# *Fluorescence Probes and labels for biomedical applications*

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# Outline

## Fluorescence Probes

### Labeling "in vitro"

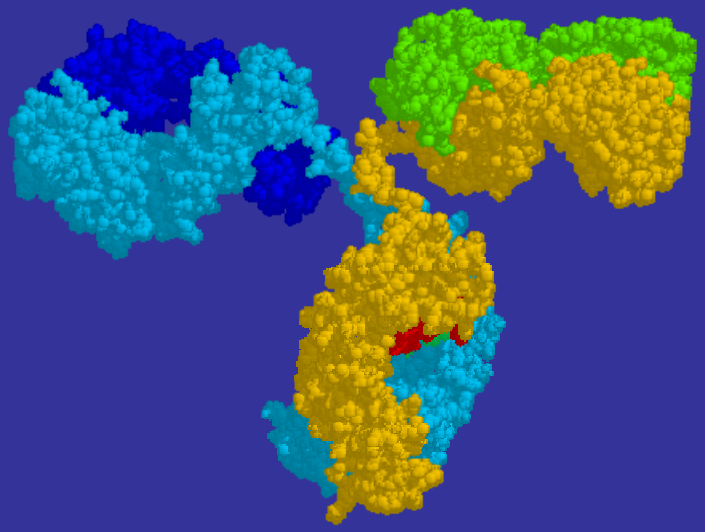
- Labeling proteins
- Labeling membranes
- Ions indicators
- Quantum dots
- Labeling DNA

### Labeling "in vivo"

- Genetic Incorporation
- Mechanical Incorporation

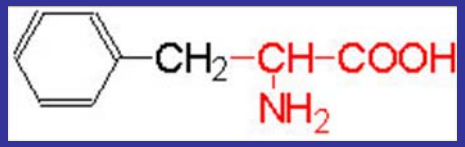
# Labeling *"in vivo"*

# Labeling proteins

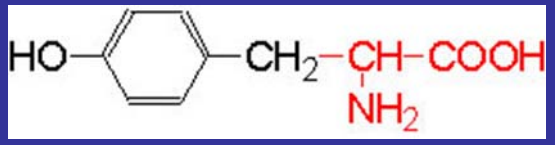


# Naturally Occurring Fluorophores

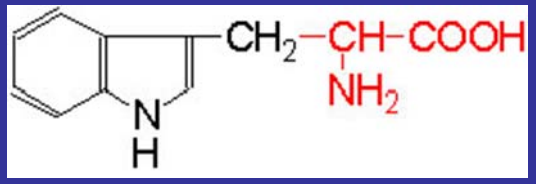
## Aromatic Amino Acids




**Phenylalanine**
Ex/Em 260 nm/282 nm

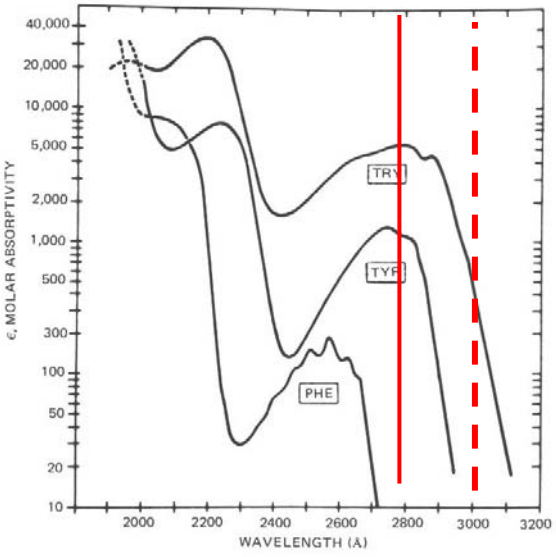



**Tyrosine**
ex/em 280 nm/305 nm

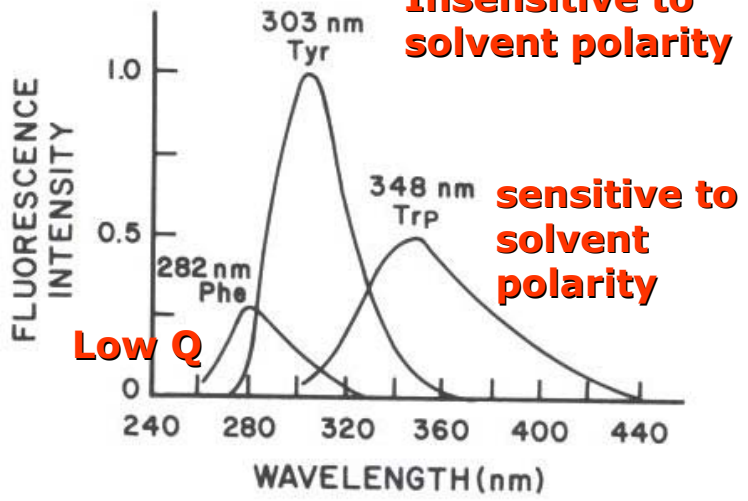



**Tryptophan**
ex/em 280, 295nm/ 305-350 nm

**Excitation**

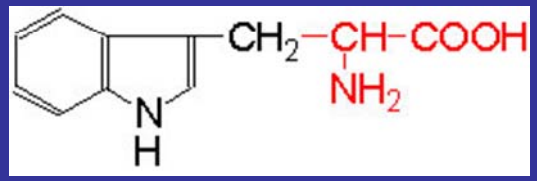



**Emission**

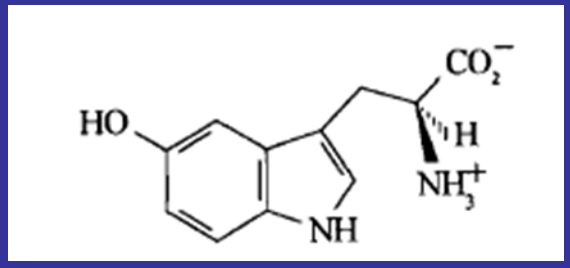


# Tryptophan derivatives

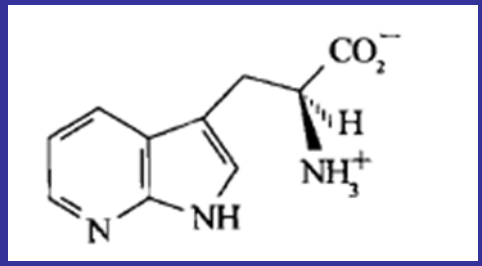
**Tryptophan derivatives may be genetically incorporated in a protein**




**Tryptophan**
ex/em 280, 295nm/ 305-350 nm




**5-Hydroxy-tryptophan**
ex/em 310nm/339 nm




**7-azatryptophan**
ex/em 320nm/403nm

**$\Phi$ =0.14**

- solvent-sensitive emission

**$\Phi$= 0.097**

- solvent-insensitive emission

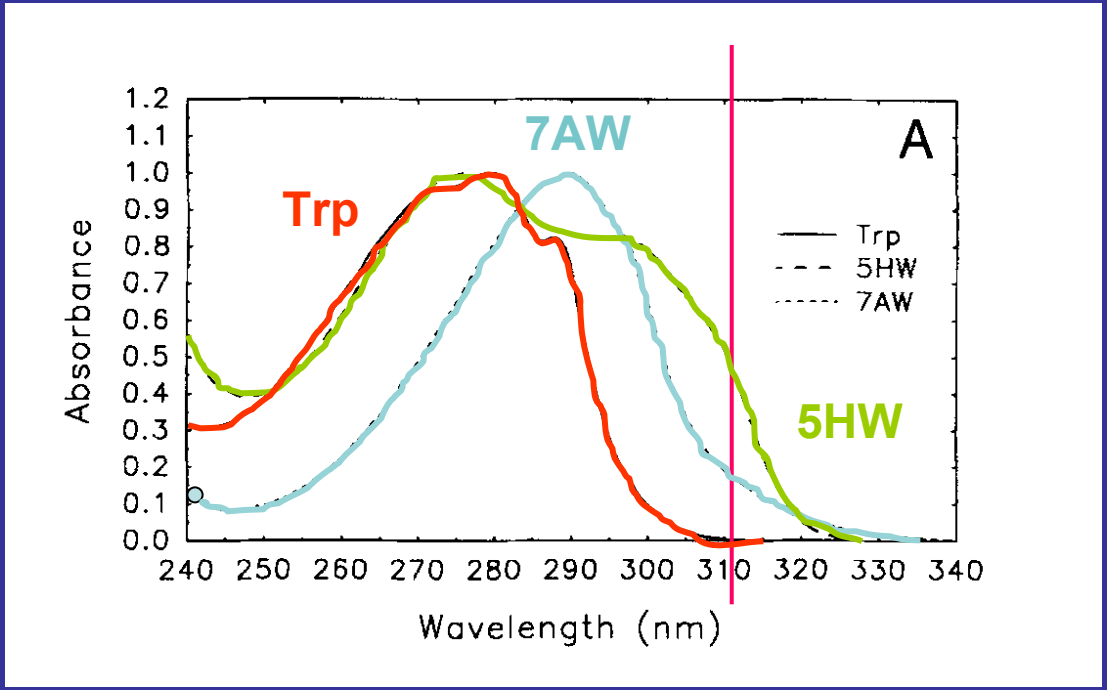
**$\Phi$ = 0.017**

- Large emission shift in water

$\Phi$ =Number of photons emitted/number of photons absorbed

***Protein Science*** *(1997), 6, 689-697.*

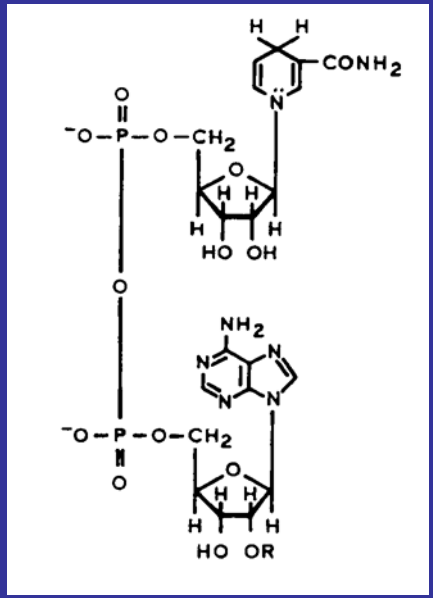
**Absorbance spectrum is red-shifted with respect to that of tryptophan.**



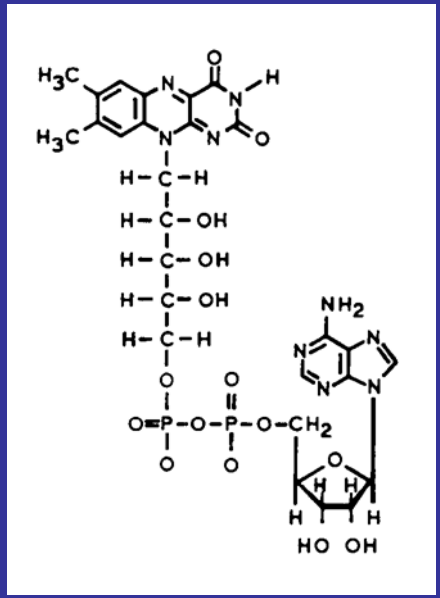

**It is possible to selectively excite them, in the presence of tryptophan of other proteins**

***Protein Science*** (1997), 6, 689-697.

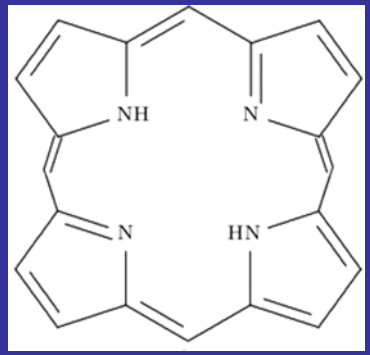
# Enzymes Cofactors




**NADH**
(oxido-reductases)
Ex/Em 340/460 nm




**FAD**
(metabolic enzymes
(ex/em 450nm/540 nm)



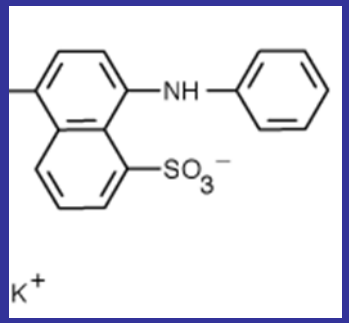

**Porphyrins**
(ex/em 550 nm/620 nm),

# Extrinsic probes

(not present in the natural molecule/macromolecule)

## Non-covalent Attachment

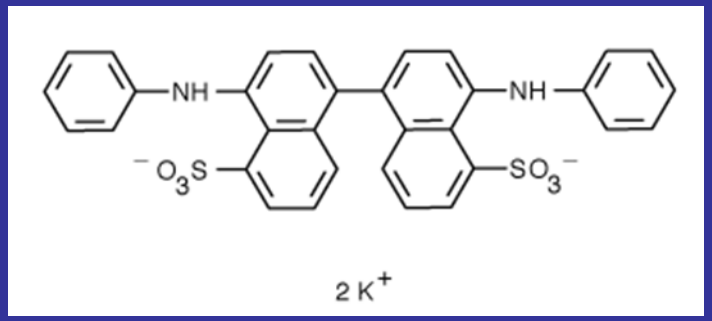
**1,8-ANS**



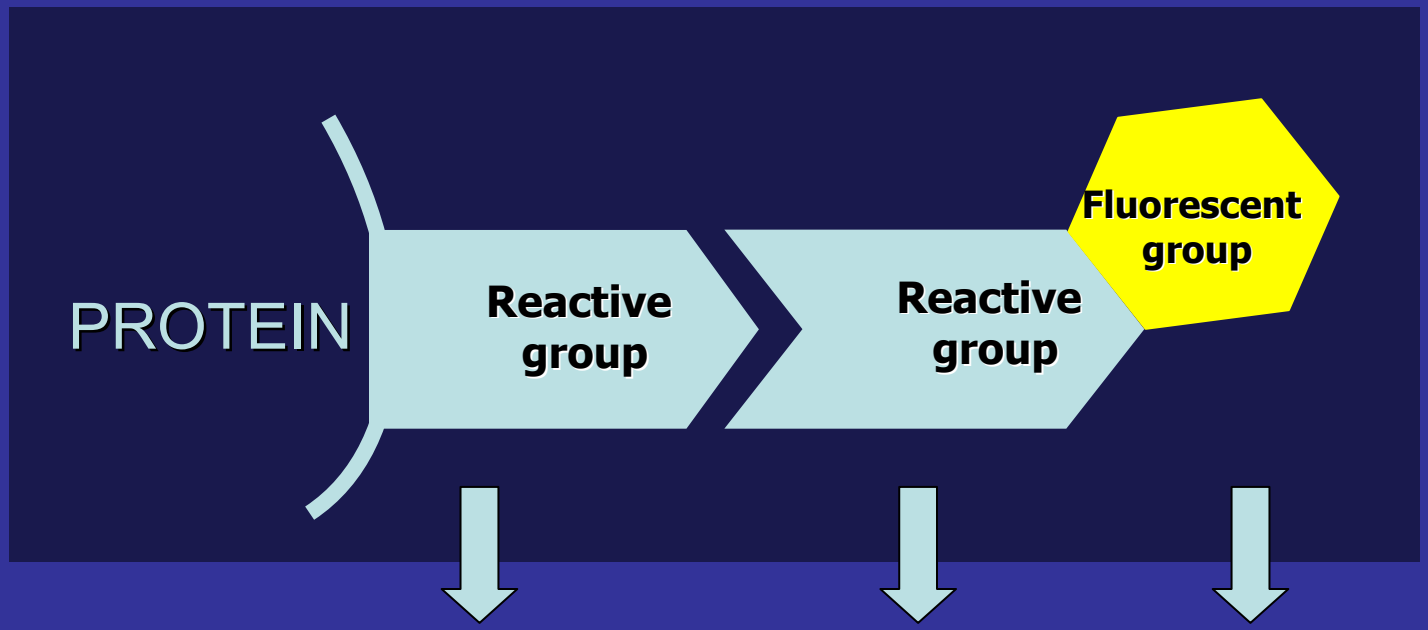

Developed by G. Weber in 1950's

Barely fluorescent in pure water but their fluorescence can be strongly enhanced if the environment becomes hydrophobic (hydrophobic patches on proteins)

**bis-ANS**



# Covalent Attachments




**Available reactive group in the protein**

**$NH_2$** Lysine Arginine

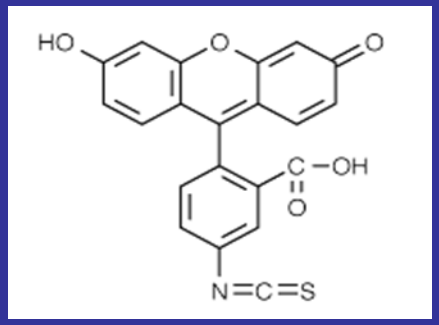
**SH** Cysteine

**Depends on the reactive group in the protein.**

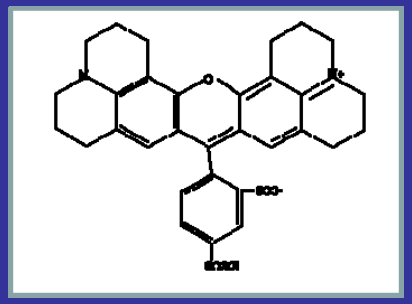
**Light source**
**Lifetime**
**Spectral properties**
**Autofluorescence**

**Labeling should not change the biological activity of the protein.**

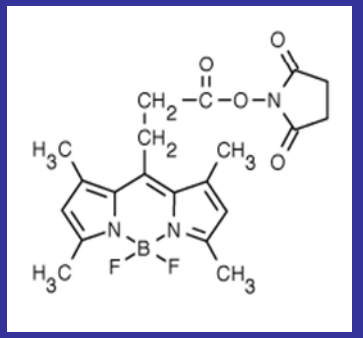
# Fluorescent groups



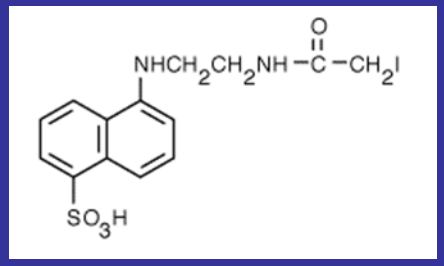

**FITC**
**(488/512) t ≈ 4.05**



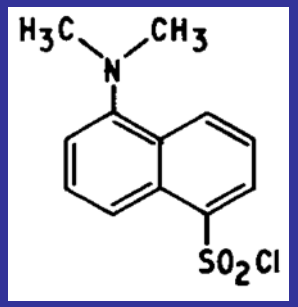
**Texas Red**
**(595-615), t ≈ 3.5 ns**



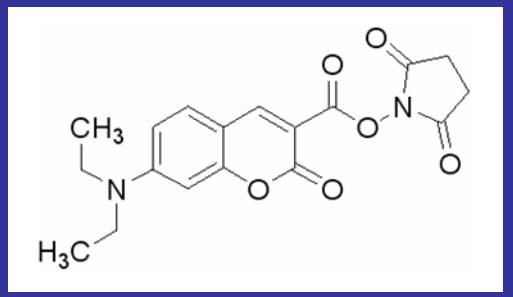

**BODIPY**
**(493/503), t = 6 ns**




**IAEDANS**
**(360/480) *t* ≈ 15 ns**

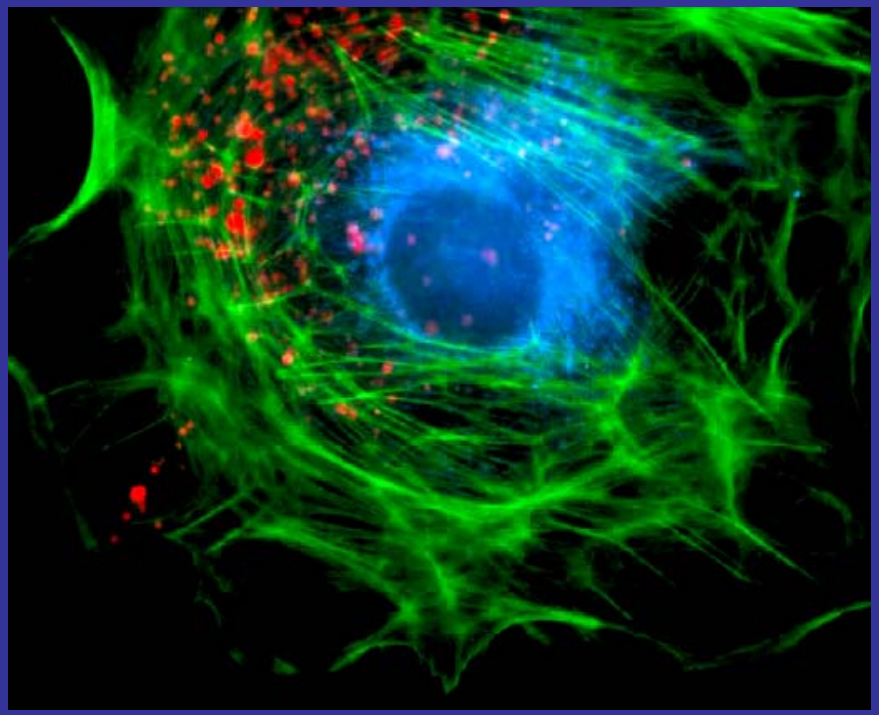



**Dansyl chloride**
**(335/518) t ≈ 10 ns**




**Coumarin-3-carboxylic acid -NHS**
**(445/482), *t* ≈ 2 -3 ns**

# The Alexa-Fluor series



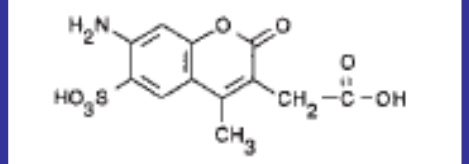
1999
"there is a need
for probes with high fluorescence quantum yield and high photostability to allow detection of low-abundance biological structures with great sensitivity and selectivity"

*The Journal of Histochemistry & CytochemistryVolume 47(9): 1179–1188, 1999.Molecular Probes, Inc., Eugene, Oregon*

# Designed to be more photostable than their commonly used spectral analogues

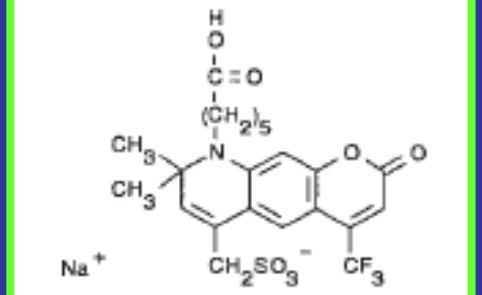
Coumarin-AMCA

**Alexa 350** 346/442



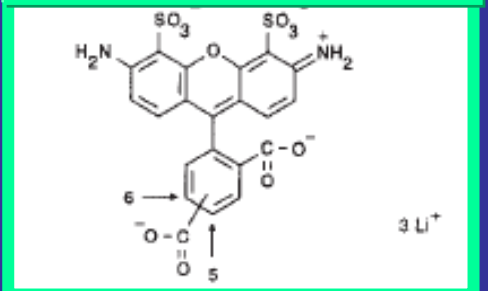
Lucifer Yellow

**Alexa 430** 434/539



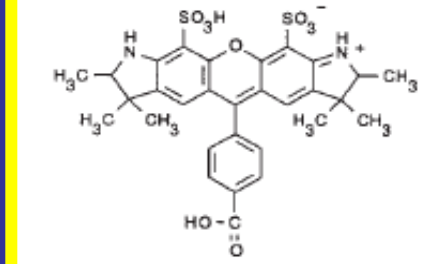
fluorescein

**Alexa 488** 495/519



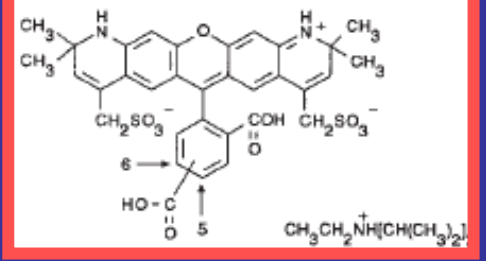
rhodamine 6G

**Alexa 532** 531/554



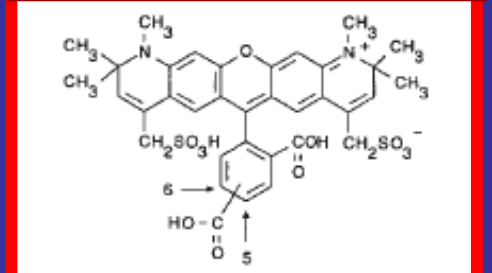
lissamine rhodamine B

**Alexa 568** 578/603



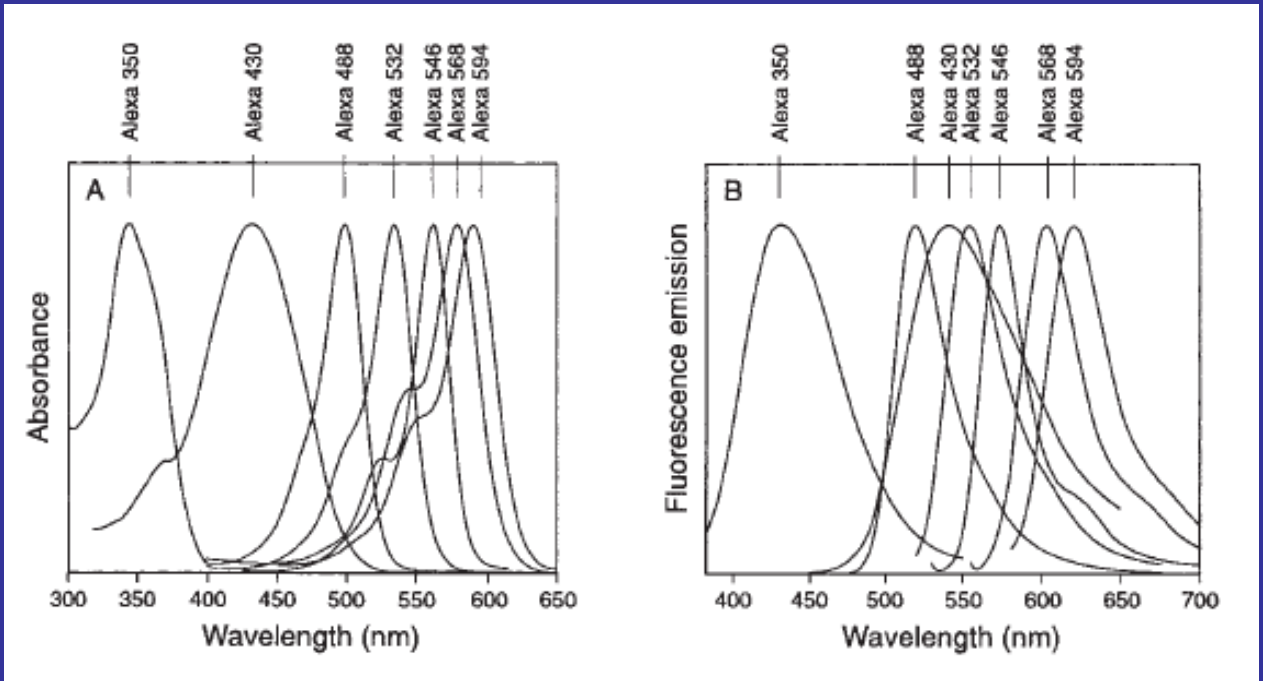
Texas Red

**Alexa 594** 590/617



All Alexa dyes and their conjugates are more fluorescent and more photostable than their commonly used spectral analogues.

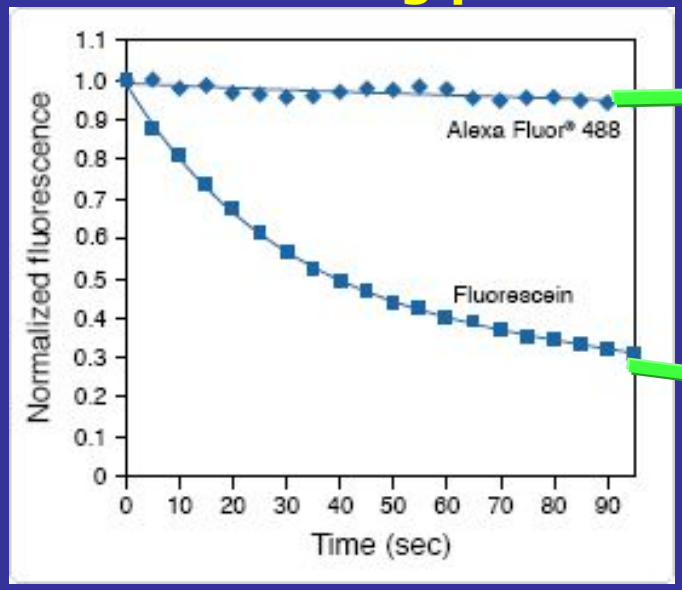
In addition, Alexa dyes are insensitive to pH in the 4–10 range.



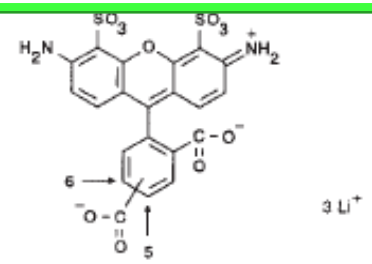

*The Journal of Histochemistry & CytochemistryVolume 47(9): 1179–1188, 1999.*
*Molecular Probes, Inc., Eugene, Oregon*

# Photostability
## Alexa Fluor 488 v/s fluoresceine

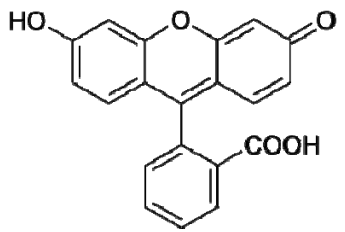
### Photo bleaching profile




**ALEXA 488**



**FLUORESCEIN**




- Cells stained with Alexa Fluor488 or fluorescein conjugates of goat anti–mouse IgG antibody

- Samples were continuously illuminated and images were collected every 5 seconds with a cooled CCD camera.

