Fluorescence Correlation Spectroscopy, FCS (i880)

FCS is a method for analyzing the signal intensity fluctuations from a stationary focused laser spot. An FCS autocorrelation curve analysis can give you information about the concentration, diffusion and binding of fluorescent molecules in solution.

Setting up a Dye-in-Solution Sample

1. Prepare calibration dye (Rhodamine Green or any of the Alexa dyes) by dissolving in a water/buffer solution. Dilute down such that the concentration is around 2 nM.

2. Put dye in a no. 1.5 glass-bottom dish or in a 6-/8- well Nunc chamber coverglass.

3. Start Zen system in standard way. Turn on appropriate laser. Use 488 nm laser if imaging Rhodamine Green.

4. Use the 40X C-Apochromat 1.2 NA water immersion lens.

5. After doing the steps outlined below, follow the same procedure for your sample dye.

Focusing Sample

1. Start with objective in the lowest position and focus up until the immersion water just makes contact with the coverglass.

2. In the Acquisition tab, use reflected laser light off the coverglass to position the focus.

3. In the *Light Path* menu, setup reflected light path. Use the T80/R20 MBS and check the "Reflection" box.

4. In the *Acquisition Mode* menu, change *Scan Mode* to *Line* and set a scan speed of around 9.

Light path configuration for Reflected light (Dichroic: MBS T80/R20)

Set averaging to one (1), bit depth to 8, and frame size to 512 by 1 for fast line scanning.

5. In the *Channels* tab, close the pinhole to 1AU to help reduce reflected light.

6. Use low laser power and decrease gain as needed.

7. Start with objective in the load position and focus up until the immersion water just makes contact with the coverglass.


Fast line scanning acquisition parameters

8. Start the line scan (*Live*) after the water makes contact. The line profile should look similar to the profile shown below.
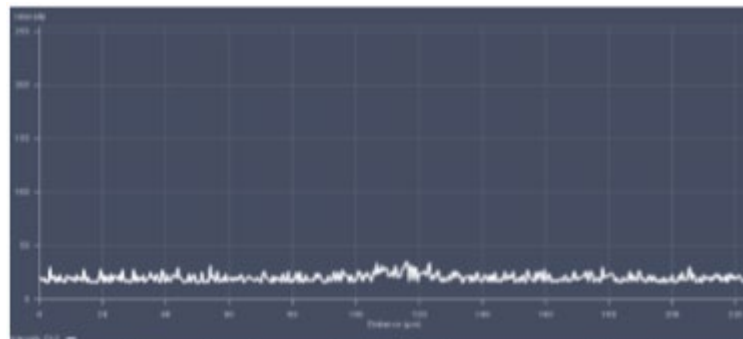

Line profile when focus is away from coverglass/sample

9. Start to move the focus up. When the first interface of the coverglass is reached, a spike in intensity will be seen. Make sure to adjust gain/laser power to avoid saturation. This interface is between the immersion water and the coverglass.
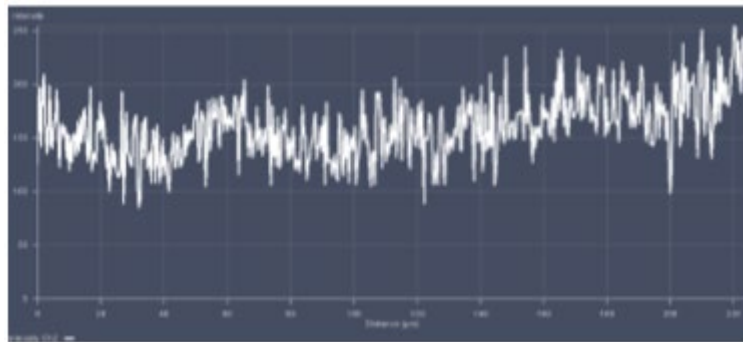
*Line profile when focus is at coverglass interface*

10. Continue to move focus up towards sample. The intensity will drop back down. The focus is now in the coverglass.

11. Move focus up slowly. Another spike in intensity will be seen at the second interface of the coverglass.

12. 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.

