1st LFD Workshop in Advanced Fluorescence Imaging and Dynamics

Computer Training

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Laboratory for Fluorescence Dynamics (LFD)
Department of Biomedical Engineering
University of California, Irvine

and

University of California, Irvine
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A. Fluorescence Correlation Spectroscopy (FCS)

Exercise 1. Simulation of single point FCS

Setting up the problem:

After opening SimFCS, select “Simulate FCS data” from the actions panel at the left hand side of the screen. In the simform that opens up, select “Parameters” from the menu at the top of the screen. A menu will pop up with seven tabs and different options for the simulation.

PSF: This tab has options for the shape of the point spread function. Select 3D Gaussian from the pull down menu. Set the Radial Waist (r or y) to 0.3 (the units are microns). Ignore the value of Radial Waist (x)—this refers only to point spread functions that are not cylindrically symmetric. Set the axial waist to 1.5 μm. The a_astig and m_astig parameters are for introducing astigmatism to the PSF and should be set to zero. The plotting threshold is simply for visualization and will not affect the outcome of the simulation. Set the box size to 64. This is actually the half size of the box. Since the simulation moves the particles in a random walk along a grid of points spaced 0.05 μm apart, this will give a box with 6.4 μm along each dimension. Set the multiple points field to 1 and ignore the slit separation as this is for more complex simulations.

Particles: This tab allows you to choose the numbers and types of particles for the simulation. We will be doing a single species simulation, so only the first row will have particles. Choose 100 molecules with a brightness of 100,000 counts per second per molecule (cpsm). This is the average intensity at the peak of the point spread function. Set the diffusion coefficient to 40 μm²/s. Note that at the bottom left hand corner of the tab, there is a Max D value in μm²/s. This maximum value is set by the simulation based on the time per channel (clock frequency) which we will set later. This D value is the value at which the probability of making two grid steps in one time step becomes significant. It is possible to set the diffusion coefficient to a value higher than this, but...
the simulation will be physically unrealistic, because grid steps greater than one are not allowed. Ignore the rotation value for now. Set Br2 to zero, since we will only be simulating one detection channel. The last column should have a 1 in it. At the bottom right of the tab is a legend showing the type of particle that this last column can designate. If 2 is selected, the particle will rotate with a time constant equal to the Rotation column which is in units of time channels. 3 and 4 refer to different shapes of macromolecular assemblies.

<table>
<thead>
<tr>
<th># mol</th>
<th>Br1 (opm)</th>
<th>D (um2/s)</th>
<th>Rotation (“Dr”)</th>
<th>Br2 (opm)</th>
<th>1, 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100000</td>
<td>40.0</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.0000</td>
<td>10</td>
<td>0</td>
<td>1</td>
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<td>0.0000</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Max D = 45.672 Type 1 = normal, 2 = rotation, 3 = stick, 4 = disk

Misc: This tab contains options for rotation, flow, background and particle confinement. For our simulation, just select “randomize data” and “Use Poisson dist for counts.” This setting is appropriate for simulating photon counting experiments (i.e. not analog).

Stick/Disk: This tab allows the simulator to change the shape and properties of the stick or disk particles selected under the Particles tab. It will be ignored for now.

Reaction: This tab allows for reactions between particles (rows) defined in the Particles section. This will also be ignored.
Time/Scan: This tab allows for the simulation of circular scanning or raster scanning FCS. For now, make sure the “Scanning” check box is unchecked and set the clock frequency to 100000 (10 μs time period). Set the cycles field to 50. One cycle is the amount of time required to collect $2^{15}$ data points. At the right hand side of the tab, the actual simulation time is shown in seconds. The “Binning Mode,” “Use sections,” and “Number of bins for linear scale” fields are for display during the simulation and will not affect the outcome.

Display: This tab allows for the change of the display during the simulation run. Ignore it for now.

TZone 2D: This tab allows for parameters related to transient confinement of particles. Ignore it for now.

The Parameters section can be closed by clicking on “Parameters” once again.

Running the Simulation:

Close the parameters tab and click on Start_simulation. Select diffusion in a volume from the pull down menu. After a moment, the simulation will start. If you like, select “Show PSF” (this will show a 3D threshold plot of the PSF). This can also be done before the simulation to demonstrate the different PSF’s. In addition, the particles can be plotted in real time (“Plot Particles”). All of these display options slow the simulation considerably, so it is advisable to turn them off for the majority of the simulation. Almost all of the plots in SimFCS can be edited by double clicking. The plotting routine is TeeChart Pro. A free editor for Tee Chart Pro, Tee Chart Office, can be downloaded for free from: http://www.steema.com/downloads/dwn_tch_office.html.

Also, during the simulation, the data will register on the main SimFCS screen similar to the way real data acquisition would register. On the top plot, the intensity per second is plotted in red, and the $g(0)$ value, calculated from the variance divided by the intensity squared, is plotted in green. On the bottom plot, the logarithmically binned
autocorrelation function is plotted. This function is calculated as the average of each cycle ($2^{15}$ points) and is calculated using the multiple tau approach. Note that this should only be used for visual purposes during the simulation. After the simulation is complete, the “Recalculate” (on the lower right of the screen) button can be pushed to get a more robust estimation of the autocorrelation function.

All of SimFCS is built around the huge vector, which, quite literally, is a huge vector containing the trajectory of data points from either experiment or simulation. This vector is made up of unsigned short integers (0 to 65536) and can contain up to 64 Mb of data (about 34 million data points). There are several links in different parts of SimFCS to the huge vector screen, where the actual data can be viewed and binned to the time resolution desired for the experiment.

