



Guide to Basic FCS Experiments on Zeiss LSM 780/Confocor3/BiG Systems Running ZEN 2010

With the Zeiss LSM 780, BiG Module or Confocor 3 systems, fluorescence correlation spectroscopy (FCS) data can be acquired and analyzed. From this data, information about particle mobility, concentration, and interaction can be measured at a single molecule level. The purpose of this document is to act as a guide for acquiring a basic FCS measurement using a dye-in-solution sample. Any questions or concerns can be directed to the Zeiss Product and Applications Support group at 1-800-509-3905 or support@zeiss.com

Outline

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- 2) Focus into the sample
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 - a) Light Path
 - i) Selection of MBS, secondary dichroics, emission filters and detectors
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1. Sample Preparation

The dye to be used should be dissolved in a water/buffer solution and diluted down such that the concentration is between 1-50 nM. After dilution, the dye solution needs to be put in a chamber that will hold media with a # 1.5 cover-glass bottom. Make sure to put enough media to cover the entire bottom of the chamber. Typically, a Mat-Tek dish or 8-well NUNC chamber work well as sample holders. Common dyes used for this kind of experiment is Rhodamine 6G or any of the Alexa dyes. The Alexa dyes are recommended as Rhodamine 6G has problem with aggregation.

Mat-Tek Dishes

<http://www.glass-bottom-dishes.com/product.html>

NUNC Chambered Cover Glass

<http://www.nuncbrand.com/us/page.aspx?ID=235>

Alexa Fluor Dyes

<http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/The-Alexa-Fluor-Dye-Series.html>

2. Focus Into the Sample

Before loading the sample onto the scope, some consideration is needed when selecting the objective to use. It is imperative for good FCS measurements that a well corrected objective is used to ensure overlap of the PSF for different wavelengths as well as the objective needs to be corrected for any spherical aberration. Also, the use of fluorescence free water as an immersion medium is highly recommended. From Zeiss there are 3 “FCS approved” objectives that meet these criteria:

- 40x C-Apochromat NA 1.2 W Corr
- 63x C-Apochromat NA 1.2 W Corr
- 40x LD C-Apochromat NA 1.1 W Corr

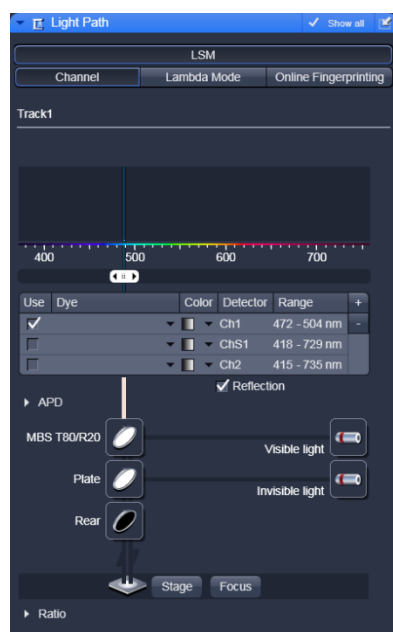
All of these steps will help ensure a well defined PSF which is crucial to accurately model the resulting FCS data.

2.a Setup and Focus with Reflected Laser Light

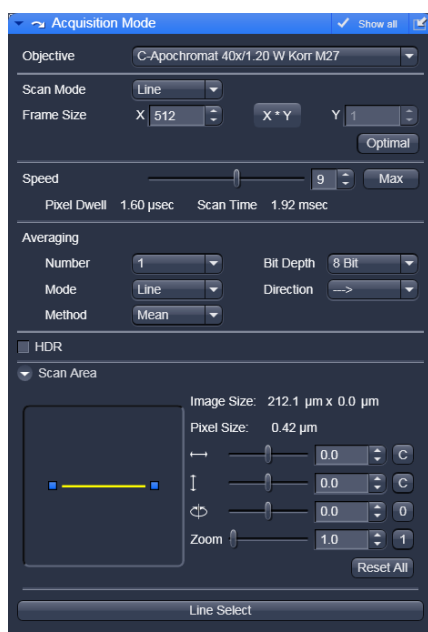
After the dye solution sample has been loaded onto the scope, the objective needs to be focused into the sample. An easy and reliable way to focus into a solution sample is to utilize reflected laser light off of the coverglass to position the focus.

1. Setup reflected light path
2. Focus up until both sides of the coverglass have been imaged
3. Move focus an additional 200 μm into sample

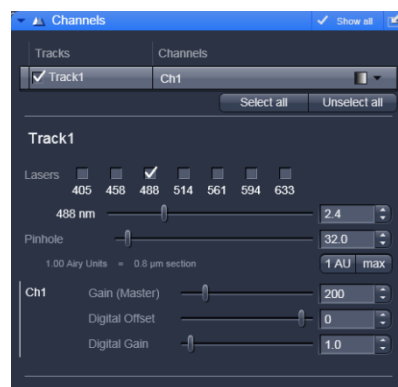
1.



Make sure to use the 80/20 MBS along with checking the “Reflection” box in the light path menu. On LSM 780 and Conforcor 3 systems, make sure to use the standard PMT in Ch 1.

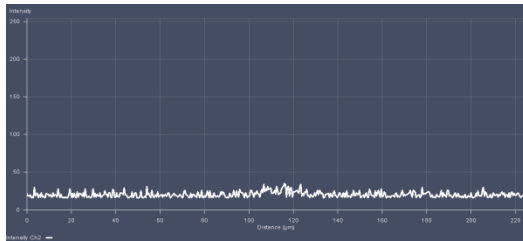


Change the scan mode to line with a scan speed of 9

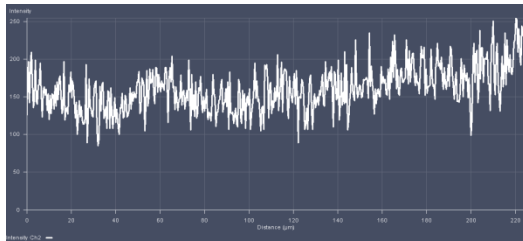


Close the pinhole to 1 AU to help reduce reflected light

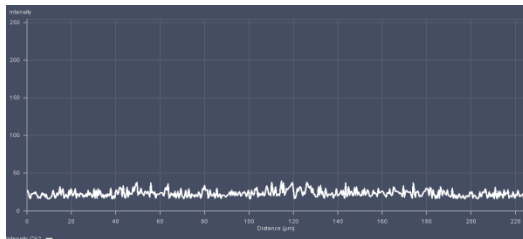
2.



