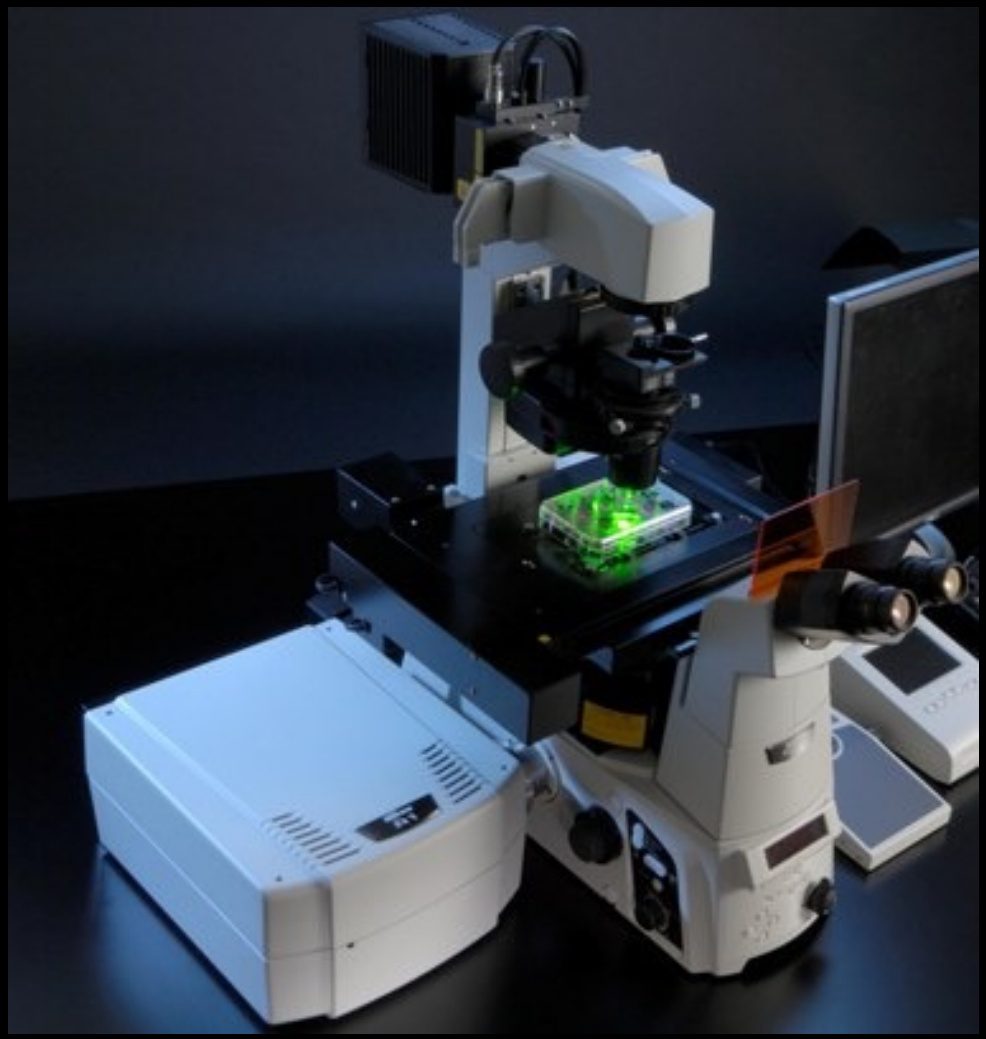
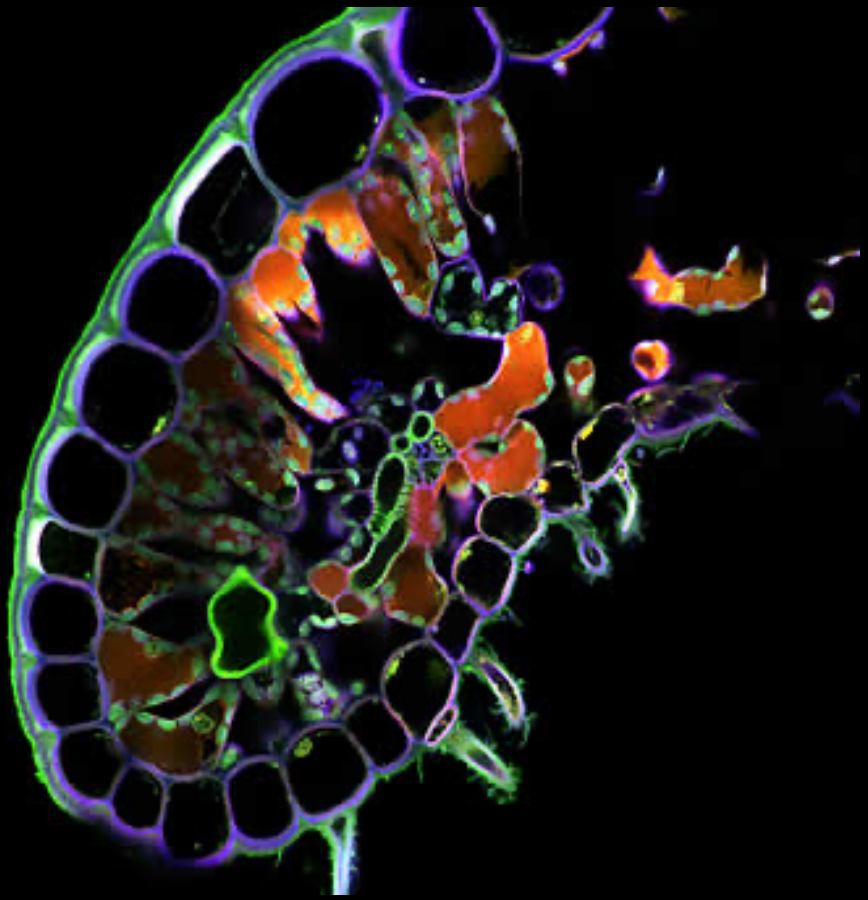
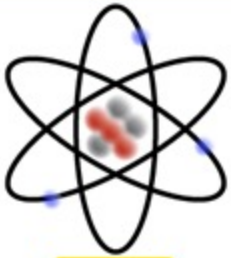
Microscopie confocale

GUILLAUD Philippe – 2021





Scales of Nature



electron

atoms

single cells

humans

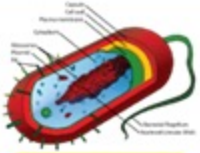
Earth

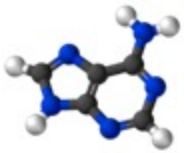
Sun

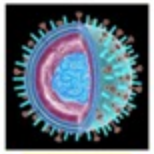
galaxies

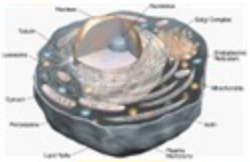
observable universe

10^{-16} 10^{-14} 10^{-12} 10^{-10} 10^{-8} 10^{-6} 10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4} 10^{6} 10^{8} 10^{10} 10^{12} 10^{14} 10^{16} 10^{18} 10^{20} 10^{22} 10^{24} 10^{26} 10^{28}









protein

bacteria

fruit fly

whale

small molecule

virus

cell

hair

mouse

human

1 nm 10 nm 100 nm 1 µm 10 µm 100 µm 1 mm 1 cm 10 cm 1 m 10 m

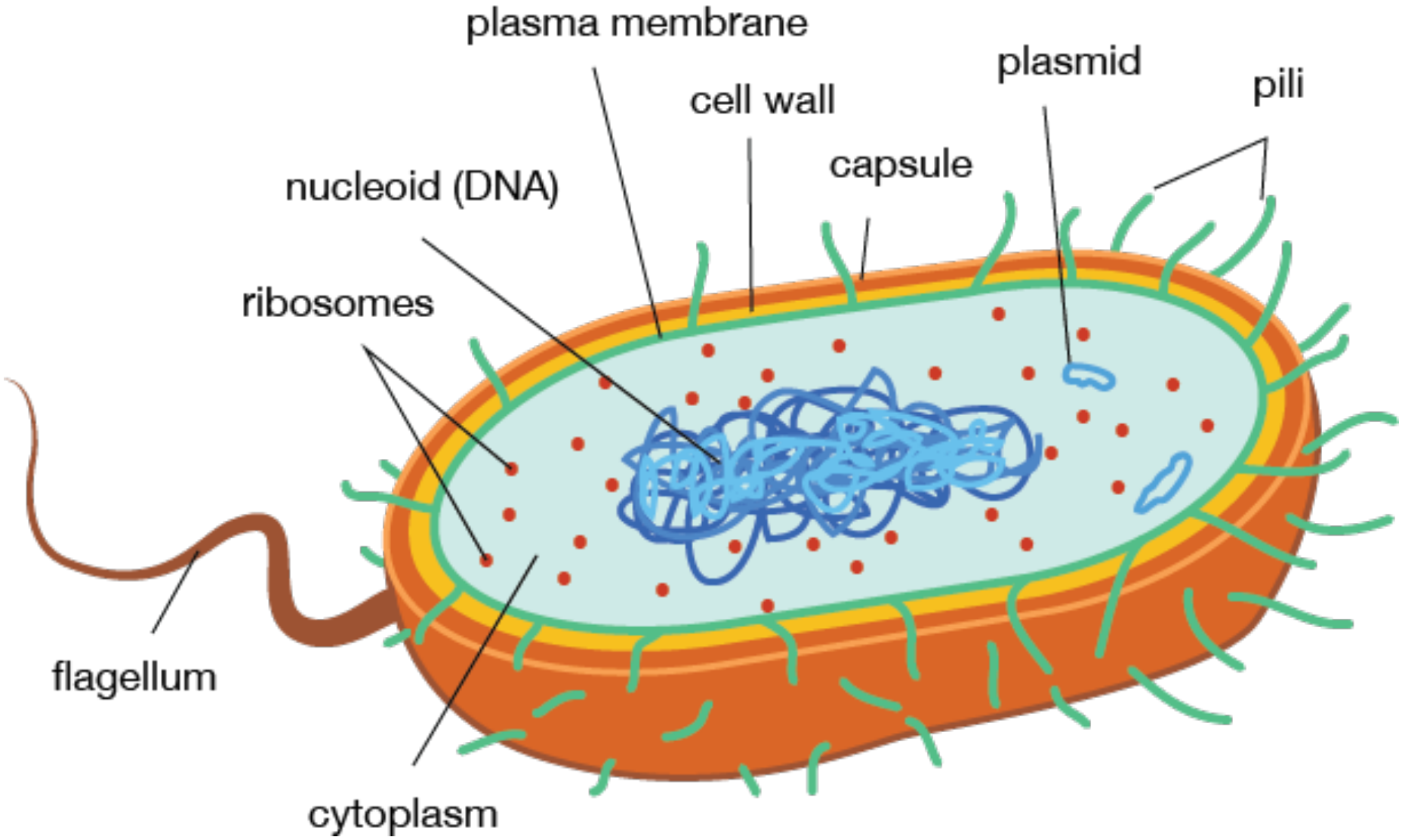
Electron Microscopy

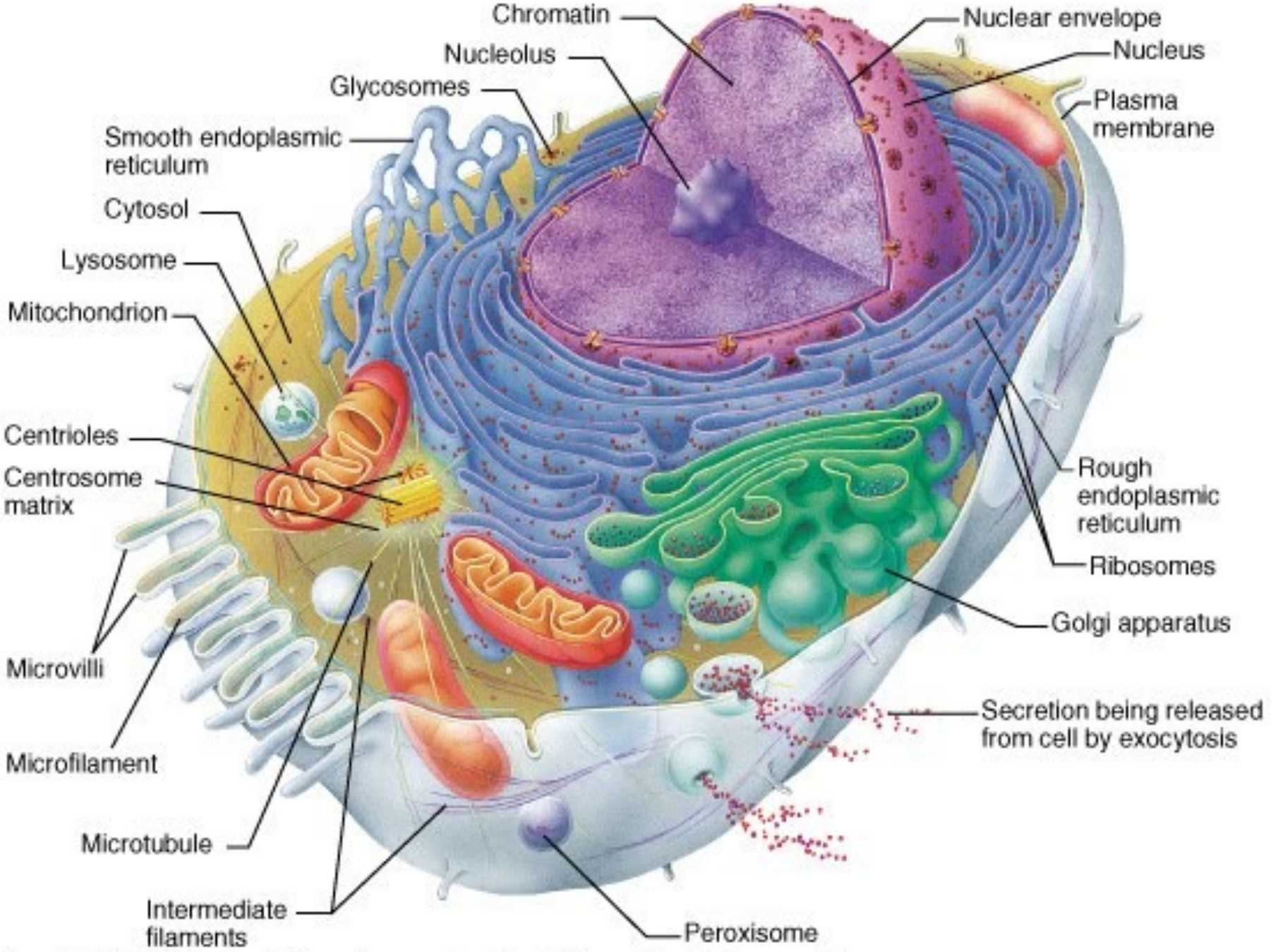
Naked Eye

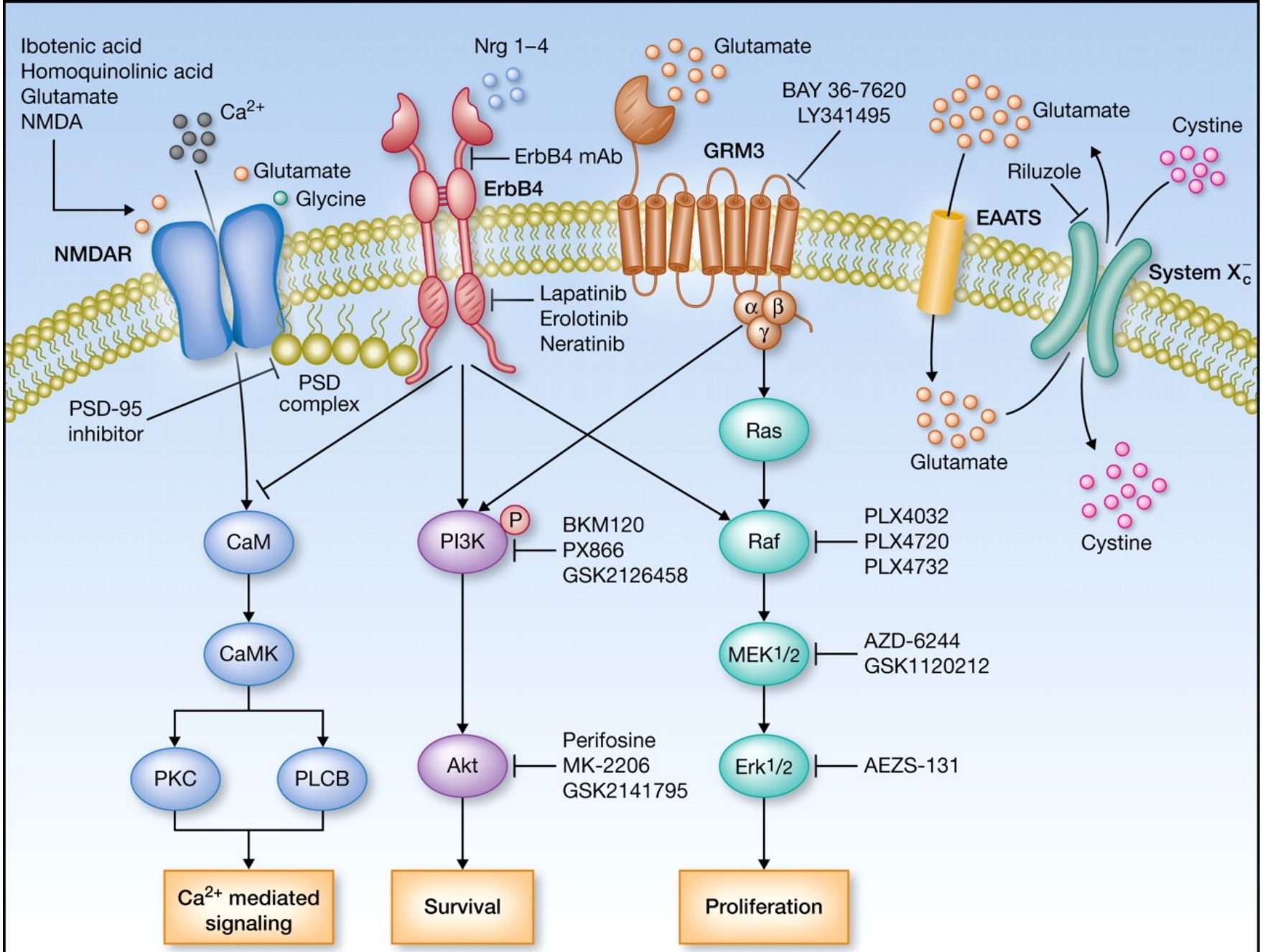
Nanoscopy

Light Microscopy

- Human eye : 100000 nm
- Simple magnifier : 10000 nm
- Optical microscope : 200 nm limits ←
- Electron microscope : 0.5 – 3 nm
- Scanning Probe microscope : 0.1 – 10 nm


plasma membrane
cell wall
plasmid
pili
capsule
nucleoid (DNA)
ribosomes
flagellum
cytoplasm



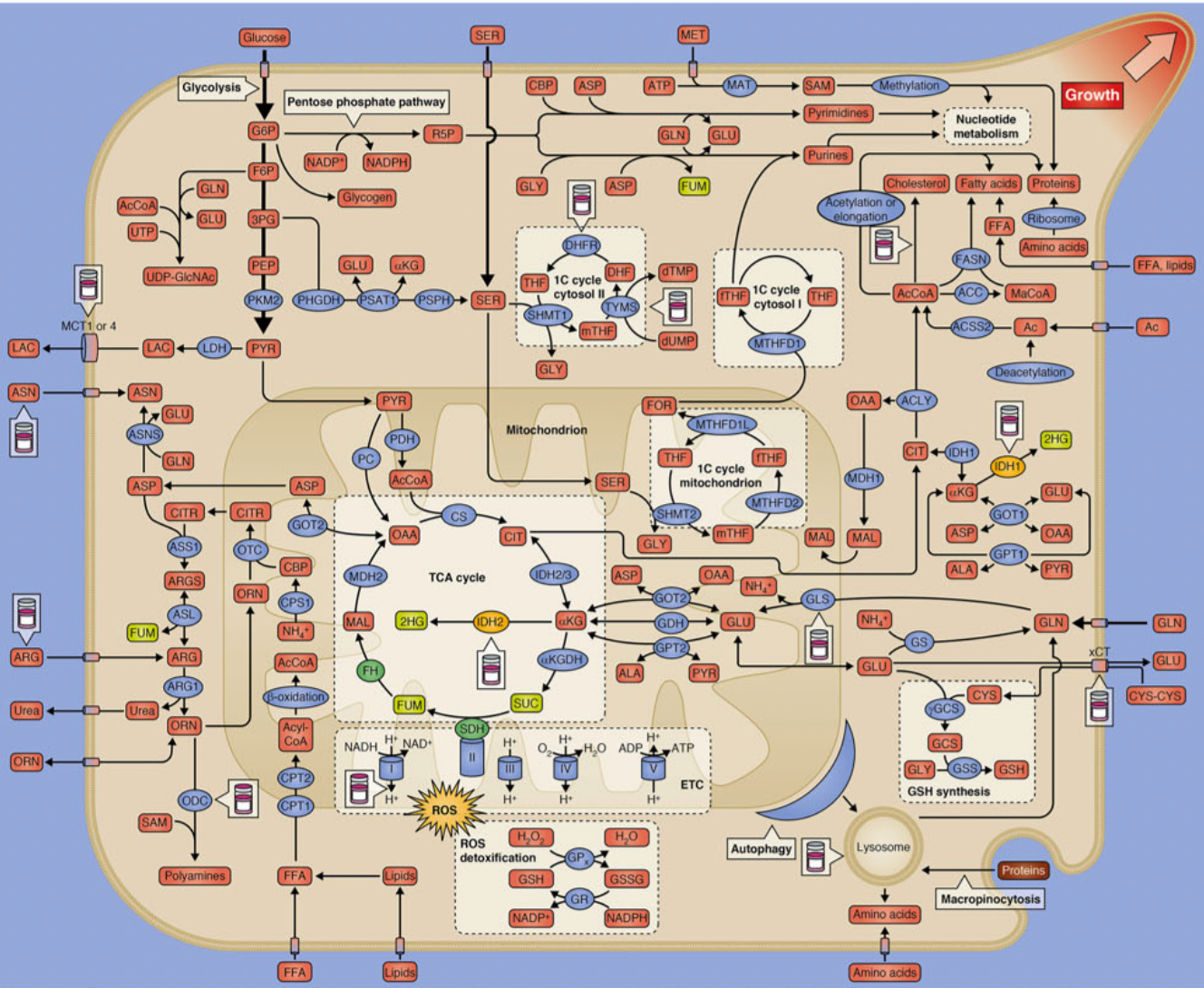


Journal of Cell Science

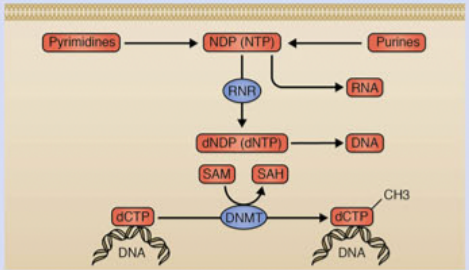
Cancer Metabolism at a Glance

Alexei Vazquez, Jurre J. Kamphorst, Elke K. Markert, Zachary T. Schug, Saverio Tardito and Eyal Gottlieb

The Company of Biologists



Nucleotide metabolism



Therapeutic cancer metabolism targets

Category	Compound	Target
Antifolates	Methotrexate Pemetrexed Raltitrexed	DHFR TYMS
Nucleotide analogs	5-Fluorouracil	
Cholesterol synthesis inhibitors	Statins	HMGCR
Fatty acids synthesis inhibitors	TVB-2640	FASN
Mitochondria inhibitors	Metformin	Complex I
Autophagy inhibitors	Chloroquine	Lysosome
Transport inhibitors	AZD3965 Sulfasalazine	MCT1 xCT
Nutrient deprivation	PEG-arginase PEG-arginine deiminase PEG-asparaginase	Plasma ARG Plasma ASN
Polyamines	DMFO	ODC
Mutant IDH inhibitors	A-120, IDH305, G peptide vaccine AG-221 AG-881	Mutant IDH1 Mutant IDH2 Mutant IDH1 or 2
Glutaminase inhibitors	CB-839	GLS

Key

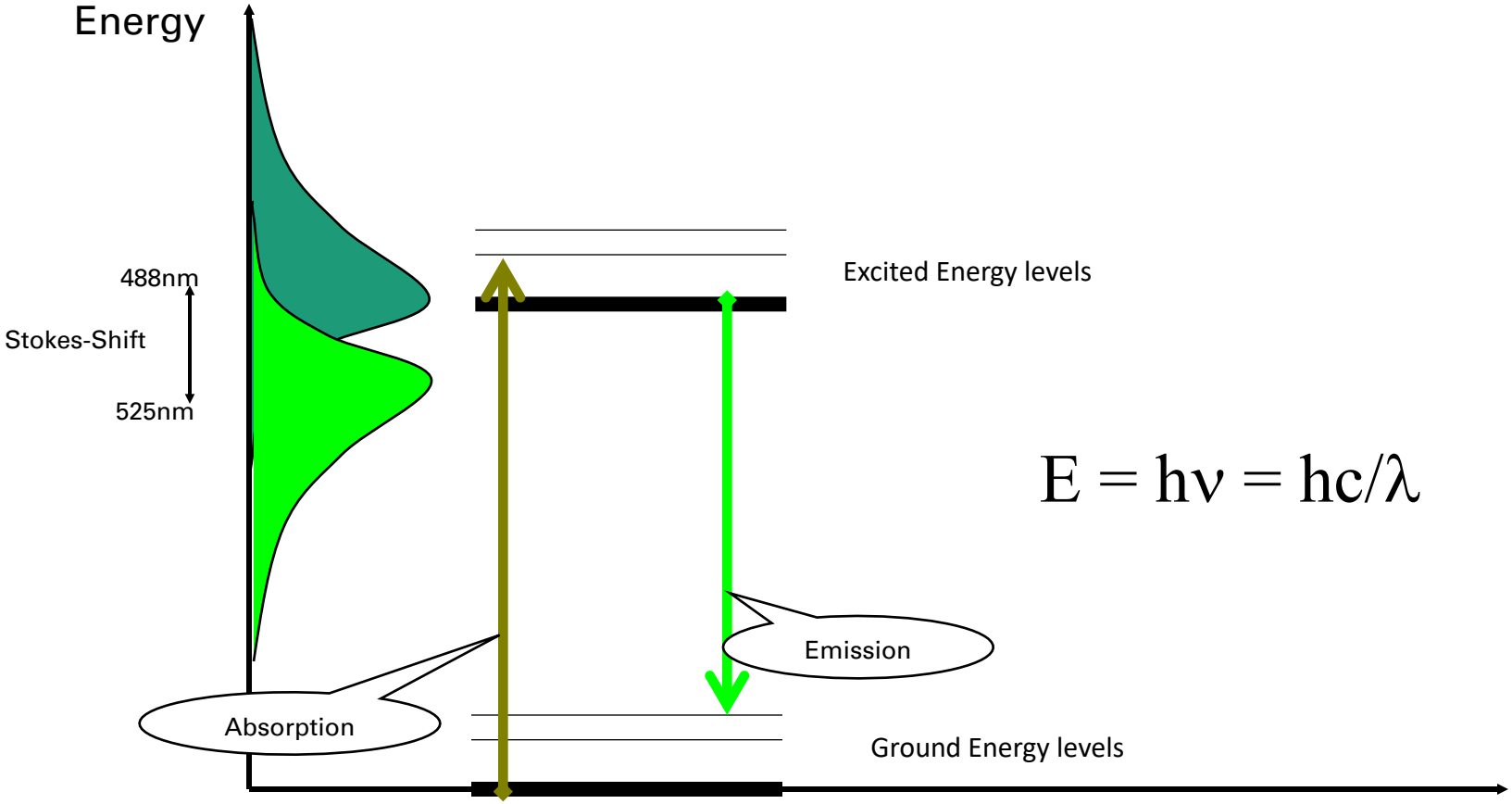
Abbreviations: αKG, α-ketoglutarate; αKGDH, α-ketoglutarate dehydrogenase; 2HG, 2-hydroxyglutarate; 3PG, 3-phosphoglycerate; Ac, acetate; ACC, acetyl-CoA carboxylase; AcCoA, acetyl-CoA; ACLY, ATP citrate lyase; ACSS2, acetyl-CoA synthetase 2; ALA, alanine; ARG, arginine; ARG1, arginase 1; ARGS, argininosuccinate; ASL, argininosuccinate lyase; ASN, asparagine; ASNS, asparagine synthetase; ASP, aspartate; ASS1, arginosuccinate synthase 1; CBP, carbamoyl phosphate; CIT, citrate or isocitrate; CITR, citrulline; CPS1, carbamoyl phosphate synthetase 1; CPT1 or 2, carnitine palmitoyltransferase 1 or 2; CS, citrate synthase; CYS, cysteine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dNDP (dNTP), deoxynucleotide diphosphate (triphosphate); DNMT, DNA methyltransferase; ETC, electron transport chain; F6P, fructose 6-phosphate; FASN, fatty acid synthase; FFA, free fatty acids; FH, fumarate hydratase; fTHF, 10-formyl-tetrahydrofolate; FOR, formate; FUM, fumarate; γGCS, γ-glutamylcysteine synthetase; G6P, glucose 6-phosphate; GCS, glutamylcysteine; GDH, glutamate dehydrogenase; GLN, glutamine; GLS, glutaminase; GLU, glutamate; GLY, glycine; GOT1 or 2, glutamate oxaloacetate transaminase 1 or 2; GPT1 or 2, glutamate pyruvate transaminase 1 or 2; GPx, glutathione peroxidase; GR, glutathione reductase; GS, glutamine synthetase; GSH, glutathione; GSS, gluthatione synthetase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IDH1, 2 or 3, isocitrate dehydrogenase 1, 2 or 3; LAC, lactate; LDH, lactate dehydrogenase; MaCoA, malonyl-CoA; MAL, malate; MAT, methionine adenosyltransferase; MCT, monocarboxylate transporter; MDH1 or 2, malate dehydrogenase 1 or 2; MET, methionine; mTHF, 5,10-methenyl-tetrahydrofolate; MTHFD1, 1L or 2, methelenetetrahydrofolate dehydrogenase 1, 1L or 2; NDP (NTP), nucleotide diphosphate (triphosphate); OAA, oxaloacetate; ODC, ornithine decarboxylase; ORN, ornithine; OTC, ornithine transcarbamoylase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PHGDH, 3-phosphoglycerate dehydrogenase; PKM2, pyruvate kinase isoform M2; PSAT1, phosphoserine transaminase 1; PSPH, phosphoserine phosphatase; PYR, pyruvate; R5P, ribose 5-phosphate, ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; RNR, ribonucleotide reductase; SAM, S-adenosylmethionine; SDH, succinate dehydrogenase; SER, serine; SHMT1 or 2, serine hydromethyltransferase 1 or 2; SUC, succinate or succinyl-CoA; THF, tetrahydrofolate; TYMS, thymidylate synthase; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; xCT, cystine-glutamate antiporter.

doi: 10.1242/jcs.181016

Contrast using Fluorescence

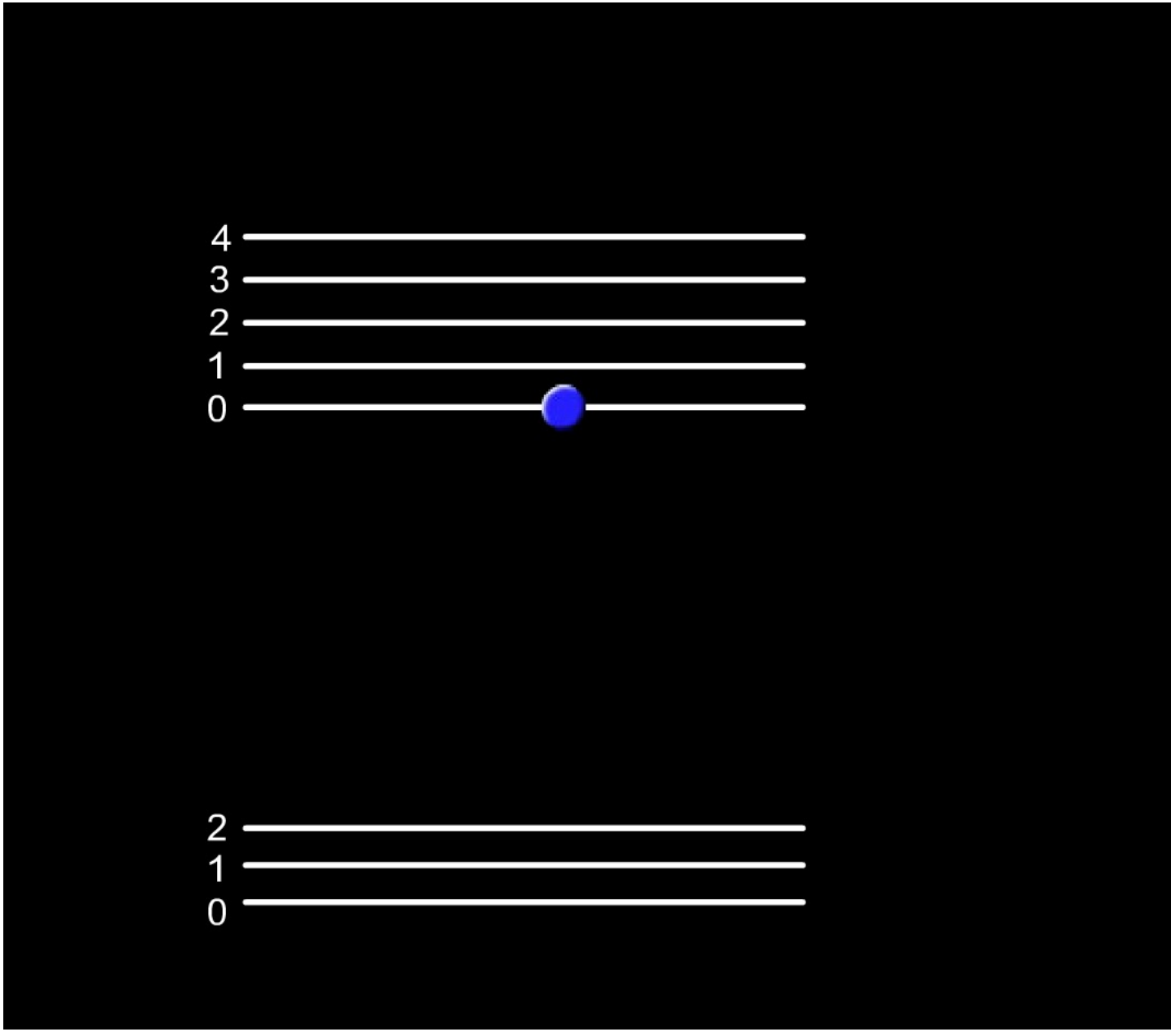
- Only specific structures are stained and images
- Unwanted structures remain are not visible
- Detail can be seen even if smaller than resolution limits
- With the advent of special dyes, staining of living cells is now possible

