FRET and FLIM: monitoring biosensor activities and protein interactions in living cells

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Overview

• Förster (Fluorescence) resonance energy transfer (FRET):
  ➢ Why FRET?
  ➢ Spectral overlap and spectral bleedthrough.

• Methods used to detect FRET:
  ➢ FRET by spectral bleedthrough correction.
  ➢ Acceptor photo-bleaching FRET.
  ➢ Fluorescence lifetime imaging microscopy (FLIM).

➢ Motive - introduce FLIM method for intravital imaging.

Resolving power of the light microscope

• The wavelength of the illuminating light and the numerical aperture (NA) of the objective limit optical resolution of the conventional light microscope to ~200 nm:

  Rayleigh equation for resolution:
  \[ r = \frac{1.22 \lambda}{2NA} \text{ or more simply } r = \frac{0.61 \lambda}{NA} \]

  where \( r \) is the distance separating two adjacent particles that can be resolved.


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What is FRET and why use it?

- Newer super-resolution techniques offer several fold improvement in resolution (40 – 100 nm; SIM, STED, PALM...).
- Much greater resolution is necessary to detect protein-protein interactions or biosensor conformational changes in living cells.
- FRET microscopy measures the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor that can only occur over a distance less than 80Å.
- FRET microscopy can provide measurements of the spatial relationship between fluorophores on the scale of angstroms.

FRET measures the spatial relationship between fluorophores

- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore (the acceptor - A) enters the electric field, energy can be transferred directly by dipole-dipole coupling.
- No intermediate photon!

Förster resonance energy transfer

- Theodor Förster described how FRET efficiency decreased as the inverse of the sixth power of the separation distance.

\[ E_{\text{FRET}} = \frac{R_0^6}{R_0^6 + r^6} \]

- The detection of FRET indicates the fluorophores are separated by less than ~ 80Å.

Overview

- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.
Spectral overlap is necessary for FRET

- Förster recognized that the spectral overlap determines the probability of resonance.
- The donor emission spectrum must significantly overlap the absorption spectrum of the acceptor;
- The more overlap → more efficient energy transfer.

Spectral bleedthrough background signals

- The more overlap, the more background → spectral bleedthrough (SBT).
- Acceptor cross-talk: direct acceptor excitation
- Donor bleedthrough: emission in FRET channel
- SBT: 
  - Quenched emission
  - Sensitized emission
  - Direct acceptor excitation
  - Donor emission bleedthrough

Spectral bleedthrough background signals

- The accurate measurement of FRET by sensitized acceptor emission requires removal of SBT!
- Alternative methods that detect the effect of FRET on the donor are not subject to SBT.*
- There can be acceptor back-bleedthrough if the donor band-pass is too wide.
Overview

- Förster (Fluorescence) resonance energy transfer (FRET):
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Methods used to detect FRET:

The genetically encoded ‘FRET Standards’

- Numerous methods have been developed to measure FRET, but their accuracy is difficult to determine.

- A simple solution was to develop FRET reference proteins derived from genetically encoded FP fusion proteins.

- Several different FRET methods are used to acquire measurements from cells expressing the fusion proteins to verify their use as standards.

- Model to compare FRET donors and acceptors.

The use and abuse of FRET imaging

- The genetically encoded FPs led to a dramatic increase in the use of FRET measurements to detect protein interactions in living cells.

- A consequence of this newfound popularity has been the “degradation in the validity of the interpretations” of these experiments.
  

- It is often difficult to directly compare the accuracies of different methods.

  - Use “FRET Standards” to characterize the experimental system.

  - Use more than one FRET method to verify the results.

Methods used to measure FRET

- There are many different ways to measure FRET (here are a few):

  1. Spectral bleedthrough correction:
     
     \[ E = \text{FRET} - \text{[Spectral cross-talk]} \]

  2. Acceptor photo-bleaching:
     
     \[ E = \frac{(I_{\text{post}} - I_{\text{pre}})}{I_{\text{post}}} \]

  3. Donor lifetime measurements:
     
     \[ E = 1 - \frac{I_{\text{DA}}}{I_{\text{D}}} \]

- For intravital imaging - ratiometric imaging of biosensors (1:1 donor:acceptor) or lifetime are the most useful methods to measure FRET.
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Spectral overlap is necessary for FRET

- mTurquoise and mVenus are among the better FP FRET pairs because they have substantial spectral overlap:
  
  \[
  J(\lambda) = \left( \frac{2}{3} \right) (92,200) (538)^4 = 2.2 \times 10^{18}
  \]

  \[
  R_0 = 0.211 \left( \frac{92,200}{(1.4)^4 \times (0.84) \times (2.2 \times 10^{18})} \right)^{1/4} = 56\text{Å}
  \]

  mTurquoise QY = 0.84
  mVenus EC = 92,200 M\(^{-1}\) cm\(^{-1}\)

Spectral bleedthrough correction methods

- mTurquoise and mVenus are among the better FP FRET pairs because they have substantial spectral overlap:

- but spectral bleedthrough into the FRET channel is a significant problem.

