

# Causes of fluorescence and bioluminescence

- **Luciferase / luciferin**
- **Fluorescent proteins**
  - Also mediate color without fluorescence
- Orange – phycoerythrin in red algae, cyanobacteria, some corals
- Pigments

# Fluorescent proteins

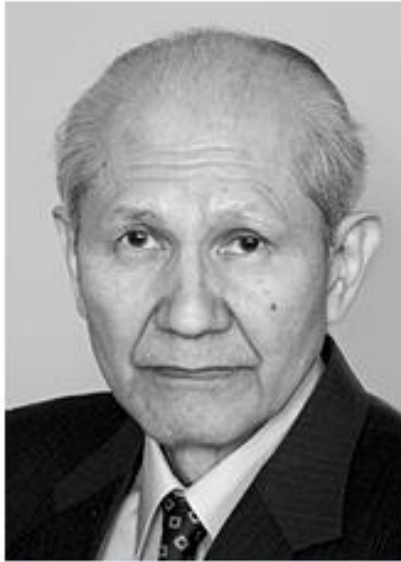


Photo: U. Montan

**Osamu Shimomura**



Photo: U. Montan

**Martin Chalfie**



Photo: U. Montan

**Roger Y. Tsien**

Chemistry



## The Nobel Prize in Chemistry 2008

"for the discovery and development of the green fluorescent protein, GFP"



Photo: U. Montan

**Osamu Shimomura**

🕒 1/3 of the prize



Photo: U. Montan

**Martin Chalfie**

🕒 1/3 of the prize



Photo: U. Montan

**Roger Y. Tsien**

🕒 1/3 of the prize

 Printer Friendly

 Comments & Questions

 Tell a Friend

**The 2008 Prize in:**

Chemistry

🕒 Prev. year   Next year 🕒

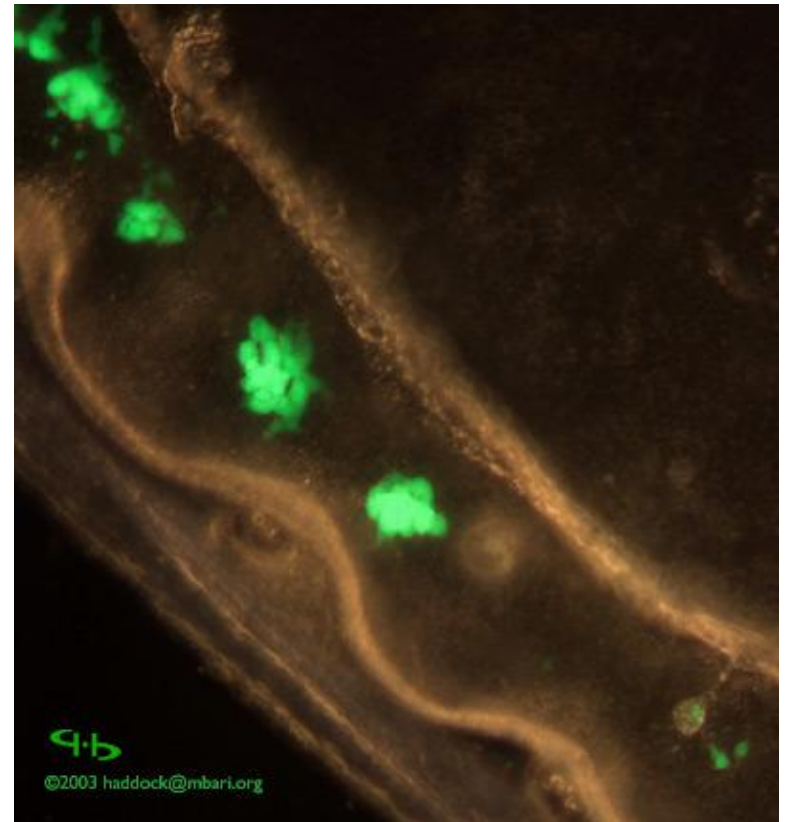
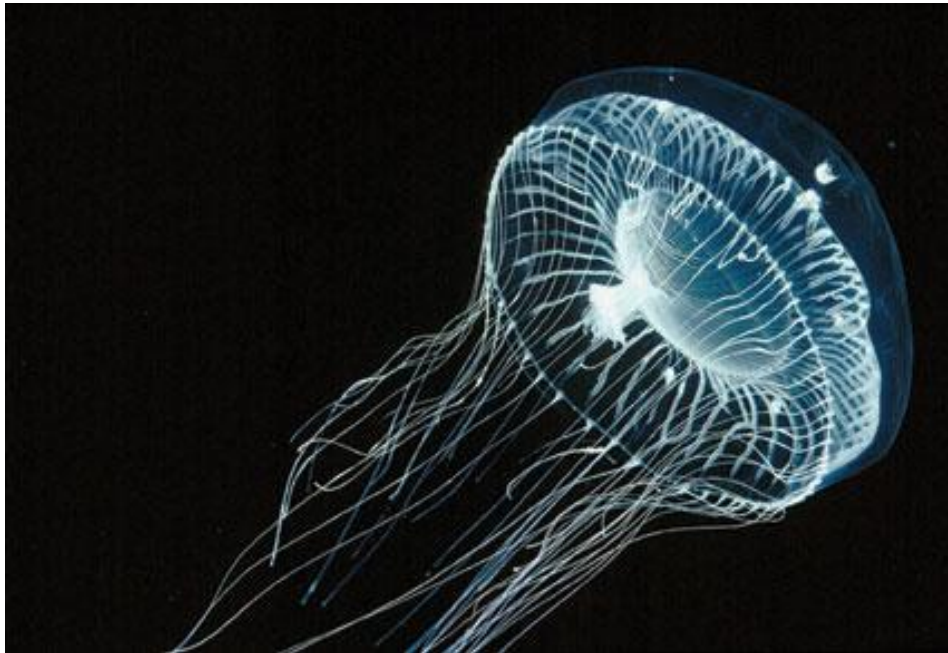
**The Nobel Prize in Chemistry 2008**

- Prize Announcement
- Press Release
- Scientific Background
- Information for the Public
- Presentation Speech
- Illustrated Presentation
- Speed Read

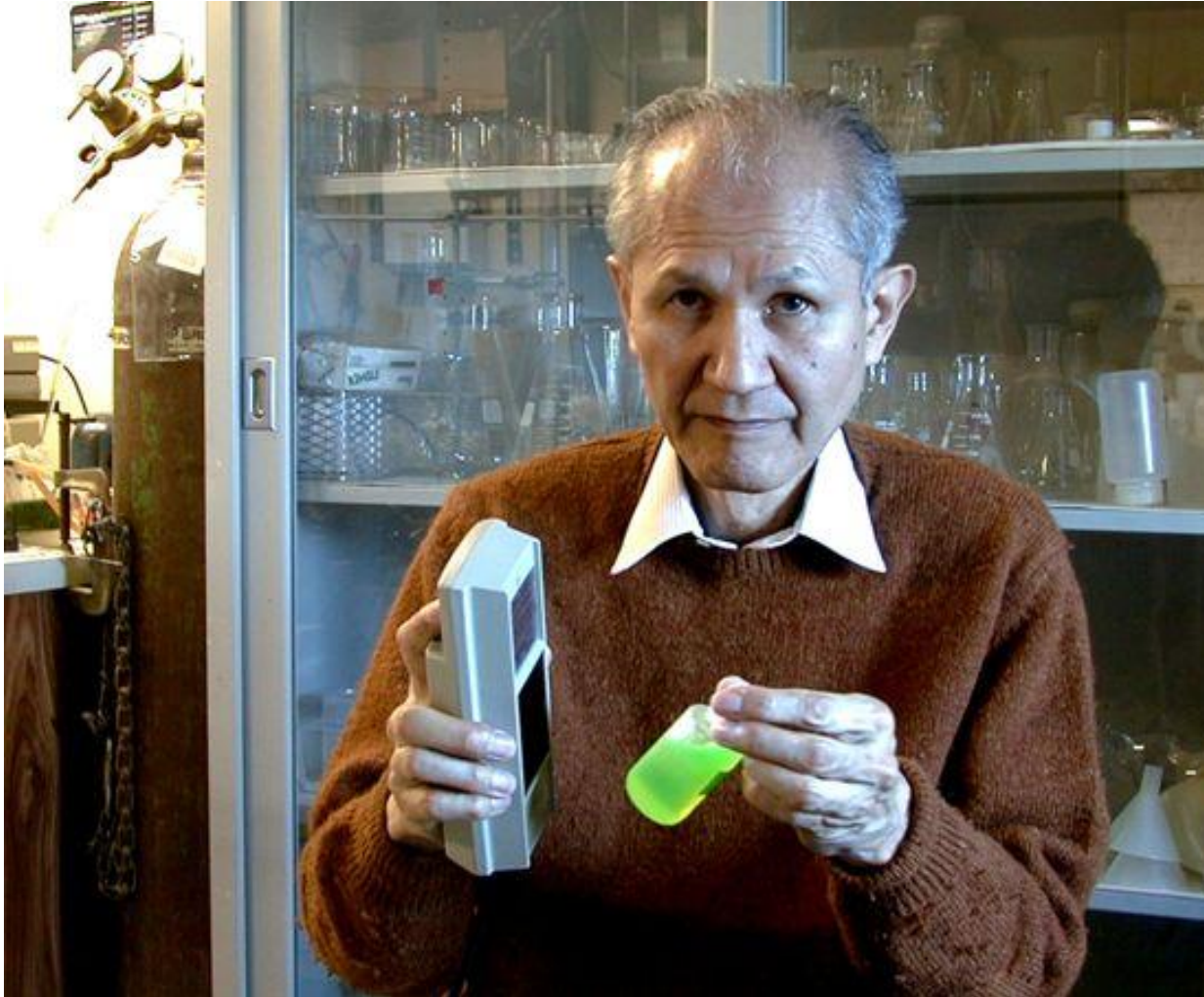
**Osamu Shimomura**

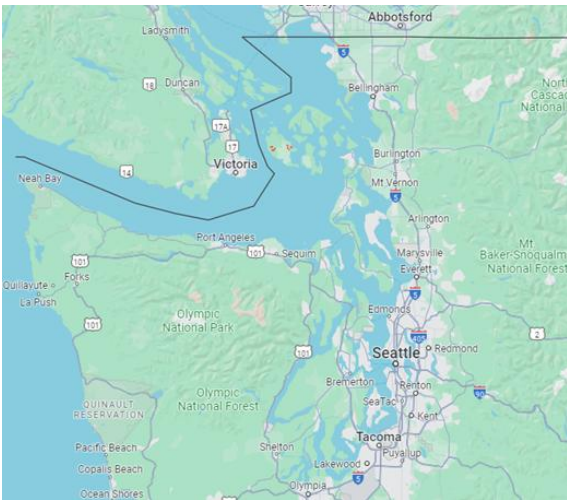
- Autobiography
- Nobel Lecture
- Interview
- Documentary
- Nobel Diploma
- Photo Gallery

Where **G**REEN **F**LUORESCENT **P**ROTEIN  
- **GFP** comes from



# Osamu Shimomura (1928 - 2018)



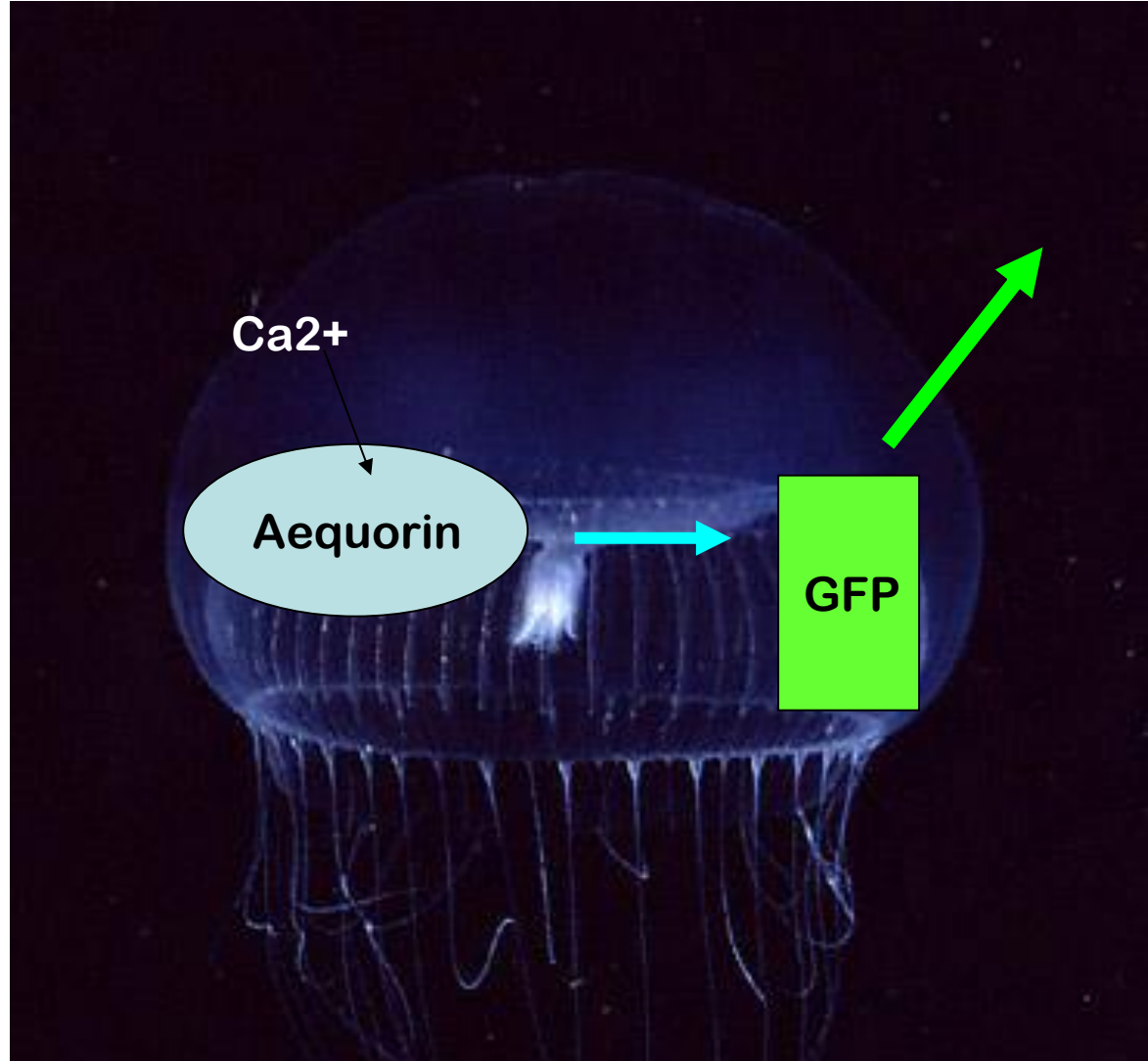


Over the summers of **19 years**, Shimomura and co-workers collected **85.000** jellyfish off Friday Harbor in Washington on the West Coast of North America.

An undertaking it is difficult to contemplate in modern day science.

# GFP natural function

- Aequorin emits **blue light** in the presence of calcium
- Aequorin binds to GFP
- Blue light from Aequorin excites GFP and GFP emits **green light**



# The Bioluminescence of Green Fluorescent Protein in *Aequorea victoria*

*Aequorea victoria* is known for its naturally occurring green fluorescence around the ring of its bell, in large thanks to the presence of two fluorescent proteins: Aequorin and Green Fluorescent Protein (GFP).

The process begins when calcium ions bind to the chemiluminescent Aequorin, a protein with  $Ca^{2+}$  in the middle of each of its two subunits. Once the calcium is bound, the chromophores begin to emit blue light at 470nm.

**Aequorin:**



**Chromophore**



**Coelenterazine:** a luciferin compound that fluoresces upon oxidation by calcium

Blue light is emitted at 470 nm

The light emitted by Aequorin's chromophore causes the chromophore of GFP to become excited.



**GFP**



**GFP Chromophore**

GFP contains a naturally built chromophore consisting of a special sequence of 3 amino acids: Serine, Tyrosine, and Glycine. When the chromophore receives blue light from aequorin, it becomes excited and emits its very own green light.

**GFP - excited state**



*Dipso*

# GFP

- **1962** - GFP purified from 10,000 jellyfish *Aequorea victoria*
- **1969** "Green protein" named **green fluorescent protein**.
  