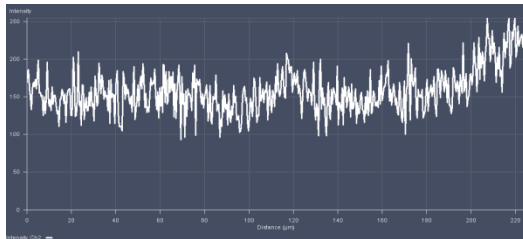
Start with objective in the load position and focus up until the immersion water just makes contact with the coverglass. Start the line scan after the water makes contact. The line profile should look similar to the profile shown on the left.



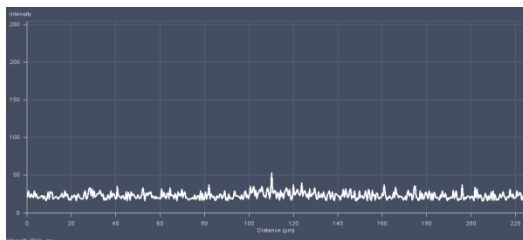
Start to move the focus up. When the first interface of the coverglass is reached a spike in intensity will be seen. This interface is between the immersion water and the coverglass.



The intensity will drop back down as the focus continues to move up toward the sample. The focus is now in the coverglass

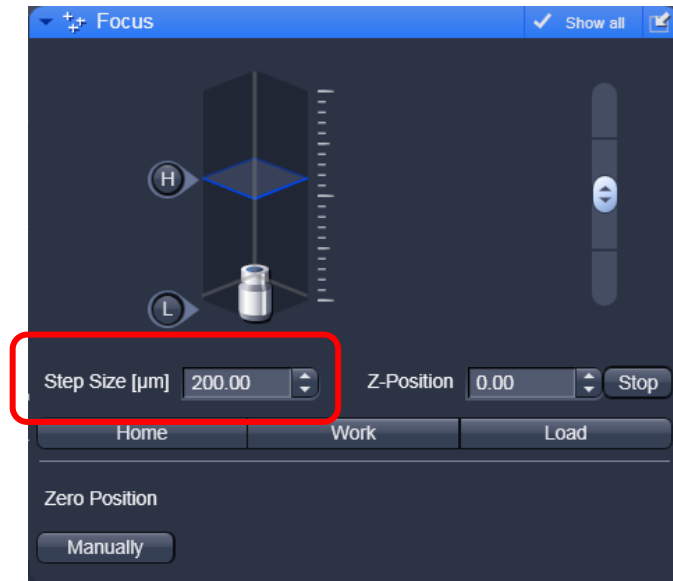


Another spike in intensity will be seen at the second interface of the coverglass with the sample.



After the second spike, the focus should be moved such the intensity from the second spike just returns to the base level. At this point, the focus is just beyond the coverslip in the sample.

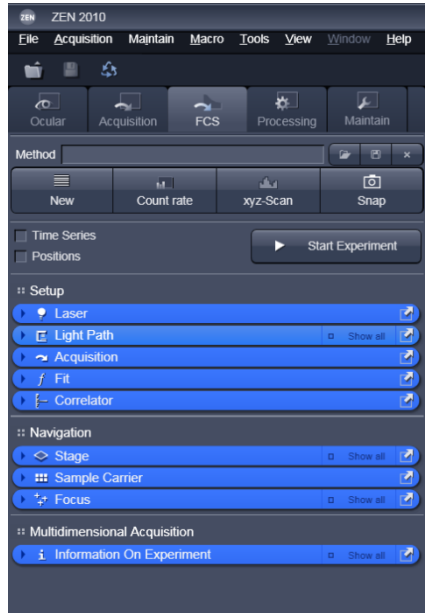
3.



After focusing just beyond the cover slip (shown in step 2), the focus menu should be opened and the step size set to 200 μm . Use the Step Size up arrow to move the additional 200 μm into the sample. The focus should be completely in the dye solution.

3. Setup of LSM 780/Confocor 3/BiG for FCS

In ZEN 2010 all options for FCS data acquisition and data analysis can be found under the FCS Tab. For the purpose of this manual, only a select number of the FCS menus are covered.

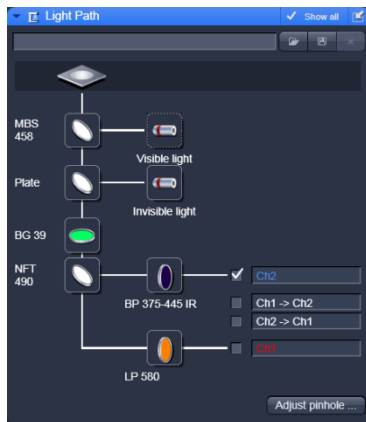


3.a Light Path

3.a.1 Selection of MBS, secondary dichroics, emission filters and detectors

The selection of appropriate secondary dichroics, emission filters, and fluorescence collection is done under the FCS Light Path menu.

Confocor 3/BiG

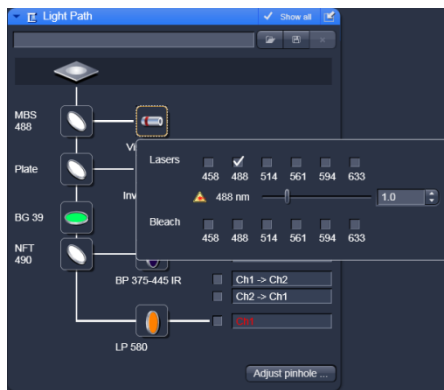


The beam path configuration for the Confocor 3/BiG is found under the Light Path menu in the FCS tab

