Laboratory Training

Introductory Course
in Fluorescence Techniques, Spectroscopy and Microscopy

March 23–25, 2009
Laboratory for Fluorescence Dynamics
University of California, Irvine
Fluorescence Spectra and Polarization

Samples
ANS and ANS- BSA
In 1954, Gregorio Weber and D. J. R. Laurence reported on the unusual environmental sensitivity of the fluorescence of certain anilinonaphthalene compounds. In particular they observed that the emission of 1,8-anilinonaphthalene sulfonic acid (1,8-ANS) was very weak in aqueous solutions but increased markedly in less polar solvents (such as ethanol) or when bound to bovine serum albumin (BSA).

In these experiments we shall study the excitation and emission spectra of 1,8-ANS in water, in ethanol and bound to BSA. We shall distinguish between uncorrected (technical) and corrected (molecular) spectra. We shall also measure the polarization of 1,8-ANS in these three systems. We shall then discuss and interpret our results.

FITC -BSA
Labeling of BSA with FITC (Figure). Prepare two solutions: FITC in 0.01M NaOH~1mg/ml (~2.5x10^{-3} M) and Bovine Serum Albumin in 50mM Phosphate buffer pH ~8.0 ~1mg/ml (~1.5 x10^{-5} M).
- Add ~ 5ul of FITC solution to 0.5 ml BSA solution.
- Let sit at room temperature for ~ 1 minute
- Pass over small gel filtration column.
- Monitor separation using UV handlamp
- Collect separately the two fluorescent peaks (free and bound)

References


Materials and Instruments
To save time, pre-made solutions of ANS (approximately 10^{-6} M concentration) in buffer, in ethanol (EtOH) and in the presence of excess BSA will be provided.

Solutions of FITC and BSA (for the covalent labeling experiments) will also be provided.

All steady-state experiments will be carried out on an ISS PC1 instrument equipped with a xenon arc lamp light source, Glan-Thompson polarizers and photon-counting electronics.
Steady-State Fluorescence Experiments. Effect of solvent environment on ANS fluorescence spectra and polarization

**Excitation and emission technical spectra of ANS in ethanol**
We will first measure the uncorrected (technical) excitation and emission spectra of ANS in ethanol. For the excitation spectrum we will use an emission wavelength of 470 nm; for the emission spectra we will use an excitation wavelength of 370 nm.
(Note: to obtain the uncorrected excitation spectrum we will not use the ratio acquisition mode). We will also note the effect of changing the excitation wavelength (to 280 nm) on the emission spectrum.

![Figure 2. Absorption (dotted line) and uncorrected excitation spectra for ANS in EtOH.](image)

![Figure 3. Absorption (dotted line) and “ratio” corrected excitation spectra.](image)
Absorption (dotted line) and excitation spectra - corrected by a lamp curved as described in Jameson et al Methods in Enzymology (2003) article

**Questions:**
Where are the excitation peaks (nm) located in your spectra?
What is the best excitation wavelength?

**Experiment 2: excitation and emission corrected spectra of ANS in ethanol**
We will now obtain the corrected (molecular) excitation and emission spectra for ANS in ethanol (EtOH). To obtain the corrected excitation spectra we will use the instrument’s ratiometric mode with a quantum counter (concentrated rhodamine B in ethanol) in the reference path – this quantum counter measures the total intensity of the exciting light at all wavelengths utilized. To obtain the corrected emission spectrum we will place a polarizer – principle polarization direction oriented parallel to the vertical lab axis and in this way to the monochromator grating grooves- in the emission path and then utilize the appropriate correction factors stored in the software.

**Figure 5.**
Emission correction factors for ISS PC1; parallel (vertical) polarizer factors – solid line; perpendicular (horizontal) polarizer – dotted line.
Questions: Where is the peak emission (nm) in your spectrum? What is the Full Width Half Maximum (FWHM) in nm?

emission technical spectrum of ANS-buffer sample
Next we will measure the uncorrected emission spectrum of the ANS-buffer sample using 370 nm excitation.

You will note that the fluorescence is much weaker than for the ANS/ethanol solution and that the Raman peak due to the O-H bond symmetric and asymmetric stretch vibration of water is now visible (note: calculate where the water Raman peak is expected for 370nm excitation).

