FLUORESCENT LABELING

Susana Sanchez
Laboratory for Fluorescence Dynamics. UCI

5th Annual Principles of Fluorescence Techniques, Genova, Italy, Jun 25-29, 2007

How to choose the labeling protocol?

In vivo or in vitro

Spectroscopy or Microscopy

Light source available

Lifetime and Spectral Properties of the fluorescent probe

Outline

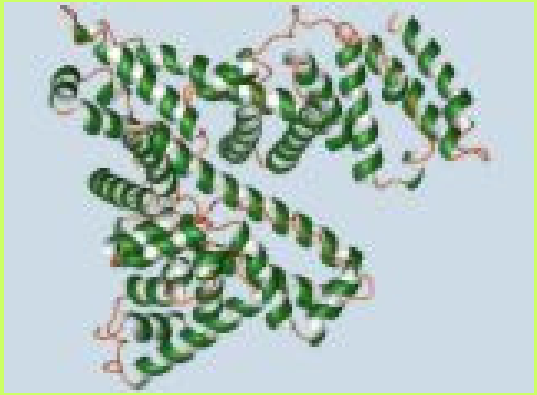
Labeling "*in vitro*"

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

Labeling "*in vivo*"

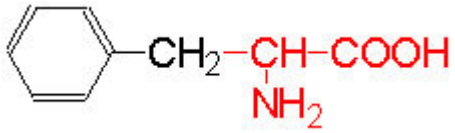
- Genetic Incorporation
  (GFP, FLASHtag)
- Mechanical Incorporation
  (Electroporation, Microinjection
  Agrobacterium-med- transfection

Labeling proteins



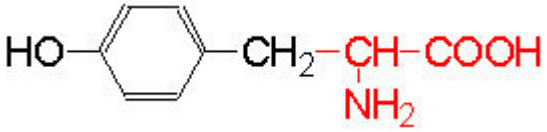
Proteins: Naturally Occurring Fluorophores

aromatic amino acids



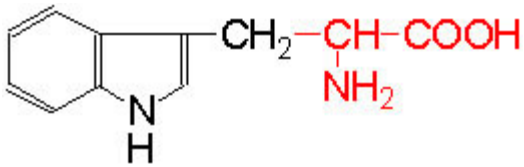
Phenylalanine (Phe – F)

Ex/Em 260 nm/282 nm



Tyrosine (Tyr – Y)

ex/em 280 nm/305 nm



Tryptophan (Trp-W)

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

Abs/Ex

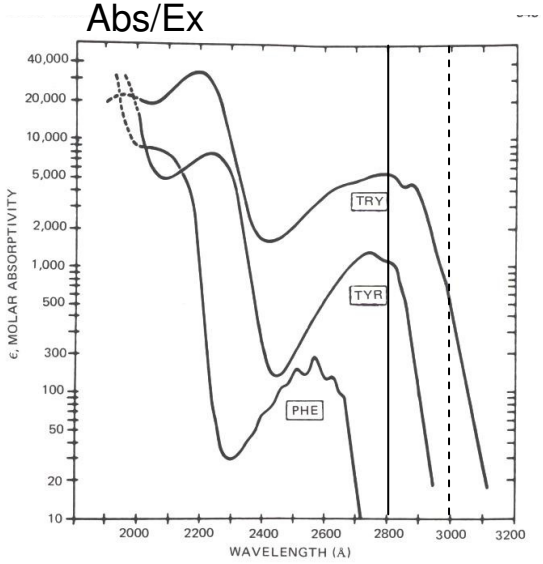
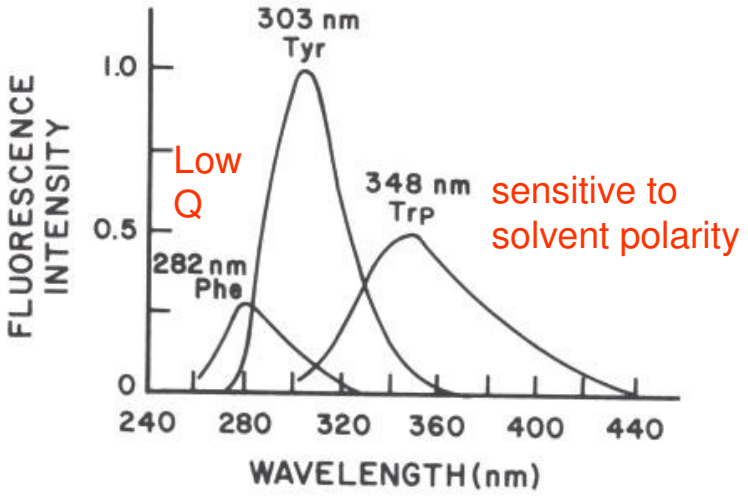


Figure 11.1. Absorption spectra of the aromatic amino acids. (From Ref. 6.)

Insensitive to solvent polarity

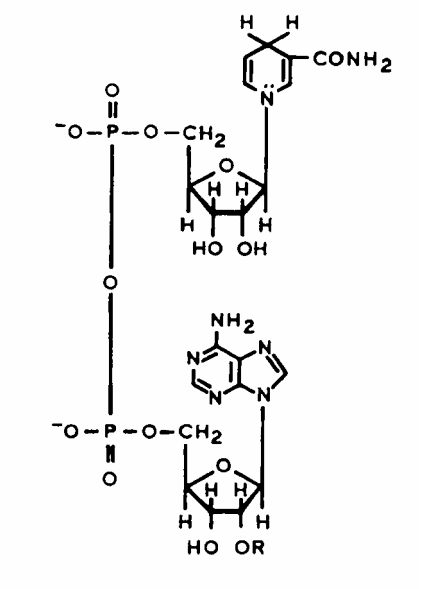


Low Q

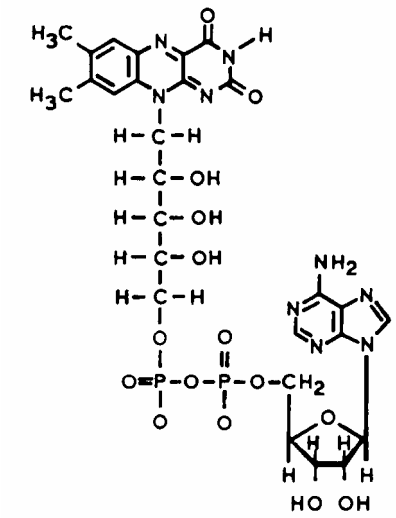
sensitive to solvent polarity

Proteins: Naturally Occurring Fluorophores

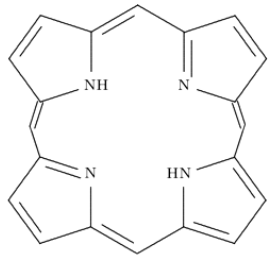
Enzymes Cofactors



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



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



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

Fe+2 (Heme)
Myoglobin,hemoglobin
cytochromes b and c,
cytochrome P450 and
cytochrome oxidase

Mg+2 chlorophylls

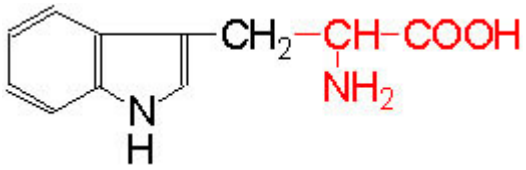
metal free pheophytins

J. Agric. Food Chem. **2003,** *51,* 6934-6940

Proteins: Synthetic Fluorophores

(genetically incorporated in the protein)

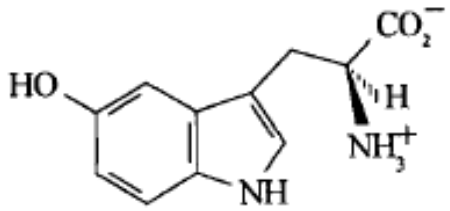
Tryptophan derivatives



Tryptophan (Trp-W)
ex/em 295nm/ 305-350 nm

Φ =0.14

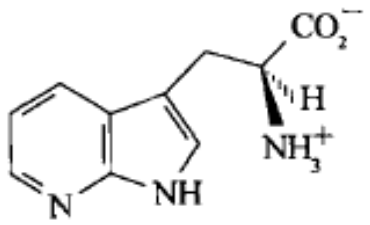
•solvent-sensitive emission



5-Hydroxytryptophan
ex/em 310nm/339 nm

Φ= 0.097

•solvent-insensitive emission



7-azatryptophan
ex/em 320nm/403nm

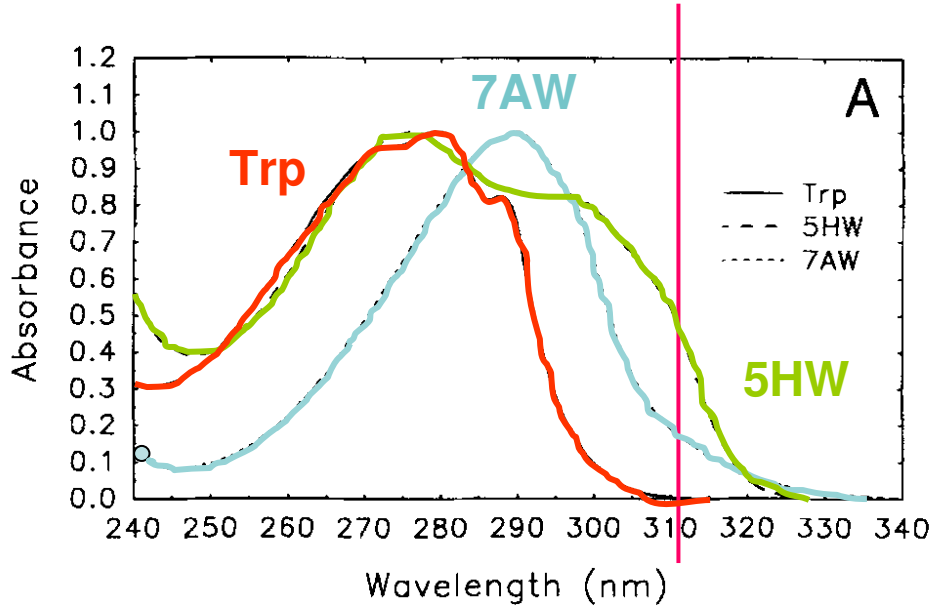
Φ = 0.017

•Large emission shift in water

Φ =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.



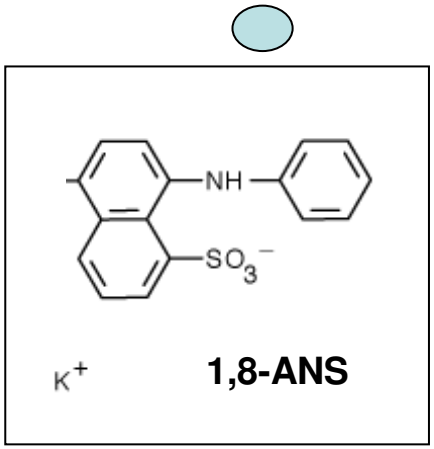
It is possible to selectively excite them, in proteins, in the presence of tryptophan of other proteins

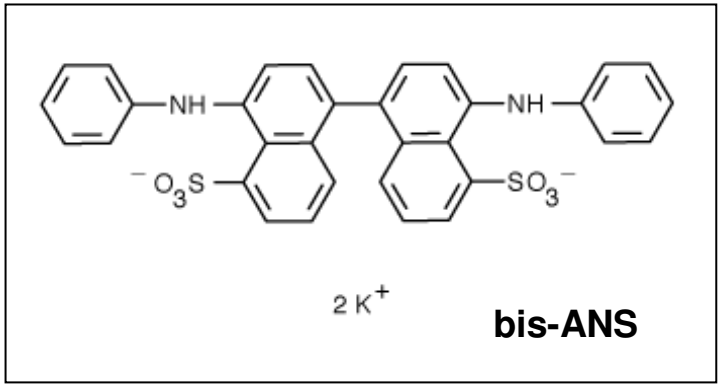
Protein Science (1997), 6, 689-697.

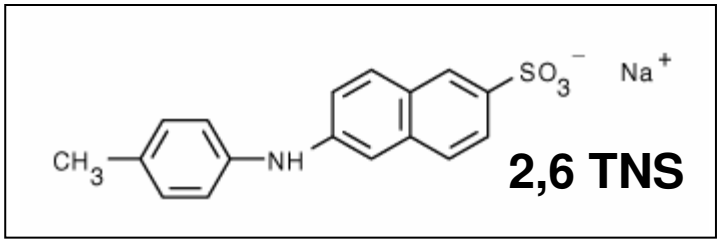
Proteins: Extrinsic probes

(not present in the natural molecule/macromolecule)

Non-covalent Attachments

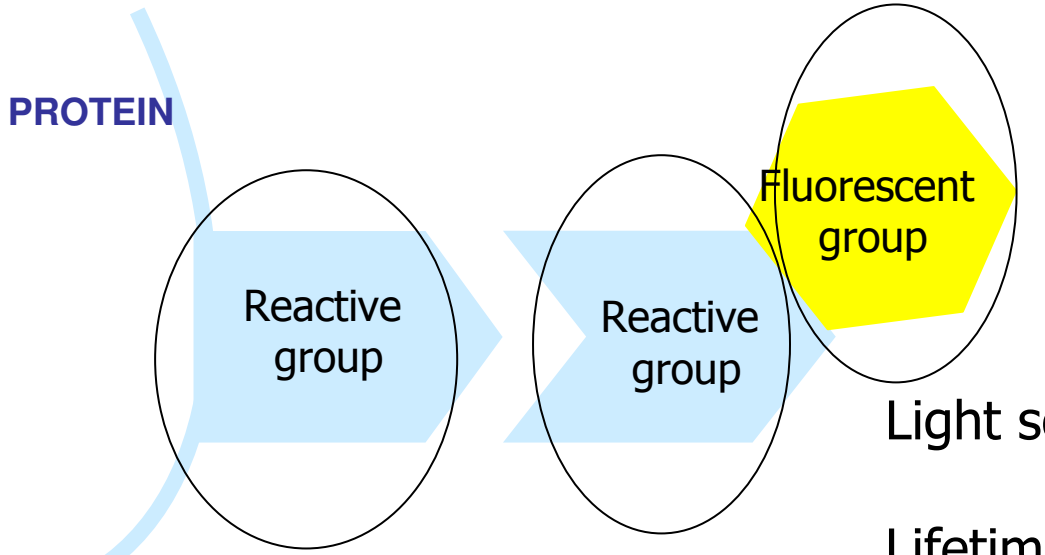






barely fluorescent in pure water but their fluorescence can be strongly enhanced if the environment becomes hydrophobic (hydrophobic patches on proteins)

Covalent Attachments



Available reactive group in the protein

NH_2 Lysine
Arginine

SH Cysteine

Depends on the reactive group in the protein.

Light source

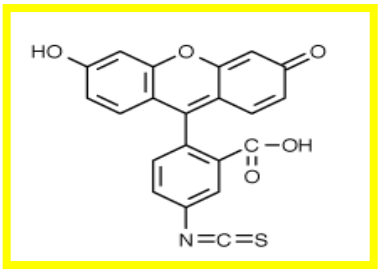
Lifetime of the fluorescent group

Spectral properties

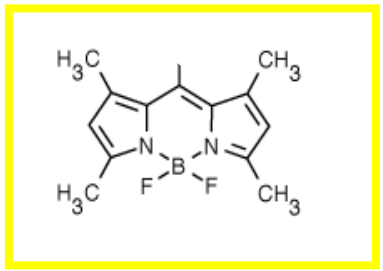
Autofluorescence

Labeling should not change the biological activity of the protein.

