BIFL Data Analyzer 1.5
Short description

BIFL Data Analyzer (BIFLDA) is intended for sliding analysis of fluorescence decays and correlation functions built from BIFL data (B&H SPC, PicoQuant TimeHarp 200 and PicoHarp 300) using Maximum Likelihood (ML) method. Inter arrival time distribution (IATD) coincidence analysis is also supported.

Data management.

BIFLDA software is designed to process BIFL data obtained either from polarized measurements with the two detectors collecting 0 and 90 degrees traces (Polarization experiment) or from two detectors collecting two different colors (Two Color experiment). The software supports importing four detector data but the idea is to use every time just two of them. Things that can be analyzed are 1) the polarizations of the two colors (same analysis as the polarized measurements), 2) the sum of two polarizations for each colour (this is the magic angle trace: (s+2Gp)), and 3) the total sum of all four detectors which is build up by summing the two magic angle traces from the two colours. The first analysis can be done separately for each colour, the second and the third analysis is in fact the same as two-colour experiment.

The source data that are imported by BIFLDA software are located either in one or in a series of files that have B&H SPC 402/432, SPC 401/431, SPC 6x0/256ch, SPC 6x0/4096ch, SPC 830 data format or in a file that have PicoQuant TimeHarp 200 (versions 5 and 6) and PicoHarp 300 data format. One unit of data (BIFL data set) that can be imported at one step consists of up three sets of BIFL files:

1. first set of data files represents the continued sample BIFL trace;
2. second set of data files represents the continued scatter BIFL trace;
3. third set of data files represents the continued background BIFL trace.

It is also possible to use a part of the sample BIFL trace as a background.

On the basis of imported BIFL data set for Two Color experiments the following three traces are built up:

- Channel 1 - trace containing the data from first detector.
- Channel 2 - trace containing data from the second detector.
- Total - trace containing data from the two detectors together. This trace is calculated from intensities of Channel 1 and Channel 2 as [channel1]+D*[channel2], where D is a correction for efficiency and wavelength dependence.
- Energy transfer efficiency (E) function according to the formula:

$$E = \frac{1}{1 + \frac{\Phi_A}{\Phi_D} \frac{D}{\left(\frac{I_A}{I_D} - L \right)}}$$

where:

D is the ratio between the different detector efficiencies. This parameter can be defined by user;

L is the proportion of the donor signal I_D leaking into the acceptor channel;

Φ_A and Φ_D the quantum yields of fluorescence for the acceptor and donor; I_D and I_A are the intensities of donor (channel 0) and acceptor (channel 1).

- Fractional intensity(F_2) function that is a measure for the emission maximum in a trace. This function is built according to the following formula:

$$F_2 = \frac{I_2}{I_1 + I_2}$$

where:

I_1 is intensity of channel 0;

I_2 is intensity of channel 1.

On the basis of imported BIFL data set for Polarization experiments the following five traces are built up:

- Channel 1 (S) - trace containing the data from first detector (S-polarized light).
- Channel 2 (P) - trace containing data from the second detector (P-polarized light).
- Total (Sum S + 2G*P) - trace containing data from the two detectors together. This trace is calculated from intensities of S and P as (S+2G*P).
- steady state polarization trace according the following formula

$$p = \frac{S - G * P}{S + G * P}$$

- steady state anisotropy trace according the following formula:

$$r = \frac{S - G * P}{S + 2G * P}$$

After building the required macrotime traces the BIFLDA software provides the possibility to create fluorescence decays and autocorrelation functions available for the fit and also inter arrival time distributions available for further IATD coincidence analysis. There is an option* to calculate absolute photon arrival times combining macro and micro time information**. Fluorescence decays and correlation functions are made only from Total-trace for the polarization measurements and from all traces for the two color measurements. Inter arrival time distributions can be calculated either from total trace or from each channel independently. There are three options for calculation of inter arrival time distributions from the total trace: independently from router, i.e. for each photon; for channel 1 to channel 2 time delay and for channel 2 to channel 1 time delay.

