FluoFit

PicoQuant GmbH
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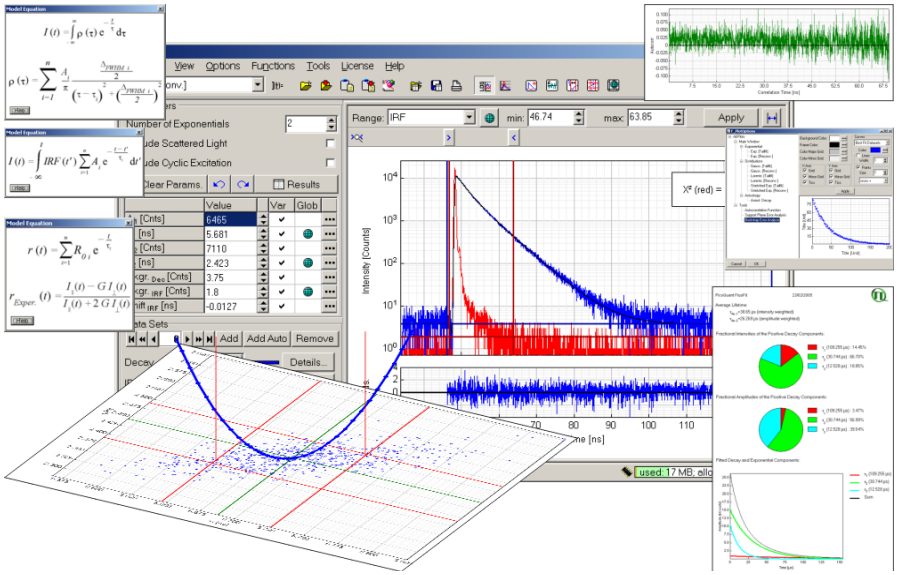
Global Fluorescence Decay Data Analysis Software



User's Manual and Technical Data

Version 4.6

Contents

1. Introduction to FluoFit

Having acquired fluorescence decay curves, a major task is to extract the kinetic information from the raw data. While a simple mono-exponential fit may be sufficient for quick analysis in some cases, the situation is often more complicated. For instance, the observed decay may originate from a superposition of various emissions with various lifetimes, for example: dyes in heterogeneous media or exciplex systems in solution. In most cases, the width of the instrument response function of the recording system is not negligible. This will result in observed decays that represent the convolution of the true exponential decay with the instrument response function. Furthermore, effects such as time shift due to the photomultiplier colour effect, or light scattering leading to unwanted contributions to the signals complicate the interpretation. Fluorescence decay fit tools such as the FluoFit package address these problems. Since direct deconvolution is not possible in a straightforward manner, such programs typically use iterative reconvolu-tion of the theoretical model decay with the instrument response, until an optimum agreement with the observed data is reached.

FluoFit is a global fluorescence decay fit software for PCs. The program implements an iterative reconvolution of the instrument response using nonlinear least-squares error minimisation based on the Levenberg-Marquardt algorithm. Standard exponential (up to four-exponential terms) and multimodal lifetime distribution models can be fitted to the recorded data. IRF and decay backgrounds, time shift, as well as other optional parameters can be included as fit parameters. Robust algorithms and solid implementation lead to quick and reliable convergence after few iterations. Additionally, any model parameter can be adjusted manually and kept fixed during fitting. The fit limits are selected by graphical sliders. Reduced χ^2, plot of weighted residuals and their autocorrelation function are available to facilitate the assessment of the fit quality. A flexible data weighting scheme allows fitting not only photon counting data, but also decay curves obtained by analogue hardware. User preferences are widely adjustable and can be automatically stored and retrieved. The 32-bit software is available for Windows 2000 / XP / Vista and features a modern and easy to use graphical user interface. The results can be printed and saved, numerical values can be saved for later reference. The program supports data files from the TimeHarp, PicoHarp, NanoHarp, HydraHarp, B&H SPC and MSA photon counting systems as well as single or multiple-column ASCII data files.

This manual provides information on how FluoFit works. It cannot replace a theoretical introduction to photon counting and statistical data analysis. If you are new to this area, please refer to appropriate textbooks to learn about the basic principles and explanations of terms.

1.1 What Is New in Version 4.6

- TimeHarp 260 support

1.2 Recommended Literature

Comprehensive textbooks about photon counting and related data analysis:

D. V. O'Connor and D. Phillips: *Time-Correlated Single Photon Counting*.
Academic Press, London, 1984; ISBN 0-12-524140-2

J. N. Demas: *Excited State Lifetime Measurements.* Academic Press, New York, 1983

J. R. Lakowicz: *Principles of Fluorescence Spectroscopy. Second Edition.*
Kluwer Academic/Plenum Publishers, New York, 1999; ISBN 0-306-46093-9

J. R. Lakowicz: *Topics in Fluorescence Spectroscopy. Volume 1 and 2.*
Plenum Press, New York, 1991; ISBN 0-306-43874-7 and ISBN 0-306-43875-5

Easy-to-read explanation of the mathematics behind:

P. R. Bevington and D. K. Robinson: *Data Reduction and Error Analysis for the Physical Sciences*.

McGraw-Hill, 1992

W. H. Press, S. A. Teukolsky, W. T. Vetterling and B. R. Flannary: *Numerical Recipes in C.* Cambridge University Press, 1992

Anisotropy and interpretation of decay data:

D. J. S. Birch and R. E. Imhof: *Kinetic Interpretation of Fluorescence Decays.* Analytical Instrumentation, Vol.14, p. 293-329, 1985

P. Kapusta, R. Erdmann, U. Ortmann and M. Wahl: *Time-resolved Fluorescence Anisotropy Measurements Made Simple.* Journal of Fluorescence, Vol.13, p.179-183, 2003

Data analysis methods:

D. V. O'Connor, W. R. Ware and J. C. Andre: *Deconvolution of Fluorescence Decay Curves. A Critical Comparison of Techniques*. Journal of Physical Chemistry, Vol. 83, p. 1333-1343, 1979

A. Grinwald and I. Z. Steinberg: *On the Analysis of the Fluorescence Decay Kinetics by the Method of Least-Squares.* Analytical Biochemistry, Vol. 59, p. 583-598, 1974

J. C. Andre, L. M. Vincent, D. V. O'Connor and W. R. Ware: *Applications of Fast Fourier Transform to Deconvolution in Single Photon Counting.* Journal of Physical Chemistry, Vol. 83, p. 2285-2294, 1979

A. Gafni, R. L. Modlin and L. Brand: *Analysis of Fluorescence Decay Curves by Means of the Laplace Transformation.* Biophysical Journal, Vol. 15, p. 263-280, 1975

J. Enderlein and R. Erdmann: *Fast Fitting of Multi-exponential Decay Curves*. Optics Communications, Vol. 134, p. 371-378, 1997

1.3 Disclaimer and Copyright Notice

2. Software Deployment

2.1 Requirements

FluoFit is designed to run on PCs with the Windows 2000 / XP / Vista operating system installed. The software takes approximately 10 MB, however, not including the storage space for data files. The screen resolution should be 1024×768 or better.

The FluoFit package is protected by a Hardlock protection module (dongle) that must be connected to a USB port of the PC (or server, if it is a network dongle) during operation. In order to recognise its presence and to use the Hardlock, a software driver needs to be installed. (See the next chapter.)

2.2 Installation and Uninstallation

FluoFit is supplied on CD, together with a protection module (Hardlock). In some cases the software may also be distributed via e-mail, e.g. for updates.

The setup distribution files are:

Readme.txt	general information
Setup.exe	self-extracting installation file

Install the program as follows:

1. Make sure you are logged in with administrator privileges.
2. Attach the Hardlock module to a USB port of your PC.
3. Start the installation by running `setup.exe`.
4. The setup starts with a welcome screen showing a logo and version information. The next screen contains the text of the License Agreement. You need to accept this agreement in order to continue.
5. In the following dialogs you can revise the destination drive and directory (default: `C:\Program Files\PicoQuant\FluoFit`) and the name of the *Start Menu* folder (i.e. program group) where the shortcuts will be placed (default: *PicoQuant - FluoFit*).
6. After installing FluoFit, the setup program will launch the installer for the Hardlock drivers, unless you removed the ✓ from the "☑ Install Hardlock Drivers" section of the last screen. Note that these drivers must be correctly installed in order to use the program.

The subfolder named `sampledata` contains data files with real experimental results in various file formats. You can load these files to try out FluoFit even before you have collected experimental data of your own. Refer to chapter *7. Fitting Examples and Tutorials* starting at page 36 for explanations regarding these data sets. The subgroup *tutorials* of the FluoFit group in the Windows start menu provides links to the sample data for the corresponding tutorial chapters of this manual. There is a second subgroup *tutorials (analysed)*, which contains the readily analysed examples for a comparison.

In order to uninstall the program correctly, please use the Windows *Start Menu*.

1. Make sure you are logged in with administrator privileges.
2. From the FluoFit group in the Windows start menu select *Uninstall /Uninstall FluoFit*.

Removal of the FluoFit package does not affect the Hardlock drivers. If necessary, they can be uninstalled separately:

1. Make sure you are logged in with administrator privileges.
2. Open the *Control Panel* and select *Add or Remove Programs.*
3. Locate *Hardlock Device Driver* and click on *Change/Remove.*

2.3 License Information and Upgrade

To display information on your FluoFit license, select *License*, then *Info...* from the main menu. The following dialog will appear:

The serial number of the Hardlock module, the location of the current license and the software package available are shown in the upper region of the dialog. Currently there are two packages available for FluoFit, called "Basic" and "Pro".

For backup purposes the configuration can be saved to a file (`*.pqu`) by pressing the *Extract License Info...* button. Usually there should be no need to do so.

When planning a license upgrade, contact the support for assistance with the selection of suitable upgrades. License upgrades have to be purchased. The usual procedure is that you get a license upgrade file on a diskette, CD, or via e-mail.

License files are unique to the Hardlock module, for which they have been compiled. An attempt to load a mismatched license file will be rejected by the program. Attempts by the user to circumvent this rejection will most probably result in a damaged Hardlock module.

To load a license file, select *License*, then *Upgrade...* from the main menu. A Windows standard file open dialog will show, where you can specify the license file provided by the support or by any other authorised distributor.

If the license upgrade file (extension `*.pqu`) was successfully opened, the program will prompt you with a message stating that the program has to be restarted to register the changes. Finally, the *License Info...* dialog will show the new license information. Exit FluoFit and restart the software. The new license will be used from now on.

2.4 Supported Data File Formats

General remarks

FluoFit stores its results in a proprietary binary format with a file name extension of `*.ffr`. However, the complete result report or any of its sub-sections can be exported in ASCII format, as described in *5.4 Managing the Results* starting at page 30. The default file name extension of such an export is `*.dat`, and the format is self-explanatory.

The date format and decimal symbol usually depend on the actual Windows regional settings as well as on the language version of Windows.

TimeHarp binary files

TimeHarp interactive mode data files (`*.thd, *.thi`) are binary files containing both the measurement setup parameters and the array of intensity (counts) data. The TimeHarp 100 format (ver. 2.x and 3.0), the TimeHarp 200 format (ver. 2.x up to 6.0) and the TimeHarp 260 (ver. 1.0) format are supported in this release of FluoFit. Continuous mode and TTTR mode data files cannot be loaded directly. You can use the TimeHarp software for conversion to the interactive mode data format before loading by FluoFit. For details about the TimeHarp file formats see the TimeHarp manual.

NanoHarp binary files

NanoHarp data files (`*.nhd`) are binary files containing both the measurement setup parameters and the array of intensity (counts) data. The format ver. 1.0 is supported in this release of FluoFit. For details about this file format see the NanoHarp 250 manual.

PicoHarp binary files

PicoHarp interactive mode data files (`*.phd`) are binary files containing both the measurement setup parameters and the array of intensity (counts) data. The format vers. 1.x and 2.0 are supported in this release of FluoFit. For details about the format see the PicoHarp 300 manual.

HydraHarp binary files

HydraHarp interactive mode data files (`*.hhd`) are binary files containing both the measurement setup parameters and the array of intensity (counts) data. The format ver. 1.0 is supported in this release of FluoFit. For details about the format see the HydraHarp 300 manual.

Clipboard format

The TimeHarp, NanoHarp, PicoHarp and HydraHarp control software export plain, tab separated ASCII data to the Windows clipboard. The structure is self-explanatory and can be easily viewed and processed by pasting the content to a suitable text or spreadsheet processor.

Becker&Hickl SDT files

These binary files (`*.sdt`) are created by the control software of Becker&Hickl SPC-xxx series TCSPC boards or MSA-xxx series multichannel scalers. Refer to the manual of the relevant board for the exact file format specification.

ASCII Data

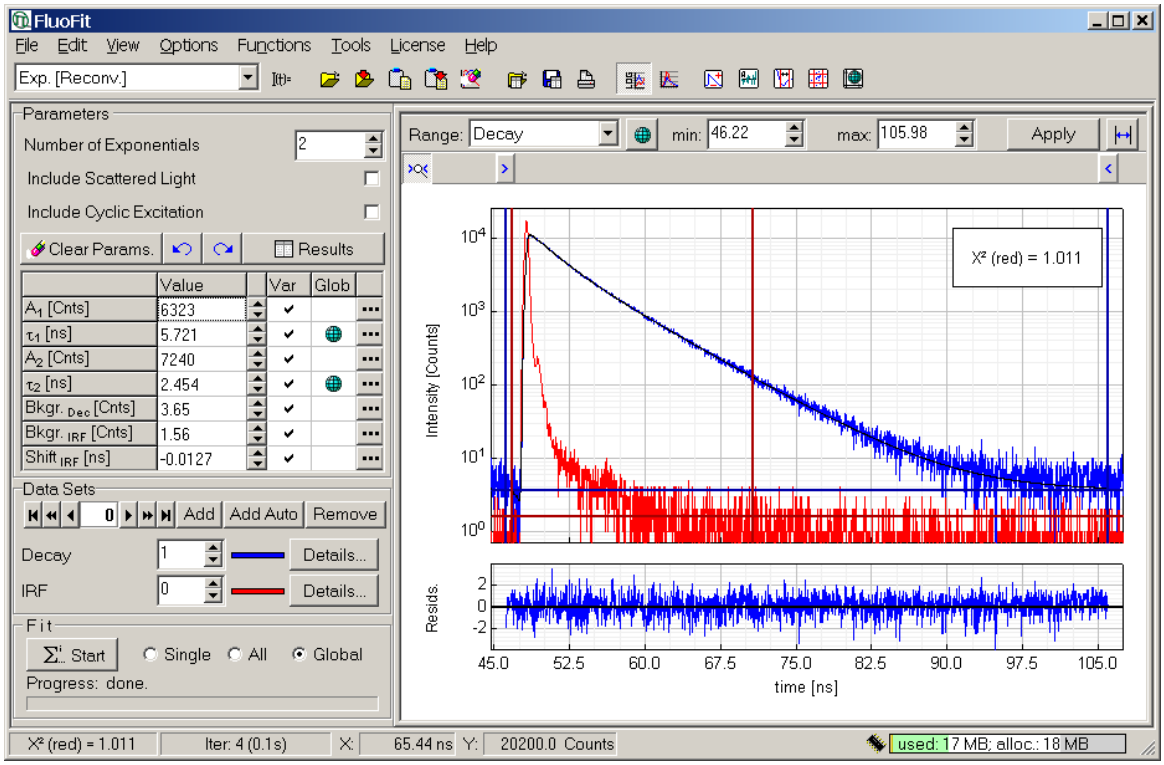
For general purposes, an ASCII import function is available. The data must be provided in single or multiple column(s) with an optional time column. If a time column is provided, it is expected to be the first column. The time axis values must define ascending, equally spaced time intervals. Columns can be separated only by space, semicolon or tab. The column values may be floating point values but, except for the time column, they will be rounded to the nearest integer when importing the data. The file name extension must be `*.dat`. For files not providing the time calibration, i.e. data bin width or time spacing, the correct value should be entered manually. (See page 23).