Analyzing the Data:

Under the main SimFCS screen, click “Analysis” on the top menu and select “Large vector correlation” from the pull down menu, a new window opens. This section of the program calculates the autocorrelation function based on the fast fourier transform. Since the FFT requires power of two size chunks of data, the autocorrelation function is calculated in sections (in this example select 256K). Before starting the calculation, by pressing the “calculate” button, you may select “Plot each segment” to view these segments as they are calculated. The individual segments can be selected and deleted, for example, if one section of the data contains a spike (often due to a diffusing aggregate). This anomalous autocorrelation segment can be selected by clicking on it. It will then appear red. Clicking “Delete segment” removes that segment. Press “Recalculate average” to recalculate the autocorrelation without that segment. Note that the errors are
also calculated and can be visualized by selecting “Plot errors.” When the data is saved (“Save Correlation Files”), the errors are saved as well. They can then be used as weighting factors for fitting. At the end of the calculation, the average is shown on the top graph (may be difficult to see if you are plotting each segment). The bottom graph shows the logarithmically binned data. This format is generally accepted for FCS data. On the right hand side of the page is the photon count histogram (PCH) and its average.

If you desire, you can fit the autocorrelation data from the Large vector correlation screen, but you can only fit to the 3D Gaussian model, and you cannot fit the PCH. The preferred method for fitting is to use “Globals”. This program has a long standing tradition as a fitting program for fluorescence decay kinetics, and now has been extended to fit FCS data as well. First, save the data from SimFCS. To do that, click on the “Save correlation files” button below “Calculate”. The following window will open:
Make sure to select Correlation 1 and PCH 1 to save. To select a specific destination folder for your file double-click on the “Common file name” path. The Huge Vector can be saved as a binary file (unsigned short integer) to analyze separately if you like. The comments as well as other specifications are saved in a journal (*.jrn) file (open with a text editor) with the same name as the data file. Subsequent files with the same name are saved with an incremented number identifier (*1000, *1001, etc.). The first number is the channel number. The clock frequency and number of cycles will be automatically saved. The PCH data will be saved with the extension *.his and the correlation data will be saved with the extension *.cor.

Now, open Globals to analyze the data. You can have Globals and SimFCS open simultaneously and go between them for different analyses as long as your computer has enough memory available to handle it. In Globals, double click on the worksheet cell under “Filename.” This will bring up an open file dialog to select the data file. First, select the correlation file (with the extension *.cor – check if the dialog window is set to display files of this type, and if it is in the correct folder). Make sure the record (rec) number is set to zero (record numbers correspond to different data file formats).
Next, click on Options from the menu at the top of the page. On the Program Defaults tab, set the minimization algorithm to “Port 3” (the Marquardt will work for correlation fitting but not so well for PCH) and make sure that the “Use Two Photon Formulas” is not selected (if this is selected, it is assumed that the PSF is squared). Also, select “Variable” under the errors section. This uses the errors calculated by SimFCS to weight the chi-squared value. If this is set to fixed, all data points are weighted equally. Close the window.
In the main "Globals" screen click on the “Select model” button to the right. The following screen appears:

In this screen, select “Fluctuation Spectroscopy” and then “diffusion-G” and finally “single species.” There are several ways to add species in Globals. You can select a model with multiple species, or you can click the add species button at the bottom of the screen and then configure the second species as you desire. (The simple Jablonski diagram visualization that you see on the right can be ignored. It is a carry over from fluorescence decay kinetics.)

Now that the model is set up, close that window and open the “Values/Linking” window from the main Globals screen to set the initial guesses for the fitting procedure.
The table shows the different parameters, their initial-guess values and whether they are fixed (F) or variable (V) for the fitting procedure. “fract_1” is an overall amplitude associated with file 1, it should be fixed to one for our analysis. “Backgnd” should be fixed to 0 in the example. WaistG_r is the waist diameter (1/e) of the focus assuming a Gaussian intensity distribution. In this exercise the value is fixed to 0.3 (in microns). W_ratio is the w_o/w_f ratio, which should be fixed to 5 for our simulation. The G(0) and D values can be initialized to 1 and left variable. It is possible to obtain a better initial guess for G(0) from the data. This value can be set to “fixed” the first analysis (by typing F in the cell next to the value) and then allowed to vary once the D value is reasonable. Click on “Start Minimization” to do one round of minimization and on “Report” for the results. The blue triangle at the top of the report page allows the user to change the format of the graph. If you set the errors to variable, the chi-square value should be approximately 1.
Fit the PCH data in the same way. Here set the model to PCH_G. Make sure that the Brightness and Number are set to variable (i.e. they have a “V” in the initial guess worksheet). It is advisable to fix the background value (here fix it to zero), as it can have a large affect on the brightness and number if it is allowed to vary. Again, the fraction (amplitude of file 1) should be fixed to 1. You may need to change the Report graph format to observe the fit. Here the chi square can deviate significantly from one. This is a result of incomplete data sampling. The average chi-squared from many simulations or from a long simulation should be 1.
Equations:

\[ V_{PSF} = \int PSFdr^3 \]

\[ \gamma = \frac{\int |PSF|^2(r)dr^3}{\int |PSF(r)dr^3} \]

3D Gaussian:

\[ PSF(r, z) = \exp \left( -\frac{2r^2}{\omega_r^2} + \frac{-2z^2}{\omega_z^2} \right) \]

\[ V_{3DG} = \omega_r^2 \cdot \omega_z \cdot \left( \frac{\pi}{2} \right)^{3/2} = 1.9687 \cdot \omega_r^2 \cdot \omega_z \]

\[ \gamma_{3DG} = 2^{3/8} = 0.3536 \]

Gaussian-Lorentzian Squared:

\[ PSF(r, z) = \frac{4\omega_r^4}{\pi^2 \omega_z^2} \exp \left( -\frac{2r^2}{\omega_r^2} \right), \text{ where } \omega_z^2 = \omega_r^2 \left( 1 + \left( \frac{z\lambda}{\pi \omega_r^2} \right)^2 \right) \]

\[ V_{GL} = \frac{\omega_r^4 \cdot \pi}{\lambda} \]

\[ \gamma_{3DG} = \frac{3}{(4\pi^2)} = 0.076 \]
Questions:

1. Calculate the number of particles from the autocorrelation fit \( G(0) = \gamma / N \). How does this compare to the PCH results?