- **1992** - sequence of GFP published in *Gene*

*Gene*, 111 (1992) 229–233

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225

GENE 06296

## **Primary structure of the *Aequorea victoria* green-fluorescent protein**

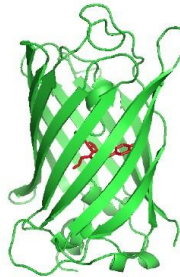
(Bioluminescence; Cnidaria; aequorin; energy transfer; chromophore; cloning)

**Douglas C. Prasher<sup>a</sup>, Virginia K. Eckenrode<sup>b</sup>, William W. Ward<sup>c</sup>, Frank G. Prendergast<sup>d</sup> and Milton J. Cormier<sup>b</sup>**

- **1994** - GFP gene sequence alone mediates fluorescence
  - No need for co-factors
  - Expressed in neurons

- **1995** - **S65T** mutation discovered
  - Improved emission

- **1996** - structure determined

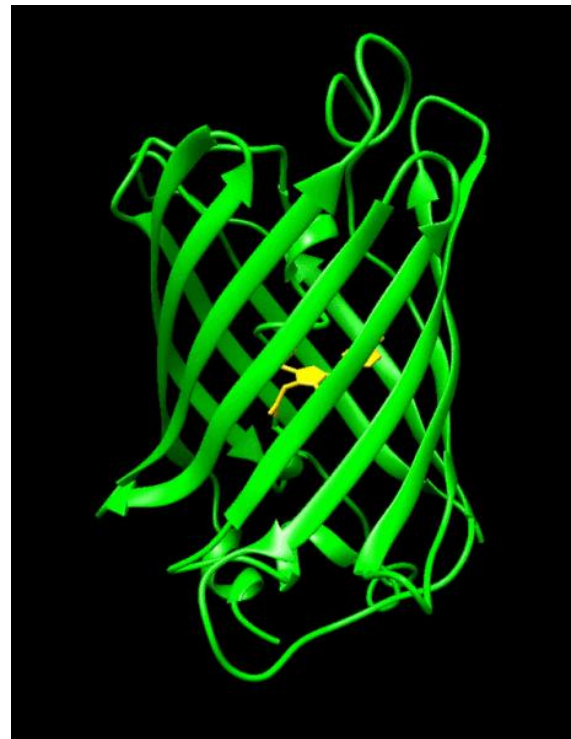


- **1996** - Improved version evolved by DNA shuffling

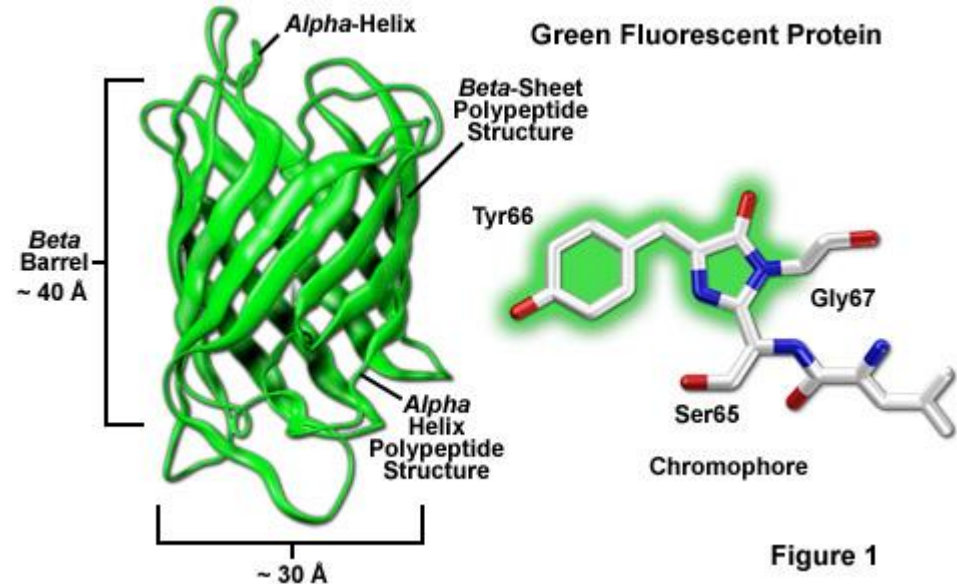
- **1999** - fluorescent proteins from coral

# GFP properties

- 239 aa, 28 kDa
- 11 stranded beta can
- 6 loops at each end



- **Chromophore** protected from Outside
- Chromophore **forms alone**



# GFP structure

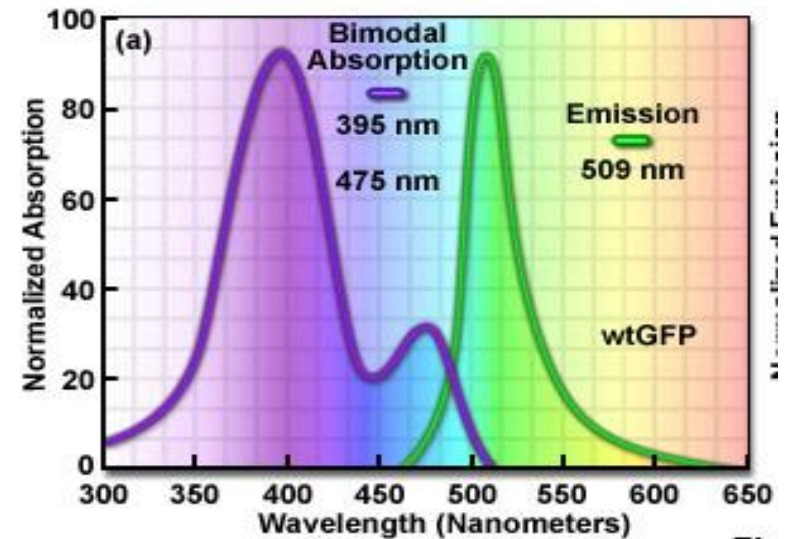
- **Stable:** 10M urea, 85° C, pH 4-11
- **very well Expressed** 80 mg / liter -> 5g / liter
- Monomeric when expressed in cytoplasm
  - Can be dimeric at high concentrations
- **Folds slowly** - t/2 **5-50 minutes** from denatured and preoxidised
- Does not tolerate insertions
- Stable chromophore **requires no other cofactors**
- Detection limits (no. GFP molecules):
  - CHO 10,000 cytoplasm; 2,000 on surface
  - Bacteria 1,000 cytoplasm; 400 on surface

**The original wt GFP had several major drawbacks** that reduced its effectiveness as a tool for cell imaging.

- Its **folding efficiency** and thus fluorescent signal drops dramatically at physiologically relevant temperatures such as 37°C,
- its **maturation rate** is very slow and it had a strong tendency to aggregate.

- **Two separate excitation peaks** were also observed due to the coexistence of neutral ( $\lambda_{ex} \sim 395$  nm) and phenolate ( $\lambda_{ex} \sim 475$  nm) forms of the chromophore.
- Excitation at  $\sim 475$  nm was preferred as its lower energy is less damaging to the cell. However, the 475 nm excitation wavelength was only a minor contributor to fluorescence ( **$\sim 15\%$  compared to excitation at  $\sim 395$  nm**).

**Absorption and Fluorescence Emission Spe**



**Fig**

Che approccio Biotecnologico usereste per migliorare le «qualità» della GFP ?

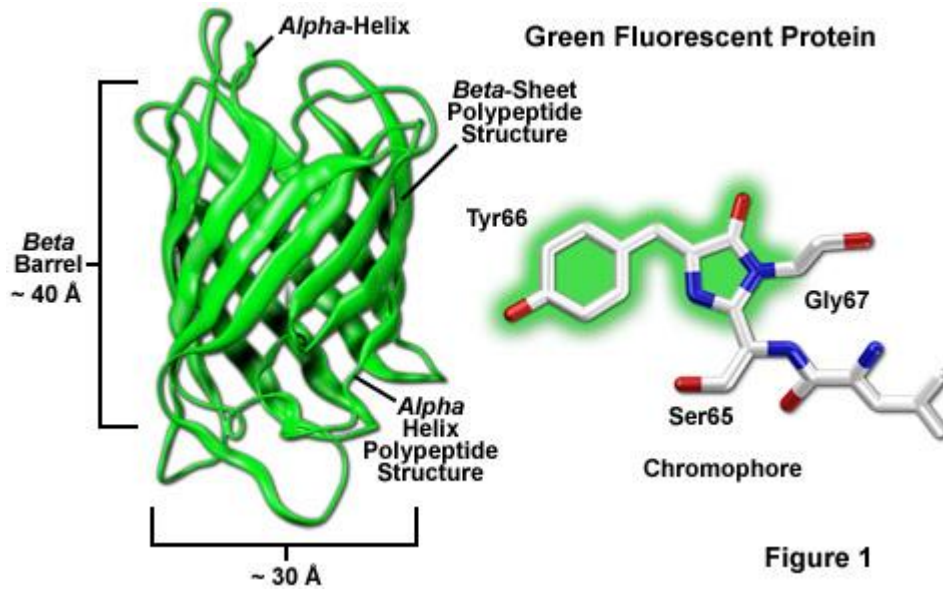
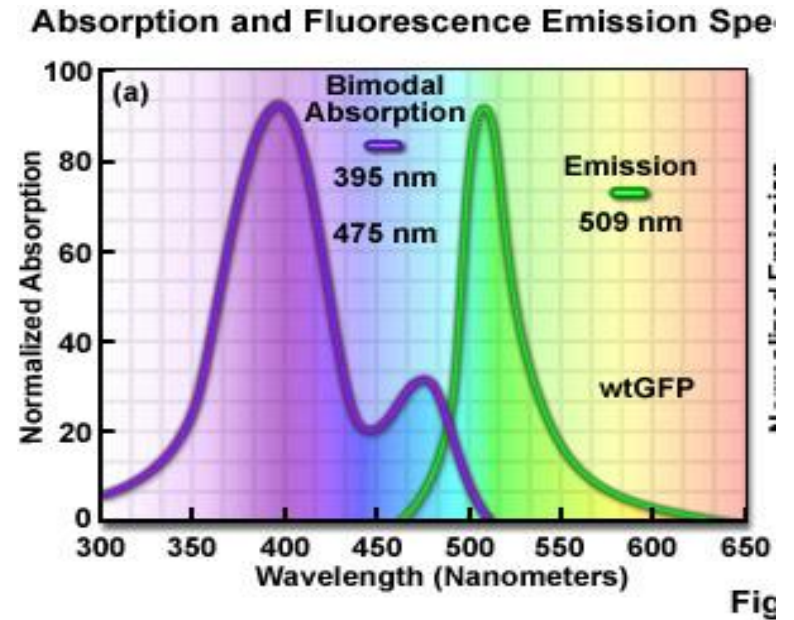


Figure 1



## Absorption and Fluorescence Emission Spect

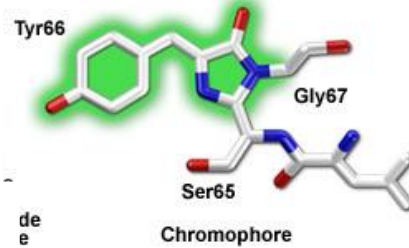
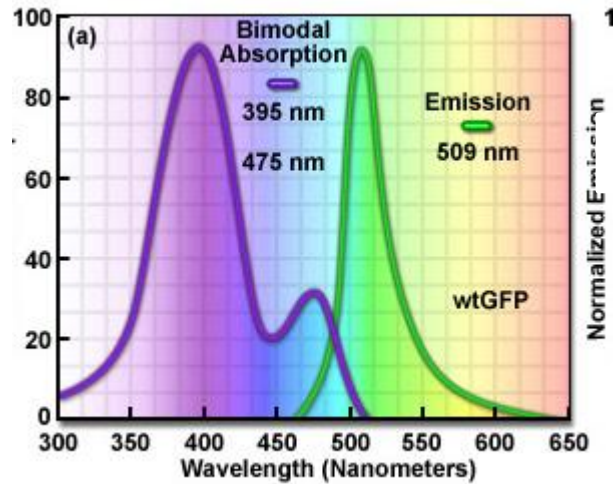
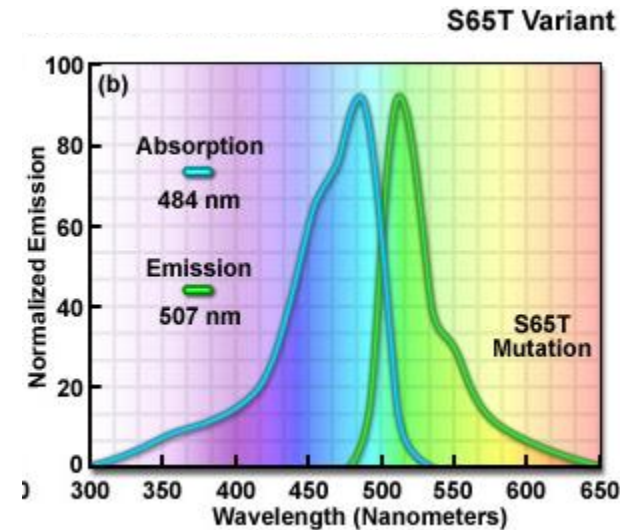
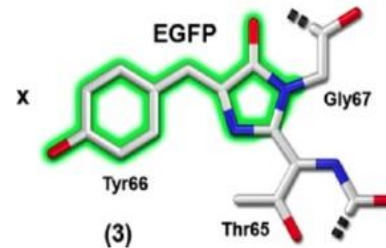


Figure 1



The excitation maximum of green fluorescent protein is readily shifted to **488 nanometers** (in the cyan region) by introducing a single point mutation altering the serine at position 65 into a threonine residue (**S65T**).

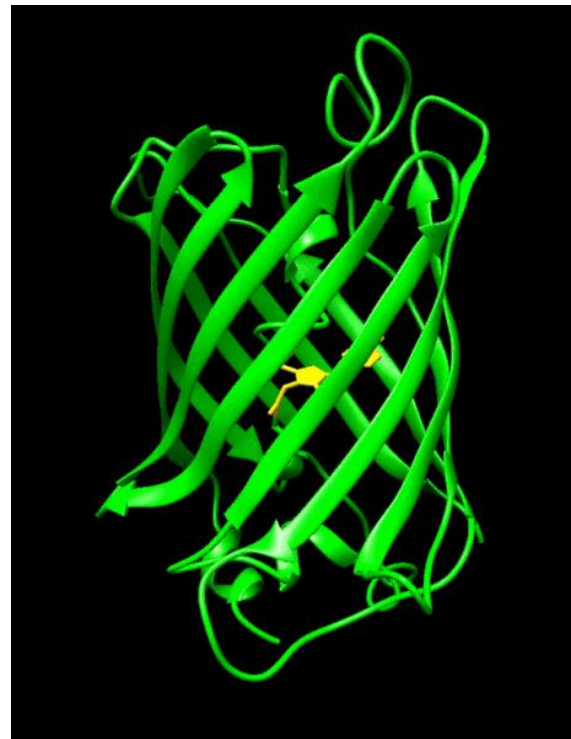
This mutation is featured in the most popular variant of green fluorescent protein, termed **enhanced GFP (EGFP)**,

This matched the spectral characteristics of commonly available FITC filter sets, **increasing the practicality of use by the general researcher**

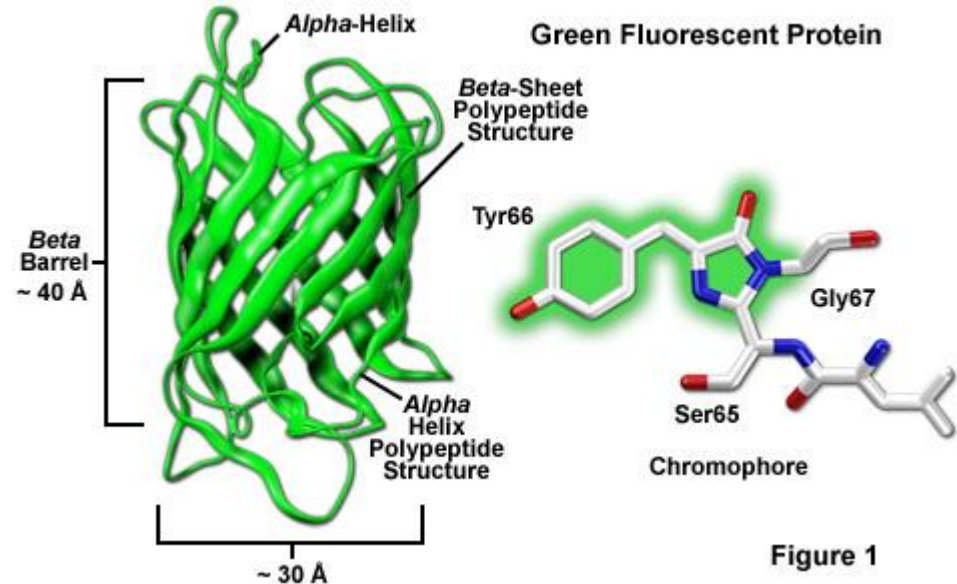


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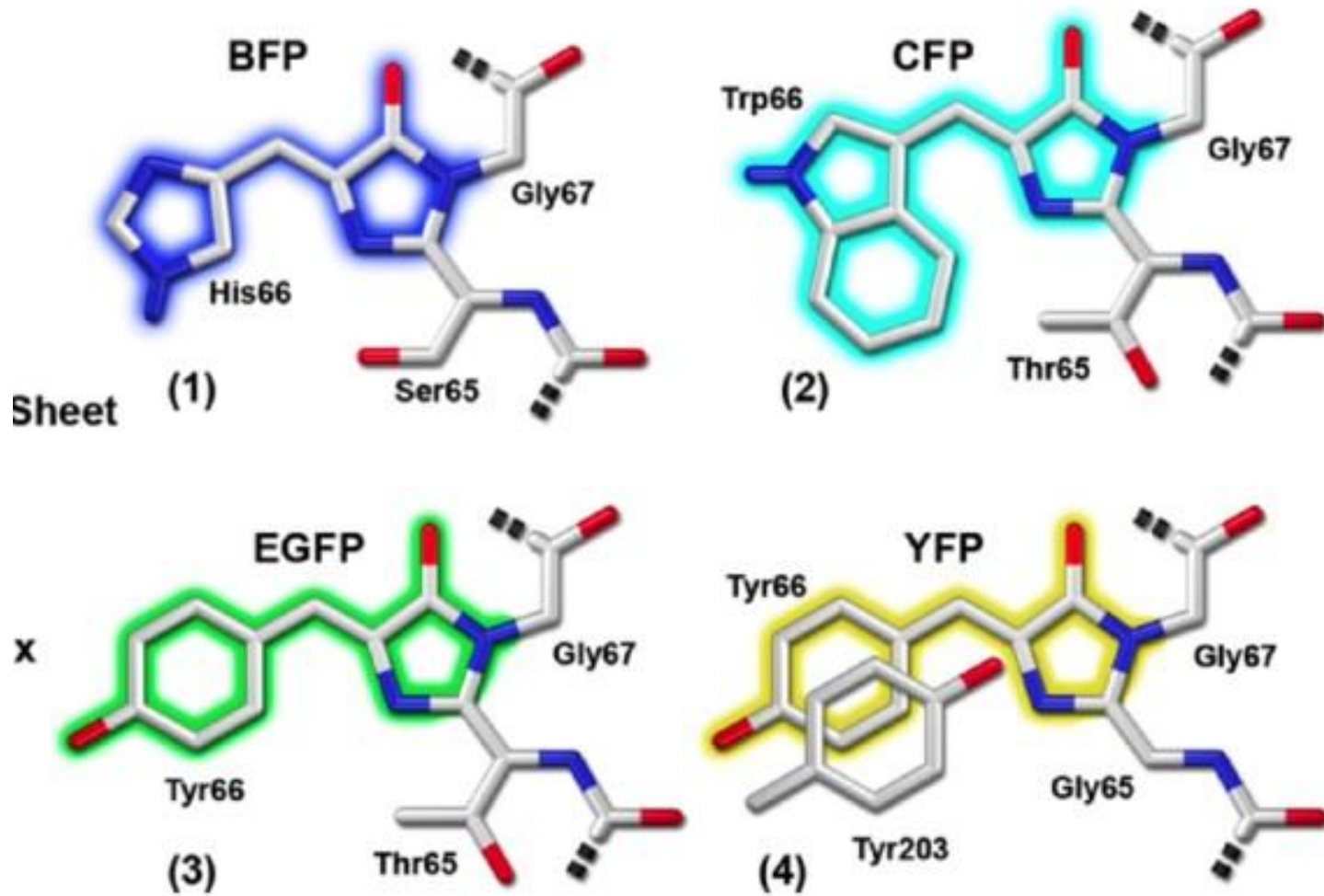
- **Chromophore** protected from Outside
- Chromophore **forms alone**