**http://www.invitrogen.com/**

**BRIGHTNESS:** Alexa Fluor conjugates exhibit more intense fluorescence than other spectrally similar conjugates

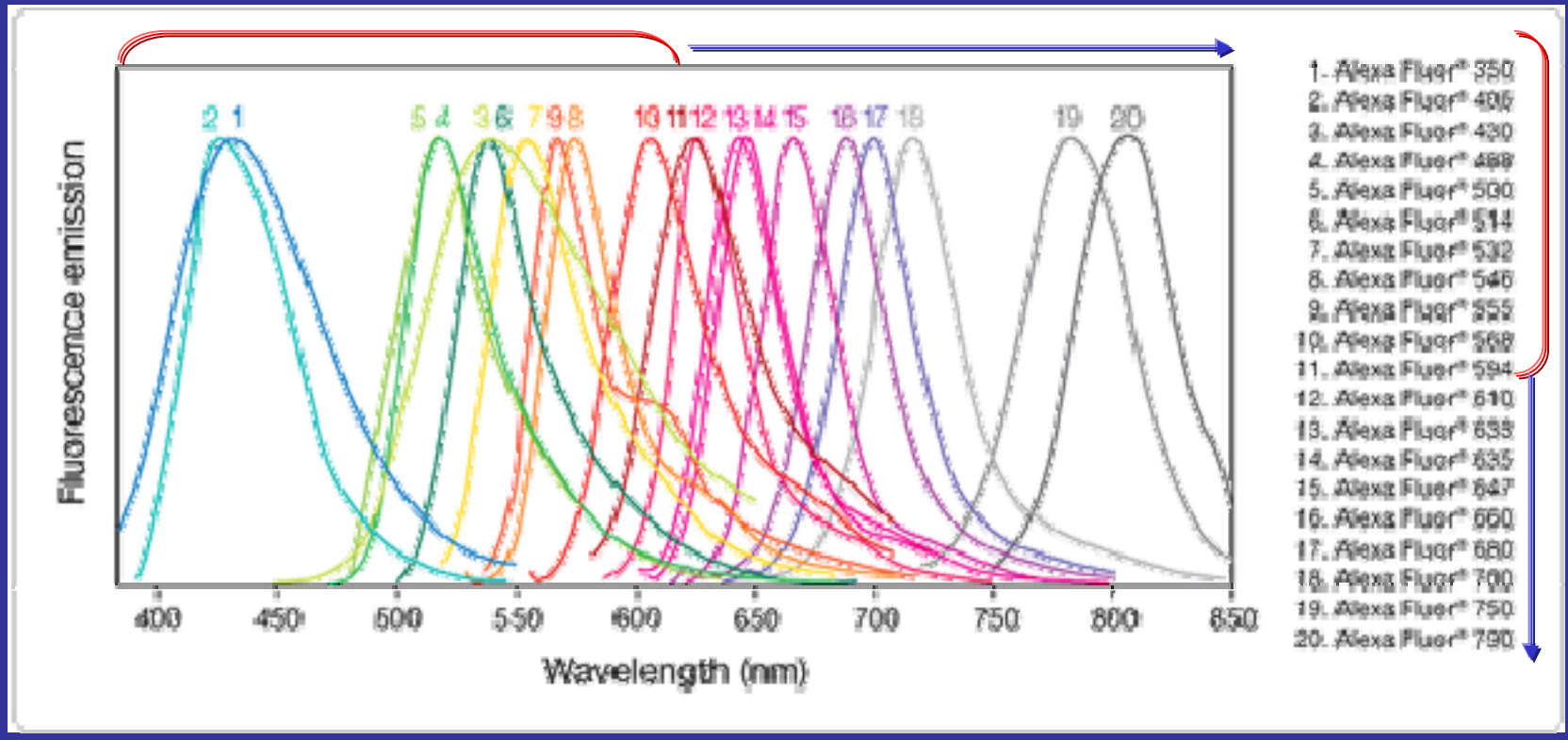
**PHOTOSTABILITY:** Alexa Fluor conjugates are more photostable than most other fluorescent conjugates

**COLOR SELECTION:** Alexa Fluor conjugates are available in several distinct fluorescent colors, ranging from blue to red to near-infrared

****WATER SOLUBILITY:** Alexa Fluor reactive dyes have good water solubility, so protein conjugations can be performed without organic solvents

Conventional fluorophores and their conjugates can be replaced with spectrally similar Alexa Fluor dyes without affecting optical filter choices or other instrumentation considerations

# The Alexa series expanded




**Alexa Fluor dyes are available as amine-reactive succinimidyl esters**

# Spectral properties of Alexa Fluor dyes

| Alexa Fluor Dy | Absorpt Max (nn | Emission Max (nm) * | Emission Color † | Extinction Coefficient ‡ |
|---|---|---|---|---|
| Alexa Fluor 350 | 346 | 442 | Blue | 19,000 |
| Alexa Fluor 405 | 402 | 421 | Blue | 35,000 |
| Alexa Fluor 430 | 434 | 539 | Yellow-green | 15,000 |
| Alexa Fluor 488 | 495 | 519 | Green | 73,000 |
| Alexa Fluor 514 | 518 | 540 | Green | 80,000 |
| Alexa Fluor 532 | 531 | 554 | Yellow | 81,000 |
| Alexa Fluor 546 | 556 | 573 | Orange | 112,000 |
| Alexa Fluor 555 | 555 | 565 | Orange | 155,000 |
| Alexa Fluor 568 | 578 | 603 | Red-orange | 88,000 |
| Alexa Fluor 594 | 590 | 617 | Red | 92,000 |
| Alexa Fluor 610 | 612 | 628 | Red | 144,000 |
| Alexa Fluor 633 | 632 | 647 | Far-red | 159,000 |
| Alexa Fluor 635 | 633 | 647 | Far-red | 140,000 |
| Alexa Fluor 647 | 650 | 668 | Far-red | 270,000 |
| Alexa Fluor 660 | 663 | 690 | Near-IR § | 132,000 |
| Alexa Fluor 680 | 679 | 702 | Near-IR § | 183,000 |
| Alexa Fluor 700 | 702 | 723 | Near-IR § | 205,000 |
| Alexa Fluor 750 | 749 | 775 | Near-IR § | 290,000 |
| Alexa Fluor 790 | 782 | 805 | Near-IR § | 260,000 |

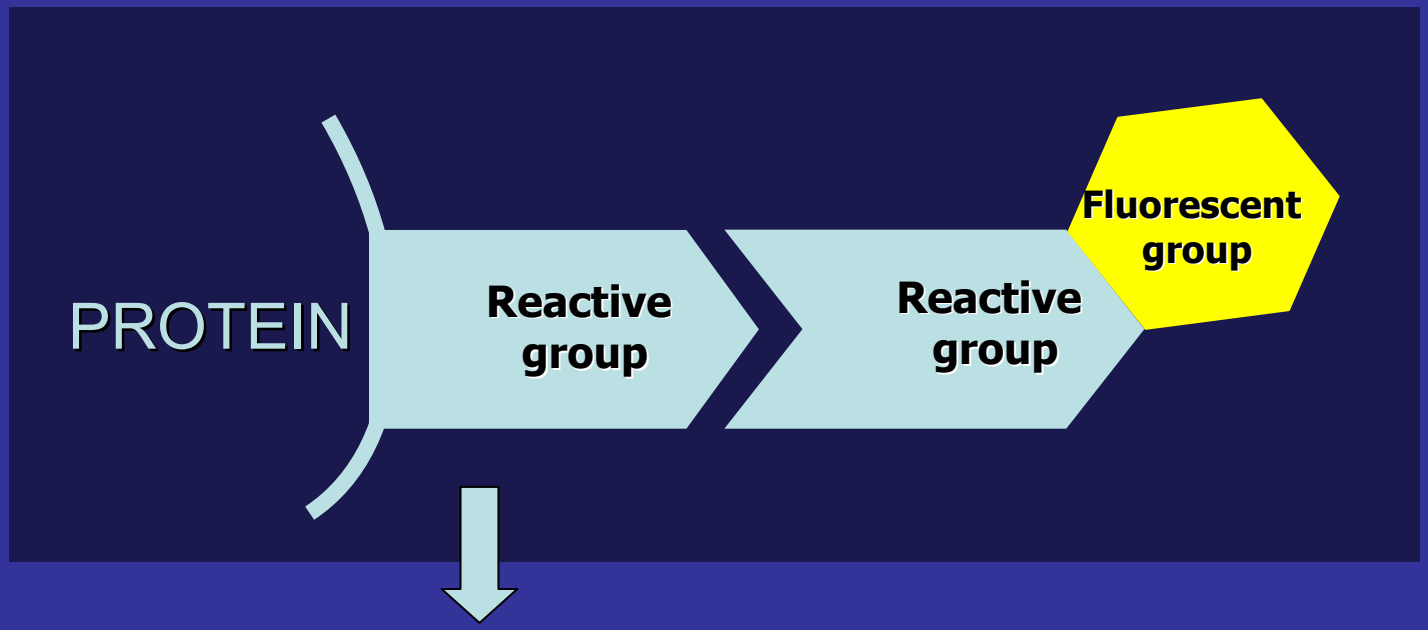
**http://www.invitrogen.com**

**Fluorescence quantum yields (QY) and lifetimes (τ) for Alexa Fluor dyes–**

| Alexa Fluor Dye * | QY † | τ (ns) ‡ |
|---|---|---|
| Alexa Fluor 488 | 0.92 | 4.1 § |
| Alexa Fluor 532 | 0.61 | 2.5 |
| Alexa Fluor 546 | 0.79 | 4.1 |
| Alexa Fluor 555 | 0.10 | 0.3 |
| Alexa Fluor 568 | 0.69 | 3.6 § |
| Alexa Fluor 594 | 0.66 | 3.9 § |
| Alexa Fluor 647 | 0.33 | 1.0 |
| Alexa Fluor 660 | 0.37 | 1.2 ** |
| Alexa Fluor 680 | 0.36 | 1.2 |
| Alexa Fluor 700 | 0.25 | 1.0 |
| Alexa Fluor 750 | 0.12 | 0.7 |

**http://www.invitrogen.com**

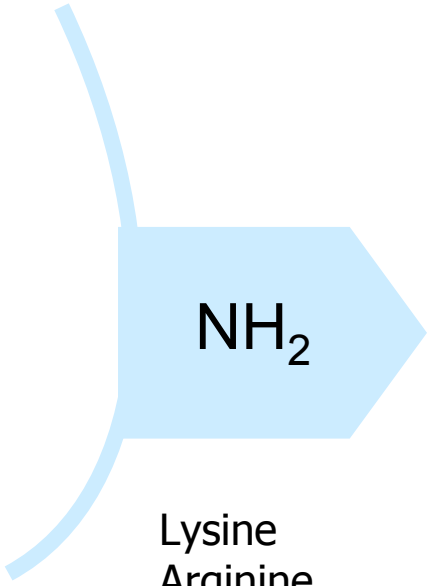
# Covalent Attachments




**Available reactive group in the protein**

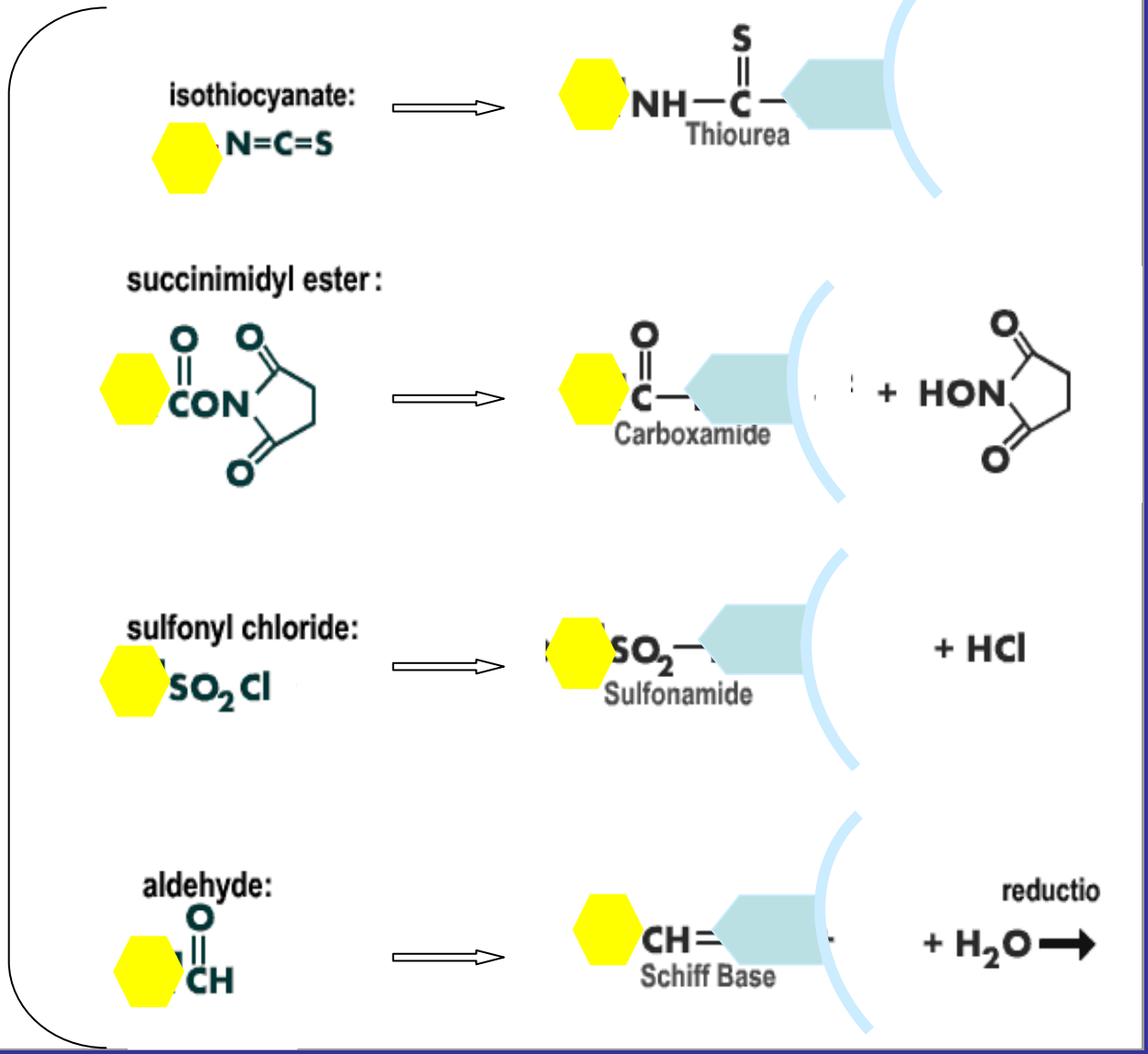
| | |
|---|---|
| $NH_2$ | Lysine<br>Arginine |
| SH | Cysteine |

# Targeting amino groups




Lysine
Arginine

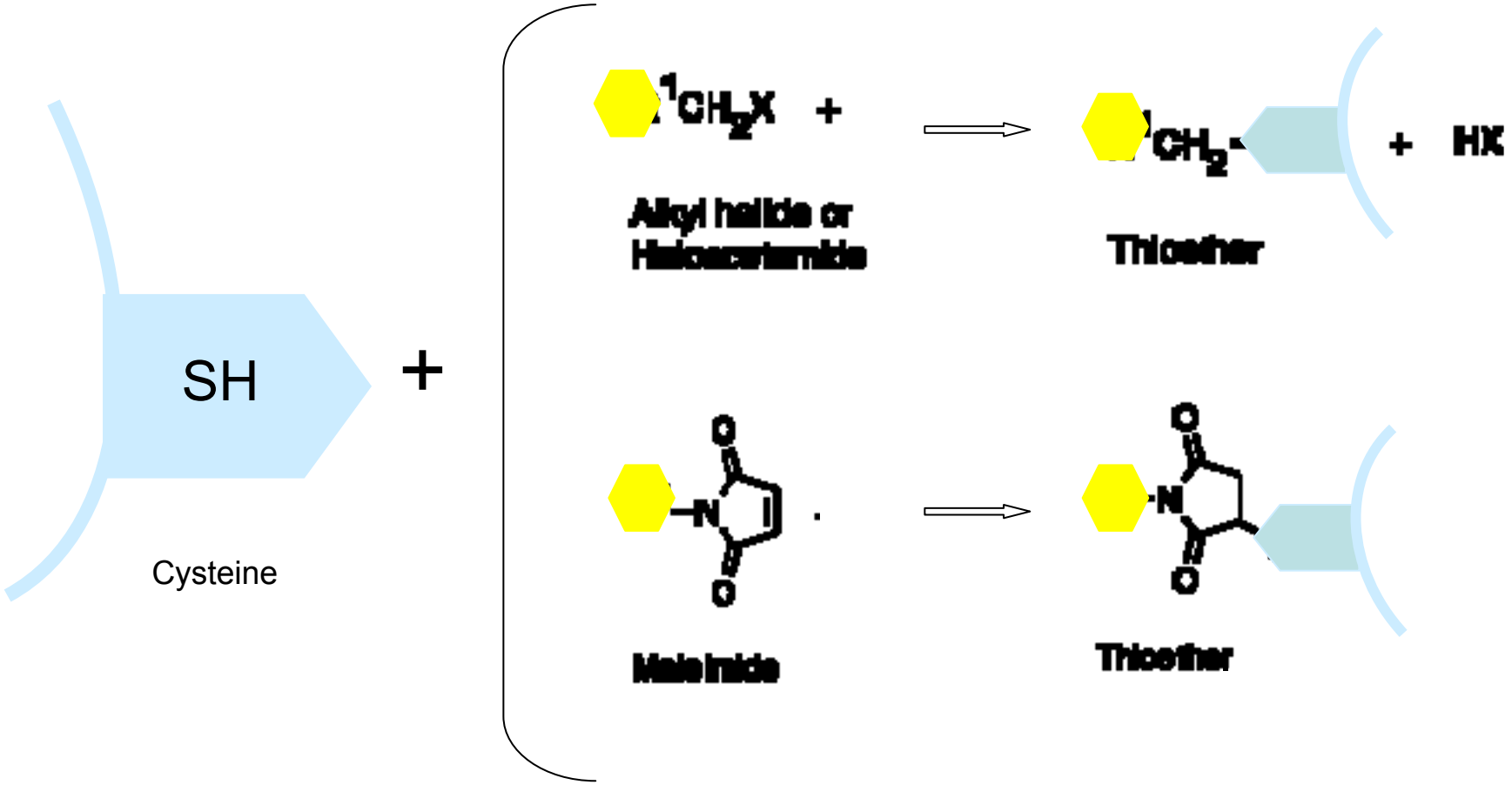
+



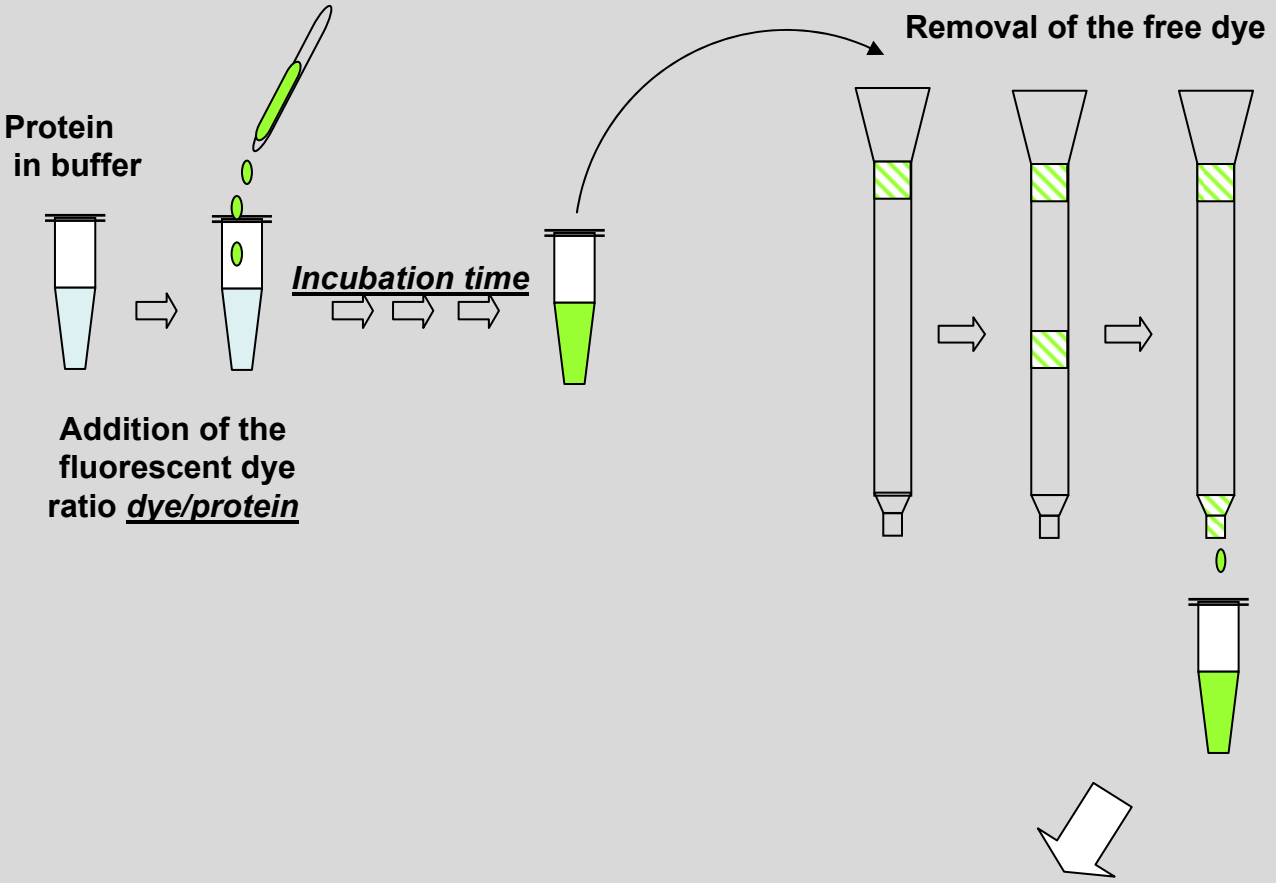

pH 8.5 to 9.5 :or modifying lysine residues.

near neutral pH: labeling of N-terminus .(pKa 7)

# Targeting thiol groups:



# General labeling protocol for extrinsic labeling




**Labeling ratio**

$$\frac{[\text{protein}]}{[\text{fluorescent dye}]}$$

**Sample characterization**

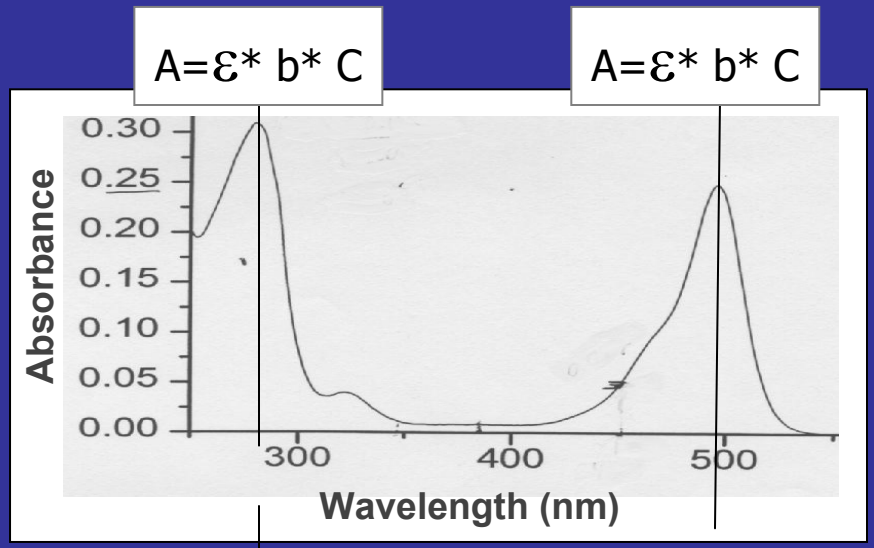
Absorption spectra
Protein determination

**Biological testing**

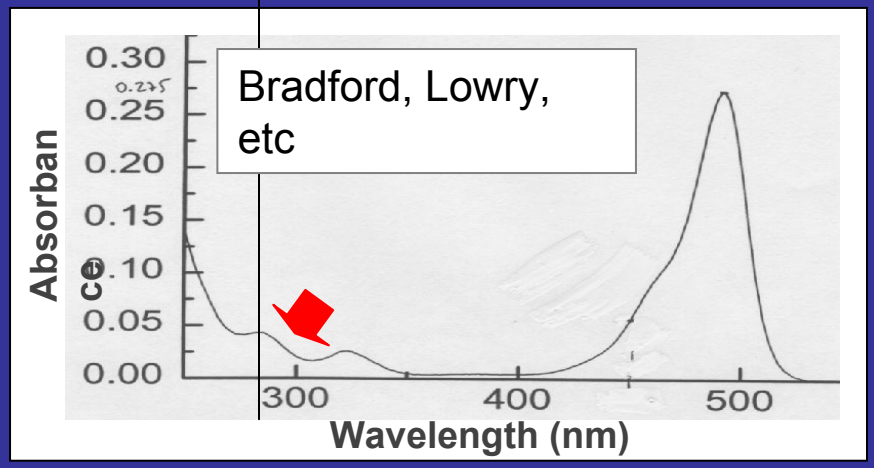
Activity measurements
SDS or native gel
Denaturation exp etc.

# Characterization after the labeling

**Protein-Fluorescein**



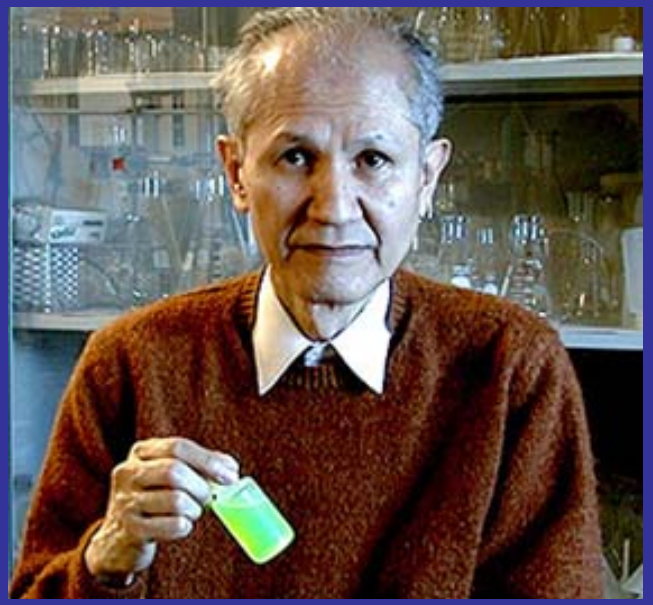

**Fluorescein**




**Labeling should not change the biological activity of the protein.**

# Green Fluorescent Protein

Purified by Osamu Shimomura in 1961

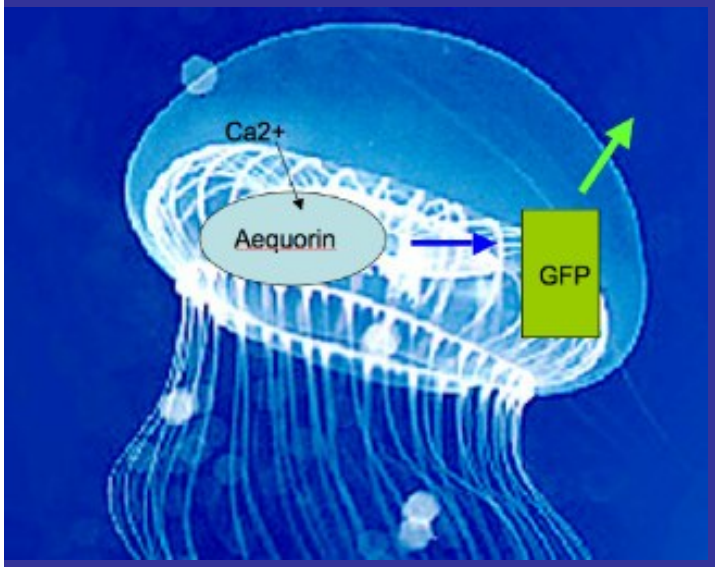


***Aequorea victoria*** jellyfish **Osamu Shimomura**

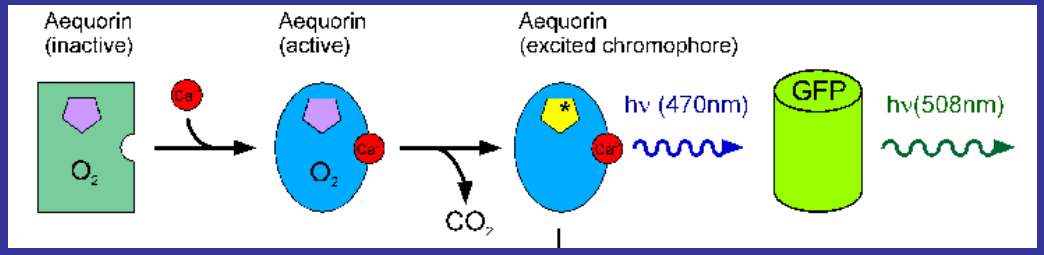
***Shimomura O, Johnson F, Saiga Y (1962). "Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea". J Cell Comp Physiol 59: 223-39.***

# Green Fluorescent Protein

**GFP is produced by *Aequorea victoria.***




**Upon mechanical stimulation *A. victoria* emits a green light after excitation of GFP by Aequorin ($Ca^{2+}$).**

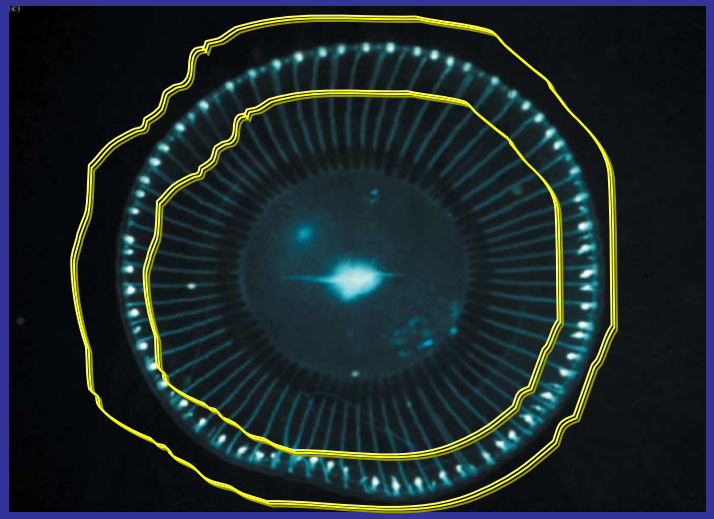



**Presumably a defense mechanism to blind attackers**

***Shimomura O, Johnson F, Saiga Y (1962). "Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea". J Cell Comp Physiol 59: 223-39.***

# Green Fluorescent Protein

**The protein is purified from the luminescent organs in the ring**

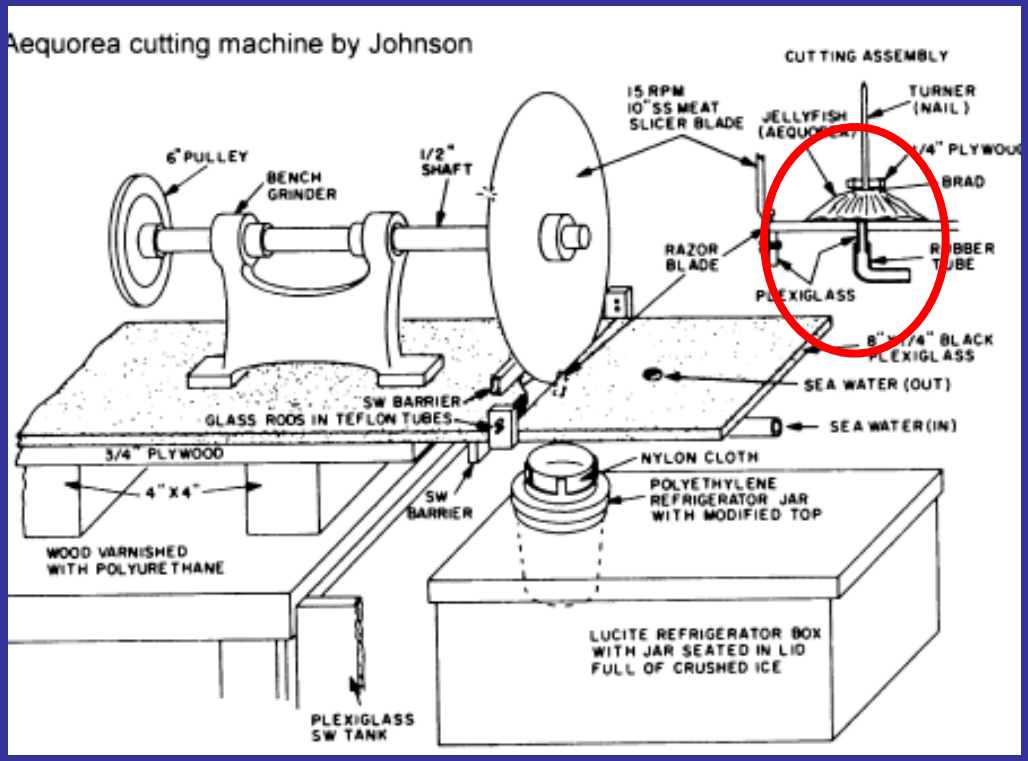


**The ring can be manually removed with scissors**

***Shimomura. The discovery of aequorin and green fluorescent protein. Journal of Microscopy, 217: 3–15 (2005).***

# The "ring cutting machine"

- To obtain 1mg of GFP they required 50,000 animals (2.5 tons of jellyfish)
- The job was done in one summer processing 3,000 jellyfish each day.