Question: What do you think happens to the emission maximum and the Raman peak position and intensity when the excitation wavelength is altered to the blue and to the red with respect to 370 nm?
Experiment 4: emission technical spectrum of ANS with BSA
Now we will measure the corrected excitation and uncorrected emission spectra of ANS in the presence of excess BSA.

Discussion on spectra results
We will now discuss our findings. In particular, we will compare the excitation and emission spectra of the three samples and discuss the reasons for the differences.

Polarization (anisotropy) measurements on the 3 samples
We will now measure the polarization/anisotropy of the three samples using an excitation wavelength of 370 nm and viewing the emission through an appropriate cutoff filter (i.e., one that blocks 370 nm light but which passes wavelengths greater than 385 nm).

The polarization is defined as:

\[ P = \frac{I_{VV} - gI_{VH}}{I_{VV} + gI_{VH}} \quad [1] \]

Where:
$I_{VV}$ is the measured fluorescence intensity with the polarizer in the excitation channel in the (V)ertical position and the polarizer in the emission channel in the (V)ertical position.

$I_{VH}$ is the measured fluorescence intensity with the polarizer in the excitation channel in the (V)ertical position and the polarizer in the emission channel in the (H)orizontal position.

The number $g$, called the $g$-factor, is given by the following relation, where the letters $V$ and $H$ refer to the positions of the polarizers in the excitation and emission channel, respectively.

$$g = \frac{I_{VV}}{I_{HH}} \quad [2]$$

While the anisotropy is defined as:

$$r = \frac{I_{VV} - gI_{VH}}{I_{VV} + 2gI_{VH}} \quad [3]$$

The following relations allow the switching between polarization and anisotropy values:

$$P = \frac{3r}{2 + r} \quad [4]$$

$$r = \frac{2P}{3 - P} \quad [5]$$

**Question:** The total intensity is defined as $I_{vv} + 2gI_{vh}$. What approach will result in the simplest formula: Descriptions using $P$ or one utilizing $r$?

**Question:** What is the meaning of a $P = 1$ value? and similarly for a $P = 0$? Can $P$ be equal to $-1$?

**Discussion on polarization results**

We will discuss the significance of the polarization/anisotropy values obtained for the three samples.

**Relation between polarization and lifetime**

In a solution, molecules rotate while in an excited state. The value of polarization (anisotropy) measured in steady-state mode depends on the value of the decay time and the value of rotational correlation time. The expression discovered by Perrin establishes that:

$$\frac{1}{P} - \frac{1}{3} = \left( \frac{1}{P_0} - \frac{1}{3} \right) \left( 1 + \frac{RT}{\eta V} \tau \right) \quad [6]$$

Which can be re-written in terms of anisotropy as:
\[ \frac{r_0}{r} = 1 + \frac{\tau}{\tau_c} \]  \hspace{1cm} [7]

With \( \tau_c \):

\[ \tau_c = \frac{\eta V}{RT} \]  \hspace{1cm} [8]

Where:
- \( R = 8.314 \times 10^7 \) erg mol\(^{-1}\) K\(^{-1}\), is the molar gas constant
- \( T \) is the absolute temperature (K)
- \( \tau \) is the decay time (nsec)
- \( \tau_c \) is the rotational correlation time (nsec)
- \( \eta \) is the viscosity of the solution (N / (m\(^2\) sec)). In SI units N(newton) = kg / (m sec\(^2\))
- \( V \) is the molar volume of the rotating molecule (m\(^3\))
- \( r_0 \) is the limiting anisotropy, that is the anisotropy when no rotation is present
- \( r \) is the measured value of the anisotropy

Reworking above equation 7, one can estimate the decay time of the molecule by measurements of the steady-state polarization.

\[ \tau = \tau_c \left( \frac{r_0}{r} - 1 \right) \]  \hspace{1cm} [9]

First we start by estimating the quantity \( \tau_c \). This quantity is defined as the rotational correlation time for a molecule and depends upon the solvent viscosity \( \eta \), the molar volume \( V \) of the rotating molecule, the gas constant \( R \) and the absolute temperature \( T \).

