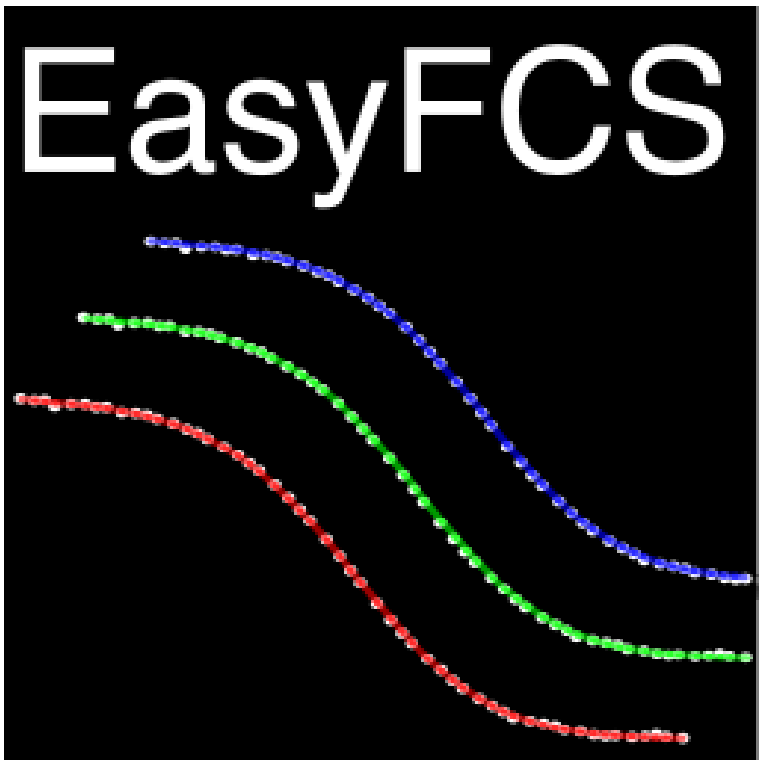
EasyFCS manual

Simple Analysis Software

for FCS Data

version 2.1



https://github.com/ayleray/EasyFCS

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1 Introduction

This manual describes the installation and operation of EasyFCS. This software gives you access to several tools for analyzing data of fluorescence correlation spectroscopy (FCS). For instance, with this software, you can easily calculate the auto-correlation of fluorescence traces and fit the data with different models.

EasyFCS is under constant development. We appreciate any feedback on bugs, missing features or other inconveniences. We hope you will enjoy analyzing your FCS data with this software.

2 Installation

When you install EasyFCS for the first time, you have to install first the Matlab Runtime and then EasyFCS.

2.1 System requirements

Before installing EasyFCS, please make sure that your PC meets the following requirements :

— Windows11 or Windows 10 (version 21H2 or higher) operating system

— Any Intel or AMD x86-64 processor with two or more cores

— 8 GB internal memory (RAM). 16 GB is recommended

— 4-6 GB for storage for a typical installation

2.2 Matlab Component Runtime

The Matlab Component Runtime should be installed before installing EasyFCS for the first time. It can be find here :

https://fr.mathworks.com/products/compiler/matlab-runtime.html

Please download, extract and install the Matlab Runtime by following the instructions. Once it is done, you can launch EasyFCS.

2.3 EasyFCS

To launch the MAPI software, double click the `EasyFCS.exe` file located in the same folder.

The following window will appear (Figure 1) :

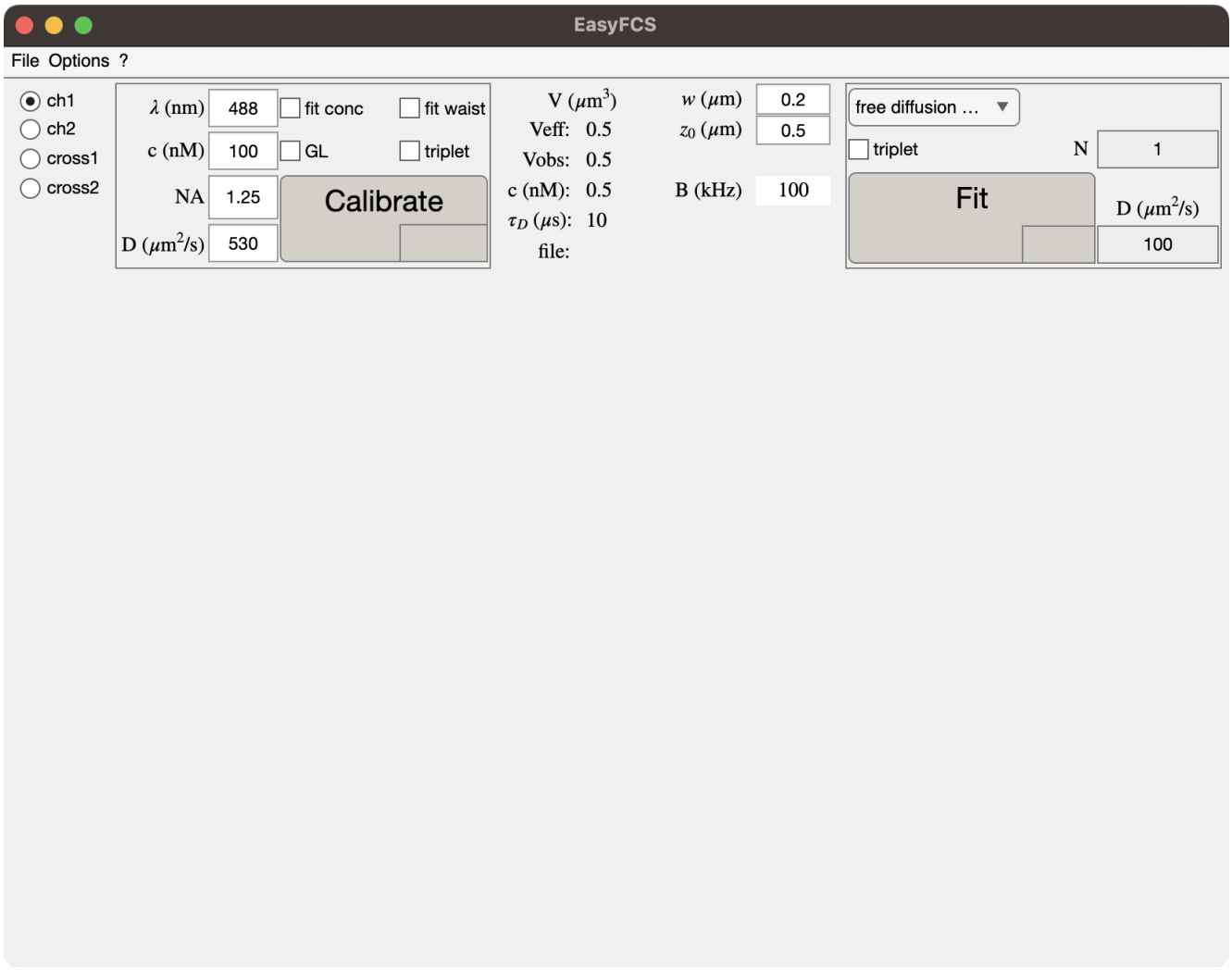

Figure 1: *The Main EasyFCS software window*

For having access to the documentation file with EasyFCS, you just need to click on the help menu (" ?") and select "Documentation".

You are now ready to use EasyFCS.

3 Quick start guide

This tour will guide you through the process of analyzing FCS data with EasyFCS. The purpose of this chapter is to introduce you the most common tools that you can do with EasyFCS : opening a file and using the interactive interface.

If you come across some menu options or toolbar buttons in MAPI software, that are not explained in this quick start guide, please refer to the section 4 for a complete reference of all options, features and functionality of EasyFCS.

If you want to know more details about the mathematical equations enabling the fluorescence lifetime data analysis, you should read the section 5.

