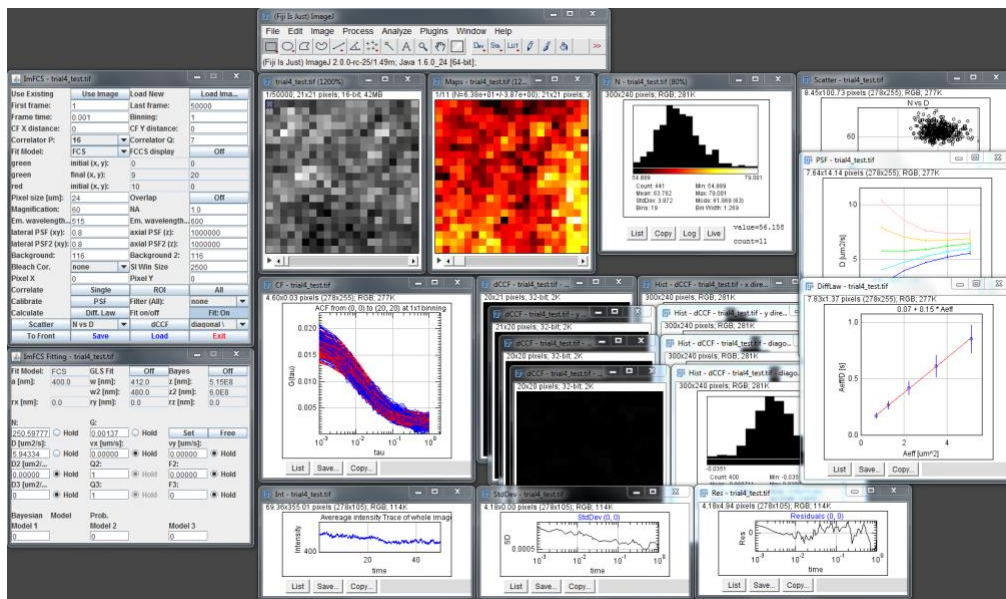


# Imaging FCS

## A plugin for ImageJ

June 26, 2022



Imaging\_FCS 1.612 is a basic ImageJ plugin to calculate and view spatio-temporal correlation functions from 16 bit GRAY TIFF STACK files. It was written under FIJI (ImageJ 1.53f; Java 1.8.0\_201) using the math library commons-math3-3.6.1 and requires ImageScience.jar for statistical functions and Apache Poi for file reading and writing. It calculates correlations for each pixel or group of pixels in the image along the time axis (which is the z-axis in the stack). It contains a wide range of functions including, bleach correction, point spread function (PSF) calibration, and calculation the mean square displacement, among others.

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## 1. Introduction<sup>1</sup>

### 1.1 What does Imaging FCS 1.612 do?

Imaging FCS 1.612 is an ImageJ plugin that allows calculating spatio-temporal auto- and cross-correlations of an image stack (16 bit gray value TIFF). It can perform the following tasks:

1. Calculation of the temporal autocorrelation function (ACF) either at each pixel (or square areas produced by  $n \times n$  binning) of an image. Or at any manually selected region of interest (ROI).
2. Calculation of the spatio-temporal cross-correlation function for any two pixels (or square areas produced by  $n \times n$  binning or manually selected ROIs) of an image. This can also be used for dual-colour fluorescence cross-correlation spectroscopy (DC-FCCS) if the image is split on the camera according to wavelength (fit models for both cases are provided).
3. Perform different bleach corrections of the data.
4. Analysis of the correlations by fitting with the appropriate fit function (1). The program can fit auto-correlations, spatial cross-correlations (diffusion and transport), and dual-colour cross-correlations. Models can include 1 triplet and up to 3 different diffusive components. We provide simple non-linear least squares or a more time consuming general least squares algorithm.
5. Calculate Model probabilities of fit functions using Bayesian Model Selection (2-4).
6. Possible fit models, with manually chosen parameters, can be visualized.
7. Depiction of parameter maps (e.g. Diffusion coefficients, number of particles, flow velocities,  $\chi^2$  values) from the fit results.
8. Creation of histograms for the fit parameters.
9. Production of scatter plots of fit parameter pairs to check for correlations and trends in the fit parameters.
10. Calibrating the point spread function (PSF) by using a freely diffusing sample (5).
11. Calculating the FCS diffusion law for an FCS image (6).
12. Produce dCCF plots (dCCF or  $\Delta$ CCF: difference between forward and backward correlation functions between neighbouring pixels; (1, 7)).
13. The mean square displacement (MSD) can be calculated from the correlation function.
14. Saving of experimental data and reading and reconstitution of experiments. Since version 1.4 the saving of the data is done in .xlsx files. Saved data of earlier versions cannot be read anymore.

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<sup>1</sup> This document does not explain the theory of correlation analysis but is only concerned with the use of the Imaging FCS plugin. For details on the theory please see the references.

15. Simulations of data in 2D and 3D are supported. Domains and meshworks can be simulated in 2D
16. Store and read configuration file for the user convenience of loading experimental parameters specific to a workflow.
17. Generate parameter video maps from an imagestack.
18. Direct access to camera for real-time Imaging FCS.

Please note, Imaging FCS does not calculate pure spatial correlations; all correlations are always performed in time.

## 1.2 GPU capability

GPU mode is introduced in Imaging FCS version 1.612, which enables computation of ACF, CCF, polynomial bleach correction, Number & Brightness analysis, diffusion law and PSF calibration to be done on an NVIDIA GPU. The code currently runs on 64-bit Windows and Linux. We tested on Windows 10, Ubuntu 18.04 and Elementary OS 5.

The Java library `gpufitImFCS.jar` must be placed in `jars` folder in `Fiji.app`. In terms of utilization, it is seamless to the user because the code will perform necessary background checks to determine if a computation can be done on a GPU. Furthermore, if an error is encountered while running a computation on GPU, the process will switch to a CPU automatically and a message is typically printed on `Fiji.app` console window for information.

We adapted and expanded the fitting code from *Gpufit: An open-source toolkit for GPU-accelerated curve fitting* (8), which is available at <https://github.com/gpufit/Gpufit>. We leveraged on this existing framework to perform fitting of polynomial bleach correction and ACF curves. Moreover, we added a CUDA kernel to perform ACF and CCF computations. The gain in computation speed comes from the parallelisation of calculations for each pixels onto GPU threads, which otherwise is done sequentially on a CPU. We consolidated the CUDA and Java codes into `agpufit.cu` and `gpufitImFCS.java` files respectively for ease of compilation and distribution. The dynamic link library (.dll) and shared object (.so) library files are generated from `agpufit.cu` file, which are bundled together with the `gpufitImFCS.jar` file so that the library can be loaded at runtime.

The bridge from Imaging FCS to CUDA code, which are written in C++, is made possible through Java Native Interface (JNI). The JNI static functions are coded in `gpufitImFCS.java`. In the Imaging FCS plugin, pixels data are passed to GPU in an 1D array in order to speed up the process. We observed that the JNI function `SetFloatArrayRegion` breaks down when the output array size is large, which is why we hardcoded a limit of 96 pixels x 96 pixels x 50000 frames for binning output size, beyond which the binning operation will be done on CPU.

Calculations are parallelized in the GPU along the pixels. The GPU kernel dimensions is set based on the dimension of output array. For example, if binning or cross correlation is needed, the output dimension is smaller than input image stack. The number of threads per block (a `dim3` parameter declared with a prefix “`blockSize`”) is set at 16x16 in `agpufit.cu` to avoid “too many resources requested

for launch” error. On the same token, please see function `Info::configure()` in `agpufit.cu` also. The number of blocks per grid (a `dim3` parameter declared with prefix “`gridSize`”) is set correspondingly.

We used `nvcc` compiler version 9.2 for compilation of CUDA codes. NVIDIA driver should be kept up to date. For Windows users, a copy of `cublas64_92.dll` is bundled in `gpufitImFCS.jar`. This cuBLAS library is essential for fitting functions to run properly, as per the documentation of Gpufit. Should you encounter an issue running the calculations on the GPU, you may try installing CUDA Toolkit 9.2 or newer, which would install necessary CUDA libraries to your computer. Error messages and warnings are printed on Fiji.app console. If an error is encountered while running on a GPU, the code will switch to the CPU to perform the calculations automatically.

Do note that `agpufit.dll` and `cublas64_92.dll` would be loaded at runtime in order for GPU codes to run on a Windows machine. For efficiency reason, a subfolder called `gpufitImFCS-cublas` would be created in Fiji.app, jars folder, where these dlls would be saved. In the event these dlls could not be saved, they will be placed in the system Temp folder whenever the plugin is launched. For Linux, the shared object library is named `libagpufit.so`. It is statically linked to `cublas_static` during compilation. Similarly, it is saved to a subfolder `gpufitImFCS-cublas` or in the system tmp folder.

The numerical calculations by CPU and GPU are similar. A comparison of computed ACF values and average diffusion coefficients for the bilayer file for example:

Settings	Largest difference in computed ACF values	Diffusion coefficient ( $\mu\text{m}^2$ )			
		Average		Standard Deviation	
		CPU	GPU	CPU	GPU
Default: <code>cfX = 0, cfY=0, binning = 0x0, bleach correction = none</code>	$8.722 \times 10^{-9}$	0.4094	0.4094	0.1994	0.1994
<code>cfX = 1, cfY = 1, binning = 2x2, polynomial bleach correction = 4</code>	$2.064 \times 10^{-6}$	0.6715	0.6716	0.3105	0.3093
<code>cfX = 0, cfY = 0, binning = 5x5, polynomial bleach correction = 6</code>	$1.922 \times 10^{-6}$	0.9711	0.9657	0.4311	0.4256

A comparison between the execution times for files of different sizes in the CPU and GPU is provided below. The files had already been loaded in ImFCS using the “load” button before measuring the execution time in the CPU and GPU. The tests are done on a computer operating Windows 10 v18.09 with Intel Core i7-7800X CPU @ 3.50 GHz, 32GB of RAM and a NVIDIA Titan XP GPU of 12GB of memory.

The execution time taken to calculate the autocorrelations, fit the autocorrelations and display the parameteric maps:

Number of Pixels	Execution time [s]	
	CPU*	GPU*
4	$0.5 \pm 0.05$	$1.7 \pm 0.2$

441	$26.7 \pm 0.2$	$4.3 \pm 0.4$
3600	$249.4 \pm 4.6$	$17.0 \pm 0.4$
8100	$579.2 \pm 3.4$	$19.4 \pm 0.6$
16384	$1186.1 \pm 180.5$	$36.5 \pm 0.9$

\*The files were analysed 5 times to obtain the mean execution time along with its standard deviation.

The execution time taken to calculate diffusion law:

Number of Pixels	Execution time [s]	
	CPU <sup>#</sup>	GPU <sup>#</sup>
100	$13.9 \pm 0.3$	$15.85 \pm 0.02$
3136	$539.6 \pm 1.4$	$68.4 \pm 0.4$
6384	$1051.8 \pm 10.1$	$123.0 \pm 0.5$

The execution time taken to calculate N&B:

Number of Pixels	Execution time [s]	
	CPU <sup>#</sup>	GPU <sup>#</sup>
3136	$39.65 \pm 0.06$	$9.20 \pm 0.02$
6384	$81.4 \pm 0.1$	$17.8 \pm 0.1$

<sup>#</sup> The files were analysed 3 times to obtain the mean execution time along with its standard deviation.

### 1.3 How to install the plugin?

Imaging FCS works under ImageJ, FIJI, or Micromanager. In the rest of the document we refer only to FIJI as Imaging FCS has been written under FIJI. However, the same applies to ImageJ and Micromanager.

#### 1.3.1 Installation from the ImageJ update site

The easiest way to install Imaging\_FCS\_1\_612 is by using the ImageJ update site. In ImageJ chose *Help->Update*. This opens the “ImageJ Updater” window. Click on *Manage update sites*. To open the “Manage update sites” window. Please tick both “Image Science” and “ImagingFCS”. Then close the “Manage update site” window and click “Apply Changes” in the “ImageJ Updater” window.

Please note that the update site will also upload the latest Apache POI files necessary for writing Excel files.

### 1.3.2 Manual Installation

1. You need a functional installation of ImageJ, Fiji, or Micromanager (the plugin was compiled under ImageJ 1.52n, Java 1.8.0\_201 (64-bit), using Apache Commons “commons-math3-3.6.1” math library<sup>2</sup>).
2. You need to download and install Apache POI in the jars folder of Fiji (<https://poi.apache.org/download.html>). At the time of writing “poi-3.17” was the latest stable release. However, we had tested versions between 3.11 – 3.17 and all worked equally well. These files are required for writing and reading .xlsx files.
3. You need the Imaging\_FCS\_1\_612.jar. Place the .jar files to plugins folders in Fiji.app.
4. We provide the Java source file for your information. However, as Fiji updates relatively fast as the migration to ImageJ2 is happening, there can be compilation problems. But the .jar file should work. So, we suggest using the .jar file.
5. We provide a 21x21 pixels test file measured on a fluorescently labelled lipid bilayer, which contains 20,000 frames and was recorded with 1 ms time resolution.
6. If there are problems with loading/using images from the plugin, please switch SCIFIO off (see section 14. Known Issues).

To start Imaging FCS, install Imaging\_FCS\_1\_612.jar in the “plugins” folder of FIJI. On PCs you find this under “Fiji.app/plugins”. On Macs, go to the application folder and “control-click” the Fiji application. Select “Show package contents” and copy the Imaging\_FCS\_1\_612.jar to the “plugins” folder. Then launch Fiji. You can start Imaging FCS under the “Plugins” tab in FIJI.

### 1.3.3 Compiling the files yourself

Other ways to start Imaging FCS: You can compile the Java source file yourself using the following steps.

To compile the CUDA code, you will need CUDA Toolkit 9.2 or newer installed. Identify the CUDA library is located for instance, in C:\Program Files. Do check that your GPU driver is compatible with the CUDA Toolkit version that is used. nvcc compiler is used to compile the agpufit.cu file to agpufit.dll or libagpufit.so for Windows and Linux OS respectively. Java Development Kit 8 (JDK) is also required. The path variable JAVA\_HOME points to the location of this Java JDK. You can specify the NVIDIA GPU architecture for which the code is compiled by using the flag gencode. You can choose to make use of cuBLAS library, which helps speeding up the fitting process, by including the flag -D USE\_CUBLAS, the file path to the cuBLAS library and flag -lcublas.

- a) For Windows, launch Command Prompt, navigate to the location of agpufit.cu and enter the following command:

```
nvcc agpufitjni.cu -O3 -D USE_CUBLAS -rdc true -shared -o agpufit.dll -gencode
arch=compute_35,code=[sm_35,compute_35] -I"%JAVA_HOME%/include" -
```

---

<sup>2</sup> Note, if you want to compile the program yourself from the Java source code you will need to install “commons-math3-3.6.1” in the jars folder of Fiji (Fiji.app\jars) and possibly remove the older “commons-math3-3.2”. You can download this jar file at [http://commons.apache.org/proper/commons-math/download\\_math.cgi](http://commons.apache.org/proper/commons-math/download_math.cgi). In addition, you will need to install the Apache POI poi-3.15 in the jars folder of Fiji. Poi-3.15 can be downloaded at <https://poi.apache.org/download.html>.

```
I"%JAVA_HOME%/include/win32" -I"C:\Program Files\NVIDIA GPU Computing
Toolkit\CUDA\v9.2\include" -lcublas
```

- b) For Linux, launch Terminal, navigate to location of agpufit.cu and enter either of the following command:

```
nvcc agpufitjni.cu -O3 -D USE_CUBLAS -rdc true -shared --compiler-options -Wall --
compiler-options -fPIC -o libagpufit.so -gencode
arch=compute_35,code=[sm_35,compute_35] -I"$JAVA_HOME/include" -
I"$JAVA_HOME/include/linux" -L"/usr/lib/x86_64-linux-gnu" -lcublas_static -lcublas
```

To compile Direct Camera Readout C++ source code, Java Development Kit 8 (JDK) is required to generate C++ header file from AndorSDK2v3.java, AndorSDK3v2.java, Hamamatsu\_DCAM\_SDK4.java, Photometrics\_PVCAM\_SDK.java. Subsequently, generate 4 cameras specific .dll (we recommend Visual Studio 2019 IDE).

To compile the program in its entirety, we need Java Development Kit 8. Let “builddir#” be the location where .class file will be placed, “sourcedir#” will be the location where corresponding .java will be placed and “outdir” be the output directory where Imaging\_FCS\_1\_612.jar will be placed. There are 5 such java files: GpufitImFCS.java, AndorSDK2v3.java, AndorSDK3v2.java, Hamamatsu\_DCAM\_SDK4.java, and Photometrics\_PVCAM\_SDK.java

- 1) To compile GpufitImFCS.java to GpufitImFCS.class:  

```
javac -d “builddir1” “sourcedir1”\GpufitImFCS.java
```
- 2) Repeat for 4 others .java
- 3) Copy corresponding .dll and/or .so to “builddir1”, “builddir2”, ... “builddir5” respectively.
- 4) Compile Imaging\_FCS\_1\_612.java to generate Imaging\_FCS\_1\_612.jar, run:  

```
jar cf “outdir”\Imaging_FCS_1_612.jar -C “builddir1”; “builddir2”; “builddir3”;
“builddir4”; “builddir5”;
```

Repeat only step 4) for changes to source code other than GPU and Direct Camera Readout related.

You can also use a Java IDE (e.g. Netbeans, Eclipse, JSource etc.; we tried only Netbeans up to now but that worked fine).

**Note:** Do not install the .jar file and try running the Java file in Fiji at the same time. This can lead to some errors in the execution. We suggest to just using the .jar file.



## 1.4 Starting the plugin and loading images

After you started the program, you will see the ImFCS control panel:

Image		Use	Load	Batch
First frame:	1	Last frame:	30000	
Frame time:	0.00106	Exp Set	Write Conf	
CCF distance:	0 x 0	Binning	1 x 1	
Correlator P:	16	Correlator Q:	8	
Fit Model:	ITIR-FCS (2...	FCCS Disp Off	Overlap Off	
Bgr NUM	90	Background 2:	90	
LiveReadout	PVideo			
Options	N&B Off	Threshold	Average	
Scatter	N vs D	Bleach Cor	none	▼
dCCF	x direction	Filter (All):	none	▼
PSF	Diff. Law	Fit Off	All	
Sim Off	Res. Table	MSD Off	ROI	
To Front	Save	Read	Exit	

**Fig. 1.1:** ImFCS Panel. The description of all parameters is given in Annex 1 “Control Panel ‘ImFCS’ Elements”. Note that the title of the control panel contains also the name of the image (here “trial4\_test.tif”) which was used at that moment by the plugin.

### 1.4.1 Loading an Image

Click the “Load” button on the upper right. A dialogue will appear that will let you load any image stack. Make sure the image stack you load is a 16 bit GRAY TIFF STACK file.

### 1.4.2 Use an existing Image

In case the image stack you want to analyse is already open in FIJI, click on that window to make sure it is the uppermost window and then click the “Use” button. The image will then be resized and placed along the ImFCS control panel for further use.

### 1.4.3 Required Parameters

Experimental Settings			
Pixel size [um]:	24		
Magnification:	100	NA	1.45
$\lambda_1$ [nm]:	583	$\lambda_2$ [nm]:	600
PSF (xy):	0.8	PSF (z):	1000000
PSF2 (xy):	0.8	PSF2 (z):	1000000
Resulting Fit Parameters			
a [nm]:	240.00	w [nm]:	321.66
z [nm]:	402068965.52	w2 [nm]:	331.03
z2 [nm]:	413793103.45	rx [nm]:	0.00
ry [nm]:	0.00	rz [nm]:	0.00

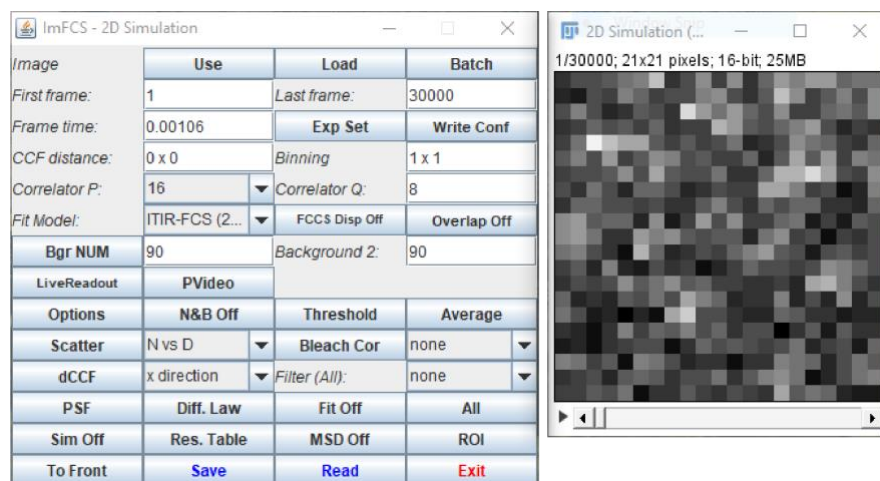
**Fig. 1.2:** Experimental Settings Panel. The description of parameters pertinent for fitting curve fitting.