2. The PCH analysis didn’t allow for variation of beam shape parameters. Why not?

3. Calculate the concentration of particles in the simulation box. Hint: 1 \( \text{um}^3 = 1 \times 10^{-15} \text{Liters} \). Now calculate the concentration of particles from the simulations. How do these compare?

4. For a typical confocal experiment, the radial waist is 200 nm and the axial waist is 600 nm. For a typical two photon experiment at 800 nm, the radial waist is 300 nm. Calculate the expected \( G(0) \) for these two experiments. If you like verify the results with a simulation. Comment on the signal to noise of single photon vs. two photon measurements.

5. Attempt to fit the autocorrelation function to different \( \omega_z / \omega_r \) ratios. Comment on the sensitivity of FCS to the z dimension of the PSF. Why do you think this is?

6. Attempt to fit the autocorrelation function to different beam (radial) waists. Does the goodness of fit change? Explain.

7. Imagine a spherical point spread function with amplitude 1 inside radius, \( r \), and amplitude zero outside this radius. Calculate the gamma factor for this PSF. Comment on the effect of the point spread function shape on the gamma factor.

8. Attempt to fit the PCH and the autocorrelation curves from the above simulation to the Gaussian Lorentzian squared model. Comment on the sensitivity of the two techniques to the shape of the point spread function.

9. Using the equation for the volume of the 3D Gaussian, calculate the \( \omega_r \) and \( \omega_z \) values for a spherically symmetric PSF \( \omega_r = \omega_z \) with a volume equal to that for the previous simulation. Run a simulation with these parameters and compare the PCH and the autocorrelation to what we observed previously.
Exercise 2. Simulation of homodimerization and global analysis.

Setting up the problem:

Set up the simulation in the same way as in exercise 1, except for the particles section. For the formation of a dimer, the brightness and volume might both be expected to double. The diffusion coefficient is inversely proportional to the cube root of the volume (the particle radius), so it will be multiplied by a factor of \((0.51/3)\) or approximately 32 \(\mu\text{m}^2/\text{s}\) if 40 \(\mu\text{m}^2/\text{s}\) is the diffusion coefficient of the monomer. Set up the simulation with 75 particles at 100000 cpsm (monomers) and 25 particles at 200000 cpsm (dimers). Run the simulation as in exercise 1.

Analyzing the Data:

Calculate and fit the autocorrelation and the PCH data as before, except this time using the “3 species with brightness ratio” model. Under Values/Linking, set the R1-Bright1 parameter (the brightness of species 2 relative to species 1) to 2, since we expect the brightness of the dimer to be double that of the monomer. Fix the relative brightness and number of species 3 to 0 since we only have two components in this system.

Note that the autocorrelations will likely not support a two component fit, since the diffusion coefficient has changed a relatively small amount. The PCH will support...
this fit, but the brightness values will be somewhat different than the simulated values. This is because there is a large amount of parameter correlation: the particle numbers and the brightnesses have a large amount of interplay. The accuracy in determining parameters can be improved by Global analysis. Add the PCH file from our first exercise to the second row of the starting Globals screen. Under the Values/Linking screen, there are now two columns, one representing each file for analysis. Selecting a row and clicking the link across button forces the parameters to be varied as a single parameter for both files. Link the first brightness parameters together. The brightness ratio should be fixed to zero for the second file (here we only expect a single species) along with its accompanying number parameter. Now repeat the analysis. The added restraint of the single species file will aid in the analysis resulting in a much better estimate of the parameters for the two species analysis.
Equations:

\[ G(0) = \sum_{m=1}^{M} \left( \frac{\langle I_m \rangle}{\langle I \rangle} \right)^2 G(0)_m, \]  
where \( I_m \) is the intensity of species, \( m \).

\[ \langle I_m \rangle = \varepsilon_m \cdot N_m, \]  
where \( \varepsilon_m \) and \( N_m \) are the brightness and number of species, \( m \).

\[ \langle I \rangle = \sum_m \varepsilon_m \cdot N_m. \]

Questions:

1. Calculate \( G(0) \) from the above equations for the simulation performed earlier. How does this compare with the simulation results?

2. One can imagine an experiment where the brightness of each species is known as well as the total number of particles (concentration). It is now possible to calculate the concentration of each species. Perform this analysis for the above simulation (note that the total number of molecules in the simulation is 125, not 100).

3. In the analysis of the simulation PCH, we used the constraint that the dimer was twice as bright as the monomer. This is not always the case (e.g. self quenching, etc…). Try to do the analysis without this constraint. Now do the global analysis without the constraint.

4. How much different do you think the molecular volumes would have to be in order to resolve the two species in an autocorrelation analysis? If desired, test your hypothesis with a simulation.

Setting up the problem:

Set up the simulation with three types of particles as seen below. Here we have entries under Br2 which refers to the brightness in channel 2. The first two particles are our monomeric species. They happen to both have the same diffusion coefficient, so the simulation is essentially the same as for Exercise 2. The third species is the heterodimer. Run the simulation as before.

Analyzing the data:

Under the large vector correlation screen, you will notice that at the upper left of the dialog, there is a set of radio buttons grouped under “function”. Before we had Ch1 autocorrelation checked. Repeat this analysis as before. Then select Ch2 autocorrelation and do that analysis the same way as Ch1. The autocorrelation for channel 2 should appear in blue in the bottom graph. The information in the upper graph is overwritten when a new analysis is done, so make sure that you delete any outliers and recalculate the average before analyzing a new channel. Now select Ch1-Ch2 cross-corr and do that analysis. The cross correlation will appear in green along with the two autocorrelations. You can also do the Ch2-Ch1 cross-corr if you like, but the result will be the same and the Ch1-Ch2 data will be overwritten. Also, only one cross correlation can be saved at a time.