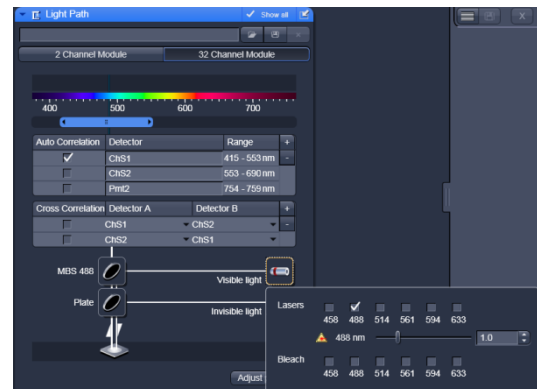
LSM 780



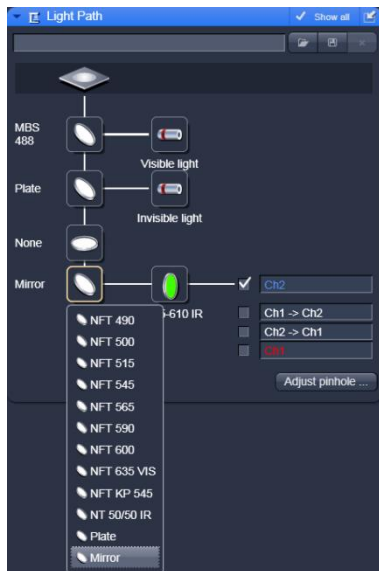
The beam path configuration for the LSM 780 is found under the Light Path menu in the FCS tab



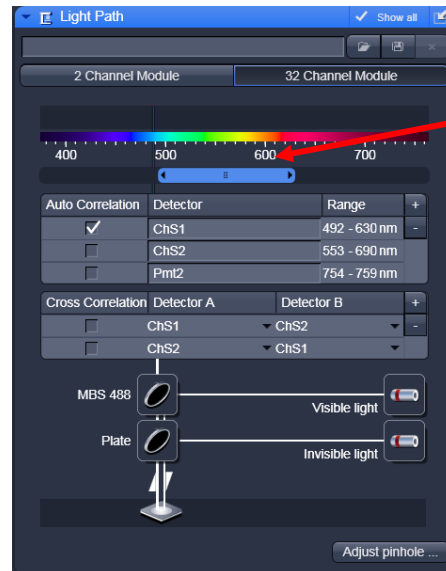
Choose the excitation laser under the “Visible Light” button. The correct MBS for the chosen laser line can be selected from the MBS button



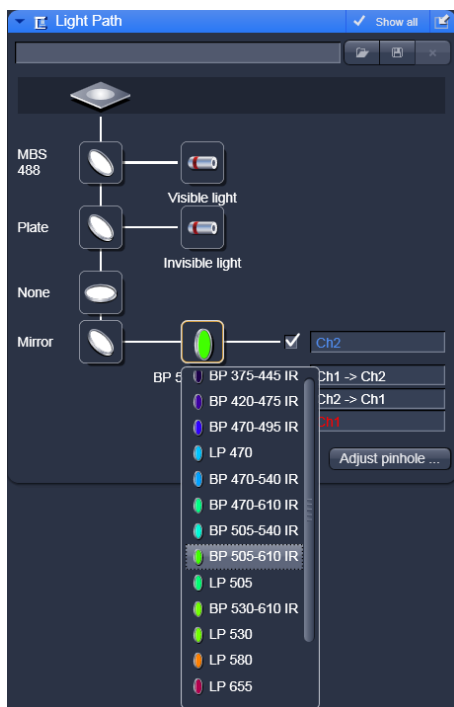
Choose the excitation laser under the “Visible Light” button. The correct MBS for the chosen laser line can be selected from the MBS button



The appropriate secondary dichroic can be chosen based on which detector will be used. For blue-green fluorophores use Ch2 and Ch1 for red fluorophores (Confocor 3). A choice of secondary dichroic is not available on systems with the BiG module as the secondary dichroic is dictated by the filter cube placed in the module.



The appropriate channel can be selected in the Auto Correlation area of the Light Path menu. For blue-orange fluorophores it is best to use the internal 32-Channel GaAsP array (ChS1,ChS2). For far red fluorophores, Pmt2 is the best choice. The collection range can be set with the slider shown above.



Emission filters can be chosen by selecting the button in front of the active channel. It is best to use band pass filters if possible to reject any unwanted light.

3.a.2 Count Rate

Once the beam path for FCS has been setup, select the count rate button to view how much fluorescence is reaching your detector based on the beam path setup and the amount of laser used. There are three options in the count rate menu: Count Rate, Correlation, and CPM.

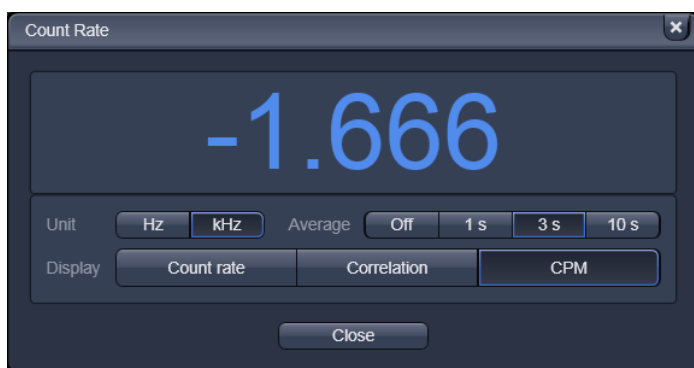




With the Count Rate button selected the read out of the system will be in kHz (photons/sec). This gives a measure of how much fluorescence is coming back from the sample based on the beam path used. It is ideal to keep the count rate under 500 kHz to avoid any saturation or damage to the detector.



The Correlation button gives information on whether the system detects any moving molecules in the sample. If the average value is above 1.0, then the observed moving fluorophores are correlating.



