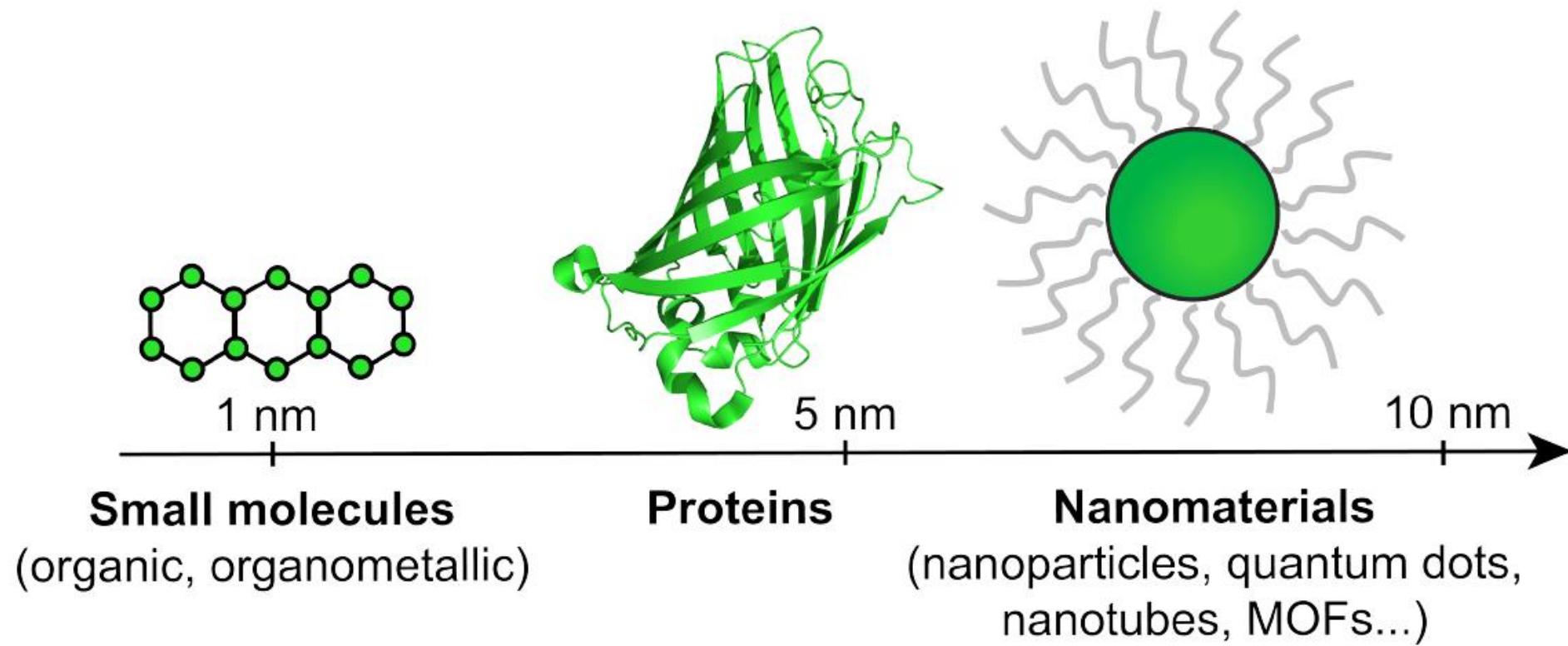


# Introduction to fluorophores

FPs, organic dyes, labeling and photophysics

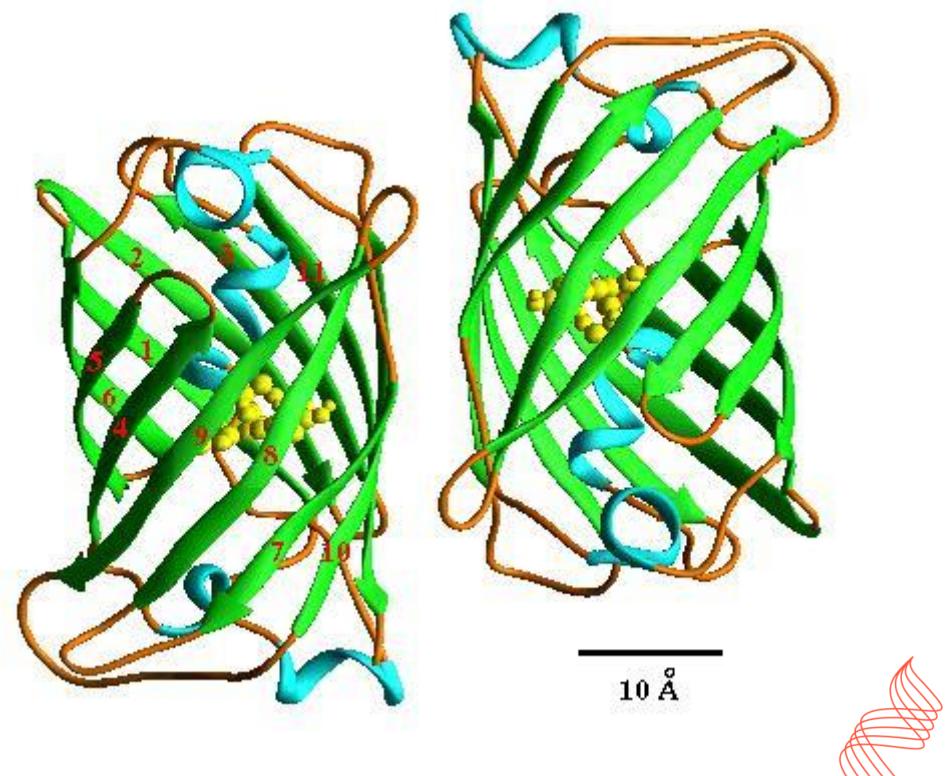




## Introduction to fluorescent proteins

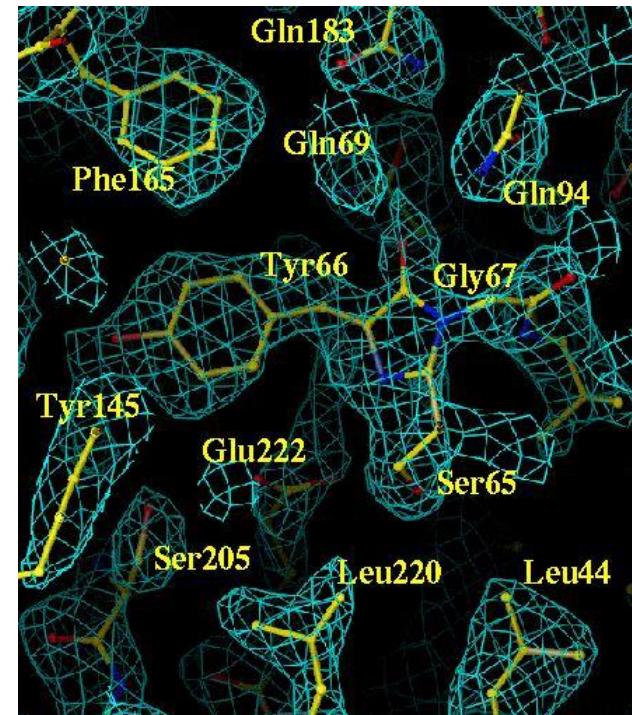
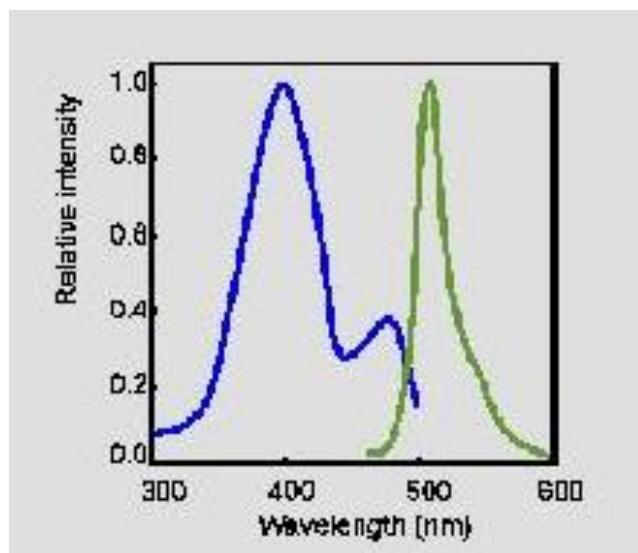
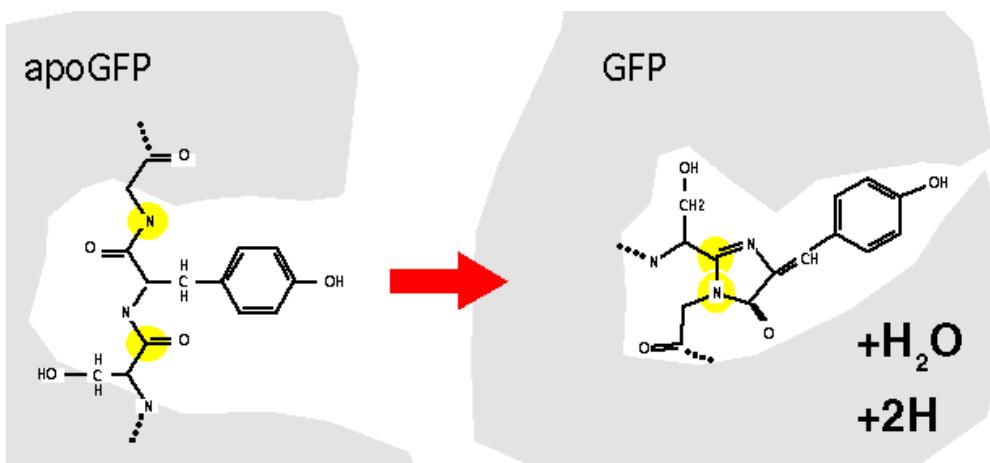
## Green Fluorescent Protein :

- from *Aequorea victoria* jellyfish (1962, Shimomura et coll)
- gene cloned in 1992 by Prasher et coll.
- 238 AA protein, compact and globular. MW : 27 kDa
- monomeric at low concentrations



# Introduction to GFP

Ser65, Tyr66 and Gly67 → maturation

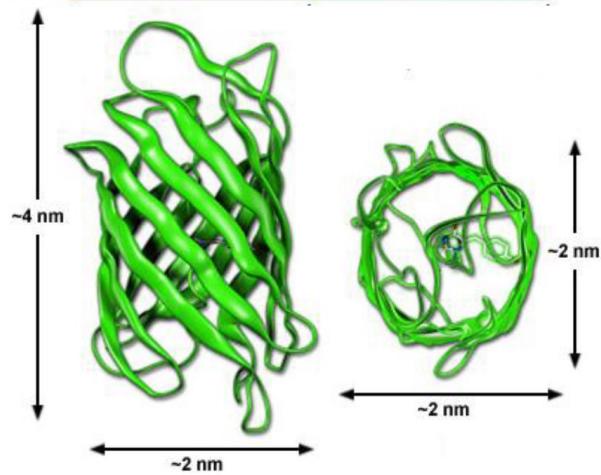


Protection of the fluorophore within the barrel allows for a relatively good photostability

