Operating Instructions

FAST

Advanced Analysis of Fluorescence Kinetics

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for version 3.4.0

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1. About the Program

FAST is a new and unique software package for the analysis of complex fluorescence kinetics using state-of-the-art Fluorescence Analysis Software Technology (FAST). The program combines a user-friendly graphical interface with sophisticated mathematical algorithms for solving mathematically ill-posed optimisation problems emerging from the evaluation of time-resolved fluorescence.

The FAST software is based on proprietary and well-proven computational algorithms of Global Least Squares Analysis (GLSA) and Non-negative Truncated Singular Value Decomposition (NTSVD) developed by Dr. Alexander Goldin. FAST is now exclusively owned and further developed by Edinburgh Instruments Ltd.

These algorithms are particularly powerful for solving unstable, non-linear least squares optimisation problems with multiple local minima, a situation that is often observed in fluorescence decay kinetics. Compared with the traditionally used Marquardt-Levenberg algorithm the Goldin algorithm is 100% convergent, virtually guaranteeing a fit result consistent with the global minimum instead of some local minimum.

Comprehensive testing with simulated data shows that FAST significantly outperforms other commercially available software packages in terms of both the reliability of fit results and the speed of analysis.

The exceptional robustness of the Goldin algorithm is achieved by:
1) Ensuring that only “reliable” directions are considered at each iteration step.
2) Employing an adaptive step-length strategy for computing the Jacobian matrix.

FAST enables the user to fit raw fluorescence kinetics with the following fluorescence kinetic models:
- Exponential Components Analysis (up to 4 exponential terms).
- Global Analysis of exponential decay kinetics.
- Lifetime Distribution Analysis.
- Stretched Exponential Components Analysis.
- Förster Kinetics.
- Micellar Quenching Kinetics.
- Time Resolved Anisotropy Kinetics (four different rotational diffusion models).

These models can be fitted to the raw data by means of either tail fits (fits that do not include an Instrument Response Function, IRF) or reconvolution fits (fits that use both the model function and the IRF to calculate the theoretical fluorescence decay curve).

Most of the fits can be performed in a “batch mode”, where the same model function is sequentially fitted to a set of individual decays.

1.1. Lifetime Distribution Analysis

The robust and easy-to-use Lifetime Distribution Analysis allows lifetime distribution functions to be determined on a grid of up to 200 logarithmically spaced time intervals. Shift and background may be left as free fit parameters (standard) or may be optionally fixed. An optimal level of "regularisation" (stabilisation) is set automatically.

The FAST Lifetime Distribution Analysis does not require a preset shape of the resulting distribution functions, such as Gaussian or Lorentzian distribution functions. However, the analysed decays must be free of growth kinetics as the resulting distribution function allows only for positive amplitudes.

The Lifetime Distribution Analysis is an advanced fitting routine that yields reliable results when the data are of sufficiently good quality, i.e. a narrow instrument response function, high number of data channels, high number of counts in the peak of the decay, no systematic errors.

1.2. Exponential Components Analysis

The Exponential Components Analysis routine provides a robust multi-exponential analysis with up to four exponential terms. The user selects the number of lifetime components (1-4) and the software automatically sets the start parameters (Automated Fit). For the experienced user the option exists to edit the start values before the fit is initiated (Manual Parameter Control).

The fit algorithm ensures fast and robust convergence with a maximum of independence of the start parameters. A shift between the measured sample kinetics and the IRF as well as a constant background of both the IRF and the sample decay can be computed automatically or can be fixed to predefined values.

With Exponential Components Analysis the option is given to calculate the lifetime confidence intervals by means of a Support Plane Analysis. This option also provides parabolas in a chi-square – lifetime graph, allowing the user a graphical assessment of the validity of the chosen multi-exponential model and its lifetime parameters.

1.3. Global Exponential Components Data Analysis

The Global Analysis routine is a powerful tool to simultaneously analyse a set of multi-exponential decay curves (each curve having up to four exponential terms) with one or more common parameters.

With this analysis one or more fit parameters may be linked, i.e. they remain free floating, but they are identical in all individual decay curves.

Global Lifetime Data Analysis is the method of choice for the analysis of a series of decay curves measured when varying a secondary parameter such as wavelength, temperature, sample concentration, etc. Often only a limited number of lifetime parameters change with the secondary parameter, so that those parameters that remain constant can either be linked or even fixed during the iteration process. This increases the accuracy of the fitted parameter and also serves as a validation for the applied model.

1.4. Stretched Exponential Components Analysis

Some fluorescence phenomena can be described with a model function that is known as Stretched Exponential Function or Kohlrausch Quenching Model. This is a model with a faster initial decay than standard exponential processes, and a longer tail.

FAST offers the fitting of fluorescence decays with up to four stretched exponential terms. Shift and background may be left as fitting parameters, or may be fixed.

Literature:

M. Berberan-Santos, E.N. Bodunov, B. Valeur, Ann. Phys. 17, 7 (2008), 460-461 (historical review)
M.N. Berberan-Santos, E.N. Bodunov, B. Valeur, Chem. Phys. 315 (2005) 171-182 (mathematical)

1.5. Förster Kinetics

Most Förster Kinetics (FRET Kinetics) can be modelled with a series of exponential decays (refer to 1.1. Exponential Components Analysis). This is true in particular for a "two-state" model of unquenched (large distance – no Förster transfer) and quenched (distance short – effective Förster transfer).

However, complex Donor-Acceptor distributions require a more advanced analysis. FAST provides the classical model for a homogeneous and rigid (diffusion negligible) distribution of Donor-Acceptor pairs with random distance. Background and shift are optional parameters, as well as the fixing of individual parameters.

Literature:

Th. Förster, Ann. Phys. 2 (1948) 55-75 (original work)
B.D. Wagner, W.R. Ware, J. Phys. Chem. 94, 9 (1990) 3487-3494

1.6. Micellar Quenching

Fluorescence kinetics of Fluorophores embedded in micelles can often be described by the "Micellar Quenching" model. This model is based on the assumption that a localised high concentration of fluorophores (in micelles) causes quenching between the fluorophores. The fitting of measured fluorescence kinetics of micelles results in the average micellar aggregation number and the rate constant of association and dissociation of quenchers.

Like the other models, the Micellar Quenching routine allows the shift and the background to be fitted. Initial parameter estimates are standard, but manual parameter input is possible.

Literature:

M.H. Gehlen, F.C. De-Schryver, Chem. Rev. 93 (1993), 199-221 (mathematical)

1.7. Time Resolved Fluorescence Anisotropy Analysis

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Analysis of time-resolved fluorescence anisotropy is based on the global minimisation of the parallel and perpendicular linearly polarised fluorescence lifetime measurements with linked parameters. Four different models can be used for analysis:

- Spherical rotor
- Two spherical rotors
- Spherical rotor with restricted rotation
- Elliptical rotor

1.8. Weighting of Data

FAST has been specifically designed for the advanced analysis of data obtained using the photon counting techniques. Thus, FAST is applicable to fluorescence lifetime data measured using Time Correlated Single Photon Counting (TCSPC) as well as for phosphorescence decays measured using the multi-channel scaling technique (time-resolved photon counting) and chemical reaction kinetics monitored by Photon Counting.

In all counting methods individual data points exhibit Poissonian Data Statistics. The Poissonian weighting is an ideal scenario for curve fitting, as even for a single data point the standard deviation is known and is given by the square root of that data point. This special feature of Poissonian distributed noise has the consequence that an ***absolute*** fit quality parameter is available. This quality parameter is the normalised chi-square, χ^2, which has the value of unity for an ideal fit.

For small number of counts in data channels (<~10) the chi-square statistics are inconsistent with the Poissonian distribution of noise. For decay processes with poor data statistics (insufficient number of counts in the peak channel, decays with extended tails, improper selection of the fitting range) the use of chi-square as the optimisation parameter yields results that are skewed towards shorter lifetimes. FAST has now implemented a proprietary algorithm to reduce the effect of the chi-square optimisation for low count data channels. This yields fit results that have significantly higher accuracy than those obtained by other programs.

1.9. Additional Features

FAST includes several additional features that increase its usefulness as an analytical tool. These include:

- Overlapping presentation of several time-dependent fluorescence kinetic curves and their fitted curves, which greatly simplifies visual data comparison and examination.
- Simultaneous plotting of time-dependent fluorescence kinetic curves, instrument response function and residuals as well as a graphical and numerical representation of the fitting results in a flexible report format.
- Export of the program's ***Report*** in a text format or in a range of popular image formats including HTML allows creation of high-quality figures for posting on a customer web-site.
- Export of results into file or clipboard for easy import into documents, presentations and image handling software.

2. Loading Data

2.1. Single Files

Figure 1 shows the demo file ***4-exponential decay.FL*** in the FAST main window. This file contains data for both an instrument response function (IRF) and a measured sample kinetics. This file can usually be found in the directory ***C:\Users\Public\Public Documents\FAST Demo Files*** in Windows 7 and in the directory ***C:\Documents and Settings\All Users\Shared Documents\FAST Demo Files*** in Windows XP.

When loading this data-file ensure that the file-type filter (***Files of Type***) is set to the Edinburgh Instruments type (*.fl).

The file can be loaded with IRF (***Import data with IRF***) or without IRF (***Import decay data only (without IRF)***). Select the option ***Import data with IRF***.

The file ***4-exponential decay.FL*** contains a time column, an IRF data column and a decay data column. Therefore, both the IRF and the decay will be loaded into the program.

If the option ***Import data with IRF*** is selected, but only two data columns are contained in the file (time coordinates and intensities), then this second data column will be interpreted as a sample time-course. The dialogue box will open one more time for the user to select a file containing the IRF.

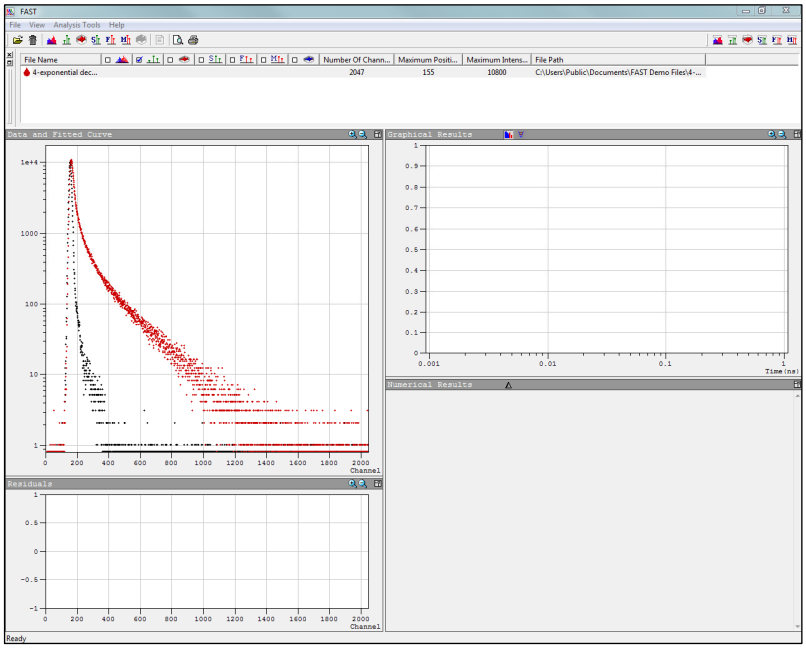


Figure 1
Layout of the FAST main window loaded with the demo file ***4-exponential decay.FL***.

The IRF and the sample time-course will be displayed in the ***Data and Fitted Curve*** window. An IRF is always shown in black and a sample time-course in a different colour.

同样的通道数所以必须得同时测量

Sample kinetics and corresponding IRF should contain the same number of channels. By default the X-axis shows channels. The last channel value of the X-axis corresponds to last channel number of the loaded measurement.

The default layout of data presentation can be changed by using the ***Zoom*** option. If only a fraction of a loaded measurement is zoomed in the window, a newly loaded measurement will also be shown zoomed in the same proportion. For example, if the Zoom option is used to show only the first 1/3 of the first measurement, then the same 1/3 part of the second loaded measurement will also be shown.

Several measurements can be loaded simultaneously. The section on the top of the main screen, the File Manager, will contain the file names, the file paths and important properties (total number of channels, a number of channels containing the maximal number of photons and the number of counts in that channel). Only highlighted files are displayed in the ***Data and Fitted Curve*** window. If more than one data file is highlighted the corresponding IRF will not be shown (Figure 2).

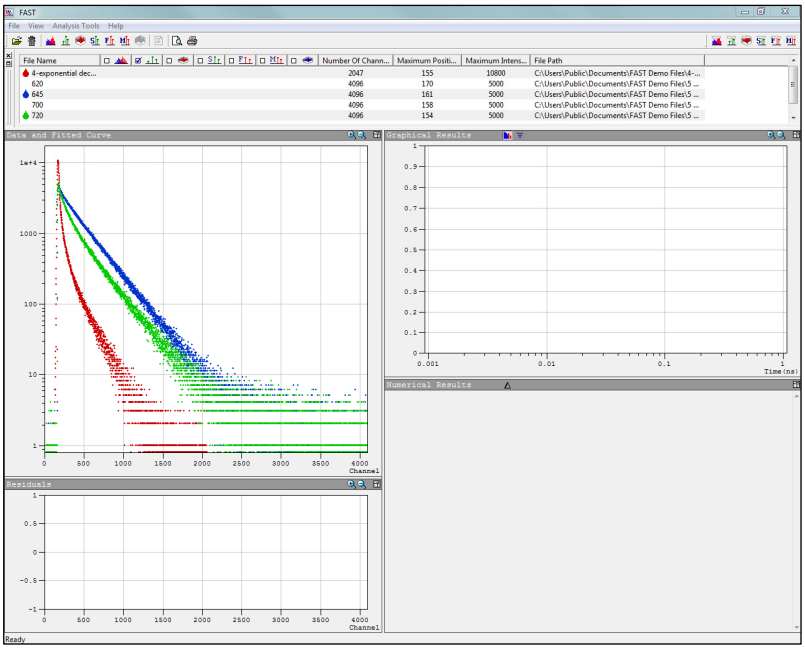


Figure 2
FAST main window with several measurements displayed.

2.2. Multiple Files

FAST can process several decays individually or collectively. The user interface provides a convenient way to load several time-courses into the program. FAST allows loading of “multiple files”, i.e. files that contain more than one sample decay.

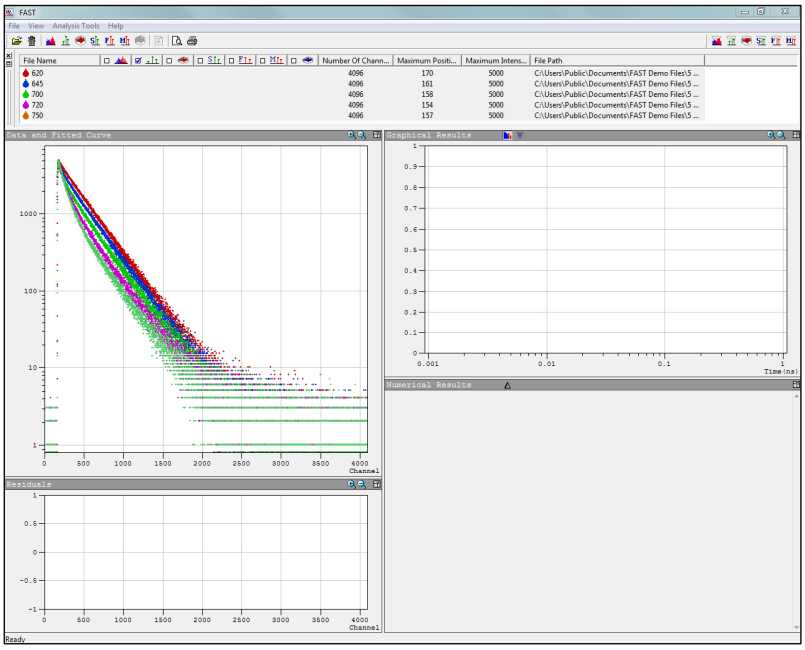


Figure 3
Loading of “multiple files” into FAST.

第一列作为IRF

The file ***…FAST Demo Files\5 global decays link3.FL*** consists of six data columns: the first is for the IRF while the five others are sample decays. If the option ***Import data with IRF*** is selected, the first data column will be interpreted as an IRF and the remaining five data-strings as sample measurements. Each of the decays is labelled. In the given file ***5 global decays link3.FL*** the label refers to the wavelength at which the decay was recorded. The label is shown in the first column of the File Manager.

Note that the instrumental response function is not shown in the graph display when more than one file is highlighted (see previous section).

Loading the file with ***Import decay data only (without IRF)*** selected will result in 6 individual data files being shown with the IRF measurement interpreted as one of the decays. This file may have to be deleted from the list prior to analysis, and then only tail fit analysis will be possible.

2.3. Different Import Formats

FAST allows data in various formats to be loaded. These include file formats generated by software from different manufacturers as well as a simple ASCII file format.

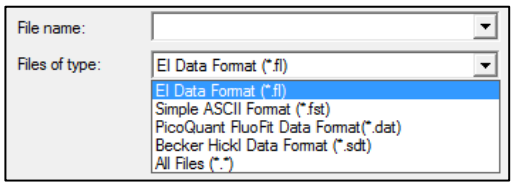


Figure 4
Filtering of data files.

The ***.fst*** extension is reserved for ASCII files. The files can contain two or three data columns. The first column represents the x values in nanoseconds. The second column contains IRF while the third one contains the data of the sample time-course. If the IRF column is omitted the option ***Import decay data only (without IRF)*** should be used.

A template of a .fst file is shown below:

10.937472	3.000E+0	2.000E+0
11.035128	2.000E+0	0.000E+0
11.132784	2.000E+0	3.000E+0
11.230440	4.000E+0	2.000E+0
11.328102	5.000E+0	2.000E+0
11.425752	8.000E+0	2.000E+0
11.523408	1.200E+1	6.000E+0
11.621064	7.000E+0	5.000E+0
11.718720	1.500E+1	6.000E+0
11.816376	1.000E+1	9.000E+0
11.914032	2.600E+1	1.400E+1

.sdt files (files saved by Becker & Hickl software) have no flags for decay data and IRF data. Therefore FAST offers the way to import the data of these files correctly:

If ***Import data with IRF*** has been selected and an appropriate file containing the decay data has been highlighted in the file menu, a small selector dialogue box will be displayed after clicking the ***Open*** button. Here three options are given:

1. Import of an IRF that is contained in the same file. If this is selected the number of the page containing the IRF must be specified.

2. Import of a single IRF from a different file. If this is selected the ***Open*** file menu will appear again asking for a different file containing the IRF to be selected. Note that in this case the IRF must be on the first page of the selected IRF file.

3. Import of multiple IRFs from a different file. If this is selected the user will be ask to specify the file containing the IRFs. Note that in this case the sequence of pages with different IRFs must match the sequence of pages in the file containing the decays.

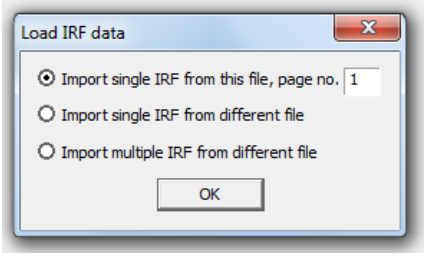


Figure 5
Selector dialogue box for .sdt file import.

3. Understanding the Main Screen

The main program window loaded with the ***4-exponential decay.FL*** file from the ***…FAST Demo Files*** directory is shown in Figure 1. To explain the features of the main screen we will now perform an Exponential Components Analysis reconvolution fit. To fit this time-course select the ***Exponential Components*** menu option from the ***Analysis Tools*** menu, leave the fitting range unchanged (***From*** 1 … ***To*** -1), set the ***Number of Components*** to 4 and start the fitting by clicking the ***Calculate*** button as shown in Figure 6.

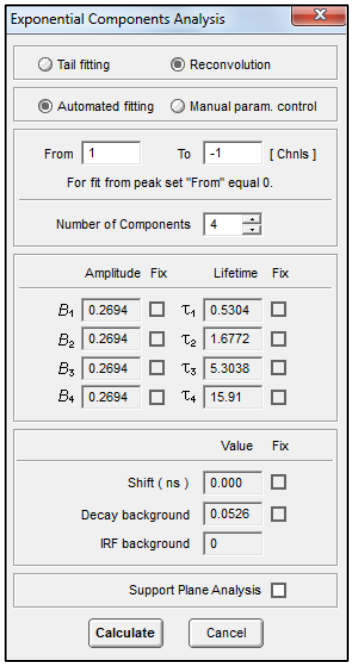


Figure 6
Dialogue box for setting up a fit with Exponential Components Analysis. Set the fitting range ***From*** / ***To*** and the ***Number of Components*** as shown and start the fit by clicking on ***Calculate***.