The plugin reads in the basic information from the file, including the number of frames, dimensions of the frames (not shown in the plugin but shown by ImageJ), background value (the lowest counts found in the entire stack; for other correction see chapter 9 for number and brightness analysis). However, experimental parameters have to be properly entered for the fit information to make sense. This

includes the following: *Frame time*, *Pixel Size*, *Magnification*, *NA*, *Emission wavelength*, *lateral PSF*, *axial PSF*. The definition of all these parameters is given in Annex 1. In the case of two-colour measurements, *Emission wavelength 2*, *lateral PSF 2*, *axial PSF 2* have also to be provided. The values for the axial PSF can be obtained by measuring the light sheet thickness ( $1/e^2$  value, see Annex 1). The lateral PSF can be obtained by a calibration measurement and using the *Calibrate PSF* function of the plugin (see section 5). Toggle “*Exp Set*” to make changes to *Pixel size*, *Magnification*, *NA*, *Emission wavelength*, and *PSFs*.

## 1.5 Starting multiple instances of the plugin

Fiji allows running multiple instances of the same plugin. This means one can run the Imaging FCS plugin several times and in each use a different image. To make differentiating between the windows possible, each window of the plugin carries its title plus the title of the image loaded. E.g., in Fig. 1.1 the control panel is called “ImFCS”. In Fig. 1.3, the name “trial4\_test.tif” has been added to yield “ImFCS – trial4\_test.tif” to indicate that the control panel uses the image stack “trial4\_test.tif”. The same will be true for all other windows (correlation functions, parameter maps etc.). The “*To Front*” button of the plugin allows bringing all windows connected to that panel to the front.



**Fig. 1.3:** After selecting a file with “Use Image” or “Load image”, the selected image is adjusted in size and placed alongside the ImFCS Panel. The ImFCS panel is renamed to contain the image name.

## 2. Calculating Correlations

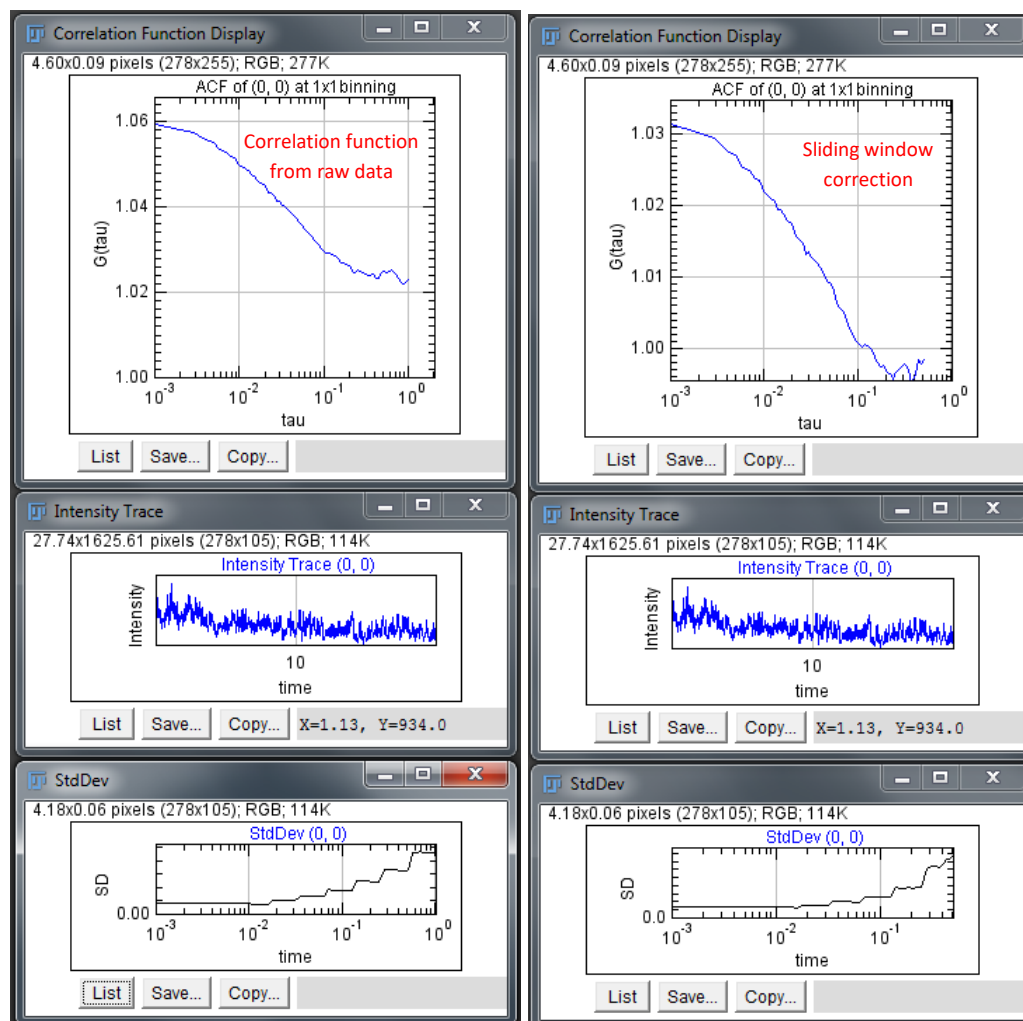
### 2.1 Performing individual correlation analysis

After the image stack is loaded, perform one of the following two actions to calculate the correlations as specified within the Control panel (for details on the different options see below):

1. Use the mouse to click any pixel within the image and the program will calculate and depict the correlation, standard deviation, and intensity trace.
2. If you have a number pad, you can use the “2”, “4”, “6”, and “8” keys to move within the image (the number or cursor keys don’t work as they have already other uses in FIJI).

You should see the correlation and intensity trace windows as on the left in Fig. 2.1. In addition, the standard deviation (SD) of the autocorrelation function is depicted. As the ACF is the mean value of

many individual intensity/photon count products, the standard deviation of the ACF is calculated as the standard error of the mean of these products (9) and is then corrected by a blocking procedure (3).



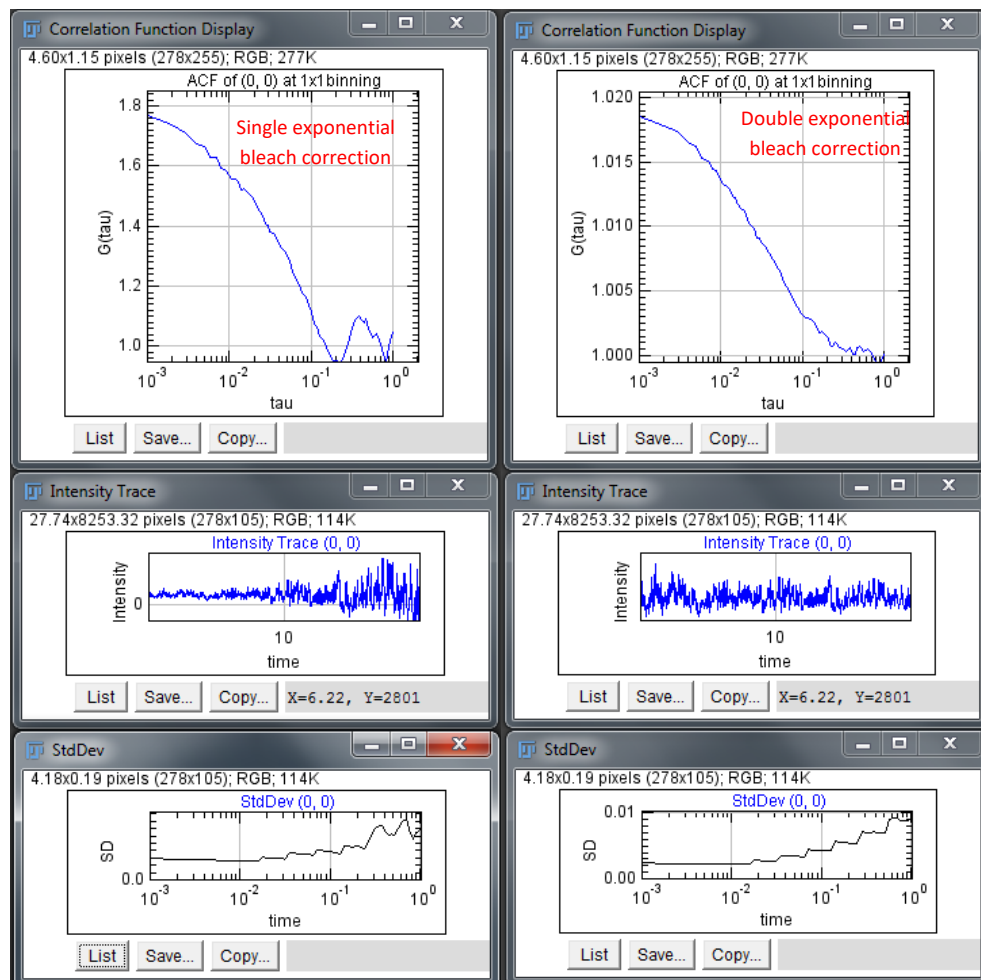
**Fig. 2.1:** Correlation functions and intensity trace. On the left the correlation is performed on the raw data. On the right side a “sliding window” bleach correction has been applied. Note that the intensity trace is the same (for more information see the text). The standard deviation in the lower panels is the standard deviation calculated for the correlation functions.

If bleaching is present, the data can be corrected by one of four methods.

- 1) Either a sliding window approach can be used. In this case, the total intensity trace for each pixel is divided into subsets. The correlation is then performed on each subset and the results are averaged. Note, that this will reduce the overall time available for correlations (the correlator P and Q values are automatically adjusted by the program) and increase the variance especially at long times making fitting of slow particles and possibly  $G_{\infty}$ , i.e. the correlation value at long times, more difficult. As default, the traces are divided into 20 subsets. However, the program will provide a general dialog when choosing *Sliding Window* as bleach correction, asking for the sliding window size. The panel on the right in Fig. 3 shows the ACF of the left panel after a sliding window

bleach correction. Note that the SD changes accordingly. However, the intensity trace stays the same as it is not corrected.

**Note:** If the sliding window is narrow and cannot cover enough time to allow the calculation of a full correlation function, then the results of the fits will be biased (6).



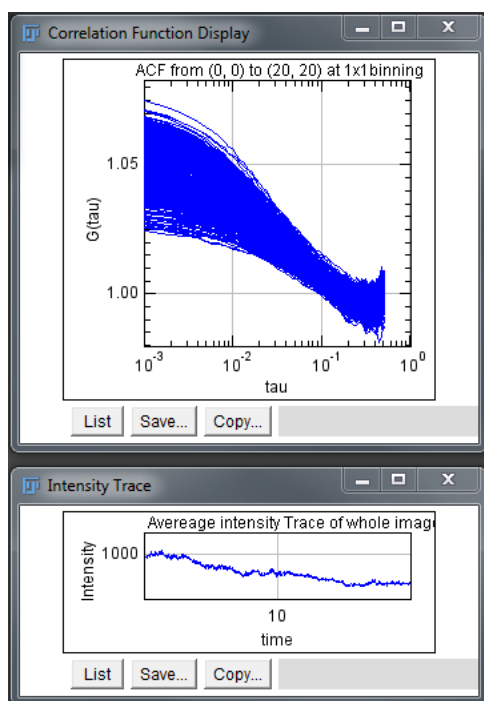
**Fig. 2.2:** Single and double exponential bleach corrections. The data is the same as in Fig. 3 for comparison of the three bleach correction approaches.

- 2) In addition to the sliding window approach, the data can also be corrected by a single or double exponential fit to the intensity trace (see Fig. 2.2). In that case the intensity trace is corrected by a single or double exponential fit, which is reflected in the change of the intensity trace plot. The data chosen for Fig. 2.2 is the same as for Fig. 2.1 for better comparison.
- 3) The third method is fitting the data by a polynomial fit. When this is selected, then the user is asked for the degree of the polynomial. In our experience a degree in the range of 4 to 8 is sufficient for bleach correction. The polynomial degree should not be selected too high as otherwise the correction can influence also the fast fluctuations which are investigated and the resulting diffusion coefficients will be biased.
- 4) The last method is correction by line segments. In this case the user will be asked for a window length. The intensity trace will be divided in stretches of that window length and the intensity will be corrected with a liner fit.

Except the exponential corrections, all other methods can take account of general slow fluctuations, and not only bleach decays. The sliding window approach, however, as it uses subsets of the total measurement can lead to distortions and biases of results (10) if the subsets are too small. So sliding window approaches should be used for long data sets and for sets which show changes other than just bleaching. The polynomial and line segment corrections are the most widely used approaches at the moment, especially when fluctuations are seen that are not only due to photobleaching (11).

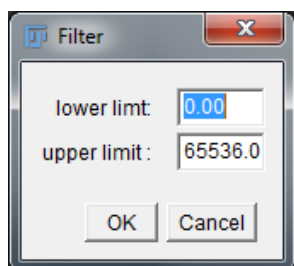
### 2.3 Performing analysis of regions of interest or the whole image

To calculate all correlations simultaneously click the button “All” on the lower right. It will take ~20 seconds to calculate all correlations for a 20x20 image. Larger or smaller images will take less or more time accordingly. Note that now the intensity trace shows the average intensity of the whole image:



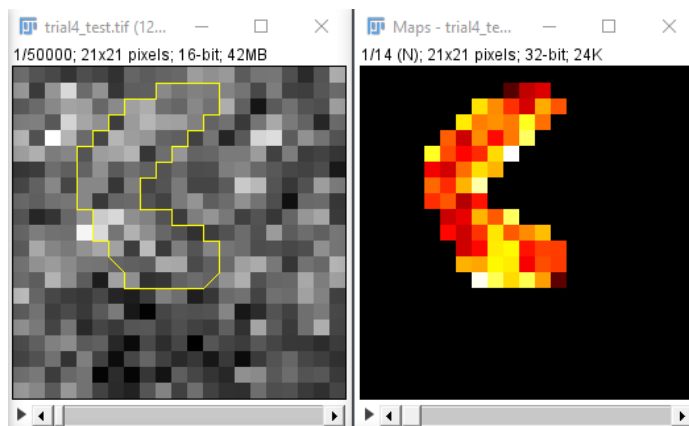
**Fig. 2.3:** All correlations calculated for a 21x21 pixels image. Here a sliding window bleach correction was used. It took ~ 8 seconds for the calculation of all ACFs. The intensity trace is the average intensity for the whole image.

Below the “All” button you can choose a possible filter. The default is “none”. If you choose “Intensity” or “Mean”, you will be asked to provide upper and lower thresholds for the intensity (of the first frame) or for the mean intensity value. The filter dialogue will pop up each time you press the “All” button (Fig. 2.4). Only pixels whose intensity/mean intensity falls between these thresholds will be



**Fig. 2.4:** Selection of lower and upper threshold for pixel intensities/mean intensities as a selection filter for which pixels ought to be correlated.

correlated. This feature is useful if one wants to avoid fitting background pixels. This speeds up the calculations and reduces fitting problems as background pixels have no correlations and the fit will return meaningless values (and sometimes can hang the program for longer times).



**Fig. 2.5:** Correlations and parameter map calculated for an arbitrary ROI within a 21x21 pixels image.

The same procedure can be applied to regions of interest (ROIs). Just select a ROI in the image by using the ROI tools of FIJI and then press the button “ROI” (Fig. 2.5). Filters can be set in the same way as for the “All” button.

## 2.4 Average correlation functions

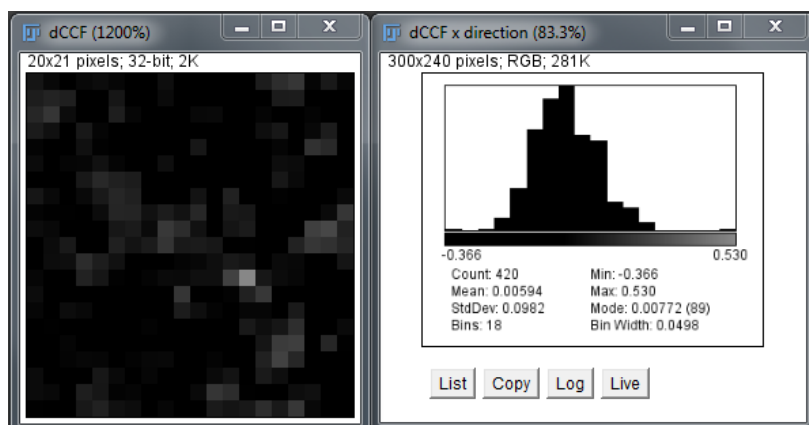
When pressing the button “Average” on the main panel, the average correlation function of all valid pixels will be calculated. Valid pixels can mean two different things. First, if “fit” is switched off, then all pixels that were correlated are valid, others that were not correlated by the user are invalid. Second, if “fit” is switched on, then only those pixels are valid that had a converged fit and which fall within the thresholds, if any were set, are valid. An individual pixel can be set to invalid status if it is double clicked in the parameter map window. If “fit” is switched on, the average FCS is also fitted. The fit results are shown in the fit window. The average correlation function is plotted in the Correlation Function plot window.

## 2.5 Difference in cross-correlation functions ( $\Delta CCF$ or dCCF)

The difference in cross-correlation functions ( $\Delta CCF$  or dCCF) is the integrated difference between the forward and backward correlation functions between two spatially different areas. It contains information about active transport of molecules and sample heterogeneity (1, 5, 7):

$$\Delta CCF = \int_0^T (CCF_{AB}(\tau) - CCF_{BA}(\tau)) d\tau$$

In the case of diffusion the mean value of the dCCF is close to 0. In the case of flow or active transport the dCCF is positive or negative depending on the dCCF direction and the flow/transport direction. The width of the distribution is a measure of the heterogeneity.

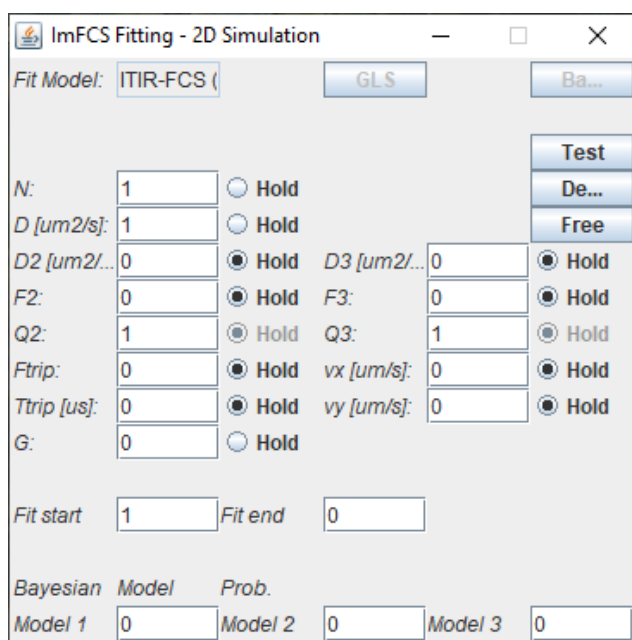


**Fig. 2.5:** dCCF image calculated in the x direction. Note that the dimension of the original image was 21x21 pixels. But as the dCCF has been calculated in the x direction it contains now 20x21 pixels only. On the right side the dCCF histogram is shown.

### 3. Data Fitting

#### 3.1 The Fit panel

To fit the data press the “Fit” button. This is a toggle button that switches data fitting on or off. When switched on a Fit parameter window is shown which carries the title “ImFCS Fitting”:



**Fig. 3.1:** Fit parameter window “ImFCS Fitting”.

There are now two possibilities for fitting data. One can use either a weighted non-linear least squares (NLS) fit or a generalized least squares (GLS) fit. One can choose between the two by switching the GLS button on or off.