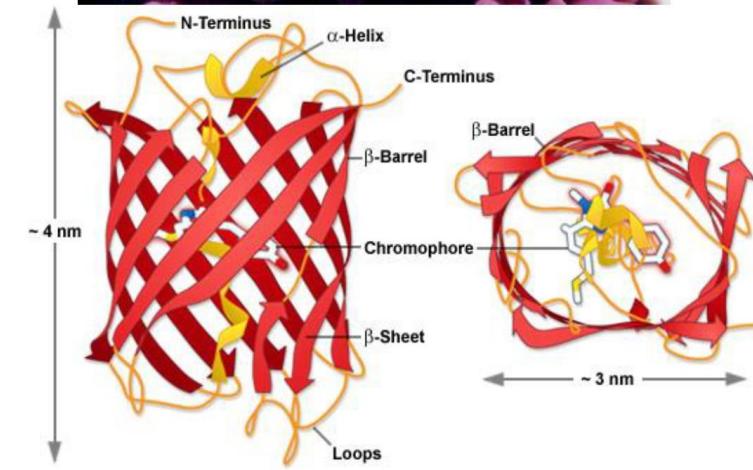


# The two families of FPs

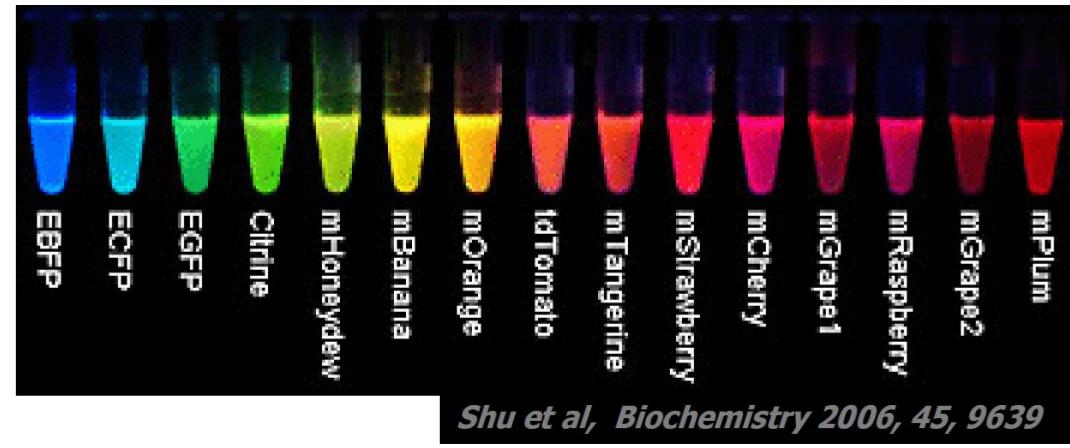
*Hydrozoan FPs*



*Anthozoan FPs*



# Creating new FPs

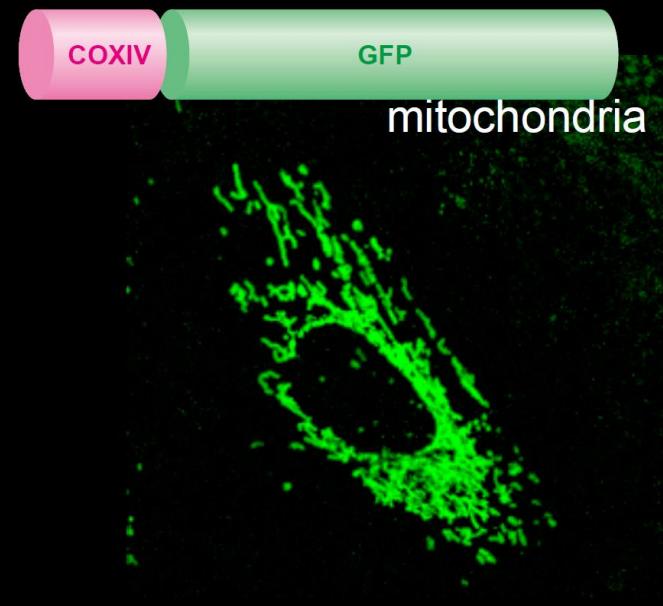
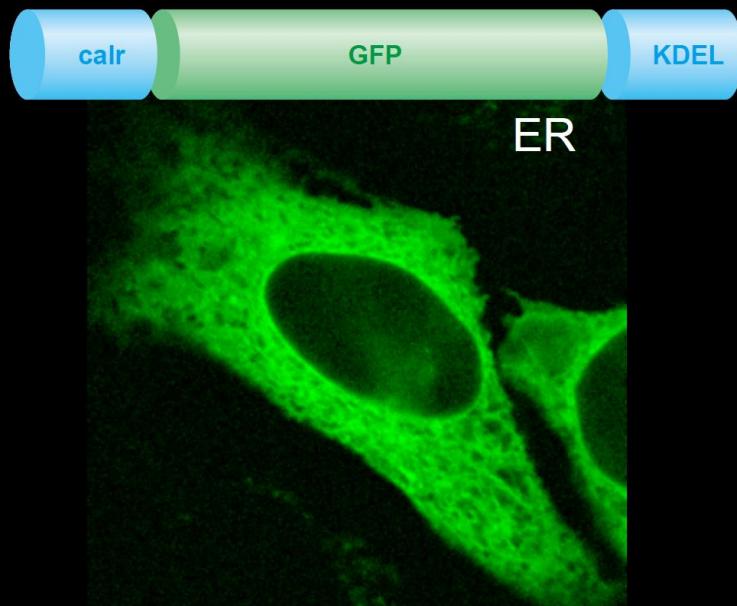
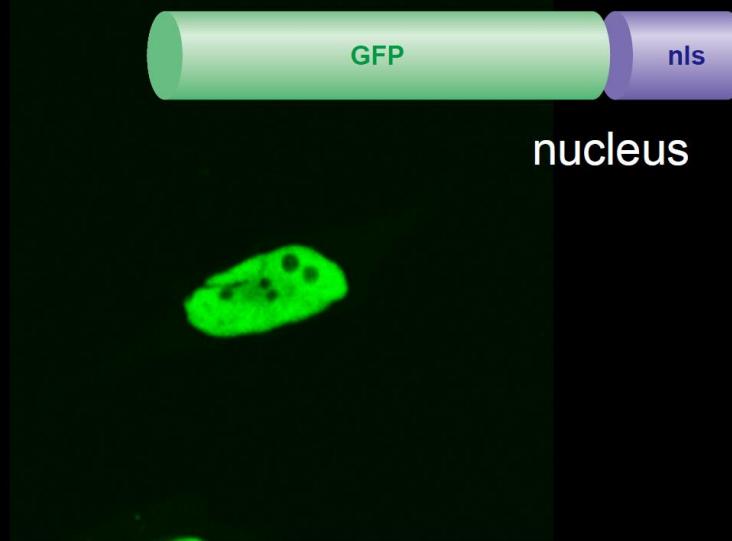
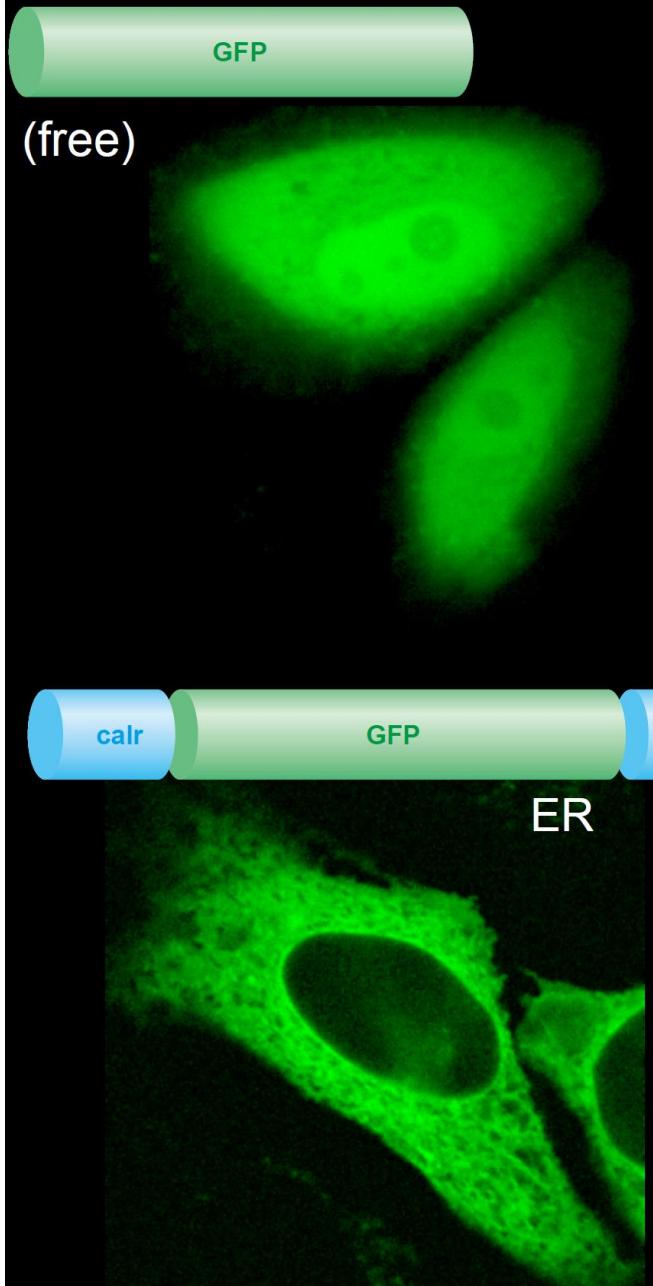


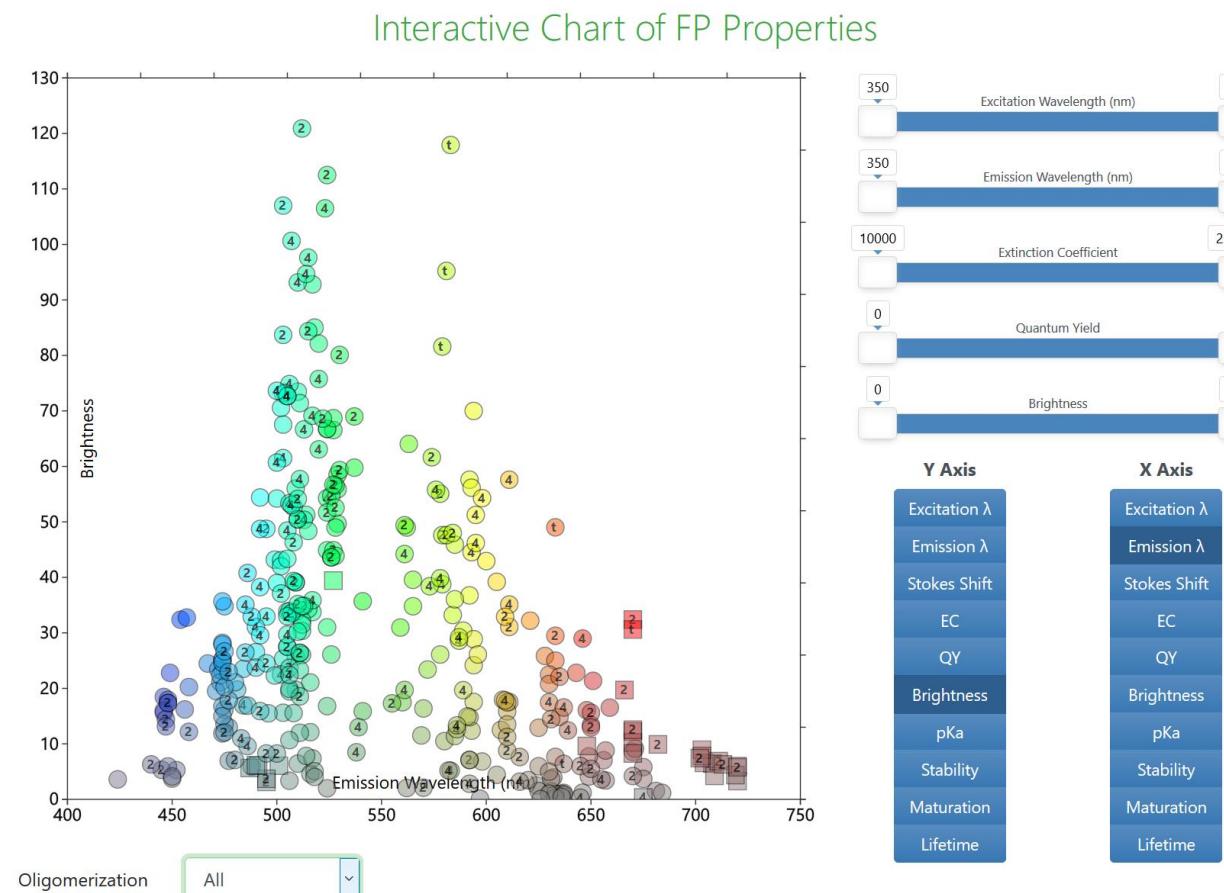
MANY VARIANTS OF THE GFP HAVE BEEN DEVELOPED :

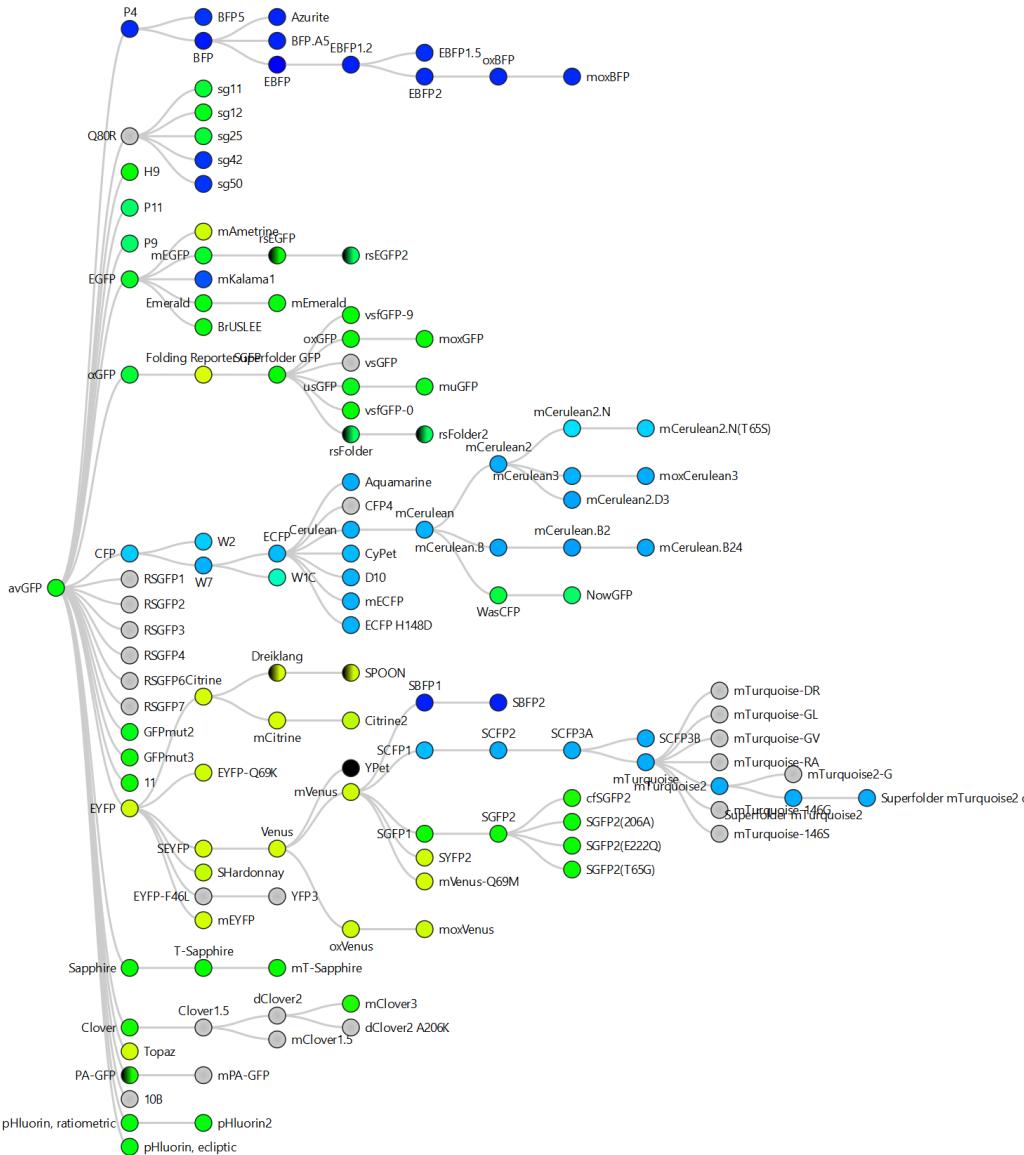
- higher brightness
- higher photostability
- less pH sensitive
- better eukaryotic / prokaryotic expression
- monomeric forms
- photoactivable / photoconvertible forms



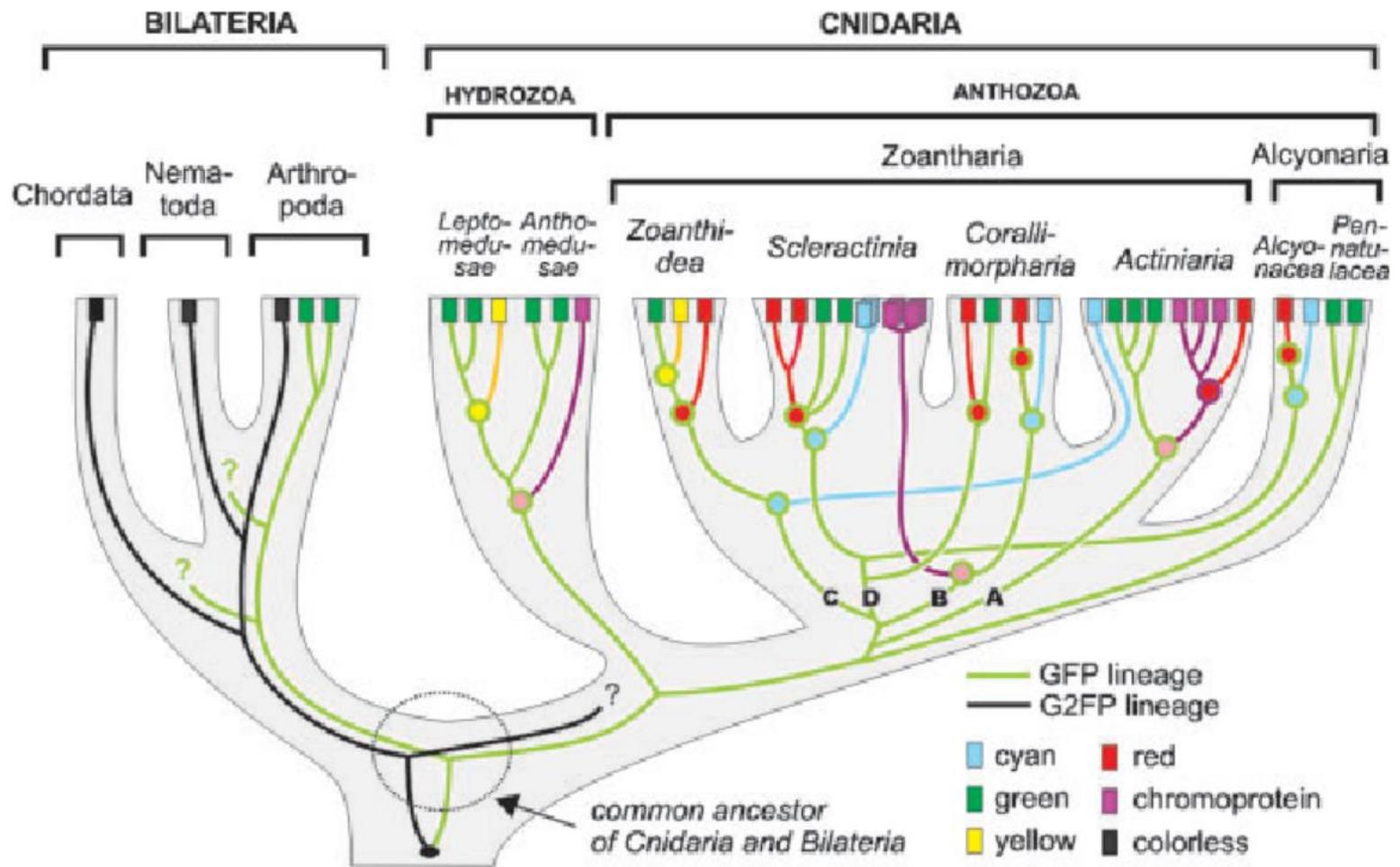
# Examples of FP targeting to subcellular organelles