# What about other colors

- Mutate GFP to different colors
  - **BFP** - **CFP** - **YFP**
- Look for other natural proteins, and mutate if necessary
  - dsRed, Fp611, mFruit series

# Other colors based on GFP



## Chromophore Modification Effects on Fluorescent Protein Emission Spectra

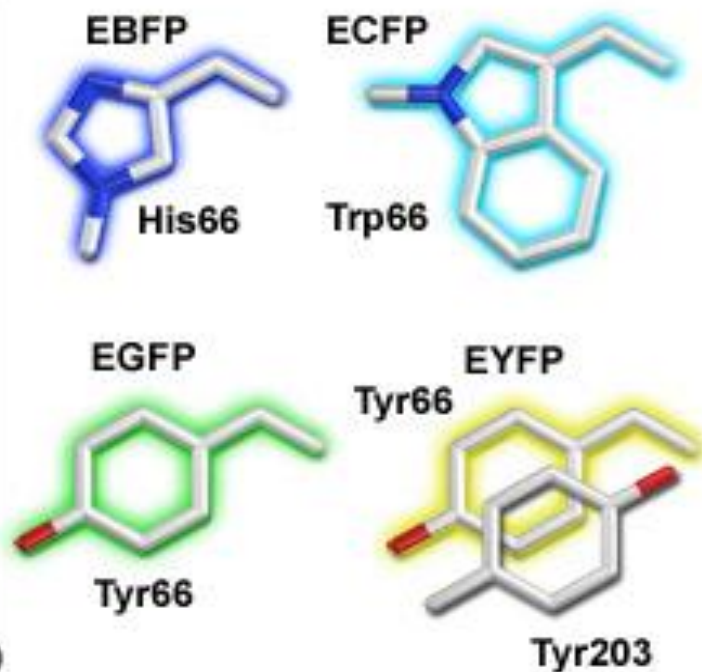
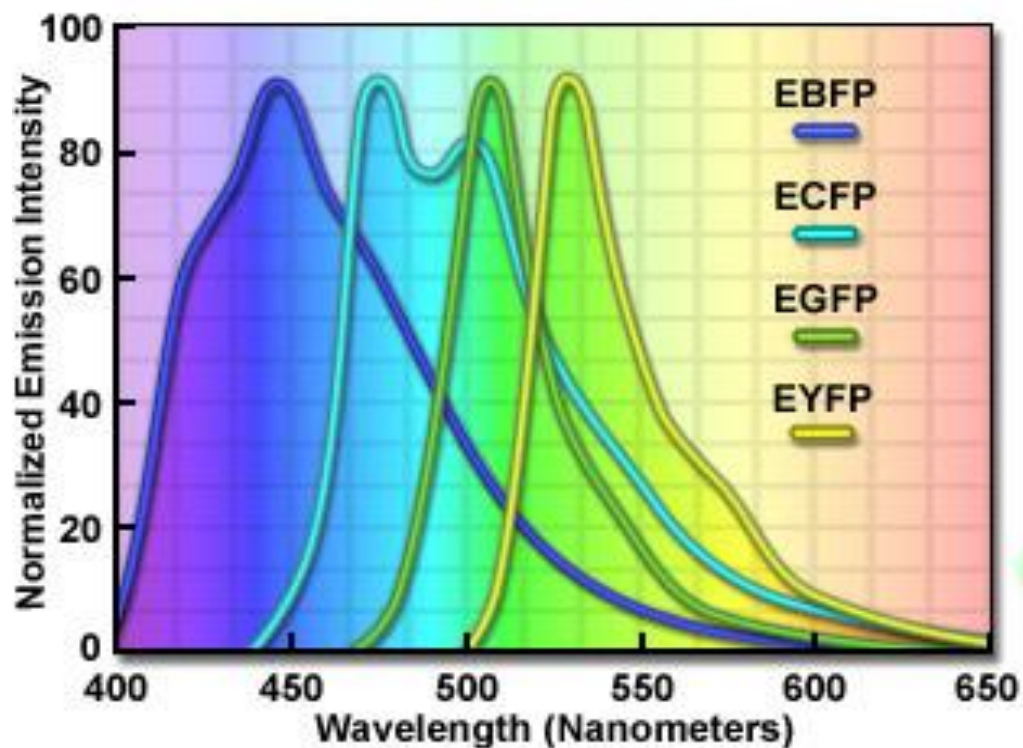


Figure 6

The construction of **red-shifted** mutants from the *Aequorea victoria* jellyfish green fluorescent protein beyond the yellow spectral region has proven **largely unsuccessful**,

investigators have turned their search to the **tropical reef corals**.

The first **coral-derived** fluorescent protein to be extensively utilized was derived from *Discosoma striata* and is commonly referred to as **DsRed**.



# Excitation / emission curves

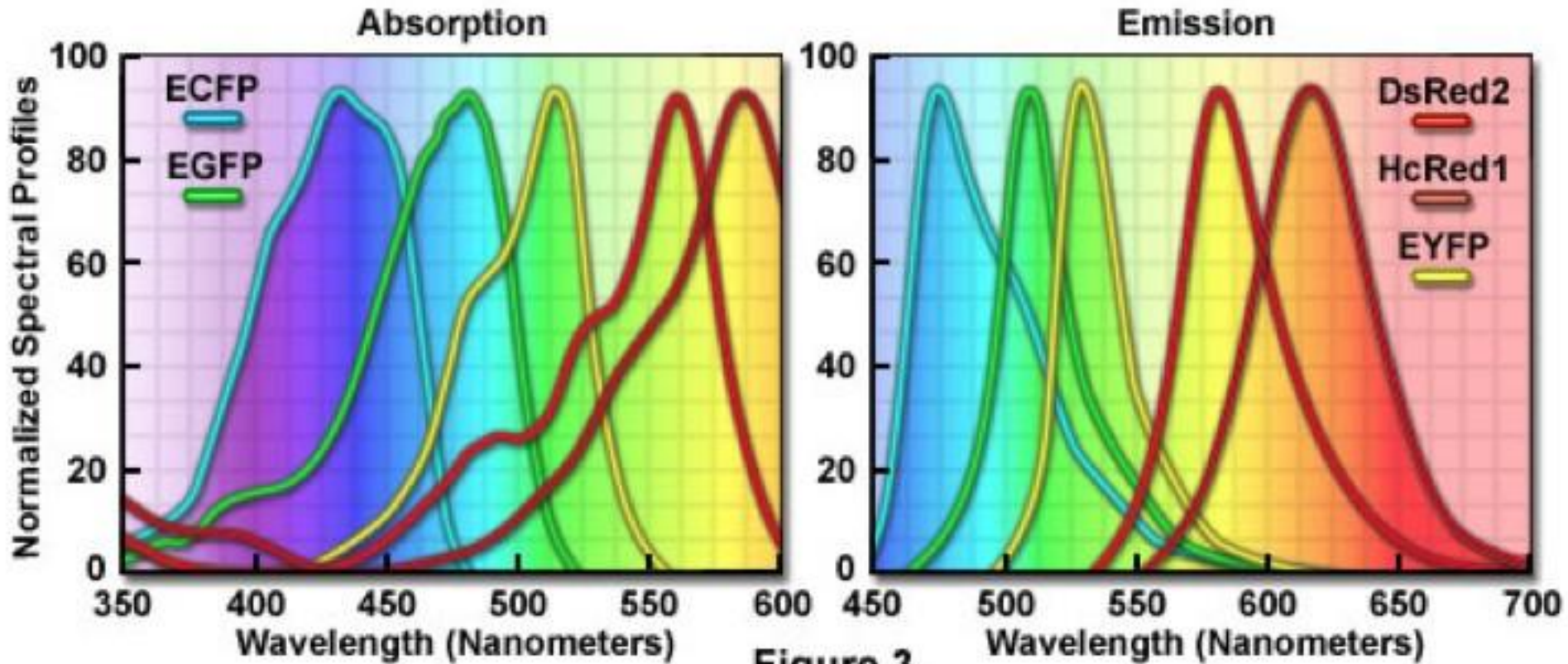
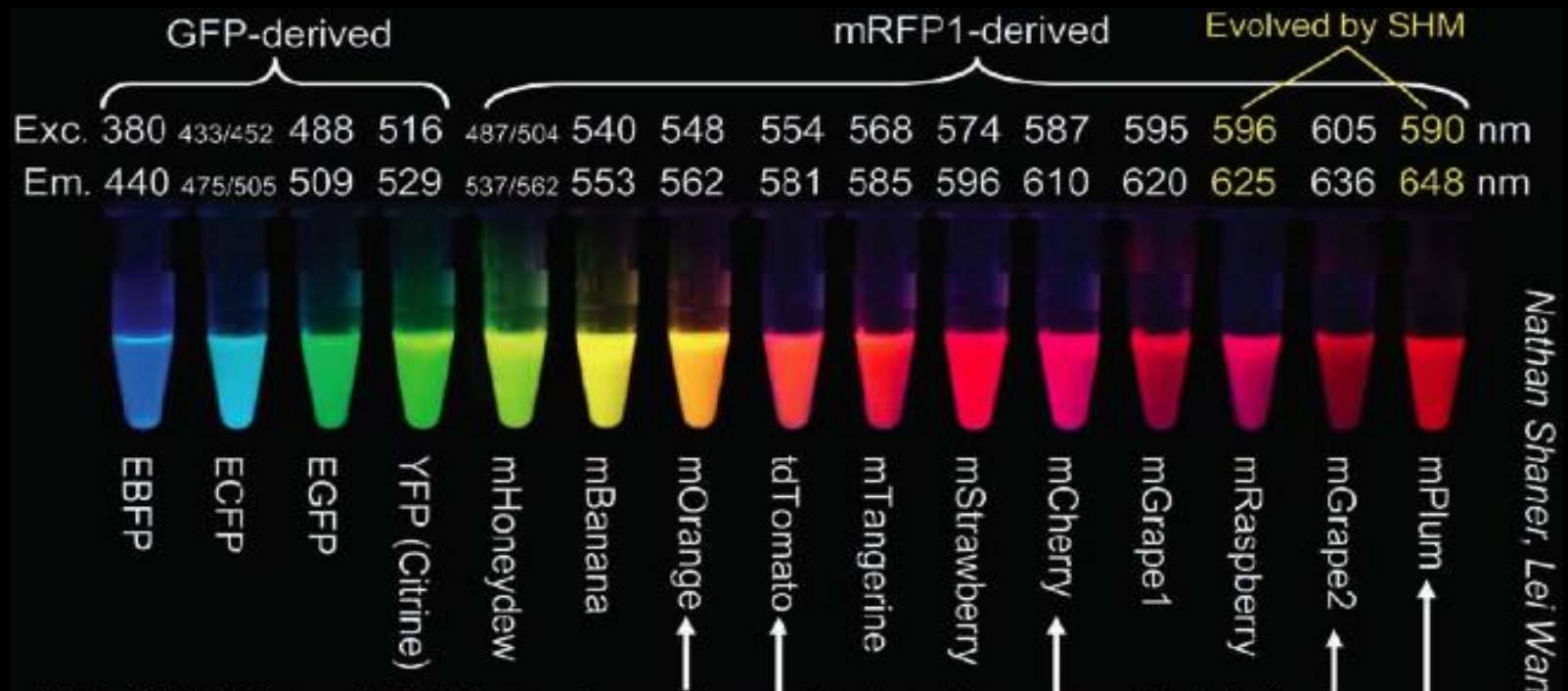


Figure 3



# The fluorescent protein palette



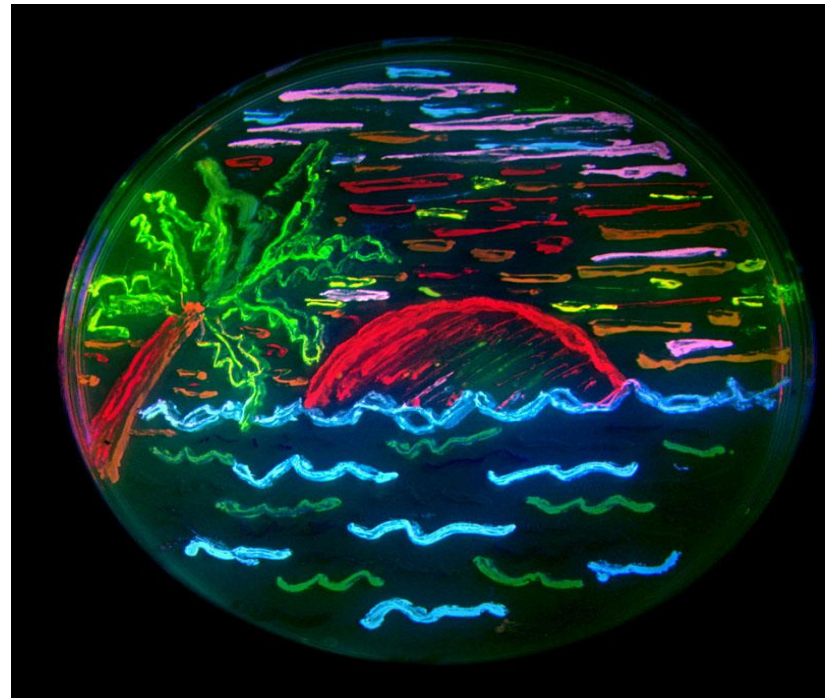
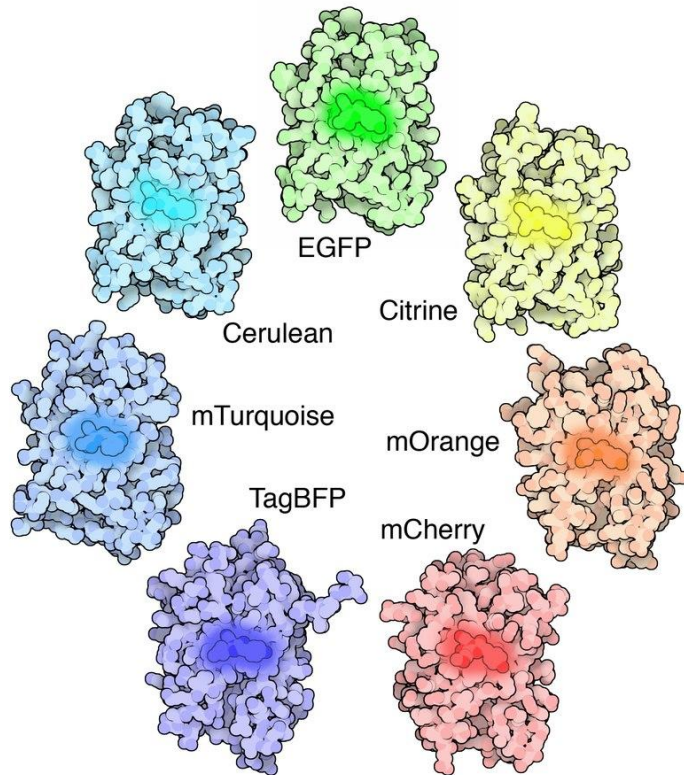
Nathan Shaner, Lei Wan

Excitation	380	433	488	516	487	540	548	554	568	574	587	595	596	605	590	nm
Emission	440	475	509	529	537	553	562	581	585	596	610	620	625	636	648	nm

