FLUORESCENCE CORRELATION SPECTROSCOPY

ABSTRACT

Fluorescence correlation spectroscopy (FCS) is a technique that detects diffusion of fluorescent species through a small focal volume. From FCS measurements, quantitative data such as particle size, binding constants for interactions of biological molecules, and local viscosity can be probed. Because this technique relies on the detection of small numbers of fluorescent species at a time, it is well suited to probing the heterogeneity of behaviours in a system.

I. OBJECTIVES

- Learn the physics principles behind fluorescence correlation spectroscopy
- Understand the concept of a correlation function
- Use the signal detected from FCS of standard-sized particles to determine the extent of the focal volume of a laser beam
- Determine the expected FCS signal for particles of different size

II. BACKGROUND

Fluorescence correlation spectroscopy (FCS) detects diffusion of a fluorescent species through a focussed laser beam in solution[1–3], as illustrated in Fig. 1. The fluorophores are chosen to absorb the wavelength of the laser (or, if studies of a particular fluorescent species are desired, the laser wavelength is chosen such that it is absorbed by the fluorophore of interest). When these particles diffuse into the region of solution illuminated by the laser, they can absorb the light and fluoresce, emitting light at a longer wavelength. (See subsection below for description of fluorescence.) The intensity of fluorescent photons emitted as a function of time relate directly to the number of fluorescent particles illuminated by the laser beam. Since the laser beam follows a path through a sample chamber, it illuminates a relatively large volume. However, by tightly focussing the laser beam, and then setting up the optics in the FCS instrument to detect only light originating from the focal volume, it is possible to constrain the detection volume to a small region immediately surrounding the tightest focus of the beam. This volume is illustrated schematically in Fig. 1 and can be approximated as an ellipsoid.

Experimentally, the small volume in solution is defined by a tightly focussed laser beam and by detecting only fluorescence emitted from within the tight focal volume. This is achieved by using a high-numerical-aperture (high-NA) objective lens to focus the laser

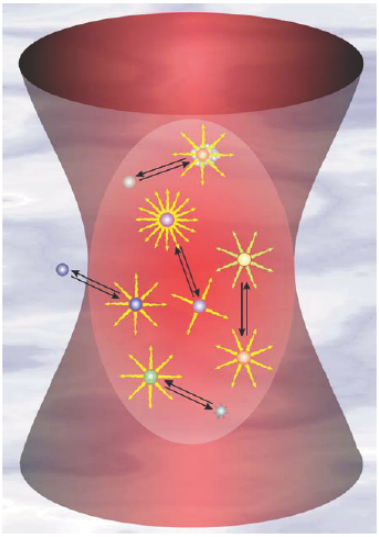

FIG. 1. Illustration of fluorescent particles diffusing through the focal volume of an illuminating laser beam. Figure from [3].

and by positioning a spatial filter (pinhole) to detect light from the correct image plane. Numerical aperture relates to the focus angle by NA $= n \sin\theta$, where n is the index of refraction ($n_{\text{air}} = 1.00$; $n_{\text{water}} = 1.33$; $n_{\text{oil}} \approx 1.55$) and θ is the angle of the highest-angle rays (see Fig. 2a.) In contrast to the simple picture shown in Fig. 2a; however, the light rays do not focus to a spot. Because light is an electromagnetic wave, it cannot be compressed to a single point but is limited by the diffraction limit of light to a spot size of $\approx \lambda/2$.[1] That sets the rough theoretical limit to the smallest possible cross-sectional size of the focal volume. By correctly placing a spatial filter in the detection path of the fluorescent light, it is possible to detect only photons that have arisen from a particular volume element. This is the second key aspect to successful FCS detection and is illustrated below in Fig. 2b.

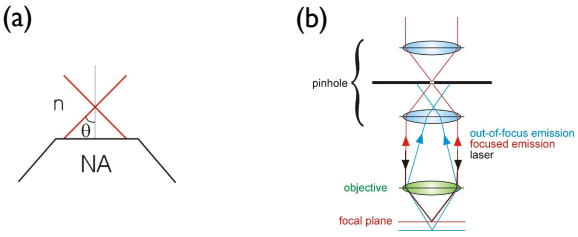

FIG. 2. a. The numerical aperture NA of a lens is given by NA $= n \sin\theta$ where θ is defined as illustrated here. b. Light emitted from the focal plane can be selectively detected by placing a pinhole in the image plane of the focus. As illustrated here, out-of-focus emission is not focussed by the first lens onto the aperture of the pinhole and thus is not detected further downstream. (Figure courtesy of Marcia Levitus, Arizona State University.)

