Lecture 4
Fluorescent Labels

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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

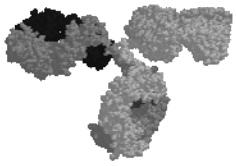
Fluorescence Probes

- **Labeling "in vitro"**
  - Labeling proteins
  - Labeling membranes
  - Ions indicators
  - Quantum dots
  - Labeling DNA
- **Labeling "in vivo"**
  - Genetic Incorporation
  - Mechanical Incorporation

Contribution to the slides

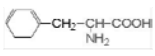
- Theodore Hazlett
- David Jameson
- Ewald Terpetschnig
- LFD people

Labeling proteins

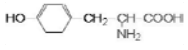


Naturally Occurring Fluorophores

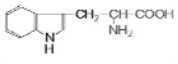
Aromatic Amino Acids



Phenylalanine
Ex/Em 260 nm/282 nm

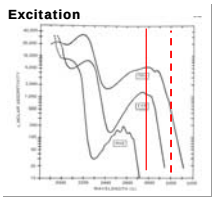


Tyrosine
ex/em 280 nm/305 nm

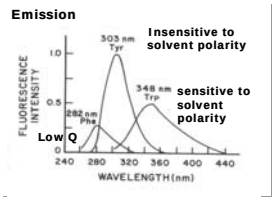


Tryptophan
ex/em 280, 295nm/ 305-350 nm

Excitation

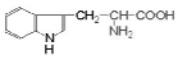


Emission

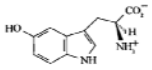


Tryptophan derivatives

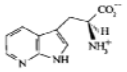
Tryptophan derivatives may be genetically incorporated in a protein



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



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



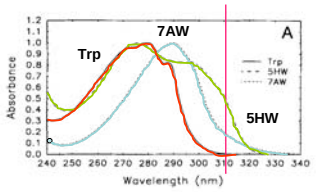
7-azatryptophan
ex/em 320nm/403nm

Φ =0.14	Φ= 0.097	Φ = 0.017
•solvent-sensitive emission	•solvent-insensitive emission	•Large emission shift in water

Φ =Number of photons emitted/number of photons absorbed

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

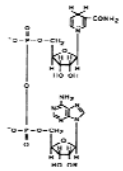
Absorbance spectrum is red-shifted with respect to that of tryptophan.



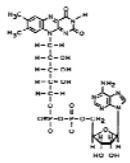
It is possible to selectively excite them, in proteins, in the presence of tryptophan of other proteins

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

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),

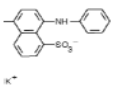
Extrinsic probes

(not present in the natural molecule/macromolecule)

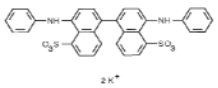
Non-covalent Attachment

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

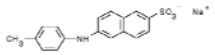
1,8-ANS



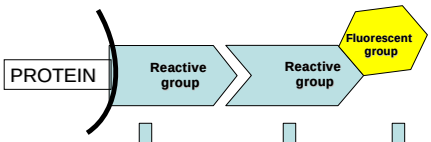
bis-ANS



2,6 TNS



Covalent Attachments



Available reactive group in the protein

NH_2 **Lysine Arginine**

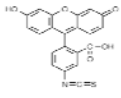
SH Cysteine

Depends on the reactive group in the protein.

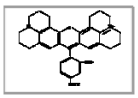
Light source
Lifetime
Spectral properties
Autofluorescence

Labeling should not change the biological activity of the protein.

