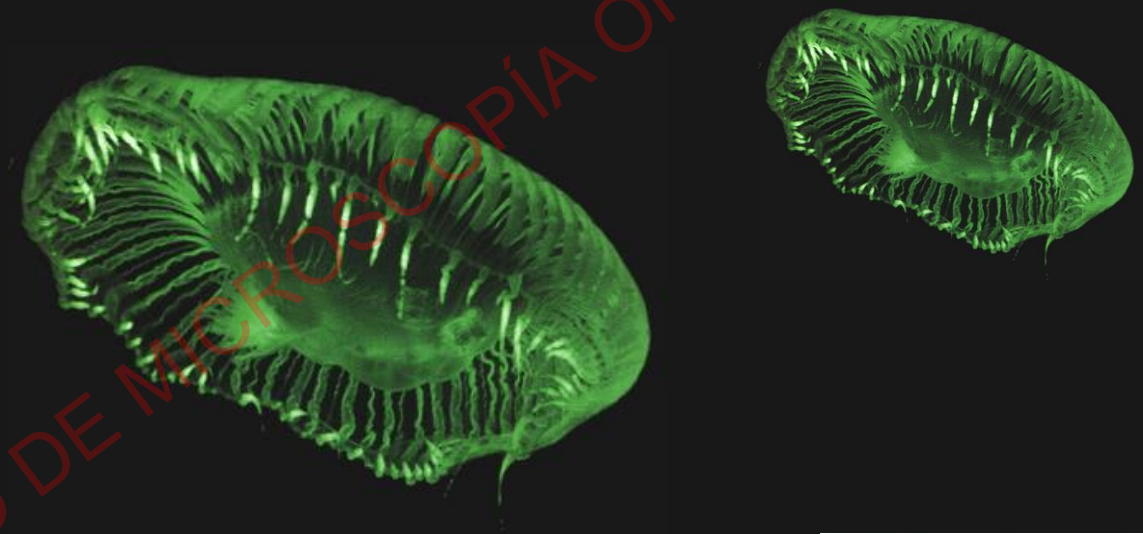
Fluorescent proteins and related assays

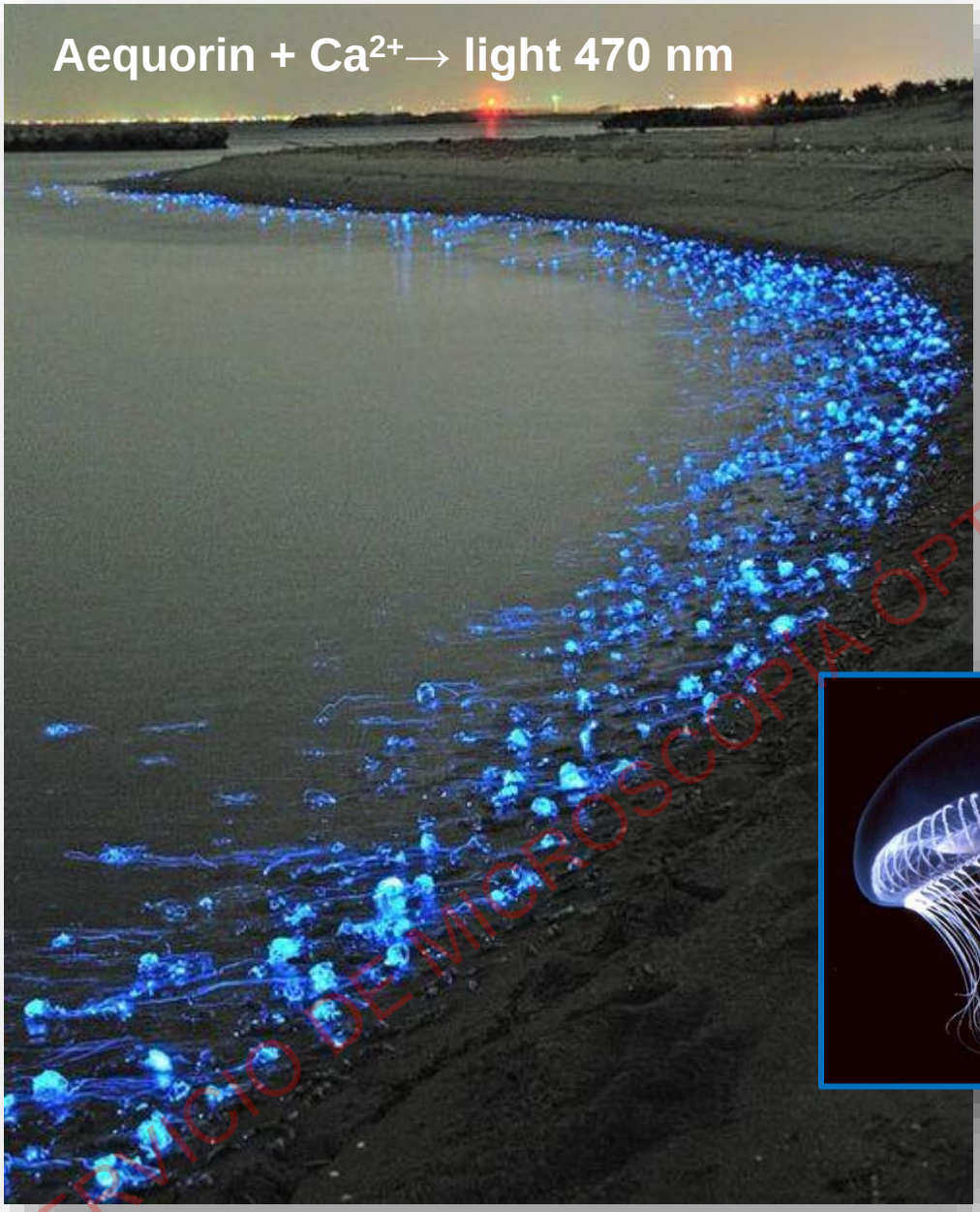


Carmen Sánchez Jiménez
Servicio de Microscopía Óptica y Confocal (SMOC)
Centro de Biología Molecular Severo Ochoa (CBMSO)

Outline

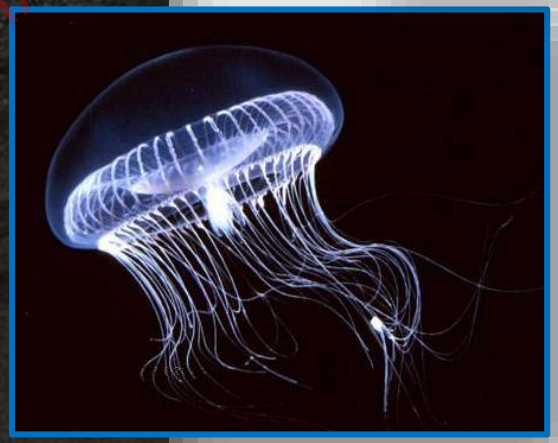
- **The discovery of fluorescent proteins (FPs). FP families**
- Advantages of using a recombinant protein
- Spectra profile and important parameters in a FP
- The color palette of FPs. Unmixing
- Dynamic events: FRET, photobleaching, photoactivation and photoconversion
- Precautions in *in vivo* experiments

Aequorea victoria



Friday Harbor (Washington)

Green Fluorescent Protein (GFP)

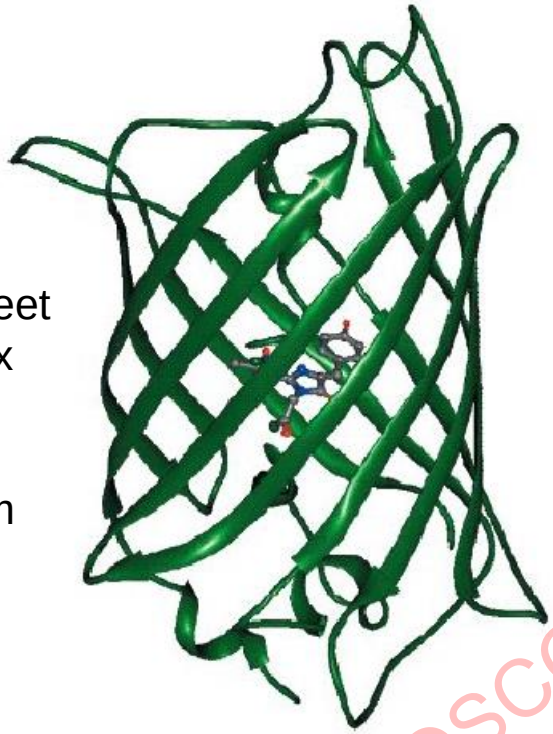


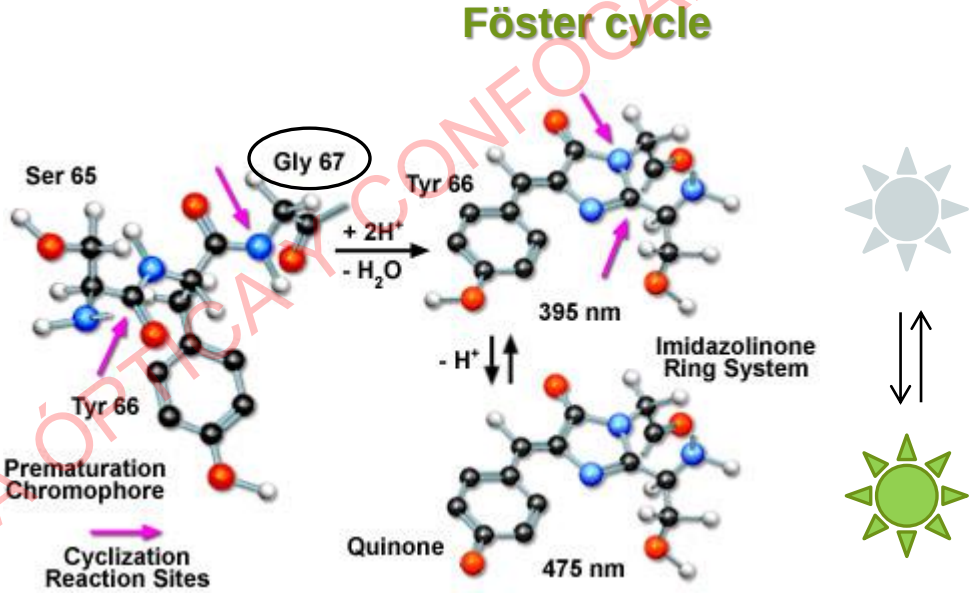
Quantum efficiency of Cypridina Luminescence, with a note on that of Aequorea .
J Cell Comp Physiol 1962 Aug;60:85-103.
Shimomura O, Johnson FH, Saiga Y et al.

Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea.
J Cell Comp Physiol 1962 Jun;59:223-39.
Shimomura O, Johnson FH, Saiga Y

wtGFP

11 β-sheet
1 α-helix
238 aa
27 kDa
4 x 3 nm





wtGFP → EGFP (enhanced)

S65T, F64L and a Kozak translation initiation site (A/GCCAT) to enhance its expression in mammalian cells (brightness, maturation at 37ºC, excitation wavelength…)

The Nobel Prize in Chemistry 2008

Photo: U. Montan

Osamu Shimomura

Prize share: 1/3

Photo: U. Montan

Martin Chalfie

Prize share: 1/3

Photo: U. Montan

Roger Y. Tsien

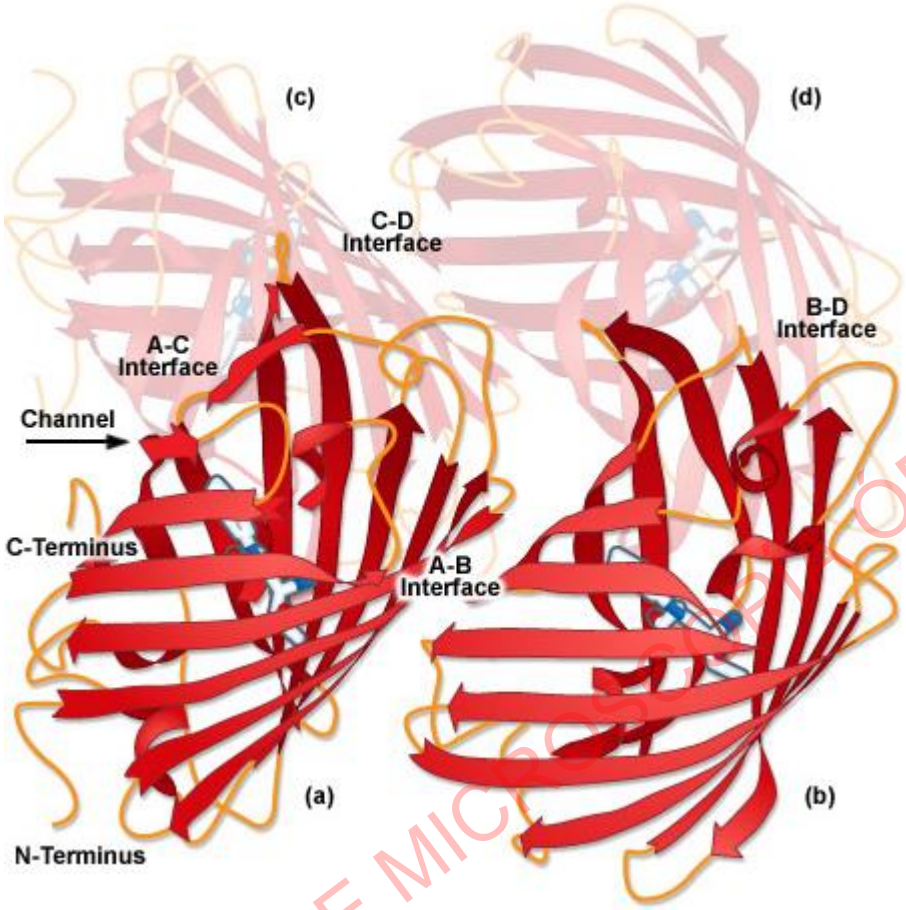
Prize share: 1/3

The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien *"for the discovery and development of the green fluorescent protein, GFP"*.

Douglas Prasher

Reported the cloning and nucleotide sequence of wtGFP

DsRed. Inherent oligomerization

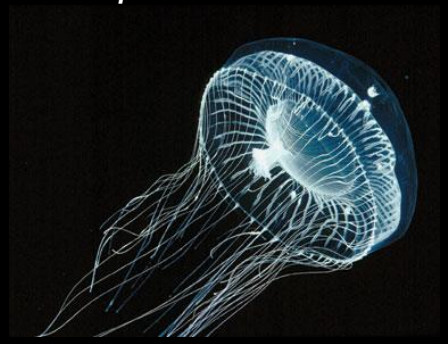


- Larger than monomeric proteins
- Does oligomerization affect protein localization?
- Reporter for whole cell labeling or labeling of subcellular compartment. Worse as a tag for proteins.

⇨ Monomeric red proteins by mutagenesis

Hydrozoa

Aequorea victoria



Green (GFP) → EGFP → Emerald

PA- GFP

Blue (BFP) → Azurite, EBFP2

Cyan (CFP) → Cerulean

Yellow (YFP) → Venus, Citrine

Sapphire

Anthozoa

Discosoma striata

DsRed → mRFP1, tdTomato → mBanana, mOrange, mCherry, mStrawberry, mTangerine, PA-mRFP1

Others *Anthozoa*

→ Kaede, Dronpa …

https://www.fpbase.org/lineage/

Sometimes the same antibody can recognize other family members

Leptocardii

Branchiostoma Lanceolatum

lanYFP
lanRFP

→ **mNeonGreen**

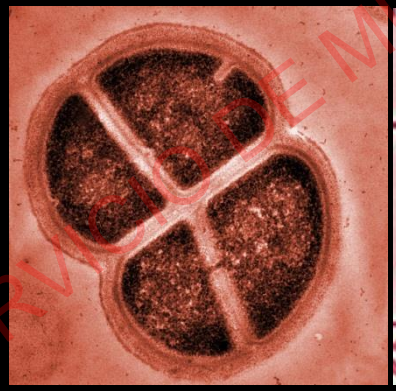
Actinopterygii

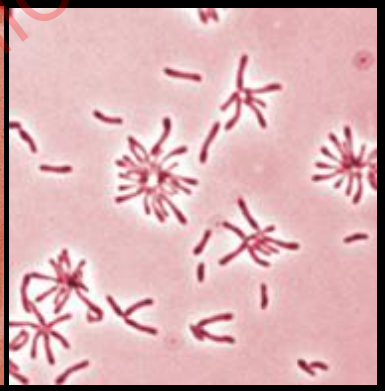
Anguilla japonica

UnaG (hypoxia)
(bilrubin-inducible)

more and more…

Bacteria





iRFPs
(biliverdin-inducible)

https://www.fpbase.org/lineage/

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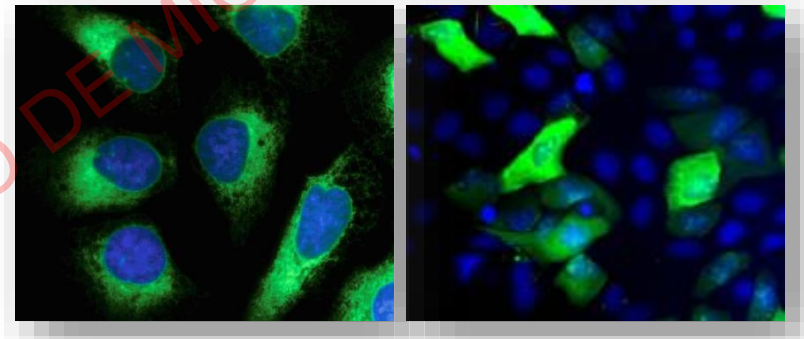
Advantages of using a recombinant fluorescent protein

- Easiest *in vivo* experiments
- No manipulation for staining

Advantages of using a stable cell line/organism

- Homogeneity (*vs*. transient transfection)
  - Intensity (not always)
  - All your cells/organisms are positive stained



