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Laboratory for Fluorescence Dynamics
(LFD)
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A. **Fluorescence Correlation Spectroscopy**
   - Autocorrelation
   - Photon Counting Histogram (PCH)
   - Cross-Correlation
   - References

   - **LAB 1.**
     - *Experiment 1:* Calculating concentrations: volume calculations
     - *Experiment 2:* Instrument and sample artifacts.
     - *Experiment 3:* Multiple Species: PCH & Autocorrelation
     - *Experiment 4:* Two Color FCS

B. **Raster Image Correlation Spectroscopy (RICS)**
   - Temporal and spatial fluctuations in microscope images.
   - Raster Scan Image Correlation Spectroscopy.

   - **LAB 2:** Performing RICS *in vitro* and *in vivo*.
     - *Experiment 1:* Measurement and analysis of Fluorescein in buffer pH 10 solution
     - *Experiment 2:* Measurement and analysis of 20 nm fluorescent beads in a buffer solution.
     - *Experiment 3:* Measurement and analysis of paxilin-GFP expressed in CHO-k1 cells

C. **Fluorescence Lifetime Imaging/ Förster Resonance Energy Transfer (FLIM/FRET)**
   - The Fourier transforms approach in the context of TCSPC data analysis.
   - Representation of fluorescence decays in the phasor plot.
   - FRET in the phasor plot.

   - **LAB 3:** FRET measurements *in vivo*.
     - *Experiment 0:* Measure the reference lifetime. Vary the amount of counts in each pixel and discuss how it changes the distribution of the lifetime.
     - *Experiment 1:* Lifetime Measurement and analysis for cells transfected with the donor (Cerulean).
     - *Experiment 2:* Lifetime Measurement and analysis for cells transfected with the donor and the acceptor (Cerulean-Venus).
     - Analysis.

   - **LAB 4** (Optional): Concentration measurement.
     - *Experiment 0:* Measure the first letter “L”. Locate the lifetime on the phasor plot.
     - *Experiment 1:* Measure the third letter “D”. Locate the lifetime on the phasor plot.
     - *Experiment 2:* Measure the middle letter “F”. Locate the lifetime on the phasor plot. What are
Fluorescence Correlation Spectroscopy (FCS)

General Information
Fluctuation spectroscopy has been utilized for 100 years, but the use of fluorescence in these studies was only introduced in 1972 (Elson and Magde 1974; Magde and others 1972; Magde and others 1974). Since that time, new developments in confocal microscopy, in detectors, and in computer speeds have greatly improved the usefulness of this method that has seen an explosion of interest over the past few years. There are a number of different manners in which fluorescence fluctuations may arise, but fluctuations deriving from particles diffusing in and out of a defined excitation volume are most common. From data collected in this way, information on the particle density (concentration), brightness, and diffusion constant can be obtained.

Autocorrelation
(see Rigler and Elson 2000; Thompson 1991)

The autocorrelation below was calculated from data collected on a 2-photon FCS microscope. The data were fit to a single diffusion constant model:

\[ G(\tau) = \frac{\langle \delta F(t)\delta F(t-\tau) \rangle}{\langle F(t) \rangle^2} \]

where

\[ \delta F(t) = F(t) - \langle F(t) \rangle \]

And with \( \tau = 0 \) we get:

\[ G(0) = \left( \frac{\langle F(t) \rangle - \langle F \rangle}{\langle F \rangle} \right)^2 = \frac{\text{variance}}{\text{mean}^2} = \frac{1}{\langle N \rangle} \]

where \( \langle N \rangle \) is our average particle number in the observation volume. Thus, \( G(0) \) is related to the reciprocal of the particle number \( N \). This relationship between \( G(0) \) and the particle number \( N \) holds for simple systems in which the particles all have the same brightness. The actual fluorescence observed will depend upon the particle density at time \( t \) and position \( r \), \( \langle C(r,t) \rangle \), the excitation profile and the volume observed \( \langle W(r) \rangle \) and instrument factors and particle brightness \( Q \).
\[ F(t) = \kappa Q \int dr \, W(r) C(r, t) \]