3. User Interface

This manual only provides a short introduction to the main features of the software. For reference information, step-by-step instruction and a collection of short articles on the scientific background please refer to the extensive help file. It can be accessed via the *Help* command of the main menu. Furthermore, the help file is context-sensitive. Pressing <F1> brings up the help page that describes the dialog or control element that is currently being active.

3.1 Main Window

The FluoFit main window provides a menu, a toolbar and a parameter panel for setting the model parameters and to control the fitting process. The figure below shows a screenshot with some real data loaded and fitted.



The data and all curves calculated in the fit are shown in the main plot on the lower right side of the dialog. Above the main plot is the toolbar. Here you can access frequently used commands by a simple mouse click. To display hints on the tool buttons simply move the mouse cursor over the button. The corresponding hint will be displayed on the status bar at the bottom of the dialog, beneath the main plot. Above the toolbar is the menu for access to additional commands.

On the right side of the status bar, there is an indicator showing the current status of the memory used by the program:

The bar-chart-like display of the indicator scales up to a maximum of 2 GB, which is the maximum address space allowance for data storage.

The orange fraction corresponds to used memory, whereas the red one indicates allocated, but unused address space.

3.2 Menu

The menu bar provides access to various functions of the program via a pull-down menu system. The most frequently used functions can also be accessed via the toolbar.

File menu

Load Data	Load TCSPC data All previously loaded curves are removed from the memory.
Import Data	Import TCSPC data The imported curves are appended to the previously loaded curves.
Load Results	Load a previously saved FluoFit binary results file (`*.ffr`) All previously loaded curves are removed from memory.
Save Results	Save the current results as a FluoFit binary results file (`*.ffr`)
Print Results	Show and print a summary of the current results
Exit	Exit FluoFit

Edit menu

Clear Parameters	Clear all fitting parameters
Clear Data Curves	Clear all loaded and imported data
Paste (Add)	Paste TimeHarp, NanoHarp, PicoHarp or HydraHarp data previously saved to clipboard. The imported curves are appended to any previously loaded curves.
Paste (Replace)	Paste TimeHarp, NanoHarp, PicoHarp or HydraHarp data previously saved to clipboard. All previously loaded curves are removed from memory.
Undo (Fitting Parameters)	Recall the previous fit parameters of the current model
Redo (Fitting Parameters)	One step forward in the history of fit parameters

View menu

Lin / Log Intensity Scale	Toggle the linear / logarithmic scale of the main plot Logarithmic scale may be disabled for some models (e.g. anisotropy).
Normalised Intensity Scale	Normalise the IRF intensity to the decay maximum This command is meaningless for tailfitting.
Zoom (Time Scale)	Zoom the time axis to the selected fitting range(s)
Tool Bar	Show the tool bar
Status Bar	Show the status bar
Parameters	Show the parameter panel

Options menu

Fitting Preferences...	Customisation of the fitting settings for the current model
Plot Options...	Customisation of all plot components. Each single component or group of plot components can be customised.

Functions menu

Exponential	Select a (multi-)exponential model
Distributions	Select a lifetime distribution model
Anisotropy	Select an anisotropy model

Tools menu

Data Operations	Curve management, i.e. deleting of single curves and simple curve arithmetics (i.e. +, −, /, and *)
Autocorrelation Function	Calculation and visualisation of the autocorrelation function of the weighted residuals trace
Support Plane Error Analysis	Calculation of the fitting parameters' confidence intervals using the support plane method
Bootstrap Error Analysis	Calculation of the fitting parameters' confidence intervals using the Bootstrap method
Nonglobal Parameter Diagram	For batch and global analysis: The nonglobal parameters (i.e. the parameters that may have different values for each data set) are either plotted as a function of the index of the corresponding data set or (with TRES data) as a function of the wavelength associated with the data set.

License menu

Info...	Shows and/or exports the current license configuration.
Upgrade...	Loads license upgrade files. You should contact PicoQuant when planning to upgrade your license.

Help menu

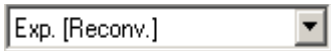
Contents... *Index...* *Find...*	Bring up the contents, index, resp. full text search for this help file
What's new...	Version history, key features and the corresponding help topics
About FluoFit	Software version information

3.3 Toolbar

The toolbar provides quick access to the most frequently used functions of the program. The main menu provides complete access to the functionality.

To hide or display the toolbar, choose *Toolbar* from the *View* menu.

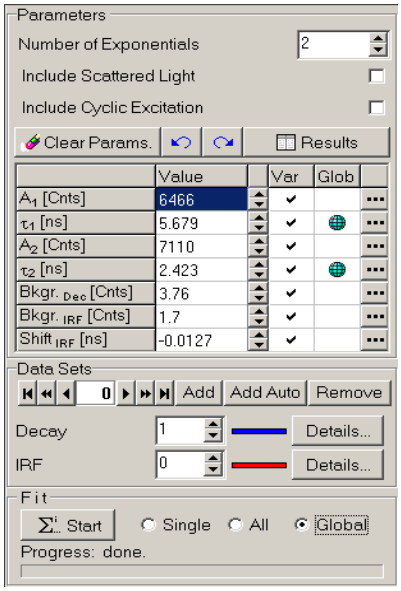
The following table explains the individual buttons.

	Select the fitting model A more structured list is available from the main menu (*Functions*).
 *Show Model Equation*	Show the model equation for the current fitting model
 *Load Data*	Load TCSPC data All previously loaded curves are removed from memory.
 *Import Data*	Import TCSPC data The imported curves are appended to the previously loaded curves.
 *Paste (Add)*	Pastes TimeHarp, NanoHarp, PicoHarp or HydraHarp data previously saved to clipboard. The imported curves are appended to the previously loaded curves.
 *Paste (Replace)*	Pastes TimeHarp, NanoHarp, PicoHarp or HydraHarp data previously saved to clipboard. All previously loaded curves are removed from memory.
 *Clear Data Curves*	Clear all loaded and imported data from the memory
 *Load Results*	Load a previously saved FluoFit binary results file (`*.ffr`) All previously loaded curves are removed from memory.
 *Save Results*	Save the current results as a FluoFit binary results file (`*.ffr`)
 *Print Results*	Show and print a summary of the current results; export results as ASCII
 *Lin / Log Intensity Scale*	Toggle the linear / logarithmic scale of the main plot Logarithmic scale may be disabled for some models (e.g. anisotropy).
 *Normalised Intensity Scale*	Normalise the IRF intensity to the decay maximum This command is meaningless for tailfitting.
 *Data Operations*	Curve management, i.e. deleting of single curves and simple data arithmetics (i.e. +, −, /, and *)
 *Autocorrelation Function*	Calculation and visualisation of the autocorrelation function of the weighted residuals trace
 *Support Plane Error Analysis*	Calculation of the fitting parameters' confidence intervals using the support plane method
 *Bootstrap Error Analysis*	Calculation of the fitting parameters' confidence intervals using the Bootstrap method
 *Nonglobal Parameter Diagram*	For batch and global analysis: The nonglobal parameters (i.e. the parameters that may have different values for each data set) are plotted as a function of the index of the corresponding data set.

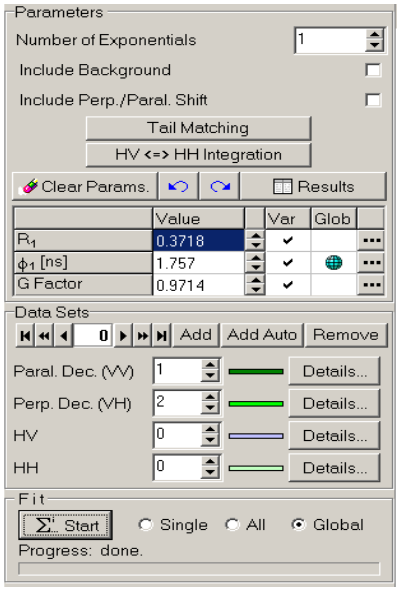
3.4 Parameter Panel

The parameter panel comprises several groups of edit-boxes, check boxes and buttons allowing the setting and adjusting of various fit parameters, as well as controlling the calculations. Its layout depends on the analysis type selected.

Intensity decay analysis



Anisotropy decay analysis



Parameters

This section is divided into two major parts. The topmost part allows setting of all model parameters, which are not subject to optimisation, for example the number of exponentials or similar. Editing these parameters affects the appearance of the lower part, since it determines the number of fitted parameters. For anisotropy analysis, the upper part contains buttons providing access to functionality special to the model (calculation of the *G*-factor).

The table in the lower part of the *Parameters* section lists all model parameters, which may be optimised during the fit. The first column shows the name and the unit of the corresponding parameter, the second column shows its current value. The value can be directly edited in this column. The spin control to the right of the parameter value can be used to vary the value in steps. The third column, *Var*, is used to control the behaviour of the parameter in the fit. Checking the appropriate cell will enable optimisation for the relevant parameter while unchecking it will keep it fixed at the value entered in the second column (*Value*). The *Glob* column can be used to select global scope for a parameter. The ▪▪▪ button can be used to display details about the parameter and to set or unset limits for its value.

Directly above the table, a group of buttons provides access to the following functions:

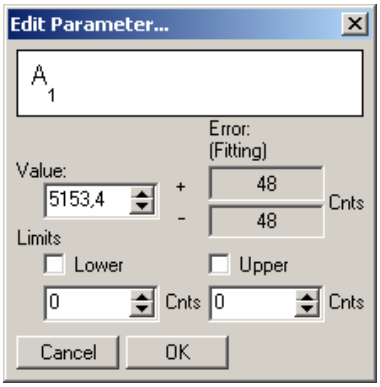
Clear all fitting parameters

Undo: Return to the previous best fit parameter set

Redo: One step forward in the history of best fit parameter sets

Opens the *Report* dialog with the results of the last fit

To show an overview of the properties of a fitting parameter, press the button marked with three dots in the corresponding row of the fitting parameter table. A dialog similar to the following one will be brought up:



The name of the parameter is displayed in the topmost panel of the dialog. The controls below display the current confidence intervals for this parameter and allow editing the parameter value. This can probably be done more conveniently in the fitting parameter table itself.

If the range of the parameter value is to be limited in the fit, activate the *Lower* or *Upper* limit by checking the corresponding tick box. Enter the value for the corresponding limit in the edit box below the checkbox. If limits are set for a parameter, its details button in the fitting parameter table shows three red dots instead of black ones.

Data Sets

The data sets section is used to select the decay IRF etc. curves from the loaded data sets. The index and name of the curves to be selected here varies with the fitting model. For example, a tailfit will only display the decay there, while the anisotropy decay model lists not only parallel and perpendicular polarised decay, but also HV and HH trace, which may be used for G-factor calculation.

The number that can be edited in the spin edit box to the right of the curve name (e.g. 'Decay') corresponds to the index of the curve in the set of all loaded curves. This may not be identical to its index in an imported file, especially if more than one file is imported. To show the file name and file index of the currently selected curve, press the corresponding Details... button on the right side. This will open the *Curve Details* dialog, described in detail on page 23.

The topmost group of controls of the Data Sets section can be used to configure a batch or global fit:

A single data set contains all curves necessary for a nonglobal fit, e.g. the decay and the IRF for a reconvolution fit. For a batch analysis or a global fit, there will be more than one data set. To add a data set press the *Add* button. The program will add the new data set, auto increment some of the curve selection spinedits and display the added data set. The automatic increment will facilitate to add curves, for example, from a TRES measurement. To add all currently imported curves to the data sets, press the *Add Auto* button.

The buttons showing arrows are for navigation through the data sets. The main plot, the display of the fitting parameters and the curve selection controls correspond to the currently displayed data set selected here.

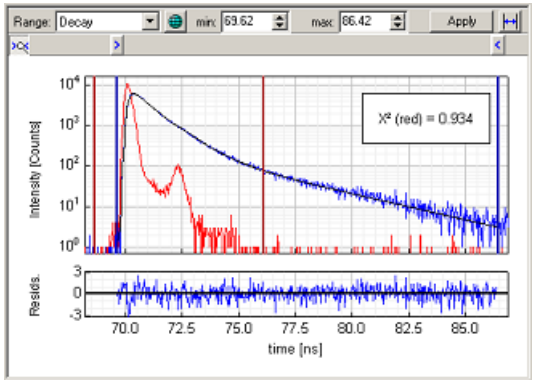
Fit

Having edited all settings in the sections above, the fit can be started by pressing the *Start* button of the fit section. The progress is monitored by the progress indicator to the right of the button. While the fit is running, the *Start* button will be replaced by a button with the caption *Stop*. The fitting process can be interrupted pressing this button. Note that with some models the calculation of a single χ^2 can be rather time consuming. Since the current χ^2 calculation will be completed before terminating the fit, the termination might be delayed.

Having defined more than one data set, the radio buttons on the right of the *Start* button can be used to select the way the program treats these data sets in the fit. Select *Single* to exclusively fit the currently selected and displayed data set. *All* corresponds to running a single curve fit for all defined data sets in a batch-like way. *Global* will start a global fit involving all currently defined data sets.

3.5 Main Plot

The main plot next to the parameter panel shows the analysed experimental data and the calculated curves. This plot facilitates the immediate visual assessment of the fit. For example, when a exponential decay analysis model is selected, the plot displays the following curves: decay data, IRF (when applicable) and the fitted model decay. A separate plot shows the weighted residuals.



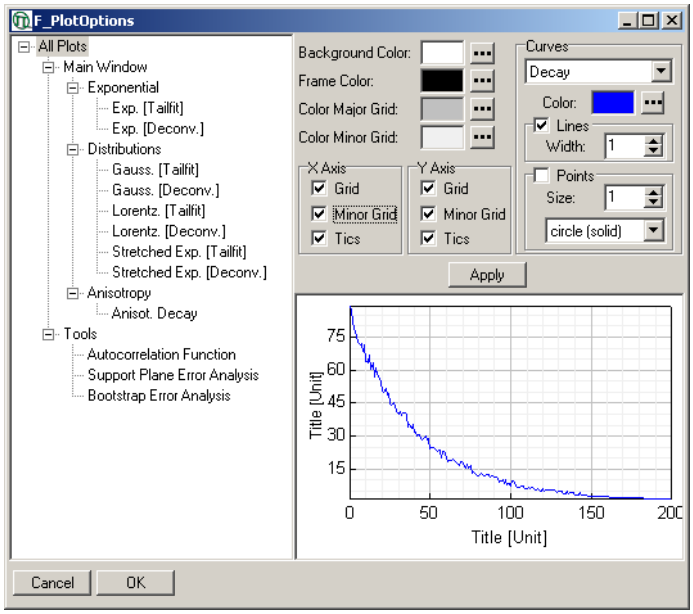
Directly above the plot two sliders allow you to set the data ranges by a click and drag mouse action. Alternatively, the range boundaries can be entered in time units (not channels) in the *min* and *max* edit boxes. To apply the values entered here either press <RETURN> or click the *Apply* button. When you perform a batch analysis or a global fit, this button can be used to apply the fitting ranges of the currently displayed data set to all data sets: 

For simple tailfit models only one data range needs to be set: the range of the decay curve data points to be fitted. For other models there may be more ranges. For example, a reconvolution model uses not only a decay curve but also an IRF (sometimes called “lamp function” or "pulse response"). In this case the IRF range selects the points of the IRF for the reconvolution. The data range to be edited can be selected by the *Range* box on the upper left side of the visual panel.