Stable vs. transient transfection

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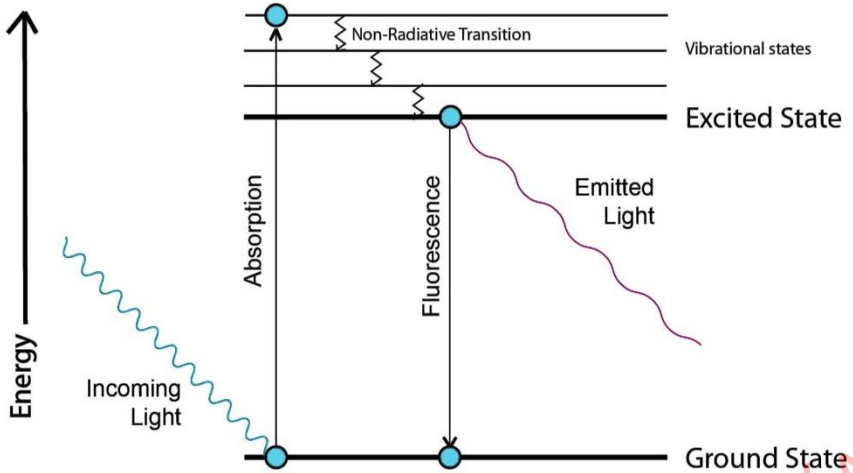
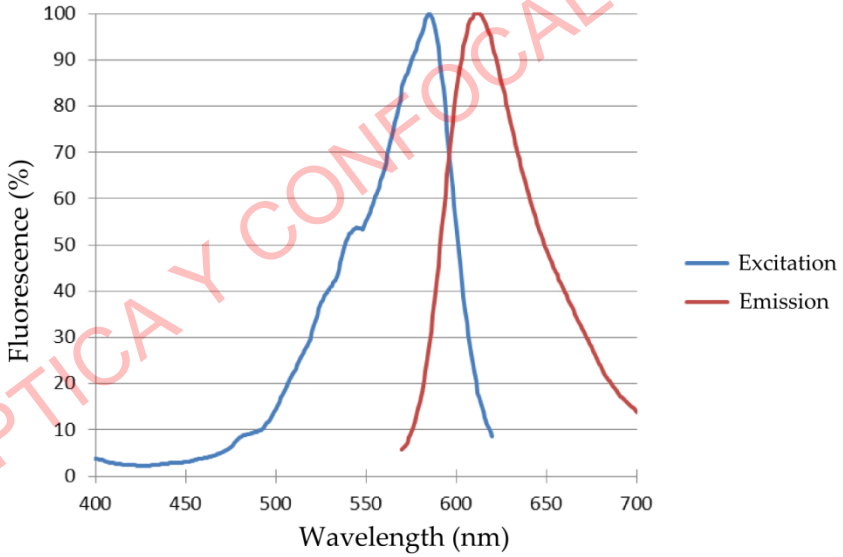
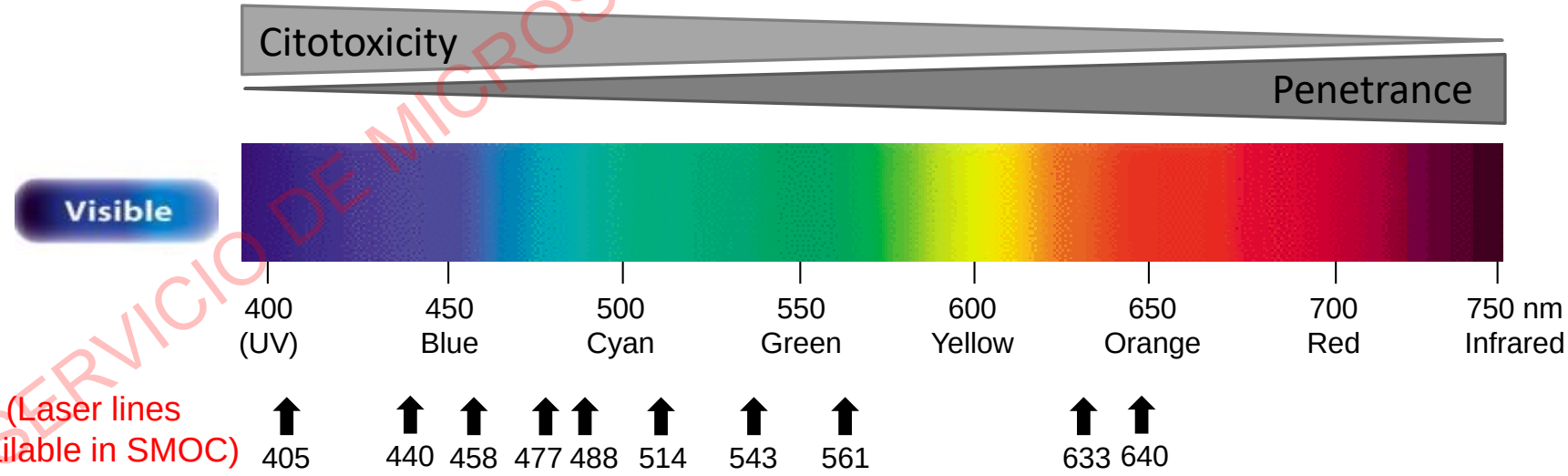
Fluorescent protein spectra profile

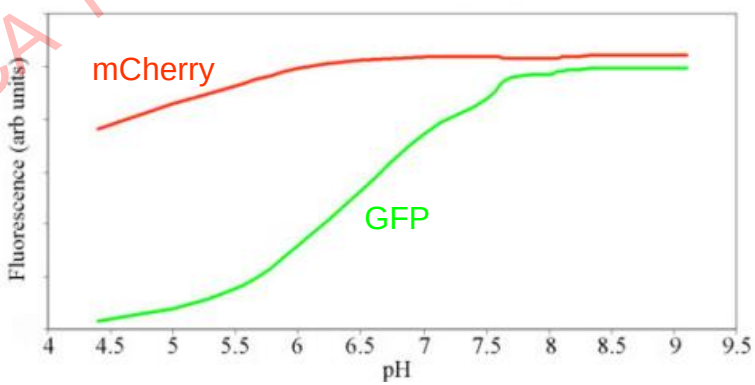






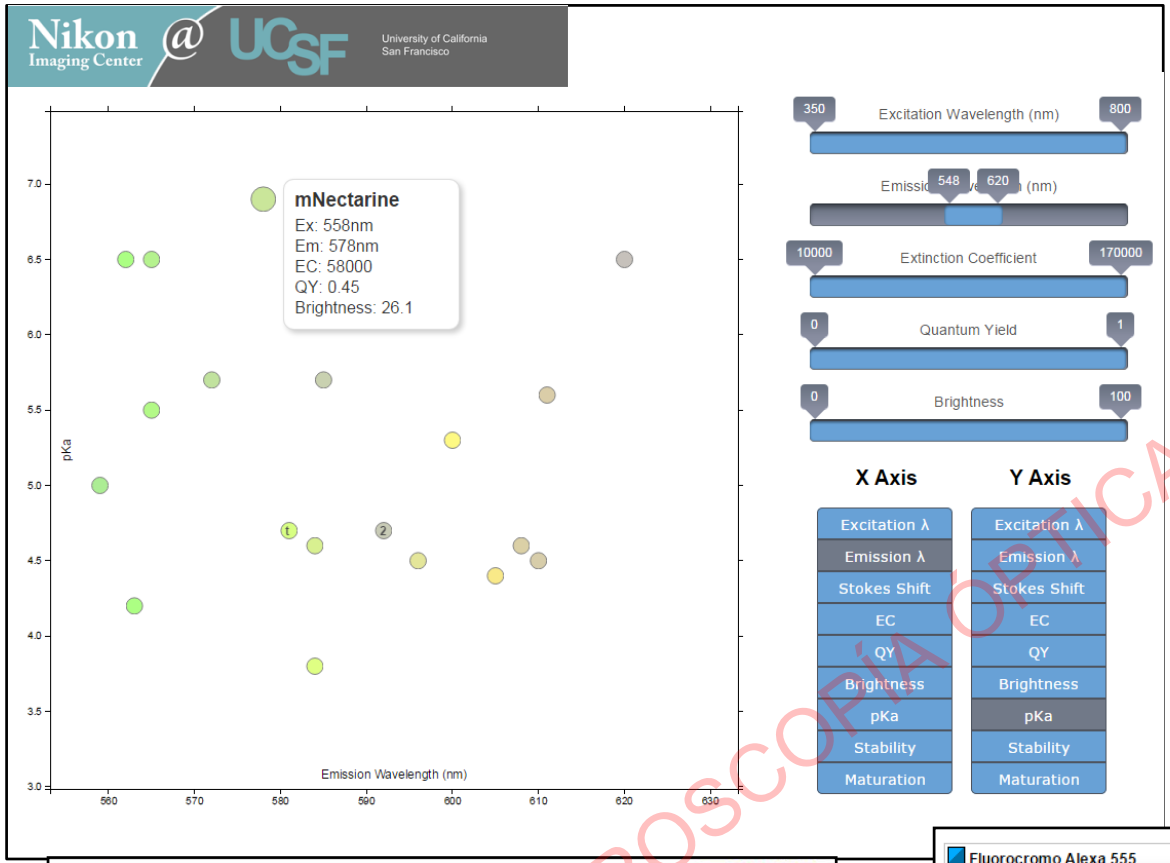
Parameters to consider when choosing a fluorescent protein

- **Brightness:** QY x EC
  - **Molar extinction coefficient (EC):** measurement of how strongly a chemical species absorbs light at a given wavelength.
  - **Quantum yield (QY)** $= \frac{photons\ emitted}{photons\ absorbed}$
- ***In vivo* structure (monomer, dimer, tetramer…)**
- **Low photobleaching rate**



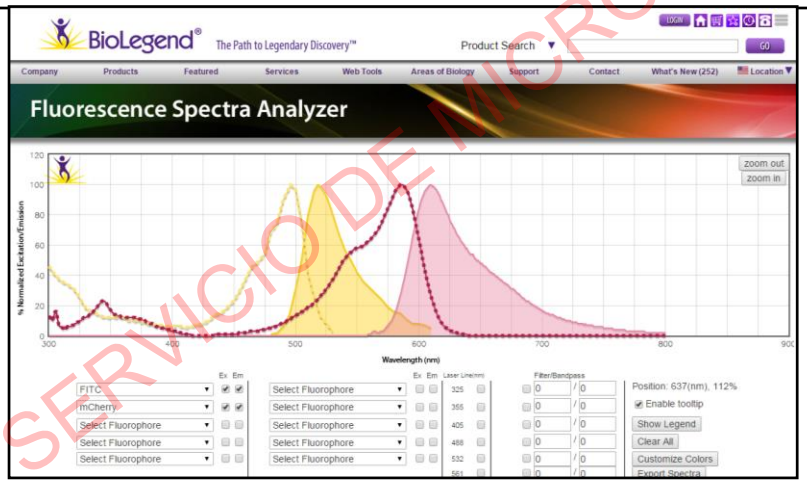
- **Toxicity, autofluorescence and penetrance:** the closer to far red, the less toxicity. Also, far red proteins avoid the natural green auto-fluorescence and they penetrate better into the sample.
- **Full absorption and emission spectrum:** not only maximum peaks, to avoid cross-talk if you need to image more than one fluorophore .
- **Others (depending on the experiment… e.g. pH fluctuations):** can alter fluorescence intensity.

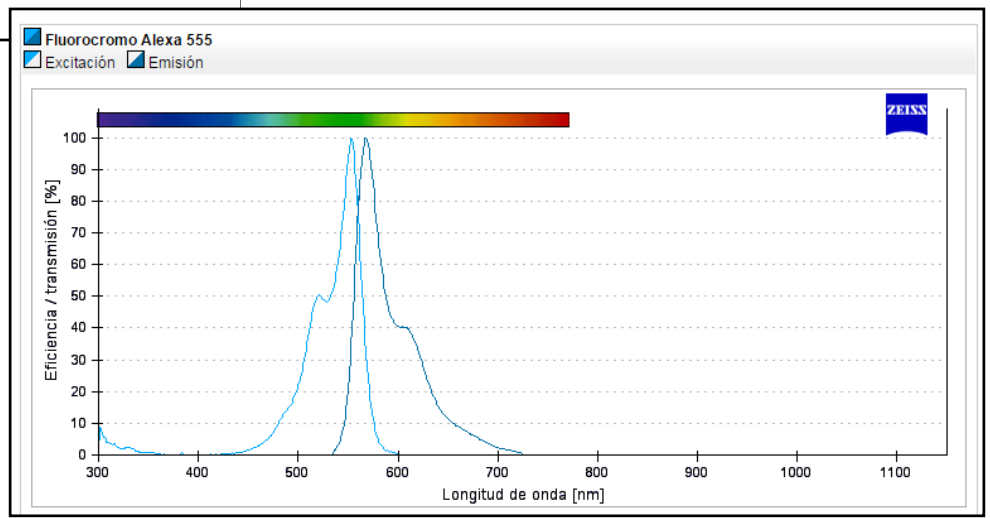
Spectra viewers for fluorochromes and FPs



Web SMOC → Reactivos → Excitación/Emisión fluoróforos y proteínas fluorescentes

Web SMOC → Reagents → Excitation/Emission spectrum for fluorophores and fluorescent proteins





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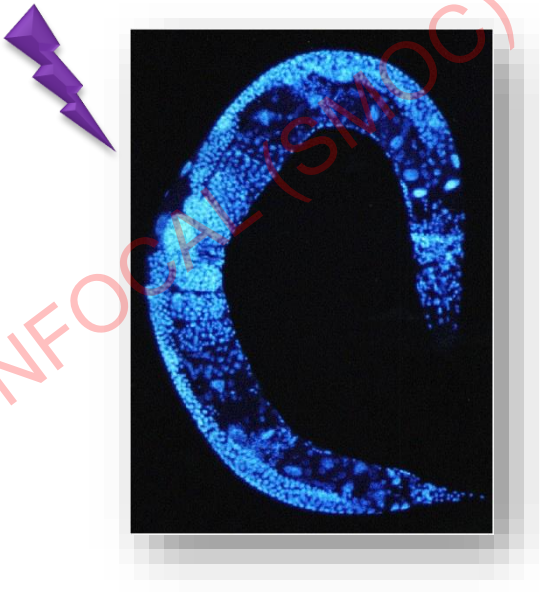
FLUORESCENT PROTEINS

The perfect protein doesn't exist

(brightness, photostability, maturation speed, pH stability…)

https://www.fpbase.org/

Blue fluorescent proteins



mTagBFP2

Ex/Em: 402/457 nm | Brightness = 33 | Monomer | pKa = 2.7

EBFP2

Ex/Em: 383/448 nm | Brightness = 18 | Monomer | pKa = 5.3

Blue proteins shoud be avoided for *in vivo* experiments (low photostability and high phototoxicity).

Also, 405 nm laser line could photoconvert some proteins

Others BFPs: https://www.fpbase.org/table/

All BFPs can be imaged with DAPI filter sets (in widefield) and excited with 405 nm laser line (in confocal)

Cyan fluorescent proteins

ECFP

Ex/Em : 439/476 nm Brightness = 13 Monomer pKa = 4.7

mTurquoise2

Ex/Em : 434/474 nm Brightness = 28 Monomer pKa = 3.1

mCerulean3 (↑↑ photostable)

Ex/Em: 433/475 nm Brightness = 35 Monomer pKa = 3.2

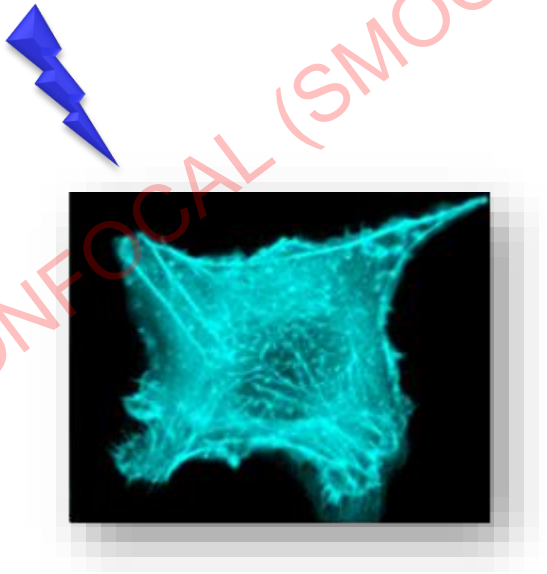
Aquamarine

Ex/Em: 430/474 nm Brightness = 23 Monomer pKa = 3.3

mTFP1 (attention to the filter set configuration)

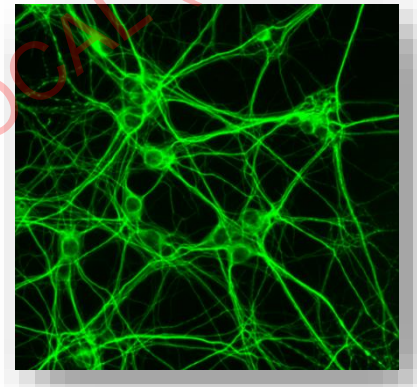
Ex/Em: 462/492 nm Brightness = 54 Monomer pKa = 4.3

Others CFPs: https://www.fpbase.org/table/



All CFPs can be imaged with CFP filter sets (in widefield) and excited with 440/458 nm laser line (in confocal).

Green fluorescent proteins



EGFP

Ex/Em : 488/507 nm Brightness = 34 Weak dimer pKa = 6

mEGFP

Ex/Em : 488/507 nm Brightness = 34 Monomer pKa = 6

mNeonGreen

Ex/Em : 506/517 nm Brightness = 93 Monomer pKa = 5.7

TagGFP2

Ex/Em : 483/506 nm Brightness = 34 Monomer pKa = 4.7

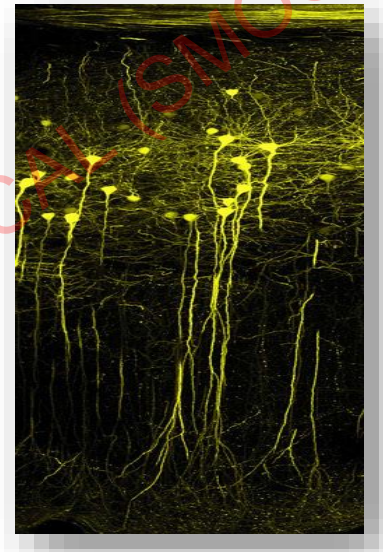
Superfolder GFP

Ex/Em : 485/510 nm Brightness = 54 Weak dimer pKa = 5.5

Others GFPs: https://www.fpbase.org/table/

All GFPs can be imaged with GFP/FITC filter sets (in widefield) and excited with 488 nm laser line (in confocal)

Yellow fluorescent proteins



EYFP

Ex/Em : 513/527 nm Brightness = 51 Weak dimer pKa = 6.9

mEYFP

Ex/Em : 513/527 nm Brightness = 51 Monomer pKa = 6.9

mVenus

Ex/Em : 515/527 nm Brightness = 66 Monomer pKa = 5.5

SYFP2

Ex/Em: 515/527 nm Brightness = 69 Monomer pKa = 6

mCitrine

Ex/Em: 516/529 nm Brightness = 70 Monomer pKa = 5.7

Others YFPs: https://www.fpbase.org/table/

All YFPs can be imaged with GFP/YFP filter sets (in widefield) and excited with 488/514 nm laser line (in confocal)

Orange fluorescent proteins

DsRed

Ex/Em: 558/583 nm	Brightness = 49	Tetramer	pKa = 4.5

mKOk

Ex/Em: 551/563 nm	Brightness = 64	Monomer	pKa = 4.2

tdTomato

Ex/Em: 554/581 nm	Brightness = 95	Dimer-tandem	pKa = 4.7

TagRFP-T

Ex/Em: 555/584 nm	Brightness = 33	Monomer	pKa = 4.6

Others Orange-FPs: https://www.fpbase.org/table/

All Orange-FPs can be imaged with Rhodamine filter sets (in widefield) and excited with 543 or 561 nm laser lines (in confocal)

Red fluorescent proteins

mCherry

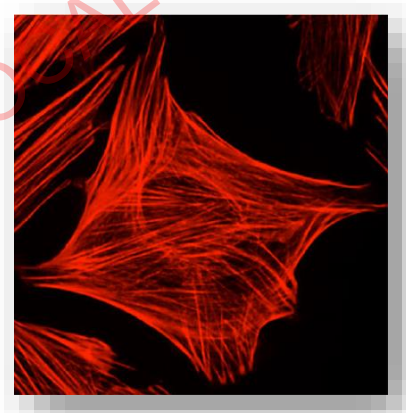
Ex/Em : 587/610 nm Brightness = 16 Monomer pKa = 4.5

mRuby

Ex/Em : 558/605 nm Brightness = 39 Monomer pKa = 4.4

mRuby2

Ex/Em : 559/600 nm Brightness = 43 Monomer pKa = 5.3



Others RFPs: https://www.fpbase.org/table/

All RFPs can be imaged with TexasRed-DsRed filter sets (in widefield) and excited with 543 or 561 nm laser lines (in confocal)

Far-red fluorescent proteins

mKate2

Ex/Em : 588/633 nm Brightness = 25 Monomer pKa = 5.4

mCardinal (↑↑ photostable)

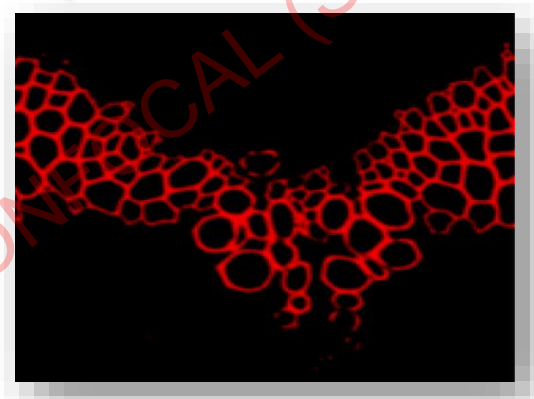
Ex/Em : 604/659 nm Brightness = 17 Monomer pKa = 5.3

Katushka

Ex/Em : 588/635 nm Brightness = 22 Dimer pKa = 5.5

mNeptune (↑↑ photostable)

Ex/Em : 600/650 nm Brightness = 13 Monomer pKa = 5.4



Others far-RFPs: https://www.fpbase.org/table/

All Far-RFPs can be imaged with Cy5 filter sets (in widefield) and excited with 633/640 nm (or 561nm) laser line (in confocal).