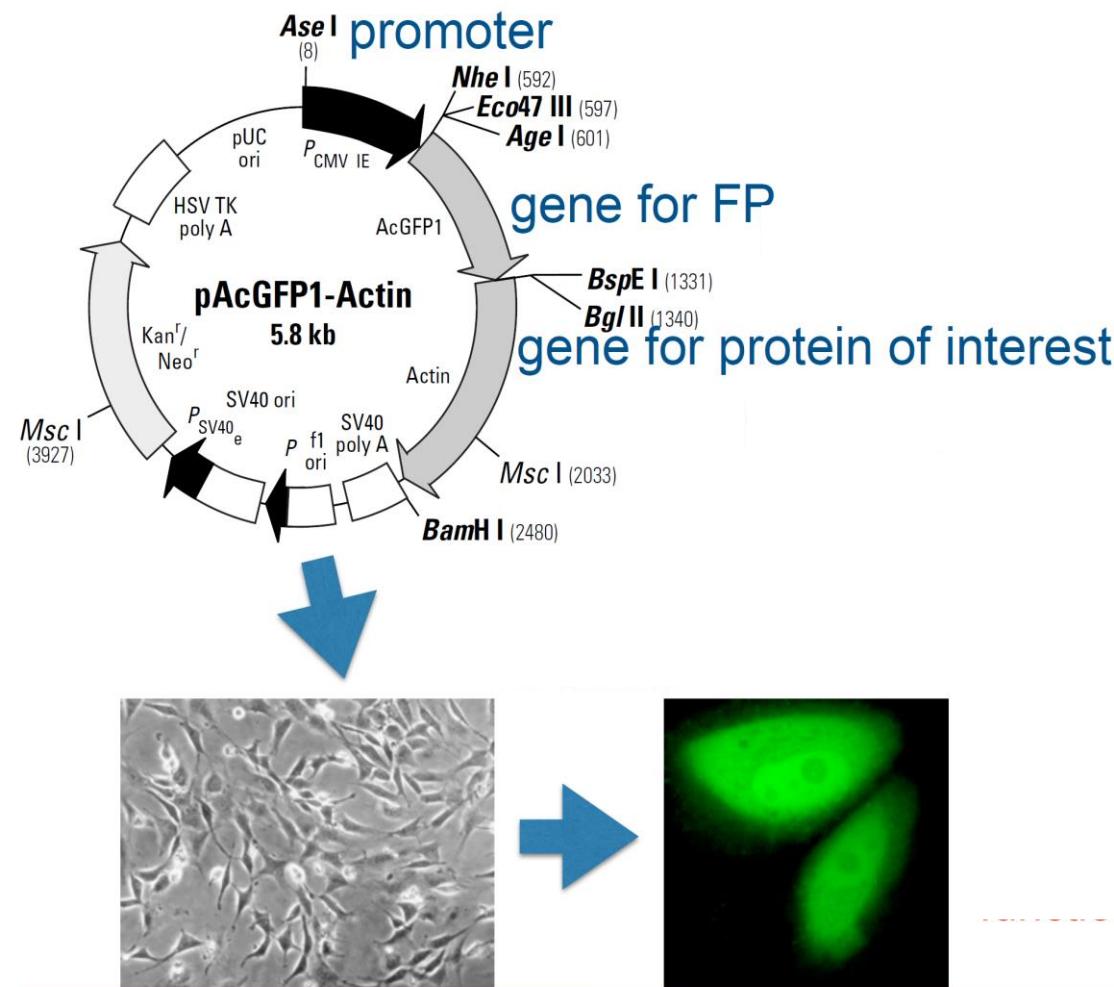




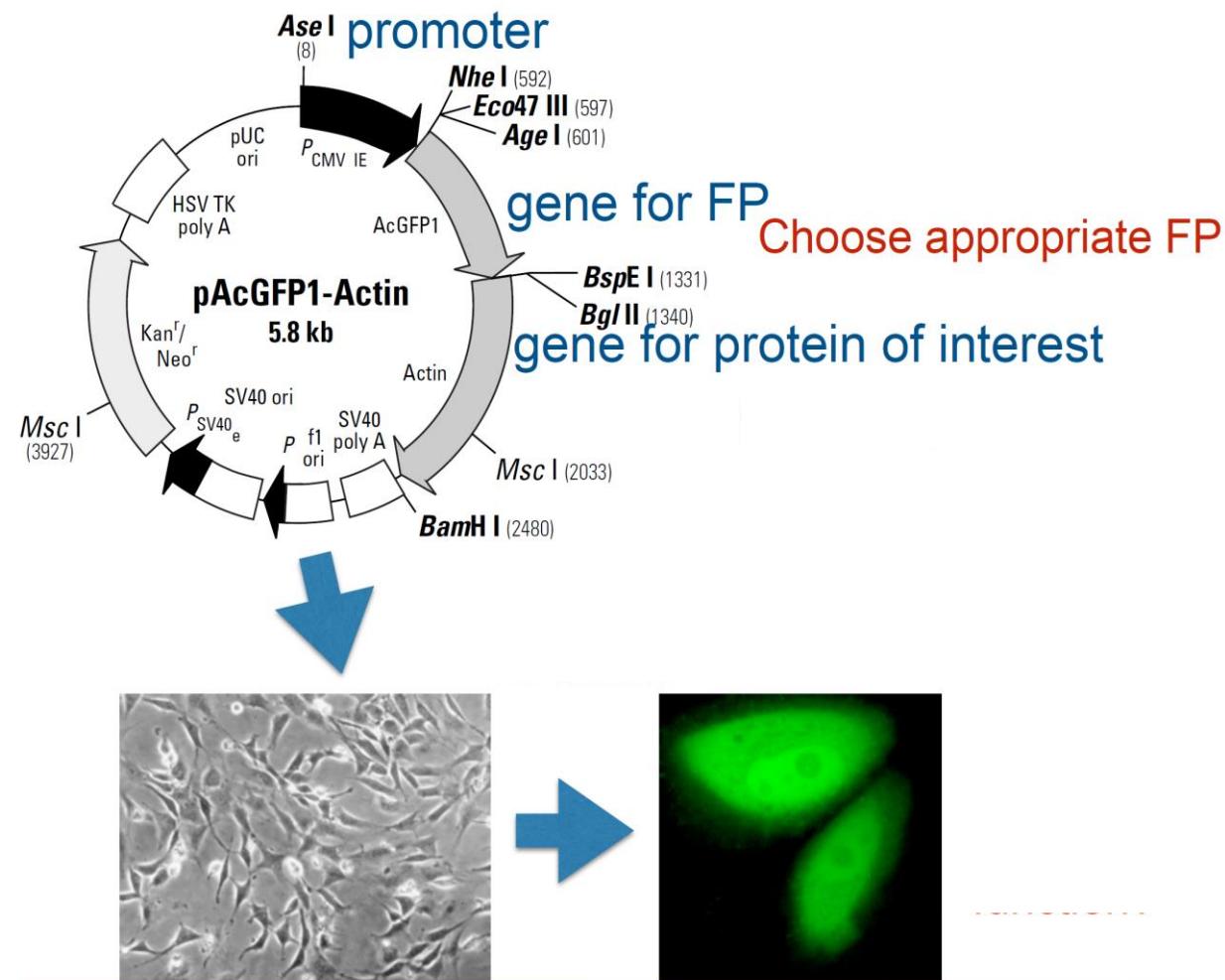




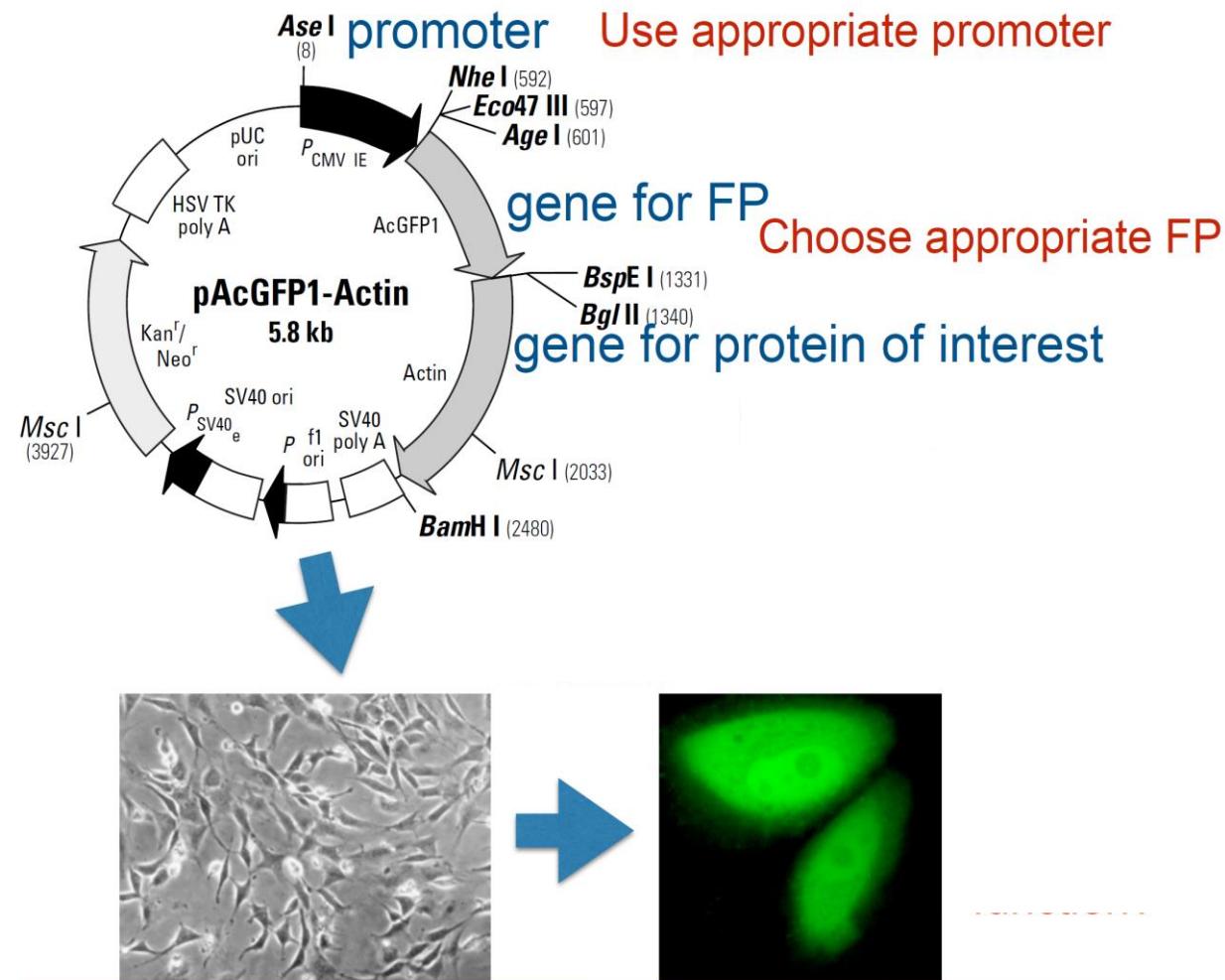
# Labeling your POI with a GFP



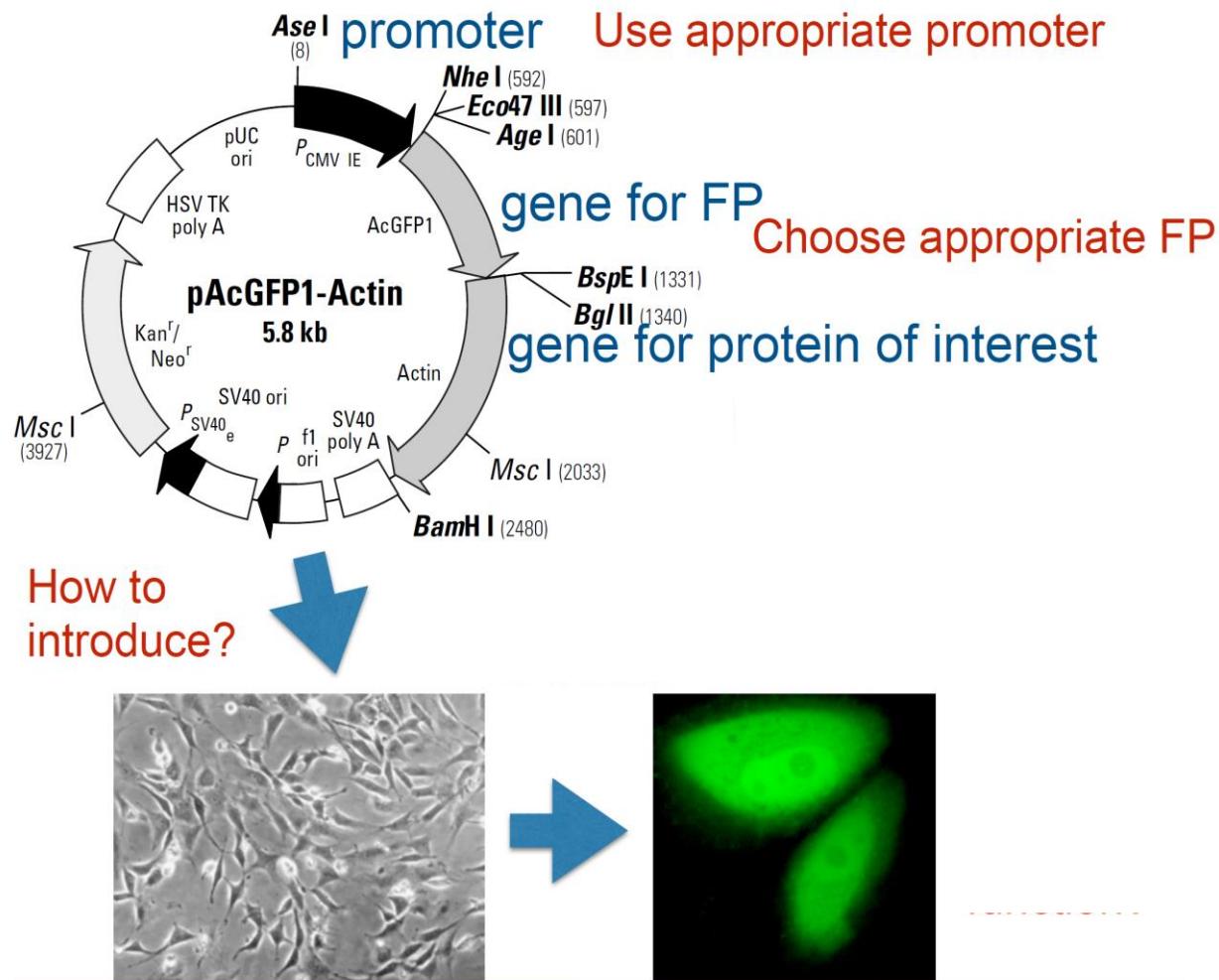
# Labeling your POI with a GFP



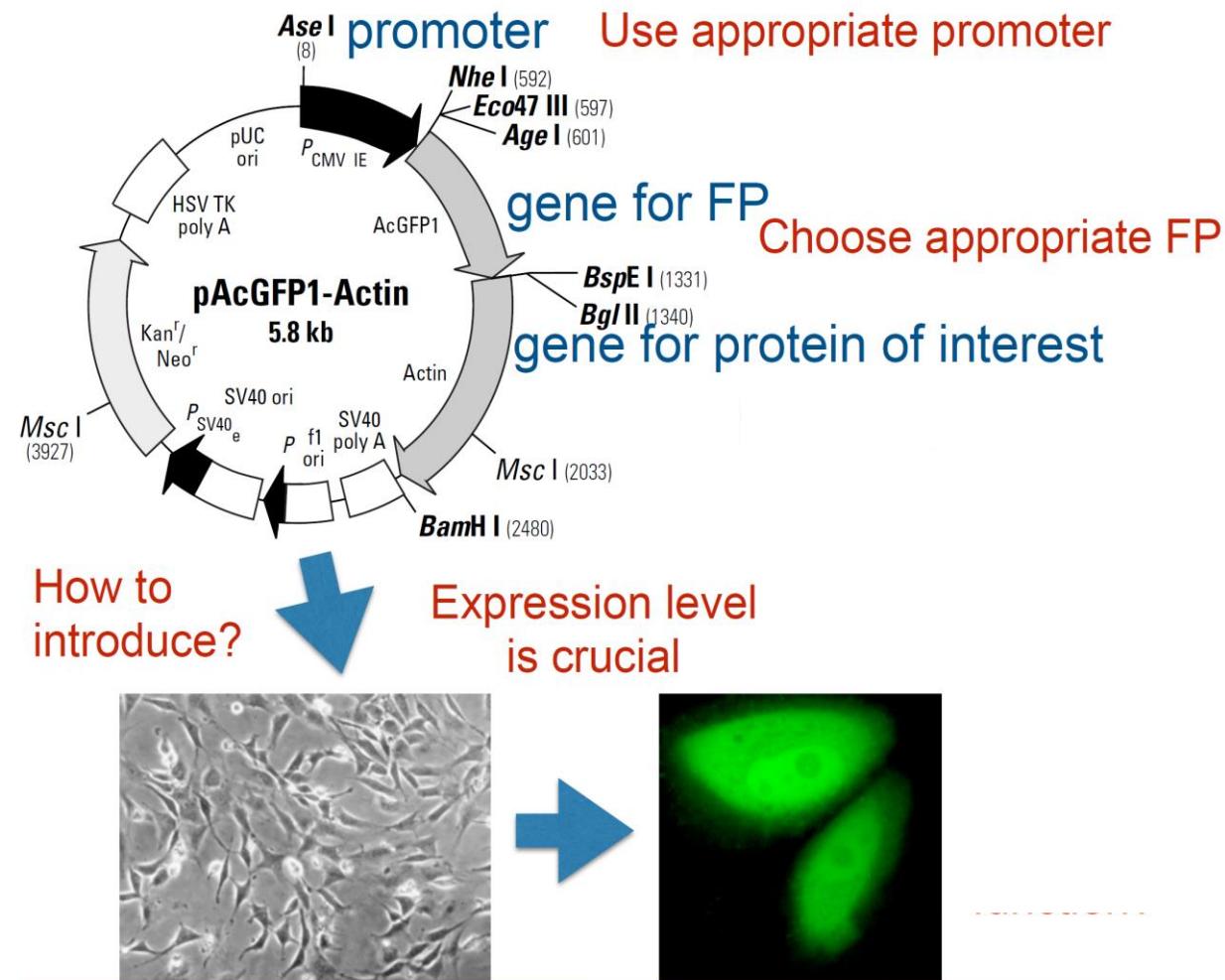
# Labeling your POI with a GFP



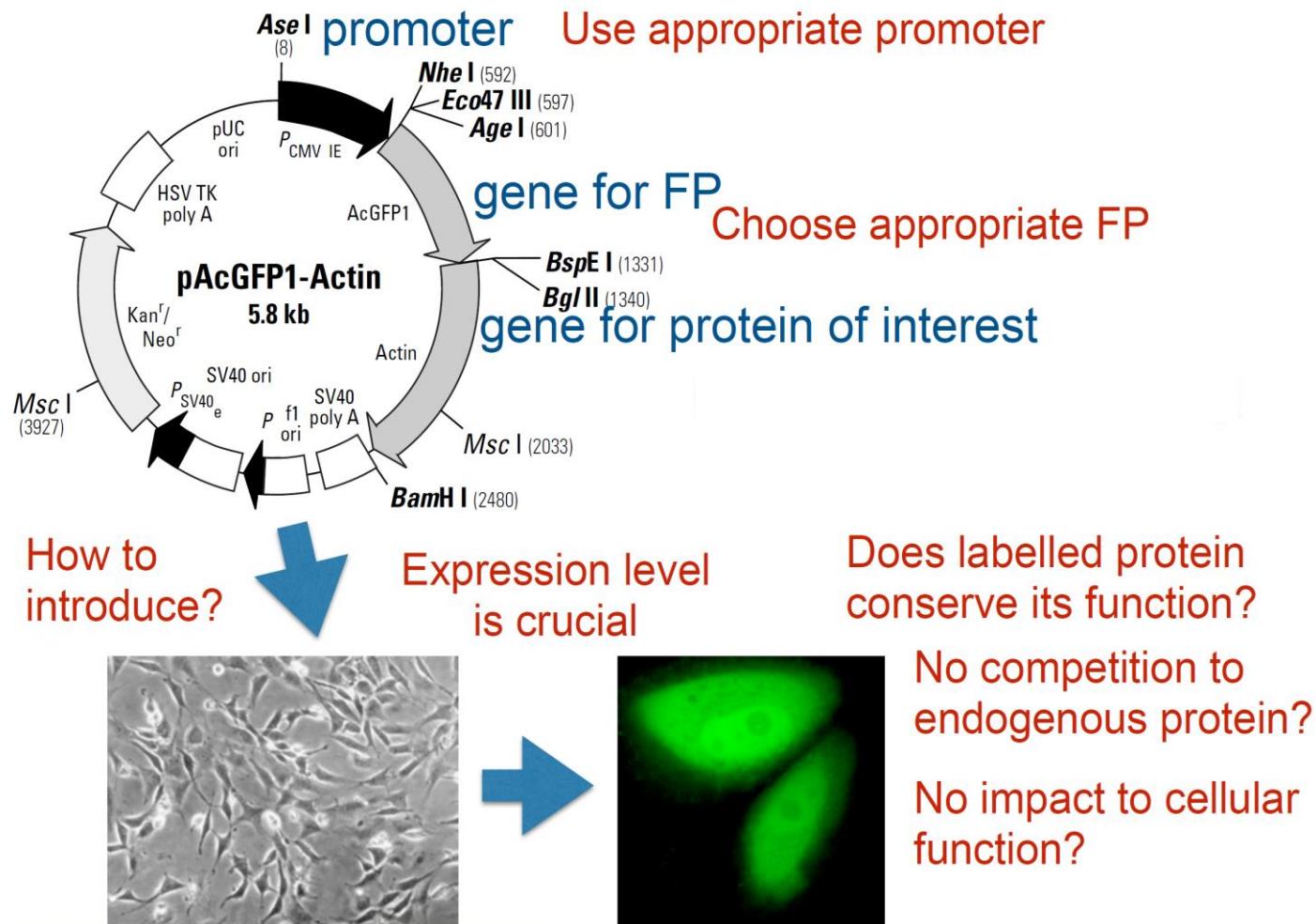
# Labeling your POI with a GFP



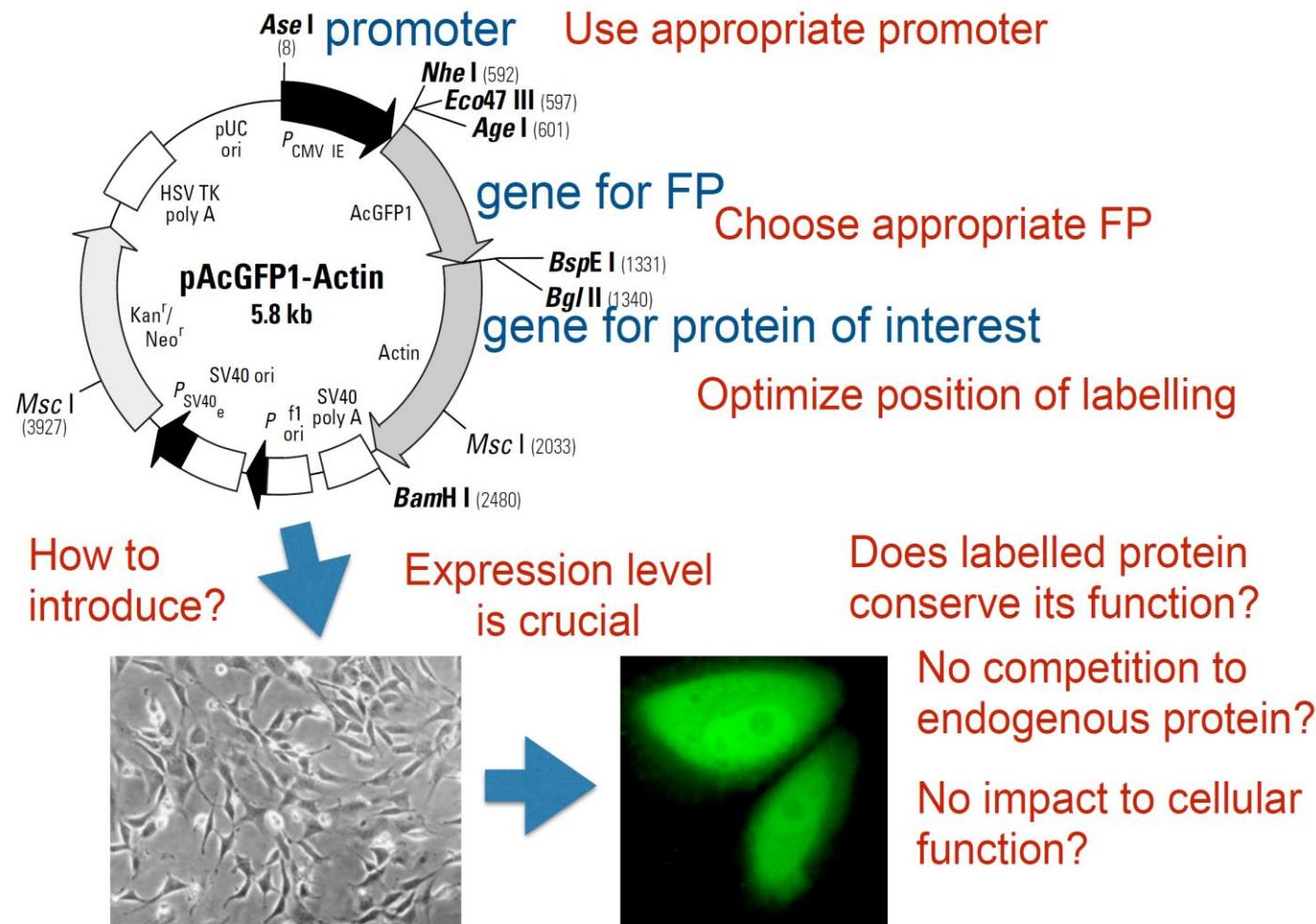
# Labeling your POI with a GFP



# Labeling your POI with a GFP



# Labeling your POI with a GFP



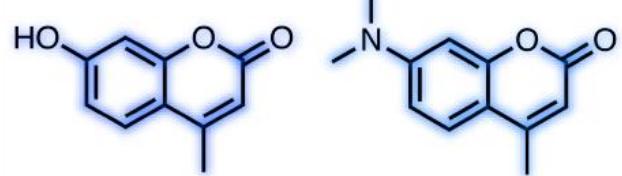