*Shimomura. The discovery of aequorin and green fluorescent protein. Journal of Microscopy, 217: 3–15 (2005).*

# Shimomura: daily schedule
## summer 1961

**Our task was to catch and process as many jellyfish as possible.**

**- 6 am - 8 am: collect jellyfish**
**- Quick breakfast**
**- Cut rings from the jellyfish until noon.**
**- Afternoon devoted to the extraction.**
**- Dinner**
**- 7 pm - 9 pm: collect jellyfish and keep it in an aquarium for the next day.**

***Shimomura. The discovery of aequorin and green fluorescent protein. Journal of Microscopy, 217: 3–15 (2005).***

**1974, Morize et al**
**GFP was completely purified and crystallized**

**1979, Shimomura**
**the structure of the GFP chromophore was elucidated**

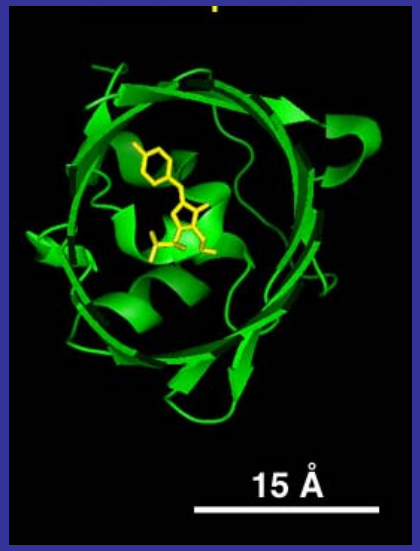
**1992, Prasher et al**
**cDNA of GFP was cloned**

**1994, Chalfie at al and Inouye & Tsuji.**
**cDNA of GFP was expressed in living organisms**

***Matz et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol. 17:969-73 (1999)***

# GFP chromophore

- β-barrel structure, with chromophore housed within the barrel.

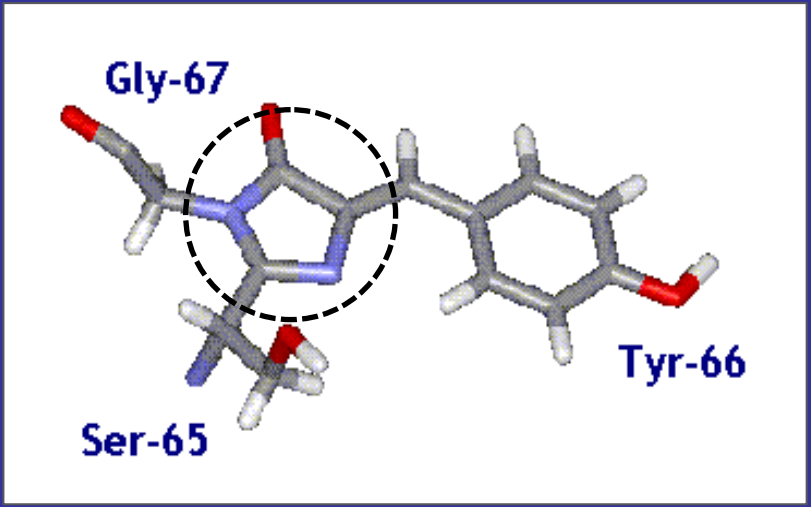



- The chromophore is formed spontaneously from

Serine-65, Tyrosine-66, Glycine-67

upon folding of the polypeptide chain, without the need for enzymatic synthesis.

# GFP chromophore



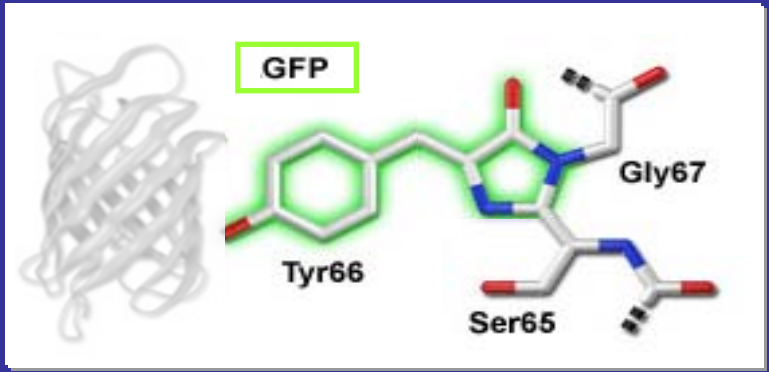

**The Ser-Tyr-Gly sequence is post-translationally modified to a 4-(p-hydroxybenzylidene)- imidazolidin-5-one structure**

**The fluorescence is not an intrinsic property of the Ser-Tyr-Gly tripeptide. The cyclized backbone of these residues forms the imidazolidone ring.**

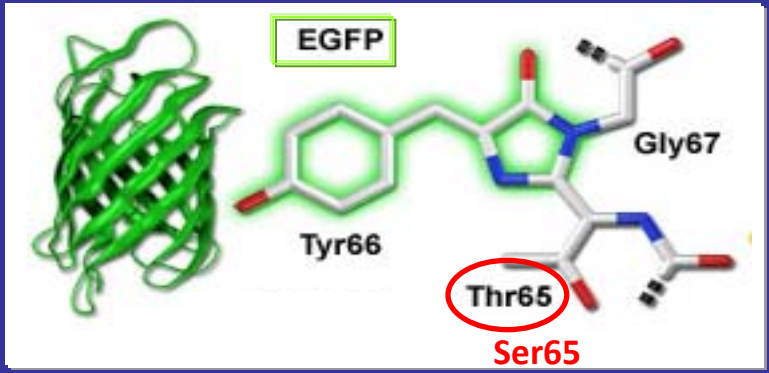
***http://www.scivee.tv/node/11255***

# FPs of different colors

**A number of new GFP proteins have been made using site directed mutagenesis to alter the amino acids near the chromophore and thus alter the absorption and fluorescence properties.**



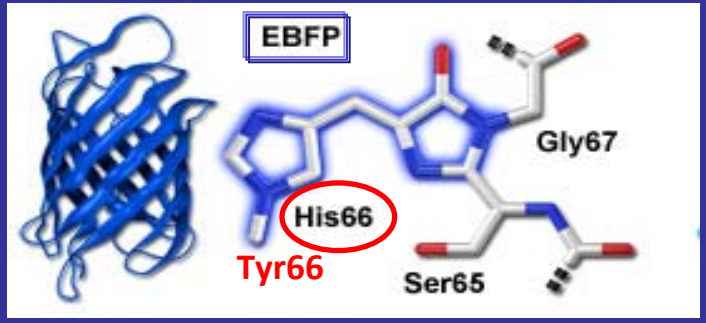

**GFP from *A. victoria***
**Matures at low temperature**
**Excitation peak = 395 nm**
**Emission peak = 509 nm**
**MW:25-30 KDa**

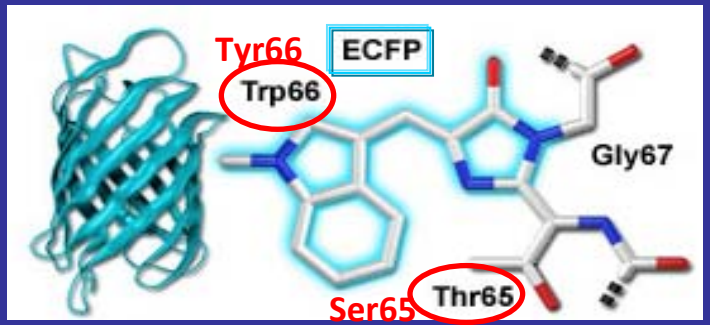


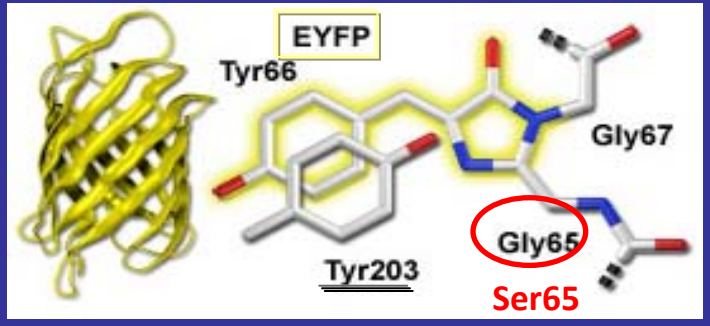

**1995 Ole Thastrup EGFP (37°C folding GFP) allowed the use of GFPs in mammalian cells**
**E = 55,000 $M^{-1}cm^{-1}$**
**QY = EGFP is 0.60**
**Excitation peak = 487 nm**
**Emission peak = 507 nm**

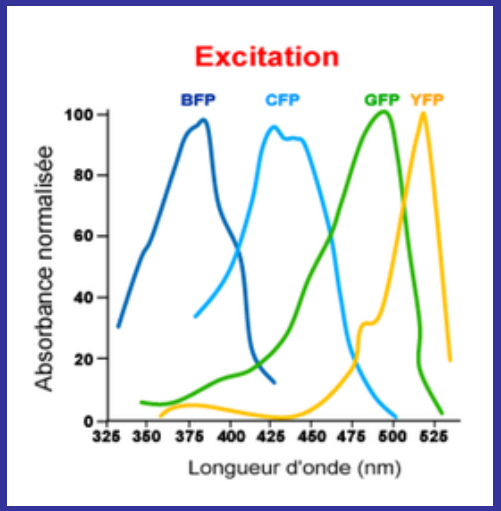
***http://www.olympusfluoview.com/applications/fpcolorpalette.html***

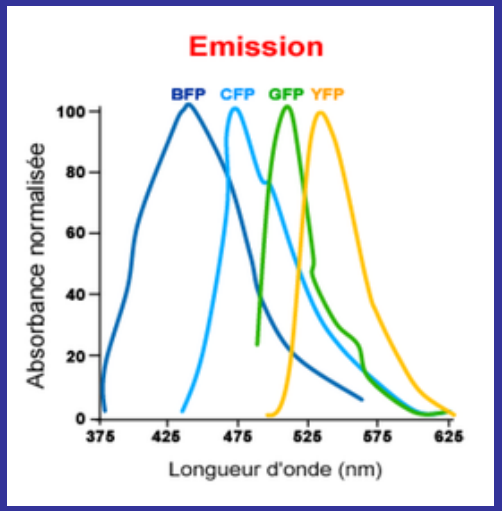
# Other mutations



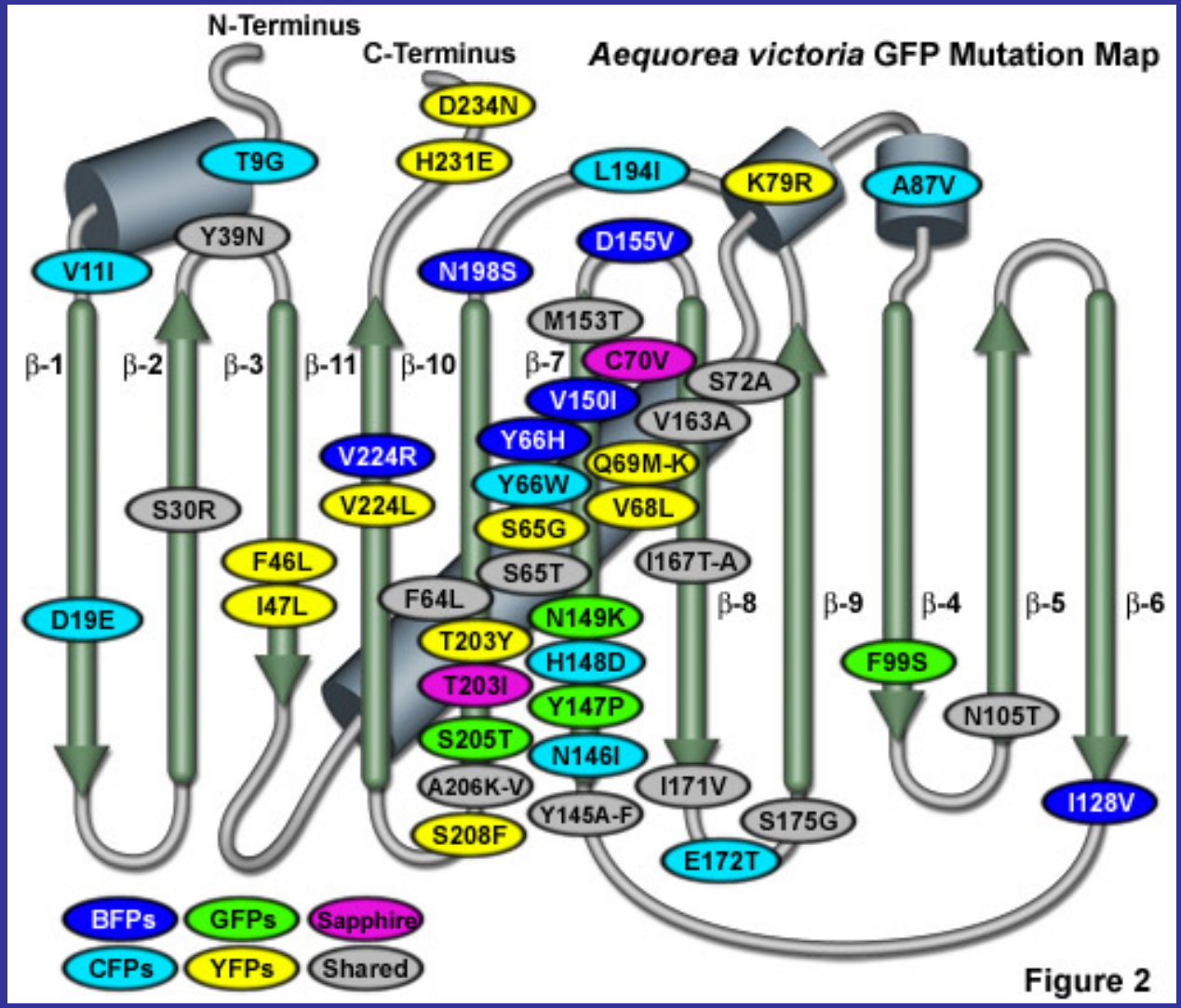




*http://www.olympusfluoview.com/applications/fpcolorpalette.html*

# GFP mutations map




*http://www.olympusfluoview.com/applications/fpcolorpalette.html*

**It is possible to insert the gene for GFP into cells and use the resulting protein as a reporter for a variety of applications.**

**from the *Aequorea*-based fluorescent proteins, the yellow fluorescent proteins (YFPs) remain the most red-shifted of the GFP derivatives. Em/ex = 520/530 nm**

## The search for a red-emitting fluorescent protein

- **Provide an important tool for multicolor imaging**
- **Generate new FRET biosensors with spectral profiles in the longer wavelength regions.**
- **Cellular auto-fluorescence is significantly reduced at longer wavelength regions.**
- **Living cells and tissues better tolerate illumination by the longer excitation wavelengths, allowing extend periods for imaging.**

**http://zeiss-campus.magnet.fsu.edu/articles/probes/anthozoafps.html**

# Red fluorescent proteins

**Matz et al, 1999 DsRed purified from Disco*soma striata***
fully matured DsRed fluorescent protein
excitation/emission = 558 / 583 nm.

- **The maturation of DsRed is slow**
- **Maturation goes through an intermediate green chromophore stage**
- **DsRed is an obligate tetramer with the tendency to form oligomers.**

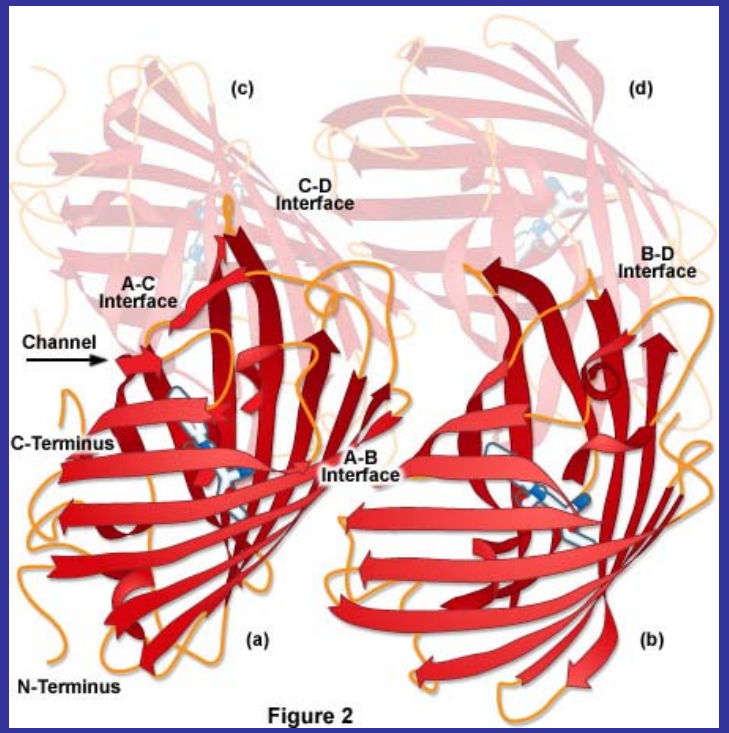
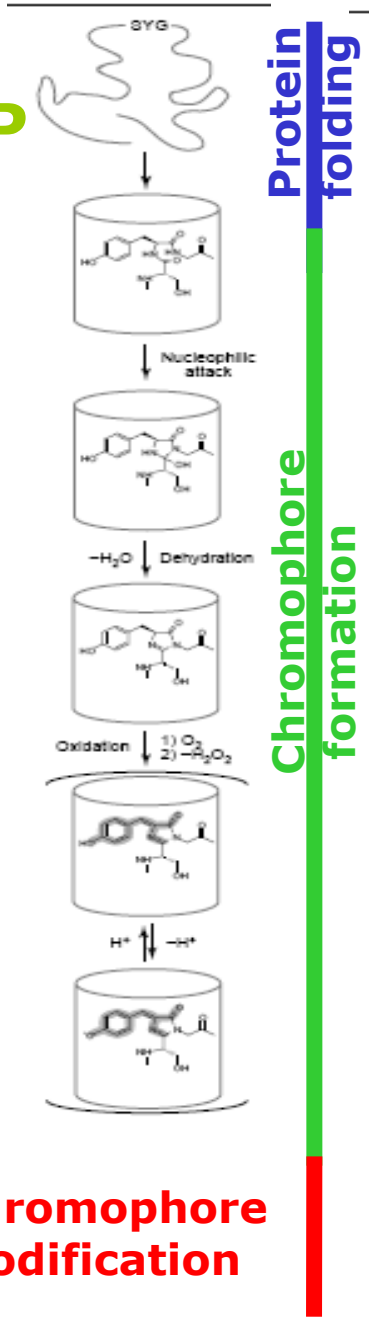


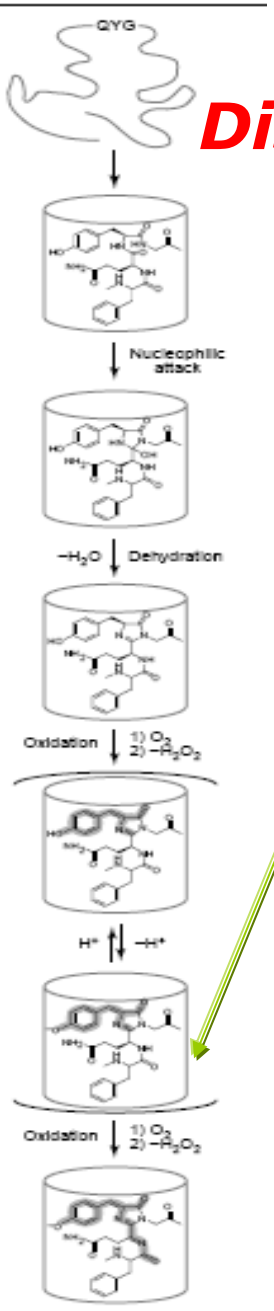
Figure 2

## *Aequorea* GFP

**Matures at low temperature**

**Dimeric**




## *Discosoma* DsRed

**Slow maturation**

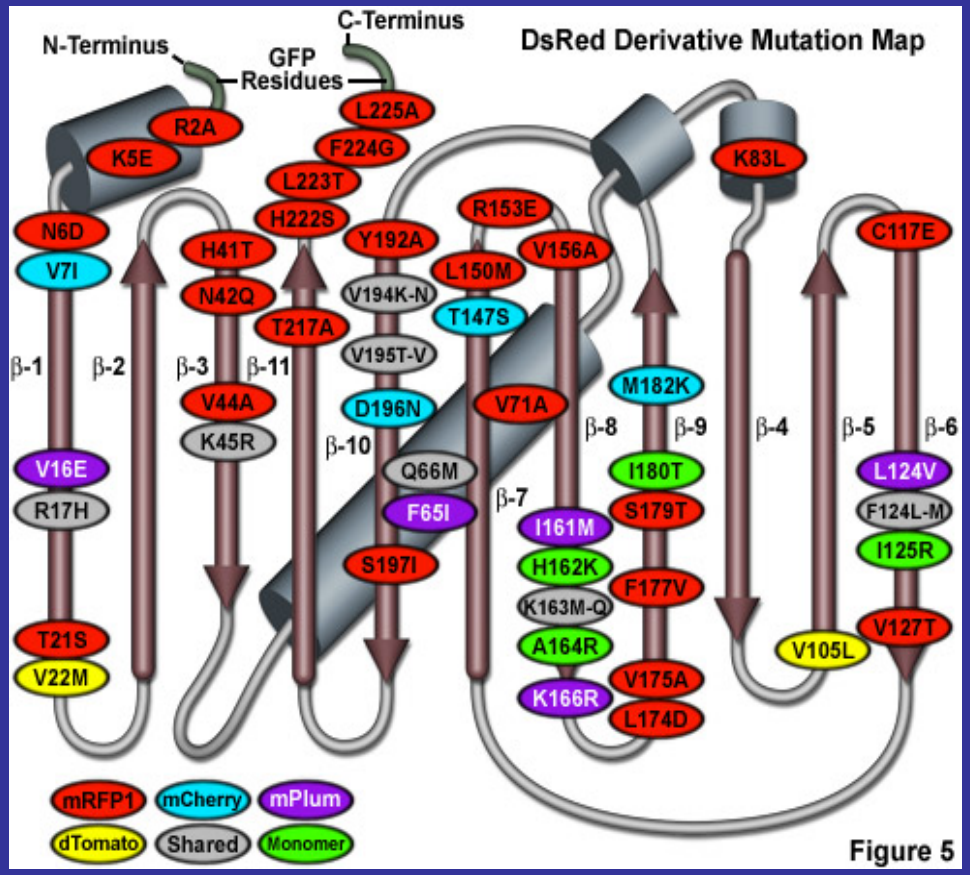
**Tetrameric**

**DsRed forms a chromophore identical to GFP to generate its green intermediate**

**EGFP mutant**
**Mature at 37°C**
**Monomeric**

**mRFP1 mutant**
**Faster Maturation**
**Monomeric**

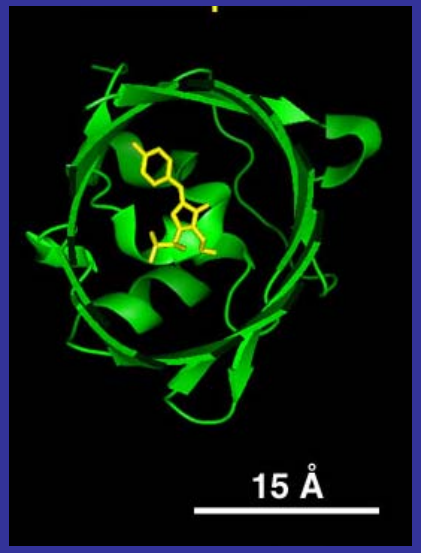
**Over the past several years, extensive mutagenesis efforts, successfully been applied to mRFP1 to yield a new generation of fluorescent protein variants.**
**THE mFRUIT FAMILY**