- Correction techniques are needed to identify and remove spectral bleedthrough from the FRET channel.

FRET by spectral bleedthrough correction

- Most imaging systems come with a computer algorithm for SBT correction (there are Image J plugins).
  - SBT background images:
    A. Donor alone - Don Channel
    B. Donor alone - FRET Channel
    C. Acceptor alone - FRET Channel
    D. Acceptor alone - Acc Channel
  - Experimental images:
    E. Experimental - Don Channel
    F. Experimental - FRET Channel
    G. Experimental - Acc Channel
FRET by spectral bleedthrough correction

• The two spectral crosstalk components, determined from the control cell measurements, are removed from the FRET image.

Spectral FRET measurements

• Energy transfer results in quenching of donor emission and sensitized emission from the acceptor.

• Exciting the donor and acquiring the complete emission spectrum detects both events.

• This approach is called spectral FRET.

FRET by spectral bleedthrough correction: negative control

• Spectral FRET uses reference spectra obtained from cells expressing either the donor alone or acceptor alone.

• The method of linear unmixing removes donor spectral bleed through (DSBT).

• Bleedthrough correction removes acceptor spectral bleedthrough (ASBT) from each image pixel.
FRET by spectral bleedthrough correction

• Advantages:
  ◦ Simple algorithms available on most imaging systems.
  ◦ Compatible with most types of imaging (difficulties with 2-photon).

• Limitations:
  ◦ Very sensitive to quality of the control data.
  ◦ Subject to artifacts of cell movement.

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    ◦ Acceptor photo-bleaching FRET.

Acceptor photo-bleaching

• Energy transfer results in quenching of the D emission and sensitized emission from the A.

• Photo-bleaching the acceptor relieves donor quenching (de-quenching).

• The increase in the donor signal is detected in the donor channel, which is free of SBT background.*

* There can be acceptor back-bleedthrough if the donor band-pass is too wide.
• C/EBPα dimerizes only when bound to DNA in the cell nucleus.
• C/EBPα binds to repeated DNA elements in heterochromatin.

FRET quenches the donor emission

• C/EBPα binds to repeated DNA elements in heterochromatin.

Acceptor photo-bleaching dequenches the donor

Confirming spectral FRET using acceptor photo-bleaching

• pb-FRET is commonly used to verify other FRET-based measurements.
• pb-FRET can be used in combination with other FRET methods – e.g., verify SBT correction methods.
• But, pb-FRET is an end point assay.
FRET by acceptor photo-bleaching

- **Advantages:**
  - Simple approach that uses each cell as its own control; it can be very accurate.
  - Commonly used to verify results from other methods.

- **Limitations:**
  - Requires selective bleaching; dark-states and photo-switching can be problematic.
  - Subject to artifacts of cell movement – limited to stable protein interactions in living cells.
  - Endpoint assay - no dynamics.

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- **Methods used to detect FRET:**
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  - Fluorescence lifetime imaging microscopy (FLIM).

Spectral bleedthrough background signals

- **Methods that detect the effect of FRET on the donor are not subject to SBT.**

  - Fluorescence lifetime imaging microscopy (FLIM) measures the effect of FRET on the donor.

  - Spectral bleedthrough background signals

  - A mixture of these probes with overlapping emissions can be separated by their individual lifetimes!
FRET by FLIM

- Events in the probe environment that affect the excited state can change the lifetime – molecular stop watch.
- FRET is a quenching event (k_{ET}), allowing transition to the ground state without fluorescence emission.
- Quenching events cause the fluorescence lifetime to shorten.

- Fluorescence lifetime imaging microscopy (FLIM) can accurately measure the change in lifetime resulting from FRET.

FRET by FLIM

- To measure FRET by FLIM, only two measurements are required:
  - donor lifetime in the absence of acceptor (\( t_D \)).
  - donor lifetime in the presence of acceptor (\( t_{DA} \)).

\[ E_{FRET} = 1 - \left( \frac{t_{DA}}{t_D} \right) \]

- Two different FLIM methods:
  - Time domain - uses a femtosecond pulsed laser and gated camera, and determines lifetimes by exponential decay fitting methods.
  - Frequency domain - uses excitation light modulated at radio frequencies, and determines lifetimes directly by phasor analysis.

- The physics that underlies the two methods is the same – they only differ in how the signals are analyzed.

Frequency domain FLIM measurements

- In frequency domain, a continuous light source is modulated at high radio frequencies (usually 1-400 MHz) to excite the fluorophores.
- The emission signal from the fluorophores will also be modulated:

\[ \Phi \] and change in \( M \) are used to extract the fluorescence lifetime.

