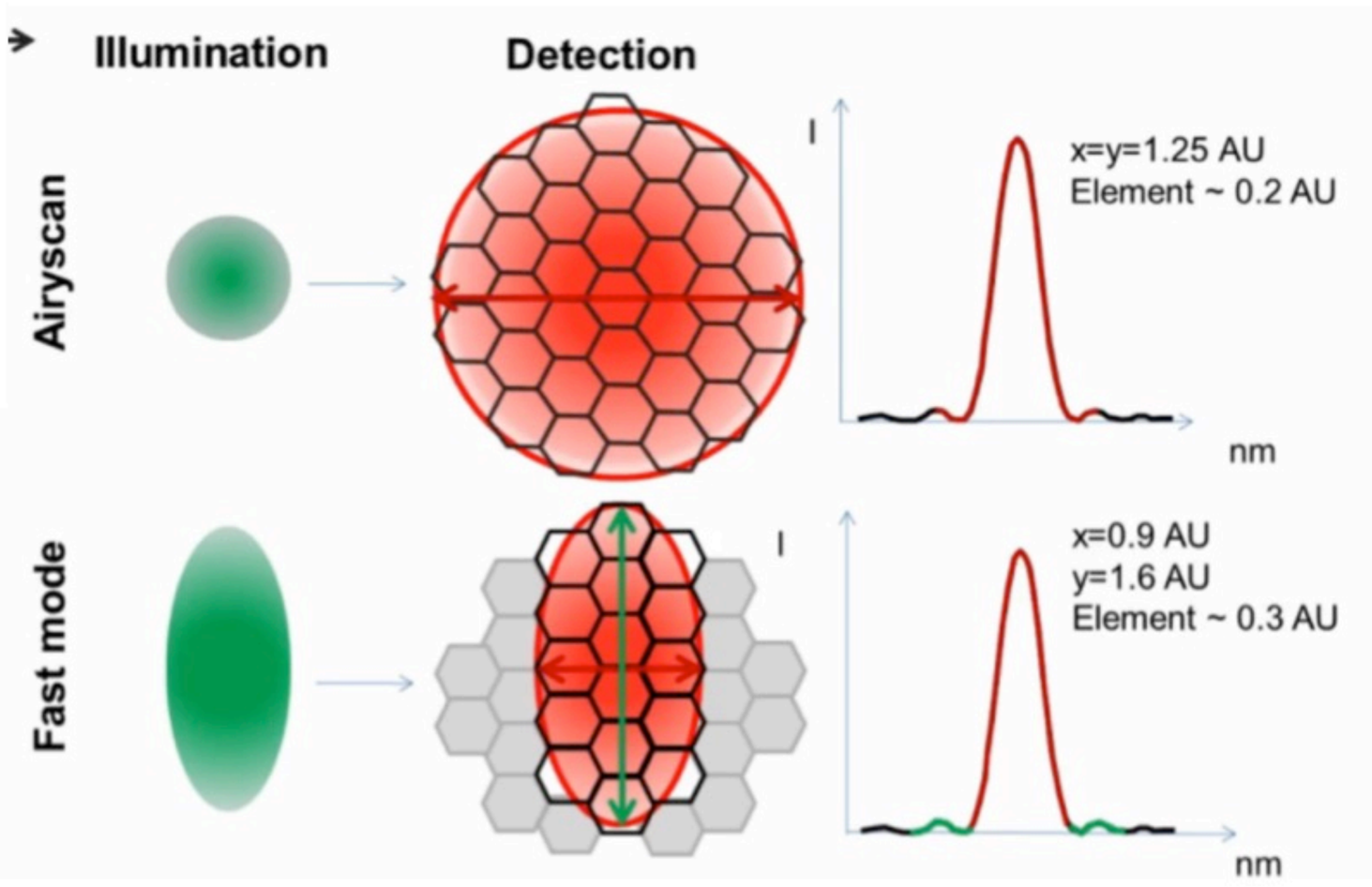


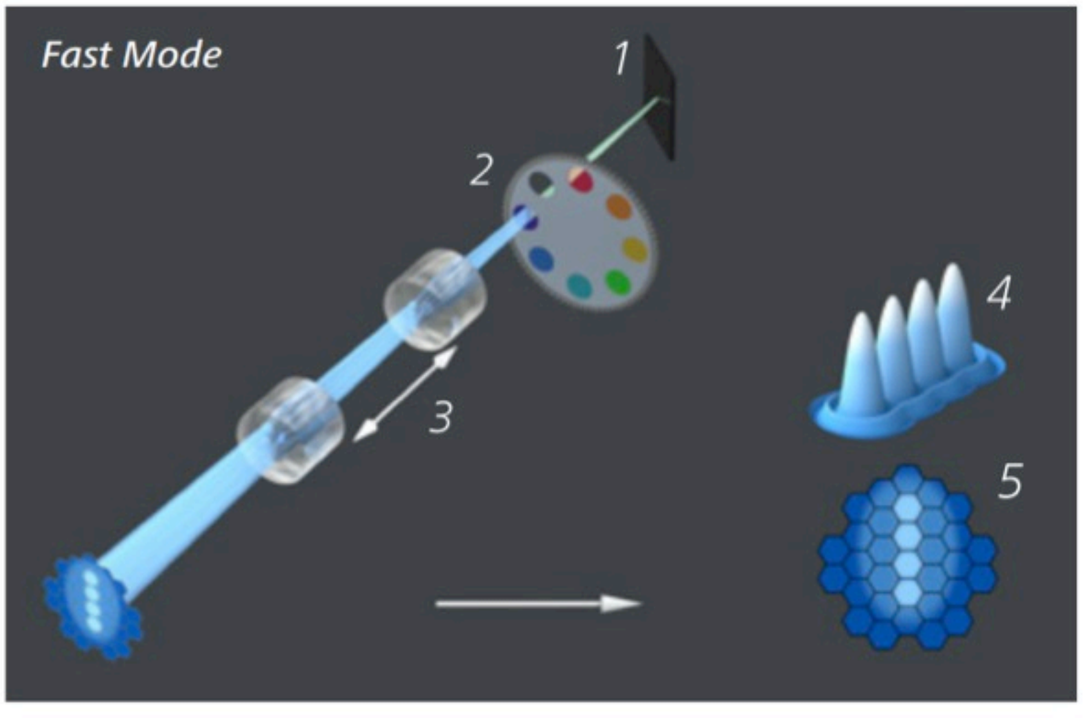




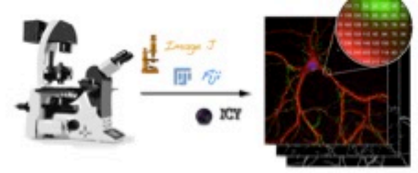
Zeiss fast Airyscan for live cell imaging







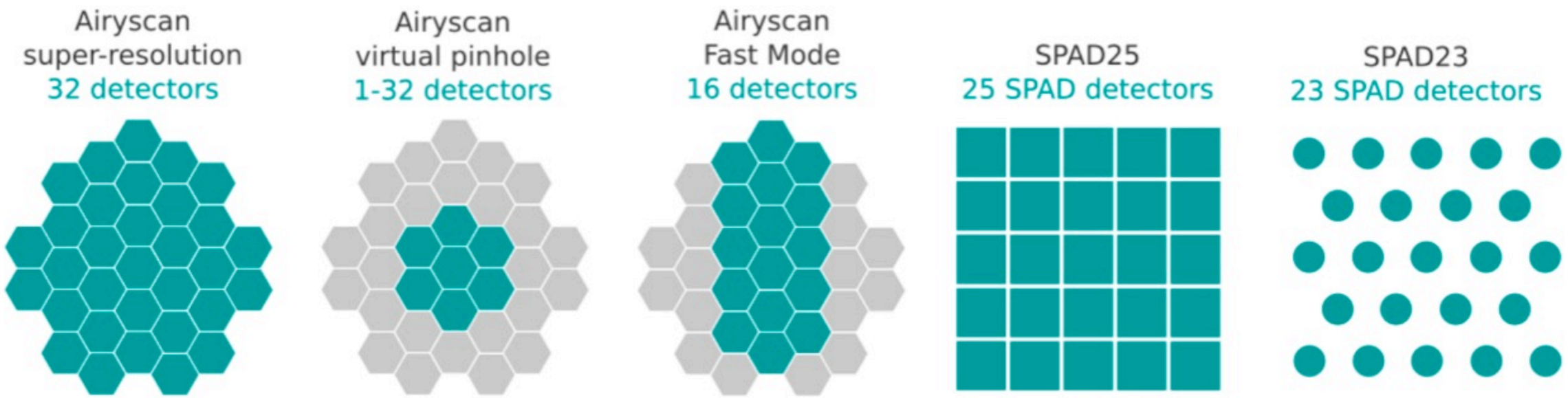
From http://crl.berkeley.edu/2016/06/14/the-fast-module-for-zeiss-lsm-880-airyscan-is-here/



Zeiss fast Airyscan for live cell imaging

About array-detector confocal systems

Within array-detector confocal systems, an array of multiple detectors replaces the conventional pinhole and single detector typical for a regular confocal system. Each individual detector acts as a small pinhole, where all but the central detector are slightly displaced with respect to the original pinhole center. The signal from each detector is used to build up an image, and each image is a slightly shifted version of the central detector image. The total (summed) signal collected by all detectors is comparable to that of a single large pinhole in 'classic' confocal. Consequently, an array-detector system has the advantage of combining the benefit of high-signal of a large pinhole confocal system with the high- resolution aspect of a small pinhole confocal system.



The **Huygens Array Detector Optical Option** allows you to obtain high resolution and contrast from array detector data, such as that acquired on a Zeiss® Airyscan system. The Airyscan microscope can be used in 4 modes, and Huygens now offers high quality deconvolution for each single mode:

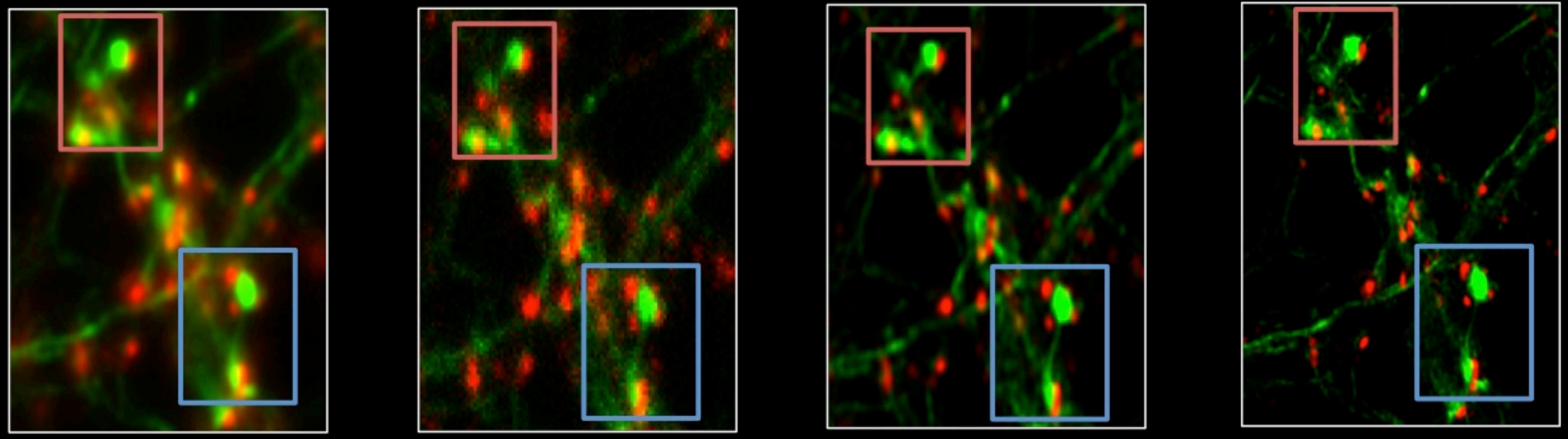
- **Standard mode**: this is the conventional confocal mode of the Airyscan. For Huygens deconvolution you can use the Confocal Optical Option in Huygens setting the pinhole size at the size of the pinhole used during acquisition.
- **Virtual pinhole mode**: in this mode you can decide for the size of the pinhole in post-processing. For Huygens deconvolution you can use the Confocal Optical Option in Huygens setting the pinhole size at the size of the pinhole decided in post-processing.
- **Super Resolution mode**: these datasets include all 32-detector images. The Array-detector confocal option in Huygens includes various modes to intelligently combine the information from these 32-detector datasets and deconvolve data acquired in Airyscan super-resolution mode.
- **Fast mode**: this mode uses elongated laser excitation in combination with 16 detectors (the central 3 'vertical' detector columns). The fast mode allows for the acquisition of 4 lines simultaneously, thereby speeding up the image acquisition with a factor of 4. The Array-detector confocal option in Huygens includes a special Fast Airyscan mode to optimally process the 16-detector datasets from Fast Airyscan.

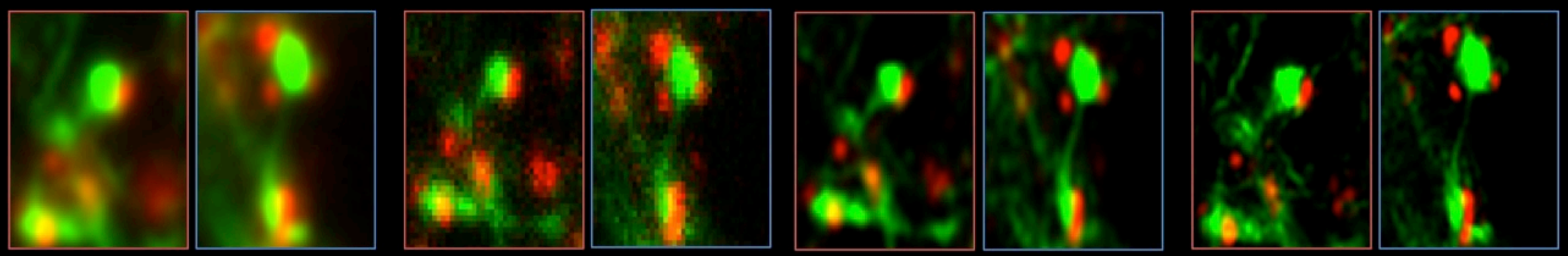
Adapted from https://svi.nl/Array-Detector

From widefield to SIM

F-actin Bassoon

Widefield Confocal Airyscan SIM





Resolution (nm)

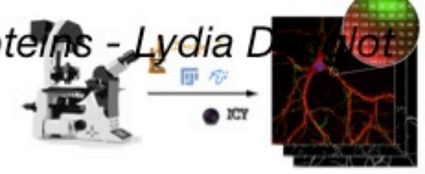
around 350 around 200 up to 140 up to 120

ZEISS LSM880
LEICA SP5, SP8

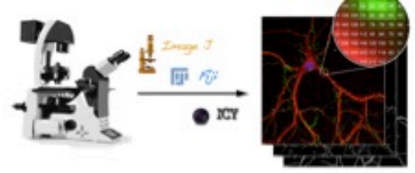
ZEISS LSM880
LEICA SP5, SP8
Spinning-disk

ZEISS LSM880

ZEISS LSM880

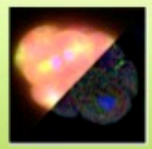


Super resolution microscopy



Resolution:

- Recalage
- PSF, deconvolution

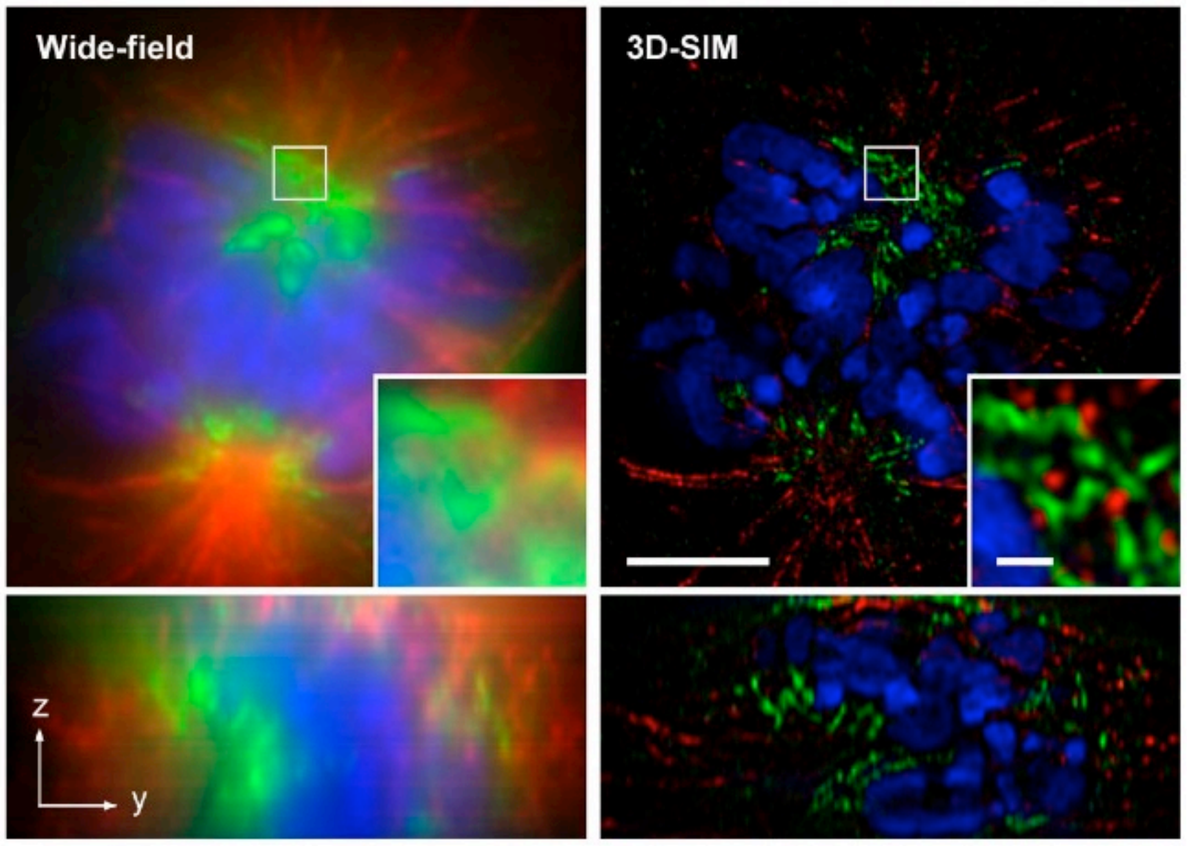


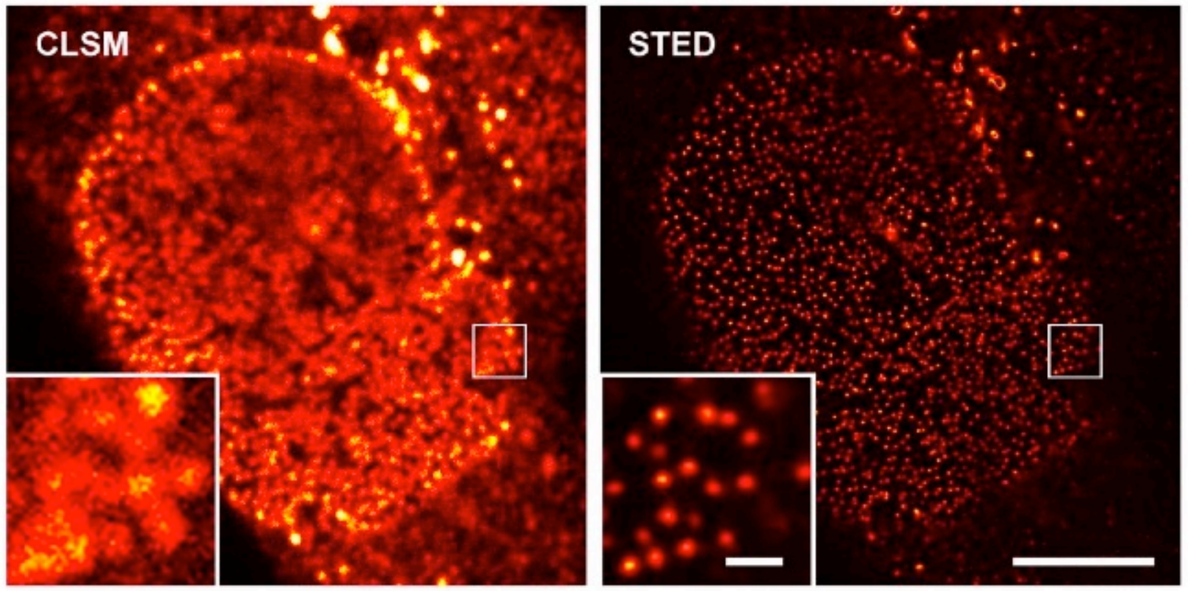
A guide to super-resolution fluorescence microscopy.

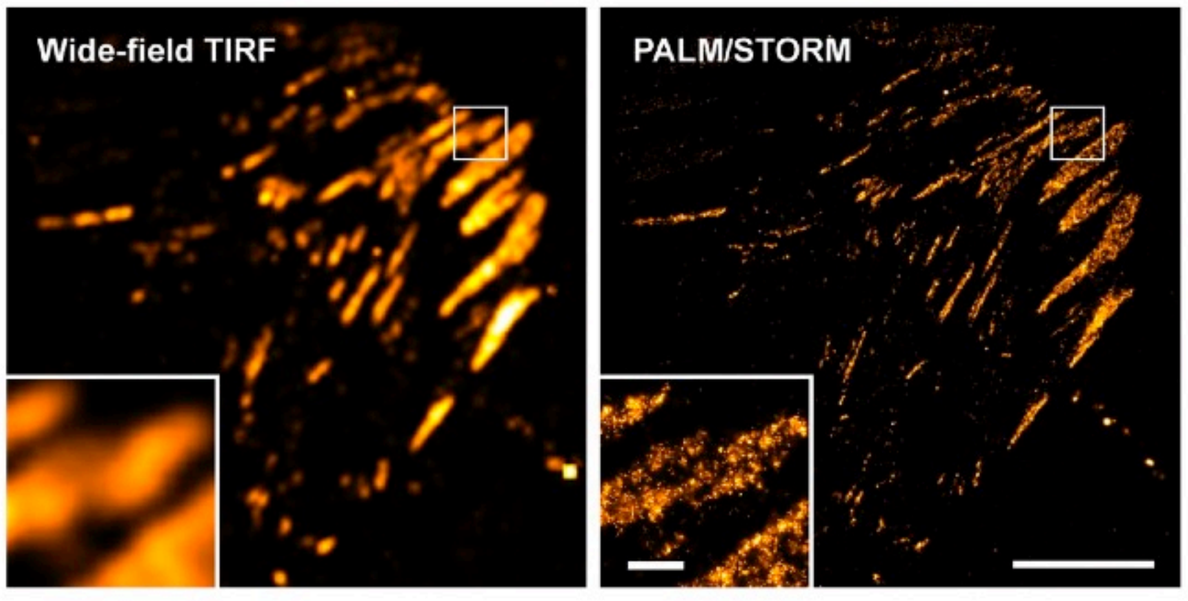
Schermelleh L[1], Heintzmann R, Leonhardt H.

