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, 295 nm/305-350 nm

Excitation Insensitive to solvent polarity

Emission Sensitive to solvent polarity

Low Q
Tryptophan derivatives may be genetically incorporated in a protein. Tryptophan derivatives such as 5-Hydroxy-tryptophan and 7-azatryptophan can be used.

### Tryptophan derivatives

- **Tryptophan**
  - Ex/Em: 280, 295 nm / 305-350 nm
  - Fluorescence quantum yield: \( \Phi = 0.14 \)

- **5-Hydroxy-tryptophan**
  - Ex/Em: 280, 295 nm / 305-350 nm
  - Fluorescence quantum yield: \( \Phi = 0.097 \)

- **7-azatryptophan**
  - Ex/Em: 310 nm / 339 nm
  - Fluorescence quantum yield: \( \Phi = 0.017 \)

- **7-AW**
  - Ex/Em: 320 nm / 403 nm

### Enzymes Cofactors

- **NADH**
  - (NADH: NADH dehydrogenase)
  - Ex/Em: 340/460 nm

- **FAD**
  - (FAD: Flavin adenine dinucleotide)
  - Ex/Em: 450 nm / 540 nm

- **Porphyres**
  - Ex/Em: 440 nm / 450 nm / 520 nm

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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.
**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)

![Image of Extrinsic probes](image)

**Covalent Attachments**

Protein → Reactive group → Reactive group → Fluorescent group

Available reactive group in the protein → Depends on the reactive group in the protein → Light source

NH₂ Lysine  Arginine  Cysteine

Labeling should not change the biological activity of the protein.

![Image of Covalent Attachments](image)

**Fluorescent groups**

<table>
<thead>
<tr>
<th>Fluorescent group</th>
<th>λ max (nm)</th>
<th>λ em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLUORESCINE</td>
<td>515</td>
<td>540</td>
</tr>
<tr>
<td>Texas Red</td>
<td>630</td>
<td>640</td>
</tr>
<tr>
<td>Dansyl chloride</td>
<td>510</td>
<td>520</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>360</td>
<td>480</td>
</tr>
<tr>
<td>BODIPY</td>
<td>503</td>
<td>600</td>
</tr>
<tr>
<td>Coumarin-3-caboxlylic acid-NHS</td>
<td>450</td>
<td>500</td>
</tr>
</tbody>
</table>

![Image of Fluorescent groups](image)
Targeting amino groups

Lysine Arginine

Targeting thiol groups:

Cysteine

General labeling protocol for extrinsic labeling
**Characterization after the labeling**

- Protein: Fluorescein
- Wavelength (nm)

**Absorbance**

- $A = \varepsilon \cdot b \cdot C$

**Fluorescein**

- Bradford, Lowry, etc.
- Wavelength (nm)

**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

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 Mg²⁺ 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 (1 min, 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 (2 min, 72°C).

Then Taq polymerase synthesize the new DNA strands. Only dNTP’s.

**Commercially labeled dUTP**

succinimidy-l-ester derivatives of fluorescent dyes

| Fluor | dUTP 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.**


**Nonpolar probes**

*example: Laurdan.*

(environment-sensitive spectral shifts)


**Laurdan Generalized Polarization (GP)**

GP in the cuvette
MLVs, SUVs, LUVs

Lipid Phase Transition

- DPPC
- DPPS:DPPC (2:1)
- DPPG:DPPC (2:1)
- DMPA:DMPC (2:1)
- DPPG:DLPC (1:1)
- DPPC:DLPC (1:1)

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

GP in the microscope
(2-photon excitation)

LAURDAN emission spectra

SimFCS software

GP image
GP histogram

Ch1: Blue filter
Ch2: Red filter

Two-photon excitation microscopy

Model systems: GUVs
- DOPC:DPPC 1:1 mol/mol
- DOPC:DPPC:CHOL 1:1:1

Natural systems
- Hela
- Erythrocytes
- Living T. brucei (ec)
Quantum dot emission is typically weak and always unstable. The shell material Zinc Sulfide (ZnS) has been selected to be almost entirely unreactive and completely insulating for the core. 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".

**Quantum Dots**

- 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.

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

- 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.