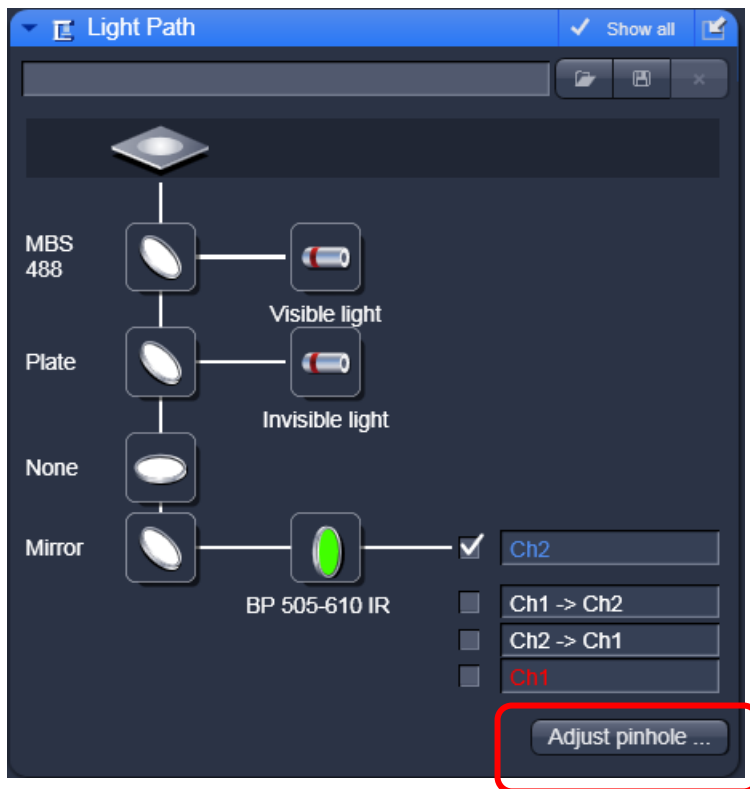
The CPM button in the Count Rate menu is the most telling about the quality of the sample for FCS. The average CPM represents the Counts per Second per Molecule. In other words, the CPM value is a direct measure of the amount of fluorescence per molecule the system detects (the signal-to-noise of the sample). A reasonable number for this value would be in the range of 1kHz-15kHz. If the count rate value is high and the CPM value is low (below 1kHz CPM), this

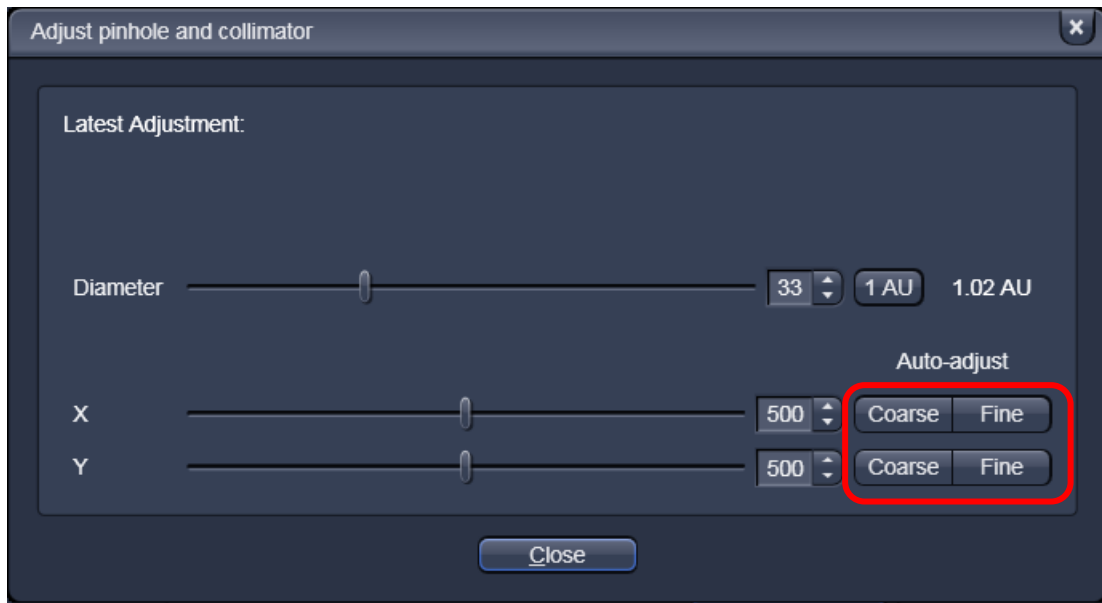
indicates the sample is too concentrated for FCS. With the CPM menu open, the correction collar of the objective should be adjusted to maximize the CPM value. This process corrects for any variation in the coverglass thickness.

3.a.2 Pinhole Alignment

To ensure a good FCS experiment the fidelity of the PSF must also be ensured. A major component of shaping the PSF is the confocal pinhole. Thus it is imperative that the alignment of the pinhole to the FCS detector be checked frequently. A pinhole alignment should be performed before every FCS session (allow the system to warm up for an hour) and when a new beam path created.

Open the FCS Light Path Menu and select the Adjust Pinhole button at the bottom right hand corner

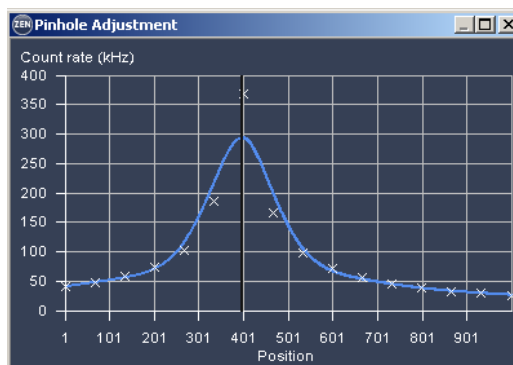




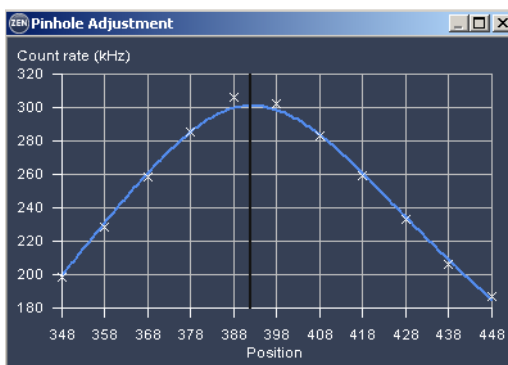
The pinhole adjust menu will give the data of the last adjustment for the current beam path and will give the current pinhole size and position. Make sure the pinhole is at 1AU before starting the adjustment. There are 2 options of the pinhole alignment, Coarse and Fine. The coarse alignment will move the pinhole over the entire travel range looking for a maximum count rate value. The fine adjustment covers a smaller travel range with finer movement looking for the peak count rate. The coarse option should be used if the beampath is new, or if the beampath has not been used for a long period of time.

Important: After the pinhole alignment has been run, the CPM should be checked and the correction collar of the objective adjusted again.

Hint: If the shape of the pinhole alignment curve is NOT a tight Gaussian, then check to make sure the beampath has appropriate dichroics and filters. If it still is not Gaussian in shape, then there might be an alignment problem that will require further diagnosis by service.