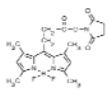
Fluorescent groups



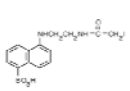
FLUORESCEINE
(488/512) t ≈ 4.05



Texas Red
(595-615), t ≈ 3.5 ns

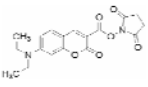


BODIPY
(493/503), t = 6 ns



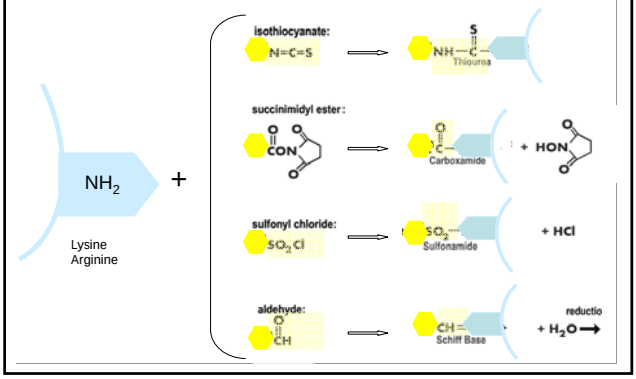
IAEDANS
(360/480) t ≈ 15 ns

Dansyl chloride
(335/518) t ≈ 10 ns

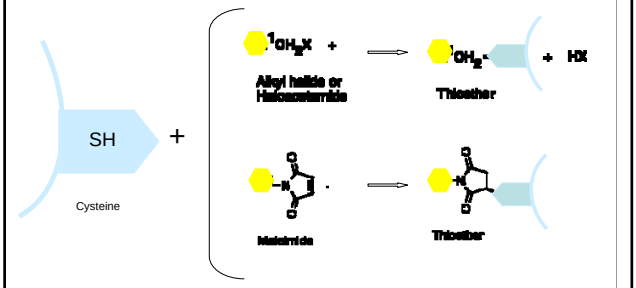


Coumarin-3-carboxylic acid -NHS
(445/482), t ≈ 2 -3 ns

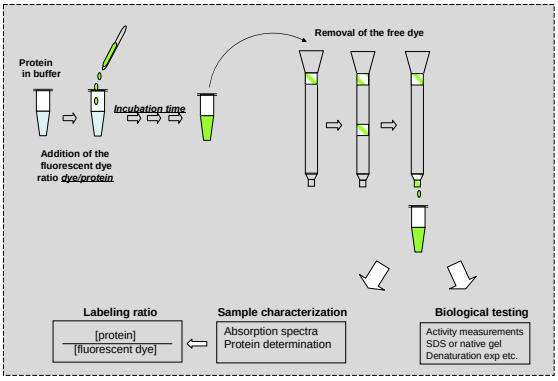
Targeting amino groups



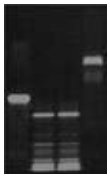
Targeting thiol groups:



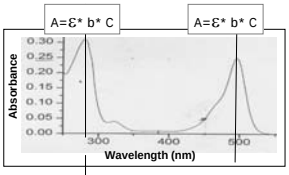
General labeling protocol for extrinsic labeling



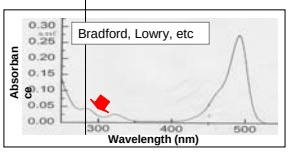
Characterization after the labeling



Protein-Fluorescein

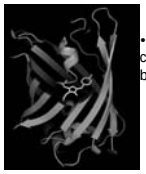


Fluorescein



Fluorescent Protein (FP) Example: GFP

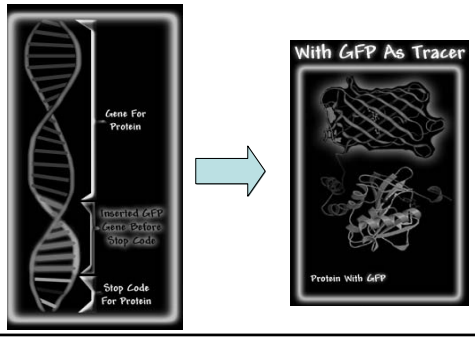
• From the bioluminescent jellyfish *Aequorea victoria*.



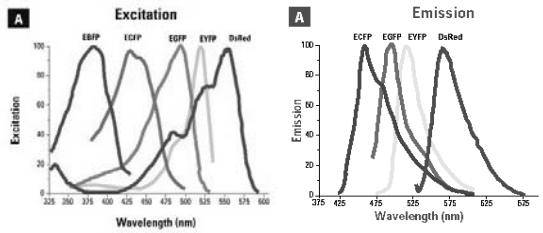
• β-barrel structure, with chromophore housed within the barrel.

• The chromophore is formed spontaneously (*from Serine-65, Tyrosine-66, Glycine-67*) upon folding of the polypeptide chain, without the need for enzymatic synthesis.

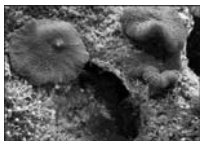
It is possible to insert the gene for GFP into cells and use the resulting protein as a reporter for a variety of applications.



