Fluorescence quenching, Fluorescence anisotropy, Fluorescence resonance energy transfer (FRET)

Special Application in Fluorescence Spectroscopy

Biophysics seminar
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Timescale of fluorescence processes
The excited electron decay possibilities

excitation
fluorescence
10^{-16} seconds
phosphorescence
10^{-8} seconds

ground state
relaxation
k_f
k_q
k_t
k_{qc}

quenching or energy transfer
internal conversion (heat)
Fluorescence basics

- **Quantum yield**: number of the emitted photons divided by the number of the excited photons

  \[ Q_f = \frac{k_f}{k_f + k_{nr}} \]

- **Lifetime**: 63% of the originally excited photons are already emitted the energy

  \[ \tau = \frac{1}{k_f + k_{nr}} \]

Fluorescence quenching

- The decrease of the fluorescence intensity by molecules able to interact with the fluorophore.

- **Quencher** is a special molecule which is responsible for the quenching. It can take away the excited energy of the fluorophore.

- The quenching process competes with the fluorescence emission.

**Decrease of the fluorescence intensity!**
Example

Two samples of quinine dissolved in water with a violet laser (left) illuminating both. Typically quinine fluoresces blue, visible in the right sample. The left sample contains chloride ions which quenches quinine's fluorescence, so the left sample does not fluoresce visibly (the violet light is just refracted laser light).

Types of fluorescence quenching

1. Static quenching

   Due to the formation of dark complex between the ground state fluorophore and the quencher some of the fluorophore behave as a non-fluorescence molecule.

   Is not affected by diffusion
   Lifetime is not sensitive for the static quenching

   ![Diagram](attachment://diagram.png)
   - Fluorophore + Quencher → Dark complex (strong complex) → No emission
   - Excitation → $h^*u$
2. Dynamic quenching

Due to the collision between the excited state fluorophore and the quencher some of the fluorophore become de-excited.

- Diffusion controlled process
- Decrease of fluorescence intensity and lifetime!

Fluorophore + Quencher → Collision complex (weak complex) → Fluorophore + Quencher

Excitation $h^*\nu$

How to measure the quenching?

Intensity vs. Concentration of quencher
The slope of the straight line gives the Stern-Volmer constant ($K_{SV}$).

Stern-Volmer equation

If the lifetime of the fluorophor is analysed as the function of quencher concentration, the result will be linear correlation.

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0$$

$\tau = 1 + K_{SV} q$

**St ern-Volmer constant ($K_{SV}$):**

Informs about the accessibility of the fluorophor

**Dynamic quenching**

$$K_{SV} = k_q \tau_0$$

$k_q$: bimolecular rate constant, informs about the diffusion ability of the fluorophor and quencher, the accessibility of the fluorophor
**Stern-Volmer equation**

\[
\frac{F_0}{F} = 1 + K_{sv}[Q]
\]

- \(F_0\): Fluorescence intensity without quencher
- \(F\): Fluorescence intensity with quencher
- \(K_{sv}\): Stern-Volmer constant
- \([Q]\): Concentration of quencher

**Modified Stern-Volmer equation (Lehrer-equation)**

More than one population of the fluorophores with different accessibilities to the quenchers.

\[
\frac{F_0}{F_0 - F} = \frac{F_0}{\Delta F} = \frac{1}{\alpha} + \frac{1}{\alpha \cdot K_{sv} [Q]}
\]

\(\alpha\): the fraction of the initial fluorescence which is accessible to the quencher
How can we decide the type of the quenching?

1. Neutral:
   - acrylamid, nitroxis
   - maping steric behavior

2. Charged:
   - iodide, cesium, cobalt
   - maping the charged surroundings

<table>
<thead>
<tr>
<th>Types of quenchers</th>
<th>Typical fluorophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Bases</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Neutral</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Iodide</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Halogen</td>
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</tr>
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</tr>
<tr>
<td>Hydrosilylating</td>
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</table>
Quenching of tryptophan

Application of quenching

• Membrane permeability
• Determination of diffusion constant
• Investigation of conformational states of proteins
Fluorescence anisotropy

Polarized light is used in anisotropy measurements

Absorption vector shows the direction in which the fluorophore prefer to absorb light

Light emission

If a fluorescent molecule is excited with polarized light the emission will also be polarized. The extent of polarization of the emission is usually described in terms of anisotropy ($r$).

The emission vector shows the direction in which the fluorophor prefers to emit.

From a random organized population select a subpopulation, which absorption vector is parallel to the electric vector of the excitation light.