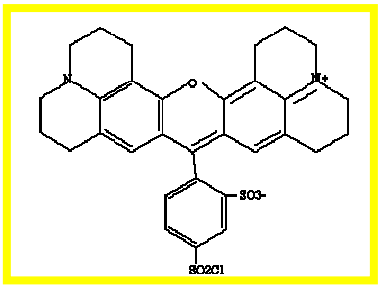
Fluorescent group



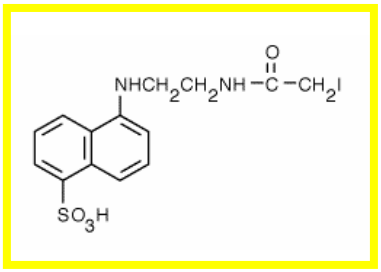
FLUORESCEINE
(488/512) $\tau \approx 4.05$



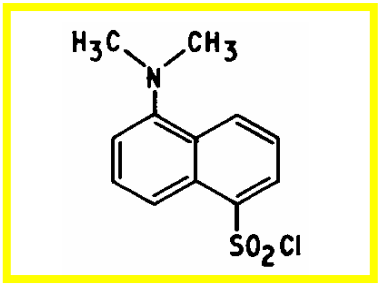
BODIPI
(493/503), τ=6 ns



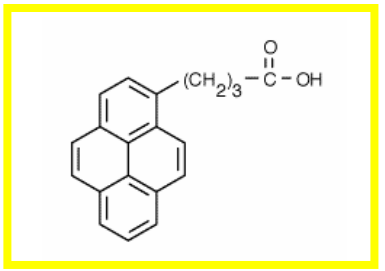
Texas Red
(595-615), $\tau \approx 3.5$ ns



IAEDANS
(360/480) $\tau \approx$ 15 ns



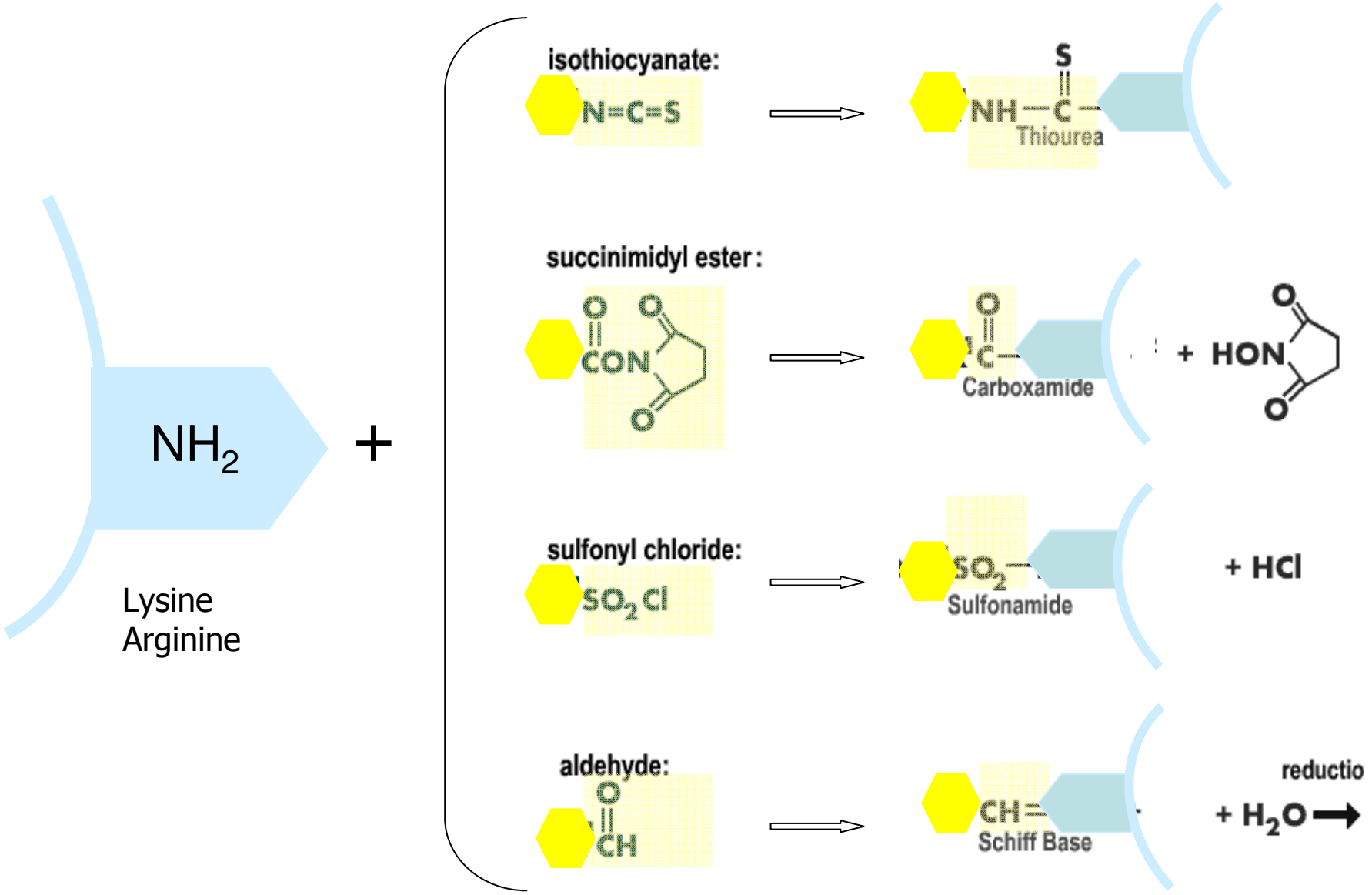
Dansyl chloride
(335/518) t ≈ 10 ns



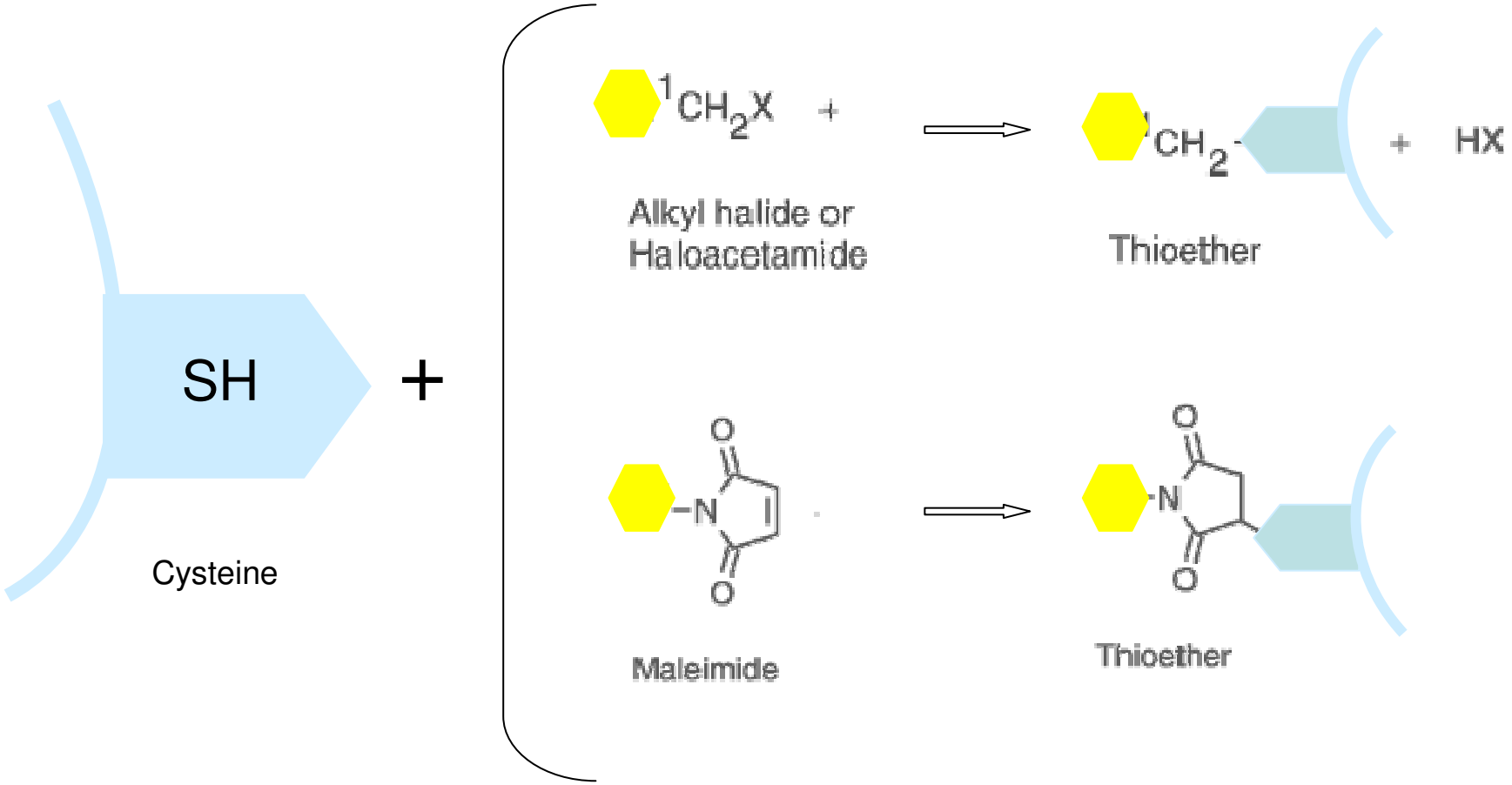
Pyrenebutyric acid
(340-376), $\tau \approx$ ns

etc…..

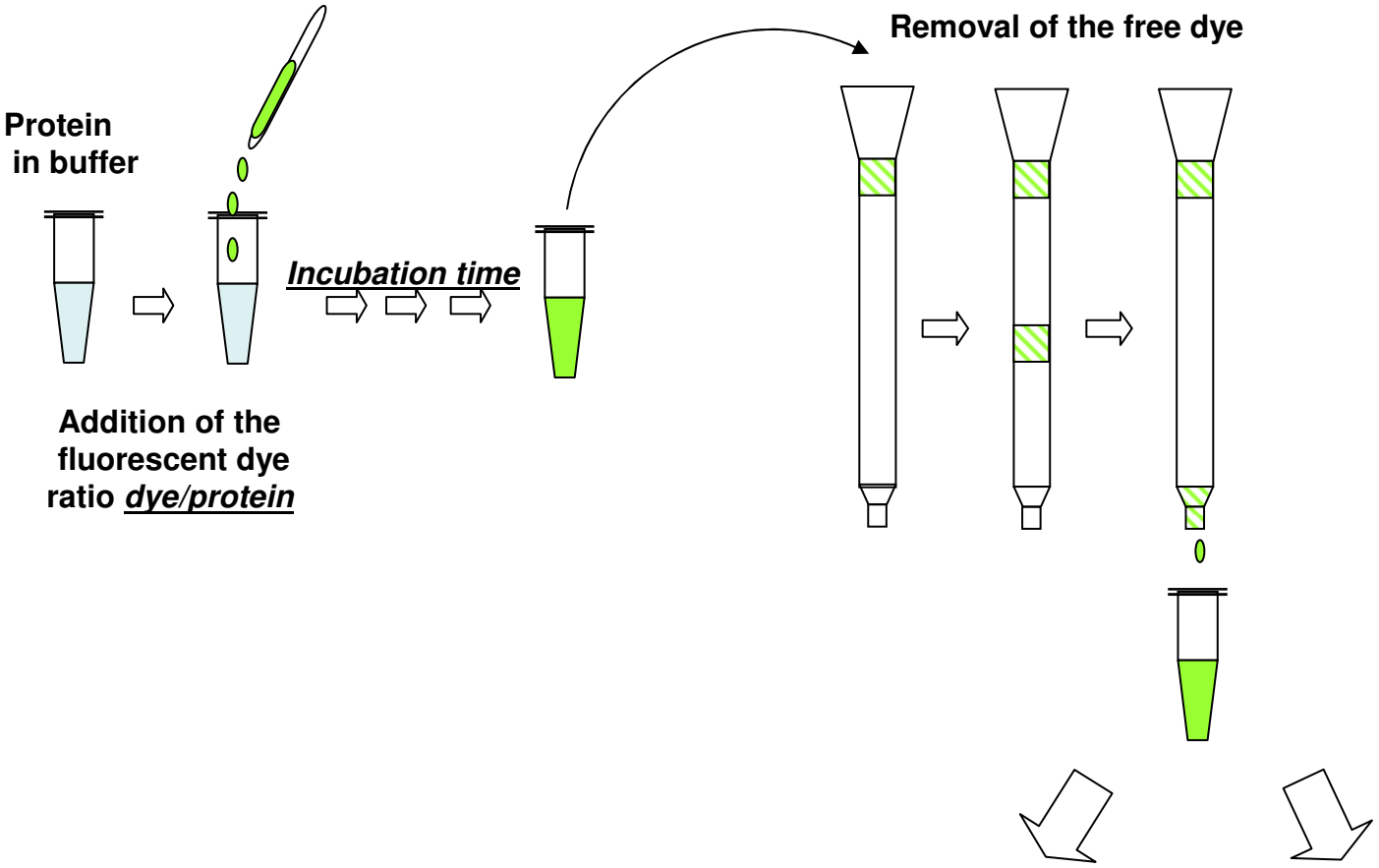
Targeting amino groups



Targeting thiol groups:



General labeling protocol for extrinsic labeling



Labeling ratio

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

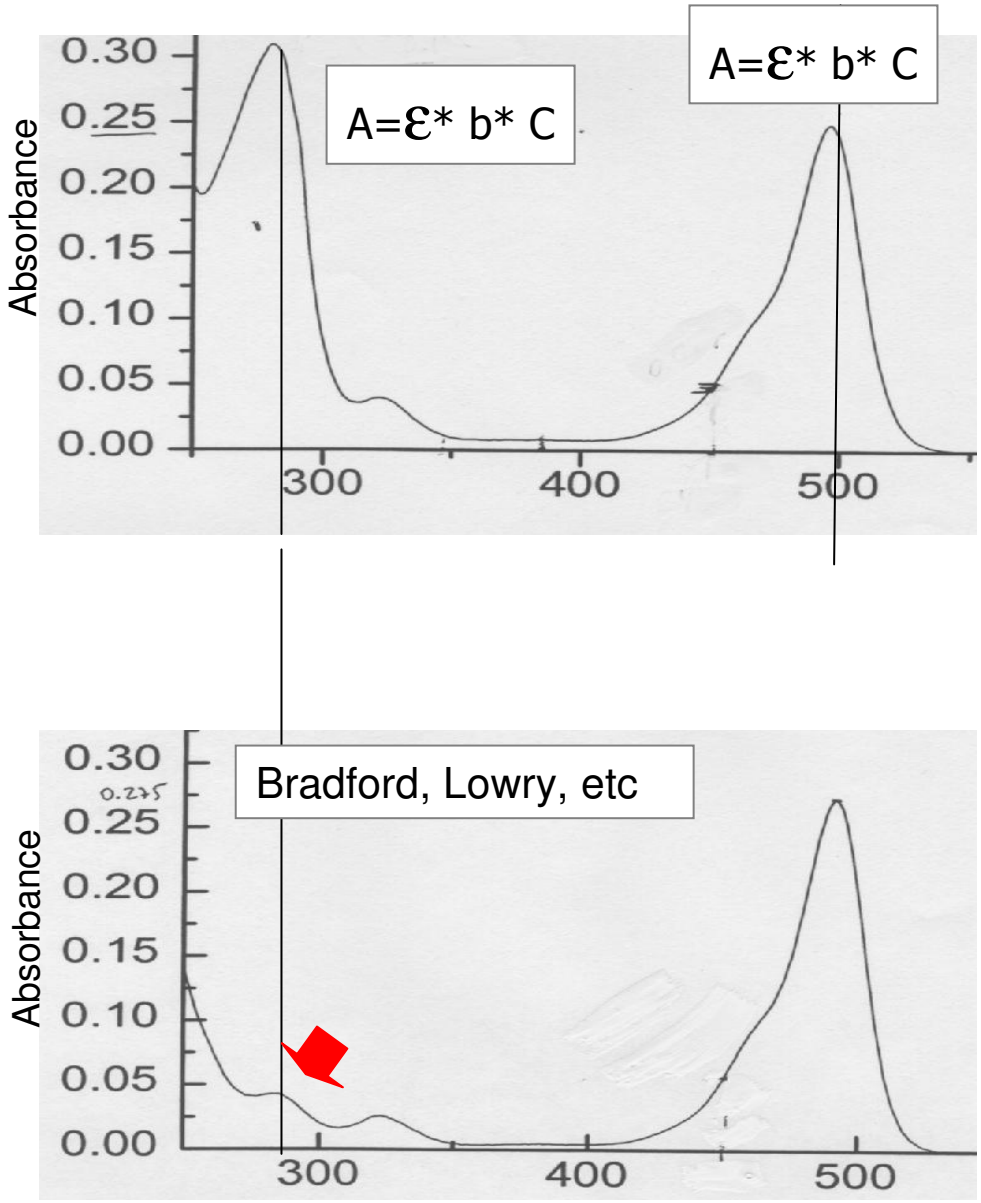
Sample characterization

Absorption spectra
Protein determination

Biological testing

Activity measurements
SDS or native gel
Denaturation exp etc.

Characterization after the labeling Absorption spectra



Protein-Fluorescein

Fluorescein

Fluorescent proteins

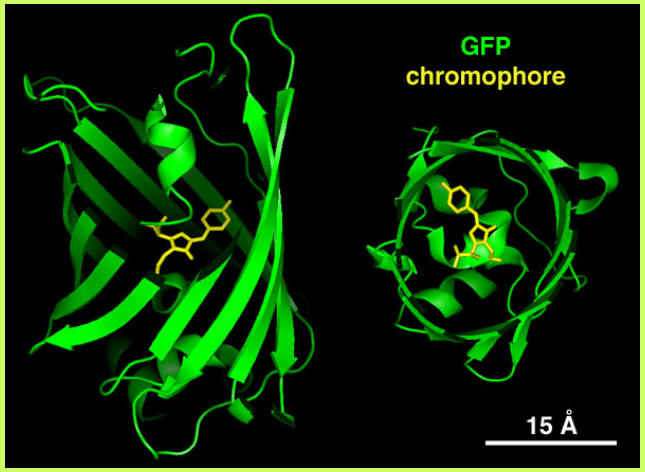
Phycobiliproteins

- From red algae and cyanobacteria (blue-green algae).
- Absorb strongly between 470 and 650 nm.
- In vivo they are only weakly fluorescent, due to efficient energy transfer to photosynthetic reaction centers.
- Highly fluorescent *in vitro*.

Four main classes of phycobiliproteins.

Protein	Subunit Composition	Approx. mol. wt.	ε (M^{-1} cm^{-1})	Total bilins per protein	λ_{abs}^{max} (nm)	λ_{em}^{max}(nm)
Allophycocyanin	$(\alpha\beta)_3$	100,000	700,000	6	650	660
R-Phycocyanin	$(\alpha\beta)_3$	110,000	1,000,000	9	555, 618	642
B-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,400,000	34	543, 562	576
R-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,200,000	34	495, 536, 565	576

Fluorescent Protein (FP)- example GFP



- From the bioluminescent jellyfish *Aequorea victoria*.

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

- The chromophore is formed spontaneously (*from Ser-65, Tyr-66, Gly-67*) upon folding of the polypeptide chain, without the need for enzymatic synthesis.

