# Phasor Plots for the Analysis of Time-resolved Fluorescence

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## 1. Principles

Phasor plots, or polar plots, have been utilized in science for a long time to describe dielectric constants relaxation. Recently, the methodology has been rediscovered for the analysis of fluorescence decay times in fluorescence microscopy imaging (FLIM) due to its simplicity and straightforwardness. In order to introduce them, we will start reviewing basic concepts of fluorescence decay times.

## 2. The Measurement of Fluorescence decay times

Upon absorption of photons, a population $N$ of molecules goes from the ground state $S_0$ to the upper excited states. They relax to the lowest level of the first excited state $S_1$ and hence they decay with rate $k_R$ to emit fluorescence. There are additional decays routes that not necessarily emit photons and they are indicated by $k_{NR}$ (non radiative paths). In general, if $N_1$ is the population of the excited level $S_1$, its population's changes are described by the relation:

$$\frac{dN_1}{dt} = -(k_R + k_{NR})\,N_1 + f_1 \qquad [2.1]$$

where $f_1$ is a function that describes the excitation source. By solving (and ignoring the effect of $f_1$) we find that the population $N_1$ changes in time according to the following relation:

$$N_1 = N_1(0)\,e^{-t/\tau_S} \qquad [2.2]$$

where

$$\tau_S = \frac{1}{k_R + k_{NR}} \qquad [2.3]$$

The quantity $\tau_S$ is the decay time of the excited state $S_1$.

## 2.1 Measurements at a single point

Upon excitation with a short pulse of light of very short duration (δ-function), the time course of the fluorescence emitted by a multi-components sample containing $i$ fluorescent species that absorbed the excitation radiation is described by the relationship:

$$I(\lambda,t) = I_0 \sum_i \alpha_i(\lambda)\, \mathrm{e}^{-t/\tau_i} \qquad [2.4]$$

where the coefficient $\alpha_i(\lambda)$, called the pre-exponential factor, is the amplitude and $\tau_i$ is the fluorescence decay time of the $i$ th component of the mixture. The amplitudes $\alpha_i(\lambda)$ are related to the the fractions of the total fluorescence emitted by the $i$ -component of the mixture (the fractional contributions $f_i$) by the following relation:

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \qquad [2.5]$$

## 2.2 Fluorescence Lifetime Imaging (FLIM)

In fluorescence lifetime imaging (FLIM) the decay times are determined at each pixel location $(h,k)$ of an image. At each location, in a multi-components environment containing $i$ fluorescent molecules, the fluorescence is described by the relationship:

$$I_{h,k}(\lambda,t) = I_0 \sum_i \alpha_i(\lambda)\, \mathrm{e}^{-t/\tau_i} \qquad [2.6]$$

# 3. Data Analysis

The instrumentation measures the decay of the fluorescence and its phase shifts and demodulation. Yet, for the determination of the decay times we need additional mathematical tools. Through the years several approaches have been developed for the determination of the decay times. A partial list includes: the non-linear least square analysis, the method of moments, maximum entropy, the Laplace transform, the phase plane, the Prony's method, the sine transform. For statistical robustness, the method of choice is today the non-linear least square analysis in both time-domain and frequency domain analysis. The technique is based upon a set of assumptions on the acquired data:

- The major error is on the dependent variable while the error on the independent variable is negligible;
- The systematic errors are negligible;
- The errors follow a Gaussian distribution;
- The data points are the results of independent measurements;
- The number of data points acquired is sufficient to make the parameters over-determined.