An inset in the top right corner displays the reduced χ^2 value of the fit. The vertical axis of the weighted residuals plot is linear and scaled in units of standard deviation.

3.6 Plot Options

Each single plot or groups of plots can be customised. Select *Options*, then *Plot Options...* from the main menu to bring up the following dialog:



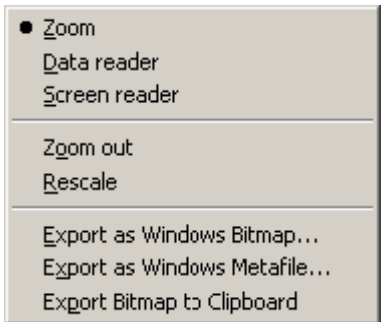
On the left side of the dialog a tree structure provides access to each individual plot or groups of plots. Highlight any tree node to select the corresponding plot (or group of plots) for customisation. On the lower right side of the dialog the current appearance of the selected plot is shown.

On the upper right side of the dialog a group of controls allows customisation of the plot. *Background Color*, *Frame Color*, *Color Major Grid*, *Color Minor Grid* and the *X Axis* and *Y Axis* control groups are concerned with the appearance of the plot as a whole, whereas the *Curves* control group is concerned with the settings for the individual curves in the selected plot.

The number and names of the curves depend on the plot type selected. For example, if the plot of the exponential deconvolution fitting model is selected, there are four curves present: Decay, IRF, Residuals and Fitted Curve, corresponding to all curves present in the main plot for this model. The topmost combo box selects the curve to be customised. The controls below can be used to set the colour of this curve, whether the curve will be drawn as a polygon (*Lines* checked) with a certain thickness (*Width*), whether each data point will be marked with a symbol (*Points* checked) and which symbol will be used for this purpose as well as the size of the symbol. These settings can be combined, for example it is possible to draw the data points as small hollow circles connected with thick lines.

3.7 Plot Components

Place the mouse pointer within any plot and click the right mouse button. The following dialog will appear:



Zoom	This function is enabled by default. The user can interactively zoom into almost any plot. There are two exceptions: The zoom is not active on the residuals' trace and unavailable in the *Report* dialog. To zoom into a subregion of the plotted curve(s), simply draw a selection rectangle of the size of the desired zoomed region by a mouse (left-) click and drag action.
Data reader	If this function is enabled, a cross is displayed on the data point next to the mouse cursor when the mouse cursor is placed over the plot and the left mouse button is down. The coordinates of the cross are displayed in the plot component.
Screen reader	If this function is enabled, a cross is displayed at the position of the mouse cursor when it is placed over the plot and the left mouse button is down. The coordinates of the cross are displayed in the plot component.
Zoom out	Moves one step backward in the zoom history.
Rescale	Undo all zooming operations.
Export as Windows bitmap	Brings up a file save dialog allowing to save the plot as a Windows bitmap file.
Export as Windows metafile	Brings up a file save dialog allowing to save the plot as a Windows metafile.
Export bitmap to clipboard	Exports the current plot in bitmap format to the Windows clipboard. The clipboard content can be directly pasted to most word processors.

4. Kinetic Models

The following chapter gives an overview on the currently implemented decay models, their adjustable parameters and the required data curve types necessary to perform the relevant analysis. Chapter 4.2 provides hints to facilitate the selection of the proper model and describes some basic strategies how to deploy them.

4.1 Definition of Model Parameters

4.1.1 Exponential Model [Tailfit]

This model is for performing multi exponential tailfits.

$$I(t) = \sum_{i=1}^{n} A_i \, e^{-\frac{t}{\tau_i}}$$

Parameters:

A_i	Amplitude of the ith component, in counts, in the first fitting range channel
τ_i	Lifetime of the ith component
$Bkgr._{Dec}$	Decay background, in counts

Data Curves:

Decay	Experimentally measured decay curve

4.1.2 Exponential Model [Reconvolution]

This model is for performing multi exponential fits with reconvolution.

$$I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i=1}^{n} A_i \, e^{-\frac{t-t'}{\tau_i}} \, dt'$$

Parameters:

A_i	Amplitude of the ith component, in counts, at time zero
τ_i	Lifetime of the ith component
$Bkgr._{Dec}$	Decay background, in counts
$Bkgr._{IRF}$	IRF background, in counts
$Shift_{IRF}$	Time shift between IRF and decay

Optional parameters:

A_{Scat}	Amplitude of the scattered light contribution, in counts. This is a multiple of the normalised IRF added to the convolved model curve.

Period $_{Rep}$ Time period between two consecutive excitation pulses

Data Curves:

Decay Experimentally measured decay curve

IRF Experimentally measured IRF (lamp function)

4.1.3 Gaussian Distribution [Tailfit]

This model fits multimodal Gaussian distributed exponentials to the decay curve tail region. Each peak is approximated by a finite number of exponentials.

$$I(t) = \int_{-\infty}^{\infty} \rho(\tau)\, e^{-\frac{t}{\tau}}\, d\tau$$

$$\rho(\tau) = \sum_{i=1}^{n} \frac{A_i}{\sigma_i (2\pi)^{1/2}} e^{-\frac{1}{2}\left(\frac{\tau-\tau_i}{\sigma_i}\right)^2} \; ; \; \sigma_i = \frac{\Delta_{FWHM\,i}}{(8 \ln 2)^{1/2}}$$

Parameters:

A_i Amplitude of the ith distributed component, in counts, in the first fitting range channel

τ_i Center lifetime of the ith distributed component

$\Delta_{FWHM\,i}$ Distribution width (full width at half maximum) of the ith distributed component

T_0 Time zero for the decay time distribution. A selection of a time zero with a tailfit is somewhat arbitrary and will induce additional uncertainty in the fitting parameters, which cannot be monitored by error analysis.

Bkgr. $_{Dec}$ Decay background, in counts

Data Curves:

Decay Experimentally measured decay curve

4.1.4 Gaussian Distribution [Reconvolution]

This model fits multimodal Gaussian distributed exponentials to the decay curve applying a reconvolution. Each peak is approximated by a finite number of exponentials.

$$I(t) = \int_{-\infty}^{t} IRF(t') \int_{-\infty}^{\infty} \rho(\tau)\, e^{-\frac{t-t'}{\tau}}\, d\tau\, dt'$$

$$\rho(\tau) = \sum_{i=1}^{n} \frac{A_i}{\sigma_i (2\pi)^{1/2}} e^{-\frac{1}{2}\left(\frac{\tau-\tau_i}{\sigma_i}\right)^2} \; ; \; \sigma_i = \frac{\Delta_{FWHM\,i}}{(8 \ln 2)^{1/2}}$$

Parameters:

A_i	Amplitude of the ith distributed component, in counts, in the first fitting range channel
τ_i	Centre lifetime of the ith distributed component
$\Delta_{FWHM\ i}$	Distribution width (full width at half maximum) of the ith distributed component
Bkgr. $_{Dec}$	Decay background, in counts
Bkgr. $_{IRF}$	IRF background, in counts
Shift $_{IRF}$	Time shift between IRF and decay

Optional Parameters:

A_{Scat}	Amplitude of the scattered light contribution, in counts. This is a multiple of the normalised IRF added to the convolved model curve.
Period $_{Rep}$	Time period between two consecutive excitation pulses

Data Curves:

Decay	Experimentally measured decay curve
IRF	Experimentally measured IRF (lamp function)

4.1.5 Lorentzian Distribution [Tailfit]

This model fits multimodal Lorentzian distributed exponentials to the decay curve tail region. Each peak is approximated by a finite number of exponentials.

$$I(t) = \int_{-\infty}^{\infty} \rho(\tau)\, e^{-\frac{t}{\tau}}\, d\tau$$

$$\rho(\tau) = \sum_{i=1}^{n} \frac{A_i}{\pi} \frac{\frac{\Delta_{FWHM\ i}}{2}}{(\tau - \tau_i)^2 + \left(\frac{\Delta_{FWHM\ i}}{2}\right)^2}$$

Parameters:

A_i	Amplitude of the ith distributed component, in counts, in the first fitting range channel
τ_i	Centre lifetime of the ith distributed component
$\Delta_{FWHM\ i}$	Distribution width (full width at half maximum) of the ith distributed component
T_0	Time zero for the decay time distribution. A selection of a time zero with a tailfit is somewhat arbitrary and will induce additional uncertainty in the fitting parameters, which cannot be monitored by error analysis.
Bkgr. $_{Dec}$	Decay background, in counts

Data Curves:

Decay	Experimentally measured decay curve

Lorentzian Distribution [Reconvolution]

This model fits multimodal Lorentzian distributed exponentials to the decay curve applying a reconvolution. Each peak is approximated by a finite number of exponentials.

$$I(t) = \int_{-\infty}^{t} IRF(t') \int_{-\infty}^{\infty} \rho(\tau)\, e^{-\frac{t-t'}{\tau}}\, d\tau\, dt'$$

$$\rho(\tau) = \sum_{i=1}^{n} \frac{A_i}{\pi} \frac{\frac{\Delta_{FWHM\ i}}{2}}{(\tau - \tau_i)^2 + \left(\frac{\Delta_{FWHM\ i}}{2}\right)^2}$$

Parameters:

A_i	Amplitude of the i[th] distributed component, in counts, in the first fitting range channel
τ_i	Centre lifetime of the i[th] distributed component
$\Delta_{FWHM\ i}$	Distribution width (full width at half maximum) of the i[th] distributed component
Bkgr. $_{Dec}$	Decay background, in counts
Bkgr. $_{IRF}$	IRF background, in counts
Shift $_{IRF}$	Time shift between IRF and decay

Optional Parameters:

A_{Scat}	Amplitude of the scattered light contribution, in counts. This is a multiple of the normalised IRF added to the convolved model curve.
Period $_{Rep}$	Time period between two consecutive excitation pulses

Data Curves:

Decay	Experimentally measured decay curve
IRF	Experimentally measured IRF (lamp function)

4.1.6 Stretched Exponentials [Tailfit]

This model fits a multicomponent stretched exponential function to the decay curve tail region.

$$I(t) = \sum_{i=1}^{n} A_i\, e^{-\left(\frac{t}{\tau_i}\right)^{\beta_i}}$$

Parameters:

A_i	Amplitude of the i[th] component (in counts) in the first fitting range channel
τ_i	Lifetime of the i[th] component
β_i	Distribution parameter of the i[th] component. Usually $0 < \beta < 1$. $\beta = 1$ is equivalent to an exponential decay, while $\beta \rightarrow 0$ converges to an infinitely broad distribution.

T_0 Time zero for the decay time distribution. A selection of a time zero with a tailfit is somewhat arbitrary and will induce additional uncertainty in the fitting parameters, which cannot be monitored by error analysis.

Bkgr. $_{Dec}$ Decay background, in counts

Data Curves:

Decay Experimentally measured decay curve

4.1.7 Stretched Exponentials [Reconvolution]

This model fits a multicomponent stretched exponential function to the decay curve applying a reconvolution.

$$I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i=1}^{n} A_i \, e^{-\left(\frac{t-t'}{\tau_i}\right)^{\beta_i}} \, dt'$$

Parameters:

A_i Amplitude of the i[th] component (in counts) in the first fitting range channel

τ_i Lifetime of the i[th] component

β_i Distribution parameter of the i[th] component. Usually $0 < \beta < 1$. $\beta = 1$ is equivalent to an exponential decay, while $\beta \rightarrow 0$ converges to an infinitely broad distribution.

Bkgr. $_{Dec}$ Decay background, in counts

Bkgr. $_{IRF}$ IRF background, in counts

Shift $_{IRF}$ Time shift between IRF and decay

Optional Parameters:

A $_{Scat}$ Amplitude of the scattered light contribution, in counts. This is a multiple of the normalised IRF added to the convolved model curve.

Period $_{Rep}$ Time period between two consecutive excitation pulses

Data Curves:

Decay Experimentally measured decay curve

IRF Experimentally measured IRF (lamp function)

4.1.8 Anisotropy [Tailfit]

This model is for performing multi-exponential fits on a calculated anisotropy decay. Since the anisotropy decay is a nonlinear function of the parallel and perpendicular polarised decay curves, reconvolution fitting is not possible when analysing the anisotropy decay directly.

$$r(t) = R_{INF} + \sum_{i=1}^{n} R_i \, e^{-\frac{t}{\phi_i}}$$

$$r_{Exper.}(t) = \frac{I_{\parallel}(t) - G\, I_{\perp}(t)}{I_{\parallel}(t) + 2\, G\, I_{\perp}(t)}$$

Parameters:

R_i	Anisotropy contribution of the i[th] component in the first fitting range channel, also called initial anisotropy
ϕ_i	Rotational correlation time of the i[th] component
G Factor	Ratio of the sensitivities of the detection system for vertically and horizontally polarised light.

Optional Parameters:

R_{INF}	Anisotropy background, also called residual anisotropy
Shift $_{Par/Prp}$	Time shift between parallel and perpendicular polarised decays

Data Curves:

Perp. Dec.	Decay of the horizontally polarised emission component measured with vertically polarised excitation, i.e. perpendicular polarised decay
Paral. Dec.	Decay of the vertically polarised emission component measured with vertically polarised excitation, i.e. parallel polarised decay
HV	Decay of the vertically polarised emission component measured with horizontally polarised excitation. This curve is not used in the fit, it is optionally included for *G*-factor calculation.
HH	Decay of the horizontally polarised emission component measured with horizontally polarised excitation. This curve is not used in the fit, it is optionally included for *G*-factor calculation.

4.1.9 Anisotropy [Reconvolution]

This model is for performing multi-exponential anisotropy reconvolution fits. Parallel polarised and perpendicular polarised decay are reconvolved separately with the anisotropy parameters as a kind of "global" parameters for both decays.

$$I_{\parallel}(t) = G\int_{-\infty}^{t} IRF_{\parallel}(t')\,\frac{1}{3}\sum_{i=1}^{n}\alpha_i\, e^{-\frac{t-t'}{\tau_i}}\left[1+2\left(R_{INF}+\sum_{j=1}^{m}L_{ij}\beta_j\, e^{-\frac{t-t'}{\phi_j}}\right)\right]dt'$$

$$I_{\perp}(t) = \int_{-\infty}^{t} IRF_{\perp}(t')\,\frac{1}{3}\sum_{i=1}^{n}\alpha_i\, e^{-\frac{t-t'}{\tau_i}}\left[1-\left(R_{INF}+\sum_{j=1}^{m}L_{ij}\beta_j\, e^{-\frac{t-t'}{\phi_j}}\right)\right]dt'$$

Parameters:

α_i	Amplitude of the i[th] fluorescence decay component.
τ_i	Lifetime of the i[th] fluorescence decay component.
β_j	Initial anisotropy of the j[th] anisotropy decay.
ϕ_j	Lifetime (correlation time) of the j[th] anisotropy decay.
G-Factor	Ratio of the sensitivities of the detection system for vertically and horizontally polarised light.