[1] See *Microscopy* labscript for an explanation.

The technique of FCS has application to a wide range of problems in the field of biophysics, and is particularly well suited to probing events at the sub-cellular scale. The types of questions addressed by this technique are described in more detail in the accompanying document. These include studies of protein aggregation, protein-substrate interaction, cellular diffusion, active transport and conformational changes (e.g., protein folding/unfolding, DNA binding).

A. Fluorescence

A fluorescent molecule, or *fluorophore*, absorbs light of energy corresponding to allowed transitions of electrons in the molecule, and then emits light at longer wavelengths when the electron that was excited by the absorbed light relaxes to a low-lying state. This process is described in detail in the *Spectroscopy and Light Scattering* labscript. Figure 3 shows the absorbance and emission spectra for the dye Rhodamine 6G, which will be one of the species studied in this laboratory. Illumination with the common laser wavelength 532 nm excites rhodamine near the peak of its absorbance spectrum. By selectively detecting light at longer wavelengths (above 550 nm), it is possible to observe only emitted light and none of the 532 nm excitation.

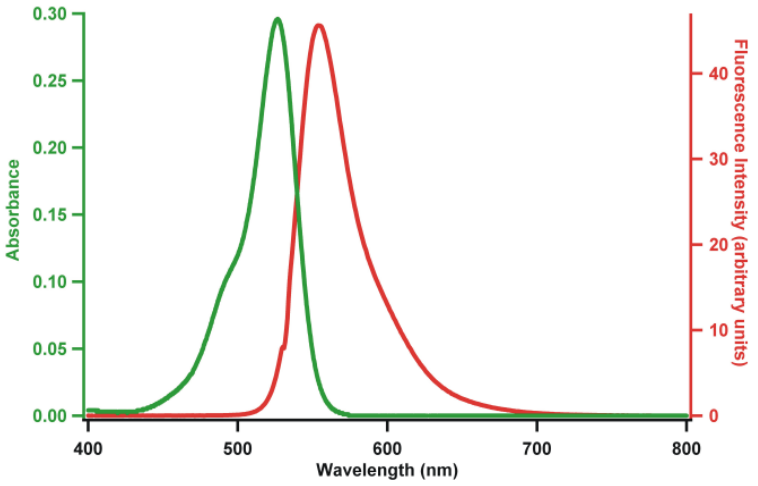

FIG. 3. Absorption (green) and emission (red) curves of Rhodamine 6G dye.

B. Experimental set-up

The layout of the FCS instrument you will be using is shown in Figure 4. The green line represents the path travelled by the laser light while the red line represents the path travelled by the fluorescence emission. The lens pair L1 and L2 acts as a telescope to expand the laser beam to give a cross-sectional area slightly larger than the size of the back aperture of the focussing objective lens. This configuration provides the tightest lateral focus of the

excitation light. The dichroic mirror DM is so named because of its different responses to two colours. Here, it is coated to reflect the green 532 nm light while transmitting light at wavelengths longer than 550 nm. The spatial filter SF1 cleans up the profile of the excitation beam (which is initially low-quality, from a laser pointer!), while the lens L3 and second spatial filter SF2 are responsible for reimaging the illuminated focal volume, as depicted in Figure 2. The photo-multiplier tube PMT sends an output pulse to the computer (not shown) whenever a photon impinges on it. The arrival times of these photons are what is saved and analysed by the LabVIEW software.