After completion of the fit the results are displayed as shown in Figure 7.

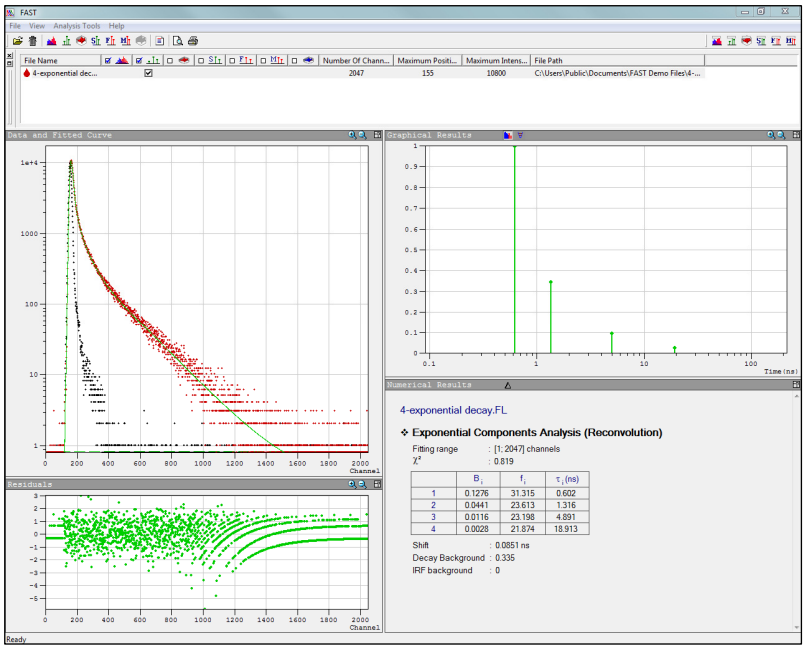


Figure 7
Illustration of the main display of the FAST software.

The main FAST screen is split into the following sub-windows:

1. The ***File Manager*** sub-window on the top, contains the file names and properties of the corresponding measurements.
2. The ***Data and Fitted Curve*** sub-window on the top left contains the graphical display of the instrumental response function, sample decay, and fitted curves.
3. The ***Residuals*** sub-window on the bottom left shows the weighted residuals for a visual assessment of the fit quality.
4. The ***Graphical Results*** sub-window on the top right is a graphical representation of lifetimes, amplitudes and lifetime distributions.
5. The ***Numerical Results*** sub-window on the bottom right lists the calculated parameters.

3.1. Graphical and Data Windows

Three of the five sub-windows, i.e. ***Data and Fitted Curve***, ***Residuals*** and ***Graphical Results,*** are graphical presentations.

These sub-windows can be resized by clicking and dragging the frame. The information about the window size and whether the sub-windows are active or not is stored by the software. Thus, the window layout is preserved when the program is closed and will be reinstated at the next start-up.

It has been found practical to reduce the height of the ***Residuals*** sub-window to give more room for the raw data and fitted curves. It may also be convenient to reduce the height of the ***Graphical Results*** sub-window to open up room for the list of the fitted parameters.

3.1.1. Right Mouse Click Functions

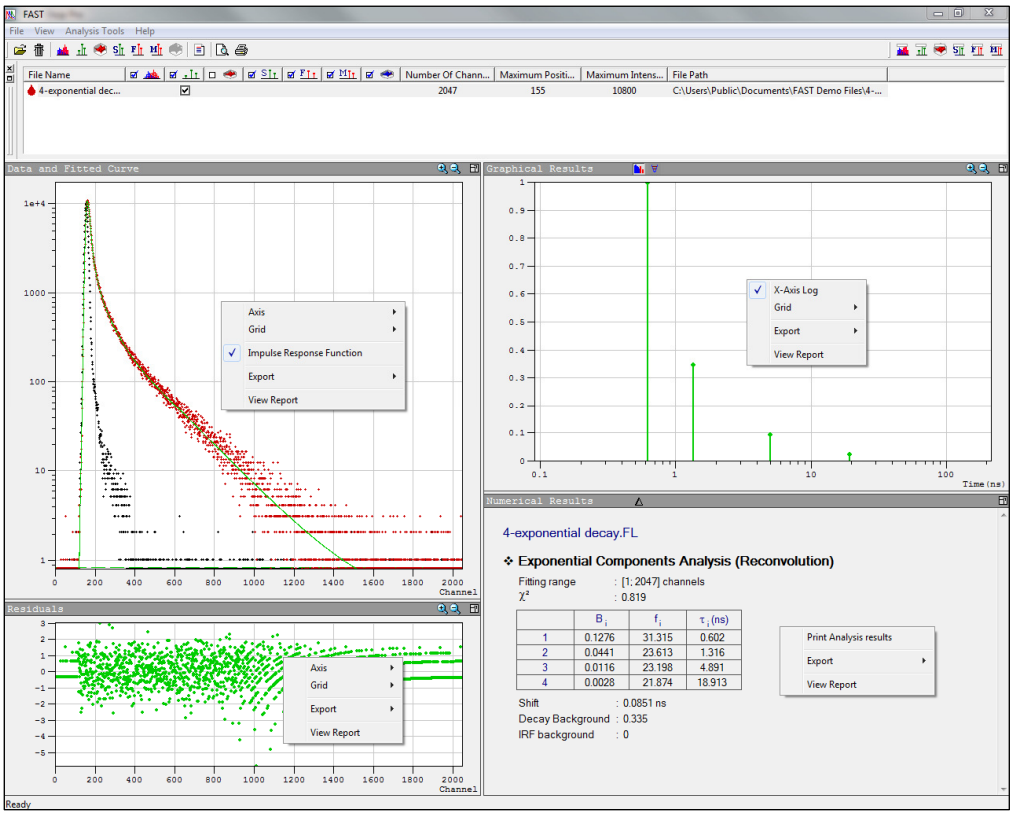


Figure 8
Main Display showing Right Mouse Click functions for each window.

Each of the sub-windows (except for the ***File Manager***) has associated right mouse button click functions. Some of them are common for all sub-windows, while others are specific to just one. The right button menus of all four sub-windows are shown in Figure 8. The meaning of each respective function is as follows:

Axis:

(applicable only to the three graphical sub-windows)

The ***X-axis*** can be changed between ***Channel*** and ***Time*** only for the ***Data and Fitted Curve*** and ***Residuals*** sub-windows.

The ***X-axis*** can be changed between ***Linear*** and ***Logarithmic*** for all three graphical sub-windows.

The ***Y-axis*** can be changed between ***Linear*** and ***Logarithmic*** only for the ***Data and Fitted Curve*** sub-window.

Note that the ***X-axis*** can be shown in the ***Logarithmic*** mode only when it is in ***Time*** (and not in ***Channels***).

Typically, the ***X-axis*** of the ***Graphical Results*** display is shown in the ***Logarithmic*** mode.

Grid:

(applicable only to the three graphical sub-windows)

The choice is between ***Detail Grid***, ***Rough Grid*** and ***No Grid***.

Impulse Response Function:

(applicable only to the ***Data and Fitted Curve*** sub-window)

The choice is given to either display the IRF or not. Note that the IRF is not shown if more than one data file is highlighted in the File Manager, even when the option to display the IRF is selected.

Export:

The export facility of the three graphical sub-windows allows exporting the data in either an image or text format. If the option ***File (Image)*** is selected a bitmap file of the entire graph (as displayed, including the aspect ratio) will be saved. If instead the option ***File (Text)*** is selected, an ASCII file with two columns separated by tabs will be saved.

If the ***File (Text)*** option is selected in the ***Data and Fitted Curve*** sub-window, the first column of the file will contain time-coordinates while the second column contains the data of the fitted curve.

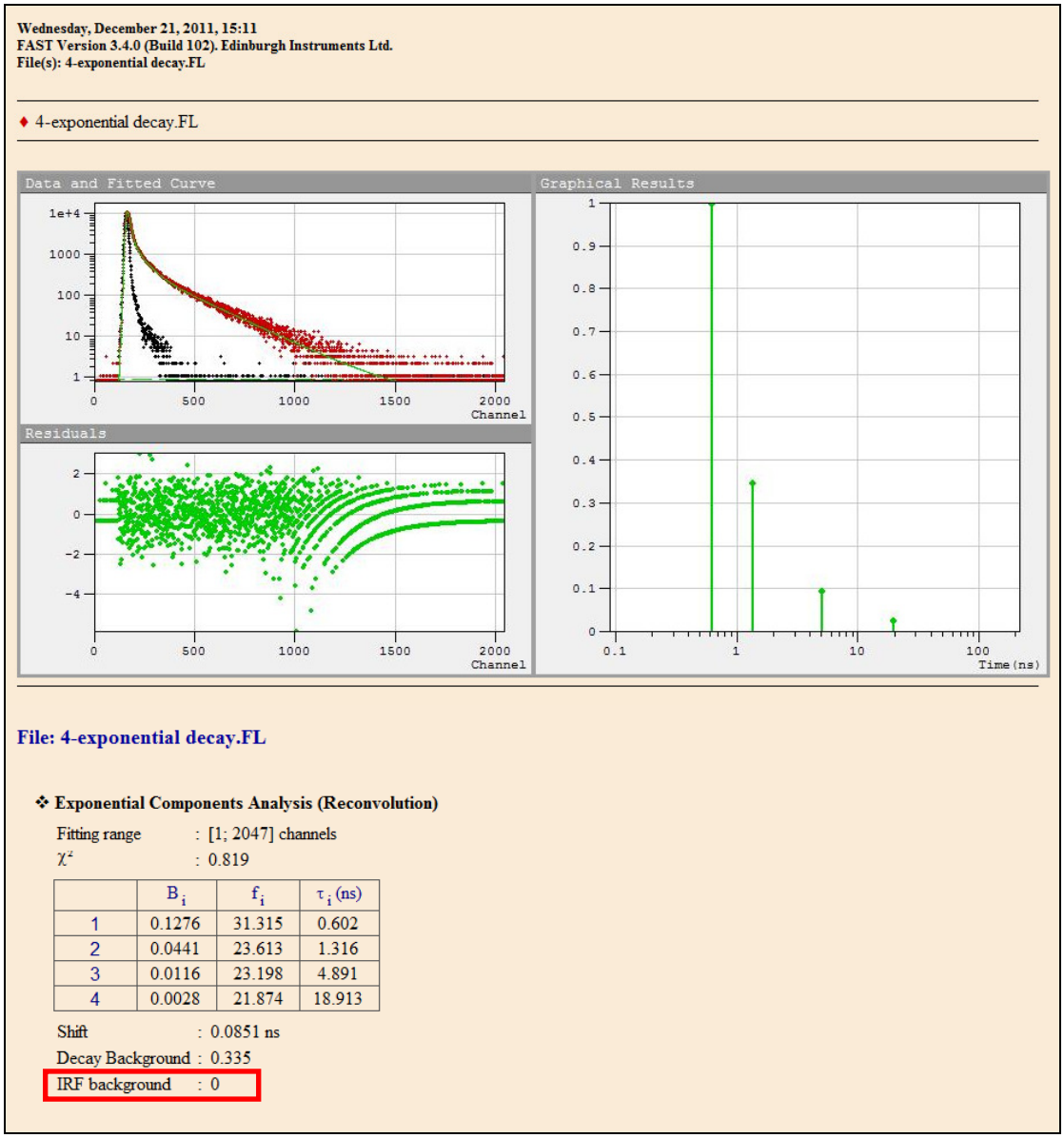
If the same option is chosen in the ***Residuals*** sub-window, the second column will contain the residuals.

If the ***File (Text)*** option is selected in the ***Graphical Results*** sub-window, a table of calculated parameters will be saved in text format. This will be a table of parameters for data obtained with Exponential Components Analysis or Global Analysis, or two columns of data in case of results from a Lifetime Distribution Analysis with the first column being the grid of lifetimes and the second column being the corresponding distribution.

The options ***Clipboard (File)*** and ***Clipboard (Text)*** allow easy transfer of graphical or text data from FAST to other programs.

View Report:

This function creates a detailed report in web page format which can be viewed with a web browser. Figure 9 shows an example of a report.



	B_i	f_i	τ_i (ns)
1	0.1276	31.315	0.602
2	0.0441	23.613	1.316
3	0.0116	23.198	4.891
4	0.0028	21.874	18.913

Figure 9
Sample View Report browser contents.

3.1.2. Zoom and Pan

The three graphical sub-windows have tool buttons on the upper right panel of the sub-window. The user can zoom into a part of the full graphical display by clicking the “+ magnifying glass” button and subsequently selecting the rectangular area of interest using “click and drag”. The mouse cursor carries the “+” symbol when the “zoom-in” option is active.

Figure10
“Zoom-in”, “Zoom-out” and “Page” icons allow control of the graphical sub-window.

Once zoomed in, a click on the now displayed “hand” button will enable the viewer to pan the zoomed window through the entire graph. While this option is chosen the mouse cursor shows the “hand“ symbol.

In order to return back to the full graph the “page” button should be clicked. If instead the “- magnifying glass” button is clicked only a fractional zoom-out will take place and several mouse clicks might be required to see the full graph.

The grey “page” button on the outer top right corner enables the user to switch between full screen view and fragmented screen view.

The ***Numerical Results*** window can also be maximised by using the “page” button.

3.2. The “View” menu

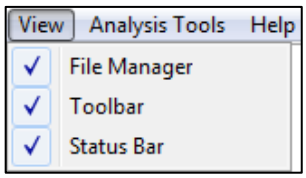


Figure 11
The ***View*** menu.

The ***View*** menu provides options for the displays of some features of the main FAST window.

File Manager. Activate or deactivate the display of the File Manager sub-window.

Toolbar. Button to select the toolbar to be displayed or not. The icons in the tool bar provide quick access to the tools that are available from the ***Analysis Tools*** menu. This option provides an alternative way for selection of the analysis subroutines.

Status Bar. A status bar on the bottom of the screen can be made active. The information displayed in the status bar may be useful when monitoring the progress of a more complex (and longer lasting) analysis.

3.3. Toolbar Icons

The buttons provided in the tool bar provide quick access to certain options from both the ***File*** menu and the ***Tools*** menu. Some buttons may be inactive if the option is not available due to insufficient number of curves loaded, or if no fit result is displayed.

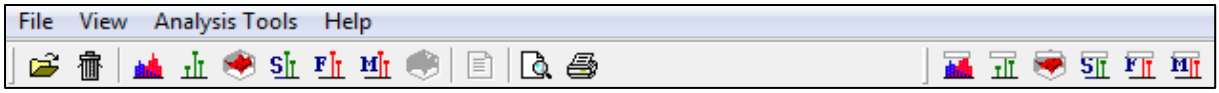


Figure 12. The Toolbar functions.

Open file

Delete file

Dialogue box for Lifetime Distribution Analysis

Dialogue box for Exponential Components Analysis

Dialogue box for Global Exponential Components Analysis

Dialogue box for Stretched Exponential Components Analysis

Dialogue box for the analysis of Förster Kinetics

Dialogue box for the analysis of Micellar Quenching Kinetics

Dialogue box for Fluorescence Anisotropy Analysis

Generation of a Fit Report

Print preview

Print

Formula Display of Lifetime Distribution Analysis

Formula Display of Exponential Components Analysis

Formula Display of Global Exponential Component Analysis

Formula Display of Stretched Exponential Component Analysis

Formula Display of Förster Kinetics

Formula Display of Micellar Quenching Kinetics

4. Basics of Fluorescence Lifetime Analysis

Kinetic parameters can be recovered from raw sample decay data by means of numerical analysis. Some basic principles of this data analysis are described below.

4.1. The Sample Decay Model

Any method of extracting kinetic parameters from raw sample decay data requires some knowledge of the underlying kinetic process. This kinetic process is described in the form of a mathematical expression, *R(t)*, that contains the variable "time" and appropriate kinetic parameters. In practice, the variable "time" is measured in discrete channels, and these channels (or time bins) may be used as the x-coordinate.

A "natural" decay process (spontaneous emission) follows an exponential decay law. In addition, many quenched decay processes are also of exponential nature.

The single exponential model is taken here as an example model function (to be analysed with Exponential Components Analysis) to explain the basics of Fluorescence Lifetime Analysis:

$$R(t) = B\, e^{-\frac{t}{\tau}} \quad , \qquad \textbf{(1)}$$

with a pre-exponential factor *B* and a characteristic lifetime τ.

R(t) is often called the ***sample decay model***. It is a theoretical expression for the response of the sample to an infinitely short excitation pulse. *R(t)* must be distinguished from the complex, convoluted (smooth numerical) ***sample response function*** *S(t)* and from the (noisy) ***raw decay data*** *F(t)*.

The characteristic lifetime, τ, is the time it takes the fluorescence to decay to a level of 1/e (about 37%) of the original value. The characteristic lifetime τ is an intrinsic sample parameter; it is solely dependent on the sample and is not affected by instrumental parameters (assuming there is no systematic error due to a wrong calibration of the time axis).

The pre-exponential factor, *B*, is a value which includes technical (instrumental) parameters and sample parameters. Instrumental features such as efficiency of the system, geometrical conditions of the sample, intensity of the excitation source, etc, will increase or decrease the measured sample signal, which effectively will result in an increase or decrease of the *B* values, but will not effect τ.

The pre-exponential factors can be either positive or negative. A positive *B* value represents a decay process, while negative *B* values are characteristic of growth processes.

4.2. Reconvolution

In practice, lifetime measurements are carried out with an instrument response function (IRF), *E(t)*, that is not infinitely short. The width and shape of *E(t)* are determined by the duration of the excitation light pulse and also by the response time of the detection system. When the half-width of the IRF is comparable with the characteristic time of the process under study the initial part of the sample measurement deviates from the theoretical sample function *R(t)* described by equation **(1)**. In this case the sample response function *S(t)* represents a convolution of *E(t)* with the sample decay model *R(t)*. Hence, in order to get valid information about the fast processes the raw data must be deconvolved.

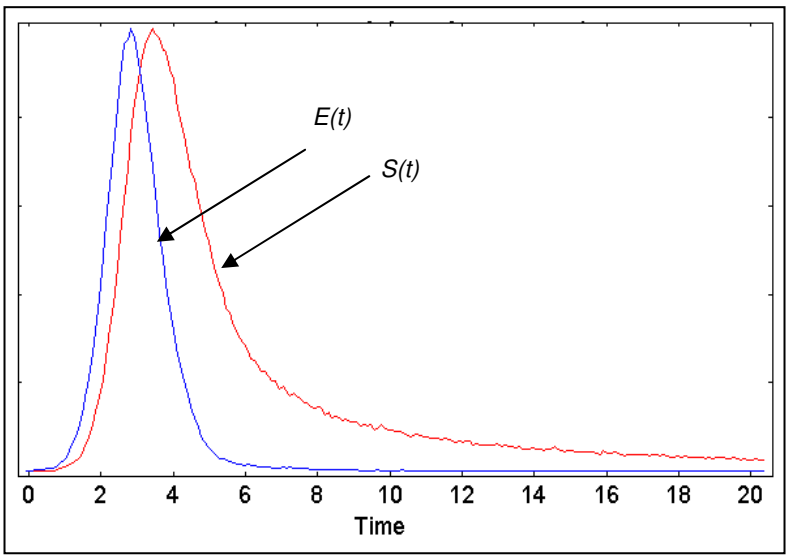


Figure 13
The measured sample kinetic (red line) is a convolution of IRF (blue line) and the sample decay model function R(t).

The mathematical relationship between the sample decay model function, *R(t)*, the instrumental response function, *E(t)*, and the sample response function, *S(t)*, is given by the convolution integral:

$$S(t) = \int_0^t E(t')\ R(t-t')\ dt' \qquad \textbf{(2)}$$

Apart from the random noise, *S(t)* fully describes the measured data, i.e. the initial rise and the subsequent decay of the measurement. In order to calculate *S(t)* one needs to know both the theoretical model for the decay, *R(t)*, and the separately measured IRF, *E(t)*. Only in the case of an infinitely short excitation the sample time-course *S(t)* is identical to the model function *R(t)*.

4.3. Shift and Background – Instrumental Parameters

Not only can the finite width of the excitation pulse influence the raw decay data, but other instrumental parameters can do so, too. They need to be taken into account when fitting the data.