* absolute photon arrival times are always calculated for PicoQuant PicoHarp data.

**calculation of absolute photon arrival times can not be performed for PicoQuant TimeHarp data due to asynchronous macro time clock.

Building fluorescence decays, autocorrelations and IATD can be done from a selected time region (large time region) in the macro time trace. Visual selection of the time region with help of special data markers is possible. Sliding method for building fluorescence decays, autocorrelations and IATD from selected time region by either selected bursts or constant time shift or constant amount of photons is implemented.

It is possible to select large time regions in all macro time traces (Sample, Irf, Bg, and also Channel 1, Channel 2, Total) with help of data markers so that this time region is applied to all traces, but it is also possible to select manually different large time regions in each trace (sample, irf, bg). The sample irf and bg is operated freely. But within the sample (channel 1, channel 2 and total), the selected time region(s) is the same for all traces.

Building fluorescence decays.

Constant time shift or constant number of photons is possible to set for sliding. From the photons belonging to each small sliding time interval the separate decay is made.

To reduce number of channels and increase signal to noise ration fluorescence decays can be binned. Binning factor can be set by user (see Options/Binning).

Building autocorrelation functions.

Constant time shift or constant number of photons is possible to set for sliding. From the photons belonging to each small sliding time interval the autocorrelation function is calculated by either formula (*) or formula (**)

$$g(j) = \frac{\frac{1}{M-j}\sum_{i=1}^{M-j} n_i \cdot n_{i+j}}{\left[\frac{1}{M}\sum_{i=1}^{M} n_i\right]^2}, \qquad (*)$$

$$G_i(m\Delta\tau_i) = \frac{\frac{1}{M-m}\sum_{k=1}^{M-m} n(k\Delta\tau_i)n(k\Delta\tau_i + m\Delta\tau_i)}{M_{\mathrm{dir,i}} \cdot M_{\mathrm{del,i}}},$$

with

$$M_{\mathrm{del,i}} = \frac{1}{M-m}\sum_{k=m}^{M} n(k\Delta\tau_i)$$

and

$$M_{\mathrm{dir,i}} = \frac{1}{M-m}\sum_{k=1}^{M-m} n(k\Delta\tau_i). \qquad (**)$$

where *j* is the number of channel where correlation is calculated; *M* is total amount of channels; *n* is the number of photons in a bin, $\Delta\tau$ is a minimal time lag. The bin width (time lag) can be set by user.

An alternative formula ** is used when quasi-logarithmic time scale is set.

Building anisotropy decays.

For each small sliding time region a time resolved anisotropy decay is built by the formula

$$r(t) = \frac{I_{//}(t) - GI_{\perp}(t)}{I_{//}(t) + 2GI_{\perp}(t)}.$$

The only time resolved anisotropy decays are displayed (in the case of polarization measurements in solution). Decays of S and P are not plotted.

Building inter arrival time distributions.

Inter arrival time distributions can be calculated either in linear or in quasi-logarithmic time scales. It is possible to set an offset in order to calculate histogram starting from the predefined value.

Data management scheme for measurements in solution.

In this case experiment was done in solution and not immobilized on coverglass or polymer. The measurement is the actual Burst Integrated Fluorescence. A molecule passes the focus and generates a burst. These burst can be detected by software. Therefore minimum and maximum thresholds is set. Minimum to discriminate between dark noise and a burst, maximum threshold to discriminate between single molecule passing through focus or two or more at same time giving rise to higher intensity. Intention is to select in this way only those burst coming from single molecule.

The selection of bursts can be done in total trace and applied also to S and P. The same procedure is valid for two colour experiment (first selection in the total trace and then applied to colour 1 and colour 2).

Sliding analysis in this case will occur over several bursts. All photons from different burst can be used if put one after each other (no combinations of photons from different bursts are used for building autocorrelation).

Analysis algorithms.