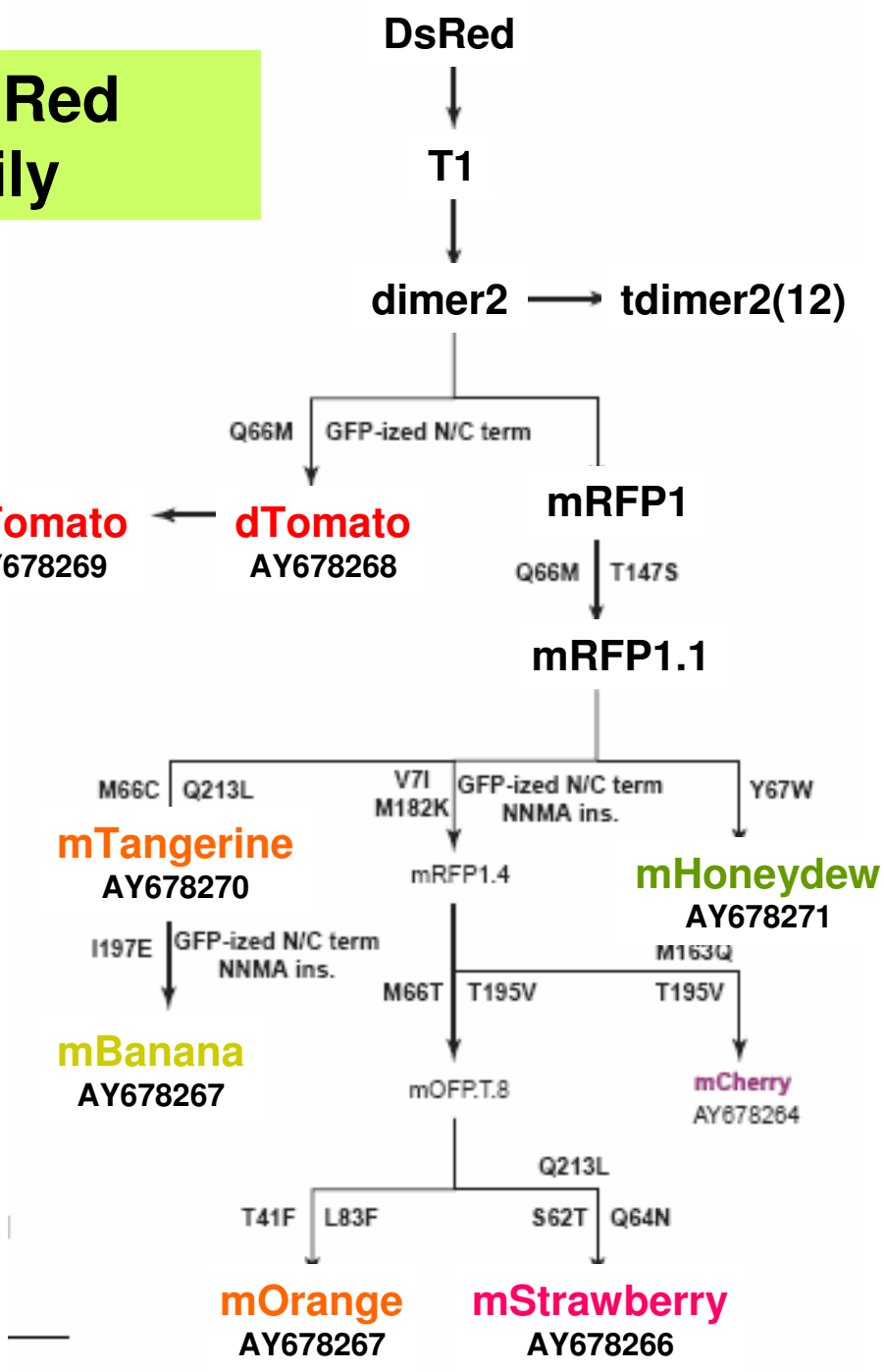
- Therefore, it is possible to insert the gene for GFP into cells and use the resulting protein as a reporter for a variety of applications.

Ds Red fluorescent proteins and derivatives

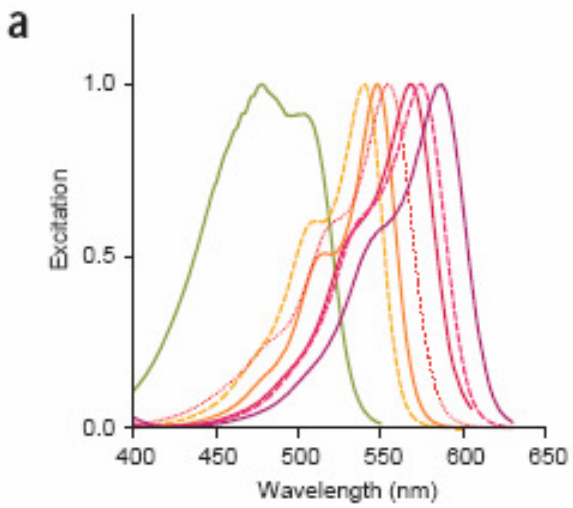
- Extracted from the Coral *discosoma sp*
- tetrameric
- mRFP is the improved monomeric form

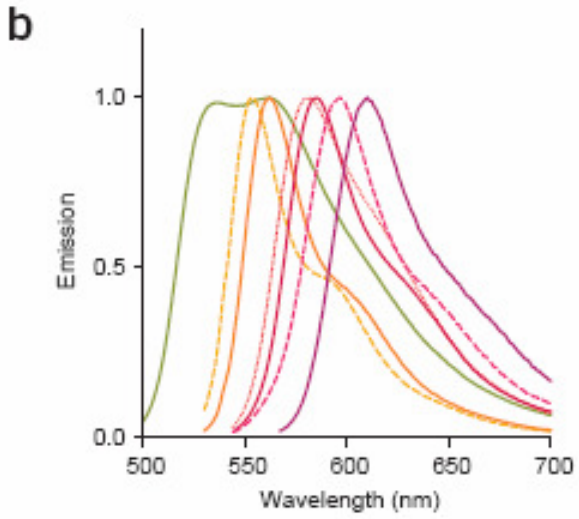
Mutants of DsRed form the mFruits proteins

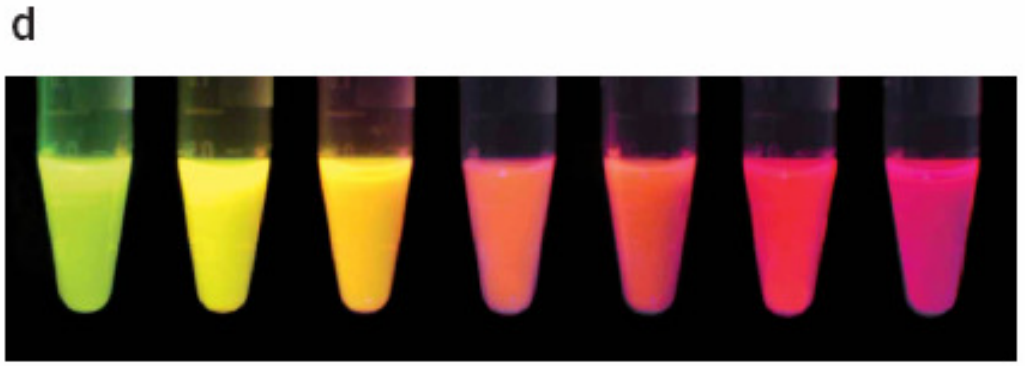
Mutants of Ds Red mFruits family



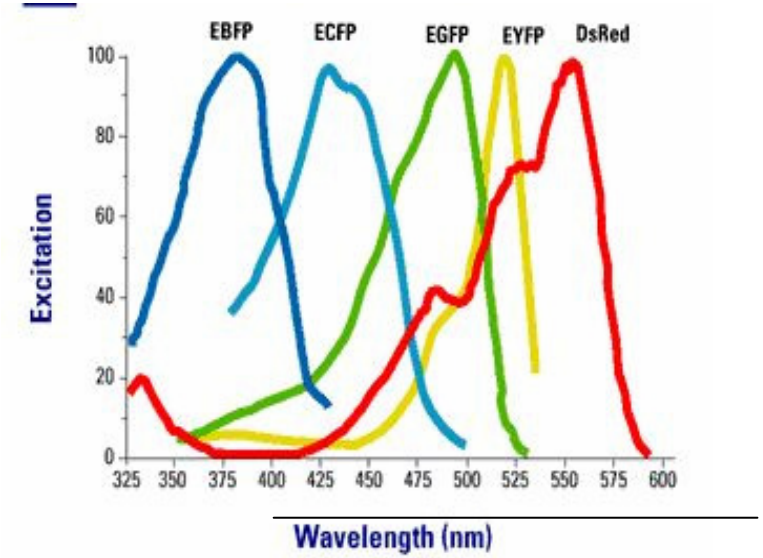
mFruits fluorescent proteins

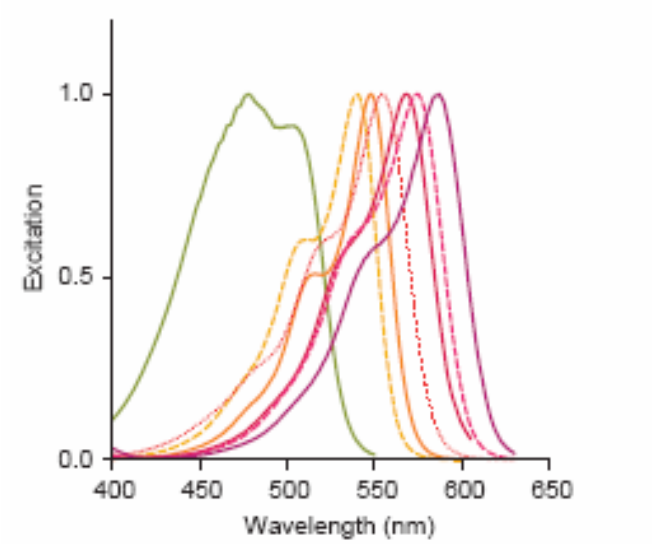


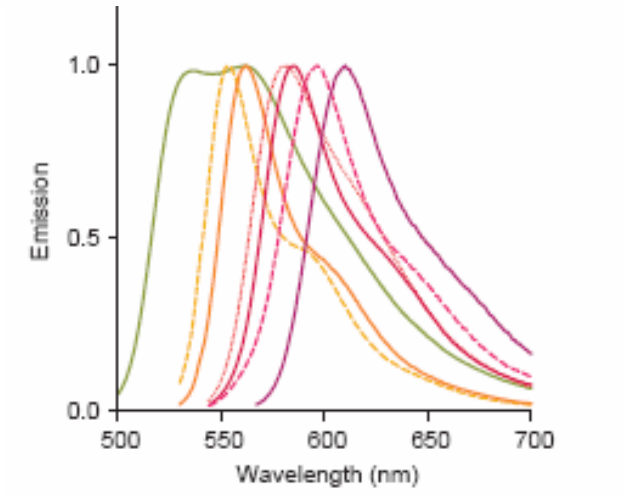




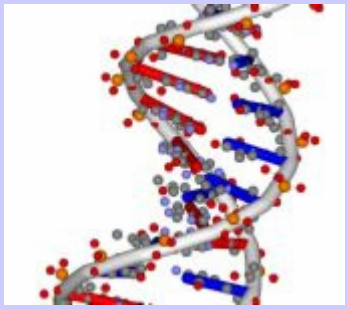
mFruits may replace or be good pairs for GFP in energy transfer experiments







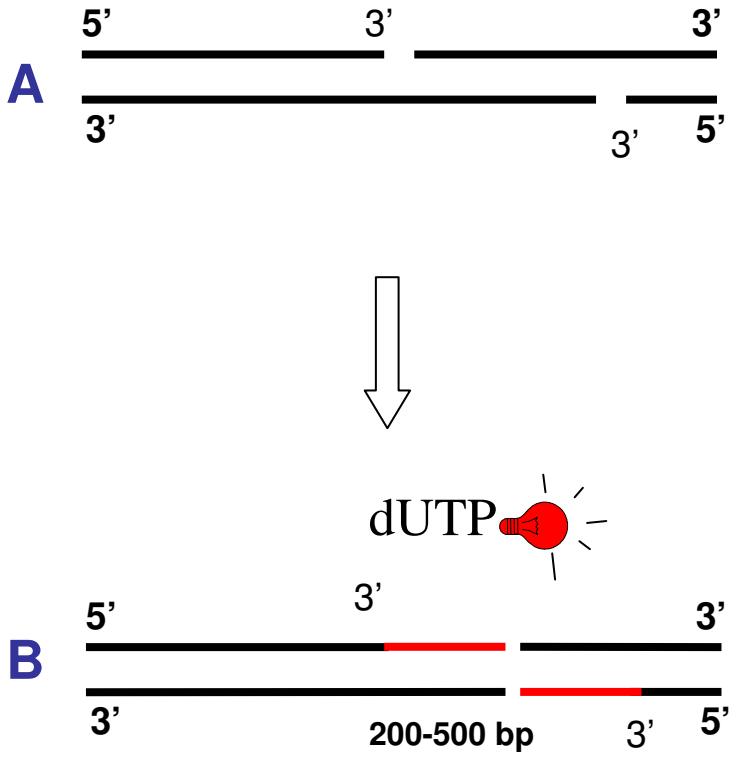
Labeling DNA



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

Nick translation

End labeling of fragments



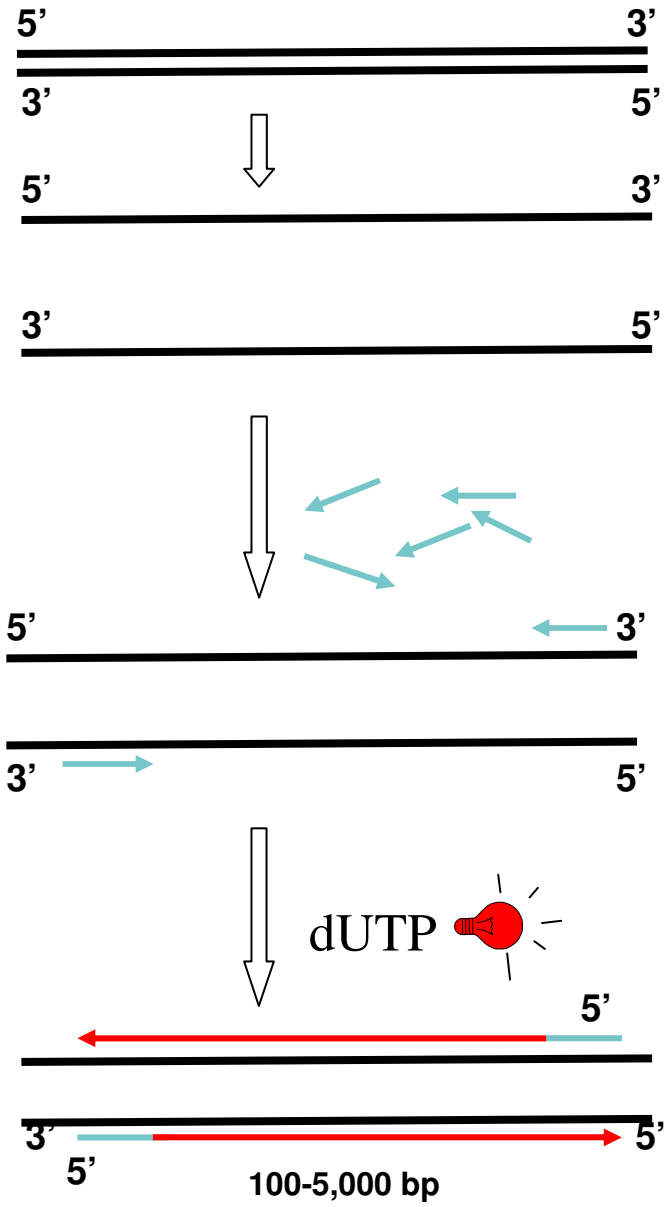
DNase I, which in the presence of Mg++ ions becomes a single stranded endonuclease creates random nicks in the two strands of any DNA molecule.

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)

Higher labeling efficiency by PCR. Requires decreased amount of probe.



30-40 cycles of 3 steps

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.

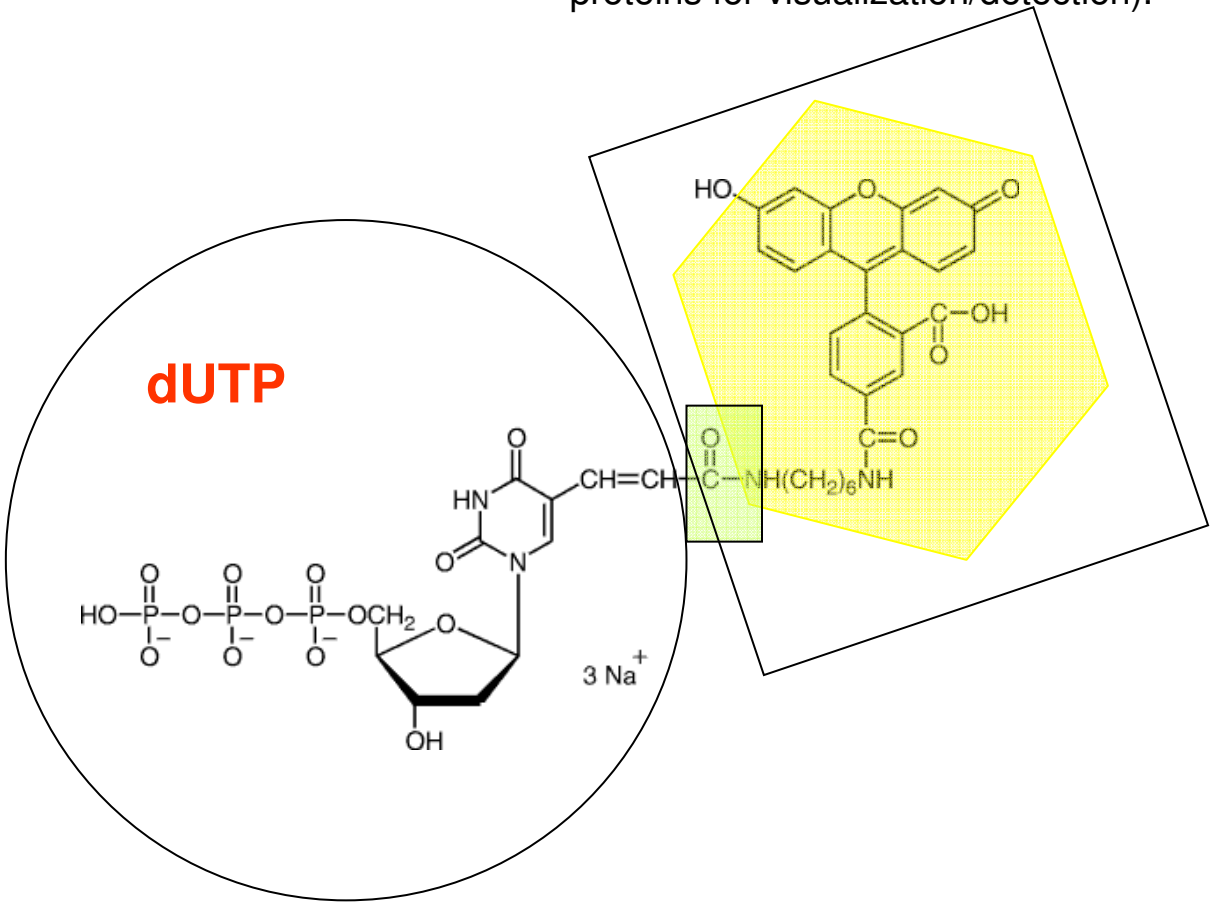
3- Extension (2min, 72ºC).
Then Taq polymerase synthesize the new DNA strands. Only dNTP's.

