Fluorescence quenching, Fluorescence Resonance energy transfer (FRET)

Biophysics seminar
29. 02. 2012.

http://en.wikipedia.org/wiki/Quenching_%28fluorescence%29
Fluorescence quenching

• The decrease of the fluorescence intensity by molecules able to interact with the fluorophor.

• Quencher is a special molecule which is responsible for the quenching. It can take away the excited energy of the fluorophor.

• The quenching process competes with the fluorescence emission.

Decrease of the fluorescence intensity!
**Types of fluorescence quenching**

### Static quenching
- Due to the formation of dark complex between the ground state fluorofor and the quencher some of the fluorofor behave as a non-fluorescence molecule.
- Decrease of fluorescence intensity!
- Lifetime is not sensitive for the static quenching!

#### Diagram: Static quenching

- **Fluorofor** + **Quencher** → **Dark complex (strong complex)** → **No emission!**
- Excitation: $h \nu$

### Dynamic quenching
- Due to the collision between the excited state fluorofor and the quencher some of the fluorofor become de-excited.
- Diffusion controlled process.
- Decrease of fluorescence intensity and lifetime!

#### Diagram: Dynamic quenching

- **Fluorofor** + **Quencher** → **Collision complex (weak complex)** → **Fluorofor** + **Quencher**
- Excitation: $h \nu$
How to plot the results of the quenching measurement?

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.

\[
\frac{\tau_0}{\tau_D} = 1 + k_q \tau_0 \quad \text{q} = 1 + K_{SV} \quad \text{q} \quad \frac{F_0}{F} = 1 + K_{sv}[Q]
\]

**Stern-Volmer constant** ($K_{sv}$)

Inform 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.

Otto Stern (1888-1969)
Physical Nobel Prize (1943)
How can we decide the type of the quenching?

Static quenching

Dynamic quenching
Application of quenching

- Membrane permeability
- Determination of diffusion constant
- Investigation of conformational states of proteins
The access of the fluorophor

Actin monomer

How does the binding side change in the presence of an actin binding protein?
\[ K_{sv_2} = 53.6 \text{ M}^{-1} \text{ (free } \varepsilon\text{-ATP)} \]
\[ K_{sv_1} = 0.28 \text{ M}^{-1} \]
\[ K_{sv_{cofilin}} = 0.09 \text{ M}^{-1} \]
\[ K_{sv_{profilin}} = 1.02 \text{ M}^{-1} \]

The nucelotide binding cleft shifted into an open conformation in the presence of profilin. The nucelotide binding cleft shifted into a closed conformation in the presence of cofilin.
Perrin suggested that energy could be transferred over distances longer than the molecular diameters.

Theodor Förster developed the theoretical basis of FRET (1946).

- **Förster type energy transfer**: energy transfer between a donor and an acceptor molecule through dipole-dipole interactions.

- **Radiationless**: photons are not involved.

- **Fluorescence Resonance Energy Transfer (FRET)**: energy transfer between fluorophores.
• *Apolar* molecule: uniform charge distribution.
• *Polar* molecule: non-uniform charge distribution (the positively and negatively charged parts are separated).

→ **Dipole-molecule**: polar molecule with two poles.
**FRET** is a special type of quenching of the fluorescence which can decrease the fluorescence intensity of a given fluorophore (donor).

**Donor**: the source of the fluorescence.

**Acceptor**: a fluorophore that can absorb the energy accumulated in the donor molecule.
Molecular mechanism of FRET
Conditions has to be fulfilled

- **Fluorescent** donor and acceptor molecule.
- The distance (R) between the donor and acceptor molecule **2-10 nm**!
- Appropriate orientation of the dipoles of the fluorophores
- **Overlap** between the donor’s emission spectrum and the acceptor’s absorption spectrum.
Distance dependence of FRET

Förster distance in energy resonance transfer

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

distance between the fluorophores
Distance dependence of FRET

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

\[ E = \frac{R_0^2}{R_0^2 + R^2} \]
How to determine the efficiency of FRET?

1. Time-dependent measurements (fluorescence lifetime)

\[ E = 1 - \left( \frac{\tau_{DA}}{\tau_D} \right) \]

2. „steady-state” measurements

\[ E = 1 - \left( \frac{F_{DA}}{F_D} \right) \]
The lifetime of the donor

\begin{tabular}{|c|c|c|c|c|}
\hline
Lifetime & [ns] & Intensity Fraction & Pre-Exponential \\
\hline
\(\tau_1\) & 1.67 & 0.598 & 35.7 \\
\hline
\(\tau_2\) & 5.08 & 0.575 & 11.3 \\
\hline
\(\tau_3\) & 0.358 & 0.173 & FIXED \ \\
\hline
\end{tabular}
The lifetime of donor and acceptor

<table>
<thead>
<tr>
<th>Lifetime ( [\text{ns}] )</th>
<th>Intensity Fraction ( f )</th>
<th>Pre-Exponential ( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_1 ) 2.61 ± 0.06</td>
<td>0.22 ( \pm 0.01 )</td>
<td>8.42</td>
</tr>
<tr>
<td>( \tau_2 ) 1.05 ± 0.03</td>
<td>0.521 ( \pm 0.01 )</td>
<td>49.7</td>
</tr>
<tr>
<td>( \tau_3 ) 0.271 ± 0.01</td>
<td>0.259 FIXED</td>
<td>95.6</td>
</tr>
</tbody>
</table>
Calculation of FRET efficiency

In case of lifetime measurement

\[ E = 1 - \left( \frac{\tau_{DA}}{\tau_D} \right) \]

Average lifetimes:

\[ \tau_D = 2.959 \text{ ns} \]
\[ \tau_{DA} = 1.191 \text{ ns} \]

\[ E = 59.8\% \]
2. The fluorescence intensity of the donor

![Graph showing fluorescence intensity vs. wavelength (nm)]
The fluorescence intensity of the donor and the acceptor

![Fluorescence intensity graph](image)
Calculation of the FRET efficiency

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

\[ F_{DA} = 4738510 \]

\[ F_D = 10740400 \]

\[ E = 55.88\% \]
How to calculate the distance between the fluorophores?

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

Förster critical distance \((R_0)\):

Distance between the donor and acceptor where the energy transfer is half of the maximum.
How to calculate the Förster critical distance?
The emission spectrum of the donor (tryptophan)

The absorption spectrum of the acceptor (IAEDANS)
Calculate the distance between the fluorophores!

$R_0 = 2,473 \text{ nm}$

$R = 2,158 \text{ nm}$
Application of FRET

- Measuring distance (molecular tape)
- Conformation of proteins
- Interaction of proteins
- Dissociation of macromolecules (pl. DNA)
Investigation of protein-protein interaction

FRET Detection of *in vivo* Protein-Protein Interactions

- **Blue Fluorescent Protein**
  - 380 Nanometer Excitation
  - Separated Protein Molecules
  - BFP

- **Green Fluorescent Protein**
  - No Green Fluorescent Protein Emission at 510 Nanometers
  - GFP

Figure 2

Intermolecular Association

- **Blue Fluorescent Protein**
  - 380 Nanometer Excitation
  - BFP

- **Green Fluorescent Protein**
  - Emission at 510 Nanometers
  - GFP
Movies show two examples of HeLa cells microinjected with 10 ng/μg Cyclin A-Cer and Cdk1YFP with images taken every 4 minutes starting 2 hours after microinjection. Images are overlays of the DIC image (greyscale) and the acceptor-normalized FRET efficiency (colour).
Thank you for your attention!