BIFLDA software provides the possibility to fit single fluorescence decays, anisotropy decays and autocorrelation functions described in previous section. Global analysis is not supported. One fit session can process fluorescence decays or correlation functions or anisotropy decays built from one macro-trace. In case of two color experiment, in one fit session three decays or autocorrelation functions built from channel0, channel1 and total macro-traces are fitted. It is possible to uncheck any of characteristics mentioned above in order to prevent them from being analyzed.

Analysis of all fluorescence decays, autocorrelation functions and anisotropy decays is done using MLE method with multinomial statistics.

The MLE method with multinomial statistics consists of target fit criterion and optimization method. Target fit criterion is defined by the following formula:

$$\chi^2_{MLE}(\mathbf{a}) = \frac{2}{\nu}\sum_{i=1}^{n} x_i \ln\left(x_i / y_i(\mathbf{a})\right)$$

where:

x - measured curve;

$y(\mathbf{a})$ - theoretical curve calculated with corresponding model;

ν - number of degrees of freedom;

The Marquard-Levenberg algorithm is used as the standard minimization algorithm for MLE.

Analysis of fluorescence decays:

Analysis of fluorescence decays is done using theoretical model of following general form:

$$y_i(\mathbf{a}) = \left(irf_{i+\delta} - b\right) \otimes m_i(\mathbf{a}) + \gamma bg_i + c \qquad (1)$$

where parameters δ, b, γ and c can be fitted by the software.

The multiexponential model is used as a pure fit model. This model is defined by the following equation:

$$m_i(\mathbf{a}) = \sum_{j=1}^{N} p_j e^{-t_i/\tau_j} \qquad (2)$$

where:

N is number of exponents;

p_j and τ_j are amplitudes and fluorescence lifetimes that can be fitted.

Analysis of anisotropy decays:

Analysis of anisotropy decays is done using the following theoretical model:

$$r(t) = r_0 e^{-t/\varphi} + r_\infty \qquad (3)$$

where:

r_0, φ and r_∞ are parameters to be fitted;

Only anisotropy is fitted (not S, P or T decays separately). The software fits the anisotropy decays calculated from BIFL data (see data management section) directly using the Eq. 3.

Analysis of autocorrelation functions:
Analysis of autocorrelation functions is done using the following theoretical model:

$$y_i(\mathbf{a}) = 1 + \sum_{k=1}^{M} c_k e^{-t_i/\tau_k} \tag{3}$$

where:

c_k and τ_k are amplitudes and triplet lifetimes that should be fitted;
M is number of exponents in the sum.

In the case if autocorrelation function was built with FCS experiment the following model is used for fitting:

$$G(t) = 1 + \frac{(1 + \frac{F_{trip}}{1 - F_{trip}} e^{-t/T_{trip}})}{N} \left(\sum_i \frac{F_i}{\left(1 + \frac{t}{T_{D_i}}\right)\sqrt{1 + \frac{t}{a^2 T_{D_i}}}} \right), \quad \sum_i F_i = 1 \tag{4}$$

where:

$N, F_{trip}, T_{trip}, F_i, T_{D_i}, a$ are parameters to be fitted;

In the case if fluorescence decays, anisotropy decays and autocorrelation functions were prepared from BIFL data without applying sliding method they are analyzed independently one by one.
In the case if sliding method was used the following analysis steps are performed during one fit session:

1. initial curve prepared from separate macro-time region (see Data management section) is analyzed with a corresponding model. In the case of fluorescence decays analysis on this step parameters δ, b, γ and c are fitted. Also the start and end channels for analysis are defined on this step;
2. curves prepared from sliding window are analyzed automatically one after another. The start and end channels for analysis are taken from previous step and are same for all curves analyzed on this step. In the case of fluorescence decays analysis on this step parameters δ, b are fixed to the values obtained on the previous step. For each fluorescence decay analyzed on this stage parameters γ and c are fixed to different values. These values are obtained by multiplying values of parameters γ and c taken from step 1 by ratio of time duration of sliding window to time duration of large time region. Also before starting analysis on this step the values of all parameters obtained on step 1 can be changed by user manually.

Generation of initial guesses for model parameters just as minimum and maximum for each parameter is same while analyzing all curves during one fit session. In the case if sliding analysis method was applied generation of initial guesses is done for both initial decay and decays prepared from sliding window.