The shape of the observation volume in a 2-photon instrument is the point spread function of the 2-photon spot (PSF) (Berland and others 1995). The region of interest defined in a 1-photon, confocal microscope is formed by size and placement of the emission pinhole. The 3D Gaussian and Gaussian-Lorentzian shapes have been commonly used to describe the PSF in both 1- and 2-photon instruments. The volume calculation for a three dimensional Gaussian shape is,

\[ V_{3D-Gaussian} = \frac{2}{\pi} \cdot w_0^2 \cdot z_0 \]

where \(w_0\) and \(z_0\) are the 1/e² radial and axial wastes, respectively. The equation for the 2-photon case relating the autocorrelation \(G(\tau)\) to the particle diffusion (\(D\)) is given as:

\[ G(\tau)_{3DG} = \frac{\chi}{\langle N \rangle} \left( 1 + \frac{8D\tau}{w_{3DG}^2} \right)^{-\frac{1}{2}} \left( 1 + \frac{8D\tau}{z_{3DG}^2} \right)^{-\frac{1}{2}} \]

The \(w\) and \(z\) parameters are the beam x-y and z half-axis elements (1/e² radii), \(\langle N \rangle\) is the average particle number and \(\gamma\) is the shape factor (theoretically define by the shape which is 0.354 for the 3D Gaussian PSF and 0.076 for the Gaussian-Lorentzian (see (Berland and others 1995)). (The 1-photon case is similar but the “8”s in the above equation are changed to “4”s). The diffusion constant shifts the decay of the autocorrelation function in time while the number of particles (for a simple one specie system) describes the amplitude at time zero (see below).

**Multiple Species Autocorrelation**

The analysis for multiple species with the autocorrelation function can work quite well, but there are several points that one must keep in mind.

Firstly, \(G(0)\) is no longer related to the particle number in the same way. The sample \(G(0)\) is a weighted sum of the \(G(0)\)s for the different species present.
where $f_i$ is the fractional contribution to the intensity by species $i$. The general equation for multiple species with a given point spread function would be:

$$G_{psf}(\tau) = \sum_{i=1}^{M} f_i^2 \cdot G(0)_i \cdot g_{PSF}(D_i, \tau)$$

where the shape of the PSF is taken care of in the $g_{PSF}(D, \tau)$ term (see above for the 3D Gaussian shape).

**Photon Counting Histogram (PCH)**

*(Chen and others 1999; Muller and others 2000)*

If the diffusion constants for the species are not significantly different, we need a second parameter to help distinguish the separate species. Brightness of the particle (e.g. number of fluorophores on a protein, for example) could be used but the autocorrelation function cannot, in one experiment, solve for this parameter. For this reason, a new approach was sought to use particle brightness as the defining parameter. Photon counting histogram method (PCH) was then developed for this purpose. The basis for the PCH method is statistical. Though the autocorrelation function best describes the timing of a system, PCH describes the probability function of the fluctuations.

The photon counting histogram has a shape, a distribution, which is the result of particle diffusion through an inhomogeneous excitation profile and the particle number fluctuations. It is well known that a Poissonian distribution describes the particle occupancy fluctuations and that this distribution has the characteristic that the mean$^2$/variance is equal to the particle number. In our case, we are measuring photons, not particles, but related to the particle number. These histograms are broadened by the presence of an inhomogeneous excitation. In fact, this broadening effect gives us information on the average particle number ($<N>$) and the particle brightness values for single and multiple specie samples. The deviation from a pure Poissonian distribution, and the effect of the particle brightness on the photon histogram (left to right increases in brightness) can be seen in the plot below.

**Poisson Distribution**

$$p(N) = \frac{(N)^N \cdot e^{-N}}{N!}$$
The particle number also influences the PCH histogram. However, as the particle number increases, the histogram approaches a pure Poisson distribution where we will lose the information content.

**Fluctuation Cross-correlation Spectroscopy (FCCS)**

**Cross-Correlation**

(Eigen and Rigler 1994; Rigler and Elson 2000; Schwille and others 1997)

**Cross-correlation FCS to eliminate detector noise.**

The signal from the excitation volume can be measured simultaneously in two detectors. By cross-correlating these signals one can eliminate major sources of background noise not present in both channels. This is particularly true at fast data collection rates (< $10^{-6}$ s) where detector noise can be the predominant signal. The form for the cross-correlation function is:

$$G(\tau)_{i,j} = \frac{\langle \delta F_i(t) \delta F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle}$$

, where $i$ and $j$ are the two signal vectors.