Fluorescence
Jablonski Diagram



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

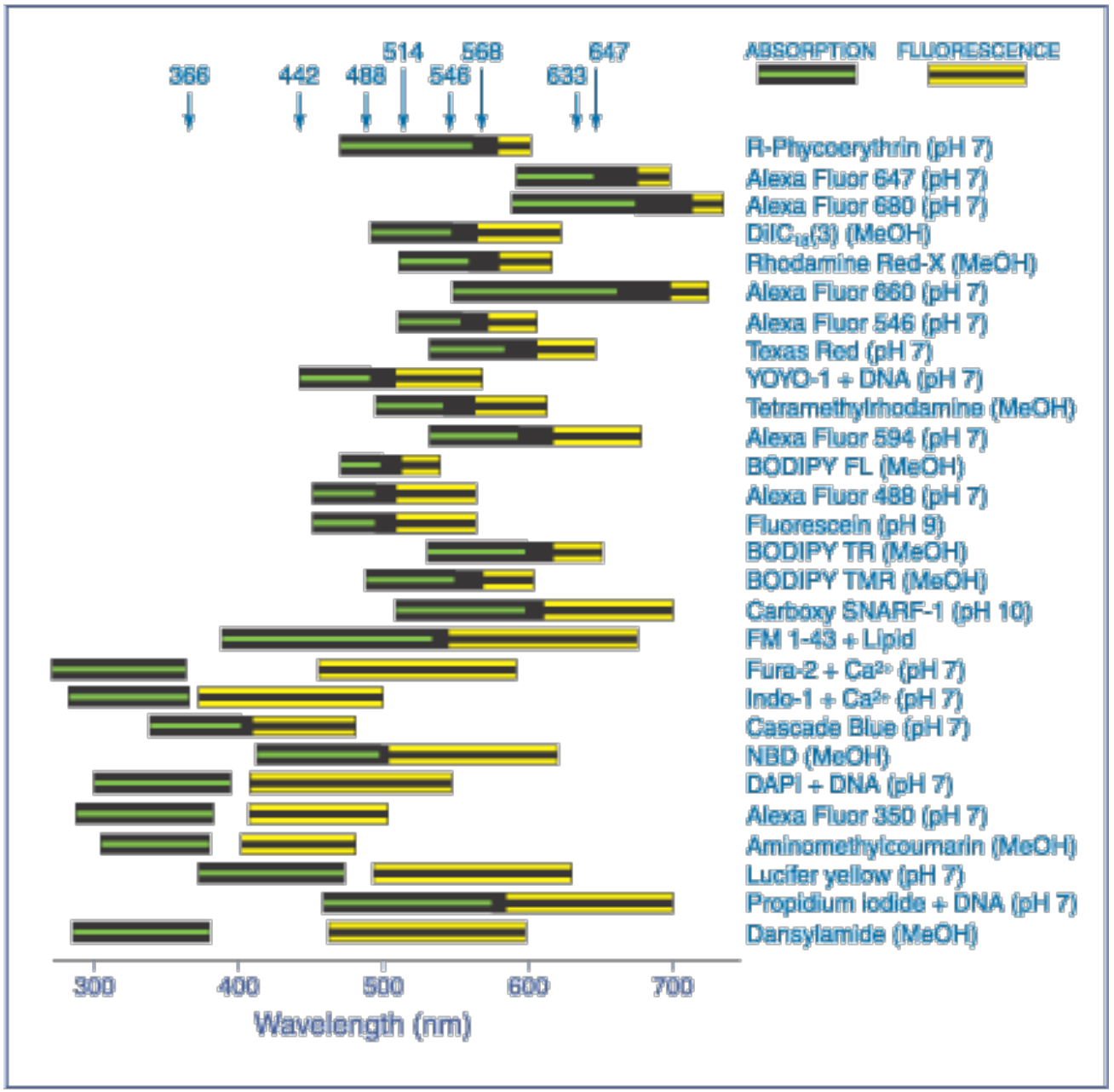
Prof. Alexander Jablonski, 1935



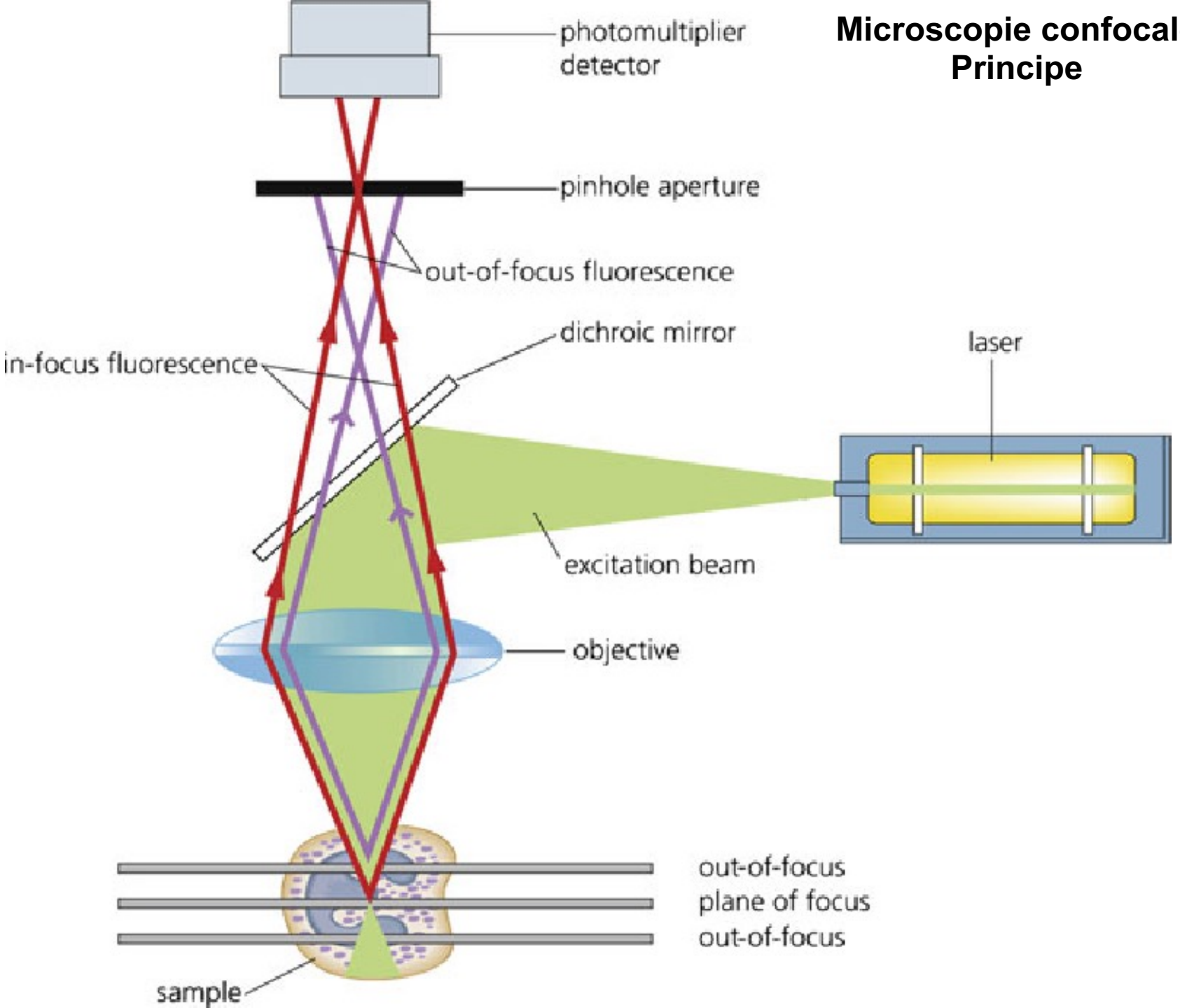
Prof. Alexander Jablonski, 1935

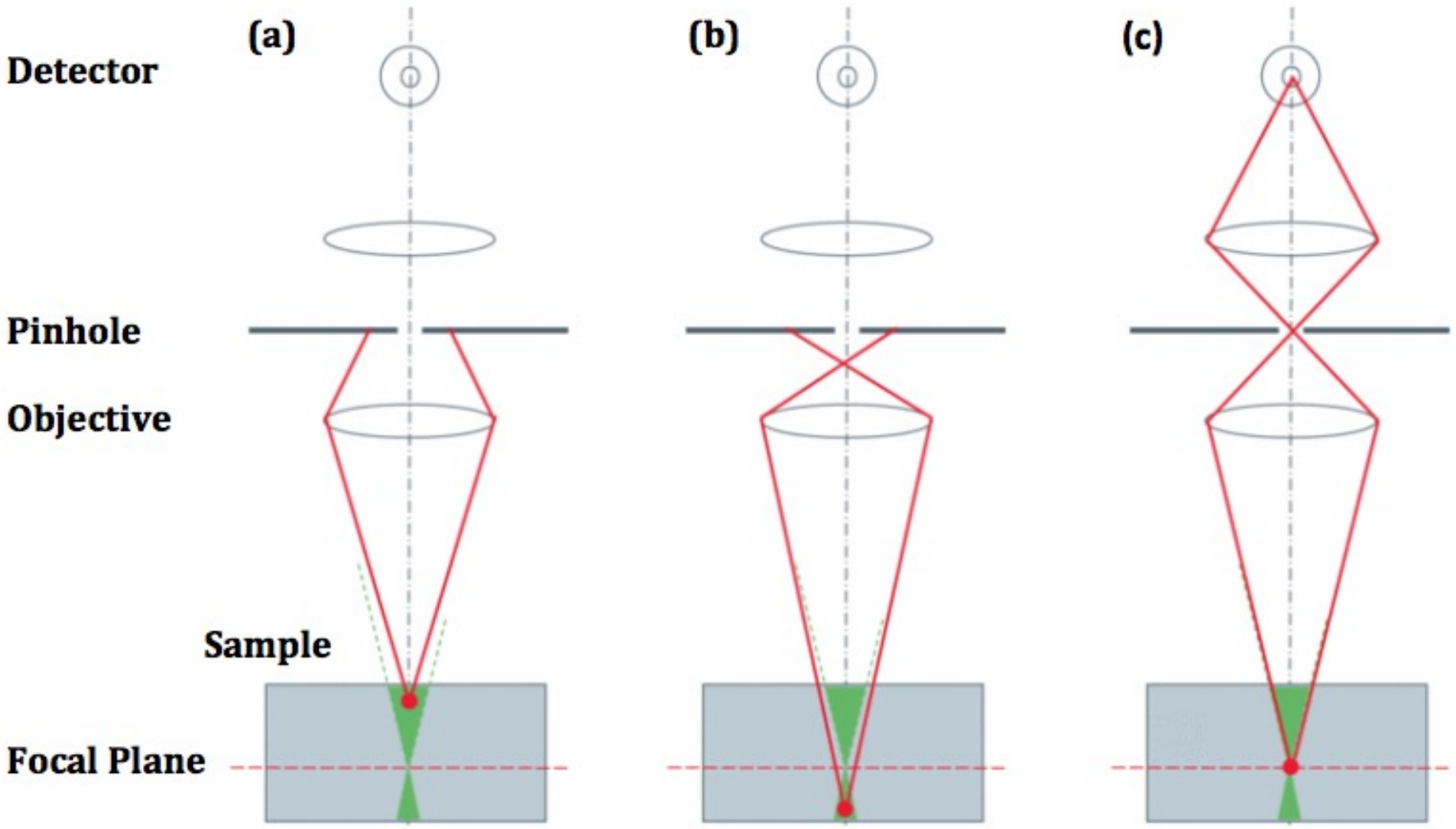
$$E = h\nu = hc/\lambda$$

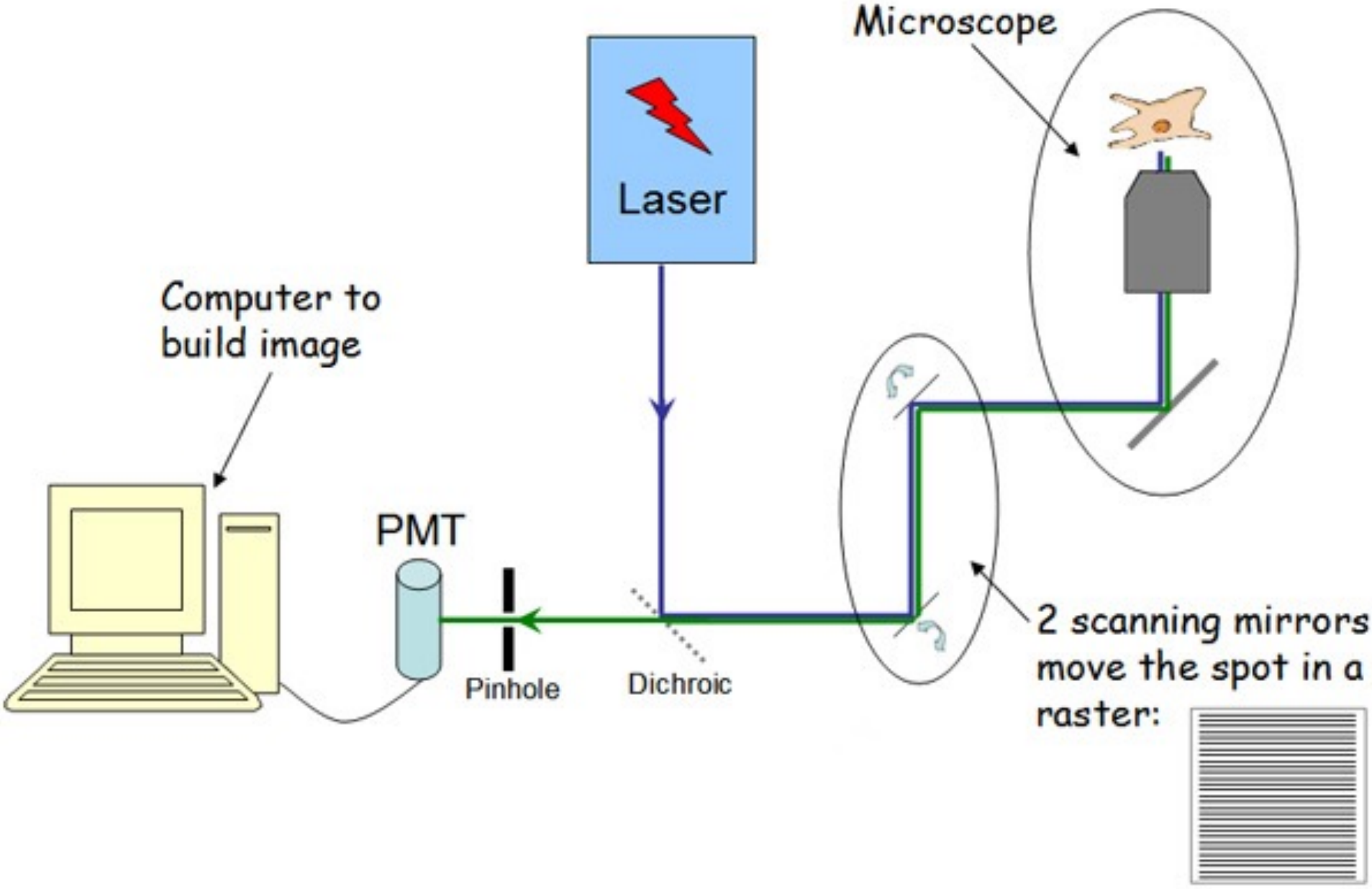
Sondes fluorescentes et intérêts en biologie

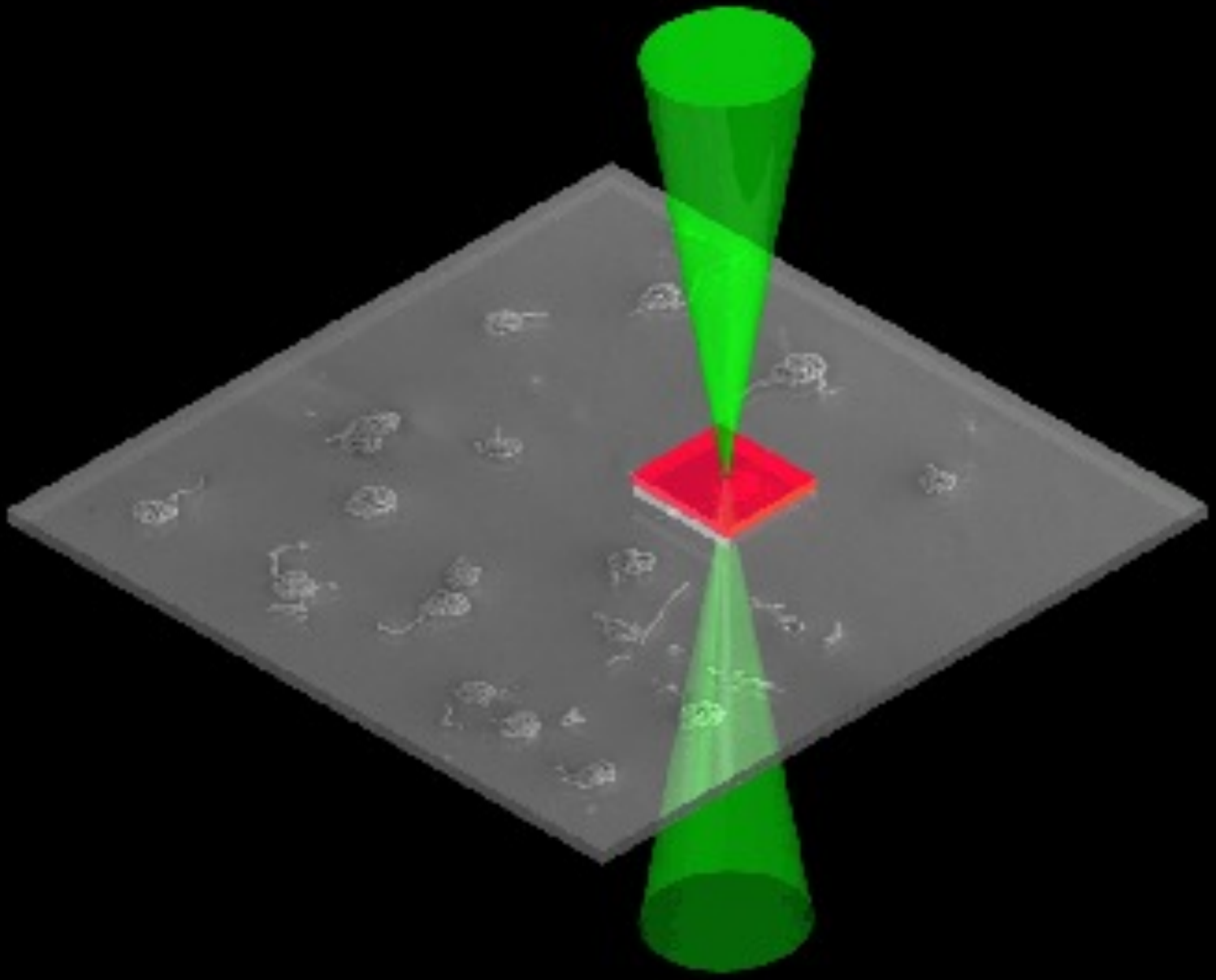


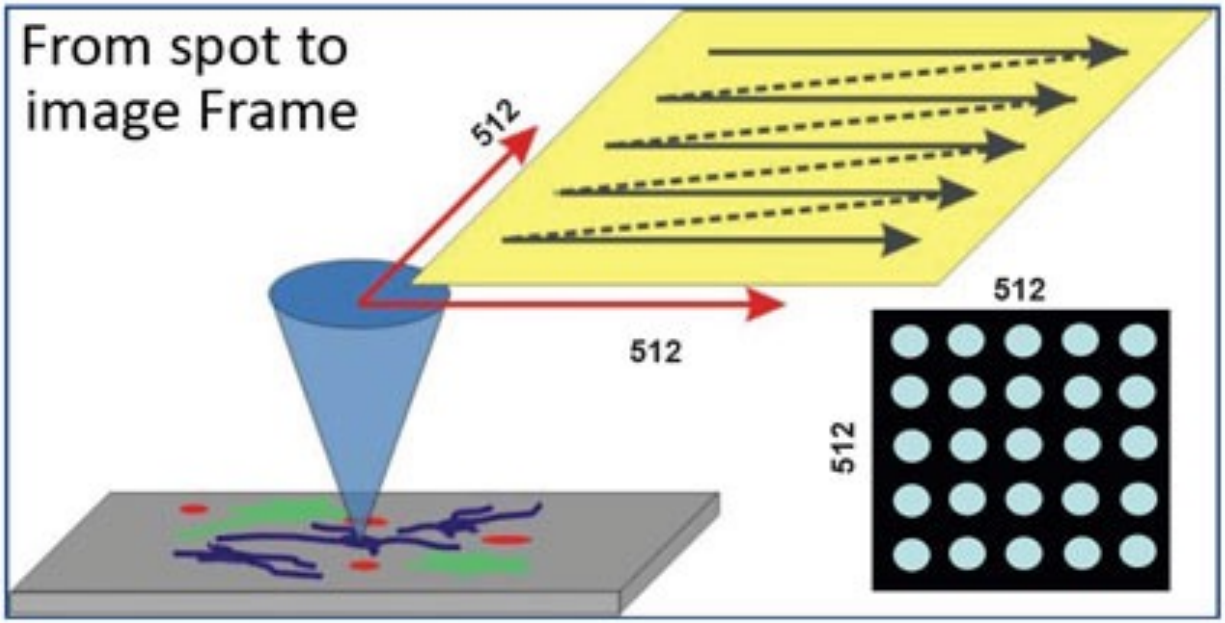
Microscopie confocale
Principe

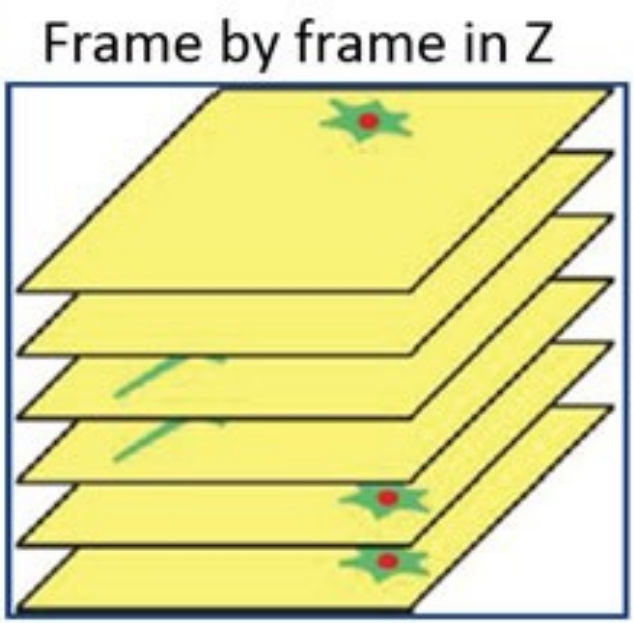


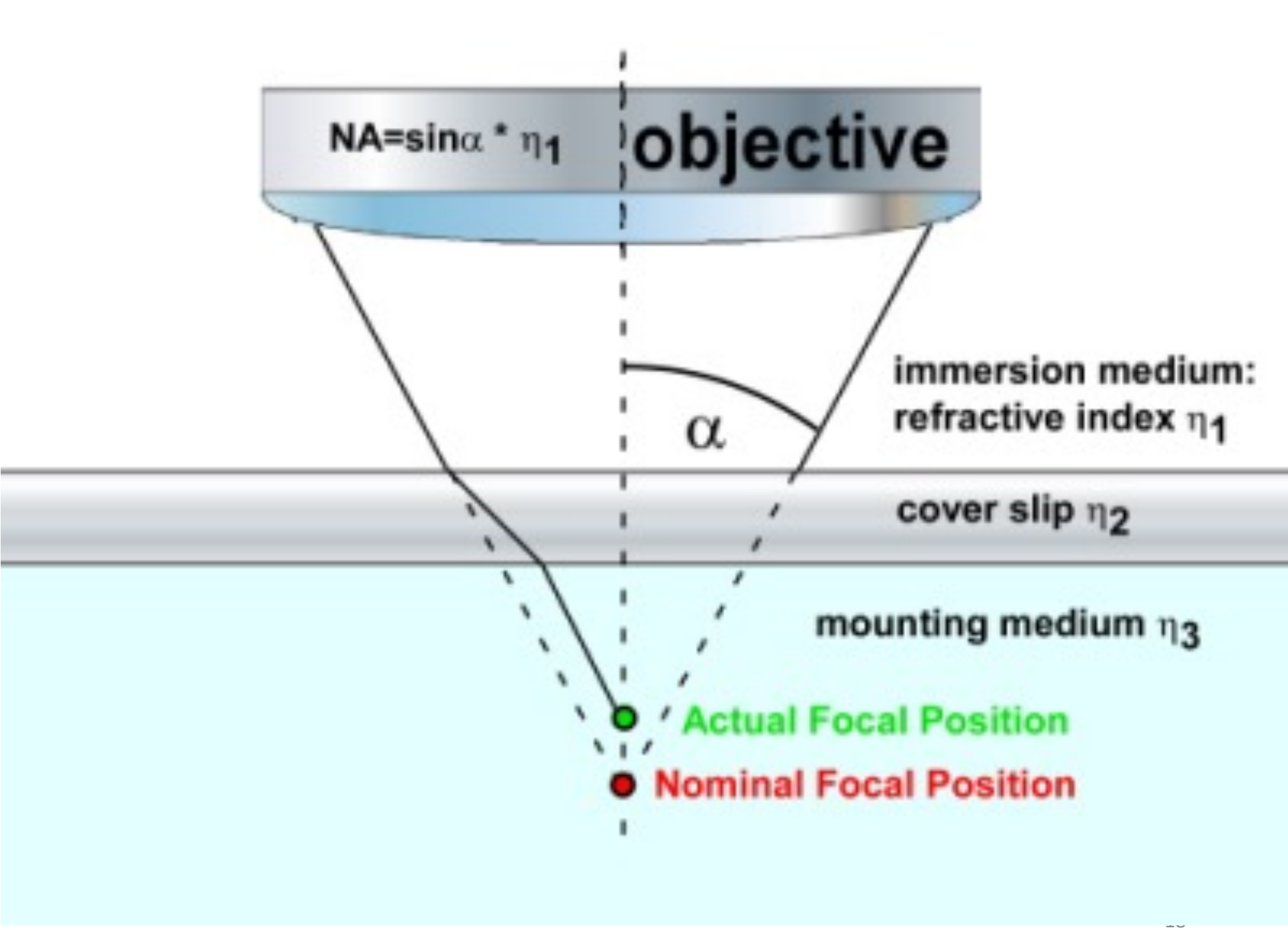

(a)
(b)
(c)
Detector
Pinhole
Objective
Sample
Focal Plane

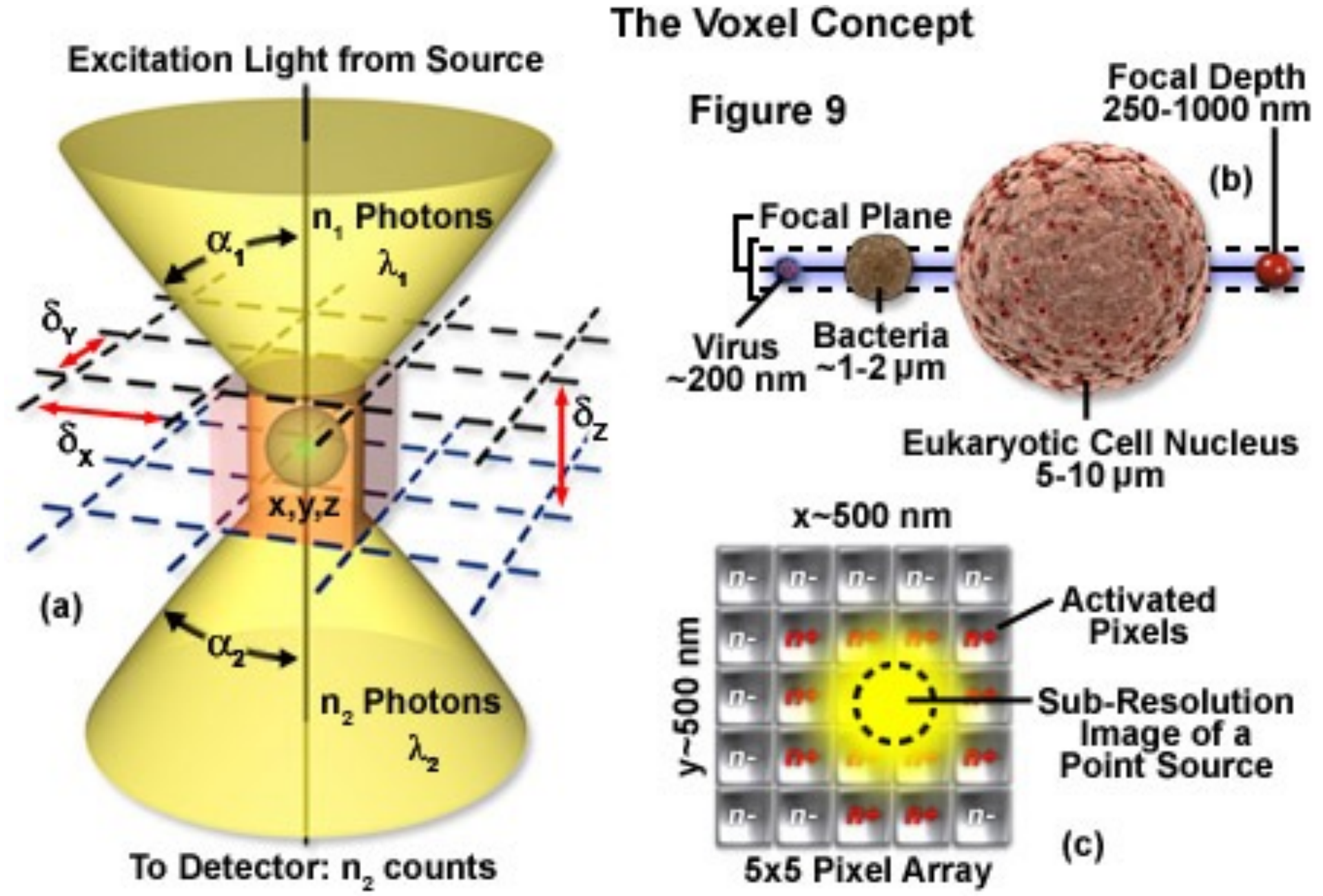

Microscope
Laser
Computer to build image
PMT
Pinhole
Dichroic
2 scanning mirrors move the spot in a raster:

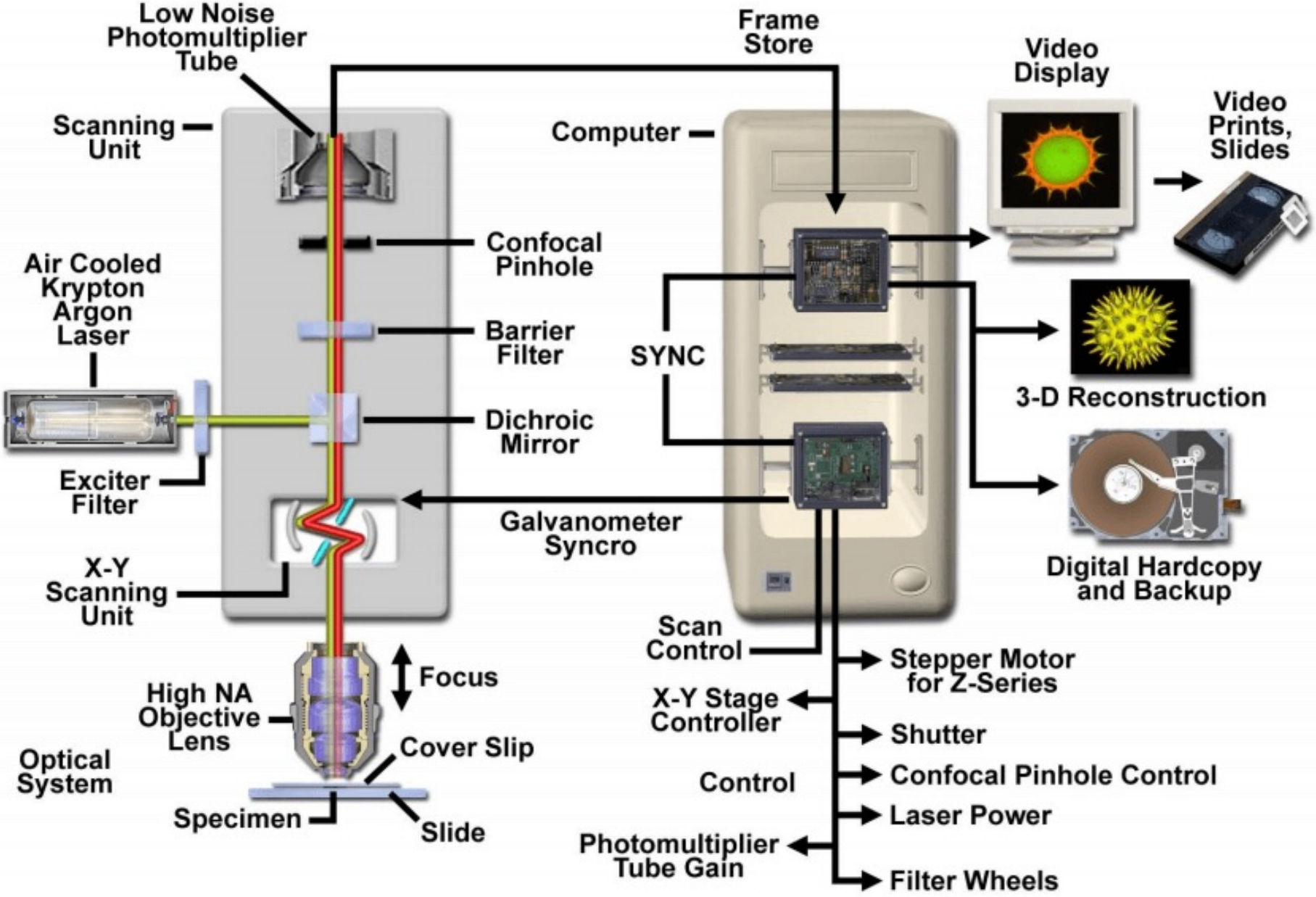



From spot to
image Frame
512
512
512
512

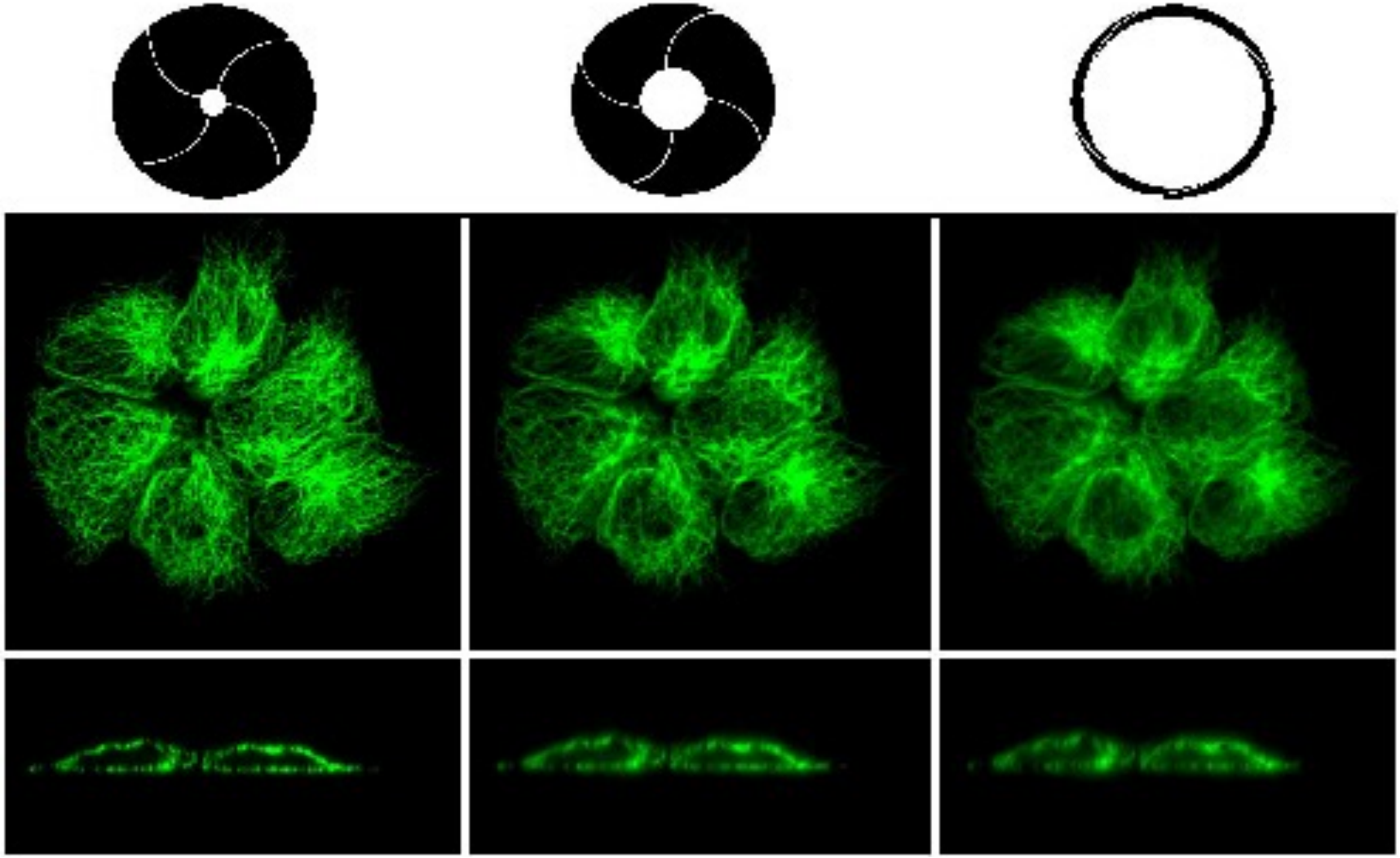

Frame by frame in Z


NA=sinα * η1
objective
immersion medium:
refractive index η1
α
cover slip η2
mounting medium η3
Actual Focal Position
Nominal Focal Position


The Voxel Concept
Figure 9
Excitation Light from Source
n_1 Photons
λ_1
α_1
δ_Y
δ_X
δ_Z
x,y,z
(a)
α_2
n_2 Photons
λ_2
To Detector: n_2 counts
Focal Depth
250-1000 nm
(b)
Focal Plane
Virus
~200 nm
Bacteria
~1-2 µm
Eukaryotic Cell Nucleus
5-10 µm
x~500 nm
y~500 nm
Activated
Pixels
Sub-Resolution
Image of a
Point Source
(c)
5x5 Pixel Array

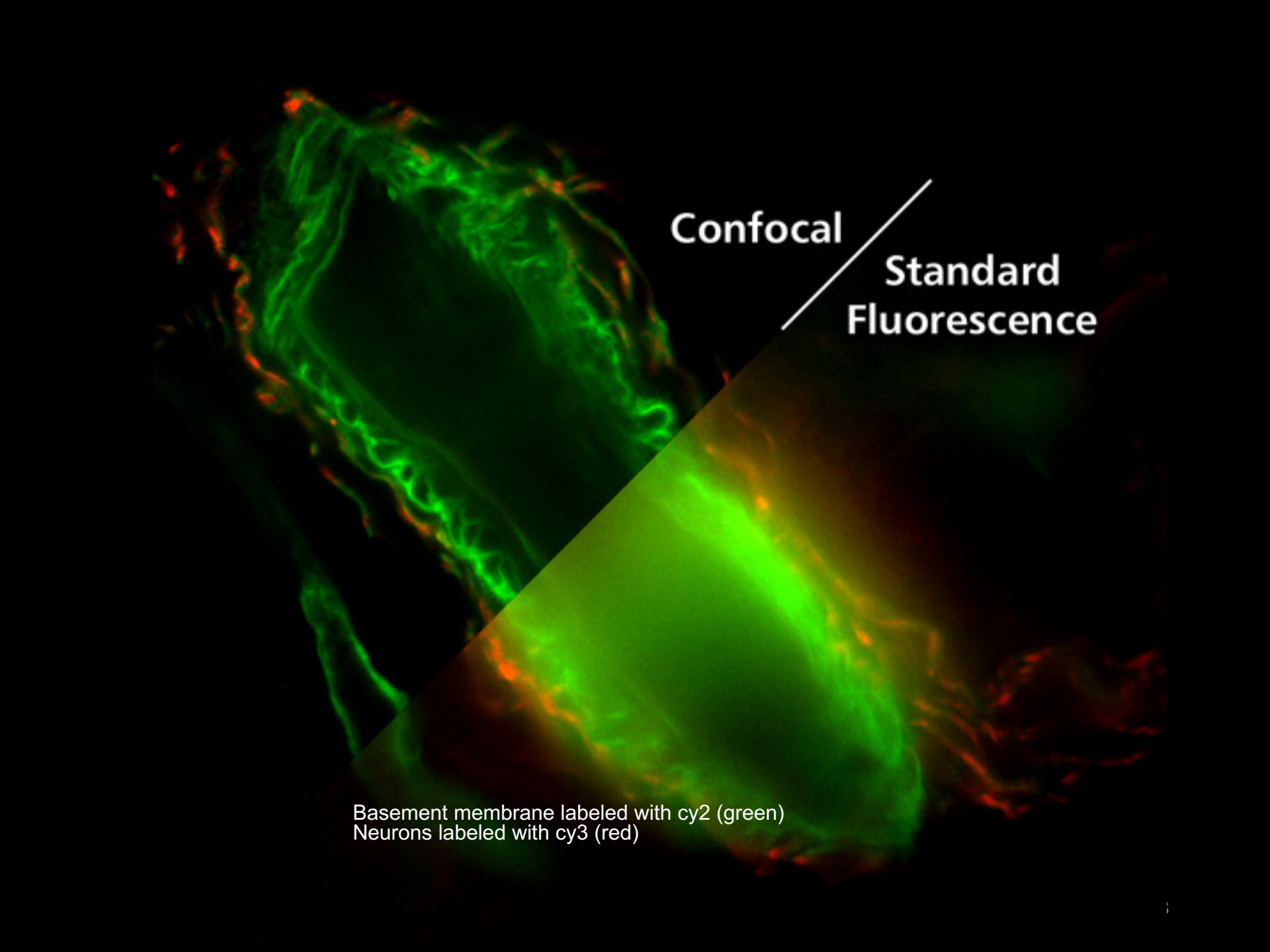

Low Noise Photomultiplier Tube
Frame Store
Video Display
Video Prints, Slides
Scanning Unit
Computer
Confocal Pinhole
Air Cooled Krypton Argon Laser
Barrier Filter
SYNC
3-D Reconstruction
Dichroic Mirror
Exciter Filter
Galvanometer Syncro
X-Y Scanning Unit
Digital Hardcopy and Backup
Scan Control
Stepper Motor for Z-Series
Focus
High NA Objective Lens
X-Y Stage Controller
Shutter
Cover Slip
Optical System
Control
Confocal Pinhole Control
Laser Power
Specimen
Slide
Photomultiplier Tube Gain
Filter Wheels