Shift (δ)

A shift parameter is fitted alongside the lifetime parameters. This shift characterises the delay of the decay process in respect to the IRF measurement. Often this shift originates from run-time effects of secondary electrons inside the photomultiplier, from propagation delays of light within the monochromator, or even from differences in the refractive index between “filter in beam” and “filter out of beam”. The shift parameter is often in the order of a few picoseconds.

Decay background ($Bgrd_{Dec}$)

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The fluorescence decay might be superimposed by a constant background. This background often originates from dark count rates of the detector, but might also be caused by light leaks to ambient light. The background is more dominant for measurements that require long data acquisitions because of small sample signal. Novel Infrared photomultiplier intrinsically have high dark count rates. A constant background of hundreds or even thousands of counts in average is not unusual. Note that also a very long decay process may be confused with an underlying background, in particular when the laser excitation rate is high and the long decay is not left enough time to decay to zero before the next pulse excites the sample again.

IRF background ($Bgrd_{IRF}$)

For most measurements an IRF background can be experimentally avoided, or it is negligibly small. However, there are cases when this is not the case, e.g. IRF measurements with an Infrared photomultipliers.

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The IRF background can not be fitted, but it still can be numerically accounted for. FAST has a proprietary algorithm to discriminate the constant IRF background from the true IRF.

There are rear cases where the light source itself produces a noticeable background. Also those special cases can be fitted, using the manual parameter control in FAST.

4.4. Principles of the Optimisation Algorithm

The principal concept for fitting the convoluted sample response function, *S(t)*, to the raw data, *F(t)*, is the minimisation of the sum of least squares:

$$\chi_g^{\,2} = \sum_k w_k^2 \, (F_k - S_k)^2 \qquad \textbf{(3)}$$

where the square of the difference between the measured values F_K and the data points of the fitted curve S_K multiplied by the weighting factor w_K is summed for all data of the fitted range. The best fit is obtained when the value of $\chi_g^{\,2}$ is at the minimum.

4.5. Fit Quality Parameters

The quality of the fit result can be evaluated in several ways.

While, in some extreme cases, a simple visual comparison between the raw data and the fitted curve could be sufficient to find the reason for the mis-fit it is more usual that other parameters need to be calculated to allow a more precise evaluation of a fit.

The most common parameters used to assess the quality of a fit are the following:

The Reduced Chi-Square

Using the expression of χ_g^2 outlined in section 4.4. and dividing it by the number of free parameters n (which is approximately the number of fitted data points subtracted by the number of lifetime parameters used in the fit) results in

$$\chi^2 = \sum_k w_k^2 \frac{[F_k - S_k]^2}{n} . \qquad \textbf{(4)}$$

χ^2 is called the "reduced chi-square" or "goodness of fit". The reduced chi-square has a distinct advantage as a measure of the quality of fit over χ_g^2 discussed in the previous section. This is because it's value is (to a large extend) independent of the number of data points and the number of fitting parameters. This allows different fits to be quantitatively compared.

For Poissonian distributed data ($w_k = 1 / \sqrt{F_k}$) the reduced chi-square has the theoretical limit 1.0.

In general, χ^2-values rising above unity indicate a bad fit result, although values of about 1.1, 1.2 or even 1.3 are acceptable under certain conditions. If the fitting range has been inappropriately chosen , χ^2 can be slightly less than 1.0.

[Principally it is necessary to distinguish between the chi-square (χ_g^2) and the *reduced* chi-square (χ^2). However, in this manual it is the latter which is used to evaluate the quality of a fit even though the word "reduced" is omitted.]

The Weighted Residuals

The weighted residuals are the difference between the original data and the fitted curve weighted by the standard deviation of each data point.

$$X_k = w_k \, (F_k - S_k) \qquad \textbf{(5)}$$

A good fit should give a residual curve that only contains random noise distributed around zero. In excess of 99% of all data points should lie within a range of +/- 3 standard deviations.

A significant deviation from a random distribution of residuals will indicate a mis-fit, either because the model that has been chosen was inadequate or because of instrumental artefacts.

4.6. Tail Fitting and Reconvolution Analysis

The analysis routines of FAST offer two fitting options: Tail Fit and Reconvolution Fit.

Tail fitting is only applicable to data which are fitted in a region with no further sample signal generation, either by the excitation light pulse or by sample formation (e.g. excimer generation). Namely, this is where *S(t)* is essentially identical to *R(t)*.

In Reconvolution Analysis the (convoluted) sample response *S(t)* is fitted to the data. This procedure allows fitting over the whole range of the rising edge and decay of the data. In other words, the Tail Fit procedure eliminates the statistical noise from the raw data, but can not handle the region in which sample excitation takes place, while the Reconvolution Fit procedure eliminates both the noise and the effects of the exciting light pulse.

The curves below show typical examples. The data regions which can be analysed with Tail Fits are also indicated. As stated above Tail Fits should only be performed in a region with no further sample excitation, i.e. in a region where the exciting light pulse has disappeared. Consequently the Tail Fit routine can only be used to analyse those samples with long decay times. The measurements in Figure 14 below show decay curves (red) and also the instrument response function (blue). For a Tail Fit the IRF is not required and therefore does not need to be measured. Nevertheless, as the measurements of Figure 14 show, some knowledge of the IRF is needed for setting up the correct start channel of the fitting range.

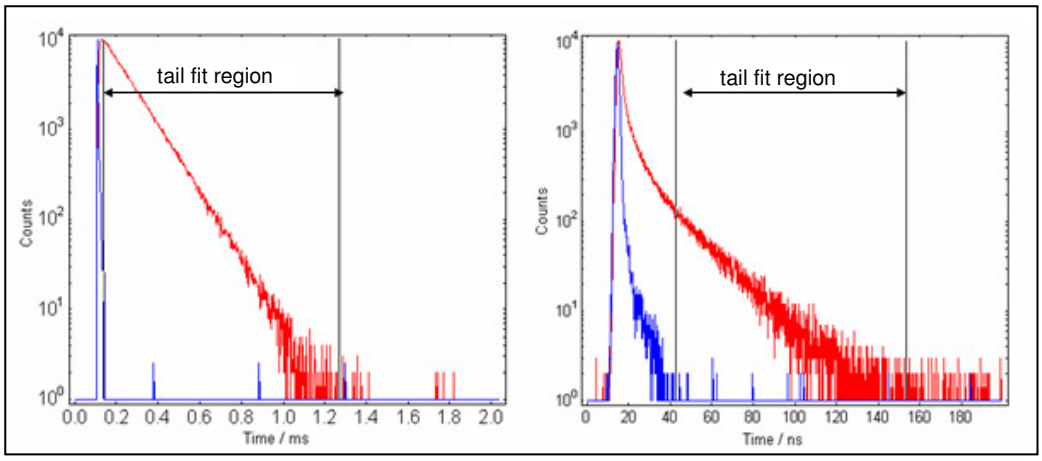

Figure14 Illustration of the Tail Fit region.

With the ***Tail Fit*** applied to the measurement in the left picture it will yield all lifetime information characteristic to the decay measurement. In contrast, Tail Fit applied to the measurement in the right picture will only result in lifetime parameters which have a dominating effect in the longer time region. The lifetime parameters in the shorter region (outside the indicated Tail Fit fitting range) can not be recovered by using the Tail Fit routine. For this data it is far more appropriate to use the Reconvolution Fit routine.

5. Analysis Tools

FAST provides users with seven different types of analysis:

- Lifetime Distribution Analysis.
- Exponential Components Analysis.
- Global Analysis.
- Stretched Exponential Components Analysis.
- Förster (FRET) Analysis.
- Micellar Quenching Kinetics Analysis.
- Analysis of Time Resolved Anisotropy Data.

It is always advisable to start with the simplest model containing a minimal number of parameters. The simplest model is a single Exponential Components Analysis, although the available knowledge about the sample and the experiment might indicate a likely double or even triple exponential fit.

On the other hand, the most general approach for fitting fluorescence lifetime kinetics is a Lifetime Distribution Analysis. This analysis type, however, is mathematically far more challenging and the interpretation of the result is not as straightforward as for the Exponential Components Analysis or indeed all other model functions.

5.1. Lifetime Distribution Analysis

5.1.1. Theoretical Model

The Lifetime Distribution Analysis provides the most general approach of data evaluation as it does not require prior knowledge of a specific model for the decay. This analysis assumes that any fluorescence (phosphorescence or kinetic) decay can be fitted by a large series of exponential functions with fixed characteristic lifetimes and a smooth distribution of pre-exponential factors.

This theoretical model has the mathematical form

$$R(t) = \sum_{i=1}^{N} B(\tau_i)\, e^{-\frac{t}{\tau_i}} \qquad \textbf{(6)}$$

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The abscissa of the distribution is the fluorescence lifetime, separated into N (up to 200) logarithmically scaled intervals. As this algorithm uses a set of *fixed* lifetimes, τ_i, the calculation of the pre-exponential factors B_i is reduced to a linear mathematical problem.

The routine also fits a constant decay background that can be caused by constant (ambient) background light, detector background, or superimposed very long sample decay processes. In addition the routine accounts for a constant background of the IRF, and a shift between IRF and decay.

If Lifetime Distribution Analysis with Reconvolution (as opposed to Tail fit) has been chosen, an additional parameter will be fitted: a shift parameter for a temporal delay between the IRF and the sample decay.

The Lifetime Distribution optimisation algorithm comprises a “search for the smoothest distribution of pre-exponential factors”, and a “search for non-negative distributions of the pre-exponential factors”. Hence, sample kinetics that include a growth in emission (for example due to excimer production) cannot be adequately fitted. However, the unique feature of this analysis is that the resulting distribution of pre-exponential factors can have any shape and is not forced into predefined forms, such as Gaussian or Lorentzian curves.

5.1.2. Performing the Fit

Figure 15 shows the layout of the dialogue box for setting up the parameters for a Lifetime Distribution Analysis.

Firstly, the fitting range (in channels) can be selected.

The convention used is that the first data point on the x-axis is denoted “1”, the channel of maximum signal is denoted “0” and the final data point is denoted “-1”. Hence if the range “From 1 To -1” is selected the fit will be obtained over the whole range of data points.

Generally for Reconvolution analysis, the start of the fitting range is selected to be any value between 1 and the peak of the decay (displayed as one of the file properties in the File Manager). Typically, a start value at the beginning of the rising edge of the decay is chosen.

A special case is the selection of a start value of “0”. When “0” is chosen, the fit will be evaluated from the peak of the decay. For Reconvolution this is typically not selected. For Tail fitting, however either “0”, or a number that is greater than the peak channel number, as displayed in the File Manager, is chosen.

The end of the fitting range should be either -1 (see above), the number of the largest data channel, or somewhat smaller. A smaller value than the maximum data channel will be appropriate, if the decay ends well within the range of displayed data. A constant background, or even a Zero background, does not contain information of the decay, and fitting over such a range should be avoided.

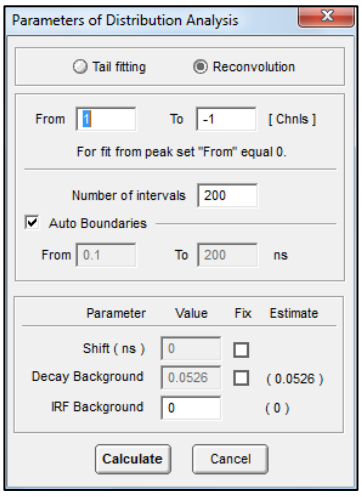

Figure 15
Dialogue box for set-up of the Lifetime Distribution fit.

It is important to check whether ***Tail fitting*** or ***Reconvolution*** has been selected. If an IRF is present, the software will select ***Reconvolution*** by default, but ***Tail fitting*** can still be selected. If no IRF is present only ***Tail fitting*** will be possible, and the option ***Reconvolution*** will be disabled.

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Number of intervals defines the grid of fixed lifetimes. 200 is the maximum value and this is typically chosen for the analysis.

Most fits will be made with ***Auto Boundaries*** active, where the software automatically selects the range of lifetimes (shown in nanoseconds) derived from the data. The advanced user can deactivate ***Auto Boundaries*** and manually enter the shortest and the longest lifetime of the logarithmically scaled X-Axis where X-Axis Time is currently selected.

The boxes for Shift, Decay Background and IRF Background show the best estimates that will be used as start values. If the user wishes to fix the Shift or the Decay Background, this can be made by ticking the corresponding box and subsequently entering the (known) value.

Figure 16 below shows the fit result for the demo file ***4-exponential decay.FL***. All blue data of this display are results of the Distribution Analysis, i.e. fitted curve, residuals, and distribution of pre-exponential factors. For comparison, the result of a Exponential Components Analysis with 4 exponential terms is shown in green. The file ***4-exponential decay.FL*** contains data of a true 4-exponential decay (Appendix A) with lifetimes and amplitude as shown by the 4-exponential (discrete) fit. The main four individual spikes of the Distribution Analysis represent those lifetimes.

Result of the Lifetime Distribution Analysis are available for three different resolution levels: low, normal and high. Switching between resolution levels using the tool buttons  in the tool bar of the ***Graphical Results*** sub-window will impact on all the results of this fit, including the fit, the residuals and the numerical results table.

The default resolution is “normal”. This is the resolution for which the chi-square is at the minimum. However, extra knowledge about the sample may be available, and by adjusting the resolution level to either “high” or “low” the fit result may be slightly modified, with very little impact on the chi-square or none, and the resulting distribution may be more consistent with the extra knowledge available.

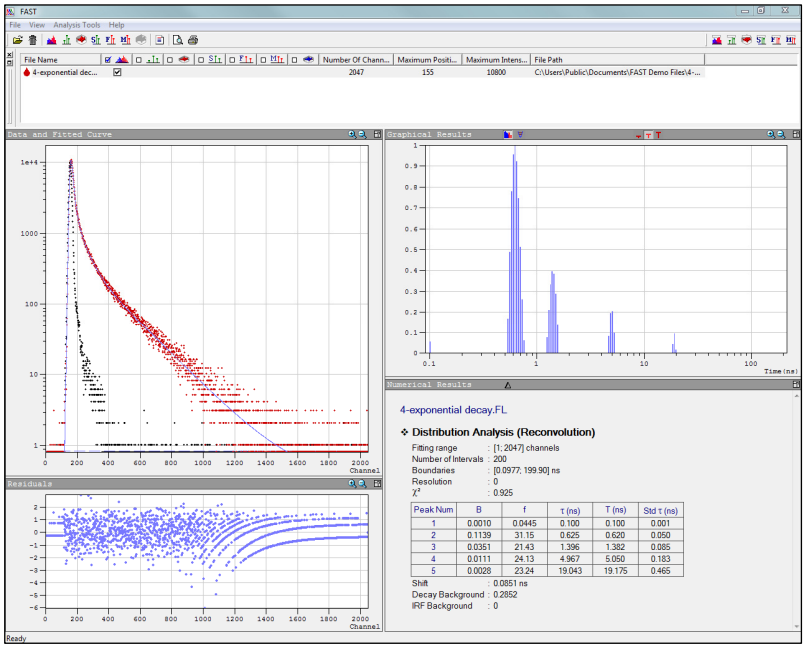

Figure 16
Results of Lifetime Distribution (blue graphs). Note the spike at 100ps is a fit artefact and is not representative for this sample.

The ***Numerical Results*** sub-window shows the fit results in numerical format. As well as showing the *Fitting Range*, the *Number of Intervals*, and the *Boundaries*, which were set prior to the fit, it shows the resolution level, the (reduced) chi-square, χ^2, the fitted decay *Background*, the fitted *Shift* between IRF and decay, together with a table of specific lifetime parameters extracted from the distribution shown graphically in the ***Graphical Results*** sub-window.

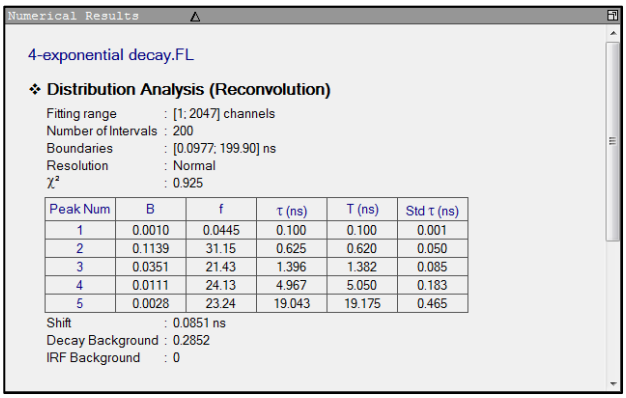

Figure 17
Results of Lifetime Distribution Analysis in numerical format. Note, the  button in the title bar of this window does not have an associated function for Distribution Analysis results.

Note that the analysis of this particular fluorescence decay ***4-exponential decay.FL*** results in a distribution that has five, not four, spikes. Peaks 2-5 represent the true lifetimes of this decay, whereas peak 1 is an small artefact (see table and graph) that should be discarded in the interpretation of the result.

The representation of the distribution in the ***Graphical Results*** sub-window shows normalised curves. The maximum of any result of distribution analysis is normalised to unity.

An unscaled (original) distribution of pre-exponential factors can be obtained by a right mouse click on the picture and selecting ***Export >> File (Text)***, see Figure 18. The data is then copied on to the clipboard, and from there the results graph can be loaded into a separate software package for further analysis and presentation.

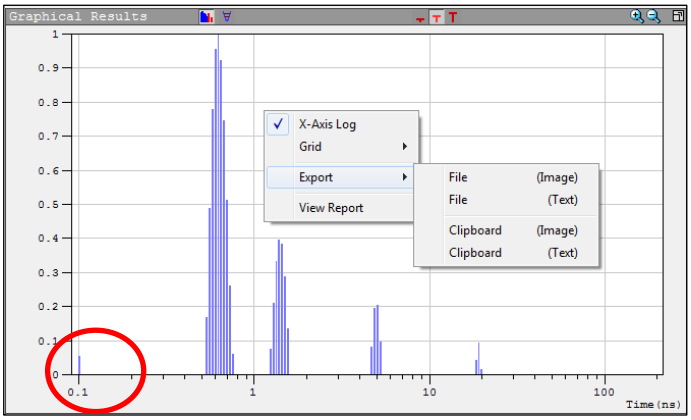

Figure 18
Distribution data as shown on the screen – normalised to Unity.

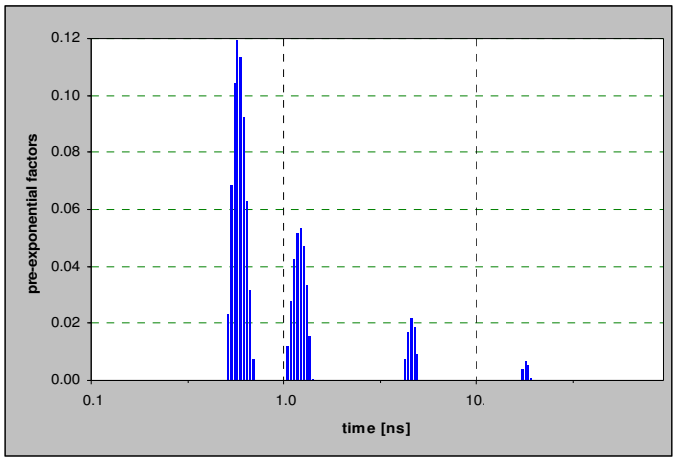

Figure 19
Data of Figure 18, exported. The data are in the original (non-scaled) form.

It should be noted that the fit shown in Figure 16 requires improvement: Data have been fitted in regions that contain no information about lifetime parameters. Looking at the curves in the ***Data and Fitted Curve*** window identifies the data region before the start of the decay, i.e. data channels 1-100, and the data region behind the tail of the decay, i.e. data channels 1500-end. An indication for an inappropriately selected fitting range is a chi-square smaller that Unity, as in the case of Figure 16 0.925.

An adequate fitting range for ***4-exponential decay.FL*** data would therefore be 100-1500. Fitting over this range will result in a chi-square that is above Unity and broader distribution spikes.

5.1.3. Interpretation of Fit results

The data of the file ***4-exponential decay.FL*** is a true 4-exponential decay, not a real distribution of lifetimes. However, this data was used here to show the capability of the Lifetime Distribution Analysis. The Distribution Analysis resulted in four major spikes, with the centre of gravity of each spike precisely matching to known lifetimes (refer to Figure 16). The width of each lifetime distribution spike, as shown in Figs. 18 and 19, is caused for two fundamentally different reasons:

1. The "true" distribution of the pre-exponential factors of the sample measurement.
2. The statistical precision of the data.

