How to Analyze Fluorescence Lifetime Images:
An introduction to phasor analysis

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What is Fluorescence Lifetime?

Excit. state: \[ \langle \tau \rangle \]

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

Ground state:

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

(\( \tau \) usually a couple nanoseconds for fluorescent proteins)
Why Use Fluorescence Lifetime?

- The lifetime of a fluorophore can be sensitive to:
  - Chemical environment (pH, ion concentration, water, etc.)
  - Proximity to other probes (energy transfer)

- A lifetime measurement is independent of concentration.

- A lifetime measurement is more resistant to background contributions and cell autofluorescence than an intensity measurement.

- In summary: Images can be obtained with contrast based on an intrinsic property of a probe, rather than simply light intensity, which can come from many things.
How is it Measured?

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

Example methods:

- **Time Correlated Single Photon Counting** – Measures the arrival time of each photon with respect to the laser.
- **Digital Frequency Domain** – Uses heterodyning to evenly sample the shape of the decay distribution.
- **Camera Methods** – Block part of the time information to accumulate one part of the decay distribution at a time for the whole image.
In a time domain analysis, one attempts to fit the data to a sum of exponentials, one for each lifetime component.

This works okay with millions of photon counts, like in the above example. But this is much more difficult in microscopy, where we only have a few hundred counts per pixel at best.
Phasor Analysis

Mathematically speaking: The phasor coordinates, \( g \) and \( s \), are the normalized real and imaginary components of the first harmonic of the Fourier transform of the data.

\[
g_H = \frac{1}{N} \sum_{p=0}^{n_p-1} H(p) \cos\left(\frac{2\pi p}{n_p}\right) \quad s_H = \frac{1}{N} \sum_{p=0}^{n_p-1} H(p) \sin\left(\frac{2\pi p}{n_p}\right)
\]

Practically speaking: The raw lifetime data is automatically transformed by software into a visual representation of that same raw lifetime data, showing the distribution of lifetime values in an image.

Phase Histogram (Shows lifetime for a pixel)

The “phasor plot”: A histogram of phasor coordinates for every pixel in an image:

All single-exponential lifetimes are along a curve called the “Universal Circle”.

Short lifetimes and scattering, show up at the bottom right.

Long lifetimes and uncorrelated room light, show up at the bottom left.
Phasors Combine Linearly

Single exponential $\tau_1$

Single exponential $\tau_2$

All possible double exponentials combining $\tau_1$ and $\tau_2$

$$g_F = \sum_i f_i g_i$$
$$s_F = \sum_i f_i s_i$$
$$1 = \sum_i f_i$$

fractional intensity
Fractional Contribution

\[ g_F = \sum_i f_i g_i \quad s_F = \sum_i f_i s_i \quad 1 = \sum_i f_i \]

\( \tau_1 = 4.05 \text{ns} \quad \tau_2 = 1.7 \text{ns} \)

f\(_1\) is fraction of intensity due to \(\tau_1\)

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\[ (\text{Microsc Res Tech 71(3): 201-213, 2008}) \]
Förster Resonance Energy Transfer (FRET)

FRET functions as a nanometer scale “molecular ruler”. It can tell us the distance between two fluorophores. (On DNA, on proteins, aggregated, etc)

The FRET Efficiency can be determined by an intensity ratio.

It can also be determined from the lifetime of the donor.

\[ E = \frac{cI_A}{cI_A + I_D} = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \]

'c' is a spectral correction factor

(Fluorescence Imaging Spectroscopy and Microscopy, Ch. 7, 1996)
The Ideal FRET Curve

Donor-only phasor position
$E = 0$

Increasing FRET, lifetime becoming shorter.
(Donor and acceptor are closer)

(In the ideal case of zero background)

Completely quenched
$E = 1$
The Real FRET Curve

Donor-only phasor position
$E = 0$

Increasing FRET, lifetime becoming shorter.
(Donor and acceptor are closer)

As FRET increases, the donor's intensity decreases.

Emission dominated by background / autofluorescence

E = 1
In many systems one is actually observing a linear combination of donors which are not experiencing FRET, and donors which are. (Or sometimes between two FRET states.)

Then the distribution of phasors for the image will be along the purple line.
The Ideal Acceptor FRET Curve

(Same as acceptor phasor if directly excited.)
Acceptor with complete FRET
E = 1

Acceptor with no FRET
E = 0

The phasors for the acceptor emission are just like the donor emission, but rotated and scaled.

The intensities are different, however. The acceptor is bright near E=1 (more energy transferred to the acceptor), and dim near E=0.
The Real Acceptor FRET Curve

Donor emission bleedthrough (due to spectral overlap) shows up here, and dominates when no FRET occurs. $E = 0$

The donor in the presence of the acceptor would be at this position in the donor channel.

While this curve may appear complicated, it can be computed immediately by software for the given parameters.

Then all you have to do is observe where the distribution of acceptor phasors is with respect to the acceptor's FRET curve.
Combinations of Acceptor States

- Just as with the donor case, combinations of FRET states combine linearly.
- The difference with the acceptor case appears when the low-FRET state in the acceptor channel is dominated by bleedthrough from the donor emission.

Donor emission bleedthrough (due to spectral overlap) shows up here, and dominates when no FRET occurs. E = 0

Acceptor phasor position in presence of donor E = 0.6
Cartoon Example #1

Donor channel:
Two FRET States + autofluorescence

Distribution of phasors in image, as a linear combination of three contributors.

Donor-only phasor position
E = 0

Donor-acceptor phasor position
E = 0.6

Cell autofluorescence

If we select the phasors inside this circle, and highlight those parts of the image, then we can immediately see which parts of the image are largely dominated by donors that are in the presence of an acceptor.
Acceptors channel: 
Acceptor with FRET + bleedthrough + autofluorescence

Acceptors in presence of donor
E = 0.6

Donors bleedthrough
E = 0

Cell autofluorescence

If we select the phasors inside this circle, and highlight those parts of the image, then we can immediately see which parts of the image are largely dominated by acceptors that are in the presence of a donor.