L_{ij}	Fluorescence / anisotropy component association matrix: L_{ij}=1: lifetime i associated with anisotropy correlation time j L_{ij}=0: lifetime i not associated with anisotropy correlation time j Homogeneous (non-associative) case: all L_{ij}=1,otherwise inhomogeneous (associative)
Bkgr. $_{Dec}$	Homogeneous (i.e. time independent) decay background in counts (paral. and perp. trace).
Bkgr. $_{IRF}$	Homogeneous (i.e. time independent) IRF background in counts (paral. and perp. trace).
Shift $_{IRF}$	Time shift between IRF and decay (paral. and perp. trace).

Optional Parameters:

R_{INF}	Anisotropy background, also called residual anisotropy

Data Curves:

Paral. Dec. (VV)	Experimentally measured decay curve (paral. polarised).
Paral. IRF (VV)	Experimentally measured IRF (paral. polarised).
Perp. Dec. (VH)	Experimentally measured decay curve (perpend. polarised).
Perp. IRF (VH)	Experimentally measured IRF (perpend. polarised).
HV	Decay of the vertically polarised emission component measured with horizontally polarised excitation. This curve is not used in the fit, it is optionally included for *G*-factor calculation.
HH	Decay of the horizontally polarised emission component measured with horizontally polarised excitation. This curve is not used in the fit, it is optionally included for *G*-factor calculation.

4.2 Which One Is Appropriate?

Selecting the proper kinetic model is not a simple task. Generally, the best model gives reasonable fit quality with the smallest number of floating (adjustable) parameters. It is important to note that any useful fitting equation must include parameters that are not related to the pure, undistorted sample response kinetics. Only a subset of fitted parameters is useful for plotting against concentration, temperature or similar experimental variables. The table below summarises the number of iterated parameters of the decay models, as implemented in FluoFit version 4.2.

Model	Number of iterated parameters	
	Reconvolution fit	Tailfit
n-exponential	3+2n to 5+2n	1+2n
n-modal lifetime distribution, or stretched exponential with n-terms	3+3n to 5+3n	2+3n

In cases of unknown sample and/or decay kinetics, start with the simplest appropriate (usually single-exponential) model and proceed step-by-step to more complicated ones if necessary. What holds for the exponential series, also holds for any other model: Whatsoever complicated the physical reality might be for your experiment, you stand absolutely no chance of resolving all the parameters of a complex equation, when a simple one already describes the data sufficiently. Remember that e.g. increasing the number of exponential terms always improves the quality of the fit. However, this does not necessarily mean that the new model is more appropriate. Critically evaluate whether the more complicated model leads to a significant improvement. For example, three or four exponential fit results are often mathematical representations of decay curve artefacts rather than real kinetic parameters.

When the decay curve is complex (e.g. three or more exponentials are needed to describe it), it is sometimes more sensible to fit a monomodal lifetime distribution model because it uses fewer adjustable parameters. FluoFit implements two such models: Gaussian and Lorentzian lifetime distributions. Compare the quality of a monomodal distribution fit to those obtained by fitting higher order exponential models. Note that the stretched exponential model can be also regarded as a (somewhat unusual) lifetime distribution model.

Multimodal distribution models, on the other hand, load a huge amount of freely varying parameters on the experimental data. Quite a lot of photons are needed to resolve more than one peak, here. Never apply more than two peaks, except when some of the parameters are known in advance and can be kept fixed in the fit. The two major parameters of these models are the central (or “characteristic”) lifetime and a parameter describing the width of the distribution, denoted as τ_i and Δ_i, respectively. It is recommended to start the fitting procedure with an ordinary single exponential fit and to use the recovered lifetime as a starting estimate for τ_i. One third of τ_i is usually a reasonable starting estimate for Δ_i.

The fitting model can be selected either by use of the toolbar or the *Functions* menu item. Please note that unlike in older FluoFit versions, tailfit and reconvolution are regarded as separate models. This is because reconvolution fitting now offers new options like scattered light correction and cyclic re-excitation, which are not applicable to tailfitting.

Let us stress once again: selection of the correct model is a step of crucial importance. Make sure that the model is actually the one which describes the physics behind the experiment. To state it more drastically: yes, it might be technically possible to fit a stretched exponential model to the perpendicular polarised decay obtained in an anisotropy measurement, but it does not make any sense.

5. Using the FluoFit Software

5.1 Starting the Program

Once installed, you can start FluoFit using the **Start** menu, clicking on FluoFit in the appropriate program group (the default is *PicoQuant - FluoFit*).

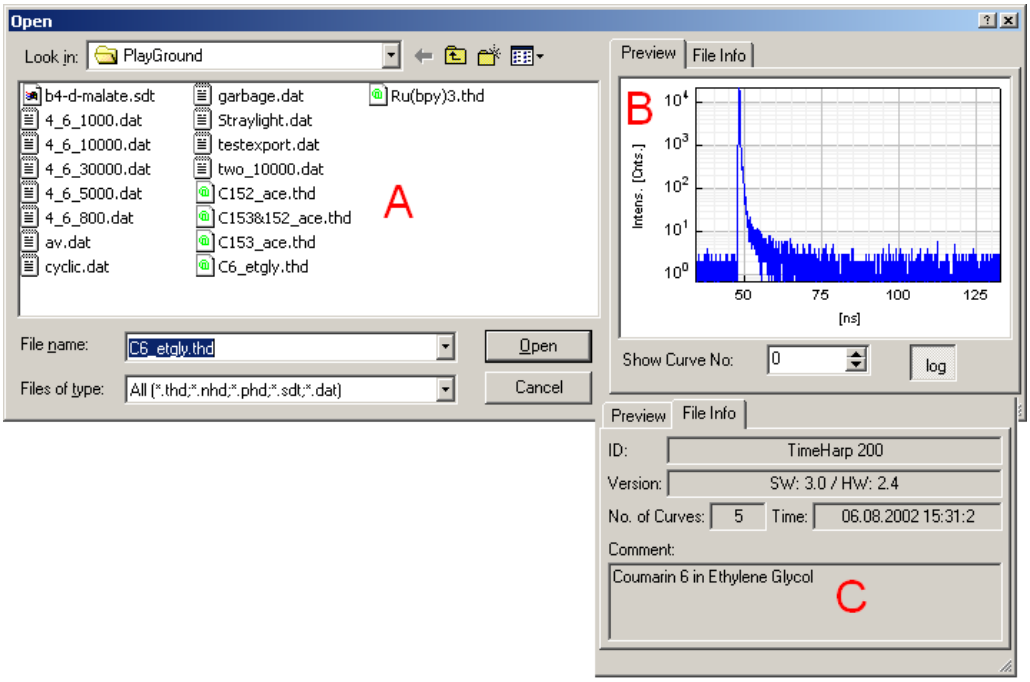
5.2 Loading Measurement Data

Press one of the following tool buttons to bring up a file open dialog:

Load Data All previously loaded curves are removed from memory.

Import Data The imported curves are appended to the previously loaded curves.

In the following dialog select the file you would like to open (**A**). On the right side of the dialog you can either select a preview to show the curves present in the file (**B**) or show the header information of the file (**C**).

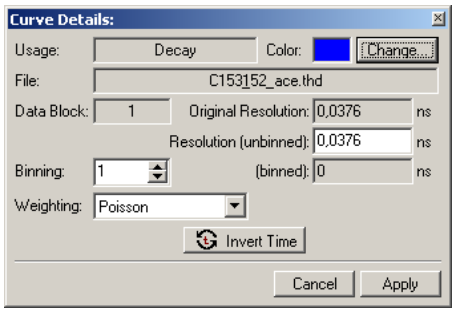


Press the *Open* button to import the selected file.

If you are a TimeHarp, NanoHarp PicoHarp or HydraHarp user, the Windows clipboard provides a quick way of data transfer to the FluoFit. It is a good idea to run the relevant control software and the FluoFit simultaneously. Using the control software, copy the content of the board's memory into the clipboard by clicking on the appropriate *Copy* button or by pressing <CTRL+C>. Switch to the FluoFit (use the Windows taskbar or press <CTRL+TAB>). Paste the curves into FluoFit by clicking on the *Paste* button or by pressing <CTRL+V>. When pasting curves, these not necessarily appear immediately in the main plot, especially when the curves have already been imported previously. To get an overview of all imported curves, use the *Data Operations* dialog, described in detail on page 32.

Users of older FluoFit versions may have noticed that no curve selection is done during data loading or import. This is not necessary any more, since FluoFit is now able to hold an arbitrary number of curves simultaneously. Curve selection is done after the import using the *Data Sets* section of the *Parameter* panel. The number, which can be edited in the spin edit box to the right of the curve name (e.g. “Decay”), corresponds to the index of the curve in the set of all loaded curves. This may not be identical to its index in an imported file, especially when more than one file is imported. To show the file name and file index of the

currently selected curve, press the corresponding Details... button on the right side. This will open the *Curve Details* dialog:



In the topmost row the usage of the curve in the current fitting model, the file from which the curve was imported, its original time resolution, and the curve colour in the main plot are displayed. The colour can be changed by pressing Change... to the right of the colour panel.

The time resolution can be edited in the *Resolution* edit box. This is very useful e.g. for ASCII files, which contained no time resolution information.

It is recommended that curves containing more than, say, 4000 data points, should be binned. Usually an amount of more than 4000 data points per curve does not provide additional information on the decay behaviour of the underlying process and will only increase the computation time. To avoid this, use the *Binning* edit box of this dialog. A binning of <n> means that <n> raw data channels are combined to one channel in the binned data. The intensity of the new channel is the sum of the <n> binned channels.

Photon counting data are subject to Poisson statistics. Data weighting is set to “Poisson” by default. However, for data acquired by other experimental setups than single photon counting devices, the Poisson weighting will probably not describe the experimental noise. In this case the weighting can be turned off by selecting <none> instead of “Poisson” in the *Weighting* combo box.

Sometimes decay data are recorded and/or stored with a reversed time axis. Because FluoFit always treats the channels' content in a normal, causal way, such a time axis should be inverted again. This can be accomplished by pressing Invert Time.

5.3 Performing a Decay Fit

The following chapters provide basic information concerning the necessary steps to get the useful kinetic information out from the raw channel content. This manual cannot replace a theoretical introduction to photon counting and statistical data analysis. If you are new to this area, please refer to appropriate textbooks to learn about the basic principles and explanations of terms. Some of the comprehensive literature available today is listed in chapter *1.2 Recommended Literature* at page 1.

The general procedure of decay analysis by means of FluoFit is as follows:

1) Load experimental data.

2) Choose an appropriate kinetic model and a fitting technique (i.e. reconvolution or tailfit).

3) Select the data range(s) for fitting.

4) Perform the iterations, i.e. fit.

5) Assess the fit, evaluate the results. If unsatisfied, restart the process from step 2 or 3.

5.3.1 Basic Concepts, Terms and Definitions

Data fitting by FluoFit means optimisation of a set of adjustable model parameters to achieve the “best possible” agreement between the selected (theoretical) model and the experimental data. To achieve this goal, one needs a quantity that characterises the degree of agreement. FluoFit uses the most popular quantitative measure of fit quality, the so-called “reduced chi-squared” parameter (χ^2 hereafter):

$$\chi^2 = \frac{1}{N-p} \sum_{i=1}^{N} W(i)^2 \left[Decay(i) - Fit(i) \right]^2$$

Here *N* is the number of fitted channels (i.e. data points) and *p* is the number of fitted parameters. The difference, *N−p* is called degree of freedom. *Decay* is an array of *N* intensity values, that is, the experimentally measured decay curve. The *Fit(i)* values are calculated using the selected kinetic model and the iterated values of model parameters. *W(i)* is called weighting factor, as explained below.

Maximum Likelihood Estimation (MLE)

In TCSPC data analysis it refers to the fact that one is about to analyse data following a Poisson distribution in contrast to a Gaussian distribution, which would be analysed by a least squares fit. The goodness-of-fit parameter of MLE fitting is defined as:

$$\chi_{MLE} = \sum_{i=1}^{N} \left[Fit(i) - Decay(i) \ln\left(Fit(i) \right) \right]$$

One has to keep in mind that the term 'MLE' is a bit misleading: For Gaussian distributed data (or for Poisson distributed data in the Gaussian limit) least squares is the maximum likelihood estimator.

Data Weighting

In order to get a meaningful χ^2 and correctly fitted parameters, the value of each residual [*Decay(i)−Fit(i)*] must be properly weighted. FluoFit was developed primarily to analyse photon counting data governed by Poisson statistics, when the weighting factor *W(i)* can be estimated as:

$$W(i) = 1/\sqrt{Decay(i)}$$

where *Decay(i)* is a measured intensity (count) value in channel *i*. If you have collected your data with a current sampling technique or a transient digitiser (e.g. oscilloscope), they are probably governed by Gaussian statistics and you should use *W(i)* = 1, i.e. uniformly weighted residuals. The appropriate weighting scheme can be selected in the *Curve Details* dialog. (See page 23.)

Iteration Algorithms and Fitting Techniques

The fast Marquardt-Levenberg optimisation algorithm needs sensible initial parameter estimates, otherwise it is possible for the fitting process to get stuck in a local minimum and not find the best fit. Initial parameter estimates are provided by a fast, automatic Monte Carlo search. Manual fitting is possible and very useful to test the mathematical behaviour of the decay model. Change the value of any fit parameter and see the effect on the fitted curve and on the residuals' distribution. This can be done conveniently using the spin controls in the fitting parameter table.

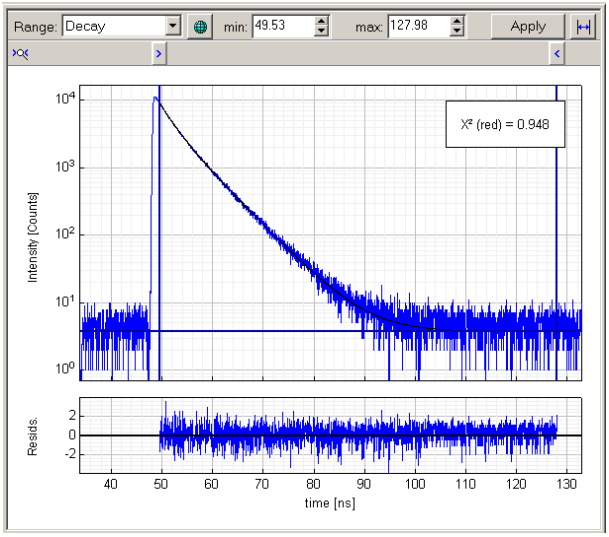
5.3.2 Data Range Selection

In most cases, not all data points of the imported curves are to be used in the fit, i.e. data ranges have to be defined. This is done in the main plot. Select the range to be fitted with the *Range* combo box. Use the sliders directly above the main plot to set the range boundaries with a mouse click-and-drag action.