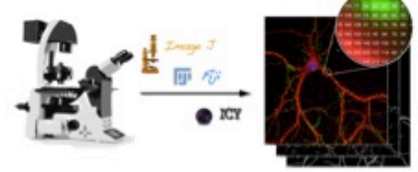
J Cell Biol. 2010 Jul 26; 190(2): 165–175.

JCB



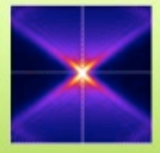


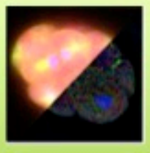




Resolution:

- Recalage
- PSF, déconvolution





Breaking the Resolution Limit in Light Microscopy

Rainer Heintzmann*, 1, Gabriella Ficz†, 2

Methods in Cell Biology
Volume 114, 2013, Pages 525–544

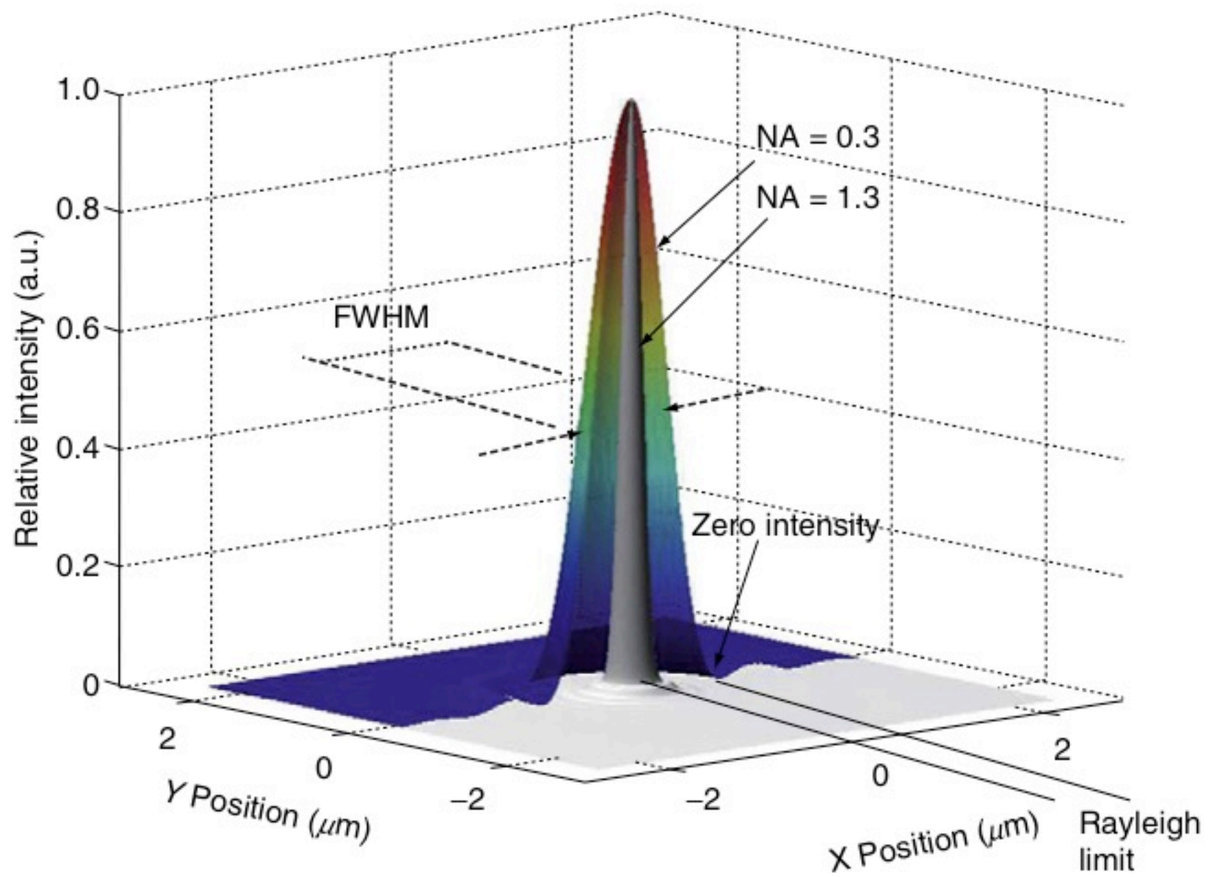
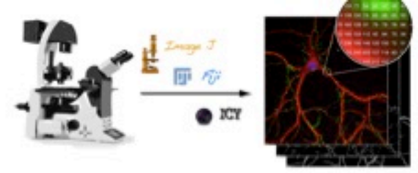


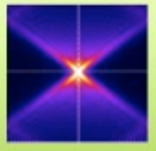
FIGURE 22.1

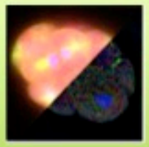
The point spread function (PSF). Two PSFs are shown, one for high numerical aperture (NA = 1.3 in gray) and one for NA = 0.3 (slightly transparent shades). As can be seen, the low-NA PSF is wide and has well-defined positions of zero intensity, leading to the definition of the Rayleigh limit. For this, PSF also the definition of full width measured at half the maximum (FWHM) is shown. The high-NA PSF (uniformly gray peak in the middle) is much finer but does not have the rings of zero intensity.



Resolution:

- Recalage
- PSF, déconvolution





A guide to super-resolution fluorescence microscopy.

Schermelleh L[1], Heintzmann R, Leonhardt H.

J Cell Biol. 2010 Jul 26; 190(2): 165–175.

JCB

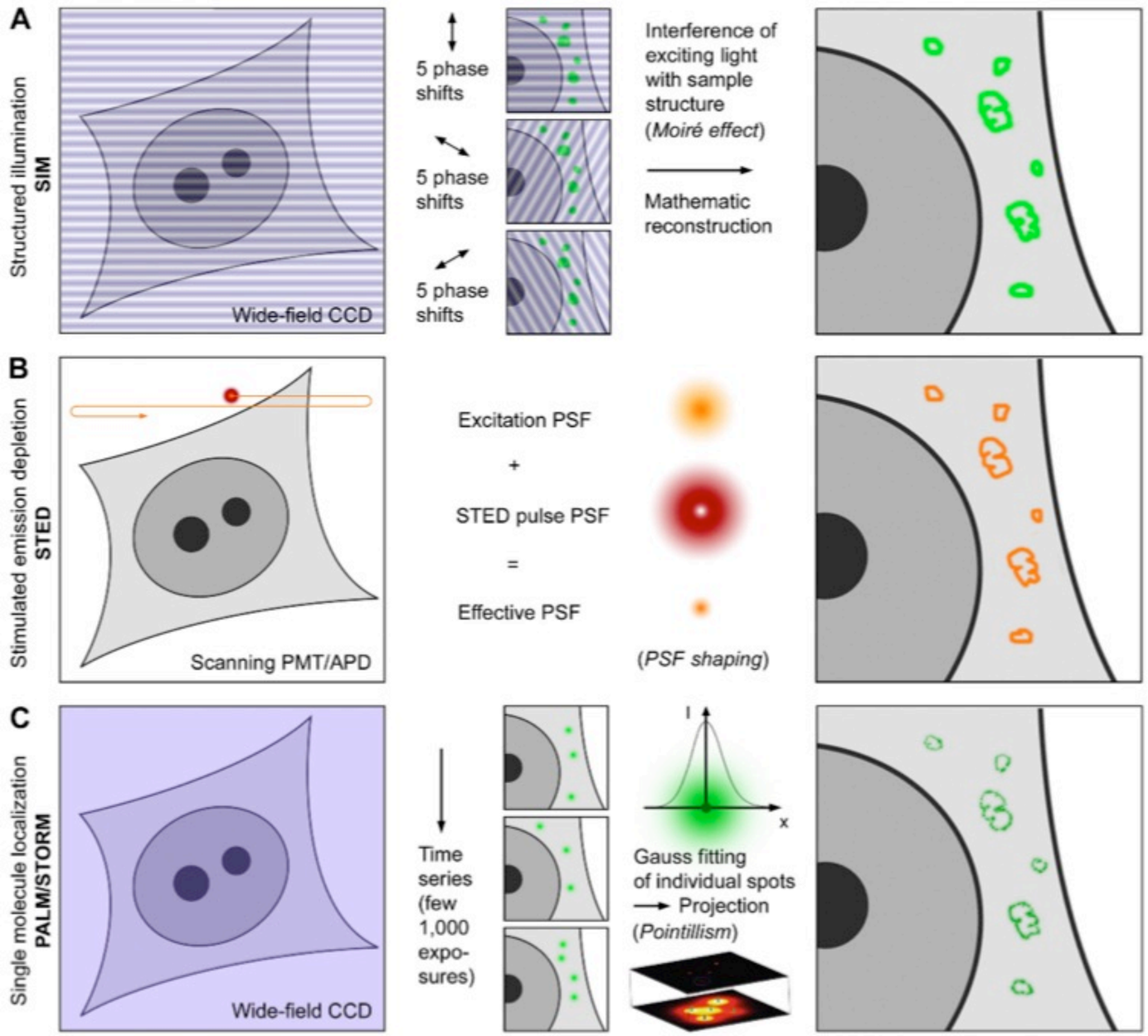
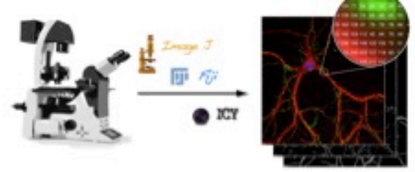
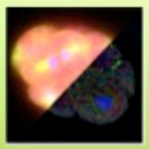


Figure 1. **Super-resolution imaging principles.** (A) In SIM the sample plane is excited by a nonuniform wide-field illumination. Laser light passes through an optical grating, which generates a stripe-shaped sinusoidal interference pattern. This combines with the sample information originating from structures below the diffraction limit to generate moiré fringes. The image detected by the CCD camera thus contains high spatial frequency sample information shifted to a lower spatial frequency band that is transmitted through the objective. A mathematical reconstruction allows, from a series of 15 raw images per slice, to reconstruct a high-resolution image with doubled resolution in xy compared with wide-field resolution. In 3D-SIM additional doubling in the axial resolution is achieved by accounting for an additional modulation introduced along the axial direction. (B) In STED microscopy the focal plane is scanned with two overlapping laser beams, typically being pulsed with a mutual time delay. While the first laser excites the fluorophores, the second longer wavelength laser drives the fluorophores back to the ground state by the process of stimulated emission. A phase plate in the light path of the depletion laser generates a donut-shaped energy distribution, leaving only a small volume from which light can be emitted that is then being detected. Thus, the PSF is shaped to a volume smaller than the diffraction limit. (C) Single molecule localization microscopy assures that only a relatively low number of fluorophores are in the emitting (active) state. This is achieved either by photoactivation, photoswitching, triplet state shelving, or blinking. These molecules are detected on the CCD camera as diffraction-limited spots, whose lateral position is determined with very high accuracy by a fit. Single molecule positions from several thousand raw images, each with a different subset of emitters, are then used to generate a density map featuring several hundred thousand single molecule positions within the plane of focus.



Resolution:

- Recalage
- PSF, déconvolution



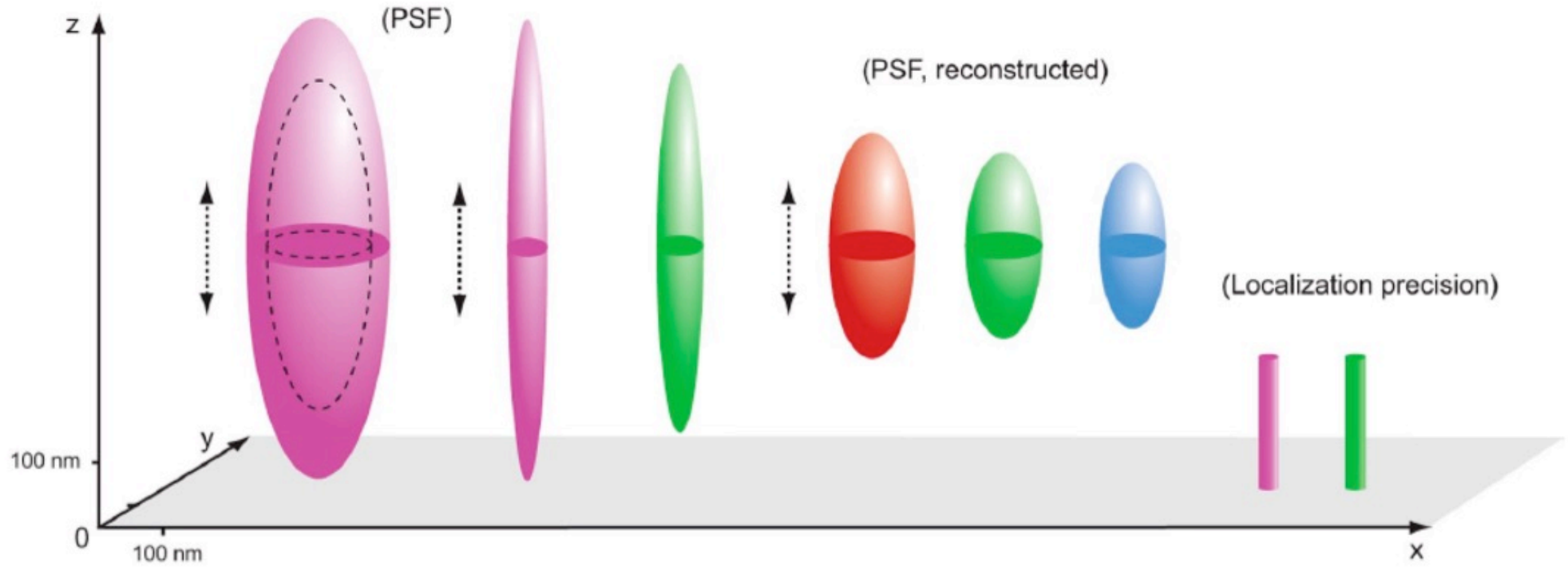
A guide to super-resolution fluorescence microscopy.

Schermelleh L[1], Heintzmann R, Leonhardt H.

J Cell Biol. 2010 Jul 26; 190(2): 165–175.

JCB

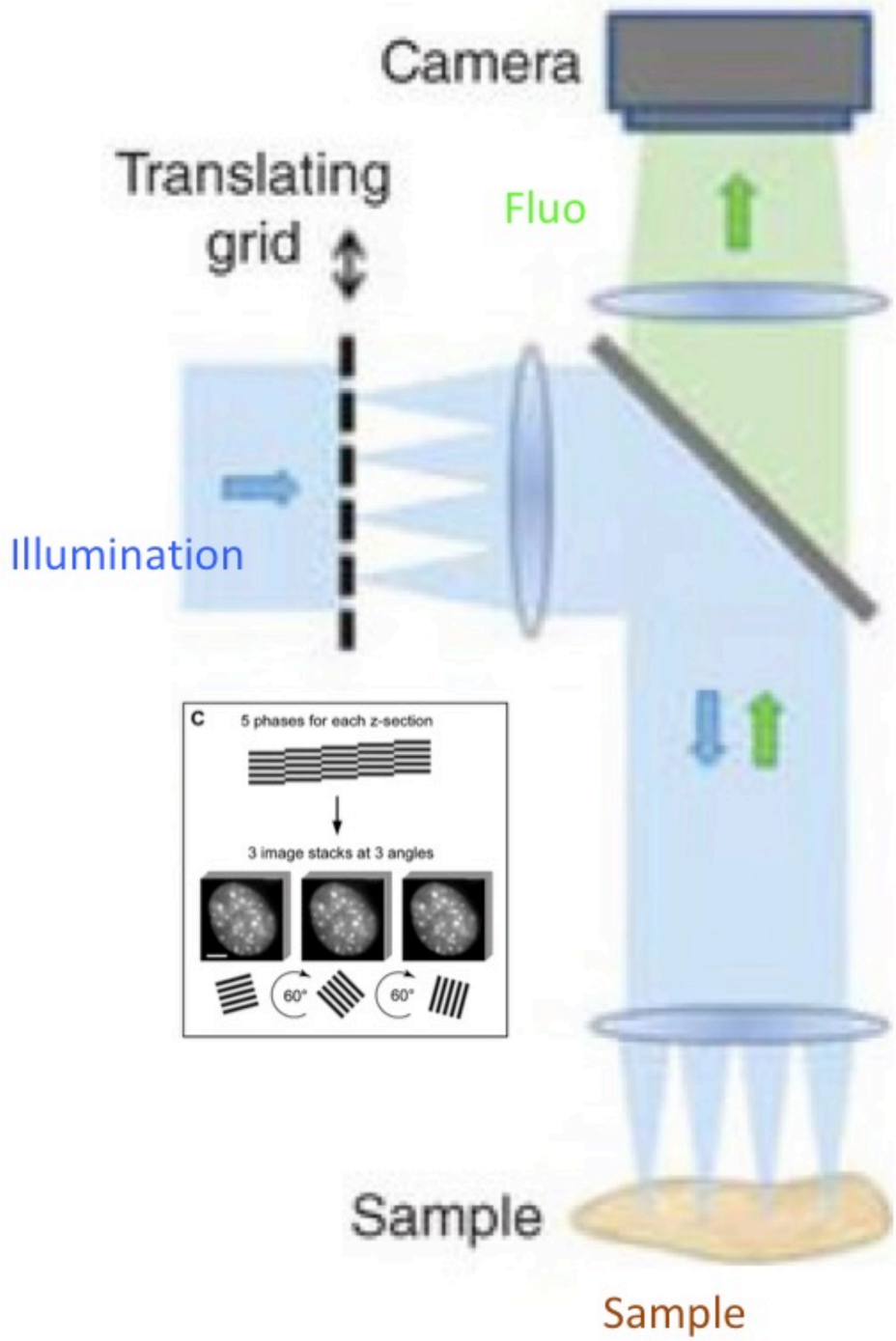
	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λem [nm]	460-670	670	520	620	520	460	670	520



$\sim D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
$\sim D_z$ [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
$\sim V_{x,y,z}$ [$*10^{-3}$ µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Figure 2. **Resolvable volumes obtained with current commercial super-resolution microscopes.** A schematic 3D representation of focal volumes is shown for the indicated emission maxima. The approximate lateral (x,y) and axial (z) resolution and resolvable volumes are listed. Note that STED/CW-STED and 3D-SIM can reach up to 20 µm into the sample, whereas PALM/STORM is usually confined to the evanescent wave field near the sample bottom. It should be noted that deconvolution approaches can further improve STED resolution. For comparison the "focal volume" for PALM/STORM was estimated based on the localization precision in combination with the z-range of TIRF. These indications do not necessarily constitute actual resolution as many other effects (e.g., fluorophore orientation, local refractive index variations, flatfield quality of the camera, local aberrations, and statistical selection bias) influence image quality and final resolution.

Superesolution : Structured Illumination Microscopy

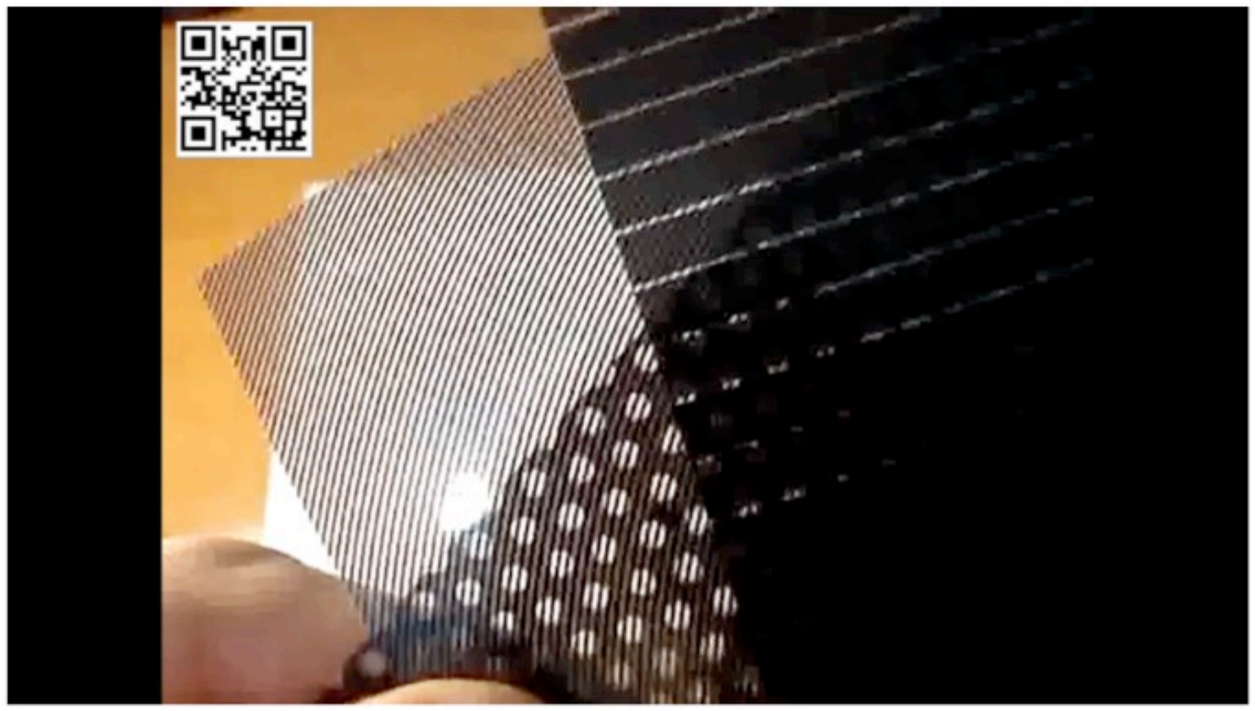


Sample

The sample is illuminated through a grid pattern.