13. Open the focus menu. Click on *Manually* to set position to zero. Set the step size set to 200 um. Use the Step Size up arrow to move an additional 200um into the sample. The focus should now be completely in the dye solution.
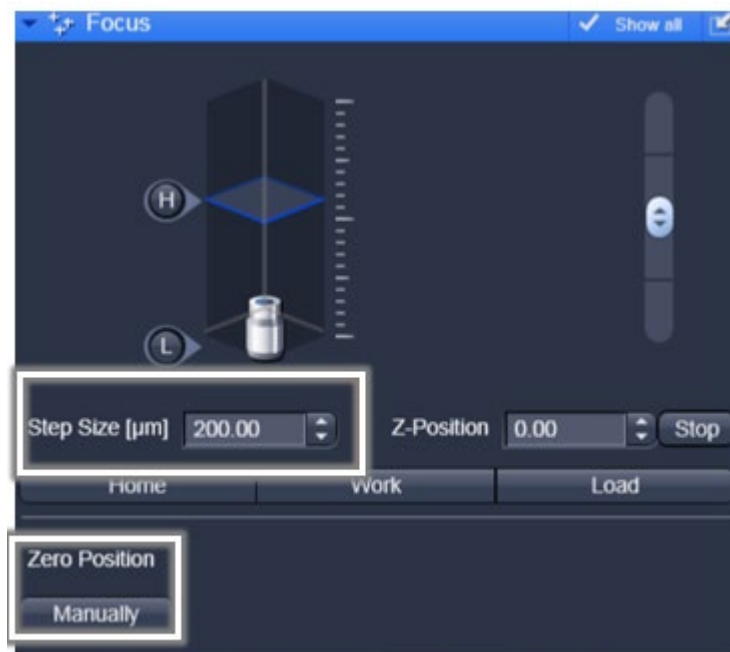
Focus adjustment and Zeroing Position

FCS Setup

1. Go to FCS tab.

2. Under *Light Path*, set up beam path configuration. You can use either ChS/Ch 2 or the BiG detectors.

3. Choose the excitation laser under the "Visible Light" button. The correct MBS for the chosen laser line can be selected from the MBS button.
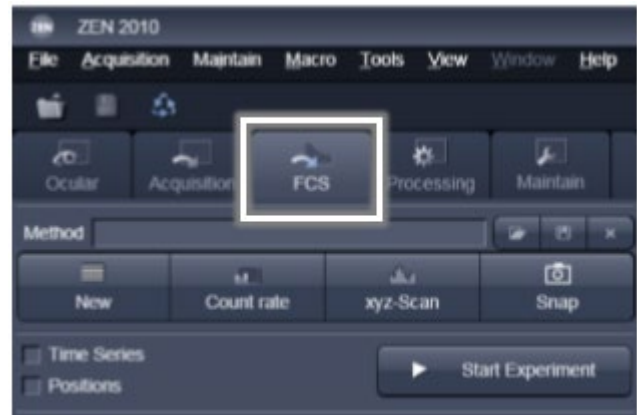
FCS tool tab

Count Rate

1. Select the count rate button to view how much fluorescence is reaching the detector based on the beam path setup and the amount of laser used.

   There are three options in the count rate menu:

   a. *Count Rate*

   b. *Correlation*

   c. *Counts per Molecule (CPM)*

Count Rate button

2. The readout of the system is displayed 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.*

Count Rate read out in KHz

3. Adjust laser power for ~*5-10 kHz* (usually ~1% laser power, acquisition tab).

4. 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.*

FCS Correlation button

5. The CPM button in the Count Rate menu signifies the quality of the sample for FCS. It is a direct measure of the amount of fluorescence per molecule the system detects (the signal-to-noise of the sample). The average CPM represents the Counts per Second per Molecule. A reasonable number for this value would be in the range of **1kHz - 15kHz**.

FCS CPM (counts per molecule) button

- *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, try to adjust the correction collar of the objective to maximize the CPM value. This process corrects for any variation in the coverglass thickness.

Pinhole Alignment

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

2. 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.