The NLS fit is a weighted fit, where the weight is given by the standard deviation (SD) of the correlation functions. The SD is now calculated by the blocking transform (3). For the GLS fit the covariance matrix is calculated from the blocked correlation data and is regularized ((3) and references therein).

If the button “*Bayes*” is switched on then the program fits a one- and a two-component model to the data and calculates the model probabilities of each model. The results will be given in the Bayesian Model Probability section at the bottom.

The “*Test*” button allows to draw a theoretical correlation function with the parameters as provided in the fit window. So one can adjust these parameters manually and see how they change the correlations function.

We have introduced a “*Free/Fixed*” toggle button. The *Fixed* selection sets the current fit parameters as fixed and all fits always start with these starting values. Each time toggle button is set to *Fixed* the fit parameters are set new to the current values. To undo that the “*Free/Fixed*” button can be clicked. If it is set to free, then each fit starts with the resulting fit parameters from the last fit. This is useful where the correlation functions have a trend to shift over the image so that the first and last pixel have very different values but there is a smooth transition for the pixels in between

Note that the values for  $a$ ,  $w$ ,  $w2$ ,  $z$ ,  $z2$ ,  $rx$ ,  $ry$  and  $rz$  are calculated from the parameters given in the ImFCS Panel and they cannot be edited by the user. If these parameters need to be changed, change the parent parameters (*Pixel size*, *magnification*, *PSF (xy)*, *PSF2 (xy)*, *PSF (z)*, *PSF2 (z)*,  $\lambda_1$ ,  $\lambda_2$ , *NA*, *binning*, *CF X distance*, *CF Y distance*) in the ImFCS Panel.

The definition of these parameters are explained here:

**Fit Model:** This can be either “FCS” or “DC-FCCS”

**GLS Fit:** Switch between weighted NLS or GLS fits.

**a [nm]:** pixel size in image space, defined as

$$\text{Pixel size/magnification}$$

**w [nm] and w2 [nm]:**  $1/e^2$  radius of the lateral PSF. This is calculated from

$$PSF(xy) * \lambda_1 / NA \quad \text{or} \quad PSF2(xy) * \lambda_2 / NA$$

**z [nm] and z2 [nm]:**  $1/e^2$  radius of the light sheet thickness profile (this is typically measured and has to be input into the program as the *PSF (z)* or *PSF2 (z)* value).

$$PSF(z) * \lambda_1 / NA \text{ or } PSF2(z) * \lambda_2 / NA$$

**rx [nm]:** Distance in x direction between pixels to be correlated. This is calculated from

$$CF X distance * Pixel size * binning / magnification$$

**ry [nm]:** Distance in y direction between pixels to be correlated. This is calculated from

$$CF Y distance * Pixel size * binning / magnification$$

**rz [nm]:** This is a possible shift of the two light sheets against each other in DC-FCCS. This is qualitatively different from the  $rx$  and  $ry$  values which just describe the distance of the pixels to be correlated.  $rz$  accounts for a possible misalignment of the light sheets in z-direction.



### Fit parameters:

All fit parameters can be either free or fixed (see the radio buttons called “hold”). By setting certain parameters to 0 and clicking hold at the same time, one can use different fit models (diffusion only, flow only, diffusion and flow, one component, two components etc.).

**N:** Number of particles in observation area or volume. Note that this parameter makes no strict sense in terms of particle numbers for spatial cross-correlations.

**G:** Convergence value of the correlation function for long times. This is expected to be 1. Deviations from 1 can indicate problems with bleaching. After bleach correction this values should be close to 1.

**D, D2, D3:** Diffusion coefficients of species 1, 2 and 3.

**vx** and **vy:** flow speeds in x and y directions. If no flow is to be fitted, they can be set to 0 and put on hold. Note, at the moment only one flow component (vx, vy) is allowed. So it is assumed that even if several particle species are present, they can have different diffusion but have the same flow.

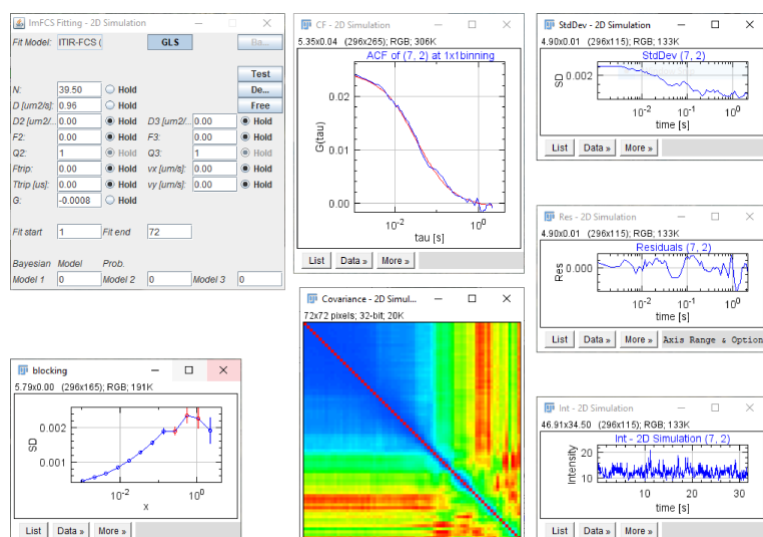
**F2, F3:** Fraction of species 2 or 3 if present; one can set these selectively to 0 and put on hold for one-, two-, or three-component fits. The fraction of species 1 is  $F1 = 1 - F2 - F3$ .

**Q2, Q3:** This is the brightness of the second and third particle in respect to the first particle. For a proper fit this needs to be known and the correct values have to be entered here. E.g. if the second particle is 50% brighter, this value has to be set to 1.5. If the value is set incorrectly the fit will give wrong values for F2. The same is true for Q3 and F3. If one of the components is set to 0 ( $F = 0$  and put on hold) then the corresponding Q parameter has no influence.

**Ftrip:** Fraction of molecules in a triplet state.

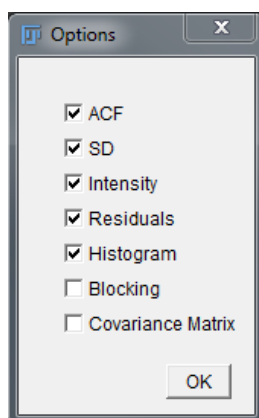
**Ttrip:** Characteristic time of the triplet.

After the parameters have been set one can then click on any pixel in the window to obtain the correlations and the fit. When a fit is performed, the residuals will be plotted in an additional window below the intensity trace window. After fitting the function for one pixel you should see any of the following windows:



**Fig. 3.2:** Data fit. The program shows the fit with the correlation function in the “Correlation Function Display” and creates plots for the standard deviation of the ACF, residuals, intensity trace, blocking results and covariance matrix. Note that the blocking and covariance matrix are not shown by default but have to be selected under “Options”. The covariance matrix is only calculated for GLS fits

Which of these windows are actually shown depends on the selection in “*Options*” in the main panel. By default the ACF, SD, Residuals, and Intensity trace are shown. But the blocking and covariance matrix (only for GLS fits) can be shown. To choose options press the “*Options*” button in the main panel:



**Fig. 3.3:** Options window to select which data to show.

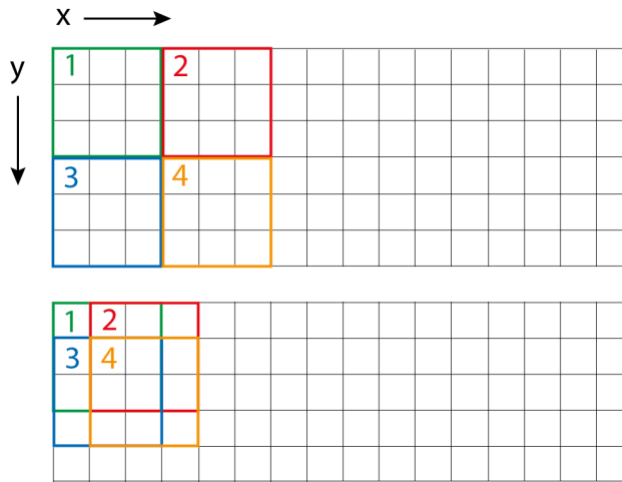
In addition, a new stack window with a parameter image map will be opened as described in the next section.

If the “*ROI*” or “*All*” button is pressed all relevant pixels are correlated and then fitted. Which pixels are fitted depends on binning and whether one uses autocorrelations, spatial cross-correlations, or dual-colour cross-correlations, and whether overlap is switched on or off. It is important that before pressing the “*ROI*” or “*All*” button you check that you have sensible fit parameters in the Fit panel as they will be used as starting values. If the parameters are too far off, fitting can take longer and might result in many pixels without fits as fits might not converge. If fits don’t converge a log window will be opened with a message giving the pixel at which problems were encountered and what the problem was<sup>3</sup>. For membrane diffusion, a good start is mostly  $N = 1$ ,  $D = 1 \mu\text{m}^2\text{s}^{-1}$ , and  $G = 0$  (all three free) and all other values 0 and on hold. You can refine models and parameters subsequently. The correlation and fitting on CPU takes about 40 seconds for a 20x20 pixel image with 50,000 frames (MacAir 1.7 GHz Intel Core i5 or PC Intel i7-3770 3.4 GHz, 64 bit, 16 MB RAM).

### 3.2 Binning and Overlap mode

When binning is used,  $n \times n$  pixels will be added together before the correlation is calculated. Binning can be used to provide better signal in some cases. In the case of binning the user can decide between two modes, overlap and non-overlap by selecting the Overlap On/Off button. In overlap mode neighbouring pixels will overlap while in the non-overlap mode only non-overlapping pixels will be calculated and correlated. The advantage of the overlap mode is that it provides more correlation functions and that correlation functions can be calculated for any position and any binning on the image. The non-overlap mode always starts with the left upper most image point and thus allows only certain pixels to be defined. At the moment the program allows only square binning, i.e.  $n \times n$  binning.

<sup>3</sup> We have limited iterations and evaluations to 2000 in the program as that was a good compromise and reaching fits for the most number of pixels without taking too much time for the fit. This can be changed in the Java file by changing the variables “fitMaxIterations” And “fitMaxEvaluations”.



**Fig. 3.3:** Explanation of Overlap mode. In the upper panel four pixels for 3×3 binning in non-overlap mode are shown. In the lower panel the 4 pixels are shown in overlap mode.

### 3.3 Parameter Maps and the Histogram Window

If fit is selected and a correlation is calculated a new stack window with a parameter image map will be opened and a histogram window for the parameters will be created. The parameter map window is a new stack window that contains as many maps as there are parameters plus a map of the  $\chi^2$  values for all fits, and an additional map indicating whether blocking was successful for the calculation of the standard deviation (3)<sup>4</sup>. Using the scrollbar at the bottom one can switch between the different parameter maps. In the histogram window the corresponding histogram to the selected parameter map is shown. However, histograms are shown only for the free fit parameters (including  $\chi^2$ ). The number of bins ( $n_{bins}$ ) in the histogram are calculated by the Freedman-Diaconis rule (12):

$$n_{bins} = \frac{\sqrt[3]{n_{points}}(Max - Min)}{2 IQR}$$

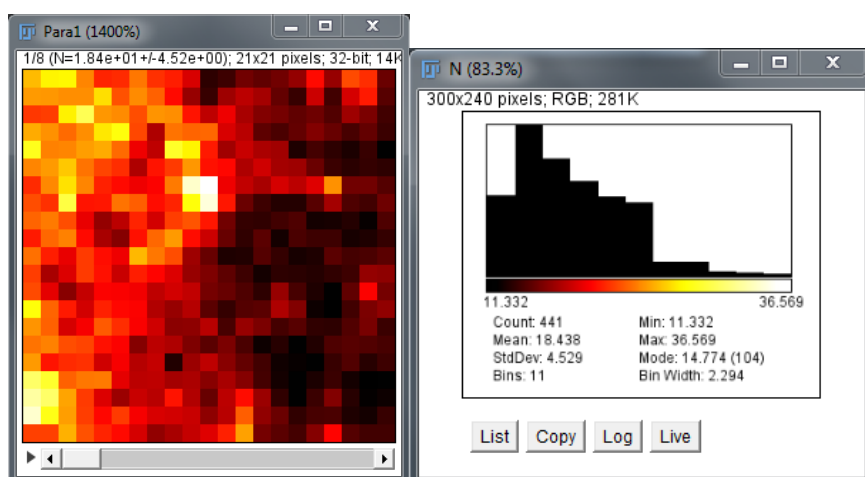
Where the number of points are given by  $n_{points}$ ,  $Max$  and  $Min$  are the maximum and minimum values, and  $IQR$  is the interquartile range.

Here is the map for the number of particles  $N$  shown (in this case all correlation functions have been fitted to show a full map). The title contains the name of the parameter plus its mean and standard deviation.

If single fits are performed, the data of these single fits will be updated in these maps. This is useful if some of the batch fits are not good and have to be individually refitted.

**Note:** If the fit parameters for one of the pixels are required, click on that pixel in the parameter map window. The fitted parameters will then be displayed in the ImFCS Fitting panel, and the correlation function and its fit will be shown in the correlation function window. If data from an ROI is required, draw an ROI in the parameter map and the selected data will be shown. For filtering and removing data points see the section on Thresholding.

<sup>4</sup> If blocking is not successful, i.e. the standard deviation does not converge to a stable value, this typically indicates that the measurements weren't sufficiently long and noise correlations exist for times longer than the correlation times. In this case so-called maximal blocking will be used as described in the cited article.



**Fig. 3.4:** Parameter and histogram window.

Here we show the case in which all correlation functions have been calculated and fitted. Note that the program calculates the correlations each time new, so that any changes in parameters in the control panel are taken into account. The exception here is binning as in this case the whole map needs to be changed. The combined correlation and NLS and GLS fits take each about 15 - 20 seconds for a 20x20 pixel image with 50,000 frames.

**Note:** If binning is changed, all existing fit results are discarded, as the program at the moment does not support the use of different binning simultaneously.

You can remove single fits from the evaluation by double clicking that pixel in the parameter map. This removes all fit values and correlations but not the raw intensity data in the image. By clicking the same pixel in the image the correlation and fit can be done again.

### 3.4 Scatter Plots

Imaging FCS allows depicting scatter plots of parameters against each other. This is useful to check for correlations between parameters. Correlations between parameters can be a sign of problems in the fit (e.g. the fraction and characteristic time can sometimes be correlated if the data is not sufficient to distinguish between two different particles/processes). But more importantly the correlation can also stem from the sample. E.g. the diffusion coefficient and number of particles can be correlated when in areas where there the concentration of particles is high are also areas where the particles interact/bind or the density is higher.

The program allows the depiction of 7 different scatter plots: “N vs D”, “N vs F2”, “D vs F2”, “N\*(1-F2) vs D”, “N\*F2 vs D2”, “D vs  $\sqrt{(v_x^2+v_y^2)}$ ” and “D2 vs  $\sqrt{(v_x^2+v_y^2)}$ ”.

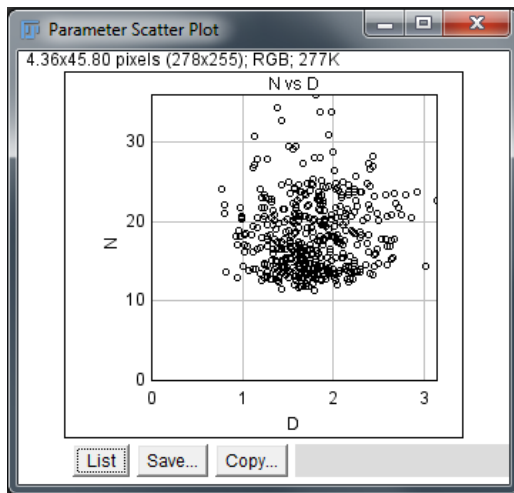


Fig. 3.5: Scatter Plot example of “N vs D”.

### 3.5 Recommendations for fitting

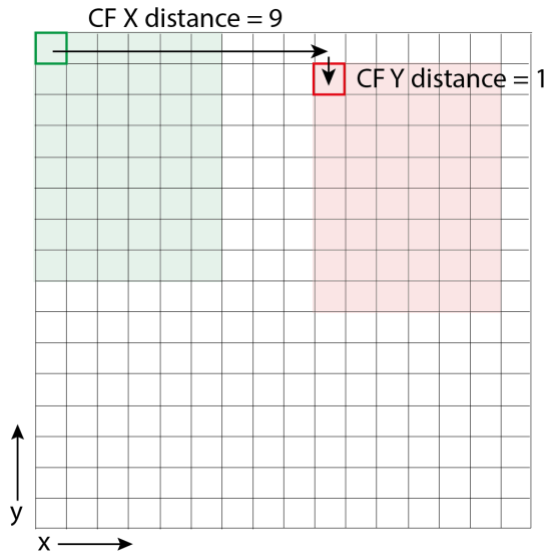
We tested the weighted NLS and the GLS fits on simulations. Both fits need a minimum number of frames to converge to the simulated diffusion coefficient. This is about 50,000 frames for the NLS fit and somewhat longer for the GLS fit. However, for the same number of frames the GLS fit will have an about 20 - 30 % smaller standard deviation for the diffusion coefficient. Bayesian Model Selection uses GLS fits and fits multiple models. So it will take correspondingly longer and probably should be used only in the final evaluation. In future versions we will try to speed up the calculations.

## 4. Dual-Colour Fluorescence Cross-Correlation Spectroscopy (DC-FCCS)

DC-FCCS measurements assume that the image was split according to wavelength onto two parts of the image sensor. For DC-FCCS measurements, please switch the fit model in the “Fit Model” field in the control panel to “DC-FCCS”. The distance between the “green” and “red” pixels has to be provided in the “CF X distance” and “CF Y distance” fields of the control panel.

Clicking onto any pixel within the green channel (green shaded area in Fig. 4.1) will then provide the cross-correlation function in the Correlation plot window. If one wants to depict both ACFs and the CCF simultaneously, one can switch on the toggle button “FCCS Display”. If this button is set on “On”, the cross-correlation is plotted in blue and the ACFs in green and red. Switching on FCCS also automatically switches the fit model to the DC-FCCS model.

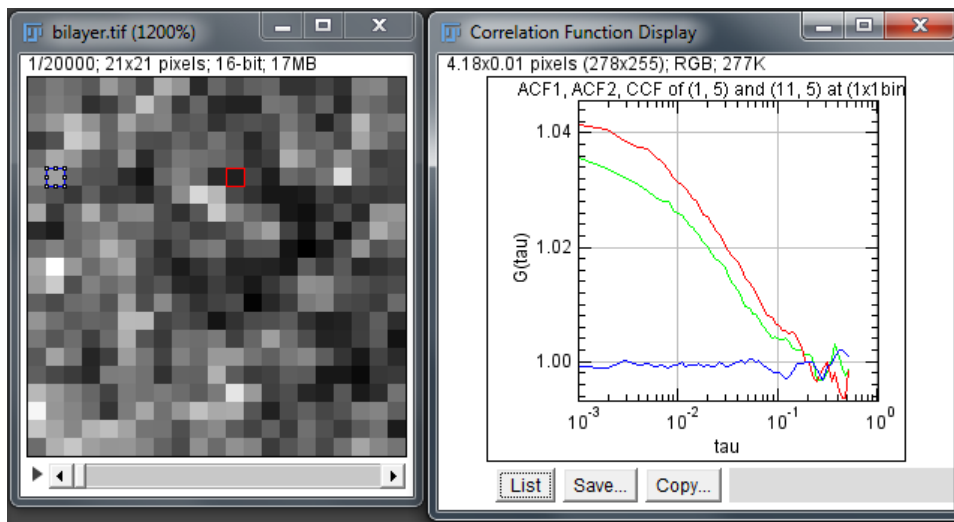
For the calculation of multiple DC-FCCS functions, chose a ROI in the region of the green channel, set the “CF X distance” and “CF Y distance” and then press “ROI”.



**Fig. 4.1:** On the left is shown the principle for DC-FCCS. The green and red shaded areas represent the images split according to wavelength on a single array detector. The user has to provide the distances between the green and red pixels. The program can calculate the cross-correlation between the corresponding pixels then.

**Important:** Of course DC-FCCS can also be calculated for all relevant pixels with fitting. However, you should first make sure that there are cross-correlations. Otherwise, calculations can take long, as the fits do not converge, and many fits will not provide fitting parameters. In addition, make sure that the starting values in the Fit panel are sensible (e.g. no negative numbers). If the starting values are too far off, the fits will not converge (see also 3.1 Fit Panel).