These questions are valid for FPs, and for other labeling strategies



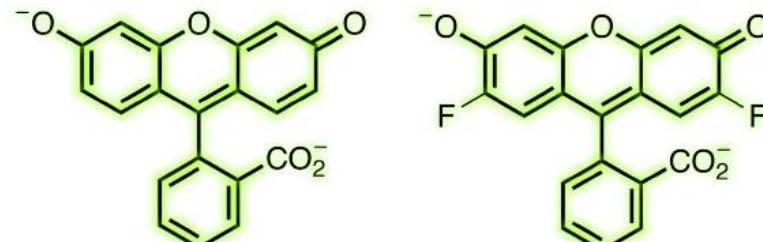
# Introduction to organic/synthetic fluorophores

# Organic fluorophores of different classes

Coumarins (~1884)



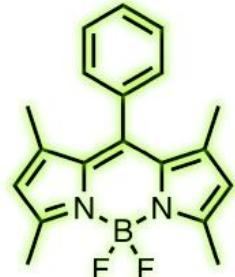
Fluoresceins (~1871)



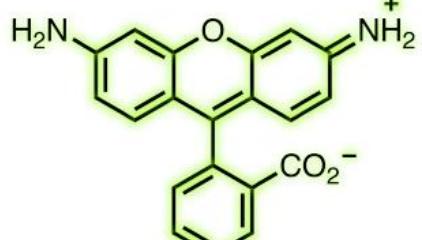
Fluorescein

Oregon Green

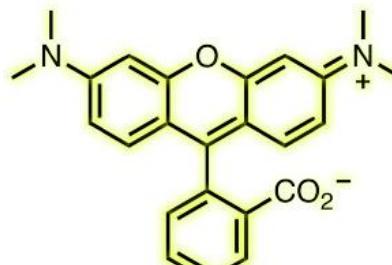
BODIPY (~1968)



Rhodamines (~1887)