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

UV hand lamp...
Ions indicators

Fluorescent probes for Ions

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

**Cations:**
H⁺, Ca²⁺, Li⁺, Na⁺, K⁺, Mg²⁺, Zn²⁺, Pb²⁺ etc.

**Anions:**
Cl⁻, PO₄²⁻, 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

<table>
<thead>
<tr>
<th>Parent Fluorophore</th>
<th>pH Range</th>
<th>Typical Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNARF indicators</td>
<td>6.9–8.0</td>
<td>Emission ratio 580/640 nm</td>
</tr>
<tr>
<td>HPTS (pyranine)</td>
<td>7.0–8.0</td>
<td>Excitation ratio 450/405 nm</td>
</tr>
<tr>
<td>BCECF</td>
<td>6.5–7.5</td>
<td>Excitation ratio 490/440 nm</td>
</tr>
<tr>
<td>Fluoresceins and carboxyfluoresceins</td>
<td>6.0–7.2</td>
<td>Excitation ratio 490/450 nm</td>
</tr>
<tr>
<td>LysoSensor Green DND-160</td>
<td>4.5–6.0</td>
<td>Single emission 520 nm</td>
</tr>
<tr>
<td>Oregon Green dyes</td>
<td>4.2–5.7</td>
<td>Excitation ratio 510/480 nm or excitation ratio 490/440 nm</td>
</tr>
<tr>
<td>LysoSensor Yellow/Blue DND-160</td>
<td>3.5–6.0</td>
<td>Emission ratio 450/510 nm</td>
</tr>
</tbody>
</table>

Molecular Probes’ pH indicator families, in order of decreasing pH

### Example: BCECF

![Fluorescence Intensity](image1)

![Fluorescence Lifetime](image2)

### Experimental protocol

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

Labeled skin is removed

Imaging

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-SN)
- CALCIUM GREEN (CG-1, CG-5N, CG-2)
- OREGON GREEN 488-BAPTA

**Ratiometric**
- Most used in microscopic imaging
- Good excitation shift with Ca$^{2+}$
- Rationed between 340/350 and 380/385 nm

**Non Ratiometric**

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Ratiometric: 2 excitation/1emission

**FURA-2**

<table>
<thead>
<tr>
<th>Indicator</th>
<th>K$_{Ca}$(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fura-2</td>
<td>0.8 μM</td>
</tr>
<tr>
<td>Fura-5F</td>
<td>0.49 μM</td>
</tr>
<tr>
<td>Fura-6F</td>
<td>0.07 μM</td>
</tr>
<tr>
<td>Fura-FF</td>
<td>5.30 μM</td>
</tr>
<tr>
<td>Fura-FF (50%)</td>
<td>35 μM</td>
</tr>
</tbody>
</table>
**Ratiometric: 1excitation /2emission**

**Indo-1**

<table>
<thead>
<tr>
<th>Indicator</th>
<th>( K_i (^{2+}\text{Ca}) ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo-1</td>
<td>0.23</td>
</tr>
<tr>
<td>Indo-51</td>
<td>0.47</td>
</tr>
</tbody>
</table>

**CalciumGreen-5N**

**Non-Ratiometric**

**Fluorescence Intensity**

**Fluorescence Lifetime**

**rats**

- Glass slide
- Cover glass
- Skin
- Skin surface

**Intensity image** → **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

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

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.

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. (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).

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

Introduction into different organisms

GFPGFP---fusion proteins

GFP encoding plasmid

P2b:
Your gene (ex: P2b)
The human histone H2B gene fused (GFP) and transfected into human HeLa cells

**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.

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, whereupon it becomes strongly fluorescent.

**FL Ash-EDT2 labeling (FLASH tag)**

transfected cells  nontransfected  nontransfected, brightened 4.5x

Non-Homogeneous labeling
Transfected cells have to be selected
Protein interactions in vivo
Visualizing the localization of protein interactions in living cells.

Two principal methods have been used

Förster resonance energy transfer (FRET) analysis

BiFC analysis

Förster 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

(a) INTERMOLECULAR FRET:
FRET between a donor and acceptor fluorophore, each attached to 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.

Proteins can be labeled in vitro with small fluorescent dyes.
Mechanically incorporated
Proteins can be genetically bound to fluorescent proteins

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.

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

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.
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. 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.


Activation of the GTPase Rac in a living motile fibroblast.
Rac Localization (GFP signal) Rac activation (FRET)


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 L. Rapoport 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.