**Two-color cross-correlation FCS.**

Two-color cross-correlation is a method to help identify and collect data on particles which are doubly labeled. This can be extremely useful in getting information on a small population of weakly associating macromolecules (associating proteins, proteins & DNA...). In correlating the two channels we then examine the fluctuations of only the particles having both colors (below).

Let us consider three common types of systems: uncorrelated, correlated, and interconverting. Uncorrelated is the state in which the different colored species diffuse separately.
In this case, the cross-correlation will show no correlation or only the correlation due to leakage of one channel fluorescence into the other detector. It is difficult to completely eliminate this artifact. The two cases are show below in which the left graph shows a small autocorrelation (black line) from leakage while the right shows a larger autocorrelation (black line) from a true correlated system.

Resonance energy transfer occurring between two different fluorophores would be an example of interconverting system. Monitoring the emission of both probes will not give us the usual functions because the fluorophores will change their brightnesses depending upon how close they are to each other. If these probes are at fixed distances, we have a constant change of brightness and would then treat the system as a correlated system (above). However, if we had a fluctuating distance due to macromolecular dynamics, we would see intensity fluctuations that could be measured. The (2-photon) equation to analyze the cross-correlation function is shown below adding the rate of the fluctuations, R, and the associated amplitude, F.

The presence of a fluctuation rate will lower the amplitude of $C(0)$ (time zero cross-correlation point) and distort the cross-correlation decay in the time frame of the rate. An example is given below. The dashed red line is a fit to a pure diffusion model.
References

LAB 1:
1) Calculating concentrations: volume calculations
   Rhodamine 110 calibration of the system.
      a. G(0) analysis
      b. PCH analysis

2) Instrument and sample artifacts.
   a. Poor statistics: improper sampling leading to erroneous autocorrelation curves.
      Collect several short time collections on a relatively dilute solution of spheres.
      The autocorrelation curves will be markedly different and oddly shaped.
   b. Effect of detector/sample saturation.
      PSF shape distortion: look at 10 nm yellow-green fluorospheres at several intensity levels. Plot brightness vs. excitation intensity (PCH) and D vs. excitation intensity (autocorrelation). Examine PCH histogram data for saturation.

3) Multiple Species: PCH & Autocorrelation
   Rhodamine 110 (300 um2/s) and yellow-green beads (20nm).

4) Two Color FCS
   a. independent species (green and red fluorospheres)
      1.) 2-photon systems: 790 nm red/green
      2.) (for 1-photon systems) excite at 488 nm AND 567 nm while collecting both channels.
B. Raster Imaging Correlation Spectroscopy (RICS)

**Temporal and spatial fluctuations in microscope images**

To introduce RICS, we first describe how to measure fast diffusion dynamics of molecules in a dilute, homogenous solution using a conventional laser scanning microscope (LSM). While images may appear uniform, there are hidden temporal and spatial fluctuations that can be analyzed to recover the diffusion coefficient and number of molecules in the instantaneous (scanning) excitation volume. Molecular processes such as conformational transitions, quenching associated with aggregation, molecular rotations, and diffusion can cause fluctuations in fluorescence intensity. However, for this mathematical derivation, we only consider signal fluctuations due to diffusion of particles in a homogenous medium since most of the processes in the cell involve transport (either by diffusion or directed motion) of particles from one location to another. Diffusion of a particle in a uniform medium can be described by the following relationship:

\[
C(r,t) = \frac{1}{(4\piDt)^{1/2}} \exp(-\frac{r^2}{4Dt})
\]

where D is the diffusion coefficient, and C(r,t) is proportional to the probability of finding the particle at position r and time t, when the particle was originally at the origin, r = 0, at time t = 0. The temporal and spatial autocorrelations are derived from Equation (1). There are two distinct parts of this equation, the temporal part and the spatial exponential Gaussian term. If a particle was at the origin at t = 0, it can be found at a distance r from the origin with a Gaussian distribution where the variance depends on time and the diffusion coefficient of the particle. If the concentration is sampled at one position, as is usually done with single point FCS, the temporal autocorrelation function of the fluorescence intensity decays with a characteristic time that depends on the diffusion coefficient and the size of the illumination volume. Alternatively, the concentration can be sampled at different spatial locations.

