Single point fluctuations analysis with the LSM880 Airy detector

Pipeline for the processing of the Airy detector data for FCS, iMSD, pCF, N&B and diffusion law.

Note: in this tutorial we discuss data acquisition in the single point FCS mode of the SLM 880 with the Airy detector. RICS cannot be done with the data acquired with the Airy detector when operating in the nanocamera mode since the data are not acquired using the Raster Scan mode.

Important: The Airy detector needs to be centered before starting data acquisition. The cross correlation between channels needs to be checked at least once every several months using a Chroma slide or the Zeiss calibration objective. If there is cross correlation, the detector must be recalibrated by Zeiss technical personnel.

Data files: Open the data file, most of the time in CZI format or ome.tiff and translate into TIF. The TIF file contains 32 channels of Uint16 in a sequence. They must be converted to a single file in the format “.B64”. Routines for the conversions are available in SimFCS. If the data from the SLM 880 were stored as ome.tiff, you can use these files but first they need to be converted into B64. The ome.tiff to B64 format conversion routines are also available in SimFCS.

Convert the data to a carpet of 32 columns (corresponding to the 32 detectors of the Airy detector) and of length equal to the length of acquisition, typically about 1Gb. The data will be represented as a carpet with 32 columns and a large number or row, generally over $10^7$ rows. For data in the carpet format, all the scanning FCS routines to analyze carpets are available. For example, you could calculate the ACF at each column that corresponds to the ACF at each detector. However, for the purpose of calibration, special routines have been added to the Airy analysis page.

Detrend: The time record typically of 10-20MB (each point is acquired in 2.5μs). The input data are detrended by default using an exponential recursive filter which maintains the variance of the signal since the data from the Airy detectors have a lot of bleaching. After detrending, the data at each column are high pass filtered. The MAV default value (50000) for the exponential smoothing corresponds to a time constant of 50000*2.5us=125 ms. This value of MAV detrends for bleaching without changing very much the fluctuations due to molecules which are much faster. You can change the MAV to other values if need.

Calibration procedure for fluctuation analysis: Acquire single point FCS with the Airy detector of a suspension of beads in a concentration of about 10nM ($G(0)$ should be around $10^{-2}$)

Calibration. The purpose of the calibration is to determine the effective waist of each detector. We fit each column of the carpet of the bead data calibration as 32 single point FCS curves, keeping the D value fixed to the value of the beads used for the calibration ($D=6μm2/s$ in this example) to obtain the waist of each of the 32 detectors. The values of the 32 waists as well as the S parameters for each detector to be used for the brightness analysis are stored once forever in a calibration file in the SimFCS executable directory.

Generally we found that the waist depends on the distance of each detector from the center, which has the smallest waist (Figure 1) as expected for the Airy detector.
Figure 1. Map of the Airy detectors waist. The range is from 0.113 um to 0.166 um. The value of the G(0) decreases for the detectors further from the center, probably due to the increased waist for the detectors far from the center. The panel at right shows a typical FCS curve and the fitting using one diffusion component.

Once the waist of each detector is obtained, we can process the data for FCS, iMSD, pCF calculations and all other fluctuation calculations.

Single point FCS.

The basic operation here is to calculate the diffusion coefficient at each detector using the detector waist determined using the calibration routine.

Figure 2. In the left, the analysis of the diffusion ans G(0) is done for each detector. At right the apparent time to cross the detector is shown for each detector. Of course the data fall in a line since we use the same set of data used for the calibration of the waist.

In the FCS mode, we can exploit the different size of each detector to construct a graph of the apparent crossing time as a function of the waist^2. Of course, if we use the same calibration file we should get a perfect alignment with a slope of 4D. The value of the diffusion coefficient is calculated and displayed in the memo of the Airy analysis page. Since the line is passing through the origin, a pure diffusion model fits the data. In the next figure we use another sample of beads.
Figure 3. Analysis of a file of diffusing beads. In the right panel, the diffusion coefficient in this case is about 4.69um²/s. The number of particles is similar to the previous sample and the time vs waist-square is relatively linear with an intercept around zero. The purpose of the plot of apparent time vs waist-square is to determine the value of the intercept. The following figure from the paper cited below illustrates the principle.

Figure 4. From “Fluorescence Correlation Spectroscopy Diffusion Laws to Probe the Submicron Cell Membrane Organization” by Laure Wawrezinieck, Hervé Rigneault, Didier Marguet, Pierre-François Lenne [https://doi.org/10.1529/biophysj.105.067959]. This figure illustrates the information that can be obtained from the plot of the apparent time vs the waist-square.

How to calculate the iMSD?

The software provides a iMSD classic analysis in which the data are treated as if they were acquired with a camera. However, for the Airy detector the camera is not illuminated with the same PSF so that this analysis will give erroneous results. Instead for the Airy iMSD analysis, the detector is divided in concentric layers of detectors (3 layers), each layer is assigned a given waist and a given average distance from the center as shown in Figure 5.
Figure 5. Right panel: All detectors shown and numbered. Left panel, only the central and first layer is shown.

