

The Ultimate Phasor Plot and beyond

Yuansheng Sun and Shih-Chu Liao
ISS

1 Introduction to the phasor plot

Fluorescence Lifetime Imaging Microscopy (FLIM) is a useful technique that provides, with its peculiar selectivity of fluorophores, a higher contrast of confocal images; moreover, it is used for providing quantitative information of the cell environment (ions, pH, oxygen content, electrical signals, index of refraction). When used in Förster Resonance Energy Transfer (FRET) applications, the measurement of the decay times provide indirect information about the proximity of target fluorophores up to a distance of about 10 nm.

The data analysis approach utilized to extract the decay times information hampers the applications of FLIM and, in several cases, provides information that are just a rough approximation of the phenomena occurring on a molecular scale. The typical approach consists in separating the region of interest (ROI) in the image and, by using a chi-square minimization technique, extracting a 1-, 2- or 3-decay times. Yet, the limitation to one or three decay rates of fluorophores is in many cases an arbitrary one and in most cases limited as, in a cellular environment, several decay rates are present and active at the same time. Additionally, the process is fairly complicated. It has always been a challenge for many users to apply the traditional non-linear least square fitting for FLIM data analysis: questions like *which decay model should be chosen – single, double, or triple exponential?*, and *how to make initial guesses for the fitting parameters to get a reliable convergence?*, and after the analysis, *how to evaluate the goodness of the fit and is my “Chi Square” good enough?* The answers to these questions require a deep knowledge of the statistical analysis and the minimization technique algorithm functionality. The phasor plot leaves all of these questions behind and presents an intuitive simple interface for you to get instantaneous and quantitative results. Does it sounds too good to be true?

What is the phasor plot? The phasor plot is a graphical representation of all the raw fluorescence lifetime imaging microscopy (FLIM) data in a vector space; that is, each pixel in a FLIM image is transformed to a point in the phasor plot. No assumption is made on the number of decay rates present in the environment as well as on the specific modeling of the decay (exponential, non-exponential). This transformation of the pixels of a FLIM image into the phasor plot can be equally applied to both time- and frequency-domain FLIM data. Since ISS provides both time- and frequency-domain FLIM instrumentation solutions, the phasor plot analysis routines for both are included in the VistaVision software by ISS.

An in-depth mathematical description of the phasor plot (the phasor plot for FLIM data analysis) is available on the ISS website – www.iss.com/resources/research/technical_notes. In this Note we only present the relevant results as applied to FLIM.

The phasor space is constructed by using two phasor vectors (G, S), where each component is represented as shown in Eq. [1].

$$g_{x,y}(\omega) = m_{x,y} \cos(\varphi_{x,y}) \quad \text{and} \quad s_{x,y}(\omega) = m_{x,y} \sin(\varphi_{x,y}) \quad [1]$$

In a frequency-domain FLIM measurement, $m_{x,y}$ and $\varphi_{x,y}$ are the modulation ratio and the phase delay measured given a particular modulation frequency (ω) at a pixel location (x,y). In a time-domain FLIM measurement (such as TCSPC - time correlated single photon counting), the two phasors can be obtained through the sine and cosine transforms of the raw decay data, given by Eq. [2], where $I_{x,y}(t)$ is the photon count of each time bin " t " at a pixel location (x,y), and ω is the angular frequency of harmonic content of the excitation light and is equal to " $2\pi f$ ", where f is the basis repetition rate of the pulsed excitation light.

$$g_{x,y}(\omega) = \frac{\int_0^{\infty} I_{x,y}(t) \cos(\omega t) dt}{\int_0^{\infty} I_{x,y}(t) dt} \quad \text{and} \quad s_{x,y}(\omega) = \frac{\int_0^{\infty} I_{x,y}(t) \sin(\omega t) dt}{\int_0^{\infty} I_{x,y}(t) dt} \quad [2]$$

For a single-lifetime (τ) species, the expressions [2] are resolved analytically and the values of g and s are given by Eq. [3].

$$g(\omega) = \frac{1}{1 + (\omega\tau)^2} \quad \text{and} \quad s(\omega) = \frac{\omega\tau}{1 + (\omega\tau)^2} \quad [3]$$

From this relationships, the lifetime of any single exponential decay is displayed on a semicircle curve centered at ($g=0.5, s=0$) with a radius of 0.5 in the phasor plot. The plot is called the *Universal Circle*. On it, for a fixed frequency, longer lifetimes tend to be displayed to the left and shorter lifetimes to the right (see Figure 1).

