Fluorescence spectroscopy

- **Principles**
- **Instrumentation**
- **Applications**
  - Solvent effects
  - Non-radiative energy transfer (NRET or FRET)
  - Anisotropy
  - Excimer formation
The fluorescence process:
Energy Diagram (Jablonski diagram)

S₁': singlet excited state
S₁: lowest singlet state
S₀: Ground state

1: excitation
2: deactivation
3: emission
Light absorption and emission processes

- **Absorption**
- **Emission**
  - (Fluorescence)
  - (Phosphorescence)

**Excited Singlet State**

**Excited Triplet State**

Energy
The Franck-Condon Principle

Electronic transitions are vertical:
The atoms of a bond do not move during an electronic transition
**Nomenclature:**

0→0: transition between vibrational states 0 (fundamental) and 0 (excited)

Loss of spectral resolution as a result of chromophore-solvent interactions
Excitation and emission spectra (definitions)

The *excitation spectrum* should be the same as the *absorption spectrum* when both spectra are measured for the same solution.

The *emission intensity* depends on the *excitation wavelength*, but the shape of the emission does not vary with emission intensity.
How does an excited state (M*) lose its energy?

1. Fluorescence
2. Thermal deactivation
3. Photochemical reaction
4. Quenching

Quantum Yield Definition:

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

$$\Phi_f < 1$$
**Wavelength dependence**

**Fluorescence spectrum**

**Time dependence**

**Fluorescence decay**

**Excited state life time**
Fluorescence spectrometer

- Detector
- Lamp
- Sample Holder
SELECTED APPLICATIONS

Pyrene probe experiment
(Determination of a surfactant critical micelle concentration)

Excimer formation

Fluorescence energy transfer

Fluorescence polarization
Py in water

Why Py?

- High quantum yield
- Long life time
- Low water solubility
- Environment-dependent emission

The intensity of the (0,0) band of the pyrene fluorescence shows a strong dependence on the polarity of the environment (Hamm effect).
Emission of pyrene in water and in micelles

Water: $I_1/I_3 = 1.7$

Micelles: $I_1/I_3 = 1.2$
Determination of the critical micelle concentration (cmc) of a surfactant

\[ \text{cmc} \]

\[
C_{16}H_{33}N^+(CH_3)_3
\]

**hydrophilic environment**

**hydrophobic environment** (micelle core)

**Constant Py concentration (~ 7 x 10^{-7} M)**

**Variable surfactant concentration**
Pyrene excimer formation

Pyrene excimer formation is a process where two chromophores (Py) in the ground state (S0) are excited to the first excited singlet state (S1) by a photon of energy $h\nu_{exc}$. The excited state Py* can then form an excimer (E*) through a dynamic process, which is characterized by a lower energy state $h\nu_{E}$.

- **Py**: Chromophore in the ground state.
- **Py***: Chromophore in the first excited singlet state.
- **E***: Dynamic excimer.
Examples of excimer formation in PNIPAM

PNIPAM-PC: \( n = 50, \ m = 50, \ p = 0 \)

PNIPAM-PC-C_{18}: \ R_1 = H, \ R_2 = C_{18}H_{37} \)

PNIPAM-PC-C_{18}Py: \ R_1 = (CH_2)_4Py, \ R_2 = C_{18}H_{37} \)

PNIPAM-PC-F: \ R_1 = H, \ R_2 = CH_2-(CF_2)_6 CF_3 \)
Assembly in water

Fluorescent hydrophobic dye (Pyrene)

excimer

Hydrophobic microdomains

Ion pair interactions

phosphorylcholine pyrene octadecyl group
Effect of solvents on the fluorescence of PNIPAM-PC-C$_{18}$Py

Clusters of hydrophobic groups

Clusters of zwitterions

Fluorescence intensity

wavelength (nm)
Fluorescence of PNIPAM-PC-PyC$_{18}$ in CHCl$_3$/CH$_3$OH mixtures
The fluorescence process: Energy Diagram (Jablonski diagram)

- $S_1'$: singlet excited state
- $S_1$: lowest singlet state
- $S_0$: Ground state

1: excitation
2: deactivation
3: emission
Non-radiative energy transfer (NRET or FRET)

- 1922 – Cario and Frank: “illumination of a mixture of mercury and thallium vapors at a wavelength absorbed only by mercury resulted in fluorescence emission from both atoms”

- 1927 – J.-B. Perrin: “transfert d’activation” up to the intermolecular distance of 100 nm

- 1932 – F. Perrin: influence of the solvent, reduction of the intermolecular distance to 10-15 nm