Overall, the detector covers about 1 Au which is on the order of 300nm so that the central detector waist is about 120nm. First we apply the pCF algorithm that calculates the cross correlation among each pair of detectors for a total of $32 \times 32 = 1024$ pair correlation function calculations. Starting from the central detector (#0 in the figure above), from the 1024 pCF’s we select all these pCF functions corresponding to a shift of 1, for example, 4 detector pairs (3,2), (0,1), (4,0) and (5,6) at 60 degrees angle and continuing with 4 pairs for each angle at 120 degrees, 180, 240 and 300 and 0 degrees, for the entire first central layer and we average all the shifts along the 6 major angles at 0 degrees (with respect to the x axis) at 60, 120, 180, 240 and 300 degrees. There are total of 24 shifts of one detector step, 4 for each direction, for all possible time delays. Next we project the 6 correlation functions obtained at each time delay along the microscope axis taking into account the positive and negative directions. From the projections along the axis we calculate the amplitude and the width of the iMSD correlation functions at each time delay using the STICS equation where the spatial averaging is done for all shifts of 1. For the time averaging we use 256 time delays calculated in a linear scale.

The plots of the variance and the amplitude of the STICS correlation as a function of time give us the kind of motion detected in the first central layer. The type of plot could be linear, confined or partially confined.

In principle we can add all shifts of 1 unit included in a second layer or to consider spatial shifts for all pairs of detectors to further extend the calculation to larger radii from the origin. However, when including one more layer, we should account for the different waist of the next concentric layer which could make the calculation rather complicated. So, we only included in the algorithm the calculation for the first layer.

In the following figure we show the result of the 256 projections of the STICS correlation function used to determine the change in the amplitude and the width of the correlation function.
**Figure 6.** Left panel: Values of the variance and 1/G0 for the STICS correlation function at one detector shift for the central corona. The squares are the result of the best fit using a linear diffusion model. Right panel: Normalized ratio between the variance in the x and y directions. For the beads files we expect that the anisotropy of the motion to be zero.

This algorithm also provides the displacement from the center and the difference between the projection along the x and y axis. These values are shown in the memo and eventually can be copied and pasted in Excel for further analysis (right panel in figure 6). This graph shows the normalized ratio (GP) of the x axis and y axis. In the case of beads we are not expecting any major difference.

In the case of beads, the 1/g0 is relatively linear as well as the variance of the Gaussian.

In the case of EGFP in cells, the confined model seems to give a much better fit of the iMSD curve for the variance while the N plot is linear.

**Figure 7.** Left panel: Values of the variance and 1/G0 for the STICS correlation function at one detector shift for the central corona. The squares are the result of the best fit using a linear diffusion model for the 1/g0 curve and the confined model for the variance curve. The values of the fit parameters (intercept, diffusion and confinement) are found in the memo. Right panel: Normalized ratio between the variance in the x and y directions. For GFP in the nucleus we found that there is very small anisotropy of the diffusion at this specific measurement point.

In the iMSD correlation function there is a spatial average. For a normal camera, we assume that each pixel is at the same distance from each other and that the PSF is constant over the surface of the
detector. This is not the case for the Airy detector. The iMSD is computed only in the inner layer made of a total of 7 detectors, which are the brightest and their waist is small and homogeneous. The option iMSD classic uses the standard algorithm implemented for large cameras. The results are not identical to the specific algorithm designed for the Airy detector.

**Diffusion laws**

These plots from the fit of the correlation functions using the iMSD equation, properly converted to displacements and time give to “law of diffusion” in the volume explored by the Airy detector. These plots are directly fit to the linear and confined model.

![Graphs showing diffusion laws](image)

**Figure 8.** Diffusion laws for beads 1 (left), beads 2 (central) and for EGFP (right) in the nucleus. In all cases the intercept is about zero indicating pure diffusion for these 3 cases.

**How to calculate the pCF for all points of the Airy detector?**

The pCF algorithm is applied to each of the 32x32 pairs of detectors for a total of 1024 pCF calculations. Before the pCFs are calculated, each column of the starting 32 intensity columns in the carpet is detrended using the exponential filter algorithm with a value of MAV that can be changed by the user. The default MAV value is 50000, which means that the exponential filter has a time constant of 125 ms for data originally at 2.5us per frame. All fluctuation faster than 125ms are retained while slow changes due to bleaching are detrended.

![Graphs showing anisotropy maps](image)

**Figure 9:** Left: diffusion anisotropy map and right anisotropy angle for the bead 1 sample.
**Figure 10:** Left: connectivity map. Right: pCF angular distribution map for the bead 1 sample.

**How to calculate N&B for the Airy detector?**

We use the algorithms developed for the carpet analysis to calculate for each column of the carpet the average intensity and the variance. Note that we use the G1 rather than the G0, which results in the subtraction of 1 from the value of B. Then we calculate B and N for each of the detector locations. Since we are using the carpet analysis page, we could generate the brightness sequence that is needed to implement the pCOMB analysis as described in Hinde E, Pandžić E, Yang Z, Ng IHW, Jans DA, Bogoyevitch MA, Gratton E, Gaus K. Quantifying the dynamics of the oligomeric transcription factor STAT3 by pair correlation of molecular brightness. Nat Commun. 2016; 7: 11047. PMCID: PMC4820838

**Figure 11.** Beads 1 sample. Left: calculation of B and N using the G1 method. There is no correction applied for the S values for each detector for this image. Center: Brightness map. Right: Number map.

Each detector must be calibrated. For this purpose we use a Chroma slide which should give the nominal value of B=1 at each detector. Below is the record for the Chroma slides
In these 3 cases the Chroma slides are reasonably close to zero as B values. These files were acquired with few frames and a better statistics will give us confidence about the calibration values to be used.