If particles are either not moving or are moving very slowly, as we sample different locations in a raster scan image, the intensity at one point is correlated to the intensity of the adjacent points if there is superposition of the PSF at the adjacent points (figure 1, situation 1). In this case the 2-D spatial correlation of an image just reflects the extent of superposition of the PSF in the adjacent points. To explain spatial correlation for fast moving particles, consider two points separated by a given distance r. If this distance is less than the width of the PSF then there may be some correlation of the fluorescence between the two points (figure 1, situation 2) but the spatial correlation will be less than that for immobile particles. However, for points that are not superimposed by the PSF, there could be some correlation due to the diffusion of the particle to these points.

In this case, the spatial autocorrelation function decays with a characteristic length that depends on the diffusion coefficient and the size of the illumination volume. LSMs operating in the line or raster scan mode acquire data from different spatial locations at different times. Spatial correlations for small diffusing particles depend on the spatial overlap and the time interval between adjacent pixels. Random diffusion of the particle or longer time intervals between data
points decrease correlation at shorter spatial scales and increase correlation at long distances (see Figure 1 and the discussion in the figure caption). It is precisely this change of shape of the spatial correlation function that contains the information about molecular diffusion.

For the most common scan configurations, circular or line scan and raster scan microscopy; the adjacent volumes along the scanning line are sampled very rapidly, while the adjacent volumes in two consecutive lines are sampled at a much slower rate. This difference in sampling time can be exploited to measure a range of diffusion coefficients, from very fast molecular motions occurring on the timescale of microseconds to slower diffusion occurring in the range of milliseconds to seconds (Figure 2).

Starting from Equation 1, the relationship between the concentration of a particle and fluorescence intensity F is given by:

\[ F(t) = \kappa Q \int d\mathbf{r} \ W(\mathbf{r}) \ C(\mathbf{r}, t) \]  

(2)

where the integral is calculated over the entire excitation volume, Q is the quantum yield, \( \kappa \) a factor that accounts for the instrument sensitivity and \( W(\mathbf{r}) \) describes the PSF of the microscope system. The temporal autocorrelation function is then given by the following expression:

\[ G(\tau) = \frac{\langle \delta F(t)\delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \]  

(3)

In this equation the angled brackets indicate time averages, \( \delta F \) is the fluorescence intensity fluctuation with respect to the average and \( t \) is the delay time between successive samplings of the fluorescence signal. By inserting Equations 1 and 2 into Equation 3 and assuming time sampling at the same spatial locations, we can obtain the familiar autocorrelation function used in FCS for the analysis of a particle freely diffusing in a volume.

Equations for the spatio-temporal correlation function for data acquired using raster scan modes are shown here and they will be used to fit experimental spatio-temporal correlation functions. Diffusion coefficients and concentrations of molecules in dilute samples are obtained from these fits.

**Raster scan image correlation spectroscopy (RICS)**
One of our goals is to make RICS accessible to most researchers using standard confocal microscopes. For this reason we expanded the theory developed for circular scan to line scan and 2-D spatial raster scan images.

The overall correlation function is written as,

\[ G_S(\xi, \psi) = S(\xi, \psi) \cdot G(\xi, \psi) \]

In this case, the spatial part of the correlation function is expressed directly in terms of the pixel size, \( \delta r \) (typically in the range 0.05 to 0.2 \( \mu m \)), pixel resident time, \( \tau_p \) (typically in the range 2-100 \( \mu s \)), and the line repetition time, \( \tau_l \), typically in the 3ms to 100 ms range.

\[
S(\xi, \psi) = \exp \left( -\frac{1}{2} \left[ \frac{2\xi \delta r}{w_0} \psi \delta r \psi \right] \right)
\]

\[
G(\xi, \psi) = \frac{\gamma}{N} \left( 1 + \frac{4D(\tau_p \xi \psi + \tau_l \psi)}{w_0^2} \right)^{-1} \left( 1 + \frac{4D(\tau_p \xi \psi + \tau_l \psi)}{w_0^2} \right)^{-1/2}
\]

References:

**LAB 2: Performing RICS in vitro and in vivo.**

RICS will be used to determine the diffusion coefficient of fast moving molecules such as fluorescent dyes in solution and slow molecules such as protein inside cells. In these experiments we will learn the strategies to take and to analyze the data to measure fast and slow fluctuations.