The phasor plot is used to display frequency characteristics of the emission signal.

The phasor is the projection of the emission signal waveform as a vector rotating about the origin in a complex plane.

The cosine function projects a vector onto the real axis, and the phase angle (α) formed with the real axis measures the phase delay.

The phasor plot maps the Φ and M from every image pixel using the Fourier transform components S and G.

For fluorophores with single component decays, the relationship:

\[ M = \cos \Phi \]

describes vectors with an endpoints falling somewhere on a universal semicircle.

Coumarin 6 has a single-component lifetime of 2.5 ns.

The phasor plot is a vector representation of the Φ and M at every pixel in the image.
FLIM analysis by phasor plot

- HPTS has spectral characteristics similar to Coumarin6, but a single-component lifetime of 5.3 ns.
- The phasor plot is made directly from the frequency data at each pixel – there is no fitting to determine lifetimes.

Phasor plot measurements of probe mixtures

- The lifetime distribution for mCerulean falls inside the universal semicircle, indicating the presence of more than one lifetime component; it is best fit to a two-component decay, with an average lifetime of 3 ns.
- The lifetime distribution for the mixture falls on a straight line between the distributions for the pure components. The position on the line determines their relative contribution.
- Despite the identical emission profiles for these two FPs, their distinct lifetimes allow phasor analysis to be used to determine the relative contribution of each to a mixture.

Intensity image: 256 x 256 – 65,536 pixels

HPTS

Phasor plot measurements of probe mixtures

- Cerulean and Turquoise2 have identical spectra, but different lifetimes.
- The lifetime distribution for mTurquoise2 falls on the semicircle, indicating a single component lifetime decay of 4.5 ns.
- The lifetime distribution for the mixture falls on a straight line between the distributions for the pure components.
Intramolecular FRET: measurements of the FRET standards

- To validate measurements of FRET, FRET standards were generated using Turquoise coupled to Venus through progressively longer linkers.

<table>
<thead>
<tr>
<th>FRET Standard</th>
<th>Linker composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turquoise-5 aa - Amber</td>
<td>-SGLRS-</td>
</tr>
<tr>
<td>Turquoise-5 aa - Venus</td>
<td>-SGLRSPPVAT-</td>
</tr>
<tr>
<td>Turquoise-10 aa - Venus</td>
<td>-SGLRQAASNADVGT-</td>
</tr>
<tr>
<td>Turquoise-17 aa - Venus</td>
<td>-SGLRSENLYFGPREFPGGTRGSLGRTADGVAT-</td>
</tr>
<tr>
<td>Turquoise-27 aa - Venus</td>
<td>-SGLRSENLYFGPREFPGGTRGSLGRTADGVAT-</td>
</tr>
<tr>
<td>Turquoise-36 aa - Venus</td>
<td>-SGLRSENLYFGPREFPGGTRGSLGRTADGVAT-</td>
</tr>
<tr>
<td>Turquoise-46 aa - Venus</td>
<td>-SGLRSENLYFGPREFPGGTRGSLGRTADGVAT-</td>
</tr>
</tbody>
</table>

- Fusions were also created with Amber - the Y66C mutant of Venus that folds correctly, but does not act as a FRET acceptor.

FLIM analysis by phasor plot: FRET standards in living cells

- Turq-5aa-Amber has a single lifetime of 3.8 ns.
- Turq-46aa-Ven is weakly quenched: EFRET = 1 – (t_{DA}/t_{D}) = 24%
- Turq-5aa-Ven is strongly quenched: EFRET = 1 – (t_{DA}/t_{D}) = 43%
Verifying FRET with acceptor photo-bleaching

- The Turquoise-5aa-Venus is highly quenched because of FRET.

![Graph showing donor intensity image](image)

- Photobleaching Venus results in de-quenching and a return to the radiative lifetime of mTurquoise (3.8 ns).

![Graph showing FRET by FLIM](image)

**FRET by FLIM**

- Advantages:
  - Time-resolved measurements are not affected by excitation intensity, probe concentration, or light scatter.
  - The frequency characteristics are directly measured from each image pixel - there is no fitting, and no a priori knowledge of the biology is required.
  - Quenched and unquenched probe populations can be quantified.
  - Label-free intravital imaging of metabolic states.

- Limitations:
  - System and analysis are complex.
  - Photon-intensive - but only if you need to separate the component contributions – the phasor plot provides a method to visualize cell physiology.

**Intravital Imaging**

- Goal: To use intravital imaging of the kidney to evaluate the molecular and cellular basis of physiology and pathophysiology.

- Challenges:
  - Extreme heterogeneity – vascular and epithelial components with highly coordinated functions.
  - Unique structure-function relationships that are dynamic.
  - Study of pathophysiology and disease require longitudinal measurements.
  - Probe delivery.

- Intravital FLIM imaging of complex cellular environments can overcome these challenges.