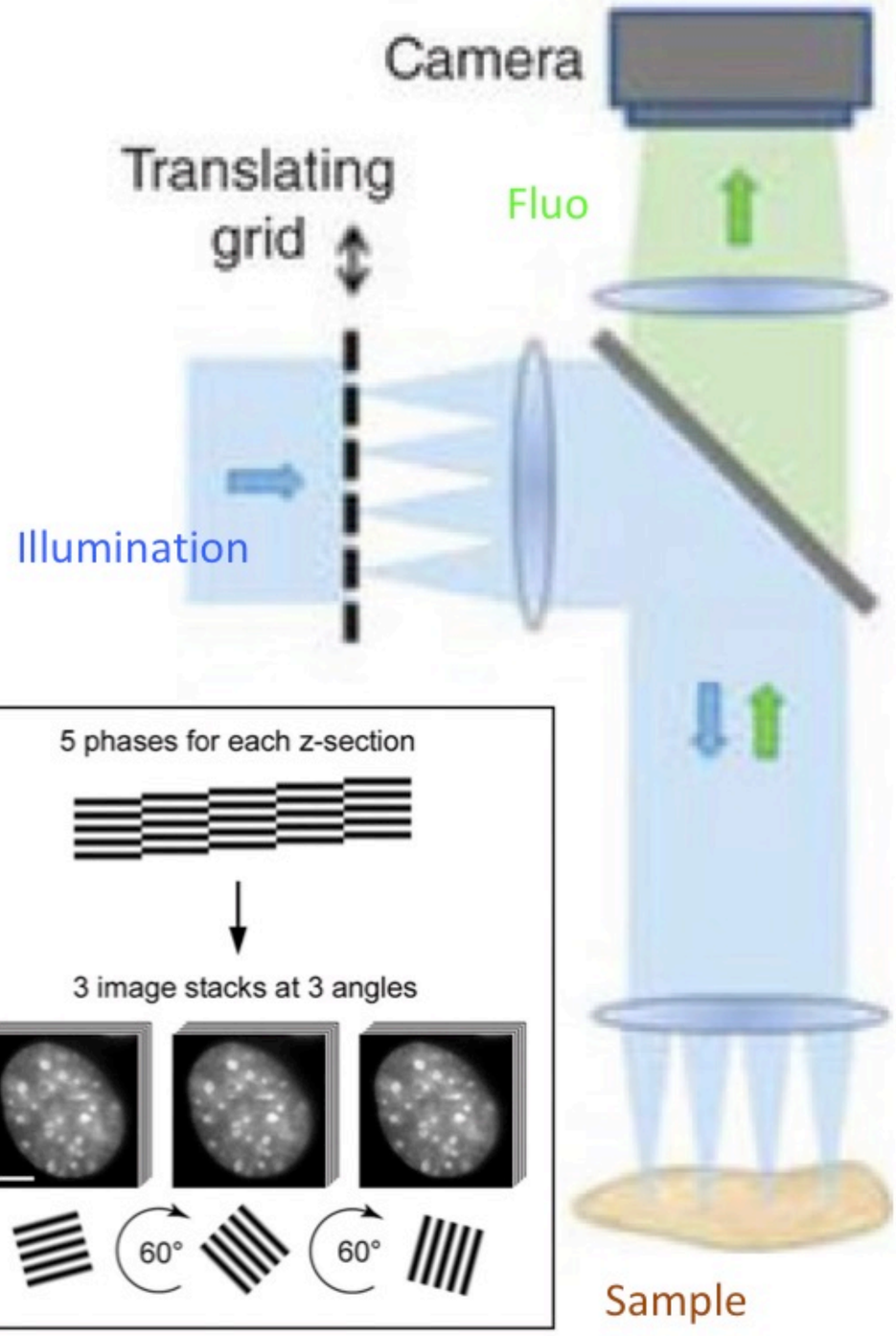
Interference between illumination pattern and the structure pattern produces a third characteristic, the Moiré fringes.

From D. Agard, iBiology.org



Nature Methods **8**, 811–819 (2011)

Superesolution : Structured Illumination Microscopy (SIM)



The sample is illuminated through a grid pattern.

Interference between illumination pattern and the structure pattern produces a third characteristic, the Moiré fringes.

From D. Agard, iBiology.org

The Moiré fringes have a lower spatial frequency than the original structure of the sample and thus can be transmitted through the objective lens and can have their image.

To have a gain of isotropic resolution :
- 3 differents orientations of the grid and
- 5 differenst phases of the illumination pattern.

Original image is then reconstructed using Fourrier transform.

Resolution SIM : 100 nm (vs 200 nm with Confocal)

Nature Methods **8**, 811–819 (2011)

Superesolution : Structured Illumination Microscopy

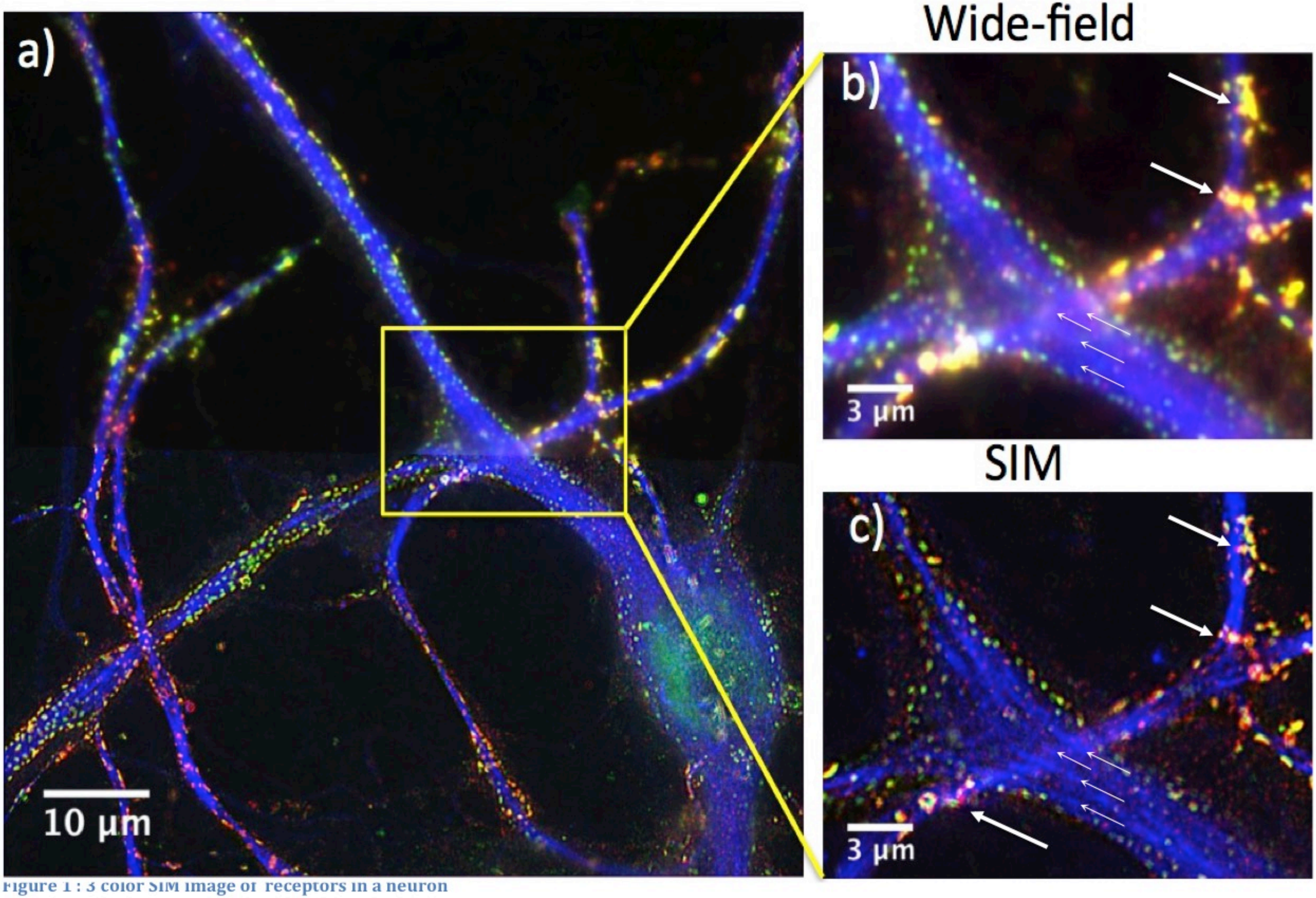


Figure 1 : 3 color SIM image of receptors in a neuron

SIM *microscopy*

Widefield microscopy

SIM *microscopy*

Widefield microscopy

SIM *microscopy*

Increased signal to noise ration

Widefield microscopy

STED microscopy

KUNGL. VETENSKAPS AKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

The Royal Swedish Academy of Sciences has decided to award the Nobel Prize in Chemistry for 2014 to **Eric Betzig**, **Stefan W. Hell** and **William E. Moerner** åfor the development of super-resolved fluorescence microscopyé.

The Nobel Prize 2014 **in Chemistry**

Their microscopes crossed the threshold

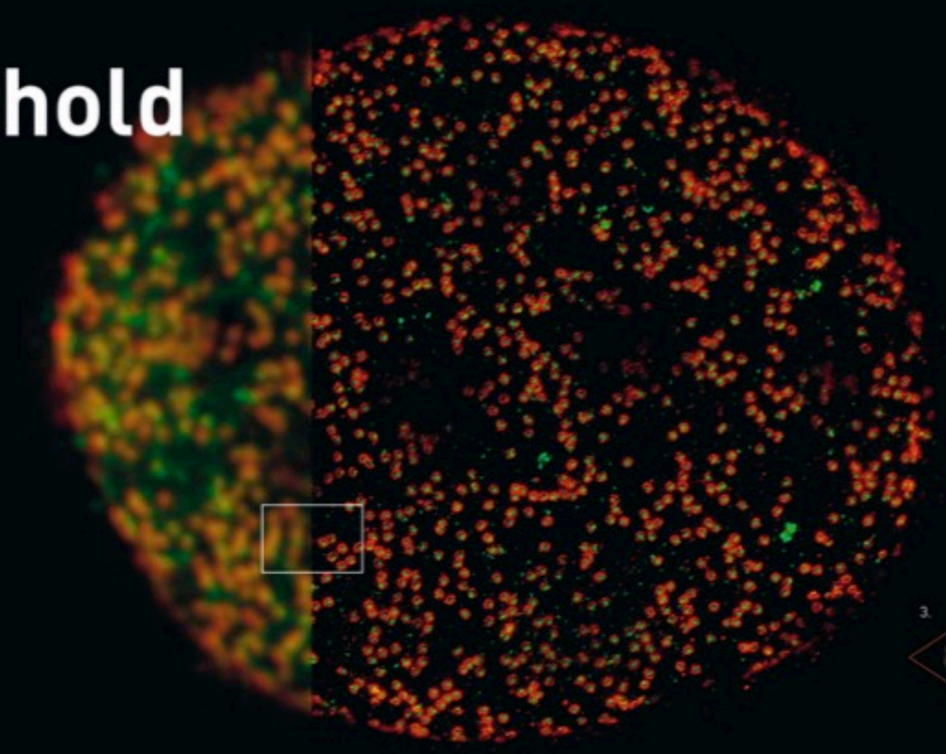
Optical microscopy had long been hindered by a presumed limitation: that it was impossible to achieve a resolution better than half the wavelength of light. Eric Betzig, Stefan W. Hell and William E. Moerner are awarded the 2014 Nobel Prize in Chemistry for ingeniously bypassing this limitation. Their revolutionary work has taken optical microscopy to nano dimensions.

Using what is now called nanoscopy, researchers can see the paths of individual molecules inside living cells. For example, they can see how molecules form synapses between the brainäs nerve cells or follow aggregating proteins in cases of Parkinsonäs, Alzheimeräs or Huntingtonäs disease.

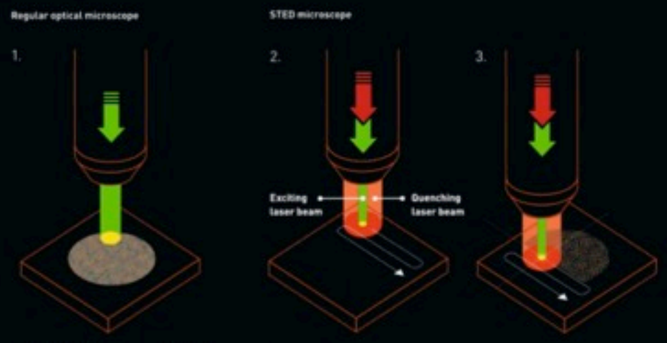
Optical microscopy is one of the most important tools in the life sciences, allowing researchers to observe processes inside living cells. However, it had long been thought that it would be impossible to discern a cell in molecular detail. In 1873, microscopist Ernst Abbe determined a threshold for optical microscopy: its resolution would never be better than half the wavelength of light, approximately 0.2 micrometres Å but, thanks to the 2014 Nobel Laureates in chemistry, optical microscopy can now be used to observe the nano world.

Two different principles are being recognised and rewarded; both build upon researchers labelling the objects to be studied with fluorescing molecules. The idea for one of the microscopy methods, *stimulated emission depletion (STED)*, came to Stefan Hell in 1993, and he realised it experimentally in 2000.

The theory behind the second method, *single-molecule microscopy*, was laid out by Eric Betzig in 1995 Å but it was William Moerner who built its practical foundations in 1989, when he was the first person to detect a single fluorescing molecule. The second decisive step was taken by Moerner in 1997, when he developed a tiny molecular lamp that he could turn on and off. Thanks to these successes, Betzig was able to actualise single-molecule microscopy in 2006.



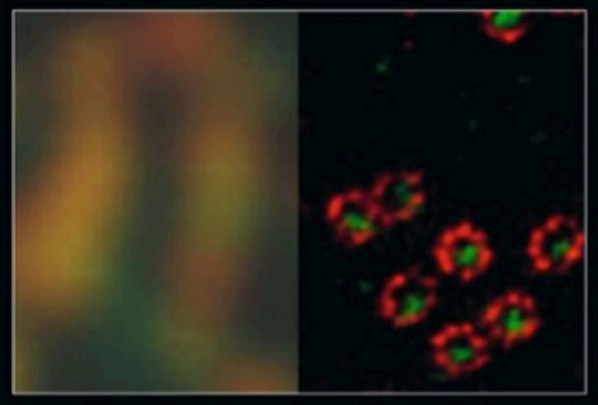
The principle of STED microscopy



2000 å Hell develops STED microscopy

1. In a regular microscope the light beam is broad and the resolution is never better than 0.2 micrometres.
2. Stefan Hell started to use two laser beams in a microscope. One excites all the fluorescing molecules which make them glow. The second, which is ring-shaped, quenches all the fluorescing molecules apart from those within a nanosized volume.
3. The laser beams sweep across the sample, nanometre by nanometre. The researchers know exactly where the beam hits the sample and can use this information to process the image, resulting in a resolution that is far better than 0.2 micrometres.

Conventional microscopy **Super-resolved microscopy**



2006 å Betzig develops single-molecule microscopy

1. In 2006, Eric Betzig circumvented Abbeäs limit using a variant of GFP similar to that produced by Moerner. Using a weak pulse of light he turned on a fraction of all the fluorescing GFP in a sample. Because so few were excited the distances between them were large and the microscope could discern every single GFP. They remained lit until they faded while an image was registered.

Betzig then turned on a new subgroup of GFPs and took a new picture. The procedure was repeated until all the GFPs in the sample had been observed and a thousand images had been registered.

2. The blurry images were processed using probability theory so that they became much sharper.

3. When Betzig layered all the images on top of each other, they produced a high-resolution image in which individual proteins could be discerned.

The principle of single-molecule microscopy

↑ The picture shows a cell nucleus and proteins that form pores leading into and out from the nucleus. Important molecules are transported in and out of the nucleus through these pores. The picture was taken using STED microscopy.

1989 å Moerner lays the foundation for single-molecule microscopy ↑

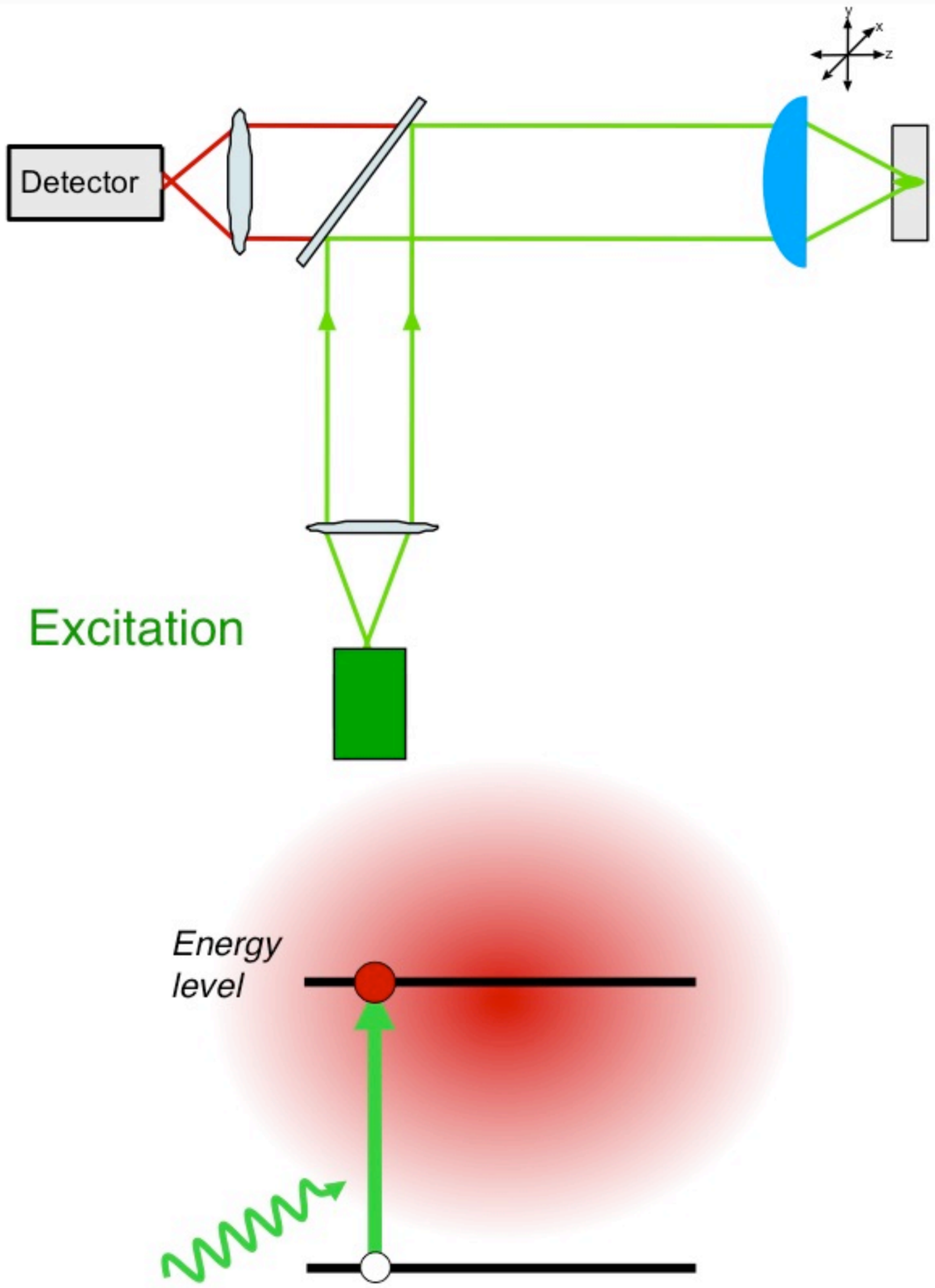
Single-molecule microscopy builds upon a discovery that was awarded the Nobel Prize in Chemistry 2008: the *green fluorescent protein (GFP)*. Using gene technology, researchers couple GFP to other proteins they want to study in a cell. GFP functions as a molecular lamp that shows where the protein is. In 1992, William E. Moerner found a variant of GFP that could be turned on and off, like a tiny molecular lamp with a switch.

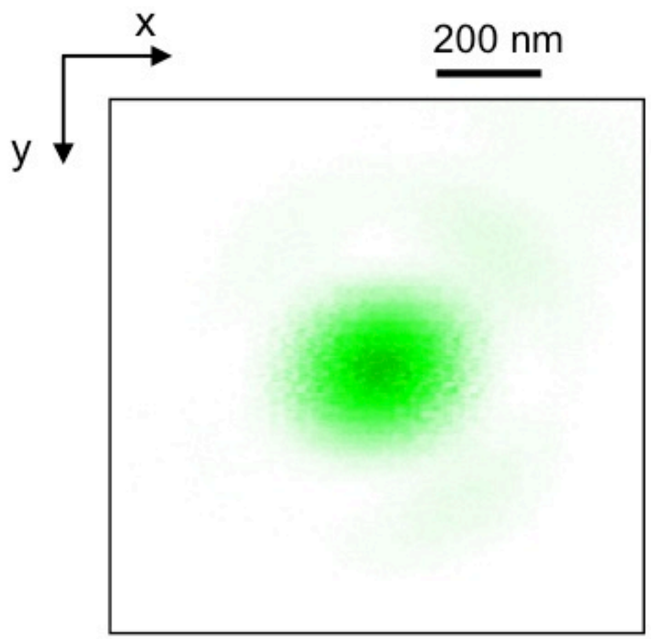
Eric Betzig
U.S. citizen. Born 1960 in Ann Arbor, MI, USA. Group Leader at Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA.

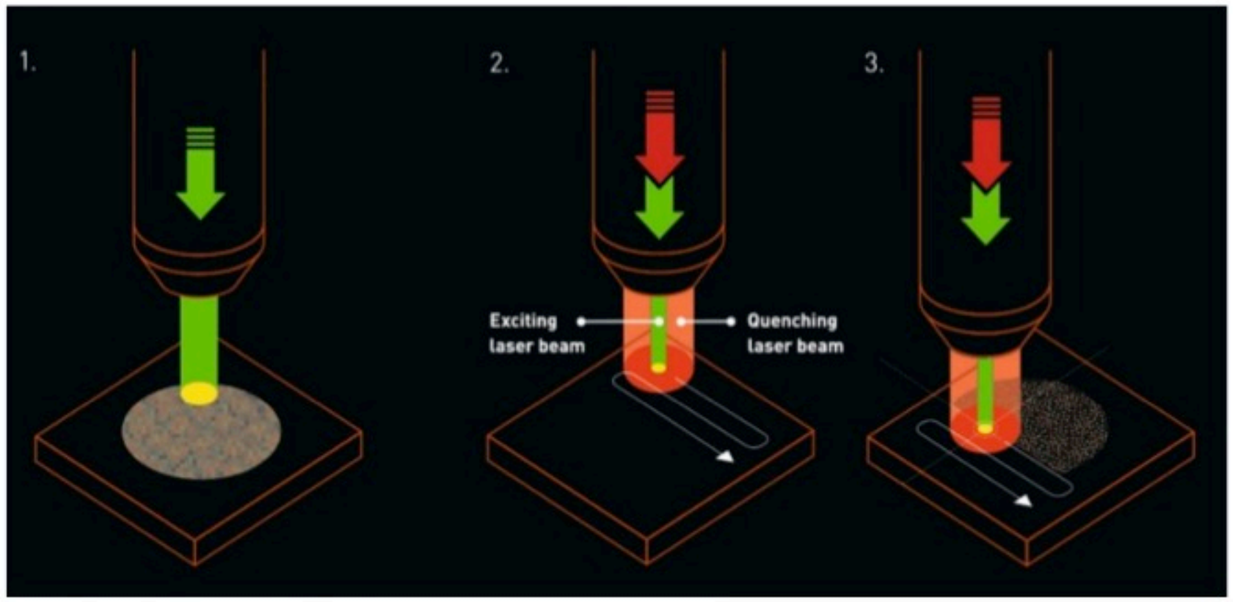
Stefan W. Hell
German citizen. Born 1962 in Arad, Romania. Director at the Max Planck Institute for Biophysical Chemistry, Göttingen, and Division Head at the German Cancer Research Center, Heidelberg, Germany.