## 3.1 Definition of Chi-Square $\chi^2$ for TCSPC data

The observables to be measured in TCSPC are the number of photons in each acquisition bin $\{N(t_1), N(t_2),... N(t_k)\}$. As we assume that the number of photons acquired in each bin follows a Poisson distribution, the standard deviation is approximated by $\sigma_k = \sqrt{N(t_k)}$. The reduced chi-square $\chi^2$ function is defined as:

$$\chi^2 = \frac{1}{n-p-1}\sum_{k=1}^{n}\frac{\left[N(t_k)-N_c(t_k)\right]^2}{\sigma_k^2} = \frac{1}{n-p-1}\sum_{k=1}^{n}\frac{\left[N(t_k)-N_c(t_k)\right]^2}{N(t_k)} \qquad [3.1]$$

Where $n$ is the number of acquisition channels (or bins), $N(t_k)$ is the number of photons collected in the bin $k$ and $N_c(t_k)$ is the number of photons in the bin $k$ assuming the photon distribution is described by the selected model.

## 3.2 Definition of Chi-Square $\chi^2$ for frequency-domain data

In frequency-domain the observables are, for each modulation frequency $\omega$, the phase shift and the demodulation $\{\varphi_\omega, M_\omega\}$. The reduced chi-square $\chi^2$ function is defined in frequency-domain as:

$$\chi^2 = \frac{1}{2n-p-1}\left\{\sum_{j=1}^{n}\left[\frac{\varphi_j-\varphi_{cj}}{\sigma_j}\right]^2+\sum_{j=1}^{n}\left[\frac{M_j-M_{cj}}{\sigma_j}\right]^2\right\} \qquad [3.2]$$

where $n$ is the number of modulation frequencies, $\varphi_j$ and $M_j$ are the measured phase shift and demodulation at each frequency, while $\varphi_{cj}$ and $M_{cj}$ are the phase shift and demodulation given by the selected model of the decay.

## 3.3 User approach to the non-least square analysis

When using the non-linear square analysis, the approach utilized by the user encompasses the following sequential steps:

1. The user makes a guess about the fitting model and proceeds with the analysis.
2. If not, the user starts from the simplest model, the single exponential decay.
3. Upon minimization, the user looks at the value of the chi-square function. If the value is close to "1", the analysis stops.
4. If the value of the chi-square function is not close to "1", the user adds a second component to the model describing the decay. Upon minimization, the user checks the value of the chi-square function;
5. … and so on

This approach requires indeed a fair amount of expertise in the choice of the model (exponential, non-exponential, etc.) and a full appreciation of the statistical tools that are to be utilized in ambiguous cases.

The situation becomes almost desperate in fluorescence lifetime imaging. Here the user is confronted with two limiting effects. On one side the signal is often limited as the number of photons per pixel is too small to carry out a significant analysis. On the other side, one should repeat at each pixel the above procedure in order to determine the decay times at each pixel of the image. Of course simplification techniques have been introduced, such as binning (the grouping of nearby pixels assumed to have the same decay time); yet a simplification often translate into an approximation that may have unexpected consequences when analyzing FRET effects in a cellular environment.

The phasor plot approach addresses these concerns:

- No expertise is necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface

# 4 The Phasor plot

## 4.1 The Fourier Transform of the decay

We calculate the Fourier Transform of function [2.4]:

$$\widehat{F} = \int_{-\infty}^{+\infty} I(\lambda,t)\, e^{i\omega t}\, dt \qquad [4.1]$$

and denote with $g(\omega)$ and $s(\omega)$ the real and imaginary components of the Fourier Transform:

$$g(\omega) = \frac{\int_0^\infty I(t)\, \cos\omega t\, dt}{\int_0^\infty I(t)\, dt} \qquad [4.2]$$

$$s(\omega) = \frac{\int_0^\infty I(t)\, \sin\omega t\, dt}{\int_0^\infty I(t)\, dt} \qquad [4.3]$$

By carrying out the calculations, the equation above can be transformed to:

$$g(\omega) = \sum_i f_i \frac{1}{(1+\omega^2\tau_i^2)} \qquad [4.4]$$

$$s(\omega) = \sum_i f_i \frac{\omega\tau_i}{(1+\omega^2\tau_i^2)} \qquad [4.5]$$