For this analysis, fit only the cross correlation in Globals. The cross correlation is only sensitive to species that diffuse together in both channels, so you can fit the data to a Gaussian single species model as in Exercise 1.

Equations:

\[
G_{cc}(\tau) = \frac{\langle \delta I_{ch1}(t) \cdot \delta I_{ch2}(t+\tau) \rangle}{\langle I_{ch1}(t) \rangle \langle I_{ch2}(t) \rangle} = \frac{\langle I_{ch1}(t) \cdot I_{ch2}(t+\tau) \rangle}{\langle I_{ch1}(t) \rangle \langle I_{ch2}(t) \rangle} - 1 \approx \frac{\langle I_{ch2}(t) \cdot I_{ch1}(t+\tau) \rangle}{\langle I_{ch1}(t) \rangle \langle I_{ch2}(t) \rangle} - 1,
\]

where \(ch1\) and \(ch2\) refer to channel 1 and channel 2 respectively.

\[
G_{cc}(0) = \gamma \sum_{m=1}^{M} \varepsilon_{m,ch1} \cdot \varepsilon_{m,ch2} \cdot N_m, \quad \text{where } N_m \text{ is the number of type } m \text{ particles in the focal volume and } \varepsilon_{m,ch1} \text{ and } \varepsilon_{m,ch2} \text{ are the brightnesses of species } m \text{ in each channel.}
\]
\[ \langle I_{\text{ch1}} \rangle = \sum_m \varepsilon_{m,\text{ch1}} \cdot N_m. \]

Questions:

1. Using the above equations, calculate the concentrations and brightnesses of each of the three species in our system.

2. In most two color experiments, the microscope filters do not 100% isolate the signals of the different species from one another. This is referred to as crosstalk or bleed through. Assuming that you know the percentage of bleedthrough for each species, is it possible to calculate the concentration of all of the species? Verify this with a simulation if you like.

3. Verify that the above equation for \(G(0)\) of the cross correlation is identical to the \(G(0)\) for the autocorrelation (see previous exercise) if the \(\text{ch1}\) and \(\text{ch2}\) refer to the same channel.

4. Mueller has developed a technique referred to as 2D PCH to look at PCH for 2 color experiments. Globals does not yet have the capability to analyze these results. What to do for this?
Exercise 4. Simulation of fluorescence kinetics: FRET fluctuations

Setting up the problem:

In this exercise, we will simulate a FRET experiment where a system is fluctuating between two states: one with high FRET efficiency (acceptor is highly fluorescent, donor is quenched) and the other with low FRET efficiency. Create a simulation with two types of particles (100 particles of each type), one with 100000 cpsm in channel 1 and the other with 100000 cpsm in channel 2. Under the “Reaction” tab, type 100000 for both the forward and backward rate. (Note that if you don’t want equal concentrations in the two channels, you will have to adjust the ratio of these rates to match the equilibrium constant). Due to the way the simulation is written, this is not the actual rate for the reaction. The actual rate (in reciprocal seconds) is given by:

\[
\text{input rate} \times \text{clock frequency}/3 \times 10^6
\]

Analyzing the data:

In the case of cross correlation kinetics data, we have the following form for the autocorrelation: \(G(\tau) = K(\tau) \cdot G_{\text{Diffusion}}(\tau)\), where \(K\) describes the kinetics and \(G_{\text{Diffusion}}\) is the familiar autocorrelation due to the kinetics that we have seen before.

\[
K(\tau) = 1 + s \cdot f \cdot \exp \left( \frac{-\tau}{\tau_{\text{kinetics}}} \right)
\]

where \(s\) is the sign of the exponential (negative for anticorrelation and positive for correlation), \(f\) is the fraction of the species undergoing the kinetics, and \(\tau_{\text{kinetics}}\) is the reciprocal of the kinetic rate. This equation form is essentially the same as that for triplet kinetics aside from the sign term for the cross correlation. One can immediately see that this equation lends itself to the global analysis, since the only thing that differs between the autocorrelation trace and the cross correlation trace is the value of \(s\) (-1 for cross correlation, 1 for autocorrelation). Since Globals doesn’t have this exact form of the equation, we will enter a custom formula for the analysis. Load one (or both) of the autocorrelation files from our simulation, as well as the cross correlation file. Go to Select Model and choose User Formulas and then time domain. This will bring up a screen where you can create custom formulas and load previous formulas that you have created.

The formula parser is similar to others you may have seen in the past (e.g. Origin), but has some added functionality, like the ability to do integrals. Specific help on custom formulas can be found under “Formula Parser” in the Help Contents. For our application, use the following formula:

\[
p1+p4*(1/(1+(4*p5*x)/(p2*p2)))*sqrt(1/(1+(4*p5*x)/(p2*p2*p3*p3)))*(1+p6*p7*exp((-x)/(p8*0.000001)))
\]

If you were previously fitting with the Gaussian diffusion model, most of the parameters will be in place already. Enter the descriptions and limits for the parameters as shown below. Press the Test function button with the appropriate parameters in the boxes above it to output some values of this function. If you like, for simplicity, split the above equation into parts and assign these to the A, B, etc… for use in the final equation.
Save your custom formula in the Globals folder under Program Files. Note that there are some other formulas already created. For example, corr_2dgaussian.frm is the correlation function for a 2D Gaussian point spread function. Go to the Values/Linking screen and fix the beam waist parameters as we did before. Fix the background to zero. You will notice that at the top of each column there is a sas parameter. This is identical to the fract 1 that we saw when fitting the autocorrelation and PCH curves and is simply an overall amplitude factor for that file. Link all other parameters between the two files, except for the Kinetics_Sign parameter. This should be fixed to 1 for the autocorrelation and -1 for the crosscorrelation. Perform the fitting as we have done in the past.
Questions:

1.) Most FRET experiments are plagued by cross talk (i.e. fluorescence from one species overlapping with the filter band for the other species). In addition, FRET quenching is usually not complete (the donor is not totally quenched in the high FRET state). What effect do you think that will have on the auto and crosscorrelations? Is it possible to calculate the FRET efficiency from the kinetic fraction? If desired, run a simulation to demonstrate these principles.