William E. Moerner
U.S. citizen. Born 1953 in Pleasanton, CA, USA. Harry S. Mosher Professor in Chemistry and Professor, by courtesy, of Applied Physics at Stanford University, CA, USA.

STED microscopy

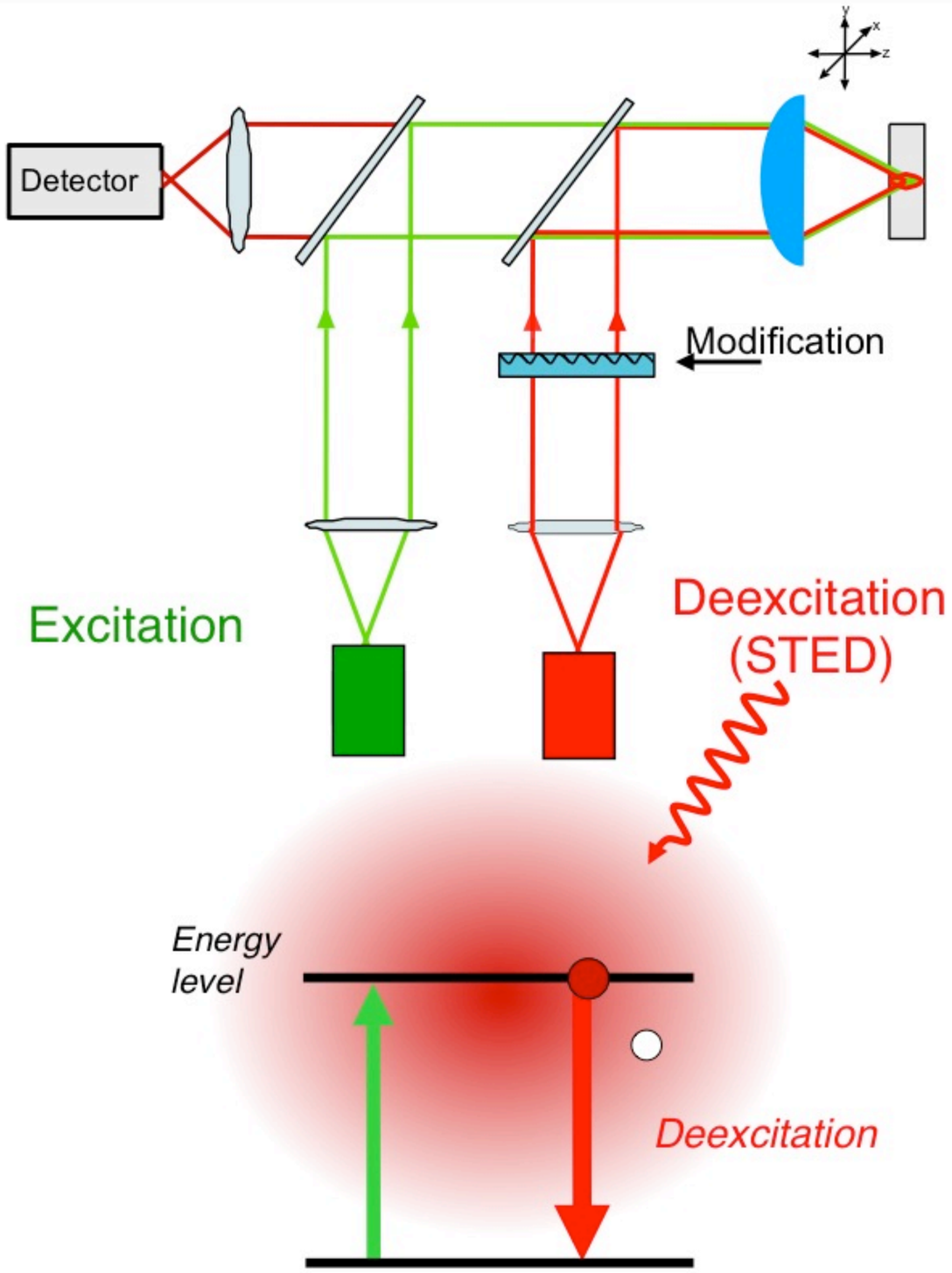


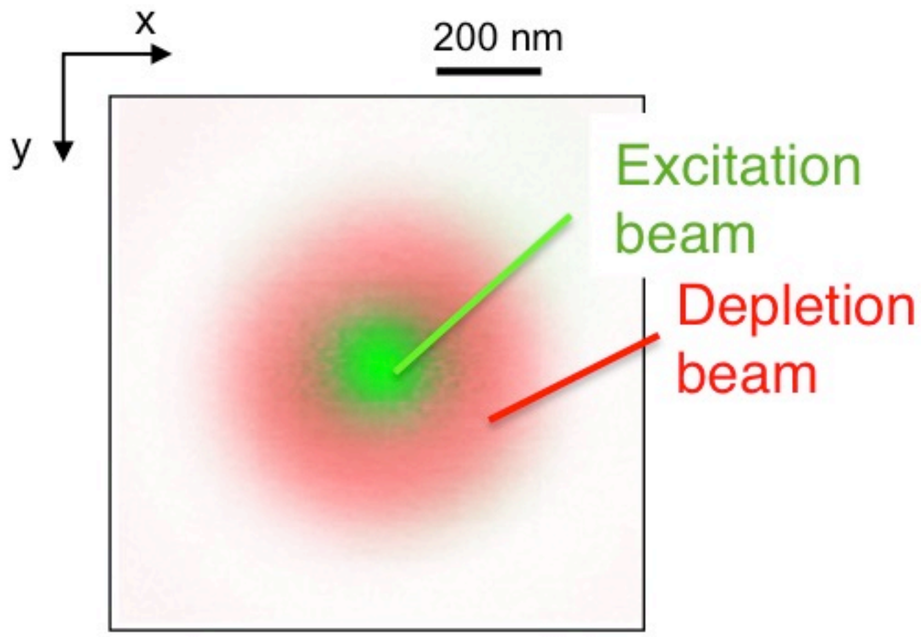


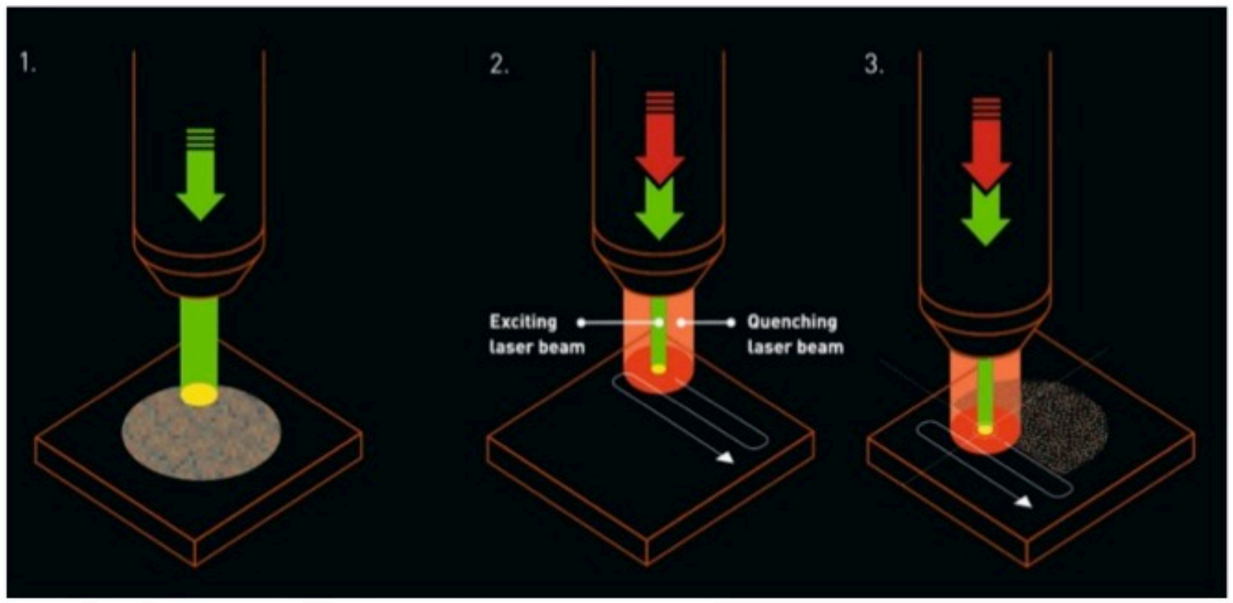


Source: MPI for biophysical chemistry, Göttingen

STED microscopy

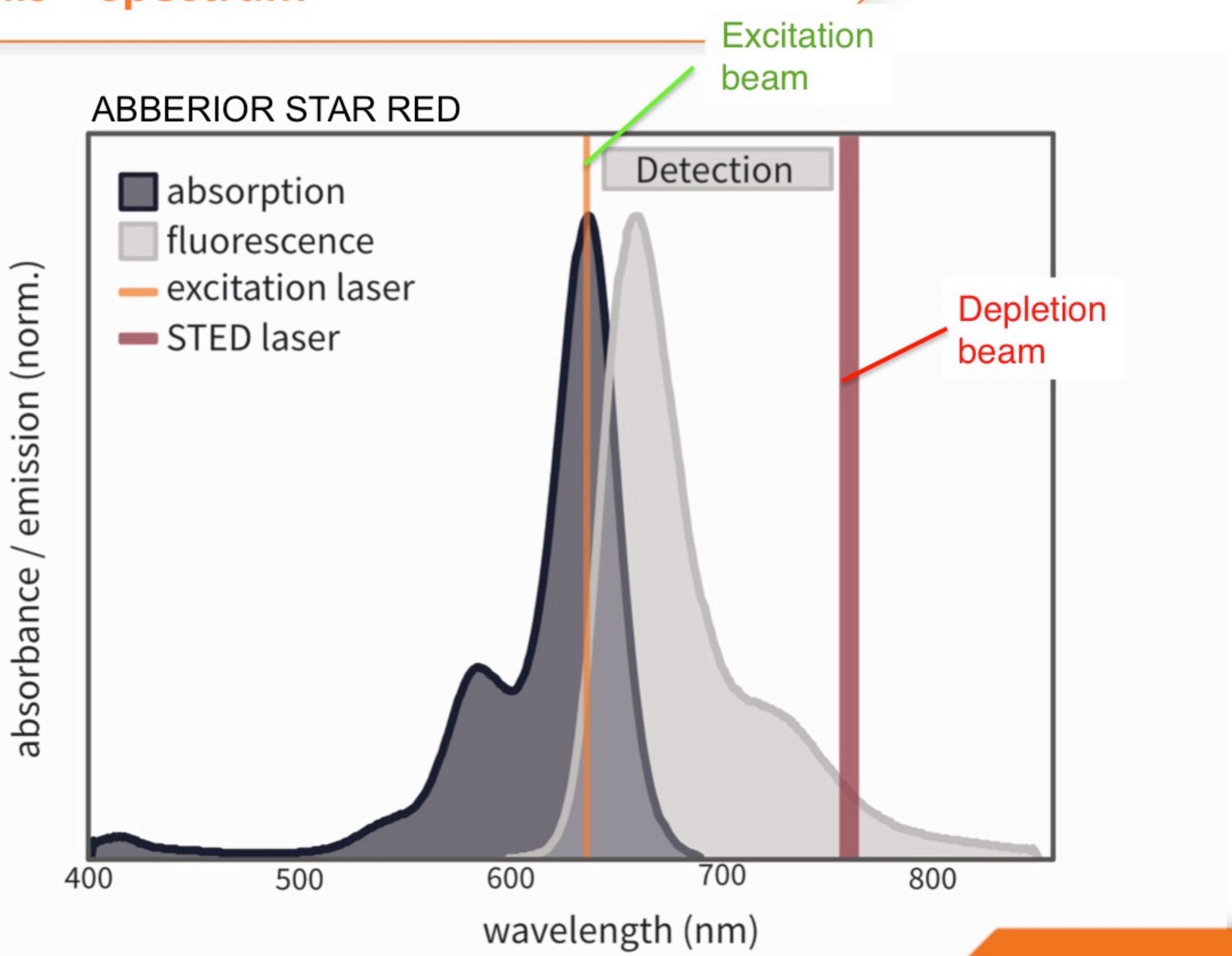




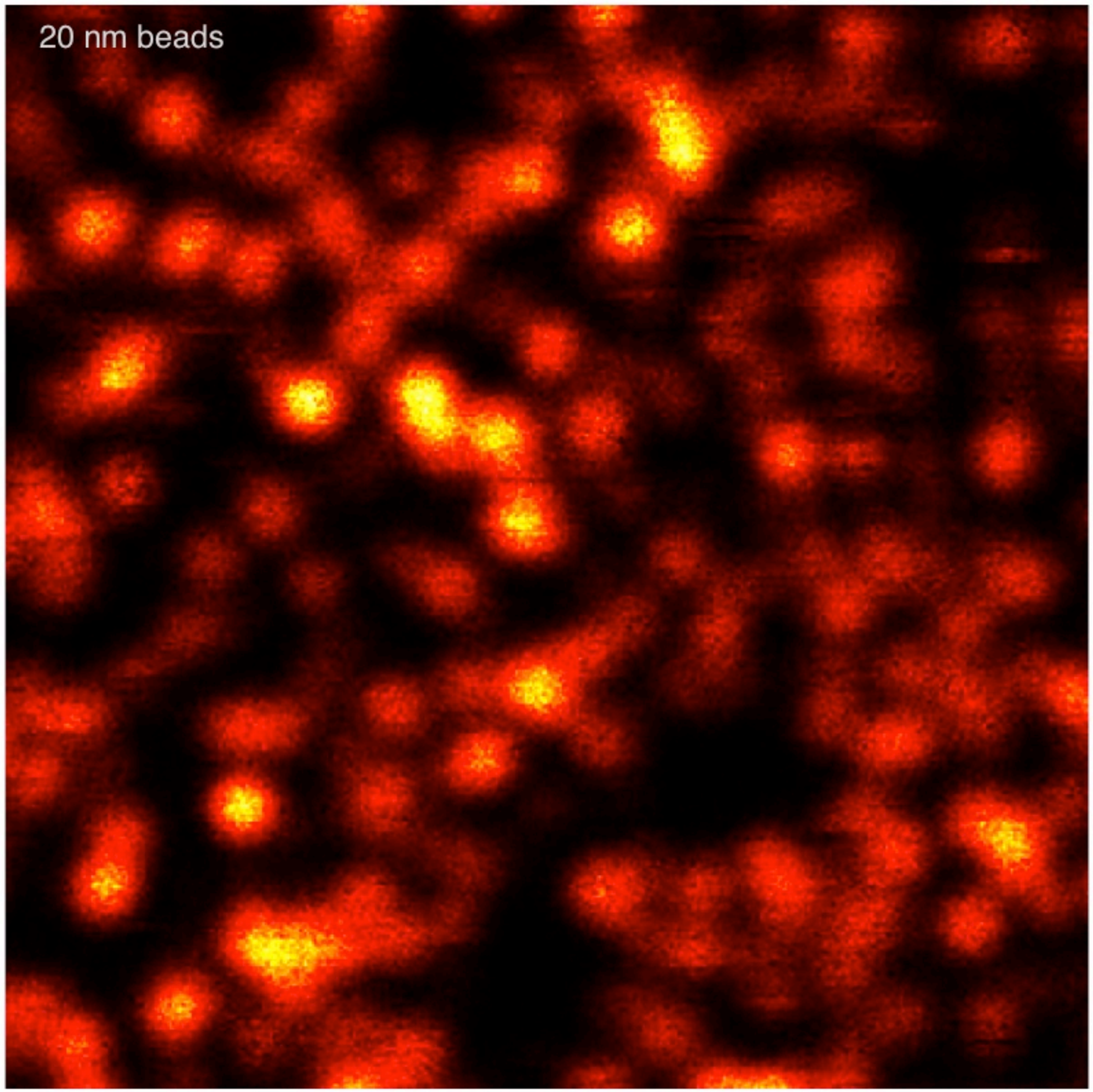


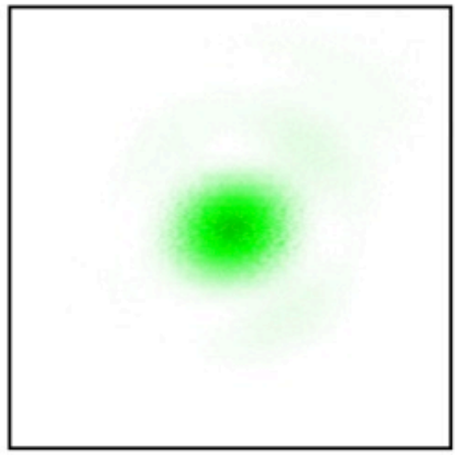
Source: MPI for biophysical chemistry, Göttingen