Tailfit

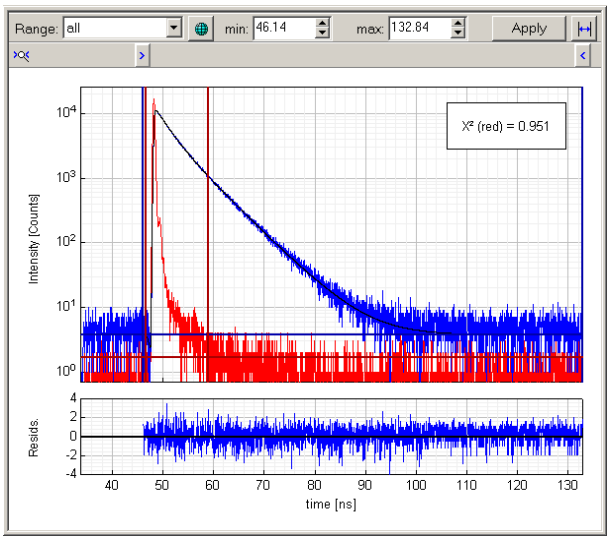
The lower range boundary should be placed slightly to the right of the decay curve maximum to make sure that the part influenced by the IRF is not contained in the fitting range. The upper boundary should be set to contain at least some of the time independent background (if possible), as in the example on the following page.

When performing a global tailfit it might be desirable to set the fitting ranges of all data sets to identical boundaries, say, to have comparable exponential amplitudes, or similar. To achieve this, press the button beside the range selection combo box: 



Reconvolution fit

The range boundaries for the decay curve as well as for the IRF should be set so that the fitting range contains some of the time independent background to the left of the rise and to the right of the tail. It is possible to set two separated, independent boundaries for IRF and decay, respectively. While the data range set for the decay curve defines all points used to calculate the χ^2, the IRF range selects the points of the IRF, which will be used for the reconvolution. Selection of the data range to be edited is done by the *Range* combo box on the upper left side of the main plot. An example is shown below:



5.3.3 The Fitting Procedure

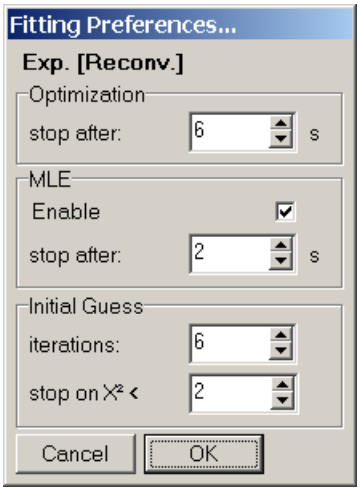
Except for very sophisticated models, starting the parameter optimisation is trivial. Press Clear to erase any previous results and start the fit by pressing Start in the *Fit* section of the parameter panel.

The program will start with a Monte Carlo search to estimate initial parameters and in a second step these will be optimised with the Marquardt-Levenberg iteration algorithm. Of course, the initial Monte Carlo search can be replaced by manually entering initial values. However, FluoFit will check these values. Some non-linear parameters, particularly the lifetimes, cannot have zero values. If such zeros are found, the program will replace them (and only them) by Monte Carlo estimates. It would be a waste of time to search for linear parameters (e.g. amplitudes, decay background), since they can be directly calculated from the rest of the model parameters and from the decay curve by a simple inversion of a linear set of equations.

Experienced users may customise the search and iteration routines using the dialog shown on the next page (select *Options*, then *Fitting Preferences...* or simply press <CTRL>+U).

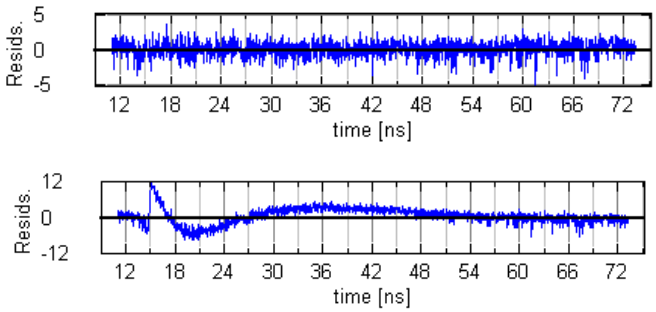
The current model is displayed in bold print in the topmost row of the dialog. In the *Optimisation* section the maximum iteration time for the Marquardt-Levenberg optimisation is specified. Checking the tick box in the *MLE* section enables Maximum Likelihood Estimation. Similar to the Marquardt-Levenberg optimisation the maximum iteration time for MLE may be configured. The *Initial Guess* section allows setting of the maximum number of iterations of the preceding Monte Carlo search as well as specifying a minimum χ^2 value as an "convergence criterion" for the Monte Carlo search.

During the fitting, the progress of the calculations is shown next to the $\sum_{...}^{i}$ Start button. At the same time, the status bar indicates the number of successful iteration cycles and the χ^2 of the current iteration. The iterations stop after reaching a stable (but not necessarily the lowest!) χ^2 value, or after the timeout specified in the *Fitting Preferences...* dialog. The parameter panel and the main plot are immediately updated with the new results. Press the $\sum_{...}^{i}$ Start button again. If the fit has already achieved a χ^2-minimum (local or global), this second run stops immediately with negligible changes of parameter values. Less likely, the optimisation has stopped somewhere on the way to the minimum, due to the timeout. In this case, subsequent runs allow to continue the fitting from the just achieved stage.



As it was mentioned in chapter *5.3.1* (on page 23) already, the iteration algorithm tries to find the parameter set that yields the lowest possible reduced χ^2, regardless of the meaning of those parameters. The resulting χ^2 value is displayed in the main plot as well as in the status bar. For photon counting data, $\chi^2 \approx 1$ indicates a good, but not necessarily meaningful fit. The acceptable χ^2 is not the only criterion!

Somewhat more informative is the plot of weighted residuals *R(i)* = *W(i)* [*Decay(i)-Fit(i)*] versus time, shown at the bottom of the main plot. The particular advantage of this plot is that it shows where the misfit occurs. Ideally, the weighted residuals are below 4 standard deviations and randomly fluctuate around zero with no obvious trend. In practice you will often find spikes or oscillations at early times (around the rising edge of the IRF and decay trace) due to various instrument artefacts. The picture below illustrate a satisfactory (top) and an obviously bad residual distribution (bottom).



Regular oscillations in the weighted residuals are indicative of RF noise pick-up. Spikes and/or trends in the residuals may be due to systematic errors in the measurement, but most probably mean that you need a more complicated model. Try to further improve the fit by selecting a more appropriate kinetic model, refining the data range selection, etc.

Tips and tricks

Sometimes a fit fails to converge to the global optimum and sticks to a local minimum resulting in a stable but elevated χ^2, although the kinetic model is correct. The most prominent example is a two component exponential fit resulting in two almost identical lifetimes, while the residuals still show serious deviations. In this case, set one of the identical lifetimes manually to zero and restart the fit. For the zero parameter a Monte Carlo search is invoked, while all other (probably reasonable) parameters are only re-optimised.

It is possible to constrain the range of possible values for any model parameter. If a parameter is to be constrained in the fit, activate the *Lower* or *Upper* limit by checking the corresponding tick box in the *Edit Parameter* dialog. (Refer to page 11.) Enter the value for the corresponding limit in the edit box below the check box. If limits are set for a parameter, its details button in the fitting parameter table shows three red dots instead of black ones. Please note that setting limits will reduce the ability of the Marquardt-Levenberg algorithm to converge, even if the best fit parameter set will be located within these parameter limits.

Wise fixing of a parameter by unchecking it in the *Var* column of the *Parameter Panel* can lead to more sensible kinetic interpretation of the results, in spite of the increased χ^2. For example, when fitting a multiexponential model to a decay of a sample containing several different fluorophores, try to fix the lifetime parameters to their real or expected values. These can be often obtained from independent measurements of single fluorophores under the same conditions. Double-exponential exciplex decays often show amplitude changes but not lifetime changes when the detection wavelength is varied. Yet another example is the reconvolution analysis of parallel and perpendicular polarised decays. For the same sample, the various lifetime components in those decay pairs are not independent. Furthermore, the major decaying component usually corresponds to the pure population decay with a lifetime which can be independently determined in a separate experiment.

Very short, apparent decay components (with lifetimes on the order of the system IRF) may be an artefact resulting from insufficient suppression of the scattered excitation light. Instead of varying the lifetime and the amplitude of such a component, try to include the correction for scattered light. However, it is always preferable to remove the scattered excitation light (or similar artefacts, such as back-reflections) during the experiment, rather than including them in the reconvolution fit.

Dynamic anisotropy effects (e.g. due to rotational relaxation) or solvent relaxation also distort the pure, exponential population decay. These additional, fast decaying or rising components can be easily misinterpreted.

5.3.4 Polarisation / Anisotropy Decay Analysis

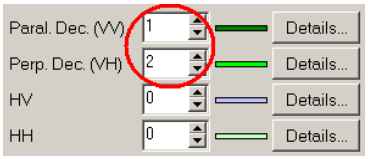
The anisotropy decay $r(t)$ is calculated from the parallel and perpendicular polarised decay curves in the following way:

$$r_{Exper.}(t) = \frac{I_{\parallel}(t) - G\,I_{\perp}(t)}{I_{\parallel}(t) + 2\,G\,I_{\perp}(t)}$$

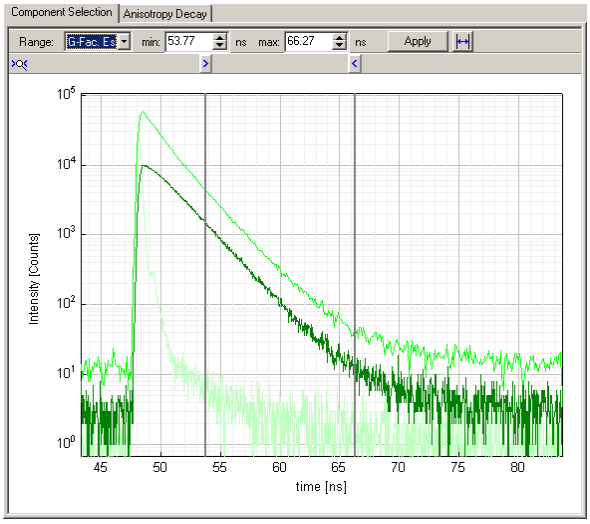
where G is a correction factor to compensate for the polarisation bias of the detection system. The G-factor can be varied as a fitting parameter, but this is only advisable as long as no anisotropy background R_{INF} is used in the analysis. The anisotropy background (also called residual anisotropy) and the G-factor are strongly correlating. If a non-zero anisotropy background can be expected (e.g. hindered rotation), the G-factor should be kept fixed. In this case the correct G can be either entered manually (if known from independent measurement) or calculated from experimental data. For the latter, there are two possibilities:

Tail matching

Tail matching is applicable in cases where the anisotropy decay is much faster than the intensity (e.g. fluorescence) decay and the (expected) residual anisotropy is zero. In addition, the measured parallel and perpendicular polarised decays must contain a sufficient amount of anisotropy-free intensity decay. Assign the appropriate decay curves for the anisotropy analysis using the *Data Sets* section of the parameter panel:



Select the appropriate parts of the parallel and perpendicular decay curves, using the *G-fac. Estim.* range. To obtain the correct result, make sure that the selected parts of the curves are not influenced by the anisotropy decay. The range has been correctly selected when the portions of the parallel and perpendicular decay curves are parallel in a logarithmic plot, as shown in the following example:



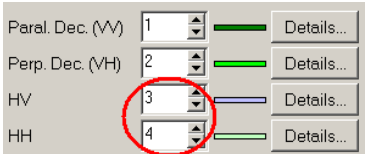
Having selected the range, click on the *Tail Matching* button. The *G*-factor will be calculated and displayed in the corresponding row of the parameter panel. If you wish to keep this value, make sure that the *Var* box of the parameter is unchecked, otherwise the *G*-factor will be optimised during the fit. (The optimisation would lead most probably to reasonable results, since the estimation of the *G*-factor by the fit is equivalent to the tail matching method.) Note that tail matching is not applicable when there is a non-zero anisotropy background (residual anisotropy = R_{INF}).

HV & HH integration

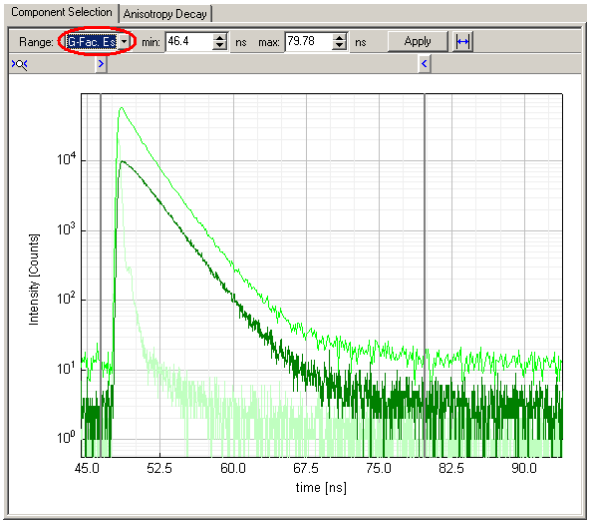
When tail matching is not feasible, the *G*-factor can be calculated from HV and HH decays:

$$G = \frac{\int \mathrm{HV}(t)\,dt}{\int \mathrm{HH}(t)\,dt}$$

Assign the appropriate decay curves for the anisotropy analysis using the *Data Sets* section of the parameter panel:

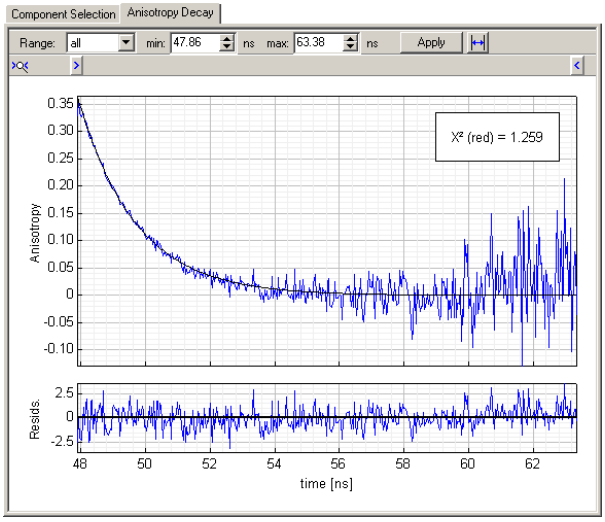


Select the appropriate parts of the HV and HH decay curves, using the *G-fac. Estim.* range. To obtain the correct result, make sure that the selected parts of the curves do not contain too much of the regions dominated by the background, as shown in the following example:



Then press the *HV <=> HH Integration* button. The *G*-factor will be calculated according to the above definition and displayed in the corresponding row of the parameter panel. Please make sure that the *Var* box of the parameter is unchecked, otherwise the *G*-factor will be optimised during the fit.

Having determined the *G*-factor, you can preview the calculated anisotropy decay by clicking on the *Anisotropy Decay* tab of the main plot. The following picture shows an example of an anisotropy decay curve as a result of calculation:



Do not become confused by a noisy curve. The anisotropy information is contained in a small difference of very large intensity values containing inherent noise. *r*(*t*) values are calculated for the "Decay" range selected in the *Component Selection* tab. Always exclude the channels before the rising edge and after the tail, because here the anisotropy is simply not defined due to lack of emission intensity.