3.1 Opening a file

To launch EasyFCS, double click the `EasyFCS.exe` file. Then, you will see the main window (Figure 1). To open some data file, click on the “File” menu and the “Open...” menu item. The following window will appear (Figure 2) :

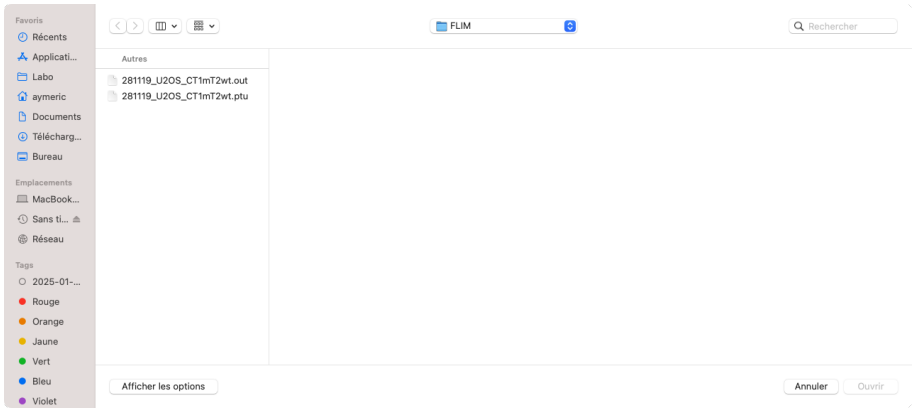

Figure 2: *The “Select the File” window*

Then, select a data file and click on the “Open” button. The following window will then pop up (Figure 3) :

You need to enter the different parameters for calculating the auto-correlation. The parameters “max time” and “min time” correspond respectively to the maximal and minimal times of the auto-correlation.

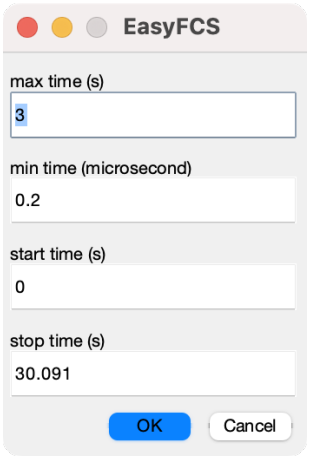

Figure 3: *The "File" option window*

The parameters "start time" and "stop time" correspond to minimal and maximal experimental times of the data to use in the auto-correlation calculation. In this example, the time trace was recorded during almost 30 s and we will use the complete time trace for calculating the auto-correlation. When all the values are fixed, click on the "OK" button, the main window of EasyFCS becomes as indicated in Figure 4).

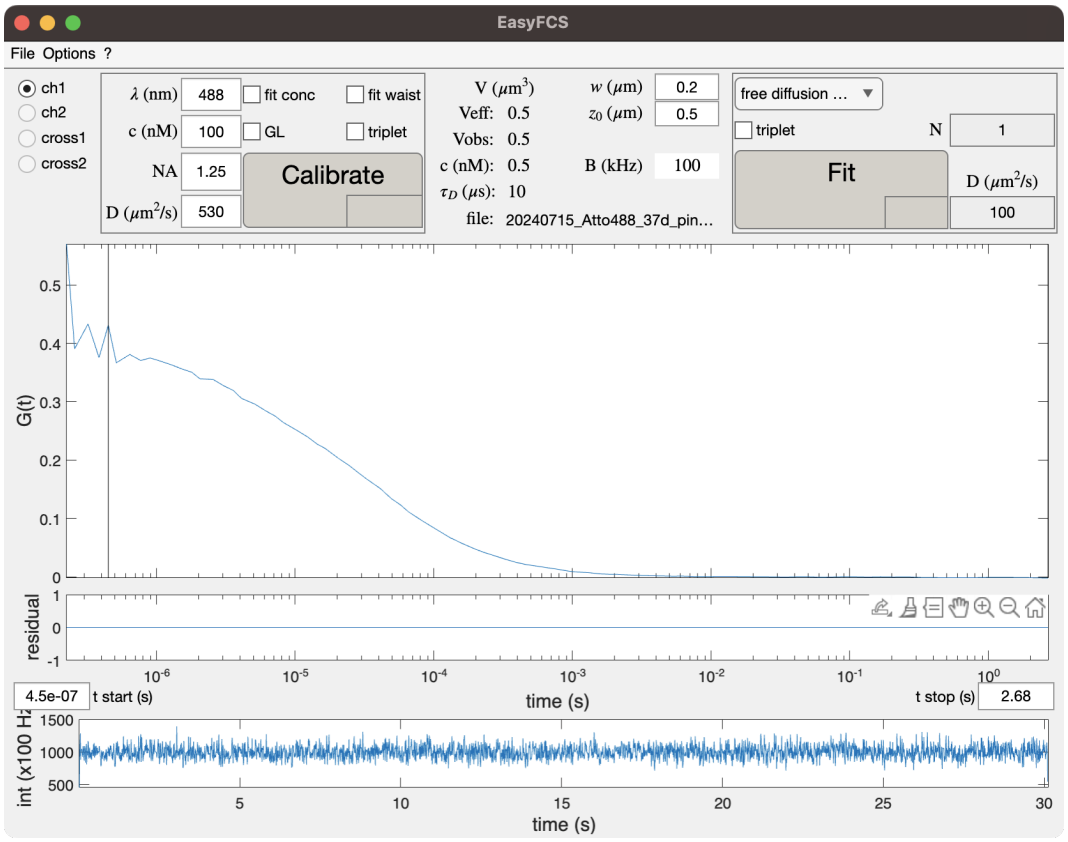

Figure 4: *The Main window of EasyFCS*

This main window consists of numerous buttons and options (Figure 4). If you want more detail about all of them, please refer to the section 4. In this section, we will just briefly describe the image and accomplish some basic tasks.

The main image shows the auto-correlation function of your acquisition.

The bottom part of the GUI shows the fluorescence time trace of your data in Hz (or counts/s).

The residual part is null for the moment because we did not applied any model.

Finally, you can see the name of the data file in the "file" part above the autocorrelation window.

3.2 First calibration with the auto-correlation function

In this section, we will describe how to calibrate your FCS experiments with an auto-correlation function (ACF).

To try a first calibration fitting, click on the "Calibrate" button. Then you will see the following window (Figure 5) :

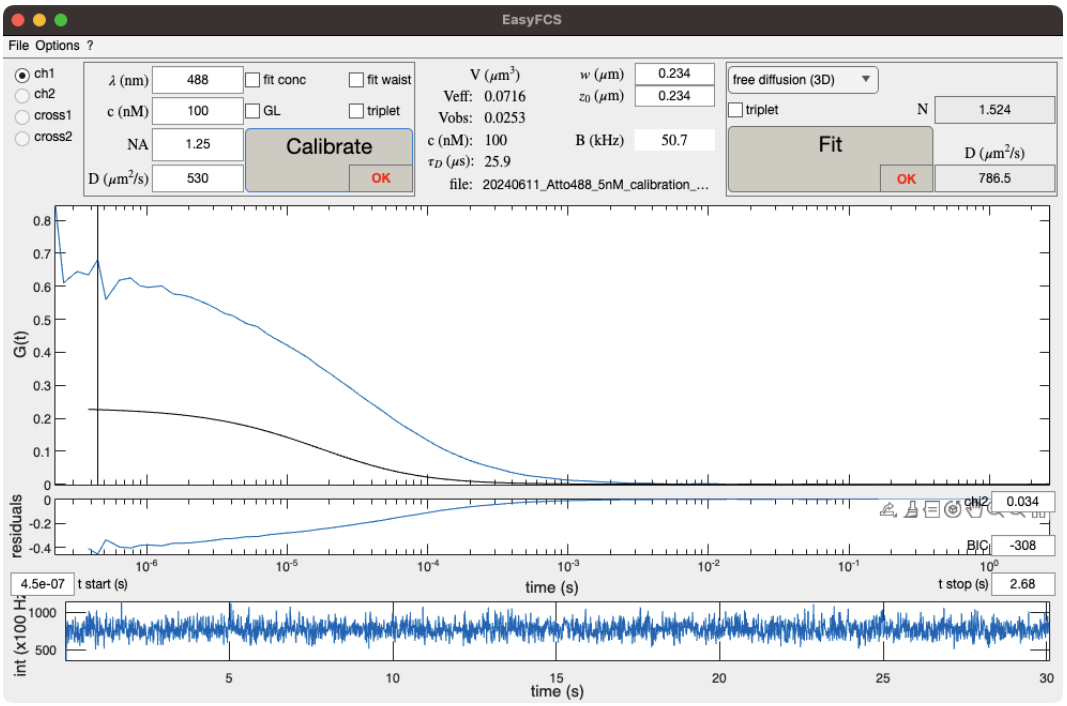

Figure 5: *The main window after first calibration*

You can see now a black curve corresponding to the fitting curve and you can see also that the residuals are not null but they are quite large. The reduced χ_2 is displayed at the right part of

the residual curve (see Equations 1 and 2 fore more details). The Bayesian information criterion (BIC) is also indicated whose expression is reported in the Equation 3.