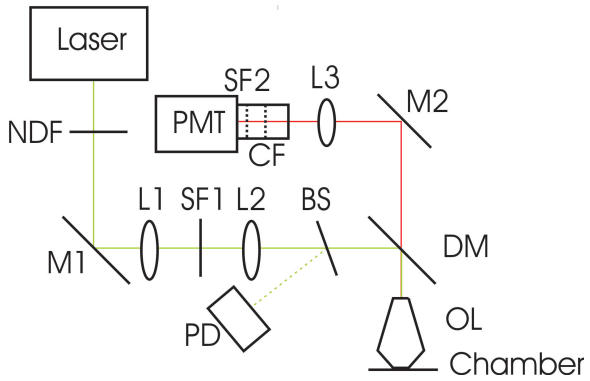

FIG. 4. Experimental set-up. The laser beam (532 nm, 5 mW diode laser) is expanded by lenses L1 and L2 and steered by two mirrors (M1 and DM) into the objective lens OL, which focuses the light in the sample chamber. The spatial filter SF1 serves to clean up the laser beam, as illustrated in Figure 6. The beam splitter (BS) picks off a small fraction of the light and directs it to a photodiode (PD), which is used to monitor the laser light intensity, which should remain constant throughout an experiment. The objective used in these experiments is a water-immersion lens with a numerical aperture of 1.23. The sample chamber is mounted to a one-axis translation stage (not shown), which allows for correct positioning of the chamber along the optic axis so that the laser focuses in the sample chamber. Emitted fluorescence passes through the dichroic mirror DM, and is steered by mirror M2 towards the photomultiplier tube PMT. The lens L3 focuses the fluorescent light, and the correct positioning of spatial filter SF2 along the optic axis constrains the light passing to arise only from the focal volume in the sample chamber. The colour filter CF blocks any stray/transmitted 532 nm light. (Both SF2 and CF are enclosed within the tube leading to the PMT.) The PMT sends a pulse each time a photon hits it; the arrival times of these pulses are read by the data acquisition card on the computer.

C. Fluctuations in fluorescence and autocorrelation analysis

For a high concentration of fluorophores in solution, the fluorescence signal detected by the PMT should be high and essentially constant as a function of time. As the concentration is lowered, a decrease in average fluorescence intensity is observed, along with an increase in the fluctuations about this average value. The increase in fluctuations is best understood by

taking the limit of a very dilute sample: in the case where there are usually no fluorophores in the focal volume, the signal detected by the PMT would be zero most of the time (in the absence of electronic noise). If a fluorophore diffuses into the focal volume, then it is repeatedly excited by the laser and emits fluorescence. The duration of the observed burst of fluorescence is given by the time the fluorophore takes to diffuse in and out of the focal volume (Fig. 5a). On dimensional grounds, we expect this time, $\tau_D \approx r_0^2/D$, where r_0 is the beam size and D is the diffusion coefficient. The interval between intensity "bursts" is then a measure of the concentration C (number / volume) of fluorophores in solution. In practice, it would take a long time to accumulate enough events to make accurate estimates of D and C if we were to work in this low-concentration limit. It turns out, however, that we can get the same information much more quickly by working in a regime where the bursts overlap slightly, so that the signal has maximum relative variation. The mathematical tool that we will use is the *autocorrelation function* of the intensity. It gives a way to determine D and C, which in turn can be exploited to give information about conformations, aggregation, binding, etc. on the molecular scale.

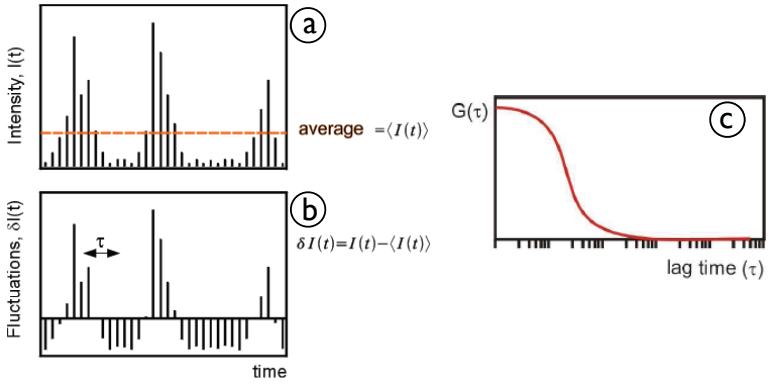

FIG. 5. Determination of fluorescence autocorrelation signal. a. Fluorescent light is emitted and detected with intensity $I(t)$. For low fluorophore concentrations, most of the time $I(t)$ is less than the time-averaged value $\langle I(t)\rangle$ and fluorescence is observed in discrete bursts. b. Fluctuations in fluorescence are observed by finding the difference between the intensity at a particular time and the time-averaged value. c. The autocorrelation function (plotted on a semi-log scale) displays a transition from strongly correlated signal (large $G(\tau)$) to no correlation ($G(\tau) = 0$) at a characteristic time τ_D. Figures courtesy M. Levitus.