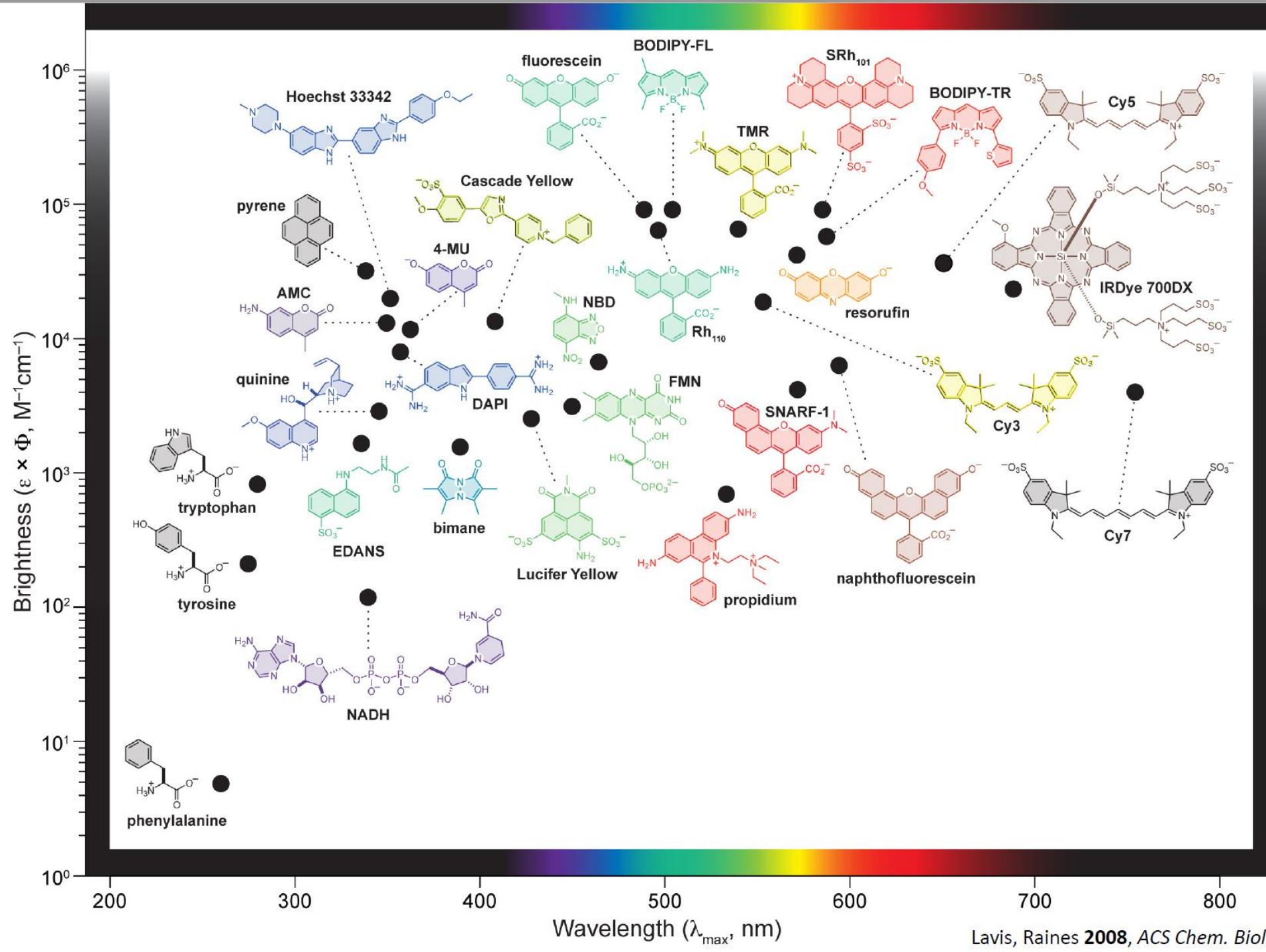
Rhodamine 110



Tetramethylrhodamine

Cyanines (~1922)





- Various brand names
  - Cy dyes (Cy 3, Cy5) : Amersham
  - Alexa fluor (Invitrogen)
  - DyLight (Thermo / Pierce)
  - Atto dyes (Atto-Tec)
  - Fluoprobes (Interchim)
  - Abberior
  - Dy and megastockes (Dyomics)
  - .....

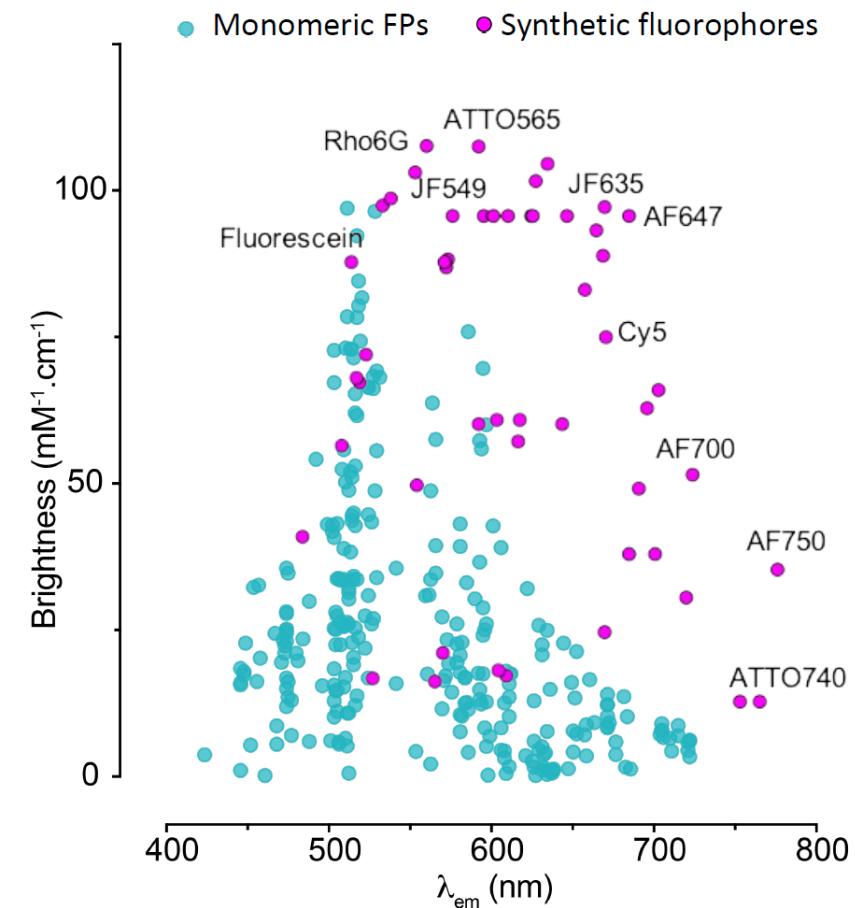
**THERE IS NO CORRELATION BETWEEN THE NAME  
AND THE STRUCTURE, AND THUS, THE  
PROPERTIES !!!**



# Synthetic fluorophores vs FPs

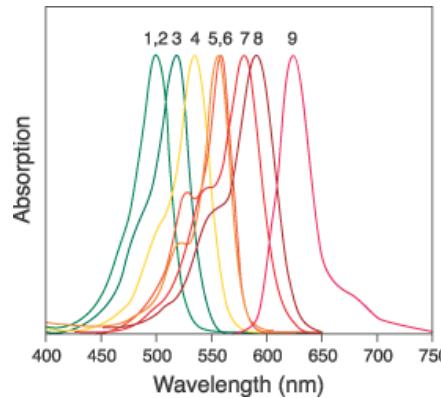
## Synthetic fluorophores

- Small size
- Brightness, photostability
- Relatively narrow exc/em spectra
- Tunability
- Functionalization
- Possible use without genetic engineering

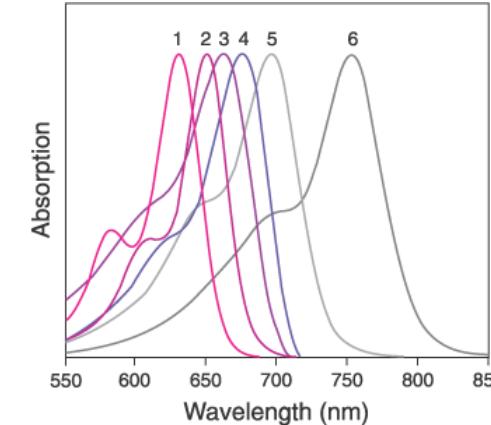


# Example of Alexa dyes

Derivatization of existing dyes to make them more soluble, more photostables, or to circumvent patents...

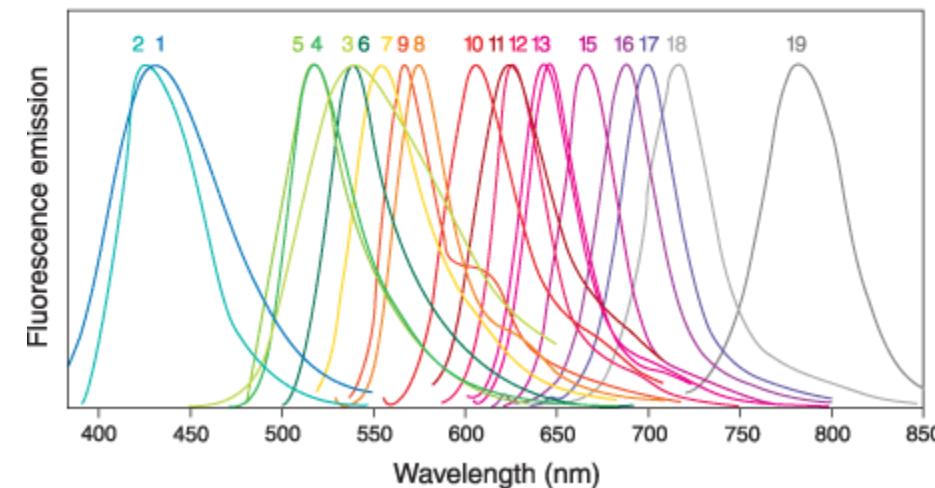


- 1 Alexa Fluor 488
- 2 Alexa Fluor 500
- 3 Alexa Fluor 514
- 4 Alexa Fluor 532
- 5 Alexa Fluor 555
- 6 Alexa Fluor 546
- 7 Alexa Fluor 568
- 8 Alexa Fluor 594
- 9 Alexa Fluor 610



- 1 Alexa Fluor 633
- 2 Alexa Fluor 647
- 3 Alexa Fluor 660
- 4 Alexa Fluor 680
- 5 Alexa Fluor 700
- 6 Alexa Fluor 750

- |                    |                     |                     |                     |
|--------------------|---------------------|---------------------|---------------------|
| 1. Alexa Fluor 350 | 6. Alexa Fluor 514  | 11. Alexa Fluor 594 | 16. Alexa Fluor 660 |
| 2. Alexa Fluor 405 | 7. Alexa Fluor 532  | 12. Alexa Fluor 610 | 17. Alexa Fluor 680 |
| 3. Alexa Fluor 430 | 8. Alexa Fluor 546  | 13. Alexa Fluor 633 | 18. Alexa Fluor 700 |
| 4. Alexa Fluor 488 | 9. Alexa Fluor 555  | 14. Alexa Fluor 635 | 19. Alexa Fluor 750 |
| 5. Alexa Fluor 500 | 10. Alexa Fluor 568 | 15. Alexa Fluor 647 |                     |

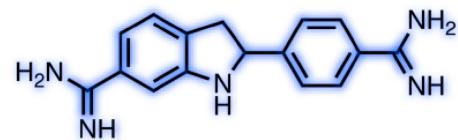


# How to turn a dye into a useful tool ?

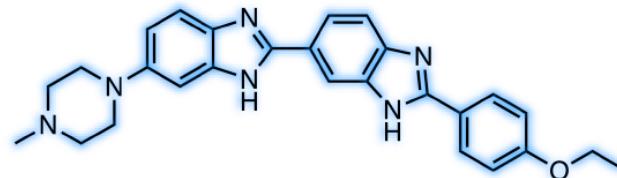
- To be useful, your fluorophores needs to have some « activity »
  - Cellular stains : DNA Intercalating dyes, dyes attached to specific small molecules (ex : lipids)
  - Dyes attached to self labeling tags
  - Dyes conjugated in vitro to your protein of interest (for in vitro experiments), to an antibody or a nucleic acid (for your cell microscopy experiments)

# Cellular stains

Fluorophores that accumulate at specific subcellular localization due to their intrinsic biochemical properties

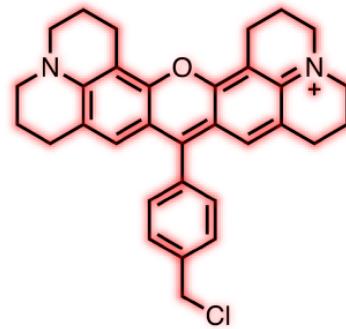


**DAPI**



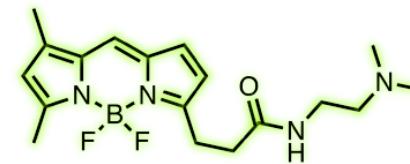
**Hoechst33342**

Bind to DNA  
➤ Nucleus



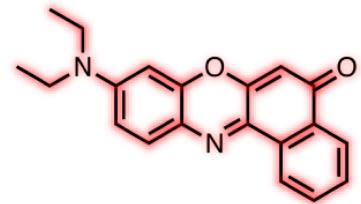
**Mitotracker Red**

Positively charged  
➤ Mitochondria



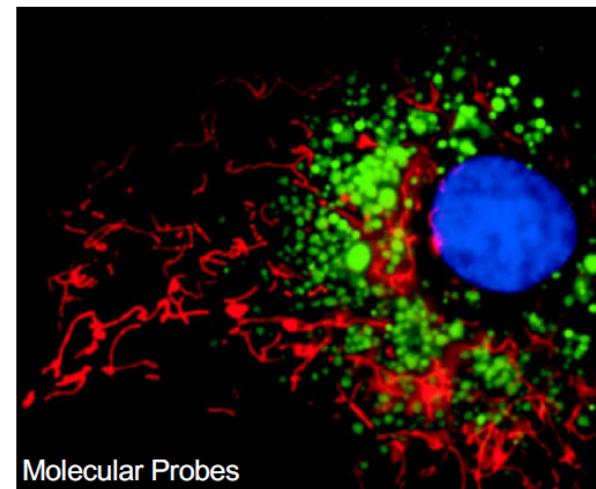
**Lysotracker Green**

Stains acidic compartments  
➤ Lysosomes



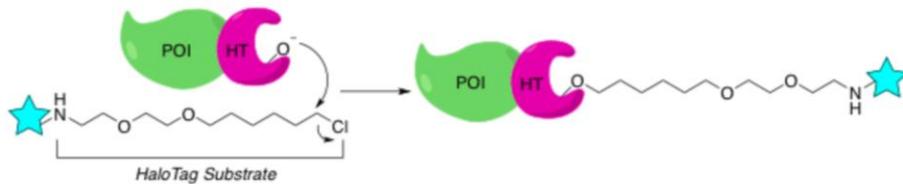
**Nile Red**

Lipophilic  
➤ Membranes, lipids



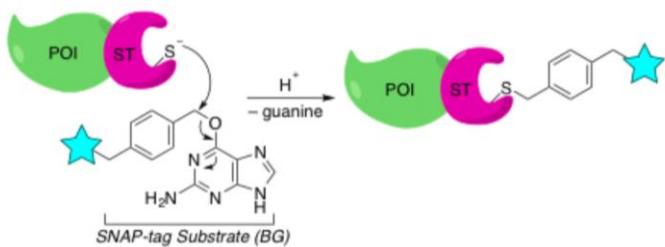
# Self labeling Tags

## Halo-Tag



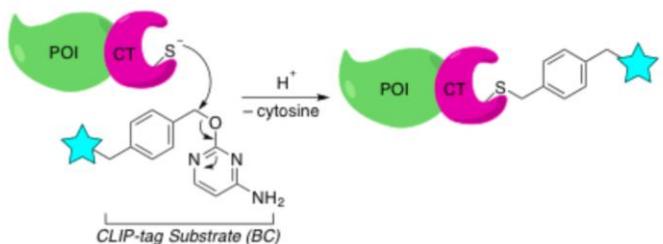
- Express your POI as a fusion with the self labeling protein

## SNAP-Tag



- Incubate with the fluorophore having the right reactive group
- Wash to remove excess dye