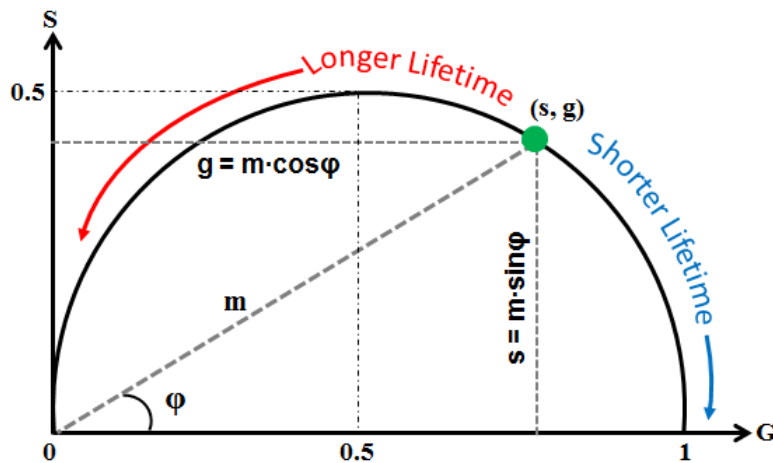


Figure 1: The phasor plot vector space and the universal circle. A single decay component is represented by a point (s,g) on the universal circle.

2 Exploring the phasor plot properties

The phasor plot represents the FLIM raw data. This great feature makes the phasor plot a model-free approach, which provides an unbiased way for data analysis requiring no expertise in the related physical and mathematical modeling for FLIM data analysis. Let us explore a few basic features of the phasor plot and see what it can do for you.

- **You can easily identify any single-lifetime species with its lifetime value.**
 - This is simply done in the ISS VistaVision software by using the mouse to place a circular cursor to cover a distribution on the universal circle; the software also provides a registration tool that can automatically identify and localize a population of the phasor points for one species whose lifetimes follow a Gaussian distribution. [Figure 2](#) displays the lifetimes of Rose Bengal in different solvents.

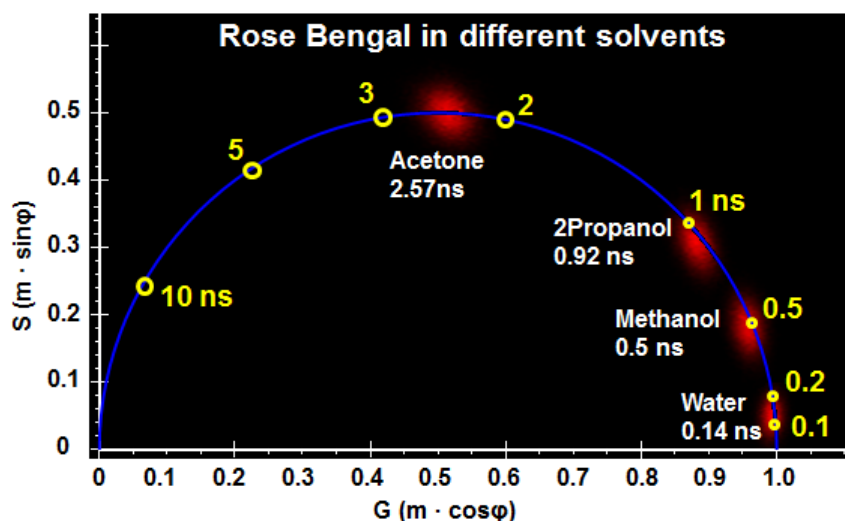


Figure 2: Decay time of Rose Bengal in different solvents displayed in the phasor plot. Data was acquired using the ISS Alba V system using the 561 nm laser as excitation wavelength (courtesy of Dr. Ammasi Periasamy, University of Virginia).