Microscopie confocale
Diaphragme (pinhole) et résolution

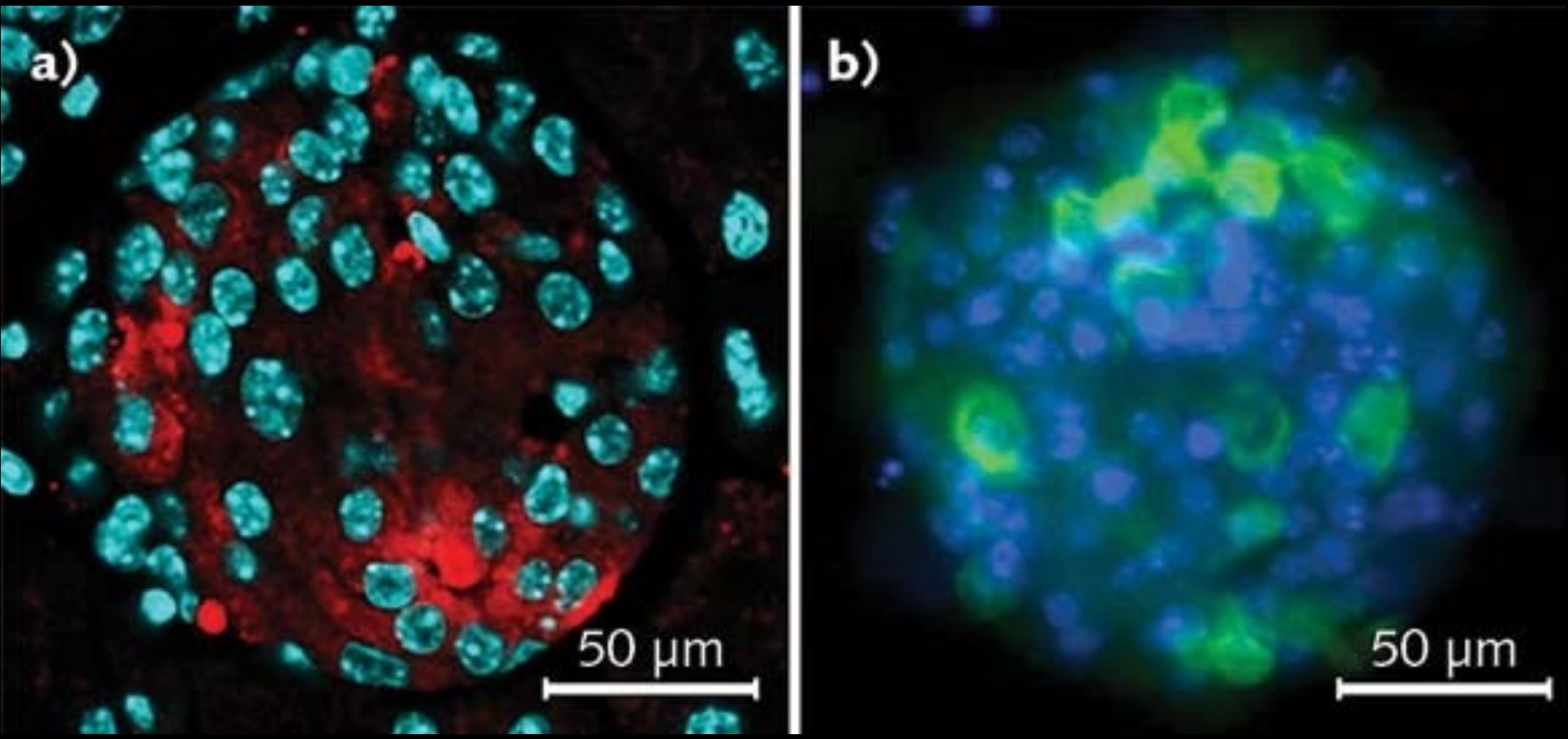


Résolution optimale

Intensité de fluorescence maximale



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


a)
50 µm
b)
50 µm

Breveté par Minsky en 1957 :

“Elimination of the out of focus flare observed in fluorescence in thick sections”

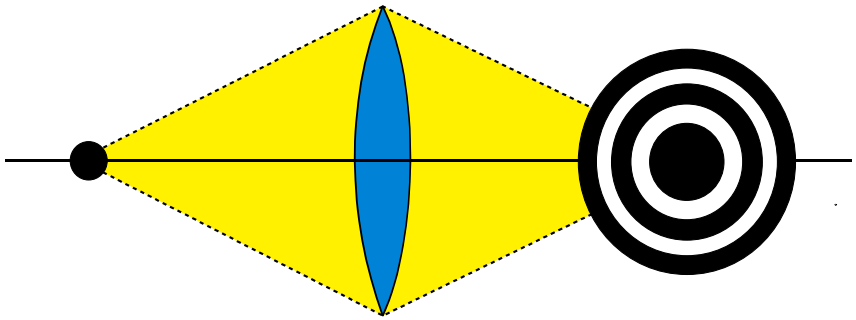
Confocal Microscopy Benefits

Suppression of out of focus light :
The confocal detection pinhole acts as a barrier to light outside the focal plane of the objective

Optical sectioning :
Specimen is monitored slice by slice (3D-resolution)
Each slice produces a sharp image by confocal optics

Improved resolution power :
Lateral resolution improved by appr. 1.4x.
Significant improvement in axial resolution

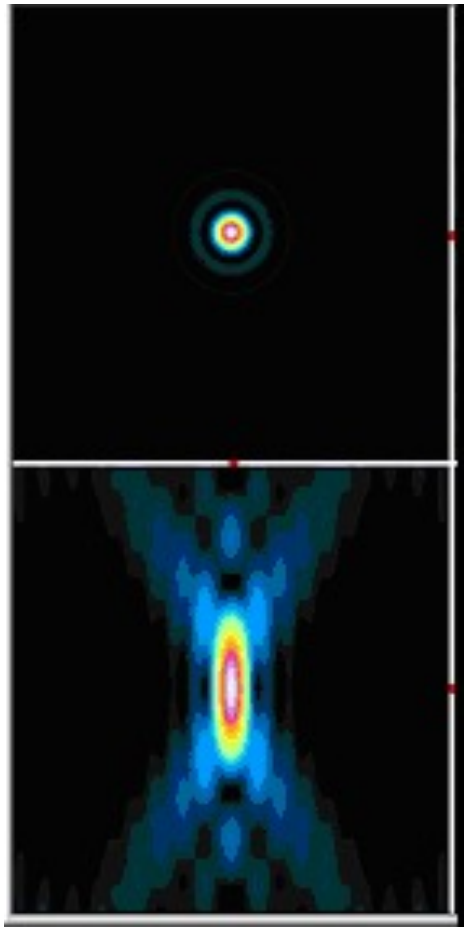
Improved contrast:
Rasterizing the specimen, straylight due to scattering is suppressed



Airy disc

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

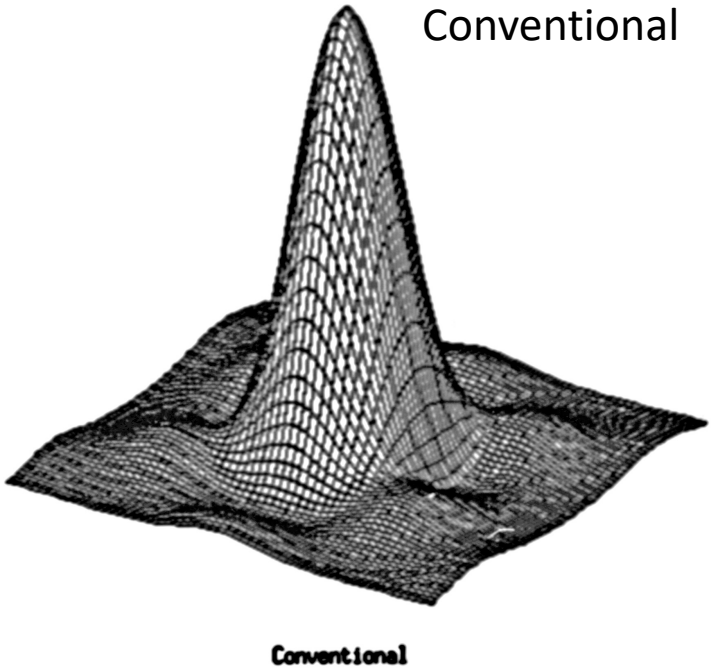
Limited by Diffraction



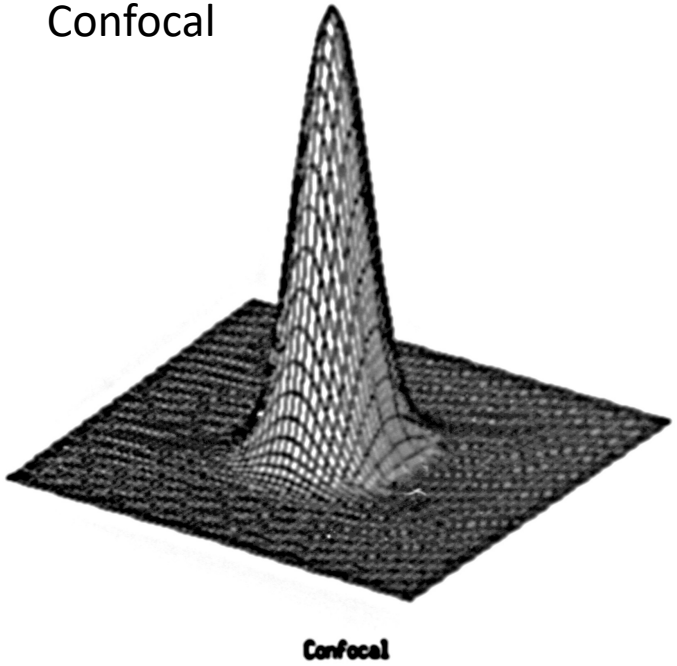
XY Plane

XZ Plane

Conventional



Confocal

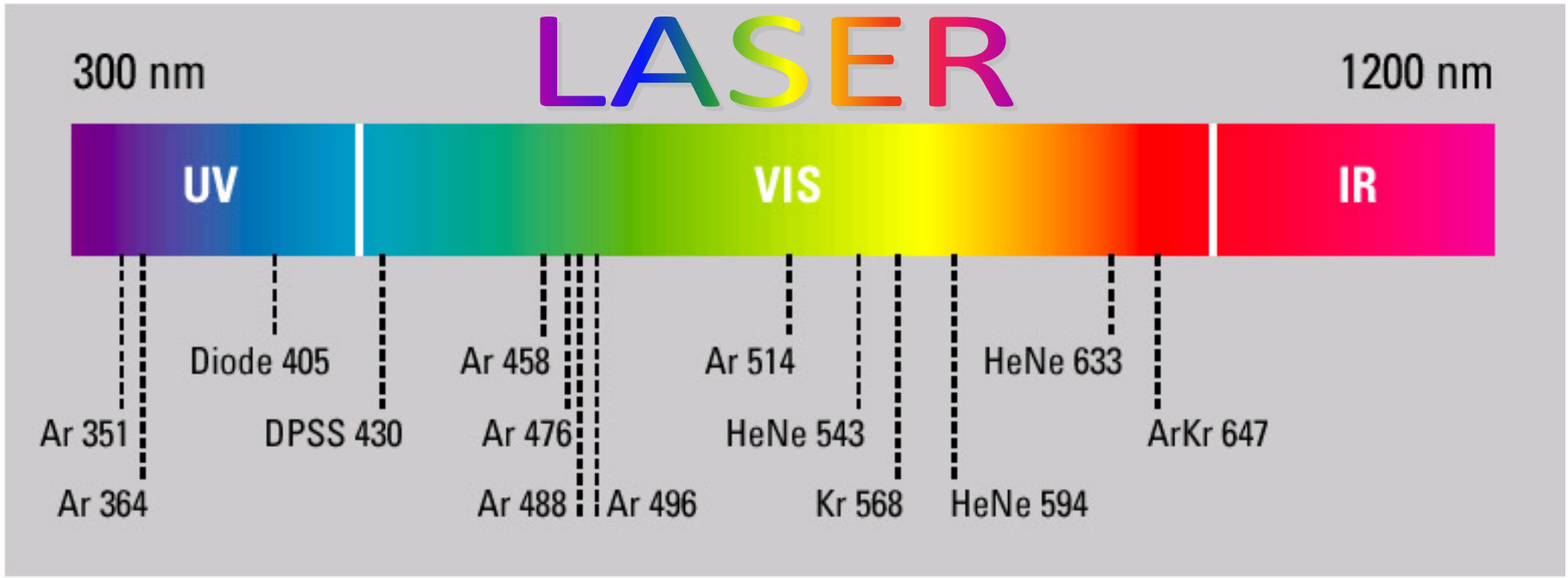


Confocal Implementation

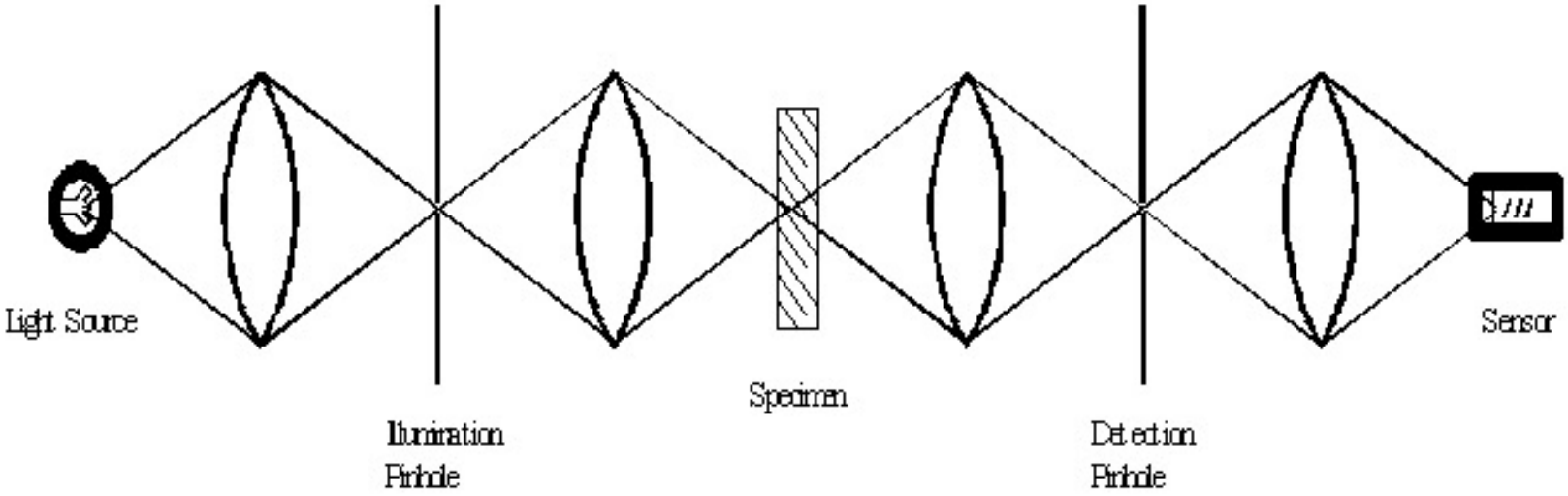
Requires to excite and observe as small a volume as possible on the sample.

This in turn requires a different way of illumination and observation of the samples

Laser types


LASER
300 nm
1200 nm
UV
VIS
IR
Ar 351
Ar 364
Diode 405
DPSS 430
Ar 458
Ar 476
Ar 488
Ar 496
Ar 514
HeNe 543
Kr 568
HeNe 594
HeNe 633
ArKr 647

Confocal Beam Path

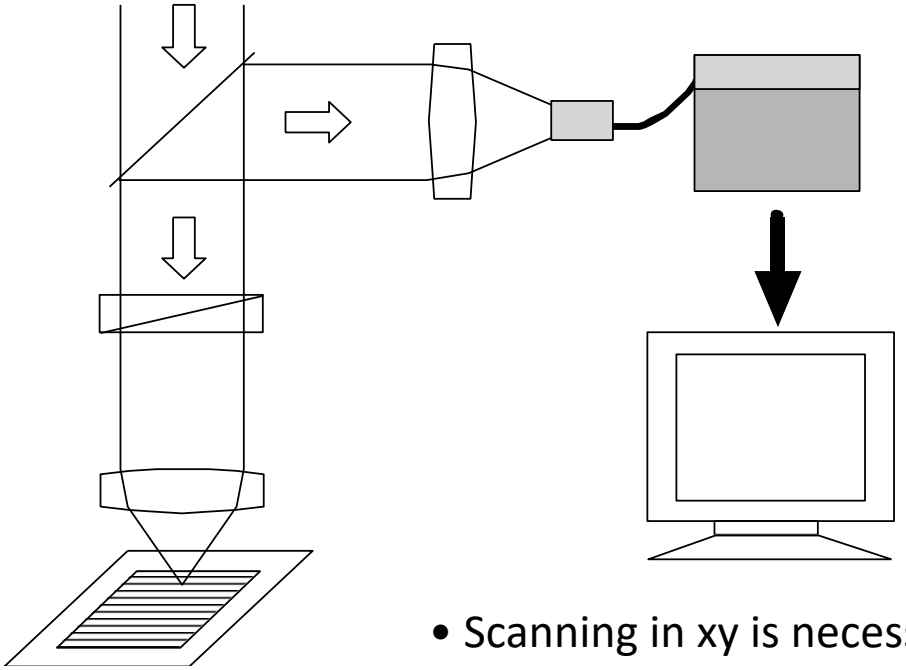


Why Laser ?

- Intense light source confined to small beam size
  Easily focused to diffraction limited spot

- As different fluorescence dyes have different spectral characteristics, many laser lines are required

Why scanning?



- Scanning in xy is necessary in order to form an image
- The scanning device is positioned between the beam splitter and the objective
- The beam is scanned (made to move) on the excitation side and descanned (made stationary) on the detection side

Why a standard microscope ?

- Greatly facilitates the use of the system
- Provides a platform for mounting samples
- The application determines what type of microscope to be used

Microscope types

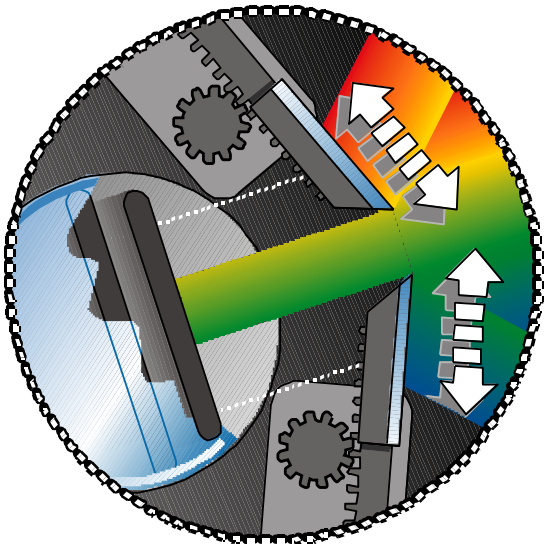
Inverted :

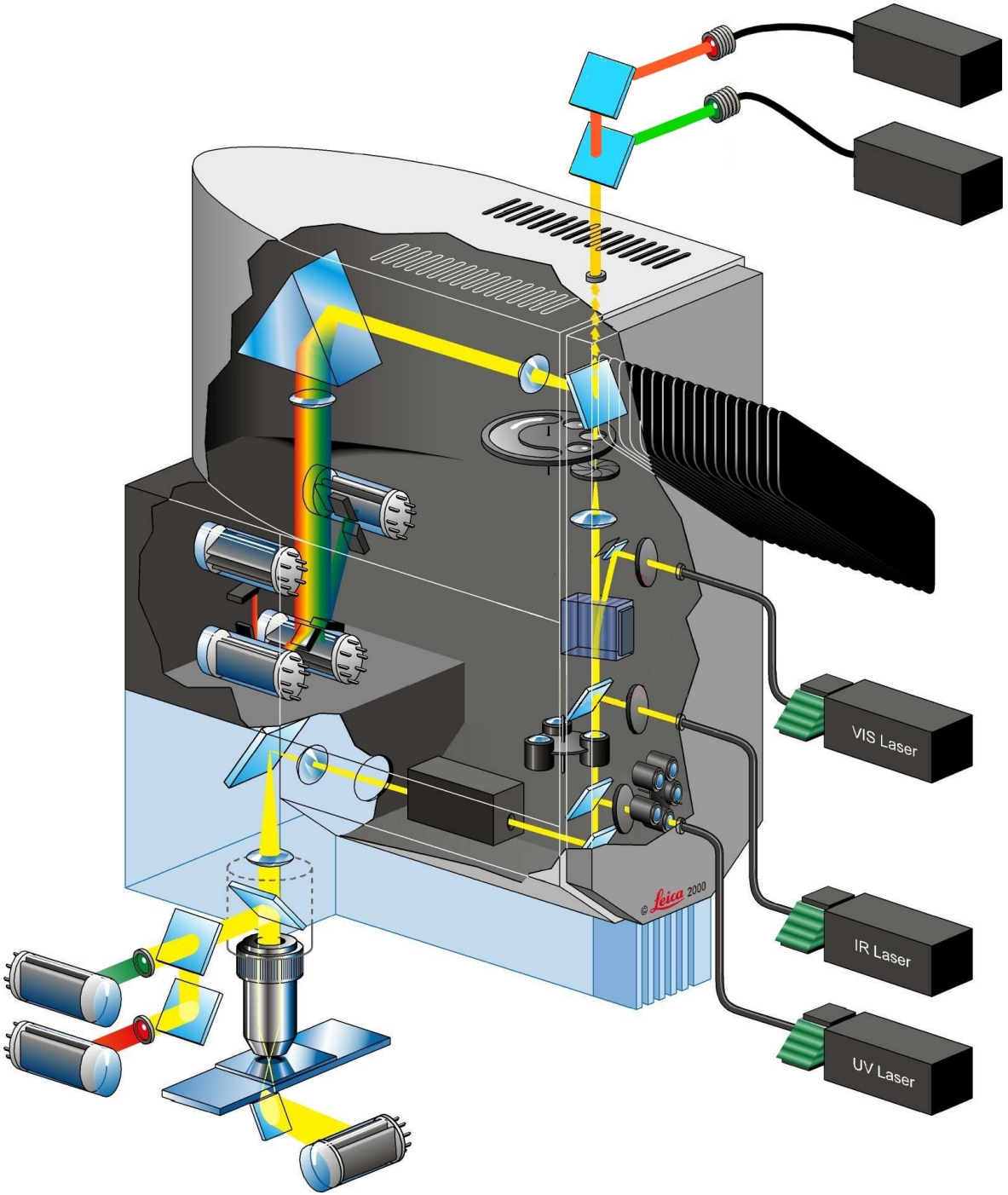
Live specimens

Upright : fixed samples

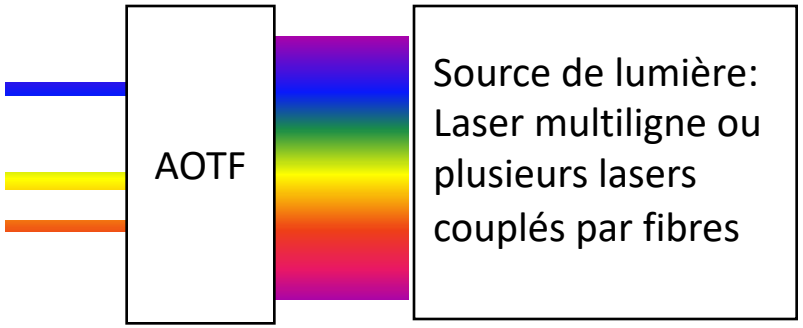
Fixed stage :

Patch clamping



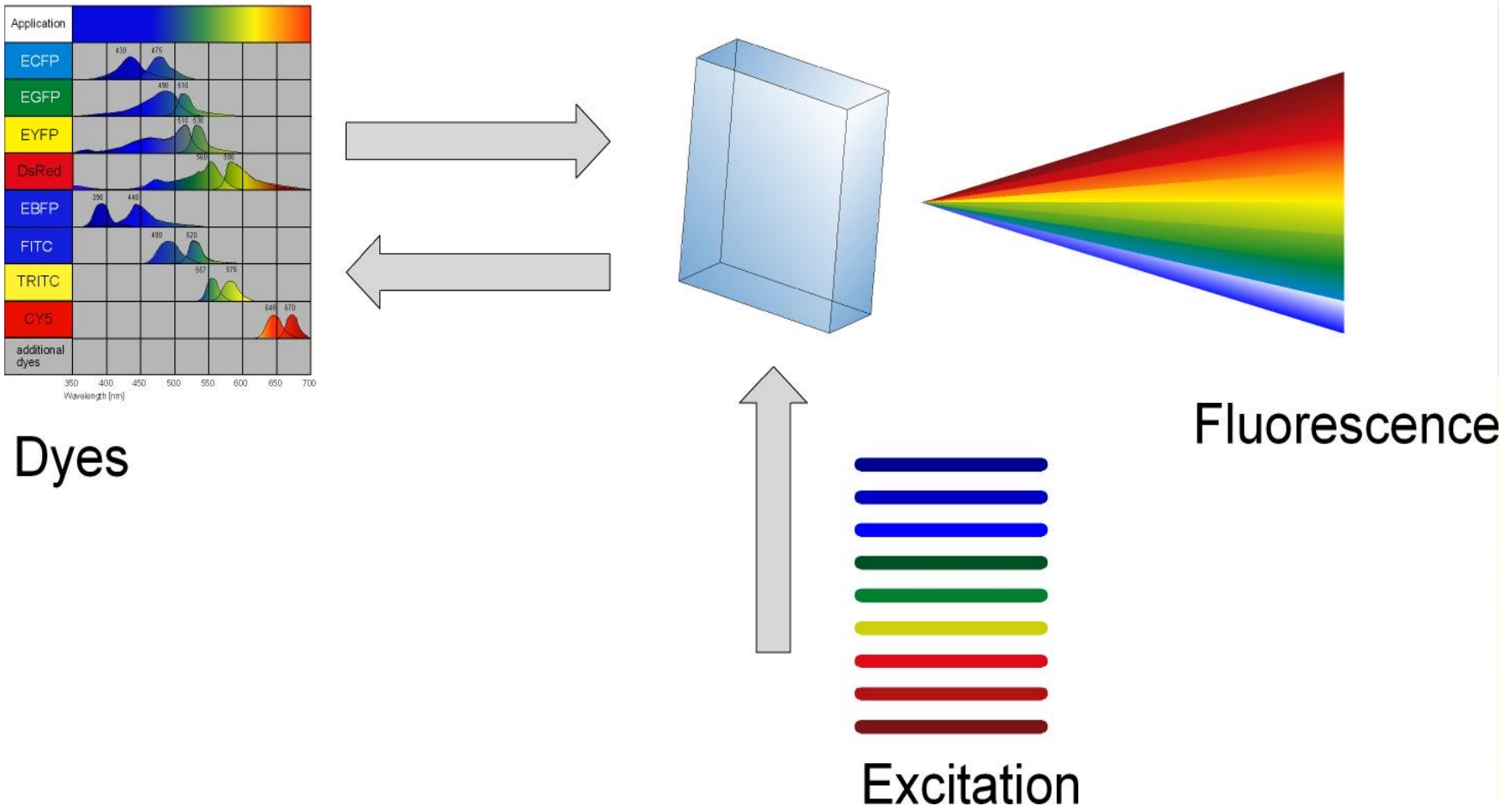

VIS Laser
IR Laser
UV Laser
© Leica 2000

AOTF (Acousto Optical Tunable Filter)
Module de sélection d'excitation

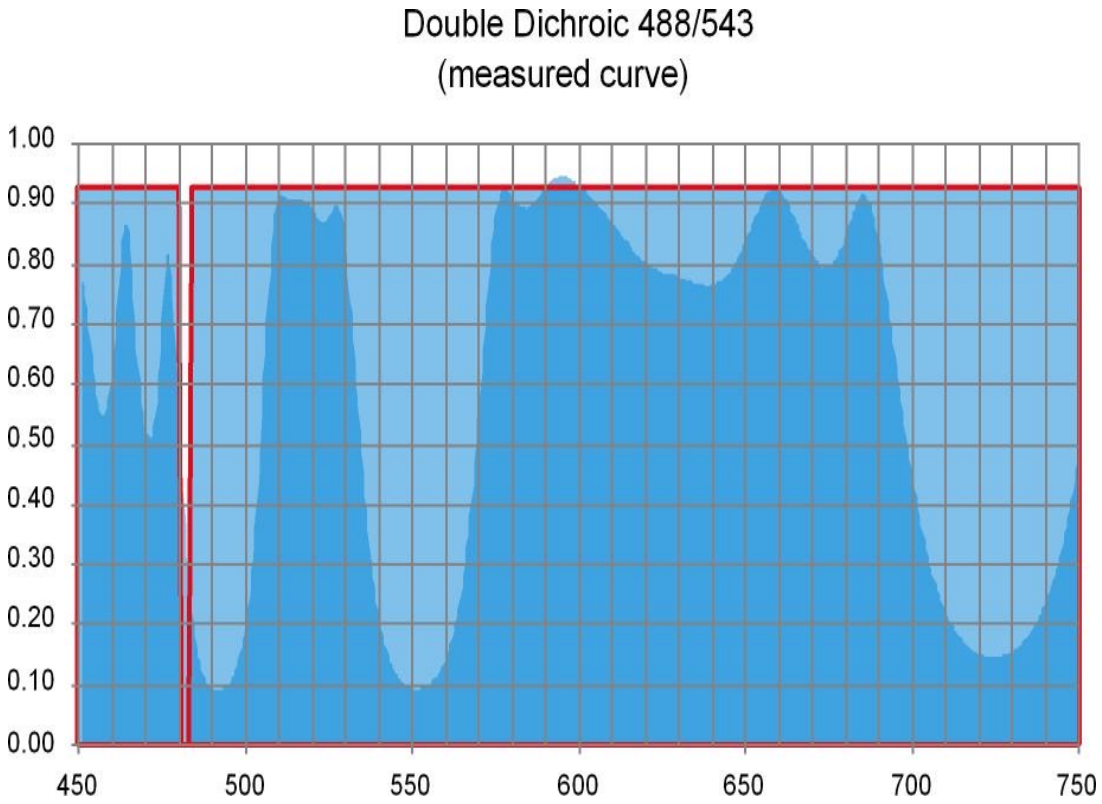


- Libre sélection de ligne
- Contrôle indépendent de l'intensité sur chaque ligne

Acousto Optical Beam Splitter: Dichroïque Électronique



AOBS transmission

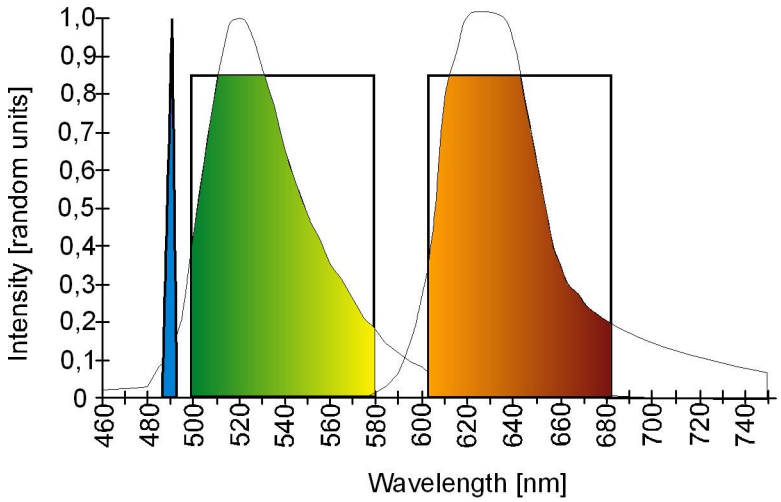


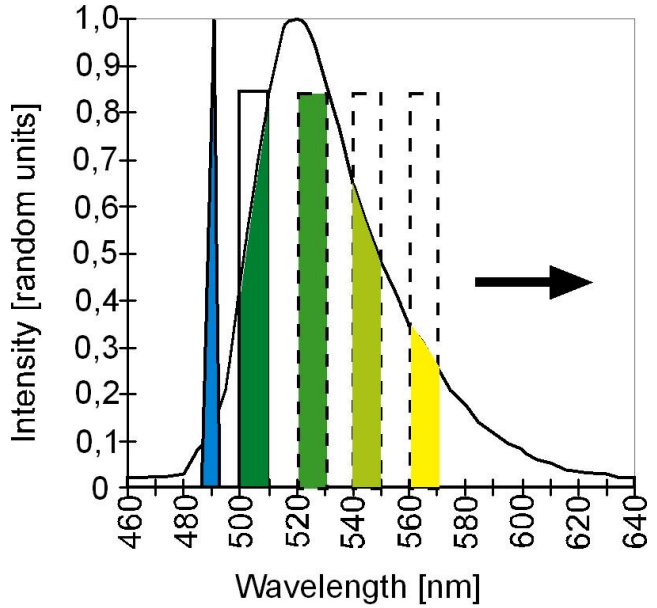
- Selectivité parfaite

- Transparence
le plus haut possible

- Plus “d’espace” pour détecter les émissions

SP Detector - Advantages





- N'importe quelle spécification de filtre est librement programmable
- Caractéristiques très étroite permet la détection de plus de fluorescence
- Aucun élément filtre dans le trajet – plus de transmission
- Balayage spectral avec haute résolution (2-5nm, 200 steps)

Microscopie confocale

Sectionnement optique de l‘échantillon

Suppression de la fluorescence en dehors du plan focal

Amélioration de la résolution latérale et axiale

Amélioration du contraste

How is the optical image relayed on to a screen ?