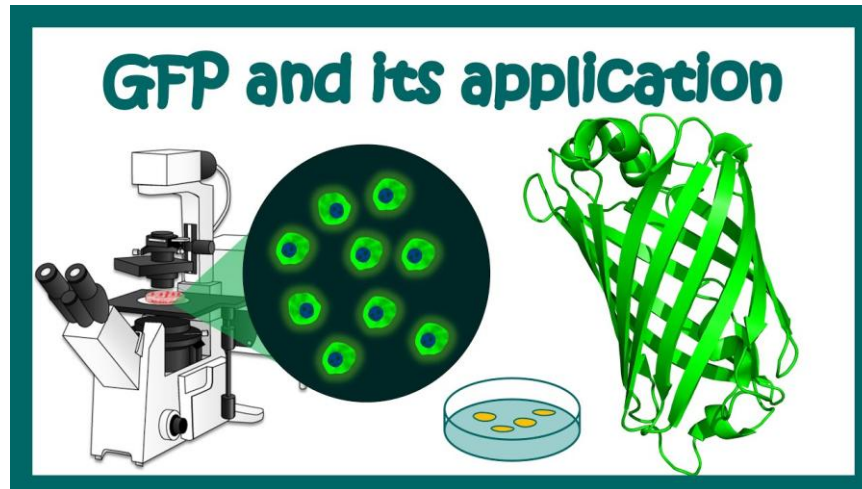


EBFP  
ECFP  
EGFP  
YFP(Citrine)  
mHoneydew  
mBanana  
mOrange  
tdTomato  
mTangerine  
mStrawberry  
mCherry  
mGrape1  
mRaspberry  
mGrape2  
mPlum

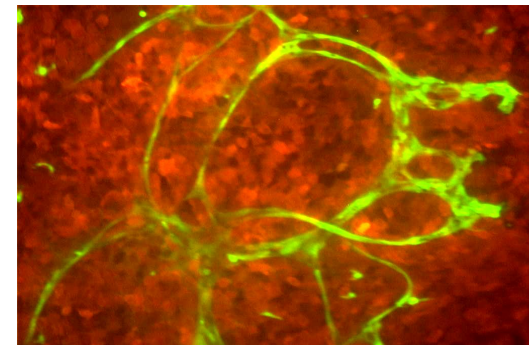
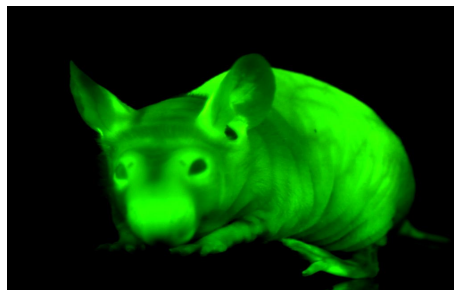
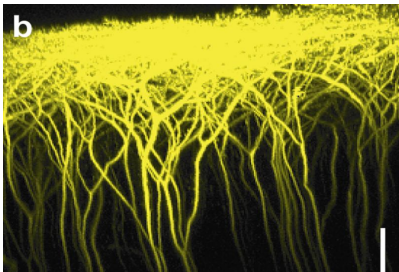
<https://www.fpbases.org/>



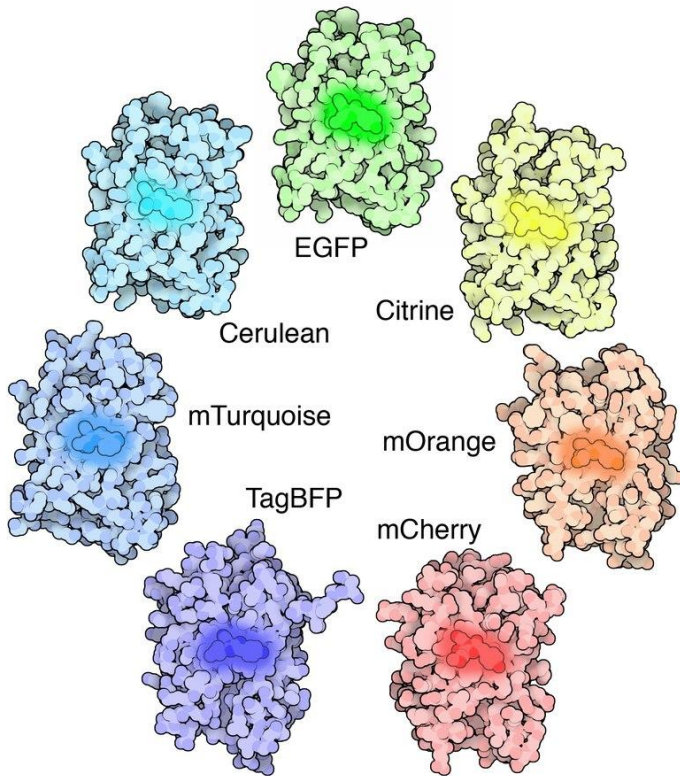
# Fluorescent Protein in cell and Molecular biology



Fluorescent proteins are quite versatile and have been successfully employed in almost **every biological discipline from microbiology to systems physiology**.



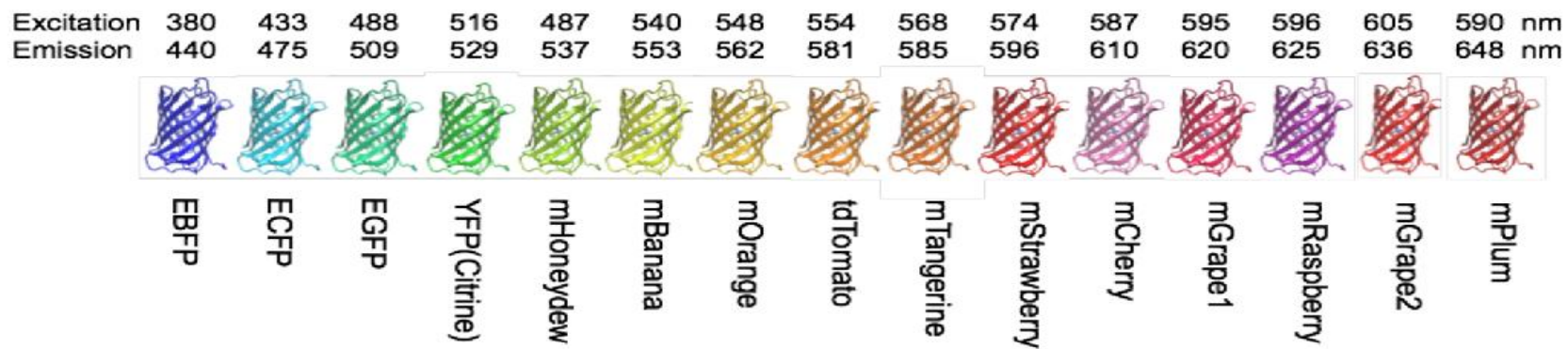
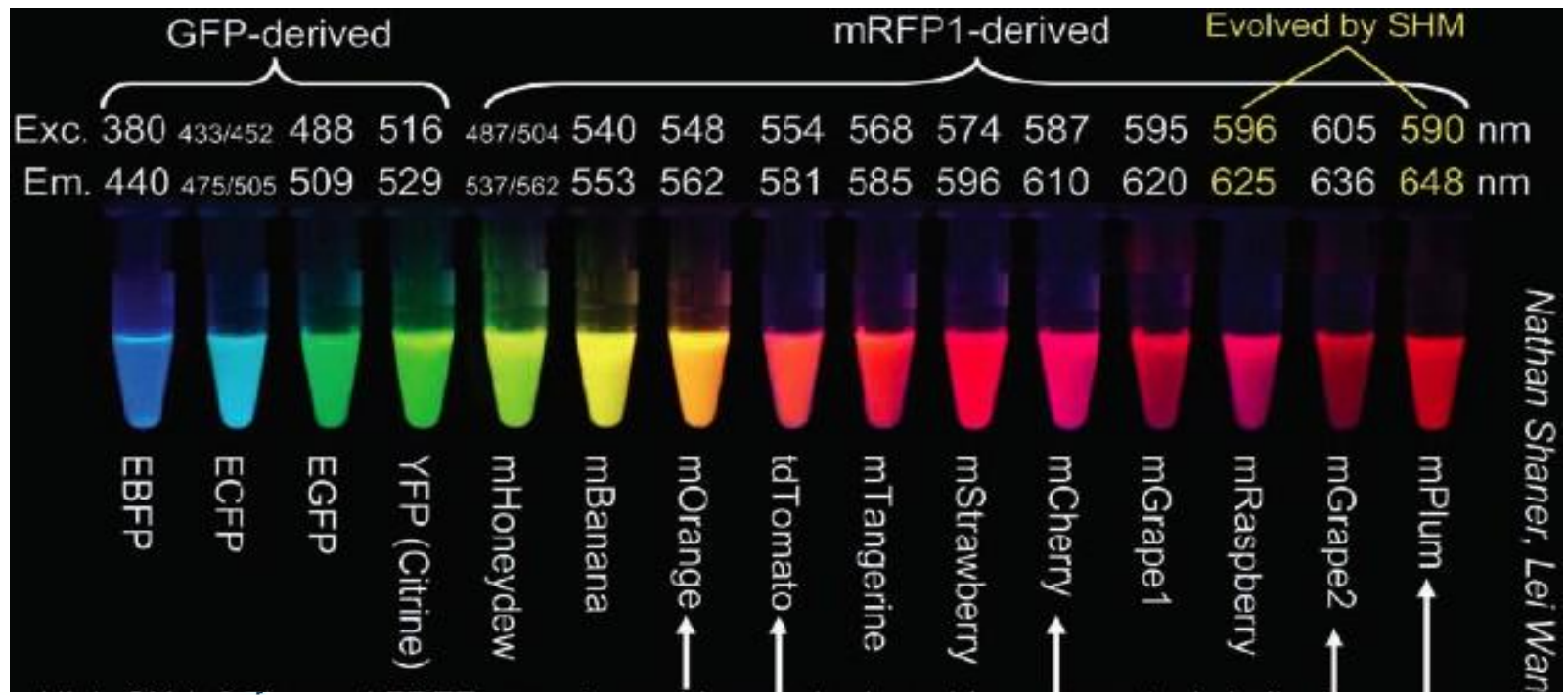
## Two ways to use fluorescent proteins



- As **gene reporter**
- As **fusion-protein**

## ***Use of Green Fluorescent Protein (GFP)***

- As a **reporter** molecule to **monitor gene expression**
  - Transgenic organism made with the **GFP-coding sequence** under the **transcriptional control of the promoter belonging to the gene of interest**

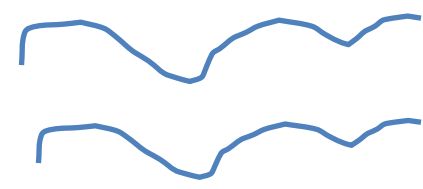


Promoter  
for gene A

Gene A



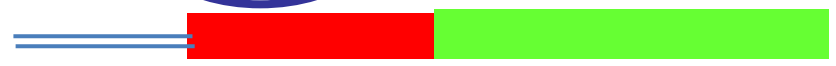
Coding region



RNA for gene A

**GFP-reporter**

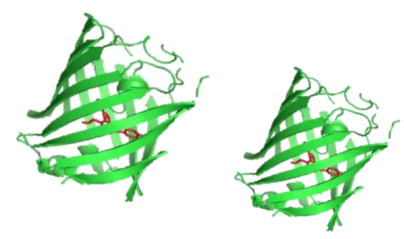
Promoter  
for Gene A



**GFP GENE**

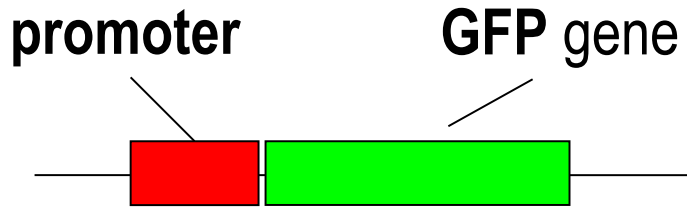


Promoter for Gene A  
regulates the expression  
of **GFP**

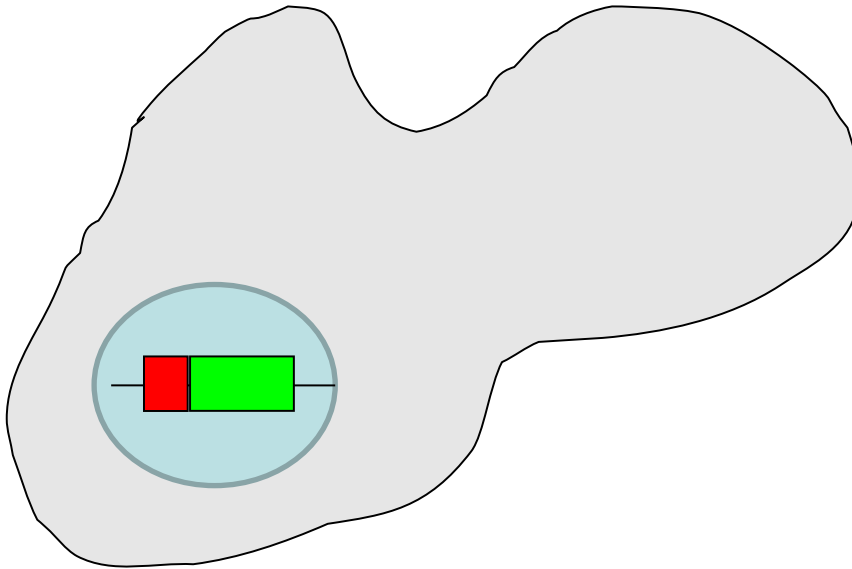


Can be used to **visualize** the  
expression of **Gene A**

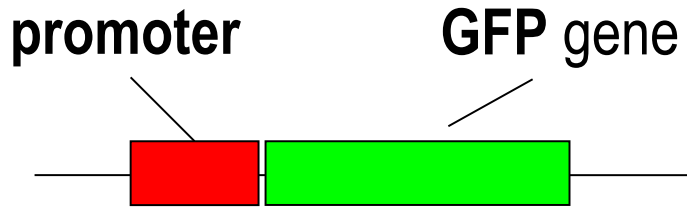
# Using fluorescent proteins as gene reporters



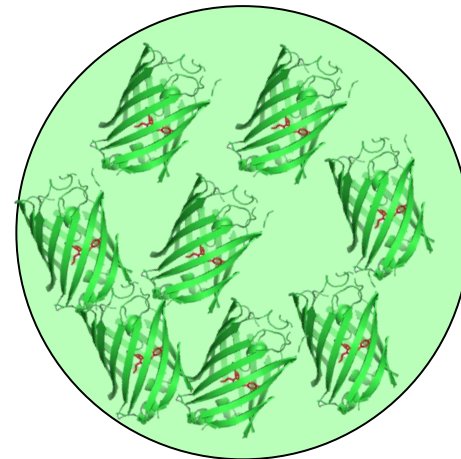
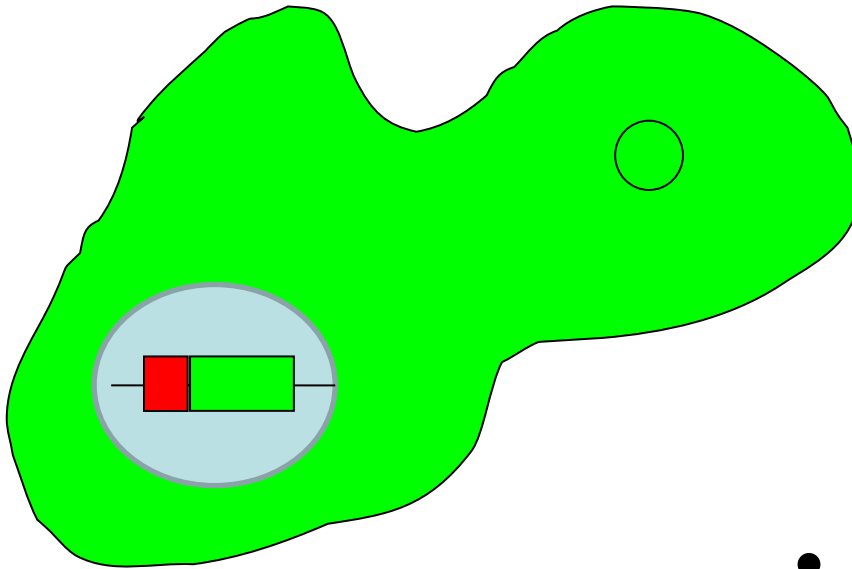
- GFP expressed wherever **original promoter is active**