- **You can easily distinguish between the single- and multi-lifetime species.**
 - The pixels distribution of a single-lifetime species is centered at a point on the universal circle.
 - The distribution of a multi-lifetime species or a mixture of different single-lifetime species producing a multi-exponential decay falls inside the universal circle.
- **You can decompose a complex mixture into individual species in a graphical way.**
 - The phasor point of a mixture composed of two species is located on the line connecting the two phasor points of representing each individual species in the phasor plot; the contribution from one species of the mixture to the total fluorescence signal is proportional to the distance from the mixture point to the other species point (see [Figure 3](#)). Note that the individual species do not have to be a single-lifetime species. This is a key concept of using the phasor plot to analyze a complex system, where it is almost impossible to solve individual lifetime components.

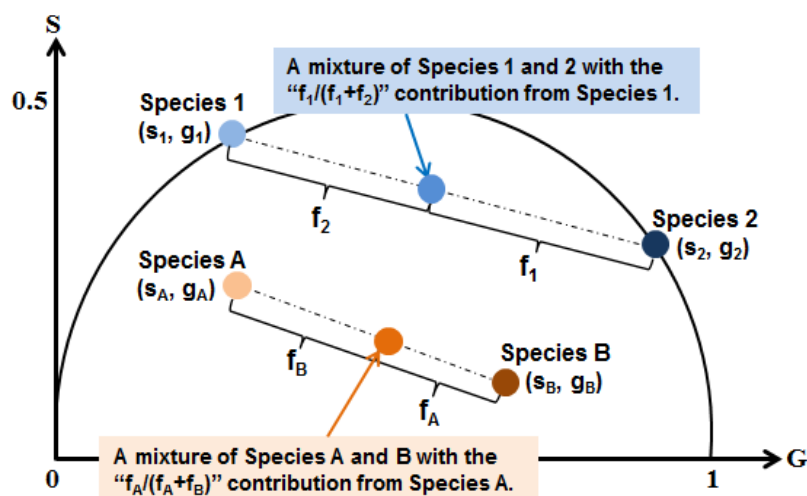


Figure 3: Decomposition of a mixture composed of two species in the phasor plot - each of the two species could have a single lifetime (the 1-2 mixture case) or multiple lifetime components (the A-B mixture case).

- **Figure 4** shows an example of using the phasor plot to compare purified mCerulean, mTurquoise2 and three mixtures of the two purified fluorescent protein solutions. As expected, the mixtures are lying on the line connecting the two phasor distributions measured from each species and their contributions in each mixture are indicated by the location of the mixture on the line.
- The rule also applies to a more complex system, for example the phasor point of a mixture of three species is located inside the triangle that connects three phasor points representing each individual species; the shape will become a polygon for more than three species. Knowing the exact locations of the mixture and every individual species in the phasor plot, the contribution from each species to the mixture can be explicitly solved.

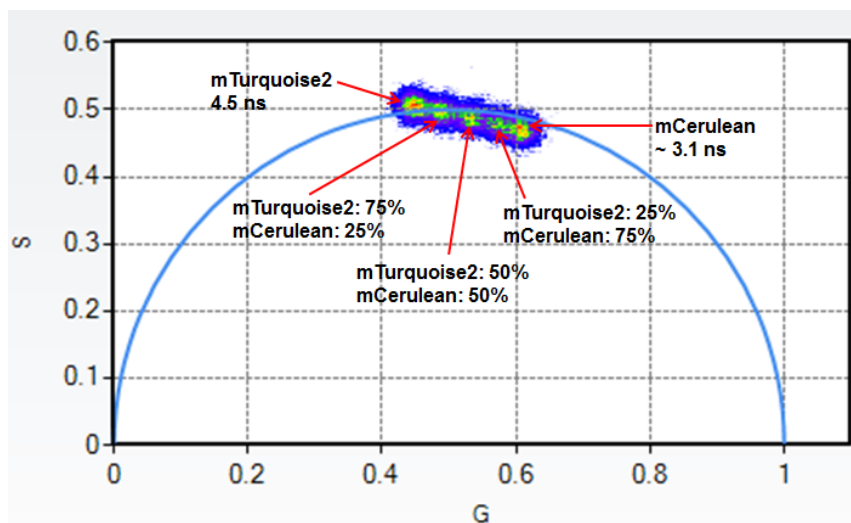


Figure 4: Comparison of mCerulean, mTurquoise2 and three mixtures of the two species in the phasor plot. Data was acquired using the ISS Alba V system using as excitation source the 440 nm laser line (courtesy of Dr. Richard N. Day; Indiana University School of Medicine).