If a larger molecule interacts with the fluorescent molecule the rotation of the complex will be slower than of the unbound molecules and result in an increase in the fluorescence anisotropy.
Fluorescence anisotropy

Emission anisotropy:
Difference of intensity vectors normalized to the total intensity
Anisotropy of different fluorophores is additive

\[ I_{\text{sum}} = I_x + I_y + I_z \]
\[ I_{\text{sum}} = I_x + I_y + I_z \]
\[ I_{\text{sum}} = I_x + 2I_y \]

\[ r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2GI_{VH}} \]

\( r \): limiting anisotropy ("frozen molecule", if \( r \rightarrow \infty \))
\( \tau \): fluorescence lifetime
\( \phi \): rotational correlation time ("flexibility")
\( D \): diffusion coefficient

Stokes – Einstein relationship

\[ \phi = \frac{V\eta}{RT} \]

\( V \): fluorescent volume
\( \eta \): viscosity of the medium
\( T \): absolute temperature
\( R \): universal gas constant

"V" could be determined

Perrin equation

Francis Perrin - 1926

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

\( I_{VV} \): The observed intensity when the emission polarizer is oriented parallel to the direction of the polarized excitation.
\( I_{VH} \): The observed intensity when polarizer is perpendicular to the excitation.
\( G \): G factor, correction factor
\( G = I_{\text{vert}}/I_{\text{hor}} \), the sensitivity of the detector and the monochromator is not the same for the vertically and horizontally polarized light.
Application of anisotropy

- Determination of rotation diffusion
- Detection of intermolecular interaction
- Observation of conformational changing

Förster/Fluorescence Resonance Energy Transfer (FRET)

Non-radiative dipole-dipole interaction between a donor and an acceptor fluorophore. The donor fluorophore gives the excited state energy to the acceptor fluorophore.

\[ k_f \]

<table>
<thead>
<tr>
<th>( k_{ex} )</th>
<th>( k_{nf} )</th>
<th>( k_f )</th>
<th>( k_t )</th>
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Theodor Förster (1910-1974)
The criteria for FRET

- Donor and acceptor fluorophores
- Distance range between 2-10nm
- Appropriate orientation of the dipoles of the fluorophores
- Spectral overlap between the emission of the donor and the absorption of the acceptor

\[ k_t = \text{const.} \times J(\lambda) \, n^{-4} k_t R^{-6} k^2 \]

How can we calculate the energy transfer?

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]

- $\tau_{DA}$: lifetime of donor in the presence of acceptor
- $\tau_D$: lifetime of donor in the absence of acceptor

\[ E = 1 - \frac{F_{DA}}{F_D} \]

- $F_{DA}$: fluorescence intensity of donor in the presence of acceptor
- $F_D$: fluorescence intensity of donor in the absence of acceptor
Förster critical distance \((R_0)\):

Distance between the donor and acceptor where the energy transfer is half of the maximum.

\[
E = \frac{R_0^6}{R_0^6 + R^6}
\]
**Types of energy transfer**

- **Heterotransfer**: between different fluorophore
- **Homotransfer**: between identical fluorophore
The access of the fluorophore

The nucleotide binding cleft shifted into an open conformation in the presence of profilin. The nucleotide binding cleft shifted into a closed conformation in the presence of cofilin.
Applications of FRET

- Measuring distance (molecular ruler)
- Conformation of proteins
- Interaction of proteins
- Dissociation of macromolecules (e.g. DNA)

FRET Detection of *in vivo* Protein-Protein Interactions

**Figure 1**

- Protein Labeled with Two Fluorochromes
- Protein Conformational Change
- Donor Excitation
- No Acceptor Fluorescence
- 12 Nanometer Separation Distance

**Figure 2**

- Blue Fluorescent Protein 380 Nanometer Excitation
- BFP
- Separated Protein Molecules
- GFP
- No Green Fluorescent Protein Emission at 510 Nanometers

- Intermolecular Association
- BFP
- GFP
- Green Fluorescent Protein Emission at 510 Nanometers
1. The lifetime of the donor

![Graph showing the lifetime of the donor with intensity, IRF, and fit lines along with lifetime decay data.

<table>
<thead>
<tr>
<th>Lifetime (ns)</th>
<th>Intensity Fraction</th>
<th>Pre-Exponential</th>
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<tbody>
<tr>
<td>$\tau_1$</td>
<td>1.67 ± 0.02</td>
<td>0.596 ± 0.006</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>5.98 ± 0.02</td>
<td>0.575 ± 0.005</td>
</tr>
<tr>
<td>$\tau_3$</td>
<td>0.358 ± 0.01</td>
<td>0.173</td>
</tr>
</tbody>
</table>

2. The lifetime of donor and acceptor

![Graph showing the lifetime of donor and acceptor with intensity, IRF, and fit lines along with lifetime decay data.

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<tr>
<td>$\tau_1$</td>
<td>2.61 ± 0.06</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.05 ± 0.03</td>
<td>0.521 ± 0.01</td>
</tr>
<tr>
<td>$\tau_3$</td>
<td>0.271 ± 0.01</td>
<td>0.259 FIXED</td>
</tr>
</tbody>
</table>

Where $\tau_1$, $\tau_2$, and $\tau_3$ are the lifetimes of the donor and acceptor, respectively, with their corresponding intensity fractions and pre-exponential factors.
Calculation of FRET efficiency

In case of lifetime measurement

\[ E = 1 - \left( \frac{T_{DA}}{T_D} \right) \]

Average lifetimes:

\[ T_D = 2.959 \text{ ns} \]
\[ T_{DA} = 1.191 \text{ ns} \]

\[ E = 59.8\% \]

2. The fluorescence intensity of the donor

![Fluorescence intensity graph](image)
The fluorescence intensity of the donor and the acceptor

Calculation of the FRET efficiency

\[ E = 1 - \left( \frac{F_{DA}}{F_D} \right) \]

\[ F_{DA} = 4738510 \]

\[ F_D = 10740400 \]

\[ E = 55.88\% \]
Thank you for your attention!