If you know some experimental parameters such as the wavelength used for the acquisition, the concentration of the fluorophore solution, the numerical aperture of the microscope objective or the diffusion coefficient of the solution, you can enter them in the corresponding edit boxes.

If you do not know them, you can fit them by clicking the "fit conc" and the "fit waist" boxes. If there is some triplet state, you need also to click the "triplet" box to include it in the model.

After that, you should see an improvement of the fitting (Figure 6) :

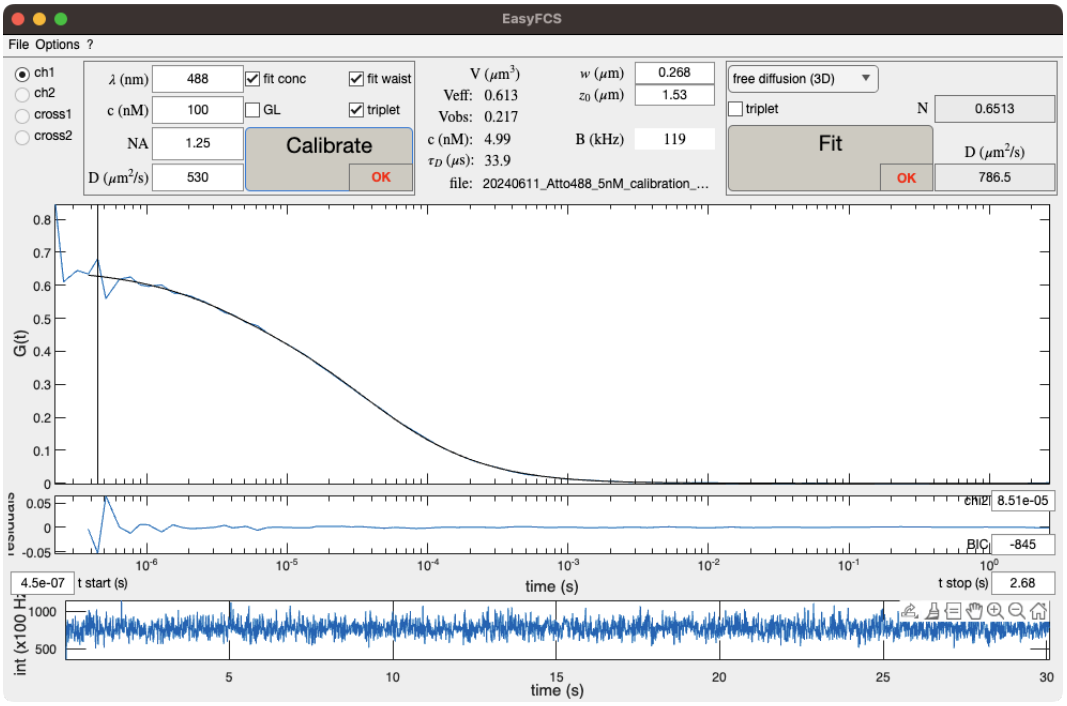

Figure 6: *The main window after better calibration*

Now, the residues are smaller and they are flat indicating that the fitting is better. The reduced χ_2 and the BIC are lower (see Equations 1 and 2 fore more details).

You can also change the values of "t start" and "t stop" if you need to exclude some parts of the ACF in the fitting.

The outputs of the Calibration process are the lateral and axial extents of the confocal volume. They are indicated in the fields : "w" and "z_0".

You can also see other parameters such as the size of the volumes : effective volume "Veff" and

observation volume "Vobs" in μm^3. You can see the concentration of the fluorophore "c" in nM and the residence time "τ_D" in μs.

You can finally have access to the brightness of the fluorophore "B" in kHz.

All the models are detailed in the section 5.

If you want to save your results, you can click on the "File" menu and the "Export..." menu item. The following window will appear (Figure 7) :

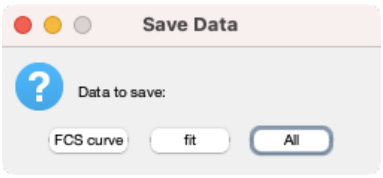

Figure 7: *The "Export Option" window*

You have then the possibility to save only the experimental ACF, or the fitting curve or both. After, you will have the possibility to save it on a new folder. The files are saved in text format only. With this action, you will only save the current data. With the data, an other file called `info.txt` will be saved. This file contains numerous information about the parameters used for the fitting (or the calibration), such as the model, the fitting paramters or the ACF limits. An example is shown in Figure 8.

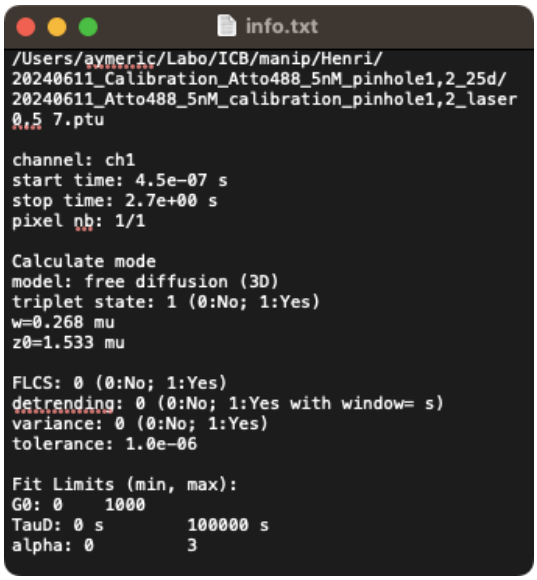

Figure 8: *The "info.txt" file*

3.3 First fitting of the auto-correlation function

In this section, we will describe how to fit your ACF with a model.

To try a first fitting with a 3D Brownian model, click on the "Fit" button. Then you will see the following window (Figure 9) :

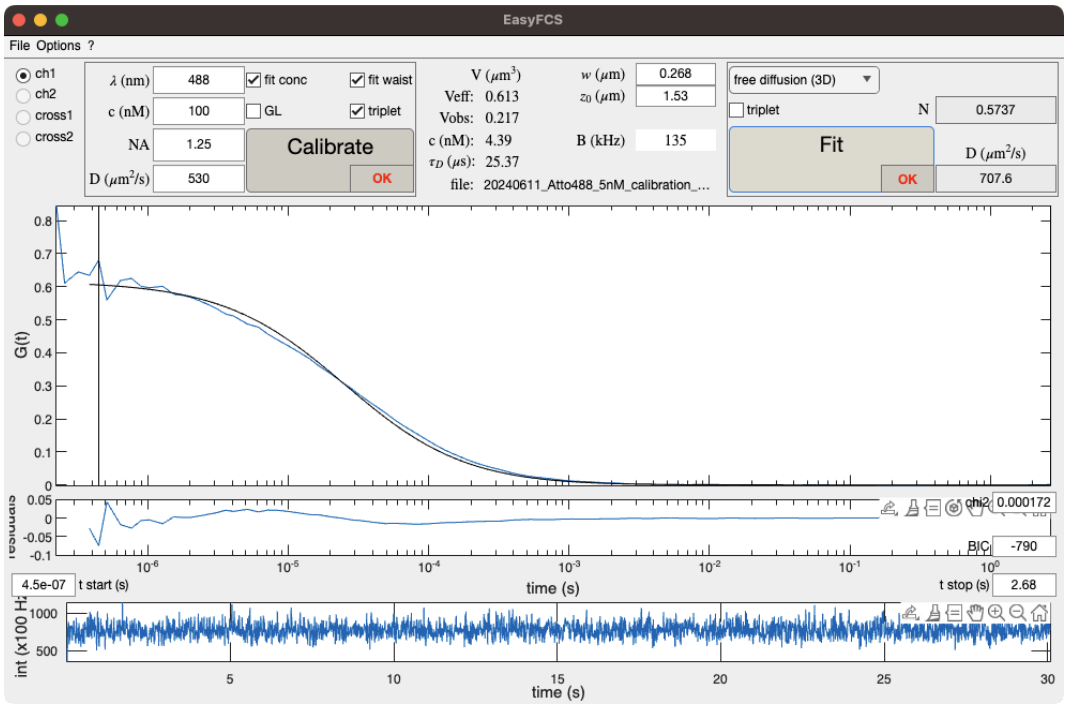

Figure 9: *The main window after first fitting*

You can see that the fitting curve does not coincide exactly with the ACF and you can see also that the residuals are not flat. You can also check the reduced χ_2 and the BIC values (see Equations 1 and 2 fore more details).