Coarse Adjustment

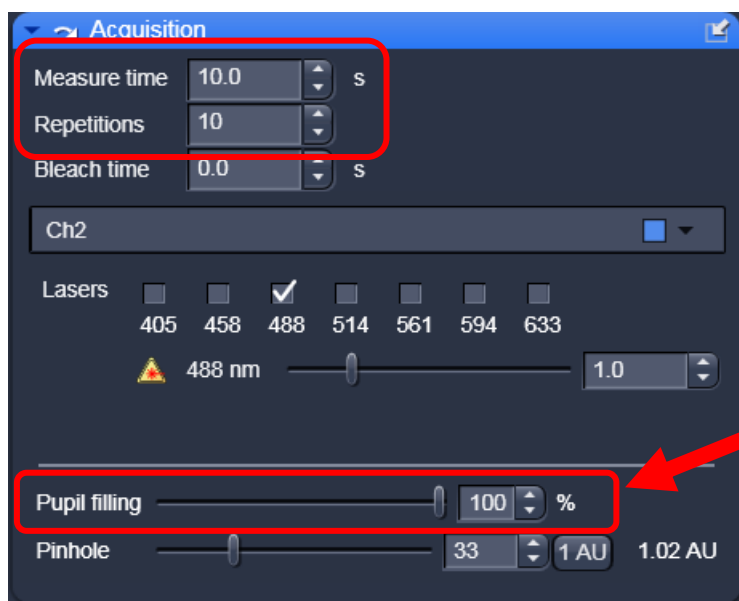


Fine Adjustment

3.b Acquisition Parameters

The FCS Acquisition menu will allow for specification of measurement parameters such as measurement time and number of measurements (Repetitions). As with any experiment, it is ideal to collect as much data as possible in order to have good statistics. For FCS experiments this can be achieved in two ways. Long measurement times with few repetitions or shorter measurement times with many repetitions. It is advantageous to have shorter measurement times with many repetitions to allow for the possible exclusion of repetitions from the final average if needed.

For dye in solution measurements, a typical set of parameters would be a measurement time of 10 seconds with 10 repetitions.

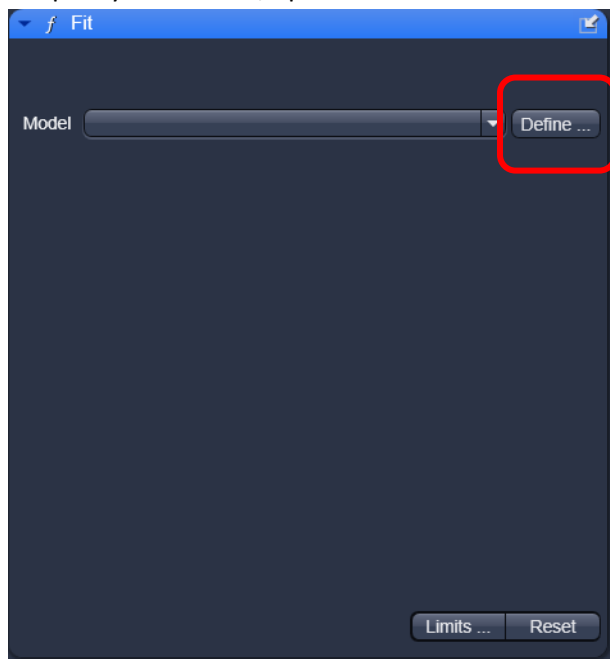


Always leave the pupil filling at 100%. Any deviation from this can cause problems with data analysis if you are not familiar with the theory of FCS and the optical components of the system.

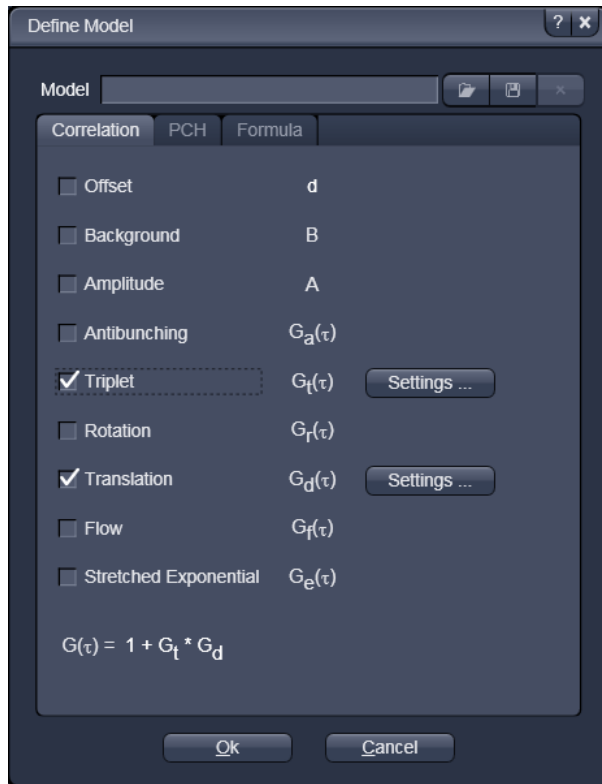
3.b Creation of Basic Fit Model

Before any data is acquired it is helpful to build the theoretical model in the software to allow for immediate data analysis later on. The FCS fit model menu will allow for the specification of many different types of biological models. For the purpose of this document, the focus will be on the modeling of free diffusion of a dye molecule in solution with the possibility of the dye molecule going in to the triplet state while the molecule is being observed.

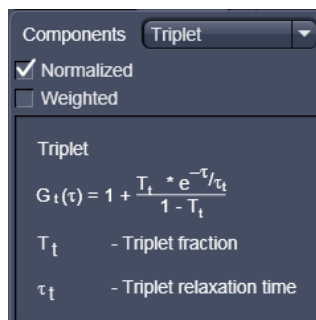
To specify a fit model, open the FCS Fit menu and select the define button.



Under the define model menu, select the triplet and translation options under the correlation tab.



Select the settings button for the triplet option and make sure only the normalized option is selected. Notice the formula for the triplet component is given.



Select the settings button for the translation option and ensure that only one component is selected and that the fractional intensities and diffusion coefficients check boxes are not checked. The free/anomalous option should be set to free and the dimension should be set to 3D.