**DsRed Derivative Mutation Map**

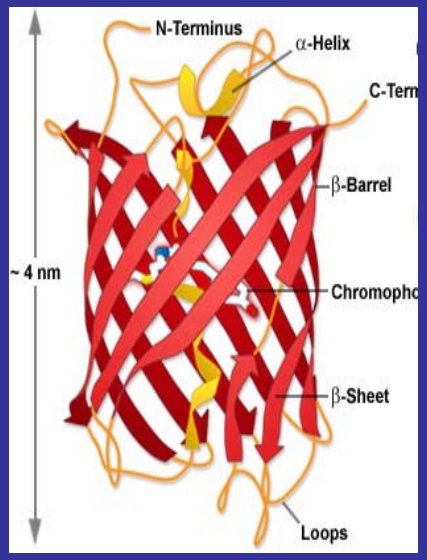
# Chromophore

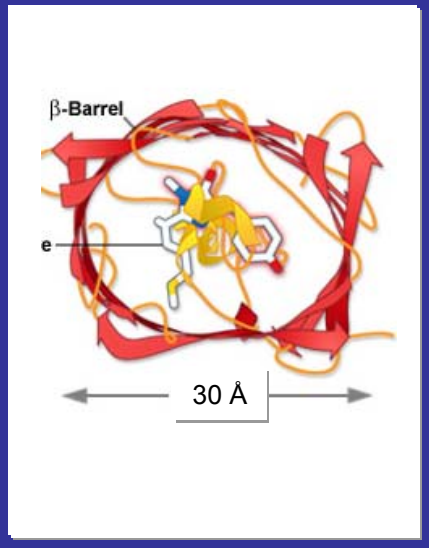
## GFP




Serine 65
Tyrosine 66
Glycine 67
MW= 20-30 KDa

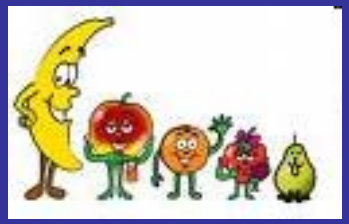
## mCherry

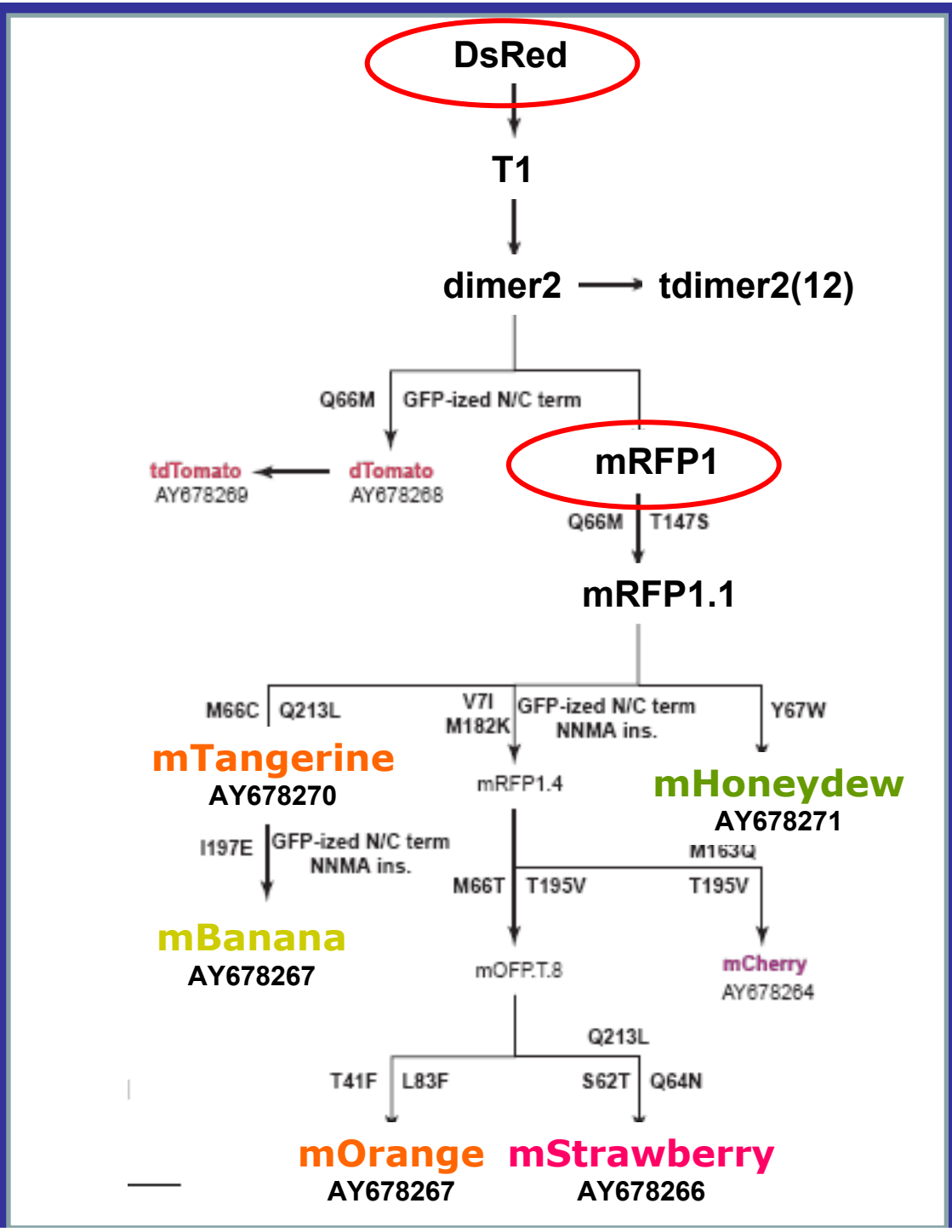


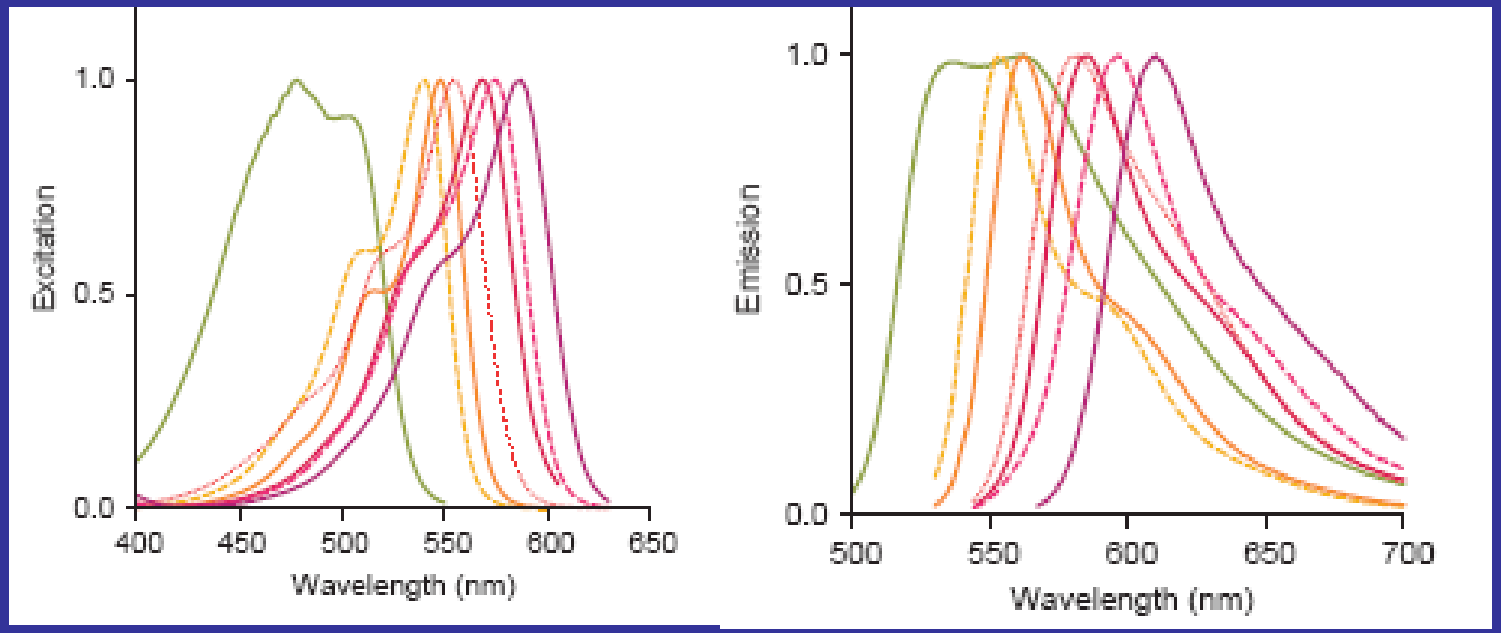

Glutamine 66
Tyrosine 67
Glycine 68
MW= 30-35 KDa

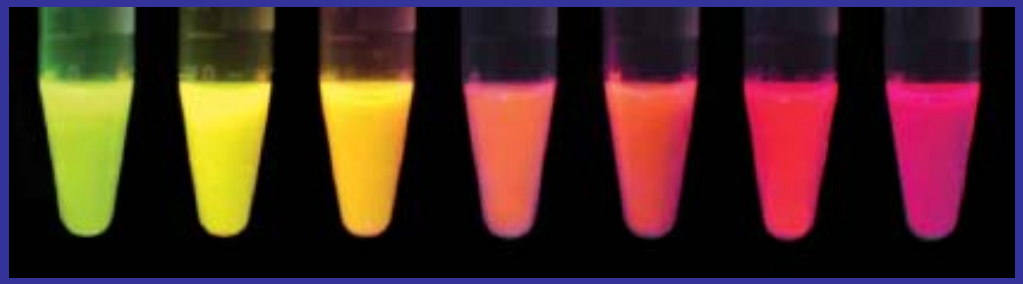
# THE mFruits family





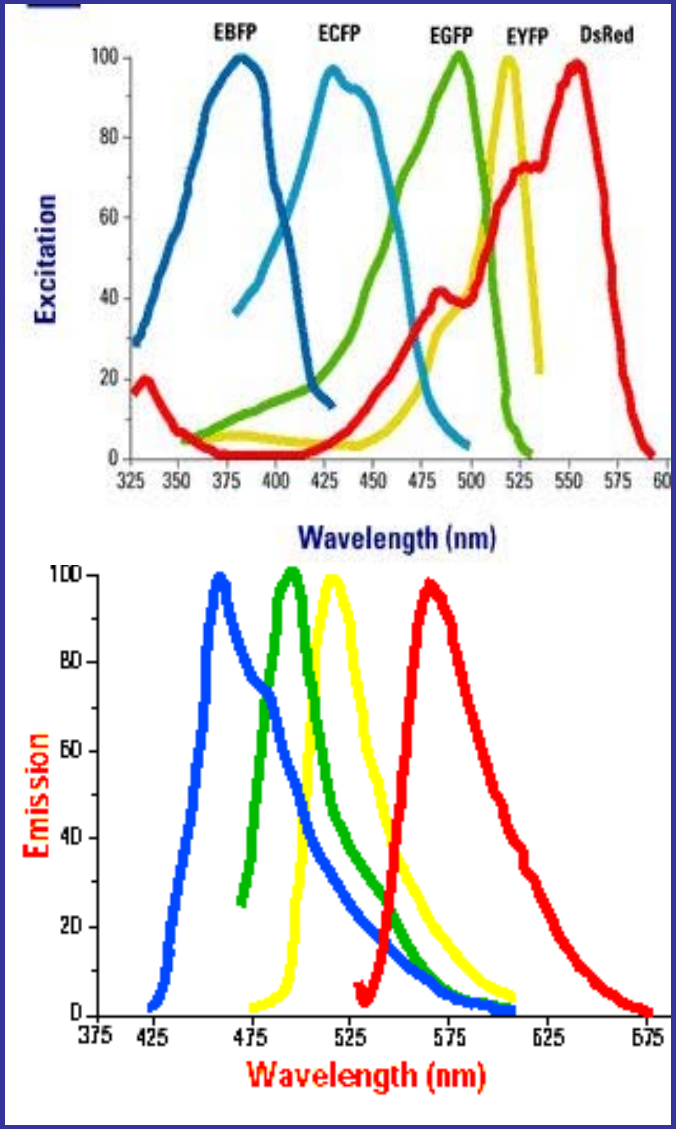
# mFruits fluorescent proteins



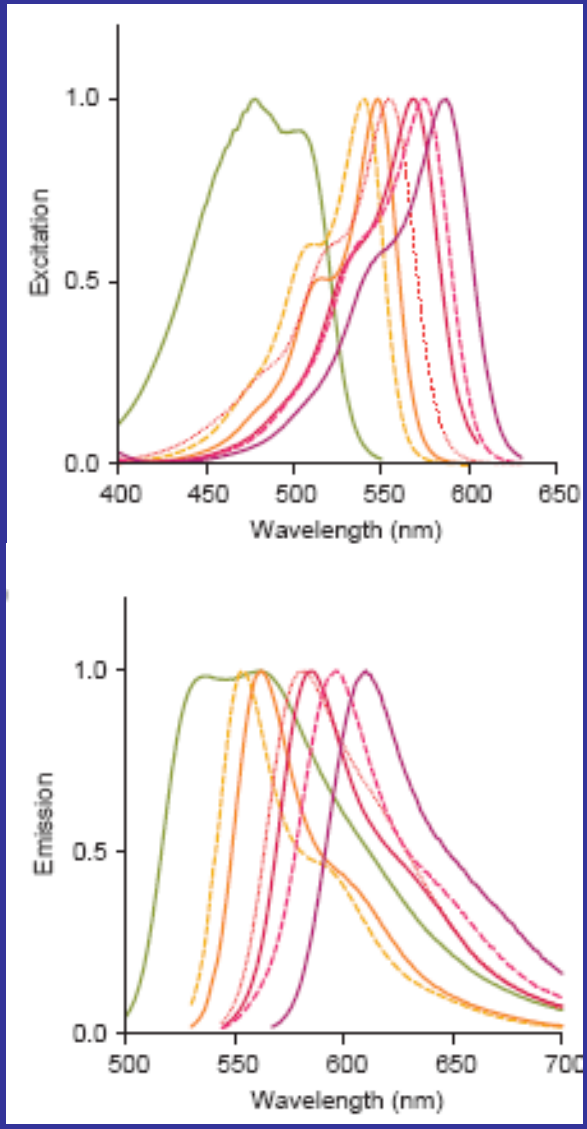



# mFruits may replace or be good pairs for FPs in energy transfer experiments

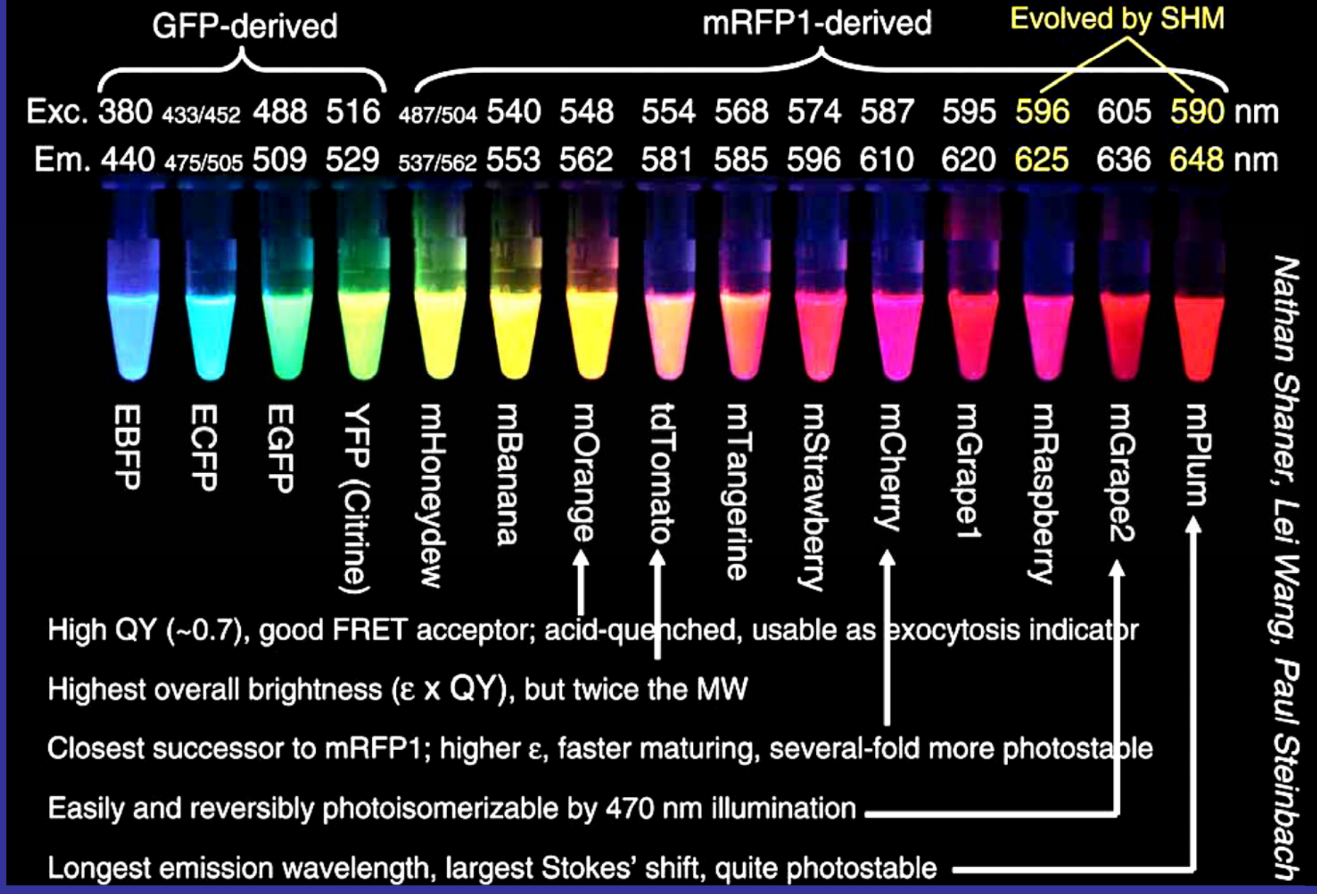
FPs

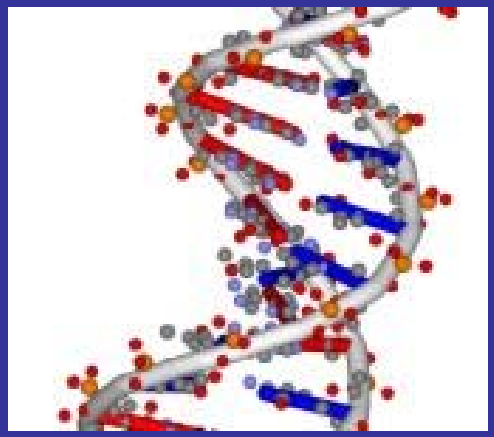



mFruits



# The 2004 palette of nonoligomerizing fluorescent proteins



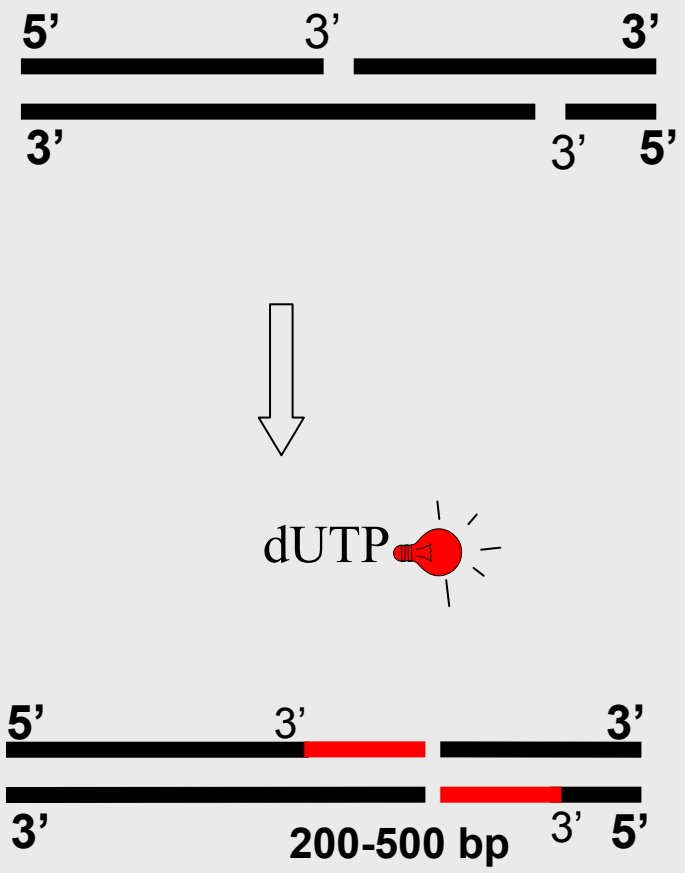


# Labeling DNA

*http://info.med.yale.edu/genetics/ward/tavi/n_coupling.html*

# Nick translation

## End labeling of fragments

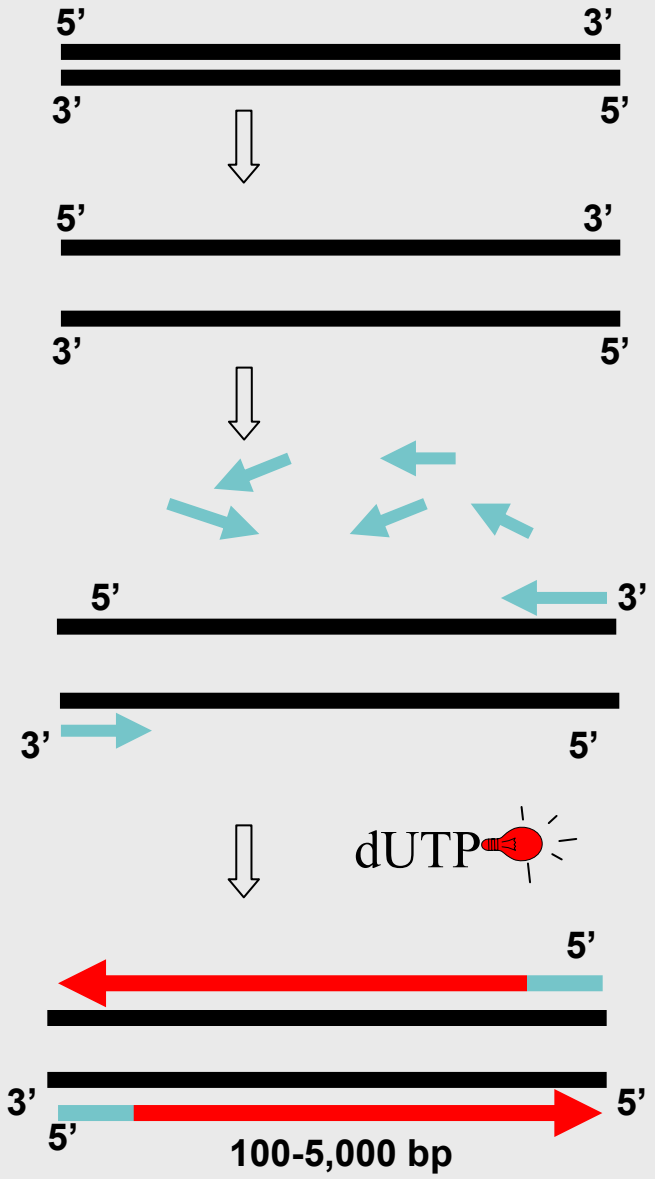



**DNase I**, which in the presence of $Mg^{2+}$ ions becomes a single stranded endonuclease creates random nicks in the two strands of any DNA molecule.

**E. coli polymerase I**, 5'-3' exonuclease activity removes nucleotides "in front" of itself.

5'-3' polymerase activity adds nucleotides to all the available 3' ends created by the DNase.

# Polymerase Chain Reaction (PCR)



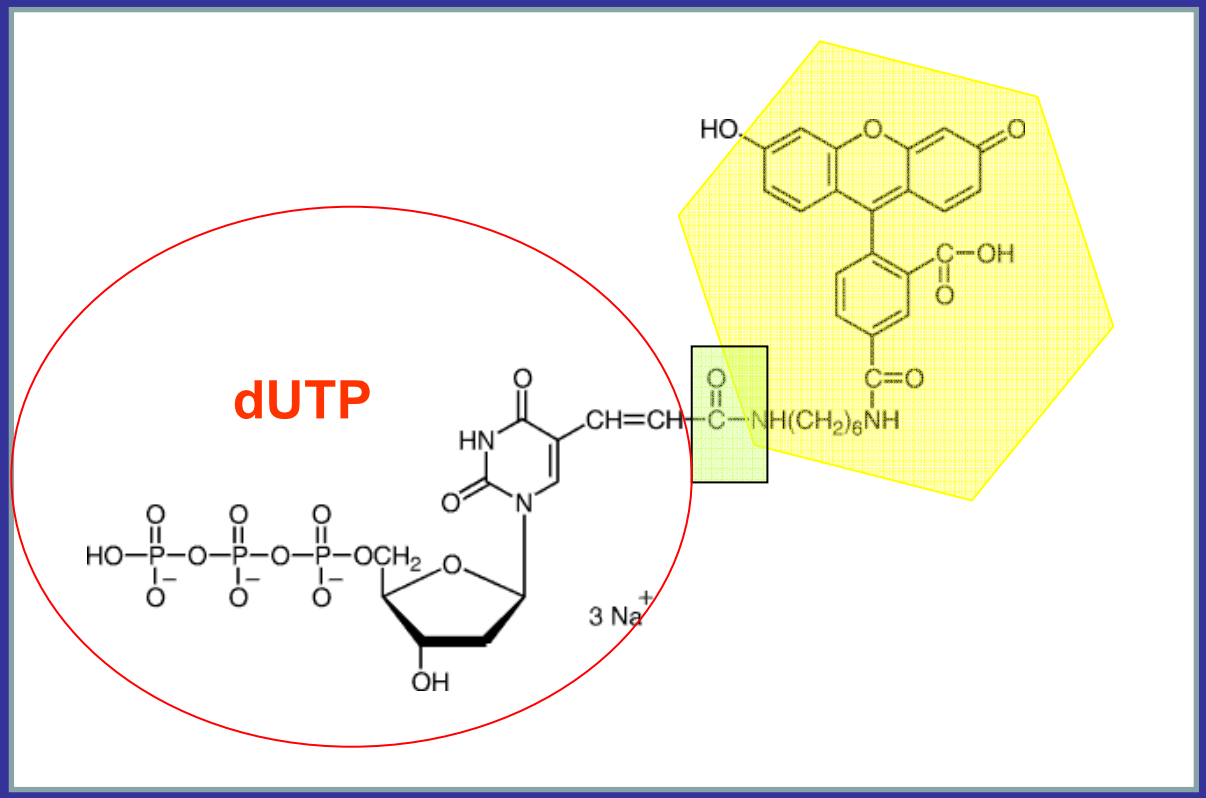

**1- Denaturation step (1min, 95ºC).**
During the denaturation, the double strand melts open to single stranded DNA

**2- Annealing (45 sec, 54ºC).**
Single stranded DNA primers (18-30 bp long), forward and reverse are synthesized (blue arrows). Then, the primers are allow to anneal to their target sequences.

**3- Extension (2min, 72ºC).**
Then Taq polymerase synthesize the new DNA strands. Only dNTP’s.

# Commercially labeled dUTP

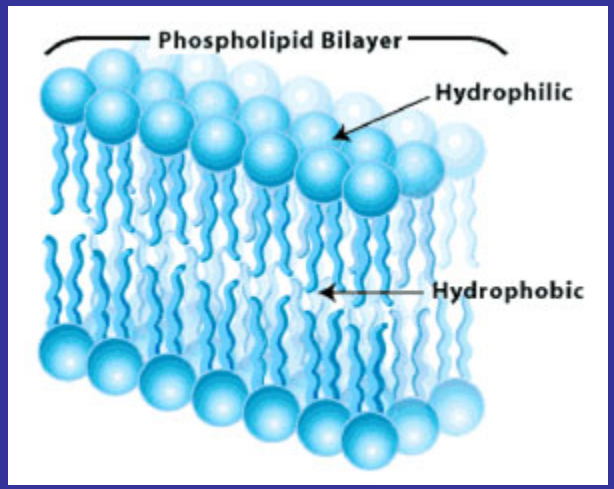
## succinimidyl-ester derivatives of fluorescent dyes




| No | Dye | MW | Abs (nm) | Em (nm) |
|---|---|---|---|---|
| 1 | DAPI | - | 350 | 456 |
| | AMCA | 450 | 353 | 442 |
| | CB | 600 | 396 | 410 |
| 2 | DEAC | 350 | 432 | 472 |
| 3 | FITC | 600 | 491 | 515 |
| | OG-488 | 510 | 495 | 521 |
| | A-488 | 650 | 493 | 517 |
| | RGr | 620 | 515 | 530 |
| 4 | R6G | 550 | 524 | 552 |
| | Cy3 | 750 | 550 | 570 |
| | TAMRA | 640 | 547 | 573 |
| 5 | TAMRA | 640 | 547 | 573 |
| | TxR | 800 | 583 | 603 |
| | Cy3.5 | 1100 | 581 | 596 |
| 6 | Cy5 | 800 | 649 | 670 |
| 7 | Cy5.5 | 1100 | 675 | 694 |
| 8 | Cy7 | 1000 | 743 | 767 |
| H1 | BIO | 550 | - | - |
| H2 | DIG | 600 | - | - |
| H3 | DNP* | 400 | - | - |
| H3 | DNP** | 400 | - | - |

**fluorescein-aha-dUTP from Molecular Probes**

# Labeling membranes

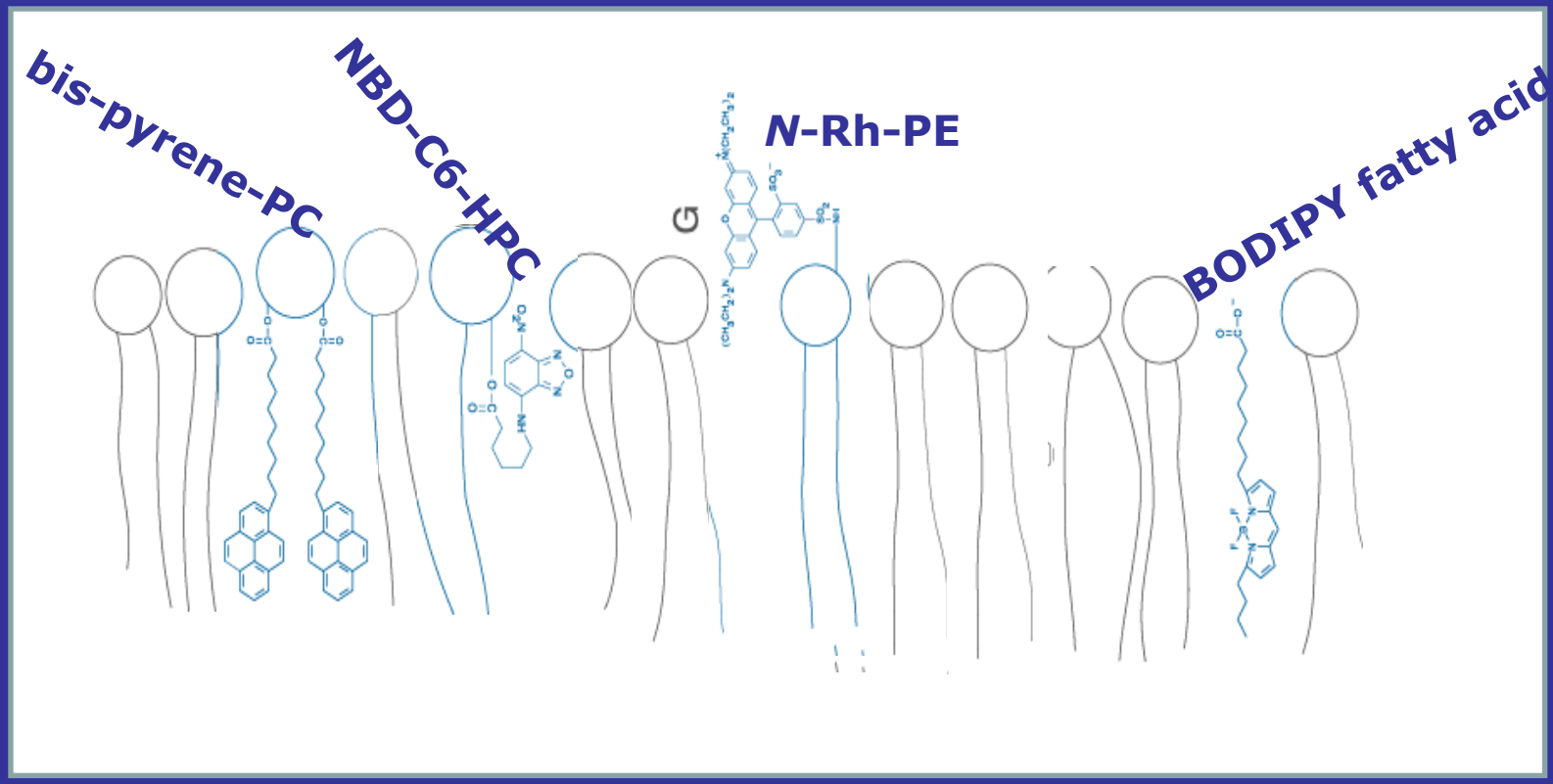



- Analogs of fatty acids and phospholipids

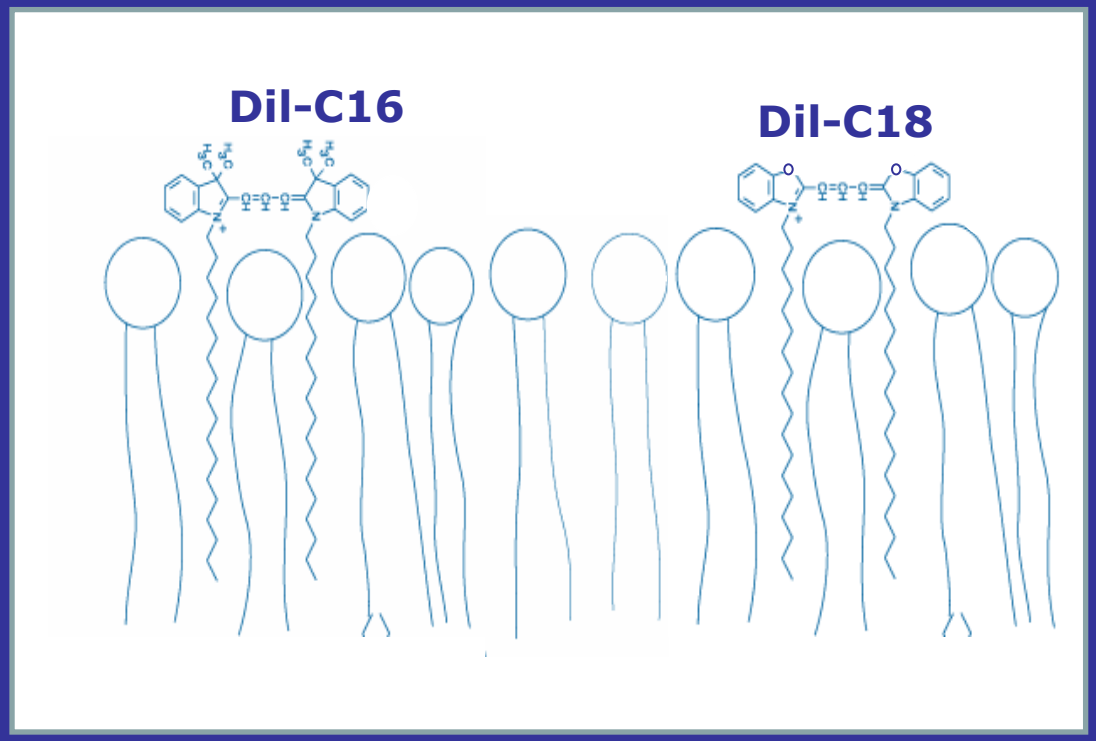
- Di-alkyl-carbocyanine and Di-alkyl-aminostyryl probes.

- Other nonpolar and amphiphilic probes.
Laurdan, Prodan, Bis ANS

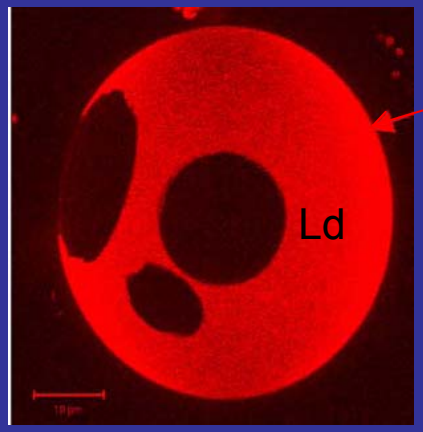
# Fatty acids analogs and phospholipids



# Di-alkyl-carbocyanine probes.

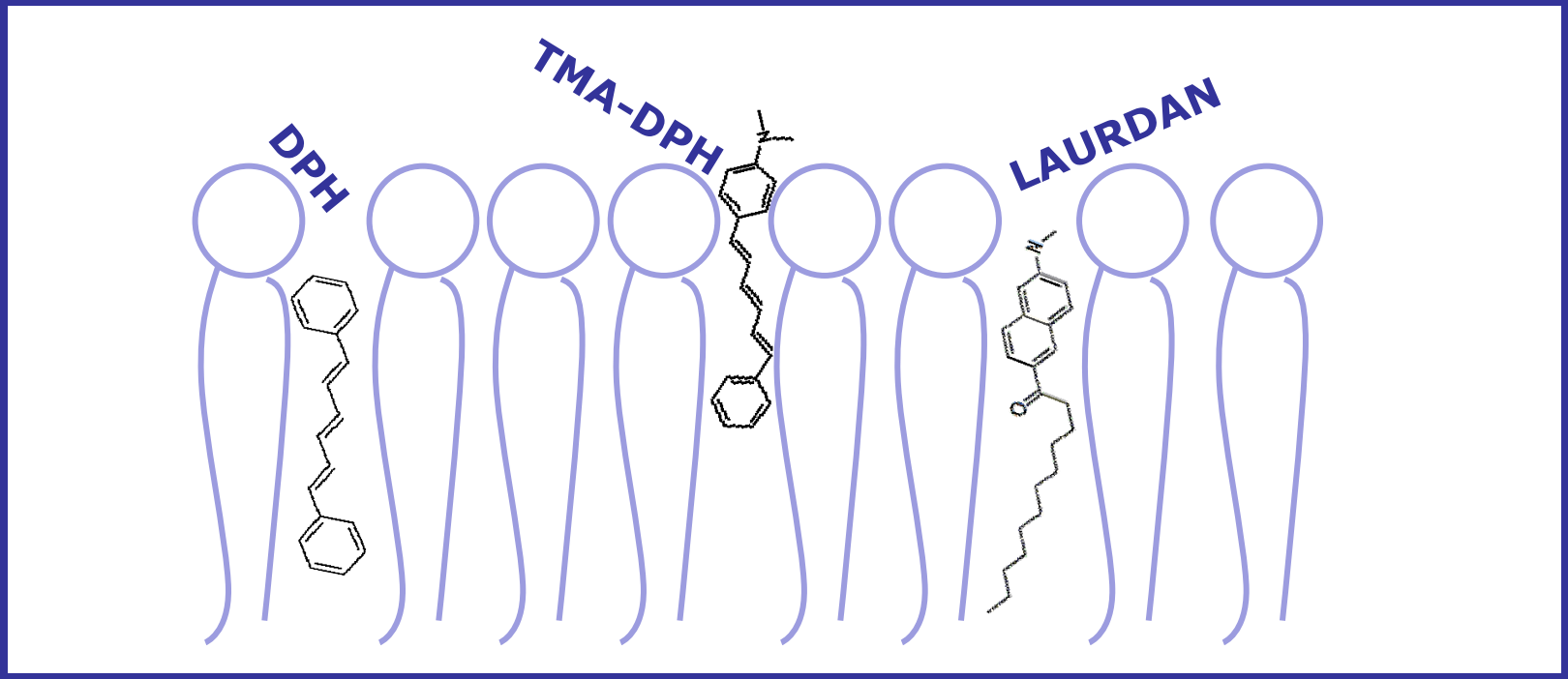



SM/DOPC/Chol (1:1:1)




*Chem.and Phys. of Lipids 141 (2006) 158–168*

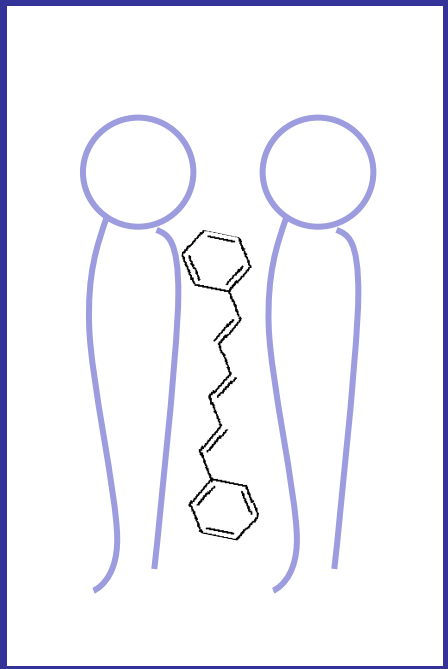
# Non polar and amphiphilic probes.