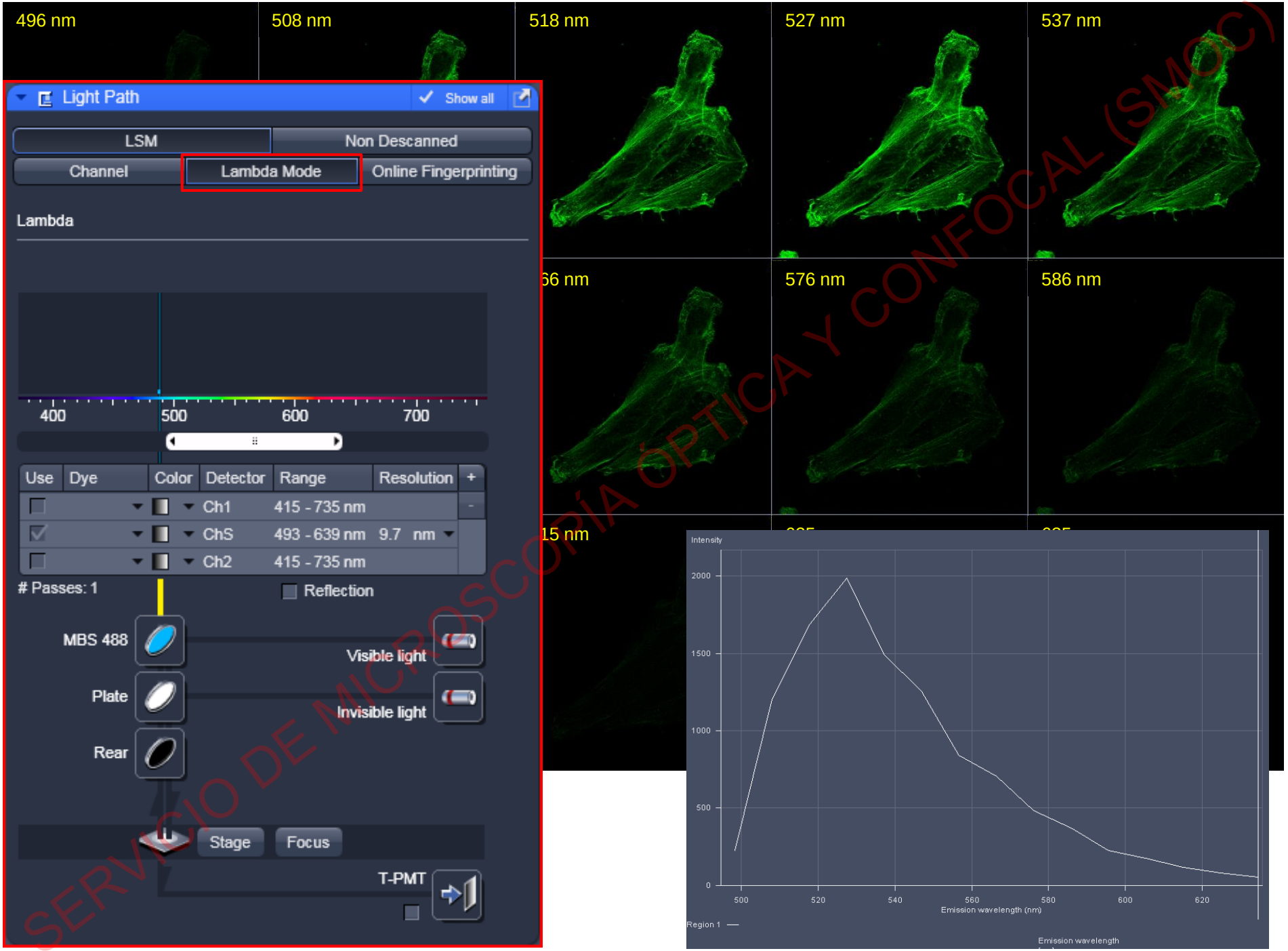
Human eyes hardly detect far-red signal, but detectors/cameras do

Our advice!

Check if your protein is what it's supposed to be

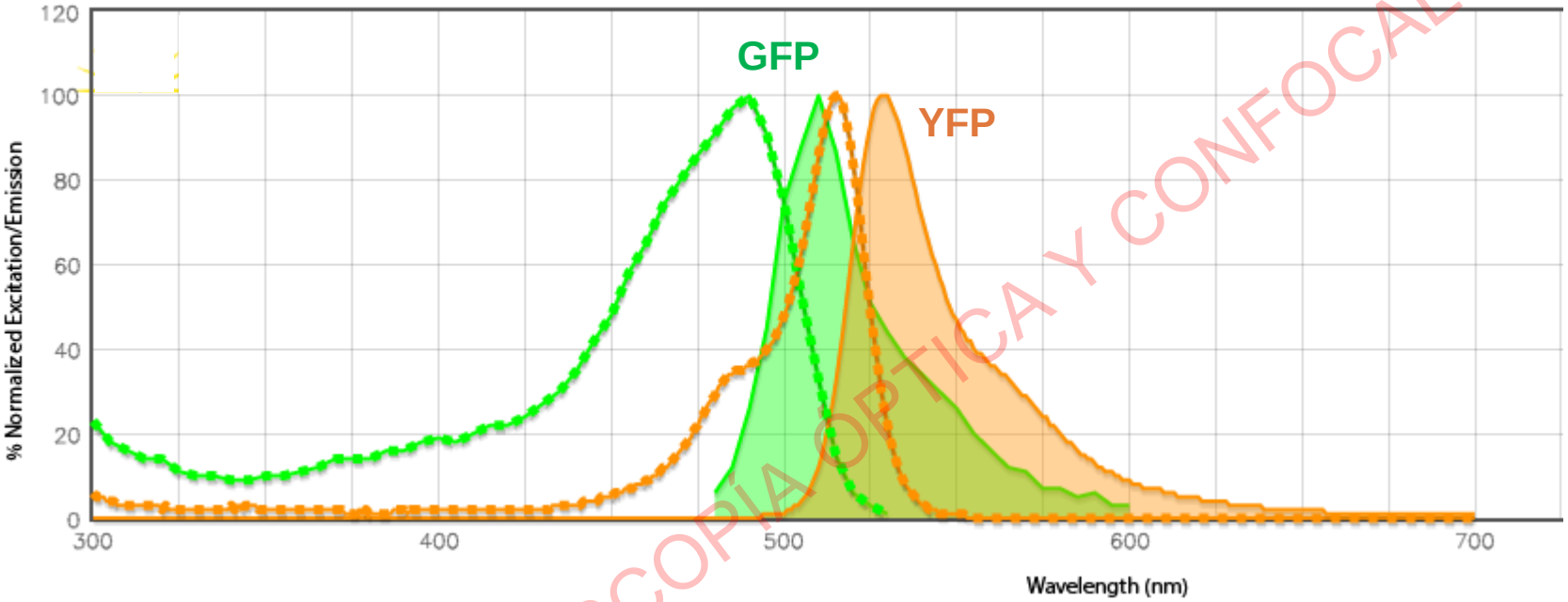
Confocal systems provided with spectral detectors in SMOC:

LSM710 inverted
LSM710 upright
Nikon A1R+

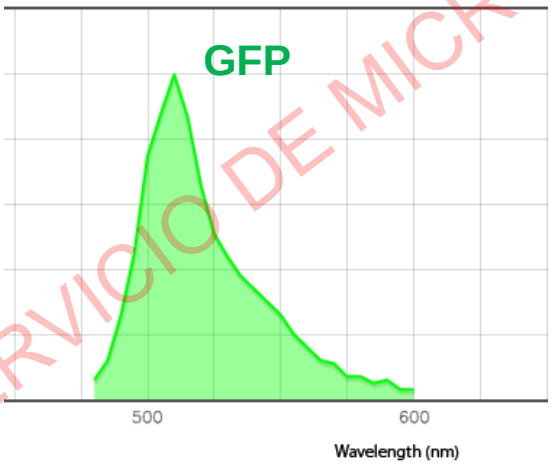


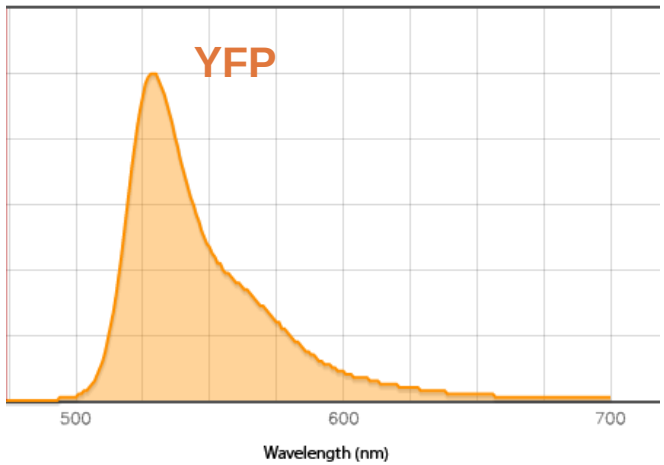
Spectral separation

If two or more fluorophores have a closer emission spectra:

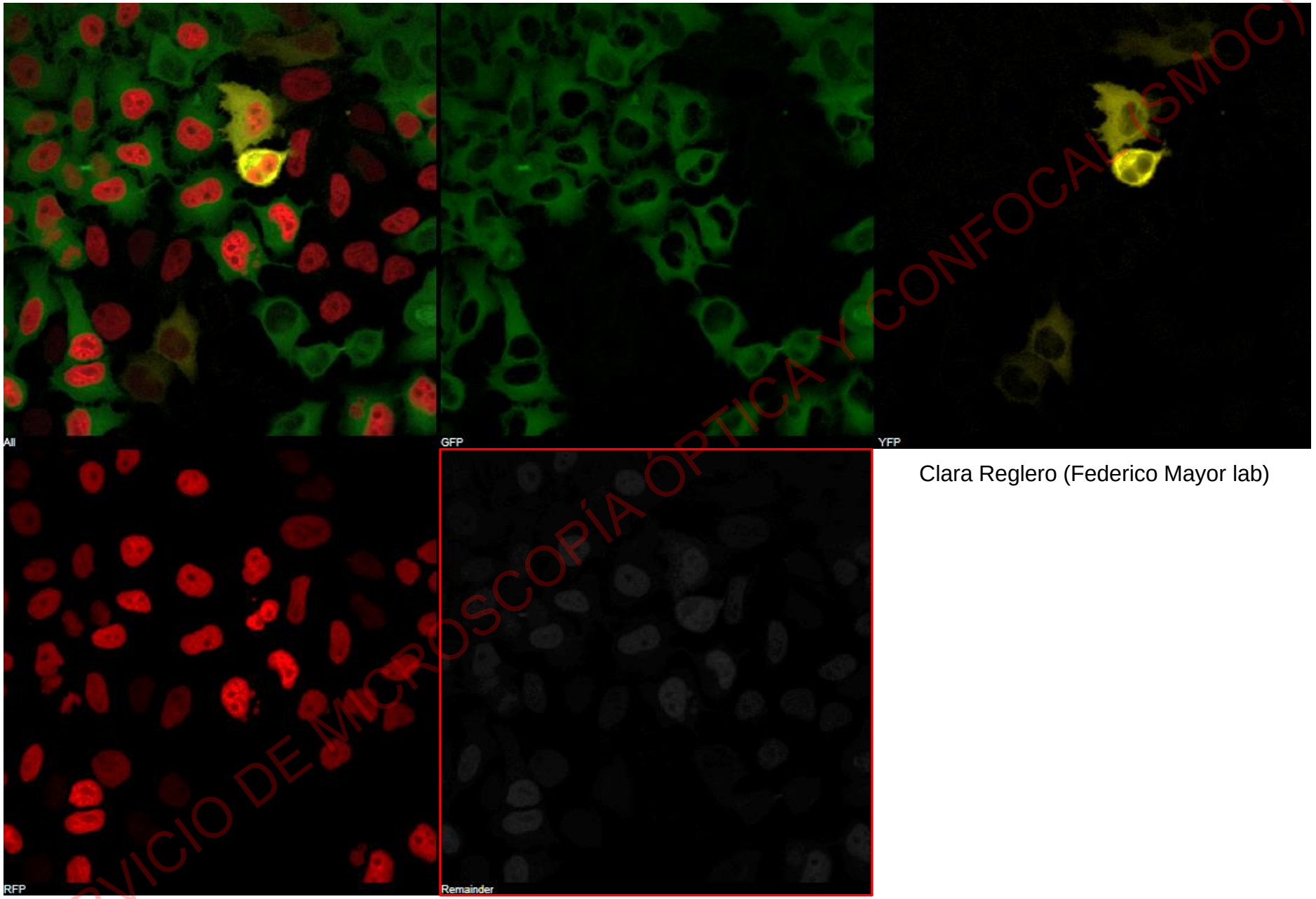


Characterization of different emission spectra:





Unmixing



Clara Reglero (Federico Mayor lab)

It is not the best option if you need to quantify fluorescence (look at the **remainder channel**)

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FRET

Föster Resonance Energy Transfer

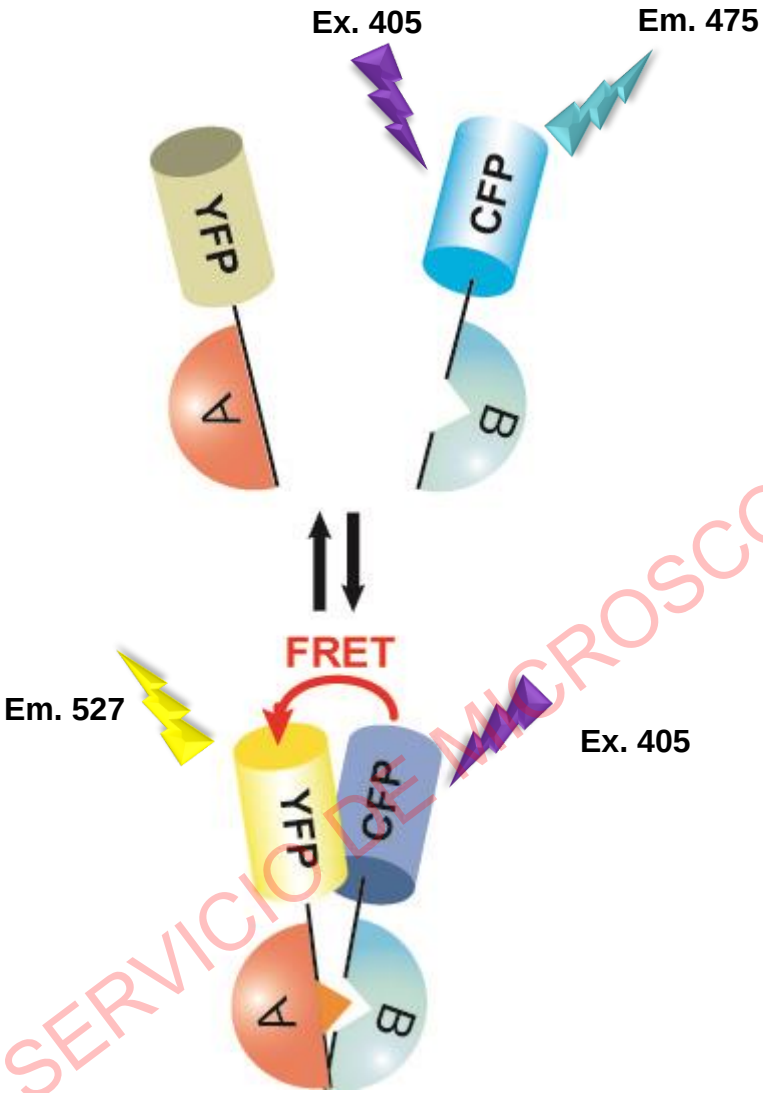
Protein-protein interaction

Conformational change

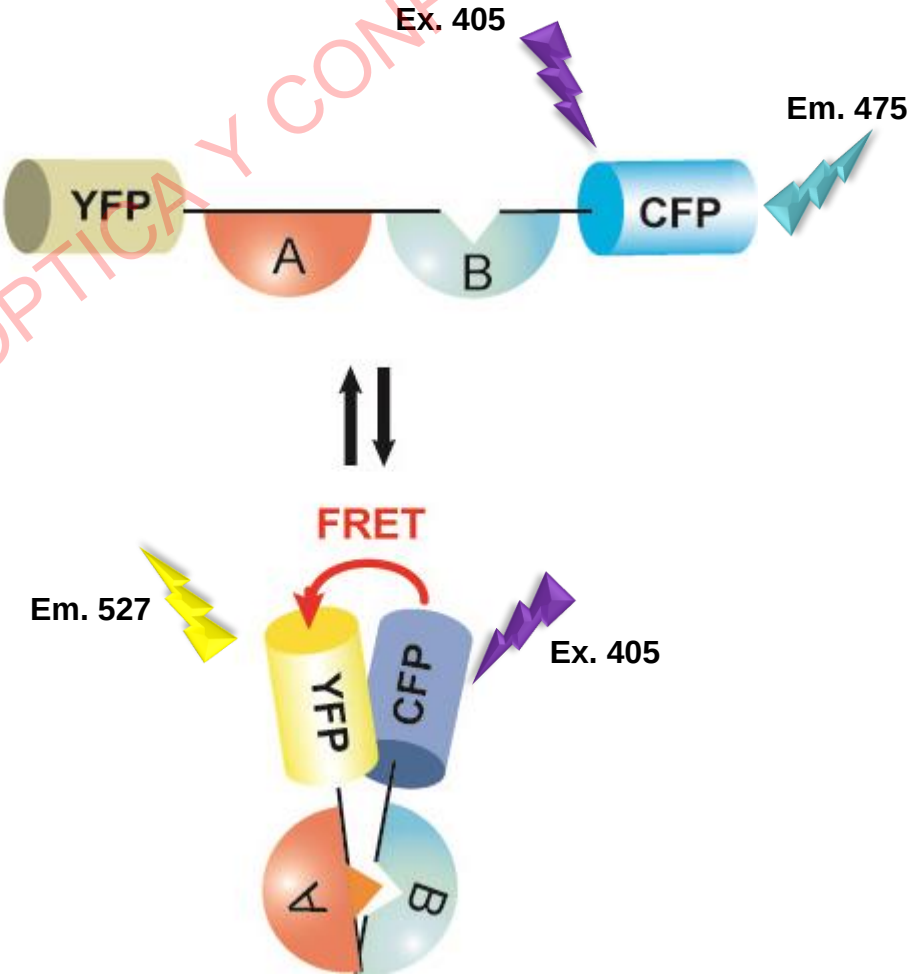
Proteolysis

Föster Resonance Energy Transfer (FRET)