Mutants of different colors have been developed.



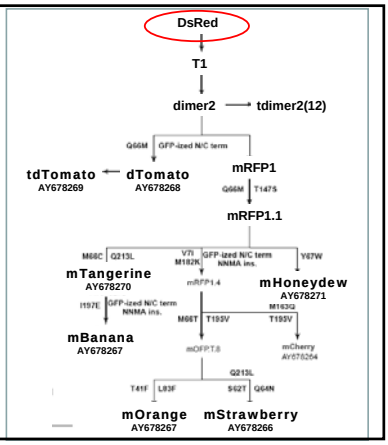
Ds Red fluorescent proteins and derivatives



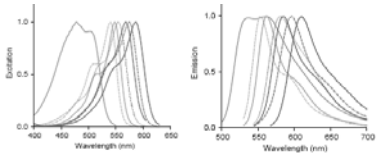
- Extracted from the Coral *discosoma sp*
- tetrameric
- mRFP is the improved monomeric form

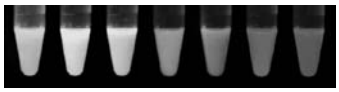
Mutants of DsRed form the mFruits proteins

THE mFruits family



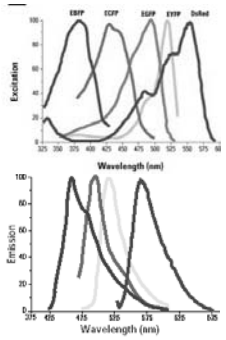
mFruits fluorescent proteins



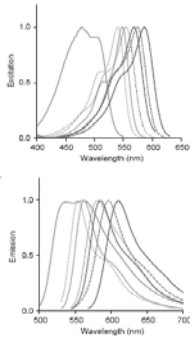


mFruits may replace or be good pairs for FPs in energy transfer experiments

FPs



mFruits

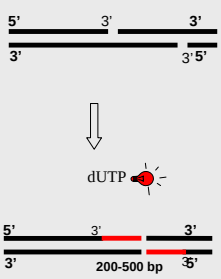


Labeling DNA

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

Nick translation

End labeling of fragments

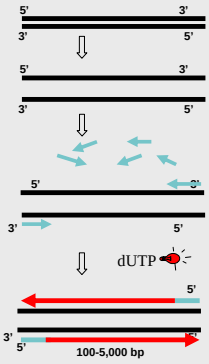


DNase I, which in the presence of Mg^{2+} ions becomes a single stranded endonuclease creates random nicks in the two strands of any DNA molecule.

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)



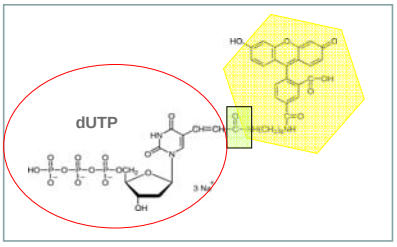
1- Denaturation step (1min, 95°C).
During the denaturation, the double strand melts open to single stranded DNA

2- Annealing (45 sec, 54°C).
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, 72°C).
Then Taq polymerase synthesize the new DNA strands. Only dNTP's.

Commercially labeled dUTP

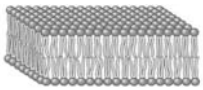
succinimidyl-ester derivatives of fluorescent dyes



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	RGG	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	-	-

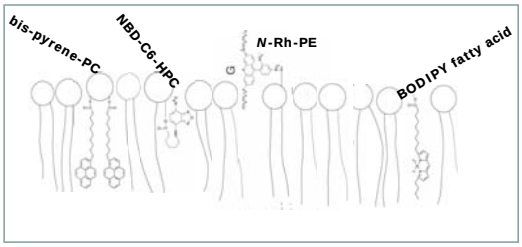
fluorescein-aha-dUTP from Molecular Probes

Labeling membranes

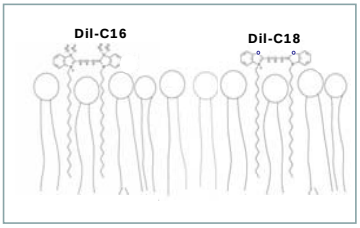


- Analogs of fatty acids and phospholipids
- Di-alkyl-carbocyanine and Di-alkyl-aminostyryl probes.
- Other nonpolar and amphiphilic probes. Laurdan, Prodan, Bis ANS

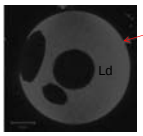
Fatty acids analogs and phospholipids



Di-alkyl-carbocyanine probes.



