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Introduction to Time-Resolved Fluorescence and Phasors
David M. Jameson
University of Hawaii
Time-resolved data are usually obtained using either time-domain or frequency domain methods.

As will be discussed later, Phasor plots can be constructed using either time-domain or frequency domain data.
Warning – the data discussed in this talk were obtained using cuvettes – not microscopes!
In the time domain (impulse) method, the sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally these short light pulses were generated using flashlamps which had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter.

![Diagram showing the relationship between exciting pulse and fluorescence intensity over time. The time axis is labeled in nanoseconds (ns), and the intensity axis shows the natural logarithmic decay (1/e) of the fluorescence signal after the exciting pulse.]
As shown in the intensity decay figure, the *fluorescence* lifetime, $t$, is the time at which the intensity has decayed to $1/e$ of the original value. The decay of the intensity with time is given by the relation:

$$I_t = \alpha e^{-t/\tau}$$

Where $I_t$ is the intensity at time $t$, $\alpha$ is a normalization term (the pre-exponential factor) and $\tau$ is the lifetime.

It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.
If the decay is multiexponential, the relation between the intensity and time after excitation is given by:

\[
l(t) = \sum_{i} \alpha_i e^{-\frac{t}{\tau_i}}
\]

One may then observe data such as those sketched below:

Here we can discern at least two lifetime components indicated as \(\tau_1\) and \(\tau_2\). This presentation is oversimplified but illustrates the point.
Here are pulse decay data on anthracene in cyclohexane taken on an IBH 5000U Time-correlated single photon counting instrument equipped with an LED short pulse diode excitation source.

$\tau = 4.1\text{ns}$  
$\text{chi}^2 = 1.023$

36.8%  
73 channels  
$73 \times 0.056\text{ns} = 4.1\text{ns}$  
56ps/ch
Pulsed Light Source

Timing Electronics or 2nd PMT

Constant Fraction Discriminator

Time-to-Amplitude Converter (TAC)

Multichannel Analyzer

Sample Compartment

Filter or Monochromator
Neutral density (reduce to one photon/pulse)

Photon Counting PMT

Counts
Time
In the harmonic method (also known as the phase and modulation or frequency domain method) a continuous light source is utilized, such as a laser or xenon arc, and the intensity of this light source is modulated sinusoidally at high frequency as depicted below. Typically, an electro-optic device, such as a Pockels cell is used to modulate a continuous light source, such as a CW laser or a xenon arc lamp. Alternatively, LEDs or laser diodes can be directly modulated.

In such a case, the excitation frequency is described by:

\[ E(t) = E_0 \left[ 1 + M_E \sin \omega t \right] \]

\( E(t) \) and \( E_0 \) are the intensities at time \( t \) and \( 0 \), \( M_E \) is the modulation factor which is related to the ratio of the AC and DC parts of the signal and \( \omega \) is the angular modulation frequency.

\( \omega = 2\pi f \) where \( f \) is the linear modulation frequency.
Due to the persistence of the excited state, fluorophores subjected to such an excitation will give rise to a modulated emission which is shifted in phase relative to the exciting light as depicted below.

This sketch illustrates the phase delay ($\phi$) between the excitation, $E(t)$, and the emission, $F(t)$. Also shown are the AC and DC levels associated with the excitation and emission waveforms.
One can demonstrate that:

$$F(t) = F_0 \left[ 1 + M_F \sin (\omega t + \phi) \right]$$

This relationship signifies that measurement of the phase delay, $\phi$, forms the basis of one measurement of the lifetime, $\tau$. In particular one can demonstrate that:

$$\tan \phi = \omega \tau$$

The *modulations* of the excitation ($M_E$) and the emission ($M_F$) are given by:

$$M_E = \left( \frac{AC}{DC} \right)_E \quad \text{and} \quad M_F = \left( \frac{AC}{DC} \right)_F$$

The *relative modulation*, $M$, of the emission is then:

$$M = \frac{(AC/DC)_F}{(AC/DC)_E}$$

$\tau$ can also be determined from $M$ according to the relation:

$$M = \frac{1}{\sqrt{1 + (\omega \tau)^2}}$$
Using the *phase shift* and *relative modulation* one can thus determine a *phase lifetime* ($\tau_p$) and a *modulation lifetime* ($\tau_m$).