For globular proteins, the expression is written as:

\[ \tau_c = \frac{\eta V}{RT (\nu + h)} \]  \hspace{1cm} [10]

Where
- \( \nu \) is the partial specific volume
- \( h \) is the degree of hydration (typically is about 0.2 g of water per 1 g of protein)

Formula [10] cannot be utilized for ANS/BSA as the molecule is not of a globular shape; still, we can estimate the value of the rotational correlation time. In water at 20 °C, and using the following values:

\[ \eta = 0.01 \text{ Poise} \]  \hspace{1cm} (1 Poise = 1 dyne x sec /cm\(^2\) = 1 gr / (cm x sec))

\[ M = 66,000 \text{ Daltons} \]

\[ \nu = 0.74 \]

\[ h = 0.3 \]

we estimate the rotational correlation time to be:
The correct rotational correlation time is about 35 nsec (Anderson and Weber, 1969, estimated the rotational relaxation time $\rho$ to be 105 nsec. Please note that $\rho = 3 \tau_c$).

Also, using for ANS the following values for the anisotropy, $r$, and the limiting anisotropy, $r_0$:

$$r_0 = 0.36$$
$$r = 0.23$$

we can estimate the decay time of the fluorophore in water to be:

$$\tau \approx 35 \cdot 10^{-9} \left( \frac{0.36}{0.23} - 1 \right) = 20 \cdot 10^{-9} \text{ sec}$$

The decay time is estimated to be about 20 nanoseconds.
Fluorescence Lifetime Experiments and Dynamics Polarization (Time-resolved Anisotropy)

The samples we will examine will be 1) fluorescein 2) fluorescein coupled to Lysozyme (14.3 kDa enzyme), and 3) EGFP. The fluorescence lifetime will be measured using the phase and modulation technique on an ISS Chronos with a modulated 471 nm laser diode. For the determination of the decay times of fluorescence, the excitation light beam, $E(t)$, is modulated at an (angular) frequency $\omega$, and phase and modulation of the resulting fluorescence, $F(t)$, is measured.

The fluorescence exhibits a shift in the phase as well as a decrease in amplitude: demodulation; only for a single exponential decay, both quantities are related to the decay time by the following relations:

$$\phi = \tan^{-1}(\omega \tau_p)$$

$$m = \frac{1}{\sqrt{1 + \omega^2 \tau_M^2}}$$

where

$$\omega = 2\pi \nu \quad \text{with } \nu \text{ the frequency of the modulation signal in Hz}$$

In these experiments, the quantities ($\phi$, $m$) are measured at a series of different values of the modulation frequency. A data table containing measured phase and demodulation values as well as their accuracy is obtained; the data are fitted, using a non-linear least squares (NLLS) minimization technique, versus a model selected by the experimenter.
Fluorescence lifetimes: Comparison of fluorescein and fluorescein tagged to Lysozyme (a 14,300 Da enzyme found in egg white)

The following plot displays data corresponding to a 4 ns decay time of fluorescein.

![Figure 10. Phase and modulation data, versus frequency, of free fluorescein in buffer. Excitation was at 472 nm using a diode laser.](image)

The following plot displays data corresponding to a 3.5 ns decay time of fluorescein tagged Lysozyme.

![Figure 11. Phase and modulation data, versus frequency, of Lysozyme tagged with fluorescein. Excitation was at 472 nm using a diode laser.](image)

Time-Resolved Anisotropy

Anisotropy decay, like the lifetime data, can be measured using time-domain or frequency domain. In time-domain, or time-correlated methods, the sample is excited with single pulses polarized light (normal to the laboratory plane) and an intensity decays are collected for: (1) the emission through a polarizer parallel to the excitation and (2) for the emission through a polarizer perpendicular to the excitation pulse. The two emission decays are then used to calculate the decay of anisotropy which can, be fit to an exponential (or multiexponential) model:
\[
A(t) = \frac{I(t)_{\text{parallel}} - I(t)_{\text{perpendicular}}}{I(t)_{\text{parallel}} + 2I(t)_{\text{perpendicular}}} = A_0 e^{-t/\phi}
\]

where the parameters of interest are the rotational correlation time, \(\phi\), and the time-zero anisotropy, \(A_0\).