# DPH
## 1,6-diphenyl-1,3,5-hexatriene

Excitation: 350 nm
Emission: 452 MeOH

Information on the physical state of the phospholipid bilayer is obtained from the changes in the **fluorescence polarization** and **lifetime**.



It is oriented parallel to the lipid acyl chain axis .

- DPH shows no partition preference between coexisting gel- and fluid-phase phospholipids
- DPH fluorescence is practically negligible in water and intercalation into membranes is accompanied by strong enhancement of its fluorescence.
- Fluorescence decay data are often analyzed in terms of continuous lifetime distributions and interpreted as being indicative of lipid environment heterogeneity.

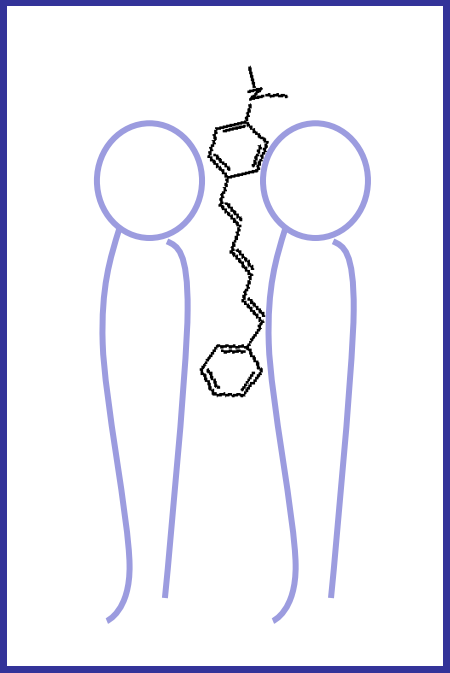
**Parasassi et al. J. of Biol. Chem. (1984) 259:14011-14017**
**Shinitzky et al. Biochemistry (1971) 10:2106-2113**

# TMA-DPH

**1-(4-trimethylammoniumphenyl)-6-phenyl -1,3,5-hexatriene *p*-toluenesulfonate**

Excitation: 355 nm
Emission: 430 Meoh

Designed to improve the localization of DPH in the membrane, TMA-DPH contains a cationic trimethylammonium substituent that acts as a surface anchor

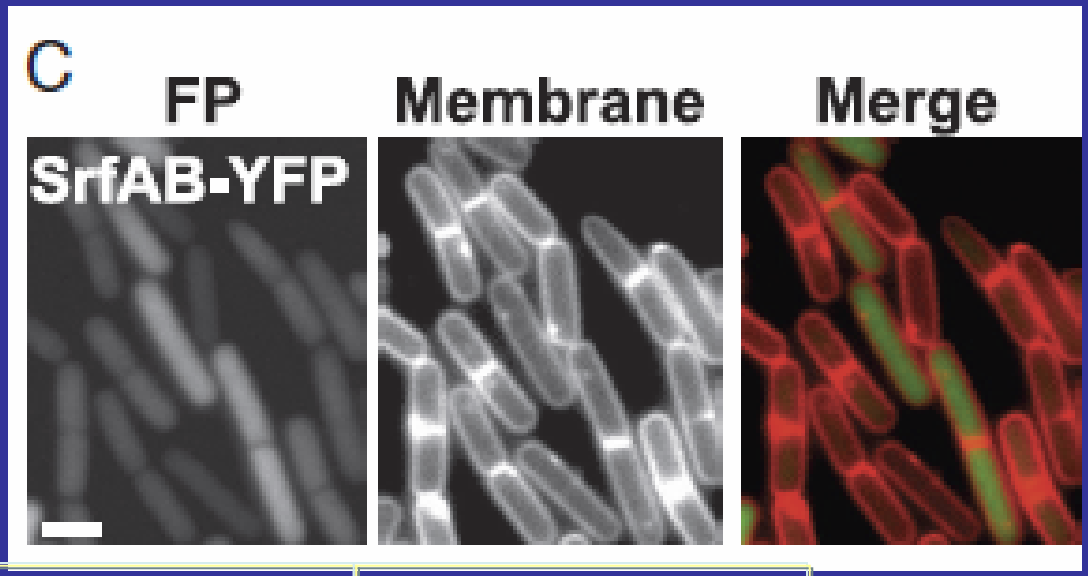


It partitions from aqueous dispersions into membranes, accompanied by strong fluorescence enhancement.

the duration of plasma membrane surface staining by TMA-DPH before internalization into the cytoplasm is quite prolonged

TMA-DPH fluorescence polarization measurements can be combined with video microscopy to provide spatially resolved images of phospholipid in large liposomes and single cells

# Localization of Pks proteins in *B. subtilis 3610*




| | |
|---|---|
| ***The SrfAB-YFP protein is diffuse in the cytoplasm.*** | ***fluorescence from membrane stained with the dye TMA-DPH*** |

**Instrument:** Olympus (Melville, NY) BX61 phase-contrast microscope equipped with an UplanF1 100 objective and a CoolSnapHQ digital camera (Photometrics, Tucson, AZ). TMA-DPH was used at a final concentration of 10 M.

**Straight et al. A singular enzymatic megacomplex from Bacillus subtilis. PNAS (2007), 104: 305-310**

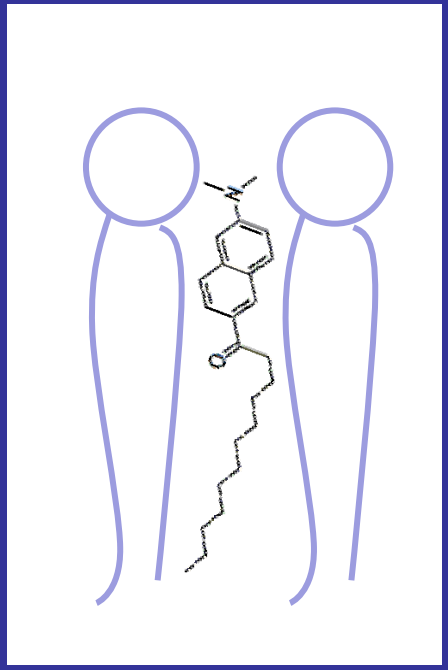
# LAURDAN

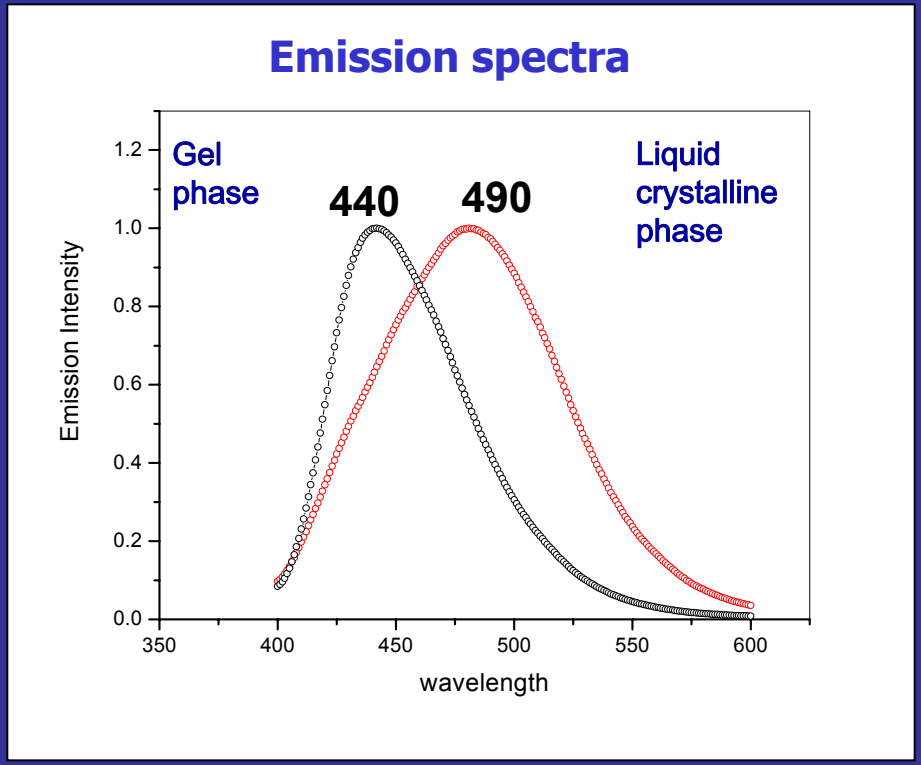
## 6-dodecanoyl-2-dimethylaminonaphthalene

Excitation: 364 nm
Emission: 497 nm
*(Methanol)*

**Information on the physical state of the phospholipid bilayer is obtained from the shift in the emission spectra.**

***(environment-sensitive spectral shifts)***

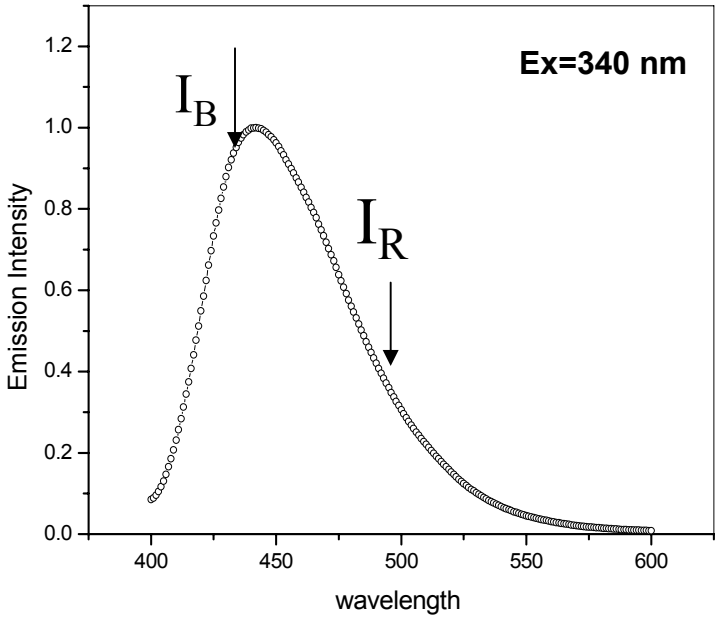





*Weber, G. and Farris, F. J.Biochemistry, 18, 3075-3078 (1979) .*

# Laurdan Generalized Polarization (GP)

## Emission spectra



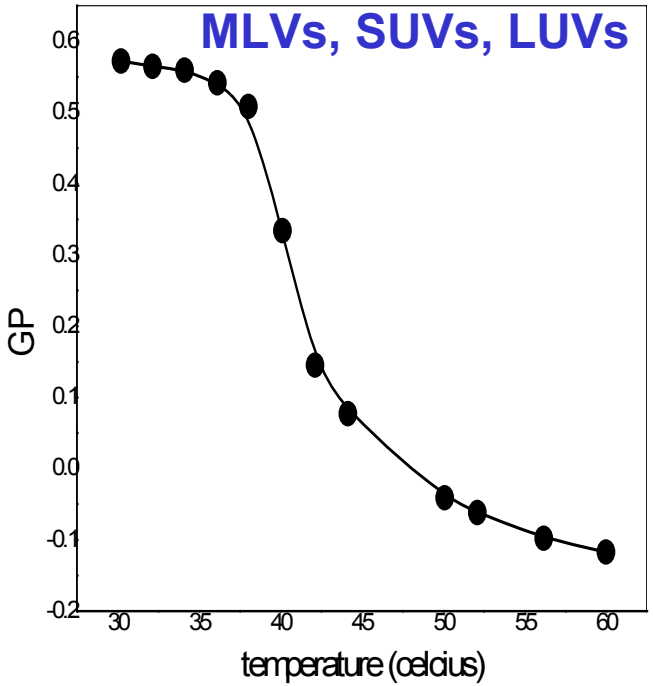

$$GP_{ex} = \frac{I_B - I_R}{I_B + I_R}$$

**-0.2**
**loose lipid packing**

**0.6**
**tight lipid packing**

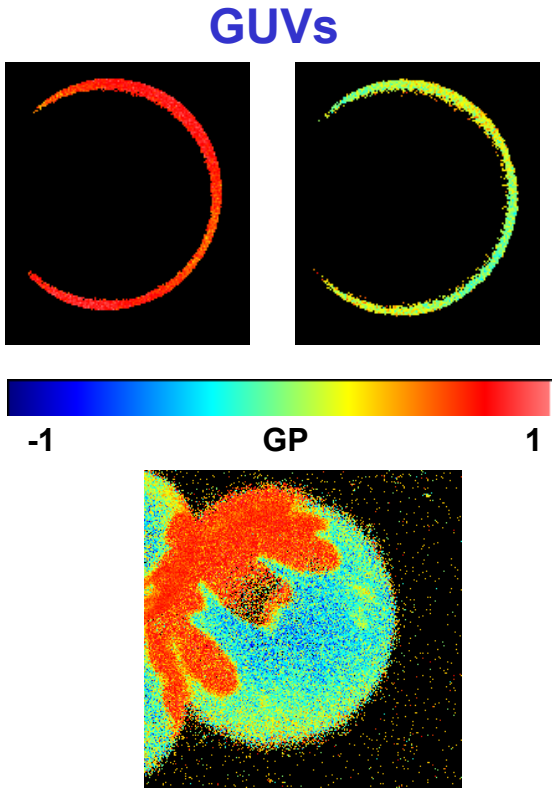
*Parasassi et al. Biophysical J., 60, 179-189 (1991).*

# GP in the cuvette



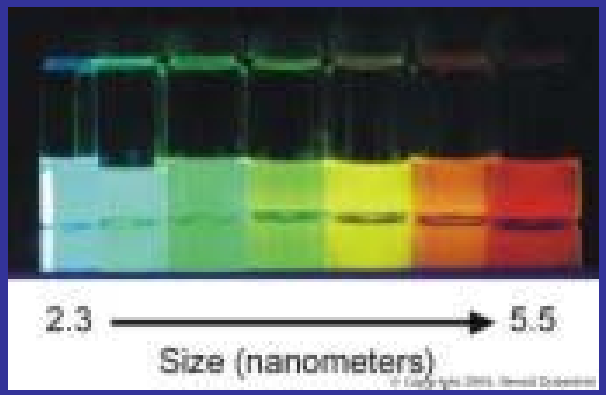

**Changes of GP for DPPC with temperature, Tm=41ºC.**

# 2Ph- microscopy




**GP value and spatial resolution**

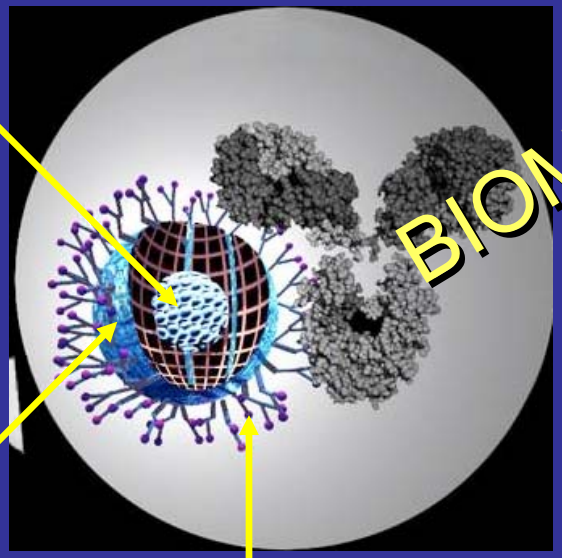
# Quantum dots



# Quantum Dots

## CORE

-Cadmium selenide (**CdSe**), or Cadmium telluride (**CdTe**)
-Semiconductor material is chosen based upon the emission wavelength.
-The **size** of the particles that **tunes the emission wavelength**.




## SHELL

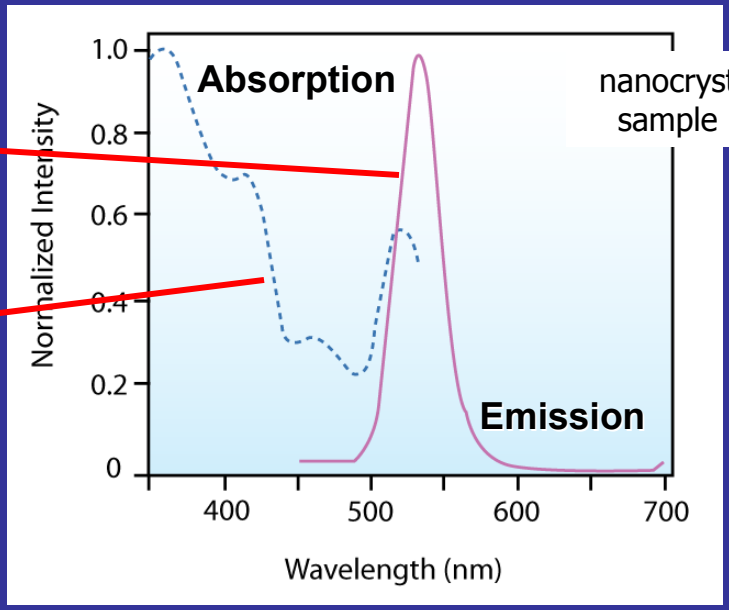
In the core emission is typically weak and always unstable.
The shell material Zinc Sulfide (**ZnS**) has been selected to be almost entirely un-reactive and completely insulating for the core.

## COATING

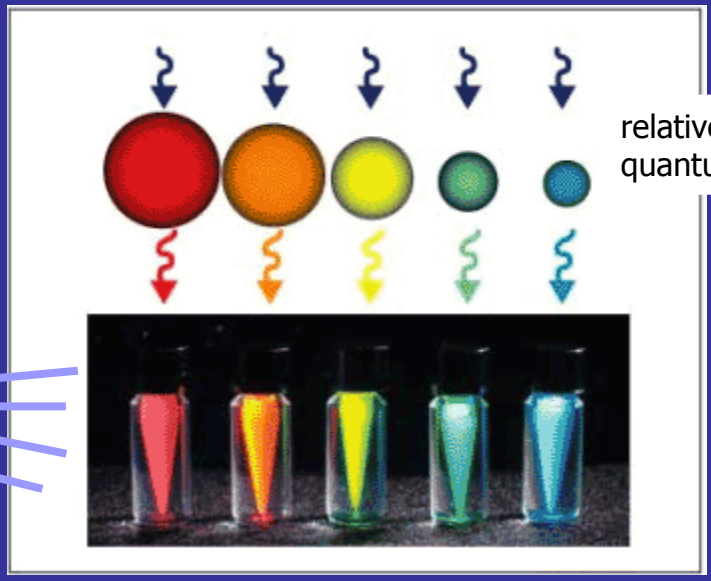
A layer of organic ligands covalently attached to the surface of the shell. This coating provides a **surface for conjugation** to biological (antibodies, streptavidin, lectins, nucleic acids) and nonbiological species and makes them "water-soluble"

•**Q-dots:** emission spectra is narrow and symmetrical.

•**Q-dots:** broad absorption spectra, making it possible to excite all colors of QDs simultaneously with a single excitation light source….




nanocrystal (NC) sample in PBS

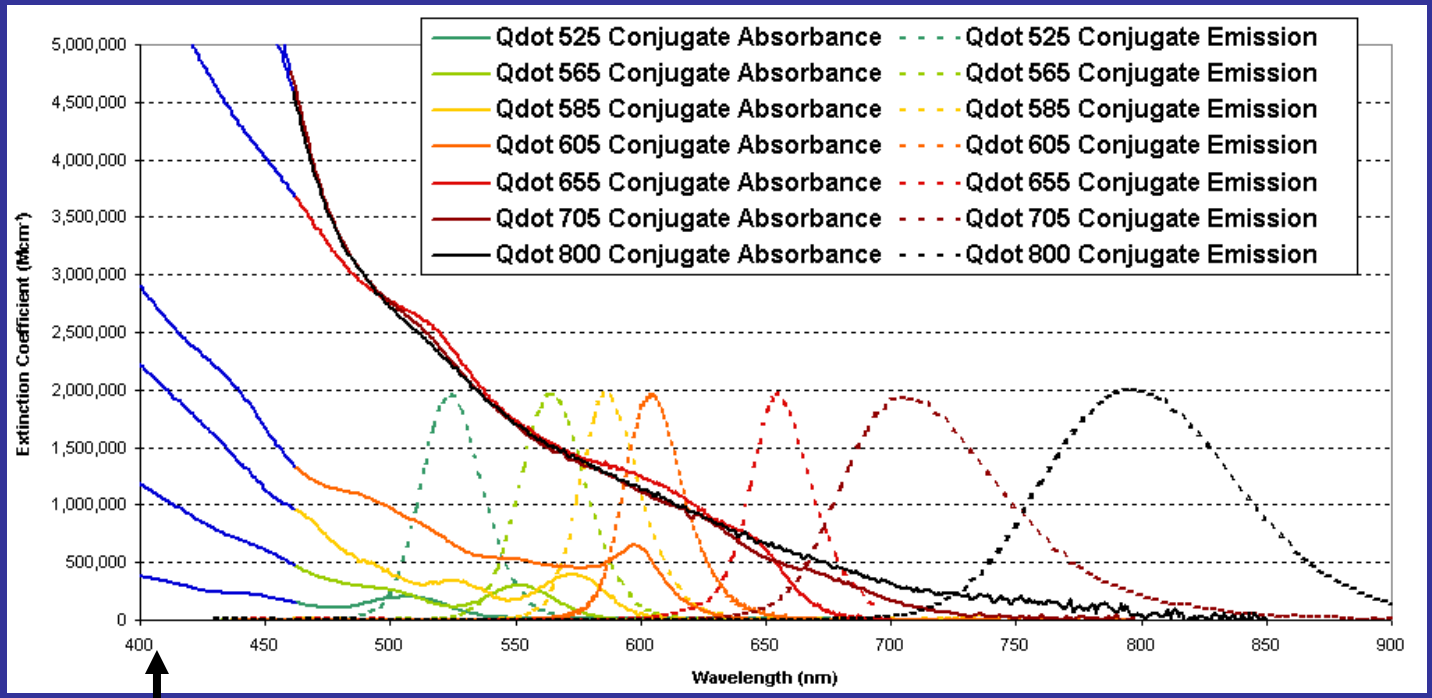


relative sizes of the CdSe quantum dots in the vials.

**UV hand lamp.**

**The emission is tunable according to their size and material composition**

# Q-dot Optical Spectra




**Violet excitation**

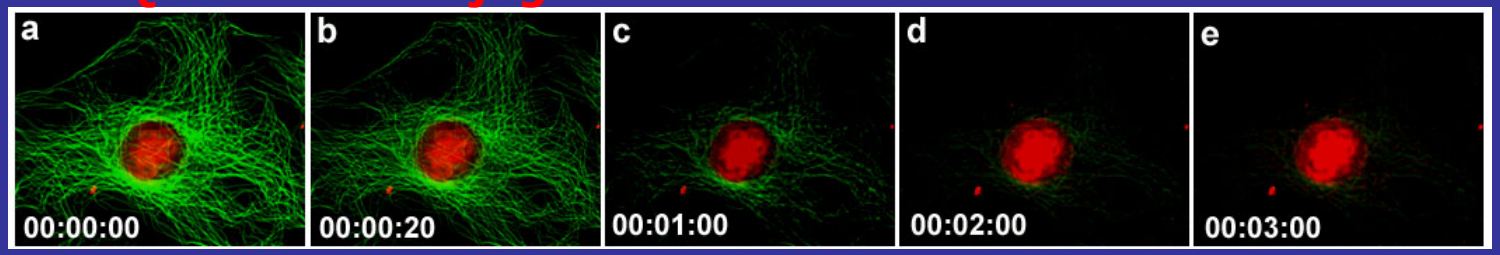
**Broad range of emissions**

**High absorbance means increased brightness**
**Single-color excitation, multicolor emission for easy multiplexing**

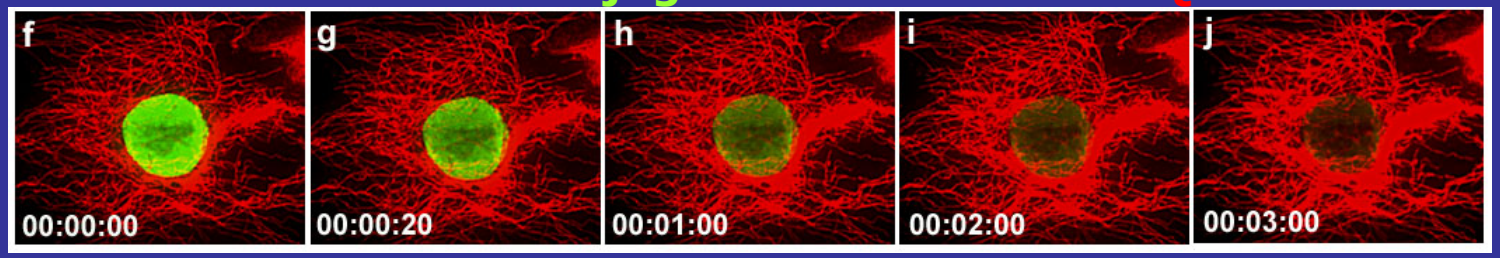
# Photostability of Qdots

## 3T3 cells

**Nucleus: Qdot® 605 conjugate** **Microtubules: Alexa Fluor® 488 conjugate**

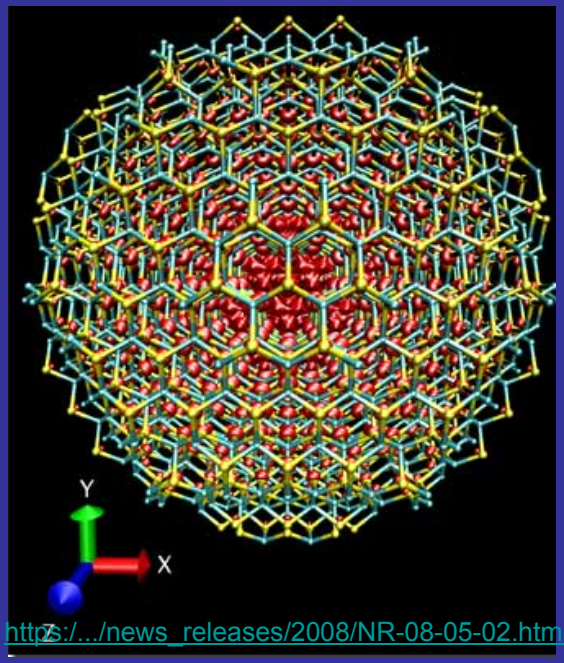



**Nucleus: Alexa Fluor® 488 conjugate** **Microtubules: Qdot® 605 conjugate**




**Photostability results in sensitivity, and sample permanence**

# Qdot Summary



https:/.../news_releases/2008/NR-08-05-02.html

## Advantages:

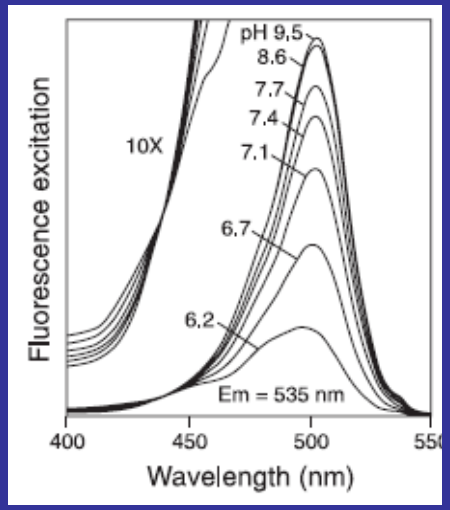
- Broad absorption spectra, making it possible to excite all colors of QDs simultaneously with a single light source - Multiplexing
- Narrow and symmetrical emission spectra Emission tunable with size and material composition
- Exhibit excellent photo-stability

## Disadvantages

Large size and high mass limit their use in applications requiring high diffusional mobility

| Quantum Dot Material System | Emission Range | Quantum Dot Diameter Range | Quantum Dot Type | Standard Solvents | Example Applications |
|---|---|---|---|---|---|
| CdSe | 465nm - 640nm | 1.9nm - 6.7nm | Core | Toluene | Research, Solar Cells, LEDs |
| CdSe/ZnS | 490nm - 620nm | 2.9nm - 6.1nm | Core-Shell | Toluene | VisibleFluorescence Applications, Electroluminescence, LEDs |
| CdTe/CdS | 620nm - 680nm | 3.7nm - 4.8nm | Core-Shell | Toluene | Deep Red Fluorescence Apps. |

# Fluorescent probes for Ions




**Fluorescence probes have been developed for a wide range of ions:**

## Cations:

$H^+$, $Ca^{2+}$, $Li^+$, $Na^+$, $K^+$, $Mg^{2+}$, $Zn^{2+}$, $Pb^{2+}$ *etc.*

## Anions:

$Cl^-$, $PO_4^{2-}$, Citrates, ATP, *and others*

# How do we choose the correct probe for ion determination?

**1-DISSOCATION CONSTANT (Kd)**

- Must be compatible with the concentration range of interest.
- Calibration. The Kd of the probe is dependent on pH, temperature, viscosity, ionic strength etc.

**2- MEASUREMENT MODE**

- Qualitative or quantitative measurements.
- Ratiometric measurements.
- Illumination source available.