If the fluorescence decay is a single exponential, then $\tau_p$ and $\tau_m$ will be equal at all modulation frequencies.

If, however, the fluorescence decay is multiexponential then $\tau_p < \tau_m$ and, moreover, the values of both $\tau_p$ and $\tau_m$ will depend upon the modulation frequency, i.e.,

$$\tau_p (\omega_1) < \tau_p (\omega_2) \quad \text{if} \quad \omega_1 > \omega_2$$

To get a feeling for typical phase and modulation data, consider the following data set.

<table>
<thead>
<tr>
<th>Frequency (MHz)</th>
<th>$\tau_p$ (ns)</th>
<th>$\tau_m$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6.76</td>
<td>10.24</td>
</tr>
<tr>
<td>10</td>
<td>6.02</td>
<td>9.70</td>
</tr>
<tr>
<td>30</td>
<td>3.17</td>
<td>6.87</td>
</tr>
<tr>
<td>70</td>
<td>1.93</td>
<td>4.27</td>
</tr>
</tbody>
</table>
These differences between $\tau_P$ and $\tau_M$ and their frequency dependence form the basis of the methods used to analyze for lifetime heterogeneity, i.e., the component lifetimes and amplitudes.

In the case just shown, the actual system being measured was a mixture of two fluorophores with lifetimes of 12.08 ns and 1.38 ns, with relative contributions to the total intensity of 53% and 47% respectively.

Here must must be careful to distinguish the term *fractional contribution to the total intensity* (usually designated as $f$) from $\alpha$, the pre-exponential term referred to earlier. The relation between these two terms is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}$$

where $j$ represents the sum of all components. In the case just given then, the ratio of the pre-exponential factors corresponding to the 12.08 ns and 1.38 ns components is approximately 1/8. In other words, there are eight times as many molecules in solution with the 1.38 ns lifetime as there are molecules with the 12.08 ns lifetime.
Multifrequency phase and modulation data are usually presented as shown below:

The plot shows the frequency response curve (phase and modulation) of Fluorescein in phosphate buffer pH 7.4 acquired on an ISS Chronos using a 470 nm LED. The emission was collected through a 530 high pass filter. The data is best fitted by a single exponential decay time of 4 ns.
Fig. 2. Phase (circles) and modulation (squares) data corresponding to ethidium bromide free in solution (blue) and bound to transfer RNA (red).
FIGURE 6.8 Simulation of phase and modulation data for a one-component system \((\tau = 4.05\, \text{ns})\) and a two-component system \((\tau_1 = 4.05\, \text{ns}, f_1 = 0.5; \tau_2 = 1.0\, \text{ns}, f_2 = 0.5)\). (I thank Carissa Vetromile for this figure.)
Frequency domain data can also be acquired using the harmonic content of pulsed sources.

Fig. 2. (a) Schematic representation of the light pulses emitted by the storage ring operating in the single-bunch mode. (b) The power spectrum of the pulse train shown in (a).

Fig. 9. Multifrequency phase (+) and modulation (×) data for tryptophan at 20°C pH = 6.9. Solid lines correspond to the best fit using two exponential components: $\tau_1 = 3.106 \pm 0.013 \text{ ns}$, $\tau_2 = 9.00 \text{ ns}$, and $f_1 = 0.968 \pm 0.005$. 

Phasors

Phasor representations have been used since the late 19th century in electrical engineering applications. (the word phasor comes from “phase vector”)

For example, phasors are used to describe alternating current (AC) circuits

\[ A(t) = A_m \sin(\omega t + \phi) \]
The phasor approach in fluorescence has been around for since 1984 but was dormant until the last decade or so when several laboratories began to apply it to FLIM (although with different names)

Tom Jovin
Andrew Clayton
Quentin Hanley

Bob Clegg

Enrico Gratton
Michelle Digman

AB plots
polar plots
phasor plots
The Measurement and Analysis of Heterogeneous Emissions by Multifrequency Phase and Modulation Fluorometry

DAVID M. JAMESON  
Department of Pharmacology  
The University of Texas Health Science Center at Dallas  
Dallas, Texas 75235  

ENRICO GRATTON  
Department of Physics  
University of Illinois at Urbana-Champaign  
Urbana, Illinois 61801  

ROBERT D. HALL  
Laboratory of Molecular Biophysics  
National Institute of Environmental Health Sciences  
Research Triangle Park  
North Carolina 27709

