The Phasor approach: Application to FRET analysis and Tissue autofluorescence

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Outline

• Background: Lifetime

• Intro to Fluorescence Lifetime Imaging Microscopy

• Motivation for FLIM

• The Phasor approach

• Rac Activation using FLIM

• Conclusions
Lifetime: Background

Energy

$S_0$

$S_1$

Ground state

Emitted fluorescence light

photons

$I(t) = A \exp(-t/\tau)$
Time Domain and Frequency Domain FLIM

A sample is flashed many times by a short duration laser source.

The histogram of the time intervals between the excitation flash, and 1st emitted photon is measured.

A sample is excited by a modulated light source.

The fluorescence emission has the same frequency but is modulated and phase-shifted from the excitation source.
Why do FLIM?

FLIM is used for:

• FRET

• Intracellular mapping of ion concentration and pH imaging

• Biochemical reactions (oxidation/reduction) processes
  • NAD and NADH

• Long lifetime imaging (phosphorescence).
  • For example $O_2$ concentration in the cell or in tissues
Conceptual approaches to Spectroscopy

1) Identification of Molecular Species

2) Demixing of multiple species in a pixel

3) Identification of processes: FRET

Using the Spectra

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission</th>
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<tbody>
<tr>
<td>λ</td>
<td>λ</td>
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Using the fluorescence decays

- Lifetime Components
- Multiexponential analysis
- Quenching of donor lifetime
- Ratio of acceptor/donor spectral intensity
The challenges of FLIM

- At every pixel there are contributions of several fluorescent species, each one could be multi-exponential.

- To make things worse, we can only collect light for a limited amount of time (100-200 microseconds per pixel) which result in about $500-1000$ photons per pixel.

- This is barely enough to distinguish a double exponential from a single exponential decay.

- Resolving the decay at each pixel in multiple components involves fitting to a function, and is traditionally a complex computational task “for experts only”.

A major problem is data analysis and interpretation
Major issues with FLIM

- Rather difficult technique
- Fitting is slow
- Results depend on initial conditions
- Interpretation requires expertise

Can we avoid all these problems?

- No expertise necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface
A new approach: no more fits!

We propose a change in paradigm: Use a different representation of the decay where each molecular species has its own unique representation and where each process (FRET, ion concentration changes) is easily identified.

We need to go to a new “space”
The phasor space and the universal circle
(From Star-Trek)

• Where does this concept come from??
• We need some math.

This is what we need: the phaser!
How to calculate the components $g$ and $s$ of a phasor from the time decay?

A sample is flashed many times by a short duration laser source.

The interval between the excitation flashes, and 1st excited photon is measured.
How to calculate the components \( g \) and \( s \) of a phasor from the time decay?

\[
g_i(\omega) = \frac{\int_0^T I(t) \cos(\omega t) \, dt}{\int_0^T I(t) \, dt}
\]

\[
s_i(\omega) = \frac{\int_0^T I(t) \sin(\omega t) \, dt}{\int_0^T I(t) \, dt}
\]

Time-domain components of a phasor. \( I(t) \) is measured

Note that \( I(t) \) can contain raw multiexponential data!!
What happens if you don’t have the decay over a period? 80 MHz repetition laser frequency: period is 12.5 ns

The TAC range of 50 ns. Using a gain of 4 results in measurement over 12.5 ns.

But the useful range is only from channel 50 to 250 out of 256 bins (78%), and the entire period not being used makes the time record incomplete.

Using a gain of 2 makes the total range 25 ns, but an entire period can be extracted!

Example using the FLIMbox. The laser repetition period is 12.5 ns and it is divided into 256 bins, resulting in a 100% duty cycle
Fig. 2: SPCIImage main panel. Intensity image, lifetime image, lifetime histogram, decay curve at selected position, and decay parameters at selected position. Recorded by bh Simple-Tau 152 FLIM system with Zeiss LSM 880.
Are other vendors of FLIM cards any better?