As the data used have no real distribution, the widths of the spikes must be caused by the accuracy of the data and can be seen as an expression of the standard deviation of the four lifetimes of this decay. If the same measurement was repeated with only 1000 counts accumulated in the peak, the apparent distribution of lifetimes would be broader while, if the measurement was made with more counts in the peak, the four spikes would become narrower.

Consequently, in order to obtain a true distribution of lifetimes, the contribution to the width arising from the noise on the data must be minimised by using high precision data. High precision data typically means measurements that have a sufficiently high number of data points on the X-Axis (1000 channels minimum) and sufficiently high number of counts in the peak of the decay (10,000 counts minimum). In addition no instrumental artefact (such as systematically distorted decays by radio interference) and a minimum of detector background are required. While high data precision is desirable for any type of fit, it is an even more important requirement for a meaningful analysis of Lifetime Distributions.

The numerical display of the fit results of the Lifetime Distribution Analysis (refer to Figure 17) contains a number of parameters that are extracted from the calculated Lifetime Distribution:

Each peak in the distribution is numbered (starting with the shortest lifetimes), and the derived parameters of this distribution spike are summarised in rows of the table. The choice of derived parameters enables easy comparison with results from fits with discrete exponential components.

B

The B value shown in the table (Figure 17) is the sum of all pre-exponential factors of a given distribution spike. In the example of the distribution fit of ***4-exponential decay.FL*** the distribution spike for the 0.625ns lifetime (refer to the table in Figure 17) consists of 10 individual non-zero B_i values (Figure 20). The sum of these 10 values is 0.1139.

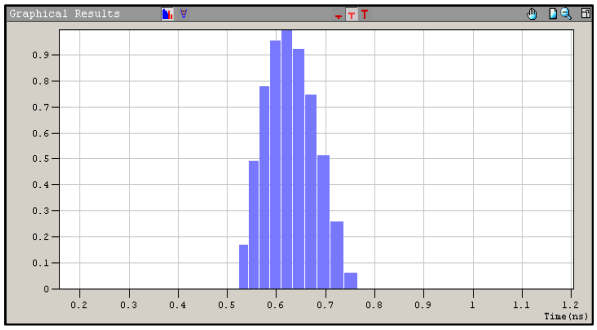

Figure 20
The first "distribution spike" of the result displayed in Figure 18, shown in linear time scale.

τ

This parameter is calculated from the centre of gravity of the lifetime distribution spike and is the equivalent to the fluorescence lifetime calculated by Exponential Components Analysis.

For the example of the first spike of the distribution of ***4-exponential decay.FL*** (Figure 20) the centre of gravity is at 0.625ns, a little larger than the maximum of this distribution spike.

f

"f" stands for "fraction" and is the equivalent of the fraction of the total fluorescence intensity (in %) within that distribution spike, given by :-

$$f_1 = \frac{B_1 \cdot \tau_1}{B_1\tau_1 + B_2\tau_2 + B_3\tau_3 + B_4\tau_4} * 100\% = 31.15\% \qquad \textbf{(7)}$$

For the first major component of the 4-exponential decay f_1 is 31.15%.

T
T is the maximum of the distribution spike. For the spike in Figure 20 it is 0.620ns, the mid- value of the 5th bar in the distribution .

Std τ
This value, given by half the width of the lifetime distribution spike, is the equivalent to the standard error calculated by Exponential Components Analysis.

The user might wish to test the Lifetime Distribution Analysis on a real distribution of lifetimes. As it is difficult to get example files with real "known" data, a distribution has been simulated and is available for evaluation: ***distribution of lifetimes.FL*** (Appendix A).

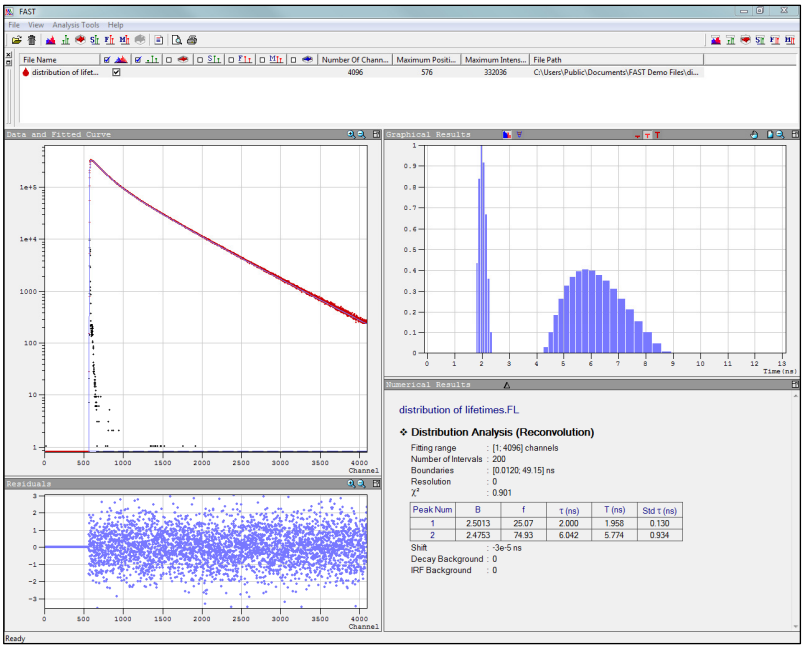

Figure 21
Lifetime Distribution Analysis of a known bi-modal distribution.
Refer to Appendix A for the set of known parameters.

5.2. Exponential Components Analysis

5.2.1. Theoretical Model

The theoretical model of the Exponential Components Analysis is a decay of up to four fluorophores of each having a discrete lifetime. The natural (unquenched) decay of an excited fluorophore follows an exponential law. In addition, the vast majority of quenching phenomena can be expressed with exponential terms (of shorter lifetimes compared to the unquenched situation). Furthermore, growth kinetics often also obey exponential kinetics; any growth is immediately recognised by a negative pre-exponential factor.

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The mathematical form for this model, uses up to four exponential terms with characteristic lifetimes, τ_i, corresponding pre-exponential factors, B_i

$$R(t) = \sum_{i=1}^{4} B_i \; e^{-\frac{t}{\tau_i}} \quad . \qquad \textbf{(8)}$$

The analysis also includes a fitting of a constant background level of the decay curve.

If Exponential Components Analysis with Reconvolution (as opposed to Tail fit) has been chosen, a shift parameter for a potential temporal delay between the IRF and the sample decay can also be fitted. If a background of the IRF is present, this will be removed automatically before fitting.

The fit algorithm will search for the global minimum of chi-squared (equation 4). Generally, the more free parameters are used in the fit, the less pronounced will be the global minimum and additional local minima will appear. The user should try to “help” the algorithm by using the smallest number of free parameters. This includes choosing the smallest possible number of exponential terms and fixing known values, such as background or shift.

5.2.2. Performing the Fit

Exponential Components Analysis fits can be performed in two ways: ***Automated fitting*** or with ***Manual parameter control***. The two dialogue boxes for setting up these fitting modes are shown in Figure 22.

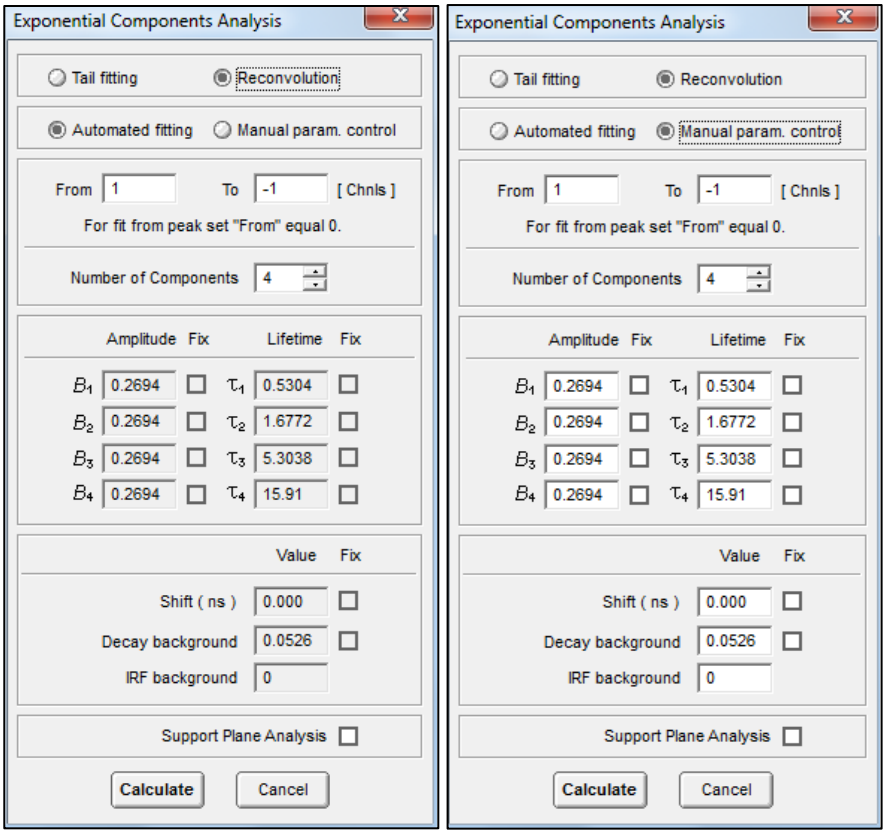


Figure 22
Set-up dialogue boxes for automated fitting (left) and fitting with manual parameter control (right).

Automated fitting provides the safest route to a good fit result. New users should try to fully exploit all options of the ***Automated fitting*** mode first, before attempting to force fit results in one or the other direction by using ***Manual parameter control***.

At the top of the screen the user has the choice between ***Tail Fitting*** or ***Reconvolution***, provided the file selected in the File Manager contains both the IRF and the decay. If no IRF is present, only ***Tail Fitting*** will be possible, and the option ***Reconvolution*** will be disabled.

The ***From*** and ***To*** editable boxes are used to choose an appropriate fitting range, to be entered in channels. As before, a typical start value at the beginning of the rising edge of the decay should be chosen.

A special case is the selection of a start value of “0”. When “0” is entered, the fit will be evaluated from the peak of the decay. For reconvolution this is typically not the best choice. For tail fits, however, either “0” should be entered as the start value, or a number that is greater than the peak channel displayed in the file manager.

The end of the fitting range should be either the number of the largest data channel, or somewhat smaller. A smaller value than the maximum data channel will be appropriate, if the decay ends well before the last data channel of the displayed data. A constant background, or even a Zero background, does not contain information of the decay, and fitting over such a range should be avoided.

For convenience, “-1” can be entered into the ***To***-box. If the value of “-1” is entered, the fit will automatically extend to the maximum data channel.

The field ***Number of Components*** allows the number of exponential terms of the model to be entered. The user will soon get a feeling whether a sample decay may be best modelled by a single exponential term, or by a number of exponentials. The simpler the model the better. And when testing the data for an appropriate model, often it will be appropriate to start over again, i.e. repeating the lifetime measurement with cleaner samples, cuvettes without finger prints, with a solvent that has been purified one more time, etc. It should always be remembered that the data that are analysed here have Poissonian noise and are demonstrated in a semi-logarithmic scale. A small additional component might not be detectable with Gaussian data that are often viewed in linear scale (typically produced by standard absorption and emission spectrometers).

Upon opening the Exponential Components Analysis dialogue box, estimates for all start parameters will be calculated, and they are displayed in the parameter windows on grey background (***Automated fitting***), or on white background (***Manual parameter control***) to indicate that editing of these numbers are possible. Automated fits will always use the estimated start parameters. They are appropriate for the vast majority of decay processes. However, in some cases the start parameters should be modified (***Manual parameter control*** – no parameter fixing). Only then will a good fit result be obtained. Typically this is required for kinetics that contain a rise as well as decay(s).

In Manual parameter control, parameters can be fixed during the fitting process, thus giving the user not only control over the start conditions, but also over the end result. Parameter fixing can be a powerful tool, as it reduces the number of free fit parameters. With the right choice of the value of the fixed parameters, this can improve the accuracy of the fitted parameters. The most obvious parameter that may be a candidate for fixing is the decay background. If there are no signal counts before the time of the excitation pulse, the background is zero and can safely be fixed accordingly.

Parameter fixing becomes problematic, if parameters are fixed with wrong values. This will skew the other (free) parameters, and at the end of the fit none of the parameters will be accurate.

If a parameter was fixed during the fit, this will be indicated in the ***Numerical Results*** window: The word “fixed” is attached to the displayed parameter. In addition the corresponding error will be Zero.

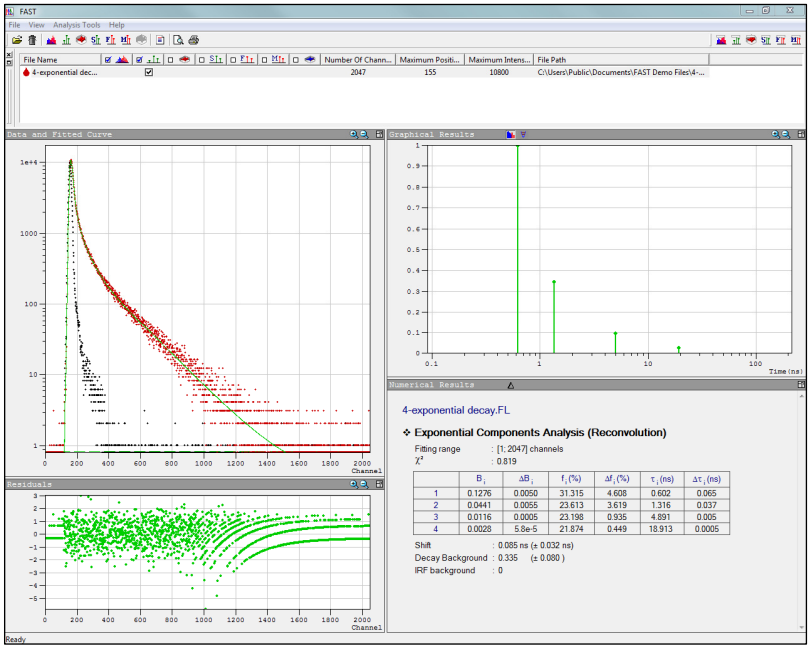


Figure 23
Fit result with errors shown.

The lifetimes and associated other parameters of the individual exponential terms will be shown in order, starting with the shortest lifetime. Therefore, if some lifetimes were fixed, the order might change when the result is displayed.

The ***Graphical Results*** window shows a graphical display of the pre-exponential factors versus their lifetimes. As was the case in the display Lifetime Distribution results, the B-values are normalised to Unity.

Note the  button in the title bar of the ***Numerical Results*** window. This button allows the user to toggle between the errors, for B, f and τ values, being displayed and not being displayed. If data are being copied into text format (***click*** and ***drag***), then this will copy the data that are actually displayed. The Numerical Results display of Figure 23 shows the table with the errors being displayed.

The errors for B, f, and τ are "asymptotic standard errors". This means they are independent of the other parameters (and their errors). FAST can also calculate the confidence intervals. These intervals reveal the range of a particular lifetime that is generated as a result of compensating errors for the other lifetimes (and pre-exponential factors).

The confidence intervals will be calculated when the ***Support Plane Analysis*** is turned on (bottom section of the dialogue boxes shown in Figure 22.) Adding the Support Plane Analysis to the Exponential Components Analysis will extend the calculation time. The benefit will be a more detailed understanding about the "level of trust" for the calculated lifetimes. This is shown in section 5.2.4.

5.2.3. Interpretation of Fit Results of Exponential Components Analysis

The table of results in the ***Numerical Results*** window contains a list of parameters that are in order of the calculated lifetimes. The number of rows is identical to the number of exponential terms requested for the fit.

The meaning of the displayed parameters are as follows:

B
Pre-exponential factor. B_1 is the amplitude of the first exponential term, B_2 that of the second, etc.

If the fit was performed in ***Tail fitting*** mode, the sum of all *B* values of a decay is the amplitude of the fitted curve (at the first channel of the fitted curve). Therefore the absolute values of the *B* values will be in the order of the number of data counts of the raw data curve.

If the fit was performed in the ***Reconvolution*** mode, *B* values are scaled by the integrated IRF, i.e. if the same decay would be analysed with a 10 times bigger IRF, the numerical values of the *B* values would be reduced by a factor of 10. If one determines the total number of counts of the IRF, and then multiplies the *B* value with this number, this will result in a value that will be similar to the *B* value for a tail fit, extrapolated to the channel of the peak of the IRF.

ΔB
This figure is a representation of the standard deviation of the associated *B* value. It is important to know that all errors of lifetime parameters are derived from one single data curve only, and many other sources of errors might have to be considered. *B* values in particular are affected by concentration effects and instrumental parameters.

f
"*f*" is the "fraction of fluorescence intensity" in %. *f* is calculated from *B* and τ. For the first component of the 4-exponential decay (Figure 23)

$$f_1 = \frac{B_1 \cdot \tau_1}{B_1\tau_1 + B_2\tau_2 + B_3\tau_3 + B_4\tau_4} * 100\% = 33.55\% \qquad \textbf{(9)}$$

f characterises the brightness of the fluorescence component (which scales with the pre-exponential factor and the lifetime).

Δf
This is the associated error of ***f***, like ***f*** itself given in %.

τ
τ is the characteristic lifetime of the presumed exponential model. For a single exponential decay model, τ would be the time it takes to decay from the initial amplitude to a value of 37% of this amplitude (1/e = 0.3675).

For a model with multiple exponential terms one can define an "average lifetime" $\langle\tau\rangle$. The average lifetime is not automatically calculated by the FAST software, but it can be calculated by the user using the displayed parameters:

$$\langle\tau\rangle = \frac{B_1\tau_1^2 + B_2\tau_2^2 + B_3\tau_3^2}{B_1\tau_1 + B_2\tau_2 + B_3\tau_3}$$, as an example for a 3-exponential decay **(10)**

Δτ
This is the standard deviation of the calculated lifetime.

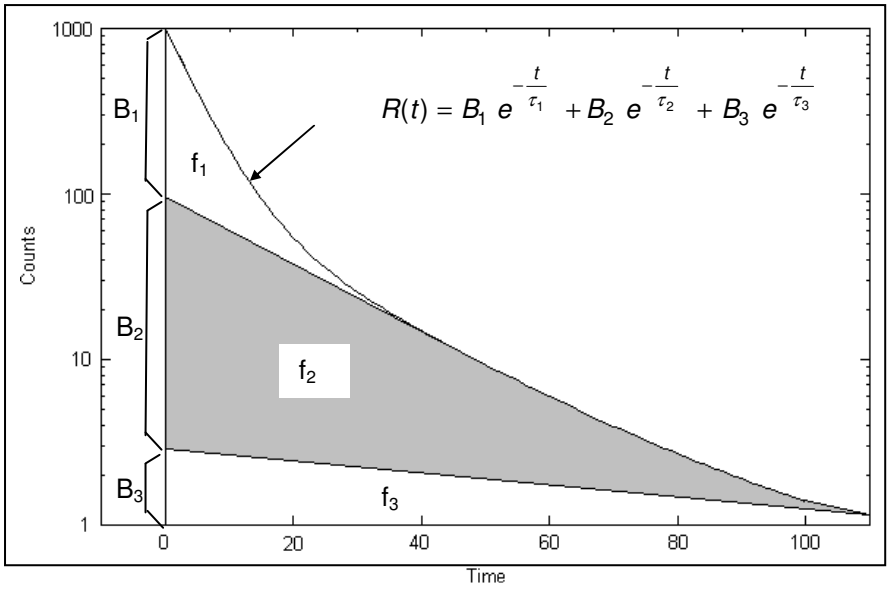


Figure 24
Graphical illustration of the lifetime parameters using a model of a 3-exponential decay.

5.2.4. Support Plane Analysis

An advanced assessment of the errors for parameters calculated with the Exponential Components Analysis is available using the ***Support Plane Analysis***. This analysis calculates a graph that shows the chi-squares versus the lifetimes, while each lifetime is systematically forced away from the fitted (and therefore optimal) value. This procedure is repeated for each lifetime of a multi-exponential fit. The result is a display of parabolas, with the number of parabolas set by the number of free exponential terms.

According to *f* statistics, confidence intervals can be calculated using the ***Support Plane Analysis***. If the chi-square parabolas cross certain thresholds which will depend on the probability with which one can "trust" the calculated lifetime, then this sets the confidence interval. FAST calculates the confidence intervals for 2 probability levels: 68% and 95%. These thresholds are shown in the graphs as dashed horizontal lines, the lower line referring to the 68% confidence level and the upper line showing the 95% confidence level.

The confidence intervals are shown in numerical format in a table below the standard result table of the Exponential Components Analysis.