## CLIP-Tag

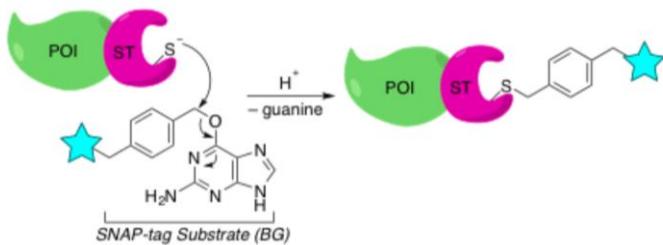


# Self labeling Tags

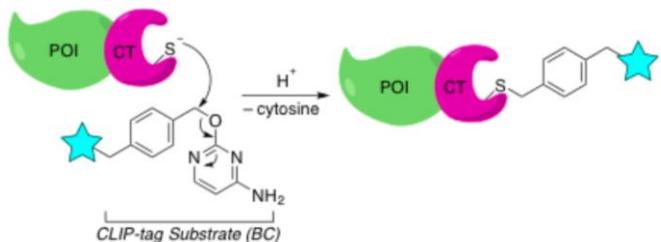
## Halo-Tag



## SNAP-Tag



## CLIP-Tag



- Broad choice of fluorescent dyes

- Higher fluorescence intensity and photostability

- Fluorescence initiated when the label is added (temporal control)

- A single construct is sufficient to label with different fluorophores (pulse chase)

- Live cell labeling



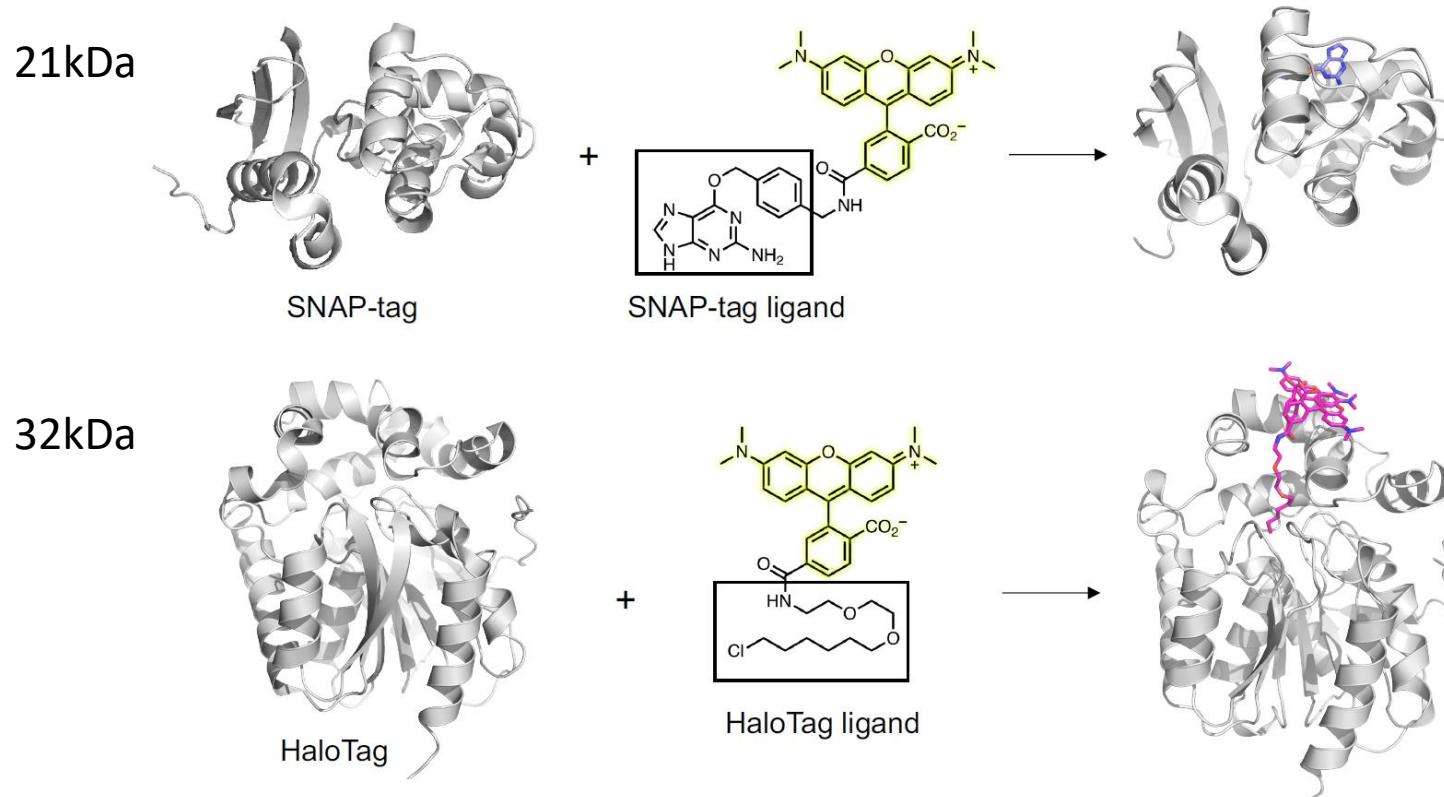
- Non-specific staining

- Bulky ( 20-33 kDa) fusion

- N or C-ter fusion preferred



# Self labeling Tags

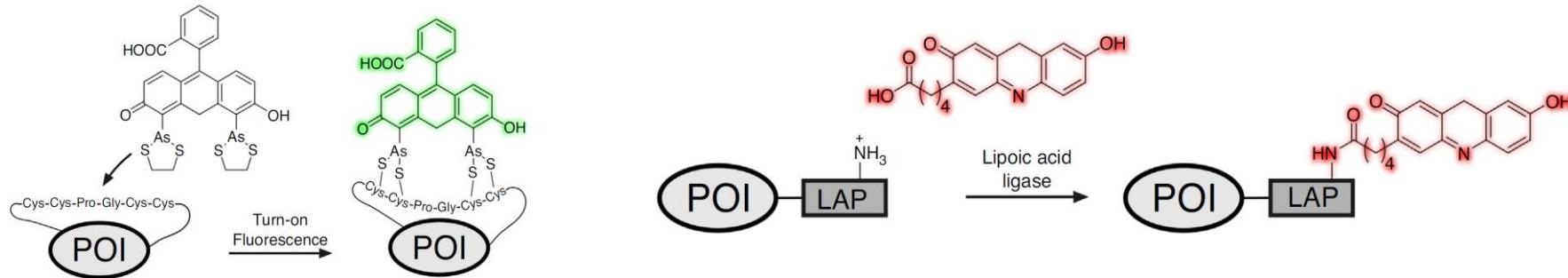


Keppler *et al*, 2003 *Nat. Biotechnol.*; Los *et al*, 2008 *ACS Chem. Biol.*



# Peptide-based labeling Tags

His tag – Ni <sup>2+</sup> – NTA	One-step	Non-covalent	10 amino acids
D4 tag – DpaTyr	One-step	Non-covalent or covalent	4–12 amino acids
Tetracysteine (CCXXCC)	One-step	Covalent	6 amino acids
dC10 $\alpha$	One-step	Covalent	22 amino acids
Sortase A	Two-step	Covalent	6 amino acids
LpIA	One-step or two-step	Covalent	13 amino acids



- Small size of the fusion
- Fluorescence initiated when the label is added (temporal control)
- Live cell labeling



- Very limited choice of dyes
- Non-specific staining
- N or C-ter fusion required (except Tetracys & LpIA)
- No experience at CBS 😊

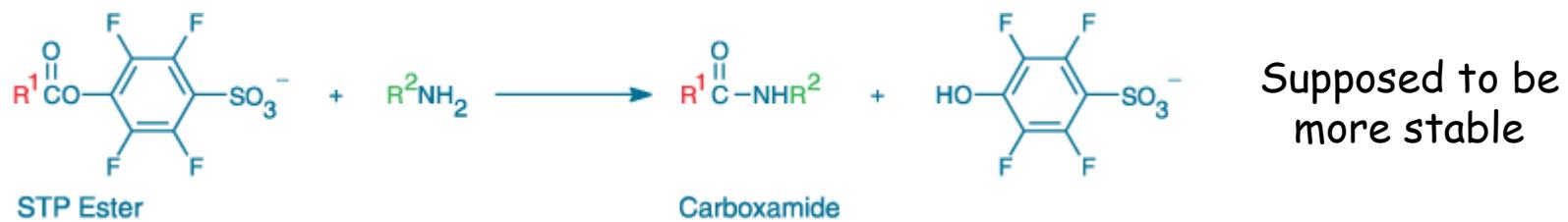
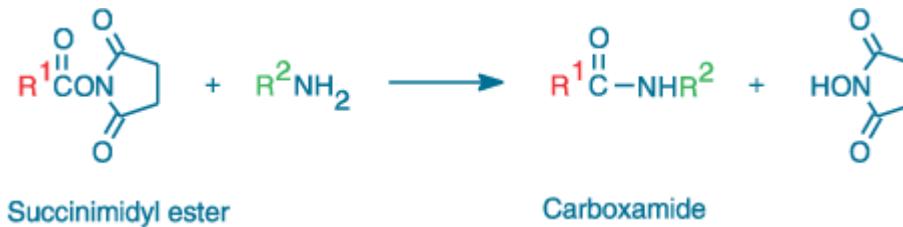


# How to label your molecule of interest with fluorophores

## REACT ON PRIMARY AMINES

Proteins : N-terminal or lyzine

Oligonucleotides : NH<sub>2</sub> added during synthesis



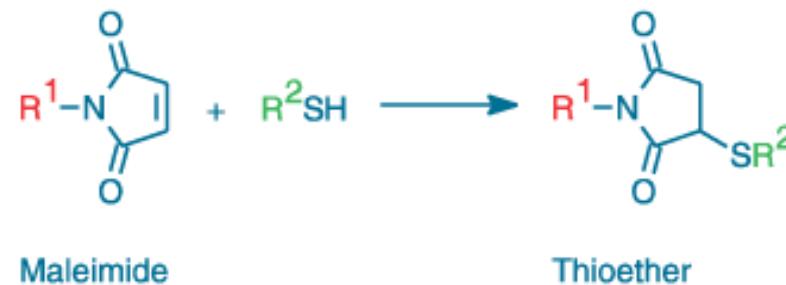
Around pH7, the lyzines are protonated, and thus only the N-terminus is reactive.



# How to label your molecule of interest with fluorophores

## REACT ON THIOLS

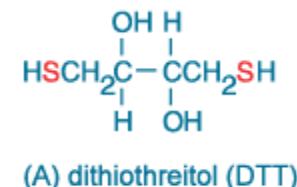
Proteins : cysteins



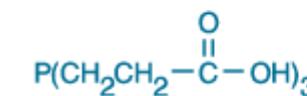
Maleimide

Thioether

TO REDUCE CYSTEINS, USE TCEP



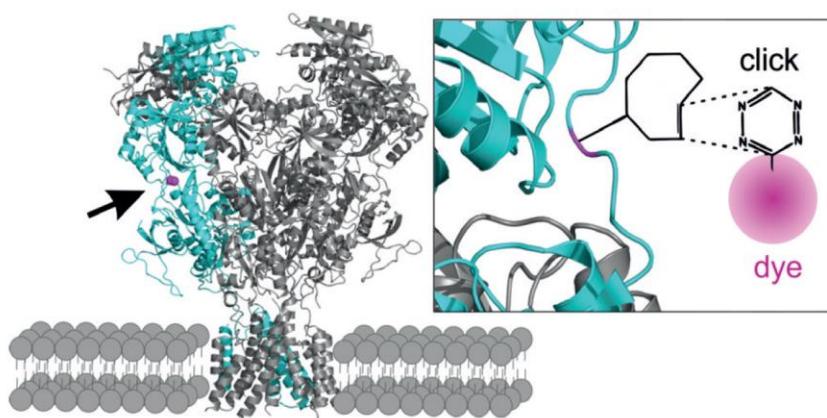
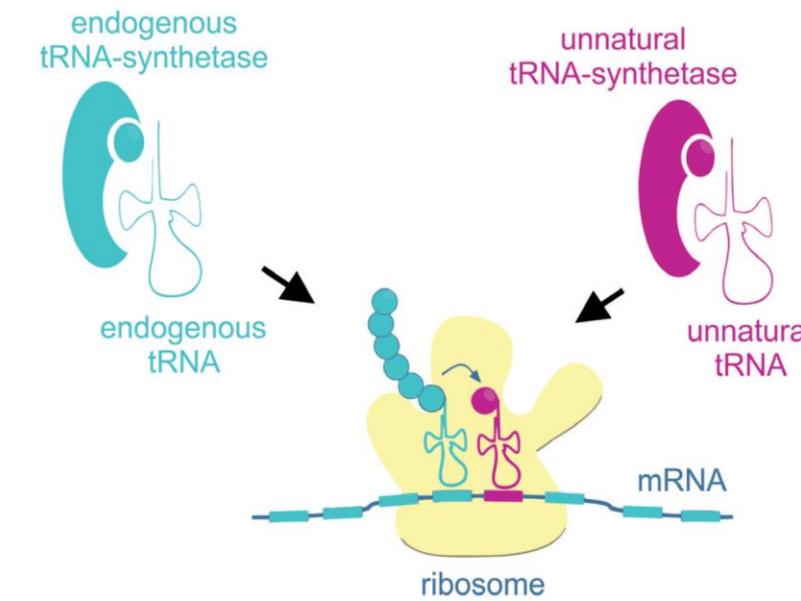
(A) dithiothreitol (DTT)



(B) tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)



# Labeling unnatural aminoacids



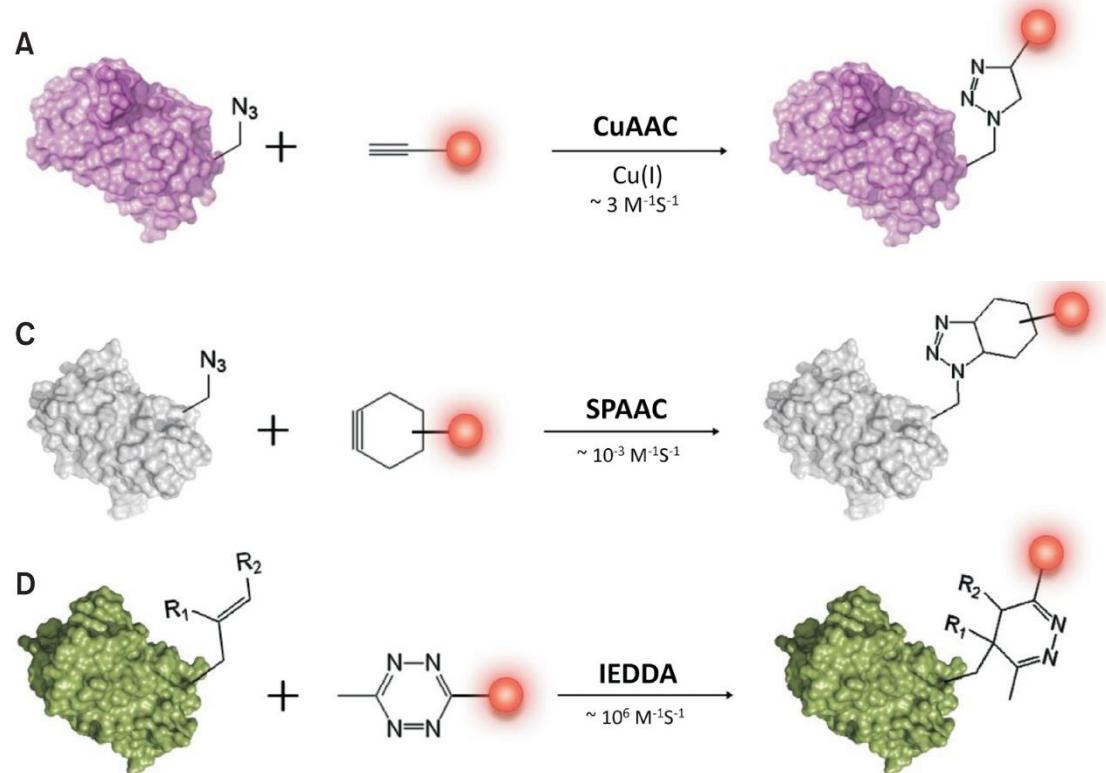
- Smallest size of the fusion
- Fluorescence initiated when the label is added (temporal control)
- Vast (and increasing) choice of fluorophores
- Orthogonal labeling possible
- Live cell labeling (beware of  $\text{Cu}^{2+}$ )