No, as far as I know. All the TCSPC card have the same problem.

B&H show the problem, the other manufactures hide it.

What is the consequence? You lose the fast and the slow decay.

So, long decay components do not appear in their instruments.

The results obtained with the Frequency domain FLIMbox cannot be replicated.
The algebra of phasors

Simple rules to the Phasor plot:

1) All single exponential lifetimes lie on the “universal circle”
2) Multi-exponential lifetimes are a linear combination of their components
3) The ratio of the linear combination determines the fraction of the components
From decay data

Fitting of exponentials

Multiexponential analysis

Lifetime distribution
Separating Single exponential lifetimes using the phasor approach

Fluorescein

Mixture

Rhodamine B1
Pax-eGFP CHO-k1 in collagen

Lifetime of EGFP

Combinations of Lifetimes

Lifetime of Collagen
Phasors for common fluorophores. EGFP (green), autofluorescence (blue) and mRFP1 (red) (at 880 nm -2photon excitation). In any given pixel, mixture of EGFP and autofluorescence must be on the yellow line, mixtures of EGFP and mRFP1 must be on the red line. Mixtures of three of them must be inside the triangle with the corner in the 3 phasors.
How to distinguish two multi-exponential components from FRET?

Simple Rules for FRET:

1) If the experimental point lies on a straight line then it is NOT FRET

2) FRET efficiencies follow a “quenching trajectory”

3) Quantitative FRET efficiencies can be obtained from the position on the quenching trajectory
1. Click on the phasor plot (or enter the coordinates of the phasor manually) and assign the phasor to species 1.

2. Click to assign the phasor to the phasor 2.

3. The fractional (intensity weighted) contribution of the two phasors is calculated according to the sum rule of the phasor.

Moving this cursor, the circle will move in the phasor plot and automatically display the relative fraction of the two species (of the two phasors), independently on the number of exponential components necessary to describe the decay.
Delay of the excitation of the acceptor due to FRET moves the acceptor phasor to the left (yellow arrow). If the delay is sufficiently long, the phasor could fall outside the semicircle. The donor phasor moves to the right (red arrow) due to quenching (shorter lifetime).
The principle of the calculator

Purpose: to generate trajectories in the phasor’s plot corresponding to different processes

At present there are two functions programmed

The calculator for FRET efficiencies from

\[
\text{FRET efficiency} = \frac{\tau_D - \tau_{\text{FRET}}}{\tau_D}
\]

The calculator for ion concentrations form the relative contribution of the free and bound phasor.
If we have a donor with a single exponential decay that is quenched by the presence of an acceptor. What should we expect?

The lifetime of the donor is quenched.

The FRET efficiency can be calculated by the ratio of the two lifetimes.

The lifetime of the donor is along a different “trajectory”. Why is the trajectory an arc rather than a line to the (1,0) point?
The FRET Calculator

• Can we quench up to zero lifetime?

• Even if we quench all the DONOR, we still are left with the autofluorescence.

• The final point is not at zero but at the autofluorescent phasor!!!
As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence.

If there is a fraction of Donor that cannot be quenched, the final point will be along the line joining the Donor with the autofluorescence phasor.
The FRET calculator

Information needed to calculate FRET:

- Donor phasor
- Autofluorescence phasor
- Amount of Donor that can’t be quenched
- fractional contribution of autofluorescence and donor lifetimes
Example of FLIM analysis using phasors

Several regions the image can be identified corresponding to a) background (2 exponentials) b) cell 1 bright (2 exponentials) c) cell 2 dim, d) cell junctions dim.