As described in the lectures, anisotropy measurements are used to determine the rotation diffusion of a fluorophore. If the fluorophore is attached to a macromolecule then information on the rotational properties (size and shape and aggregation state) can be obtained. In time-resolved measurements, time-domain or frequency domain, the researcher can tease apart fast and slow motions by resolving the decay of anisotropy in time. Typically, the data are analyzed and rotational correlation times are extracted. The relationship between the rotational correlation time, \(\phi\), and the volume, \(V\), of a spherical macromolecule is given:

\[
\phi = \frac{V\eta}{kT}
\]

where, \(\eta\), the viscosity, \(k\), Boltzmann’s constant, and \(T\), the temperature. The volume for a protein will involve its specific volume and its hydration state and molecular weight through the following expression,

\[
V = \frac{M(v + d)}{N_a}
\]

where \(M\) is the molecular weight, \(v\) the specific volume (0.73 cc/g is average for proteins), \(d\) is the hydration (0.4 cc/g is a reasonable value for protein) and \(N_a\) is Avogadro’s number.

In the frequency domain an excitation light is polarized, again normal to the laboratory plane, and the modulation ratio of, and the phase shift between, the emission polarized parallel to the excitation beam and the emission polarized perpendicular to the excitation beam are then determined. These data are collected across a wide frequency range and analyzed for rotational correlation times. For more details on the frequency domain calculations, consult the included references.

Our three samples are fluorescein, lysozyme (enzyme) and EGFP that have molecular weights of 400 Da, 14,300 Da and 26,000 Da, respectively. Assuming spherical shapes and using the specific volume and hydration given above, the expected rotational correlation times for these samples would be: 0.19 ns, 6.7 ns, 12.1 ns.
Figure 12. Phase shift and modulation data showing the anisotropy decay of Lysozyme tagged with fluorescein.

Figure 13. Phase shift and modulation data showing the anisotropy decay of free fluorescein in solution.
**Laboratory Training**

**“Laudan GP imaging”**

Introduction to Fluorescence Techniques Workshop  
UCI Spring 2009, March 32, 24, 25  
Susana Sanchez (Laboratory for Fluorescence Dynamics)

**LAURDAN Generalized Polarization (LAURDAN GP)**

LAURDAN belongs to the family of polarity-sensitive fluorescent probes first designed and synthesized by G. Weber for the study of the phenomenon of solvent dipolar relaxation. The unique characteristics of LAURDAN to determine lipid lateral organization in bilayers are particularly useful in studying lipid-phase coexistence. These characteristics can be divided into four fundamental properties. (1) the electronic transition dipole of LAURDAN is aligned parallel to the hydrophobic lipid chains; (2) LAURDAN shows a phase dependent emission spectral shift, that is blue in the ordered lipid phase and blue-green in the disordered lipid phase (this effect is attributed to the reorientation of water molecules present at the lipid interface near the fluorescent moiety of LAURDAN; (3) LAURDAN distributes equally into the solid and liquid lipid phases; and (4) LAURDAN is negligibly fluorescent in water.

Unfortunately, the extent of LAURDAN photobleaching under the epi-fluorescence microscope is severe, making it almost impossible to collect images of LAURDAN-labeled specimens for more than few seconds. However, the use of two-photon excitation fluorescence microscopy helps circumvent this problem. The fact that two-photon absorption in the microscope is confined to the focal volume without excitation in areas above and below the focal plane (because of insufficient photon flux) dramatically reduces the extent of probe photo-bleaching during image collection.

**LAURDAN Generalized Polarization Function**

LAURDAN is used as a membrane probe because of its large excited state dipole moment, which results in its ability to report the extent of water penetration into the bilayer surface due to the dipolar relaxation effect. Water penetration has been correlated with lipid packing and membrane fluidity. The emission spectrum of LAURDAN in a single phospholipid bilayer is centered at 440 nm when the membrane is in the gel phase and at 490 nm when in the liquid crystalline phase. The GP gives a mathematically convenient and quantitative way to measure the emission shift. The function is given by:

\[
GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}
\]
where $I_{440}$ and $I_{490}$ are the emission intensities at 440 and 490 nm respectively. If LAURDAN is inside a lipidic structure, such as a liposome, its spectrum will move according to the water content in the bilayer. To calculate the GP value in a two-photon microscope an excitation wavelength of 780 nm and a two channel system with the corresponding filters on the emission is used.

