Digital Frequency Domain Fluorescence Lifetime Imaging

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Lifetime, the Principle

Excit. state:

<τ>

Decay rate $k_F = 1/\tau$ for fluorescent emission.

Ground state:

Probability distribution of fluorescence emission: $e^{-t/\tau}$

(τ usually a couple nanoseconds for fluorescent proteins)
Measuring Lifetime

- The exponential decay curve for fluorescence lifetime is just a probability distribution.
- Real data is very sparse.

Therefore, to figure out the lifetime from photon counts, we have to build up a histogram of photons.
Building a Histogram for Lifetime

Time Correlated Single Photon Counting Systems (TCSPC)

- Uses a Time-to-Amplitude Converter (TAC) or Time-to-Digital Converter (TDC) to measure photon arrival times with picosecond precision.

- Histogram is built from the time delays.

- Precise, but expensive components!
Analog Frequency Domain Systems

- Modulates the gain of a PMT, or an image intensifier
- Throws away photons
- Uses photons inefficiently

\[ f_{cc} = \left| f_s - f_{ex} \right| \]

Excitation:

Emission prob.:

PMT Gain: (heterodyning)

Cross-correlated signal:

(Integrated by analog electronics)
Is there a better way?

- Digital Frequency Domain FLIM (DFD-FLIM)
  - Uses heterodyning like the analog frequency domain approach.
  - Uses digital counters to mark when photons arrive.
  - Accumulates photon counts to construct a histogram.
  - Precise and cheap!
DFD-FLIM: Heterodyning

- Samples entire decay curve.
- The emission is translated to a cross-correlation frequency:
  \[ f_{cc} = |f_s - f_{ex}| \]
A fast counter tags photon arrival times with an arrival “window” number.
Cross-correlation Phase Histogram

- Each photon count is assigned a bin number, based on the window it arrived in, and the phase offset between the sampling and excitation clocks:

\[ p = 63 - \left[ \left( p_{\text{ecc}} + \frac{64 w_{\text{arrival}}}{n_w} \right) \bmod 64 \right] \]

- A histogram of these photon counts is called a “phase histogram”, and it shows the lifetime response.

Experimental Fluorescein Phase Histogram:

- The phase histogram is a convolution of the exponential decay, the square sampling window, and the system jitter.
Modulated diode laser or 2-photon laser.

Two fully parallel channels.

This is the part we change for DFD-FLIM.
To maximize the precision of our measurements, we must minimize the uncertainty of the phasor position for each measurement.

This means we must reduce the uncertainty of the phase, $\phi_F$, and the uncertainty of the modulation, $m_F$. 
This “Quality Factor” is a complicated function of the modulation of the instrument, and the modulation from the fluorescence lifetime (tau & frequency).

This behavior holds true for all data acquisition methods!
Full Uncertainty Equations

Standard deviation of phase and modulation:

\[
\sigma_{\phi,F,h} = \sqrt{1 - m_{H,2h} \cos(2\phi_{H,h} - \phi_{H,2h})} \frac{m_F,h}{m_{IR,h} \sqrt{2N}}
\]

\[
\sigma_{m,F,h} = \sqrt{1 - 2m_{H,h}^2 + m_{H,2h} \cos(2\phi_{H,h} - \phi_{H,2h})} \frac{m_{IR,h}}{\sqrt{2N}}
\]

For a given number of counts, the uncertainties are primarily determined by the **modulation of the instrument response**: 

\[
m_{IR,h} = m_{E,h} m_{J,h} m_{S,h}
\]

Uncertainty reduces by the square root of N, the number of counts.
The Precision Plateau

Excitation

Sampling Windows

Jitter

$m_{E,1} = 0.9745$

$m_{S,1} = 0.9003$

$m_{J,1} = 0.9702$

$m_{IR,1} = 0.8512$

Calculated from Fourier Transform

Calculated from Fourier Transform

Measured, fluorescein @ 48MHz
Lifetime Measurements

Fluorescein (pH 10)
- 538 counts per pixel
- 200,000 counts/s

$\tau_p = 4.05 \pm 0.522 \text{ ns} \quad (\pm 2.0 \text{ ps})$
$\tau_m = 4.05 \pm 0.379 \text{ ns} \quad (\pm 1.5 \text{ ps})$