Fitting this calculated *r*(*t*) curve is equivalent to a standard exponential tailfit, however the parameters have a different meaning. See chapter *4.1.8 Anisotropy [Tailfit]* on page 18.

Of course the numerical value of the initial anisotropy R_0 cannot be very reliable when not taking into account the effects of the IRF. This would introduce the necessity of simultaneous reconvolution analysis on perpendicular and parallel decay. Please see chapter 4.1.9 Anisotropy [Reconvolution] on page 19 for a short introduction.

5.4 Managing the Results

5.4.1 Saving and Loading

The preferred way to store the hard-won computational data is to save them, using the menu of the main window. This way, everything will be saved, including all the experimental data currently in memory and the results of various optional calculations (e.g. the outcome of advanced error analysis).

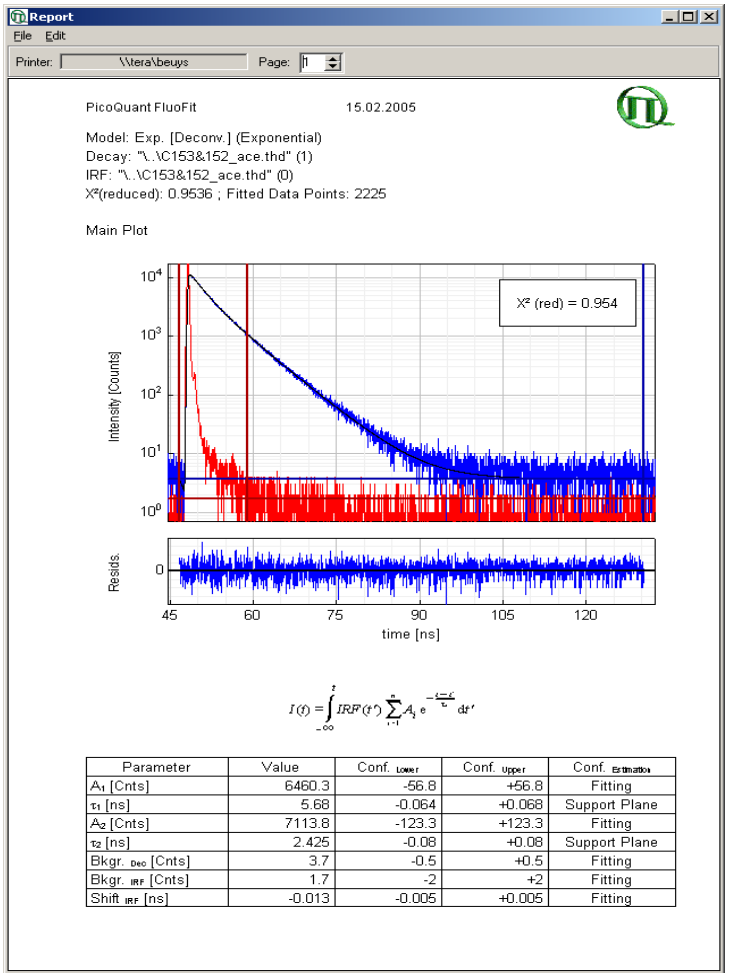
Clicking on , or selecting *File*, then *Save Results* from the main menu opens a Windows standard dialog where you can select the file location. A similar standard dialog appears when *File*, then *Load Results* is selected from the main menu, or is pressed. Loading of a result file will reset FluoFit to the stage it was at the moment of saving these results. Beware that all the curves loaded and/or imported so far during the session will be erased and replaced by those saved in the result file.

FluoFit stores its results in a proprietary binary format with a file name extension of `*.ffr`. To get the data in ASCII form, use the *Report* dialog described in the next chapter.

5.4.2 Reporting, Printing, and Exporting

To show a summary of the current fit results, select *File* then *Print Results...* from the main menu or click on . Alternatively, you can use the Results button of the parameter panel. The *Report* dialog, similar to the one depicted on the next page will be opened.

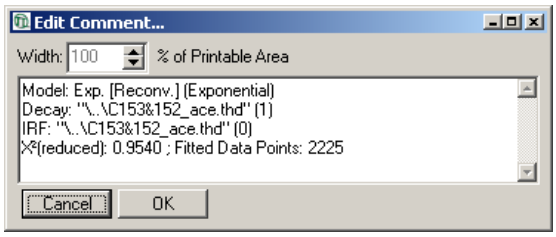
At the top, the currently selected printer is displayed and a spin control allows stepping through the report pages, if there should be more than one. FluoFit calculates quite a variety of useful things and there are usually more pages.



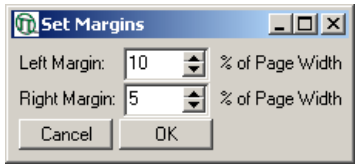
Parameter	Value	Conf. Lower	Conf. Upper	Conf. Estimation
A_1 [Cnts]	6460.3	-56.8	+56.8	Fitting
τ_1 [ns]	5.68	-0.064	+0.068	Support Plane
A_2 [Cnts]	7113.8	-123.3	+123.3	Fitting
τ_2 [ns]	2.425	-0.08	+0.08	Support Plane
Bkgr. Dec [Cnts]	3.7	-0.5	+0.5	Fitting
Bkgr. IRF [Cnts]	1.7	-2	+2	Fitting
Shift IRF [ns]	-0.013	-0.005	+0.005	Fitting

The report can be customised. The ***Edit*** menu offers the following possibilities:

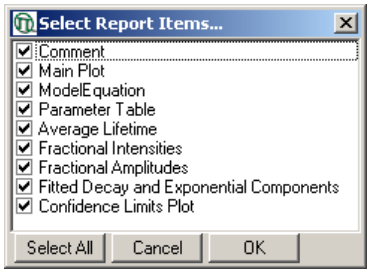
Edit Comment... The comment is the topmost report item displaying some general information about the fit. This command is equivalent to a double click on the comment. A dialog similar to the one depicted below will be opened. You can edit the text in the central edit field of the dialog. The height of the comment will be automatically set to match the the amount of text entered, while the width of the comment field is always as wide as the printable area.



Margins... This command will open the following dialog, where you can set the margins. In order to avoid conflicts when changing the printer, the margins are entered in percent of the page width.

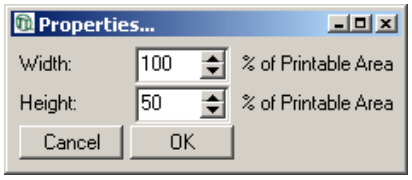


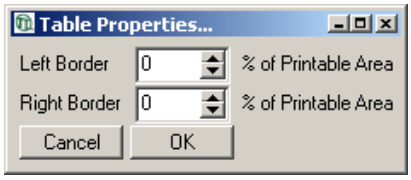
Show/Hide Report Items... Using the following dialog, you can select or unselect any report item in the list. Only the selected items will be displayed and printed. Beware that this selection applies also to data export, when the *Export ASCII...* command is selected from the ***File*** menu.



Hide Selected A report item can be selected by clicking on the check box left to the item.

In addition to the above commands available from the menu, plots and tables can be further individually customised. Double clicking on any of them will open one of the following dialogs:





The width and height are entered in percent of the printable region, which is the width of the report page minus the margins. Likewise, the table border distances are measured from the margins, i.e. “0% left and right border” means that the table will be exactly as wide as the printable area.

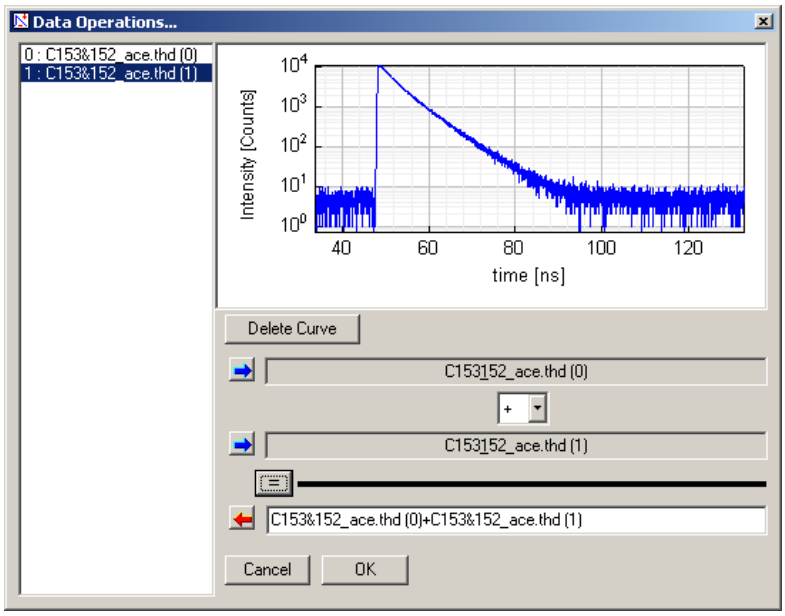
The ***File*** menu offers the following commands:

Printer Setup...	Printer selection and configuration is possible using the standard Windows dialog that will be opened.
Print	The current report, that is the displayed report items, will be printed on the current printer. See also the *Show/Hide Report Items...* command of the ***Edit*** menu described above.
Export ASCII...	This command exports the current report as an ASCII file using the Windows standard file saving dialog. This corresponds to the *Save Results* function of older versions of FluoFit. The default file name extension of such an export is `*.dat`, and the format is self-explanatory. Beware that only the displayed items will be exported. See also the *Show / Hide Report Items...* command of the ***Edit*** menu described above.
Export Selected as ASCII...	This command is available only when an item is selected with a mouse click. (Multiple selection is also possible by holding down the <CTRL> key when clicking on the items.) The result is a simple ASCII file with a default extension of `*.dat` and self-explanatory format.

6. Tools

6.1 Data Operations

Select *Tools*, then *Data Operations* from the main menu, or click on to bring up the following dialog:



On the left side of the dialog is a list of all curves currently imported into FluoFit. The highlighted curve in the list is displayed at the upper right side of the dialog. If you wish to remove a dataset, highlight it and then press the *Delete Curve* button. Changes made in this dialog will be discarded when closing the dialog by pressing the *Cancel* button.

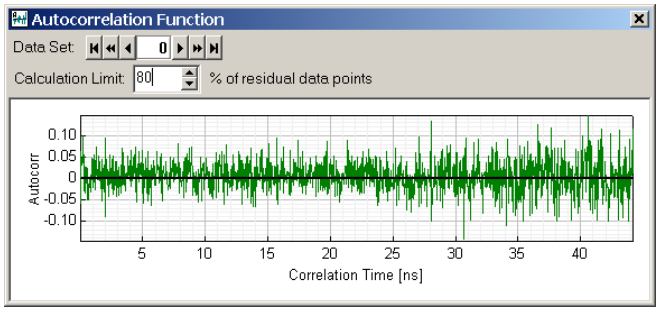
In some cases it might be convenient to do simple arithmetics on curves, e.g. to subtract an experimentally estimated background. Please note that the statistical noise of the result after such an operation is not a simple function of the noise of the operands. Due to moire effects, this mostly holds even for the sum. Therefore, FluoFit automatically sets the weighting for the resulting curves to <none>.

To do arithmetical operations on curves, highlight a curve in the list and press one of the => buttons to select it as one of the two possible operands. Do the same for the other operand. Select an operator in the corresponding combo box, then press the = button to calculate the result. The operands do not need to have the same time resolution. If a resolution mismatch occurs, the calculator takes the data point of the second operand as the next in time to the corresponding data point of the first operand.

The result curve will be automatically named after the two operands and the operator. This name can be edited in the edit box nearest to the bottom. To transfer the result curve to the curve list press <= to accept the new curve for the FluoFit data set, close the dialog by pressing *OK.*

6.2 Autocorrelation of Residuals

The residuals' autocorrelation function is a tool for assessing the fit quality. It is a measure of correlation (in the sense of similarity) between the weighted residuals in distinct channels separated by various (correlation) times. Selecting *Tools*, then *Autocorrelation Function* from the main menu or pressing opens a new window with a plot of the residuals' autocorrelation function versus the correlation time:



Since the weighted residuals are randomly distributed for a good fit (in other words: they are not correlated), the values of the autocorrelation function should be small and also randomly distributed around zero for times > 0. Unlike the plot of weighted residuals displayed at the bottom of the main plot, this window does not show where (i.e. at which channel) the misfit occurs, but provide information about the characteristic time scale of the misfit. The autocorrelation plot is very sensitive to hidden trends in residual values and provides a rigorous test of the fit quality.

The *Calculation Limit* edit box allows to specify the maximum correlation time considered in the calculation of the autocorrelation. The value is entered as a percentage of fitting range length. For example, suppose you have selected a time range for a decay fit from 10 ns to 72.5 ns. This is a 62.5 ns long time interval. Then a calculation limit of "80 % of the residual data points" means that the residuals' autocorrelation values will be plotted for correlation times in the interval from 0 to 50 ns, e.g. like in the picture above.

6.3 Advanced Error Estimation and Analysis

Calculating confidence intervals for the best fit parameters is a non-trivial problem. FluoFit makes a guess for the parameters' errors by examining the derivatives of the χ^2 surface up to second order in the neighbourhood of the best fit parameter set. This is a quick procedure and often gives quite reasonable results. In some cases it is advisable to investigate the errors more thoroughly. For example, when a multiple exponential fit leads to decay times that are only narrowly separated, it is not certain that all exponentials are really needed in the model.

The program automatically calculates and lists the standard deviations of all iterated (i.e. not fixed) model parameters. These so called asymptotic standard errors (ASE) are a reasonable measure of the parameters' reliabilities if and only if the parameters are uncorrelated regarding their errors. For all other cases FluoFit offers more sophisticated methods to estimate errors: support plane and Bootstrap analysis.

6.3.1 Support Plane Method

The idea behind support plane analysis is to vary a parameter and study the consequences of this alteration on the χ^2 value. As long as χ^2 is smaller than a given tolerance level of the best fit χ^2, the corresponding parameter set can be regarded as "also acceptable". The range of those "also acceptable" parameter sets defines a confidence interval.

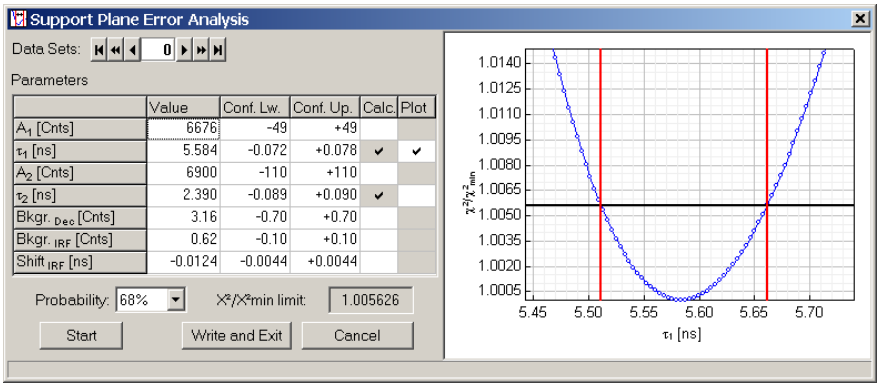
Asymptotic standard error and support plane algorithms both calculate χ^2 as a function of each single parameter and estimate the region of this function, which is smaller than a given tolerance value. This tolerance level is a function of the probability of the true parameter to be located within the confidence interval, the number of freely varying model parameters and the number of data points. It is usually given in units of the best fit χ^2:

$$\frac{\chi^2_{max}}{\chi^2_{opt}} = 1 + \frac{p}{\nu} F(p, \nu, P)$$

Here $F(p, \upsilon, P)$ is the F-statistics with p parameters and υ degrees of freedom with a probability of P. ASE and support plane analysis differ only in the way the other parameters are treated. ASE is a quicker approach to error estimation: The other parameters are kept fixed at their best fit values. This procedure gives good results only if the parameters are more or less independent.