Please also note that the “FCCS Display” mode is set to off if the “All” button is pressed as that results in too many curves to be plotted within one graph. In that case only all cross-correlation curves are plotted, with their fits, if applicable, without the autocorrelations.



**Fig. 4.2:** Cross-correlation display in dual colour mode.

Besides fitting the three individual correlation functions for each pixel, an additional quantity called cross-correlation amount ( $q$ ) is calculated.  $q$  is defined according to (13) as:

$$q = \frac{\max(N_g, N_r)}{N_x}$$

and provides a semi-quantitative measure of the fraction of co-diffusing green and red particles. Theoretically,  $q$  is expected to assume values between 0 (no cross-correlation) and 1 (complete cross-correlation). However, due to imperfect fitting of noisy curves, values  $> 1$  or  $< 0$  can be occasionally encountered. Values  $> 1$  are usually caused by errors in determination of  $N$  by fitting very noisy correlation curves, especially in situation where ACF amplitudes are very low. Since such values usually suffer from very large errors, they are excluded from further analysis by setting  $q$  in such pixels to NaN. Values of  $q < 0$  can result from errors in fitting CCFs with amplitudes close to 0;  $q$  can be set to 0 in such pixels by applying thresholds (see the respective section)

## 5. Calibration of the lateral Point Spread Function (PSF)

The program requires the axial dimensions of the PSF in the field “*axial PSF [um]*” in the “*ImFCS*” control panel. The axial dimensions, i.e. the  $1/e^2$  radius of the light sheet, needs to be measured and provided. If a 2D correlation is desired, the value can be set to a large number (the default is 1000000) in which case the thickness plays no role in the correlation and essentially a 2D fit is performed (e.g. for imaging total internal reflection FCS or ITIR-FCS).

Once that is entered the user can press the Calibrate “*PSF*” button and a small dialog will appear. This will ask the user first for a range of PSF values to be used (start value, end value, step size).

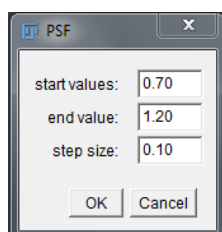


Fig. 5.1: PSF dialog.

Typically one should not use more than 5 parameter values at the same time as the calculation can become slow (e.g. Start 0.7, end is 1.1, step size 0.1). The program will then plot the diffusion coefficients fitted by a simple one component model to the sample for binning values from 1-5. As the diffusion coefficient is a constant the correct PSF value should lead to a straight line.

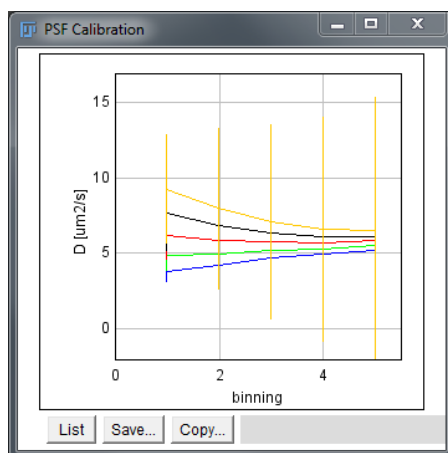
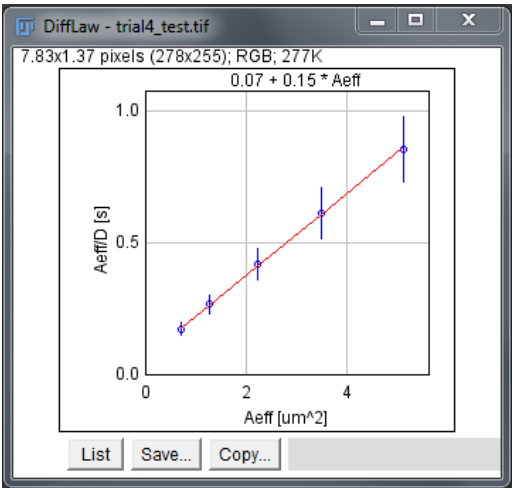


Fig. 5.2: Plot to determine the PSF size.

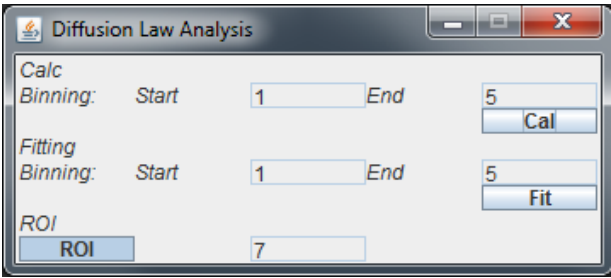
## 6. FCS diffusion law

The FCS diffusion law can also be calculated (5, 6). Pressing the button “*Diff. Law*” in the control panel will plot the FCS diffusion law for an FCS image. As this requires the calculation of correlations over the whole image several times, this is a bit slower than e.g. fitting all correlations in an image. Note also, that bleach correction can be selected and will be taken into account in the calculations.



**Fig. 6.1:** Diffusion law plot. Note that the results of the diffusion law plot are given above the frame, here the line was best fit by  $0.07 + 0.15 * A_{eff}$ .

appear which will allow choosing from which site that the options will change if overlap or no overlap mode many more binned (but calculated in the diffusion law plots. However, selection of points of larger binning. After the calculation, the diffusion law plot can then be fitted with the controls in the second row. The user can choose the start and end points of the fit and fit the data by pressing “*Fit*”. The fit results will be shown in the Diff Law panel (Fig. 6.1).



**Fig. 6.2:** Diffusion law panel. The user can decide which binning to use and afterwards can fit the data choosing start and end-points with a linear fit.

The user can also calculate diffusion laws for small sub-regions of the image (14). By clicking the button on the lower left the user can choose between “*All*” and “*ROI*”. When all is selected the diffusion law over the whole image is calculated. When “*ROI*” is selected then the diffusion law is calculated for each 7x7 pixel area of the image for binning from 1-5. For the binning overlap mode is automatically assumed to make sure there is sufficient statistics for the different points in the diffusion law.



## 7. Mean Square Displacement - MSD

When the MSD button is switched on, then the MSD is directly calculated from the ACF (15) for the part of the ACF at which its value is at least 90% of its amplitude. This means the MSD is only calculated for the first points up to about the diffusion time (i.e. the time a particle needs to cross the observation volume). When switching MSD on, a dialogue will appear and request whether 2D (ITIR-FCS) or 3D (SPIM-FCS) MSD calculations are required. Note that for 3D (SPIM-FCS) MSD calculations, the  $\sigma_z$  in the ImFCS panel has to be provided, otherwise a fit error will be produced.

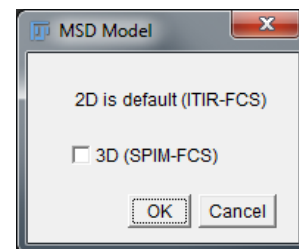


Fig. 7.1: MSD dialogue

The MSD is calculated by inverting the ACF. As this is non-trivial for the Error Function, this is done numerically (see Annex 18). No fitting procedures are provided as yet but the data is saved in the experimental .x/sx files. Also note, that if the ACF contains photophysics or triplet states or the area is non-square, the MSD for this part will still be calculated, but these points should be excluded in any later evaluation.

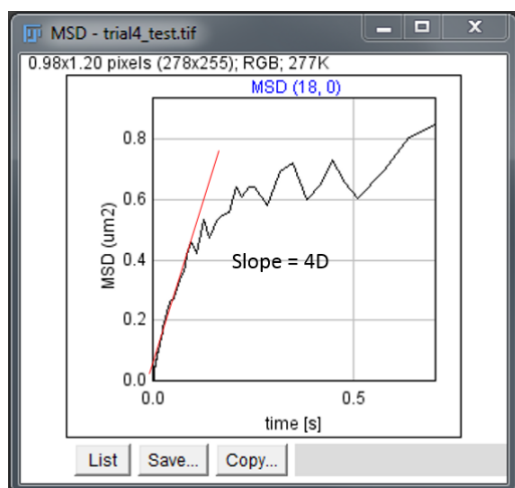


Fig. 7.2: MSD plot calculated from a correlation function of a DOPC bilayer.

## 8. Thresholds for fit results

This functionality can be used to filter out outliers in the parameter maps. Parameter maps contain the fitted parameters multiplied by a mask consisting of values either 1.0 (valid pixel) or NaN (rejected pixel) and displayed for each channel (cross-correlation, green, red) in a slice called "valid pixels". By multiplying the outliers in parameter maps by NaN, they do not contribute to the respective histograms and other statistics (mean, standard deviation, etc.). Nevertheless, the original parameter values are kept and saved in the output files and can be at any time displayed and included in the statistics by resetting the thresholds. The thresholds are defined in "Threshold settings" (Fig.8.1) panel which is opened by the button "Threshold" in the main panel. There are two ways of applying the thresholds:

- First performing the correlation and fitting and then opening “Threshold settings” panel, defining the thresholds therein and applying them on the active parameter map.
- First defining the thresholds in “Threshold settings” panel and then performing the correlation and fitting while “Threshold settings” panel is open. The thresholds are applied on all parameter maps created or updated while “Threshold settings” panel is open. Note that in this way thresholds can be applied during batch processing.

The column on the left contains the individual fitting parameters and  $\chi^2$ , for which thresholds can be defined. The next column contains radio buttons for selecting on which parameters thresholds will be applied. The following columns contain the settings of the minimum and maximum threshold for each parameter in the auto- (green and red) and cross-correlation channels, respectively. Note that the text fields are editable only for those parameters selected for thresholding by the respective radio buttons; text fields for the cross-correlation channel are active only when DC-FCCS fit model is selected. When opening the “Thresholds settings” panel, the text fields are pre-filled with values which are either:

	filter	ACF Min.	ACF Max.	CCF Min.	CCF Max.
N	<input type="radio"/>	-0.00	0.00	-0.00	0.00
D [um2/s]	<input checked="" type="radio"/>	0.3	5	-0.00	0.00
vx [um/s]	<input type="radio"/>	-0.00	0.00	-0.00	0.00
vy [um/s]	<input type="radio"/>	-0.00	0.00	-0.00	0.00
G	<input checked="" type="radio"/>	-0.0000	0.0000	-0.0000	0.0000
rel. G	<input checked="" type="radio"/>	+/- G*N	0.2		0.00
F2	<input type="radio"/>	-0.00	0.00	-0.00	0.00
D2 [um2/s]	<input type="radio"/>	-0.00	0.00	-0.00	0.00
F3	<input type="radio"/>	-0.00	0.00	-0.00	0.00
D3 [um2/s]	<input type="radio"/>	-0.00	0.00	-0.00	0.00
Chi2	<input type="radio"/>	-0.00	0.00	-0.00	0.00
Filter		CCF	same as	ACF	<input type="radio"/>
Reset		If CCF	invalid	q=0	<input type="radio"/>

Fig. 8.1: Threshold Window

- The maximum and minimum values for the respective parameter in the current parameter map (or 0.0 if no parameter map exists). This is always the case when opening “Thresholds settings” for the first time after starting the plugin.
- The last used threshold settings. When the users set their own threshold settings and apply them on a parameter map, the settings (including the minimum and maximum thresholds and all the radio button settings) are remembered even after the “Thresholds settings” panel has been closed and are recalled when the panel is opened again. The settings are remembered until the plugin is closed or until they are reset by the user. Note that if the user changes any settings and then closes the “Thresholds settings” panel without applying the new settings on any parameter map, the

settings are not remembered after closing the panel. Since the settings are different in the FCS and DC-FCCS modes, any threshold settings are reset by switching between the modes.

Note that the thresholds for the asymptotic correlation  $G$  can be set in two different ways: either in absolute values (such as is the case with all other parameters) or as a fraction of the correlation function amplitude (relative  $G = G/N$ ). Note that the radio button for selecting the relative threshold for  $G$  is active only after the radio button for  $G$  has been selected.

Below the parameter names are two buttons: “Filter” and “Reset”. Pressing “Filter” applies the current threshold settings on the active parameter map. “Reset” resets all the settings to their default values and also resets the “valid pixels” to 1.0 for all pixels and updates the parameter map accordingly.

Bellow the thresholds settings for the cross-correlation channel there are two additional radio buttons active only in DC-FCCS mode. The one labelled as “*CCF same as ACF*” sets the thresholds for cross-correlation channel to be equal to those for autocorrelation channels. The text fields for the minimum and maximum threshold for CCF then cease to be editable and any values in them are disregarded. The other radio button is labelled as “*If CCF invalid  $q=0$* ”. When selected, the values of cross-correlation amount  $q$  are set to 0.0 in those pixels for which the ACF fits passed the thresholds (the “valid pixels” are 1.0 for both ACF channels) but the CCF did not (“valid pixels” are NaN). This is important when fitting data exhibiting no cross-correlation. In that case the CCFs (which contain almost solely noise) are prone to erroneous fits giving parameters which fail to pass the thresholds. Rejecting such pixels from the analysis is likely to cause a bias in  $q$  towards larger values (CCFs with higher amplitudes are more likely to be fitted correctly).

If individual data points are to be removed, you can double click any point in the parameter maps. That data will then be set to NaN and will not be used in statistics and evaluations.

## 9. Number and Brightness analysis (N&B)

N&B analysis can be a powerful tool but has its problems in EMCCD based measurements. For a thorough discussion, please see the article by Unruh and Gratton (16). We implement here two possibilities to calculate the N&B analysis. When pressing *N&B* a new panel will open as shown in Fig. 9.1. Note that in any case a background file has to be loaded over the “Bgr NUM” button in the panel before this analysis works. The background should be taken under the same conditions as the experiments, including the size and area of the ROI. In this mode the button will change from “Bgr NUM” to “Bgr FILE” and will calculate for each pixel correction factors for mean and variance.

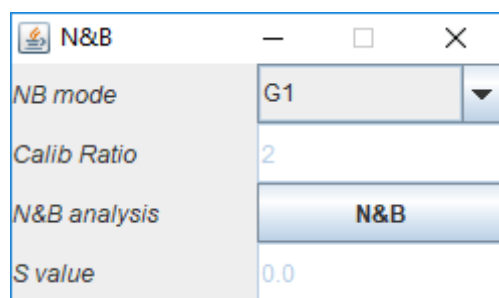


Fig. 9.1: N&B panel

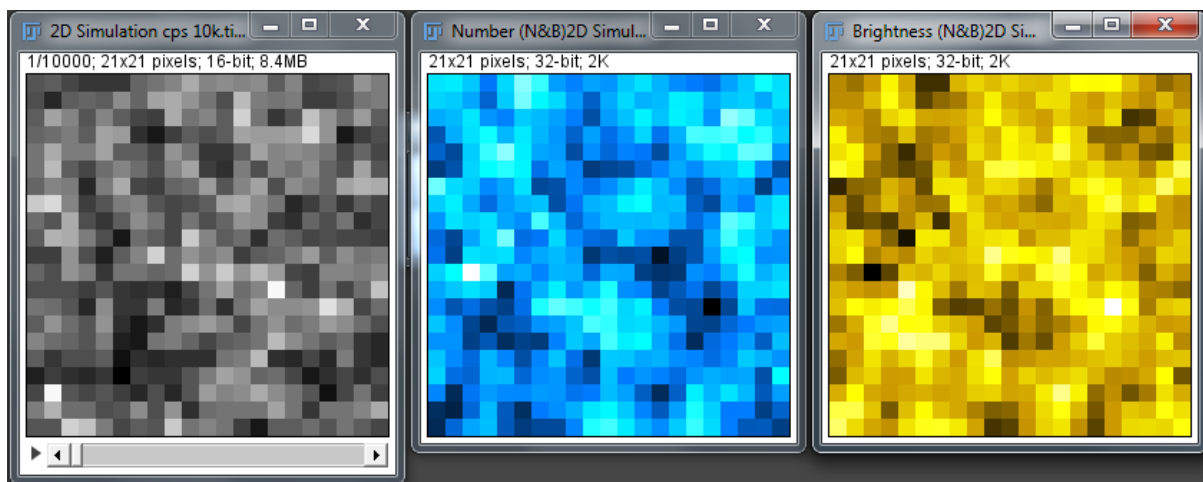
**NB mode:** There are two modes. The first mode is the so-called G1 analysis and is similar to the estimation of the amplitude of a correlation function of the first point at a non-zero lag time. The zero lag time contains shot noise and is therefore not used in correlation analysis. Essentially the G1 analysis is the estimation of the amplitude over the second point of the autocorrelation function. It is achieved by calculating the correlation for next-neighbour frames. This scheme works if the acquisition time per frame is much shorter than the average time a particle needs to diffuse through the observation volume of a pixel. We have tested this mode and it provides the same brightness values as the confocal case.

Pressing the “N&B” button will calculate the N and the B values for each pixel according to the following equations (equations 1 in (16)):

$$N = \frac{(\langle I \rangle - offset)^2}{\sigma^2 - \sigma_0^2}$$

$$B = \frac{\sigma^2 - \sigma_0^2}{\langle I \rangle - offset}$$

Where  $\langle I \rangle$  is the mean intensity in a pixel, *offset* is the offset of the camera,  $\sigma^2$  is the variance of the intensity of the pixel, and  $\sigma_0^2$  is the variance of the camera.



**Fig. 9.2:** Examples of an image and the corresponding Number and Brightness images. If “CamCor” is on, then another two images with  $n$  and  $\varepsilon'$  (see text) will be displayed.

While these expressions provide an idea of the brightness of molecules they do not give correct number and brightness values. Unruh and Gratton have shown that a value  $\varepsilon'$ , which is proportional to the molecular brightness  $\varepsilon$ , can be calculated if the parameter  $S$  is known. In that case

$$\varepsilon' = \frac{B}{S} - 1$$

$$n\varepsilon' = \frac{\langle I \rangle - offset}{S}$$

If  $S$  is unknown, the camera can be calibrated if one has two files with probes that have a known factor brightness ratio. In this case the user can use “calibrate” which is in the drop-down-menu of the NB

*mode*. The user will then be asked for two files, where the first one is assumed to be the one with the smaller brightness. Note that for this calibration to work the two calibration files and later the actual image to be treated have to be recorded with the exact same parameters and illumination intensities. With the two calibration files, with a calibration ratio  $r$ , one has then

$$B_1 = (\varepsilon' + 1)S$$

$$B_2 = (r\varepsilon' + 1)S$$

$$rB_1 - B_2 = r(\varepsilon' + 1)S - (r\varepsilon' + 1)S = (r\varepsilon' + r - r\varepsilon' - 1)S = (r - 1)S$$

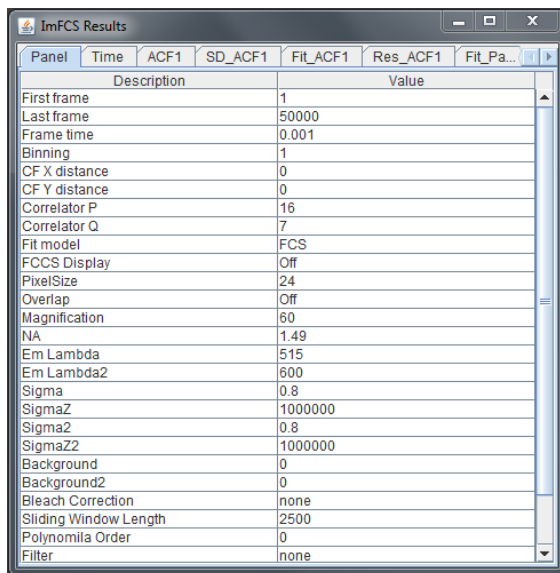
$$S = (rB_1 - B_2)/(r - 1)$$

This way  $S$  is determined and will be provided in the text field. Then one can press N&B to process the actual file.

*Note: The calibration mode has only been tested with simulations and not real data as yet.*

## 10. Data table

The data can be visualized in a data Table by pressing the Table “Create/Update” button. The data table is not automatically updated. The table can be updated by pressing again the “Create/Update” button.