The intensity autocorrelation function $G(\tau)$ is defined by

$$G(\tau) = \frac{\langle \delta I(t)\, \delta I(t+\tau)\rangle}{\langle I(t)\rangle^2}\,. \tag{1}$$

Here, $\delta I(t)$ represents the fluctuation of fluorescence intensity at time t from its average value $\langle I(t)\rangle$. Note that $\delta I(t)$ can be positive or negative. Figure 5 shows how an initial trace of fluorescence intensity as a function of time leads to an autocorrelation curve with a characteristic decay time τ_D. Before proceeding further, we note a couple of subtleties:

- Mathematicians and engineers often normalize their autocorrelation function so that $G(0) = 1$. In our definition, $G(0)$ is the square of the (standard deviation of intensity / mean of intensity).

- The brackets, $\langle\cdot\rangle$ are known as *ensemble averages*. They are defined as $\langle f(x)\rangle \equiv \int f(x)\,P(x)\,dx$, where $P(x)$ is the probability distribution of the quantity x. To measure an ensemble average, one would have to repeat an experiment many times and average each realization. This is usually not practical.

  If the system is *ergodic*—if it explores, over time, all the values of x at the frequency expected from $P(x)$—then we can replace the ensemble average with a *time average*:

$$\langle f(x)\rangle = \lim_{T\to\infty} \frac{1}{T}\int_0^T f[x(t)]\,dt\,. \tag{2}$$

  Thus, we approximate averages such as $\langle I\rangle$ by $\langle I\rangle \approx \frac{1}{T}\int_0^T I(t)\,dt$ for some long period of time T, and similarly for δI.

Pre-lab question 1: Determine the autocorrelation curve for a square pulse. This represents, in a very simplified manner, the type of signal you might see from a single fluorophore entering and exiting the focal volume, and hopefully will provide some intuition about what one sees in an FCS experiment. To get a sense for the shape of this autocorrelation curve, this question simplifies the analysis. First, start by sketching a pulse that has a duration of 10 msec, such that its intensity is $I(t) = +1$ from 10–20 msec and $I(t) = 0$ everywhere else (up to say 100 msec). Because the height of the signal differs from zero only during the pulse, take $\delta I(t) = I(t)$ in determining the numerator of Eq. (1). We will also disregard the denominator in Eq. (1), which provides a normalization factor but does not alter the shape of the resulting autocorrelation curve. Now, determine $G(\tau)$, by correlating the curve $\delta I(t)$ with itself shifted by different amounts in time $\delta I(t+\tau)$. Start by aligning these at $\tau = 0$ to determine $G(\tau = 0)$ $(= \langle I(t)\,I(t)\rangle)$. Note that only those portions of the curve that have intensity $\neq 0$ will contribute to $G(\tau = 0)$. Then determine $G(1\text{ msec})$ by shifting the fluctuation curve by 1 msec and multiplying $I(t)$ by $I(t+1\text{ msec})$. (Again, only those portions that have non-zero signal in both the original and the shifted data will contribute to the autocorrelation signal.) Do likewise for $G(2\text{ msec})$, etc. Plot $G(\tau)$ vs. the lag time τ on a graph with linear axes, then on a semi-log plot. What is the characteristic correlation time and how does it relate to the width of the square pulse?

Pre-lab question 2: Determine the autocorrelation curve for a square-wave signal. While this is not the type of signal one sees in a typical FCS experiment, it directly relates to the first experiment you will do with the FCS instrument, which involves modulating a highly fluorescent solution using a chopper. To determine the autocorrelation curve for a square-wave signal, start by sketching a wave that has a period of 20 msec, such that its intensity is +2 for 10 msec, followed by intensity 0 for 10 msec, followed by +2 for 10 msec, etc.) In this example, you will include the numerator and denominator from Eq. (1) in your calculations. To do so, first determine the time-averaged intensity over a period, $\langle I(t)\rangle$. Next, determine and plot the fluctuations $\delta I(t)$ as a function of time. Finally, determine $G(\tau)$. To avoid boundary effects, and because the signal is periodic, you can consider the correlation only within one 20 msec interval (one period). First, determine $G(\tau = 0)$ (two curves aligned) by multiplying $\delta I(t)$ by $\delta I(t)$ and dividing by $\langle I(t)\rangle^2$. Then determine $G(\tau = 1$ msec) by shifting the fluctuation curve by 1 msec, multiplying $\delta I(t)$ by $\delta I(t + 1$ msec), and dividing by $\langle I(t)\rangle^2$. Do likewise for $G(\tau = 2$ msec), etc. Continue up to at least $G(\tau = 50$ msec). Plot $G(\tau)$ vs. the lag time τ on a graph with linear axes, then on a semi-log plot. What is the characteristic correlation time and how does it relate to the width of the signal bursts?