Intermolecular

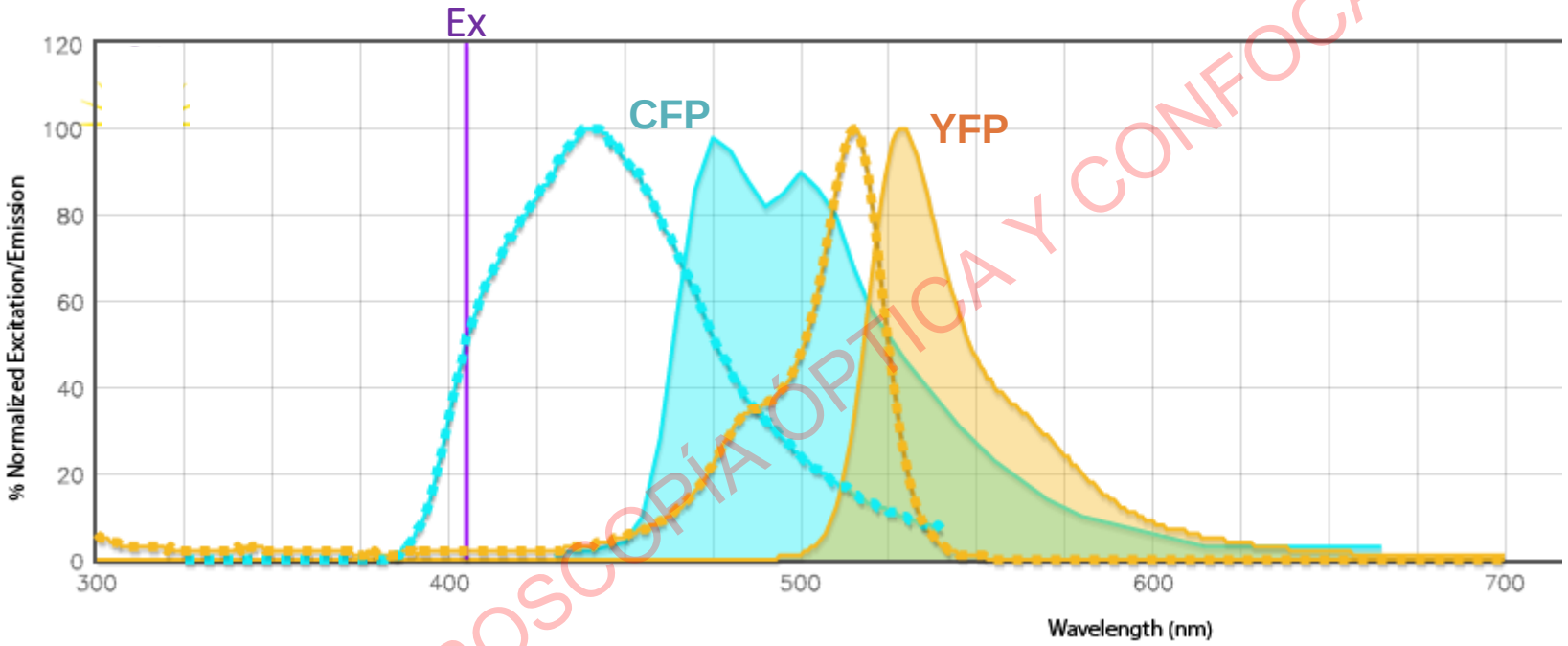


Intramolecular: conformational change or proteolysis



Föster Resonance Energy Transfer (FRET)

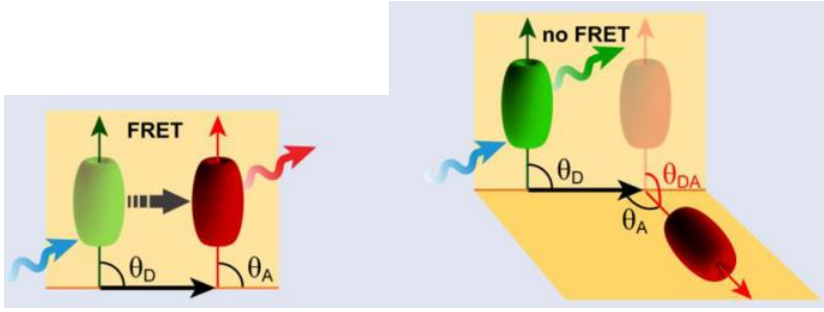
- The emission spectrum of the donor should overlap the excitation spectrum of the aceptor



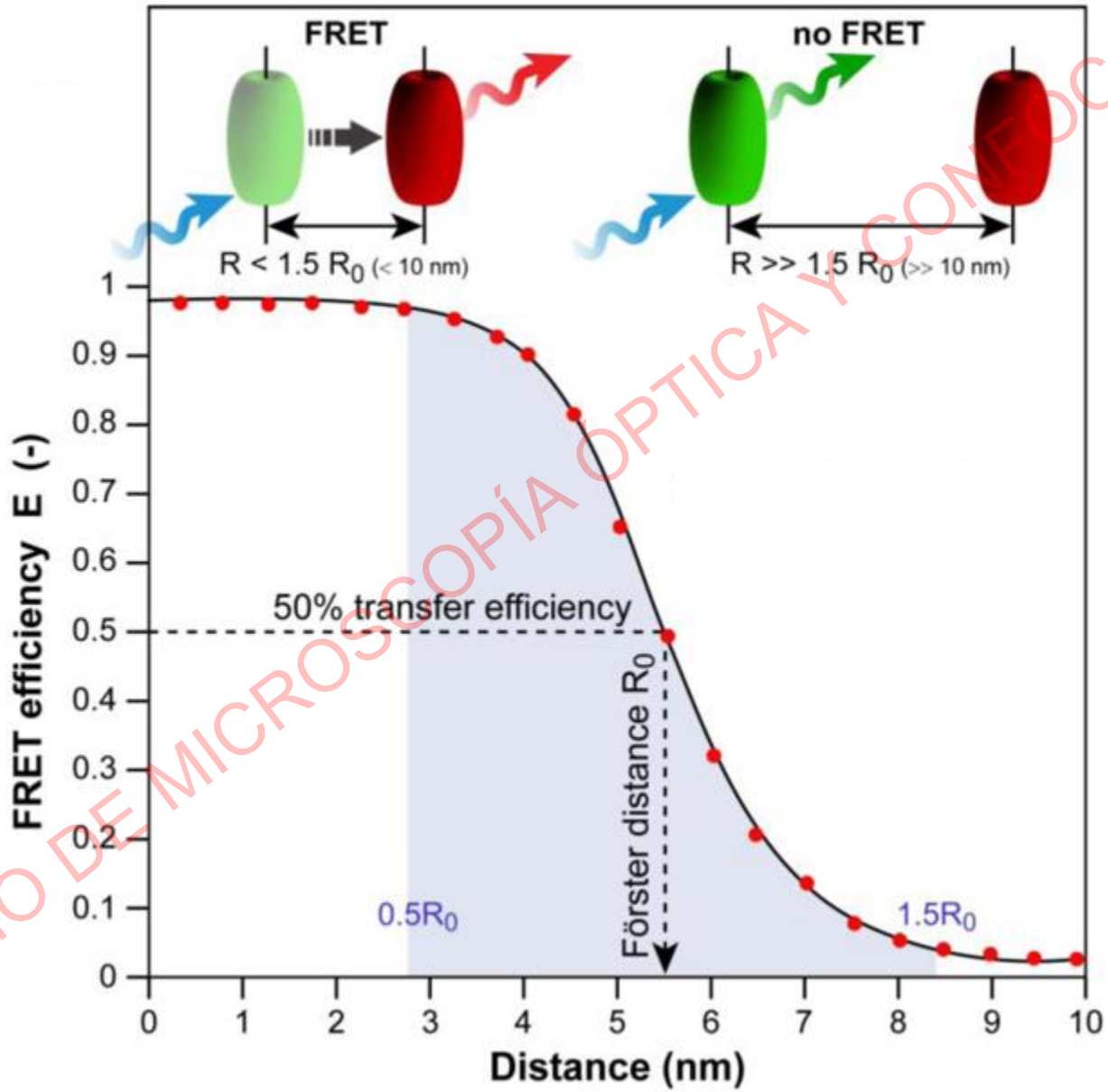
- FRET occurs if the distance between donor and aceptor is less than 10 nm

  Improves the resolution of the microscope (≈ 200 nm)

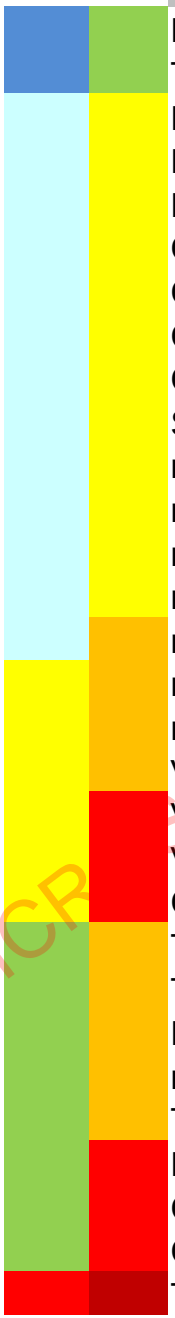
- Relative orientation is also important



Föster Resonance Energy Transfer (FRET)



FRET pairs

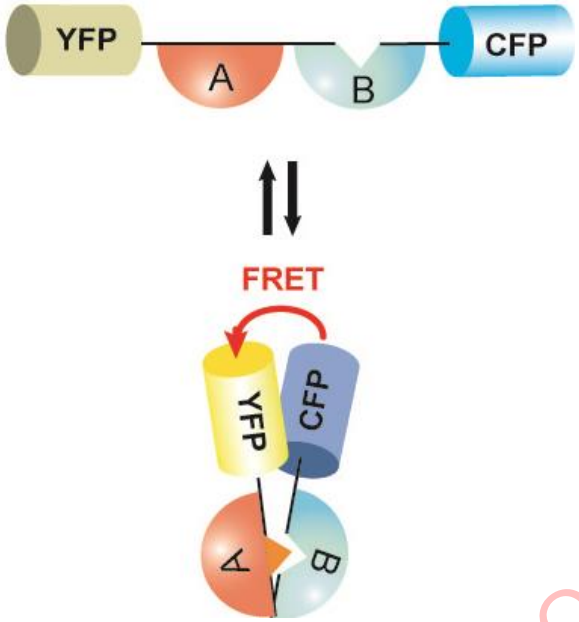


Donor	Aceptor	QY_D	EC_A	R0 (nm)
EBFP2	EGFP	0.56	57500	4.8
TagBFP	TagGFP2	0.63	56500	5.3
ECFP	Citrine	0.36	77000	4.8
ECFP	EYFP	0.40	83400	4.9
ECFP	Venus	0.36	92000	5.0
Cerulean	Citrine	0.49	77000	5.4
Cerulean	Venus	0.49	92000	5.2
Cerulean3	Citrine	0,80	77000	
CyPet	Ypet	0.51	104000	5.1
SECFP	SEYFP	0.58	101000	5.4
mTurquoise2	Citrine	0,93	77000	
mTurquoise1	SEYFP	0.84	101000	5.8
mTurquoise2	SEYFP	0.93	101000	5.9
mTFP1	Citrine	0.85	77000	5.7
mTFP1	mOrange	0.85	71000	5.7
mAmetrine	TagRFP	0.58	100000	-
mAmetrine	tdTomato	0.58	138000	-
Venus	tdTomato	0.57	138000	5.9
Venus	mCherry	0.57	72000	5.7
Venus	mPlum	0.57	41000	5.2
Citrine	mKate2	0.76	63000	5.8
TagGFP	TagRFP	0.59	100000	5.7
T-Sapphire	DsRed	0.60	43800	-
MiCy	mKO	0.90	51600	5.3
mUKG	mKO	0.72	51600	-
T-Sapphire	tdTomato	0.60	138000	-
EGFP	mCherry	0.60	72000	5.4
Clover	mCherry	0.76	72000	5.8
Clover	mRuby2	0.76	113000	6.3
TagRFP	mPlum	0.48	41000	-

https://www.fpbase.org/fret/

Föster Resonance Energy Transfer (FRET)

Intramolecular



RATIOMETRIC:

Simultaneous acquisition:

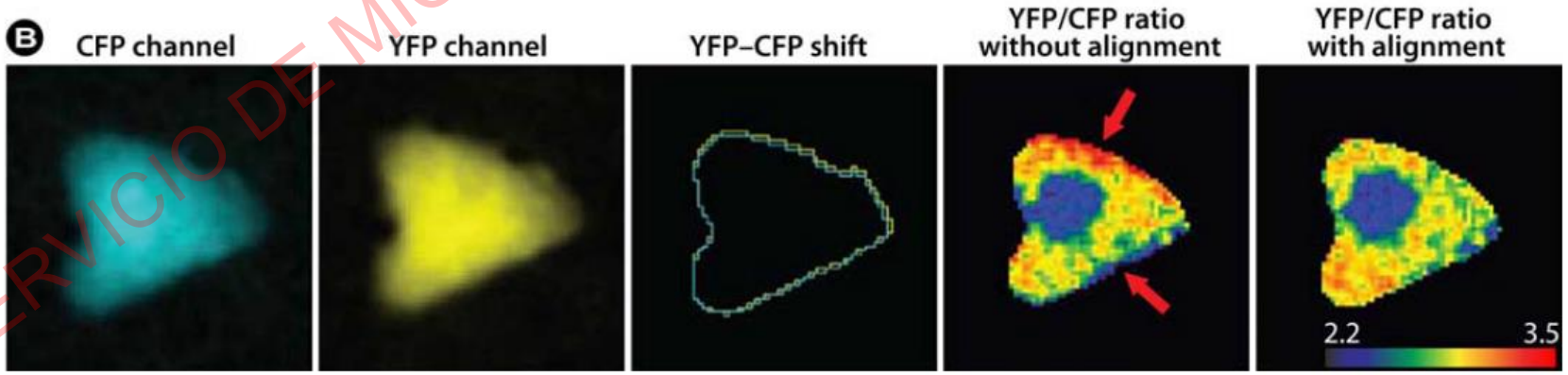
Track	Excitation	Emission
1	CFP (405 nm)	CFP (to 490) and YFP (from 530 nm)

Analysis:

$$\frac{FRET\ (YFP)}{CFP} = \text{Ratio}$$

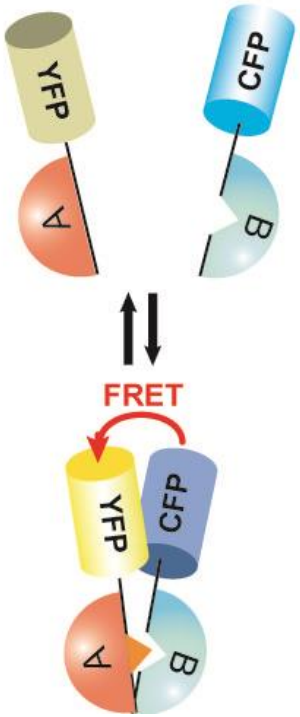
Controls:

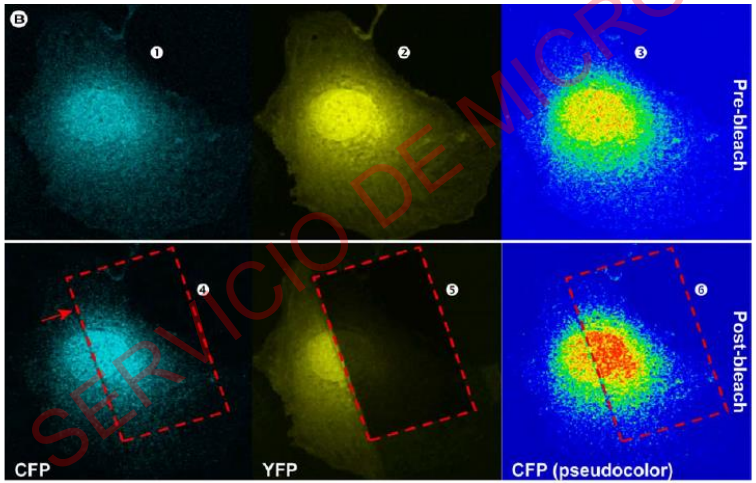
Positive FRET
Negative FRET



Föster Resonance Energy Transfer (FRET)

Intermolecular





ACCEPTOR PHOTOBLEACHING:

Sequential acquisition:

Track	Excitation	Emission
1	CFP (405 nm)	CFP (to 490)
2	YFP (514 nm)	YFP (from 530 nm)

Analysis:

$$E_{FRET} = \frac{ID - IDA}{ID}$$

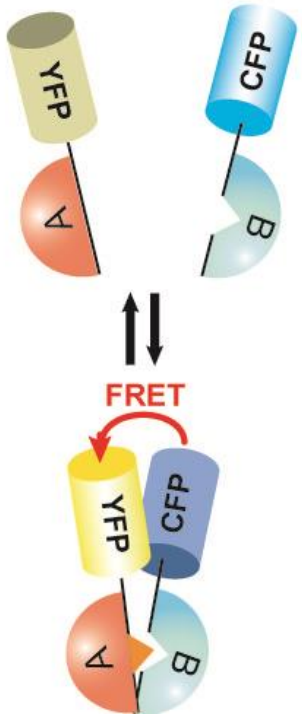
ID: donor before photobleaching of the acceptor (track 1 pre)
IDA: donor after photoblaching of the acceptor (track 1 post)

Controls to test bleaching settings:

Sample only donor
Sample only acceptor

Föster Resonance Energy Transfer (FRET)

Intermolecular



SENSITIZED EMISSION:

Sequential acquisition:

Track	Excitation	Emission
1	CFP (405 nm)	CFP (to 490)
2	YFP (514 nm)	YFP (from 530 nm)
3	CFP (405 nm)	YFP (from 530 nm)

Analysis:

$$F^c = \frac{F^b - \left(F_d^b / D_d^b\right) \times D^b - \left(F_a^b / A_a^b\right) \times A^b}{\sqrt{D^b \times A^b}}$$

Controls for calculating correction factors:

Sample only donor
Sample only acceptor
(Positive FRET)
(Negative FRET)

…it depends on the method

Fortunately, we have free software to do that!!!!