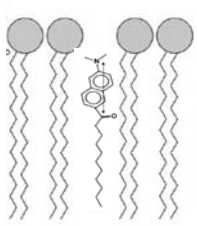
SM / DOPC / Chol (1:1:1)

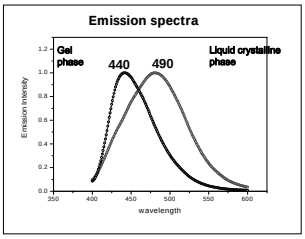


Chem.and Phys. of Lipids 141 (2006) 158–168

Nonpolar probes

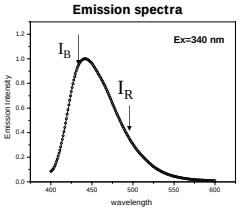
example: *Laurdan.*
(environment-sensitive spectral shifts)





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

Laurdan Generalized Polarization (GP)



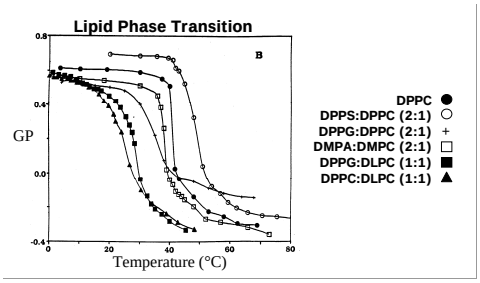
$$GP_{ex} = \frac{I_B - I_R}{I_B + I_R}$$

-0.2
loose lipid packing → **0.6**
tight lipid packing

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

GP in the cuvette

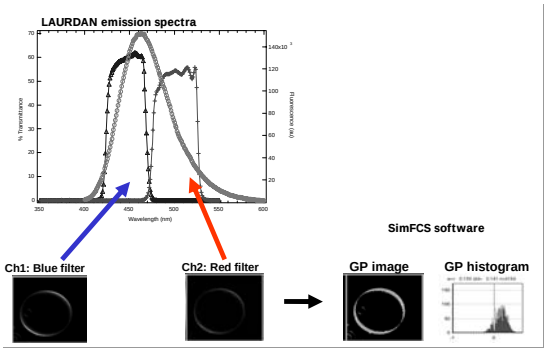
MLVs, SUVs, LUVs



Parassassi et al. Biophys. J. 60, 179 (1991)

GP in the microscope

(2-photon excitation)

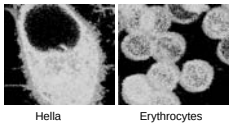


Two-photon excitation microscopy

Model systems: GUVs

DOPC/DPPC 1:1mol/mol **DOPC:DPPC:CHOL 1:1:1**

Natural systems

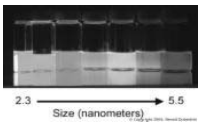


Hella Erythrocytes

Living T. brucei (ec)

-1 GP 1

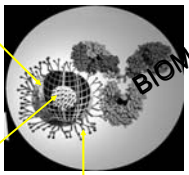
Quantum dots



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

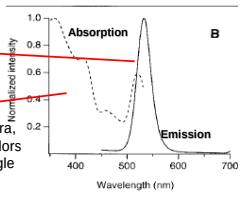
In the core emission is typically weak and always unstable.
The shell material Zinc Sulfide (**ZnS**) has been selected to be almost entirely un-reactive and completely insulating for the core.

COATING

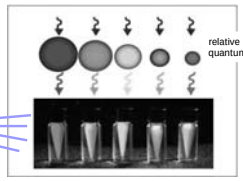
A layer of organic ligands covalently attached to the surface of the shell. This coating provides a **surface for conjugation** to biological (antibodies, streptavidin, lectins, nucleic acids) and nonbiological species and makes them "water-soluble"

•**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

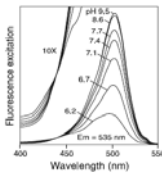


relative sizes of the CdSe quantum dots in the vials.

UV hand lamp.