**Changes in LAURDAN GP by cholesterol**

Cholesterol is found in every cell. It is especially abundant in the membranes, where it helps maintain their integrity, and plays a role in facilitating cell signaling. Cholesterol is an amphipathic molecule, meaning, it contains a hydrophilic and a hydrophobic portion. Cholesterol's hydroxyl (OH) group aligns with the phosphate heads of the phospholipids. The remaining portion of it tucks into the fatty acid portion of the membrane.

On the other side, the Naphthalene moiety of Laurdan locates in the membrane also at the level of the glycerol backbone. Therefore the presence of cholesterol in the bilayer will change the Laurdan emission spectra. The effect of cholesterol on membrane physical properties has been studied for many years. To study the effect of cholesterol in the plasma membrane, cholesterol content in the cells has to be manipulated by the use of organics compounds such as Methyl beta Cyclodextrins (MBCD). In this laboratory training, we will use red blood cells from Rabbit, to show the changes in membrane fluidity measured by GP imaging when cholesterol is removed from the cell plasma membrane by MBCD.

**Laurdan GP imaging**

Laurdan GP measurements in the microscope are done using two-photon excitation. Traditional one photon confocal excitation must be avoided because it induces severe photobleaching of the probe making it impossible to collect images under the confocal microscope for more than few seconds. GP measurements in the microscope are shown in the Figure on the left. a) The microscope set up for GP measurements includes the two-photon excitation source (Ti:Sapphire laser pumped by a Argon ion laser), Mirror scanner and two detectors on the emission to register the filtered signal. b). Transmittance of the GP-filters, centered at 440 and 490 nm respectively (in black), overlapped with the emission spectra of Laurdan (in grey). The GP image is generated using the images acquired simultaneously in the two channels.
**Experiments:**
In this practical we will see the effect of removing cholesterol from plasma membrane using methyl cyclodextrins. Rabbit red blood cells will be used.

**Protocol:**
1- Blood preparation: take 500 μL of whole blood in an eppendorf tube. Centrifuge for 2 minutes in a bench centrifuge (1,000 g) and removed the plasma. Rinse the red blood cells with buffer 134mMNaCl, 6.2mMKCL, 1.6mMCaCl2, 1.2mMMgCl2, 18mM HEPES, 13.6 mM glucose, pH 7.4. and incubate the stock at 37C.

2- From the stock solution take 100uL and add 900 uL of a buffer containing 0 and 3.5 mM MBCD, incubate for half hour at 37C. Following incubation, cells were washed and re-suspended in fresh buffer. Add LURDAN to a final concentration of 1 uM. Incubate for 20 min at 37C to equilibrate the probe with cell membranes. Cells treated with MBCD must be wash by centrifugation (1,000 g for 2 min) 3 times.

3- Take several images of the two samples at 37C.

4- Analyze the data and discuss the effect of MBCD on the membrane fluidity.

**References**
FCS Lab

Introduction to Fluorescence Correlation Spectroscopy (FCS)

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.

*Just what is fluorescence correlation spectroscopy?*

![Diagram of fluorescence correlation spectroscopy](image)

The measurement of fluorescence fluctuations in an observation volume and determining fluctuation rates and fluctuation numbers from the collected data stream. The analytical concepts central to the extraction of information from this “noise” are embodied in the autocorrelation function and the photon counting histogram (PCH).

**Autocorrelation (Rigler and Elson 2000; Thompson 1991)**

The autocorrelation below was calculated from data collected on a 2-photon FCS microscope on a simple single component system.

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

![Graph of G(t) vs. Log [t (ms)]](image)

\[
G(t) = \begin{cases} 
0.20 & \text{if } t = 0 \\
0.15 & \text{if } t = 1 \\
0.10 & \text{if } t = 2 \\
0.05 & \text{if } t = 3 \\
0.00 & \text{if } t = 4 
\end{cases}
\]

- Log [t (ms)]
And with $\tau = 0$ we get:

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

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 $r$, $(C(r,t))$, the excitation profile and the volume observed $(W(r))$ 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 due to the size and placement of the emission pinhole. The three dimensional Gaussian shape has been commonly used to describe both 1- and 2-photon instruments. The equation for the 2-photon case relating the autocorrelation $G(\tau)$ to the particle diffusion ($D$) is given as:

$$G(\tau)_{3DG} = \frac{\gamma}{\langle N \rangle} \left( 1 + \frac{8D\tau}{w_{3DG}^2} \right)^{-1} \left( 1 + \frac{8D\tau}{z_{3DG}^2} \right)^{-v/2}$$