Photobleaching

Photoactivation

Photoconversion

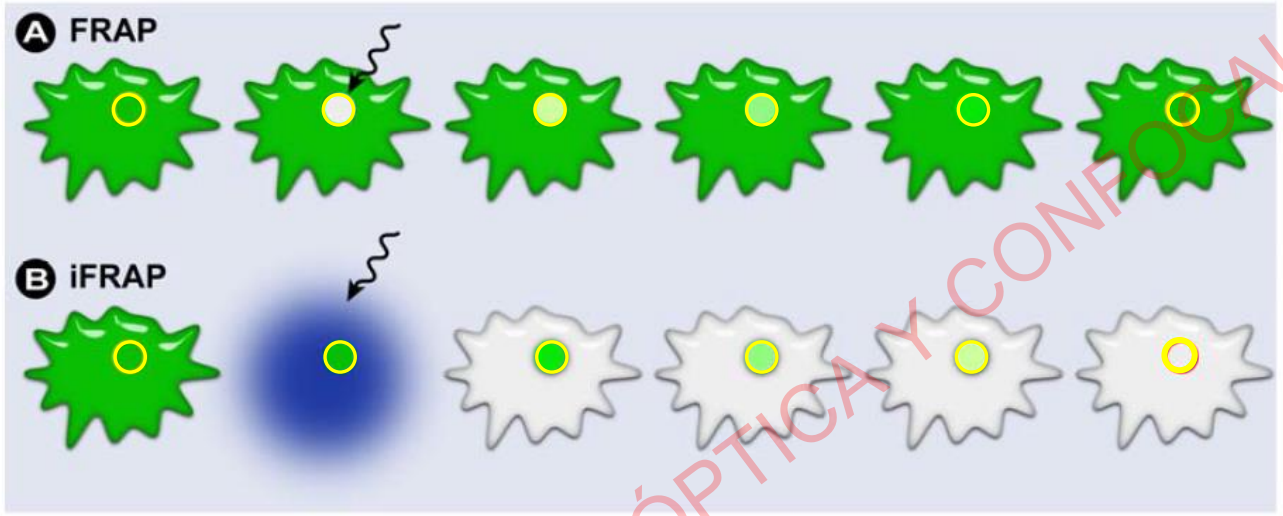
Photoswitch

Motion

Diffusion

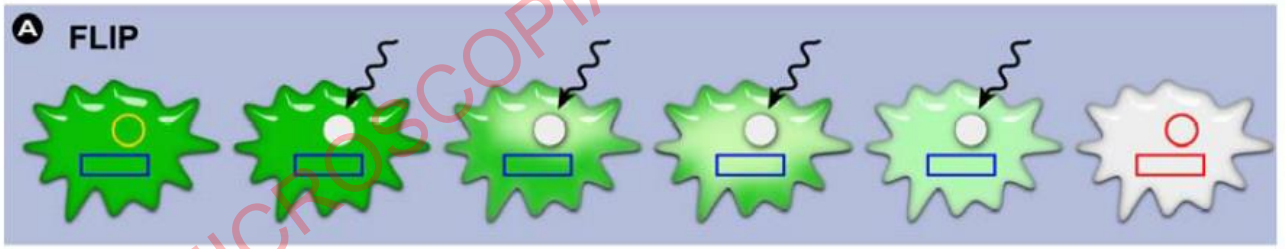
Photobleaching

Fluorescence Recovery After Photobleaching

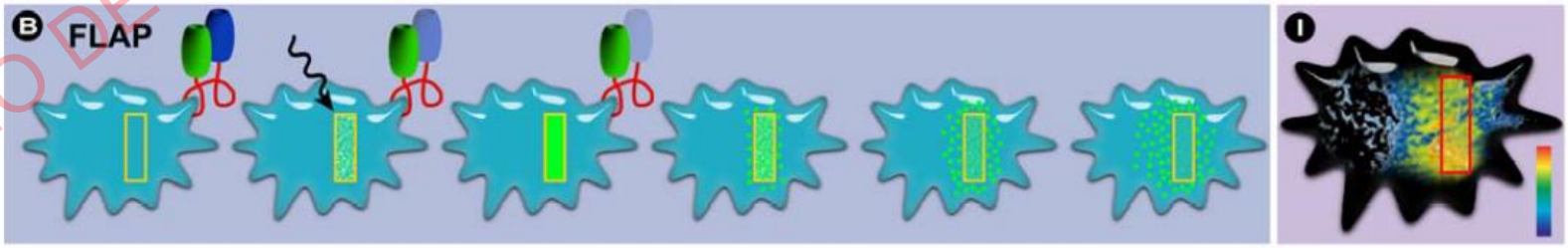


Inverse FRAP

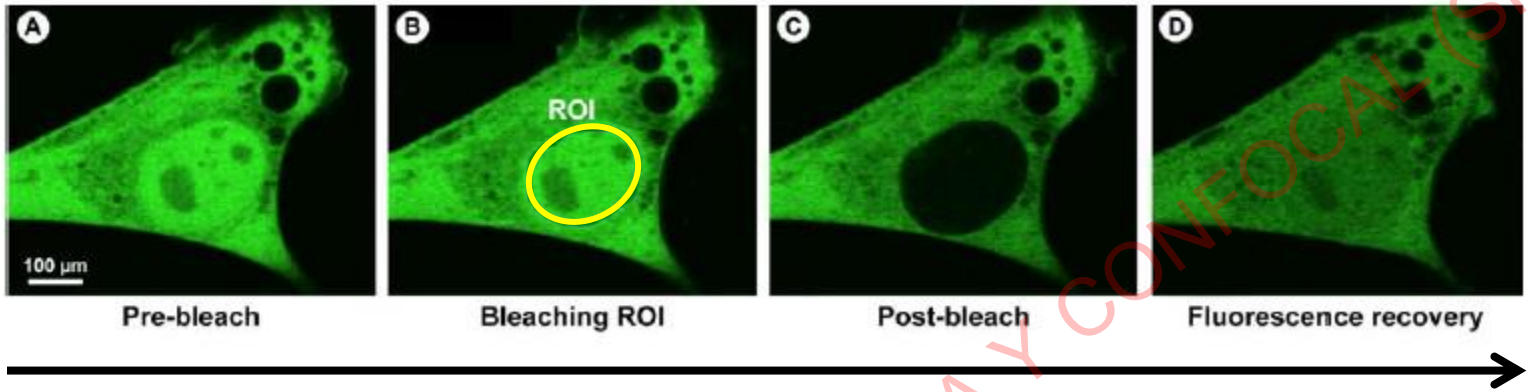
Fluorescence Loss in Photobleaching

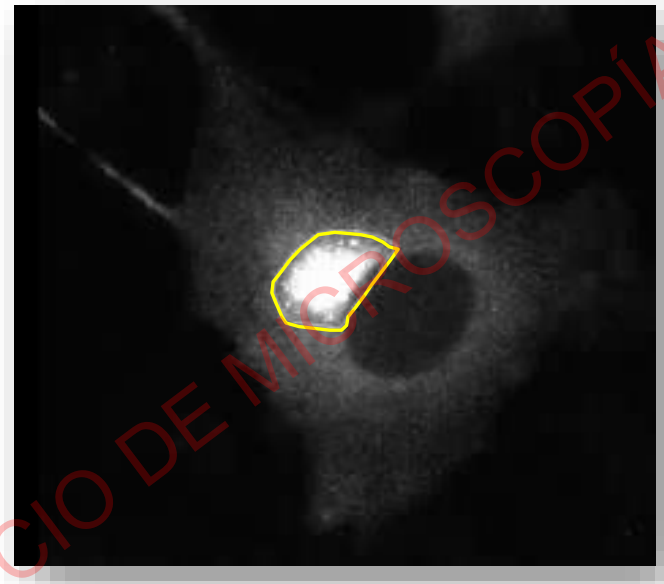


Fluorescence Location after Photobleaching



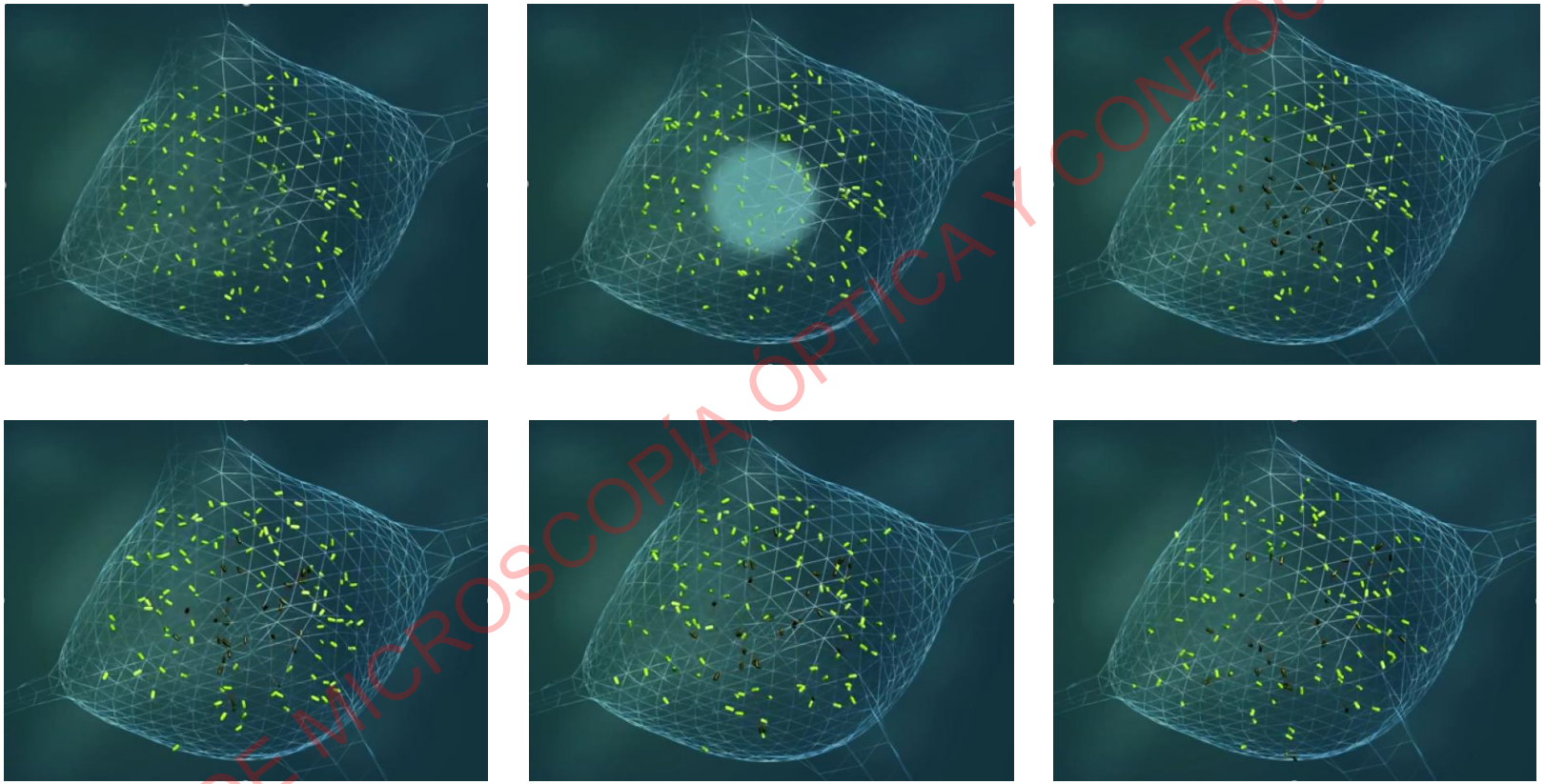
FRAP: Fluorescence Recovery After Photobleaching





Fluorescent molecules are destroyed by high light exposure (the same light that is necessary for fluorescing)
e.g. GFP is photobleached with laser line 488 at high power, usually 100%

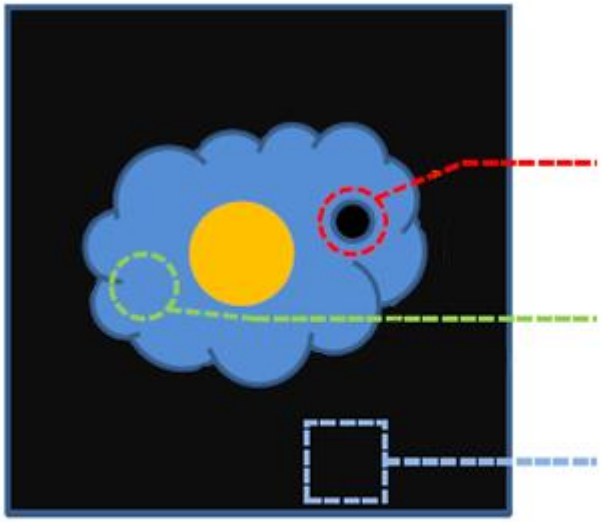
FRAP does not remove protein, only bleaches its fluorescence



Ablation with IR laser at high power (MaiTai 800 nm) is necessary for knocking down biological components

FRAP analysis

Be careful with the size of the ROI. Should be equal between samples because the final FRAP result is dependent on it



ROI for bleaching and measurement of recovery

Control ROI (for bleaching correction). Better in a neighbouring cell.

Background ROI (for background correction)

Analysis:

1- Alignment algorithm

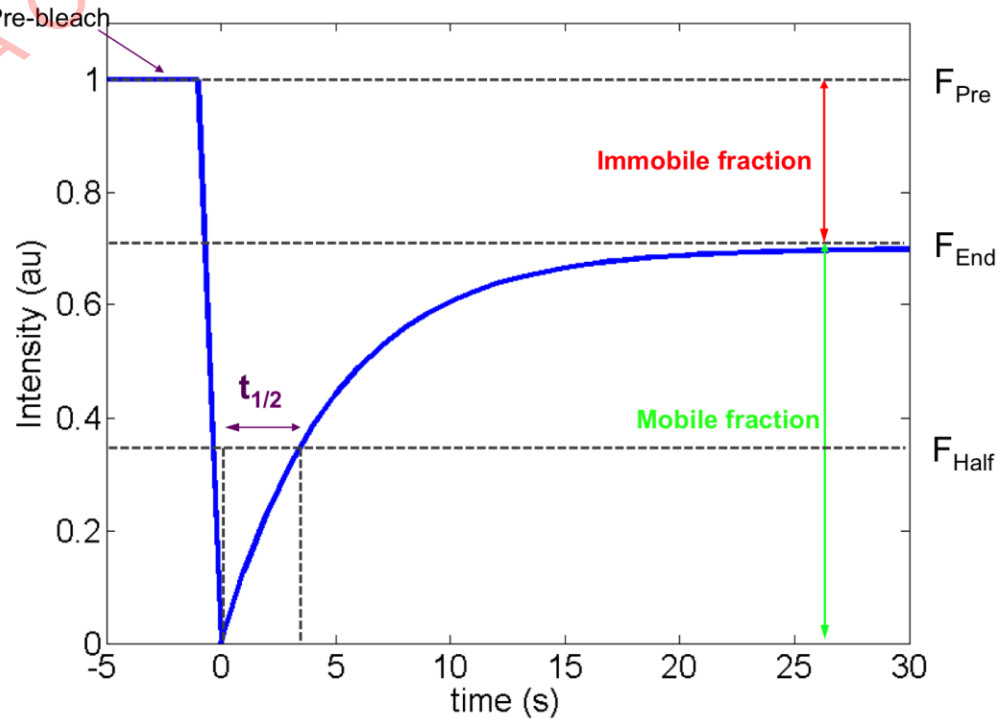
2- Background correction and

3- Bleaching correction

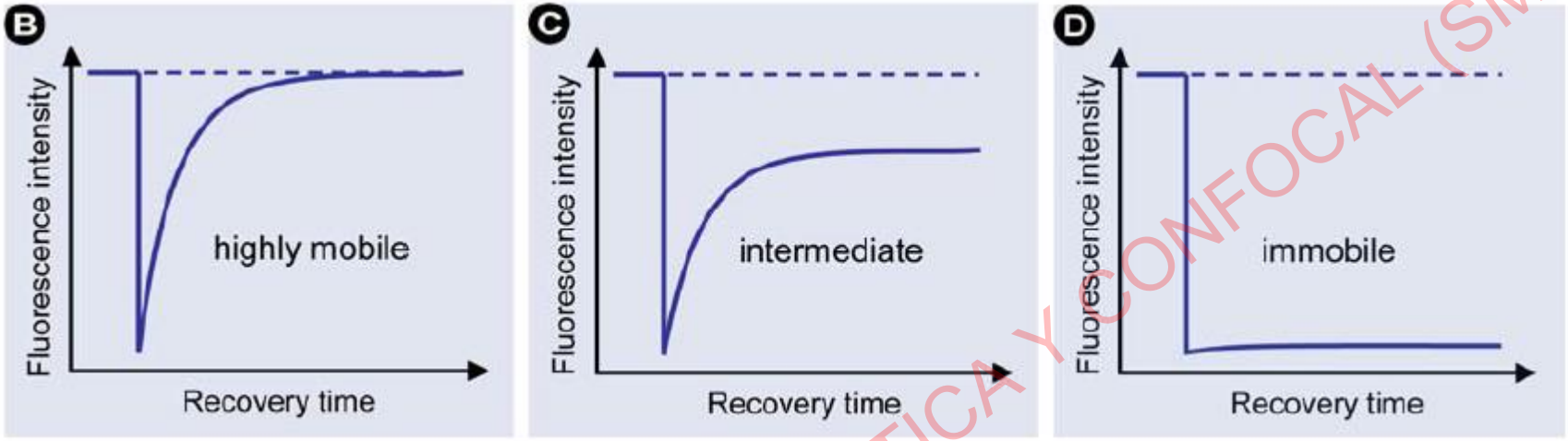
(FRAP – Background) / (Control – Background)