The emission is tunable according to their size and material composition

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+} *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

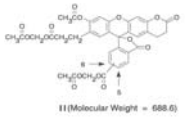
- Cell loading and distribution of the probe.
- Salt and dextran...microinjection, electroporation, patch pipette.
- AM-esters ....cleaved by intracellular esterases

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

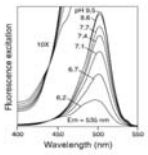
Molecular Probes' pH indicator families, in order of decreasing pK_a

Example: BCECF

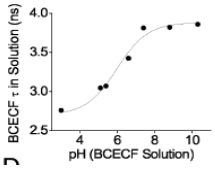


(Molecular Weight = 688.6)

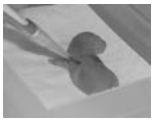
Fluorescence Intensity



Fluorescence Lifetime



Experimental protocol



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

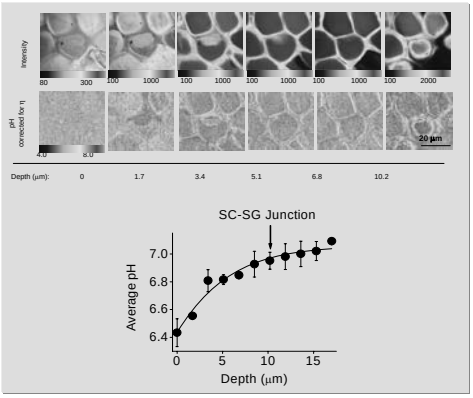
⇩

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)

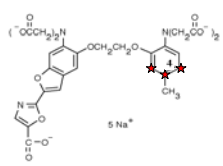
Ratiometric

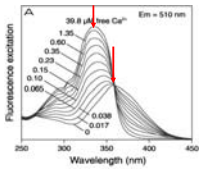
VISIBLE
FLUO
(Fluo-3, Fluo-4, Fluo5F, Fluo-5N, Fluo-4N)
RHOD (Rhod-2, Rhod-FF, Rhod-5N)
CALCIUM GREEN (CG-1, CG-5N,CG-2)
OREGON GREEN 488-BAPTA

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

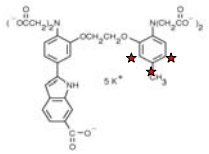
Most used in microscopic imaging

Good excitation shift with Ca^{2+}

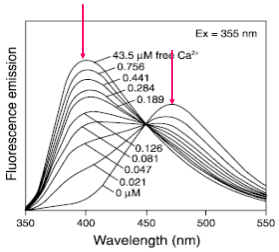
Rationed between 340 / 350 and 380 / 385 nm

Ratiometric: 1excitation / 2emission

Indo-1

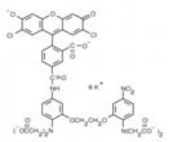


Indicator	K_d (Ca^{2+}) (μM)
indo-1	0.23
indo-5F	0.47

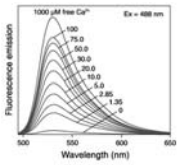


CalciumGreen-5N

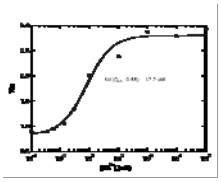
Non-Ratiometric



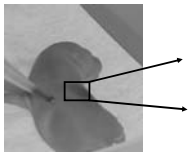
Fluorescence Intensity

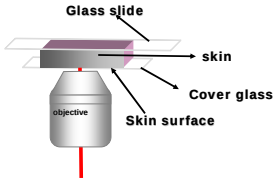


Fluorescence Lifetime

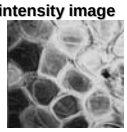


rats



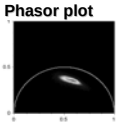


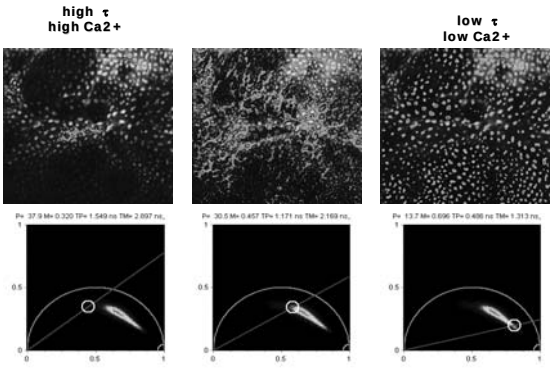
intensity image



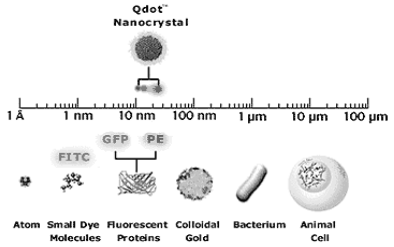
analysis

Phasor plot





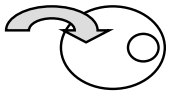
Comparing the size of the fluorescence probes and the bio-molecule being labeled