Description	Value
First frame	1
Last frame	50000
Frame time	0.001
Binning	1
CF X distance	0
CF Y distance	0
Correlator P	16
Correlator Q	7
Fit model	FCS
FCCS Display	Off
PixelSize	24
Overlap	Off
Magnification	60
NA	1.49
Em Lambda	515
Em Lambda2	600
Sigma	0.8
SigmaZ	1000000
Sigma2	0.8
SigmaZ2	1000000
Background	0
Background2	0
Bleach Correction	none
Sliding Window Length	2500
Polynomila Order	0
Filter	none

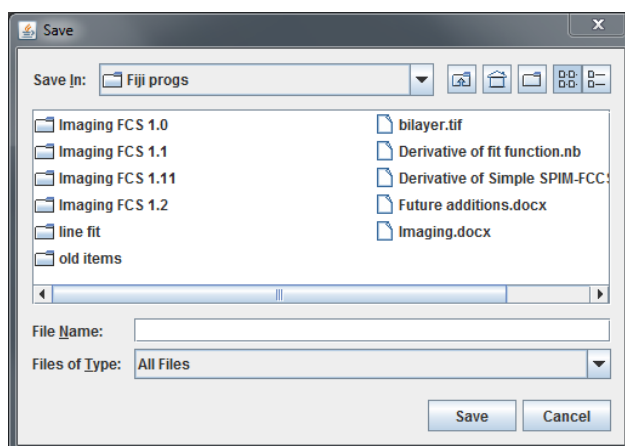
**Fig. 10.1:** Data table. The first tab contains the panel parameters. The other tabs contain the numerical values of calculations and fits. The data in this table is the same that is saved in the .x/sx files.

## 11. Saving and loading data

All data is saved in .x/sx files. This provides a spreadsheet format which can be read by many other programs and facilitates the transfer of the data to different programs.

**Note:** In general the plugins are NOT backwards compatible to earlier versions and experiments.

When pressing the button “Save” a dialog will open asking the user which data to save:



**Note:** To open an experiment the image for that experiment has to be already loaded, otherwise the program will not recreate the experiment!

**Fig. 11.1:** File selection window.

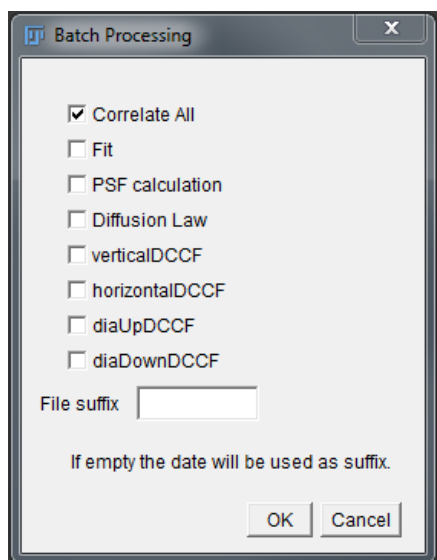
There is a limit to the number of columns in an Excel file, which is currently 16,384 columns in xlsx format. After discounting a column used for row categories, we can save up to fitting results of up to most 16,387 pixels at the moment. On a related note, the memory consumption increases as the number of pixels increases and furthermore it takes a longer time to process. A log prompt is shown when saving is completed. This issue of saving large data file will be addressed in the future when final version of SXSSF functionality in POI library is available.

## 12. Batch Mode

In batch mode, a dialogue window will be opened (Fig. 12.1) which allows the user to select which calculations are to be performed. In addition, a “suffix” text field allows the user to specify a suffix that will be added to the image file name to create the new output file name for the .xlsx file. If no suffix is specified the plugin adds the date and time to the image file name to create a unique filename for saving.

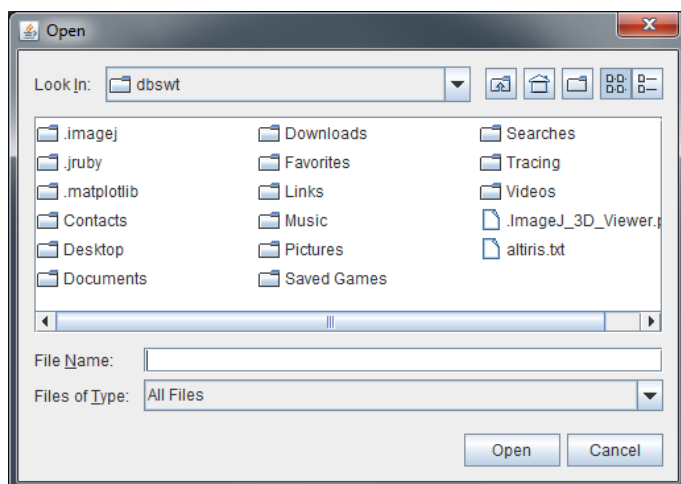
For instance if an image file is called “bilayer.tif” and you specified the suffix “-evaluated”, then the output filename will be “bilayer-evaluated.xlsx”. If you did not specify a suffix and assuming the program was run on May 11, 2015 at 10:30:12 (h:m:s), the output file will be named “bilayer2015\_05\_11-10\_32\_12.xlsx”.

After this selection an Open Dialog Window will be displayed (Fig. 12.2), which allows the user to select multiple files to be treated in the batch mode. Note that at the moment only files can be selected, no directories.



**Fig. 12.1:** Batch processing dialog.

The resulting .x/sx files are saved in the same directory as the image files.



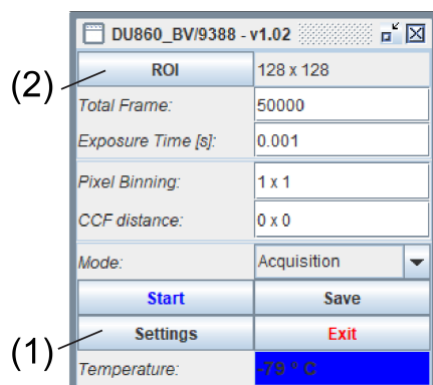
**Fig. 12.2:** The Open Dialog Window for Batch processing.

**Note:** For the FCS diffusion law the batch mode will use the current settings of the plugin. So if “All” has been chosen, the diffusion law will be calculated for the whole image. If “ROI” has been chosen, then the diffusion law will be calculated for 7x7 ROIs for the whole picture.

## 13. Direct Camera Readout

### 1.1 General Description

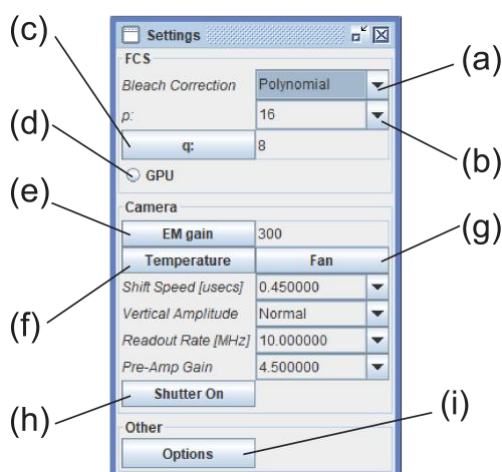
ImFCS-Direct Camera Readout is an image acquisition software that does real-time visualization of correlation functions without the need for specialized hardware as all incoming pixels are streamed to RAM and processed in a CPU/GPU. We have tested the tool so far on Andor iXon DU860, DU888, DU897, Sona 11, Hamamatsu Orca Flash 4.0, Photometrics Evolve 512 and Prime 95B. To activate, press the “LiveReadout” button located on the first column of ImFCS main panel in Fig. 1.1.



**Fig. 13.1:** Direct Camera Readout main panel. This shows the example of an Andor DU860 with full frame 128x128 pixels to be recorded.

A selector window will appear if multiple camera models are detected. Otherwise, the Direct Camera Readout main panel (Fig. 13.1) will appear on the screen. The interface has been designed to be similar, despite the camera model connected. Some variations to accommodate camera settings can be found under the Settings panel.

- (1) Click on Settings toggle button to show/hide Setting window (Fig. 13.2)

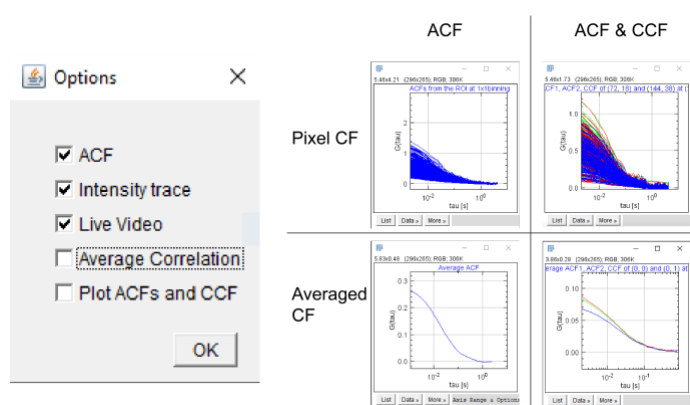


**Fig. 13.2:** Settings panel containing control for FCS evaluation, options for data visualization, and camera settings.

- (a) Enable or disable polynomial **bleach correction** on intensity trace or spatial binned intensity trace depending on whether pixel binning is selected.
- (b) **Correlator P**: The number of correlation channels for the first group of bin times.
- (c) **Correlator Q**: The number of groups of bin times (including the first). The bin time within a group is constant but will be twice as large as the bin time of the preceding group.



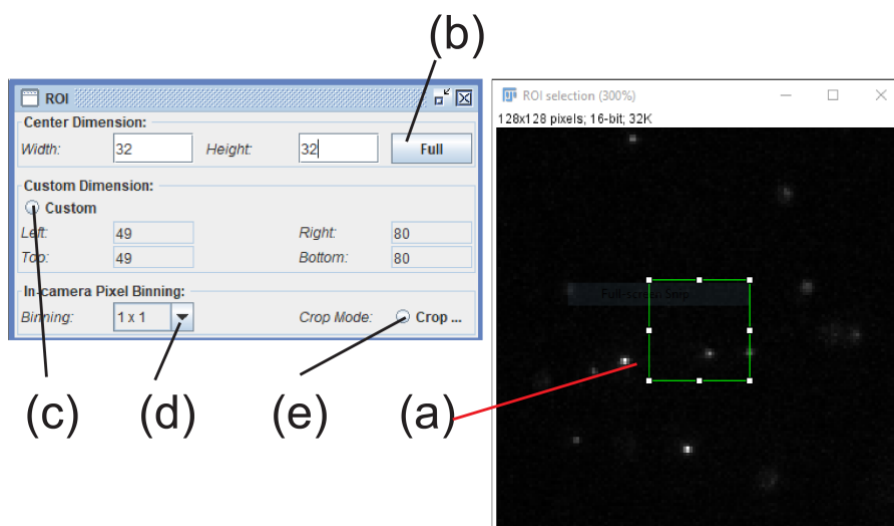
- (d) Enable **GPU** to speed up bleach correction and CF calculation. We recommend sticking with CPU for analysing small regions ( $< 64 \times 64$  pixels) as GPU outperforms CPU only when handling large amount of data, due to the overhead of loading data onto and off the GPU.
- (e) **EM gain** can be varied depending on camera model.
- (f) **Temperature** to which detector can be cooled.
- (g) Speed of **cooling fan** can be controlled depending on the system.
- (h) **Mechanical shutter** can be used to take a reference or background on selected cameras.
- (i) A dialog box with multiple checklists for live plotting ACF and intensity trace within user defined ROI. In addition, an option to average CF and/or overlay  $ACF_{green}$ ,  $ACF_{red}$ , and  $CCF$  are included (Fig. 13.3)



**Fig. 13.3:** The Options dialog box (left) to visualize FCS data with default values.

**Note:** Camera settings window (e) – (h) varies depending on camera model.

- (2) ROI toggle button reveal/hide ROI panel and selector window (Fig. 13.4)



**Fig. 13.4:** ROI panel to selecting region of camera chip for recording.

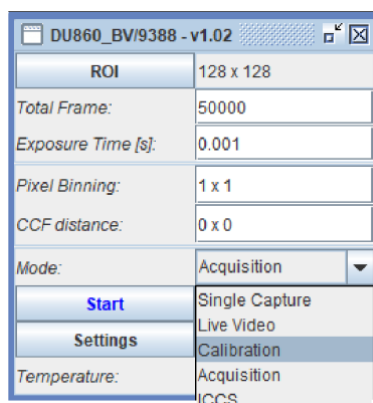
ROI selection can be made in three ways:

- Annotating ROI selector window (a) via Fiji rectangular selection tools
- Moving the selected region highlighted in green around the screen
- Keying specific values under ROI panel

Full button (b) ensure full-frame is selected. Centralized ROI can be set by keying value under width and height text box. Customized coordinates can be activated with a radio button (c). Combo box (d) starts in-camera pixel binning for better SNR at the expense of worse spatial resolution. Crop mode (e) reduces the height of CCD by excluding some rows to achieve higher frame rates.

## 1.2 Modes

The application currently supports five modes (Fig. 13.5): *Calibration mode*, *Acquisition mode*, *Live video mode*, *ICCS mode*, and *Single capture mode*. The modes can be run as follows:



**Fig. 13.5:** Drop down menu showing 5 different mode

**Step 1:** Select your preferred mode from the drop-down menus

**Step 2:** Make changes to “ROI” via toggle button

**Step 3:** Set camera “Exposure Time”

**Step 4:** Set “Total Frame” (acquisition mode only)

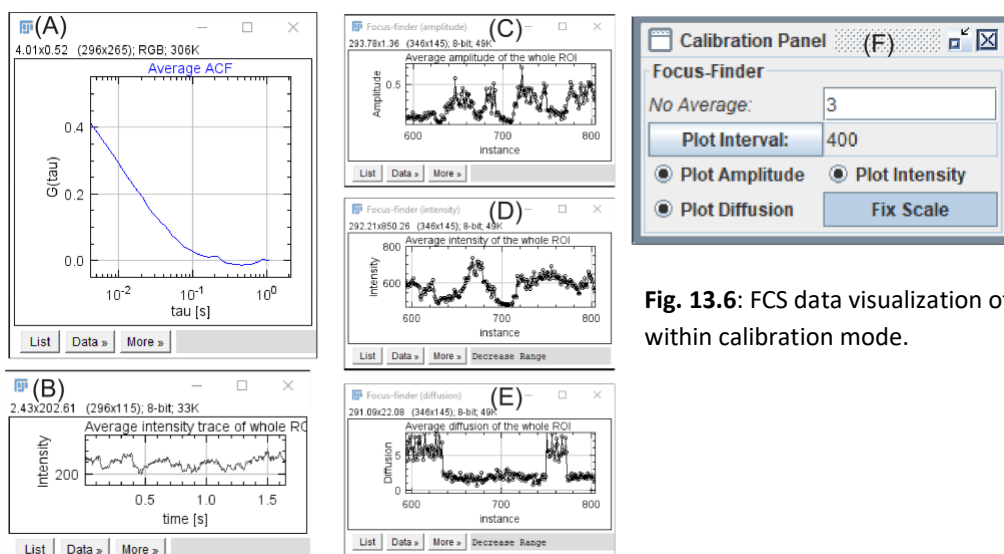
**Step 5:** Press “Start” button

**Step 6:** Press “Stop” button to abort

**Step 7:** Press “Save” button (acquisition mode only)

**Note:** Pixel Binning, CCF distance, Settings >> Bleach correction, Settings >> Correlator P, Settings >> Correlator Q, Settings >> Options can be altered while camera is recording.

### 1.2.1 Calibration mode



**Fig. 13.6:** FCS data visualization offered within calibration mode.

Calibration mode runs under an infinite loop until the stop button is pressed. The user is presented with a Live Video window at 25 fps to make ROI selections for analysis. Non-cumulative display of CF (Fig. 13.6 A), average non-bleach corrected intensity trace (Fig. 13.6 B), and plot of  $G(0)/\text{Intensity}/\text{Diffusion}$  (Fig. 13.6 C-E) updates at every cycle defined by  $\text{Exposure Time} \times \text{Plot Interval}$  (Fig. 13.6 F).

A calibration panel (Fig. 13.6 F) will appear for the user to set plot interval (number of frames in which CF within ROI is calculated) and options to display plot of  $G(0)/\text{Intensity}/\text{Diffusion}$  from the preceding instance of CF.

Usage of calibration mode includes fine focus tuning (TIRFM and SPIM), critical angle calibration (TIRFM), Dual-View alignment for simultaneous dual-colour experiment recorded on a single camera chip, sample screening, and long term sample observation.

#### Focus alignment:

- Step 1:** Make selection of ROI on the screen
- Step 2:** Set CCF distance to (0 x 0) for autocorrelation
- Step 3:** Set Pixel binning value to (1 x 1)
- Step 4:** Set “Plot Interval” (500 – 1000 frames is a good start), the number of frames where CF is calculated from selected ROI
- Step 5:** Adjust the position of objective (manually or electronically depending on the system you are using)
- Step 6:** Monitor correlation value (A and C)
- Step 7:** Repeat step 4 and step 5 till maximum correlation achieved.

Note: If no correlation observed, proceed to angle calibration and make sure TIR is achieved. If persist, increment “Plot Interval”, Exposure Time, size of ROI, or Pixel binning to improve SNR. Proceed to increase laser power if all attempt fails.

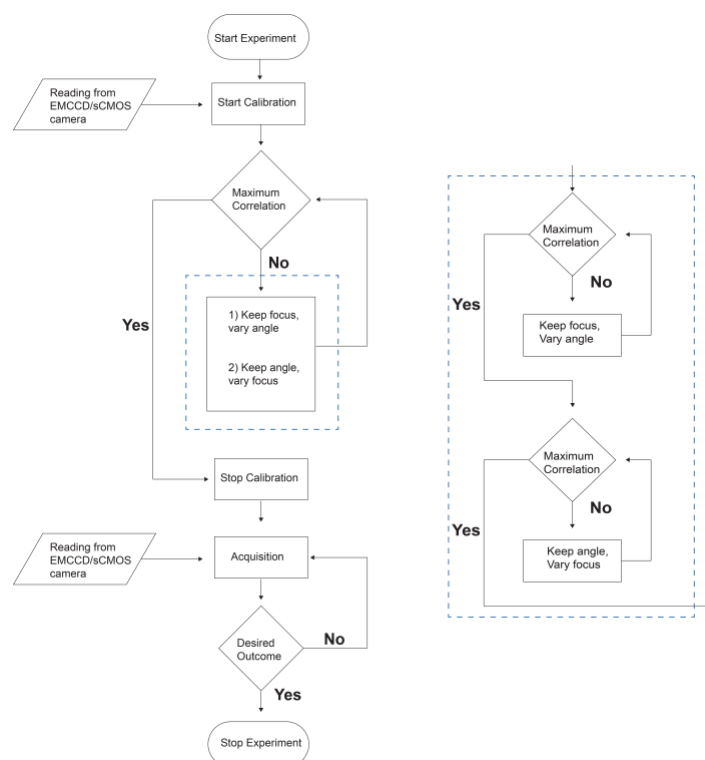
#### Critical angle alignment:

- Step 1:** Visually locate the focal plane of the sample
- Step 2:** Make selection of ROI on the screen
- Step 3:** Set CCF distance to (0 x 0) for autocorrelation
- Step 4:** Set Pixel binning value to (1 x 1)
- Step 5:** Set “Plot Interval” (500 – 1000 frames is a good start), the number of frames where CF is calculated from selected ROI
- Step 6:** Make changes to incidence angle while maximising for correlation amplitude (A and C)
- Step 7:** Optimize focussing by maximising correlation amplitude. (Refer to focussing step described previously)
- Step 8:** Repeat Step 6 and Step 7 a few times
- Step 9:** Note down position corresponding to maximum correlation function and calibrate the system against the critical angle.