For FLIM, we can define the $g(\omega)$ and $s(\omega)$ components of the Fourier Transform at each $(h,k)$ pixel of the image:

$$g_{h,k}(\omega) = \frac{\int_0^\infty I_{h,k}(t)\,\cos\omega t\,dt}{\int_0^\infty \mathrm{I}_{h,k}(t)\,dt} \qquad [4.6]$$

$$s_{h,k}(\omega) = \frac{\int_0^\infty \mathrm{I}_{h,k}(t)\,\sin\omega t\,dt}{\int_0^\infty I_{h,k}(t)\,dt} \qquad [4.7]$$

## 4.2 Definition of Phasor Plot

The two values $g(\omega)$ and $s(\omega)$ identify in the complex plane a point $(g,s)$ represented by a vector $\vec{m}(\omega)$ making an angle $\phi(\omega)$ with the $g-$axis; the vector's modulus is the modulation of the fluorescence at the frequency. This is the phasor plot.

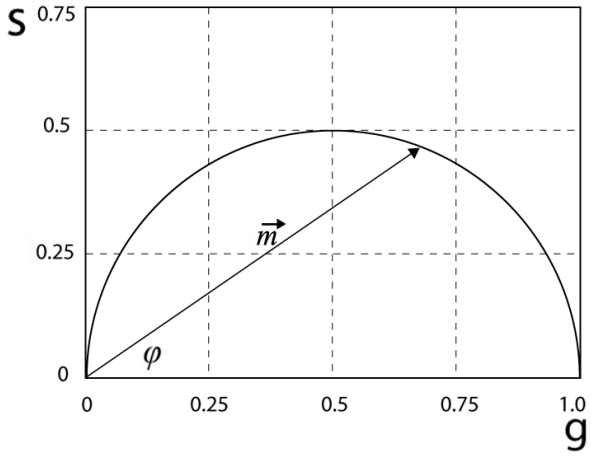


**Figure 4.1** Phasor plot for a single-exponential decay. The vector $\vec{m}$ has modulation m as modulus and the phase $\phi$.

The phase and the module of the vector $\vec{m}(\omega)$ are given by:

$$\phi(\omega) = \arctan\left(\frac{s}{g}\right) \qquad [4.8]$$

$$\left|m(\omega)\right| = \sqrt{s^2 + g^2} \qquad [4.9]$$

# 5. Data Representation

## 5.1 Universal circle

According to the expressions for the coordinates of a phasor for a single exponential decay, the following relation is obtained:

$$s_{h,k}^2 + \left( g_{h,k} - \frac{1}{2} \right)^2 = \frac{1}{4} \qquad [5.1]$$

That is, all single exponential components are represented by a semicircle of center $\left( \frac{1}{2}, 0 \right)$ and radius $\frac{1}{2}$ in the phasor plot. On this circle, called the *universal circle*, a phasor corresponding to a very short decay time (small phase angle) is close to the point (1, 0), whereas a phasor corresponding to a long decay time will be close to the coordinates (0, 0).

## 5.2 Single-exponential decay times

Using the values of [3.4] and [3.5] for a single exponential decay we find:

$$\tau_P = \frac{1}{\omega} \tan \phi \qquad \tau_M = \frac{1}{\omega} \sqrt{\frac{1}{m^2} - 1} \qquad [5.2]$$

These are the expressions for determining the decay time for a single exponential decay in frequency domain.

## 5.3 Frequency-domain data

When acquiring fluorescence signal for a single exponential decay at different modulation frequencies $(\omega_1, \omega_2, ..., \omega_n)$ the points on the universal circle move counter-clockwise as the modulation frequency increases.

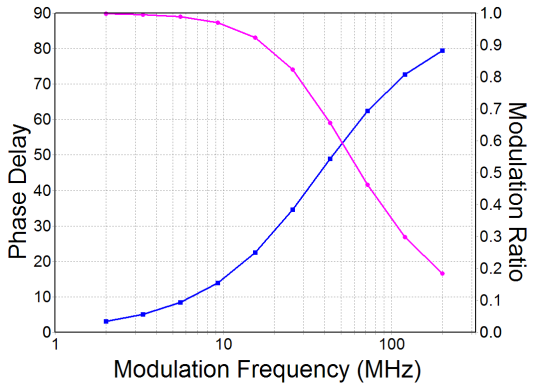


**Figure 5.1** Phase and modulation data for a solution of Anthracene in ETOH; the excitation light source is a cw LED emitting at 370 nm. The measured decay time is 4.25 ns.