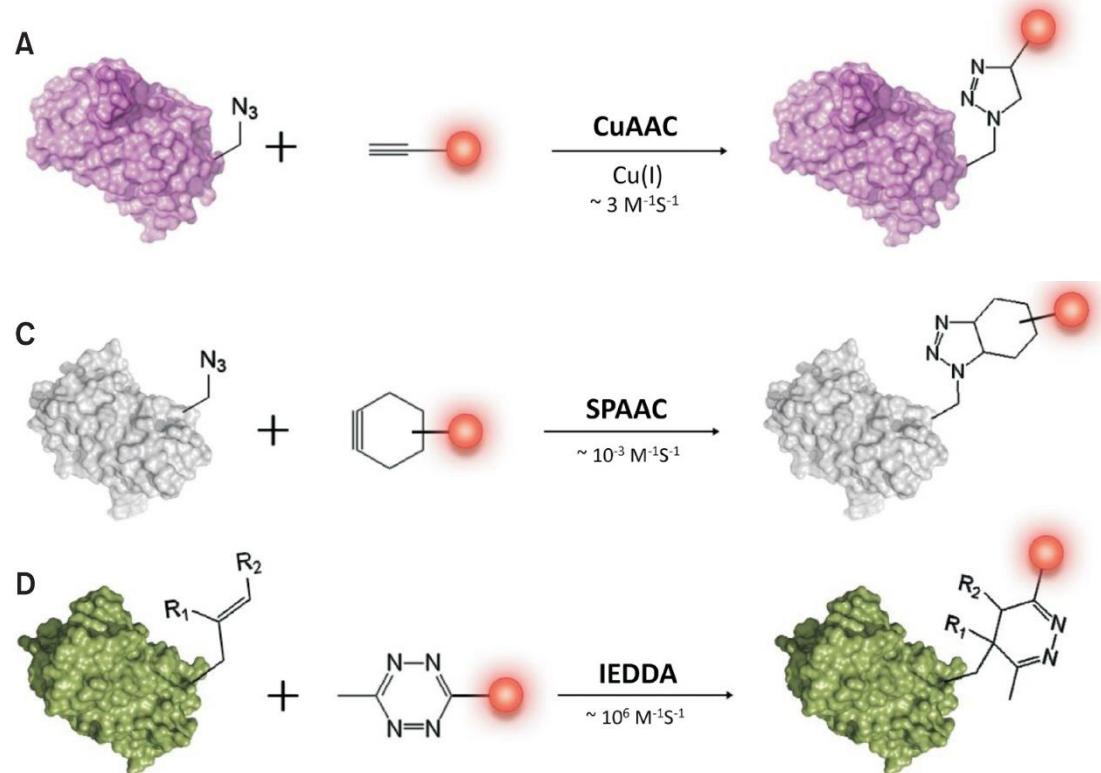
- Non-specific staining
- Non specific incorporation of the UAA
- Reagents are expensive



# Labeling unnatural aminoacids



# Labeling unnatural aminoacids

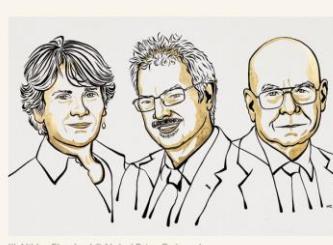


The Nobel Prize in Chemistry 2022

The 2022 chemistry laureates

The Nobel Prize in Chemistry 2022 was awarded to Carolyn R. Bertozzi, Morten Meldal and K. Barry Sharpless "for the development of click chemistry and bioorthogonal chemistry".

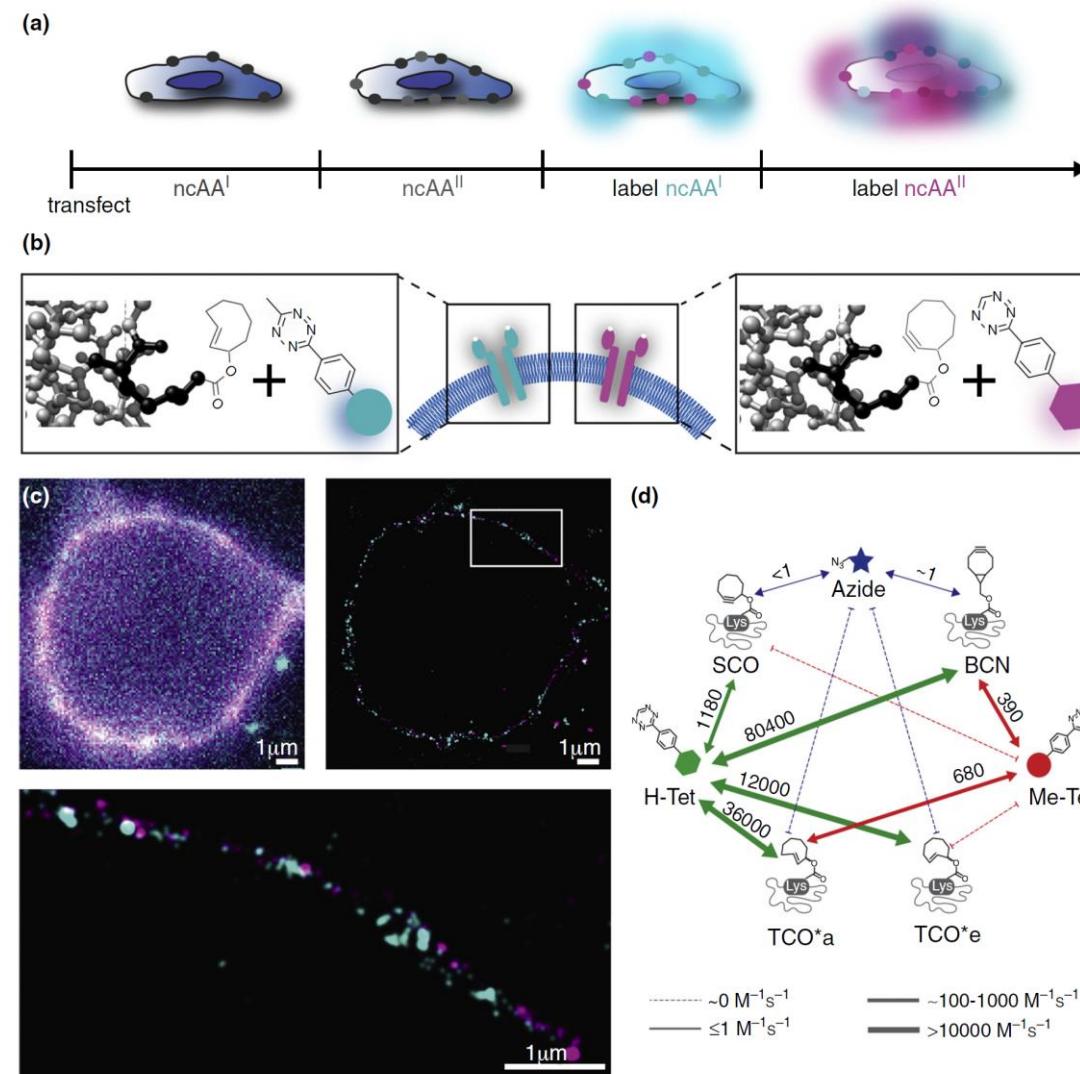
Sharpless and Meldal have laid the foundation for a functional form of chemistry – *click chemistry* – in which molecular building blocks snap together quickly and efficiently. Bertozzi has taken click chemistry to a new dimension and started utilising it in living organisms.



III, Niklas Elmehed © Nobel Prize Outreach



# Labeling unnatural aminoacids

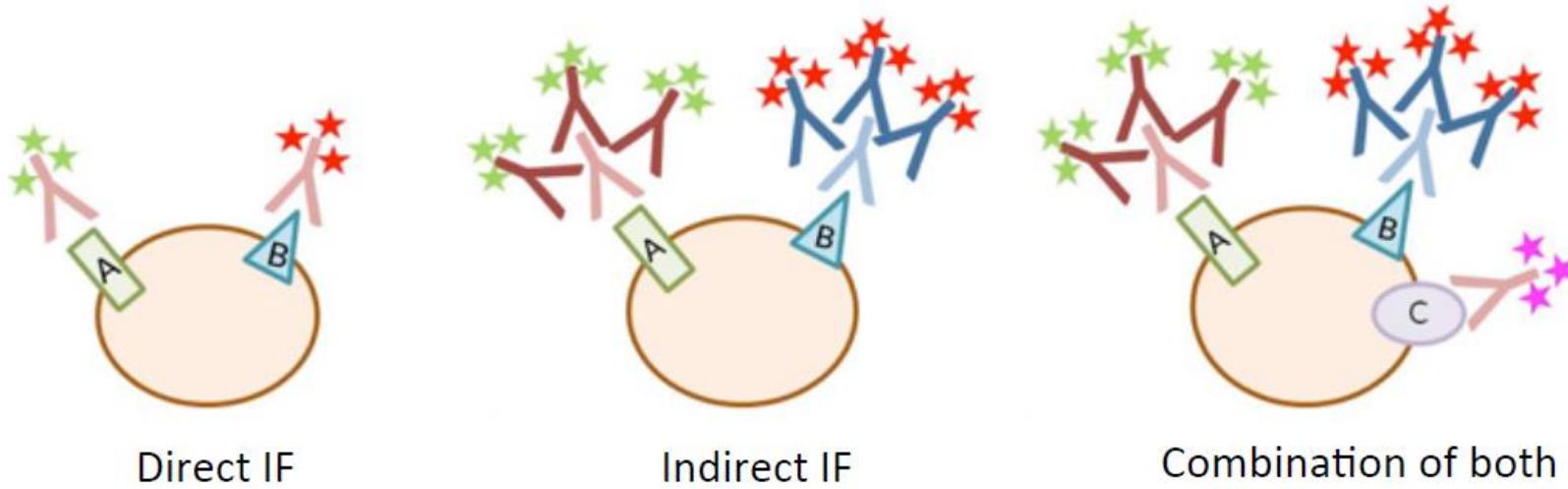


# Fluorescence & Labeling Strategies

- *Basis of fluorescence & Properties of fluorophores*
- *Fluorescent proteins*
- *Organic dyes*
- *Fluorophores photophysics*
- *Protein labeling in live cells (FPs, Self-labeling tags, UAA)*
- ***Labeling in fixed cells (Immunostaining, non-specific stains  
(mb, nucleic acids, etc...), FISH***



# Immuno-fluorescence (IF)



Endogenous proteins

Highly flexible

Multi-colour labeling (if Abs are raised in different hosts)

Signal amplification



Fixed cells

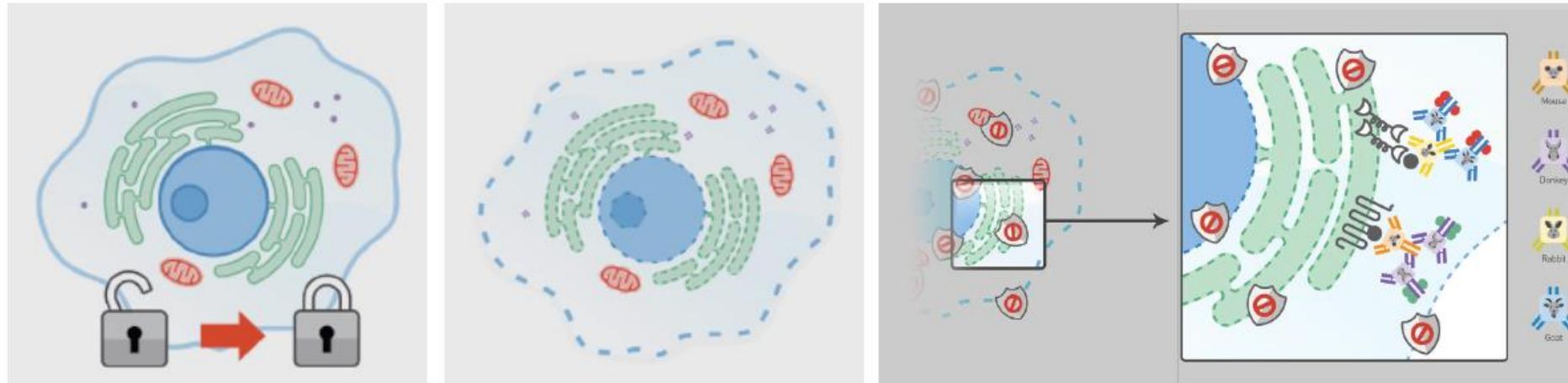
Antibody availability + epitope accessibility

Non-specific background

Size of antibody => loss in spatial resolution / image reconstruction



# Immuno-fluorescence : experimental workflow



## 1) Chemical fixation

PFA, glutaraldehyde,  
methanol

## 2) Permeabilization

Detergents (Triton, SDS,  
saponin, digitonin...)

## 3) Blocking

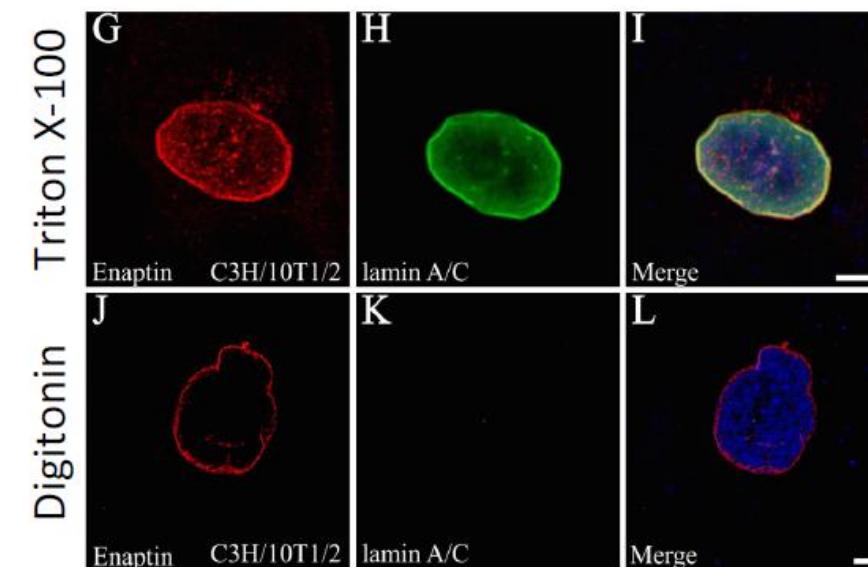
BSA, goat serum...