Commercially labeled dUTP

succinimidyl-ester derivatives of

1- fluorescent dyes

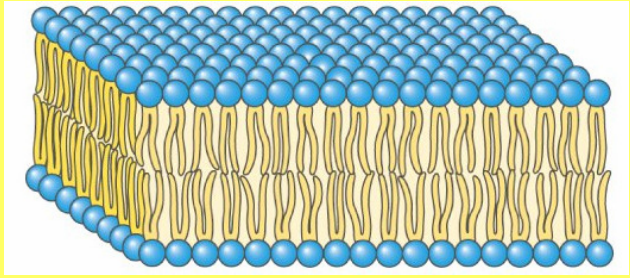
2- haptenes (Biotin, Digoxigenin, Dinitrophenyl - these require fluorescently-labeled antibodies or specific proteins for visualization/detection).



fluorescein-aha-dUTP from Molecular Probes

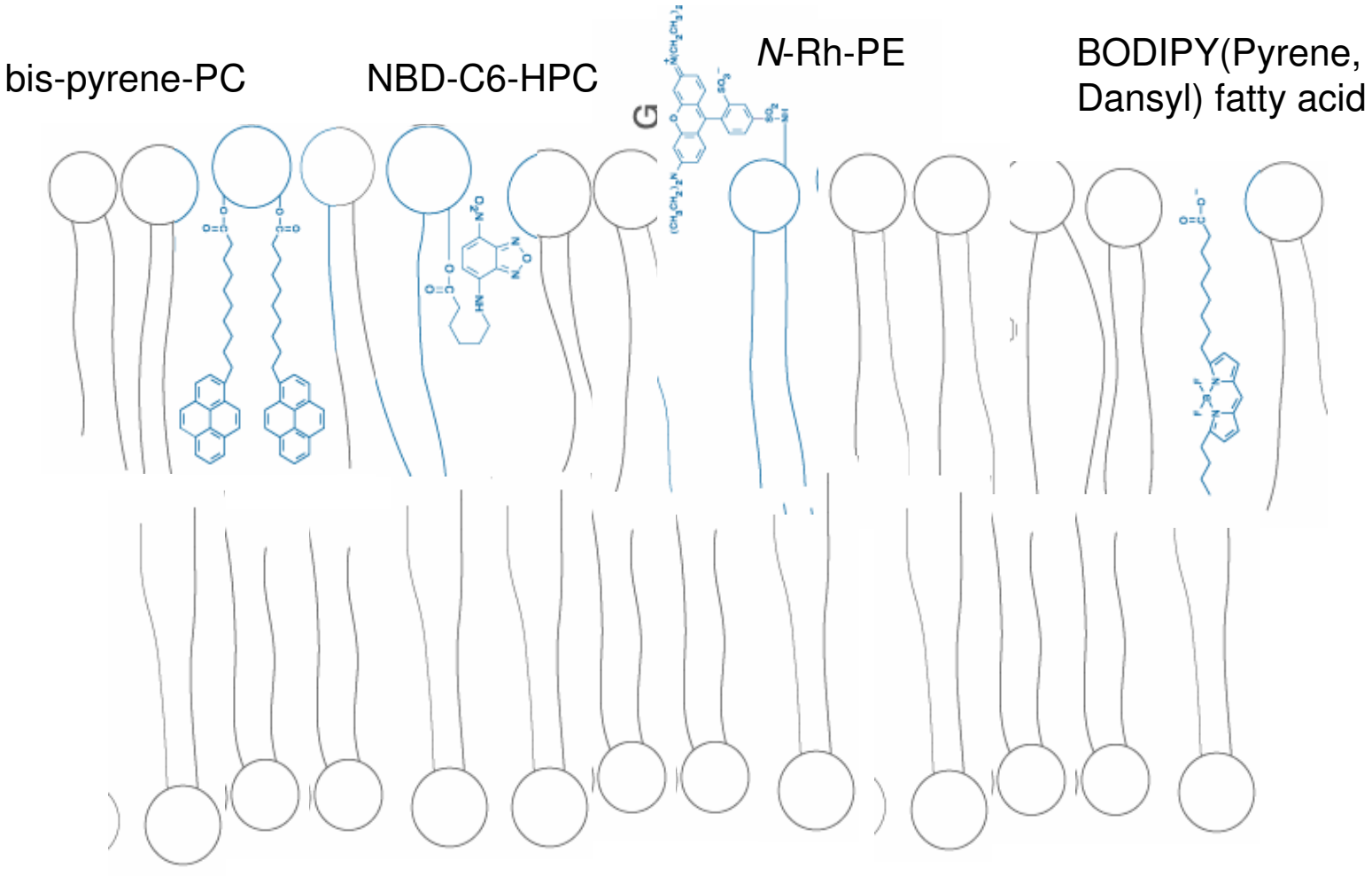
No	Dye	MW	Abs (nm)	Em (nm)
1	DAPI	-	350	456
	AMCA	450	353	442
	CB	600	396	410
2	DEAC	350	432	472
3	FITC	600	491	515
	OG-488	510	495	521
	A-488	650	493	517
	RGr	620	515	530
4	R6G	550	524	552
	Cy3	750	550	570
	TAMRA	640	547	573
5	TAMRA	640	547	573
	TxR	800	583	603
	Cy3.5	1100	581	596
6	Cy5	800	649	670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

Labeling membranes



- **Fatty acids analogs and phospholipids**
- **Sphingolipids, sterols,Triacylglycerols etc.**
- **Dialkylcarbocyanine and Dialkylaminostyryl probes.**
- **Other nonpolar and amphiphilic probes.**
  Laurdan, Prodan, Bis ANS

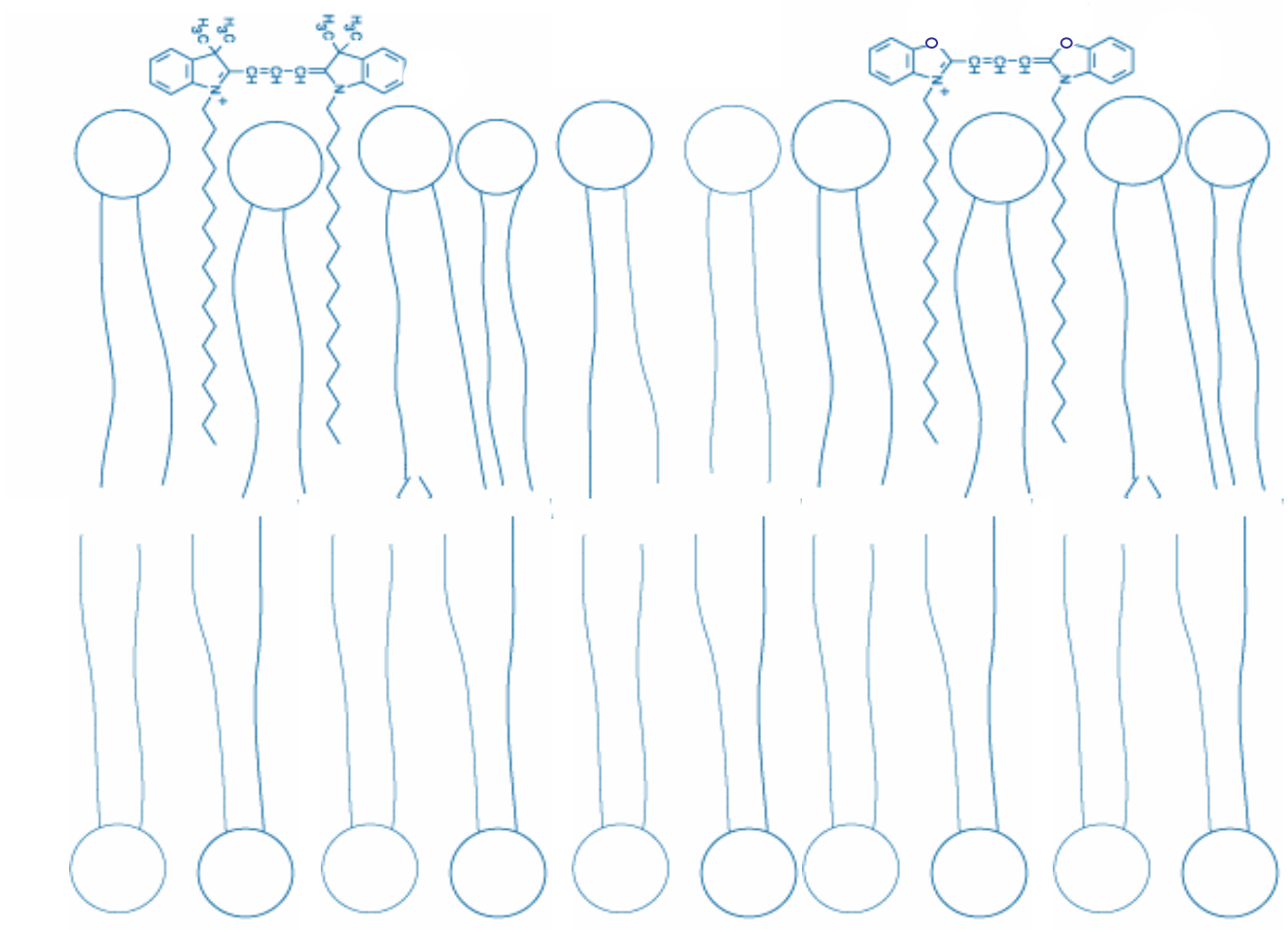
Fatty acids analogs and phospholipids



Dialkylcarbocyanine probes.

DilC18

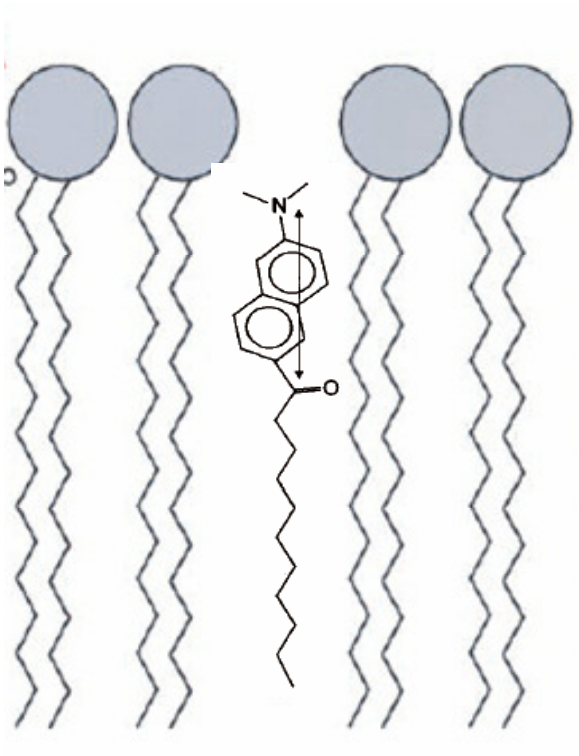
DiOC18

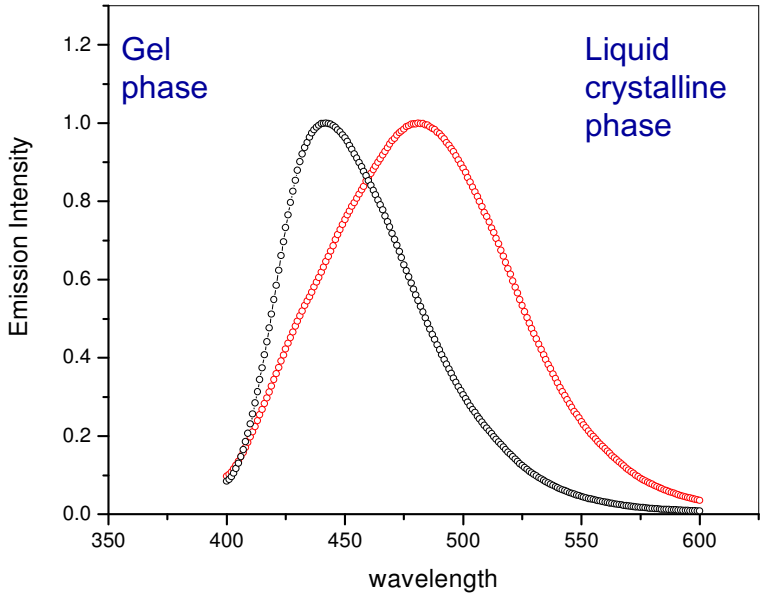


Nonpolar: *Laurdan.*

(environment-sensitive spectral shifts)

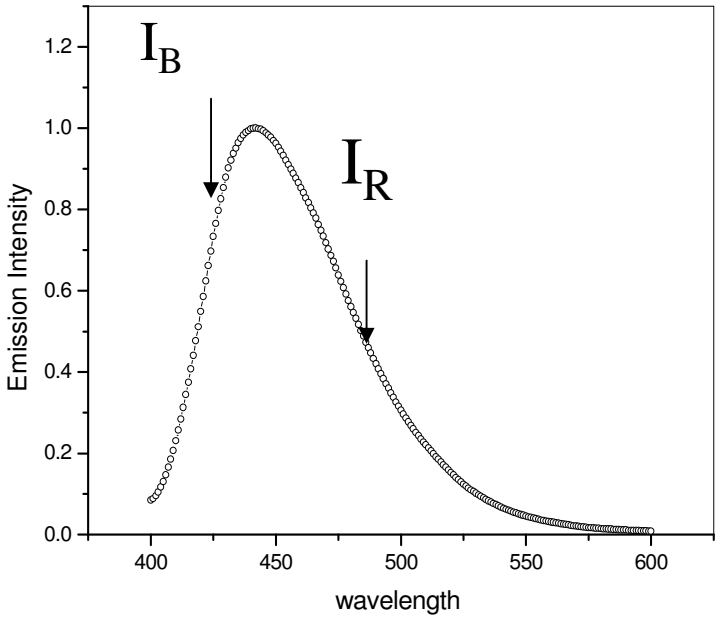
Weber, G. and Farris, F. J.Biochemistry, 18, 3075-3078 (1979) .





Laurdan Generalized Polarization (GP)

Ex=340 nm



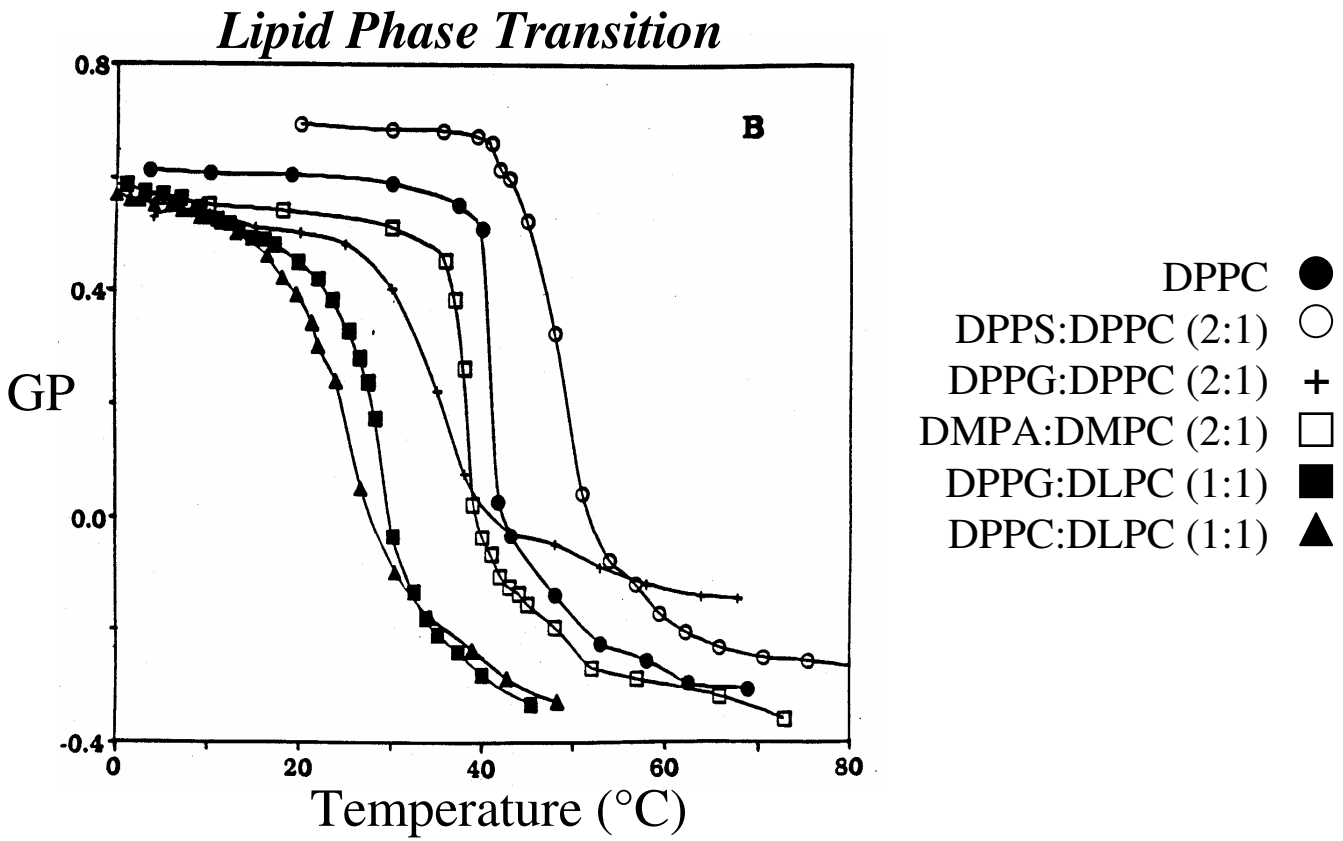
$$GP_{ex} = \frac{I_B - I_R}{I_B + I_R}$$

-0.2 loose lipid packing → 0.6 tight lipid packing

Parasassi, T., G. De Stasio, G. Ravagnan, R. M. Rusch and E. Gratton. Biophysical J., 60, 179-189 (1991).