Image of cell expressing uPAR-EGFP and uPAR-MRFP receptor. Upon addition of a ligand, the receptor aggregates. FRET should occur at the cell junctions.
The pitfall of “conventional” FLIM analysis

Donor+acceptor+ligand. A) intensity image after background subtraction, B) $\tau_p$ image

Image obtained using B&H 830 in our 2-photon microscope

Shorter lifetime region could be interpreted to be due to FRET
Identification of FRET using the phasor plot

Selecting regions of the phasor diagram. Selecting the region in A’ (donor + acceptor) the part in white lights up (A). Selecting the region in B’ (autofluorescence) the part in white in lights up (B). The color scale in B’ has been changed to better show the region of the autofluorescence. Selecting the region in C’ (along the donor quenching line as shown in D) the part in white in at the cell junction lights up in C.
Phasor Fingerprint of pure chemical species....

- GFP
- Porphyrin IX
- Retinol in DMSO
- Collagen
- Free FAD
- Bound NADH
- Free NADH
- Retinoic acid in DMSO
Label free FLIM in Living tissues

Intrinsic Fluorophores

- Extracellular
- Cellular
  - NADH and FAD: metabolic coenzymes involved in oxidative phosphorylation and glycolysis.
  - Complex distribution of autofluorescence
  - Redox ratio (NADH/NAD+ ~ free/bound NADH) reports on metabolic changes associated with cell carcinogenesis and differentiation
C. Elegans germ line: a model for stem cell biology

**Mitotic region**: stem cells niche

- The distal pool: undifferentiated cells maintained in a “stem cell-like state”
- Proximal pool contains cells that are closer to differentiating

**Transition zone**: cells that have differentiated and entered meiotic prophase (crescent-shaped DNA)

**Meiotic pachytene region**: cells that have further progressed through meiosis.

Crittenden et al. Mol Biol Cell, 2006; Cinquin et al. PNAS 2010
Image Segmentation: from pixel phasor plot to cell phasor plot

- Phasor average value of cells
- Better resolution
- Metabolic state of cells
- Cell phasors can be statistically attributed to the same or different average phasor value
C. Elegans germ line: a model for stem cell biology

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**Experimental set-up**

*C. elegans* histone-GFP fusion in germ line nuclei

\[ \lambda @ 880 \text{ nm and } 740 \text{ nm} \]

Ti: sapphire laser, 80 MHz, Zeiss 710,

ISS A320 FastFLIM, GaAs PMT,

40 x 1.2 NA,

Power \sim 5 \text{ mW}, Pixel dwell time=25 \mu s,

SimFCS software

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Cinquin et al. PNAS 2011
Stem cell metabolic “states” in *C. elegans*

In the large phasor cluster we distinguish statistically different subclusters.

Stringari et al. PNAS 2011
Mapping relative concentration of metabolites

Redox balance and modulation of stem cell self-renewal and differentiation

✓ Growth factors that promote self-renewal cause stem cell to become more reduced.

✓ Signaling molecules that promote differentiation cause progenitor to become more oxidized.

In agreement with in vitro study:
Guo et al 2008 JBO
Stringari et al. PNAS 2011
Single cell phasor plot distinguishes metabolic states of cells

- Evolution of the cell phasor fingerprints during differentiation
- Gradient of metabolic states of cells.
- Phasor fingerprint heterogeneity among mitotic cells could reveal symmetric and asymmetric divisions occurring at the level of the niche.
Conclusions

- Image segmentation: Cell phasors
- Better resolution
- Discrimination of different metabolic states of cells, small differences in redox ratio
- We identify and map relative concentration of intrinsic fluorophores

Current work and future directions

- Identify asymmetric and symmetric divisions and predict stem cell fate
- Metabolic evolution of differentiation pathways to different cell lineages.
- Metabolic pathways in colon cancer (Wnt signaling)
Conclusions

By representing “molecular species” rather than sum of exponential decays, the phasor approach reduces the problem of fitting exponential components to the exploration of regions of the phasor plot.

The analysis of the trajectories in the phasor plot provides a quantitative resolution of “processes” such as linear combination of two (or more species) and the calculation of FRET efficiency to a simple arithmetic.

You don’t need to be an expert spectroscopist to resolve the molecular species present and to calculate ion concentration or FRET efficiencies.

It globally analyzes many cells (different experiments) simultaneously.