The $w$ and $z$ parameters are the beam x-y and z half-axis elements ($1/e^2$ 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 (see (Berland and others 1995)) and 0.076 for the Gaussian-Lorentzian-squared PSF. (The 1-photon case is similar but the “8”s 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 and this weighting is proportional to the fractional intensity of each species, squared. The \( G(0) \) of the sample is then,

\[
G(0)_{\text{sample}} = \sum_{i=1}^{M} f_i^2 \cdot G(0)_i,
\]

where \( f_i \) is the fractional contribution to the intensity by species \( i \). One can see that the numerator and the denominator are equivalent to the variance and mean of the fluorescence signal, respectively, which is proportional to the reciprocal of the average particle number within the PSF. 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(s) (e.g., separate \( G(\tau)_{3DG} \) terms for each species).

**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 \( \langle N \rangle \) and the particle brightness values for single and multiple species 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](image)

\[
p(N) = \frac{\langle N \rangle^N \cdot e^{-\langle N \rangle}}{N!}
\]
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.

**Using Cross-Correlation**

**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
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)

$$C(\tau) = \frac{\gamma}{N} \left( 1 - \frac{F}{1 - F} e^{-\gamma \tau} \right) \left( 1 + \frac{8D\tau}{w^2} \right)^{-1/2} \left( 1 + \frac{8D\tau}{z^2} \right)^{-1/2}$$

point) and distort the cross-correlation decay in the time frame of the rate. An example of an extreme case is illustrated below. The dashed red line is a fit to a pure diffusion model while the data points contain an additional rate..
References


LAB : FCS

1) **Collecting FCS data and calculating concentrations**
   - Using 5nM rhodamine 110 (a "green" rhodamine) to calibrate the microscope radial waist. Assume 300 um²/s for the diffusion constant of rhodamine 110 and fit using an autocorrelation function leaving the radius free. The radius should be approximately 0.15 um.
   - Look at two additional concentrations of rhodamine 110. Look at the calculate reciprocal relationship between G(0) and the rhodamine 110 concentration? Example fits are below.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>G(0)</th>
<th>&lt;N&gt;</th>
<th>D</th>
<th>&lt;F&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 nM</td>
<td>2.18</td>
<td>0.85</td>
<td>350 um²/s</td>
<td>8.9 kHz</td>
</tr>
<tr>
<td>15 nM</td>
<td>1.29</td>
<td>3.5</td>
<td>305 um²/s</td>
<td>23 kHz</td>
</tr>
<tr>
<td>45 nM</td>
<td>1.10</td>
<td>10.1</td>
<td>304 um²/s</td>
<td>79 kHz</td>
</tr>
</tbody>
</table>

2) **Instrument and sample artifacts.**
   - **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.)
   - **Effect of detector/sample saturation:** Look at 5 nM fluorescein. Use a "normal" and then much higher powers. How do the curves compare? How does the apparent beam waist of diffusion constant change? (-- possibly due to triplet state, bleaching, & probe saturation)
   - **Detector noise:** set up a two channel detection and collect data fluorescein or beads in both channels. Look at the correlation and cross-correlation signals. The detector noise is essentially gone from the cross-correlation.

3) **Multiple Species (single channel)**
   - **Big differences in Diffusion:** Lysozyme-Fl alone and then mixed with Rhodamine 110. Can we separate the species?
   - **Huge Differences in Diffusion:** Look at rhodamine 110 (300 um²/s) and yellow-green beads (20 nm). Collect FCS data on the individual materials and the mixture under the same instrument settings. Can you see the autocorrelation of rhodamine 110 over that of the spheres? Why is this?

4) **Two Color FCS (dual channel): Cross-correlation**
   - **Uncorrelated species (green and red fluorospheres):** 2-photon systems: 790 nm red/green (for 1-photon systems, excite at 488 nm AND 567 nm while
collecting both channels). Collect FCS data and analyze the individual channels and calculate the cross-correlation curve. The cross-correlation is not zero: why is this?

- **Correlated sample:** Orange spheres will show in both channels (Green spheres will also show enough crosstalk) demonstrating 100% cross-correlation. The cross-correlation curve should overlap with the two autocorrelations.