GP in the cuvette

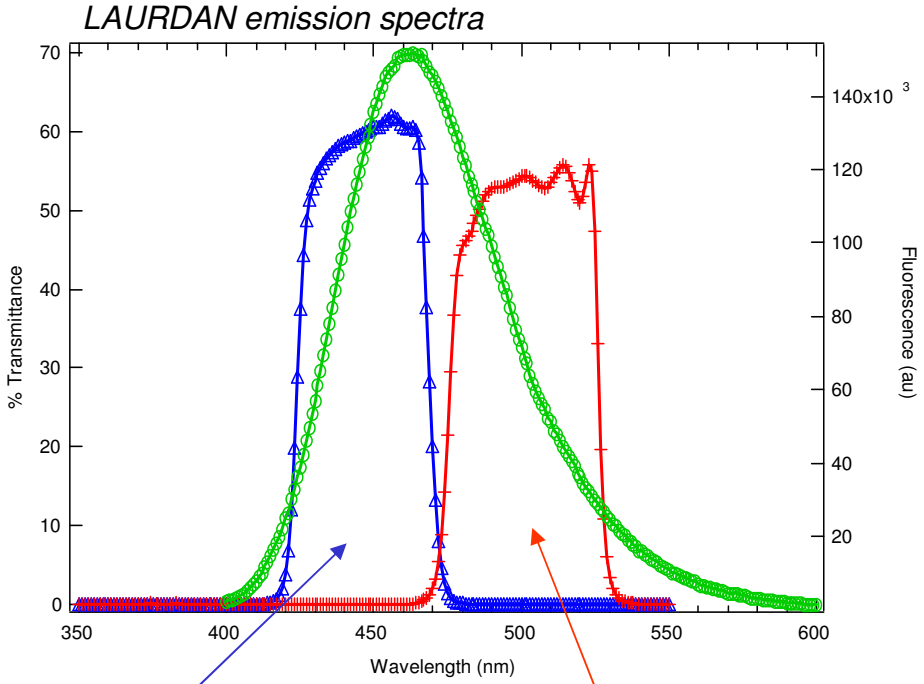
MLVs,SUVs,LUVs



Parassassi, Stasio, Ravaganan, Rusch, & Gratton (1991) Biophys. J. 60, 179

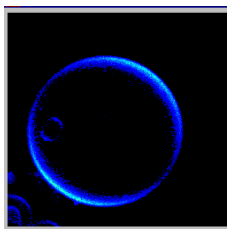
GP in the microscope

(2-photon excitation)

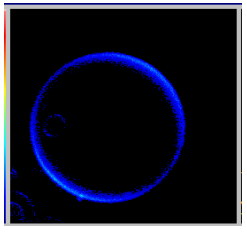


Measurement of Laurdan in the GUVs using SimFCS software

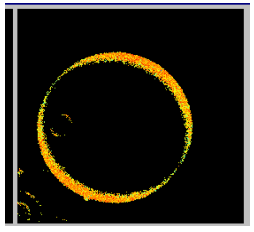
ch1
Blue filter



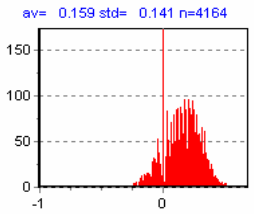
ch2
Red filter



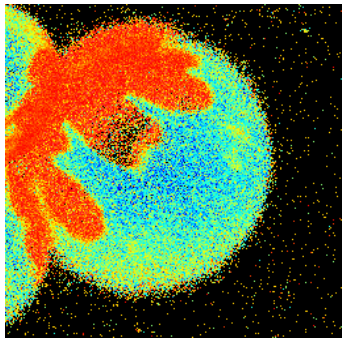
GP image

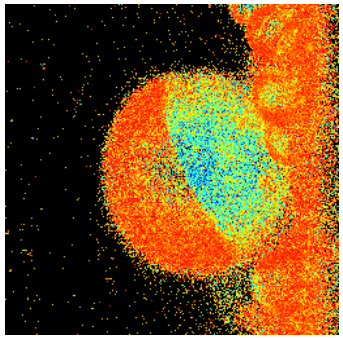


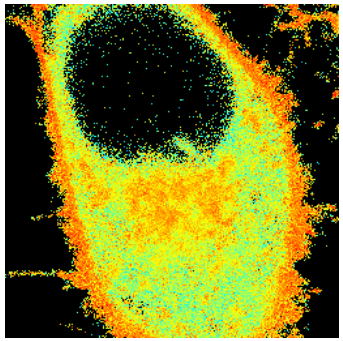
GP histogram

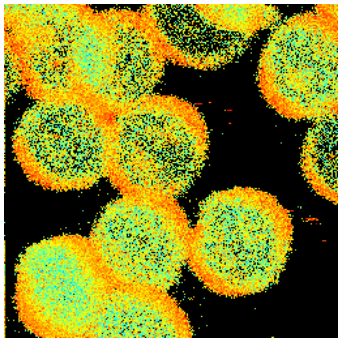


DOPC/DPPC 1:1mol/mol



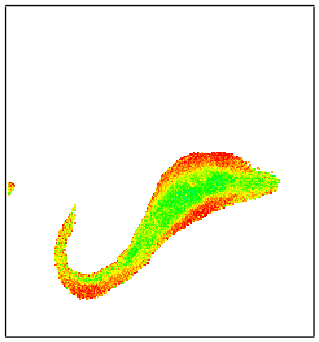






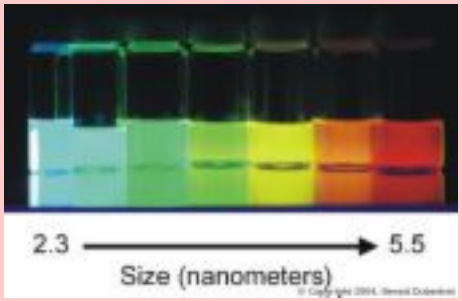
Hella

Erythrocytes



Living T. brucei (ec)

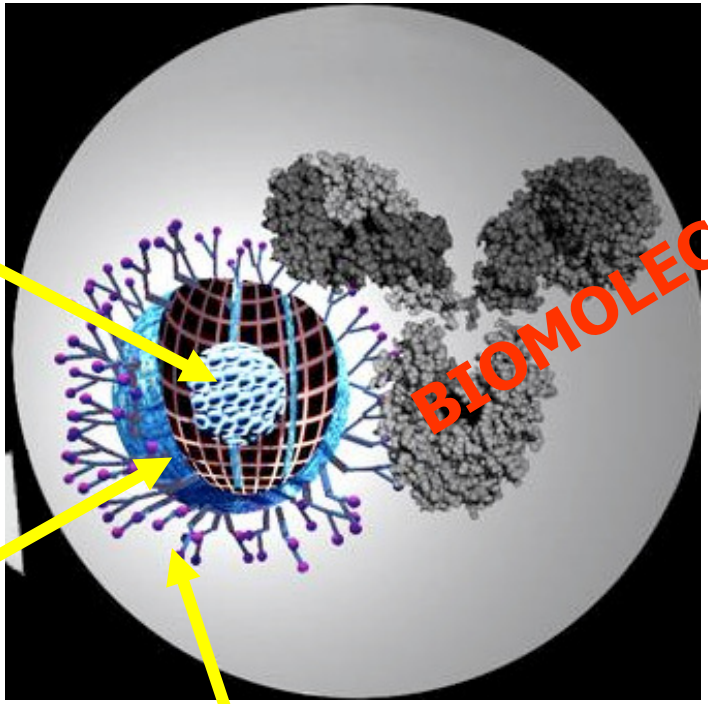
Quantum dots



CORE

cadmium sulfide (CdS), cadmium selenide (CdSe), or cadmium telluride (CdTe).

The semiconductor material is chosen based upon the emission wavelength, however it is the size of the particles that tunes the emission wavelength.

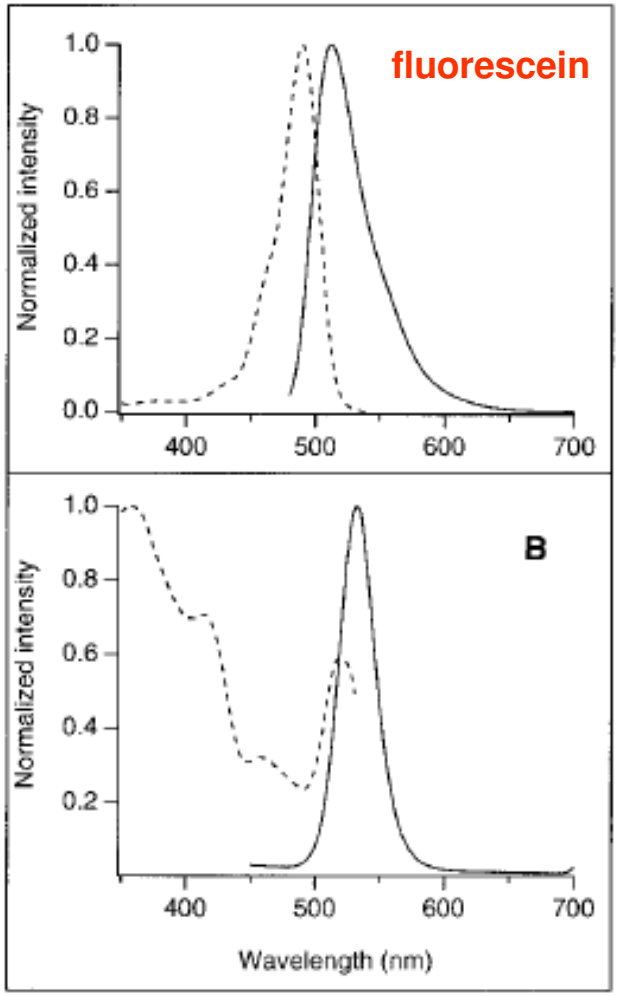


SHELL

In the cores emission is typically weak and always unstable.
The shell material (typically ZnS in Qdots) has been selected to be almost entirely unreactive and nearly completely insulating for the core.

COATING

a layer of organic ligands covalently attached to the surface of the shell.
This coating provides a flexible carboxylate surface to which many biological and nonbiological moieties can be attached.
The resulting surface is derivatizable with antibodies, Streptavidin, lectins, nucleic acids, and related molecules of biological interest.



nanocrystal (NC) sample in PBS

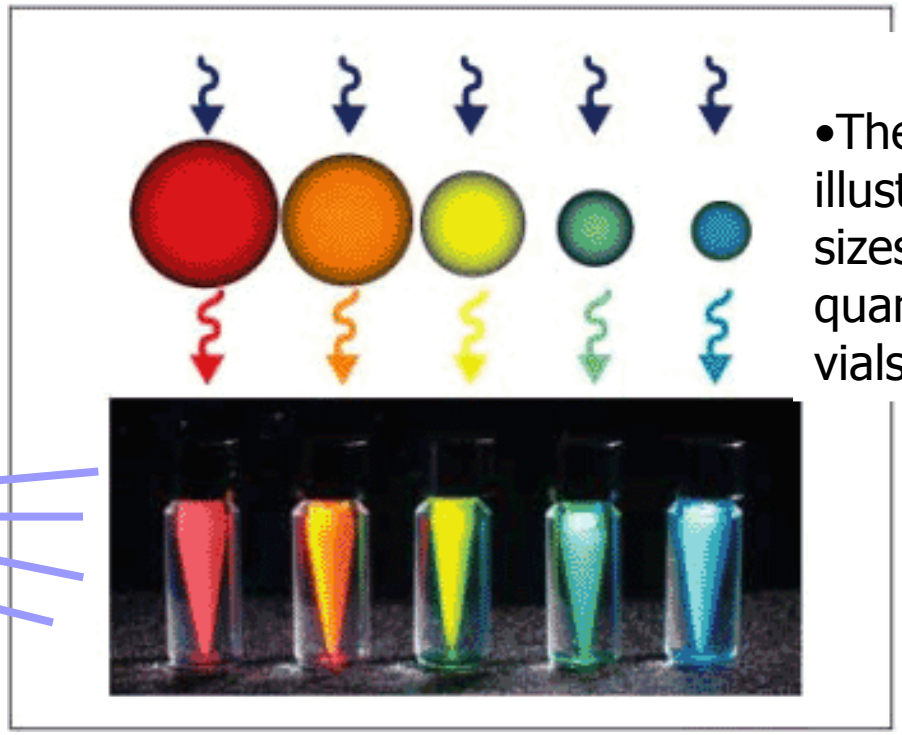
•**Q-dots:** broad absorption spectra, making it possible to excite all colors of QDs simultaneously with a single excitation light source….

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

•**Q-dots**: emission tunable according to the size and material composition, allowing closer spacing of different probes without substantial spectral overlap.

•**Q-dots:** exhibit excellent photo-stability.

The emission is tunable according to their size and material composition

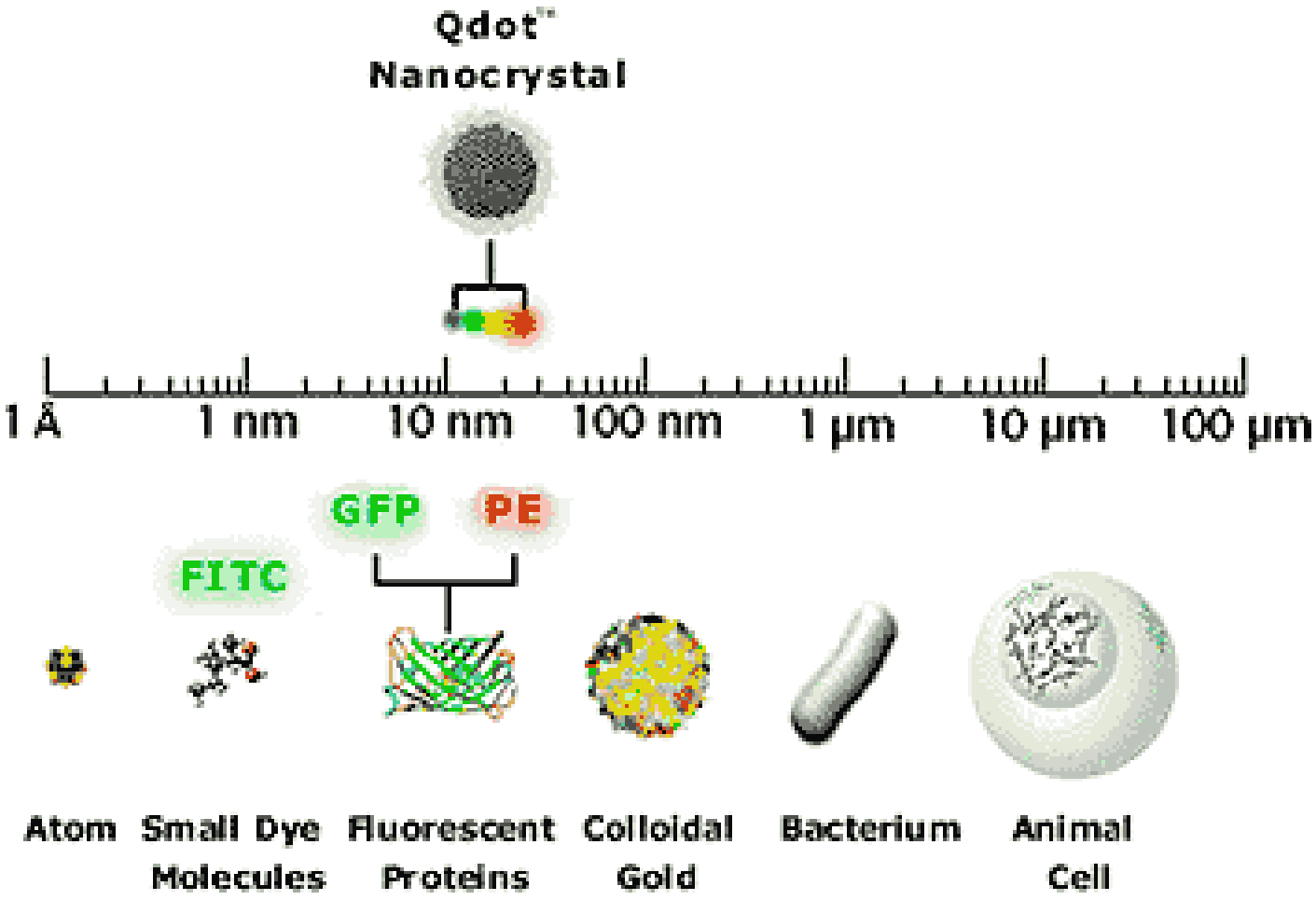


UV hand lamp.

