FLIM: FRET Applications

The Raichu-rac protein

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FLIM: Outline

• Assigning phasor values to
  • Background
    – Media
    – Glass
    – Fibronectin coating
    – autofluorescence

• Assigning phasor values to
  • Donor only in cells

• Defining the FRET trajectory

• Determining the FRET Efficiency
• “Radiation-less transfer of excited state energy from an initially excited donor to an acceptor”

• FRET dependent on $\kappa^2$, the “orientation factor” and $r^{-6}$, the distance between the two fluorophores (to the 6th power)

• When used with fluorescent proteins, can detect conformational changes and binding events (<10nm).

http://www.biomed.miami.edu/~sost/robin
There is >30% overlap of the Donor (CFP) emission and the Acceptor (YFP)
Lifetime of the glass coating

$\text{Tau}_P = 2.4$

$\text{Tau}_M = 4.6$
Determining the cell autofluorescence phasor

Cells only, no CFP

TauP=1.55
TauM=3.03
Locating the CFP phasor

DONOR:

CFP
TauP=2.7
TauM=3.1
FRET trajectory

$$E = 1 - \frac{\tau}{\tau_D}$$
Raichu FLIM/FRET analysis
In MEF cells

http://www.biken.osaka-u.ac.jp/biken/shuyouvirus/e-phogemon/raichu-Rac.htm
Wt Raichu (1011 variant) in MEF

\[ \text{Tau}_P = 2.2 \]
\[ \text{Tau}_M = 2.9 \]
Wt Raichu 1011
In MEF

TauP=1.9
TauM=2.6
Raichu 1012 in MEF
( constitutively active )
Raichu 1013 in MEF

N17 mutant: Not active

\[ \text{TauP} = 2.4 \]
\[ \text{TauM} = 2.9 \]

\[ \text{TauP} = 2.0 \]
\[ \text{Tau} = 2.7 \]
## Summary

<table>
<thead>
<tr>
<th></th>
<th>Phasor Lifetimes</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$ Phase</td>
<td>$\tau$ Mode</td>
</tr>
<tr>
<td><strong>wtRaichu</strong></td>
<td>2.2</td>
<td>2.9</td>
</tr>
<tr>
<td>1011</td>
<td>1.9</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Raichu</strong></td>
<td>1.9</td>
<td>2.6</td>
</tr>
<tr>
<td>1012 (always FRET)</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Raichu</strong></td>
<td>2.4</td>
<td>2.9</td>
</tr>
<tr>
<td>1013 (no or little FRET)</td>
<td>2.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>
This is a camera based wide-field microscope. It uses a modulated image intensifier at 40 MHz and a diode laser as the light source in the homodyne mode.

The original data is a series of images obtained at 3 or more different phases.

The camera is a 16 bit camera that operates up to 12 frames/s. Other faster cameras can be supported in that system.

Using this method, a complete FLIM recording can be obtained in only 250 ms.

Clegg, Holub and Gohlke have previously developed a system that can acquire lifetime images at 30 frames/s.
Data are imported into SimFCS. In the example below, this is the import screen. The sample is a fixed sample in which there are two types of cells, one expressing only **CFP** and the other cells expressing a construct which contains both **CFP** and **YFP**. The emission filter is set to measure only the CFP emission (donor emission). Data acquisition time was 1s (10 images exposed each for 100 ms at 36 degrees interval).

**QUESTIONS:** which cells express CFP only and the CFP-YFP construct? What is the FRET efficiency in this sample?
A fluorescein standard is collected before each measurement. Data are read into SImFCS and converted into pixel phasors. Of course, data can also be converted in the classical lifetime histograms.

For the aficionados of frequency domain lifetime these are the tauP and tauM images and corresponding pixel histograms.

Clearly, the lifetime histogram is broadened and there is an hint of a bimodal distribution.
The phasor representation

One spot in the phasor plot identifies the CFP expressing cell and the other spot identifies the CFP-YFP expressing cell.

In a few seconds, you can perform this analysis!
FRET efficiency calculation

The assignment for the unquenched (not FRET) sample is done using the phasor of the CFP only cell.

The assignment of the background phasor is done by decreasing the threshold to 3000 counts. With this threshold a new spot appears in the phasor plot that can be assigned to the background (note that the unlabeled sample was not available).

The calculated FRET efficiency is \textbf{0.163}. Note that the line for increasing autofluorescence is clearly visible in the phasor plot (black line joining the phasors of the CFP-YFP cells), but for most of the pixels the contribution to autofluorescence is small.

This analysis is done in a few minutes using the phasor approach!! No fit to exponential decays was necessary.