Labeling *"in vivo"*

Mechanical incorporation

Labeled proteins
Labeled DNA
Q-dots
Genetic material



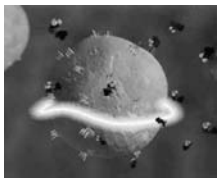
Electroporation

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. (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

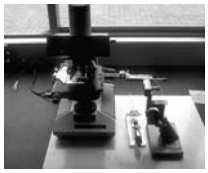
Microinjection

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. (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)

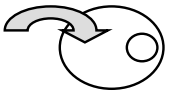
As the cells repair their injuries, they integrate their DNA into their genome, thus allowing for the host cell to transcribe and translate the gene.

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

Genetic Incorporation

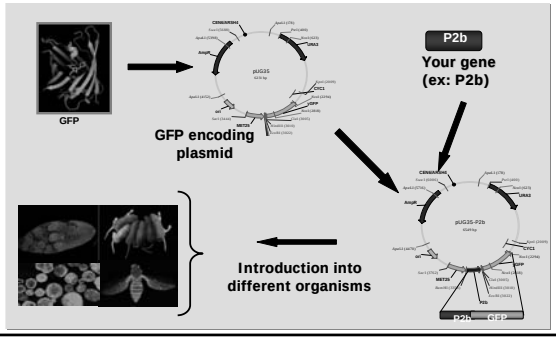


Protein localization in vivo
- **GFP fusion**
- **FLAsh**

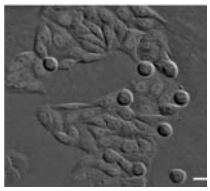
Protein Interaction in vivo
- **FRET analysis**
- **BiFC analysis**
- **Multicolor BiFC analysis**

Protein Localization in vivo

GFP-fusion proteins



GFP-fusion proteins



FCS
RICS
N&B

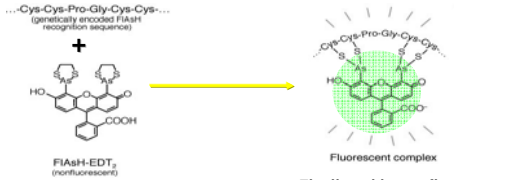
The human histone H2B gene fused (GFP) and transfected into human HeLa cells
Current Biology 1998, 8:377–385

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

Protein Localization in vivo

FL Ash-EDT2 labeling (FLASH tag)

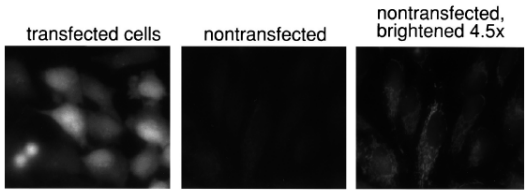
Receptor domain composed of a few as six natural amino acids that could be genetically incorporated into proteins of interest.



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

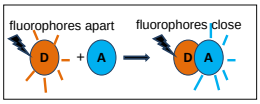
Visualizing the localization of protein interactions in living cells.

Two principal methods have been used

Föster resonance energy transfer (FRET) analysis

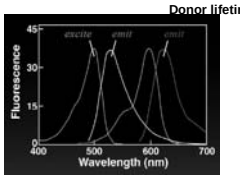
BiFC analysis

Föster resonance energy transfer (FRET) analysis

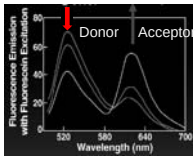


Based on changes in the fluorescence **intensities** and **lifetimes** of two fluorophores that are brought sufficiently close together.