A confidence interval cannot be calculated for fixed components. Therefore, if one or more of the lifetimes has been fixed the support plane will only show an "***x***" – mark at the position of the fixed lifetime.

An example of a ***Support Plane Analysis*** of the data file ***4-exponential decay.FL*** is shown in Figure 25.

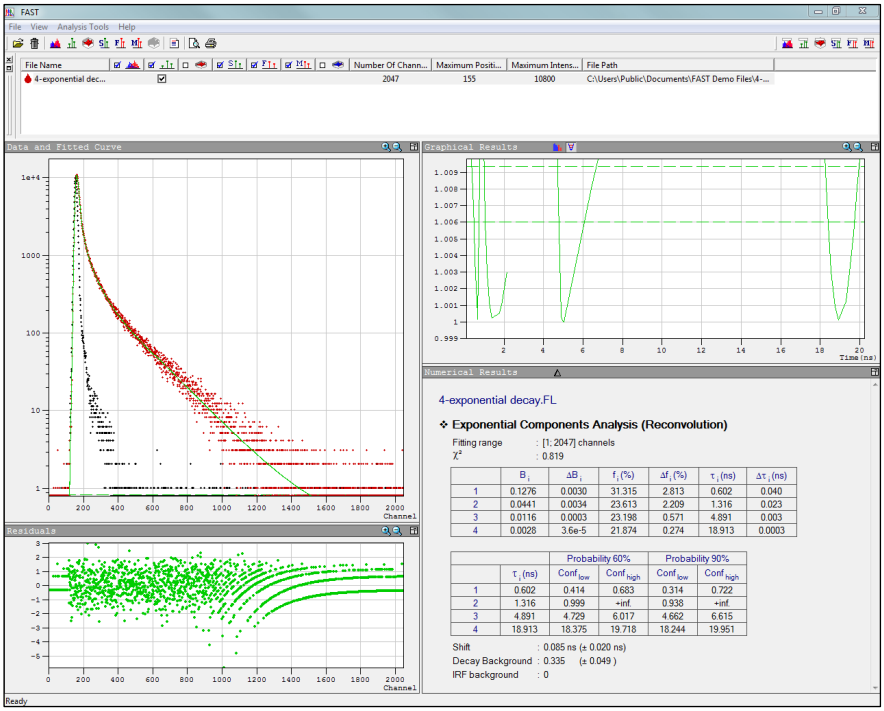


Figure 25
Results of ***Support Plane Analysis*** with demonstration of the chi-square plane and the list of confidence intervals.

At the end of each ***Support Plane Analysis*** fit the ***Graphical Results*** window switches automatically to the support plane view. Using the button reverts to the familiar lifetime view mode. The support plane view can be activated again by clicking the button.

5.3. Global Exponential Components Analysis

Global Analysis performs joint analysis of a set of experimental data with up to 100 individual sample decays. Unlike a batch analysis of the same set of data files by Exponential Components Analysis, the Global Data Analysis has two types of floating parameters: One set of fit parameters that is unique to each individual decay, the other set that applies to all individual decays of the family. The latter set of parameters is referred to as "linked" parameters. The absolute value of a linked parameter will be a result of the fit, and this parameter will be identical for all decay curves of the set.

A typical example where global analysis is a very useful tool is the study of a mixture of fluorophores and the separation of the individual spectral components by means of their lifetimes. The lifetimes will remain constant while the amplitudes will change by changing the emission wavelength.

There is a whole range of possible secondary parameters: Excitation wavelength, emission wavelength, temperature, concentration, (reaction) time, pH, etc.

5.3.1. Theoretical Model

The general mathematical expression for the sample decay model is

$$R(x,t) = \sum_{i=1}^{4} B_{x,i}\, e^{-\frac{t}{\tau_{x,i}}} \quad , \qquad \textbf{(11)}$$

where "x" refers to the secondary parameter.

Similar to Exponential Components Analysis, up to four lifetimes and the pre-exponential factors are fit parameters (for each individual curve), as well as a constant background (for each individual decay). In addition a shift parameter between the IRF and the decay is available for reconvolution fits.

The pre-exponential factors, B_i, and the lifetimes, τ_i, may optionally be linked during the fit.

As an example for a theoretical model, a homogeneous system (liquid sample) with two different emitting species with fluorescence lifetimes τ_1 and τ_2 may be considered. A fluorescence lifetime measurement is made at a set of different emission wavelengths, λ. The mathematical form of this model is:

$$R(\lambda_i,t) = B_1(\lambda_i)\, e^{-\frac{t}{\tau_1}} + B_2(\lambda_i)\, e^{-\frac{t}{\tau_2}} \quad , \qquad \textbf{(12)}$$

with the index ***i*** referring to the (discrete) wavelength steps.

The set of lifetime measurements is then analysed using Global Analysis. During the fit procedure the two lifetimes τ_1 and τ_2 are linked. The result of the analysis is two columns of pre-exponential factors. The multiplication of pre-exponential factors with the corresponding lifetime results in two columns of fluorescence intensities. A plot of these two columns of compound intensity versus wavelength shows the lifetime discriminated spectra of the two individual components, the sum of the two columns versus wavelength will be an equivalent to a (spectrally uncorrected) steady state emission spectrum.

It should be noted, that the Global Analysis routine within the FAST package does not require the same instrumental response function for each sample decay. Each of the data files of a family may have it's own IRF, and may even have a (slightly) different time range.

5.3.2. Performing the Fit

Global Analysis fits generally require that more than one data file is highlighted in the FAST file manager box (although single files can be analysed, but this may be better done using Exponential Components Analysis).

Once the files to be analysed have been selected, the Global Analysis dialogue box may be opened. The layout of this dialogue box is similar to the dialogue box of Exponential Components Analysis, and for a description of most of the functionality the user is referred to section 5.2.2.

The only differences compared to the dialogue boxes of Exponential Components Analysis are the tick-boxes for linking of lifetime parameters and the absence of the Support Plane Analysis option.

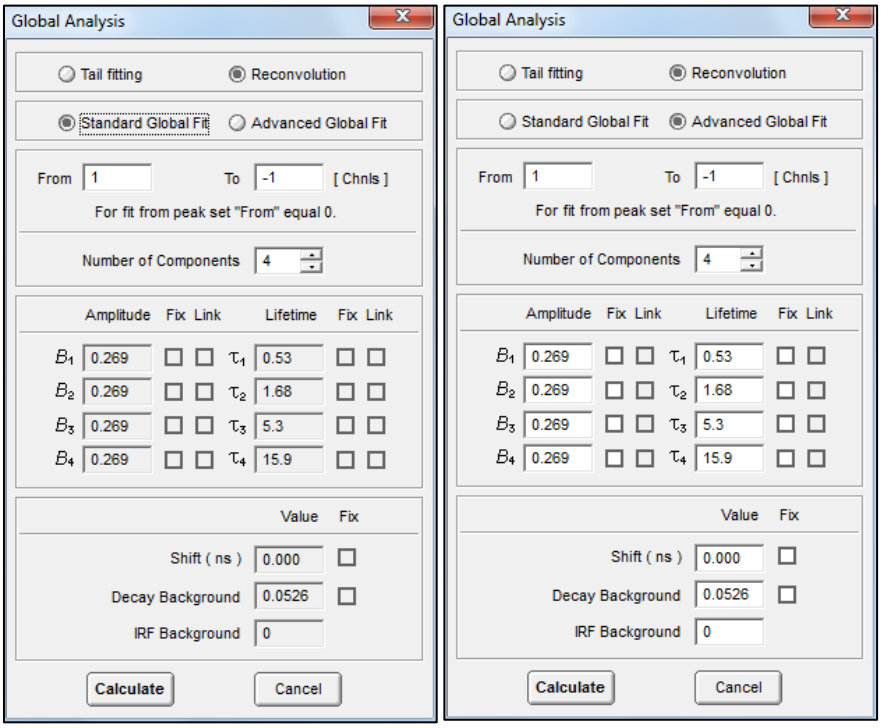


Figure 26
Set-up dialogue boxes for Standard Global Fits (left) and Advanced Global Fits (right).

Depending on the number of different curves to be fitted, and the number of data channels within each curve, fits can take substantially longer than single file analysis. If required, the fit can be aborted using the "***Stop processing***" button.

5.3.3. Interpretation of Fit Results

All lifetime parameters shown in the ***Numerical Results*** window have the same meaning as those of the Exponential Components Analysis. The user is referred to section 5.2.3. for a detailed description.

For fit results obtained with Global Analysis two types of chi-squared values are calculated: a ***Global*** χ^2 and a χ^2 that is specific to each data file. The ***Global*** χ^2 characterises the goodness of fit of the entire set of data, whereas the "normal" (local) χ^2 qualifies the individual data curve.

$$Global\ \chi^2 = \sqrt{\frac{\sum_{i=1}^{N}\left(\chi_i^2\right)^2}{N}}, \qquad \textbf{(13)}$$

with N being the number of individual decay curves.

Linked parameters are marked in the table of results with the word "linked". Linked parameters will have errors attached. In cases where parameters have been fixed, the corresponding value will be marked with "fixed" and no error will be available (error shown = 0).

A full interpretation of the fit results may require the results to be exported into a graphics package for suitable visualisation. The easiest way to export the data is to click and drag on a limited area in the Numerical Results window. Once a part of the text is highlighted ***Ctrl C*** will send the text to the clipboard, from which it may then be imported into the separate software package. Refer to Appendix A as an example for the analysis of the data set ***5 global decays link3.FL***.

5.4. Stretched Exponential Components Analysis

5.4.1. Theoretical Model

The theoretical model of Stretched Exponential (or Kohlrausch) kinetics is a modified exponential decay in the form of

$$R(t) = B\, e^{-\left(\frac{t}{\tau}\right)^{\beta}}, \qquad \textbf{(14)}$$

where β is the "stretching" exponent ($0 < \beta \leq 1$). The value β modifies the exponential function in a way that the initial slope decays faster than a normal exponential, whereas the tail extends longer. The smaller β, the more the exponential is deformed.

FAST offers the analysis of up to four stretched exponential terms, with background and shift fitting as standard. As with the other analysis tools, manual setting of start values and fixing of individual parameters is optional:

$$R(t) = \sum_{i=1}^{4} B_i\, e^{-\left(\frac{t}{\tau_i}\right)^{\beta_i}}. \qquad \textbf{(15)}$$

5.4.2. Performing the Fit

Fits are made in a similar way as with the previous analysis tools. As an example select the file ***Stretched Exponential.FL*** and then open up the dialogue box for setting up the start parameters for the Stretched Exponential Data Analysis (Figure 27).

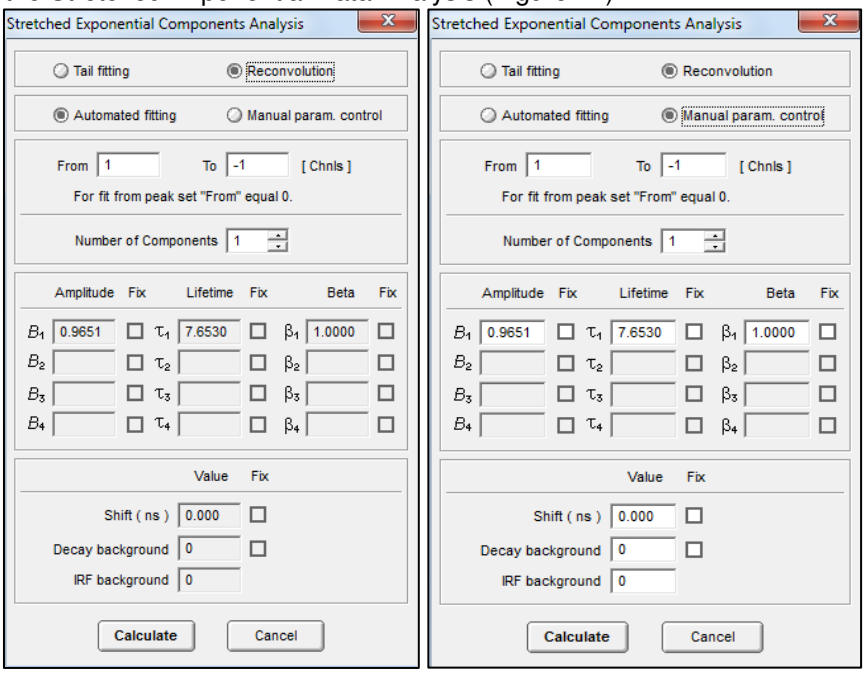


Figure 27
Set-up dialogue boxes for automated Stretched Exponential fits (left) and Stretched Exponential fits with manual parameter control (right).

5.4.3. Interpretation of Fit Results

The stretched exponential function is more flexible than the simple exponential analysis. As an example Figure 28 shows the fit result of the file ***Stretched Exponential.FL***. This decay can be satisfactorily fitted with just one stretched exponential component, whereas even a two-component exponential function does not fit the data properly.

As with the other analysis results, a graphical demonstration of the τ values is shown in the ***Graphical Results*** window. The table in the **Numerical Results** window contains the B, τ and β values.

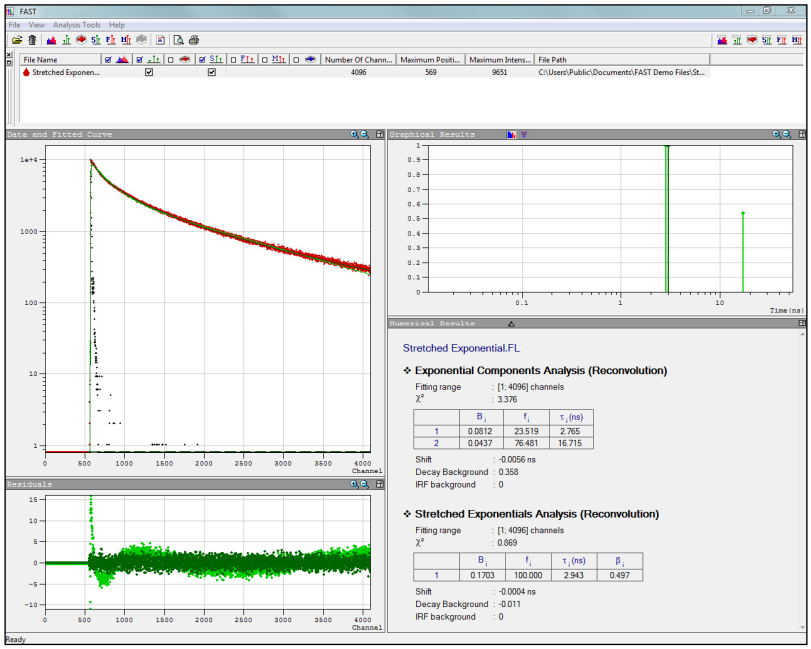


Figure 28
Fit result of a one-component stretched exponential fit, and comparison to a two-component exponential fit.

5.5. Förster Kinetics

5.5.1. Theoretical Model

Fluorescence Resonance Energy Transfer (FRET), is a phenomenon involving the transfer of excited state energy from an optically excited donor molecule to an acceptor molecule. The transfer takes place without the appearance of a photon by means of long-range dipole-dipole interactions between donor and acceptor.

Despite the fact that no "transfer photon" is involved, the condition for the mechanism to happen is that the donor emission spectrum must overlap with the acceptor absorption spectrum. The transfer efficiency depends on the extent of the overlap (the overlap integral between donor emission and acceptor absorption), the orientation of the donor emission dipole with respect to the acceptor absorption dipole, the donor quantum yield, and – of significant importance – on the distance between the two molecules.

From the viewpoint of fluorescence kinetics, one can distinguish the following scenarios:

1. All donor-acceptor pairs have the same distance and the same orientation of dipoles.

This leads to fluorescence kinetics of the form

$$R(t) = B\, e^{-\frac{t}{\tau}} \quad , \qquad \textbf{(1)}$$

With τ being the (by Förster energy transfer) quenched fluorescence lifetime:

$$\tau = \frac{\tau_0}{1 + \left(\frac{R_0}{R}\right)^6} \, , \qquad \textbf{(16)}$$

where τ_0 is the unquenched fluorescence lifetime, R_0 is the Förster radius that can be calculated from the overlap integral, orientation factors, refractive index, donor fluorescence quantum yield, and R is the (common) distance between donors and acceptors.

It is important to note the exponential nature of this kinetic model.

The analysis of this type of kinetic is currently of significant importance, as the Förster process is now suitably used as a "Molecular Ruler". A change in fluorescence lifetime signals a change in distance between donors and acceptors.

The Exponential Components Analysis of FAST is the ideal tool for the analysis of this type of kinetic.

2. All donor-acceptor pairs have similar (but not identical) distances and the same orientation of dipoles.

This causes a distribution of lifetimes of the form

$$R(t) = \sum_{\tau} P(\tau)\, e^{-\frac{t}{\tau}} \qquad \textbf{(17)}$$

By means of equation 16 the distribution of lifetimes can be converted into a distribution of distances.

The Distribution Analysis of FAST can be used for this type of analysis.

3. All donors are surrounded by randomly distributed acceptors

This model assumes that donors and acceptors are randomly distributed in a "rigid" three dimensional solution such that no diffusion takes place during the donor's excited state lifetime. It also assumes that the donor concentration is significantly lower than the acceptor concentration such that donors can not interact between each other. At those experimental conditions the individual donor molecules are surrounded by many acceptor molecules at random distance and with random orientations of the acceptor absorption dipoles with respect to the orientation of the donor emission dipole.

The mathematical expression for the donor fluorescence emission has the form

$$R(t) = B\, e^{-\left(\frac{t}{\tau} + 2\Gamma\sqrt{\frac{t}{\tau}}\right)} \qquad \textbf{(18)}$$

Here, τ is the fluorescence decay time of the donors in the absence of acceptors, and Γ is a characteristic constant of the form

$$\Gamma = \frac{[A]\, R_0^3}{447} \qquad \textbf{(19)}$$

With *[A]* being the concentration of acceptors and R_0 the Förster radius with the same meaning of that in equation **16**.

This non-exponential model (equation **18**) requires a special routine of fitting, and this is provided by the FAST Förster Analysis. The model fitted by FAST also includes a constant background of the decay, (not shown in equation **18**) and a shift between IRF and the sample decay in the case of a reconvolution fit.

5.5.2. Performing the Fit

Load the file ***Forster kinetic.FL***, select the file in the file manager and then select ***Forster Kinetics*** in the ***Analysis Tools*** menu list.

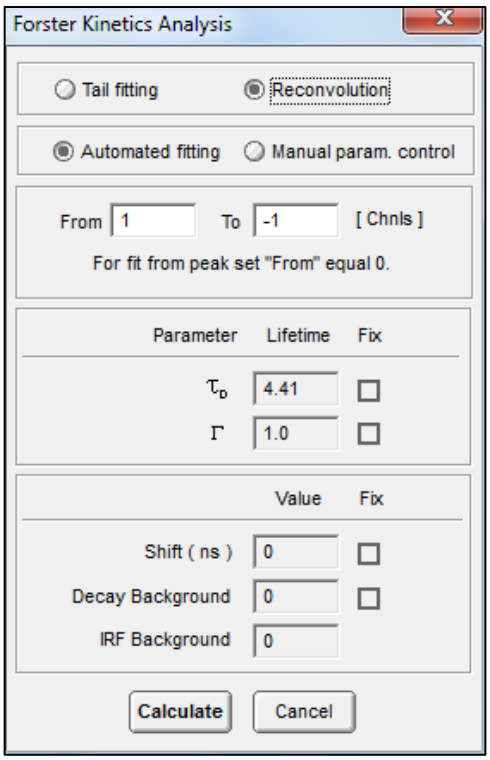


Figure 29
Dialogue box for the set up of a Förster Kinetics Analysis fit.

Although the layout of this setup dialogue box is slightly different to that of Exponential Components Analysis and Global Analysis, the functionality of the setup is the same.

After the fitting range has been selected a decision has to be made between reconvolution fit and tail fitting. Manual parameter control is possible by checking the ***Fix*** boxes.

The output parameters of this fitting routine are the fluorescence lifetime, τ_D, the characteristic constant Γ, the constant decay background and the shift between IRF and decay, if applicable.