- You can interactively map between the image pixels and the phasor points.
 - Each image pixel is mapped to a phasor point. This one-to-one mapping provides an “interactive-play” feature such that one can circle a distribution on the phasor plot to find the corresponding pixels in the image. This nice feature allows for you to easily localize and differentiate the distributions of species of different lifetimes in a sample (an example is shown in [Figure 5](#)).
 - Each location on the phasor plot gives you the lifetime information and depending upon the look-up table you choose, the intensity or the color shown at a location on the phasor plot tells you the frequency (the number) of the pixels which have the same lifetime – this means that the phasor plot directly shows you the statistics of the lifetime distribution.
- You can plot not only a whole image but also as many images as you want in one phasor plot.
 - This would significantly reduce the time for analyzing a large amount of data, compared to the traditional non-linear least square fitting analysis, which typically runs on a pixel-by-pixel base.
 - More importantly, different data sets can be directly compared and analyzed on the same graph at the same time, allowing you quickly extract and differentiate biological meanings.
 - This function is very useful for Förster Resonance Energy Transfer (FRET) measurements, as discussed below.
 - This function is provided by the ISS VistaVision “Multi-Image Phasor Analysis” module.

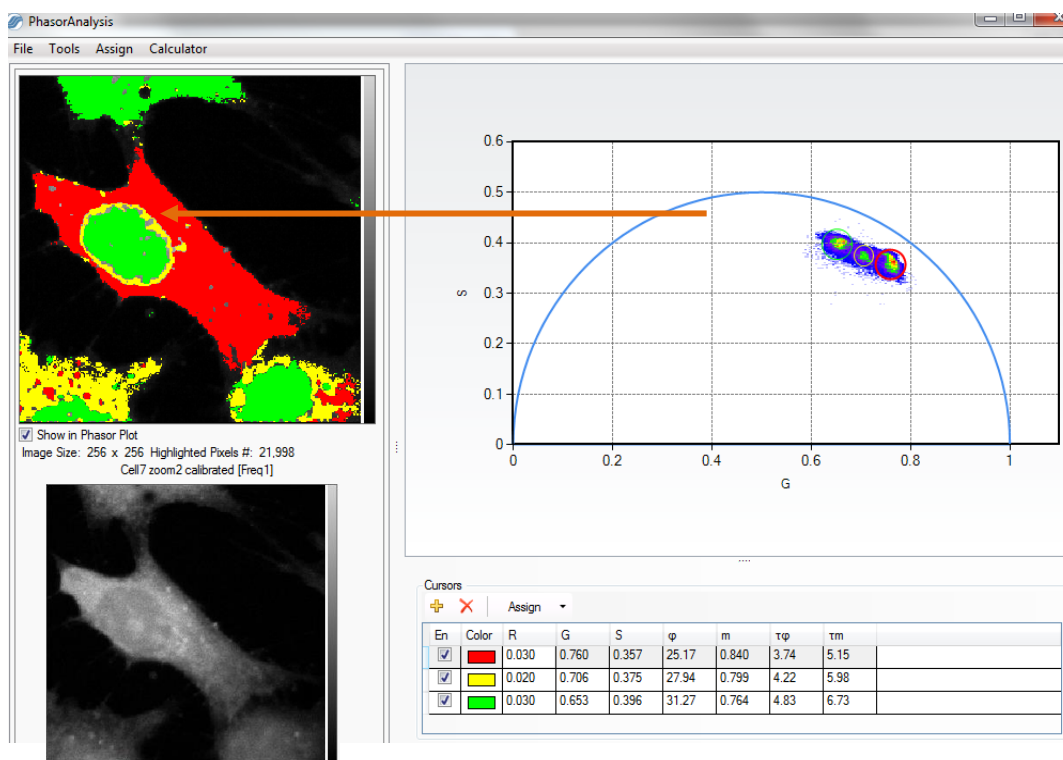


Figure 5: Differentiate different lifetimes in nucleus vs. cytosol in the phasor plot. Three populations resolved in the phasor plot are mapped back to the image pixels, marked by red (mainly in the cytosol), green (in the nucleus) and yellow (between cytosol and nucleus).