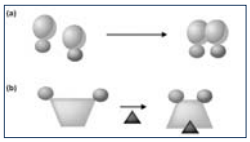
Donor intensity decrease
Donor lifetime decrease



Acceptor intensity increase



Föster resonance energy transfer (FRET)



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

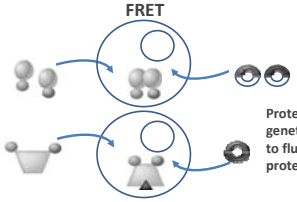
(a) INTERMOLECULAR FRET:
FRET between a donor and acceptor fluorophore, each attached to a different protein, reports protein–protein interaction.

(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.

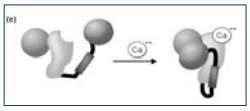
FRET

Proteins can be labeled in vitro with small fluorescent dyes.

Mechanically incorporated

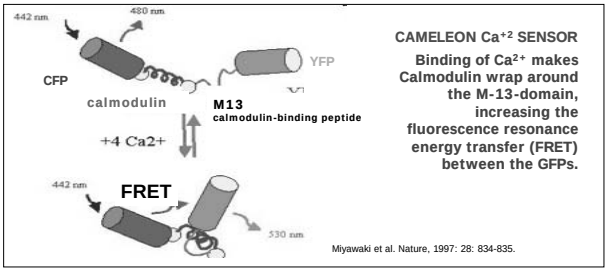


Proteins can be genetically bounded to fluorescent proteins



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

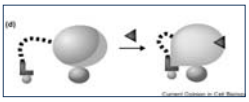
(c) Protein 'transducer'. A protein is engineered to produce a large change in the distance between an attached donor and acceptor upon ligand binding. In this example, calcium binding generates a hydrophobic pocket to which the blue peptide binds. Peptide binding brings the two GFP mutants together,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

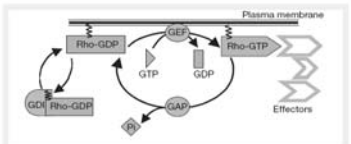


Klaus Hahn et al. Current Opinion in Cell Biology 2002, 14:167–172

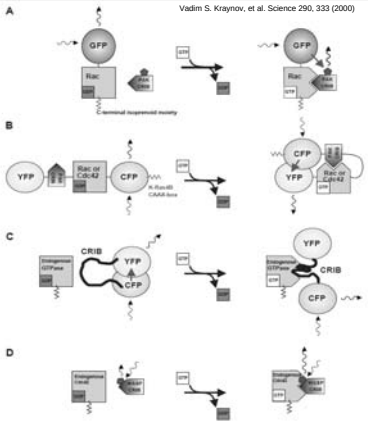
(d) Domain/antibody biosensor. A protein or antibody fragment (blue) binds only to the activated state of the protein. The protein fragment bears a dye which undergoes FRET when it is brought in close proximity to the GFP on the protein. In some examples, the domain is part of the same polypeptide chain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

Rho / Rac Biosensors

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

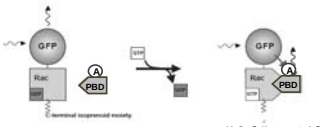


Vadim S. Kraynov, et al. Science 290, 333 (2000)



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.

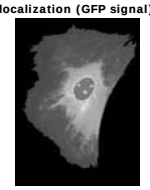


The Alexa and GFP fluorophores undergo FRET when brought close together.

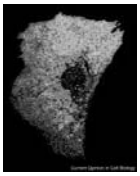
Vadim S. Kraynov, et al. Science 290, 333 (2000)

Activation of the GTPase Rac in a living motile fibroblast.

Rac localization (GFP signal)



Rac activation (FRET)



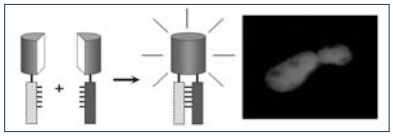
Warmer colors indicate higher levels of activation. A broad gradient of Rac activation is visible at the leading edge of the moving cell, together with even higher activation in juxtanuclear structures.

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)



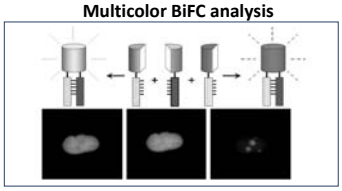
THE PRINCIPLE: Based in the association between two fluorescent proteins fragments when by an interaction between proteins fused to the fragments. The individual fragments are non-fluorescent.

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.