Equation (1) for $G(\tau)$ provides a way to calculate the autocorrelation function and from it, to determine a characteristic decay time. Intuitively, the timescale for the decay of the autocorrelation function is related to the diffusion constant. Let's make this more quantitative.

First, consider the cause of these fluctuations in fluorescence intensity. Let us assume that they are all due to concentration fluctuations; i.e., we ignore any contributions due to laser-intensity variations, photochemistry, detection electronics, etc. Then $\delta I(t)$ is related directly to the concentration of fluorophores in the excitation volume at any point in time:

$$\delta I(t) = \iiint \Phi(\mathbf{r})\, \delta C(\mathbf{r}, t)\, d\mathbf{r}\,, \tag{3}$$

where $\Phi(\mathbf{r})$ represents the detection efficiency for photons emitted from each volume element and includes contributions from the optics of the microscope, the quantum efficiency of the dye, etc., which are all assumed to be constant. The other quantity, $\delta C(r, t)$, represents the fluctuation in fluorophore concentration at location r and time t (i.e., the concentration at time t is $C(r, t) = \langle C\rangle + \delta C(r, t)$). The fluctuations in concentration $\delta C(\mathbf{r}, t)$ are described by the diffusion equation:

$$\frac{\partial\, \delta C}{\partial t} = D\nabla^2\, \delta C\,, \qquad \text{where } \nabla^2 \equiv \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} + \frac{\partial^2}{\partial z^2}\,. \tag{4}$$

Here, D is the diffusion constant of the species. For free diffusive motion in one dimension, one can show that the mean-square-displacement (MSD) between time 0 and time t is $\langle x^2\rangle = 2Dt$. In three dimensions, the magnitude of the MSD in x, y, and z is given by $\langle x^2\rangle + \langle y^2\rangle + \langle z^2\rangle = 6Dt$, since the fluctuations in each dimension are independent

and variances then just add. Finally, solving Eqs. (1)–(4), leads to an expression for the autocorrelation function in terms of experimental parameters:

$$G(\tau) = \frac{1}{V_{\text{eff}}\langle C\rangle}\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\frac{1}{\sqrt{1+\frac{\tau}{\tau_D}\left(\frac{r_0}{z_0}\right)^2}}\,. \tag{5}$$

Equation (5) describes the autocorrelation function for particles diffusing through an elliptical focal volume of radius r_0 and height along the optical axis z_0 (see Fig. 1). See the attached hand-out for an outline of the derivation [3]; the original 1972 article about FCS is also worth a look [1]. In Eq. (5) , V_{eff} is the effective focal volume, $V_{\text{eff}} \approx \pi^{3/2} r_0^2 z_0$ and corresponds to the volume of the ellipsoid depicted in Fig. 1, and τ_D is the correlation time for translational diffusion across the isotropic part of the ellipsoid,

$$\tau_D = \frac{r_0^2}{4D}\,. \tag{6}$$

The size of the effective focal volume can be determined experimentally using fluorescent particles of known diffusion constant D. Recall from the Stokes-Einstein relation for freely diffusing spheres in an unbounded medium that

$$D = \frac{k_B T}{\gamma} = \frac{k_B T}{6\pi\eta R}\,, \tag{7}$$

where spheres of radius R diffuse in a solution of viscosity η at temperature T. From a nonlinear fit to the observed autocorrelation curve, we can determine r_0 and z_0. These values then hold for future measurements on unknown species in the same set-up, where τ_D and possibly $\langle C\rangle$ are the unknown parameters to be fit in Eq. (5). The average concentration of a fluorescent species $\langle C\rangle$ can be determined from the autocorrelation function at $\tau = 0$,

$$G(0) = \frac{1}{V_{\text{eff}}\langle C\rangle}\,. \tag{8}$$

Equation (8) has a simple interpretation: the product $V_{\text{eff}}\langle C\rangle \equiv N_{\text{eff}}$ is just the average number of particles in the detected volume at any one moment in time. The autocorrelation function is the ratio of the variance to the square of the mean. Assuming a Poisson process where the variance equals the mean, we have

$$G(0) = \frac{\langle \delta N^2\rangle}{\langle N\rangle^2} = \frac{N_{\text{eff}}}{N_{\text{eff}}^2} = \frac{1}{N_{\text{eff}}}\,. \tag{9}$$