•The colored spheres illustrate the relative sizes of the CdSe quantum dots in the vials.

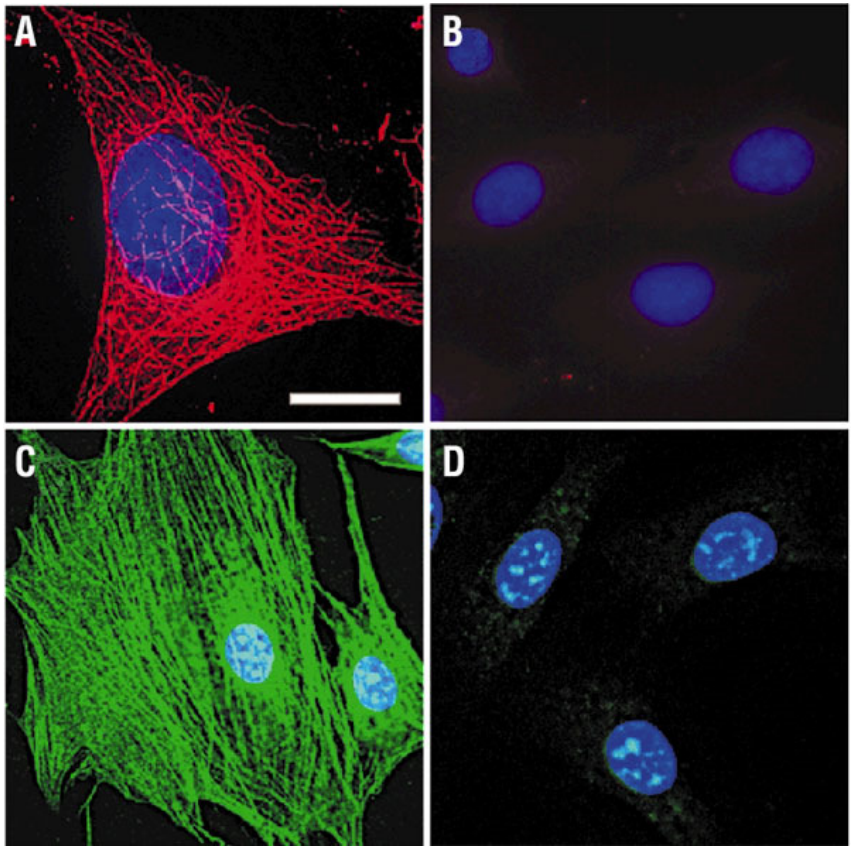
•All samples are induced to emit their respective colors even though a single source was used to excite them.

Quantum Dot Size



Example

Wu et al. Nature Biotechnology **21**, 41 - 46 (2002)



(A) Microtubules were labeled with
1-monoclonal anti-tubulin antibody.
2- biotinylated anti-mouse IgG and
QD 630−streptavidin (red).

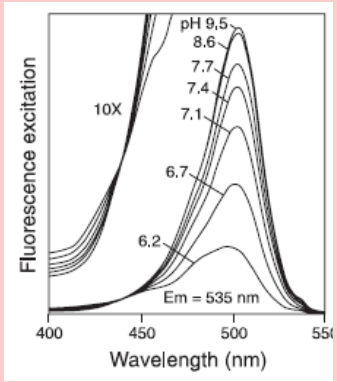
(B) Control for (A) without primary antibody.

(C) Actin filaments were stained with
1-biotinylated phalloidin and
QD 535−streptavidin (green).

(D) Control for (C) without biotin-phalloidin.

The nuclei were counterstained with Hoechst 33342 blue dye.

Ions indicators



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+} ***and others***

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

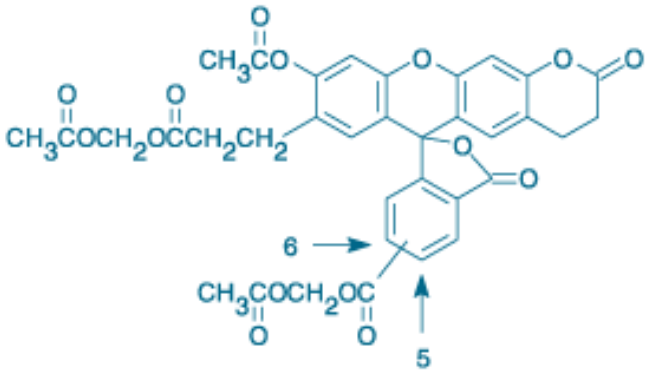
- Cell loading and distribution of the probe.
- Salt and dextran…microinjection, electroporation, patch pipette.
- AM-esters ….cleaved by intracellular esterases

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

Table 20.1 — Molecular Probes' pH indicator families, in order of decreasing pK_a

BCECF



II (Molecular Weight = 688.6)

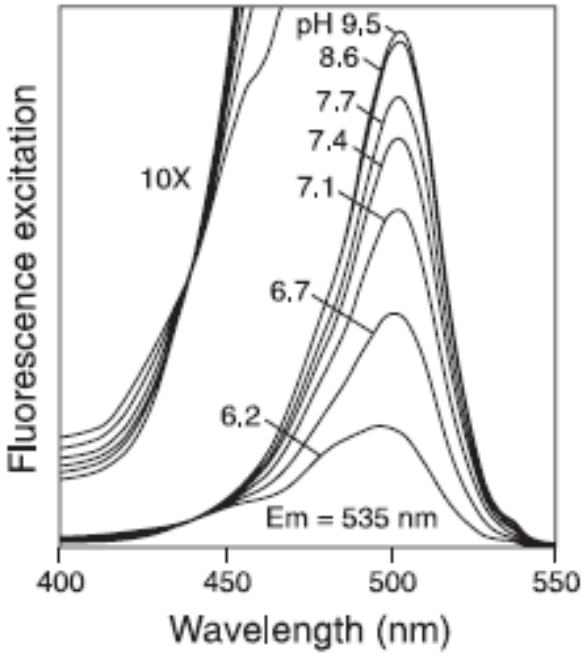
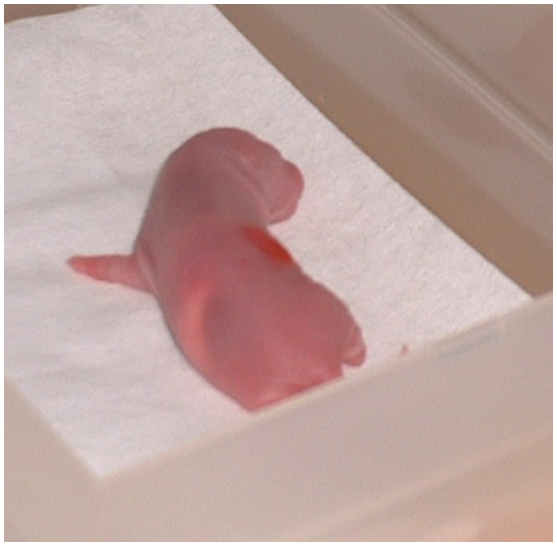


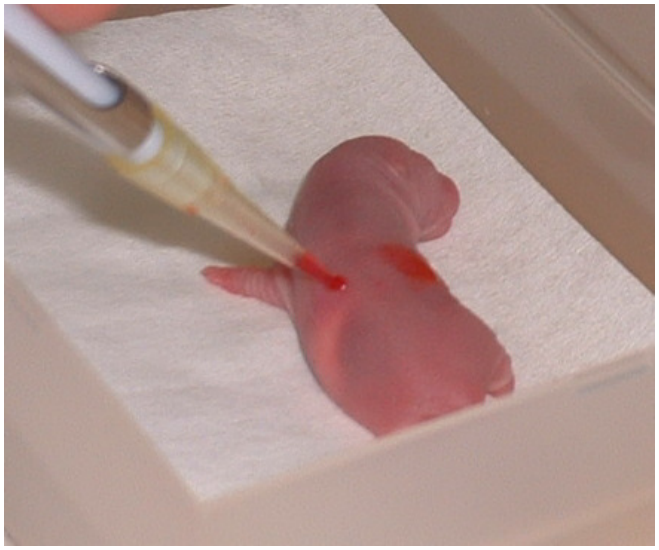
Figure 1. The pH-dependent fluorescence excitation spectra of BCECF. The 10X enlargements of the region below 470 nm clearly illustrate the excitation isosbestic point at ~439 nm.

In situ calibration: ionophore nigericin (N1495) at a concentration of 10~50 μM in the presence of 100~150 mM potassium (to equilibrate the intracellular pH with the controlled extra cellular medium)

Example 1

K.Hanson, M.J.Behne, N.P.Barry, T.M.Mauro, E.Gratton.
Biophysical Journal. 83:1682-1690. 2002.

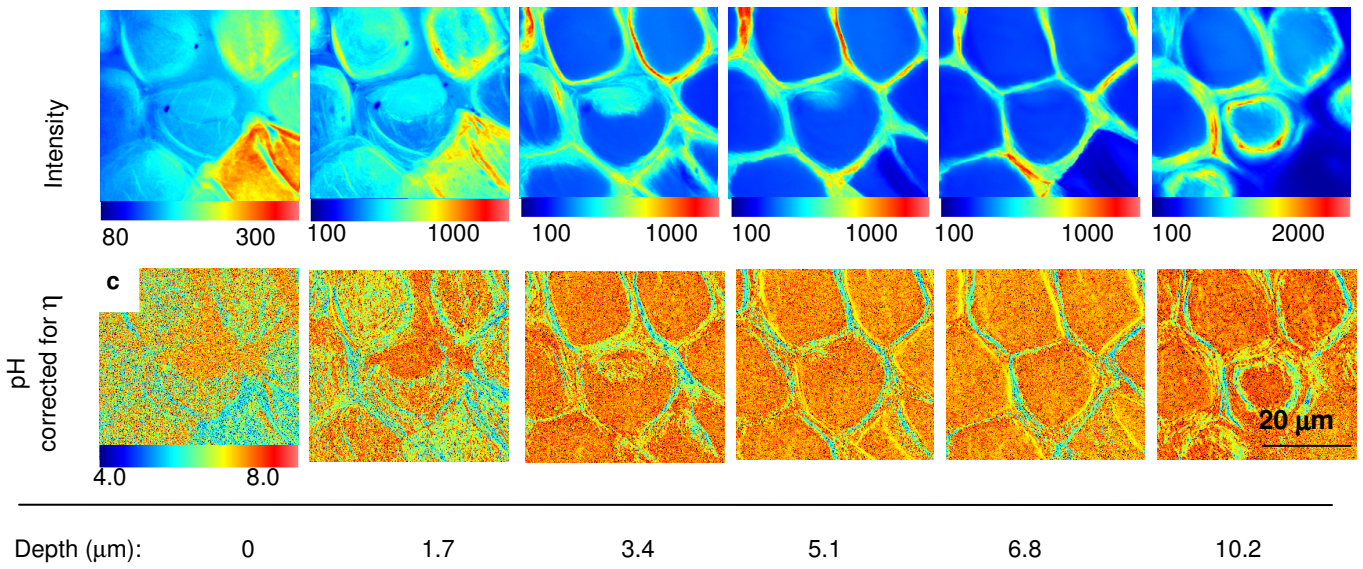


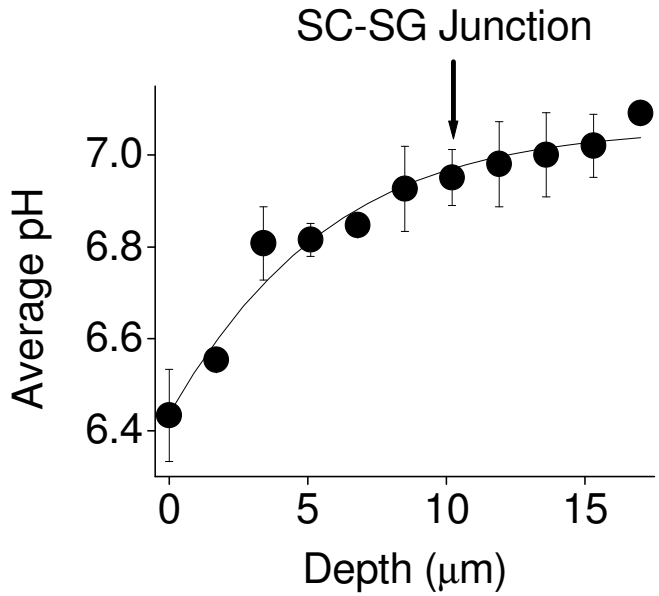


Dye in DMSO is applied to the a live animal and incubated.

Labeled skin is removed

imaging





K.Hanson, M.J.Behne, N.P.Barry, T.M.Mauro, E.Gratton. 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)

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 (OgB-1, OgB-6F, OgB-5N, OgB-2)

Cameleon system

Ratiometric: 2 excitation /1emission

FURA-2

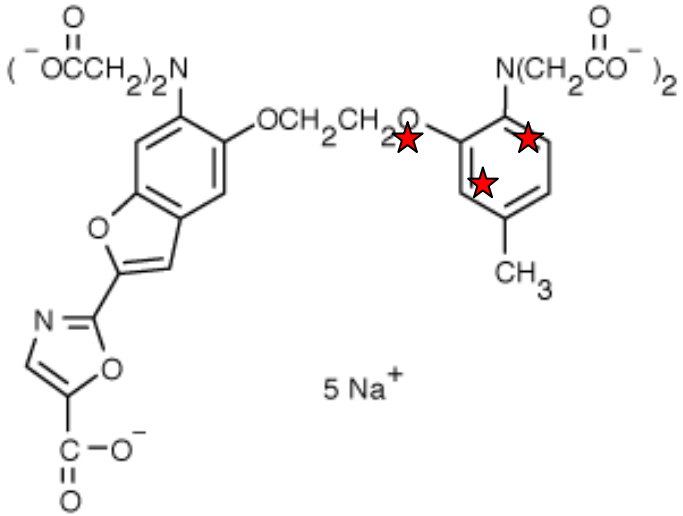
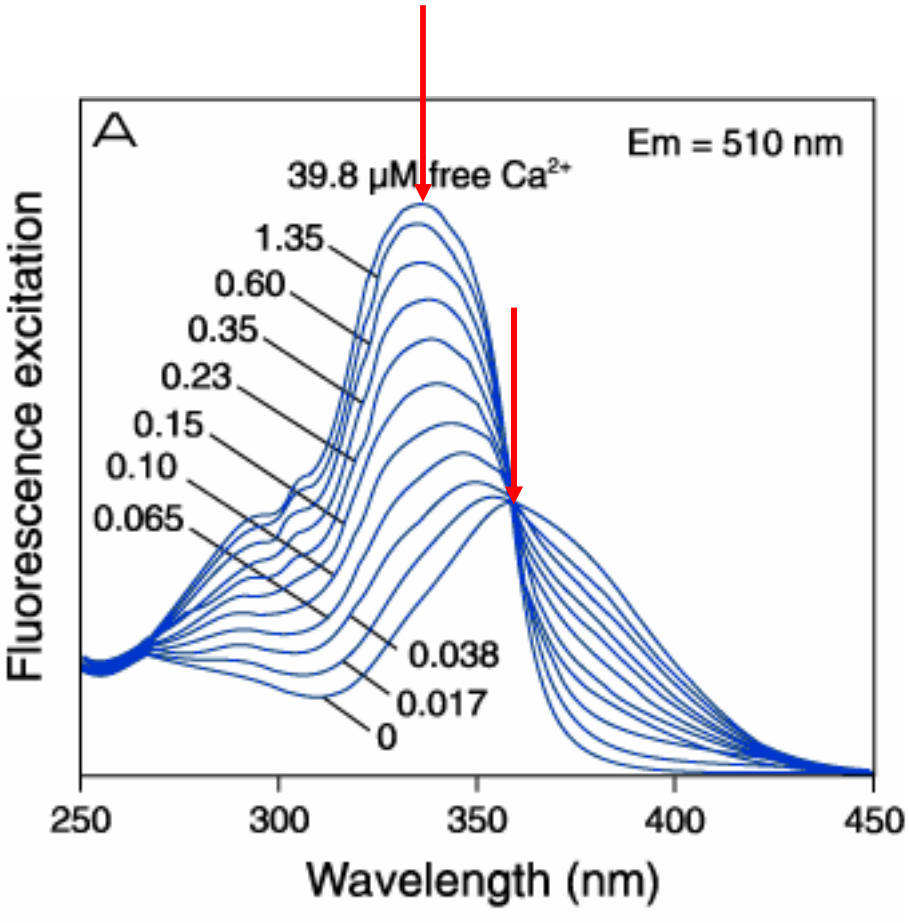