3. There are 2 options of the pinhole alignment: Coarse and Fine. Let it automatically adjust the pinhole for X and Y by first selecting Coarse for X and Y. Select Fine for X and Y after coarse adjustment.
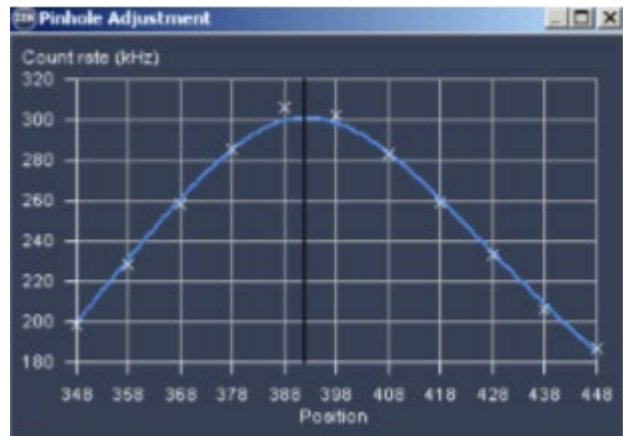
Coarse and Fine Pinhole Adjustment tool

- *Coarse* alignment will move the pinhole over the entire travel range looking for a maximum count rate value.

Coarse Adjustment Gaussian Profile

- *Fine* adjustment covers a smaller travel range with finer movement looking for the peak count rate.
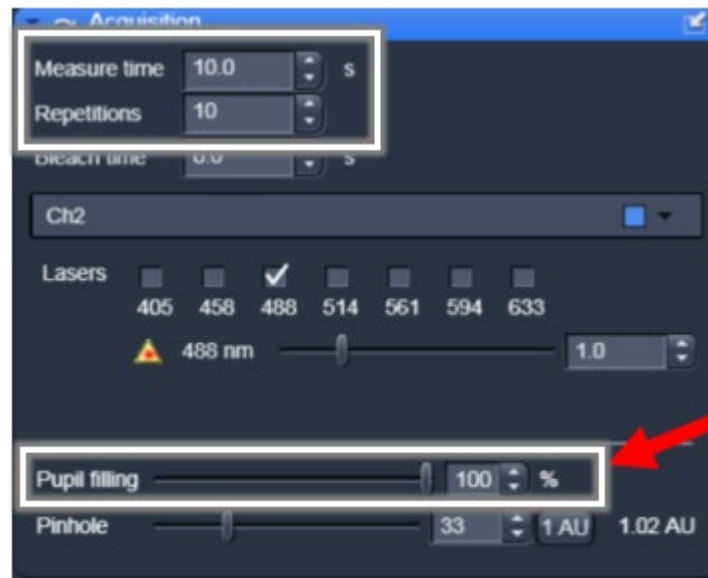

Fine Adjustment Gaussian Profile

4. Important: After the pinhole alignment has been run, check the CPM and if needed, adjust the correction collar of the objective again.
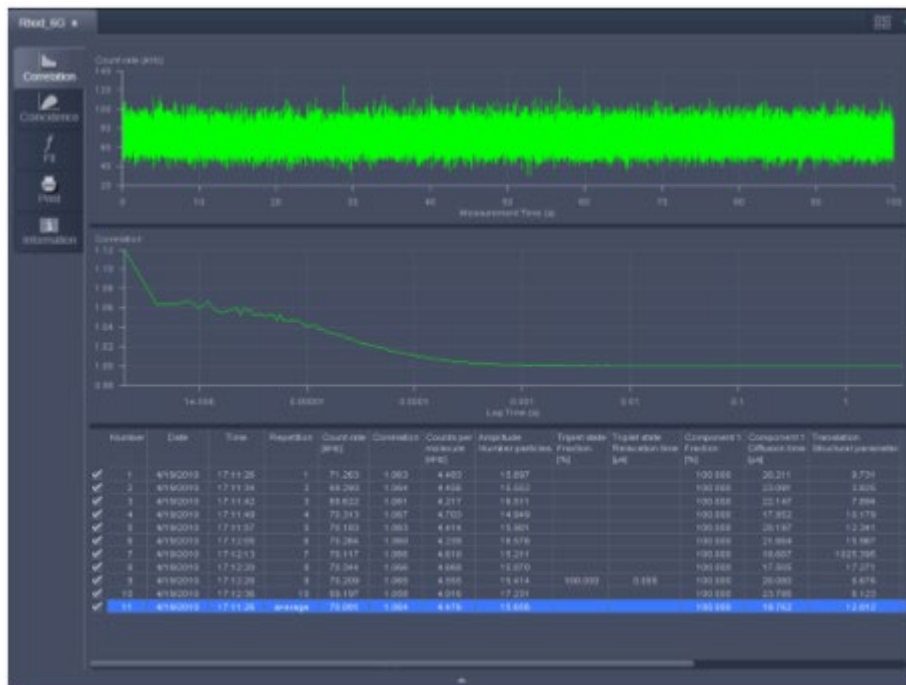
Data Acquisition