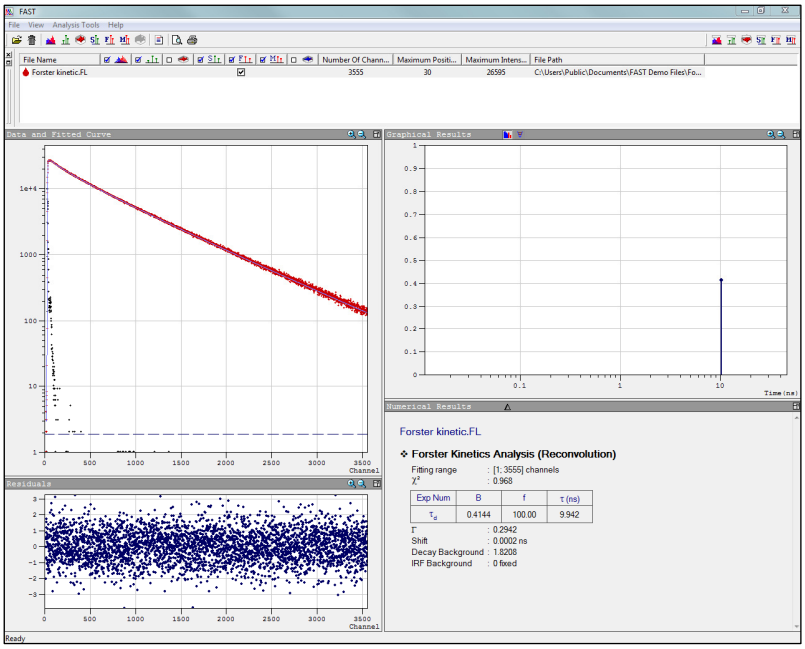


Figure 30
Förster Kinetics Analysis fit result.

Note that currently FAST does not display errors of τ_D and Γ.

5.5.3. Interpretation of Fit Results

The dimensionless constant Γ must be a positive, non Zero value. For values close to Zero the Förster kinetic degenerates into a single exponential fit.

Like all complex decays Förster kinetics of the type represented by equation 18 can typically be fitted satisfactorily by Exponential Components Analysis, using a high number of exponential terms. For example, the above data ***Forster kinetic.FL*** can be fitted with the same quality by a 4-exponential model, judged by the chi-square and the residuals of the fit result.

The value of this Förster Kinetics Analysis fitting routine is only exploited when the experimental conditions suggest the applicability of this model.

5.6. Micellar Quenching

5.6.1. Theoretical Model

The micellar quenching model describes the fluorescence kinetic of fluorophores embedded in micelles. It is based on the assumption that localised high concentrations of fluorophores in micelles cause quenching between the fluorophores.

The complex decay kinetic is expressed in the form

$$R(t) = B\,exp\left[-\frac{t}{\tau_1} + C\left(exp\left\{-\frac{t}{\tau_2}\right\} - 1\right)\right] \qquad \textbf{(20)}$$

$\tau_1\ ,\ \tau_2,\ B,\ C\ >\ 0$

5.6.2. Performing the Fit

Fits are made in a similar way to the previous analysis tools. As an example load the file ***10 indep Micellar kinetics.FL*** and then select the first data set, called ***mic3_1***. Open up the dialogue box for setting up the start parameters for the Micellar Quenching Analysis (Figure 31).

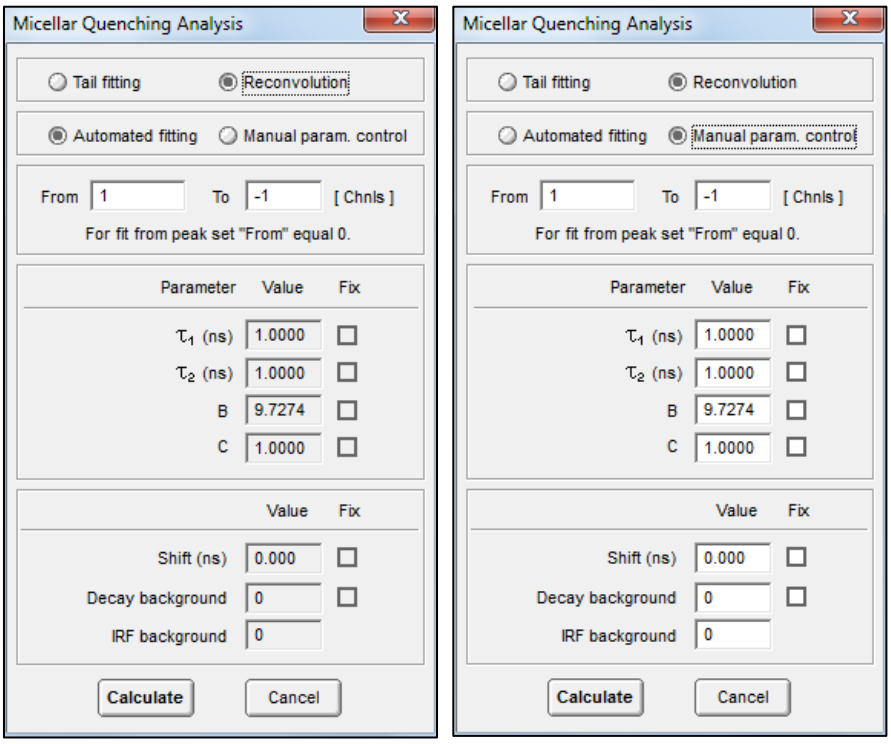


Figure 31
Dialogue box for the setup of a Micellar Quenching Analysis fit.

5.6.2. Interpretation of Fit Results

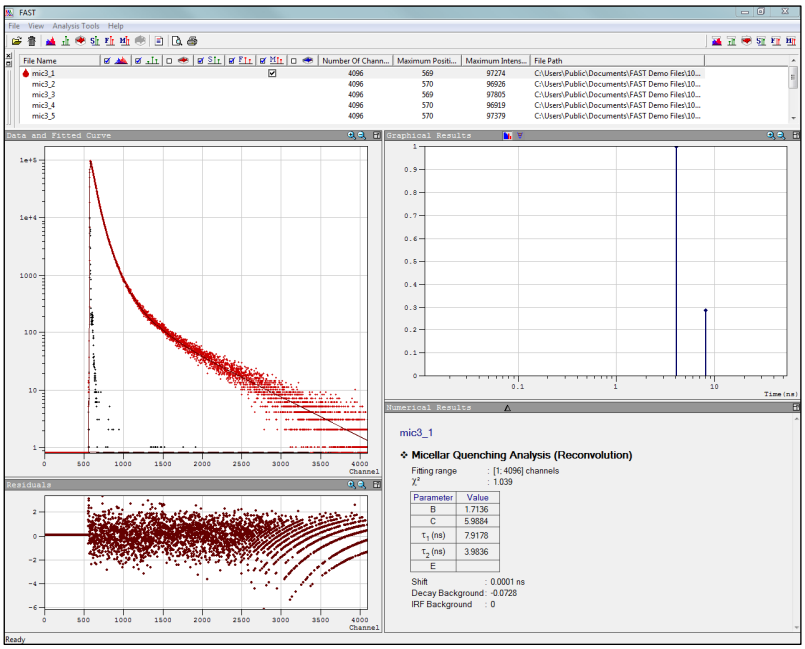


Figure 32
Micellar Quenching Analysis fit result.

The parameters τ_1, τ_2, *and* C contain the information on the average micellar aggregation number and the rate constant of association and dissociation of quenchers. The user is referred to ***M.H. Gehlen, F.C. De-Schryver, Chem. Rev. 93 (1993), 199-221*** for further details.

5.7. Anisotropy Analysis

FAST provides routines for the analysis of time resolved fluorescence anisotropy data. In the analysis of this type of data (obtained by polarised fluorescence lifetime measurements) two distinctly different models have to be combined into one: a model of the fluorescence decay (that is free of rotational effects), and a model for the rotational diffusion of dissolved fluorophores (which is not directly connected to the fluorescence emission). The mathematical expressions for the fluorescence decay model and the rotation diffusion model are ***R(t)*** and ***r(t)***, respectively.

Four different models for the rotational diffusion of molecules are supported by FAST: spherical rotor, two spherical rotors, spherical rotor with restricted rotation and ellipsoidal rotor. All four analysis routines assume that the fluorescence decay is of a single exponential nature.

The analysis of time resolved fluorescence anisotropy is based on the Global Analysis algorithm with the parallel and perpendicular fluorescence decay curves as input data and lifetime parameters linked appropriately.

5.7.1. Basics of Time Resolved Fluorescence Anisotropy Analysis

Time Resolved Fluorescence Anisotropy Analysis is applicable to those experiments where homogeneous (liquid) samples are involved with the fluorescing molecules being able to either freely rotate or rotate to a limited degree (restricted rotation).

In anisotropy experiments typically two separate linear polarised measurements are performed, one with both the excitation beam and the emission beam vertically polarised ($I_{//}(t)$) , the other with the excitation beam vertical and the emission beam horizontally polarised ($I_{\perp}(t)$). These two measurements not only contain the information of fluorescence relaxation, but also information on the rotational diffusion of the participating molecules. Unfortunately, $I_{//}(t)$ and $I_{\perp}(t)$ can often not be measured directly. They are unequally attenuated by instrumental artefacts. The kinetic curves that are measured instead are $I_{VV}(t)$ and $I_{VH}(t)$, and one of the curves, $I_{VV}(t)$, need to be re-scaled by a correction factor G.

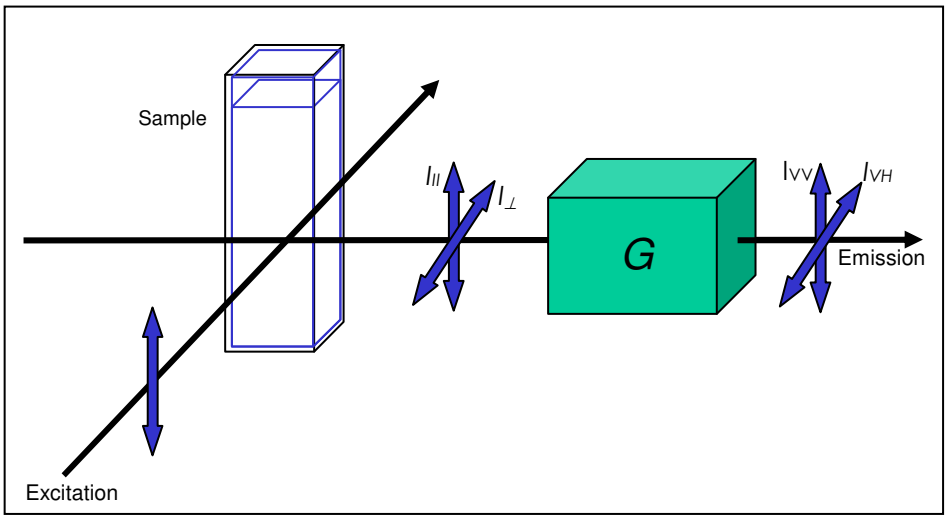


Figure 33
Principle of anisotropy measurements.

The G-factor can be measured. If the excitation polariser is put in the horizontal position, molecules excited by the polarised excitation light will have to rotate 90 degree in order to be “seen” by the emission polariser in either position, horizontal or vertical. Thus, a difference in the scaling of both measured curves, $I_{HH}(t)$ and $I_{HV}(t)$, is the correction factor G.

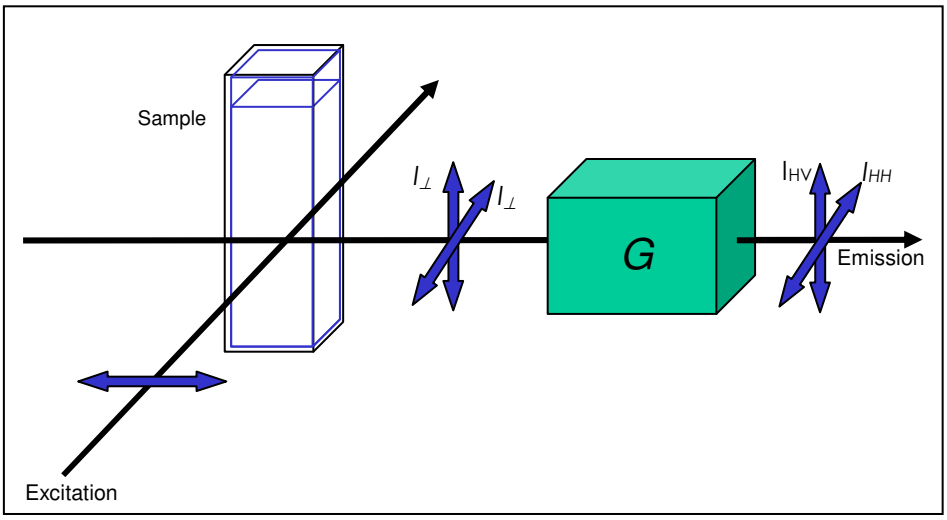


Figure 34
Experimental setup for measuring the G-factor.

The time resolved fluorescence anisotropy, $r^{raw}(t)$, can be calculated from the experimental data $I_{VV}(t)$ and $I_{VH}(t)$, using the formula

$$r^{raw}(t) = \frac{G I_{VV}(t) - I_{VH}(t)}{G I_{VV}(t) + 2\, I_{VH}(t)}, \qquad \textbf{(21)}$$

Where G was calculated using

$$G = \frac{I_{HH}}{I_{HV}} \qquad \textbf{(22)}$$

with I_{HH} and I_{HV} being the integral (summed over all time channels) values of $I_{HH}(t)$ and $I_{HV}(t)$, respectively.

The anisotropy function (equation **21**) only represents rotational diffusion parameters, not fluorescence relaxation.

In addition to the time resolved fluorescence anisotropy, the total fluorescence emission can be calculated:

$$I_T^{raw}(t) = G\, I_{VV}(t) + 2\, I_{VH}(t) \qquad \textbf{(23)}$$

This function is free of rotational effects and solely represents the fluorescence relaxation.

The task for the analysis of time resolved fluorescence anisotropy analysis is to fit the theoretical models to the experimental data. The mathematical expressions for those models are $R(t)$ and $r(t)$ for the fluorescence relaxation and the fluorescence anisotropy, respectively.

Fitting $r^{raw}(t)$ and $I_T^{raw}(t)$ “in the usual way” is not possible. By using the equations **21** and **23** one “loses” the Poissonian noise statistics of the data points and proper error propagation is required for obtaining correct fit parameters. The more serious problem however, is the fact that $r^{raw}(t)$ can not be directly reconvoluted as an integral involving a ratio function and can not be separated into a ratio of integrals (equation **21** – note that the typical rise during the duration of the excitation pulse is missing, refer to Figure 32).

Several methods are known to fit $r^{raw}(t)$ and $I_T^{raw}(t)$. The most straight forward method is offered by FAST by fitting the experimental data $I_{VV}(t)$ and $I_{VH}(t)$ directly.

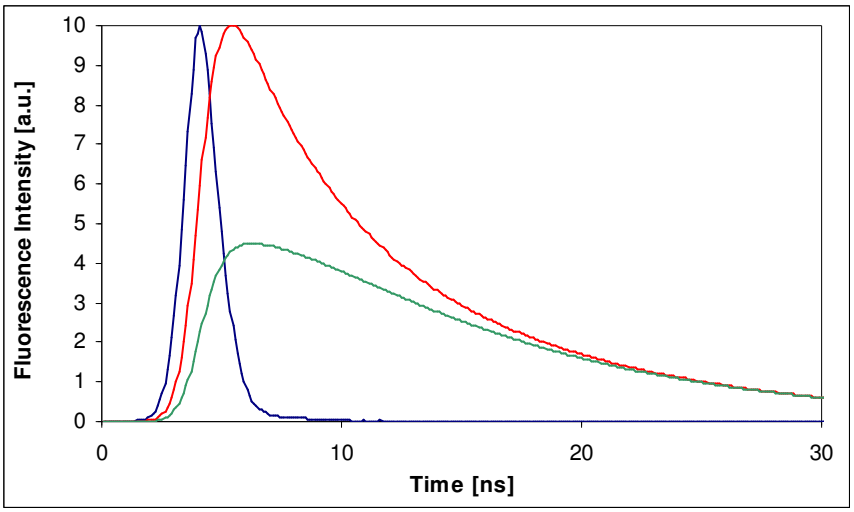


Figure 35
Schematic of polarised fluorescence lifetime measurements for the generation of the time resolved fluorescence anisotropy.

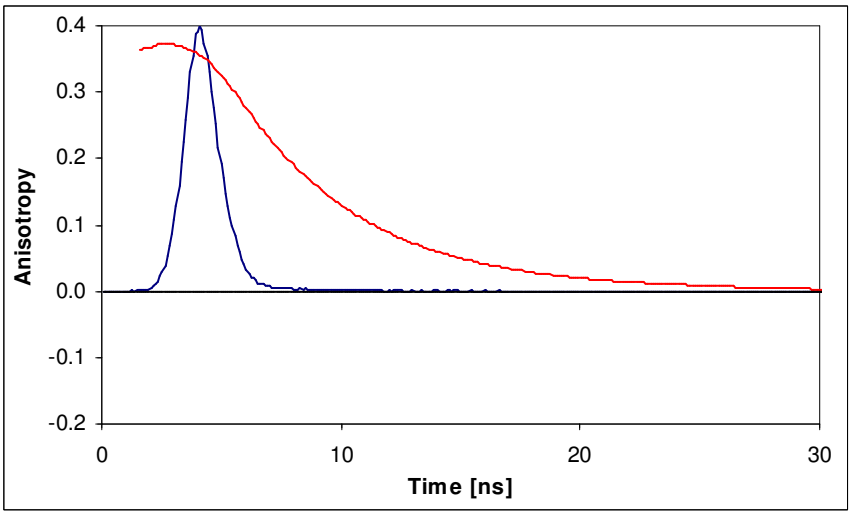


Figure 36
Time resolved fluorescence anisotropy decay, r(t), calculated from $I_{VV}(t)$ and $I_{VH}(t)$ above.

The theoretical model for the two measurements $I_{VV}(t)$ and $I_{VH}(t)$ can be derived from the model of the fluorescence decay, $R(t)$, and the model of the rotational diffusion, $r(t)$. It can be shown, that the mathematical expressions are:

$$I_{VV}^{m}(t) = \frac{R(t)}{G}\left[1 + 2\,r(t)\right] \qquad \textbf{(24a)}$$

$$I_{VH}^{m}(t) = R(t)\left[1 - r(t)\right] \qquad \textbf{(24b)}$$

The measurement with parallel orientated polarisers, $I_{VV}(t)$, is typically a multi-exponential decay, with the two processes overlapping: the energy relaxation (fluorescence) and excited dipoles rotate out of the field of view of the emission polariser. In contrast, the measurement with crossed polarisers, $I_{VH}(t)$, is typically a rise followed by a decay. The rise of the fluorescence signal is caused by the rotation of emitting dipoles into the field of view of the emission polariser, and the decay is caused by the fluorescence of the then homogeneously distributed molecules.

By obtaining the lifetime parameters of the two linked curves $I_{VV}(t)$ and $I_{VH}(t)$ one also obtains the lifetime parameters of $R(t)$ and $r(t)$.

All current anisotropy analysis routines of FAST are based on the assumption that all participating fluorescing molecules have a single exponential fluorescence decay time, i.e.

$$R(t) = I_0 \; e^{-\frac{t}{\tau}} \quad , \qquad \textbf{(25)}$$

Here the pre-exponential factor B (equation **1**) has been replaced by I_0 for reason of consistency with generally accepted nomenclature in anisotropy formulations.

The method for the analysis of fluorescence anisotropy decays offered by FAST principally does NOT require the G-factor to be measured. G is an output parameter, just like the characteristic sample decay parameters. However, in common with all fitting routines, it is always an advantage to utilise as much knowledge as possible, in particularly when the fitted function has many fit parameters (which is typical for fluorescence anisotropy calculations). It therefore is highly recommended to measure the G-factor, so that this can be fixed during the fit.

5.7.2. Models for Time Resolved Fluorescence Anisotropy

5.7.2.1. Spherical Rotor

The simplest model for rotational diffusion of molecules is based on the assumption that rotational diffusion is entirely isotropic. This is the case if the molecules can rotate freely (and with the same speed) in all directions. With a homogeneous solvent surrounding the molecules this is only given for spherically shaped molecules. This model is called the "Spherical Rotor Model", and the mathematical expression of the model is:

$$r(t) = r_0 \; e^{-\frac{t}{\phi}} \qquad \textbf{(26)}$$

where r_0 is anisotropy value at time zero, and ϕ is the rotational diffusion time. Note that r_0 is directly related to the angle between absorption and emission dipole of the ensemble of participating molecules; it can either be positive or negative and lies in the interval between -0.2 and 0.4.