**3- INDICATOR FORM**

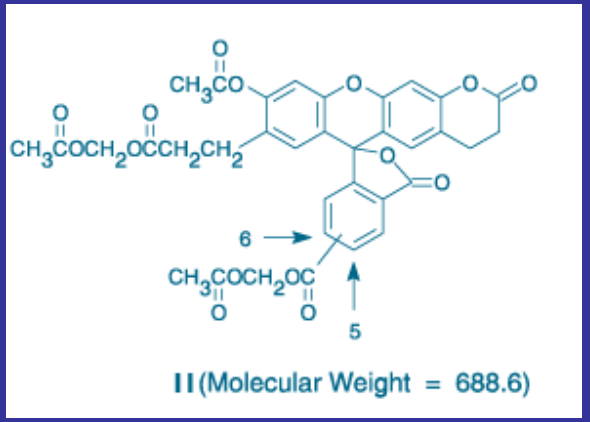
- Cell loading and distribution of the probe.
- AM-esters: cleaved by intracellular esterases.

# Probes For pH determination

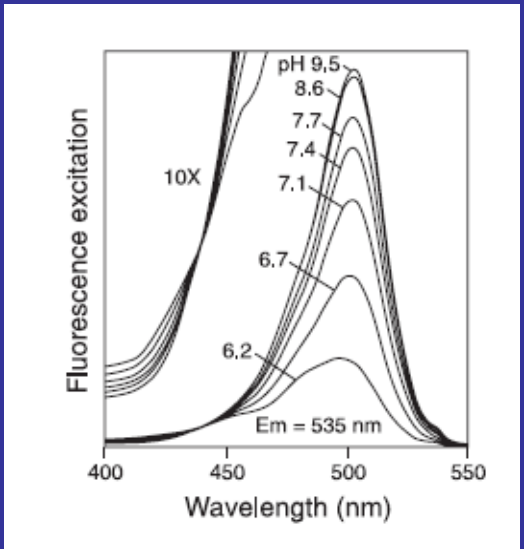
| Parent Fluorophore | pH Range | Typical Measurement |
|---|---|---|
| SNARF indicators | 6.0–8.0 | Emission ratio 580/640 nm |
| HPTS (pyranine) | 7.0–8.0 | Excitation ratio 450/405 nm |
| **BCECF** | **6.5–7.5** | **Excitation ratio 490/440 nm** |
| Fluoresceins and carboxyfluoresceins | 6.0–7.2 | Excitation ratio 490/450 nm |
| LysoSensor Green DND-189 | 4.5–6.0 | Single emission 520 nm |
| Oregon Green dyes | 4.2–5.7 | Excitation ratio 510/450 nm or excitation ratio 490/440 nm |
| LysoSensor Yellow/Blue DND-160 | 3.5–6.0 | Emission ratio 450/510 nm |

**Molecular Probes' pH indicator families, in order of decreasing $pK_a$**

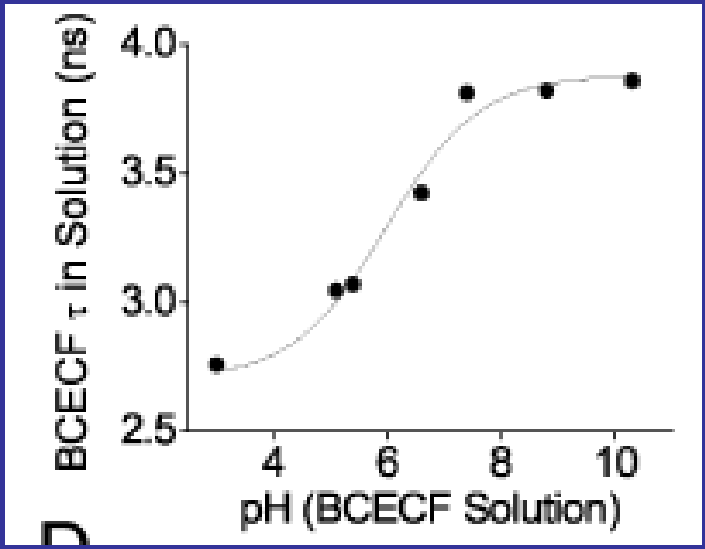
# Example: BCECF



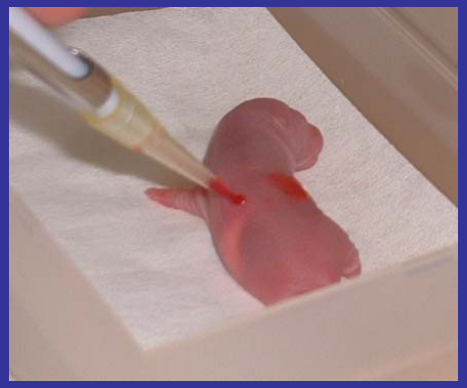

## Fluorescence Intensity




## Fluorescence Lifetime



# Experimental protocol

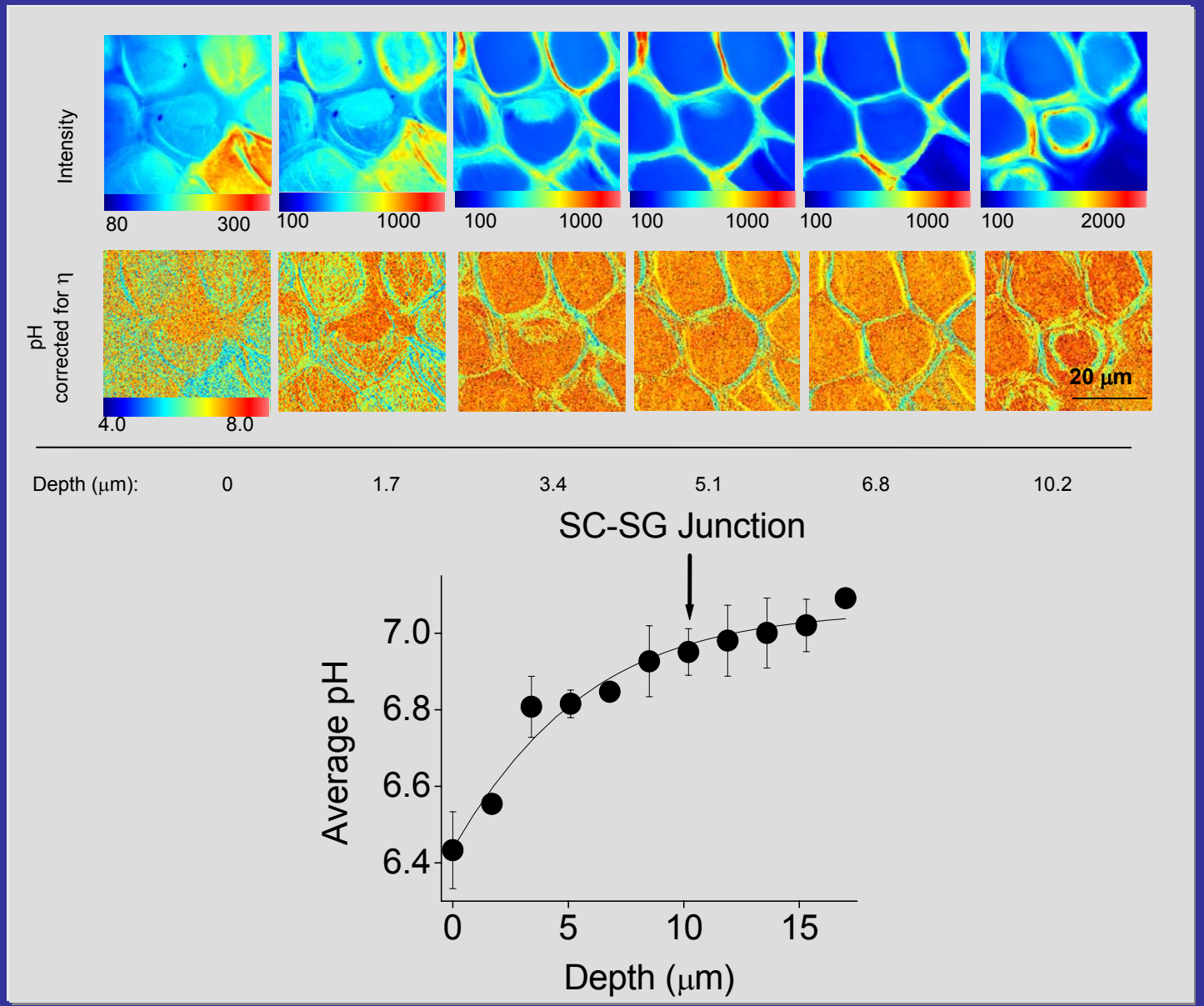


**Dye in DMSO is applied to the a live animal and incubated**

**Labeled skin is removed**

**imaging**

K.Hanson et al. Biophysical Journal. 83:1682-1690. 2002.




**K.Hanson et al. Biophysical Journal. 83:1682-1690. 2002.**

# Probes For Calcium determination

## UV

**FURA**
(Fura-2, Fura-4F, Fura-5F, Fura-6F, Fura-FF)
**INDO**
( Indo-1, Indo 5F)

**Ratiometric**

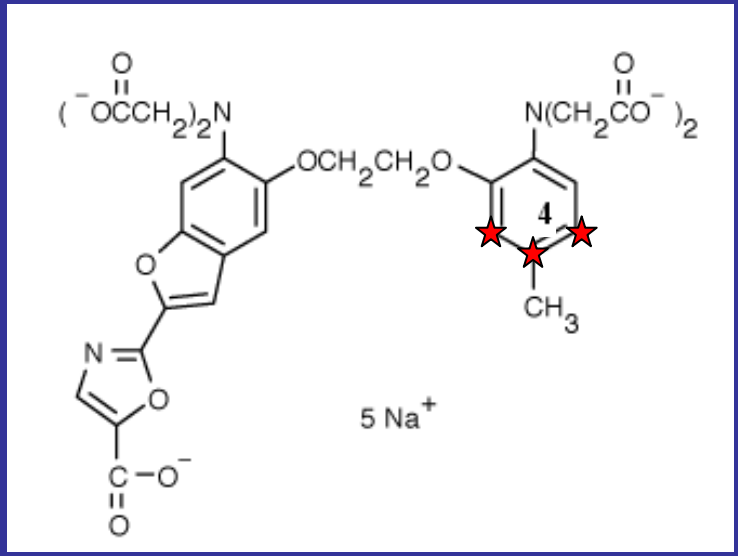
## VISIBLE

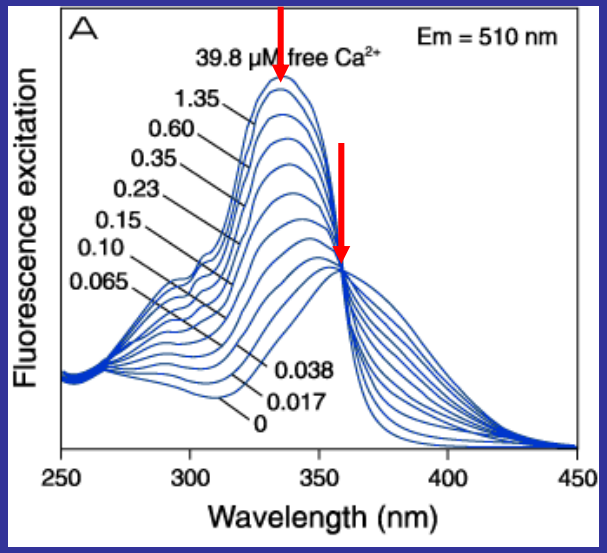
**FLUO**
(Fluo-3, Fluo-4, Fluo5F, Fluo-5N, Fluo-4N)
**RHOD** ( Rhod-2, Rhod-FF, Rhod-5N)
**CALCIUM GREEN** (CG-1, CG-5N,CG-2)
**OREGON GREEN 488-BAPTA**

**Non Ratiometric**

# Ratiometric: 2 excitation/1emission

## FURA-2




| Indicator | $K_d(Ca^{2+})$ |
|---|---|
| Fura-2 | 0.14 μM |
| Fura-5F | 0.40 μM |
| Fura-4F | 0.77 μM |
| Fura-6F | 5.30 μM |
| Fura-FF (5,6) | 35 μM |

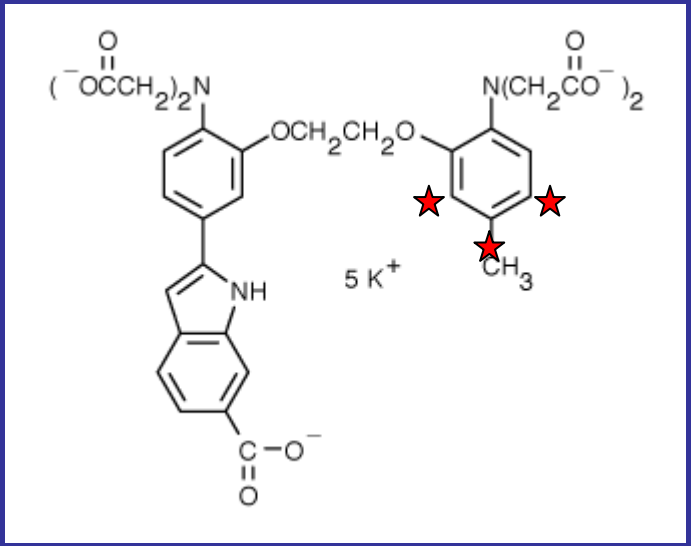
**Most used in microscopic imaging**

**Good excitation shift with $Ca^{2+}$**

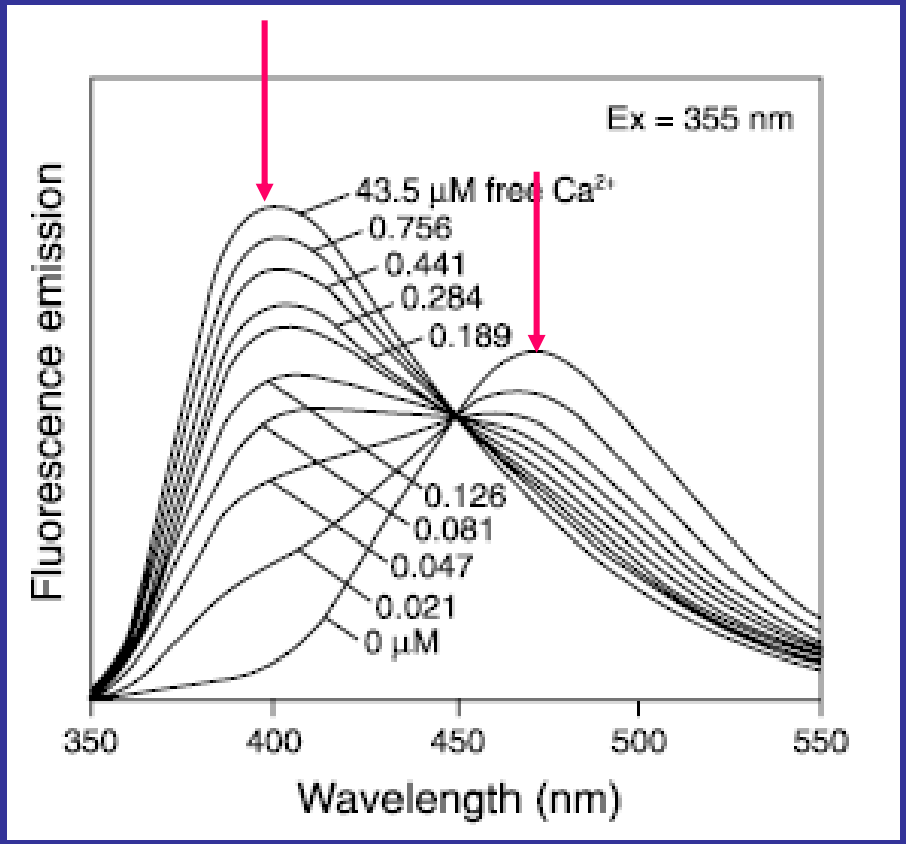
**Rationed between 340/350 and 380/385 nm**

# Ratiometric: 1excitation /2emission

## Indo-1

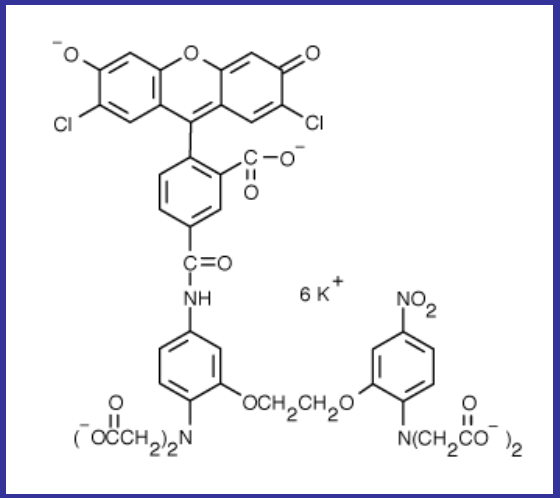



| Indicator | $K_d$ ($Ca^{2+}$) (μM) |
|---|---|
| indo-1 | 0.23 |
| indo-5F | 0.47 |

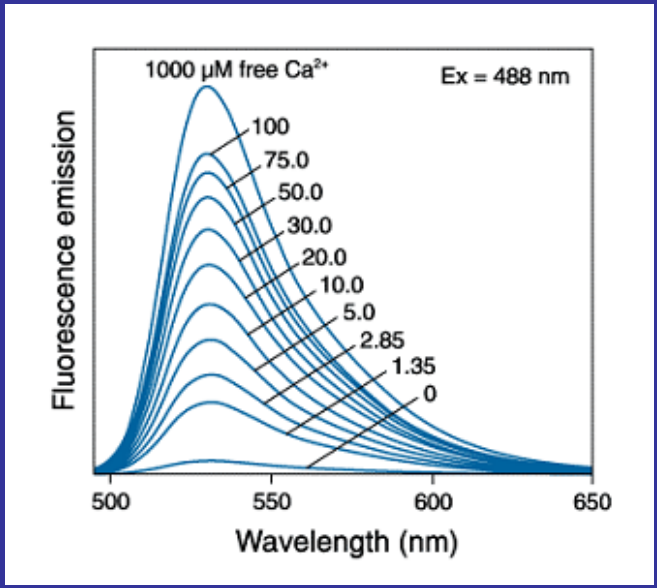


# CalciumGreen-5N

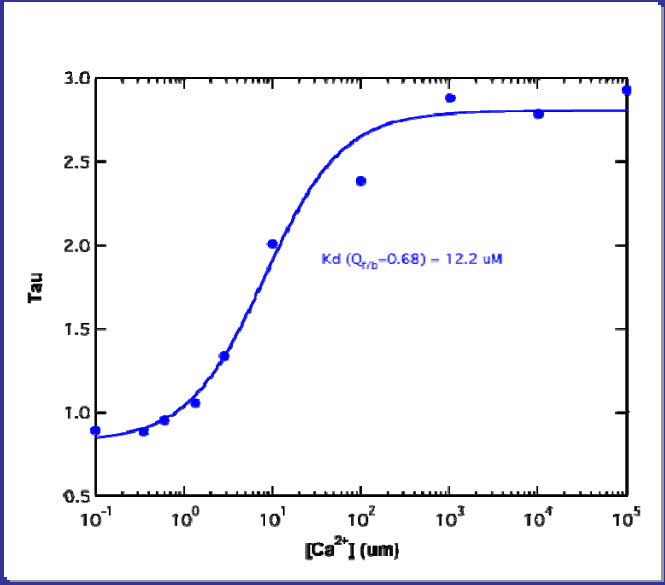
## Non-Ratiometric



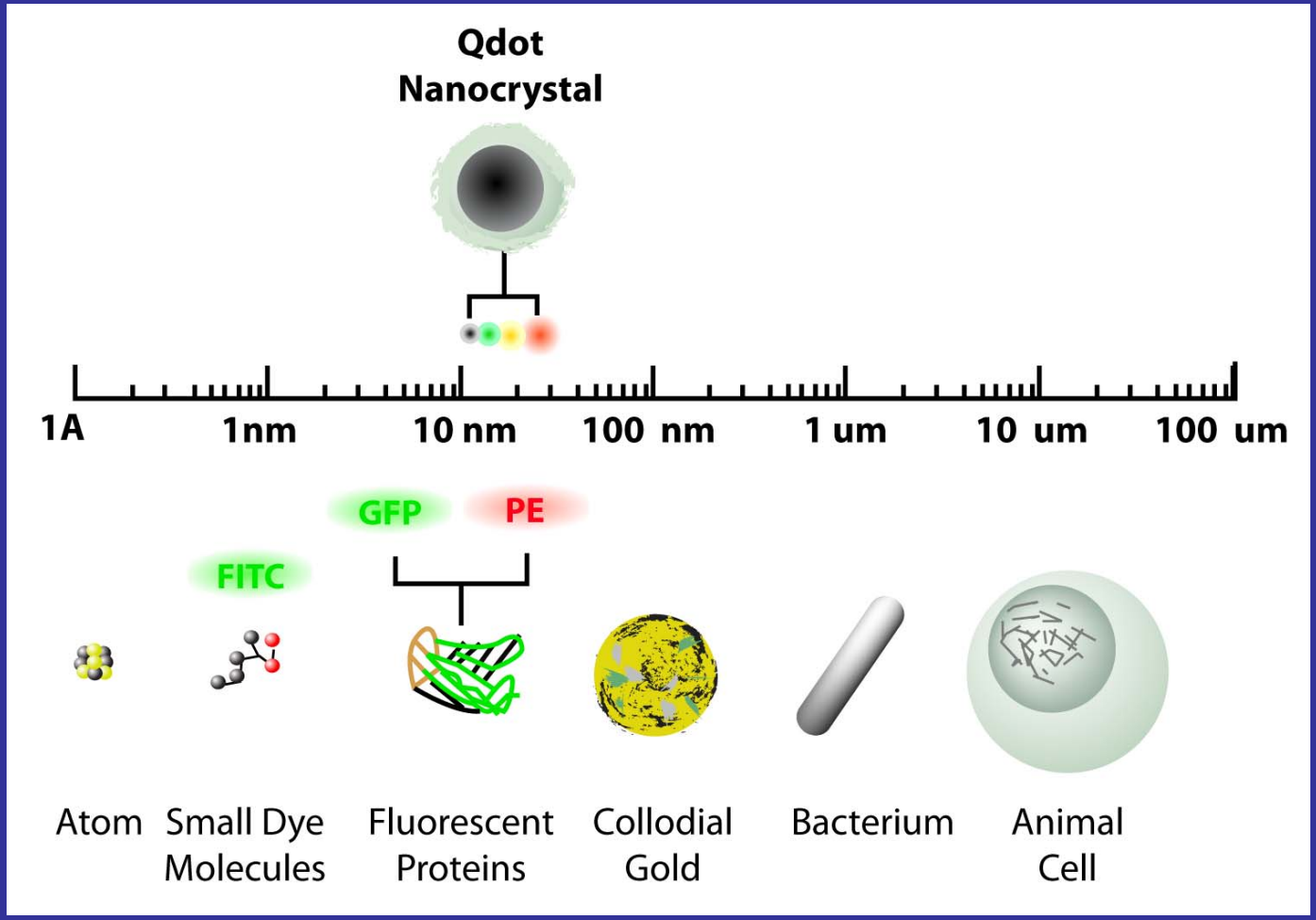

### Fluorescence Intensity




### Fluorescence Lifetime



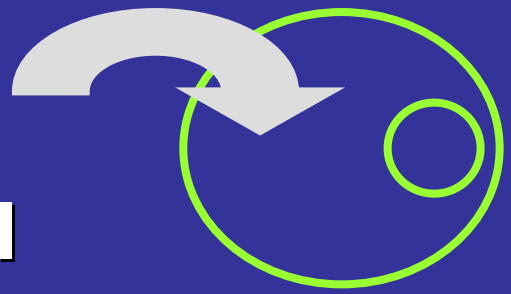
# Comparing the size of the fluorescence probes and the bio-molecule being labeled



# Labeling *"in vivo"*

# Mechanical incorporation

Labeled proteins
Labeled DNA
Q-dots
Genetic material



# Electroporation

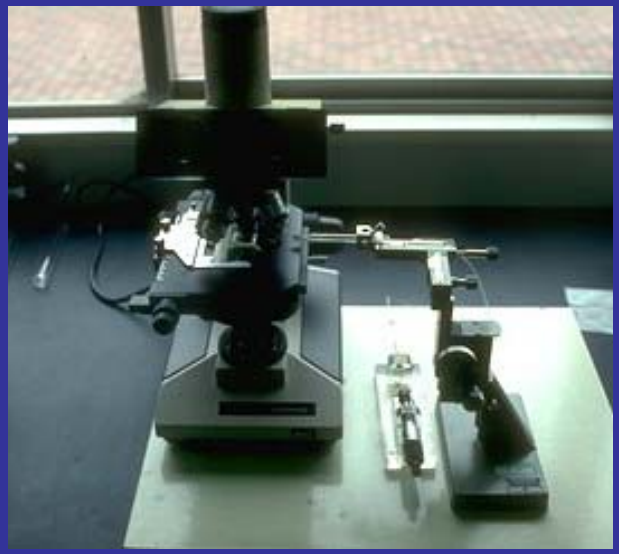
- Cells are mixed with a labeled compound.
- The mixture is exposed to pulses of high electrical voltage.
- The cell membrane of the host cell is penetrable allowing foreign compounds to enter the host cell.
- Some of these cells will incorporate the molecule of interest (new DNA and express the desired gene).

**Non-homogeneous labeling**
**Transfected cells have to be selected**

Source: http://dragon.zoo.utoronto.ca/~jlm-gmf/T0301C/technology/introduction.html

# Microinjection

- Direct injecting foreign DNA into cells.
- Under a microscope, a cell is held in place with gentle suction while being manipulated with the use of a blunt capillary.
- A fine pipette is used to insert the DNA into the cytoplasm or nucleus.
- This technique is effective with plant protoplasts and tissues.



*-Photo of a Microinjection apparatus(courtesy of A. Yanagi)*

**Non-homogeneous labeling**
**Transfected cells have to be selected**

***Source: http://dragon.zoo.utoronto.ca/~jlm-gmf/T0301C/technology/introduction.html***

# Biolistics

- **Biolistics is currently the most widely used in the field of transgenic corn production.**
- **The DNA construct is coated onto fine gold/tungsten particles and then the metal particles are fired into the callus tissue.**
- **As the cells repair their injuries, they integrate their DNA into their genome, thus allowing for the host cell to transcribe and translate the gene.**
- **Selection of the transfected cells, is done on the basis of the selectable marker that was inserted into the DNA construct**

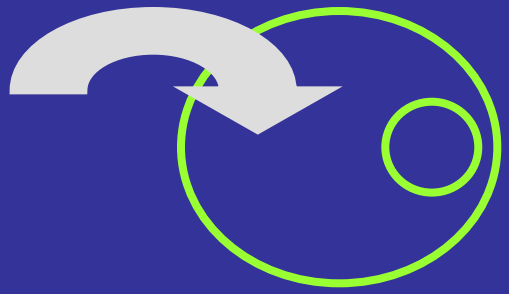
**Non-homogeneous labeling**
**Transfected cells have to be selected**

***Source: http://dragon.zoo.utoronto.ca***

# Genetic Incorporation

**Protein localization in vivo**

- GFP fusion
- FLAsh



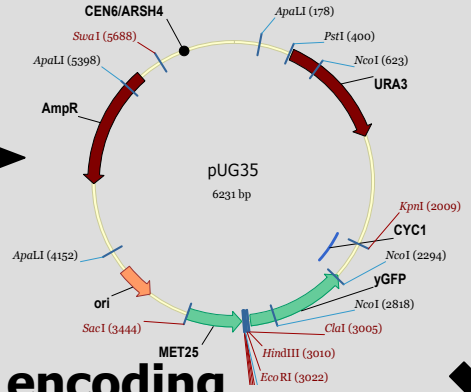
**Protein Interaction in vivo**

- FRET analysis
- BiFC analysis
- Multicolor BiFC analysis

# Protein Localization in vivo

## GFP-fusion proteins

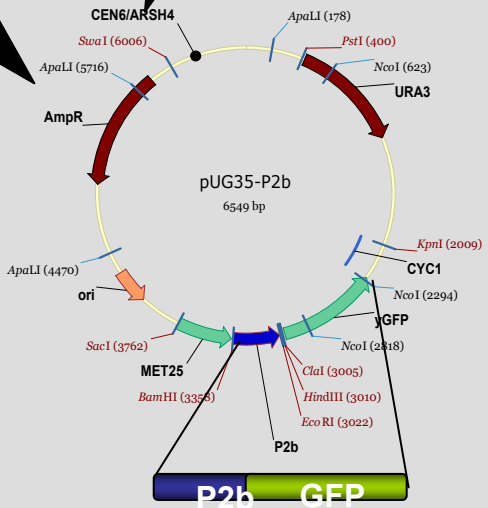
GFP




GFP encoding plasmid

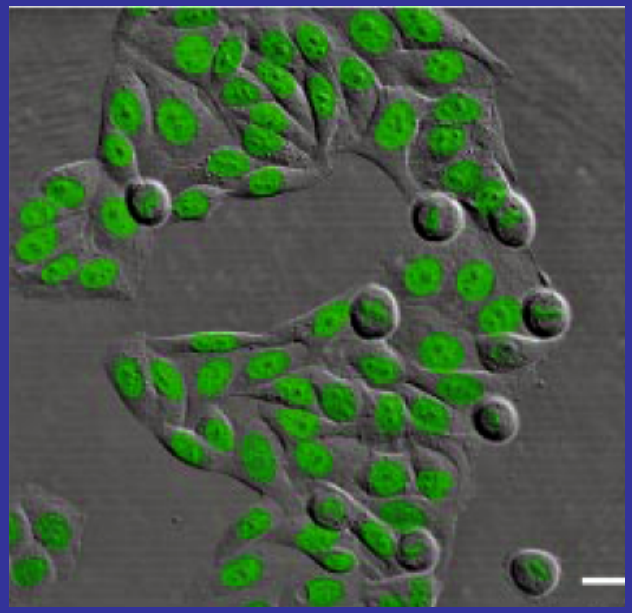
P2b

Your gene (ex: P2b)




Introduction into different organisms

# GFP-fusion proteins



The human histone H2B gene fused (GFP) and transfected into human HeLa cells

**FCS**
**RICS**
**N&B**

**Homogeneous labeling (if stable line)**
**Regulation of the expression can be a problem for FCS**

**Current Biology 1998, 8:377–385**

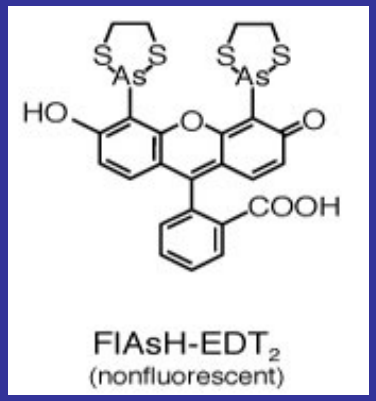
# Protein Localization in vivo

## FL Ash-EDT2 labeling (FLASH tag)

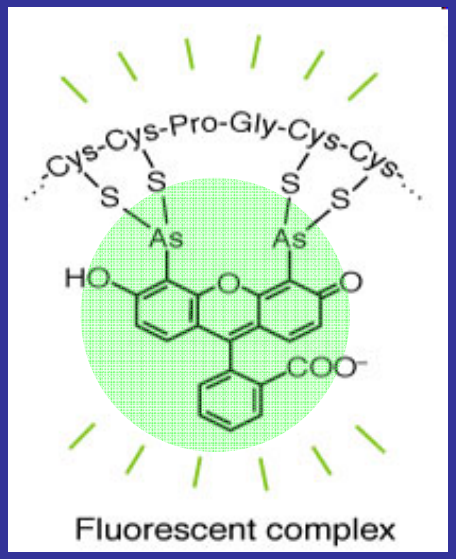
**Receptor domain composed of a few as six natural amino acids that could be genetically incorporated into proteins of interest.**

…-Cys-Cys-Pro-Gly-Cys-Cys-…
(genetically encoded FlAsH recognition sequence)

+




FlAsH-EDT$_2$
(nonfluorescent)

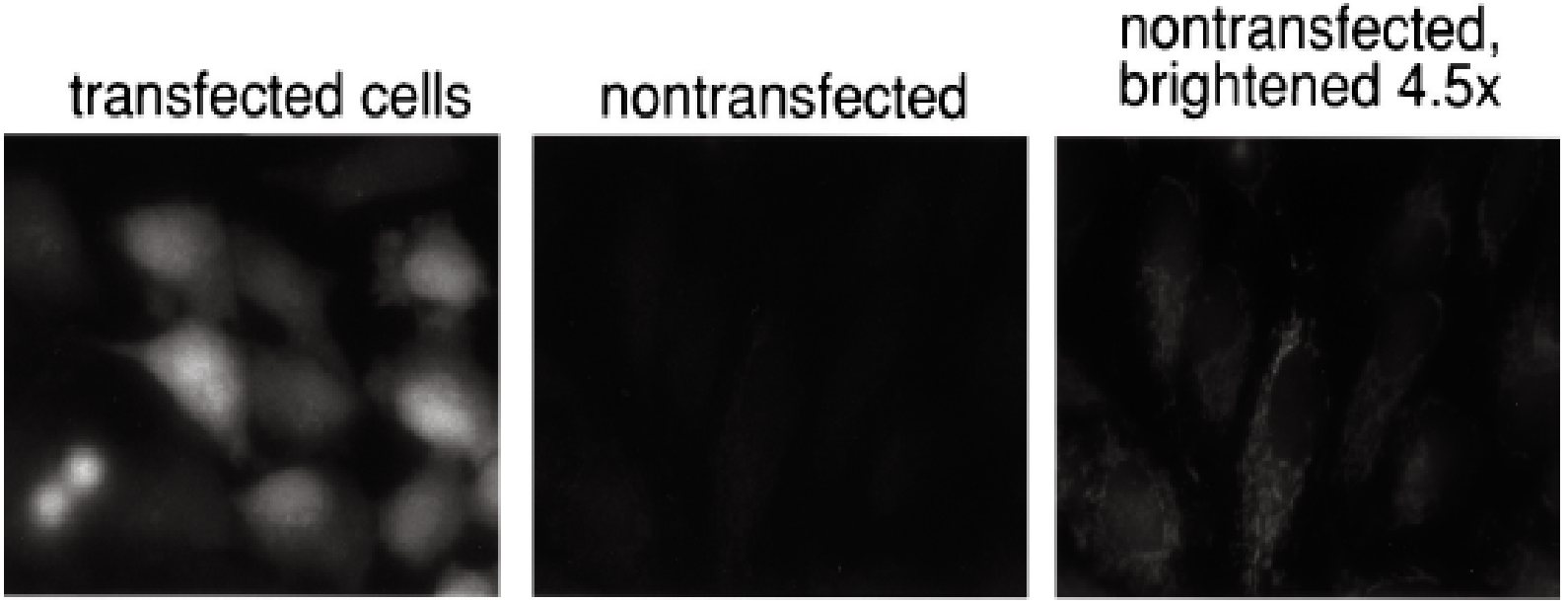



Fluorescent complex

**A small (700-dalton), synthetic, membrane-permeant ligand that could be linked to various spectroscopic probes or crosslinks.**

**The ligand is non fluorescent until it binds its target, where upon it becomes strongly fluorescent.**

# FL Ash-EDT2 labeling (FLASH tag)




**Non-Homogeneous labeling**
**Transfected cells have to be selected**

Griffin et al. SCIENCE VOL 281, 1998, 269-272

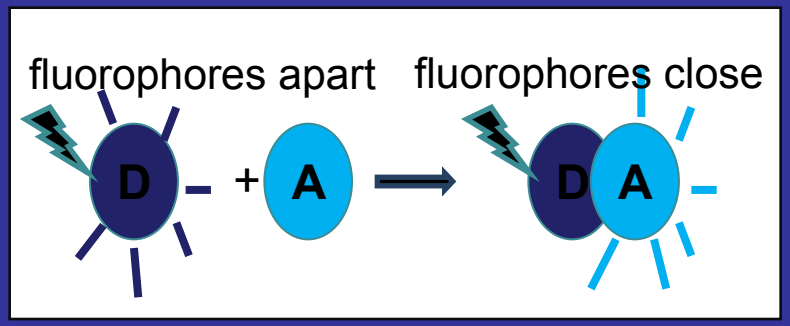
# Protein interactions in vivo

Visualizing the localization of protein interactions in living cells.