The screenshot shows a software interface for configuring a model. At the top, there are three tabs labeled 1, 2, and 3. Below the tabs, there are three dropdown menus for 'Free / anomalous' (all set to 'free') and three dropdown menus for 'Dimension' (all set to '3D'). Below these are several checkboxes: 'Fractional intensities' (unchecked), 'Diffusion coefficients' (unchecked), and '2 Photon' (unchecked). The main area contains a mathematical formula for $G_d(t) = \sum_{i=1}^3 \frac{\Phi_i}{\left(1 + \left(\frac{\tau}{\tau_{d,i}}\right)^{\alpha_i}\right)^{e_{d1}} * \left(1 + \left(\frac{\tau}{\tau_{d,i}}\right)^{\alpha_i} \frac{1}{S}\right)^{e_{d2}}}$. Below the formula are definitions for $\Phi_i = \frac{f_i * \eta_i^2}{\sum_{i=1}^3 (f_i * \eta_i)^2}$ and $C = \frac{N}{L_A * \pi^{3/2} * \omega_r^2 * \omega_z}$. A table at the bottom left shows the values for e_{d1} and e_{d2} for 1D, 2D, and 3D dimensions. To the right of the table are definitions for various parameters: i (Index of component), Φ_i (Fractional intensity), f_i (Fraction of molecules), η_i (Molecular brightness), $\tau_{d,i}$ (Diffusional correlation time), S (Structural parameter), ω_z (Axial focus radius), ω_r (Lateral focus radius), α_i (Anomaly parameter), c (Concentration), N (Total number molecules), and L_A (Avogadro number).

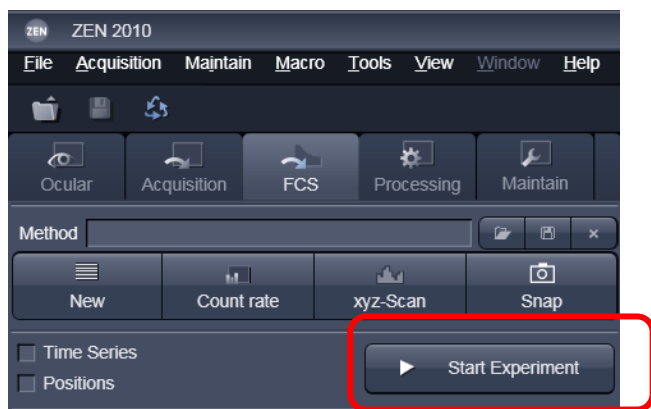
	e_{d1}	e_{d2}
1D	0.5	0
2D	1	0
3D	1	1

After making these changes, the model should be saved under the define model menu. It is recommended to give a name to the model that describes the active components in the fit model. In this case the model is fitting for 1 translation component with a triplet state.

The screenshot shows a 'Configuration' dialog box with a close button (X) in the top right corner. The text 'Save current model configuration as' is followed by a text input field containing '1Comp_Triplet'. Below the input field are two buttons: 'Ok' and 'Cancel'.

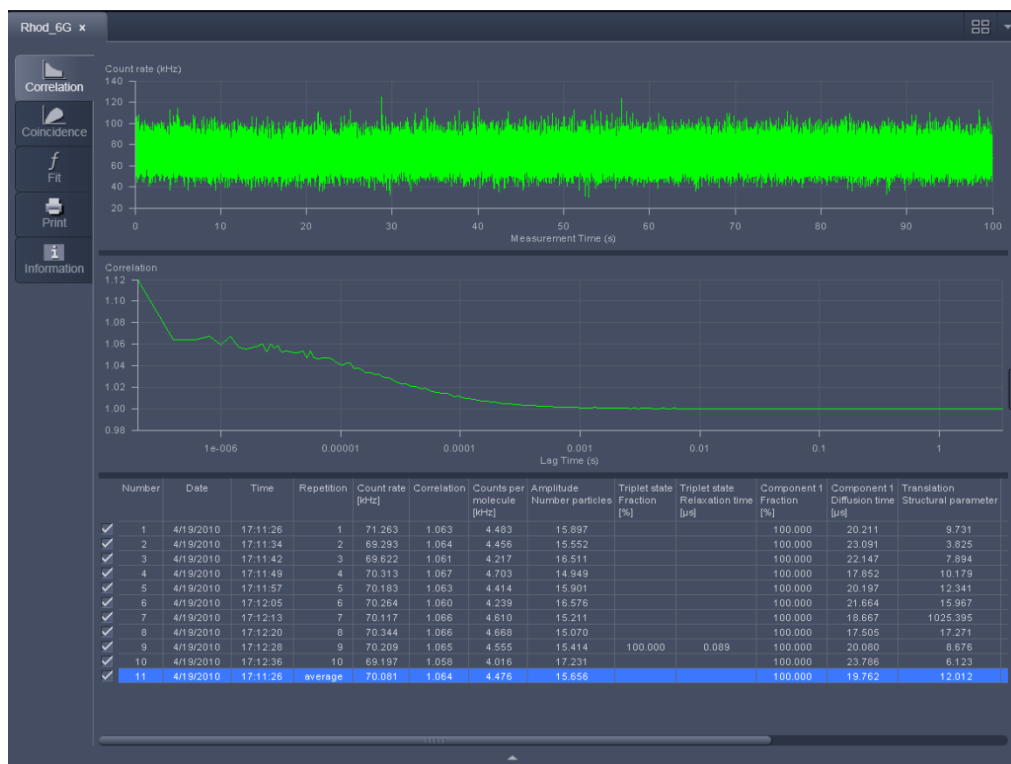
4. Data Acquisition

To start acquiring data, click the Start Experiment button in the FCS tab. This will open a new window that will show the count rate as a function of time and will show the resulting correlation curve.



4.a Navigation of the Data Table

Once an experiment is started, the new window created will show several graphs and a data table. Within the data table, the data for each repetition can be viewed by highlighting the table entry. The repetition can be included or excluded from the final averaged curve (at the bottom of the table) by checking or unchecking the box next to the repetition.