# Using fluorescent proteins as gene reporters



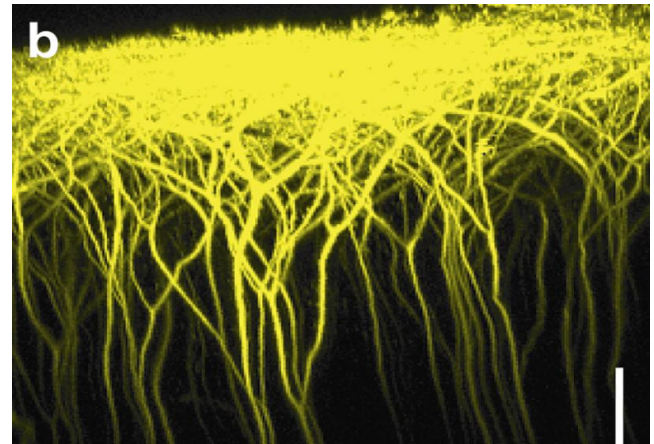
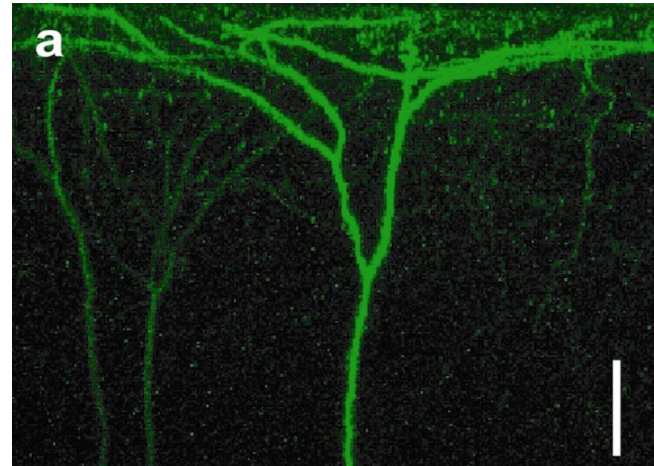
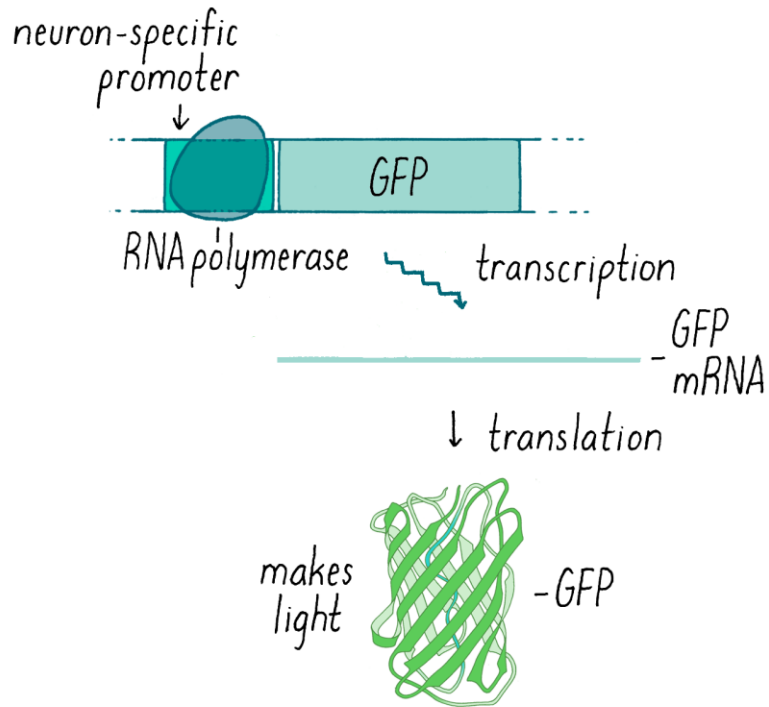
- GFP expressed wherever **original promoter is active**



- Whole cell is fluorescent

# Using fluorescent proteins as gene reporters

## GFP TRANSCRIPTION AND TRANSLATION IN NEURONS



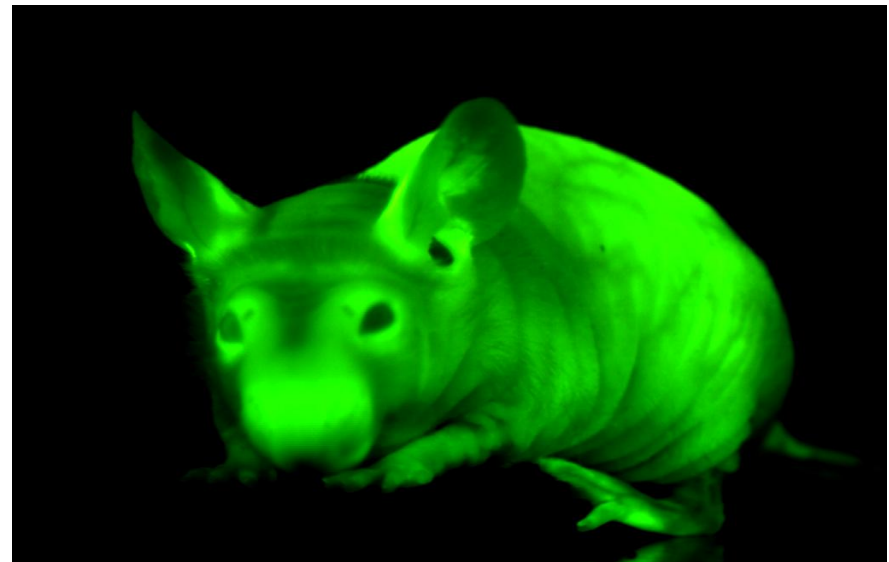
- GFP expressed wherever original promoter is active

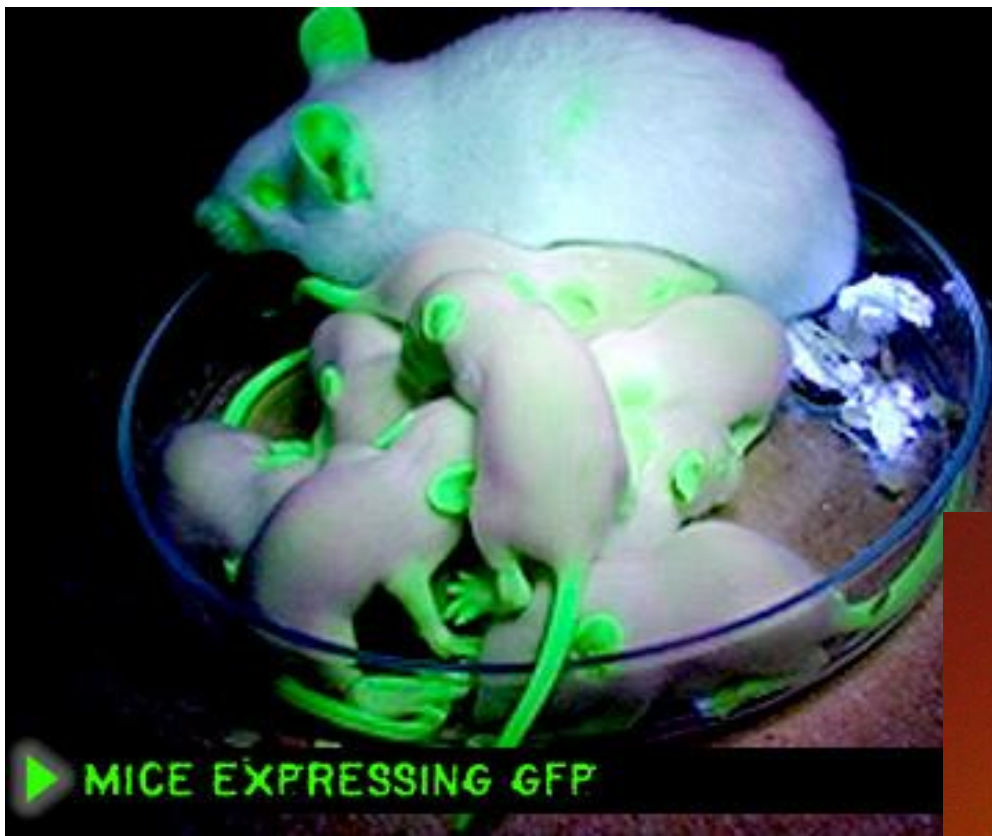
# Transgenic GFP mice

Under normal light



Under **blue light**





▶ MICE EXPRESSING GFP

Courtesy of Advanced Cell Technology



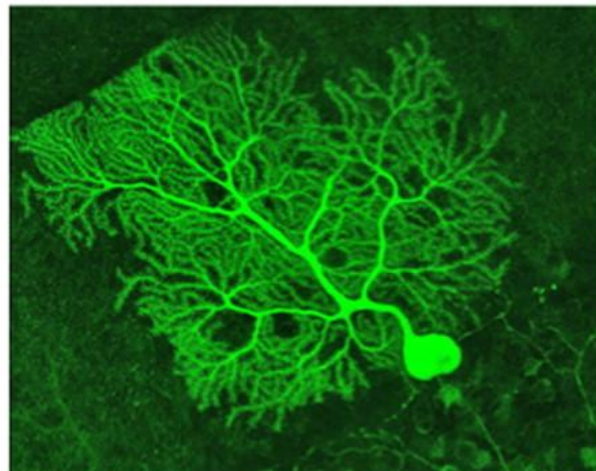
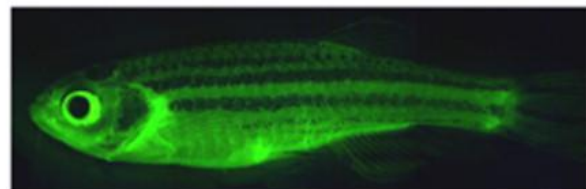
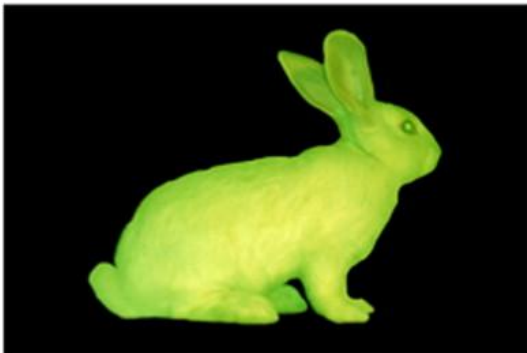
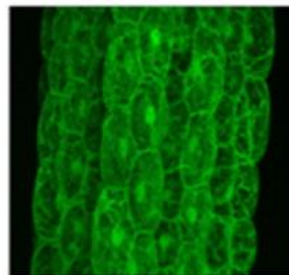
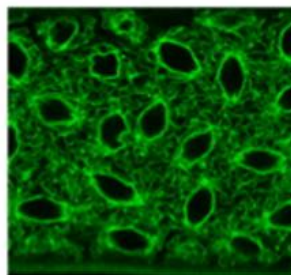
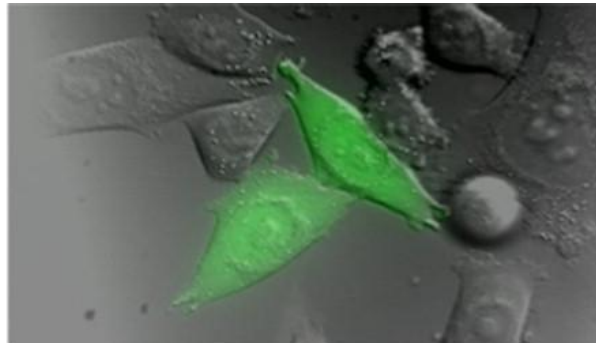
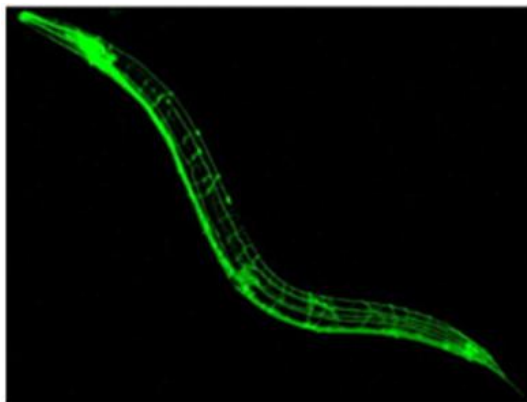
# Transgenic pigs





...and cats





# Rosa26<sup>FP</sup> mice



Rosa26<sup>EN.CFP/EN.Cherry</sup>

Rosa26<sup>EN.CFP/+</sup>

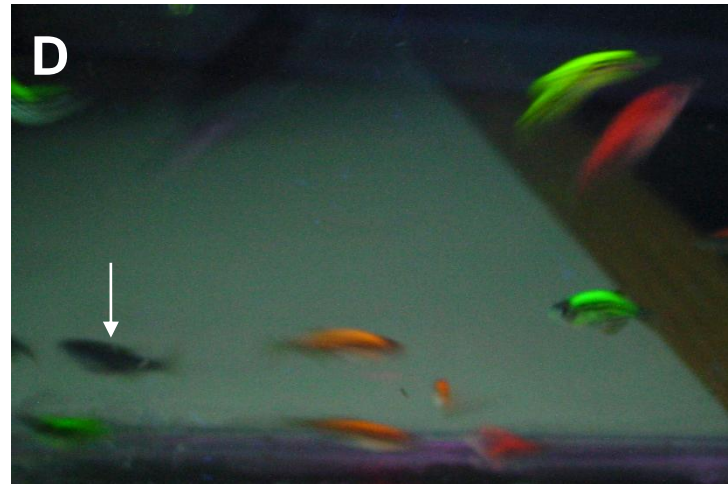
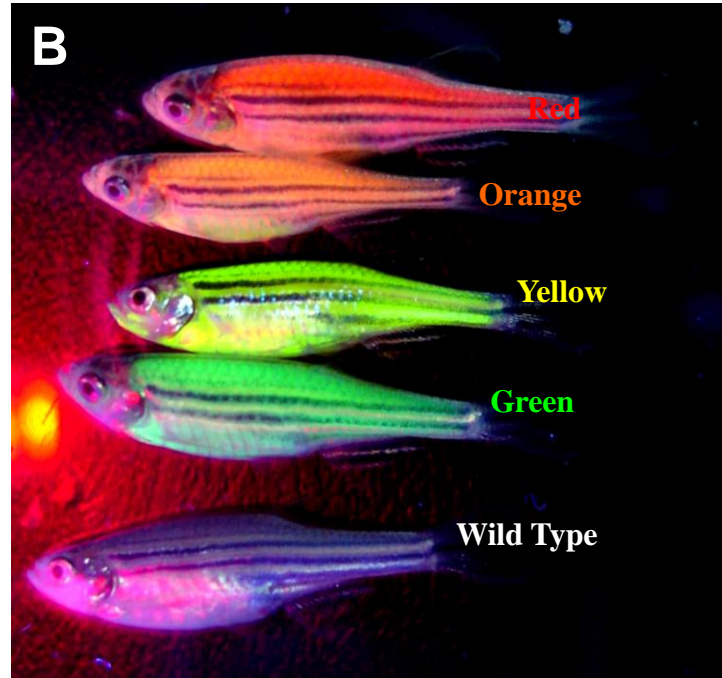
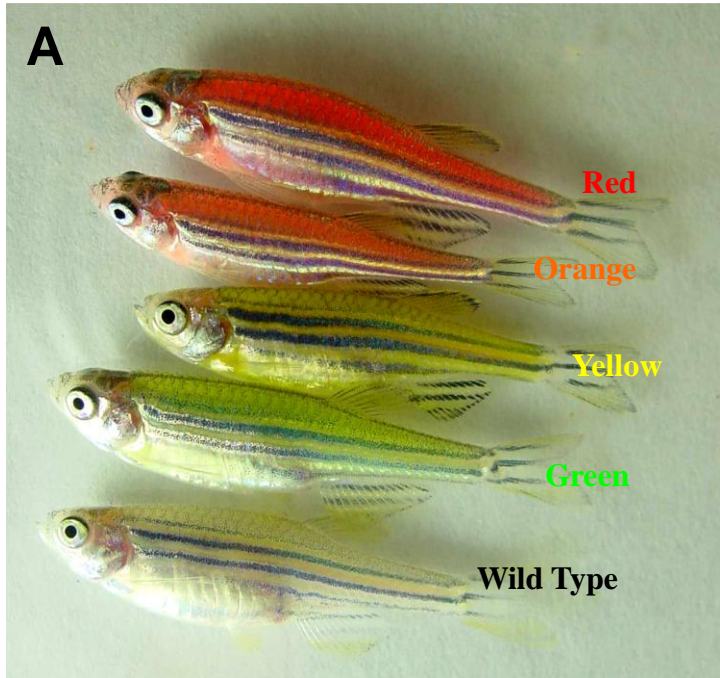
Rosa26<sup>EN.CFP/EN.YFP</sup>

Rosa26<sup>EN.YFP/+</sup>

Rosa26<sup>EN.YFP/EN.Cherry</sup>

Rosa26<sup>EN.Cherry/+</sup>

# ...and fish





TETRA



LONGFIN TETRA



LONGFIN BARB



BARB



THERE'S A  
**GLOFISH FOR  
EVERYONE!**

BETTA



DANIO

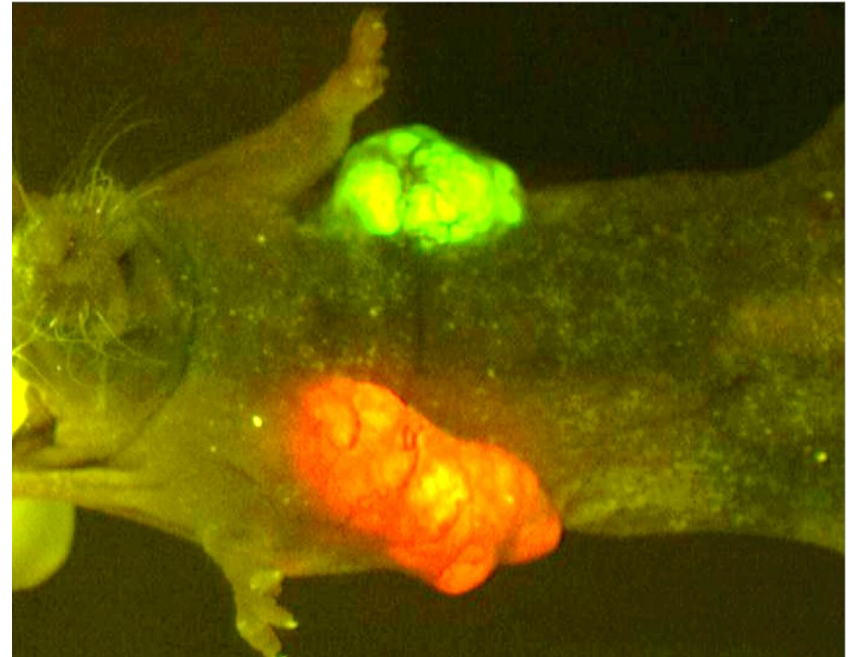
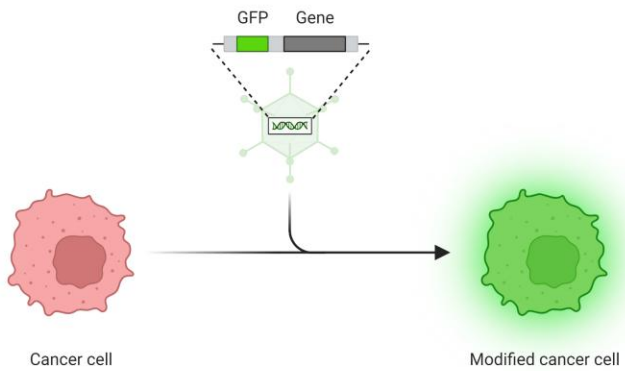