Using this model for the rotational diffusion (equation **26**) and the model for the fluorescence decay (equation **25**) the polarised fluorescence kinetics (equation **24a** and **24b**) will have the following form:

$$I_{VV}^{m}(t) = \frac{I_0}{G}\left[e^{-\frac{t}{\tau}} + 2\, r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi}\right)} \right] \qquad \textbf{(27a)}$$

$$I_{VH}^{m}(t) = I_0 \left[e^{-\frac{t}{\tau}} - r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi}\right)} \right] \qquad \textbf{(27b)}$$

The theoretical model for the measurement with parallel oriented polarisers is a double exponential decay, and the model for the measurement with crossed polarisers is an initial rise followed by a decay. Both the pre-exponential factors and the two lifetimes $\tau_1 = \tau$ and $\tau_2 = \frac{\tau\phi}{\tau+\phi}$ of the two individual measurements are linked and the data can be suitably fitted by Global Analysis. All fluorescence and rotational parameters can then be extracted from the pre-exponential factors and lifetimes of the two double exponential functions.

The output parameters of this analysis routine are:

Instrumental	rotational	fluorescence
G – G-factor	r_0 – initial anisotropy	I_0 – pre-exponential factor
	ϕ - rotational diffusion time	τ - fluorescence lifetime

5.7.2.2. Two Spherical Rotors

In some binding experiments (binding assays) the ratio of free and bound species is important. If both, the free and the bound species have rotational diffusion times that are comparable to their fluorescence lifetime, the two spherical rotor model might be applicable. In this model, both rotors are considered to be spherical.

In general, with the two rotational diffusions

$$r_1(t) = r_{01}\, e^{-\frac{t}{\phi_1}} \qquad \text{and} \qquad r_2(t) = r_{02}\, e^{-\frac{t}{\phi_2}} \tag{28}$$

and the two single exponential fluorescence decays

$$R_1(t) = I_{01}\, e^{-\frac{t}{\tau_1}} \qquad \text{and} \qquad R_2(t) = I_{02}\, e^{-\frac{t}{\tau_2}} \tag{29}$$

if follows:

$$I_{VV}^{m}(t) = \frac{1}{G}\left[I_{01}\, e^{-\frac{t}{\tau_1}} + I_{02}\, e^{-\frac{t}{\tau_2}} + 2I_{01}r_{01}\, e^{-\left(\frac{t}{\tau_1}+\frac{t}{\phi_1}\right)} + 2I_{02}r_{02}\, e^{-\left(\frac{t}{\tau_2}+\frac{t}{\phi_2}\right)} \right] \tag{30a}$$

$$I_{VH}^{m}(t) = \left[I_{01}\, e^{-\frac{t}{\tau_1}} + I_{02}\, e^{-\frac{t}{\tau_2}} - I_{01}r_{01}\, e^{-\left(\frac{t}{\tau_1}+\frac{t}{\phi_1}\right)} - I_{02}r_{02}\, e^{-\left(\frac{t}{\tau_2}+\frac{t}{\phi_2}\right)} \right] \tag{30b}$$

In many cases the fluorescence lifetime as well as the initial anisotropy of the free and bound species remain the same, only the rotational diffusion of the two species is different. For this more special case equations **30** become

$$I_{VV}^{m}(t) = \frac{1}{G}\left[I_{01} + I_{02}\, e^{-\frac{t}{\tau}} + 2I_{01}r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_1}\right)} + 2I_{02}r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_2}\right)} \right] \tag{31a}$$

$$I_{VH}^{m}(t) = \left[(I_{01} + I_{02})\, e^{-\frac{t}{\tau}} - I_{01}r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_1}\right)} - I_{02}r_0\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_2}\right)} \right] \tag{31b}$$

The output parameters of this analysis routine are:

Instrumental	rotational	fluorescence
G – G-factor	r_0 – initial anisotropy (of both species)	I_{01} – pre-exponential factor of the first species
	ϕ_1 - rotational diffusion time of the first species	I_{02} – pre-exponential factor of the second species
	ϕ_2 - rotational diffusion time of the second species	τ - fluorescence lifetime (of both species)

5.7.2.3. Spherical Rotor with Restricted Rotation

If a fluorophore is attached to a very large molecule or to an immobile surface, the molecule typically will not lose all mobility. Instead those molecules can rotate within a cone angle. This “wobbling” is called restricted rotation and the model is called the restricted spherical rotor model.

The mathematical expression for this model is

$$r_1(t) = (r_0 - r_\infty)\, e^{-\frac{t}{\phi}} + r_\infty \qquad \textbf{(32)}$$

The clear difference to the previous models is that the anisotropy does not fully decay to zero, instead it remains steady (at r_∞) after an initial decay with the rotational diffusion time constant ϕ. ϕ is generally larger than the rotational diffusion constant of the same molecule under entirely free conditions.

The parallel and perpendicular components can be written as follows:

$$I_{VV}^m(t) = \frac{I_0}{G}\left[(1+2r_\infty) e^{-\frac{t}{\tau}} + 2(r_0 - r_\infty) e^{-\left(\frac{t}{\tau}+\frac{t}{\phi}\right)} \right] \qquad \textbf{(33a)}$$

$$I_{VH}^m(t) = I_0\left[(1 - r_\infty)\, e^{-\frac{t}{\tau}} - (r_0 - r_\infty)\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi}\right)} \right] \qquad \textbf{(33b)}$$

The output parameters of this analysis routine are:

Instrumental	rotational	fluorescence
G – G-factor	r_0 – initial anisotropy	I_0 – pre-exponential factor of the fluorescence decay
	r_∞ – steady anisotropy at infinity	τ - fluorescence lifetime
	ϕ - rotational diffusion time of the restricted rotation	

5.7.2.4. Ellipsoidal Rotor

For molecules that strongly deviate from the spherical shape a more general model is required to describe the molecular rotation. Molecules that have the form of an ellipsoid with three different axes can be described with a three-exponential rotational model:

$$r(t) = r_{01}\, e^{-\frac{t}{\phi_1}} + r_{02}\, e^{-\frac{t}{\phi_2}} + r_{03}\, e^{-\frac{t}{\phi_3}} \qquad \textbf{(34)}$$

With the single exponential fluorescence decay (equation **17**) the parallel and the perpendicular fluorescence components will have the form

$$I^m_{VV}(t) = \frac{I_0}{G}\left[e^{-\frac{t}{\tau}} + 2r_{01}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_1}\right)} + 2r_{02}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_2}\right)} + 2r_{03}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_3}\right)} \right] \qquad \textbf{(35a)}$$

$$I^m_{VH}(t) = I_0\left[e^{-\frac{t}{\tau}} - r_{01}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_1}\right)} - r_{02}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_2}\right)} - r_{03}\, e^{-\left(\frac{t}{\tau}+\frac{t}{\phi_3}\right)} \right] \qquad \textbf{(35b)}$$

Special forms of this model are rod-like or disk shaped molecules which will show two rotational correlation times, one (fast) around the symmetry axis of the molecule, the other (slow) perpendicular to the that axis. Spherical molecules will have one rotational correlation time (ref to section 5.7.2.1.). In the majority of cases the initial anisotropies will be equal.

The output parameters for the ellipsoidal rotor model are:

Instrumental	rotational	fluorescence
G – G-factor	r_{01} – initial anisotropy for first rotor axis	I_0 – pre-exponential factor of the fluorescence decay
	r_{01} – initial anisotropy for second rotor axis	τ - fluorescence lifetime
	r_{01} – initial anisotropy for third rotor axis	
	ϕ_1 - rotational diffusion time of the restricted rotation	
	ϕ_2 - rotational diffusion time of the restricted rotation	
	ϕ_3 - rotational diffusion time of the restricted rotation	

5.7.3. Performing the Fit

In order to perform Anisotropy Analysis, two files containing the data of the measured curves $I_{VV}^{raw}(t)$ and $I_{VH}^{raw}(t)$ must be highlighted within the FAST file manager. If this is the case the dialogue box ***Anisotropy Analysis*** will be available from the ***Analysis Tools*** menu.

Open the file ***Spherical Rotor.FL***, the file can be found in the directory ***…/FAST Demo Files/Anisotropy***. In the FAST file manager highlight both files, ***I(VV)*** and ***I(VH)***. From the ***Analysis Tools*** menu list select ***Anisotropy***. The following dialogue box will appear:

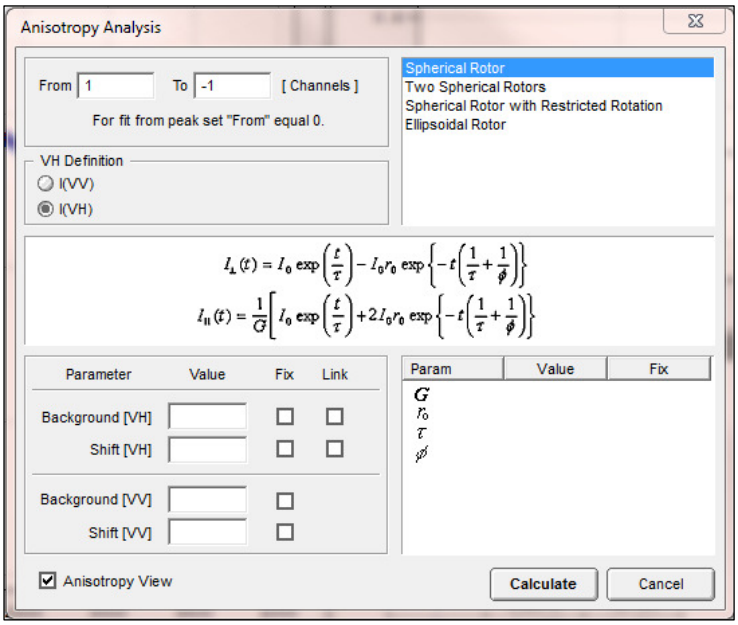


Figure 37
Anisotropy Analysis dialogue box.

After opening this dialogue box, the user must specify which of the two data files is $I_{VV}(t)$ and which is $I_{VH}(t)$. This specification is made by selecting the $I_{VH}(t)$ file in the ***VH Definition*** section of the dialogue box.

In a second step the user should select the model that should be tried with the data.

After this the fitting range should be selected, special features of this fitting range selection have been described previously (e.g. sections 5.1.2. and 5.2.2.).

If one or more parameters are known, they can be entered and fixed, or – for background and shift – they can be linked between the two data files.

To visualise the fitted anisotropy curve and the total fluorescence decay the box ***Anisotropy View*** must be checked.

At the end of the fitting procedure the results of the two linked data curves $I_{VV}(t)$ and $I_{VH}(t)$ will be shown on the main screen of FAST. If ***Anisotropy View*** had been selected in the dialogue box, an additional graphical window will open that shows either the raw and fitted anisotropy or the raw and fitted total fluorescence emission.

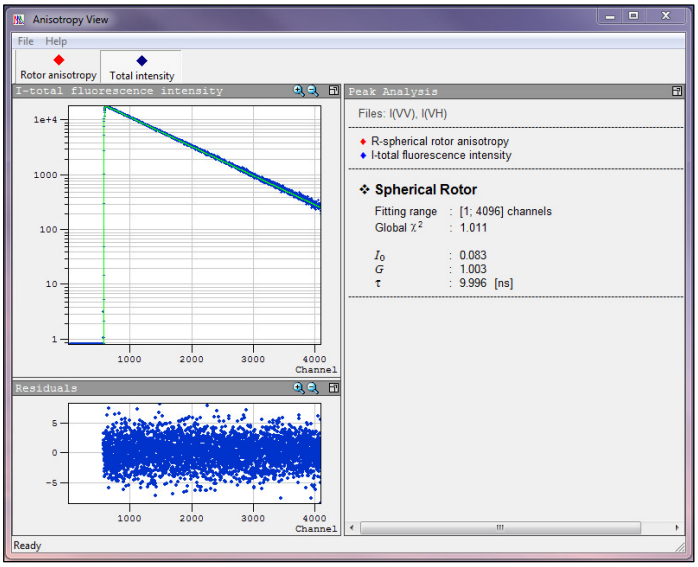


Figure 38
Total fluorescence decay of spherical rotor anisotropy data: calculated from original data and fit results.

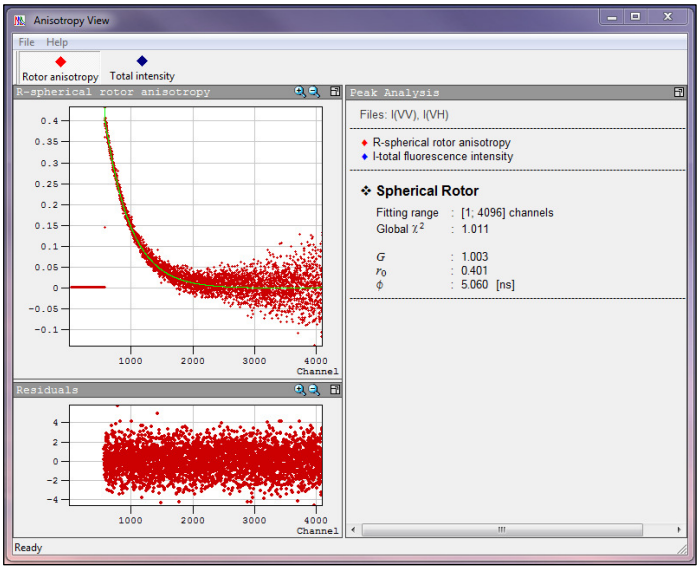


Figure 39
Time resolved fluorescence anisotropy of spherical rotor anisotropy data: calculated from original data and fit results.

The Numerical Results window will show the fitting range, the global chi-square of the anisotropy analysis, and a list of output parameters that depends on which of the anisotropy models had been selected.

Appendix A: Properties of Test Data

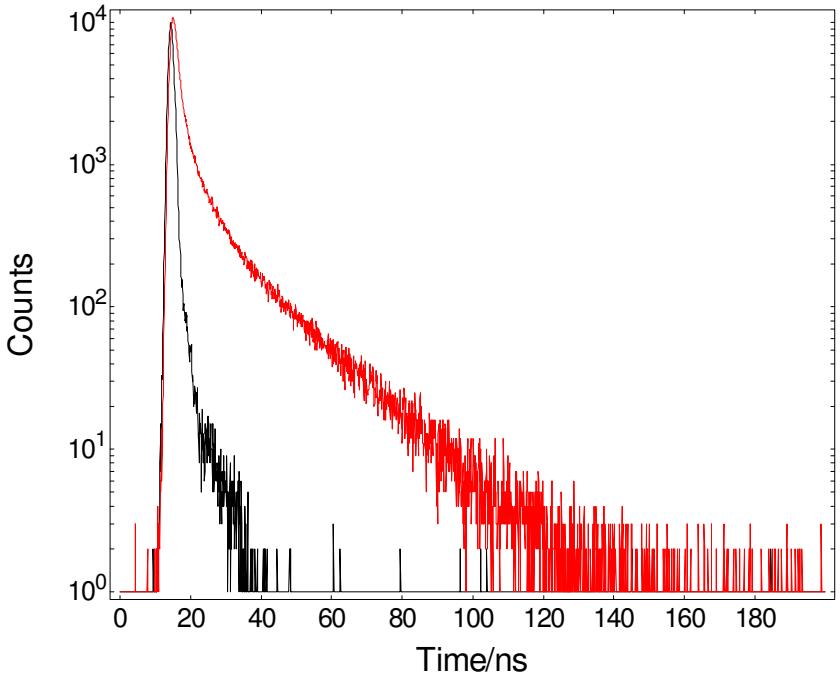
Test file ***4-exponential decay.FL***

Experimental data to simulate a four-exponential decay. The experiment was performed as follows: Four individual samples were prepared: DPB (1,4-Diphenyl-1,3-butadiene) in cyclohexane, PPO in cyclohexane, anthracene in cyclohexane (degassed), and pyrene in cyclohexane. These samples were measured individually to confirm the single exponential nature of the decay and to confirm the lifetimes of the samples. Instrumental parameters were chosen so that each sample showed the same number of average counts.
After this, the four samples were measured again for the same length of time and the sample data were accumulated into one data file – the equivalent of subsequently adding the four sample curves. Thus, an artificial four-exponential fluorescence decay has been generated with approximately equal relative fluorescence intensities and PRECISELY KNOWN lifetimes.

$$R(t) = B_1\, e^{-\frac{t}{\tau_1}} + B_2\, e^{-\frac{t}{\tau_2}} + B_3\, e^{-\frac{t}{\tau_3}} + B_4\, e^{-\frac{t}{\tau_4}}$$

τ_1 = 0.56ns ±0.03ps (DPB)
τ_2 = 1.30ns ±0.04ms (PPO)
τ_3 = 5.10ns ±0.05ns (Anthracene)
τ_3 = 19.5ns ±0.4ns (Pyrene)

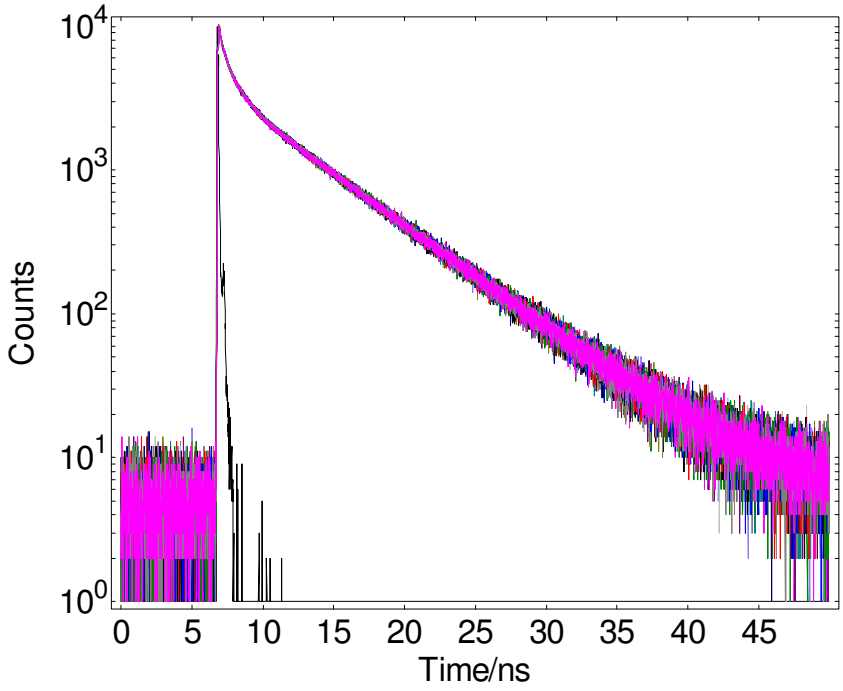
The excitation source was a nanosecond flashlamp (nF900) and the detector was a cooled R928P photomultiplier.



Judging by the chi-square and the residuals, a 3-exponential fit would appear satisfactory. However, a four-exponential Exponential Components Analysis fit recovers the known four lifetimes astonishingly well. Also the Distribution Analysis shows four distinct spikes that are in good agreement with the known lifetimes.

Test file ***20 indep 4-exp decays.FL***

Simulated data. A measured instrumental response function (Ti:Sapphire laser excitation, MCP-PMT detector) was used to generate convoluted four-exponential decays. 20 data curves have been generated, all with the same four-exponential model, but with different superimposed Poissonian noise. An artificial decay background has been added, too. 人工衰减背景
Thus 20 statistically independent decay curves are made available for testing the convergence and the search for the global minimum.

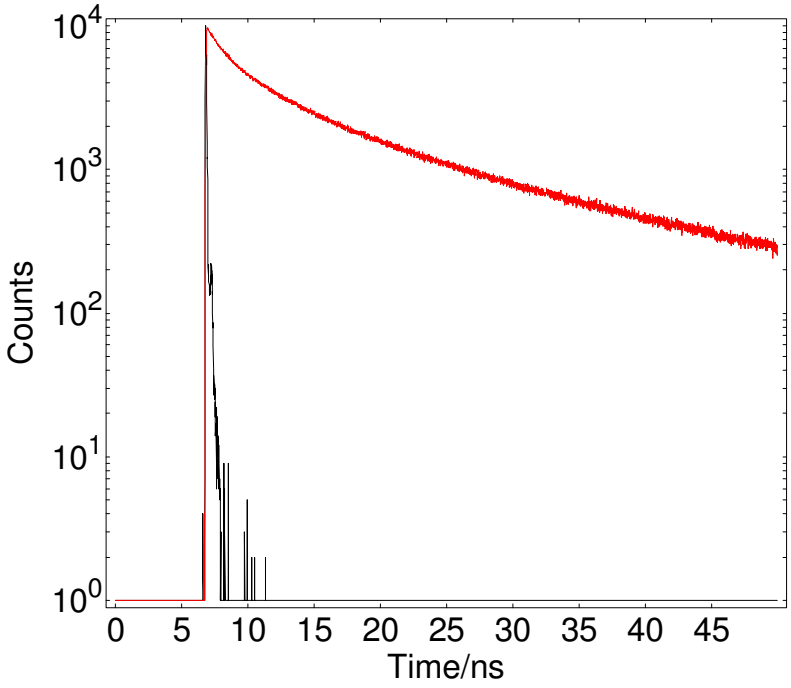


$$R(t) = B_1\, e^{-\frac{t}{\tau_1}} + B_2\, e^{-\frac{t}{\tau_2}} + B_3\, e^{-\frac{t}{\tau_3}} + B_4\, e^{-\frac{t}{\tau_4}}$$

	τ [ns]	B
1	0.1	0.048
2	0.3	0.048
3	1.0	0.048
4	6.0	0.048

Test file ***Stretched Exponential.FL***

Simulated data. A measured instrumental response function (Ti:Sapphire laser excitation, MCP-PMT detector) was used to generate a convoluted stretched exponential decay function, based on two stretched exponential components.