This is due to the fact that the triplet state has not been taken into account in the model. For including this triplet state, you need to click on the "triplet" box.

After that, you should see an improvement of the fitting (Figure 10), as indicated by the lower values of the reduced χ_2 and the BIC (see Equations 1 and 2 fore more details).

For the fitting algorithm, you need to enter 3 parameters : the model and the lateral and axial extents of the confocal volume which are indicated in the fields : "w" and "z_0".

The outputs of the Fitting process are the number of molecules in the observation volume indicated in the field "N" and the diffusion coefficient shown in the field "D".

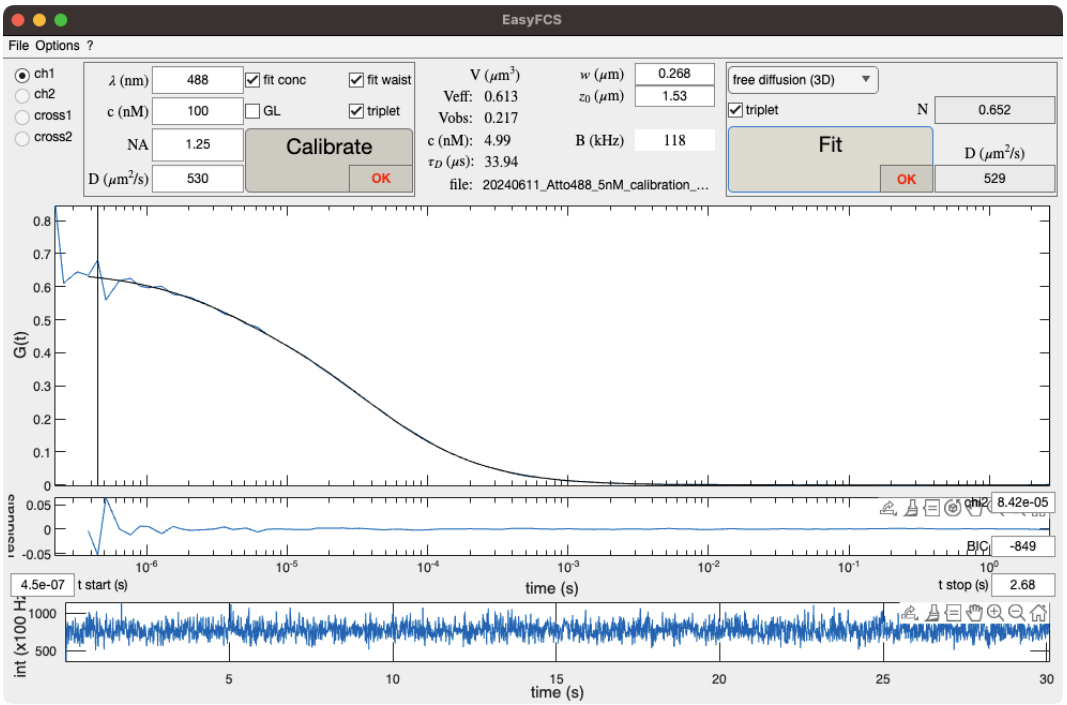

Figure 10: *The main window after better fitting*

You can also see other parameters such as the size of the volumes : effective volume "Veff" and observation volume "Vobs" in μm^3. You can see the concentration of the fluorophore "c" in nM and the residence time "τ_D" in μs.

You can finally have access to the brightness of the fluorophore "B" in kHz.

All the models are detailed in the section 5.

If you want to save your results, you can click on the "File" menu and the "Export..." menu item. The same window already shown in Figure 7 will appear.

You have then the possibility to save only the experimental ACF, or the fitting curve or both. After, you will have the possibility to save it on a new folder. The files are saved in text format only. With this action, you will only save the current data. With the data, an other file called `info.txt` will be saved. This file contains numerous information about the parameters used for the fitting (or the calibration), such as the model, the fitting paramters or the ACF limits. An example is shown in Figure 8.

4 Reference guide

This section describes the main menu options and the different buttons groups of the main window of EasyFCS. All buttons and other functional items which are not described here have been described in the section 3.

4.1 The Main menu

The main menu bar (Figure 11) sits at the top of the main window.

Figure 11: *The Main menu*

4.1.1 The File menu

The "File" menu is illustrated in Figure 12 and it is constituted of several items.

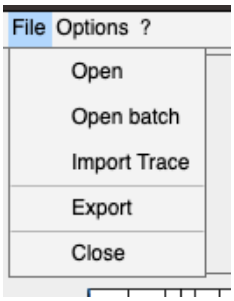

Figure 12: *The "File" menu*

- The "Open..." menu item allows you to open several types of data files : TXT, DAT, SIN, RAW, HDF5, BNY, LSM, HT2, PT3 and PTU formats.

  The TXT and DAT file format are standard ascii format with a tab as separator. The file should include two columns separated with a tab. The first column is the lag time (in microsecond).The second column is the auto-correlation function. In this case, you will not see the time trace of the experimental data. You only see the ACF in the main window and the auto-correlation is not calculated by the software.

With the SIN format, you will not see the time trace of the experimental data. You only see the ACF in the main window and the auto-correlation is not calculated by the software.

The RAW and HDF5 formats are used by the company Zeiss. With these formats, the Figure 3 permitting to choose the options for the auto-correlation calculation will pop up. After that, you will see the time trace of the experimental data on the main GUI window because the auto-correlation is calculated by the software from the arrival time of each photon with the time-tag-to-correlation algorithm developed by [Wahl2003].

The LSM format is used by the company Leica. With this format, you will see the the Figure 3 permitting to choose the options for the auto-correlation calculation. The time trace of the experimental data will be shown in the main window because the auto-correlation is calculated by the software from the fluorescence intensity.

The HT2, PTU and PT3 formats are the PicoQuant file formats. With these formats, all the experimental parameters are saved and consequently you will directly see the Figure 3 permitting to choose the options for the auto-correlation calculation. After that, you will see the time trace of the experimental data on the main GUI window because the auto-correlation is calculated by the software from the arrival time of each photon with the time-tag-to-correlation algorithm developed by [Wahl2003].

- The "Open Batch..." menu item will open a window to select one file in the requested folder. All the files in the folder will be treated automatically by EasyFCS. The procedure is identical to the procedure for opening one file. The only difference is that an other file window will pop up for saving the data. One text file will be saved containing the mean parameters for each file. With the data, an other file called `info.txt` will be saved. This file contains numerous information about the parameters used for the fitting (or the calibration), such as the model, the fitting paramters or the ACF limits. An example is shown in Figure 8.
- The "Import Trace..." menu item is reserved for simulations data.
- The "Export..." menu item will save the data that are currently opened. You have the possibility to save only the experimental ACF, or the fitting curve or both. After, you will have the possibility to save it on a new folder. The files are saved in text format only. With

this action, you will only save the current data. It will create a new folder whose name is chosen by the user. In this new folder, a text file (called `info.txt`) containing numerous information about the parameters used for the fitting (or the calibration), such as the model, the fitting parameters or the ACF limits. An example is shown in Figure 8.