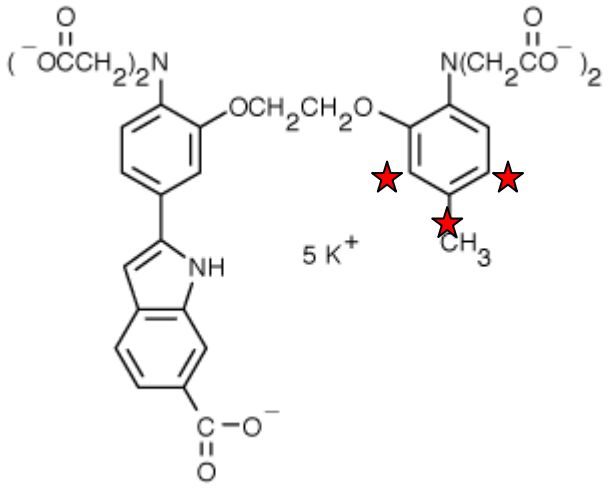
Table 1. Fura-2

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	35 μM

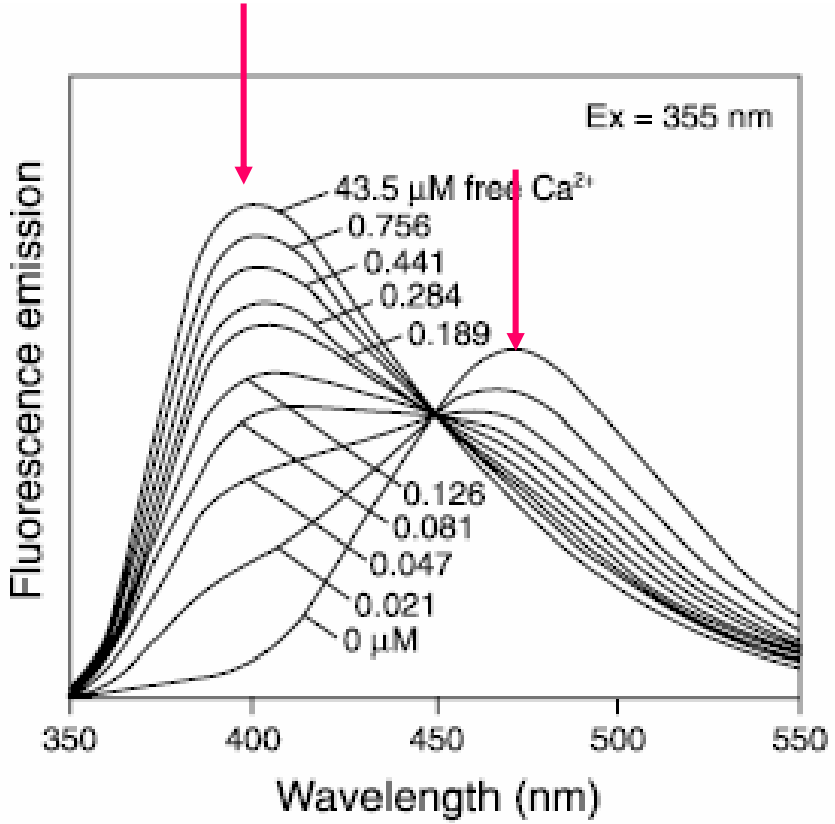


Ratiometric: 1excitation /2emission

Indo-1

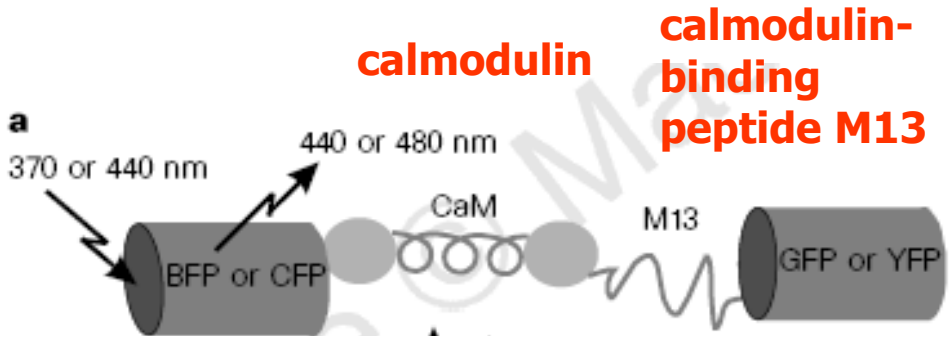


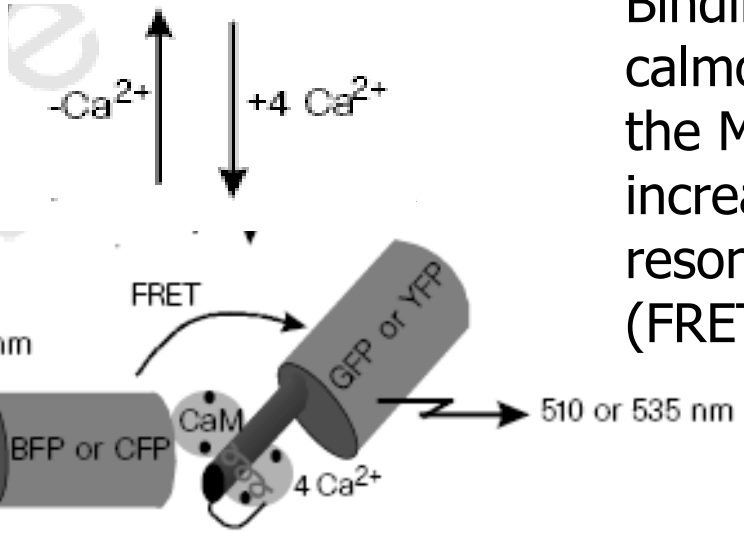
Indicator	K_d (Ca^{2+}) (μM)
indo-1	0.23
indo-5F	0.47



Cameleon construct

A. Miyawaki, J. Llopis, R. Heim, J. M.McCaffery, J. A. Adams, M. Ikura, R.Y. Tsien. Nature, 1997: 28: 834-835.



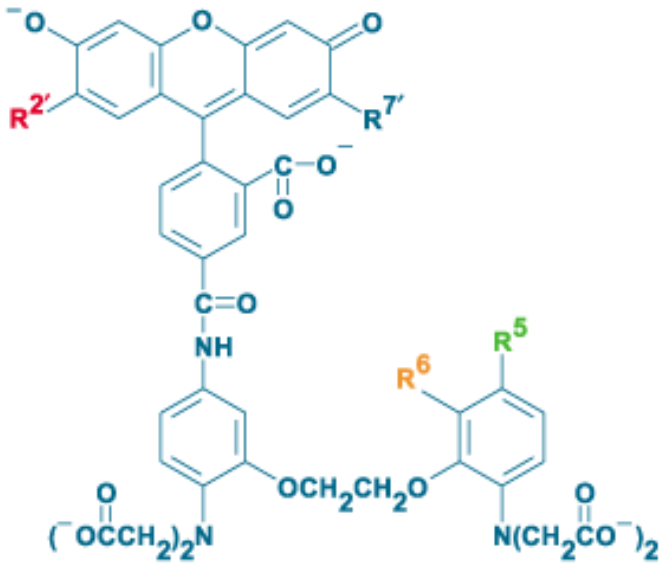


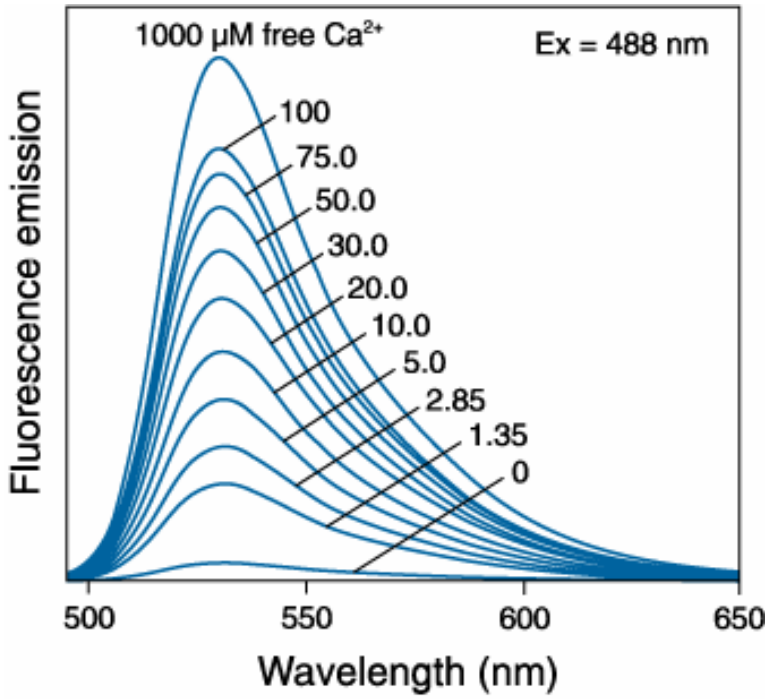
Binding of Ca^{2+} makes calmodulin wrap around the M-13-domain, increasing the fluorescence resonance energy transfer (FRET) between the GFPs.

Example 2

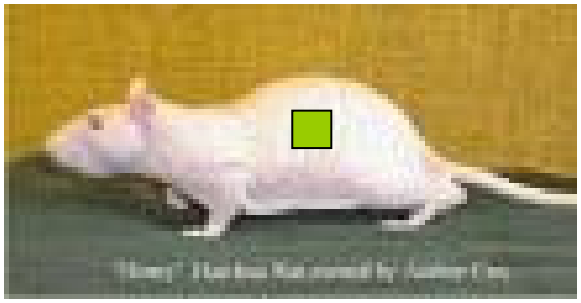
Martin Behne. University Medical Center. Hamburg, Germany.

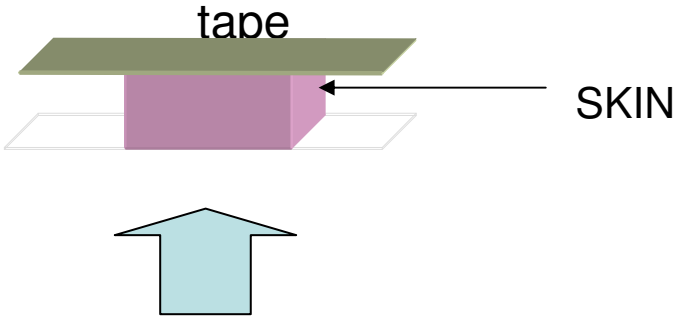
Calcium Green-5N



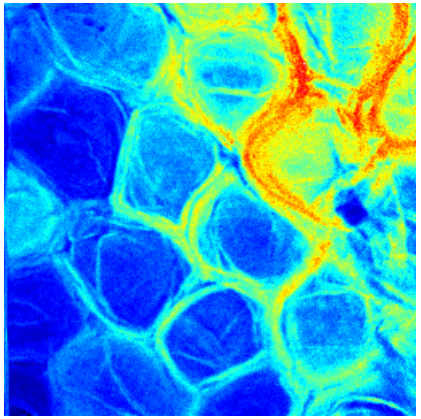


Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Calcium Green-5N	14 µM	Cl	Cl	NO_2	H

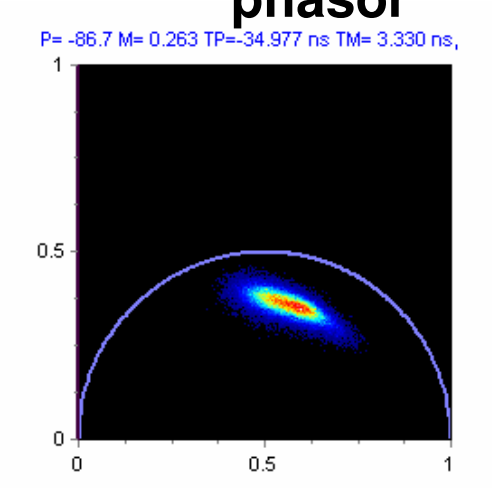


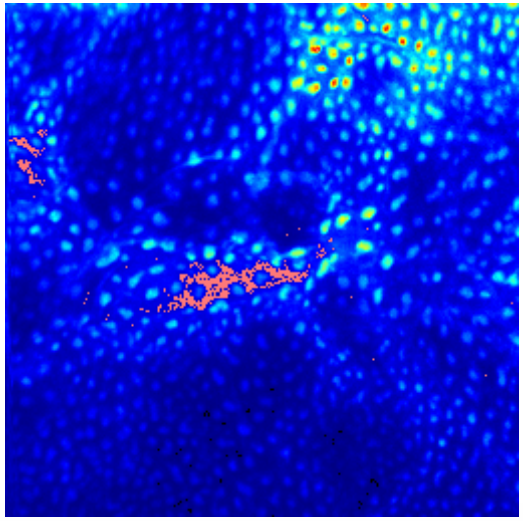


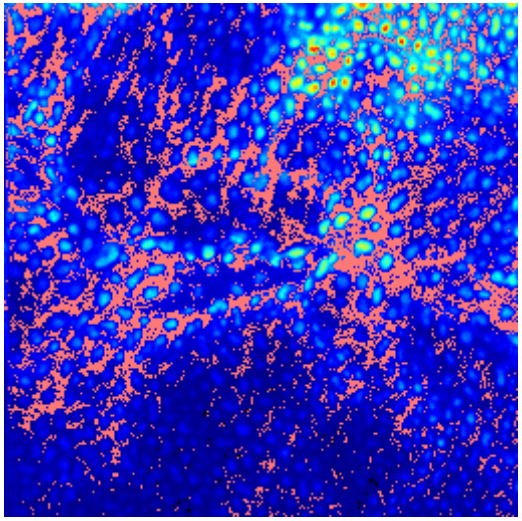
intensity image

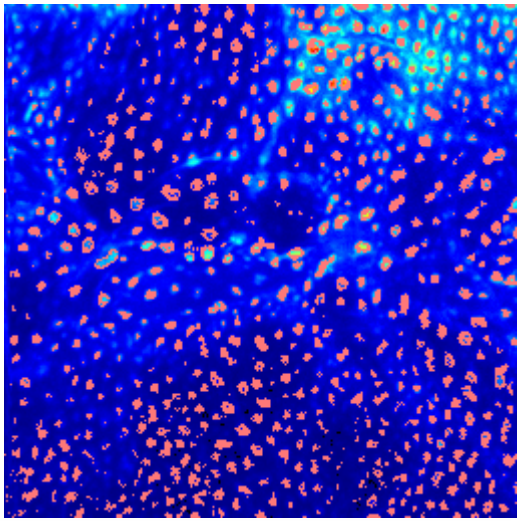


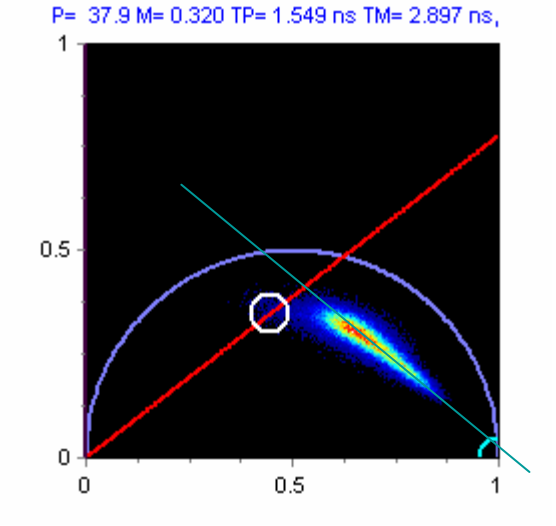
phasor







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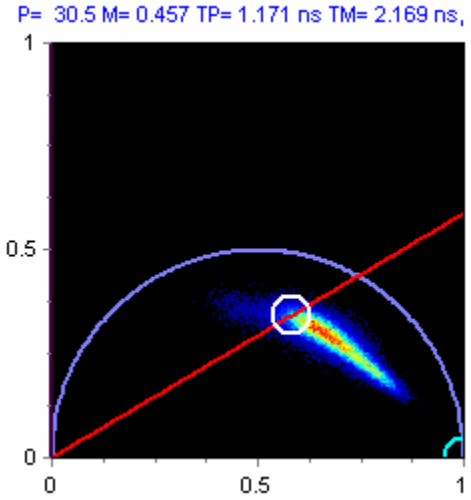
0
0.5
1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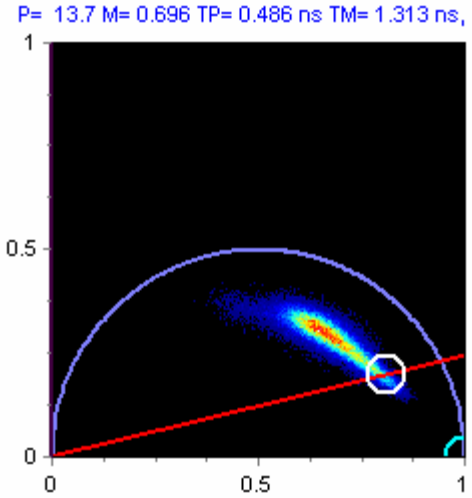
0.5
0
0
0.5
1