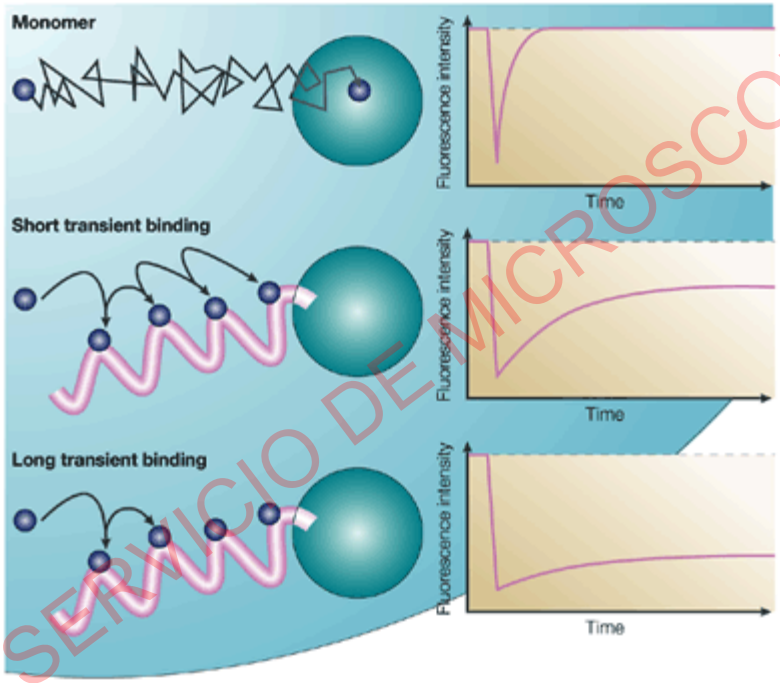
4- Normalization

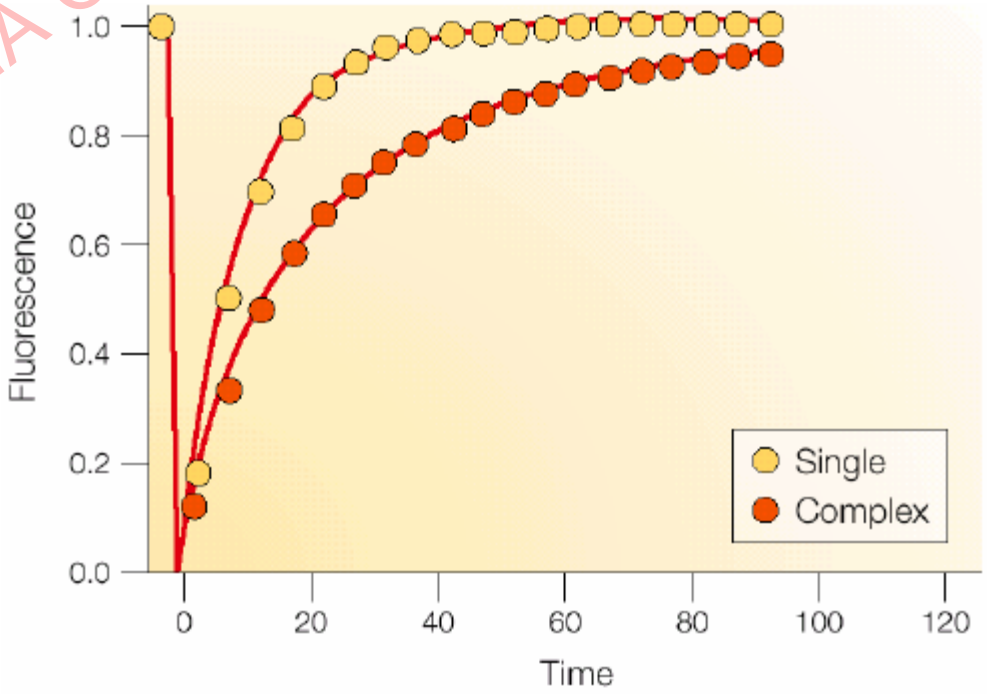
5- (Fit an equation)



FRAP analysis



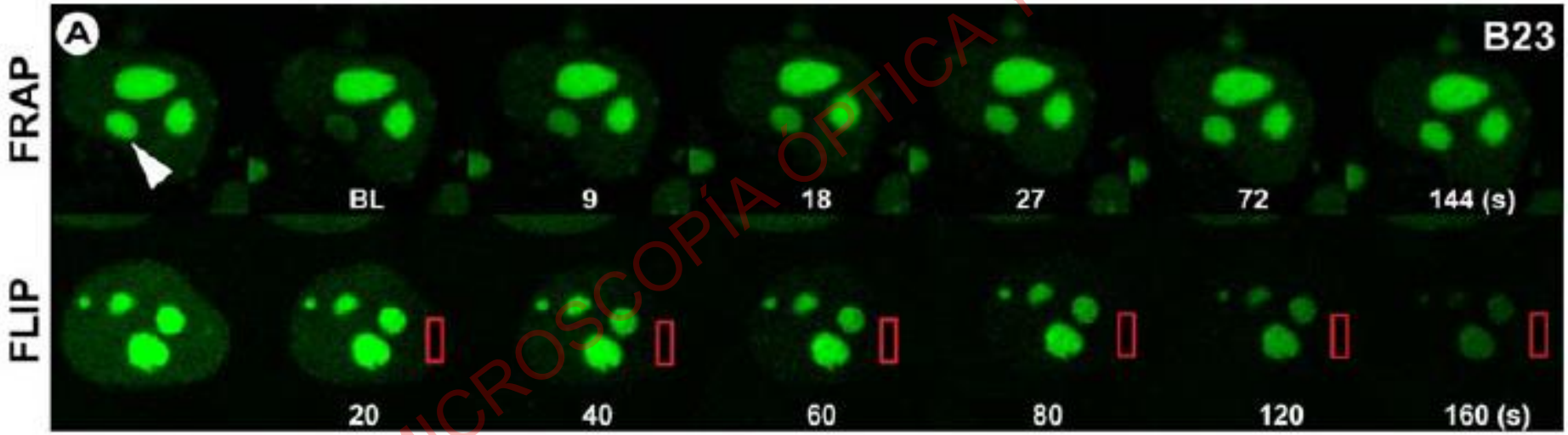




Combined FRAP and FLIP

FLIP can be used as a control of FRAP experiments

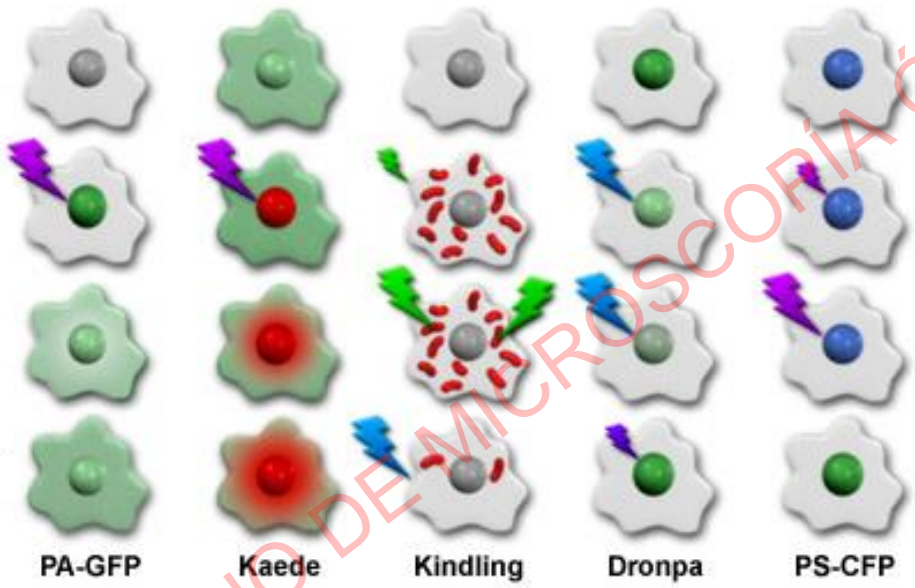
FLIP demonstrates the conectivity between two regions/compartments

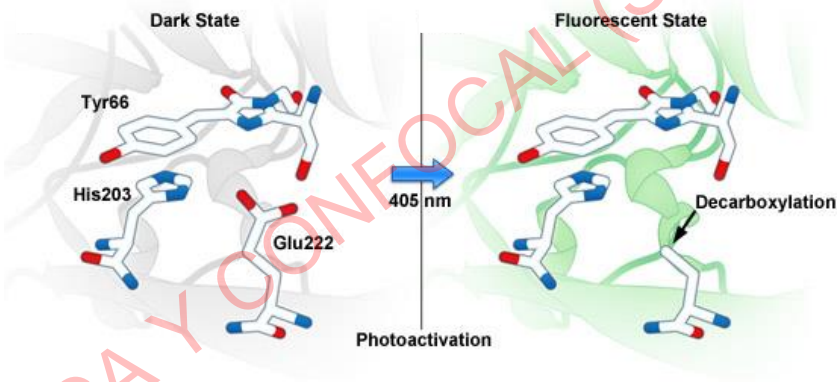


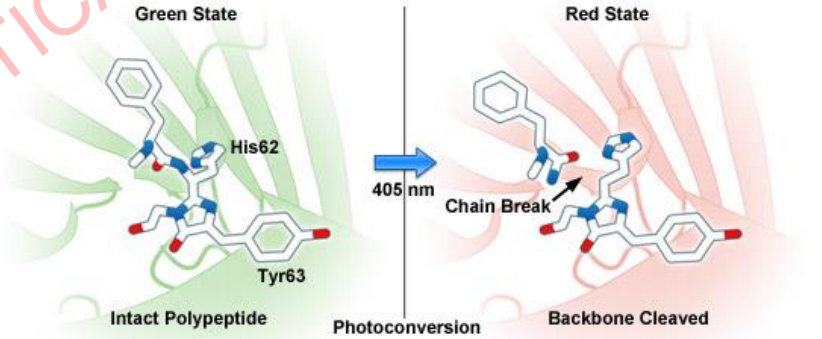
Chen, D. et al 2001

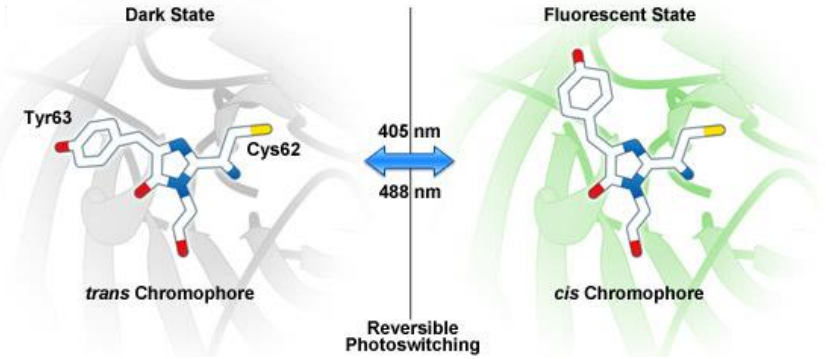
Photoconversion reactions in optical highlighters

- Photoactivation (PA)
- Photoconversion
- Photoswitch







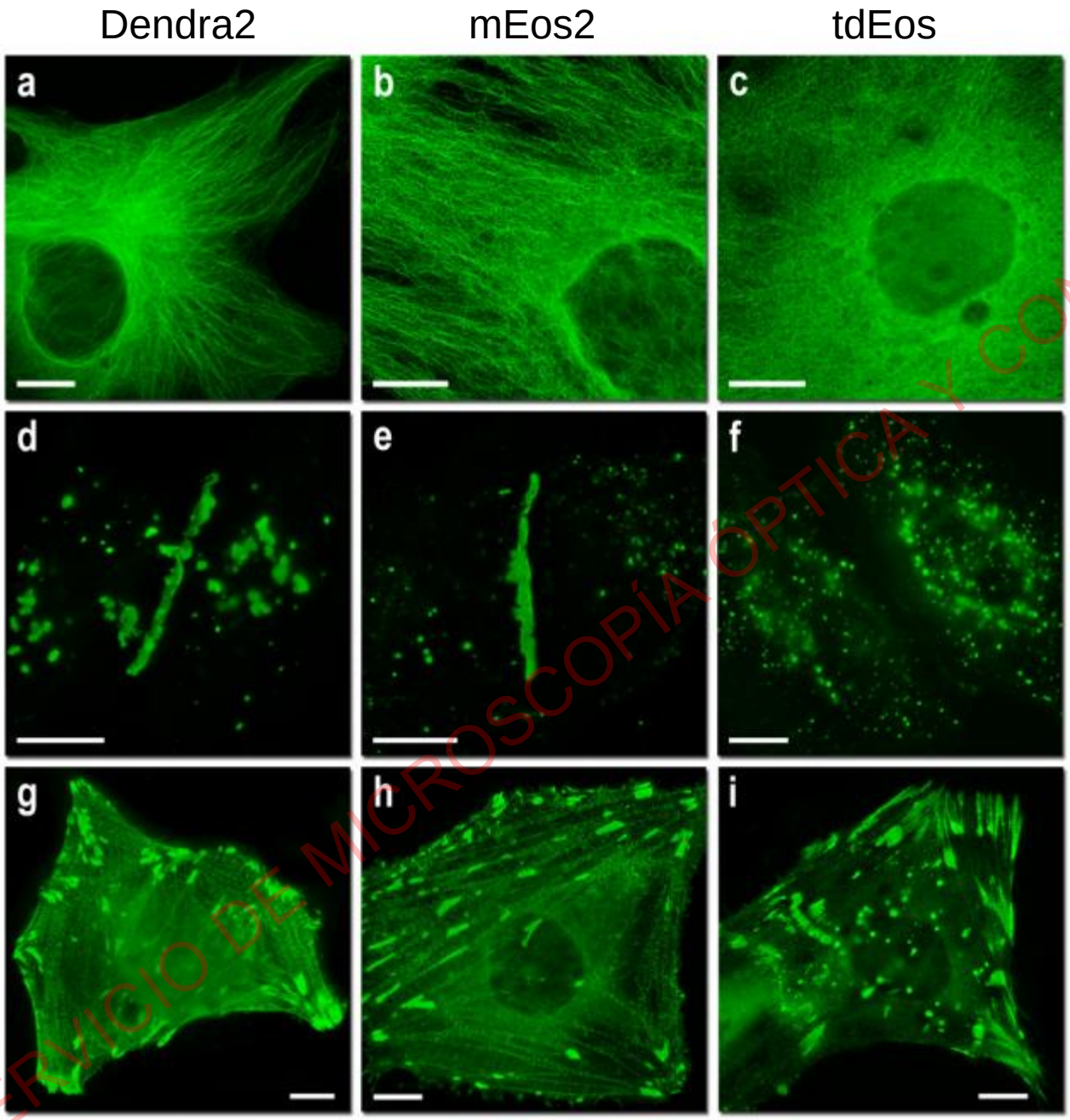


Warning!

Focus on brightfield or at a wavelenght which does not activate your protein

https://www.fpbase.org/table/

(filter bySwitch Type)



Localization precision is not the same between different proteins. Dendra2 & mEos2 > tdEos

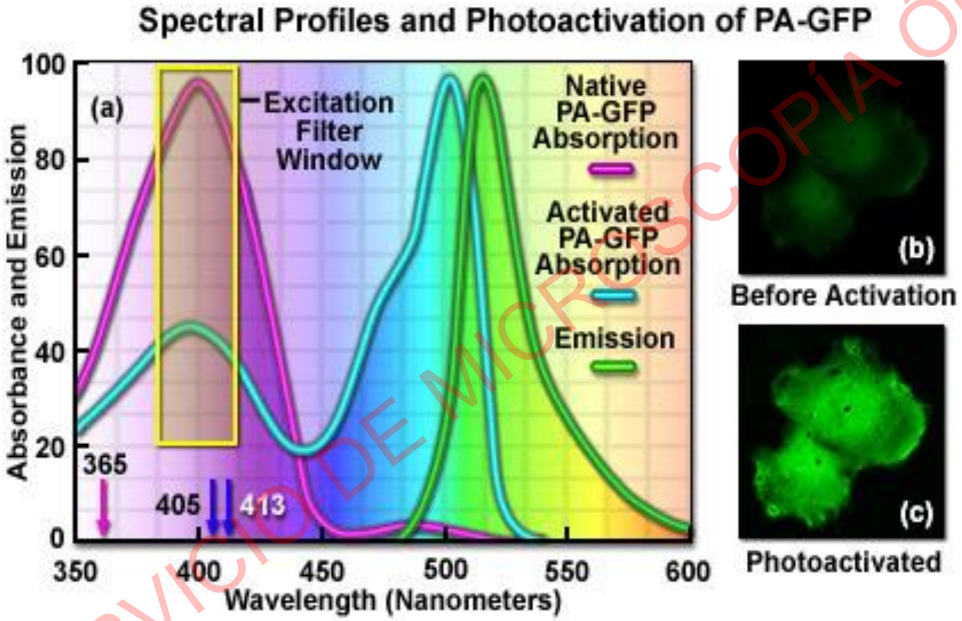
A bright and photostable photoconvertible fluorescent protein for fusion tags
Sean A. McKinney et al.
Nat Methods. 2009 Feb;6(2):131-133.

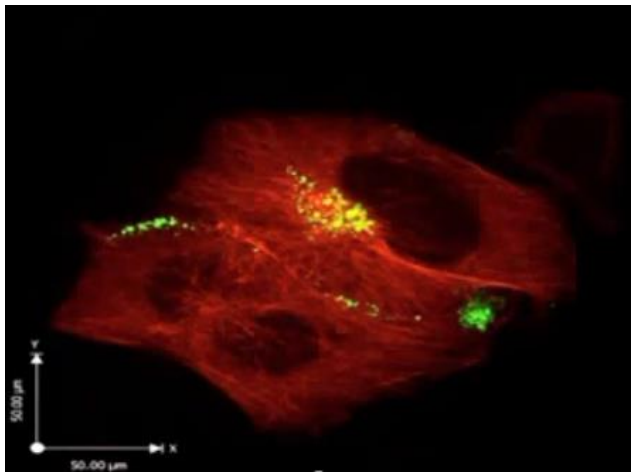
Photoactivation

Ex. 405

No fluorescence ⇒ Fluorescence (irreversible)

e.g. PA-GFP doesn't emit green fluorescence until irradiation at 405 nm





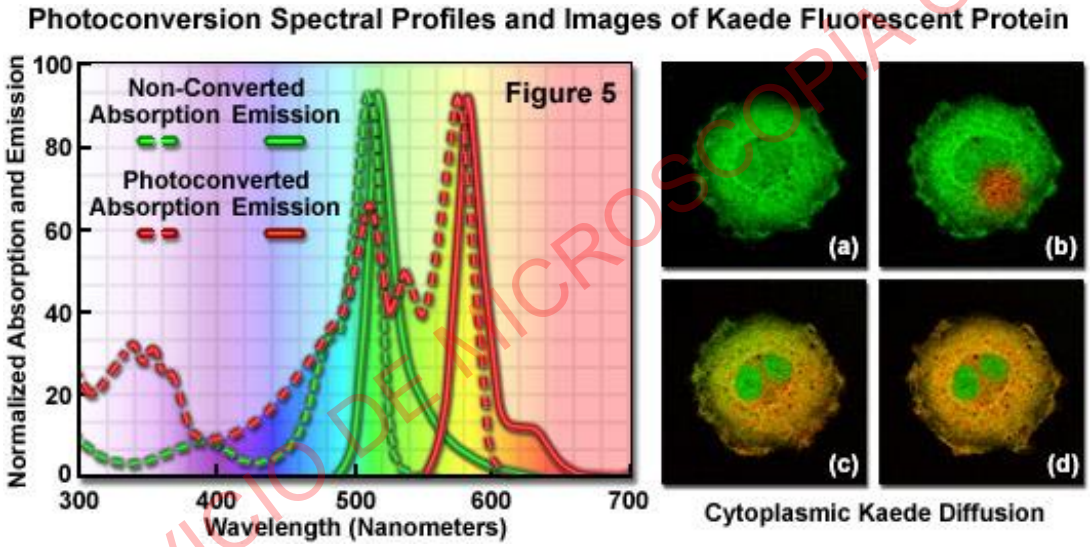
Cytoskeleton stained cells (red) + GAG HIV-1 protein (PA-GFP)

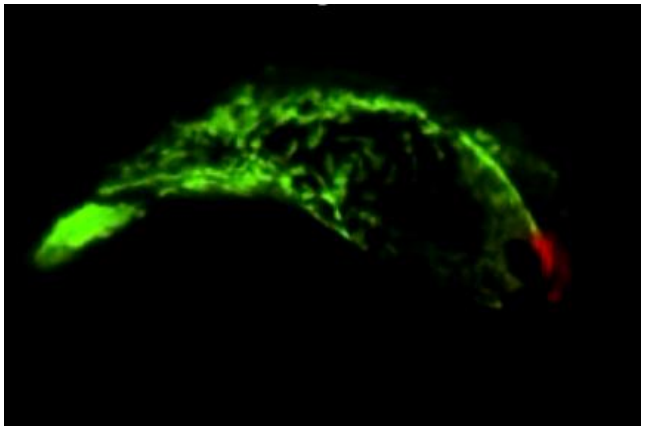
Photoconversion

Ex. 405

Fluorescence ⇨ Fluorescence in other color (irreversible)

e.g. Kaede emits green fluorescence until irradiation at 405 nm. Then, Kaede emits red fluorescence