3 Get more quantitative with the phasor plot – the “FRET Trajectory” approach

Many users question if the phasor plot can produce quantitative results in a further depth, more than just the lifetime – “Particularly, can I use the phasor plot to analyze my FRET data and get quantitative FRET efficiencies?” This had been a challenge, until Dr. Enrico Gratton and his group members in the Laboratory for Fluorescence Dynamics (LFD) at the University of California Irvine developed the “FRET trajectory” approach (2). This approach is implemented in VistaVision. So, **the answer is yes.**

FRET is non-radiative energy transfer from an excited molecule (the donor) to another nearby molecule (the acceptor) through a long range dipole–dipole coupling mechanism. As given by Eq. [5], the efficiency of energy transfer (E) from the donor to the acceptor is dependent on the inverse of the sixth power of the distance separating them (r), subject to their Förster distance (R_0) at which E is 50% (3-4). FRET is typically limited to distances less than about 10 nm, making it a powerful tool for investigating a variety of phenomena that produce changes in molecular proximity.

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad \text{and} \quad r = R_0 \left(\frac{1-E}{E} \right)^{1/6} \quad [5]$$

FLIM is among the most accurate methods of measuring FRET (5). In a FLIM-FRET experiment, the quenched lifetimes of the donor (τ_{DA}) in the samples containing both the donor and the acceptor are measured to map the FRET efficiencies based on Eq. [6], where the unquenched donor lifetime (τ_D) is typically determined from the donor-only control sample. As only donor signals are measured by FLIM for determining the FRET efficiency, the method does not require the corrections for spectral bleedthrough that are necessary for intensity-based measurements of sensitized emission from the acceptor.

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad [6]$$

In the phasor plot, it is easy to visualize the difference of the lifetimes from unquenched vs. quenched donor samples. Figure 6 shows a nice example of plotting four FLIM data sets, measured from live cells expressing Cerulean-alone (unquenched donor) and Cerulean in three different FRET-standard constructs (quenched donor), in the phasor plot. These genetic constructs were developed by the Vogel’s laboratory at NIH/NIAAA through encoding fusions between donor and acceptor fluorescent proteins separated by defined amino acid linker sequences (6). The C5V, C17V and C32V constructs shown here were generated by encoding Cerulean (FRET donor) and Venus (FRET acceptor), directly coupled by either a 5, 17 or 32 amino acid linker. It is clearly shown by the phasor plot that the lifetimes of Cerulean in the FRET-standard constructs are shorter than that of Cerulean-alone due to quenching by Venus. The phasor plot also reveals more quenching (shorter lifetime) as the linker gets shorter.