- The "Close" menu item closes EasyFCS. Note that it does NOT warn you if you have unsaved images opened in the main window.

4.1.2 The "Options" menu

The "Options" menu represented in Figure 13 gives you access to several options.

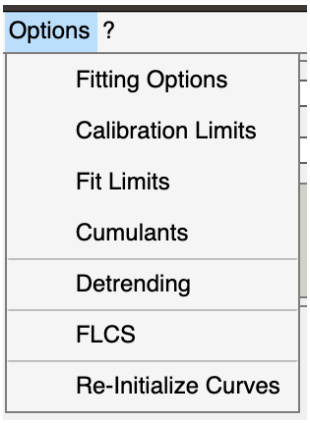

Figure 13: *The "Options" menu*

- When you select the "Fitting Options" menu item, the following window will pop up (cf. Figure 14).

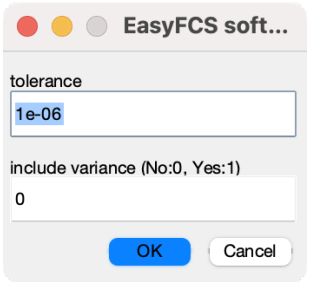

Figure 14: *The "Fitting Options" parameters*

You can the modify the tolerance of the non linear least square algorithm. When the difference between the model $G(T_i)$ and the experimental data $G^{\exp}(T_i)$ are less than the tolerance, the iterative minimization of the fitting algorithm stops.

You have also the possibility to include the variance of the experimental data $\sigma^2(G^{\exp})$ in the chi square function χ^2 that will be minimized by the fitting algorithm. The reduced chi square χ^2_ν is defined by :

$$\chi^2_\nu = \frac{1}{N_T - p} \sum_{i=1}^{N_T} \frac{\left(G^{\exp}(T_i) - G(T_i)\right)^2}{\sigma^2\left(G^{\exp}(T_i)\right)} \tag{1}$$

Where N_T is the number of experimental data points and p is the number of fitted parameters.

By default, the variance $\sigma^2(G^{\exp})$ is not included in the chi square (input set to « 0 »). It means that χ^2_ν is defined by :

$$\chi^2_\nu = \frac{1}{N_T - p} \sum_{i=1}^{N_T} \left(G^{\exp}(T_i) - G(T_i)\right)^2 \tag{2}$$

If you want to include the variance, you need to enter the input « 1 ». The variance of the autocorrelation function $\sigma^2\left(G^{\exp}(T_i)\right)$ was estimated by calculating the standard error of the mean for each point $G^{\exp}(T_i)$, as detailed in [Wohland2001].

The Bayesian information criterion (BIC) is deduced from the reduced χ^2_ν following :

$$BIC = N_T \ln\left(\frac{\chi^2_\nu (N_T - p)}{N_T}\right) + p \ln(N_T) \tag{3}$$

- The “Calibration Limits” menu item allows you to to change the limits of the fitting parameters used in the Calibration process, as shown in Figure 15 :

  You can then modify the minimal and maximal values of G_0, of the waist w (in μm) and the ratio w/z_0.

- The ‘Fit Limits” menu item allows you to modify the limits of the fitting parameters used in the Fitting process, as shown in Figure 16 :

  You can then modify the minimal and maximal values of G_0, of the residence time τ_D (in second) and the alpha value α used in anomalous diffusion model (for more details, see section 5).

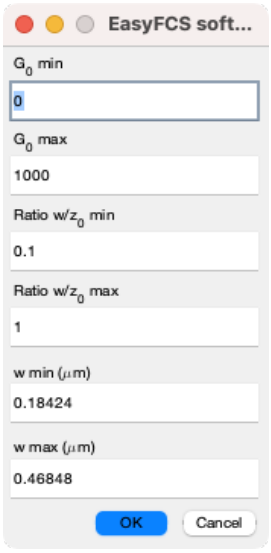

Figure 15: *The "Calibration Limits" menu*

- The "Cumulants" menu item is still in development.
- The "Detrending" dialog box of Figure 17 appears when you click on the "Detrending" menu item.

  After entering the requested time window (in second), the calculation starts and the modified time trace and auto-correlation function will be displayed in the main window. It apply a standard moving average detrending with a chosen sliding window.
- The "FLCS" menu item requires to use a file format including the arrival times of each event (PicoQuant formats for instance). Otherwise, an error message appears. By selecting this "FLCS" menu item, the following window will pop up (Figure 18) :

  This interface enables to modify the values of the minimal and maximal channels (called « channel min » and « channel max » respectively) that will be included in the FLCS filter. You can also modify the "offset" that will be subtracted to the experimental data. Once you have click on the OK button, an other window will pop up (cf. Figure 19) :

  As indicated in the window, you need to precise which filter you want to use for the data.If you want to apply a filter based on the fluorescence lifetime, you need to enter « 1 ». If you want to apply a filter based on the background, you need to enter « 2 ». After entering the requested filter, the calculation starts and the modified time trace and auto-correlation function will be displayed in the main window.

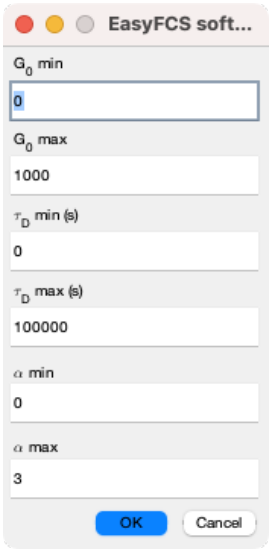

Figure 16: *The "Fit Limits" menu*

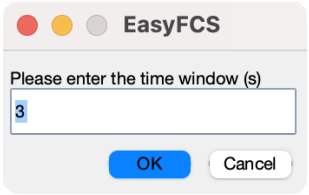

Figure 17: *The "Detrending" dialog box*

A complete description of the ACF filtering can be found here : [Enderlein2005]. In this work, the fluorescence lifetime filtering was used to separate the true fluorescence signal from the scattered light, allowing to reduce the auto-fluorescence.

- Finally, the last menu item of the "Options" menu is the "Re-Initialize Curves" menu item which enables to clear all filters and to suppress the detrending process.

  After selecting this menu item, the curves come back to their initial raw data, when you open the experimental data.