The measured fluorescence decays can be fitted to theoretical models with and without convolution.

After fit is done the weighted residuals and their autocorrelation function are calculated.

IATD coincidence analysis (by Weston et al. 2002)
Allows to determine the number of independent emitters by determining the ratio N_C/N_L of the number of photons in the coincidence IATD peak N_C, to the average number in the neighboring lateral peaks (each for user defined peaks count k), N_L.

Bursts selection
Bursts can be selected automatically or manually either in intensity or in time lag macro time plot. At first step one has to apply Lee smoothing filter. Lee filtration is described as following. The raw data n_k with n_k being the number of photons (count numbers) in the bin k of the macrotime trace with $1<=k<=N$ can be smoothed by a Lee filter of window width $2m+1$ which is defined as follows: first a running mean and variance are calculated using

$$\overline{n_k} = \frac{1}{(2m+1)} \sum_{j=-m}^{m} n_{k+j} \quad , m<k<=N-m$$

$$r_k^2 = \frac{1}{(2m+1)} \sum_{j=-m}^{m} (n-\bar{n})_{k+j}^2 \quad , 2m<k<=N-2m$$

The range of the k values is limited by the window width. The filtered data $\tilde{n}_k$ are given by

$$\tilde{n}_k = \bar{n}_k + (n_k - \bar{n}_k)\frac{r_k^2}{r_k^2 + r_0^2}$$

where r_0 is a constant characterizing the filter (determined from background photons selected using a pair of blue markers).
The resulting smoothed data $\tilde{n}_k$ are used to define a burst related to fluorescence photons. A burst is automatically defined by any continuous number of bins with $n_{\max} > \tilde{n}_k > n_{th}$ where n_{th} is a threshold value that is usually set equal to the estimated mean background count number and n_{max} is maximal threshold intended for selection of single molecule events*. The burst size is obtained by summing all the raw data count numbers n_k over the burst range. The burst range is determined from macrotime position for the first and the last photon in the burst. Bursts containing less photons then predefined number are not automatically selected. Bursts are marked in color in the corresponded macrotrace after their selection. Range and size of each burst are listed in the grid. Range of any burst can be edited by yellow markers. One can also select manually a burst using these yellow markers.
*the relation between $\tilde{n}_k$, n_{max} and n_{th} is inverse for time lag plot.

Application structure and interface.

BIFLDA application is able to perform the following operations:

1. importing BIFL data from source files. The format of the files is described in Data management section;
2. exporting in ASCII file macro-time dependencies described in Data management section;
3. selecting user-defined time region for macro-trace and build corresponding fluorescence decay or correlation function. This option is available only for Total-trace in the case of polarization experiment and for Channel 1, Channel 2, and Total-traces in the case of two color experiment. At one time decays or autocorrelation functions can be made only from one time region of one sample macro-trace;
4. selecting user-defined time region for macro-trace and build corresponding anisotropy decays. This option is available only for Polarization experiments;
5. automatic and manual selection of bursts in a macro time;
6. creation series of decays or autocorrelation functions for given macro-trace according to the sliding method described in Data management section. To do this software will support the sliding window width and shift selection;
7. creation series of inter arrival time distributions for given macro-trace;
8. selecting start and end analysis points with cursors;
9. setting values, minimum, maximum and fixing for fit parameters;
10. performing analysis of fluorescence decays, anisotropy decays or autocorrelation functions as it is described in Analysis section. While performing parameters estimation, the software takes into account several possible instrumental distortions that can present in measured data (time shift, background, G-factor);
11. displaying the measured and theoretical curves, weighted residuals and their autocorrelation in separate pop-up window;
12. viewing MLE values for each analyzed curve and average MLE value for all curves within current analysis session;
13. displaying fluorescence lifetimes histogram;
14. displaying triplet lifetimes histogram;
15. displaying IATD coincidence values and ratios;
16. exporting analysis results in ASCII file;
17. exporting autocorrelation functions in ConfoCor format.