**Two principal methods have been used**

- **Föster resonance energy transfer (FRET) analysis**
- **BiFC analysis**

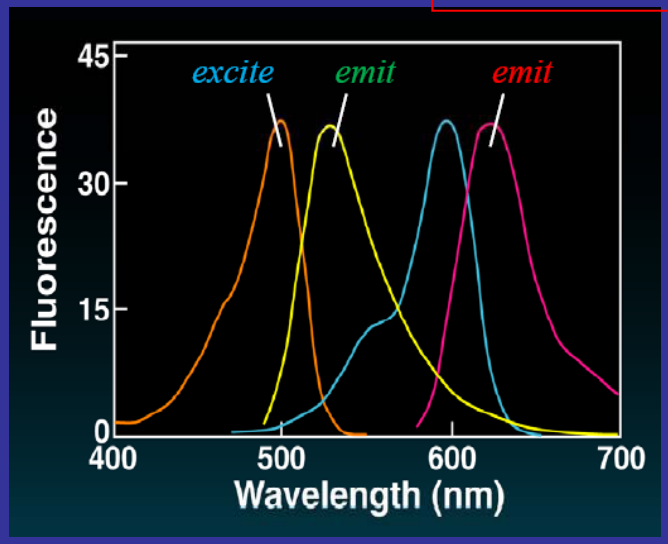
# Föster resonance energy transfer (FRET) analysis

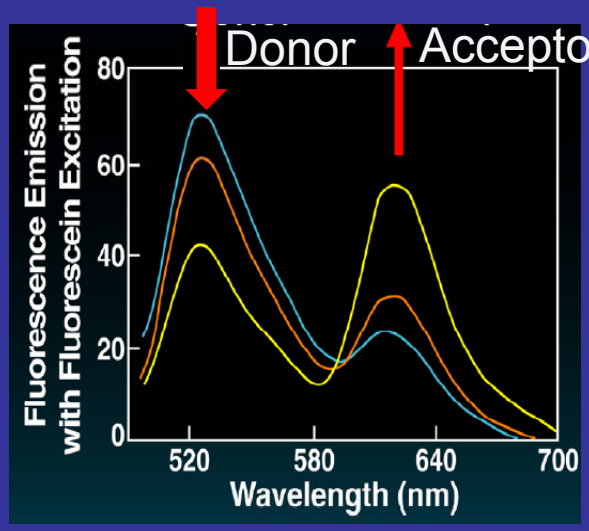



Based on changes in the fluorescence **intensities** and **lifetimes** of two fluorophores that are brought sufficiently close together.

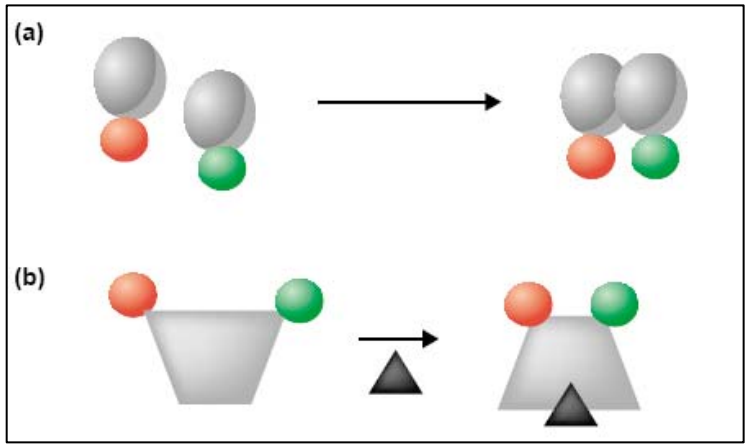
**Donor intensity decrease**
**Donor lifetime decrease**

**Acceptor intensity increase**



# Föster resonance energy transfer (FRET)




Klaus Hahn et al. Current Opinion in Cell Biology 2002, 14:167–172

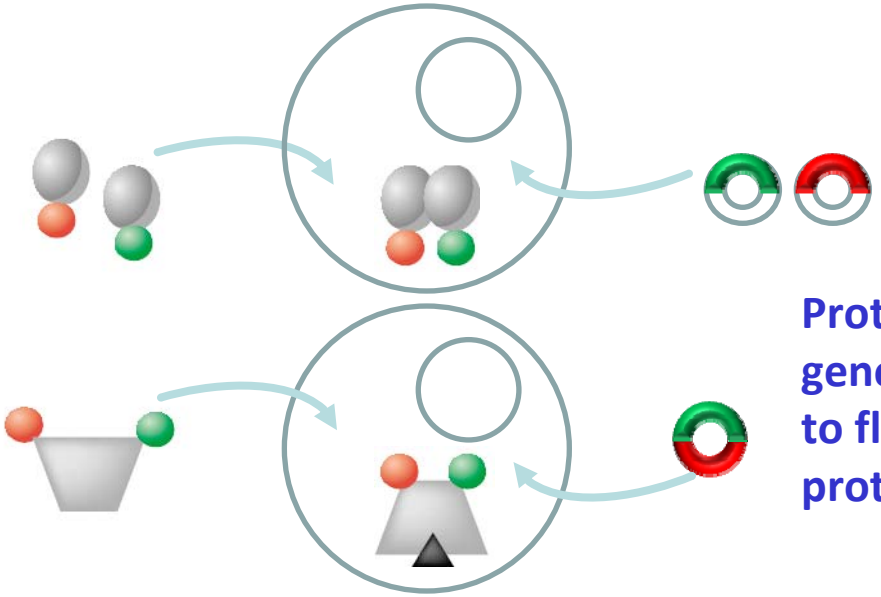
**(a) INTERMOLECULAR FRET:**
**FRET between a donor and acceptor fluorophore, each attached to a different protein, reports protein–protein interaction.**

**(b) INTRAMOLECULAR FRET:**
**two fluorophores attached to the same protein. Changes in distance between them reflect alterations in protein conformation, which in turn indicates ligand binding or post-translational modification.**

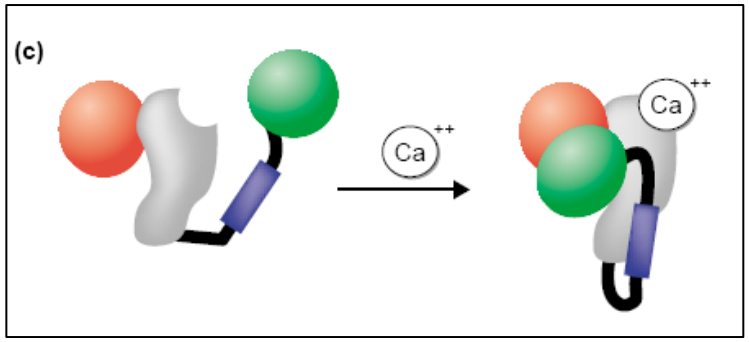
## FRET

**Proteins can be labeled in vitro with small fluorescent dyes.**

**Mechanically incorporated**

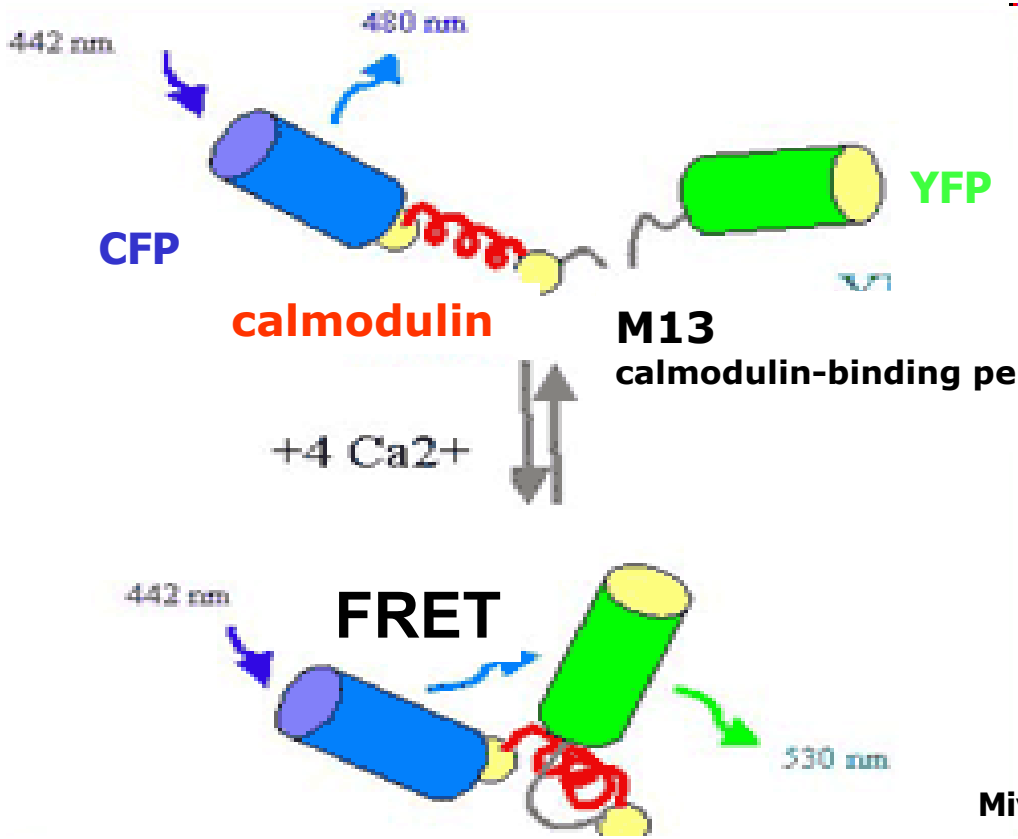


**Proteins can be genetically bounded to fluorescent proteins**




Klaus Hahn et al. Current Opinion in Cell Biology 2002, 14:167–172

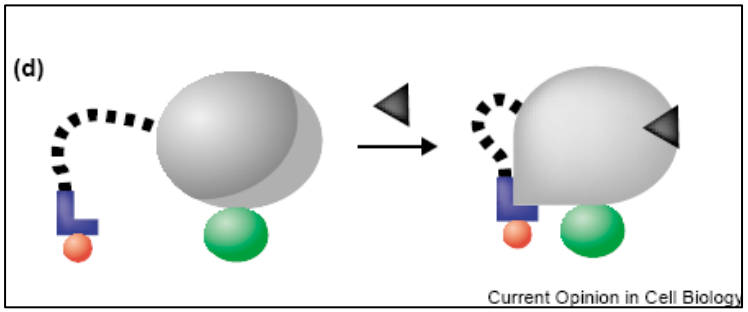
**(c) Protein 'transducer'. A protein is engineered to produce a large change in the distance between an attached donor and acceptor upon ligand binding. In this example, calcium binding generates a hydrophobic pocket to which the blue peptide binds. Peptide binding brings the two GFP mutants together, producing FRET.**




## CAMELEON $Ca^{+2}$ SENSOR

**Binding of $Ca^{2+}$ makes Calmodulin wrap around the M-13-domain, increasing the fluorescence resonance energy transfer (FRET) between the GFPs.**

**Miyawaki et al. Nature, 1997: 28: 834-835.**

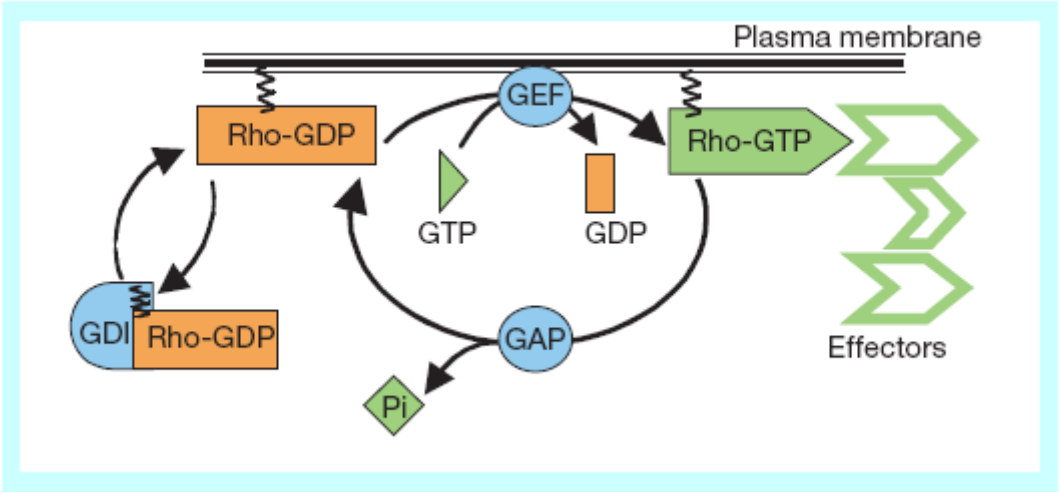



Klaus Hahn et al. Current Opinion in Cell Biology 2002, 14:167–172

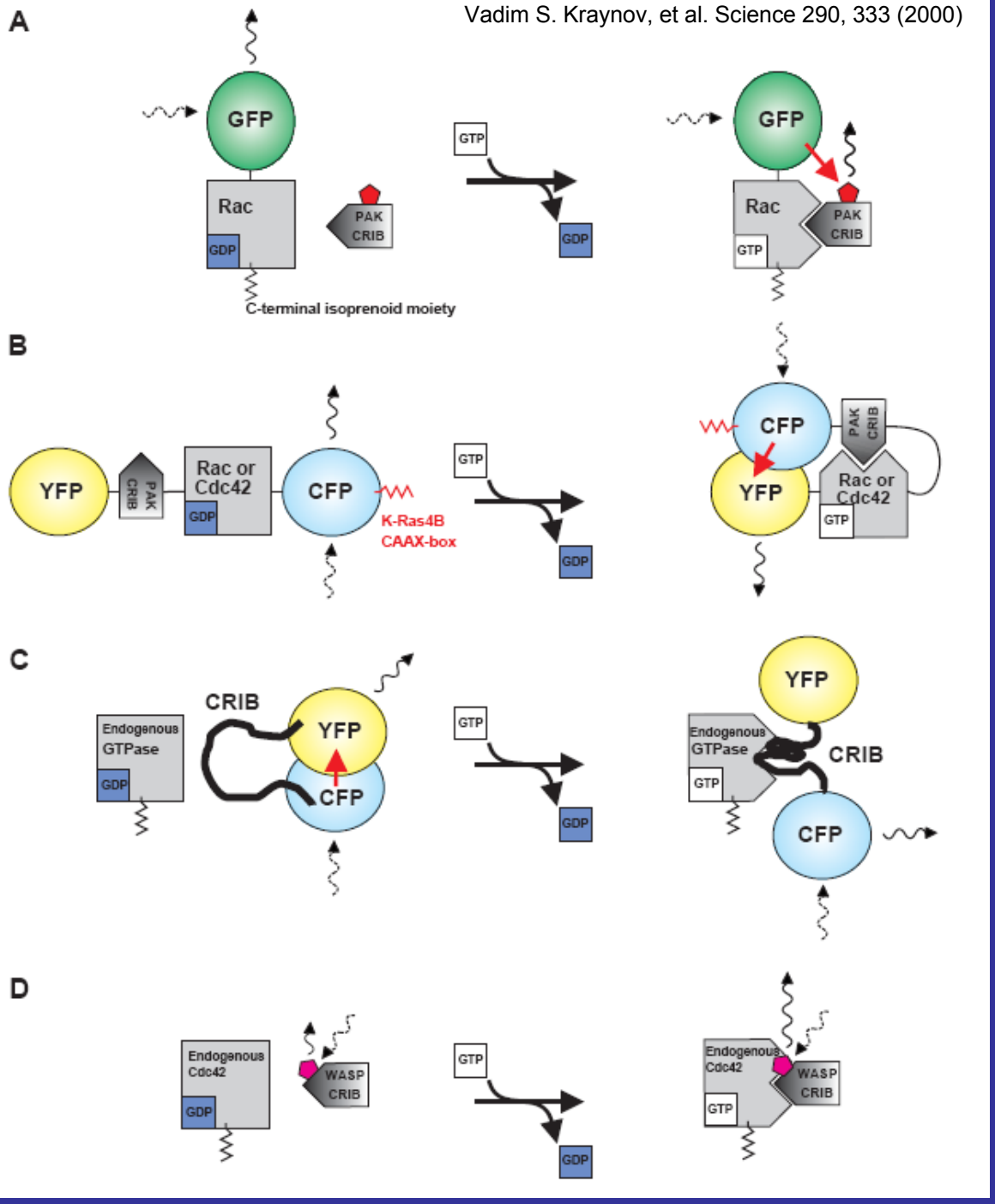
**(d) Domain/antibody biosensor**. A protein or antibody fragment (blue) binds only to the activated state of the protein. The protein fragment bears a dye which undergoes FRET when it is brought in close proximity to the GFP on the protein. In some examples, the domain is part of the same polypeptide chain as the protein (dashed line)

## Rho/Rac Biosensors

Design of different fluorescent probes for detection of Rho family GTPase activity in living cells.

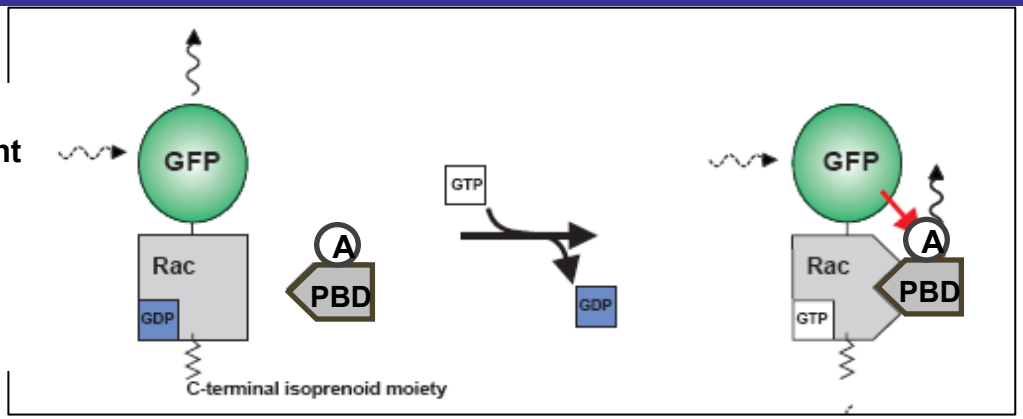


Vadim S. Kraynov, et al. Science 290, 333 (2000)



# The Rac nucleotide state biosensor.

Cells expressing GFP-Rac are injected with a fragment of p21-activated kinase (PBD) labeled with Alexa-546 dye (PBD-A), which binds selectively to GFP-Rac-GTP.

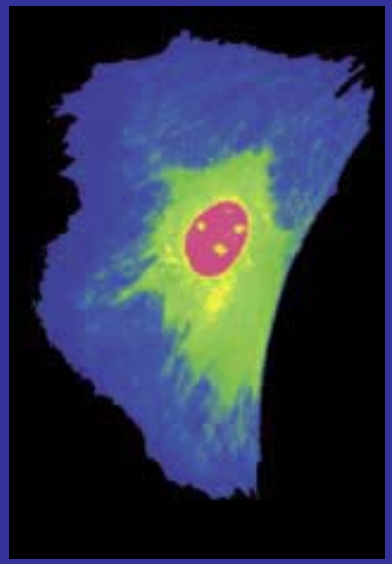



The Alexa and GFP fluorophores undergo FRET when brought close together.

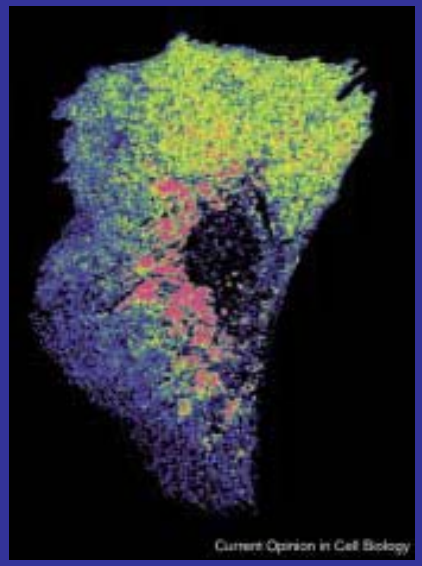
Vadim S. Kraynov, et al. Science 290, 333 (2000)

## Activation of the GTPase Rac in a living motile fibroblast.

**Rac localization (GFP signal)**



**Rac activation (FRET)**



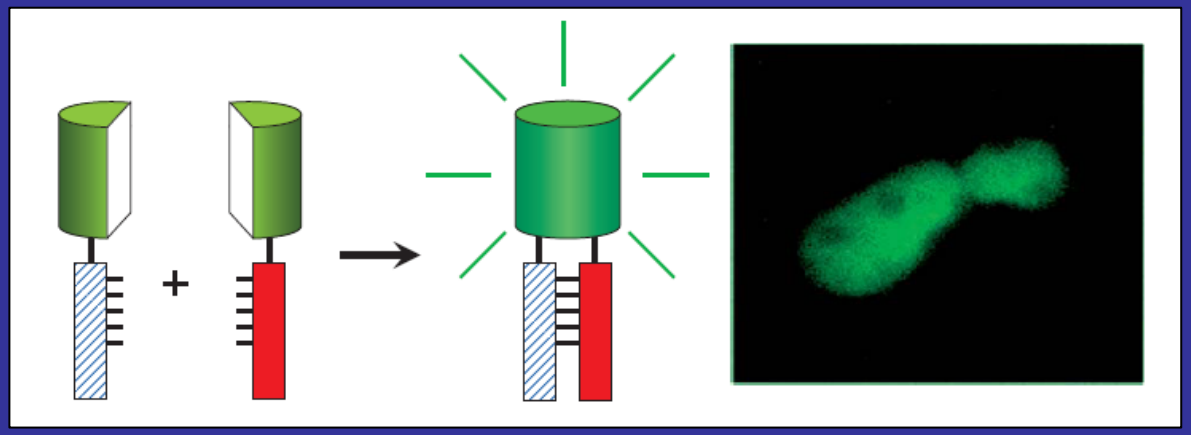

Warmer colors indicate higher levels of activation. A broad gradient of Rac activation is visible at the leading edge of the moving cell, together with even higher activation in juxtanuclear structures.

Only a specific subset of the total Rac generates FRET. This pool of activated protein is sterically accessible to downstream targets such as PAK.

Klaus Hahn et al. Current Opinion in Cell Biology 2002, 14:167–172

# BiFC analysis

## (Bimolecular Fluorescence Complementation)



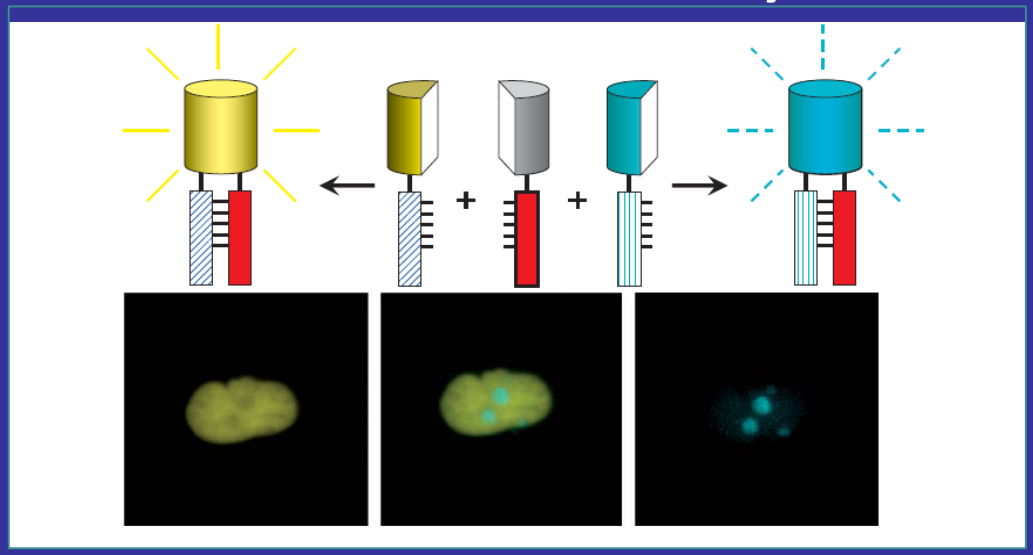
**THE PRINCIPLE: Based in the association between two fluorescent proteins fragments when by an interaction between proteins fused to the fragments. The individual fragments are non-fluorescent.**

**REQUIREMENT: fluorescent protein fragments do not associate with each other efficiently in the absence of an interaction between the proteins fused to the fragments.**

**CONTROLS: Spontaneous association between the fluorescent protein fragments can be affected by the characteristics of the proteins fused to the fragments. It is therefore essential to test the requirement for a specific interaction interface for complementation by each combination of interaction partners to be studied using the BiFC approach.**

Tom K. Kerppola Methods in cell biology, VOL. 85, 431-470

# Multicolor BiFC analysis

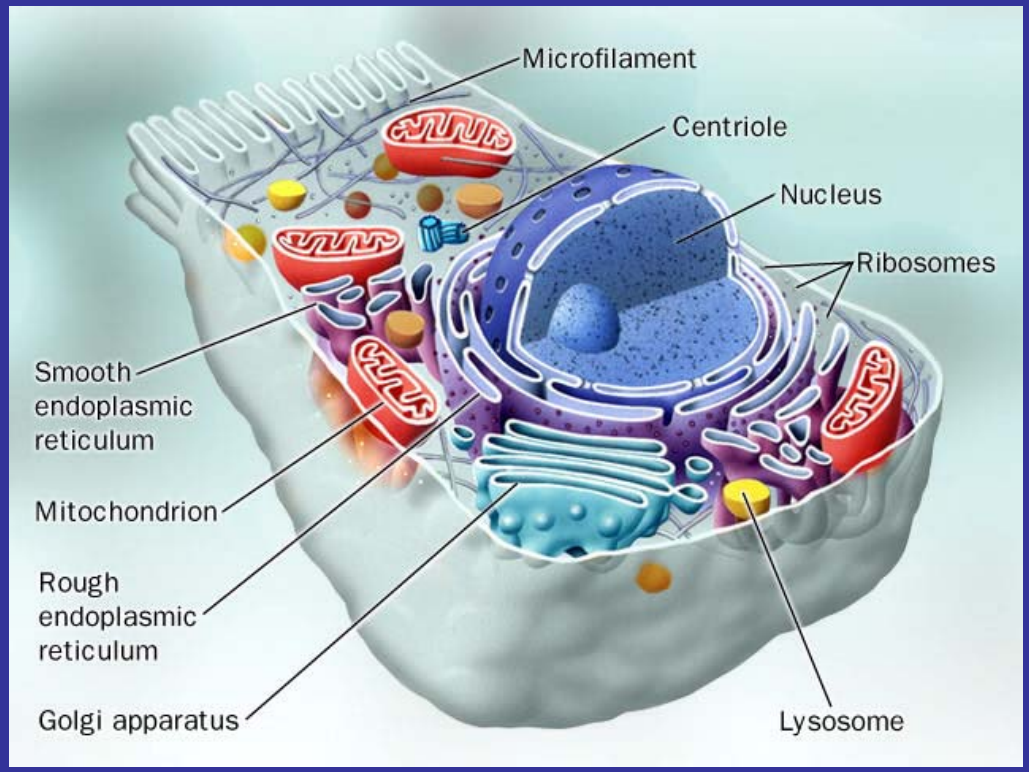


**THE PRINCIPLE: enhanced association of different fluorescent protein fragments through interactions between different proteins fused to the fragments.**