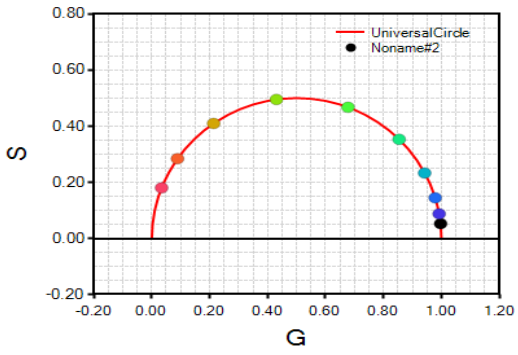


**Figure 5.2** Polar plot representing the data.

## 5.4 Time-domain data

When acquiring time-domain data (time-correlated single photon counting), we need to apply the Fourier transform to both the IRF and the histogram of the arrival time of photons. The base frequency of the data is the repetition rate of the pulsed source.

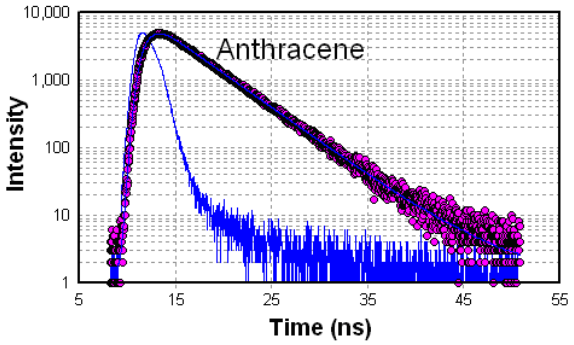


**Figure 5.3** Decay time of a solution of Anthracene in ETOH; the excitation source is a pulsed LED emitting at 335 nm. The measured decay time is 4.24 ns.

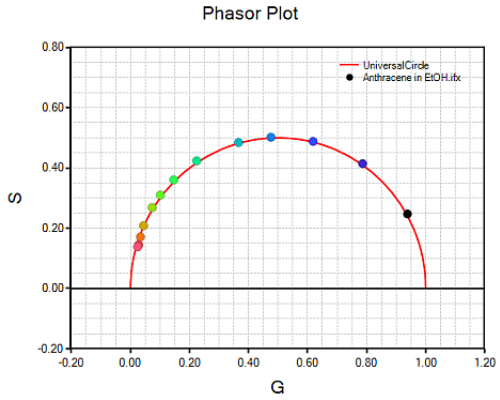


**Figure 5.4 –** Polar plot representing the data. The main frequency is 20 MHz; the other points are the harmonics, 40, 60, … MHz.

## 5.5 The Optimal frequency

Typically, data at one modulation frequency are utilized when comparing data from several records. In frequency-domain the optimal modulation frequency is given by:

$$\omega_{OP}^2 = \frac{1+\sqrt{3}}{2\tau^2} \qquad [5.3]$$

In time-domain we use the repetition rate of the pulsed laser. For a set optimal frequency, components with increasing longer decay times are positioned sequentially counter-clockwise on the universal circle (Figure 5.5).