2.) Run a simulation where significant photobleaching is a problem (i.e. species 2 is non fluorescent and starts with a low number of particles). The rate should be less than one reciprocal second for photobleaching under normal excitation conditions. How does the photobleaching affect the autocorrelation? How about the PCH?
B. Line scanning, ICS and RICS

Exercise 1. RICS and line scanning simulations

Setting up the simulation:

After opening SimFCS, select “Simulate FCS data”. In the Simform that opens up, select “parameters” and then PFS.

The parameters should like the one above.

Select Particles

You should have 100 particles of brightness 1,000,000 and of type 1. Set the D to 10um²/s.

Select the Time/Scan tab

In this page you are selecting to scan a grid of 128 points with a pixel dwell time of 1/64000 s.
Select the Start simulation/diffusion in 3D.

After few minutes, the simulation is done. Go to Analysis/ RICS

Change the size of the image and the ROI fields to 128. Set the number of frames field to 100 (in the figure you have 25, but it should be 100). Press the image 1 spin button up and down. The different frames of the simulation can be scrolled. Go back to frame 1.

Select Tools/Spatial correlation. The spatial correlation operation is performed for the 100 frames of the stack and the correlation for each image is averaged to produce the screen below.
Note that in the average of the spatial correlation, the shape of the 2-D correlation function is obliged, indicating that there is correlation along adjacent pixels, but little or not correlation at adjacent line.

To determine the diffusion coefficient of the particles, press the fit button. Set the fit values as shown below.
You should understand the values you have used:

Raster scan: selects models related to the RICS mode.
Size of image to analyze: Limits the size of the 2D function to be calculated. This value should be the smallest that contains the region where the correlation function is different from zero.
Pixel time: this is in microseconds, the pixel dwell time.
Line time: this is the time in millisecond between consecutive lines. This value is not simply the product of the pixel dwell times and the number of a pixel in a line.
Generally, there are times in which the trace in not active, which can add a substantial amount to the total time for a line.
Frame time: only used for time correlation. Also, there is a time delay between lines and at the end of frame. This value must be given to you by the software you are using.

Radius: only used for the circular scanning FCS. It calculates the distance between pixels along the circular orbit.

Points in x to jump: This is a very important parameter for the fit!!! In most analog diction system, the intensity at one pixel carries over the intensity at the next pixel. This gives the appearance of a spatial correlation, which of course is only due to the electronics of the microscope. We found that a value between 3 and 5 is sufficient to delete the effect of this electronic correlation.

Analog tau: this field is not active anymore. It will be deleted in future versions.

Pixel size: this is in microns, the distance between pixels. The software of your microscope should give you this value. Remember that this value should be smaller than the waist of the PSF for spatial correlations to be detectable.

Wo: this is the $1/e^2$ value for the Gaussian approximation of the PSF. It has recently been reported to me that this value could be different from the usual definition. I will check about this report. For the moment assume it is correct. It is consistent with the simulation part of SimFCS.

All the other parameters refer to the particular fit to be performed. In the RICS mode of data analysis, you can fit 2 diffusion components and one exponential component.

The analog filter variable is no longer active. The x-y coordinates are used to center the ACF in case that the function to be fitted is non-symmetric.

The use weight flag, uses the standard deviation matrix that was calculated during the averaging of the many frames that contributed to the average spatial correlation.

The use 2D diffusion and the use 2-photon formulas are used to load the 2D diffusion term into the function and the 2-photon diffusion term instead of the 3-D term and single photon diffusion formula.

The 3 buttons calculate function, perform fit and STOP, just do what they say.

The fitting radio button, allows choosing the entire 2D correlation or only the horizontal or vertical section of the surface. For cross-correlation, the function is non-symmetric, so that the button should be in the non-symmetric function choice.

The STICS spin edit field, allows visualizing the different data and fitting planes of the STICS operation.

The check limits check, allows performing fit without checking for unphysical values, such as negative diffusion values or similar impossible cases. However, during minimization, the computer could try an impossible value during the search for a minimum. By check this flag, the search will be stopped when an impossible value is reached. We suggest that you experience with different conditions, but always check for the validity of the results.
The chi-square value is normalized only if the use weights flag is set. Otherwise the software uses a value of 1 for the weights, giving very low chi-square values. In this case, only the ratio of the chi-square when using different models should be used as a comparison.

When the fit is done, the best fit 2-D function is shown. You can select showing the residues of the fit or the original data. In either case, the resulting 2D surface can be manipulated, rotated and copied to the clipboard, using the chart editor.

You can play with some of the parameters for the fit. For example, try the use weights flag. In this case the chi square should be normalized and should be closer to one for a good fit.
Exercise 2: Simulation using circular scanning

Set up the simulation as you did for exercise 1, but at the tab for time/scan, select scan circle and select a radius for the orbit of 20. Since every unit is 0.05 um, a radius of 20 is equal to a radius of 1 um in “physical” space. The length of the orbit is then 6.28 um. Since we have 64 points along the orbit (why?), the sampling points are spaced at $6.28/64 = 0.981$ um. The points are overlapping with respect to the PSF which has a waist of 0.25 um.