In the case of strongly dependent parameter sets, error estimation by support plane analysis leads to more accurate results, although the computation time is decidedly longer. Here χ^2 is also calculated as a function of each parameter, but the other parameters are fitted to allow compensation for its change. The resulting function is broader, since the calculated χ^2 values may only be smaller than without fitting the other parameters, as done by the ASE method. In the extreme case, where two parameters are completely dependent, there will not be a minimum at all, instead the χ^2 function will remain constant at the minimum value. As support plane calculations involve fitting, they are not only time consuming, but there may also be some irregularities in the χ^2 functions of the parameters such as kinks or jumps.

To perform a support plane error analysis on the current fit, select *Tools* and then *Support Plane Error Analysis* from the main menu or click on . A dialog similar to this will open:



The table on the left side shows the current confidence intervals of the fitting parameters. Initially this table holds the errors estimated by the fitting algorithm, which is equivalent to the asymptotic standard errors. Fixed parameters will be greyed.

Select the parameters, for which the error analysis is to be performed, by checking them in the *Calc.* column of the table. To show a slice of the χ^2 hypersurface for a calculated parameter check the *Plot* column. Due to incompatible units for amplitudes and lifetimes not all parameters can be visualised at the same time. The *Probability* combo box allows to set the probability of the parameters of being located in the calculated confidence intervals. The resulting χ^2/χ^2_{min} limit is a function of the number of data points, number of parameters and, of course, the *Probability* value selected. It is displayed in the plot as a horizontal black line.

Press *Start* to perform the analysis. To accept the results as the new parameter confidence limits press *Write and Exit* or press *Cancel* to discard them. Parameter errors of nonglobal parameters are calculated only for the data set selected by the *Data Sets* control above the parameter table.

As the support plane analysis is based on the least squares definition of χ^2 it cannot be used with MLE analysis. Use the bootstrap analysis instead.

6.3.2 Bootstrap Method

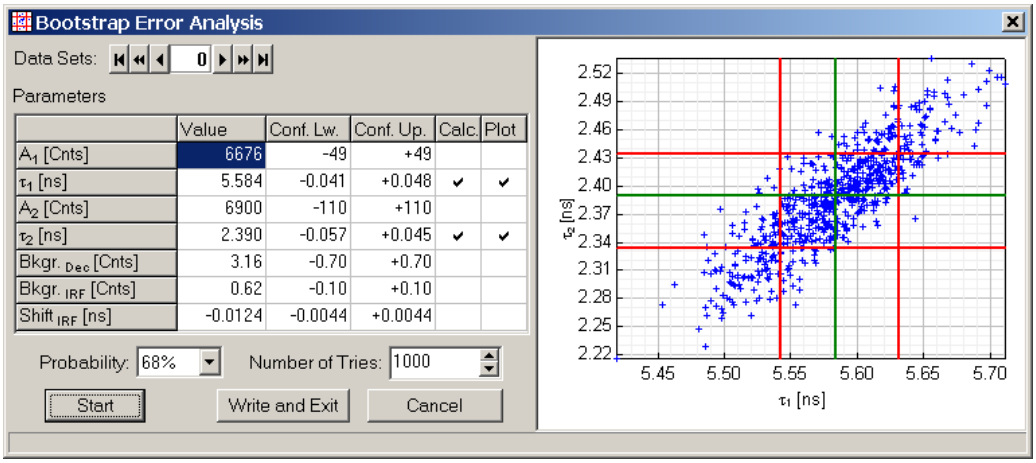
One possibility of estimating parameter uncertainties is to repeat the measurements. The uncertainty of the parameters can then be estimated from the spread of the results. This is the idea behind various Monte Carlo methods for fitting parameter error estimation. Starting from the measured data set, a number of synthetic (or simulated) data sets are derived simulating a realistic noise. The fit is then repeated for each simulated data set. Due to the stochastic nature of the simulated noise, fitting will produce slightly different results for each set. The parameter uncertainty can thus be estimated from the spread of the parameter distribution of this series of fits.

The problem with the Monte Carlo error analysis lies in the simulation process for the synthetic data sets. A thorough knowledge of the underlying statistics is needed to simulate the noise. The simulation process itself is time consuming, and it has to be repeated ~1000 times. The method of choice to overcome these problems is the Bootstrap analysis, which is often referred to as “quick and dirty Monte Carlo”. The idea behind this approach is to use the original data set directly to produce realistic synthetic data sets.

Let the original data set consist of *N* data points. Then a subset of *N* randomly chosen data points is selected from the original data set to form the new, synthetic data set. This may appear somewhat confusing, since *N* points out of *N* points seem to produce an identical data set. The “dirty trick” is to allow data points to be picked more than once. Thus, in the resulting data set a random fraction of points (~1/*e*, i.e. approx. 37%) are replaced by duplicated original points. The process can be visualised as randomly shooting *N* times at the array of original data points and taking whatever point is hit. Since *N* points are taken out of *N* points, the statistical properties of the original data set are more or less preserved in the simulated data sets.

Each new, simulated data set is fitted just like the original one to get a new best fit parameter set. The “cloud” of these parameter sets gives insight into correlations between the parameters regarding their errors. Estimating confidence regions for the parameters corresponding to a probability of P % is just picking a subregion of the cloud containing P % of all best fit parameter sets. The actual shape of the confidence region is generally arbitrary. The choice of the confidence region done with the FluoFit package is to define such a region for each parameter of the best fit parameter set: a strip parallel to the axis. The width and the position of the strip is determined by the confidence intervals, which are chosen as the smallest possible for the given parameter distribution at the cost of not being symmetrical around the best fit value. This is equivalent to stating that the true value of this parameter is located within this confidence interval with a probability of P %, regardless of the behaviour of all other parameters. This is not equivalent to stating that the probability of all parameters being located within their confidence intervals would be P %. For defining your own confidence regions, for example to match the before mentioned criterion, use the ASCII export function of the *Report* dialog to export the parameter sets of the Bootstrap analysis as an ASCII file.

The Bootstrap error analysis on the current fit is invoked by selecting *Tools*, then *Bootstrap Error Analysis* from the main menu or by clicking on . A dialog similar to this will open:



The table on the left side shows the current confidence intervals of the fitting parameters. Initially this table contains the errors estimated by the fitting algorithm, which is equivalent to the asymptotic standard errors. Fixed parameters are greyed.

Check the parameters for which the error analysis is to be performed in the *Calc.* column of the table. To show a 2D projection of the "cloud" of parameter sets, select exactly two parameters in the *Plot* column. The resulting plot visualises the correlations between pairs of parameters.

The *Probability* combo box allows to set the probability of a single parameter of being located in its corresponding calculated confidence intervals. The *Number of Tries* edit box sets the number of data sets to be generated and fitted during the analysis.

Press *Start* to perform the analysis. To accept the results as the new parameter confidence limits, press *Write and Exit* or press *Cancel* to discard them. Parameter errors of nonglobal parameters will be calculated only for the data set selected by the *Data Sets* control above the parameter table.

7. Fitting Examples and Tutorials

The FluoFit software package contains sample data files to facilitate the learning of basic fitting techniques. Analyses of these test files as described below demonstrate various software features. Each datafile is briefly described, the correct result is given and important notes are made where necessary.

The following fitting procedures are described:

- Single exponential tailfit
- Single exponential reconvolution
- Double exponential reconvolution
- Simple anisotropy analysis

7.1 Single Exponential Tailfit

`Ru(bpy)3.thd`

The luminescence decay of diluted aqueous solution of Tris(2,2'-bipyridyl)ruthenium(II) chloride was measured by a FluoTime 100 spectrometer. The sample was excited with a 425 nm LDH-C-400 violet diode laser, triggered externally with a frequency of 250 kHz. The absorbance of the sample was below 0.1 at the excitation wavelength. The emission was detected through a 580 nm long pass filter. The count rate was about 3000 cps, well below the pile-up limit.

Load the file and select the decay no. 0 or 1 for analysis. Select the "Exp. [Tailfit]" model. To set the proper range, cut off the rising edge and exclude the last channels that contain background counts only.

Results:

Decay 0 fitting range from 0.85 µs to about 3.5 µs, A_1 = 1085 counts, $\tau_1 \approx 0.354$ µs with $\chi^2 \approx 0.914$
Decay 1 fitting range from 0.85 µs to about 4.5 µs, A_1 = 13049 counts, $\tau_1 \approx 0.355$ µs with $\chi^2 \approx 1.021$

7.2 Single Exponential Reconvolution

`C152_ace.thd` and `C153_ace.thd` contain single exponential decays of Coumarin 152 and 153, respectively, both dissolved in acetone. The dyes were purified by thin layer chromatography (TLC) before use, and the solutions were diluted to have an absorbance of approximately 0.1 at the main absorption band maxima. For data acquisition, a FluoTime 100 spectrometer equipped with sheet polarisers was used. The excitation source was a 407 nm LDH-C-400 violet diode laser operated at 10 MHz, set to vertical polarisation. The magic angle polarised emission was detected through a 520 nm long pass filter. The count rate was about 40–50 kcps, well below the pile-up limit.

Make sure that the "Exp. [Reconv.]" model is set. Load the file and assign the curves properly: curve 1 for "Decay" and curve 0 for "IRF". Set the proper time ranges for the IRF and the decay, respectively. No scattered light correction, nor cyclic re-excitation correction is necessary.

Results:

Coumarin 152 fitting range from 46 ns to 80 ns, $\tau \approx 2.40$ ns with $\chi^2 \approx 0.912$
Coumarin 153 fitting range from 46 ns to 110 ns, $\tau \approx 5.55$ ns with $\chi^2 \approx 0.995$

7.3 Double Exponential Reconvolution

C153&152_ace.thd

The solutions of the previous example were mixed together. The measurement conditions were the same as above. The mixture of the two dyes shows a double exponential decay, and each lifetime component should be in proper accordance with the previous single exponential fits.

Results:

Fitting range from about 46 ns to 108 ns, $\tau_1 \approx 2.38$ ns and $\tau_2 \approx 5.57$ ns with an overall $\chi^2 \approx 1.006$. The amplitudes (A_1 and A_2) are about equal.

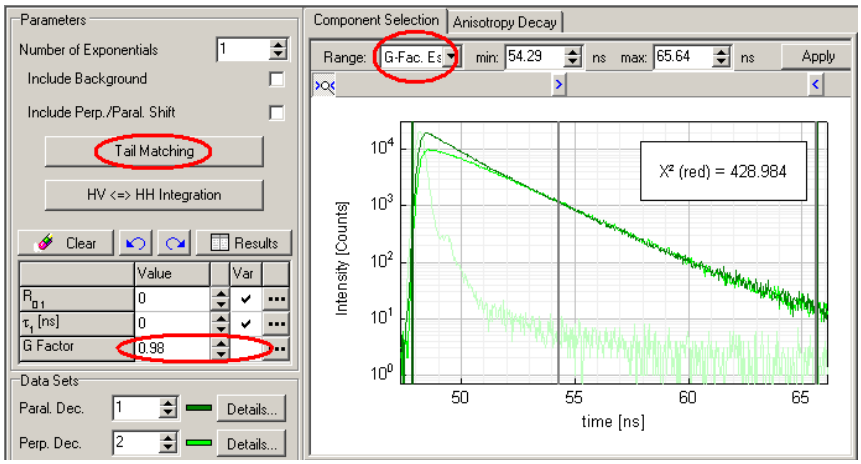
This datafile is well suited to try out the advanced error analysis features of FluoFit. Please refer to chapter *6.3 Advanced Error Estimation and Analysis* starting at page 33 to learn how to evaluate the fitted parameters further.

7.4 Simple Anisotropy Analysis

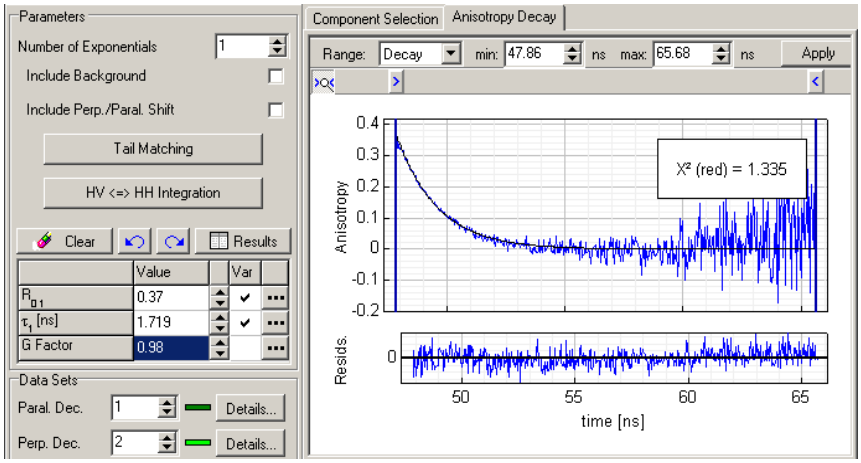
`C6_etgly.thd` contains polarised fluorescence decays of 2µM Coumarin 6 solution in ethylene glycol at 30°C. A FluoTime 100 spectrometer equipped with a 407 nm LDH-C-400 violet diode laser was used for data acquisition. The laser was operated at 10 MHz and the polarisation plane of the beam was set to vertical. Polarised components of the fluorescence were selected by a sheet polariser and detected through a 470 nm long pass filter. Emission count rate was about 60–70 kcps, measurement runtime 15 seconds for each decay. The file content is as follows: curve #0 is an IRF, which is not used here, curve #1 is the parallel polarised decay (VV in the notation of older FluoFit versions), curve #2 is the perpendicular polarised decay (VH), and curve #3 is a magic angle polarised decay.

The simplest anisotropy analysis approach uses the parallel and perpendicular polarised decays to calculate the anisotropy decay directly, channel by channel. The resulting curve is then analysed by the “Anisotropy [Tailfit]” model.

Refer to chapter *4.1.8 Anisotropy [Tailfit]* starting at page 18 for instructions how to handle polarised decays. The first step is to determine the *G*-factor. In our case the tail matching is applicable. (After all, the HV and HH decays are not available in this data file.) Owing to the filter based detection, a *G*-factor close to 1 can be expected. Ignore the high, apparent χ^2 value displayed right after the first tail matching, since nothing has been optimised yet.