Uncertainty per pixel

<table>
<thead>
<tr>
<th></th>
<th>$\sigma_\phi$</th>
<th>$\sigma_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental:</td>
<td>3.50°</td>
<td>0.035</td>
</tr>
<tr>
<td>Analytical Est.:</td>
<td>3.50°</td>
<td>0.035</td>
</tr>
</tbody>
</table>

(Used as a reference, 4.05ns)
Lifetime Measurements

Rhodamine B
- 202 counts per pixel
- 50,000 counts/s

(Has 3% uncorrelated background)

\[ \tau_p = 1.723 \pm 0.287 \text{ ns} \]
\[ \tau_m = 2.001 \pm 0.448 \text{ ns} \]
\[ (\pm 1.1 \text{ ps}) \]
\[ (\pm 1.8 \text{ ps}) \]

Experimental:

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Analytical Est.</th>
<th>Uncertainty per pixel</th>
<th>Uncertainty of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.86°</td>
<td>3.83°</td>
<td>( \sigma_\phi )</td>
<td>( \sigma_m )</td>
</tr>
<tr>
<td>0.049</td>
<td>0.049</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These measurements show that the precision model is complete, and that it accounts for all parameters which affect the system precision.
System Stability

- Because precision improves arbitrarily with counts, the final precision is limited only by how long counts can be accumulated.

- Accuracy then becomes more important. With a photodiode for an optical phase reference, we have observed long-term phase fluctuations of around 0.1°, and long-term modulation fluctuations of less than 0.001.

- For fluorescein, this corresponds to an accuracy of around 10ps.

- Note: Both the precision (arbitrary) and the accuracy (10ps) are much smaller than the sampling window size of 5.2ns. Only the modulation value of the sampling window affects the precision, and its size does not affect the accuracy.
### A Challenge Question

Which system is more precise for a fluorescein (4.05ns) measurement?

<table>
<thead>
<tr>
<th>System 1</th>
<th>System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>- An 80MHz repetition rate.</td>
<td>- A 48MHz repetition rate.</td>
</tr>
<tr>
<td>- A femtosecond pulsed laser.</td>
<td>- A diode laser with 2.6ns pulses.</td>
</tr>
<tr>
<td>- An acquisition card which can determine each photon arrival time to within 12ps.</td>
<td>- An acquisition card which can determine each photon arrival time to within 5.2ns.</td>
</tr>
</tbody>
</table>
Comparison of Systems

Lower uncertainty, more precision.

System 1
(TCSPC, 80MHz)

System 2
(FLIMBox, 48MHz)

\[
\sigma_{\phi_h} = \sqrt{1 - \frac{m_{H,2h} \cos(2\phi_{H,h} - \phi_{H,2h})}{m_{F,h} m_{IR,h} \sqrt{2N}}}
\]
Photon Efficiency and Frequency

\[ F = \frac{\sigma_{t}}{\tau} \sqrt{N} \]

4.05ns, 48MHz
4.05ns, 80MHz

Less relative uncertainty for given counts.

Theoretical minimum for phasor analysis:

Less relative uncertainty for given counts.

(anomalous clustering, due to dead zones)
Jitter: Friend, not Foe

- As we have seen above, jitter of one or two nanoseconds has negligible impact on precision.

- For very short lifetime measurements, jitter can actually provide a substantial benefit.

- Jitter forces a narrow response to be oversampled in several bins, which improves sensitivity to small differences.

- APDs, even with their intrinsic jitter, can yield better lifetime resolution due to their higher quantum efficiencies (more counts).
Conclusions

- Digital frequency domain FLIM (DFD-FLIM) provides a cost-effective approach to FLIM for phasor analysis. Do try this at home.

- Phasor precision for a given number of counts depends on the modulation values. Consider these when evaluating a system or choosing experimental parameters.

- Accuracy is limited by the phase and modulation stability of a system. Use a good phase reference.

- Don't be afraid of a little jitter. It helps!