Run the simulation. Go back to the RICS page and check the line scan flag. Move the spine edit for frame 1 and note that the pattern on the screen is different now. Of course, these are not raster scan data, but circular scan data!!

Calculate the spatial correlation function using the tools menu
Note the different shape of the spatial correlation with respect to the raster scan case.

Go to the fit page

Chose the circular scan function and enter 20 for the scan radius. Perform the fit.

The value of D for my simulation is not exactly on target, but still close. The residues are small and the chi-square very good. It will be interesting to see what you obtain!!

**Question:** Could we have analyzed the same data using the scanning-FCS screen?? What will have been the major difference??
Lest go to the scanning FCS screen. Close the RICS page and in the analysis menu select the scanning FCS function. Press the calculate button. The intensity at each line should appear as a carpet representation.

In this screen, we can analyze the columns of the carpet. For example, we can select one column, calculate the ACF at that column and then perform a fit of the column ACF.

Select 32 at the Points from edge field. Press the ACF at trace button. You are automatically transferred to the large vector correlation page with the buffer filled up with the data from the column you selected.

Set the FFT length to 16K and calculate the autocorrelation at channel 1. Then press the fit button, that brings you to the ACF curve-fitting interface.
Set all parameters as shown above and then perform the fit. The fit is excellent and the recovered value for D is 8.4 μm²/s. If all lines are used, the recovered value of D is 9.0 μm²/s.

What about the G(0)? Will the G(0) obtained using the RICS approach and the G(0) from fitting each column be the same?
Exercise 3. Line scan analysis of adhesions

In your CD ROM you should find the file pax1023.bin. This is a file collected by Michelle Digman using the circular scan mode at the focal adhesion shown in the figure below. The protein is Paxillin EGFP. Data taken with the 2-photon microscope.

Field of view 15.6 mm
Scanning radius is 7 pixel=0.426 mm
Length of the orbit is 2.68 mm
64 points is the orbit
Distance between adjacent points is 0.044 mm
Orbit period 1ms.

Go to Analysis/ scanning FCS

From the menu file open the file in *.bin format. Set the period to average field at 1000, points per period 64 and press the “calculate image” button. You should see the scanning FCS carpet shown below.
Clearly, the intensity increases as a function of time. The first question is how to get rid of this slow trend as to recover the “presumably” fast fluctuations due to the occupation number of the paxillin molecules.

In the menu, select “carpet analysis”. Change the moving average field to 20 points. Press the “set detrend button”. Select the option segment random numbers and store the detrend data in huge 2.

What is going to happen here is that every column will be divided in 20 segments. For each segment the average will be calculated. The largest average will be stored. For each segment, random “uncorrelated” numbers will be added in a quantity that will make the average counts in each segment to be the same. After this operation all trends should have disappeared. However, since different columns have different average, the difference in intensity in the columns will be preserved.

In which way, if any, the addition of random numbers will affect the statistics?
Questions: In the detrend option screen there are various manner in which the columns could have been filtered. For example, calculating a moving average and subtraction the moving average at each point in the column, or subtracting an exponential average. Instead, you were told to use the random number addition mode.

1. Will any other method of data detrend have worked?
2. What is special about this data?
3. If the data was produced in the confocal microscope with analog data detection, which method should I use?

Be sure you understand what is going on here, otherwise the entire analysis could be messed up!

Now select “Column ACF detrend” in the drop window of the carpet analysis and press the Analysis button. In about 1-2 minutes the detrend operation and the ACF carpet with the columns after detrend will be shown. To see the data, go back to the “carpet analysis” page from the menu and press “exchange channels”. The date after detrend was saved in channel 2. After this operation the data is in channel 1 and you can press the calculate image button to display the data after detrend.
Note that the intensities in the columns have been very much preserved but the trends along the columns are gone.

Now we can select columns and perform the ACF calculation in selected columns, in selected columns range or in the entire carpet. For example, select points from edge 34 and edge width=4. This means that column 34 plus 4 columns in each side are selected and combined for the ACF analysis. Press ACF at trace. You are now in the large vector correlation page.

Select a length of 16K for the ACF calculation. Perform the ACF calculation. If you had previous plots in that page, thing can appear messy. Ask for help how to erase previous graph. Otherwise just proceed with the fit procedure. After the fit, the plots will be autoscaled and the figure will appear nice.
Clearly we can easily observe the fast relaxation due to the occupation number of the paxillin molecules at the adhesion. The apparent diffusion is about 0.96 um2/s and the G(0) is very small.

Try to do the same analysis without detrend. The ACF will be dominated by the slowly varying intensity and the fast process will be very difficult to discern.

In this analysis we averaged several columns. It is possible to analyze each column individually, save the data and import in Globals for analysis. It also possible to automatically calculate the ACF at each column and perform a single component analysis at each column and display the G(0) and D in a form of a plot.

If you are interested, got to the Carpet analysis page. Select the function Column ACF. The ACF will be calculated at each column. It can take 1-2 minutes. Then press Fit carpet. The fit page will appear. This is to initialize the fit. All columns have been averaged so that the “average fit” can be done. Select the function you want, for example single component fit. Perform the fit. Only when you are satisfied with this average fit, close the fit window. Now each column will be fitted separately. The results of the fit are shown in a form of a graph in the scanning FCS page.
Outside columns 20 to 45, the fit gives very unstable results. The reason is that there is no adhesion in these region and we are just fitting the noise. However, in the region with the adhesion, $D$ is in the range of $1 \text{ um}^2/\text{s}$, on the average smaller in the right part of the adhesion and the $G(0)$ is around 0.02, again being smaller on the average in the right part of the adhesion.
Exercise 4. RICS analysis of cell images

Go to the RICS page and select the “For beginners” button. On the prompt, select the image sequence 4-22-03-#2-A5-CHO-CELL3B.tif. The is a stack of 100 images of an alfa5-EGFP integrin in a CHO cell collected by Claire Brown on an Olympus Fluoview 300 at the University of Virginia. Close the TIF verify windows. This window tells you some of the parameters found in the header of the file.