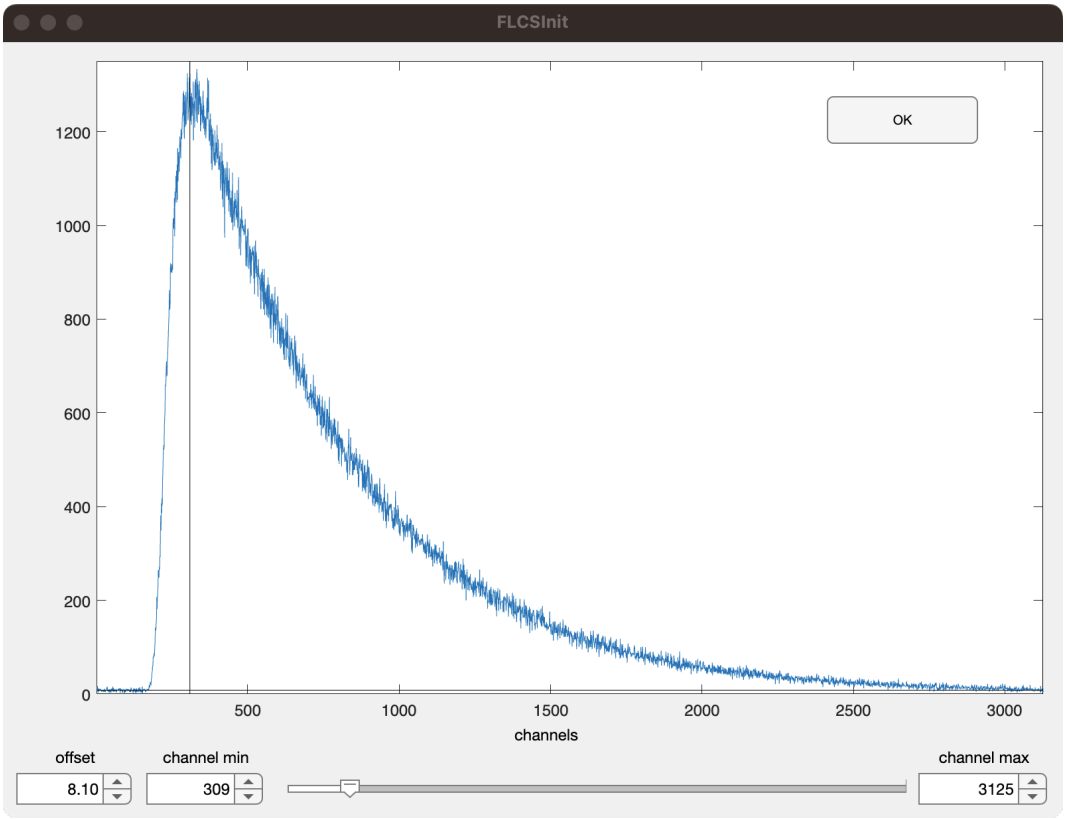

Figure 18: *The "FLCS" interface*

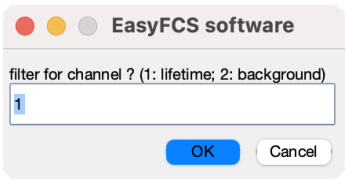

Figure 19: *The "FLCS" dialog box*

4.1.3 The " ?" menu

The Help menu which is constituted of 2 items is shown in Figure 20 :

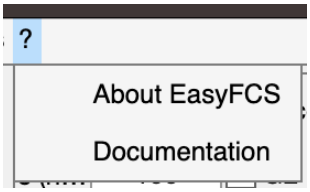

Figure 20: *The " ?" Menu*

- To display information about EasyFCS, click on the "About EasyFCS" menu item. The following window will pop up (Figure 21) :

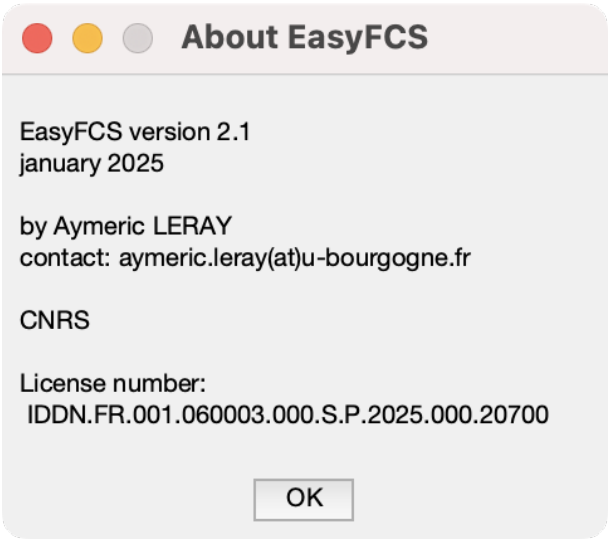

Figure 21: *The "About EasyFCS" information*

  In this window, you can see the version of EasyFCS that you are currently using. If you need further information, you can contact the team work by visiting the website : https://github.com/ayleray/EasyFCS.

- The "Documentation..." menu item should launch the Adobe Acrobat Reader and display the manual that you are looking now. If you do not have the Acrobat Reader installed, this option will not work. To download and install the latest version of the free reader, please visit the website of Adobe : https://www.adobe.com/.

4.2 The EasyFCS main window

The main window of EasyFCS is represented in Figure 22 :

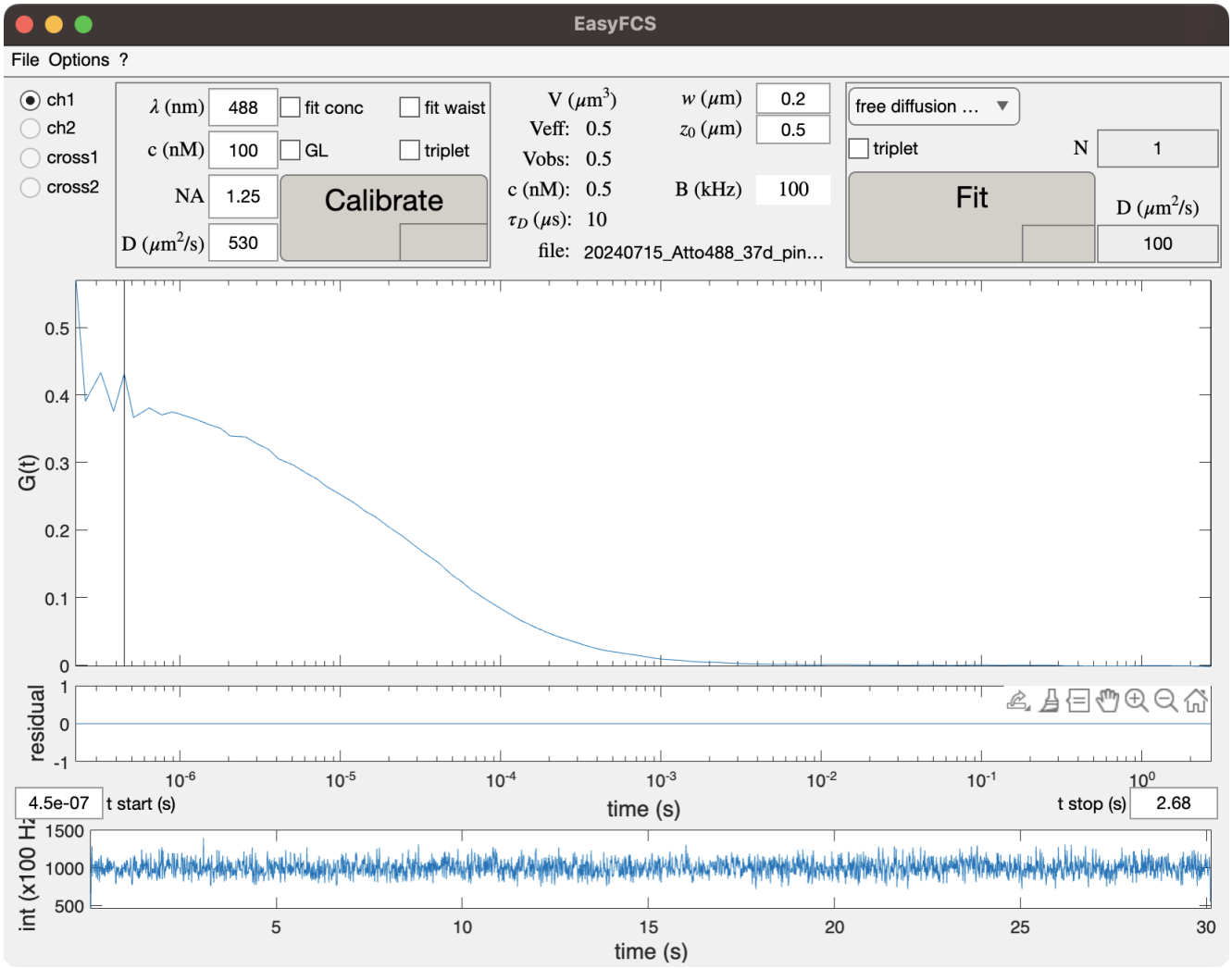

Figure 22: *The Main window of EasyFCS*

This main window consists of numerous buttons and options (Figure 22).

The main image shows the auto-correlation function of your acquisition.

The bottom part of the GUI shows the fluorescence time trace of your data in Hz (or counts/s).

The residual part is null for the moment because we did not applied any model.

You can also change the values of “t start” and “t stop” if you need to exclude some parts of the ACF in the fitting.

Finally, you can see the name of the data file in the “file” part above the autocorrelation window.

All the other groups of the main window will be described in the next sections of this documentation.

4.2.1 The "Channel" group

The "Channel" group (illustrated in Figure 23) is located on the left part of the EasyFCS main window. This group allows you to modify the channel that will be displayed on the main window.

Note that if your data contains only one channel, it will not be possible to modify this option. By default, the channel 1 is selected.