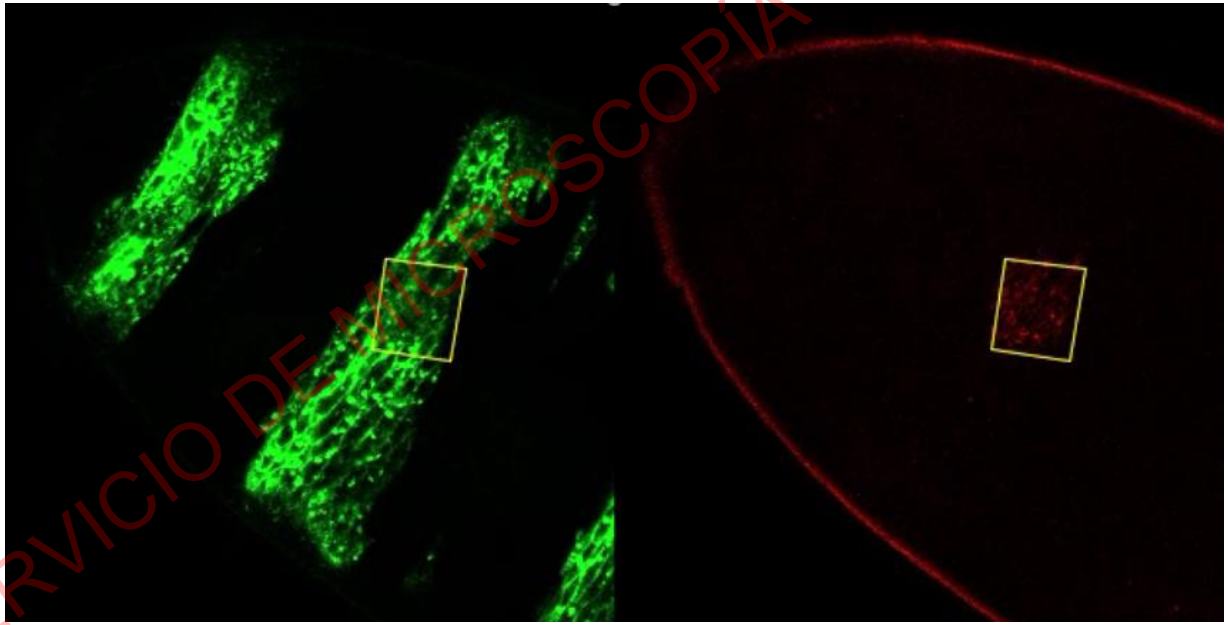
(Cranial neural crest cell migration)

EGFP protein can be photoconverted from green to red by blue light

- **Low oxigen environment**
- **Oxidation agents** (potassium ferricyanide, benzoquinone , and MTT)

EGFP protein can be photoconverted from green to red by violet light

- **pH dependent**



Fly embryo. Adrian Aguirre & Gustavo Aguilar (Isabel Guerrero lab)

Green to red photoconversion of GFP for protein tracking in vivo.
Sci. Rep. 2015 Jul;5, 11771
Sattarzadeh, A. et al.

Photoswitch - Kindling

Ex. 543

No fluorescence ⇨ Fluorescence (irreversible)

Ex. 543 Ex 457

No fluorescence ⇨ Transient fluorescence ⇨ No fluorescence (reversible)

Tracking Organelle Movements with Kindling Fluorescent Protein

e.g. KFP1

(a) 3 % 543 nm laser line: display all mitochondia (reversible)
40 % 543 nm laser line (yellow box): irreversible fluorescence in mitochondria 1-6

(b) 2 % 457 nm laser line: reversible quenching

(c) tracking mitochondria 1-6

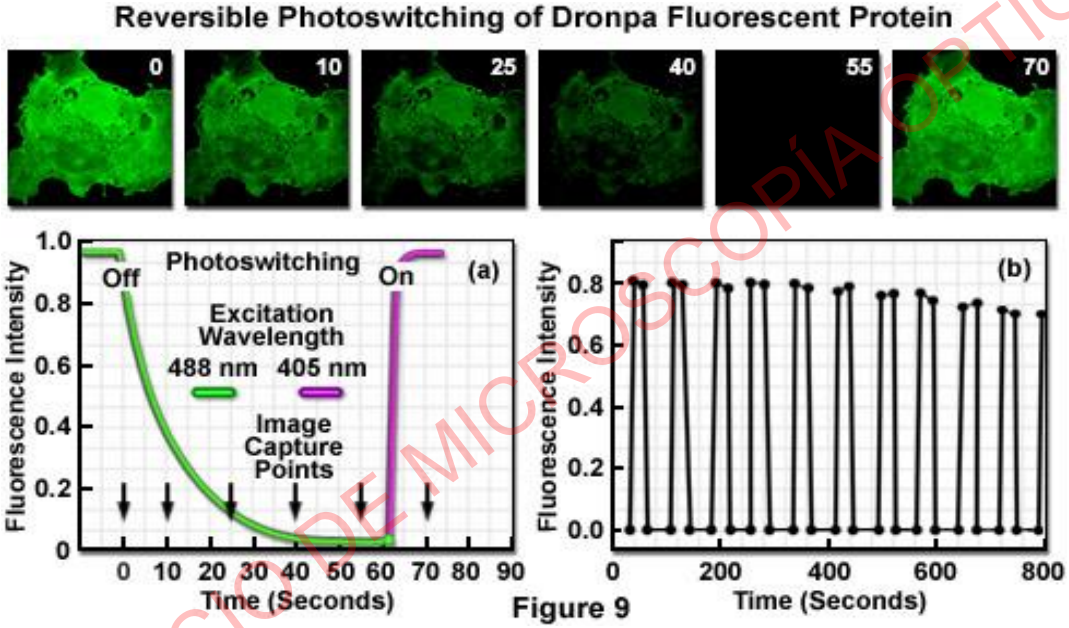
Photoswitch

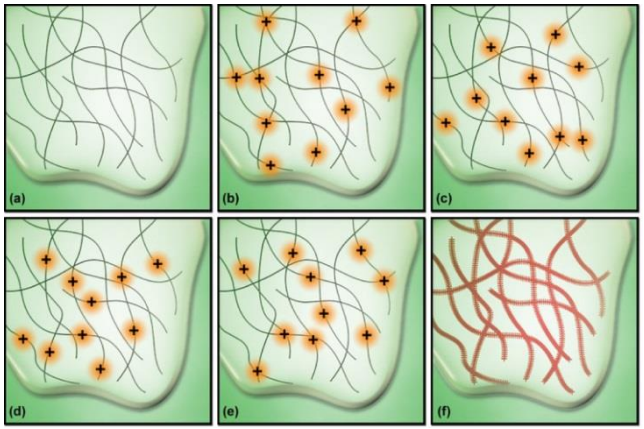
Smart fluorescent proteins: Innovation for barrier-free superresolution imaging in living cells.
Develop. Growth Differ. 2013 May;55:491–507
Dhermendra K. Tiwari and Takeharu Nagai

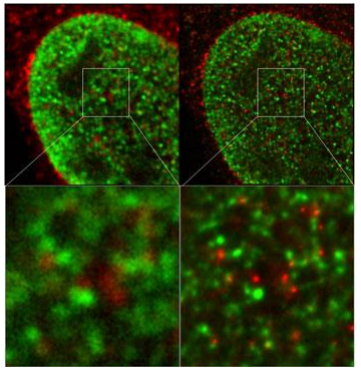
Ex. 488 ⇨ Fluorescence ⇨ Ex. 405 ⇨ Fluorescence decay ⇨ Fluorescence (reversible)

e.g. Dronpa

Used in single molecule localization (super-resolution techniques)



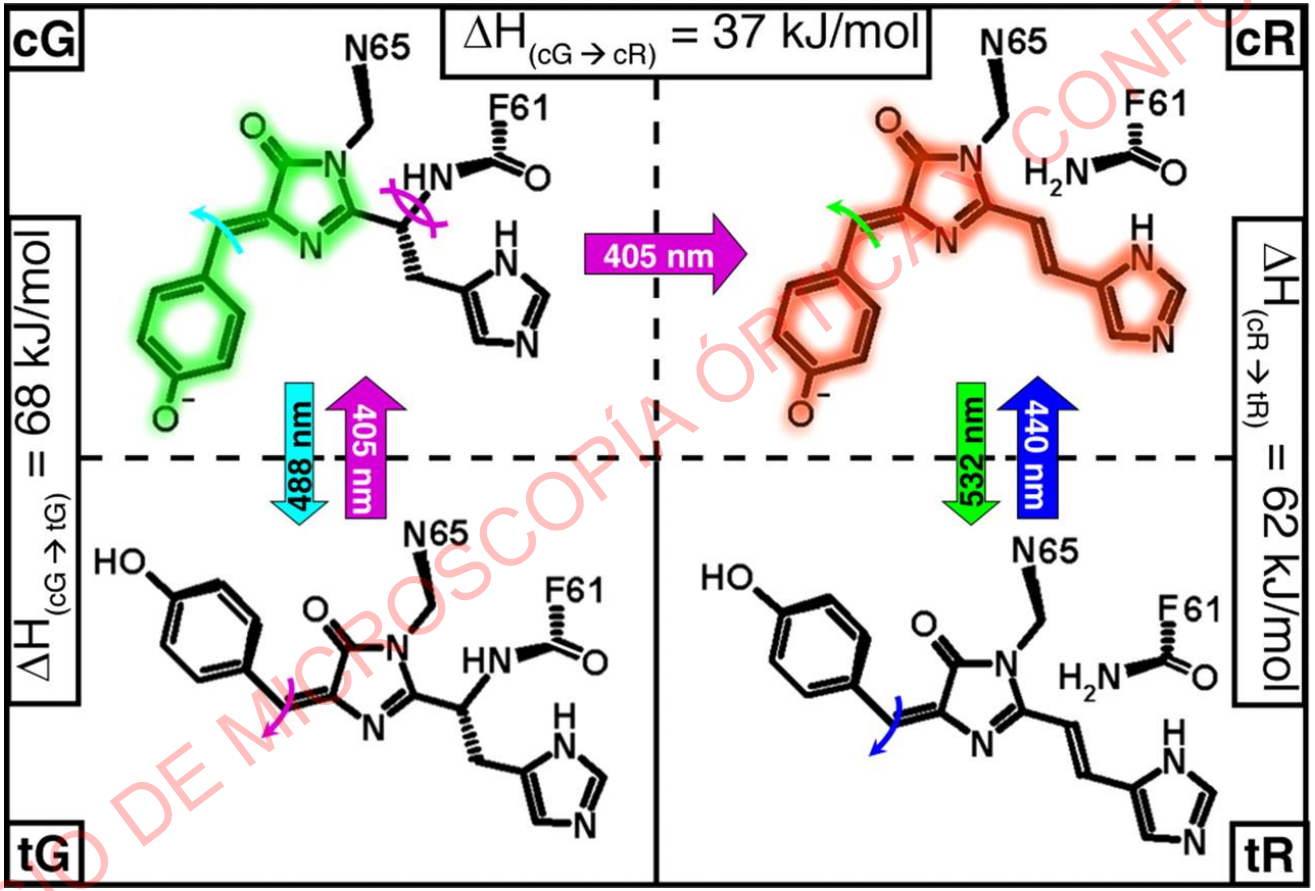




Photoconversion & Photoswitch

e.g. IrisFP, mIrisFP

Used in single molecule localization (super-resolution techniques)

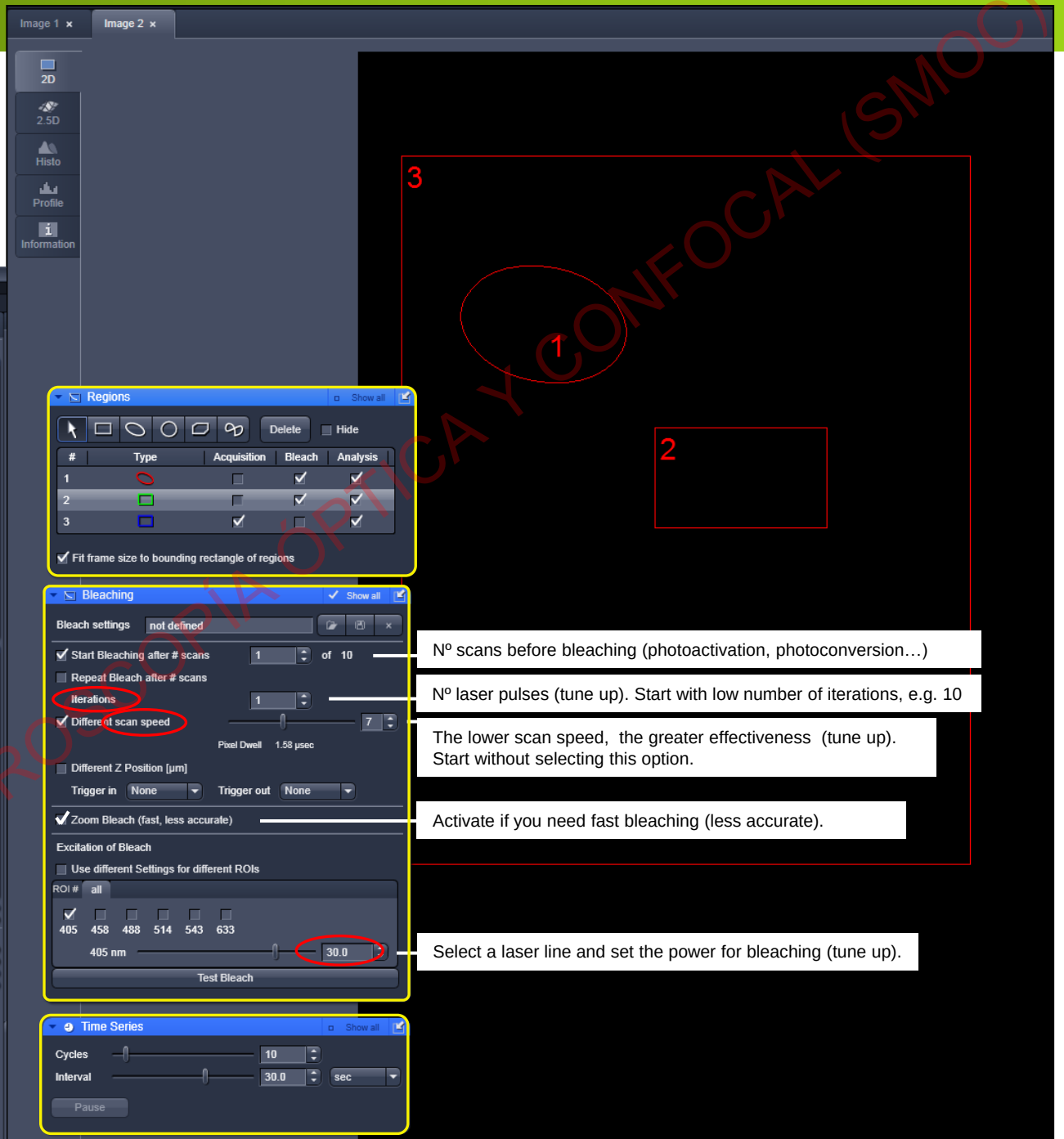


Structural characterization of IrisFP, an optical highlighter undergoing multiple photo-induced Transformations
PNAS 2008
Virgile Adam et al.

A photoactivatable marker protein for pulse-chase imaging with superresolution
Nature Methods 2010
Jochen Fuchs et al.

How is it made?

Ask SMOC staff if you need help to confugure the settings



Nº scans before bleaching (photoactivation, photoconversion…)

Nº laser pulses (tune up). Start with low number of iterations, e.g. 10

The lower scan speed, the greater effectiveness (tune up). Start without selecting this option.

Activate if you need fast bleaching (less accurate).

Select a laser line and set the power for bleaching (tune up).

Outline

- The discovery of fluorescent proteins (FPs). FP families
- Advantages of using a recombinant protein
- Spectra profile and important parameters in a FP
- The color palette of FPs. Unmixing
- Dynamic events: FRET, photobleaching, photoactivation and photoconversion
- **Precautions in *in vivo* experiments**

Precautions I

Biological:

- **Misfunction**
- **Mislocation**

Check the correct function and location of the exogenous protein in the cell *vs.* the endogenous protein.

- **Artifacts:** avoid protein overexpression. Select medium-intensity expressing cells.

Precautions II

Experimental (*in vivo*):

- **Temperature**
- **pH (≈ 20 mM Hepes or 5% CO_2)**

Equilibrate the sample for 30 min

- **FoilCover PeCon:** reduces evaporation, permeable to gases

- **Glass (or special polymer) bottom dishes or slides (0.17 mm No 1.5)**

- **Growth medium without phenol red and low serum:** less background fluorescence
- **Characterize the spectra of your FP:** especially if 405 nm laser line is used during the image adquisition (because of undesirable photoconversions)

Precautions III

Biosafety:

Talk to SMOC and Biosafety Core staff before using **pathogens**

Samples should be **fixed** in most of the cases

Laboratorio 310
(Ext. 4643)

confocal-cbm@listas.csic.es

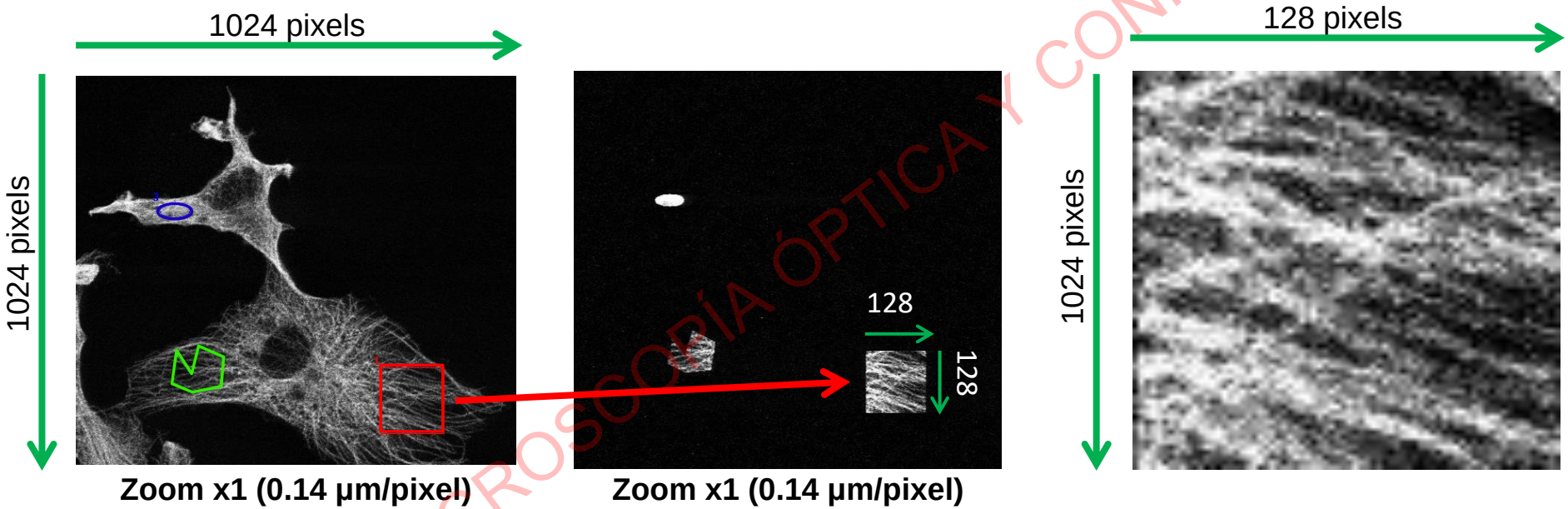
www.cbm.uam.es/confocal

Extra information

Precautions IV – Imaging adquisition ROI *vs*. ZOOM

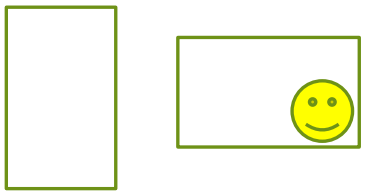
❑ Regions of interest (ROIs):

Number of pixels proportional to the entire image. Faster image scanning speed.