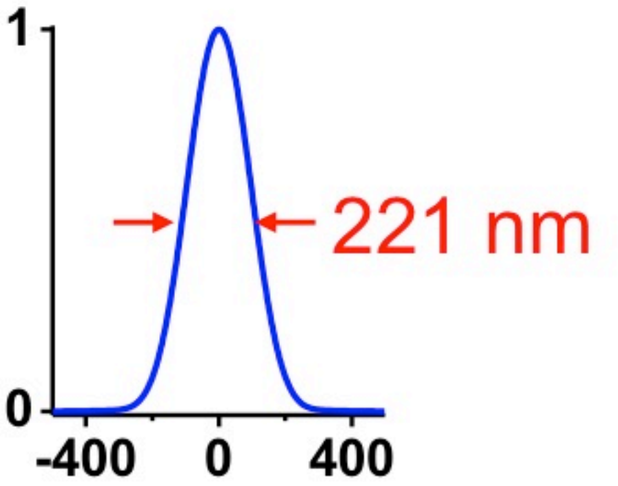
STED details – spectrum



STED intensity and resolution

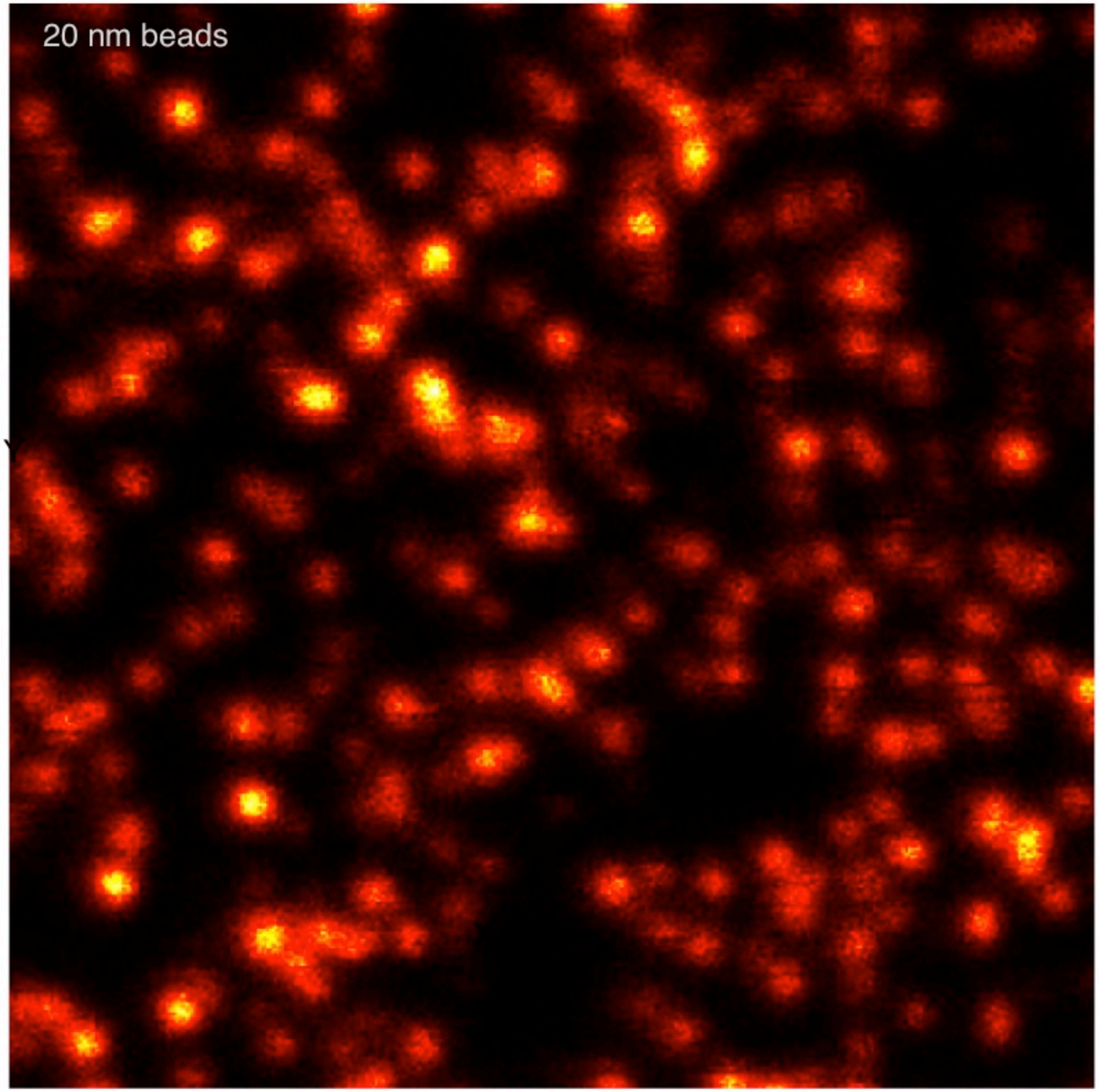


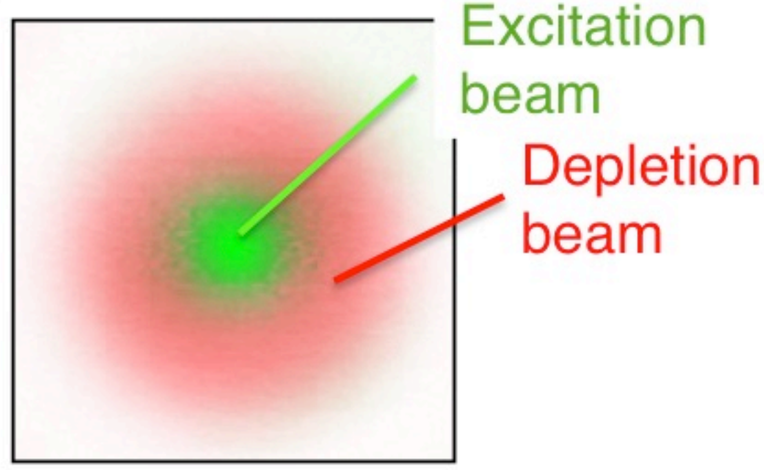


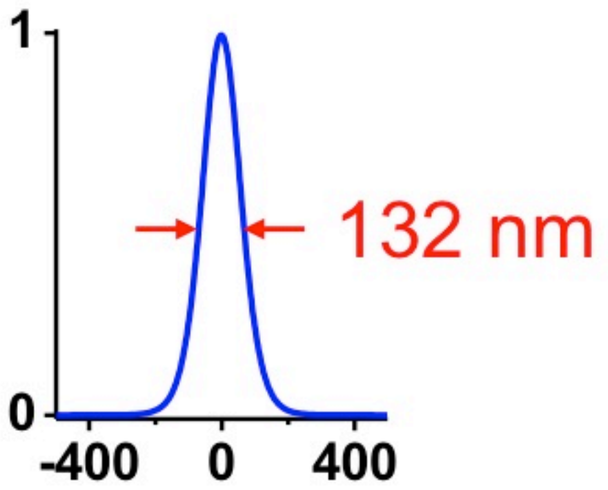


Source: MPI for biophysical chemistry, Göttingen

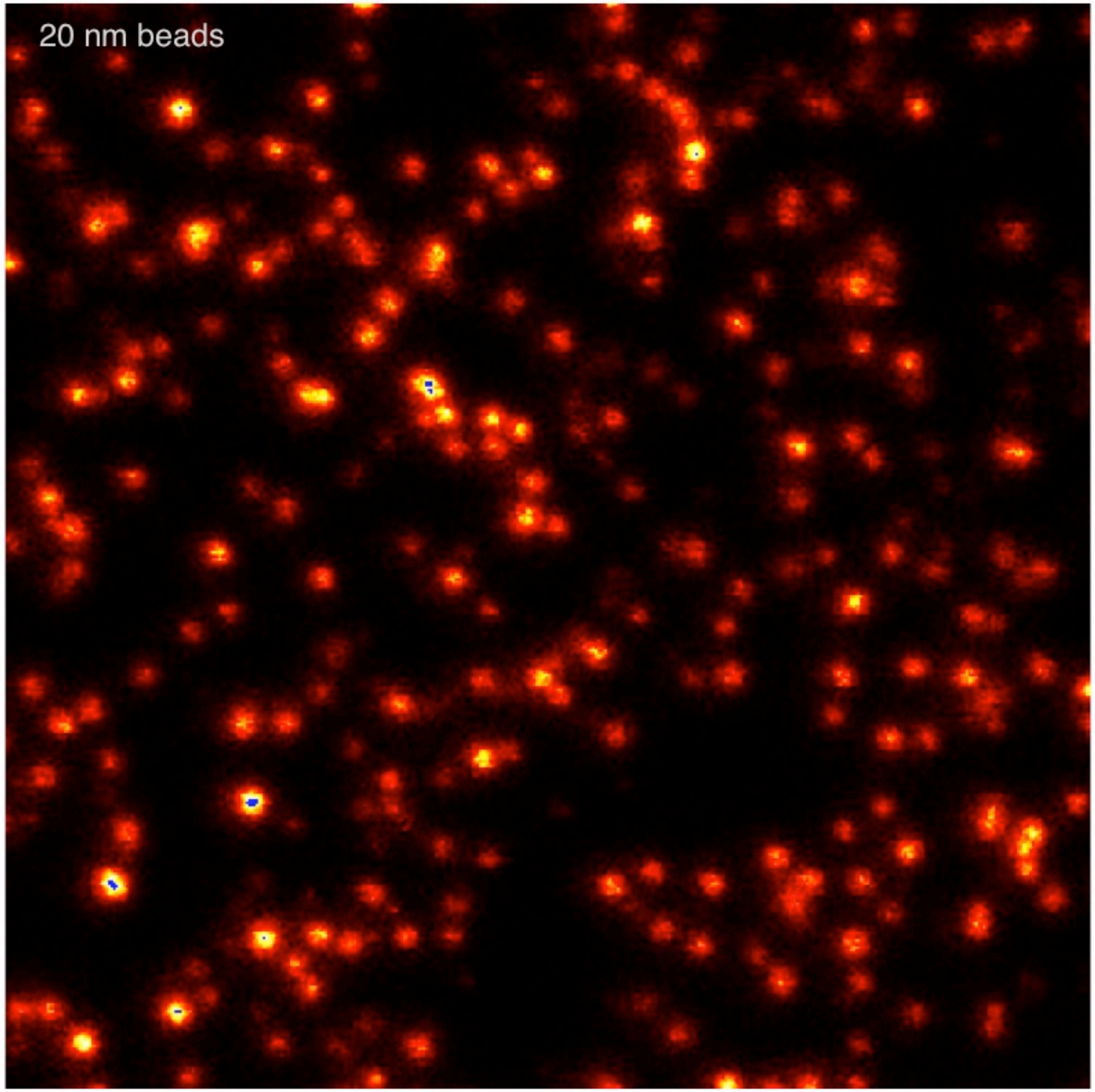
STED intensity and resolution

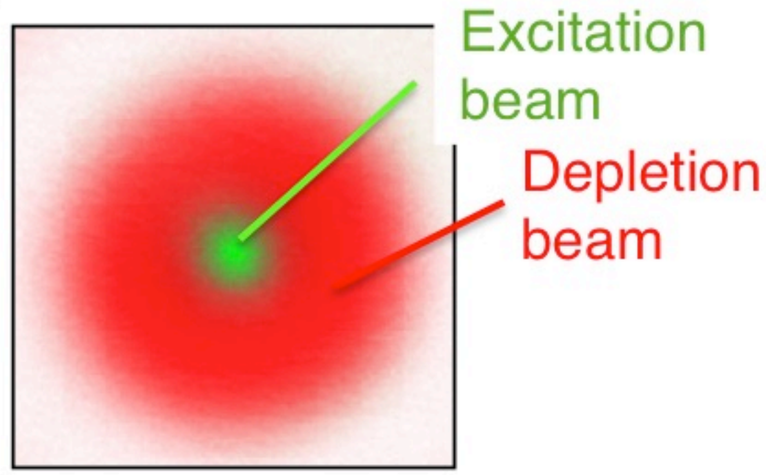


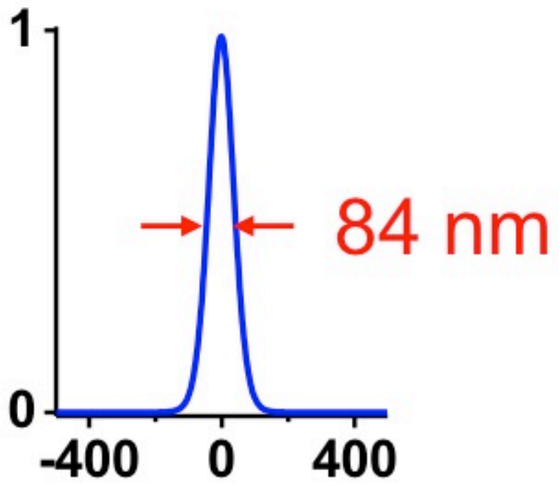




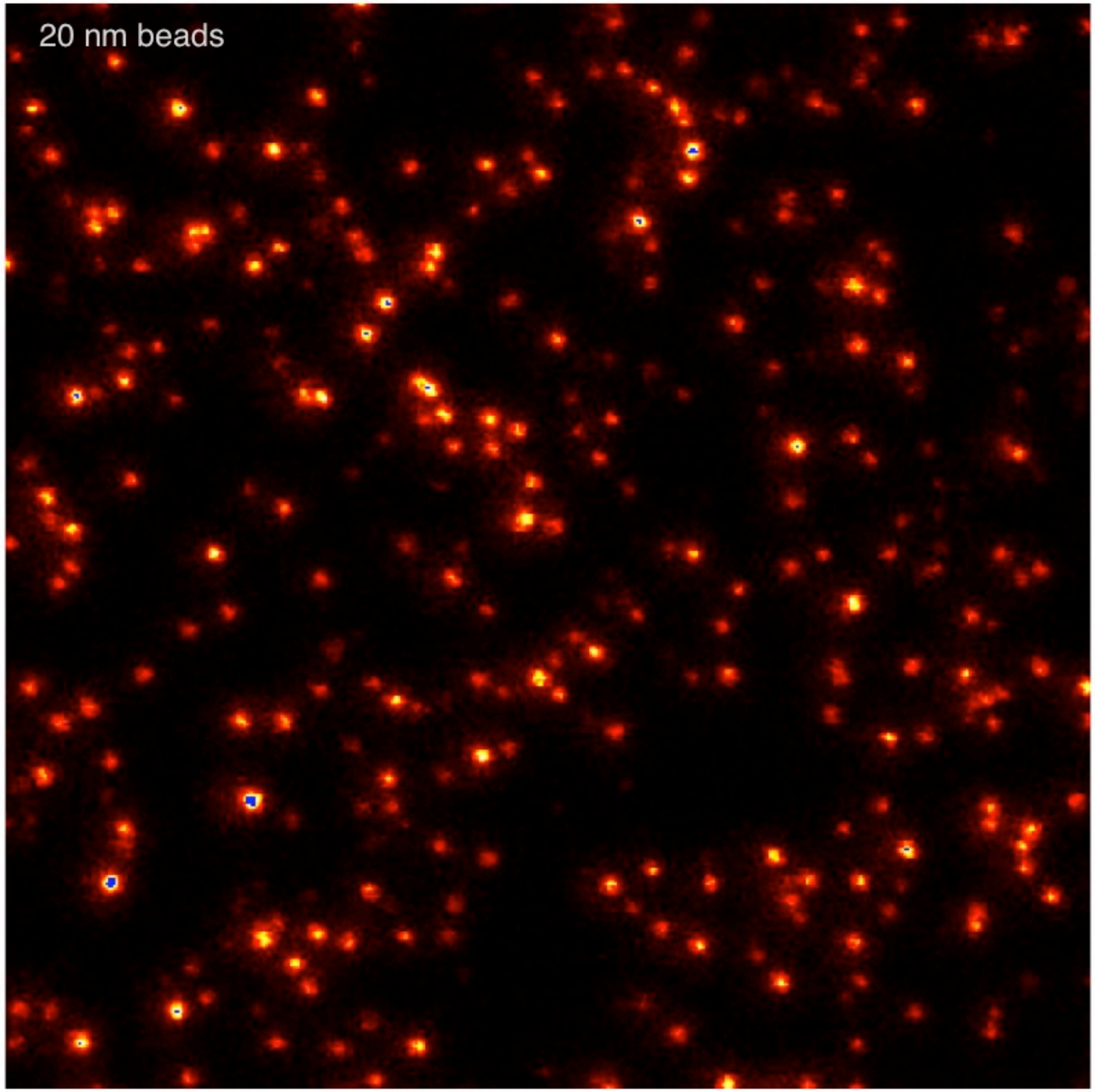
STED intensity and resolution

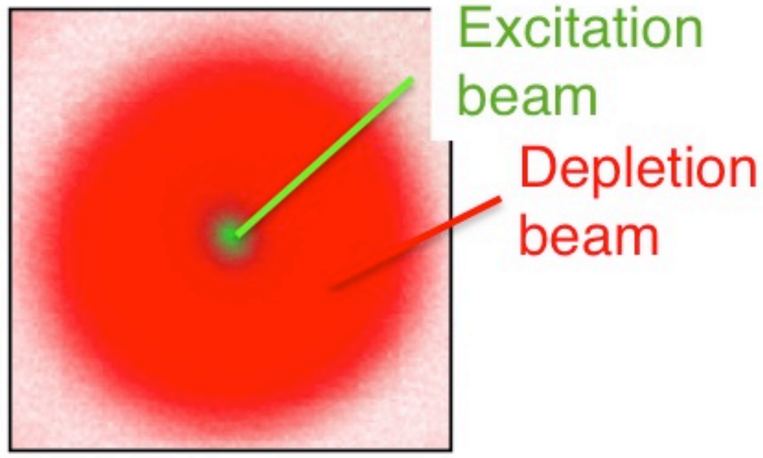


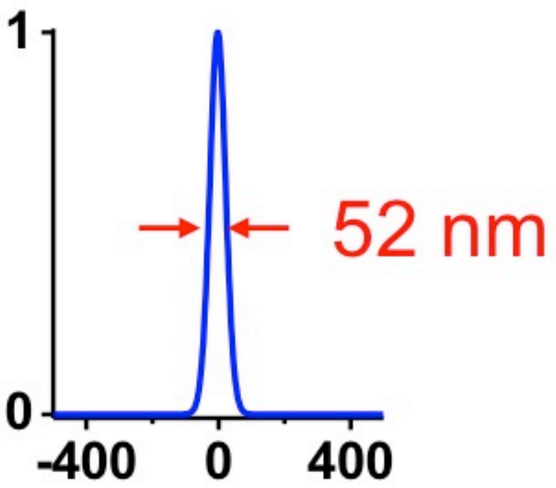




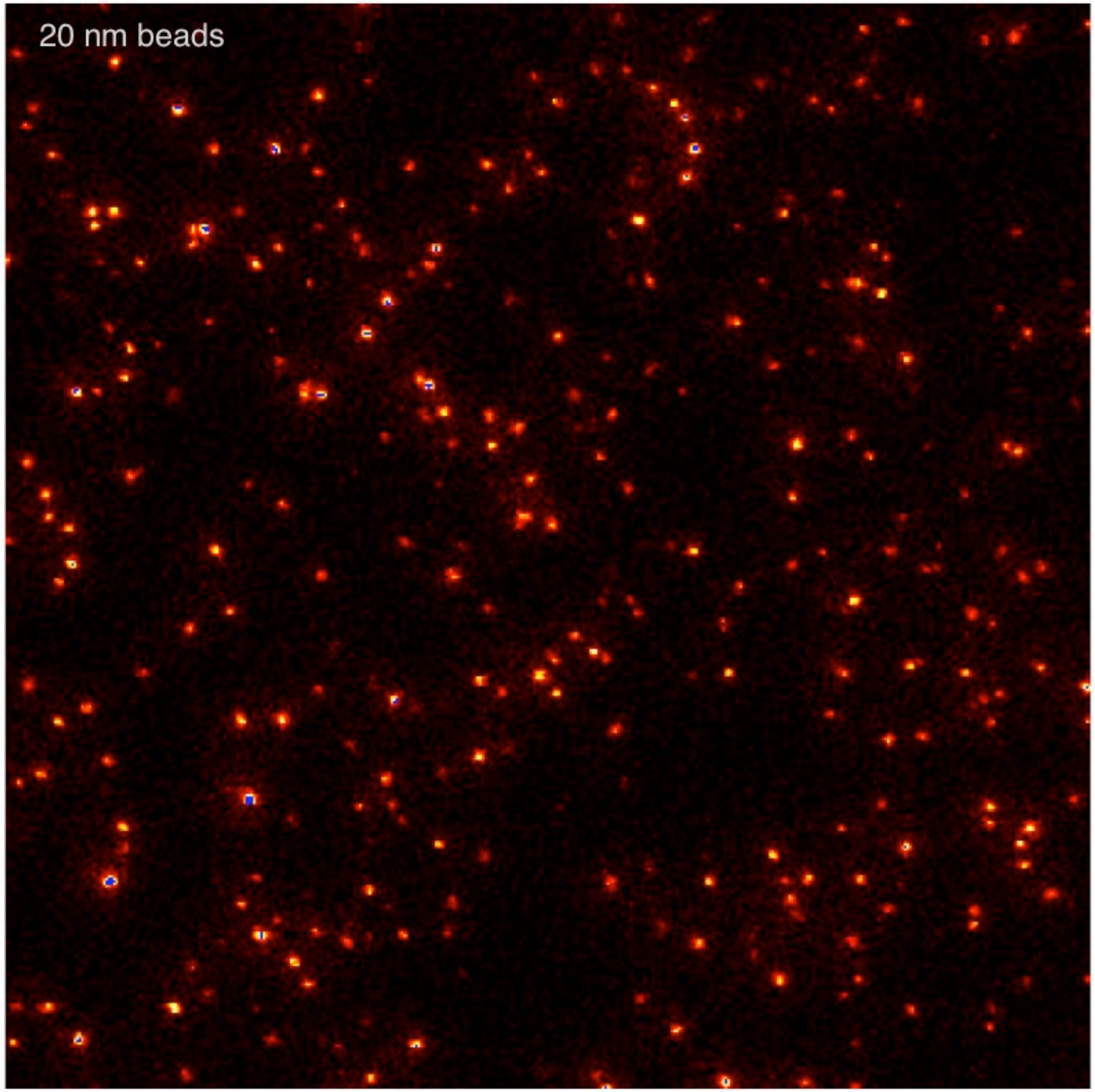
STED intensity and resolution

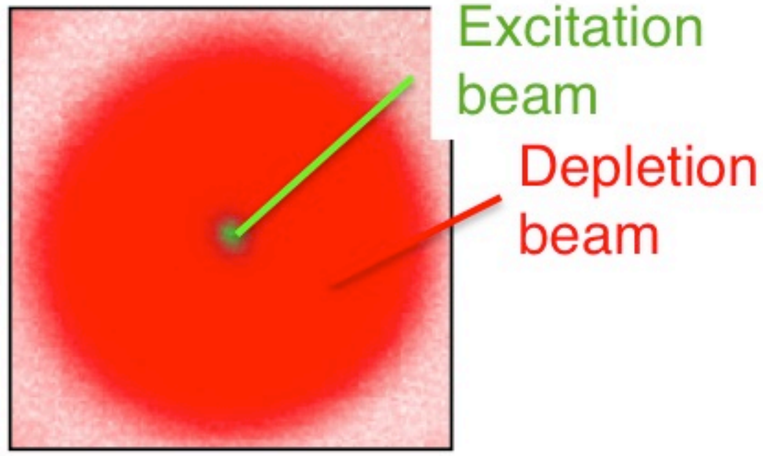


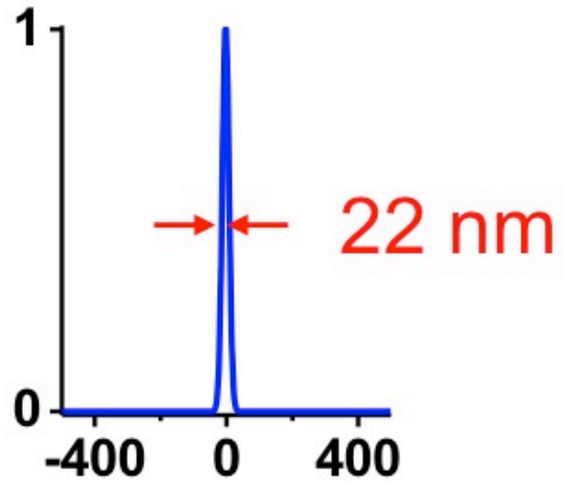




STED intensity and resolution



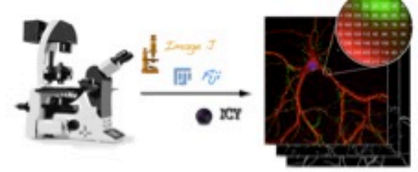




STEDYcon - dyes

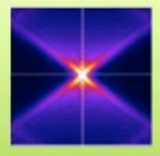
	640	561	488	405
Exc				
Dyes	STAR RED	STAR 580	STAR 488	DAPI
	STAR 635P	STAR 600	Alexa 488	Hoechst33258
	STAR 635	Alexa 594	Oregon Green	Alexa 405
	Atto 647N	Atto 594	Atto 488	
	Atto 633	Atto 590	FITC	
	Alexa 647	Cy3	STAR470SXP	
	Cy5		STAR520 SXP	
	SiR	Live 580	GFP	CFP
		Atto 590	YFP	

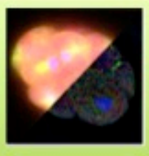
abberior INSTRUMENTS



Resolution:

- Recalage
- PSF, déconvolution

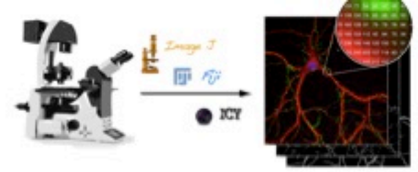




Widefield microscopy

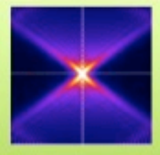
After deconvolution

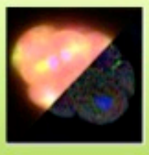
http://www.biology.wustl.edu/imaging-facility/images/deconbeforeafter.jpg

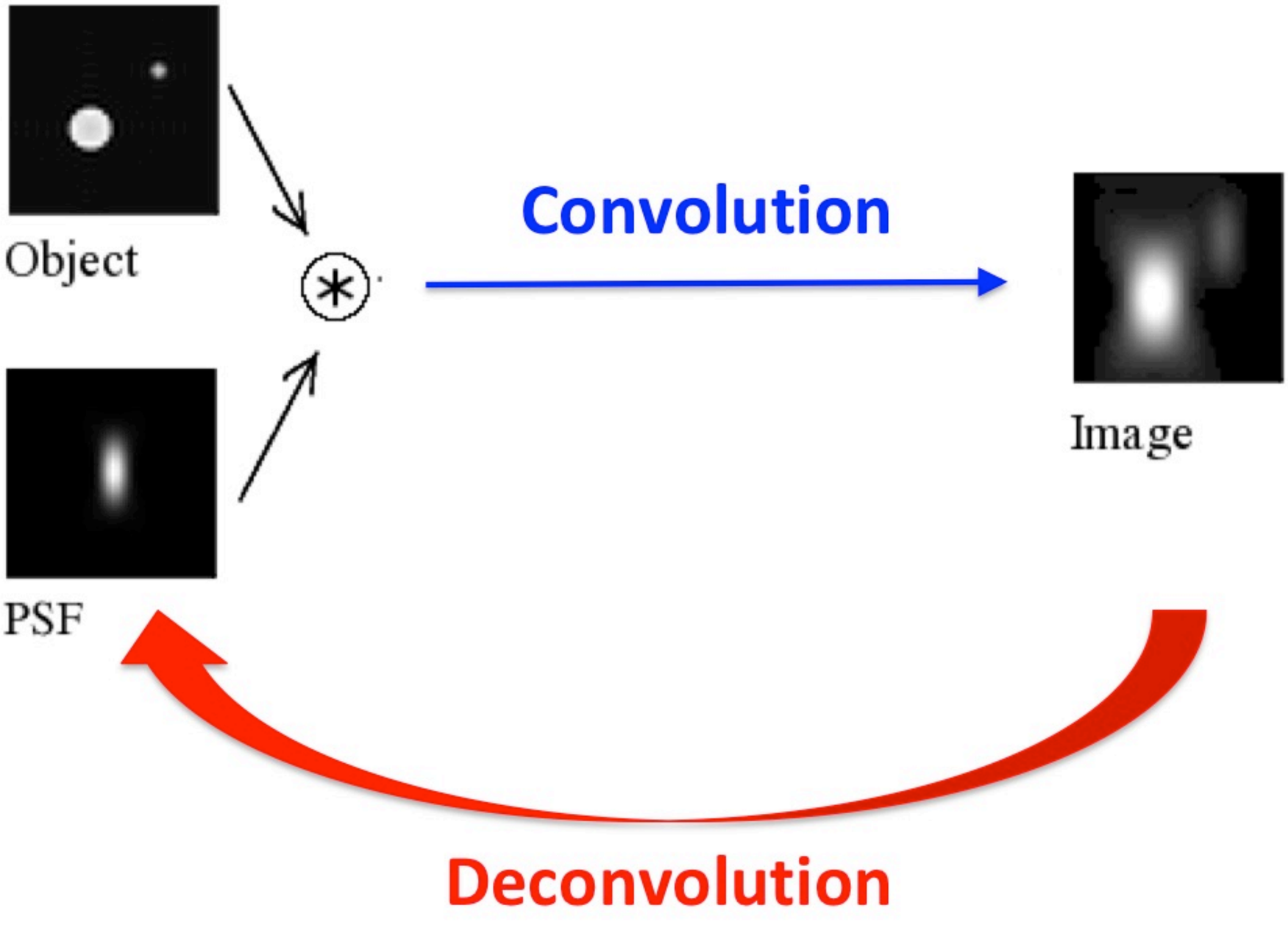


Resolution:

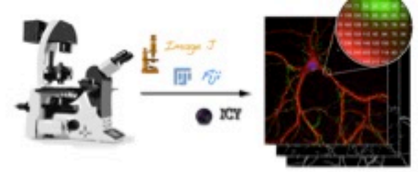
- Recalage
- PSF, déconvolution







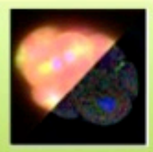
Point Spread Function ou Fonction d'étalement du point : réponse du système optique à une source ponctuelle. Les abérations du système conduise à un étalement de l'image du point.