SHARK

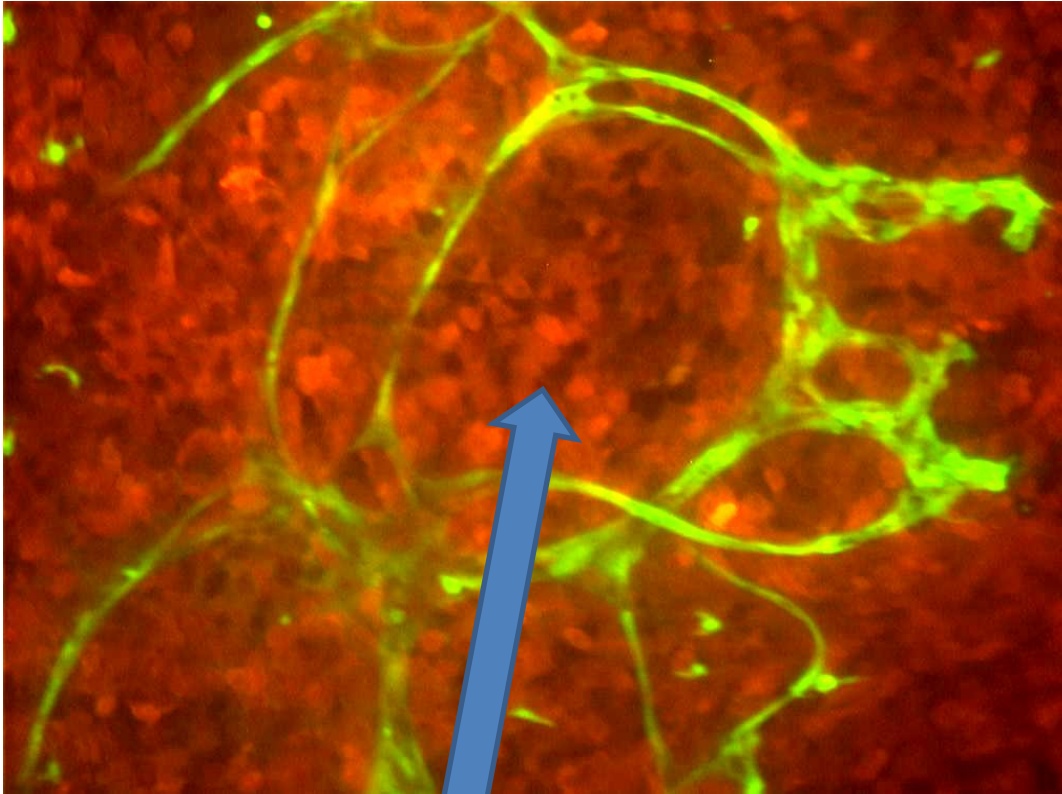


# Mice with FP expressing tumors

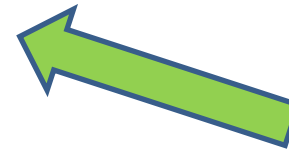
## Green Fluorescence Protein (GFP) Reporter in Cancer Cell



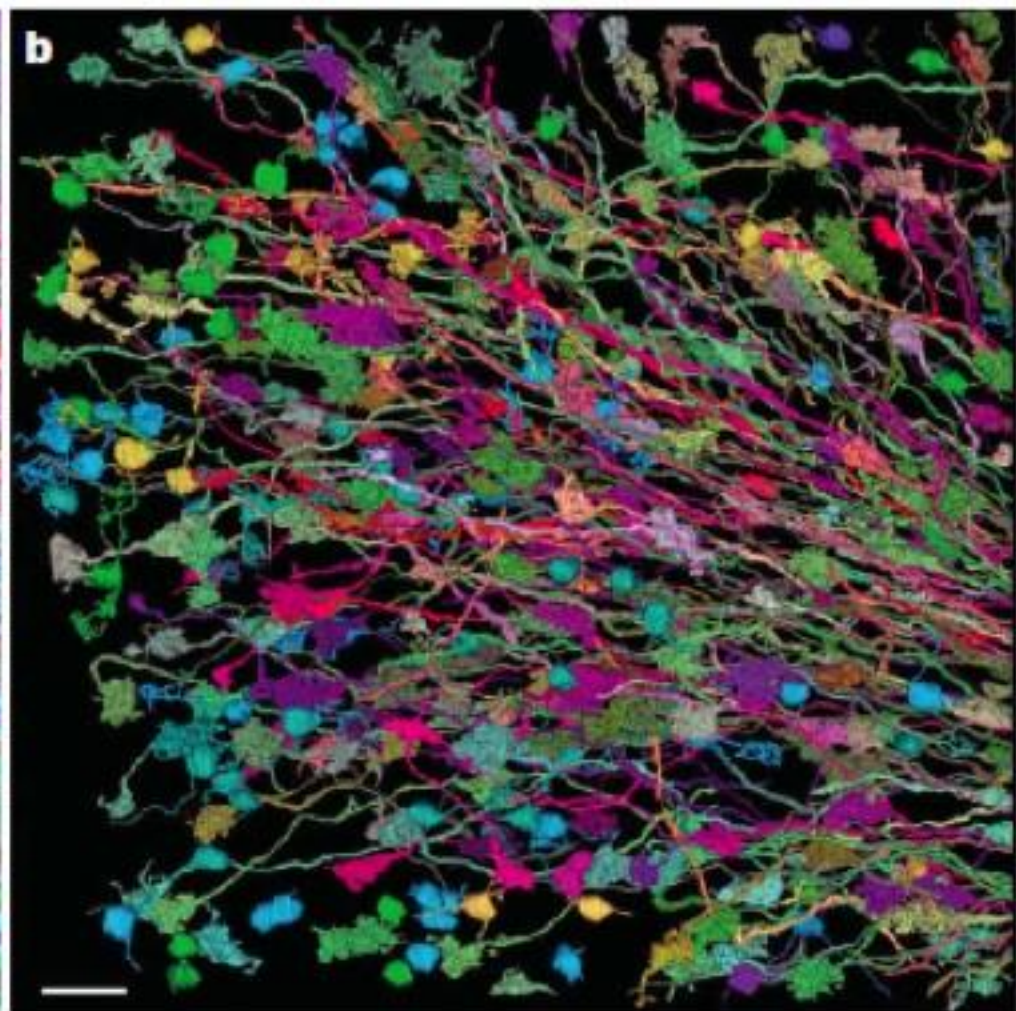
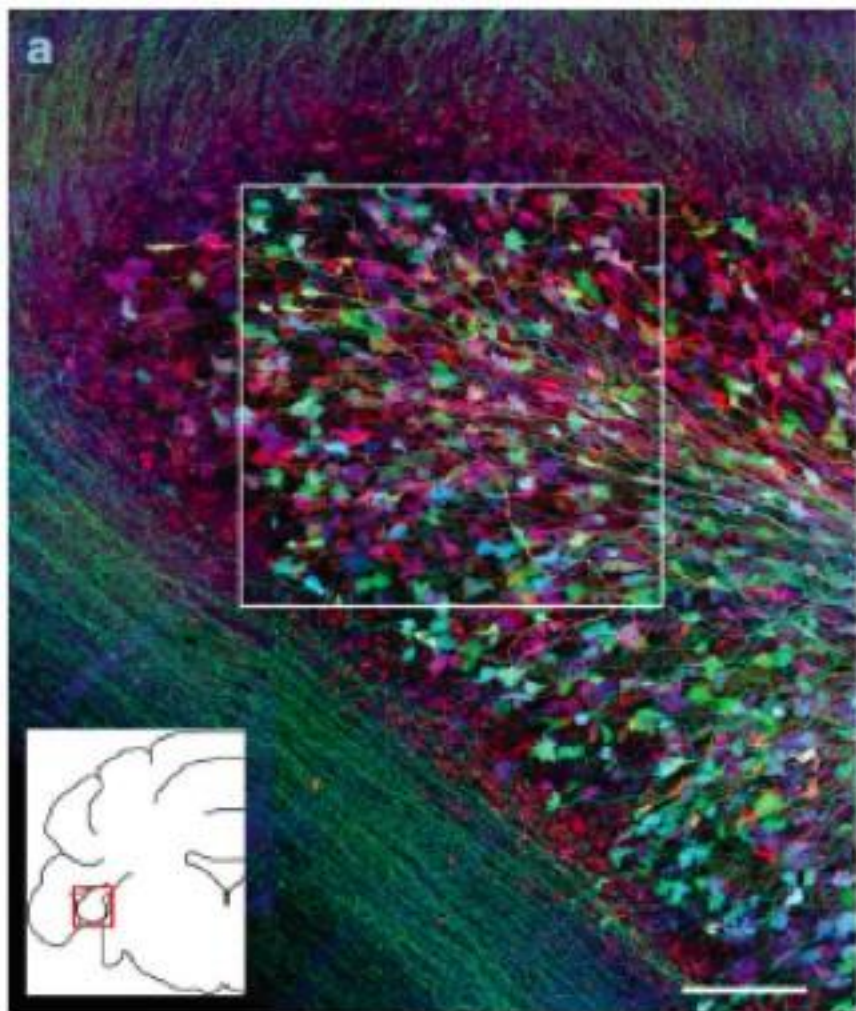
## Visualizing tumor angiogenesis



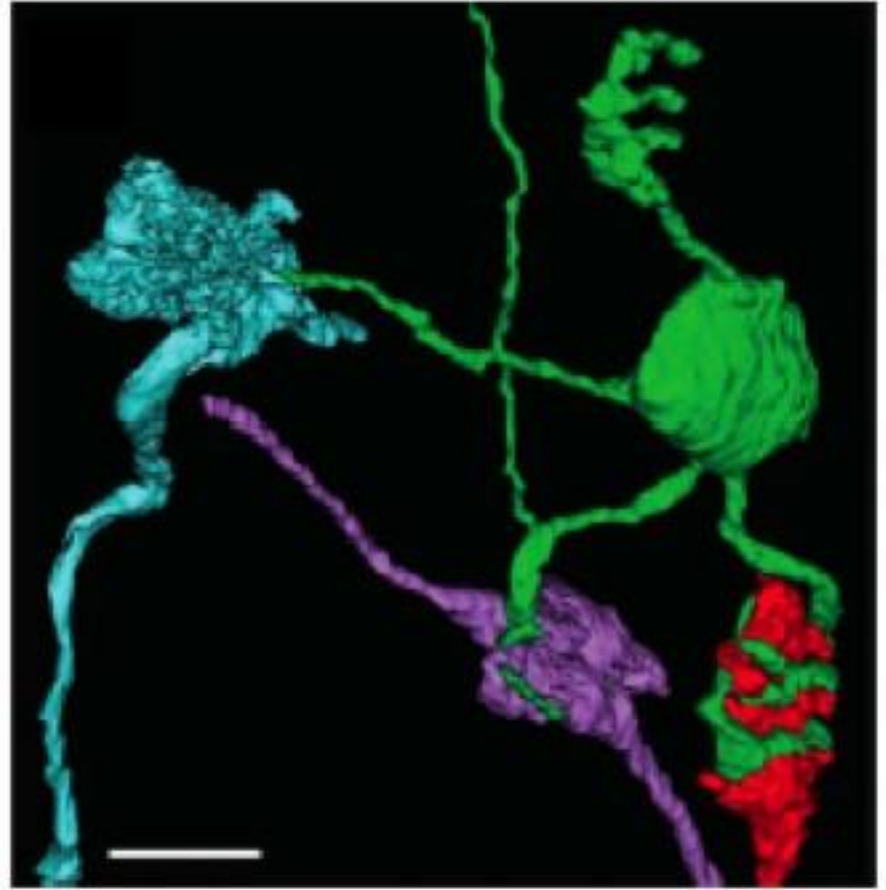
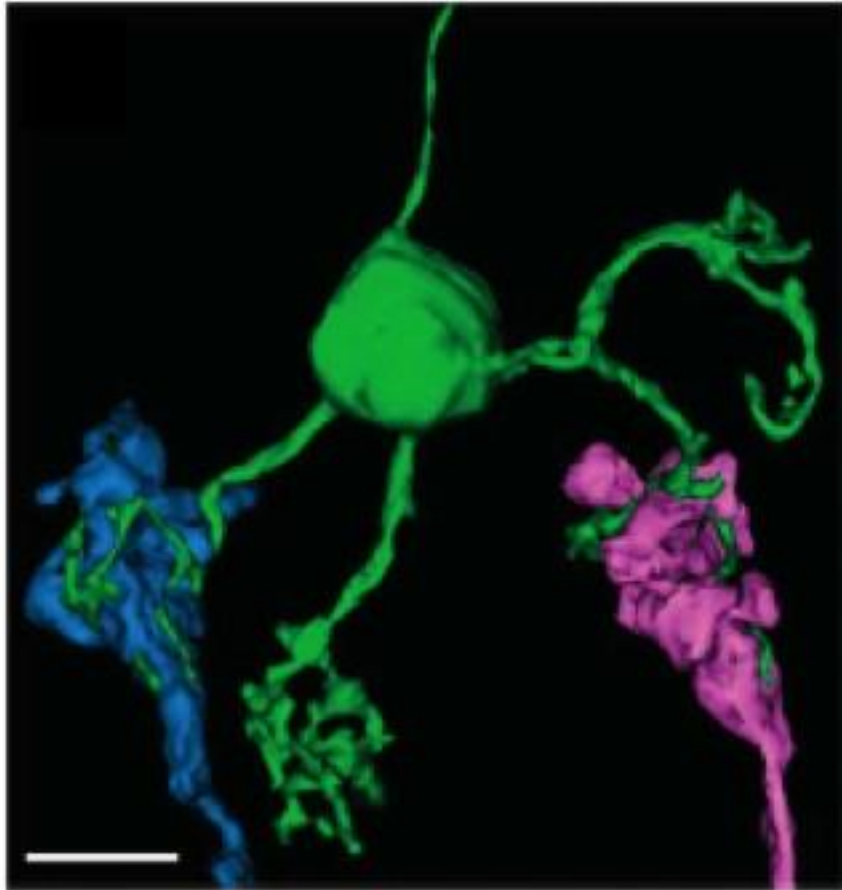
**GFP** expressing  
blood vessels