Date are read in a 256x256 ROI, choose for you. The RICS operation is then performed automatically. The fit page is activated and the fit is performed and displayed. The sequence of operation has been preprogrammed for you as a demonstration. Now we will go back to the reading part and repeat the operations with you so that you can understand the logical steps to follow.

Open again the same file using the open, tif files and pointing to this file in the open window screen. After closing the verify TIF window, the data are read in. At the end of the reading operation, in the lower left image, you should have a region of interest selected by a box. Move the box to the center of the image and press the reprocess button. This selects the data in a 256x256 square in the region of interest.

After data reprocess, you can select the RICS calculation in the Tools menu. Chose the RICS –average, after selecting moving average and 20 in the RICS page as shown below.

Perform the RICS operation. You should obtain something like the 2D ACF shown below.
Only the correlation due to the mobile part is left. All the immobile part is compensated. To convince yourself, just perform the same operation without removing the immobile fraction.

**Question:** What was the criterion used to chose moving average using 20 frames? Can we just have removed the immobile fraction? Try the different modes to see the differences caused by these modes of data analysis.

Go to the fit page. Pay attention to the set up of the parameters, since you must know the pixel time, the line time and the pixel size.
The result of the fit is shown below.

The fit is excellent; the apparent diffusion constant recovered is in the 1 um2/s range. You can play with other parameters for the fit and select different models. The next question now is about determining of there are small region of the image in which we can see aggregation of the integrins. For this, we go to the next exercise.
Exercise 5. N&B analysis in line scanning and in cells

Using the same example data file we can go to the N&B analysis. To detect α5-integrin clusters, we must examine the periphery of the cell. Open again the file and select the lower part region as shown below.

Now, in the tools menu, select <N> and brightness map. Perform the analysis. A new screen should appear with a new form of 2D histogram display.

The 2D histogram of N vs B has like two branches indicated in the figure by the square blue rectangle and the red oval. The points in the blue rectangle are contributed by the image background. That can be eliminated in two different ways. One is to offset the image intensity and the other is to threshold the image intensity. The two operations are very different in nature.
Enter an image background of 20 (digital levels), close the window and perform again the N&B analysis. For the moment close the 2D histogram page and return to the RICS page. The two images in the lower left and upper right positions, are the Brightness and N images

We can start to distinguish the bright spots at the cell border where the integrins are presumably aggregating. Now recalculate the N7B analysis and return the 2D histogram page.

Move the cursor in the right side in the histogram figure. The points contained in the cursor (the circle) will light up in the image. For better view, select the parameters as shown in the figure below. If you don’t see the image as shown, you must go back to the RICS page, select for image 1, no auto-scale, min 20 and max 200. Perform the N&B analysis and return to the 2D histogram page.

Now you can explore the region of large brightness and low number of particles.
There many other features and possibilities in this screen. Too many to explore in this brief time allocated for this exercise. If you are curious, you can ask the assistants.

Have fun!!!
C. Fluorescence Lifetime Imaging (FLIM)

Phasor Properties

The Phasor is a frequency domain representation of the fluorescence lifetime at a single frequency. For single exponential lifetimes, this completely describes the system. For multiple exponentials, the Phasor represents some intensity weighted linear combination of the lifetimes in the system. Therefore, given the Phasor for a single multiexponential measurement, we cannot determine the lifetimes for the system. Nevertheless, given multiple measurements (e.g. multiple pixels in an image), heterogeneity becomes obvious. To demonstrate this effect, use the attached Microsoft Excel worksheet which calculates the multifrequency fluorescence modulation and phase curves, the time domain fluorescence decay, and the Phasor at a single frequency for a combination of four exponentials.

Two State Equilibria

Fluorescent ion indicators often exist in two distinct states with different emission spectra and fluorescence lifetimes (and sometimes absorption spectra). The lifetime may be multiexponential in both the free and ion-bound state. Binding to the ion of interest shifts the equilibrium between these states, changing the spectral properties and the fluorescence lifetimes. Therefore, the free and bound states will be characterized by different “Phasors”. Close to the dissociation constant, the fluorescence lifetime is characterized by a linear combination of the two Phasors. Simulate the Phasor for different combinations (relative amplitudes) of a 4 ns lifetime state and a 1 ns lifetime state (e.g. amplitude 1 = 0.2, amplitude 2 = 0.8). Record S (the Phasor y coordinate) and G (the Phasor x coordinate) values for each combination. Plot S vs. G to see how the Phasor changes with different combinations. When half of the population is in the 1 ns state, has the Phasor moved halfway between the Phasor for the two states? Does the position of the Phasor change linearly with concentration? What about with fractional intensity? What information would you have to have in order to calculate concentration from the position of a Phasor?

The file, capillaries1001.ref, contains phase and modulation data on three capillaries filled with different dye solutions. Open the FLIM module in SimFCS. Under the File menu, click “Read referenced and add” and then open the above file. Referenced simply indicates that this file has been corrected for phase shifts and detection efficiency relative to a reference with a known lifetime (e.g. fluorescein). At the top of the screen the image is shown in false color (you can change this to grey scale by unchecking “Color points at the bottom of the screen). At the bottom of the screen, a histogram of Phasors is shown, also in false color.