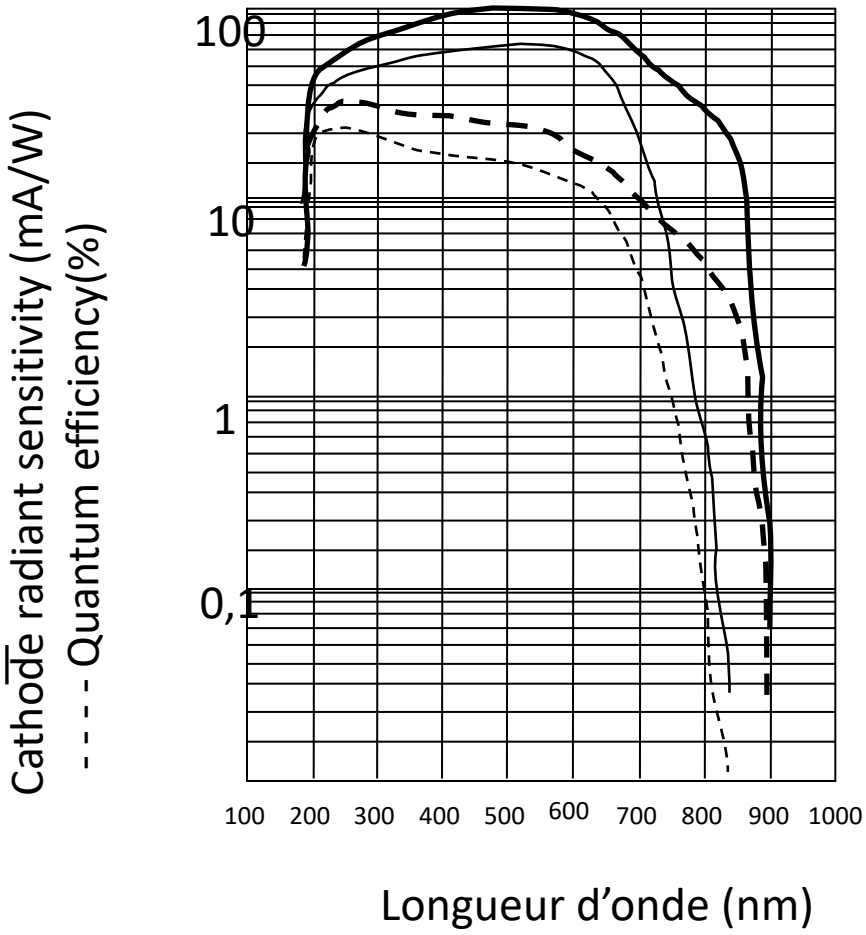
Light ➔ Current

Photomultiplier Tubes (PMTs)

- High efficiency
- Wide spectral sensitivity
- Low dark current
- High dynamic range

Détection : photomultiplicateurs

Conversion d'une détection de photons en signal électrique



APD

- higher sensitivity
- low background
- smaller dynamic range
- only a certain amount of photons/time allowed

⇩

weak samples

PMT

- lower sensitivity
- higher background
- bigger dynamic range

⇩

bright samples

Current ➔ Digits (Digitazitation)

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

Frame Store
Data Files (e.g. TIF)

About Data:

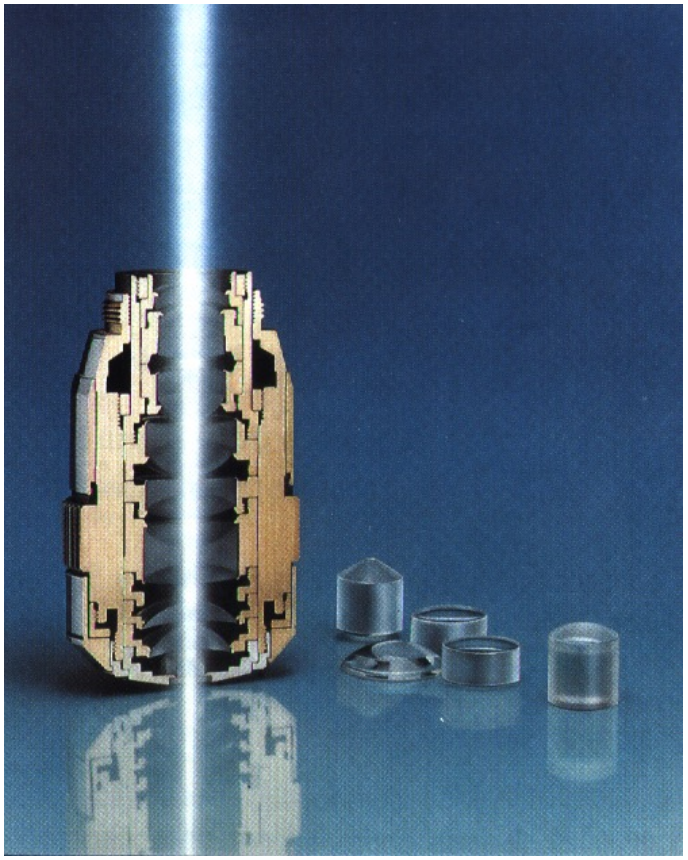
8 Sections	512x512	8bit 1ch:	2 MB
100 Sections	4096x4096	12 bit 5ch:	6000 MB

Objective Lens

Needs for confocal imaging:

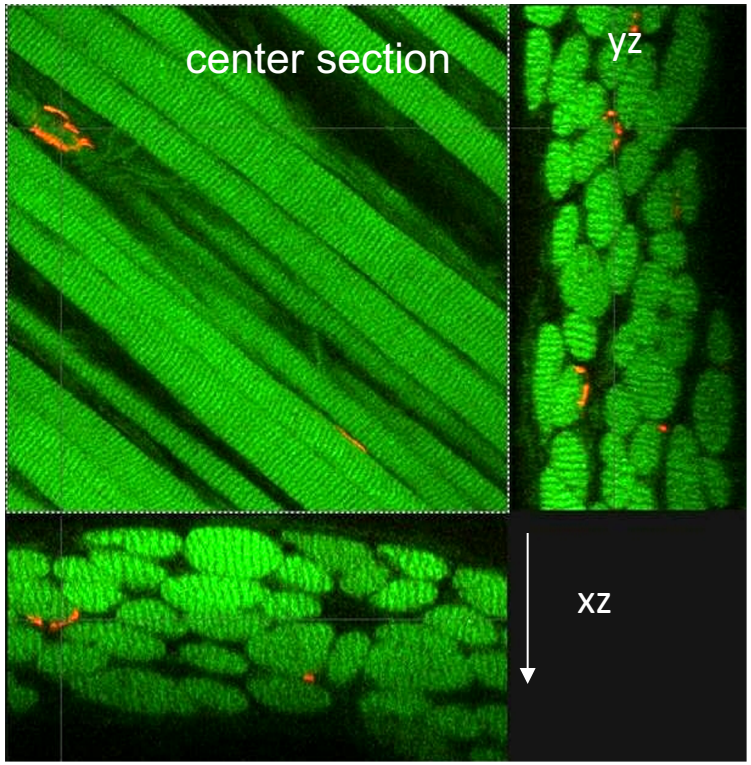
- High aperture
- High colour correction
- Flat field
- Long working distance
- High transmittance
- Variety of coupling media

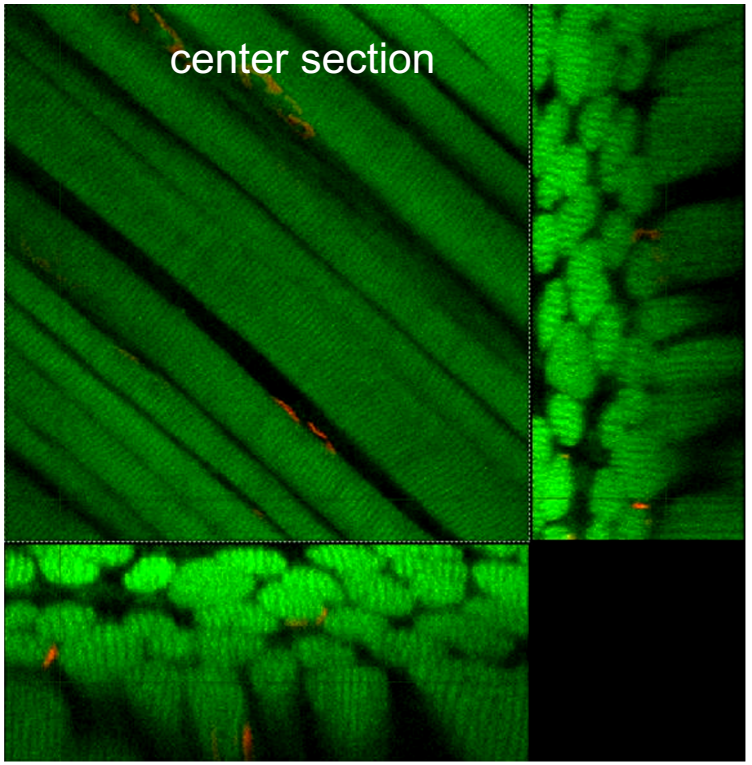
i.e. Plan Apochromat &
Plan-Fluotar



Objectif Glycérol :

Comparaison: Objectif à huile vs. Glycérol





Objectif Glycérol PL APO 63x1.3 Objectif huile PL APO 63x1.32

Sample: muscle fibers embedded in Glycerol (80/20%) thickness ca. 100µm

Paramétrage d’image

Mode de scan

Fréquence de balayage laser

Objectif utilisé

Zoom optique appliqué

Format d’image

Rotation de scan

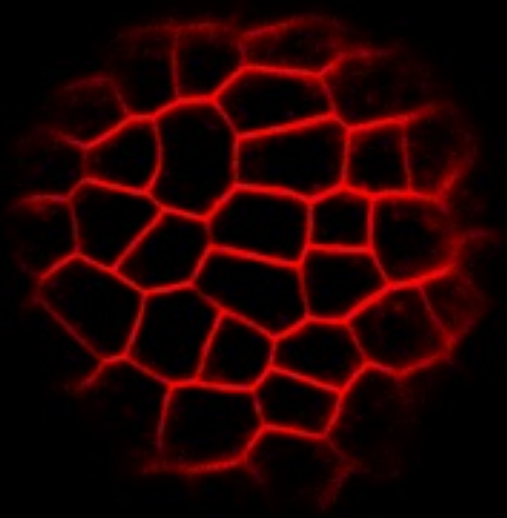
Moyennage d’images

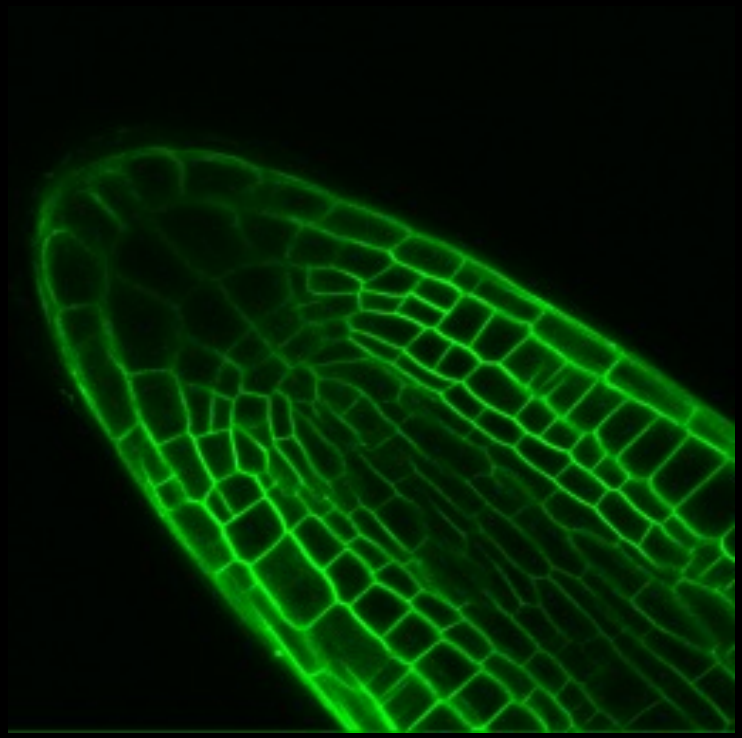
Accumulation

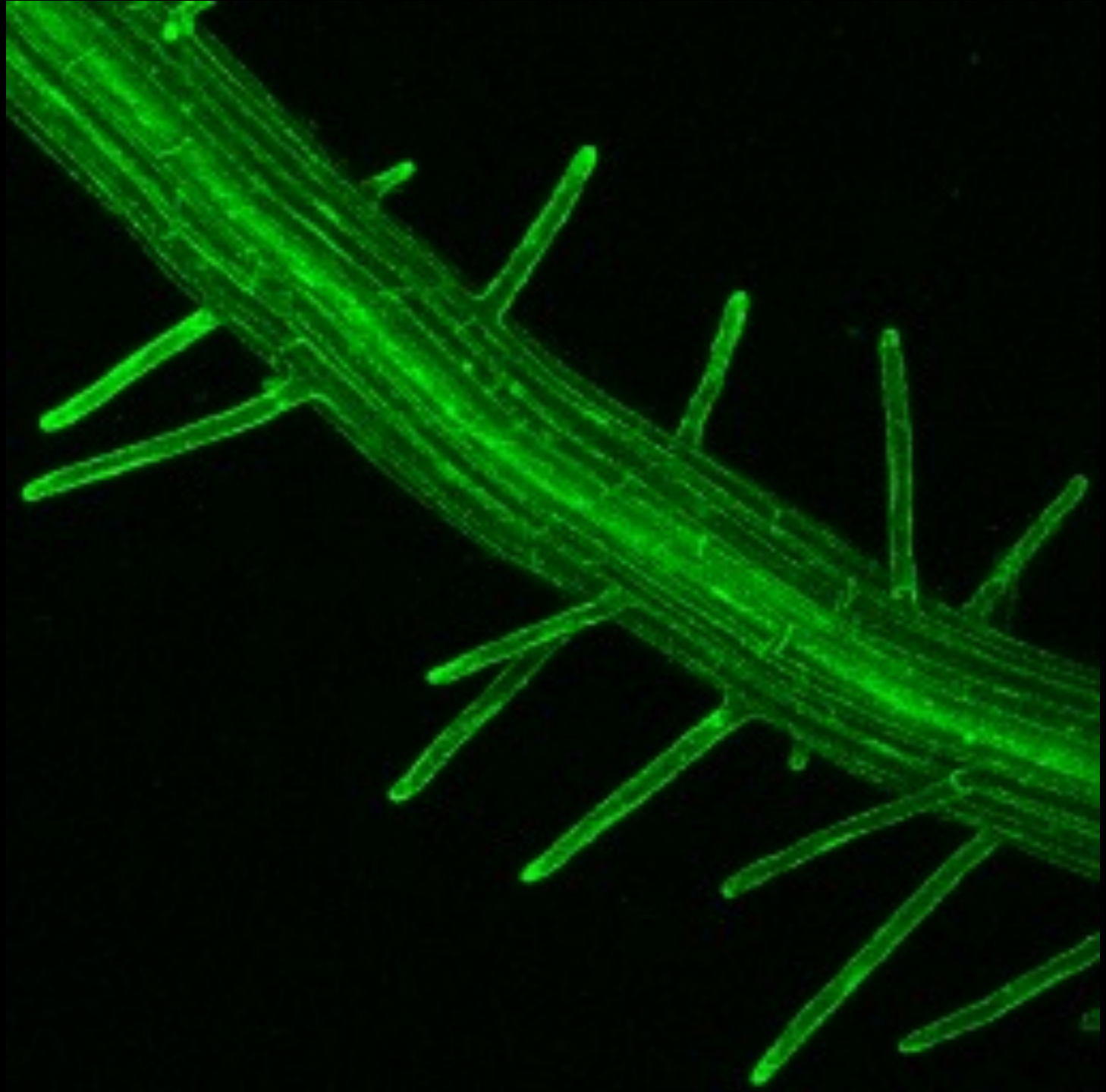
Gain du PMT

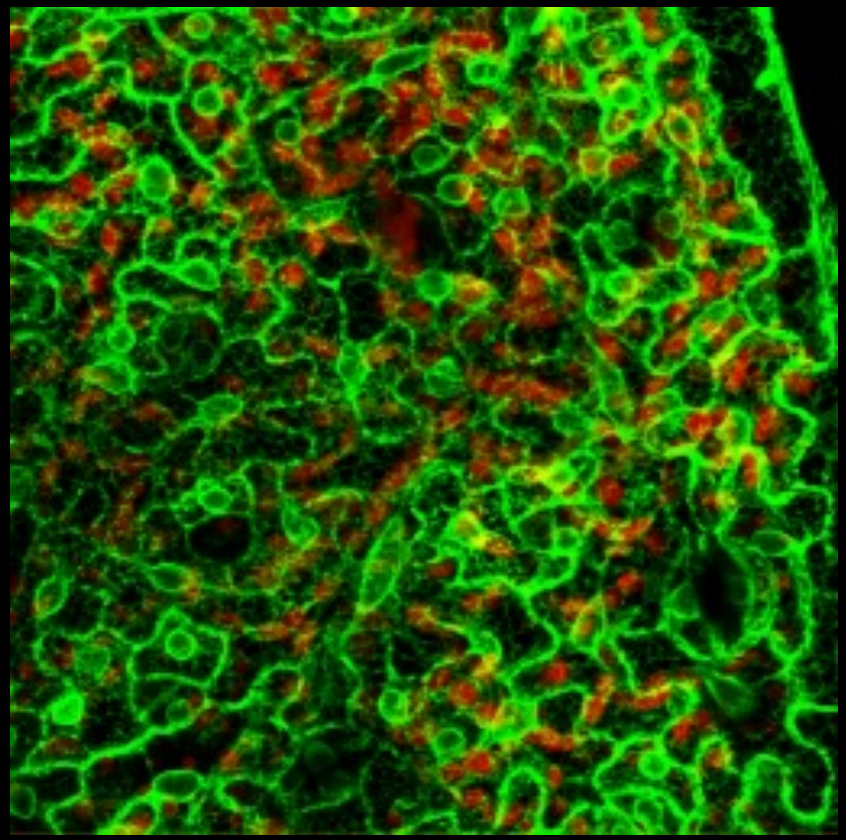
Offset du PMT

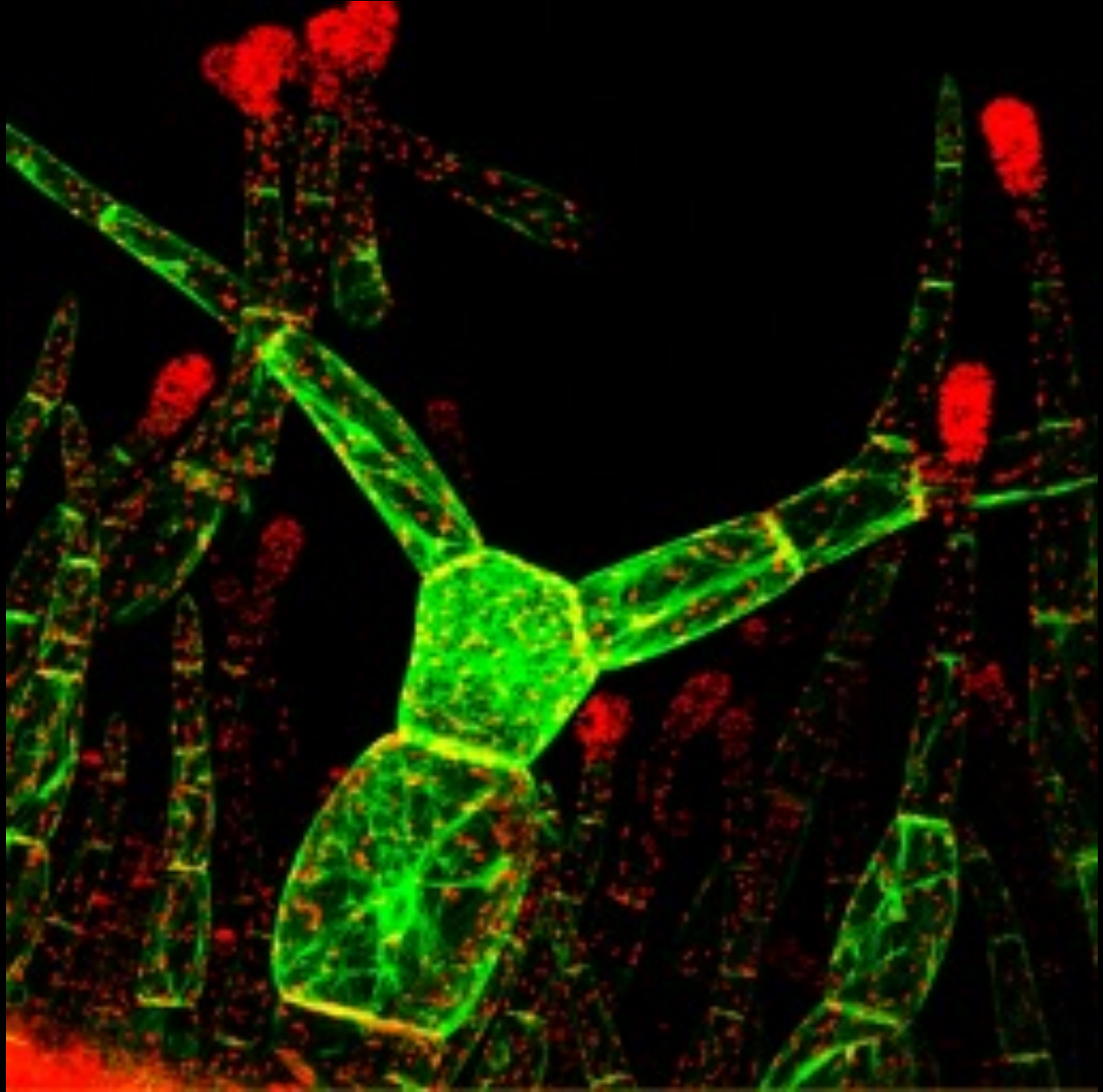
EXEMPLES D'APPLICATIONS DE LA MICROSCOPIE CONFOCALE



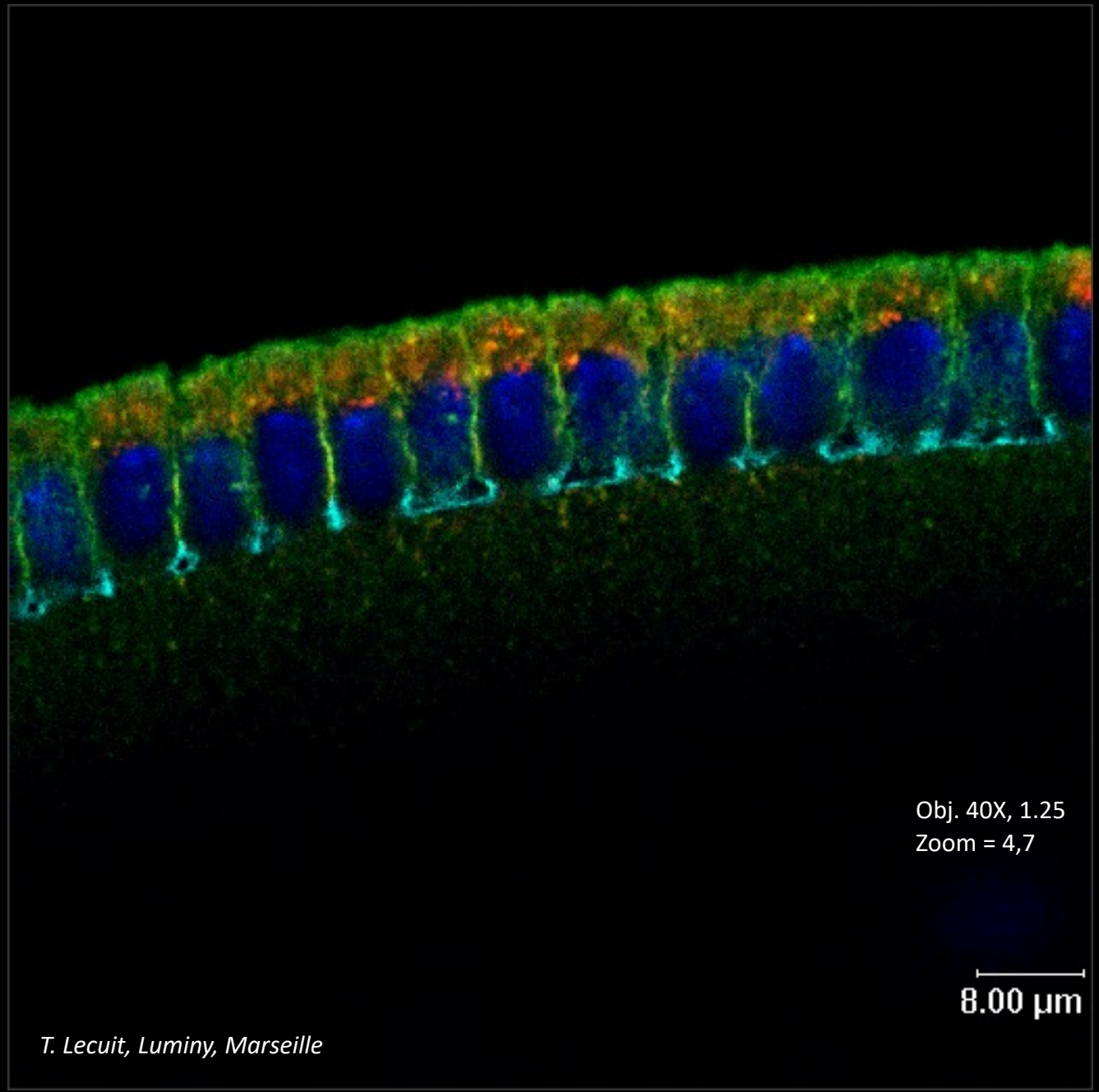








Embryon Drosophile



λ exc = 405 nm
λ ém. = 422-496

λ exc = 488 nm
λ ém. = 496-533

λ exc = 543 nm
λ ém. = 556-628 nm

λ exc = 633 nm
λ ém. = 661-690 nm

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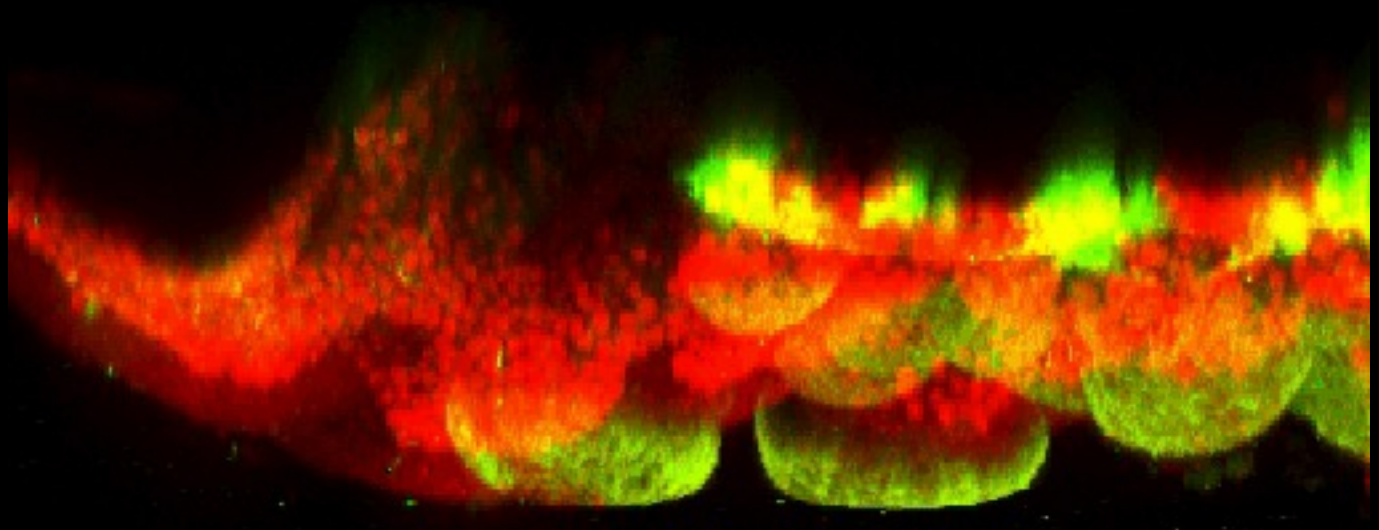
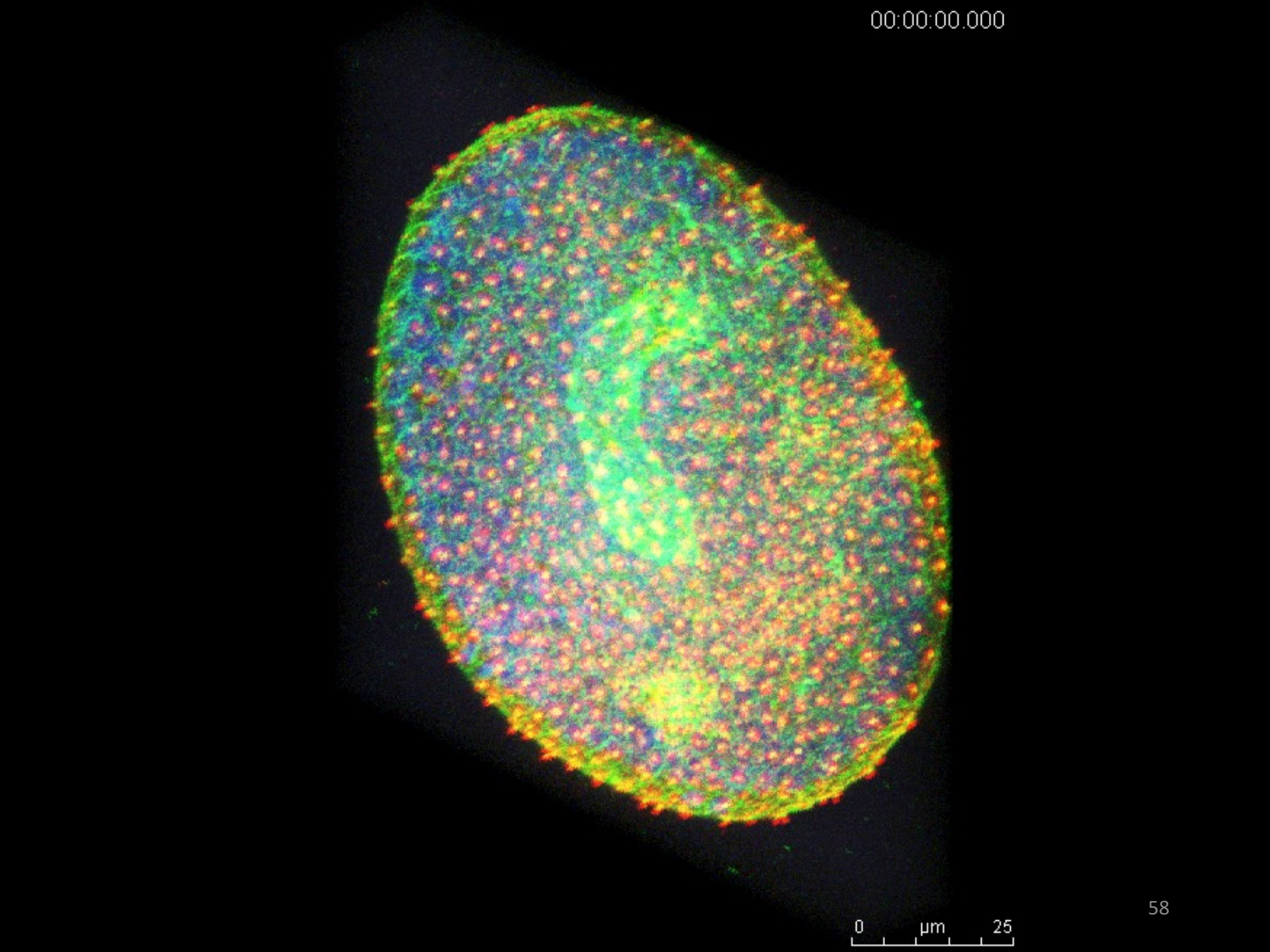
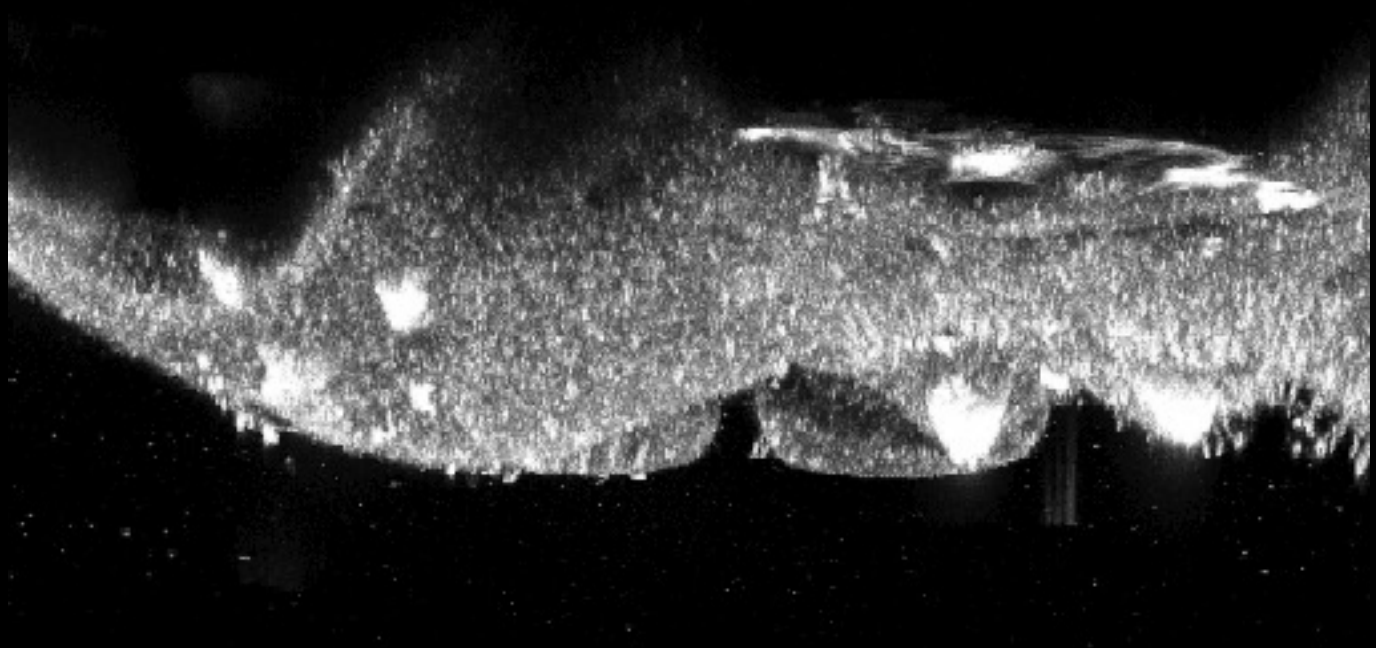



00:00:00.000
0
µm
25

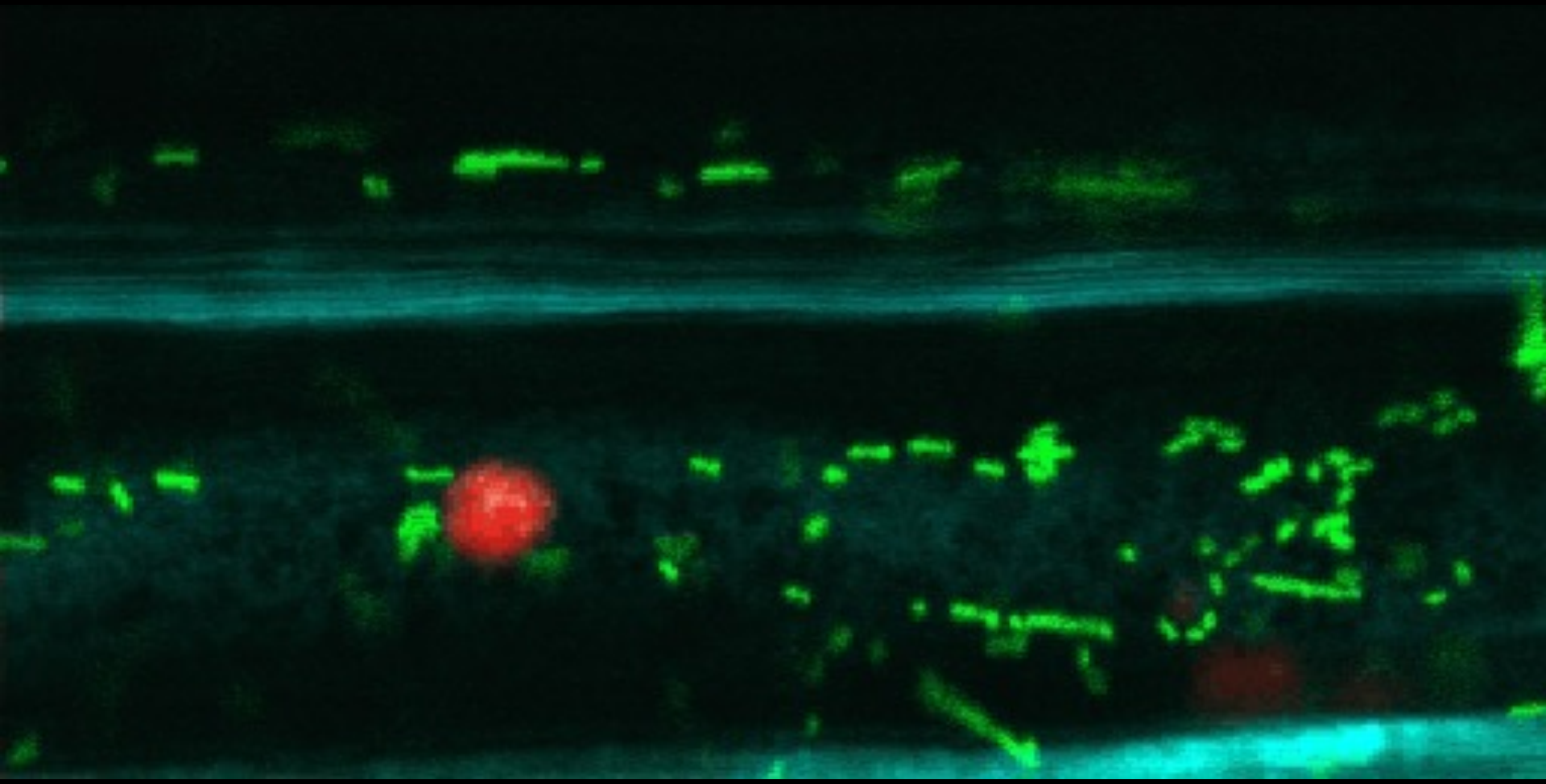
Réflection

Etude de surface



Réflection

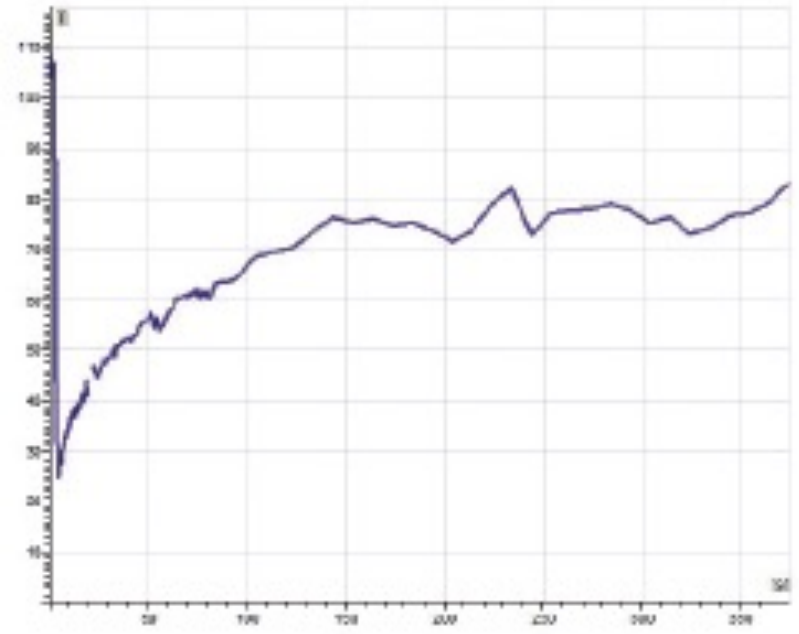
Time-Lapse

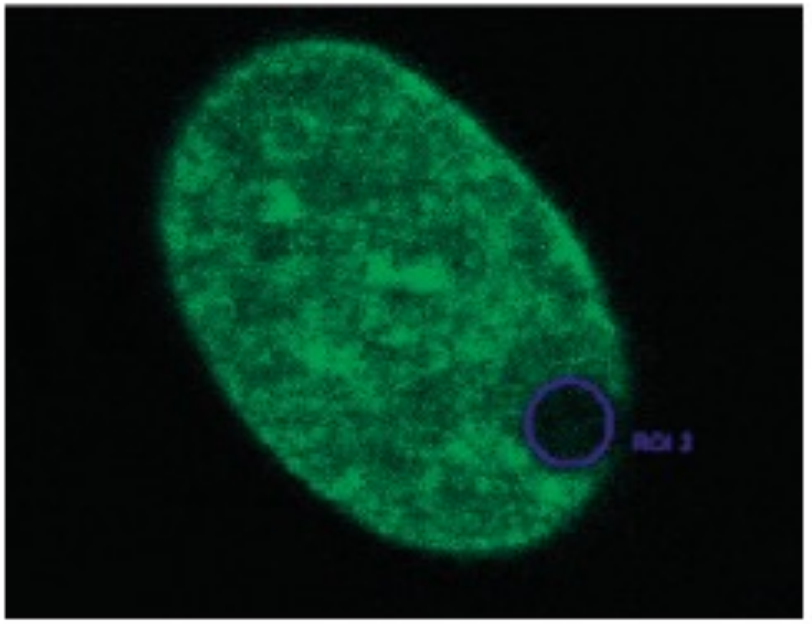


Arabidopsis thaliana
First channel: Cell wall in reflection.
2 & 3 channel: Monitoring mitochondrial (GFP-green) and plastid (autofluorescence-red) movement.