Count rate plotted as a function of time

Autocorrelation curve

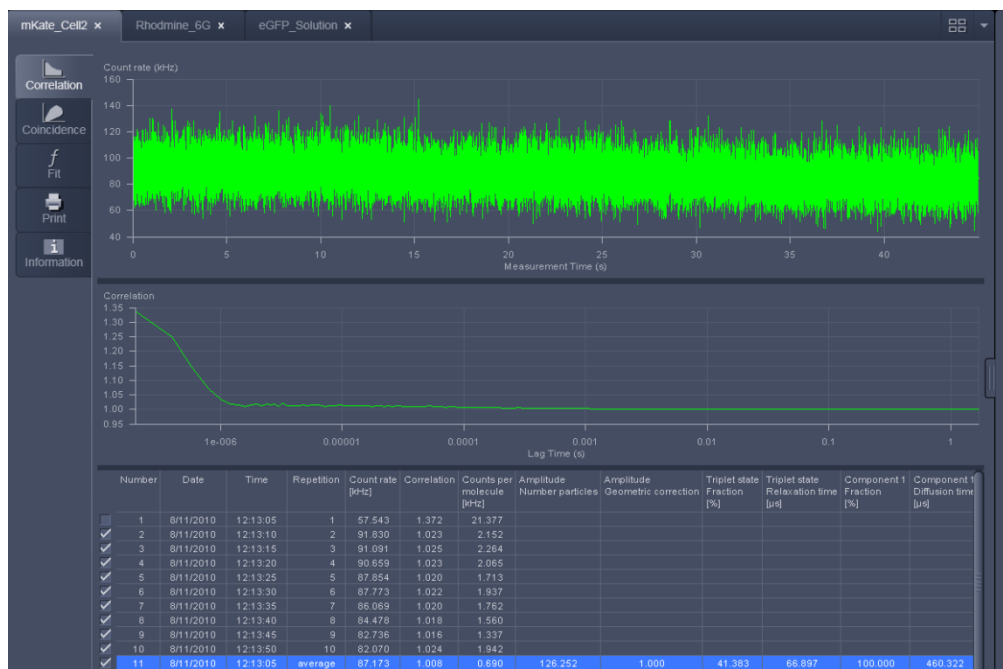
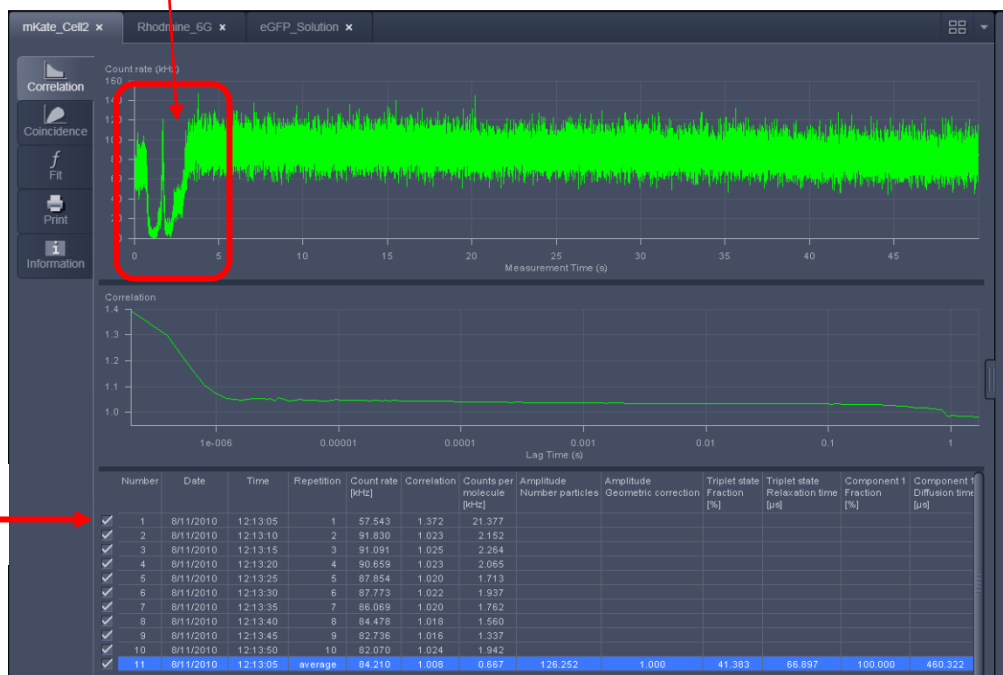
Data table

4.b Creation of Average Correlation Curve

In some cases it might be necessary to exclude repetitions from being included in the final average correlation curve. To exclude the repetition simply uncheck the box next to the repetition. Once the average has been created the data can be analyzed.