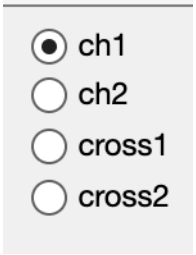

Figure 23: *The "Channel" group*

- The "ch1" corresponds to the channel one. This is the default selection. The auto-correlation is defined by :

$$G(\tau) = \frac{\langle \delta F_1(t+\tau)\delta F_1(t)\rangle}{\langle F_1(t)\rangle^2} \tag{4}$$

- The "ch2" corresponds to the channel two. This option can be selected only if your data contains two channels. If you select this channel, the auto-correlation of the channel 2 will be displayed, which is defined by :

$$G(\tau) = \frac{\langle \delta F_2(t+\tau)\delta F_2(t)\rangle}{\langle F_2(t)\rangle^2} \tag{5}$$

- The "cross1" displays the cross-correlation between the channel 1 and the channel 2. This option can be selected only if your data contains two channels. This cross-correlation is defined by :

$$G(\tau) = \frac{\langle \delta F_1(t+\tau)\delta F_2(t)\rangle}{\langle F_1(t)\rangle \langle F_2(t)\rangle} \tag{6}$$

- The "cross2" corresponds to the cross-correlation between the channel 2 and the channel 1. This option can be selected only if your data contains two channels. If you select this channel, it will display the cross-correlation defined by :

$$G(\tau) = \frac{\langle \delta F_2(t+\tau)\delta F_1(t)\rangle}{\langle F_1(t)\rangle \langle F_2(t)\rangle} \tag{7}$$

4.2.2 The "Calibration" group

The "Calibration" group is placed on the left part of theEasyFCS main window and it is represented in Figure 24 :

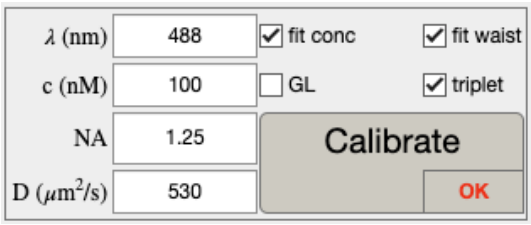

Figure 24: *The "Calibration" group*

This group gives access to numerous parameters concerning the Calibration process. All the theoretical models will be detailed in the section 5.

- The "λ" edit box enables to enter the wavelength used during your experiments. This parameter is used only if you do not fit the waist w of the observation volume, which will be fixed according to :

$$w = \frac{0.6\lambda}{NA} \tag{8}$$

  where NA is the numerical aperture of your microscope objective.

- The "c" edit box enables to enter the fluorophore concentration (in nano molar). This parameter is used only if you do not fit the concentration w of the fluorophore (see section 5 for more details).

- The "NA" edit box enables to enter the numerical aperture of your microscope objective. This parameter is used only if you do not fit the waist w of the observation volume, which

will be fixed according to :

$$w = \frac{0.6\lambda}{NA} \tag{9}$$

- The "D" edit box enables to enter the diffusion coefficient of your fluorophore (in $\mu m^2/s$). This parameter is critical because it is used with all calibration models (see section 5 for more details). It is important to know the diffusion coefficient of you calibration sample. You can find more details about this topic in the PicoQuant application note : https://www.picoquant.com/images/uploads/page/files/7353/appnote_diffusioncoefficients.pdf
- The "fit conc" check box enables to modify the model used. When the box is unchecked, the concentration is fixed (it will be not fitted). If you check this box, it means that the concentration will be fitted ; it will be an additional fitting parameter that will be optimized by the non linear least square algorithm (see section 5 for more details).
- The "fit waist" check box enables to modify the model used. When the box is unchecked, the waist is fixed following the Equation 8. If you check this box, it means that the waist w will be fitted ; it will be an additional fitting parameter that will be optimized by the non linear least square algorithm (see section 5 for more details).
- The "triplet" check box enables to modify the model used. When the box is unchecked, the triplet kinetics is not taken into account. If you check this box, it means that the triplet state will be included in the model (see section 5 for more details).
- The "GL" check box is an option that is still under development.
- Finally, the "Calibrate" button enables to apply the fitting model and display both the fitting curve and the residuals curve in the main window of EasyFCS. The obtained parameters will be displayed in the Parameters group (see next section).

4.2.3 The "Parameters" group

The "Parameters" group (illustrated in Figure 25) is positioned in the central upper part of the main window of EasyFCS. It allows you to display several useful parameters.

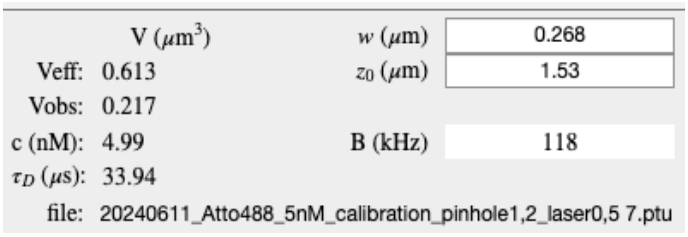

Figure 25: *The "Parameters" group*

- In the left part of this group, you can see the value of the effective volume V_{eff} and the observation volume. V_{obs} When the normalised spatial distribution of the emitted light (or normalised total point spread function) is approximated by a three-dimensional Gaussian, they are defined by :

$$V_{eff} = \pi^{3/2} w^2 z_0 \tag{10}$$

$$V_{obs} = \left(\frac{\pi}{2}\right)^{3/2} w^2 z_0 \tag{11}$$

  The concentration of the fluorophore is defined by :

$$c = \frac{1}{G_0 N_A V_{eff}} \tag{12}$$

  where N_A is the Avogadro's constant ($N_A = 6.022 \times 10^{23}$ mol^{-1}) and G_0 is the auto-correlation at lag time t=0.

  Finally, we can see the residence time τ_D which is defined by :

$$\tau_D = \frac{w^2}{4D} \tag{13}$$

  where D is the diffusion coefficient.

- In the right part of the "Parameters" group, you can see the lateral and axial extents of the

Gaussian beam called respectively w and z_0 (in μm) and defined as the distances where the intensity is reduced by a factor $1/e^2$. These two parameters are editable and they are used in the fitting process that wil be detailed in the next section.

You can see also the brightness B which is defined by :

$$B = \frac{\mu}{N} \tag{14}$$

where μ is the mean of the fluorescence intensity time trace and N is the number of molecules in the observation volume.

- Finally, the name of the opened file is indicated in the bottom of this "Parameters" group.

4.2.4 The "Fitting" group

The "Fitting" group (represented in Figure 26) is visible in the right part of the main GUI interface of EasyFCS.

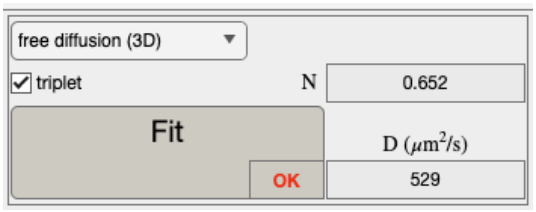

Figure 26: *The "Fitting" group*

It is important to note that the fitting process used the values of w and z_0 indicated in the "Parameters" group and detailed previously.

The "Fitting" group contains several parameters that will be detailed now :

- The drop down button enables you to select the diffusion model. Several models are available, as you can see in Figure 27 :

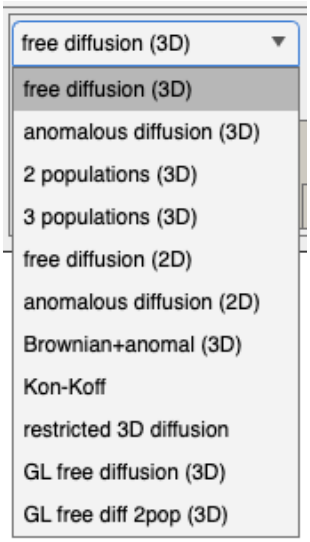

Figure 27: *The drop down button with the different available models*

  These models will be detailed in the next section.

- The "triplet" check box enables to modify the model used. When the box is unchecked, the triplet kinetics is not taken into account. If you check this box, it means that the triplet state will be included in the model (see section 5 for more details).