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

Fluorescence Recovery After Photobleaching (FRAP)



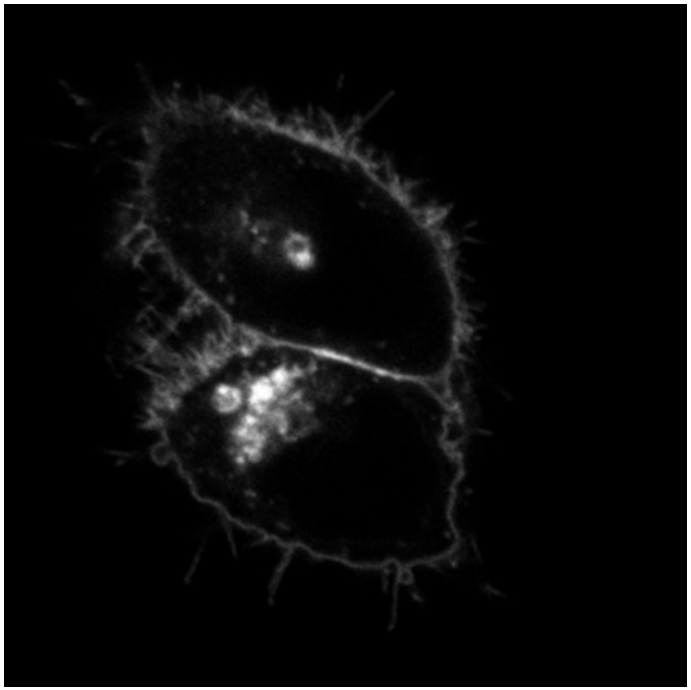


	FD464	$FD464_{theor}$	$H1\text{-}GFP_{arbitrary\ ROI}$	$H1\text{-}GFP_{circular\ ROI}$	$H1\text{-}GFP_{literature}$
M_f [%]	103	100	91	–	~ 90
$t_{1/2}$ [s]	2.3	(1.0)	138.6	59.9	~ 55
τ [s]	3.3	(1.4)	200.9	41.5	–
D_{eff} [$\mu m^2/s$]	1.6	3.7	–	0.01	–

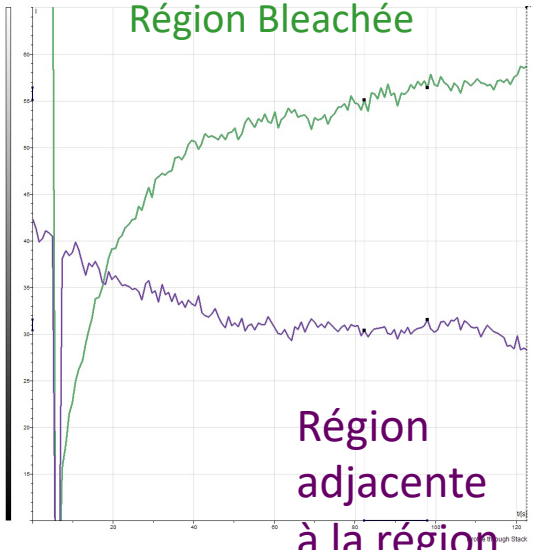
τ: Time constant of recovery (calculated by LCS, circular ROI)

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

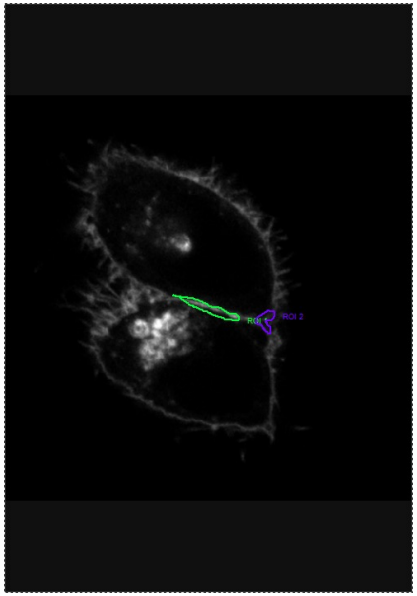
Fluorescence Recovery After Photobleaching



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



I= f(t)



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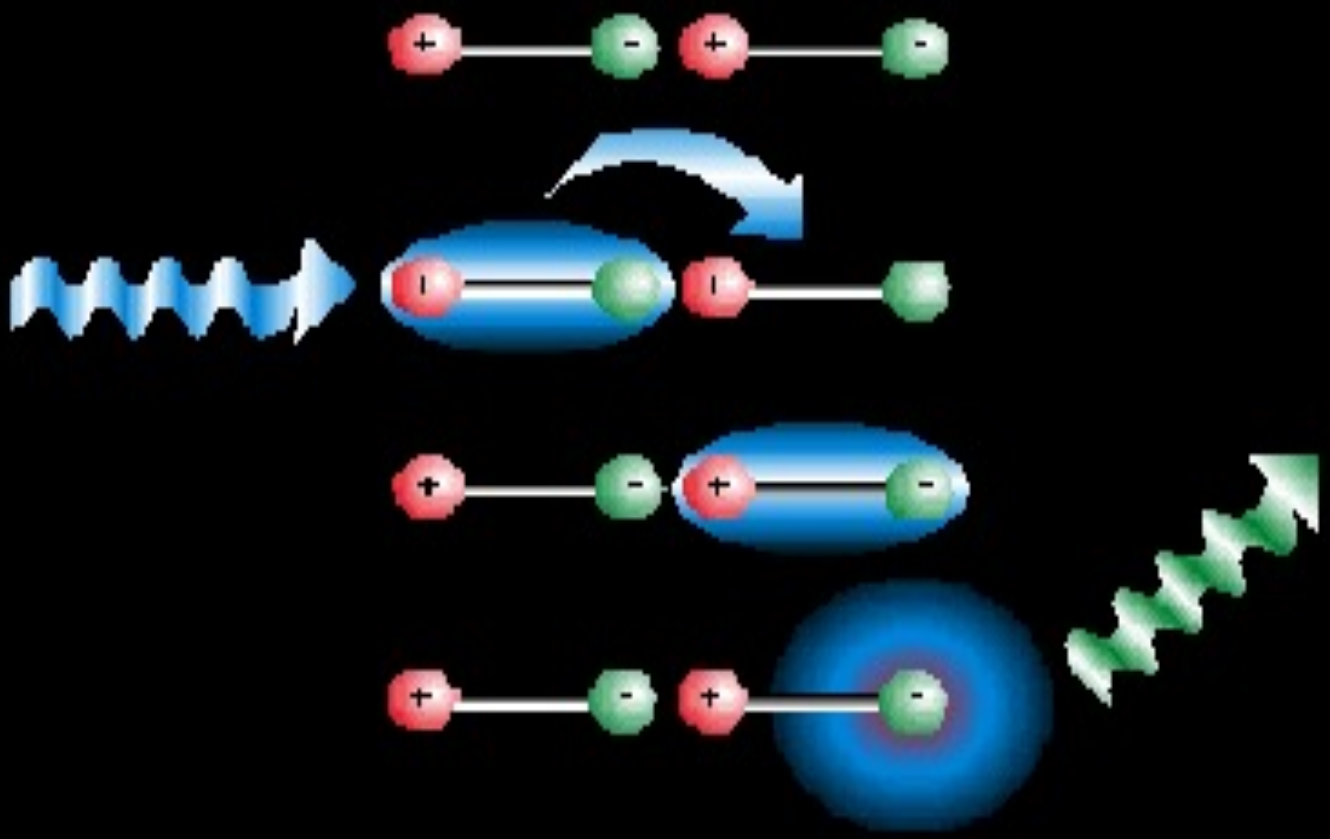
Fluorescence Resonance Energy Transfer



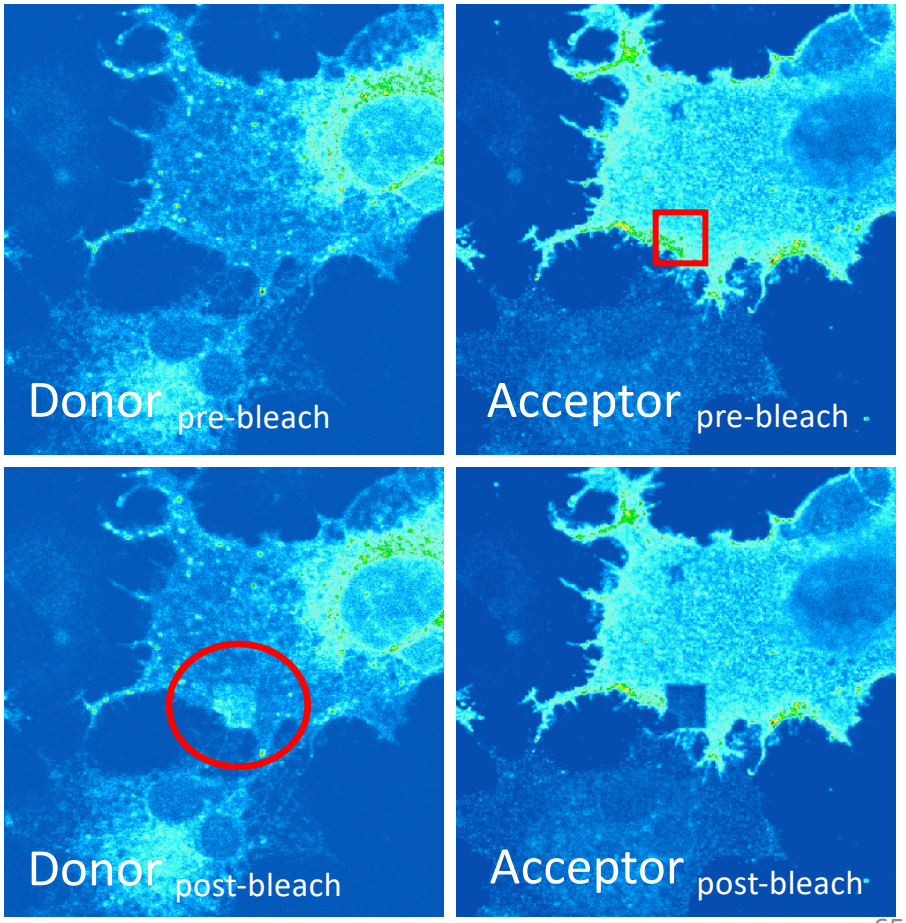
Fluorescence Resonance Energy Transfer

Bleaching de l'accepteur

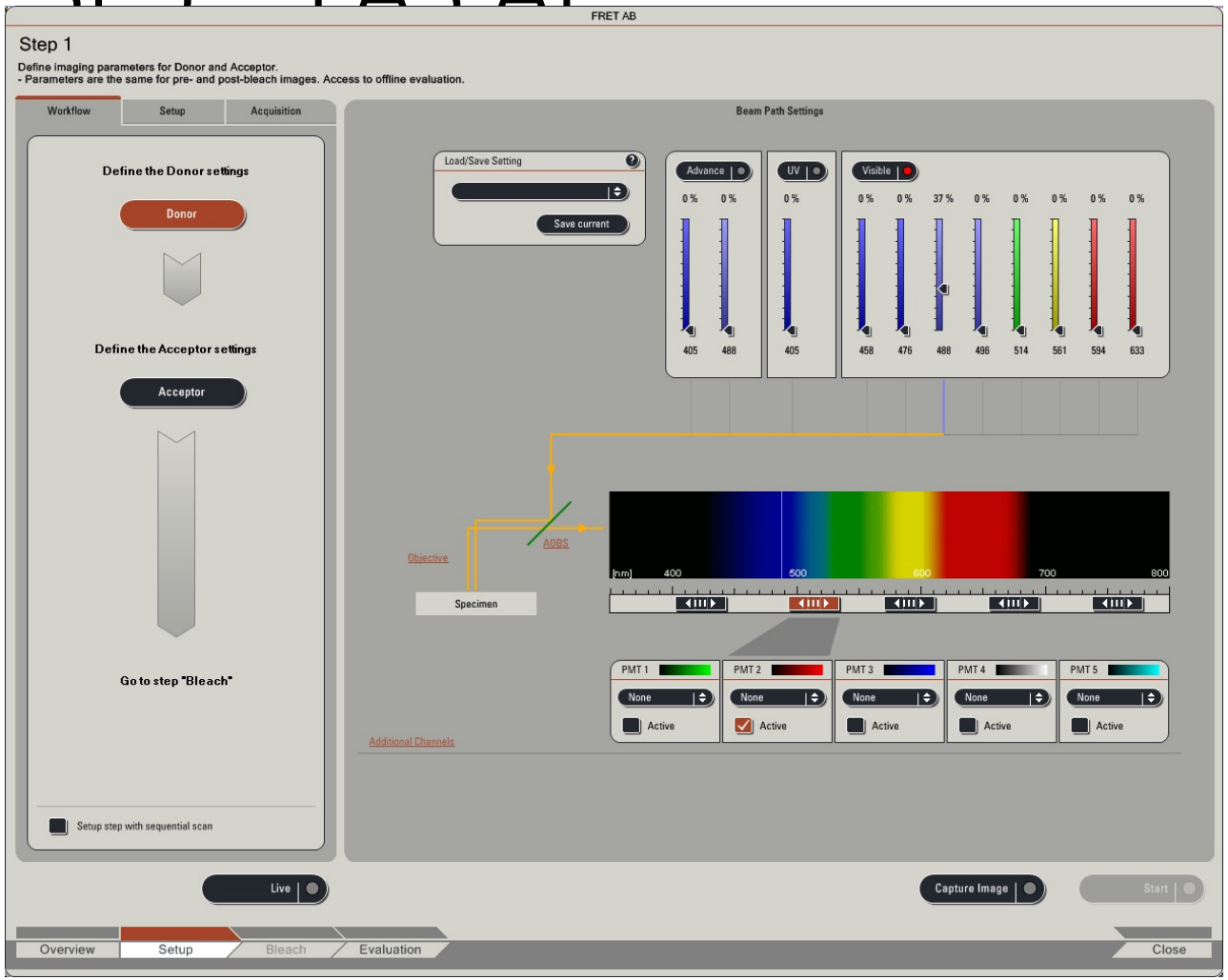
Etude des modifications des interactions moléculaires

Proximité donneur-accepteur 10–100 Å

Augmentation de l'intensité de fluorescence du donneur

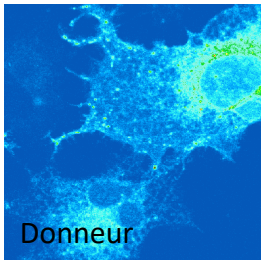


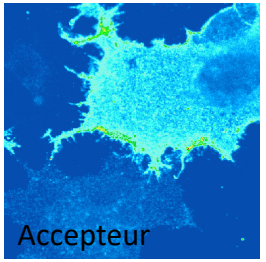
SP5 – LAS AF



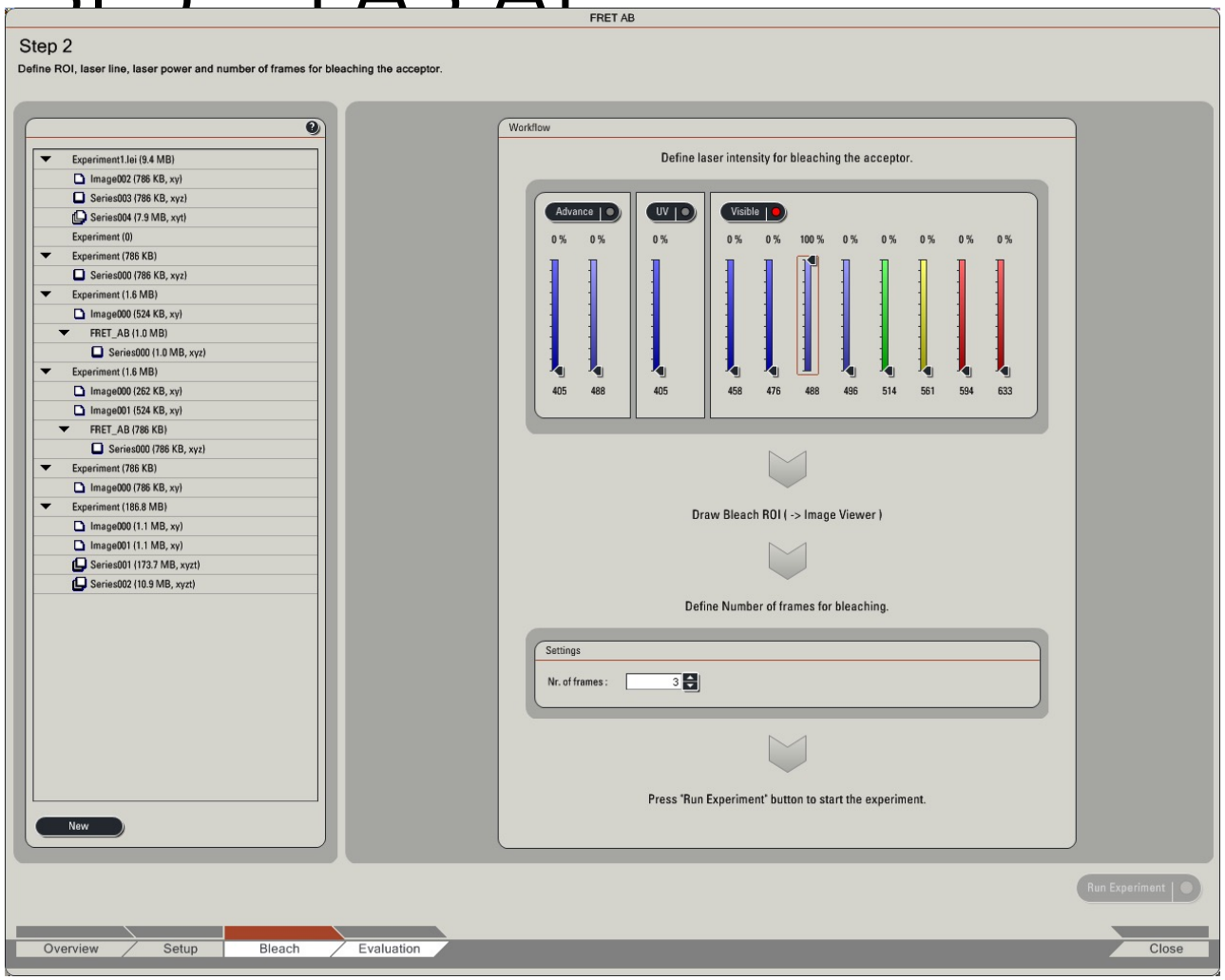
Application FRET-AB

Setup pour imager
le donneur et l'accepteur



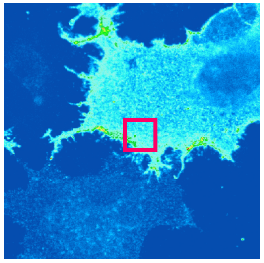


SP5 – LAS AF

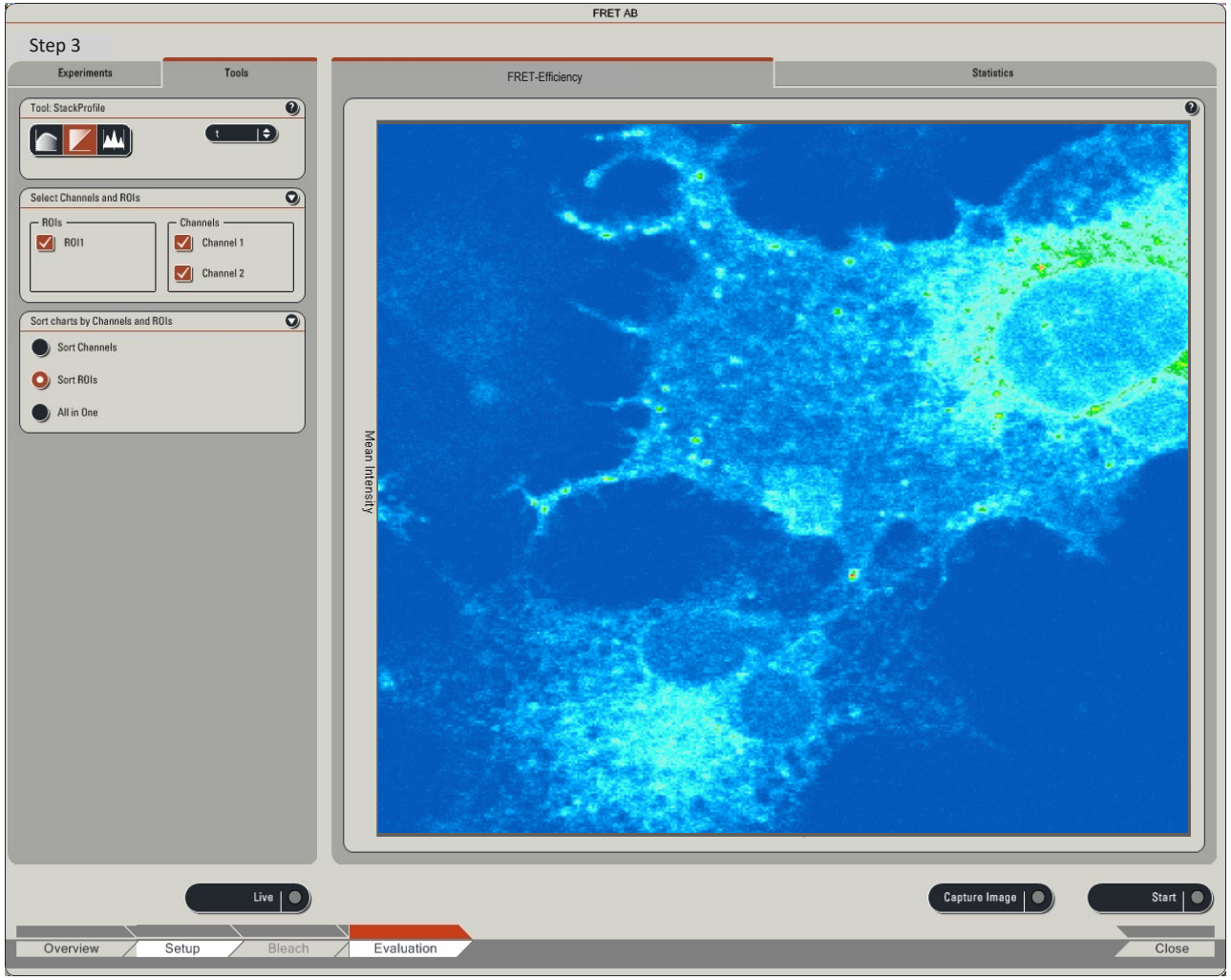


Application FRET-AB

Setup du bleach
pour l'accepteur



SP5 – LAS AF

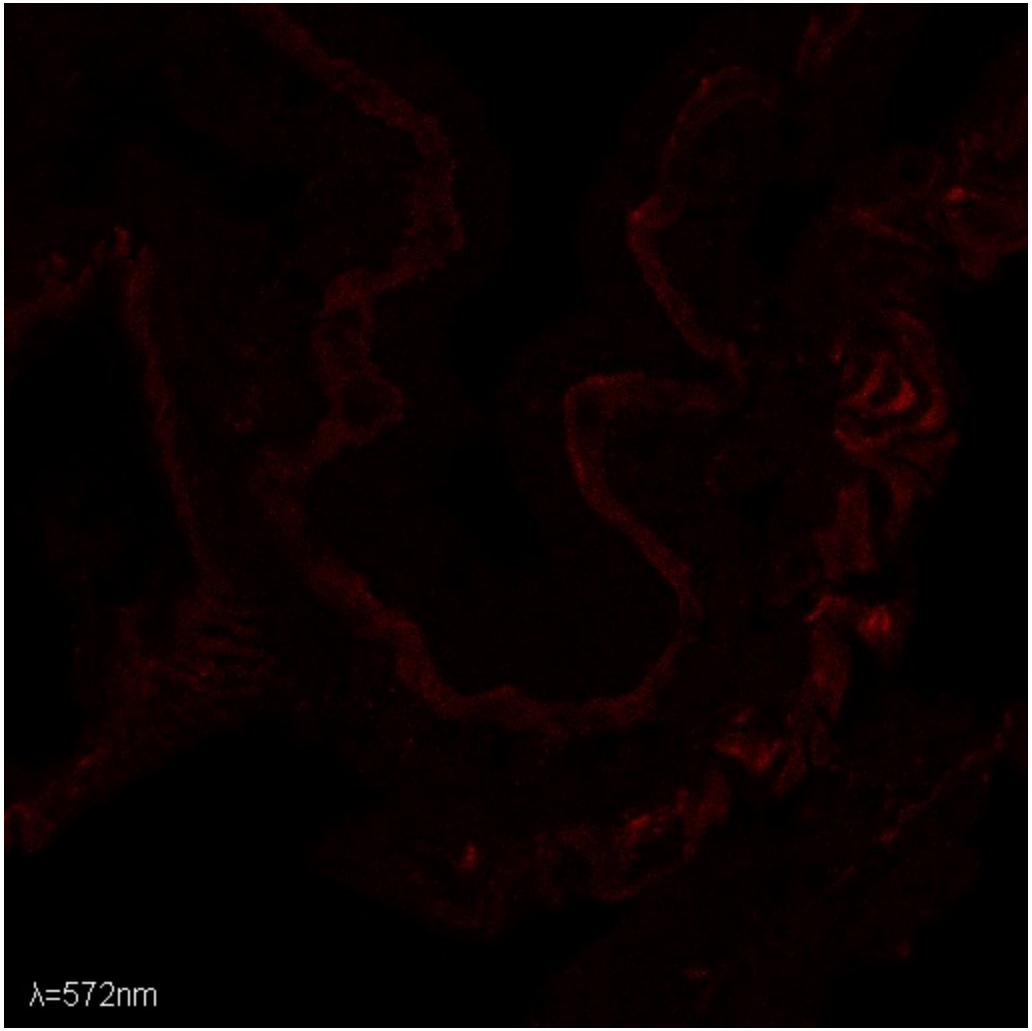


Application FRET-AB

Quantification

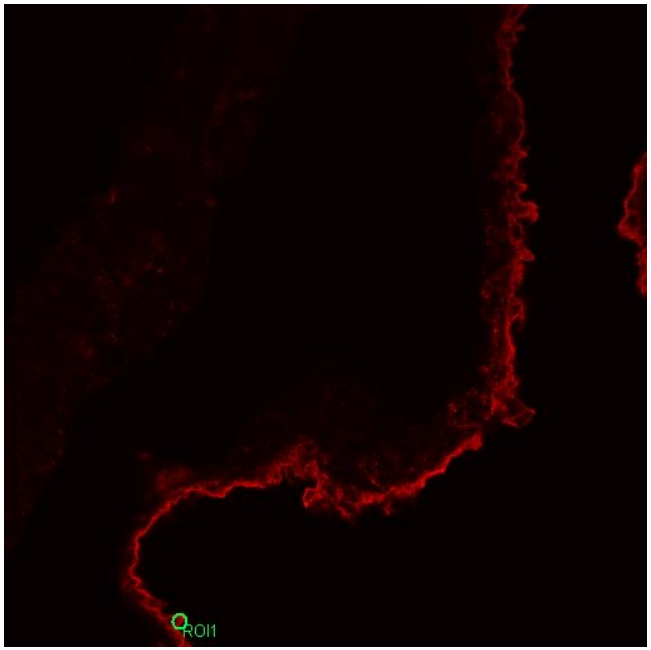
Etudes Spectrales

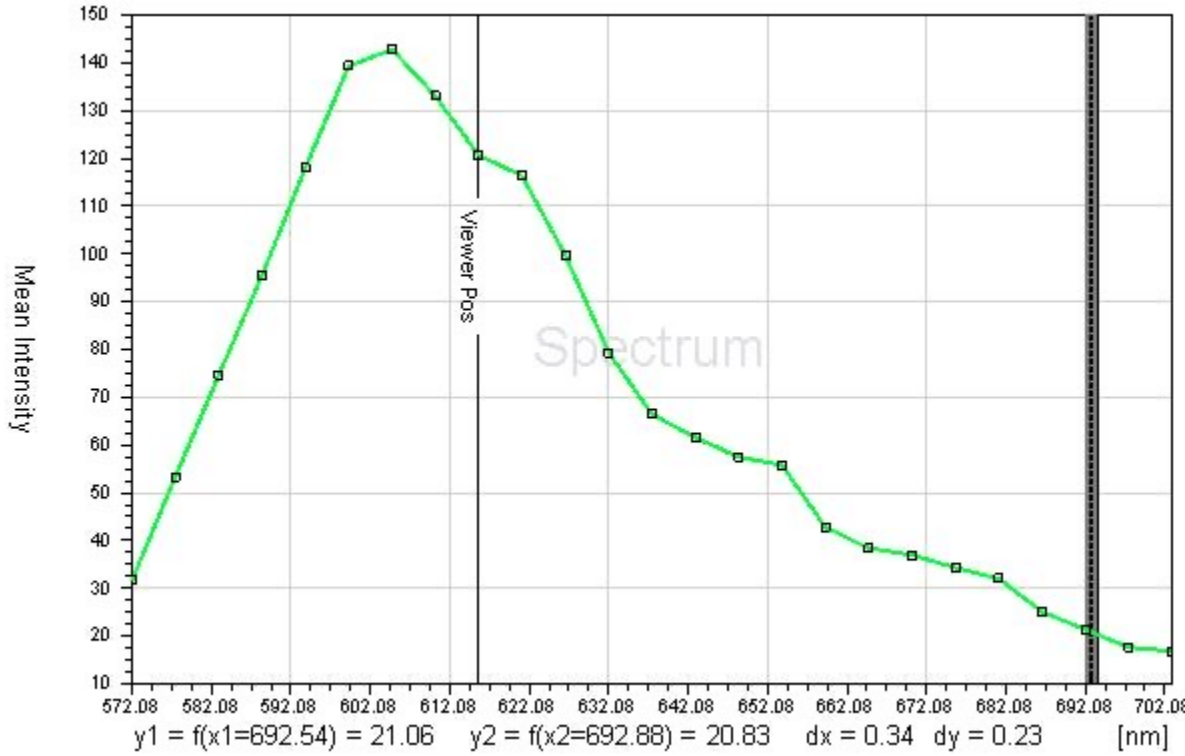
Spectre d'émission de fluorescence



Etudes Spectrales

Spectres d’émission de fluorescence



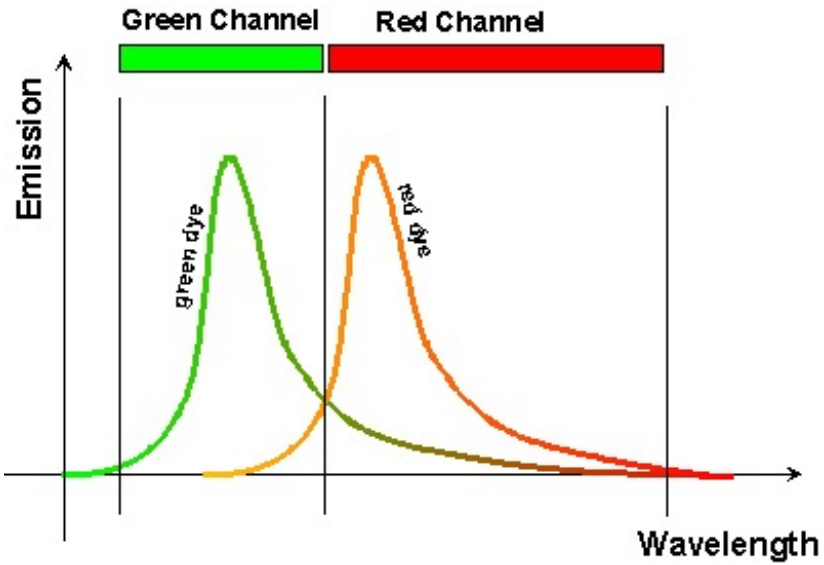


Etudes Spectrales

Séparation spectrale

Alexa 488 – GFP

Example

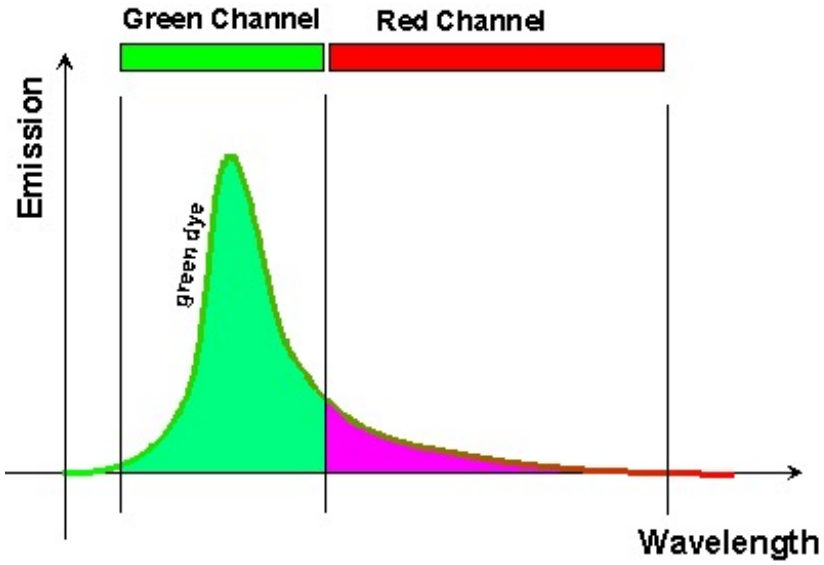


FITC/TxR sample

2 channel recording:

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

FITC



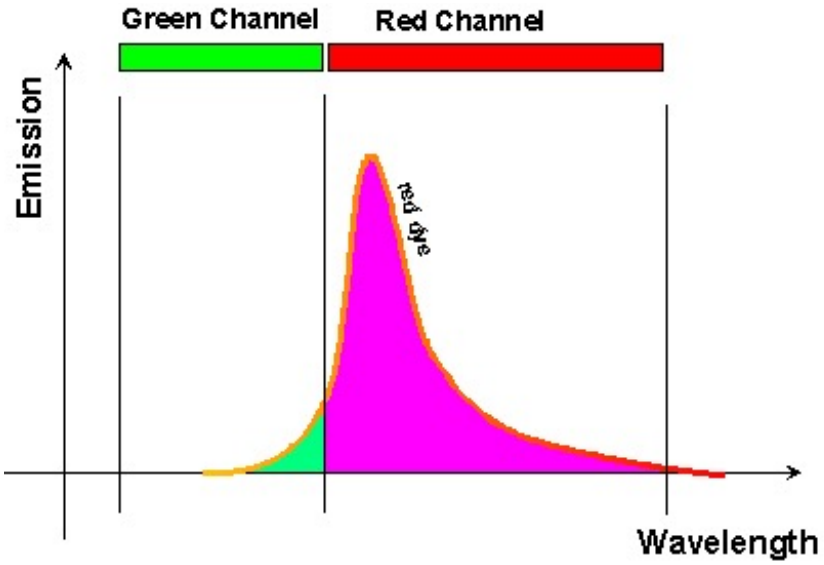
The total of all light collected from FITC molecules will be distributed into both channels.

We assume here:

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

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

TxR



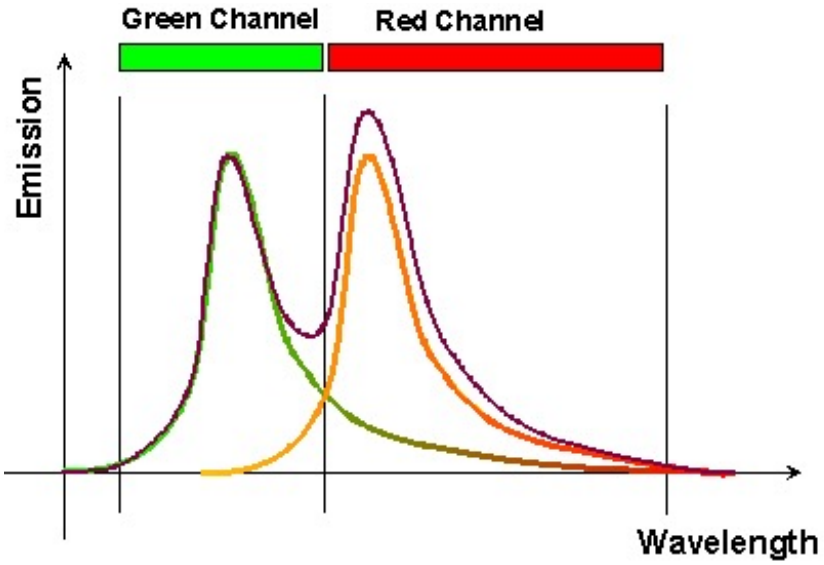
The total of all light collected from TxR molecules will be distributed into both channels.

We assume here:

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

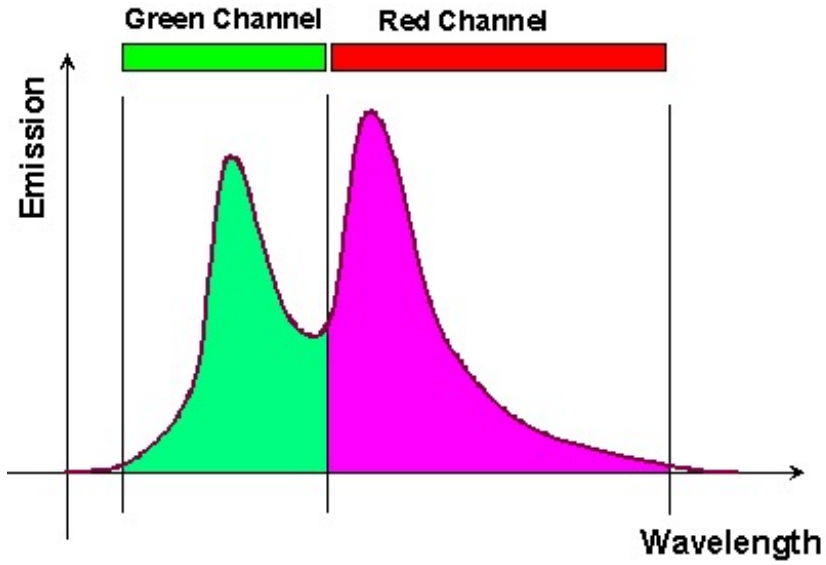
4/5 of all FITC emission goes into the red channel (R)

Both dyes



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

A calculated measurement



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

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

Spectra: # and δ

Important Note:

Theoretical spectrum: infinite number of datapoints.
Reality: maybe 50 channels, or just 2

Equidistant measurement is a good thing. But it is not always necessary.

In the unmix case, equidistance is no good: better to tune the bandwidth and center wavelength. So we optimize S/N for separating data later.

Requires important hardware features as tunable bands, any filter characteristic etc. which you find only with the Leica SP design.

What we dont‘t need:

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

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

This is just a set of n linear equations with n unkowns.
You may remember from primary school.