Dual-View alignment:

- Step 1:** Proceed to align for *Critical angle* and *Focussing*
- Step 2:** Set CCF distance (distance between red and green channel) in a desired arrangement (in our case we set CCF  $x = 0$ , CCF  $y = 64$  since we are arranging the red channel south of green channel on a  $128 \times 128$  sensor)
- Step 3:** Set Pixel binning value to  $(1 \times 1)$
- Step 4:** Make selection of ROI on the screen
- Step 5:** Traverse one channel with respect to another
- Step 6:** monitoring cross-correlation amplitude (A and C)
- Step 7:** Repeat step 5 and step 6 for maximum cross-correlation amplitude
- Step 8:** The two channels are now spatially equivalent. Proceed to experiment

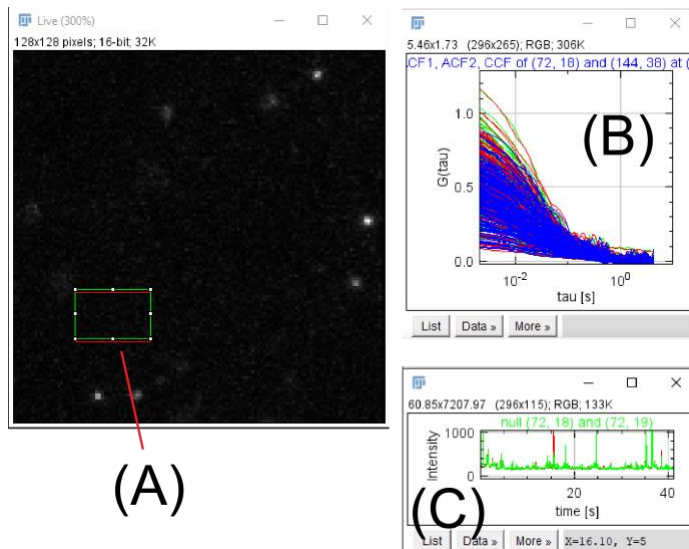
The procedure for focus and angle alignment is presented in Fig. 13.7. Critical angle calibration requires focal plane of the imaging system to overlap that of 2D plane of interest (near cover slip for TIRFM) In order to accurately determine the incidence angle and evanescent field penetration depth, system need to be calibrated against critical angle position. We recommend to proceed with Fig. 13.7 (*right*) till the maximum amplitude of correlation functions is reached, Once TIR illumination has be setted, fine focus tuning can be performed before measurement.



**Fig. 13.7:** Recommended procedure for alignment.

### 1.2.2 Acquisition mode

Use this mode when data saving is required. As you select acquisition mode you need to provide the total number of frames to be saved. The user can, however stop the acquisition at any time using “Stop”. The so far acquired data can be saved, pressing “Save”, which triggers a dialog box to save the imagestacks (.tif) and metadata (.csv). Note the maximum number of frames are limited by the number of pixels per frame and the available RAM.



**Fig. 13.8:** Acquisition mode with live development of correlation functions.

To display correlation functions live, create an ROI in the “Live” window (Fig. 13.8 A). The correlation functions and the corresponding non-bleach corrected intensity trace are displayed (Fig. 13.8 B)

### 1.2.3 Live video mode

A basic functionality to let user start recording videos and display frames on the screen at 25 fps. Note that refresh rate drops below 25 fps if exposure time is set above 40 ms.

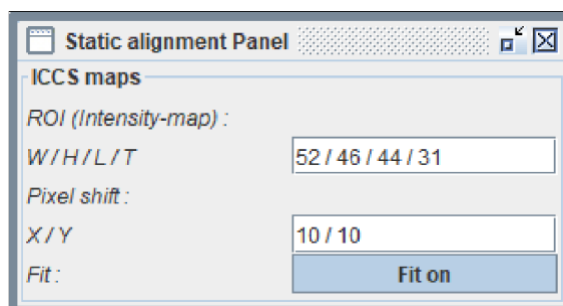
### 1.2.4 ICCS mode

**Step 1:** Prepare a fiducial marker, e.g. immobilized fluorescence beads on cover glass

**Step 2:** Find focal plane and adjust incidence angle  $>$  critical angle if you are using TIRFM

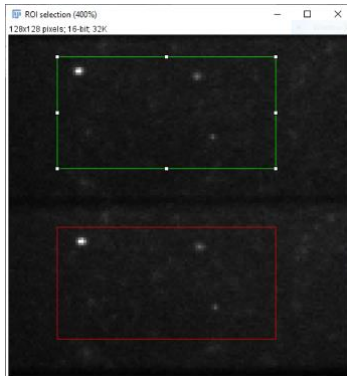
**Step 3:** Set CCF distance (distance between red and green channel) in a desired arrangement (in our case we set CCF  $x = 0$ , CCF  $y = 64$  since we are arranging the red channel south of green channel on a 128 x 128 sensor)

**Step 4:** Set the spatial shift ( $x, y$ ) for cross-correlation matrix (CCM)



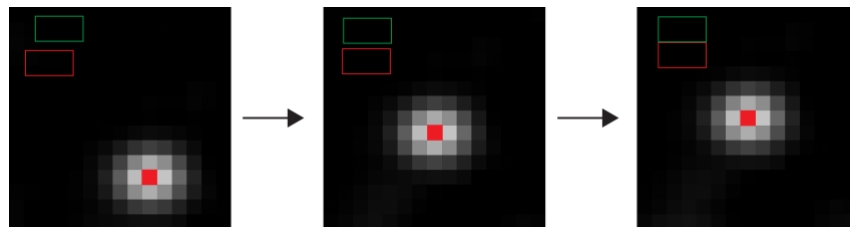
**Fig. 13.9:** ICCS control panel

**Step 5:** Make selection on the screen (Fig. 13.10)



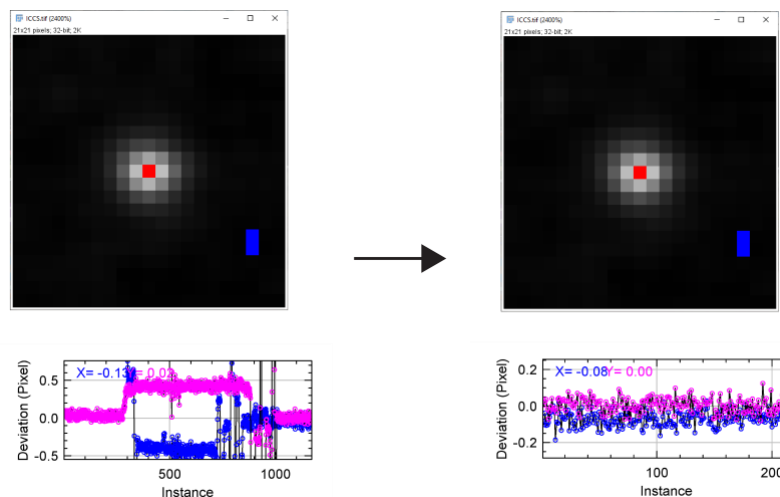
**Fig. 13.10:** 25 fps live video window where user can make selection on the screen

**Step 6:** Center the maxima of CCM between two channels (Fig. 13.11) as you correct for linear drift by manually altering the projection from one of the channel. The vector position of the maximum indicate the linear drift



**Fig. 13.11:** CCM between two region. Red pixel indicate vector position of maximum correlation value.

**Step 7:** For sub-pixel precise alignment, fit the cross-correlation matrix with 2D Gaussian fitting model by pressing “Fit on” and monitor the vertical and horizontal drift curves while aligning. An aligned system should gives you a near 0 drift value



**Fig. 13.12:** Slight misalign indistinguishable from CCM. Toggle “Fit on” to localise for centroid.

**Step 8:** Prepare sample and proceed for measurement

### 1.2.5 Single capture mode

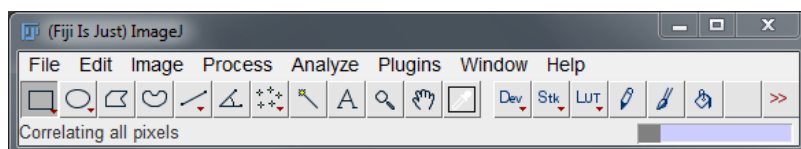
Capture a single frame and display images on the screen. Use this mode to update the ROI selector window.

## 14. Exiting the plugin

Press the “Exit” button and the plugin will be terminated and all windows and data will be removed.

## 15. Checking Progress

Progress for all calculations is shown in the ImageJ status bar. It is given as a progress bar and a short message what is being done (here: “Correlating all pixels”).



**Fig. 14.1:** Progress is indicated by a progress bar including a short message about what is

## 16. Theoretical ACFs and Simulations

The plugin allows to plot theoretical FCS functions or to simulate a 2D diffusive system (a 3D version is planned for later).

By pressing the “Test” button in the Fit panel (see Fig. 3.1) the theoretical ACF with the parameters from the fit panel will be plotted in the “Correlation Function Display” window. This can be useful to obtain estimates for initial parameters or in general to get a feeling for the changes of ACF functions with parameters.

The button “Simulation” in the control panel will bring up a dialog with adjustable parameters for a simulation (for details see Annex 4). A simulation for 100,000 frames with 20x20 pixels and 400 simulated particles takes about 40 seconds to run. The simulator creates an Image which is automatically set as the active window for the plugin. All operations of the plugin can be applied to this image.

## 17. Known Issues

1. In Micromanager use ImageJ version 1.47v or later (which come with Micromanager version 1.4.16 and later). In earlier Micromanager versions 1.4.14 and 1.4.15, which are bundled with earlier versions of ImageJ, the program can be slow or possibly does not support the output of plots.

2. In the ImageJ2 update from June 2014 (ImageJ 2.0.0-fc-SNAPSHOT) switch SCIFIO off for loading images. This does not work properly with the plugin. You can do that under the menu item Edit/Options/ImageJ2 and then make sure that the option “Use SCIFIO when opening files” is not ticked.
3. If “Fit” is switched on but the data cannot be properly fit, it can happen that FIJI stops responding, which then requires to stop FIJI (task manager on PC, or “Force Quit” on Mac). This problem has now been mitigated by limiting the fit to 2000 iterations and evaluations each and by running all correlations and fitting on a background thread in Java. The program will now open a FIJI log window and reports all pixels at which fitting exceptions occurred.
4. If the plugin/.jar file creates error messages when started, check that you run ImageJ 1.50a or higher.
5. Sometimes the installation of the POI folder in the Fiji/jars folder does not work and the user gets error messages. In this case copy the jar files from the POI folder directly into the Fiji/jars folder or use the ImageJ update site for this plugin.
6. Direct camera readout – Upon update to ij.-1.53q, creation of image e.g, ROI panel, ICCS mode slows down. Temporary solution is to put ij.-1.53f.jar in the Fiji/jars folder.
7. Imaging FCS analysis software – GPU fails to correlate when binning is not 1x1 when user select ROI mode.

## 18. Changes to ImagingFCS

### a. Changes from 1.61 to 1.612 (version 1.61 patch no 2)

1. Drop metadata information on internal line speed and line interval for Hamamatsu Orca Flash 4.0 (not supported).
2. Fixes bug where ACF and fits are displayed even though pixels are invalid/outside threshold range.
3. Compiled with ij.-1.53f
4. Increase maximum byte per image stack to 3.7GB. File larger than 3.7GB will be save as 2 or more separate stacks.

### b. Changes from 1.60 to 1.61

1. Added support for Hamamatsu ORCA-Quest C15550-20UP.
2. Fixes file saving issue (program stalls-out when saving large amount of dataset > 4GB).
3. Fix bug on ROI selection with Hamamatsu camera. Now automatically select to nearest valid ROI.
4. Enhance file saving routine to remember the last save location.



5. Prompt user to enter experimental parameters on starting camera.

#### **c. Changes from 1.52 to 1.60**

1. Direct Camera Readout (v 1.1) has been added as a data acquisition tools with real-time cumulative and non-cumulative spatio-temporal correlation visualization. It supports Andor DU860\_BV, DU888\_BV, DU897\_BV, SONA-4BV11, Hamamatsu Orca Flash 4.0 C11440-22CU, C11440-22C, C13440-20CU, C13440-20C, Photometrics EVOLVE- 512, Prime 95B GS144BSI.
2. Added '*Pvideo*': a routine calculating parameter video maps from an image stack (performs segment wise bleach correction).
3. Added bleach correction parameter: number of intensity points to be averaged before bleach correction is performed.
4. Introduced 'Experimental Settings' Panel to store experimental specific settings and declutter main panel.
5. Write and read a configuration file in user home directory storing ImFCS settings.
6. Saved experiment file no longer backwards compatible (version 1.52 and before).
7. Fit panel: added default button to set all fit parameters to their default value
8. Cursors can now be used to move through the image and correlations and fits are automatically done Fit panel: added default button to set all fit parameters to their default value

#### **d. Changes from 1.51 to 1.52**

1. ACF, CCF, N&B, polynomial bleach correction, diffusion law and PSF calibration can be computed on NVIDIA GPU.
2. Improve stability of Save and Read functions.
3. 3D free diffusion simulation has been modified to correct for the rounding off errors in the number of photons emitted from particles across the z plane. The extension in z plane has also been increased to have a more uniform distribution of particles for a free diffusion simulation.

#### **e. Changes from 1.50 to 1.51**

1. A batch simulation mode has been introduced (with limited functionality which allows simulating a batch of data with a range of D1, D2, and F2).

2. Rectangular binning is now supported and the fit models have been updated accordingly. At this point in time, square binning is much faster when fits are performed.

#### **f. Changes from 1.49 to 1.50**

1. The saving and loading of experiments were improved. Unfortunately, that also means that there is no backward compatibility for the loading of saved experiments with older versions.
2. N&B analysis has been improved.
3. The PSF calibration was improved and one can select now the binning to be used.
4. An exponential term is now included in the fitting function to account for triplets or photophysics.
5. We have included an ImageJ update site for “ImagingFCS” to facilitate installations.
6. A mistake in the simulations was corrected. If the fractions for the first and second diffusive particles did not add up to N, the total number of simulated particles, then it could happen that one particle with diffusion coefficient 3 was simulated along. This is a rounding error as only integer numbers are allowed. This is corrected now and if the fraction for the third particle is set to 0, then no third particle will be simulated.

#### **g. Changes from 1.48 to 1.49**

1. Users can now calculate an average correlation function over all valid pixels in an image.
2. The diffusion law can now be calculated for sub-regions of an image. At the moment this is implemented for 7x7 pixel areas with binning from 1 to 5 using overlap mode.
3. Note that now older experiments saved with versions 1.48 and earlier cannot be loaded with this plugin anymore

#### **h. Changes from 1.47 to 1.48**

1. In DC-FCCS we calculate now for each pixel the cross-correlation amount ( $q$ ).
2. A threshold window was included which allows to filter results within certain parameter ranges. This can be useful to exclude outliers.
3. The parameter maps contain additional slices to depict the  $q$  value (DC-FCCS) and the validity of pixels for applied thresholds.

#### **i. Changes from 1.46 to 1.47**

1. The values in the intensity filter function to select pixels for data treatment was incorrectly calculated for binning  $> 1$ . This has been corrected.

2. The program allows now correcting the data with a background image. This removes patterned noise, which can be important at low EM gains.
3. A number and brightness analysis routine is now included in the program based on the article by Unruh et al. (16).

#### **j. Changes from 1.45 to 1.46**

1. The simulations were extended and can now correctly perform domain and meshwork simulations in 2D (but not yet domains and meshworks simultaneously).
2. Diffusion law fits have now their own panel where the diffusion law calculation and the fitting can be controlled.
3. An option panel was added which allows to choose which windows the user wants to see. The user can now also see the covariance matrix for the ACF and the blocking functions. In addition, some windows can be switched off if they are not of interest to avoid cluttering of the screen.

#### **k. Changes from 1.43 to 1.45**

1. The ACF fits were made considerably faster (see typical performance values as given on the website).
2. A bug was corrected which lead to repeated pop-up windows if parameters were set out of realistic bounds for the fits. If parameters are unrealistic there is now a single message and the fits are all terminated.
3. The simulations were improved to contain more options and a new panel.
4. A bug in the display of dCCF functions when using pixel binning was corrected.

#### **l. Changes from 1.42 to 1.43**

1. The simulator was upgraded and allows not 2D and 3D simulations.
2. The parameter maps are now set overall to "NaN" so that the statistics of the histograms takes only account of the actually fitted values. Therefore, the average values in the title of the parameter windows have been removed.
3. Clicking a pixel in the parameter window now updates all windows including the intensity and residual windows.

4. One can now remove single fitting and correlation results by double clicking on a pixel in the parameter maps.
5. “Singular Matrix Error” messages are not anymore displayed. The fit is unsuccessful and the values in the parameter windows are set to NaN (not a number).
6. The linear fit for the diffusion law is now weighted correctly with the variance (before it was weighted with the standard deviation).

#### **m. Changes from 1.41 to 1.42**

1. An FCS simulator was included. However, at the moment it is restricted to 2D diffusion.

#### **n. Changes from 1.4 to 1.41**

1. Bug fixes regarding the calculation of some of the areas for ROIs and the creation for irregular ROIs for the spatial cross-correlation.
2. MSD was originally calculated only for 2D samples, i.e. for ITIR-FCS. MSD is now also calculated for SPIM.
3. Documentation was corrected, as in the SPIM function a factor 4 was missing in the last term.

#### **o. Changes from 1.3 to 1.4**

1. The program now saves all data in the .x/sx spreadsheet format and reconstitutes experiments from these files. Note that now older experiments saved with versions 1.3 and earlier cannot be loaded with this plugin anymore.
2. The mean square displacement (MSD) can now be directly calculated from the correlation function (2D diffusion only at the moment).
3. A Data table file can be created to visualize the numerical values of the results.
4. Multiple bugs relating to the depiction of the FCCS functions were corrected.
5. “Overlap” mode can now also be used for the calculation of diffusion laws.
6. Additional bleach correction modes were added.
7. “NullPointerExceptions” that crept up with one of the recent Fiji updates were corrected.

#### **p. Changes from 1.29 to 1.3**

1. The program allows now also to calculate correlations and fits in a region of interest (ROI). Just select any region of interest and click "ROI" in the panel to obtain the correlations.
2. The FCS fit model was updated to have now up to 3 diffusing components. While this is of limited use as the Imaging FCS data (or any other FCS data for that matter) does typically not have sufficient S/N to resolve 3 components, it can be useful for comparison in Bayesian Model Selection. Note that the FCCS model contains still maximally 2 components.
3. The FCS model has been corrected as there was an error in the calculations of the derivatives for the fraction of the second component. This however did not affect any one component fits done up to now.
4. Fitting: Note that we do not support anymore non-weighted fits. We have introduced two new options in fitting, "GLS" and "Bayes". If both are off, fits are performed by weighted non-linear least squares fitting. If Generalized Least Squares (GLS) is on then fits take the covariance matrix into account and not only the variances. The fits however take much longer. So for initial evaluations we still recommend to use the normal weighted fits. If "Bayes" is on then multiple models (at the moment only 1 and 2 component models; in the future we will introduce options to select and include more models) are fit and the likelihood of each model is provided. "Bayes" works with non-linear least squares and also with GLS.
5. In the Fit panel we have introduced a "Set" and "Free/Fixed" button. The set button sets the current fit parameters as fixed and all fits always start with these fit parameters. Each time the set button is pressed the fit parameters are set new to the current values. To undo that the "Free/Fixed" button can be clicked. If it is set to free, then each fit starts with the resulting fit parameters from the last fit. This is useful where the correlations functions have a trend to shift over the image so that the first and last pixel have very different values but there is a smooth transition for the pixels in between.
6. All correlations and fitting is done now on a background thread in Java. This allows the user to continue working while the calculations are done.
7. A progress bar is now indicated instead of the counting of calculations to show the progress.

#### **q. Changes from 1.26 to 1.29**

1. A bug was fixed in the sliding window bleach correction which caused the correlation to be always calculated for a sub-stack starting with slide 1 (irrespective of what value was entered in the field "First frame").
2. A bug in DC-FCCS was fixed which was preventing fitting of the ACFs in the red channel, causing the program to abort calculating and fitting the correlation functions.
3. Updating of window names was introduced for all output windows; when output windows are re-used for results of correlation of a newly loaded image, their names are updated to match the name of the current image.

4. A bug was fixed in creating parameter maps in DC-FCCS; when x or y coordinate of the initial pixel of the green region was larger than 0, the bug resulted in parameters for only a part of the selected region being displayed in the map, the rest of the pixels in the parameter map containing zeros.
5. In DC-FCCS, parameter maps are shown for the cross-correlation as well as for the autocorrelations in both the green and the red channel in the following order: parameters for cross-correlation, parameters for green channel, parameters for red channel, Chi2 of CCF fit, Chi2 of green ACF fit and Chi2 of red ACF fit.
6. The fit model for 2 diffusive components was corrected as in the normalization the denominator was not raised to the second power. This influenced if at all only amplitude, no diffusion coefficients.
7. We introduced a new “Overlap” toggle button. This is important for binning. Previously only non-overlapping binned pixels were allowed. In this version the user can chose between overlapping and non-overlapping pixels.
8. We introduced the blocking Transform (3) to calculate the standard deviation of the ACFs. The blocked standard deviation also takes account of the noise correlations and should lead to better fits. Whether blocking was successful (and thus the data collection was sufficient) is shown in an own panel in the parameter maps. It is also saved along with the Fitting parameters.
9. The exponential bleach correction has been improved and now conserves the average and variance (17).
10. Fit parameters and fit functions can now be re-called by pressing on a pixel in the parameter maps.