- 1948 – Th. Förster: “the absorption and fluorescence spectra of similar molecules are far from completely overlapping”, resulting in further reduction of the NRET distance to ~ 5 nm (fluorescein) in agreement with experimental data
Non-Radiative Energy Transfer Process
Jablonski Diagram

1. Donor excitation
2. Donor emission
3. Acceptor excitation by NRET
4. Acceptor emission
The FRET efficiency is determined by:

1. The *distance* between the donor and the acceptor

2. The *spectral overlap* of the donor *emission spectrum* and the acceptor *absorption spectrum*

3. The relative *orientation* of the donor emission dipole moment and the acceptor absorption dipole moment
Direct Non-Radiative Energy Transfer (NRET)

\[ \text{D}^* \xrightarrow{\text{R}} \text{A} \]

\[ \text{D}^* + \text{A} \xrightarrow{\text{E}_{\text{et}}} \text{D} + \text{A}^* \]

The efficient of energy transfer \((E_{\text{et}})\) is a well defined function of the \(D / A\) separation distance \(R\):

\[ \text{E}_{\text{et}} = \frac{R_0^6}{R_0^6 + R^6} \]

\(R_0\): Characteristic distance of a \(D / A\) pair
Fürster radius

Fürster distance depends on

1. Overlap integral of the donor emission spectrum with the acceptor absorption spectrum
2. Mutual orientation of donor and acceptor

\[ R_0^6 = 8.8 \times 10^{23} \kappa^2 n^{-4} Q_0 J \]

\( \kappa^2 \) - dipole orientation factor,
\( n \) - refractive index of the medium,
\( Q_0 \) - fluorescence quantum yield of the donor in the absence of the acceptor
\( J \) - spectral overlap integral

\[ J = \int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda \]

\( f_D \) – normalized intensity of donor emission spectrum in the range from \( \lambda \) to \( \lambda + \Delta \lambda \)
\( \epsilon_A \) - acceptor extinction coefficient

If either the donor or the acceptor is freely rotating (or both), \( \kappa^2 = 2/3 \) is assumed (randomization by rotational diffusion prior to NRET).
Overlap integral can be calculated from absorption and emission spectra of the chromophore.

**Typical Values of $R_0$**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>$R_0$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>Tetramethylrhodamine</td>
<td>55</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>Fluorescein</td>
<td>46</td>
</tr>
<tr>
<td>EDANS</td>
<td>DABCYL</td>
<td>33</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>Fluorescein</td>
<td>44</td>
</tr>
<tr>
<td>BODIPY FL</td>
<td>BODIPY FL</td>
<td>57</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>QSY-7 dye</td>
<td>61</td>
</tr>
<tr>
<td>Donor</td>
<td>Acceptor</td>
<td>$R_0$ (Å)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Dansyl</td>
<td>22</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Pyrene</td>
<td>29</td>
</tr>
<tr>
<td>Dansyl</td>
<td>Fluorescein</td>
<td>33-41</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>Rhodamine</td>
<td>49-54</td>
</tr>
<tr>
<td>Pyrene</td>
<td>Coumarin</td>
<td>39</td>
</tr>
<tr>
<td>Terbium</td>
<td>Rhodamine</td>
<td>65</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Dansyl</td>
<td>21-24</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Pyrene</td>
<td>28</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>DPH</td>
<td>40</td>
</tr>
</tbody>
</table>
Applications

- Dynamics in biophysics:
  - protein-protein interactions
  - protein-DNA interactions
  - protein conformational changes

The most popular FRET pair for biological use is the cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) pair. Both are colour variants of green fluorescent protein (GFP). While labelling with organic fluorescent dyes requires troublesome processes of purification, chemical modification, and intracellular injection of a host protein, GFP variants can be easily attached to a host protein by genetic engineering.

<table>
<thead>
<tr>
<th></th>
<th>λ_{ex} (nm)</th>
<th>λ_{em} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>395, 475</td>
<td>509</td>
</tr>
<tr>
<td>CFP</td>
<td>439</td>
<td>476</td>
</tr>
<tr>
<td>YFP</td>
<td>475, 514</td>
<td>527</td>
</tr>
</tbody>
</table>

![Spectral Profiles of Common Fluorescent Proteins](image)
Monitor Changes in Polymer Association Through NRET

Donor Emission

A: Acceptor: Pyrene
D: Donor: Fluorene
FLUORESCENCE ANISOTROPY

- Polarization of light
- Fluorescence polarization and anisotropy
- Applications
- Examples
Polarization of Light

Plane Polarized Light
The light ray passes through a filter which has a single, preferred vibration direction.