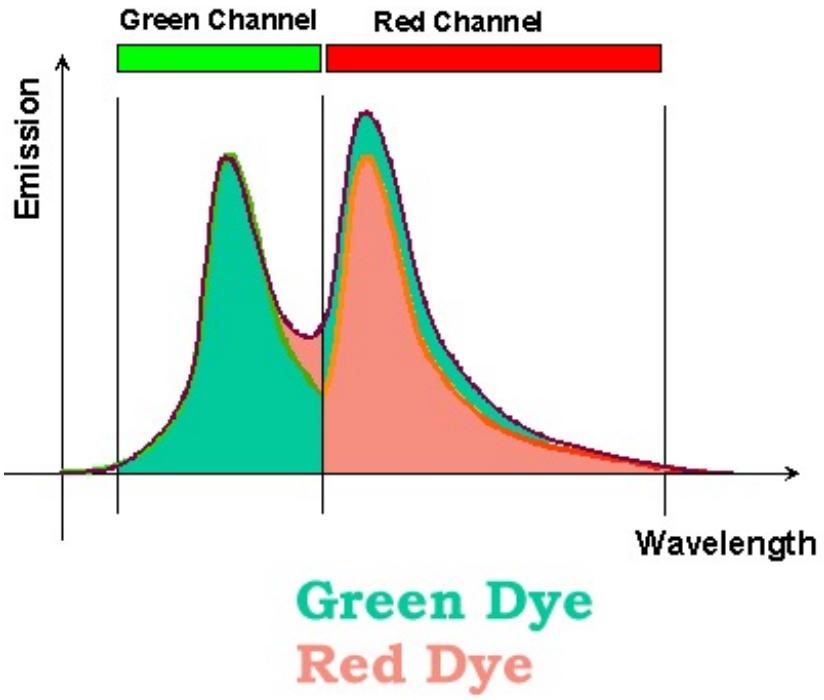
3y+2x=7
7y+ x=2

There are methods, to solve these by computers

Important note: it is sufficient to have two equations for 2 unknowns.

Those systems providing more and equidistant channels (with gaps in between) are technically massively inferior to the Leica SP AOBS concept.

Contributions



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

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

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

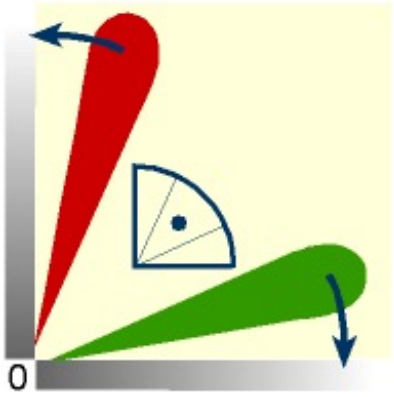
Math Solution

1. Guess coefficients and subtract manually bleed through
   ➔ Manual Dye Separation

2. Take n channel image, have pure dyes somewhere and let the computer solve
   ➔ Channel Dye Separation

3. Take a spectrum, have the spectra from the pure dyes and let the computer solve
   ➔ Spectral Dye Separation

Stat Solution



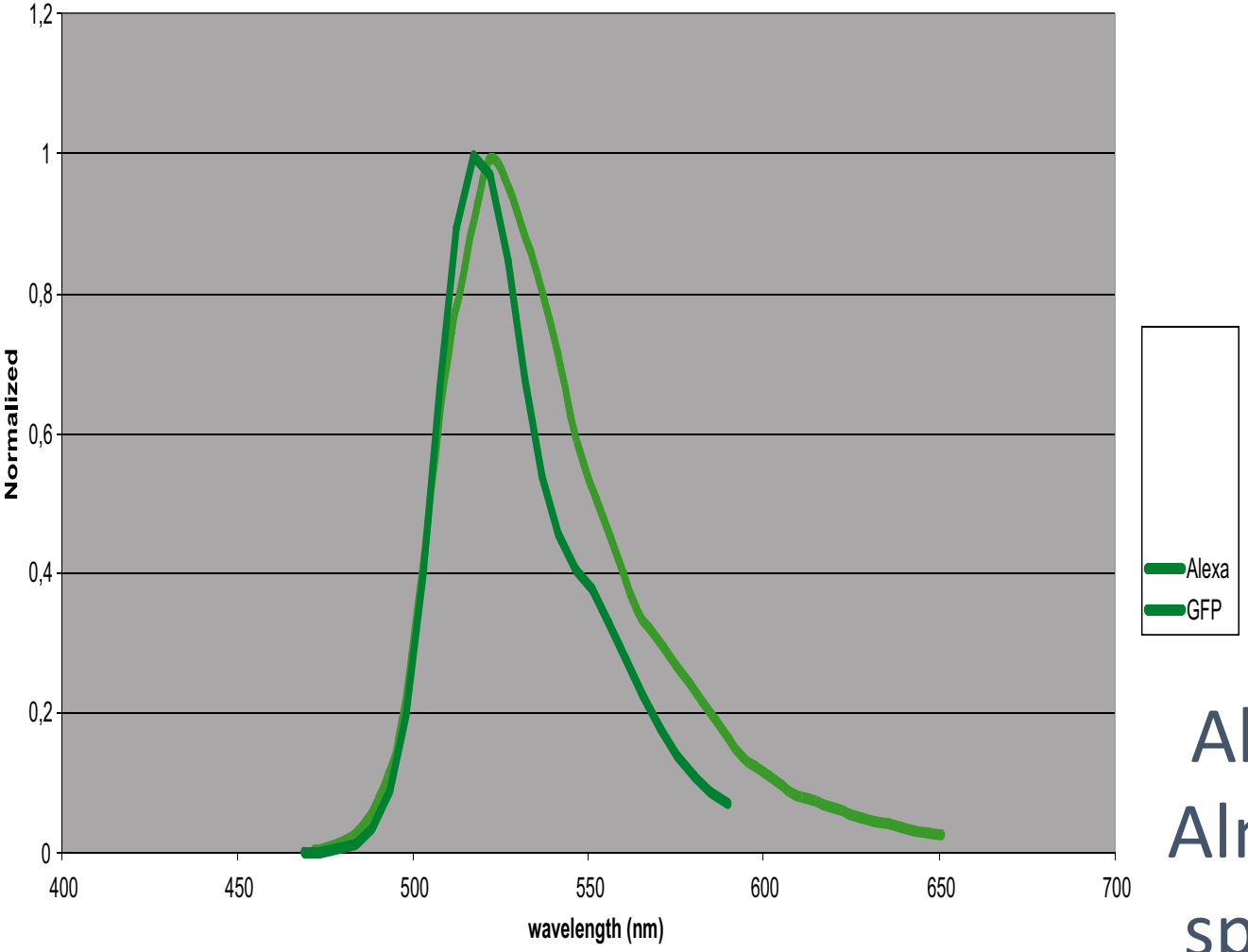
Let computer find best fitting line for clouds. Angle corresponds to coefficients.
➔ Adaptive Dye Separation

1. Let computer bend lines to coincide with axes
   ➔ strong

2. Let computer bend until first non-empty bin hits the axis
   ➔ weak

3. Find projection of n dimensions, when n dyes around
   ➔ Dimensionality reduction (this is not an unmixing method)

Emission control:
SP performance test

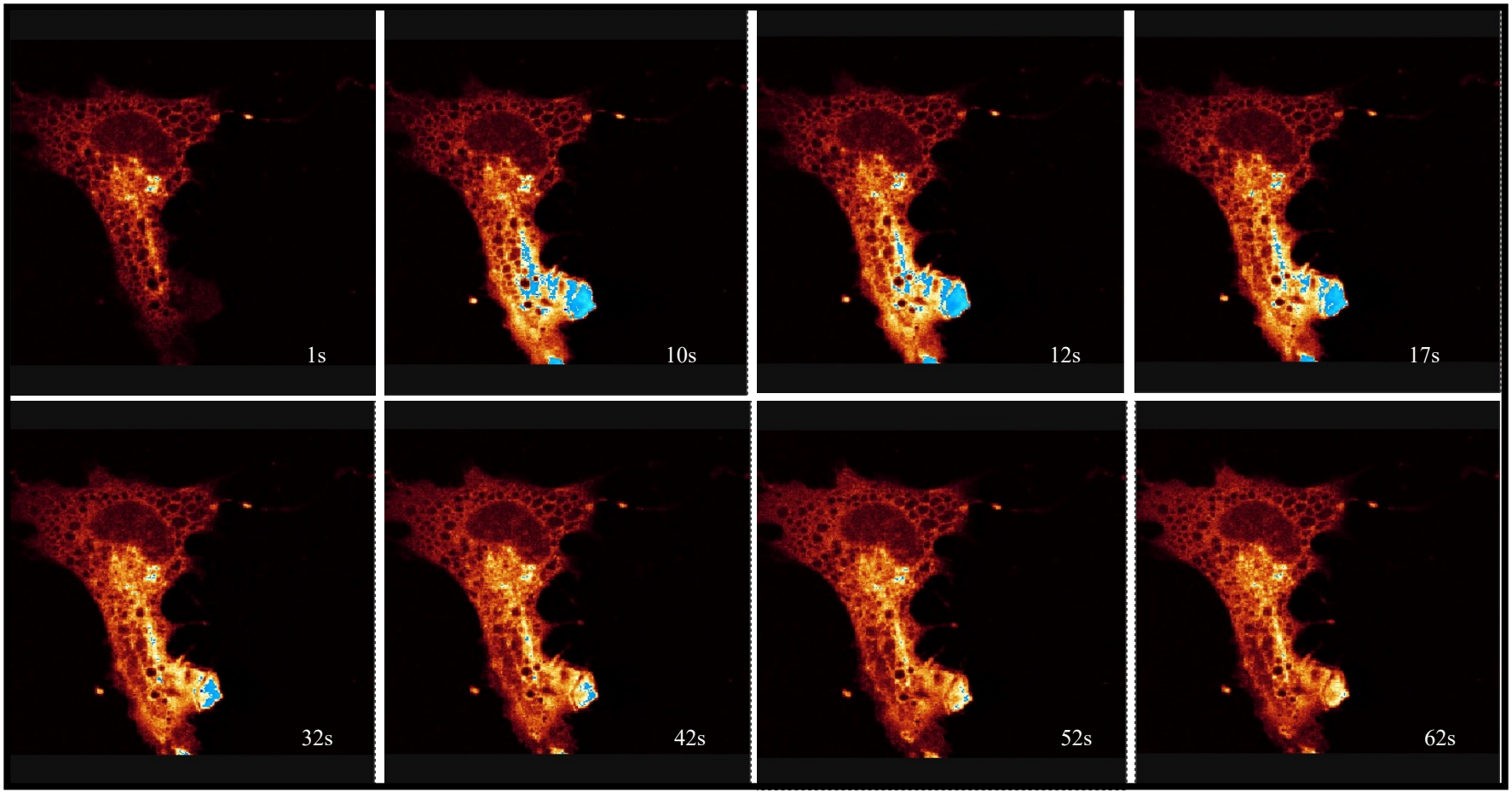


Alexa 488 – GFP:
Almost complete spectral overlap

GFP Photoactivable

Pulse laser IR 60 ms 800 nm

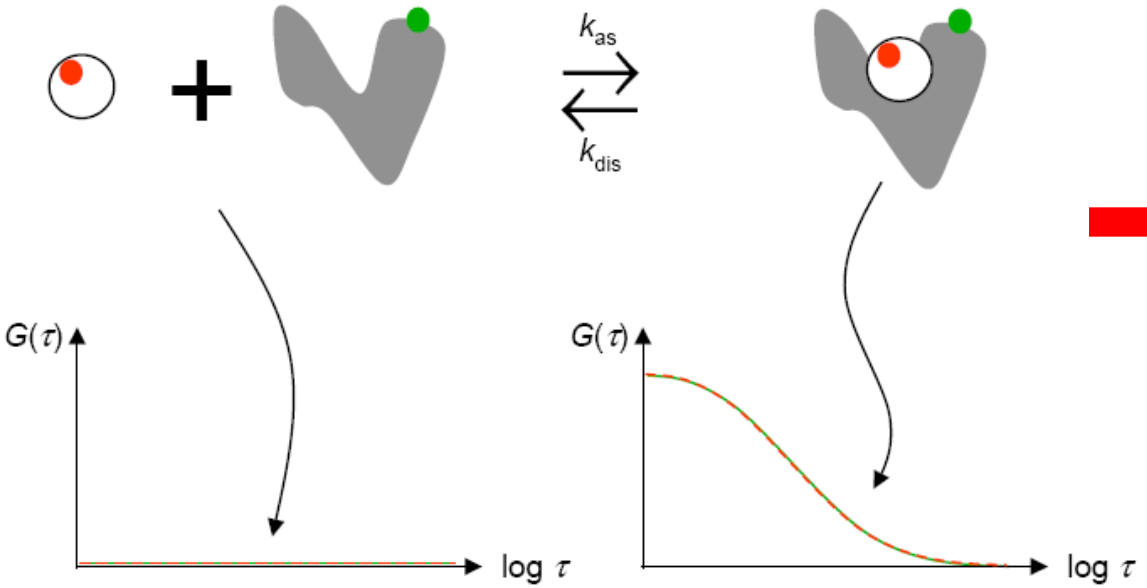
D. Choquet, Institut François Magendie, UMR 5091 CNRS, Bordeaux.
Physiologie cellulaire de la synapse



Fluorescence Correlation Spectroscopy

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

- Concentration
- Coefficient de diffusion

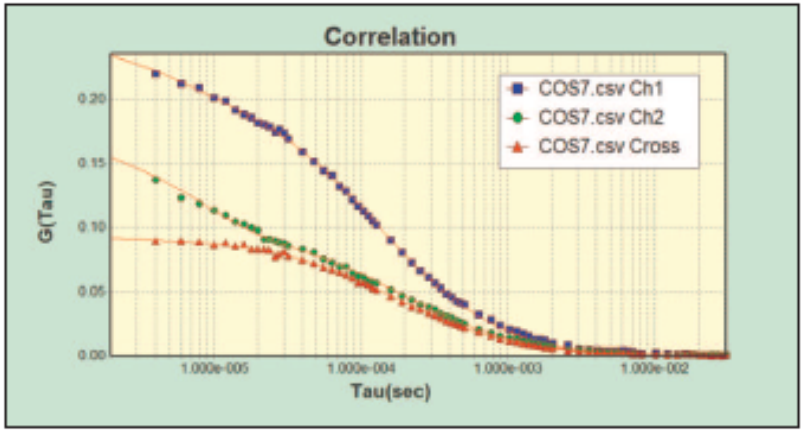


- Constante de dissociation

Fluorescence Correlation Spectroscopy

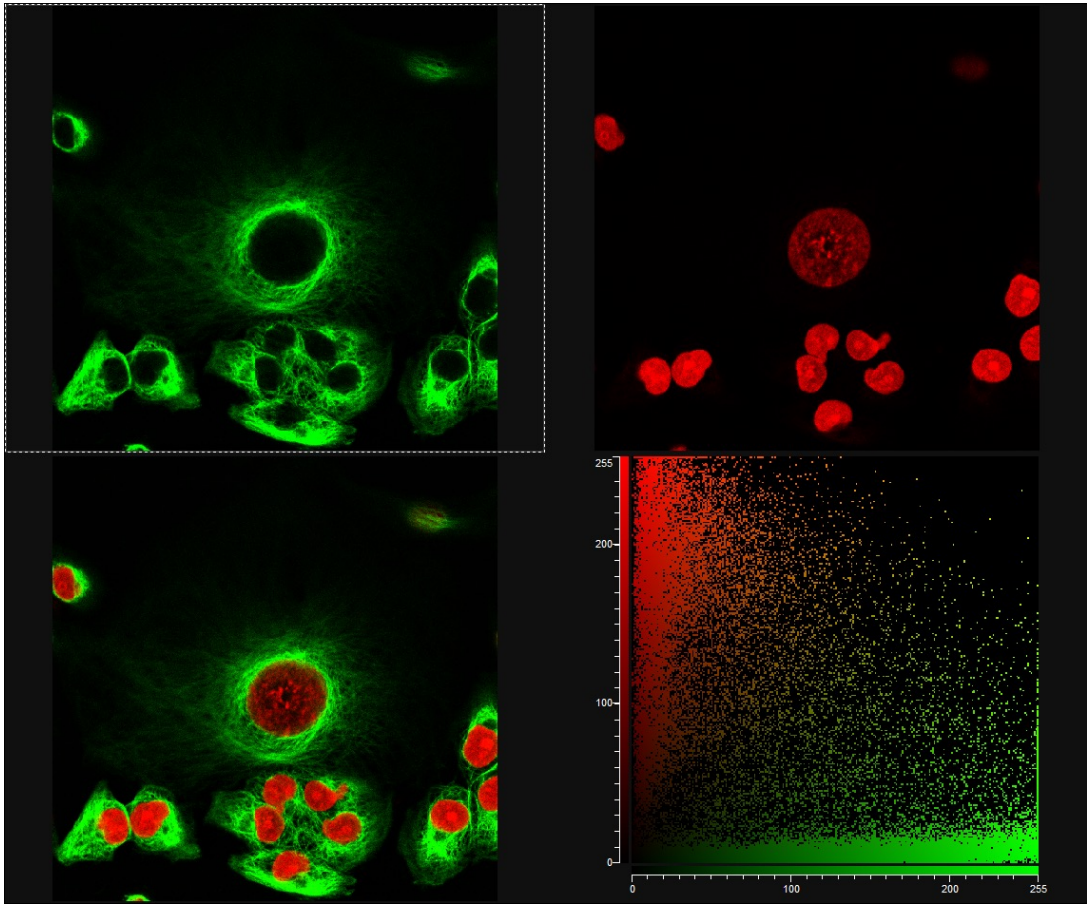
Measurement of molecule interaction within living cells using cross correlation analysis

Cross correlation analysis can also be applied to living cells. To evaluate system performance an GFP HcRed Tandem fusion protein was expressed in COS cells. The fluorophores were excited with 488 and 594 nm leading to an expected ratio of diffusion times between both channels of 1.48.



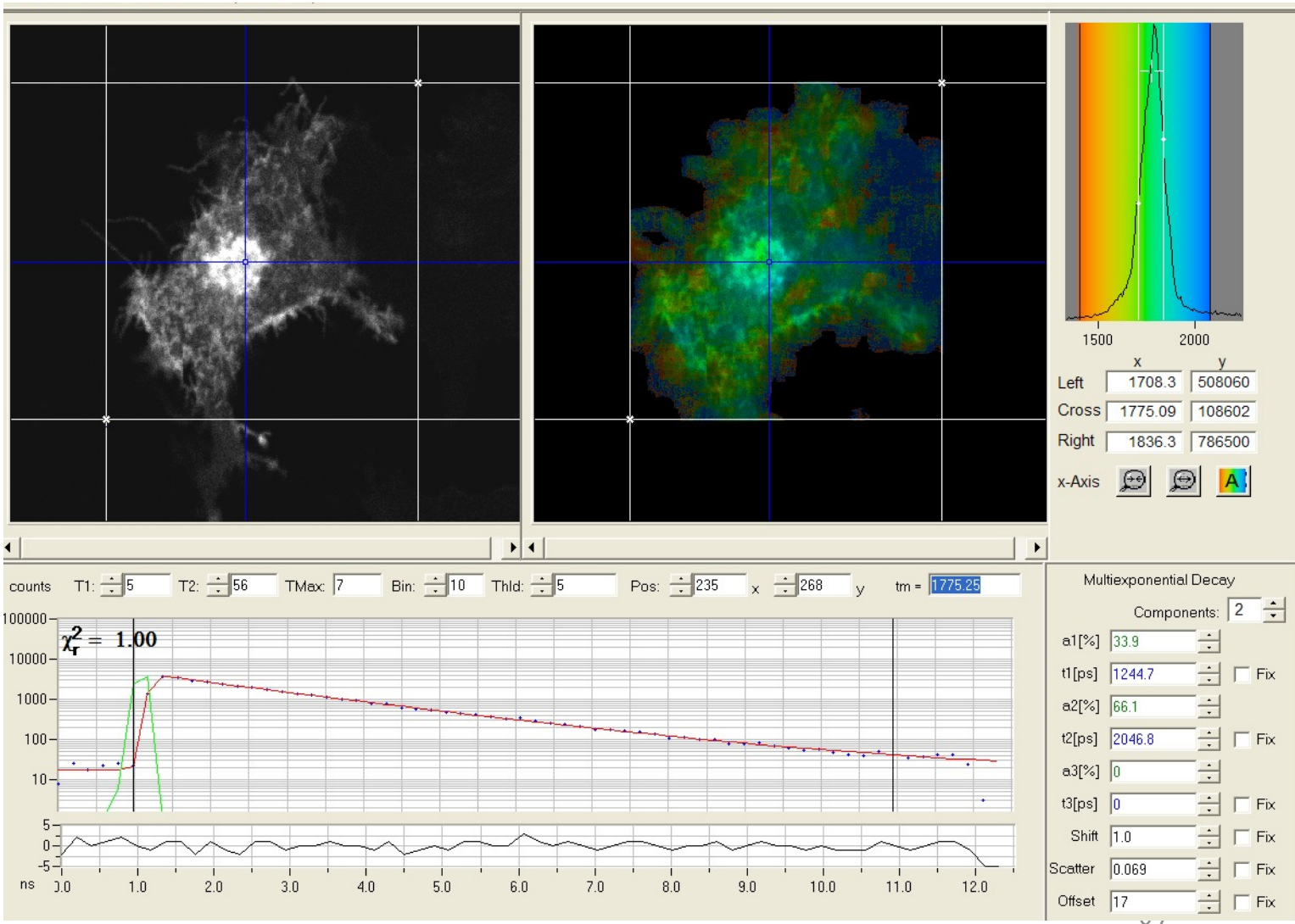
The high cross correlation amplitude as well as a cross correlation diffusion time (tau_{Cross} = 176 µs) between the two autocorrelation diffusion times (tau_{ch1}= 129 µs tau_{ch2} = 188 µs) clearly show the perfect alignment and high optical performance of the system that allows to perform this kind of analysis in living cells as well.

Etude de colocalisation

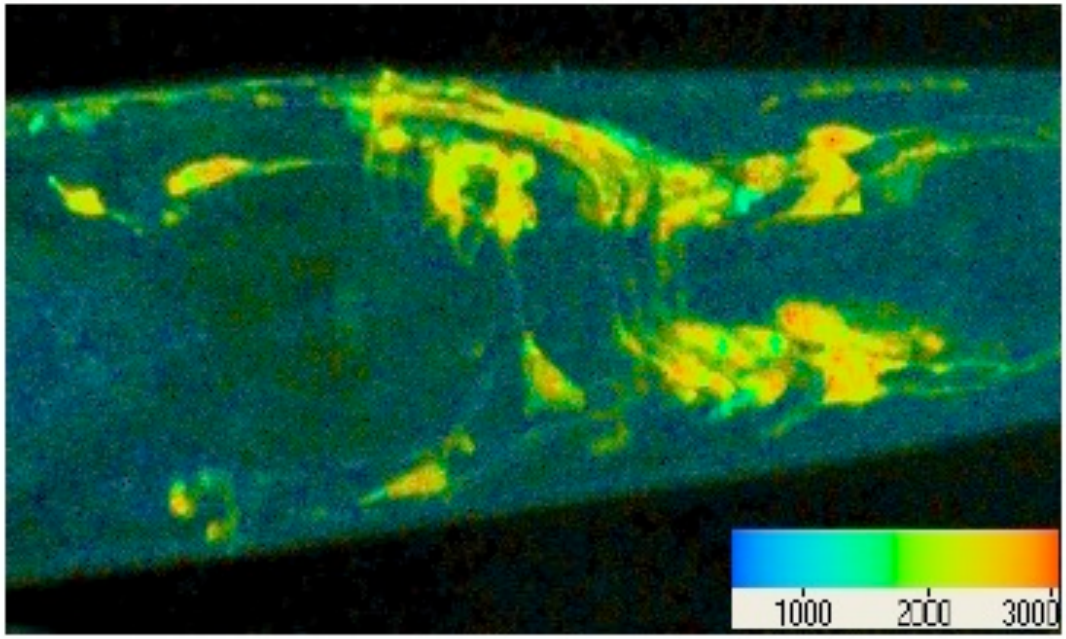


Geometric Analysis	Image
#Pixels	262,144
#Pixels, mask	21,397
Area [µm²]	5,112.01
Area, mask [µm²]	417.26
Mask area rate	8.16%
Densitometric Analysis:	
Channel 1	
Intensity sum	7,744,006
Intensity sum, mask	1,624,968
Mean intensity	29.54
Mean intensity, mask	75.94
Mask intensity rate	20.98%
Channel 2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

Fluorescence Lifetime IMaging FLIM



Fluorescence Lifetime IMaging FLIM

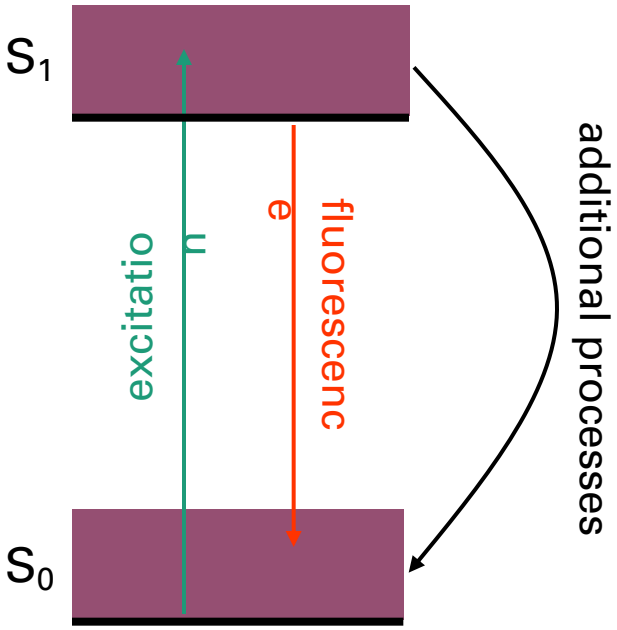


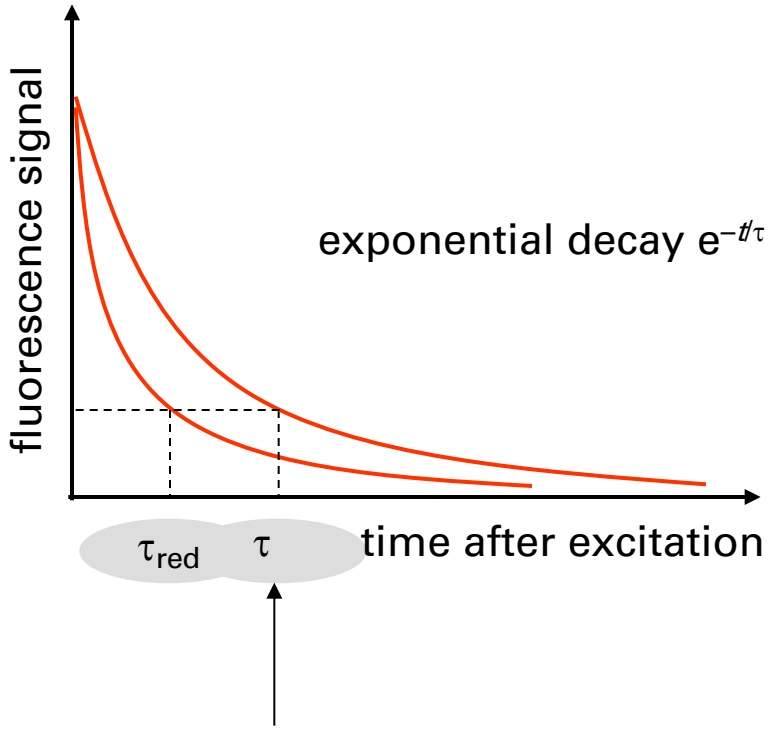
Fluorescence lifetime image of a C. *elegans*. The different lifetime colours derive from various fluorescent proteins (CFP, GFP, YFP). Courtesy H. Hutter.

Fluorophores and lifetimes

Probe:	$\lambda_{exc}/\lambda_{em}$	τ_a (ns)	τ_b (ns)
BCECF	490/520	3.0 (acid)	3.8 (base)
Fluo-3	490/520	2.44 (no Ca^{2+})	0.79 (Ca^{2+})
Lucifer Yellow		3.3	
Sodium Green		1.1 (low Na^{+})	2.4 (high Na^{+})
Hoechst		2.2 (no acc., 7-AAD)	1.4 (acceptor,7-AAD)
FITC	490/520	4.0 (pH > 7)	3.0 (pH < 3)
TRITC	543/	2.0	
Rhodamine 700	659/669	1.6 (pH 9)	1.55 (pH 6)
Rhodamine 700		1.55 (H_2O)	2.99 (Ethanol)
Cy3	550/570	0.27	0.5 (antibody conjug.)
Cy5	633/	1.0	
GFP free (S65T)	488/507	2.68	
CFP		1.3	
YFP		3.7	

Fluorescence lifetime

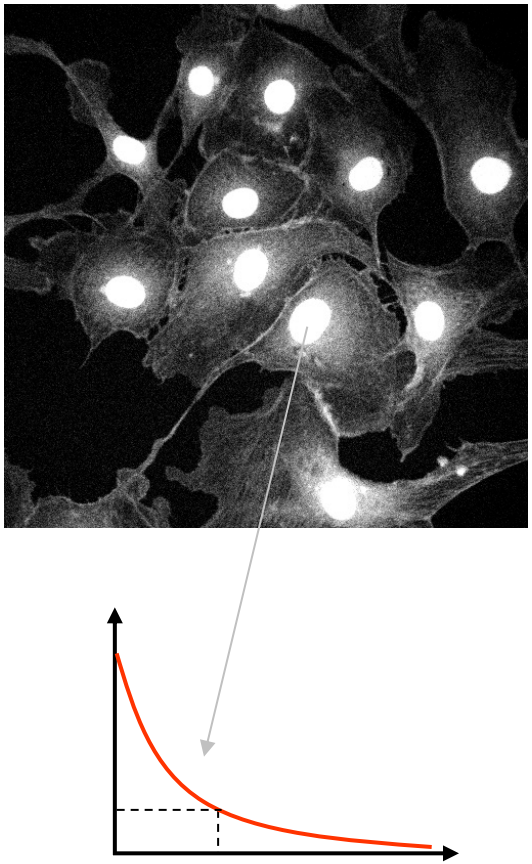




fluorescence lifetime:

- ave. time betw. excitation and emission
- characteristic property of dyes, ~ns
- depends on environment (ions, pH,

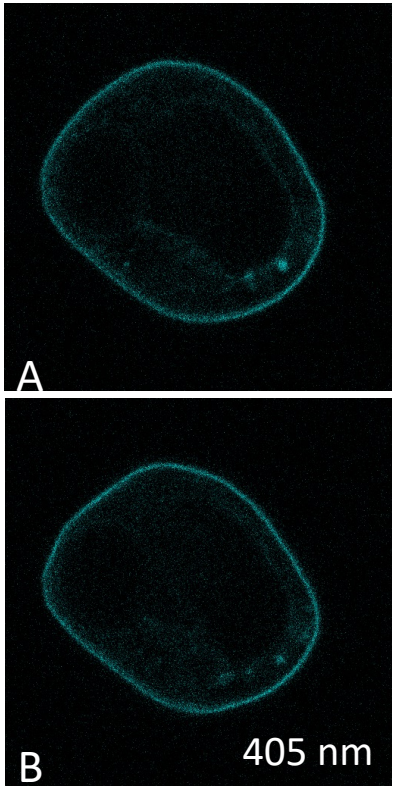
Fluorescence lifetime imaging (FLIM)

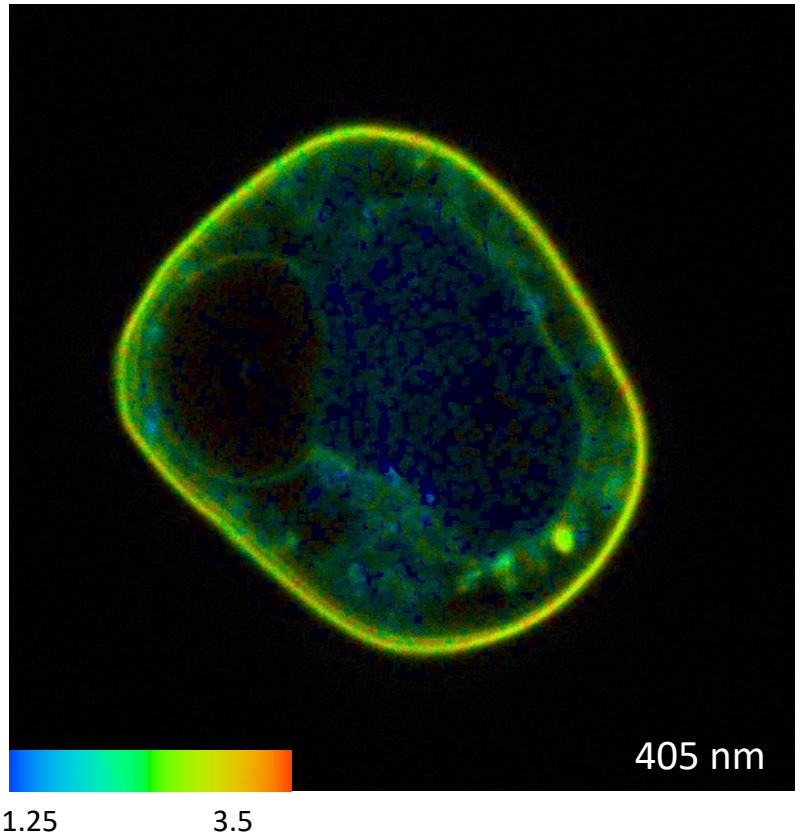


- measurement of lifetime with spatial resolution

- transformation of nanoseconds in color code

Leica TCS SP2 and D-FLIM CFP-MHC





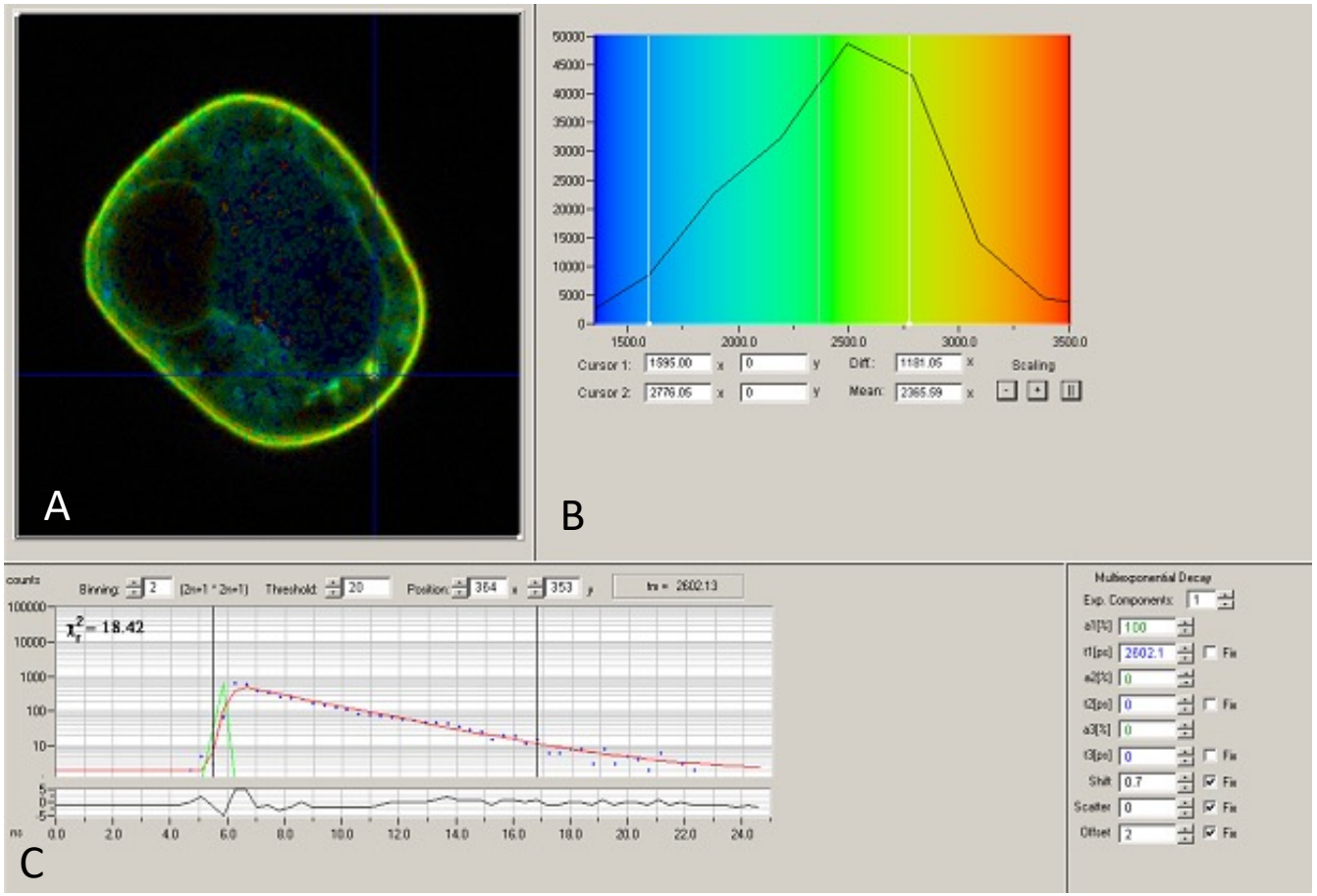
Cyan: CFP tagged to MHC
(Major Histocompatibility Complex)