P= 13.7 M= 0.696 TP= 0.486 ns TM= 1.313 ns,
1
0.5
0
0
0.5
1

Labeling *“in vivo”*

Genetic Incorporation

GFP

FLAsh

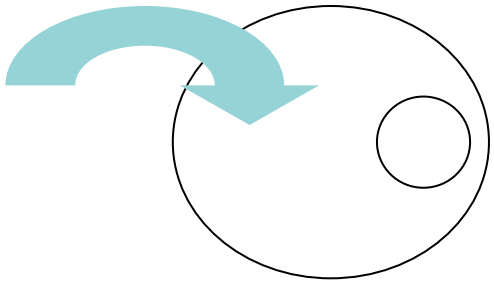
Mechanical incorporation

Labeled proteins

Labeled DNA

Q-dots

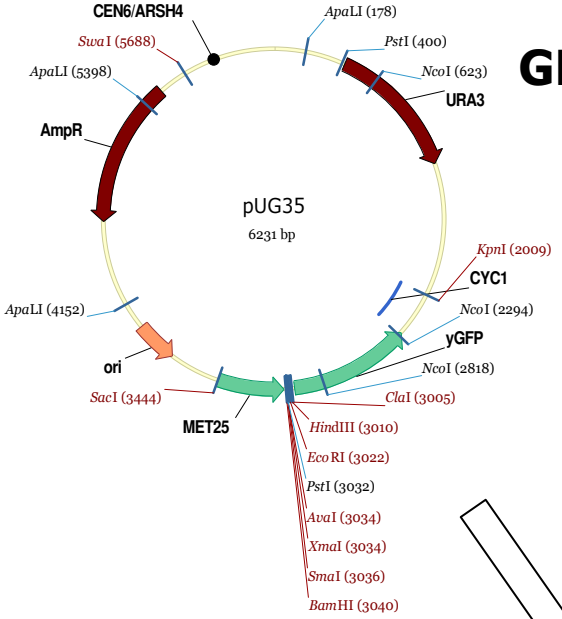
Genetic material



GFP-fusion proteins

GFP

GFP encoding plasmid



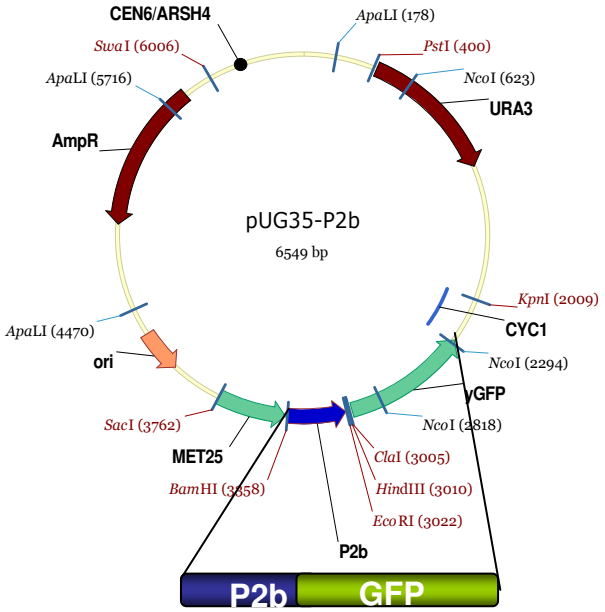
Your gene

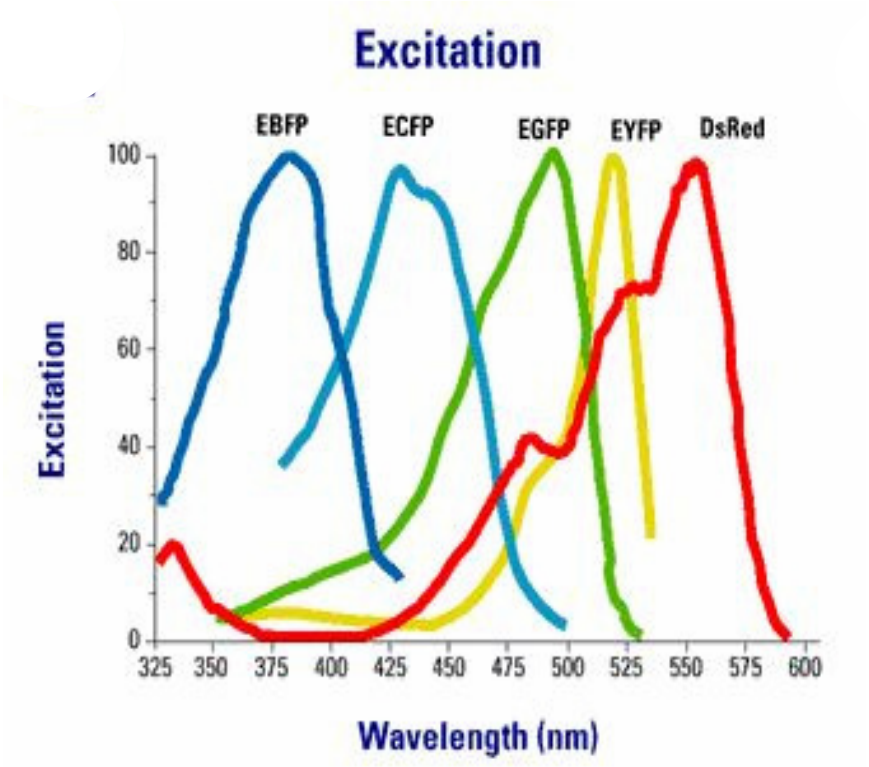
P2b

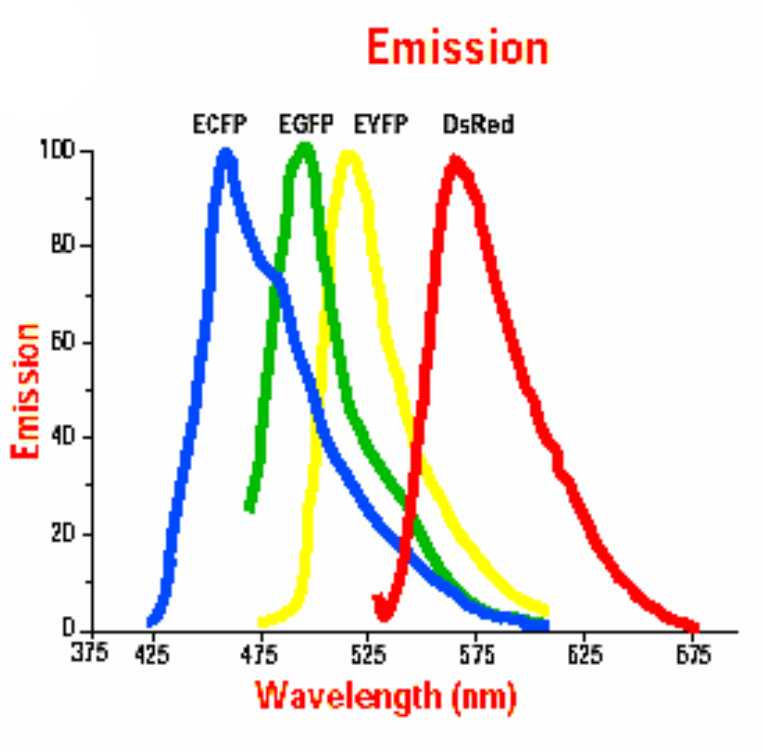
(example. P2b)

Introduction into

different organisms




Excitation
EBFP
ECFP
EGFP
EYFP
DsRed
Excitation
100
80
60
40
20
0
325 350 375 400 425 450 475 500 525 550 575 600
Wavelength (nm)


Emission
ECFP
EGFP
EYFP
DsRed
Emission
100
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20
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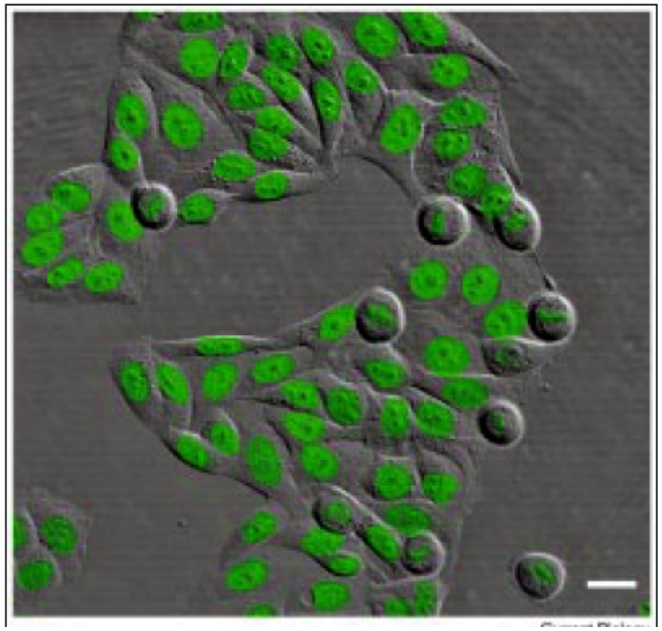
375 425 475 525 575 625 675
Wavelength (nm)

GFP-fusion proteins



The human histone H2B gene fused (GFP) and
transfected into human HeLa cells
Current Biology 1998, 8:377–385

Homogeneous labeling
Regulation of the expression can be a problem for FCS

FLASH-EDT2 labeling (FLASH tag)

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

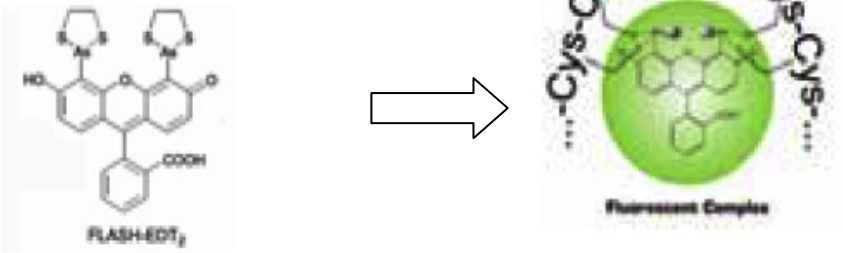
a small (700-dalton), synthetic, membrane-permeant ligand that could be linked to various spectroscopic probes or crosslinks.

The ligand has relatively few binding sites in nontransfected mammalian cells but binds to the designed peptide domain with a nanomolar or lower dissociation constant.

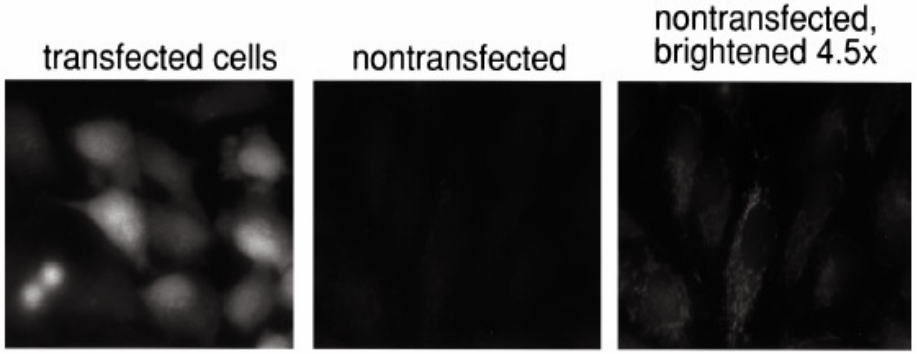
An unexpected bonus is that the ligand is nonfluorescent until it binds its target, whereupon it becomes strongly fluorescent.

Tetra-cys motif

…-Cys-Cys-Pro-Gly-Cys-Cys-…



bis-arsenical fluorophore FLASH-EDT2



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

Non-Homogeneous labeling
Transfected cells have to be selected

Electroporation

Electroporation is the process where cells are mixed with a labeled compound and then briefly exposed to pulses of high electrical voltage.

The cell membrane of the host cell is penetrable allowing foreign compounds to enter the host cell. (Prescott *et al.*, 1999).

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

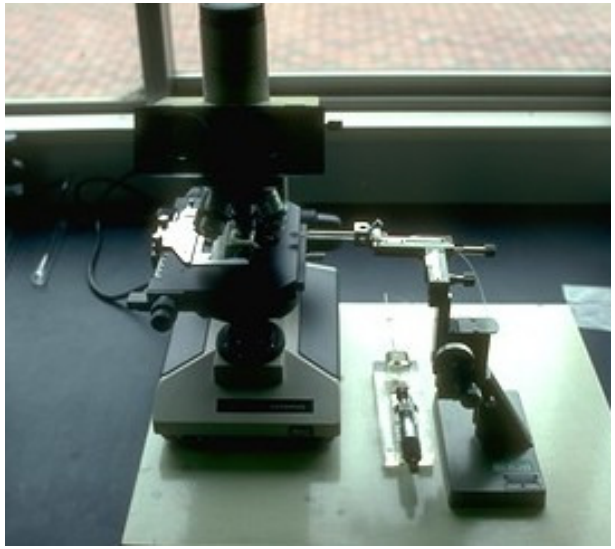
Microinjection

Microinjection is the process of directly injecting foreign DNA into cells.

By examination with a microscope, a cell is held in place with gentle suction while being manipulated with the use of a blunt capillary.

A fine pipet is then used to insert the DNA into the cytoplasm or nucleus. (Prescott *et al*. 1999)

This technique is effective with plant protoplasts and tissues.



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

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

Non-homogeneous labeling
Transfected cells have to be selected

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. (Rasmussen *et al.*, 1994)

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.

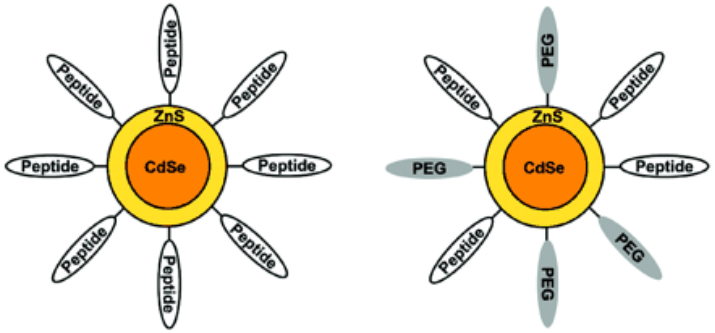
Selection of the transfected cells, is done on the basis of the selectable marker that was inserted into the DNA construct (Brettschneider et al., 1997).

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

Non-homogeneous labeling
Transfected cells have to be selected

Nanocrystal targeting in vivo

Blood vessels express molecular markers that distinguish the vasculature of individual organs, tissues, and tumors.
Peptides that recognize these vascular markers have been identified, purified and attached to a Q-dot.



Each of the peptides directed the Qdots to the appropriate site in the mice, showing that nanocrystals can be targeted *in vivo* with an exquisite specificity.

Fig. 1. Schematic representation of Qdot targeting. Intravenous delivery of Qdots into specific tissues of the mouse. Qdots were coated with either peptides only or with peptides and PEG. PEG helps the Qdots maintain solubility in aqueous solvents and minimize nonspecific binding.

Åkerman et al.PNAS | **October 1, 2002** | vol. 99 | no. 20 | **12617-12621**

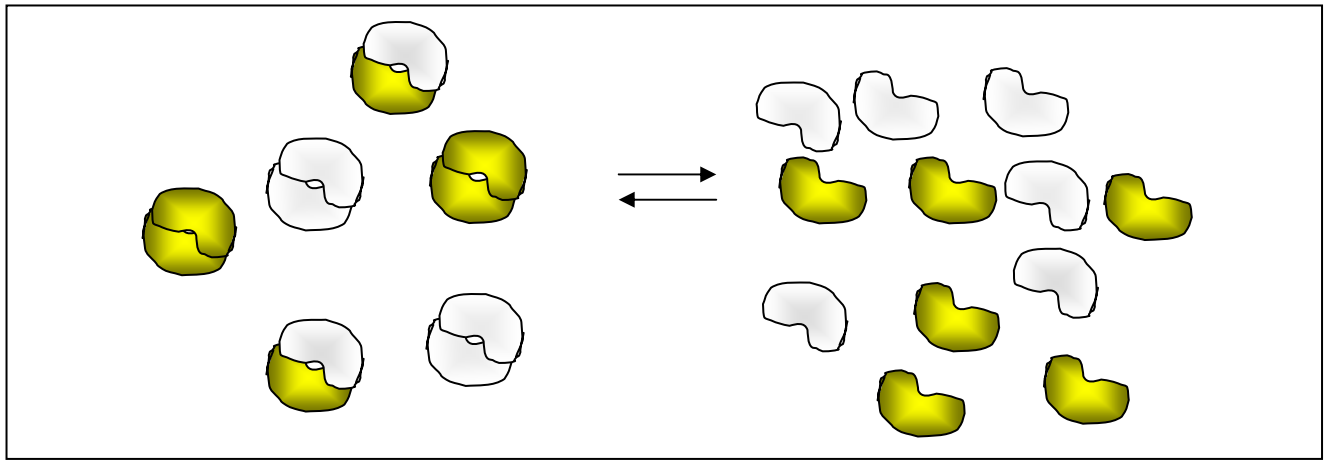
Can the inappropriate labeling induce errors in interpretation?

Experimental considerations

Correct labeling for the chosen technique

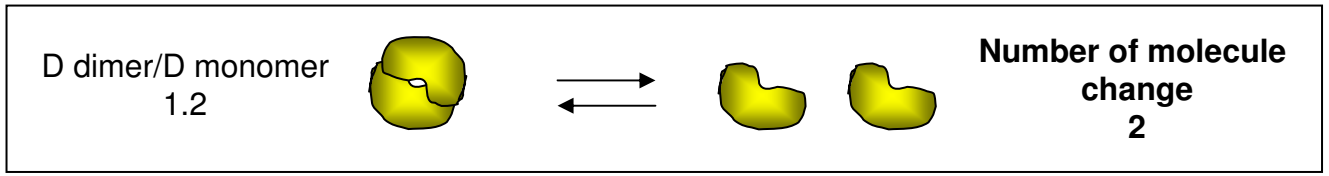
Example: dimer dissociation

Spectroscopy: Polarization measurements

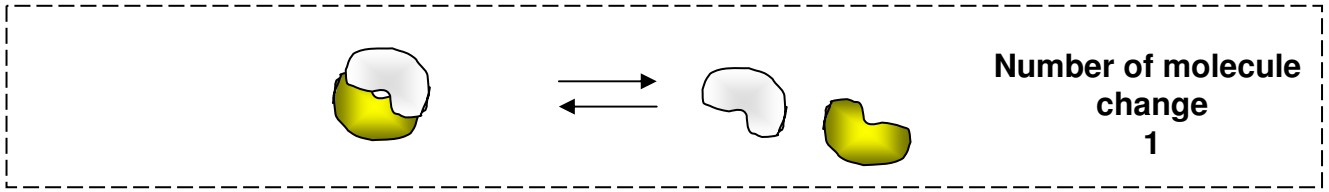


Measuring a population of molecules

Microscopy: FCS measurements



Measuring single molecules level



The end