**Experiment 1:** Measurement and analysis of Fluorescein in buffer pH 10 solution. For observation of fast moving molecules we must chose a pixel dwell time which is faster than the...
characteristic time of diffusion of the molecule through the laser beam. Also the pixel size must be small enough as to sample several times the PSF. For example, the dwell time for the fluorescein sample must be in the order of 8 to 16 μs. The pixel size should be on the order of 0.05 μm. In the Olympus microscope the zoom is achieved by moving the cursor on the zoom bar. A field on the screen gives the pixel size as you change the zoom. In the LFD instruments, the zoom is achieved by changing the full-scale voltage of the scanner. In the M3 microscope, the zoom factor for 4000mV is 0.058 um/pixel. The pixel size is also displayed in the image acquisition screen. The fluorescein solution should be in the 10-20 nM range. A frame size of 256x256 is adequate for this experiment. This experiment will be used to obtain the value of the size of the PSF. It will be assumed that the diffusion coefficient of fluorescein at 20C is 300 μm²/s.

**Experiment 2:** Measurement and analysis of 20 nm fluorescent beads in a buffer solution. The instrument conditions should be the same as those used in experiment 1. For this experiment the pixel dwell time can be in the 20 to 50 μs/pixel range. The pixel size should always remain at about 0.05 μm.

**Experiment 3:** Measurement and analysis of paxilin-GFP expressed in CHO-k1 cells. The instrument conditions should be modified to have a dwell time in the 50 to 64μs. The pixel size should remain the same at 0.05μm. First you have to zoom out to select a particular cell in the plate and then zoom in to measure the desired area of the cell. Both frame scanning and line scanning will be performed. The measurements will then be analyzed to calculate the diffusion of the paxillin molecules in different parts of the cell.
C. Fluorescence Lifetime Imaging/ Förster Resonance Energy Transfer (FLIM/FRET)

The Fourier transforms approach in the context of FLIM data analysis

In our development of FLIM data analysis, we have followed a radically different approach that has the potential to resolve the very complicated spectroscopic situations that frequently arise in FLIM. In this application, we describe the use of the Fourier transform of the time decay to rapidly analyze the decay at each pixel from images obtained using the time-correlated single photon counting method TCSPC method.

A major outcome for us was to found a method to transform the histogram of time delays at each pixel into a global representation of the decay. In a universal plot, which we name the “Phasor Plot,” each molecular species is represented by a point. In this plot we can immediately distinguish whether we have mixtures of molecular species or changes in lifetime arising from quenching (FRET), without having to resolve the decay at each pixel into exponential components.

Our analysis method is based on the frequency domain equations. These equations are well known (Spencer and Weber, 1969) and express the value of the lifetime in terms of the phase and modulation of the modulated light at each pixel (Figure 3)

\[ \tau_p = \frac{\tan(\phi)}{M} \] \hspace{1cm} (1)

\[ \tau_m = \frac{\sqrt{1/M^2 - 1}}{\omega} \] \hspace{1cm} (2)

where \( \phi \) is the phase delay between emission and excitation, \( M \) is the modulation ratio of the emission with respect to the excitation and \( \omega \) is the angular frequency of the light modulation. The formulas above are sufficient to determine the average value of the lifetime at each pixel and to test if there is more than one exponential component at that pixel. The lifetime determined by phase (\( \tau_p \)) and modulation (\( \tau_m \)) should be identical if the decay is single exponential ("\( \tau \) rule"). This criterion gives a simple way to estimate if the sample has one or more lifetimes. Note that once we have the “phase” and “modulation” at each pixel, the calculation of the lifetime image is “instantaneous” since it only requires the calculation of formulas (Eqs. 1 and 2).

The Fourier transform approach provides a simple, fast and elegant algorithm for the determination of lifetimes in FLIM. In our system, data acquired using the TCSPC technique is Fourier transformed and then we apply the equations as if the data was collected in the frequency domain. Our approach is radically different than the methods used.
to analyze the time domain data; since we just need to calculate formulas, the results are instantaneous.