Zoom x1 (0.14 µm/pixel) **Zoom x1 (0.14 µm/pixel)**

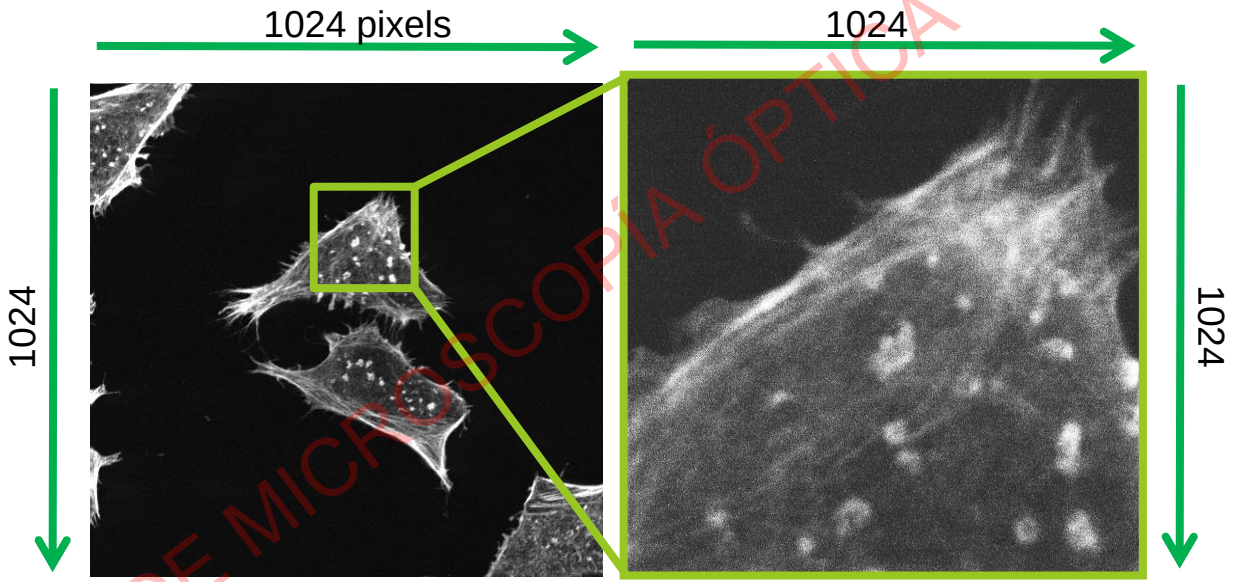
Vertical regions are slower than horizontal regions. The scanner moves faster in X than in Y.



Precautions IV – Imaging adquisition ROI *vs*. ZOOM

❑ Zoom:

Same number of pixels than the entire image but concentrated in a small area.
Same image scanning speed.
It increments the photobleaching and photodamage risk.

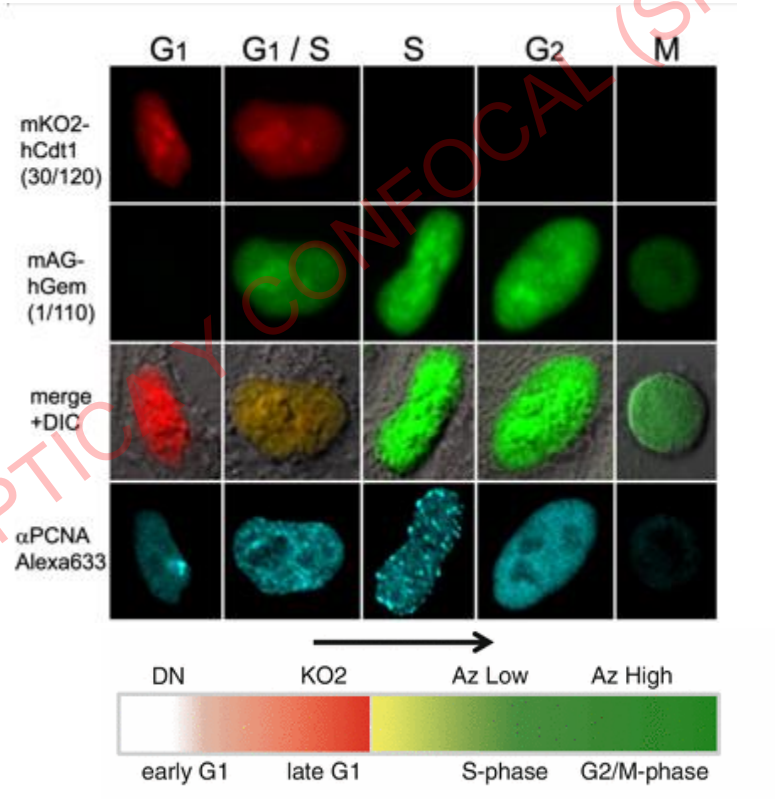


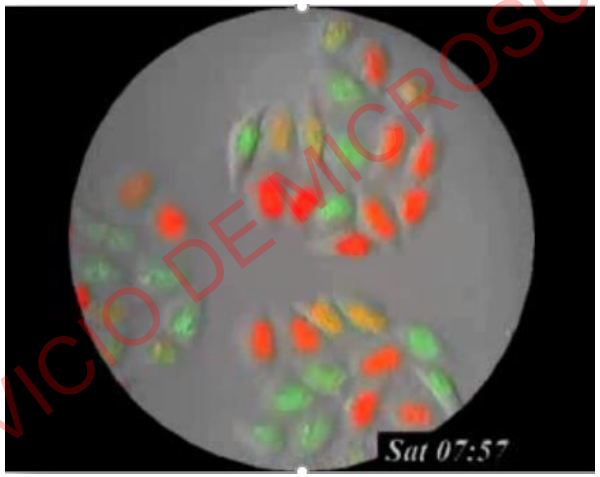
Fucci. Fluorescent ubiquitination-based cell cycle indicator

Fucci-G_1 Orange: mKO2-hCdt1 (30-120)

Fucci-S/G_2/M Green: mAG1-hGem (1-110)

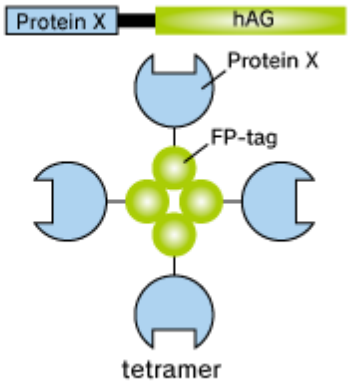
Fucci-S/G_2/M Green (N+C) : mAG1-hGem (1-60)



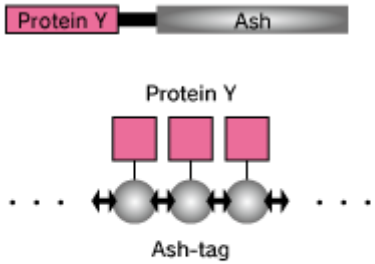


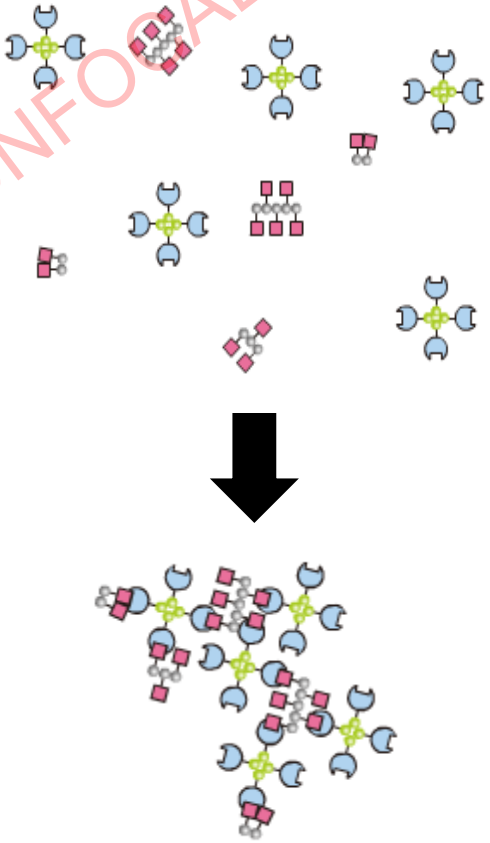
Fluoppi. Protein-protein interaction (PPI)

Azami Green (AG) tetramer

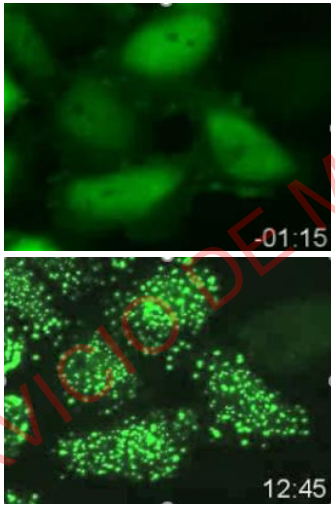


PB1 domain of p62
electrostatically homo-oligomerization



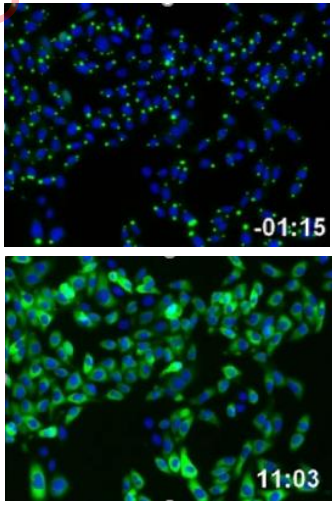


PPI induction



mTOR(FRB) and FKBP12

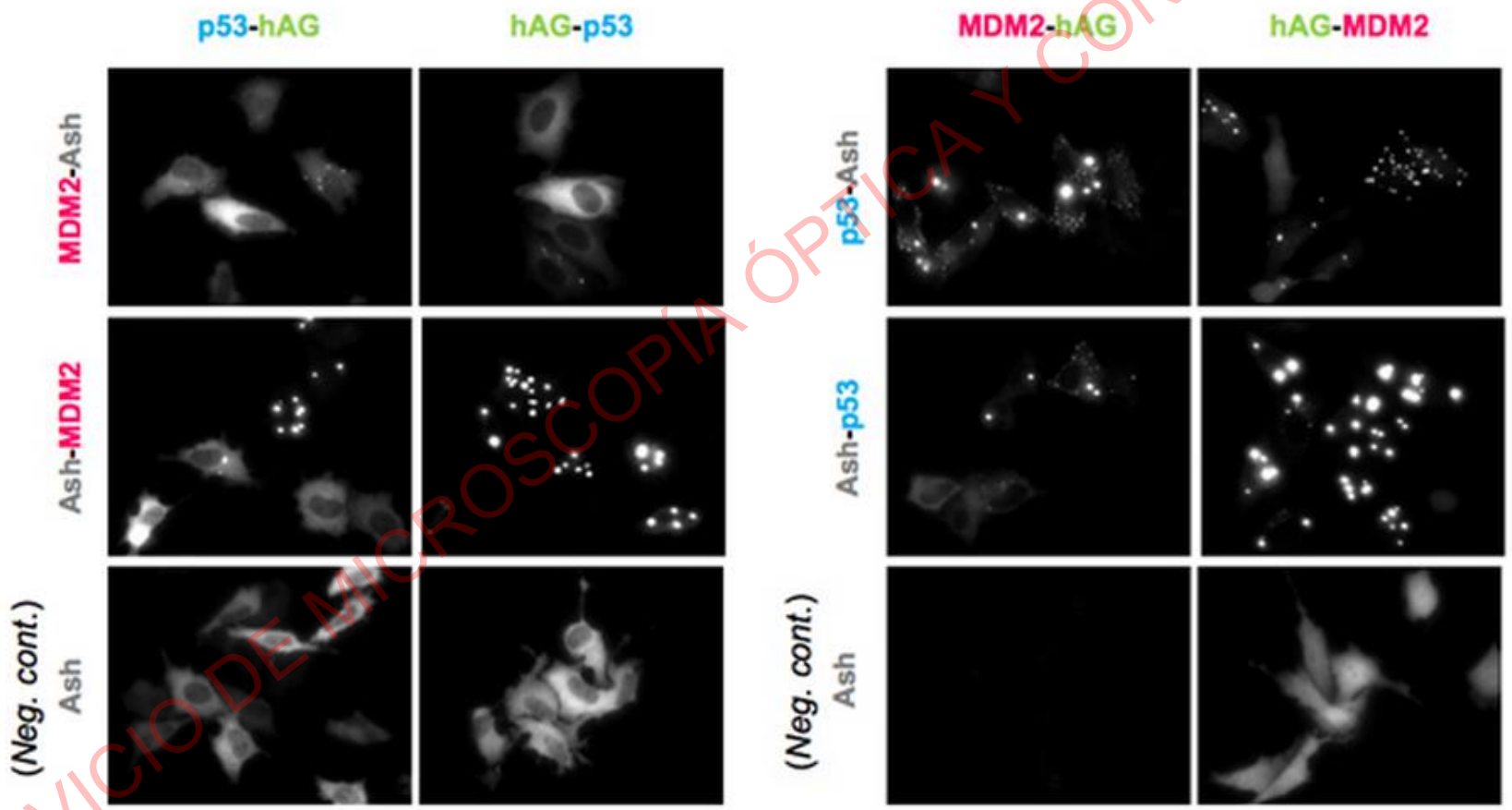
PPI inhibition



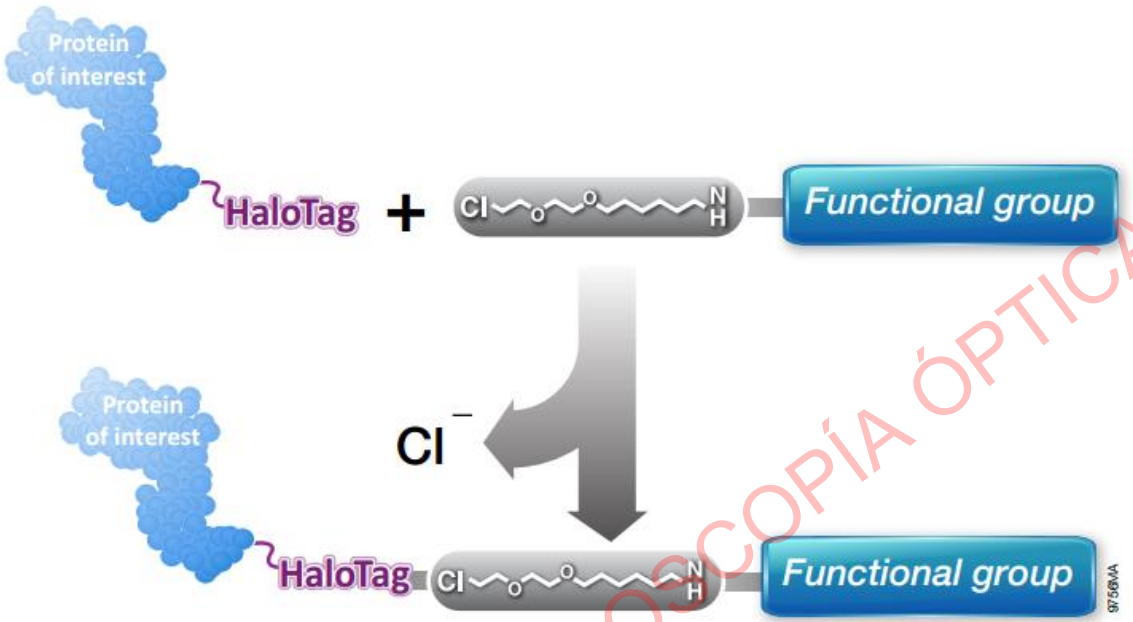
p53 and MDM2

Check all the eight possible constructs to identify the best working combination

An example of p53-MDM2 PPI



Halo-tag, SNAP-tag, TMP-tag and CLIP-tag. Versatility

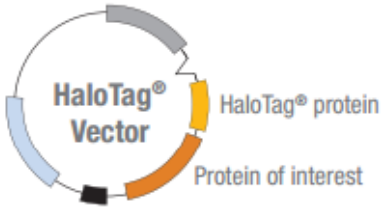


Applications

(depending on the interchangeable functional group):

Protein localization
Protein trafficking
Protein capture
Cell-to-gel analysis
Protein:protein interaction analysis…

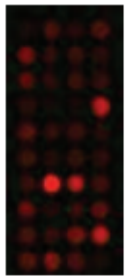
Multilabeling is possible combining these available constructions



Express HaloTag® fusion protein.

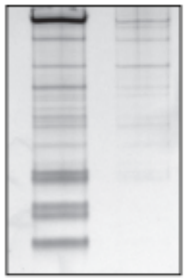
Covalent capture on resin or surfaces

Protein labeling using HaloTag® fluorescent ligands



Protein display

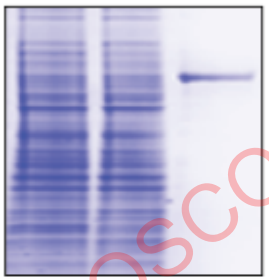
Immobilization of proteins on surfaces



Protein interactions

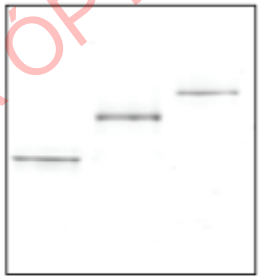
Pull-down of protein: protein complexes

Capture of protein: DNA interactions (CHIP)



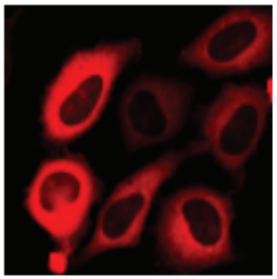
Protein purification

Protein Purification (from *E. coli* or mammalian cells)



Protein detection

Direct protein detection with fluorescent ligands without Western blotting

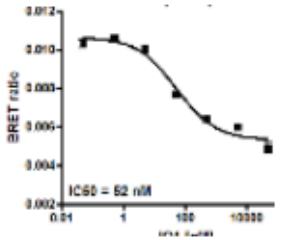


Cellular imaging

Live and fixed-cell imaging

Protein localization and translocation (spatial and temporal)

High content screening



Bioluminescence resonance energy transfer (BRET)

Detection of protein: protein interactions in living cells using BRET (Bioluminescence Resonance Energy Transfer)