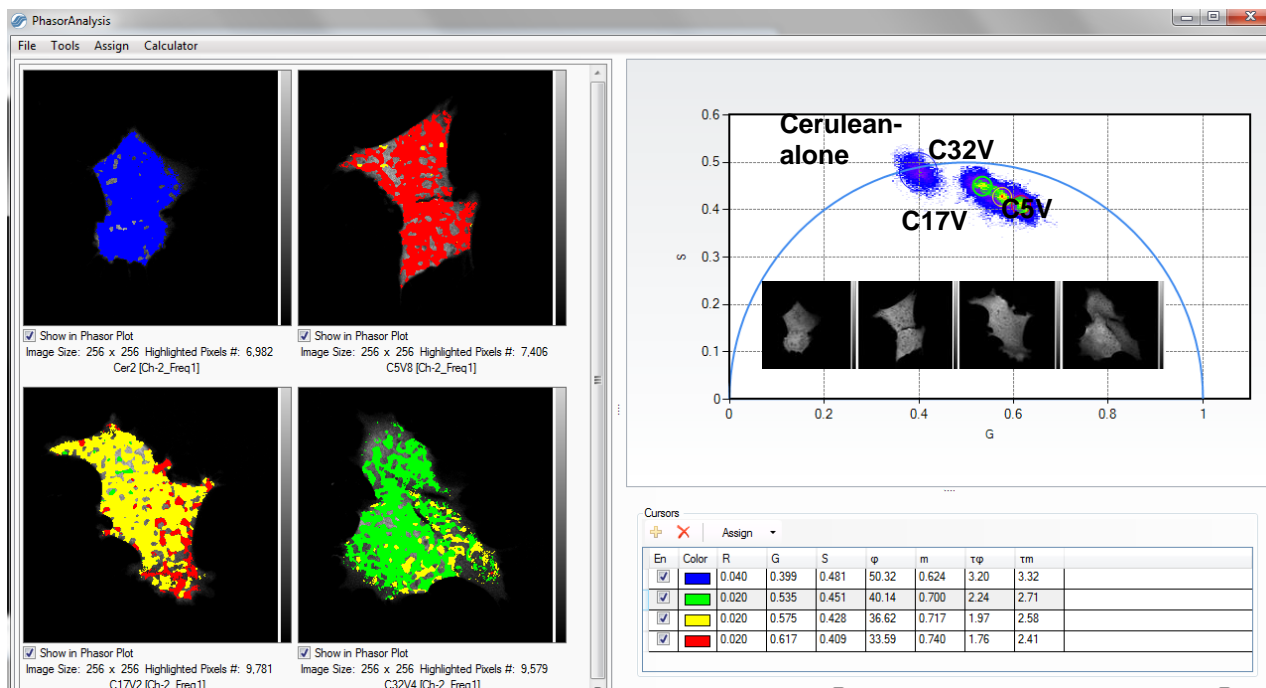


Figure 6: Differentiate different lifetimes of Cerulean in cells expressing Cerulean alone and three different FRET-standard constructs (C32V, C17V and C5V; see text for details). (Data acquired using ISS Alba V system with the 440-nm laser excitation wavelength by Yuansheng Sun during the 2014 KCCI workshop on FLIM and FRET, organized by Dr. Ammasi Periasamy at the University of Virginia).

However, it does not seem to be straightforward to link both the data of both unquenched and quenched donors to produce the FRET efficiency in the phasor plot. Fortunately, Dr. Gratton's LFD group came up an elegant solution, called the "FRET trajectory" approach (2). Figure 7 shows how the FRET efficiency for C5V is determined using the Cerulean-alone and C5V data shown in Figure 6, based on the "FRET trajectory" method, implemented in the ISS VistaVision software.

- Load both unquenched and quenched data (images) into the phasor plot using the "Multi-Image Phasor Analysis" module (Figure 7-left panel; also see Figure 6).
- Select the population of the unquenched donor (Cerulean-alone in this case) phasor points using the circle shown on the phasor plot and assign it to be the unquenched donor phasor position. Use the arrow buttons to finely adjust the circle position, and a higher "Score" indicates a better circle position that matches the selected population. The circle size is adjustable.
- Select the population of the phasor points originated from background (e.g. caused by the scatter of the excitation light or the sample auto-fluorescence) using the circle and assign it to be the background phasor position. In this case, the background is assumed to be from the scatter of the excitation light and its phasor position is defined at the ($g=1, s=0$) for zero lifetime.
- Click the "FRET Calculator" button in the "Multi-Image Phasor Analysis" module to plot the FRET trajectory (Figure 7-right panel). The trajectory is plotted depending upon the contributions from the background and the unquenched donor, which can be determined from control samples and then assigned to the software by the users. A circle lies on the trajectory and travels along the trajectory by sliding the "Efficiency" bar. The "Efficiency" is the FRET efficiency. By sliding "the "Efficiency" value, one can move the circle to the position

that best matches the population of the quenched donor (C5V in this case) phasor points – here, its FRET efficiency is obtained from reading the value shown in the “Efficiency” bar. Again, a higher “Score” indicates a better circle position that matches the selected population and the circle size is adjustable.