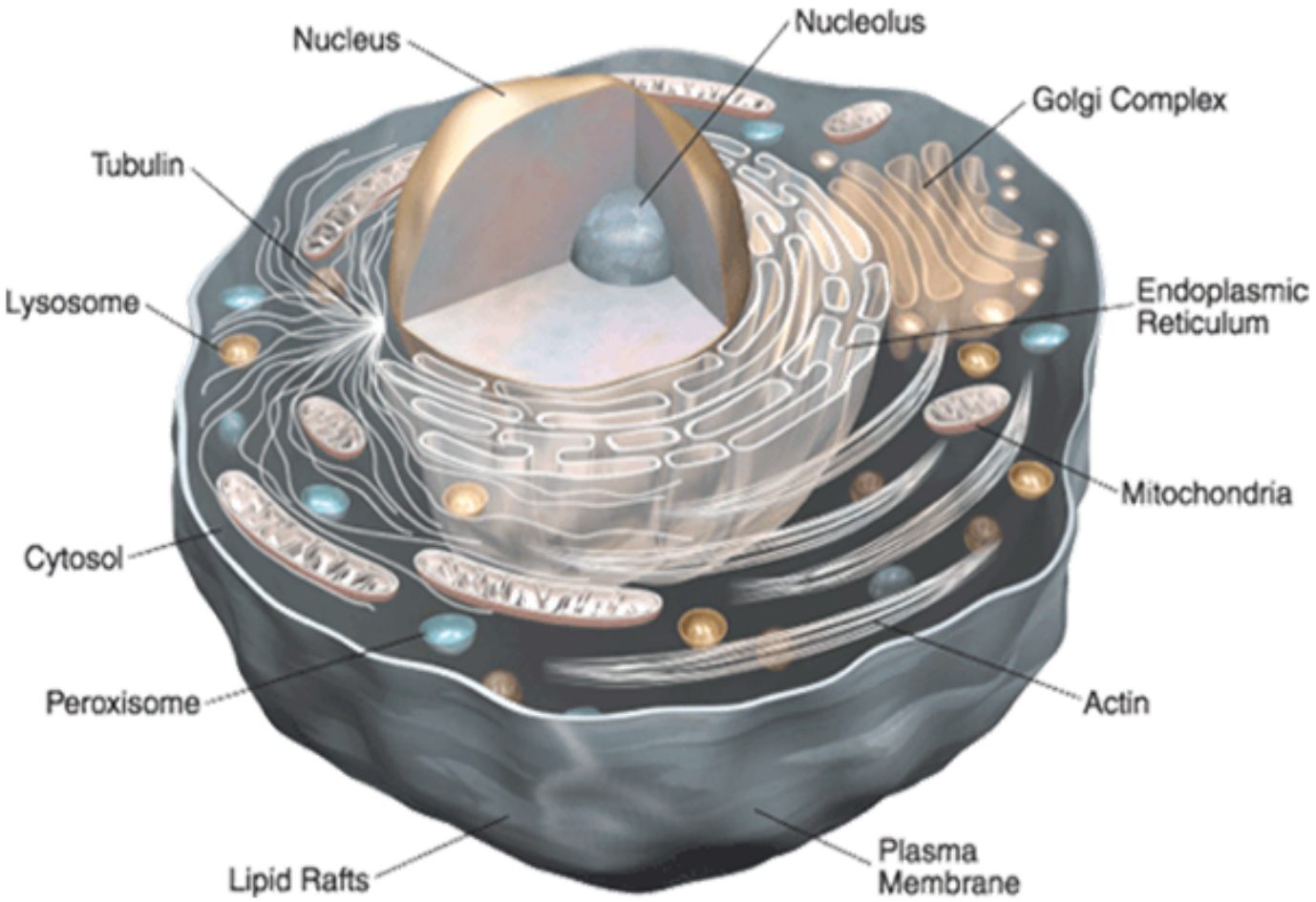
**Since bimolecular fluorescent complex formation can stabilize protein interactions at least in vitro, the relative efficiencies of complex formation do not necessarily reflect the equilibrium binding affinities of the interaction partners in the cell.**

**Quantitative comparison of the efficiencies of complex formation between alternative interaction partners requires that the fluorescent protein fragments can associate with the same efficiency within each complex.**

# Labeling cellular organelles



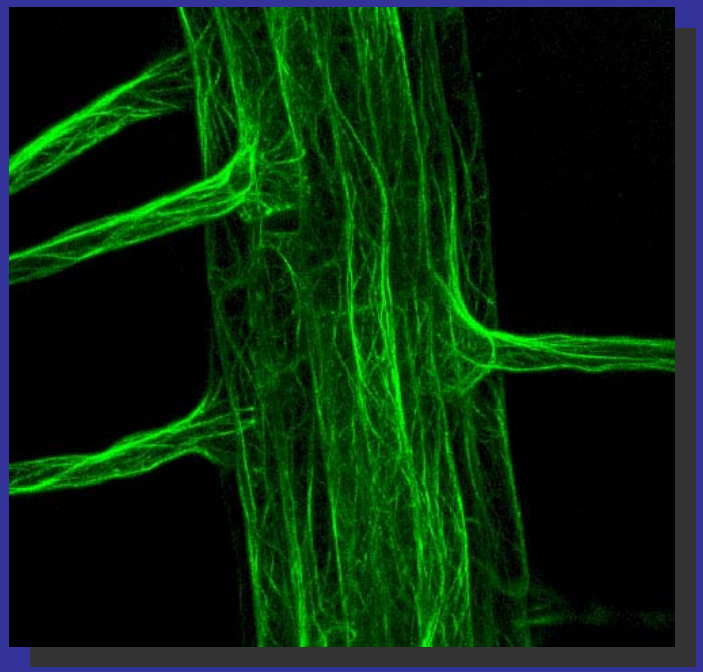
# Organelle Stains for Fluorescence Imaging Selection Guide




- Adiposomes
- Cytoplasm
- Cytoskeleton
- Endoplasmic reticulum
- Golgi complex
- Intracellular membranes
- Lysosomes
- Membrane trafficking
- Mitochondria
- Nuclear envelope
- Nucleoli
- Nucleus
- Peroxisomes
- Plasma membrane
- Whole-cell stains for image segmentation in HCS applications

invitrogen™

Quick Order



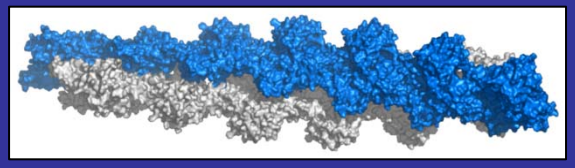
# Probes to label Actin

- Labeled Phallotoxins for F-Actin
- DNase I Conjugates for G-Actin
- Fluorescent Actin Conjugates
- Probes for Studying Actin Dynamics

# Phallotoxin Conjugates
## for Labeling F-Actin

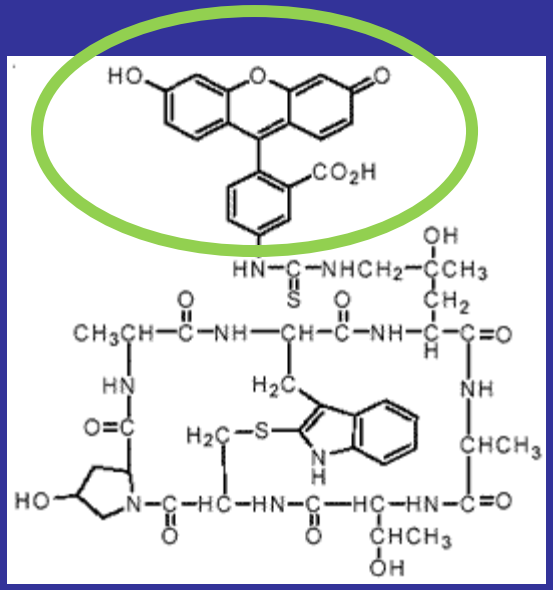
- **Phalloidin**, a heptapeptide toxin from the poisonous mushroom ***Amanita phalloides***.
- Though highly toxic to liver cells, it has since been found to have little input into the death cap's toxicity as it is not absorbed through the gut
- Binds tightly and specifically to polymerized actin, stabilizing the filaments from a variety of depolymerizing agents and conditions

Phallotoxins bind to filamentous actin (F-actin)



F-Actin; surface representation of 13 subunit repeat based on Ken Holmes' actin filament model

# Fluorescein-phalloidin



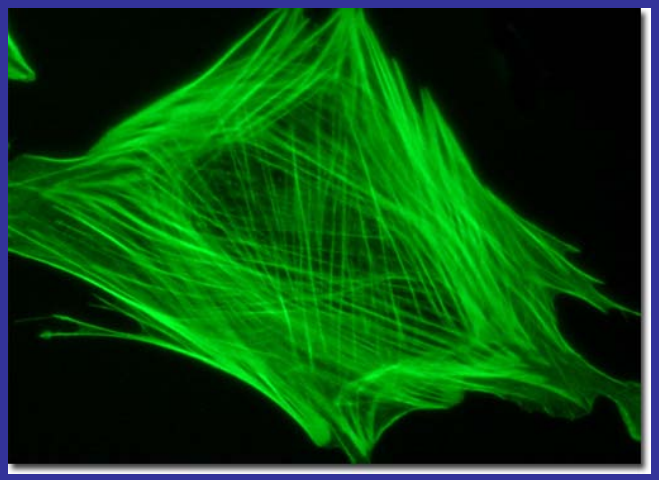


*Table 1. Spectral characteristics of our phallotoxin probes.*

| Cat # | Conjugate | Abs * (nm) | Em * (nm) |
|---|---|---|---|
| C-606 | coumarin phallacidin | 355 | 443 |
| N-354 | NBD phallacidin | 465 | 536 |
| A-12379 | Alexa™ 488 phalloidin | 495 | 518 |
| F-432 | fluorescein phalloidin | 496 | 516 |
| O-7466 | Oregon Green™ 488 phalloidin | 496 | 520 |
| B-607 | BODIPY® FL phallacidin | 505 | 512 |
| O-7465 | Oregon Green 514 phalloidin | 511 | 528 |
| E-7463 | eosin phalloidin | 524 | 544 |
| B-7491 | BODIPY R6G phalloidin | 529 | 547 |
| R-415 | rhodamine phalloidin | 554 | 573 |
| B-3475 | BODIPY 558/568 phalloidin | 558 | 569 |
| A-12380 | Alexa 568 phalloidin | 578 | 600 |
| A-12381 | Alexa 594 phalloidin | 581 | 609 |
| B-3416 | BODIPY 581/591 phalloidin | 584 | 592 |
| B-7464 | BODIPY TR-X phallacidin | 589 | 617 |
| T-7471 | Texas Red®-X phalloidin | 591 | 608 |
| A-12382 | BODIPY 650/665 phalloidin | 647 | 661 |
| B-7474 | biotin-XX phalloidin | — | — |
| P-3457 | phalloidin | — | — |

* Absorption (Abs) and fluorescence emission (Em) maxima.

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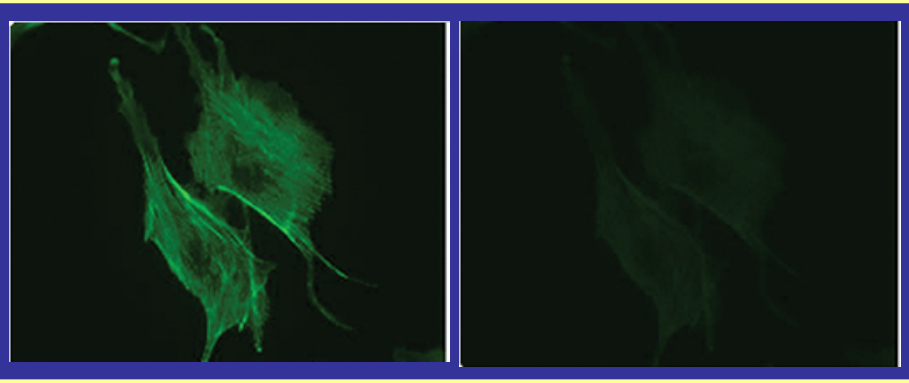
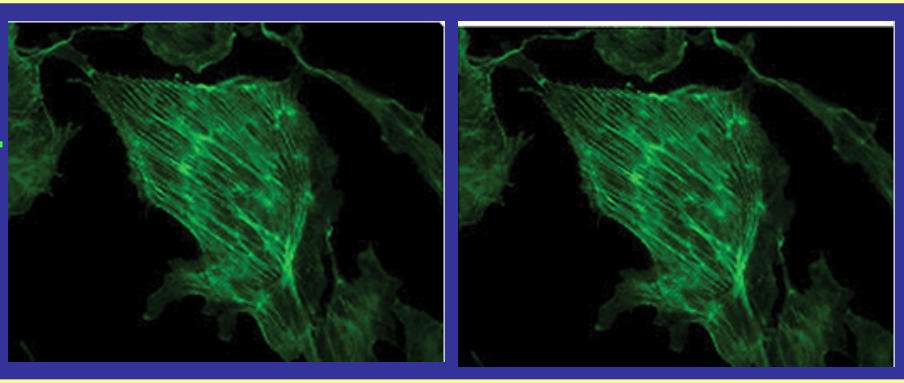
# The photostability of the Alexa-Fluor dye conjugates is particularly apparent when compared to traditional fluorophores such as fluorescein

**Fluorescein-phalloidin**



**photobleached to about 20% of its initial value**

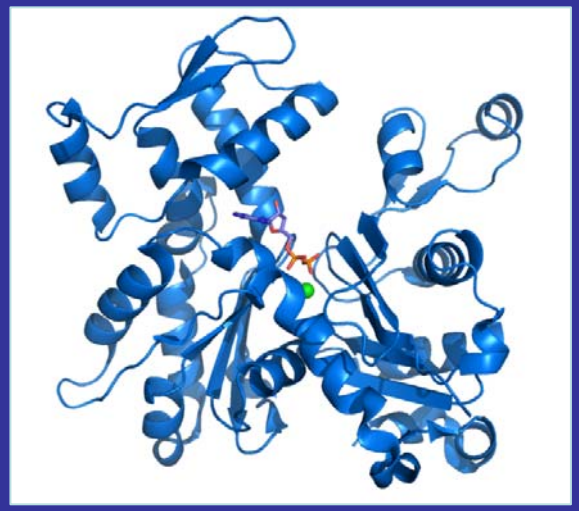
**Alexa Fluor 488-phalloidin**



**Intensity stayed at the initial value**

The cells were placed under constant illumination on the microscope. Images were acquired at one-second intervals for 30 seconds.

# DNase I Conjugates
## for Labeling G-Actin



G-Actin

**fluorescent conjugates of bovine pancreatic DNase I selectively label monomeric G-actin.**

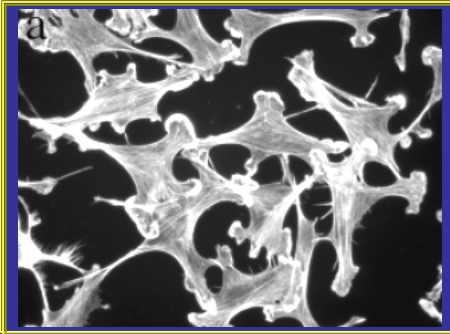
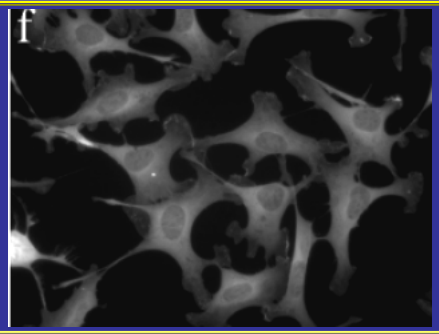
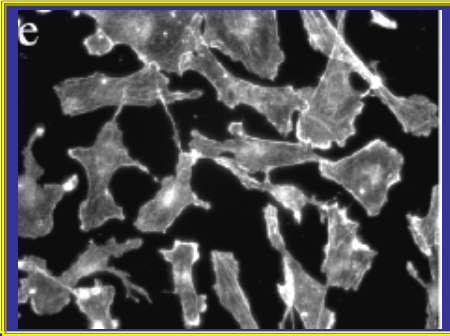
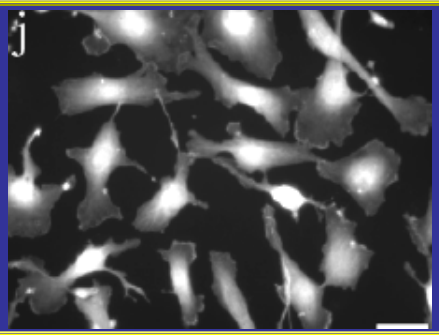
***Table 2.*** *Spectral characteristics of our fluorescent DNase I conjugates.*

| Cat # | Conjugate | Abs * (nm) | Em * (nm) |
|---|---|---|---|
| D-970 | fluorescein | 494 | 517 |
| D-12371 | Alexa™ 488 | 495 | 519 |
| D-7497 | Oregon Green™ 488 | 496 | 524 |
| D-971 | tetramethylrhodamine | 555 | 580 |
| D-12372 | Alexa 594 | 590 | 617 |
| D-972 | Texas Red® | 597 | 615 |

* Absorption (Abs) and fluorescence emission (Em) maxima.

**DNase I conjugates are used in combination with fluorescently labeled phallotoxins to simultaneously visualize G-actin pools and filamentous F-actin**

***Effect of Latrunculin*** **(inhibits actin polymerization) on Swiss 3T3 cells.**

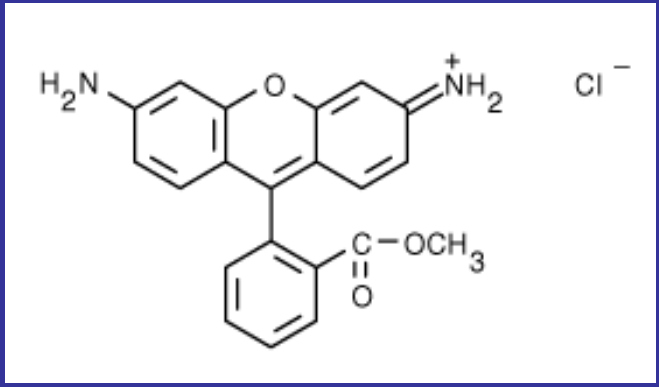
| Fluor- phalloidin | Alexa488-DNase I | |
|---|---|---|
|  |  | **CONTROL**<br>**Incubated in media for 30 m**<br>**Fix and double labeled** |
|  |  | **EXPERIMENT**<br>**Incubated in media plus**<br>**Plus2 M Latrunculin for 30 m**<br>**Fix and double labeled** |

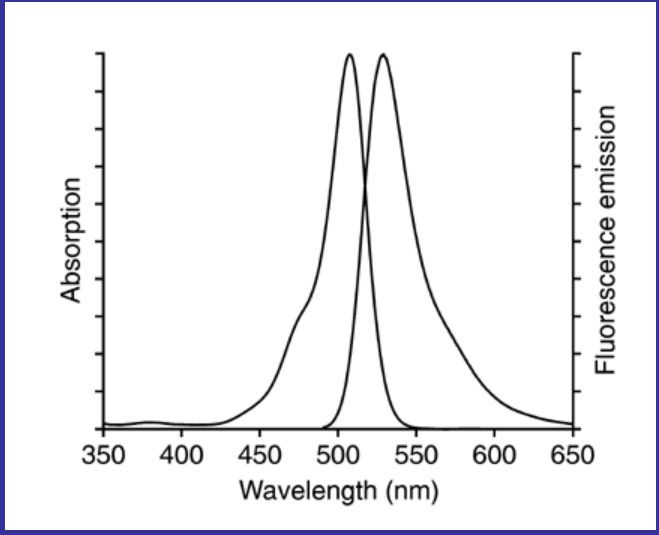
***Cramer et al.Cell Motility and the Cytoskeleton 51:27–38 (2002)***

# Probes to label Mitochondrion

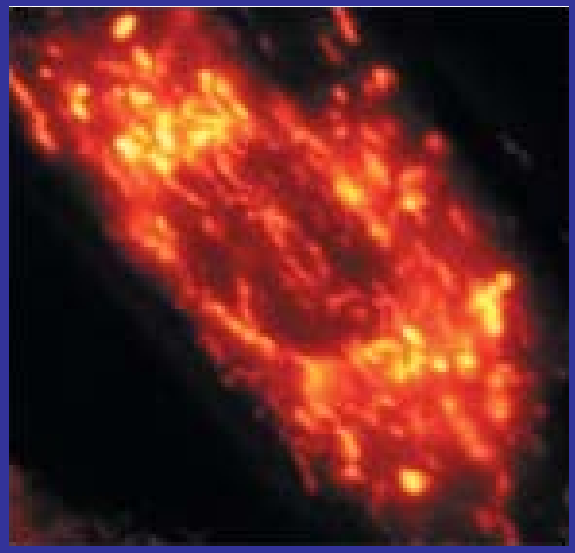
- Rhodamine 123 and Tetrabromorhodamine 123
- MitoTacker probes
- Antobodies to Mitochondrial proteins
- MitoFluor probes
- Potential sensitive probes

# Rhodamine 123



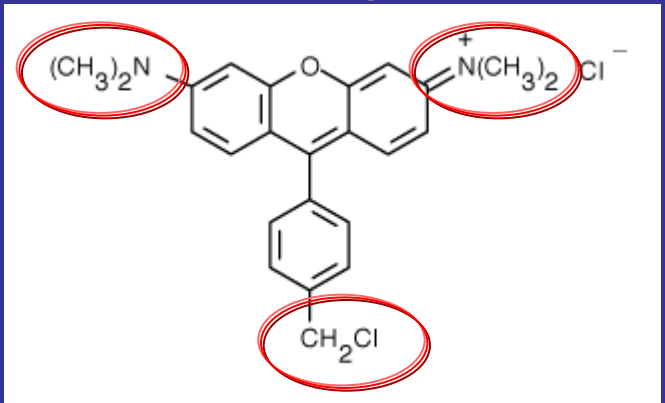

- **Cell-permeant, cationic,**
- **Sequestered by active mitochondria without inducing cytotoxic effects.**
- **Uptake and equilibration takes a few minutes**

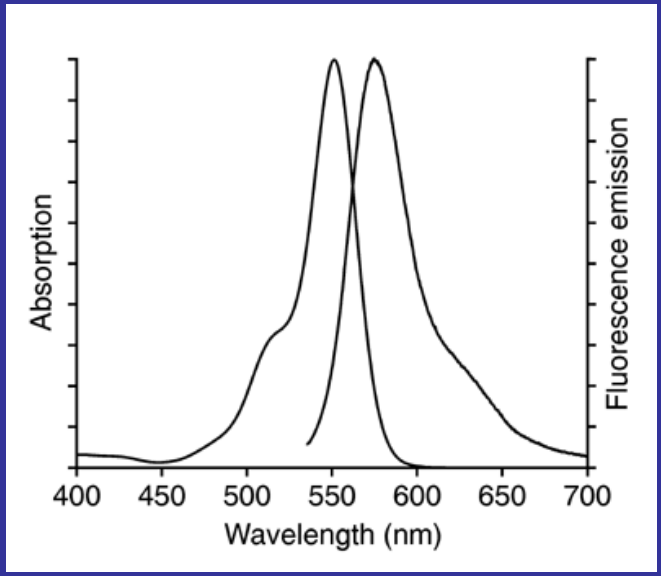


Staining of rat cortical astrocytes by rhodamine 123

# Mitotracker

MitoTracker Orange CMTMRos



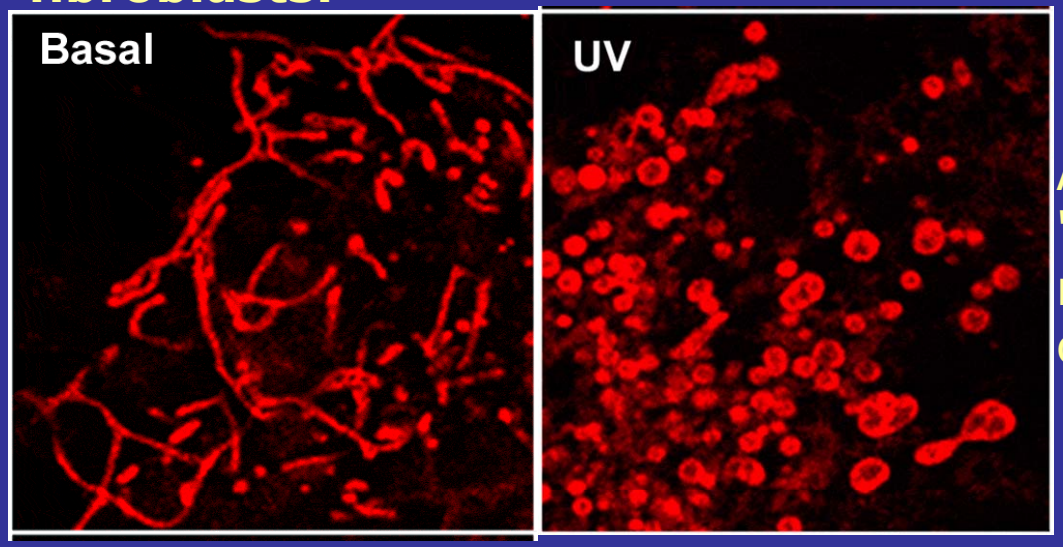




- **Cell-permeant mitochondrion-selective dyes which passively diffuse across the plasma membrane and accumulate in active mitochondria**
- **The dye remains associated with the mitochondria after fixation.**

*Giulia Ossato. LFD-2010*

**MitoTracker allows direct real-time registration of mitochondrial alterations in response to stressful stimuli (i.e. oxidative bursts, UV treatment etc.) in "in vitro" cultured cells.**

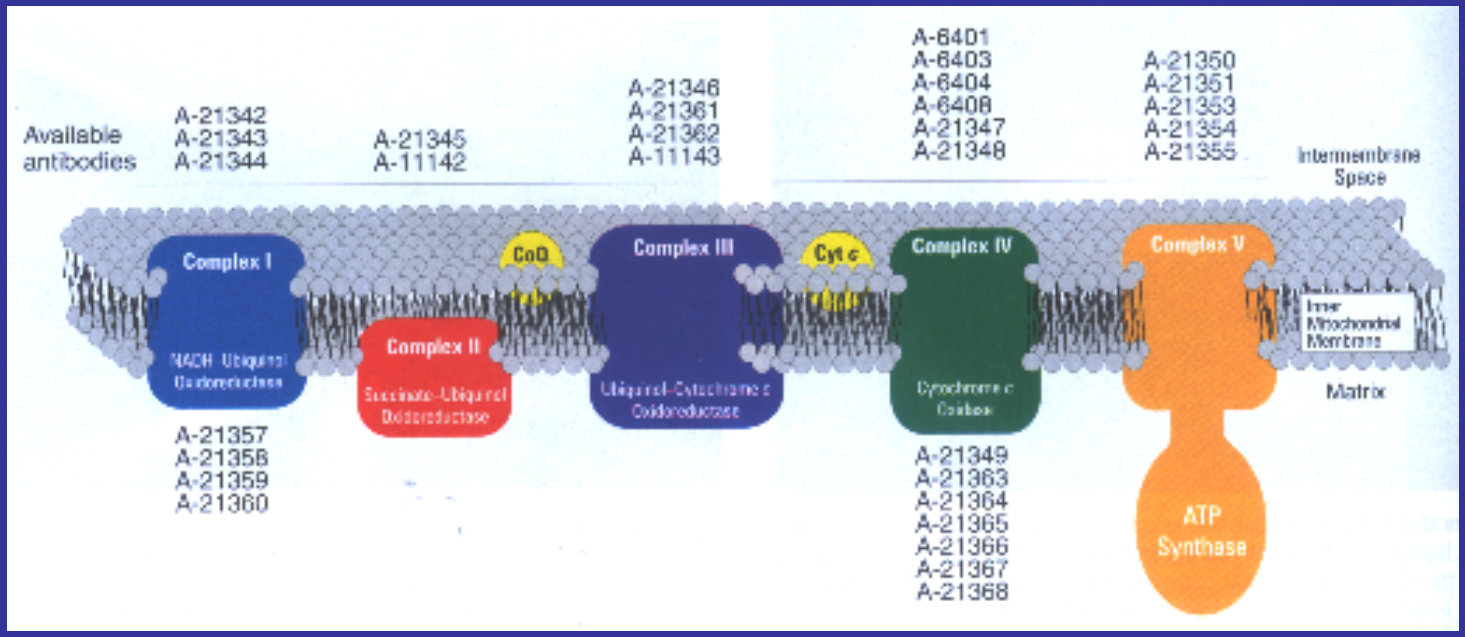
**EXPERIMENT: Mitochondrial behavior in response to UV treatment in NIH 3T3 fibroblasts.**




After UV an evident "ballooning" of the mitochondria can be observed.

**http://www.med.unipg.it/imagelab/mitoch.html**

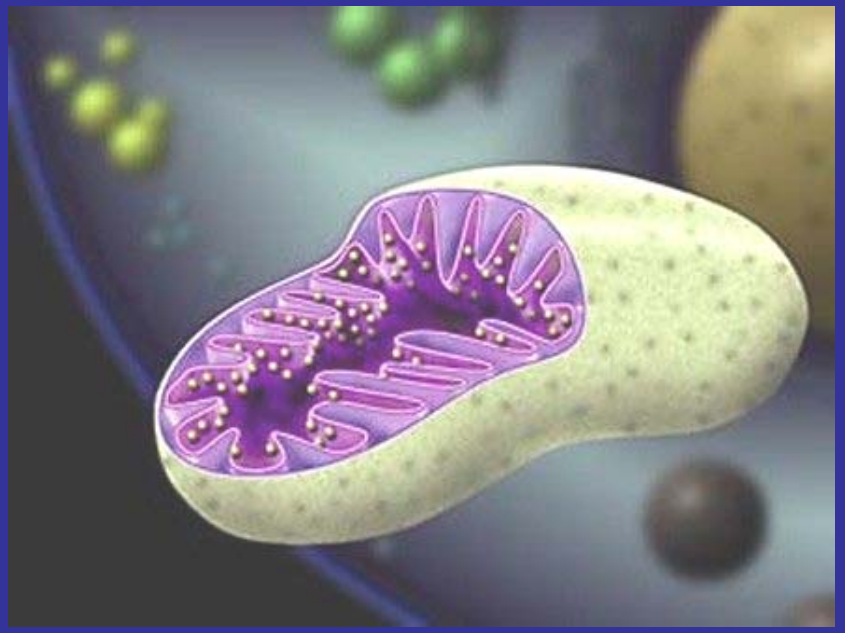
# Antibodies to Mitochondrial proteins




The mammalian oxidative phosphorylation system, and specific nonoclonal antibodies available from Molecular Probes

***www.invitrogen.com***

## Antibodies for the different complex are available in different colors



| *Conjugate* | *Goat anti-rabbit IgG (H+L)* | *Goat anti-mouse IgG (H+L)* | *Ex/Em** | *Fluorescence similar to--* |
|---|---|---|---|---|
| Alexa Fluor® 488 | A11008 | A11001 | 495/519 | FITC |
| Alexa Fluor® 555 | A21428 | A21422 | 555/565 | Cy3 |
| Alexa Fluor® 594 | A11012 | A11005 | 590/617 | Texas Red |
| Alexa Fluor® 647 | A21244 | A21235 | 650/668 | Cy5 |
| HRP | 81-6120 | 81-6520 | NA** | NA |
| AP | 81-6122 | 81-6522 | NA | NA |
| Biotin | B2770 | B2763 | NA | NA |

*Excitation/emission (nm); **Not applicable

# Contribution to the slides

- Theodore Hazlett
- David Jameson
- Ewald Terpetschnig