Before (A) and after (B) FLIM acquisition

Duration for acquisition: 200 s

Courtesy
Dan Davis, Imperial College London

Leica D-FLIM: CFP-MHC



CFP tagged to MHC

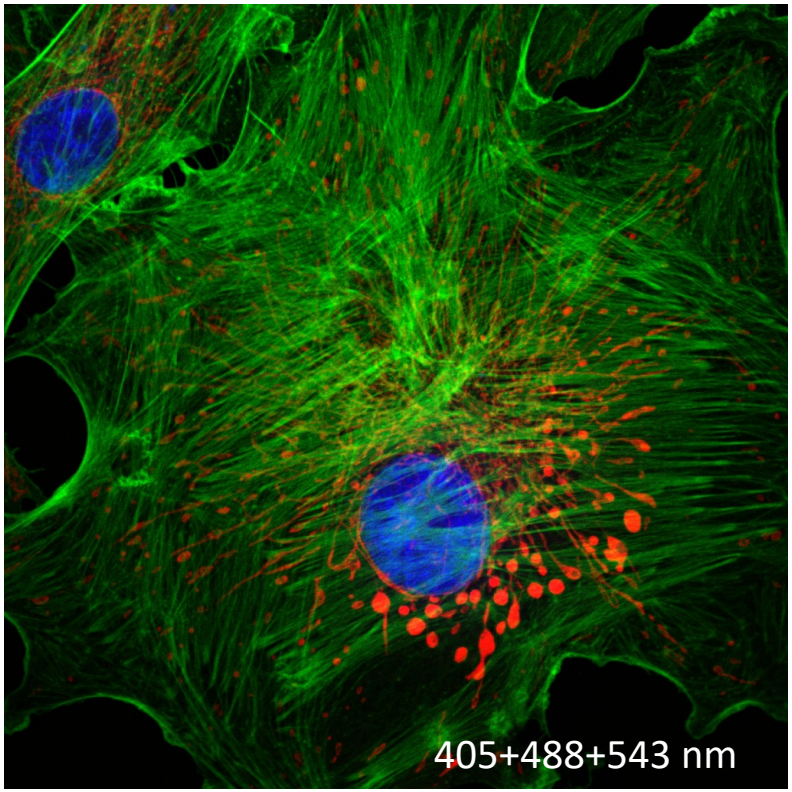
A: Lifetime image

B: Lifetime distribu-tion over the image area

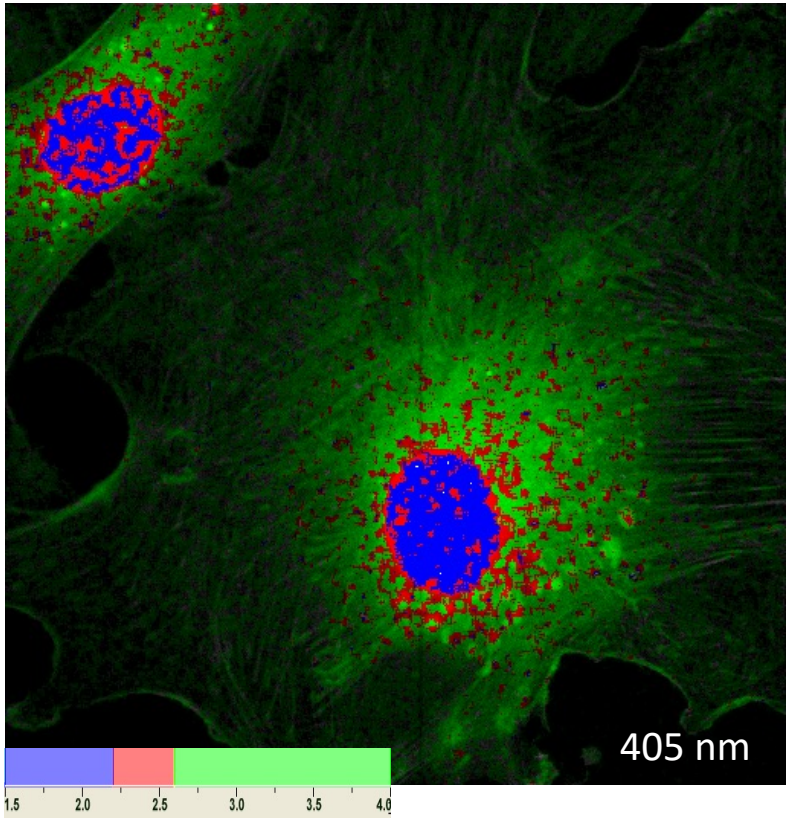
C: Fitted decay curve

(corresponding to the pixels selected by the blue cursor in A)

Leica TCS SP2 and D-FLIM FluoCells (BPAE)

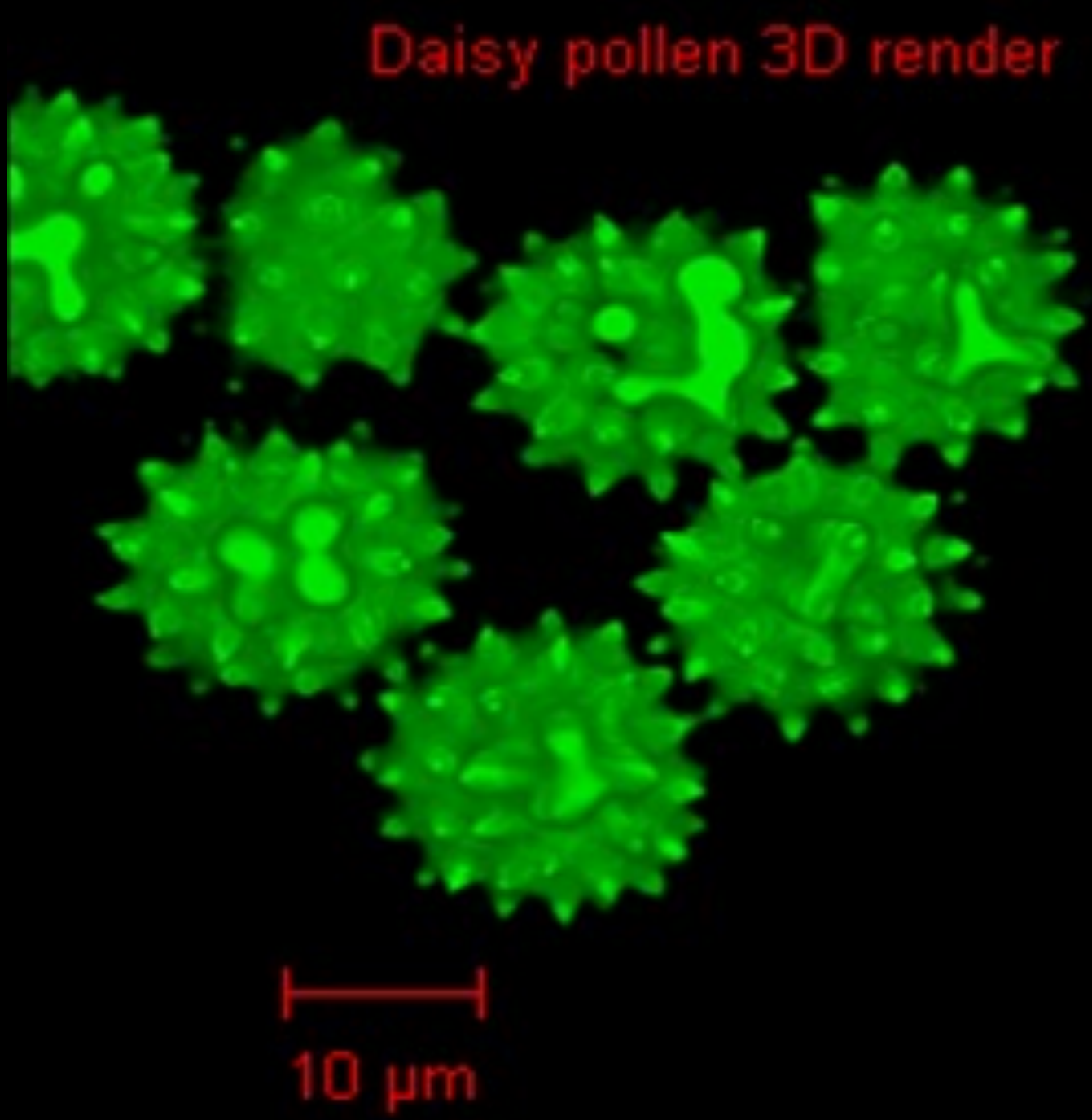


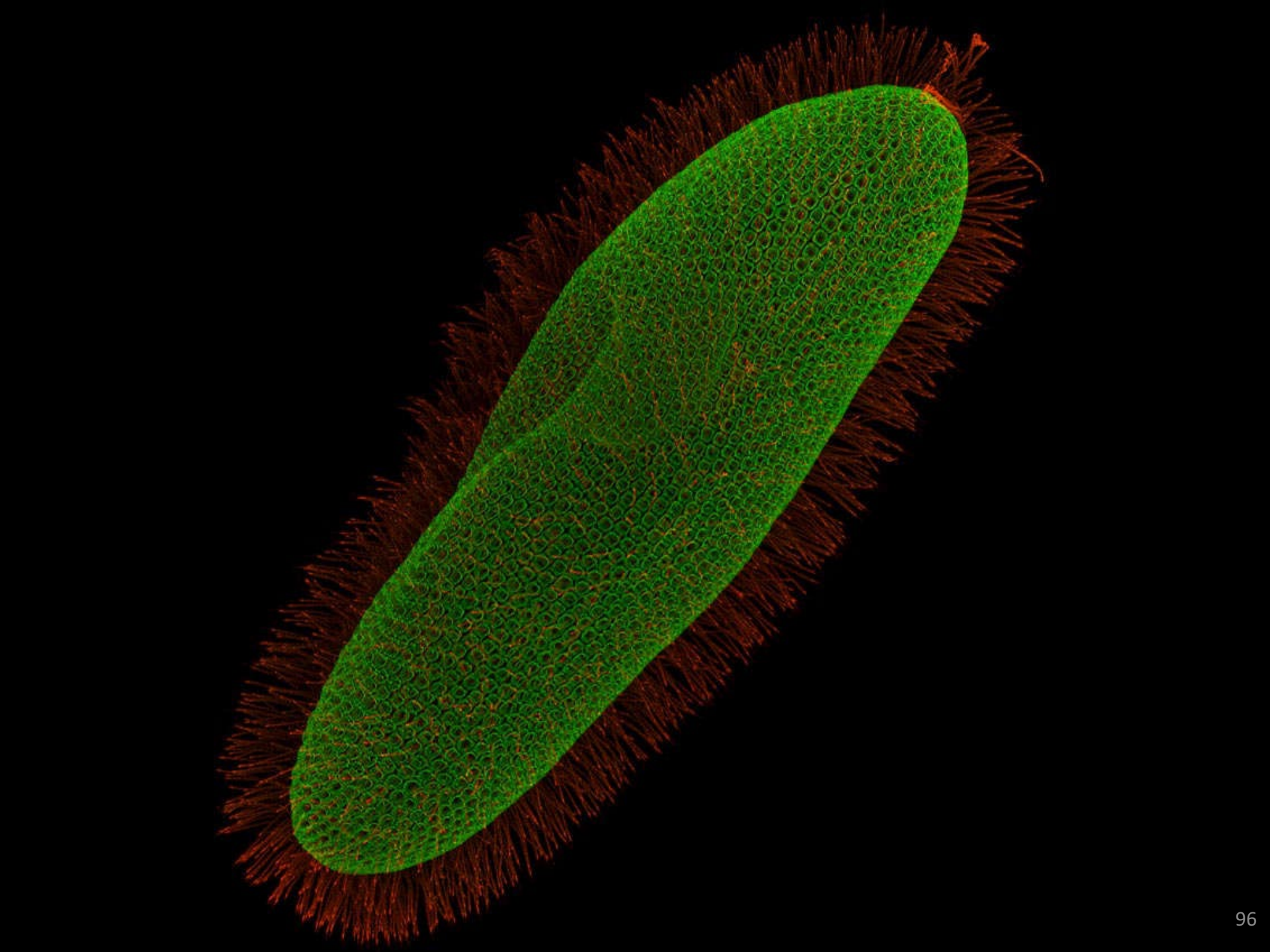
Blue: DAPI (nucleus)
Red: Mitotracker Red (mitochondria)
Green: BODIPY FL phallacidin (actin)

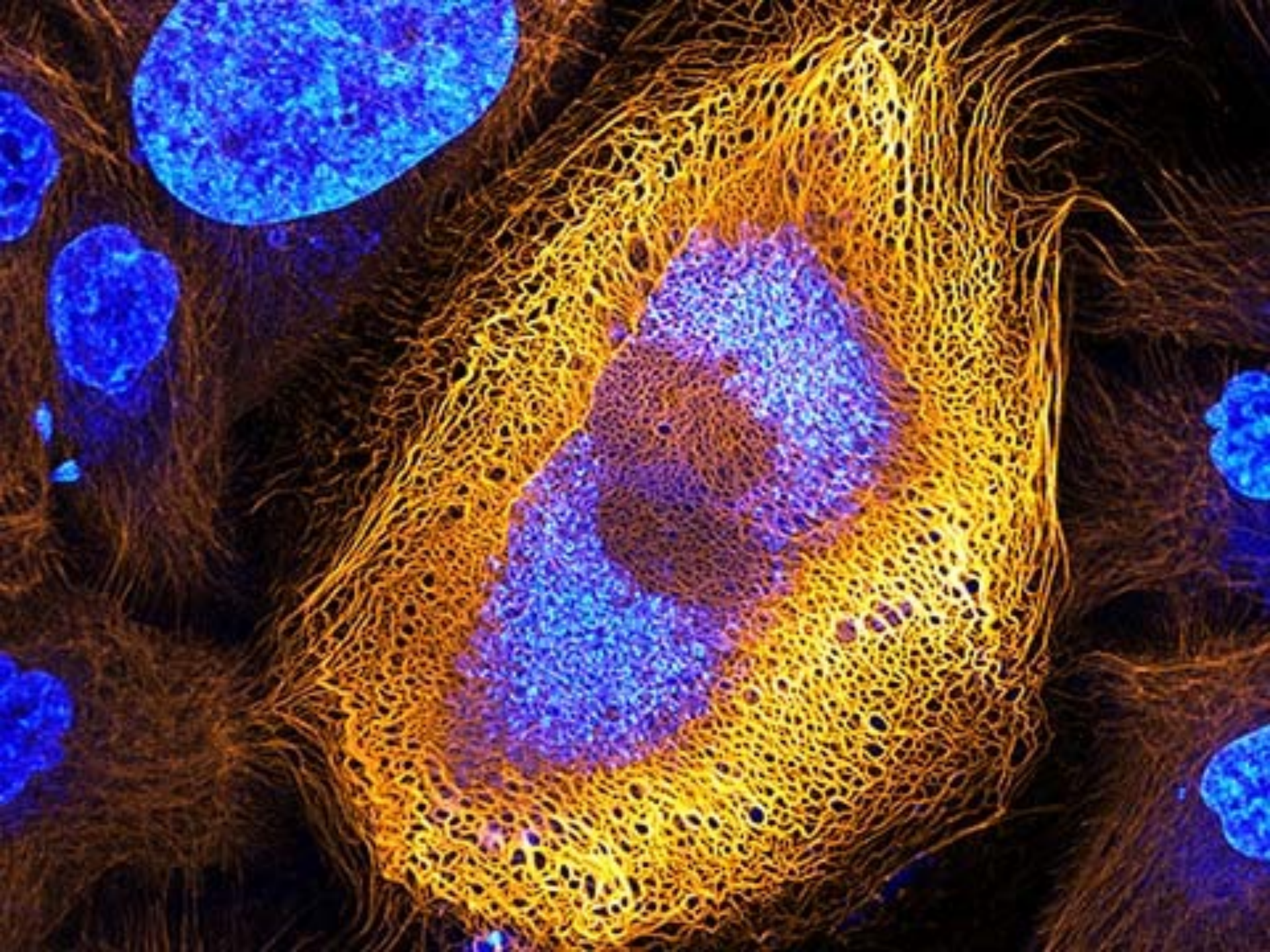


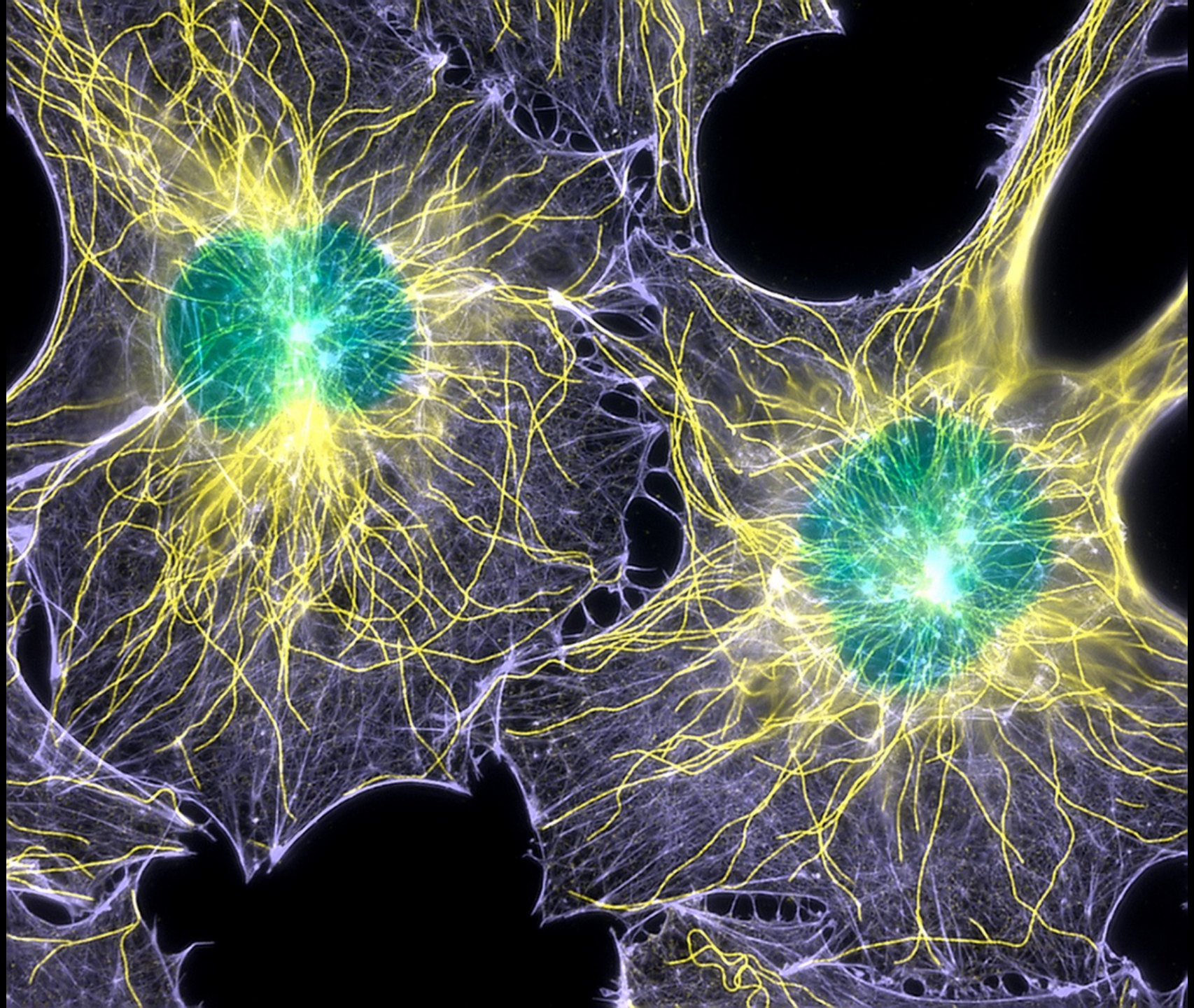
Blue: 1.5 - 2.2 ns
Red: 2.2 - 2.6 ns
Green: 2.6 - 4.0 ns