A single light ray with the electric component of the light vibrating in all directions.

Preferred Vibration (Polarization) Direction
A polarizing filter acts like a picket fence. It only lets certain direction vibrations pass through it.
Fluorescence Anisotropy

1. Photoselection
   Using linear polarized light (here, vertical)

2. Rotational Diffusion during some time, $t$

3. Detection
   Using parallel and perpendicularly linear polarized light.
• Excitation with polarized light

• Preferential excitation of the fluorophores with absorption transition moments oriented along the vertical component of the electric vector of the excitation light

• The resulting excited-state population is not oriented at random

• Rotational diffusion causes loss of preferential orientation and results in depolarization of the emitted light (or partial loss of polarization

• Anisotropy measurements reveal the rotational displacement of a molecule in the time between the absorption and emission events
Vertical excitation

Light source

Detection in vertical and perpendicular directions

\[
r = \frac{I_{II} - I_\perp}{I_{II} + 2I_\perp}
\]

\[
P = \frac{I_{II} - I_\perp}{I_{II} + I_\perp}
\]

r: anisotropy  P: polarization
Anisotropy of A Molecule

• Emissions at $I_{II}$ and $I_{\perp}$ depend only on $\Theta$, the angle between the absorption and emission dipoles.

$$r = (3\cos^2\Theta - 1)/2$$

<table>
<thead>
<tr>
<th>$\Theta$</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0^\circ$</td>
<td>$1$</td>
</tr>
<tr>
<td>$54.7^\circ$</td>
<td>$0$</td>
</tr>
<tr>
<td>$90^\circ$</td>
<td>$-0.2$</td>
</tr>
</tbody>
</table>
Anisotropy of Rigid Samples in an Isotropic Solution

- One assumes that there is no movement of the sample during the fluorescence lifetime, then:

  \[ r = \frac{(3\langle \cos^2 \Theta \rangle - 1)}{2} \]

- Maximum value of \( \langle \cos^2 \Theta \rangle \) (colinear adsorption) is 0.6

- If absorption and emission dipoles are colinear, \( r = +0.4 \) (maximum value)
Anisotropy depends on the excitation wavelength

Perylene in propylene glycol at -60 °C

\[ \lambda = 260 \text{ nm} \quad r = -0.2 \quad \Theta = 90° \]
\[ \lambda = 282 \text{ nm} \quad r = 0 \quad \Theta = 54.7° \]
\[ \lambda = 430 \text{ nm} \quad r = 0.35 \quad \Theta = 16.8° \]
Anisotropy of **Freely Rotating Fluorophores in Isotropic Solution**

- Additional depolarization (increase in isotropy) of excited samples occurs as emission dipoles rotate through angle $\theta$. Thus, the total anisotropy depends on intrinsic properties and environmental factors.

- If the fluorophore rotates fast enough, all memory of the original photoselection is lost and $I_{II} = I_{\perp}$, hence the anisotropy will be 0 for common samples, $-0.2 < r < 0.4$.

(rigid and oriented matrix)
Anisotropy of Macromolecules

- Value of anisotropy depends on molecular motion
- Faster motion will yield lower values, slower motion will yield larger values
- Thus anisotropy can be used to measure motion, and molecular properties related to motion (size, shape, etc...)

Anisotropy of Macromolecules: Perrin equation

- Polarization is related to rotational diffusion constant of the polymer chain $D_{rot}$

- $1/6D_{rot} = \theta$ rotational correlation time
  
  also: $\theta = V_h \eta / RT$
  
  - $V_h$ = Molar volume of molecule
  - $\eta$ = Viscosity

- Perrin equation relates $r$ to $V_h \eta$:

  $1/r = 1/r_0 (1+\tau/\theta) = 1/r_0 \left(1+\tau RT/ \eta V_h \right)$
PNIPAM400-Np
$M_n = 49,000$
$n+m \sim 450$
ca. 1 Np group/chain
Cloud point = 32.0 °C (DSC)

PNIPAM400-Py
$M_n = 74,000$
$n+m \sim 650$
ca. 1-2 Py groups/chain
Cloud point = 32.0 °C (DSC)

Naphthalene-labeled PNIPAM in water: Fluorescence anisotropy ($r$)

Perrin equation:

$$\frac{r}{r_0} = 1 + 6D\tau$$

- $r_0$: fundamental anisotropy
- $D$: rotational diffusion coefficient
- $\tau$: fluorescence lifetime

increase in anisotropy

Increase in microviscosity
Naphthalene-labeled PNIPAM in water: Fluorescence anisotropy

~ 31 °C

~ 33 °C
Recalling light scattering results….