**RFP** expressing tumor



# Individual neurons



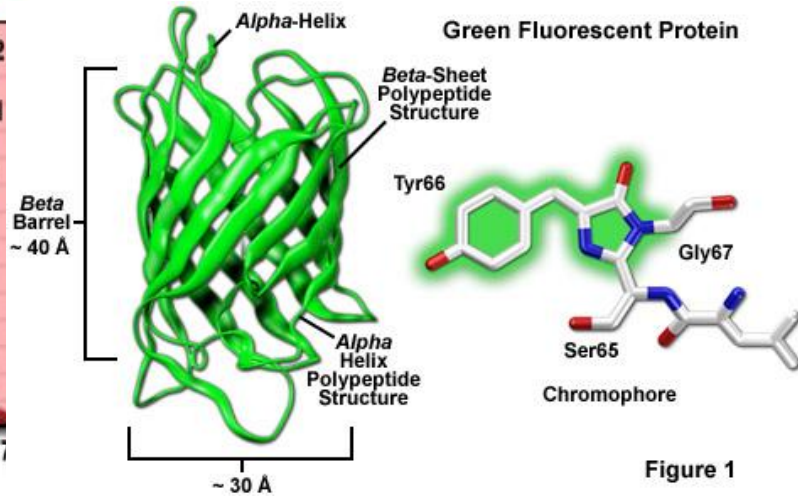
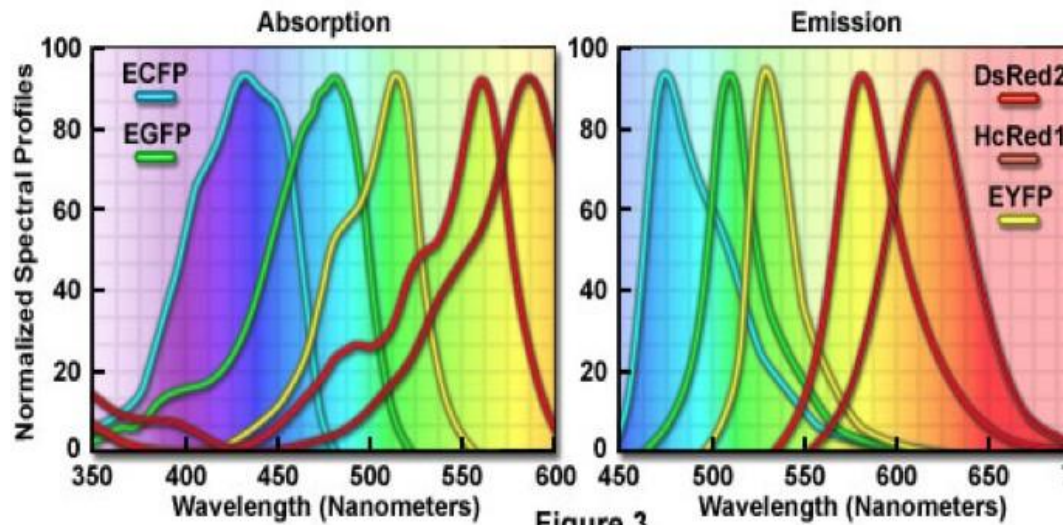
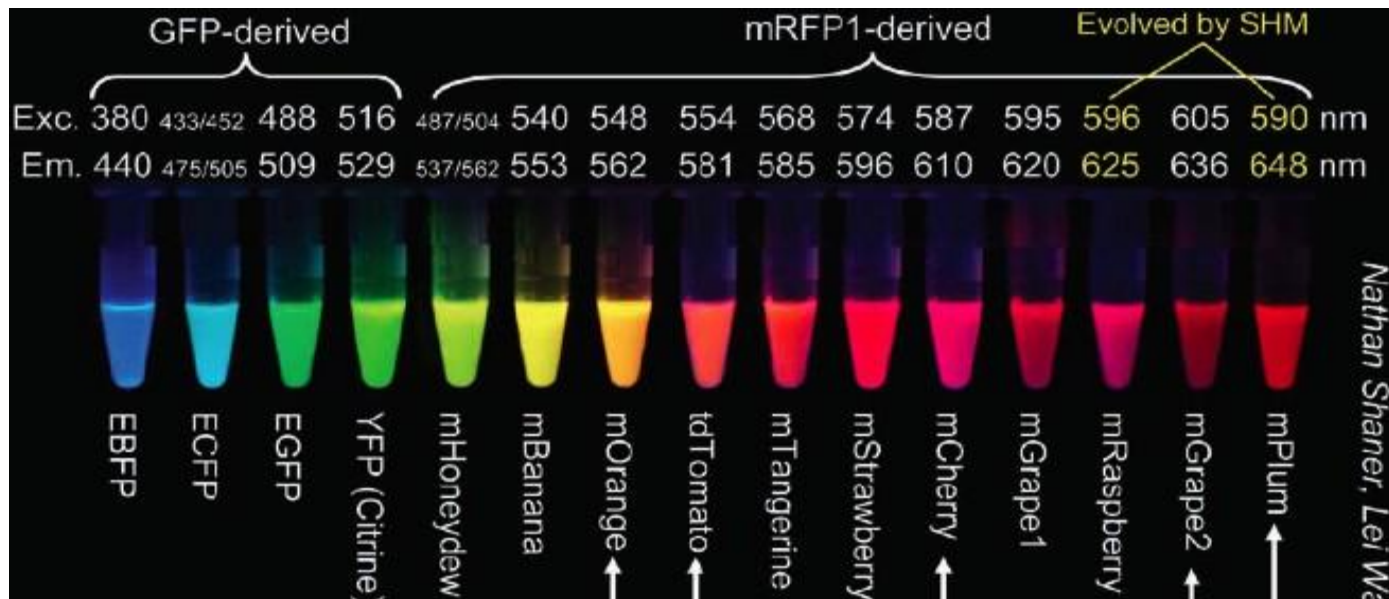
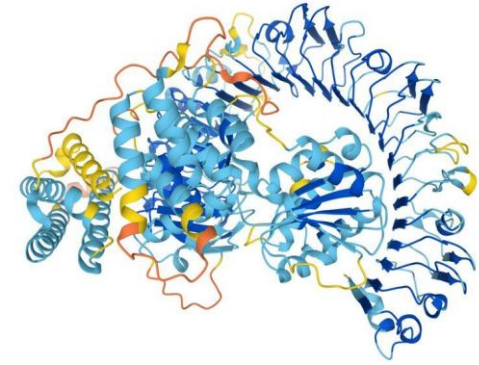


Figure 1

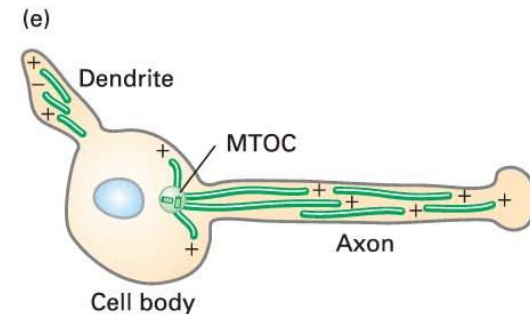
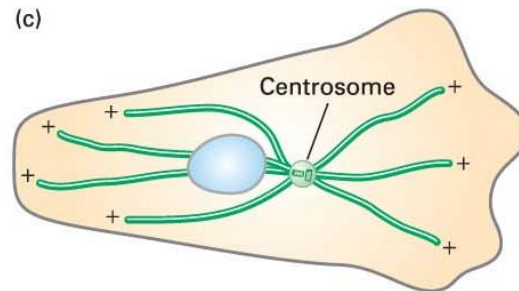


Durante la vostra tesi avete identificato la proteina XYZ come sovraespressa nei tumori del polmone



Da una analisi bioinformatica risulta che la proteina **potrebbe essere una proteina associata ai microtubuli**

Proponete la vostra migliore strategia sperimentale per verificare l'ipotesi

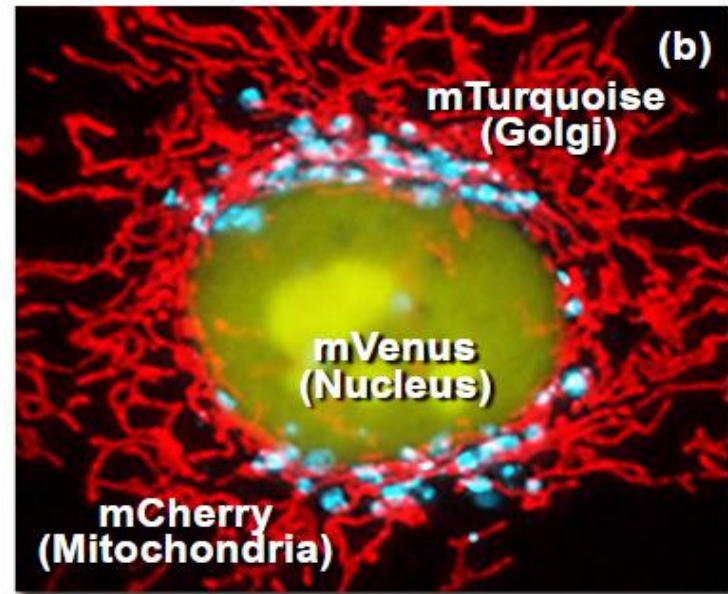
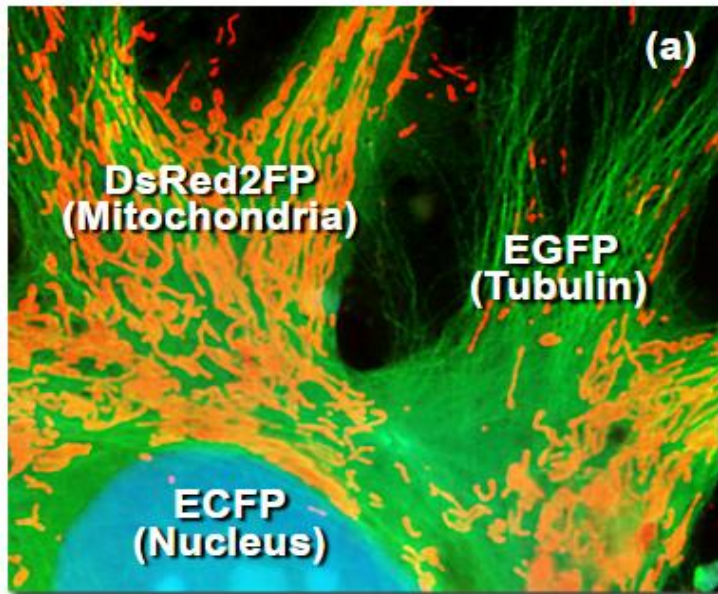


# Use of Green Fluorescent Protein (GFP)

- As a **tag to localize proteins**



*Figure 1 - Fluorescent Protein Labels in Living Cells*

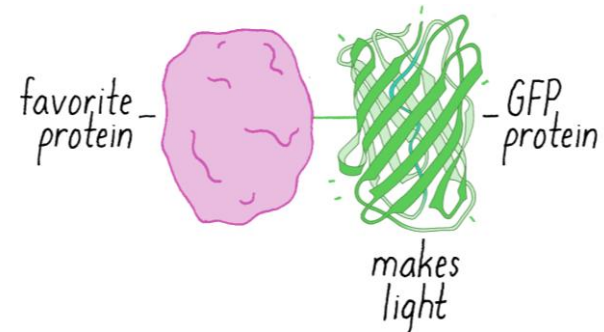


# Use of Green Fluorescent Protein (GFP)

- **As a tag to localize proteins**

- The GFP-encoding sequence is placed at the **beginning** or **end** of the gene for another protein
- This yields a **chimeric protein** consisting of the protein of interest with a GFP domain attached

FAVORITE PROTEIN MADE FLUORESCENT

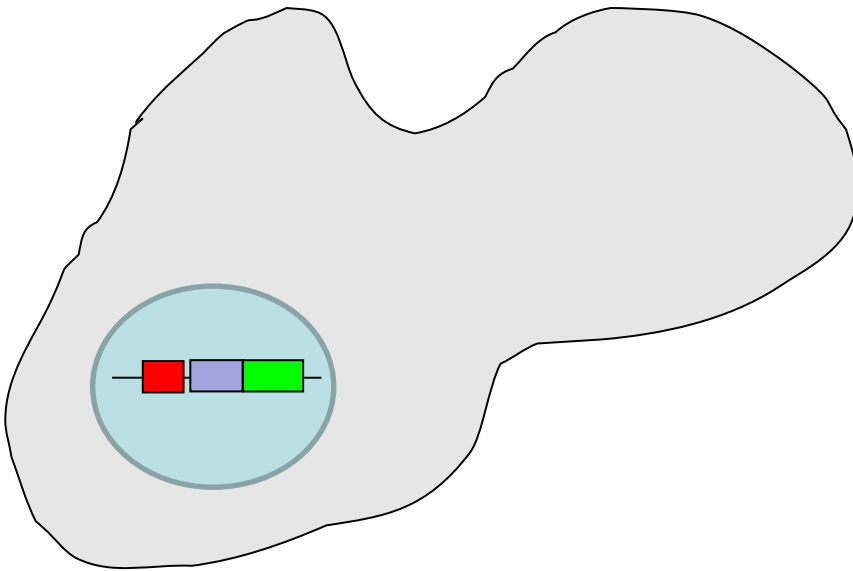


GFP-fusion protein often behaves like the original protein, **directly revealing its subcellular location**

# Using fluorescent proteins as fusion proteins



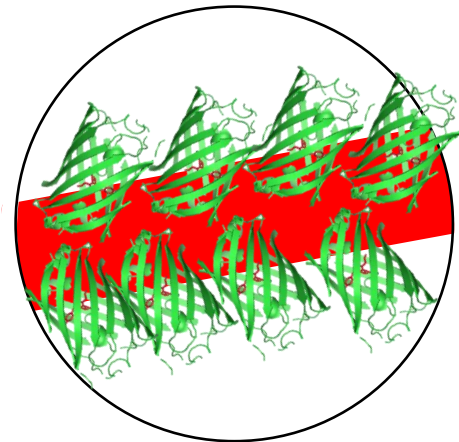
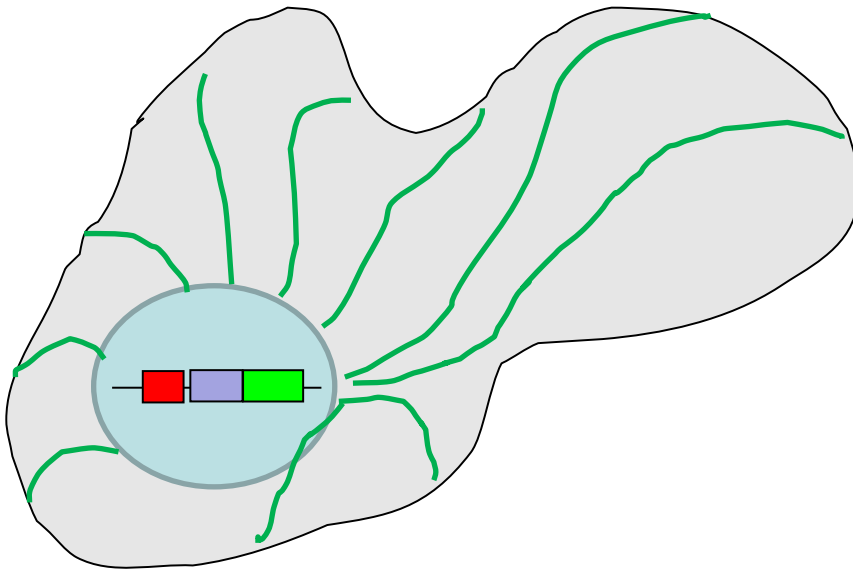
- protein X - GFP- Fusion  
expressed wherever (*original*) promoter  
is active



# Using fluorescent proteins as fusion proteins



- GFP- Fusion  
expressed wherever (*original*) promoter is active



# Three ways to make Green fluorescent protein “GFP” fusion constructs:



C-Ter Fusion



N-Ter Fusion

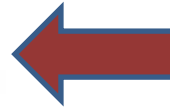
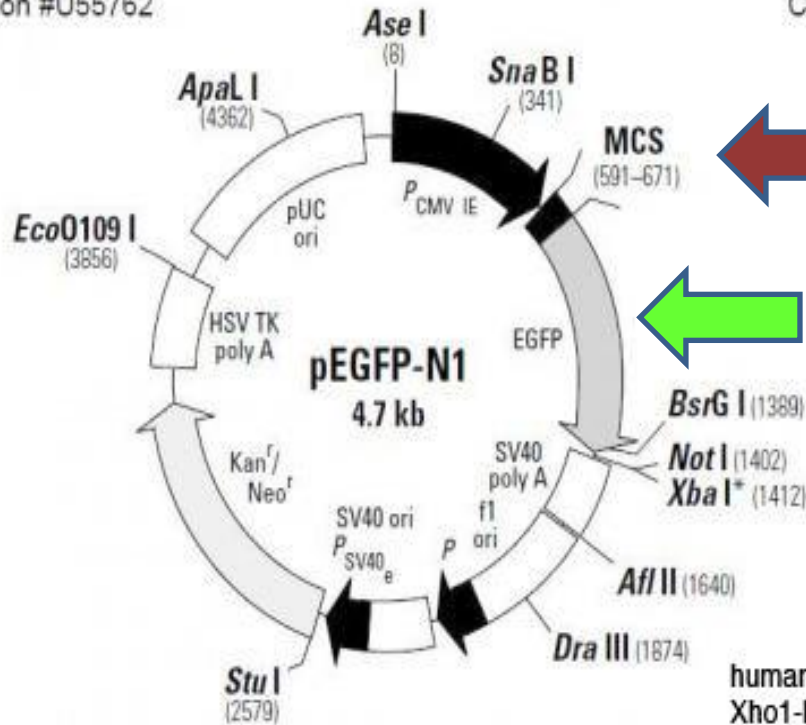


pEGFP-N1 Vector Information

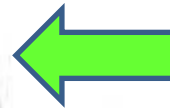
GenBank Accession #U55762

PT3027-5

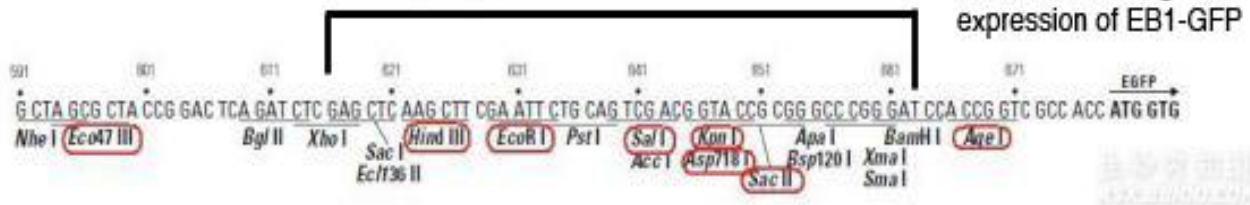
Catalog #6085-1



Clone protein HERE

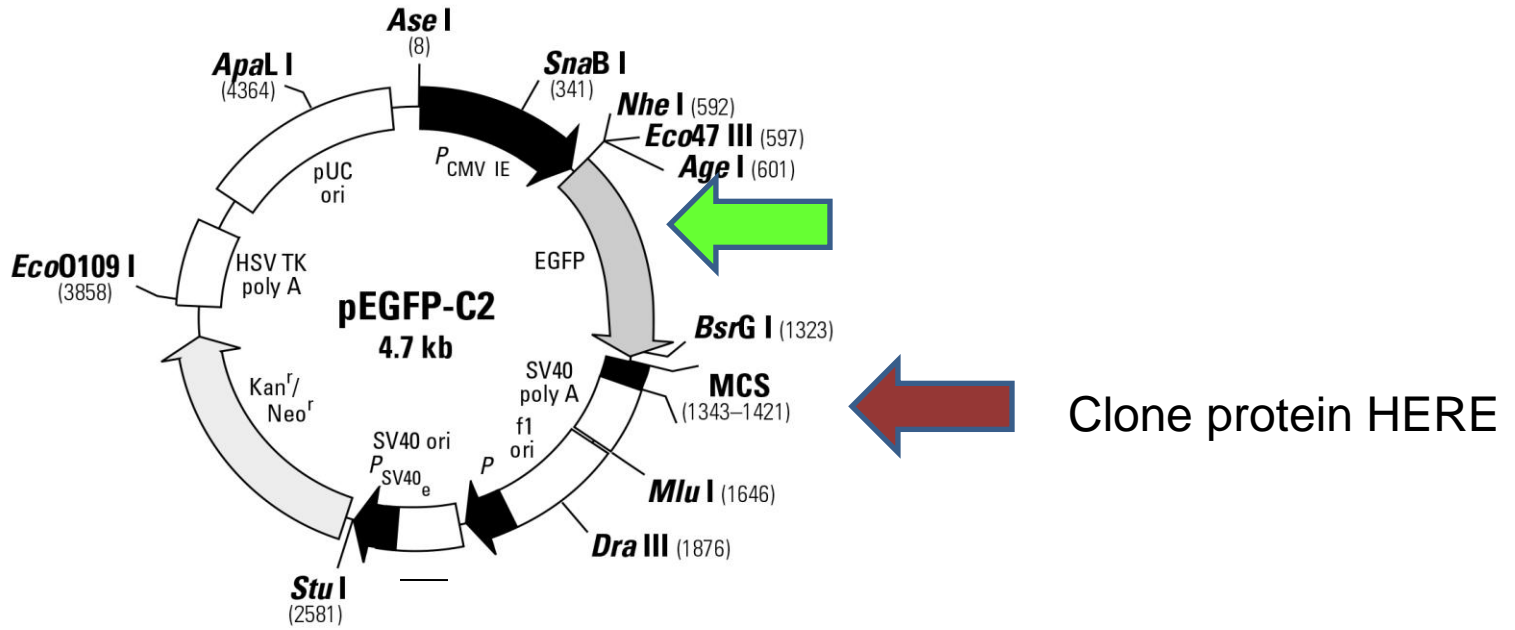


human EB1 cloned as an Xho1-BamH1 fragment for expression of EB1-GFP



## pEGFP-C2 Vector Information

GenBank Accession #: U57606



EGFP → 1330 1340 1350 1360 1370 1380 1390 1400 STOPs

TAC AAG TCC GGC CGG ACT CAG ATC TCG AGC TCA AGC TTC GAA TTC TGC AGT CGA CGG TAC CGC GGG CCC GGG ATC CAC CGG ATC TAG ATA ACT GAT CA