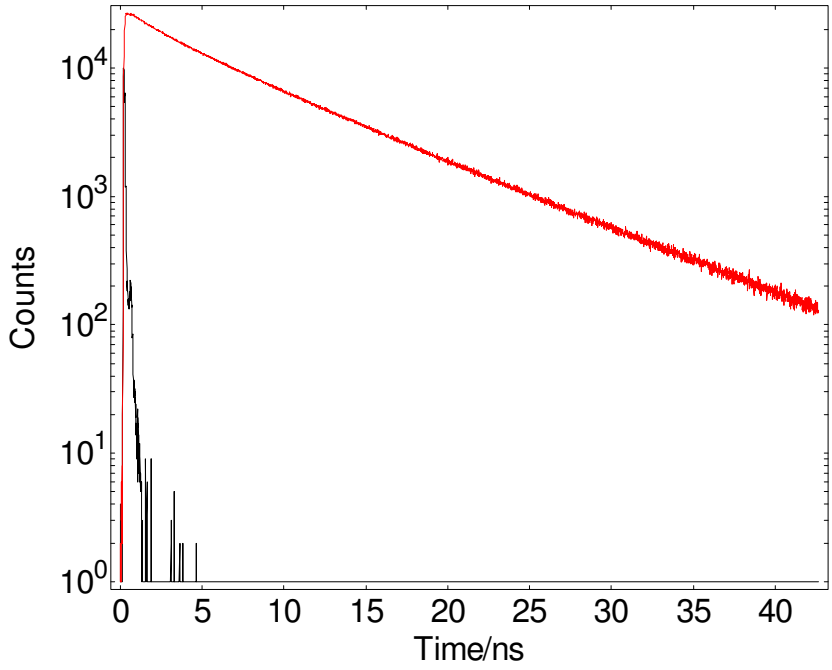


$$R(t) = B_1 \, e^{-\left(\frac{t}{\tau_1}\right)^{\beta_1}} + B_2 \, e^{-\left(\frac{t}{\tau_2}\right)^{\beta_2}}$$

	τ [ns]	B	β
1	0.1	0.048	0.048
2	0.3	0.048	0.048

Test file ***Forster kinetic.FL***

Simulated Förster kinetics, based on a measured instrumental response function (Ti:Sapphire laser excitation + MCP-PMT detection).

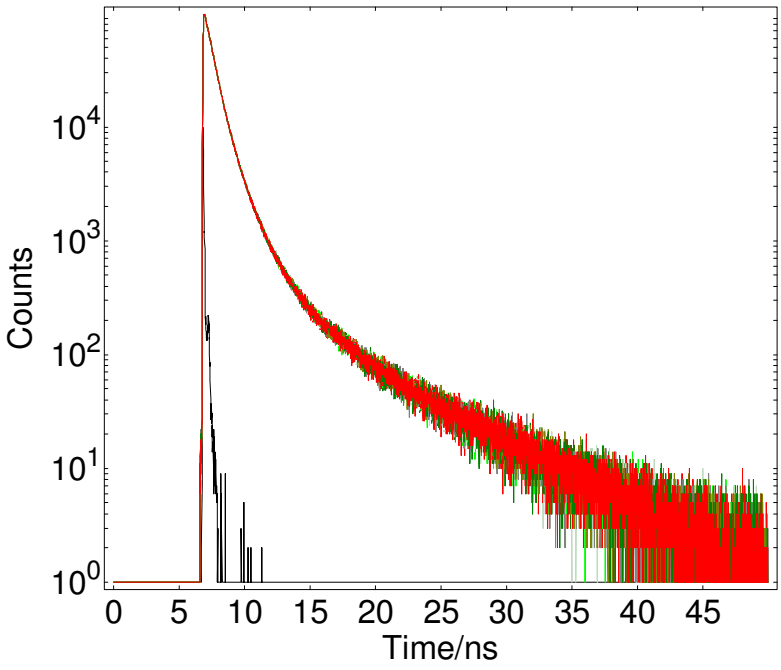


$$R(t) = B\, e^{-\left(\frac{t}{\tau} + 2\Gamma\sqrt{\frac{t}{\tau}}\right)}$$

τ = 10ns,
Γ=0.3

Test file ***10 indep Micellar Kinetics.FL***

Simulated Micellar kinetics, based on a measured instrumental response function (Ti:Sapphire laser excitation + MCP-PMT detection).



$$R(t) = B\ exp\left[-\frac{t}{\tau_1} + C\left(exp\left\{-\frac{t}{\tau_2}\right\} - 1\right)\right]$$

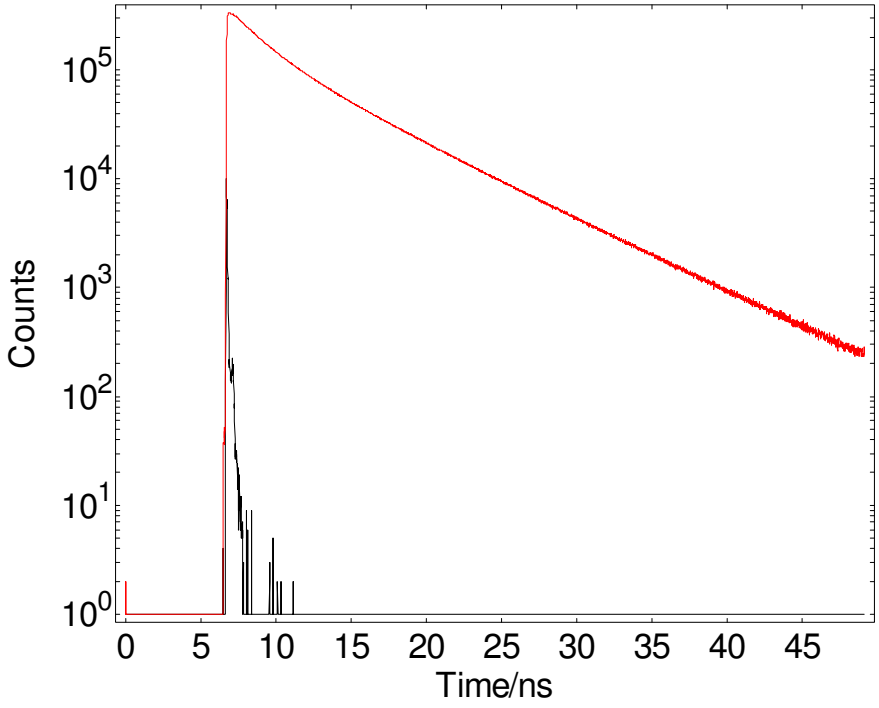
τ_1 , τ_2, B, $C > 0$

τ_1 = 8ns,
τ_1 = 4ns,
C = 6

Test file ***Distribution of Lifetimes.FL***

Simulated data, based on a measured instrumental response function (Ti:Sapphire laser excitation + MCP-PMT detection).

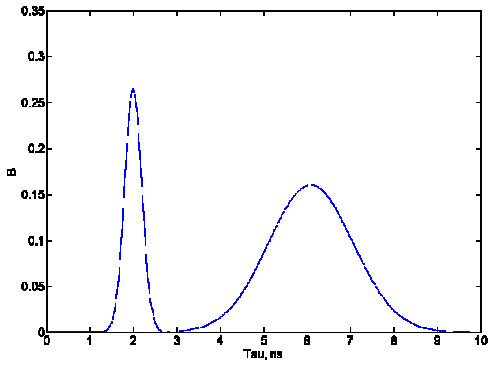
A bi-modal Gaussian distribution of fluorescence lifetimes has been generated for testing the distribution analysis.



The distribution has the form

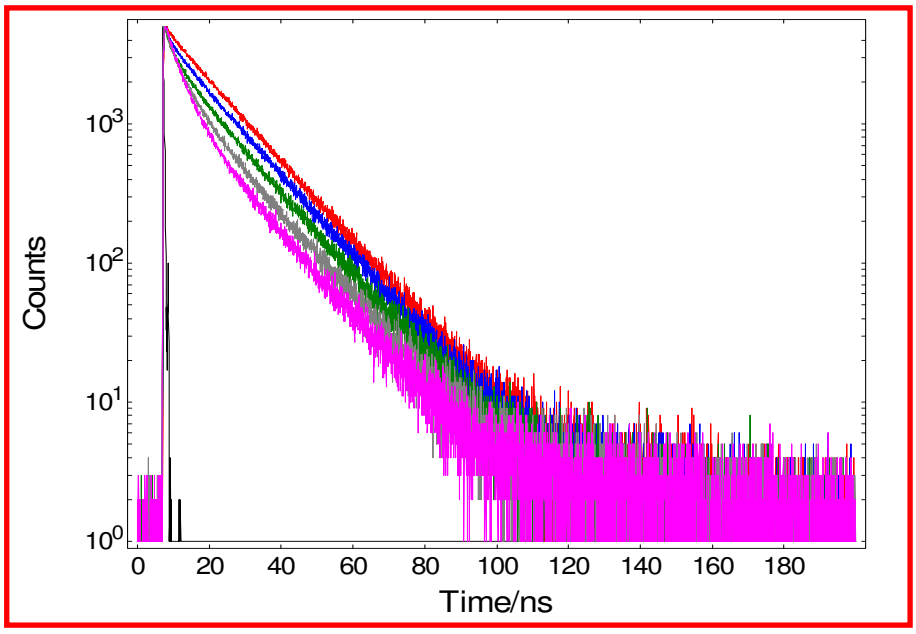
$$B(\tau_i) = \sum_{i=1}^{N} \left\{ \frac{1}{\sqrt{2\pi}\sigma_1} \exp\left[-\frac{(\tau_1 - \tau_i)^2}{2\sigma_1^{\,2}} \right] + \frac{1}{\sqrt{2\pi}\sigma_2} \exp\left[-\frac{(\tau_2 - \tau_i)^2}{2\sigma_2^{\,2}} \right] \right\}$$

$\tau_1 = 2$, $\sigma_1 = 0.2$, $\tau_2 = 6$, $\sigma_2 = 1$.

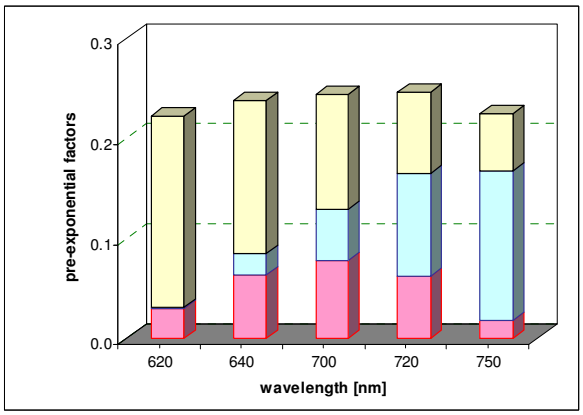


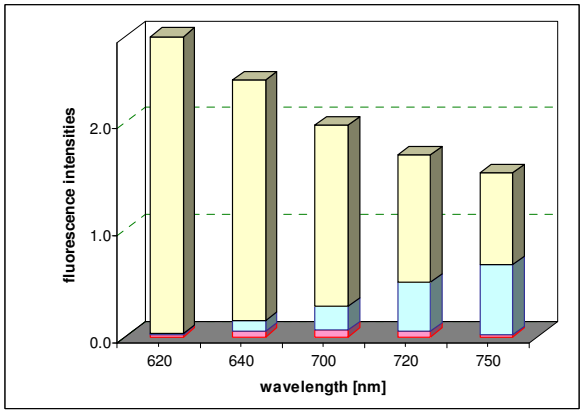
Test file ***5 global decays link3.FL***

Experimental data of Hematoporphyrin IX in aqueous solution. Excitation by 405nm picosecond pulsed diode laser, detection by MCP-PMT. Decay curves measured at 620nm (red), 645nm (blue), 700nm (green), 720nm (grey), 750nm (magenta). All curves measured to the same peak height.



The best fit is obtained by global analysis with three exponential terms, all three lifetimes linked. A graphical presentation of the change of pre-exponential factors (proportional to concentration) and fluorescence intensity (B x τ) is shown below.



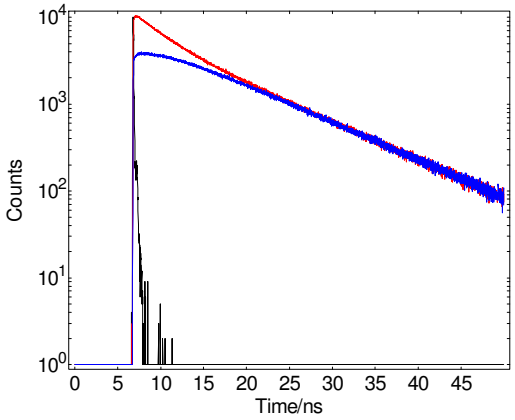


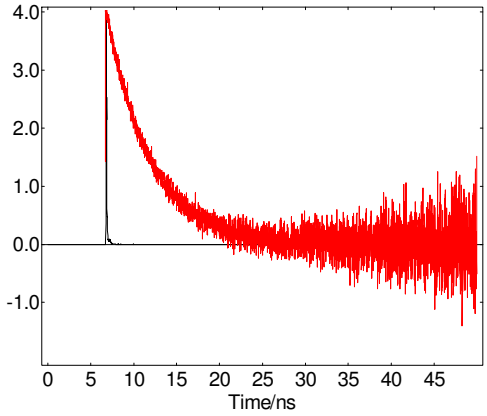
Note that the fluorescence intensities are artificially skewed as the decay curves were all measured to the same peak height.

Appendix B: Properties of Anisotropy Test Data

All test curves for time resolved fluorescence anisotropy are simulated data. The parameters used for the simulation are given in the tables below. The Instrumental Response Function used for convolution is real (measured) data with Ti:Sapphire laser for excitation and MCP-PMT detector.

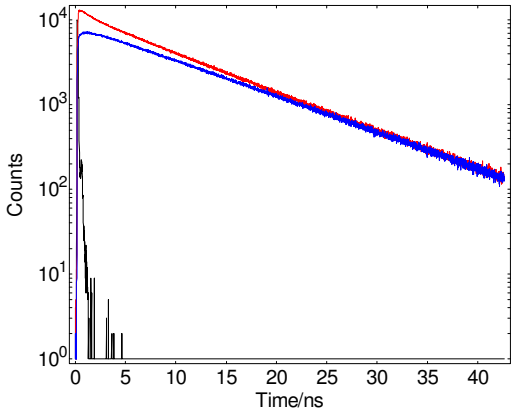
Test file ***Anisotropy - Spherical Rotor.FL***

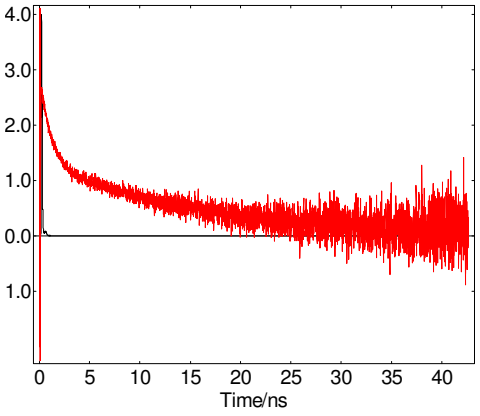




G	1
r_0 – initial anisotropy	0.4
ϕ - rotational diffusion time	5 ns
I_0 – pre-exponential factor	0.083
τ - fluorescence lifetime	10 ns

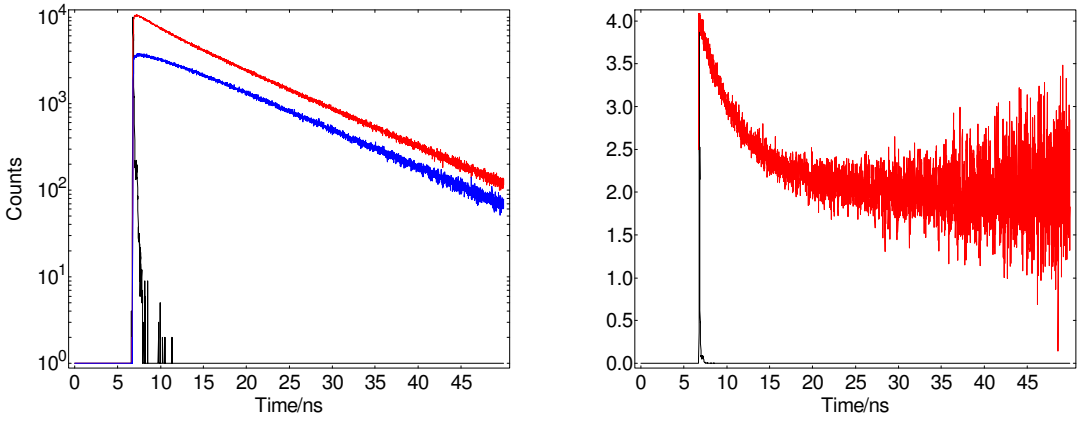
Test file ***Anisotropy -Two Spherical Rotors.FL***





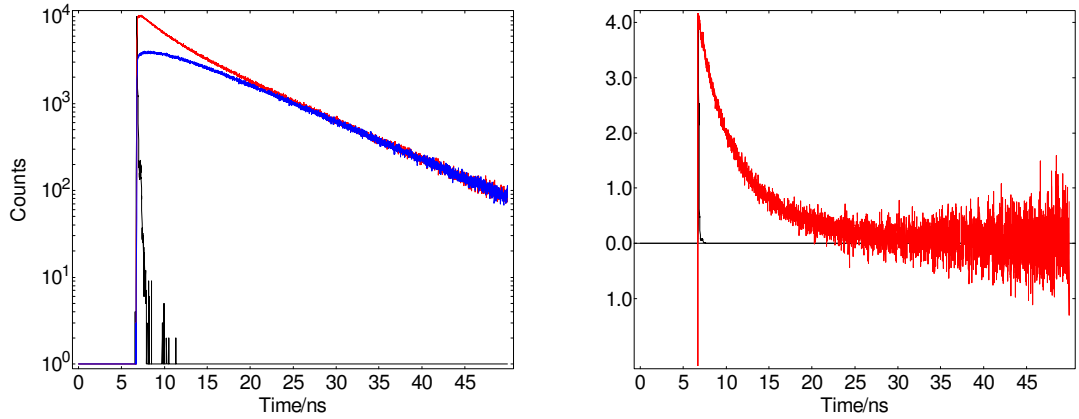
G	1
r_0 – initial anisotropy	0.265
ϕ_1 - rotational diffusion time, first rotor	15 ns
ϕ_2 - rotational diffusion time, second rotor	1 ns
I_{01} – pre-exponential factor, first rotor	0.062
I_{02} – pre-exponential factor, second rotor	0.062
τ - fluorescence lifetime	10 ns

Test file ***Anisotropy - Sph Rotor Restd Rotation.FL***



G	1
r_0 – initial anisotropy	0.4
r_∞ – anisotropy at infinity	0.2
ϕ - rotational diffusion time	5 ns
I_0 – pre-exponential factor	0.083
τ - fluorescence lifetime	10 ns

Test file ***Anisotropy - Ellipsoidal Rotor.FL***



G	1
r_{01} – initial anisotropy, first axis	0.1
r_{02} – initial anisotropy, second axis	0.1
r_{03} – initial anisotropy, third axis	0.2
ϕ_1 - rotational diffusion time, first axis	10 ns
ϕ_2 - rotational diffusion time, second axis	2.67 ns
ϕ_3 - rotational diffusion time, third axis	4.47 ns
I_0 – pre-exponential factor	0.083
τ - fluorescence lifetime	10 ns