Because of the nature of the sample, we can safely expect zero residual anisotropy, which means the *Include Background* option should remain unchecked. (In this case it is possible to allow the determined *G*-factor value to vary during the fitting; it should recover the manually determined value.) Otherwise, iterating the *Bkgr.*$_{Aniso}$ parameter may improve the χ^2 value, but the interpretation of the resulting fitted parameters becomes questionable. The same holds for introducing a second exponential term. There is no reason to expect time shift between the decay curves, so the *Include Perp./Paral. Shift* option better remain unchecked as well. However, it is valuable to experiment with the FluoFit and to try out all these options. The result of the simplest possible analysis is shown below:



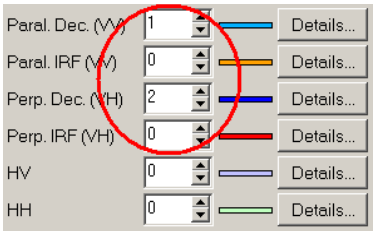
Curve #3 of `C6_etgly.thd` contains a magic angle polarised decay, which is free of anisotropy effects. This allows for proper fluorescence lifetime to be determined by reconvolution fitting. The resulting single lifetime can be used in a more advanced anisotropy analysis involving reconvolution fitting of the parallel and perpendicular polarised decays. The following tutorial provides a short introduction to this approach. Details can be found for in the literature listed in chapter *1.2 Recommended Literature*.

7.5 Anisotropy Reconvolution Analysis

The same data file as in the previous example is used (`C6_etgly.thd`). Curve #0 is the IRF, applicable for parallel and perpendicular polarisation, curve #1 is the parallel polarised decay (VV in the notation of older FluoFit versions), curve #2 is the perpendicular polarised decay (VH), and curve #3 is a magic angle polarised decay.

The model used for anisotropy reconvolution fitting is described in chapter *4.1.9 Anisotropy [Reconvolution]* starting at page 19.

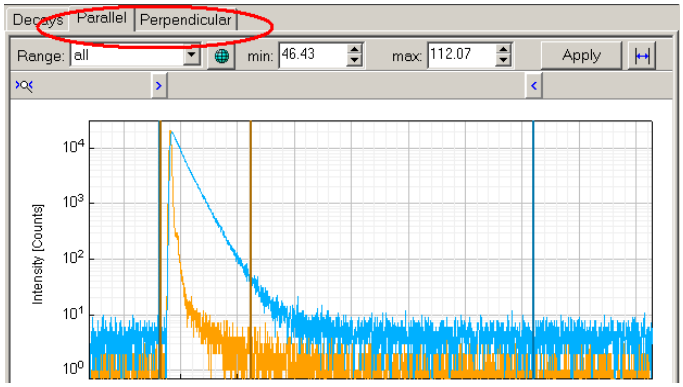
Assign the curves contained in the file the following way:



As in the previous example the G-factor can be estimated by tail matching or by fitting; the HV and HH decays are not available in this data file. Owing to the filter based detection, a *G*-factor close to 1 can be expected.

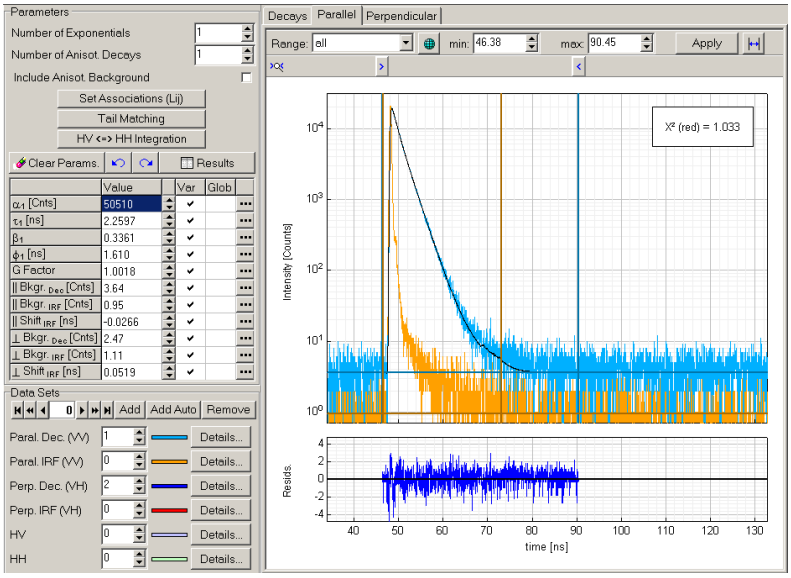
Because of the nature of the sample, we can safely expect zero residual anisotropy, which means the *Include Background* option should remain unchecked.

An anisotropy reconvolution analysis will reconvolve parallel and perpendicular decay separately with the model decays determined by the common fluorescence and anisotropy parameters. Thus two reconvolution data sets have to be set up (like shown in chapter *7.2 Single Exponential Reconvolution* on page 36): the fitting ranges can be edited on the *Parallel* and *Perpendicular* page of the main plot:

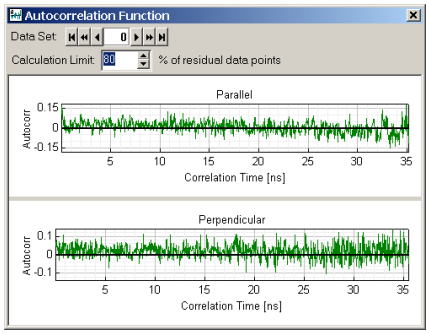


In this particular example the IRF is the same for both polarisation directions, thus the IRF range will be identical for both data sets. Nevertheless, it can be edited on both pages.

For this data set a single exponential model is appropriate both for the fluorescence as well as the anisotropy decay. The results for this analysis are shown below:



Please note that no anisotropy decay is calculated while analysing the data. Consistency of the model with the data can be checked by means of the parallel and perpendicular residuals trace. As usual the autocorrelation function of the residuals traces can be calculated. It will show an autocorrelation function for both the parallel and perpendicular residuals:



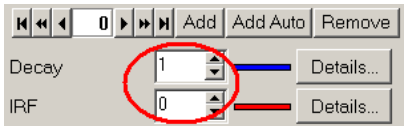
The anisotropy decay as calculated from the reconvolved data is shown on the results report pages.

7.6 Global Reconvolution Fitting

`Ox1-4_TRES01.thd` contains 15 decay curves (data blocks 1-15) and an associated IRF in block no.0. Two laser dyes with slightly different emission spectra and lifetimes, Oxazin4 and Oxazin1, were dissolved in ethanol and excited with a 636 nm pulsed diode laser. The FluoTime 200 lifetime spectrometer was operated in a so-called TRES measurement mode. The monochromator scanned the wavelength range from 610 to 680 nm in 5 nm steps. At each step, a 10-second decay measurement was automatically performed. These decay curves document the wavelength dependence of the mixture's decay kinetics. This dependence is governed by the amplitude changes of the two lifetime components. The relative contribution of the faster Oxazin1 fluorescence increases with the wavelength.

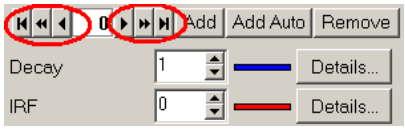
In preparation of a global fit the data sets corresponding to a single wavelength each have to be set up. The most convenient way of doing this follows the course outlined below:

1. For data set zero select curve #0 as IRF and curve #1 as decay:

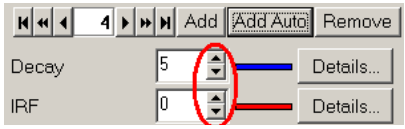


2. Press *Add Auto*:

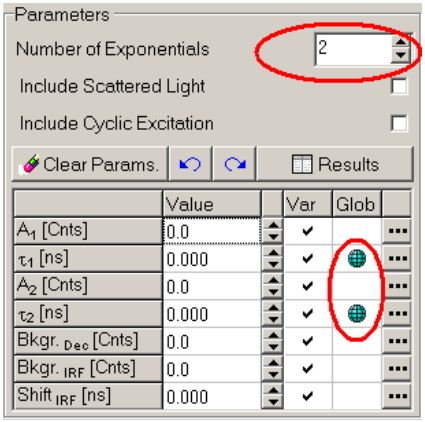
3. The program will automatically add 14 new data sets and will assign the remaining curves in the file (#2 to #15) to the decays of these data sets. The IRF will be set to #0, as in the data set zero.
4. Use the spin buttons of the data set control to navigate through the defined data sets:



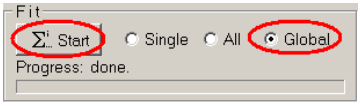
5. The curve assignment for each data set can be edited separately (this will not be necessary for this data file):



For this example a double exponential model is expected to describe the data. Both lifetimes will be of global scope, i.e. will be the same for all data files. Please select a double exponential model and set the lifetimes as global parameters by clicking on the *Glob.* column of the τ_1 resp. τ_2 row:



Set the fitting mode to *global* and start the fit:



The program performa a global Monte Carlo search and a global optimisation for the defined data sets. Two points may become apparent:

1. Global fitting is more time consuming than a simple fit.
2. Convergence is a more complex task for a global fit. The probability of getting caught in a local minimum of the χ^2 hypersurface is larger than for a simple fit.

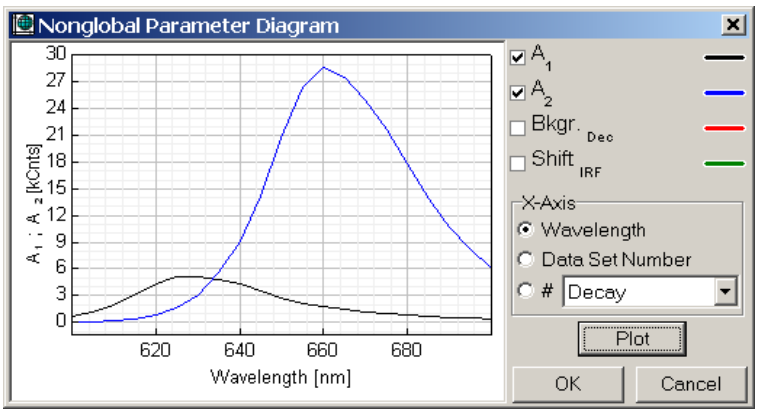
If the fit got stuck in a local minimum there are two possible ways out. Pressing the *Clear Params.* button and restarting the fit is the most straight forward measure. If the data set is a really tricky one, the fit can sometimes be "persuaded" to converge by finding initial values for the global parameters using a single data set, for example, use data set #14, clear the parameters, set the fitting mode to *single* and start the fit. Switch back to *global* fitting mode and start the global fit.

Assessing the goodness of fit

While the assessment of the goodness of fit follows the same lines as for a nonglobal fit, there are some technical details worth mentioning. First of all, there are two kinds of χ^2 values: the global one for the complete fit, and a local one for each single data set. The global χ^2 is displayed in the first panel of the status bar, while the local ones are displayed in the upper right corner of the main plot.

To estimate the accordance between data and model, it is of course insufficient just to examine the χ^2 values. A quick test is skimming through the data sets and examining each residuals trace. Use the data set navigation control for this purpose. Similarly, the autocorrelation function can be used for assessment.

Often a global data set is the result of a TRES measurement, i.e. of an emission wavelength scan. The wavelength dependency can be visualised by the "nonglobal parameter diagram". It can be brought up by pressing the following tool button:



Since the global parameters are not dependent on the data set index, only plotting of nonglobal parameters makes sense. In the above case the wavelength dependence of the amplitudes is plotted. The plot resembles the steady-state spectrum of the sample. On the X-axis the *Wavelength* (only for TRES measurements), the *Data Set Index* or the data block number (#) of a data set curve may be displayed. Selection of the X-axis parameter may be done by the radio buttons on the right side of the dialog.

Estimation of fitting parameter accuracy can again be done by use of the bootstrap or support plane analysis. Please note that for all nonglobal parameters the confidence intervals are calculated for the selected data set only.

Since global fitting is an advanced technique a closer look at the literature listed in chapter *1.2* is strongly recommended to users new to this field before applying this technique.

8. Appendix

8.1 Technical Reference Data

Decay Models and Parameters

Exponential decay models.....up to 4th order, tailfit or reconvolution

Lifetime distributions.....Gaussian or Lorentzian distributed lifetimes with up to 4 peaks or up to 4 stretched exponential terms, tailfit or reconvolution

Anisotropy.....up to 4th order exponential, tailfit of the anisotropy decay and anisotropy reconvolution

Decay parameters.....amplitudes, lifetimes, distribution width, background

Reconvolution parameters.....background, time shift, scattered light contribution, pulse repetition rate

Anisotropy parameters.....G-factor, amplitude, background, time shift between polarised decays

Algorithms

Nonlinear least squares fitting / MLE.....Marquardt-Levenberg, Monte Carlo or manual parameter variation

Correction for finite IRF.....iterative reconvolution

Error test/assessment.....χ^2, distribution and autocorrelation of weighted residuals

Error analysis.....asymptotic standard errors, Bootstrap and support plane analysis

Global analysis/batch mode fitting.....for all fitting models, number of data sets only memory limited

User Interface

Graphical user interface.....Windows® GUI, menu or mouse driven

Display.....linear or logarithmic scale

Data import.....file or Windows clipboard

Preferences.....saved in Windows registry

Data Formats

Number of channels.....any

Channel width.....any

Supported formats.....TimeHarp, NanoHarp, PicoHarp, HydraHarp (binary or clipboard)
B&H SPC and MSA binary data
ASCII

Operating Environment

Required PC.....minimum 400 MHz CPU clock and 256 MB memory
recommended (global fitting) >=512 MB

Display.....1024 × 768 or better

Disk space.....10 MB (except data storage)

Operating system.....Windows 2000 / XP / Vista / 7 / 8

Protection module port.....USB (parallel on request)

Printer.....any printer supported by Windows

® Registered trademark of Microsoft Corp.

8.2 Abbreviations

ASCII	American Standard Code for Information Interchange
ASE	Asymptotic Standard Errors
CPU	Central Processing Unit
FWHM	Full Width at Half Maximum
GUI	Graphical User Interface
INF	Infinity; infinite
IRF	Instrument Response Function; Lamp Function
ISBN	International Standard Book Number
LDH	Laser Diode Head
LPT	Line Printer, sometimes for Line Printer Port
MLE	Maximum Likelihood Estimation
RAM	Random Access Memory
TCSPC	Time Correlated Single Photon Counting
TLC	Thin Layer Chromatography
TRES	Time Resolved Emission Spectra
TTTR	Time-Tagged Time-Resolved
USB	Universal Serial Bus
WEEE	Waste Electrical and Electronic Equipment

8.3 Support

The FluoFit software has gone through extensive testing by PicoQuant. It is now very stable and reliable. Nevertheless, we will continually make improvements and fix bugs where they can be identified. The results of these efforts will be incorporated into upcoming versions of the program.

In any case, we would like to offer you our complete support. Please do not hesitate to contact PicoQuant if you need assistance with your data analysis.

If you observe any errors or bugs, please try to find a reproducible error situation. E-mail a detailed description of the problem and relevant circumstances, together with the data in question to photonics@pq.fta-berlin.de Your feedback will help us to improve the product and documentation.

Of course, we also appreciate good news: If you have obtained exciting results or published a scientific paper, we would like to know! Please send us an e-mail containing the appropriate citation to info@picoquant.com. Gain additional publicity! PicoQuant maintains a database of publications mentioning PicoQuant devices and/or written by us. It can be found at our website at http://www.picoquant.com/_scientific.htm and it is a valuable source if you would like to know which laboratories are using PicoQuant products or how broad is the field of various applications.

Thank you very much in advance for your kind co-operation!

Retraction of old devices

Waste electrical products must not be disposed of with household waste. This equipment should be taken to your local recycling centre for safe treatment.

WEEE-Reg.-Nr. DE 96457402

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