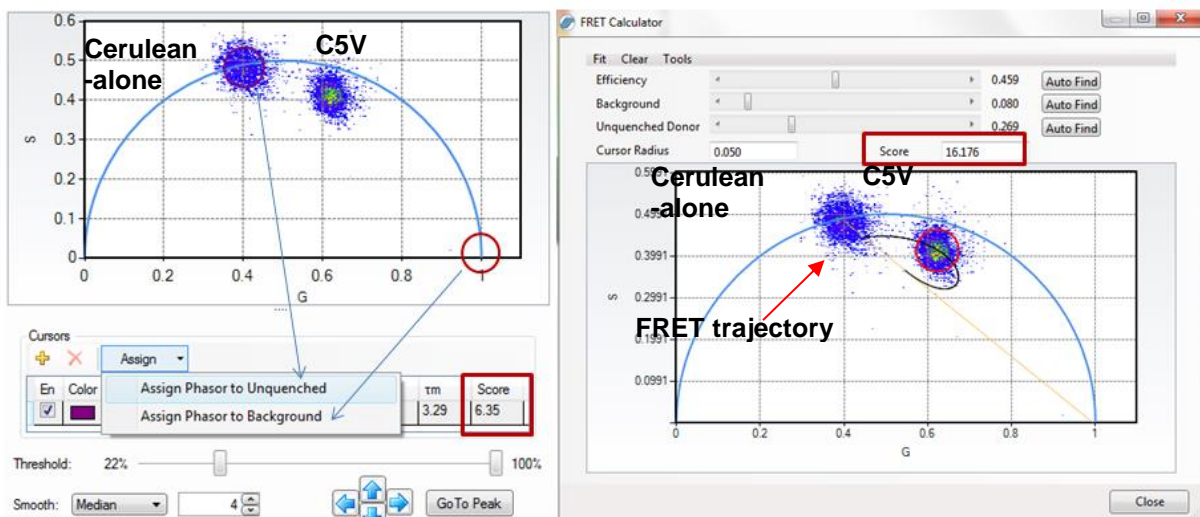


Figure 7: Determine the FRET efficiency using the “FRET trajectory” approach in the ISS VistaVision software. The C5V FRET efficiency is determined using the Cerulean-alone and the C5V data, shown in Figure 6.

4 Conclusions

The traditional non-linear fitting for FLIM data analysis requires some level of expertise in understanding the physics of fluorescence lifetime and a full appreciation of the statistical tools that are to be utilized in cases that are at best disputable. Undoubtedly, this projects a challenge for many biologists who have to focus on solving their biological questions rather than their tools. In contrast, the phasor plot approach provides a simple interface to analyze the raw FLIM data quantitatively.

A summary of the merits of the phasor plot given by Dr. Enrico Gratton are - **“No expertise necessary; Instantaneous results; Independent of initial choices; Quantitative results; and Intuitive simple interface”**.

We hope that more people will be encouraged and start to apply FLIM to their research after knowing what the phasor plot can do and realizing the FLIM data analysis is not that scary. Then, your application of using the phasor plot will drive it **beyond** what we can imagine today.

Acknowledgements

We thank Dr. Richard N. Day, Dr. Ammasi Periasamy, Dr. Enrico Gratton and members in LFD for their help during the preparation of this application note.

References

1. Richard N. Day 2014. *Measuring protein interactions using Förster resonance energy transfer and fluorescence lifetime imaging microscopy*, *Methods* 66 (2) 200-207.
2. Digman, M. A., V.R. Caiolfa, M. Zamai and E. Gratton 2008. *The phasor approach to fluorescence lifetime imaging analysis*. *Biophys. J.* 94, L14-6.
3. Förster, T.1965. *Delocalized excitation and excitation transfer*; In *Modern quantum chemistry*, Sinanoglu, O., editors. Academic Press Inc., 93-137.
4. Clegg, R. M.1996. *Fluorescence resonance energy transfer*. In *Fluorescence imaging spectroscopy and microscopy*, Wang, X. F., Herman, B., editors. John Wiley & Sons Inc., New York. 179-251.
5. Sun, Y., R.N. Day and A. Periasamy 2011. *Investigating protein-protein interactions in living cells using fluorescence lifetime imaging microscopy*. *Nat. Protoc.* 6, 1324-1340.
6. Koushik, S. V., H. Chen, C. Thaler, H.L. Puhl 3rd and S.S. Vogel 2006. *Cerulean, Venus, and VenusY67C FRET reference standards*. *Biophys. J.* 91, L99-L101.

For more information please call (217) 359-8681
or visit our website at www.iss.com



ISS[™]

1602 Newton Drive
Champaign, Illinois 61822 USA
Telephone: (217) 359-8681
Fax: (217) 359-7879
Email: iss@iss.com