*Eag I* *Bgl II* *Xho I* *Sac I* *Hind III* *EcoR I* *Pst I* *Sal I* *Kpn I* *Apa I* *BamH I* *Xba I\** *Bcl I\**

*Acc I* *Asp118 I* *Sac II* *Bsp120 I* *Xma I* *Sma I*

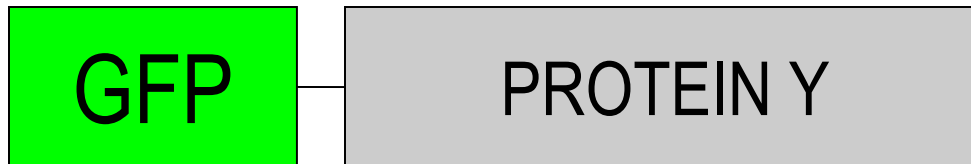
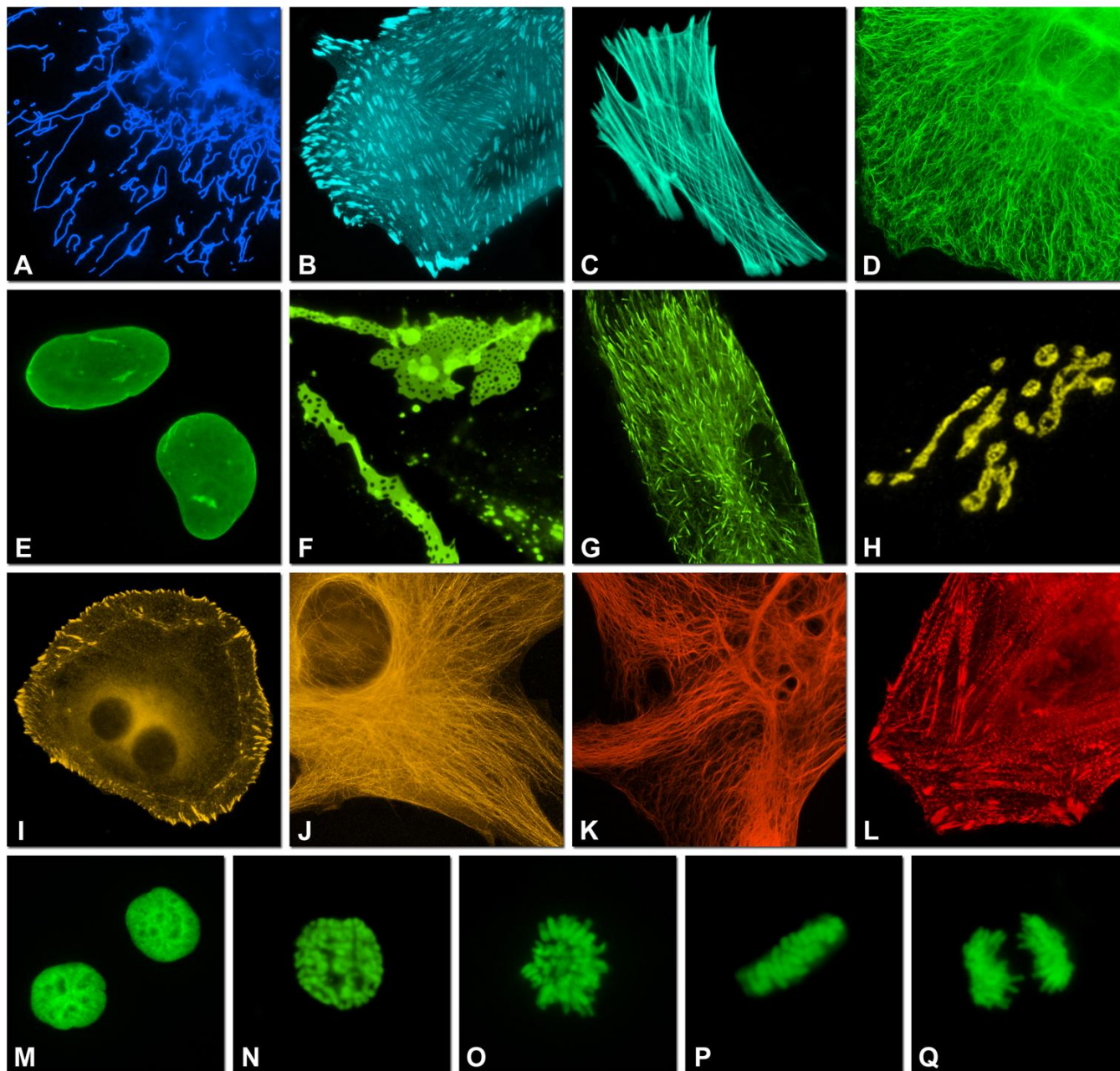
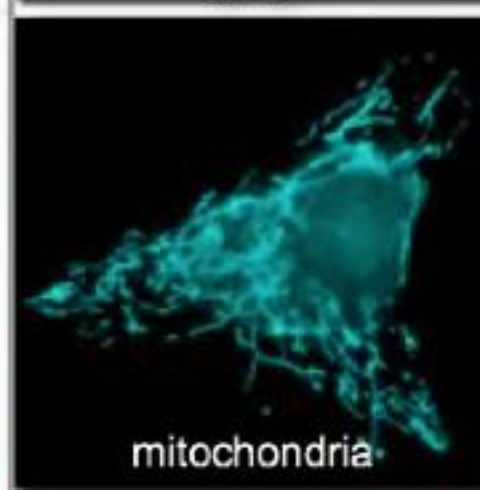
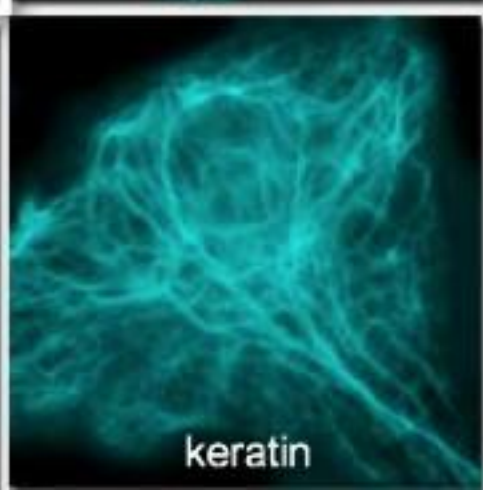
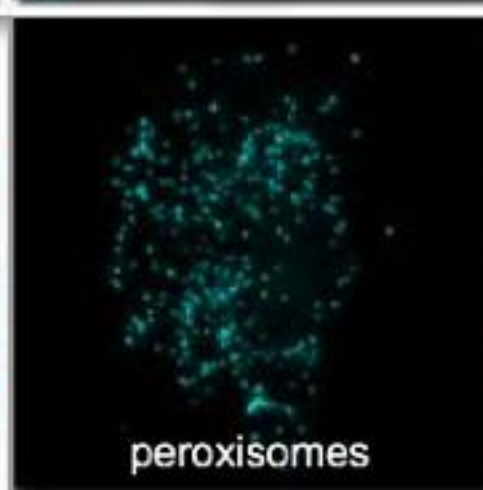
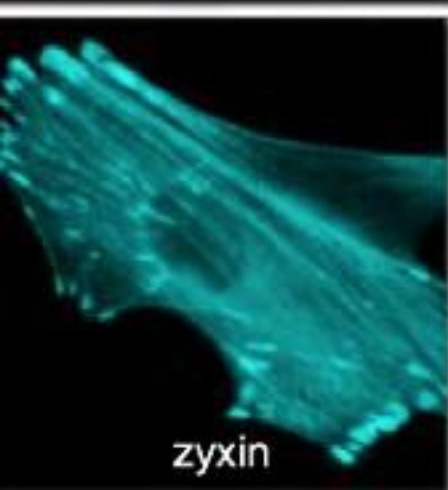
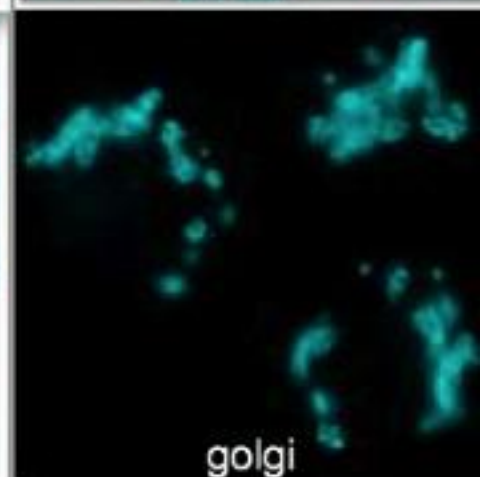
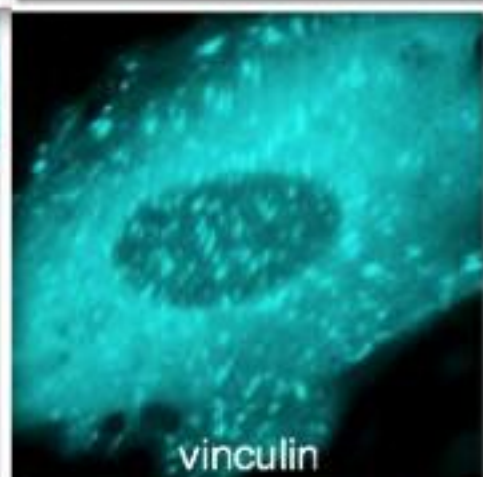
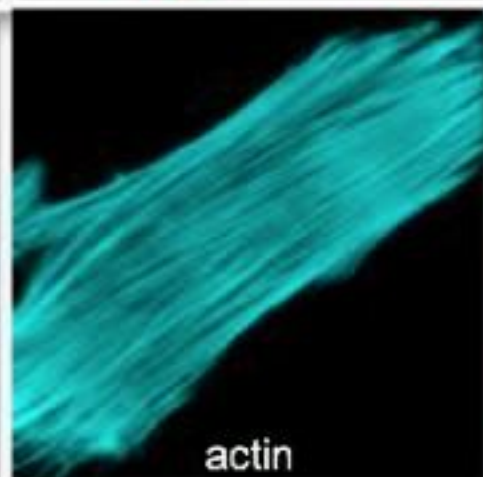
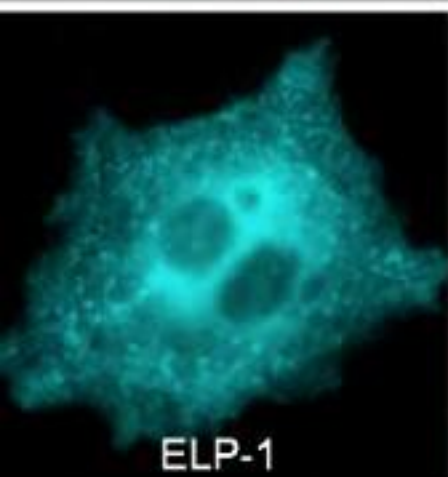
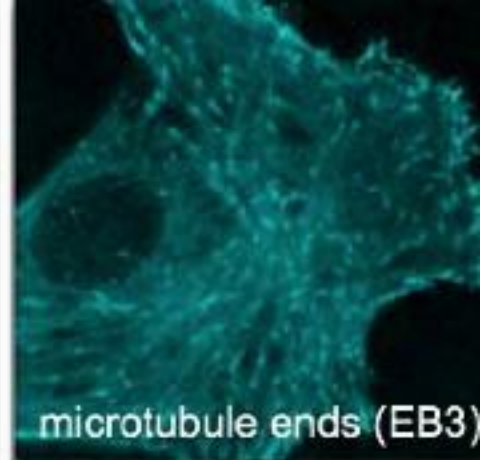
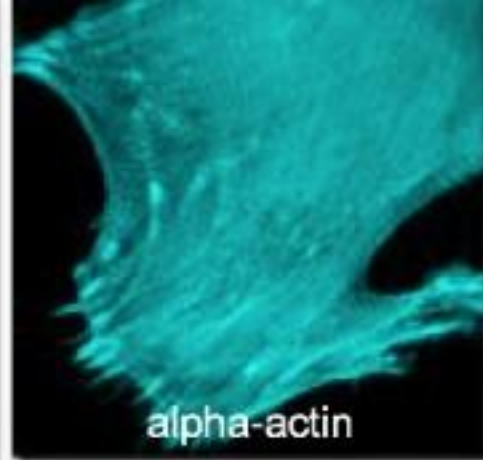
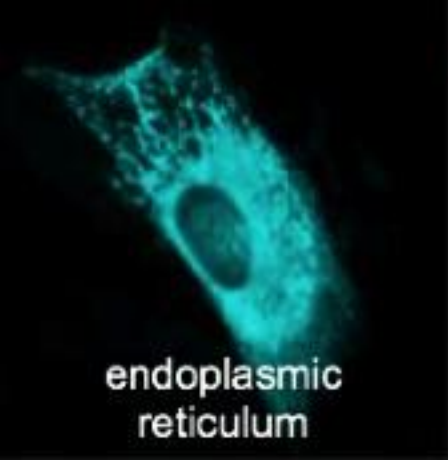
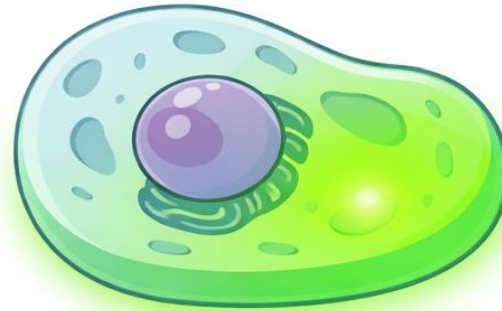
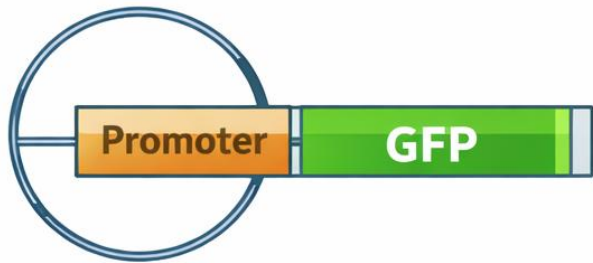


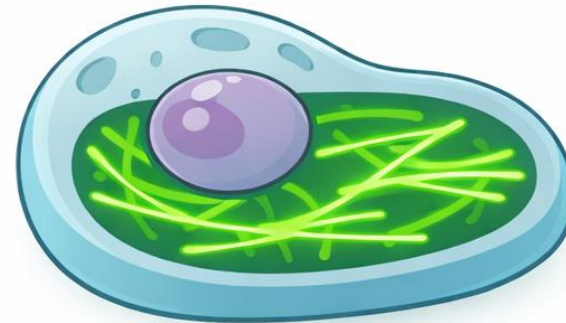
Fig. 3. (A-L) Subcellular localization of selected monomeric FP fusions (listed in Table 1) with targeting proteins imaged in widefield fluorescence. Images are pseudocolored to match the FP emission profile.







**Fluorescent Cell**



**Fluorescent Microtubules**