The amplitude of $G(\tau)$ is thus *larger* when the solution studied is more dilute. Of course, if there are extremely few particles, then the detected fluorescence will be very low and the estimate of $G(\tau)$ will be extremely noisy. Thus we need to balance signal (the amplitude $G(0)$) and noise (the precision we can measure $G(\tau)$ in a finite observation time). A good compromise to is to design experiments so that $N_{\text{eff}} \approx 1$. If N_{eff} must vary during an experiment, try to keep it between 0.1 and 10.

Pre-lab question 3: Estimate the diffusion constant D for spheres of diameter 100 nm in water. Give your answer in units of $\mu m^2/s$.

Pre-lab question 4: Equation 5 provides the functional form for the autocorrelation model you will use to fit your data. How many independent, unknown parameters are there in this equation? Write a simplified form of Eq. 5 for use in fitting, which involves only independent, unknown parameters.

Note that the discussion so far has focussed on analysis of measurements of fluorescence intensity as a function of time (Fig. 5). In the experiments to be performed here, the data actually recorded are the arrival times of photons at the PMT (Fig. 4). By choosing timing bins, we can convert the arrival times into an estimate of the intensity $I(t)$, which can be used to calculate $G(\tau)$ in Eq. (1) in the usual way, as outlined in Questions 1 and 2. An alternative, more elegant method for determining the autocorrelation function directly from photon arrival times is described in a paper by Laurence et al.[4] This is the technique implemented in the program you will use.

III. INVESTIGATIONS

Determine the experimental autocorrelation curves for three different systems:

1. A concentrated solution of Rhodamine 6G dye, where the time dependence of rhodamine fluorescence is modulated by "chopping" the excitation laser to give pulses of excitation light as a function of time. Investigate the relation between the autocorrelation curve and the chopper wheel frequency.

2. A dilute solution of fluorescently labelled 100-nm beads, where the time dependence of fluorescence is modulated by diffusion of beads into and out of the focal volume. For this system, determine the effective focal volume from your measurements.

3. A dilute solution of fluorescently labelled beads of a different size, where the time dependence of fluorescence is modulated by diffusion of beads into and out of the focal volume. Here, using the focal volume parameters found in Step 2, estimate the size of these beads.

Phys 833: Determine the experimental autocorrelation curve also for fluorescently labelled DNA of a defined length.

IV. MATERIALS AND EQUIPMENT

- Fluorescence correlation spectroscopy instrument with photomultiplier tube for detecting arrival times of photons
- Computer for data acquisition and data analysis
- Chopper wheel
- Glass slides
- Number 1 cover glasses
- Parafilm
- Heat block
- Stock solution of Rhodamine 6G (10 μM in water)
  IMPORTANT: Rhodamine is a health hazard.
  - **Dispose in chemical waste.**
  - **Wear gloves and eye protection.**
- Concentrated solution of 100 nm-diameter fluorescently labelled polystyrene nanospheres (0.1% w/v)

- Concentrated solution of undisclosed-diameter fluorescently labelled polystyrene nanospheres (0.1% w/v)
- Sample chambers, one for each solution studied. See Protocol: Making Sample Chambers for information on how to prepare these.
- Phys 833: Solution of linearized pBluescript DNA + SybrSafe dye