## 19. Acknowledgements

The original program was written by Thorsten Wohland. Radek Machan was responsible for many bug fixes in the newer versions and work on the DC-FCCS part. Jagadish Sankaran wrote the MSD calculation algorithm and improved the data fitting part of the program. Mark Bathe and Syuan-Ming Guo wrote the first GLS and Bayesian Model Selection algorithms in Matlab (<http://fcs-bayes.org/>) and they helped Thorsten with the coding of the algorithm in Java. Jagadish Sankaran and Tang Wai Hoh wrote the CUDA code that enables computations on NVIDIA GPU. Daniel Aik wrote the Direct Camera Readout program interfacing with several cameras for real-time evaluation of FCS data. Many people in the lab checked the program for bugs which improved the program considerably. Any feedback or help in improving the program is welcome.

## Annex 1: Control Panel “ImFCS” Element

<b>Image</b>	<b>Use</b>	<b>Load</b>	<b>Batch</b>
First frame:	1	Last frame:	30000
Frame time:	0.00106	<b>Exp Set</b>	<b>Write Conf</b>
CCF distance:	0 x 0	<b>Binning</b>	1 x 1
Correlator P:	16	Correlator Q:	8
Fit Model:	ITIR-FCS (2...	FCCS Disp Off	Overlap Off
<b>Bgr NUM</b>	90	Background 2:	90
<b>LiveReadout</b>	<b>PVideo</b>		
<b>Options</b>	<b>N&amp;B Off</b>	<b>Threshold</b>	<b>Average</b>
<b>Scatter</b>	N vs D	<b>Bleach Cor</b>	none
<b>dCCF</b>	x direction	<b>Filter (All):</b>	none
<b>PSF</b>	<b>Diff. Law</b>	<b>Fit Off</b>	<b>All</b>
<b>Sim Off</b>	<b>Res. Table</b>	<b>MSD Off</b>	<b>ROI</b>
<b>To Front</b>	<b>Save</b>	<b>Read</b>	<b>Exit</b>

**Use:** Pressing the “Use” button will tell the program to use the uppermost image window in ImageJ for the correlations. This image has to be a 16 bit grey value stack, otherwise the program will provide an error message. If unsure which window is on top, you can just click the window you want to use before pressing the use button.

**Load:** This button will open a dialogue for loading an image file, which is then used in ImFCS.

**Batch:** Allows to evaluate several files with the same options. First the user will be asked which evaluations to perform. Then the user will be asked to select one or multiple files. The files will then be sequentially loaded and evaluated and the data will be saved in .x/sx files (see section 8).

**First Frame:** This is the first frame at which the correlator will start. This parameter is typically 1 but can be changed so that the correlations will start from any frame you chose. This allows the user to analyse correlations at any time within the image series. This might be important if the camera has an unstable baseline at the beginning of an image series, or if there are any other artefacts at the start of the imaging series.

**Last Frame:** This is the last frame the correlator will use. This parameter is set to the last frame of the loaded image. It can be changed if only part of the stack is to be correlated.

**Frame Time:** The user has to provide the acquisition time per frame which was used in the acquisition of the image stack. This will influence the time axis and the fit results. **Note:** *Changing this value at any time will lead to the reformatting of the parameter window and the deletion of all fit results.*

**Binning:** This allows binning multiple pixels into a larger pixel. Pressing this button will open a dialog where x- and y-binning values can be specified. **Note:** *Changing this value at any time will lead to the reformatting of the parameter window and the deletion of all fit results.*

**CF X Distance and CF Y Distance:** If spatial cross-correlations are to be calculated, this provides the distance in pixels (including binning) between the pixels to be correlated. These pixels are indicated in

the image as a blue and red frame (correlations are calculated from blue to red). **Note:** *Changing this value at any time will lead to the reformatting of the parameter window and the deletion of all fit results.*

**Correlator P:** The number of correlation channels for the first group of bin times. The bin time is equal to the Frame time/acquisition time. This can be set to 16 or 32. All following bin times will have half the number of channels, i.e. 8 or 16.

**Correlator Q:** This is the number of groups of bin times (including the first). The bin time within a group is constant but it will be always twice as large as the bin time of the preceding group.

Example: Using a Correlator P of 16 and a Correlator Q of 6 with a Frame time of 1 ms results in the following time points for the correlation:

Group 1: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16

Group 2: 18, 20, 22, 24, 26, 28, 30, 32

Group 3: 36, 40, 44, 48, 52, 56, 60, 64

Group 4: 72, 80 ...

Group 5: 144, 160, ...

Group 6: 288, 320 ... 512

ImFCS actually also calculates the 0 lagtime channel. It is an indication of the shot noise, but actually it is not used in data fitting.

**Fit Model:** There are two choices. First there is "FCS" which provides a fit for autocorrelation functions and spatial cross-correlation functions. The second option is "DC-FCCS" and provides dual-colour fluorescence cross-correlation fits. It is assumed that for DC-FCCS the image was split into two different wavelength ranges which were then imaged onto one camera so that a part of the camera contains the data for the shorter wavelength (green) and another part contains the data for the longer wavelength (red). The distance between the "green" and "red" pixels has to be provided in the "CF X distance" and "CF Y distance fields".

**FCCS Display:** If this is switched on (can only be used if the "DC-FCCS" fit model is chosen) then both ACFs and the CCF will be depicted. Otherwise only the CCF will be shown.

**Pixel Size [µm]:** Size of the actual pixel on the camera chip. This is typically in the range between 5 – 25 µm and can be obtained from the camera datasheet.

**Overlap:** This is a toggle button to switch overlap on/off. This has meaning only when binning is used. In the case of binning, one can choose whether one wants to obtain only non-overlapping pixels, or whether one wants to have overlapping pixels. The overlap mode can be practical in spatial correlation or FCCS measurements.

**Magnification:** Magnification of the optical system. This is required to calculate the pixel size in object space (=pixel size/magnification).



**NA:** numerical aperture of the microscope objective, used to calculate the PSF.

**Em. Wavelength 1:** Wavelength of emission,  $\lambda_{em}$ , for the fluorophore used. This value is used to calculate the PSF.

**Em. Wavelength 2:** (DC-FCCS only) Wavelength of emission,  $\lambda_{em}$ , for the second fluorophore used in the case of DC-FCCS. This value is used to calculate the PSF.

**lateral PSF (xy):** This value, which we will call  $\sigma_0$ , characterizes the  $1/e^2$  radius of the PSF in the lateral direction (along the light sheet). Which is given by  $\sigma_0 \lambda_{em}/NA$ . A typical value is 0.8 (note that in the past we also used the  $1/\sqrt{e}$  radius which is just half of the  $1/e^2$  radius; so in that case the value would have been 0.4; we decided to switch to the usage of the  $1/e^2$  radius as this is the common figure used in FCS).

**axial PSF (z):** This value, which we will call  $\sigma_z$ , characterizes the  $1/e^2$  radius of the PSF in the axial direction (across the light sheet), which is given by  $\sigma_0 \lambda_{em}/NA$ . To obtain good values the light sheet thickness needs to be measured. If a 2D correlation is desired, the value can be set to a large number (the default is 1000000) in which case the thickness plays no role in the correlation and essentially a 2D fit is performed (e.g. for imaging total internal reflection FCS or ITIR-FCS).

Assuming a Gaussian light sheet cross section in the z-direction, the following relations hold between the  $1/e^2$  radius ( $\omega$ ) and the full width half maximum (FWHM):

$$\omega = FWHM/\sqrt{2\ln(2)} = \sigma_0 \lambda_{em}/NA$$

$$\sigma_0 = NA*FWHM/(\lambda_{em} * \sqrt{2\ln(2)})$$

Here NA is the numerical aperture of the detection objective and  $\lambda_{em}$  is the emission wavelength of the fluorophore observed.

Note that the observation volume is defined by the light sheet thickness and the pinhole effect of the pixel. Similar to the pinhole in a confocal system, the pixel will detect light from different planes differently. The user should take account of this fact when providing the axial PSF (REF).

**lateral PSF2 (xy):** (DC-FCCS only) Same as above for the second, red wavelength range for DC-FCCS.

**axial PSF2 (z):** (DC-FCCS only) Same as above for the second, red wavelength range for DC-FCCS.

**Bgr NUM (button):** For this to work the user needs a “background” file, which is a file recorded with the shutter closed at the exact same location, with the exact same parameters and the same number of frames as the actual data file. EMCCDs show often patterns even on images taken with the shutter closed. This pattern can depend on the column, row or pixel (18). Here, we decided to correct every pixel for its noise individually. After this correction the Background (text field) and Background2 (text field) are automatically set to 0. Overall this correction is not crucial in FCS as the signal-to-noise ratio is typically quite large and thus the patterned noise is negligible. However, at very low intensities, in N&B analysis, one can sometimes see that pattern and a correction is required.

**Background (text field):** This value is automatically determined as the smallest pixel value in the stack to avoid negative values. But it can be changed manually. This will influence the amplitude and thus the parameter N in the fits.

**Background2 (text field):** (DC-FCCS only) This can be adjusted in case the data for the red channel has a different background. The default value is the same as Background. If the two channels have different backgrounds, then both, Background and Background 2 have to be adjusted manually.

**Options:** Select which plots (ACF, SD, residuals, histograms etc.) should be shown.

**N&B:** Number and Brightness analysis.

**Threshold:** This will open a window in which the user can filter the fit results by parameter ranges. This allows the exclusion of outliers from the data.

**Average:** Calculate the average correlation function for all valid pixels, and if fit is on, fit the data. The results will be shown in the fit window.

**Scatter:** Button to perform a scatter plot of fit parameters. The drop down list next to it provides the different parameter combinations possible. Possible selections are "N vs D", "N vs F2", "D vs F2", "N\*(1-F2) vs D", "N\*F2 vs D2", "D vs  $\sqrt{v_x^2 + v_y^2}$ " and "D2 vs  $\sqrt{v_x^2 + v_y^2}$ ". After selection press scatter to produce a scatter plot.

**Bleach Cor.:** If the intensity traces show bleaching, this can be corrected by three different methods. The different methods will ask for separate inputs if needed. A) sliding window: by defaults this divides the intensity trace in 20 smaller windows of equal size (this can be changed in the accompanying dialog by giving the desired window size). For each set of frames the correlation function is calculated and then all correlation functions are averaged. As the intensity is not changed the intensity trace in the "Intensity Trace" plot will still show the decay due to bleaching. Note: if the windows are set too small, this can lead to biased diffusion coefficients. B) single exponential: a single exponential is fit to the intensity trace and the trace is corrected. The corrected intensity trace will be shown in the "Intensity Trace" plot. C) double exponential: a double exponential is fit to the intensity trace and the trace is corrected. The corrected intensity trace will be shown in the "Intensity Trace" plot. D) polynomial fit: a dialog is shown in which the order of the polynomial to be used can be given. Typically one should stay with 4 or less. E) line segment: As in sliding window, the window size can be set here. Within the window size the intensity is corrected for bleaching by linear fits.

**DCCF:** DCCF function which calculates the difference of the forward and backward correlation function. The DCCF can be calculated in different direction (x, y, and diagonal up or diagonal down) as selected from the drop down menu next to the button. A DCCF image and a histogram is produced.

**Filter (All):** One can correlate all pixels which fall into a certain intensity range. This is useful if one wants to avoid fitting background values, if e.g. not all pixels in an image contain the sample. One can choose either to filter according to the intensities of the first frame in the stack ("Intensity") or to the mean value ("Mean") of the whole stack. This selection has to be made before the "All" button is pressed. Accompanying dialogs will ask for the limits.

**PSF:** This will ask the user first for a range of PSF values to be used (start value, end value, step size). Typically one should not use more than 5 parameter values at the same time as the calculation can become slow (e.g. Start 0.7, end is 1.1, step size 0.1). The program will then plot the diffusion coefficients fitted by a simple one component model to the sample for binning values from 1-5. As the diffusion coefficient is a constant the correct PSF value should lead to a straight line.

**Diff. law:** This will open a diffusion law panel to plot the FCS diffusion law for an FCS image. As this requires the calculation of correlations over the whole image several times, this is a bit slower than e.g. fitting all correlations in an image.

**Fit on/off:** The user can decide whether a fit is to be performed or not. The fits will be performed each time a correlation is done. This is used for “Correlate Single”, “Correlate All”, or when the user clicks a pixel in the window. If the fit is switched on a fit parameter window is opened where starting values can be given and results are shown. In addition a new image stack is created in which the fit results are stored in parameter maps. Averages for the parameter maps are given in the title line above the map.

**“All”:** Correlate all pixels and plot the correlation functions. This works for auto- and cross-correlations and calculates all possible correlations. If fit is on, then the correlations will also be fitted. If MSD is on the MSDs will also be calculated.

**Sim on/off:** Provides a simulator to simulate 2D or 3D data with up to 3 components and free choice of experimental conditions.

**Res. Table:** Creates or updates the result table with the current values of all evaluated data (ACF, SD, fits etc.). Note that the table is not automatically updated. The user has to press this button again to update the table.

**MSD:** If this button is set to “MSD On” then MSD is calculated along with the correlation functions and displayed in a separate window. A dialogue will be called when switching MSD on to determine whether 2D or 3D MSD calculations are to be done.

**“ROI”:** After having chosen a region of interest (ROI) pressing the button “ROI” will correlate all pixels within the ROI. If fit is on, then the correlations will also be fitted. If MSD is on the MSDs will be calculated along.

**To Front:** Brings windows related to this instance of the plugin to front. This is useful for finding the windows; in particular in the case that several instances of the plugin are run simultaneously.

**Experiment “Save”:** This button opens a file dialog window in which the location and name of the .x/sx file, which contains all evaluation data, can be chosen.

**Experiment “Read”:** This button opens a file dialog window in which the experimental .x/sx file can be chosen. Note that to load an experiment one has to load the correct image from which the fits were produced.

**Write Conf:** Store ‘ImFCSconfig’ file at user.home directory

**PVideo:** Opens a dialog box to produce video of parameter maps (currently D and N) from the image stack loaded previously

**LiveReadout:** This button opens a window in which camera can be controlled.

**Exit:** Closes the program and all windows, except the image window. But it removes all items (listeners and overlays in Java) that were added to the image window.

## Annex 2: Fitting Functions

Note that the full calculations for the fitting functions have been provided in the literature (10, 13, 19-24) and can also be obtained from interactive CDF files on our website. Therefore, we do not provide the full derivations here. In particular, we left out all calculations of the effective volumes and just included their results in the final functions.

**A note on the SPIM model:** At the moment, the given fitting function does not take into account cross-talk. It will fit ACFs but the recovered D is slightly biased (up to 30%). But it will give incorrect and distance dependent D values for spatial CCFs. A new model will be provided in the next upgrade.

The autocorrelation function  $G(\tau)$  is typically expressed as

$$G(\tau) = g(\tau) + G_{\infty}$$

where  $g(\tau)$  is the temporal part of the autocorrelation function and  $G_{\infty}$  is the value of convergence for  $\tau \rightarrow \infty$ . The value of  $G_{\infty}$  is 1 or 0 depending on the definition of the autocorrelation function (see our website [here](#); note that you need the CDF plugin from Wolfram, which is freely downloadable, to see this interactive pages). It is typically left as a fit parameter as the condition  $\tau \rightarrow \infty$  cannot be fulfilled. The value of  $G_{\infty}$  differs from its ideal value usually very little, within a range of a few %.

If the amplitude  $G(0)$  of the autocorrelation function  $G(\tau)$  is inversely proportional to the number of particles in the observation volume, then

$$G(0) = \frac{1}{N} + G_{\infty}$$

For a full derivation see (10, 23). Therefore, we can write

$$G(\tau) = \frac{1}{N} \frac{g(\tau)}{g(0)} + G_{\infty}$$

Below we will give the functions of  $g(\tau)$  from which  $g(0)$  and  $G(\tau)$  can be derived. Note that this definition is used in all model function in this plugin. However, it makes strictly only sense for the temporal auto- and cross-correlations without a spatial shift. For spatial cross-correlations N is more difficult to interpret as it depends on the particles that pass from one area to the next and the other particles that contribute to the fluorescence but not to the correlations.

We provide in this plugin 2 different general correlation functions which can then be modified by fixing different parameters to obtain one and two component models with diffusion, flow or both.

The model for diffusion and flow for one particular component is given by

$$\begin{aligned}
g_i(\tau) = & \left[ \sqrt{\frac{4D_i\tau + \sigma_{xy}^2}{\pi}} \left( e^{-\frac{(a+r_x-\tau v_x)^2}{4D_i\tau + \sigma_{xy}^2}} + e^{-\frac{(a-r_x+\tau v_x)^2}{4D_i\tau + \sigma_{xy}^2}} - 2e^{-\frac{(r_x-\tau v_x)^2}{4D_i\tau + \sigma_{xy}^2}} \right) + (a+r_x-\tau v_x) \operatorname{Erf} \left( \frac{(a+r_x-\tau v_x)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right. \\
& \left. + (a-r_x+\tau v_x) \operatorname{Erf} \left( \frac{(a-r_x+\tau v_x)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) - 2(r_x-\tau v_x) \operatorname{Erf} \left( \frac{(r_x-\tau v_x)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right] \\
& \times \left[ \sqrt{\frac{4D_i\tau + \sigma_{xy}^2}{\pi}} \left( e^{-\frac{(a+r_y-\tau v_y)^2}{4D_i\tau + \sigma_{xy}^2}} + e^{-\frac{(a-r_y+\tau v_y)^2}{4D_i\tau + \sigma_{xy}^2}} - 2e^{-\frac{(r_y-\tau v_y)^2}{4D_i\tau + \sigma_{xy}^2}} \right) + (a+r_y-\tau v_y) \operatorname{Erf} \left( \frac{(a+r_y-\tau v_y)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right. \\
& \left. + (a-r_y+\tau v_y) \operatorname{Erf} \left( \frac{(a-r_y+\tau v_y)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) - 2(r_y-\tau v_y) \operatorname{Erf} \left( \frac{(r_y-\tau v_y)}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right] \\
& \times \left( 1 + \frac{4D\tau}{\sigma_z^2} \right)^{-1/2}
\end{aligned}$$

Here the first two terms describe the diffusion and flow in the xy directions along the pixel, and the last term describes the diffusion (no flow is incorporated at the moment for the SPIM case) in the z direction, orthogonal to the pixels and across the light sheet.

The final function for 2 particles, which takes account of the molecular brightness (24) is then:

$$G(\tau) = \frac{(1-f_2) \frac{g_1(\tau)}{g_1(0)} + q^2 f_2 \frac{g_2(\tau)}{g_2(0)}}{N(1-f_2+f_2q)^2} + G_\infty$$

Simplified models can be obtained by setting particular parameters to 0 (1, 10). A simple autocorrelation model without flow and spatial shift, for instance, can be achieved by setting all velocities  $v$  and displacements  $r$  to 0.

$$\begin{aligned}
g_i(\tau) &= \left( \frac{\sqrt{4D_i\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D_i\tau + \sigma_{xy}^2}} - 1 \right) + \operatorname{Erf} \left( \frac{a}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right)^2 \times \left( 1 + \frac{4D\tau}{\sigma_z^2} \right)^{-1/2} \\
g_i(0) &= \left( \frac{\sigma_{xy}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{\sigma_{xy}^2}} - 1 \right) + \operatorname{Erf} \left( \frac{a}{\sigma_{xy}} \right) \right)^2
\end{aligned}$$

and

$$G(\tau) = \frac{1}{N} \frac{g_i(\tau)}{g_i(0)} + G_\infty$$

$$= \frac{1}{N} \frac{\left( \frac{\sqrt{4D_i\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{\frac{a^2}{4D_i\tau + \sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D_i\tau + \sigma_{xy}^2}} \right) \right)^2 \times \left( 1 + \frac{4D\tau}{\sigma_z^2} \right)^{-1/2}}{\left( \frac{\sigma_{xy}}{a\sqrt{\pi}} \left( e^{\frac{a^2}{\sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sigma_{xy}} \right) \right)^2} + G_\infty$$

The **observation volumes** in imaging FCS are given by

$$A_{eff}(\tau) = \frac{a^2}{\left( \frac{\sigma_0}{a\sqrt{\pi}} \left( e^{\frac{a^2}{\sigma_0^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sigma_0} \right) \right)^2}$$

for ITIR-FCS, and by

$$V_{eff} = \frac{\sqrt{\pi} a^2 \sigma_z}{\left( \frac{\sigma_{xy}}{a\sqrt{\pi}} \left( e^{\frac{a^2}{\sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sigma_{xy}} \right) \right)^2}$$

for SPIM-FCS.