**Representation of fluorescence decays in the Phasor Plot**

To understand the inner working of the frequency domain method and its relevance to FLIM for the identification of molecular components, we follow an approach originally developed by G. Weber in 1981 for the analysis of frequency domain data, which was then further expanded and given a geometrical interpretation by Jameson et al. (1985). In the original work by Weber, the focus is on the determination of the lifetimes of a multi-component system. In our approach, we expand this concept with the goal of producing images of species based on decay properties, rather than resolving the lifetimes of molecular species.

Each species could have a complex decay such as the decay of EGFP, which is double exponential. In imaging, it is important to spatially separate different molecular species, even if they have complex decays. Our goal is to display the spatial map of EGFP as being distinct from the spatial map of other fluorescent species, rather than resolving the decay components of the EGFP. Of course, it is also important to determine the lifetimes of individual species. However, this task becomes easier after one species has been spatially isolated and subtracted from the contributions of background and other contaminants.

In our approach, we first transform the decay into a set of orthogonal coefficients and then we apply the linear algebra to these coefficients. In the frequency domain (or in the time domain after Fourier transform of the decay), at any given modulation frequency each decay component can be thought of as a “phasor”. The concept of phasor comes from electrical engineering. If a linear system is excited by a sinusoidal signal, the response of the system is also sinusoidal but with a phase and amplitude difference relative to the excitation. The sinusoidal excitation can be written in the form:

\[ E_{ex} = A \sin(\omega t) \]  

In the Cartesian plane, Equation 3 represents a rotating vector at angular velocity \( \omega \). Since the response of the system is sinusoidal and has the same frequency, the response is also a rotating vector, but with a different phase \( \varphi \) and relative amplitude \( M \) with respect to the excitation. If we only consider the difference between the two vectors, then we have a phasor (Figure 3 above).

The phasor is stationary and directly reflects the phase and amplitude difference between the excitation and response. Each decay component at a given frequency is represented by a phasor that has a direction (phase delay relative to the excitation) and a length (the modulation ratio of amplitude relative to the excitation). In the frequency domain, we measure the phase and modulation of the emission with respect to the excitation. The components of the phasor are given by the following equations in terms of phase and modulation, which are the quantities directly measured by the instrument:

\[
g_i(\omega) = m_i \cos(\varphi_i) \\
\sin (\varphi_i) = m_i \sin (\varphi_i) \quad (4)
\]

In the time domain we measure the decay curve \( I(t) \). In terms of the decay curve, the phasor components are given by:

\[
g_i(\omega) = \frac{\int_0^\infty I(t) \cos(\omega t) dt}{\int_0^\infty I(t) dt} \\
\sin (\varphi_i) = \frac{\int_0^\infty I(t) \sin(\omega t) dt}{\int_0^\infty I(t) dt} \quad (5)
\]
The time-domain equations show that the $g_i$ and $s_i$ functions are the cosine and sine moments of the decay. The first and second Fourier components can efficiently be computed using an FFT algorithm. Since our approach only requires the use of the first, or at most second, Fourier coefficient, our results will be relatively stable and maximally exploit the count statistics.

Using Equations 1 and 2 we can see that there is a direct relationship between a phasor (phase and modulation) and the lifetime. If we plot the phasor coordinates in a plane where the $g$ component is the x coordinate and the $s$-component is the y coordinate, every possible lifetime can be mapped into this universal (normalized) representation of the decay (Phasor Plot). If the phasor represents a single exponential component, then Equations 1 and 2 must be equal. The points in the phasor plot that satisfy this relationship must lie along a semicircle of radius $\frac{1}{2}$ centered at $(\frac{1}{2},0)$, i.e., $M=\cos\phi$ (see Figure 3B). In this case, the phase angle (or modulation) is directly related to the (single) lifetime through Equations 1 and 2. The longer the lifetime is, the larger the phase angle and the smaller the length of the vector will be.