I. INTRODUCTION .......................... 56  
A. Statement of the Problem ............. 56  
B. Measurement of Fluorescence Lifetimes 57

II. INSTRUMENTATION ....................... 59  
A. Brief History ......................... 59  
B. State of the Art ..................... 60

III. DATA ANALYSIS ....................... 66  
A. Single Exponential Decay ............. 66  
B. Multieponential Decays ............... 67

APPENDIX 2. PHASE AND MODULATION 
LIFETIME RELATIONS

We have asserted that a heterogeneous emitting population, in the absence of excited state reaction, will demonstrate a phase lifetime which is always less than the modulation lifetime. The algebraic demonstration of this fact is somewhat cumbersome [11, 68]. We present here a brief and more intuitive demonstration of the phenomenon.

One may make a simple geometrical representation of the phase delay and relative modulation as shown in Fig. 11. Here we depict a vector of length M making an angle $\phi$ with the x-axis where $\phi$ represents the phase delay and M the relative modulation. Since for a single exponential decay we have the relation $M = \cos \phi$, the endpoint of the vector is constrained to be on the circle of radius 1/2 with a center at (1/2, 0). The intercept of the extension of this vector with the line through x = 1 equals $\omega t$ (since $\tan \phi = \omega t$). This circle is universal for single exponential systems irrespective of the lifetime or modulation frequency. We note that the X and Y intercepts of the vector correspond to our previously defined G and S functions (since $G = M \cos \phi$ and $S = M \sin \phi$).

Figure 12 represents the case of two exponential decays with phase delays and relative modulations of $M_1$, $M_2$, and $\phi_1$, $\phi_2$, respectively. These decays contribute to the total emission intensity decay with fractional weights of $f_1$ and $f_2$, respectively. The total fluorescence observed is represented by the vector sum, $M$, of the two components and gives an observed phase delay of $\phi$. Again we see that the intercept of the extension of the M vector with the x = 1 line corresponds to $\omega t^P$ (since $\tan \phi = \omega t^P$). The value of $\omega t^M$, however, corresponds to the line segment BD. This observation follows from the fact that the triangle OAB,

![Fig. 11. Geometrical representation of phase delay ($\phi$) and modulation ratio ($M$) for a single exponential decay.](image-url)
Resolution of the Fluorescence Lifetimes in a Heterogeneous System by Phase and Modulation Measurements

Gregorio Weber

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801 (Received: August 12, 1980)

A closed-form procedure is described for the determination of the decay constants and the relative contributing intensities of the $N$ independent components of a heterogeneous fluorescence emission employing measurements of the phase shift and relative modulation of the total fluorescence at $N$ appropriate harmonic excitation frequencies. At each frequency the phase and modulation measurements yield the real part of the Fourier transform of the fluorescence impulse response, $G$, and its imaginary part, $S$. It is shown that the moments of a distribution of the lifetimes are linear combinations of the $G$s (zero and even moments) or the $S$s (odd moments), and the rule for the construction of the coefficients of $G$ and $S$ in these linear combinations is derived. The classical de Prony method is used to obtain the lifetimes and fractional contributions of the components from the moments. For binary and ternary mixtures the numerical computations required are trivial. In the present state of the art, the lifetimes of the components of a binary mixture should be derivable with a loss in precision somewhat smaller than 1 order of magnitude with respect to the overall measured lifetimes.

$$G_r = M_r \cos \phi_r = [(1 + (\omega \tau_r^p)^2)(1 + (\omega \tau_r^M)^2)]^{-1/2} \quad (9)$$

$$S_r = M_r \sin \phi_r = G_r \omega \tau_r^p \quad (10)$$

$$G(\omega) = \int_0^\infty I(t) \cos \omega t \, dt \quad (55)$$

$$S(\omega) = -\int_0^\infty I(t) \sin \omega t \, dt$$

Frequency domain

Time domain
A Method for On-Line Background Subtraction in Frequency Domain Fluorometry

Gregory D. Reinhart,¹,² Pasquina Marzola,³ David M. Jameson,²,⁴ and Enrico Gratton⁵,⁶

Fig. 1. (A) Phasor representation of the AC part of the fluorescence signal. The length of the phasor is proportional to the AC value, and the angle is equal to the phase angle as indicated. (B) Vector composition of background and sample signal to give the overall measured signal.
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
Methodological measurements I