The calculation of the phase and modulation is strongly dependent on the signal-to-noise. Therefore it is a good idea to smooth noisy data by a moving average. This can be done using the Smooth button at the right hand side of the screen. Note that this will

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also reduce your spatial resolution in the Phasor. If you need high spatial resolution, make sure you have at least 100 photons in each pixel of the image. In addition, small amounts of room light will appear towards the lower left hand corner of the Phasor (room light is uncorrelated, so it has zero modulation depth). This can be eliminated by setting a threshold. Click Recalculate to update the image with the new threshold. Note that if the room light is significant compared to the fluorescence signal, it will bias the fluorescence Phasors by pulling them toward the lower left hand corner of the Phasor plot, with Phasors corresponding to lower intensity regions of the image being pulled more toward the lower left hand corner than those of higher intensity regions of the image. Different components of the Phasor can be selected in the image by clicking and dragging the cursor in the Phasor plot. Above the Phasor, the phase and modulation for the cursor is listed as well as the apparent phase and modulation lifetimes for these components.

All of the capillaries contain 10 mM Tris buffer, pH 8.0. Fluorescein has a lifetime of 4.05 ns in basic solution. Which capillary contains fluorescein? The shortest lifetime capillary contains Rhodamine B. Rhodamine B has a single exponential lifetime around 1.5 ns in phosphate buffer at pH 7.0. Is Rhodamine B single exponential in this solution? What can you say about the pKₐ of Rhodamine B? Can you guess what the center capillary contains?

SimFCS contains a calculator to determine more quantitative information about the Phasor histogram. In order to use the calculator, you have to provide some information. The terminology of the calculator comes from the terminology of FRET, so calculating fractional intensity requires a little creativity. Under “Assign”, the researcher has the options to “Assign Phasor to unquenched” or “Assign Phasor to background”. To the right of the screen the tau phase and tau mod for each of these assignments is shown. To calculate fractional intensity, we will actually calculate fractional autofluorescent background where the background is assigned to one of our species. The FRET efficiency will be set to zero, since we don’t have quenching in this experiment. Assign the Phasor for the fluorescein containing capillary as unquenched. Assign the short lifetime Phasor as background. Under Calculator on the menu, select low res. This brings up a contour map of the Phasor. At the top of this map there are sliders for FRET efficiency, % autofluorescence, and % donor unquenched. Since we are not doing a quenching experiment, the first and last sliders mean nothing. The Scan check boxes simply display the phasor “trajectory” for different values of the parameter in question. For a linear combination, this trajectory is simply a line between the two species. Moving the slider for % autofluorescence changes the fractional intensity combination of the “unquenched” and “background” species that we assigned earlier. Above the plot, F_backg displays the percent intensity contribution of the species we assigned earlier to background. Calculate the percent intensity contribution of each species in the center capillary. What would you have to know in order to calculate concentration from this information?
Quenching.

In this section, we will discuss quenching due to FRET. The efficiency of energy transfer is related to the fluorescence lifetime as follows:

\[
E = 1 - \frac{\tau_{DA}}{\tau_D}, \quad \tau_{DA} = \tau_D (1 - E),
\]

where \( \tau_{DA} \) is the lifetime of the donor in the presence of acceptor (quenched) and \( \tau_D \) is the lifetime of the donor in the absence of acceptor. Calculate the Phasor for a fluorophore with a 4 ns lifetime. Now calculate its Phasor under different efficiencies of energy transfer. How does this differ from what we saw with the two state system?

If one can preferentially excite the donor fluorophore (no direct excitation of the acceptor) and observe the acceptor fluorescence, a very unusual phenomenon is observed. The fluorescence of the acceptor rises with the same time constant as the quenched donor fluorescence. Then it decays with the characteristic lifetime of the acceptor. For example, the case where the donor lifetime has been quenched to 0.5 ns and the acceptor lifetime is 4 ns would be simulated for the acceptor by a lifetime of 0.5 ns with an amplitude of -1 and a lifetime of 4 ns with an amplitude of 1. Simulate this case.

Often in FRET experiments, only a fraction of the donors experience FRET. The others continue to fluoresce with an unquenched lifetime. Simulate the situation where 50% of the donors remain unquenched. Rationalize the results in terms of the quenching simulations done above and the two state experiments we did earlier.
The file, CFPpax8651866.ref, contains referenced FLIM data for a cell transfected with a CFP paxillin construct. In the SimFCS FRET analysis page, open the file using the “Read referenced and add” command as before. The file, 1011rac1002.ref, contains referenced FLIM data for a cell transfected with a CFP-YFP fusion protein. Load this file with “Read referenced and add”. The second image will appear at the top of the screen, and the Phasor histogram points from that file will be added to the Phasor plot. At the right you can select or deselect images from the plot (and the Phasor). Deselect the “current” image so that only two images are shown. Again, threshold out any room light, and smooth the Phasor. It may be helpful to toggle one of the images for visual comparison. What percentage of the species are FRETing in the CFP-YFP image? Is the FRET efficiency high or low?

Assign the unquenched Phasor using the image of the cell containing donor only (CFP paxillin). Assign the background Phasor to the lower left hand corner of the image (room light). Use the calculator to determine if background is contributing significantly to the image. Check the scan box next to the FRET efficiency slider. Assume that there is no unquenched donor. Estimate the percentage of autofluorescence and the FRET efficiency. If there is unquenched donor, can you determine the FRET efficiency accurately? Suggest a control experiment that would allow you to determine the FRET efficiency accurately in the presence of unquenched donor.

Equations:
Relationship between G (x on the Phasor plot), S (y on the Phasor plot) and M and φ:
\[ G = M \cos(\phi), \quad S = M \sin(\phi), \quad M = S^2 + G^2, \quad \phi = \tan^{-1}\left(\frac{S}{G}\right) \]

Relationships between lifetime and M and φ:
\[ \tau_M = \frac{1}{2\pi\omega} \left( \frac{1}{M^2 - 1} \right), \quad \tau_\phi = \frac{\tan(\phi)}{2\pi\omega}, \quad M = \frac{1}{\sqrt{1 + (2\pi\omega\tau)^2}}, \quad \phi = \tan^{-1}(2\pi\omega\tau) \]