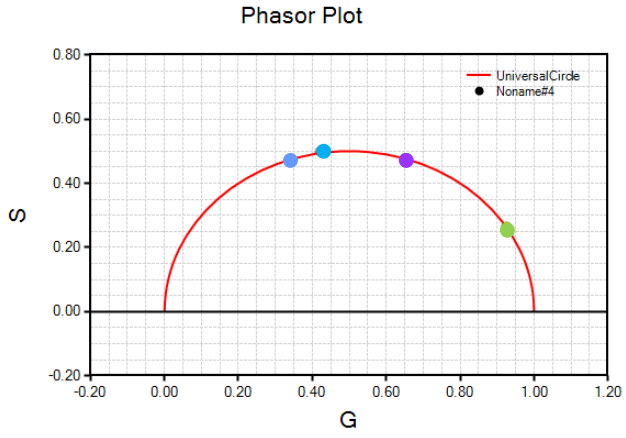


**Figure 5.5** The following fluorophores are displayed for 48 MHz. The shortest decay time (green dot) is located to the right on the universal circle; as the decay time gets longer, the points are displayed counter-clockwise. In the order from right to left:

- p-terphenyl, 1.05 ns
- naphthalene, 2.7 ns
- perylene, 4.3 ns
- anthracene, 5.1 ns

## 5.5 Multi-exponential decay times

For a two-exponential decay, we can write the following vector:

$$\vec{m} = f_1 \vec{m}_1 + f_2 \vec{m}_2 \qquad [5.4]$$

where $f_i$ is the intensity-weighted fractional contribution of the component with lifetime $\tau_i$.

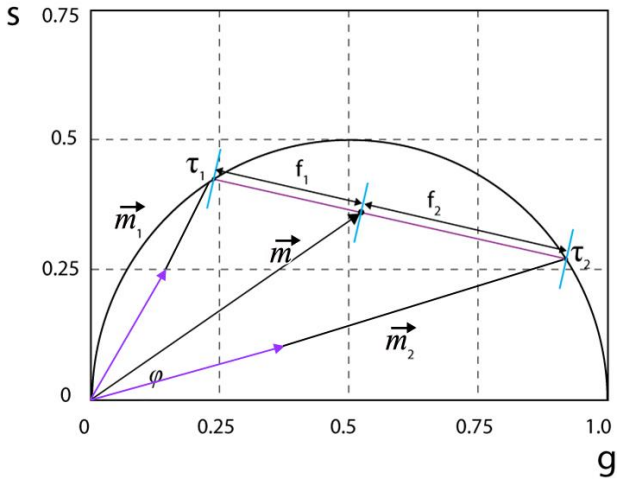


**Figure 5.5** Phasor plot for a two-exponential decay process. The modulus of vector $\vec{m}$ reaches an area within the semicircle on the line connecting the two decay times. Their fractional contributions $f_1$ and $f_2$ are given by the intersection of the vector with the connecting line.

In general, the following relations apply:

$$g = \sum_i f_i \, g_i \qquad s = \sum_i f_i \, s_i$$
$$\sum_i f_i = 1 \qquad [5.5]$$

That is, the phasors for multiexponential lifetime decays or combinations of single-exponential lifetime decays are given by the normalized linear combination of the component phasors, making them fall below the universal circle.

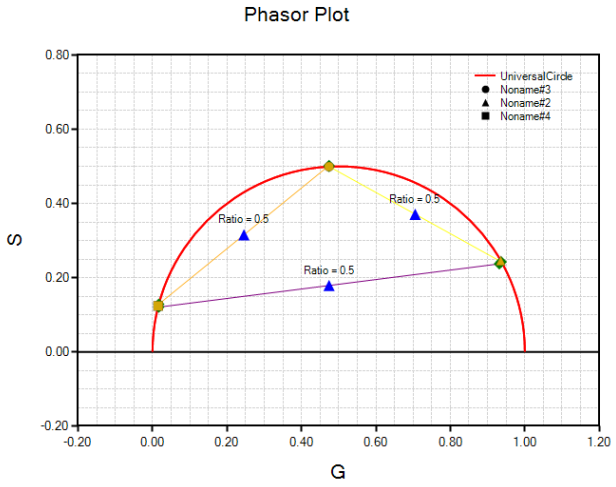


**Figure 5.6** Phasor plot for a solution containing 3-component decay times (1 ns, 30%), 4 ns, 50% and 1 ns, 20%). The experimental point falls in the middle of the triangle.

## References


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