Fluorescence Lifetime Imaging Microscopy (FLIM)

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Optical Technology Development

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FRET-FLIM course, May 2009
Fluorescence Resonance Energy Transfer (FRET) Microscopy

$\text{FRET efficiency} = 1 - \frac{\tau_{\text{FRET}}}{\tau_{\text{noFRET}}}$

$\tau_{\text{FRET}} < \tau_{\text{noFRET}}$

$E = 1 - \frac{\tau_{\text{FRET}}}{\tau_{\text{noFRET}}}$
Fluorescence lifetime

Question: how quickly do excited molecules relax back to the ground state?

Since emission is a spontaneous process, its rate is proportional to the concentration of molecules in the excited state \([N^*]\):

\[
\frac{d[N^*]}{dt} = -(k_f + k_x)[N^*] \implies N(t) = N_0 \exp(-t/\tau_{ex})
\]

**Exponential decay:**

- Intrinsic or natural lifetime: \(\tau_f = \frac{1}{k_{\text{fluor}}}\)
- Experimental lifetime: \(\tau_{ex} = \frac{1}{k_{\text{fluor}} + k_{\text{nonfluor}}}\)

The experimentally determined excited state lifetime is always smaller than the theoretical one:

\[\tau_{ex} = \tau_f \cdot Q\]

The larger the quantum yield, the longer \(\tau_{ex}\).
Fluorescence lifetime

\[ I = I_o e^{-t/\tau} \]
Lifetime measurements

**Pulse or Time-Domain**

\[
\frac{dN}{dt} = -(k_f + k_x)N = -\frac{1}{\tau} N
\]

\[
I(t) = I_0 e^{-t/\tau}
\]

**Phase-modulation or Frequency-Domain**

\[
\tan \Phi = \omega \tau_p
\]

\[
m = \left[1 + \omega^2 \tau_m^2\right]^{-1/2}
\]

\[
\tau_p = \frac{\tan \Phi}{\omega}
\]

\[
\tau_m = \left[\frac{1}{m^2} - 1\right]^{1/2}
\]

\[
\tau_p = \tau_m \quad \text{single exponential decay}
\]

\[
\tau_p \neq \tau_m \quad \text{multi-exponential decay}
\]
2 Approaches to Measure FLIM in Time-Domain

**Time Correlated Single Photon Counting (TCSPC)**
- Upgrade for scanning microscopes
- High temporal and spatial resolution
- Slow (30-60 s per FLIM image)

=> useful for fixed samples

**Timegated Intensified CCD Camera**
- Upgrade for all camera based microscope (wide-field, TIRF, spinning-disc, 2-photon)
- Lower spatial resolution due to intensifier
- Fast (0.5 – 2 s per FLIM image)

=> suitable for live cell imaging
Measurement of fluorescence lifetimes: TCSPC

- Measurement of delay times between absorption and fluorescence of a photon.
- Plot in logarithmic scale to yield a histogram, which is in the simplest case fitted by a straight line.
- Caution! Limited to 1 photon per pulse! Doesn't work at high emission signals.
- Usually 1 emitted photon per 50 - 100 excitation pulses.
Fluorescence lifetime imaging microscopy (FLIM) with a time-gated CCD camera
Measurement of fluorescence lifetimes: Time-gated CCD camera

Fluorescence lifetime can be extracted from only 2 images (timepoints)

$$\tau = \frac{\Delta t}{\ln(I_{t1} / I_{t2})}$$
Dynamics and interaction by live-cell FLIM

- Hela cells imaged every 5 min for 10 h @ 37°C
- FLIM stack acquired in approx. 1 s
# Fluorescence Lifetime of XFPs

## Curve Fit

<table>
<thead>
<tr>
<th></th>
<th>FLIM</th>
<th>Curve Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tau / ns</td>
<td>sd</td>
</tr>
<tr>
<td>CFP</td>
<td>2.22</td>
<td>0.03</td>
</tr>
<tr>
<td>Cerulean</td>
<td>1.93</td>
<td>0.02</td>
</tr>
<tr>
<td>GFP</td>
<td>2.05</td>
<td>0.13</td>
</tr>
<tr>
<td>YFP</td>
<td>2.38</td>
<td>0.15</td>
</tr>
<tr>
<td>Venus</td>
<td>2.47</td>
<td>0.13</td>
</tr>
</tbody>
</table>

### XFPs fluorescence decay

**HeLa cells**

- **CFP**
- **Cerulean**
- **GFP**
- **YFP**
- **Venus**
CFP and Cerulean show a multi-exponential decay
Observing protein-RNA interaction via FRET-FLIM inside cells

- Protein is tagged with a yellow version of GFP
- RNA is stained with a red intercalator dye

Fluorescence lifetime imaging microscopy (FLIM)

\[
\tau_{\text{FRET}} < \tau_{\text{no FRET}}
\]
Examples of lifetime measurements

Time-resolved data can provide information not available from steady-state fluorescence measurements.

- Distinguish static and dynamic quenching
- Separate dyes with similar spectral properties by their lifetime
- Distinguish population of dyes rather than an average value (e.g., in FRET)

A protein contains two tryptophan residues, each with a distinct lifetime. Because of spectral overlap of the absorption and emission, it is not possible to resolve the emission from the two residues from steady-state data. However, time-resolved data can distinguish between both of them indicating a quenching of one of them (shorter lifetime).
Surface Plasmon Resonance: Reduction of the fluorescence intensity and lifetime close to metallic surfaces

\[ \tau = \frac{1}{k_{rad} + k_{SPP} + k_{LM}} \]
Measuring the height of microtubules by SPR-FLIM

Lifetime of Alexa488-labeled microtubules

- Glass surface: \( \tau = 3.1 \text{ ns} \)
- Gold surface: \( \tau = 2.0 \text{ ns} \)

(collaboration with M. Berndt & S. Diez)
Auto-fluorescence has typically a shorter lifetime (<0.5-1 ns) than fluorescence dyes or fluorescent proteins (> 2 ns).

Time-gated imaging decreases auto-fluorescence and improves S/N.
Unmixing of GFP, YFP, and Venus expressing cells by fluorescence lifetime imaging microscopy
FLIM microscope can be used for ...

... lifetime measurements
- observe environment (e.g. pH, membrane lipids composition)
- ion imaging (e.g. Ca$^{2+}$, Zn$^{2+}$, Na$^{+}$, K$^{+}$)
- separation of spectral similar fluorophores (e.g. GFP & YFP)

... FRET
- protein-protein interactions
- protein activity due to conformational changes
- DNA-protein interactions
- RNA-protein association
- Several interaction in parallel (???)

... time-resolved fluorescence microscopy
- separation of spectral similar fluorophores
- reduction of autofluorescence (tau < 0.5ns)

... time-resolved anisotropy measurements
... ???
Literature

Review

Widefield-FLIM setup
Lorenz, RNA (2009), 97.