The phasor is like a vector and it follows vector algebra. Therefore, phasors can be added and subtracted using simple rules. This is especially important for decomposing the decay into components, background subtraction and differential measurements. Given two exponential lifetime components, they must lie on the semicircle. Their composition, i.e., the phasor resulting from the linear combination of these two components, must be on the line joining the two lifetime points as shown in Figure 3C. For every decay curve, we can plot the phasor coordinates in the phasor plot using Equations 4 or 5, depending on whether measurements are done in the frequency or time domain. It is a simple graphical task to determine the fractional contributions of two components if they are known single exponentials (Figure 3C). Also, if one of the lifetimes is known, we can graphically determine the other lifetime component and fractional contributions with a simple graphical construction (see discussion in Figure 3C).

In a fluorescence system with multiple components, the overall decay is a phasor that is the sum of many independent phasors. Note that a phasor can represent a decay component, which can be multi-exponential or even a distribution of lifetimes. At each modulation frequency we have a different phasor diagram. In general, we have a phasor given as the sum of several component phasors:

\[
G(\omega) = \sum_{i} g_i, \\
S(\omega) = \sum_{i} s_i
\]

For example, suppose that a fluorescent molecule has a double exponential decay. In the phasor plot, this will correspond to a point inside the semicircle. If we have a second molecular species, which can also be multi exponential, the linear combination of the two molecular species must lie on the line joining the two phasors (Figure 4A). It is a general result that the possible values for a mixture of two components must be in a very specific straight line in the phasor plot. For example, the phasors of a population of cells that have different levels of expression of one protein (e.g., EGFP) and background autofluorescence in different ratios must all be along a specific line in the phasor plot. This geometrical property allows us to recognize and interpret (in terms of molecular species) the lifetime.
values found in cells, as will be shown later in this work.

**FRET in the Phasor Plot**

FRET analysis is one of the most common applications of the lifetime technique in microscopy. Quenching of the lifetime of the donor in the presence of the acceptor is considered one of the most convincing pieces of evidence that there is FRET among the donor-acceptor pair. We have studied in great detail various microscopy methods (Breusegem, Thesis, 2002) to determine FRET and we agree with this common knowledge that FLIM is one of the best ways to determine and quantify FRET inside of cells.

In a typical FRET experiment, we would like to answer the following question: is FRET occurring in some parts of the image? Our method of visual inspection using the phasor plot directly answers this question.

**References**


**LAB 3: FRET measurements in vivo.**

In this laboratory we will measure FRET by measuring the decrease in the donor lifetime when the acceptor is present. For this we will measure the lifetime of the donor in the absence and in the presence of the acceptor. The lifetime data and the FRET efficiency will be analyzed using the phasor plot representation.
The system to be used is cells that have been transfected with Cerulean (donor, mutated CFP) and with Cerulean-Venus (mutated YFP) (donor-acceptor).

![Absorbance and emission of CFP and Cerulean](image1)

Nature Biotechnology V22 No.4 April 2004

The absorbance and emission of CFP and Cerulean

![Emission spectrum of Cerulean and Venus](image2)

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**Experiment 0**: Measure the reference lifetime. Vary the amount of counts in each pixel and discuss how it changes the distribution of the lifetime.

**Experiment 1**: Lifetime Measurement and analysis for cells transfected with the donor (Cerulean).

**Experiment 2**: Lifetime Measurement and analysis for cells transfected with the donor and the acceptor (Cerulean-Venus).

**Analysis**:
1. Identify background in the phasor plot.
2. Identify unquenched donor in the phasor plot. Observe how the background shifts the unquenched donor.
3. Identify the FRETing pixels in the phasor plot. Calculate the FRET efficiency.

**(Optional) LAB 4**: Concentration measurement.
In this laboratory we will measure the lifetime of two fluorescent solution and the mixture of the two solutions in micro-fluidic channels. The goal is to figure out the ratio of each component in the mixture.
**Experiment 0:** Measure the first letter “L”. Locate the lifetime on the phasor plot.

**Experiment 1:** Measure the third letter “D”. Locate the lifetime on the phasor plot.

**Experiment 2:** Measure the middle letter “F”. Locate the lifetime on the phasor plot. What are the distances between LF and FD on the phasor plot? What is the relative brightness of the fluorescence in L and D? Assume that the relative brightness of each fluorophore does not change after mixing, determine the ratio of the two fluorophores in the mixture.