A. Setting up the FCS instrument

1. Turn on the laser by plugging it in to the power source. Because it is a cheap module from a laser pointer ($50!), it must be turned on and allowed to warm up for at least 20 minutes before its power stabilizes.
2. Turn on the computer and open the LabVIEW program that collects and analyzes the data. It is accessed via "Shortcut to FCS Main" on the Phys 433 folder on the desktop.
3. Turn on the photodiode with the switch on its top. (A green light will illuminate when it is on.)
4. Temporarily remove the neutral density filter NDF (see Fig. 4).
5. Clamp the sample chamber to the positioning stage using the magnets provided. Slowly move the chamber towards the objective lens using the stage micrometer. When it is approximately 1 mm away, apply a thin layer of clean water between the cover glass and the objective lens using the squirt bottle. This is the immersion medium for the "water-immersion" objective. Then move the chamber a slight bit closer to the objective in order to trap the water droplet between the chamber and objective lens.
6. Turn off the room lights so it is easier to see the next step, and leave them off for the rest of the experiment to keep the background light intensity low.
7. As you move the chamber closer to the lens, you should see, consecutively, two bright, focussed spots of the green laser light by looking at the scattered beam between the dichroic mirror and colour filter 1 (Fig. 4). The first spot is the reflection of the laser focus off the front surface of the front cover glass, and the second is its reflection off the back (interior) surface of the front cover glass. After this second reflection, the laser focus should be just into the sample chamber. Adjust the chamber's depth so that the laser focus is a little deeper, towards the middle of the chamber solution. Do not move the chamber too close to the objective, or you risk cracking the cover glass of your chamber. The micrometer screw will generally lose traction before this point, and you will feel no resistance when you turn the screw. Stop turning at this point. For the concentrated solution of R6G, it is usually possible once you are focussed in the sample chamber to observe the fluorescence as an orange spot on a piece of white paper positioned after the dichroic mirror.

8. Replace the NDF after the laser.

9. Turn on the PMT by plugging it in. IMPORTANT: Only turn on the PMT when there are low levels of light intensity. The PMT can be damaged if excessive photons impinge upon it while its on.

10. Run the LabVIEW program to monitor the fluorescence signal as a function of time. You can view the real-time autocorrelation curve and see the signal develop with time.

11. Note the warning button halfway down the left side of the LabVIEW panel. If it is red, this means that there are more photon counts arriving in the specified sampling window than you have specific on the front panel (Number of samples $<$ Average rate $\times$ max τ). You should either reduce the length of your sampling window (decrease max τ) or increase the number of counts detected (increase Number of samples).

12. Use the concentrated solution of R6G to optimize the alignment of the system. This involves translating the spatial filter SF2 (entrance to optical fiber) to maximize the light entering the PMT, a value read as the Average Rate (cps) on the upper left plot in the LabVIEW panel. For reference, a 10 μM R6G solution should give $\approx 10^5$ cps using an OD=1 neutral density filter at the laser. If your count rate is significantly lower, then there is an alignment problem.

13. If the LabVIEW program gives you a warning about the data buffer being full and it is unable to read the data, this generally means that you are close to optimal alignment and the count rate is higher than the program can manage. In this case, make a new solution of 1 μM R6G in water, and repeat steps 2–11.
    • Stop the program and unplug the PMT before turning on the room lights.

14. When you are finished with measurements for the day, turn off the PMT (it should also be turned off between each set of measurements). Also, turn off the laser and the photodiode.

When you are making measurements for the experiments in this lab, you will save and later analyze the averaged autocorrelation data. (You do not have to determine the autocorrelation curve yourself from the raw data—you can, if you wish, by saving the raw data, but that is not necessary for this lab.) To save, hit `save autocorrelation data` while the program is running. (It will not save the data after the program has been stopped.) This saves the averaged autocorrelation curve as you see it on the screen. It is advisable to collect data for long enough that the autocorrelation signal does not change when each new set of data points is averaged into it. The data is saved as three columns: τ, averaged $G(\tau)$, standard error of the mean of $G(\tau)$. For more information on the LabVIEW program, see the comments on its block diagram (Control-`E`).

For the data acquisition with the chopper wheel, position the chopper after the NDF. (There is a screw for holding the mount on the table.) It is turned on with its controller. When recording autocorrelation curves, be sure to collect sufficient photons such that the curves appear smooth and there is no observable change in the averaged $G(\tau)$ curve as each new $G(\tau)$ sample curve is included in the average.

V. SUGGESTED ORDER OF EXPERIMENTS

1. Fill a sample chamber with a 10 μM or 1 μM solution of Rhodamine 6G and mount in the FCS instrument. Measure and save the autocorrelation curve of this sample. Using the chopper wheel at 1 kHz, measure the fluorescence signal as a function of time. From this, determine and save the autocorrelation curve of the observed signal. Repeat for at least two other chopping frequencies (e.g., 500 Hz and 2 kHz).