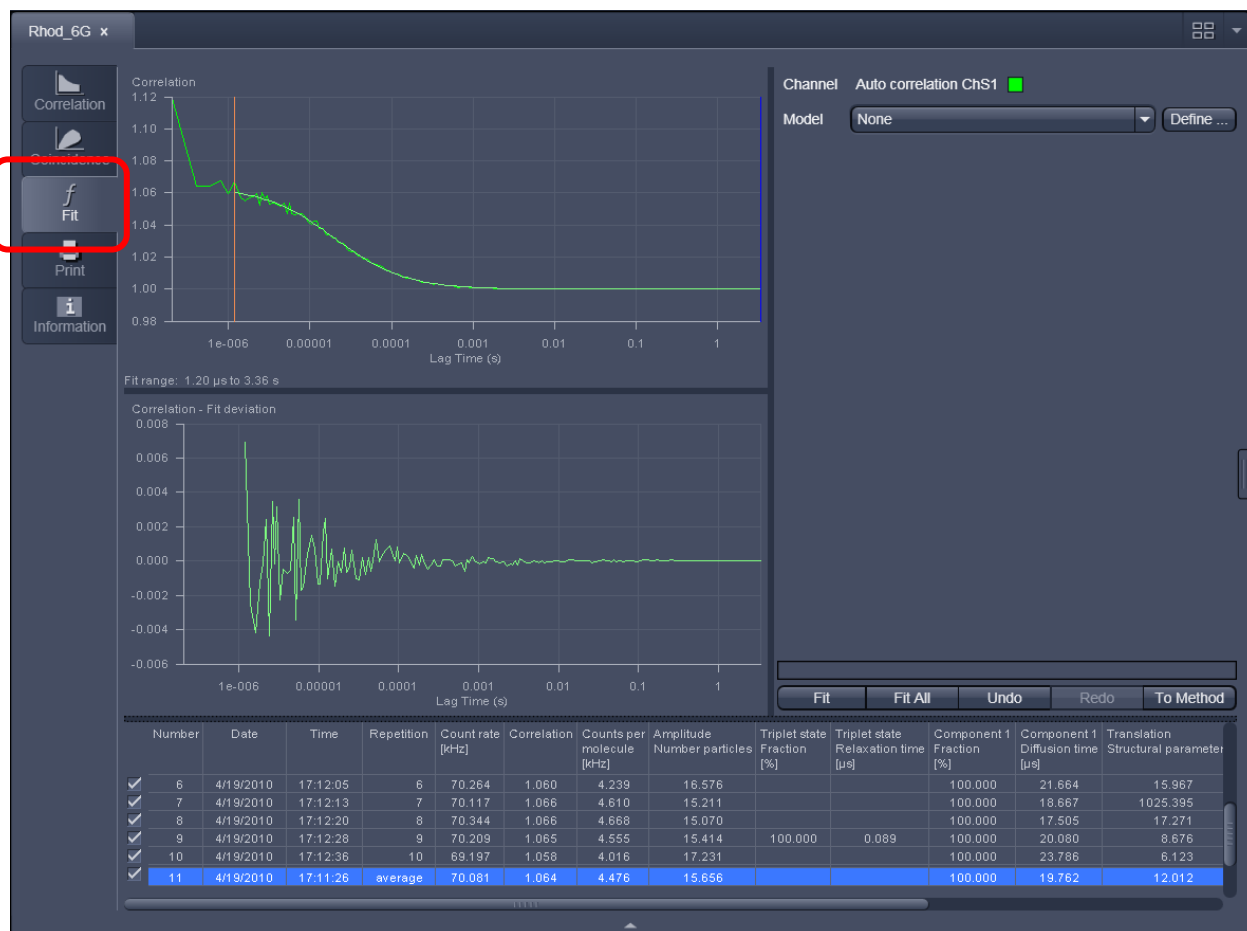
Abnormality to be removed

Uncheck



5. Data Analysis

After the average has been constructed, it is time to analyze the data using the model defined earlier. To analyze the data, select the fit tab on the left hand side of the image container



Next, use the orange line on the left hand side of the correlation graph and move it in to about 1.2us-3.0us. The data points to the left of this line can be ignored as any data in the region can be attributed to the after-pulsing of the detectors and is not real data.

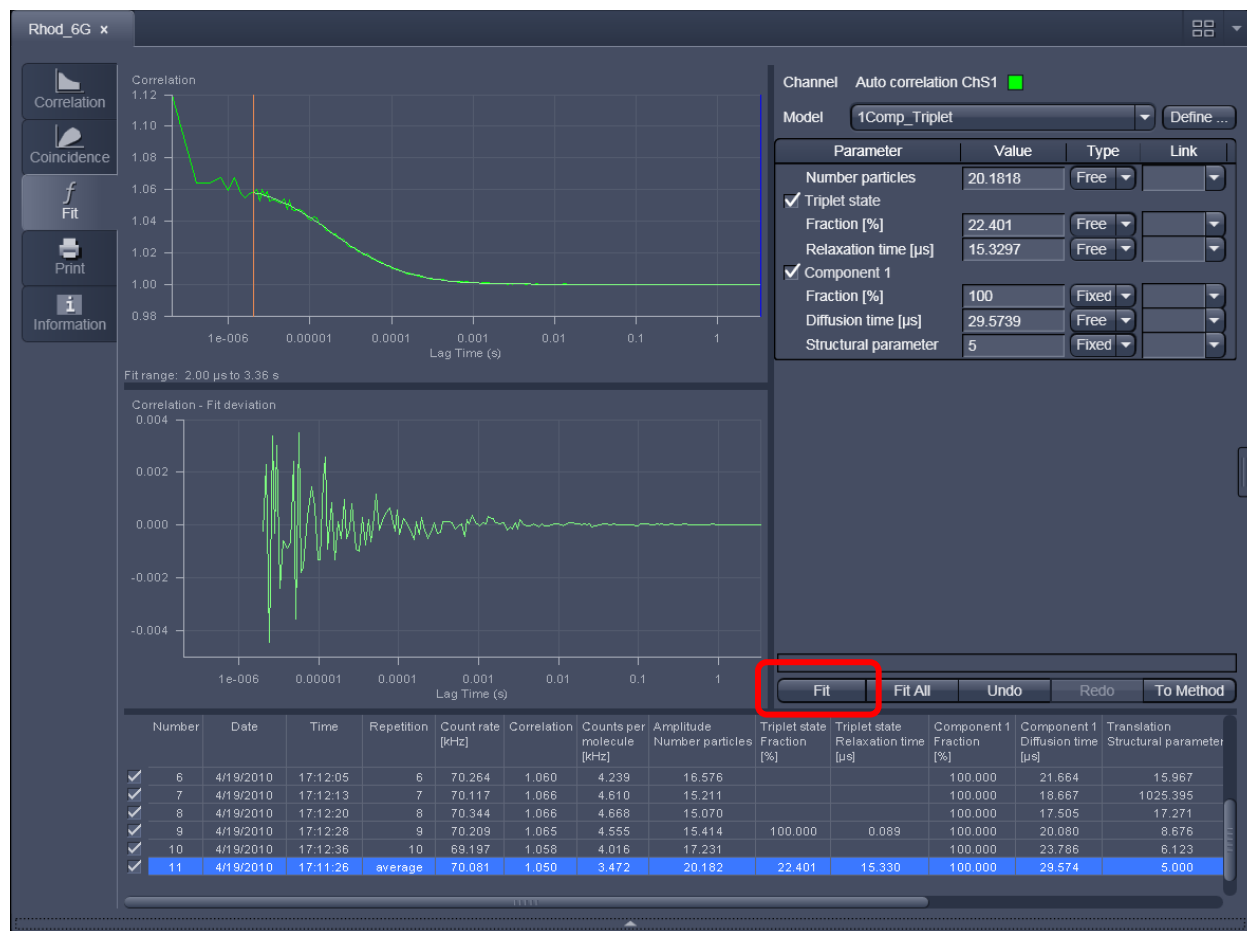
5.a Selection of Fit Model

To load the fit model use the drop down list of models on the right to load in the model saved earlier.



5.b Fitting the Data

After the model is loaded, press the Fit button at the bottom right of the image container. The fit results will be populated in the fit table on right and in the data table below the correlation curve.



Typically in a well aligned system the structure parameter can be fixed to a value between 5 and 7 before fitting. The structure parameter is the ratio of the axial resolution to the radial resolution. If the fit is good, then the graph in the fit deviation window (the residuals) should not have many sequential points about zero and should look like a random distribution of points about zero. It is up to the end user to determine what a good fit is and what is not.