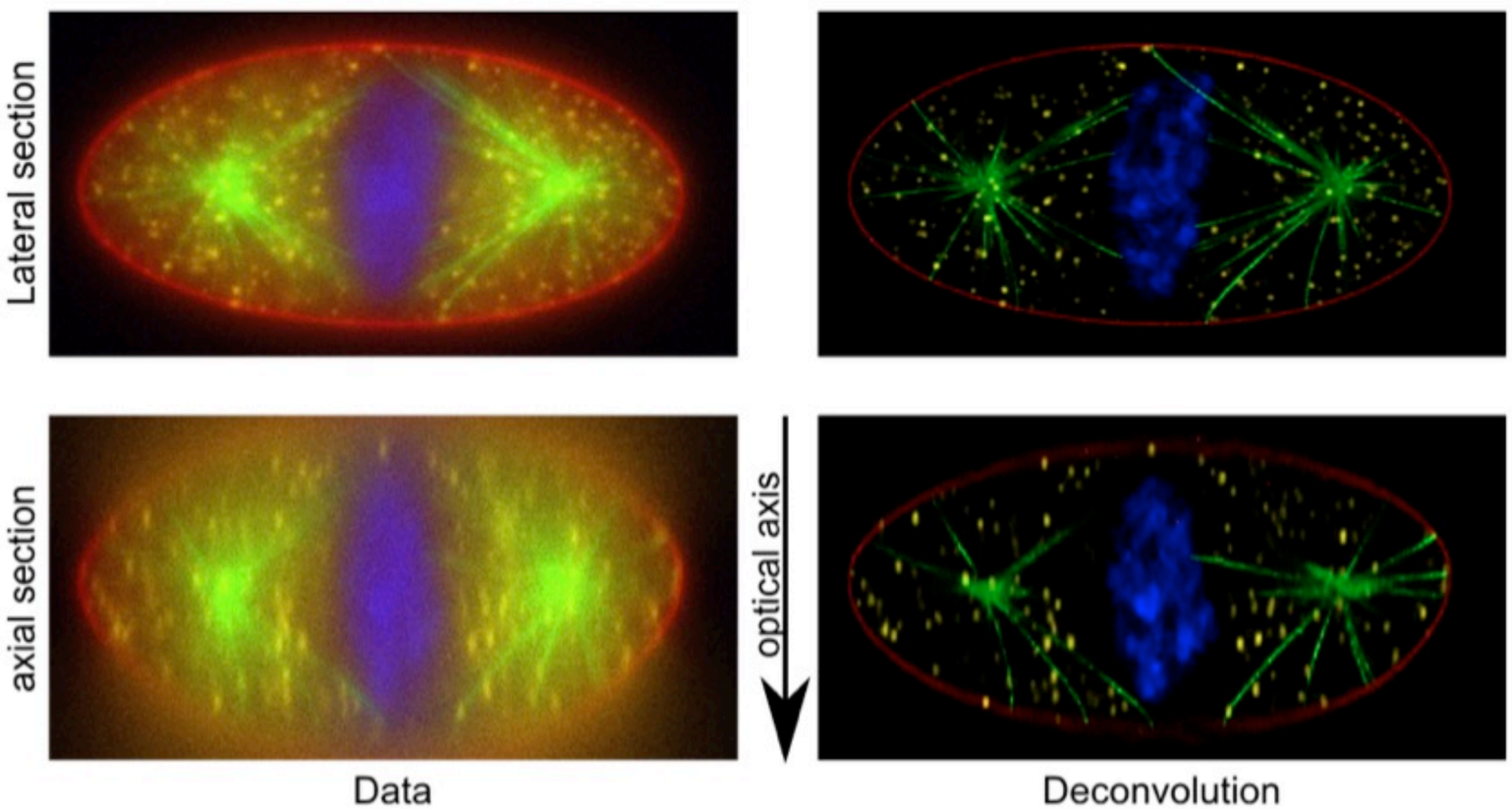
Deconvolve

The deconvolution method receives the 1st prize of ISBI 3D Microscopy Challenge

Resolution:
- Recalage
- PSF, déconvolution



The deconvolution method proposed by F. Soulez receives the 1st prize of "3D Microscopy Deconvolution Challenge" that took place during ISBI 2013.



Lateral and axial cross-section of the simulated 3D micrograph used in the challenge (left) and corresponding deconvolution (right).

The deconvolution result show a strong improvement both in term of resolution and signal to noise ratio.

Aquiring a PSF:

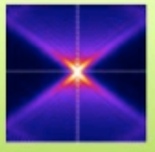
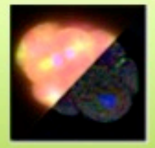
Principle : *to image on punctual object (a bead) whose diameter is lower than the resolution of the system used to image.*

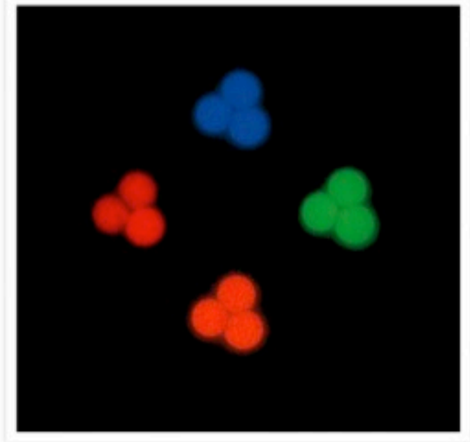
***Ex:* TetraSpeck™ Microspheres,**
0.1 µm, fluorescent

4 wavelength
350/440 nm (blue),
505/515 nm (green),
575/585 nm (orange)
655/685 nm (dark red)

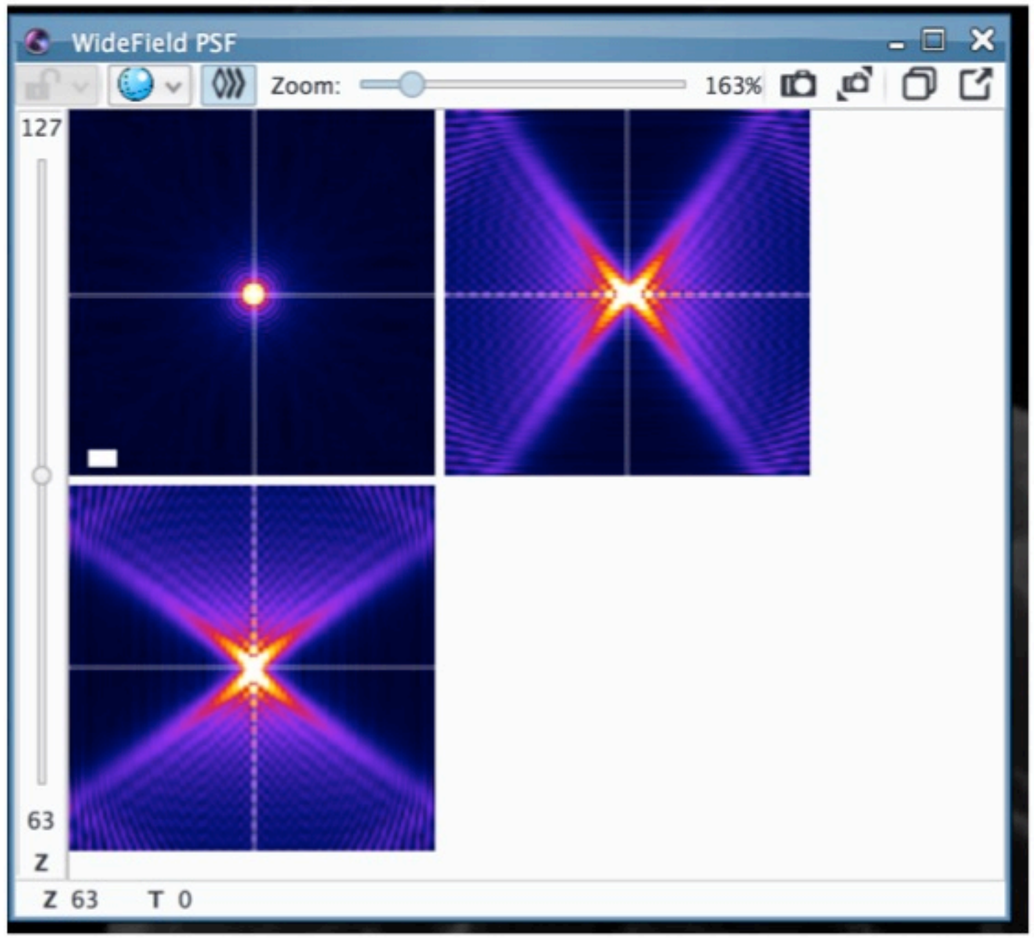
5 size
0.1 µm (T7279)
0.2 µm (T7280)
0.5 µm (T7281)
1.0 µm (T7282)
4.0 µm (T7283)

Resolution:
- **Recalage**
- **PSF, déconvolution**

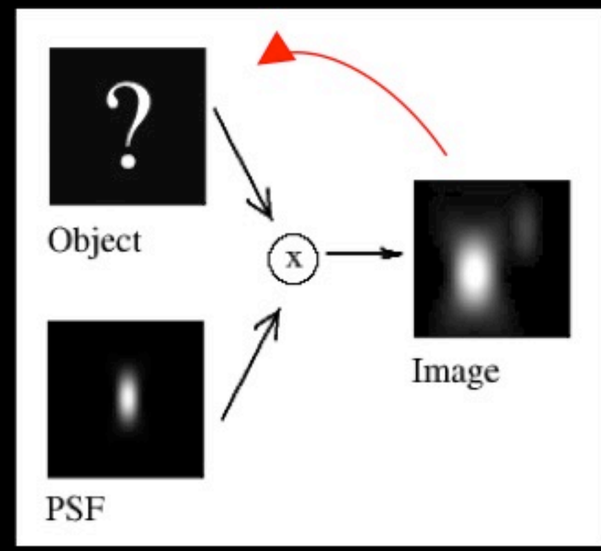



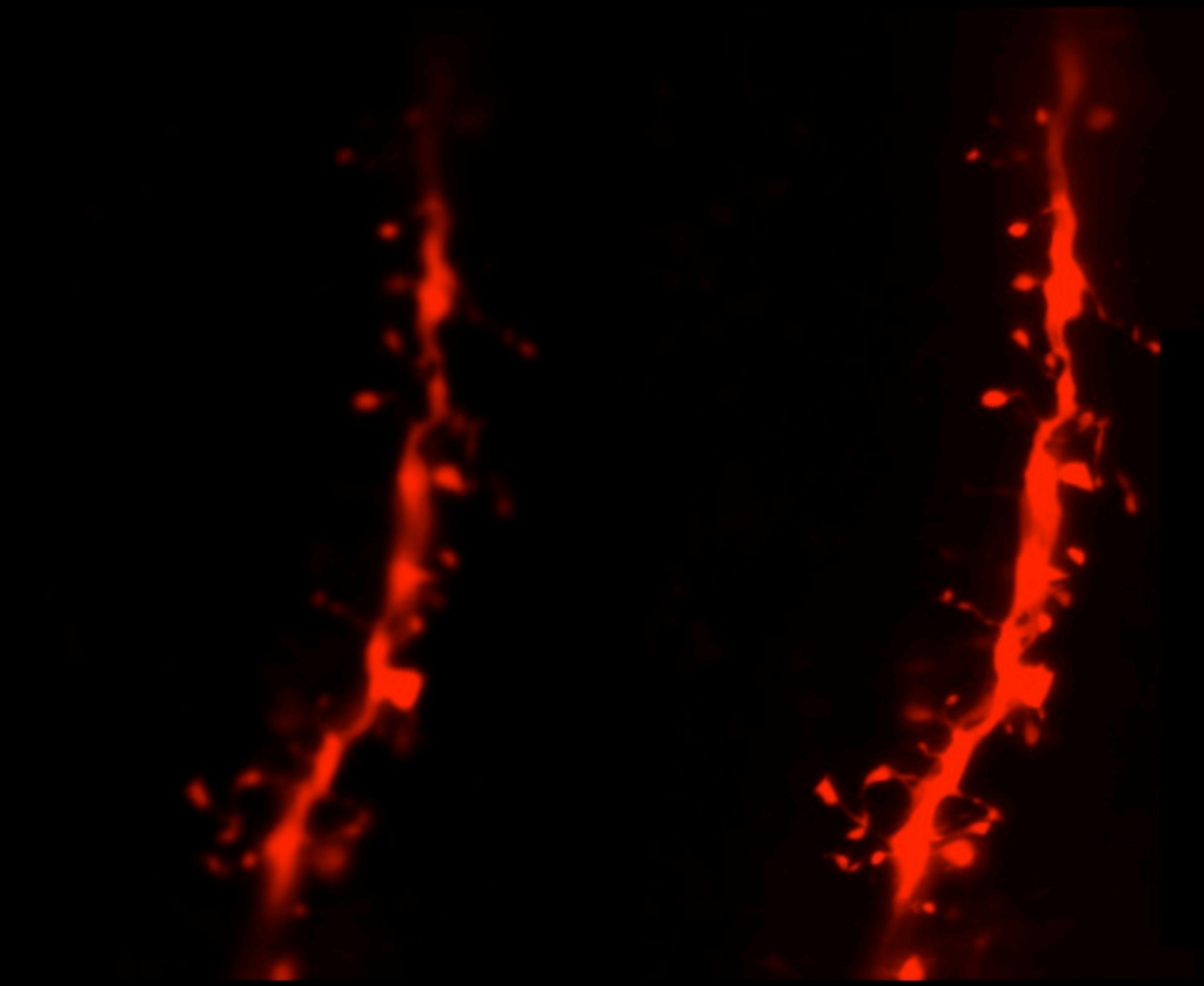


https://www.thermofisher.com/fr/fr/home/technical-resources/research-tools/image-gallery/image-gallery-detail.altid.g001048.html



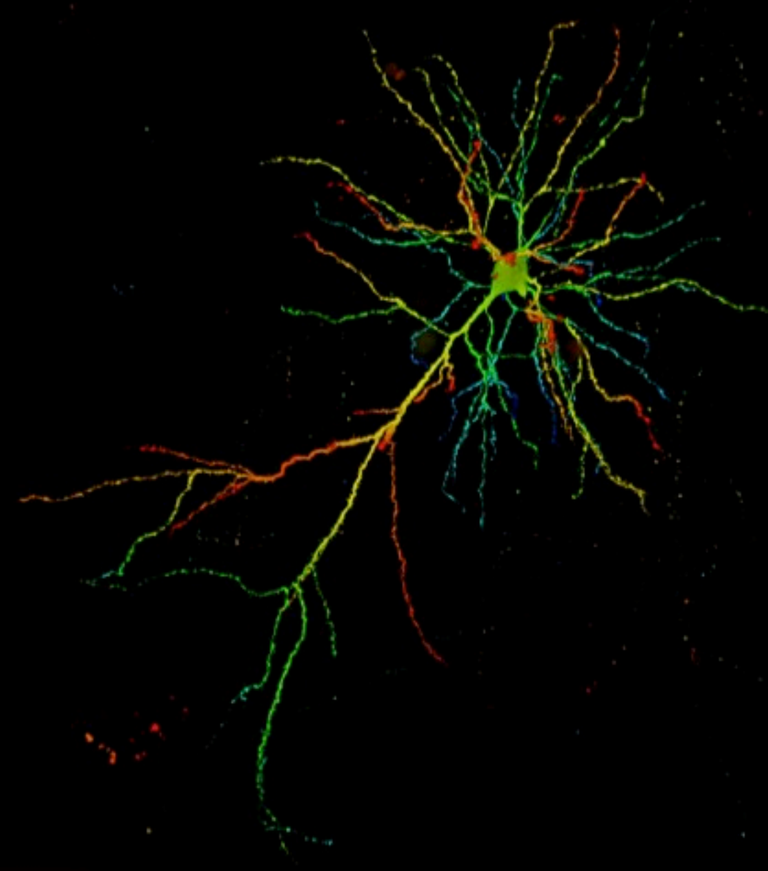
Deconvolution

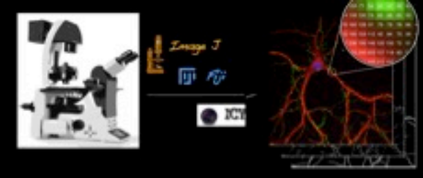




Confocal image

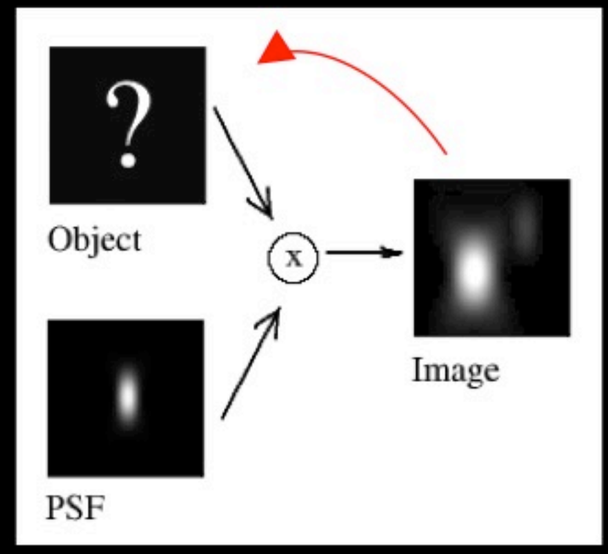
STED image

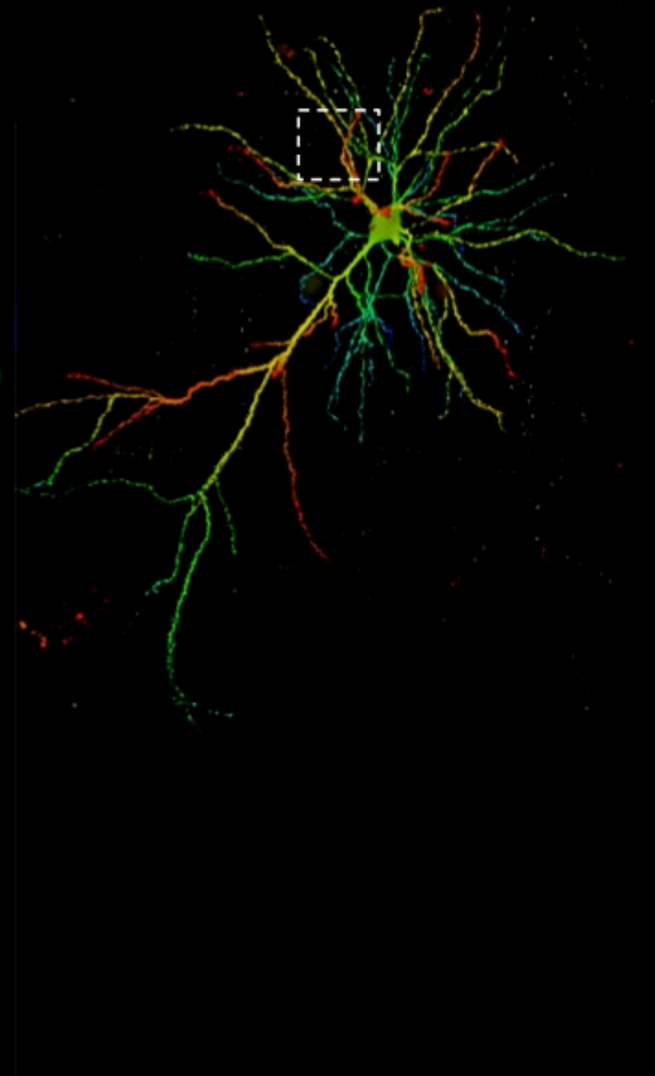




Confocal image

STED image





Deconvolved
Confocal image

Deconvolved
STED image

SUPER-RESOLUTION TECHNIQUES

Optimal

Not optimal

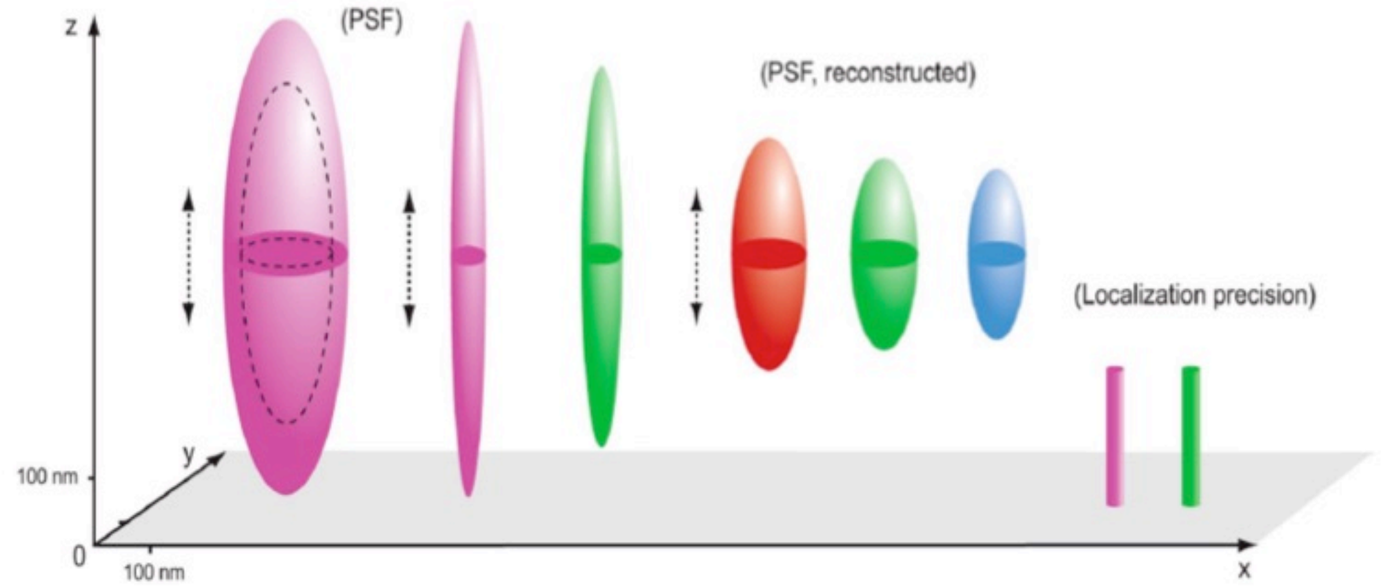
Not recommended

Dye/probe consideration

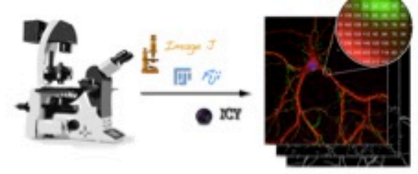
Sample preparation

Heavy post-processing

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520

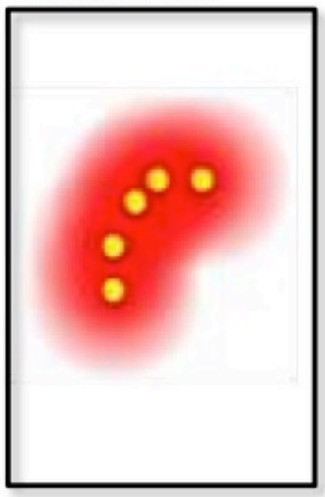


	SIM	STED	PALM	STORM
Typical res. XY	100 nm	50 – 80 nm	20 – 50 nm	20 – 50 nm
Typical res. Z	200 – 250 nm	150 – 300 nm	50 – 100 nm	50 nm – 100 nm
Probe availability		Specific	Specific	Specific
Multi-color imaging	4-color	2- to 3-color	1- to 2-color	2- to 3-color
Live-cell imaging	Sample-dependent (e.g. slow dynamics)		Single Particle Tracking	Fixed samples
3D	Zeiss LSM880	Leica SP8 – STED 3Dx	Zeiss LSM880	Bruker Vutara