2. Fill a sample chamber with water only and mount in the FCS instrument. Measure the fluorescence signal as a function of time. The count rates here should be very low, as the only signal observed is due to instrumental noise. The autocorrelation curve of this signal shows you the contribution of the instrument response function (IRF). The range of delay times τ where the IRF generates a significant autocorrelation signal $G(\tau)$ should be excluded from your fitting of $G(\tau)$ from the nanospheres, as it is arises from the instrument (generally PMT afterpulsing[2]) rather than diffusion of the spheres.

3. Fill a chamber with a dilute sample of the 100-nm-diameter fluorescent nanospheres. Generally a 500-fold dilution of the 0.1% w/v stock of spheres into water works well. Be sure to vortex the stock solution so it is well mixed, before pipetting the bead solution to dilute it. Measure the fluorescence signal as a function of time. If it is appropriately dilute, you should observe a characteristic autocorrelation curve (Fig. 5c). If it is not, repeat your experiment with a different dilution until you can obtain a characteristic autocorrelation curve. Generally, you should try diluting by factors of 10 until you see signal.

Pre-lab question 5: What is the final bead concentration (in beads/ml and in moles/litre) of this 500-fold dilution of the 0.1% weight/volume initial stock of 100 nm spheres? The density of polystyrene is ρ =1.05 g/ml. For this molar concentration, how many spheres on average would you expect in the focal volume of the FCS instrument, if the effective focal volume were defined by $r_0 = 500$ nm and $z_0 = 2r_0$? Repeat the calculation for a 10 μM rhodamine solution. How many molecules of rhodamine are typically in the observation volume?

Phys 833: See additional directions on how to prepare DNA for these measurements.

VI. ITEMS TO INCLUDE IN LAB WRITE-UP

- Answer all of the pre-lab questions.

[2] Sometimes a detected photon produces *two* PMT pulses rather than one; the extra "afterpulse" arrives typically about few μs after the "real" pulse and leads to spurious peak(s) in $G(\tau)$ at that timescale. One cause is residual gas in the PMT that gets ionized by the real photoelectrons. The positive ions are pulled back to the metal cathodes and emit new photoelectrons on impact [6].

- Plot the autocorrelation signal for water and identification of the range of τ values at which the instrument response function (IRF) contributes significantly.

- Plot the autocorrelation signal determined for a solution of R6G and for R6G where the laser signal is chopped at each frequency.

- Relate quantitatively the positions of peaks in the autocorrelation signal to the pulse frequency arising from the chopper wheel and discuss what this means.

- Plot the autocorrelation signal determined for the solution of dilute 100-nm fluorescent beads. Include a fit to these experimental data. Identify the range of delay times τ used in fitting and the relevant parameters extracted from the best fits.

- Determine the correlation time τ_D and effective focal volume parameters r_0, z_0 from the beads' autocorrelation curve, using your calculated value for the diffusion constant D for 100 nm beads.

- Discuss how your calculated effective focal volume relates to the minimum possible focal volume.

- Plot the autocorrelation signal determined for the solution of dilute fluorescent beads of undisclosed size. Include a fit to these experimental data. Identify the range of delay times τ used in fitting and the relevant parameters extracted from the best fits.

- Determine the size of these beads, using the focal volume parameters determined from the 100-nm beads.

- Discuss at least one of the points from the following section.

VII. POINTS TO PONDER

- FCS is often used to determine binding and unbinding constants for proteins and ligands, and for nucleic acid and protein structures. Sketch plots of the expected autocorrelation function for a fluorescent protein of diameter 5 nm and for this protein bound to a DNA of length 3kb. (See the Electrophoresis labscript for a discussion of how DNA coils up in solution and how to calculate the approximate radius of this coil.)

- How would the formula for the autocorrelation curve, Eq. (5), change if you had a mixture of two fluorescent species of differing diffusion constants? As an example, consider a sample that has equal numbers of beads of two sizes, one ten times the diameter of the other. Plot the expected autocorrelation curve.

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[6] Hamamatsu Corporation, *Photomultiplier Tubes: Basics and Applications*, 3rd ed., Sec. 4.3.8.
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