For DC-FCCS we provide here the simplest cross-correlation function. It assumes one diffusion coefficient and light sheets with different size. But both light sheets are perfectly centred:

$$G_{SPIM}(\tau) = \frac{1}{N} \frac{\left( \frac{\sqrt{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}}{a\sqrt{\pi}} \left( e^{\frac{a^2}{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} \right) \right)^2 \times \left( 1 + \frac{8D\tau}{\sigma_{z,01}^2 + \sigma_{z,02}^2} \right)^{-1/2}}{\left( \frac{\sqrt{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}}{a\sqrt{\pi}} \left( e^{\frac{a^2}{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} \right) \right)^2} + G_\infty$$

In the plugin we allow a possible shift in the light sheet direction ( $r_z$ ) but assume that the pixels for the cross-correlations are perfectly aligned. This leads to the following function (13).

$$G_{SPIM}(\tau) = \frac{1}{N} \frac{\left( \frac{\sqrt{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D\tau + \frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} \right) \right)^2 \times \left( 1 + \frac{8D\tau}{\sigma_{z,01}^2 + \sigma_{z,02}^2} \right)^{-1/2} e^{-\frac{2r_z^2}{8D\tau + \sigma_{z,01}^2 + \sigma_{z,02}^2}}}{\left( \frac{\sqrt{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{\frac{\sigma_{01}^2}{2} + \frac{\sigma_{02}^2}{2}}} \right) \right)^2 e^{-\frac{2r_z^2}{\sigma_{z,01}^2 + \sigma_{z,02}^2}}} + G_{\infty}}$$

The Java code for Imaging FCS contains the functions for the most general two component cases and their derivatives for all parameters for data fitting.

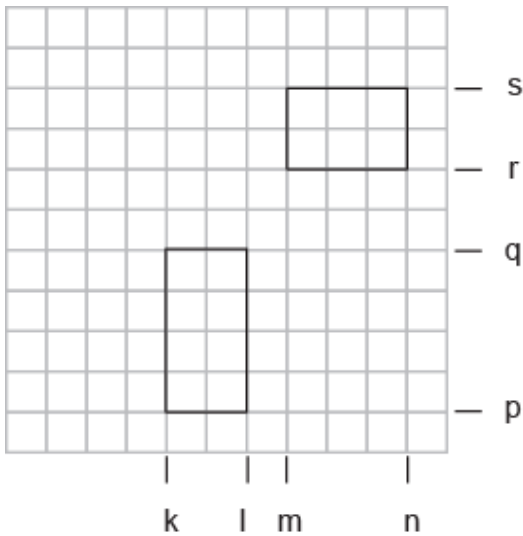
### Triplet

For all functions a triplet can be added by setting the  $F_{trip}$  fraction to a value between 0-1 and freeing the parameter for the fit (switch off the “Hold” radio button). Then the following function will be multiplied to the correlation function (excluding  $G_{\infty}$ ):

$$G_{Trip}(\tau) = 1 + \frac{F_{Trip}}{1 - F_{Trip}} \exp \left( -\frac{t}{\tau_{Trip}} \right)$$

### Non-square, rectangular binning

In Fig. A2-1 we show the general definition for the derivation of the function. However, we implemented at the moment rectangular areas of the same dimensions and orientations only. This means that  $(k-l) = (m-n)$  and  $(q-p) = (s-r)$ .



**Fig. A2-1:** Schematic representation of the regions on a CCD chip which were used for the derivation of the generalized cross-correlation functions of ITIR-FCCS.



The function was originally derived in ref [1]:

$$g_{general}(\tau) = \frac{1}{(4 \langle C \rangle (k-l)(m-n)(p-q)(r-s))} \times$$

$$\left[ \begin{aligned} & \frac{2\sqrt{D\tau + \sigma^2}}{\sqrt{\pi}} \left( e^{-\frac{(l-m+v_x\tau)^2}{4(D\tau + \sigma^2)}} + e^{-\frac{(k-n+v_x\tau)^2}{4(D\tau + \sigma^2)}} - e^{-\frac{(l-n+v_x\tau)^2}{4(D\tau + \sigma^2)}} - e^{-\frac{(k-m+v_x\tau)^2}{4(D\tau + \sigma^2)}} \right) \\ & + (l-m+v_x\tau) \operatorname{erf}\left(\frac{l-m+v_x\tau}{2\sqrt{D\tau + \sigma^2}}\right) + (k-n+v_x\tau) \operatorname{erf}\left(\frac{k-n+v_x\tau}{2\sqrt{D\tau + \sigma^2}}\right) \\ & - (l-n+v_x\tau) \operatorname{erf}\left(\frac{l-n+v_x\tau}{2\sqrt{D\tau + \sigma^2}}\right) - (k-m+v_x\tau) \operatorname{erf}\left(\frac{k-m+v_x\tau}{2\sqrt{D\tau + \sigma^2}}\right) \end{aligned} \right] \times$$

$$\left[ \begin{aligned} & \frac{2\sqrt{D\tau + \sigma^2}}{\sqrt{\pi}} \left( e^{-\frac{(q-r+v_y\tau)^2}{4(D\tau + \sigma^2)}} + e^{-\frac{(p-s+v_y\tau)^2}{4(D\tau + \sigma^2)}} - e^{-\frac{(q-s+v_y\tau)^2}{4(D\tau + \sigma^2)}} - e^{-\frac{(p-r+v_y\tau)^2}{4(D\tau + \sigma^2)}} \right) \\ & + (q-r+v_y\tau) \operatorname{erf}\left(\frac{q-r+v_y\tau}{2\sqrt{D\tau + \sigma^2}}\right) + (p-s+v_y\tau) \operatorname{erf}\left(\frac{p-s+v_y\tau}{2\sqrt{D\tau + \sigma^2}}\right) \\ & - (q-s+v_y\tau) \operatorname{erf}\left(\frac{q-s+v_y\tau}{2\sqrt{D\tau + \sigma^2}}\right) - (p-r+v_y\tau) \operatorname{erf}\left(\frac{p-r+v_y\tau}{2\sqrt{D\tau + \sigma^2}}\right) \end{aligned} \right]$$

### Annex 3: Mean Square Displacement

For the sake of simplicity for the derivation, the following definition of  $G(\tau)$  is used ignoring  $G_\infty$ . In the case of SPIM-FCS

$$G(\tau) = \frac{1}{N} \frac{g(\tau)}{g(0)}, \text{ where}$$

$$g(\tau) = \left( \frac{\sqrt{4D\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D\tau + \sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}} \right) \right)^2 \times \left( 1 + \frac{4D\tau}{\sigma_z^2} \right)^{-1/2} \text{ Hence } G(0) = \frac{1}{N}$$

$$G(\tau) = \frac{G(0)V_{eff}}{\sqrt{\pi}a^2w_z} \left( \frac{\sqrt{4D\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D\tau + \sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}} \right) \right)^2 \times \left( 1 + \frac{4D\tau}{\sigma_z^2} \right)^{-1/2}$$

$$G(\tau) = \frac{G(0)V_{eff}}{\sqrt{\pi}a^2w_z} \left( \frac{\sqrt{4D\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \frac{\left( e^{-\frac{a^2}{4D\tau + \sigma_{xy}^2}} - 1 \right)}{\sqrt[4]{1 + \frac{4D\tau}{\sigma_z^2}}} + \frac{\text{Erf} \left( \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}} \right)}{\sqrt[4]{1 + \frac{4D\tau}{\sigma_z^2}}} \right)^2$$

This equation needs to be solved for  $4D\tau$

Substituting  $x = \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}}$ ,  $p = 1 - \frac{\sigma_{xy}^2}{\sigma_z^2}$  and  $q = \frac{a^2}{\sigma_z^2}$  sequentially, we get

$$G(\tau) = \frac{G(0)V_{eff}}{\sqrt{\pi}a^2w_z} \left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}\sqrt[4]{p + \frac{q}{x^2}}} + \frac{\text{Erf}(x)}{\sqrt[4]{p + \frac{q}{x^2}}} \right)^2$$

$$\left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}\sqrt[4]{p + \frac{q}{x^2}}} + \frac{\text{Erf}(x)}{\sqrt[4]{p + \frac{q}{x^2}}} \right)^2 - \text{coeff}_0 \times G(\tau) = 0, \text{ where } \text{coeff}_0 = \frac{\sqrt{\pi}a^2w_z}{G(0)V_{eff}}$$

Taylor series expansion yielded a diverging series. Hence a Pade approximation was used. The function used in Mathematica® is called PadeApproximant. The details of the function can be found here (<http://reference.wolfram.com/language/ref/PadeApproximant.html>). A discussion on the effectiveness of Pade Approximation in Math overflow is found here (<http://mathoverflow.net/questions/122539/the-unreasonable-effectiveness-of-pade->

[approximation](http://en.wikipedia.org/wiki/Pade%20approximation)). The article explaining Pade Approximation in Wikipedia can be accessed here. (<http://en.wikipedia.org/wiki/Pade%20approximation>).

Terms up to order 5 were needed to approximate the function. The relative error between the approximation and the original function is 0.7%.

Performing a Pade approximation for  $\left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}\sqrt[4]{p + \frac{q}{x^2}}} + \frac{\text{Erf}(x)}{\sqrt[4]{p + \frac{q}{x^2}}} \right)^2$  using Mathematica®, we get

$$\frac{\text{coeff}_5 \times x^5 - \text{coeff}_3 \times x^3}{\text{coeff}_4 \times x^4 + \text{coeff}_2 \times x^2 - 1}$$

$$\text{coeff}_5 = \frac{p_2 + 126pq^2 - 44q^3}{126\pi q^{\frac{3}{2}} p_1}$$

$$\text{coeff}_4 = \frac{14175p^4 + 37800p^3q - 30240p^2q^2 - 3840pq^3 - 1132q^4}{7560q^2 p_1}$$

$$\text{coeff}_2 = \frac{p_2 - 420pq^2 - 64q^3}{63qp_1}$$

$$\text{coeff}_3 = \frac{1}{\pi\sqrt{q}}$$

$$p_2 = 945p^3 - 630p^2q \text{ and } p_1 = -15p^2 + 20pq + 2q^2$$

$$\text{coeff}_5 \times x^5 - \text{coeff}_4 \times \text{coeff}_0 \times g_i(\tau) \times x^4 - \text{coeff}_3 \times x^3 - \text{coeff}_2 \times \text{coeff}_0 \times g_i(\tau) \times x^2 + \text{coeff}_0 \times g_i(\tau) = 0$$

This polynomial in  $x$  is solved using a Brent solver. The upper bound of  $x$  at  $t=0$  is  $\frac{a}{\sigma_{xy}}$ . The lower

bound of  $x$  as  $t$  tends to  $\infty$  is 0. Hence the Brent solver is programmed with these two limits.

The solution obtained for  $x$  is back transformed to yield  $4D\tau$ . This value is multiplied by 1.5 to yield the mean squared displacement in 3D ( $6D\tau$ ) since these samples are in 3D.

### TIRF approximation

Similar to the SPIM case, in the case of TIRF,  $G(\tau) = \frac{1}{N} \frac{g(\tau)}{g(0)}$  where

$$g(\tau) = \left( \frac{\sqrt{4D\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D\tau + \sigma_{xy}^2}} - 1 \right) + \text{Erf} \left( \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}} \right) \right)^2$$

Hence  $G(0) = \frac{1}{N}$

$$G(\tau) = \frac{G(0)A_{eff}}{a^2} \left( \frac{\sqrt{4D\tau + \sigma_{xy}^2}}{a\sqrt{\pi}} \left( e^{-\frac{a^2}{4D\tau + \sigma_{xy}^2}} - 1 \right) + Erf\left(\frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}}\right) \right)^2$$

This equation needs to be solved for  $4D\tau$

Substituting  $x = \frac{a}{\sqrt{4D\tau + \sigma_{xy}^2}}$

$$G(\tau) = \frac{G(0)A_{eff}}{a^2} \left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}} + Erf(x) \right)^2$$

$$\left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}} + Erf(x) \right)^2 - coeff_0 \times G(\tau) = 0$$

Where  $coeff_0 = \frac{a^2}{G(0)A_{eff}}$

Performing a Taylor series expansion of up to power 8 of  $\left( \frac{(e^{-x^2} - 1)}{x\sqrt{\pi}} + Erf(x) \right)^2$

yields  $\frac{29}{1260\pi}x^8 - \frac{17}{180\pi}x^6 + \frac{1}{3\pi}x^4 - \frac{1}{\pi}x^2$

The relative error between the approximation and the original function is less than 0.5%.

$$\frac{29}{1260}x^8 - \frac{17}{180}x^6 + \frac{1}{3}x^4 - x^2 + \pi \times coeff_0 \times G(\tau) = 0$$

Substitute  $t = x^2$

$$t^4 - \frac{\frac{17}{180}}{\frac{1260}{1260}}t^3 + \frac{\frac{1}{3}}{\frac{1260}{1260}}t^2 - \frac{1}{\frac{1260}{1260}}t + \frac{\pi \times coeff_0 \times G(\tau)}{\frac{1260}{1260}} = 0$$

This equation is quartic in  $t$  and a solver has been implemented based on the algorithm provided in IgorPro 6 (Wavemetrics®) to solve for  $t$  (solvequartic and getonecubicroot functions). The obtained values of  $t$  are later used to calculate  $x$  which yields the value of  $x$  later.

## Annex 4: Simulation

The simulation module offers the opportunity to simulate different situations and test the results against real measurements. At the moment it offers 2D and 3D simulations for ITIR-FCS and SPIM-FCS situations. A number of the simulation parameters are taken from the ImFCS panel. This includes pixel size, the NA, magnification, wavelength, and PSF and light sheet thickness (see lateral and axial PSF). Other parameters can be directly controlled in the simulation panel.

Simulation Panel		Triplet off	
Mode	2D (free) ▼	Particle #	1000
Seed	1	Bleach time	0
CPS	10000	Extension	1.5
Pixel #	21	Time res	0.001
Frame #	30000	Step Size [nm]	20
Steps per Frame	10	Dout/Din	1.0
D1 [um2/s]	1.0	F2	0.0
D2 [um2/s]	0.1	F3	0.0
D3 [um2/s]	0.01	koff (triplet)	700
kon (triplet)	300	Cam Noise	3
Cam Offset	100	FRAP Frame	1000000
FRAP Radius [...]	3.0	Dom Density	30
Dom Rad [nm]	30	Pout	0.6
Pin	1.0	Hop Prob	1
Mesh Size [nm]	100		
Batch		Stop	Simulate

**Mode:** Diffusive modes to be simulated (2D, 3D, free, domain trapping, hop diffusion).

**Triplet:** A triplet can be included in the simulations, although a triplet can be seen in measurements only for very fast array detectors.

**Seed:** Seed for the random number generator. This ensures reproducibility. Has to be an integer.

**Particle #:** The number of particles to be simulated. Has to be an integer.

**CPS:** Counts per Particle and Second; this provides a measurement for the brightness of the probes. Has to be an integer.

**Bleach Time:** Average bleaching time to simulate photobleaching. This is determined by an exponential  $\text{Exp}[-t/\text{bleachtime}]$ .

**Pixel #:** The pixel number  $n$  provides the dimension of the detection area ( $n \times n$ ). At the moment only square areas are allowed. Has to be an integer.

**Extension:** The factor by which the simulated area is larger than the detection area.

**Frame #:** number of frames to be simulated. Has to be an integer.

**Time res:** This is the frame time.

**Steps per frame:**

**Step Size [nm]**

**D1, D2, D3:** diffusion coefficients in  $\text{um}^2/\text{s}$  of up to 3 components.

**F2, F3:** fraction of the last two components (has to be between 0 and 1).  $F1 = 1 - F2 - F3$ . Please note that as the number of particles must be an integer, the values  $F1 \cdot N$ ,  $F2 \cdot N$ ,  $F3 \cdot N$ , have to be rounded. When simulating many particles this effect will be negligible. But when simulating few particles it can lead to slight deviations.

**Dout/Din:** If domains are simulated this provides the ratio between the diffusion coefficient outside and the inside of domains.

**Camera offset:** A constant offset for the camera.

**Camera noise factor:** Mean of Poisson Generated Noise

**FRAP Radius:** Only available in 2D. Radius of a circular area to be bleached in the centre of the image.

**FRAP Frame:** Only available in 2D. Frame at which bleaching happens. Bleaching is instantaneous.

**Dom Rad [nm]:** Average domain radius. The program creates domains with this value as average and a 10% standard deviation in sizes. *Note: The system can only simulate domain sizes that are smaller than 3 times the pixel size. For larger domains the results will be incorrect.*

**Dom Density:** Number of domains per  $\mu\text{m}^2$ .

**Pin, Pou:** Probability to enter or exit a domain.

**MeshSize [nm]:** If a meshwork is simulated this is the mesh size. At the moment only square meshes are allowed.

**Hop Prob:** Probability to hop over a barrier in a meshwork.

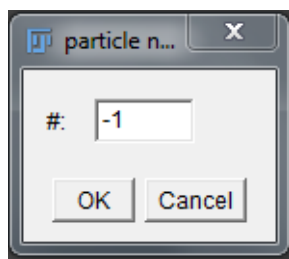
**Batch:** Allows to produce multiple simulations. At the moment this allows only a variation of D, D2 and F2. This will be further extended in the future.

The simulator also offers to plot the coordinates of one of the particles in the log window. The format is:

$\{\{x_1, y_1\}, \{x_2, y_2\}, \dots, \{x_n, y_n\},\}$

For that purpose after the start of the simulation a dialog window is opened where the number of the particle, for which the coordinates are to be plotted, can be provided. The default is -1, which means no particle. Putting a number in between 0 and the number of particles simulated minus 1 will then result in the coordinates to be printed in the log window. This is a temporary measure to allow users to have access and check some of the trajectories.

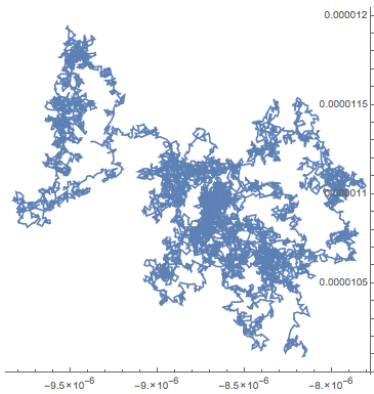
**Note:** Use this feature with care as the printout slows down the simulations and can lead to large printout lists. We suggest to use not more than 1000 values to print out, e.g. simulate 100 frames with 10 steps per frame.



Simulation output: The user can choose one particle for which the coordinates are printed out in the log window.

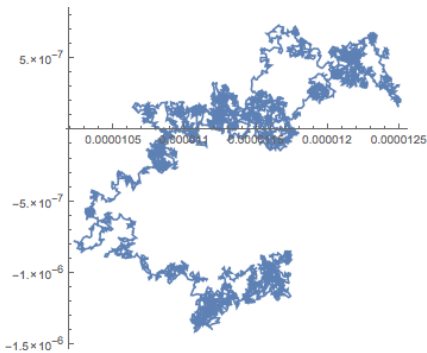
### Example trajectories of simulations:

#### *Free Diffusion*

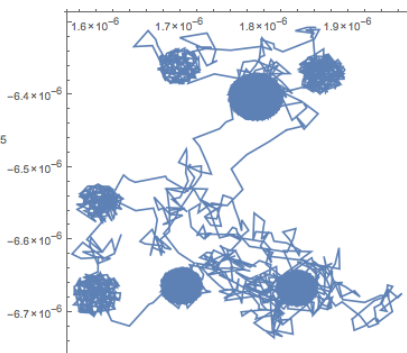


**Domain Diffusion**

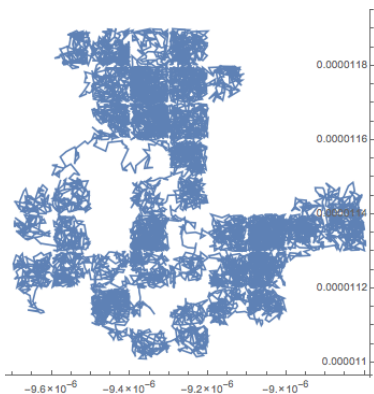
Large field of view (~2 um)



small field of view (~400 nm)



**Meshwork Diffusion**



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