## 4) Incubation with primary ± secondary antibody

**Variables to optimize:** fixation agent, time and temperature, detergents, blocking agent, antibody concentration and incubation time

These depend on

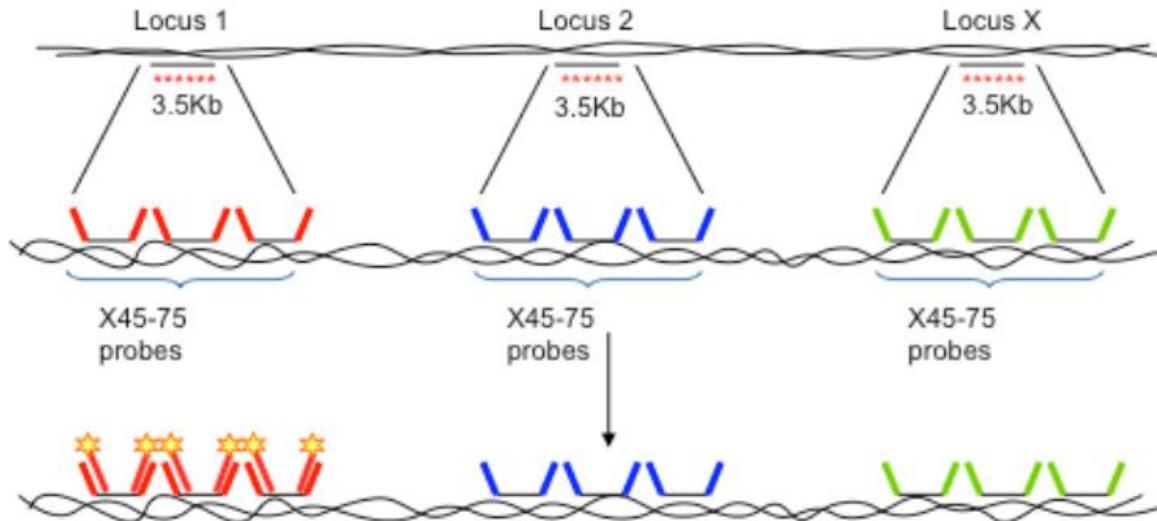
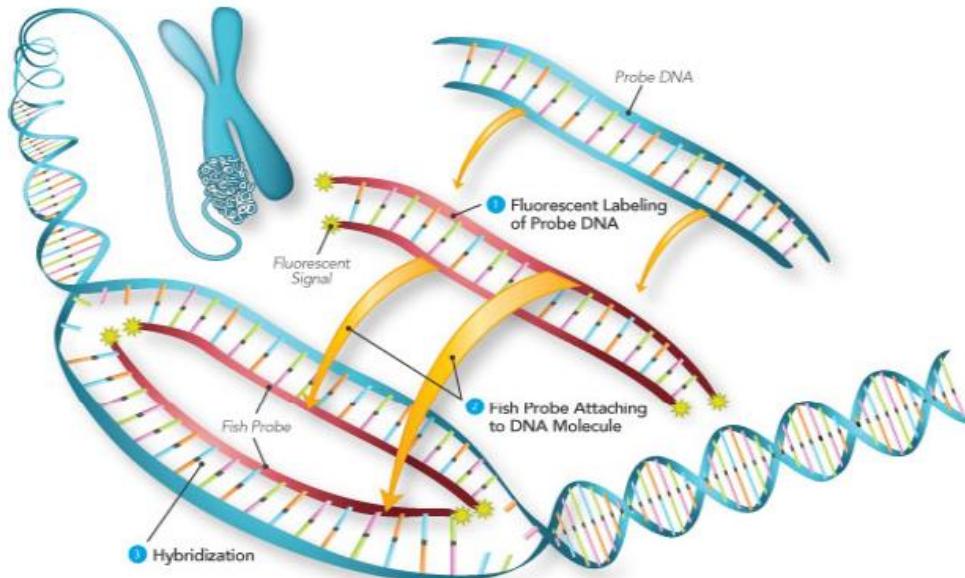
- sample
- antibody
- imaging method



# Labeling specific genomic loci: DNA FISH

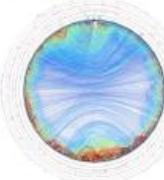
## Fluorescent *in situ* hybridization

- 1- Design a set of DNA probes for target locus
- 2- Fix cells
- 3- Denaturation and annealing
- 4- Post-fixation
- 5- Imaging

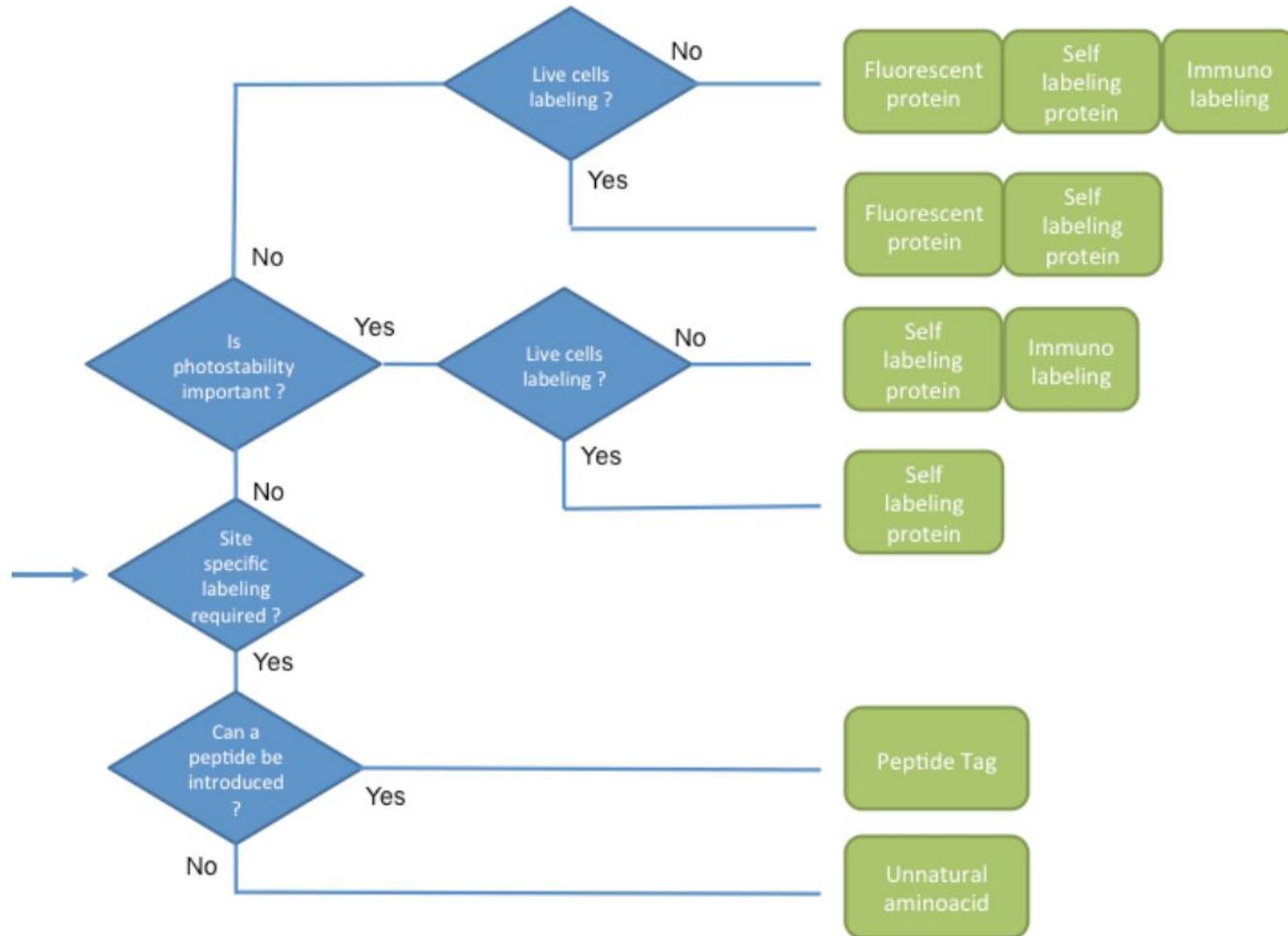


Multiplexed DNA FISH

Marcelo Nollmann's team



# What should I ask myself before choosing a labeling strategy ?



# Introduction to fluorophores photophysics

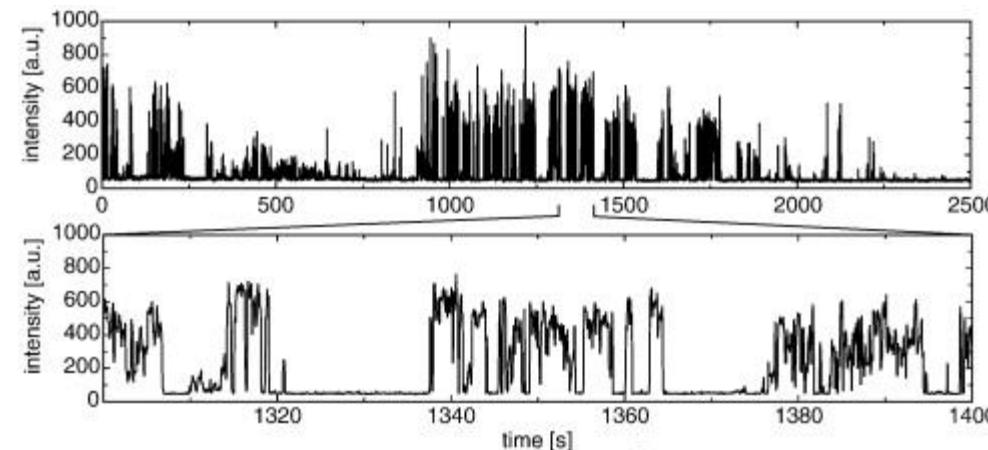
## Photodestruction of the fluorophore :

- Mainly due to  $O_2$ 
  - Interaction with long lived excited states (Triplet)
  - Production of radicals



# Blinking

All fluorophores show blinking, except nitrogen vacances in nanodiamonds, and some specially engineered QD



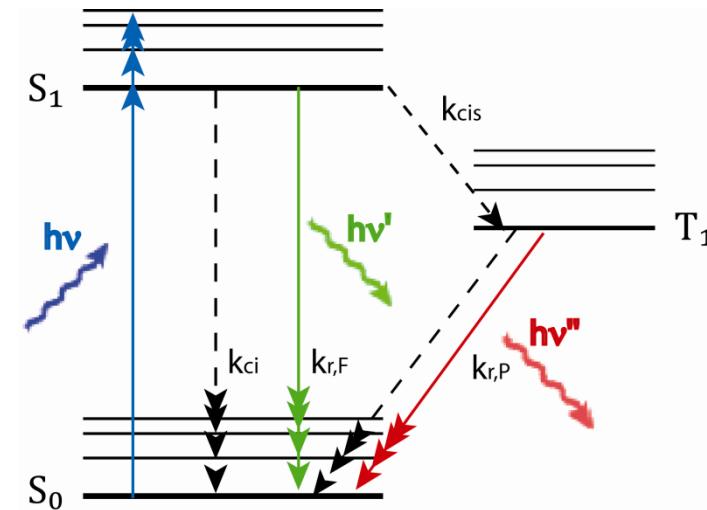
QD blinking at various time scales



# Blinking

- Triplet blinking

- Random event
- Triplet state is long lived( $\mu\text{s}$ -ms) . appear as blinking
- Quenched by  $\text{O}_2$
- Quenched by reducing agents (MEA, BME, Trolox)



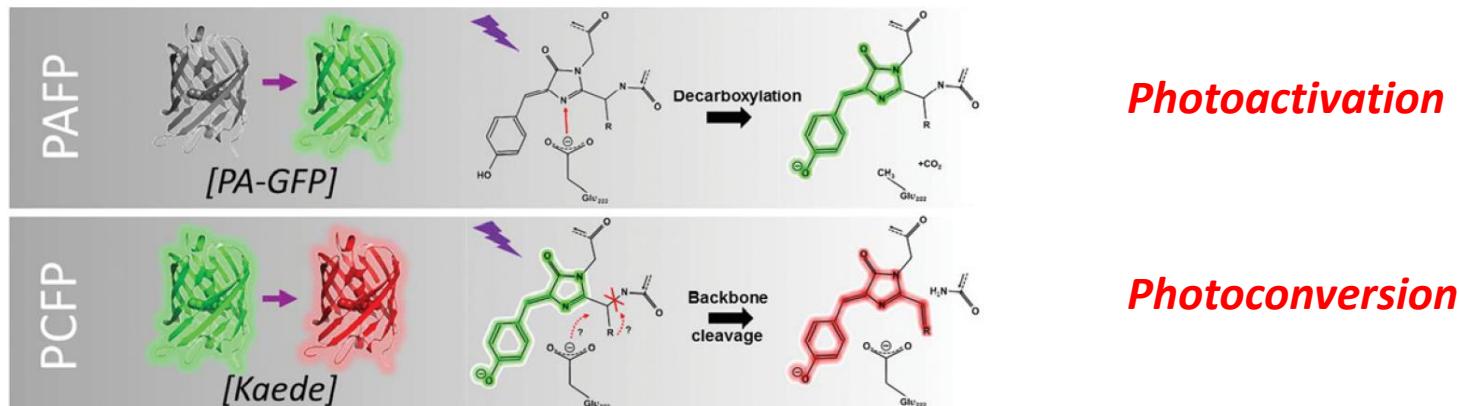
- Redox blinking

- Photoinduced electron transfer
- Higher probability in the triplet state
- The ions are not fluorescent
- Triplet state quenchers help restoring the ground state

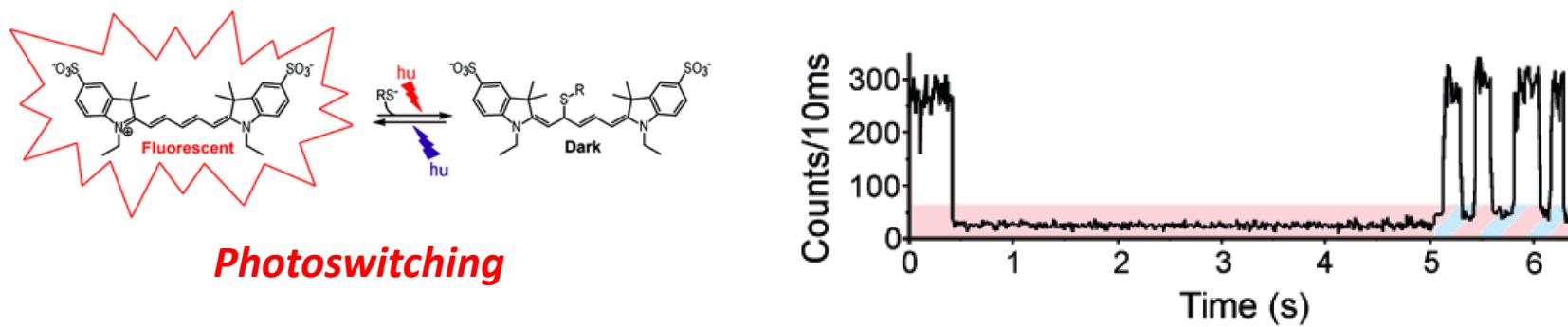


# Photochromism

- Reversible photoinduced reaction between 2 chemical species
- Seen in the FPs

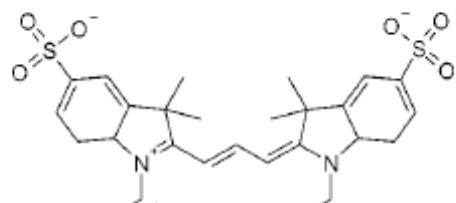


- Cy5 / thiol : dark state, that can be restored by UV light



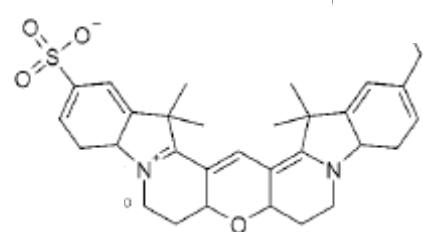
# Photoisomerization

- Cyanines



- FPs

Cy3 : only the *cis* is fluorescent



- Rigidified version
- no cis-trans isomerization
- higher QY

cy3B