- The results of the fitting process are indicated in the right part of the "Fitting" group. For instance, you can see the number of molecules in the observation volume N and the value of the diffusion coefficient D.

5 Models

5.1 Calibration models

5.1.1 Without triplet state

In the Calibration part, several functions can be fitted according to the chosen options. The unknown parameter are noted x_n.

- if no options are checked, it means that the waist and the concentration are fixed. The function has only one unknown and it is given by :

$$G(\tau) = \frac{x_1}{cN_A\pi^{3/2}w^3}\left(1+\frac{4D\tau}{w^2}\right)^{-1}\left(1+x_1^2\frac{4D\tau}{w^2}\right)^{-\frac{1}{2}} \tag{15}$$

- If only the "fit conc" box is checked, the concentration will be fitted. The function has 2 unknowns :

$$G(\tau) = x_1\left(1+\frac{4D\tau}{w^2}\right)^{-1}\left(1+x_2^2\frac{4D\tau}{w^2}\right)^{-\frac{1}{2}} \tag{16}$$

- when only the "fit waist' box is checked, the waist will be fitted but the concentration is fixed. The function has 2 unknowns :

$$G(\tau) = \frac{x_1}{cN_A\pi^{3/2}x_2^3}\left(1+\frac{4D\tau}{x_2^2}\right)^{-1}\left(1+x_1^2\frac{4D\tau}{x_2^2}\right)^{-\frac{1}{2}} \tag{17}$$

- If the two options "fit conc" and "fit waist" are checked, meaning that the waist$w$ and the concentration c are fitted, there are 3 unknown parameters and the function is :

$$G(\tau) = x_1\left(1+\frac{4D\tau}{x_2^2}\right)^{-1}\left(1+x_3^2\frac{4D\tau}{x_2^2}\right)^{-\frac{1}{2}} \tag{18}$$

5.1.2 With triplet state

If the "triplet" box is checked, it will add a term before the previously defined functions. This term will add two supplementary unknowns parameters (noted $x_1^{triplet}$ and $x_2^{triplet}$) :

$$G^{triplet}(\tau) = \left(1 + x_1^{triplet} \frac{e^{-\frac{\tau}{x_2^{triplet}}}}{1 - x_1^{triplet}}\right) \times G(\tau) \tag{19}$$

5.2 Fitting models

5.2.1 Without triplet state

In the Fitting part, several models can be used. They are detailed after. The unknown parameter are noted x_n.

- The "free diffusion (3D)" model is the well known Brownian diffusion model defined by :

$$G(\tau) = x_1 \left(1 + \frac{4x_2\tau}{w^2}\right)^{-1} \left(1 + \frac{4x_2\tau}{z_0^2}\right)^{-\frac{1}{2}} \tag{20}$$

- The "anomalous diffusion (3D)" model contains 3 unknown parameters. It is defined by :

$$G(\tau) = x_1 \left(1 + \left(\frac{4x_2\tau}{w^2}\right)^{x_3}\right)^{-1} \left(1 + k^2 \left(\frac{4x_2\tau}{w^2}\right)^{x_3}\right)^{-\frac{1}{2}} \tag{21}$$

  where $k = \frac{w}{z_0}$.

- The "2 populations (3D)" model is the well known Brownian diffusion model with two populations which is defined by :

$$G(\tau) = x_1 \left(x_3 \left(1 + \frac{4x_2\tau}{w^2}\right)^{-1} \left(1 + \frac{4x_2\tau}{z_0^2}\right)^{-\frac{1}{2}} + (1 - x_3) \left(1 + \frac{4x_4\tau}{w^2}\right)^{-1} \left(1 + \frac{4x_4\tau}{z_0^2}\right)^{-\frac{1}{2}}\right) \tag{22}$$

- The "3 populations (3D)" model is the well known Brownian diffusion model with three

populations defined by :

$$G(\tau) = x_1\left(x_3\left(1+\frac{4x_2\tau}{w^2}\right)^{-1}\left(1+\frac{4x_2\tau}{z_0^2}\right)^{-\frac{1}{2}} + x_4\left(1+\frac{4x_5\tau}{w^2}\right)^{-1}\left(1+\frac{4x_5\tau}{z_0^2}\right)^{-\frac{1}{2}} + (1-x_3-x_4)\left(1+\frac{4x_6\tau}{w^2}\right)^{-1}\left(1+\frac{4x_6\tau}{z_0^2}\right)^{-\frac{1}{2}}\right) \tag{23}$$

- The "free diffusion (2D)" model is the well known Brownian diffusion model defined by :

$$G(\tau) = x_1\left(1+\frac{4x_2\tau}{w^2}\right)^{-1} \tag{24}$$

- The "anomalous diffusion (2D)" model is defined by :

$$G(\tau) = x_1\left(1+\left(\frac{4x_2\tau}{w^2}\right)^{x_3}\right)^{-1} \tag{25}$$

- The "Brownian+anomal (3D)" model contains 5 unknown parameters. It is defined by :

$$G(\tau) = x_1\left(x_3\left(1+\frac{4x_2\tau}{w^2}\right)^{-1}\left(1+\frac{4x_2\tau}{z_0^2}\right)^{-\frac{1}{2}} + (1-x_3)\left(1+\left(\frac{4x_4\tau}{w^2}\right)^{x_5}\right)^{-1}\left(1+k^2\left(\frac{4x_4\tau}{w^2}\right)^{x_5}\right)^{-\frac{1}{2}}\right) \tag{26}$$

where $k = \frac{w}{z_0}$.

- The "Kon-Koff" model was detailed by [Michelman-Ribeiro2009]. It is defined by :

$$G(\tau) = x_1\int\int \frac{(1-\phi)e^{-\frac{k_1\tau}{2}} + (1+\phi)e^{-\frac{k_2\tau}{2}}}{2}\left(e^{-\frac{w^2}{4}q_r^2-\frac{z_0^2}{4}q_z^2}\right)2\pi q_r dq_r dq_z \tag{27}$$

The lateral and axial Fourier transform variables are noted respectively q_r and q_z and the parameter ϕ is given by :

$$\phi = \frac{(x_3 * + x_4)^2 + x_3 - x_4)(q_r^2+q_z^2)x_2}{(x_3+x_4)\sqrt{(x_2(q_r^2+q_z^2)+x_3+x_4)^2 - 4x_2(q_r^2+q_z^2)x_4}} \tag{28}$$

and the k_1 and k_2 values are given by :

$$k_1 = x_2(q_r^2 + q_z^2) + x_3 + x_4 + \sqrt{(x_2(q_r^2 + q_z^2) + x_3 + x_4)^2 - 4x_2(q_r^2 + q_z^2)x_4} \quad (29)$$

$$k_2 = x_2(q_r^2 + q_z^2) + x_3 + x_4 - \sqrt{(x_2(q_r^2 + q_z^2) + x_3 + x_4)^2 - 4x_2(q_r^2 + q_z^2)x_4} \quad (30)$$

- The "restricted 3D diffusion" model is detailed by [Piskorz2014]. It is defined by :

$$G(\tau) = x_1 \left(1 + \frac{2\left\langle r^2(\tau)\right\rangle}{3w^2}\right)^{-1} \left(1 + \frac{2\left\langle r^2(\tau)\right\rangle}{3z_0^2}\right)^{-1/2} \quad (31)$$

where the mean square displacement $\langle r^2(\tau)\rangle$ is defined by :

$$\left\langle r^2(\tau)\right\rangle = 6x_2\tau + \frac{6}{5}x_4^2\left(1 - e^{-\frac{x_5(5x_3\tau)}{x_4^2}}\right) \quad (32)$$

5.2.2 With triplet state

If the "triplet" box is checked, it will add a term before the previously defined functions. This term will add two supplementary unknowns parameters (noted $x_1^{triplet}$ and $x_2^{triplet}$) :

$$G^{triplet}(\tau) = \left(1 + x_1^{triplet}\frac{e^{-\frac{\tau}{x_2^{triplet}}}}{1 - x_1^{triplet}}\right) \times G(\tau) \quad (33)$$

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