1. In the FCS *Acquisition* menu, set the measurement time for 10 sec with 10 repetitions. Leave the pupil filling at 100%.

FCS Acquisition Parameters

2. 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 and data table.


Count Rate vs Time plot

Autocorrelation Curve

Data Table

Data Fitting

1. An Average correlation curve can be created by excluding repetitions (simply uncheck the box next to the repetition). Once the average has been created, the data can be analyzed.

2. Go to *Fit* (tab on left of viewing panel). Move orange line to ~1 usec. (Afterpulsing causes artifacts below this timescale.)
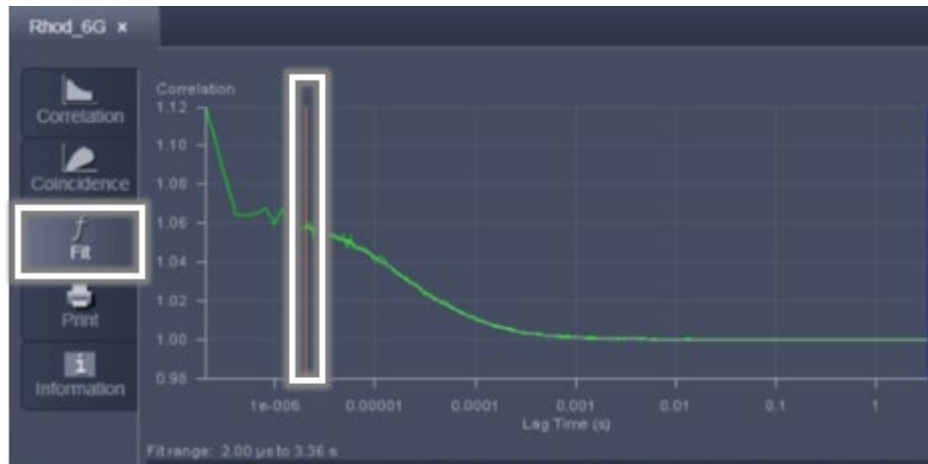
Autocorrelation Curve showing where to exclude afterpulsing effects

3. Choose your fit model (probably 1 comp) and click *Fit All*.

   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.
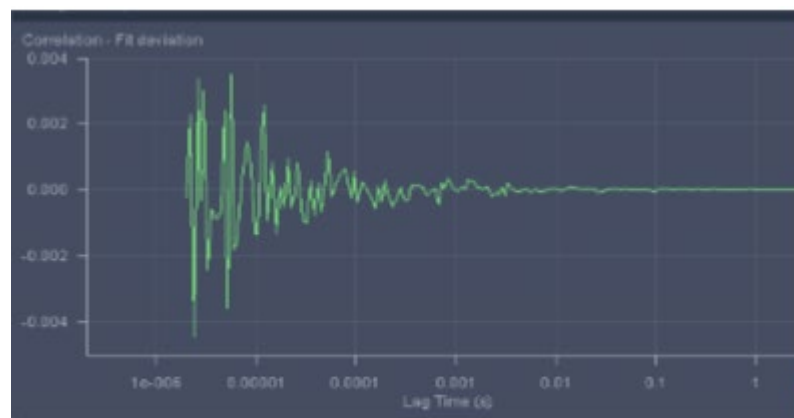
Fit deviation or Residual plot

4. Rhodamine Green should exhibit a $\tau D \sim 30$ μsec with 40x/1.2 water objective.