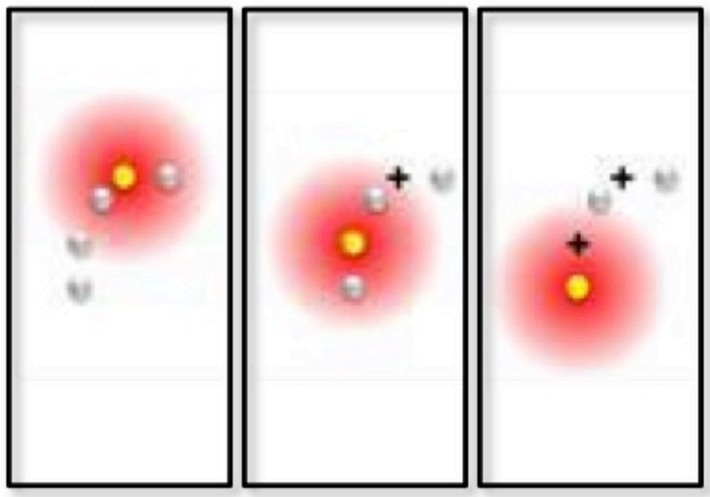
Stochastic Optical Reconstruction Microscopy

PROBLEM

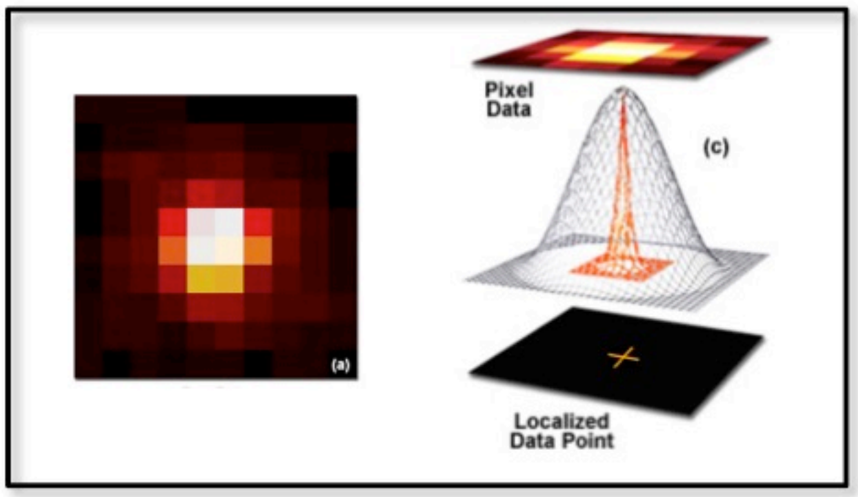


Molecules are to close to be resolved

SOLUTION



Stochastic activation of individual molecules
10 000 to 30 000 images !!



Fitting position to gaussian function

Reconstructed Super-resolution image from localization

Modified from B. Huang, H. Babcock, X. Zhuang, "Breaking the diffraction barrier: super-resolution imaging of cells", *Cell*, 143, 1047-1058 (2010)

Stochastic Optical Reconstruction Microscopy :

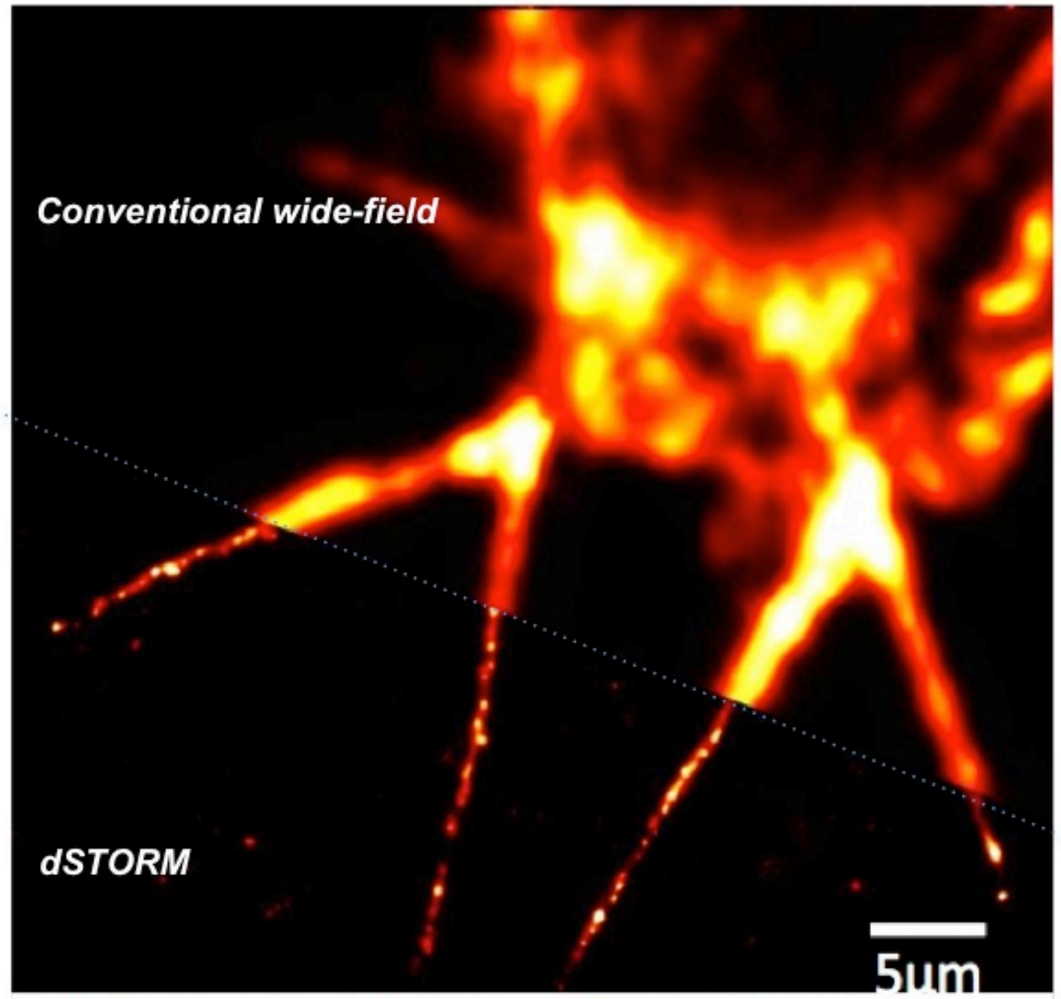


Figure 2 : Conventional microscopy and dSTORM image of actin filaments.

→ **Widely used for imaging cytoskeleton**

→ **Or to localized individual molecule within clusters**

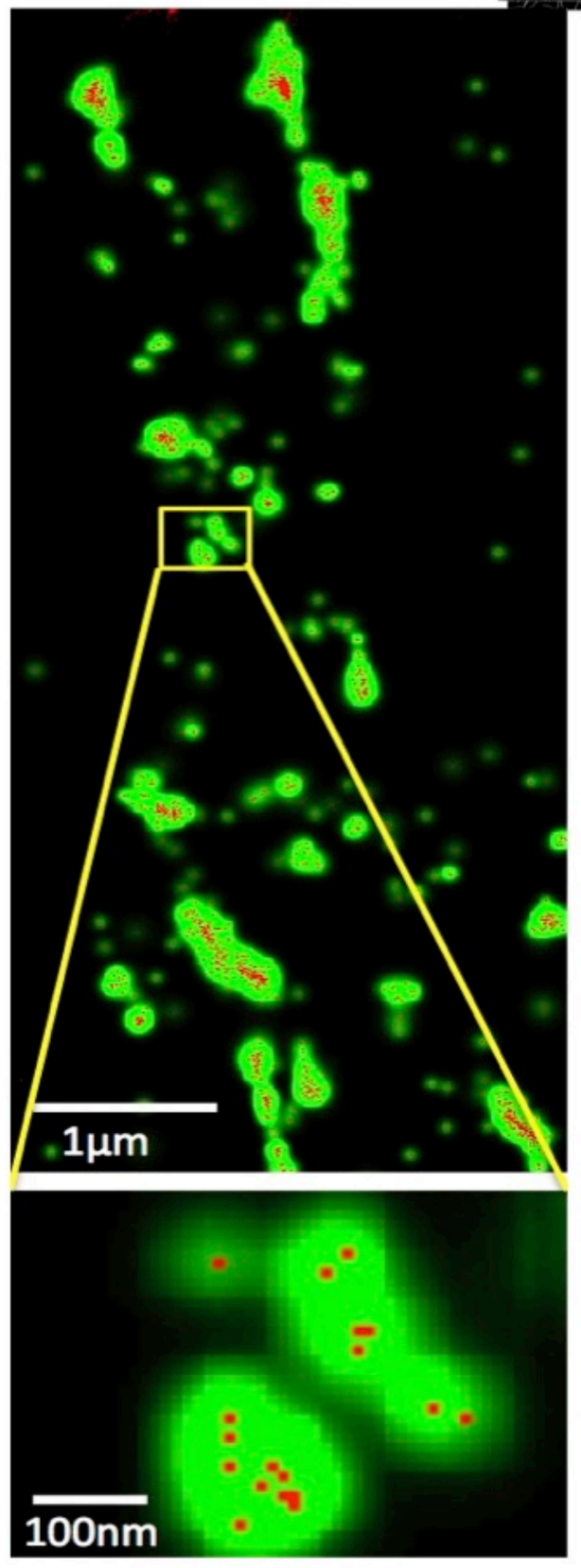


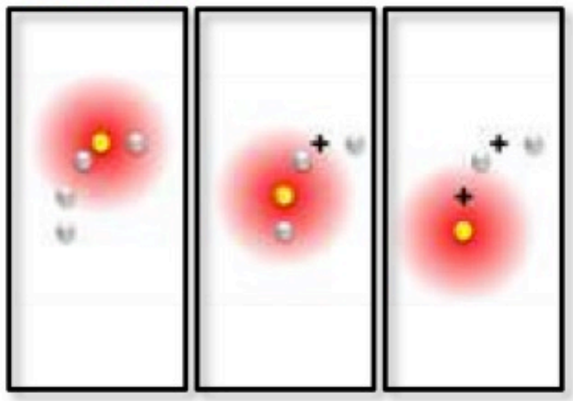
Figure 3 : dSTORM image of Glutamate receptor on a dendrite.

STORM : how to get them blink as a christmass tree ?

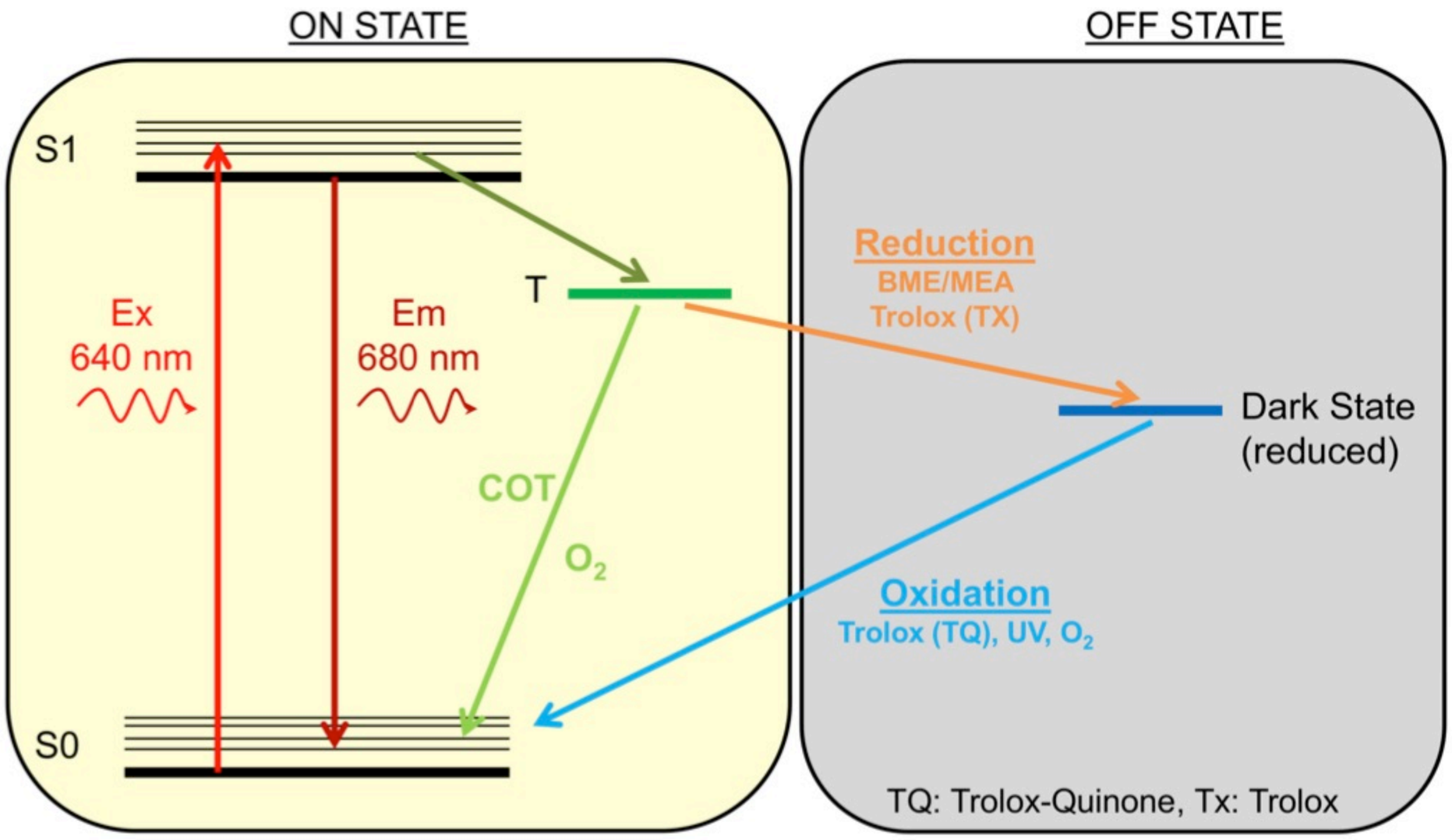
High laser power

STORM : how to get them blink as a christmass tree ?

SOLUTION



Stochastic activation of individual molecule
10 000 to 30 000 images !!



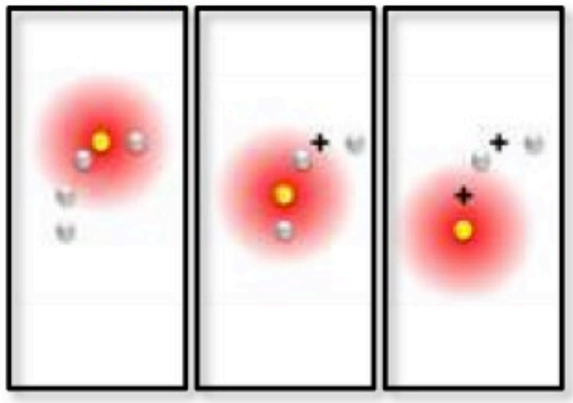
Oxidized dyes are no longer fluorescent

Needs a buffer with reducing agents (BME, MEA) and oxygen scavenger systems (Glc + Oxydase+ Catalase)

Olivier, N., Keller, D., Gönczy, P., and Manley, S. (2013). Resolution Doubling in 3D-STORM Imaging through Improved Buffers. PLoS ONE *8*, e69004.

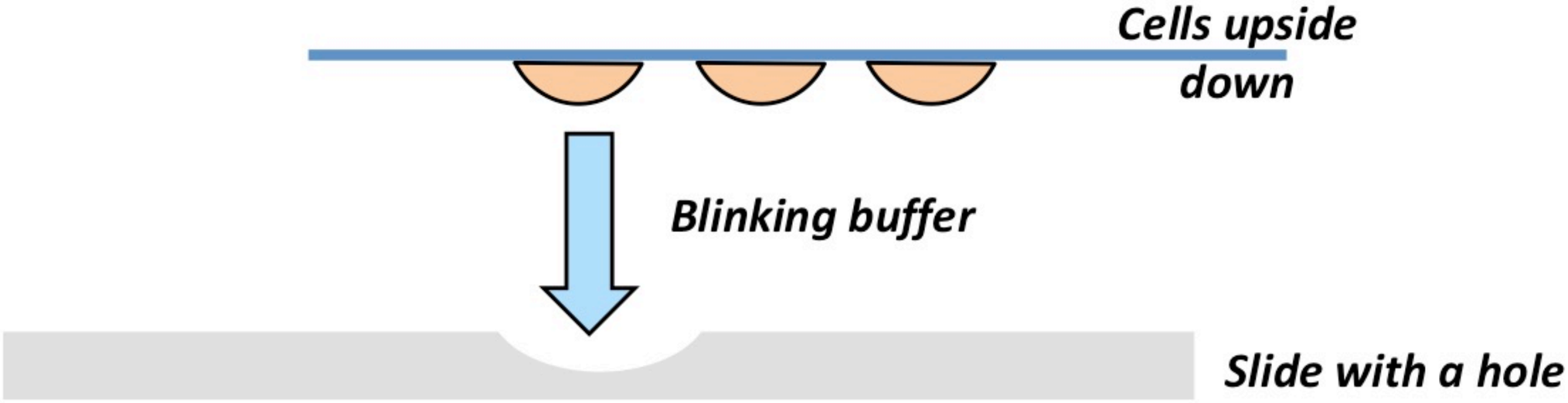
STORM : how to get them blink as a christmass tree ?

SOLUTION



Stochastic activation of individual molecules
10 000 to 30 000 images !!

Cells on Coverslip : thickness 170 µm for super-resolution
 # 1.5

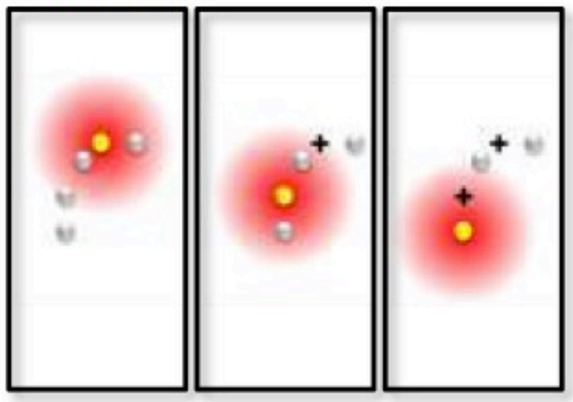


Needs a buffer with reducing agents (BME, MEA) and oxygen scavenger systems (Glc + Oxydase+ Catalase)

Olivier, N., Keller, D., Gönczy, P., and Manley, S. (2013). Resolution Doubling in 3D-STORM Imaging through Improved Buffers. PLoS ONE *8*, e69004.

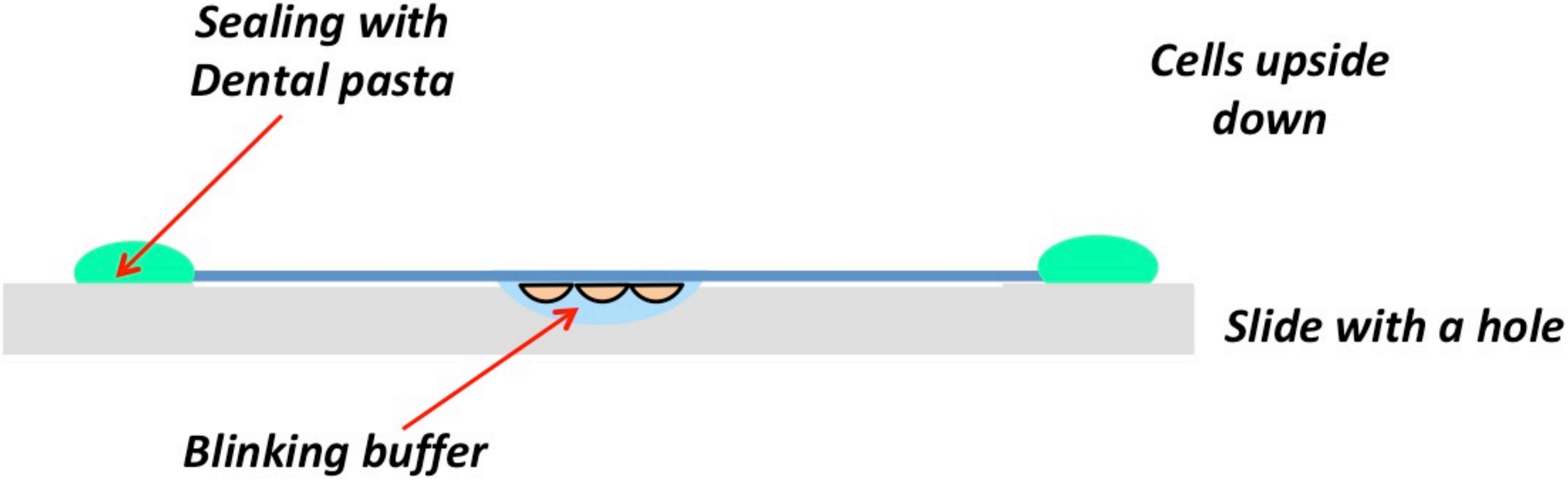
STORM : how to get them blink as a christmass tree ?