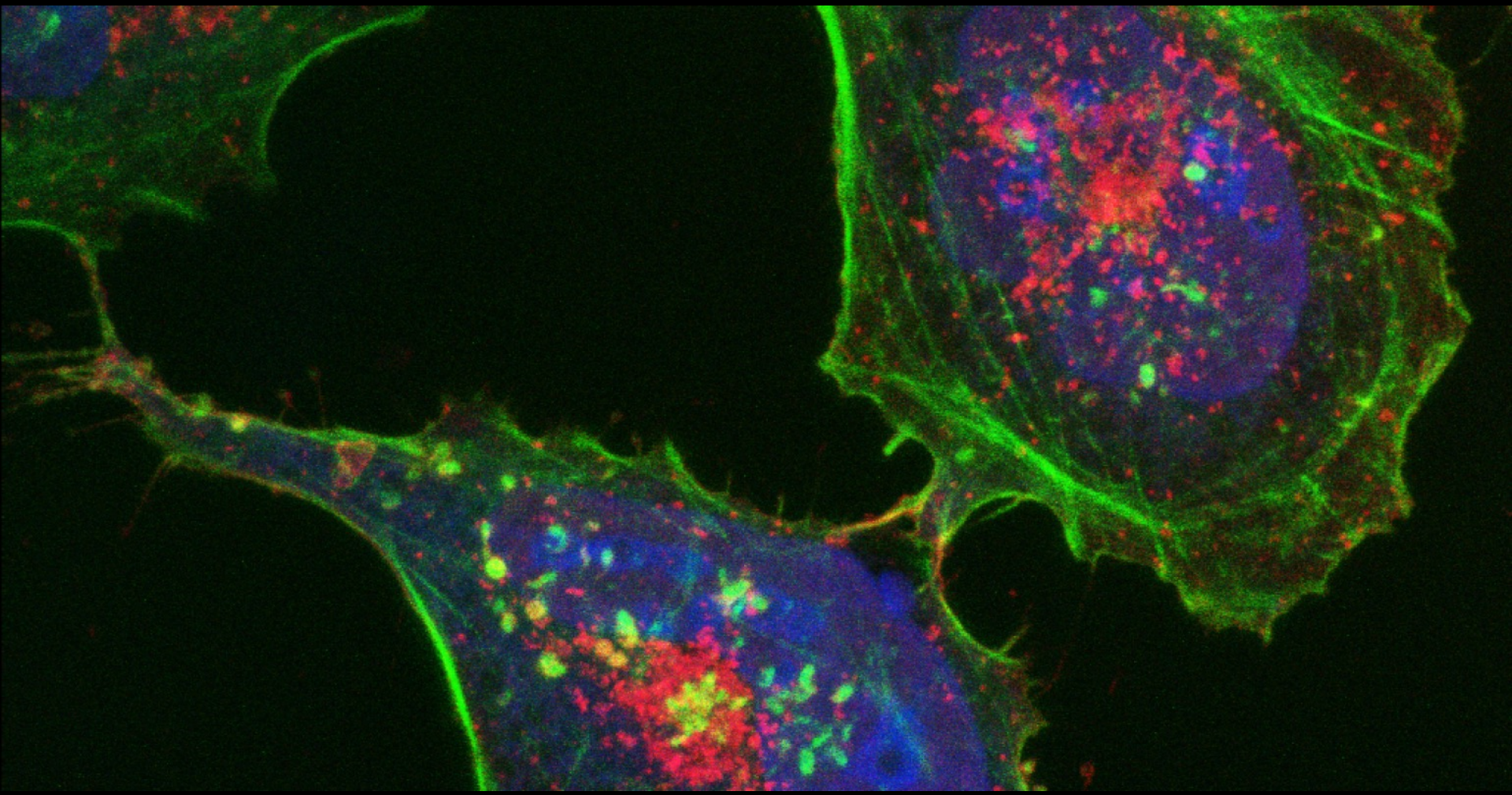
Molecular Probes, F-

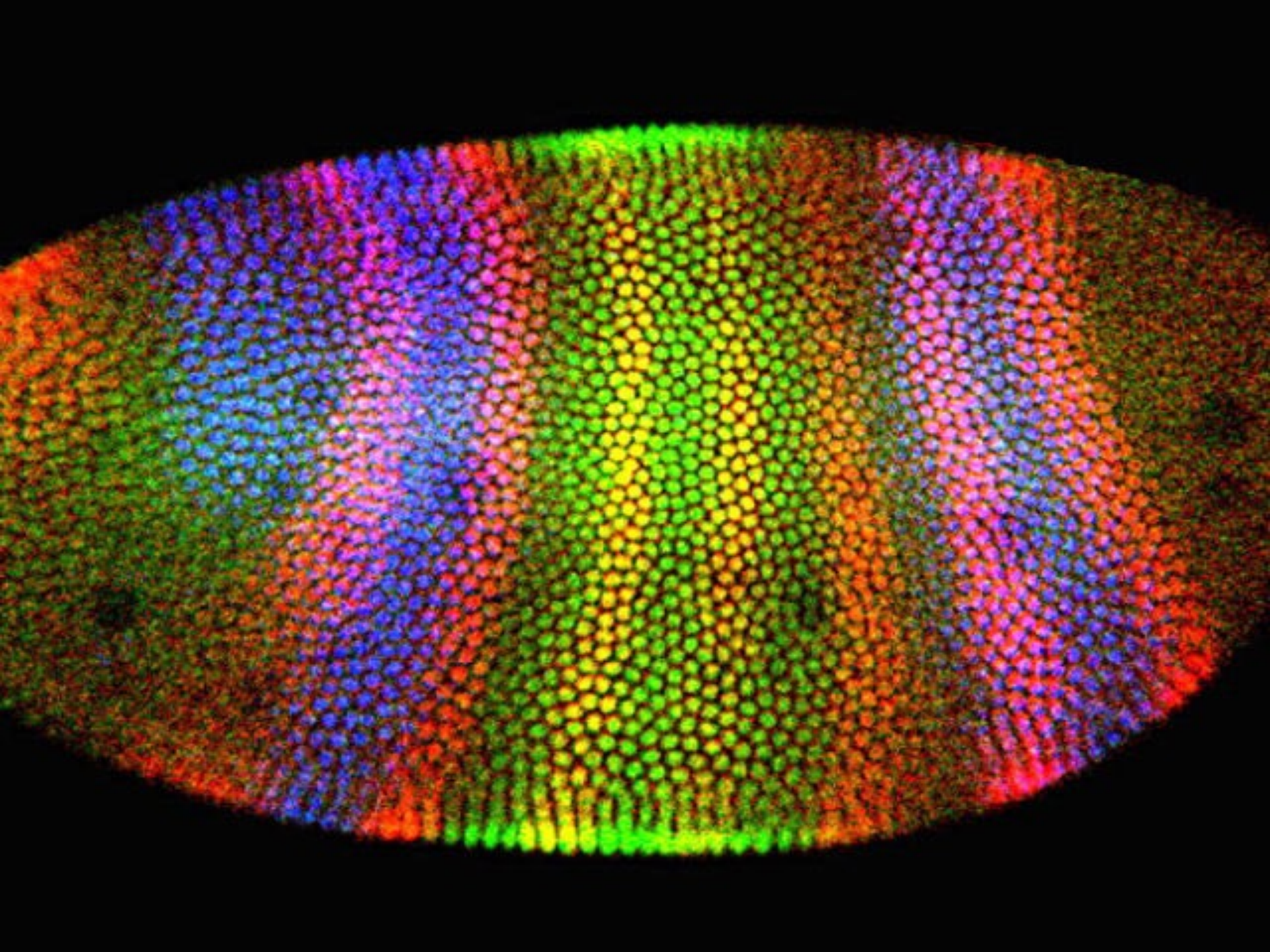

Daisy pollen 3D render
10 µm

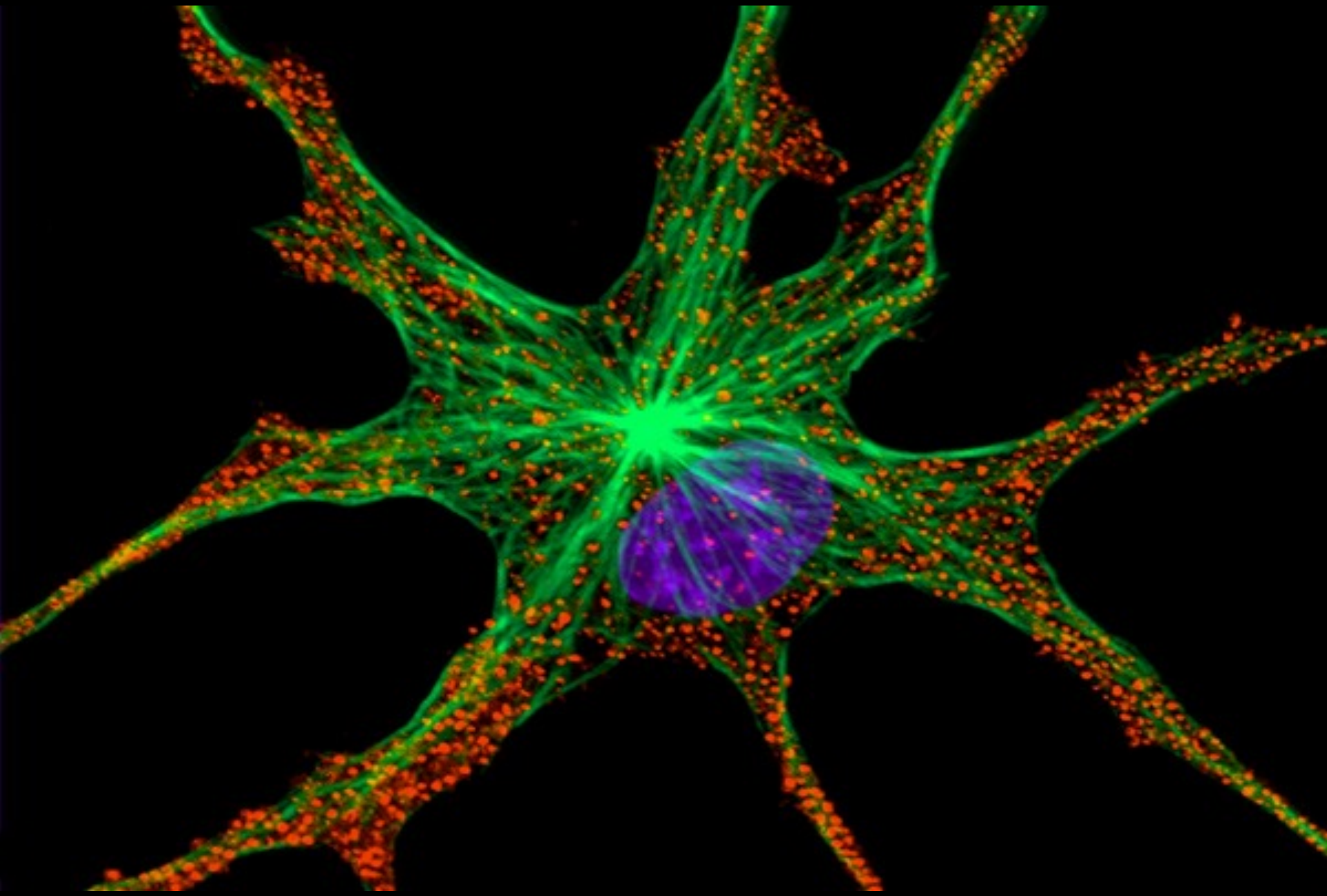


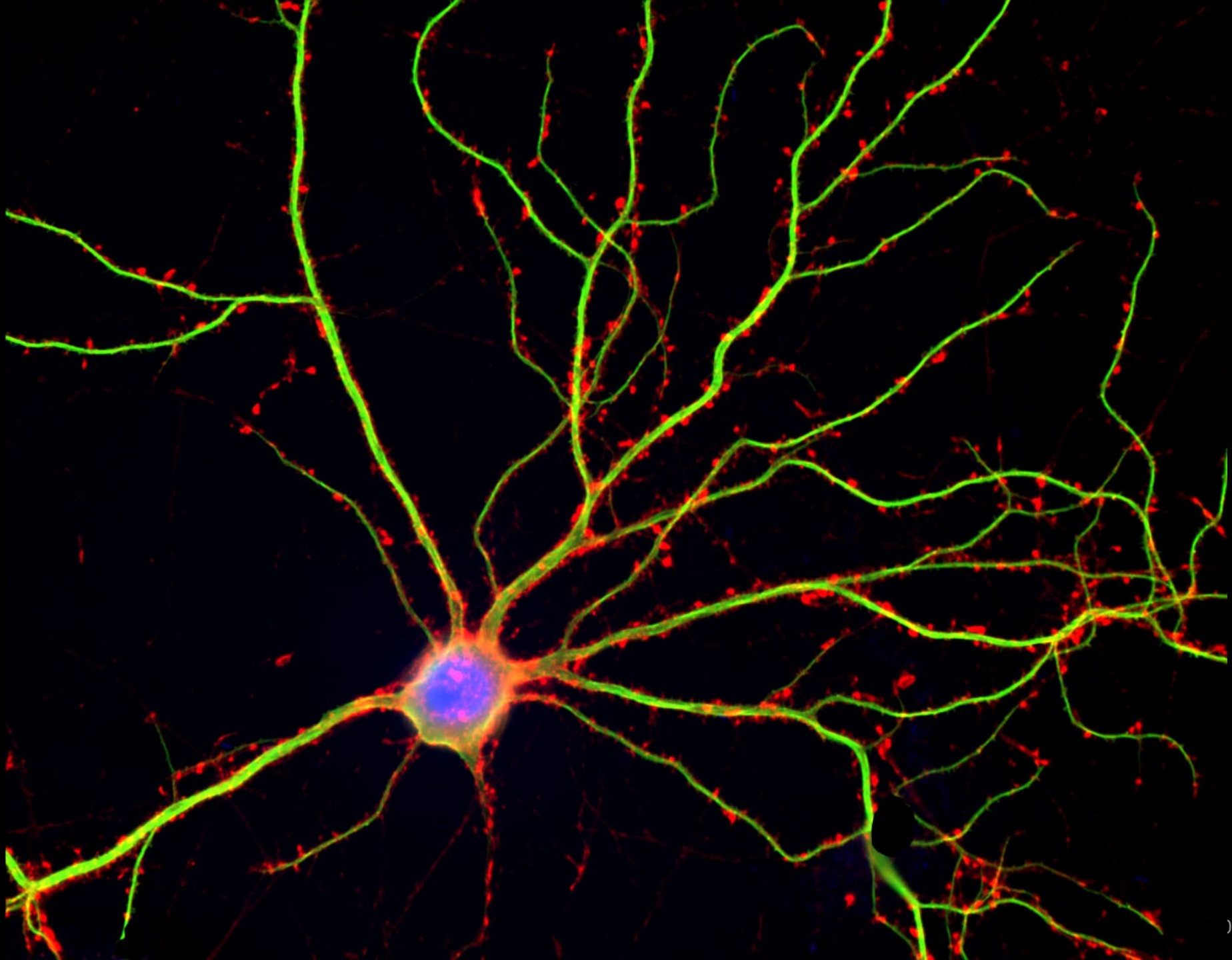


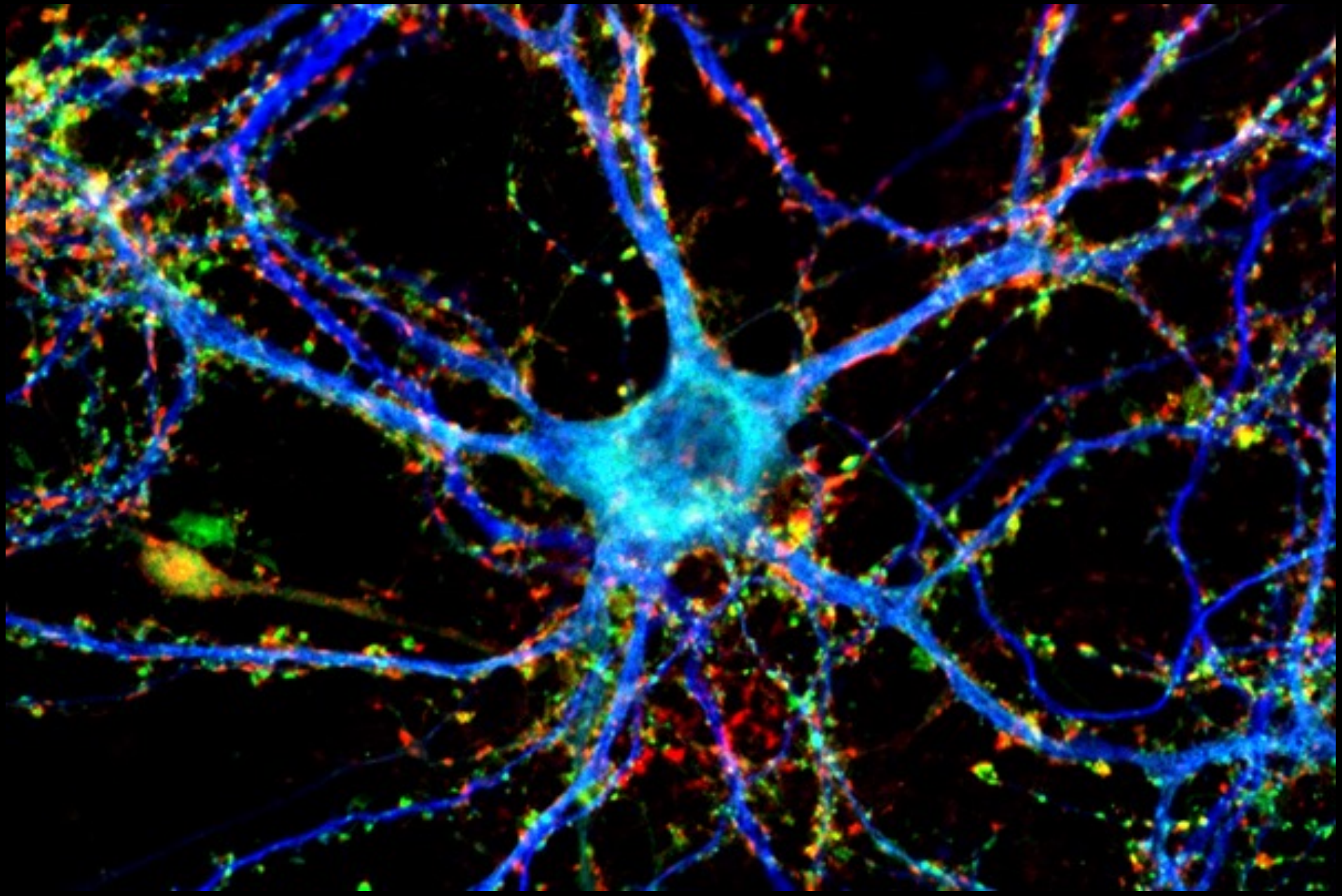


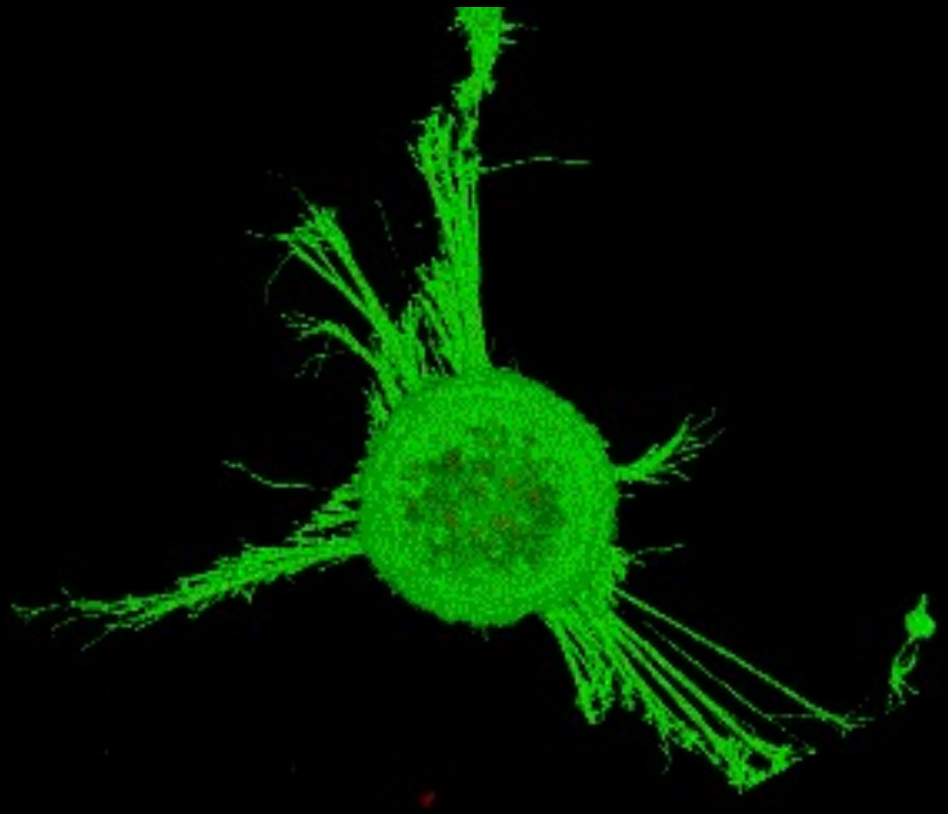


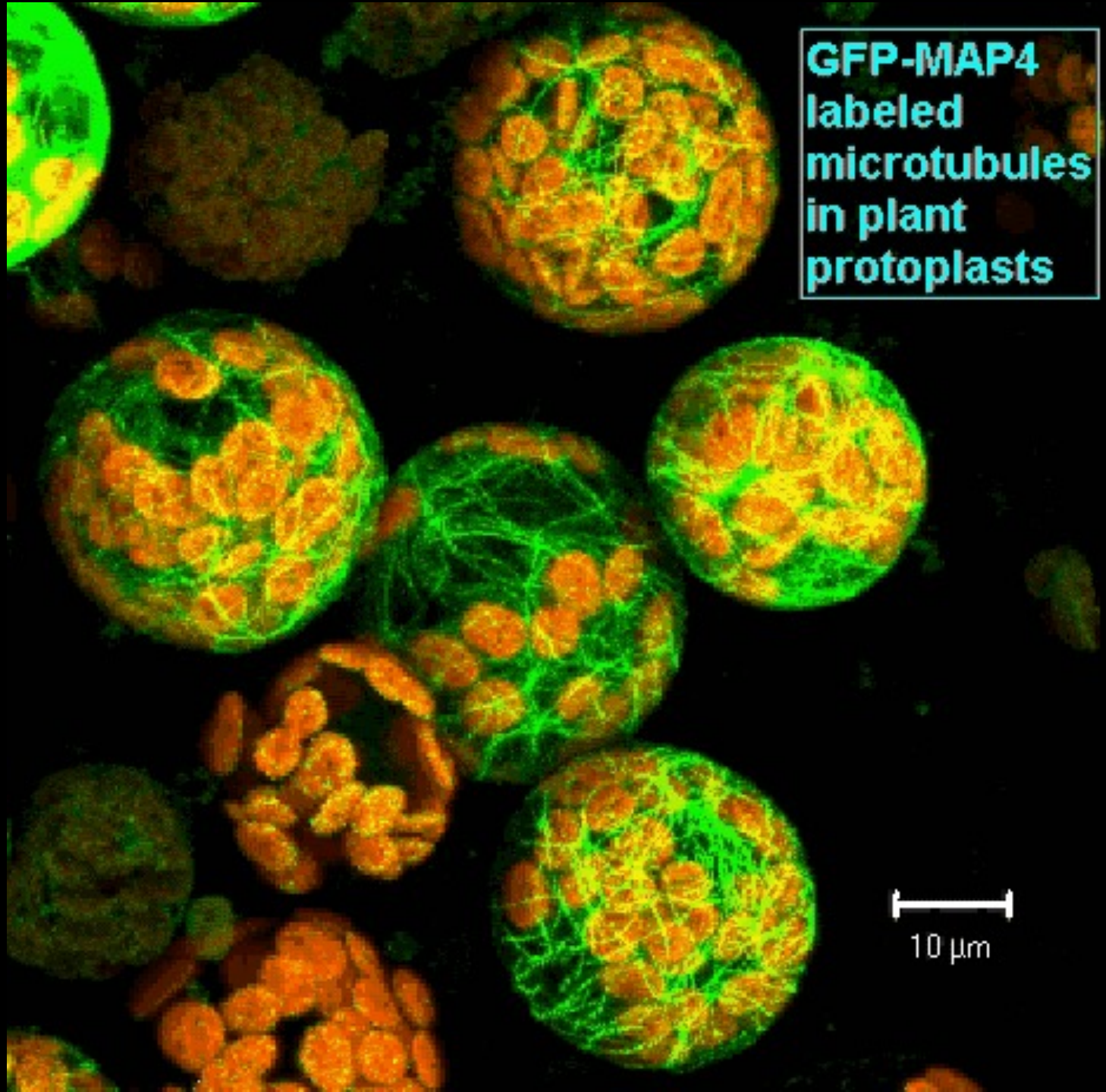


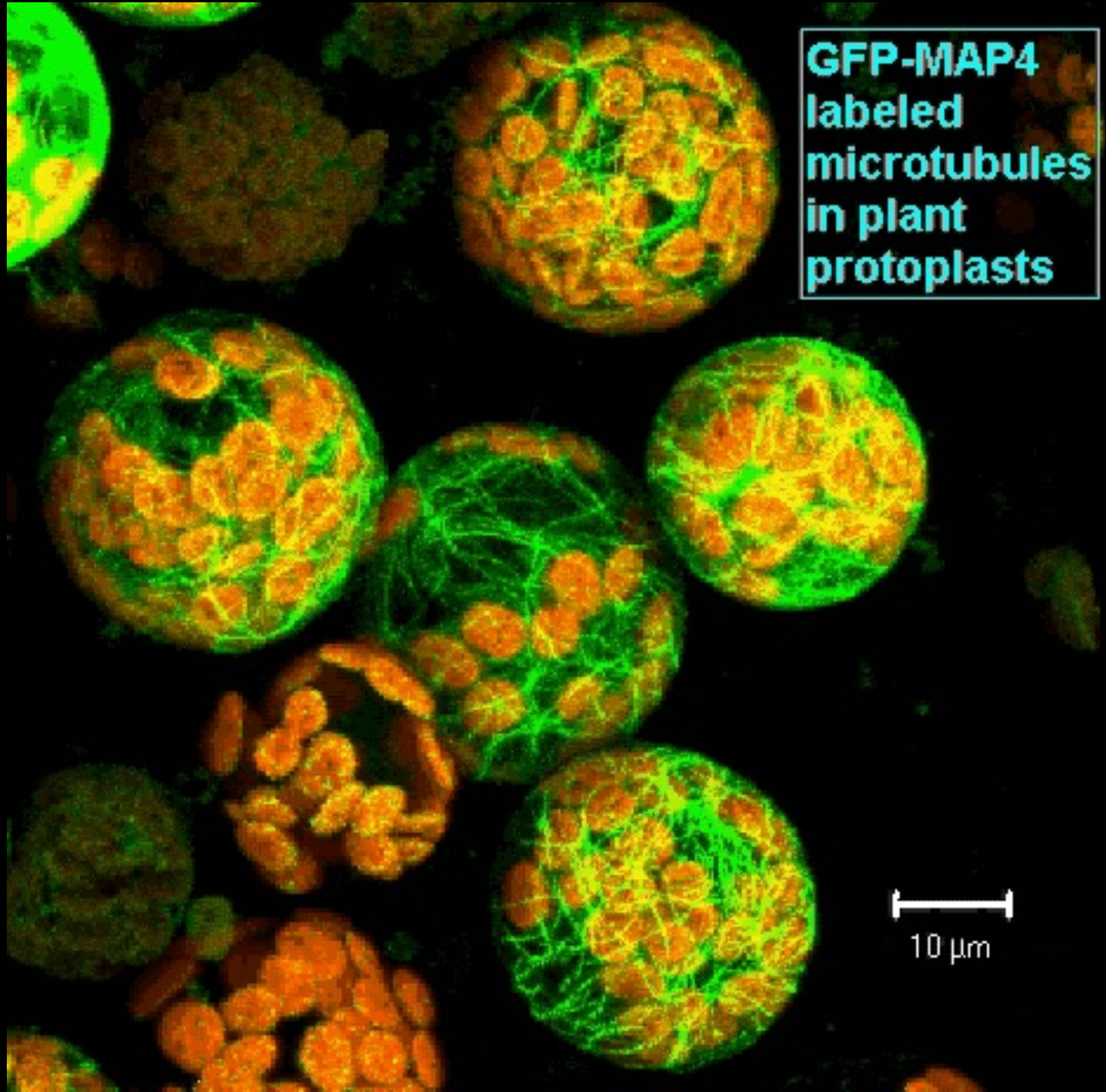


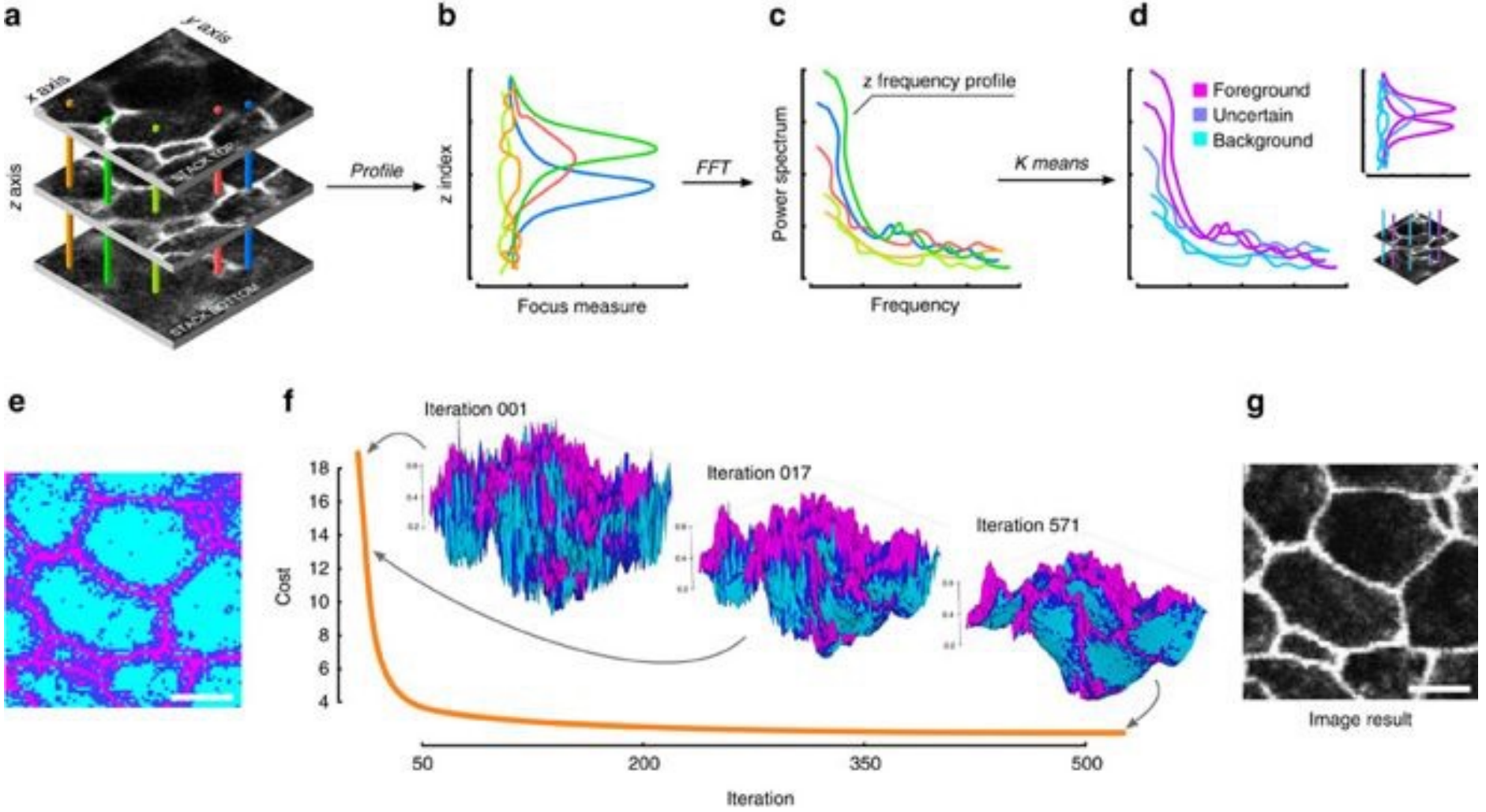


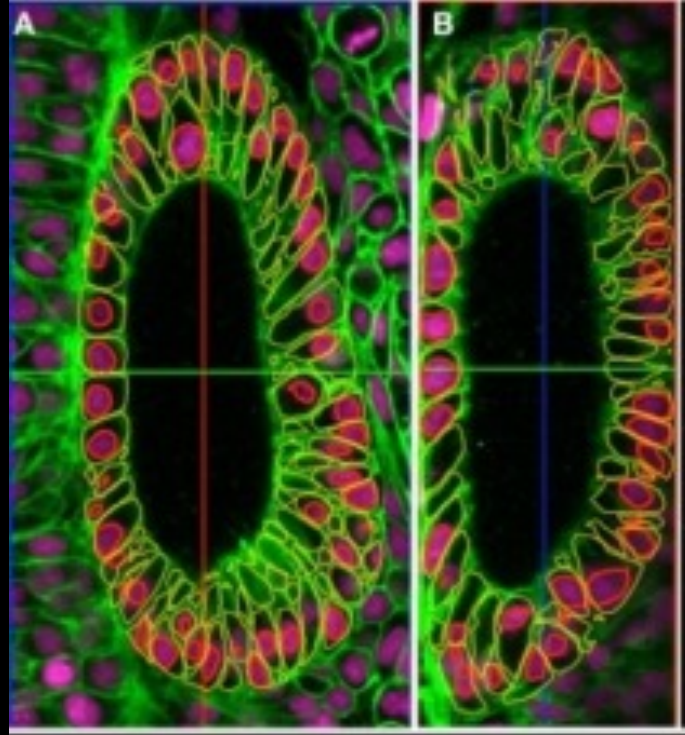


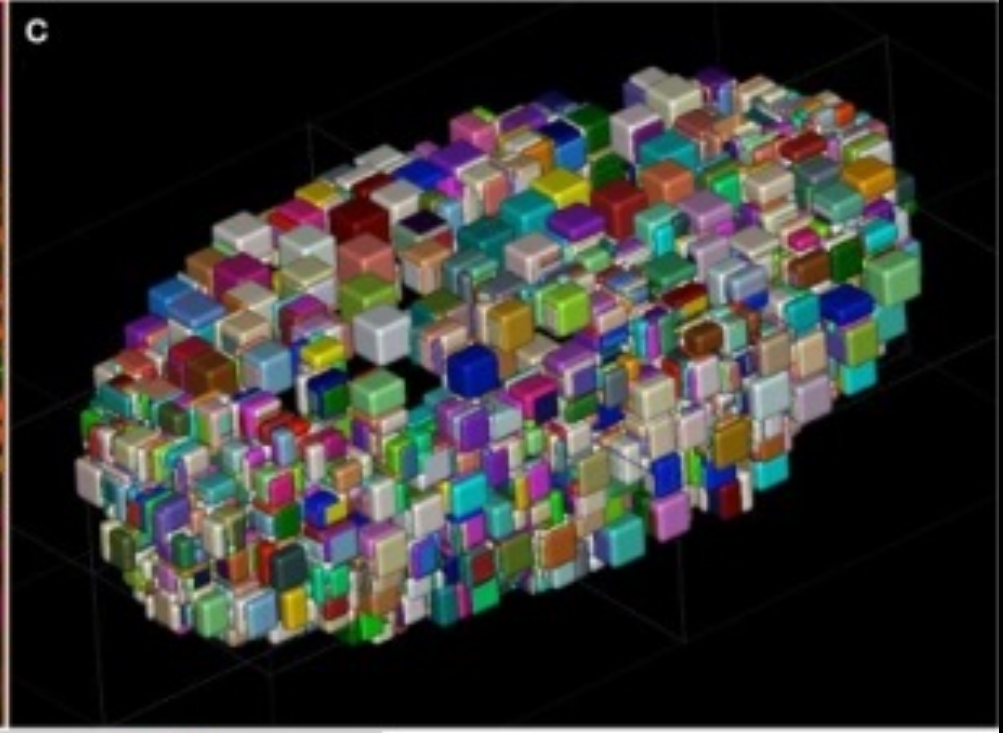


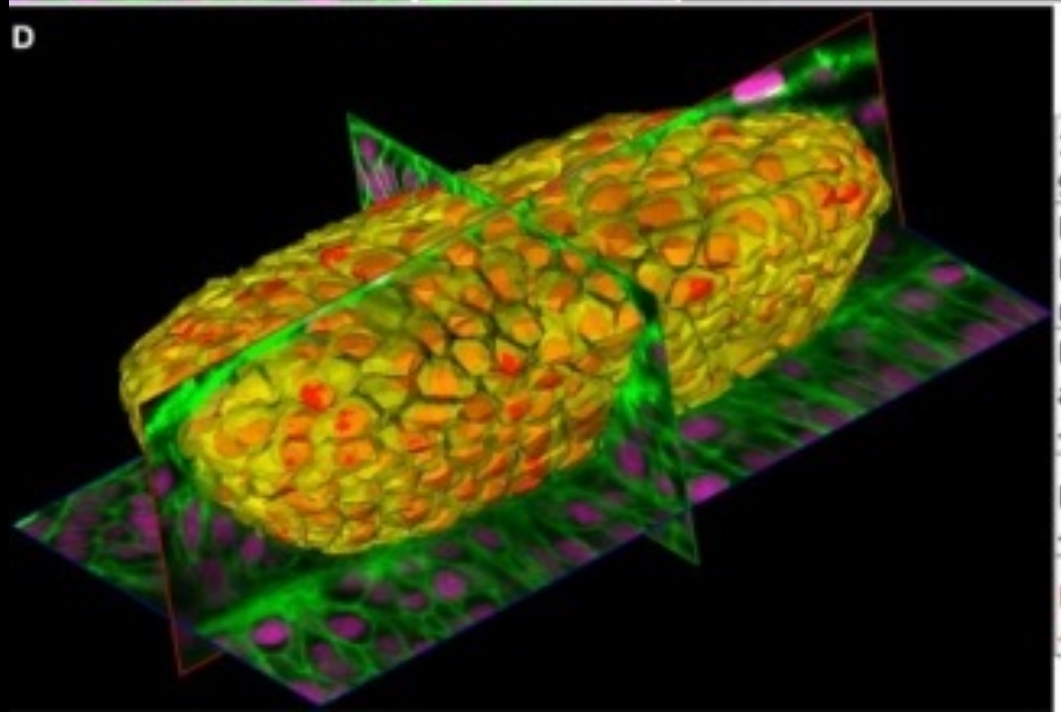

GFP-MAP4
labeled
microtubules
in plant
protoplasts
10 µm


GFP-MAP4
labeled
microtubules
in plant
protoplasts
10 µm


a
y axis
x axis
z axis
Profile
b
z index
Focus measure
FFT
c
z frequency profile
Power spectrum
Frequency
K means
d
Foreground
Uncertain
Background
e
f
Iteration 001
Iteration 017
Iteration 571
Cost
18
16
14
12
10
8
6
4
50
200
350
500
Iteration
g
Image result

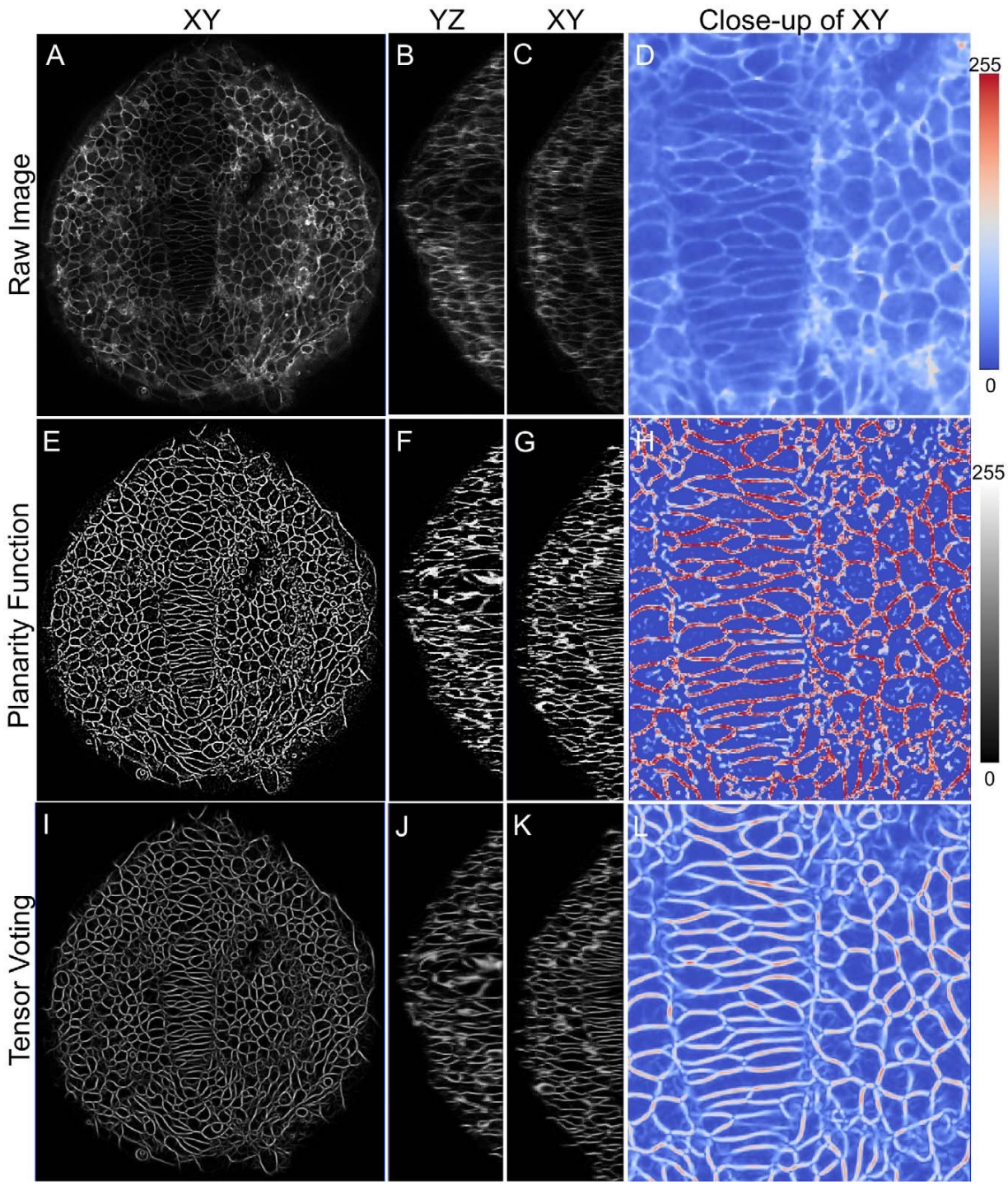


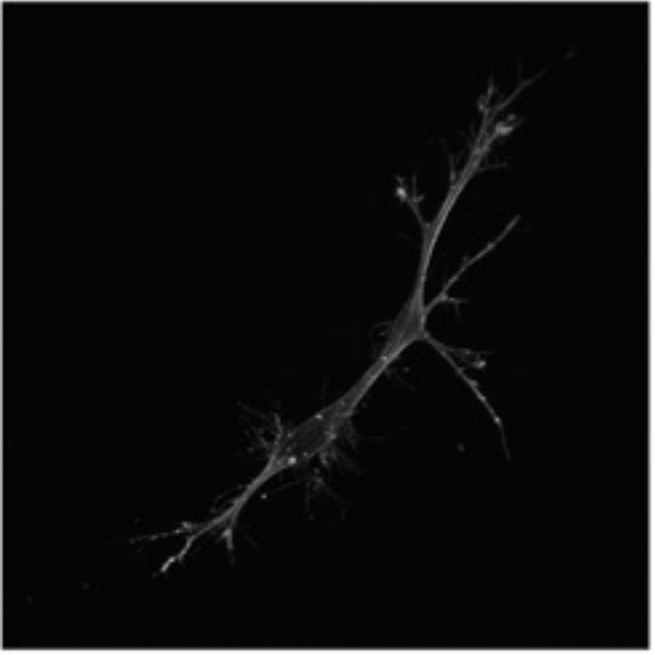




E

100 iterations with 945 cells	> 1 function	Inter-actions	Time (min)
ITKv3 (T = 1) + no DD + no overlap	NA	NA	NA
ITKv3 (T = 1) + DD apriori + no overlap	NA	NA	40.64
ITKv4 (T = 12) + DD + no overlap	Yes	Yes	18.61
ITKv4 (T=12) + DD + overlap	Yes	Yes	20.83


XY
YZ
XY
Close-up of XY
Raw Image
Planarity Function
Tensor Voting
A
B
C
D
E
F
G
H
I
J
K
L
255
0
255
0

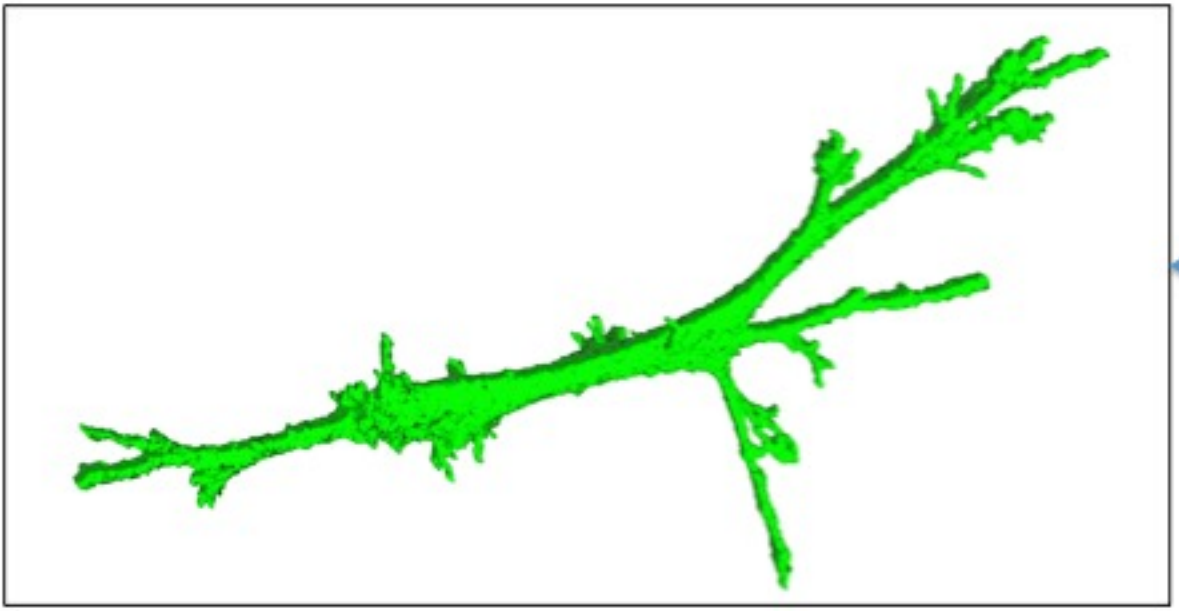


(1)

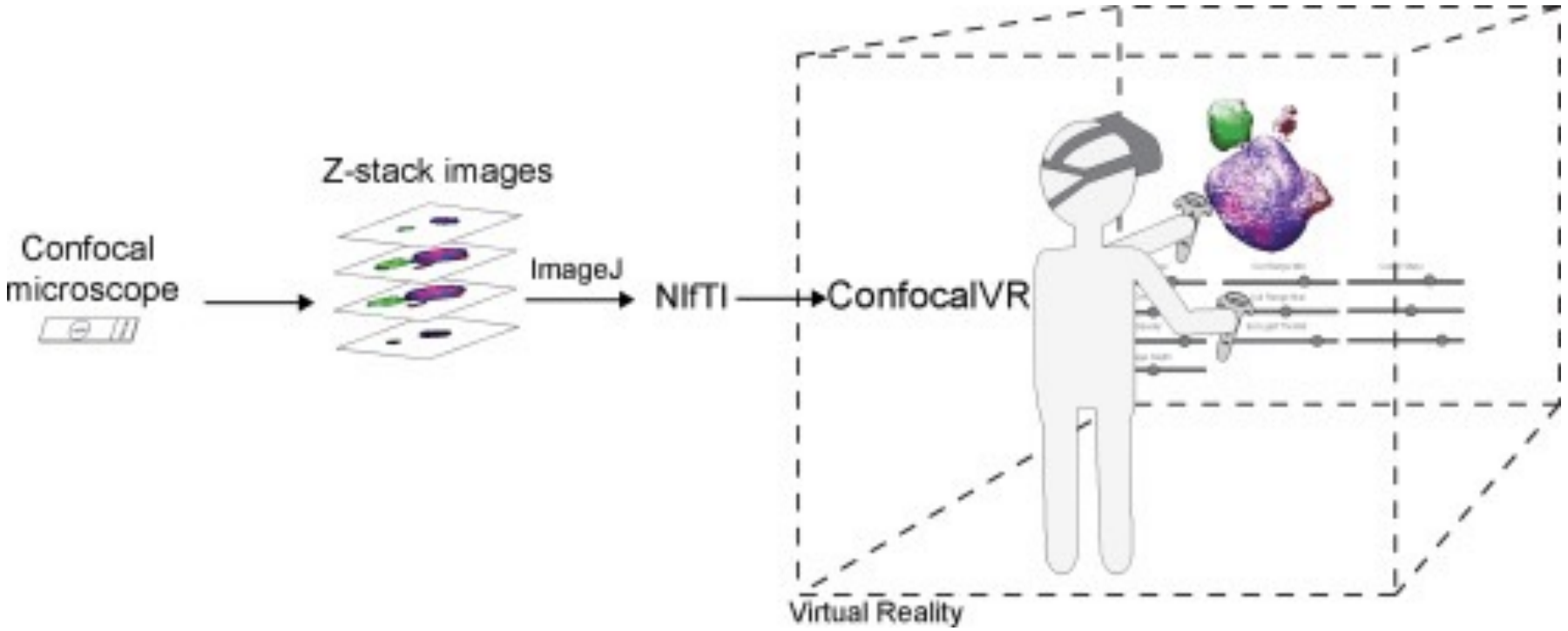
3D segmentation

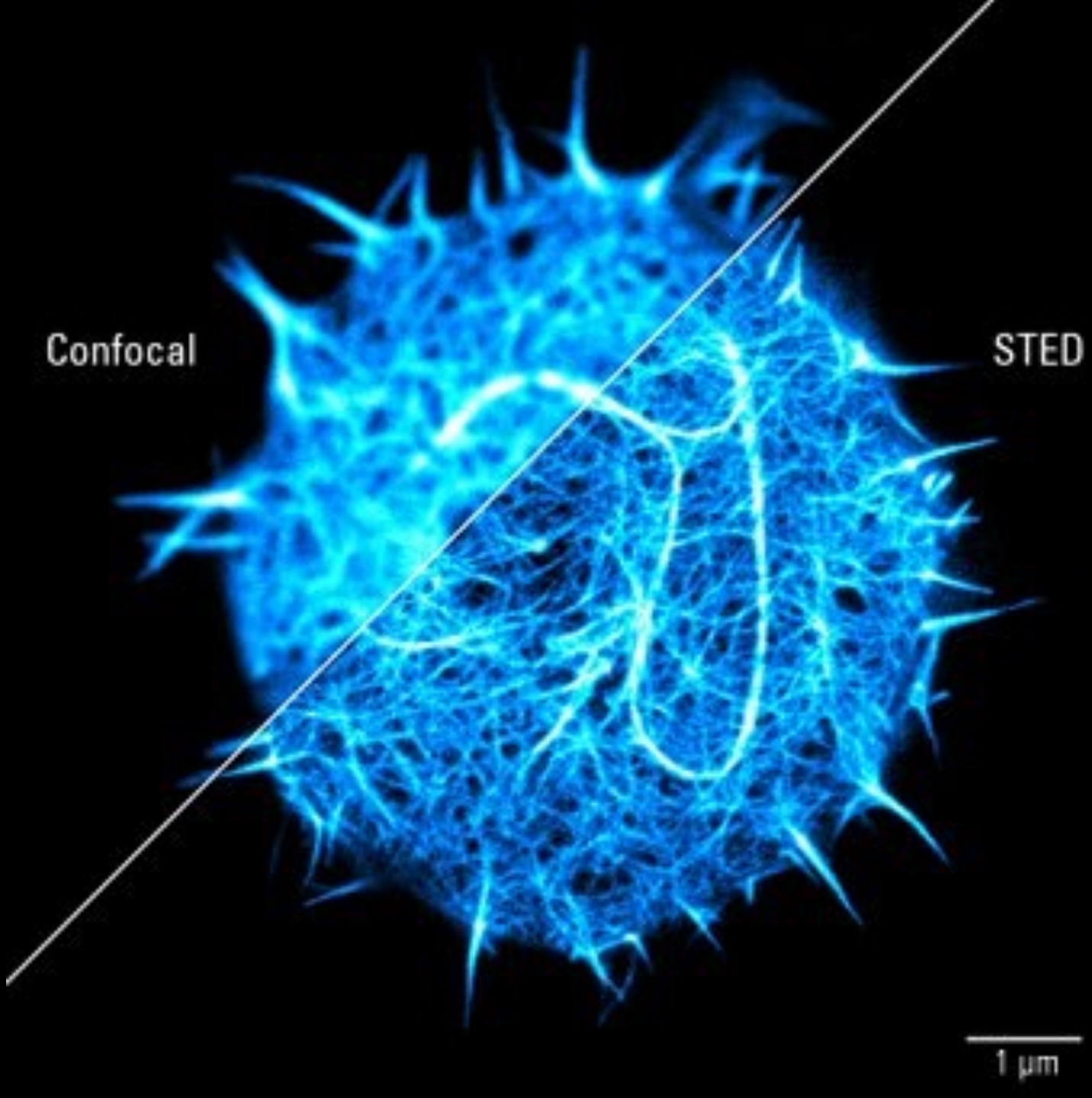
(2)

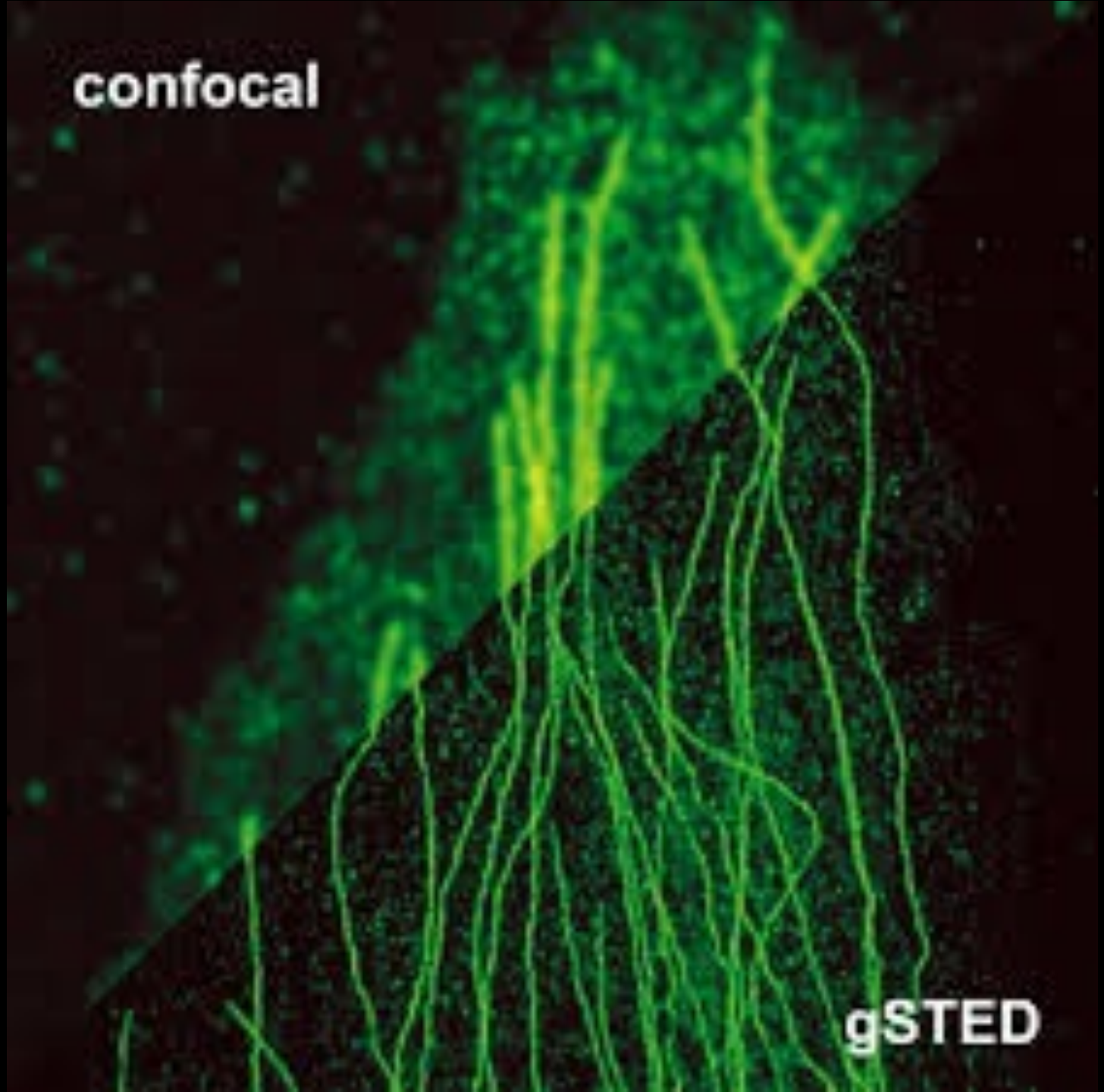
3D visualization



(3)


Z-stack images
Confocal microscope
ImageJ
NIfTI
ConfocalVR
Virtual Reality


Confocal
STED
1 µm


confocal
gSTED