- Map the position of the mixture of three different single-exponential dyes (Rhodamine B, AEDANS, DENS)

![Graph showing positions of dyes at 20 MHz](image)
Methodological measurements

- Map the position of the mixture of three different single-exponential dyes (Rhodamine B, AEDANS, DENS)
- The mixture lies inside the hypothetical triangle
- When some of the dye is added to the mixture – the point is moved towards the appropriate triangle vertex
Methodological measurements II

- When analyzed for different frequencies – position of the hypothetical triangle in universal circle is anticlockwisely shifted when modulation frequency is increased.
- Middle points copy the curvature of the universal circle.
The pH dependent forms of tryptophan in solution are the zwitterionic and anionic forms due to the protonation of the amine and carboxyl groups – the pKa of the amine group is 9.39.
The pH dependent forms of tryptophan in solution are the zwitterionic and anionic forms due to the protonation of the amine and carboxyl groups – the pKa of the amine group is 9.39.
NATA

89 MHz; 20°C

0 M acrylamide

0.2 M acrylamide

3.0 ns

0.7 ns
lysozyme

89 MHz; 20C
Urea-induced unfolding FtsZ-F40W

Data of Felipe Montecinos
Data of Felipe Montecinos
Spectral resolution in conjunction with polar plots improves the accuracy and reliability of FLIM measurements and estimates of FRET efficiency

Y.-C. CHEN* & R.M. CLEGGE*†
*Bioengineering Department, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.
†Department of Physics, Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.
FRET in GFP

Fig. 1. 3D structure of GFP (blue) with the tryptophan in red and the fluorophore in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
FRET in GFP

Exc 280nm; em >500nm

Exc 471nm; em >500nm

280 MHz

250 MHz

25 MHz

Fig. 1. 3D structure of GFP (blue) with the tryptophan in red and the fluorophore in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Utilization in lipid vesicles

- Vesicle – small membrane enclosed sac
- Laurdan in DMPC vesicles (transition temperature 23 °C)
- Measured for temperature range from 5 to 55°C
- Similar behaviour to glycerol solution

![Graph showing fluorescence intensity vs. wavelength and temperature transition](image)
Phasors analysis:
Phase transition of complex systems

- DPPC
- DPPC:PC:Chol (1:1)+20% mol Chol
- DPPC:DOPC:Chol (1:1)+20% mol Chol

Good models for Pulmonary Surfactant

Data of Leonel Malacrida
Methods and Applications in Fluorescence

Investigation of the conformational flexibility of DGAT1 peptides using tryptophan fluorescence

Jose L S Lopes\textsuperscript{1,2}, Ana P U Araujo\textsuperscript{1} and David M Jameson\textsuperscript{2}

\textsuperscript{1} Institute of Physics of Sao Carlos, University of Sao Paulo, Sao Carlos, SP, Brazil
\textsuperscript{2} Department of Cell and Molecular Biology, University of Hawaii at Manoa, Honolulu, HI, USA

DGAT1 peptides

Sit1 (FGDREFYRDWNNES) $\rightarrow$ N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (HPS)
The protein Prdx 1 (a peroxiredoxin) is a decamer at high concentrations which dissociates into dimers upon dilution.

(done in collaboration with Matias Möller)
Each monomer has 2 tryptophan residues
Normally, to study a protein’s tryptophan lifetime one excites at >300nm to avoid tyrosine to tryptophan energy transfer. But the absorption at 300nm is much less than at 280nm. But using phasors we don’t care if we also excite tyrosines! In fact we don’t care how complex the lifetime is since we will only get one phasor point at any given concentration. We hope that the lifetime of the protein will change upon the decamer to dimer transition.

Which it does!
The degree of dissociation will then be mapped along the hypotenuse.
Enrico Gratton’s phasor approach (implemented in the SimFCS software) plots a phasor point for each pixel in the image.
Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in a live tissue

Chiara Stringari, Amanda Cinquin, Olivier Cinquin, Michelle A. Digman, Peter J. Donovan, and Enrico Gratton

Laboratory of Fluorescence Dynamics, Biomedical Engineering Department, Department of Developmental and Cell Biology, Center for Complex Biological Systems, and Department of Biological Chemistry and the Sue and Bill Gross Stem Cell Research Center, University of California, Irvine, CA 92697
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