SOLUTION



Stochastic activation of individual molecules
10 000 to 30 000 images !!

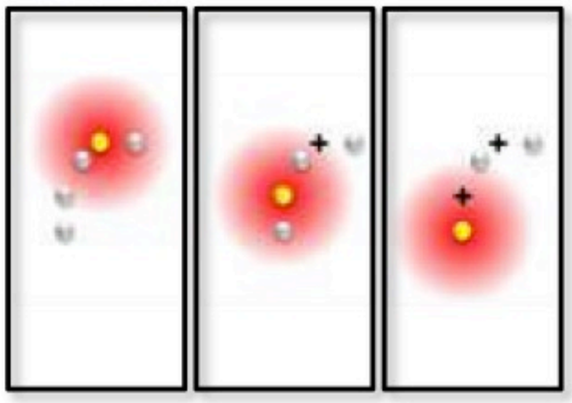
Cells on Coverslip : thickness 170 µm for super-resolution
 # 1.5



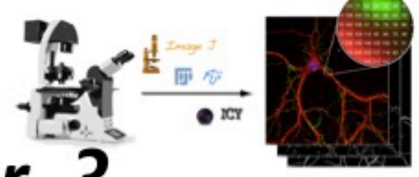
Needs a buffer with reducing agents (BME, MEA) and oxygen scavenger systems (Glc + Oxydase+ Catalase)

Olivier, N., Keller, D., Gönczy, P., and Manley, S. (2013). Resolution Doubling in 3D-STORM Imaging through Improved Buffers. PLoS ONE *8*, e69004.

SOLUTION



STORM : Which fluorophores in which buffer ?



24 A User's Guide to Localization-Based Super-Resolution Fluorescence Imaging

Graham T. Dempsey
stry and Chemical Biology, Harvard University, Cambridge, Massachusetts, USA

Table 24.1 Probes and imaging buffers for different applications

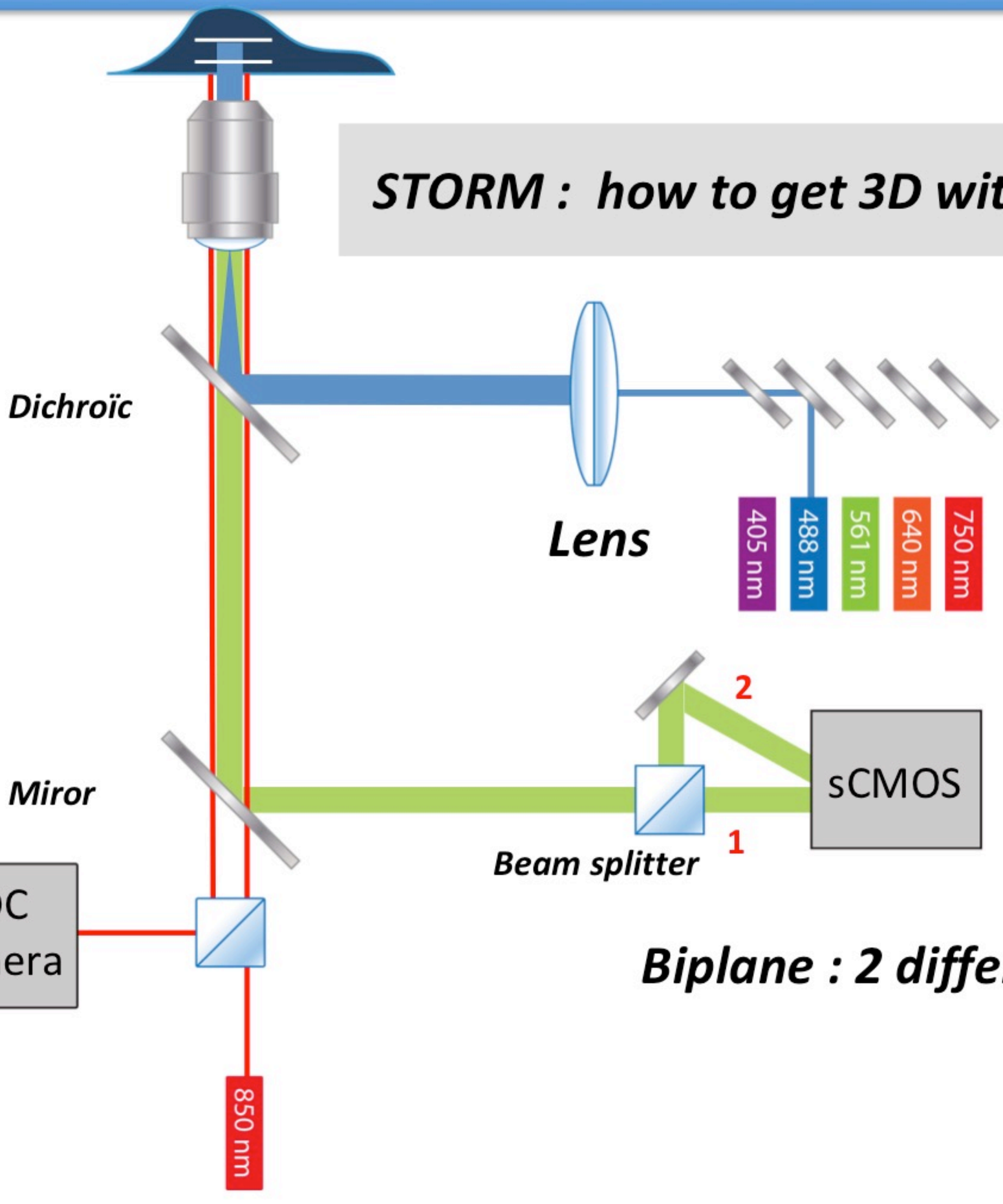
Application	Probe(s)	Imaging solution						
		Buffer	GLOX (v/v) (%)	Glucose (w/v) (%)	MVAA (mM)	BME (mM)	MEA (mM)	TCEP (mM)
Fixed-cell, single-color imaging	Alexa 647, DyLight 750, or Atto 488	pH 7–9	1	1–10	–	100	–	–
		pH 7–9	1	1–10	–	–	10	–
		pH 7–9	1	1–10	1	–	–	25
	mEos2 or PA-mCherry	PBS	–	–	–	–	–	–
Highest resolution, fixed-cell, single-color imaging	Reduced Cy3B	pH 7–9	1	1–10	1	–	–	–
Fixed-cell, multireporter imaging	Alexa 647 and DyLight 750 and/or Atto 488	pH 7–9	1	1–10	–	100	–	–
		pH 7–9	1	1–10	–	–	10	–
		pH 7–9	1	1–10	1	–	–	25
	Alexa 647 and mEos2 and/or DyLight 750	pH 7–9	1	1–10	–	100	–	–
		pH 7–9	1	1–10	–	–	10	–
Fixed-cell multiactivator imaging	Alexa 405–Alexa 647 and/or Cy2–Alexa 647 and/or Cy3–Alexa 647	pH 7–9	1	1–10	–	–	10	–
Live-cell, single-color imaging	Alexa 647	DMEM; low serum; no phenol red	1	2.5	–	70	–	–
	mEos2 or PA-mCherry	DMEM; low serum; no phenol red	–	–	–	–	–	–

This list is intended to be a starting point, as other probes are available. Note that 1% GLOX is 0.5 mg/mL glucose oxidase (Sigma–Aldrich) and 40 μg/mL catalase (Sigma–Aldrich).

Methods in Cell Biology, Volume 114

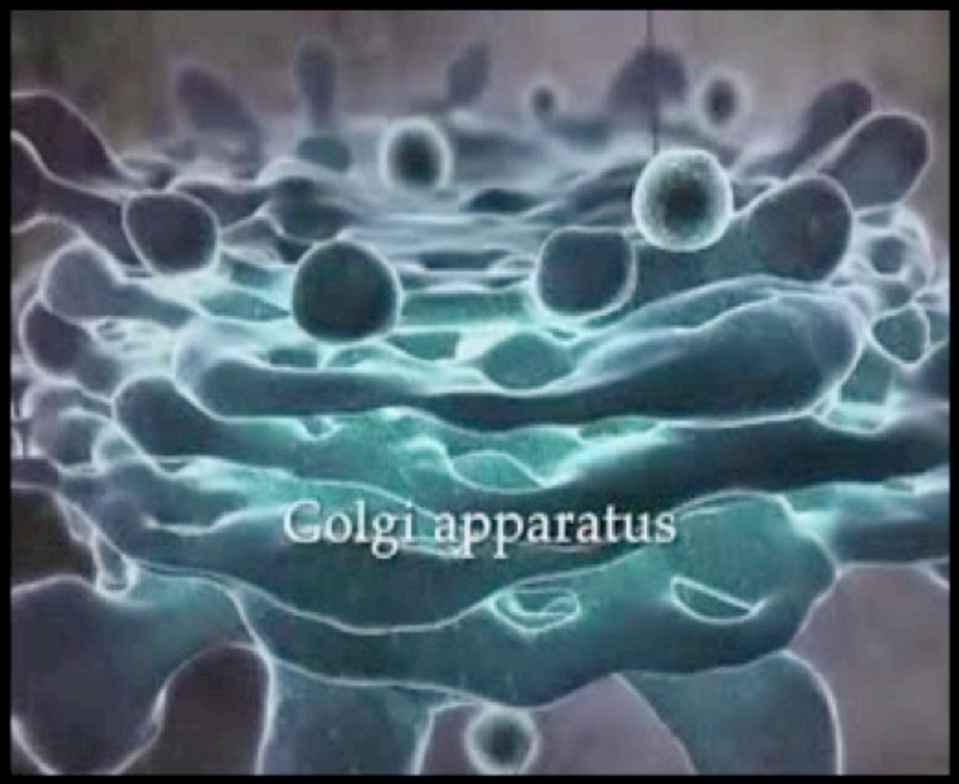
BRUKER

STORM : how to get 3D with biplane ?



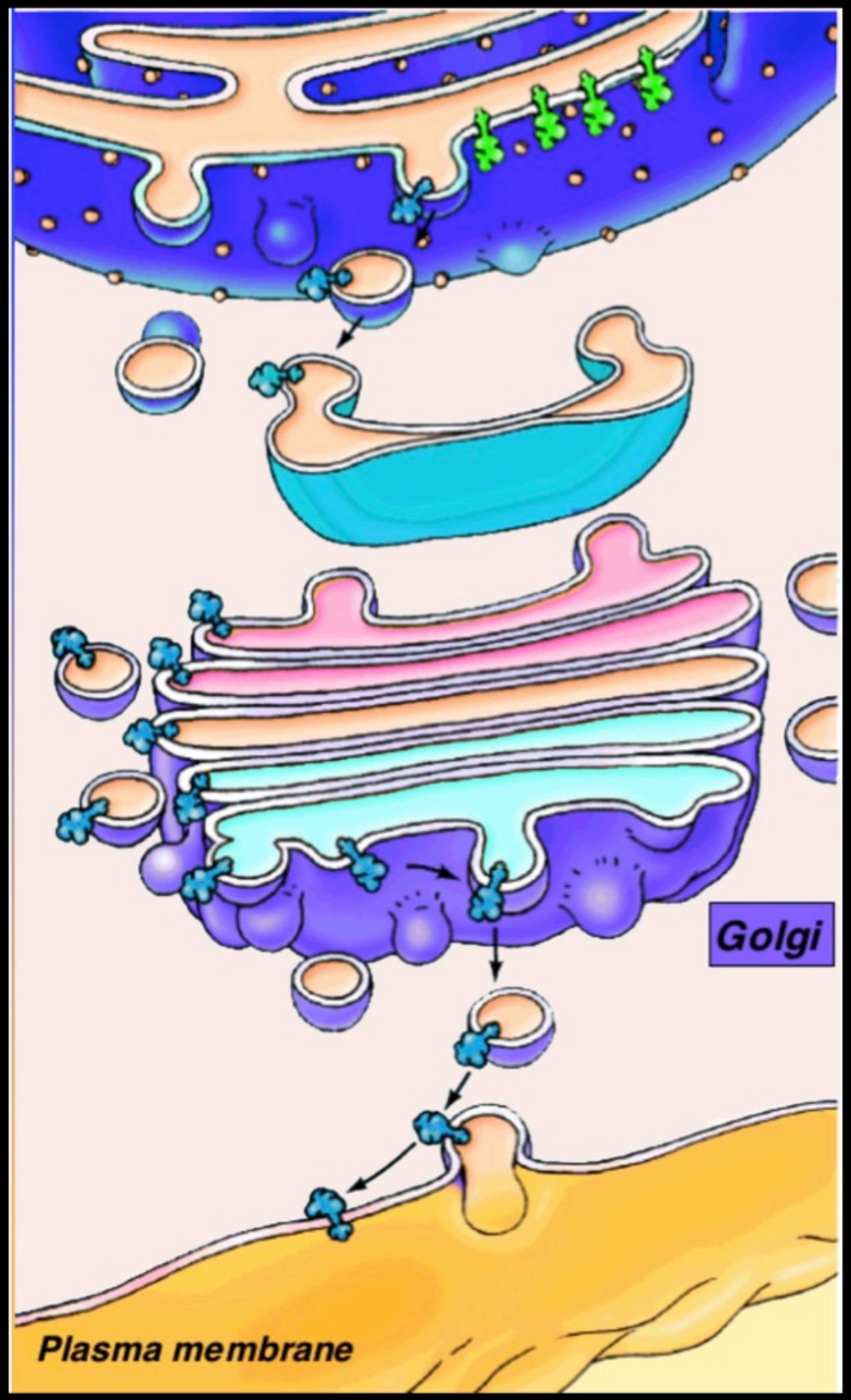
Path1 Path2

Biplane : 2 different optic path



Golgi apparatus :

golgi proteins are closed
but not in the same compatment

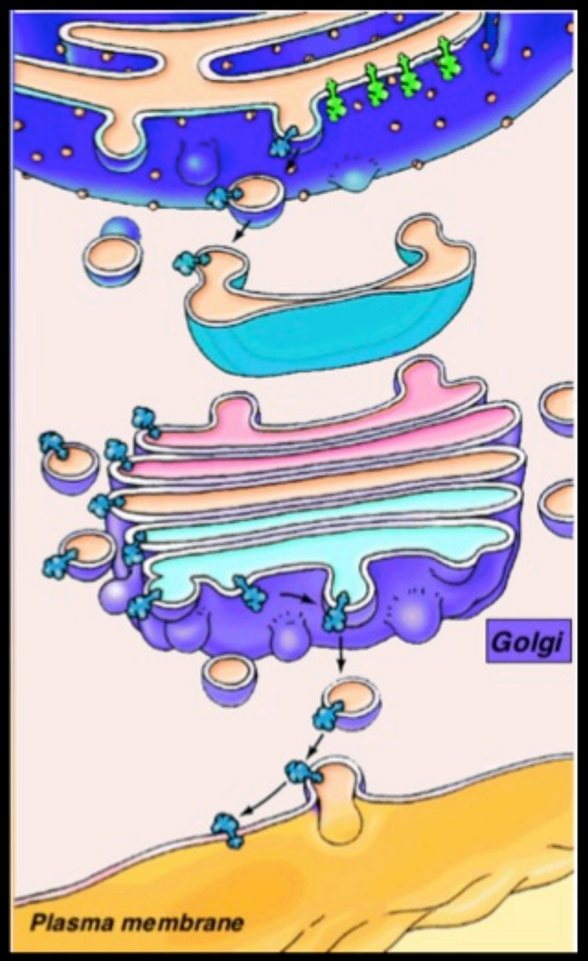


ER

Cis-Golgi

median-Golgi

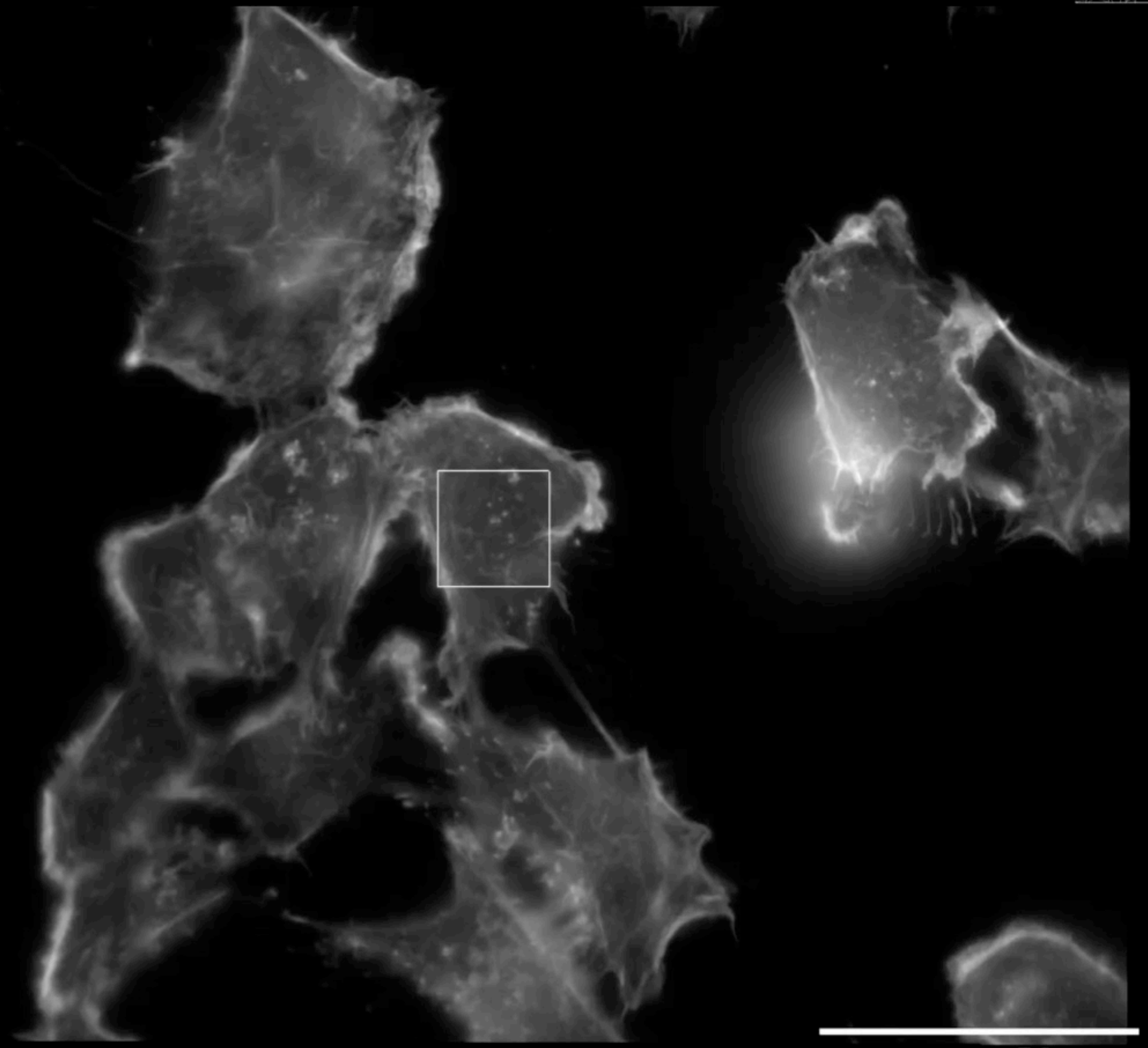
Trans-Golgi



Actin 568

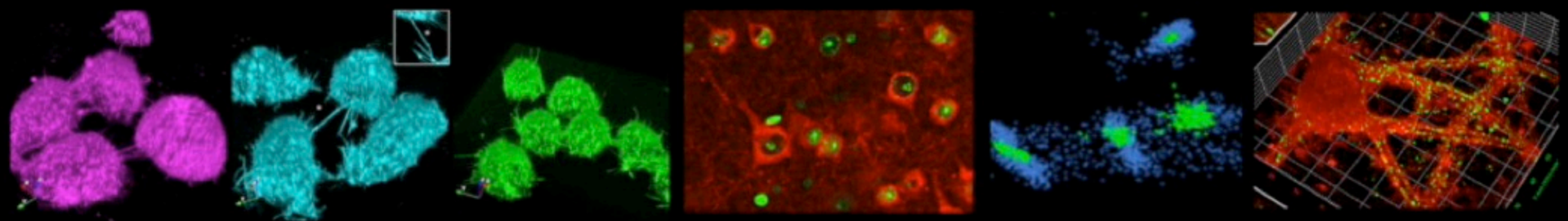
Cis Golgi 647

Trans Golgi 488



Multiscale imaging with MemBright probes

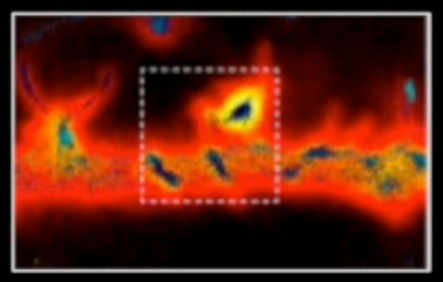
- **BioXRiv (2018)** 380451
- **Cell Chemical Biology 2019, in collaboration with M. Collot (Chemist)**



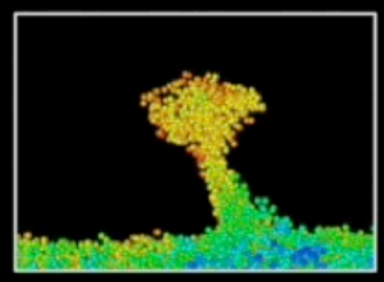
MEMBRIGHT

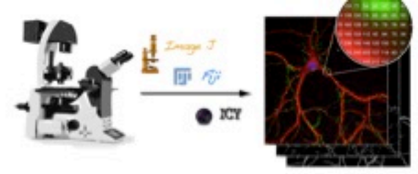
FLUORESCENT MEMBRANE PROBES DESIGNED FOR MULTISCALE IMAGING

Episode 1



Cell Chemical Biology 2019
https://doi.org/10.1016/j.chembiol.2019.01.009





Introduction to microscopy :

from conventional …

… to Super Resolution

How to choose the better objective for your sample ?
- Refraction index
- Numerical aperture
- Resolution
- Immersion medium
- Mounting medium
- Depth of field
- Working distance

Illumination mode :
- Bright-field microscopy
- Phase contrast microscopy

Fluorescence microscopy
- Matrix, Bit depth, pixel size, histogram, RGB color pictures
- Confocal microscopy
- Spinning-disk microscopy
- Airyscan

Super-resolution microscopy
- Structured illumination microscopy (SIM)
- Stimulated emission depletion (STED)
- Deconvolution
- STORM, PALM